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Pivotal Ideas

From Membrane Structure to Bacteriorhodopsin

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Jeder Mensch erfindet sich früher oder später
eine Geschichte, die er für sein Leben hält.
Max Frisch
(Frisch, 1964)

Hamburg, 1946

After escaping from a Russian prisoner of war camp in Poland early in 1946 at age 25, I began to study medicine at the University of Hamburg. Instruction in the first two years consisted mainly of lectures or following cookbook recipes in the few laboratory courses. Histology was the only course in which one had the opportunity to do a bit of exploring on one's own, browsing through a large collection of slides available in the Institute of Anatomy. I enjoyed it, and was soon asked by the professor, if I might be interested in undertaking a research project on the differentiation of islet cells in the fetal pancreas. I accepted the offer and began practicing classical histology, somewhat to the detriment of my further medical education. I was fascinated by the functionally, largely unexplained structural details in cells that could be observed with the light microscope, and also frustrated by the resolution limit of the microscope and the problem of distinguishing artifacts introduced by the techniques from "true" structures.

For my doctoral thesis, prepared, as customary, during the last two years of the medical curriculum, I wanted to change fields and asked a professor in the Department of Pharmacology for a topic. He was glad to encounter a student with a background and interest in morphology, and I soon found myself back in Anatomy attempting to detect effects of local anesthetics on myelinated nerve fibers using in vivo fluorescence microscopy and a recently acquired phase contrast microscope. These techniques reduced to some extent the concern about fixation and staining artifacts, but emphasized the problem of optical artifacts. For instance, when the question arose whether or not the axon of the myelinated nerve fibers was surrounded by its own membrane, I found that the problem could not be resolved by morphological techniques at that time. Experimental work was hampered by lack of funds and the availability of high intensity light sources and high sensitivity film to record images. I sought refuge in the literature trying to find out as much as I could about the nerve axon and membranes in general.

Fortunately, the library of the Institute of Anatomy had survived the war intact, was well equipped, and microfilm copies of the missing recent literature were available without charge from the U.S. Library of Congress. Thus I became familiar with the relatively recent work of W.J. Schmidt, and F.O. Schmitt on the molecular structure of myelin, and its implication for the structure of cell membranes. Going back to earlier studies, I learned about Overton's correlation of lipid solubility with permeability and R. Höber's impedance measurements on erythrocytes, which led to the conclusion that a thin lipid layer covers the cell surface (Höber, 1910, 1912). These papers, together with the measurements on surface films by Gorter and Grendel (1925) and the Davson/Danielli work with model systems (Danielli & Davson, 1935), greatly impressed me because they showed a way to obtain information about the molecular structure of cell components while minimizing the artifact problem. They also convinced me that the bimolecular lipid layer with adsorbed protein was the best available model for the molecular structure of the cell membrane, especially since the same struc-

ture could be independently derived from the work on myelin. This conclusion was soon to be vindicated by Betty Geren's electron micrographs, which demonstrated that the myelin sheath was derived directly from the Schwann cell membrane (Geren, 1954), and by David Robertson's extension of this work to his general unit membrane model (Robertson, 1959). What I learned in pharmacology about the just developing concept of cell surface receptors further emphasized the importance of membranes, and I was hooked. The main problem now seemed to be to explain the function of membranes, e.g., excitation and impulse conduction in nerves or selective ion permeability in erythrocytes on the basis of molecular structure.

After completing my thesis and passing the final examination, I continued my reading and some pharmacology research while doing clinical work as a "Pflichtassistent" (the equivalent of an internship in the U.S.), but now it was time to find a job. This was difficult in 1952, because I wanted to continue doing research and was not impressed by the research I had encountered in the clinical departments. That left either a Max-Planck Institute or a "theoretical" department, as disciplines not directly dealing with patients (but conducting experiments!) were called. There, positions were scarce, indeed. I therefore jumped at the opportunity when my thesis advisor told me about an opening for a postdoctoral fellow to do electron microscopy at the "Tropeninstitut" in Hamburg, even though the head of the Anatomy Department, like many other biologists at that time, dismissed electron microscopy as nothing but the most expensive way to produce artifacts. The position was funded by the Deutsche Forschungsgemeinschaft and carried the princely salary of DM 160 a month. On the one hand, this was an improvement over the DM 136 one was paid as a Pflichtassistent, but on the other, it lacked the friendly nurses, who were usually happy to feed a hungry Pflichtassistent.

The opening was in the Virology Laboratory of the Institute, headed by Dietrich Peters, a chemist who had obtained his degree working with Adolf Butenandt. The laboratory had recently acquired an electron microscope and an air-driven analytical ultracentrifuge, which had actually been ordered in 1938. So, I spent the next two years investigating the structure of the vaccinia virus and trying to establish the chemical nature of its morphological components by selective digestion with nucleases and proteases. These were exciting times in virology and electron microscopy, when progress was rapid as basic concepts and techniques were developed. However, both microscope and centrifuge kept breaking down frequently and we spent as much time repairing them as working with them. That we succeeded at all was mainly due to the efforts of two talented technicians, R. Geister and W. Giese, both chemistry students, who in the difficult postwar years had been unable to finish their studies. Thanks mainly to them, in those two years I learned more new biology, experimental skills, chemistry, and physics than during any other two years of my career. My interest in membranes, in the meanwhile, had to be satisfied by following the literature.

