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RECOLLECTIONS

The story of a toxic protein, 1888–1992

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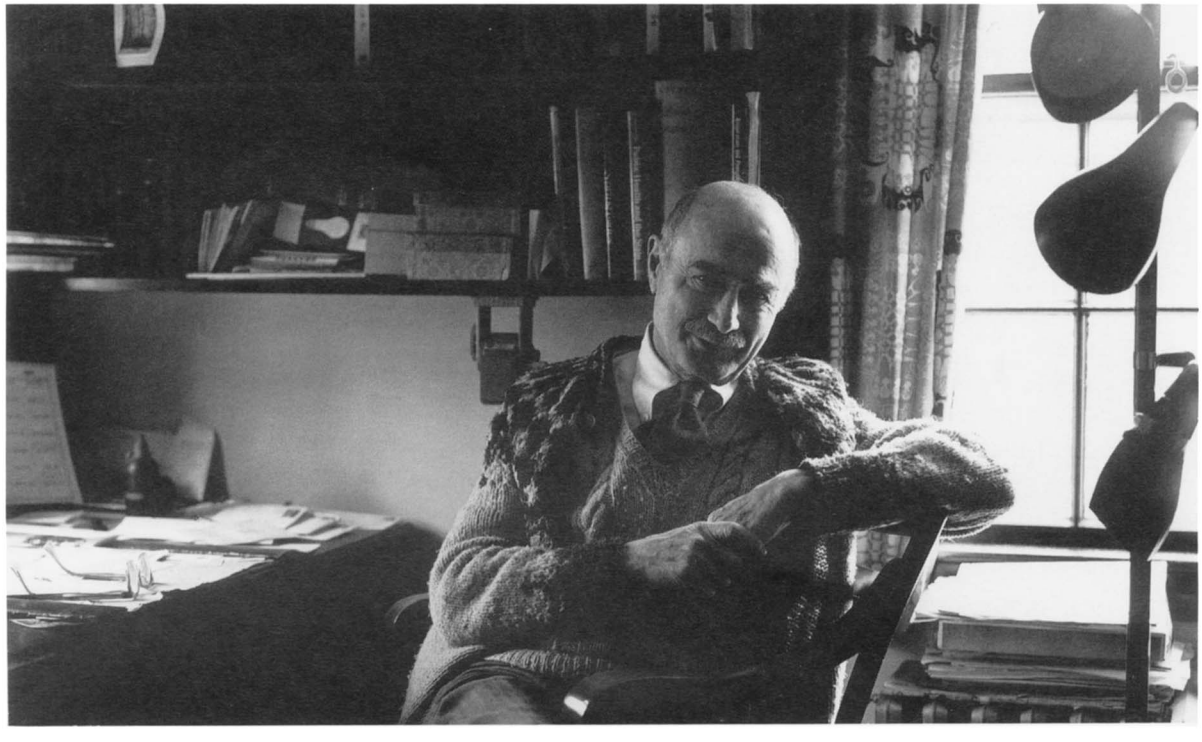

Fig. 1. A.M. Pappenheimer, Jr., in the earlier days of his career.

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A.M. Pappenheimer, Jr. received his Ph.D. in organic chemistry from Harvard University, where he was instructor and tutor in Biochemical Sciences (1930–1939). Following a National Research Council Fellowship at the National Institute for Medical Research in London (1933–1935) and a position in the Antitoxin & Vaccine Laboratory in Jamaica Plain, Massachusetts (1936–1939), Dr. Pappenheimer was Assistant Professor of Biochemistry & Bacteriology at the University of Pennsylvania (1939–1941). He then joined the New York College of Medicine, where he rose through the ranks from Assistant Professor to Professor and Chairman of Microbiology (1941–1958). From 1958 until his retirement in 1979, he was Professor of Biology at Harvard University, where he is continuing his work as Professor Emeritus. Dr. Pappenheimer is a member of the National Academy of Sciences and Fellow of the American Academy of Arts and Sciences. He received the Eli Lilly Award in Bacteriology in 1947 and a Guggenheim fellowship in 1966–1967. In 1990 Professor Pappenheimer was awarded the Paul Ehrlich Prize and Gold Medal, shared with John F. Collier, for his fundamental studies on diphtheria toxin.

