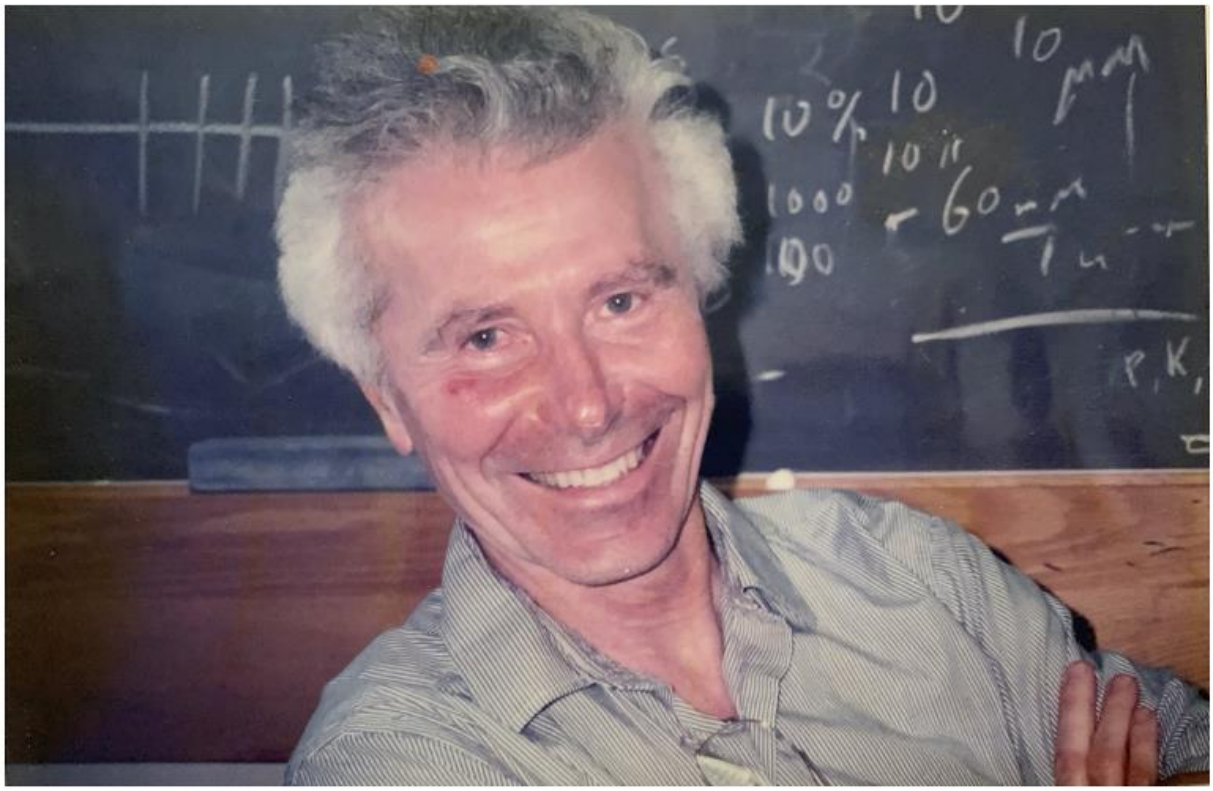

Guido Guidotti - a biochemist's biochemist (and sometimes geneticist): curious, fearless, brilliant, imaginative, intuitive, rigorous and modest.

by Nancy Kleckner
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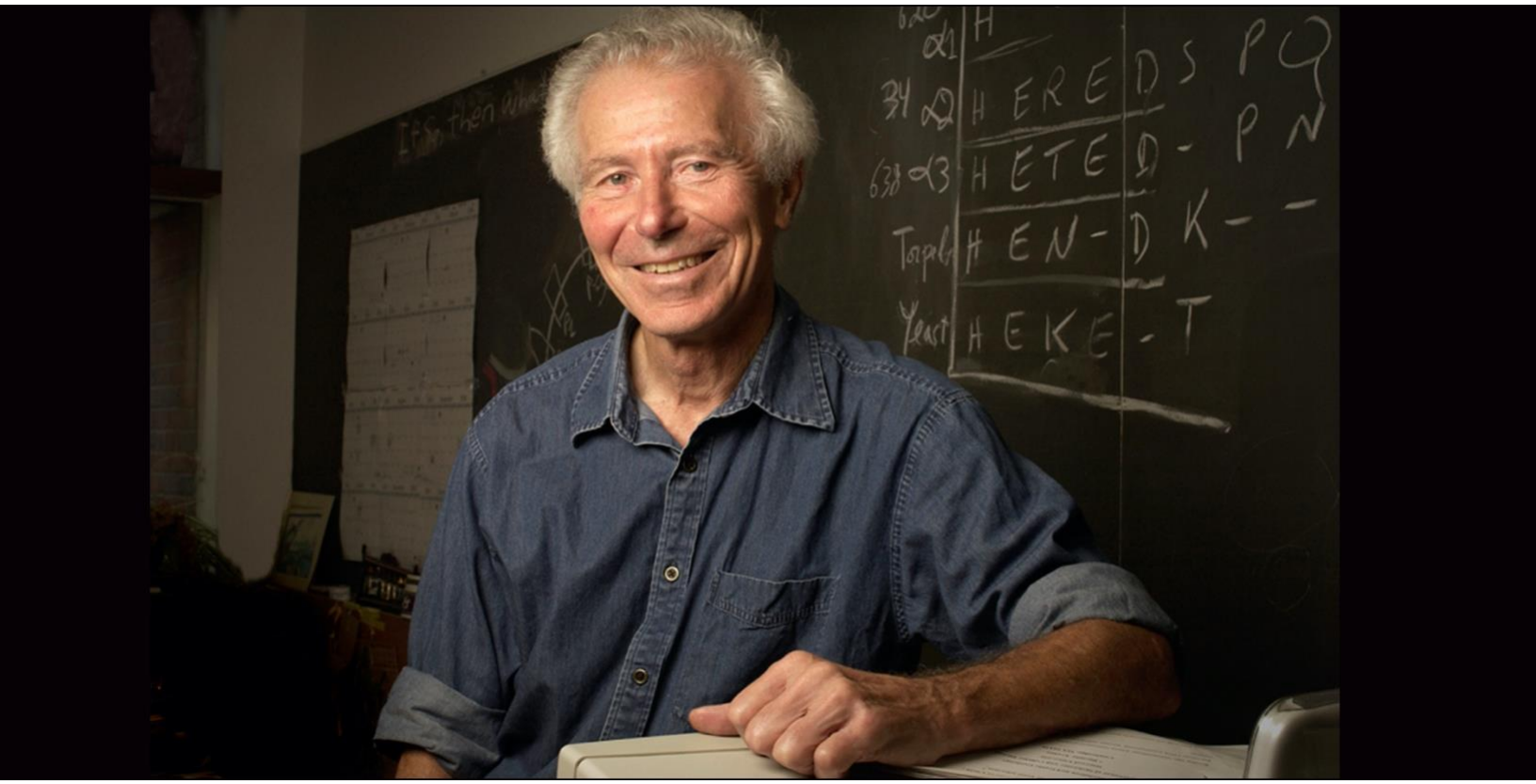

Photo by Stu Rosner

Contents

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Appendices

I. Introduction

Guido Guidotti was born on November 3, 1933, in Florence, Italy. He died on April 5, 2021, in Newton, Massachusetts after a long and courageous battle with cancer (the family's obituary is given in *Appendix I*). Guido lived in Florence and then Sienna until the age of three, at which point his family moved to Naples. His early life, during which he distinguished himself as a brilliant student even despite World War II and its aftermath, has been described by his brother Mario (*Appendix II*). After the war, Guido came to the United States for a year as an American Field Service student in Decatur, Illinois (*Appendix III*). Upon returning home to Naples, he decided then to go back to Decatur for college at Millikan University. He then trained as a physician at Washington University, St. Louis. Upon completing his MD and internship, he was told that a career in academic medicine required him to do research. To satisfy this requirement, he went to Rockefeller University in 1958 as a PhD student. Guido originally intended to return to Washington University as a resident; however, his path instead was diverted to Harvard University, where he remained for 58 years, from 1963 until the time of his death. Tenured in 1969, he was the Higgins Professor of Biochemistry. His CV and list of publications, including his research interests at the time of his death, is attached (*Appendix IV*). In the description below, his publications are referred to by the corresponding numbers in this CV. Papers that he denoted as "especially important" are indicate with an (*).

During his research career of more than 60 years, Guido trained in his laboratory roughly 100 undergraduate, graduate and post-doctoral students. He was also renowned as a teacher of Harvard undergraduate students, in recognition of which he was awarded the Phi Beta Kappa Prize for Excellence in Teaching in 2000. He taught undergraduate biochemistry of all types as well as physical chemistry and physical biology. He also taught a freshman seminar entitled "What is Life" in which he imparted his long-accumulated wisdom and perspective. In this way, Guido trained not only future scientific researchers but also literally thousands of pre-medical students in the intricacies of biochemistry. As we discovered in person, it was literally impossible to go to a hospital emergency room or a doctor's office in the Boston area without someone piping up "I took your biochemistry course". Remembrances of Guido as teacher are provided in *Appendix V.*

Guido had an unmatched intellectual curiosity and brilliance and a passion for educating and empowering everyone around him. This was combined with off-scale warmth, humor, and modesty - a most unusual combination in any person, let alone an accomplished scientist and Harvard professor.

This is the story of Guido's research career as seen through the eyes of someone who lived with him, loved him, and talked science with him for more than 40 years. Insights and contributions from many of Guido's laboratory members and colleagues are gratefully acknowledged, including the many quotations provided in email communications. Two excellent articles provide complementary insight into Guido's life and career, one written as an ASCB member profile in 2003 (*Appendix VI*) and another, beautifully written to capture many of Guido's essential qualities, by then Harvard undergraduate Menting Qiu (*Appendix VII*).

Four contributions from Guido himself are also appended here. First, he wrote a history of biochemistry at Harvard (*Appendix VIII;* see also *Appendix XII*). Second, Guido described his views on scientific publication in an interview for Open Science, where he published the last paper describing experimental work done in his laboratory (*Appendix IX)*. Third, one of the most

important findings of Guido's lab was the discovery of CD39 as the first identified human ecto-ATPase. An overview of early critical findings related to this discovery is reproduced in *Appendix X* = ref 202). Also appended is one of the last experimental papers from Guido's lab (*Appendix XI* = ref 225*). This paper describes how the extracellular active site of CD39 is regulated by mechanical effects that rotate its two terminal transmembrane domains. This paper also included the implications of this process for modulating the active site in different versions of the protein and different cellular and extracellular contexts, thus bringing to bear Guido's entire scientific experience.

Much of the intellectual trajectory of Guido's work can be traced via a series of review and perspectives articles that he wrote, intermittently, at critical junctures (e.g. 9, 20, 25, 32, 33, 36, 68, 85, 93, 103, 112, 144, 162, 176, 184, 202, 224). This thread is also apparent in the papers from his laboratory in which rigorously performed and described Results were framed at the beginning by a meaningful Introduction as to why the work should be important and at the end by a Discussion. In the Discussion, current dogma was considered and then it was noted that "on the other hand," some different interpretation of the data was favored by the authors; and finally, at the very end, some entirely new idea, insight or conclusion that had emerged from the described work was presented. These insights often foreshadowed future work in the lab. Guido's own writing also reveals the ways in which he combined rigorous research with the master diagnostician's ability to assimilate and integrate diverse clues to intuit what was really going on.

Overview. From the perspective of the outside world, Guido's scientific work was seminally important in many respects, and he was an acknowledged pioneer and leader in every area he investigated.

His early work involved early analyses of hemoglobin and allostery, for which he developed a model that, in my opinion, remains viable.

He then tackled the general problem of membrane proteins. At that time there were no purification methods, and also no assays for such molecules (which would require reconstitution of membrane vesicles). Notably, also, it would be decades before any membrane protein was crystallized. As Lew Cantley describes it: "At the time Guido started, there were functions of membrane proteins but no information on the protein; and there were membrane proteins, but with no idea of function; and there was no information about the topography of membrane proteins within the membrane, let alone the nature of the conformational changes involved in their functioning. Guido was adventurous enough to tackle problems that no one else had the courage to tackle. His laboratory developed the necessary expertise. Trainees and guest scientists came to work on these complicated problems."

Guido's most well-known foundational contributions to the membrane protein field are: (1) The first purification of plasma membrane proteins from the red blood cell and the resulting identification of the red blood cell anion exchange protein (Band 3). This molecule mediates the exchange of chloride ions with bicarbonate across plasma membranes and thereby permits red cells to exchange carbon dioxide for oxygen. (2) The purification of the (Na^+,K^+)-ATPase and subsequent elucidation of its topography and mechanism. This so-called "sodium pump" is one of the most important proteins in the body. It is responsible for ensuring the unequal distribution of the two ions inside and outside of cells. Cells use the resulting Na^+ gradient to move ions and molecules into and out of the cytoplasm; and the resulting K^+ gradient to maintain electrochemical charge across the plasma membrane, thereby controlling membrane potential and cellular excitability, differently in different tissues. In addition to these two lines of research, Guido's laboratory also carried out the purification and analysis of other centrally

important ion transporters, early studies of how hormones (vasopressin and insulin) stimulate transport of nutrients into cells, and, finally, purification and analysis of the insulin receptor itself.

The latter two decades of work in Guido's laboratory were initiated by his discovery that CD39, a molecule known to play central roles in brain function, blood clotting, immunity, inflammation and other basic physiological processes, was in fact a divalent cation-stimulated ecto-ATPase. CD39 converts ATP to AMP, which then is converted by another molecule to adenosine, a major cell signaling molecule. Guido intuited that ecto-ATPases and related ecto-apyrases function in coordination with programmed secretion of ATP in the cognate tissues. His lab then went on to investigate the nature of such secretion. Finally, he intuited and then showed that activity of CD39 is governed by conformational changes of mobile, interacting trans-membrane domains, mechanically modulated by membrane elasticity. These findings brought his work full circle, back to his early love of allostery but in a novel and interesting new form. The lab also made it possible to understand how catalytic activity of CD39 and its relatives can be somewhat different from one another and how these activities can be adjusted to respond to local conditions.

It can also be noted that, throughout all of his work, he drew on his medical training and interests to address multiple aspects of normal biological states and disease states, including (but not limited to) consideration of the mechanistic basis of specific conditions, e.g. hypokalemic periodic paralysis (164).

At the time of his death, Guido listed two of his major research interests as: (1) Structure and function of membrane proteins, in particular proteins involved in ATP secretion and in the hydrolysis of extracellular ATP; and (2) Membrane tension and elasticity. Guido was characteristically also interested in the big question of how life evolved. He also listed as a major research interest: the existence of polymer amides and their evolutionary role before the appearance of RNA-encoded proteins, and he was using his resources as a Harvard faculty member to support the investigations of Julie and Malcolm McGeoch in this area (231-234).

Guido's group also made countless other contributions within and beyond all of these areas, reflecting his wide-ranging interests and his willingness to have laboratory members come up with their own lines of research.

Not coincidentally, for many of his undergraduates, graduate students and post-doctoral fellows, the work in Guido's laboratory was the springboard to their own future independent studies. His many scientific progeny have populated the faculties of major Universities across the country and around the world and of major research institutions (notably NIH) as well as many biotechnology companies.

Comment. Full context for the scientific discoveries is elusive, being as it is highly personal and subjective. It can also be invisible when, as in Guido's case, the crucial insights were calmly stated and were often decades ahead of their time. Guido's insights often led and permeated the field, but in such a subtle way as to be easily ignored. In addition, Guido did not put his name on any of the papers from his laboratory for which he did not perform bench work with his own hands, until 1985, when he was forced to do so by the NIH grant system (see discussion of this point in ASBC Member Profile, *Appendix VI*). Part of the goal of this article is to connect the invisible dots of Guido's thinking and research.

An attempt has been made to cite virtually all of the published work from Guido's lab and to mention nearly all of his students. Intentionally, there are no citations to the work of other

groups. However, the interested reader will easily find relevant background and/or current updates from the attributions given, by perusal of Google or PubMed.

Guido's research was motivated only by his intellectual curiosity, his delight in figuring out how life works, and his joy in enabling the work and lives of the people he trained and with whom he worked. Scientific credit was not a priority. I suspect that he would have tolerated, but probably would not have really approved of, the below summary.

Phase I. Hemoglobin. At the Rockefeller, Guido chose to work on hemoglobin, a molecule of intense interest and study then and now. Guido carried out his work in the laboratory of Lyman Craig, working with Bill Konigsberg and Bob Hill (1-4, 5*, 6, 6a, 7*, 8-9). He and his colleagues were the first to determine the complete amino acid sequence of the alpha and beta chains of hemoglobin. As Bill reports, there was intense competition from other labs but Guido *et al*. worked 16 hours a day and weekends to try to finish first. Guido had the advantage of working in a lab where there was a thousand tube countercurrent distribution instrument that was not available elsewhere. This device enabled Guido *et al.* to directly sequence peptides of interest without further purification. Guido and his coworkers were successful in completing the work before other labs. Guido separated and determined the amino acid compositions of the alpha and beta chains and began an investigation of how the molecule dissociated, providing clear biochemical evidence for dimer/tetramer and monomer/dimer conversions (5*, 7*). The complete amino acid sequence was essential for many of the discoveries that followed including the 3-dimensional structure of hemoglobin determined by Max Perutz at Cambridge University using X-ray diffraction, and identification of the mutation that causes sickle cell disease. It also paved the way for the discovery of mutations in humans responsible for thalassemias.

Guido of course recognized that the most interesting and challenging aspect of hemoglobin is its function and thus the allosteric conformational change that allowed for the cooperative binding of oxygen. To this end, Guido's initial work at Harvard comprised a series of biochemical studies that followed on his earlier work (10-14). Aside from their scientific importance, these studies are notable for three reasons. First, they were performed by Guido with his own hands. Second, they relied on a counter current distribution apparatus that Guido brought with him from the Rockefeller. This apparatus now occupies a place of honor in Harvard's Northwest Building, not far from Guido's laboratory. Third, the studies were carried out, famously, on Guido's own blood (for commentary see *Appendix VII*).

Guido's Harvard hemoglobin work focused on the fundamental importance of the prominence of the $\alpha\beta$ dimer and the dimer/tetramer equilibrium. His experiments showed that, "for all practical purposes, two molecules of ligand combine with the $\alpha\beta$ dimer at one time over most of the intermediate range of saturation with ligand" (14). He proposed that this effect occurs whether the dimer exists alone in solution or is present within the $\alpha_2\beta_2$ tetramer and that, in the latter case, "once the first $\alpha\beta$ unit in a tetramer has combined with ligand, its conformation is altered and it forces the associated $\alpha\beta$ unit into a new conformation" (14). Guido's model was published in 1967 in the context of two competing models: the Monod-Wyman-Changeaux model, which assumed that, upon ligand binding, the hemoglobin tetramer undergoes a concerted change from one conformation to another, and the Koshland-Nemethy-Fillmer model which assumed that ligand binding to each subunit is transmitted sequentially to the rest of the subunits. His

hypothesis was closely aligned with the latter model. In fact, a more general formulation of the situation embodied by Guido's model was published in the same year by Jeffries Wyman.

Guido continued to investigate the αβ/αβ interface and other aspects of his hypothesis in subsequent studies of hemoglobin (15, 21, 22, 24, 25, 31, 34, 42, 57, 60, 61, 70). Most notably, he and Frank Bunn published work in 1972 showing that: "there is no obvious relationship between oxygen affinity and cooperativity. In fact, the Bohr effect [the fact that hemoglobin's oxygen affinity increases with the partial pressure of CO_2 and/or blood pH] and cooperativity are largely independent phenomena, which means that the stabilizing interactions in hemoglobin are not related in any simple fashion to oxygen affinity." (34), a finding that matches his earlier hypothesis. Subsequently, work by Connie Park in Guido's lab included development of isoelectric focusing as a way to rapidly define different subunit species. This made it possible, as a first example, to provide additional evidence of "cooperative interaction across the boundary between dissociable dimers" (42), in accord with Guido's hypothesis.

In fact, the nature of hemoglobin allostery remains the subject of active debate to the current day, with more definitive information awaiting application of methodologies that allow to see conformational changes in real time. If history repeats itself, Guido's fundamental insight may yet ultimately prove to be correct.

Overall, Bill Konigsberg comments that "Guido is considered one of the founders of this field and many papers have relied on his findings as a basis for their studies."

Phase II. A new challenge: membrane proteins. Upon completion of his own hemoglobin work, and the emergence of Perutz crystal structures of hemoglobin, Guido sought a new, bigger, and broader challenge. He decided to work on membrane proteins because, at that time, almost nothing was known about these molecules. The barrier to progress, but then also the reward, was the involvement of the membrane. There was no known way to purify membrane proteins away from their milieu nor were there ways of assaying their activities within that milieu. On the other hand, if those technical challenges could be solved, a whole new world could be explored from many different perspectives. This was the perfect challenge for a biochemist's biochemist.

As quoted above, "Guido was adventurous enough to take on problems that no one else wanted to work on." At the time Guido embarked on this work, membrane proteins couldn't be crystallized, and a membrane was needed to reconstitute activity. Thus, function could be demonstrated but with no protein to analyze; and proteins could be analyzed but without analysis of function; and how the protein was arranged in the membrane, and what types of conformational changes were involved in transport functions were unknown.

By way of confirmation, Harvard colleague Vicki Sato recalls "listening to Guido give a plenary lecture at the Cell Biology meetings. He started by drawing two parallel lines on the chalkboard, and wrote ‘inside’ to the far left and ‘outside’ to the far right. He commented that this summarized, to his knowledge, the state of understanding of how membrane proteins were structured in the membrane and how they functioned. It blew me away."

Guido and his group not only developed ways of purifying membrane proteins but also ways of reconstituting them into membrane vesicles to allow mechanistic dissection and functional dissection in their normal context.

In addition, one of Guido's first graduate students to work on membrane proteins, Jack Kyte reports that "Guido mused to me that he was thinking of studying the brain because almost nothing was known about it." In fact, many of the publications from Guido's lab specifically considered membrane proteins in the brain, including the sodium pump (59, 88, 102*, 154), activation of the sodium pump by insulin (157, 158); insulin receptor (148, 149); the glucose transporter (152, 153); tubulin (35, 47, 48); channels in the eye (89, 117, 123, 179, 195); synergy between ATPase activity and serotonin uptake (183); and, ultimately, key roles for the ecto-ATPase CD39 (189, 198).

Guido was also attracted by other aspects of his new field, all of which he pursued throughout his entire research career. First, membrane proteins play central roles in every aspect of a cell's and the body's physiology. Study of these molecules thus allows linking of biochemical function to medical aspects of the human condition in normal and then, inevitably, diseased states. Second, every physiological process is linked by complicated interconnections to other processes, and Guido was a master at integrating the effects of many interacting systems to provide new insights. Third, the *raison d'être* of membrane proteins is to mediate movement of various substances across membranes. This activity, as for hemoglobin, would involve allosteric conformational changes but with the added contribution of interplay with the membrane. The fundamental properties of this little-understood ensemble were thus brought into the equation with respect to both topology and function. Fourth, as an offshoot of this aspect, Guido was interested in mechanical inputs into biochemical processes. In Wyman's formulation of allostery, pre-ligand protein states were considered "taut" (*i.e.* with potential energy stored in strain within the molecule) and ligand bound states were considered "relaxed" (with the accompanying energy difference driving the conformational change). The same principle, of course, operates at all length scales, including macroscopic features of membranes and membrane/cytoskeleton interactions, whose existence and significance Guido also intuited and studied. Indeed, the door of our shared laboratory at Harvard bears the title "Physical Biology of Membranes and Chromosomes." Guido was also a major (albeit, as usual, largely invisible) driving force creating and fostering our Department's PhD track in Engineering and Physical Biology.

Guido's intelligence, plus the unique expertise of his laboratory attracted generations of trainees and visiting scientists to work on these complicated problems. Every new person coming to the lab could either try to advance existing problems under study in the laboratory or choose a new problem, as long as Guido found it interesting. As a result, Guido's laboratory made important contributions in a dazzling diversity of areas.

Guido's entry into the field of membrane proteins involved two prongs. As he described himself (36): "One approach to the study of membrane proteins [is] the isolation of components and then an investigation of their arrangement in the membrane and of their function. This approach is possible with components...which are present in large amounts. Another approach to the problem is to attempt the isolation and characterization of proteins that have a definite function even though they may be present in very small amounts." Guido took both approaches as initiated by three graduate students, Steve Rosenberg, Bob Bloch and Jack Kyte,

First prong: red blood cell membranes. Guido and Steve made use of Guido's old friend the red blood cell which, conveniently, comprises a plasma membrane bag filled with hemoglobin and only trace amounts of other subcellular components. Reinhard Reithmeier comments that: "To study hemoglobin, Guido had lysed red cells and centrifuged the lysate, keeping the supernatant and throwing away the membrane pellet. Now he kept the pellet and threw away the supernatant!" With the appearance of SDS gels, Guido appreciated that the way was open to identification of individual membrane proteins in RBC membrane "ghosts." At the time Steve initiated these studies, no one had purified any integral membrane protein and no one knew what any such protein did. Prevailing dogma, based on analysis of an uncharacterized aggregate, held that the plasma membrane held only one protein, a putative "structural protein." Steve's seminal work, published in 1968-69 (16, 18, 19*, 20) revealed, instead, a large number of plasma membrane components ("at least 12"). Guido was particularly interested in two molecules identified in this initial study as well as the RBC membrane glucose transporter (32, 33, 36; below).

Band 3. The first molecule of interest was "Band 3", so named by Rosenberg because it was the species of the third largest molecular weight in his SDS gel profile. He demonstrated that this molecule was a glycoprotein but, importantly, was distinct from a prominently discussed "major" glycoprotein (now known as glycophorin A) (e.g. 20; see also later work of Findlay on glycoproteins (52).) Rosenberg also showed that Band 3 was exposed on the outer surface of the membrane but also was intimately embedded within, and thus likely spanning, the membrane. In 1975, Guido and Michael Ho made the seminal finding, in parallel with Cabanchik and Rothstein, that this molecule was the red blood cell anion exchange protein, the single most central player in red blood cell function (56*). This molecule, the most abundant membrane protein in the red cell, is a chloride/bicarbonate antiporter. CO_2 produced in tissues throughout the body by glycolysis diffuses into the red cell where it is converted to bicarbonate by carbonic anhydrase. Bicarbonate is then exported into the blood in exchange for chloride by Band 3, thus increasing the blood's capacity to transport CO_2 as plasma bicarbonate. Then, in the lungs, bicarbonate enters the red cell via Band 3 to be converted back to CO_2, which diffuses out of the red cell to be expired.

Guido's lab continued to study Band 3 as a powerful system for analysis of membrane protein topology, about which, of course, at the time, little or nothing was known. In a particularly important study, Kurt Drickamer fragmented the protein, with and without concomitant localization with residue-specific labels. Amino acid analysis of the fragments revealed that the N-terminus was located inside the cell, that the C-terminus was located outside the cell, and that between these two regions, the polypeptide traversed the membrane an odd number of times (71, 80*). Additional studies by Kurt, Anjana Rao, Reinhart Reithmeier and Lew Cantley (91, 97, 98, 100, 101, 106*) led to a more complete description of Band 3 topology and its functional dimeric state as summarized by Guido in 1980 (112). Kurt's work on Band 3 was preceded by his identification, by γ-^{32}P-ATP labeling, of a previously unknown plasma membrane Mg^{2+} ATPase (58). Reinhart Reithmeier continued to work on Band 3 once he had set up his own lab back in Canada, providing a lasting legacy of his time in the Guidotti lab.

Dan Jay also studied Band 3, in chicken erythrocytes (126, 145). He also developed, and applied to human Band 3, a novel general procedure for end labeling of proteins which, along with targeted fragmentation, allows one to determine the positions of specifically-tagged amino acids along the sequence (132, 145).

The cytoskeleton. The second molecule of interest identified in Rosenberg's analysis was remarkably similar in its size, subunit composition, and hydrodynamic properties to the actomyosin complex of muscle tissues. Guido understood the potential for important interplay between such a molecule and membranes and, thus membrane proteins. He therefore proposed

that an actomyosin-like system was responsible for ATP-dependent changes in cell shape (32, 36). The identified molecule turned out to be spectrin. It is now known that the biconcave disk shape and deformability of the mammalian RBC, which are vital to its ability to circulate through arteries and veins, rely upon a viscoelastic spectrin–F-actin network attached to the membrane. Guido's early intuition then informed future studies of his group. Bob Bloch investigated why cytochalasin B, an inhibitor of cell movement, also inhibited sugar transport (44). Betty Eipper carried out studies of brain tubulin and its phosphorylation (35, 47,48). And Feig and Guidotti (51) investigated the interplay between the plasma membrane Ca^{2+}-dependent adenosine triphosphatase activity, which regulates cellular calcium levels, and hereditary spherocytosis (HS), a common cause of hemolytic anemia. In HS, the RBC is spherical rather than having its normal disc-like shape, and thus does not move efficiently through the vascular system. Jim Anderson continued to investigate the nature and roles of spectrin and its phosphorylation (94, 108). And Guido's lab continued to investigate the possibility of membrane/myosin-actin interactions through the work of David Brandon (69, 235) and in collaboration with Dan Branton (110), and, more broadly, to investigate the roles of structural components in general (113).

A Link. In further confirmation of Guido's intuitions, subsequent work by other groups showed that a key component of membrane/cytoskeleton interactions and cell mechanical support is the direct linkage of Band 3 and Guido's actomyosin molecule (aka spectrin) as mediated by a third molecule, ankyrin. Mutations in Band 3 or the cytoskeletal components that disrupt this linkage cause red cell shape changes associated with various diseases, including HS.

The Glucose Transporter. Bob Bloch initiated studies of glucose transport at a time of many confusing and conflicting results. The only firm foundation was that the process is mediated by a protein in the red cell membrane. His studies led to Bob and Guido's formulation that transporters are gated pores that undergo conformational changes as they move substrate across the membrane. This view comprises one of Guido's many critical insights.

As Bob describes it: "The major issue at the time (although the resolution seems obvious now) was whether the glucose "carrier" shuttled from one side of the membrane to the other, moving sugar down its concentration gradient, in a process then referred to as "facilitated diffusion", or whether the "carrier" spanned the membrane, allowing glucose to move through a channel or pore. My studies of carrier kinetics and the effects of protein modifiers on transport strongly pointed to the latter model, which, under Guido's guidance, expanded to include the concept that conformational change in the "carrier" was required for translocation."

These ideas are articulated in the Discussions to two papers (45, 46). "The main features of this transport system are that the carrier spans the membrane and that there is a binding site for sugar on each surface of the membrane on the same carrier molecule. The process of translocation moves sugar from the external binding site to the internal one, or vice versa. An important feature of the translocation, and the central point of this transport system, is that the process does not require rotation of the carrier in the plane of the membrane, nor movement of the protein backward and forward across the membrane. Translocation is envisaged as a conformational change in a fixed protein that spans the bilayer, i.e. in a pore. The details of this process are not known and possible mechanisms are not useful at this point." (46) Moreover, "To explain data [obtained with protein modifiers], the transport system would have to be fixed, with the low K_M, low V_{MAX} site always on the outer surface of the membrane, and the high K_M, high V_{MAX} site always on the inner surface. The assumption that the carrier rotates from one side of the membrane to the other would therefore have to be eliminated. The transport process can be considered to operate instead by a "gating" or "pore" mechanism." (45)

Other studies in the lab would subsequently show that the same formulation holds for Band 3 (above) and the (Na^+,K^+)-ATPase (below).

The second prong: the (Na+,K+)-ATPase. The (Na^+,K^+)-ATPase is one of the most important proteins in the body. It is found universally in all cells and is responsible for maintaining cation gradients in which Na^+ is at high concentration outside the cell and low concentration inside, due to the action of the pump, and K^+, which is exchanged for Na^+, is present at low concentrations outside the cell and at high concentrations inside. The energy of ATP hydrolysis drives the action of the pump to create these gradients. The sodium gradient is used to move diverse molecules, from calcium to amino acids to hydrogen ions, into and out of cells. Furthermore, the gradients of sodium and potassium ions are used to run the entire functioning of the brain. In this case, potassium movement out of cells, through constitutively open channels, sets the resting interior negative membrane potential, while transient opening of chemical or voltage gated sodium channels causes the initiation or propagation of nerve signals. The (Na^+,K^+)-ATPase is essential for creating the ion gradients that run these processes. Activity of the sodium pump is also important in maintaining blood glucose levels through sodium-dependent uptake systems in the kidney and intestine, among many diverse roles in organs throughout the body.

Thirty years of the sodium pump**.** At the time Guido tackled this subject, nothing was known about the corresponding protein. Jack Kyte purified the (Na^+,K^+)-ATPase (28*). He provided the first evidence of the structure of the protein, which he showed to comprise two subunits, and began to further characterize its structure and activity (28*, 29, 37, 38). Jack recalls: "When I first joined the lab in the summer after my freshman year as a graduate student, Guido said to me one day that he thought I should work on sodium potassium ATPase. That was about it. I, of course, had never heard of the enzyme so rather than ask him what it was and show my ignorance, I simply said OK and spent the next two weeks in the library of the Biological Laboratories figuring out what it was and what it did."

By 1976, work in Guido's lab and others fully validated the inferences above. As Guido again stated: "Solutes are carried across eukaryotic plasma membranes by oligomeric glycoproteins which span the cell membranes and conduct transport by undergoing conformational changes" (68). This conclusion was deduced from analysis of only a handful of membrane proteins, three of which were the (Na^+,K^+)-ATPase, the anion exchange protein (Band 3) and the glucose transporter, as studied by Guido's lab (above).

Guido and colleagues continued to analyze the (Na^+,K^+)-ATPase with respect to its mechanism of action and conformation(s) within the membrane. A critical advance occurred in 1977 when Stan Goldin in Guido's lab was able to reconstitute membrane vesicles containing purified renal (Na^+,K^+)-ATPase, thereby showing that the two involved polypeptides comprised the entire active transport system, and also rigorously defining the catalyzed ion movements (53, 59, 79*). Further studies by Giotta (63, 67ab, 78) helped define the quaternary structure of the enzyme. This body of work inspired two reviews of the structure and arrangement of membrane proteins within the membrane (85, 93).

Guido's work on the sodium pump also attracted to his laboratory Lew Cantley who, as a post-doctoral fellow, investigated its detailed mechanism of action. With Lee Josephson, Lew first identified a slow conformational change, the presence of both high and low affinity sites for ATP (76), and the presence of a tyrosine at the active site (86). Analysis of this conformational change led to their identification of the inhibitor of the pump, present in muscle tissue which they ultimately identified as an oxidized state of the metal vanadium (81, 82*). This finding ultimately provided insight into the mechanism of ATP hydrolysis by the (Na^+,K^+)-ATPase. Vanadate forms a trigonal bipyramidal structure analogous to the transition state for phosphoryl

transfer reactions and phosphate hydrolysis. Lew and Marilyn Resh showed that vanadate is transported into the red cell through the band 3 protein where it acts on the cytoplasmic side (87*, 95), consistent with it acting at the ATP hydrolysis site. Detailed biochemical studies further revealed a mechanism in which the enzyme hydrolyzes ATP in a two-step process with vanadate as a mimic of the intermediate phosphate-bound state. More specifically, they proposed an "alternating site" model in which the low affinity ATP site of one cycle becomes the high affinity site where phosphorylation occurs in the subsequent cycle (90). Interestingly, vanadium is essential to mammalian life. Vanadate was thus analyzed as a modulator of (Na^+,K^+)-ATPase activity *in vivo*, including elucidation of interactions with norepinephrine (92), and Lew proposed that its action might underlie cation movements in the nervous system linked to spreading depression (90). This insight into vanadate as an inhibitor of enzymes with covalent phospho-protein intermediates led to the use of vanadate as an inhibitor of phospho-tyrosine phosphatases by Nick Tonks and Ed Krebs as they began to purify and characterize this family of enzymes that, like (Na^+,K^+)-ATPase, have phosphoprotein intermediates. Vanadate has been explored as an insulin sensitizer because of its ability to prolong the activity of the insulin receptor Tyr kinase by inhibiting the phosphotyrosine phosphatases that turn it off.

These breakthroughs were followed by a large number of further studies in Guido's lab by Kathy Sweadner, Bob Farley, Mike Forgac, Gilbert Chin, Julie McGeoch, Frank Gorga, Kyunglim Yoon, Maddalena Coppi, Jia Liu, and Kyunglim Lee (84, 88, 96, 107, 109, 116, 119, 120, 125*, 129, 141, 142, 161, 177, 188, 193, 196, 201, 203). This work defined and refined the sodium pump topology and mechanism as well as the discovery of three distinct isozymes with different physiological roles (e.g. 102*, 134*, 154; below). Among these discoveries were three key contributions from Forgac and Chin. First, the enzyme is able to move sodium ions uncoupled to the movement of potassium ions under potassium-free conditions, suggesting that the enzyme is able to complete its catalytic cycle with sodium ions replacing potassium ions in stimulating dephosphorylation (116). Second, proteolytic sites were located intracellularly, implying that insertion of the sodium pump into the membrane was analogous to "a cork in a bottle" (125*). Third, the activity of the pump was used to create density differences in reconstituted vesicles that permitted the isolation and study of (Na^+,K^+)-ATPase populations in inside-out and right side-out vesicles (129).

Studies of the sodium pump also allowed Guido to continue to pursue his interest in membrane protein/cytoskeleton interactions with the demonstration that the alpha subunit of the (Na^+,K^+)-ATPase interacts with actin binding protein cofilin (210) and to investigate the role of the sodium pump in paralysis that occurs under conditions of potassium depletion, as described below (164).

Extensions. Guido's laboratory also carried out studies of the roles and mechanism of sodium fluxes beyond those mediated by the sodium pump, in two different parts of the body.

The liver. Irwin ("Win") Arias, a world-renowned expert on the liver, was interested in the fact that intracellular pH in hepatocytes is highly regulated, particularly in response to growth stimuli after partial hepatic resection. These pH changes were known to be sodium dependent. So, he came to Guido's lab to investigate the involvement of ion channels in this phenomenon. Together with Mike Forgac, Win was able to demonstrate the existence of a Na^+/H^+ antiport activity in vesicles derived from hepatocytes, the first time such an activity had been seen in the liver (130).

The heart. Sisto Luciani, cardiologist and Professor of Pharmacology at the University of Padova, came as a Fulbright Scholar to spend a year in Guido's laboratory. He

chose to study the membrane protein that exchanges sodium and calcium. Lew Cantley explains Sisto's choice as follows. "The Na^+/Ca^{2+} exchanger explains the ability of cardiac glycosides to stimulate heart muscle contraction. An elevation of cytosolic calcium in cardiac muscle is critical for the contraction of the muscle. After every contraction, the cytosolic calcium is either: (i) pumped into the sarcoplasmic reticulum or (ii) pumped out of the cell, back into the blood (an uphill gradient that requires Ca^{2+}-ATPases (pumps) at the sarcoplasmic reticulum or at the plasma membrane) or (iii) moved out of the cell via Na^+/ Ca^{2+} exchange proteins that take advantage of the sodium gradient established by (Na^+,K^+)-ATPase. Sodium moving down the gradient from the blood into the muscle cell is exchanged for cytosolic calcium providing yet a third way to deplete cytosolic calcium in between contractions of the heart."

