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PHYSICAL CHEMISTRY OF NUCLEIC ACIDS

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■ **Abstract** The Watson-Crick double helix of DNA was first revealed in 1953. Since then a wide range of physical chemical methods have been applied to DNA and to its more versatile relative RNA to determine their structures and functions. My major goal is to predict the folded structure of any RNA from its sequence. We have used bulk and single-molecule measurements of thermodynamics and kinetics, plus various spectroscopic methods (UV absorption, optical rotation, circular dichroism, circular intensity differential scattering, fluorescence, NMR) to approach this goal.

MADISON

In 1950, I was a 19-year-old junior at the University of New Mexico at Albuquerque when the Korean war started. The likelihood that I would soon be drafted encouraged me to get my B.S. degree a year early. Guido Daub, my organic chemistry professor, was able to get me into graduate school at the University of Wisconsin, although I had applied very late. All the teaching assistantships had already been assigned, but Professor John Ferry offered me a research assistantship, which I gladly accepted. My project was to study viscoelastic and dynamic mechanical properties of synthetic polymers. I knew nothing about viscoelasticity or polymers, and I am sure if I had been given time to pick a research director I would have ended up with someone like Paul Bender, a classical physical chemist who studied problems I understood, such as the heat of reaction of HCl with NaOH.

My first job in the lab was to clean up a water bath used for measuring viscosities. The temperature was controlled by a large bulb of mercury connected to a capillary. An adjustable wire in the capillary was the thermostat. When the mercury expanded, contact with the wire turned off the heating element in the bath. The constant making and breaking of the electrical contact at the surface of the mercury led to oxidation, which had to be removed periodically. I took all the mercury out of the bulb and washed it carefully with dilute acid, followed by water. To finish the job, I put the beaker of mercury into the drying oven for glassware to get rid of the water. I do not remember how long the mercury cooked before one of my lab partners removed it. I was told that mercury vapor was not good

for you, and that it was sufficient to rinse with acetone and let the mercury dry at room temperature. At that time the proper action for a mercury spill was to sweep up the big drops and throw sulfur on the rest.

I spent the next few months measuring the viscosities of polyisobutylene solutions. But that fall one of Ferry's second-year students failed his qualifying exam, leaving a hole in the project to study the conversion of fibrinogen to fibrin. I was assigned to fill the hole. Actually, I was asked to fill in for awhile, and if I really did not like biological polymers, I could switch back.

The mechanical rigidity of a blood clot is provided by the polymerization of activated fibrinogen to form a fibrin gel. The activation is caused by the removal of a peptide from fibrinogen by the proteolytic enzyme thrombin. We wanted to characterize the mechanism of the polymerization by measuring concentrations of activated fibrinogen monomers, dimers, etc. as a function of time of polymerization. The first requirement was to slow the reaction down enough so that we could use methods like ultracentrifugation and light scattering to study it. Hexamethylene glycol in water was the preferred solvent, but I measured the effects of different chemicals on the clotting times to look for a better one. We wanted a reagent that would slow the reaction but not denature the protein. Some of Ferry's biochemical colleagues thought that studying clotting in nonbiological solvents could never yield any biologically relevant information. However, once the main intermediates in the polymerization process were identified in hexamethylene glycol, we found the same species in more physiological solvents. The nonaqueous solvent made it easier to study the reaction, but the same mechanism seemed to occur in an aqueous buffer.

Nearly 50 years later there is still discussion and disagreement about the relevance to biological function of structures of biological macromolecules measured in crystals or of mechanisms of reactions studied in nonbiological environments. We biophysical chemists are considered naive reductionists because we mainly study one or two species in vitro. The molecular biologists at least study reactions in bacteria or yeast. Of course, the real biologists insist on studying whole multicellular organisms. I am convinced that whatever we learn about biological molecules—no matter how simple the system or how unusual the environment—can be applied to understand their biological functions. Intermolecular interactions do not distinguish between biologically relevant and biologically irrelevant reactions. Whenever someone questions the biological relevance of an experiment, I respond that anything of interest to a human is clearly biologically relevant. I hasten to add that NIH requires more justification than this for funding. The correct answer for NIH is that the proposed experiment will eventually lead to a cure for cancer, AIDS, and old age.