On looking back over my scientific and academic career, I realize how much I owe to good fortune and to chance. I was fortunate from the very outset by my choice of parents, who not only provided me with an excellent education, but encouraged me to be interested in things for their own sake, rather than for what I might gain from them, and finally who instilled in me a sacred regard for the truth. I was fortunate too, to have received my education and to have become established in my field at a time when the competition was not as severe or as frenzied as it appears to be today. My father was a Professor of Pathology at Columbia Medical School and often complained that his background in chemistry was insufficient for his research. At Harvard I concentrated in the new tutorial field—Biochemical Sciences—in fact, I believe I was the first student to sign up. My tutor was Ronald Ferry, an

Assistant Professor of Biochemistry at Harvard Medical School. There was no biochemistry department in Cambridge at that time. The only biochemistry then available at the college was a half-course given by Professor L.J. Henderson. I will always remember a lecture in which he wrote the formulas of the naturally occurring fatty acids on the blackboard. He stared at them for what seemed a long time. Finally, he said: "That's funny. The carbon atoms are all multiples of two. I wonder why that is." My section man in the course was John Edsall. In my senior year Dr. Ferry urged me to go to medical school, but I decided that graduate school would give me a better preparation for biomedical research.

Through the help of Ronald Ferry, I obtained a summer job at the end of my senior year in 1929 to work as a technician with the Harvard Fatigue Laboratory. We set up a laboratory at 10,000 feet near Leadville, Colorado, to study blood and fatigue during adaptation to moderately high altitudes. Upon my return home at the end of that summer, I discussed my plans for graduate study with my father's friend Hans Clarke, Professor of Biochemistry at Columbia Medical School. He suggested that I seek the advice of James B. Conant, Professor of Chemistry at Harvard, who was becoming interested in the chemistry of biological molecules. I went to see him in the fall and he accepted me as a student, despite the fact that my undergraduate record in chemistry was far from brilliant and that I told him frankly that I was not interested in becoming a chemist, but wished the training to go into biological research. Things are far more democratic today. The student applies a year before, usually to many graduate schools, where the grades are carefully screened by a committee. Committees rarely like to take chances, and I probably would not even have been granted an interview if today's procedures had been in effect at that time.

I received my Ph.D. in 1932 and wrote my thesis on the effect of certain organic bases, such as imidazole derivatives, on the oxidation potential of heme. I also worked on the oxidation potential of the hemoglobin–methemoglobin system. The latter studies suggested that, like oxygenation of hemoglobin to oxyhemoglobin, methemoglobin formation was what we today would call a "cooperative" system. Our studies also led us to propose a structural model for the hemoglobin molecule in which each heme was linked to imidazole rings of two histidine residues in the globin molecule. This speculation was verified many years later when the three-dimensional structure was determined by X-ray crystallography.

I spent the following postdoctoral year working in the Department of Microbiology at the Harvard Medical School on pneumococcal polysaccharides and learning something about the biology and the immunology of infectious disease, which was to become my major life's interest. I then, fortunately, received a National Research Fellowship and spent 2 years in Sir Henry Dale's laboratory at the National Institute for Medical Research in London working on an anaerobic bacterial growth factor known as the "sporogenes vitamin." I attempted to isolate the factor first from yeast and then from a concentrated fraction derived from more than a ton of pregnant women's urine. This material was sent to me from Paris by the French chemist André Girard, after he had removed the steroid hormones. My work was done in collaboration with B.C.J.G. Knight and Sir Paul Fildes of the Middlesex Hospital who analyzed my fractions for growth factor using *Clostridium sporogenes*. Although I finally managed to isolate about a gram of an oil and showed it to be an acid with specific activity many thousand times the activity of the starting material, I failed to obtain the factor in crystalline form. Although I did not have much in the way of publications to show for my 2 years spent in London, I learned a good deal and made many lasting friendships. What was more, I knew exactly what I wanted to do upon my return home. I wished to isolate a pure potent bacterial toxin and to find out what made it so toxic.

It is interesting to reminisce about what biological research was like in the early 1930s. For example, Fildes and Knight had just claimed to have succeeded in "training" a tryptophan-requiring bacterium to learn how to make its own tryptophan! Bacterial genetics did not exist. Nevertheless, the ubiquity of biochemical reactions was beginning to be recognized in a few forward-looking laboratories, such as that of Sir F. Gowland Hopkins in Cambridge, and there were a few persons who realized that growth factors for microbes might turn out to be identical to vitamins required by mammalian species (Mueller, 1922). There were no federal research grants and there were no drug companies, such as Sigma with 800 numbers to supply, overnight, the vast variety of purified enzymes, metabolic substrates, monoclonal antibodies, etc., etc., that are now available on the market. There were no radioactive elements to make analyses easy. If one wanted an enzyme one had to isolate it oneself using the very primitive methods then available for fractionation of proteins. If one wanted an antibody, one had to immunize a rabbit and obtain evidence that it was specific for the antigen used to immunize. Most of our equipment was homemade. We certainly did not publish so many papers in those days, but I think we may have had more fun than many of those who enter biological research today.

