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The Excitement of Discovery

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■ Abstract I had the good luck to start research at the dawn of molecular biology when it was possible to ask fundamental questions about the nature of the nucleic acids and how information is transferred in living systems. The search for answers led me into many different areas, often with the question of how molecular structure leads to biological function. Early work in this period provided some of the roots supporting the current explosive developments in life sciences. Here I give a brief account of my development, describe some contributions, and provide a hint of the exhilaration in discovering new things. Most of all, I had the good fortune to have inspiring teachers, stimulating colleagues, and excellent students.

CONTENTS

EARLY FAMILY LIFE	2
HARVARD YEARS	3
CALTECH: 1949–1954	4
SCIENCE AT NIH	9
CAMBRIDGE, ENGLAND, AND THE STRUCTURE OF COLLAGEN 1	0
RNA STRUCTURE AND THE DISCOVERY OF HYBRIDIZATION 1	2
MOLECULAR BIOLOGY AT MIT	3
INFORMATION TRANSFER AND DNA-RNA HYBRIDIZATION 1	4
EARLY THOUGHTS ON THE ORIGIN OF LIFE	5
DNA IS FOUND IN ORGANELLES	5
SINGLE-CRYSTAL STRUCTURES 1	6
The Double Helix at Atomic Resolution	7
POLYSOME DISCOVERIES AND PROTEIN SYNTHESIS	9
The Mechanism of Protein Synthesis	1
Polypeptides and Polyesters	3
CRYSTAL STRUCTURE OF tRNA	3
Tracing the Backbone of Yeast tRNA ^{phe} and Solving the Structure 2	4
SEARCH FOR LIFE ON MARS 2	7
INTERNATIONAL SCIENCE AND PUGWASH MEETINGS 2	8
DNA TURNS LEFT-HANDED	9
Does Z-DNA Have a Biology?	0
Z-DNA Binding Proteins 3	2
Viruses Use Z-DNA Binding Proteins	3

EARLY FAMILY LIFE

In the years just before and immediately after World War I, my parents immigrated separately as young adults to the United States from Russia. My father arrived in 1913 and lived first in New York and Connecticut. The chaos surrounding immigration and the necessity to support himself and his family made it impossible to go to high school. Instead, he began to learn the dry cleaning trade. His parents had a farm in the Berkshire Mountains in western Massachusetts, and it was there that he met my mother who had immigrated in 1920 and lived with her family in Hartford, Connecticut. They married and settled in Hartford. A first son David was born in 1922, and I followed in 1924. In early 1925, the family moved to Springfield, Massachusetts, where my father had set up a dry cleaning business. Most of my childhood was spent in Springfield, which was at the time a heavily industrialized city on the banks of the Connecticut River. It had a number of industries, including gun manufacturers, especially in the U.S. Armory, which had produced guns since 1794.

Even though neither of our parents had a high school education, they valued education enormously. My father had a difficult time with his business and once the Depression set in, he, like many others, struggled just to find employment. However, he admonished my brother and me to get an education because, as he stressed, you can never lose the education, even though you may lose jobs or your possessions. Although our financial position was precarious, my brother and I never felt deprivation; after all, everyone else we knew was in a similar situation.

David was precocious, and even though we were only two years apart in age, during our childhood the psychological distance was much greater. He read widely, seemed to understand everything and usually interacted with older people. All of this, I suspect, provided a spur for me to catch up. The Springfield public schools that we attended provided a sound education. The school system was populated by a large number of very capable female teachers—in that era women did not have access to many other professions. I attended a technical high school that provided a good background in mathematics but largely taught its students how to operate machine lathes and other industrial equipment. It was taken for granted that we would work to supplement the family income whenever we could.

As a young boy, I seemed to have a great thirst for information and knowledge in many different fields. I was especially intrigued by the wonders of Nature and can clearly recall meeting regularly with two or three neighborhood boys at about age nine. I described to them what I knew about the organization of the Universe and pondered about what really was beyond the stars that we could see. Such questions left me with a strong sense of wonder and awe. In high school, I was befriended by an English teacher who was very interested in science. He sponsored an after-school group to discuss relativity theory. Certainly, we did not go very far into the theory, but this reinforced my enthusiasm for learning new things. In the summer of 1941, after my junior year at high school, I applied for and obtained a position working in the U.S. Armory in Springfield. My job was that of a barrel rifler. Thus, I ran the machines that put the helical grooves in the inside of rifle barrels. This was a highly skilled operation and required a knowledge of many machine manipulations. I worked the shift from 11:00 P.M. to 7:00 A.M. and kept the job during my entire senior year at high school. At age 17, it did not seem too difficult to keep to this schedule, and I found that I could do some of my homework while running the machines, especially memorizing Milton's poems.

The high school English teacher urged me to apply to college, and I was awarded a tuition scholarship to Harvard College. In August 1942, I resigned from my position as a Master Rifler in the U.S. Armory and moved 90 miles east to Cambridge, Massachusetts, to begin a college education.

HARVARD YEARS

Entering college in the fall of 1942 had a heightened sense of tension and excitement. Most of us knew we would be going off to the military soon. My academic interests were not fixed, but on an intuitive level I knew that I wanted to study chemistry, physics, and mathematics, as well as philosophy. However, the real fascination came from learning about atoms and molecules. After some uncertainty, I finally decided to major in biochemical sciences. This allowed me to take a large number of physical chemistry courses.

After a short time I enlisted in the Navy V12 Program, which was designed to train Naval officers. The program kept us at Harvard but had interesting features, such as a 6:00 A.M. wake-up time, followed by a three- to four-mile run along the Charles River before breakfast. Many of the instructors complained that the Navy students seemed to fall asleep in their 9:00 A.M. classes. Because there was a shortage of graduate teaching assistants, I volunteered to grade papers in physics courses, and that brought me in contact with several outstanding professors. By June 1944, I had completed the accelerated Navy program and was then sent to work in a hospital at a submarine base for six months. After that, I was sent to Syracuse Medical School. Upon being discharged from the Navy in January 1946, I returned to Harvard College to complete the requirements for my undergraduate degree and also to start a research program.

John T. Edsall, a prominent protein biochemist, was my tutor. Working with him was a great stimulus. Edsall had been studying amino acids by Raman spectroscopy and had demonstrated the zwitterion nature of amino acids. For my undergraduate thesis I extended these studies to a number of rarer amino acids, as well as dipeptides, which allowed us to identify the Raman bands associated with stretching vibrations of the peptide bond (1). In addition, I was able to carry out an infrared spectral analysis of urea, working with E. Bright Wilson in the Chemistry Department. This involved a point-by-point measurement using a homemade IR machine, slowly ratcheting through the spectrum. It took me 30 h of continuous operation to obtain the complete spectrum of urea. In two years, that machine was replaced by an automated system that shortened the time for a similar spectral analysis by almost three orders of magnitude.

At that point I faced a career dilemma. I had a well-developed interest in pursuing the physical chemistry of biological materials, but at the same time, medical school was interesting, giving me a detailed view of the biology of one organism. After much internal debate and discussions with John Edsall, I applied to complete my last two medical school years at Harvard Medical School. This decision had the added advantage that I could continue to work with John Edsall during that time since his office and laboratory were then in the medical school. It also gave me an excellent opportunity to learn how to fractionate and characterize proteins.

Many people visited John Edsall during this period, and one such visitor was J.D. Bernal from Cambridge, England. I was impressed by Bernal's intensity and the breadth of his knowledge. I came to know Bernal several years later when I was in Cambridge, England, where he was appropriately known as Sage.

During my senior year at medical school, I had discussions with John Edsall about my future. John Edsall had never taken an internship after medical school, but rather had gone on directly to do biophysical work in muscle proteins. Having decided to follow a similar path, I wrote to a number of scientists whom Edsall recommended, including Linus Pauling. I was pleased to receive an acceptance from Linus Pauling and was delighted when my application for a National Research Council Fellowship was accepted. This was before the NIH had a postdoctoral fellowship program, and the NSF was yet to be invented. Thus, I graduated in high spirits, spent a few months in Europe, and then showed up in Pasadena, California, on October 1, 1949.

CALTECH: 1949–1954

When I was ushered into Linus Pauling's office shortly after my arrival at Caltech, I was dressed in a formal suit and tie appropriate for an East Coast medical school graduate. Sitting behind a desk in a flamboyant Hawaiian shirt was Linus Pauling, who greeted me with a warm smile. It was clear that this was a different world from the one I had left in Boston. At the time of my arrival, Pauling had already published the important paper dealing with sickle cell anemia and its characterization as a molecular disease. This was a revolutionary description of disease, and it changed the way research was interpreted. Pauling's accomplishments at this time were already legendary. He had applied quantum mechanics to chemistry and written his monumental work, *The Nature of the Chemical Bond*, the first full treatment of the relationship between molecular structure and the properties of substances. The predicted quantum mechanical orbitals made possible a rational understanding of the way molecules were built

up, both organic and inorganic. My initial interests were in pursuing chemical theory, and Pauling suggested that I do some valence-state calculations for carbon atoms. At the same time, he suggested that I use my medical background to look for other cases of blood diseases that might also be associated with molecular defects in hemoglobin. In surveying a number of patients at the Los Angeles Children's Hospital, I found a young patient who had Cooley's anemia, now known as thalassemia, whose blood contained almost 100% fetal hemoglobin (2). I developed a method for analyzing the blood; this was one of my first publications while at Caltech. However, the fetal hemoglobin was normal, so it was not useful for studying molecular defects.

