H.R. Kaback

June 5, 1936–December 20, 2019

ANNUAL REVIEWS

Annual Review of Biochemistry

It's Better To Be Lucky Than Smart

H.R. Kaback*

Department of Physiology and Department of Microbiology, Immunology and Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, California, 90095, USA

Annu. Rev. Biochem. 2021. 90:1–29

First published as a Review in Advance on January 20, 2021

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

https://doi.org/10.1146/annurev-biochem-011520-105008

*June 5, 1936–December 20, 2019

Keywords

chemiosmosis, proton electrochemical gradient, membrane vesicles, lactose permease, proton symport, site-directed mutagenesis, autobiography

Abstract

Bacterial cytoplasmic membrane vesicles provide a unique experimental system for studying active transport. Vesicles are prepared by lysis of osmotically sensitized cells (i.e., protoplasts or spheroplasts) and comprise osmotically intact, unit-membrane-bound sacs that are approximately 0.5–1.0 μm in diameter and devoid of internal structure. Their metabolic activities are restricted to those provided by the enzymes of the membrane itself, and each vesicle is functional. The energy source for accumulation of a particular substrate can be determined by studying which compounds or experimental conditions drive solute accumulation, and metabolic conversion of the transported substrate or the energy source is minimal. These properties of the vesicle system constitute a considerable advantage over intact cells, as the system provides clear definition of the reactions involved in the transport process.

This discussion is not intended as a general review but is concerned with respiration-dependent active transport in membrane vesicles from *Escherichia coli*. Emphasis is placed on experimental observations demonstrating that respiratory energy is converted primarily into work in the form of a solute concentration gradient that is driven by a proton electrochemical gradient, as postulated by the chemiosmotic theory of Peter Mitchell.

Contents

THE PATH TO SERENDIPITY

As a student at Overbrook High School in Philadelphia, I did well academically as a mechanical arts major, which means that I took four academic courses along with mechanical drawing and a shop class. But my major interest was in football as a quarterback, with a secondary interest in baseball as a catcher. Between the shop courses and the athletics, I knew many of the African American kids, and by the time I was a senior, I was captain of both the football and the baseball teams. This prompted my Caucasian friends to nominate me for school president, and because I was well known to both the African American and the white kids, I won by a landslide. My slogan was "If you want just another Bible reader for president, don't vote for me." However, what I remember most about my presidency was reading the Bible every morning at the school assembly. I was certainly not cut out to be a politician!

When it was time to apply to college, my father's cousin, a respected Philadelphia lawyer, recommended Haverford College, a small, all-male, prestigious Quaker school on the Philadelphia main line. It was only a 30-minute drive from Chestnut Hill, where we lived behind the Hill Pharmacy, which was run by my father the pharmacist. My memory about what transpired at the time is somewhat blurred, but I ended up being accepted by Haverford in all probability because the Dean of Admissions liked football players who were "well rounded"—certainly not for any particular academic prowess. My high school advisor even said that Haverford probably would not accept me. To her disappointment—she favored another student for Haverford—I was accepted in the class of 1958. As I graduated from Overbrook in February, my mother convinced me to go to business school to learn typing and shorthand, which did me in good stead. In addition, I was slave labor behind the soda fountain in the Hill Pharmacy.

In September 1954, I matriculated early at Haverford College for football practice but was approximately 30 pounds overweight from eating as many Philly cheesesteak sandwiches as I sold from behind the soda fountain. Until I threw a football, the one thing I did really well, the coach was sure I was a lineman, and I was demoted to the junior varsity. The football coach also coached baseball and was an alcoholic anti-Semite, but I foolishly kept trying to make it in both sports and only succeeded in ruining my knees—tearing the left ACL at a time when no repair was available. My athletic career at Haverford was a total flop and had little to do with later athletic developments, as I fell in love with tennis.

When classes started, I was particularly uneasy because there were guys in my class who got perfect scores in the SAT exams. I worked my backside off, fearing that I would flunk out and never become the surgeon I thought I wanted to be. As a freshman, I took Biology, which was taught by the chairman of the department, Professor Emmett Reid Dunn, a classical herpetologist. I survived my freshman year. Biology was boring, but I loved Western Civilization, a course that involved reading a famous book every week or so. At that time, fewer than 400 male students were enrolled in the school, so the ratio of teachers to students was very high, and the 4 years at Haverford College became the formative years of my life.

During the summer following my first year, Professor Dunn died unexpectedly, and the department fell into the hands of Ariel Loewy, who was fresh from a postdoctoral fellowship with John Edsall at Harvard, the world authority on protein chemistry at the time. Loewy was a tall, slim, loose-jointed Romanian-born fellow with a slight accent. He was very extroverted and only a few years older than the students. Loewy immediately hired Melvin (Mel) Santer, who had just finished a postdoctoral fellowship with Wolf Vishniac at Yale, a well-known microbiologist, followed thereafter by Irving (Irv) Finger, who had just finished training in Elvin Kabat's immunology laboratory at Columbia. These three tremendously enthusiastic young scientists taught a full-year course to six or seven biology students, making Haverford College the first institution to teach undergraduates what is now known as molecular biology. In 1955–1956, we learned about the Watson–Crick double-helical structure of DNA, Pauling's α-helical proteins, Palade's electron micrographs of mitochondria and ribosomes, the Krebs cycle, and other early underpinnings of molecular biology. No textbooks were available to cover the material, which was taught experimentally, so we used the *Scientific American* and scientific journals. Like a good student, I took copious notes during lectures but never looked at them until it was time to study for an exam. Thus, I discovered how neat the Embden–Meyerhof glycolytic pathway is only when I looked at my notes. The material and how it was taught were incredibly heady. But Loewy and Santer (Finger was not there yet) wanted their students to go to graduate school, and if you aspired to medical school and were also a football player, you had to be a moron. Therefore, one of the clear driving forces that got me into science was to prove that you could be a football player and go to med school without being intellectually compromised.

It is also notable that Loewy, Santer, and Finger each had research grants, and senior students majoring in biology worked on one of their projects during the senior year. I spent many hours in Mel Santer's laboratory setting up experiments on a Warburg respirometer, which was a pain. However, we also grew cells, did experiments with radioisotopically labeled compounds, used paper and column chromatography, measured absorption spectra, and learned most of the other techniques used in a state-of-the-art biochemistry laboratory of that era. As a result, my first scientific publication is on the intermediary metabolism of *Thiobacillus thioparus* with my mentor Mel Santer and my classmate Norman Klineman (1).

In the summer of 1958, Mel Santer sent me to the laboratory of Britton Chance at the University of Pennsylvania to determine whether pyrimidine nucleotide is involved in thiosulfate metabolism in *T. thioparus*. Chance was one of the powers in biochemistry: an independently wealthy, twice-gold-medaled Olympic yachtsman and physical chemist who could build instruments—mostly spectrometers—that could measure anything. He was also known for having a wry, sarcastic sense of humor. The Chance laboratory was in the Mahoney Clinic, which included two groups of scientists: physicists who built the spectrometers and biochemists who used them. The only individual who was master of both was Chance. When I arrived in the laboratory, a graduate student from Yale (whose name I forget) and I were introduced to Dr. Chance (henceforth known as Brit), and we were ushered into his own laboratory, a small room with black walls and crammed with hand-built instruments and their components. There was hardly room to

walk around. Brit put the two of us in front of a hand-built, split-beam spectrophotometer known as the Yang Machine (later marketed by the Applied Physics Corporation, which became the Cary Instruments Corporation) and said, "Okay boys, maybe two minuses will make a plus," and left the room. We did nothing for a couple of days, because we could not find the light switch and were afraid to ask. Subsequently, I grew *T. thioparus* for experiments, which involved some real work. The bugs were grown in 20-L carboys, and the medium contained thiosulfate. Oxidation of thiosulfate caused the medium to become acidic, which caused sulfur to precipitate, and the medium had to be neutralized for the bacteria to continue growing. This step had to be done in the middle of the night. During the summer, Brit would spend the day sailing off the coast of southern New Jersey and come to the laboratory very late at night, usually around the time that I showed up to neutralize my cultures. I think Brit slept in the laboratory, because early the next morning, he had already left again for South Jersey. In any case, it turned out that pyrimidine nucleotide is involved in thiosulfate metabolism, so the summer was a success. Moreover, as a result of our meetings in the middle of the night, I think Brit never forgot my name. I should also add in passing that this was the summer that he discovered respiratory control in mitochondria, which convinced Brit that a high-energy phosphate intermediate was involved in oxidative phosphorylation. Peter Mitchell and chemiosmosis later famously overturned the hypothesis.

In the early 1950s, a Haverford alumnus, William Pyle Philips, endowed the College with the Distinguished Visitors Fund for the expressed purpose of bringing world-class scientists to the campus to give lectures and talk with the faculty and students. Remarkably, between 1957 and 1958, for only seven biology majors, we had David Bonner, Arthur Kornberg, Joshua Lederberg, Salvatore Luria, Linus Pauling, Roger Stanier, and others who escape my memory give talks and hobnob with us. We even got to take them to meals without our professors. On one occasion, we were having lunch with Joshua Lederberg, who had a mouthful of spaghetti when my classmate Norman Klineman asked if his mother had ever forgiven him for not finishing medical school. Lederberg almost choked on the spaghetti. It was quite an experience for us to get this close to the famous scientists who authored many of the articles we were reading!

Senior biology majors had to write a thesis to graduate, and I chose to write on an obscure topic, Gram's stain in bacteria, which in 1957 was thought to be due to a ribonucleate in the gram-negative cell membrane. The last of the Philips Visitors that year was Arthur Kornberg, who was still at Washington University in St. Louis and had not yet become a Nobel laureate. At that time, Paul Berg was a postdoctoral fellow in his laboratory, and Berg had just discovered the pH 5 fraction, which turned out to be transfer RNA. Berg would add adenosine triphosphate (ATP) and a radiolabeled amino acid (e.g., alanine) and observe time-dependent incorporation of labeled alanine to a maximum, when no more was incorporated. But if he then added a different radiolabeled amino acid, it would be incorporated much like alanine, and so forth with each amino acid.

Although I was near the top of my class scholastically, I managed not to do well on multiple-choice exams, and I apparently did particularly poorly on the MCATs (about which no one informed me). Therefore, medical schools like Harvard, Penn, or Yale, which were supposed to be shoo-ins for me, rejected my applications. But after an anxious wait into late spring, the Albert Einstein College of Medicine, only in its fourth year and anxious to have students from Haverford, admitted me into the class of 1962. By the time I matriculated at Einstein, I felt I was not going to practice medicine. I had gotten so turned on to molecular biology at Haverford that I reckoned that, although I was programmed to be a medical doctor, I would also become a biochemist. Therefore, I attended all the biochemistry seminars I could squeeze in, and one of the first was given by Werner Maas, a bacterial geneticist from New York University (NYU). Maas described *Escherichia coli* mutants that became resistant to growth inhibition by D-serine or canavanine by

losing the ability to take up these amino acid homologs. Moreover, the D-serine–resistant mutants also lost the ability to take up structurally related glycine or alanine, and the canavanine-resistant mutants could no longer take up lysine or arginine. To this naïve young medical student, the findings sounded similar to what Berg had found with Kornberg. A light bulb lit in my head with the notion that the RNA responsible for Gram's stain might be involved in amino acid transport. Because I wanted to do research that summer, I proposed to obtain the mutants from Maas, prepare a membrane fraction, extract the RNA, and perform a Berg-type experiment. Moreover, Adele Kostellow, a young assistant professor in the Department of Physiology and Biophysics, a bacterial geneticist turned developmental biologist who knew Maas, allowed me to work in her laboratory and supplied the materials I needed.

Adele Kostellow obtained the D-serine- and canavanine-resistant mutants from Maas, and I went to work. Fortunately, I prepared membranes by osmotically lysing penicillin-induced spheroplasts, and to be certain the cells lysed, I used a phase-contrast microscope. Essentially, all I saw was a population of little empty bubbles—membrane vesicles or ghosts—floating past the lens with very few intact, dark, rod-shaped *E. coli*. At this point, I got the bright idea of incubating the vesicles from the normal *E. coli* or from each of the mutants with the appropriate radioactive amino acid to see if there might be differences. From this first, amateurish experiment, a clear difference was apparent. Relative to vesicles from normal bugs, less-radioactive L-serine was associated with the vesicles from the D-serine–resistant mutant, and less lysine was associated with the vesicles from the canavanine-resistant mutant. Incredibly, an original experiment had worked in my own hands, and I was completely hooked! Throughout medical school, I spent most weekends and many vacations in Adele Kostellow's laboratory playing scientist with my membrane vesicles. When I added DNase, the viscosity decreased dramatically and the vesicles from normal *E. coli* took up more radioactive amino acid. And amazingly, when I added RNase, the difference between the mutant and the wild-type vesicles seemed to disappear. So maybe my idea was correct? Unfortunately, the result was not reproducible!!!