The polymerization of fibrinogen begins after the proteolytic enzyme thrombin clips off an oligopeptide from the cigar-shaped fibrinogen. My thesis project concluded with building a transient electric birefringence apparatus to study the size and shape of fibrinogen and to learn how it changed on activation by thrombin. I applied an electric field for a few milliseconds to orient the protein molecules

and measured the buildup and decay of the orientation by the appearance and disappearance of birefringence in the solution. From the slight decrease, less than 100 Debye (D), in effective dipole moment of the fibrinogen when the peptide was removed, we concluded that the peptide with its 10 negatively charged carboxylate groups must have been near the center of the molecule. Removal of 10 charges from the end of the 600-Å molecule would change the dipole moment by about 15,000 D in a vacuum. Our solutions had no added salt so we argued that the measured decrease of 100 D meant the peptide was within a few Angstroms of the center of rotation of the protein. An atomic resolution X-ray structure of fibrinogen published recently shows that the amino-terminal ends cleaved by thrombin are indeed at the center of the molecule.

YALE

After 3 years my research progress had gone well and my wife was pregnant, so I suggested to Ferry that it was time for me to move on. John Kirkwood visited about that time and Ferry persuaded him to take me on as a postdoc. Kirkwood had recently become chairman at Yale, where he had a large group of theoretical students and a small group of experimentalists, which I would join. Thus, in 1954 we drove from Madison to New Haven just in time for our daughter to be born as Hurricane Edna was hitting New England.

A postdoctoral position is the best job that I can imagine—there is little responsibility and maximum opportunity to learn new things. At Yale there was an enthusiastic group of people thinking about proteins and nucleic acids. Jon Singer (now at University of California, San Diego) was studying antigen-antibody reactions; Peter Geiduschek (also at UC San Diego) worked on DNA and was very excited about the paper by Watson and Crick that had just appeared. Kirkwood was interested in everything. I was warned to never tell him any preliminary experimental results because he could and would create a theory to explain any result—right or wrong. He had an idea about the effect of ion fluctuations on enzymatic activity that predicted maximum catalytic activity at the pK of the contributing groups. The pH dependence of catalytic activity was not consistent with this model, but it did lead to some enzyme experiments that I continued later. A Yale undergraduate who worked on this project as a "bursary boy"—a fellowship student—was Don Crothers. He is still at Yale but no longer as an undergraduate.

As an advisor in theory, Kirkwood was superb. My thesis experiments on electric orientation and birefringence of fibrinogen had suggested two questions: What are the effective electric forces on the charged protein in an external field in the presence of counterions and added ions? How does the optical activity (circular birefringence) of the protein change with orientation and thus effect the linear birefringence? Kirkwood immediately told me that the first question was very hard and I should concentrate on the second. He showed me the paper he had published in 1937 on a quantum mechanical formula for the orientation-averaged

optical rotation. Bill Hammerle, a theoretical postdoc of Kirkwood's, and I derived the result that the rotational strength of an electronic transition was very sensitive to the direction of incidence of light. In fact the principal components of the optical rotation tensor generally had different signs. Kirkwood praised the work but said his name should not be on the paper as it was not his idea. His interest in optical activity had recently revived because of a paper published by Bill Moffitt from Harvard. Elkan Blout and Paul Doty at Harvard had found that the optical rotation of polypeptides showed large and characteristic changes when the polypeptide went from an α-helix to a coil. Moffitt's paper explaining the optical activity in terms of exciton interactions in the helix was incorrect; he had missed a term that did not contribute to absorption but was crucial for rotation. Kirkwood called and told Moffitt about the error and they published a correction in *PNAS* with Moffitt as first author (1). I thought it was a very considerate thing to do. Kirkwood seemed to agree with Norbert Wiener that unlike chess, in science the mistakes you make are not as important as whether you finally get it right.

After two very stimulating years at Yale, learning as much as I could from Kirkwood, Fuoss, Lyons, Onsager (his statistical mechanics course was called Norwegian I and II by the students), Singer, Sturtevant, and J. H. (Ray) Wang, I started applying for academic jobs. The University of Kansas interviewed me, but they hired Sherwood Rowland instead. As I was considering a second postdoc with John Edsall at Harvard, I received an offer of an instructorship from Berkeley. I learned that they had offered the job to Stanley Gill, who had chosen to go to the University of Colorado. Kirkwood recommended that I accept the Berkeley offer, although he assured me that I would not be given tenure. He said that they only hired their own Ph.D.s, but that after a few years at Berkeley I should be able to move on to an acceptable position. I also learned that the University of New Mexico would probably hire me after my stay at Berkeley. With no expectation of tenure, and with a backup job opportunity, the pressure to make it in my first academic job was minimal. So in 1956 we drove across the country to Berkeley in our 1949 Chevrolet; we bought a new radiator in Salt Lake City to make it across the desert and over the Sierra.

