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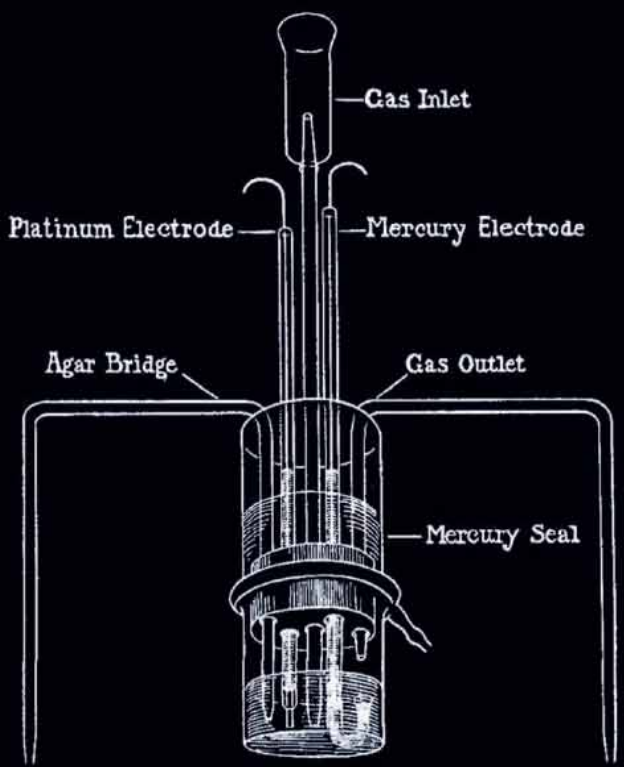

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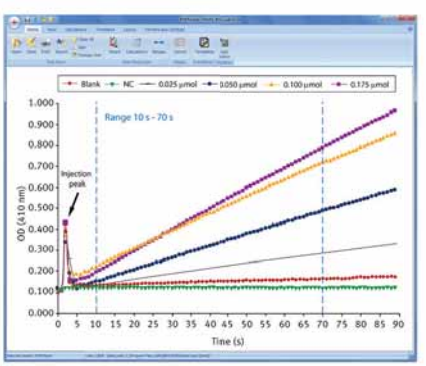

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JBC Historical Perspectives: Bioenergetics*

Nicole Kresge, Robert D. Simoni, and Robert L. Hill

Bioenergetics is a broadly defined subfield of biochemistry that deals with diverse aspects of energy transactions and transformations. The discipline covers a range of topics that once dominated biochemical literature, from redox energy to ATP synthesis to active transport to metabolism.

The *Journal of Biological Chemistry* (JBC) Classics and Reflections featured in this collection touch on various aspects of bioenergetics and the biochemists that pioneered the field.

Investigations into the energy of oxidation-reduction reactions figured prominently in the development of bioenergetics as a discipline. This area is represented by E. C. Slater's Reflections and his body of work that helped to define the mitochondrial respiratory chain and the relationship between electron transport and oxidative phosphorylation. Henry Lardy's Reflections also contributes valuable historical perspective, covering a diverse set of metabolic studies from glycolysis to the many phosphoryl-transfer reactions that characterize energetics.

Leon A. Heppel had a wide ranging career, investigating topics from nucleic acid biochemistry to energetics. His Reflections and Classic papers recount his efforts to describe the energetics of active transport as well as his studies on the ATP synthase responsible for oxidative phosphorylation.

One of the best studied systems of active transport energetics is calcium transport in the sarcoplasmic reticulum. The Ca^{2+}-ATPase of the sarcoplasmic reticulum utilizes the energy of ATP to establish a calcium gradient essential for the contraction/relaxation cycle of muscle. David MacLennan started studying the sarcoplasmic reticulum calcium pump in 1969. The work, which he continues today, allowed him to develop a theory on the mode of action of this ATP-dependent calcium pump. MacLennan's 1970 JBC Classic paper describes the experiments that resulted in his initial purification of the pump.

Central to the field of bioenergetics is the synthesis of ATP by oxidative phosphorylation. Probably no single topic occupied biochemists over a longer period of time or created more controversy. There were many theories on how the energy was provided for ATP synthesis, but in the end, it was Peter Mitchell's controversial chemiosmotic hypothesis that provided the answer. However, questions remained about how the energy of a proton gradient generated by the mitochondrial electron transport system was coupled to ATP synthesis. Efraim Racker's research explained much of the enzymology involved in the ATP synthesis complex. He was able to show that the complex consisted of two subcomplexes, the F_1 portion, which is peripheral to the mitochondrial inner membrane, and the F_o portion, which is intrinsic to the membrane. Racker published a paper in the JBC detailing the purification and properties of the F_1 portion. He followed that with a second paper showing that F_1 catalyzed the complex's coupling and ATPase activities.

Nobel laureate Paul Boyer's 1979 JBC Classic paper and his Reflections article explain the research that resulted in the elucidation of the mechanism of energy coupling in oxidative phosphorylation. After decades of failed and contentious mechanistic claims by workers in the field, Boyer was able to demonstrate that the energy of the proton gradient induced a conformational change in the ATP synthase complex that allowed the release of newly synthesized ATP from the complex. Boyer called this the "binding change mechanism," and it explained the energy coupling to ATP synthesis in oxidative phosphorylation.

* To cite articles in this collection, use the citation information that appears in the upper right-hand corner on the first page of the article.

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Classics

A PAPER IN A SERIES REPRINTED TO CELEBRATE THE CENTENARY OF THE JBC IN 2005

JBC Centennial
1905–2005
100 Years of Biochemistry and Molecular Biology

Polyribonucleotide Synthesis and Bacterial Amino Acid Uptake: the Work of Leon A. Heppel

Oligonucleotides as Primers for Polynucleotide Phosphorylase
(Singer, M. F., Heppel, L. A., and Hilmoe, R. J. (1960) *J. Biol. Chem.* 235, 738–750)

The Polymerization of Guanosine Diphosphate by Polynucleotide Phosphorylase
(Singer, M. F., Hilmoe, R. J., and Heppel, L. A. (1960) *J. Biol. Chem.* 235, 751–757)

A Binding Protein for Glutamine and Its Relation to Active Transport in *Escherichia coli*
(Weiner, J. H., and Heppel, L. A. (1971) *J. Biol. Chem.* 246, 6933–6941)

Leon A. Heppel was born in Granger, Utah in 1912. Several years later, he and his family moved to San Francisco, where Heppel attended school and became interested in chemistry. While in high school, Heppel's mother got him a job doing analytical work at the American Cream Tartar Company. The job supported him through high school and afterward when he enrolled at the University of California, Berkeley as a chemistry and chemical engineering major. Unfortunately, American Cream Tartar's parent company, Stauffer Chemical Company, merged with the Schilling Spice Corporation in 1931, and, as Heppel recalls, "A vice president of Schilling Spice undertook to effect economies, but the only economy he could find was getting rid of me. Shocked and urged by my mother to plead my case, I told the vice president how much I depended on the job. His cold reply was, 'You need Schilling Spice Company but does Schilling Spice need you?' I never forgot those cruel words. Because of them, I abandoned my plan to be a chemical engineer, turning instead to physiological biochemistry, which I thought would be a gentler profession" (1).

Fortunately, Heppel received a fellowship allowing him to complete his B.S. degree. He graduated in 1933 and entered Berkeley's graduate school as a biochemistry student. Heppel did his thesis with C. L. A. Schmidt, studying potassium metabolism in rats. He was awarded his Ph.D. in 1937 but had a lot of trouble finding a job. Luckily, Schmidt came to the rescue and got Heppel a partial fellowship to attend medical school at the University of Rochester. At Rochester, Heppel joined W. O. Fenn's laboratory, where he continued to work on potassium metabolism in young rats. He completed his M.D. and internship in 1942. Then, the entry of the United States into World War II interrupted normal peacetime activities, and Heppel joined the United States Public Health Service. He was assigned to the National Institutes of Health (NIH) where he carried out studies on the toxicity of halogenated hydrocarbons.

After the war, Heppel remained at the NIH and joined the new research section for the study of enzymes. He eventually became the chief of the Laboratory of Biochemistry and Metabolism at the National Institute of Arthritis and Metabolic Diseases. After establishing his lab at the NIH, Heppel turned his attention to the phosphorylation and hydrolysis of purine ribonucleosides, which led to an interest in enzymes that hydrolyze RNA. By 1950, Heppel and his technician Russell J. Hilmoe had begun to do experiments on enzymes that catalyze the hydrolysis and phosphorolysis of polyribonucleotides and their derivatives. They studied 5′-nucleotidase, inorganic pyrophosphatase, and the hydrolysis and phosphorolysis of purine ribosides and ATP. Then, in 1955, Severo Ochoa and Marianne Grunberg-Manago discovered polynucleotide phosphorylase (PNPase), an enzyme that converted ADP and other nucleoside diphosphates into RNA-like $(NMP)_n$ polymers. This was the subject of a previous *Journal of*

A youthful Leon Heppel. Courtesy of the Office of NIH History, National Institutes of Health and Dr. Buhm Soon Park.

Biological Chemistry (JBC) Classic (2). Ochoa and Grunberg-Manago turned to Heppel for help in characterizing the polymer products produced by PNPase.

Heppel's work with PNPase is the subject of the first two JBC Classics reprinted here. Heppel and Hilmoe, along with postdoctoral fellow Maxine Singer (who will be the subject of an upcoming JBC Classic), began to analyze the polyribonucleotides formed from nucleoside 5′-diphosphates by PNPase.

In the first Classic, Singer, Hilmoe, and Heppel show that short oligonucleotides can serve as primers for PNPase. Specifically, they found that oligoribonucleotides with an unesterified, terminal, C-3′ hydroxyl group served as primers for the polymerization of adenosine 5′-diphosphate, uridine 5′-diphosphate, and thymine ribonucleoside pyrophosphate catalyzed by polynucleotide phosphorylase. The oligonucleotides were starting points for chain proliferation but were not incorporated into the finished polymer.

In the second Classic, the researchers discuss the polymerization of guanosine diphosphate by PNPase. They showed that GDP, when present alone, is not polymerized by enzyme fractions from *Agrobacterium agile* or *Escherichia coli*. However, polymerization of GDP did take place in the presence of oligonucleotide primers with an unsubstituted hydroxyl group at carbon 3′ of the terminal nucleoside residue. Unlike polymerization reactions with adenosine 5′-diphosphate, uridine 5′diphosphate, and thymine ribonucleoside pyrophosphate, the primers were incorporated into the polymer.

These experiments showed how mixed polyribonucleotides of various kinds could be synthesized. Some time later, Singer and Heppel used PNPase to prepare the polyribonucleotides and oligoribonucleotides that Marshall Nirenberg and Heinrich Matthei used in their experiment that defined the genetic code for phenylalanine.

In 1967, Heppel left the NIH to become a professor of biochemistry at Cornell University, where he remains today as a Professor Emeritus. By the time he moved to Cornell, Heppel's research focus had shifted to the properties of bacterial membranes. His work on sugar- and amino acid-binding proteins found in the periplasmic space of *E. coli* and other bacteria is the subject of the last JBC Classic reprinted here. Along with his graduate student Joel Weiner, Heppel investigated glutamine uptake in *E. coli* and showed that the bacteria contains a specific binding protein for glutamine, which they isolated, purified, and characterized. Their data suggested that this protein played a role in the active transport of the amino acid across the bacterial membrane. Subsequent work by Heppel and others defined a large class of binding protein-dependent transport systems in bacteria.

Heppel was also instrumental in Earl Sutherland's identification of cAMP. As explained in a previous JBC Classic (3), Sutherland and Ted Rall had discovered that increased formation of phosphorylase in the liver was mediated by a heat-stable factor. Sutherland wrote to Leon Heppel hoping that he might be able to help elucidate the structure of this molecule. Around the same time, David Lipkin wrote Heppel describing a new nucleotide that was produced by treating ATP with barium hydroxide. Heppel deduced that Sutherland and Lipkin were

studying the same molecule, which turned out to be adenosine 3′,5′-monophosphate, now commonly referred to as cyclic AMP or cAMP.

Heppel has received many honors and awards in his career including the 1959 Hillebrand Prize of the Chemical Society of Washington. He is a member of both the National Academy of Sciences and the American Academy of Arts and Sciences.[1]

Heppel's co-author on the first two papers, Russell J. Hilmoe, played an important role in the history of the American Society for Biochemistry and Molecular Biology. In 1975 he succeeded Robert A. Harte as the second Executive Officer of the society and Manager of the JBC and served until 1979. Hilmoe earned his B.S. degree from South Dakota University and his Ph.D. in Biochemistry from Georgetown University. During World War II he served in the U.S. Army Medical Corps and then with the U.S. Army Chemical Corps. In 1948 he became an intramural scientist in the National Institute of Arthritis and Metabolic Diseases at the National Institutes of Health, and in 1964 he became a science administrator in the National Institute of General Medical Sciences and oversaw extramural research grant support and graduate biomedical research training.

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[1] Biographical information on Leon A. Heppel was taken from Refs. 1 and 4.

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Classics

A PAPER IN A SERIES REPRINTED TO CELEBRATE THE CENTENARY OF THE JBC IN 2005

JBC Centennial
1905–2005
100 Years of Biochemistry and Molecular Biology

Unraveling the Enzymology of Oxidative Phosphorylation: the Work of Efraim Racker

Partial Resolution of the Enzymes Catalyzing Oxidative Phosphorylation. I. Purification and Properties of Soluble, Dinitrophenol-stimulated Adenosine Triphosphatase (Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960) *J. Biol. Chem.* 235, 3322–3329)

Partial Resolution of the Enzymes Catalyzing Oxidative Phosphorylation. II. Participation of a Soluble Adenosine Triphosphatase in Oxidative Phosphorylation (Penefsky, H. S., Pullman, M. E., Datta, A., and Racker, E. (1960) *J. Biol. Chem.* 235, 3330–3336)

Efraim Racker (1913–1991) was born in the town of Neu Sandez, Poland. At the age of 2, he and his parents moved to Vienna, where Racker grew up. After finishing high school, he went to the University of Vienna to study medicine. Because his graduation from medical school in 1938 was around the time Hitler marched into Austria, Racker decided to leave while it was still possible and fled to Great Britain where J. Hirsh Quastel offered him a job at Cardiff City Mental Hospital in Wales. There, Racker tried to find biochemical causes for mental diseases. When Great Britain entered the war, Racker lost his job at Cardiff and was interned on the Isle of Man where he practiced medicine for the first time in his life. Although he enjoyed being a doctor, he decided to try his luck as a researcher in the United States.

His first appointment was as a research associate in physiology at the University of Minnesota, Minneapolis, from 1941 to 1942. There, Racker carried on his search for a biochemical basis for brain diseases and showed that polio virus inhibited glycolysis in the mouse brain. Despite spending a year doing research, Racker once again found himself working as a physician when he accepted a position at the Harlem Hospital in New York City. His career in biochemistry began in earnest in 1944 when he was appointed assistant professor of microbiology at the New York University Medical School. During his time in New York, Racker continued his glycolysis studies and found that the inhibition could be overcome by the addition of glutathione. This led to his discovery that glyoxylase converts glyoxal to glycolic acid via a carboxyl-*S*-glutathione intermediate (1). This was the first "energy-rich" thioester of biological relevance to be identified. Similarly, Racker and his technician Isidore Krimsky showed that glyceraldehyde 3-phosphate oxidation occurred through a thiol ester enzyme intermediate (2).

In 1952, Racker was offered the position of associate professor at Yale Medical School, which he accepted. There, he continued to work on carbohydrate metabolism and discovered and purified *trans*-ketolase, a key enzyme in the pentose phosphate pathway. His stay in New Haven lasted 2 years and then he accepted the position of chief of the Nutrition and Physiology Department at the Public Health Research Institute of the City of New York. At first, Racker continued to work on the mechanism of glycolysis and the pentose phosphate pathway but then turned to the regulation of glycolysis. He showed that glycolysis was dependent on the continuous regeneration of ADP and inorganic phosphate by ATPase.

Soon after Racker moved to the Public Health Research Institute, Maynard E. Pullman joined his department. Pullman had just earned a Ph.D. from Johns Hopkins University (1953) and spent a year as a fellow in pediatrics. Upon his arrival at the Public Health Research Institute he decided he wanted to determine the mechanism of ATP synthesis in mitochondria

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and chloroplasts. At that time, it was assumed that ATP synthesis was coupled to respiration through a "high energy" intermediate. Pullman and Racker, joined by Anima Datta and graduate student Harvey S. Penefsky, started by attempting to isolate the enzymes involved in ATP synthesis. They obtained fresh bovine hearts and, using a mechanical blender, isolated several grams of mitochondrial membrane fragments, which catalyzed oxidative phosphorylation. These submitochondrial particles were then vigorously shaken with tiny glass beads in a shaker built by Peter M. Nossal. The shaker was considered so dangerous that Nossal screwed it to the floor of a separate room, operated it by remote control, and allowed nobody else to touch it. When the mitochondrial fragments were sedimented in an ultracentrifuge, they still respired but no longer synthesized ATP. However, Racker and his co-workers discovered that oxidative phosphorylation could be restored if the supernatant was added back to the fragments.

While attempting to isolate the soluble component that made oxidative phosphorylation possible, Racker and co-workers discovered that an ATPase was purified together with the phosphorylation activity. The purification and properties of this ATPase are the subjects of the first *Journal of Biological Chemistry* (JBC) Classic reprinted here. Eventually, the researchers realized that this ATPase was in fact the coupling factor that restored oxidative phosphorylation. They named this first enzyme of oxidative phosphorylation Factor 1 or F_1. The second JBC Classic reprinted here provides the evidence that the coupling and ATPase activity are both catalyzed by F_1.

After the publication of these papers, Pullman remained at the Public Health Research Institute and was eventually promoted to associate director in 1983. He then left the Institute to become a senior research scientist at Columbia University's College of Physicians and Surgeons in 1989 where he remained until 1992.

Racker continued to work on ATP synthesis and together with Vida Vambutas he purified a similar coupling factor from spinach chloroplasts (3). Later, with Yasuo Kagawa, Racker subfractionated submitochondrial particles with cholate and salt and identified a membrane factor that anchored F_1 to the membrane and rendered it cold-stable and sensitive to the toxic antibiotic oligomycin (4). They named this insoluble F_1-binding factor F_o, with the subscript signifying the letter "o" for oligomycin and not zero, as is often thought.

In 1966, Racker left the Public Health Research Institute to help create and lead the biochemistry department of a new biology unit at Cornell University. By this time, Racker was convinced that oxidative phosphorylation was not mediated by a high energy chemical intermediate but by a transmembrane proton gradient as proposed by Peter Mitchell (5). Joined by Walther Stoeckenius, Racker incorporated bacteriorhodopsin, a protein that functioned as a light-driven proton pump, and the F_1F_o-ATPase into liposomes (6). They showed that the protons pumped out by the illuminated bacteriorhodopsin flowed back through the F_1F_o-ATPase and generated ATP from ADP and inorganic phosphate, proving that Mitchell's hypothesis was correct.

In the years that followed, Racker and his colleagues reconstituted a variety of different membrane enzymes into liposomes and established reconstitution as a powerful approach for determining the mechanics of pumps, transporters, and receptors. As a result of his work, numerous prestigious honors and prizes were awarded to Racker, such as the Warren Triennial Prize in 1974, the National Medal of Science in 1976, the Gairdner Award in 1980, and the American Society of Biological Chemistry's Sober Memorial Lectureship. He was elected to the American Academy of Arts and Sciences and the National Academy of Sciences and was an Associate Editor for the JBC as well as a member of the JBC editorial board. Racker also came up with the maxim "Don't waste clean thinking on dirty enzymes," which is one of the Ten Commandments of Enzymology (7).[1]

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Classics

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100 Years of Biochemistry and Molecular Biology

Ion Transport in the Sarcoplasmic Reticulum: the Work of David H. MacLennan

Purification and Properties of an Adenosine Triphosphatase from Sarcoplasmic Reticulum
(MacLennan, D. H. (1970) *J. Biol. Chem.* 245, 4508–4518)

David Herman MacLennan was born in Swan River, Manitoba, Canada in 1937. He attended the University of Manitoba, where he earned his B.S.A. in Plant Science in 1959, and Purdue University, where he received his M.S. in Plant Pathology in 1961 and his Ph.D. in Biology in 1963. After graduating, MacLennan went to the Institute for Enzyme Research at the University of Wisconsin, where he first studied as a postdoctoral fellow (1963–1964) and later became an assistant professor (1964–1968). He joined the Banting and Best Department of Medical Research at the University of Toronto in 1969, where he continues his research today. MacLennan served as Department Chairman from 1978 to 1990 and is currently the J. W. Billes Professor of Medical Research and University Professor.

At the University of Toronto, MacLennan started looking at mitochondrial electron transport components and the mitochondrial proton pump. This led him to study the sarcoplasmic reticulum calcium pump. The work, begun in 1969 and continuing today, allowed him to develop a theory on the mode of action of this ATP-dependent calcium pump. MacLennan's initial purification of the pump is the subject of the *Journal of Biological Chemistry* (JBC) Classic reprinted here.