I also married, and our first son was born. It was time to look for a job which could sustain a family. I had the choice of an Assistant Professorship in the Departments of Gynecology or Pathology at the University and picked the latter because it offered me complete freedom in the selection of my research topics. Unfortunately, the University had no electron microscope, and most of my time was occupied with autopsies, biopsies and teaching pathology to not very interested dental students. I found time for some histopathology research and became interested in the pathology of the lymphatic system and the antibody response, which I studied with standard light microscopy techniques. However, West Germany was rapidly recovering economically, some money for research at the University became available, and in 1956, the Department was able to acquire a Siemens electron microscope, which was a vast improvement over the electrostatic AEG/Zeiss instrument I had used before.

At Siemens, Ernst Ruska was still in charge of electron microscope development and production, and his latest model, the Elmiskop I, set a new standard for electron microscope performance. Equally important, he had established an applications laboratory at the factory in Berlin, where new users typically received intensive training for three days before the instrument was delivered. The laboratory was run by Cilly Weichan in a very informal and efficient way. One had access to the physicists and engineers, could learn the latest tricks, and not infrequently obtain newly developed parts to try out. This was complemented by a staff of knowledgeable and interested service people, who would usually appear on short notice and not mind working long hours. It thus became possible to routinely produce electron micrographs, which attracted attention simply through their high quality. Similarly improved were the preparation techniques, mainly through the introduction of the Porter-Blum microtome, glass and diamond knives, OsO_4 as fixative, and methacrylates as embedding media. Obtaining well-preserved thin tissue sections still required some skill and luck, but almost any section reasonably free of compression and knife marks revealed a wealth of new and interesting structures. At the time, the existence of a cell membrane had not been fully accepted by all biologists, but the electron micrographs clearly showed identical thin dark lines around all cells and unexpectedly also around most cell organelles. These could sometimes be resolved into triple-layered structures and were taken to be images of membranes by most electron microscopists. These observations, which seemed to vastly expand the role of membranes, naturally intrigued me, but I did not immediately see an

approach to test the interpretation. Cell fractionation techniques were still in their infancy; erythrocyte ghosts were the only membrane preparations accessible and their functional properties were just beginning to be explored. However, with the newly acquired electron microscope I could now continue my work on the lymphatic system at the ultrastructural level. In thin sections of spleens from rabbits that had survived a severe secondary immune reaction, I found multilayered concentric structures with a regular spacing of ~4.0 nm in macrophages, which—prepared by my earlier work on the myelin sheath—I guessed to be myelin figures. To prove it, I isolated a phospholipid fraction from brain and prepared thin sections of authentic myelin figures which, indeed, did show the same structures. Since the myelin figures were known to consist of concentric bimolecular lipid layers, and contrast was due to the deposition of osmium in the sections, I based the interpretation of the micrographs on the known reaction of osmium tetroxide with the double bonds of unsaturated fatty acids. An obvious next step was to add protein and thus prepare a physical model of the Davson-Danielli membrane. Its appearance in electron micrographs of thin sections closely resembled the triple-layered structures seen on the surface of cells and organelles prepared the same way and thus constituted a strong argument in favor of their interpretation as cell membranes with the Davson-Danielli structure.

Obviously, this work had taken me rather far afield from classical pathology, but I wanted to continue it. My interest in pathology as practiced in most German university departments at that time had also greatly diminished. I sensed that most of the exciting progress in understanding of pathological processes had shifted to other disciplines. I also wanted more time for my research than the routine work left me. Predictably, the department head did not share my point of view and our formerly good relationship deteriorated to the point where my position in the department became untenable and he refused to let me complete my "Habilitation," which was practically finished. The conflict was finally resolved with the compromise that I would obtain my "Dozent" degree and then leave.

Shortly before this happened I had met Prof. Frank Hartmann of glass knife fame at a local histochemistry symposium (Latta & Hartmann, 1950). Professor Hartmann had come from the Department of Anatomy at the University of Minnesota to explore his ancestry, while spending a sabbatical year at the University of Kiel. I had invited him to give a seminar in Hamburg, and when he arrived he could not fail to notice the strain in my relation with the department head. After I had explained the situation to him, he asked me whether I would consider coming to the States, and generously offered to recommend me to any group there. At the time, the laboratory of Keith Porter and George Palade at The Rockefeller Institute was the Mecca of all biological electron microscopists, and unabashedly I named it as my first choice.

A few weeks later a letter from Keith Porter arrived offering me a one year postdoctoral position to continue research on the ultrastructure of the liver, which an Italian postdoctoral fellow due to return home was pursuing in his laboratory. Professor Hartmann had also advised me to apply for a Fulbright fellowship, which was granted, and after acquiring the necessary tuxedo I embarked on the SS America for New York on December 28, 1958.

