RECOLLECTIONS

The hunt for mitochondrially synthesized proteins

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I sensed right away that something important had happened. The banter in my small basement laboratory at Cornell University had abruptly ceased and everybody had flocked around the window where my technician Jo Saltzgaber held a tiny sausage-like polyacrylamide gel against a brilliant shaft of spring sun. Jo had spent the winter of 1969 trying to resolve the individual proteins of mitochondrial membranes by gel electrophoresis, but when the snow started to melt outside, all she had to show was a sad collection of stained gels whose vague, smeared bands firmly reminded her that membrane proteins are insoluble in water and cannot be separated from each other in the usual gel systems. But this time, Jo had tried the new sodium dodecyl sulfate (SDS) gels that had been invented two years before by Jake V. Maizel, Jr. and his colleagues at Albert Einstein College and perfected a year later by Mary Osborn and Klaus Weber at Harvard. After she had treated the gel with a protein stain, the result was everything she had hoped for: her sample of yeast mitochondria had been resolved into sharp bands, and there were so many of these bands that quite a few of them were probably individual proteins. To me, the little gel tube appeared like a magic weapon whose power sent a pleasant tingling down my spine. Now that we could pry the water-insoluble mitochondrial proteins apart as sharp layers in a polyacrylamide gel, they could no longer hide from us by huddling together as aggregates. We knew that among these proteins there were some that were made inside the mitochondria. Although we

had not yet identified them, we soon would. Our long hunt for them was drawing to close.

The hunt had begun eight years earlier at the University of Vienna when Ellen Haslbrunner, Hans Tuppy, and I set out to find the reason why the respiration-deficiency of some yeast mutants was not inherited in a Mendelian fashion. Did these so-called *petite* mutations alter genes that resided within mitochondria, which themselves are not inherited according to Mendel's laws? If so, then mitochondria should contain DNA. When we checked this possibility, our samples of isolated yeast mitochondria indeed contained DNA, but when we purified the mitochondria further by centrifuging them to density equilibrium in a sucrose gradient, the DNA did not specifically cosediment with the mitochondrial marker enzyme cytochrome oxidase. Instead, it smeared through the entire gradient. Most of it was apparently nuclear DNA that had escaped from the nucleus during cell breakage and could not be separated from mitochondria by our usual purification method. We tried density gradients made from other sugars: no success. We used inorganic salts: unmitigated disaster. Then one of my friends at the university clinic told me about Urografin, a new X-ray contrasting agent he used for examining kidney function. I assumed that Urografin (which was sold as "Renografin" in the US) had to be gentle to biological material, because my friend injected it into his patients. And he would give it to me free of charge! We tried a density gradient made from Urografin and, for reasons I have never properly understood, it worked. Now most of the DNA sedimented to the bottom of the centrifuge tube; while the mitochondrial band contained only little DNA, the distribution of this DNA in the gradient paralleled exactly that of cytochrome oxidase. This DNA appeared to be inside the organelles because we could only digest it with DNAase after we had destroyed the integrity of the mitochondrial membranes. We submitted our findings in January 1964 under the cautious title "DNA Associated with Yeast Mitochondria" to *Biochemical and Biophysical Research Communications* (Schatz et al., 1964), the newest and "hottest" biochemical journal of the day, and soon an avalanche of reprint requests and telephone calls began to inundate our little Vienna laboratory.

But we had not been alone. After we had submitted our article, we learned that Sylvan and Margit M. K. Nass, two electron microscopists working at the University of Pennsylvania, had discovered that chicken mitochondria contained thread-like inclusions that could be removed by DNAase, but not by RNAase. They, too, had concluded that mitochondria contain DNA. Their paper ap-

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Gottfried Schatz was born on August 18, 1936, in Strem, a little Austrian village close to the Hungarian border. He grew up in Graz, the second-largest city of Austria, but spent the year 1952 as an American Field Service-sponsored high school student in Rochester, NY. After receiving his Ph.D. in Chemistry from the University of Graz in 1961, he joined the Biochemistry Department of the University of Vienna where he began his studies on the biogenesis of mitochondria. From 1964 to 1966 he worked as a postdoctoral fellow with Efraim Racker at the Public Health Research Institute of the City of New York on the mechanism of oxidative phosphorylation. After a brief interlude back in Vienna, he emigrated to the USA in 1968 to join the staff of the Biochemistry Department at Cornell University in Ithaca, NY. Six years later he moved to the newly created Biozentrum of the University of Basel (Switzerland), his present address. He has served as Secretary General of the European Molecular Biology Organization (EMBO), but has also maintained close contacts with colleagues and scientific organizations in the USA and Japan. He and his Danish wife Merete have three children.