When I returned to the Boston area in the fall of 1935, the great depression was still in full swing and jobs were hard to find. At that time it was taken for granted that toxigenic bacteria, such as *Clostridium tetanus, Clostridium botulinum*, and *Corynebacterium diphtheriae* required extremely complex media for their growth, containing proteins or their breakdown products (peptones) into which they secreted minute amounts of their potent toxins that, presumably, were also proteins. It seemed to me that if one could grow the organisms on a simple medium containing only known components of low molecular

weight, the culture supernate would contain only proteins synthesized by the bacteria themselves. I talked my project over with Howard Mueller, Associate Professor of Bacteriology at Harvard Medical School, who many years before had discovered a new amino acid, methionine (Mueller, 1923), as an essential growth factor for bacteria. Mueller thought he could find a place for me to work at the Antitoxin and Vaccine Laboratory in Jamaica Plain, provided I was willing to try to isolate diphtheria toxin, because one could not work with anaerobic bacteria at the State laboratory. Since Mueller had already worked out the conditions for massive growth of the diphtheria bacillus on a simplified peptone-free medium requiring only traces of still unidentified accessory factors present in yeast or meat extracts, this seemed a good idea. With Mueller's help, I was awarded an Edward Hickling Bradford Fellowship at the Harvard Medical School that provided me with living expenses. Elliott Robinson, Director of the Antitoxin Laboratory, kindly provided me with bench space, and Sylvia Johnson, a lab assistant, instructed me in the mysteries and folklore of toxin production, its conversion to toxoid, and in the methods of assay originally worked out by Paul Ehrlich. I immediately set to work and had no trouble obtaining heavy growth of toxigenic diphtheria bacilli on Mueller's medium. But alas, insignificant amounts of toxin were produced. So I started adding everything I could think of that might contain a "toxin-promoting factor," even peptone. After 2 months futile search, I finally decided to add toxin itself to see if it contained a factor necessary for its own biosynthesis.

The diphtheria bacillus is a strict aerobe and at that time was produced in so-called Fernbach flasks, in which a small amount of culture medium gave a large surface-volume ratio. The organisms grew as a veil or pellicle on the surface and were harvested about a week after inoculation. The laboratory had recently switched to Pyrex vessels and all of them were in use when I decided to make my toxin. But someone remembered that there were some of the old soft glass flasks stored in the attic which I could use. A further fortunate accident occurred, which caused me to leave these flasks, containing their peptone media, sitting in the incubator for 3 days before inoculation. When finally harvested a week later and the culture filtrate tested for toxin, the yield was about twice as high as Sylvia Johnson had obtained in Pyrex using the same medium. The only difference seemed to be the flasks themselves. I repeated the experiment, but this time inoculated an equal number of Pyrex flasks as controls, every one of which produced a lower yield than the soft glass ones. So I broke off a piece of the soft glass, ground it to a powder, and titrated the powder in Pyrex. The results were surprising. The mere addition of 0.3 mg powdered glass to 25 mL culture medium caused a small but significant increase in growth and toxin yield, but more surprising still, the addition of 5 or 10 mg of glass inhibited toxin production almost completely without reducing the bacterial growth at all. Obviously something was being leached out of the soft glass. I had done my Ph.D. work on heme compounds, and iron catalysis was very much in vogue at that time, so almost the first thing I tried was the addition of iron salts. To my great delight, the addition of only 1–2 μg iron (as $FeSO_4$) increased toxin production by 30% or more, whereas 15 μg gave nearly complete inhibition. The curve superposed on that of powdered glass. Suddenly everything became clear. Looking back at previous publications, it became apparent that all successful preparations of media involved a step in which a flocculent precipitate formed on boiling the alkaline medium and was removed by filtration. I guessed that the amorphous precipitate was calcium phosphate, which carried down contaminating traces of other metal phosphates including iron. All I had to do was to add Ca^{2+} to Mueller's medium, boil, and filter. The medium then gave good yields of toxin and with a few minor modifications became suitable for large-scale production, both for easy isolation of toxin in pure form and for practical production of toxoid for immunization.