BERKELEY

Four new faculty members in physical chemistry were hired at University of California (UC), Berkeley in 1956. Frank Harris, a Berkeley Ph.D., was hired as an assistant professor. Mark (Phil) Freeman, (University of Washington), Bruce Mahan (Harvard), and I were hired as instructors. Two of us made it to tenure. Frank Harris left his pregnant wife for the wife of a graduate student; this was unacceptable in the 1950s so he was not promoted. Phil Freeman had been having a continuing argument with a senior faculty member about the interpretation of Freeman's experiments. At the end of his seminar on the subject Phil pulled a large dueling pistol out of his briefcase, set it on the table and asked "Are there any questions?" Not everyone thought this was funny.

In our first year at Berkeley the new instructors were not allowed to have graduate students. We were told that they would delay us getting started in research; this is in major contrast to the present custom of establishing a large group as quickly as possible. My Berkeley research started out as a direct continuation of my thesis and postdoctoral research. I measured the transient electric birefringence of synthetic polypeptides with the usual arrangement of light incident perpendicular to the electric field. My calculations on the optical rotation of oriented molecules had predicted large differences in sign and magnitude for the rotation of polarized light incident along a helix axis and perpendicular to the helix axis. To measure this effect I used newly available, conducting transparent windows to allow light incident parallel to the electric field. No linear birefringence occurs, so changes in circular birefringence of the polypeptide helices can be measured. The measurements were consistent with the expected results, but a direct comparison with theory was difficult. Phil Freeman and I decided that a better test was to use small copper wire helices and microwave radiation; the copper helices, inserted in styrofoam cubes, could be oriented easily by hand. We borrowed a microwave oscillator, wave guide, and detector from Bill Gwinn and measured the rotation of the oriented helices in the 2–3.5 cm wavelength region.

The paper (2) appeared in 1957 in the *Journal of Physical Chemistry* with the subheading: "I. Experimental." We planned on having the "II. Theory" paper ready shortly. It took over 20 years; the problem was that the helices were the same size as the wavelength. In 1964 Bob Woody and I derived the optical properties (dipole strengths and rotational strengths) of a free electron on a helix (3). The derivation assumes the wavelength is large compared with the helix, so no comparison with our wire helices was possible. Finally, in 1980, Dexter Moore and I made calculations to compare with the 1957 experiments, using the full transition integrals for interaction with light [exp (i k • r)] that allowed any size helix (4). The results were in reasonable agreement with the experiment, but the best part of the work was that it reminded me how much fun it had been making the measurements.

In 1958, a unique scientific conference called "Biophysical Science—A Study Program" was sponsored by the Biophysics and Biophysical Chemistry study section of NIH to educate physicists, physical chemists, biochemists, and biologists about important biological problems and potential methods to solve them. About 120 of us lived in the dorms at the University of Colorado at Boulder with our families for one month. Nearly everyone stayed for the entire conference, even the senior people. Now, in spite of e-mail and satellite phones that keep you connected continuously, if you stay for the full three to four days of a meeting you are considered either unambitious or retired. The conference covered a wide range of topics from macromolecules to the physiology of vision; the participants ranged in age from their 20s to 70s. As usual, most of the important communication occurred during meals, afternoon sports, and late-night drinking. The sports groups seemed to separate by field. Most of the afternoon tennis players were in statistical mechanics including Norman Davidson, Terrell Hill, and Walter Stockmayer. David

Davies was an exception, but his young family prevented him from going mountain hiking on weekends with the other X-ray crystallographers led by John Kendrew. David and I resumed our tennis games—on luxurious grass courts—in 1964 when we were both in Kendrew's group at the Medical Research Council Laboratory in Cambridge.

The lectures were helpful, particularly after we got instant reviews from Leo Szilard. He was a portly, very visible man who sat in the front row. After listening for a few minutes he would sometimes get up and walk slowly out to sit outside to wait for the next speaker. Eventually, when Szilard left others would follow because we knew we could learn more in the conversation outside, rather than listening inside. I felt sorry for the speaker, but it did not deter me from leaving.