Melvin V. Simpson (left) and Gottfried Schatz at the State University of New York at Stony Brook in 1994, after Gottfried Schatz had delivered the First Melvin V. Simpson Lecture at the Department of Biochemistry and Cell Biology.

peared in the December 1963 issue of the *Journal of Cell Biology*, a few months before our paper (Nass & Nass, 1963). Reports on the existence and properties of mitochondrial DNA now started to appear in rapid succession, and a new field of research was born.

What was this mitochondrial DNA doing? My colleague Erhard Wintersberger found that yeast mitochondria contained not only DNA, but also RNA as well as a DNA-dependent RNA polymerase (Wintersberger & Tuppy, 1965). Later, he showed that much of the mitochondrial RNA hybridized to mitochondrial DNA and resembled ribosomal RNA. Mitochondrial DNA thus seemed to encode the RNAs of some mitochondrial ribosomes yet to be discovered. But what about genes encoding proteins? A simple calculation convinced us that there was not enough DNA in yeast mitochondria to encode all of the hundreds of mitochondrial proteins. Most of these proteins therefore had to be encoded by nuclear genes, synthesized on cytosolic ribosomes, and then imported into mitochondria—a bizarre scenario!

Yet we knew that mitochondria could also make some of their proteins themselves, and we suspected that these proteins were encoded by mitochondrial DNA. Melvin V. Simpson and his colleagues at Yale University had reported already in 1958 that isolated rat mitochondria could incorporate radioactive amino acids into protein (McLean et al., 1958). The authors went to great lengths to exclude that the observed activity reflected contaminating microsomes. Indeed, a few years later it was found that protein synthesis by isolated mitochondria was insensitive to inhibitors of microsomal protein synthesis such as cycloheximide, but sensitive to inhibitors of bacterial protein synthesis such as chloramphenicol and erythromycin. But after these landmark discoveries, things ground to a halt. Amino acid incorporation by mitochondria was very weak; because [^{35}S]-methionine was not yet commercially

available, incorporation had to be measured with tritiated or ^{14}C -labeled amino acids of low specific radioactivity; the labeled proteins were insoluble; and a fraudulent claim that one of the labeled proteins was cytochrome *c* further damaged the field's reputation, even though the fraud was quickly discovered and the record set straight. But most frustrating was the finding that the incorporated radioactivity failed to co-fractionate with any of the known mitochondrial enzymes. Because the abnormally inherited *petite* mutants lacked cytochrome oxidase and succinate-cytochrome *c* reductase, it was generally suspected that these enzymes were synthesized by mitochondria. But when a few researchers heroically purified cytochrome oxidase or cytochrome *b* from small samples of isolated mitochondria that had been incubated with radioactive amino acids, both preparations proved to be unlabeled. Scientific investigations, like the scientists themselves, can have dry spells, and the field of mitochondrial protein synthesis suffered one in the middle sixties. In 1964, it was even claimed that mitochondria could not synthesize any proteins at all and that previous claims to the contrary had to be attributed to protein synthesis by contaminating bacteria.

In the summer of 1964 I left Vienna to join Efraim Racker as a postdoctoral fellow in New York City. I was reluctant to stop working on mitochondrial DNA so soon after we had participated in its discovery, but during my first years in Vienna I had become increasingly frustrated by my ignorance of biochemistry. This ignorance had become painfully obvious after I had joined the Biochemistry Department at the University of Vienna in the spring of 1961. Our chairman was Hans Tuppy, an enormously gifted, imaginative, and dynamic young biochemist who had been a key participant in Fred Sanger's Nobel prize-winning work on the amino acid sequence of insulin. In setting up his Department, Tuppy had collected some of the best young Austrian biochemists who admired and tried to emulate him. Although funds were scarce, I am still amazed at how much this department accomplished in the early sixties. But most of my departmental colleagues were protein chemists with only a cursory interest in mitochondria, and I was very much on my own. I had to learn more about the biochemistry of membranes and mitochondria. As a chemistry student at the University of Graz in Austria, I had always been fascinated by biochemical processes, but during the poverty-stricken post-war years the University of Graz did not offer any biochemistry courses. As its library contained only musty biochemistry textbooks from the late forties and early fifties, I had created my own "Biochemistry 101" course. First, I worked my way through the Biochemistry Section of *Chemical Abstracts*, then a popular abstracting periodical that our library carried. Second, I jotted down the names and addresses of the authors whose articles interested me most. Third, I purchased several dozen picture postcards of Graz and sent them to these authors with the modest request: "Dear Dr. X, please send me reprints of all your articles." Fourth, fifth, and sixth, I waited...and, because airmail was then a luxury and I had sent my postcards by surface mail. In retrospect, I am amazed that I received any replies at all. The most generous one was from David E. Green, who had apparently taken me quite seriously and sent me a heavy CARE package containing most of his several hundred reprints on the function and structure of mammalian mitochondria. What a feast! I read all of these articles, mostly on benches in our local park, and soon lost myself in an enchanted world that was protected by two graceful membranes and populated by clever enzyme complexes that burned our food, gave us energy and warmth, and synthesized many of our key chemicals. Billions of controlled

