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A LIFE WITH BIOLOGICAL PRODUCTS[1]

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CONTENTS

In 1925, I took my first course in bacteriology. The time was propitious. In the later part of the nineteenth century, bacteriology became a distinct biologic science. The germ theory of disease had been proven and the causative organisms of many diseases had been isolated. During the first quarter of this century, many investigations focused on the role of bacteria and their antigens in infectious diseases and their immunological prevention and treatment.

In my 64 years of association with bacteria, bacteriology came of age and

[1]Dedicated to Dr. Roderick Murray (1909–1980).

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recently entered the molecular biological phase. It has been my high privilege to participate in the development and standardization of biological products both nationally and internationally to insure their safety, purity, and potency. My emphasis has been on bacterial vaccines, and antisera and general standards.

During these years, it was my good fortune to become acquainted with outstanding pioneer scientists worldwide who were devoted to problem-solving of the mysteries of how bacteria effect infectious diseases and their control, and the improvement of public health in general. I am deeply indebted to them for their stimulus and help, as well as to associates, and my staff scientists and technicians.

EARLY DAYS IN ARKANSAS

I was born January 20, 1901 in a Christian home where discipline, truth, love, and spiritual growth shaped my adult life. Both parents descended from early British emigrants. The name Pittman appeared in New England as early as 1653. In 1834, Samuel settled on a farm in the Prairie Grove valley of the Ozark mountains in northwest Arkansas. My father, James Pittman, grandson of Samuel, was born in 1871 in a large log cabin that was the first post office in the valley. He was the first of seven children. His higher education was at the University of Arkansas, in nearby Fayetteville, and St. Louis Medical College. Work as a street car conductor helped defray expenses.

My mother, Virginia Alice McCormick, the youngest of seven children, was born in 1871 in Churchville in the Shenandoah Valley of Virginia. She was a cousin of Cyprus Hall McCormick, inventor of the harvester reaper. Her parents migrated via the Ohio, Mississippi, and Arkansas rivers to Evansville, Arkansas in the Ozarks. There her father operated a sawmill. Attracted to the sawmill by the machinery, Mother learned to identify trees by bark on the logs. Later, the family moved to Prairie Grove where her parents contracted typhoid fever and died. Mother had many talents: mechanical ability was used to repair her sewing machine and the brass front 1914 Ford car (there was no garage in the township). She had a "green thumb," was very observant and made logical interpretations, e.g. weather forcasts or the character of a person; was an excellent seamstress; did fancy needle work and oil painting; was hospitable, a quiet leader, and promoter of education; kept a small lending library; and taught rudiments of piano playing.

After marriage in 1899, Father began medical practice in Allen, Cherokee Nation (Oklahoma). Malaria infection influenced their return to Prairie Grove within the year. They had three children. About 1909, the family moved to Cincinnati (114), a country village that had been a large Indian trading center before the Frisco Railroad was constructed nearby. Father was the only doctor

for miles around. He responded to calls, day or night, sunshine or rain, a true horse and buggy doctor (34) who passionately served all people. One time a cholecytectomy was performed on a patient without funds in a room of our house.

My first medical experience was administration of anesthesia during the setting of a fractured bone. The ether was poured on cotton placed over an openly woven egg beater and held over the patient's nose. Then, when a federal law required that all children be vaccinated against smallpox, I served as assistant in the vaccination of school children in about 10 rural schools.

My early education was in a two room school and at home. We were taught the fundamental subjects; most importantly, the motivation to succeed and discipline.

At home there was oral reading of current and classic novels and the Bible at night by kerosene lamp light. College education was considered essential.

Three years of high school was in Prairie Grove. I lived with grandparents on their farm. Then I spent two years at a newly organized college in Siloam Springs, 12 miles north of Cincinnati with a focus on piano music. Long hours of practice precipitated a spinal complication that has been a life companion. Music study was discontinued. This was good; I was not truly talented.

In December 1919, my father died after an appendectomy from peritonitis, said to be caused by infected catgut. On his death bed, he requested Mother to take the three children to Hendrix College, a small accredited Methodist College with a ratio of ten boys to one girl. Available money was supplemented with canned vegetables and fruit that were prepared during the summer and with Mother's sewing. Each child graduated with honors and, after graduate study, entered into medical work. My sister Helen became a public health nurse and teacher; my brother James, a general surgeon; and myself, a microbiologist engaged in the federal control of biologic products.