The organization of the Pauling lab was quite different from that which I had seen before. Pauling had an office in one corner of the Crellin Laboratory and in the other corner was a large room housing ~ 15 postdoctoral fellows, each with a desk in the open room. This was a very stimulating environment where we had intense discussions. There were a large number of talented postdoctoral fellows at Caltech. During that period I became especially close to Jack Dunitz, David Davies, and Robert Shulman, all of whom became highly productive scientists. Every few days Pauling would walk into the large room, and we would have some animated discussions, usually related to thoughts that Pauling had about different subjects. He would often make suggestions about topics that were worth pursuing.

I volunteered to work as a teaching assistant in the general chemistry course that Pauling taught. In the laboratory for the course, I joined Norman Davidson, who had recently joined the faculty and was working on shock-wave kinetics. We became close friends and talked often about biological and chemical phenomena. It may be that these discussions helped him move in later years into biological studies. During the same period, I became very friendly with Max Delbrück, who was in the Biology Department in an adjoining building. Jim Watson appeared briefly in 1949 and then left to go to Copenhagen to work with Herman Kalckar. One of the stimulating features of being a postdoctoral fellow at Caltech was the complete access that it gave to members of the faculty. Caltech was a small school at the time, and there was a great deal of interaction between young and old members of the scientific community. In the Chemistry Department, I became close to Verner Schomaker, who had an extensive knowledge of chemistry, and Jerry Vinograd, who arrived later. Among the many talented graduate students were Martin Karplus, Gary Felsenfeld, Matt Meselson, and Hardin McConnell.

One evening I was visiting Pauling at his home, where he was staying because he had a cold. I was there to discuss research, and he showed me a large book that had just arrived containing the results of a Faraday Society meeting in theoretical chemistry. He tossed the book down and said, "This is like whipping a dead horse." He explained that in the 1930s he had tried to obtain closed solutions for the equations describing the orbitals in molecules leading to the arrangement of bonding sites. However, he was unable to get exact solutions, and since then, people were using approximations piled upon other approximations, a process that Pauling thought was not very productive. This was, of course, before computers were developed, and Pauling's assessment of the futility was appropriate at that time. That evening had a great impact on me; I thought, "Here is an area where Linus Pauling was unable to solve the problems. Why am I fussing around doing valence-state calculations by approximations that are unlikely to be much better than those of many other people." After reflecting on this for some time, I decided to switch my research into X-ray structural analysis. Stimulated by Pauling's statement that knowing the structure of molecules led to their properties and function, I started by working with Dick Marsh, a crystallographer who was analyzing the structure of silk fibroins. At that same time, I started a crystal X-ray analysis with Jack Dunitz and Leslie Orgel, a theoretical chemistry postdoctoral fellow recently arrived from Chicago. We solved the three-dimensional structure of ferrocene (3), which had already been solved in projection. Today, this seems like a simple molecule, but at that time in the absence of computers or diffractometers, it required a great deal of work to obtain the data, measure the intensities visually, then carry out calculations with the aid of primitive punched-card systems that did not help very much.

During this period, I was taking a large number of courses and was very impressed by a thermodynamics course taught by Jack Kirkwood. Working with Kirkwood's graduate student Irwin Oppenheim, and Martin Karplus, we decided to take very good notes and then write Kirkwood's lectures in book form. This turned out to be a great method for learning thermodynamics, and the thick book that we produced was distributed in mimeograph form to many people. Several years later, Oppenheim expanded that book with Kirkwood to bring out his thermodynamics text.

I usually worked late into the evening in the laboratory and met Richard Feynman, who had recently arrived at Caltech. He was unmarried then, and it was also his habit to work late in the evening. We frequently got together and went to a local bar at the end of the evening where we would sit and talk about many things. He had a fund of stories that I listened to with great eagerness, many of which were eventually taped and produced as a collection. In 1952, I married my wife Jane, and we moved into a house in Pasadena near Caltech. Her interests were in childhood development. However, she did participate somewhat in my work by measuring the visual intensities of a large number of reflections in our three-dimensional X-ray analysis of the structure of ferrocene.

Quite often I would visit Pauling in his office. Invariably the secretary waved me in, and he was willing to discuss questions that I posed. On one occasion, I came into his office, and there he sat with his large feet resting on the desk. His chair was tipped back, and he was speaking into a dictating machine. I asked what he was doing. He said, "I'm writing a book on general chemistry." "Are you able to just dictate it?" I asked. "Oh, yes," he said, "I dictate the book and then get a copy back from the secretary and correct her mistakes." I considered this quite remarkable. However, over the years I adopted this same mode of writing, organizing the material for a paper in my head and then dictating it.

In December 1952, Linus Pauling suggested that I try to obtain X-ray diffraction patterns of DNA. This was after he had already generated a three-stranded model for DNA with bases on the outside. Shortly after publication, he realized it was incorrect. Pauling had been denied a passport to attend a meeting in London at which time Maurice Wilkins had displayed some diffraction photographs of DNA. Pauling wrote to Wilkins to ask if he could see these diffraction patterns. Wilkins declined because, he explained, they were still under analysis by his colleagues and himself. Thus, Pauling asked me to try to collect diffraction data.

The X-ray facilities at Caltech were not set up for fiber diffraction; I had a far less than optimal camera with a fairly weak, conventional X-ray tube. Thus, exposures took over 24 h, and it took some time to obtain oriented photographs. I began to get well-oriented photographs a few months later. Shortly after that, however, Pauling informed me that his son Peter had written to him that two "English" fellows at the Cavendish Laboratory had made a model of DNA with two strands in which the bases were on the inside. A short time later, their April publication in *Nature* was available, and it was clear that their structure both fit the data and solved an important biological problem in that it implied an obvious method for DNA replication.

In September 1953, Pauling organized a conference on the structure of proteins that included nucleic acids as well. A British group from the Cavendish Laboratory was there, including Bragg, Kendrew, Perutz, and Crick. By then, Jim Watson had returned to do postdoctoral work with Max Delbrück. Other attendees included Maurice Wilkins and John Edsall. Max Perutz presented his first evidence showing that a heavy atom could be introduced into the hemoglobin crystal, making it possible to solve the phase problem. Watson and Crick presented their work on DNA, and Wilkins showed the results of his X-ray studies. I gave a brief paper on how DNA and the alpha helix might interact, but the ideas were not notable. In particular, I failed to recognize that it would be possible for an alpha helix to enter into the major groove of the DNA molecule. During that period, my interest had changed to focus on RNA structure, to find whether RNA could form a double helix. Jim Watson, who was not fully comfortable in confining himself to work in the Delbrück lab, was interested in the same question. This made it natural to join forces and work together on this problem.

I became acquainted with and charmed by Francis Crick, who had such a bubbling enthusiasm. Crick invited me to come to Cambridge, England to use their more intense rotating anode machine, if I ever got results with the RNA project that would benefit by using this advanced facility. This turned out to be a significant invitation that I acted upon two years in the future.

One possibility for separating the DNA chains for replication was that the molecule might also turn left-handed. Working with wooden ball-and-stick

8

models that the Pauling Laboratory had in abundance, I tried to build a left-handed version of the right-handed B-DNA model of Watson and Crick. I could show that left-handed B-DNA would not be stable; however, I had occasion to revisit the problem some 25 years later. At one point, Pauling poked his head in the door of the model room and said, "Alex, work hard on this problem. I like most important discoveries to be made in Pasadena." This comment was typical of Pauling, who had a great competitive sense, especially regarding the Bragg Laboratory in Cambridge, England. Because he had already discovered the alpha helix and the beta sheet as well as the sickle cell discovery of molecular disease, I could see why he would make such a statement, even though he had clearly missed out on the double helix.

Jim Watson and I collected samples of RNA from many people. By then, I had developed reasonable skill in making oriented fibers, formed by slowly withdrawing a glass rod from a sticky nucleic acid droplet using a microscope for the slow movement. The X-ray diffraction patterns were not encouraging. They all looked similar, somewhat suggestive of a double helix but with a different distribution of intensity. Leslie Orgel was interested in the work on RNA and, together with Jim Watson, the three of us discussed these problems extensively. It was thought that RNA was important in directing the synthesis of proteins. Just how that occurred was mysterious and unknown. George Gamow, a distinguished theoretical physicist and a friend of Max Delbrück, arrived at Caltech, very much taken by the problem of how proteins are made under the direction of the nucleic acids. He proposed the first code for determining the sequence of amino acids from the DNA double helix (4). His code was incorrect, but it stimulated a great deal of work in an effort to develop the actual code used in nature. Later, we wrote a review considering various codes (5). Gamow was a lively personality and a great practical joker. He had the idea of setting up a club of people interested in the coding problem, and he decided that the group would be defined by a tie. It became known as the RNA Tie Club and consisted of some 20 members, one for each amino acid. Many of the members were friends of Gamow, but it also included many of the principal workers in the then-small field of nucleic acid research as well as a number of physicists such as Dick Feynman, Edward Teller, and others. The main purpose of the club was to circulate manuscripts dealing with the code. It was in one of these RNA Tie Club manuscripts that Francis Crick first developed the adapter hypothesis suggesting that a small RNA molecule might act as an intermediate between the amino acids and the nucleic acid molecule that codes for protein. Later, it was realized that these adapters had been discovered by Mahlon Hoagland and Paul Zamecnik (6) and eventually opened the door to the role of transfer RNA in protein synthesis.

