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From outer to inner space: Traveling along a scientific career from astro-chemistry to drug research

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obtained a B.S. in chemistry and physics from Cal-Tech. His Ph.D. was in chemical physics for work on chemical reactions on comets at the University of Chicago. After postdoctoral appointments at the University of California at La Jolla, Imperial College in London and active duty in the U.S. Air Force, he took up a faculty position in biochemistry at Columbia University where he performed biostructural research on bioactive peptides. After a sabbatical at Oxford University he took up positions as an Associate Professor and later as a Professor of Biochemistry at the University of Connecticut Health Center in Farmington, Connecticut. There, his research interests changed slowly from biostructural work to the biochemistry of opiates and opiate receptors. He pioneered the uses of anti-morphine monoclonal antibodies and anti-opiate receptor anti-idiotypic antibodies in research on the biochemistry of narcotics. In 2000 he founded Global Scientific Consulting, LLC.

Summary

This professional history describes my journey as a research scientist after my early training and experiences in the pre- and early post-World War II United States. My graduate training concentrated on a problem in astrochemistry: phenomena on comets. As my career developed, I felt confident enough in myself as an experimentalist to enter, and make contributions to, several different fields: structural biochemistry (*via* nuclear magnetic resonance spectroscopy), molecular immunology, pharmacology, neurochemistry, and cell biology. One emphasis is on the nature and quality of my scientific training that permitted me to do cross-disciplinary work. A second emphasis is on the technical and intellectual developments in late twentieth-century science and how, along with the changes in American society as it passed through three major wars, they influenced my life and thought.

"What we cannot speak about we must pass over in silence."

The last proposition from
Tractatus Logico-Philosophicus, Ludwig Wittgenstein, 1921

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1 Introduction: so few degrees of separation

This narrative describes my career as a research scientist from my beginning work to my present interests concerning the interaction of drugs with gene expression.

Depending on how you look at it, I had the great fortune or the great misfortune to become a scientist just at the time when science was becoming a profession.

After his first viewing of a rocket launch at Cape Canaveral, Harold Urey (1893–1981) told me in wonder "it was as big as a locomotive standing on end!" I think that only an American with one foot in the 19th century and the other in the 20th could have come up with this technological simile. Now I am a scientist with feet bridging two centuries: Dr. Urey was my principal Ph.D. thesis advisor and to me his remark symbolizes how few degrees of separation[1] there are between chemists of my era and origins of quantitative chemistry.

As science has become more specialized during my career, so has what might be called the languages and thought processes used in the different areas of specialization.

Looking back, I believe I can claim this: I speak physical chemistry and biophysical chemistry well, am quite fluent in molecular pharmacology and immunology, speak serviceable biochemistry and molecular biology, and really stumble along in biology. To work in these different areas one must use different languages and think along different lines. The molecular science underlying all biological disciplines is identical, but just as translation between social languages is often difficult, the translation between scientific languages is too. I was privileged to have been given the opportunity to teach myself some of the translations – an option for young scientists that is unfortunately rapidly disappearing because, while self-teaching is personally more satisfying to the individual, it is very inefficient.

Let me start at the beginning.

2 Early days

My cultural background contains two streams of Judaic tradition – Germanic and Russian – that individually have been influential in American culture, business and science. On my father's side, the Glasels came to this country

from the Tyrol in the second half of the 19th century. On my mother's side, the heritage was Russian/Hungarian farmers.

2.1 Beginning education

My family resided in New York City and Rye, New York, until I was seven years old. My formal education began on Sunday mornings, when my father taught me to read – pre-kindergarten – using the comic strips in the Sunday papers as a textbook. I also had the good fortune to subsequently attend Rye Grammar School – a very advanced school for its time. For example, it had its own student news broadcasts to classes every morning.

Aged 41 when the United States entered World War II (WWII), my father requested and obtained active duty in the Navy (he was a Chief Warrant Officer in the Naval Reserve). He was immediately transferred to the West Coast and then shipped out to the Pacific theater about the time (June, 1942) of the Battle of Midway. My mother and I followed and the rest of my education until graduate school took place in California (San Diego, Los Angeles, San Francisco). With my father away throughout the war, my mother continued my outside-school education by reading many volumes of Dickens aloud to me.

The psychological impact of WWII on me was enormous. I followed the news very closely and my father wrote me about the men he was with *via* v-mail. Although I was too young to fully understand the sweep of world events, I followed them closely in the newspapers and they became firmly imprinted in my memory. While he was on leave, I remember my father crossing out sunken submarines from a list he had and telling me about crew members he knew on them. Because of these events, I became acutely aware of the precarious nature of life and through the war years, I was never certain that I would see my father again.

In San Diego (where we landed after the war and my father's discharge from the Navy), I continued to be well served by a fully functional public school system. I still remember individual teachers at the junior high school and high school levels. In particular, in high school, I had a science teacher who allowed me to supplement formal classes with after-hours chemistry

experiments on my own, working with real chemicals, and trying reactions whose descriptions I dug out of advanced texts.

2.2 Scientific beginnings

As did many young people at the beginning of the nuclear age, I had become interested in nuclear physics and in my case, cosmic rays. I became (I think) one of the first people in San Diego after the war to have a licence from the Atomic Energy Commission (AEC #1828, Aug. 16, 1950) to purchase radioactive materials, and with it, I purchased one ounce of thorium nitrate at a cost of $ 0.83 in August, 1950. I used the nitrate as a test source of ionizing radiation for a continuous cloud chamber I constructed when I was in my junior year in high school. It was an even greater thrill observing cosmic ray tracks with the thorium source removed: it produced a great sense of mystery about the origin of these rays that I knew had traveled enormous distances to leave their calling cards in my little peanut butter jar apparatus.

At about the same time I became interested (I think because of an article I read in *Popular Science* magazine) in field emission microscopy. I wrote to Erwin Müller (1911–1977), who invented the technique that allows the visualization of individual atoms in 1937, at the Kaiser-Wilhelm Institute in Berlin telling him of my plans to build such a microscope. Müller wrote me back a very nice letter in German along with a reprint of his latest work. In the letter he gently told me that "Um gute Resultate zu erreichen, muss man allerdings einige Erfahrungen in der Vakuumtechnik besitzen." ("To get good results, one must have experience in vacuum technique"). I wasn't destined to have such technique for another 8 years.

Looking back now, it is clear to me that my early scientific interests focused on the mystery surrounding things we can't see with our own eyes: for example, cosmic rays and atoms.

When it came time for college, the tuition-free (at that time) University of California at Berkeley seemed to be the answer for a family with our income. The only drawback was the reportedly huge classes – especially in freshman chemistry. However, after a visit to an open house at The California Institute of Technology (CalTech) my father decided on the spot that no

matter what the financial sacrifice on his and my mother's part, I had to go to CalTech, where I was accepted a few months later.

3 CalTech years

Matriculating at CalTech was then meant to be (and was) intimidating. Speaking to the assembled incoming freshman before classes started, Dean Foster Strong casually said something like, "look right and left, at the end of the first term one of those classmates will no longer be here". Try saying something like that to modern college freshmen! With these beginning words we were let loose for long days of classes and homework.

The Korean War continued through my beginning undergraduate years, as did the military draft. Knowing I would soon be eligible for the military draft, I joined The Air Force Reserve Officers Training Corps (AFROTC) which recruited actively on the CalTech campus. As described later, joining the AFROTC had definite effects on my career and attitudes.

3.1 Among smart people

Including the students, there were some weighty intellects at CalTech when I was there. Linus Pauling was in his prime, Richard Feynman had recently arrived, and there were many other famous or soon-to-be-famous members of the faculty. But there were also many faculty members whom the rapid advances in the sciences had left completely behind. And, for the first time, I met men my own age (only men – CalTech then was like a Jesuit school for scientists) who were very smart and had scientific abilities far outstripping anything I had.

I still have a CalTech examination book with an essay written for – and corrected personally by – Linus Pauling during freshman chemistry. Pauling was insistent on proficiency in language so he gave a snap essay exam each year for the whole freshman class of 125 students taking the course. Pauling had this much work to do because one of the features of a CalTech education then was that future engineers and scientists took all classes together the first two years. For example, I took engineering drafting (which helped me greatly later when I had to design apparatus in graduate school).

3.2 CalTech's philosophy – then

This idea – that there is fundamental knowledge that engineers and scientists both need to know – is completely out of fashion and all students that I meet now have specialized very early and assume they will join teams of people with interlocking abilities. I don't want to enter into a debate here about the merits of this and other changes in undergraduate scientific education that have taken place since my era. But I do know that, with the preparation I got as an undergraduate, I've been able to keep up with a general understanding of the major developments in all branches of science that have taken place since I left formal training.

3.3 Humility, humiliation and teaching CalTech style

CalTech was both a place for humility and for humiliation. It was and is mainly a graduate school and regularly graduate students deemed not prepared for the CalTech standard were made to attend undergraduate courses (where they would usually slip into seats in the back of the class and attempt to be invisible). In my junior year, James Watson was on campus, just back from England where he had collaborated with Crick in the monumental work that changed biology forever. My undergraduate friends majoring in biology appreciated and understood his work (even if some of the faculty had missed its implications.) One of my friends told me how important Watson's work was going to be and I searched Watson out and got a reprint of one of the two famous *Nature* papers [1] (he said it was his last copy, and I still have it).

Pauling and Feynman were great lecturers who knew the full value of stagecraft in teaching. At that time, Feynman confined his lower-level formal lecturing to what were called "Friday Evening Demonstration Lectures" that were open to students and public alike. Some inkling of Feynman's lecture style can be obtained by reading his *Lectures on Physics* [2] (the books were based on lectures given in 1961–62) or listening to recordings of them, now available on audio cassette [3].

Constantly smiling during his dynamic lectures, Pauling obviously delighted in undergraduate education. Later on I heard it said that Enrico Fermi could lecture on physics in one continuous development of thought starting with Newton's laws and going all the way up to the edge of what is

known. Pauling could do the same thing with chemistry and I still use his textbook *General Chemistry* as a reference. I was 17 years old and being exposed to, and challenged by, some of the finest analytical minds of the 20th century.

3.3.1 My research beginnings

Beginning near the end of my sophomore year, and continuing until I graduated, I did some real research. John D. Roberts, a great and forward-looking organic chemist at CalTech, suggested to me that he thought the mechanism of an organic reaction (I forget which) that appeared in all undergraduate organic chemistry textbooks was wrong. He suggested a simple and straightforward experimental way of testing the mechanism. I proved Roberts to be correct and the textbooks wrong. This research left a lasting impression on me of how clearly a great scientist thinks about a problem. The experiment Roberts suggested to me was so simple that for the first time in science I could have kicked myself for not thinking of doing it the same way.

I went on from my research start with Roberts to solve a crystal structure (of a Li-Sn metallic compound) using X-ray diffraction. I worked on this directly with B. Gunnar Bergman (then officially Pauling's student but actually working mainly with David P. Shoemaker, another about-to-be well-known crystallographer).

In my third year at CalTech, I became friendly with another Pauling protégé, Norman Davidson, who proved to have a great influence on my career. Although he later did as I was to do – unilaterally declare himself a molecular biologist – Davidson at the time was doing research on gas phase reactions using shock tubes. However, Davidson suggested I do some research with the then relatively new and primitive electron microscope. I took up his suggestion, and although the research didn't come to anything, I learned a lot about staining samples and, as a side issue that would turn out to be lucky in my future thesis project, learned a lot about high vacuum systems (mainly, in those days, about the plenty of things that could go wrong with them).

Looking back now, my second and third years at CalTech must have been a time when I widened my mental horizons greatly. I read Harold Urey's new book *The Planets, Their Origin and History* when it came out in 1952 even

though I had no idea who Urey was or that I was to become his student and, briefly, an astrochemist!

I also read Edmund Berkeley's *Giant Brains* about the development of computers. This was several years before the first commercial stored-program computer (the UNIVAC) appeared on the market. I remember tabulating data from my X-ray diffraction experiments using Hollerith cards and IBM sorters, but all calculations were done by hand on Marchant electro-mechanical calculators. Thus I humbly entered the computer age and have followed it along like a surfer picking up a wave that gets bigger and bigger.

I was also a little unusual as a chemistry major insofar as I took enough courses in physics to officially minor in that subject. This was especially difficult for me because, much as I like physics, I was (and am) a terrible and unnatural mathematician (that is, at a level necessary to do creative physics). That problem of being fluent in another scientific language struck again: I found that I didn't speak mathematical physics well.

When my senior year started, I began looking at graduate schools. CalTech science graduates were in great demand: I even got an unsolicited invitational telegram from a well-known infrared spectroscopist at the University of Minnesota inviting me to enter the chemistry department there. Evidently, to recruit them, student-hungry departments around the country were finding the names of CalTech's about-to-graduate students. And our entire chemistry class totaled 8 students!

For graduate school, I was determined to go to a big city school in the eastern U.S. and under the influence of Norman Davidson, who had graduated from there in 1941, I decided to apply to the University of Chicago's Chemistry Department and was accepted with a teaching assistantship.

3.3.2 A 1950s CalTech education in summary

To summarize a CalTech education at that time: it produced pretty arrogant boy-men with a superb education in the foundation of quantitative science: the integration of mathematics and basic physics, and – if they wanted it – the beginnings of a liberal education. The system also produced the ability to calculate the answers to numerical problems, knowledge of what a mathematical derivation is, knowledge of what a scientific experiment is, knowl-

edge of what a control for an experiment means, and what experimental errors are and where they come from. In my case, it did furnish me with a basic liberal arts education, and allowed me to expand my interests in that direction. It was a superb school.

In June 1955, I graduated from CalTech with "Academic Honors" (granted to the upper 10% of the around 100 remaining original members of the class) with my father and grandfather present. (My mother had died the previous fall.)

4 Ph.D. candidate at the University of Chicago

In the 10 years after WWII ended, the University of Chicago had swept up many scientists who had engaged in nuclear weapons research: Enrico Fermi, Harold Urey, Leo Szilard, Joseph and Maria Mayer to name only a few. It had built its Institute for Nuclear Studies to house this faculty directly across from the grandstand under which was the famous squash court (when I got there, still standing but empty) where Enrico Fermi supervised – with a break for an Italian lunch – the criticality of the first nuclear pile. Fermi had died a year before I arrived, but his personal and scientific influence still pervaded the Institute.