New York, 1959

In the Summer of 1958, I had presented my work on the myelin figures, membrane model, and the chemistry of OsO_4 fixation at the Third International Conference on Electron Microscopy in Berlin, where, except for some nasty comments by Frank Schmitt, it was well received. I had also briefly met Keith Porter there, but we had had little time to talk, and my presentation had been in German. Upon my arrival at Rockefeller, he asked me to show him again what I had done, and after listening to my explanations in not exactly fluent English, he concluded our conversation with the remark: "You go on with what you have been doing, I'll find somebody else for the liver." A few weeks later he also offered me an Assistant Professorship and I stayed at what was just becoming Rockefeller University for the next eight years.

For someone arriving from the backwater, what Hamburg and its University had become after the war, New York and Rockefeller were overwhelming. The laboratory still located in the second basement of Smith Hall was crowded, and I could not do much until Keith had found a niche for me. So I had some time to venture forth from the plush surroundings of Caspary Hall and explore Manhattan, mostly on foot, but soon I settled in and work took over.

We were then at the beginning of a new era in cell biology. The combination of electron microscopy with biochemistry and cell fractionation was opening a new realm, bridging the gap between light microscopy and the macromolecular dimension. Rockefeller Institute and especially the Porter/Palade laboratory were at the center of these developments. A steady stream of visitors was passing through and the famous dining room was still functioning. In the laboratory much work was done at night, the microscopes were often signed up until three o'clock in the morning, and one might encounter George Palade or Keith Porter in the laboratory well after midnight.

My first two years at Rockefeller were mainly occupied with expanding, refining and correcting the bilayer model, which in its first version had interpreted the contrast distribution in the image incorrectly. I also applied the technique to other lipid structures, and took up

x-ray diffraction work to show that the liquid crystalline structure of the myelin figures and other lipid phases survived the necessary fixation, dehydration and embedding procedures essentially intact. However, it became increasingly obvious that to account for the function of biological membranes an expansion or revision of the Davson-Danielli-model was required, and that new data on natural membranes had to be obtained.

I selected the erythrocyte ghost as the best-known, clean membrane preparation available, and the mitochondrial inner membrane as an example of a highly specialized membrane with one dominant function. I expected that the basic building principles would be the same. The composition of the erythrocyte membrane was well known and its permeability characterized, but transport sites were widely dispersed over the surface and could not be detected in the microscope or isolated, at least not in functional form. The inner mitochondrial membrane had been subfractionated and functional complexes of the respiratory chain had been isolated. Working with it, resulted in frequent contacts and some collaboration with Ef Racker's and David Green's groups, and I necessarily became involved in the just beginning battle between the proponents of the chemical and chemiosmotic hypotheses of oxidative phosphorylation. I did not materially contribute to the controversy until much later, but began to favor the chemiosmotic mechanism rather early, partly because I found that functional reconstitution of oxydative phosphorylation was apparently only possible in vesicles, and probably also because I was not biased by an intensive education in biochemistry and have an inborn preference for physics over chemistry. The work led to a modification of the general membrane model. With Becca and Sidney Fleischer, I could show that totally lipid-depleted mitochondria maintain the unit membrane structure of their cristae, which led me to the conclusion that at least some of the proteins in the inner mitochondrial membrane must span the bilayer (Fleischer, Fleischer & Stoeckenius, 1967). This was a significant deviation from the classical Davson-Danielli model, which confined protein to the surfaces, but it retained its central feature, the lipid bilayer, which at that time was not a generally accepted feature, at least not by biochemists.

In the early sixties a general dissatisfaction with the Davson-Danielli model spread, prompted by its inability to explain specific membrane functions, and new models were proposed which discarded the lipid bilayer. They were mainly variations of a basic "subunit-model," which depicted membranes as planar arrays of lipoprotein particles. The situation was not helped by the transient appearance of an ill-defined "structural protein" postulated to be present in all membranes. Neither the arguments nor the sparse experimental support for these models looked convincing to me. I did not see how these structures could provide the necessary permeability barrier, and continued to favor the bilayer. However, in 1963, A.D. Brown published a paper on halobacteria, prokaryotes which are found in saturated brines. He reported that *Halobacterium halobium* does not possess the typical bacterial cell wall, and that its cell membrane disintegrates into a uniform population of macromolecular subunits when the salt concentration in the medium is reduced (Brown, 1963). The data he presented seemed to back up the conclusions, and thus supported a subunit structure for this membrane. I decided to look at it myself. Fortunately, shortly thereafter Bob Rowen, a microbiologist from Albert Einstein College of Medicine, came to spend a sabbatical year with me and agreed to start the project. We soon found that the standard preparation techniques for electron microscopy gave poor results with halobacteria, but slight modifications would preserve the structure well. The micrographs then clearly showed a cell wall, and we could identify Brown's macromolecular subunit fraction as a component of the wall, not the membrane. However, the cell membrane did disintegrate in low salt, but into fragments of different size and composition. One of the fractions had a striking, deep purple color, whereas the others were orange-colored, and an apparently membranous, uncolored fraction was also present (Stoeckenius & Rowen, 1967). When I took an x-ray diffraction pattern of a concentrated purple fraction to check for bilayer structure, I also found to my surprise a series of sharp reflections indicating the presence of some well-ordered crystalline lattice, which like the purple color, at that time we could not attribute with certainty to one of the several membranous components present in this fraction.