When I came to write up this work, I found that several other laboratories had reported the inhibitory effect of iron salts, but no one seemed to have realized its importance or significance. Two papers with Sylvia Johnson as co-worker were submitted in 1936 to the *Journal of Experimental Medicine* and came back by return mail with a curt note from the editor saying that their journal only published work of basic importance and advised sending them to some technical journal. This was most discouraging, but when I sent the manuscripts to the *British Journal of Experimental Pathology* they were accepted without modification, and all 300 reprints we had ordered disappeared almost overnight. Elliott Robinson decided that I was useful and created a new position as Senior Chemist for me to fill.

The next 3 years at the Antitoxin Laboratory were happy and fruitful ones. The isolation of highly purified toxin was now relatively easy, and in one summer I grew 300 L of culture, concentrated the supernate down at 25 °C in a home-made vacuum still, and isolated 25 g of microscopic crystals of toxin containing 10,000 lethal doses per mg per kg susceptible animal. The purified toxic protein was denatured and aggregated at pH values below 6. Today, only a few hundred micrograms are needed to characterize a protein or of its cloned DNA to determine its complete amino acid sequence. But in 1937 I hydrolyzed six 1-g samples in boiling sulfuric acid to determine the basic amino acid content of the toxin by isolation of arginine flavianate, histidine nitranilide, and lysine picrate, in yields that we now know were between 50 and 60% of the true values. This amount of purified diphtheria toxin may now be purchased from Sigma for about $500,000.

Because of its high potency, I still wished to make absolutely sure that we were really dealing with the toxin itself and not some innocuous protein containing traces of copurified toxin. I decided that a quantitative study of

its precipitation by specific antitoxin should give a definitive answer to this question. Michael Heidelberger and Forrester Kendall had just described a quantitative method for the study of specific precipitation of pure protein antigens by their homologous rabbit antibodies. Diphtheria antitoxins, however, are usually prepared from the sera of horses that have been under immunization with formalinized toxin (toxoid) for a long period of time. Once again I was very lucky, because the quantitative behavior of the toxin–antitoxin "flocculation" reaction proved to be very different from protein–antiprotein reactions in the antisera of other animal species. Instead of a sharp maximum point of precipitation at which both antigen and its antibody were specifically precipitated, there was a broad "equivalence zone" of complete precipitation during which the linear increase was solely due to the antigen, i.e., toxin. Because at least 95% of my purified toxin was precipitated within this linear zone, it was at least 95% immunologically pure.

In 1938 the first Swedish oil turbine-driven ultracentrifuge in this country was installed in Prof. J.W. Williams' laboratory in the Chemistry Department at the University of Wisconsin. Jack Williams kindly agreed to let me bring some toxin to Madison and determine its molecular weight by sedimentation and diffusion. It was homogeneous by both criteria. I made several trips to Wisconsin after that and with Williams and Harold Lundgren, we studied the toxin–antitoxin reaction in the ultracentrifuge and were able to obtain definitive evidence, for the first time, that one molecule of antibody could bind two molecules of antigen, as had been predicted by Heidelberger (1939) and by Marrack (1938). With Mary Petermann, we confirmed our earlier suggestion based on immunochemical data, that the molecular weight of protease-treated antitoxin was less than 2/3 that of the native antitoxin molecule, because an inactive piece was split off (now known as the Fc portion of IgG) and that the antigen-binding sites must be relatively close together in space. I might add that in those days, sedimentation in the ultracentrifuge was followed by photographing a scale placed behind the sedimenting proteins and measuring the displacement of the lines at the boundaries using a photomicrometer. Concentrations were calculated from the areas under the displacement curves. It was a time-consuming procedure. Nevertheless, most of our molecular weights and calculations were not very far off from what we now know to be the true values.

I had now been working at the Antitoxin Laboratory for nearly 4 years. My work had begun to attract some attention, and people had begun to write me to ask if they could work in my laboratory. This was, of course, impossible because of lack of funds and space. Besides, what I really wanted to do was to teach in a university. I knew I had no future at Harvard and therefore when I was offered an Assistant Professorship in the Department of Bacteriology at the University of Pennsylvania, I accepted. This turned out to be a mistake, because the person in charge of teaching bacteriology was only interested in taxonomy and diagnostic methods. He did not wish to have new-fangled ideas about bacterial metabolism and immunochemistry introduced into his course. I was only allowed to become a section man in the Biochemistry Department.

