Gertrude B. Elion

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# THE QUEST FOR A CURE

*Gertrude B. Elion*
Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709

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## THE BEGINNINGS

As I look back on my life and career and wonder what were the influences and events that shaped them, I realize how little one appreciates what is happening at the time it occurs. The love and support of my parents, which I took more or less for granted while I was growing up, allowed me to take the "road less traveled by" and to persist in the face of considerable obstacles. Who were these parents?

My father, who graduated from New York University School of Dentistry in 1914, had come to the United States from Lithuania as a small boy. My mother came from Russia in 1911 at the age of 14. By the time she was 19, she was married to my father who had then been a dentist for two years. A year and a half later I was born.

Although my mother never had a college education, she was a prodigious reader and the scope of her reading was astounding. My father also loved to read and I can remember many evenings during my childhood when I was exposed to a variety of literature, poetry, history, biography, and fiction, being read aloud by my father.

My mother had more common sense than anyone I have ever known. I am certain she would have been successful in any career she could have undertaken. Although she never complained about the fact that this had not been an option for her in those days, it was apparent that she wanted me to have every opportunity to be independent and to have a satisfying career. This became very important later when I was unable to find a job and continued to rely on my parents for a number of years after graduation from college.

When I was born, we lived in an apartment adjoining the dental office in what was then a middle-class neighborhood in Manhattan, 116th Street and



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Madison Avenue. My brother, Herbert, was born just as I was starting school. Shortly thereafter, we moved to the Bronx, which was then considered a suburb. I loved school. I had a great desire to learn and the mere acquisition of knowledge gave me excitement and pleasure. I had learned to read well before I entered the first grade. It was the custom at that time to skip children who were achieving more than was expected of them. By the time I had finished junior high school, I had been skipped four times and was two years ahead of most of my classmates. This presented some social problems for me during my teenage years.

My high school career was unremarkable. I went to Walton High School, an all-girls school with a good academic curriculum. We were required to take two foreign languages, mathematics, science, English, history—in essence, a college preparatory program. The Wall Street crash of 1929 had changed our life style since my father had lost very heavily in the stock market. By the time I graduated from high school, the depression was at its height and Franklin Delano Roosevelt had just been elected. It is questionable whether my brother or I would ever have gone to college if we hadn't been living in New York where Hunter College and the College of the City of New York had free tuition, if your high school grades were good enough to qualify you for admission.

The summer before I entered college, my maternal grandfather died of stomach cancer. He had been very close to me during my preschool years. The suffering I witnessed during his last months made a great impression on me. I decided that a worthwhile goal for my life would be to do something to help cure this terrible disease. It was a goal that I never relinquished. I decided I would major in chemistry in college, since that seemed to me to be the best road to the discovery of drugs that could accomplish my goal. I did not know that years later my mother would also die of cancer.

Hunter College was an excellent school and we had a remarkably large class of chemistry majors, about 75, for a woman's college. Most of the women were planning to teach, but a few of us were determined to be laboratory scientists. We were undoubtedly a little naive not to realize what was awaiting us on graduation in 1937. The depression was still with us and the few available jobs or graduate school assistantships were definitely not for women. What had made me think that graduating "summa cum laude" would open any doors for me to a research laboratory?

After an entire summer of job-hunting and of sometimes being told, "We have never had a woman in the laboratory; we think you would be a distracting influence," my perseverance began to falter. I decided I had better go to secretarial school and learn something useful. After six weeks of secretarial school, I was rescued by the New York Hospital School of Nursing. They offered me a job as a laboratory assistant, helping to teach the Biochemistry

course for nurses. It was only for a trimester and I would be unemployed again at the end of the three months, but I didn't hesitate. My secretarial career was over.

Several months after this reprieve, I felt fortunate to obtain a job working for nothing for a young chemist, Alexander Galat, whom I had met socially. He had been given a laboratory in a small pharmaceutical company as partial payment for a diagnostic test he had invented. After six months he was permitted to pay me $12 per week. By the time I left, I had one and a half years of good laboratory experience, was earning $20 per week, and had saved enough to go to graduate school at New York University for my Master's degree. I was able to do the research for my thesis on a part-time basis, while teaching chemistry and physics in the New York City high schools. My teaching career, as a so-called "permanent substitute," lasted two years. I enjoyed it some of the time, especially when I had good students, but I was still waiting for the opportunity to get into research.

World War II finally did what I had been unable to do on my own: it opened the doors for women to work in chemistry laboratories. While the men were away, employers had to take the "risk." My return to the laboratory was as a quality control chemist in a food laboratory. Testing the acidity of pickles, measuring the color of mayonnaise and determining the concentration of sugar in preserves was not very glamorous, but I was earning money and I was learning to use instrumentation I had never seen in college. After a year and a half, it was time to move on.

I was offered a job in a new research laboratory at Johnson and Johnson in New Brunswick, New Jersey. One of their vice presidents had decided that the company should go into the pharmaceutical business and had hired an experienced organic chemist, Alan Pierce, to head the laboratory. I was one of his two assistants. We were soon synthesizing sulfonamides and I was beginning to feel that I was finally on my way. Six months later there was a change in vice presidents and the decision was made not to go into the pharmaceutical business after all. I was told I could stay on, measuring the tensile strength of sutures. That was not quite what I had in mind for my future. I began job-hunting again.

## THE OPPORTUNITIES

### *Introduction to Nucleic Acids*

My job interview with George Hitchings at Burroughs Wellcome Co. was unusual in that, instead of asking me a lot of questions about myself, Hitchings proceeded to tell me what he was doing. I had not heard much about nucleic acids before, nor did I know what purines and pyrimidines were. The idea

that one might possibly interfere with cell division by making antimetabolites of the structural bases of nucleic acids sounded exciting. Hitchings apparently thought my scholastic record and laboratory experience were sufficient to fulfill his requirements for a chemist and promised to call me in a week. I went home excited at the prospect that I might get this job and determined to hold off accepting any other offers (of which there were then two) until I had heard from George Hitchings. A week later the offer came. On June 14, 1944, I went to work at the Wellcome Research Laboratories at a yearly salary of $2600.

