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Historical Review

Getting mitochondria to center stage

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ABSTRACT

The question of how eukaryotic cells assemble their mitochondria was long considered to be inaccessible to biochemical investigation. This attitude changed about fifty years ago when the powerful tools of yeast genetics, electron microscopy and molecular biology were brought to bear on this problem. The rising interest in mitochondrial biogenesis thus paralleled and assisted in the birth of modern biology. This brief recollection recounts the days when research on mitochondrial biogenesis was an exotic effort limited to a small group of outsiders.

Ever since mitochondria were discovered in 1857 by the Swiss anatomist Rudolf Albrecht von Kölliker, described more detail in 1890 by the German pathologist Richard Altman, and given their present name by the German anatomist Carl Benda in 1898 [1–4], their relationship to the cell that housed them has engendered intense debate. Were mitochondria intracellular parasites [4], highly adapted endosymbionts similar to the autonomously replicating chloroplasts [5,6], or just another type of intracellular organelle like the cell membrane or the nucleus? The American biologists Ivan E. Wallin [7] and Lynn Margulies (then named Lynn Sagan; [8]) had re-emphasized various forms of the endosymbiotic theory, but when I stumbled into the world of research in the early sixties, few biochemists seemed to give this important problem much thought. At that time, the ultrastructure of mitochondria, the composition and organization of their respiratory chain and many of their key metabolic pathways were already known and most "mitochondriacs" were fiercely determined to be the first in unraveling the mysterious mechanism by which mitochondria couple the free energy of respiration to the synthesis of ATP. Biochemistry, cell biology and genetics were still very much separate worlds with little communication between them, and the revolution sparked by molecular biology and genetic engineering was still two decades off. Peter Mitchell's concept [9] of vectorial chemical reactions and their pivotal role in metabolic control and cellular energetics had not yet gained a significant foothold among biochemists, most of whom considered mitochondria as just another chapter in the general book of metabolism.

Much of this was unknown to me when I entered the final stretch of my chemistry studies at the University of Graz in postwar Austria. I had wanted to become a biochemist, but as our university did not offer any biochemistry curriculum at that time, I had decided to master this new science on my own. By today's standards, my study method was highly idiosyncratic: I collected the names and addresses of famous biochemists whose work I thought to be interesting and sent them picture postcards with the modest request "Dear Dr. X, please send me all your papers". Some kind souls did indeed send me a few of their most recent reprints, but the renowned mitochondrial researcher David E. Green at the University of Wisconsin in Madison had apparently taken my request literally: he sent me a weighty airmail package containing all of his several hundred reprints. I read them with growing fascination and was soon hooked on mitochondria. David Green's package was the magic wand that opened the door to an enchanted world that was to hold me in its spell for my entire research career. Green's generosity will always remind me of the fact that science is a supremely communal effort that thrives on the sharing of knowledge and intellectual values between generations.

After I had obtained my PhD in chemistry, I moved to the University of Vienna where the brilliant young biochemist Hans Tuppy had accepted me into his laboratory and given me free rein to pick a research topic. Inspired by the mitochondrial fires David Green had kindled within me I decided to look into the question of how cells build their mitochondria.

Most of my institute colleagues considered this question beyond the reach of biochemistry. In 1958, Simpson and his group [10] had made the seminal discovery that isolated rat liver mitochondria incorporate labeled amino acids into protein, but all efforts to identify these labeled proteins had run into a brick wall. Mitochondrial biogenesis was apparently not a problem which a biochemist could successfully put his teeth into. But I had pinned my hope on yeast and its well-characterized genetics. In France, Boris Ephrussi and Piotr P. Slonimski had described strange yeast mutants that had lost the ability to respire [11,12]. These "*petite*" mutants never reverted to respiring cells and did not pass on their

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defects according to Gregor Mendel's laws. Ephrussi and Slonimski were convinced that these mutations reflected the inactivation or loss of a non-chromosomal genetic element that controlled the formation of the respiratory system. They took it for granted that the respiratory system of yeast was housed in typical mitochondria and suspected that this might also be true of the mysterious genetic element affected by the strange *petite* mutations.