At Hendrix, I majored in mathematics and was awarded the college Mathematic Medal. One year, I taught a class in algebra. I also majored in biology. Graduation, *magna cum laude,* was in 1923. The dedicated professors stimulated and motivated students to excel at college and throughout life. After serving for two years as teacher and principal of the Academy of Galloway Female College, I went to the University of Chicago for graduate study to prepare for some form of medical work. Funds for medical school were not available.

THE UNIVERSITY OF CHICAGO, 1925–1928

I arrived at the University of Chicago in late August 1925 with $600 saved from the $2100 received for the two years of teaching. Without prior arrange-

ments, I was accepted as a candidate for a MS in the Department of Bacteriology and Hygiene. Payment of the tuition of $75 per quarter was supplemented with funds received for babysitting at night at 35¢ per hour.

Bacteriology was a relatively new and exciting biologic science. Status of development in the field was well described by 83 contributors to *Newer Knowledge of Bacteriology and Immunology* edited by Jordan & Falk (38).

Dr. Edwin Oakes Jordan (1866–1930) (42) was selected by President Harper as one of the original faculty members of the University, in the Department of Zoology. He was a student of William Thompson Sedgwith, a leader of his day in applied biology and sanitation (100). He taught first a course in Sanitary Biology and, in 1894–95, courses in General Bacteriology. In 1900, bacteriology became a part of the Department of Pathology and Bacteriology. A number of students became outstanding leaders. In 1913, the Department of Bacteriology and Hygiene was created with Dr. Jordan as Chairman (42).

The spirit of research permeated the Department. As a student it was my high privilege to be associated with the scholarly faculty and the graduate students that were making outstanding contributions. The early days are well described in a history of bacteriology at the University by Koser (42).

My courses and research in this Department and in other Departments of the University provided a fitting foundation for post-graduate work. I was impressed with a course in vital statistics. The reference book was the PhD thesis of I. S. Falk (25) who received this degree at the age of 23. Research on the pneumococcus was done under his supervision. My MS thesis project was on the S and R colonial forms of the pneumococcus and their pathogenicity (89).

A few months before I was to receive the MS degree, the department offered me a fellowship to continue my studies for a PhD: an opportunity undreamed of. After the 1918–19 influenza pandemic, the Metropolitan Life Insurance Company had provided funds for two fellowships for investigations on respiratory diseases. The $75 received per month paid for tuition and one good meal per day.

Courses in the Department of Bacteriology and Hygiene were supplemented with very valuable courses in the Medical School. They included biochemistry, human anatomy dissection (thorax and abdomen), histology, and pathology. My thesis research was directed by Professor Mercy A. Southwith in the Department of Pathology. The title was Pathogenesis of Experimental Pneumococcus Lobar Pneumonia (97).

In the fall of 1928, I left the University to work with Dr. Rufus Cole at the Hospital of the Rockefeller Institute for Medical Research (RIH) (predecessor of the Rockefeller University). Dr. C. P. Miller, Department of Medicine, recommended my appointment after a consultation with the bacteriology

department. I returned to Chicago during my 1929 vacation, defended my thesis—written in New York after work hours. The PhD was conferred at the August Convocation.

THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH, 1928–1934

It was a privilege and great opportunity to spend six years of post graduate work at the hospital, a department of the Rockefeller Institute (RI). My life with biological products started here. The hospital was established so that disease could be studied as it actually occurs (102). It opened in 1910 with Dr. Rufus Cole as Director. Pioneers had made highly significant contributions, especially on the characteristics of the pneumococcus and treatment of pneumococcus lobar pneumonia.

In the Acute Respiratory Group, prudently led by O. T. Avery (from 1913 until retirement in 1948), there was unity of purpose and close cooperation of the staff members. How fortuitous that I was associated with this group when I discovered the capsule of *Haemophilus influenzae* and the "S" and "R" colonies of *H. influenzae* that were similar to the colonies of the pneumococcus that I had studied in Chicago and that had been extensively studied by Avery et al (4).

When I went to the RIH, the role of *H. influenzae* as the cause of influenza had not been definitely settled. My project was to determine if *H. influenzae* is the cause of influenza. Numerous studies on this organism had been made during the 1918–19 catastrophic influenza epidemic. In his 1926 comprehensive review of about 1100 articles, Jordan (37) "concluded that it could not be deducted that *H. influenzae* was the cause or not the cause of influenza." The most encouraging results had been reported by Blake & Cecil (10, 19). Blake had been on the staff of RIH and was closely associated with RI. A strain of *H. influenzae* isolated from empyema fluid of a case of influenza, passed in mice and monkeys, induced a severe respiratory infection in monkeys that clinically resembled influenza. The authors, however, cautiously declined to infer that the *H. influenzae* strain used was the cause of influenza.