My first assignment was to make 2-thioparaxanthine (2-mercapto-6-hydroxy-1,7-dimethylpurine), which someone wanted to test for its antithyroid activity. This involved following the methods of Emil Fisher described in the old German literature. Fortunately, my knowledge of German was adequate, but the reactions involved were difficult. In particular, the first step, which involved heating theobromine with phosphorus oxychloride for six hours in a sealed glass tube at 160° C and rocking the tube every hour to facilitate solution of the solid, was a bit frightening. The glass tubes were encased in metal pipes and heated in a gas-lit oven. It was a hot summer and our laboratory was not air-conditioned. Moreover, there were dryers for a dextromaltose product directly below our laboratory and the temperature of the floor was generally over 100° F. The work-up of the reaction mixture was also difficult and there were multiple steps thereafter. Nevertheless, by the end of July, I had successfully synthesized 1.5 grams of 2-thioparaxanthine.

During that first summer I learned as much as there was to learn about purines from the sparse literature on the subject. The synthetic papers were mainly in German, the works of Traube, Fisher, Isay, and Biltz published in the early 1900s and a few English papers by Bogert and Davidson. I devoured the book on *Nucleic Acids* by Levene & Bass and realized how little was really known about the structure or biosynthesis of the nucleic acids. The arrangement of the nucleotides in nucleic acid was still being debated in the early 1940s. Were they linked through the sugars or through the phosphate moieties? Was the sequence the same in all nucleic acids, with a tetranucleotide unit simply being repeated over and over again? It was known that RNA contained ribonucleotides of adenine, guanine, uracil, and cytosine, and DNA was composed of deoxyibonucleotides of the same bases, except that thymine replaced uracil. It was in the late 1940s that the work of Chargaff and his co-workers, Zamenhof, Vischer, and Magasanik, led to the conclusion that the composition of DNA was characteristic of the species from which it was derived, that different tissues of the same species yielded the same DNA, and that the tetranucleotide hypothesis was incorrect. The fact that DNA contained genetic information was also indicated by the work of Avery, who had shown

in 1944 that DNA prepared from one strain of pneumococcus could transform another.

Since so little was known at that time about the enzymes and metabolic pathways by which the nucleic acid bases were biosynthesized or incorporated, it was almost an act of faith that one might be able to synthesize antimetabolites of these bases that would actually be utilized by cells, or that could prevent the utilization of essential metabolites. Nevertheless, it was known that certain lactic acid-forming bacteria could grow and multiply on defined media if one of the four "natural" purines (adenine, guanine, xanthine, hypoxanthine) was supplied together with thymine. Thus, these bacteria obviously had the pathways for the interconversion of adenine and guanine and for the amination of hydroxypurines. The enzymes responsible for these interconversions were not isolated or named until the 1950s. Alternatively, one could supply an acetone powder of liver, the so-called "*L. casei* factor" and the microorganism would make its own purines and thymine. The structure of this growth factor, folic acid, was not known until 1946. By the time I arrived in the laboratory, Hitchings, with the aid of a young woman, Elvira Falco, whom he had enticed away from the Bacteriology laboratory, had set up an assay with *Lactobacillus casei* in which one could test a compound for its ability to substitute for or to antagonize a purine, thymine, or folic acid. This test system was valuable, not only for the identification of inhibitors of cell division, but for the accumulation of a body of knowledge from which one could deduce the existence and structural requirements of enzymes not yet known and pathways not yet elucidated.

After a few months in the laboratory, I was making other purines besides thioparaxanthine, as well as pyrimidines and pteridines. Some of these were actually compounds that had never been described before and I felt the excitement of the inventor who creates a "new composition of matter." The pteridines presented a real challenge to a young chemist. They were not crystalline, were highly insoluble, could not be re-crystallized from water or organic solvents and had no melting points. They could be synthesized from 4,5-diaminopyrimidines by closure of the pyrazine ring with diketo compounds. The 4,5-diaminopyrimidines were also intermediates for the synthesis of purines.

One of the instruments that we sorely lacked in those days was a photoelectric spectrophotometer. We did have an old Bausch and Lomb spectrometer, which had a carbon arc as a light source. The relative absorption of a solution at various wavelengths was recorded on a photographic plate, with the solvent as control. One had to match the density of silver grains visually to determine the degree of absorption. My first paper published from Hitchings' laboratory was one dealing with "The Ultraviolet Absorption

Spectra of Thiouracils." I still look at that paper with some amusement when I see the peculiar irregularities in the curves resulting from the methodology we had used. Our first Beckman DU Spectrophotometer, obtained in 1946, was cause for great celebration.

## *Leukemia*

After a little more than a year in the laboratory, the biochemistry of nucleic acids became more challenging to me than the mere synthesis of new compounds. The compound that was to teach me much about purine utilization and interconversions, as well as to excite me by its activity against human leukemia, was 2,6-diaminopurine, which I synthesized in 1948. The synthesis had been described in the German literature in the early 1900s. It seemed like such a simple analog, a possible antagonist of either adenine or guanine. According to *L. casei*, diaminopurine was an adenine antagonist, since the only purine that could reverse the inhibitory activity of high concentrations of diaminopurine was adenine. Somewhat surprisingly, diaminopurine also had the characteristics of a folic acid antagonist, since its effects could be reversed by folic acid. This fact, together with data on a number of pyrimidines and pteridines, led us to the conclusion that the 2,4-diaminopyrimidine moiety could confer antifolate properties, no matter what else was attached to the molecule. This finding was to prove an excellent lead into the development of the antimalarial and antibacterial diaminopyrimidines by others in our laboratory (1).

In 1947 Hitchings had established a link with the Sloan-Kettering Institute, then headed by Cornelius Rhoads. This connection enabled us to send compounds there for antitumor testing in mice and was the basis of many collaborative efforts in the following decade. Joseph Burchenal soon found that 2,6-diaminopurine had activity against a mouse leukemia and could produce increases in survival similar to those with methotrexate, although at a higher dose. Diaminopurine also showed activity against vaccinia virus, a DNA virus, in Randall Thompson's hands at the University of Indiana.

After Fred Philips had studied the toxicology of diaminopurine in animals and Burchenal had found that it produced remissions in two adults, one with acute myelocytic and one with chronic myelocytic leukemia, interest in the compound intensified. One of the remissions lasted over two years, during which time the young woman gave birth to a child. Unfortunately, she then relapsed and was resistant to further therapy. Two other patients failed to respond at all. Although most patients could not tolerate diaminopurine because of nausea and vomiting, it seemed as though we were on the threshold of a "breakthrough."