Two years later, I was offered an Assistant Professorship at New York University Medical School by Colin Macleod who had just been brought in as head of the Department of Bacteriology and Immunology. I will never forget the interview with Colin at the Rockefeller Institute, sitting on a balcony overlooking the East River on a beautiful spring day. Colin, who was only 32 (my own age) told me his ideas of the kind of department he wished to set up. They coincided closely with my own ideas. He also told me about the exciting work he had been doing with Avery on purification of the pneumococcal transforming principle, which appeared to be closely associated with DNA or perhaps even might be DNA itself. The only condition I laid down was that my graduate student, Alan Bernheimer, be given a teaching fellowship so that he could finish work for his doctoral thesis at N.Y.U. Bernie is still in the same department and has recently retired as Professor Emeritus.

Less than 6 months after we had moved to New York, the Japanese bombed Pearl Harbor and the United States entered World War II. Our first priority from then on was, of course, directed toward helping the war effort. The teaching of medical students was accelerated, and basic research was no longer of primary concern. I had received a defense grant from the Office of Scientific Research and Development to work on toxins elaborated by anaerobic bacteria causing gas gangrene in wound infections. This enabled me to hire Edelmira D. Hendee as my assistant. She remained my close collaborator and friend until I left N.Y.U. in 1958. However, before the end of 1942, I became convinced that my research on gas gangrene was not going to contribute significantly to the war effort. I managed to get my OSRD grant transferred and obtained a Captain's commission in the Sanitary Corps. I hoped to be assigned to a Medical General Laboratory about to be sent to North Africa, but within a few days of putting on my uniform, I received top secret orders to the First Service Command in Boston where I was to report to Professor J. Howard Mueller at the Harvard Medical School! It seemed that both the British and the Americans had grown apprehensive that the Germans and Japanese might be considering waging bacterial warfare against the Allies. For an entire year I worked behind locked doors on the production of botulinus toxin, its detoxification, and on immunization against botulism. In fact, I think I was the first person ever to be immunized against that poisonous protein. By the summer of 1943 the work had progressed sufficiently for the newly organized Camp Detrick to take over production of botulinus toxin and immunization of the personnel concerned. Somehow, I managed to get transferred to the 19th Med-

ical General Laboratory, an outfit about to be sent to the Southwest Pacific. After nearly 2 years of overseas "service?" (consisting mainly of chasing butterflies and learning to play cards) in New Guinea, the Philippines, and finally Japan, I arrived home a few days before Christmas 1945, eager to renew acquaintance with my family, including a son I had never seen, and very eager to get back to work.

When I decided back in 1935 to isolate a bacterial toxin, I had the naive idea that it would contain a tightly bound poisonous cofactor such as strychnine or the arrow poison curare, which would account for its toxicity. However, analysis of my purified diphtheria toxin indicated that it contained nothing but amino acids. In short, it was just another protein. So why was it so toxic? My early calculations suggested that a very few molecules, perhaps only one, were sufficient to kill a sensitive cell. Life in the army had given me plenty of time to think about this question. It seemed to me that the crucial role played by iron in the control of toxin biosynthesis might furnish a clue as to its mode of action. Therefore, upon returning to academic life in 1946, I decided to reinvestigate, quantitatively, the secretion of toxin and of the heme precursor, coproporphyrin III, in relation to the iron content and to the iron enzymes of the diphtheria bacillus. Over the next year or two, Edelmira Hendee and I found that toxin and porphyrin were only secreted during the terminal stages of bacterial growth, after all the external supply of iron had been exhausted and the bacteria were becoming pale and anemic. We found a quantitative relationship between the decrease in cellular iron enzymes, such as the cytochromes and catalase, and secretion of toxin and coproporphyrin III. This led us in 1947 to suggest that diphtheria toxin might be the protein moiety of a major cytochrome of the diphtheria bacillus and that it might exert its lethal action by interfering with the cytochrome system in sensitive animal cells, either its function or possibly its biosynthesis (Pappenheimer, 1947).