Around this time, the *Journal of Biological Chemistry* (*JBC*) initiated publication of *Preliminary Communications*, and Adele Kostellow suggested that I submit a short manuscript describing my initial observations. After a few weeks, I received what I considered to be an immensely complimentary letter from *JBC* stating that my findings were of such potential importance that I should do more experiments and submit a full manuscript. In addition to the cover letter, 10–20 pages of suggested experiments were included. As neither Adele Kostellow nor I knew how the game was played, and in particular that rebuttals were allowed, this began a cycle of doing the suggested experiments and submitting manuscripts to *JBC*, followed by more suggested experiments and more revised manuscripts. Moreover, the periods between submissions became increasingly longer because it felt like the situation was impossible. The problem was ultimately solved in an unusual manner (see the section titled The NIH Years).

On Saturday mornings, NYU Medical School held what were known as Honors Lectures. One Saturday, the invited lecturer was none other than Francis Crick. Maas invited me, a junior medical student from Einstein, to have a private audience with Crick to tell him about my new idea that RNA might be involved in amino acid transport. I was ushered into a room and introduced to Crick. I explained my idea to him, and he then looked down his long nose and said sternly in a British accent, "No, no, that cahn't possibly be correct." As I left the room, I said to myself, "What the hell does this guy know about transport?" To this day, it astonishes me that this giant of biological science was sufficiently insecure that he had to put down a third-year medical student! It also surprises me that I had the guts to continue to follow my own nose experimentally. In any event, Crick was correct—RNA is *not* involved in amino acid transport.

When asked by an advisor in the Department of Medicine what I was interested in with regard to internships, I flippantly answered, "Biochemistry." He responded with, "Okay wise guy, what do you want to intern in?" I answered, "Medicine, I guess." He then asked whether I was interested in pediatrics, and I said that I was just about as interested in pediatrics as I was in medicine. His response was that all the smart students go into medicine, and if I did research and was successful, I would stand out in a Department of Pediatrics. So I went to the Department of Pediatrics at Jacobi Hospital, where I was welcomed with open arms and offered a special deal. I would do a regular pediatric internship the first year but then spend 4 years as a fellow, spending 4 months on the wards and 8 months in any biochemistry laboratory I chose. At the end of the 5 years, I would have a PhD in biochemistry and also be a board-qualified pediatrician. Furthermore, during the last 4 years, I would be paid as a fellow, not a resident. I took the deal, but the Department of Pediatrics made the mistake of starting me in the newborn/premature nursery, which I despised. I also felt that I knew more biochemistry at that point than anyone in the Department of Pediatrics and was not excited by the ongoing research. The only positive things I remember about that internship year are the birth of my son George and that I managed to cajole my colleagues into covering for me so that I could to go Atlantic City to the FASEB meetings to present an article on the vesicles.

These were the days of the Vietnam War, and if you finished an internship as a physician but had no residency plans, you were immediately reclassified 1A so that you could be drafted and sent to Vietnam. Of course, I wanted out of medicine and had not signed up for a residency. Rather, I had been accepted by the so-called Berry Plan under the category of "research" but had not yet signed the acceptance when I received notice that I was reclassified 1A. I appealed the reclassification to my draft board, stating that I should be classified 2S (a student classification), because I wanted to study for a PhD in biochemistry. In response, I received both a denial of the request for reclassification as well as a notice to appear for a physical exam in downtown New York City prior to induction into the US Army. In a panic, I ran to Adele Kostellow, who called the draft board claiming that my unrepaired ACL was physically disabling. Remarkably, she convinced the official on the draft board to "lose" my file. I also went to see Alfred Gilman Sr., who chaired the Department of Pharmacology and with whom I had had many conversations, as the rare medical student with an interest in research. Gilman had asked in one of our conversations whether I might be interested in going to the National Institutes of Health (NIH). In those war years, if you were lucky enough to go to the NIH, you could do your selective service time working in a laboratory, which sounded like heaven to me. Gilman called his friend Robert Berliner, who was director of the Heart Institute (now the National Heart, Lung, and Blood Institute), and I ended up in Earl R. Stadtman's enzymology laboratory. Stadtman had a passing interest in transport, and he allowed me to bring my membrane vesicles with me. Thus, the path to serendipity began in earnest, and over the next few years, I became a real scientist.

THE NIH YEARS

The Vietnam War made the NIH the premier institution in the world for biological research. One reason was that the laboratory heads had their pick of the cream of the crop of graduates from the very best medical schools in the country. Furthermore, during those years, the NIH was home to some of the world's most outstanding biological scientists, and one could take courses in the night school from the people who wrote the textbooks.

When I arrived in Earl Stadtman's laboratory in Building 3 with my small collection of *E. coli* transport mutants, the notion that the solution to transport would come from enzymology, and the hope that membrane vesicles was the experimental system to use, I was anything but

confident. Here was this big laboratory with all these brilliant young guys headed by the famous Earl Stadtman, whose primary interest was in water-soluble enzymes and their regulation. And Earl's personality did not help! In addition to highly touted aspects, the so-called Stadtman Way involved the newcomer's impression that Earl did not talk to you until you proved yourself scientifically, which stimulated you to work harder than ever. When asked into his office to discuss work, we would sit facing each other but nothing would be said until I felt compelled to speak. Earl's knowledge about transport was not very deep, and he was heavily opinionated. For example, he felt strongly that facilitated diffusion, a well-documented phenomenon, was not possible.

However, Stadtman was truly a superb biochemist whose strength was his emphasis on the importance of controls. Initially, one of the questionable aspects of the vesicles was whether the difference in uptake of amino acids between vesicles from the normal *E. coli* and vesicles from the D-serine- or canavanine-resistant mutant could be due to the residual intact bacteria in the vesicle preparations. As I had no idea how to energize the vesicles to increase transport activity to a level anywhere near that of the parent cells, the veracity of the system was totally dependent upon the difference in transport between vesicles from the normal bugs and vesicles from a transport mutant. Earl came up with a great way to test this possibility. He suggested that I generate a curve in which transport was measured as a function of cellular DNA in samples in which normal cells were broken by increasing times of sonification. The same ratio was then determined in the membrane vesicle preparations. Although the results were not published in full, the experiments showed clearly and decisively that transport by the vesicles could not be due to intact cells. At this point, Earl and I both believed that transport by the vesicles was real, and because I had characterized a proline transport mutant in the vesicles but was still having trouble getting the original work published in *JBC*, Earl succeeded in getting Arthur Kornberg to communicate the article on vesicular proline transport to the *Proceedings of the National Academy of Sciences* (2). Publication of the original work came a bit later (see below).

Stadtman laboratory members were interested in water-soluble enzymes, which they were purifying and characterizing, and uninterested in membrane proteins, which require detergents for solubilization and are much more difficult to handle. But one of the postdoctoral fellows, Clifford Woolfolk, who worked with Thressa Stadtman, was interested in what I was doing, and as he worked across the hall, we had many conversations. Although Earl had offered him a position, Cliff joined the faculty at the new campus of the University of California at Irvine (UCI). At the end of my second year at the NIH, I had fulfilled my selective service obligation and was transferring into the civil service when I received an invitation from UCI to come to Irvine for a job interview (obviously instigated by Woolfolk). I was flattered and agreed to go. However, a few days prior to leaving, Earl called me into his office, where he told me he was aware of the invitation from UCI and offered me a position. I was totally blown away! I was not sure Earl knew my name, let alone what he thought of my work. Without talking to my wife or asking what space I would occupy or anything else, I accepted the offer on the spot and floated out of Earl's office approximately a foot above the floor.

It was then approximately 1967, and I had one bay in the Stadtman laboratory and was still working alone. The original work with vesicles from the D-serine–resistant mutant that had been back and forth to *JBC* innumerable times had morphed into two full manuscripts: the first an extended version of the *Preliminary Communication* article with Adele Kostellow and the second taking the problem further with Stadtman. When I received the last rejection from *JBC* with even more suggested experiments, I got depressed and wanted to bury the manuscripts, but I decided to try once more. This time when they were returned yet again, the reviewer recommended that I should consider submitting the first manuscript as a *Preliminary Communication*, thereby making the full circle, and I went a bit crazy. I called the *JBC* office, asking to speak with William Stein,

the editor. Stein was a Rockefeller Professor who received a Nobel Prize with Stanford Moore for amino acid analysis, and he dressed in a white shirt and tie with a tweed jacket, clearly a gentleman. When he got on the phone, I began screaming obscenities about the reviews. I owe Stein a tremendous debt, as he let me go on raving until I finally ran out of steam, when he said, "Have you ever either rebutted the comments because you don't think they are justified or agreed and made a revision or done an appropriate experiment?" I yelled that I had a file drawer full of correspondence, and he asked again if I had ever *rebutted* anything, to which I had to answer negatively. He then suggested that I either agree with a given comment and do as the reviewer recommended or rebut the comment. After I did this, I should submit the manuscripts to him personally with a detailed description of what I had done with respect to the reviews. He promised to give me an answer within 2 weeks. The reviewer's comments were either nitpicking or nonsense. For example, in one case, the reviewer took issue with my identification of phosphatidylethanolamine by migration on chromatography in comparison with a known standard. The origins were a millimeter or so different from each other, as were the positions of the chromatographed spots. In other words, the known and the experimental sample chromatographed identically. I started my rebuttal by stating that the reviewer must think that the author is either deaf, dumb, and blind or a complete moron, and I treated each of the reviewer's comments in similar fashion. Two weeks after returning the manuscript to Stein with my rebuttals, both manuscripts were accepted without modification (3, 4), and I learned one of the most important lessons of my early career—do not take crap when you know you are correct!

In 1966, an article from Saul Roseman's laboratory at Johns Hopkins University appeared in *JBC* (5) indicating that a small heat stable protein (HPr), a component of the phosphoenolpyruvate phosphotransferase system (PTS) discovered in his laboratory, might be involved in transport of sugars in *E. coli*. However, it was heretical at that time to think that phosphorylation was involved in sugar transport, because Robert K. Crane at Rutgers Medical School had shown that fluorine substitution at each position of glucose did not inhibit transport by the gut. Curious, I purchased radiolabeled α-methylglucoside (αMG), a nonmetabolizable glucose analog, and one Friday afternoon when I had nothing better to do, I added the αMG to the membrane vesicles. To my amazement, it accumulated like crazy. Furthermore, essentially all the radioactivity in the vesicles was recovered as α-methylglucoside phosphate (αMGP), and with more purified vesicles, uptake and phosphorylation were dependent specifically upon phosphoenolpyruvate (PEP). In intact cells, a very significant percentage of the αMG taken up is recovered as αMG, making it impossible to discern whether αMG or αMGP came first. How to resolve the problem? Another buddy in the Stadtman laboratory, my good friend Bernard Babior, and I came up with an ingenious approach. I first loaded the vesicles passively with [^{14}C]glucose in the absence of PEP, and the external [^{14}C]glucose was then removed. [^{3}H]Glucose was added with PEP, the radiolabeled sugars taken up by the vesicles were recovered, and the different isotopically labeled glucose and glucose-P molecules were quantified. The experiment demonstrated with remarkable clarity that the [^{3}H]glucose added externally with PEP was phosphorylated almost exclusively relative to the free [^{14}C]glucose preloaded into the vesicles, and reversing the isotopically labeled sugars yielded the same result. In other words, the experiment demonstrated unequivocally that transport of glucose in *E. coli* occurs by vectorial phosphorylation and that transport through the cell membrane and the first step in metabolism occur simultaneously (6).

But what about galactosides? Fox & Kennedy (7) had identified a membrane protein reputed to be involved in galactoside transport. Was this protein a component of the PTS? Transport of lactose in the vesicle system was not stimulated by PEP, but the answer had to wait.

By 1968, I had moved into a small laboratory with a couple of benches and half of my desk in a broom closet, and I even had my first postdoctoral fellow. Larry Milner, a physician, came to

me from Herbert Weissbach's laboratory because Herb was leaving the NIH for a new research institute financed by Hoffman-LaRoche named the Roche Institute of Molecular Biology (RIMB) (more about this later). I had become interested in the passive permeability properties of the vesicles with respect to leakage of phosphorylated glucosides accumulated by the PTS and wanted to extend the studies to proline. In the early experiments on amino acid transport, glucose was used to produce mild stimulation of transport.[1] I had tried everything I could lay my hands on to stimulate transport, but the only thing that worked about as well as glucose was lactate. By this time, I had switched from a strain of *E. coli* that made beautiful vesicles by electron microscopy (probably because they lost most of their outer membrane) to the K-12 stain in which all the genetics were done. But glucose did not do anything for transport with K-12 vesicles, so I told Milner to try lactate, and it worked. I knew from my PTS studies that glucose was not metabolized past the phosphorylated state, and I believed that the vesicles were virtually devoid of soluble, metabolic enzymes. They certainly did not have many, if any, soluble cytoplasmic proteins that would oxidize lactate. Therefore, I suggested to Milner that we purchase some [^{14}C]lactate so that we could determine what happened to it chemically when incubated with the vesicles. After a short while, he came to me with the CalBiochem catalog saying, "Hey Ron, they have [^{14}C]labeled D,L-lactate, L-lactate, or D-lactate. Which one shall I get?" I told him to get the unlabeled ones first, and we would see which one stimulates transport best.