Sisto was able to demonstrate that a Na^+/ Ca^{2+} exchange activity could be extracted and reconstituted in artificial phospholipid vesicles and partially purified, thus allowing unambiguous characterization in the absence of other factors that regulate Ca^{2+} levels (131).

Lew also points out that cardiac glycosides (e.g. the structurally related molecules, ouabain, digitalis, digoxin and digitoxin) bind to the(Na^+,K^+)-ATPase and inhibit its activity, raising the sodium level in heart muscle. This makes Na^+/Ca^{2+} exchange less effective at moving calcium out of the cell between contractions and, as a consequence, there is less stimulus of the cardiac muscle needed to reach the threshold for the next contraction. The resulting effect explains why digoxin is effective in treating patients with congestive heart failure and cardiac arrhythmia. As a cardiologist, Sisto was interested in the possibility that an inhibitor of Na^+/ Ca^{2+} exchange could be more effective and less toxic than cardiac glycosides. Now, more than 30 years later, such inhibitors are moving into clinical trials.

Cardiac glycosides have a very long history. British folk medicine, dating back nearly a thousand years, made use of a tea generated from the root of the plant foxglove for treatment of elderly men and women with ‘dropsy’ (cardiac failure) and the active ingredient (digitalis, one of the most broadly used drugs in the world) was isolated about 200 years ago. But it wasn’t found until the 1950s that its target was an enzyme that mediated sodium export from cells and, it wasn’t until the 1970s that Guido's group identified and purified that enzyme (the Na^+,K^+)-ATPase) and demonstrated its structure.

More methods. In addition to developing purification and vesicle reconstitution procedures, Guido's laboratory also developed new reagents for specifically labeling components on the cell exterior (55) and the hydrophobic segments of intrinsic membrane proteins (105). Also, Steve Clarke developed a method for determining membrane protein molecular weights in the presence of Triton X-100, thus dramatically enabling many studies, including documentation of the dimeric nature of the membrane domain of Band 3 (62*). He also showed that Triton can cause some denatured proteins to flip their conformation from hydrophobic inside to hydrophobic outside. He speculated that the ability of proteins to exist in two conformations might allow them to pass from the cytoplasm (in the first conformation) through/into the mitochondrial double membrane (in the second conformation) (83). Notably, it was the use of mild detergents such as Triton X-100 that allowed the solubilization and purification of hydrophobic membrane proteins in a native state (as opposed to the denaturing detergent SDS in common use), and the Guidotti lab was an early adopter of this break-through technology. Then, in much later work, Guido's lab developed a bioluminometric method for real-time detection of ATPase activity (212), which figured heavily in the laboratory's biochemical studies of ecto-ATPases (below). Finally, last but not least, the entire laboratory effort benefitted from John Pringle's early development of reliable methods for protease-free purification and SDS gel analysis of proteins (23, 30; 230; below).

III. The next frontier: hormonal regulation of membrane transporters. Not content with investigating the membrane topology and biochemical mechanisms of intrinsic membrane transporters, Guido also tackled the problem of how these important molecules are regulated, another subject about which, at the start of his investigations, little biochemical or molecular information was available. This line of research also had two major prongs.

First prong: regulation of adenylate cyclase by vasopressin. Post-doctoral fellow Eva Neer initiated an entirely new area of investigation in Guido's lab. Vasopressin is the "antidiuretic hormone." It constricts blood vessels and, in the kidney, promotes increased retention of fluid to combat low blood pressure, thereby providing the main regulation of water balance in the body. At the initiation of this research, the response to vasopressin was known to involve activation of plasma-bound adenylate cyclase for synthesis of the second messenger cyclic AMP. To further explore this link, Eva developed a new strategy for synthesis of biologically-active vasopressin (39) and a way to purify soluble adenylate cyclase (40), which she then combined to show that vasopressin binding was rapidly reversible (41). Moreover, via the methods developed by Pringle in Guido's lab, she determined the molecular weight of the protein (54). Perhaps because of emerging hints for the potential involvement of GTP in signal transduction pathways linked to these events, Eva then began to study another ill-defined enzyme, guanylate cyclase. She demonstrated that adenylate cyclase and guanylate cyclase were distinct enzymes (64, 75), contrary to dogma at the time. Guido also suggested that the adenylate cyclase of macrophages might be hormone-stimulated, which Remold-O'Donnell in his lab then showed to be the case (49, 50). It is again important to emphasize that at the time of Neer's work, the existence of GTP-responsive (G-)proteins and G-protein coupled receptors, which link vasopressin to adenylate cyclase, was not yet known.

Thereafter, however, Eva's interest in these problems led her to discover an entire family of G-proteins in her independent research, which then led to her appointment as only the second woman to achieve tenure at Harvard Medical School (https://www.cell.com/fulltext/S0092-8674(02)71135-5; https://news.harvard.edu/gazette/story/2003/03/remembering-dr-eva-neer-read-at-the-faculty-of-medicine-meeting-on-dec-18-2002/; https://historycambridge.org/research/did-you-know/eva-neer-my-neighbor-groundbreaking-biochemist/)

Second prong: insulin regulation of the (Na^+,K^+)-ATPase and the glucose transporter. The first discovered, and most prominent, role of insulin is to stimulate glucose transport. Guido's interest in insulin and its effects thus began with Bob Bloch's purification and investigation of the glucose transporter (above; 44-46). Subsequent studies from Guido's group clarified the fact that skeletal muscle, liver and brain have different fetal and adult forms of this protein (152); and Jeff Brodsky also showed that the brain transport process is indistinguishable from that of erythrocytes (153).

Insulin stimulation of the sodium pump**.** Diverse reports in the literature had led Guido to suspect that insulin regulated not only the glucose transporter, but also the (Na+,K+)-ATPase) (114*). If this idea were true, after a meal, insulin would co-regulate both potassium uptake and glucose uptake from the serum. This is important because, among other reasons, normal regulation of serum potassium is essential to prevent cardiac arrhythmias. In a seminal paper, Marilyn Resh, Raphael Nemenoff and Guido (114*) discovered that insulin did indeed stimulate (Na^+,K^+)-ATPase-mediated influx of potassium into cells, confirming Guido's suspicion. Importantly, also this work also showed that uptake of monovalent cations (by the (Na^+,K^+)-ATPase) and glucose are mediated by different membrane transport systems but also noted that, on the other hand, in published reports, the two processes have similar insulin stimulation

profiles with respect to several criteria. The insulin receptor had not yet been purified. Nonetheless, Resh *et al.* speculated that "hormone interaction with the insulin receptor generates a common signal which serves to activate two different membrane transport proteins", a supposition for which they subsequently provided further evidence (114*, 121, 124). Interestingly, work by Marilyn (122) also demonstrated that insulin stimulation was the result of an increase in the overall activity of (Na^+,K^+)-ATPase) transporters already in the membrane, and thus a direct mechanistic effect, not an increase in the number of transporters, which was the effect shown previously for the glucose transporter.

Guido's lab went on to investigate insulin-dependent activation of the sodium pump in more detail. In 1979, Kathy Sweadner made the key discovery that the brain contained two forms of the sodium pump whose different affinities for cardiac glycosides explained some known peculiarities of cardiac physiology, and whose differential expression in neurons and non-neuronal cells pointed to distinct functions (102*). Cloning work in Jerry Lingrell's lab identified the two forms as the (Na^+,K^+)-ATPase gene products α1 (ubiquitously expressed) and α2 (selectively expressed) and identified a third gene product, even more restrictively expressed, called α3. The corresponding α3 protein was identified in rat brain by Yen-Ming Hsu in Guido's lab (154).

Further investigation of the mechanism for insulin regulation of the sodium pump, as well as that of the glucose transporter, occupied many different Guido lab trainees, including Jonathan Lytton, Chung Wang, Bill Brennan, Diana McGill, Jeff Brodsky, Luisa DiAntonio, Maddalena Coppi and Julie McGeogh, over a significant stretch of time, resulting in many interesting research studies and publications. Overall, this work showed that the α1 isoform primarily serves a housekeeping function whereas the α2 isoform is specifically activated by insulin (114*, 134*, 135, 143, 158, 160, 163, 196). Marilyn and Raphael's original description of insulin stimulation had suggested the selective responsiveness of a pump with high affinity for the cardiac glycoside ouabain. Jonathan Lytton (134*, 140) then further demonstrated that adipocytes and skeletal muscle also have two Na^+ pump forms resembling α (α1) and α(+) (α2), and only the α(+) version responded to insulin in both tissues due to a change in its apparent Km for Na+ ion. Subsequent work by Diana McGill (167, 168*) confirmed and extended these observations, demonstrating a somewhat more complex mechanism involving both Vmax and Km regulation. Important work by Jeff Brodsky (157, 158, 159) characterized (Na^+,K^+)-ATPase isoforms, particularly α2, and regulation of their activity, in brain as well as in differentiating 3T3 cells. Diana (166) and Julie McGeogh (161) investigated roles for GTP and Ca^{2+} ion as possible components of the insulin effect. And somewhat later, Maddy Coppi used ubiquitin-mediated degradation and recombinant expression of (Na^+,K^+) ATPase mutants to further investigate the mechanism for regulating Na^+ pump activity (188, 192, 196).

These and other findings from Guido's lab are summarized in their 1996 review (176) as follows: "Insulin increases the uptake of K^+ from the blood into skeletal muscle, presumably to clear dietary K^+ from the blood. The mechanism of this effect involves the α2 isoform of the (Na^+,K^+) pump, which is the principal isoform in skeletal muscle. The critical property of this isoform is that it can be regulated by interaction with a repressor that modulates its Vmax and its $K_{0.5}$ for Na^+. The repressor-pump interaction is affected by the [Na^+] and signal transduction from the insulin receptor, leading to stimulation of the (Na^+,K^+) pump [both] by increased entry of Na^+ [via an unknown route] and by insulin action.....[We infer from these data that] there are at least four components required for the activation of the sodium pump by insulin: the α2 isoform of

the (Na^+,K^+)-ATPase, a repressor of the activity of the α2 isoform, an effector that releases the repression, and the insulin receptor that activates the effector." It can be noted that the specific mechanism of action of insulin on the sodium pump remains unknown to this day, and the work from Guido's group is still cited as the prevailing hypothesis. Kathy Sweadner suggests, based on current literature, that the proposed repressor might be a small "third subunit" FXYD1 (phospholemman) and that release of repression might result from phosphorylation by protein kinase C. Recently, Lew Cantley's laboratory has shown that the effects of insulin all go by way of PI3 kinase activity as part of a complex signaling pathway.

Hypokalemic periodic paralysis (HPP)**.** Guido had long been fascinated by this genetically-determined human disease in which "the plasma level of K^+ is normal until an attack takes place, and then it decreases to low levels; the attacks, however, can be brought about by insulin." (164). Guido and Yen-Ming Hsu finally investigated this effect directly using skeletal muscle of rats maintained on a low potassium diet (164). Yen-Ming's work confirmed the predicted specific involvement of the α2 isoform (above) and showed that the number of pumps in the membrane was dramatically reduced even though, paradoxically, messenger RNA level was concomitantly increased. Also, Guido had previously understood that there must exist an insulin-dependent mechanism for Na entry independent of the sodium pump. Resh had observed that stimulation of the (Na^+,K^+) pump by insulin can last a very long time (>60min; 114*). Thus: "an insulin-dependent Na+ transporter is needed to prevent depletion of intracellular Na+ by the insulin-activated (Na^+,K^+)-pump". Otherwise, the insulin-activated pump would ultimately have depleted intracellular Na^+ to below the Km of the pump, with resultant cessation of pump function. Yen-Ming's studies provided additional evidence to this effect. From this and other results, Guido and Yen-Ming realized that, for persons with HPP, the critical defect should be an increase in that insulin-dependent Na+ uptake: "If, [in the HPP individual], this [Na^+ uptake] channel opens so as to allow more Na^+ entry than happens in other persons, insulin will cause a greater stimulation of the (Na^+,K^+)-ATPase [due to the higher Na^+ concentration] and thus a greater uptake of K^+, thereby depleting K^+ in the extracellular spaces, and finally [inhibiting] the pump. At this stage, without pump activity, continued Na^+ entry will cause depolarization of the membrane and thus paralysis." (164). In 2018, structural studies from the Catterall Lab confirmed Guido and Yen-Ming's deduction: the genetic defect responsible for hypokalemic periodic paralysis, identified by molecular work, was found to lie in the gene for a voltage-gated Na^+ channel ($Na_v1.4$) and to result in increased Na^+ uptake.

Subsequently in Guido's lab, Julie McGeoch searched for the afore-mentioned insulin-dependent Na+ channel by biochemical approaches. She identified such a channel, which she found to be gated by cyclic nucleotides, to be of relatively broad specificity (169), and to be found in many tissues, and thus likely the principal route for Na^+ entry in many cell types (175). It was also modulated in an interesting way by nicotine in the eye (179). Eventually, Lorraine Santy identified two distinct forms of this channel by biochemical analysis, pointing to flexibility in fine tuning the cell's response to insulin (185, 197). However, the channel they identified was not that responsible for HPP.

McGeoch's work on Na+ entry also led, serendipitously, to the discovery that subunit c of the F_0 portion of the ATP synthase will, by itself, generate regular current oscillations when incorporated in a lipid membrane held at constant potential (195, 208). This finding triggered the authors' proposal that this subunit might initiate neural oscillations in the mammalian brain. They showed that the basis for such oscillations was opposing actions of cGMP and calcium (208) and further examined this phenomenon from a biophysical perspective (173,

207). Julie and Guido further suggested that the pathogenesis of Batten syndromes, which involve overaccumulation of subunit c, is linked to the protein's ion channel function (215). This hypothesis remains to be explored.

The insulin receptor. Guido's lab also investigated another major piece of the insulin signaling puzzle, the insulin receptor. Ramani Aiyer isolated and characterized the insulin receptor (127, 128) and, with Gonul Velicelebi, began to define subunit structure (133). Ken Chiacchia examined disulfide bonds in the receptor, thought to hold subunits together, and the role of insulin in their stability (150, 165). This work led to Eric Mortensen and Jonathan Drachman's studies to assess the structural requirements for activation of the beta subunit kinase. They demonstrated that the receptor exhibited hormone-responsive tyrosine kinase activity (163). This work also led to a clear and direct demonstration that guanine nucleotides regulate insulin binding. This finding led the authors to propose (contrary to suggestions in the field at that time), that a G-protein is involved in insulin receptor regulation and, specifically, that such a protein determines the affinity of insulin for its receptor (170).

Later studies on the insulin receptor in Guido's lab were carried out by Chung Wang, Bill Brennan, Ken Chiacchia, Kuanghui Lu, and James Wu. Together, their work demonstrated the lack of a correlation between the number of insulin receptors and the number of glucose transporters (137). They also rigorously documented insulin receptor autophosphorylation, thus demonstrating intrinsic receptor kinase activity, which is now accepted, and they found no role for phosphorylation in receptor activity (146). They then analyzed changes in the insulin receptor in the brain during fetal development (148, 149) and investigated the mechanism and role of disulfide bonds and dimerization (150, 165, 182, 217, 219).

IV. Pièce de Résistance: ecto-ATPase CD39 (now ENTPDase1)

In 1996, Guido's laboratory identified CD39 as the first human ecto-ATPase. Previously, CD39 was implicated in a wide diversity of basic physiological responses (and their pathologies) but no activity for the molecule was known. The journey of Guido's laboratory to this discovery is summarized in a 1998 review from the lab (202 = *Appendix X*). The implications of this seminal finding were immediate and profound and led to an explosion of research which continues to increase, exponentially, to the current day, culminating in dramatic interest in, and pursuit of, therapeutic interventions that target CD39. Coincidentally, Guido passed away on the 25th anniversary of his discovery (1996-2021).

Human cells are now known to contain several versions of CD39, all evolved from a common progenitor, which differ in their localizations and their relative activities with respect to ATP and ADP. These enzymes are now designated collectively as ecto nucleoside triphosphate diphosphohydrolases, or "ENTPDases". Guido placed the significance of these molecules in context as follows. In 2000 he wrote (206): "In addition to its role as an energy source in the cytoplasm, ATP is regularly released into the extracellular space and the lumen of intracellular organelles, where it and its derivatives, ADP and adenosine, modulate neuronal signaling, platelet clotting, immune function and other processes through interaction with extracellular receptors. A set of enzymes with active sites outside the cell, or ecto-enzymes, is responsible for the extracellular metabolism of ATP. In particular, ecto-ATPases and ecto-apyrases hydrolyze the terminal phosphate of trinucleotides and of both tri- and dinucleotides, respectively, and 5′-nucleotidase converts AMP to adenosine. The ecto-ATPases and apyrases are divalent cation-

activated, high activity enzymes expressed on a variety of cell types including neurons, endothelial cells, activated lymphocytes, and cells of skeletal muscle, secretory organs, and several types of tumors." And in 2007 he could further write that: "the ectonucleoside triphosphate diphosphohydrolases (ENTPDases) have emerged as the major family responsible and specific for breaking the terminal phosphoanhydride bonds of tri- and dinucleotides. As such, they have been shown or hypothesized to modulate many of the signaling and biosynthetic processes in which extracytoplasmic nucleotides play a role, including vascular homeostasis, cell size maintenance, neuronal signaling, immune function, and protein and lipid modification (225*).

Following the discovery of CD39's activity, Guido's laboratory continued to advance the field through his early appreciation that ENTPDase activities were coupled to regulated secretion of nucleotides in cognate tissues; his elucidation of ATP transport mechanisms; and his remarkable and elegant mechanistic studies of CD39/ENTPDase activities which revealed an entirely new mechanism of concerted allosteric control in which the mechanical properties of the membrane play a critical role.

Discovery. Guido's discovery of CD39 as ecto-ATPase grew serendipitously out of his interest in the plasma membrane Ca^{2+} ATPase, a ubiquitous molecule that uses the energy of ATP hydrolysis to actively transport Ca^{2+} out of cells. He and Stephen Feig first studied the role of the calcium pump in hereditary spherocytosis as described above (51). He and Sui Tong then addressed the structure and catalytic mechanism of the enzyme (77, 111).

The ecto-ATPase era began with arrival of Sue-Hwa Lin, whose intention was to further study Ca^{2+} transport. As Sue recently recalled: "I had purified a Ca^{2+}-Mg^{2+}-[stimulated] ATPase when I was a graduate student at Brown University. Guido allowed me to continue working on this protein in his lab. My original purpose was to reconstitute this purified protein to liposomes and characterize its transport activity. However, I could not show that this protein can transport Ca^{2+} after 8 months of trial and error. Thus, I used 'total membranes' to do reconstitution and found there was [indeed] a Ca^{2+} transporter in the original membrane[.] [H]owever, that Ca^{2+} transporter's property is different from the Ca^{2+}-Mg^{2+}-ATPase protein that I had purified (138, 139). Guido came up with the idea that this protein may be an ecto-protein....I then confirmed that my previously purified Ca^{2+},Mg^{2+}-ATPase is an ecto-ATPase (151). After I left the lab, Guido continued on the project and found out this protein is similar to human CD39."

So how did Guido come up with the idea that Sue's protein should be an ecto-ATPase? The trail of thinking is suggested by the Introduction to their paper (151*): previous work had documented ATP/ADP-dependent Ca^{2+} release and had shown that it is transient due to rapid hydrolysis of ATP by a plasma membrane ecto-ATPase. Sue's mystery enzyme was a Ca^{2+}/Mg^{2+}-stimulated ATPase, leading to the intuition that it could be the ecto-ATPase responsible for this effect, which Sue and Guido then showed to be the case (151*). As a life-long habit, Guido read everything, often late at night when the rest of the world was asleep. One can imagine that this critical intuition emerged from such a late-night session.

The quantity of apyrase in Sue Lin's preparations was inadequate for amino acid sequencing and thus for designing of primers for cloning from cDNA identification. The same challenge applied in general to other membrane-integrated enzymes of this type. Guido thus had the idea to investigate a soluble version. His idea was that the soluble versions might be evolutionarily related to the membrane-bound ones but would be much easier to purify. This intuition was

correct. An appropriate molecule was known to be present in potato, and Mas Handa purified the potato apyrase from "1 kg of peeled red skin potatoes" (180*). Degenerate primers corresponding to the amino- and carboxy-termini of the molecule enabled cloning of the corresponding gene. The deduced amino acid sequence revealed homology between the potato apyrase and several known and predicted proteins from a variety of organisms which they found to share a conserved "apyrase domain". Most notable among these proteins were GDA1 (a budding yeast Golgi protein), and human CD39.

The impact of Mas' findings was immediate. As noted in a review by Aileen Knowles: "The First International Workshop on Ecto-ATPases took place in Mar del Plata, Argentina in 1996...The small gathering of ecto-ATPase researchers at the meeting was abuzz with excitement because the molecular identity of the first ecto-ATPase was revealed in a paper by Handa and Guidotti. These authors purified and cloned a soluble E-type ATPase, the potato apyrase, and observed sequence homology with several other proteins, including a yeast GDPase (GDA1) and human CD39." [Purinergic Signal. 2011 Mar; 7(1): 21–45. Published online 2011 Jan 21. doi: 10.1007/s11302-010-9214-7].

Given these findings, Guido asked Ting-Fang Wang in his lab to purify CD39 from human cells. Ting-Fang suggested that he should first compare the ecto-ATPase/ecto-apyrase activities of CD39+ and CD39- cell lines, which he did, thus confirming the relationship. He then went on to directly clone and express CD39 and demonstrate that COS-7 cells expressing his CD39 gene did, in fact, acquire apyrase activity and thereby defined the structure of CD39 as the characteristic structure of the E-ATPase family (181*). The same finding was made in parallel by Simon Robson by a different route.

Interestingly, Ting-Fang reports that "immediately after we published this paper, I got a phone call from a Harvard lawyer. He suggested [that we] apply for a patent, because [ADP-induced platelet aggregation could be prevented by overexpression of CD39 (below)]. I turned the phone to Guido. Without any hesitation, Guido replied on the phone that: 'This is Harvard. Harvard is a place for student education and basic research. It is not a place for making money.'" In every aspect of his life, Guido set a standard for human behavior to which we all should strive but which, all too often, many of us are unable to achieve.

General conceptual framework. Being an ecto-ATPase, the catalytic site of Sue's enzyme was on the outside of the plasma membrane, rather than inside as in the (Na^+,K^+)- and Ca^{2+}-ATPases and other transporters. This situation implied to Guido a new mystery: how does ATP appear in the extracellular space, especially as it is present there in very low levels. In this regard, Guido and Sue made an important distinction (155): "In the case of cells which are not polarized and which have their entire surface in contact with the extra-cellular fluid, the most likely source of ATP is secretion into the circulation. [On the other hand, other types of cells are known to] release ATP, among which are platelets, adult heart cells, adrenal chromaffin cells, and neurons". Guido and Sue further intuited that ecto-ATPases might work specifically in these latter types of tissues to modulate ATP release. They then confirmed this idea by showing that, in liver cells, ecto-ATPase activity is substantially enriched in the canalicular membrane (155). Their ensuing suggestions were that (i) the canalicular ecto-ATPase might degrade ATP to AMP to produce a substrate for formation of adenosine and that (ii) ATP was specifically secreted into the bile canalicular region "by some mechanism which is still unknown". They therefore comment that: "According to this scheme, the ecto-ATPase would be involved not simply in the metabolism and uptake of extracellular nucleotides" but "in the mechanism of retrieval of secreted

nucleotides". This view, in fact, is correct. Moreover, the types of cells that Guido cited as specifically releasing ATP are also now known to be ones in which CD39 plays an important role, in some cases as initially implied by studies from Guido's lab.

Guido further extended this framework to include the notion that the balance between ATP secretion and CD39 activity would ultimately govern the level of adenosine and thereby regulate the diversity of processes governed by that molecule and its cognate purinurgic receptors. He investigated this possibility by pursuing his long-standing interest in the brain. He was particularly intrigued by the fact that ATP is an excitatory neurotransmitter and that its final degradation product, extracellularly, is adenosine, which is an inhibitory neurotransmitter. He reasoned that ecto-ATPases (CD39) and ecto-pyrases might be involved in modulating neurotransmission. He and Ting-Fang presented early evidence for such a link by showing that ecto-apyrase protein is expressed in primary neurons and astrocytes in cell culture (189). Additionally, their cloning of a human CD39 gene revealed its genomic colocalization with a susceptibility gene involved in human epilepsy (189). They linked this coincidence to the published finding of a deficiency of ecto-apyrase activity in the brains of humans with temporal lobe epilepsy (and in those of mice with audiogenic seizures), and with the fact that seizures result from imbalances of excitation and inhibition of nerve firing. Together these findings led them to suggest that defects in CD39 might be responsible for epilepsy susceptibility. This suggestion has since been directly verified. Ting-Fang and Guido then went on to show that CD39 is widely distributed in neurons throughout the brain, with enrichment in brain post-synaptic density membrane fractions, and localization in proximity to a marker of synaptic vesicles (198). They thus concluded: "These results, together with the observation that P2 purinergic receptors [whose ligand is adenosine] are present throughout the brain, suggest that ecto-apyrase is involved in regulating synaptic transmission mediated by extracellular ATP (198)".

Extensions. The findings and framework that Guido, Sue and Ting-Fang described are now known to underlie many other discovered roles of CD39 family members.

Platelet clotting. Platelets (thrombocytes) are colorless megakaryocyte cell fragments whose aggregation underlies blood clotting (thrombosis). The major function of CD39, now called (E)NTPDase 1, appears to be the inhibition of ADP-induced platelet aggregation when ATP is released from endothelial cells into the circulatory system. In fact, administration of a soluble form of CD39 reduces platelet aggregation. Since clotting is a key factor in heart attacks, strokes and vascular complications of diabetes, efforts to ameliorate such effects are beginning to target CD39.

Neuronal signaling. There continues to be a great deal of current excitement about CD39 and its central roles for the activity of neurons. As but one example among many, CD39 in the brain allows you to sleep. When you are awake, CD39 and CD73 are producing adenosine, which builds up during the day, and when it reaches a critical level, binding to the adenosine receptor on neurons makes you fall asleep. And caffeine acts by binding to, and blocking the function of, that receptor.

Immune function, cancer and inflammation. The activities of T-cells and natural killer cells that attack tumor cells are suppressed by adenosine. Additionally, CD39 levels are often elevated in cancer cells. Suppression of CD39 activity might thus be expected to reduce adenosine levels, thereby improving the body's ability to fight cancer. In addition, high levels of ATP create an environment that favors inflammation while adenosine favors an anti-inflammatory environment. CD39 plays a central role shifting this balance in the latter direction

with implications for e.g., multiple sclerosis and inflammatory bowel disease and during responses to bacterial and viral infections.

Pain. ATP is a ligand for the ligand-gated ion-channel receptors (P2X1-7). Cell lysis during tissue damage and disease stages release a large amount of cytosolic ATP, which could serve as a pain signal. CD39 actively degrades this pain signal. Correspondingly, adenosine receptors, and thus adenosine levels, and consequently CD39, are directly implicated in chronic pain perception.

Vascular calcification. In an aspect of CD39 function unrelated to adenosine, the two phosphates released by the CD39 ATPase conversion of ATP to AMP have been implicated as agents in vascular calcification.

COVID-19. Guido spent the last year of his life under confinement due to the COVID-19 pandemic. During that time, he suspected that there could be a link(s) between CD39 and COVID-19. He was particularly interested in reports that the disease is linked to coagulation disorders (which, by extension, could be due to and/or might be ameliorated by modulation of CD39 via its role in scavenging ADP (above)). He was reading with interest recent work in this area, e.g. by Simon Robson, among others. In addition, former lab member Ted Abraham has published work that links COVID-19, cystic fibrosis, and ATP levels (and thus inevitably CD39) (229). Guido (despite his usual objection to taking any credit) is second author on this paper, which was in press at the time of his death. As Ted notes: "This work travels a full circle from basic science back to direct clinical applications where Guido got his medical start."

Ongoing efforts to develop drug and immuno-therapies which target CD39 activity to address the pathologies in which it is implicated will be greatly assisted by Guido's analysis of CD39 structure and mechanism, and the yeast screen Guido developed for detecting expression of novel activities that result in release of adenosine (below).

ATP transport. ENDTPases act on ATP that has been released into the extracellular space. The best characterized overall pathway involves the fusion of secretory vesicles and granules containing ATP and other nucleotides with the plasma membrane. ATP can also be released by mechanical perturbation (e.g. shear stress) or, in some cell types, is released constitutively. In all of these cases, ATP must be transported through a membrane, either into the Golgi lumen for incorporation into vesicles, or directly through the plasma membrane. Guido was interested in understanding the mechanisms of such ATP transport (discussions in 184, 209).

Guido was interested in the possibility of direct transport through the plasma membrane. Ted Abraham, who showed that ATP release was coupled to the activities of two different ABC family ATPase molecules: MDR1 (also known as ABCB1 and p-glycoprotein) and CFTR, the cystic fibrosis transmembrane conductance regulator (171; 187). CFTR was not a random choice. In the lung, airway defenses are regulated by a complex purinergic (ATP/ADP/AMP/adenosine) signaling network, which includes CFTR, and is located on the epithelial surfaces, where ATP stimulates the clearance of mucin and pathogens. This constellation of features provides the perfect environment for CD39 modulation. Indeed, CD39 has since been directly implicated in both cystic fibrosis and COPD. Guido favored the notion that MDR1 and CFTR might directly export ATP. However, both effects are now known to be indirect, with several other types of channels acting as direct mediators of ATP release.

Guido also investigated the possibility of vesicle-mediated release. Laurie Bankston and Guido (183*) showed that accumulation of ATP within chromaffin granules, specialized vesicles that

store ATP in its role as a neurotransmitter, is the result of vectorial transport, rather than by exchange with intra-vesicular ATP as previously reported, thus supporting the existence of active movement of ATP across a (non-plasma) membrane. Also, Ting-Fang identified a Golgi-localized human UDPase (199).

Finally, in an elegant study that used budding yeast as an experimental system, Guido, Xiaotian Zhong and Rajeev Malhotra provided direct evidence for actively-mediated ATP uptake into the Golgi compartment (218*). It can be noted that such transport has also been documented in mammalian cells, but only some ten years later.

Guido's lab had engaged in studies of budding yeast many years earlier, via the efforts of John Pringle and Gail Willsky (23, 30, 99; below). Now, the search for ATP export systems in yeast began with James Booth. James tracked down a report of an ATP transporter in the vacuolar membrane of yeast, only to find that the original report was based on an incorrect assay (178); however, he noticed and then characterized a vacuolar phosphate uptake activity (194). Next, Rod Boyum went back to basics to demonstrate that there is, indeed, ATP export in yeast, which he also showed to be glucose dependent (186, 190*, 191).

Xiaotian then took up the cause. Following seminal work on regulation of a yeast ENDTPase, described below, Xiaotian and Rajeev Malhotra tackled the ATP transport problem directly (218*). They noted at the time (2003) that: "Extracellular nucleotides signal via a large group of purinergic receptors. Although much is known about these receptors, the mechanism of nucleotide transport out of the cytoplasm is unknown." That is: no non-mitochondrial nucleotide transporter had thus far been identified. To address this deficit, they developed a functional screen for ATP release to the extracellular space and thereby identified Mcd4p, a 919-amino acid membrane protein with 14 putative transmembrane domains. Further analysis revealed that Mcd4p mediates ATP uptake into the Golgi compartment (the lumen), after which that ATP would move through the secretory pathway, ultimately being released extracellularly via vesicular transport.

In addition, both Golgi uptake and the extracellular release of ATP were shown to be regulated by the activity of the vacuolar H^+-ATPase (V-ATPase), which therefore is providing the proton motive force required to drive Mcd4p-mediated transport. This latter finding is especially interesting for several reasons.

(i) "The V-ATPase alters the acidification in yeast intracellular organelles in response to the change in cytoplasmic conditions as affected by the carbon source. Because Mcd4p-mediated ATP uptake in the Golgi is regulated by the activity of V-ATPase, this uptake would also be modulated by the carbon source." Correspondingly, the ATP release observed in this system is glucose-dependent.

(ii) The previous work of Bankston and Guidotti in mammalian cells had also shown that ATP accumulation into chromaffin granules requires the protonmotive force generated by the V-ATPase (183*; above). This raised the possibility that mammalian Mcd4p protein family members might also have a role in ATP uptake in secretory vesicles and granules within neurons and secretory cells. More generally, given that ATP is released extracellularly in many cell types, luminal ATP may be the general source of this ATP in many cases.

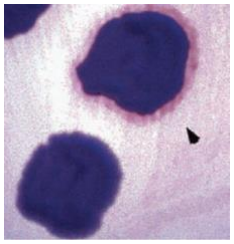
(iii) Xiaotian's earlier work had implicated yeast vacuolar H-ATPase in repressing the activity of CD39 homolog Ynd1, leading to the suggestion that this repression allows Ynd1 to move through the secretory pathway to its localization in the plasma membrane (209; below). Thus, in yeast, this one molecular complex mediates both regulated ATP release into the

extracellular space and ENDTPase-mediated modulation of ATP/ADP/adenosine levels, the two effects that Guido had, early on, considered would be tightly coupled (above).

Broader consideration of these findings also provided several hints that phospholipid metabolism is linked to ATP transport into the lumen of intracellular compartments, the ER as well as the Golgi, about which the authors made several specific suggestions.

Finally, as per typical Guido, the experimental observations were coupled with an interesting big idea: that occurrence of the Mcd4 pathway led to the evolutionary appearance of P2 purinergic receptors: "Extracellular ATP serves as a fast and potent extracellular signaling molecule that triggers a broad range of biological processes. How did this happen? If ATP release is a consequence of the presence of ATP in intracellular compartments, it has existed in the eukaryotic progenitor since the time that intracellular membranes were first used for protein secretion to drive protein folding and maturation. We suppose that eventually the release of ATP was harnessed to provide information regarding the cell and to signal to other cells through the appearance of purinergic receptors. Purinergic responses are already present in *Paramecium*. We suggest that the presence of Mcd4p led to the release of ATP extracellularly and [thus] to the evolution of purinergic receptors. The discovery [presented above] of the involvement of the Mcd4p protein family, the V-ATPase, and membrane trafficking in ATP release has opened a new avenue to study nucleotide movement across cellular membranes and the modulation of purinergic signaling." (218*)

It can also be noted that the functional screen for a gene mediating ATP release (secretion) was highly ingenious. A standard yeast strain was transformed with a yeast multicopy plasmid library. Transformants were then screened by replica plating onto a lawn of a tester strain which could not make its own adenine (Ade-) but could grow if cross-fed by an ATP-releasing transformant. Moreover, the resulting cross-fed Ade- colonies would be pink in color due to accumulation of a pigmented intermediate. Thus, the replicated colony of the desired transformant would be surrounded by a halo of pink colonies from the tester lawn (see arrow in image).



Development of this tester system was tricky because the desired outcome is dependent on a biochemical relay: (i) ATP is hydrolyzed to adenosine by acid phosphatases at the cell surface; (ii) that adenosine is then taken up by the tester strain due to the presence of an introduced mammalian adenosine transporter gene; and finally (iii) the now-intracellular adenosine is converted to adenine in the cytoplasm.

The idea for this screen, as well as for a second novel mutant screen designed for analysis of CD39 transmembrane domain relationships by Saari Paavilainen (228), means that the title of "yeast geneticist" can be added to Guido's more well-known title of "biochemist's biochemist". Notably, also, Guido came up with the main idea for the ATP release screen while looking out over Isola di Capri and the Bay of Naples, on one of his many visits to his beloved Napoli, thus illustrating the principle that time away from the grind can release the mind for creative thinking.

Regulation. Guido was intrigued by yet another important conundrum: "Since NTPDases like CD39 have extremely high ATPase activities, one wonders how they manage to transit through the endoplasmic reticulum and the Golgi on their way to the plasma membrane without hydrolyzing the organellar ATP required for chaperone function and phosphorylation of luminal proteins" (209). Guido's lab uncovered two answers to this question.

By vacuolar H^+-ATPase in yeast. Xiaotian and Raj's breakthrough in understanding ATP transport (above) was preceded by other yeast studies which provided the first evidence for specific, direct regulation of ENTPDase activity (209). This work began with Xiaotian's identification of a new yeast CD39 homolog, Apy1p (205), also found by another group and named Ynd1. Ynd1 has an unusually long cytoplasmic C-terminal tail, which Guido and Xiaotian suspected might play a regulatory role. Xiaotian and Raj therefore used this tail region as bait in a two-hybrid screen. They identified a specific physical interaction between this tail and the vacuolar H+-ATPase Peripheral Subunit Vma13, which is known to mediate the dynamic assembly/disassembly of active H+-ATPase in the membrane in response to variations in growth conditions. Their further studies showed that this interaction represses Ynd1 activity, *per se*, irrespective of H+ATPase activity, and that this regulation occurs in the Golgi lumen. And in addition, as a separate effect, Ynd1p activity is inhibited at low pH, which is the effect of active H+-ATPase. Thus, overall, assembly of H+-ATPase in the membrane results in inhibition of Ynd1 activity. These findings are remarkable in part because they were the first identification of an interacting protein for a member of the ENTPDase family. They were also the first example of regulation of the activity of an ectoenzyme, in the membrane, but by an interaction involving a cytoplasmic domain. They linked their findings to the general conundrum as follows: "...it is not obvious why Ynd1p does not hydrolyze all the ATP in the Golgi. Association with a regulatory subunit like Vma13p together with luminal acidification could be a way to control apyrase activity."(209). The generality and importance of regulation of ENTPDases by an associated partner was subsequently confirmed for CD39 by Wu and Robson.

By N-glycosylation-dependent localization in mammalian cells. CD39 is heavily glycosylated. Xiaotian, Rajeev, Rachel Woodruff and Guido showed that CD39 is not active until it reaches the plasma membrane, and that complete N-glycosylation is required for that surface localization (213). This coupling explains how this important ENTPDase is kept from depleting lumenal ATP levels in the biosynthetic pathways from the ER to the plasma membrane via its very high turnover ATPase activity. Subsequent mutational analysis by James Wu confirmed this conclusion and its general applicability to other, related enzymes (221); and Xiaotian went on the define the specific roles of ER core glycosylation and Golgi terminal glycosylation in achieving functional surface expression (222).

Xiaotian and Rajeev also investigated membrane insertion for yeast Ynd1. This molecule exhibits the longest translocated N-terminus reported. By sequential deletion analysis they showed that translocation of this 500-residue-long hydrophilic domain across the membrane to the outside of the cell requires its C-terminal transmembrane domain and showed that this domain operates as a true signal sequence because it can analogously promote translocation of large extracellular domains of two other membrane proteins, thus revealing a new and unanticipated mechanism for translocation (223*).

Mechanism. Guido's laboratory focused much of its later work on elucidating the mechanism of ecto-ATPase activity in CD39 and its relatives.