fires within us, tiny never-dying embers glowing on the hearth of life. I fell in love with mitochondria.

Few students ever received their Ph.D. degree with such an eclectic biochemical knowledge: I knew David Green's research on mitochondria, but very little else. Yet, without the charm of the Graz postcards (or was it the stamps?) and David Green's generosity I would have known even less. I have never forgotten how I waited for the replies to my messages, and to this day try to answer promptly every letter I receive.

By now it should be clear why I had to leave Vienna, even though it would have been prudent to stay and exploit our recent discovery. Why did I not join Green, who had initiated me to the field? In fact, I had first wanted to join Green, but then changed my mind when I met Racker, whom I immediately admired and liked. I only met Green several years later when he invited me to his laboratory to track down a case of "scientific misconduct." But that is another story.

During my years in New York I learned how to work with large amounts of beef heart mitochondria, gaining experience one cannot get from reading papers or textbooks. Armed with this experience, I began to study the ATPase complex of yeast mitochondria. The pioneering studies of Maynard E. Pullman, Harvey S. Penefsky, Yasuo Kagawa, and Racker had shown that this complex consists of a soluble ATPase domain (termed F_1) that is linked to a membrane-embedded proton-conducting pore termed F_0 . The ATPase of the entire F_1F_0 -ATPase complex is sensitive to the antibiotic oligomycin and, like most proteins, is relatively stable at 0°C. However, once F_1 is detached from F_0 , it becomes cold labile and its ATPase is no longer oligomycin sensitive. Together with Harvey Penefsky, I purified F_1 from yeast mitochondria and showed that it could bind to F_0 from beef heart mitochondria and regain its sensitivity to oligomycin.

When I returned to Vienna in the fall of 1966, Piotr Slonimski and others had just shown that the *petite* mutations of yeast profoundly altered the buoyant density of mitochondrial DNA, suggesting massive deletions (Mounolou et al., 1966). I decided to check whether these mutations also altered the mitochondrial ATPase complex. The result was striking: mitochondria from different *petite* mutants still had F_1 , but this F_1 was oligomycin insensitive even in intact mitochondria. The F_1 itself seemed to be normal because F_1 isolated from the mutant mitochondria became oligomycin sensitive when mixed with F_0 from beef heart mitochondria (Schatz, 1968). I concluded from these results that mitochondrial DNA encoded all or part of the membrane-embedded F_0 moiety of the mitochondrial ATPase complex. I was so close! My next steps should have been to isolate F_0 from wild-type cells, to characterize its protein subunits, and to check whether they were synthesized within mitochondria. But it was not to be. Late in 1967, just after I had submitted my paper, my wife and I decided to emigrate to the US and then spent the first half of 1968 preparing our move. By then we had two small children and a third on the way. Trying to get the necessary papers from Austrian and US offices was a descent into Kafka's netherworlds. There was no time to worry about F_0 . By then, Alexander Tzagoloff in New York City had begun to work on the biosynthesis of yeast F_0 and was making rapid progress. I knew I would have to find another project once my laboratory in the US had been set up.

In the fall of 1968 we left Vienna for Cornell University in Ithaca, NY, running smack into the hippie revolution. Love and drugs were in; my Austrian tie was out; and I greatly improved my passive knowledge of short English words, most of them com-

posed of only four letters. During my first week as a Cornell professor, I was ordered to spend two nights watching out for students who might want to improve US society by setting my laboratory on fire. Yet these were wonderful days for academics. Grants and jobs were still plentiful, and the students were fun to be with. Most of them were in permanent need of a haircut, but they were outgoing and idealistic. Everything was questioned, everything tried; what could be more scientific? It seemed that a new society was just around the corner, and that a campus like ours was its testing ground.