Although this seemed to be an attractive and reasonable hypothesis at the time, we now know that toxin has no effect whatsoever on the functioning of the animal cytochrome system, although it does block its biosynthesis. Nevertheless, we succeeded at first in obtaining what appeared to be exciting and novel circumstantial evidence consistent with our theory. During the summer of 1949, I attended a Growth Society Symposium at the University of Vermont. Carroll Williams from Harvard spoke on the metamorphosis of the large saturnid moth, *Platysamia cecropia*, from a caterpillar to a diapausing pupa that overwinters within a cocoon and finally develops into and emerges as an adult moth. Williams described how the classical cytochrome system present in the caterpillar almost completely disappears during pupal diapause and is replaced by a cyanide-insensitive respiratory system. Then during adult development there is a rapid and progressive reappearance of the complete cytochrome system once more, which can be followed day by day in the developing pupa. As soon as I returned to New York, I sent Carroll some toxin, which he injected into a few diapausing and a few developing pupae. To our great delight, relatively small amounts (<1 μg) of the toxic protein, after a short lag of only a few hours, stopped adult development dead in its tracks, but even large doses seemed to have very little effect on diapausing pupae. In contrast to potassium cyanide, which causes immediate but reversible inhibition of adult development, the action of toxin was irreversible and permanent. Upon receiving this news, I took the next train for Boston. Carroll and I agreed to work together in Cambridge during the following summer, and in May 1950, I initiated my first attempt at caterpillar farming at our place in Scotland, Connecticut, by setting out ca. 1,000 cecropia eggs on 16 wild cherry trees covered with nylon netting for protection against birds. By fall we had harvested 828 cocoons and Carroll had taught Mrs. Hendee and me how to put plastic windows into pupae so that one could follow day-by-day adult development, a process that requires exactly 21 days at 25 °C from termination of diapause to emergence of the adult moth.

On further study we found that during diapause, the classic cytochrome system could only be detected in two small sets of muscles—those involved in opening and closing of the spiracles through which the gases, O_2 and CO_2, exchange and those controlling the wiggling of the abdomen. These were destroyed by the toxin, but other tissues were unaffected and the diapausing heart kept beating slowly for weeks, provided the animals were kept in a humid atmosphere to prevent desiccation. Our studies with Williams led to a study of the properties of a new cytochrome that Sanborn and Williams had earlier discovered in the midgut of caterpillars and was present in the diapausing heart. It was named cytochrome b_5 by Britten Chance who showed it was present in all or most animal species.

While there can no longer be any doubt that diphtheria toxin does block cytochrome synthesis in developing pupae, we now know that this is not its primary target. It remained for Norman Strauss, who came to work for me as a postdoctoral fellow 1956–1958 and Edelmira Hendee using radioactive amino acids that had just become available, to show that the primary effect of the toxin was to block *all* cytosolic protein synthesis in cultured sensitive animal cells. Finally, the "coup de grace" was delivered by my graduate student John Collier after I had moved back to the Biology Department at Harvard University in 1958. John showed that minute amounts of the toxin blocked amino acid incorporation into protein in a cell-free system, provided that NAD was present as a cofactor. Later, as a postdoctoral fellow in Geneva, John proved that elongation factor 2 (EF2 or eukaryotic polypeptidyl translocase) is the specific target molecule that is inactivated. Ronald Goor, who followed Collier as a graduate student, and I then found that the inactivation of EF2 could be reversed by nicotinamide. Finally, it was

shown by Japanese workers and independently confirmed in our laboratory that the toxin was an adenine-dinucleotide-ribosyl transferase that catalyzes the reaction:

$$NAD^+ + EF2 \rightleftharpoons ADPR\text{-}EF2 + \text{nicotinamide} + H^+.$$

We had finally found the primary target of diphtheria toxin, but many new questions were raised. We soon found that the toxin molecule contained two cystine residues, the first of which was easily reduced and which subtended an exposed loop of 14 amino acids, and 3 of which were arginine residues sensitive to proteolytic cleavage. Both our laboratory and John Collier's, who was now in Los Angeles, independently showed that intact toxin is enzymatically inactive and that after reduction and cleavage of the exposed loop, split into two nontoxic fragments A and B. All the enzymic activity is located on the N-terminal fragment A, but B is required to bind to receptors on sensitive cells and for transport of A across the plasma membrane into the cytosol. How is this accomplished? When Patrice Boquet came over from the Pasteur Institute to work with us, he found that although native enzymatically inactive diphtheria toxin bound no labeled Triton X-100, after denaturation in 0.1% SDS, or at pH values below 6, or by cleavage of the exposed loop and reduction of its disulfide, more than 40 molecules were tightly bound. The lipid binding was restricted to fragment B. It was also shown that the receptor recognition site was located near the C-terminal end of B. This suggested that the molecule contained three domains: (1) an N-terminal ADPR-ribosylase domain specific for eukaryotic EF2, followed by (2) a lipid-binding region similar to that of typical membrane proteins and (3) a C-terminal positively charged receptor-binding domain. EF2 is a G protein that binds GTP, which is dephosphorylated to GDP when EF2 binds to ribosomes. As shown in Jim Bodley's laboratory ADPR is bound to a highly conserved region of EF2 that contains a unique posttranslationally modified histidine residue—diphthamide.