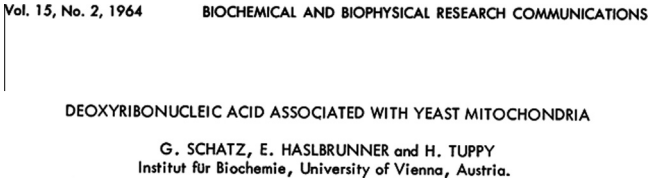
The studies by Ephrussi and Slonimski proved to be a breakthrough, yet few biochemists knew of them, and their general impact was minimal. Interest in the genetic control of mitochondrial formation was still an exotic hobby indulged in by a tiny group of believers. Had I been fully aware of this intellectual isolation as well as of my general ignorance of biochemistry, genetics, and cell biology, I would never have dared to apply to this exclusive sect. But in science, ignoring one's ignorance can pave the way to discovery. I started out by testing the idea that the genetic factor discovered by Ephrussi and Slonimski was identical with the mitochondrial structures. If so, the respiration-deficient *petite* mutants of yeast should lack mitochondria. But when I broke open the mutant cells and analyzed their subcellular particles by sucrose density gradient centrifugation and enzyme analysis, mitochondria were still present. They were, of course, respiration-deficient because they lacked several cytochromes of the respiratory chain, but they equilibrated in sucrose gradient at exactly the same density as respiring mitochondria from wild-type cells and still exhibited typical mitochondrial marker enzymes such as succinate dehydrogenase and other Krebs cycle enzymes [13]. My first working hypothesis had been wrong! It was only much later that the group of Ladislav Kováč in Bratislava [14] as well as our Basel laboratory [15] could prove that loss of mitochondria from eukaryotic cells is invariably lethal.

Could the genetic element affected in *petite* mutants be a special DNA within the mitochondria? Biochemists, histologists and electron microscopists had detected DNA in mitochondria for years, but most of them were not ready for the idea that the DNA really belonged there. This reluctance may explain why textbook accounts of mitochondrial DNA almost never tell how this DNA was discovered. In the early sixties, biochemists still embraced Christian de Duve's dogma that every macromolecule had one, and only one, intracellular location - and DNA was accepted as an unambiguous marker for the nucleus. Given this general frame of mind it is easy to understand why the presence of DNA in mitochondrial fractions was usually attributed to contamination by nuclear fragments. In 1961, at the Fifth Annual Meeting of the American Society of Cell Biology in Chicago, Hans Ris showed electron micrographs of mitochondria with inclusions resembling the DNA-containing nucleoids of bacteria and made the heretical proposal that mitochondria (and also chloroplasts) contain their own DNA. In a paper that appeared in the following year, Hans Ris and Walter S. Plaut further documented and expanded these observations [16]. Soon thereafter, biochemical and morphological evidence from several groups confirmed the presence of DNA in chloroplasts (see [17] for a review). By 1962, the ground for the concept of mitochondrial DNA was thus well prepared, but the concept itself was not generally accepted. In retrospect it seems that the scientific community was waiting for convincing studies that documented the existence of mitochondrial DNA by more quantitative approaches.

