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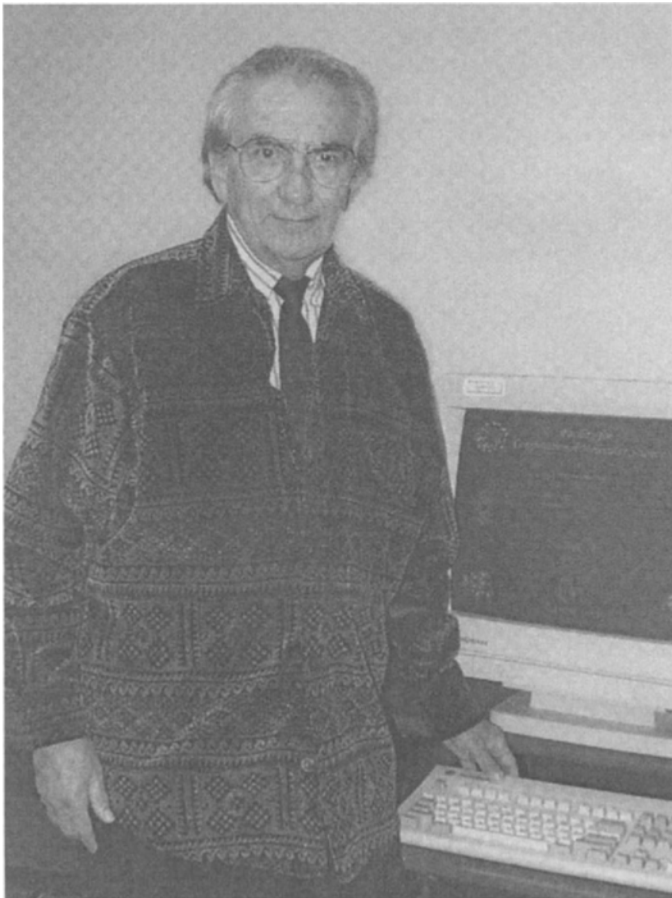

Morris H. Aprison

BORN:

Milwaukee, Wisconsin
October 6, 1923

EDUCATION:

University of Wisconsin, B.S. (Chemistry) 1945[1]
U.S. Navy (R.T. Program; Electronics) 1944–1946
University of Wisconsin, Teacher Certification, 1947
University of Wisconsin, M.S. (Physics) 1949
University of Wisconsin, Ph.D. (Biochemistry) 1952

APPOINTMENTS:

Galesburg State Research Hospital (1952–1956)
Indiana University School of Medicine (1956)
Distinguished Professor Emeritus, Indiana University School of Medicine (1993)

HONORS AND AWARDS (SELECTED):

American Society for Neurochemistry
Council, (1971–1973, 1975–1979)
Chairman, Scientific Program Committee (1972)
International Society for Neurochemistry
Council (1973–1975); Secretary (1975–1979); Chairman (1979–1981)
Gold Medal Award, Society of Biological Psychiatry (1975)
First professor to assume the title -Distinguished Professor of Neurobiology and Biochemistry- at Indiana University (1978)
The May 1992 issue of *Neurochemical Research* (Vol. 17, No. 5) was dedicated to honor Dr. Aprison

Morris H. Aprison pioneered research that identified and correlated the roles of serotonin (5-HT) and acetylcholine (ACh) in specific animal behaviors leading to a theory of depression. His interest in central nervous system (CNS) neurotransmitters resulted in the discovery that glycine, in addition to its metabolic roles, had a functional role in specific regions of the CNS as an inhibitory postsynaptic neurotransmitter. Using computational chemistry techniques, he identified the molecular mechanisms that can explain how the inhibitory neurotransmitters glycine and GABA, and the excitatory neurotransmitters ACh and 5-HT$_3$, react at their respective receptors in the CNS.

[1] Awarded while in the Navy for academic work completed in 1944 (see text).

Morris H. Aprison

Introduction and Reinforcements

When I began my education at the university level, I had no idea that I would devote most of my adult life to research in neuroscience. I did know, however, that I wanted to use the tools of chemistry, physics, and biochemistry in some way to improve the life of mankind. This strong interest in helping others developed in the mid-1930s because of two important experiences of great emotional impact. The first influence was seeing the movie *Louis Pasteur*, with the gifted actor Paul Muni playing the lead role. Learning of Pasteur's great discoveries and the resulting benefits for mankind was very inspirational to me! The second influence was a remarkable teacher of biology, Noah Shapiro, who used the contract system, a unique teaching method, to challenge his students at West Division High School in Milwaukee, Wisconsin. Two of the required "A grade" contracts contained the assignment to read two wonderful books written by the author Paul de Kruif. I eagerly read *The Microbe Hunters* and *The Hunger Fighters*. I was captivated by the research achievements and the lives of the brilliant investigators described in those books. Thus, while still a sophomore in high school, I dreamed of eventually doing similar work.

Reinforcing all of this were the most influential people in my life—my parents! I was born on October 6, 1923, to Henry and Etel Aprison in their home in Milwaukee. They were recent immigrants from Austria and Lithuania, respectively, who were struggling to become Americans. My father was trained in carpentry and had reached the level of "meister" cabinetmaker before he left Austria. He arrived in Milwaukee in 1920 and quickly obtained a position in a furniture factory because he was an excellent carpenter and spoke fluent German, an asset in Milwaukee, which had a large German population. My mother arrived shortly thereafter. Both began to learn to speak English and to adjust to life in their new country. After they met and married in 1922, their lives began to improve until my father lost his job due to anti-Semitism—he asked to take 2 days off work, without pay, to celebrate his religious holidays. To support his family, he obtained a series of noncarpentry jobs until my uncle suggested he buy a small business so as to have a more secure income; my parents

agreed and became owners of a small neighborhood grocery. I grew up a grocer's son.

My parents and I lived in four rooms behind the grocery store, and I went to public schools. They always encouraged my interests in schoolwork and in sports. As immigrants, they realized the importance of advanced schooling and developing one's skills. They were very reinforcing with their praise; I thrived on their love and support.

The Milwaukee public schools had neighborhood playgrounds that were used year-round for sports and games. It was there that I learned and excelled at basketball and chess. I won several chess championships at that time and also in college. However, I did not succeed in making the 1941 basketball team at the University of Wisconsin.

Madison, Wisconsin and the U.S. Navy (1941–1946)

I entered the University of Wisconsin (UW) on September 24, 1941, to work toward a B.S. degree in chemistry. Several months later, on December 7, 1941, while listening to the Chicago Bears–Green Bay Packers football game that Sunday afternoon, I and all other listeners learned that Japan had attacked Pearl Harbor. After the United States declared war on the axis powers the administration of UW announced a government plan to defer 62 science students. I was one of them. I was able to finish all my required course work, including my B.S. thesis (*Dissociation Constants of Some Substituted Piperidines*), but was 10 elective credits short of my degree when the government abruptly canceled all science deferments. I enlisted immediately in the Navy in order to take a special test, which was a requirement to enter a unique Navy program in electronics. I passed this EDDY test. The Navy was searching for men who could learn how to operate and repair advanced types of radar, sonar, transmitters, radios, and IFF (identification, friend or foe) equipment used aboard our ships under battle conditions. While in this program, I was pleased to learn that UW had granted me "10 elective credits for war services" and, thereafter, my B.S. degree. After graduating from this program I shipped out on the aircraft carrier Ticonderoga just as the war was ending. I served in the Pacific area on two additional ships, one of which the Navy used to transport part of the Chinese Sixth Army and some of their horses to Manchuria. After I was honorably discharged from the Navy, I returned to Milwaukee to consider my future.

Teachers Certification and Physics (1946–1950)

I chose to first get a teachers certificate and then to seek employment until I could pursue M.S. and Ph.D. degrees. I earned a teachers certificate in 1947, but I was discouraged by the low salaries and rigid rules set for

teachers. I quickly enrolled in the master's program in the Physics Department at UW, and in 1949 I received my M.S. degree. Since so many students were accepted into the physics graduate program after they had worked on the atomic bomb project in Chicago, "thesis" space for them was at a premium. However, because of my acceptable B.S. thesis, I was required only to pass a 3-hour final examination on all subject matter taught in the courses up to Theoretical Physics given in the department. Prior to taking this examination I returned to my home in Milwaukee to review my notes and study for this important event in my life. It was at this time that my best friend, Jack Manning, encouraged me to take a weekend off. He introduced me to two college coeds, and my date, Shirley, was terrific. We began to date, fell in love, and married on August 21, 1949.

I became interested in biophysics, but UW did not offer degrees in this discipline. Since I needed time to consider my next career move, I took a position at the Institute of Paper Chemistry in Appleton, Wisconsin, as an assistant in the Physics Group in order to stay in Wisconsin. The research done at this institute by the group I joined focused on developing photoelectric instruments that could be used to measure color, smoothness, and other characteristics of paper. While finishing this research, and writing two papers, I received an important letter from an old friend, Jack Clemmons, who was doing research in the Department of Pathology at UW with the then chairman, Dr. D. M. Angevine. They wanted to hire me to provide technical assistance to help build an improved historadiographic apparatus and associated electronic equipment in order to study calcification of various tissues. I called Dr. Angevine and told him that I would come back only as a graduate student. I was willing to consider his project as part of my thesis if a research committee would agree. He was very receptive to the idea and told me he would pursue this plan with the dean of the graduate school.

I was invited to meet the dean, Dr. C. A. Elvehjem, and my future major professor, Dr. R. H. Burris, in Madison. I was accepted as a graduate student, and after some discussion we agreed upon titles to the two-part thesis: *An Improved Historadiographic Apparatus* and *Nitrogen Fixation by Excised Nodules of Soybean Plants.* I returned to Appleton very delighted with my good fortune. I was again on the path I wanted to be on. I was a few months short of being 27 years old, I was married, I had a job, and I had been given an opportunity to return to UW to work toward a Ph.D. in biochemistry.