At the time MacLennan started studying the sarcoplasmic reticulum calcium pump, it was known that a calcium-activated ATPase was part of the system that transported calcium into the sarcoplasmic reticulum. Ca^{2+} was transported when ATP was hydrolyzed. A similar system was known to occur in the mitochondria, and in this system the transport function was separable from the system that hydrolyzed ATP. However, no separation had been achieved in the sarcoplasmic reticulum transport system, suggesting that the ATPase enzyme and the ion transport enzyme were one and the same. In this JBC Classic, MacLennan reports on the purification of the ATPase from sarcoplasmic reticulum via fractionation. Based on several observations, including the fact that the enzyme has sites for both Mg^{2+} and Ca^{2+}, the enzyme carries out partial reactions of Ca^{2+} transport, and the enzyme contains phospholipid and can form in membranes, MacLennan concluded that the ATPase is also the calcium transporter.

MacLennan's current research still focuses on ion transport. Specifically, he is studying how normal sarcoplasmic reticulum proteins carry out their functions of calcium transport, sequestration, and release and how mutant forms cause abnormalities or disease. In addition to his research on the sarcoplasmic reticulum, MacLennan has made important contributions to the fields of human and animal health. He and his coworkers defined the genetic basis for three important muscle diseases: malignant hyperthermia, central core disease, and Brody disease.

A Fellow of both the Royal Society of Canada (1985) and Royal Society of London (1994), MacLennan has received many awards including the Canadian Biochemical Society's Ayerst Award in 1974 and the Biophysical Society's National Lectureship Award in 1990, the Gairdner Foundation's International Award in 1991, the Canada Council Izaak Walton Killam

This paper is available on line at http://www.jbc.org

David H. MacLennan

Memorial Prize in Health Sciences in 1997, and the Glaxo-Wellcome Prize in 2000. He was an associate editor for the *Canadian Journal of Biochemistry* from 1972 to 1976 and a member of the JBC editorial board from 1975 to 1980 and 1982 to 1987. In 2001, he was appointed an Officer of the Order of Canada.

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Classics

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ATP Synthesis and the Binding Change Mechanism: the Work of Paul D. Boyer

Subunit Interaction during Catalysis. Alternating Site Cooperativity of Mitochondrial Adenosine Triphosphatase
(Hutton, R. L., and Boyer, P. D. (1979) *J. Biol. Chem.* 254, 9990–9993)

Paul Delos Boyer was born in 1918 in Provo, Utah. He attended Brigham Young University where he focused on chemistry and mathematics. During his senior year, he considered several career paths, including employment as a chemist in the mining industry, a training program in hotel management, studying osteopathic or conventional medicine, and graduate training. After seeing a notice for a Wisconsin Alumni Research Foundation (WARF) Scholarship for graduate studies he applied and was accepted, setting the stage for his career in biochemistry.

After receiving his B.S. degree in chemistry in 1939, Boyer set off for the biochemistry department at the University of Wisconsin in Madison. He was assigned to work with Paul Phillips, who encouraged him to explore metabolic and enzymatic interests. Boyer was granted his Ph.D. in 1943 and then headed to Stanford University to work on a war-related research project dedicated to the stabilization of serum albumin for transfusions.

With the completion of the project in 1945, Boyer accepted an offer of an Assistant Professorship at the University of Minnesota. However, his local War Draft Board in Provo, Utah had other plans, and he soon became a member of the U. S. Navy. The Navy did not know what to do with him so they gave him a nearly private laboratory at the Navy Medical Research Institute in Bethesda, Maryland. In less than a year he returned to civilian life and his position at the University of Minnesota.

At Minnesota, Boyer started using kinetic, isotopic, and chemical methods to investigate enzyme mechanisms. In 1956, he accepted a Hill Foundation Professorship and moved to the medical campus of the University of Minnesota in Minneapolis. Much of his group's research was on enzymes other than the ATP synthase. However solving how oxidative phosphorylation occurred remained one of the most challenging problems of biochemistry, and he could not resist its call. Boyer focused on finding a possible phosphorylated intermediate in ATP synthesis using ^{32}P as a probe. This culminated in the discovery of a new type of phosphorylated protein, a catalytic intermediate in ATP formation with a phosphoryl group attached to a histidine residue. However, he later found that the enzyme was an intermediate in the substrate level phosphorylation of the citric acid cycle and not a key to oxidative phosphorylation.

In the summer of 1963, Boyer moved his laboratory to the Department of Chemistry and Biochemistry at the University of California in Los Angeles, where he remains today as Professor Emeritus. He became the Founding Director of the Molecular Biology Institute at UCLA in 1965 and spearheaded the construction of the building and the organization of an interdepartmental Ph.D. program.

However, this institutional service did not diminish his research program, and he continued to focus on oxidative phosphorylation. In the early 1970s, Boyer recognized the first main postulate of what was to become the "binding change mechanism" for ATP synthesis: that energy input was not used to form the ATP molecule but to promote the release of an already formed ATP. When Boyer approached the editors of the *Journal of Biological Chemistry* (JBC) about publishing his new concept for oxidative phosphorylation, they declined. Instead, Boyer

This paper is available on line at http://www.jbc.org

Paul D. Boyer. Photo courtesy of UCLA photography.

took advantage of his recent membership in the National Academy of Sciences, and the hypothesis was published in the *Proceedings of the National Academy of Sciences* (1). Companion studies (2) on the ATPase activity of muscle myosin strengthened his conviction that the new concept was valid.

In the mid-1970s, Boyer proposed the second main concept of ATP synthesis: catalytic cooperativity. He deduced that the three catalytic sites in ATP synthase worked cooperatively such that ATP could not be released from one site unless ADP and P_i were available to bind to another site, or when the reaction ran in reverse, P_i could not be released from one catalytic site unless ATP was available to bind at another catalytic site.

Toward the end of the 1970s, Boyer initiated experiments that led to the recognition of rotational catalysis, the third feature of the binding change mechanism. It was known that ATP synthase contained three copies of the major α and β subunits and single copies of the γ, δ, and ϵ subunits. However it seemed unlikely that all three β subunits could have identical interactions with the single copy subunits, making it impossible for all three catalytic sites to be identical. At this time, water highly labeled with ^{18}O had become more available, and Boyer could measure the ^{18}O isotopomers of P_i containing 0, 1, 2, 3, or 4 ^{18}O atoms. By measuring the distribution of isotopomers formed during ATP synthesis or hydrolysis using highly ^{18}O-labeled substrates, Boyer and his graduate student Lee Hutton were able to characterize the catalytic sites. If all the catalytic sites behaved similarly, the distributions of ^{18}O isotopomers would conform to a statistically predicted pattern. This is the subject of the JBC Classic reprinted here. Boyer and Hutton found that the distribution of isotopomers conformed very closely to that expected for identical behavior at all catalytic sites, ruling out the possible participation of two types of catalytic sites.

Later experiments with chloroplast and mitochondrial ATP synthases also showed that all catalytic sites behave identically. In order for this to be possible, Boyer proposed that the large catalytic subunits moved rotationally around a smaller asymmetric core. The occurrence of rotational catalysis was strongly supported by the x-ray structure for F_1-ATPase from bovine heart mitochondria (3).

Thus according to Boyer's binding change mechanism for ATP synthesis, the three catalytic sites on the enzyme bind ADP and phosphate in sequence and then undergo a conformational change so as to make a tightly bound ATP. The sites then change conformation again to release the ATP. These changes are accomplished by rotational catalysis driven by the rotating inner core of the enzyme, which is in turn driven by the protons crossing the mitochondrial membrane. For this pioneering work, Boyer was awarded one-quarter of the 1997 Nobel Prize in Chemistry. John E. Walker received the other quarter, and Jens C. Skou received the other half.

In recognition of his contributions to research on oxidative phosphorylation, Boyer has been given honorary doctorates from the Universities of Stockholm (1974), Minnesota (1996), and Wisconsin (1998). He has also received the Award in Enzyme Chemistry from the American Chemical Society (1955), the Rose Award from the American Society for Biochemistry and Molecular Biology (1989), the Glenn T. Seaborg Medal (1998), and the UCLA Medal (1998). He was elected to the American Academy of Arts and Sciences in 1968 and to the National Academy of Sciences in 1970. From 1959 to 1960, he served as Chairman of the Biochemistry Section of the American Chemical Society, and from 1969 to 1970 he was President of the American Society of Biological Chemists. Boyer was Editor or Associate Editor of the *Annual Review of Biochemistry* from 1963 to 1989 and was also Editor of the classic series, *The Enzymes*.[1]

Nicole Kresge, Robert D. Simoni, and Robert L. Hill

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[1] All biographical information on Paul D. Boyer was taken from Refs. 4 and 5.

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Reflections

A PAPER IN A SERIES COMMISSIONED TO CELEBRATE THE CENTENARY OF THE JBC IN 2005

JBC Centennial
1905–2005
100 Years of Biochemistry and Molecular Biology

A Research Journey with ATP Synthase

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These reflections present a perspective of how I and my graduate students and postdoctoral fellows, over a span of many years, arrived at the concept that ATP is made by an unusual rotational catalysis of the ATP synthase. A recent sketch of the structure of this remarkable enzyme is given in Fig. 1. Such a depiction is the culmination of the efforts of many investigators.[1] The two portions of the enzyme are the membrane-imbedded F_0 and the attached F_1 that has three catalytic sites, principally on the large β subunits. ATP is formed when protons pass through the F_0, driving the rotation of the ring-shaped cluster of *c* subunits and the attached ϵ and γ subunits. Other subunits attached to outer portions of the F_0 and F_1 served as a stator. The internal rotary movement of the γ subunit is coupled to sequential changes in the conformation of the catalytic sites. During ATP synthesis these conformational changes promote the binding of ADP and P_i, the formation of tightly bound ATP, and the release of ATP.

Revealing the mechanism of the ATP synthase became a major research goal in the latter part of my long career. This paper recalls how my career developed as related to the remarkable progress in biochemical knowledge. It presents the background and results of fruitful, as well as mistaken, approaches that were explored.

The Early Years

Born and educated through college in Utah, at the age of 21 I entered graduate school in the Department of Biochemistry at the University of Wisconsin in the fall of 1939. The biochemical research and teaching there were excellent. Not until years later did I appreciate all that is necessary to create such a fine scientific environment.

I had had no previous courses or research experience in biochemistry and was uncertain about my career choice. By the end of my first year of graduate study the fascination of biochemical understanding and the addictive effect of experimental attempts to uncover new knowledge had firmly launched me toward a career in biochemical research. The Department of Biochemistry at Wisconsin was at the forefront of research in nutrition and metabolism. Recent achievements included the identification of nicotinic acid as a vitamin, the irradiation of milk to produce vitamin D, the discovery of a vitamin K antagonist (dicoumarin), and the discovery of lipoic acid as a growth factor for bacteria. At that time incoming graduate students were assigned to a mentor professor. Both Henry Lardy, from South Dakota, and I joined the group of Professor Paul Phillips whose major interest was in dairy cattle nutrition. Evidence had been obtained that vitamin C might help prevent reproductive difficulties in cattle, and one of my assignments was to find if vitamin C might ameliorate the reproductive failure that occurred in rats with vitamin E deficiency. No benefits of vitamin C were noted, but the rats

[1] Except for a few instances, the mention of important advances in information about the ATP synthase and in related areas of biochemistry is included without specific references. The objective of this contribution is not to provide a review of the field and to recognize priorities for contributions but to note how various advances impacted on studies by my group.

This paper is available on line at http://www.jbc.org

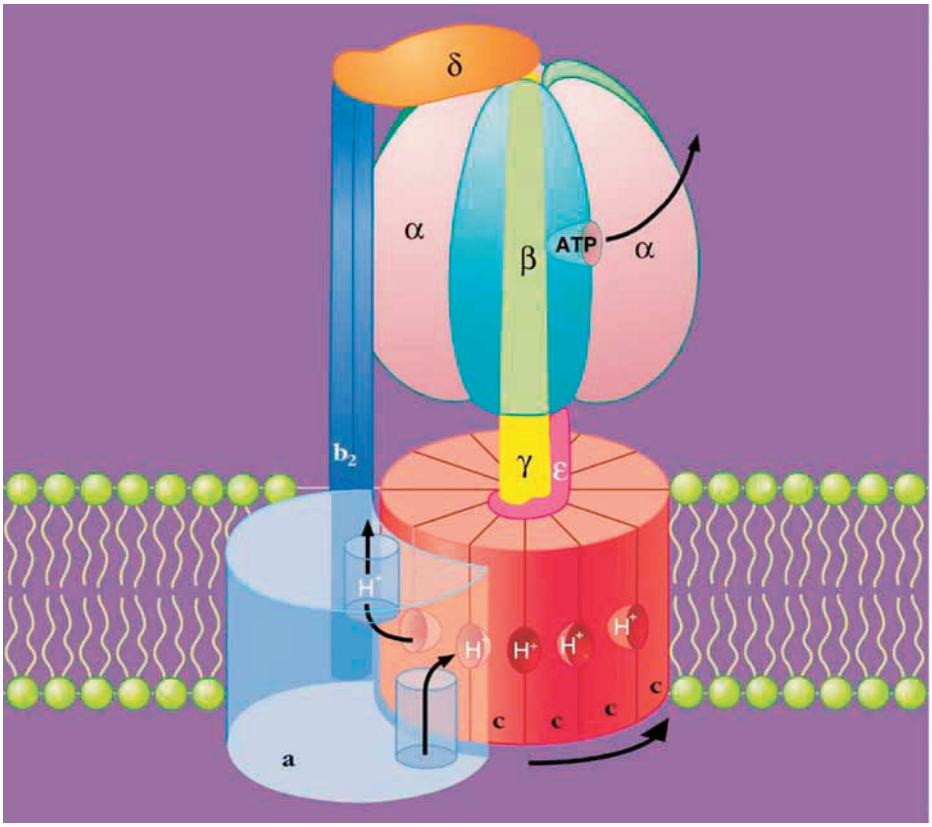

FIG. 1. **The ATP synthase.** The enzyme from *E. coli* has an F_1 portion with subunits designated as $\alpha_3\beta_3\gamma\delta\epsilon$. When separated it acts as an ATPase. The F_0 portion subunits are designated as ab_2c_{9-12}. The passage of protons, at the interface of the a subunit and the ring of c subunits, causes a rotation of the c and attached ϵ and γ subunits relative to the rest of the enzyme. The asymmetric γ subunit (*yellow* and *light green*) extends through the center of the $\alpha_3\beta_3$ cluster. The b_2 and δ subunits serve as a stator. The rotation of the γ subunit results in sequential conformational changes of the catalytic sites that promote ADP and P_i binding, ATP formation, and ATP release. The mitochondrial and chloroplast enzymes are similar, except the F_0 portion has more subunits. The three catalytic sites are principally on the β subunits at an interface with the α subunits. The α subunits also have three non-catalytic sites that bind nucleotides. The figure is from Ref. 112 (copyright 2001, National Academy of Sciences, U. S. A.).

also showed the striking muscular dystrophy characteristic of vitamin E deficiency. Exploration, together with Henry Lardy, of the possible cause of this dystrophy led me into study of ATP-related enzymes. Henry is still active in an exceptionally distinguished career that has included major contributions to the understanding of oxidative phosphorylation.

The milieu at Wisconsin (meetings where students and staff discussed recent research papers, frequent research seminars, and class instructions) introduced me to the wonder of enzyme catalysis. A prominent event was a symposium on respiratory enzymes at which the outstanding biochemists Meyerhof, Cori, Ochoa, Lipmann, Kalckar and others contributed (1). From this and other sources I learned that ATP and phosphorylations were central to the capture and use of energy derived from foodstuffs.

Perhaps defective formation of ATP might underlie the muscle dystrophy in my vitamin E-deficient rats. One approach was to measure the ability of muscle extracts to make phosphocreatine during glycolysis. No definitive defect from vitamin E deficiency was found, but in the course of these experiments, I noted a stimulation of the transfer of phosphate from 3-phosphoglycerate to creatine by K^+ ions. This was traced to a requirement of K^+ for transfer of the phosphoryl group from 2-phosphoenolpyruvate to ADP. The discovery of the K^+ activation of pyruvate kinase was the first demonstration of a K^+ requirement for an enzyme reaction. The two *Journal of Biological Chemistry* publications reporting this were the best of several from my graduate studies (2, 3). An understanding of the K^+ activation was attained at the University of Wisconsin some 50 years later from the x-ray structure of pyruvate kinase (4). The K^+, coordinated to four protein ligands, to an oxygen of the γ-phosphate of ATP, and to a water oxygen, apparently provides a requisite positive charge.

Oxidative phosphorylation was discovered only 7 years before I started graduate studies. As noted in an interesting Prefatory chapter by Englehardt in *Annual Reviews of Biochemistry* (5), ATP was discovered by Lohmann in 1927, and oxidative phosphorylation was first dem-

onstrated by Engelhardt and Liubimova in 1932. These salient contributions at that time seemed far from recent to me, and discoveries such as that of cell-free fermentation by Buchner made about 40 years earlier were relegated to the distant past—science after the escape from the Middle Ages. Now, from my present perspective, research of 30 years ago still seems fairly recent and vibrant. Time seems to go much faster, but it is I who has changed while a unit of time has retained its constant value.

An Introduction to Properties of Proteins

Some 20 amino acids linked in peptide bonds can yield proteins with a truly remarkable diversity of structural properties and the ability for specific combination and catalysis. The versatility of proteins is arguably the most important property of matter that has made life possible. Little was known about protein structure when I was a graduate student. As stated in a 1946 textbook of biochemistry (6): "Since the protein molecule is often built up of hundreds, even thousands, of these amino acids, the problem of protein structure is one of almost insuperable difficulty." In the following years, to be an observer as the wondrous properties of proteins have been revealed is one of the finest rewards provided by my profession.

My appreciation of protein structure and function arose in 1943 when I joined a small group at Stanford University that was supervised by Murray Luck, founder of the *Annual Review of Biochemistry*. Our nation was at war, and Luck's group was asked if they could find how concentrated solutions of human serum albumin, used primarily for the treatment of shock in wounded soldiers, could be heated to inactivate pathogens without denaturing the albumin. The group found that low concentrations of long chain fatty acids or other non-polar anions such as acetyltryptophan would satisfactorily stabilize the albumin. Albumin preparations used militarily and commercially are still stabilized with small concentrations of *N*-acetyltryptophan. As part of these studies, I noted that when albumin solutions were exposed to urea or guanidine hydrochloride, the large viscosity increase accompanying denaturation could be reversed by fatty acid addition; a specific combination was markedly influencing the folding of the protein (7). My interest in protein structure was firmly initiated.

Early Studies at Minnesota

In my 17 years at the University of Minnesota, I studied a wide variety of biochemical problems, including such items as the chemistry of α-tocopherol oxidation products, possible formation of antibodies by a refolding of denatured γ-globulins, sulfhydryl groups and enzyme catalysis, and the free energy of hydrolysis of ATP. Mostly my interests have concerned enzymes, and over the years names of some 25 different enzymes have appeared in titles of my publications.

An unsettled problem from my graduate studies at Wisconsin was clarified by the demonstration that mitochondria from the muscles of vitamin E-deficient rats performed oxidative phosphorylation as well as those from as normal muscle (8). Studies in my laboratory (9) and those of my graduate colleague Henry Lardy (10) independently reported that during oxidative phosphorylation oxygen uptake was decreased by the lack of phosphate acceptors. Such respiratory control was the basis for the later development, by Britton Chance and others, of the extensive use of an oxygen electrode to replace the cumbersome Warburg manometric method for measuring rates of oxygen uptake during oxidative phosphorylation.

More importantly, stimulated by the pioneering studies of Mildred Cohn (11), we initiated studies using the heavy oxygen isotope, ^{18}O, for probing phosphorylation reactions. As noted in later sections, insights into ATP synthase catalysis by my group were crucially dependent upon the use of ^{18}O. The ^{18}O isotope and mass spectrometer facilities were made available by physics professor Alfred Nier (a benefit of a research university and a cooperative faculty). Over the years we and others have modified and improved techniques for ^{18}O measurements. Yet studies with ^{18}O remain more laborious than many approaches and have not been widely used. The lack of familiarity with the ^{18}O measurements probably added to the reluctance of the field to accept our concepts, as they were later developed in the 1970s.

In our early studies with ^{18}O we demonstrated that in the glyceraldehyde-3-phosphate dehydrogenase reaction an oxygen from inorganic phosphate appears in the carboxyl group of the 3-phosphoglycerate formed (12). This was explained by a phosphorolysis of an acyl enzyme intermediate demonstrated by studies of Racker's group (13) and mine (14). The phosphorylation accompanying this oxidative step of glycolysis was a prominent basis for the widely

adopted paradigm that a phosphorylated intermediate was likely formed during the oxidative phosphorylation of the respiratory chain.

In related experiments my group showed that the enzymic catalyses for formation of phosphocreatine from 3-phosphoglycerate occurred with the retention of all 3 oxygens of the phosphoryl group. Thus such phosphoryl transfers do not involve any steps giving exchange of P_i oxygens with water (12). Also we found that syntheses coupled to ATP cleavage, such as formation of glutamine from glutamate and ammonia, occur with transfer of an oxygen from the substrate to P_i (15). No water oxygen is incorporated into the P_i.

Our initial studies of oxidative phosphorylation with ^{18}O revealed an important characteristic of the oxidative phosphorylation process. We incubated mitochondria with P_i labeled with both ^{18}O and ^{32}P and unlabeled ATP in the presence or absence of substrates or of oxidation inhibitors. We were surprised to discover that, in addition to the strikingly rapid exchange of P_i oxygens with water, a quite rapid $P_i \leftrightarrow$ ATP exchange was occurring (16). The reactions of oxidative phosphorylation appeared to be dynamically reversible. The reversibility continued even when electron carriers were inhibited or nearly fully reduced. This gave evidence for formation of some type of energized compound or state, independent of oxidation-reduction reactions that allowed the ready reversal of the reaction sequence. We thought this likely was some type of chemical intermediate; the idea of an electrochemical gradient across a coupling membrane was far from our thoughts.