4.1 Starting to become a chemical physicist and teacher

I took the usual variety of graduate chemistry courses (e.g., chemical thermodynamics, quantum chemistry, statistical mechanics, etc.) but also completely overstretched myself with courses in quantum electrodynamics (given by Richard Dalitz, a well-known particle physicist) and of all things, magnetohydrodynamics – given by the then 45-year-old S. Chandrasekhar (1910–1995) in his scientific prime.

Although I had financial support from UC, first as a teaching assistant and then later *via* fellowships, it was still not enough to live on. After taking courses for a year, I discovered I could be awarded a Master of Science degree from UC without any further work and this would enable me to teach freshman chemistry at the University of Illinois at Navy Pier (UINP) at a pretty good salary.

Teaching freshman chemistry was challenging, especially at that University and in that era: UINP was mainly attended by students who couldn't afford to go the main University of Illinois campus downstate, and included many who didn't speak English at home. Chicago was then, and still is, divided into very definite ethnic areas whose inhabitants were mixed only in certain circumstances like getting an education. It was very intriguing to learn to teach effectively at a level different from CalTech or UC.

4.2 My independent research career's foundation: Harold Urey

During this time, I also began looking for someone to direct my eventual thesis work. A visiting scientist working with Harold Urey suggested I talk to Urey about doing a thesis with him. In my interview with Urey as his prospective student, he related to me the mystery of explosions on comets (where does the stored energy come from?) and at the end of the interview he asked me whether I would like to solve this problem. The answer was yes.

I was to be Urey's last student. His previous one before me had been Stanley Miller who had received worldwide scientific and public recognition for his experiments suggesting how life on earth may have begun.

Urey had a wonderful way with any professional or would-be professional scientist he met, students included, that intimidated many of them. He treated everyone absolutely the same: we were all assumed to be on the same scientific level. There are merits and demerits to this way of treating people, but the merits far outweigh the demerits. During my thesis work and thereafter as a postdoctoral worker, and finally as a faculty member, when I would come to him with what often were half-baked scientific ideas, Urey would invariably take them seriously, consider them, and then give me succinct and dead-on *scientific* reasons why they were half-baked. The result was that I never left his presence humiliated, just chastened and with new understanding of what science was all about.

Urey was a great scientist, but a very un-complex man. He has been variously described by different authors as "boorish and thin skinned" [4], "short, intense, enthusiastic" [5], and "a bantam cock of a physical chemist" [6], among others. I think the derogatory descriptions are grossly unfair: he was just a very ordinary man outside of his scientific talents. Urey's secret weapon

in social situations was his wife Frieda who made everyone she met, students especially, feel at home and relaxed almost instantly after meeting her.

After I passed the preliminary examinations allowing me to enter thesis research, Urey arranged for me to have space in the old chemistry building (his office and laboratories were a couple of blocks away in the Institute) and gave me account numbers for purchasing equipment and chemicals. I can't remember seeing him in my laboratory for another year.

During that year I was fortunate in attending the almost daily afternoon tea sessions in his mass spectrometry labs at the Institute. After WWII, Urey had gone into geochemistry, using mass spectrometry as a tool, mainly to get away from the secrecy he had been involved with when working on separation of uranium isotopes for the nuclear weapons project. His Chicago laboratory had turned out some outstanding geochemists before I got there (among them Gerald Wasserburg, Harmon Craig and Cesare Emiliani).

4.2.1 A research problem in astrochemistry

Starting my thesis research project was heady stuff. The basic scientific problem was that comets, drawing near to the sun, experience large outbursts with energy production inconsistent with known chemical or photochemical reactions [7]. Just before I started the project, Bertram Donn and Urey [8] had postulated that comets accumulated free radicals in concentrations up to 10% *via* soft photochemical reactions as they moved away from the sun in their orbits. Upon their return to the close proximity to the sun, their idea was that the free radicals reacted with each other, causing the observed outbursts.

My job was to do experiments to prove the Donn-Urey theory of comet explosions correct.

Reactions on comets take place in the high-vacuum, low-temperature environment of deep space. My job was to design methods for studying photolytic reactions under high-vacuum, low-temperature conditions that simulated actual cometary conditions as closely as possible. The problem was that this was somewhat pioneering work. As the introduction to my thesis [9] states:

> "...it was found that the field of low temperature spectroscopy in the regions of interest had not been explored to any great extent. Until

recently there were two main reasons for this: the apparatus and refrigerants needed were not widely available, and in many cases sufficient data had not been obtained concerning exact assignments of spectral lines in molecules of interest."

My approach to the problem, approved perfunctorily by Dr. Urey, was to place a cryostat, that I would design, containing an internal liquid hydrogen-cooled window in a spectrophotometer in such a manner that the window could be rotated within the cryostat in one direction to receive gases to be condensed on it, in another direction for irradiation of the condensed gases with hard ultraviolet radiation from a hydrogen discharge lamp of my (future) design, and in a third direction to allow spectra from the ultraviolet to near-infrared (near-IR) of the condensed and irradiated material to be acquired. This was a complicated design that required the outer cryostat to have windows at room temperature through which the beam of the spectrometer passed, an inner liquid nitrogen-cooled shield, and finally the inner liquid hydrogen cryostat tipped with the cooled window. The technical problem was that the whole inner assembly had to be rotated in a high vacuum to receive the gases for condensing, irradiation, and to obtain the spectra of the irradiation products. Urey trusted me, without any evidence that I could do the job, to specify and order the spectrophotometer, to design the apparatus, to arrange its construction, and to do the experiments completely on my own.

The first entry in my lab book on Nov. 21, 1957 starts "Beckman DK-1 recording spectrophotometer delivered and installed."

4.2.2 Teaching myself free radical chemistry and low temperature physics

With the spectrophotometer at hand, I set about designing the cryostat, cooled window assembly, high-vacuum system, hydrogen discharge lamp, and the modifications to the spectrophotometer (then a relatively expensive piece of instrumentation), made engineering drawings, specified materials and supervised the fabrication by the superb professional machine shop at the Institute, and built part of it (teaching myself machining) in the student-faculty workshop. I also took a course in glass blowing given by the old world master glassblower in the Chemistry Department and then proceeded to

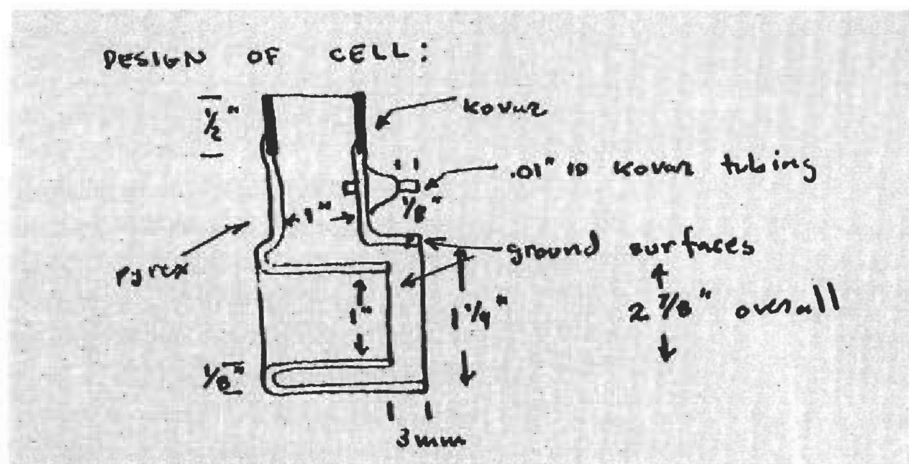

Fig. 1
Liquid-hydrogen-cooled spectrophotometer cuvette designed by the author for viewing putative cometary photolytic products. The windows that were later glued on had to be parallel, so this was a very difficult design to execute for the glassblowers in the Chemistry Department at the University of Chicago. They did a wonderful job.

make the glass manifold for the vacuum system. Figure 1 shows my first window design sketch in my lab notebook.

In my first design the windows were of CaF_2, a substance transparent to light from the far ultraviolet through the near-IR. The use of liquid hydrogen as the coolant was in the interests of technical simplicity and would never be allowed today (hydrogen gas forms an explosive mixture with oxygen in almost all proportions!). The alternative of using liquid helium as the refrigerant would have required much more extensive apparatus and the heating during irradiation of substances on the window would have boiled off much more helium than we could afford. In my design, the liquid hydrogen that boiled off was simply vented into the Hyde Park neighborhood *via* a hose that went through a hole I punched in one of my laboratory's windows. Figure 2 shows a photograph of the completed apparatus, including some of its main features.

Figure 1 shows that light from the monochromator passed through a 3 mm pathway of liquid hydrogen which should have been transparent to light of all the wavelengths I was using. However, almost from the first filling with liquid hydrogen, I found that I got a spectrum in the near-IR without any condensate on the windows of the cuvette.

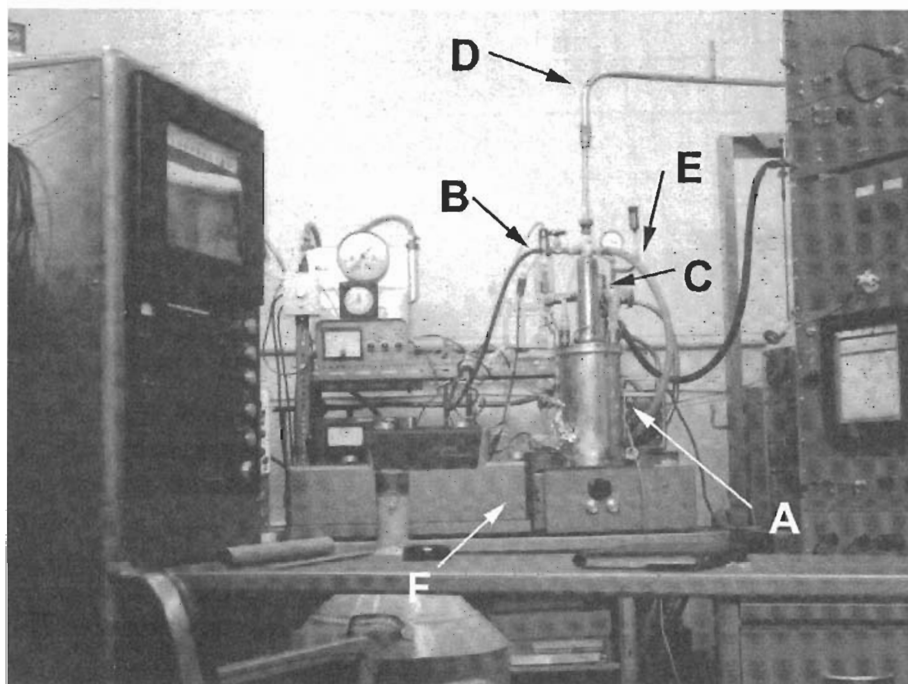

Fig. 2
The author's thesis apparatus. A, the outer cryostat; B, the vacuum gauge; C, the rotating inner cryostat; D, liquid hydrogen fill tube; E, hose to the window for liquid hydrogen boil-off; F, the Beckman DK-1's monochromator. Not visible, the hydrogen discharge lamp for irradiating samples.

4.2.3 First slip-ups, discoveries and encounters with "experts"

This led to the first important non-discovery of my career.I tried cleaning the windows, changing the vacuum pump oil, evaporating liquid hydrogen to see if there was an impurity that left a residue, everything I could think of; but the near-IR spectrum was always still there. Finally, I just concluded the spectrum was due to liquid hydrogen itself.

News of my finding excited quite a few faculty because it violated quantum theoretical spectroscopic "selection rules" pertaining to a symmetrical molecule like hydrogen: hydrogen shouldn't have an absorption spectrum in the near-IR. Willard Stout, a faculty member who was also editor of the *Journal of Chemical Physics* (a journal Urey had founded), told me that it could be "a name effect" (e.g., the "Glasel effect"; the first of many bearing my

name, I hoped, and in the same league with known ones such as the famous "Raman Effect"). I even figured out a simple theory of what was happening. Isolated hydrogen molecules shouldn't absorb IR because they have no permanent electrical dipole moments. It took me only a little thinking to see that in a liquid the molecules crash into one another frequently and create short-lived crushed molecular complexes that aren't at all symmetrical and *could* have IR spectra.

As I set out to write a short "Letter to the Editor" about this effect to the *Journal of Chemical Physics* at Stout's invitation, I came across a nasty surprise: there were several papers by a Canadian group that had recently found and reported on the same phenomenon. Alas, no Letter to the Editor, no named effect, and friends and faculty that had encouraged me about the work now said I should have looked for it in the literature *before* getting them excited. Dr. Urey shook his head and said it could happen to anyone.

I learned a lot from this affair. Perversely, I was happy with myself because I now knew I would *never* drop an observation into the mental trash bin just because I didn't understand it. It also gave me confidence that I would never miss something out of the ordinary in an experiment. Finally, I resolved to do literature searches before reporting something I thought was totally new.

I redesigned the window to be a single sheet of synthetic sapphire (which conducts heat better than copper at liquid hydrogen temperatures). When this was built, I began new condensation and irradiation experiments. Outside the problems with high vacuum leaks that gave me constant headaches, I found a new interpretational problem: as I deposited materials on the window, the recorded baseline as a function of wavelength went from flat, to one with big waves, to smaller waves, and finally flat again when there was a lot of material on the window (Fig. 3).

Because of the familiarity of wavy backgrounds to anyone with a passing knowledge of optics – and I had a good course in optics as an undergraduate – it didn't take me too long to figure out that I was observing interference patterns between light striking the vacuum-deposit interface and that striking the sapphire-deposit one. Using these interference patterns, I knew I had a way of measuring the thickness of the deposited layer. This was very exciting to me, because knowing the thickness of the deposited layer was necessary to calculate the concentration of any molecules in the layer and there was no other way I could figure out to know these concentrations.

This was my first true discovery, but alas it became a non-event.