A meeting like this has, I think, tremendous advantages for everyone involved. You meet your peers and future colleagues of all ages, which is of immense value, particularly for the young people. Everyone learns what the current exciting problems and possible solutions are. There is time to not only think and talk about research but to actually do some. Charles Townes in his autobiography *How the Laser Happened* (5) writes that he finished the Townes-Schawlow paper on the laser at this meeting.

One problem that attracted my attention at the meeting was the UV absorption of DNA. Concentrations of DNA were routinely measured using an extinction coefficient at 260 nm, but the extinction coefficient varied over 20% depending on preparation methods. Heating and cooling the DNA produced large changes in absorption. The puzzle was that the spectrum did not change. There were no large energy shifts, just changes in intensity. A native DNA had 40% less absorption than the sum of its constituent nucleotides with essentially no change in shape of the broad 260-nm band. The effect was called hypochromicity or hypochromism. I thought this effect could be treated by the same methods used to calculate circular dichroism. Perturbation of the wavefunctions by internucleotide interaction rather than changes in energy levels would be dominant. I derived the theory back in Berkeley that showed that the main contribution to hypochromism was interaction between the transition dipoles on stacked bases. The bands below 200 nm were borrowing intensity from the longer wavelength bands. A simple classical explanation is that the polarizability of two stacked disks is less than the sum of the individual polarizabilities. The paper was published in the *Journal of the American Chemical Society* in 1960 (6). An erratum appeared in 1961; I had included a term that should have cancelled. It did not change the conclusions, only the quantitative calculations. I was embarrassed by the mistake, but I took comfort in how the error was found. Norman Davidson at Caltech had asked Richard Feynman to explain the paper to his group at a seminar; Feynman immediately pointed out the mistake.

We were interested in experimentally testing theories for hypochromism and circular dichroism of polynucleotides and polypeptides. The simplest polymers were dinucleoside phosphates (two nucleosides, each consisting of a base and a ribose, joined by a phosphate) that could be obtained by hydrolyzing RNA. The

measured hypochromism and optical rotation could be interpreted in terms of the amount of stacking of the bases; adenines stacked the most and uracils the least. Higher oligomers could be obtained by the same method, but it became much harder to separate the 64 trimers compared to the 16 dimers. This is when the Unabomber came to my aid; he suddenly resigned his position in the math department. A temporary instructorship was offered to Karen Uhlenbeck, so her husband Olke also had to obtain a job in Berkeley. He was given a Miller fellowship, which he used in my laboratory.

THERMODYNAMICS OF NUCLEIC ACIDS

Olke Uhlenbeck brought with him oligonucleotides and the enzymes to synthesize others. We started on the goal of predicting thermodynamic properties of single- and double-stranded polynucleotides from measured nearest-neighbor properties. The idea is that the free energy of a folded RNA relative to the unfolded single strand can be estimated by adding the measured free energies of its components. Base-paired helices contribute negative free energies, and single-stranded loops contribute positive free energies because of their loss of entropy. Olke's method for getting everybody in the lab involved was to take bets on the predicted properties. With real money at stake (25¢), the students made sure that the sample was pure, the experiment was done properly, and the data were analyzed rapidly. Our first paper on prediction of secondary structure—base pairing—in RNA appeared in *Nature* in 1971 (7). We underestimated the biochemists (molecular biologists had not yet been invented) who were the audience for this paper. We decided that they would not understand that negative free energies meant more stable structures, so we assigned positive stability numbers instead of free energies. Base pairs had positive stability numbers; unpaired hairpin loops, interior loops, and bulges had negative stability numbers. To predict a secondary structure we simply calculated stabilities for possible structures and looked for the maximum. In spite of the stability numbers, our method was an improvement over previous methods that only maximized numbers of base pairs.

In our next paper (8) on secondary structure (published jointly with Don Crothers' group) we switched to free energies; apparently biochemists did understand that negative values could be favorable. Prediction of secondary structure in RNA has improved considerably since the 1970s. Doug Turner and his students at the University of Rochester have measured thermodynamic contributions from the 10 possible Watson-Crick nearest neighbors, many base-base mismatches, stacking at the ends of helices, and all sorts of loops and bulges. Their parameters along with Michael Zuker's algorithm for considering all possible secondary structures provide widely used predictions of RNA secondary structure from sequence. I am particularly pleased that Doug Turner is now such an expert on thermodynamics, because when he came to Berkeley as a postdoc from Columbia he claimed that he had never even heard of Lewis and Randall.