But I had not emigrated to the US to help forge a new society; I had come to do better science. For someone like me who wanted to study mitochondria, few places were then more exciting than the new Biochemistry Department that my former postdoctoral mentor Efraim Racker had created at Cornell University. Racker had attracted many scientists interested in various aspects of membrane function: internationally known senior figures such as Quentin Gibson, Leon Heppel, and André T. Jagendorf; and younger faculty members such as Peter C. Hinkle, Richard E. McCarty, David C. Wharton, and myself, who were eager to make their mark. I was given my own small basement laboratory whose address, Wing Wing G-1, would today suggest a *Drosophila* mutant or an oncogene. I was given my own grant. I even got my own electric typewriter. And I hired my first technician, Jo Saltzgaber, who already made her entrance at the opening of this story.

As her last name suggests, Jo came from a family of German immigrants. She had obtained a bachelor's degree in biology, but had then gone to work as a technician in order to raise a family and to help her husband through college. She was ambitious, hard-working, and meticulous; she ran my lab with a firm hand; she was a real friend; she was a godsend.

The excellent facilities at Cornell renewed my determination to track down the proteins made by mitochondria. As our fermentors had not yet arrived, we could not start working with isolated yeast mitochondria. Jo and I therefore checked into the possibility, much discussed at the time, that mammalian mitochondria synthesized "structural protein," an insoluble protein thought to function as a scaffold for the catalytically active membrane proteins. But when we isolated "structural protein" from beef heart mitochondria, it proved to be a mixture of denatured membrane enzymes, mostly F_1 -ATPase; it was not a biological reality, but an artifact (Schatz & Saltzgaber, 1969a).

Finally, our long-awaited New Brunswick fermentors arrived and I could continue to hunt for the proteins made by yeast mitochondria. My previous attempts to incorporate ^{14}C -labeled leucine into isolated yeast mitochondria had taught me two things. First, I would never get the protein products hot enough without five additional NIH grants. Second, the methods for preparing yeast mitochondria were so lengthy, and our common centrifuges at Wing Wing so dirty, that I would always have to worry about protein synthesis by contaminating bacteria. Why not label the mitochondrially synthesized proteins in living yeast cells? It should be quite simple: incubate yeast cells with glucose as an energy source; inhibit their cytosolic ribosomes with cycloheximide; add labeled leucine; and then isolate and analyze the mitochondria. The results made us dance: virtually all of the incorporated label was in the mitochondria, and this labeling was abolished when we inhibited the cells with both chloramphenicol and cycloheximide (Schatz & Saltzgaber, 1969b). This *in vivo* labeling protocol was fast, minimized bacterial contamination, and labeled the mitochondria at least 100 times more efficiently than when we used isolated

mitochondria. Now we could get the elusive mitochondrially synthesized proteins really hot.

With this new labeling tool we showed that all *petite* mutants of yeast lacked mitochondrial protein synthesis. Stefan Kuzela and Ladislav Kovac at the University of Bratislava (then in Czechoslovakia) had come to the same conclusion by examining the incorporation of labeled amino acids into isolated yeast mitochondria (Kuzela et al., 1969). The *petite* mutation thus prevented the expression of all the genes that were still left on the defective mitochondrial DNA molecules. This finding explained why all *petite* mutants had the same enzymic deficiencies even though they had widely different deletions in their mitochondrial DNA, or no mitochondrial DNA at all.

There matters stood in the fall of 1969, when Jo proudly displayed her beautiful SDS gel of mitochondrial proteins. The next step was obvious: we labeled yeast mitochondria in cycloheximide-inhibited yeast cells, isolated the mitochondria, ran them on an SDS-polyacrylamide gel, cut up the gel into about 100 1-mm slices, and counted each of these in a liquid scintillation counter. Labeling and electrophoresis were easy, but getting the gel slices required the combined skills of an oboe player, a snake charmer, and a brain surgeon. First, the slippery gel had to be blown out of the glass tube from which it always seemed to pop out at an unexpected moment. The usual STEP 2 of the procedure was, therefore: wait until nobody is watching you and then try to pick up the gel from the floor. Next, the gel had to be frozen to the cutting bench of a gel slicer by powdered dry ice. Then came the artistic touch: one had to remove the dry ice and let the gel soften up just enough so that it could be guillotined piecemeal by a mechanically operated razor blade. If the gel had become too soft, it got squashed to an ugly puddle. If it was still too hard, the razor blade sent it flying to the floor. Wait until nobody watches you (STEP 1); and stay calm because you are not even halfway through yet. You still must place each slice into a separate glass vial; pour 10 ml of a smelly solubilizer into each vial; cap each vial; incubate the vials overnight in an oven; let them cool; open them again; add scintillation fluid; recap the vials; place them into a scintillation counter; let them adapt to the dark; and then count them for at least 10 min each. And, oh yes, you then must empty them and wash them for the next round. Ah, the good old days!