When I first began to work with diphtheria toxin in 1935, it was well known that one must avoid ever letting the pH fall below 6. At slightly acid pH, the toxin aggregated, became insoluble, and lost more than 90% of its toxicity. However, the supernates always retained some toxicity when tested in animals. No one realized the significance of this small amount of residual toxicity for some 40 years. Then Sandvig and Olnes in Oslo and others demonstrated convincingly that a low pH is required during cell-mediated endocytosis for fragment A transport across the plasma membrane. In other words, the conformational change that takes place during denaturation is essential for entry into the cytosol. At the very low concentration of approximately one molecule per endocytotic vesicle, inactivation of toxin by aggregation at low pH is obviously impossible (for reviews see Pappenheimer, 1955, 1977, 1979).

We still did not know the complete amino acid sequence of the toxin molecule. It had been known since the work of Loeffler a century ago, that nontoxigenic bacteria, indistinguishable from toxigenic diphtheria bacilli were frequently present as part of the normal throat flora of man. However, it was not until 1951 that it was discovered by Freeman that *all* toxigenic strains are lysogenic for a bacteriophage β that carries a *tox* gene in its genome. We knew that the expression of this gene was controlled by the bacterial host. However, when Tsuyoshi Uchida came from Japan to work with us in 1969, he soon showed that the phage *tox* gene was the structural gene for toxin and not a bacterial gene as I had thought. This enabled him to isolate several nontoxigenic bacterial strains that produced nontoxic proteins (CRMs) that cross-reacted with antitoxin and have proved extremely useful in many ways. They also made possible the easy isolation and cloning of the *tox* gene. Two laboratories, one in Italy and the other in California, independently determined the sequence of the entire *tox* operon including its promoter, leader sequence, and the nucleotides coding for its 535 amino acids. The DNA was from phage carried by two bacterial strains that were markedly different and had been isolated 50 years apart from the throats of two individuals who lived in different countries. The sequence of all 1,942 nucleotides was identical!

Recently, the three-dimensional crystalline structure of the toxin molecule has been published (see Kinemage 1). In the ribbon drawing shown in Figure 2, the three domains are clearly visible. The NAD binding site on the fragment A domain (residues 1–193) is formed by β strands CB2, CB3, CB7, and by the short helix CH3 and the loop CL2. The helical lipid-binding domain contains amino acid residues 205–378 and is connected to the A domain by the TL1 loop. Several years ago, Deborah Zucker isolated and mapped a large number of monoclonal antitoxins selected for high affinity for the native, intact diphtheria toxin molecule. We concluded that one of these that inhibited *both* enzymatic activity and binding to sensitive cell receptors must be directed against a very small region where all three domains must come close together in space. Note that the CB7 β strand of the A domain lies very close to RB6 of the receptor-binding domain, as predicted, even though it lies at least 300 residues away. For someone who has worked with diphtheria toxin for more than 55 years, the beautiful studies by David Eisenberg and his coworkers on its three-dimensional structure have been particularly gratifying. So many of our speculations, based on simple old-fashioned methods, have turned out to have been correct, and other questions that puzzled us have been answered.

Ever since the discovery of diphtheria toxin by Roux and Yersin (1888), diphtheria and its toxin have served as models for other infectious diseases caused be toxin-secreting bacteria. Toxins secreted by *Cl. botulinum, Vibrio cholera, Pseudomonas, Escherichia coli* LT, pertussis, and others all contain three domains with functions analogous to those of diphtheria toxin. Their genes are all car-