H. influenzae was not a stranger at the Hospital. A number of studies, published by Stillman (110) and by Rivers (101), did not promise supporting evidence.

With this background, it was not clear where I should start. I began by getting acquainted with the characteristics of the 28 cultures that had been collected. Out of floundering experiments emerged the discovery that some strains of *H. influenzae* were capsulated (62, 63).

The Capsule of H. influenzae

I had observed that the hemophilic bacteria were lysed by serum from laboratory animals, that the bacterial strains differed in susceptibility, and that the serum of animal species differ in activity. Guinea pig serum was especially active.

In order to compare the susceptibility of different strains, the transparent medium of Levinthal & Fernbach (43), which they used to compare the colonies of the Pfeiffer bacillus and the Koch-Weeks bacillus, was selected to determine if all bacteria were killed. My able technician Salvatorus Spatolli and I simplified preparation of the medium by combining equal parts of filtered boiled 10% blood broth and 4% melted nutrient agar instead of filtering chocolate agar.

In the very first test with the medium, encapsulated *H. influenzae* was discovered. Two types of colonies were demonstratable on a plate culture from the lytic test of culture no. 35. Both were much larger than on blood or chocolate agar. One colony, "S," was opaque, mucoid, and iridescent in obliquely transmitted strong light. The other colony, "R," was smaller in size, faintly rough, more translucent, and had a bluish sheen in transmitted light.

Two more cultures in the collection had S and R forms. These three cultures had been carried for 5 to 9 months since isolation. The first culture studied, no. 35, had been isolated from the heart blood of a mouse inoculated with sputum from a case of bronchopneumonia by the routine procedure used at RIH for the isolation of the pneumococcus (5). The second culture was from a case of meningitis and the third from the blood of a case of bronchopneumonia. The latter two cultures differed serologically from no. 35. No. 35 was designated type a and the other two type b (63).

The project on the causal relation of *H. influenzae* to influenza was discontinued. Emphasis was placed on the determination of the incidence of encapsulated *H. influenzae*, their serotype specificity and relation to pathogenesis, and on the production of type b antiserum and its efficacy.

During a period of less than four years, 521 hemophilic cultures were isolated largely from the sputum of cases of pneumonia admitted to RIH for research on cause and prevention of pneumonia. Some cultures were from outside sources. Only a few were encapsulated. I identified six serotypes. Type b was dominant and the most pathogenic. I found only one strain each of type d and type e. Peculiarly, no additional types have been found during more than 50 years. My study was intensive. Three media and the mouse were used for the isolation of the bacteria in each sputum specimen from many patients. This study showed that non-type-specific (nontypable) *H. influenzae* was pathogenic. Avery et al (5) had pointed out the association of *H. influenzae* with the pneumococcus microscopically and in the heart blood of the mouse inoculated with sputum. Pathogenicity of *H. influenzae* was shown

during the 1918–19 influenza pandemic. The influenza bacillus was isolated in pure culture from lungs of patients at autopsy but not from the blood (115). This lack of blood invasion and the difference in rabbit pathogenicity of these bacteria from cases of influenza and cases of meningitis (116) confirm that *H. influenzae* strains associated with influenza were not encapsuated (M. Pittman, review in preparation). Nevertheless, there has been some confusion that nontypable *H. influenzae* is an "R" form that has lost most of its original capsule (105). Recently, Hoiseth & Gilsdorf (36) provided evidence that nontypable organisms lack genetic ability to be encapsulated. Murphy & Apicello have pointed out the role of this nonencapsulated pathogen (52).

Production of Type b Antiserum

The discovery that the strains of *H. influenzae* from meningitis patients were usually type-specific and of one type (type b), suggested that a highly immune serum might have therapeutic value. Earlier attempts by other investigators did not find *H. influenzae* antiserum effective (cf. 64). Notwithstanding, in the light of newer knowledge, a horse was immunized. Type-specific antipneumococcus serum (horse) was in use in the hospital for treatment of pneumococcus pneumonia. To produce the *Haemophilus influenzae* type b antiserum, I followed in principle the method used to produce the pneumococcus antiserum at RI (6).

During immunization of the horse, type-specific antibody progressively rose, as shown by the precipitation of type b carbohydrate purified by W. F. Goebel, anti-infectious action in mice and rabbits, and prevention of skin lesions in rabbits induced by type b bacteria (64).