Madison, Wisconsin: Biochemistry (1950–1952)

I started to take graduate courses in biochemistry and began the research and library search on instruments used in historadiography. I learned that at that time the technique of historadiography was severely limited by the

small number of historadiographs that one could take in a day; most of the lost time occurred waiting for the oil and mercury diffusion pumps to cool before removing the tissue sample from the photographic chamber and, upon introduction of the next sample, even more time was lost waiting for those pumps to produce the desired vacuum. Our improved apparatus was therefore designed and built to markedly shorten these times. The two main features of the new unit were the ability to use electrostatic focusing of the electrons in the X-ray tube and the unique design of a vacuum interlock in the photographic chamber. The former feature resulted in the use of a more intense X-ray beam and thus a shorter exposure time, whereas the latter allowed the vacuum pumps to run continuously. Five improvements were made in the new unit: (i) reduction of exposure time from 5–45 minutes to 30–40 seconds, (ii) a method for maintaining the vacuum thus permitting speed-up in changing samples, (iii) the capacity to take 10 historadiographs per hour, (iv) the addition of an automated timing circuit to make accurate time exposures for quantitative work, and (v) the incorporation of safety features allowing simplicity of operation. I was very happy with these results. A paper was published, a year had passed, and I then turned to the second half of my thesis that I suspected would be more difficult. Indeed, Dr. Burris told me that this research problem had not been solved even though many investigators had worked on it.

Professor Burris suggested that I use vigorously fixing, field-grown soybean plants for my research project. He took me to the university farms and showed me a small plot of land on which I could plant a row of inoculated seeds each week so as to have soybean plants containing many nodules on a continuous basis all summer. After numerous experiments, fixation of nitrogen by excised nodules of soybean plants could be achieved consistently. The successful demonstration of fixation was attributed to (i) the use of $^{15}N_2$ as a tracer, (ii) the use of vigorously fixing field-grown plants, (iii) the rapid treatment of the nodules with $^{15}N_2$ following excision using a special glass interconnecting system that I took to the farm, and (iv) analysis of only the soluble portion of the nodules after a basic lead acetate procedure when I returned to the laboratory. With these four factors now part of the procedure, many other parameters were then examined. Ultimately, I found a positive correlation between fixation of N_2 and the size of the nodules. The larger nodules, containing a higher percentage of tissue invaded by the rhizobia, fix nitrogen more rapidly than do smaller nodules. Interestingly, nodules 5 mm in diameter fixed N_2 best. Also, when nodules were sliced, it was found that fixation was less efficient than that with whole nodules. Moreover, when water was added to those slices, the fixation decreased to a fifth of that obtained without water. These data suggested that some necessary substrates, ions, or coenzymes were being diluted on addition of water, thus reducing the rate of uptake of $^{15}N_2$. Crushed nodules, both with and without added water,

exhibited no capacity to fix nitrogen at all. These data further suggested that cellular structure may be of prime importance in keeping labile enzymes and substrates in the proper position or proper concentration *in situ* for nitrogen fixation to take place. Based on these and other findings, the two-part thesis was finished and accepted. On August 22, 1952, I received my Ph.D. I was proud of reaching this goal and also surprised but pleased to learn that I was the first and only graduate student in the Department of Biochemistry that had minored in physics. I took a short vacation before pursuing a lead for a senior research position I had learned about earlier that year.

Galesburg, Illinois (1952–1956)

I met Dr. Harold Himwich, the research director at Galesburg State Research Hospital (GSRH), during the federation meetings in the spring of 1952. He was interested in hiring me as a biophysicist to help develop a research program with multiple approaches directed toward solving some of the functional illnesses afflicting mankind. He invited me to meet him at his laboratories after I received my Ph.D. Several months later, I visited the GSRH in Galesburg, Illinois, where I accepted Dr. Himwich's offer to be chief of the biophysics group. This hospital was one of many in Illinois that housed mental patients, but it had been used first by the U.S. Government and then by the state of Illinois for other purposes. There existed several buildings that could be used to build a "research institute or laboratory" within the grounds of the GSRH. I learned that Dr. Percival Bailey, a consultant to the state of Illinois, had recommended that a modern facility be constructed in which research could be directed toward solving the problems of mental illnesses; only two facilities of this kind existed at that time, one in New York and one in Los Angeles. Dr. Bailey believed that a third was necessary and should be built in the middle of the United States. The governor agreed, Dr. Harold Himwich was hired as the first director, the renovation of the necessary space in the GSRH was designated, and the laboratory was built. It was later named the Thudichum Psychiatric Research Laboratory (TPRL).

About the time I arrived, Dr. Paul Nathan, who had received his Ph.D. in physiology from the University of Chicago, was also hired. We were told that we could work on any project we wished as long as it was directed toward understanding mental illness.

Dr. Himwich met often with us in the first month to discuss research papers and reviews on the nervous system. We discussed the literature which contained arguments about whether neurotransmission in the central nervous system (CNS) was chemical or electrical. I was beginning to realize that conducting research in the field of mental health was more difficult than anything I had done in the past; there were very few leads in

the literature on which to base an active research program. Then we visited the wards in the GSRH—it was an era before patients were given drugs such as chlorpromazine—and these visits made a long-lasting impact on me, so much so that I decided to stay in this field of study. Perhaps it was at this time, late 1952, that I entered the "field of neuroscience" without realizing it. It also was a time when "overlapping disciplines" were not officially recognized. Journals with titles containing words such as neurochemistry, biophysics, and neuroscience would appear later (the *Journal of Neurochemistry* did not appear until May 1956, the *Biophysical Journal* in September 1960, and the *Journal of Neuroscience* in January 1981).

To a novice without psychiatric experience, it appeared that the patients we saw in the wards were displaying abnormal behaviors—certainly non-acceptable behaviors to the peer group on the outside. Why was their behavior different and how does a normal individual change so as to emit atypical behavioral patterns? If the brain is the source of biochemical and biophysical events governing the behavior of man, then one can think along lines leading ultimately to the design of key laboratory experiments. Thus, I wondered whether it was possible to correlate biochemical changes within the important and delicately balanced systems of the brain with concomitant changes in behavior of the whole person. I concurred with most of the other authors whose work we were reading that the process of neurotransmission in the brain of man was extremely important. I believed that if one could determine how to understand the neurobiological mechanisms in the brain that are involved in the generation of normal behavior, one might then find a way to correct abnormal behavior when it occurred. It was not difficult to take the next step and hypothesize that a neurotransmitter with its associated enzyme system could be important in the mechanisms of the brain that ultimately determine the behavior of an organism. Furthermore, evidence was becoming available at that time showing that acetylcholine (ACh) and its catabolic enzyme, acetylcholinesterase (AChE), were important. Anticholinesterase drugs were known to have pronounced effects on living organisms. Therefore, if we could decide on an animal model, I would first test the cholinergic system. Furthermore, since I would have to analyze cerebral tissues, we were immediately limited to the use of animals since human CNS tissue was ruled out.

Dr. Himwich directed us toward a model which he and several coworkers had studied while in the Army Chemical Corp. Following the unilateral intracarotid injection of diisopropyl fluorophosphate (DFP), a potent anticholinesterase, forced circling occurred in several species of mammals including the primate *Macaca mulatta*. These earlier studies were carried out for the purpose of finding protection from poisons such as DFP, which could easily be used to disable or even kill a soldier. We decided to use much smaller doses of DFP with rabbits and examine the brains of these animals by first measuring the AChE activity in the cerebral cortices and

caudate nuclei. In later experiments, we would measure ACh levels in the same two cerebral areas. In our laboratory, the usual response to such an injection into the right common carotid artery resulted in forced circling by the animal to its left; however, in a few cases the rabbit turned to its right, whereas rarely the animal did not circle in either direction. We distinguished these animals by calling the first group "lefters," the second "righters," and the last "neutrals." I started measuring the AChE activity in a tissue sample first because it was easier than measuring ACh content. I developed the former method and then worked on the second, which was a bioassay and very "tricky" to do unless one had much experience. The AChE data were published, as was a paper describing an improved method to measure the ACh content. We then reported on these data too. I found it very interesting that I received the most reprint requests for the paper describing the ACh method.

Based on the AChE data, we could offer explanations for the three kinds of behavior noted in rabbits after the injections of DFP; however, when writing that paper without the ACh data, we had to speculate about the ACh levels to explain some of the behaviors. When the measures of ACh were completed the data fit! Furthermore, it was possible to correlate the rate of turning by the rabbit with the amount of asymmetric ACh content in its cerebral cortices following the unilateral intracarotid injection of DFP. I published these data in 1958, and this paper contains an important figure, which shows neurochemical data on the ordinate and behavioral data on the abscissa (Aprison, 1958). This figure is a first or among the first of its kind! However, I concluded that our understanding of compulsive turning or circling left much to be desired. Furthermore, it is not a behavioral condition that lends itself easily to further study. Therefore, I became involved in other research as well as other activities at TPRL.

Many of Dr. Himwich's friends presented seminars at TPRL and then would meet with the young investigators. I was especially fond of Dr. Ralph W. Gerard, who with Himwich nominated me for membership to the American Physiological Society in 1955. We enjoyed discussing my latest results and recent data in the literature. I heard that he often referred to me as "the young biophysicist who was working in the mental health field." He even invited me to give a seminar at the new Mental Heath Research Institute in Ann Arbor, Michigan, after he left the University of Chicago. I remember that visit well because I met Dr. B. Agranoff and several other young investigators at that time. I did not know that several years later I would invite Dr. Agranoff to join me as coeditor of a new series of books titled *Advances in Neurochemistry*.

Dr. Himwich also invited many outstanding foreign doctors who wished to carry out research at TPRL. One who came from Italy via Chicago to Galesburg was Dr. E. Costa. He and I teamed up to do two interesting studies on serotonin (5-HT), a compound that began to generate much

interest in the mid-1950. In one experiment, we measured the 5-HT content in many specific areas of the human brain and spinal cord, providing the first direct evidence for the presence of serotonin in the CNS of man. In the other study, we set up cross-circulation experiments to study the distribution of 5-HT injected into the internal carotid artery of the recipient rabbit. We found that very small amounts of 5-HT were recovered from different parts of the brain suggesting that it can cross the blood–brain barrier under our experimental conditions.