Possibilities arose of pursuing interesting aspects of enzyme catalysis not related to ATP formation. For example, in 1955 while on a Guggenheim fellowship for study in Sweden with Nobelist Hugo Theorell, I noted a previously overlooked shift in the fluorescence of NADH upon binding by a dehydrogenase (17). This gave a new basis for measuring combinations of NADH with enzymes. However, the problem was not as interesting as the studies of oxidative phosphorylation that I was also pursuing in the laboratories of Olov Lindberg and Lars Ernster at the Wenner Gren Institute. In an experiment conducted in part in Sweden ^{18}O was used to demonstrate that the terminal bridge oxygen in ATP formed by oxidative phosphorylation came from ADP, not P_i. This and some other research were reported at an International Union of Biochemistry symposium in Japan (18). At that time I was a bit pessimistic about gaining a satisfactory insight into how oxidative phosphorylation occurs. In my contribution I stated: "Our basic knowledge of the chemistry involved does not appear adequate for the task, and the problem is likely to be with us for some time. Researchers who undertake indirect approaches to the problem should do so with recognition that their experiments cannot give final answers, and may not even point the way to final solutions." In retrospect, the pessimism seems appropriate.

During the next several years we undertook experiments looking for intermediates in oxidative phosphorylation, particularly by making use of ^{32}P as a tracer. We learned that radioactively induced reactions of phosphorus compounds with highly labeled $^{32}P_i$ could give rise to radioactive impurities that stick to mitochondrial components but that did not behave like intermediates. Most of my publications during this period were from some worthwhile investigations with other enzymes; one needs to keep research funding available. One of my favorite sayings is that most of what you accomplish in research is the coal that you mine while looking for diamonds.

Some of our studies concerned patterns of isotope exchanges at equilibrium with glutamine synthetase using ^{18}O, ^{32}P, and ^{14}C. It soon became apparent that covalent bond cleaving and formation may not be rate-limiting in enzyme-catalyzed exchanges. Somewhat surprisingly, adequate rate equations governing exchange reactions of enzymes were mostly lacking. I spent a fair effort in a pioneering development of appropriate relationships (19). To some reviewers these relationships were unexpected, and there is an interesting story not told here about what I needed to do to get the publication accepted. Various applications were made by my group. For example, data with glutamine synthetase revealed that the binding of ATP and glutamate was random, and such subtleties as a spatial selectivity of transfer of only one oxygen of the glutamate γ-carboxyl group to phosphate when glutamate and glutamine are readily interconverted at the catalytic site (20). The understanding obtained was useful for later measurements of isotope exchanges that helped in the discovery of compulsory sequential participation of catalytic sites of ATP synthase.

An observation of later interest was that myosin and actomyosin can catalyze an exchange of phosphate oxygens with water oxygens. This can occur with P_i in the medium without added

ATP (21) or with the P_i formed from ATP before it is released to the medium (22). We did not pursue such observations until about a decade later when we belatedly recognized their potential relationship to the mechanism of oxidative phosphorylation.

The Phosphohistidine Story

In 1961 it seemed that our searches with ^{32}P had hit pay dirt. We reported that under oxidative phosphorylation conditions a brief exposure to $^{32}P_i$ and solubilization of the mitochondria with concentrated urea and detergent gave a non-dialyzable ^{32}P-labeled substance. The rate of its formation from P_i or ATP, the disappearance in a cold P_i chase, and the effect of inhibitors and reaction conditions were consistent with its being an intermediate in oxidative phosphorylation. Our interest was heightened when my capable associates identified the substance as a phosphorylated histidine residue in a protein (23). This was the first recognition of a phosphohistidine in biochemical systems. The ability to form the bound phosphohistidine in soluble preparations from mitochondria encouraged the possibility that we could characterize details of the formation process. As the research developed, I became overly enthusiastic in regarding the phosphorylated protein as an intermediate of oxidative phosphorylation (24). In retrospect, I should have been more cautious. It was at this stage that my laboratory group moved to UCLA where we joined the Biochemistry Division of the Chemistry Department.

Our continued studies showed that dialyzable substances from mitochondria could modulate the bound phosphohistidine formation, and this led to the recognition that CoA and succinate were particularly effective. We had overlooked the substrate level phosphorylation accompanying the citric acid cycle. We became aware that a Ph.D. thesis at Illinois by Upper (25) had reported evidence of formation of a phosphoenzyme with the *Escherichia coli* succinyl-CoA synthetase and that such formation had been suggested earlier from catalysis of an ADP $\leftrightarrow$ ATP exchange by the synthetase (26). Our further studies showed that the phosphorylated protein we had detected was indeed an intermediate in the formation of nucleoside triphosphate (ATP or GTP depending on enzyme source) from P_i by succinyl-CoA synthetase (27, 28). Our bound phosphohistidine was clearly not an intermediate in oxidative phosphorylation. In Olympic analogy, we were reaching for a gold but were fortunate to have obtained a bronze.

Another Decade with Little Essential Progress

At this stage I felt that perhaps I could do more for science by accepting an opportunity to become the initial Director of the Molecular Biology Institute at UCLA. Fortunately this did not prevent reasonable continuation of laboratory studies, although I was not encouraged about the progress we and others were making toward elucidation of the major problem of how cells captured energy from oxidations to make ATP. At that time I of course did not know that a decade later we would be fortunate in developing a new concept for oxidative and photosynthetic phosphorylation.

Meanwhile my group pursued some worthwhile studies with other enzymes and continued a few probes of ATP synthesis that were useful but did not yield or point to breakthroughs. In a more sensitive search for the labeling of unidentified components with ^{32}P, a small amount of rapidly labeled lipid fraction was detected (28). However, this labeling was found to continually increase with time, not an expected characteristic of an intermediate. The independence of oxygen exchanges from oxidation-reduction reactions was more firmly established (29). A claim that a localized AMP might be the initial phosphoryl acceptor was refuted and ADP as the initial phosphoryl acceptor more firmly established (30). A sensitive search for possible substances that might transitorily bind an oxygen from P_i on its way to water was negative (31). An exploration of the source of phosphate oxygens in *E. coli* and *Bacillus subtilis* showed that only a few oxygens that entered with the P_i remained (32). Most of them came from water and substrates, undoubtedly by exchange patterns we had been investigating. Other studies gave a welcome observation that laid the base for the later extensive use of chloroplasts by my group; under appropriate conditions chloroplasts catalyzed rapid $P_i \leftrightarrow$ ATP, $P_i \leftrightarrow$ HOH, and ATP $\leftrightarrow$ HOH exchanges. Like oxidative phosphorylation, photophosphorylation was dynamically reversible and its mechanism could be probed by oxygen exchange measurements (33). From later developments the lack of the exchanges noted previously was likely because of the unusual and particularly strong Mg-ADP inhibition of chloroplast ATPase activity that can occur in the dark but is readily reversed by protonmotive force.

In an attempt to gain more insight about energy coupling we also conducted some studies on active transport by *E. coli*. We obtained convincing evidence that a common energized state or intermediate could drive transport or ATP synthesis (34), a view that had been independently developed by Harold (35) and others. However, unlike Harold, we were reluctant at that time to regard that the energized state was a protonmotive force. We were not alone in this reluctance. The field was active, and frequently reviewed. The 1967 (36), 1969 (37), 1971 (38), and 1974 (39) reviews in the *Annual Review of Biochemistry* on electron transport and phosphorylation gave brief and generally negative assessments of Mitchell's proposal that protonmotive force drove ATP synthesis. My hesitation in accepting this proposal came from the lack of a satisfying explanation as to how proton migration could drive ATP formation.

The mechanism of the ATP synthase remained unclear. As noted in the reviews mentioned above, there were a plethora of hypothetical compounds and reactions suggested for participation in ATP formation. A possibility consistent with our various experiments was that an energized state, not involving oxidation-reduction reactions, was used to drive a reaction in which an oxygen from P_i formed water as ADP was phosphorylated to yield ATP. We and others wondered if in some manner energy captured in conformational changes of proteins was involved. Remarkable advances in recognizing the versatility of protein structure were occurring. The x-ray structure of hemoglobin and other proteins and the allosteric properties of enzymes suggested the energy requirements for ATP formation might be accommodated in conformational changes of proteins. But we still had no clear idea about how the conformational changes might function.

A New Concept

In 1972, the first part of what I later called the binding change mechanism of ATP synthesis came from further considerations of past data, not new experimental findings. While attending a seminar that I did not understand, some puzzling aspects of oxygen exchange measurements were occupying my mind. Our thoughts had been that the major use of captured energy was to make the covalent structure of ATP. The realization struck me that past data could be explained if the major use of energy was not to form the ATP but to release a tightly bound ATP from the enzyme. Reversible formation of bound ATP at a catalytic site could explain why the exchange of P_i oxygens was less sensitive to uncouplers than net oxidative phosphorylation. For me it was a rare moment of insight, like suddenly reaching a summit on a mountain climb and seeing a beautiful valley spread below. All enzymes have the capacity for ready reaction reversal at catalytic sites and to bind both products and reactants. The reversal of the hydrolysis of ATP by the ATP synthase is no more remarkable than the reversal of simple hydrolyses by many enzymes, except that with the ATP synthase the product ATP is tightly bound. An additional step or steps must intervene for ATP release. This could logically be an energy-requiring conformational change of the catalytic site.

Richard Cross had joined our laboratory as a postdoctoral fellow. At UCLA he further documented the uncoupler-insensitive oxygen exchange and other aspects. We submitted a paper, "On a New Concept for Energy Coupling in Oxidative Phosphorylation Based on a Molecular Explanation of the Oxygen Exchange Reactions," to the *Journal of Biological Chemistry* for consideration. The publication was declined; at that stage our evidence was not strongly convincing. However, the concept remained appealing. I had recently been elected to the National Academy, and the paper was published in *Proceedings of the National Academy of Sciences* as the first paper I sponsored for the journal (40). A follow-up paper gave additional details (41).

The presence of multiple binding sites for ADP and ATP on the isolated F_1-ATPase and the ATP synthase had been recognized by Slater's group and others. During catalytic turnover some of these nucleotides exchanged with medium nucleotides, and Slater and associates had also suggested the possibility that energy-requiring release of bound ATP might occur in oxidative phosphorylation (42).

The validity of the concept of the role of a tightly bound ATP was strengthened by our finding that myosin ATPase would spontaneously form a tightly bound ATP from medium P_i (43). The estimated $-\Delta G^0$ of the binding of ATP from our and other data was 12–13 kcal/mol. A corresponding tight binding was anticipated for the ATP synthase. In related experiments, Bagshaw and Trentham had recently shown that the apparent ΔG for the hydrolysis of the bound ATP to bound ADP and P_i was only about -1.3 kcal/mol (44); the equilibrium was not

far from unity. In a subsequent cooperative study with these investigators an exchange of phosphate oxygens of bound ATP with water was demonstrated to accompany the ATP hydrolysis by myosin (45). The ability to form a bound ATP from P_i by the reversal of ATP hydrolysis readily accounts for the capacity of myosin to catalyze a $P_i \leftrightarrow HOH$ exchange we had observed years earlier (21). Later observations characterized how the combination of myosin with actin promotes the release of the tightly bound ATP, a conformational transition analogous to that proposed for the ATP synthase (46).

At this time I contributed a chapter on "Conformational Coupling in Biological Energy Transductions" in which the possibility that changes with ATP synthase were driven by protonmotive force was recognized. However, my preferred view was still that the conformational changes were driven by some type of interaction with oxidation-reduction enzymes (47).

Recognition of the Role of Protonmotive Force

Peter Mitchell introduced his concept of energy-linked proton translocation in 1961 (48), and in ensuing years he and others continued to present evidence and win converts. By the early 1970s even holdouts like myself were beginning to see the light. It seems probable that the role of protonmotive force would not have been recognized for a long time without Mitchell's contributions.

If proton translocation were coupled to ATP synthesis, I felt it would be accomplished indirectly by protein-linked conformational changes. In contrast, Mitchell proposed that the translocated protons reached the catalytic site and participated directly in the removal of a water molecule. I found his 1974 proposal in *FEBS Letters* (49) unattractive and called attention to some deficiencies in a *FEBS Letters* contribution (50). Without informing me, the journal allowed Mitchell to present a rebuttal following my paper (51). This seemed inappropriate, and the journal agreed to publish my subsequent paper presenting a model of how, through conformational coupling, proton translocation could drive ATP synthesis (52). The suggestions made still seem applicable.

Over the years Peter and I had extensive correspondence and shared a mutual respect. Although we were looking at essentially the same mechanism we tended to present different pictures of our views. Too often in science there is rancor between those who disagree. An important lesson that I have learned is that more will be accomplished if one can maintain cordial relations in an exchange of interpretations.

Other Developments

By the mid-seventies other investigators had provided much welcomed information about the ATP synthase that was quite relevant to mechanism studies. Hatefi and others in David Green's laboratory had shown that the mitochondrial inner membrane could be fractionated to yield separate complexes of the respiratory chain components and the ATP synthase. They (and particularly Racker[2] and associates) had separated and characterized the F_1-ATPase. The knobs visible in electron micrographs of mitochondrial membranes were identified with the F_1-ATPase, connected by a stalk to the membrane portion of the synthase. A similar ATPase had been found in a wide variety of organisms. The ATPase was known to have two or three copies of major α and β subunits and single copies of other smaller subunits. The unusual subunit stoichiometry and observations in a number of laboratories that modification of one β subunit per enzyme essentially stopped catalysis raised intriguing questions about mechanism. The portion of the synthase imbedded in the membrane, F_0, was recognized as being involved in proton transport. The addition of F_1-ATPase to F_0 preparations could restore oxidative phosphorylation or photophosphorylation. Either proton gradients or membrane potential sufficed to drive ATP formation. Beechey had shown that a buried carboxyl group on a small hydrophobic subunit of F_0, present in multiple copies, readily reacted with dicyclohexylcarbodiimide (DCCD)[3] and that this blocked oxidative phosphorylation.

Although information about the ATPase was becoming extensive, how proton translocation could be coupled to ATP formation remained poorly understood. We were encouraged some by the concept that energy-linked binding changes were involved. Fortunately, at this time we obtained evidence for an unusual catalytic site cooperativity displayed by the ATP synthase

[2] Racker's contributions were outstanding. One of his former associates, Geoffrey Schatz, has provided a splendid memoir of Racker's career (*Memoirs*, National Academy of Sciences, 1996, available on the internet from www.nationalacademies.org).

[3] The abbreviation used is: DCCD, dicyclohexylcarbodiimide.

and the isolated F_1-ATPase. There was a feeling in my research group that some important secrets about the ATP synthase were being revealed. This created an ambience that stimulated research efforts. Such occasions are an all too infrequent reward of basic research. They help soften the disappointments of the many experiments that yield little or no helpful information.

Alternating Site Participation

Many enzymes have more that one catalytic site, suggesting the possibility of a catalytic cooperativity between sites such that catalytic events at one site are promoted by substrate binding at another site. With most multicatalytic site enzymes, limited or no cooperativity has been observed. In contrast, we found that the ATP synthase showed a nearly complete dependence of continued catalytic steps at one site on the presence of substrate(s) at a second site. This was the first enzyme for which such a striking behavior had been discovered, adding to our interest in the phenomenon.

Our discovery arose from researches by Jan Rosing, a postdoctoral fellow with exceptional experimental skills from Slater's group, and Celik Kayalar, a gifted graduate student. They were symbiotically productive. We devised methods for estimating oxygen exchanges by submitochondrial particles that accompany: (*a*) the binding, exchange, and return to the medium of P_i; (*b*) the binding, exchange, and return to the medium of ATP; (*c*) the binding of P_i, intermediate exchange, and the release of ATP formed; and (*d*) the binding of ATP, intermediate exchange, and the release of the P_i formed. These measurements with ^{18}O were accompanied by measurement of the $P_i \leftrightarrow$ ATP exchange with $^{32}P_i$. The exchange patterns gave evidence that besides promoting ATP release, energy input also increased competent P_i binding. More importantly, the measurements yielded exchange patterns that Kayalar proposed could be explained if the binding of a substrate at one site was necessary for the release of a product from another site.

Whether two or three catalytic sites per enzyme were present was not known at that time. We proposed alternating behavior of two sites, although it was recognized that the results would also be compatible with sequential participation of three sites (53, 54). During net ATP formation or hydrolysis, sites were considered to proceed sequentially through the steps of binding, interconversion of reactants, and release so that at any one time each catalytic site was at a different stage of the catalysis. The concept seemed attractive, but more evaluation was needed.

David Hackney, a talented postdoctoral fellow from Dan Koshland's laboratory, had joined our group and initiated his excellent experimental and theoretical studies of the oxygen exchanges. We were proposing that P_i and ADP can bind and reversibly form bound ATP but that ATP cannot be released until P_i and ADP bind to an additional site. If dynamic reversal of ATP formation at a catalytic site continued in the absence of net reaction, then reductions in the concentration of P_i or ADP should increase the amount of intermediate oxygen exchange per ATP made. We were encouraged by a report from a former postdoctoral fellow of our group, Robert Mitchell, that he and his colleagues observed increased intermediate oxygen exchange accompanying ATP hydrolysis by submitochondrial particles when ATP concentration was lowered (55). Support for the possibility also came from the observation of Wimmer and Rose (56) that when ATP was exposed to chloroplasts in the light, the ATP showed nearly complete exchange of its oxygens before being released. This is as expected if low ADP concentration in the medium prevented the release of the ATP and many reversals occurred before its release.

Hackney observed that during net oxidative phosphorylation as either ADP or P_i concentration was decreased, there was a marked increase in water oxygen incorporation into each ATP formed (57). Additional observations made it unlikely that some type of enzyme heterogeneity or hysteresis could explain the exchange patterns. It deserves emphasis that these experiments were performed with submitochondrial particles during net ATP synthesis, giving them relevance to the actual oxidative phosphorylation process.

An interesting possibility was that catalytic site cooperativity might also be found with the isolated F_1-ATPase. Several years earlier, Ef Racker brought some of his purified F_1-ATPase to our laboratory to find if his enzyme would catalyze an intermediate $P_i \leftrightarrow$ HOH exchange. We tested this at millimolar concentrations of ATP and found that the P_i formed contained only close to the one water oxygen necessary for the hydrolysis. Now, however, with our evidence for cooperativity, it was evident that if reversible ATP formation could occur in the absence of

protonmotive force and if participation of alternating sites was necessary, then the extent of intermediate $P_i \leftrightarrow HOH$ exchange with each P_i released should increase as ATP concentrations are lowered. This was found to be so (58) and as ATP concentrations were lowered the number of reversals before the P_i was released approached a limit of over 300 (59). Tightly bound ATP at a single site was undergoing reversible hydrolysis waiting for ATP to bind to another site and promote ADP and P_i release.

The reaction rates and equilibrium characterizing the slow catalysis at a single site were determined in a widely recognized study by Cross together with Grubmeyer and Penefsky (60). They termed this "uni-site catalysis," and their results added considerably to the acceptance by others of alternating site participation. In these studies the K_d for ATP binding to one site of the F_1-ATPase was shown to be near 10^{-12} M (61), indicative of the need for energy input for ATP release and akin to the affinity of ATP for myosin.

The capacity to make bound ATP from medium P_i and ADP/ATP ratio near unity on the enzyme was nicely demonstrated with the chloroplast F_1-ATPase by Feldman and Sigman (62), a contribution that warrants wider recognition. In a slow reaction, needing relatively high P_i concentration, a tightly bound ADP became phosphorylated. Other findings made it probable that this was at the same site as the ADP that was rapidly released in the acid-base transition of thylakoid membranes and thus that this site was likely where covalent bond formation occurred during photophosphorylation.

In addition, results of various investigators established that chemical modification of only one catalytic site effectively stopped catalysis and that each of the three catalytic sites had a different capacity for derivatization. Such behavior agreed with the concept that during catalysis all three catalytic sites were in different conformations and proceeded sequentially through the conformations.

The Basis of ^{18}O Exchange

Our studies with ^{18}O are interpreted on the basis that the exchange results from a reversal of the formation of bound ATP from bound ADP and P_i. As covered in the Appendix of a review there is strong support for this interpretation (63). This includes demonstrations that the P_i oxygen exchanges catalyzed by the sarcoplasmic reticulum ATPase (64, 65) and pyrophosphatase (66, 67), as well as that of myosin ATPase as mentioned above, result from reversible formation of a phosphorylated enzyme or enzyme-bound pyrophosphate or ATP, respectively.

Probes of Initial Reaction Rates

Other evaluations of our postulates were needed. Rapid mixing and quenching techniques yielded essential information. One objective was to find if a tightly bound ADP on the chloroplast ATP synthase might react with medium P_i to form ATP in the first turnover of the enzyme. We used rapid mixing in an acid-base transition of chloroplast thylakoid membranes, as introduced by Jagendorf and colleagues, to start ATP synthesis in a few milliseconds. We found that the tightly bound ADP was not directly phosphorylated but was rapidly released to the medium and that the first ATP formed came from medium P_i and ADP (68). As substantiated in later experiments, the tightly bound ADP in such chloroplast membranes prior to release is tightly bound at a catalytic site without P_i.