SPECTROSCOPY OF NUCLEIC ACIDS

We were also working on a variety of optical properties of polymers, particularly polynucleotides. The main emphasis was on circular dichroism and circular birefringence, but UV absorption—hypo- and hyper-chromism—were also considered. The effects of electric fields and magnetic fields were treated, including orientation of the molecules (electric and magnetic birefringence), as well as direct electronic effects (Faraday effect and Stark effect). Experiments, theory, and calculations were made. Doug Turner invented fluorescence-detected circular dichroism (FDCD) (9, 10), in which the chirality in the vicinity of a fluorophore can be measured from the intensity of the fluorescence emission excited by circularly polarized light. This method has developed into a very sensitive and widely used analytical method.

The usual method of measuring circular dichroism actually measures circular extinction, the sum of absorption, and scattering. As large molecules—especially aggregates of large molecules—scatter light significantly, there can be a large contribution from scattering to circular extinction. It is important to be able to separate the effects, because scattering and absorption depend on very different size scales. Absorption depends on local interactions; scattering effects are largest for distances of the order of the wavelength of the light. The most useful structural data are obtained from the angular dependence of the scattered light when circularly polarized light is incident. For the most general experimental design, the polarization of the incident light is characterized by its four-component Stokes vector, and the angular-dependent 4-by-4 Mueller matrix is used to represent the effect of the sample on the outgoing light. Marcos Maestre, Carlos Bustamante, and I derived equations for various chiral models of coupled dipoles, helices, and liquid crystals. Experimental comparison was difficult for nucleic acid double helices because the effects are small, so when we learned that sperm from a Mediterranean octopus were helical we were overjoyed. The circular differential scattering agreed with the dimensions seen in the electron microscope (11). I proudly presented this work at a Department of Energy (DOE) site visit of the Chemical Biodynamics Division of the Lawrence Berkeley Lab. David Shirley, then director of the Lawrence Berkeley Lab, could hardly keep from laughing out loud. I do not know if he thought that research on octopus sperm emphasized the multidisciplinary nature of the lab, or if he was laughing while trying to come up with a justification for DOE support of the work. In any case the site visit did not affect my DOE budget. Actually, site visits never seemed to have any effect, either positive or negative, on budgets.

The ability of circularly polarized light to distinguish chiral objects suggested to Marcos Maestre and me that it would be useful to make images of the differential absorption of the circularly polarized light. We used a digital detector and subtracted the images for right and left circularly polarized light. We applied for a patent, but the patent examiner said they would not patent a polarimeter connected to a microscope. The lawyers advised us to apply for patents on applications of the method for detecting clinically important targets in cells, but we declined.

Magnetic circular dichroism, the differential absorption of circularly polarized light, fit in well with our applications of polarized light to nucleic acids; therefore it was natural for us to apply radio frequency magnetic circular dichroism—better known as nuclear magnetic resonance. Although it is sort of a joke to lump NMR with magnetic circular dichroism, all spectroscopies are of course similar. My colleague Bob Harris and I considered the effect of circularly polarized UV light on NMR. We showed that contrary to a published report, the effect on chemical shifts of circularly polarized light was negligible (12).

As in our work with UV light, our NMR work started with RNA dinucleotides and trinucleotides. The first studies (1977) were at the Stanford Magnetic Resonance Laboratory on a 360-MHz (for protons) machine; Berkeley at that time only had a 200-MHz machine. We have most recently (2001) studied a 56-nucleotide RNA at Stanford on an 800-MHz machine; Berkeley only has 600 MHz.

RNA STRUCTURE

The number of atomic resolution structures of RNA molecules is minuscule compared with the number of protein structures. Proteins crystallize easier, and NMR determination of protein structure is easier. In proteins, scalar couplings can be used to make sequential through-bond assignments of a ^{15}N- and ^{13}C-labeled polypeptide chain. The equivalent cannot be done on nucleic acids because of the lack of a spin 1/2 oxygen nucleus to place on both sides of the phosphorus in each nucleotide. Furthermore, RNAs are easily hydrolyzed by finger nucleases, trace metal ions, and ribozymes. In spite of this, we have been able to identify and characterize several novel RNA structures by NMR.