In the next few years we spent a great deal of time studying the utilization

and interconversion of purines, nucleosides, and nucleotides in *L. casei*, in collaboration with M. Earl Balis and George Brown, using radioactive precursors. Of great help was the investigation of the diaminopurine-resistant strain of *L. casei* that we had isolated. From these studies (2) we concluded that: "The biochemical nature of the mutation involved in diaminopurine resistance would appear to involve the alteration of an enzyme system which deals, normally, with the incorporation of adenine," and that the "assumption is that diaminopurine is incorporated into an analogue of the metabolite via the direct pathway by which adenine is incorporated and that it is the diaminopurine-containing analogue that is the active inhibitor." Two years later, Arthur Kornberg described the isolation and properties of adenine phosphoribosyltransferase and our enzyme had been identified.

The period of the 1950s was a golden age in nucleic acid biochemistry. It was the time when John Buchanan, G. Robert Greenberg, and their groups unraveled the multiple steps of purine biosynthesis and found that inosinic acid (hypoxanthine ribonucleotide) was the first purine compound formed in the biosynthetic pathway. The double helical structure of DNA was proposed by Watson and Crick. Kornberg described the enzymatic synthesis of DNA by DNA polymerase. Every Spring meeting of the Federation of American Societies for Experimental Biology revealed new and astonishing information about nucleic acid synthesis.

Excited by the wonderful knowledge that was unfolding, I decided to continue my formal education and obtain a PhD degree. Needless to say, I had no thought of giving up my job. The only solution was to attend graduate school on a part-time basis. Brooklyn Polytechnic Institute was the only school offering graduate classes in Chemistry at night. For two years I went to Brooklyn several nights a week, which involved a long commutation from Tuckahoe during rush hour, and a one hour subway ride back home to the Bronx after class, late in the evening. Suddenly, this came to an end. The Dean informed me that if I was really "serious" about my graduate work, I would have to give up my job and go to school full time: I told him this was not possible. My attempt to obtain a PhD was over. For years I wondered whether I had made the right decision. Now I know I did.

While the biochemical studies occupied some of my time, I continued to synthesize a variety of purines, pyrimidines, and pteridines. One of the synthetic procedures that was to play an important part in my work was thiation, the direct exchange of sulfur for oxygen in heterocyclic systems. In 1943, Carrington had described the thiation of hydantoins with phosphorus pentasulfide in an inert, high-boiling solvent, tetralin. I used the method successfully for a number of pyrimidines and quinazolines, but had much more difficulty applying it to the purines. Part of the problem lay in the

insolubility of guanine, xanthine, and hypoxanthine in an organic solvent like tetralin. When the reaction took place, the product coated the starting material and no further reaction occurred. There was also the problem of separating thioguanine from guanine or 6-mercaptopurine from hypoxanthine. Unlike pyrimidines, purines were difficult to chlorinate, and that route to the mercapto compounds was unsatisfactory at that time.

With persistence and some changes in solvent and reaction conditions, I finally succeeded in isolating and purifying thioguanine and 6-mercaptopurine. Both compounds behaved like purine antagonists in *L. casei*. Our attention centered on 6-mercaptopurine (6-MP) at first, since it was the easier one to synthesize. When it was tested at Sloan-Kettering in early 1951 against Sarcoma 180, Donald Clarke reported that the tumor was not only inhibited but, in many of the mice, it completely regressed or proved to be nonviable on transplantation. The compound was also active on several mouse leukemias. Burchenal was extremely anxious to test 6-MP in children with acute leukemia.

In those days children with acute leukemia had a mean life expectancy of 3 months if untreated. With the use of cortisone and the antifolate methotrexate in 1949, that average survival time was increased to 6 months. By 1953, Burchenal and his group had extended the mean survival time of leukemic children to one year by the addition of 6-MP to their treatment. By the end of 1953 the drug had been approved by the FDA for the treatment of acute childhood leukemia. At a three-day symposium sponsored by the New York Academy of Sciences in 1954, much of the data on 6-mercaptopurine was reported (3).

It took years before the work of a number of different groups revealed all of the loci of action of 6-MP. One of our early insights was based on studies with a 6-MP-resistant strain of *L. casei*. In 1953, we deduced that, since the 6-MP-resistant mutant was unable to grow with hypoxanthine as the purine source, 6-MP and hypoxanthine must require the same enzyme for utilization. In addition, we suggested "that a hypoxanthine-containing metabolite may be an intermediate in the conversion of adenine to guanine in *L. casei*, and the transformation to a guanine-containing substance is viewed as a possible site of action of 6-mercaptopurine" (4). The identification of inosinic acid as the first purine nucleotide formed by de novo biosynthesis had not yet been made. The many loci of action of 6-MP ribonucleotide, its conversion to thioguanylic acid, and the subsequent incorporation of thiodeoxyguanosine into DNA have been reviewed many times (See refs. in 5, 6). As new chemotherapeutic drugs joined the armamentarium for fighting leukemia, 6-MP remained one of the mainstays in the multi-drug regimens, passing from being a remission-inducing agent to one still used in maintenance therapy. Thioguanine (6-TG), which

was developed several years later, found its niche in the treatment of acute myelocytic leukemia in adults, particularly in combination with cytosine arabinoside.

Within a year of the time 6-MP became an accepted treatment for acute childhood leukemia, it became apparent that this drug was not going to be the cure we had all hoped for. Relapses occurred and patients often no longer responded to 6-MP and methotrexate. These were heart-breaking times. We seemed to be so close to the solution and yet the ultimate goal eluded us. We still had much to learn about leukemia, its persistence in "privileged" sites such as the central nervous system, about the need to continue treatment long after remission had occurred, and the importance of multiple drug regimens to prevent or overcome resistance.

Our approach to improving upon 6-MP was, in hindsight, somewhat simplistic. We would make a variety of derivatives of 6-MP and explore their structure-activity relationships, study the mechanism of resistance to 6-MP, and attempt something that was quite unusual at that time, investigation of the pharmacokinetics and metabolic fate of 6-MP.

In 1950, the separation of nucleic acid bases, nucleosides and nucleotides by ion-exchange and paper chromatography had been reported by Waldo Cohn and C. E. Carter. Radioactive sulfur had become available and I was able to synthesize $^{35}$S-6-MP from 6-chloropurine. The radioactive samples were counted with a Geiger flow counter, necessitating drying each sample on planchets to make infinitely thin films. The ion-exchange columns were bulky and the flow rates variable; the separations frequently took days. Nevertheless, after some metabolism studies of 6-MP in mice by Samuel Bieber and myself, the radioactive drug was studied in several leukemic patients, in collaboration with Leonard Hamilton of the Sloan-Kettering Institute (3).