In addition to people coming to TPRL, some began to leave. I too received an offer that I just could not turn down. In November 1956, after voting in the presidential election, I took my family, which now included two wonderful young sons, Barry and Robert, and left for Indianapolis, Indiana, the location of the Indiana University School of Medicine and the new Institute of Psychiatric Research. Dr. John Nurnberger, Sr., Professor of Psychiatry, chairman of the department, and director of the Institute of Psychiatric Research (IPR), had offered me the academic position of Assistant Professor of Biochemistry and Psychiatry and also Principal Investigator of Biochemistry on the research staff of IPR. I happily accepted! There, I would try to develop an area of study that I would call "neurochemical correlates of behavior."

Indianapolis, Indiana (1956–1999)

When I started at IPR in late November 1956, I met some of the new staff. By June 1958, Dr. John Nurnberger, Sr., had appointed 10 senior staff members, and in order to support the laboratory as well as clinical and basic research being developed at IPR he hired 7 noninstitute consultants and 25 administrative, technical, and maintenance staff. While I was trying to decide on a new animal model to use to continue my research started in Galesburg, a report from Sweden appeared followed by a paper published by Dr. S. Akerfeldt in *Science* (1957). This report caused excitement in many laboratories throughout the world in which investigators were working on problems devoted to mental illness. Akerfeldt described a simple blood test that he said could help physicians in their diagnosis of cases involving schizophrenia. He reported that the sera of patients with certain mental disturbances, especially the acute schizophrenic patient, had the capacity to oxidize *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (DPP) more rapidly than fresh sera obtained from healthy normal subjects. Dr. A. L. Drew and I decided to test this result using sera from 23 children hospitalized because of psychiatric illness. Statistical analysis of the data obtained from biochemical measurements of the sera of schizophrenic children and those obtained from measurements of the sera of nonschizophrenic children did not support the suggestion that the Akerfeldt test could be used to distinguish between schizophrenic and

nonschizophrenic children (Aprison and Drew, 1958). In a similar study using adults, Horwitt *et al.* (1957) were likewise unable to distinguish between normals and schizophrenics. However, since a few positive studies had been reported, I wanted to do one more study and examine the lag period that almost always occurred in the oxidation of DPP when normal serum was used. Dr. H. J. Grosz joined me in this research. We found, as I had expected, that a positive correlation exists between the lag periods in the DPP oxidation and the serum ascorbic acid concentration found in 14 controls, 16 patients with diagnoses of schizophrenic reactions, six dogs, and two rabbits. Administration of ascorbic acid to a number of normal controls and schizophrenic patients produced a prolongation of the lag period (Aprison and Grosz, 1958). These data suggested the "mental hospital diet hypothesis," i.e., such diets were thought to be deficient in some important constituents such as vitamin C. We, however, decided to let others pursue this lead.

Searching for an Animal Model to Study Depression

At that time a Skinnerian psychologist, Dr. C. B. Ferster, had joined IPR. He left a cage containing a pigeon in the lobby of IPR that really intrigued me. The morning I saw the pigeon in the plastic cage, I also saw an ink recorder and a sign that told the reader that if a one-cent coin was dropped into the slot the pigeon would work for food! I also noticed toward the back of the cage a metal board with several lights attached, some electronic equipment, a small opening, and a round Lucite disk placed on the board to the side of the opening. I immediately followed the directions on the sign and was absolutely surprised. The lights in the cage came on and the pigeon immediately moved toward the disk, which had turned green. Apparently, a green light and a red light were wired behind the disk, each meant to turn on under specific electronic conditions. The paper in the recorder also began to move at a constant speed and a straight black line was produced until the bird pecked at the disk. The bird pecked quite slowly at first, then picked up the pace, and finally pecked very quickly. I noticed that the pen on the recorder had begun to move as the bird pecked and was generating a curve whose rate of change appeared the same as the pigeon's "behavioral responses"! About 10 minutes had elapsed. Suddenly, an oblique but very short line appeared on the recording, and into the small opening appeared a scoop of bird food. The pigeon ate this food as fast as it could. After about 25–30 seconds, another short oblique line appeared because the green-colored disk had turned red and then the scoop dropped from view into the opening. The oblique lines indicated where the reinforcements occurred. The portion of the curve between the two oblique lines during the time the disk was illuminated red showed the so-called fixed-ratio (FR) performance. Whenever the disk appeared red, the pigeon seemed to peck at its fastest and most steady rate, and after a

much shorter time period the scoop with the food appeared at the opening again. The pigeon ate quickly before the food scoop dropped away, the color of the disk changed again from red to green, and the process began to repeat.

I learned later that the reinforcement occurred as a result of the 50th peck. When the disk appeared green, the reinforcement was being delivered on the basis of time rather than on the number of responses. More important, I realized that the pecking behavior of the bird was "being measured quantitatively." As the rate of pecking increased, the slope of the line being recorded also increased in direct proportion to the rate of the pecking behavior! I had not realized it at that time, but I had been watching a trained pigeon working for food, "its daily bread," on a fixed ratio (50/1)–fixed interval (10-minute) schedule of reinforcement. This pigeon was at 80% body weight and hungry; it had learned to work on this schedule every day in order to get enough food to maintain its 80% body weight. I realized immediately that I had found a way to continue the pursuit of my important goal and I was ecstatic! All I thought I had to do was to convince Dr. C. B. Ferster to join with me and to use his trained pigeons in my neurochemical experiments. When we met for the first time, I told Ferster about my research at TPRL and how impressed I was with his display in the lobby. I also explained why it was important for us to work together. I told him that my research project was based on the idea that there was a need to identify the changes in cerebral biochemical events in animals whose behavior was neuropharmacologically changed while being measured continually and quantitatively and, subsequently, to determine if time correlations in these two diverse measures could be found. The successful experiments, I pointed out, should yield data that could eventually lead to a theory of depression! We agreed not only to work together on my project but also to learn as much as we could about the other's area of expertise. However, he was worried that it would take too much time to train the pigeons that we would choose to use because we would then sacrifice them in order to measure the biochemical parameters in specific brain areas. I assured him that we could surmount this problem, and we did. We built a vertical multiple-unit pigeon apparatus that permitted the training of birds continually over a 24-hour period prior to being placed in a standard Skinner box; thus, it was possible to provide a sufficient number of animals to do the necessary research (Ferster and Aprison, 1960).

Neurochemical Correlates of Behavior

We began our collaborative research with the 5-HT-monoamine oxidase (MAO) system. Much interest had developed in this area during the prior years. Pharmacological data obtained with research on smooth muscles suggested to me, as well as to many other investigators, that abnormal levels of brain 5-HT might be the cause of the psychiatric changes in man

as well as the cause of abnormal behavioral changes in animals. The importance of 5-HT in cerebral function was based on several other kinds of data: (i) 5-HT content was found to vary in specific areas of the brain in animals and man; (ii) its biosynthetic and degradative enzymes were also found in these same areas; (iii) increasing the cerebral 5-HT content by either injecting its precursor, 5-hydroxytryptophan (5-HTP), or 5-HTP plus a MAO inhibitor or decreasing it by pyridoxine deficiency produced marked central disturbances including behavioral changes, as did studies with indole drugs such as LSD. We expected that our research would support these observations and it did (Aprison and Ferster, 1961a; Aprison, 1965; Aprison and Hingtgen, 1970).

The first step in this program was a specification of the behavior of the animal for which the biochemical correlate was sought. Because the Skinnerian psychologists had already developed techniques that could provide predictable baselines in a systematic account of animal behavior and because the reproducibility of the behavioral baseline was comparable to that obtained in pharmacological bioassay techniques, we believed that such advances made it possible to quantitatively measure the behavior of an animal objectively. Ferster and I decided to use these operant techniques and began with the multiple fixed-ratio, fixed-interval schedule (FR50/FI10) of reinforcement that I had seen in the lobby. We quickly showed that the 5-HT precursor, 5-HTP, when injected intramuscularly (i.m.) into a pigeon's breast muscle, produced quantitatively measured behavioral changes. We chose to define these behavioral changes as "depression" when a significant reduction of learned behavior occurred on food-reinforced operant schedules.

In the early studies we produced dose–response data; we described how to calculate or compute reproducible behavioral measures from the raw response time data generated by the pigeons working in their Skinner boxes; we measured brain MAO activity in birds injected with the inhibitor iproniazid, and we showed that a relationship exists between the behavioral measurement and the brain MAO activity at three different doses of i.m.-injected 5-HTP (10, 25, and 50 mg/kg). The latter data showed that at any level of cerebral MAO activity, the greatest behavioral effect was obtained at the highest 5-HTP level. More important, the data also showed that at any given dose of 5-HTP, the greatest behavioral effect was obtained at the lowest brain MAO level (Aprison and Ferster, 1961b; Aprison, 1965). These data supported the idea that if MAO activity decreased, less 5-HT was destroyed and more would be available to be released and produce behavioral effects. We reasoned that if the behavioral change is caused by the action of released 5-HT, and the latter is controlled by MAO located in the mitochondria of the presynaptic nerve endings, we should then study the kinetic relationship between the 5-HTP-induced behavioral disruption and the variation in the content of 5-HT in

different brain areas in pigeons not given a MAO inhibitor. Thus, we investigated how the 5-HT content varied in four specific brain parts (telencephalon, diencephalon plus optic lobes, cerebellum, and pons medulla oblongata) as well as in liver, heart, lung, and blood in birds killed at various time intervals during the period of behavioral disruption following an i.m. injection of 50 mg/kg 5-HTP. Because the time course of the behavioral effect in any given animal is relatively invariant with a constant dose of 5-HTP, and because there can be marked variation in the time course of response from bird to bird, a unique method of treating these data was developed by Aprison *et al.* (1962). The data on serotonin were plotted against the average percentage of the behavioral effect in each bird rather than the length of time after 5-HTP injection. Thus, the variation in behavioral disruption of the same dose of 5-HTP in each pigeon was weighted, and the variability of the data was greatly reduced. The behavioral depression was found to be temporally related to a three- or fourfold increase in 5-HT content only in the telencephalon and diencephalon. The time course of change in both measurements was remarkably similar, as was the return to normal levels, only for these two brain areas, thus confirming the original explanation of the cerebral 5-HT–MAO–behavior relationship (Aprison and Ferster, 1961b; Aprison *et al.*, 1962). For the other tissues no such correlations existed. Other neurochemical experiments showed that our observed periods of behavioral depression are associated with an increased release of 5-HT into the synaptic cleft of specific areas of pigeon and rat brains. These neurochemical/behavioral data, as well as *in vitro* and *in vivo* studies of nerve ending fractions, strongly suggested that some types of depression may be related to an excess, rather than a deficiency, of free 5-HT in the synaptic cleft.