The therapeutic action of the antiserum was examined by cooperative physicians outside of RIH. In spite of widely varying conditions, their results indicated, as did the laboratory results, that the serum had definite anti-infectious activity. One patient recovered. Septicemia of the treated patients was promptly cleared and spinal fluid clearance might last up to 14 days. Then the bacteria returned and the patients died.

Horse antiserum has a deficiency in complement activity. Pittman & Goodner (94) showed that the type b antiserum did not fix complement in the presence of type b, capsular carbohydrate. With the bacterial protein, complement was fixed. In contrast, rabbit antiserum in the presence of both the carbohydrate and protein fixed complement. The agglutinin titer with type b bacteria did not differ between the two immune sera: neither did the precipitin titer with capsular carbohydrate.

Rabbit antiserum treatment was introduced by Alexander (2). Subsequently, therapy with antiserum was replaced by sulfonamide compounds (67) and antibiotics (35). We (35, 67) showed experimentally that both agents had antibacterial action on *H. influenzae*. Currently it appears that type b

carbohydrate conjugated with a protein antigen will contribute to the prevention of type b infection (104).

TAXONOMY OF GENUS *HAEMOPHILUS* AND GENUS *BORDETELLA*

The study of the characteristics of more than 500 hemophilic cultures relative to incidence of encapsulated strains and related publications led to an invitation to prepare "Genus *Hemophilus* Winslow et al 1917" for *Bergey's Manual of Determinative Bacteriology,* editions 5 (1939), 6 (1948), and 7 (1957). In the 7th edition, *H. aegyptius* (Trevisan, 1899) Pittman & Davis, 1950 was listed as a separate species, and *H. pertussis* with related species was transferred to the new genus *Bordetella.* I prepared the chapter, "Genus *Bordetella Moreno-Lópes* 1952, 178[AL]," for *Bergey's Manual of Systematic Bacteriology* (84). As a member of the International Committee on Genus *Haemophilus,* I was closely associated with national and international culture collections, in which I deposited cultures. These worldwide contacts broadened my information on infectious diseases. Cultures were received from local, national, and international sources, often with a request for an opinion on classification. The first cultures were the hemophilic bacteria isolated from pigs with swine influenza by Dr. Richard Shope (108). The bacteria and virus acted symbiotically. Alone they were not pathogenic. Later, cultures from swine were received from Europe. According to my records, the first and latter cultures were different. There were also cultures from fish and turkeys as well as from humans. The cultures isolated from cases of epidemic acute conjunctivitis in the Rio Grande Valley of Texas (22, 88) were of special interest from the standpoint of the clinical disease, epidemiology, and taxonomic classification.

Classification of the Koch-Weeks Bacillus

Since the primary isolations of the Koch-Weeks and the Pfeiffer bacilli, opinions on their taxonomic relationship have varied (cf. 88). One confounding factor was the custom of designating all *Haemophilus* bacteria from conjunctivitis the Koch-Weeks bacillus, at least in New York City. Cultures submitted to me at the RIH did not differ from the non-type-specific *H. influenzae* that I was studying. Later, Dr. Dorland J. Davis and I studied cultures from epidemic acute conjunctivitis that differed from *H. influenzae.*

In 1947, Dr. Davis was requested by the US Public Health Service to investigate conjunctivitis in the Rio Grande Valley in Texas (22). Cultures sent to me were hemophilic but within two weeks I had a vague feeling that they differed from any *H. influenzae* that I had ever examined. The following year we isolated both the "Koch-Weeks bacillus" and the influenza bacillus

from children with conjunctivitis (88). One helpful criteria in the field was that the colony of the Koch-Weeks bacillus in semi-solid media was small and fluffy with a comet-like tail. In contrast, the colony of nontypable *H. influenzae* is small and granular, while the colony of encapsulated bacteria is large and fluffy (88).

Before publication of the identifying characters of the bacillus as *Haemophilus aegyptius* we consulted authorities on systematic bacteriology (M. Pittman, personal letters). *Haemophilus aegyptius* is on the Approved List of Bacterial Names (109) and Kilian & Biberstein (41) used this name in *Bergey's Manual of Systematic Bacteriology*. In 1982, Mazloum et al (47) noted differential criteria.