Finally everything worked. I started to set up camp in front of the scintillation counter, my glazed eyes riveted to the luminous display. I never could just go home and read the printout the following morning. I began to resemble one of the hapless creatures in a Las Vegas casino. But on plotting the results, I found about half a dozen broad radioactivity peaks, none of them coinciding with a major stained protein band. The mitochondrial protein products were clearly not major mitochondrial proteins. But at least I could specifically label them, resolve them on a gel, and count them. So far so good. But what were their properties and their function?

It was at this critical stage that Thomas L. Mason joined my laboratory as a postdoctoral fellow. Over the years I have worked with close to 100 postdoctoral fellows, students and sabbatical visitors, and I am often no longer sure who overlapped with whom. But for each of them I still remember my first reaction on meeting them face to face. Tom's face conveyed openness, intelligence, and an appealing touch of shyness. He had grown up in Wisconsin and seemed to embody much of what I admired about the US. I immediately liked him. He became one of my best postdoctoral fel-

lows and, later, a close friend who now is too far away. He, too, was a godsend.

Tom knew about my earlier work on the biosynthesis of the F_1F_0 -ATPase and was disappointed when I told him that this project was already well underway in another laboratory. We therefore decided to check whether cytochrome oxidase was made by mitochondria. Cytochrome oxidase (also termed cytochrome *aa₃*) is the last enzyme of the mitochondrial respiratory chain in the mitochondrial inner membrane, reducing oxygen to water. It had already been purified by detergent-extraction of mammalian mitochondria and been extensively studied by spectroscopic and biochemical methods. However, its subunit composition was unknown because at that time most biochemists had either never heard of SDS gels or viewed them as an exotic novelty. Tom and I conveniently ignored the earlier reports that cytochrome oxidase was not labeled by isolated mitochondria. We hoped that we would see things others before had missed, because we now had better eyes.

By a stroke of luck, our colleague David C. Wharton happened to be an internationally recognized expert on cytochrome oxidase and he agreed to help us get started. Aided by his immense experience, we quickly purified cytochrome oxidase from yeast mitochondria, monitoring the purification by the characteristic green color of the enzyme and its ability to oxidize reduced cytochrome *c*. When we ran our best cytochrome oxidase preparations on an SDS gel, we saw six to seven protein bands: three large ones of apparent molecular masses 45 to 25 kDa, and three to four small ones of 14.4 to less than 9 kDa, the resolution limit of our gels. Rabbit antisera against the enzyme precipitated the same seven bands from a detergent extract of mitochondria, and also inhibited cytochrome oxidase activity. We knew that these antisera would be crucial tools for us.

At this stage my family and I left Ithaca for an extended summer vacation in Austria. Tom, the good American, did not believe in vacations and continued to work through the hot and humid Ithaca summer. When I returned to the lab, he greeted me with this special mix of gaiety, pride, and defiance that spells good news. I did not even glance at my pile of accumulated mail, but asked him to spill the beans. With the words "Well, here it goes," Tom opened his notebook and showed me two nearly flawless experiments. In the first experiment, he had labeled yeast in the presence of cycloheximide, isolated the mitochondria, solubilized them with detergent, added the cytochrome oxidase antiserum to the extract, collected the green immunoprecipitate, ran it on an SDS gel, and counted the gel slices. The result: only the three large cytochrome oxidase subunits were labeled. In the second experiment, he had labeled yeast in the presence of chloramphenicol and then proceeded exactly as in the first experiment. Now only the small subunits were labeled. The exciting conclusion: the three large cytochrome oxidase subunits were made by mitochondria, whereas the small subunits were made on cytosolic ribosomes and then presumably imported. Today, any postdoctoral fellow would insist on publishing such a result as quickly as possible in one of the trendy scientific magazines, but the times were then different and Tom was not just any postdoctoral fellow. We presented these results orally at a Gordon Conference in the summer of 1971, and published them as a Symposium paper in 1972 (Mason et al., 1972), but Tom then spent more than a year repeating and perfecting his results before submitting them as two companion papers to the *Journal of Biological Chemistry* (Mason et al., 1973; Mason & Schatz, 1973).