The demonstration that exposure to protonmotive force caused the release of a tightly bound ADP from a catalytic site without phosphorylation had important implications for later developments. The tightly bound ADP in the presence of Mg^{2+} causes potent inhibition of ATPase activity of the ATP synthase and F_1-ATPase. Thus such inhibition in the intact synthase is readily and quickly overcome by protonmotive force. When a step of rotational catalysis occurs, the binding site with the tight ADP is opened as if it had an ATP present, while another site is binding ADP and P_i. The properties of the tightly bound ADP also aided interpretation of Walker's 1994 x-ray structure of the major portion of the F_1-ATPase, in which one β subunit has a tightly bound ADP and Mg^{2+} present (69).

Our rapid mixing experiments verified that medium ADP was rapidly bound and phosphorylated as if no phosphorylated intermediates were involved. They provided evidence that during photophosphorylation, in addition to a transitorily bound ATP, about one bound P_i and one bound ADP per enzyme are present and committed to ATP synthesis (70). Such results harmonize with the alternating site model with more than one catalytic site having bound reactants, as required if a tight site is already filled and substrates must initially bind at another site.

Research Conferences and Binding Change Mechanism

Research conferences are important to scientific progress because concepts can be freely discussed, and the publication of proceedings often allows inclusion of material not suited for the usual journals. For example, in my contribution to a 1979 conference honoring Ef Racker, I summarized our concepts and considered how to name our suggested mechanism. A name seemed desirable for ease of discussion and to identify the concept in the field. My contribution entitled "The Binding Change Mechanism for ATP Synthesis" was the first publication in which this nomenclature was used (71).

The binding change mechanism at that time included the following concepts. The first compound made from P_i is ATP itself (no intermediates); a principal requirement of energy is not for the formation but for the release of ATP; energy input also promotes the competent binding of P_i and the sequential participation of catalytic sites so that binding of substrate(s) at one site is necessary for release of product(s) from another site. Two years later, another and even more novel concept of the binding change mechanism was developed, namely the proposal of rotational catalysis. The suggestion that rotation of internal subunit(s) drives the binding changes for catalysis was first published in reports from 1981 and 1983 conferences at the University of Wisconsin (72, 73). How this concept came about is outlined next.

The Proposal of Rotational Catalysis

In the 1970s highly enriched ^{18}O was available, mass spectrometry techniques for ^{18}O analysis had improved, and Mildred Cohn had introduced an NMR method for measuring ^{18}O in phosphate compounds. David Hackney developed theoretical aspects of ^{18}O measurements relevant to observed distributions of ^{18}O isotopomers of P_i with 0 to 4 ^{18}O atoms per P_i or 0 to 3 ^{18}O atoms per ATP molecule. Measurement of the presence of ^{18}O in ATP formed by photophosphorylation showed a pronounced increase in ^{18}O loss at lower ADP and P_i concentrations (74). More importantly, the distribution of ^{18}O isotopomers corresponded to that statistically expected if all the ATP were produced by the same catalytic pathway. This eliminated the possibility that substrate modulation arose from heterogeneity of the enzyme used and made modulation by control sites unlikely. We now regarded the catalytic site cooperativity of ATP synthase to be reasonably well established.

Companion studies with the F_1-ATPase showed that when highly ^{18}O-labeled ATP was hydrolyzed by F_1-ATPase at different ATP concentrations, the distribution of ^{18}O isotopomers was as expected for a single catalytic pathway (58). At appropriate labeling and substrate concentration ranges, the distribution patterns provided a sensitive test for more than one catalytic pathway. A statistically homogeneous distribution meant that every substrate that reacted faced the same possibilities of proceeding through the same reaction steps. This means that rate constants governing the binding and release of substrate(s), their reversible interconversion, and the release of product were the same. To me, the power of this type of ^{18}O use is unusual and indeed a bit awesome.

By now essential contributions of other investigators, including Kagawa and associates in their fine studies with the F_1-ATPase from thermophilic bacteria, had established the presence of three catalytic sites with circular distribution of alternating large α and β subunits around a central core. Catalytic sites were regarded as largely on the β subunits, with the core representing the γ and possibly other small subunits. Observations in McCarty's laboratory demonstrated that modifications of –SH groups on the γ subunit markedly affected catalytic capacity of the chloroplast enzyme (75). The capacity of the F_O component for DCCD-sensitive proton transport had been established. These and other findings strengthened our view that conformational changes in the F_O were in some manner transmitted through the stalk to the catalytic sites on the β subunits to drive the binding changes for ATP synthesis.

Catalytic sites on multisubunit enzymes can be very sensitive to conformational changes in adjacent subunits. Changes in the γ subunit markedly modulated catalysis. How could all three β subunits have identical interactions with the γ subunit? Occurrence of tripartite symmetry of the γ subunit seemed unlikely. The evidence that all three sites conducted catalysis identically was compelling to me. The more I puzzled about these aspects, the more it seemed that there was only one satisfactory answer. This is that the internal asymmetric core, composed of γ and any other tightly associated minor subunits, would need to move rotationally with respect to the outer ring of catalytic subunits. Such movement would allow identical interactions with β subunits as the rotation drove the sequential conformational

changes of catalytic sites. When I first presented this concept to my research group, their acceptance was initially quite reserved (they knew all too well that I could be wrong). With further consideration, they became interested and supportive. Much remained to be explored, and some experimental approaches are summarized in the next few sections.

Modulation of Oxygen Exchanges by ATP Concentration

The modulation by ATP concentration of the ^{18}O exchange by the mitochondrial F_1-ATPase was more carefully documented (59). The chloroplast F_1-ATPase showed a similar behavior, and the distribution of the ^{18}O isotopomers in the P_i formed corresponded to a single catalytic pathway (76). Various wild type and mutant *E. coli* F_1-ATPase likewise showed increased exchange of the P_i formed with lower ATP concentrations. However, the distribution of ^{18}O isotopomers with the *E. coli* enzyme revealed more than one reaction pathway, apparently arising in part from the degree of dissociation of the inhibitory ϵ subunit (77). A question had been raised about whether the F_1-ATPase from a thermophile showed catalytic cooperativity because uni-site catalysis was not readily apparent. A cooperative experiment disclosed the expected modulation of the oxygen exchange but at a higher range of ATP concentration (78). The ATPase activity of yeast and *Neurospora* mitochondria showed distinct ATP modulation of the oxygen exchange (79). These various results meant that the increase in the extent of oxygen exchange with each P_i formed (which occurs with a decrease in the ATP concentration) is likely a general property of all F_1-ATPases and supports the probability that all ATP synthases share a common mechanism.

The ATPase of vacuolar membranes has been noted to have a composition resembling that of the ATP synthase. We felt that it should show similar oxygen exchange properties, and measurements demonstrated that this was so (79).

Some Other Assessments

We devised methods to measure bound reactants during steady-state ATP synthesis. A hexokinase accessibility method gave a measure of bound ATP, and a rapid dilution of medium $^{32}P_i$ gave a measure of bound P_i committed to form ATP. Measurements during photophosphorylation showed that even at lower substrate concentrations the total of catalytic site-bound ATP and committed P_i was greater than one per enzyme, as anticipated if the proposed catalytic site cooperativity was occurring. During photophosphorylation, $^{32}P_i$ rapidly labeled catalytic ATP, and then the medium [^{32}P]ATP formed was incorporated much more slowly into the non-catalytic sites (80). When illumination ceased, the catalytic site ATP continued to show ^{18}O exchange (81), meaning that reversible formation of bound ADP was still occurring. Within minutes the P_i dropped off, leaving a tightly bound ADP at the catalytic site (82). Such results helped explain labeling patterns we and others had observed and supported our concepts of tightly bound ATP as an intermediate and of catalytic site cooperativity.

The Insidious MgADP Inhibition

Occasionally in biochemical research one encounters a property of a system that seems designed to confuse and thwart the researcher. Such is the case with the inhibition by Mg^{2+}, which is dependent on the presence of an ADP bound without P_i at a catalytic site. Clarification of this unusual role of a tight ADP was necessary for an adequate understanding of the proposed binding change mechanism. The F_1-ATPase as conventionally isolated usually has a considerable portion with tight ADP present. In 1975, Moyle and Mitchell reported that mitochondrial F_1-ATPase was slowly inactivated by Mg^{2+} (83). Hackney noted the inhibition was slowly reversible by ATP addition (84). Observations in Vinogradov's laboratory showed that the inhibition depended on the presence of tightly bound ADP and that the Mg-ADP-inhibited form was stabilized by azide (85). Subsequent studies in our and other laboratories revealed characteristics of the inhibition. F_1-ATPases with tightly bound ADP when exposed to Mg^{2+} shows little or no initial activity upon ATP addition. Added ATP promotes slow release of the inhibitory ADP from a catalytic site as an increase to a steady-state rate is attained. At steady state, a slow interconversion of active and inactive forms continues. The bound ADP required for inhibition may arise from the cleavage of bound ATP or from medium ADP depending upon reaction conditions. P_i and various anions activate by promoting release of the ADP. The inhibitory ADP is at a catalytic site, not at a regulatory site as had been suggested.

Another important result of our continued probing was the recognition that, under some conditions, the presence of ATP at a certain non-catalytic site is necessary for the onset of

activity of the chloroplast F_1-ATPase (86). This was the first recognized function for a non-catalytic bound ATP. The action was found to result from acceleration of the release of the inhibitory ADP from catalytic sites that follows the addition of medium ATP (87). With the mitochondrial F_1-ATPase, ATP binding to the non-catalytic site could also accelerate the onset of the Mg-ADP inhibition. Upon addition of ATP and Mg^{2+} to the mitochondrial enzyme, an initial burst of activity declines to a slow rate as the Mg^{2+}-induced inhibition sets in; then the rate increases to a steady state as the non-catalytic sites slowly bind ATP (88).

From the above it is apparent that complicated rate patterns may be found. It is probable that with all F_1-ATPases, and even under favorable conditions, a fair portion of the enzyme may be in the inhibited form. Many reported and planned experiments may be undermined by an unrecognized occurrence of the Mg-ADP inhibition. A procedure for estimating the portion of the enzyme in the inhibited form, as developed by Murataliev (87), deserves wider application.

As mentioned earlier, with the intact ATP synthase the inhibitory MgADP is quickly removed by exposure to protonmotive force. This is akin to the removal of inhibitory imido-ATP that blocks hydrolysis but not synthesis. Chloroplast fragments show a light-activated ATPase that can be maintained by ATP cleavage. For unknown reasons, the activity continues even at higher Mg^{2+} concentrations that would readily result in inhibition of the separated F_1-ATPase (89).

Insights from Use of 2-Azido Nucleotides

By the mid-1980s, the sequence of the ATP synthase subunits was becoming available. An ATP derivative, 2-azido-ATP, which serves as a good substrate and upon photolysis becomes covalently attached, was described in Lardy's laboratory. We embarked on studies to find the number and clarify the location of ATP and ADP binding sites on the F_1-ATPases. That the ADP needed for the Mg^{2+} inhibition was bound at a catalytic site was readily confirmed. The 2-azido-ATP or ADP at catalytic or non-catalytic sites (known to be principally on the β or α subunits, respectively) labeled specific tyrosines not far apart on the β subunit. The sites were thus near subunit interfaces. Sites with similar conserved sequences were noted with the mitochondrial, chloroplast, and *E. coli* enzymes (90–92). Whether the liver (93) and chloroplast enzymes (see Ref. 94 and earlier references) had six nucleotide binding sites remained in question. Our data with the 2-azido nucleotides supported the probability that they, like the mitochondrial enzyme, had six total nucleotide sites (95, 96). Such results added to the already recognized similarity of structure and mechanism of the enzyme from different sources.

We were somewhat surprised to find that derivatization by 2-azido nucleotides of some catalytic or of non-catalytic sites of the chloroplast F_1-ATPase gave rise to multiple catalytic pathways. Measurements of the distribution of ^{18}O isotopomers formed revealed that partially modified enzymes retained some activity that still showed modulation of oxygen exchange by ATP concentration. With more extensive derivatization, the native catalytic pathway disappeared, and two weak, but independent, pathways were noted. Clearly some remaining catalytic sites retained weak activity independent from what neighboring sites are doing (97).

Behavior of ATP Synthase in Intact Mitochondria

Our experiments developing the binding change mechanism had been performed with isolated F_1-ATPases or fragmented membranes. The characteristics of the exchange reactions of the ATP synthase under conditions where mitochondria were capable of a high rate of tightly coupled oxidative phosphorylation were not known. To gain such information, we undertook cooperative experiments with the research group of K. LaNoue, using the ^{32}P and ^{18}O labels (98).

The tightly coupled mitochondria were incubated with oxidizable substrates. When low ADP limited net ATP synthesis, all catalytic steps continued rapidly as concentrations of P_i, ADP, and ATP remained unchanged. The expected rapid exchange of medium P_i with medium ATP with ^{32}P was observed. However, this rate was only about a fourth of the rate of interconversion of bound P_i and ATP at the catalytic site as measured by ^{18}O. Strikingly, the interconversion rate remained high even when the membrane potential was reduced considerably by dinitrophenol addition. The addition of ADP and a hexokinase and glucose trap resulted in rapid formation of glucose 6-phosphate. The distribution of ^{18}O isotopomers in the glucose 6-phosphate showed a single reaction pathway, even when some uncoupler was added. The rapid reversal of bound ATP formation continued, so that about two reversals at the catalytic

site occurred for each ATP released to the medium. Even though this reversal was occurring and some medium P_i was being formed from bound ATP, the overall reversal to form medium P_i from medium ATP ceased. This can be explained by the lack of import of ATP by the ADP-ATP translocase. During rapid ATP synthesis, unlike the rapid reaction reversal that is occurring at the catalytic site of the ATP synthase, with high ADP in the medium the translocase is a one-way street.

Another important confirmation of alternating site participation came from these experiments with intact mitochondria. In the dynamic state with no added ADP the forward and reverse rates of all steps are equal, so that there is an equal chance that medium P_i that has formed bound ATP will be released to the medium as P_i or as ATP. If release of the bound ATP can only occur when another ADP and P_i bind, then the rate that medium P_i forms bound ATP will be twice the rate that it forms medium ATP. A ratio of about 2 to 1 for these rates was found when the overall rates were varied up to 10-fold by changes in reactant concentrations or temperature. This provides evidence that alternating site participation is occurring under conditions where rapid oxidative phosphorylation is possible.

One aspect of these experiments may be pointing to an important and unrecognized property of the ATP synthase in its native environment. When the membrane potential was reduced by the addition of some uncoupler while ATP was present not only did net ATP hydrolysis occur but also the rate of reaction reversal at the catalytic site remained high. This was reminiscent of earlier observations with submitochondrial particles that the oxygen exchange accompanying ATP hydrolysis was much greater at higher ATP concentrations than with the separated F_1-ATPase and was relatively insensitive to uncouplers. In some manner, the capacity for rapid interconversion of bound reactants is retained better with the native membrane-bound synthase. I would still like to know how this is accomplished.

Site Filling and Catalysis, an Unfinished Story

It is well recognized that when only one catalytic site on the F_1-ATPase binds ATP or ADP and P_i, a slow interconversion of the substrates occurs. What remains uncertain at the time of this writing is whether a second or a second and a third site must bind ATP for the rapid release of ADP and P_i to occur. For over two decades since slow uni-site catalysis and alternating participation of catalytic sites was recognized, my group and most others felt that the binding of ATP to a second site sufficed for rapid catalytic turnover during net ATP hydrolysis. Because of binding affinities, it was recognized that all three catalytic sites would be filled at millimolar concentrations of ATP, approximating physiological conditions. I proposed that ADP during net synthesis, or ATP during net hydrolysis, entered the catalytic cycle by binding to different sites as indicated in Fig. 2. The critical need under conditions favoring rapid synthesis was regarded as the presence of interconverting, tightly bound substrates at Site 2 of Fig. 2 and ADP and P_i at Site 1. Conversely, under conditions for rapid hydrolysis the critical need was the presence of ATP at Site 3. In each case one rotational step would change the site to the tight conformation where covalent catalysis could occur. For both net synthesis and hydrolysis, the presence or lack of ADP or ATP at a third site was regarded as having a minor influence on the rates.

Several years ago, Senior and Weber and colleagues introduced a fluorometric method for estimating the amount of bound nucleotides at catalytic sites. They replaced a tyrosine at catalytic sites with tryptophan and replaced tryptophans in other locations of the *E. coli* F_1-ATPase (Ref. 99 and earlier references). The binding of nucleotides at the catalytic sites quenched the fluorescence of the tryptophan and allowed an estimation of the number of catalytic sites filled. They and Allison's group with the thermophilic F_1-ATPase (Ref. 100 and earlier references) found that three sites appeared to be filled with nucleotide as near maximal velocity was reached with an increase in the ATP concentration. They then assumed that the binding of ATP to a second and to a third site was necessary for rapid net hydrolysis. This I believe will prove to be incorrect. Instead, it still seems likely that although three sites may become filled (probably mostly with ADP), the essential need for rapid hydrolysis is the binding of ATP to a second site. In other words, bi-site activation probably occurs along with tri-site filling. These issues are considered in more detail elsewhere (101).

Evaluations of Rotational Catalysis

As the research journey proceeded, we attempted some evaluations of whether rotational catalysis indeed occurs. We found that when the chloroplast F_1-ATPase was reacted first with

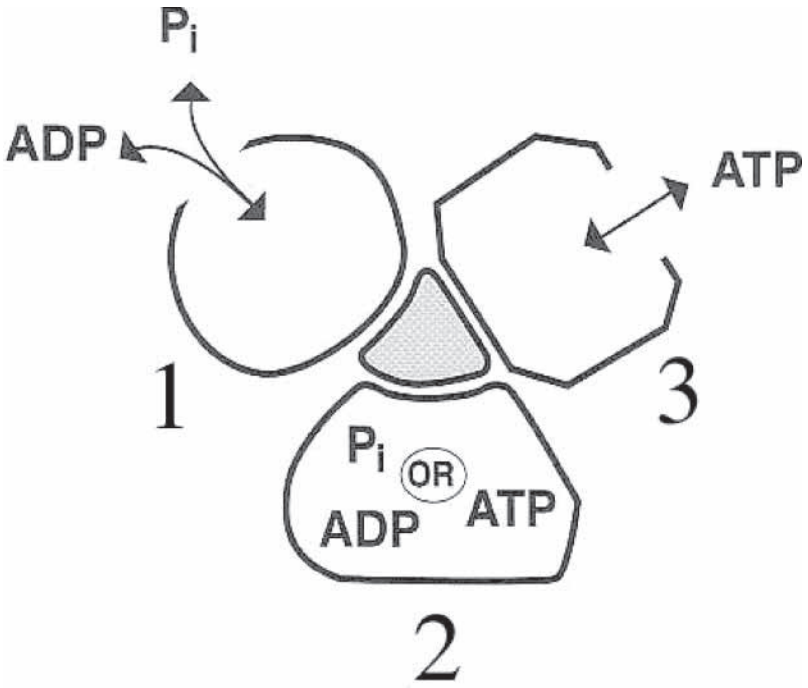

FIG. 2. **A depiction of the three major conformations of catalytic sites for bi-site activation of ATP synthesis or hydrolysis by the ATP synthase.** Three catalytic sites in different conformations are shown with asymmetric interactions to the *shaded* γ subunit. During catalysis sites are converted sequentially into three different states accompanying rotation of the γ subunit. The sequence for synthesis is *1* $\rightarrow$ *2* $\rightarrow$ *3*; for hydrolysis it is *3* $\rightarrow$ *2* $\rightarrow$ *1*. Site *1* binds ADP better than ATP and is the site at which ADP and P_i must be present for rapid synthesis to occur. Site *2* has the ability to catalyze chemical transformation and to be present as a form with ADP and P_i present or with ATP present. ATP can be released from Site *3* during synthesis and must be present at this site for rapid hydrolysis. Figure and legend are reprinted with permission from Ref. 101.

2-azido-ATP and then with [^{14}C]DCCD, two different β units were derivatized. Thus the DCCD does not label a subunit with tightly bound nucleotide present. The DCCD-labeled enzyme retained weak catalytic activity. This made it possible for us to find if catalysis changed the conformations of the β subunits that determine their chemical reactivity, as would be expected if rotational movement of the γ relative to the β subunit had occurred. When the enzyme was first reacted with DCCD, allowed to perform catalysis with 2-azido-ATP, and then the azido-ATP photolyzed, the subunits were randomly labeled (102). To us, this made rotational catalysis likely, but the weak catalytic activity of the DCCD-modified enzyme detracted from the result.

In another approach a bifunctional cross-linking agent that reacted with lysine NH_2 groups, and that had a central cleavable –S–S– linkage, was used. About three cross-links, mostly between the γ and δ subunits and the α subunit, caused loss of two-thirds of the activity, and the activity was recovered when the disulfide bonds were cleaved (103). Although the findings were consistent with rotational catalysis, they were not proof.

In contrast, in the same year (1987) Musier and Hammes reported that a cross-linking of the β and γ subunits did not inhibit catalysis and concluded that rotational catalysis did not occur (104). This at first appeared to be a to be a definitive finding. However, examination of their paper suggested some possible experimental uncertainties. Also, possibly the derivatization had uncoupled Ca^{2+}-activated hydrolysis from rotation. A more likely possibility was that the long –CH_2– chains in their cross-linkers may have allowed sufficient freedom of movements to not be restrictive. The catalytically induced movements of β subunits that we had observed with the 2-azido experiments (102) still seemed valid. I thus did not abandon the concept of rotation and looked forward to better evaluations. The concept remained controversial in the field.