Our methodology did not permit us to do pharmacokinetics in the way it can be done now with high pressure liquid chromatography and mass spectrometry. However, fractionations of the urine samples on Dowex-1 and Dowex-50 ion-exchange columns led to the isolation and identification of a number of metabolic products. We began to understand the various catabolic reactions to which 6-MP was subject, e.g. methylation and oxidation of the sulfur, removal of the sulfur to form inorganic sulfate, and oxidation of the purine ring on the 2 and 8 positions.

The methodology improved with the advent of scintillation counters, but we did not get our first high pressure liquid chromatograph until 1969. The metabolic studies were to play an important role in our understanding of species differences in the disposition of the thiopurines and consequent differences in therapeutic effectiveness. The large number of structural modifications of 6-MP and 6-TG that were made and tested in our laboratory

in the 1950s were made largely on an empirical basis. We did not yet have the kind of knowledge of the substrate and inhibitor specificities of the purine and pyrimidine metabolizing enzymes that was acquired later.

Two derivatives made with the specific intention of providing masked forms of 6-MP and 6-TG were their 6-S-(1-methyl-4-nitro-imidazolyl) derivatives, azathioprine (Imuran®) and thiamiprine (Guaneran®) These compounds were expected to be vulnerable to nucleophilic attack between the sulfur on the purine and the methyl-nitroimidazole ring because of the ortho-nitro substituent. Hopefully, this might happen preferentially inside leukemic cells, releasing 6-MP and 6-TG. These derivatives were as active as 6-MP and 6-TG against Adenocarcinoma 755 in mice, but appeared to be less toxic and, therefore, to have a better therapeutic index. We enlisted the collaboration of R. Wayne Rundles at Duke University to study their metabolism and antileukemic activities in man (6, 7). Although our laboratories were some 500 miles apart, the blood and urine samples were flown back and forth between LaGuardia Airport and Raleigh-Durham with regularity, usually packed in Rundles' little black leather doctor's bag. These studies showed that these prodrugs were indeed converted to 6-MP and 6-TG, and were as active as these thiopurines in chronic myelocytic leukemia. Although some quantitative differences were discerned between the metabolism of 6-MP and azathioprine in man, there was no indication of an improved chemotherapeutic index in leukemia.

With Rundles we were able to test the clinical efficacy of a number of other substituted derivatives of 6-MP and 6-TG that had shown good antitumor activity in mice. In patients with chronic myelocytic leukemia who were not very ill, efficacy could be evaluated within a few weeks, while the metabolic studies were in progress. Patients who did not respond to the new derivative, as measured by the granulocyte count, were given 6-MP or 6-TG to test whether the leukemia was responsive to these effective thiopurines. The 6-alkylthiopurines, which had shown good activity in the mice, were inactive in man, although the patients responded well to 6-MP or 6-TG. The reason for this discrepancy soon became evident. In mice, the alkylthiopurines were being dealkylated to the free thiopurines. In man, the alkylthio groups were being oxidized to form alkylsulfinyl groups or were removed from the purine ring completely (7). Thus, with studies in a relatively few patients, we eliminated the need for extensive clinical trials of inactive compounds.

## *Immunosuppression and Transplantation*

In 1958, we were unexpectedly precipitated into the field of immunology. Robert Schwartz, working with William Dameshek in Boston, had obtained some 6-MP from us and had tested it for its ability to suppress the antibody

response in rabbits to bovine serum albumen. Their rationale was that "immunoblasts," which were formed when lymphocytes responded to the challenge of a foreign protein, closely resembled leukemic lymphoblasts. Therefore, a drug that worked against acute leukemia might have an effect on the immunologically stimulated lymphocytes. And indeed it did. Schwartz worked out the conditions of timing and dose that could produce immunological tolerance in rabbits. In 1958, an understanding of the immune response was in its infancy. There were large lymphocytes and small lymphocytes, but it was not at all clear which were more important in the immunological response. Plasma cells and macrophages were known, but no one had yet discovered B cells and T cells, let alone different kinds of T cells. Schwartz imbued us with his own enthusiasm and convinced us of our obligation to set up an immunological screen to test some of our other antimetabolites for this interesting property. In the meantime, he offered to test compounds in his rabbit system under code numbers without knowledge of the chemical structures. Bieber, our "in house" biologist, set up a screen for immunosuppression, which consisted of injecting sheep red cells into the tail vein of mice and examining the mice for their antibody response a week later.

Having a test for immunosuppressive activity turned out to be extremely useful, not only for what it taught us about the immune response, but because it revealed some interesting differences between rabbits and mice with respect to azathioprine. In mice, azathioprine was more active than 6-MP in suppressing the immune response, whereas in rabbits azathioprine was inactive. This surprising discrepancy was explained later when we found that azathioprine was metabolized differently in the rabbit than in the mouse or man. Because of high concentrations of aldehyde oxidase in the rabbit, extensive oxidation occurred on the purine ring at position-8, before the compound could be split to 6-MP. Thus, the product released after oxidation and reaction with glutathione was 6-mercapto-8-hydroxypurine, which is biologically inert. Fortunately, mouse and man are similar in their metabolism of azathioprine.

The story of how Schwartz's report on the immunosuppressive activity of 6-MP inspired a young surgeon, Roy Calne, in England, to try 6-MP to prevent kidney transplant rejection in dogs has been told before (5, 8). Calne visited us on his way to spend a year with the transplant surgeon, Joseph Murray, at the Peter Bent Brigham Hospital in Boston, and we gave him azathioprine to try on dog kidney transplants. I often wonder whether we would have given him that compound if we had not already done the metabolic experiments with azathioprine in mouse and man or studied its immunosuppressive activity in mice. Was it Fate? The use of azathioprine to prevent kidney transplant rejection in man became a reality in 1962. The rest is history. It was 16 years

before the next successful immunosuppressive agent for use in kidney transplants, cyclosporin, became available. Over 200,000 kidney transplants have now been performed worldwide, and heart, liver, lung, pancreas, and bone marrow transplants are also common. Azathioprine is also a recognized treatment for rheumatoid arthritis.