It was only later that we understood why we had succeeded where others before had failed. The key was our labeling proce-

ture. When isolated mitochondria make the large cytochrome oxidase subunits, they cannot assemble them into the holoenzyme because they lack excess pools of the small subunits. As a consequence, the large subunits do not cofractionate with functional cytochrome oxidase and are, in fact, degraded by intramitochondrial proteases. In contrast, yeast cells contain excess pools of unassembled small cytochrome oxidase subunits that can enter mitochondria and associate with newly synthesized large subunits even when cytosolic protein synthesis is inhibited by cycloheximide. The mitochondrially made subunits are thus stably present in the functional cytochrome oxidase holo-enzyme.

Once again, we were not alone. Unknown to us, Hanns Weiss and his colleagues at the University of Munich had studied the biogenesis of cytochrome oxidase in the mold *Neurospora crassa* and had concluded that one subunit of the enzyme was made by mitochondria (Weiss et al., 1971). When Weiss visited us in Ithaca in 1971, we spent long hours of often passionate discussions trying to explain our divergent results. In the following year the Munich laboratory realized that the two other mitochondrially made subunits of the *Neurospora* enzyme had aggregated during their solubilization procedure and had remained invisible because they failed to penetrate into the polyacrylamide gel. Further work then established that the enzymes from *Neurospora* and yeast were, in fact, quite similar and that in both cases the three largest subunits were made inside the mitochondria.

While all this happened, the field received another major push from Alex Tzagoloff's work in New York City. Born in Russia, Alex had come to the US as a young boy and had finished his education with a postdoctoral stay in David Green's laboratory at Wisconsin at about the same time I worked with Efraim Racker in New York City. Green and Racker were both trying to resolve the machinery of oxidative phosphorylation, but never seemed to see eye to eye on anything, and our two laboratories maintained an uneasy truce. Alex managed to keep aloof from these often petty tensions. Soft-spoken, intelligent, and an acknowledged virtuoso in the laboratory, he was one of Green's young stars who produced a steady flow of exciting papers on the protein complexes of the mitochondrial inner membrane. In 1968, while I had briefly returned to Vienna, Alex had moved to the Public Health Research Institute in New York City to establish his own laboratory. His goal: to study mitochondrial biogenesis in yeast. I, too, had initially decided to join this Institute where I had spent such wonderful postdoctoral years, and where teaming up with Alex offered exciting possibilities. On further reflection, however, I had concluded that my family would prefer the green hills of Ithaca to the excitement of New York City, and the plans of our collaboration washed down the Hudson river and vanished in the sea.

Starting out as an independent investigator in New York City, Alex had brilliantly achieved what I should have done in Vienna: solving the biosynthetic origin of the F_1F_0 -ATPase complex. He purified the complex from yeast mitochondria, determined its subunit composition by SDS-gel electrophoresis, and showed that one of the small and hydrophobic F_0 subunits was labeled by cycloheximide-inhibited yeast cells. This subunit was thus made inside mitochondria. Alex published this finding in 1971 (Tzagoloff, 1971), the same year we first reported our results on cytochrome oxidase, but for a while his contribution did not receive the attention it deserved, perhaps because the ATPase complex was not yet considered as well defined an entity as cytochrome oxidase. Yet his 1971 paper reported the first functional identification of a mitochondrially made protein. It was a true landmark.

Alex also studied the biosynthesis of cytochrome oxidase and eventually reached the same conclusions as we. However, he was for a while sidetracked by the inability to detect the three large subunits of the enzyme on SDS gels. Like Weiss and his colleagues at Munich, he had solubilized his mitochondrial samples by boiling them with SDS in the presence of high salt concentrations, thereby inducing aggregation of the unusually hydrophobic large subunits.

This hydrophobicity was the curse by which the mitochondrial genetic system punished us for having identified some of its products. We tried to isolate larger amounts of these mitochondrially made subunits; they precipitated as balls of steel. We tried to sequence them; they merely scoffed. Strange legends started to grow around them. Some claimed that the large cytochrome oxidase subunits were in reality tight aggregates of smaller proteins. Others considered them as hydrophobic contaminants of unknown function.