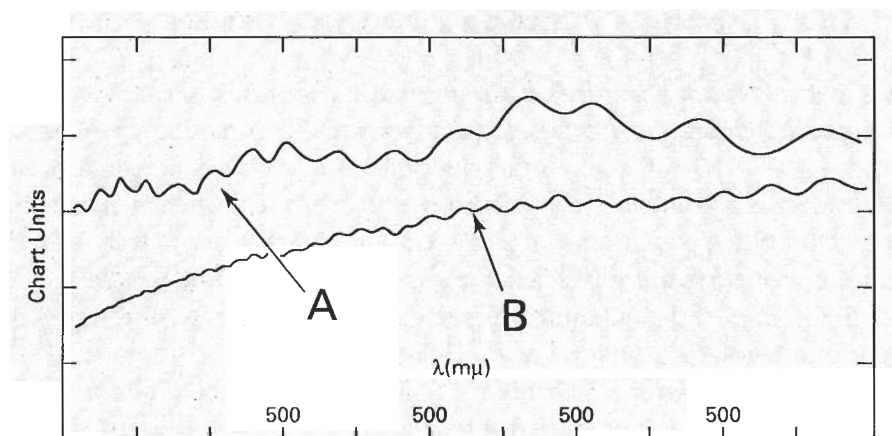

Fig. 3
Near-IR spectra of water deposited on a sapphire window at 20 K in high vacuum. A, 15′ deposition; B, 30′ deposition. Note that the waves become smaller in amplitude and closer together as the deposit becomes thicker.

This time I did do my homework: my method for determining the thickness of layers condensed from vacuum hadn't been reported yet. I submitted a quick paper to *The Review of Scientific Instruments* (where techniques of this type are best reported). It was very quickly rejected: the reviewers sternly offered their decision that the phenomenon of interference fringes had been known since Newton's time and therefore my technique was not new.

I then made a big mistake that I have never repeated in any walk of life: I believed the "experts"(in this case, my paper's scientific reviewers) and I did not fight back by rebutting the experts or arguing with the editor, or by submitting the paper to another journal. I just put the manuscript away and didn't look at it again because, at the time, it seemed like a repeat of the liquid hydrogen spectrum debacle. The problem was that the reviewers were correct in only a minor sense: interference fringes had been known since Newton's time. However, for them not to acknowledge that there were different uses for interference fringe effects, such as I had found, was like saying that the spectrum of colors from a prism had been known since Newton's time and therefore no part of spectroscopy was new.

A few years later someone else published the same use of interference fringes to measure concentrations of deposited reactants and it became

known as *their* method. I had been rabbit-punched by experts who were more opinionated than knowledgeable. Forever after, I have fought until I had run out of all recourse any opinion by an expert on any subject that is wrong in the light of my own knowledge. This includes scientific subjects, grant proposal study sections, as well as many aspects of daily living and artistic opinion. Since then, judging from the quality of oral or written reviews I've received in all aspects of my professional life, I think not more than 30% of scientific reviewers of journal, articles, or grant proposals are actually qualified by training and judgement to write intelligent reviews.

4.3 Urey leaves, Joe Mayer takes over

In 1958, without telling many people, including me, Harold Urey was recruited by the new University of California's branch in San Diego (now known as the University of California at San Diego, UCSD) and left the University of Chicago forthwith.

Searching for another faculty member who could take over my thesis research led me to Joseph Mayer (1904–1983). Joe was well known for his theoretical work in statistical mechanics and he and his wife Maria Goeppert Mayer (1906–1972), a nuclear physicist even more famous than Joe, were long-standing friends of Urey's.

I became part of Joe's small group – all theoreticians – and started attending the weekly lunches that the group had with him at local restaurants. Joe was the first scientific intellectual I had ever met (and one of the very few I have since met) and a very sophisticated thinker about many non-scientific things, including larger world issues. We became friends and the more I learned about him as a scientist and a man, the more I admired him.

4.4 Thesis and graduation

In late 1958, I told Joe that I thought I satisfied the requirements for a Ph.D. in chemical physics. Somewhat bemused, Joe said, "I still don't know what you've done, but OK". I hadn't solved the comet problem (to date, nobody has) but I had enough data to submit manuscripts for several papers on low-temperature spectroscopy [10–13].

My thesis project did not succeed in proving the Donn-Urey theory of cometary outbursts to be correct (I reviewed the whole problem at a later date [7]): the maximum concentration of free radicals I could achieve was way below what could lead to the observed energy yields in cometary explosions. I did manage to show experimentally a fact that was confirmed many years later for comets: at low temperatures, the NH radical was one of the most stable [13].

In my thesis research, I learned a good deal about spectroscopy at low temperatures and a lot of spectroscopic theory and experimental methods – almost all self-taught. The most satisfying paper to come out of this research concerned the stabilized nuclear spin states of protons in the hydrogen atoms of H_2O frozen in an argon matrix [10]. It was satisfying because it was a complete story and showed I had mastered basic spectroscopic technique and could deal with a problem that I had recognized and solved on my own.

As a result of my mainly solitary learning experience in graduate school, I left Chicago with a rock-solid conviction that has never left me: I believe there is no scientific experiment of any kind that I can't learn to do quickly and if there are meaningful results to be obtained in the experiment, I will obtain them.Urey insisted that I join him in California after getting my Ph.D. and with his help I was awarded a National Science Foundation Postdoctoral Fellowship in 1959 (at that time only 50 of these were given out yearly). I immediately traveled to the West Coast to begin work.

5 Postdoctoral work with Harold Urey: the new University of California at San Diego

The UCSD campus was so new that there was no room for my instruments (which UC had let me take with me), so Urey arranged for me to have a space in the nearby General Atomic Corp. research building.

I can't say that I had a happy time that year. The quality of the personnel in Urey's laboratory was nowhere near as good as at UC, I was isolated several miles away at General Atomic, the Air Force was trying to make me live up to my CalTech AFROTC obligation of 2 years active duty (which I was now trying to avoid as long as possible), and yes, the beach was distracting. Also, I knew that the comet problem wouldn't be solved without firing an instrument-bearing rocket right into a comet, taking spectroscopic and other data all the while, and that wasn't likely.

Since much of my later career involved me in computer technology and programming, it is significant that during my stay in La Jolla, I began learning FORTRAN II.

For reasons I can't explain I made a sudden decision to change my career and go into biochemistry. And not just biochemistry, but into applying the then new tool of nuclear magnetic resonance (NMR) spectroscopy to biochemistry. I suppose my decision was also based on what little I knew about the structures of molecules involved in biochemical reactions: the few structures known were almost always more complicated or "unusual" to me than the ones I had encountered in my training in chemistry. Since NMR was emerging as the tool for determining structures, I thought it could build a career pioneering the use of NMR in biochemistry.

With Urey's support, I received a second National Science Foundation Postdoctoral Fellowship to go to Great Britain to take up NMR.

5.1 If I can learn nuclear magnetic resonance spectroscopy I have a job at Columbia University

I obtained a position in Lloyd Jackman's laboratory in the Chemistry Department at the Imperial College of Science and Technology in London. I chose Jackman because he was young and becoming well known for his structural-organic chemical work using NMR.

While I waited to leave La Jolla, with propitious timing, Stanley Miller (by then on the UCSD faculty) introduced me to the visiting David Rittenberg (1906–1970), Chairman of the Department of Biochemistry at Columbia University's College of Physicians and Surgeons in New York. Rittenberg had been one of Urey's first graduate students at Columbia in the 1930s.

After he got his Ph.D. with Urey in Chicago, Stanley had taken an Assistant Professorial position in Rittenberg's Department, leaving there after a few years to go to La Jolla. I have always assumed that Stanley had spoken to him beforehand, because Rittenberg offered me a job as an Assistant Professor in his Department almost immediately. When informed that I had an active duty commitment to the Air Force, Rittenberg replied that he would wait the two years. Our agreement was that I would set up a laboratory at Columbia to do NMR spectroscopy applied to biochemical problems. It didn't seem to

bother Rittenberg that I had only seen an NMR spectrometer once, and knew nothing about biochemistry.

Although I was still too much into the world of physical sciences to appreciate the honor, Columbia's Biochemistry Department was then one of the most prestigious in the United States. The Department's faculty contained such notables (besides Rittenberg) as Erwin Chargaff (1905–), David Shemin (1911–1991) and David Nachmansohn (1899–1983), while elsewhere at the College of Physicians and Surgeons (P&S) were scientists such as Elvin Kabat (1915–2000). I also didn't know that it had been a long time since there was any turnover in the Department. Stanley Miller had been the last appointment before me.

6 Postdoctoral research in London learning NMR

Fifteen years after the end of WWII, England was still showing the deprivations it had suffered. There were still bomb craters visible in the City of London and coffee was still rationed. After Southern California, London was cold, dark and smelled of coal smoke, and I loved it immediately (and have never stopped).

6.1 Department of Chemistry, Imperial College

I duly reported to Imperial College (IC). It was then a great Victorian pile as far as architecture was concerned, but with a scientific reputation in America as the "MIT" of Great Britain. In fact, I had come at a time of great change in the faculty at IC: Patrick Blackett (1897–1974), Derek Barton (1918–1998) and Ernst Chain (1906–1979) had all recently joined the Departments of Physics, Chemistry, and Biochemistry respectively, making them certainly the equal of any such departments in the world.

6.1.1 Making a MUDDLE

A brilliant spectroscopist, David W. Turner was also on the Chemistry Department faculty doing NMR research, and Lloyd suggested I work closely with

him. Previous to my arrival, David had come up with a typical (for him) astute observation: the resonant frequency ratios of some nuclei (in particular, ^{1}H:^{14}N) were very nearly a rational fraction (in this case 13/180).

In those days magnetic field drifts, along with radio frequency drifts, made it difficult to accurately irradiate two nuclei simultaneously (desirable in some important experiments such as spin decoupling). David saw that if the oscillator supplying the resonant radio frequency for ^{1}H were locked to that of ^{14}N by radio frequency division by 180 followed by multiplication by 13, the job of getting the exact resonant frequency for ^{14}N could be transferred to a low-frequency oscillator (much more stable than a radio frequency oscillator). Then the two nuclei could be irradiated simultaneously, regardless of magnetic field drifts, because their resonant frequencies would be accurately locked together. David, with Lloyd's agreement, suggested I make it my job to make this idea practical.

I did two things. First, I purchased Abragam's just-published classic monograph on NMR spectroscopy [14]. Reason: I knew nothing about theoretical or practical NMR. Second, I started researching in the IC library for circuitry for dividing and multiplying radio frequencies, also something I knew nothing about. This was still the vacuum tube era in Great Britain and it was still the postwar era of frugality as far as funding for academic science was concerned (for example, IC's new Varian NMR spectrometer was shut off each evening to conserve electricity; it then required hours of wasted time in the morning to regain magnetic field homogeneity adequate for doing research). While I was able to convince the Prof that we were worthy of an expenditure (not small, given our budget) for an oscilloscope, it fell to me to get most of the electronic components from war surplus stores (then mostly located in the red-light section of Soho).

I was quickly successful in designing and building this radio frequency divider/multiplier (I believe the original is the property of London's Science Museum now; at least I saw it on display there in recent years.) Acronyms began being popular then, so in honor what I perceived to be the English attitude, I promptly named our device MUDDLE (*M*odulated *U*nclear *D*ecoupler and *D*amn *L*ittle *E*lse) and so labeled its front panel in bold lettering. We published a paper describing MUDDLE [15], but never even thought to patent the method. Several years later I was told that the method became the basis of Varian's "gyrospin" feature on their next-generation NMR instruments.

But world events finally overtook my life and four years were to pass before I published another paper in the physical sciences.

6.1.2 The Berlin Wall affects my life

During the night of August 12–13, 1961, the Berlin Wall went up and President Kennedy was faced with how to react to this provocation. As we know, despite his calling for a full war alert for the American military, no shots were fired then and the Wall became a reality for almost the next 30 years.

As a direct consequence of the war alert, six months after the Wall was built, I was called to active duty in the USAF. The reason was that "chemical physics" was not an Air Force personnel qualification descriptor so when I provided information to the USAF personnel office upon my graduation from UC, the nearest thing that seemed to apply was "nuclear chemistry", which seemed near enough to the stable isotopic chemistry most of Urey's laboratory was doing. However, the Air Force now suddenly found a need for nuclear chemists and, on paper, my qualifications fit and I was called to active duty.

I knew nothing about nuclear chemistry – the work I was about to engage in. I found out later that the outfit I was to be assigned to had detected an increase in Russian plutonium production at about the time of the Wall's construction and needed trained nuclear chemists to beef up their analytical capabilities: hence the Air Force's sudden need for me.

My orders sent me to Sacramento, California and my assignment as a First Lieutenant in the "1155th Technical Operations Squadron" of the "1066 Field Activities Group". These were innocuous cover names to shield the mission of the units.

7 A nuclear research officer, United States Air Force

The 1066th bore the responsibility in the United States for detecting and analyzing nuclear explosions – specifically above-ground Russian nuclear tests at that time – for yield, weapon design, etc., along with foreign fissionable materials production. Both testing and production were monitored largely *via* radiochemical and physical examination of airborne debris gathered by U-2 planes assigned to the Group.

7.1 Military science and life

On the basis of my smattering of Fortran II training during my La Jolla postdoctoral appointment, my first non-chemical assignment came when I was instructed to "learn to program" a new IBM 1620/1400 computer combination that arrived in Sacramento one day. Such was the hurry, I taught myself this higher-level programming language without disciplining myself to write flow diagrams for my programs, an unfortunate practice I have never been able to shed. But I got the job done and within a few weeks we could use the computer to help physically reconstruct a weapon from the sets of data available.

I learned a lot, both scientific and personal, during my active duty in the military. After my first year, I was put in charge of a small group whose responsibility was setting up and running a electron microprobe analyzer (a then newly developed apparatus for using secondary electron emissions to do elemental analysis of solid specimens – such as bomb debris). This technique was déjà vu insofar as it involved wrestling with vacuum systems but it also taught me how to teach non-commissioned officers and enlisted men with no scientific training how to do experimental physics. Also, during my off-duty time in the Air Force, I moonlighted by teaching undergraduate "physics for non-physics majors" at Sacramento State College and quantum chemistry at the graduate level for the University of California Extension Program.