These results restored my temporarily shaken faith in universal applicability of the bilayer model, but since the Brown paper had gone largely unnoticed by membrane biologists, they contributed nothing to the general acceptance of the bilayer model. That would come only several years later with Don Engelman's demonstration of a typical bilayer phase transition in the mycoplasma cell membrane (Engelman, 1970, 1971). Differential scanning calorimetry, introduced shortly before, also showed the bilayer phase transition but did not have quite the same impact.

I continued work on the halobacterium membrane, even though the evidence for a subunit structure had obviously been spurious, because this membrane seemed to offer a unique opportunity for resolution and eventual reconstitution of a multifunctional membrane under very mild conditions, at a time when the art of solubilizing functional membrane components with detergents was still in its infancy. Influenced by the important results obtained with the recently introduced "black lipid films," I had begun to develop a concept of membrane organization, which was eventually published in a review article with Don Engelman (Stoeckenius & Engelman, 1969). It postulated that the lipid

bilayer forms the structural backbone for membranes and provides the necessary basic permeability barrier. Bound to and inserted into it are other components, mainly proteins, which contribute the selective transport functions, energy-transducing systems and receptors. Influenced by my mitochondria work, I thought that some of these functional components would form complexes and be segregated in domains, and I hoped to directly demonstrate such an organization in the halobacterium cell membrane. The concept proved to be quite useful and led later also to isolation of the gap junctions (Goodenough & Stoeckenius, 1972). Another reason for continuing to work with halobacteria was the unprecedented observation of membrane sheets in the cytoplasm, which in sections showed the typical triple-layered unit membrane structure and a regularly striated surface when negatively stained. They also constituted the main contaminant of the purple fraction.

These membrane fractions were first purified and characterized by Wolf Kunau, who had come as a postdoctoral fellow from Klenk's department at the University of Cologne. He separated the orange and the intracytoplasmic membranes cleanly from the deeply purple-colored membrane fragments, which we simply called the purple membrane. (This later proved to be a somewhat unfortunate choice, because it led to a confusion of halobacteria with purple bacteria.) We got some surprises. Whereas the orange fraction appeared fairly conventional, the purple membrane contained only 25% lipid, ant the intracytoplasmic membranes looked like typical unit membranes in sections, but contained no lipid, only protein. We finally identified these as collapsed gas vacuoles with a very intriguing structure and properties that still merit further investigation (Stoeckenius & Kunau, 1968).

San Francisco, 1967

At this point, the work was interrupted by my move to San Francisco early in 1967. Julius Comroe from the Cardiovascular Research Institute of The University of California Medical School had made a very generous offer, which in addition to a large, well-equipped laboratory included $150,000/year for seven years to be used entirely at my discretion. This, together with the attractions of California, proved irresistible. In retrospect, it may also have been fortunate for the purple membrane work because later it probably would have been difficult for an investigator with no proven competence in the field to obtain grants for studying rhodopsin in a bacterium and looking for photosynthesis in the absence of chlorophyll, without substantial evidence already in hand. However, the move also caused a temporary disruption of the work with halobacterium membranes.

The cell membrane fractionation project was finally resumed in 1969 with Allen Blaurock and Dieter Oesterhelt. Allen had obtained his Ph.D. with Roy Worthington at Ann Arbor analyzing the structure of nerve myelin by x-ray diffraction. Before coming to San Francisco, he had spent two years in Maurice Wilkins' laboratory at King's College, London, with structural studies of membranes and the rod cell outer segments of the retina. Dieter was a biochemist from Munich, who had obtained his Ph.D. in chemistry with F. Lynen. Both Allen and Dieter were intrigued by the purple membrane, but also worked on other projects; Dieter looked for electron transport chain components in the other cell membrane fractions, trying to find a respiratory chain domain; Allen analyzed diffraction patterns from the cell wall and gas vacuole membranes, which also had highly ordered structures.

Allen obtained a diffraction pattern of the clean purple membrane fraction, which was essentially the same as I had seen at Rockefeller. He then prepared an oriented sample and immediately realized that the sharp reflection pattern must be due to a two-dimensional hexagonal lattice in the plane of the membrane sheets, presumably formed by the protein, and that this pattern held the promise of obtaining the structure of a membrane protein at high resolution, which was to be beautifully realized later by Nigel Unwin and Richard Henderson (Unwin & Henderson, 1975; Henderson et al., 1990). Allen went on to show that a substantial amount of protein must be present at the center of the membrane. The lattice was also clearly visible in freeze-fracture electron micrographs and allowed us to locate the purple membrane sheets as patches in the cell membrane. Allen published a detailed account of this discovery, which reflected some of the excitement we felt (Blaurock, 1982). The model structure we eventually published made the purple membrane protein the paradigm of an integral membrane protein (Blaurock & Stoeckenius, 1971).