Sue-Hwa Lin's recollections of Guido and ecto-ATPases/apyrases (above) also include the following: "A few years [after I left the lab] Guido gave a talk to the students of the "Protein Purification and Characterization" course at Cold Spring Harbor Lab. He described how he identified that CD39 has two transmembrane domains and why such a structure arrangement is important. I remember he was very excited about it." The reason for his excitement is as follows.

This story began because Guido was struck by the fact that CD39 and certain other ENTDPase family members have an unusual structure: a large extracellular domain containing "apyrase conserved regions" (ACRs) comprises the nucleoid binding/hydrolysis region but is flanked by two transmembrane helices, one at each end of the molecule. This topology is commonly observed among channels, transporters, and receptors; however, it is unusual for ectoenzymes, most of which contain a single protein or lipid link to the membrane which serves a passive anchoring function. Why the active site of CD39 would be held down by transmembrane domains on both sides was thus a mystery (202).

In a remarkable series of studies, Guido's laboratory showed that the two transmembrane domains of CD39 and relatives are intimately linked to active site function. They are required to create the enzyme active site conformation required for activity. Furthermore, this effect is achieved by rotation of the two domains relative to one another as directed by mechanical forces within the membrane. Moreover, modulation of these rotations by the membrane milieu can link rapid changes in substrate specificity in dynamic feedback loops which accommodate changing cellular environments.

Much of this work from Guido's lab is summarized in an overview article (224) and a later research paper (225* = *Appendix XI*).

Two terminal transmembrane domains. The idea that the two transmembrane domains of CD39 (and relatives) should be important for the active site function emerged from the fact that these molecules were very sensitive to detergents, whereas soluble ecto-ATPases, with essentially identical active site regions, and ectoenzymes in which membrane association simply serves passively as an anchor, are not. Guido *et al.* intuited that integration in the membrane via the transmembrane domains was not just a passive structural feature but was important for function (202= *Appendix X*). This idea, which was unprecedented, carried the overarching implication that information must somehow flow from the transmembrane domains to the catalytic domain, thereby influencing enzyme activity. It also opened up the obvious possibility that activity of such enzymes could be modulated among different molecules and/or in different conditions by changes in the state of the membrane.

Ting-Fang, Yvonne Oh and Guido provided the first indications to this effect (200*, 202 = *Appendix X*). They showed that mutant CD39 proteins lacking one or both transmembrane domains have only a fraction of the enzymatic activity of the wild-type enzyme and are no longer sensitive to detergents, implying that the detergent effect on activity was mediated by the transmembrane domains. They concluded that activity requires both of the transmembrane domains plus their presence in the membrane.

Functional interplay between transmembrane domains and the ATPase active site. Guido and Alison Grinthal carried out a series of studies which investigated in detail the mechanistic role of the transmembrane domains. The extracellular domains of ENTPDases contain five regions (*A*pyrase *C*onserved *R*egions or ACRs) which specify nucleotide binding and hydrolysis. Alison discovered that a mutation in a critical residue in ACR1, which putatively specified one of the phosphate binding loops, conferred strong effects only in the presence of both transmembrane domains (206). This finding, unexpected at the time, pointed directly to functional interplay between a phosphate binding loop of the catalytic domain and the pair of transmembrane domains - notably suggesting that the enzyme used two different NTP

binding modes depending on whether it was fully rooted in the membrane (224). Further inspection of ACR sequences across various soluble and membrane-bound ENTPDase family members, from this perspective, also identified two residues in ACR1, including one of the analyzed phosphate binding loop residues, whose identities correlate with the presence or absence of transmembrane domains, further implying communication between the two features. (For Alison's personal description of this discovery and Guido's reaction, see her "Comment" below).

This insight had broader implications which, conversely, further supported the original finding (224). ENTPDase family members catalyze hydrolysis both of NTP to NDP and of NDP to NMP. Yet the relative rate constants for these reactions vary significantly, resulting in enzymes that hydrolyze primarily NTPs ("ecto-ATPases"), primarily NDPs, or both NTPs and NDPs ("ecto-apyrases"). The original CD39 (now ENTPDase 1) is in the first category. Guido and Alison noted that, aside from the two unique residues noted above, ACR sequences are highly conserved in all members of the ectoapyrase family and, in many cases are identical between proteins with different activity spectra, suggesting that the differences in their substrate preferences lay elsewhere. On the other hand, the apyrase and ATPase subfamilies exhibit conserved differences in their transmembrane domains, consistent with the possibility that it is the transmembrane domains which determine the different substrate preferences of the different enzymes.

Guido and Alison went on to confirm additional predictions implied by these comparisons.

(i) Comparisons between CD39 (ENTPDase), an ecto-ATPase, and CD39L1 (ENTPDase 2), an apyrase, demonstrated that the two proteins exhibited identical (reduced) activities and substrate preferences when the transmembrane domains were removed or the enzymes solubilized. Thus: "the two extracellular domains have the same basic substrate activities and their respective transmembrane domains confer the distinctive specificities of the native proteins" (216).

(ii) In a particularly striking finding, when the CD39 extracellular domain is combined with the transmembrane domains of CD39-L1, the resulting chimeric protein exhibits normal CD39 activity. "Thus, for CD39, the information that determines substrate specificity appears to be in the extracellular domain and to have a nonspecific requirement for two transmembrane domains for [its] manifestation" (216). In contrast, for CD39-L1, substitution of the transmembrane domains of CD39 produced a novel substrate specificity, different from either parent molecule, thus further demonstrating the importance of interplay between the two features (216).

From these and other findings, Guido and Alison then drew the following conclusion (216): "The present evidence for a nonspecific requirement for two transmembrane domains supports the idea that the extracellular domain itself does not inherently assume the native conformation; rooting both ends in the membrane appears to alter the energetics from what might putatively be considered a relaxed state to a taut state [in which the appropriate biochemical events can occur]. More generally, Guido and Alison suggested that "double rooting in the membrane changes the energetic state of the extracellular domain and is a prerequisite for native properties and thus for the distinction between apyrase and ATPase substrate specificity." The language of this statement corresponds to the formulation of Jacob, Wyman and Changeux's allostery hypothesis where ligand binding triggers a concerted change from taut to relaxed states. In the case of ENTDPase, the soluble state would be relaxed; the two transmembrane domains would create the taut state required for activity, with different manifestations in enzymes with different activities; and ensuing ATP hydrolysis to AMP would restore the relaxed state. Thus, Guido and Alison

were essentially envisioning a new paradigm for the allostery hypothesis in which a pair of transmembrane domains played a central role in creation of the poised enzyme state. This idea brought Guido's research full circle from his early work on allostery in hemoglobin (above) and, as Alison relates: "also brought it full circle in the form of a student showing up in the then Department of Biochemistry and Molecular Biology (see *Appendices VIII and XII*) who was able to make these connections, thanks to being versed in what by then had become a basic tenet of biochemistry".

These two studies also suggested that the key active site difference between ecto-ATPase and ecto-apyrase enzymes lay in different mechanisms by which ADP was hydrolyzed on the way from ATP to AMP. Concomitant work by Chen and Guidotti (211, 214) and other groups revealed more specifically that the transmembrane domains determine whether or not ADP is released from the active site as an intermediate during ATP hydrolysis" (as in the apyrases) or not (as in the ATPases) (216, 224). Absence of ADP release in the case of CD39 also matches and explains its role in reducing clot-inducing ADP levels (discussion in 228).

Dynamic rotation of trans-membrane helices. To further study the roles of the two ENTDPase transmembrane domains, Guido and Allison turned to oxidative crosslinking of cysteines which had been introduced in pairs into the two transmembrane domains (TM1 and TM2) (220). Such crosslinking will occur only between pairs that are oriented toward each other and are within reach of disulfide bond formation. Thus, crosslinking patterns among various pairs of positions were analyzed and correlated with activity and substrate binding in the native membrane environment. This analysis revealed interactions between TM1 and TM2, with especially strong interactions occurring intramolecularly, although intermolecular interactions were also observed. Further, these interactions occurred predominantly near the extracellular side of the membrane and thus proximal to CD39's extracellular domain. In particular, they occurred near putative active site components ACR1 and ACR5, which are located a short distance from TM1 and TM2, respectively. This finding directly validated and explained earlier findings, because the critical phosphate binding loop residues involved in the responses of CD39 and CD39L1 to the presence of two transmembrane segments (above) are located in ACR1.

This study also provided an unexpected and striking discovery: the two helices were found to exhibit a high degree of flexibility with respect to which helix faces comprised the interaction surface, with full disulfide formation detectable between all faces, both within a molecule and between different molecules. That is: the helices exhibit rotational mobility (220). This was, and still is, an unprecedented observation. Notably, also, structural studies by the group of Norbert Strater support this view, documenting domain motions of the type predicted and also revealing an interaction between the extracellular domain and the membrane that would constrain free rotation of the enzyme during nucleotide hydrolysis, as required by the model.

Alison and Guido further showed that: "Analysis of activity after locking the helices in various orientations via disulfide bonds suggests that not only the arrangement but also the ability of the helices to move relative to each other is crucial for enzyme function." Moreover, these dynamic rotational motions are modulated by substrate binding. They inferred that the balance between stability and dynamic flexibility of specific interactions underlies the transmembrane domains' functional relationship with the active site (224).

Modulation by membrane elasticity and biological implications. The above results raised two further general questions: "(i) [Mechanistically], what do the transmembrane domains do

that makes them so important? and (ii) Do they ever change organization and regulate switching between enzymatic states in vivo?" (225*= *Appendix XI*). The answers to these two questions turn out to be related. Alison and Guido demonstrated that bilayer mechanical properties regulate conversion between the two enzymatic functional states of CD39 by modulating transmembrane helix dynamics (225*= *Appendix XI*). They proposed specifically that "a mechanical bilayer property, potentially elasticity, regulates CD39 activity" and, in accord with their earlier work, this regulation is achieved by "altering the balance between stability and flexibility of its transmembrane helices and, in turn, of its active site." This mechanism further implies that static differences or dynamic changes in membrane properties could underlie differences in the activities of different NTDPases, which exhibit diverse cellular localizations and specificities, and/or their activities in different cellular conditions.

The specific findings of this study were as follows (225*= *Appendix XI*): "Alteration of membrane properties by insertion of cone shaped or inverse cone shaped amphiphiles or by cholesterol removal switches CD39 to the same enzymatic state as does removing or solubilizing the transmembrane domains. The same membrane alterations increase the propensity of both transmembrane helices to rotate within the packed structure, resulting in a structure with greater mobility but not an altered primary conformation. Membrane alteration also abolishes the ability of substrate to stabilize the helices in their primary conformation, indicating a loss of coupling between substrate binding and transmembrane helix dynamics. Removal of either transmembrane helix mimics the effect of membrane alteration on the mobility and substrate sensitivity of the remaining helix, suggesting that the ends of the extracellular domain have intrinsic flexibility."

Guido and Alison then placed these findings in broader contexts.

Mechanism. "Changes in elasticity change the energy required for local membrane deformation associated with a protein conformational change and can therefore modify the total energy barrier between different transmembrane domain conformations. If, as proposed above, the balance between stability and mobility is a key feature of the interplay between the transmembrane domains and active site of [ENTDPases], anything that changes bilayer elasticity might change activity by altering this balance. In particular, changes that increase elasticity, such as detergents and cholesterol depletion, might increase mobility by lowering the barriers among different conformations and thus uncouple transmembrane domain and active site dynamics as proposed for truncated and solubilized CD39 (225*= *Appendix XI*)." More generally, differences in the elastic responses of transmembrane segments could contribute to different substrate specificities among different ENTDPase. Guido's knowledge of membrane composition and physical properties also led to contributions to work of Anand Subramaniam and Howard Stone on how glycan networks pattern the phase behavior of lipid membranes (227).

Biological implications. The envisioned mechanically based scenario also provides a direct mechanism for linking ENTDPase activity and substrate preference to underlying cellular conditions, not only in steady state, but in a dynamic, time-dependent manner in response to changing conditions. Specific cases involving mechanical effects are known. "Physiologically, extracellular nucleotide concentrations are directly linked to a number of mechanical and biochemical processes that change the properties of the membrane....ATP release is a primary response to mechanical pressure and shear stress as well as to changes in osmotic pressure; in the latter case the resulting extracellular ATP is required to restore osmotic balance. Inflammatory processes and oxidative stress, both of which rely on signaling by extracellular nucleotides, trigger release of unsaturated fatty acids and lysophosphatidylcholine, both of which can insert in the membrane and change its curvature and elasticity. High cholesterol levels are

associated with blood clotting, which is modulated by extracellular nucleotides. In each of these cases the ability to modulate the hydrolysis rate, substrate specificity, and intermediate ADP release of CD39 according to the mechanical state of the membrane would provide a direct feedback mechanism between cellular conditions that affect the membrane and the extracellular nucleotide signaling processes that respond to them. Thus, the presence of two transmembrane domains might be a key to instantaneously tailoring extracellular nucleotide signaling to the state of the cell." (225*=*Appendix XI*).

Furthermore, *in situ* regulation of ENTDPase activity, e.g. by the cytoplasmic domain in Ynd1p as described above (209), is predicted to involve modulation through effects on the transmembrane domains.

The future. Guido's discoveries of, and studies on, ENTDPase brought to bear his unique combination of imagination, knowledge and rigorous experimentation. The results illustrate what can happen when science is guided only by intellectual curiosity. This work has provided a unique and wonderful system for further exploring the complexities of protein conformational changes, the involvement of membranes and a deeper understanding of forces within membranes, e.g. membrane tension, a problem of fundamental importance and current interest. Perhaps someday this potential will be seized upon anew by someone as curious, insightful and intrepid as Guido. One could add that such efforts would best be carried out in the context of a robust community which, like Guido, embraces the generally unappreciated fact that all molecular processes are subject to mechanical effects at length scales varying from the atomic to the macroscopic subcellular, cellular and inter-cellular levels.

V. Off the beaten path.

Guido was happy to have members of his group work on any problem of their choosing, as long as he deemed it interesting. This led to many investigations which (at least superficially) did not fit neatly into the main experimental flow of the lab, aside their focus on proteins and, in most cases, membrane proteins.

Carbonic anhydrase (Pierre Henkart) **(**17, 26, 27**).** Concomitantly with the laboratory's early work on Band 3, which mediates the exchange of O_2 and CO_2/bicarbonate in hemoglobin, Pierre studied carbonic anhydrase, which catalyzes the inter-conversion of carbon dioxide and water into carbonic acid, protons and bicarbonate ions. This work also provided a happy link between Guido's work and that of John Edsall, for whom carbonic anhydrase was a major research interest, and with whom Guido shared a cramped laboratory and office space and a long scientific friendship. Subsequently, in 1998, Reinhart Reithmeier's lab demonstrated that carbonic anhydrase is physically associated with Band 3 in red cells, forming a membrane transport metabolon where imported substrates are delivered directly to the enzyme that metabolizes the substrate.

Proteolytic artifacts (John Pringle). The early days of membrane protein studies in Guido's lab also saw the arrival of John Pringle. In Matt Meselson's and Nick Gillham's genetics course, John had glimpsed the awesome potential of yeast as a model system for eukaryotic molecular and cell biology, and he was determined to work on it, but no lab in the Boston area was doing so at the time. In accord with his general scientific philosophy, Guido agreed to take John as a student so long as he worked on an interesting protein problem. John's consequent attempts to characterize yeast malate dehydrogenases were eventually supplanted by studies of the proteolytic artifacts that proved to be unexpectedly pervasive in studies of yeast (and other) proteins, as well as during use of the then-new SDS-gel electrophoresis (23, 30, 230).

Recognition of the proteolysis problem and the adoption of methods to counter it (230) spread rapidly and are now so routine that, as John says, "Almost no one now remembers that these measures, and their importance, once needed to be *discovered*. But they did, and it wouldn't have happened when it did without Guido's patience and gentle guidance of a very idiosyncratic and stubborn student."

Steve Clarke adds: "A crucial discovery of John was that when proteins were prepared for SDS gel electrophoresis, the newly unfolded polypeptides were exceptionally sensitive to very small amounts of proteases that were present and that often unfolded more slowly. Thus early studies using SDS gels to define the polypeptide composition of cells, viruses, organelles, membranes and protein complexes were often compromised by proteolytic nicking of their polypeptide components, rendering it impossible to study the protein composition. However, John found that a simple treatment of heating samples for 3 minutes at 100 °C immediately after adding SDS and mercaptoethanol resulted in rapid inactivation of protease activity (23). This treatment is now almost universally used for preparation of samples for SDS gels, but it is unclear whether all scientists understand why it is so important and where the technique came from! It is a tribute to Guido's generosity in sponsoring "off the beaten path" work in his laboratory that this technique immediately allowed for the determination of the intact polypeptide composition of red cell membranes in the lab (33, 36, 52, 56) as well as many later advances."

Yeast Mg^{2+}-ATPase (Gail Willsky). Like John Pringle, Gail Willsky came to Guido's lab wanting to work on yeast. In accord with Guido's dictate (above) she proceeded to isolate and characterize the plasma membrane Mg^{2+}-ATPase, showing that its mechanism was similar to that of the cation transporters for Na+ and Ca^{2+} (99).

Mitochondrial membrane (Steve Clarke) (65, 66, 72). In addition to his studies of detergent effects (above) Steve investigated the major protein components of mitochondrial membranes. These were of interest for two reasons: first, mitochondria being derived from bacteria, these membranes comprise both "inner" and "outer" membrane layers. Second, these organelles are responsible for respiration, which generates most of the ATP in the cell. This process is carried out by the membrane-embedded electron transport apparatus and is driven by an ion (proton) gradient across the membrane. Steve was interested to understand how this gradient is generated. He was excited to follow the lead of others in the lab using SDS gel electrophoresis to determine the protein composition of mitochondria. His excitement of finding a major large molecular weight component in liver mitochondria that he initially thought might be a myosin or spectrin homolog paid off when he found that it was instead an enzyme of the urea cycle that is a major function of this organelle (65).

Nuclear membrane (Bob Jackson) (73, 74). Bob investigated the composition of the nuclear envelope which, at that time, was totally uncharted territory.

Chloride transport (Eugene Rosenthal and Larry Coury). In accord with the fact that Guido never met a membrane ion channel he didn't like, Eugene Rosenthal reconstituted, isolated and identified the Cl- channel from *Torpedo* electroplax, suspected to be an especially rich source of this molecule (174). Then, somewhat later, Larry Coury studied chloride transport in yeast. He confirmed the absence of any such activity in the plasma membrane of that organism and went on to exploit these findings to express and analyze a mammalian chloride transporter (204).

MalK (Howard Shuman). Howie came from Jonathan Beckwith's lab with a geneticist's

background in the study of maltose transport in *E.coli.* In Guido's lab he added a biochemical dimension, identifying and demonstrating that MalK was a peripheral membrane component that directly mediated active import of maltose (115, 118).

ATPase activity of the retina (Scott Thacher). Guido's interest in the brain (above) was tackled directly by Scott Thacher in a project on the retinal rod outer segment. A then-current theory was that Ca^{++} release from disc membranes was an initial response to light. Scott, using retinas from the toad, provided the first demonstration of the existence of an ATPase, but one independent of Ca^{++} (89). He also identified a protein with properties similar to, but distinguishable from, previously described membrane-bound ion pumps (117). Finally, he showed that Mg++-ATP causes rods to undergo a structural change which, however, was not attributable to his previously identified ATPase (123).

Invertebrate oxygen carriers (Lloyd Waxman). In continuation of Guido's work on human hemoglobin, Lloyd investigated the subunit compositions of molecules with analogous functions in invertebrates. He found that annelid hemoglobins and chlorocruorins comprised small polypeptide chains; mollusc hemoglobins and hemocyanins comprise very large chains (>200,000 daltons); and arthropod hemocyanins comprise chains of intermediate size. These studies provided the first glimpse into similarities and differences among these different molecules.

Space polymers (Julie McGeoch). Julie and her husband Malcolm are interested in how life began, which they approach by analysis of polymers in objects from space. Guido shared these interests and supported their investigations into polymer amide (233) and, in work published just at the time of Guido's death, their discovery of a new polymer comprising glycine Fe, Si, Li and O (234).

Summary

The above description makes clear Guido's exceptional gifts as a scientist. In addition, Guido was never overly concerned about whether other people accepted his ideas and conclusions or not. He knew that there is a truth to science that no amount of human manipulation can change. We should all be so firm in our adherence to this principle, even though it may come with some cost.

In the words of his students and colleagues.

Guido's qualities as a scientist are best described by statements from his students and colleagues (reprinted here with their permission; in alphabetical order).

Ted Abraham: "Over the years Guido has always been generous in discussing scientific ideas. He would gently puncture scientific misconceptions. Since the onset of the COVID-19 pandemic, our discussions were by phone or email. I include below initial hypotheses on the role of the cystic fibrosis phenotype, systemic ATP levels and improved survival in the presence of Sars-COV-2 infection. The crucial data arose from the most lethal period of COVID-19 in Italy. In the midst of the somber tables was the surprising, unexpected improved survival of CF patients who had tested COVID-19 positive. The comments in the initial hypothesis manuscript are Guido's. He used a scientific Tricolore notation with

green and white (i.e. white—out for some sections) and red as his detailed recommendations. After a nine month gestation with extensive retrospective clinical testing, the obvious benefits of oral ATP as treatment for elderly patients with COVID-19 emerged. Guido was able to learn of the final paper journal acceptance although, alas, the publication did not occur until May, 2021. Our work travels a full circle from basic science back to direct clinical applications where Guido got his medical and research initiation. Guido's generous spirit and lifelong scientific mentorship and deep and kind friendship will be sorely missed by me and all who knew him."

Alessandro Alessandrini: "Maya Angelou once said, "*I've learned that people will forget what you said, people will forget what you did, but people will never forget how you made them fee*l." While Guido will be remembered by colleagues as an exceptional scientist, those who were fortunate enough to be mentored by him will always know how he made you feel – like you were the only person in the room, encouraging you every step of the way. Whether in the lab or in the classroom, Guido was an empathetic, patient, and tireless teacher. That is what made him unique and I have tried to emulate him in how I run my lab and in being a better mentor and person. I will truly miss him and I will always be thankful for his advice and guiding hand."

Laurie Bankston: "I remember when Guido proposed that I study the ATP transporter in adrenal chromaffin granules. At the time, its existence was not actually confirmed. He showed me an obscure article (I have no idea how he had found it, but he was the sort of scientist who could find such things) in which the contents of chromaffin granules was listed. It showed that not only did these secretory vesicles store as much as 500 mM catecholamines, they also contained about 250 mM ATP. Guido suggested at the time that the reason that ATP was also accumulated was to balance the positive charge of the catecholamine. This led to the idea that the secretory vesicles in the brain which store high concentrations of dopamine or serotonin also store ATP. This means that the need for ATP is likely much higher in neurons and suggests that defects in mitochondria may play a role in neurodegenerative diseases such as Parkinson's. Guido's insights always seemed to have farther reaching implications. One of the things I appreciated most about Guido was his impeccable scientific ethics. He would always say, "It is what it is". I think that really says it all. I am so grateful to have been trained by Guido. He was one of a kind, certainly one of the best."

Bob Bloch: "For me, Guido remains the epitome of what a scientist should be: inquisitive, creative, critical, enthusiastic, funny, generous, humane and, perhaps above all, humble. Everyone who trained with him benefitted from his extraordinary example. His love of science, his love of family, his affection for his trainees and everyone we brought to his attention (my wife, Lydia, and our kids, Daniel and Melissa, are also admirers), his encouragement to all of us to do our very best, wherever we might choose to direct our efforts, are my "true North". His last email to me ended with "Keep on going!". His inspiration is there for all of us who knew and loved him to do exactly that."

James Booth: "Watching Guido teach was a formative experience for me. His boundless energy, clarity, and the way he always kept his eye on the big questions set a standard for teaching that I've tried to live up to ever since. I will also always remember the way the arc of Friday teas would come around to that magical time at the end when Guido would be on the couch sharing his thoughts, with a rapt crowd and the room filled and electrified by his wonderful laugh."

David Brandon: *"* I left Guido's lab with great respect for his devotion to science and education, his brilliance as a lecturer, and his amazing flexibility with people and projects. Guido's devotion to teaching and mentoring was surely a factor that propelled me in that direction. Even though I was outside academia, I mentored many students, from high school through grad school, throughout my career, including a delivery truck driver and a former flight attendant. I've always remembered how open Guido was to backing students and their wide variety of research proposals. Also, I have a deep appreciation for Guido's encouragement of cross-training by fellow grad students and post-docs. These friends and colleagues could resolve almost any technical or conceptual problem, including how to navigate grad school toward a career."

Jeff Brodsky: "Guido's modesty was second only to his many (unsung) insights and critical discoveries....My entire project and four papers arose from an off-hand comment he once made: why would an insulin activated subunit of the sodium pump be in the brain? These gems were frequent."

Gilbert Chin: "How I tried to honor Guido as a scientist is reflected in the last research paper I wrote before moving to [be an editor at] *Science* - both in the series of experiments performed and in the dedication at the very end. I like to think that he would have understood how much I appreciated his having shared his love of membrane proteins."

"Characterization and Solubilization of the FMRFamide Receptor of Squid" Biological Bulletin November 1994 DOI: 10.2307/1542241.....Acknowledgements: G.J.C. would like to dedicate this paper to Professor Guido Guidotti."

Steven Clarke: (Birthday greeting, November, 2008)
"Happy Birthday Guido!

I will be always indebted to you for the pleasure of working with you and taking so many lessons in life from you – as well as for such wonderful support over the years.

I arrived at Harvard as a fresh graduate student interested in membranes. I looked up Joel Kirshbaum, who had also attended Pomona College as an undergraduate, and he hooked me up with Jack Kyte and Bob Bloch. It was immediately clear that there was only one lab for me! I remember first seeing Guido in the BioLabs library, cruising in as a bundle of energy and enthusiasm with some student on his tail trying to keep up with him. The Harvard rotation system had just started, and I was incredibly naive about how one navigated getting into the lab that one wanted to. I was finally assigned Guido's lab for the last rotation of the year due to an error by the Graduate Advisor who mixed me up with another first year student. I was in heaven. (The effects of my refusal to switch back with the other student was a low part of my graduate life; the idea that perhaps we both could have worked with Guido never occurred to us!).

The fractionation of membrane proteins by SDS gel electrophoresis had just become possible, and I was determined to understand the polypeptide composition of mitochondria. I realized that this wasn't a direction of the lab, but I had hoped to take advantage of what others were doing with red cell membranes. I was afraid that Guido wouldn't have room for another student, so when June came around and the students already in his lab were having him sign their forms for the next year of graduate school, I just stood in line behind Lloyd Waxman and the others with my paper. When Guido looked up and saw me, he did hesitate for a second or two, but then smiled and signed it and I was in!

I don't see how there could have been a richer intellectual environment and one that taught me more lessons on how to be a caring and critical scientist. Our group meetings and journal clubs were highlights of the week. I'm sure that as students we caused some heartache for Guido – we certainly didn't come in with the generosity of spirit that Guido always had and sometimes battled each other without much mercy. But all of us were smart enough to know what a jewel we had in Guido as an advisor – he didn't have to say much to bring us back into line.

Guido had such a sense of style – simple and direct. When I'd see him at the lunch truck near the Peabody Museum steps, his "wallet" would be bills folded into a 3 x 5 index card. And when Guido invited me to a meeting with him in Italy, his carry-on luggage was a brown paper grocery bag. While I still lug around a thick wallet, when Cathy and I travel we now often use grocery bags – whether this is just taking one more of Guido's ideas or honoring him I don't know – probably it's both.

Guido's teaching still a source of awe and inspiration to me. My biochemistry lectures at UCLA were basically the best imitations I could do of Guido in Biochem 10, borrowing very heavily on his wonderful presentations. And he was so protective of us as TA's. I once wrote a miserable exam question for the class, something where I gave them the L-form of biotin, and expected them to know that it was this was the form that did not occur in nature and might be an inhibitor rather than a substrate. Of course, I think maybe two kids in the class of two hundred figured this out, and at the next class meeting, Guido was met with a chorus of loud and angry complaints about the question. I sat in the back cringing at the thought of being exposed, but Guido simply told his story of the pessimist and the optimist – the kid in

the candy store crying because he was sure it would all be taken away from him and the kid with a load of horse manure dumped on him so gleeful because "there has to be a pony in here somewhere"! I'm not quite sure how this made them feel better about the exam, but it seemed to work, and I was off the hook!

In the first years I was there, Guido drove a very old Volkswagen beetle that had lost most of its paint in the New England winters. We used to kid him that some juvenile delinquent from Somerville might just vandalize it by painting it one night and he'd have to scrape it all back off.

As a native Californian, I would torment the lab with all of my complaints about the humid summers, wretched winters, and 3-day springs. When I met Cathy, I would tell her all of the horrible conditions I had to endure in Cambridge, including the tenement of a third floor walk up row apartment. So when we visited Boston for the first time together, I made a point to show her how bad I had it. Unfortunately, we got there on a totally gorgeous early summer day and my old neighborhood was nicely gentrified. I began to lose some credibility. I lost the rest of it when we met Guido and I asked him to tell Cathy just how unusual this nice day was – he simply said with a twinkle in his eye, "it's really like this most of the time".

Much of my scientific life has been trying to think like Guido does. When I returned to visit Guido one summer as a postdoc in Dan Koshland's lab at Berkeley, I told him what I was doing trying to figure out how signaling worked in bacterial chemotaxis. In his characteristic broad-based view of science, Guido suggested that it would probably turn out to have a mechanism with post-translational modifications like insulin signaling. It turned out he wasn't far off – protein phosphorylation is central to chemotaxis, but at aspartyl and histidyl residues. But his suggestion got me thinking about protein methylation and when I returned to Berkeley I started experiments asking if the chemotaxis receptors might be modified by methylation – and indeed this was the case. Dan always remembered to share the credit for that discovery with Guido – he would say he might need to send me again when we ran into another problem!

Over the years, I have met a lot of different kinds of scientists. But no one really ever matched Guido for his wisdom, smarts, insight, integrity, humor, generosity, and thoughtfulness. Thank you, boss! Here's to the next quarter century!"

Kurt Drickamer: "Guido was generous to many people in many ways, but to me his most influential and admirable trait was his generosity with ideas. He had so many, and for innumerable young scientists, both in his group and outside it, his sharing of these ideas was critical to getting started in science. I am sure that I was not the only one who continued to draw on this well of ideas long after moving on from his laboratory."

Betty Eipper: "One of my favorite recollections of my time in Guido's laboratory was entering his office, where he sat at a desk surrounded by stacks of the Journal of Biological Chemistry, and listening to him describe, with great enthusiasm, the results of the most recent articles he had read. By his own account, reading the pages between those light blue covers was better than reading the world's best mystery. Another recollection was the amazing confidence he had in the ability of his trainees to address the problems they deemed interesting and important - with a background in physical chemistry and my ability to survive Konrad Bloch's biochemistry course, he actually believed that I could figure out something new and exciting about microtubule protein, when I had no such confidence. Only after I left the magical environment he created in his laboratory, did I have to learn how most laboratories work. Throughout my research career, I tried to convey a similar excitement and joy for research to my students."

Adam Faye: "As an undergraduate at Harvard, I was very fortunate to have had the opportunity to work with Guido. He was passionate, engaging, generous with his time, and a brilliant scientist. The time in his lab was formative as I learned the fundamentals of benchside research and further developed my critical thinking skills. I have fond memories of discussing both science and soccer with Guido and Alison Grinthal during my time in the lab, and could not have asked for a better mentor to introduce me to the world of scientific research. He will be sorely missed!"

Mike Forgac: "The sweep of the work from Guido's lab, and the way he encouraged new members to pursue their own passions, [were extraordinary], and resulted in this truly remarkable diversity of areas of

research into membrane proteins. Where other labs would typically focus on just one of these systems, Guido didn't hesitate to take on this huge range of topic simultaneously."

Michael Gottesman: "Guido had the ability to integrate information from his own work and that of others and come up with unusual, and very often paradigm-shifting hypotheses that explained the data. Very few scientists can do this, and the ones who do have lasting impact. We have lost a very great man."

Alison Grinthal: "When I came, the lab's CD39 research was driven by two puzzling questions. (1) Why does CD39 have two transmembrane domains? And (2) How can a single active site, e.g. CD39's, accommodate so many different substrates, and hydrolyze them all with such high activity? To address the second question, I was looking at mutations in ACR1 (putatively the β-phosphate binding loop and therefore, we thought, possibly involved in ADP hydrolysis) based on comparisons to the hsp70/actin family (which are specific for NTP, and, incidentally, also soluble). I initially examined these mutations in the C-terminal-truncated version of CD39 (lacking one of the transmembrane domains) since it was easier to work with/solubilize and since, at that time, while we knew the transmembrane domains were important for activity, we still mostly thought of the soluble activity as just a mini version of the native activity. We did see some modest effects on the ADPase activity - and as a final control experiment for a "nice little paper", I was supposed to make sure the mutation results held in the full-length membrane-bound version. This "final check" failed gloriously - the mutation effects were wildly different in the presence of two intact TM domains (e.g. specifically demolished ATPase activity and converted the enzyme to an ADPase). This result provided an instant spark of combustion between the two questions. Obvious as it all seems now, it's important to note this spark could easily have died on the spot if it weren't for Guido and the atmosphere he created - with so many questions, such far-reaching perspective and ego-free curiosity, he could take any unexpected result and see its place in the whole fabric. He was away at the time, but thanks to this immensely liberating atmosphere, I spent zero time mourning the demise of the "nice story" and immediately saw the world open up into a much bigger, more fascinating one. As I was dancing at the spectrophotometer, the phone rang - Guido calling from Italy, and sure enough, as soon as I told him the news he immediately saw it too. I can't emphasize enough how lucky I felt to have an advisor where I was free to have my eyes wide open - along with his own direct insights, this gift to his students helped foster so many groundbreaking discoveries in his lab (and beyond)."

Mas Handa: "Guido was always an inspiration not only for his scientific acumen but also his ability to connect empathetically with everyone around him. He was a true role model in how he unwaveringly cultivated my passion for science, in particular through times of personal challenges and trauma. I feel very fortunate and thankful to have had the opportunity to know Guido and learn from him. I will miss him very much."

Renate Hellmiss: "Though I am not a scientist, I would like to write a few words about Guido as the person he was. Guido and Ernie Peralta, my husband, had a very deep connection. Guido was a colleague, mentor, friend, and the father figure Ernie never had. He was simply there for Ernie and unconditionally supported and loved him.

During one of the many Friday teas held by the Guidotti-, Kleckner- and Peralta labs, Guido and Ernie discovered that they probably had already met 30 years prior Ernie's arrival in BMB. As it turned out, Ernie was born in April of 1959, the same time that Guido did his residency in the same hospital.

Both men loved to talk and live science, they enjoyed to play, watch and talk about sports; soccer for Guido and basketball for Ernie.

Guido was a tad eccentric but that simply made him more special. One time, we were at a nice restaurant and Guido pulled out a cardboard case, held together with lab tape, that contained his reading glasses. He picked a very delicious Italian wine from the list. At the end of the meal, Guido removed another

cardboard case, marked with a different color of lab tape, from his pocket and this one contained the money to pay for the meal.

There are many ways to describe Guido. I would like to list a few that others might not have previously mentioned: He had his own dress code; blue jean shirt, brown corduroy pants and a good pair of running shoes. He was a great chef who with just a few ingredients could create delicious meals. He adored his mother --always wore the sweaters she had knitted for him--and simply loved his family. Guido would form his own opinions -- irrespective of whether they were popular -- and he lived by his beliefs and standards.

So, when Ernie was diagnosed with a glioma and was told that he had less than a year to live. Guido was there. He took Ernie to chemo- and radiation treatments. Later on, he took him for regular walks around Fresh Pond, and ultimately visited us daily while Ernie was in home-hospice. Guido was there the day Ernie died.

Guido was a one-of-a-kind Mensch who made this world a better place for many of us."

Dan Jay: "Guido has the best scientific mind that I have encountered. He had an ability to ask the key question or distill the problem down to the key experiment. I view my training with him as a series of my following up on one of those until it stopped working and another visit to Guido's office would set me on the next right course. Guido always conveniently would forget that they were his ideas and helped me to believe in myself."

Lee Josephson:

"Nothing is by Chance". Guido left me with the idea that "nothing is by chance, though it may appear to be and we maybe far from understanding it." The development of drugs from active compounds found in plants is not because the plant, by chance, make a structure that bound to a receptor in a human.

The counter argument to "nothing is by chance" prevalent in the 1970's, was that the plant kingdom synthesizes trillions of unique compounds so by chance a few of these interact with one or more of the thousands of human macromolecules.

Armed with the "not by chance" mantra, Guido supported a quest to find an endogenous cardiac glycoside as an inhibitor of Na-K ATPase and this led to the discovery of vanadate. The discovery of vanadate had no great impact on the development of new and better drugs, but it led to a world-wide effort to find endogenous inhibitors of the Na-K ATPase.

"Knowledge Allows Justice" The natural world is arbitrary and unjust. "Justice" is the ability of some humans, armed with knowledge, to help others unjustly afflicted. Untreated type I diabetes is a cruel injustice. The insulin permits justice.

As one of many in Guido's lab, I did not get to know him that well. But a couple of comments he made led me to believe that "the world is unjust, and only knowledge allows justice" was a factor behind his switch from medical practice to academic science. Put another way, he could not tolerate the common situation MD's encounter: being unable to help their patients."

Minjeong Kim: "One thing I remember especially about Professor Guidotti is that the reason why I treasured him was for everything that he shared with me about his family. Certainly I admired him as a scientist; but more importantly it was his appreciation of life not only in academia but also life around him (his family being the foremost) that made him such an important person in my life.

Whenever class would end, I would quickly pack my backpack and wait for the professor to grab his cane so that we could walk outside of the class together. I looked forward to asking him what his plan for the rest of the day was, and he would talk about all of you -- from going to the fish market to prepare dinner, his childhood memories in Naples, to the differences in cooking octopus that he saw between himself and

his Korean in-law. He was the grandfather on campus that God blessed me with, whose values resonated with my own.

Someone once suggested that Professor Guidotti would have won the Nobel prize had he not put science second in his life after his family. But I think that all of the comments from his students and colleagues attest to the fact that he made a lasting influence in more lives than the Nobel prize could have done. I am grateful to have been blessed by his life."

Nancy Kleckner: "While we were suffering under COVID confinement during the last year of Guido's life, I benefitted from our long discussions about the problems being investigated in my own laboratory. Most notably, in *E.coli*, post-doc Katerina Chatzi had obtained images of cells without walls (L-forms) which pointed to robust interactions of the nucleoid with the inner membrane and post-doc Maria Mukhina had envisioned that certain unique nucleoid dynamics might reflect the presence of a heat source at one end of the cell. Guido, after his usual late-night reading and thinking sessions, announced on two following mornings, a specific molecular candidate for nucleoid/membrane linkages, and a specific polarized heat source. Like so many others in Guido's sphere of influence, I can now hope to follow up on his suggestions, secure in the knowledge that his deep and far-sighted intuitions are usually correct and, at the same time, keeping his legacy alive.