I was scheduled to be discharged from active duty in the USAF on February 16, 1964. One day while I waited, I was called into the squadron commander's office and asked if I would accept a regular commission in the Air Force! To be offered this is a singular honor for any reservist in the U.S. military. Even though I was anxious to get out of uniform and back to big-league science and my own ideas for research, I hesitated. Although I finally turned it down, I've often wondered in years since whether or not I made a good decision. I haven't had many honors in my life, but this offer of a commission was certainly a sterling one, and I turned it down. I was discharged from the Air Force, resigned my reserve commission, and traveled to New York to begin my career as an academic scientist.

8 Back to academic life as a biochemist

8.1 New in biochemistry and NMR

While I was still on active duty, I had written my first grant proposal – to set up a laboratory at Columbia equipped with the best NMR instrumentation then available (the 0.141T electromagnet Varian DA-60EL proton high resolution and multi-nuclear wideband instrument), with Rittenberg as a co-investigator. We got the grant, and using my design, a temperature- and humidity-controlled room was built and the instrumentation installed within about six months after I arrived after my discharge.

After the instrument was running satisfactorily, I started an ambitious program to apply proton and multinuclear NMR to problems I thought might be of biochemical interest. My subsequent NMR work at Columbia fell into three categories: the involvement of water structure in biochemical interactions, biomolecular structures (in particular bioactive peptides), and instrumental developments.

8.1.1 Research on water and biomolecular interactions *via* NMR

My work on water structure involved the then new approach of studying the rotational motions of water molecules *via* the NMR relaxation properties of water labeled with the stable isotopes ^{2}H and ^{17}O. My decision to work on this project stemmed from my knowledge of what was happening in chemical physics as well as biochemical research.

The structure of liquids was a hot theoretical and experimental topic in chemical physics at that time and some of this focus was applied to biochemistry. In particular, both Pauling and Stanley Miller had recently published papers hypothesizing that water structure played a role in general anaesthesia [16, 17]. Their basic idea was that anaesthetics altered the structure of water around neurons so as to inhibit transmission of impulses. Furthermore, their arguments implicated anaesthetics that promoted formation of hydrates. The rare gas xenon, a good anaesthetic, is an example of a chemically unreactive species that forms hydrates.

I decided to see if NMR could be applied to the problem of how water molecules interacted with biological structures.

Rittenberg had close connections with many scientists in Israel (he had helped set up the Biochemistry Department in the Weitzmann Institute after the war). He arranged for Yeda Research and Development Co. in Rehovoth, Israel, to present me with a gift of a few milliliters of 10% enriched $H_2{}^{17}O$ – at that time worth thousands of dollars.

I wanted to see whether or not xenon in solution altered water structure as indicated by changes in water molecules' rotational reorientation times – quantities that I knew could be derived from ^{17}O NMR spin-lattice relaxation times.

In thick-bore NMR tubes, I made stoichiometric xenon-$H_2{}^{17}O$ mixtures that formed a waxy solid hydrate below 27 °C and melted to a clear solution above this temperature. The paper that appeared describing this work [18] contained all the equations necessary to interpret ^{17}O relaxation experiments and to quote its conclusion, "in essence, the xenon lowers the effective temperature of the water." I'm not aware of any papers following up this phenomenological explanation for xenon's anaesthetic properties but I followed up with another paper giving a theoretical analysis of the observed relaxation parameters for both $H_2{}^{17}O$ and ^2H_2O [19]. Using $H_2{}^{17}O$ I was also able to report (prior to many subsequent X-ray crystallographic studies showing the same thing) that proteins contained patches of relatively immobile water [20]. I confirmed this observation using ^2H_2O [21].

Even though no competitors were working with $H_2{}^{17}O$, I decided that using it was too expensive and time-consuming for one person to perform numerous measurements with because it needed to be re-purified carefully after work with each solute was completed. I therefore turned to measurements of biomolecular motions in solution using much less expensive ^2H_2O and ^2H-labeled compounds. To do the labeling work, I had to redevelop a proficiency in simple organic synthesis. Fortunately, I enjoyed doing syntheses as a respite from instrumental and theoretical NMR work and was grateful for the thorough training in organic chemistry that came from working with Jack Roberts as an undergraduate.

My Columbia work with ^2H_2O and ^2H-labeled compounds resulted in a series of papers [22–25] dealing with: a correction to the commonly assumed microviscosity value necessary to describe water molecule rotation [26], the interactions of water molecules with biopolymeric conformation [23], the roles of water-solute interactions in denaturing agent and surfactant actions [24], the motions of nucleotide bases in polynucleotides [22], and NMR relaxation times for water in highly heterogeneous systems [25].

In retrospect, the conclusions of the last two papers [22, 25] were somewhat ahead of their time. Working with deuteron-labeled polynucleotides, I showed for the first time that the librational motions of the bases are far more rapid than had previously been assumed. As I stated, "studies of this nature should reveal a large amount of quantitative data on the microscopic motions within macromolecules". Subsequently, much work along these lines has been reported.

The work on heterogenous systems contrasted ^1H and ^2H relaxation in ^1H_2O and ^2H_2O, respectively. The NMR tubes were packed with uniform-diameter glass beads from 0.2 cm down to 0.0015 M in diameter. I showed that as the surface-to-volume ratio increased (the system becoming more heterogeneous), there were very large effects on the water ^1H spin-lattice and spin-spin relaxation times (T_1 and T_2). This paper was followed up by a more quantitative treatment (see below) and I have been told that these papers were later referred to frequently during the development of magnetic resonance imaging (MRI), a technique that was then just beginning its development and which depends partly on the relaxation properties of molecules in a heterogenous system.

8.1.2 Developing instrumentation: the era of minicomputers arrives

Three largely instrumental development papers were part of my Columbia work. Two of them, a "poor man's" technique for measuring spin-lattice relaxation times [27] and a method of improving the DA-60 spectrometer [28], rapidly became obsolete as NMR spectrometers improved. However, a notable instrumentation improvement, done with a Yugoslav postdoctoral worker (the only postdoc I had during my Columbia years) in consultation with workers at Brookhaven [29], was much more interesting: we designed and made a device for the digital production of radio frequency (rf) phase shifts in the rf pulses used for NMR relaxation measurements. It was my first venture into digital technology and the paper is interesting on two counts: (1) it describes our use of some of the first commercially available integrated circuits in combination with the new Digital Equipment Corp. PDP-8/I minicomputer I had been able to obtain; (2) it also describes one of the first uses of computer-generated gated rf pulses for use in NMR – now ubiquitous on all commercial NMR instruments.

8.2 Biomolecular structural work

My first two papers [30, 31] on biomolecular structure (using high-resolution 60 MHz proton NMR) reflect my early belief that three-dimensional conformations of biologically interesting molecules could be approached by NMR methods. Both these papers drew some simple conclusions concerning intramolecular and H-bond stabilization of the molecules involved (biotin and cysteines) and discussed how these might be involved in their function. This NMR approach was new to biochemistry, and both papers continue to be cited sporadically in the literature.

8.2.1 Peptide hormones

These early biomolecular structure papers involved analyzing the NMR spectra of "off-the-shelf" chemicals using standard analytical methods. I saw a dead-end in doing routine work of this sort: work that anyone with an NMR instrument could do. Fortunately, in the late 1960s I began a collaboration with Frank Bovey's laboratory at Bell Telephone Laboratories (specifically with a member of Frank's laboratory, Anne Brewster) and Victor Hruby from the University of Arizona. The focus of our attention was the conformation of oxytocin in solution as determined by NMR using vicinal coupling constants and amide proton exchange rates. At this stage in the development of applications of NMR to bioactive peptides, one often needed a proprietary source of hard-to-get peptides. In this case Victor, who had obtained his Ph.D. under Vincent du Vigneaud (1901–1978), was the source of NMR quantities of oxytocin and some of its derivatives.

The study of peptide conformations *via* NMR was stimulated by a paper [32] from Lyman Craig's laboratory at the Rockefeller Institute (now University) analyzing the conformation of a gramicidin using one of the few superconducting NMR spectrometers available at the time: the 5.2 T instrument at Bell Laboratories. Shortly after this paper was published, another 5.2 T instrument was installed at Rockefeller and both instruments became available to outside investigators.

Although not apparent at the time, our results [33] illustrated a strength and some weaknesses of NMR structural determinations – characteristics that in my opinion persist to the present day. Oxytocin is a biologically impor-

tant cyclic polypeptide with a prolyl-leucyl-glycinamide "tail" attached to the ring at a cystinyl residue. Several groups were competing to determine the solution conformation of this peptide.

Our contribution was to show that there were two conformations for the hormone: two almost equally populated *cis* and *trans* conformers about the Cysteine-Proline bond. The conformers interchanged very slowly (theoretically the energy barrier interchange was on the order of 20 kcal/mole). The strength of NMR was to show the existence of this isomerization clearly and to explain why X-ray diffraction studies on the same peptide were difficult. The weaknesses of NMR structural methods that this [34, 35] and other groups' studies on peptide hormones showed were that although they *suggested* possible structure-function relationships, they didn't indicate how to experimentally confirm the suggestions.

It was perhaps around this time that I began to have doubts about the utility of the reductionist approach of relating biomolecular structure to solving actual biological problems. If so, I ignored the doubts for another 15 years.

In technology, a quieter revolution was going on: it was the advent of minicomputers employing the first integrated circuits. As mentioned above, I was able to buy one of the first Digital Equipment Corporation PDP-8/Is to come off the production line. The reason I wanted the computer was that it had been clear from my initial research ideas about using NMR in biochemical studies that many materials of interest were only available in small quantities or were insoluble in aqueous media, so signal-to-noise averaging was going to be a vital point.

8.2.2 Fourier-transform spectroscopy arrives: homemade

In the mid 1960s the advent of the Cooley-Tukey algorithm [36] for doing fast Fourier transforms (FFTs) with a computer changed the whole picture of doing NMR and in particular how signal-to-noise averaging would be done in the future. Using the PDP-8/I, we set about using it for relaxation measurements with a digital pulsed NMR modification we designed [29] and for programming the computer to do FFTs. The system worked well, but with only 16 kilobytes of computer memory, it was good only for single resonance relaxation studies and wide-line signal-to-noise averaging (Fig. 4).

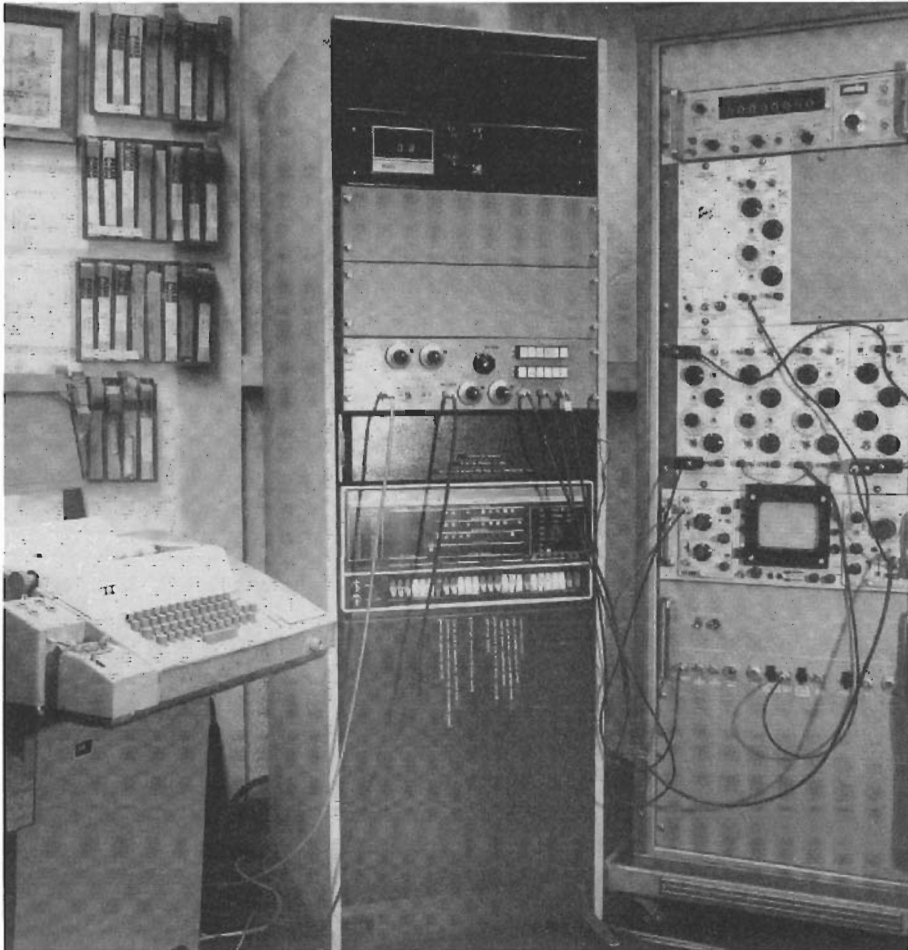

Fig. 4
Our homemade FFT spectrometer circa 1969. Note the punched tape programs to be input *via* the ASR-33 teletype unit. The PDP-8/I used octal arithmetic and the boot program had to be entered by hand using switches.

8.2.3 David Rittenberg

David Rittenberg died of another heart attack at the age of 64 on January 24, 1970. With him gone, I began searching for a job with another biochemistry department. Within a couple of months I had obtained a faculty job as an

associate professor in the Biochemistry Department at the first State Medical-Dental School in Connecticut then being constructed (The University of Connecticut Health Center, UCHC). By agreement with the administration at UCHC, I would take a sabbatical in England to allow time for a temporary building to be built at the Connecticut campus to house the NMR equipment I had been given permission to move out of P&S.

9 Sabbatical at the University of Oxford

I had obtained a place in Robert J.P. Williams's laboratory in the Inorganic Chemistry Laboratory at Oxford. Bob Williams was an expert on metalloenzymes and had published extensively on his imaginative theory of enzyme catalysis. He was also a member of the Oxford Enzyme Group that was very active in structural biochemistry.