Dieter, infected by Allen's enthusiasm, now concentrated on the purple membrane, but initially experienced difficulties with the analysis. Whatever extraction or solubilization procedure he tried either did not touch the membrane or bleached the color to a pale yellow. However, Allen knew that CTAB had been successfully used to solubilize rhodopsin, and when Dieter tried it he could soon show that the color was due to a chromoprotein of ~24 kD, which was virtually the only protein present.

We would presumably have identified the pigment eventually anyway, but probably not before Dieter would have had to return to Munich and only after I would have found another similarly competent chemist to continue the project. A lucky guess saved much time. It was prompted, I believe, by two incidents. At the 1969 Biophysical Society Meeting in Baltimore, Max Delbrück in his Plenary Lecture on the photoresponses of Phycomyces showed the absorption spectrum of a flavoprotein, which he believed to be the light re-

ceptor. It closely resembled the spectrum of a cell membrane fraction from our halobacteria. Since I did not know the dogma, which held that only photosynthetic bacteria are phototactic, it occurred to me to look for phototaxis in *H. halobium.* It was, of course, a wild idea, but since it was an easy experiment I tried it upon my return and found that I could reverse the swimming direction of the cells by removing the green filter which we customarily used in our phase contrast microscope. Everybody in the laboratory was rather surprised by this effect, but we did not immediately see a connection with our membrane work. At that time, Allen had also been talking extensively with Dieter about his earlier work on rhodopsin in rod outer segments. It probably was the coincidence of these conversations with the observation of phototaxis and the similar effect of CTAB that prompted Dieter to suggest shortly afterwards that the purple pigment might be a rhodopsin-like protein but, as far as I recall, without referring to the phototaxis or the CTAB-solubilization. I was rather skeptical and pointed out that rhodopsin only occurs in animals and also rapidly bleaches in the light, but Dieter tested his wild idea anyway, and established conclusively in a very short time that he could extract retinal from the purple membrane and that its main component was, indeed, a rhodopsin-like protein, which we named bacteriorhodopsin (Oesterhelt & Stoeckenius, 1971). Since rhodopsin was one of the most extensively investigated proteins, he could easily adapt established techniques for the characterization of bR.

Dieter returned to Munich in the fall of 1970 and there rapidly developed the necessary techniques for handling the protein, e.g., chromophore reduction, hydrolysis, and exchange, determination of the isomers, effects of pH and salt etc. I was more inclined to use physical techniques, especially spectroscopy, and since my laboratory was not well equipped for this work, I traveled for exploratory experiments to other laboratories. Early low temperature spectra of photoproducts were obtained at U.C. San Diego in Warren Butler's laboratory with his student Rich Lozier, who later joined me in San Francisco and became a long-time collaborator. Time-resolved spectra, measured with Richard Cone at Johns Hopkins, clearly showed that the protein in the membrane was immobilized and underwent a fast cyclic photoreaction. The first evidence for it, however, came from another chance observation.

Whereas most components of halobacteria require molar salt concentrations to maintain their structure and function, the purple membrane showed no obvious change in properties when suspended in distilled water. Nevertheless, whenever feasible I ran duplicate experiments at low and high ionic strength, mostly because I wanted to check whether the same reactions could be carried out in intact cells. In early chromophore extraction attempts, Dieter had observed that addition of ether to a membrane suspension in water bleached the purple color. When I returned to this experiment and repeated it in 4 M NaCl, the membrane also bleached because the absorption maximum shifted to 410 nm, but the color recovered within a few seconds in the dark. Shortly afterwards, I saw apparently the same photoproduct in the low temperature and time-resolved spectra. Dieter and I were still collaborating closely and regularly exchanged results. When he learned about the ether experiment, he reasoned that such a dramatic effect indicated a conformational change in the protein, which would have to manifest itself also in other observable parameters. Dieter explored this phenomenon further with Benno Hess, who had already become interested in bacteriorhodopsin and had suggested a collaboration. They found a transient fluorescence change and proton release, which matched the kinetics of the absorbance changes, and determined the action spectrum, which matched the bR absorption spectrum. They also measured a quantum efficiency for the proton release, which was closer to the now generally accepted value ~0.67 than most other results obtained during the following 15 years (Oesterhelt & Hess, 1973). With the discovery of phototaxis in our cells and the subsequent identification of a rhodopsin-like protein, its function as the required sensory photoreceptor seemed to be obvious and was apparently confirmed by a very crude action spectrum, which Allen had readily obtained since our light microscope happened to be equipped with a continuously variable interference filter. However, I was not completely happy with this conclusion because the cells contained rather large amounts of the pigment, which could occupy more than half of their total membrane area. Despite some nagging doubts that the large amounts, which began to accumulate late in log-phase, might simply be a storage form of the pigment, I began to seriously consider the possibility that it might also function as a light energy converter and began to look for changes of the ATP-levels in cells under illumination. After some early, equivocal results obtained with Eva Kirsten, Arlette Danon, who came from the Weizmann Institute to San Francisco for one year in 1971, pursued this inquiry. Neither of us had experience with this kind of experiments, and it took some time to establish the assay. We then clearly observed effects, but were not able to conclusively demonstrate light-driven ATP-synthesis before Arlette had to return to Rehovot, where she would continue the work.