My PhD student Ellen Haslbrunner and I decided to search for DNA in yeast mitochondria by chemical measurements. But when we did this with wild-type mitochondria isolated by differential centrifugation, we found enormous amounts of DNA which, upon density gradient centrifugation, did not co-fractionate with mitochondrial marker enzymes, but smeared through the entire gradient. Most of it was apparently nuclear DNA that had escaped from the nucleus during cell breakage and was not separated from mitochondria by our usual density gradients. After many unsuccessful trials, we finally used density gradients made from the X-ray contrasting agent Urografin – and hit pay dirt. Now most of the DNA recovered in the crude mitochondrial fraction sedimented to the bottom of the centrifuge tube, whereas the sharp band of pure mitochondria equilibrating in the middle of the gradient contained only a minute amount of DNA. To our great joy, this amount was remarkably constant between different experiments and the distribution of DNA across the mitochondrial band paralleled exactly that of the mitochondrial marker enzyme cytochrome oxidase. This DNA had to be insidethe organelles because we could only digest it with DNAase after we had destroyed the mitochondrial membranes. Its concentration was between 1 and 4 μg per mg mitochondrial protein. We submitted our findings in January 1964 (Fig. 1) with the cautious title "DNA Associated with Yeast Mitochondria" to Biochemical and Biophysical Research Communications [18], the newest and "hottest" biochemical journal of the day, and soon received a flood of reprint requests, letters, and meeting invitations.

But disappointment was not far behind. Two months before our report on mitochondrial DNA appeared, we learned that Margit K. Nass and Sylvan Nass, two electron microscopists at the University of Pennsylvania in Philadelphia, had followed up on Hans Ris' observations and described thread-like inclusions within the matrix of chick embryo mitochondria [19,20]. As these threads were sensitive to DNAase, but not to RNAase or protease, the authors had correctly identified them as containing DNA. In those days, US journals took up to half a year to reach our Vienna university library and transatlantic telephone calls were very expensive. Some reviews had already cited us as the "discoverers of mitochondrial DNA" – and so the two excellent papers by the Nass team left us crestfallen. In retrospect, however, these concordant reports led to the general acceptance of mitochondrial DNA and stimulated many of our colleagues to join our efforts towards understanding mitochondrial biogenesis.

What was this mitochondrial DNA doing? It seemed to represent the non-chromosomal genetic element controlling the formation of the respiratory system in yeast because Slonimski and others showed by the relative crude methods then available that the *petite* mutation of yeast profoundly altered the buoyant density of mitochondrial DNA, suggesting massive deletions [21]. Slonimski and his colleagues could not explain, however, why all *petite* mutants exhibited exactly the same biochemical defects, even though their defective mitochondrial DNA molecules differed dramatically between different *petite* strains. As will be mentioned below, the explanation of this puzzle came from measuring mitochondrial protein synthesis in intact yeast cells. And there was another surprising fact about this mitochondrial DNA: it could not possibly encode all of the mitochondrial proteins. This conclusion was particularly compelling for mammalian mitochondria which, after purification in Urografin density gradients, contained almost ten times less DNA than yeast mitochondria, i. e. between 0.2 and 0.6 μg DNA per mg protein [22]. We calculated that a typical mammalian mitochondrion contained 3×10^{-17} g DNA. Assuming that the DNA was double-stranded, it could encode at most 1.2 MDa of polypeptide chains. Today, our early calculations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DEOXYRIBONUCLEIC ACID ASSOCIATED WITH YEAST MITOCHONDRIA

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Fig. 1. Caption of our report on the presence of DNA in mitochondria.

could be challenged on several grounds, yet they came remarkably close to the values obtained two decades later by sequencing mitochondrial DNA. Clearly, most mitochondrial proteins were encoded by nuclear genes, synthesized on cytosolic ribosomes, and then imported into mitochondria. In order to identify the proteins made inside the mitochondria (presumably under the direction of mitochondrial DNA), we avoided working with isolated mitochondria because this approach had led Simpson and his excellent team down the garden path. As an alternative, we devised a simple method for labeling mitochondrially-made proteins in living yeast cells [23]. We incubated the cells with glucose as an energy source, inhibited their cytosolic ribosomes with the antibiotic cycloheximide, added radioactive leucine and, after 10–60 min, isolated the mitochondria. We then resolved the mitochondrial proteins by SDS–polyacrylamide gel electrophoresis, which had just then made its triumphant entry into the biochemical arena. When we cut up the gel tubes into thin slices and counted each of these in a scintillation counter, we found about half a dozen radioactivity peaks, none of which coincided with a major stained protein band. The mitochondrial protein products were clearly not major mitochondrial proteins.