The last entry in my laboratory notebook at Columbia was July 26, 1970. It concerned spin-lattice relaxation times for polymers [23]. The first notebook entry at Oxford on September 9, 1970, described measurements using the only NMR instrument conveniently available to Williams at the time, a tiny Varian A-60 spectrometer. The experiments described used the lanthanide ion Eu (III) as a chemical shift perturbation agent for the NMR spectrum of ribose-5-phosphate. These measurements, which continued for a week or so, introduced a new method for NMR structural determination.

9.1 Lanthanide ion chemistry

Lanthanide ions have ionic radii that are roughly the same size as many metal ions found in native metalloenzymes. They also have electron paramagnetic properties that are completely different from transition metal ions. Not only do their magnetic moments vary from zero for La (III) and Lu (III), at the beginning and end of the rare-earth element series in the periodic chart (with high values in between), but their electronic states vary so that members of the series, such as Eu(III) and Gd(III), with large paramagnetic moments, affect NMR signals in different ways. For example, Eu(III) causes electron paramagnetic-induced NMR chemical shift perturbations without line broadening in molecules where the lanthanide comes into close proximity to the

nuclei whose resonances are being measured. On the other hand, Gd(III) causes NMR line broadening but no chemical shifts situated in the same place in the same molecule.

Bob's idea was that these lanthanide ion properties could be used somehow to determine rigid active site structures of metalloenzymes. The idea followed from the recent widespread adoption of lanthanide-containing organic molecules as "shift reagents" to reduce the complexity of second-order NMR spectra. Upon my arrival, Bob Williams suggested that I work with a Ph.D. student of his, Antonio Xavier, on using lanthanide ions in the metalloenzyme work already in progress.

Other than an introduction in an undergraduate course in inorganic chemistry, I had no prior knowledge of the chemistry of the lanthanide ions. However, I did know that one of the current NMR problems being worked on was the functional requirement nucleotides and oligonucleotides had for Mg(II). The structural problem to be solved was what were the conformations of these flexible molecules *in solution* when they interacted with the metal ion. So far NMR work by several other groups had been done using Mn(II) (a transition metal ion with a large electron paramagnetic moment and an ionic radius similar to Mg(II)) as an NMR line-broadening probe. Their published results had so far been very equivocal.

9.1.1 Determining biomolecular structures using NMR and lanthanide ions

My contribution to the ensuing work was simply to suggest that, instead of working on lanthanide-substituted metalloenzymes, we should concentrate on using the lanthanides – which had about the same ionic radii as Mg(II) and Mn(II) – to look at the conformations of flexible nucleotides. Specifically, I suggested that we start out using the shift-inducing properties of Eu(III) on the simple nucleotide, adenosine monophosphate (AMP). It seemed an easy enough thing to do on an A-60 (which anyhow was completely inadequate for any enzyme work) and the reagents were readily available.

The basic experiments consisted of measuring the chemical shifts of the various resonant protons (later ^{13}C was added) as a function of Eu(III) concentrations and forming shift ratios from the various frequency-shifted res-

onances. Within two weeks after beginning these experiments we had acquired enough data, followed by theoretical analysis, to make it evident that we could obtain conformational information from this type of measurement. We also knew that the derived ion-proton distances were quite accurate, because theory told us that the shift ratios vary as the inverse cubes of the distance between the nuclei and the metal ion.

The method we developed is formally analogous to the present NMR method of obtaining structures of flexible molecules using the nuclear Overhauser effect (NOE). It was clear to us at this time (10–15 years before the start of widespread reporting of NMR-derived structures) that with a limited number of shift ratios there were going to be, in general, many conformations (a "family" of conformations) that differed but which fit the ratios within experimental error. The lanthanide ion structural method we developed has been reviewed [37].

To make the method practical, we needed a way to delineate the families of structures that fit the available data (in the form of shift ratios) for each molecule we worked on.

9.1.2 Families of structures and computer data fitting

A few minutes toying with measuring interatomic distances on a Dreiding model of AMP and calculating shift ratios by hand convinced me that this was no way to do the job – automation was needed. This was long before the day when computer molecular modeling was routine and available to anyone with a minimal desktop computer. Computer molecular modeling was not unknown at Oxford; it was just unknown at the Inorganic Chemistry Laboratory where Bob's laboratory was located. In fact, there was a very good modeling programmer who was part of the Oxford Enzyme Group, C. David Barry.

Working with David Barry was a great pleasure throughout my stay at Oxford. Although not a "wet" chemist or an NMR spectroscopist, he immediately realized the problem we were trying to solve. In effect, as we developed our method, Dave acted as a daily language translator (he spoke computer language), taking our equations and structures and translating them into software that searched out structures that fitted the data. This was accomplished by algorithms that wiggled the molecule (his program was

eventually called "Burlesque") through conformation space to find conformational families that fit the data.

9.2 Putting it all together: our first lanthanide-derived structural family

The family we found for AMP, shown in Figure 5, differed from the conformation of AMP in crystals. Our first paper on this method was written in early December 1970, a little over two months after I arrived at Oxford [38]. This was the first paper in a series that extended the method to other molecules, nuclei and solvent systems [39–42].[2]

The least noticed, but to me one of the most important, of these lanthanide papers was one showing that conformations of AMP differed greatly between dimethylsulfoxide and aqueous solutions [41]. This work planted a seed in my mind that conformations of flexible molecules in solution possibly had little relevance to their biologically active conformations: I reasoned that since biomolecules function in so many different environments, if just changing the solvent changes a molecule's conformation, how can we have confidence that any solution conformation predicts the biologically active one?

Despite the development of this new structural method, subsequent advances in determining structures by NMR without perturbation probes that took place several years later seemed to attract more attention around the world. At any rate, very little subsequent lanthanide-perturbation work by any laboratory has been reported, even though it still would be potentially very useful as an adjunct to direct methods to increase conformational accuracy.

10 Connecticut years – Part I, 1970–1980

Shortly after taking up my job at UCHC in 1971, I took out of storage the NMR spectrometer and other equipment I had moved from Columbia. These instruments enabled measurements of proton high-resolution spectra, spin-lattice and spin-spin relaxation times at 60 MHz and broadline and Fourier transform deuteron spectroscopy.

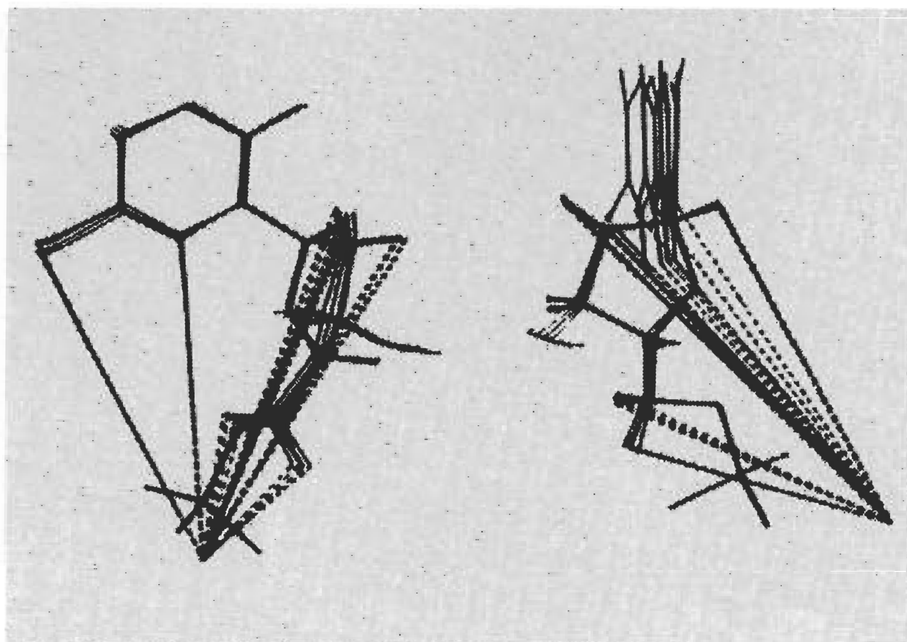

Fig. 5
Orthogonal views of the family of solution conformations for AMP from [38]. Dotted lines indicate metal-ion proton distances for the family that are consistent with the NMR data.

10.1 Water relaxation in heterogeneous systems – pre-magnetic resonance imaging

The first project I started in Connecticut was an extension of the work on water structure begun at Columbia. With a postdoctoral student, Ku Ho Lee, I looked in detail at the spin-lattice relaxation times of deuterons in a series of ^2H_2O-glass microbead systems. The purpose of this work was as we stated in the beginning sentence of the paper that resulted [43], "In spite of its importance, the state of water at foreign surfaces is not well understood". We succeeded in quantitatively analyzing the results in terms of magnetic field inhomogeneity effects at the surfaces of the beads resulting from magnetic susceptibility differences between the beads and bulk water.

While not of seemingly great importance at the time, in the next decade scientists interested in interpreting the roles of spin-lattice relaxation in

obtaining good clinical MRIs began to refer to the paper frequently because the same phenomenon is operative in tissues within the body.

10.2 Peptide hormones redux: encountering a central problem in structural drug research

At the same time the new water experiments were going on, I continued the collaboration I had begun with Victor Hruby and Anne Brewster on the conformations of oxytocin and its derivatives. This work was to carry me deeper and deeper into trying to solve a central problem that still exists at the heart of drug research: how do drugs structurally interact with macromolecules? Later this interest would take the form of trying to understand how drugs interacted with membrane-bound receptors, but 30 years ago structural concepts of drug receptors were in their infancy and I took a simplistic approach.

Oxytocin is found in concentrations on the order of 0.03 M within neurosecretory granules, along with similar concentrations of proteins of low molecular weight (approximately 10 kDa) called neurophysins. The role of the neurophysins was unknown but many workers thought that their purpose was to protect oxytocin from enzymatic degradation by binding to the peptide. Fortuitously, we also began a collaboration with Jeffrey McKelvy who was in the Anatomy Department at UCHC, familiar with neurophysin protein chemistry, and interested in NMR techniques.

Since I had one of the few instruments available to do NMR experiments on deuteron-labeled compounds, I proposed that we look at changes in overall rotational and intramolecular motions in deuterated oxytocin and derivatives as they interacted in aqueous solutions with purified neurophysin II.

Our first reported results were on the microdynamical molecular motions of five deuterated derivatives of oxytocin prepared by Victor's laboratory [44]. These experiments showed directly that the "conformation" for flexible molecules can only be regarded as a time-averaged one, whose applicability to biological processes depends on whether or not the motions take place on the same time scale as the physical process being studied. The paper ended with the sentence that summed up our main point: "We believe that these measurements represent the first time that important intramolecular motion has been directly demonstrated in an active hormone." Next, we went on to detailed experiments on the interaction of deuterated oxytocin and deriva-

tives with neurophysin II [45]. This work showed that the Pro-Leu-Gly-NH_2 "tail" of oxytocin was not involved in its interaction with neurophysin II and that there was a rapid exchange between free oxytocin and its neurophysin II bound form.

10.2.1 A regional NMR facility

In 1973 my proposal to the NIH to establish The Southern New England High Resolution NMR Facility was funded for a duration of five years with me as Principal Investigator.

I immediately purchased a 2.35T, JEOL PFT-100 high-resolution Fourier transform spectrometer that incorporated a Texas Instruments 980A computer and had both ^{1}H and ^{13}C probes. To staff the laboratory, I was fortunate to attract Hermann Bleich, a physicist-by-training and NMR-experienced scientist, to run the facility. John Cutnell (on sabbatical leave from the physics faculty at Southern Illinois University) soon joined the laboratory along with Christine Regula as a technician. These five years saw a multiplicity of projects started and my interests switch over rapidly to "wet" biochemistry as I saw our NMR efforts go further and further away from engaging in biological (as opposed to physical biochemical) problems.

In the laboratory during those five years, Hermann, John, Christine and I represented, in our varying interlocking skills, what was to be a dying generation of scientists, "all-arounders". Hermann could switch from designing and building advanced electronic circuits, to NMR theory, then to NMR experiments and back with no effort at all. John could equal Hermann in NMR theory and experiments, along with the ability to do machine and higher-level computer language programming. Christine had the know-how to take a biochemical project that I wanted to carry out from the general idea to performance almost without my help.

In a 1973 talk I gave at the NIH as part of a symposium on recent research methods, I'm pleased that I could see clearly what developments were coming several years later. While Intel had introduced the world's first microprocessor in 1971, the first commercial one, the 8-bit 8080, wasn't available until 1974. In my talk I told an audience of biochemists that "anyone with even a little knowledge of computer technology realizes that inexpensive, advanced microprocessors are very near... one can easily foresee the day

when the computer part of the spectrometer will be completely integrated into the experimental part." This came true several years later.

The different research paths I set out on are better explained by category than chronology because most of the time they were all going on in parallel.

10.3 Research developments, 1970–1980

10.3.1 Instrumentation

The PFT-100 was already obsolete when we purchased it because it didn't have quadrature detection (i.e., two-phase sensitive detectors) soon to be used on all commercial spectrometers. Using a design suggested by Alfred Redfield (Hermann Bleich's thesis advisor), Hermann quickly built us a quadrature detector. According to a basic design I suggested, Herman also built water resonance suppression circuitry that worked with an FT spectrometer by selectively saturating the water resonance line before applying the non-selective 90° pulse [46]. During the same period John Cutnell had an idea about how to eliminate annoying phase shifts in FT spin-lattice relaxation measurements that were caused by the invariably imperfect rf pulses that must be used in FT spectroscopy which Herman also built: it proved to be one of the first group of devices built by spectroscopists of that era to use phase alternation to eliminate instrumental artifacts [47].

Later on, when we got interested in rotating-frame spin-lattice relaxation as a way of estimating intramolecular motions in flexible molecules, Herman designed and built a simple modification [48] that allowed these experiments to be done without modifying the software for our spectrometer. This instrumental work was not terrifically innovative, but was in advance of what was then commercially available and so allowed us to do many experiments that only a few other laboratories could do.