It has often been asked whether azathioprine has any real superiority over 6-MP as an immunosuppressant. That question really cannot be answered by in vitro tests since, in the absence of red blood cells, the conversion of azathioprine to 6-MP is very slow. In a variety of in vivo test systems, the difference between the two drugs is not large but is always in favor of azathioprine, i.e. immunosuppressive activity occurs at a lower dose and the maximum tolerated dose is higher. This difference may be due to pharmacological factors such as the slow release of 6-MP from the red blood cells after the reaction of azathioprine with glutathione. However, it is more likely that azathioprine reacts with other sulfhydryl and amino groups on cell membranes or enzymes, thereby blocking some receptors, in addition to releasing 6-MP.



## *Gout and Hyperuricemia*

One of the principal catabolic products of 6-MP is its oxidation product, thiouric acid. We had shown in the early metabolic studies in 1954 that this compound was formed by the enzyme xanthine oxidase. Doris Lorz had studied this enzyme in depth from 1950 to 1956, examining it for both substrate and inhibitor specificity. The time now seemed ripe to determine whether inhibitors of this enzyme could function in vivo and could change the catabolic fate of 6-MP. We chose 4-hydroxypyrazolo(3,4-d) pyrimidine (allopurinol), a compound synthesized by Falco in the mid-50s, for our first in vivo trials in mice. This compound was a potent inhibitor of xanthine oxidase, had no cytotoxic activity on bacteria or tumors, and was well tolerated by the mice. Had we tested a number of the other available inhibitors (as we did subsequently), we would have concluded that this approach was not viable. In mice and in dogs, the oxidation of 6-MP to thiouric acid was markedly inhibited by concomitant use of allopurinol. This increased the antitumor and immunosuppressive activities of 6-MP three- to fourfold. Toxicity was also increased but, in mice, there was an improvement in the therapeutic index. In man, the effect of allopurinol on the metabolism of 6-MP was similar to that in the mouse and the antileukemic efficacy of 6-MP was also increased three- to fourfold, but there appeared to be no change in the therapeutic index. Our initial disappointment gave way to excitement at the thought that a nontoxic in vivo inhibitor of xanthine oxidase might be useful for inhibiting uric acid formation. Indeed, in one of Rundles' leukemic patients in whom the combination of 6-MP and allopurinol was studied, the levels of serum and urinary uric acid did decrease.

The disease in which excess uric acid is a major problem is gout. The next clinical studies followed quickly in collaboration with Rundles and James Wyngaarden at Duke (5, 9). The findings of efficacy were unequivocal. Uric acid tophi dissolved under treatment, serum and urinary uric acid decreased, and side effects were minimal. Although allopurinol has a short half-life in the serum, its oxidation product, oxypurinol, has a long half-life, about 18–24 hr. Moreover, oxypurinol is also a potent xanthine oxidase inhibitor, so that enzyme inhibition is prolonged and the drug needs to be given only once a day. Vincent Massey found that oxypurinol binds very tightly to the reduced form of xanthine oxidase and is released only after reoxidation of the enzyme. With Alfred Gutman and T'sai-Fan Yu, we showed that oxypurinol is reabsorbed in the proximal kidney tubule in a manner similar to uric acid. This accounts for its long plasma half-life. Hypoxanthine and xanthine, the oxypurine intermediates in purine degradation, are excreted by the kidney at close to glomerular filtration rate, or are reutilized for ribonucleotide synthesis. In 1966, the year that allopurinol was approved by the FDA, a symposium held in London under the auspices of the Heberden Society brought together many of the investigators from various parts of the world to present their experiences with allopurinol (10). A new and effective treatment for gout had been launched.

## *Expanding the Horizons*

Although by this time our laboratory had been responsible for a number of important chemotherapeutic agents, only two enzymes of nucleic acid metabolism had been studied in any depth: xanthine oxidase and dihydrofolate reductase. The 2,4-diaminopyrimidines synthesized by Elvira Falco, Peter Russell, and Barbara Roth had led to the antimalarial pyrimethamine and to the wide-spectrum antibacterial trimethoprim (1). When James Burchall joined the department in 1962, he began the isolation and characterization of the dihydrofolate reductases from many different species. As the differences between these unfolded, the particular sensitivities of the enzyme from different sources to different diaminopyrimidines, which had previously been delineated empirically, began to be understood. Moreover, it laid the foundation for the search for new selective dihydrofolate reductase inhibitors.

In 1966 we hired Thomas Krenitsky, a young biochemist whose postdoctoral work had been involved with uridine phosphorylase. Our program to investigate in detail the enzymes of purine and pyrimidine metabolism was now launched in earnest. This investigation was to give us, in the years that followed, an understanding of the substrate and inhibitor specificities of the phosphotransferases, phosphoribosyltransferases, kinases, oxidases, and deaminases, as well as of the enzymes of nucleotide interconversion. This would

help us to understand and capitalize on some of the exciting selective activities that we later encountered with protozoa and viruses.

In 1967 Hitchings was promoted to Vice President in charge of Research. The Biochemistry Department was divided into the Department of Experimental Therapy, of which I became the head, and the Department of Microbiology headed by Burchall.

A year later the announcement was made that Burroughs Wellcome Co. would be vacating the premises at Tuckahoe, New York, and moving in 1970 to a new research facility to be constructed in Research Triangle Park, North Carolina. The factory would also move to North Carolina. Both facilities would be much larger and, thus, we could begin to recruit additional staff, which had not been possible in our inadequate laboratory space. While we were able to squeeze a few new people into the Tuckahoe laboratories during the last year before our move, many joined us in North Carolina. The new building was not ready when we arrived and waiting for a few new laboratories to be available each week required a good deal of patience and stamina. Fortunately, the universities in the area, Duke University, the University of North Carolina at Chapel Hill, and North Carolina State University, were able to make some space available for our chemists for that first year. The rest of the research staff coped as well as it could.

A sad feature of the move for me was the loss of almost all of a group of young, dedicated, talented women who had worked tirelessly with me to study the metabolism of 6-mercaptopurine, thioguanine, azathioprine, and allopurinol. They were unable to move to North Carolina because of family obligations. I also lost several of my young chemical assistants, some of whom went on to graduate school, some to other jobs in the New York area.