To measure transport, cells or membrane vesicles are incubated with a radiolabeled transport substrate like [^{14}C]proline, and at a desired time, the sample is rapidly diluted and immediately filtered on a membrane filter that retains the cells or vesicles. The filters are then assayed for radioactivity, and in the mid-1960s, this was done by gas-flow counting to measure β-particle emission from decay of ^{14}C. Without going into detail, the radioactivity is detected by discharge of an anode wire, and when the anode wire aged, it would break and had to be replaced. When Milner and I tested the filters from the vesicle samples incubated with [^{14}C]proline and D,L-lactate, we observed mild stimulation over the control sample incubated with radiolabeled proline alone. L-lactate produced less stimulation, and with the sample incubated with D-lactate, the anode wire discharged and broke. We replaced the anode wire, and the same thing happened again. After the third anode wire broke, we decreased the radioactivity of the labeled proline, and the counter still went wild, but the anode wire remained intact. Furthermore, we found that the D-lactate was converted only into pyruvate. Thus, we discovered that oxidation of D-lactate to pyruvate specifically causes the vesicles to transport not only proline but also various other amino acids, sugars, and other goodies. Although the vesicles oxidize other substrates like NADH, α-glycerol-P, and succinate, some even better than D-lactate, and they reduce the same respiratory chain in the membranes as D-lactate, these oxidations do relatively little to stimulate transport (12).

As discussed above, the 1966 article from the Roseman laboratory (5) suggested that β-galactoside transport might occur by vectorial phosphorylation utilizing the PTS, and Fox & Kennedy (7) had identified a membrane protein associated with the famous *lac* operon that could possibly be a PTS component. It is important in this context to realize that when François Jacob and Jacques Monod (see 13) showed that the *lac* operon comprises three structural genes–*lacZ*, *lacY*, and *lacA*—and Georges Cohen and Howard Rickenberg (14) showed that *lacY* is important

[1]The original mild stimulatory effect by glucose was resolved when Kazunobu Matsushita, then years past his postdoctoral tenure in my laboratory, described a pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) (8) on the outer surface of right-side-out (RSO) vesicles from *E. coli* (9). GDH is constitutively produced, but *E. coli* does not synthesize PQQ (10); thus, GDH is present mostly as an apo-enzyme on the membrane. Once PQQ is supplied exogenously, GDH is activated to oxidize glucose to gluconate on the outer surface of the membrane, concomitant with a rapid generation of large $\Delta\tilde{\mu}H^+$ (11).

for transport, it was the first indication that transport might involve a protein. This was at a time when arguments were still being made about the actual existence of cell membranes, let alone proteins responsible for transport (15).

Eugene Barnes, one of my first postdoctoral fellows, and I prepared membrane vesicles from *E. coli* devoid of β-galactosidase, but with or without lactose permease, as Monod had dubbed it (henceforth denoted LacY). We then demonstrated that D-lactate oxidation caused vesicles with LacY to accumulate lactose against a 50- to 100-fold concentration gradient (16). Moreover, the system had all of the properties of LacY in intact cells such as inactivation by *N*-ethylmaleimide and protection against inactivation by β-galactosides (17).

Now we had a well-defined system in which to study transport, a single-step oxidation of D-lactate to pyruvate that drives accumulation of galactosides by a genetically encoded component that is likely a protein. The question was, "What is actually going on?" But this question had to wait, because Herbert Weissbach, head of biochemistry at the newly established RIMB in Nutley, New Jersey, had offered me a position, and deciding was no simple matter. Although I loved working at the NIH and my family was happy living in Rockville, I still did not feel comfortable with Stadtman, and I could have looked for another position at the NIH. Furthermore, pharmaceutical companies had a history of supporting basic science for a while but then dropping it. However, RIMB was the brain child of Sidney Udenfriend and Herbert Weissbach at the NIH and John Burns and Alfred Pletscher at Roche with the blessings of V.D. Mattia, then CEO of Hoffman-LaRoche Nutley, and the company was riding very high on the profits from Librium and Valium. Although located on the campus of the company in Nutley, New Jersey, RIMB had a separate charter, and our only obligation was to do basic science. I reckoned that we would have carte blanche for a minimum of 5 years, and if I did not succeed scientifically, I could always practice pediatrics. Fortunately, the lives and suffering of hundreds of children were alleviated by my success as a scientist. This, plus my younger brother Michael Kaback's pioneering of the genetic screening program for Tay-Sachs Disease, became the Kaback family's contributions to American pediatrics.

RIGHT-SIDE-OUT MEMBRANE VESICLES

As described in *Adventures in Serendipity* (18), the membrane vesicles were made first from *E. coli* (**Figure 1**) treated with penicillin to induce formation of spheroplasts (**Figure 2**), which are

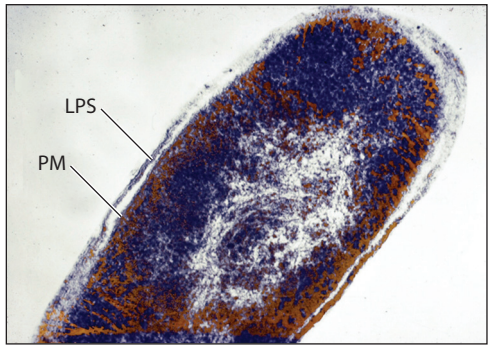

Figure 1

An intact *Escherichia coli* cell visualized in cross section by electron microscopy, showing the outer membrane, consisting of LPS, and the inner plasma membrane. Abbreviations: LPS, lipopolysaccharide; PM, plasma membrane. Figure reproduced with permission from Reference 110; copyright 1972 Elsevier.

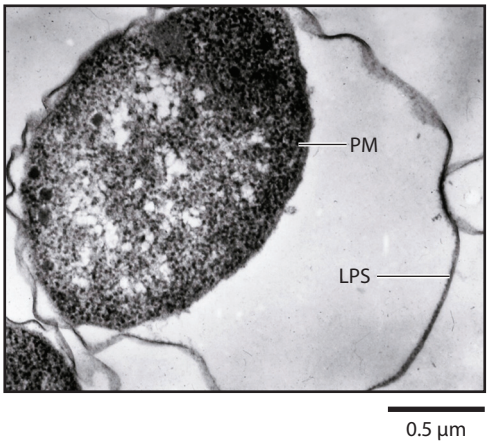

Figure 2

An electron micrograph of a spheroplast prepared from *Escherichia coli* W by treating cells with penicillin. Penicillin blocks the synthesis of peptidoglycan, which is required for the *E. coli* cell wall that protects the bacteria from lysis in hypotonic environments. When grown in the presence of an appropriate osmotic stabilizer, penicillin-treated *E. coli* will not lyse but instead becomes spherical. The interior of the cell remains enclosed by the plasma membrane, while the LPS layer of the cell wall becomes enlarged. Abbreviations: LPS, lipopolysaccharide; PM, plasma membrane. Figure reproduced with permission from Reference 111; copyright 1971 Elsevier.

osmotically sensitive because peptidoglycan synthesis is stopped and the cells outgrow this rigid layer of the cell wall. However, this method is inconvenient for making vesicles on a large scale. Therefore, I switched to lysozyme, which degrades the peptidoglycan layer enzymatically. With gram-positive bacteria, which do not have an outer membrane, lysozyme is used directly. But gram-negative cells have an outer membrane containing lipopolysaccharide (LPS), the integrity of which is dependent upon divalent cations like Mg^{2+}. Thus, with gram-negative bacteria, for lysozyme to gain access to the peptidoglycan layer of the cell wall, ethylenediaminetetraacetic acid (EDTA) is used to chelate divalent metal in addition to lysozyme to make the cells osmotically sensitive. This treatment is done in the presence of high sucrose concentrations or another osmolyte to stabilize the spheroplasts (from gram-negative bacteria) or protoplasts (from gram-positive bacteria). These osmotically sensitized cells are then lysed by rapid dilution in buffered medium without the stabilizing osmolyte to form empty vesicles (**Figure 3**) that are osmotically sealed (19).

During lysis, the cell membrane actually tears, and the inside of the vesicles—or ghosts, as they were originally called—equilibrates with the medium. Therefore, the bigger the dilution during lysis, the lower the content of soluble cytoplasmic constituents in the preparations. Relatively high concentrations of DNase and RNase are also present during lysis, and EDTA is also utilized, as it destabilizes the ribosomes for digestion.

The lysis phenomenon was first visualized by phase-contrast microscopy by placing a drop of water at the side of the cover slip. Although streaming occurs, which makes focusing difficult, the phase-dense spheroplasts swell, the membrane ruptures at a single site, the structures suddenly become transparent, and the membrane reseals. Thus, it was obvious from the beginning that the membranes do not invert. It is also apparent that the small amount of contamination caused by intact bacteria is easily removed by low-speed centrifugation or centrifugation through 58% sucrose.

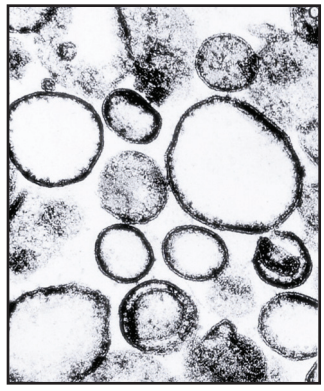

Figure 3

Electron micrographs of spheroplasts produced from *Escherichia coli* ML 308-225 (magnification × 77,000). *E. coli* were treated with ethylenediaminetetraacetic acid and lysozyme to degrade the peptidoglycan layer of the cell wall, causing the cells to become osmotically sensitized. The cells were lysed, and the membranes were isolated then sealed osmotically to form right-side-out vesicles. Figure reproduced with permission from AAAS from Reference 112.

The contention that a hole opens in the membrane when the cytoplasmic contents are released was supported by electron microscopy with colloidal gold particles (S.C. Silverstein & H.R. Kaback, unpublished observations). When mixed with *E. coli* spheroplasts or vesicles, the colloidal gold particles were observed only in the surrounding medium—not inside the spheroplasts or vesicles. However, when the spheroplasts were lysed in the presence of colloidal gold, the colloidal gold was observed both in the medium and within the vesicles. Although these experiments provide a strong indication that the vesicle membrane has the same orientation as the membrane in the intact cell, the conclusion was disputed initially, and a great deal of effort was put into testing the sidedness of the preparations.

Vincent Marchesi first used freeze-fracture electron microscopy (**Figure 4**) to demonstrate that the texture of the convex and concave surfaces is very similar to that of the freeze-fractured *E. coli* cytoplasmic membrane. It was then shown immunologically with antibodies against D-lactate dehydrogenase (D-LDH) or F_1F_O-ATPase that these antigens are inaccessible to antibody unless the vesicles are subjected to sonification, vigorous homogenization, or mistreated otherwise (20). Subsequently, in a series of collaborative experiments with Peter Owen (initially a postdoctoral fellow in the Salton laboratory at NYU and then at Trinity College, Dublin) using rocket electrophoresis, we were able to identify ~14 distinct immunoprecipitates. By sequential adsorption with intact or disrupted vesicles, 11 of the identified proteins, each of which is a membrane-bound enzyme, were essentially inaccessible to antibody unless the vesicles were disrupted. The remaining 3 proteins were accessible whether or not the vesicles were disrupted, and each is associated with either LPS or Braun's lipoprotein, which are expected to be on the outer face of the cytoplasmic membrane (21–23).