Phylogenetic studies, however, have failed to show a difference in the DNA of the two organisms (29) and the name *Haemophilus influenzae* biotype aegyptius is used by some scientists (33). The gene that effects the marked differences in pathogenicity has not been identified. *H. aegyptius* infection is limited to the conjunctiva and occurs only during the hot climate breeding season of the "sore eye" gnat or fly (9). Whereas *H. influenzae* infections occur in many sites, eye infection is an extension from the nasopharynx (88). Between epidemics no carrier has been found (3). Is there a nonculturable stage? Colwell et al (20) reported that from Bangladesh mud no *Vibrio cholerae* grew on culture media but they were recovered from inoculated animal and humans. *Campylobacter jejuni* also has a nonculturable stage (103). It appears that *H. aegyptius* and *H. influenzae* are engaged but marriage has not, and probably will not be consummated.

THE NATIONAL INSTITUTES OF HEALTH, 1936–1972

Regulations of Biological Products

The regulations of biologicals in the United States began with the Congressional Biologics Control Act in 1902. The purpose of the Act was to insure the continued safety, purity, and potency of all vaccines, toxins, antitoxins, therapeutic serums, or analogous products applicable to the prevention, treatment, or cure of diseases or injuries (83). The year before, 10 children had died from tetanus after treatment with diptheria antitoxin.

The National Institute of Health (successor of the Hygienic Laboratory) was designated to regulate the Control Act. This responsibility was transferred to the Food and Drug Administration in 1972. I prepared a short history of the Regulation of Biological Products, 1902–1972 for the NIH Centennial Activities (1987) (83). Developments in regulations were made possible by advancements in knowledge. I was Chief, Laboratory of Bacterial Products, Division of Biologics Standards from 1957 until official retirement in 1971. By invitation, I have remained as a Guest Worker without official

responsibilities or pay. Apparently, I was the first woman to be named chief of a NIH laboratory and among the first to be promoted to super grade GS-16.

The story of the Biologic Control Laboratory is a story of an amazing safety record, as told in 1950, by R. D. Goldthrope, Scientific Branch, NIH, (unpublished). "The painstaking work and perpetual vigilance of its small group of scientists and technicians have protected the lives of millions of sick and well Americans." I was privileged to be a member of this group. The group remained small, not above 50, until the introduction of tissue culture of viruses for viral vaccines made a larger staff necessary. The Division of Biologics Standards was established at NIH in 1955 to meet the growing needs. Dr. Roderick Murry ably served as Director until retirement in 1972. He published an excellent and comprehensive view of the activity of DBS in 1968 (53).

My experiences in the development of requirements and standards for bacterial products and general requirements include the collateral research that supported the development and improvement of standards for bacterial vaccines.

REQUIREMENTS FOR BACTERIAL ANTISERA *Meningococcus Antiserum* In 1936, when the US was coming out of the Great Depression, funds from the Social Security Act were made available to expand medical research. I was among the microbiologists employed. My assignment was to work with Sara E. Branham (14). She had been one of my instructors at the University of Chicago. The project was to develop a potency test for meningococcus antiserum (horse) that was less efficacious in the current meningitis epidemic than in earlier epidemics (13).

A mouse potency assay was developed (15, 16) but never promulgated. The newly introduced sulfonamide derivatives were more efficacious than this antiserum (13). However, much was learned about mouse potency assay techniques (66). Most important was the application of the Petri technique to quantitate the level of specific meningococcus antibodies (86). With 100 lots of antiserum, there was a 94% correlation between the mouse protection assay value and the precipitation estimate (68). The discrepancy was obtained with a serotype of the meningococcus, which at that time had not been clearly defined. Some 30 years later, Mancini (45), unaware of our study (68), referred to a practical method for quantitation of antigens by the Ouchterlony technique. Our precipitin test has been adopted for the isolation of *H. influenzae* (105) and *Salmonella typhi* (59).

Study of *Neisseria meningitis* was facilitated by the procurement in 1937 of the first freeze-dry apparatus at NIH. Dr. Branham had >100 cultures that had to be transferred at least twice a week. Cultures of *H. influenzae* that I dried at that time are still viable.

Haemophilus influenzae *antiserum* Requirements were also developed for type b *H. influenzae* antiserum (rabbit): 1000 units/mg of capsular carbohydrate was specified. Then production of the therapeutic rabbit antiserum was discontinued. Sulfonamide derivatives were antibacterial against both type b (67) and non-type-specific bacteria (65). Later, antibiotics were used (35). To assure that the capsular serotype of diagnostic sera were properly labeled, requirements were issued. The quellung or precipitin test was specified and type-specific strains were supplied.