In 1959, a proposal titled *Neurochemical Correlates of Behavior* was submitted to the National Institutes of Mental Health as a 3-year grant in which I described many experiments designed to further test the role of cerebral 5-HT in depression. Although these studies were to be performed on animals, our ultimate goal was to understand the specific biochemical mechanisms causing certain types of abnormal human behavior, such as depression. As the principle investigator, I received a call from the study section secretary in 1959 who informed me that the grant was approved, and the committee recommended that it be extended for 2 additional years. I quickly accepted the report and asked how that decision had been made. He told me that the committee not only thought the experiments were important but also they were pleased that two young and proven investigators from different fields of study had chosen to work together in an important overlapping area of research that should be supported. This experience proved important to me and helped shape many of my other studies later in my career. Therefore, I was concerned some time later when Dr. Ferster decided to leave IPR. I was very fortunate to get

Dr. J. N. Hingtgen, a young psychologist with strong interests in research, to agree to join our faculty and to work collaboratively with me. Over the years, my collaborators and I generated enough important new data to write about 240 publications in this specific area of science as well as to renew this grant for 21 consecutive years. Cross-disciplinary study can be very important when working on complex issues in psychiatry and neuroscience.

A Concept of Hypersensitive Serotonergic Receptors

As we published more data, we developed a conceptual framework of neurobiological mechanisms which could lead to abnormal behavioral states (Aprison and Hingtgen, 1970, 1981; see also Figs. 1–1 and 1–2 in Aprison and Hingtgen, 1993). We were aware of the serotonin deficiency theory of Lapin and Oxenkrug (1969), which associates depression with lowered levels of 5-HT in the synaptic cleft and postulates that the therapeutic mechanism of antidepressive drugs is to increase amine levels by means of uptake blockade. This idea did not fit with our results nor with the results of Takahashi and his group in Japan. One of my young postdoctoral students, Dr. K. Tachiki, went to work with that group, and when he returned three of us wrote a chapter describing the research from both our groups (Aprison *et al.*, 1978). This chapter was dedicated to Dr. H. E. Himwich, who had died on March 4, 1975.

The early concept of hypersensitive serotonergic receptors involved in clinical depression was developed in that chapter. Critical to our thinking were two interesting and important sets of clinical data that appeared in the literature. Papeschi and McClure (1971) had reported that the probenecid-induced accumulation of 5-hydroxyindoleacetic acid (5-HIAA) in the CSF of depressed patients was significantly decreased during the 1- to –3-week treatment with amitriptyline or imipramine when compared with the pretreatment values for these patients. Goodwin and Post (1974) noted that probenecid-induced accumulation of 5-HIAA in the CSF of depressed patients is lower than that of controls. These findings suggest that (i) the turnover of cerebral 5-HT in depression is lower than in normals, and (ii) when the depressed patients are clinically improving after taking one of the tricyclic antidepressants, there is apparently a still lower turnover of cerebral serotonin. These two observations, when added to the data from our animal model research, together with the fact that data from other laboratories did not support the Lapin–Oxenkrug theory (Nagayama *et al.*, 1981), prompted us to complete more basic studies, leading us to formulate the hypersensitive postsynaptic serotonergic receptor theory of depression. This theory was originally presented by Aprison *et al.* (1978) but later expanded in scope as more data accumulated (Aprison and Hingtgen, 1981, 1983, 1986, 1993).

A New Theory of Depression

This new Aprison–Hingtgen theory explains depression as follows: Persons who are prone to become depressed release less 5-HT at serotonergic synapses than normal persons. The consequence of prolonged reduced release of 5-HT should result in the formation of a hypersensitive receptor in the postsynaptic membrane of the serotonergic synapse. During the developmental stages of the disease and prior to the onset of depression, the decrease in the level of released 5-HT is probably compensated for by an increase in sensitivity of the receptor (hypersensitivity). This can occur when the number of 5-HT receptors increases or when the receptor becomes hypersensitive. An individual with this malady probably does not show all of the usual signs of depression; the hypersensitive receptors would handle the information as though a normal amount of 5-HT had been released. Since this illness is predominantly associated with adults, it could be suggested that the process of reduced release probably occurs over a long period of time or that the patient inherits such a system from birth and it becomes critical only during times of stress. Thus, later if a "psychiatric precipitating factor" such as the death of a loved one or loss of a job occurs, the event probably results in the increased release of several transmitters including 5-HT; in the case of 5-HT, however, this increase does not reach its original levels. We call this increase "subjective," and its impact on the hypersensitive receptor system should be comparable to that noted in the animal studies after an injection of 5-HTP large enough to produce behavioral depression. Hence, since the 5-HT system in man appears to be the same as that in animals, depression should occur in humans by a similar mechanism. Furthermore, we reasoned that the release of 5-HT could be reduced not only in individuals predisposed to depression but also in those under constant or severe stress (Aprison and Hingtgen, 1981; Hellhammer *et al.*, 1983). Continual stress may cause such a reduction due to the presence of serotonergic autoreceptors in key areas of the CNS. Since it is known that the release of 5-HT is under inhibitory control of autoreceptors, and if the initial stress that increased the release of 5-HT lasts long enough, the serotonergic system would adjust by decreasing this release. The net effect of this reduction over time could be the development of a hypersensitive receptor system. Animal experiments supported these explanations.

Because all the psychiatrists in our department were exceedingly busy, we could not test this theory clinically. Therefore, we continued our basic research approach with animals, designing experiments that could test predictions of the Aprison–Hingtgen theory. If we were using a "correct animal model" to study human behavior, certain predictions could then be tested easily. If these predictions were proven correct, we would be reinforced to continue this line of research. Initially, we showed in a study with

rats that were pretreated with *p*-chlorophenylalanine, an inhibitor of tryptophan hydroxylase, the biosynthetic enzyme for making 5-HT in CNS, that a significantly increased behavioral depression occurred after administration of low and previously ineffective doses of 5-HTP. Other similarly treated rats provided receptor-binding data demonstrating that the 5-HT receptors in the cerebral cortex became hypersensitive (Fleisher *et al.*, 1979), data which we believed supported our theory.

Next, we wished to determine if the antidepressive drugs that psychiatrists prescribe for patients, meant to increase the synaptic 5-HT levels by blocking its uptake, would increase or decrease the quantitatively measured behavior in our animals. We predicted that these drugs would not only block the 5-HTP-induced behavioral depression but also do so in proportion to the drug's ability to bind to 5-HT receptors or to block 5-HT molecules from interacting with its normal serotonergic receptor. To test this prediction, we established a behavioral basis for distinguishing between pre- and postsynaptic events by using fluoxetine (Prozac), a specific presynaptic uptake blocker of 5-HT, and methysergide, a known postsynaptic 5-HT receptor blocker. Fluoxetine potentiated the depressive effects of low doses, whereas methysergide almost abolished the depressive effect of large doses of 5-HTP. When we tested this prediction in our animal model, we found that 5 mg/kg methysergide as well as 1.5 mg/kg iprindole, 2.5 mg/kg imipramine, 2.5 mg/kg amitriptyline, 1.0 mg/kg mianserin, and 2 mg/kg trazodone, when administered in a single dose within a range comparable to a daily clinical dose, resulted in varying percentage blockade of 5- HTP-induced depression in rats. Starting with methysergide, these six percentages were found to be 70, 22, 40, 47, 52, and 62%, respectively. Furthermore, we found an excellent inverse linear relationship with these calculated behavioral neuropharmacological data obtained in Indiana and the data on the *in vitro* binding of lysergic acid diethylamide (^{3}H-LSD) to membranes isolated from the dorsal neocortex of rats in Sweden and reported as IC_{50} (nM) values in the presence of each of these drugs. These data are best explained by a postsynaptic event, which we believe is what characterizes these five drugs as antidepressives (Aprison and Hingtgen, 1993, Fig. 1-3). Since fluoxetine acts presynaptically and not with postsynaptic receptors, we suggested that its long-term clinical use may result in a beneficial effect due to the increased accumulation of 5-HT in the cleft, which in turn would result in a slow downregulation of key hypersensitive serotonin receptors.