But the most convincing evidence that the vesicles are all right-side-out (RSO) came from an experiment that took ~20 years to conceive, which demonstrated directly that every vesicle in the population carries out active transport. The story starts when Bob Abeles called from Brandeis University to tell me he had developed a suicide acetylenic substrate for lactate oxidase from *Mycobacterium smegmatis*, 2-hydroxy-3-butynoic acid (HBA), and asked me if I would like to try it on the D-LDH in *E. coli* membrane vesicles. This was one of those unusual experiments where everything worked immediately. Chris Walsh, Abeles's postdoctoral fellow, brought the HBA to

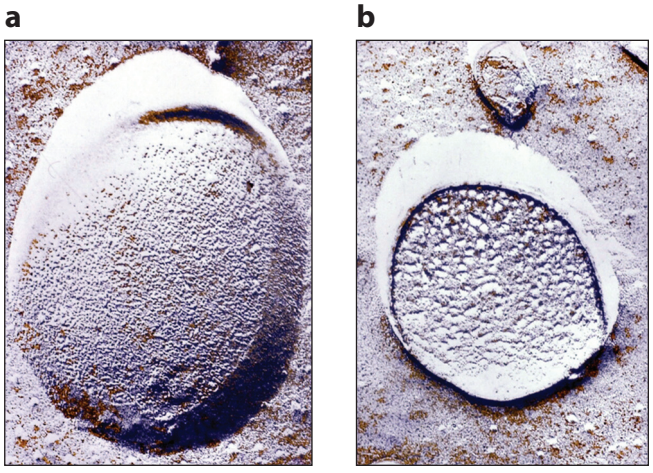

Figure 4

Freeze-fracture microscopy was used to visualize the texture of the (*a*) outer and (*b*) inner surface of membrane vesicles from *Escherichia coli* ML 308-225 (magnification approximately × 140,000). Figure reproduced with permission from Reference 113.

my laboratory. With Chris looking over my shoulder, it was shown that preincubation with HBA completely killed D-LDH, but the double-bond analog 2-hydroxy-3-butenoic acid [vinylglycolate (VG)] had no effect whatsoever (24). This finding was consistent with the mechanism proposed by Abeles. Approximately once in every 30 turnovers, oxidation of HBA leads to extraction of the proton from the α-carbon, followed by rearrangement and formation of a highly reactive allene intermediate that reacts with the FAD in the active site of D-LDH (25). However, with VG, such an allene intermediate cannot be formed, and the oxidation product is the analog of pyruvate.

But I wanted to do more control experiments, so for a lark, I tested the effect of HBA on the PTS, and to my astonishment, HBA killed PEP-driven αMG transport and phosphorylation. Even more remarkably, VG, which did nothing to D-LDH, killed the PTS at much lower concentrations than that of HBA (26). When VG is oxidized by D-LDH, the product is analogous to pyruvate, but it has conjugated double bonds at the carboxyl oxygen and between carbons 3 and 4 that are attacked by nucleophiles such as cysteine to form covalent adducts (**Figure 5*a***).

Arnold Liebman, head of the Isotope Synthesis Group at Hoffman-LaRoche, then synthesized [^{3}H]VG, and we showed that ascorbate/phenazine methosulfate (PMS)–driven transport of VG by the vesicles occurs via a lactate permease. Moreover, it was demonstrated that the rate-limiting step for covalently labeling the membrane vesicles is transport of VG (27) (**Figure 5*a***). For many years, I had wanted to test whether all of the vesicles in the preparations are active, but it is impossible to do this with substrates like lactose that are accumulated against a concentration gradient and likely in free solution. But now we had a transport substrate that stuck chemically to the inside of the vesicles once transported. Liebman then made us very high specific activity [^{3}H]VG, and Sam Silverstein did radioautography in the electron microscope. Each and every membrane vesicle exhibits the telltale worms of radioactive decay (**Figure 5*b*, *i***), and vesicles labeled indiscriminately with [^{3}H]acetic anhydride (**Figure 5*b*, *ii***) are indistinguishable from those labeled with [^{3}H]VG. Therefore, it is apparent that every vesicle in the preparation carries out respiration-driven active transport (27). This experiments remains one of the most satisfying of my career.

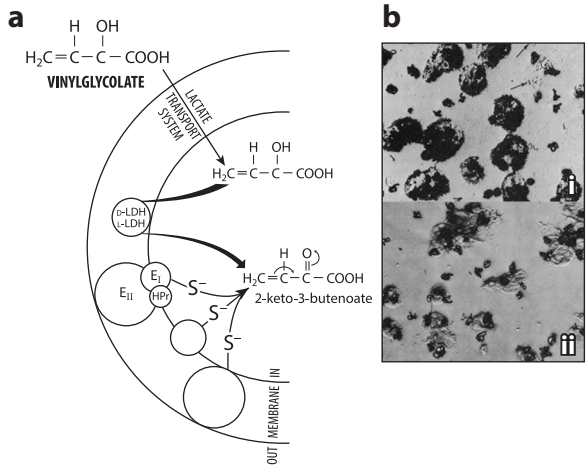

Figure 5

(*a*) Model for the transport of VG. VG is transported across the *E. coli* membrane by the lactate transport system, then oxidized to 2-keto-3-butenoate by membrane-bound D- and L-LDHs. Almost all of the VG that is taken up is covalently bound by the membrane vesicles. (*b*) Membrane vesicles visualized by electron microscopic radioautography after incubation with (*i*) [^{3}H]VG in the presence of ascorbate-PMS or (*ii*) the nonspecific stain [^{3}H]acetic anhydride. Evidence of radioactive decay is near universal among vesicles from both preparations, indicating that all vesicles participate in the transport of VG. Abbreviations: E_I, Enzyme I of the phosphoenolpyruvate-phosphotransferase system; E_{II}, Enzyme II of the phosphoenolpyruvate-phosphotransferase system; HPr, histidine-containing protein of the phosphotransferase system; LDH, lactate dehydrogenase; VG, vinylglycolate. Figure reproduced from Reference 27.

THE ROCHE INSTITUTE OF MOLECULAR BIOLOGY

At its inception, Sidney Udenfriend was the director of the RIMB, and there were three departments: Molecular Pharmacology, headed by Udenfriend; Biochemistry, headed by Herbert Weissbach; and Cell Biology, headed by Arthur Weissbach.

The move to RIMB took place in the spring of 1970, and by fall, my laboratory focused on β-galactoside transport, or more specifically, how D-lactate oxidation specifically drives lactose accumulation against a concentration gradient via LacY. As mentioned previously, although the vesicles oxidize various substrates quite well, D-lactate is far and away best for transport.[2] Furthermore, each oxidizable substrate reduces the same respiratory chain at rates that are commensurate with the rate of oxidation. Therefore, it seemed reasonable to suggest that the energy-coupling site was between D-LDH and cytochrome *b*. It was also apparent that neither oxidative phosphorylation, ATP, ADP or other nucleotide di- and triphosphates, nor PEP is involved.

The Kaback and Barnes Model

It was also clear that a membrane-embedded respiratory chain is involved, as oxygen is required and various respiratory chain inhibitors block D-lactate–driven transport. An experiment was then done utilizing inhibitors of respiration that worked at different levels of the respiratory chain: anoxia or KCN to block the terminal oxidase(s); hydroxyquinoline-*N*-oxide to block cytochrome *b*;

[2] Subsequently, it was discovered by Wilhelmus N. Konings (28) that ascorbate/PMS drives transport in *E. coli* vesicles even better than D-lactate does and is applicable to bacterial vesicles that do not have D-LDH.

amytal to block at the level of NADH dehydrogenase; and oxamate or oxalate, potent competitive inhibitors of D-LDH. Each of these inhibitors effectively blocked transport driven by D-lactate oxidation. Remarkably, however, if the inhibitors were added after a steady-state level of lactose accumulation was achieved, only those inhibitors that blocked the respiratory chain after D-LDH caused the lactose concentration gradient to dissipate. Oxamate or oxalate, in particular, despite almost complete inhibition of D-lactate oxidation and transport did not cause efflux of lactose, whereas cyanide or anoxia, for example, caused rapid efflux of the accumulated galactoside. These observations plus the evidence that cysteine(s) appeared to be essential for the activity of LacY led to the so-called Kaback and Barnes model for transport (29), which postulated that LacY was an intermediate in the respiratory chain between D-LDH and cytochrome *b* and that transport by LacY involved sulfhydryl/disulfide interconversion. But one thing the model did not explain was why uncoupling agents like dinitrophenol or carbonylcyanide-*m*-chlorophenyhydrazone (CCCP), which make membranes permeable to H^+ (protonophores), killed transport and caused efflux without affecting D-lactate oxidation. However, Ting et al. (30) had shown that no correlation exists between uncoupling activity in mitochondria and proton permeability in planar bilayers, and my laboratory (31) had shown that CCCP can react with Cys residues, albeit at relatively high concentrations. Therefore, I simply looked upon this as an unresolved issue. In any event, as the Kaback and Barnes model began to fall into disrepute, it became the Barnes and Kaback model, and since it has become clear that the model is completely incorrect (read further), it is now known as the Barnes model. (This is intended as humor, dear reader!)

Peter Mitchell and Chemiosmosis

In the early 1960s, Peter Mitchell, an iconoclastic Englishman, published a series of articles (32–34) that appeared to most to be about an absurd theory derived from his interest in phosphate transport in *Staphylococcus aureus* to explain oxidative phosphorylation. I first came across what Mitchell called "chemiosmosis" when I saw his 1961 article in *Nature* (35), which I tried to present to the journal club in Stadtman's laboratory that met every day over lunch. All I could understand was that Mitchell was postulating that oxidative phosphorylation is driven by a pH gradient. This was a radical new idea at the least, and Mitchell made things worse by inventing his own vocabulary. Moreover, all of the big powers in biochemistry were convinced that oxidative phosphorylation involved a high-energy phosphate intermediate (X~P, as it was called). During the 1960s, we would go to the mitochondrial sessions at the FASEB meetings in Atlantic City for comic relief, as each big shot in bioenergetics would do the equivalent of throwing rotten tomatoes at Peter Mitchell.

According to chemiosmotic theory, the two basic energy-trapping devices on earth—sunlight and respiration—give rise to a transmembrane electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$) comprising both an electrical potential ($\Delta\Psi$) and a pH gradient (ΔpH), and it is this $\Delta\tilde{\mu}_{H^+}$, sometimes called proton-motive force, that is the immediate driving force for phenomena like oxidative phosphorylation, active transport, transhydrogenase, and the flagellar motor as well as other cellular activities (**Figure 6**).

By the mid-1960s, the tide had begun to turn. André Jagendorf & Uribe (36) demonstrated with chloroplasts that an acid jump can cause ATP synthesis, the first experiment that caused people (but not me!) to take notice. And people who "got it," like Guy Greville (37) and Franklin Harold (38), wrote reviews in which Mitchell's ideas were explained in understandable terms. Frank Harold and I became friendly enemies, as he was convinced that the vesicle system worked chemiosmotically. When I would present him with experimental findings that did not fit, Frank would say they simply could not be correct, and I would speak louder and louder.

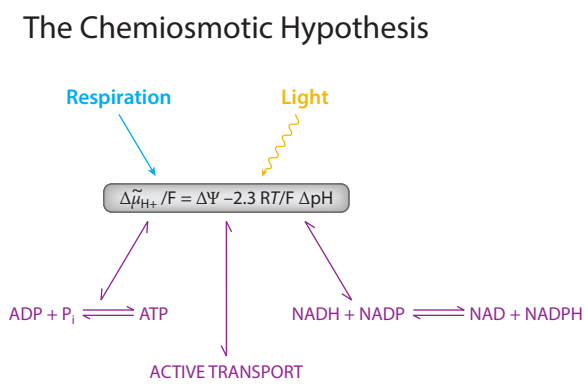

Figure 6

The chemiosmotic hypothesis. The immediate driving force for oxidative phosphorylation, photophosphorylation, secondary active transport, and transhydrogenation is a proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) that is generated from the respiration or absorption of light. Abbreviations: $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical gradient of protons; ΔpH, transmembrane pH difference; ΔΨ, transmembrane electrical potential; F, Faraday constant; R, gas constant; *T*, temperature (K). Figure adapted from Reference 114.

But John Reeves (39) showed that D-lactate oxidation by the vesicles causes transient acidification of the medium. Therefore, it seemed reasonable to think that D-lactate oxidation might generate a pH gradient across the membrane. (I talked John out of this silly notion!) In addition, West (40) and West & Mitchell (41) showed that galactoside transport by nonmetabolizing *E. coli* occurred with alkalinization of the external medium, thereby indicating that lactose might be transported across the membrane with an H^+. Thus, the tide was now turning for me, too.

However, Simon Silver and colleagues (42) showed that D-lactate oxidation in membrane vesicles from *E. coli* drives accumulation of K^+ or Rb^+, but only in the presence of valinomycin, and that valinomycin rescues certain K^+ transport mutants, but not others, thereby suggesting that valinomycin might be a cofactor for a potassium transporter that was lost during vesicle preparation. We confirmed the basic observations and extended them significantly (43).

When I presented a talk at the Albert Einstein College of Medicine in the early 1970s, Alan Finkelstein called my attention to an article from the laboratory of Liberman and Skulachev (44) in which lipophilic ions were used to measure the polarity of membrane potentials in mitochondria and chloroplasts. One such lipophilic cation is dimethyldibenzylammonium (DDA^+), which Frank Harold had shown qualitatively is accumulated by *Streptococcus faecalis*, indicating that it has an internal negative ΔΨ. I reckoned that DDA^+ should be easy to synthesize in radioactive form by reacting [^{14}C]methyl iodide with methyldibenzylammonium, which Jeff Lombardi accomplished in an afternoon. No accumulation of [^{14}C]DDA^+ by the vesicles was seen, and we concluded that valinomycin may be a cofactor (43).