10.3.2 Starting to branch out from NMR

As I started becoming a wet protein biochemist, I was able to purchase newly developed equipment: a high-pressure liquid chromatograph (HPLC), the

first one at UCHC. While we were using the instrument routinely to characterize peptides using reverse-phase columns, I started wondering about the validity of the then received truth that proteins could only be separated using open column size-exclusion or ion-exchange chromatography. Using the neurophysins as examples, I quickly showed that at least proteins of this molecular mass (about 10 kDa) could be separated by reverse-phase HPLC. I believe the resulting paper [49] was the first report of what has become a standard method of protein separation. The manuscript was originally turned down when a referee stated that "this would never be a practical method of protein separation", thus keeping my opinion of "experts" alive.

10.3.3 NMR and bioactive peptides

By far the major projects we worked on in the period 1973-79 were directed toward learning how NMR relaxation times could contribute to determination of bioactive peptide structures and how these structures interacted with the macromolecules they bound to. We started this work on peptides like oxytocin, vasopressin and tetragastrin, and later branched out to enkephalins. These peptides seemed an ideal way to get at what I believed (and still do) was a major scientific problem to be solved: structurally, wherein lies specificity of binding of bioactive substances to their receptors?

We were doing this work at a time when the possible use of relaxation times and the NOE in determining peptide structures was under increasing investigation by several groups throughout the world.

Our projects differed from other groups insofar as we were focused largely on detailed theoretical analyses of the effects of intramolecular motions on measured relaxation times and NOEs [50–58].

This series of papers established to our satisfaction that in the absence of intramolecular motions in peptides, relaxation times and NOEs could be used to extract main and side-chain torsion angular information. However, we also showed that accurate information of this kind depended upon using dipolar relaxation formalism and that it was deficient where there are intramolecular motions. Since our work showed that *intra*molecular motions *were* important in the peptides we were looking at, the conclusion dawned on me slowly that NMR solution structures of flexible molecules weren't going to contribute much to solving the problem of where their specificity lay.

10.3.4 Beginnings of opiate work

After the existence and identification of the opiate peptides were established in 1975, we were one of three groups to report the first NMR work on one of these, methionine-enkephalin (mENK) [59]. It was satisfying at the time that one of the other contestants made incorrect resonance assignments and the other published their resulting structure in mirror-image stereochemistry.

In subsequent work, we reported on intramolecular motions in mENK, the differences in conformation of mENK in dimethylsulfoxide and water, and the lack of direct interaction of mENK with phospholipid membranes [58, 60, 61] using more and more sophisticated NMR techniques. We also found that lanthanide ions catalyze amide proton exchange in aqueous solutions of peptides [62], an observation we never followed up.

I thought it was not a very satisfactory initial venture into drug-related research: we worked hard but the results showed to my satisfaction that solution conformations of small peptides had *no* implications for their bound conformations on macromolecules, so this body of work actually was another step in my disillusionment with NMR structures of peptides.

However, work which did have important implications for structural determinations of larger, and less flexible, molecules *did* come out of these projects.

10.3.5 Determination of structural families of flexible molecules from spectral density functions

To aid in interpreting the NMR relaxation measurements in structural terms, I began developing a Fortran II source program that included many machine language subroutines for displaying large molecules, determining internuclear distances in them, and printing out ball-and-stick, wire and solid-sphere molecular models of their structures. Most of this was done by a college student, Leonard Spain, under my direction and was done long before computer modeling programs were available for purchase and execution on mini-computers. Our program was in parts called "PEPTID, WOBBLE and WOESS" (Fig. 6), also allowed input of NMR relaxation data and contained some linear programming features that preceded other similar programs by many years.

Since the laboratory was concentrating heavily on the interpretation of ^{1}H and ^{13}C spin-lattice relaxation times and NOEs in peptides, the basic idea of these computer programs was that we could combine these measured NMR parameters with van der Waals filters and vicinal coupling constants to determine self-consistent structures. This idea was already familiar because it was completely analogous to the work that came out of my stay at Oxford 8 years earlier. Just the equations were different.

Jim Visintainer, a new postdoctoral worker, started in on this project along with myself and our programmer. We called the method that developed the SDFM (for spectral density function method) approach to peptide structural analysis. Using one-dimensional NMR, the paper that resulted [63] anticipated the principles and problems of structural determination *via* two- and three-dimensional NMR now in common use.

In the paper, we introduced an NMR "R" value, in analogy to X-ray diffraction models, to estimate the goodness-of-fit of our structures to the NMR data – something later workers in this field didn't get around to doing until the mid-1980s. As we pointed out, the basic problems in determining structures from NMR relaxation or NOE data are the same: determining the spectral density functions $J(h4_i)$; it doesn't make any difference if these appear as a sum (as in spin-lattice relaxation) or a division of sums (as in NOEs) in the equations relating the observables (T_1s or NOEs) to interproton distances. Historically, NMR structure determinations have taken a different course, but I think we were at least among the first groups to put the principles on a firm theoretical and experimental foundation.

Despite our solid NMR work on peptides, it was all getting very boring to me because it seemed farther and farther away from attacking real biological problems. So, with this becoming more important to me, I consciously began teaching myself "wet" biochemistry. The starting point was learning protein chemistry.

When Jeff McKelvy moved away from UCHC in the mid-1970s, he left me with a large quantity of acetone-dried bovine pituitary powder – a rich source of hypophyseal hormones and neurophysins. I decided to teach myself how to isolate and purify neurophysins from this source. Because of some confusions in the literature about the properties of these proteins, it was of interest at the time to characterize them and their interactions with oxytocin at the high concentrations at which they were present in neurosecretory granules.

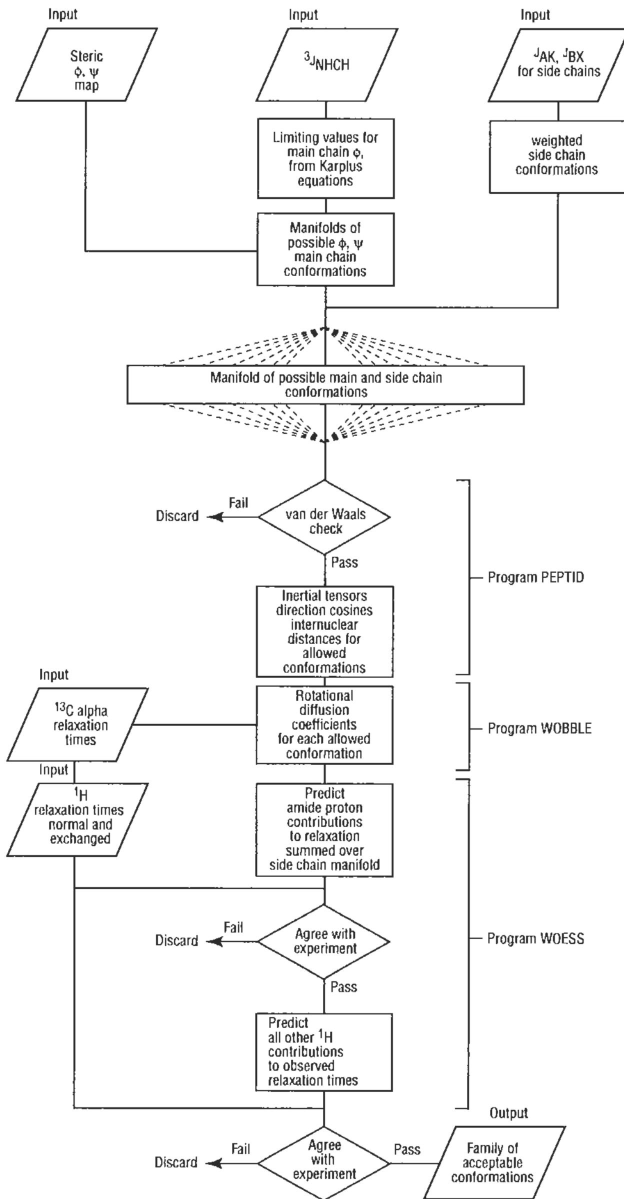

Fig. 6
Flow diagram of the functioning of our 1979 peptide structural determination procedure and programs (given acronyms PEPTID, WOBBLE and WOESS).

11 Beginning wet biochemistry

11.1 Neurohypophyseal proteins: physical and biochemical properties

With Christine Regula's help, I found that protein chemistry was surprisingly easy to pick up: in those days of open column chromatography, all one had to do was master the necessary plumbing! We soon obtained good yields of purified neurophysins.

While we did some work with the neurophysins, using an analytical ultracentrifuge to measure their polymeric associations, similar work was being done by other groups and we never published our results (although I learned a lot about the technique). Instead, we concentrated on determining binding parameters of radiolabeled hormones to the neurophysins (a problem that hadn't been worked on very extensively), choosing equilibrium dialysis as the measurement technique. These experiments went very well and the resulting papers [64, 65] confirmed in my mind my new-found status as a "biochemist."

In one of these papers [65] we dealt with a problem still evident in the literature: common errors in the quantitative analysis of binding data. Many biochemists using Scatchard model analysis of binding were (and are) mathematically incorrect in their analysis of the case of more than one binding site. For example, a later survey paper on analyses of binding experiments that exist in the literature (citing ours as an exception) reported a very large percentage of misinterpretations [66] and I continue to see the same errors made.

As the decade of the 1970s drew to a close we published two papers that typify the way my interests had split. On the one hand I was collaborating in a review of the biochemistry of the hypothalamus with Jeff McKelvy [67] (albeit still concentrating on the physical biochemical aspects), and on the other still trying to use every morsel of NMR information in accurate determinations of solution peptide structures [68].

When a renewal of our "New England NMR Research Resource" grant was not funded in 1979, the staff of the NMR Facility left and I obtained a Fogarty International Fellowship to study the biochemistry of opiate receptors at Imperial College in London.

12 Sabbatical at Imperial College: neurochemistry and opiates

I chose IC because I had decided to work on the biochemistry of opiates and opiate receptors and Eric Barnard and John Hughes were both in the Biochemistry Department there. Hughes had been a co-discoverer of the enkephalins and Barnard's laboratory was trying to isolate opiate receptors using photoaffinity labeling with derivatized enkephalins.

12.1 Opiate receptors

This was the pre-molecular biology era of "receptology." Isolation and characterization of receptor proteins depended largely on choosing the right starting biological material and the right detergent for solubilization. In correspondence prior to my arrival at IC, Barnard had suggested that I work with his group on their efforts to solubilize affinity-labeled opiate receptors and I initially agreed. My lab notebook in July, 1979, when I started work at IC, is filled with notes on previous attempts by other workers to isolate "the" opiate receptor. To optimize the tissue source of receptors, I began learning a new scientific dialect: subcellular fractionation.

12.1.1 Learning subcellular fractionation

The then accepted premise was that opiate receptors were located in synaptosomes and Barnard's group was using rat brain synaptosomal preparations as starting materials for their receptor isolation work. Reading the literature, and one of Barnard's student's lab notebook, I had found an inconsistency in the assumed synaptosomal location for the majority of opiate receptors.

Harry Bradford's laboratory at IC was noted for work on subcellular fractionation and I began learning how to do this from his people. Within a couple of weeks I had learned to do crude brain dissections to obtain tissues from the caudate nucleus (known to be the anatomical location of maximum opiate ligand binding in the brain) and to make good synaptosomal, microso-

mal and myelin subcellular fractions, analyze for total membrane-bound proteins in each fraction, and do marker enzyme analyses to characterize the fractions. I was fortunate in having Bradford's group to help me learn these techniques but, in fact, I found that these procedures didn't involve much scientific skill (I later found the same to be true for most molecular biological techniques) – it was more like painting by numbers.

After confirming the purity of my subcellular fractions *via* transmission electron microscopy (calling on my undergraduate research background), I learned how to do filter binding assays using radiolabeled opiate ligands and confirmed what I had suspected: the major location of stereospecific opiate binding was in the microsomal fraction, not the synaptosomal one [69]. Shortly thereafter, the results were confirmed [70].

The importance of these papers was their suggestion that central nervous system (CNS) opiate receptors were not localized to pre- and post-synaptic membranes. To my knowledge, this problem has still not been addressed by neuroanatomists and electrophysiologists.

There were many good scientists in the Biochemistry Department at IC doing new things and talking about new ideas. One of the ideas – the existence of monoclonal antibodies (Mabs) – that were being discussed proved important for my future career.

12.1.2 Monoclonal antibodies and the germ of an idea about anti-idiotypic antibodies

I became friendly with a colleague in the Biochemistry Department who began to fill me in on the, then new, technique for production of Mabs which had first been reported four years previously. Very soon I was writing to Elmer Becker, an immunologist colleague at UCHC, about the feasibility of producing anti-opiate Mabs when I returned home. Elmer had collaborated with us on our NMR structural study of the chemotactic tripeptide [63] and was always interested in new methods. In an April, 1980 letter to him I outlined my sabbatical plans, my views on NMR-determined peptide structures, and ideas I had about how Mabs might be used as receptor structural analogs.

In his answer to this letter, Elmer mentioned another concept that was new to me: anti-idiotypic antibodies (a-Ids) and their properties of binding

to idiotypic antibodies (Ids). I had never heard of the concept of immune networks involving Ids and a-Ids and began to learn as much as was known about the concept. As described below, a-Ids were to become an important focus for my research.

I immediately began puzzling about the structural implications of Id-a-Id binding. Antibodies were supposed to have a "cleft" which formed their binding sites. How do two antibodies, each with a binding site in a cleft, bind to each other, I wondered? I still wonder about this question because, after all the subsequent hubbub about a-Ids and their possible uses as therapeutic agents, it is still important but unanswered.

Also, to a lapsed structural biochemist (as I would have referred to myself at the time), the idea that a small organic molecule (a hapten) could be structurally mimicked by a large protein's (the a-Id) binding site was just astounding. I asked myself: wherein lies the structural complementarity between an a-Id's binding site (made up of amino acid residues) and the non-peptide hapten? This seemed to me another fundamental, formidable, and very important structural and biological problem. It has never been solved, or, to my knowledge, been worked on.