Because I cannot review here all of our research on this specific topic, a listing of the "milestones" that led to the development of the Aprison–Hingtgen "Hypersensitive Serotonergic Receptor Theory of Depression" is given in Table 1. The dates listed in Table 1 refer to pertinent references in Aprison *et al.* (1978) and Aprison and Hingtgen (1993). I believe that when this theory is eventually tested clinically, it will be correct. I have suggested to a number of psychiatrists some procedures

Table 1 Development of the Hypersensitive Serotonergic Receptor Theory of Depression

1. Quantitative studies deomonstrated that 5-HTP can induce behavioral depression in pigeons: first suggestion that the free or released 5-HT causes the behavioral depression (1960, 1961).
2. Monoamine oxidase (MAO) inhibition enhanced 5-HTP-induced depression, which was negatively correlated with brain MAO levels (1961).
3. Initial study using quantitiative behavioral–neurochemical measures demonstrated a direct relationship between elevated 5-HT levels in discrete brain areas and 5-HTP-induced depression (1962, 1965).
4. Description of behavioral effect of α-methyl-metatyrosine (α-MMT) supported the first suggestion that behavioral depression was related to released 5-HT (1963, 1965).
5. Serotonin–behavioral correlations were replicated in the rat: Dopamine and norepinephrine changes were not correlated to behavioral depression following 5-HTP in the pigeon (1965, 1966).
6. Relationship between decreased levels of total cerebral 5-HT and behavioral depression following α-MMT was established (1966).
7. First study published showing that 5-HTP-induced depression and α-MMT-induced depression are both related to increased release of 5-HT from serotonergic nerve endings (1973, 1974).
8. L-Tryptophan and 5-HTP administration was shown to yield similar serotonergic–behavioral correlations (1975, 1976).
9. First complete description of the hypersensitive serotonergic receptor theory of depression was presented (1978).
10. A model of a hypersensitive serotonergic receptor was produced in the rat with chronic parachlorophenylalanine treatment (1979).
11. Psychopharmacological data were published implicating postsynaptic action of clinically used antidepressive drugs with our animal model of depression (1980, 1981).
12. Additional descriptions were provided regarding postsynaptic serotonergic hypersensitive receptor theory of depression (1981).
13. Evidence was provided indicating that both acute and chronic antidepressant treatment blocks 5-HTP-induced depression (1981).
14. Chronic activity-wheel stress without drug administration produced a significant decrease in 5-HT levels in specific areas of the rat brain (1983).
15. A selective and potent 5-HT_2 receptor blocker was shown to be effective in eliminating depression following 5-HTP administration in our animal model (1985).
16. Chronic reserpine treatment potentiated 5-HTP-induced behavioral depression suggesting the development of supersensitive serotonin receptors (1987).
17. Three different behavioral stress procedures resulted in hypersensitivity to 5-HTP administration (1988).
18. Central 5-HT mechanisms in the 5-HTP animal model of depression were further demonstrated with microinjection of 5-HTP directly into the lateral hypothalamus of rats (1988).
19. Chronic activity-wheel stress resulted in development of hypersensitivity of 5-HT_2 receptors (1989).
20. 5, 7-Dihydroxytryptamine-induced lesions of the raphe reduced 5-HT and 5-HIAA levels in specific brain regions and potentiated 5-HTP-induced response suppression (1991).

that could be used to confirm it, including a technique reported by Wong *et al.* (1987) describing the localization of 5-HT receptors in living human brain by positron emission tomography. With time and funds, it is hoped that the clinical research will be carried out to finally test our theory. I am pleased that Dr. Hingtgen and I worked so well together in our research; I thank him very much. I also thank Drs. K. Tachiki, H. Nagayama, D. Hellhammer, and E. Engleman for their important contributions.

Identifying New Transmitters

While the work on neurochemical correlates of behavior was in progress, another major area of study was established. This area of research involved searching for new or undiscovered neurotransmitters. Based on my belief that scientific exploration of exceedingly complex systems such as the mammalian CNS requires a multifaceted attack, I wished to find a neurophysiologist who might care to join me in using a combined neurochemical–neurophysiological approach to investigate such a problem. Dr. John Nurnberger, Sr., had hired Dr. R. Werman as the principal investigator in neurophysiology, and I had noticed how hard Werman was working on his own research. I was determined to find the "right" way to approach him. I wanted to explain to him why I thought that a logical expansion of the work on neurochemical correlates of behavior was the development of a research program which could lead to the identification of new neurotransmitters in the CNS of vertebrates, and that such research should be important to him too. Months later, after a seminar by a guest neurophysiologist that Werman had invited, I asked many questions involving mechanisms of action during the transmission process, especially regarding how the neurotransmitter action was terminated. I was beginning to believe that the action at a cholinergic synapse (via the postsynaptic action of AChE) was not a general case, as I once had thought, but a special case. This event led to a discussion the next day between Werman and myself; he asked me why I had not addressed similar questions to him. We decided to talk more about our mutual interests, and soon after we decided to work together. We decided that in view of the advanced state of feline lumbosacral spinal cord physiology, the spinal cord seemed to be the site to begin our search for CNS transmitters. Furthermore, Werman preferred to study the spinal cord of the cat since his expertise was in that system and not in the brain. I agreed since I believed the area of research was more important at that time than the tissue system. It turned out to be an excellent decision! After several years of joint research, we were able to present data (i) that strongly supported a functional role for glycine, the simplest amino acid, as a phylogenetically older postsynaptic inhibitory neurotransmitter released from small interneurons within the lumbosacral gray area of the cat spinal cord, and (ii) that glutamic acid (glutamate) as well as aspartic acid (aspartate) also had neurotransmitter roles with glutamate acting as a noncholinergic excitatory neurotransmitter released from dorsal root fibers onto motoneurons, and aspartate acting as an excitatory transmitter released from excitatory interneurons. Later work by Aprison, Werman, and others provided a neurochemical basis that glycine and glutamate were transmitters in other areas of the vertebrate CNS (Graham *et al.*, 1967; Aprison and Werman, 1968; Shank and Aprison, 1988; Aprison, 1990).

How did these amino acids become candidates for postsynaptic transmitters? This is an important question. A reader might find it difficult to imagine the basis by which one can conclude whether or not a specific compound is a neurotransmitter in the CNS. Thus, in the case of glycine, structurally the simplest of all amino acids—an amino acid with no asymmetric carbon atoms but with well-known and important metabolic roles—it was not easy to accept its role as a postsynaptic inhibitory neurotransmitter. When we began, the task seemed difficult since it was well-known that (i) it had been estimated and reported in the literature that an enormous number of neurons were located within the CNS; (ii) in addition, there were a large number of synaptic contacts between neurons; and (iii) a large number of chemical compounds had already been identified as well as localized in the brain and spinal cord. Therefore, how did we start this line of research?

First, we discussed at great length what criteria would be necessary to conclude that a specific compound was a transmitter (or could act like a transmitter) if it had more than one role. I concluded that compelling evidence for such identification must consist of a combination of three kinds of specific data. The first set was neurochemical. That is, one had to show that the candidate was present in the presynaptic neuron of the synapse. The second set was neurophysiological. One had to demonstrate that the candidate could reproduce the ionic membrane processes evoked by transmitter action. Finally, the third set was both neurochemical and neurophysiological, and the researcher would have to show that the putative transmitter could be collected from the extracellular fluid in response to stimulation of the presynaptic nerve. I prefer to refer to these three pieces of data as the presynaptic criterion, the identity of action criterion, and the release criterion, respectively (Aprison, 1978). Data to support these three criteria are given in the first three figures in Aprison (1978) as well as in a chapter titled *The Discovery of the Neurotransmitter Role of Glycine* (Aprison, 1990). Both of these publications also present and discuss in detail most of the important published supportive neuropharmacological, autoradiographic, and neuroanatomical data. I will not present these data here because of space constraints, but instead note that it was soon clear that our data were repeatable, and glycine was accepted to function as an inhibitory transmitter in specific areas of the CNS.

We also published many papers on methods and distribution of glycine in the CNS of various species, glycine metabolism, its distribution in isolated subcellular fractions, neuropharmacological and neurophysiological studies involving glycine and strychnine, biochemical aspects of transmission at inhibitory synapses, and the determination of the number of glycine binding sites in areas of the rat CNS. It is this latter area that I wish to comment on. After discussing the newly discovered role of glycine with Dr. Jay Simon, who had joined the IPR staff in 1978, we decided that

it was very important to develop a new binding assay that was specific, saturable, pH sensitive, and reversible since such an assay would provide meaningful data to characterize glycine receptors. Dr. Hideji Kishimoto had arrived from Japan in 1981 and joined us in this research. We finished this work and published our paper (Kishimoto *et al.*, 1981) which was the first detailed study using [^{3}H]-glycine to document sodium-independent, glycine binding to CNS elements, and included the determination of K_D and B_{max} values in several areas of the rat CNS. The fact that the assay was not dependent on the presence of extracellular sodium was strong support that glycine was not binding to a transporter. An additional important observation that came from these studies was the result that although the cerebral cortex had the highest B_{max} value, it was known to have very few inhibitory glycinergic synapses. The significance of these data would not be recognized for nearly a decade. However, we suggested that glycine was binding to sites other than the glycine receptor, and that these other glycine binding sites were sensitive to D-serine. In our original paper, we referred to these "other" binding sites as "non-postsynaptic receptor binding sites." As it turns out, this "other" binding site probably represented the now well-documented glycine binding site of the important *N*-methyl-D-aspartate (NMDA) receptor complex, where it has been shown that D-serine acts as an agonist, thereby explaining its ability to compete for glycine in our original assay system. Since Werman left shortly after the neurophysiology studies (Werman *et al.*, 1968), I continued this research without him and was very fortunate to have had many other collaborators in the neurochemistry work whom in addition to Simon and Kishimoto, I also thank: R. Shank, L. Graham, E. Daly, N. Nadi, W. McBride, P. Shea, T. Kariya, and M. Toru. I conclude this chapter with a discussion of the last joint effort in my research career—a project involving computational chemistry.

Using Quantum Theory to Study Receptor–Neurotransmitter Interactions (1986–1999)

As I mentioned earlier, once I became interested in mental illness and how to solve this problem, I became very involved with research on transmitters. However, when I began, I was aware that at some time in the future I would be led to investigate the atomic mechanisms operative at the receptor–neurotransmitter interactions. When I had pretty well finished the research work discussed previously, I was in my early sixties and ready for my second sabbatical, the first having been taken during 1978 at the Salk Institute. I had started to inquire about locations and investigators in theoretical chemistry or biochemistry, when I learned from a colleague in Bloomington, Indiana, that one answer to my query could be found right on our own IUPUI campus (Indiana University and Purdue University had

agreed to merge facilities in Indianapolis to form a new combined city–university with the abbreviated reference IUPUI). I learned that Dr. Kenneth Lipkowitz, an organic chemist by degree work and a computational chemist by further study and training, was well-known and might take on an "older student." I made an appointment to see him, and after we discussed my program he agreed to teach me the necessary computer techniques. I arranged to take a 6-month sabbatical with Kenny (he liked first names), and from February 1986 to August 1986 I spent full-time on the other side of our campus, where the chemistry department was located. We would continue to work together, I full-time and he part-time, for over 10 years and publish 12 research papers (1987–1996) and one minireview (1996); eleven of these publications appeared in the *Journal of Neuroscience Research*.