Guido also had a related characteristic behavior. He had a deep, long-range understanding not only of science but of almost everything, including politics, human behavior and life in general. In every venue - from the dinner table to joint Friday afternoon lab "tea" to Department meetings, as well as during scientific discussions, he would let the assembled parties discuss a topic, usually giving whichever knee-jerk reactions came into their minds; but then, finally, Guido would say something brief which crystallized the fundamental essence of the problem and/or the solution. Those who knew this pattern and appreciated Guido's depth of thinking, divested themselves of their own opinions but then waited expectantly to hear "the truth". Even his young grandson Nicholas appreciated how much wisdom Guido would impart even as we consumed the Neapolitan meal that he had just prepared for the family.

These and others of Guido's qualities were directly related to Guido's strong affinity for Napoli, as is explained by Sophia Loren (born Sofia Scicolone) who grew up not far from where Guido grew up, also during the war (she is one year younger). A recent interviewer recently stated: "Loren has always defined herself as a Neapolitan first and an Italian second. I asked what the difference is and she laughs at my ignorance and says it would take too long to explain. 'Naples is so strong, so vital. It's about music and dance. Books and books of history. Read all the books first and then we can discuss.' " https://www.theguardian.com/film/2020/nov/06/sophia-loren-the-body-changes-the-mind-does-not I can hear Guido, a lifelong devotee to music, art and ballet, and a voluminous reader, saying exactly these same words."

Christine Li: "I am one of the lost graduate students that Guido helped. I had transferred from a toxic lab at the medical school to start anew at the Bio Labs. My advisor, Ron Calabrese, was a crackerjack neurophysiologist, but wanted to branch into neuropeptide biochemistry, which I would spearhead. We understood what we wanted to do, but not quite how to do it. I stumbled through some of the techniques but couldn't understand all my results. That was when a Guido-ite, Gilbert Chin, suggested that I consult Guido. I showed up unannounced, but Guido, who knew nothing about me, was immediately gracious, and, of course, extremely helpful, answering my questions and making suggestions as to how to improve my experiments. He guided me throughout my project, and I always considered him an unofficial advisor to my thesis. I am forever grateful at how kindhearted Guido was to take an unknown student under his wing.

Lastly, in these days of social awareness, Guido was always supportive of me as a scientist, interested in helping me with my experiments and getting me to the finish line with no expectation of anything for him. Guido was the consummate "good" guy."

Sue-Hwa Lin: "Guido played a big part in my life, both as an advisor for my academic career and as a fatherly figure who guided me through many difficult times. He was always there to advise me and to cheer me up not only when I was in his lab but also after I left his lab. He is an educator who wants to see his students succeed and he had successfully trained a good group of scientists. He made a difference in many people's lives. We all respect him and appreciate him. He will be always in my heart.

I always communicated with Guido on his birthday. Here are three messages that I sent him on these occasions to tell him stories that I remember from the past.

Nov. 3, 2013
Dear Guido, Happy 80th birthday!!!
When I was a postdoctoral fellow in your lab, I knew I would like to have my own lab someday. Thus, I was quietly practicing "what if I were the boss of this lab". I watched how you handle many things that came up. Many of the things that you said are so true that I talk about them all the times and I use them as my guide. Here is one example of your advice. Research presentation: Make a good story. One thing for sure about research is that it is never straightforward. As I was following the clues from my experiments, I found my research direction was quite far away from what I originally planned. As I was about to go for an interview, Guido asked me what my plan was for my research presentation. I said I would describe my work the way it happened and I asked him whether that would be okay. Instead of answering my question directly, Guido said "If you walked on a street, and the street has a big hole. You accidentally fell into the hole and found there was gold in it. Do you need to tell people that was how you found the gold? Can't you tell them that you knew there was gold in the hole because you researched it? Then, you went there and indeed found the gold."

Nov. 3, 2014.
When I just started as a postdoc in your lab in 1983, I saw an old gentleman who was often absent-minded and thought out loud while walking in our lab area. Later, I was surprised to learn that he is Dr. John Edsall, Guido's mentor and the founder of the Journal of Biological Chemistry. Mentors and mentees in sciences form a unique form of family, in which the members are bound by respect and appreciation for each other and the love of sciences. I saw that relationship between Guido and Dr. Edsall. We invited Dr. Edsall to the Cold Spring Harbor protein purification course in 1997. Dr. Edsall arrived around 5 pm, when I was taking my group down the hill to the dining hall. Dr. Edsall had some difficulty walking up the hill, and Guido carefully helped him walk up the hill to our laboratory area. After staying around the lab area for a short time, Guido again helped him down the hill to the dining hall. There, Guido selected foods for Dr. Edsall and brought the foods to him. During the dinner, Guido asked Dr. Edsall whether he wanted some more foods and got up several times to get foods for Dr. Edsall. They were like father and son. What a wonderful relationship. Thus, I felt that Dr. Edsall was my grandfather, and I was observing my father taking care of my grandfather.

Nov 3, 2019
In Chinese saying, a person's life can be significantly promoted if he/she is helped by a "noble person." You are the noble person in my life. Your kindness and wisdom have significantly changed my life. Every word you said and every opinion you expressed are so true and so logical; they are important guidance for me to go through many tough times in my life. Now, [my son] Albert starts his scientific career and he frequently asks me "what to do" when he encounters issues in the lab. I always give him the "Guidotti" answers and tell him that those "Guidotti" answers have been proven correct by my own experience.

Jia Liu, agreeing with a comment from Bill Tsai: "I will always remember Guido as that vibrant, cheerful, and energetic mentor who modeled kindness and humility to everyone he touched."

Sisto Luciani: "The time I spent in Guido's lab was the most relevant experience in my scientific work. I still remember the meetings in Guido's office to organize my hard research project on the sodium/calcium exchanger from cardiac sarcolemmal vesicles."

Jonathan Lytton: "My enduring memories from my time as a graduate student come from Guido's broad smile and easy laugh as he provided a large tube of his own blood – with the assistance of Michael Ho and a tygon tubing torniquet – for the perennial "band 3 affinity purification" project he had assigned me as a rotation student. Although I failed at this quest, I was nevertheless granted entry into the Guidotti lab kingdom which was a privilege like no other. That laugh and smile were frequently on display as Guido coached us students through the complex and exciting process of learning science and research. Guido provided the support, the encouragement, the confidence to do our best work. His guidance was kind, principled and insightful. He joined us, and we were family, whether doing research at the bench, or socializing on Friday at "tea", or on the soccer field, the beach, or the ski slopes. The integrity and intelligence with which Guido led his lab family had lasting impact."

Rajeev Malhotra: "If I think back over the past 25 years since I started college, the person who has had the greatest impact on my life has been Guido. Guido has been everything to me: friend, teacher, mentor, role model. When I was a freshman and decided to major in Chemistry and Physics, my interests were initially more inclined towards quantum physics. I even spent a year doing research with some of the particle physicists on campus trying to understand neutrino oscillations. It was not until I took Guido's Biochemistry (BS-11) class in the Spring of Sophomore year when I realized that the interplay of biochemistry, molecular biology, genetics, and biomedical research was the field for me. Guido was the best teacher that I've ever had: the way he could distill difficult principles into intuitive concepts, bring in evidence directly from the primary literature, and how he taught us to always think about the next question to ask in the field, the next important experiment to perform. It was because of him that I realized my true passion was in biomedical research and I felt like the luckiest person in the world when Guido agreed to let me join his lab.

Working in Guido's lab has been one of the greatest privileges of my life. To be at the forefront of research, learning about ectoapyrase and extracellular nucleotide regulation and membrane protein biology from Guido and Xiaotian along with yeast chromosome biology from Nancy and the members of her lab; it just could not have gotten any better. No matter how busy Guido was, he always made time for me and his door was always open. Learning how to write manuscripts and grants from Guido has carried me to where I am today. And hearing his stories about his time at Washington University as a medical intern inspired me to follow the path that I have taken. Guido's compassion and caring helped me to survive and thrive during a challenging time in college and medical school. He made the lab and everyone in it feel special. I will always remember our weekly Friday teas and the fun camaraderie at lunch time and the watching of World Cup soccer together. My aspiration has always been to run a lab in the same way that Guido ran his lab. I can only hope to be the type of PI and mentor that he has been for all of us. Guido's legacy will continue to reverberate across many generations of scientists to come. "

Julie E M McGeoch: "Guido was a brilliant philosopher and a scientist you could trust, meaning a difficult research topic could be pursued in his group. I worked in his group from 1987 to the present. He tolerated my idea since 2008 that a simple protein molecule might form in gas phase space. All through COVID lockdown I devised a method using extracted polymer from 4.5billion year old asteroid material to crystalize 3 structures. With the help of Malcolm McGeoch we deduced the 3D structure of a polymer of glycine Fe, Si, Li and O from X-ray data from APS Argonne. We submitted the work (and were invited to do so) to the Physics of Fluids the day before Guido died – it is to be published with acknowledgments to Guido on June 9th 2021 (234). This work could not have happened without the support of Guido and by **support I mean support** - lab space, instrument fees, computers and endless listening to my ideas however outlandish and many tries at grants that did not make it – he had faith it would come through."

Diana McGill: "Guido's curiosity and exuberance for studying proteins is what first drew me to him and his lab – it was infectious. But it was his kindness and humanity that has stuck with me all these years. I was such a novice as a first-year student, so when I went to Guido to ask him if I could join his lab, I was incredibly nervous. The first question he asked me was if I had ever run an SDS gel. When I answered, very embarrassed, that I had not ….and here I still smile when I hear his voice in my head….. Guido

dramatically threw his hands in the air and exclaimed “Whaaat?! You’ve never run and SDS gel?!” At this moment I remember thinking for sure he was not going to allow me to join his lab. But his demeaner shifted immediately, as I now understand he probably read my body language perfectly, and he said so kindly “Well, we’ll have to fix that when you join the lab.” Joining Guido’s lab was the best professional decision I have ever made and he truly changed my life. I hope he knew that decades worth of my own students have benefited immensely from all I learned from him about the art of teaching and mentoring."

Eric Mortensen: "Most scientists don’t have the creativity or the principles of Guido. As an example, Guido believed my data when I showed, way before another more prominent group, that binding of insulin to the insulin receptor fit a negative cooperatively model. But since Guido didn’t play the game most lab leaders did of using their name to push the publication of students' articles, this finding was unfortunately never published. I consider that Guido’s willingness to be driven by data was one more demonstration of his integrity and it has been a driving factor throughout my career. While I have since entered the field of applied biochemistry (clinical trials) my time in Guido’s lab has been one of the most important planks in my platform for clinical development."

Yvonne Ou: Guido was my first scientific mentor, and like many, I was drawn to his lab after experiencing his epically energetic and charismatic lectures in Biochemistry 11, and I ultimately decided to concentrate in Biochemical Sciences because of Guido. How lucky I am to have joined his lab! When I think about how many students I meet now who are turned off by laboratory research due to an unfulfilling experience, I am ever so grateful that I had the fortune to be a small part of the Guidotti lab and legacy. Guido was remarkable in his incisive commentary but generosity towards his students; I now wish I could've returned to his lab at a more mature stage because I'm sure I missed out on recognizing many nuggets of wisdom as an undergraduate. Guido taught me how to think scientifically, logically, and created a lab environment where doing experiments was purposeful and exciting. Learning molecular biology and protein biochemistry with Ting and Guido, as well as contributing to experiments exploring the role of CD39's transmembrane domains, were absolute highlights of my college career. Even more importantly, Guido set me on a path towards becoming a physician-scientist. I truly believe that had it not been for Guido's mentorship and my experience in his lab, I likely would have veered off -- not because Guido dictated that a life dedicated to scientific inquiry was the right path for me, but because he led by example and made such a life seem completely fulfilling, impactful, and joyful.

Sari Paavilainen: I feel myself very lucky and privileged that I have been a post doc in Guido’s lab and a member of his research team. I remember Guido as a nice and enthusiastic person, scientist and a mentor. The time in his lab was very inspiring and I learned new perspectives and insights in the research field of membrane proteins.

John Pringle: "I was Guido's second student and a real mentoring challenge. Although motivated, hard-working (mostly), and not drawn to the wilder sides of 1960s life, I was young and still trying to sort myself out as a person, and I had a modest chemistry background and almost no research experience. Nonetheless, I had idiosyncratic ideas about what I wanted to study and the stubbornness of a mule about pursuing these ideas in my own way. For nearly four years, I had little progress to show, and most advisors would have kicked me out or tried to force me to change projects (which, given my personality, probably would have had the same effect). But Guido stayed calm and supportive, nudged only gently (although I did get – and deserve – the occasional "Enough of this bullshit!" at lab meetings), and answered questions only when asked. The story had a happy ending, as a late rush of discoveries led to a decent thesis, an enduring effect on the methods of protein chemistry, and the launch of a successful subsequent career. But I feel now, as I felt when I finished in 1970, that this was by no means foreordained, and that without Guido's deft mentorship I might well have left science altogether, and I really don't know what would have become of me. So my gratitude is boundless, and I will continue to cherish Guido's memory and to try to follow his shining example of curiosity, integrity, and a focus on the science rather than on who gets credit for it."

Anjana Rao: "Guido was the most remarkable person I have ever met. His quantitative approach to science, his ability to cut through the convoluted arguments in papers to arrive at a sensible and rational hypothesis or conclusion, his integrity in all things, and his disinterest in self-promotion were notable and are still encountered together only rarely. We were all fortunate to have been trained by him and to have known him personally."

Reinhart Reithmeier: "When I was looking for a post-doc position in 1976, a mentor who was a post-doc with Konrad Bloch recommended Guido Guidotti as a leader in the field of membrane proteins. I went to the library (pre-Google days!), but all I could find was a 1972 review in Annual Reviews in Biochemistry and some papers on hemoglobin. I learned from my mentor that Guido didn't put his name on papers unless he did some experimental work. He told me to look in any issue of the JBC (Guido's favourite journal) for papers on membrane proteins from Harvard and you will find his name in the acknowledgements - and there they were! I wrote a letter and Guido replied that I could work on any number of red cell membrane proteins. I picked Band 3, an abundant membrane protein with no known function, that ended up being my life's work."

Marilyn Resh: "Guido was a "Prince": A one of a kind mentor who truly cared about his students and Postdocs and always took the time to make sure we understood what we were doing in the lab and why. Guido's favorite phrase when one of us got a great lab result: "Excitement beyond belief", still resonates with me. He leaves behind a legacy of deeply devoted trainees who will continue to carry the torch of science."

Simon Robson: "Guido was renowned as the Biochemist's Biochemist and in my view was the most perfect gentleman and an understated, modest genius; in the truest sense of both words. After our interactions and parallel discoveries as to the nature and biology of the dominant ectonucleotidase CD39 in the mid-1990's, he and I organized the Third International Workshop on Ecto-ATPases and Related Ectonucleotidases, at the Marine Biological Laboratory, Woods Hole, MA, in September, 2002. This very successful meeting rested on his inspiration and it brought together all the other scientists involved in this rapidly growing area to enthusiastically develop this research in a collaborative manner. Much has been achieved over the past two decades with the use of CD39 not only as an anti-platelet agent but more importantly as a checkpoint inhibitor target in cancer - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5338647/. His pioneering discoveries have culminated in these innovative therapies being tested clinically as cancer drugs at the time of his untimely death. This is a wonderful legacy and it is one we are all proud of."

Howie Shuman: "I was finishing grad school and looking for a postdoc. Guido was giving a seminar at HMS about membrane proteins; I think my PhD adviser Jon Beckwith invited him due to the lab's obsession with secretion and signal sequences. Although I definitely had the whole genetics thing down pretty well, I recognized that I knew nothing about membrane protein biochemistry.

I remember becoming completely enraptured as Guido explained basic principles and overarching questions of membrane protein structure. Basically it was a transformative moment; the intellectual equivalent of falling in love. I made an appointment to visit Guido and I clearly remember arriving at the Biolabs and finding him washing glassware at the lab sink outside his office. Up until that point, my experience of Harvard professors was that they existed in a much more rarified atmosphere. When we went into his office to chat and he popped onto his wooden stool at the steelcase desk, I felt that I had arrived at heaven on earth.

Thankfully things worked out well. The time in the Guidotti Lab changed my whole outlook on what problems to work on and how to approach them. Hearing about the Na/K ATPase forced me to think about mechanistic models for the maltose transporter, and the silly cartoon that I drew in the Biolabs' library for a JBC paper, is not all that generally different than the detailed atomic models published thirty years later.

As far as I can tell, the ratio of Guido's accomplishments to his modesty is so far off scale, no one has or will likely ever come close. It was truly a privilege to know him."

Scott Thacher: "As a biophysics student, I was fascinated by photoreception. I proposed to Guido that I look for a calcium pump in the disc membranes of rod outer segments. (I think it was my suggestion—time has dimmed my memory. The important thing is that I wanted to work with Guido.) We didn't find one, and I characterized some other ATPase activities. The studies weren't in the mainstream of photoreceptor signaling so haven't been followed up. The only reason to be embarrassed by this is that I wasn't wise enough at the time to work with someone else in his lab on a project where some foundational work had already been completed. But I'm delighted to say that the training in self-reliance has been very useful in the long run. There's no question that Guido was not only willing to give me this latitude but was supportive as well."

Bill Tsai: "Guido, your personal immigrant story has always been inspiring to me, likely because I am an immigrant myself. I'd always imagine the fear and excitement in your heart as you landed in Decatur, Illinois, starting your new life in Millikan University as a foreign exchange student in 1950.

Well, since then, it seems to me that you have really lived out a wonderful - and deeply meaningful - life in the US, both in establishing your scientific legacy as a professor at Harvard and having a close family with Nancy, Guido Jr., and Nicholas. Some might argue that you truly lived out an immigrant's dream in the US.

My favorite memory is you recounting the days when you played soccer with Guido Jr. and his friends in your neighborhood, and how you fed all of them homemade pasta, and how this reminded you of your childhood in Naples, Italy.

You once told me the story of being baptized as an infant in "El Duomo" of Florence, Italy – this was in response to my own visit to this celebrated church with Josephine (my wife). I know you to be an intensely private person, so I hold this vignette to be very special.

Although I'd admit that it has been challenging to watch you slowly age in the past few years, my memory of you will be someone who is young, full of life, energy, and possibilities. So I guess somewhere between baby Guido being baptized in the El Duomo and the 17-18 year old Guido coming to the US will always be how I will remember my mentor."

Gonul Velicelebi: "The years I spent as a postdoc in Guido's lab (1978-83) were the most formative years of my career. Guido's persona and approach to science profoundly influenced how I matured as a scientist and even as a person. Guido's lab was a very stimulating environment for postdoc training, with a diverse group of highly motivated young scientists working on a diverse set of innovative projects. He was constantly training us, sometimes in subtle ways. We had two lunchtime seminars every week: someone from within the group on Mondays and an invited speaker on Wednesdays. I would listen to these seminars with great interest, but more often, I was eager to hear Guido's questions - this was my glimpse into his thought process, always insightful, incisive, questioning dogma, analyzing and synthesizing data in real-time. Guido made science fun and personal. After 40 years, I still remember vividly his depiction of cellular proteins in almost cinematic terms, his undying admiration for the hemoglobin molecule and its mechanism, his amazing ability to reduce complicated concepts to first principles, and many more. Finally, I was deeply influenced by Guido's larger-than-life personality and whole-hearted, reductionist approach to life and events. Guido found common ground with everyone and connected with each of us beyond our research projects – with me, that was a shared a passion for soccer and the shared experience of coming to the US as AFS high school exchange students, albeit 18 years apart. Guido's friendship enriched my life. I will be grateful for his legacy and cherish his memory until the end of time."

Chung Wang: "My recollection of Guido: Guido not only is an outstanding biochemist, but also is a great mentor. I was trained as a cell biologist before joining Guido's laboratory. I actually learn most of the fundamentals of biochemistry of membrane proteins through occasional conversations with Guido. As I recalled, while those discussions might be brief, they were precise and truly informative. After going back to Taiwan, I used Guido's way (i.e., allowing people to run their own show) to manage my laboratory. Interestingly, the outcome is that a number of my former students become good friends of mine, which gives me real satisfaction."

Ting-Fang Wang: Guido was a real educator. He was not only an outstanding mentor of science and research in general but also a moral model for all scientists. I really miss him.

Gail Willsky: "My experience in the Guidotti lab scientifically guided my professional career and also how I ran my lab.

It has been noted that Guido allowed those working in his lab to pursue interests not directly related to the lab's main projects. Yet Guido's current research seminars made it seem that he had purposefully set out to find people studying these outlying areas. Guido's thinking outside current dogma helped frame my research in the lab. In 1976 the question I was pursuing was whether the proton ATPase in the yeast plasma membrane was an F (mitochondrial) or P (plasma membrane ion pump) type ATPase. The current dogma at the time was to purify membrane proteins, which proved difficult, and then study them. The F ATPase did not have a stable phosphoprotein intermediate, while the P type ATPase did. Our strategy, which successfully identified the proton ATPase as a P type ATPase, was to use only a partially purified enzyme prep and look for the phosphoprotein intermediate. F-ATPase contamination would not matter.

It was readily apparent in the Guidotti lab that collegiality and nurturing even the non-research goals of the people in the lab, was more productive than promoting intra-lab competition. Guido was supportive of my longtime interest in teaching undergraduates. While at Harvard I was tutor in Biochemical sciences and a TA under Konrad Bloch in Biochemistry 10, as I did not want my first exposure to teaching undergrads to be as an Assistant Professor. Guido promoted a very collegial non-competitive attitude, not always found in high profile labs, that enabled me to spend four happy productive years there. While other faculty used extra funds for being Dept. Chair for research/administrative projects, Guido took us all to Killington VT for a day of skiing with overnight lodging. Bob Farley and I were both looking for jobs at the same time studying plasma membrane ion pumps and would interview at the same place sometimes. We would joke about giving the audience a two-part plasma membrane ion pump course and have remained close friends to this day."

Xiaotian Zhong: "Besides his profound yet modest scientific insights, Guido gave scientific luxury to his lab members in pursuing projects off his own research agenda and made them more valuable by adding his own scientific perceptions/merits. During my postdoc research in Guido's lab between 1998-2002, my main project was the investigation and identification of genes involved in extracellular ATP release from yeast cells, but I also wanted to study a yeast CD39 homolog uncharacterized at the time to be my "safety project". Guido didn't discourage me on this side project. Instead he pointed out to me that this homolog protein had no N-terminal signal sequence and might have an unusual membrane topology. Indeed, my later research confirmed his prediction (205) and also identified a novel carboxyl terminal signal sequence that could translocate a large hydrophilic domain across membrane (223). Recent research in this field has reported a new set of membrane protein complexes involved in such type of membrane insertion and translocation. Guido's genius and insights also helped me and Raj [Malhotra] to design an elegant screening strategy (218*) for cloning genes involved in ATP release from yeast cells. We uncovered novel mechanistic involvements of secretary trafficking (from Golgi to plasma membranes) and proton-gradient in Golgi (set up by V-H^+-ATPase) in the constitutive ATP release from yeast cells, which could also be reflected in mammalian cells. The scientific training and critical thinking I obtained from Guido's lab are tremendous and have benefited me greatly in my latter application research at Pfizer. Should I be given an opportunity in conducting academic research again in the future, I would pick up the unfinished task of cloning ATP transport genes Guido offered me generously 20 years

ago. Hopefully new research could elucidate the molecular mechanisms of the processes for which Guido laid groundbreaking work for the scientific community".

Appendix I

Family Obituary

Guido and his grandson Nicholas

2006

Guido Guidotti
Pioneer in Membrane Protein Biochemistry

Guido Guidotti, Higgins Professor of Biochemistry at Harvard University, passed away on April 5, 2021, from prostate cancer. Guido trained as a physician and then devoted 50+ years of curiosity-driven pioneering scientific research to understanding how proteins embedded in biological membranes perform their physiological functions in transport and signal processing. He was a giant in this field. He was also renowned as an inspiring teacher and as a gentle and truly remarkable human being whose smile could light up a room.

Guido was born in Firenze, Italy in 1933 and grew up in Siena and then in his beloved Napoli. He came to the United States at the end of World War II for a year, as an American Field Service student in Decatur, Illinois. His host family then supported his return, originally to pursue a medical career. He completed pre-medical studies at Millikin University in Decatur, obtained an M.D. from Washington University School of Medicine in St. Louis and was an intern and resident in internal medicine at Barnes Hospital. He then obtained a PhD from Rockefeller University and never looked back. Guido came to Harvard University in 1963, remaining there until his death.

The trajectory of Guido's discoveries is the wandering, yet mysteriously coherent, path of a scientist, and a person, who was not afraid of anything. After early seminal studies on hemoglobin, whose sequence and biochemical properties he famously determined using his own blood, Guido initiated his membrane protein research. At that time, almost nothing was known. A colleague relates that: "I saw Guido give a plenary lecture at a meeting. He started by drawing two parallel lines on the chalkboard, [corresponding to the two sides of the membrane], and wrote ‘inside’ to the far left and ‘outside’ to the far right, [denoting the inside and outside of the cell]. He commented that this summarized, to his knowledge, the state of understanding of how membrane proteins were structured in the membrane and how they functioned." Guido's ensuing research filled in his chalkboard drawing. By 1976, Guido could write, largely on the basis of work from his own laboratory, that: "Solutes are carried across eukaryotic plasma membranes by oligomeric glycoproteins which span the cell membrane and conduct transport by undergoing conformational changes". He identified the structures and topologies of the protein responsible for the red blood cell ion transport that enables removal of carbon dioxide from tissues into the lungs, of the enzyme that maintains the electrical properties of neurons by sodium and potassium ion transport, and many others. He also carried out pioneering studies into hormone regulation of membrane protein activity. Finally, Guido's intuition over decades of research culminated in his discovery that a protein called CD39 sits in the cell membrane and hydrolyzes extracellular adenosine triphosphate (ATP), as a so-called "ecto-ATPase", thereby ensuring that this extracellular ATP is present at an appropriate concentration. When CD39 activity is aberrant or missing, neurons in the brain do not recover after they fire; blood does not know when to clot (and when not); inflammatory responses in cancer, infection and disease are compromised; veins and arteries calcify; and pain responses are perturbed. Guido's discovery lit up the research community, and his findings in this area set the stage for currently exploding therapeutic efforts which target CD39 as a way to ameliorate the above pathologies. Guido's later research also revealed a remarkable mechanism in which CD39 activity is governed by mechanical effects within the membrane. Finally, both CD39 and ATP have been implicated in COVID-19 infection, in part by work to which Guido recently contributed.

Guido was not only a world-renowned scientist but was off-scale as both a laboratory mentor and a teacher. Many of the thousands of undergraduates to whom he taught biochemistry went on to be doctors. He could never go into a hospital setting in Boston without someone saying "I took your biochemistry course". In the last year of his life, he taught three popular courses, in biochemistry, in membrane proteins, and a freshman seminar entitled "What is Life".

Guido was known by everyone with whom he came in contact as a very special person. Above all, he was kind, generous, and enabling of everyone in his sphere, beginning with the members of his research group. Additionally, scientific fame and credit were never the point. Most famously, Guido would not put his name on his students' or post-doctoral fellows' publications unless he had contributed to the experiments with his own hands, until ultimately forced to do so by funding organizations. He was also an accomplished soccer player and bicyclist and had extensive knowledge of, and interest in, art, music and books. He was unusually insightful, wise and foresighted, not only in his scientific work but in life in general.

Guido's spirit and influence live on in the beloved members of his family, who always came first in his life: his brother Mario and Mario's wife Ludi Borello; their daughter Alice and her two children Gaia and Fabio; Guido's son Guido Jr., his wife Anna Yoo and their son Nicholas Yoo Guidotti, who was the sunshine of Guido's later life; and Nancy Kleckner, his soul mate, wife and Harvard colleague for more than 40 years. Guido also remains an important presence in the lives and research of the many trainees and visiting scientists who passed through his laboratory. The community of Guido will always cherish his spirit and his love, which will help in the difficult times ahead when everyone he touched must somehow now manage without him.

A detailed summary of Guido's research and documentation of other aspects of his life, plus comments from many of his students and colleagues, can be found at https://nrs.harvard.edu/URN-3:HUL.INSTREPOS:37368811. Donations in Guido's memory can be sent to "Death with Dignity" (https://deathwithdignity.org/).

Appendix II

Guido's early life as told by his brother Mario Guidotti

Guido Guidotti: Early Life.
by Mario Guidotti (brother)

Guido Guidotti was born in Florence (Italy) on 3 November 1933 to a family whose members were the mother, Jiun Alice Casano, aka Mimi, the father Mario, a Captain of the "Bersaglieri", the Italian elite infantry troops, and a brother born the previous year on 29 April.

The Guidotti family, originally from Lucca, Tuscany, had moved to Naples more than a generation before and was intrinsically neapolitan. Not so the mother's Casano family, whose father Admiral Salvatore Casano was sicilian and whose mother Jiun Leland, was a Bostonian (a proper one); they had met in Boston at a dinner party for the Italian fleet on visit there and married shortly after, following an evidently passionate correspondence; after having been stationed in various Italian ports, when the Admiral retired from the Navy, they settled in Naples in the 1920s.

The Guidottis were in Florence because the Captain had been assigned to the Istituto Geografico Militare (Military Geographical Institute) which had the task of reconnaitring from the air parts of the country and of drawing the relative maps; they lived for two years in Florence until 1935 when the Captain was sent to Somalia, then an Italian african colony. The family did not follow him there but returned to Naples to be near Mimi's family; they rented an apartment with garden at Pizzofalcone where they stayed until 1938. Of this period Guido remembered the Admiral's visits on Saturdays for lunch after his work: he always came with a packet of delightful neapolitan cakes.

After leaving the Navy, the Admiral had found a job at the American consulate in Naples, thanks to his good English and the relations the family had with the anglo-american colony in Naples. In this period the Casanos who in Naples had always lived in Posillipo, a residential area interspersed with small villages of fisherman and peasants, found a beautiful apartment in Villa Maisto, an estate about half a mile off the main road down viale Costa; it comprised several acres of farmland that sloped downwards to the sea; there an extent of tufaceous rock (one of the best, if not the best in Posillipo) provided excellent bathing conditions and also had a spring of mineral water with relative pumping apparatus.

In 1938 Captain Guidotti was back from Africa and was posted in Siena; he rented an apartment there in Via Sallustio Bandini 2 near the centre of the town. The family occupied the apartment in early autumn in time for the school. Mario entered the 2nd class of the primary school, while Guido studied with a teacher at home for the exam of admission to the 2nd class, because you could not start school before you were 6 years old, but once you were 6 you could be admitted to a class above the 1st after taking an exam. Guido entered the 2nd class of primary school in September 1939. The state school was about a half hour walk from the Guidotti's home; the brothers usually went there and back accompanied by their father's orderly, but not always, as Siena was a small town, then with no traffic. The two years of schooling in Siena gave good results. The boys during their stay in Siena picked up a sienise accent which in Italy has more or less the same prestige of Oxford English; alas, it was soon lost when they returned to their neapolitan milieu.

Life in Siena was very pleasant; there was a large park, the “Lizza”, where the knights of the time had their tournaments, on the ramparts of the medieval fortress, the “Fortezza”: Guido rode his bicycle and played with friends, some (the Principes) from Naples. There were also very nice walks to take in the beautiful countryside and visits to museums, country houses, inns and wine cellars of the famous Chianti wine. In the summer of 1939 before going to Naples for the summer holidays at their grandparents' the brothers attended the”Palio”which is a horse race around Piazza del Campo ,the main square in Siena, in the shape of a shell. Some, “contrade”, (districts Siena is divided in), chosen by lot, (there are too many of them for all to participate), take part in the race around the piazza to win the “Palio”, a picture of the Madonna embroidere on cloth, that is given to the winner to keep and honour until the next race (there are two per year normally). Both the horses and the jockeys of the contrade are drawn by lot. It is difficult to imagine the attachment and the passion of the Sienese for the event and it is very interesting to participate in the preliminaries and the trials prior to the race, lasting a week or more. The brothers went there accompanied by their dear orderly Elio Lamberti (they kept in touch with him also after the war).

In May 1940 Major Guidotti (he had been promoted Major on 1 January 1940) was once more sent to Somalia. On 10 June of the same year Italy entered in the 2nd World War on the side of the Nazis. Mimi decided to leave Siena and to go to Naples to stay with her parents in Villa Maisto. Although recovering from chicken-pox and measles the boys had passed on to her, she put all the furniture of their home in a store house in Siena and moved to Naples. The apartment there was large so there were no problems in settling down. Guido now in the 3rd class of the primary school attended together with his brother the school “Domenico Cimarosa”, in Posillipo not far from Villa Maisto.

Soon the effects of the war started to be felt: the food for which they had ration cards was bad and not sufficient; airplanes, few in number and said to be british, came at night to bomb the city; all the antiaircraft guns and searchlights placed on the hills above Naples entered into action. The next day children went around collecting the splinters of the burst shells. During the raids the family got up, dressed and went to a shelter under the house: the grotto where the peasants working the land of the villa lived. The grownups carried a small case with precious documents and some valuables. At first Grandma did not go to the shelter because she thought it useless; but soon she had to go too because rumours spread that she, an American, stayed at home to signal to the Allies. Back home after the raid Grandma provided biscuits and drinks; back to bed all went hoping the siren would not send them running down to the shelter once more.

Guido amused himself with the bicycle he had brought from Siena, playing football on the road to Villa Maisto and also took part in stone fights with bands of neighboring boys, probably stimulated by the widespread wartime atmosphere. In April 1941 the Military informed Mimi that Major Guidotti had been taken prisoner by the British and was to be sent to a prisoner of war camp in India: soon letters, often censored by the British, began arriving from him through the Red Cross.

In the middle of 1942 one lone airplane at night bombed the boys' school destroying most of it and rendering it unusable: notwithstanding the distance of about one mile between the school and Villa Maisto the blast was impressive. The boys then had to go to another school at Marechiaro, distant from home more than 30 minutes on foot and have classes in the afternoon. In the meantime the air-raids intensified and changed timing: they took place during daytime often at dusk and were conducted by a great number of Liberators in bombing formation, which flew at great height, apparently invulnerable; they produced great damage to the city and its infrastucture, mainly the port and the industrial area.

The bombing grew always more dangerous, so it was decided to shut all the schools at the beginning of 1943: once the schools were closed, Mammy decided to move with the boys to the house of our friends Principe, who lived nearer the centre of the city but also close to a very good

air-raid shelter; the grandparents remained at their home and hid for a while two important members of the Resistance against Nazifascism.

On 8 September 1943 the Italian government (Mussolini had been removed on 25 July) signed an armistice with the Allies, whose armed forces were slowly moving north through southern Italy and were then not far from Naples. In Naples the Germans, together with the fascists still faithful to Mussolini, took control of the situation and ordered the people living in Posillipo within 300m from the sea to leave their homes. Mimi, the boys and the grandparents were given hospitality in an apartment of the Principes at Arco Mirelli: all five in a single room. It was not possible to leave the apartment because all around the city guerilla warfare was going on against the Nazis carried out by the young Italians who did not want to be taken to Germany to work or to fight for them, as the Germans had ordered.

The warfare started soon after the 8 September and reached its highest point in the last four days (27-30 Sept) before the arrival of the Allied troops on 1 October 1943. Meanwhile as regards the refugees at Arco Mirell, on 30 September the Admiral, afraid that his house would be ransacked, decided to return to villa Maisto, which he did in the late evening: he met some Germans on the way but they did not bother him. The next morning Grandma and Mario left for Villa Maisto: the sacking had already begun: two hotels passed on the way home were being emptied of everything they contained including WCs. In the evening the Admiral and Mario went for a walk in Posillipo and met the first of our liberators, two Canadians in a jeep. They greeted them warmly: the incubus of the German occupation was over and also that of the Allied bombardments.

From then on, although the situation at home was still very difficult: no water, no light, no telephone, luckily there was some coal for cooking, things started to improve. G radually, the bread became edible and the food more abundant. Guido and his brother both spoke good English, which their mother and Grandmother had taught them as children, so they made friends with many of the allied troops and received sweets and comics from them and sometimes were invited to lunch at their canteens. USIS (the United States Information Service) opened a library in Naples which the brothers frequented with pleasure.

Guido had now been for a whole year without schooling; he had been promoted to the 1st class of secondary school, without having to pass the specific exam that had been in force up to then to limit access to higher education. The schools were starting to reopen at the end of 1943, but there was no transportation to the centre of the city where the schools were located. Luckily the catholic priests Barnabiti, resident in Posillipo, decided to start a school there; it was within walking distance from home, so it was decided that with the new year Guido was to go to school, because he had to start the studying Latin. Mario instead was to stay home to help the family, fetching the water, going to the market, etc. because in the meantime Mimi had found a job with the Allied forces as translator. Guido started school at the beginning of 1944. A few months later, the waterworks having been partially repaired and electric light restored, Mario too returned to school.

Guido did very well at school and had a number of other interests: sports, he played in the school football (soccer) team; as a good catholic he served mass at the local parish of Bellavista; he was a Boy Scout and went often camping near Naples but also in other parts of the country; he participated in several mountain climbing competitions, winning some. He also had many friends both at school and at the Scouts and enjoyed going to the movies and to the theatre, especially the Opera. Grandma took the family to the neapolitan opera house San Carlo to see Carmen,and all of Wagner whom she loved and many other operas.

In January1946, Mario, the father, who had in the meantime been promoted lieutenant-colonel, returned home from India. He was not in very good shape as life had been hard in the prisoner of

war camps. He was naturally accommodated in the apartment at villa Maisto. The boys had to renew their acquaintance with him after such a long absence. The Colonel decided to leave the Army because he was fed up with it; and he tried various business activities without success. Meanwhile he had convinced Mammy to give up her job with the Allied administration; she was sorry to return to being only a housewife.

One day Mario and Guido were returning from school and they stopped to help some gentlemen who were trying to open a gate for their car. Speaking english with them, they found out that they were old friends of the family, the Frothinghams, who were in Naples to bring back into service the Mobil Oil refinery. They came to visit the family, and in due time offered the Colonel a job at the Refinery as responsible for public relations, which the Colonel was pleased to accept and held for the rest of his working life.

Time was passing: Guido finished the 5 years of secondary school and in 1948 started the 3 years of classical (with Latin and Greek) "liceo", that ended with a very tough examination, the bugbear of all Italian students; passing the final exam gave access to the University. In 1949 the two brothers received an offer of a scholarship from the American Field Service for a year at an American High School. Mario could not accept the offer because in 1950 after his final liceo exam he would have been more than 18 years old and could not have expatriated on account of his compulsory military service. Guido who was more than a year younger did not have that problem; but he did not want to lose a year postponing his final liceo exam, so he decided to take the exam in 1950 together with Mario before going to the US. He started studying very hard, with a professor for the classical subjects and with his brother Mario for the scientific ones, to complete the 2nd liceo and to prepare for the final exam. He made it; not only, but he was also one of the best that passed the exam.

In the summer of 1950, he sailed to the US by ship and went to a high school in Decatur, Illinois where he lived with the Williams family. He had a wonderful time that year in the US and also graduated from the Decatur High School. He came back to Naples again by ship in the summer of 1951 with a very important problem to solve. The Williams and other families in Decatur asked Guido to return to the US and to go to the University there; until graduation they would help him with expenses exceeding his scholarships. Guido, who until 3 November was not 18 years old, and thus could expatriate without any problem, had to take a very difficult decision: leave his family, his country and his beloved Naples in view of possibilities that Italy in the 1950s did not offer (the Italian boom was still to come). He decided for the US: this time he travelled by plane. Guido's new life in the US then started: College at Millikin University in Decatur; Medical School and M.D. at Washington University, St Louis, Mo; Ph.D at Rockefeller Institute New York, NY; and Professor at Harvard University, Cambridge, MA.