Our thoughts in 1953 and 1954 were much more primitive. We wondered whether it was possible for the RNA molecule to have a form that would bind specific amino acids in some kind of a linear array. Such models seem quite naïve today, but that is where thinking about this problem first began. In the work on RNA fibers, Jim Watson and I used RNA preparations in which the base ratios varied widely. Some were nearly complementary, as found in DNA, and others were far removed. Nonetheless, the patterns remained about the same. This continued to be a puzzle. Of course, at the time, we did not understand the variety of different types of RNA in the preparations. And there was some question about whether RNA was a linear polymer, as was the case for DNA. In the literature, there was discussion of the fact that chains could also be attached to the 2' hydroxyl group of the ribose, thereby giving rise to a complicated net, instead of a linear polymer. Watson and I published two papers describing these results, one in *Nature* (7) and another sent to *PNAS* (8) by Linus Pauling. We included a diffraction pattern but had no clear interpretation of it.

The National Research Council Fellowship was awarded for a three-year period. At the end of that time in 1952, I enlisted in the U.S. Public Health Service so that I could continue my research. Having finished my medical training after World War II, I was subjected to the doctor draft for the Korean War. However, working as a "yellow beret" at NIH offered an alternative. I was hired by Seymour Kety, who was then organizing the research groups for the newly formed National Institute of Mental Health. He appointed me Chief of the Section on Physical Chemistry, but because the large research Building 10 was being built in Bethesda, I remained at Caltech for another two years. As a Section Chief, I was required to hire coworkers. Jack Dunitz was interested in continuing work in the general biological area, and he agreed to join me at NIH. By June 1954, the building had been completed. Jane and I packed our belongings and started out on the long trip from Pasadena to Bethesda, Maryland. As I reflected on my five years of work at Caltech, I concluded that I learned quite a bit but actually accomplished very little. The publications I had were what I considered routine, and I had serious doubts about whether I could, in fact, make important discoveries.

SCIENCE AT NIH

The contrast between Caltech and NIH was very great. Whereas Caltech was small in size and had people from all branches of science, NIH was extremely large and was staffed predominantly by experts in biomedical research. As I moved into the new laboratory in Building 10, I was faced with the job of assembling the right equipment to carry out research. In a short time Jack Dunitz arrived from Caltech. By then, I had written a letter to David Davies, who had left Caltech to return to England,where he was working in a chemical company. I urged him to come and join the research at NIH, since I thought the phosphates that we would be looking at (the nucleic acids) would be more interesting than the inorganic chemicals that he would be working with. A letter was also sent to former Pauling graduate student Gary Felsenfeld, urging him to join the lab.

I determined to continue working on the question of RNA structure, and the question of whether it could form a double helix. With somewhat better X-ray

cameras than those available at Caltech, I continued to study the diffraction patterns from oriented RNA preparations. However, they did not provide insight. This research effort took an abrupt change early in 1955 when Severo Ochoa came to give a seminar at NIH, describing the enzyme polynucleotide phosphorylase that had just been isolated by Marianne Grunberg-Manago in his laboratory (9). It converted nucleoside diphosphates into long RNA chains. He agreed to make some of these polymers available to me, and within a short time with some help from Leon Heppel at NIH, we were able to make our own enzyme and our own polymers. I found that the random copolymer containing adenine and uracil residues produced a diffraction pattern very similar to that found in naturally occurring RNA. These synthetic chains were linear and that made me feel that the likelihood of a significant amount of branched RNA molecules was not very great. The preparation of polyadenylic acid began to produce an interesting pattern highly suggestive of a helical complex. After working on that for some time, it became apparent that more data could be obtained with a more powerful X-ray source and better cameras. Accordingly, I wrote to Francis Crick, and he invited me to Cambridge, England, where I could use the improved equipment that had been developed at the Medical Research Council unit in the Cavendish Laboratory.

CAMBRIDGE, ENGLAND, AND THE STRUCTURE OF COLLAGEN

In July 1955, I flew to Cambridge, England, for a short visit. At roughly the same time, Jim Watson arrived in Cambridge, as well as Leslie Orgel. We were thus able to resume intensive discussions on RNA structure that we had begun at Caltech, with the significant addition of Francis Crick. Improved diffraction patterns of polyadenylic acid were obtained by myself and by Jim Watson. Eventually, we solved its structure working with Francis Crick and David Davies (10). However, the direction of my research would soon change rather abruptly.

Francis Crick had generously offered to have me stay at his house in Portugal Place, together with his wife Odile and their two young daughters. One Saturday morning the new issue of *Nature* arrived, and Francis looked through it. Over breakfast I asked him if there was anything interesting in the journal. He replied that there was an article by Bamford and colleagues at the Courtauld's Laboratory, describing a new form of polyglycine called polyglycine II. They did not know the structure but presented IR spectra and a powder X-ray diagram. As we continued eating breakfast, the idea developed that perhaps we could solve the structure by working with skeletal brass molecular models that were available in the laboratory. The usual form of polyglycine has an extended polypeptide chain with each residue related to the previous one by a translation and a rotation of 180°. Once we were in the laboratory, we realized that, if we changed the rotation

from 180° to 120°, we could build a trigonal lattice in which each polyglycine chain formed hydrogen bonds with six other chains around it. The lattice so constructed clearly predicted the position and intensities (11) observed in the published powder pattern. We realized that we had solved its structure by early afternoon of the same day. This was a striking example of the power of using a molecular model to interpret the structure of regular macromolecules.

After we had written the paper and sent it in, Francis and I were having afternoon tea outside the laboratory. I mentioned to him that the C-H bond of the glycine residues where longer side chains would be attached for other amino acids was in the same plane as the N-H bond in the adjoining peptide linkage. To me this meant it would be easy to form a pyrollidine ring. We wondered if we could take three chains from the polyglycine lattice and make a repeating sequence of glycine-proline-proline as a model for the unsolved structure of collagen. It was known at the time that collagen had a large amount of glycine and dipeptide sequences of Gly-Pro and Hydroxypro-Gly. Thus, a repeat of this type seemed reasonable. We returned to the laboratory and found that by building such a model, then twisting it so that we had a coiled coil, we could make a model that predicted some of the known reflections of the highly characteristic collagen fiber diffraction pattern. In our haste we almost overlooked the fact that, because of the symmetry, there were two such models that could be generated from the same lattice. After building six-foot-high models, we placed them at one end of a long basement corridor in the Cavendish with a powerful point light source at the other end. On a sheet of paper we plotted the projection of the model and its approximate atomic coordinates. A reducing pantograph was used to drill small holes at atomic centers in a metallic strip about 12 cm in length. With this "mask," we made a trip to another laboratory that had a large optical diffractometer. By looking at the diffraction patterns, we could see that one of the two similar models seemed a better fit to the collagen diffraction pattern. We published a short note in Nature (12) describing the structure and later a longer, detailed article comparing the diffraction pattern of this model with the observed collagen pattern (13).

The effect of this work was that my stay in the Crick household, originally planned to be a few weeks, was extended to somewhat over six months, during which time my wife came and joined us. I felt bad about imposing on the Cricks for such a long period, but my wife and I had the opportunity to reciprocate several years later when the Cricks spent a term staying with us in our house in Cambridge, Massachusetts.

Although the collagen work had a different direction from the focus on the nucleic acids, it had a strong positive effect on my psyche. For one thing, I began to develop some self-assurance in my ability to carry out research and make discoveries. I believe that a form of "scientific maturation" is an important component in developing a confident thrust into research work. Some time later, Linus Pauling was invited to attend a meeting held in England on collagen, and I presented the results of our work in this area. At an intermission, a number of

the English scientists asked if it was true that I had trained to do research in the Pauling laboratory. Pauling replied that I had spent a long time in the laboratory without accomplishing very much, but I must have learned quite a bit. He smiled at me as he made this comment. I felt that it was probably a fair assessment.

RNA STRUCTURE AND THE DISCOVERY OF HYBRIDIZATION

On returning to NIH from England, work on RNA structure continued with David Davies. In 1956, we mixed solutions of polyriboadenylic acid (poly rA) and polyribouridylic acid (poly rU) and discovered a remarkable transformation revealed by fiber X-ray diffraction. These two molecules actually reacted with each other to produce a double helix! A brief note was sent to the *Journal of the American Chemical Society* in June of that year (14), describing the work with a preliminary interpretation of the diffraction pattern. At the same time, Bob Warner published a short note, describing hypochromism when the two polymers are mixed (15). Our X-ray patterns clearly indicated the formation of a helical complex that was not present in either of the two individual polymers. Furthermore, the pattern had significant differences compared with those produced from DNA.

This result, which seems so obvious today, generated a great deal of skepticism at the time. While walking down a long corridor at NIH, I met Herman Kalckar, an eminent Danish biochemist. I mentioned that we discovered that poly rA and poly rU formed a double helix. Kalckar was incredulous. "You mean without an enzyme?" he asked. His attitude was justified, since the only double helix known at that time was one made with the DNA polymerase enzyme that Arthur Kornberg had purified (16). Other critics thought it was highly unlikely that polymers containing over 1000 nucleotides would be able to disentangle themselves and form a regular structure. They believed it would be hopelessly entangled. Somewhat later a distinguished polymer chemist told me that my interpretation was wrong since both polymer chains were highly charged and that they would never combine. These were examples of the thinking at that time.