It was becoming clear that structural data could provide the base for critical assessment of rotational catalysis. I was aware that Walker's group was attempting to obtain suitable crystals for x-ray analysis. In the meantime, it seemed that my group might accomplish more by studies that were under way with the 2-azido derivatives and by trying to define the location and function of bound nucleotides. As these and related studies progressed I prepared a comprehensive 1993 review of the status of research on how ATP is made under the title "The

Binding Change Mechanism for ATP Synthase—Some Probabilities and Possibilities" (63). The literature at that time was regarded as giving strong support to the concepts proposed in the binding change mechanism, with the exception that rotational catalysis was regarded as likely but definitely not established.

Fortunately progress continued in Walker's laboratory (105). This culminated in the 1994 report of the structure of a major portion of the mitochondrial F_1-ATPase (69). Receipt of an advance copy of the report from Walker was an occasion for gratifying emotion. The reported structure showed that the three β subunits were indeed in different conformations, and one had poor nucleotide affinity. The γ subunit was centrally located with structural associations consistent with its rotation driving sequential conformational changes of the β subunits. The authors interpreted their data as strongly supporting the binding change mechanism. Other x-ray studies interpreted as inconsistent with the binding change mechanism (106) appear mistaken.

The availability of high resolution structural data made more critical assessments of rotational catalysis possible. Richard Cross, my former postdoctoral associate, noted residues in the γ and β subunits that were closely adjacent. When these were replaced with cysteines by mutagenesis good catalytic activity was retained. Oxidation of the –SH groups to form a disulfide cross-link blocked catalytic capacity, which was regained when the disulfide was reduced. With the disulfide linkage present, two β subunits that were not cross-linked were replaced by β subunits from an enzyme labeled with radioactivity during growth of the *E. coli*. Cleavage of the disulfide and catalysis resulted in randomization of the position of the γ subunit relative to the labeled β subunits (107). Similar loss of catalytic capacity upon disulfide cross-linking and related salient findings were reported from Capaldi's laboratory (108). Such results and other related findings were considered by the field to establish the occurrence of rotational catalysis.

In the spring of 1997, a stunning visual conformation of rotational catalysis came from the laboratories of Yoshida and Kinosita in Japan. In a novel experimental approach, they attached a long actin side chain with a fluorescent label to the γ subunit and, through inserted histidine residues, attached the modified enzyme to solid support. Upon hydrolysis of ATP, the rotational movement of γ was observable in the microscope (109). Important characteristics of the catalysis were shown and are still being studied. I remember the thrill when I saw the rotation from a VCR recording that Yoshida kindly sent me. The dramatic experiment has gained wide recognition and removed nearly all doubt about the existence of rotational catalysis. Independently, Junge and associates developed a sophisticated fluorescence polarization technique that showed rotation accompanying ATP cleavage (110). This progress added to the near certainty of rotational catalysis. I was able to include references to their papers in press in a contribution entitled "The ATP Synthase—A Splendid Molecular Machine" that appeared in the 1997 *Annual Review of Biochemistry* (111).

A Life Style Change

By 1994, my research laboratories were essentially closed. ATP synthase and bioenergetics and enzymology had yielded center stage to biochemistry related to genetics and development. Postdoctoral fellows were no longer seeking my laboratory. It was over 50 years since I had received my Ph.D. My mental capacities seemed to have slipped more than my physical capacities. I felt it would be difficult to continue to be at the research forefront and that unfunded U. S. Public Health Service applicants might make better use of funds than I, so I gave 2 years of committed research support back to the U. S. Public Health Service and bought a summer home in a mountain valley in Wyoming with accompanying golf and tennis facilities.

In October of 1997, while on my way to back to my winter haven in a home I love in the hills above UCLA, I received that electrifying early morning call informing me that I had been chosen to share the 1997 Nobel Prize in Chemistry. This of course was personally very gratifying. It increased my stature with my grandchildren. More importantly, it was a recognition of the many fine investigators that had contributed to the unveiling of the ATP synthase mechanism. It gave additional meaning to the careers of postdoctoral fellows and graduate students of my group without whom there would have been no prize, and it has made my life since more vital and interesting. I recommend that if you are going to receive a Nobel prize, have it come late in your career when you no longer have the rewards that come from participation with a fine research group. Also, then you do not need to answer the question, "What research are you going to do now?"

Some Final Comments

The contributions of many scientists were essential for the gaining of our present insights into the ATP synthase catalysis. They should share in the satisfaction that comes from knowing much about how this important biological catalyst appears to operate. As the scientific enterprise grows ever larger, many fine contributors do not receive the appreciation they deserve. They should share in a pride for what has been achieved.

The scientific accomplishment of the Twentieth Century that I admire most is the revealing of the multifaceted capabilities of proteins and of their structures that make these capabilities possible. Perhaps I am a bit more infatuated with enzymes than some. I do not know any enzyme that I could not learn to love, although I will admit that some are more attractive than others.

To all who created our stable and prosperous country and its research universities, which made a career such as mine possible, and mostly to my colleagues, I give my thanks.

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Reflections

A PAPER IN A SERIES COMMISSIONED TO CELEBRATE THE CENTENARY OF THE JBC IN 2005

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1905–2005
100 Years of Biochemistry and Molecular Biology

Happily at Work

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It is a great privilege to be asked for a "Reflections" essay; I admire those prepared by my predecessors. My teachers were less prestigious than Arthur Kornberg's (1), and there was no single major theme in my research as was the case with several previous contributors to this series. Instead we studied a wide variety of metabolic phenomena that I have described in a summary of my first 50 years of biochemical research (2).

Our findings included a treatment for selenium poisoning in livestock (undergraduate thesis; selenium-containing mercapturic acids are excreted in the urine) that was applied successfully to a human case; our studies of spermatozoa will be described in a following section. We elucidated the mechanism by which L-glyceraldehyde inhibits glycolysis (3). That disproved Needham's non-phosphorylating glycolysis in embryos and tumors. Could that have encouraged him to drop experiments and to devote his talents to prepare his magnificent history of Chinese science instead? We found that the function of biotin was to fix CO_2 in heterotrophic organisms (4); cellular respiration rates varied with the availability of inorganic P and phosphate acceptor (5, 6); propionate was metabolized by CO_2 addition to ultimately yield succinate (7, 8). My students purified and crystallized some 10 phosphate-transferring enzymes, and we demonstrated that most of them required MgATP as substrate and were inhibited by free ATP; we found 16 different antibiotics that affected oxidative phosphorylation (9, 10) and a dozen that acted as ionophores (11), some of which are still being used in experiments. We also found that caffeine increased respiration and dramatically induced whiplash-type motility in sperm by increasing cyclic AMP (12, 13); the respiratory response was dependent on the utilization of acetylcarnitine (14). Thyroid hormone and also dehydroepiandrosterone induced the synthesis of mitochondrial glycerol-3-phosphate dehydrogenase to as much as 20 times the normal concentration (15–17) and formed part of a thermogenic system (17, 18). The path of carbon in gluconeogenesis was found to involve carboxylation of pyruvate (Utter reaction) in mitochondria, reduction of oxalacetate to malate, malate transport to cytosol in exchange for pyruvate, oxidation of malate to oxalacetate (the precursor of phosphopyruvate) together with the generation of the NADH required to reduce 3-phosphoglycerate to triose phosphate (19, 20); serine was found to be converted to glucose by an entirely different pathway, probably the reverse of its synthesis from hydroxypyruvate (21). We also found that levels of liver cytosolic phosphoenolpyruvate carboxykinase (PEPCK) are regulated by the need for gluconeogenesis; they are increased by fasting and decreased in well fed animals; PEPCK is activated by ferrous ion, and in liver free calcium activates PEPCK by releasing Fe^{2+} from mitochondria to the cytosol (22); feeding tryptophan inhibits gluconeogenesis because its metabolite, quinolinate, forms a complex with ferrous ion that blocks PEPCK (23, 24). The widely reported enhancement of liver mitochondrial respiration following exercise or the administration of glucagon or adrenaline to rats was found to be mediated by elevated malate concentration in the liver (25, 26). Malate is known to facilitate mitochondrial uptake of substrates by exchange across the mitochondrial membranes.

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Naturally there was also an abundance of studies that yielded useful facts but not new concepts and many experiments undertaken to test hypotheses that turned out to be without merit! One reason for the diversity of research is that we wanted graduate students to have their own thesis research problems. Sixty-four candidates earned their Ph.D. degree in our group between 1945 and 1989 and more than 100 postdoctorate fellows conducted their research in our laboratories at the Institute for Enzyme Research. Relationships with these scholars and friends have always meant a great deal to me.

At the time my research history was written (2), we were studying an intriguing class of Janus-like proteins, caltrins, that function in fertilization. Because the work was in progress it was not described in that essay. The caltrins have not been widely publicized and therefore are probably not familiar to most biochemists. The caltrins of different species have widely different structures and their multiple functions are achieved by disparate mechanisms.

The Caltrin Story

No aspect of living processes is more awe-inspiring than the union of a microscopic spermatozoon with an egg of the same species to initiate a new life. In this process the contribution of the male is to present a set of haploid chromosomes to join those of the egg. However, this presentation is a complex ceremony involving "capacitation," *i.e.* alteration of sperm plasma membranes to permit penetration by Ca^{2+}. Calcium uptake is followed by disruption of the acrosome, a sac containing hyaluronidase and proproteinases that autocatalytically are converted to active acrosins. The sperm attach to the protective layer of the egg, a glycoprotein matrix (zona pellucida), and the calcium-activated acrosomal enzymes attack the zona to provide a path for sperm entry. Calcium uptake by the contractile components in the sperm tail facilitates the acquisition of "hyperactivated" motility characterized by rapid lashing and wider excursion of the sperm tail. This causes the sperm to swim in tight arcs to drive through the zona and then penetrate the egg. The role of caltrins in regulating each of these processes is complex and fascinating.

Our work with spermatozoa had been continuous since 1939 when my professor, Paul Phillips, and I developed a medium for the preservation of animal sperm (27). It permitted the retention of motility and fertility for 8–12 days and launched the artificial insemination industry in livestock. Because we had solved the practical problem, I was free to study basic aspects of sperm metabolism and the regulation of energy capture for motility. The findings during that period included the first clear statement concerning the mechanism by which 2,4-dinitrophenol functions: "the fact that DNP decreased the motility of the spermatozoa, while the processes of glycolysis and oxidation are increased, indicates an interference of the energy-coupling mechanism with the result that oxidation and glycolysis run rampant, while the energy is lost as heat rather than as work" (28).

During that same period we discovered that, unlike most substrates that increased both respiration and motility (29, 30), "β-hydroxybutyrate was unusual in that it depressed endogenous respiration slightly, but supported an excellent degree of motility. It is possible that the oxidation of this metabolite, in spermatozoa, is more efficiently coupled with phosphorylation than is the oxidation of the endogenous lipid reserve" (29). This explanation seems also to apply to the working heart (31) and may have therapeutic implications (32).

Nearly a century ago the eminent physiologist Jacques Loeb demonstrated that fertilization of sea urchin eggs does not occur in the absence of Ca^{2+} (33, 90). This failure is based on the need for Ca^{2+} to promote the lysis of the acrosomal membranes on the sperm head (acrosomal reaction) of both invertebrates (34) and vertebrates (35, 36).

We had been investigating the role of calcium transport in the regulation of sperm behavior, including the acrosome reaction and enhancement of motility for some years (35, 37, 91) when Donner Babcock found that the rapid uptake of calcium by bovine epididymal sperm did not occur in sperm separated from ejaculates (38). Epididymal sperm contain 6 ± 1 nmol of calcium/10^8 cells and will accumulate up to 50 nmol/10^8 sperm when incubated in a medium containing 0.2 mM calcium and an oxidizable energy source such as β-hydroxybutyrate (37, 38, 91). Ejaculated bovine sperm have the same low calcium content despite being bathed in 9 mM calcium in seminal fluid. When washed free of seminal fluid and suspended in media containing calcium, ejaculated sperm still do not take up this divalent cation. The obvious next experiment was to add seminal fluid to epididymal sperm, which demonstrated the presence of a calcium transport inhibitor that we termed caltrin. The inhibitor was purified to homo-

Bovine Caltrin **10** **20**
Ser.Asp.Glu.Lys.Ala.Ser.Pro.Asp.Lys.His.His.Arg.Phe.Ser.Leu.Ser.Arg.Tyr.Ala.Lys.Leu.Ala.Asn.Arg.Leu.
Ala.Asn.Pro.Lys.Leu.Leu.Glu.Thr.Phe.Leu.Ser.Lys.Trp.Ile.Gly.Asp.Arg.**Gly.Asn.Arg.Ser.**Val

Rat Caltrin 10 20
Lys.Val.Ile.Gly.Lys.Lys.Ala.Asn.Cys.Pro.Asn.Thr.Leu.Val.Gly.Cys.Pro.Arg.Asp.Tyr.Asp.Pro.Val.Cys.Gly.
Thr.Asp.Gly.Lys.Thr.Tyr.Ala.Asn.Glu.Cys.Ile.Leu.Cys.Phe.Glu.Asn.Arg.Lys.Phe.Gly.Thr.Ser.Ilu.Arg.Ile.Gl
n.Arg.Arg.Gly.Leu.Cys

Guinea Pig Caltrin I **10** **20**
Ala.Phe.Ala.Pro.Ser.Lys.Val.Asp.Ser.Asp.Arg.Pro.Asn.Cys.Ser.Arg.Tyr.Val.Gln.His.Leu.Tyr.Met.Cys.Thr.
Lys.Glu.Leu.Asp.Pro.Val.Cys.Gly Thr.Asp.Gly.His.Thr.Tyr.**Gly.Asn.Arg.Ser**.Ile.Phe

Guinea Pig Caltrin II **10** **20**
Arg.Arg.Leu.His.Gly.Gln.Ala.Ile.Asn.Arg.Pro.Gly.Ser.Cys.Pro.Arg.Val.Met.Ile.Tyr.Cys.Pro.Ala.Arg.His.Pro
.Pro.Asn.Lys.Cys.Thr.Ser.Asp.Tyr.Asp.Cys.Pro.Lys.Pro.Gln.Lys.Cys.Cys.Pro.Gly.Tyr.Cys.Gly.Lys.Gln.
Cys.Tyr.Gln.Pro.Glu

Mouse Caltrin 10 20
Leu.Ile.Cys.Asn.Ser.Cys.Glu.Lys.Ser.Arg.Asp.Ser.Arg.Cys.Thr.Met.Ser.Gln.Ser.Arg.Cys.Val.Ala.Lys.Pro.
Gly.Glu.Ser.Cys.Ser.Thr.Val.Ser.His.Phe.Val.Gly.Thr.Lys.His.Val.Tyr.Ser.Lys.Gln.Met.Cys.Ser.Pro.Gln.
Cys.Lys.Glu.Lys.Gln.Leu.Asn.Thr.Gly.Lys.Lys.Leu.Ile.Tyr.Ile.Met.Phe.Gly.Glu.Lys.Asn.Leu.Met.Asn.Phe

Stallion Caltrin (very preliminary) 10 20
Ala.Leu.Leu.Ser.Pro.Gly.Thr.Ala.Pro.Asp.xxx.His.Val.Tyr.Lys.Asp.Arg.Leu.His.Leu.xxx.Asn.Arg.Glu.Arg.
Lys.Leu.Ile.Cys.Ala.Thr.Asn.Gly.GlnThr.Tyr.Arg.Asn.Pro.Cys.Ile.Phe.Cys.Arg.Glu.Cys.Ile.Ala.Ser------

Bold: Bovine vs Guinea Pig I
Underlined : Rat vs Guinea Pig I

FIG. 1. **The amino acid sequences of caltrins from bull, rat, guinea pig, and mouse.**

geneity (39) and the amino acid sequence was determined (Fig. 1) (40). A protein with the properties of caltrin was demonstrated to be bound to plasma membranes of ejaculated sperm and was not detected on the membranes of epididymal sperm (41). The sequence also disclosed that a similar protein, termed bovine seminal plasmin, had been isolated from bovine semen and was described as having antimicrobial activity (42). Errors in the sequencing of seminal plasmin (43) were later corrected (44), thus confirming our structure and the identity of seminal plasmin and caltrin. Analyses for bovine caltrin based on inhibition of calcium uptake by epididymal bull sperm indicated that bull seminal fluid contained about twice as much caltrin as was required to inhibit calcium uptake 90% by the sperm present in an ejaculate.

On storage, caltrin lost activity as a blocker of calcium transport and became an *enhancer* of calcium uptake (45). This transformation could be accomplished rapidly by binding the fresh inhibitory caltrin to a cation exchanger. The recovered caltrin *increased* both the rate and extent of calcium uptake. The acidic components of the eluate contained ether-extractable phospholipids that restored calcium transport inhibition to the enhancer caltrin protein. Among the pure phospholipids tested only phosphatidylserine converted enhancer caltrin to a calcium transport inhibitor. Phosphatidylcholine, phosphatidylinositol, and citrate abolished the stimulation of calcium uptake but did not change the enhancer to an inhibitor (46).

With the aid of anti-bovine caltrin antiserum, two caltrin proteins were detected and isolated from guinea pig seminal vesicle secretions (47, 92). There were no common amino acid sequences in these peptides designated G.P. caltrins I and II, and the only identity with bovine caltrin was a group of four (Gly-Asn-Arg-Ser) near the carboxyl terminus of bovine caltrin and G.P. caltrin I (Fig. 1); nonetheless, the anti-bovine caltrin antiserum recognizes these guinea pig proteins. Both G.P. caltrins contain carbohydrate residues as detected with concanavalin A (47, 92); bovine caltrin does not (39). The molecular weights of the peptide portion of G.P. caltrins I and II are 5082 and 6255, respectively. The maximal inhibition of calcium uptake into guinea pig sperm by each of the G.P. caltrins approached 50%. Deglycosylation of G.P. caltrins using trifluoromethanesulfonic acid caused both I and II to enhance the rate of calcium uptake by guinea pig epididymal sperm, *i.e.* they become enhancer caltrins (48).

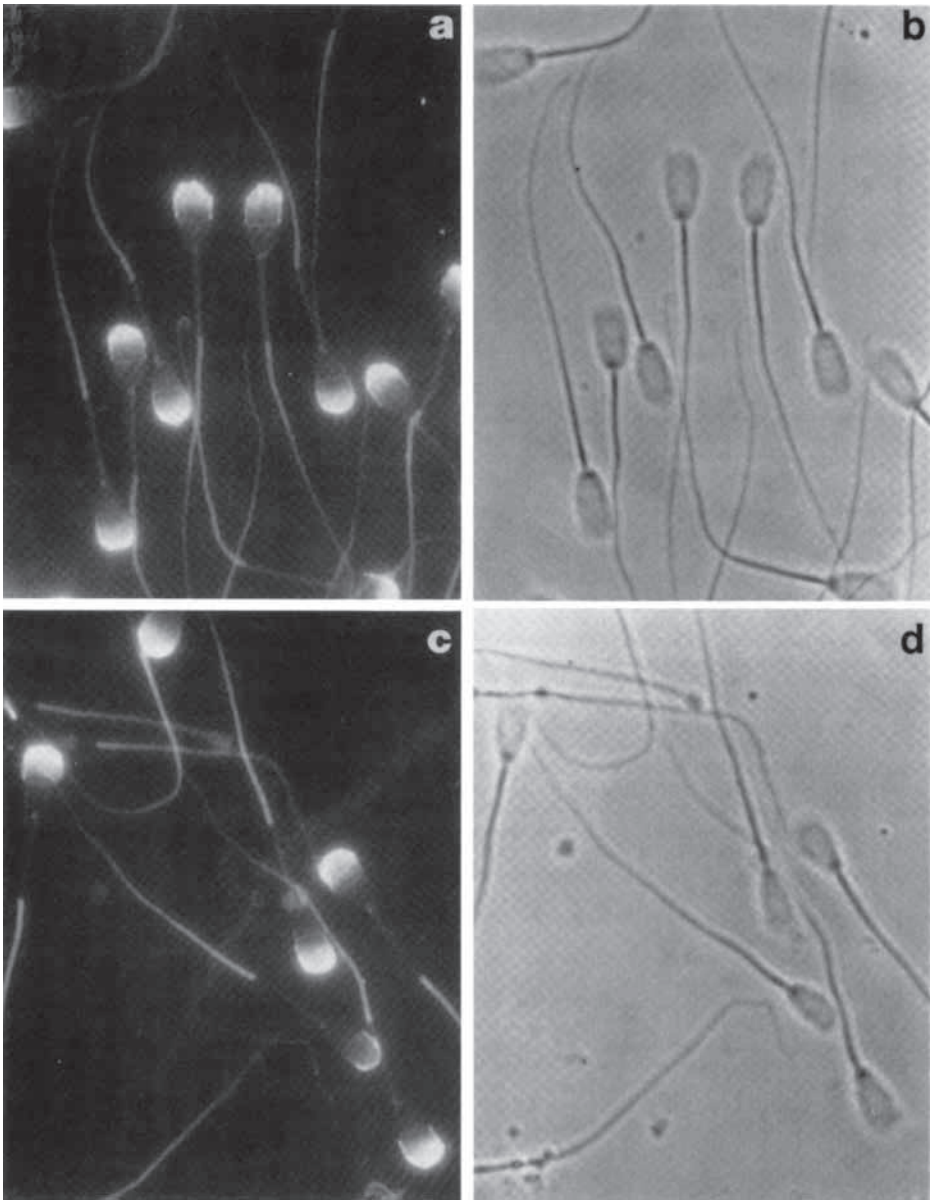

FIG. 2. **Bull sperm bind caltrin to the tail and over the acrosome.** *a*, epididymal sperm treated with 0.40 mg of caltrin/10^8 cells in 1 ml and washed. *c*, ejaculated sperm washed free of seminal fluid and not exposed to purified caltrin. Sperm were spread and dried on glass slides, treated in succession with rabbit monospecific caltrin antiserum and goat anti-rabbit IgG that had been labeled with fluorescein isothiocyanate. Slides were washed to remove excess protein and viewed with a Zeiss fluorescence microscope (*a* and *c*); *b* and *d* are corresponding phase contrast photomicrographs. Epididymal sperm not exposed to caltrin did not bind the fluorescent-labeled antibody.