Appendix III

Guido as an AFS student in Decatur, Illinois

Guido arrives in Decatur, Illinois as AFS student

(1950; age 17)

Italian Student Greeted on Arrival

A group of Decatur high school students welcome Guido Guidotti of Italy arriving from Chicago yesterday to attend D. H. S. for a year. At the station are, left to right, Anne Baker, Connie Kull, Glenna Flanders, John Luallen, Guido, Lucien Kapp, Carolyn Holmstrand and Mary Lou Evans. The student council is sponsoring the 16-year-old youth and with the American Field service will finance his visit. Guido will be in the senior class. He is staying with Mr. and Mrs. Ralph Williams of 1455 West Decatur street.

(Photo by Floyd Stradley).

Guido at Decatur High School

STUDENT
COUNCIL

GUIDO GUIDOTTI
Foreign Student

Appendix IV

Guido Guidotti CV and List of Publications

Guido Guidotti

Guido Guidotti
Higgins Professor of Biochemistry
Department of Molecular and Cellular Biology
Harvard University
52 Oxford Street
Cambridge, MA 02138

Place of birth : Florence, Italy.

Education:

James Millikin University, Decatur IL		1951-1953	Premedical
Washington University	M.D.	1957	Medicine
Rockefeller University	Ph.D.	1963	Biochemistry

Faculty positions :

Assistant Professor of Biology	Harvard University	1963-68
Associate Professor of Biology	Harvard University	1968-69
Professor of Biochemistry	Harvard University	1969-present

Editorial Boards:

Journal of Biological Chemistry 1971-76
Journal of Membrane Biology 1981-91
Molecular Biology of the Cell 1992-2005

Grant Review Sections:

Physiological Chemistry Study Section (NIH) 1971-75
Biochemistry Review Group (NSF) 1976-1982
American Cancer Society 1976-1980
Physical Biochemistry Study Section (NIH) 1983, 1987-1991
Medical Foundation 1991-2001

Professional Societies :

American Society for Biochemistry and Molecular Biology
American Association for the Advancement of Science-Fellow
American Society for Cell Biology
American Academy of Arts and Sciences

Teaching Activities:

Biology 191.	Biochemistry of Cellular Structure and Function	1963-67
Biology 16.	General Biochemistry	1966-70
Biochemistry 10	Introductory Biochemistry	1971-76
Biochemistry 10	Introductory Biochemistry	1981-89
Biochemistry 176	Biochemistry of Membranes	1976-80, 1983, 1986,1989, 1991, 1995,
MCB 176	Biochemistry of Membranes	1999, 2001-present

Biological Sciences 11	Introductory Biochemistry and Cell Biology	1992-2000
Biological Sciences 56	Biochemistry and physical properties of macromolecules	2003-2008
Freshman Seminar 26z	What is Life ?	2009- present
MCB 178	Biochemistry of Protein Complexes	2011-present

Teaching Award :
2000 Phi Beta Kappa Prize for Excellence in Teaching (Iota and Alpha Chapters of Phi Beta Kappa).

2007 Alumni Achievement Award Washington University Medical School

2003 ASCB profile

Publications

(*) indicates publications that Guido designated as most significant.

NB that up to 1985, Guido did not put his name on any publication from his laboratory to which he did not contribute experimental bench work. After that point he was forced to do so by failure of NIH study sections to understand his approach.

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6a. Craig, L.C., Guidotti, G. (1963) Dialysis Behavior of Peptides and Proteins Which Associate. Science 140, 380.

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10. Guidotti, G. (1965) The rates of reaction of the sulfhydryl groups of human hemoglobin.

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19*. Rosenberg, SA, Guidotti, G. (1969) Fractionation of the protein components of human erythrocyte membranes. J Biol Chem. 244, 5118-5124.

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Never written:

23x. Jones, S.L., Thorn, K.S., and Guidotti, G. FRET Reveals the Dominant Form of CD39 to Be a Monomer in Living Cells.

23z. Wu, J.J., and Guidotti, G. The interrelationship of N-glycosylation and disulfide bond formation in CD39.

Note: the following papers were added to this compendium by the authors to acknowledge Guido's contributions even though his name was not on the paper....

230. (added by John Pringle, whose contribution was entirely from work in Guido's lab) Weber, K., Pringle, J.R. and Osborne, M. Measurement of Molecular Weights by Electrophoresis on SDS-Acrylamide Gel. Methods Enzymol. 26, 3-27 (1972).)

231-234. Papers from J.E.M. McGeoch as sponsored by Guido Guidotti.

231. McGeoch, J.E.M Topology of the mammalian cell via Cryo-FIB etching. J Microscopy 227, 172-184 (2007).

232. McGeoch J. E. M. & McGeoch M. W. Entrapment of water by subunit c of ATP synthase. J. Roy. Soc. Interface 5, 311-318 doi:10.1098/rsif.2007.1146 (2008).

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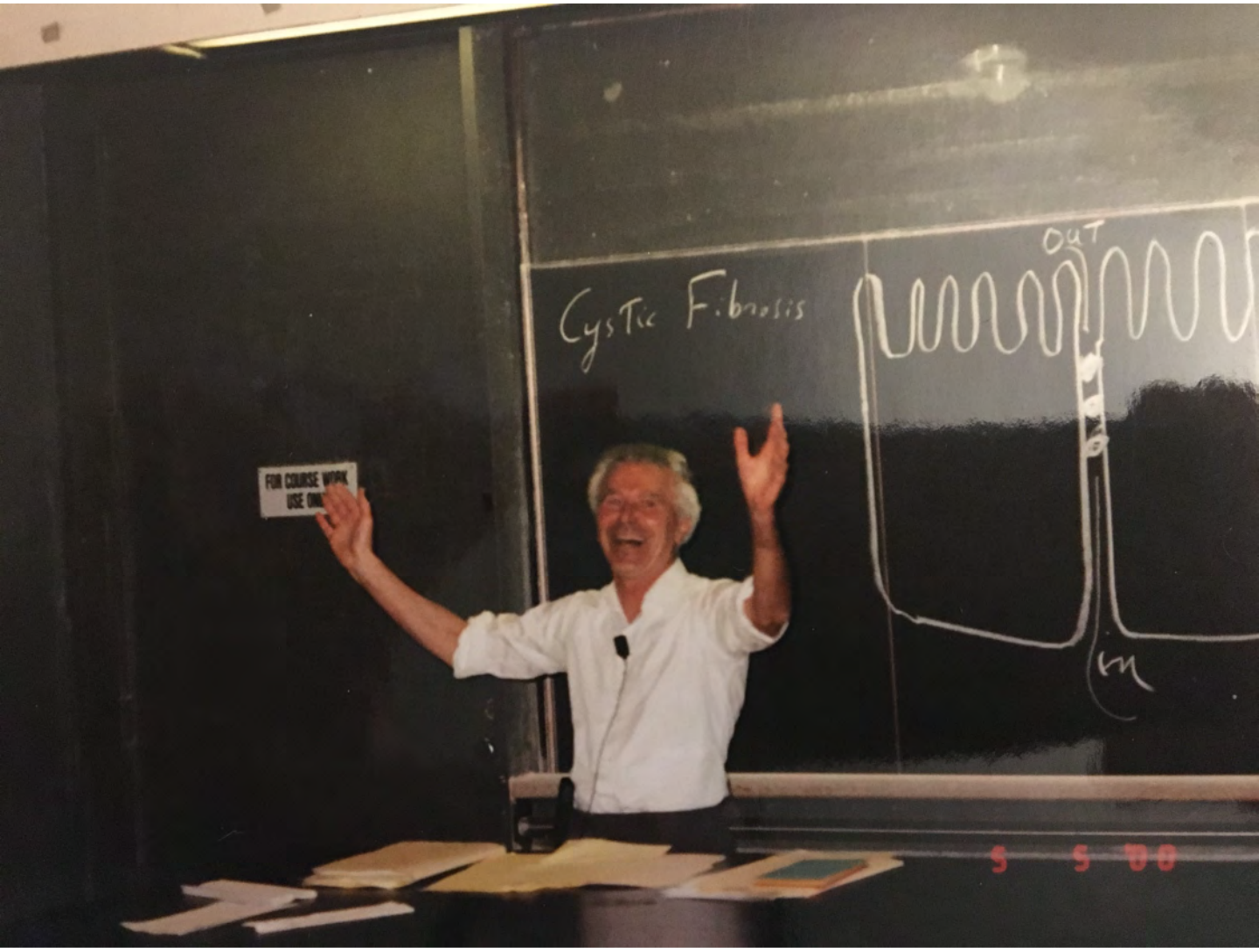
Appendix V

Guido as a teacher at Harvard University and as a BMB professor with his laboratory

Guido at 40, with Guido Jr (age 5)

Guido giving group seminar… ~1973

Guido’s car: the “Diamond Grey”



from Raj Malhotra

Professors Carroll Wood (Biology), Guido Guidotti (Biochemistry), and John Dowling (Biology) entertain questions during Monday's Danforth Center session on lecturing in the sciences. (Photo: Joe Wrinn)

Thrown Geraniums Keep Lectures Lively

Biochemistry may be one of the most exciting fields of scientific endeavor today, but learning some of the everyday mechanics—like intermediary metabolism—can be downright boring, said Professor **Guido Guidotti** (Biochemistry).

Making such fundamental information interesting to students, he said Monday evening, is the challenge of teaching an introductory science course.

Guidotti, who gets rave reviews from students for his lectures on intermediary metabolism, was offering pointers on "Lecturing in the Sciences" along with Professor **John Dowling** (Biology) and Professor **Carroll Wood** (Biology). The discussion was part of a professional training series sponsored by the Harvard-Danforth Center for Teaching and Learning.

Wood, Curator of the Arnold Arboretum, told of a professor at Cornell University who transfixed students in his botany course by throwing a potted [illegible] across the room. While an in[illegible] must be somewhat of a performer, [illegible] off a large lecture course, he said, the ability to relate information to a central theme and to the students' own experience is equally important.

"I'll start with an overview, then go back and give details and specifics," said Dowling, who is also Associate Dean of the Faculty of Arts and Sciences. In teaching neurobiology (*Biology 25*), he tries to explain the central concept of the lecture first, then build up evidence piece by piece with examples, diagrams (he practices drawing them in advance), experiments, and some slides.

"If an experiment is elegant, do it," agreed Guidotti. Otherwise, he said, don't.

Dowling offered the following nuts-and-bolts teaching suggestions for large lecture courses:

—Go at a pace that allows everyone to understand information. The key, he said, is repetition.

—Home in on a few students. "Some faces are very responsive and can give you a clear idea of how things are going," he said.

—Tell students when you are about to make an important point, particularly if rustling noises let you know their attention is wandering. (Wood cautioned that there are hazards to this approach. His brother, also a professor, once told his students that no matter what they learned from the course, they should remember three key points. After expounding on the first two, he forgot the third.)

—Continually relate new information to material you have discussed in the past.

—Be available to answer questions after the lecture. This not only helps students, he said, but also lets the instructor know which points were not easily understood.

Guidotti added that there is only one way to keep introductory lecture material equally alive to professor and student and that is to rewrite it every year.

Opinions differed on the subject of notetaking. Guidotti said that he distributes lecture notes later. Dowling, on the other hand, advocates student notetaking. "I like to have students work very hard during the lecture to understand the concepts we are dealing with."

Thrown Geraniums Keep Lectures Lively
Harvard Crimson ~1977

Professors Carroll Wood (Biology), Guido Guidotti (Biochemistry), and John Dowling (Biology) entertain questions during Monday's Danforth Center session on lecturing in the sciences. (Photo: Joe Winn)

Thrown Geraniums Keep Lectures Lively

Biochemistry may be one of the most exciting fields of scientific endeavor today, but learning some of the everyday mechanics--like intermediary metabolism--can be downright boring, said Professor **Guido Guidotti** (Biochemistry).

Making such fundamental information interesting to students, he said Monday evening, is the challenge of teaching an introductory science course.

Guidotti, who gets rave reviews from students for his lectures on intermediary metabolism, was offering pointers on "Lecturing in the Sciences" along with Professor **John Dowling** (Biology) and Professor **Carroll Wood** (Biology). The discussion was part of a professional training series sponsored by the Harvard-Danforth Center for Teaching and Learning.

Wood, Curator of the Arnold Arboretum, told of a professor at Cornell University who transfixed students in his botany course by throwing a potted geranium across the room. While an instructor must be somewhat of a performer, to carry off a large lecture course, he said, the ability to relate information to a central theme and to the students' own experience is equally important.

"I'll start with an overview, then go back and give details and specifics," said Dowling, who is also Associate Dean of the Faculty of Arts and Sciences. In teaching neurobiology (*Biology 25*), he tries to explain the central concept of the lecture first, then build up evidence piece by piece with examples, diagrams (he practices drawing them in advance), experiments, and some slides.

"If an experiment is elegant, do it," agreed Guidotti. Otherwise, he said, don't.

Dowling offered the following nuts-and-bolts teaching suggestions for large lecture courses:

--Go at a pace that allows everyone to understand information. The key, he said, is repetition.

--Home in on a few students. "Some faces are very responsive and can give you a clear idea of how things are going," he said.

--Tell students when you are about to make an important point, particularly if rustling noises let you know their attention is wandering. (Wood cautioned that there are hazards to this approach. His brother, also a professor, once told his students that no matter what they learned from the course, they should remember three key points. After expounding on the first two, he forgot the third.)

--Continually relate new information to material you have discussed in the past.

--Be available to answer questions after the lecture. This not only helps students, he said, but also lets the instructor know which points were not easily understood.

Guidotti added that there is only one way to keep introductory lecture material equally alive to professor and student, and that is to rewrite it every year.

Opinions differed on the subject of notetaking. Guidotti said that he distributes lecture notes. Dowling, on the other hand, advocates student notetaking. "I like to have students work very hard during the lecture to understand the concepts we are dealing with."

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from the Harvard Crimson ~1977

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last lecture before COVID-19 confinement

▼ What would you like to tell future students about this class?

MCB 176: Biochemistry of Membranes Fall, 2016

A gem of a class. Guido is incredible, learned so much, and was not overwhelming.

Informative

I loved this course! I randomly came across this class and was pulled in by the first lecture. Guido is an amazing professor and definitely knows what he's talking about. I learned a lot from this course about membranes, and I'm glad I took it!

Guido is a great professor and you'll learn a lot. The course is very dependent of reading and writing about papers and giving presentations. By the end of the course, you'll know a lot about current research and have to opportunity to think critically about it. Take the course!!!! Won't regret it.

This course teaches you a lot about how to think in science (specifically in structural biology). I learned a lot about strengths and weaknesses of different experimental methods as well as how to critically evaluate publications. This course also teaches you a lot about how to read papers, and summarize/explain them to others succinctly. You also get a lot of opportunities to explore your specific interests in the field through presentations and the final project. The course staff were very supportive, and I loved the seminar style of the class. Sometimes the material can be quite challenging, but I think the goal is to understand a

FRSEMR 26Z: What is Life? Fall, 2016

this class was fantastic

It was difficult to understand the lectures because the professor went into such great detail about every aspect of the topics we learned. I made friends however, which was nice.

Enjoy every second of the experience because it goes by quickly.

Be ready to have your perspective of what life is change.

This seminar has an extremely light workload while still being interesting

---

MCB 178: Biochemistry of Protein Complexes Spring, 2017

it's really easy and pretty much no work. a short weekly summary about a paper, two short presentations, and a final presentation/research proposal. great to balance out with harder classes

---

A great course with a great professor. The workload is lighter than most--the class is seminar style, so mostly reading papers and presenting on them. However, you you will need a very good grasp on biochemistry to keep up with the material.

It's an interesting overview of diverse topics. Informative, but not overly taxing.

I loved this course! Consider taking this class to appreciate biochemistry more.

It's a pretty good course and the work is not to extensive. Nonetheless, it's still a very informative course depending on the amount of work you are willing to put into it. Guido is an awesome professor!

Appendix VI

ASCB member profile of Guido Guidotti (2003)

[<< back]

2003

Guido Guidotti

"The first thing Guido Guidotti taught me about doing science was that little things matter," says John Pringle, who was one of his first graduate students at Harvard University in 1968. "Early in my graduate studies when Guido was still working on hemoglobin and the binding constants of oxygen, I went up to his lab and found his technician doing the experiment while Guido was over by the sink washing the glassware. Later, I asked him about this and he said that the experiment was interesting enough that he could trust the technician to do it right, but he just couldn't trust her to get the glassware clean enough."

But what matters to Guidotti is not always what matters to others. For example, Guidotti's CV lists a modest number of publications during the 1970s and 1980s, a time when his lab was making major advances on membrane protein transport. "That's this business of his not taking credit on papers," says Pringle, who is now at the University of North Carolina at Chapel Hill. "That's why it's so hard to figure out what he's done over the years. If you do a search for Guido, you don't find as much as you should," says Pringle. Guidotti's name was neither on Pringle's first published papers nor on other major papers from early Guidotti graduate students and post-docs who launched their careers from his lab. "Guido just wouldn't take credit unless he'd been actively involved in the creative part or had put in a lot of other work himself," Pringle recalls. "For a while, everyone knew about Guido's not taking credit. We all thought, 'this is cool.' Then everyone forgets what Guido actually did."

Lewis Cantley, now at Harvard Medical School, corroborates Pringle's assessment that Guidotti's impact far exceeds his bibliography. As a post-doc in 1978, Cantley says he only managed to convince Guidotti to add his name to papers on vanadate as a NaK ATPase inhibitor by arguing that the Guidotti lab itself deserved the recognition. Guidotti is well-known for turning out extremely confident and well-trained people who go on to major careers. A partial list of Guidotti students and post-docs might include Eva Neer, Jack Kyte, Steven G. Clarke, Kurt Drickamer, Anjana Rao, Marilyn Resh, Michael Forgac, Daniel Jay, Robert Bloch, Gilbert Chin, Betty Eipper, James M. Anderson, Jonathan Lytton and Chung Wang. Their mentor shrugs off any undue credit. In any case, Guidotti says his days as an unacknowledged collaborator ended abruptly in 1985 when he was called on the carpet before an NIH study section. "They said I was plagiarizing by using these articles that were published without my name on them," Guidotti recalls. "So now I put my name on all the papers, even the ones I didn't work on."

Even today, papers may not be the best way to follow the life of Guido Guidotti. Despite his name and the faint trace of an Italian accent after more than fifty years in America, Guidotti admits, when cornered, that his grandmother was "a lady from Boston," a Brahmin by birth, even if she was born in Tokyo. Her father (that is, Guidotti's great-grandfather) was George Adams Leland, Sr., a distinguished Boston ear-nose-and-throat specialist who was teaching in Japan by invitation of the Imperial Court, when she was born there in 1880. At the suggestion of a Japanese princess, the baby was named Jiun, supposedly "obedience" in Japanese. After three years in Japan, the Lelands returned to Boston (via Germany where her father stopped to study new surgical techniques). In 1907, Miss Jiun Leland was seated next to a young Italian naval officer, Salvatore Casano, at a formal dinner in Boston to honor the arrival of his warship on a goodwill mission. One thing led to another and they married and moved to Italy in 1908. Salvatore Casano rose to the rank of admiral in the Italian Navy, according to Guidotti, before he was forced out during "the fascist time." Jiun Leland Casano lived in Italy to the end of her life in 1975, speaking English to her daughter and grandchildren, and Italian (with a pronounced Boston accent) to everyone else.

Guido Guidotti first came to the U.S. in 1950 as an American Field Service exchange student bound for a final high school year in Decatur, Illinois. He was born in Florence in 1933 but weathered the war with his mother and older brother at his Yankee grandmother's home in Naples. His father, an officer in the Italian Army, was captured by the British in North Africa in 1941 and held as a POW in India until 1946. Even with the return of peace, Decatur seemed like welcome relief. "Naples was a chaotic large city with over one million people," Guidotti recalls; "Decatur was a town of 50,000. In Naples, the war hadn't really gone away.

The port of Naples was completely destroyed by the bombs so that by 1950, things were just getting back to normal, although life in Naples is always different from any other place."

Guidotti found Illinois "very different and very cold but the people were very cordial and friendly." He was taken in by Ralph and Dot Williams with whom he remained close ever after. It was the Williamses who arranged for Guidotti's admission to Millikin University in Decatur. After two years at Millikin, Guidotti was "miraculously" accepted into the Washington University School of Medicine, earning his MD in 1957. He served a residency in Internal Medicine, and was looking toward a career in academic medicine when Sputnik shook up American science. Suddenly a stint in basic research was de rigeur for a would-be medical academic and Guidotti was advised to join Lyman Craig at the Rockefeller as a way of adding a little hemoglobin research to his resume. After two years in New York, he was so engrossed in the

problem of identifying the amino acid sequence of the beta-chain of hemoglobin that he thought he might as well finish his doctorate. (He had also become the personal source of the lab's beta-chain hemoglobin).

In 1963, Guidotti was of- fered a faculty position at Harvard University under the "Committee on Higher Degrees in Biochemistry," which also included James Watson, Matthew Meselson, Walter Gilbert, Konrad Bloch, Paul Doty and John Edsall. The name and orientation of his depart- ment has morphed several times over the decades. These days, the sign outside says "Department of Molecular and Cellular Biology." As the Higgins Professor of Bio-chemistry, Guidotti is highly regarded by Harvard students, who voted him the 2000 Phi Beta Kappa Prize for Excellence in Teaching.

In 1990, Guidotti married molecular biologist and Harvard colleague Nancy Kleckner. They live in Newton. Guidotti has a son, also named Guido, by a previous marriage. The younger Guidotti is 34, married, and an MD who is a consultant in the Boston area. Guidotti has been a member of ASCB since 1992 and an Associate Editor of the Society's journal, Molecular Biology of the Cell, since its inception.

In forty years of research, Guidotti has watched an entire field—membrane protein transport and signaling—coalesce before his eyes. The work he is most closely identified with is the characterization of the NaK pump and the inhibition of NaK ATPase, work credited with explaining the mechanism behind the clinical use of cardioglycosides to control heart failure. Yet Guidotti is no longer sure his old molecular explanation is complete. "The NaK ATPase has been a major actor in the control of the force of cardiac contraction, but the more I read about it, the more difficult it is to understand how it works. There is a quandary emerging in more recent work that shows the concentrations of cardioglycosides used to treat heart failure would inhibit only a small fraction of the sodium pumps in the body. So it isn't clear how this would affect the heart un- less there were special high affinity receptors there," he says.

The idea of a hypothetical secondary receptor that might undermine one of the major discoveries of his lab seems to delight Guidotti. "Nature teases you. It makes you think you know something whereas you don't know the whole story. The first time you make a discovery, you think you understand everything, but as you do more work on the subject, you realize that there are more and more actors involved in this business. When you collect all the actors together, you realize you don't know who has the principal role."

Says Guidotti, "The reason cells work is that they have enormous back-up arrangements so that any perturbation that comes along can be dealt with. You have to be ready for this change and that change so you can escape one way or another." And so, says Guidotti quite cheerfully, "you solve one problem and that always brings up another."

Appendix VII

The Journey of a Researcher: An Interview with Guido Guidotti in THURJ, the Harvard undergraduate research journal, by Menting Qiu

thurj
the harvard undergraduate research journal

The Journey of a Researcher: An Interview with Guido Guidotti

Menting Qiu '18, THURJ Writer

In a back office room of a quietly busy laboratory on the first floor of the Northwest Building, Professor Guido Guidotti sits with his MacBook Air in his lap, thoughtful. When I knock on the door, he looks up cheerfully and calls out, "Hi there! How do you do?" "I'm well, thank you, and you?" I respond. He flashes me his bright smile: "Well, I'm still here!"

More often than not, Guidotti's radiant energy and constant enthusiasm belie his advanced years, 50 of which he has dedicated to teaching and researching at Harvard. Now the Higgins Professor of Biochemistry, Guidotti teaches "MCB 176: Biochemistry of Membranes," "Freshman Seminar 26z: What is Life?," "MCB 178: Biochemistry of Protein Complexes," and "MCB 391: Biochemistry"; in the meantime, he focuses his research on the structure and function of membrane proteins.

A Winding Path to Harvard

Guidotti describes his education background, with a laugh, as "a story with no object in sight." Growing up in Naples, Italy, Guidotti first came to the United States as an exchange student in 1950 (Johnson 2011). He attended high school in Decatur, Illinois, for one year, after which he accepted a scholarship to attend Millikin University in the same city. Wanting to pursue a medical profession, Guidotti applied to several medical schools in the United States, including Harvard Medical School. On his decision to apply to medical school, Guidotti says he thought, "I'll apply now and see what happens. I didn't expect anything to happen." In the end, he was accepted to Washington University, "and so I went to Washington University! It was very easy!"

However, according to Guidotti, towards the end of the 1950s when "Sputnik had just gone up," many people became interested in scientific research. He says that everyone, including medical schools, got "psyched up about science" and "how everybody had to be a scientist." Told that he needed to learn how to do research in order to "have a career in medicine," Guidotti took an opportunity to attend the Rockefeller Institute in New York, and ended up completing a graduate degree there. However, by the time he was "ready to come back" to Washington University, the school no longer had a spot for him to complete his residency in medicine. "So I had to look for someplace else to go," says Guidotti. A protein chemist at Harvard had asked two other protein chemists at the Rockefeller if they had anyone to recommend for a "position in protein chemistry in the Committee of Higher Degrees in Biochemistry" at Harvard; they recommended Guidotti. So, Guidotti was invited up to Harvard to see if "they were interested in me and I was interested in them. And so I came up and eventually it worked out…So rather than going back to Washington to do my residency…I came [to Harvard]."

Sequencing his own Hemoglobin

Before coming to Harvard, Guidotti had been part of a lab at the Rockefeller Institute that was working on the amino acid sequence of hemoglobin, which was one of the largest proteins being sequenced at the time. However, due to the limitations of technology at the time, a project of such magnitude also required much larger quantities of substances on which to perform experiments than would be needed today. Says Guidotti, "In those days, to do chemistry, you actually needed gram amounts of stuff, rather than micrograms." In this case, Guidotti's lab required large amounts of one particular substance: blood.

Rather than obtaining "expired blood in a blood bank," Guidotti explains that he offered to donate his own blood to his lab. "When I was [working at the hospital], every six weeks or so, I gave blood to the blood bank…So I said, I can give blood, I'm used to it! So I got somebody in the lab, we boiled a syringe, and I helped the person put the needle in the vein and draw out the blood. And then we

got 50mL at a time…and put it through the machine, the countercurrent distribution [which separates the two chains of hemoglobin], and we got several grams of the [hemoglobin] chains. And then when we needed it again we'd do the same thing all over again." His selfless donation in pursuit of scientific progress is unusual and commendable, but Guidotti tells the story as if it were just a routine part of his life. Even after many years of formidable research, he remains remarkably humble.

Further Research at Harvard

After coming to Harvard, and ten years or so of working on hemoglobin, Guidotti's research migrated from proteins inside the red blood cell to those in the membrane. Eventually, he came to focus on other cells' membrane proteins, such as the insulin receptor and the Na+/K+ ATPase. As Guidotti's research shifted, one thing has remained constant throughout his years of research: his passion for proteins. It was not known "in the early times," says Guidotti, "that proteins could change conformation, and have a behavior depending on conformational change." To this day, Guidotti is still fascinated by the unknowns in protein chemistry: "How can a protein, that seems to be sort of fixed in its arrangement, end up being so flexible and do so many things? How does it manipulate that? We have a protein that sits in the membrane with transmembrane domains. The transmembrane domains you'd think would be there holding the protein in, but they're moving around all the time, and they flip back and forth. Why? I mean, it's completely crazy business!"

A Legacy of Humility

When asked which aspects of his career he is most proud of, Guidotti makes no mention of any research achievements he and those in his lab have made (such as successfully sequencing hemoglobin and being the first to isolate the sodium pump), or teaching awards he's won, such as Harvard's Phi Beta Kappa Prize for Excellence in Teaching in 2000 (Johnson 2011). Instead, he humbly highlights his passion for his work, and the contributions of others to his career: "All the research has been interesting…and I was glad that the graduate students in general did well." And on how his graduate students were able to perform such great work? Guidotti cites the fact that his department, with powerhouses like James Watson and Matthew Meselsohn, "was a very eminent department and students came and flocked in." According to Guidotti, when other labs in his department were full, students ended up asking him to join his lab. "And so I got lots of great students," he says with a laugh.

It's not just through his words that Guidotti under-credits his work; in fact, he is well-known for having a small bibliography that is largely misrepresentative of all that he has contributed to the scientific world, because he refused to take credit for work that he didn't put as much manual work into (ASCB 2009). Says Guidotti, "I wasn't even on the papers [my graduate students] published unless I had worked manually in the operation. Because I mean, advice is easy to give, but the work is tough. Unless you work, you shouldn't, in my opinion, get your name on the paper." In a setting where publishing is key to academic survival, such a view is rare and unique. Yet, perhaps it is a simple demonstration of Guidotti's pure drive and energy to perform good scientific work regardless of credit, and further, what he believes should be the emphasis in research: the fascinating process of discovery, not the accolades and recognition that may result.

To Prospective Researchers

According to Guidotti, research is all about asking questions, and then searching for the answers to these questions in order to find out more about the unknown. "Research is [done on] something that is not known, and [when] you think you have a way of answering that question, you go ahead and do it, and if you get an answer of course it may be wrong, because you don't always see the whole picture. [But] at least you got an answer to something, and usually when you get an answer, it opens up another view of what you might do next."

Aside from emphasizing the question-and-answer aspect of research, Guidotti advocates for basic science research. According to Guidotti, applied research is important, "but if one wants to do it with knowledge, one has to understand what one is doing…Curing cancer doesn't mean just making drugs and applying them to cancer and seeing if you can kill the cancer. You have to understand what the cancer, what the cell is doing." Ultimately, Guidotti advocates for medical research methodology that does not attack disease before achieving a careful, rigorous understanding of its foundations. He emphasizes that curing cancer, for example, should not be "a war on cancer…Science doesn't work by wars. Anytime you make a war on something, it never works…Because one has to understand the physiology, the behavior of a cell." Guidotti concludes: "People who are interested in research…have to think about what the normal behavior of the system they are looking at is."

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Appendix VIII

History of Biochemistry at Harvard College, by Guido Guidotti

Biochemistry at Harvard College

by Guido Guidotti

Although the Department of Biological Chemistry (now Biological Chemistry and Molecular Pharmacology) at Harvard Medical School was established in 1908, the Department of Biochemistry and Molecular Biology in the Faculty of Arts and Sciences was started only in 1967 and lasted until 1994. Nevertheless, biochemistry was taught at Harvard College since the early 1920s. Lawrence J. Henderson, for whom the Department of Physical Chemistry at Harvard Medical School was set up in 1920, taught a biochemistry course in Cambridge, Chemistry 15, from about 1919 to 1940; this course had a great influence on the scientific choices of John Edsall and Jeffries Wyman. Henderson also was involved in the development of the Biochemical Sciences concentration and tutorial program in 1925-26. This concentration, administered by the Board of Tutors in Biochemical Sciences whose members were principally at Harvard Medical School, was designed to introduce Harvard undergraduates to biochemistry. In 1967 the tutorial program became the responsibility of the Department of Biochemistry and Molecular Biology.

Shortly after Jeffries Wyman returned from England in 1927 to an appointment in the Harvard Zoology Department, he started to give a course in physical biochemistry which eventually included studies on the properties of hemoglobin. When in 1940 Henderson ceased giving Chemistry 15, John Edsall, who was in the Department of Physical Chemistry at Harvard Medical School, joined Wyman in teaching the physical biochemistry course. The lectures in the course were the basis for the book Biophysical Chemistry published in 1958. John Edsall continued to give the course after Wyman left Harvard in 1952 ; eventually he taught it with Paul Doty. The most recent edition of the course was taught by Don Wiley as MCB 112.

In 1935, the Department of Biology, by then in the Biological Laboratories, realized that biochemistry was an important subject; George Wald, who had come to Harvard from Columbia University, began to give a course in general biochemistry (Biology 190), initially with Kenneth Thimann, a plant biochemist also in Biology, and continued to do so until 1960. Associated with this course, there was an independent laboratory course in biochemistry (Biology 191) taught even after the end of the lecture course until 1975.

In the period after the second world war, President Conant did try to establish biochemistry in Cambridge through the formation of a Department of Biochemistry with three subdivisions in 1946. Attempts were made first to recruit Carl Cori from Washington University in 1947 and then Edwin J. Cohn from the Department of Physical Chemistry at Harvard Medical School in 1951. These initiatives failed and the vote establishing the department was rescinded in 1955.

Nevertheless, in the late 1940s and early 1950s, biochemistry became a growing activity in Cambridge. In 1948, Paul Doty came to the Department of Chemistry. His interests in polymers expanded to include studies of polypeptides, proteins and nucleic acids. In 1954, John Edsall moved from the Department of Biophysical Chemistry at Harvard Medical School to the Biological Laboratories in Cambridge, and Konrad Bloch and Frank Westheimer joined the Chemistry Department from the University of Chicago. Finally, a successful initiative in Cambridge began in 1954-55 with the formation of the Committee on Higher Degrees in Biochemistry by this group of biochemist, together with Wald and Thimann, so as to have a graduate program leading to the PhD degree in biochemistry. In 1956 James Watson arrived in the Biological Laboratories from CalTech, shortly after his discovery of the structure of DNA with Crick in 1953, and brought molecular biology to Cambridge. In 1958, the Committee was awarded a training grant from the National Institutes of Health to support the graduate program. By the early 1960s, Matthew Meselson joined the Committee from CalTech, Walter Gilbert was

attracted to Watson's laboratory from the Physics Department, Mark Ptashne came as one of the early graduate students and stayed on and Guido Guidotti joined the Committee from the Rockefeller University. The graduate program was very successful and attracted many superb students. In 1965, the name of the Committee was changed to Committee on Higher Degrees in Biochemistry and Molecular Biology. Edsall (1954-57), Bloch (1957-60), Watson (1960-63), Meselson(63-65), and Edsall (65-67) served as Chairmen of the Committee. Howard Berg was Chairman of the Board of Tutors in Biochemical Sciences. Because it was clear that biochemistry and molecular biology were vigorous academic endeavors and the approach to research of the members of the Committee on Higher Degrees in Biochemistry and Molecular Biology was different from that of the members of the more traditional Department of Biology, the Committee decided to form the Department of Biochemistry and Molecular Biology (BMB) so that it could become an independent entity and recruit its own faculty. Under the impetus of Paul Doty , the Department was approved by the Faculty of Arts and Sciences on 11 April 1967. Paul Doty was the first Chairman of BMB and Jack Strominger was the first member recruited specifically to the new Department; the other members of BMB were Berg, Bloch, Edsall, Gilbert, Guidotti, Meselson, and Watson. Ptashne became a Lecturer in 1968. In 1971, Stephen Harrison and Don Wiley joined the Department, followed by James Wang and Nancy Kleckner in 1977, Tom Maniatis and Doug Melton in 1981, Ernest Peralta in 1989 and Sam Kunes in 1993. Jeremy Knowles became a joint member of Chemistry and of BMB in 1980. After Paul Doty, the following members of BMB served as Chairman : Strominger (1970-73), Watson (1973), Meselson (1974), Bloch (1974-75), Meselson (1975-77), Guidotti (1977-80), Ptashne (1980-83), Wang (1983-85), Maniatis (1985-88), Harrison(1988-92) and Wiley (1992-95).

With approval of the University, plans were made in 1968 for a new building to house members of BMB and of the Chemistry Department; however, funds for the project were not forthcoming. In 1976, Matt Meselson, while chairman of the

Department, was able to identify a source of moneys and to convince Henry Rosovsky and Derek Bok of the urgent need for new space for the Department. The result was the construction of the Fairchild Biochemistry building. In 1981 Harrison, Wiley, Ptashne, Wang, Kleckner, Guidotti, Meselson, Doty, Strominger, Maniatis and Melton moved into the building. Ernie Peralta and Sam Kunes came to Fairchild later. Konrad Bloch, although a member of BMB, remained in Conant Laboratory, as did Jeremy Knowles in Mallinckrodt.

Members of BMB were involved in undergraduates courses on biochemistry and molecular biology. John Edsall was a tutor in Biochemical Sciences from 1928 to 1968, and Chairman of the Board of Tutors from 1931 to 1957. Stephen Harrison was the Chairman from 1972 to 2001. Edsall's course on proteins, now MCB 112, has been mentioned above. Konrad Bloch taught biochemistry for advanced undergraduates and graduate students, Chemistry 190, from 1955 to 1974. Jim Watson taught introductory molecular biology as part of Biology 2 in the 1960s; the lectures in the course were the basis for his book The Molecular Biology of the Gene. In 1971, Biochemistry 10, an introduction to biochemistry and molecular biology, was started by Jim Watson and Guido Guidotti as the first undergraduate course of the new Department. The course eventually became Biological Sciences 10 (now Biological Sciences 52) and 11 (biochemistry was dropped from the curriculum in 2000). Matt Meselson for many years taught the undergraduate genetics course, initially as Biology 140, now Biological Sciences 50. Paul Doty started the course on physical chemistry Chemistry 61, which became MCB 61 and now Biological Sciences 56.

Several members of BMB were awarded the Nobel prize between 1962 and 1980 : Jim Watson (1962) and Konrad Bloch (1964) for Physiology and Medicine; Wally Gilbert (1980) for Chemistry. George Wald, who remained in Biology, got the Nobel prize for Physiology and Medicine in 1967.

At the time that BMB became a Department in 1967, there remained in Biology a small group of faculty with cellular rather than organismic interest : Lawrence Bogorad, Woody Hastings, Fotis Kafatos, Paul Levine, Keith Porter, Alwin Pappenheimer, John Raper, George Wald and Carroll Williams. Keith Porter, who had come to Biology from the Rockefeller University in 1962, had a major role in promoting the field of cell biology. As Chairman of Biology in the late 1960s, he also oversaw a revision of the undergraduate curriculum with the introduction of a course in cell biology which he taught, Biology 15 (now Biological Sciences 54) and the appearance of a new course on biochemistry, Biology 16, which then became part of Biochemistry 10. Pappenheimer, who came to Biology in 1956, was Chairman of the Board of Tutors in Biochemical Sciences from 1958 to 1964; in 1959, he started an undergraduate course on immunology, Biology 169, now MCB 169. Over a period of 10 years, the group in Biology evolved with the departure of Levine, Porter, Raper and Wald and the arrival of Dan Branton, John Dowling, William Gelbart and Richard Losick. In 1978, Biology split again into Cellular and Developmental Biology (CDB) and Organismic and Evolutionary Biology (OEB), with CDB occupying space in the Biological Laboratories. Additional faculty members joined CDB during the next 10 years : Howard Berg, Ray Erikson, Walter Gilbert, Lawrence Goldstein and Markus Meister. Fotis Kafatos was the first Chairman of CDB, followed by Branton, Losick, and Gilbert.