Bordetella pertussis *antiserum* Beginning with the first trial use of pertussis antiserum therapy (12), the results of treatment and prophylaxis with various antipertussis products were enigmatic (93). Nevertheless, an intracerebral challenge potency assay was developed and a Standard Antipertussis Serum (rabbit) was designated. The assigned unit value was based on the Netherlands unit of Antipertussis Reference. Manufacturers of antipertussis serum products were notified of the US Reference Standard Lot 2 and of the proposed potency assay. The realization that the clinical symptoms of pertussis were effected by pertussis toxin (80) cast light upon the variable results obtained with pertussis antiserum and the nontherapeutic effect obtained with pertussis immune globulin (93). Like with other bacterial-toxin diseases, pertussis antitoxin is not therapeutic after the toxin has attached to cells and altered their regulatory function (113).

Serotype antibodies of *B. pertussis* were considered essential for immunity against whooping cough (98). Their role in prolonged immunity in humans, however, remains uncertain. To insure that the types were represented in pertussis vaccine, Dr. Grace Eldering, an authority on typing (23), prepared, on contract, type specific antiserum for each of the six types. She also typed each of the production *B. pertussis* strains used by each manufacturer (G. Eldering, unpublished data). The role of agglutinogens in prevention of pertussis in humans remains controversial.

REQUIREMENT FOR BACTERIAL VACCINES Following the early successful treatment with diphtheria antitoxin, attention turned to use of antisera and vaccines prepared from nontoxin producing bacteria. Licenses were issued for bacterial antisera, followed by vaccines against practially all known pathogenic bacteria. Today, the Code of Federal Regulation (27) specifies specific requirements (Additional Standards) for only five bacterial vaccines—pertussis, typhoid, cholera, anthrax, and BCG.

Although concerned with regulations of all bacterial vaccines at one time or another, my research on both the development of potency assays and participation in the correlation of potency with human efficacy was largely limited to pertussis, typhoid, and cholera vaccines.

Pertussis vaccine In 1943, Dr. Milton V. Veldee, Director, Biologics Control Laboratory, requested that I develop a potency assay for pertussis vaccine. Others had failed, including Dr. J. W. Hornibrook, formerly assigned to this laboratory, and Dr. Pearl Kendrick, Michigan State Bureau of Laboratories. Both had tried the intranasal route of challenge and Kendrick had also tried the intraperitoneal route (40).

My acquaintance with *Bordetella pertussis* was largely limited to whooping cough at age 4 years and the confusing literature I had reviewed in 1926 for a term paper at the University of Chicago. Where should I start? The answer came from Dr. John Foote Norton, one of my teachers at the University of Chicago. He had observed encouraging pertussis-vaccine protection of mice against the intracerebral (ic) route of challenge. He and Dr. John Dingle (59a), both then at Upjohn Laboratories, were using this route in experimental potency tests of typhoid vaccine. In January, 1944, Dr. Norton suggested to Dr. Kendrick and to me that we try this route of challenge in potency testing of pertussis vaccine. Results were good. We worked independently but with free exchange of developments. Dr. Kendrick used two doses of vaccine to immunize mice (40) and I used one dose to avoid an inflated estimate of potency (57).

Manufacturers were informed of progress in 1945 (55), and in January, 1946, a tentative mouse protection test was sent to them (56). This test became official when Minimum Requirements were issued in 1948 and became effective on January 1, 1949 (57). Manufacturers quickly made adjustments in the preparation of their product. Samples that I tested in 1945–46 showed that three products had no potency and others varied as much as 10 fold within and between products.

Standardization of pertussis vaccine 1. Opacity. The first step in the development of the potency test was to prepare an opacity standard to be used in estimating the bacterial content of the vaccine and of the challenge culture for the potency test. Together, Dr. Kendrick and I adjusted the turbidity of a suspension of Pyrex glass particles (18) to be equivalent to that of a specified number of bacteria in an aged vaccine determined by direct count (78). The preparation was designated as the US Opacity Standard, and subsequently it was used as the International Opacity Reference Preparation (120), with an assigned value of 10 units without bacterial equivalence. The use of this Standard reduced significantly the variation in the turbidity of vaccines claimed to have equal numbers of bacteria (78).