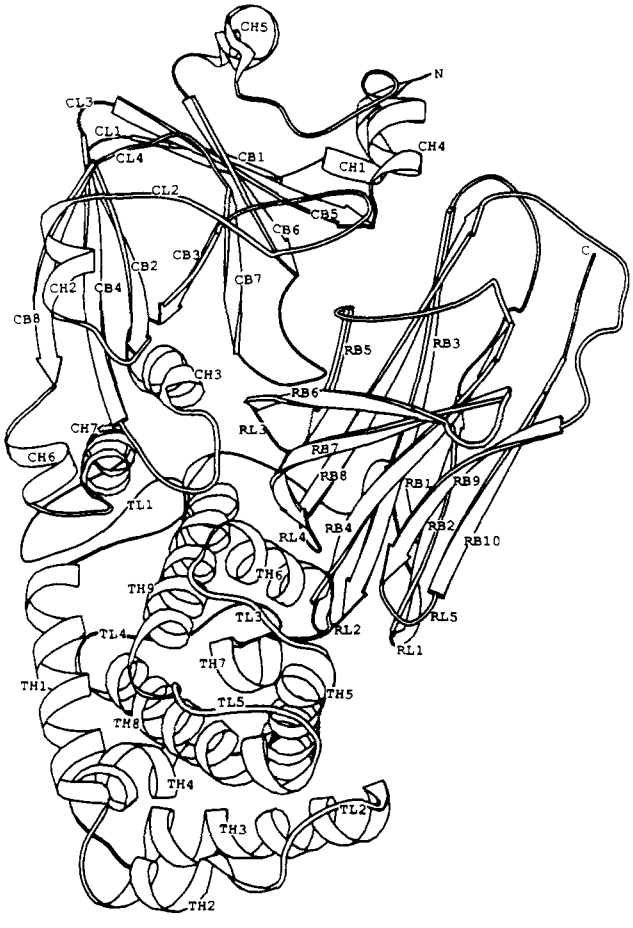

Fig. 2. Ribbon drawing of diphtheria toxin, labeling each secondary segment. The first letter denotes the domain: C for catalytic, T for transmembrane, and R for receptor binding. The second letter denotes the secondary structural class: H for helix, B for β strand, L for loop. The third symbol is the sequential number from the N-terminus of each domain. The residue numbers in each segment are as follows: CH1: 2-7, CB1: 11-14, CB2: 16-24, CH2: 28-34, CB3: 52-57, CH3: 58-66, CB4: 76-86, CB5: 88-96, CH4: 99-106, CH5: 120-126, CB6: 130-136, CB7: 147-152, CB8: 159-166, CH6: 168-173, CH7: 176-186; TH1: 205-221, TH2: 225-231, TH3: 238-257, TH4: 258-269, TH5: 274-288, TH6: 297-307, TH7: 310-315, TH8: 326-346, TH9: 356-378, RB1: 386-390, RB2: 393-399, RB3: 412-424, RB4: 428-438, RB5: 447-453, RB6: 455-465, RB7: 467-480, RB8: 483-495, RB9: 513-520, and RB10: 525-534. (Reproduced from Choe, S., Bennett, M.J., Fujii, G., Curmi, P.M.G., Kanterjieff, K.A., Collier, R.J., & Eisenberg, D., 1992, The crystal structure of diphtheria toxin, *Nature 357*, 216-222, with permission of the publisher.)

ried on mobile elements such as phage, transposons, or transposon-like DNA. Their targets are specific arginine, asparagine, or diphthamide residues on eukaryotic G proteins.

As I write these reminiscences, almost every branch of biology has succumbed to the molecular and genetic approach that is now in fashion. Standardized routine methods are now at hand for isolating, cloning, and rapidly determining the nucleotide sequences of genes from any animal, plant, or bacterial species, including their promoters and regulatory regions. By genetic engineering, one can now construct modified genes and hybrid genes coding for chimeric protein molecules at will. Obviously, the more graduate students and postdoctoral fellows that can be put to work on problems of this kind, the faster one can obtain publishable results. It seems to me that the current urge to build up a large team in order to "get ahead" and gain a desirable tenured position often leads to rather unhealthy competition among younger investigators and to exploitation of students and postdoctoral fellows. Under our present system of funding, the leader of a large and expanding research team must spend a major portion of his or her time applying for research grants and does not have much spare time to spend with students.

I have always enjoyed working in the laboratory and when administrative duties kept me away from the bench for any considerable length of time, I would begin to worry that I might have lost the ability to work with my own hands. Not only is lab work fun (especially when things go well), but it keeps one in closer and more intimate contact with one's students and one's coworkers. It also serves to remind one constantly of how much easier it is to sit in a chair and suggest experiments for others to carry out than it is to go out and do them oneself. It has always been my feeling that research should be fun, and I like to think that even in this day and age, important and innovative contributions can still be made by individuals working in small groups. In hindsight, I believe that I have received as much satisfaction from the friendship and contributions made by my students as from any of the honors that may have come my way, and I like to think that I may have had something to do with starting them off on the road to success. If I had to do it all over again, I do not think I would wish my scientific life to be very different.

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References

Pappenheimer, A.M. (1947). Bacterial toxins. *Fed. Proc. 6*, 479.

Pappenheimer, A.M. (1955). The pathogenesis of diphtheria toxin. In *Mechanisms of Microbial Pathogenicity*, pp. 40-56. Cambridge University Press, Cambridge.

Pappenheimer, A.M. (1977). Diphtheria toxin. *Annu. Rev. Biochem. 46*, 69.

Pappenheimer, A.M. (1979). Transport of diphtheria toxin A fragment across the plasma membrane. In *Transmembrane Signaling*, pp. 669-674. Alan Liss, New York.