Two weeks after sending off the 1956 *JACS* note, I wrote a letter to Linus Pauling, describing these results. The letter reveals a sense of incredulity on my part that this reaction could happen and that it was "completely reproducible." This was the first demonstration that RNA molecules could form a double helix. It was also the first hybridization reaction and, as we pointed out in the *JACS* letter, "this method of forming a two-stranded helical molecule by simply mixing two substances can be used for a variety of studies" (14).

An important method for studying the nucleic acids was measuring their absorbance in the ultraviolet. For some time, it had been known that polymerization of nucleotides resulted in a decrease in absorbance at 260 nm. Bob Warner had described the hypochromism on mixing (15). Although the mecha-

nism of hypochromicity was not understood at the time, it was a useful tool for analysis. Insight into the reaction of poly rA and poly rU was obtained by Gary Felsenfeld, who carefully measured hypochromicity in mixtures of varying composition and showed that it fell to a very sharp minimum at a 1:1 mol ratio (17). We also discovered that addition of divalent cations such as magnesium would change the picture dramatically (18), leading to the formation of a three-stranded molecule. We concluded that a second strand of polyuridylic acid bound in the major groove of the poly rA-poly rU duplex. Addition of this strand did not increase the diameter of the molecule and neatly accounted for the 50% increase in sedimentation constant. It was proposed that the uracil in the second poly rU strand bound N3 and O4 to the N7 and N6 of adenine. This proposal was considerably strengthened two years later by the X-ray analysis of the co-crystal containing 9-methyl adenine and 1-methyl thymine by Karst Hoogsteen (19) which had the same hydrogen bonding. The 1957 discovery of the RNA triplex pointed out the structural complexity inherent in RNA molecules. Over the next several years, a variety of polynucleotide interactions were studied (20-23), leading to the formation of other two- and three-stranded molecules.

MOLECULAR BIOLOGY AT MIT

In the 1950s when biomedical research was expanding rapidly, the NIH was regarded as a prime recruiting ground for universities. After receiving several offers for faculty positions in university biology or chemistry departments, I decided to move so that I could work with students. I was particularly attracted by an offer from the then rather small Biology Department at MIT because it could attract excellent students, and several people at MIT, such as Jerry Wiesner and Victor Weisskopf, felt strongly that molecular biology would grow at the Institute. Accordingly, in 1958, I left NIH and moved to the Biology Department at MIT. Once again, I found myself moving into empty rooms, but the generous program of NIH grant support soon allowed me to equip the rooms and resume research.

Shortly after I arrived, I was asked if I would be willing to have Antioch Work Study students come to work in my laboratory for a three- or six-month period. I readily accepted, as these students were known to be highly motivated and well qualified. This program continued at MIT for several years during which time I had as undergraduates people such as Joan Steitz and Mario Capecchi, among others, who made valuable contributions to the laboratory's research program. At the same time a number of talented people came to the MIT laboratories for postdoctoral work. David Blow, originally from Cambridge, England, began working with me at NIH and moved to MIT where he continued working for another year. Among others from England were researchers such as Tony North and David Green, both of whom were trained crystallographers. Ken-ichi Tomita from Japan, Paul Knopf, and Hank Sobell were also among the early postdoctoral fellows.

INFORMATION TRANSFER AND DNA-RNA HYBRIDIZATION

In the late 1950s, a key question was, How does DNA "make" RNA? Several investigators at that time had shown that crude preparations of an RNA polymerase activity would incorporate ribonucleotides into RNA using a DNA template, but the mechanism was not at all clear. It was widely believed that information transfer went from DNA to RNA, but how did that happen? When I was at NIH, I met Gobind Khorana, who was then visiting the laboratories of Arthur Kornberg and Leon Heppel. We became friends, and when I later learned that he had been able to synthesize oligomers of poly deoxythymidylic acid (poly dT) (24), I asked if he would be willing to make a sample available. I told him I wanted to study the possibility that this could make a double helix with an RNA molecule, even though it was known that the RNA backbone was significantly different from the DNA backbone due to the 2' hydroxyl group in RNA. At the time it was not obvious that they could combine.

Nonetheless, in 1960 I showed that these two molecules could accommodate each other to form a hybrid helix containing one strand of poly dT and one strand of poly rA (25), as seen from hypochromism and other studies. This was the first experimental demonstration of a hybrid helix, and the discovery of messenger RNA was still one year in the future. It immediately provided experimental support for a model of how DNA could "make" RNA, using complementary base-pairing, as in DNA replication. A year later in 1961, experiments by J. Hurwitz with a purified RNA polymerase preparation demonstrated that this was the mechanism underlying information transfer from DNA to RNA (26). The reaction between poly dT and poly rA was the first experimental demonstration that the two different backbones could adapt to each other in this method of information transfer. The reaction was also the first hybridization of a DNA molecule with an RNA molecule. The same hybridization reaction is widely used today in the purification of eukaryotic mRNA by hybridizing poly dT to their poly rA tails.

Later in 1960, Marmur, Doty, and their colleagues demonstrated that it was possible to renature naturally occurring denatured DNA duplexes by incubating them at an intermediate temperature that would allow the single strands to anneal together with the correct sequence (27, 28). A year later, this annealing method was also adopted to form DNA–RNA hybrids in viral systems (29). Together with my graduate student Howard Goodman, we established, using hybridization experiments, that tRNA was encoded by DNA (30). Our knowledge of RNA at the time was so primitive that we had to rule out RNA replication for tRNA synthesis.

EARLY THOUGHTS ON THE ORIGIN OF LIFE

In 1961, I was asked to contribute an article to a volume dedicated to Albert Szent-Gyorgyi, and I decided to write about the evolution and origin of life. This was prompted by the current ideas largely espoused by the Russian biochemist Oparin, who believed that life began through the creation of primitive protein molecules that provided the necessary environment for developing cells and cell replication. However, the greatest weakness in a theory of this type was it did not really explain the evolution of nucleic acid–mediated protein synthesis. It seemed more reasonable to suggest that life began with nucleic acids.

I suggested that primitive polynucleotide chains could act as follows: "We postulate that the primitive polynucleotide chains are able to act as a template or a somewhat inefficient catalyst for promoting the polymerization of the complementary nucleotide residues to build up an initial two-stranded molecule.... It may be reasonable to speculate that the hypothetical stem or parent polynucleotide molecule was initially an RNA-like polymer..." (31). It was apparent by that time that RNA molecules could contain genetic information, as in the RNA viruses. They also were known to play a central role in the synthesis of proteins. DNA was thus regarded as a specialized derivative molecule that evolved later in a form that contained specific information and carried out the molecular replication inherent in propagating living systems.

At the time it was not known how mRNA was made in vivo. Thus, I suggested, "mRNA may be made in vivo as complementary copies of one or both strands of DNA. If both strands are active, then the DNA would produce two RNA strands that are complementary to each other. Only one of these might be active in protein synthesis, and the other strand might be a component of the control or regulatory system" (31). This is perhaps the first statement of what we now know as "antisense" RNA. It also suggested that RNA molecules might have a control or regulatory function, which now appears to be the case with the newly emerging information about the role of micro-RNAs or RNAi.

This article, published in 1962, is probably the oldest statement regarding the role of RNA in the origin of life. Only after many years did the concept of "The RNA World" develop together with the enormous growth and recognition of its ribozyme activities. It is interesting that in the early days of the development of molecular biology, very large features of biological systems were unknown. It was possible to speculate then and anticipate features that could be investigated only several years in the future.

DNA IS FOUND IN ORGANELLES

In 1962, a student, Mike Vaughn, came into my office, saying that electron micrographs of organelles showed string-like features that might be DNA. Together with a postdoctoral fellow, Ed Chun, we decided to explore the

possibility, using the newly developed method of density gradient centrifugation. Chloroplasts were isolated from several organisms and all had a DNA species distinct from the nucleus. Another band appeared in some pelleted preparations that we suggested came from contaminating mitochondria (32). This approach was widely adopted, opening a vast field of research. This is an example of how new areas of biology opened up during that period.

SINGLE-CRYSTAL STRUCTURES

X-ray diffraction studies of nucleic acid fibers were carried out extensively by M. Wilkins, R. Franklin, and colleagues in the 1950s and 1960s, but it was realized that the limitations of such studies were enormous. In fiber X-ray diffraction, a rather small number of reflections are registered. However, the number of variables needed to define the structure (at least 3N, where N is the number of atoms) is so great that it was clear that fiber diffraction could not "prove" a structure. It could only say that a particular conformation was compatible with the limited diffraction data from fibers. Starting in the early 1960s, there were many single-crystal X-ray diffraction studies involving co-crystals of purines and pyrimidine derivatives such as those initiated by Karst Hoogsteen (19). These studies were useful in obtaining information about components of nucleic acid structure. For example, together with Hank Sobell and Ken Tomita, we solved a co-crystal of cytosine and guanine derivatives showing that they were held together by three hydrogen bonds (33), not two, as initially suggested by Watson and Crick. Linus Pauling had already emphasized this point based on general structural considerations (34). Several co-crystals were solved of derivatives of adenine and uracil or adenine and thymine during this period in our MIT laboratory (35-37), as well as elsewhere (38). At the same time selective base-pairing could be demonstrated in solution. The center of the double helix is hydrophobic owing to base stacking. Together with Yoshimasa Kyogoku and Dick Lord, we studied the hydrogen bonding of purine and pyrimidine derivatives by their IR spectra in chloroform solution. It showed that adenine selectively paired with thymine or uracil derivatives, while guanine paired with cytosine derivatives (39).