The seminal vesicles of rats and mice contain caltrins that have been purified and sequenced (Fig. 1) (49). Their calculated molecular weights are 6217 and 8476, respectively. Rat caltrin is derived from a 54-kDa inactive precursor produced in the seminal vesicles (50); the biosynthesis of rat caltrin and its precursor is androgen-dependent (51). The active rat protein has a sequence of 13 amino acids nearly identical with a segment of G.P. caltrin I. Neither rat nor mouse caltrins have any significant sequence similarity to G.P. caltrin II or bovine caltrin. Each of the caltrins, with the exception of the bovine, contains cysteine residues that are not reactive with thiol reagents until the protein has been treated with reducing agents such as dithiothreitol. Reducing the cystine disulfide bonds of rat caltrin and carboxymethylating the protein diminishes, but does not eliminate, the effect on calcium transport. The locations of the disulfide bonds are known (52). In the case of mouse caltrin, which contains 7 cysteine residues, the protein appears to be a disulfide dimer formed between the odd cysteines. Reduction converts the 17-kDa native mouse caltrin to 8.5 kDa. Bovine caltrin also behaves as a dimer of M_r 9600–10,500 by gel permeation and gel electrophoresis, but from its amino acid content we found a M_r of 5411 and no cysteine.

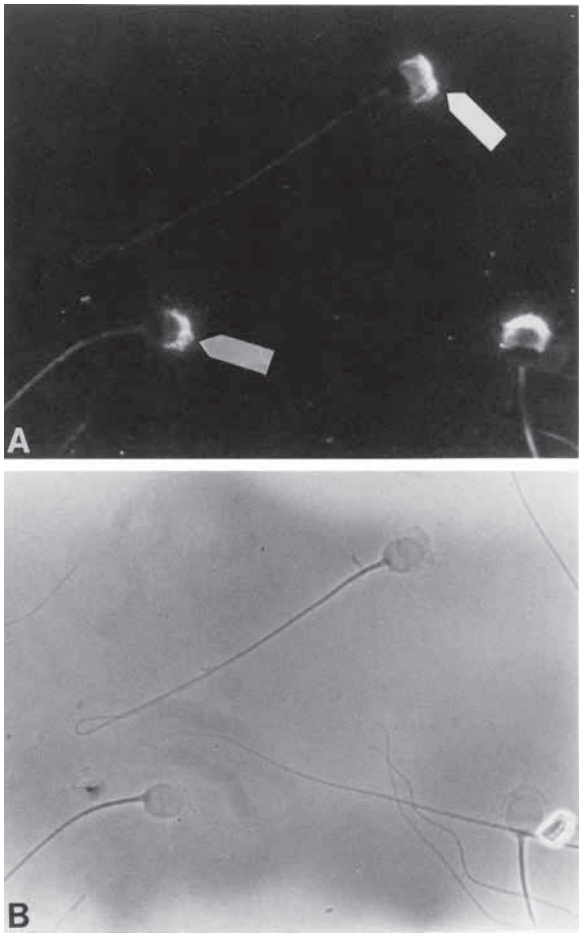

FIG. 3. **Binding of guinea pig caltrin I to guinea pig epididymal sperm.** Epididymal sperm was treated with 0.5 mg of caltrin I/10^8 cells in 1 ml for 1 h and then washed twice with phosphate-buffered saline. Further treatments are described in the legend to Fig. 1 except that the antiserum had been prepared from rabbits treated with caltrin I. *A*, immunofluorescence photomicrograph. *Arrows* designate caltrin binding to the acrosome. *B*, corresponding phase-contrast photomicrograph.

A caltrin protein designated SVS VII has been purified from mouse seminal vesicles by Yee-Hsiung Chen and co-workers (53). It has 76 amino acids, 71 of which are identical with our sequence for mouse caltrin (49). Luo *et al.* (53) determined the sequence of their protein from the corresponding cDNA and the first 18 amino acids by automated Edman degradation; they ascribe the differences to errors in our structure. Our sequence was determined by the Edman procedure applied to five different peptides isolated from caltrin subjected to partial proteolysis. Four of the peptides contained the amino acids in question; each of these fragments contained the same sequence. Therefore we are confident of our structural assignment and assume that there are genetic differences between CD-1 and Swiss white mice. Three of the differences can be explained by single base changes. The Chen group (54) reported another caltrin-like protein, P12, from mouse seminal vesicles with no appreciable sequence similarity to our mouse caltrin.

Bovine caltrin binds over the acrosome and the entire tail of bull sperm but does not bind to the posterior part of the head nor to the midpiece, which contains the mitochondria (45). Washed sperm separated from bull semen show the same fluorescence staining pattern as epididymal sperm treated with caltrin (Fig. 2), but epididymal sperm not exposed to caltrin do not bind the fluorescence-labeled antibody (45). Caltrin binding at these two sites was assumed to regulate the acrosome reaction and the hyperactivation of motility, respectively. These separate functions were clearly defined in the case of guinea pig sperm (see below).

The immunofluorescence test showed G.P. caltrin I binding to the G.P. sperm over the acrosome but not to other parts of the sperm (Fig. 3). Caltrin I (0.5 mg/10^8 sperm) nearly completely inhibited hyaluronidase release from the acrosome during 30 min of incubation in the presence of 1 mM calcium; many of these sperm were hyperactive but had intact acrosomes (48). Guinea pig epididymal sperm not treated with caltrins released hyaluronidase and were

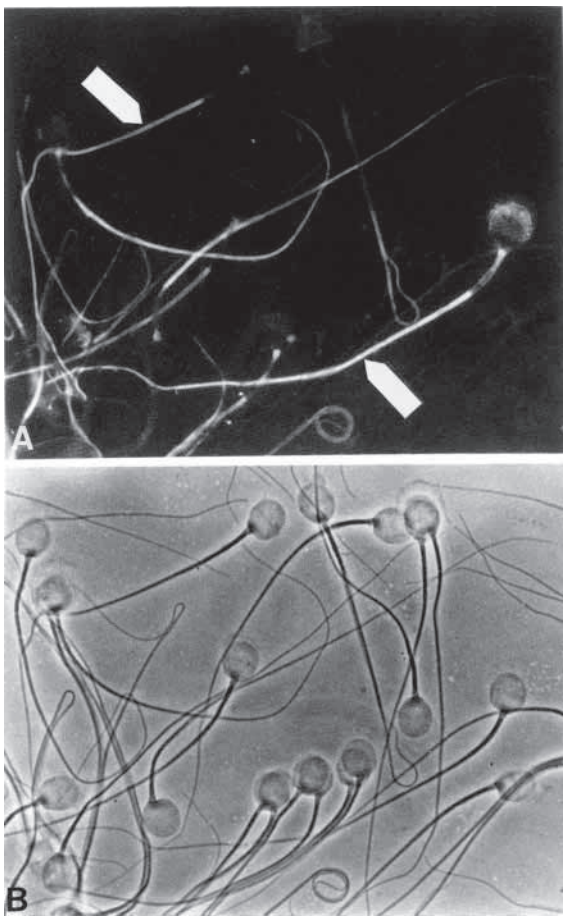

FIG. 4. **Binding of guinea pig caltrin II to guinea pig epididymal sperm.** Conditions are the same as described in the legend to Fig. 3 except that sperm were treated with purified guinea pig caltrin II and the rabbit antiserum had been prepared with caltrin II. *A*, immunofluorescence photomicrograph. *Arrows* designate caltrin binding to the sperm tails. *B*, corresponding phase-contrast photomicrograph. Reprinted with permission from Ref. 48.

hyperactive, indicating calcium access to both the acrosome and the tail. Guinea pig caltrin II (0.5 mg/10^8 sperm) bound to the sperm tail and very sparingly to the head (Fig. 4). It depressed hyaluronidase release from the acrosome only partially, and during 2 h of incubation the sperm maintained linear motility because calcium access to the contractile mechanism was blocked (47, 92). These separate sites of binding and function account for the fact that each of the GP caltrins inhibits about 50% of the calcium uptake that occurs in the absence of these seminal vesicle proteins (48).

The "Eureka!" announcement (55) that "observations of differences in the effect of seminal plasma contamination on hyperactivation and capacitation made in the present study provide further evidence for the existence of functionally separated, distinct regions in the spermatozoon" in effect confirmed for the human sperm what had been demonstrated much earlier in guinea pig sperm (48).

Some years ago Tschesche *et al.* (56) reported the presence of proteolytic inhibitory proteins in mammalian seminal plasma, and their observation has been confirmed by several laboratory groups. Rat caltrin and guinea pig caltrin I are also trypsin inhibitors (52), and the former is identical with the inhibitors from the pancreas isolated by Uda *et al.* (57) and from liver by Kido *et al.* (58). There is extensive homology between rat caltrin and trypsin inhibitors isolated from pancreatic secretions of many different mammalian species. Guinea pig caltrin II, bovine, and mouse caltrins are not trypsin inhibitors (50). The gene for caltrin (seminal plasmin) has been characterized (59) and, remarkably, has been shown to be a member of the extensive neuropeptide Y gene family (60). Seminal plasmin/caltrin was the subject of an excellent review (61).

In summary, the role of caltrins in fertilization can be postulated from their known functions. On ejaculation, spermatozoa bind caltrins as well as other seminal vesicle products. Bound caltrins prevent calcium movement into the acrosome and thus prevent a premature acrosome reaction. The hydrolytic and proteolytic enzymes are retained until needed. By

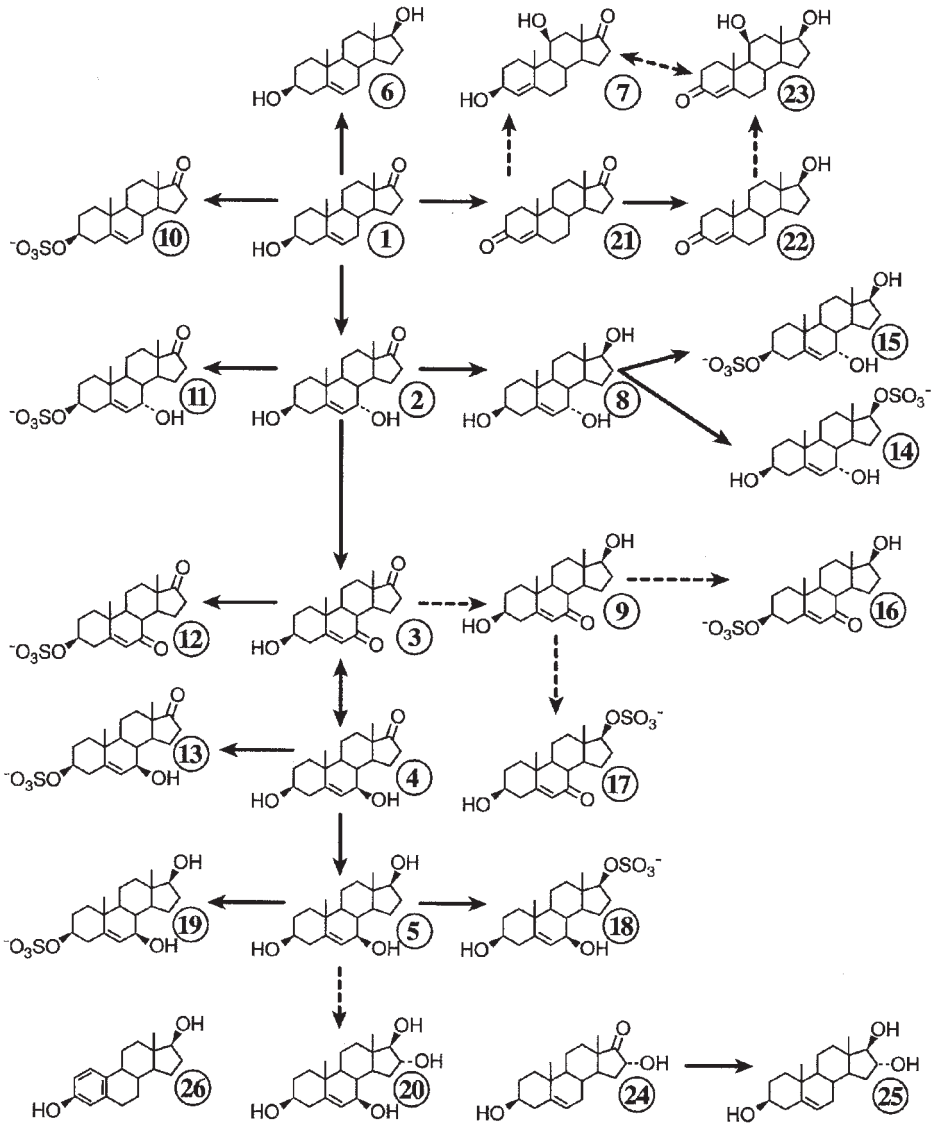

FIG. 5. **Steroids produced from DHEA (1) in liver homogenate fortified with ATP, NADPH, and malate.** The *broken arrows* are postulated enzyme-catalyzed reactions.

preventing calcium uptake by the tail, caltrins keep the sperm moving forward. After some time the sperm will have moved up the female reproductive tract and encountered the egg(s); the phosphatidylserine will have been dissociated from the caltrin protein in the case of bovine and the carbohydrate residues hydrolytically removed in the case of rodents. The enhancer forms of caltrin then stimulate calcium uptake at the acrosome where it activates membrane discomposition and at the tail where it induces whiplash movement of the sperm tail. Species that produce two caltrins have one that acts at the acrosome and controls hyaluronidase and acrosin release; the other binds to the sperm tail and regulates motility.

Investigation of these proteins in our laboratory was dropped for lack of laboratory space on my reaching emeritus status in 1988 but is being continued by Carlos Coronel in Argentina.

Dehydroepiandrosterone—A New Chapter

The University of Wisconsin treats its retirees more hospitably than Columbia treated Professor Chargaff (62); I was allowed to retain a small laboratory, enough for chemistry but not for extensive metabolic research.

A problem that was inviting concerned possible active steroid hormones derived metabolically from dehydroepiandrosterone (DHEA). This steroid was known as an intermediate in the conversion of cholesterol to testosterone and estrogens. Administered in large amounts, it caused fat and weight loss in obese mice (63), rats, and dogs, decreased blood sugar in diabetic mice (64), decreased the incidence of spontaneous and carcinogen-induced tumors in mice (65),

enhanced immune responses (66), and improved memory in old mice (67). We, like some others, assumed that DHEA was converted metabolically to more active steroids that exerted these beneficial effects. Beginning in the 1960s many investigators had studied the conversion of DHEA to other steroids by animals, humans, and tissue preparations, but only a few of the products were tested for any biological activity. We initiated a program of synthesizing derivatives of DHEA that were logical metabolites in the hope of finding one or more new hormones. Such a search requires an assay for biological activity, and our earlier research provided one. Administering extra thyroid hormone to rats induces the formation of mitochondrial glycerophosphate dehydrogenase (GPDH) to 20 times the normal level in liver (15, 16) and somewhat less in other tissues (16). After Tagliaferro *et al.* (68) reported that DHEA enhanced metabolism and thermogenesis, we found this steroid induced the formation of hepatic GPDH but not that of other tissues (17, 69). Cytosolic malic enzyme is also increased by these hormones (70), and the response of these two enzymes to administered steroids thus provides a semiquantitative assay of activity. The two enzymes comprise a thermogenic system regulated by calcium and other factors (17, 18, 71, 72).

We found that hydroxylation of DHEA at any position other than 7 abolished the ability to increase the thermogenic enzymes (73). 7α-Hydroxy-, 7-oxo-, and 7β-hydroxy-DHEA were more active than DHEA. Because activity increased in that sequence we postulated that the same sequence was involved in converting DHEA to an active hormone. By incubating DHEA with liver homogenate fortified with ATP, NADPH, and malate and assaying the products at short time intervals, that sequence was indeed established (74) and is shown in Fig. 5. The detection and quantitative measurement of the many products formed from DHEA (Fig. 5, **1**) by liver were possible because of the analytical prowess of Dr. Ashok Marwah (75–79). Several additional products remain to be identified including some glucuronides.

DHEA derivatives bearing oxo- or hydroxyl groups at position 7 do not serve as precursors of androgens or estrogens and therefore are potential therapeutic agents. 7-Oxo-DHEA had no detectable toxicity in rats (80) or monkeys (81) even in massive doses and in a phase I clinical trial was well tolerated by normal men given doses up to 200 mg/day for 28 days (82). Tested at that dose for 8 weeks in obese subjects (body mass index of 31.9 $\pm$ 6.2 kg/m^2) who were restricted to 1800 calories/day, subjects receiving 7-oxo-DHEA lost significantly more body weight and fat than those receiving placebos (83). 7-Oxo-DHEA was far more effective than DHEA as an enhancer of memory in old mice and in restoring memory in mice treated with scopolamine (84). Androstenediol (Fig. 5, **6**), one of the main products of DHEA metabolism in liver, has been known for many years to have estrogen activity. It also activates androgen receptor transcriptional activity in prostate cancer cells (85). This function is not inhibited by hydroxyflutamide or bicalutamide, two agents used for treating prostate cancer (86). This raises the question whether androstenediol, produced in adrenals and liver, accounts for the failure of orchidectomy to be an effective long term treatment for prostatic cancer. In a collaborative study, we have also found that DHEA has activity in adipose cells not displayed by its metabolites (87).

Structure/activity comparisons show that ring D of DHEA can be altered in several different ways without abolition of activity. The ring can be expanded by insertion of oxygen at 17a (88) with retention of the ability to induce the formation of both GPDH and malic enzyme. Hydroxylation at position 15 or introduction of 15–16 unsaturation, nearly abolishes the response of GPDH, but induction of malic enzyme is retained (89). Substitutions at position 16 yield steroids with varying activity; some are highly active and point the way to possible routes to true hormones. The search goes on.

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Reflections

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Keilin, Cytochrome, and the Respiratory Chain

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The defining episode in my scientific career was my close association with David Keilin in the nearly 10 years (1946–1955) during which I was a member of the Molteno Institute at the University of Cambridge. Not only did my work in Cambridge determine the direction of my subsequent research, but Keilin's character, way of working and thinking, and his integrity as a scientist and human being were a continuing inspiration. I remained in contact with him until his death, and one of my proudest moments was when, during his first venture abroad since the War for the conferring of an honorary degree in Utrecht, I was able to show him my laboratory in Amsterdam.

David Keilin (1887–1963)

David Keilin was born in Moscow on March 21, 1887, of Polish parentage; his father was a businessman and small landowner. The family returned to Warsaw where he graduated from the Górski High School in 1904. He studied medicine at the University of Liège in Belgium for a year, but in 1905, being advised that his health would not stand the strain of medical studies, moved to Paris to study biology. In 1915, he obtained his doctorate with a thesis on the biology of insect larvae. In the same year he was invited by G. H. F. Nuttall to be his assistant at the Quick Laboratory of Parasitology in Cambridge, England, the forerunner of the Molteno Institute, where he was to spend the rest of his working life. He was appointed Lecturer in Parasitology in 1925 and in 1931 succeeded Nuttall as Professor and Director of the Molteno Institute. He had to relinquish both posts in 1952 upon reaching the compulsory retirement age of 65 but was able to continue working in the Institute until his death in 1963. He received many honors, including election as Fellow of the Royal Society in 1928 and the award in 1952 of the highest honor of the Society, the Copley Medal. Many do not understand why he was never awarded the Nobel Prize.

Keilin's paper in the *Proceedings of the Royal Society* in 1925 with the title "On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants" (1) marked the beginning of studies of what Warburg later called the respiratory chain (atmungskette), many of us called the electron transfer chain, and David Green, with some prescience, the electron transport chain. The story of how Keilin came upon cytochrome when studying hemoglobin in the horse intestinal parasite *Gastrophilus intestinalis* is told in his posthumously published book (2).

Already 75 years ago there was quite a lot known about biological oxidations. The word "oxidase" had already been introduced by Gabriel Bertrand (3) in the 19th century to describe the enzyme responsible for the hardening of lacquer, now known as laccase. In 1910–1912 Battelli and Stern made thorough studies of the oxidation of a number of substances by oxygen in the presence of ground-up tissue and showed the sensitivity of this process to cyanide (4). They referred to the enzyme responsible as indophenol oxidase from the color reaction they used to measure its activity. In the early 1920s, Thunberg (5) showed that the oxidation of a large number of organic compounds such as succinic acid is catalyzed by enzymes, each specific for its substrate, named dehydrases and later dehydrogenases by Wieland (6). As is well

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FIG. 1. **David Keilin (1887–1963).**

known, a controversy developed concerning the mechanism of biological oxidations. Wieland and Thunberg, impressed by the ability of dehydrogenases to catalyze the oxidation of organic compounds by artificial acceptors such as methylene blue, proposed that the fundamental action is the activation by the dehydrogenases of hydrogen atoms, otherwise inert, so that they can react with oxygen. Warburg, impressed by the presence of iron in respiring cells and the ability of cyanide both to combine with iron and to inhibit cell respiration, proposed that the fundamental process is the activation of oxygen by an iron-containing respiratory enzyme (atmungsferment) (7).