Within a year or two of the move, my department had greatly expanded. Krenitsky's section of Enzymology had added three PhD's, Richard Miller, James Fyfe, and Thomas Spector. A section of Metabolic Studies had been formed, with Donald Nelson and Paulo de Miranda as senior investigators, with Thomas Zimmerman soon to follow. There was an Immunology Section, with Gerald Wolberg and Richard Quinn, and a Chemistry Section with Janet Rideout and Lowrie Beacham. We also established a tissue culture laboratory, headed by Naomi Cohn. The knowledge, expertise, and dedication of this team and those who joined them in the 1970s, was responsible for the work which followed.

In addition to a large increase in staff, there was an upgrading of our instrumentation and facilities. We bought our first high pressure liquid chromatograph in 1969; within five years we had four of them. Recording infra-red and ultraviolet spectrophotometers, nuclear magnetic resonance, and mass spectrometry were taken for granted and computerization of instruments

became commonplace. The revolution in methodology had made some of our early experiments seem archaic and it became almost embarrassing to publish the data obtained by those old methods.

The exploration of new fields to conquer with our antimetabolites continued, together with basic studies of mechanism of action and resistance, pathways of metabolism, transport, and attempts to regulate immunological reactions.



## *Leishmaniasis*

I would like to digress somewhat at this point from the chronological sequence to relate our experiences with allopurinol in an area into which we were thrust unexpectedly in the mid-1970s. Joseph Marr, who was then in the Department of Medicine in St. Louis, called one day to inform me that he had found allopurinol to be toxic to leishmania, a protozoan which causes a disease, leishmaniasis, that is widespread in tropical countries. I was skeptical at first. Our previous studies with allopurinol had shown it to be amazingly inactive with respect to any of the anabolic pathways of nucleic acid synthesis. Krenitsky had found it to be a substrate for purine nucleoside phosphorylase, but the allopurinol ribonucleoside formed accounted for only 10% of an allopurinol dose in humans, was inert, and was excreted in the urine. Allopurinol was a very poor substrate for human hypoxanthine phosphoribosyltransferase (HPRT) and the amount of allopurinol ribonucleotide formed in rat tissues was so low that it could only be detected after using large intravenous doses of $^{14}$C-allopurinol of high specific activity. Even under the latter conditions, allopurinol was not incorporated into the nucleic acids. Marr's findings with leishmania were, therefore, intriguing and challenging. They required an explanation. We set up a productive collaboration with Marr during which we learned a great deal about the difference between protozoan and mammalian enzymes (See refs. in 5). In our group the principal players were Nelson, Krenitsky, and Spector.

The first surprise was that the leishmania could convert allopurinol very efficiently to a ribonucleotide. In fact, the amount of allopurinol ribonucleotide formed on incubation of allopurinol with the promastigote forms of *L. donovani* or *L. braziliensis* for 24 hr in culture was more than three times the concentration of ATP in these protozoa. This was due to very high levels of a leishmanial HPRT with a lower $K_m$ and higher $V_{max}$ than for the mammalian enzyme. Even more unexpected was the finding that the leishmania had converted allopurinol ribonucleotide to the corresponding amino analogue, and formed 4-aminopyrazolo (3,4-d)pyrimidine mono-, di- and triphosphates. These latter compounds (APP-P, APP-DP, and APP-TP) are not formed in

mammalian cells. Studies on the aminating enzyme, succinoadenylate (SAMP) synthetase, showed that the leishmanial enzyme could indeed convert allopurinol ribonucleotide to the succinylamino derivative, which is then converted by SAMP lyase to the amino compound. The mammalian SAMP synthetase does not act upon allopurinol ribonucleotide. The toxic compound is apparently the APP-TP since it can act as a substrate for the leishmanial RNA polymerase and be incorporated in the RNA.

Another unusual finding with the leishmania was its ability to utilize allopurinol ribonucleoside and convert it directly to the nucleotide, a reaction not found in man. Therefore, it seemed possible that allopurinol ribonucleoside could be used as a treatment for leishmaniasis in place of allopurinol. This substitution might be an advantage since allopurinol ribonucleoside is quite soluble and is excreted in the urine essentially unchanged in man, whereas allopurinol is converted quite rapidly to oxypurinol, which is inactive on leishmania. Unfortunately, we were unable to test allopurinol ribonucleoside in the available rodent models for leishmaniasis since rodents, unlike man, oxidize allopurinol ribonucleoside to oxypurinol ribonucleoside, which is inactive.

Another protozoan that is responsible for a widespread tropical disease is *Trypanosoma cruzi,* the etiologic agent of Chagas' disease. Like the leishmania, *T. cruzi* can also convert allopurinol to APP-TP, which is toxic to the parasite. The clinical efficacy of allopurinol in the treatment of Chagas' disease (11) and in cutaneous leishmaniasis has recently been demonstrated (12). Our excursion into the biochemistry of protozoa was intriguing and useful.

## *Herpesvirus Infections*

Another field, which we had only touched upon in 1949, that of antiviral chemotherapy, was inviting. The current dogma was that one could not hope to interfere with the replication of viral DNA without toxicity to the DNA of the host cell. Idoxuridine (5-iodouracil deoxyriboside) had found utility as a treatment for herpetic keratitis by topical application to the eye. The compound was, however, too toxic and too metabolically unstable to be given parenterally. In 1968 there was a report that adenine arabinoside, isolated from the fermentation filtrates of a strain of streptomyces, had activity against DNA viruses. This rang a bell. Since 2,6-diaminopurine had shown antiviral activity against vaccinia virus, a DNA virus, and all our studies on diaminopurine had shown it to be a close analog of adenine, perhaps 2,6-diaminopurine arabinoside would also have antiviral activity. Although we did not at that time have an antiviral screen operating in-house, our colleague John Bauer, in the Wellcome Research Laboratories in the U.K., had kept a number of

antiviral screens going, in vitro and in vivo. We synthesized 2,6-diaminopurine arabinoside and, in 1969, sent it off to Bauer to test. The answer came back by telegram. The compound was active against herpes simplex virus and against vaccinia virus in mice. A new field was open to us!

The synthesis and antiviral studies of a number of purine arabinosides were expanded. The synthetic work was performed by Janet Rideout, Bauer and Peter Collins continued the antiviral screening in the U.K. Of particular interest was the finding by de Miranda that 2,6-diaminopurine arabinoside was deaminated in vivo to guanine arabinoside, which was as potent an antiviral as the diamino derivative (13). This was good news since the deamination of adenine arabinoside resulted in a large decrease in antiviral potency. Our move to North Carolina disrupted the work temporarily, although the delay turned out to be a blessing in disguise. Better compounds were just beyond the horizon.