During the 1980s, some of the research interests of BMB and CDB started to overlap in the areas of molecular and developmental biology. In 1988, the graduate admission procedures for the two programs were integrated into a successful program in Biochemistry, Molecular, Cellular and Developmental Biology (BMCDB); after the first year of graduate school, the students could choose in which Department to do research. In the early 1990s Andrew McMahon and Elizabeth Robertson came to BMB and CDB as joint appointments in order to bring vertebrate development to Harvard University in Cambridge. In 1994, BMB and CDB were fused into the Department of Molecular and

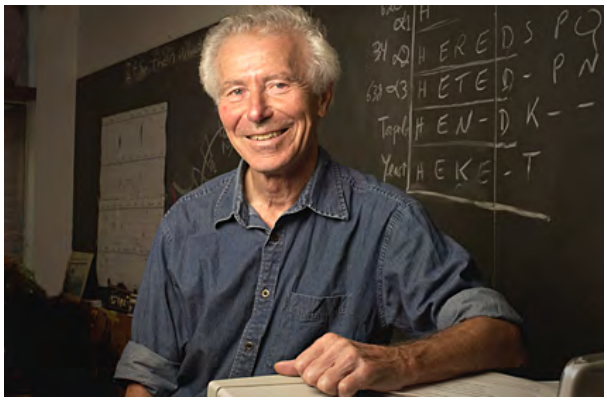
Cellular Biology with faculty members in both Fairchild Biochemistry Building and the Biological Laboratories.

Appendix IX

Interview of Guido Guidotti by *scienceopen.com*

Guido Guidotti.

September 29, 2017 * Author: Elizabeth Allen



It's true to say that all our Board Members have a first-rate academic pedigree and this is the case for Guido Guidotti (ORCID/0000-0002-0499-3412). He obtained an MD at Washington University School of Medicine and was a resident in internal medicine at Barnes Hospital. He received a PhD in Biochemistry from The Rockefeller Institute. He then joined the Committee on Higher Degrees in Biochemistry and Molecular Biology at Harvard University, where he is now the Higgins Professor of Biochemistry.

Together with author Sari Paavilainen, they published their first article entitled "Interactions between the transmembrane domains of CD39: identification of interacting residues by yeast selection" in October and we're delighted to share some background information on this review article and the reasons why Guido and Sari choose to publish with us.

Q. Can you tell us a little bit about your current research and the article that you published with ScienceOpen?

A. Nearly all the research done in my laboratory is concerned with the properties of protein molecules. During the past 20 years, we have studied enzymes that are attached to the lipid membrane by transmembrane domains and have the active site on the extracellular domain, so-called ecto-enzymes. The central question for the ecto-ATPase of interest, CD39 or E-NTPDase 1, is why is the enzyme attached to the membrane by two transmembrane domains rather than by one, as most other ecto-enzymes are. A possible answer is presented in this article, which describes a mutational analysis of the residues in the transmembrane domains, suggesting that the domain movements of the extracellular part of the protein during catalysis are coupled to rotational movement of the transmembrane domains.

Q. What are your thoughts on Open Access scientific publishing and the likely changes to the future publishing landscape?

A. Since the results of investigations financed by public money should be open to all interested parties, the evolution towards Open Access is inevitable. The only consideration is whether the fee for publication paid by the investigators is sufficient to pay for the cost of publication

Q. In particular, what do you think about the possibility of changing or replacing the traditional model of pre-publication anonymous peer review?

A. In my opinion, reviews should not be anonymous because a reviewer should be prepared to support the remarks made about the paper in a straightforward and candid fashion, and not hide behind the shield of anonymity. However, there is the view that a paper should be vetted for accuracy before publication and it will take time to convince authors that transparent discussion after publication is

Q. Why did you choose ScienceOpen as a venue for your recent publication?

A. I am in favor of post-publication review by identified scientists as a more transparent way to achieve dissemination of information and support the effort of ScienceOpen in this regard. Disclaimer : approximately 220 articles describing work done in my laboratory have been published, 110 in the Journal of Biological Chemistry and 30 in Biochemistry which are publications of the American Society of Biochemistry and Molecular Biology and the American Chemical Society, respectively. The process of publication in these journals has been straightforward and in general problems were resolved rationally. I have a high opinion of these journals.

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Appendix X

T.-F. Wang, M. Handa and G. Guidotti (1998). Structure and Function of Ectoapyrase (CD39). Drug Developmental Research 45: 245-252.

DRUG DEVELOPMENT RESEARCH 45:245–252 (1998)

Research Article

Structure and Function of Ectoapyrase (CD39)

Ting-Fang Wang, Masahisa Handa, and Guido Guidotti*

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts

Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Although the presence of nucleotidase activities on the extracellular surface of cells has been known for many years, the enzymes responsible for these activities in animal cells have only recently been identified. Here, we describe how we cloned the gene for potato apyrase and, thus, found the way to identify CD39 as the animal cell ectoapyrase. CD39 has two transmembrane domains, one at each end of the molecule, small cytoplasmic NH_2- and COOH-terminal domains, and a large extracellular domain with the enzymatic activity. One of the characteristic features of this enzyme is the loss of activity caused by solubilization with detergents. Exploration of this phenomenon revealed that ectoapyrase (CD39) is a tetramer in the membrane; solubilization with Triton X-100 causes dissociation of the tetramer to monomers with concomitant loss of activity. On the other hand, the enzyme can be solubilized with digitonin without loss of activity and retention of the tetrameric state. The conclusion is that the transmembrane segments of the protein are involved in association of the monomers to tetramers. Drug Dev. Res. 45:245–252, 1998.

Key words: ectoapyrase; CD39; nucleotidase; cross-linking; detergent solubilization; oligomerization

INTRODUCTION

The existence of nucleotidase activities on the outer surface of the plasma membrane of cells has been known for approximately 50 years [Rothstein et al., 1953]. However, the molecular identification of the proteins of this class, called ecto-ATPases [E-ATPases; Plesner, 1995] or ectoapyrases, is recent [Handa and Guidotti, 1996]. Since the latter discovery, several genes for E-ATPases have been cloned. Our interests in ecto-ATPase molecules started with the isolation of a nucleotidase with a broad substrate specificity from rat liver cell membranes, Table 1 [Lin and Fain, 1984; Lin, 1985]. Lin subsequently showed that the active site of the protein was located on the extracellular surface of the plasma membrane [Lin and Russell, 1988]. Interestingly, the majority of the enzyme was found on the canalicular surface of the liver cell, indicating that the activity was principally in the lumen of the bile canaliculus [Lin, 1989].

In an attempt to determine the molecular structure of the enzyme, we obtained the amino acid sequence of peptides of the potato apyrase, an enzyme with an activity similar to that of the rat liver enzyme, and cloned the gene for the potato enzyme [Handa and Guidotti, 1996]. Upon examination of the database for proteins similar to the potato apyrase, Handa found several related proteins present in plants, yeast, parasites, and human cells (Fig. 1). The striking finding was the relationship between a surface antigen present in activated B cells, called CD39 [Maliszewski et al., 1994], and the potato apyrase. This result suggested that CD39 might have apyrase activity and be the animal cell version of the ectoapyrase.

Indeed, Wang and Guidotti [1996] soon showed that CD39 expressed in COS-7 cells endowed the intact cells with apyrase activity and defined the structure of CD39 as the characteristic structure of the E-ATPase family. In subsequent work, several members of the E-ATPase class of proteins have been cloned [Kaczmarek et al., 1996; Wang et al., 1997; Kirley, 1997; Kegel et al., 1997; Chadwick and Frischauf, 1997; Marcus et al., 1997].

The amino acid sequence of the ectoapyrase (CD39) reveals that there is a transmembrane segments at each end of the molecule. Because it has been show that the

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TABLE 1. Substrate Specificity of Ca^{2+}-ATPase and Mg^{2+}-ATPase Activities[a]

Substrate[b]	Ca^{2+}-ATpase (%)	Mg^{2+}-ATPase (%)
ATP	100	100
ADP	32.8	53.6
AMP	0	0
AMP-PNP	0	0
GTP	92.3	120.3
GDP	52.3	81.6
UTP	81.1	111.7
CTP	51.9	129.2
p-Nitrophenyl phosphate	0	0
Ap5A	0	0
Ap5A + ADP	20.3	56.3
Ap5A + ATP	92.3	108.8

[a]The results represent the average of duplicate determination. The specific activities of Ca^{2+}-ATPase was 10.46 µmol/mg/min. The specific activity of Mg^{2+}-ATPase was 5.68 µmol/mg/min.
[b]Final concentration of 2 mM of each substrate was used except for Ap5A, which was 0.4 mM. From Lin (1985) with permission.

NH_2- and COOH-terminal ends of the protein are in the cytoplasm [Maliszewski et al., 1994], the protein has most of its mass on the extracellular side of the plasma membrane, where the active site of the enzyme is located, whereas small NH_2- and COOH-terminal cytoplasmic domains precede and follow the transmembrane segments that anchor the protein in the membrane. This arrangement is unusual for ectoenzymes, because these are usually attached to the membrane by a single protein or lipid link [Semenza, 1986; Kyte, 1995]. The central question in this paper is why animal cell ectoapyrases are anchored in the membrane with two transmembrane segments: is it a coincidence with no functional significance or is it because the transmembrane segments have functions in addition to anchoring?

A clue to the involvement of the transmembrane domains in the function of the ectoapyrase comes from the observation that the enzymatic activity is sensitive to most detergents. This sensitivity is unexpected, because the activity of other ectoenzymes is not affected by detergent solubilization of the membrane [Sigrist et al., 1975; Hooper and Turner, 1988]. The interpretation of the latter finding is that the transmembrane segment serves only to anchor the protein in the membrane and is not involved in enzymatic function; a supposition supported by the ability to detach the enzymatic part of the protein from the membrane by protease or phospholipase activity [Semenza, 1986; Hooper et al., 1997].

We approached the question of the function of the transmembrane domains by constructing mutants of the ectoapyrase lacking either one or both of the transmembrane segments and measuring the enzymatic activity of these mutants in the membrane and after solubilization with detergents. The structures of the mutants are shown in Figure 2.

The effect of detergents on the various constructs is shown in Table 2. Solubilization of the membrane with Triton X-100, C12E9, dodecyl maltoside, and CHAPS caused a substantial loss of enzymatic activity (88%) of the wild-type enzyme. Digitonin on the other hand had only a small effect on activity (10% decrease). In contrast, Triton X-100 and similar detergents had a negligible effect on the enzymatic activities of the soluble ectoapyrase and of the COOH- and NH_2-terminal deletion mutants.

These results suggested that the presence of two transmembrane domains is necessary for the inhibitory effect of some detergents. Accordingly, we wondered whether detergents have an effect on the structure of the ectoapyrase.

The quaternary structure of the ectoapyrases expressed in COS-7 cells was determined by glutaraldehyde cross-linking and sucrose density gradient centrifugation. Glutaraldehyde cross-linking experiments on crude membranes with wild-type ectoapyrase resulted in a ladder of bands with Mr 70,000, ~ 150,000, ~ 230,000, and ~ 300,000 corresponding to dimers, trimers, and tetramers of the Mr 70, 000 polypeptide (Fig. 3A). The indication that wild-type ectoapyrase is a tetramer in cell membranes was supported by the sedimentation behavior of the solubilized enzyme. As can be seen in Figure 4, ectoapyrase solubilized with digitonin had a sedimentation coefficient of 9.8 S, whereas in Triton X-100, the protein had a sedimentation coefficient of 4 S. It is important to point out that digitonin had little effect on the activity of wild-type ectoapyrase, whereas in Triton X-100, 88% of the enzymatic activity was lost (Table 2). On the basis of results obtained with other membrane proteins [Clarke, 1975; Neer, 1974; Aiyer, 1983; Lu and Guidotti, 1996], we estimate that the protein components of the 9.8 S and the 4 S peaks have Mr values of ~330,000 and ~ 75,000, respectively. The relative values of 9.8 S and 4 S for the species of ectoapyrase are the predicted ones for proteins with relative sizes of 4:1 [Tanford, 1961]. The straightforward conclusion is that in digitonin the ectoapyrase is tetrameric, whereas in Triton X-100 it is monomeric. This conclusion is supported by cross-linking done on the gradient fraction and shown in Figure 3B,C. Glutaraldehyde cross-linking of the digitonin-solubilized ectoapyrase tetramers (fraction 5 in Fig. 4B) produced the ladder of bands indicative of monomers, dimers, trimers, and tetramers after a 2-min exposure to 8 mM glutaraldehyde (Fig. 3C); whereas with Triton X-100–solubilized monomeric protein (fraction 13 in Fig. 4B), no cross-linked product was visible after a 10-min exposure to 8 mM glutaraldehyde (Fig. 3B). We conclude that ectoapyrase is a tetrameric protein, and detergents

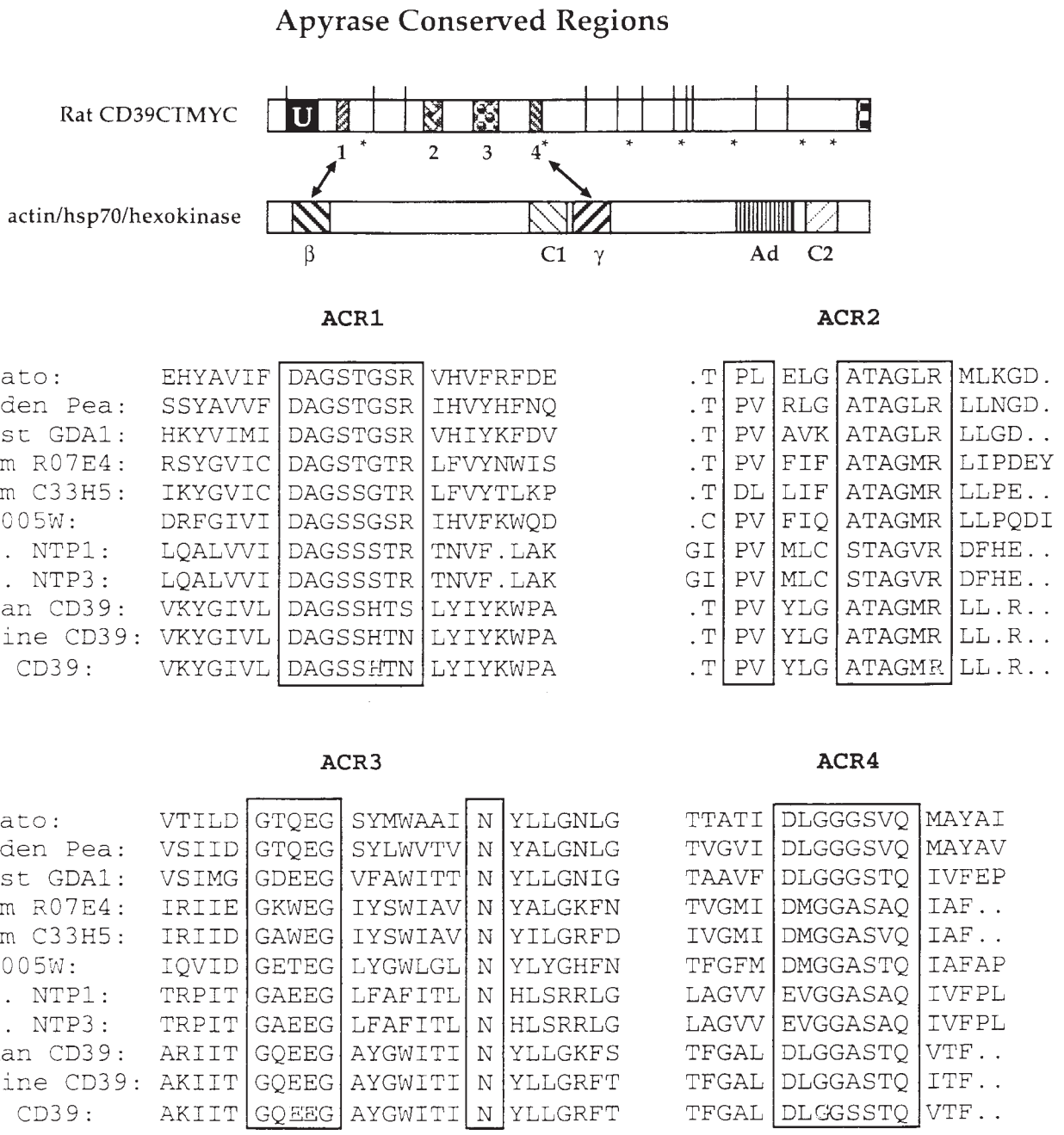

Fig. 1. Four regions of the ectoapyrase are similar to those in several proteins in the data base. The amino acid sequences of potato apyrase, garden pea nucleoside triphosphatase, *Saccharomyces cerevisiae* Golgi guanosine diphosphatase, two *Caenorhabditis elegans* hypothetical 61-kDa proteins, a yeast hypothetical 71.9-kDa protein, *Toxoplasma gondii* nucleoside triphosphate hydrolase isoforms (NTP1 ands NTP3), human cd39, murine CD39, and rat CD39 were aligned by using the GCG Pileup program.

that inhibit ectoapyrase activity (e.g., Triton X-100) dissociate the ectoapyrase tetramer into monomers.

Because the native ectoapyrase is a tetramer, we inquired whether oligomerization is a result of interactions between the extracellular domain or the transmembrane domains. Soluble ectoapyrase, lacking the transmembrane and cytoplasmic domains, sedimented more slowly than bovine serum albumin (BSA) on a sucrose density gradient (Fig. 5A), and there were no cross-linked products found after treatment with glutaraldehyde (Fig. 5B). These results indicate that soluble ectoapyrase is a monomer. The conclusion is that the extracellular domain of wild-type ectoapyrase is not sufficient for oligomerization.

Figure 6 presents the results of sucrose density gra-

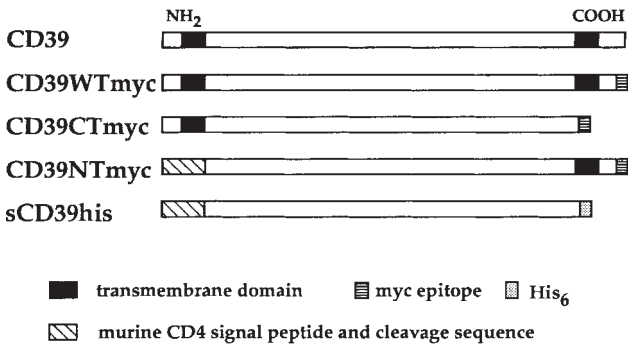

Fig. 2. Construction of the soluble and mutant ectoapyrase proteins. Schematic representation of wild-type CD39, the myc-tagged CD39 (CD39WT-Myc), the NH_2-terminal deletion mutant (CD39NT-Myc), and the COOH-terminal deletion mutant (CD39CT-Myc). The transmembrane domains of CD39, the CD4 signal peptide and cleavage sequence, the Myc epitope, and the hexahistidine peptide (His_6) are indicated. From Wang et al. [1998] with permission.

dient sedimentation of CD39CT-Myc and CD39NT-Myc mutant ectoapyrases. Like wild-type ectoapyrase, CD39CT-Myc and CD39NT-Myc behaved as monomeric proteins after solubilization with Triton X-100 (Fig. 6A,B). Interestingly, most of CD39CT-Myc and a fraction of CD39NT-Myc were tetrameric after solubilization with digitonin, indicating that either one of the two transmembrane domains of ectoapyrase is sufficient for ectoapyrase oligomerization. We conclude that the transmembrane domains of ectoapyrase are responsible for the formation of ectoapyrase tetramers.

The dissociation of the tetrameric enzyme by Triton X-100 is reversible. When the monomers in fraction 13 of the Triton X-100 gradient in Figure 6B were sedimented through a sucrose density gradient containing 0.2% digitonin, the enzyme had the sedimentation behavior of the tetrameric proteins and the total activity of the applied monomer increased by five- to sixfold (Fig. 7). Together, these results suggest that ectoapyrase wild-type tetramers have higher catalytic activity than do the wild-type monomers.

We conclude that interactions between the NH_2- and COOH-terminal transmembrane domains of the four monomers are required for stabilization of the tetramers in a conformation with an increased enzymatic activity compared with that of the monomers. Because mutant ectoapyrases with only one transmembrane domain (CD39CT-Myc and CD39NT-Myc) are tetramers, one transmembrane domain appears to be sufficient for ectoapyrase oligomerization. However, these interactions are not able to promote the conformation with a high enzymatic activity. Without interactions between the NH_2- and COOH-terminal transmembrane domains of the four monomers, the conformation of the tetramer is altered so that the enzymatic activities of CD39NT-Myc and CD39CT-Myc are smaller than that of the wild-type tetramer and similar to those of the wild-type monomer and the soluble CD39. Because the mutants have a conformation with a low enzymatic activity, they are not affected by detergent solubilization.

Two other members of the E-ATPase protein family [Plesner, 1995], human ecto-ATPase [CD39L1; Chadwick and Frischauf, 1997] and human Golgi lumenal UDPase [Wang and Guidotti, 1998], are also tetrameric proteins. However, other E-type ATPases that are not membrane bound such as potato apyrase (Table 2), *Toxoplasma gondii* NTPases [Bermudes et al., 1994], and *Tetrahymena* ecto-ATPase [Smith et al., 1997] are not sensitive to detergents; and the potato apyrase has been shown to be monomeric [Kettlun et al., 1982]. This finding is a further indication that the transmembrane segments are the key elements in the oligomerization of the enzyme and in the effect of detergents on the activity of the enzyme. The effect of detergents is to disrupt the interactions between the NH_2- and COOH-terminal transmembrane domains, dissociating the oligomers into monomers with a reduced enzymatic activity.

The membrane topology of ectoapyrase and ecto-ATPase is unusual for ectoenzymes, because these are usually attached to the membrane by a single transmembrane domain or lipid link [Semenza, 1986; Kyte, 1995]. There are, however, several membrane proteins with two transmembrane domains separated by a large protein

TABLE 2. Detergent Effects on the Enzymatic Activities of Various Apyrase Proteins[a]

	Control (%)	Triton X-100[b] (%)	DIgitonin (%)
CD39 (WT) crude membrane	100 ± 1	12 ± 1	90 ± 2
Soluble CD39	100 ± 2	92 ± 3	95 ± 3
Potato apyrase	100 ± 1	155 ± 4	ND
CD39CT-Myc crude membrane	100 ± 2	145 ± 1	ND
CD39NT-Myc crude membrane	100 ± 1	144 ± 2	ND

[a]Soluble CD39, potato apyrase, and the crude membranes of COS-7 cells transfected with wild-type CD39, CD39NT-Myc, or CD39CT-Myc cDNAs were treated with or without 1% detergents at 4°C for 1 h then assayed for calcium-dependent. ATPase activity in the presence of 1mM NaN_3, 0.5mM Na_3 VO_4, and 1mM ATP (see text). The calcium-dependent ATPase activities of samples treated with detergents are presented as the percentage of the actvitities of control samples. Values are means ± standard deviation of three independent experiments. ND, not determined.
[b]Similar results were obtained with C12E9, dodecyl maltoside, CHAPS, and octylglucoside. From Wang et al. (1998) with permission.

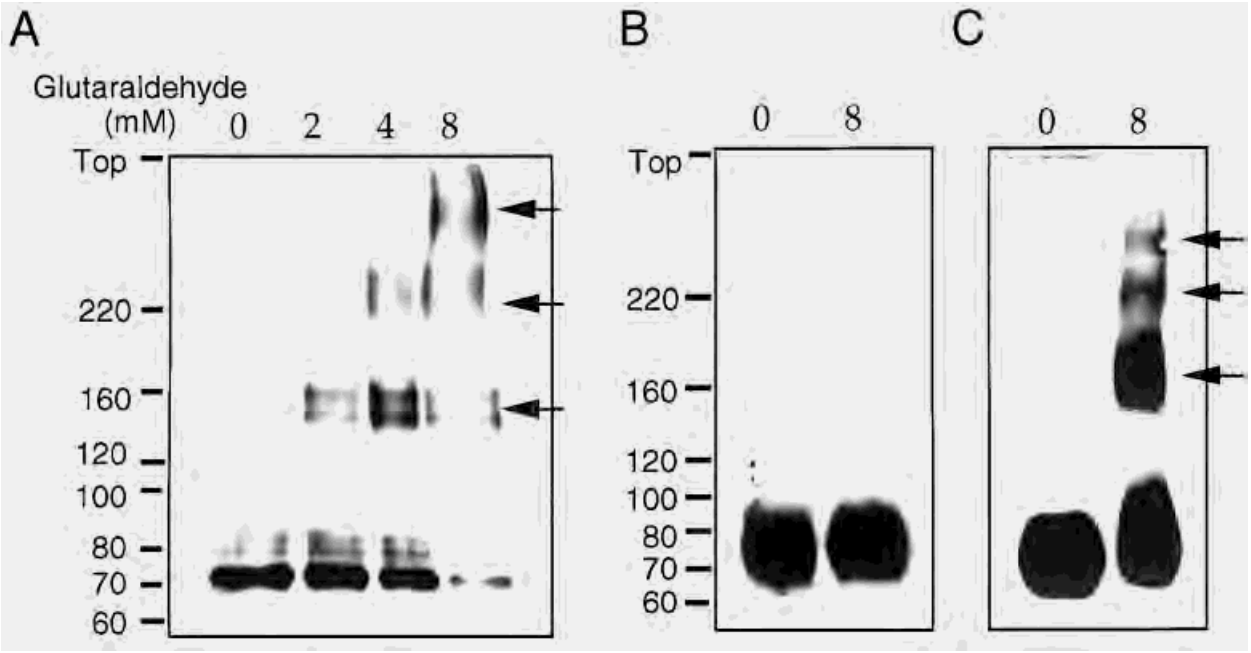

Fig. 3. Glutaraldehyde cross-linking of wild-type ectoapyrase before and after solubilization with detergents. **A:** Crude membranes (25 ml) of COS-7 cells transfected with wild-type CD39 cDNA were incubated with 0–8 mM glutaraldehyde for 2 min. **B:** Triton X-100 solubilized ectoapyrase monomers (fraction 27 in Fig. 6A) were incubated with 0 or 8 mM glutaraldehyde for 10 min. The samples were separated by 5.5% nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was detected by blotting with anti-CD39 antibody. Positions of molecular size markers are shown in kilodaltons. **C:** Digitonin-solubilized ectoapyrase tetramers (fraction 20 in Fig. 5A) were incubated with 0 or 8 mM glutaraldehyde for 2 min and analyzed by SDS-PAGE as in B. From Wang et al. [1998] with permission.

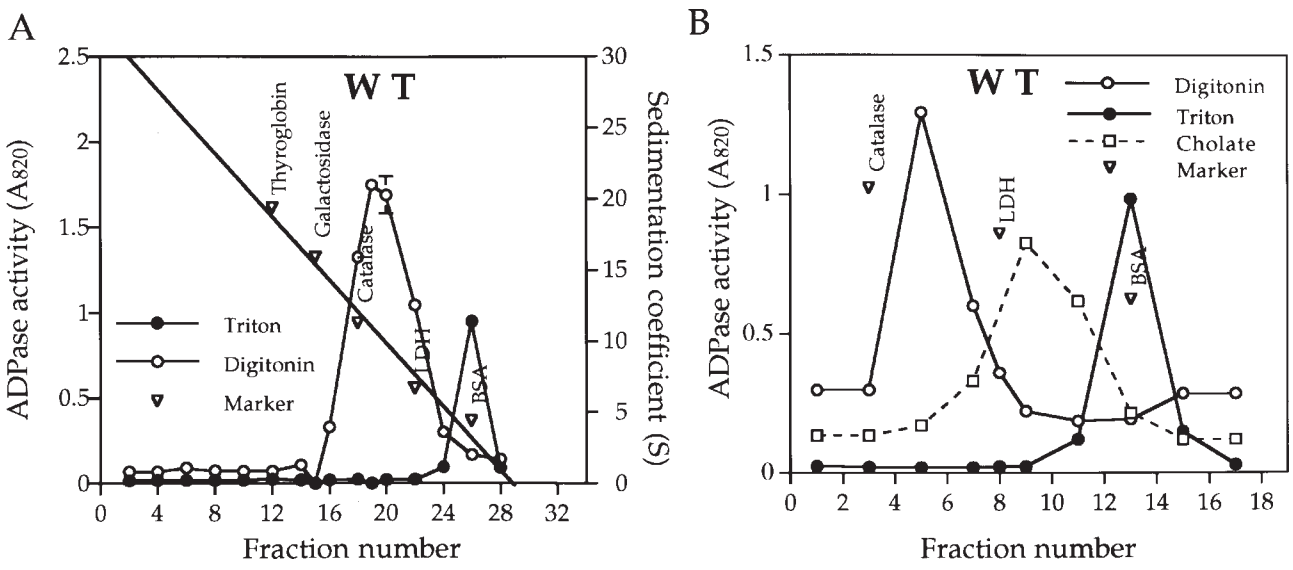

Fig. 4. Sucrose density gradient sedimentation of detergent solubilized ectoapyrase proteins. Crude membranes (40 ml) of COS-7 cells transfected with wild-type CD39 cDNA were solubilized with 1% digitonin, 1% Triton X-100, or 2% cholate for 15 min on ice, and then separated on 5–25% (w/v) (A) or 5–20% (w/v) (B) sucrose density gradients as described in the text. Each fraction was assayed for Ca^{2+}-dependent ADPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4. The positions of marker proteins were determined by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis. The calibration curve was obtained by plotting the fraction number vs. the sedimentation coefficients of the marker proteins. The molecular weights of detergent solubilized ectoapyrases were estimated by their sedimentation coefficients based on the results of Liu and Guidotti [1997]. From Wang et al. [1998] with permission.

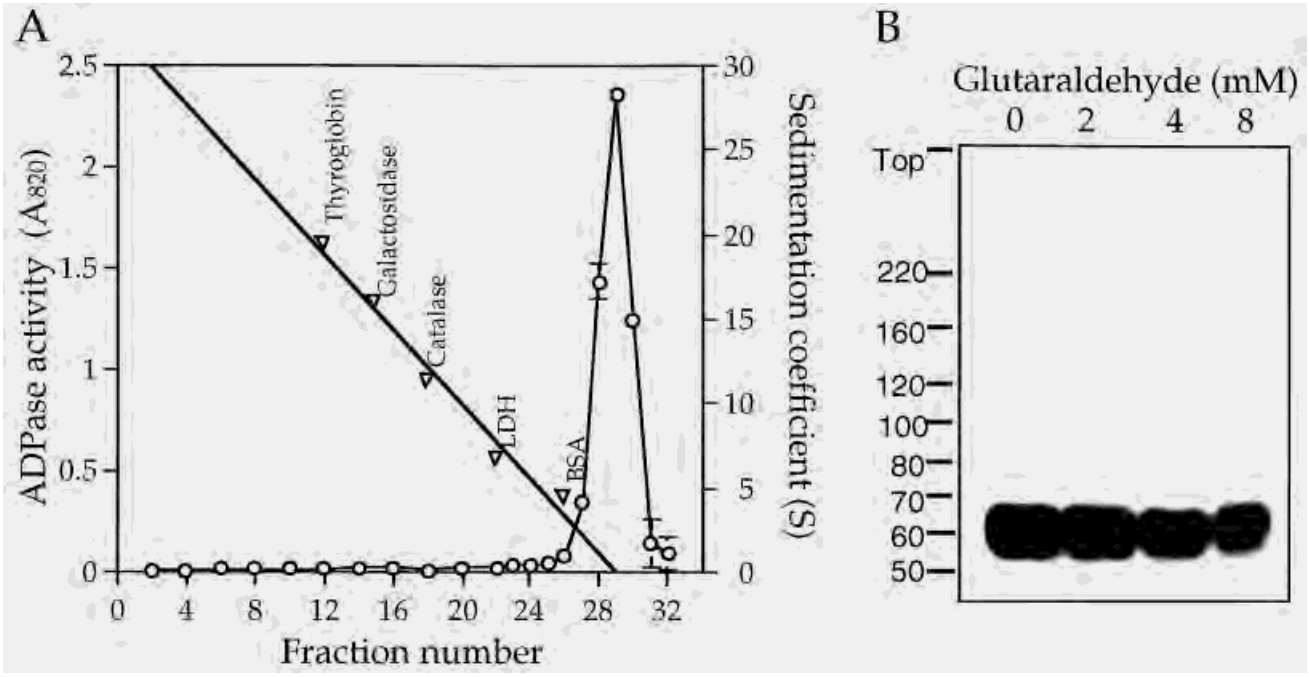

Fig. 5. Soluble ectoapyrase is a monomeric protein. **A:** Sucrose density gradient sedimentation of soluble ectoapyrase. The purified protein (20 mg) was sedimented on 5–25% (w/v) sucrose density gradient as described in the text. Fractions were collected and assayed for Ca^{2+}-dependent ADPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4. The positions of marker proteins were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). B. Glutaraldehyde cross-linking of soluble ectoapyrase. Three milligrams of soluble ectoapyrase were incubated with 0–8 mM glutaraldehyde for 10 min. The samples were separated by 7.5% nonreducing SDS-PAGE, and soluble ectoapyrase was detected by immunoblotting with anti-CD39 antibody. Positions of molecular size markers are shown in kilodaltons. From Wang et al. [1998] with permission.

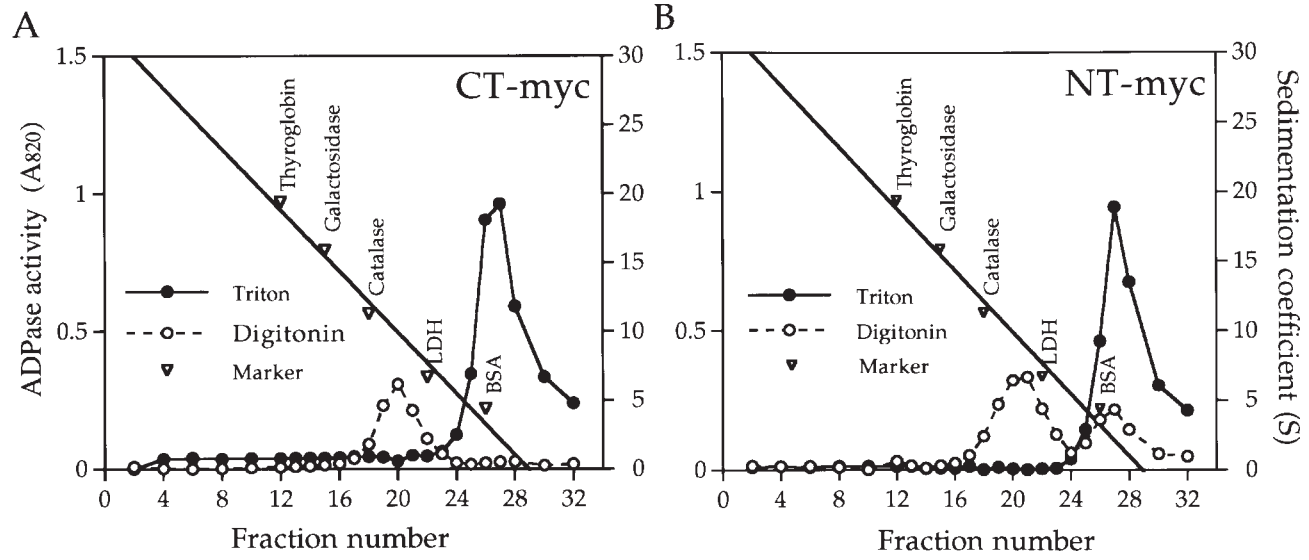

Fig. 6. Sucrose density gradient sedimentation of detergent solubilized mutant ectoapyrase proteins. Crude membranes from COS-7 cells transfected with CD39CT-Myc (**A**) and CD39NT-Myc (**B**) cDNA were solubilized with 1% digitonin or 1% Triton X-100 for 15 min on ice and then separated on 5–25% (w/v) sucrose density gradients as described in the text. Each fraction was assayed for Ca^{2+}-dependent ADPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4. The positions of marker proteins were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular weights of detergent solubilized mutant ectoapyrases were estimated by their sedimentation coefficients based on the results of Liu and Guidotti (1997). From Wang et al. [1998] with permission.

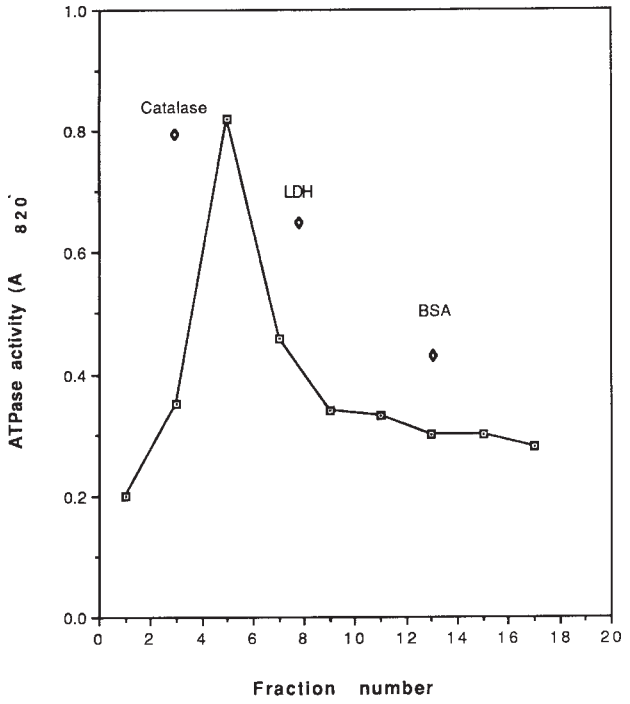

Fig. 7. Reconstitution of ectoapyrase tetramers from monomers by sucrose density gradient sedimentation. Triton X-100 solubilized ectoapyrase monomers (100 ml of fraction 13 in Fig. 6B) were sedimented on a 5–20% (w/v) sucrose density gradient containing 0.2% digitonin as described in the text. Fractions were collected and assayed for Ca^{2+}-dependent ATPase activity in the presence of 1 mM NaN_3 and 0.5 mM Na_3VO_4. The position of marker proteins was determined by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis. The total Ca^{2+}-dependent ATPase activity of the reconstituted ectoapyrase tetramers was integrated (10–12 absorbance units) and compared with that of the ectoapyrase monomers applied to the gradient (2 absorbance units). From Wang et al. [1998] with permission.

segment, i.e., a family of receptors participating in sensory systems [Swanson et al., 1994] and a class of channel proteins [North, 1996]. The bacterial aspartate receptor is an example of the former group; it has one transmembrane segment at the NH_2-terminus and the second one in the middle of the molecule, so that the extracellular and cytoplasmic domains are of approximately equal size [Wang and Koshland, 1980]. The channel proteins include P2X purinergic receptors [Surprenant et al., 1996], inwardly rectifying K^+ channels [Doupnik et al., 1995], an epithelial sodium channel (ENaC) [Canessa et al., 1994], and the mscL mechanosensitive channel of *Escherichia coli* [Sukharev et al., 1994]. Interestingly, P2X receptors [Kim et al., 1997], inwardly rectifying K^+ channels [Kubo et al., 1993], and the epithelial sodium channel [Firsov et al., 1998] are also tetrameric proteins. We wonder whether the subunit interactions in these channels are similar to those in the ectoapyrase tetramer. Conversely, it is possible that the ectoapyrase also has channel or pore activity.