But a disturbing trend emerged from the crystallographic studies. Only Hoogsteen base-pairing was found between adenine and thymine/uracil derivatives. This led some investigators to suggest that the double helix might be held together by Hoogsteen pairing. The calculated diffraction pattern of such a helix had many similarities to that predicted by a double helix held together by Watson-Crick base pairs, even though the fit was not good (40). Thus, the question remained: What is the real structure of the double helix?

The Double Helix at Atomic Resolution

The first single-crystal structures of a double helix were solved in 1973 by my students and postdoctoral fellows, including John Rosenberg and Ned Seeman. This was before it was possible to synthesize and obtain oligonucleotides in significant quantities suitable for crystallographic experiments. However, we succeeded in crystallizing two dinucleoside phosphates, the RNA oligomers GpC (41) and ApU (42). The significant point in this analysis was that the resolution of the diffraction pattern was 0.8 Å. Atomic resolution allowed us to visualize not only the sugar phosphate backbone in the form of a double helix, but also the positions of ions and water molecules. It could be shown that extending the structure using the symmetry of the two base pairs made it possible to generate RNA double helices that were quite similar to the structures that had been deduced from studies of double-helical fibers of RNA. The bond angles and distances and, in addition, gave rise to the nomenclature for identifying torsion angles in the sugar phosphate backbone.

The GpC structure had the anticipated base pairs connected by three hydrogen bonds. However, the ApU structure showed for the first time that Watson-Crick base pairs formed when the molecule was constrained in a double helix (Figure 1), as opposed to the Hoogsteen base pairs that were favored in the single-crystal complexes of adenine with uracil derivatives. I mailed preprints of these to several people, including Jim Watson. He phoned me, saying that after having read the ApU manuscript, he had his first good night's sleep in 20 years! This indicated to him that the uncertainty about the organization of the double helix was resolved. The significance of the double helix at atomic resolution was recognized by the editors of *Nature* who, in their "News and Views" commentary, called it the "missing link" and recognized that "the many pearls offered" helped resolve one of the big uncertainties in nucleic acid structure ["News and Views" (1973) *Nature*, 243:114].

These structures capped the effort that I had started some 20 years earlier, which started to make progress in 1956 with the recognition that poly rA and poly rU would form a double helix. Here, at last, was the demonstration at atomic resolution of the details of that structure. High-resolution crystallographic analysis of larger fragments of the double helix (DNA or RNA) did not emerge until almost a decade later with the availability of chemically synthesized and purified oligonucleotides in large enough quantities to permit single-crystal diffraction analysis.

A single-crystal X-ray structure of a hybrid helix did not appear until 1982 when, together with Andy Wang and colleagues, we solved the structure of a DNA–RNA hybrid linked to double-helical DNA (43). This was 22 years after the hybrid helix was first observed (25). It showed that the dilemma of two different backbone conformations was resolved by having the DNA strand adopt the RNA duplex conformation. This had been inferred from fiber diffraction



Figure 1 The 0.8 Å resolution double helical structure of the dinucleoside phosphate ApU, as displayed on the cover of *Nature* magazine (42).

studies and has remained a constant feature, reflecting the relative conformational flexibility of the DNA backbone compared with the less flexible RNA strand.

POLYSOME DISCOVERIES AND PROTEIN SYNTHESIS

Experiments in 1961 revealed the presence of a rapidly metabolizing fraction of RNA called mRNA. Evidence supporting the triplet code (44) suggested that very large mRNA molecules could be formed. While attending a Gordon Conference in 1961, I began to think about how ribosomes would be organized on mRNA. The ~150 amino acids in hemoglobin chains, for example, required 450 nucleotides or an mRNA over 1500 Å long if the bases were stacked. This was much larger than the ~200 Å-sized ribosomes in which protein synthesis occurred.

On returning to MIT, I talked with my first graduate student Jon Warner and suggested that he try to see if there were several ribosomes associated with individual mRNA strands. His first experiments using Escherichia coli were unsuccessful. He then joined Paul Knopf to work with rabbit reticulocytes, which have the advantages of containing no nucleus; they can be lysed by gentle methods and they synthesize mostly hemaglobin. Their experiments were enormously successful. Sedimenting the lysate on a sucrose density gradient revealed that polymerized radioactive amino acids were found in a rapidly sedimenting fraction but were not found with individual ribosomes. In a shallow sucrose gradient, a series of peaks of both optical density and radioactivity were found. By simple counting it was possible to separate fractions containing two, three, four, or five ribosomes. Although electron microscopy studies were not very common in those days, we brought these samples to Cecil Hall, an electron microscopist in the department, and the organization of what we called polysomes was clearly visible. A cluster of five ribosomes that synthesize hemaglobin was found acting on the same mRNA strand. A thin strand could be observed running between the ribosomes, both in shadowed electron microscope preparations and in preparations negatively stained with uranyl acetate (Figure 2). They could be readily dissociated by a brief exposure to ribonuclease.

Two papers were sent in for publication: one dealing with the electron microscopic characterization (45) and the other with biochemical experiments (46). *Science* put the electron microscopic field of polysomes synthesizing hemoglobin on the cover of the magazine. The article described our interpretation with ribosomes moving along the mRNA strand at the same time as the polypeptide chains were elongating. This was a radically different view of protein synthesis, and many *Science* readers objected vigorously to this paper. Editor Phil Abelson sent me a sheaf of these letters. Most of the comments required no answer since the second paper with the biochemistry came out the following month. However, some of the readers' comments afforded a certain



Figure 2 (*Top*) Negatively stained electron micrograph of rabbit reticulocyte polysomes synthesizing hemoglobin, showing the thin mRNA strand (43). (*Bottom*) A schematic model of polysome function is shown, as demonstrated experimentally (47).

level of amusement. For example, several stated that these were "artifacts," and that they had been seen in electron microscopic sections for years.

The model suggested that longer mRNA strands coding for larger polypeptide chains would have larger polysomes. At about that time Jacques Monod visited Salvador Luria at MIT and came to my laboratory where I showed him these recent results. He expressed great delight because it solved a problem that he had been pondering, namely, how could you have the coordinate synthesis of several different peptide chains in unison. It was apparent from the model that the mRNA could be polycistronic, and the ribosomes moving along the chain would synthesize one polypeptide chain after the other, insuring a stoichiometric synthesis.

The discovery of polysomes changed thinking in the field of protein biosynthesis in several ways. First, the electron microscopic pictures offered a graphic visualization of the assembly mechanism, implying that ribosomes move along the message and the polypeptide chain elongates. Although there had been limited previous discussion of ribosomal movement along the message, images of the polysomes clearly indicated a dynamic assembly structure, strongly reinforcing the movement of ribosomes. Further experiments were done with polysomes to investigate this mechanism. Experiments in vitro with labeled single ribosomes by my student Howard Goodman (47) showed that they could attach to the end of polysomes, while further incubation led to the release of individual ribosomes and completed polypeptide chains (Figure 2). Although movement of ribosomes over mRNA seems obvious now, this notion required a reordering of the thinking of many people in the field. There was some resistance to these ideas for the first year or so, but subsequent experiments in my laboratory and several others showed the universality of the mechanism.

An example of the extent to which the polysome discovery jolted the thinking of scientists in the field was graphically illustrated to me at a meeting on hemoglobin at Columbia University's Arden House in November 1962, shortly after submitting the two polysome papers. After I presented the polysome data, including my interpretation, Fritz Lipmann took strong exception. He told me that I had misinterpreted the data. I was surprised at the vigor of his criticism and a little puzzled at the same time. Several weeks later Lipmann came to Boston and visited me at MIT. He apologized and told me that he had been mistaken when he objected to my interpretation. I was quite impressed that such a distinguished scientist would go out of his way to tell a younger colleague that he had been mistaken.

The Mechanism of Protein Synthesis

The isolation of polysomes on a sucrose gradient meant that the ribosomes actually involved in protein synthesis could be separated from the large number of inactive monomer ribosomes. This made it possible to uncover further aspects of the translation machinery. For example, it was generally assumed that there was one polypeptide chain per ribosome. Isolating active ribosomes made it possible to demonstrate experimentally that there is one growing polypeptide chain per ribosome (48). Of far greater interest, however, was our ability to analyze the number of transfer RNA (tRNA) molecules per active ribosome, as this had a direct bearing on the mechanism of protein synthesis.