Keilin's paper made it clear that the electrons derived from the activation of the hydrogen atoms by the dehydrogenase are transferred via three hemoproteins, which he named cytochromes *a, b*, and *c*, to an oxygen-activating oxidase. He did not name the oxidase in his 1925 paper, but in 1927 identified it, on the basis of its sensitivity to cyanide, with Battelli and Stern's indophenol oxidase and on the basis of its sensitivity to both cyanide and carbon monoxide with Warburg's atmungsferment (8). Much to Warburg's chagrin he continued to call it indophenol oxidase and, in retaliation perhaps, Warburg refused to accept the role of the cytochromes. This became one of the controversies of the 1930s (see Ref. 9), matching the vigorous confrontations in this field 30 or 40 years later at the annual meetings of the ASBC.

The basic features of our present picture of the respiratory chain were established by Keilin and his co-workers in the 1920s and 1930s. Already in his first paper, he showed that cytochrome *b* is the first acceptor of electrons from substrate. Making use of the exceptional stability of cytochrome *c*, Keilin and Hartree (10) extracted it from heart muscle. Most importantly, in 1939 (11) they showed that what had hitherto been thought of as a single cytochrome *a* consists of two components that they now called cytochromes *a* and a_3. In contrast to the other cytochromes, including cytochrome *a*, cytochrome a_3 combines with carbon monoxide and cyanide and has, therefore, all the properties ascribed to Warburg's atmungsferment.

By 1939, it was possible to write the respiratory chain as a simple chain: dehydrogenase → cytochrome *b* → cytochrome *c* → cytochrome *a* → cytochrome a_3 → oxygen. Strictly speaking the order of cytochrome *c* → cytochrome *a* had not been established (it could have been reversed), but Keilin was convinced, correctly as it is now known, that cytochromes *a* and a_3 are closely associated.

Expansion of Keilin's Respiratory Chain

That was still the situation when in 1946 I joined Keilin as a Ph.D. student (rather mature in age by English standards but not in biochemical knowledge (see Ref. 12)), and more than a half-century later, this description of the respiratory chain is still valid although additional electron-transferring components have been added to it. The first of these was cytochrome c_1, already discovered by Okunuki in 1939 (13) but not generally accepted until Keilin and Hartree in 1955 showed that the absorption band initially ascribed to cytochrome *c* is derived from two components, one the classical cytochrome *c* and the other Okunuki's cytochrome c_1 (14).[1]

The second addition to Keilin's respiratory chain, proposed in 1948 before cytochrome c_1 was accepted, was an electron-transferring factor acting in the chain between cytochromes *b* and *c* that was irreversibly and specifically destroyed by aerobic incubation with a dithiol compound, called BAL (17). After the discovery by Van Potter that the powerful respiratory chain inhibitor antimycin also inhibits electron transfer between cytochromes *b* and *c*, which he ascribed (incorrectly as it transpired) to its binding to the factor, he kindly gave it the name Slater factor (18).

In the late 1950s, F. L. Crane (19) in David Green's laboratory discovered ubiquinone (coenzyme Q) as a new hydrogen carrier between the dehydrogenases and the electron transfer chain proper, but it was not until much later that it was recognized that ubiquinone is also involved in electron transfer within the respiratory chain (see below).

After many earlier proposals that copper is involved as well as iron in the oxidation of cytochrome *c*, this was finally established in the 1960s by Helmut Beinert, using paramagnetic resonance spectrometry (EPR) (20). Bob van Gelder (21) in my Amsterdam laboratory showed that the cytochrome *c* oxidase takes up 4 electrons per molecule, one each into the hemes of cytochromes *a* and a_3 and two into the copper atoms.[2]

The application by Beinert of EPR spectrometry revealed also a whole new class of electron carriers, the iron-sulfur centers (22). With one exception, these centers are involved in the transfer of reducing equivalents from the flavin, by then recognized as a component of all ubiquinone-reducing dehydrogenases, to ubiquinone, rather than in Keilin's respiratory chain itself. The one exception was not in fact discovered by Beinert but by his colleague Rieske and is generally known as the Rieske iron-sulfur protein (23). The high redox potential, around about that of cytochrome c_1, made it an attractive site of action of antimycin and a candidate for my old factor. However, there was no experimental evidence for a reaction with antimycin, and for many years in Amsterdam we did not know quite what to do about the Rieske protein until Simon de Vries found that its EPR spectrum is affected by ubiquinone (24). The breakthrough was made after Bernie Trumpower (25) showed that, after extraction of the Rieske protein, antimycin inhibits the *reduction* of cytochrome *b*, instead of its *oxidation*, as it was supposed to do if it inhibits the chain between cytochromes *b* and *c*. This reminded me of an old observation by Deul and Thorn (26) in my laboratory that this is exactly what antimycin does after destruction of the factor, what we called the "double kill" experiment. Sure enough Simon de Vries showed that the treatment I had used in the 1940s to destroy the factor has a drastic effect on the EPR spectrum of Rieske's iron-sulfur protein (27). By establishing the identity of my factor and the Rieske protein, the number of possible components of the respiratory chain was at least reduced by one.

The double kill experiment is nicely explained by Mitchell's Q cycle (26) to which I had paid insufficient attention when it was proposed, despite a friendly letter from Peter saying that it would give him great pleasure if it turned out that the Q cycle explained the Slater factor. It does. According to this cycle, there are two possible entries of electrons from ubiquinol to cytochrome *b*, one coupled with the reduction of the Rieske iron-sulfur protein and therefore susceptible to BAL treatment and one via a separate antimycin-sensitive ubiquinol-binding site, which (when the cycle is functioning) operates in the opposite direction by accepting electrons from cytochrome *b*. I soon became an enthusiastic supporter of the ubiquitous Q cycle (29).

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[1] To my everlasting embarrassment, I had published a paper in 1949 (15) in which I concluded that Okunuki's evidence for the existence of cytochrome c_1 was unsatisfactory (see also Ref. 16).

[2] That it was much later shown that cytochrome *c* oxidase contains 3 atoms of copper per molecule is not inconsistent with van Gelder's titrations, because two of the copper atoms are coupled and take up only a single electron.

I have now got a bit ahead of myself chronologically. Just as is the case with Keilin's c and a absorption bands, the b band turned out also to be double, but in this case it is derived from two protoheme prosthetic groups bound to a single polypeptide chain. The first clue of the existence of two components came from Britton Chance and was established in his laboratory in a redox titration by Wilson and Dutton (30). There was quite a lot of what turned out to be rather cloudy work on cytochrome *b* in the 1970s, but the dust settled with Fred Sanger's determination of its molecular weight via DNA (31), which told me that it is a two-heme cytochrome (32). Its function was established by the Q cycle as a transmembrane subunit of ubiquinol-cytochrome *c* reductase with the lower potential heme, denoted b_{566}, accepting electrons from ubiquinol on the outside of the inner membrane and transferring them to the higher potential heme (b_{562}) on the inside of the membrane and eventually to ubiquinone.

Fractionation of the Respiratory Chain

Keilin and his students used for their studies of the respiratory chain a suspension of small particles obtained by grinding heart muscle with sand in weak phosphate buffer that became known as the Keilin and Hartree heart muscle preparation (33). I do not think that much attention was given in early studies to the nature or origin of these particles. Indeed I think that I was the first to show that they contain about 30% lipid, an accidental observation made when I was looking for a method of determining the dry weight of the preparation, since in those days the activity of a respiratory preparation was expressed by the Q_{O_2} (μl of O_2/h/mg, dry weight). When as a newcomer I asked Ted Hartree how to measure the dry weight of the suspension in the buffer, he suggested that I precipitate it with trichloroacetic acid, centrifuge, wash the precipitate, dry it, and weigh it. This I did, but I decided to speed up the drying process by washing with ethanol. I found that this decreased the weight by 30%, compared with washing with water, and changed my definition of Q_{O_2} to base it on fat-free dry weight. I did observe that the ethanol extract was bright yellow but did not give this any thought, thereby missing the opportunity of discovering ubiquinone.

The significance of the lipid became clear when at about this time Albert Claude (34) showed that the site of intracellular respiration is the mitochondrion and, when the mitochondrion was viewed by thin section electron microscopy by Palade (35), more precisely in the inner membrane or cristae. We now recognize that the Keilin and Hartree preparation consists of submitochondrial particles, or vesicles, derived from the inner membrane.

No attempt was made by the Keilin school to fractionate the chain apart from the isolation of cytochrome *c*. The first success was obtained by Wainio (36) and Lucile Smith (37) using deoxycholate and cholate, respectively, to disperse the membrane and allow its components to be separated by conventional ammonium sulfate fractionation. David Green's school (38) importantly expanded this technique to the separation of what he called four complexes, catalyzing, respectively, the reduction of ubiquinone by NADH (Complex I) or succinate (Complex II), the reduction of ferricytochrome *c* by ubiquinol (Complex III), and the oxidation of ferrocytochrome *c* by oxygen (Complex IV). I have always thought it a pity that he gave the name Complex to these multisubunit proteins, each of which has a clearly defined enzyme function.

Function of the Respiratory Chain

In the 1920s Keilin and Warburg envisaged that the function of the respiratory chain is to catalyze the oxidation of intermediary metabolites by the transfer of electrons derived from hydrogen atoms to oxygen. That it might have an additional function in ion transport was suggested in 1939 by Lundergårdh (39), specifically that in plants the cytochromes act as electron carriers in one direction and as anion carriers in the opposite direction. The primary function of the respiratory chain, oxidative phosphorylation, was discovered by Engelhardt in 1931 (40). Measurements of the stoichiometry (P:O ratio), made independently in 1939–1940 by Belitzer and Tsibakowa (41) in Leningrad in the USSR and Severo Ochoa (42) in Oxford in England, established that phosphorylation must be coupled not, or not only, to the dehydrogenation of substrate but to electron transfer along the respiratory chain.

Oxidative Phosphorylation and Topography of the Respiratory Chain

After completing my Ph.D. in 1948 with a thesis on the succinate oxidase system and a subsequent study of the NADH oxidase system (43), oxidative phosphorylation was the logical next topic for my research, especially after Al Lehninger's paper on oxidative phosphorylation

coupled to the oxidation of NADH (44). This was a new field for the Molteno Institute, and given the opportunity by the Rockefeller Foundation to study in the United States, I spent about 6 months working in Severo Ochoa's laboratory at New York University learning the new techniques.[3] Severo's interests were then mainly on carbon dioxide fixation, but in the same building, Ef Racker was developing the concept of an acyl intermediate in the oxidative phosphorylation reaction of glycolysis (46). Adapting an enzyme assay that he had described, I developed a procedure that enabled me to measure oxidative phosphorylation between substrate and cytochrome *c* (47), the first direct demonstration of what later became known as "site 2 oxidative phosphorylation." I continued these studies after returning to the Molteno Institute and in 1953 published what became known as the "chemical hypothesis" of oxidative phosphorylation (48) in which, by analogy with substrate-linked phosphorylation, the energy of electron transfer is conserved primarily in non-phosphorylated high energy forms of components of the chain.

Around this time, the two functions of the respiratory chain, ion movements and oxidative phosphorylation, were beginning to coalesce. Workers on gastric secretion favored a simple redox pump mechanism, according to which the secreted protons were those liberated from hydrogen carriers by transfer of electrons to the cytochromes. In 1951, however, both salt accumulation in plants (49) and gastric secretion (50) were found to be inhibited by 2,4-dinitrophenol, known to uncouple oxidative phosphorylation from electron transfer. This focused attention on ATP instead of electron transfer as the source of charge separation, and Davies and Krebs (51) proposed in 1951 that "ionic concentration differences, *i.e.* osmotic energy . . . may play a role in the synthesis of ATP." Williams (52) proposed that protons could bring about condensation reactions such as polyphosphate formation.

These concepts were developed by Mitchell (53) into a coherent hypothesis encompassing a functional link between electron transfer in the respiratory chain and the translocation in the opposite direction of protons across the inner mitochondrial membrane, whereby the energy is conserved as an electrochemical proton gradient. To accommodate experimental evidence of an H^+:e ratio of about 2, in 1966 he introduced the concept of loops in the respiratory chain with two electrons crossing the membrane from one side to the other followed by two hydrogen atoms in the opposite direction (54). This very important concept of the sidedness of the membrane with the specific location of the electron acceptors and donors was not at first generally accepted, not only because many did not at first accept (or possibly understand) the precise role of protons envisaged by Mitchell but also because those of us more specifically interested in the respiratory chain knew that the order of electron transfer originally proposed was wrong. Mitchell's brilliant proposal of the Q cycle (28), made in answer to these criticisms, as a description of how the oxidation of ubiquinol by ferricytochrome *c* is coupled to the net production of protons on one side of the membrane and their consumption on the other side was soon given solid experimental support.

As more became known of the structure of the two large multisubunit proteins involved in the respiratory chain, namely ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase, as well as of the ubiquinone-reducing dehydrogenases, such as succinate-ubiquinone reductase and NADH-ubiquinone reductase, it became clear that their dimensions are in fact greater than a phospholipid bilayer and that they are embedded and specifically orientated across the phospholipid layer, which confirmed in structural terms Mitchell's sidedness concept.

Mechanism of Electron Transfer

The discovery in the 1960s and early 1970s of more and more electron-transferring centers in the respiratory chain, particularly the multiplicity of iron-sulfur centers, gave a lot of headaches to those of us who found even the 1948 sequence of dehydrogenase $\rightarrow b \rightarrow$ factor $\rightarrow c \rightarrow a \rightarrow a_3 \rightarrow O_2$ longer than necessary to accommodate a P:O ratio of 3 in oxidative phosphorylation. At the time of the International Congress in Switzerland in 1970, I remember that, in desperation, we proposed double chains.

The real function of the multiplicity of electron transfer centers has only recently become understood as a result of the structural information that tells us where the centers are located in the protein, together with a fundamental increase in our understanding of the nature of electron transfer. Dutton and co-workers (55) have demonstrated that, by virtue of electron

[3] A biographical note on Severo Ochoa is to be found in Arthur Kornberg's "Reflections" (45).

tunneling, electrons can readily travel through the protein medium a distance of up to 14 Å between redox centers but that transfer over greater distances is facilitated by a chain of electron carriers. Within this distance of 14 Å, rapid electron tunneling takes place even if the electron transfer is endergonic, provided that the centers are sufficiently close. It is the proximity of the redox centers in chains that provides highly directional electron transfer.

The role of distance between the redox centers in controlling the rate and therefore the specificity of electron transfer is beautifully illustrated by the mobility of the Rieske iron-sulfur protein subunit in ubiquinol-cytochrome *c* reductase, as shown by the x-ray crystallographic studies of Berry, Crofts, and their colleagues (56). In one conformation, stabilized by the ubiquinol inhibitor stigmatellin, the Fe-S cluster is close enough to the ubiquinol-binding site to allow its reduction by ubiquinol. In a second conformation, it is close enough (about 8 Å) to the heme in cytochrome c_1 to permit rapid electron transfer. The important point is that in neither conformation can both reactions occur at a suitable rate. For example, in the stigmatellin-stabilized conformation, the iron-sulfur cluster is about 27 Å from the heme. Thus, the reaction mechanism must involve movement of the Rieske iron-sulfur protein. Keilin would have enjoyed this paper. X-ray crystallography of proteins was not strange to him. He supported Kendrew and Perutz in their work and lived to see the solution of the structures of myoglobin and hemoglobin.

We now know that one of Keilin's cytochromes, cytochrome *b*, as well as Okunuki's cytochrome c_1, are subunits of a single protein, ubiquinol-cytochrome *c* reductase, and that his cytochromes *a* and a_3 are also bound to a single subunit of cytochrome *c* oxidase. Cytochrome *c* remains a single polypeptide. In his earlier papers, Keilin often used the singular "cytochrome" to refer to the cytochrome system, and I think that he regarded them as acting as a single unit. In 1947, he and Hartree stated that "the catalysts in the particles, as in the intact cells, are more or less rigidly held together in a framework that assures their mutual accessibility and a consequent high catalytic activity" (33). This idea of an ordered macromolecular assembly, under the name of the "solid state" model of the respiratory chain, seems to be winning favor over the "liquid state" model that envisaged independent free diffusion of the multisubunit proteins in the membrane and of cytochrome *c* in the space between the inner and outer membranes of the mitochondrion (see *e.g.* Ref. 57).

In any case, the function of the cytochromes is to transfer electrons. It is the function of the protons, freed by this removal of the electrons from the hydrogen atoms of intermediary metabolites, to drive ion transport and the synthesis of ATP. As Mitchell pointed out in the conclusion to his Nobel Lecture in 1978, "David Keilin's chemically simple view of the respiratory chain appears now to have been right all along." (58).

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Reflections

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100 Years of Biochemistry and Molecular Biology

Reminiscences of Leon A. Heppel

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My parents were converted Mormons who had emigrated from Germany to Utah planning to live on a farm. The oldest of five children, I was born in Granger, Utah, in 1912. Farm life proved difficult and after 10 years our family moved to San Francisco. There, the city encouraged interesting local activities particularly for poor people, and life was more pleasant than in Utah.

In school, I became interested in chemistry. While a high school student, my mother, who was ambitious on my behalf, persuaded John Stauffer, president of Stauffer Chemical Company, to give me a job doing analytical work at the American Cream Tartar Company in San Francisco. This supported me through high school and afterward when I enrolled at the University of California, Berkeley to major in chemistry and chemical engineering.

At Berkeley

Unhappily, my job at the American Cream Tartar Company and the support it provided did not last. In 1931, the Stauffer Chemical Company merged with the Schilling Spice Corporation and the combined company owned American Cream Tartar. A vice president of Schilling Spice undertook to effect economies, but the only economy he could find was getting rid of me. Shocked and urged by my mother to plead my case, I told the vice president how much I depended on the job. His cold reply was, "You need Schilling Spice Company but does Schilling Spice need you?" I never forgot those cruel words. Because of them, I abandoned my plan to be a chemical engineer turning instead to physiological biochemistry, which I thought would be a gentler profession. Fortunately I received a fellowship that allowed me to complete a B.S. degree in 1933. That same year I entered Berkeley's graduate school as a biochemistry student.

Living in midtown San Francisco and commuting each day to Berkeley was a tiring chore. The Bay Bridges had not been built. In early morning, I took a streetcar to the Ferry Building where I boarded a boat for Oakland; on good days this took half an hour, but if the fog was intense, it was a much longer trip. From Oakland an electric train went to Berkeley and the university. In midafternoon, I returned across the Bay and spent a few hours working in one of the several Stauffer Chemical factories. Aside from the commute, however, life and science in Berkeley were exciting. During this period, Ernest O. Lawrence and others were doing great work and were anxious to talk about it. I made good friends among the chemists, one of whom discovered ^{14}C (Martin D. Kamen in 1940).

Nutrition was a major subfield of biochemistry in the 1930s, and I decided to do my thesis in that subject under Professor C. L. A. Schmidt. Schmidt was harsh and domineering but helpful. In later years when he became dean of the College of Pharmacy at the University of California, San Francisco, he hired my mother to take charge of equipment and supplies.

For my thesis research, I decided to work on potassium (K^+) metabolism in white rats. The experiments showed that K^+ was essential for the growth and survival of young rats, and there was some evidence that sodium (Na^+) could partially replace K^+. Rubidium (Rb^+) supported

good growth in K^+-free diets for a month, but thereafter the rats developed sudden tremors and died. My Ph.D. degree in biochemistry was awarded in 1937, a year when there were no jobs available for a biochemist. Luckily, Schmidt came to my rescue. He remembered a promise that George Whipple had made when he left Berkeley to start a new medical school in Rochester, New York. Whipple had told Schmidt that if he ever had a Ph.D. student who decided to come to medical school in Rochester, the student would receive partial support from the school. Right after receiving the Ph.D., I boarded a train for Rochester.

At Rochester

Good fortune in the shape of a mentor came my way in Rochester. My work at Berkeley had attracted the attention of W. O. Fenn, a brilliant young physiologist who was a very quiet person and unusually kind. Fenn spent much of the day doing experiments with the help of a cheerful but somewhat talkative young woman. He gave me a position and suggested that I continue to study K^+ metabolism in young rats. My initial results replicated my earlier finding that the rats grew well for a while when Rb^+ replaced dietary K^+ but then quickly developed tremors and died. In the early phase, although the rats appeared to be healthy, 7.5% of their muscle K^+ was replaced by rubidium. Other experiments demonstrated that Na^+ could replace K^+ to some extent, and studies with radioisotopes confirmed that K^+ and Na^+ were able to cross an animal cell membrane. This was an astonishing finding, as German physiologists believed that the lipid cell membrane prevented passage of hydrophilic metal ions. Thanks to the generous spirit of Fenn, I was the sole author on three papers describing this work (1–3).