However, at a 1974 New York Academy of Sciences meeting, Frank Harold approached me with a Cheshire cat smile and informed me that [^{14}C]DDA he had obtained commercially was accumulated by *E. coli* membrane vesicles during D-lactate oxidation. But to do so, a lipophilic anion tetraphenylborate (TPB^-) was also required (45), and Karl-Heinz Altendorf, then a postdoctoral fellow with Frank, came to my laboratory and reproduced the observations with my very own vesicles. But the need for TPB^- was unexpected and muddied the waters, particularly when John Reeves showed that DDA^+ accumulation in the presence TPB^- was inhibited by

N-ethylmaleimide (46). But at this point, I had to acknowledge that evidence for Mitchell's chemiosmotic concept was mounting with the vesicles, although I was still skeptical.

One of the advantageous things about RIMB being associated with Hoffman-LaRoche was that investigators at the Institute had use of the Isotope Synthesis Group headed by the late Arnold Liebman, a synthetic chemist whom I have already mentioned. Because lipophilic cations should act as uncoupling agents and collapse a membrane potential in a concentration-dependent manner, I tested all of the phosphonium- or arsonium-based lipophilic cations I could find, and tetraphenyl phosphonium (TPP^+) or tetraphenyl arsonium (TPA^+) were best, followed by the methyltriphenyl cations, and I asked Liebman if he could make them radioactive, which he did by catalytic exchange with tritium gas. (This will be important!) Although [^{3}H]triphenylmethylphosphonium ($TPMP^+$) accumulated in response to D-lactate or ascorbate/PMS oxidation, TPP^+ was not touched. So on the one hand, it seemed clear that D-lactate or ascorbate/PMS oxidation generates a membrane potential ($\Delta\Psi$) of ~100 mV, as determined with $TPMP^+$ (47). But, on the other hand, the tetraphenyl cation did not appear to equilibrate with $\Delta\Psi$. Moreover, the $\Delta\Psi$ measured with $TPMP^+$ was insufficient to account for the level of accumulation if the lactose/H^+ stoichiometry is 1:1.

At another New York Academy of Sciences meeting, in 1975, Erich Heinz from the Johann Wolfgang Goethe University in Frankfurt, Germany, gave a talk in which he used [^{3}H]TPP^+ to measure $\Delta\Psi$ in Ehrlich ascites cells (48), but the TPP^+ he had was at a very low specific activity. Therefore, relatively high concentrations had to be used, which could have partially dissipated $\Delta\Psi$. In any case, Heinz kindly provided me with a sample of his [^{3}H]TPP, and remarkably, it was accumulated by *E. coli* vesicles under appropriate conditions. Now we had a puzzle! Heinz's low specific activity TPP^+ appeared to equilibrate with $\Delta\Psi$, but our much higher specific activity [^{3}H]TPP did not, although both samples behaved identically on thin-layer chromatography. I then got the bright idea of having Sofia Ramos pass each compound over a Dowex 50 cation exchange column, and the results were absolutely clear-cut—Erich Heinz's TPP^+ stuck beautifully, while ours flowed right through! Apparently, although the triphenylmethyl cations behave nicely during catalytic tritium exchange, tetraphenyl cations do not. Rather than try to determine precisely why, Liebman synthesized [^{3}H]TPP^+ or [^{3}H]TPA^+ with triphenylphosphine or triphenylarsine and [^{3}H]phenylmagnesium bromide (Grignard reagent), and both lipophilic cations equilibrated with $\Delta\Psi$ in the vesicles as well as in various cultured eukaryotic cells (49–51). For a significant period of time, we provided the world with [^{3}H]$TPMP^+$ or [^{3}H]TPP^+.

As for the problem involving the low magnitude of the measured $\Delta\tilde{\mu}_{H^+}$, the obvious answer was that we were measuring only the $\Delta\Psi$ component and not ΔpH. But my laboratory had been trying hard for more than 5 years to measure ΔpH with a permeant weak acid by standard assays like filtration or centrifugation through silicon oil and had failed completely. However, Etana Padan and her colleagues showed that intact *E. coli* generate a ΔpH (interior alkaline) and that the magnitude of the ΔpH is dependent upon external pH, exhibiting a maximal value of ~2 pH units at pH 6.0 or below and zero pH units at pH ~7.5 (52, 53). In addition, Hagai Rottenberg (54) had utilized acetate to determine ΔpH in mitochondria, and he suggested that this weak acid might be more useful than the generally used permeant weak acid (5,5′-dimethyloxazolidine-2,4-dione) because it might be less apt to leak from the vesicles during filtration assays, which required dilution. [^{14}C]acetate was then used with the usual filtration assay, but the level of accumulation was far too low to account for much of a ΔpH.

Gary Rudnick and Shimon Schuldiner had been using flow dialysis (55) in the laboratory to measure binding/transport of azidophenyl- and dansyl-galactosides by the vesicles (56, 57), a technique that does not involve separating the vesicles from the bathing medium. One Friday afternoon, it occurred to me that flow dialysis might be perfect for measuring steady-state concentration gradients of weak acids, and I suggested the idea to Shimon. The next morning, I received

an excited phone call from him telling me that I had to come to the laboratory to see the flow dialysis run he had done, and it was indeed gratifying. At pH 5.5, he measured an ~100-fold concentration gradient of acetate or 2 units of ΔpH (inside alkaline). Sofia Ramos then went on to use flow dialysis to measure ΔpH with various weak acids, as well as $\Delta\tilde{\mu}_{H^+}$ (i.e., ΔpH + ΔΨ), which amounts to approximately −220 mV at pH 5.5 (approximately half ΔpH and half ΔΨ) (58, 59). We could also show that internal pH is constant at pH 7.6, and that ΔpH and ΔΨ are related reciprocally, so that collapsing ΔΨ with valinomycin led to an increase in ΔpH and collapsing ΔpH with nigericin at acid pH led to an increase in ΔΨ. Furthermore, unlike in mitochondria, these ionophores had no effect on respiration (i.e., the rate of H^+ pumping), indicating that respiratory control is not responsible for the reciprocal phenomena. We also used flow dialysis to determine steady-state concentration gradients of many different solutes and determined how they are coupled to the components of $\Delta\tilde{\mu}_{H^+}$ (60). Quantitative support for the weak-acid determinations of intracellular or intravesicular pH has come from high-resolution ^{31}P nuclear magnetic resonance spectroscopy (61, 62), and separate support for the lipophilic cation determinations of ΔΨ has come from electrophysiological measurements with oversized *E. coli* (63).

As shown with intact *E. coli* (52, 53) and RSO membrane vesicles (58, 59), ΔpH (interior alkaline) is constant at pH 7.6. Therefore, ΔpH is highest at acidic external pH values (at pH ~5.5) and decreases to zero as external pH approaches 7.6. Above pH 7.6, internal pH becomes acidic relative to external pH owing to the activity of the Na^+/H^+ antiporter, which exchanges 2 H^+ in for 1 Na^+ out (64). In an effort to understand the effect of external pH on ΔΨ and ΔpH, permeant lipophilic anions and weak bases, respectively, were used to measure the effect of external pH in inside-out membrane vesicles (65). Astoundingly, exactly the opposite effects were observed. ΔpH (interior acid) is maximal at pH ~7.6 and decreases to zero at pH ~5.5. Thus, it appears that no specific regulatory mechanism is in operation, but this remains an open question.

Although the overall impact of these studies provides convincing evidence that chemiosmosis is central to respiration-driven active transport in *E. coli*, the specificity for D-lactate oxidation in the vesicles remains a puzzle, particularly because Kazunobu Matsushita was able to reconstitute active lactose transport in proteoliposomes with high efficiency using only purified LacY and purified cytochrome *o* (66). Jeroen Hugenholtz was also finally able to demonstrate $\Delta\tilde{\mu}_{H^+}$-driven transport generated by ATP hydrolysis via F_1F_O-ATPase from ATP synthesized inside of RSO membrane vesicles by imaginative "vesicle engineering" (67). In addition, Hajime Tokuda showed that the melibiose permease catalyzes Na^+/galactoside transport by using H^+/Na^+ antiport to convert $\Delta\tilde{\mu}_{H^+}$ into $\Delta\tilde{\mu}_{Na+}$ (68, 69).

Where Do We Go From Here?

We could now clearly see a fork in the road. My laboratory could try to determine why D-lactate oxidation is specific for generating $\Delta\tilde{\mu}_{H^+}$ or measure $\Delta\tilde{\mu}_{H^+}$ or $\Delta\tilde{\mu}_{Na+}$ in other bacterial membrane vesicles or intracellular organelles of eukaryotic cells. Alternatively, we could focus on a single permease and try to work out its mechanism. Having started out believing that enzymology was key to understanding transport, I could not resist the alternative. Moreover, the obvious choice was LacY, as Benno Müller-Hill and colleagues (70) had cloned the *lacY* gene, the first gene encoding a membrane protein to be cloned and sequenced. This advance also allowed overexpression of LacY (71, 72).

Playing God with a Blindfold

In the summer of 1980, I took a 3-month sabbatical leave at the Hebrew University in Jerusalem primarily to learn rudimentary molecular biology in Amikam Cohen's laboratory and to try overexpressing LacY in Etana Padan's laboratory.

A short while after my return from Israel, Amikam Cohen visited RIMB and gave a seminar in which he mentioned a new technique called site-directed mutagenesis developed by Zoller & Smith (73), and I immediately recognized that although the technique would allow us to play god, we would do so with a blindfold, as we had no high-resolution structure. However, by this time, Beyreuther et al. (74) had demonstrated that Cys148 was the famous side chain in LacY that was protected against alkylation by substrate, thereby providing an indication that this Cys residue was in the substrate-binding site. Therefore, I got Bill Trumble, Paul Viitanen, and Hemanta Sarkar together and, using a synthetic mutagenic oligonucleotide synthesized by Mohindar Poonian with M13 phage, Cys148 was replaced with either Ser or Gly (75), the idea being that we would kill transport but learn how to do site-directed mutagenesis. The mutants were made twice, and twice C148S and C148G LacY exhibited good activity, leading me to accuse the guys of incompetence. But the mutants were made a third time and sequenced, and the mutations were clearly present. Thus, we drew the surprising conclusion that although Cys148 is essential for substrate protection against alkylation, it is not important for activity. However, transport was still partly inhibited by *N*-ethylmaleimide in the Cys148 mutants, which led to mutation of the other seven native cysteines in LacY, none of which were essential for activity (76). Finally, all eight cysteines were replaced in the same molecule, and a Cys-less LacY with reasonably good transport activity was obtained (77), thereby putting the final nail in the coffin of the Kaback and Barnes model for transport as well as other notions that Cys residues play a role in regulation.

Because no transporter had been successfully crystallized at this time, a *lacY* gene encoding Cys-less LacY with unique restriction sites at approximately every 100 base pairs was constructed, and over the next decade or so, each residue was changed initially to a Cys (78) [with the exception of the 17 C-terminal residues, which can be deleted with no effect on expression or activity (79)]. By this means, a library was built with the primary intention of carrying out extensive site-directed thiol cross-linking and other studies to obtain a model for helix packing without crystallization. Although such a model was constructed (80), once a crystal structure was obtained, it became clear that no amount of site-directed studies would yield a realistic structure because of the inherent flexibility of LacY and because thiol cross-linking gives the closest distance between two Cys side chains.

However, Cys-scanning mutagenesis (78) was particularly valuable for determining which residues are irreplaceable in the symport mechanism for stability and for all sorts of dynamic studies. Cys is average in bulk, of intermediate hydropathy, and amenable to highly specific modification with biochemical and biophysical probes. As evidenced by the widespread use of Cys mutagenesis with many different membrane proteins, the technique has gained great favor in general (e.g., 81).

To determine which residues play an obligatory role in the mechanism and to create a library of mutants with a single-Cys residue at each position of LacY for structure/function studies, each residue in Cys-less LacY was replaced individually with Cys. The great majority of the 401 single-Cys mutants are expressed normally in the membrane and catalyze accumulation of lactose against a significant concentration gradient, thereby demonstrating that Cys replacement at most positions does not induce severe perturbations in the structure of the permease or in the symport mechanism. Mutation of 92 residues inhibits the steady-state level of accumulation by >50%, but residual accumulation against a gradient is still evident (78). However, only nine side chains are absolutely irreplaceable for symport as shown by mutagenesis: Glu126 (helix IV), Arg144 (helix V), Trp151 (helix V), Glu269 (helix VIII), Asn272 (helix VIII), His322 (helix X), Tyr236 (helix VII), Arg302 (helix IX), and Glu325 (helix X). Furthermore, conservative replacement of these irreplaceable residues is worse than replacement with a neutral side chain (82), except for Trp151, where a Trp is preferred but Tyr or Phe replacements are still active (83); Asn272,

where replacement with Gln is fully active (84); and Glu126, where the Asp replacement has partial activity. Although it was suggested that Tyr236, Glu269, and His322 are involved in coupled H^+ transport, X-ray crystal structures with occluded galactosides show directly that each irreplaceable residue with the exception of Arg302 and Glu325 makes direct contact with the galactopyranosyl moiety of two different, relatively high-affinity lactose homologs (85, 86).