I learned that computational chemistry is a relatively new area of science that allows one to explore at the atomic level aspects of nature that are not directly amenable to experimentation; moreover, it is a multidisciplinary area of research that transcends traditional barriers separating chemistry, physics, mathematics, biology, and neuroscience. Using such computational methods, we established a long-term research program based on first principles to understand and identify the mechanism to explain how a neurotransmitter, once released from the presynaptic neuron, can react at its receptor site in the synapse. I believed that this study was important since it was known that such a mechanism initiates ion flow through channels in the postsynaptic membrane such that if the transmitter is inhibitory hyperpolarization occurs, and if it is excitatory depolarization occurs. We started with glycine and strychnine for obvious reasons.

First, we obtained molecular structures from published crystallographic studies of these two compounds and fed the data into our VAX 11/780 computer. It had been shown that the crystal structures were minimum-energy structures that could be used directly for such comparisons. All calculations in the late 1980s were performed with this computer with molecular modeling software written in-house. All graphical representations were displayed on a Tektronix 4107 high-resolution color graphics terminal and plotted in stereo on a Tektronix 4662 interactive digital plotter. A structural comparison of glycine and strychnine was made by minimizing the six translational and rotational degrees of freedom of the atoms that map glycine onto strychnine. Since I had learned in my training period that molecular graphics together with quantum chemistry could be used to identify similarities between dissimilar structures, an exhaustive comparison of topological and electronic features of both molecules was made. However, in order to do these comparisons, a step was taken to facilitate the molecular orbital calculation. We truncated the strychnine molecule with methyl or hydrogen substitutions and successfully located a glycine-like three-atom fragment in the strychnine molecule that, when compared

to glycine (the two oxygen atoms and the nitrogen atom), exhibited both topological and electronic charge congruence in three pairs of atoms. In a later publication we reported on finding a second glycine-like fragment on the strychnine molecule, also with excellent topological and electronic charge congruence, but this time with three pairs of atoms different from those found in the first study. The topological congruence in the second glycine-like fragment was much better than that with the first fragment for which we had used the truncated strychnine molecule in the quantum analysis. We were now doing our molecular modeling with the Quanta/CHARMm molecular modeling software from Molecular Simulations,Inc., running on a Silicon Graphics Workstation. This time we were able to use the full strychnine molecule, publishing the derived atomic charges of all 25 atoms in Table 1 of a paper by Aprison *et al.* (1995a).

In the late 1980s, we had begun to notice, and report, the existence of a fourth negative site in glycine and GABA antagonists. Perhaps this observation was important for distinguishing agonists from antagonists of the same transmitter system. This same pattern was found when we began to study three weak glycine antagonists: *N,N*-dimethylmuscimol, *N*-methyl-THIP, and iso-THAO (see Appendix 1; Aprison and Lipkowitz, 1991, 1992). Thus, we suggested that from theoretical concepts, agonists and antagonists of inhibitory neurotransmitters such as glycine and GABA possess two characteristics which a neuroscientist could use to distinguish an agonist from an antagonist (Aprison *et al.*, 1987; Aprison and Lipkowitz, 1989). First, each antagonist has at least three binding sites (two negative and one positive) that complement the CNS receptor, and these sites are similarly spaced as in three such binding sites in agonists as well as the natural neurotransmitter. Second, each antagonist has an additional important binding site, i.e., a negatively charged fourth atom or group of atoms, that can attach to the top of the chloride channel in the CNS receptor, serving as a mechanism to inhibit chloride ion flux.

As we continued our research, several new concepts were discovered. Again using molecular modeling methods, we identified a molecular mechanism that could explain how the incorporation of two methyl groups in place of two hydrogen atoms on the terminal nitrogen atom of muscimol could not only convert this potent agonist at GABAnergic receptors to an inactive molecule at these receptors but also convert this new derivative to an antagonist of glycine at glycinergic receptors (Aprison and Lipkowitz, 1992). In our paper we showed that our theoretical concepts were correct when we identified three new attachment sites of proper charge within *N,N*-dimethylmuscimol that fit with three glycine atoms, yielding again three pairs of atoms with an average deviation of 0.052 Å for this fit. In addition, a fourth negative site was identified that could serve as the possible point of attachment to the top of the chloride ion channel. The interested neuroscientist is referred to this paper for the extensive important details.

In 1994, Dr. E. Galvez-Ruano, a visiting professor from Spain and a scholar in pharmacology and organic chemistry, joined Kenny and me in some additional studies. Using our molecular modeling techniques, we investigated nine glycine antagonists in order to try to identify the molecular descriptors that characterize strychnine as a strong antagonist and *N,N*-dimethyl-muscimol, iso-THIA, THIA, *N*-methyl-THIP, iso-THAZ, THAZ, iso-THPO, and iso-THAO (see Appendix 1 for chemical names) as weak antagonists. As expected, all nine compounds had the three-atom regions (two negative and one positive) that we had postulated are necessary to permit such compounds to attach to the recognition site in the glycinergic synapse. Each of these nine antagonists also had a fourth negative atom in about the right position to give each their antagonistic characteristic. Furthermore, we described how our data led to molecular calculations of angles defined by the three-dimensional spacing of specifically placed atoms as well as the distance between pairs of specific atoms, which in turn led to theoretical insights to explain (i) why strychnine is a strong glycine antagonist and (ii) how to rank the nine antagonists in order of decreasing potency. The former consideration led to the realization that a special bidentate binding is occurring at the positive region of strychnine. The special binding to the proper portion of the glycine recognition site appeared to be possible as an extended positive grouping containing a carbon–nitrogen bond and the associated hydrogen atoms in strychnine. Thus, at this positively charged region the positive charge extends to cover an area that could bind through electrostatic domains to a tertiary carboxyl group in an amino acid such as aspartate in the polypeptide (Aprison *et al.*, 1995b). This negative region in the receptor is apparently larger than we originally thought, and the region it covers is probably as wide as the carboxyl group, where resonance delocalizes the charge density over the two equivalent oxygen atoms. The larger anionic region permits interactions with antagonists that are structurally more complicated, thereby suggesting that another mechanism may be operative in some antagonists which we had not considered. The interested reader can find the data in Figs. 4 and 5 of that paper to determine which descriptors previously referred to permitted the ranking (the list at the top of this paragraph gives the ranking in decreasing potency).

Next, we studied the phylogenetically newer inhibitory neurotransmitter, GABA. We identified agonistic and antagonistic mechanisms which we theorized were operating at the $GABA_A$ receptor, and our data suggested that there exists a slightly different agonistic mechanism (Galvez-Ruano *et al.*, 1995). In addition, we found a remarkably different antagonistic mechanism operative than that for glycine. Using GABA and six GABA agonists, we found that each of the agonists have three clearly defined atoms that can serve as attachment points at the $GABA_A$ receptor site. One of the three attachment points included a carbonyl or carboxylate

oxygen atom that has an important role. To explain the operative mechanism, we theorized that first a rapid two-point attachment occurs at the recognition site in the receptor where GABA or a GABAergic agonist binds, one from the positive end and one from one of the other two negative atoms in the ligand. We believed that the positive end of the agonist perhaps associates through hydrogen bonding to a β-carboxyl group in one of the aspartate molecules in the polypeptide at the receptor, whereas the negative attachment points probably bind through hydrogen bonding to arginine molecules in this polypeptide. The data suggested that the second negative site in the agonist immediately triggers a conformational change by pulling together the aforementioned groups by electrostatic attraction and thus opening the chloride channel. We proposed that the carbonyl oxygen is partly responsible for triggering the opening by formation of a double hydrogen bond to arginine. We think that this attraction is probably the first step inducing the conformational change. I refer the interested reader to Fig. 5 in Galvez-Ruano *et al.* (1995), which shows color-coded molecular electrostatic potential energy surfaces of GABA, two GABA agonists, DHMUSC, and THIP, as well as the GABA antagonist iso-THIP. These data clearly show that GABA, DHMUSC, and THIP have a prominent positive domain (seen as a red area) and two well-defined negatively charged regions (seen as a violet areas) and act as agonists, whereas iso-THIP has a prominent positive domain (red) but only one negatively charged binding region (violet) and acts as an antagonist. In the case of the five important GABAergic antagonists that we investigated in that paper, a fourth attachment site was not found and only two sites have been identified, i.e., one at the positive cationic part of the molecule and the other at the negative part, a carbonyl oxygen atom in many cases. These data support a hypothesis for the antagonists to simply bind to the recognition site, thereby blocking GABA from entering this site and subsequently preventing the opening of the chloride channel. Our research to this date (1996), especially regarding the mechanisms postulated for GABA and its agonists and antagonists which function at the GABA recognition site in the GABA receptor, was a first attempt to bridge atomic-level detail from molecular modeling data with published molecular neurobiological data for this important and phylogenetically newer inhibitory system. We had provided a highly probable answer to the question "How does GABA and a GABA agonist open the chloride channel?" I now wished to expand this approach to answer a similar question about glycine, the neurotransmitter in a phylogenetically older inhibitory system.