Rabbit reticulocyte cells have already lost their nucleus and no longer synthesize RNA. This made it possible for Jon Warner to do a rather clean analysis of the system by incubating the reticulocytes with radioactive adenine or cytosine (49). These molecules could penetrate into the interior, where they were incorporated into ribonucleoside triphosphates. Although RNA was not synthesized, the 5' CCA ends of tRNA molecules were continually cleaved off and then enzymatically readded to tRNA. The radioactive nucleotides thus labeled the ends of tRNA molecules and nothing else. This showed that two tRNA molecules were bound to active ribosomes, but only one was bound to inactive ribosomes at the top of the gradient. Several investigators had shown previously that ribosomes would bind one tRNA in an exchangeable fashion, and attempts were made to formulate a mechanism whereby a single tRNA would be involved in protein synthesis; however, the mechanism seemed implausible. With the presence of two tRNA molecules, it was possible to make a different interpretation. We postulated that the two tRNA-binding sites occupy adjacent codons. We called one Site A, which bound aminoacyl tRNA, and the other Site P, which bound peptidyl tRNA. We envisioned these two sites acting in a coordinated manner to transfer the growing polypeptide chain and to move the mRNA codon from Site A to Site P. We suggested that it constituted the basis of ribosomal movement relative to the messenger strand. This statement, formulated in 1964, rapidly became the standard description for interpreting ribosomal movement and tRNA activity in protein synthesis. The recent determination of the threedimensional structure of the ribosome has confirmed the interpretation and provided a structural basis for understanding how this mechanism works.

The statement describing the movement of tRNA molecules in the ribosome postulated engagement of the transfer RNA molecule to the mRNA, and at the same time, it is the substrate for the ribosomal peptidyl transferase enzyme that transfers the peptide chain from the tRNA in the P site onto the amino acid of the aminoacyl tRNA in the A site. The structural and geometric background for understanding where these two events occur had to await determination of the three-dimensional folding of transfer RNA molecules.

In 1967, Leonard Malkin and I asked to what extent the growing polypeptide chain in rabbit reticulocyte polysomes was accessible to externally added proteolytic enzymes (50). These experiments demonstrated that a resistant fragment shielded by the ribosome was the most recently synthesized segment, and it was attached to the tRNA molecule. It was shown that the shielded segment contained 30–35 amino acids. An extended conformation at 3.6 Å/amino acid implied a protected segment of about 100 Å in length. The structure protecting the chain came to be referred to as the polypeptide tunnel. The recent three-dimensional structure determination of the 50S subunit at high resolution has revealed a tunnel of that approximate size (51). The large shielded section of polypeptide probably stabilized its attachment to the ribosome. For very large polypeptide chains, it was possible that the chains could start to fold in a native conformation while they were still attached to the ribosome. This had been observed at an earlier date as active β -galactosidase could be detected on bacterial polysomes synthesizing that protein (52).

Polypeptides and Polyesters

The first indication that the ribosomal peptidyl transferase could catalyze the formation of esters as well as peptides was obtained in experiments with analogs of puromycin in which the α -amino group was replaced by a hydroxyl group (53). This study revealed that many of the characteristics of ester formation were similar to those found in the peptide formation of the puromycin reaction itself. These experiments encouraged my student Steve Fahnestock to incorporate an ester linkage in the viral coat protein of the R17 bacteriophage by providing all of the amino acids needed in a segment of the viral coat protein, except for one provided by chemically modified phenylalanyl tRNA^{phe}. Treatment with HONO yielded an α -hydroxy phenyllactyl tRNA (54). The coat protein was synthesized with an ester linkage specifically introduced at one position in the chain, identified explicitly by hydrolyzing the synthesized chain in the presence of alkali and characterizing the fragments produced by the reaction.

This 1971 experiment was the first example of a nonnatural residue incorporated into a protein. In recent years, a large number of nonnatural residues have been incorporated into proteins, and this field is now burgeoning to produce many proteins with specialized features. Finally, experiments were carried out incorporating α -hydroxyl phenyllactyl tRNA^{phe} in an in vitro synthesis directed by polyuridylic acid (55). This yielded ribosome-catalyzed polyester formation in which every residue contained ester linkages rather than peptide linkages. It was the first completely nonnatural polymer made by ribosomes directed by mRNA.

CRYSTAL STRUCTURE OF tRNA

Methods for purifying tRNA improved during the 1960s, and there was an increase in attempts to form single crystals. This effort was very frustrating because it was easy to fail, and most people involved in the effort failed repeatedly. In 1968, working with postdoctoral fellow Sung Hou Kim, we were able to obtain single crystals of *E. coli* tRNA^{phe} (56). Three other groups also obtained single crystals of various tRNAs in that same year, and all of these crystals were poor in that they were somewhat disordered and the resolution was limited. Our earliest crystals diffracted to ~ 20 Å. By the next year, we were able to get crystals that diffracted to 6 or 7 Å resolution, and a study of the three-dimensional Patterson function using the 12 Å data from the crystals of *E. coli* tRNA^{Fmet} yielded approximate molecular dimensions of 80 \times 25 \times 35 Å (57). These crystals represented progress of a sort, but the frustration was great because they were not suitable for solving the structure.

Aided by Gary Quigley and others in the lab, we spent the next two years looking at many different purified tRNA preparations and explored many different crystallization procedures. By 1971, we reached an exciting turning point: Yeast tRNA^{phe} could be crystallized in a simple orthorhombic unit cell

with a resolution of 2.3 Å (58)! These were the first crystals of tRNA suitable for analysis. The key event in making these crystals was the incorporation of spermine, a naturally occurring polyamine. The spermine bound specifically to yeast tRNA^{phe} and stabilized it so that it made a high-resolution crystal. This was an important discovery at the time. The stabilization effect of spermine on yeast tRNA^{phe} made it possible to form good crystals in other lattices as well. Analysis of the crystal diffraction pattern showed that it had a characteristic helical distribution of diffracting intensities when viewed in one direction but did not show a helical distribution when viewed at right angles. This was taken as evidence that short helical segments containing 4–7 base pairs were found in the molecule, a result entirely consistent with the cloverleaf folding of tRNA molecule (59). This discovery opened the door to the ultimate solution of the structure of yeast tRNA^{phe}.

Tracing the Backbone of Yeast tRNA^{phe} and Solving the Structure

Myoglobin was the first protein whose three-dimensional structure was solved. The structure was revealed at various levels of resolution. An important milestone was the tracing of the polypeptide chain that showed how the myoglobin molecule is organized as a series of α -helical and single-stranded regions folded together. This was our first glimpse of how a protein molecule is folded, even though the high-resolution structure had not been completed.

The structure of yeast tRNA^{phe} was revealed in a similar, gradual way. Crystallographic research moved more slowly in the early 1970s than today. Computers were primitive; advanced area detectors, cryo-crystallography, and synchrotron beams were things in the future. In the lab, Bud Suddath built a cold room around our primitive diffractometer to stabilize the crystals. However, before work could continue, heavy-atom derivatives had to be discovered that would be useful for phasing the diffraction pattern of a crystalline nucleic acid molecule. This had never been done before, and it took considerable time to discover appropriate derivatives. Three different types of heavy atoms were developed containing platinum, osmium, or samarium ions. The osmium residue was very important because it was known to form complexes with ribonucleotides involving both the 2' and 3' hydroxyl groups. Only one pair of cis hydroxyl groups was found at the 3' CCA end of the tRNA molecule. In order to gain some appreciation of the geometry, the structure of an osmium-adenosine complex was solved, which enabled us to visualize the interaction (60). The single osmium derivative in the tRNA crystal made it possible to identify the 3' end of the tRNA chain (61). The samarium ions were very useful, and they occupied more than one site. The platinum residue was somewhat less valuable since it was only useful for 5.5 Å data. An interim electron density map at 5.5 Å made it possible to uncover the external shape of portions of the molecule. However, the true shape and fold of the molecule was not revealed until a map at 4 Å was produced in 1973 (61).

At 4 Å resolution, peaks were seen throughout the electron density map that were due to the electron-dense phosphate groups. We knew a great deal about the distance constraints between adjacent phosphate groups in a polynucleotide chain, and this made it possible to look for peaks between 5 and 7 Å apart. Tracing the chain led to the discovery that the tRNA molecule had an unusual L-shape. The CCA acceptor helix was collinear with the T pseudo-U helix, and it is almost at right angles to the anticodon stem that is collinear with the dihydro U stem. The molecule had the shape of an L, with the amino acid acceptor 3' hydroxyl group at one end of the L and the anticodon loop at the other over 70 Å away. At the corner of the L, there was a complex folding of the T pseudo-U and dihydro U loops. A perspective diagram of the L-shaped fold was published (Figure 3), as well as a sample of the electron density map showing double-helical regions in which the intense peaks associated with phosphate groups are separated from each other by a region of lower electron density due to the base pairs.

The L-shaped folding of the tRNA polynucleotide chain was a dramatic and surprising discovery, especially the separation between the acceptor site and the anti-codon. The backbone tracing (Figure 3) was published on the front page of *The New York Times* on January 13, 1973, together with a discussion of its role in protein synthesis. No one had anticipated that the molecule would organize in this fashion. Even at 4 Å resolution, this folding was compatible with much experimental data concerning tRNA molecules. For example, it was known that photo activation of *E. coli* tRNA^{val} resulted in the formation of a photo dimer involving the 4-thio-U residue in position 8 and the cytosine in position 13. In the 4 Å folding of the polynucleotide chain, these two bases were in close proximity, and the distance between the phosphate groups of these two residues was short enough to allow formation of the photo dimer (61).