Nancy Carrasco, a most intelligent, creative, and highly motivated colleague, demonstrated that Glu325 is directly involved in coupled H^+ transport after I advised her not to mutate it (87, 88). Neutral replacements resulted in a molecule in which all reactions involving H^+ transport are abolished, but the mutants bind galactosides and catalyze transmembrane exchange of lactose (i.e., equilibrium exchange and counterflow) at least as well as wild-type LacY does. Moreover, although the mutants exhibit uniport of lactose (equilibration of lactose across the membrane without H^+ translocation), they do so only at high concentrations [the major kinetic effect of $\Delta\tilde{\mu}_{H^+}$ is to decrease the K_m (89)]. Another highly productive and very bright postdoctoral fellow, Miklós Sahin-Tóth, showed that replacement of Arg302 with Ala or Ser produces a similar phenotype (90). Therefore, it was postulated that this Arg residue may play a role in deprotonation of Glu325.

In the early 1980s, we also obtained our first bit of structural insight into LacY when David Foster, Milos Boublik, and I showed by circular dichroism that LacY is ~85% helical (91), a result that comes satisfyingly close to 86%, as determined later from the X-ray crystal structure (92). We also concluded from hydropathy profiling that LacY probably comprises 12 transmembrane α-helices with both the N and C termini on the cytoplasmic side of the cell membrane. Although the first 6 and the last 2 hydrophobic segments were clear-cut, segments 7–10 were not, which led others to predict 14 transmembrane helices. However, the studies of Calamia & Manoil (93) with alkaline phosphatase fusions strongly supported the 12-helix model, and this number of transmembrane helices was ultimately fully confirmed by the X-ray crystal structures. Here is an instance where it was clearly better to be lucky than smart.

Back to Biochemistry

During this same period of time, a number of biochemical observations were made that were not fully appreciated until we finally obtained X-ray structures and began to think seriously about what $\Delta\tilde{\mu}_{H^+}$ is actually doing mechanistically as well as thermodynamically. For example, imposition of $\Delta\tilde{\mu}_{H^+}$ (ΔpH or $\Delta\Psi$) has little or no effect on transmembrane lactose exchange (94–96), although it decreases K_m 50- to 100-fold with little effect on V_{max} (89). As β-galactosidase has a very high turnover number, no free lactose is observed in the cytoplasm of *E. coli*, because each molecule transported is immediately cleaved into galactose and glucose. So why does *E. coli* need to transport lactose against a concentration gradient? The answer lies in the kinetics. An *E. coli* lacking a $\Delta\tilde{\mu}_{H^+}$-coupled LacY will be outgrown in an environment where the lactose concentration is low because the functional affinity of the system is low (i.e., K_m is 50–100 times higher in the absence of $\Delta\tilde{\mu}_{H^+}$).

In 1980, Michael Newman & T.H. Wilson (72) made a seminal discovery by solubilizing LacY from the membrane with the detergent octyl-β-D-glucopyranoside and reconstituting the protein functionally in proteoliposomes. They also demonstrated that adding exogenous phospholipids during solubilization is essential to obtain functional LacY. In addition, Greg Kaczorowski et al. (97) showed that LacY can be photolabeled specifically with radiolabeled *p*-nitrophenyl-α-D-galactopyranoside (α-NPG), which was particularly useful for following the protein during purification. These advances led to a collaboration between the Wilson and Kaback laboratories that yielded purified LacY in a fully functional state (98). Unmodified LacY was purified

by a simple procedure employing differential solubilization and ion-exchange chromatography and reconstituted into proteoliposomes by octylglucoside dilution. The proteoliposomes exhibited both $\Delta\Psi$-driven lactose transport and transmembrane lactose exchange. Furthermore, the purified protein was identified as the product of the *lacY* gene. Subsequent experiments by Paul Viitanen and colleagues (99, 100) primarily demonstrated that purified, reconstituted LacY is fully functional. When affinity purification techniques came into vogue, a biotin acceptor domain was engineered onto the C terminus of LacY, which was biotinylated in vivo, and LacY was purified by avidin affinity chromatography (101). However, only ~15% of the protein was biotinylated unless a second step utilizing biotin ligase and ATP was used to completely biotinylate the protein (102). However, in the interest of simplicity, we ultimately settled on engineering 6 to 10 His residues on to the C terminus of LacY and using metal chelate chromatography (Talon) to obtain highly purified LacY in a single step, as demonstrated by mass spectrometry (103). It is frequently possible to overexpress soluble cytoplasmic proteins to 50% or more of the cell protein. But with membrane proteins like LacY, 10% of the membrane protein (i.e., 1% of the cell protein) is considered to be good overexpression.

WESTWARD HO

One winter day in 1988, I received a phone call from Dean Kenneth Shine at the University of California, Los Angeles (UCLA), inviting me to consider taking the Chair of Physiology in the Medical School. I responded that I was not interested, but Dean Shine convinced me that the weather was gorgeous in Los Angeles and I should come and enjoy some time in the sun. At the end of the visit, I had an appointment with Dean Shine, who asked me what would bring me to UCLA. Without knowing much about it except rumors, I answered that I would seriously consider moving if I became an Investigator of the Howard Hughes Medical Institute (HHMI). I returned to New Jersey, and approximately a year later, I received another call from Dean Shine offering me positions as an HHMI Investigator and Professor of Physiology at UCLA.

Prior to the move from New Jersey, I hired Kerstin Stempel as laboratory manager and a couple of technicians to outfit the new but temporary laboratory, and I flew to Los Angeles every month or so to see how things were going. For the first year or so, the laboratory was in the Molecular Biology Institute (Paul Boyer Hall) awaiting construction of the MacDonald Research Laboratories, where we would eventually end up on the sixth floor. But as I was relatively late appearing at UCLA, some of my postdoctoral fellows, one in particular, arrived before me. That postdoctoral fellow was a terrifically creative Israeli named Eitan Bibi, presently chairman of biochemistry at the Weizmann Institute. Within a month or so, Eitan came into my office and said in his Israeli accent, "Ron, I vunt to tayk de lac vy gene an' express it in two pieces." My immediate response was, "You're out of your mind. That will never work, but if you're determined, give it a try."

By November, Eitan had constructed two plasmids, one encoding the N-terminal half of LacY and the other encoding the C-terminal half. When he transformed indicator cells with either plasmid alone, the colonies were white, indicating that each fragment by itself either was degraded or had no activity. But when he transformed with both plasmids, the colonies were red, indicating that the fragments were made, somehow found each other in the membrane, and formed a functional split molecule (104). Kevin Zen and colleagues (105) later extended the finding and showed that functional splits can be made at many places in LacY, not just in the middle of the molecule.

One of the attractions of Southern California was that I could play tennis virtually any time I wanted, and UCLA has superb tennis facilities. I would leave the laboratory in the early evening a couple of times during the week and in the late mornings on weekends and play tennis for a couple of hours before going home. Also, as my knees were shot from playing football, I worked out

three times a week religiously, and I thought I was in pretty good shape despite a previous cardiac problem secondary to hypercholesterolemia that had responded well to treatment. However, just prior to Labor Day weekend in 1991, while my wife was on the East Coast, I developed a strange cough with an uncomfortable feeling in my neck and jaw that increased when I walked faster. A confirmed denier, I suppressed what I knew was very likely angina for a week, but on Friday afternoon I told a physician working in my laboratory, and he refused to leave the office until I called my cardiologist. When I described the symptoms, the cardiologist advised me to go to the emergency room immediately, but when I refused, he had me come to his office where a resting electrocardiogram was normal. He still wanted to admit me to the hospital, but as I would have had to call my wife, who would immediately fly home and nothing would be done in the hospital over the weekend anyway, I talked him out of it. However, we made a deal. The cardiologist gave me a bottle of nitroglycerin pills. The deal was that if the symptom recurred, I was to put one under my tongue, and if it disappeared, I would call him immediately. I was also to show up for a stress visualization of my heart on Monday morning. I lasted approximately 30 seconds on the treadmill, and visualization indicated that I was hardly perfusing my myocardium. At this point, it was clear that I was a candidate for bypass surgery, and I had to call my wife to return. The surgery was done a couple of days later, and more than 25 years have now passed.

From 2000 to 2007, a huge amount of experimental work came from highly committed, superlatively intelligent individuals like Lan Guan and two superb senior biochemistry colleagues, Irina (Smirnova) Kasho and Vladimir Kasho, almost all of it focusing on LacY as a model membrane transport protein. From 1989 to 2004, essentially all of the work was funded by the HHMI, and although I do not want to overemphasize the point, no NIH study section would have financed the studies that produced breakthroughs. But being an HHMI Investigator was not always a pleasure. Each year, HHMI scientists must attend a meeting at HHMI headquarters, then a lovely place in Chevy Chase, Maryland, that was like a five-star hotel. However, the atmosphere was tense, as everyone was concerned that they might lose HHMI support if their presentation was deemed inferior, and every 5 years, an outside advisory committee reviewed individual research programs. However, it was here that Chris Miller courageously and comically credited me with the invention of sulfur! My last review took place in 2002, when we had finally obtained crystals of LacY in collaboration with Jeff Abramson and So Iwata at Imperial College London but did not yet have the structure, and it seemed to me that the presentation went quite well. However, the day I returned to the laboratory, I received a fax from Jerry Rubin, vice president for research at HHMI, informing me that I was being dropped and had 2 years to obtain other support. I was totally shocked and even thought a mistake had been made, but a call to Rubin confirmed the fax. As we were clearly on the verge of obtaining the first X-ray crystal structure of this type of membrane protein, I called Tom Cech, then president of HHMI. But when he pronounced after a couple of conversations that "there will be no more paradigm shifts with Lac permease," I realized that the phone calls and letters from supporters were hopeless.

Although I was a lame duck, I still had to attend the yearly research meeting at headquarters. The last one was in May 2004, and I even had to make a presentation. When my turn to speak came up, I approached the stage with a fedora in hand and placed it upside down on the podium. I then proceeded to give my talk, focusing on the recently published X-ray structure of LacY, which made the cover of *Science* (92). At the end of the talk, I pointed to the hat and said, "For those of you who would like to see this work continue, please leave money in the hat as you leave." This brought the house down with laughter. To this day, I have no idea why I was dropped; one reason may be that I had turned 65, but the HHMI cannot be guilty of age discrimination—they might get sued!

SUMMARY

I have been very lucky indeed, and much of what has transpired has indeed been an adventure in serendipity. To have met my wife, the love of my life, when we were children is certainly lucky in itself, and to have maintained our relationship for more than 60 years borders on the miraculous in this day and age. To a great extent, the success of our marriage has involved mutual compromise, and it is certain that she compromised to a much greater degree, thereby allowing me to pursue my obsessions.

Had I not attended Haverford College at the specific time that I did, I doubt very much that I would have become a scientist. Not only did I get turned on to modern biology by Ariel Loewy and Mel Santer, but because of Kornberg's visit and my senior thesis on Gram's stain, I would never have gotten the crazy idea that there might be another species of RNA in the membrane that is involved in amino acid transport. This led in turn to my "discovery" of membrane vesicles as a model system in which to study transport. It took more than another dozen years to discover serendipitously how to make them transport like intact cells with D-lactate or ascorbate/PMS.

Then came what I call the "Chemiosmotic Wars," which raged within the bioenergetics community. On one side were the chemically oriented, who believed in X~P or conformational change, and on the other side was Peter Mitchell, a loner, and his chemiosmotic theory. Having shown that glucose transport in *E. coli* involves a covalent reaction and for other reasons, I initially postulated that LacY might be a respiratory intermediate, but I also began to take the Mitchell hypothesis seriously, certainly prodded by Shimon Schuldiner and particularly by my relationship with Frank Harold. This reevaluation led to the development of the methodology to quantify the membrane potential and the pH gradient in the vesicles and relate them to concentration gradients of various solutes. Mitchell, the dark horse, was the winner and was awarded the Nobel Prize in Chemistry in 1978.

Along came the cloning of *lacY* by Müller-Hill and colleagues, which yielded the amino acid sequence of LacY and enabled its solubilization and purification. Site-directed mutagenesis was developed or, in the absence of structure, we played God with a blindfold. No study section on Earth would have funded either the construction of a Cys-less LacY or the replacement of essentially each residue in LacY with a Cys. Although the idea behind this work was to obtain a structure by thiol cross-linking and other site-directed methods, important information was obtained. It was found that only 9 out of the 417 residues in LacY are irreplaceable with respect to transport. Furthermore, these residues were found to be clustered in the approximate middle of the LacY molecule.

EPILOGUE

Although space considerations preclude discussion of the most recent findings leading to a hypothesis for a molecular mechanism of H^+/lactose symport, the reader is referred to a recent "Viewpoint" article in the *Journal of General Physiology* (106).