The usual form of DNA (B-DNA) is a right-handed double helix with 10 Watson-Crick base pairs per turn. RNA is also right-handed, but with 11 base pairs per turn; this RNA structure is similar to A-DNA, the dehydrated form of B-DNA. However, alternating purine-pyrimidine sequences of DNA had been found to switch to a left-handed helix (Z-DNA) in high salt concentrations (>4 *M* NaCl). A *Scientific American* article (13) stated that steric hindrance from the 2′-hydroxyl group on ribose would prevent RNA from going left-handed, so we had to try it. Kathi Hall synthesized the ribo-polynucleotide with an alternating G–C sequence with the help of Mike Chamberlin, the RNA polymerase expert from the Molecular and Cell Biology Department. We needed even higher salt concentrations, but left-handed Z-RNA did form. What its function in biology is—in addition to attracting the interest of some humans—is unknown.

While on sabbatical at the University of Colorado I learned from Larry Gold about an RNA hairpin loop of four nucleotides, UUCG (a tetraloop), that blocked the enzyme reverse transcriptase that synthesizes DNA from an RNA template. Because the UUCG tetraloop's melting temperature to the single strand is higher than loops of other sequences, it became known as an extra-stable tetraloop. The obvious question was what is special about the structure? At Berkeley, Gabriele

Varani and Joon Cheong found that the guanine, G, in the loop is in a rare *syn* conformation relative to the ribose instead of the usual *anti*; it also forms an unusual type of G • U base pair. Except for the middle U, the bases make a compact structure knit together by hydrogen bonds and electrostatic attractions. The middle U is dangling outside the loop, consistent with the fact that any base can substitute for the U without decreasing stability or the reverse transcriptase stop. The structure thus actually did help in understanding the function. As a test of our NMR structure we gave some of the RNA to Steve Holbrook at the Lawrence Berkeley Lab to get a crystal structure. However, the RNA would only crystallize as a double-stranded helix with non-Watson-Crick base pairs; no hairpin loop formed in any crystal. We tried to study the double helix in solution, but it always precipitated, so we were never able to compare the NMR and X-ray structures.

Another benefit of my sabbatical at Colorado was learning about pseudoknots from Olke Uhlenbeck. Although we both heard the same lecture on pseudoknots at a meeting in the Netherlands, he paid attention. He convinced me that pseudoknots were the most important new structural motifs found in RNA. In a pseudoknot, one end of the chain in a hairpin loop (a stem and loop) forms a second loop and stem by folding back to base pair with the first loop. If each stem had 11 or more base pairs (one turn of helix), a knot could form. Jackie Wyatt and Jody Puglisi measured the thermodynamic stabilities of pseudoknots with different loop and stem sizes and determined the NMR structure of one of them. They both got theses from Olke's insight. A few years later Harold Varmus, then at University of California, San Francisco, called to ask if I was interested in studying the pseudoknot reponsible for programmed frameshifting in mouse mammary tumor virus. This is a typical retrovirus that synthesizes its essential enzymes plus a viral coat protein as one long polypeptide chain; the enzymes are reverse transcriptase to synthesize its DNA template, integrase to incorporate this DNA into its host, and protease to cut the polyprotein into its active parts. The polyprotein is made by a series of frameshifting signals in the viral RNA that synthesizes the enzymes and the coat protein as one chain. The virus needs much less of the enzymes than of the coat protein, so most of the time synthesis stops after the first protein. Ten percent of the time, synthesis continues to make the polyprotein containing the vital enzymes. In most retroviruses, but not HIV, a pseudoknot is required for this to occur.

In collaboration with the Varmus group we studied the effect of changes in sequence and structure of the pseudoknot on frameshifting efficiency (14). We learned what characteristics of the pseudoknot were required for frameshifting. I presented the work at Yale once, and afterwards Peter Moore said, "Now all you have to do to understand how this works is to determine the structure of the ribosome." He and others have since done this, so we hope that we will eventually be able to study the interaction of the pseudoknot with the ribosome and thus understand how pseudoknots cause frameshifting.

As should be clear, we and many others are determining the structures and thermodynamic stabilities of the building blocks that make up a functional RNA. I mentioned earlier the 56-nucleotide RNA molecule whose structure Ming Wu