After my sabbatical ended, my attempted solution to the problem was to carry me far into molecular immunology and away from where structural NMR was headed.

13 Connecticut years – Part II, 1980–2000

13.1 Structural work on opiates

Upon my return to UCHC my plan was to teach myself molecular immunology while continuing to produce papers on NMR and receptor biochemistry. To this end, I brought over one of Barnard's students, Richard Venn, as a postdoctoral worker to work on the biochemistry.

Only two NMR papers came out of my laboratory during the five years after I returned from IC [71, 72]. They were good papers – we showed that intramolecular motion on a biologically significant time scale existed in morphine, a molecule previously assumed to be rigid – but the quantity was too low to sustain my credibility as an NMR spectroscopist.

13.2 Anti-morphine monoclonal antibodies, opiate receptors

The first thing Venn and I did was to look at the sensitivity of sheep brain opiate receptor-bearing membranes to UV light. As I had suspected from the first days in Barnard's laboratory, these opiate-receptor preparations were extremely sensitive to UV of the same wavelengths that his laboratory and other workers were using for photolabeling [73]. This paper had some influence on ending time-wasting photoaffinity labeling of the opiate receptor because work in that area by others soon halted.

I still had hopes that non-photoaffinity methods could result in a successful opiate-receptor isolation. To do this, I thought we needed to know more about the receptors' properties. Second, to raise opiate-mimicking a-Ids, we needed a starting point. I decided that since polyclonal anti-morphine antibodies had been previously produced, we should use analogous methods to produce anti-morphine Mabs (to be the Ids) which could then be used later to produce morphine-mimicking a-Ids.

Workers in receptology at that time were not attempting to use molecular biological methods to isolate or sequence opiate receptors. Dick Venn and I spent much time, as did other groups, unsuccessfully attempting to solubilize the receptor using conventional detergent methods. To do this we had to set up our own filtration binding assays, which led to an encounter (analogous to the equilibrium dialysis problem mentioned above) with how sloppy some investigators were in performing binding isotherm measurements and analysis. Our critique of this literature contained examples of some of these errors and I'm sure it did little to endear us to many people in the field [74].

Most of the biological assays on agonist and antagonist effects were done using the twitching responses of rodent *vas deferens* or *ileum* tissue preparations. On the other hand, most of the binding assays or attempts to solubilize the receptor used subcellular fractions or membrane preparations from cerebral cortex preparations. Collaborating with Jack Cooper and Jim Reese at Yale, we attempted to meet this logical disconnect directly by performing subcellular fractionation on *ileum* preparations and determining opiate binding parameters on the fractions.

The results succeeded in confusing us completely because they showed that in these rodent autonomic system membrane preparations, the opiate receptor binding was predominantly in the synaptosomal fraction, thus sug-

gesting that the receptors were localized to the pre- and post-synaptic membranes (in contrast to the case in brain) [75]. Since one classical action of opiates (as an anti-diarrhoetic) presumably results from its binding to *ileum* opiate receptors, it is possibly important biologically that CNS receptors are found in the microsomal fraction and *ilium* receptors in the syanaptosomal one. Research on the functions of opiates in the enteric system have lagged far behind the CNS effects and, to this day, few studies have concentrated on the functions of either exogenous or endogenous opiates in the functions of the gastrointestinal system.

13.3 Radiation inactivation studies of opiate receptors – knowledge before its time had come

By now we had published two binding studies and a review of the literature on opiate-receptor binding. The end result was that I had become very disillusioned with binding studies telling much about the molecular properties of the receptor.

We tried another direction: in cooperation with Ellis Kempner, we tried using the radiation inactivation technique he had largely developed by himself [76] to determine a molecular mass of the receptor. When the work was finally published [77], my mathematical analysis of the data predicted that the receptor recognition protein was complexed with other proteins, although my derived mass of the recognition unit (50 kDa) came close to the average receptor protein mass predicted from the receptors' gene sequences (41 kDa) reported 5 years later [78–80], even without taking into account presumed glycosylation of the protein to form the native unit.

The radiation inactivation work was published more than a decade before recent work indicating that, indeed, opiate receptors function as complexes [81]. However, I was still thinking as a physical chemist, and that put me very far apart from the way most other workers in the opiate field regarded progress.I decided to drop the receptor isolation work and, seeking a way out of the research impasse I found myself in, we pushed ahead with re-creating ourselves as molecular immunologists. Part of my reasoning was that nobody else in the opiate field seemed to want to do this type of work.

13.4 Molecular immunology and opiates

Without any previous experience in the area, Venn and I started work to produce anti-morphine Mabs which we then hoped to use to produce morphine-mimicking, anti-receptor, a-Id antibodies.

We soon found that the main requirements to do Mab work were simple manual procedures (sterile technique, freezing and thawing cells, mixing up media accurately, immunizing mice and later performing splenectomies on them) with little intellectual involvement required.

Since both of us were experienced chemists, and polyclonal anti-morphine antibodies had previously been produced by other workers using the hapten, morphine hemisuccinate, conjugated to bovine serum albumin, it was easy for us to make the conjugate. We were able to report the first production of high-affinity anti-morphine Mabs by 1983 [82] and immediately set about characterizing their binding properties.

The cross-binding properties of these Mabs with other opiate agonists were interesting insofar as the antibodies were quite specific for the parts of the morphine molecule that pharmacologists had long associated with opiate potency. But none of the anti-morphine Mabs bound the "universal opiate antagonist," naloxone, significantly. Also, contrary to our expectations, none of our anti-morphine Mabs bound Enks significantly (a fact later independently confirmed with other anti-morphine Mabs produced by a different group) [83].

Taken as a whole, all these results make it very clear that our ideas about the structural features of the interactions resulting in drug binding to proteins are still very cloudy.

For example, after their discovery, structural biochemists had supposed that the opiate peptides and the previously known plant/synthetic opiates had structural similarities; how else could they bind to the same receptor and initiate the same intracellular responses (or in the case of naloxone, block the responses?), it was reasoned. Despite cloning the receptors, and despite innumerable structure-activity-relation (SAR) studies on opiates, I believe the key structural relationships between morphine, opiate peptides and naloxone that result in their all binding to the same receptor with high specificity and affinity remain to this day not only a mystery but a challenge to the applicability of structural chemistry to pharmacology.

At the same time as we were producing our anti-morphine Mabs, there was similar activity in several neuroscience laboratories directed toward produc-

ing anti-Enk antibodies. It is illustrative of how much my thinking about the use of these antibodies differed from all the other groups that, when they successfully produced anti-Enk Mabs, not one of them looked at cross-reactivities with plant/synthetic opiates [84, 85]. In fact, to my knowledge the question has been avoided to this day.

With our anti-morphine Mabs in hand we started into, for us, completely unknown territory by attempting to produce the putative morphine-mimicking a-Ids.

13.5 Are binding sites for drugs on antibodies good models for drug-receptor binding?

By 1985 we were able to report the production of polyclonal preparations of morphine-mimetic a-Ids raised by immunization of rabbits with Fab fragments of our anti-morphine Mabs [86–88]. The cross-binding properties of these antibodies were another surprise to me: They blocked both morphine *and* Enk binding to membrane-bound opiate receptors. Here was another paradox: the idiotypic anti-morphine Mabs didn't bind Enks, but the a-Ids mimicked both Enks and morphine. Where did the structural similarities lie? Since at this time there was a great deal of interest by pharmaceutical firms in the development of a-Ids as therapeutic agents, it seemed to me that we had happened onto a structural paradox, the solution of which could lead us to some major insights into the nature of binding-site specificities.Even today, with the push toward "structural biology," our ideas on the origins of antibody binding-site specificity and affinity are largely qualitative and based on very few quantitatively solved structures.

I thought (and still do) that structural studies on a series of Mabs directed against a single hapten form an ideal system for beginning to understand receptor binding specificities and affinities. My reasoning is that this system is, in effect, the result of nature performing site-directed mutagenesis to present us with the best binding sites for each particular hapten. Why guess at trying to produce the same result *via* site-directed mutagenesis when we can have nature's cooperation? Unfortunately, I was unable to obtain funding for a project of this nature and therefore turned further toward molecular biological research.

13.6 Learning molecular biological skills

I taught myself enough molecular biology to enable us to infer sequences of our anti-morphine Mabs from sequencing cDNA we made from the hybridomas producing the Mabs [89], and enough immunology and immunochemistry to contribute papers on new techniques [90–93] and reviews [94] during these years.

13.7 More structural work on opiates

I kept on with NMR work on opiate ligand conformations because I was still hoping to attack the problem that obsessed me: wherein lie specificity and binding affinities of drugs for receptors? With the help of a medical student researcher, and the generosity of Aksel Bothner-By at the NMR laboratory at the University of Pittsburgh we first followed up my paper on Walden inversion in morphine with a more comprehensive treatment of both our 100 MHz and Bothner-By's 600 MHz NMR instruments. In this work [72] we showed that dynamic nitrogen inversion also takes place in the three different opiate agonists and antagonists we examined, and probably takes place in all of the many piperidine ring-containing drugs. These results suggested that a complication in understanding the interactions of agonists and antagonists with receptors is determining which invertomer (or both?) is the biologically active form.

I followed up this NMR work with 500 MHz studies done with the cooperation of Phil Borer, who was working with George Levy at Syracuse University in New York. This combined our anti-morphine antibody work with the NMR studies. My idea was that in the absence of purified soluble receptor, the interactions of ligands with antibodies directed against them might yield information on the specificity/affinity problem that preoccupied me. *Via* transferred NOE measurements, we showed [95] that the bound invertomeric conformations of opiate drugs can be different from their solution conformations, suggesting that the binding can stabilize an invertomeric form that is energetically unfavorable in solution.

The problem with this work was that transferred NOEs can only be used with ligands undergoing rapid exchange between solution and bound forms.

The real biological interest was for the high-affinity binding (and therefore slow exchange) case that could be attacked by the isotope editing technique: work I couldn't get funding for.

I decided to try to attack the problem of bound conformations of drugs by spending a concentrated time at a well-equipped (and well-funded) NMR laboratory.

14 Sabbatical at the Australian National University

In 1988 I took a six-month sabbatical leave with Larry Brown's NMR group at the Australian National University (ANU) in Canberra. Larry's laboratory was well equipped with a new 500 MHz instrument and well staffed with talented people. I had grand plans to finally use the isotope editing scheme to look at two types of binding, the opiate-anti-opiate Mab case and oxytocin-neurophysin binding. This latter was of interest because it was biologically relevant and could be done because some years before Victor Hruby had synthesized some oxytocin peptides specifically labeled with ^{13}C. For the opiate work, I spent several weeks synthesizing morphine specifically labeled with ^{13}C, thus again putting on the hat of an organic chemist. I arrived in Canberra with NMR quantities of all the materials I needed.

14.1 Transferred NOEs

Sadly, I got very little done at ANU. Larry and I just spoke different scientific languages. He didn't see the interest in binding studies, and I didn't understand his lack of interest. I wound up with not much time on the instrument and never got the modifications to the probe necessary to do the isotope editing experiments. I managed to complete the preliminary transferred NOE work from two years earlier in a definitive way [96], but didn't get any data on tightly bound ligands. I didn't know it then, but my NMR days were over.

I decided to go back home and concentrate on using our a-Ids to study the receptors and dropped the idea of using the anti-morphine Mabs as models of receptor binding sites.

15 Research 1990–2000

15.1 Using anti-idiotypic antibodies to observe opiate receptors *via* confocal microscopy

One of the problems with opiate receptors is that they are not very immunogenic. The anti-receptor a-Id approach enabled us to bypass trying to make anti-receptor antibodies by conventional methods. The initial problem I wanted to attack with our a-Ids was the distribution of receptors on cell surfaces. One of the laboratories at UCHC had recently received a new instrument that I knew nothing about – a confocal microscope. Confocal microscopy, which had just come into relatively common use in several laboratories around the world, seemed ideal for the opiate receptor fluorescence labeling experiments I had in mind.

I had the good fortune to be able to employ a Polish national living in the United States, Margaret Ornatowska, M.D. Margaret couldn't practice medicine here, and didn't really want to; she was interested in learning how to do research.

Margaret and I learned how to culture NG-108-15 hybridoma cells (which express large numbers of -subclass opiate receptors) and to biotinylate our a-Ids so that they could be fluorescence labeled with FITC- and Texas Red-conjugated avidin. We were also fortunate in this work to have the cooperation of a computer programmer, Mr. Frank Morgan, who worked in a colleague's laboratory at UCHC. In those early days of confocal microscopy, three-dimensional reconstruction software was not widely available. Frank developed a very useful program to do this reconstruction and made it available to us for one of its first applications (Fig. 7).

Margaret and I published two very thorough papers describing the use of confocal microscopy to study opiate receptor distributions on both asynchronous and synchronized cultures (we had to learn how to do the synchronization) of NG-108-15 cells [97, 98]. Our overall conclusion from this work was that reversibility of anti-Id binding to the receptors by opiate congeners and the stereospecificity of the binding were not connected. That is, we concluded that stereospecific and non-stereospecific receptors co-existed in asynchronous cultures and that in synchronous cultures the percentage of cells displaying stereospecific binding of the anti-Ids was dependent on

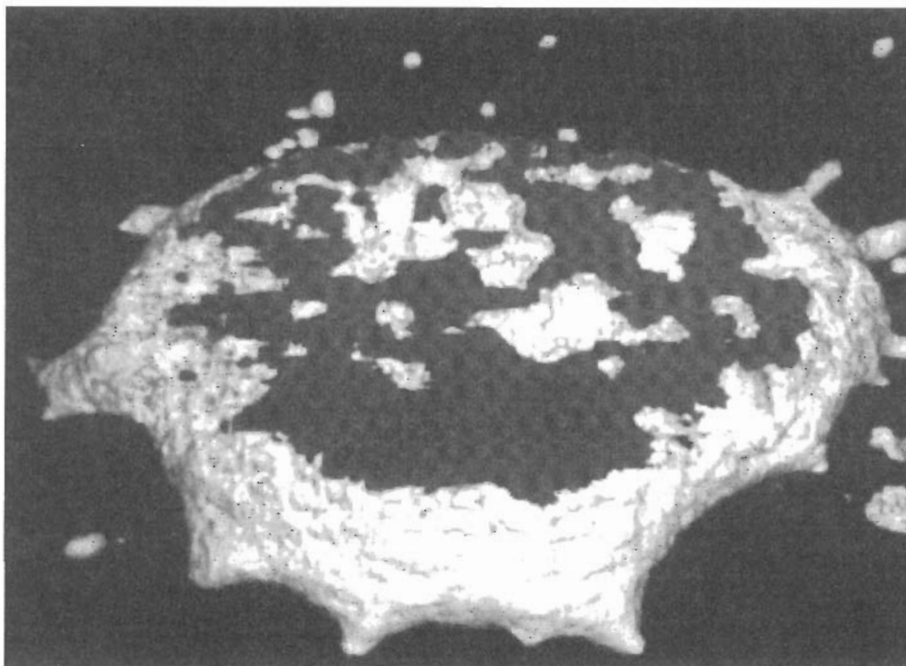

Fig. 7
Confocal fluorescence microscopy of NG108-15 cells. Three-dimensional reconstruction through a middle section of a cell stained with FITC-avidin-a-Id. Note internalization of a-Id binding material. From [97].

cell cycle. This implied that the receptor's binding site(s) mature(s) as the cell grows.