The principal conclusion of this work is that the transmembrane domains of the ectoapyrase mediate ectoapyrase oligomerization and affect enzymatic activity through heterologous interactions. Detergent inhibition of ectoapyrase activity is due to the dissociation of tetramers into monomers. Ectoapyrase mutants lacking one or both transmembrane domains have lower enzymatic activity than the wild-type enzyme, and the activity is not affected by detergents.

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Appendix XI

A. Grinthal and G. Guidotti (2007). Bilayer Mechanical Properties Regulate the Transmembrane Helix Mobility and Enzymatic State of CD39. Biochemistry 46: 279-290.

Bilayer Mechanical Properties Regulate the Transmembrane Helix Mobility and Enzymatic State of CD39†

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ABSTRACT: CD39 can exist in at least two distinct functional states depending on the presence and intact membrane integration of its two transmembrane helices. In native membranes, the transmembrane helices undergo dynamic rotational motions that are required for enzymatic activity and are regulated by substrate binding. In this study, we show that bilayer mechanical properties regulate conversion between the two enzymatic functional states by modulating transmembrane helix dynamics. Alteration of membrane properties by insertion of cone-shaped or inverse cone-shaped amphiphiles or by cholesterol removal switches CD39 to the same enzymatic state that removal or solubilization of the transmembrane domains does. The same membrane alterations increase the propensity of both transmembrane helices to rotate within the packed structure, resulting in a structure with greater mobility but not an altered primary conformation. Membrane alteration also abolishes the ability of the substrate to stabilize the helices in their primary conformation, indicating a loss of coupling between substrate binding and transmembrane helix dynamics. Removal of either transmembrane helix mimics the effect of membrane alteration on the mobility and substrate sensitivity of the remaining helix, suggesting that the ends of the extracellular domain have intrinsic flexibility. We suggest that a mechanical bilayer property, potentially elasticity, regulates CD39 by altering the balance between the stability and flexibility of its transmembrane helices and, in turn, of its active site.

Of the various enzymes that process nucleotides at the cell surface and in the lumen of intracellular organelles, the ectonucleoside triphosphate diphosphohydrolases (eNTPDases)[1] have emerged as the major family that is responsible and specific for breaking the terminal phosphoanhydride bonds of tri- and dinucleotides (*1*, *2*). As such, they have been shown or hypothesized to modulate many of the signaling and biosynthetic processes in which extracytoplasmic nucleotides play a role, including vascular homeostasis, cell size maintenance, neuronal signaling, immune function, and protein and lipid modification (*3*−*8*). Consistent with the variety of tasks they perform on and in the cell, different family members exhibit different localizations and specificities: some reside on the plasma membrane and others in the Golgi, lysosomes, or endoplasmic reticulum, while each has a characteristic hierarchy of preferences for di- versus trinucleotides as well as for different bases (*1*, *9*−*11*). The defining structural feature shared by all family members is a set of five short sequences called apyrase conserved regions (ACRs) (*12*, *13*), two of which are thought to constitute phosphate binding loops on the basis of homology to the nucleotide binding domain of the actin/hsp70/hexokinase ATPase superfamily (*12*, *14*) and all of which are required for enzymatic activity (*15*−*18*). Like the enzymatic activity, all five ACRs are located in the extracytoplasmic domain, consistent with their putative identity as the active site.

Like most other ectoenzymes (*19*), some eNTPDases are secreted into the extracytoplasmic space or anchored in the membrane by a single transmembrane helix (*20*). However, several family members exhibit a topology commonly observed among channels, transporters, and receptors but unusual for ectoenzymes: a large extracellular domain flanked by two transmembrane helices (*9*−*11*, *20*, *21*). Why the active site would be held down by transmembrane domains on both sides rather than by a single protein or lipid link was originally a mystery, but in recent years, it has become clear that the two transmembrane helices are intricately linked to active site function rather than simply serving as anchors (*22*). In particular, CD39 (eNTPDase1), a plasma membrane-bound apyrase, loses 90% of its activity upon removal of either transmembrane helix or both as well as upon disruption of their native state by detergent solubilization (*23*). Furthermore, kinetic and mutational analysis of the native and truncated forms has revealed that they differ not only in total activity but also in hydrolysis mechanisms, the role of a putative phosphate binding loop ACR1, substrate specificity, and the presence or absence of release of intermediate ADP during ATP hydrolysis (*24*−*26*). All of these enzymatic features line up one way when CD39 is in

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[1] Abbreviations: eNTPDase, ecto-nucleoside triphosphate diphosphohydrolase; ACR, apyrase-conserved region; TM1, transmembrane helix 1; TM2, transmembrane helix 2; AA, arachidonic acid; OA, oleic acid; DOHA, docosahexaenoic acid; LPL, lysophospholipid; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; LPG, lysophosphatidylglycerol; LPE, lysophosphatidylethanolamine; CuP, copper phenanthroline; CD, cyclodextrin; NT, N-terminally truncated CD39; CT, C-terminally truncated CD39.

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its native state and a different but consistent way regardless of whether the native state is disrupted via removal of the N-terminal, the C-terminal, or both transmembrane helices or by detergent solubilization. Thus, native CD39 function requires both transmembrane helices to be present and in the membrane, and the state of the transmembrane helices governs which of at least two distinct functional states the active site occupies (*24*).

These studies established that several physiologically important features of the enzyme depend on the transmembrane domains, but the fact that these insights were won by wholesale removal of the transmembrane domains or complete extraction from the bilayer left open two major questions. (1) What do the transmembrane domains do that makes them so important? (2) Do they ever change organization and regulate switching between enzymatic states in vivo? Recently, using disulfide cross-linking of cysteines substituted for TM1 and TM2 residues, we found that the transmembrane helices interact strongly both within and between subunits, in particular near the extracellular side of the membrane, suggesting a potential role for specific intra- and intermolecular helical interactions (*28*). However, each helix exhibits a high degree of rotational mobility within the packed structure; significant cross-linking takes place between all faces of TM1 and TM2 within a molecule as well as between all faces of TM1 and TM1′ and of TM2 and TM2′ of different subunits. Specific primary interaction surfaces were identified by differences in temperature dependence, supporting a distinct packing arrangement rather than complete randomness. Nevertheless, locking the helices in the primary or any other orientation results in an activity loss comparable to that induced by solubilization or by complete removal of the transmembrane domains. These results indicate that specific helix interactions and orientation do matter but suggest that the ability of the helices to move relative to each other is at least as important if not more important than their ability to stabilize one optimal arrangement. Substrate binding in turn regulates mobility, further suggesting that a balance between stability and flexibility of specific interactions underlies the functional relationship of the transmembrane domains with the active site.

Are any of these features subject to regulation by the environment? Several lines of reasoning suggest that the transmembrane helices might be primed to respond to changes in the membrane and to translate them into changes in the enzymatic functional state. Energetically, altering membrane physical properties may alter the bilayer deformation energy for a given protein conformational change (*29*); if the energy difference between protein conformations is relatively low compared to the associated bilayer deformation energy, appropriate alteration of membrane properties can be sufficient to allow the conformational change. For CD39, the extracellular domain appears not to pose a barrier to whatever conformational change alters its function, since it achieves the altered state by default in the absence of transmembrane domains (*24*). The mobility discussed above suggests that the transmembrane helices themselves are also not optimized for strong association (*28*), potentially leaving the membrane as a significant factor keeping the protein in its native state. Physiologically, extracellular ATP is a first-line response to and normalizing agent for a wide range of alterations in membrane physical properties, such as those due to swelling, sheer stress, or inflammatory responses (*3*−*5*); as a primary modulator of cell surface ATP concentrations, CD39 activity might be expected to be tailored to the state of the membrane as well. A recent report provided direct evidence that CD39 activity does indeed vary on the basis of the cholesterol content of the membrane (*30*), although whether this reflects a specific requirement for cholesterol or a response to mechanical bilayer properties is unknown.

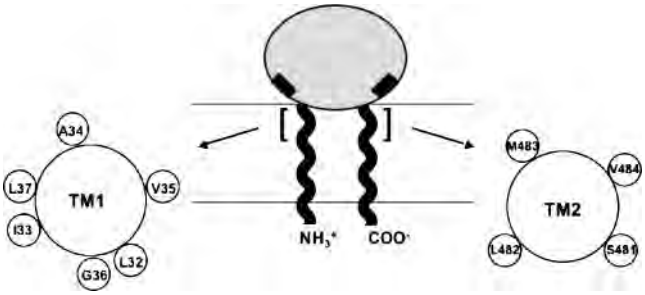

FIGURE 1: Organization of CD39 extracellular and transmembrane domains. The shaded oval represents the extracellular domain. N- and C-terminal helical strands represent transmembrane domains TM1 and TM2, respectively. Black rectangles adjacent to TM1 and TM2 represent active site regions ACR1 and ACR5, respectively. The TM1 and TM2 residues examined in this study are highlighted with brackets, and their relative positions around the helix are shown in helical-wheel format.

In this study, we ask whether mechanical properties of the membrane regulate CD39 function and, if so, what changes in the transmembrane helices translate membrane properties to the active site. We find that a range of amphiphiles with unrelated structures that alter membrane curvature in opposite ways all convert CD39 to the same functional state that removal of the transmembrane helices does. In parallel they, as well as cholesterol removal, increase the rotational mobility of both transmembrane helices. We suggest that a general mechanical bilayer property, potentially elasticity, regulates CD39 by altering the balance between the stability and mobility of its transmembrane helices, thereby modulating the coupling between transmembrane domains and the active site.

EXPERIMENTAL PROCEDURES

DNA Construction. Full-length CD39 forms containing single cysteine substitutions in TM1 or TM2 or paired cysteine substitutions in TM1 and TM2 were constructed previously as described in ref *28*. Figure 1 shows the positions of the cysteine-substituted residues in the context of the entire CD39 molecule as well as their organization in helical-wheel format. CD39 lacking TM1 (NT) and containing single cysteine substitutions in TM2 was constructed by replacing the *Sac*II−*Not*I fragment from pcDNA3-NTmyc (described in ref *23*) with the *Sac*II−*Not*I fragment from the TM2 cysteine-substituted versions of pcIneo-CD39HA. This procedure also replaced the C-terminal myc tag with an HA tag. CD39 lacking TM1 (NT) and containing single cysteine substitutions in TM2 was constructed by PCR amplification of TM1 and extracellular domain coding regions from pcIneo-CD39HA containing TM1 cysteine substitutions. T7EEV (Promega) was used as the forward primer, and the reverse primer annealed to the CD39 sequence encoding MIPAEQP prior to TM2 (as in ref *23*) and contained the coding sequence for an HA tag followed by a *Not*I site. The

resulting fragment was digested with *Nhe*I and *Not*I and inserted into pcIneo.

Preparation of COS7 Cell Crude Membranes. COS7 cells were transiently transfected with 6 μg of plasmid per 100 mm dish using Lipofectamine (Invitrogen). Cells were harvested 72 h after transfection, and crude membranes were prepared as described in ref *31*. Membranes were resuspended in 50 μL of Tris-HCl (pH 7.8) per 100 mm plate, and aliquots were flash-frozen in liquid nitrogen and stored at −80 °C.

Nucleotidase Assays. Nucleotidase assays were carried out in a 200 μL solution containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM HEDTA, 2 mM ATP or ADP, and $MgCl_2$ or $CaCl_2$ to give the indicated concentrations of free Mg^{2+} or Ca^{2+} as calculated and described in ref *24*. In most cases, $MgCl_2$ was used to be consistent with the fact that the cytoplasmic side of the membrane is not usually exposed to high Ca^{2+} concentrations and thus to avoid any potential nonphysiological interference of Ca^{2+} with amphiphile insertion or distribution. Nevertheless, similar results were obtained with Ca^{2+}. Amphiphiles were diluted in assay buffer from stock solutions as follows. An AA (Sigma) stock solution was 300 mM in EtOH. OA (Sigma) was 200 mM in EtOH. DOHA (Sigma) was 300 mM in EtOH. Stearic acid (Sigma) was 80 mM in EtOH. Arachidic acid (Sigma) was 150 mM in chloroform. All LPLs were 10 mM in H_2O (LPC and LPI from Sigma and LPG and LPE from Avanti). Triton X-100 was 10% in H_2O (solution from Pierce). Cyclodextrin was 6% in H_2O. Membrane concentrations were approximately 5 μg/mL. Reaction mixtures containing all components except the nucleotide were preincubated for 10 min at 37 °C, and reactions were started by addition of nucleotide, mixtures incubated for 20 min at 37 °C, and reactions stopped by addition of 300 μL of 10% SDS. The phosphate concentration was determined by the colorimetric method (*32*). For the reversibility experiments, membranes were preincubated for 10 min at 37 °C in reaction solutions containing the indicated amphiphiles, 10 mg/mL BSA in assay buffer was added to a final concentration of 0.5 mg/mL, solutions were preincubated for an additional 10 min at 37 °C, and reactions were carried out as described above.

Oxidative Cross-Linking. Cysteine-substituted CD39 constructs were cross-linked in crude membranes using copper phenanthroline (CuP) as the oxidizing agent. Copper phenanthroline was prepared by combining cupric sulfate and 1,-10-phenanthroline (Sigma) at a 1:3 molar ratio in water and used at a final concentration of 0.3 mM (expressed as the Cu^{2+} concentration) except where otherwise indicated. For all constructs, time courses were initially carried out at 0.03, 0.3, and 3 mM CuP to confirm that CuP concentration was not the rate-limiting factor. Reactions were carried out in 12 mM Tris-HCl at 37 °C for 30 s or 1, 2, or 5 min, except for intramolecular cross-linking of A34C/V484C, which was cross-linked at 22 °C to obtain an observable rate. Reactions were performed at the same membrane concentrations that were used for activity measurement; to facilitate using the dilutions of membranes and amphiphiles as described above, a 500 μL reaction volume was used. Membranes were added to buffer-containing amphiphiles, and reactions were started by addition of a 100× solution of CuP to prevent significant changes in amphiphile concentrations. Reactions were stopped by adding 20 μL of 0.5 M EDTA for a final EDTA concentration of 20 mM, and membranes were collected by centrifugation in a Ti70.1 rotor at 40 000 rpm for 30 min at 4 °C. The centrifugation step also served the purpose of confirming that protein was not extracted from the membrane during any of the amphiphile treatments. Membranes were resuspended in 30 μL of nonreducing SDS loading buffer containing 20 mM *N*-ethylmaleimide (NEM) and 20 mM EDTA and used immediately for SDS−polyacrylamide gel analysis.

Western Blot Analysis. Samples resuspended as described above were resolved on a 5.5% SDS−polyacrylamide gel as described in ref *33* and transferred to nitrocellulose at 250 mA for 2.5 h. Nitrocellulose membranes were probed with anti-HA11 monoclonal antibody (Covance) in 2% milk in Tris-buffered saline and 0.1% Tween, followed by a secondary anti-mouse horseradish peroxidase-conjugated antibody (Sigma) in 3% milk in Tris-buffered saline and 0.1% Tween. The protein was visualized by chemiluminescence (substrate from Pierce) and exposure to film, and the percent cross-linked was determined by quantitation of the dimer band as a percent of the sum of monomer and dimer bands in a given sample.

RESULTS

Mechanical bilayer properties can be altered by direct pressure, by reversible insertion of amphiphilic compounds, or by variations in lipid composition. CD39 most likely encounters each of these situations in vivo; here we have employed a series of amphiphilic compounds that have been shown to mimic the effects of physical pressure and to alter the bilayer deformation energy for several types of mechanosensitive channels (*29*, *34*−*37*). Compounds were added to isolated native membranes to allow direct comparison of the native and altered states and to prevent potential indirect effects mediated by cytoplasmic components.

Unsaturated Fatty Acids Reversibly Alter the Enzymatic Functional State. Cis-unsaturated fatty acids are thought to alter bilayer properties by virtue of their inverse cone shape; the combination of a wide hydrocarbon tail and a narrow headgroup alters the packing of the native lipids, introducing negative spontaneous curvature as well as changes in membrane tension and elasticity. As shown in Figure 2A, adding arachidonic acid (AA) to membranes from COS7 cells transfected with CD39 reduces the ATPase activity by approximately 80%, similar to the activity loss that occurs when the transmembrane domains are removed. Although the molar fraction of AA in the membrane is unknown, the K_i of approximately 3 μM is comparable to that observed for other proteins known to be sensitive to membrane mechanical properties (*34*, *35*). The same effect is observed for other unsaturated fatty acids with varying degrees of unsaturation; oleic acid (OA), which has 18 carbons and one degree of unsaturation, and docosahexaenoic acid (DOHA), which has 22 carbons and six degrees of unsaturation, inhibit ATPase activity to the same extent that AA does, which has 20 carbons and four degrees of unsaturation. OA, the smallest and least unsaturated molecule in the series, has the highest K_i of approximately 6 μM.

As shown in Figure 2B, the transmembrane domains are required for sensitivity to AA. The membrane-bound construct lacking TM1 (NT) is only slightly sensitive to AA up

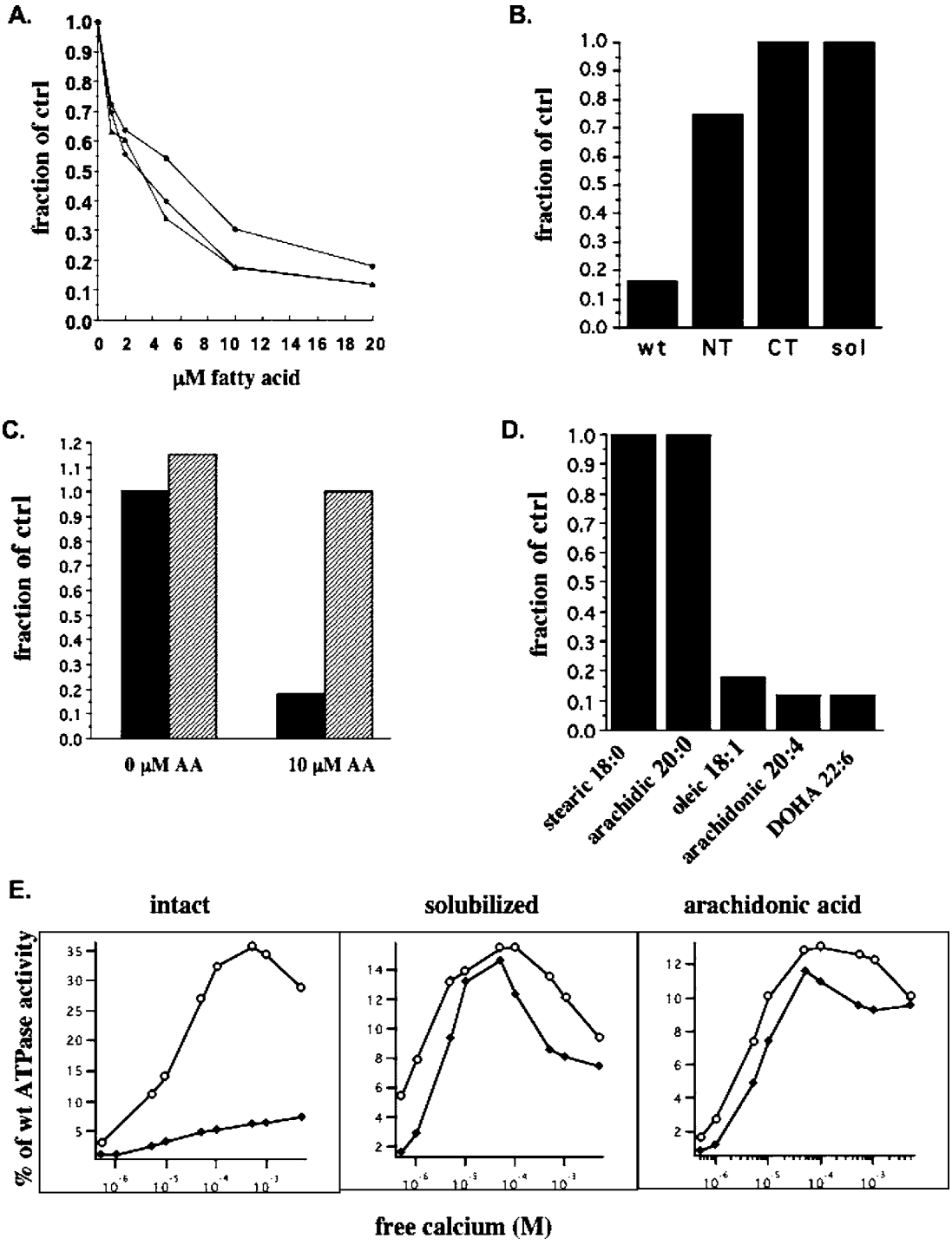

FIGURE 2: ATPase activity in the presence of unsaturated fatty acids. (A) The ATPase activity of full-length CD39 was measured in isolated membranes in the presence of the indicated concentrations of arachidonic acid (◆), oleic acid (●), or docosahexaenoic acid (▲). (B) The ATPase activity in the presence of 10 μM AA was measured for full-length CD39, singly truncated NT lacking TM1, singly truncated CT lacking TM2, and soluble CD39 lacking TM1 and TM2. Activities are expressed as a fraction of the native full-length CD39 ATPase activity. (C) Full-length CD39 was exposed to 0 or 10 μM AA followed by direct measurement of ATPase activity (black bars) or followed by treatment with BSA prior to activity measurement (gray bars). (D) The full-length CD39 activity was compared in the presence of saturated fatty acids stearic and arachidic acid and their unsaturated counterparts OA and AA as well as DOHA, each at 20 μM. (E) ATPase (◆) and ADPase (○) activities in the presence of the indicated free Ca^{2+} concentrations were measured for full-length CD39-H59G in intact membranes (panel 1), solubilized in 1% Triton X-100 (panel 2), and in the presence of 10 μM AA (panel 3) and expressed as a percent of maximal native wild-type CD39 ATPase activity.

to concentrations as high as 100 μM, similar to the minor degree of inhibition observed upon detergent solubilization. Both CT, the membrane-bound counterpart lacking TM2, and soluble CD39, lacking both TM1 and TM2, are completely insensitive to AA up to the same concentrations. The same results were obtained with DOHA and OA. In contrast, inhibitors that are expected to inhibit via the extracellular domain, such as FSBA, trinitrophenol, and low concentrations of SDS, have comparable effects on both native and truncated constructs (data not shown). These results suggest that low concentrations of unsaturated fatty acids mimic removal of the transmembrane helices or detergent solubi-

lization, consistent with the idea that they inhibit via their effects on the membrane.

To verify that unsaturated fatty acids act via a reversible process rather than by destroying the membrane, we pretreated membranes with AA and either measured activity directly or used bovine serum albumin (BSA) to extract AA from membranes before measuring activity. Figure 2C shows that pretreatment and activity measurement in the presence of AA produces the same inhibitory effect that has been observed previously; however, pretreating with AA, adding BSA, and measuring activity in the presence of both produce the same result that not adding AA does. The same result was obtained with OA and DOHA. Unsaturated fatty acids therefore appear to inhibit activity via reversible insertion in the membrane, consistent with the conditions under which they alter the membrane mechanical properties discussed above.

As summarized in Figure 2D, unsaturation is required for inhibition of activity. The saturated counterparts of OA and AA, stearic and arachidic acid, respectively, have no effect despite their expected ability to partition in the membrane. The wide hydrocarbon chain and resulting inverse cone shape therefore appear to underlie the effects of unsaturated fatty acids on the membrane and on CD39.

Do unsaturated fatty acids convert CD39 to the same altered functional state that solubilization or removal of the transmembrane domains does? A key distinction between the two states is their difference in ATP and ADP hydrolysis mechanisms; in the native state, residue H59 in ACR1 is critical to ATPase activity but less critical to ADPase activity, while in the solubilized state, both activities are independent of this residue. As a result, native CD39 with an H59G substitution is an ADPase but solubilized CD39-H59G has equal ATPase and ADPase activities each comparable to those of solubilized wild-type CD39 (*24*). We therefore compared the kinetic profiles of CD39-H59G in intact membranes, when solubilized in 1% Triton X-100, and in native membranes treated with 10 μM AA. As illustrated in Figure 2E, AA mimics solubilization in its effect on the ATPase:ADPase ratio as well on total ATPase and ADPase activities. Activities are expressed as the percent of wild-type CD39 ATPase activity in intact membranes, as determined previously for native and solubilized CD39-H59G; like solubilization, AA decreases ADPase activity, increases ATPase activity, and changes the ATPase:ADPase ratio from 1:6 to approximately 1:1. AA, like 1% Triton X-100, also reduces the apparent K_m for Ca^{2+} by ~1 order of magnitude. The identity of the kinetic profile to that of the solubilized state and its marked difference from the native state suggest that, rather than denaturing or otherwise incapacitating the enzyme, addition of AA to native membranes converts CD39 to the same mechanistic functional state that solubilization or truncation does.

Cone-Shaped Amphiphiles Mimic Unsaturated Fatty Acids. In contrast to unsaturated fatty acids, lysophospholipids (LPLs) and Triton X-100 have narrow hydrocarbon tails and wide headgroups. As a result, their insertion into the membrane at concentrations below their critical micelle concentrations (CMC) also alters packing of the native lipids; like inverse cone-shaped molecules, they change various mechanical features of the membrane, including tension and elasticity, but they induce positive rather than negative spontaneous curvature. In Figure 3A, we show that addition of lysophosphatidylcholine (LPC) to native membranes reduces CD39 ATPase activity by 80%, similar to unsaturated fatty acids, solubilization, and truncation. The K_i of approximately 2 μM is below the CMC and is comparable to that observed for mechanosensitive channels (*34*, *35*).

As shown in Figure 3B, both transmembrane domains are required for sensitivity to LPC; as with unsaturated fatty acids and solubilization, NT is only slightly sensitive to LPC and CT and soluble CD39 are completely insensitive, suggesting that LPC acts via the membrane. LPC inhibits activity in a reversible manner, as illustrated in Figure 3C; treating membranes with LPC followed by extraction by BSA and measurement of activity in the presence of both reveals that the inhibitory effect is completely reversible up to 8 μM LPC and 90% reversible at 9 and 10 μM LPC, most likely due to the fact that LPC approaches its CMC at the latter concentrations. As with fatty acids, the shape of the molecule rather than its specific identity correlates with its inhibitory potential; as summarized in Figure 3D, LPLs with large headgroups, lysophosphatidylinositol, lysophosphatidylcholine, and lysophosphatidylglycerol, all inhibit activity, while lysophosphatidylethanolamine, which has a small headgroup and is not considered to be cone-shaped, has an only slight effect. Kinetic analysis of CD39-H59G in the presence of LPC produces results identical to those shown for AA in Figure 2E (data not shown), suggesting that LPC also converts CD39 to the same altered functional state that solubilization and truncation do.

The effect of LPLs is mimicked by sub-CMC concentrations of Triton X-100, an amphiphile that has no structural features in common with LPLs other than an overall cone shape. As shown in Figure 4A, Triton X-100 inhibits CD39 ATPase activity in a dose-dependent manner when added to native membranes; maximal inhibition comparable to that observed with LPLs is reached by 0.005%, below the CMC. The comparison of effects on full-length, NT, CT, and soluble CD39 in Figure 4B indicates that, as with solubilization, unsaturated fatty acids, and LPLs, both transmembrane domains are required for sensitivity to nonsolubilizing concentrations of Triton X-100. The same kinetic analysis and results as described above for AA and LPC suggest that addition of Triton X-100 to native membranes also converts CD39 to the previously characterized altered functional state (data not shown).

Bilayer Alterations Increase Transmembrane Helix Mobility. The results given above reveal that the functional state of CD39 is altered in a transmembrane domain-dependent manner by a variety of structurally unrelated compounds that have little in common other than their ability to insert into the membrane and alter its mechanical properties. Consistent with the work of Papanikolaou et al. (*30*), we found that removal of cholesterol, an inverse cone-shaped molecule, by cyclodextrin also reduces activity by approximately 80%. Together, these data point toward a common mechanism by which alteration of membrane mechanical properties acts via the transmembrane helices to convert the active site to a different functional state.

If so, the same membrane alterations would also be expected to change some structural or dynamic feature of the transmembrane helices. We therefore used our previously reported set of cysteine-substituted TM1, TM2, and TM1−

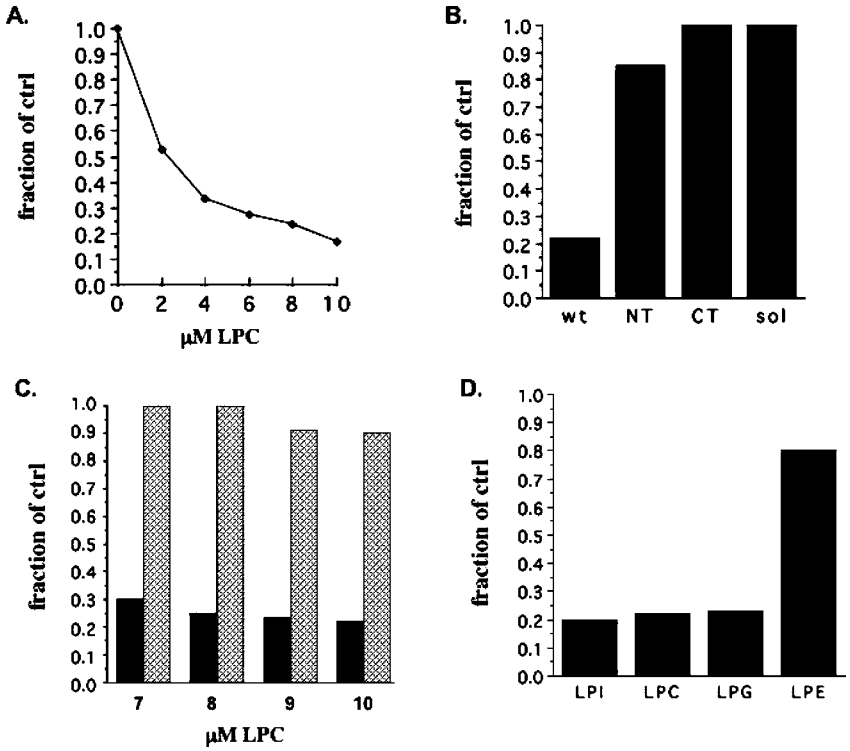

FIGURE 3: ATPase activity in the presence of lysophospholipids. (A) The ATPase activity of full-length CD39 was measured in membranes in the presence of the indicated concentrations of lysophosphatidylcholine (LPC). (B) The ATPase activity in the presence of 8 μM LPC was measured for full-length CD39, NT, CT, and soluble CD39 and expressed as a fraction of the native full-length CD39 activity. (C) Full-length CD39 was exposed to the indicated concentrations of LPC followed by direct measurement of ATPase activity (black bars) or followed by treatment with BSA prior to activity measurement (gray bars). (D) The full-length CD39 ATPase activity was compared in the presence of 8 μM lysophospholipids with large (lysophosphatidylinositol, LPC, and lysophosphatidylglycerol) and small (lysophosphatidylethanolamine) headgroups and expressed as a fraction of the native CD39 activity.

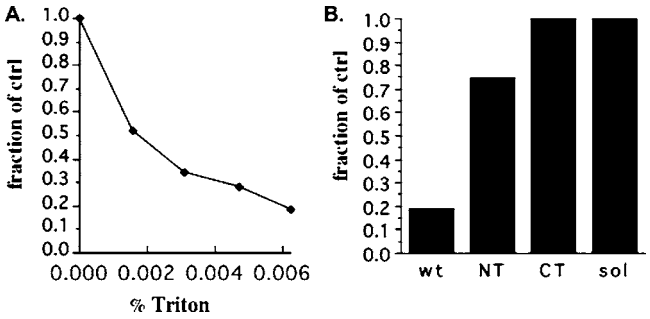

FIGURE 4: ATPase activity in the presence of Triton X-100. (A) The ATPase activity of full-length, membrane-bound CD39 was measured in the presence of the indicated nonsolubilizing concentrations of Triton X-100. (B) The ATPase activity in the presence of 0.005% Triton X-100 was measured for full-length CD39, NT, CT, and soluble CD39 and expressed as a fraction of the native full-length CD39 activity.

TM2 constructs to examine how inter- and intramolecular disulfide cross-linking patterns respond to the membrane treatments described above. As described previously (*28* and references cited therein), relative cross-linking rates reveal the relative proximity of different cysteine pairs and are commonly used to determine helicity and interacting surfaces of transmembrane domains. The technique has further been established as a means of detecting dynamic motions within a protein (*38*). While the method does not provide direct information about the rates and frequency of such motions, it has been established as a means of detecting qualitative differences in mobility for a given protein under different conditions (*38*, *39*).

Rather than using temperature sensitivity to distinguish among helix interfaces as in our previous report, we used the cross-linking rate at 37 °C to monitor changes in cross-linking propensity. As shown in Figure 5A, intermolecular TM1−TM1′ and TM2−TM2′ cross-linking rates at a series of positions near the extracellular side of the membrane correlate with the helical patterns previously identified by temperature dependence. On TM1, positions L32, V35, and G36, which are insensitive to temperature, exhibit the fastest cross-linking rates, the latter two with complete cross-linking by 30 s, while L37, which is partially sensitive to temperature, exhibits an intermediate rate with cross-linking complete by 2 min, and I33 and A34, both of which fail to cross-link at 4 °C, are the slowest, with cross-linking complete by 5 min. While generally slightly slower than those on TM1, relative cross-linking rates on TM2 also reflect a helical pattern. L482, the position least sensitive to temperature, is nearly completely cross-linked by 30 s; M483, a partially temperature sensitive position, is intermediate, and S481 and V484, neither of which cross-links at 4 °C, are the slowest.

To verify that the observed rates reflect cysteine−cysteine collisions rather than accessibility to the cross-linking reagent, we compared cross-linking rates at a series of copper phenanthroline (CuP) concentrations. As shown in Figure 5B for position I33, the cross-linking rate plateaus between 0.03 and 0.3 mM CuP; adding a 10-fold excess of CuP has no further effect on the rate. The maximal rate was reached before this point for all other positions as well, indicating that the cross-linking rates measure helix−helix collis-

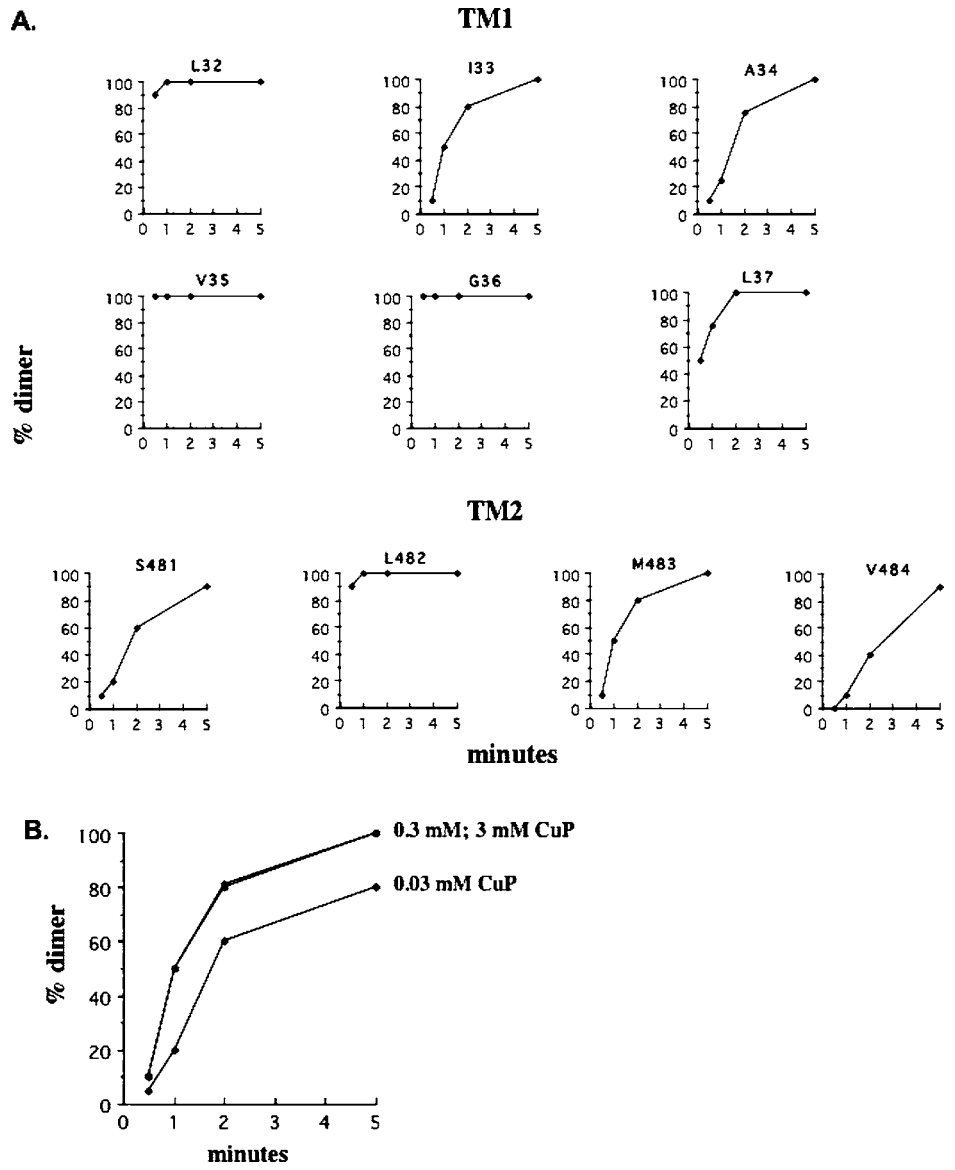

FIGURE 5: TM1 and TM2 cross-linking rates. (A) Full-length CD39 constructs containing single cysteine substitutions at the indicated positions in TM1 or TM2 were cross-linked in isolated membranes by being exposed to 0.3 mM copper phenanthroline (CuP) for 30 s or 1, 2, or 5 min at 37 °C. Cross-linking efficiency is expressed as the percent dimerized. (B) Cross-linking rates for I33C were compared at 0.03 (◆), 0.3 (●), and 3 mM CuP (▲) at 37 °C.

ion rates, rather than helix−reagent collision or reaction rates.

To determine the effects of membrane properties on cross-linking rates, we measured rates in the presence of 20 μM OA or 0.005% Triton X-100 or following cholesterol extraction by cyclodextrin as representatives of inverse cone-shaped amphiphiles, cone-shaped amphiphiles, and alteration by removal rather than addition of a membrane component. As summarized in panels A and B of Figure 6, each of these treatments increased the cross-linking rate at every slow position such that all were completely cross-linked by 30 s. For both TM1 and TM2, comparison of the percent cross-linked at 30 s reveals the distinction between the primary interface and those of other orientations; OA, Triton X-100, and cyclodextrin each produce the same result of abolishing this distinction. Although we cannot determine whether the rates become equal, the fact that the rate increase at the slow positions is not accompanied by a decrease at other positions to the previously observed slow rates suggests that these treatments increase the time spent in the alternate orientations rather than shifting the helices to a different primary conformation.