A second approach to the problem arose from my interest and belief in chemiosmosis, for which the evidence had been increasing with Tom Thompson's demonstration that the classical uncoupler dinitrophenol increases the proton permeability of black lipid films (Bielawsky, Thompson & Lehninger, 1966) and, in my opinion, became irrefutable with the Jagendorf experiment (Jagendorf & Uribe, 1966). Therefore, I also measured effects of light on pH and respiration of halobac-

teria cell suspensions. Again, I had no experience with the techniques and the results were poor. I saw respiration inhibition fairly consistently but the pH often changed rather wildly and irreproducibly. It never occurred to me to measure pH and O_2 concentrations separately, and it took a long time before I learned that current leakage from the oxygen electrode was the main cause of the trouble. Only in 1972, when Roberto Bogomolni joined us in a new laboratory, which I established in the NASA facility at Moffet Field, was that problem satisfactorily solved thanks to the combination of Roberto's exceptional experimental skills, his training as a graduate student in the Berkeley Biodynamics Laboratory, and the NASA workshop facilities.

A short note on my work style may be in order here. The training in physics, chemistry, biochemistry, and physiology I received as a medical student was woefully inadequate for a career in research. It was largely restricted to lectures, which outlined the present state of the field or, in physics and chemistry, not even that. Except for electron microscopy, where I had started when the field was developing, I essentially worked as an amateur, acquiring some basic knowledge in the process. I had realized that shortcoming very soon, but having already lost six years due to the war, I felt that I could ill afford the time necessary to remedy the situation. Also, as long as I was mainly using electron microscopy my lack of training did not appear that serious; I was obviously doing quite well. The situation changed when my research began to require other tools and basic knowledge. Not only my insufficient training but also a lack of the appropriate equipment impeded progress. To compensate for these shortcomings, I traveled for preliminary experiments to laboratories with the necessary expertise and equipment, and at the same time looked for postdoctoral research fellows with suitable training, who could do the work and simultaneously educate me. This approach necessarily involved some misappropriation of grant money obtained for other projects, but it worked. Fortunately, NIH rules were more liberal 20 years ago and less strictly enforced.

While I was struggling with O_2 and pH electrodes, Dieter, who had not complicated his life by trying to measure both parameters simultaneously, had obtained very clean data showing proton ejection from illuminated cells and the collapse of the resulting proton gradient by added protonophores. Together with my data on the respiration inhibition by light and a difference spectrum demonstrating the light-induced transient bleaching of bR in intact cells, we felt that we had enough evidence to postulate that bR functions as a light-driven proton pump, and it was time to publish our results (Oesterhelt & Stoeckenius, 1973).

This obviously was an important discovery and the presentation would require care and extensive discussions. Consequently, I traveled to Munich to do a few additional experiments and to write a first draft of the paper. It was the Summer of 1972 and the time of the Olympic Games. Fortunately, a friend of mine, who lived in a beautiful apartment overlooking the park along the Isar river, had fled the Olympic crowds, and offered me his apartment for my stay. He had thoughtfully also left the key to his wine cellar. The weather was pleasant, the crowds were not in evidence near the University or the apartment. The work went well, and we spent long days in the laboratory writing, conducting some experiments, and discussing the new field that was opening up before us. Late in the evenings, when I was too tired to do much else, I would watch a replay of the day's games on TV with a glass of good wine. I have never written a paper under more pleasant circumstances.

Our results unequivocally demonstrated that light energy absorbed by bR generated the electrochemical proton gradient across the cell membrane. Furthermore, the transient release of protons from the isolated purple membrane during the photoreaction strongly suggested that bacteriorhodopsin was solely responsible for the effect. However, one could not rigorously exclude that additional components were also required because the isolated purple membrane consists of membrane sheets, not vesicles, so that the vectorial character of the proton release and uptake could not be proved with purified membranes. If functional bR could be incorporated with preferential orientation into lipid vesicles, the remaining doubts could be removed. Efraim Racker had accomplished that feat with mitochondrial inner membrane components, and was the acknowledged expert in the field. I knew Ef well, and we had collaborated before when we both were still in New York. I called him in Ithaca and arranged for a visit with seminar and a demonstration of his technique. I took a purple membrane preparation with me and we obtained at the first try vesicles that generated a proton gradient upon illumination. This result eliminated the last doubt that bR is indeed a light-driven proton pump. Ef has told the story of that visit before, and his account largely agrees with my recollection.

After I had left, Ef continued the work and accomplished the additional incorporation of mitochondrial ATP-ase into the bR-vesicles, which proved to be a far more difficult task. The demonstration of photophosphorylation in this preparation was a beautiful validation of Mitchell's chemiosmotic theory (Racker & Stoeckenius, 1974). That it is also regarded by many as the decisive factor in the general acceptance of chemiosmotic energy transduction, as I have often been told, surprised me. I considered the then existing evidence to be quite convincing already.