I reasoned that if transmitters and their antagonists fit into their receptor sites, each should be "supported" in this position even if the interaction is very fast. Therefore, I was not surprised to find that such data were reported in published molecular biological experiments, which were directed toward examining and identifying the effects of changing specific

amino acids in the polypeptides located in the glycine and GABA receptors in the CNS. We examined glycine and strychnine as well as GABA and R5135 again but used more sophisticated theoretical techniques to investigate the hydrogen bonding that yielded pseudo-rings (Aprison *et al.*, 1996c). In Figs. 1, I and 1, II, diagrams of the critical recognition elements that we identified as being important between receptor components interacting with glycine and strychnine, respectively, are shown. Note that in Fig. 1, I, the key recognition elements for binding by glycine at its recognition site appear to involve electrostatic interactions at regions A–C, with ARG 218, ARG 271, and aspartate (ASP) 148, respectively (Barnard *et al.*,1987; Devillers-Thiery *et al.*, 1993). At region C, the negative β-carboxylate group of ASP 148 binds to the positive nitrogen region of glycine, whereas at regions A and B there is double hydrogen bonding between the positive guanidinium groups in ARG 218 and ARG 271 with the two negatively charged oxygen atoms of glycine.

In addition to the data from the molecular biological studies, we believed that arginine had three characteristics that suggested it played an important role at inhibitory receptors. First, the three methylene groups in the side chain allowed flexibility for the guanidinium group to rotate and attach to negative atoms such as oxygen atoms in carboxyl groups or to free hydrated chloride ions floating in synaptic spaces. Second, the guanidinium group is positively charged at physiological pH. Third, and most important, the formation of a pseudo-ring arising from the positive guanidinium group interacting with an oxygen atom results in a planar positive region (Fig. 1), which can further interact with aromatic benzene groups of phenylalanine (PHE) and/or tyrosine (TYR) via charge-transfer complex. This is not shown in Fig. 1, but Schmieden *et al.* (1993) demonstrated that the aromatic rings in PHE 159 and TYR 161 are part of the recognition site, and we suggested that the pseudo-ring formed by the positive portion of the guanidinium group in ARG 218 and the oxygen atom O #1 in glycine can fit between PHE 159 and TYR 161, thus forming the charge-transfer complex at the recognition site. Data reported by Vandenberg *et al.* (1992a) also suggest that threonine (THR 204) is important, and we placed this amino acid (Fig. 1, I) to hydrogen bond at region D between one of the nitrogen (N #4) hydrogen atoms located at the positive end of glycine and the hydroxyl oxygen atom of THR 204. We believed that these hydrogen bonds and the pseudo-ring formation's ability to form a charge-transfer complex stabilize the glycine molecule at the neurotransmitter recognition site. Furthermore, when in this position, we believe the negative chloride ion, which is known to be hydrated, can bind to one of the two hydrogens attached to each N in the guanidinium group of ARG 271 and in the process lose some of its hydrating water molecules (Fig. 1, I, E).

The hydrated chloride ion is too large to enter the channel, but it can attach by electrostatic binding to the positive region of the guanidinium

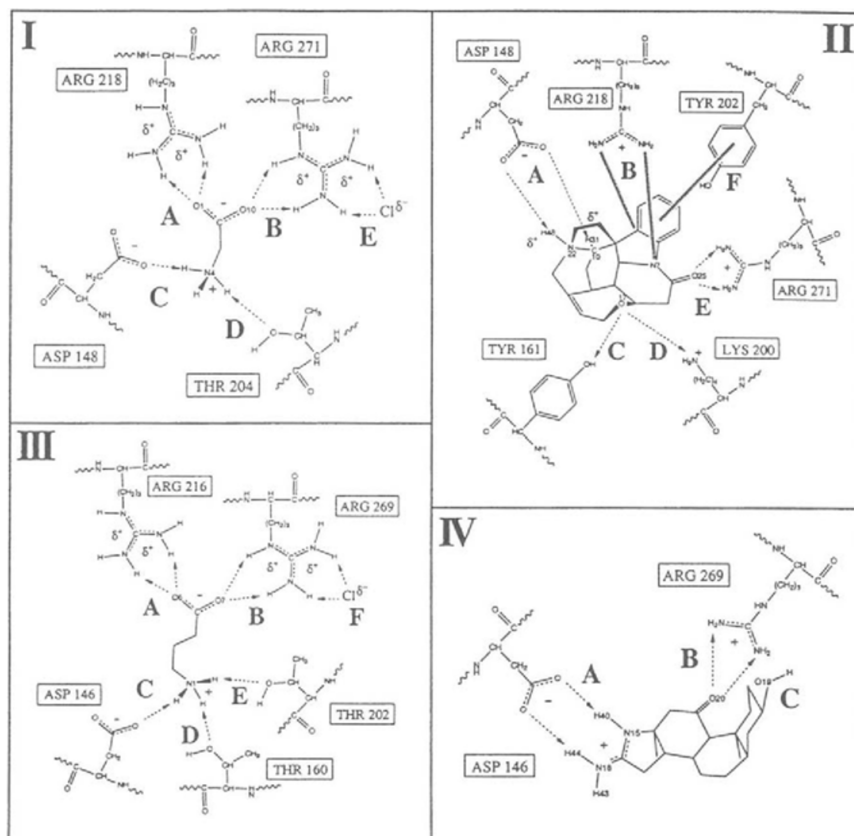

Figure 1. I – Representation of zwitterionic glycine binding to side chains ARG218, ARG271, ASP148, and THR204 of the glycinergic receptor. Regions A and B show complementary electrostatic associations of receptor side chains with the neurotransmitter caboxylate, while regions C and D show electrostatice attraction and hydrogen bonding, respectively, with the neurotransmitter ammonium group. Region E is where the partially solvated chloride ion associates, and is near the chloride channel orifice. II – Representation of the strychnine binding in the glycine receptor site. The key side chains involved are labeled. Interactions A–F stabilize the binding of the antagonist. A: A bidentate electrostatic interaction of ASP148 with two positive charged atoms. B: A bidentate electrostatic attraction with ARG218 guanidinium ion with two negatively charged atoms. C: A hydrogen bond from TYR161 and the lone pair electrons on O17. D: An electrostatic attraction between LYS200 and the lone pair electrons on O17. E: A bidentate electrostatic attraction of ARG271 with the negative carbonyl oxygen. F: A charge-transfer complex between TYR202 and the aromatic moiety on strychnine. III – Representation of zwitterionic GABA binding to its receptor site. Key electrostatic and hydrogen bonding interactions are labeled A–F as in I & II. IV – Representation of R5135 binding in the GABA receptor site. Stabilizing interactions from electrostatic and hydrogen bonding are labeled as sites A–C. *Adapted from* M.H. Aprison, E. Galvez-Ruano, D.H. Robertson, and K.B. Lipkowitz, *J. Neuros. Res.*, 43:372–381 (1996). *Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.*

group of ARG 271 even before glycine binds to its recognition site. However, only when glycine attaches at its recognition site in the receptor can glycine pull the ARG 271 to a position where chloride probably ends up near the top of the channel. We reasoned that the attraction of the negatively charged carboxyl oxygen of glycine and the two positively charged hydrogen atoms of the guanidinium group weakens the chloride attraction, allowing it to be released. Our quantum mechanical calculations support this explanation when we found that the –1.00e charge on the chloride ion is changed to –0.72e after it binds to the guanidinium group of ARG 271, but not in the presence of glycine. However, when glycine interacts with this group, the negative charge on the chloride ion changes to –0.79e, supporting our suggestion that chloride binding is weakened. Moreover, we speculated that once glycine binds with all of its receptor sites, the critical polypeptide side chains are pulled together by electrostatic attraction, toward the bound glycine, creating more space at the nearby channel opening for the partially solvated chloride ion to move through. Thus, we believe that the chloride ion is released in concert with the opening of the channel, allowing it to move efficiently into the channel for transport via a gradient of electrostatic forces downward toward the bottom of the channel, where it finally moves into the interior of the neuron, thereby making that cell more negative.

In Fig. 1, II, I again consider the antagonistic interaction by strychnine at the glycine receptor but this time show the specific amino acids located in the sequence of the polypeptide at the receptor and shown to be important (Ruiz-Gomez *et al.*,1990; Vandenberg *et al.*,1992b,c). Note that (i) the key recognition elements for binding appear to involve electrostatic interactions at regions A and B; (ii) one pendant group is negatively charged and the other is positively charged; (iii) the two positively charged nitrogen atoms in the guanidinium group of ARG 218 fit well with the two complementary negatively charged atoms in strychnine that we had previously proposed as two of the three critical binding sites (the reader should also recall that similarly charged atoms had been earlier identified in each of eight weak antagonists; Aprison *et al.*, 1995a); (iv) at the positive region of strychnine, the pendant carboxyl group of ASP 148 in the peptide, which is a carboxylate, can bind to it; and (v) we postulate that hydrogen bonding at C and D helps to stabilize the binding of the large strychnine molecule at the glycine receptor, as does the carbonyl oxygen atom, O #25, which binds to the positive region of the guanidinium group of ARG 271 by double hydrogen bonding. We found three more interesting items in this section. First, at region A in Fig. 1, II, two electrostatic binding domains are bidentate and we have proposed that such double-binding sites are superior to single-site attachments (Aprison *et al.*, 1995a). Second, when O #25 binds as already noted, the previously bound chloride ion to ARG 271 is not in an ideal position to be released near the top of the chloride

channel. We believe that the chloride ion becomes rehydrated, and since it cannot pass through the chloride channel in this state the glycinergic receptor is effectively blocked. Third, the benzene ring in strychnine is important because its presence allows the formation of a pseudo-ring in only one way, between O #25 in strychnine and the positive region in the guanidinium group of ARG 271 after hydrogen bonding occurs.

In Fig.1, III, the key recognition elements for the stable binding of GABA at its recognition site in the GABAergic receptor is shown. As noted for glycine, the amino acids believed to be important at this recognition site also have been reported. From the previous discussion of glycine, the reader should be able to see how we integrated the five identified amino acids located in the GABA polypeptide, ARG 216, ARG 269, ASP 146, THR 160, and THR 202, into our model of this site. As explained for the operative mechanism for glycine, the negative chloride ion at F can interact with ARG 269 at the GABA receptor via its guanidinium group; the chloride ion attraction is weakened when GABA binds and is similar to that described previously for glycine. Thus, both mechanisms we propose for glycine and GABA are the same in terms of opening the chloride ion channel, except two different but equivalent arginine molecules are involved at the GABA receptor. Furthermore, our calculations of the physical–chemical changes occurring when glycine interacts at its receptor are similar to those of the changes occurring in the pseudo-ring formation at A, B, and F in Fig. 1, III supporting the evidence of interactions between specific amino acids in the polypeptide and GABA. Finally, we believe that a charge-transfer complex mechanism as explained previously for glycine occurs also for GABA but with a slight difference. Since Amin and Weiss (1993) reported that the aromatic ring in TYR 157 and TYR 205 is essential for GABA-mediated activation, we postulated that the pseudo-ring formed at A in Fig. 1, III fits between these two tyrosine rings to form the complex and help stabilize it.