Very slowly the fog lifted. First, Eberhard Ebner isolated nuclear yeast mutants that specifically lacked cytochrome oxidase activity as well as one or two mitochondrially made subunits of the enzyme (Ebner et al., 1973). Then Robert O. Poyton showed that an anti-serum specific for the second largest cytochrome oxidase subunit strongly inhibited the activity of the purified yeast enzyme (Poyton & Schatz, 1975). Both findings strongly implied that these large subunits were essential for the function and the biosynthesis of cytochrome oxidase. The nuclear mutants isolated by Ebner were later ingeniously used by Thomas D. Fox for unraveling the mechanisms by which the nucleus controls the expression of mitochondrial DNA (Fox, 1986). In 1979, Buse and his colleagues at the University of Aachen in Germany finally managed to sequence the first large subunits of mammalian cytochrome oxidase, establishing them as hydrophobic, but otherwise "normal" proteins (Steffens & Buse, 1979). Perhaps the most decisive breakthrough was the discovery of specific mitochondrial DNA mutations that altered only a single mitochondrially made protein (Bunn et al., 1970; Wilkie 1970; Tzagoloff et al., 1975; Douglas & Butow, 1976). Some of these mutations made the F_1F_0 -ATPase or cytochrome *b* resistant to antibiotics; others selectively inactivated one of the enzyme complexes of oxidative phosphorylation; and still others were naturally occurring mutations that had no physiological effect, but altered the electrophoretic mobility of a mitochondrially made protein. Unlike the massive *petite* mutations that knocked out mitochondrial protein synthesis, these specific mutations could be used to map genes on mitochondrial DNA. Soon the first map of yeast mitochondrial DNA appeared (Tzagoloff et al., 1979); it was circular, just like that of a bacterium! This map did not tell us whether the mapped genes were regulatory or structural genes for the mitochondrially made proteins, but Slonimski's laboratory and ours had studied the effects of mutations and reversions in a mitochondrial gene affecting cytochrome oxidase function and the results strongly implied that this gene was, in fact, the structural gene of the second largest cytochrome oxidase subunit (Cabral et al., 1978).

Now recombinant DNA methods entered the scene and all of us were sure that the mitochondrial genome was as good as conquered. We had all the tools; what could stop us? We had only to sequence all of its genes, accept the scientific awards, and move on to other things. But the mitochondrial genome fought back. It could not stop us from sequencing its genes, but it garbled the message of these genes by using a genetic vernacular that we did not understand. We had taught our students that TGA was a "non-sense" codon that signaled the end of a polypeptide chain, but

when yeast mitochondria said "TGA," they meant tryptophan. Even worse, mitochondria from different organisms spoke different vernaculars. The code could finally be cracked by comparing gene sequences with the amino acid sequences of the protein products (Barrell et al., 1979; Fox, 1979; Li & Tzagoloff, 1979). But mitochondria from plants and some parasites had prepared a second line of defense: they "edited" their primary messenger RNAs by replacing specific nucleotides with others (Benne, 1989). To know the amino acid sequence of a protein made by these mitochondria, we must either predict the editing steps (which we cannot yet do with certainty), or sequence the mature mitochondrial mRNA (which is not a job for the faint-hearted).

After Sanger and his colleagues published the complete sequence of human mitochondrial DNA (Anderson et al., 1981) the battle was largely over, although the mitochondrial genome continued to hold on to some unconquered niches. Armed with Sanger's sequence information, Attardi and his colleagues made peptides corresponding to some of the still unidentified open reading frames on human mitochondrial DNA, and used antibodies against these peptides to show that our mitochondria also make seven hydrophobic subunits of the NADH dehydrogenase complex (Attardi et al., 1986). Today, the complete sequences of many different mitochondrial genomes are known and at the click of a mouse button my computer reads to me the proteins encoded by these genomes. Even allowing for the question marks just mentioned, most of these proteins are clearly identified as hydrophobic subunits of the oxidative phosphorylation system in the mitochondrial inner membrane. Fortunately, mitochondria of mammals and yeast do not edit their mRNAs, and Melvin Simpson can take pleasure in the knowledge that the radioactive leucine he fed to isolated rat mitochondria in 1958 incorporated the precious label into 13 proteins: 3 subunits of cytochrome oxidase, 1 subunit of the cytochrome *bc*₁ complex, 7 subunits of the NADH dehydrogenase complex, and 2 subunits of the *F*₀ part of the ATPase complex. The mitochondria of the yeast *Saccharomyces cerevisiae* make only eight stable proteins, and those of plants at least twice as many. Now that the amino acid sequences of many of these hydrophobic proteins are known through the nucleotide sequences of their genes, they no longer strike terror into the hearts of protein chemists. Starting in 1988, Butow, Fox and their co-workers (Fox et al., 1988; Johnston et al., 1988) began to shoot DNA into mitochondria of living yeast cells, ushering in the era of mitochondrial transformation. The atomic structure of bovine cytochrome oxidase has recently been unraveled (Tsukihara et al., 1995) and, once it is published in full, will show us in exquisite detail the arrangement of the three mitochondrially made subunits in the holoenzyme and their probable role in enzyme activity.