Arlette Danon had returned to the Weizmann Institute in September of 1972 and initially had difficulties in finding a place and support for her work. To look for photophosphorylation in an organism which lacked chlorophyll apparently did not strike her superiors as a

very promising project. She finally managed to set up her experiments and get new data, which were similar to what we had obtained in San Francisco. Only after she realized that the cells needed a long recovery period after harvesting, and began to work under anaerobic conditions in simple salt solutions, did the results become reproducible and improve dramatically. I got a letter with a very excited and exciting description of the new data and decided to fly to Israel with some additional pieces of equipment. There I found a very happy Arlette working in a utility room in the second basement amid a maze of pipes. During the next two weeks we did additional experiments, managed to obtain a crude action spectrum, and wrote the first draft of a paper describing our results (Danon & Stoeckenius, 1974). It was delayed by skeptical referees, but when it finally appeared in 1974 it succeeded in lifting Arlette from the second basement to the first floor, a change she accepted with mixed feelings. The first questions about the chemistry and function of the purple membrane fraction were thus answered, and we began to investigate its role in the energy metabolism of the cells to an extent which sufficed to show that halobacterium is the chemiosmotic organism *par excellence,* because the high salt concentrations in its environment allow it to store much larger amounts of energy in ion gradients than is possible for cells and organisms living at more conventional ionic strength. When energy-deficient cells are exposed to light only a small fraction of the energy is used for ATP-synthesis, most of it builds up the ion gradients—a fact still not appreciated by most investigators working with purple membrane. However, the opportunity to isolate a light-driven ion pump easily in gram quantities was too tempting, and we soon concentrated our efforts on exploring its mechanism beginning with the photoreaction cycle. Work was begun by Rich Lozier and Roberto Bogomolni who assembled a flash spectrophotometer in the Astrophysics Department at Moffet Field, where a suitable laser was available. The main intermediates and their kinetics were soon identified (Lozier, Bogomolni & Stoeckenius, 1975); Rich continued to analyze the cycle for more than fifteen years, first with Werner Niederberger, later with Luis Parodi and crucial help from John Nagle, who spent a sabbatical year in our laboratory in 1980 (Nagle, Parodi & Lozier, 1982).

Even though after 20 years of work and exciting new findings the cycle is still not satisfactorily understood, the results of our first analysis and data from a few other laboratories have proved to be essential for all efforts to understand the molecular mechanism of proton transport. Later additions and refinements did add comparatively little until the amino acid sequence had been determined and extensive efforts to obtain site-specific mutants and use time-resolved Resonance Raman (RR) and Fourier transform infrared (FTIR) spectroscopy began to bear fruit and could be combined.

Roberto suggested in 1973 that we should try to obtain RR spectra of bacteriorhodopsin and its photocycle intermediates. He traveled to Ithaca and initiated a collaboration with Aaron Lewis from Cornell's Department of Applied Physics. We could soon show that the Schiff's base, which links the retinal to the protein, is protonated and transiently deprotonates during the photocycle (Lewis et al., 1974). This approach was to be developed by others, mainly Rich Mathies at Berkeley and his collaborators, into one of the most important tools for exploring the photocycle. We have enjoyed a long and fruitful collaboration with Rich. With Koji Nakanishi's group at Columbia University, we obtained slightly later the first evidence that the retinal undergoes an all-*trans* to 13-*cis* isomerization during the photocycle (Pettei et al., 1977). With these data in hand in 1976, we could propose a first model for the transport mechanism (Stoeckenius, 1976), which was fully published only in 1979 (Stoeckenius, 1979). It postulated that the Schiff's base proton is transported, driven by pK changes of the Schiff's base, and moves along chains of proton-exchanging groups, and that conformational changes of the molecule switch the connection of the Schiff's base from the external to the internal surface thus rendering the process vectorial. This first sketch of a model has withstood the test of time quite well.

Since then, additional, closely related rhodopsins have been discovered in halobacteria, halorhodopsin (hR), which is a light-driven Cl^--pump (Lanyi, 1990), and two sensory rhodopsins (sRI and sRII), which govern the phototactic responses of the organism (Bogomolni & Spudich, 1991). In addition to nearly identical chromophores, these pigments share extensive sequence homologies with bR, indicating very similar tertiary structure and suggesting that the molecular mechanisms for these different functions are closely related. Recent work confirms this conclusion, too. Under special conditions, hR can also transport protons, even though it requires two photons, and the direction of charge transport is reversed (Bamberg, Tittor & Oesterhelt, 1993); and at very low pH bR may translocate chloride inward, i.e., in the same direction as hR. We have recently proposed a model to explain this switch from anion to cation transport (Dér et al., 1991). Signal transduction by sR may also be attributed to essentially the same molecular process (R.A. Bogomolni et al., *in preparation*). These latest results may still require confirmation and refinement; nevertheless, a very satisfying unified picture explaining the function of the bacterial rhodopsins is evolving. It is a nice example showing how relatively small modifications have adapted a molecule to serve different functions in the same organism. Much remains to be done to understand the details of these mechanisms, and as usual the results will only broaden the scope of the inquiry. The bacterial rhodopsins will provide interesting work for some time to come, not only for the exploration of some basic cel-

lular functions, but also in evolution, since they occur in one of the earliest known life forms, and show a striking similarity to light sensors in eukaryotes and G-protein-coupled receptors in general.