In Fig.1,IV, we show that the interaction of R5135 with the recognition sites appears to be mainly through two principal attachments at A and B, and perhaps C. The negative β-carboxyl group of ASP 146 attaches to the very positive end of R5135 at N #15 and N #18 through the attached positive hydrogen atoms H #40 and H #44, whereas at site C hydrogen bonding provides stabilization. At the negative end, the carbonyl oxygen O #20 attaches to the two positive regions of the guanidinium group of ARG 269 through a double hydrogen bond, a mechanism similar to the O #25 attachment in strychnine. The pseudo-ring formed at B, after minimization, resulted in changes in charge. It is interesting that due to the bidentate binding, and the presence and position of two methyl groups in R5135, its position at the GABA receptor allows the formation of a pseudo-ring in only one way, between O #20 and the positive guanidinium region of arginine. Before binding and minimization, O #20 had a charge of –0.22e; afterwards, it decreased to –0.40e. Our data explain that this antagonist is firmly bound

at the positive end by bidentate binding to ASP 146 in the recognition site and at the negative end through double bonding to the guanidinium end of ARG-269. Since the chloride ion is no doubt rehydrated and not moving through the channel, its antagonistic role is apparent.

The reader may wish to see Aprison *et al.* (1996c) for two color figures which show (i) zwitterionic glycine and GABA and illustrate the quantum-mechanically, geometry-optimized complex formed with the receptor side chains described previously; (ii) the van der Waals model of these two complexes which illustrates that no steric repulsions exist; (iii) a molecular electrostatic potential energy map; and (iv) a van der Waals model of each antagonist. I believe that our research has brought neuroscientists perhaps one step closer to clarifying the three-dimensional configuration of these two important inhibitory neurotransmitter receptors (glycinergic; GABAergic)!

In 1991, Maricq *et al.* reported the amino acid sequence for the $5HT_3$ receptor ($5HT_3R$) and compared this sequence with the sequence for three other members of a recently identified ligand-gated ion channel superfamily (nAChR, GABA, and glycine). In 1987, it was shown that the ligand-binding subunit of the glycine receptor shares homology with the nicotinic acetylcholine receptor (nAChR) polypeptide, and the comparison of the strychnine-binding subunit of the glycine receptor (Gly,48K) with both subunits of the $GABA_A$ receptor yielded data that supported the existence of a gene family for neurotransmitter-gated ion channel receptors which comprises both anionic and cationic channels, including the excitatory nAChR (Grenningloh *et al.*, 1987a,b). We then published a minireview to show how two excitatory neurotransmitters, ACh and 5-HT, and two inhibitory neurotransmitters, GABA and glycine, can bind to their respective recognition sites. We presented models for each transmitter interaction with specific amino acids previously identified from molecular biological studies. Furthermore, we identified molecular mechanisms that can explain how the binding process initiates ion flow through channels located within the postsynaptic membrane such that if the transmitter is excitatory depolarization occurs, and if it is inhibitory hyperpolarization occurs. Our molecular modeling data and the similarities of specific amino acids in the sequence in all four receptor polypeptides used to construct the four models support glycine, GABA, ACh, and 5-HT as being members of the same ligand-gated ion channel superfamily (Aprison *et al.*, 1996a). We were pleased to receive so many reprint requests for this minireview (>550) that we ran out of reprints.

I briefly describe my last research paper. Our model of nAChR presented in the minireview was new. However, several new molecular biological studies appeared while our paper was in press. Since I have been very interested in ACh for a long time, we incorporated the new data into our model and published an updated computer-generated model of this cholinergic receptor (Aprison *et al.*, 1996b). From the data in the literature,

12 amino acids were identified as being important in the polypeptide for ACh to function at the nicotinic cholinergic receptor. After studying possible three-dimensional configurations and paying attention to regions of enhanced or deficient π-electron density as well as stereo and electronic considerations such as hydrogen bonding and van der Waals forces, we realized that it was too difficult to show a three-dimensional molecular display of all 12 amino acids at the same time. We therefore used three figures in our paper to describe this model; although not reproduced here, I will attempt to describe it. We suggested that five amino acids, TRP 86, ASP 89, TYR 93, ASP 138, and THR 191, are associated with the cationic end of ACh, which is electron deficient. Three others, ARG 209, TYR 190, and TYR 198, are associated with the ester end, where an enhanced electron density is found. That left ASP 200, TRP 149, TYR 151, and THR 150 to be accounted for. Once ACh interacts at its recognition site and is in its quantum-mechanically most stable geometry, ARG 209, which is in the polypeptide and close to the recognition site, is attracted through hydrogen bonding between its guanidinium end and with the ester oxygens at the negative end of ACh. The oxygen atoms of ASP 200 hydrogen bond to the other side of the guanidinium group, forming a pseudo-ring. Two aromatic amino acids, TRP 149 and TYR 151, and not TYR 190 and TYR 198 as previously suggested, enhance the binding at this pseudo-ring through additional hydrogen bonding and charge-transfer complexation, with THR 150 functioning to further stabilize this evolving complex. We have postulated that this latter process allows the ion channel to twist, thus opening it.

I cite from Aprison *et al.* (1996b)[2]

> Since Maricq *et al.* (1991) report that glutamic acid (GLU 262) is present at the top of the Na^+ channel, we postulate that the negative β-carboxyl in this amino acid attracts the aqueous Na^+ and initiates the removal of a number of hydration water molecules. The process reported by Unwin (1995) fits beautifully with this concept, and his explanation and data support the idea that Na^+ is thus made available to move into the cation channel. Unwin (1993, 1995) proposes that the helices bend toward the central axis, allowing leucine side chains to project inward, forming a tight ring, thus resulting in closing the channel, which in turn prevents ions from crossing the membrane barrier. Once ACh acts at the receptor, localized disturbances occur at the receptor, resulting in small rotations of the subunits, which in turn are communicated to the structure in the membrane. Unwin (1995) eloquently shows that such events lead to an open channel.

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I thank Kenny Lipkowitz for joining me in these molecular modeling investigations. His expertise and interest were greatly appreciated. I also thank Daniel Robertson, the manager of the Computational Molecular Science Facility we used, for his help and keen insights in helping us solve some of our computer problems. Joseph Hingtgen and Jay Simon helped me experience "closure," each through his own discipline, and I am very grateful. In addition, I find that this occasion has given me an opportunity to review my life's research endeavors. In pursuing my very early dreams and goals, I was afforded the unique opportunity by two research directors, Dr. H. Himwich and Dr. J. Nurnberger, Sr., not only to pursue my own research in several different areas of neuroscience which led to some important contributions but also to meet and work with many intelligent, dedicated, and unique scholars, investigators, professors, and even "dedicated" administrators! Many of these encounters have resulted in long-lasting friendships for which Shirley and I are very grateful! Equally satisfying has been the opportunity given to me to teach eager medical and graduate students as well as brilliant postdoctoral students. Lastly, the opportunity to (i) serve in some of my societies as well as in numerous editorial positions and (ii) partake in peer review grant experiences has been very sobering if not rewarding!

As I finish this chapter, it is November 1999. I am into my 76th year, Shirley and I celebrated our 50th wedding anniversary last August, my son Barry has been married to Erin for 14 years and they have Margaret and Nathan, and my youngest son Robert has been married to Mary Kay for 15 years and they have Evan and Jennifer! We are very happy, pleased, and think that Henry and Etel Aprison would be proud.

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Appendix I

Chemical nomenclature	
4,5,6,7-Tetrahydroisoxazolo [5,4-*c*]pyridin-3-ol	THIP
4,5,6,7-Tetrahydro-4H-isoxazolo [3,4-*c*]pyridin-3-ol	iso-THIP
5,6,7,8-Tetrahydro-4H-isoazolo [4,5-*d*]azepin-3-ol	THAZ
5,6,7,8-Tetrahydro-4H-isoazolo [3,4-*d*]azepin-3 ol	iso-THAZ
5,6,7,8-Tetrahydro-4H-isoazolo [5,4-*c*]azepin-3-ol	THIA
5,6,7,8-Tetrahydro-4H-isoazolo [3,4-*c*]azepin-3-ol	Iso-THIA
5,6,7,8-Tetrahydro-4H-isoazolo [4,3-*c*]azepin-3-ol	iso-THAO
4,5,6,7-Tetrahydroisoazolo [4,3-*c*]pyridin-3-ol	Iso-THPO
Methyl (4,5,6,7-tetrahydroisoxazolo [5,4-*c*]pyridin-3-ol)	*N*-methyl-THIP
N,N-dimethyl-3-hyroxy-5-aminomethyl isoxazole	*N,N*-dimethyl-muscimol
3α-Hydroxy-16-imino-5β-17-aza-adrostan-11-one	R5135
[3-(Piperazinyl-1)-9H-dibenz(*c,f*) triazolo(4,5-*a*)azepin]	Pitrazepin
1,5-Diphyenyl-3,7-diazaadamantan-9-ol	Diaza
3-Hydroxy-5-aminomethyl isoxazole	Muscimol or DHMUSC
S-5-aminomethyl-2-isoxazolin-3-ol	*S*-(+)-dihydromuscimol
Amino acid identification	
Arginine	ARG
Aspartic acid	ASP
Glutamic acid	GLU
Leucine	LEU
Threonine	THR
Phenylalanine	PHE
Tryptophan	TRP
Tyrosine	TYR