The L-shaped tRNA molecule is now a standard feature of molecular biology, having been found in virtually all tRNA molecules, even when they are complexed to aminoacyl synthetase enzymes. The significance of the folding is twofold. First, it revealed that the 3' acceptor end is over 70 Å away from the anticodon loop, which has implications for understanding the interaction between tRNA molecules and tRNA aminoacyl synthetases. Second and most important, it suggested that the interaction of the tRNA molecules with mRNA occurs at one end of the L, whereas the segment responsible for forming the peptide bond is considerably removed from the site. This makes it possible to have great specificity with many interactions at either end of the molecule due to this separation.

At that time I made a proposal regarding the movement of tRNA molecules in the ribosome (62). A codon occupies ~ 10 Å (3 \times 3.4Å) when the bases are stacked. Thus, the center-to-center distance between the A-site and P-site codon is 10 Å. The chain tracing showed that there was an anticodon stem, and it was ~ 20 Å in diameter. Thus the paradox: How can two tRNAs simultaneously occupy the A and P sites? The answer, I suggested, was that the mRNA "bends or turns a corner" between the A and P site, allowing both tRNAs to make contact. In the recent paper tracing the path of mRNA in the ribosome (63), a kink of $\sim 45^{\circ}$ is seen between the A-site and P-site codons. Thus, the tRNA chain tracing was valuable in anticipating some features of the system.



Figure 3 (*Left*) At 4 Å resolution the fold of the tRNA^{phe} chain could be visualized, as shown in this perspective diagram (61). (*Right*) The 3 Å tRNA^{phe} structure reveals the complete interactions of the L-shaped molecule, as shown on the cover of Science (64).

Today, we are accustomed to seeing a variety of complex ribonucleotide molecules in which double-helical segments and single-chain segments are juxtaposed to make complex structures with a variety of functions, especially in ribozymes. The beginning of our understanding of the manner in which complex polynucleotide chains can fold started with this first tracing of yeast tRNA^{phe} visualized at 4 Å resolution. This tracing was seen in more detail a year later in our 3 Å analysis of the folding of yeast tRNA^{phe} in the orthorhombic lattice (64). Simultaneously, Aaron Klug and colleagues published the 3 Å structure of the same spermine-stabilized yeast tRNA^{phe} in the monoclinic lattice (65). Both papers confirmed the L-shaped folding of the polynucleotide chain, even though the lattice was different. These 3 Å structures were very similar and revealed in great detail the manner in which base-pairing of nucleotides, both in the double-helical regions and in the single-stranded regions, stabilizes the molecule. The folding was held together by a variety of hydrogen-bonding interactions, including many in the nonhelical regions of the molecule. These hydrogenbonding interactions included the formation of triplexes and other interactions. Recognition of the importance of these alternative types of hydrogen bonds explained why the model builders of that period, trying to anticipate the structure of tRNA, were all incorrect. They relied excessively on Watson-Crick base-pair interactions and did not recognize the stabilizing effect of many other types of hydrogen bonds. The L-shaped folding was predicted to be a general conformation found in all tRNA molecules (66). Subsequent work amply verified the relative constancy of the hydrogen-bonding networks (67, 68).

We have known of the L-shaped folding of tRNA molecules since 1973. The full understanding of why this particular folding is robust, and of the manner in which modified nucleotides are important to this folding, is still a work in progress. However, for understanding the interaction of tRNA molecules with the ribosome during protein synthesis, the L-shaped folding provides the central information regarding its interactions and movements in protein synthesis.

I have focused on the work of my colleagues and myself in uncovering some basic aspects of RNA structure and protein biosynthesis over the 20-year period 1954–1974. However, the detailed mechanism could not be understood until the more recent developments elucidating the three-dimensional structure of the ribosome. The earlier work in our lab and others set the stage for understanding the mechanisms that we now are beginning to envision in more detail in the machinery of protein synthesis.

SEARCH FOR LIFE ON MARS

My earlier interest in problems related to the origin of life led me to participate in the biological experiments carried out on the Mars Landers in the Viking Mission. Starting in 1969, a team was assembled that included Joshua Lederberg, Norman Horowitz, Chuck Klein, and others. We met periodically in California to design the experiments in the search for life on Mars. The discussions leading to the selection of experiments often had a philosophic character since we asked what were the fundamentals for life and how might they be transformed in the harsh environment of the Martian surface. For example, would a Martian "tree" largely grow underground to shield itself from hard UV radiation, much in the manner of the endolithic organisms that are found in the Antarctic? For this reason, I visited the Antarctic when I was a member of the National Science Board and was able to retrieve rock samples with photosynthetic organisms embedded in the rock.

In July 1976, I was living in Pasadena as a Visiting Professor at Caltech and was present at the Jet Propulsion Lab with other Viking Mission scientists as the first pictures came back from the surface of Mars. It was an extremely tense gathering because we did not know whether the Lander would actually land safely and be functional. It was electrifying to watch the slow, line-by-line creation of the first photograph taken on the surface of Mars.

In August 1976, I was scheduled to present a paper at the International Biochemistry Congress in Amsterdam, and I brought to the Congress a report of experimental results from the first month or so of the Mission. A great deal of excitement was generated at the time since our first findings looked as if they might be positive. Only later did we begin to understand the complexities of what we observed. Although our first publication from the Mission (69) suggested both positive and negative results, in the end we concluded that much of what we were looking at had to do with the chemistry of the Martian surface due to either the addition of water or heat. Overall, this experience helped to maintain my enthusiasm for research on extraterrestrial life.

INTERNATIONAL SCIENCE AND PUGWASH MEETINGS

In the late 1950s, together with many others, I was strongly concerned about the threat of nuclear war. When I was invited to attend a Pugwash meeting in 1959, I readily assented. These were meetings of scientists from the East and the West. Linus Pauling and John Edsall both attended the meeting in Pugwash, Nova Scotia. Up to 1972, I attended 13 of these meetings in various countries, attended by scientists from the Soviet Union, the People's Republic of China, and many other countries. These served as useful vehicles for discussing new ideas and helped to minimize the tension then growing between East and West. At a meeting in Moscow in 1960, I met a number of scientists from the Soviet Union and adopted a mode of operation. I usually wrote joint papers with the Soviet scientists, presenting suggestions on alleviating tensions in the nuclear arms race. At a meeting in London in 1962, a Russian colleague and I developed the idea that we might use automated seismographs to monitor nuclear testing, the so-called "black boxes." The proposal that we drafted was then signed by several other American and Soviet scientists and became a document of the conference.

It was also sent to world leaders. At one stage, the Soviet Union contemplated using such automated seismographs, and it was one of the elements that facilitated the eventual signing of the Limited Test Ban Agreement in 1963. Extending over many years, I developed warm friendships with a number of Russian scientists, including V.A. Engelhardt and A.A. Bayev, who was active in the field of transfer RNA sequencing. The meetings contained elements of both politics and science. Although these meetings took considerable time, both in preparation and execution, I felt the effort was worthwhile since it addressed a major problem facing our society—the prevention of nuclear war.

DNA TURNS LEFT-HANDED

It was not until the late 1970s that the development of DNA synthesis made it possible to carry out single crystal X-ray diffraction studies that could "prove" the structure. In 1978 I met a Dutch organic chemist, Jacques Van Boom, who could synthesize DNA oligomers. He made $d(CG)_3$, and Andy Wang crystallized it and discovered it diffracted to 0.9 Å resolution. Heavy atoms were used to solve the structure, which revealed, remarkably, a left-handed double helix with two antiparallel chains held together by Watson-Crick base pairs (70). Every other base had rotated around the glycosyl bonds so that the bases alternated in *anti* and *syn* conformations along the chain. The zigzag arrangement of the backbone (hence, Z-DNA) was different from the smooth, continuous coil seen in B-DNA (Figure 4). The general response to this unusual structure was amazement, coupled with skepticism.

The relationship between Z-DNA and the more familiar right-handed B-DNA began to be apparent from the earlier work of Pohl & Jovin (71), who showed that the UV circular dichroism of poly (dG-dC) nearly inverted in 4 M sodium chloride solution. The suspicion that this was due to a conversion from B-DNA to Z-DNA was confirmed by examining the Raman spectra of these solutions and the Z-DNA crystals (72). The conversion to left-handed Z-DNA was associated with a "flipping over" of the base pairs so that they were upside down in their orientation relative to what would be found in B-DNA. Sequences that most readily converted had alternations of purines and pyrimidines, especially alternations of C and G (73). Alfred Nordheim showed that it also occurred quite easily with alternations of CA on one strand and TG on the other strand (74), and many other sequences were shown to be capable of forming Z-DNA (75).

This discovery stimulated a burst of research from a large number of chemists who were very interested in studying DNA conformational changes. It tended to leave most biologists rather puzzled, since the ionic conditions suitable for stabilizing Z-DNA were very far from those present in a cell. This view changed somewhat with the discovery that negative supercoiling would also stabilize Z-DNA (76). Supercoiling was known to be a part of biological systems, and it



Figure 4 A diagram showing a comparison between B-DNA and Z-DNA with solid lines running from phosphate to phosphate, as shown on the cover of *Nature* (70).

suggested a connection between this alternative conformation and biological phenomena.

Does Z-DNA Have a Biology?