In brief, the first X-ray structure of LacY was obtained in 2003 (92), which confirmed much of the biochemistry and molecular biology. The initial structure contained two bundles of six mostly irregular helices surrounding a water-filled cavity open on the cytoplasmic side and tightly sealed on the periplasmic side. For the next decade, we continued to get crystal structures in the same conformation until X-ray structures of a double-Trp mutant obtained in collaboration with Robert Stroud's laboratory were found to contain an occluded galactoside and to be narrowly open on the periplasmic side and tightly sealed on the cytoplasmic side. Comparison with another structure

containing a covalently bound galactoside provides an indication that sugar binding by LacY may involve induced fit (107).

By utilizing eight independent biochemical/spectroscopic approaches, it was shown that LacY catalyzes symport by an alternating access mechanism that allows the sugar- and H^+-binding sites to become alternatively accessible to either side of the membrane. Additional experimental findings indicate the following:

- The limiting step for lactose/H^+ symport in the absence of $\Delta\tilde{\mu}_{H^+}$ is deprotonation, whereas in the presence of $\Delta\tilde{\mu}_{H^+}$, the limiting step is probably opening of apo LacY on the other side of the membrane.
- Glu325 in helix X must be protonated to bind galactoside (the pK of Glu325 is ~10.5, as is the apparent pK for galactoside binding) (108).
- Galactoside binding and dissociation—not $\Delta\tilde{\mu}_{H^+}$—are the driving forces for alternating access.
- Galactoside binding involves induced fit, causing transition to an occluded intermediate that undergoes alternating access.
- Galactoside dissociates, releasing the energy of binding.
- Arg302 (helix IX) comes into proximity with protonated Glu325, causing deprotonation. Accumulation of galactoside against a concentration gradient does not involve a change in K_D on either side of the membrane (109), but the pK (the affinity for H^+) decreases markedly. Thus, transport is driven chemiosmotically, but contrary to expectation, $\Delta\tilde{\mu}_{H^+}$ acts kinetically to control the rate of the process.

In a collaborative effort with Jan Steyaert's group in Brussels, Belgium, single-domain camelid nanobodies that bind to the periplasmic side of LacY and stabilize outward–open conformations have been utilized. Four Nb/LacY X-ray crystal structures have been obtained thus far.

In conclusion, I visualize scientific endeavor as an infinite number of closed doors behind which a dim light (the "good idea") is randomly distributed at the end of a difficult circuitous pathway. To see the light, an investigator must open as many doors as possible, because the light is dim and randomly distributed. If a light is perceived, the investigator then has to be willing to struggle through that difficult circuitous pathway to reach the objective, which is on solid ground only so long as it is not disproven. Thus, if the good fairy arrives waving a magic wand and offers you a choice between being lucky or being smart, always pick lucky—but work your backside off in addition!

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This article is dedicated to Teenchy, my wonderful wife of more than 60 years. I am also deeply indebted to the members of my research group and my collaborators over the past 50 years, who contributed their minds, hearts, and hands to this work.

In addition, I am grateful to Chris Miller for editorial suggestions. At one time or another, the studies were supported financially by the National Heart Institute, the Roche Institute of Molecular Biology, and the Howard Hughes Medical Institute and by National Institutes of Health and National Science Foundation grants. I also thank Ruth and Bucky Stein for a generous gift.

LITERATURE CITED

1. Santer M, Margulies M, Klineman N, Kaback HR. 1960. Role of inorganic phosphate in thiosulfate metabolism by *Thiobacillus thioparus*. *J. Bact.* 79:313–20
2. Kaback HR, Stadtman ER. 1966. Proline uptake by an isolated cytoplasmic membrane preparation of *Escherichia coli*. *PNAS* 55(4):920–27
3. Kaback HR, Kostellow AB. 1968. Glycine uptake in *Escherichia coli*. I. Glycine uptake by whole cells of *Escherichia coli* W^+ and a D-serine-resistant mutant. *J. Biol. Chem.* 243(7):1384–89
4. Kaback HR, Stadtman ER. 1968. Glycine uptake in *Escherichia coli*. II. Glycine uptake, exchange, and metabolism by an isolated membrane preparation. *J. Biol. Chem.* 243(7):1390–400
5. Kundig W, Kundig FD, Anderson B, Roseman S. 1966. Restoration of active transport of glycosides in *Escherichia coli* by a component of a phosphotransferase system. *J. Biol. Chem.* 241(13):3243–46
6. Kaback HR. 1968. The role of the phosphoenolpyruvate-phosphotransferase system in the transport of sugars by isolated membrane preparations of *Escherichia coli*. *J. Biol. Chem.* 243(13):3711–24
7. Fox CF, Kennedy EP. 1965. Specific labeling and partial purification of the M protein, a component of the β-galactoside transport system of *Escherichia coli*. *PNAS* 54:891–99
8. Ameyama M, Nonobe M, Shinagawa E, Matsushita K, Taimoto K, Adachi O. 1986. Purification and characterization of the quinoprotein D-glucose dehydrogenase apoenzyme from *Escherichia coli*. *Agric. Biol. Chem.* 50:49–57
9. Yamada M, Sumi K, Matsushita K, Adachi O, Yamada Y. 1993. Topological analysis of quinoprotein glucose dehydrogenase in *Escherichia coli* and its ubiquinone-binding site. *J. Biol. Chem.* 268:12812–17
10. Matsushita K, Arents JC, Bader R, Yamada M, Adachi O, Posta PW. 1997. *Escherichia coli* is unable to produce pyrroloquinoline quinone (PQQ). *Microbiology* 143:3149–56
11. Matsushita K, Nonobe M, Shinagawa E, Adachi O, Ameyama M. 1987. Reconstitution of pyrroloquinoline quinone-dependent D-glucose oxidase respiratory chain of *Escherichia coli* with cytochrome *o* oxidase. *J. Bacteriol.* 169:205–9
12. Kaback HR, Milner LS. 1970. Relationship of a membrane-bound D-(—)-lactic dehydrogenase to amino acid transport in isolated bacterial membrane preparations. *PNAS* 66(3):1008–15
13. Müller-Hill B. 1996. *The* Lac *Operon: A Short History Of A Genetic Paradigm*. Berlin: Walter de Gruyter
14. Cohen GN, Rickenberg HV. 1955. Etude directe de la fixation d'un inducteur de la b-galactosidase par les cellules d'*Escherichia coli*. *C. R. Hebd. Séances Acad. Sci.* 240:466–68
15. Ling GN. 1962. *A Physical Theory of the Living State: The Association-Induction Hypothesis*. London: Blaisdell
16. Barnes EM Jr., Kaback HR. 1970. β-galactoside transport in bacterial membrane preparations: energy coupling via membrane-bounded D-lactic dehydrogenase. *PNAS* 66(4):1190–98
17. Barnes EM Jr., Kaback HR. 1971. Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and β-galactoside transport in *Escherichia coli* membrane vesicles. *J. Biol. Chem.* 246(17):5518–22
18. Kaback HR. 2016. *Adventures in Serendipity*. Bloomington, IN: Xlibris
19. Kaback HR. 1971. Bacterial membranes. In *Methods in Enzymology*, ed. NP Kaplan, WB Jakoby, NP Colowick, Vol. XXII, pp. 99–120. New York: Elsevier
20. Short SA, Kaback HR, Kohn LD. 1975. Localization of D-lactate dehydrogenase in native and reconstituted *Escherichia coli* membrane vesicles. *J. Biol. Chem.* 250(11):4291–96
21. Owen P, Kaback HR. 1978. Molecular structure of membrane vesicles from *Escherichia coli*. *PNAS* 75(7):3148–52
22. Owen P, Kaback HR. 1979. Antigenic architecture of membrane vesicles from *Escherichia coli*. *Biochemistry* 18(8):1422–26
23. Owen P, Kaback HR. 1979. Immunochemical analysis of membrane vesicles from *Escherichia coli*. *Biochemistry* 18(8):1413–22
24. Walsh CT, Abeles RH, Kaback HR. 1972. Mechanisms of active transport in isolated bacterial membrane vesicles. X. Inactivation of D-lactate dehydrogenase and D-lactate dehydrogenase-coupled transport in *Escherichia coli* membrane vesicles by an acetylenic substrate. *J. Biol. Chem.* 247(24):7858–63

25. Schonbrunn A, Abeles RH, Walsh CT, Ghisla S, Ogata H, Massey V. 1976. The structure of the covalent flavin adduct formed between lactate oxidase and the suicide substrate 2-hydroxy-3-butynoate. *Biochemistry* 15(9):1798–807
26. Walsh CT, Kaback HR. 1973. Vinylglycolic acid. An inactivator of the phosphoenolpyruvate-phosphate transferase system in *Escherichia coli*. *J. Biol. Chem.* 248(15):5456–62
27. Short SA, Kaback HR, Kaczorowski G, Fisher J, Walsh CT, Silverstein SC. 1974. Determination of the absolute number of *Escherichia coli* membrane vesicles that catalyze active transport. *PNAS* 71(12):5032–36
28. Konings WN, Barnes EM Jr., Kaback HR. 1971. Mechanisms of active transport in isolated membrane vesicles. *J. Biol. Chem.* 246:5857–61
29. Kaback HR, Barnes EM Jr. 1971. Mechanisms of active transport in isolated membrane vesicles. II. The mechanism of energy coupling between D-lactic dehydrogenase and β-galactoside transport in membrane preparations from *Escherichia coli*. *J. Biol. Chem.* 246(17):5523–31
30. Ting HP, Wilson DF, Chance B. 1970. Effects of uncouplers of oxidative phosphorylation on the specific conductance of bimolecular lipid membranes. *Arch. Biochem. Biophys.* 141(1):141–46
31. Kaback HR, Reeves JP, Short SA, Lombardi FJ. 1974. Mechanisms of active transport in isolated bacterial membrane vesicles. XVIII. The mechanism of action of carbonylcyanide *m*-chlorophenylhydrazone. *Arch. Biochem. Biophys.* 160(1):215–22
32. Mitchell P. 1963. Molecule, group and electron transport through natural membranes. *Biochem. Soc. Symp.* 22:142–68
33. Mitchell P. 1967. Translocations through natural membranes. *Adv. Enzymol.* 29:33–87
34. Mitchell P. 1968. *Chemiosmotic Coupling and Energy Transduction*. Bodmin, UK: Glynn Research Ltd.
35. Mitchell P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* 191:144–48
36. Jagendorf AT, Uribe E. 1966. ATP formation caused by acid-base transition of spinach chloroplasts. *PNAS* 55(1):170–77
37. Greville GD. 1969. A scrutiny of Mitchell's chemiosmotic hypothesis. *Curr. Top. Bioenerg.* 3:1–78
38. Harold FM. 1972. Conservation and transformation of energy by bacterial membranes. *Bacteriol. Rev.* 36(2):172–230
39. Reeves JP. 1971. Transient pH changes during D-lactate oxidation by membrane vesicles. *Biochem. Biophys. Res. Commun.* 45(4):931–36
40. West IC. 1970. Lactose transport coupled to proton movements in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 41:655–61
41. West IC, Mitchell P. 1972. Proton-coupled β-galactoside translocation in non-metabolizing *Escherichia coli*. *J. Bioenerg.* 3:445–62
42. Bhattacharyya P, Epstein W, Silver S. 1971. Valinomycin-induced uptake of potassium in membrane vesicles from *Escherichia coli*. *PNAS* 68(7):1488–92
43. Lombardi FJ, Reeves JP, Kaback HR. 1973. Mechanisms of active transport in isolated bacterial membrane vesicles. XIII. Valinomycin-induced rubidium transport. *J. Biol. Chem.* 248(10):3551–65
44. Bakeeva LE, Grinius LL, Jasaitis AA, Kuliene VV, Levitsky DO, et al. 1970. Conversion of biomembrane-produced energy into electric form. II. Intact mitochondria. *Biochim. Biophys. Acta Bioenerg.* 216(1):13–21
45. Harold FM. 1974. Chemiosmotic interpretation of active transport in bacteria. *Ann. NY Acad. Sci.* 227:297–311
46. Lombardi FJ, Reeves JP, Short SA, Kaback HR. 1974. Evaluation of the chemiosmotic interpretation of active transport in bacterial membrane vesicles. *Ann. NY Acad. Sci.* 227:312–27
47. Schuldiner S, Kaback HR. 1975. Membrane potential and active transport in membrane vesicles from *Escherichia coli*. *Biochemistry* 14(25):5451–61
48. Heinz E, Geck P, Pietrzyk C. 1975. Driving forces of amino acid transport in animal cells. *Ann. NY Acad. Sci.* 264:428–41
49. Kiefer H, Blume AJ, Kaback HR. 1980. Membrane potential changes during mitogenic stimulation of mouse spleen lymphocytes. *PNAS* 77(4):2200–204