In the early eighties, mitochondrially made proteins also entered the big league. Synthesis of aberrant mitochondrial protein products by mitochondria of several crop plants was found to cause male sterility (Huang et al., 1990), a trait of great economic importance to plant breeders. And a surprisingly large and still growing number of inherited human diseases was traced to mutations in mitochondrially synthesized proteins (Holt et al., 1988). I now find myself giving talks to blue-jeaned plant molecular biologists at the huge land grant colleges in the Midwest, or to sartorially resplendent clinicians in Grand Ballrooms of metropolitan hotels. Some of the work on mitochondrially synthesized proteins is moving from university campuses to small companies, results are patented, and there are the first whiffs of commercial secrecy. As the hunt for mitochondrially synthesized proteins draws to a close, the field has lost its innocence.

Although I had originally intended to live out my days in the US, I moved to the Swiss city of Basel in the spring of 1974. Basel was rapidly becoming one of the premier biology centers of Europe, and the newly created Biozentrum of the University of Basel was to be one of the city's scientific flagships. When my Swiss colleague Max M. Burger phoned me at Cornell one night to ask me whether I was interested in joining the Biozentrum, I did not engage in the usual rituals of academic courtship, but simply said yes. I had grown up in war-torn Austria and Switzerland had always seemed like a blissful island to me. I also sensed that it was time for a change. The hippie years had faded away; US society had returned more or less to what it had been before; my old Austrian tie was again fashionable; and the oil shock of 1973 had rudely chilled the adventurous spirit of the late sixties; and helping to build a new scientific institute in the heart of Europe was an exciting challenge. Soon after this move my laboratory stopped working on mitochondrially synthesized proteins and began to study how mitochondria import proteins from the cytoplasm. Yet another story, to be told another time. But many of the old questions still haunt me, like adolescent yearnings never quite fulfilled. How does the nucleus control mitochondrial protein synthesis? How are mitochondrially synthesized proteins inserted into the mitochondrial inner membrane? How does mitochondrial protein synthesis work and why has nobody yet succeeded in making the process work outside an intact mitochondrion? And then there are the whys: *Why* does a cell devote more than a hundred of its nuclear-encoded proteins to synthesizing a few inside the mitochondria? *Why* are some mitochondrial genomes extremely compact whereas others seem outrageously large in relation to the number of genes they carry? *Why* RNA editing?

I have just turned 60, and if I were to follow the old Hindu advice, I should long have retreated to the woods to contemplate. But I have Western roots and have always been inspired by Homer's world. As an adolescent, I worshipped Achilles, whose power and recklessness fascinated me. Later, I began to appreciate the intellectually supple Odysseus who served me as a guide in running research groups and scientific institutions. Should it now be Nestor whom Homer portrays with such keen insight and tenderness? Should I now spend more time telling my students and post-doctoral fellows how it had been before, how the discoveries had happened, but also how much knowledge is getting lost? I do enjoy telling them why the adenine nucleotide they add to their mitochondrial sample will be quickly altered; why an uncoupler often fails to abolish respiration-driven ATP synthesis; why an unstirred mitochondrial suspension can be an invitation to disaster; and how mitochondria of *petite* mutants can generate a membrane potential even though they lack both a respiratory chain and the *F*₁*F*₀-ATPase. Most of the papers describing these effects were published before 1970; they are not included in today's computer bases and are rapidly being lost from our collective consciousness. Adding these early articles to electronic databases would forestall many failed experiments and be a genuine service to science.

But in Homer's Nestor, wisdom collocates with the self-complacency of old men. I must never forget that, despite a life's work on mitochondria, I know very little about these mysterious structures, and that much of what I do know may be wrong. I must encourage my students to challenge the dogmas of our field, and to remember that "experto crede" is for scholiasts, not scientists. Scientists are not made to be residents. They are travelers that never quite arrive.

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