Research work on the biology of Z-DNA progressed very slowly. By the mid-1980s after several years of research in which nothing definitive emerged

about Z-DNA, most biologists were very skeptical about its role. Many felt that Z-DNA was a nonfunctional conformational phenomenon. My conviction was very simple. Here was an alternative DNA conformation, which could, in principle, form under in vivo conditions, and I felt it likely that it would be used because evolution is opportunistic. The challenge was to find out how it was used.

The first indications of a biological role for Z-DNA came from immunological work. In collaboration with David Stollar, we found that, unlike B-DNA, Z-DNA is highly antigenic, yielding polyclonal (77) and monoclonal (78) antibodies. Characterization of these antibodies by Eileen Lafer led to the discovery that Z-DNA-specific antibodies are found in human autoimmune diseases, especially systemic lupus erythematosus (79). Antibodies to Z-DNA also provided a useful tool for characterizing chromosome organization. They bound specifically to the interband regions of the *Drosophila* polytene chromosomes; the binding was particularly strong in the puff regions, the sites of enhanced transcriptional activity (80). Ciliated protozoa have two nuclei: the macronucleus, which is the site of transcription, and the micronucleus, which contains DNA involved in sexual reproduction. Anti-Z-DNA antibodies stained the macronucleus of the ciliated protozoan *Stylonychia*, but not its micronucleus (81). These were the first data to suggest a connection between Z-DNA and transcriptional activity.

An important advance came with the work of Liu & Wang (82) in 1987 on the interaction of RNA polymerase with DNA during transcription. They pointed out that the moving complex does not rotate around helical DNA, but instead plows straight through. Because the ends of the DNA molecule are fixed, the DNA behind the moving polymerase was unwound and subjected to negative torsional strain, while positive torsional strain developed in front. Further evidence came later from the work of P. Shing Ho and colleagues, who found a high concentration of sequences favoring Z-DNA formation near the transcription start site (83). To study the association with transcription more directly, I collaborated with Burkhardt Wittig and colleagues, using a technique developed by Peter Cook at Oxford. Mammalian cells were encapsulated in agarose microbeads; mild detergent treatment lysed the cytoplasmic membrane, permeabilizing the nuclear membrane but leaving the nucleus otherwise intact. The resulting "entrapped" nuclei replicated DNA at nearly the in vivo rate, and they were able to carry out transcription (84). Using biotinylated monoclonal antibodies against Z-DNA, the level of Z-DNA was shown to be regulated by torsional strain (85). An increase in transcriptional activity of the embedded nuclei resulted in a parallel increase in the amount of Z-DNA (86). Using a UV laser pulse for protein-DNA cross linking, the biotinylated anti-Z-DNA antibodies were linked to DNA. This made it possible to isolate DNA restriction fragments bound to the antibody. With cultured human cells, three regions upstream of the *c-myc* gene formed Z-DNA when *c-myc* was expressed. However, these regions quickly reverted to B-DNA upon switching off c-myc transcription (87). Nonetheless, the actin gene control retained its Z-DNA at all times.

31

The picture that then emerged was that the negative torsional strain induced by the movement of RNA polymerase stabilized Z-DNA formation near the transcription start site. Even though topoisomerases tried to relax the DNA, the continued movement of RNA polymerases generated more negative torsional strain than the topoisomerases could relax. However, upon cessation of transcription, topoisomerases rapidly converted it back to the right-handed B conformation. Thus, Z-DNA was seen as a metastable conformation, forming and disappearing depending upon physiological activities.

Z-DNA Binding Proteins

If Z-DNA were to have biological functions, it seemed highly likely that a class of proteins should bind to it specifically. The challenge was to isolate such proteins that bound selectively to Z-DNA with high affinity. The first successful method was developed in the lab by Alan Herbert (88). A gel shift assay was used with radioactive-labeled, chemically stabilized Z-DNA in the presence of a \sim 20,000-fold excess of B-DNA and single-stranded DNA. A Z-DNA-binding protein was found to be a nuclear RNA editing enzyme (89), called doublestranded RNA adenosine deaminase (or ADAR1). This enzyme acts on doublestranded segments formed in pre-mRNA, binding to the duplex and selectively deaminating adenosine, yielding inosine. Ribosomes interpret inosine as guanine. Thus, ADAR1 can alter the amino acid sequence of a DNA-encoded protein. The functional properties of the edited protein (with the amino acid alteration) are often different from those of the unedited protein. The editing enzyme is found in all metazoa; it acts to increase the functional diversity of proteins transcribed from a given locus (90).

Proteolytic dissection of the editing enzyme ADAR1 led to a domain from the N terminus called $Z\alpha_{ADAR1}$ (91). $Z\alpha_{ADAR1}$ was found to contain all of the Z-DNA binding properties associated with the editing enzyme, and it bound Z-DNA tightly with a low nanomolar K_d . This domain was used to create a conformationally specific restriction endonuclease that would only cut Z-DNA. The naturally occurring restriction enzyme Fok1 has a DNA recognition domain and a separate nuclease domain attached to it. A chimeric molecule was made by Yang Kim in which the Z α domain of the editing enzyme ADAR1 was covalently linked with the nuclease domain of the Fok1 restriction enzyme (92). This restriction enzyme bound and cleaved plasmids only when they were supercoiled and Z-DNA was present.

In 1999, Thomas Schwartz, a graduate student, discovered that the purified $Z\alpha_{ADAR1}$ domain could be co-crystallized with d(CG)₃. The crystal structure solved at 2.1 Å resolution (93) revealed that the DNA was in a form virtually identical to that seen in the first Z-DNA crystal (70). The 70-amino acid binding domain was found to adopt a helix-turn-helix β -sheet motif (winged helix) in which the recognition helix and the β -sheet bound to five successive phosphate groups in the zigzag backbone of Z-DNA, and it recognized the *syn* conformation of guanine.

It is possible that the Z-DNA binding domain of ADAR1 targets Z-DNA forming regions of some transcriptionally active genes, as only they have Z-DNA. $Z\alpha_{ADAR1}$ appears to be active in vivo in the editing of certain transcripts where it may target the gene (94); however, its role in RNA editing is not resolved. Bernie Brown and Ky Lowenhaupt showed that $Z\alpha_{ADAR1}$ also binds tightly to left-handed Z-RNA (95). This may be related to the role of the editing enzyme in modifying measles and other negative-strand RNA viruses, which are extensively hyperedited.

The co-crystal structure of $Z\alpha_{ADAR1}$ and Z-DNA made it possible to identify those amino acids important for Z-DNA recognition. A computer search rapidly revealed other proteins with similar sequence motifs. One is the protein DLM1 which is up-regulated in tissues in contact with tumors and is also interferoninduced. The co-crystal structure of a domain of DLM1 ($Z\alpha_{DLM1}$) and d(CG)₃ was solved at a resolution of 1.85 Å, and it showed that this second protein domain recognizes Z-DNA in a manner very similar to that found with $Z\alpha_{ADAR1}$, but with a few variations (96). This second structure clearly indicated that a family of such proteins exists.

Viruses Use Z-DNA Binding Proteins

Another member of this family of proteins is E3L, found in poxviruses such as vaccinia. These are large DNA viruses that reside in the cytoplasm of cells and produce a number of proteins that help to abort the interferon response of the host cell. E3L is a small 25-Kd protein necessary for pathogenicity. When vaccinia virus is given to a mouse, the mouse dies in about a week. However, in a virus that has a mutated or missing E3L, it is no longer pathogenic for the mouse, even though the virus can still reproduce in cell culture (97). To demonstrate the pathogenicity of the vaccinia virus in the mouse and its relationship to possible Z-DNA binding activities of E3L, a collaboration was set up with Bertram Jacobs. Chimeric viruses were created in which the N-terminal domain of vaccinia E3L (Z_{E3L}) was removed, and either the domains $Z\alpha_{ADAR1}$ or $Z\alpha_{DLM1}$ were inserted. In carrying out these domain swaps, a little more than a dozen amino acids in the domain remained unchanged, but over 50 other amino acids were changed. Nonetheless, the chimeric viruses were as pathogenic for mice as the wild type (98). Other experiments were carried out in which mutations in the chimeric virus that weakened Z-DNA binding were also shown to weaken pathogenicity. Similar mutations in the wild type weakened mortality. Loss of Z-DNA binding led to loss of pathogenicity. It is likely that the Z_{E3L} domain binds to Z-DNA formed near the transcription start site of certain genes, thereby impairing the antiviral response of the host cell (98). This is a new example of the way in which viruses seek to exploit features of the host cell in order to overcome the host defense mechanisms.

A small molecule or drug can probably be made that will bind to the Z-DNA binding pocket of the E3L molecule. This drug should prevent mice from dying

when infected with vaccinia virus. It may also be active in humans to prevent untoward effects due to vaccination. More significant is the fact that the E3L protein of the closely related variola virus, the agent of smallpox, is virtually identical to the vaccinia E3L (98). Hence, such a drug binding to E3L may develop into a treatment of smallpox.

To my great surprise, work on Z-DNA and its binding proteins has led us to the possibility of developing a therapy for certain viral diseases, including smallpox. This is a striking example of serendipity in scientific research. And, of course, it helps to maintain the excitement that is an inherent part of the scientific enterprise.

In this outline, I have described the gradual unfolding of a research trail. My driving force has always been curiosity, and one of the intrinsic rewards of a life in science is the excitement of uncovering some aspect of nature.

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