Comparison of rates in the presence of OA and its saturated counterpart stearic acid suggests a correlation between the effects on enzymatic activity and cross-linking. As shown in Figure 6C, despite its ability to insert into the membrane, stearic acid has no effect on the cross-linking rate at position I33, consistent with its lack of an effect on activity. The correlation between effects on activity and on transmembrane helix behavior is further supported by the similar concentration dependence of the two effects. As summarized in Figure 6D, the dose dependence for the increase in percent cross-linked at 30 s at position I33 is nearly identical to the dose dependence for the loss of ATPase activity; the correlation is observed for both OA and Triton X-100. These results are consistent with a potential relationship between activity and the observed changes in transmembrane helix interactions.

We previously demonstrated that intermolecular cross-linking at the slower positions is due to rotational mobility

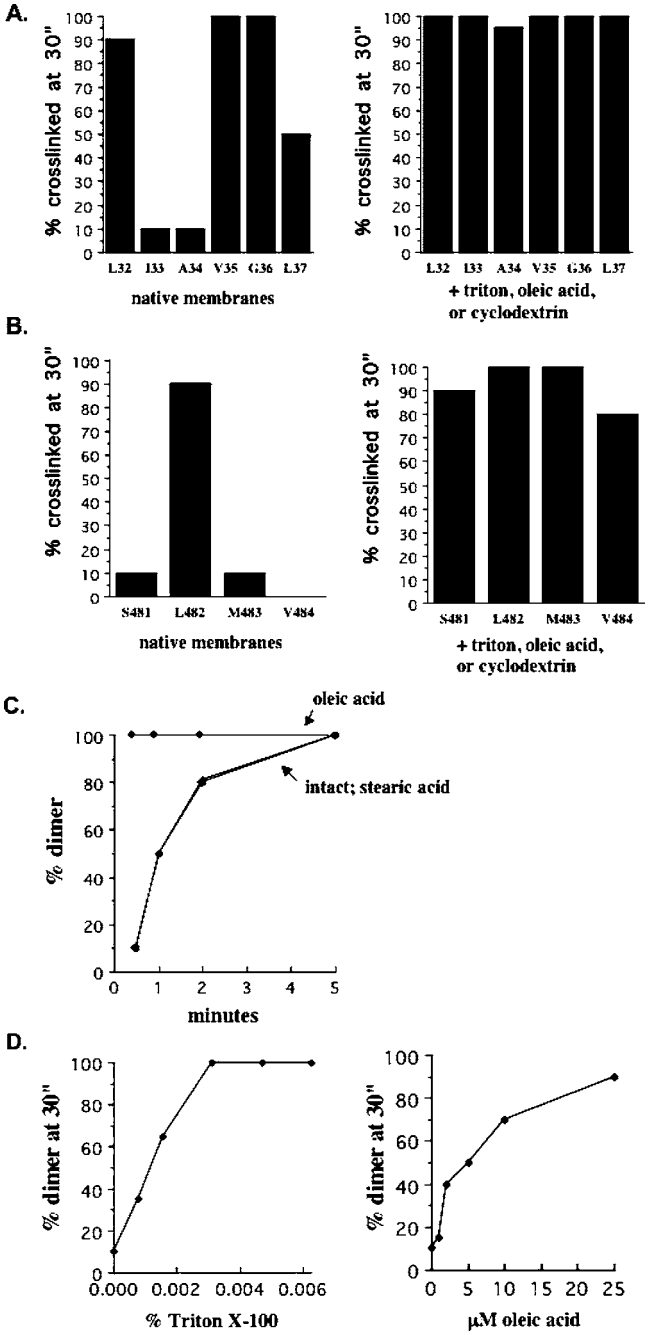

FIGURE 6: TM1 and TM2 cross-linking efficiency in membranes treated with Triton X-100, OA, or cyclodextrin. (A) The percent cross-linking at 30 s was compared for the single TM1 cysteine-substituted contructs in native membranes (left) and in the presence of either 0.005% Triton X-100, 20 μM OA, or 3% cyclodextrin (right). The three treatments yielded identical results. (B) The percent cross-linking at 30 s was compared for TM2 positions as described for panel A for TM1. (C) Cross-linking rates for I33C were compared in intact membranes (◆) and in the presence of 20 μM OA (●) or its saturated counterpart, stearic acid (▲). (D) The percent cross-linking at 30 s was determined at the indicated concentrations of Triton X-100 (left) or OA (right) for I33C.

within dimers rather than to collisions between dimers or formation of higher-order oligomers (*28*). If these results apply to the modified membranes in these experiments, the increased cross-linking rates at the slower positions would be expected to result from an increase in the rotational mobility of each helix. To test directly for an increase in rotational mobility, we examined the rate of intramolecular

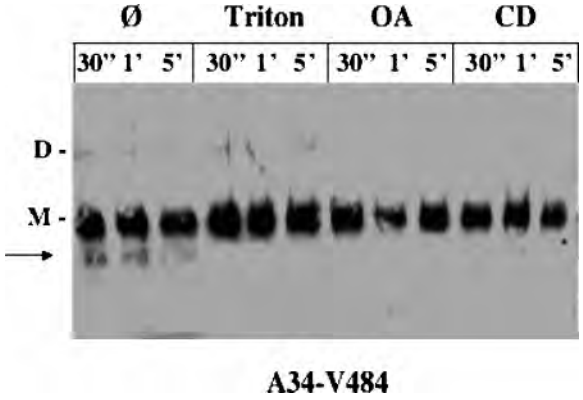

FIGURE 7: TM1−TM2 cross-linking in intact and modified membranes. CD39 containing paired cysteine substitutions at positions A34 in TM1 and V484 in TM2 was cross-linked for the indicated times in intact membranes and in membranes treated with 0.005% Triton X-100, 20 mM OA, or 3% cyclodextrin. The disappearance of the 56 kDa C-terminal fragment (arrow) correlates with intrasubunit TM1−TM2 cross-linking as determined in ref *28*. M stands for monomer and D for dimer.

TM1−TM2 cross-linking between positions A34 and V484. As shown in Figure 7, the cross-linking rate can be observed over the 5 min time course in native membranes but, as for intersubunit cross-linking, the rate is increased so that cross-linking is complete by 30 s in the presence of OA, Triton X-100, or cholesterol removal. Furthermore, despite the potential for this construct to form cross-links between dimers or among higher-order oligomers due to its two cysteines, no higher-order cross-linking is observed under any conditions. These results demonstrate that membrane physical properties increase the rotational mobility of the transmembrane helices and that, while formation of higher-order oligomers is not completely ruled out, the increase in rotational mobility appears to predominate over other potential effects.

Membrane Alterations Uncouple Substrate Binding and Transmembrane Helix Dynamics. Our previous work revealed that ATP binding decreases the rotational mobility of the transmembrane helices and stabilizes their primary orientation (*28*). To gain insight into how membrane physical properties might affect the relationship between substrate binding and transmembrane helix mobility, we measured the extent of cross-linking at 5 min in the presence of ATP and Triton X-100, OA, or cyclodextrin. For position I33, cross-linking is complete by 5 min in native membranes in the absence of ATP but is almost completely abolished in the presence of ATP. As summarized in Figure 8, Triton X-100, OA, or removal of cholesterol counteracts the ability of ATP to inhibit cross-linking and restores cross-linking to at or near the level in the absence of ATP. As discussed below, the ability of membrane alterations to interfere with coupling between active site and transmembrane helix dynamics may have implications for the mechanism by which they regulate enzymatic function.

Removal of Either Helix Mimics the Effect of Membrane Alteration on Another Helix. To gain further insight into the relationship among helix mobility, helix interactions, and membrane properties, we measured intermolecular cross-linking rates for versions of CD39 lacking TM1 (NT) or TM2 (CT). As shown in Figure 9, TM1−TM1′ cross-linking rates in the CT construct lacking TM2 display none of the rate

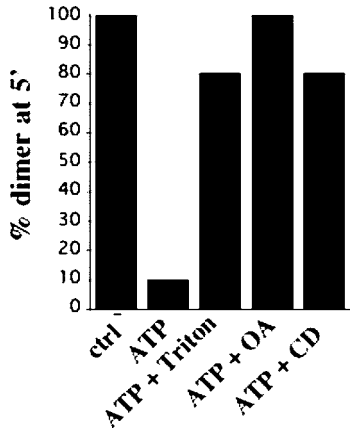

FIGURE 8: Regulation by substrate in intact and modified membranes. The percent cross-linking at 5 min was determined for I33C in intact membranes in the absence of ATP and in membranes left intact or treated with 0.005% Triton X-100, 20 μM OA, or 3% cyclodextrin in the presence of 5 mM ATP.

variation observed at the corresponding positions in full length CD39; instead, all positions cross-link completely by 30 s. The absence of TM2 furthermore renders all positions insensitive to ATP. Similar results are observed for TM2−TM2′ cross-linking in the absence of TM1; while none of the positions achieve complete cross-linking, consistent with the previous report that a portion of NT remains monomeric as measured by sucrose density gradient sedimentation, no distinctions among positions are observed and maximal cross-linking occurs by 30 s All positions are insensitive to ATP, in contrast to the sensitivity of all but L482 in full-length CD39. Furthermore, neither Triton X-100 nor OA has any effect on rates or the maximal degree of cross-linking for either CT or NT. Removing one helix therefore appears to mimic the changes in cross-linking profiles induced by alterations in membrane properties. These results support the idea that helix interactions rather than diffusion in the membrane are the primary factor limiting intermolecular cross-linking rates in full-length CD39. In addition, they indicate that TM1−TM2 interactions are responsible for limiting the rotational mobility of each helix; in the absence of the other helix, each has significantly more freedom to rotate despite its attachment to the extracellular domain.

DISCUSSION

These experiments reveal that membrane mechanical properties regulate the CD39 enzymatic functional state, the rotational mobility of its transmembrane helices, and coupling between transmembrane helix motions and substrate binding. A variety of amphiphilic compounds that alter bilayer features such as tension, spontaneous curvature, and elasticity were all found to convert CD39 to the same functional state that removal of transmembrane helices or detergent solubilization does, as indicated by identical kinetic profiles, activities, substrate specificities, and hydrolysis mechanisms with respect to residue H59 in ACR1. Both transmembrane domains are required for sensitivity to these reagents; in contrast, inhibitors that bind to the active site inhibit both full-length and truncated CD39. The latter result, in conjunction with the structural diversity of the amphiphiles, supports the interpretation that these agents work via the membrane. The change in functional state is correlated with an increase in the rotational mobility of both transmembrane helices; bilayer alterations increase interhelical cross-linking rates such that the helical dependence of cross-linking rates observed in native membranes is abolished on the observable time scale. Bilayer alteration furthermore counteracts the ability of ATP to restrict transmembrane helix mobility, thus uncoupling transmembrane helix dynamics from substrate binding.

While a concomitant change in the monomer−oligomer equilibrium is not excluded as an explanation for the change in cross-linking rates, the change in intramolecular mobility appears to predominate as demonstrated by an increase in the intramolecular TM1−TM2 cross-linking rate, the lack of cross-linked dimer formation by the double cysteine-substituted construct, and the fact that removal of one transmembrane helix mimics the effects of bilayer alteration. The possibility that the change in the cross-linking pattern might result from local unwinding or other deviation from helicity at the ends of the transmembrane domains is also not excluded. A change in lipid packing or interaction with the various reagents might in theory promote such a change if it changes the local hydrophobicity. Nevertheless, this explanation is unlikely to account for the fact that removing either transmembrane domain produces the same results without manipulation of the membrane or for the ability to modulate this parameter with substrate or temperature without losing the overall helical cross-linking pattern. In addition, both transmembrane domains, including the regions studied here, are predicted with more than 90% confidence to be helical, independent of membrane environment. In any case, the observed change in the cross-linking pattern is inconsistent with a shift to any other equally rigid conformation and therefore suggests an increase in mobility whether or not the transmembrane domains remain helical (*40*). Thus, the most straightforward interpretation of our data is an increase in conformational mobility, most likely dominated by but not limited to a change in the rotational mobility of TM1 and TM2.

This study suggests that the critical structural difference between the previously identified enzymatic functional states is the degree of conformational mobility. Our original work established that the enzyme reverts to an alternate functional state upon disruption of the transmembrane helices by any of a diverse set of truncation and solubilization approaches (*24*). On the basis of these results, we proposed that the active site can exist in a relaxed or tense conformation and that the two transmembrane domains are required to maintain the tense, or native, conformation (*41*). The subsequent discovery of a high degree of TM1 and TM2 rotational mobility, of the ability of substrate to modulate mobility, and of a reduction in activity upon locking the helices in any single orientation suggested that dynamic motions, rather than simple stabilization of a tense conformation, might be the key to the requirement for transmembrane domains (*28*). The results presented here reconcile these two proposals by suggesting that, for full-length, membrane-bound CD39, the tense and relaxed states correspond to moderate and large degrees of transmembrane helix mobility. For the case of interconversion between functional states by bilayer alteration, mobility is the only transmembrane domain feature we observe to be altered; both TM1 and TM2 remain present and in the membrane and do not appear to dissociate or to

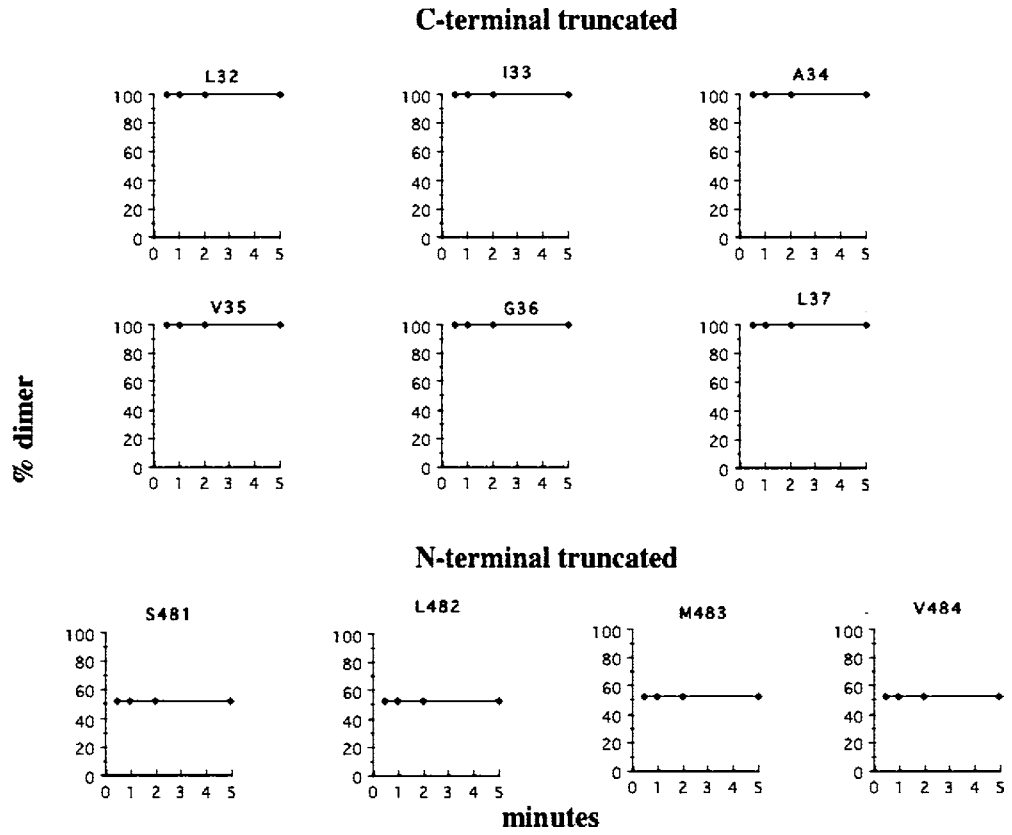

FIGURE 9: Cross-linking of constructs lacking either TM1 or TM2. CT constructs lacking TM2 and containing single cysteine substitutions at the indicated positions in TM1 and NT constructs lacking TM1 and containing single cysteine substitutions in TM2 were cross-linked for the indicated times as described for full-length CD39 in the legend of Figure 4. Identical results were obtained in the presence of 5 mM ATP or in the presence of 0.005% Triton X-100 or 20 μM OA.

convert to a different primary conformation. Conversion to the relaxed state via removal of TM1 or TM2 also increases the rotational mobility of the remaining helix, suggesting that when not restrained by both the opposite helix and the membrane each helix is relatively unconstrained by the extracellular domain. Removing both TM1 and TM2 may thus achieve the same functional conversion by relieving the two remaining ends of the extracellular domain of restrictions on their own mobility. Thus, the native functional state appears to exist when the transmembrane domains and the membrane itself work together to establish an optimal balance between stability and mobility; the relaxed state exists when either the transmembrane domains or the membrane is altered to allow too much mobility, allowing the intrinsic properties of the presumably more flexible extracellular domain to dominate.

The relaxed state is correlated not only with a change in enzymatic properties but also with a loss of coupling between substrate binding and helix mobility. In the native state, the transmembrane helices move relative to each other, but a substrate can reduce such motions; in contrast, when the enzyme is converted to the relaxed state via the alteration of membrane properties, a substrate can no longer stabilize the transmembrane helices. Previous experiments have indicated that soluble CD39 has a substrate affinity even higher than that of native CD39 and that both have a K_m well below the concentrations used here (*17*, *24*, *25*), suggesting that the loss of coupling more likely reflects a change in the relationship between binding site and transmembrane domain dynamics than a lack of binding. One potential explanation is that altering the membrane reduces the energy barriers among helix orientations such that thermal energy is sufficient to overcome the stabilizing influence of substrate binding. Alternatively, the mode of substrate binding itself may change in the relaxed state. A change in binding mode would be consistent with the documented change in the hydrolysis mechanism with respect to ACR1 (*24*). Since ACR1 and ACR5 are adjacent to TM1 and TM2, and since we observe that both helices are required for the substrate sensitivity of either TM1 or TM2, it might be of interest to compare the role of ACR5 in substrate binding and hydrolysis in the tense and relaxed states.

Why a membrane-induced increase in mobility would change the enzymatic properties of CD39 is unknown. If, as documented for P-glycoprotein (*42*, *43*), the active site and transmembrane domains undergo discrete coordinated motions during nucleotide hydrolysis, the observed loss of coupling between CD39 substrate binding and helix stabilization suggests that membrane alteration would also abolish coordination between helices and the active site at other stages of nucleotide hydrolysis. Enzymatic behavior may also be directly related to the degree of active site mobility, as reported for other enzymes as well as for other proteins that recognize a broad array of substrates (*44*). The native ability to hydrolyze ATP and ADP in succession without intermediate ADP release might potentially be particularly dependent on an appropriate balance between stability and flexibility.

The fact that structurally diverse amphiphiles and cholesterol removal all produce the same enzymatic and conformational results suggests that the transmembrane domains sense a mechanical property of the bilayer rather than the direct presence of a specific molecule. Each of the molecules

alters several parameters such as fluidity, tension, curvature, and elasticity; in general, these effects are difficult to separate, and thus, distinguishing exactly which parameter a given protein senses is not always possible. Nevertheless, our results in combination with previous studies suggest that CD39 is more sensitive to certain parameters than to others. The fact that cone-shaped and inverse cone-shaped molecules have identical effects despite their opposite influences on spontaneous curvature indicates that curvature is not the primary determinant of CD39 behavior. Previously, fluidity has been proposed to regulate the related two-transmembrane domain family members, eNTPDase2 and -8 (*45*); however, the conformational changes we observe are intramolecular, and fluidity has recently been somewhat discounted as an explanation for changes in protein activity since it cannot change the equilibrium among states (*46*). In addition, Papanikolaou et al. (*30*) showed that adding cholestenone to cholesterol-depleted membranes does not restore CD39 activity despite the fact that it restores fluidity. However, our results parallel those of Lundbaek and colleagues, who have shown that the same variety of unsaturated fatty acids, cone-shaped molecules, and cholesterol depletion all have similar effects on voltage-dependent sodium channels as well as on gramicidin channels (*29*, *37*). On the basis of their study of the springlike behavior of the bilayer, they proposed that while these molecules have opposite effects on curvature they all increase bilayer elasticity and consequently lower the total energy for changes in protein conformation (*29*). Since CD39 responds to the same array of membrane treatments and exhibits an increased propensity to change transmembrane domain conformation, bilayer elasticity may also be the predominant physical property that regulates CD39.

Our observation that CD39 is highly responsive to changes in bilayer mechanical properties suggests that its functional state can be regulated by the state of the membrane in living tissues. Bilayer properties are altered in vivo, particularly in vascular endothelial cells in which CD39 resides, directly by mechanical pressure, shear stress, and changes in osmotic pressure, by release and insertion of unsaturated fatty acids and LPC during inflammatory processes and oxidative stress, and by changes in cholesterol levels. Furthermore, extracellular nucleotides play a central role in cellular responses to and modulation of many of these processes (*3*−*5*). Mechanical pressure, shear stress, and osmotic stress trigger ATP release, and in at least the latter case, the resulting extracellular ATP is required to restore osmotic balance. Signaling by extracellular nucleotides also modulates inflammatory processes and responses to oxidative stress, and the balance among extracellular ATP, ADP, and adenosine regulates blood clotting, which can be associated with cholesterol levels. The ability of CD39 to respond instantaneously to such changes in the membrane by altering its hydrolysis rate, substrate specificity, and intermediate ADP release during ATP hydrolysis might thus provide a direct feedback mechanism by which extracellular nucleotide signaling is tailored to the state of the cell.

Modulation of transmembrane helix mobility may also have implications for CD39 functions other than nucleotidase activity. Bodas et al. (*47*) have shown that CD39 can serve as an ATP channel, and Wu et al. (*48*) have found that the short cytoplasmic tail can bind Ran binding protein M and thereby regulate nucleotidase activity, suggesting a potential role for the transmembrane helices in relaying information between the cytoplasm and the active site. Regulation of transmembrane helix mobility according to the state of the membrane might thus regulate their ability to transport nucleotides or signals across the membrane; in the latter case in particular, our observation of uncoupling between substrate binding and helix mobility suggests that coupling between Ran binding protein interaction and nucleotidase activity might also be regulated by membrane properties. Regulation of the balance between the stability and mobility of the transmembrane helices according to the physical state of the membrane may therefore provide a mechanism for interdependence among the active site, the bilayer, and the internal state of the cell.

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Appendix XII

Harvard Magazine article about the Department of Biochemistry and Molecular Biology (BMB) upon inauguration of its Sherman Fairchild Biochemistry Building (1980).

The Sherman Fairchild Biochemistry Building was built through the seminal efforts of Matt Meselson, who identified a funding source, and Guido Guidotti who, as then Chairman of the Department of Biochemistry and Molecular Biology, managed the building's design and construction.

HARVARD Magazine

September-October 1980

September-October 1980
$3.50

"The greatest single achievement of nature to date was surely the invention of DNA. We have had it from the very beginning, built into the first cell to emerge, membranes and all, somewhere in the soupy water of the cooling planet . . ."

LEWIS THOMAS: ON THE UNCERTAINTY OF SCIENCE

PROBING THE CHEMISTRY OF LIFE ***

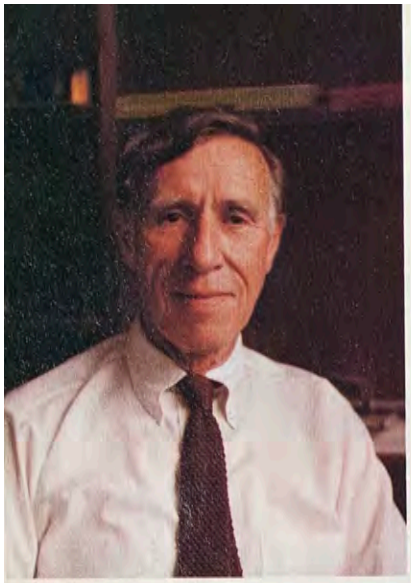
THE NICOTINE FIX, II

DISCOVERY: ATROCITY IN AFRICA

*** Article about the new Department of Biochemistry and Molecular Biology on the occasion of the dedication of its especially-created home, the Sherman Fairchild Biochemistry Building

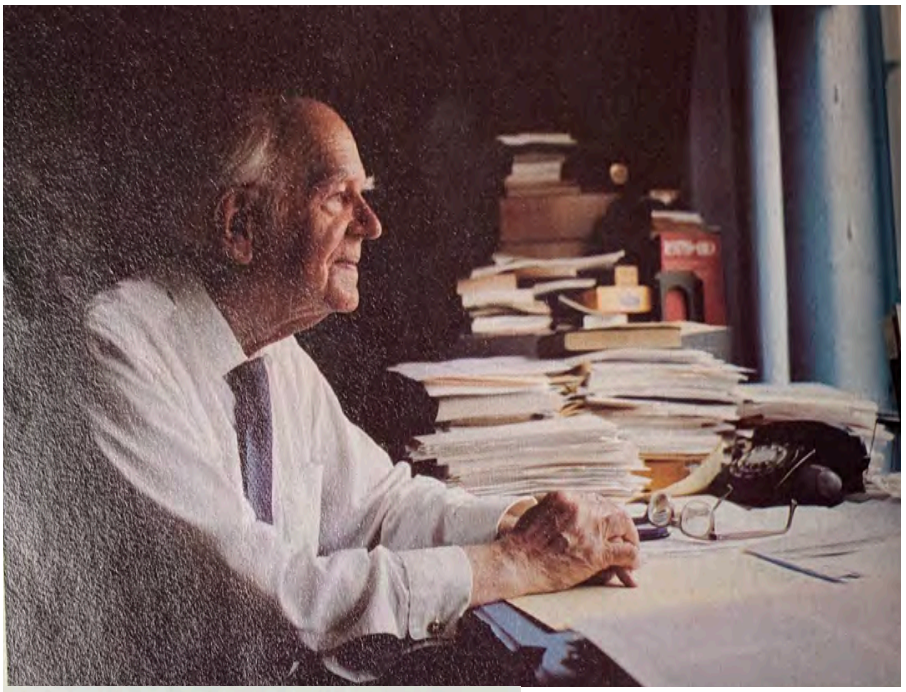
GUIDO GUIDOTTI

Famous among students for his brilliant and enthusiastic lectures on intermediary metabolism, Professor Guidotti now works on proteins that transport substances across membranes into and out of cells.



KONRAD E. BLOCH

Konrad Bloch's research elucidated the biosynthetic pathways that lead to cholesterol, steroids, and other complex natural products. He pioneered in the application of radioisotope markers to biochemical problems, and received the Nobel Prize in 1964. After more than thirty years dedicated to the determination of how cells make substances such as cholesterol, Bloch, who is Higgins professor of biochemistry, intends to determine *why* they do so in such a distinctive and specific way. His recent findings shed new light on the evolution of changes in the chemical structure of small molecules. Bloch hopes to analyze every structural detail of the cholesterol molecule from both an evolutionary and a functional perspective. The aesthetic of many an accomplished scientist is reflected in one aspect of what he expects to find in his research, that "nature has reached perfection"; Bloch's work begins to tell us how.



JOHN T. EDSALL

Nowadays an undergraduate in biochemical sciences takes the basic nature of polypeptide or protein molecules as much for granted as the formula for water (H_2O). But it was not so long ago that John Edsall, who was chairman of the Board of Tutors in Biochemical Sciences for more than 25 years, now emeritus, contributed to the research that provided the basis for our understanding of what polypeptides really are. Without it, later progress in research on enzymes, such as those that digest food, and hormones, such as insulin, could not have taken place.

The undergraduate program in biochemical sciences is largely a product of Edsall's vision, but his influence on the entire science of biochemistry reaches far beyond Harvard. As an early editor of the Journal of Biological Chemistry, from his office in the basement of the Biological Laboratories, he set high standards for scientific publications and profoundly influenced the shape of a growing science.

PAUL M. DOTY

Now Mallinckrodt professor of biochemistry, Paul Doty came to the Chemistry Department in 1948 and was one of the key figures in the Committee on Biochemistry, which eventually led to the establishment of a department in the late Sixties. His work on denaturation and renaturation of DNA—the separation and rejoining of the DNA strands—provided an understanding of some of its fundamental properties and led to work on DNA hybridization, which has become a major tool of modern molecular biology.

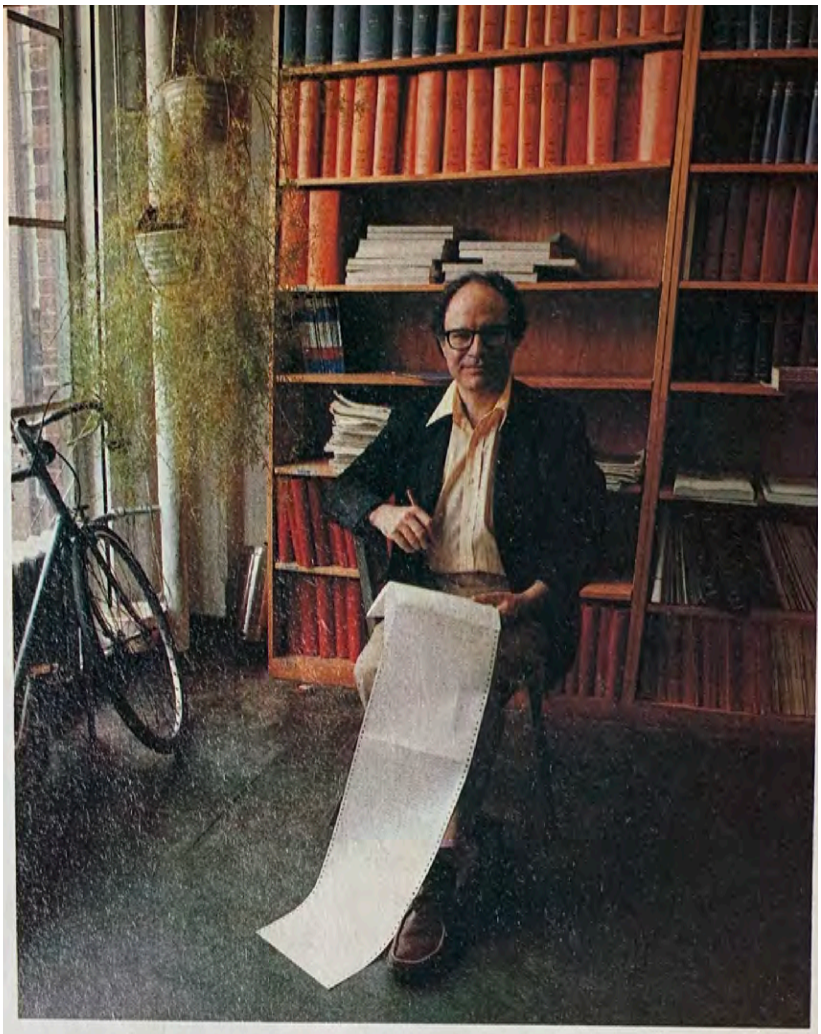
In the early Sixties Doty was a science-policy adviser to President Kennedy, and has since become a major figure in disarmament-policy formulation and negotiations, an interest that led him to a joint appointment in Harvard's Kennedy School of Government. The scintillation counter in the background is an example of the expensive, high-technology apparatus that will be easier to share among the entire department in the new facility.

JAMES C. WANG

A recent addition to the department, Professor Wang was previously in the department of chemistry at Berkeley. His work represents an incisive and intricate approach to the enzymology of DNA metabolism, especially as it relates to topological factors that arise as a consequence of the coiled, double-helical structure.

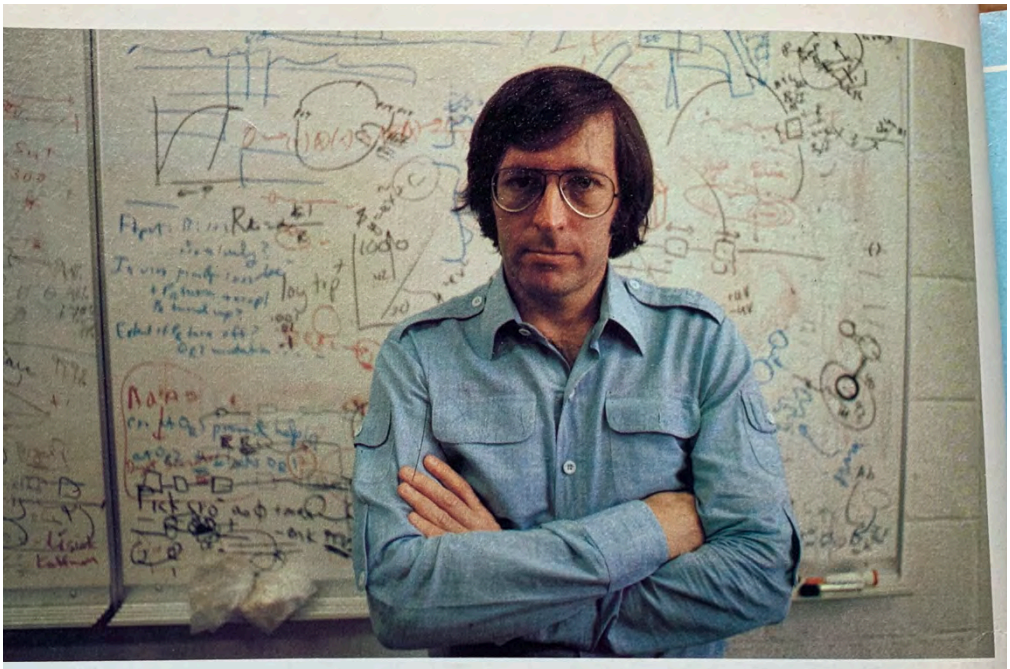
MATTHEW S. MESELSON

Major advances in science often require elements not only discovery, but invention. To see the problem, to pose the right question, may be the first step, but solutions are sometimes impossible without new tools or technology. On occasion, a technique developed to answer a single question becomes as consequential as the solution to the original problem. The famous "Meselson-Stahl experiment" was the first major proof of the general nature of DNA replication as proposed by Watson and Crick. It was also the first application of cesium chloride density-gradient centrifugation, a technological breakthrough pioneered by Meselson, Stephen Stahl, and Jerome Vinograd that was to become one of the main working tools of molecular biology. Meselson is Thomas Dudley Cabot professor of the natural sciences. His many contributions to molecular biology are complemented by a devotion to the cause of chemical- and biological-weapons disarmament. His activities outside the ivory tower reflect the hope, as he stated in a recent article in Scientific American , that "the increasingly profound knowledge of life processes be directed solely to beneficial purposes."



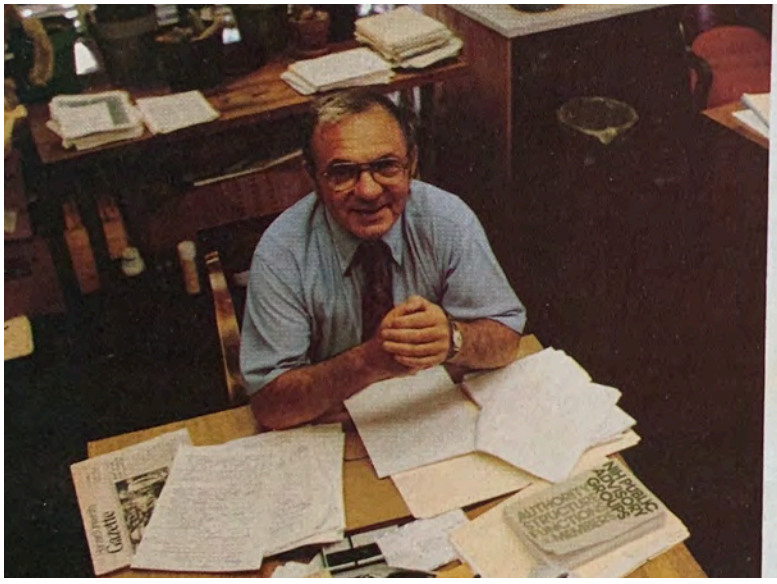
WALTER GILBERT

Walter Gilbert is the American Cancer Society professor of molecular biology. His early studies on ribosomes, the subcellular particles that synthesize proteins from amino acids, and on messenger RNA, which conveys the genetic specifications for proteins from DNA to the ribosomes, is considered classic, as is his work at the molecular level of gene control. With Allan Maxam, he recently perfected a technique for sequencing DNA—or determining its nucleotide sequence—a tool that has already led to an understanding of genetic interactions at a level inconceivable only a few years ago. It has also contributed to the burgeoning field of recombinant DNA, and to techniques of cloning genes for such medically important molecules as interferon.



MARK PTASHNE

The drawing behind the present chairman of the Department of Biochemistry and Molecular Biology depicts parts of the scheme by which a virus controls its genetic destiny. As a Junior Fellow, Professor Ptashne isolated the repressor molecule that controls the development of a virus called bacteriophage lambda. His work since then has led to a nearly complete picture of how certain viral genes are regulated—turned on and off—at the biochemical level. The regulatory proteins interact with each other and with the DNA according to a remarkable set of rules, and as a consequence, the viral infection can proceed along one or the other of two very different life cycles.



JACK L. STROMINGER

Professor Strominger's work has elucidated the major stages in bacterial cell-wall synthesis and the way penicillin works by interfering with the process. His recent work has extended to an investigation of transplantation antigens, the molecules recognized as foreign when a recipient organism rejects a transplanted organ.

STEPHEN C. HARRISON

Professor Harrison is chairman of the Board of Tutors in Biochemical Sciences, a group that antedates the Department of Biochemistry and Molecular Biology by more than thirty years. His pioneering application of X-ray crystallography to the structure of viruses provides a paradigm for the investigation and understanding of architectural features of complex biological assemblies. In the new laboratories he will share space and equipment with associate professor Don Wiley, who has applied similar techniques to determine the structure of a protein found in the membrane of the influenza virus. In this kind of high-technology work, involving such complex and costly apparatus as X-ray diffractors and computers, the new laboratory will enhance opportunities for cooperation and lessen the need for wasteful duplication. Here Harrison is pictured in front of a mural that schematically represents the icosahedral symmetry of the Tomato Bushy Stunt Virus. The ability to look at the structure of viral particles in such minute detail may provide major insights into how cells, and finally organisms, are built.

SARAH C. R. ELGIN

One of a number of biochemists who have come to Harvard from Cal Tech through the years, including Matthew Meselson and J.D. Watson, Sarah Elgin studies the control of gene expression in higher organisms. Elgin's work has begun to unravel the nature of dynamic changes in chromosome structure that are thought to play a crucial role in the orderly expression of their genes. Her investigations of the Drosophila (fruit fly) polytene chromosome may eventually help explain the genetic basis for such complex biological programs as the development of an embryo.

DAVID DRESSLER

David Dressler's research has concentrated on how DNA molecules replicate themselves and recombine with one another. His ingenious experiments led to the "Rolling Circle" model of DNA replication, which is utilized not only by certain viruses but in the amplification of certain genes in higher organisms.

Along with J.D. Watson, Guido Guidotti, and Konrad Bloch, Dressler was a founder of Biochemistry 10, now the major undergraduate course in biochemistry and molecular biology. His skill as a lecturer has been a major factor in making Biochem 10 one of the College's most highly rated and popular courses. The massive contraption at the right is an electron microscope.