2. Potency. From the beginning of the use of bacterial vaccine, the human dose was expressed in numbers of bacteria. Hence, the potency of the bacteria was estimated, which was not good. The number of bacteria per total human immunizing dose (THD) varied between products. Adjustment was made by

specifying that the THD be 12 units (74, 75). The value of 12 units was estimated to be equivalent to the potency of the vaccine used by Kendrick & Eldering (39) that was protective in a field study. *Minimum Requirements: Pertussis Vaccine* were revised effective May 1, 1953 (74).

To determine if the mouse potency estimate reflected human protection and to assess the efficacy of 12 U per THD, a field trial was designed. No money was available for a trial. Felton & Verwey (26) were the first to show with a noncellular, nontoxic vaccine (15 U/THD), the relationship between unitage and human protection. The extensive field trials of the Medical Research Counsel of England showed that there was a direct correlation between mouse potency and protection against home exposure (48). My estimate of the unitage of some of the trial vaccines, determined relative to the US Standard Pertussis Vaccine Lot 4, showed that unitage reflected the level of protection against home exposure (76). In the United States and other countries, efficacy has been $\geq$ 90%.

The International Standard Pertussis Vaccine was calibrated against the US Standard Vaccine and the requirements followed largely the US requirements except not less than 4 PU per single dose was specified (123). The US requirement has an upper limit on potency.

3. Statistics. At the time the potency test was being developed, statistical methods were not in general use by manufacturers to estimate the ED_{50} from the results of the test. The manufacturers were provided with the Wilson-Worcester Method (58).

Pittman & Lieberman (96) had analyzed the results of a large number of protection tests using the methods of Wilson & Worcester and of Reed & Muench in comparison with the Probit estimates. The Wilson-Worcester estimates of the ED_{50} were as exact as and consistent with the Probit estimates. The formulae of the former method are simpler, calculation less complicated and less time consuming. The Reed-Muench estimates were not entirely consistent with the Probit estimates.

The *Memorandum* revised in 1956 (58), with two tables from which the $ED_{50} \pm SD$ may be obtained directly when 16 or 32 mice are used per immunizing dose, were widely used until computers were readily available. They were reproduced by the World Health Organization (WHO) and distributed worldwide. They are still being used in some countries (30).

4. Toxicity and Antigenicity. Before isolation, *B. pertussis* showed one of its abnormalities, nongrowth on medium containing peptone. The next anomaly was that rupture by *B. pertussis* caused high parental fatality and local dermonecrosis in animals (11), whereas intact bacteria were not dermonecrotic (12). In 1951, Gordon & Hood (28) pointed out many epidemiological abnormalities.

Relative to NIH responsibility for the requirements for pertussis vaccine, I

participated in a number of studies of reactivity. These and studies of others (cf. 81) pointed out the nature of the two-stage disease and of the reactions in animals. Reactions included increased susceptibility to a number of agents (histamine, serotonin, endotoxin, and bacterial infections); metabolic alterations (hypoglycemia, refractoriness to epinephrine hyperglycemia, and hyperinsulinemia); and potentiation of immune response to protein antigens (cf. 80). Four or more toxins had been described. Did one or more toxins effect the multiple responses?

I was a Guest Scientist at the University of Glasgow and was invited to present a paper entitled "Antigenic Specificity of Bacterial Infections" at the seminar on Epidemiology of Communicable Diseases, May 10, 1977. Some bacteria had a capsule, others secreted an exotoxin. What was the specific antigen of *B. pertussis?* It suddenly dawned on me that it was an exotoxin. I was chagrined. Of course, there was a thread of evidence running through the literature. However, not until my fourth presentation of this hypothesis was its significance recognized (79).

Shortly after Parfentjev & Goodline (60) reported histamine shock in mice injected with *Hemophilus pertussis* vaccine, I published several articles on histamine sensitivity (HS) of the mouse. Mice intranasally infected with *H. pertussis* showed that the degree of HS was directly related to inoculum and severity of infection, that duration paralleled paroxysmal coughing of the child, and that after recovery from infection, mice were immune to ic challenge (70). With plain pertussis vaccine, the HS paralleled protective activity (72), and female mice were more susceptible to HS than male mice (71).

Another study (111) demonstrated, in contrast to the marked increase in HS in the mouse induced by pertussis vaccine, that both the rabbit and the guinea pig showed a slight but significant decrease in normal HS; the guinea pig more than the rabbit. The mouse showed a decrease in blood glucose, while the rabbit showed no change. Why?

Numerous studies support the essential role of pertussis toxin (PT) in whooping cough. Foremost is the molecular structure of PT and its activity that is effected by catalytic action of ADP-ribosyltransferase on a specific membrane protein that controls the function of cells (107), leading to multiple types of clinical reactions.