In the confocal microscopic work, in the previous work with radiation inactivation, and in a subsequent paper using non-confocal fluorescence microscopy, I was probably too unimaginative to synthesize the results correctly. Re-reading the papers and the methods, particularly those used to define stereospecificity in the fluorescence work, indicates that what we were really observing were the changes in binding specificities of the receptors as they aggregated into complexes – a conclusion drawn from very new work based on completely different methods than we used [81, 99, 100]. I remain proud of Margaret's and my work with confocal microscopy; it was very new at the time.

In the early 1990s, with a new government in Poland, Margaret returned home, and Dianne Agarwal became my first graduate student.

15.2 Beginning to understand the molecular biology of opiate receptors

Dianne began her thesis work by learning to do cell culture and continuing our fluorescence labeling work. The first mature work she collaborated in was a study of co-localization of δ- and μ-subclass opioid receptors in SK-N-SH cells [101]. Our conclusions were that the results were consistent with a model where μ- and δ-binding sites reside on different subunits of a multimeric complex. In the light of the recent work quoted above [81], this work also seems before its time.

At about this time, opiate receptor research was changed drastically with the cloning and sequencing of the δ-subclass of opiate receptor [78, 79] (followed shortly thereafter by similar work on μ- and κ-receptors). This immediately made obsolete all the solubilization efforts still being made by several groups around the world and opened new avenues of research.

I was determined to try to get a jump on other workers by doing something new in opiate research. In choosing this new direction, I fell back on a habit formed years earlier when I was a full-fledged chemical physicist: I tried to simplify.

From extensive reading about research being done on the effects of drugs, including opiates, on mammalian cells, I had come to the conclusion that there were no good model cell systems; workers in this area got different results depending on what cell lines they were using. This situation resulted in lots of papers, but without much commonality that could be discerned. I believed (and still do) that one of the main problems is the plethora of signals, particularly growth factors, that every cell is bombarded with constantly and which each different cell type responds to differently.

I set Dianne the task of developing a cell system that grew in a *defined* medium and whose growth and proliferation depended on what I hoped would be only a limited number of defined growth factors. Neither Dianne nor I had any real training in mammalian cell culture and biology and in entering this field I was, as usual, depending on attitude and native savvy, not prior training. Dianne rapidly became adept at the manipulations required to culture mammalian cells. Within a year from starting, she had developed a cell line whose origin was the familiar NIH 3T3 fibroblast cell line but which could be cultured in a defined medium containing only insulin as a growth factor. In fact, this cell line (which we named 3T3DA) could be syn-

chronized by withdrawing insulin from the medium. This arrested cell proliferation without killing them and reintroducing insulin started them proliferating in synchronization.

15.2.1 A good tool for determining drug signaling pathways to the nucleus?

With this cell system, I thought we had a very good tool for studying drug effects on cells (once we transfected the cells with receptor-encoding genes), without the confounding effects of multiple exogenous growth signals. This was a typical way of thinking for a physical scientist – proceed from a simple model system to more complex ones – but not for biological scientists; I was unable to get funding for work with our cells.

When I began to have funding difficulties several years before, I had turned to making proposals to pharmaceutical firms for support of the type of work I have been describing in this section. Although I continued to make such proposals, I quickly found that drug firms were even more concerned with fashionable research than academic reviewers. Proposal reviewers from biotechnology and pharmaceutical firms were a bit more polite than those employed by the government, but they universally saw what I proposed as coming up with potential rewards too far in the future for their plans.

Still, using some support from UCHC, small commercial sales of our antimorphine Mabs, and generous gifts of several receptor-encoding genes from original investigators, Dianne and I plowed on and gathered sufficient data to publish a very complete paper on the mitogenic (as opposed to transforming) effects of drugs on the DA cell line transfected with the receptor-encoding genes and expressing the receptors [102]. In this paper, we showed that proliferation of the DA cell line was down-regulated by chronic morphine treatment, and that the second-messenger signaling by the δ-receptor upon acute morphine treatment was the same as had long been recognized to exist in other cells expressing the same receptor. But we found that the second-messenger signaling pattern changed upon chronic treatment with the drug. We logically surmised that we would have to understand the "downstream" signaling pathways of activated opiate receptor into the nucleus to understand what genes the initial signals were controlling.

I decided that we had to jump ahead directly to finding what genes were affected by chronic morphine treatment. My reasoning was that this is the

clinically relevant case. The question was: can chronic treatment with narcotics temporarily or permanently alter the expression of certain proteins that have subsequent effects on cell phenotype? For example, we had found a phenotypic response to withdrawal of DA cells from chronic morphine treatment: they underwent a transient up-regulation of cellular proliferation. I became interested in a quantitative analysis of this up-regulation prior to undertaking work to understand its molecular basis.

15.2.2 Still thinking quantitatively

I found a reference to a computer program (CELLSIM) that analyzed cell proliferation using numerical methods (as opposed to solving differential equations analytically) and was able to obtain a version of the 25-year-old program from its author, Charles Donaghey of the University of Houston. CELLSIM allows a theoretical curve for cell proliferation based on cell cycle variables to be compared with experimental curves. While not mathematically unique (because some variables had to be assumed), the results of the analysis indicated [103] that under chronic morphine treatment, the decrease in proliferation of DA cells was due almost entirely to an increase in the length of time spent in the G1 phase of their cell cycle. On the other hand, we concluded that when withdrawn from chronic morphine treatment, the DA cells undergo an increased rate of proliferation due to a shortening of time spent in G1.

Having published several papers, Dianne had done enough research to write a thesis. She left in 1998 and, continuing to be funded by UCHC with some additions from my own pocket by myself, I continued the work I thought important.

15.2.3 High school algebra pays off

During this period, I published a paper [104] that had nothing to do with the direction I wanted my research to go, took me a half-hour to write, generated controversy and work by other groups, and has proved to be one of the most cited of my biochemical papers. These events convinced me that I really had no place in academic science.

Murray Deutscher, a fellow faculty member at the Health Center, and I were editing a text book on biophysical chemistry methods. He dropped by my office one day and said he thought the chapter on UV spectroscopic methods should contain more practical material and suggested I add an explanation of the popular use by molecular biologists of UV spectroscopy – monitoring the purity of nucleic acid preparations from the ratio of UV absorbances taken at 260 nm and 280 nm. Everybody in molecular biology, including me, used this ratio constantly because it was recommended in all the molecular biology recipe books.

In the library I looked up the original publication that was the origin of the method and was surprised to find that it was developed to determine DNA impurities in *protein* preparations, not the other way around. As it would to anyone trained in high school algebra who examined the problem, it was immediately clear to me that since maximum protein molar extinction coefficients at 280 nm were much smaller than DNA molar extinction coefficients at 260 nm, the A260/A280 was a terrible method for determining purity of DNA preparations. I solved the simple equation for the ratio as a function of the extinction coefficients and showed the ratio's insensitivity by numerical example. I wrote the paper up and it was accepted immediately.

When published it provoked a storm of protest and several subsequent papers attempting to show I couldn't be correct. Even Murray wouldn't believe the results because he told me (as did most of the protesters) that his laboratory's DNA preparations couldn't be as dirty as my results indicated and still be used. Some months later, a scientist who was editing a review on the subject asked me if I was sure of my results and then told me in the next breath that he was going to give a "balanced" account of the pros and cons in the review.

My paper's conclusions, being the result of a simple mathematical relation, couldn't really be refuted and have come to be accepted now. What the protesters didn't think about was that the protein impurities in most DNA preparations are probably complex mixtures that, depending on the subsequent reactions the "purified" DNA was subjected to, sometimes interfere with those experiments and sometimes don't.

I considered the whole incident bizarre and representative of how far away I was from how molecular biologists thought about the world of natural things; group opinion and faith seemed to carry as much weight to workers in this area as (what I call) science. If scientific colleagues didn't believe in

the validity of a simple equation – relying on word-of-mouth instead – what hope was there for me who always leaned in exactly the opposite direction.

My further experiences in molecular biological research on opiate receptors drove home this message even more forcibly.

15.3 Final work: out of funds but not out of ideas

In the previous few years Dianne and I had found that DA cells transfected with the δ-opiate receptor gene became active in expressing their (previously not expressed) β_2-adrenergic receptor at high levels. We also found that chronic exposure to procaterol (a specific β_2 agonist) up-regulated proliferation of the cells. Tracing the signaling pathways, we found that separate chronic morphine and chronic procaterol treatments differentially affected the mitogen-activated protein kinase (MAPK) isozymes known to be involved in growth regulation (as it should if one drug up-regulated proliferation and one down-regulated it). We also found that simultaneous chronic treatment with both receptors' agonists had non-additive effects on the levels of these isozymes and on cell proliferation. This meant that the signaling pathways into the nucleus due to simultaneous drug treatments contained a certain amount of "cross-talk." I had been puzzled for a long time over how linear signaling could take place in response to each different drug when the intermediate signaling molecules were identical in many cases.

Our work had, it seemed to me, developed into a clean-cut system to study many phenomena mediated by drug-activated receptors. True, we didn't know why the adrenergic receptor's expression was stimulated by having an opiate receptor introduced into the cell's genome but, however it happened, it gave us a wonderful tool, I thought; we had a cell line that depended on only one media growth factor, expressed two G-protein coupled receptors that could be specifically activated, and responded with different phenotypes to the different agonists and to mixtures of the agonists. These results appeared in another complete paper [105].

15.3.1 Pleotropic effects of drugs: a rapidly developing field

The field of opiate research in the late 1990s had come around strongly to using molecular biological methods. In particular, laboratories interested in

studying chronic effects of endogenous and exogenous opiates were concentrating on the effects of drugs on the signaling pathways to the nucleus: something we had started on 4 years previously but could never get funded for.

I decided to try to leap ahead and find out as much as possible what transcripts were differentially regulated by drug treatments. I reasoned that the most important thing was to identify the proteins differentially changed by chronic drug treatments, since some of them would be involved in the phenotypic changes we were observing in cells subjected to the treatments.

Although this problem has become a major focus of modern drug research (prompted by the development of "high-throughput" techniques such as cDNA microarrays), at the time I started the work there was a great deal of controversy over how to observe differential transcription efficiently. For example, the differential display technique was known to discriminate against low-abundance transcripts [106] and to produce many "false positives". However, a newly developed polymerase chain reaction (PCR)-based differential library technique had just been described [107, 108] that appeared to eliminate many of the previous problems.

This new technique proved to be very easy to perform and, within two weeks, I had begun to identify some up-regulated transcripts from DA cells subjected to chronic morphine treatment. Database searching revealed that most of these genes had already been identified and encoded proteins that had been previously related to cell proliferation. In particular, one transcript that was up-regulated as a result of chronic morphine treatment was known to encode a protein that is important in the proliferation of trophoblast giant cells during human embryonic development.

The placenta has been called a "pharmacy" because of its production of a wide diversity of hormones and growth factors, and *via* the literature I found that the trophoblast giant cells not only expressed δ-opiate receptors and opiate peptides, but were subject to damage by exogenous narcotics. To me, it seemed important that even in our model cells, chronic morphine treatment up-regulated a gene that encoded a protein that affected the proliferation of trophoblast giant cells.

I thought that I had been lucky again and jumped into a new and relatively uncrowded area of basic drug research: the effects of narcotic drugs on placental development.

15.3.2 "Not innovative"

Instead, it was a dead end; for the first time in my entire career, a proposal based on the data I had gathered was described by a reviewer as "not innovative." I had been turned down before by reviewers who didn't believe that I could technically do the experiments I proposed, or for not referring to previous grant support in papers (true!) and for various other reasons, but never because I was not innovative. Furthermore, the same proposal was turned down by my colleagues at UCHC when I applied for further support to widen the search for morphine-regulated genes using the newly available cDNA microarrays. The reason given was that I would obtain a lot of data that I wouldn't know how to use.

Faced with very long odds for getting funded to do the things I believed important in my chosen field, and unwilling to try to get funding for work I didn't think important, I decided to offer my generalist experience in science as a consultant.

Science as an intellectual and manual occupation was still wonderful; the practice of academic scientific research became intolerable to me. I had kept the faith as long as I could, and then said goodbye to all that.

Annotations

1 The idea that everyone in the world is connected to everyone else *via* not more than six human contacts. See J. Guare, *Six Degrees of Separation: A Play* and more recently, Duncan J. Watts and Steven H. Strogatz, "Collective Dynamics of 'small-world' Networks", Nature *393*: 440–442 (1998) and references therein.

2 A note on authorship of these and later papers from my laboratory is in order: Bob Williams convinced me that to avoid seniority arguments, authors from each institutional affiliation should be in alphabetical order. At the time this made sense to me so I adopted this method. In reality, for the rest of my career it caused confusion: depending on the name of a co-author my name has been first or last author on subsequent papers with no connotation of who was responsible for the work.

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