When we moved, our previous head of Organic Chemistry retired and we acquired a new head, Howard Schaeffer, who came from the University of Buffalo. Schaeffer had for years been studying the substrate and inhibitor specifities of the enzyme adenosine deaminase. He had found a good inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and had also found that a portion of the riboside moiety of adenosine could be altered to an acyclic side chain without elimination of substrate activity. He continued to expand the series of acyclic nucleosides when he came to Burroughs Wellcome. The compounds were sent to the U.K. for antiviral screening. The activities of both EHNA and 9-(2-hydroxyethoxymethyl)adenine against the herpes simplex virus (HSV) were interesting and warranted further synthetic efforts. Schaeffer and Lilia Beauchamp extended the series both with respect to the heterocyclic base and the side chain. The 2,6-diamino-9-(2-hydroxyethoxymethyl)purine was more active than the adenine derivative. Moreover, as had happened in the arabinoside series, the diaminopurine compound was deaminated in vivo to the guanine derivative. No one could have anticipated that this guanine derivative (acyclovir) would be 100 times more active than the diamino compound. Indeed, the diamino derivative was active because of its conversion to the guanine analog by adenosine deaminase. In the presence of an adenosine deaminase inhibitor, it was inactive.

From the time we first knew that we had an exciting new antiviral agent in hand, a large group of diligent and devoted scientists worked as a team to obtain both the basic and practical information needed to make the compound a successful treatment for herpesvirus infections. The first challenge was to discover the reason for the high degree of selectivity of acyclovir (originally called acycloguanosine). Its ability to inhibit replication of herpes simplex viruses, types 1 and 2, (HSV-1, HSV-2) and varicella zoster virus (VZV) at

concentrations between 0.1 μM–4 μM, while the 50% inhibitory values for the cells in which the viruses were grown were 300 μM–3000 μM, posed an important challenge. The answer would undoubtedly yield important information about the biochemical differences between herpes viruses and mammalian cells.

To do the kind of mechanistic and metabolism studies that we had in mind, we established a virus laboratory in our building in 1975 and hired Phillip Furman and later Karen Biron, as well as several junior scientists to undertake the viral studies. They worked closely with the other members of the Department. Our colleagues in the U.K. continued to explore the activity of acyclovir in a variety of animal models and with a variety of viruses.

The first definitive experiments on mechanism of action were those which showed that $^{14}$C-acyclovir was converted in HSV-infected cells to three new radioactive metabolites. These appeared in a high pressure liquid chromatogram of the cell extract in the region of the nucleoside mono-, di-, and triphosphates. These compounds were not formed to any noticeable degree in uninfected cells. The identity of these new compounds was relatively easy to establish since they were convertible enzymatically to the original acyclovir (ACV).

Some elegant biochemistry by Fyfe identified the first enzyme in the conversion of ACV to its phosphates as a herpes virus-specified thymidine kinase. Although this enzyme was known to exist, its substrate specificity had never been investigated to any extent. The idea that an acyclic nucleoside analog of guanosine could serve as a substrate for this enzyme would certainly not have seemed likely to any knowledgeable chemist. Yet it was unquestionably true. When the viral enzyme was isolated and purified, its ability to phosphorylate ACV was confirmed. The cellular thymidine kinase isolated from uninfected Vero cells was inactive on ACV. Thus, the first major basis for the selectivity of the compound was established.

The substrate specificity of the herpes virus-specified thymidine kinase was examined in detail by Paul Keller. A certain latitude in the size and nature of the acyclic side chain was permissible but a terminal hydroxyl group was essential. The enzyme did not tolerate changes in the purine base to any extent, e.g. xanthine, adenine or 2,6-diaminopurine with a 9-(2-hydroxyethoxymethyl) side chain were not substrates. However, 2-methylthio-6-aminopurine with the same side chain was a substrate for the herpes thymidine kinase. Interestingly, it did not have antiviral activity. This finding reminded us that phosphorylation to a monophosphate was a necessary but not a sufficient requisite for antiviral activity.

The remaining steps in the activation of ACV to a triphosphate were investigated by Richard and Wayne Miller. They found that the cellular

guanylate kinase transformed ACV monophosphate to the diphosphate and that several cellular enzymes could convert the di- to the triphosphate.

The next selective step in the activity of ACV was the effect of its triphosphate on the viral and cellular DNA polymerases. Furman, Biron, and St. Clair conducted this investigation. The viral DNA polymerases of HSV-1, HSV-2, and VZV proved to be much more sensitive to inhibition by ACV triphosphate than were the cellular α and β DNA polymerases. Moreover, the viral polymerases were able to incorporate ACV into the growing viral DNA molecule, thereby causing chain termination, since ACV lacked a 3′-hydroxyl group. There was also an inactivation of the viral DNA polymerase by ACV triphosphate that was not seen with the cellular enzymes.

While the synthetic and mechanistic studies were progressing, there was much to be learned about the therapeutic potential of acyclovir in animal model systems, and its absorption, tissue distribution, metabolism, pharmacokinetics, and toxicology. When we published the first two papers in *Proceedings of the National Academy of Sciences* and *Nature* in late 1977 (14) and early 1978 (15), the studies had been going on for over three years. While the first two papers did not appear to attract a great deal of attention, the thirteen posters presented at the Interscience Conference on Antimicrobial Agents and Chemotherapy in October 1978 created a major stir. I remember the occasion well for personal as well as professional reasons. I was in the process of recovering from back surgery, which had been performed at the beginning of September. The surgeon thought it inadvisable for me to fly to Atlanta for the meeting on October 1st. I assured him that nothing could keep me away, even if, as did happen, I had to be carried onto the plane on a pallet and taken off in a wheel chair. We had waited too long for this moment and I was not going to miss all the fun. My back eventually healed anyway.

One reason for the excitement at the 1978 meeting was that we had finally shown that antiviral drugs could be selective and that one could capitalize on the differences between the viral and cellular enzymes. Within months, all the large pharmaceutical companies had initiated antiviral programs and the competition became fierce. Perhaps it was a good thing for, when the AIDS epidemic broke several years later, scientists were already geared up to think that antiviral chemotherapy was not impossible.