In Retrospect

Looking back I have to conclude that the way to bacteriorhodopsin was largely determined by a series of chance encounters and lucky accidents. The work on the myelinated nerve fiber, which led to my interest in membrane structure, was not my choice, and when I began to study the immune reaction, I did not foresee any connection with the myelin sheath and membrane structure problems. Picking the inner mitochondrial membrane for structural exploration acquainted me with the just developing field of membrane bioenergetics, which was to prove very advantageous once I stumbled upon the purple membrane. Finally, the selection of halobacterial membrane was prompted by a paper reporting poor experimental data with incorrect interpretations. Only from a decision to continue with the exploration can I derive some intellectual satisfaction, because it was based on a valid, new concept of membrane structure. The outcome, however, was entirely unexpected and radically changed the direction of my research. The other correct choice, which I consider important, is less directly linked to the bacteriorhodopsin work. It was my insistence in the face of waning acceptance that the lipid bilayer is the basis for the structure and function of membranes, combined with my willingness to modify the membrane model as required by new experimental results. It is gratifying to see that the lipid bilayer has become increasingly recognized as one of the basic structures required for life, perhaps preceding and comparable in importance to the DNA double helix.

Recalling the early days of the work on bacteriorhodopsin, what strikes me most vividly is how we groped for the right way to approach the problem. Even when we clearly made the correct choice, I was often unsure and beset by doubts. While this experience may be true more often than acknowledged for most original research, it was clearly reinforced by the abrupt change in research direction to an entirely new field. Many of the approaches we had to search for and selected only hesitantly would have been immediately obvious to an experienced photobiologist. That our laboratory managed to maintain a leading position for some time is to a large extent due to the initial reluctance of most professional photobiologists to accept bacteriorhodopsin either as a rhodopsin or as a light energy transducer. To illustrate: I submitted a paper on bacteriorhodopsin for the Sixth International Photobiology Conference at Bochum in 1972, and found my talk scheduled for the session on DNA-repair mechanisms. I inquired how this had happened, and learned that the chairmen for the vision and photosynthesis sessions had maintained that bacteriorhodopsin had no connection to their topics and, after all, DNA repair was also studied in bacteria.

It is risky to radically change research direction relatively late in one's career; not infrequently it appears to be the expression of a scientific mid-life crisis, and fails. I believe I escaped that fate because the change was clearly dictated by the results of our work and broke new ground. In the new field I was less encumbered by established concepts and enjoyed the advantage of the nonspecialist not to be biased in favor of the results from my own technical specialties. I could consider all available evidence equally critically, and before accepting any conclusion I was more inclined to look for contradictory or corroborative evidence obtained with different techniques. Also, it was probably easier for me to think of critical tests for a hypothesis because it would not be "my" technique that might be shown to have yielded deceptive results. Such prejudices and emotions should, of course, not influence one's work, but they do.

One may wonder why I did not concentrate on the structural instead of the functional exploration of the purple membrane. The approach to be followed was obvious because I was well aware of the development in electron crystallography, and that the purple membrane was an ideal object for it, but I also knew that I would have been out of my depth in that field. Early on, I tried to persuade at least two competent colleagues to try it, but neither mounted a serious effort, which, I believe, both now regret. However, we probably could not have matched anyway the beautiful structure analysis that Richard Henderson has carried out over the last twenty years.

The other determinants on the way to bacteriorhodopsin were, of course, the people I encountered. The not quite voluntary termination of my career in pathology, though somewhat traumatic at the time, clearly pushed me in the right direction. Obvious are the importance of Frank Hartmann's help and Keith Porter's generous decision to support my project rather than his own, for which he brought me to the States. This supportive attitude was continued by George Palade after Keith had left Rockefeller University and similarly at U.C. San Francisco. Even though for a Cardiovascular Research Institute at a medical school, light energy transduction may not be considered to be the most appropriate research topic, both Julius Comroe and Richard Havel as directors supported and encouraged the work when I decided to concentrate all research effort on the purple membrane.

Finally, I must explicitly acknowledge my collaborators at other institutions and in my own laboratory, few of whom could be mentioned here. It is difficult and often impossible to clearly attribute credit for results

in a research group. However, in my case the contributions of my collaborators were certainly more important than usual, because I selected them whenever possible for their training and experience in the many areas essential for the research where my own expertise was minimal. I was especially lucky when Roberto Bogomolni joined me in 1971. His exceptional intellectual and experimental skills, combined with his excellent training in biophysics and photobiology, promoted new approaches and soon became indispensable for most of the work carried out and remained so until the laboratory closed in 1989. We are still collaborating.

I have shown this manuscript in various stages of completion to most of the persons named in it that could be reached, and also to some colleagues, who have followed the field from the beginning. I am grateful for their corrections and advice, most of which have been incorporated. Where, inevitably, some discrepancies in our recollection of events remained, I have relied mostly on my own notes and memory.

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