Our new *in vivo* labeling method showed clearly that all *petite* mutants of yeast lacked mitochondrial protein synthesis, regardless of the alterations in their mitochondrial DNA. The group of Ladislav Kováč had come to the same conclusion by examining the incorporation of labeled amino acids into isolated yeast mitochondria [24]. The *petite* mutation thus prevented the expression of all the protein genes that were still left on the defective mitochondrial DNA molecules. As was shown later by several different groups, yeast mitochondrial DNA carries the genes for the mitochondrial tRNAs and ribosomal RNAs widely scattered over its entire length, so that any significant DNA deletion or rearrangement is bound to inactivate the synthesis of proteins within mitochondria.

But which proteins did mitochondria make? It took us two years to solve at least part of the riddle: three of the labeled protein bands were identical with the three largest and highly hydrophobic subunits of cytochrome oxidase. The smaller subunits of this oligomeric enzyme were made on cytosolic ribosomes, imported into the mitochondria, and then assembled there into the functional holo-enzyme [25–27]. Alexander Tzagoloff and his group in New York had obtained analogous results for the oligomeric ATP synthase of yeast mitochondria [28]. Now we finally understood why Simpson and his colleagues had been unsuccessful in identifying the proteins labeled by isolated mitochondria: mitochondria from yeast and mammals do not synthesize complete enzymes, but only hydrophobic subunits of oligomeric enzyme complexes.

From then on, things were starting to happen fast. Tzagoloff and his team isolated yeast mutants with mutations in individual mitochondrial genes, opening the way to a detailed map of yeast mitochondrial DNA [29,30]; the map was circular, just like that of a bacterium! It did not tell us whether the mapped genes were regulatory or structural genes for mitochondrially-made proteins, but together with Slonimski's laboratory we could prove that a mitochondrial gene affecting cytochrome oxidase activity was, in fact, the structural gene of the second largest, mitochondrially-made cytochrome oxidase subunit [31]).

In the late seventies recombinant DNA methods entered the scene and all of us were convinced 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 "mitospeak" that we did not understand. We had taught our students that TGA was a "nonsense" codon that signaled the end of a polypeptide chain, but when mitochondria said "TGA", they meant tryptophan [32]. Even worse, mitochondria from different organisms spoke in different vernaculars. Comparing gene sequences with the amino acid sequences of the protein products finally cracked the code. After Fred Sanger and his colleagues published the complete sequence of human mitochondrial DNA [33] and Giuseppe Attardi's group matched this feat with the complete transcription map of mitochondrial DNA [34], the battle was largely over. Today we know that human mitochondrial DNA encodes 13 very hydrophobic proteins, all of them essential subunits of oligomeric enzymes mediating oxidative phosphorylation [35].

Once mitochondria had become a playground for recombinant DNA methods, their role in disease, in aging and in shaping the history of human evolution started to attract the attention of an ever widening community of biologists of all stripes [36]. Gone were the times when I had to start my seminars by explaining what mitochondria were, what they looked like, and what they did. Gone, too, was the habit of congress organizers to schedule sessions on mitochondrial biogenesis for the last day of the meeting when most congress participants were already preoccupied with catching their flight back home. And when, in the 1970s, Walter Neupert's group in Munich and our group in Basel started to unravel the immensely complex pathways by which mitochondria import proteins from the cytoplasm [37,38], mitochondria became part of the general effort to understand how proteins move within cells or from the cell's interiorinto the extracellular space. Most of the papers my collaborators and I published during my active research career and most of the prizes and honors that came my way cited my work on mitochondrial protein import – and yet I feel a special nostalgia for those early years when a small group of us, well outside the general limelight, tried to pry open the door to the mysteries of mitochondrial biogenesis. It felt good to push mitochondria to center stage, but I also cherish the time in the wings.

Acknowledgments

This article is dedicated to Ernesto Carafoli on the occasion of his 80 birthday. It draws on three earlier recollections providing more detailed accounts [35,39,40].

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