The pharmacological properties of ACV were better than we could rightfully have expected: it was metabolically stable and was excreted in the urine, essentially unchanged, in man; was distributed in all tissues, including the brain; and had a plasma half-life of about 3 hr. Its oral absorption varied greatly from species to species, being very good in the mouse and dog, poor in the rat, and intermediate (about 15–20%) in man. The monkey absorbed almost none of the drug orally and the plasma half-life was very short. The

data on pharmacokinetics and metabolism in different animal species was very helpful in understanding why the drug had to be given at much higher doses in some animal model systems for herpes virus infections than in others (5, 16).

As we had hoped and anticipated, ACV was nontoxic at doses that far exceeded those needed for antiviral activity. The thorough toxicology work-up by Walter Tucker and his team was very reassuring and was important to convince the skeptics that an effective antiherpetic agent really could be safe.

During the next few years we had many collaborators who were outstanding in the preclinical and clinical evaluation of the antiviral activities. Bauer and Collins tested acyclovir in a number of herpes virus infections in mice and rabbits; Earl Kern examined its activity in HSV-2-infected guinea pigs; Ken Soike tested its effect on the Simian varicella zoster virus in monkeys. The intravenous pharmacokinetics in man was investigated by de Miranda, Harvey Krasny, and Steven Good, in collaboration with Richard Whiteley, Paul Lietman, and James Connor. The early clinical trials against genital herpes infections were conducted by Lawrence Corey.

In September 1981, Burroughs Wellcome Co. held a three-day international symposium on acyclovir in Washington, D.C., under joint sponsorship with the National Institute of Allergy and Infectious Diseases. The proceedings of that symposium were published as a supplement to the *American Journal of Medicine* in 1982 (16). The 77 papers that were presented covered chemistry, mechanism of action, antiviral spectrum, preclinical pharmacology and toxicology, efficacy in animal models, antiviral efficacy in the normal and in the immunocompromised host with mucocutaneous, ocular, and genital herpes infections. In 1982, the FDA approved the ointment and intravenous formulations of ACV (Zovirax)® and several years later the oral form. The number of indications for the use of ACV has continued to increase and now include mucocutaneous herpes infections, genital herpes, herpes encephalitis, shingles, and chicken pox. Prophylactic use of the drug has been of great help in preventing the activation of latent virus in immunosuppressed patients, e.g. those undergoing bone marrow or organ transplantation or cancer chemotherapy. In patients with genital herpes who suffer from frequent recurrences, prophylactic use of ACV has markedly reduced the number of recurrences or prevented them entirely (17). Antiviral chemotherapy has come of age.

## THE PRIZE

In 1983 I had to make a decision whether to retire or go on working: I chose to do both. With acyclovir now launched, I thought it might be a good time to retire as head of the Department before a new drug turned up that I couldn't

bear to leave. The members of the group were such excellent scientists and I knew they would all go on to do well, with or without me. For years I had the pleasure of being an orchestra conductor of some fine musicians. They could indeed make beautiful music together. I was fortunate that I could leave the group in the capable hands of Tom Krenitsky, while I remained at Burroughs Wellcome as a Scientist Emeritus and consultant. I felt I had the best of both worlds.

The first year of my "retirement" was a very busy one and made me realize that I would probably never have time to wonder about what to do next. I was president of the American Association for Cancer Research that year and had just been appointed to the National Cancer Advisory Board for a six-year term. I was also serving on a Steering Committee for Filariasis for the World Health Organization. There was still time to go to scientific meetings and give lectures around the world. I was also in the happy position of remaining close to the work going on at Burroughs Wellcome, watching the expansion of the antiviral program in particular. It was satisfying to watch the same team that had done so well with the development of ACV now expand and proceed to use their skills to develop azidothymidine (AZT, zidovudine, Retrovir®) for the treatment of AIDS.

In mid-October 1988, to my complete surprise, I was notified that I had been awarded the Nobel Prize in Physiology or Medicine, together with George Hitchings and Sir James Black. People often ask whether this wasn't what I had been aiming for all my life. Nothing could be farther from the truth. It had never occurred to me that I might be considered for this award. My rewards had already come in seeing children with leukemia survive, meeting patients with long-term kidney transplants, and watching acyclovir save lives and reduce suffering. The Nobel Prize was a wonderful international recognition not only for me but for our whole team, for whom it was a great morale booster.

The Nobel Prize ceremony itself is, of course, a memorable event, and the week of celebration that surrounds it is, as my five-year old grandniece described it, a fairy tale. It was wonderful to be able to share the occasion with eleven members of my family, my brother's children and grandchildren. Unfortunately, my brother was ill at the time and could not come.

What I did not anticipate was how much the receipt of the Nobel Prize would change my life. Suddenly, I was greatly in demand by the press, television, universities, committees, and Boards. Honors began to come in a steady stream. I was elected to the National Academy of Sciences, the National Inventors' Hall of Fame, National Women's Hall of Fame, and received the National Medal of Science. I now have many honorary degrees, but I still remember the feeling of pride when I received the first two, in 1969, from

George Washington and Brown Universities. They represented the PhD I had never received and the vindication of my parents' faith in me. Unfortunately, neither of them lived long enough to see it.

## PASSING THE TORCH

Shortly after my retirement, I was asked to be a Research Professor of Pharmacology and Medicine at the Duke University Medical Center. I agreed to work with a third-year medical student each year on a research project. The students are allowed this optional year of research in place of course work. The students have worked in the laboratory of Henry Friedman in the Department of Pediatrics, while I have acted as mentor, helping to delineate the problem, design the experiments, analyze the results, and plan the next steps. We have worked on brain tumor biochemistry, pharmacology and chemotherapy both in vitro and in human xenografts in nude mice. It has been challenging for me and, I think, for the students. They are all so bright, so motivated and so energetic that I find myself needing to read and study along with them. I have had seven students to date, three women and four men, and all have done exceedingly well in their final year and in their medical residencies. It is the kind of teaching I thoroughly enjoy and I hope to continue it as long as I am able.

As I look about me and see young people shying away from science, I feel an almost missionary zeal to do something to change that trend. My hope is that I can not only help students already committed to science, but that I can spread the news among the younger generation that science is fun. I would like to see them experience the same excitement and fulfillment that I have had in my career. However, I try not to minimize the difficulties they may encounter or the perseverance it may require. If the goal is worthwhile and its pursuit enjoyable, the reward will be inevitable.



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