50. Lichtshtein D, Dunlop K, Kaback HR, Blume AJ. 1979. Mechanism of monensin-induced hyperpolarization of neuroblastoma-glioma hybrid NG108-15. *PNAS* 76(6):2580–84
51. Lichtshtein D, Kaback HR, Blume AJ. 1979. Use of a lipophilic cation for determination of membrane potential in neuroblastoma-glioma hybrid cell suspensions. *PNAS* 76(2):650–54
52. Padan E, Zilberstein D, Rottenberg H. 1976. The proton electrochemical gradient in *Escherichia coli* cells. *Eur. J. Biochem.* 63(2):533–41
53. Zilberstein D, Schuldiner S, Padan E. 1979. Proton electrochemical gradient in *Escherichia coli* cells and its relation to active transport of lactose. *Biochemistry* 18(4):669–73
54. Rottenberg H. 1975. The measurement of transmembrane electrochemical proton gradients. *J. Bioenerg.* 7(2):61–74
55. Colowick SP, Womack FC. 1969. Binding of diffusible molecules by macromolecules: rapid measurement by rate of dialysis. *J. Biol. Chem.* 244(4):774–77
56. Rudnick G, Schuldiner S, Kaback HR. 1976. Equilibrium between two forms of the *lac* carrier protein in energized and nonenergized membrane vesicles from *Escherichia coli*. *Biochemistry* 15(23):5126–31
57. Schuldiner S, Weil R, Kaback HR. 1976. Energy-dependent binding of dansylgalactoside to the *lac* carrier protein: direct binding measurements. *PNAS* 73(1):109–12
58. Ramos S, Schuldiner S, Kaback HR. 1976. The electrochemical gradient of protons and its relationship to active transport in *Escherichia coli* membrane vesicles. *PNAS* 73(6):1892–96
59. Ramos S, Kaback HR. 1977. The electrochemical proton gradient in *Escherichia coli* membrane vesicles. *Biochemistry* 16(5):848–54
60. Ramos S, Kaback HR. 1977. The relationship between the electrochemical proton gradient and active transport in *Escherichia coli* membrane vesicles. *Biochemistry* 16(5):854–59
61. Navon G, Ogawa S, Shulman RG, Yamane T. 1977. High-resolution ^{31}P nuclear magnetic resonance studies of metabolism in aerobic *Escherichia coli* cells. *PNAS* 74(3):888–91
62. Ogawa S, Shulman RG, Glynn P, Yamane T, Navon G. 1978. On the measurement of pH in *Escherichia coli* by ^{31}P nuclear magnetic resonance. *Biochim. Biophys. Acta Bioenerg.* 502(1):45–50
63. Felle H, Porter JS, Slayman CL, Kaback HR. 1980. Quantitative measurements of membrane potential in *Escherichia coli*. *Biochemistry* 19(15):3585–90
64. Hunte C, Screpanti E, Venturi M, Rimon A, Padan E, Michel H. 2005. Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. *Nature* 435(7046):1197–202
65. Reenstra WW, Patel L, Rottenberg H, Kaback HR. 1980. Electrochemical proton gradient in inverted membrane vesicles from *Escherichia coli*. *Biochemistry* 19(1):1–9
66. Matsushita K, Patel L, Gennis RB, Kaback HR. 1983. Reconstitution of active transport in proteoliposomes containing cytochrome *o* oxidase and *lac* carrier protein purified from *Escherichia coli*. *PNAS* 80(16):4889–93
67. Hugenholtz J, Hong JS, Kaback HR. 1981. ATP-driven active transport in right-side-out bacterial membrane vesicles. *PNAS* 78(6):3446–49
68. Tokuda H, Kaback HR. 1977. Sodium-dependent methyl 1-thio-β-d-galactopyranoside transport in membrane vesicles isolated from *Salmonella typhimurium*. *Biochemistry* 16(10):2130–36
69. Tokuda H, Kaback HR. 1978. Sodium-dependent binding of *p*-nitrophenyl α-d-galactopyranoside to membrane vesicles isolated from *Salmonella typhimurium*. *Biochemistry* 17(4):698–705
70. Büchel DE, Gronenborn B, Müller-Hill B. 1980. Sequence of the lactose permease gene. *Nature* 283(5747):541–45
71. Teather RM, Müller-Hill B, Abrutsch U, Aichele G, Overath P. 1978. Amplification of the lactose carrier protein in *Escherichia coli* using a plasmid vector. *Mol. Gen. Genet.* 159:239–48
72. Newman MJ, Wilson TH. 1980. Solubilization and reconstitution of the lactose transport system from *Escherichia coli*. *J. Biol. Chem.* 255(22):10583–86
73. Zoller MJ, Smith M. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* 100:468–500
74. Beyreuther K, Bieseler B, Ehring R, Müller-Hill B. 1982. Identification of internal residues of lactose permease of *Escherichia coli* by radiolabel sequences of peptide mixtures. In *Methods in Protein Sequence Analysis*, ed. M Elzinga, pp. 139–48. Experimental Biology and Medicine, Vol. 3. Clifton, NY: Humana

75. Trumble WR, Viitanen PV, Sarkar HK, Poonian MS, Kaback HR. 1984. Site-directed mutagenesis of cys_{148} in the *lac* carrier protein of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 119(3):860–67
76. van Iwaarden PR, Driessen AJ, Menick DR, Kaback HR, Konings WN. 1991. Characterization of purified, reconstituted site-directed cysteine mutants of the lactose permease of *Escherichia coli*. *J. Biol. Chem.* 266:15688–92
77. van Iwaarden PR, Pastore JC, Konings WN, Kaback HR. 1991. Construction of a functional lactose permease devoid of cysteine residues. *Biochemistry* 30(40):9595–600
78. Frillingos S, Sahin-Tóth M, Wu J, Kaback HR. 1998. Cys-scanning mutagenesis: a novel approach to structure function relationships in polytopic membrane proteins. *FASEB J.* 12(13):1281–99
79. McKenna E, Hardy D, Kaback HR. 1992. Evidence that the final turn of the last transmembrane helix in the lactose permease is required for folding. *J. Biol. Chem.* 267(10):6471–74
80. Sorgen PL, Hu Y, Guan L, Kaback HR, Girvin ME. 2002. An approach to membrane protein structure without crystals. *PNAS* 99(22):14037–40
81. Karlin A, Akabas MH. 1998. Substituted-cysteine accessibility method. *Methods Enzymol.* 293:123–45
82. Kaback HR, Sahin-Tóth M, Weinglass AB. 2001. The kamikaze approach to membrane transport. *Nat. Rev. Mol. Cell Biol.* 2(8):610–20
83. Guan L, Hu Y, Kaback HR. 2003. Aromatic stacking in the sugar binding site of the lactose permease. *Biochemistry* 42(6):1377–82
84. Jiang X, Villafuerte MK, Andersson M, White SH, Kaback HR. 2014. Galactoside-binding site in LacY. *Biochemistry* 53(9):1536–43
85. Kumar H, Kasho V, Smirnova I, Finer-Moore JS, Kaback HR, Stroud RM. 2014. Structure of sugar-bound LacY. *PNAS* 111(5):1784–88
86. Kumar H, Finer-Moore JS, Kaback HR, Stroud RM. 2015. Structure of LacY with an α-substituted galactoside: connecting the binding site to the protonation site. *PNAS* 112(29):9004–9
87. Carrasco N, Antes LM, Poonian MS, Kaback HR. 1986. *Lac* permease of *Escherichia coli*: histidine-322 and glutamic acid-325 may be components of a charge-relay system. *Biochemistry* 25(16):4486–88
88. Carrasco N, Puttner IB, Antes LM, Lee JA, Larigan JD, et al. 1989. Characterization of site-directed mutants in the *lac* permease of *Escherichia coli*. 2. Glutamate-325 replacements. *Biochemistry* 28(6):2533–39
89. Robertson DE, Kaczorowski GJ, Garcia ML, Kaback HR. 1980. Active transport in membrane vesicles from *Escherichia coli*: the electrochemical proton gradient alters the distribution of the *lac* carrier between two different kinetic states. *Biochemistry* 19(25):5692–702
90. Sahin-Tóth M, Kaback HR. 2001. Arg-302 facilitates deprotonation of Glu-325 in the transport mechanism of the lactose permease from *Escherichia coli*. *PNAS* 98(11):6068–73
91. Foster DL, Boublik M, Kaback HR. 1983. Structure of the *lac* carrier protein of *Escherichia coli*. *J. Biol. Chem.* 258(1):31–34
92. Abramson J, Smirnova I, Kasho V, Verner G, Kaback HR, Iwata S. 2003. Structure and mechanism of the lactose permease of *Escherichia coli*. *Science* 301(5633):610–15
93. Calamia J, Manoil C. 1990. *Lac* permease of *Escherichia coli*: topology and sequence elements promoting membrane insertion. *PNAS* 87(13):4937–41
94. Kaczorowski GJ, Robertson DE, Kaback HR. 1979. Mechanism of lactose translocation in membrane vesicles from *Escherichia coli*. 2. Effect of imposed $\Delta\Psi$, ΔpH, and $\Delta\mu H^+$. *Biochemistry* 18(17):3697–704
95. Kaczorowski GJ, Kaback HR. 1979. Mechanism of lactose translocation in membrane vesicles from *Escherichia coli*. 1. Effect of pH on efflux, exchange, and counterflow. *Biochemistry* 18(17):3691–97
96. Garcia ML, Viitanen P, Foster DL, Kaback HR. 1983. Mechanism of lactose translocation in proteoliposomes reconstituted with *lac* carrier protein purified from *Escherichia coli*. 1. Effect of pH and imposed membrane potential on efflux, exchange, and counterflow. *Biochemistry* 22(10):2524–31
97. Kaczorowski GJ, Leblanc G, Kaback HR. 1980. Specific labeling of the *lac* carrier protein in membrane vesicles of *Escherichia coli* by a photoaffinity reagent. *PNAS* 77(11):6319–23
98. Newman MJ, Foster DL, Wilson TH, Kaback HR. 1981. Purification and reconstitution of functional lactose carrier from *Escherichia coli*. *J. Biol. Chem.* 256(22):11804–8
99. Viitanen P, Garcia ML, Kaback HR. 1984. Purified reconstituted *lac* carrier protein from *Escherichia coli* is fully functional. *PNAS* 81(6):1629–33

100. Viitanen P, Newman MJ, Foster DL, Wilson TH, Kaback HR. 1986. Purification, reconstitution, and characterization of the *lac* permease of *Escherichia coli*. *Methods Enzymol*. 125:429–52
101. Consler TG, Persson BL, Jung H, Zen KH, Jung K, et al. 1993. Properties and purification of an active biotinylated lactose permease from *Escherichia coli*. *PNAS* 90:6934–38
102. Pouny Y, Weitzman C, Kaback HR. 1998. In vitro biotinylation provides quantitative recovery of highly purified active lactose permease in a single step. *Biochemistry* 37(45):15713–19
103. Whitelegge JP, le Coutre J, Lee JC, Engel CK, Privé GG, et al. 1999. Toward the bilayer proteome, electrospray ionization-mass spectrometry of large, intact transmembrane proteins. *PNAS* 96(19):10695–98
104. Bibi E, Kaback HR. 1990. In vivo expression of the *lacY* gene in two segments leads to functional *lac* permease. *PNAS* 87(11):4325–29
105. Zen KH, McKenna E, Bibi E, Hardy D, Kaback HR. 1994. Expression of lactose permease in contiguous fragments as a probe for membrane-spanning domains. *Biochemistry* 33(27):8198–206
106. Kaback HR, Guan L. 2019. It takes two to tango: the dance of the permease. *J. Gen. Physiol.* 151(7):878–86
107. Chaptal V, Kwon S, Sawaya MR, Guan L, Kaback HR, Abramson J. 2011. Crystal structure of lactose permease in complex with an affinity inactivator yields unique insight into sugar recognition. *PNAS* 108:9361–66
108. Grytsyk N, Sugihara J, Kaback HR, Hellwig P. 2017. pK_a of Glu325 in LacY. *PNAS* 114:1530–35
109. Guan L, Kaback HR. 2004. Binding affinity of lactose permease is not altered by the H^+ electrochemical gradient. *PNAS* 101(33):12148–52
110. Kaback HR. 1972. Transport across isolated bacterial cytoplasmic membranes. *Biochim. Biophys. Acta Rev. Biomembr.* 265:367–416
111. Kaback HR. 1971. Bacterial enzymes. *Methods Enzymol.* 22:99–120
112. Kaback HR. 1974. Transport studies in bacterial membrane vesicles. *Science* 186:882–92
113. Kaback HR. 1972. Bacterial transport mechanisms as studied in cytoplasmic membrane vesicles. *Ann. N. Y. Acad. Sci.* 195:407–11
114. Kaback HR. 1986. Active transport in *Escherichia coli*: passage to permease. *Annu. Rev. Biophys. Biophys. Chem.* 15:279–319