and Minxue Zheng recently determined at Berkeley. It is an independently-folding domain of the first RNA ever found to be catalytic (by Tom Cech). The complete ribozyme has over 400 nucleotides; it is presently too big for NMR. The structure of the small domain is significantly different in aqueous solution (200 mM Na^+) from its structure in a crystal as part of a larger piece of the ribozyme. As the crystalline RNA was in the presence of divalent ions, we added magnesium ions to the solution to more closely mimic the biological environment, and maybe to induce a structure similar to the crystal. Unfortunately, Mg^{2+} muddles the NMR spectrum beyond assignment; peaks split, broaden, and overlap. It is clear that multiple species are formed. This is not uncommon in RNA; it may be that RNA has evolved to be flexible and dynamic so it can change easily and respond to small changes in its environment. But it does make it difficult or impossible to determine its structure by any standard method. Multiple species produce either sums or averages of spectra depending on whether their exchange rates are slow or fast compared with the measurement time scale. In either case, or in the even worse case of intermediate exchange, determination of structure is very difficult. An obvious solution to the problem is to study a single molecule. One molecule at one time will have one conformation; a measured property will not be an average or sum of many species. It would be great to measure NMR on a single molecule, but at present we are content with using mechanical properties (force times distance) to measure thermodynamics and kinetics of single RNA molecules.

SINGLE-MOLECULE THERMODYNAMICS AND KINETICS

Carlos Bustamante, a graduate student in my group from 1976–1980, returned to Berkeley as a faculty member in 1998. He was now a leader in single-molecule studies, but he had only worked on DNA and proteins; he had ignored RNA. We agreed to rectify this. We (Jan Liphardt, Bibiana Onoa, and Steve Smith did the work) started with the 56-nucleotide domain from the first ribozyme and simpler versions of it. Micron-sized polystyrene beads are attached to the ends of the RNA by 500-base-pair DNA/RNA handles. One bead is held by a micropipet; the other is in a laser light trap. The distance (in nanometers) between the beads is measured and the force [in piconeutons (pN)] acting on the bead in the light trap is measured from its position relative to the center of the trap. Thus, the work necessary to unfold the RNA is measured; 1 pN times 1 nm (a zeptojoule) is equal to 0.6 kJ mol^{-1}. If the work is reversible at constant temperature and pressure, it is equal to the Gibbs free energy. The usual way to unfold an RNA is to heat it, add a denaturant such as 7 *M* urea, or remove all ions except the counterions needed to neutralize the phosphates. By using force, however, we can measure the free energy of unfolding the RNA at any temperature or solvent; we can study the effect of divalent ions, proteins, and any ligands on its unfolding and refolding (15).

The first RNA studied—a hairpin of 22 basepairs closed by a loop of 4 nucleotides (a tetraloop)—performed perfectly. It folded and unfolded (broke and

reformed the base pairs) reversibly at a force of 14.5 pN with a change in length of 20 nm, thus revealing a free energy of transition of about 175 kJ mol^{-1} at room temperature. This free energy is in good agreement with that expected for unfolding the RNA without force (after correction for the loss of entropy of the stretched out single strand held by beads in our experiment). The change in length corresponds to breaking the 22 basepairs. When we hold the force constant in the folding-unfolding transition region we can see the molecule jump back and forth between folded hairpin and extended single strand. We actually watch the distance between the beads oscillate between two values. By increasing the force we shift the equilibrium toward the long single strand; decreasing the force favors the short hairpin. A plot of the logarithm of the equilibrium constant versus force gives a straight line with slope equal to the change in length of the RNA divided by kT.

$$\frac{\partial \ln K}{\partial F} = \frac{\Delta l}{kT}$$

The length from the slope agrees with the length seen in the force versus extension curves. The kinetics lessons are just as compelling. Increasing force speeds the rate of hairpin-to-single-strand reaction and slows the rate of single-strand-to-hairpin reaction. The lifetimes of the hairpin and single strand vary but have an exponential distribution of values. The logarithm of each first-order rate constant (the reciprocal of the average lifetime for each species) is linear in force. We interpret the slope (multiplied by kT) as a measure of the distance between the ends of the hairpin and the transition state, or the ends of the single strand and the transition state. The position of the transition state depends on the sequence of the hairpin, and the number of bases in the loop. We are obtaining detailed knowledge about the free energy landscapes for folding and unfolding RNA molecules with different secondary structures.

The most exciting result is that these single-molecule experiments illustrate so many concepts of freshman physical chemistry so directly:

1. Reversible mechanical work is equal to free energy.
2. At equilibrium there really are reactions going in opposite directions.
3. Force and distance are equivalent to pressure and volume; they have similar effects on equilibria as in the familiar van't Hoff equation.
4. For a first-order kinetic process the lifetimes of a species are distributed exponentially.
5. A reaction coordinate as understandable as the distance between the ends of a molecule can characterize the extent of a reaction.