The War Years

By 1942 when I completed the M.D. degree and internship at Rochester, my work there had drawn considerable attention, and I received three offers for assistant residency positions from schools where interest in electrolytes was great: Yale Medical School with John Peters, Columbia University with Robert Loeb, and San Francisco Medical School. However, the entry of the United States into World War II interrupted normal, peacetime activities. Arthur Kornberg, a close medical school friend, and I joined the United States Public Health Service. Kornberg received sea duty while I was assigned to the National Institutes of Health (NIH). At NIH under orders from the Navy, I carried out tedious studies on the toxicity of halogenated hydrocarbons. Most importantly, the future began to take shape when I made a new friend, the enzymologist Bernard Horecker. Also, I persuaded Rolla E. Dyer, Director of NIH, to bring Kornberg to Bethesda. Together with Kornberg and Herbert Tabor and with the help of Horecker, I began to learn enzymology. Kornberg then left to spend a year (1946) in the laboratory of Severo Ochoa in New York and another (1947) with Gerty and Carl Cori in St. Louis. When he returned to NIH, he started a new research section for the study of enzymes and invited Horecker and me to join.

Enzymology at NIH

Leaning on my background in toxicology, I began to examine the behavior of enzymes in toxic situations. Also, I investigated the metabolic reactions that convert inorganic nitrite to nitrate and nitroglycerines. I also purified inorganic pyrophosphatase and crystallized it with the help of Moses Kunitz (of the Rockefeller Institute (now University)) and purified 5′-nucleotidase.

A Sabbatical Year in England

Then, in about 1951, my attention turned more generally to the phosphorylation and hydrolysis of purine ribonucleosides. This led, quite naturally, to an interest in enzymes that might hydrolyze RNA. Accordingly, my technician, Russell Hilmoe, and I purified from spleen an enzyme that partially solubilized RNA. The next step was to determine which linkages in RNA were split and which were resistant to the enzyme action. Roy Markham and J. D. Smith in Cambridge, England had demonstrated that fragments produced by RNA hydrolysis could be separated using paper chromatography and paper electrophoresis. Fortunately, I succeeded in obtaining a year's leave of absence from NIH, one of the first sabbaticals to be offered there, and spent a profitable year abroad in the laboratory of Markham. My work in England included the demonstration that the natural configuration of purine nucleotides in RNA was 3′–5′ rather than the alternative 2′–5′ (4). Further evidence for this linkage was obtained from a study of the action of nucleases on mononucleotide esters carried out with Daniel Brown and

Lord Alexander Todd (5). Also, the early steps in the hydrolysis of RNA by pancreatic ribonuclease were worked out in a collaboration with Paul R. Whitfeld (6). This work lead to the isolation, by paper chromatography and paper electrophoresis, of cyclic terminal oligonucleotides. Whitfield, an Australian graduate student in the laboratory, was an excellent colleague in research and deserving of the credit he received when his name appeared on five of our publications (for example, Refs. 6–8).

Later on, I had an interesting interaction with Markham and Sutherland. Dr. Markham found that heating ATP with dilute alkali caused the formation of substantial quantities of a new compound whose properties puzzled him, as he related in a letter to me. At a later date, Dr. Sutherland wrote about a compound isolated from liver in minute quantities. It was biologically active. The two letters ended up in different parts of a pile of mail. However, one day I chanced to re-read both letters and I figured that these compounds were the same. This turned out to be so, and thus cyclic adenylic acid became readily available.

Nucleic Acid Biochemistry at NIH

I returned to NIH in January of 1954. Interesting and stimulating visitors began to come to the laboratory to learn techniques and collaborate. Henry Kaplan, a very distinguished Professor of Radiology at Stanford spent a sabbatical in the laboratory. Three joint papers were published with Horecker and Jerard Hurwitz, then a beginning researcher and now a distinguished biochemist. Jack Strominger was also a welcome visitor; the two of us, together with Elizabeth Maxwell, studied the phosphorylation of nucleoside monophosphates by nucleoside triphosphates. At this time, there was considerable interest in the results and methods I had obtained during my stay in England. A good deal of attention was being paid in particular to the demonstration that "synthetic" oligonucleotides could be synthesized by enzyme-catalyzed nucleotide exchange reactions (7). Before long, I learned about the discovery of polynucleotide phosphorylase in *Azotobacter vinelandii* by Marianne Grunberg-Manago and Ochoa at New York University. The same enzyme was independently discovered in *Escherichia coli* by Uri Littauer and Kornberg.

At the time, I was one of only a few individuals who had the knowledge and experience required to study this enzyme and its products. Ochoa proposed that we collaborate and I accepted. Early in the course of the collaboration, a very able and pleasant postdoctoral fellow, Maxine Singer, joined my laboratory. She contributed greatly to the studies and made the association enjoyable. We put to good use all that I had learned in England about polyribonucleotides. One of our important findings was that short oligonucleotides could serve as primers for polynucleotide phosphorylase (9). Some time later, Singer and I used polynucleotide phosphorylase to prepare polyribonucleotides and oligoribonucleotides that Nirenberg used in his work on the genetic code. Singer continued to work on polynucleotide phosphorylase when she became an independent investigator.

The elegant organic synthesis of oligonucleotides by Khorana was not available until a later period. Therefore, when working on the genetic code, it was an advantage to be able to use enzymatic methods.

Russell Hilmoe remained my able and intelligent technician for many productive years; he was particularly good at adapting to new situations. Marie Lipsett, who had a good grasp of physical chemistry, joined the laboratory group; she collaborated with Dan Bradley on the study of complex formation between oligonucleotides and homopolymers. The flow of visitors continued as many people began to investigate nucleic acid enzymology. Littauer and I. R. (Bob) Lehman visited from Kornberg's department in St. Louis. Gobind Khorana's occasional visits were a joy as they gave me a chance to observe the development of his work and share in his good company as well as collaborate. Several times I also visited in Khorana's laboratory. Audrey Stevens was an especially brilliant postdoctoral fellow; all on her own she was one of the people who simultaneously discovered RNA polymerase. Altogether, it was an enjoyable and exciting time. After some years, however, I decided to turn to a different problem: the properties of bacterial membranes.

New Fields

Harold Neu, a medical postdoctoral fellow, joined me in the new investigations. The first problem he tackled was the location of ribonuclease in *E. coli*. At that time, a ribonuclease had been found associated with the 30 S ribosomes of the bacteria. Neu showed that the ribonuclease was actually in the periplasmic space between the cell membrane and the cell wall but

binds to the 30 S ribosomes when the cell is split open (10, 11). With special care, it was possible to obtain ribosomes free of ribonuclease. Thus, the ribonuclease is a periplasmic enzyme with no connection to ribosomes. In the course of this work, Nancy Nossal, a postdoctoral fellow, contributed to the development of Neu's procedure for the osmotic shock of the cells (12). The protocol made it possible to recover enzymes in high yield from the periplasmic space of Gram-negative bacteria. The procedure has since been used in many laboratories. Neu, and later others, discovered a number of other periplasmic enzymes, all located in the space between the cell membrane and cell wall.

Anraku, a visitor from Japan, was very quiet but very effective and productive. He observed that Gram-negative bacteria able to transport D-galactose contain a specific periplasmic protein that can bind that sugar. A similar observation was made in the laboratory of Arthur Pardee. In the next few years, a large number of binding proteins were discovered in my laboratory and elsewhere. At NIH, several additional postdoctoral fellows contributed to this work. H. R. Dvorak, an M.D., had a special interest in metalloproteins. He and R. W. Brockman, a hard worker who visited the laboratory from Alabama, also worked on phosphatases released from *E. coli* by osmotic shock.

The Years at Cornell

In 1967, Efraim Racker induced me to join the Department of Biochemistry at Cornell University. The move was the beginning of more than 30 pleasant and productive years in Ithaca. The first postdoctoral fellow to join the laboratory, George Dietz, was an able and pleasant young man who studied the uptake of hexose phosphates by *E. coli*.

Joel Weiner, a graduate student from Canada, and Clem Furlong, a postdoctoral fellow, worked on amino acid transport in *E. coli* including leucine-specific and glutamine-specific (13) periplasmic binding proteins. Furlong was an especially good experimentalist and was helpful with equipment problems. Weiner later became an outstanding member of the Canadian Biochemical Society. Ed Berger, a graduate student, carried out a landmark study showing that there are different mechanisms of energy coupling for the active transport of proline and glutamine in *E. coli* (14); this work received much favorable attention. Another member of the early group at Cornell was postdoctoral fellow Barry Rosen. He studied basic amino acid transport in *E. coli*, another process that involved a binding protein.

Other students, postdoctoral fellows, and visitors contributed to our growing understanding of the periplasmic space and transport. Susan Curtis looked at the mechanism of ribose uptake, which involved energy from ATP rather than an energized membrane. James Cowell noted a similar result for glycylglycine. Janet Wood, a very able Canadian, worked on L-leucine transport. J. B. Smith and a graduate student, Paul Sternweis, purified the two "minor" subunits of F_1-ATPase and examined their properties (15). I was able to help Smith during a period when jobs were difficult to get and was delighted when he began doing independent work. T. Kitagawa made an interesting finding when he showed that the osmotic shock procedure does not necessarily kill the cells; some cells remain viable. Stanley Dunn and Masamitsu Futai used their time in the laboratory purifying and reconstituting the *E. coli* F_1-ATPase (16). Nizar Makan from India spent several postdoctoral years on exhaustive work that yielded evidence for metabolic processes that might be involved in permeabilization.

In 1975, I decided to gain more experience in animal cell research. A half-year sabbatical was granted and I spent it with Henry Rozengurt in London. In the ensuing years, I made six additional visits of several months each to the Rozengurt laboratory. On one of these visits, I observed that 3T6 cells, which are spontaneously transformed, leaked nucleotides when 50 μM ATP is added to the medium; the effect is highly specific for ATP. Many excellent investigators have since studied this phenomenon, and G. Weisman, I. Friedberg, and I reviewed this work in 1986 (17). Friedberg received his degree for the work in my laboratory in about 1980. The most recent years in my laboratory included Ding-ji Wang and Ning-na Huang. They showed that ATP, in concentrations of a few micromolar, was a mitogen and explored this important effect of extracellular ATP in a series of papers (18).

I want also to mention a few other people who were in my laboratory at various times and whose collaboration I value. They include R. G. Alfonzo from Venezuela, K. Jacobson, a skilled organic chemist, and the productive Fernando Gonzalez, a graduate student and postdoctoral fellow. Barun De was a persistent and hard worker. Ahmed Ahmed came to the United States on a number of occasions to learn modern biology; he is a well known Professor of Plant Science

and Toxicology in his native Egypt. I was also fortunate to know Gary Weisman and to watch with pleasure as he developed into a leader in his field.

In the early 1980s I was able to spend 13 months (divided into short periods) back at NIH as a Fogarty Scholar in the laboratory of Claude Klee. It was good to be able to spend the entire day doing experiments at the bench. Klee is remarkable for being able to do experiments at the same time that she was running the Laboratory of Biochemistry in the National Cancer Institute.

Conclusion

These reminiscences cover about 75 years. They are based on what I remember and no claims for accuracy are made. Selected references and reviews are included for the interested reader, and these sources also describe similar work done in other laboratories.

I have never forgotten how Professor W. O. Fenn arranged that I would be sole author on three papers describing work that I carried out in his laboratory. After all, he was the department head and I was only a medical student on a part-time physiology fellowship. On occasion, I tried to do the same for a student or postdoctoral fellow of mine. However, I stopped when a reviewing editor accused me of removing my name because I had no interest in the work.

Wonderful friendships are formed in research laboratories. Bernard Horecker was a friend for many years and a good source of advice; several of our joint papers are still referred to on occasion. Arthur Kornberg gave no end of guidance and inspiration, especially in the early years. My wife Adelaide and I will always have a special place in our hearts for Herb and Celia Tabor.

I have mentioned here nearly all of those who held positions in my laboratory over the years. The list is small. I prefer to work with a small group and always to do some experimental work myself.

I am especially pleased with the performance of women in my laboratory. They had difficulties in obtaining positions in my day.

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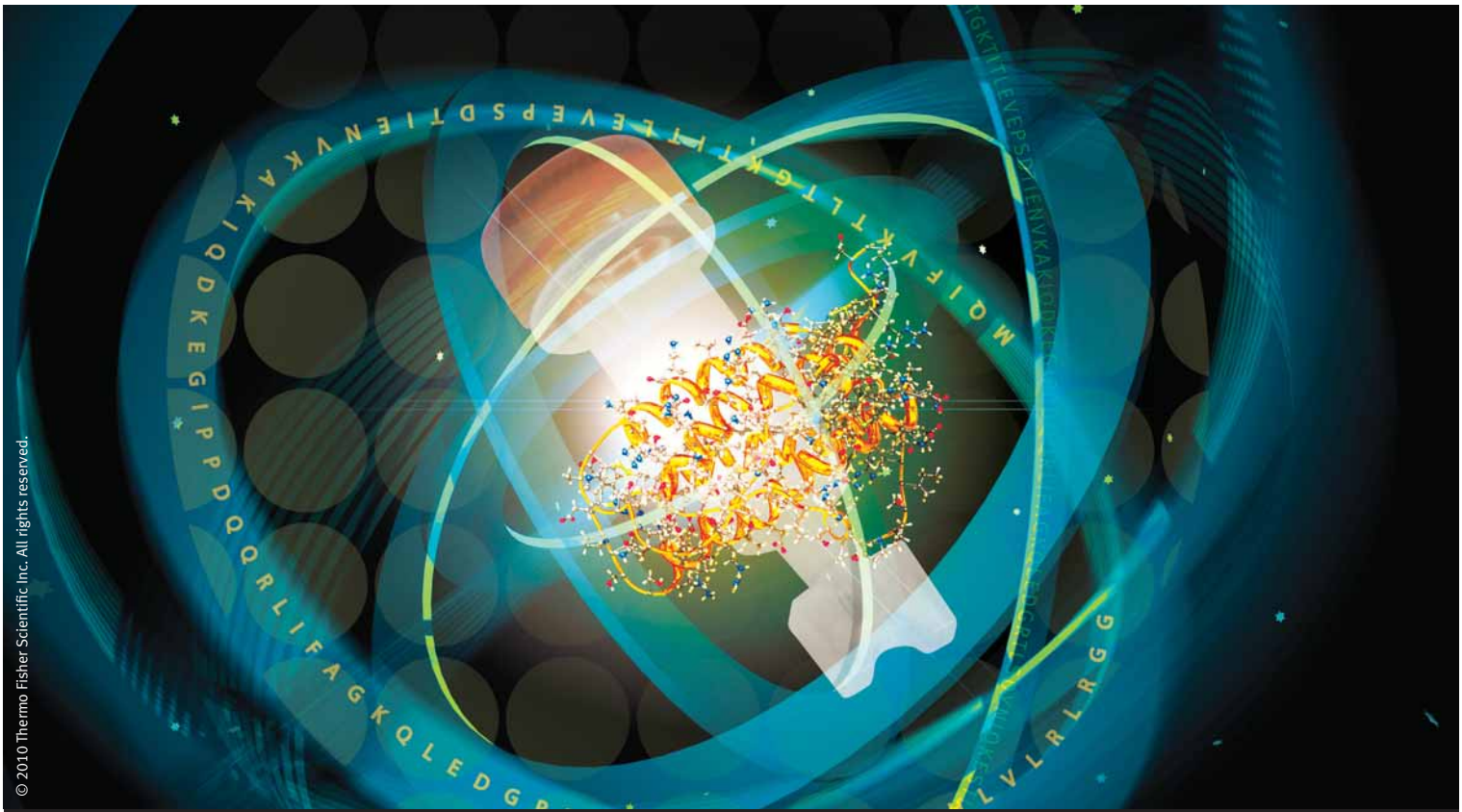

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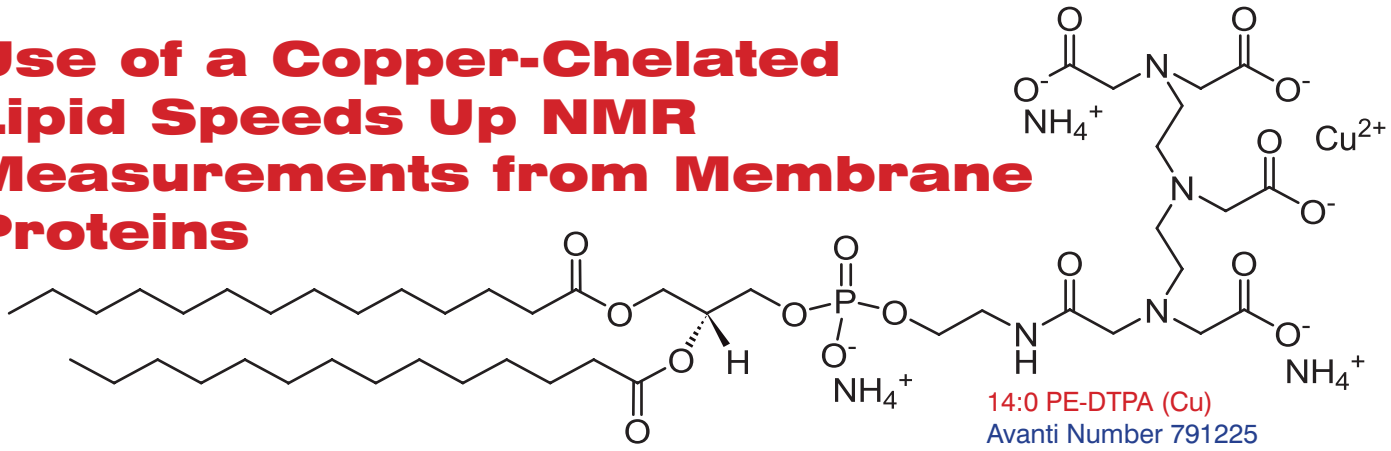

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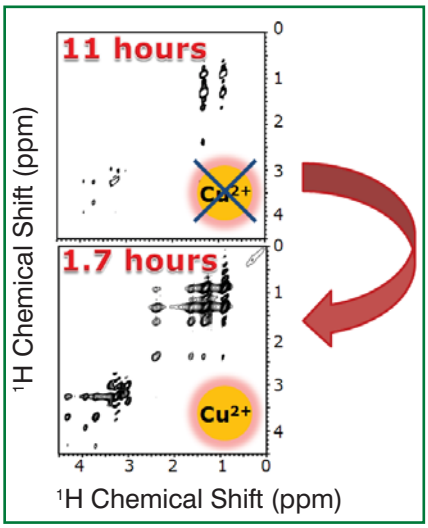

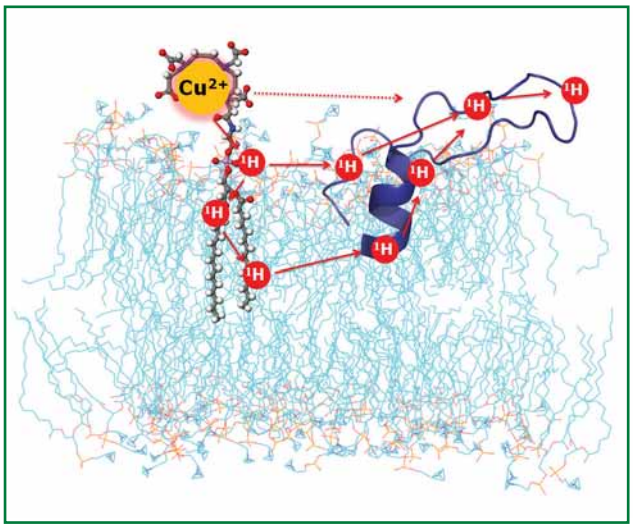

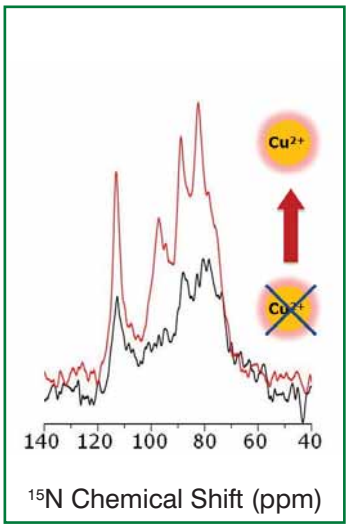

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Recent studies have demonstrated the abilities of solid-state NMR techniques to solve atomic-level-resolution structures and dynamics of membrane-associated proteins and peptides. However, high-throughput applications of solid-state NMR spectroscopy are hampered by long acquisition times due to the low sensitivity of the technique. In this study, we demonstrate the use of a paramagnetic copper-chelated lipid to enhance the spin-lattice relaxation and thereby speed up solid-state NMR measurements. Fluid lamellar-phase bicelles composed of a lipid, detergent, and the copper-chelated lipid and containing a uniformly (15)N-labeled antimicrobial peptide, subtilosin A, were used at room temperature. The use of a chelating lipid reduces the concentration of free copper and limits RF-induced heating, a major problem for fluid samples. Our results demonstrate a 6.2-fold speed increase and a 2.7-fold improvement in signal-to-noise ratio for solid-state NMR experiments under magic-angle spinning and static conditions, respectively. Furthermore, solid-state NMR measurements are shown to be feasible even for nanomole concentrations of a membrane-associated peptide.
Yamamoto, K., J. Xu, K.E. Kawulka, J.C. Vederas, and A. Ramamoorthy. (2010).
Use of a copper-chelated lipid speeds up NMR measurements from membrane proteins. *J Am Chem Soc* 132:6929-31.

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