Larger and more complex RNAs introduce new phenomena including irreversible unfolding, multiple unfolding paths, and so forth. However, one very pleasant property of mechanical unfolding experiments is that it does not matter how large the molecule is. Delphine Collin has unfolded a ribosomal RNA with 1500 nucleotides. It will be a while before all the transitions are assigned.

A FACULTY POSITION

I was hired at Berkeley in 1956 through the old boy network. The acting chairman, Jim Cason, telephoned his organic colleague, Jim English, at Yale, asking for a recommendation for a physical chemist. The chairman at Yale, Kirkwood, recommended me, and after a short discussion with English I was offered a job at Berkeley. As an Hispanic (both my parents were born in Mexico), I have since become a valuable addition to the diversity statistics. As far as I could tell, hiring was pretty haphazard in the 1950s and 1960s, although in biophysical chemistry, the department did very well. Dick Powell, our chairman from 1960–1966, seemed to choose people based on the cost of their interview trip. John Hearst, then a postdoc at Dartmouth, was interviewed through his research director, Jerome Vinograd, who was invited up from Caltech to serve as proxy. Ken Sauer was a postdoc in Melvin Calvin's lab at Berkeley, and Jim Wang was a postdoc at Caltech when they were hired.

In the 1970s increasing pressure was put on Berkeley to hire women and minorities. I remember one faculty meeting where one after another of my senior colleagues made impassioned pleas for obtaining a woman. Taken out of context it might have sounded like a group of sex-deprived old men begging for relief. I mention old men because it surprised me that the younger faculty were less enthusiastic; it was the older, more politically aware, men who recognized the need for women on the faculty. We hired the first faculty woman—with tenure—in 1977; the first African American—also with tenure—was hired in 1981 while I was chairman. It is very clear that the women and minorities on our faculty have been a tremendous asset to the department; it is also clear that they would not have been hired without the strong push from outside the department.

I am sometimes asked how the students and the campus have changed over the past 45 years. The answer is that the students have stayed pretty much constant, while I have changed. When I arrived in Berkeley the graduate students and I were in our twenties; they still are. We used to play tennis, rock climb on Indian Rock, run in Redwood park, backpack in the Sierra, and party—at some of the parties just breathing would get you high. Indian Rock, a 30-foot pile of rocks in Berkeley used as a practice area by beginners and expert rock climbers, provided our routine Friday afternoon outing. Curt Johnson and Arlene Blum introduced me to the sport. Curt and I moved up to bigger mountains including Mt. Hood in Oregon and the Breithorn rear Zermatt. Arlene graduated to Annapurna and Everest. We carried RNA flags—in Arlene's case a tRNA flag—up several mountains.

The students and I now, rarely, walk in Tilden park. We have both grown more conscious of funding and of the importance of publishing in high impact journals (as first or last author). Now students are more apt to choose nonacademic jobs because they fear the five-year uncertainty before a tenure decision and the recurring anxiety at each grant renewal period. Tenure and grants were not necessarily easier to get earlier, but now many students do not think the pain and effort of getting a job, getting tenure, and getting funded are worth the minor pleasures of a faculty position. Of course, I disagree.

It seems to me that by working hard the first few years you can get a grant and earn tenure while doing some good science. After that it is all fun and you cannot be fired. Students and postdocs do the research, write the papers, and most importantly of all, entertain and teach you. An ex-student, who became a patent lawyer after receiving her Ph.D., said that she liked hearing about exciting new inventions without having to spend long hours in the lab on experiments that did not work. I agree; I have not worked in a lab since my sabbatical at Oregon State in 1971. Yet I not only get to learn about exciting new results nearly every day, but I eventually get credit for them. A tenured position at a university can't be beat. A class is three 50-minute or two 80-minute lectures a week; yet my colleagues think more than one class a term is excessive. Although we all complain, correctly, of being overworked and underpaid, the fact is we can pretty much work as much or as little as we like. We get expense-paid trips all over the world, and we can significantly augment our university salary if we consult, or get patent royalties, or found a company. I love my job and plan to continue it until I get bored or die, whichever comes first.

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I greatly appreciate everything I learned from everybody, and all the fun we had in and out of the lab. I trust the next few years will be as exciting as the previous ones. It was suggested a while ago in *Physics Today* that the termination of mandatory retirement might encourage the most dedicated professors to be buried with a few students and postdocs to continue their research. I have not discussed this yet with my current research group.

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