The mouse strain selected for potency (77) and toxicity (80a) evaluation is very important. With the N:NIH (SW) strain I routinely used, there was a direct correlation between the histamine sensitizing dose (HS_{50}) and the ED_{50} responses. By selective breeding, high and low HS strains (HSF-S and HSF-R) were developed (46). The ratio, R/S, of the SD_{50} and the ED_{50} of the test vaccine remained constant (cf. 81). Mouse strains with highest sensitizability have the highest immunizability for pertussis vaccine, as well as for diphtheria and tetanus toxoids, which was shown by Csizmas (see 77).

An analysis of the results of 9.5 years of toxicity testing (87) and other studies point out the relationship between mouse weight gain and human toxicity, both of which are related to the HS unitage (cf. 81), and that stability of potency of the vaccine is effected by the preservative (87). Merthiolate is the preservative of choice. Whooping cough is a neurotoxic disease (82). The pharmacologic mode of action of the metabolic changes is only beginning to be revealed.

Typhoid vaccine The participation of Pittman & Bohner (85) in the laboratory assessment of the potency of typhoid vaccines that were being tested in WHO field trials contributed to promulgation of US Standards for Typhoid Vaccine. The mouse potency test in routine use differentiated two trial vaccines (K, acetone killed and dried, and L, heat-killed, phenolized and dried) in the same order as their efficacy for humans. The ratios, K/L, were the same, 3.69. Melikova et al (49) confirmed our findings that mouse potency and human protection reflected the same difference as the mouse in protective activity of K and L vaccines. They used the same mouse test we used.

The US typhoid vaccine reference was tested in the laboratory concurrently with vaccines K and L (85). It was much lower in potency and was replaced with a K-type vaccine that was comparable in potency to the K vaccine that was efficacious in protection of humans. Publication of "Additional Standards for Typhoid Vaccine" in the Federal Register followed.

Studies on typhoid vaccine continued with colleagues. One study showed the importance of the mouse strain used for the potency test (24). Two studies indicated that Vi antigen was the most important protective antigen for the mouse (117, 118). Purified Vi was prepared shortly after I retired (119).

Dr. John B. Robbins and associates have been promoting the assessment of the Vi capsular polysaccharide for protection of humans against typhoid. In a recent study in Nepal, the Vi antigen, tested in comparison with pneumococcus capsular polysaccharide, provided about 75% protection (1). Both antigens were nonreactive. It is anticipated that Vi convalently bound to a protein (112) will increase immunogenicity and the use of this antigen will contribute to the control of typhoid fever in endemic areas. It is gratifying that our earlier studies on the protective activity of the Vi antigen for the mouse supported the studies that have shown the protective activity of Vi vaccine for humans.

Cholera vaccine My interest in cholera vaccine started in 1958 when I participated in the development of the first international requirements for biological substances—yellow fever vaccine and cholera vaccine. After further considerations, the requirements for cholera vaccine were published in 1959 (121). Other meetings with World Health Organization and cooperative tests followed. Committee members were concerned with the relation of the

laboratory assessed potency of the vaccine and its prevention of cholera. The requirements were revised in 1968 (121).

The years from 1960 to 1970 were of special interest, stimulated by the Southeast Asia Treaty Organization (SEATO)-Pakistan Cholera Research Laboratory (CRL) in Dacca, East Pakistan (now Dhaka, Bangladesh). SEATO, after effecting an improvement of small pox vaccine in Southeast Asia, decided to focus on cholera. Dr. Joseph Smadel, an eminent research scientist, especially on Southeast Asian infectious diseases, was the power behind the project. I had known him at RIH. Dr. John C. Feeley, a new staff member, and I were brought in to help design the laboratories and equipment for CRL that would be located in a building erected by the US Public Health Service.

Through the activities of two committees, I was closely connected with the cholera investigations. The NIH Cholera Advisory Committee members were actively engaged in cholera research. Their evaluation and coordination of the projects were extraordinary and very beneficial.

As a member of the Panel of Expert Consultants to Technical Committee for Pakistan-SEATO Cholera Research Laboratory, I followed closely the work at this laboratory. All aspects of the disease were under investigation. Twice I visited CRL and the CRL hospital at Matlab.

In 1965, after Dr. Smadel's death from cancer, I served as Project Officer for CRL until 1970. Responsibility was largely related to NIH financial agreement.

With Dr. Feeley, I participated in several studies on *Vibrio cholerae* and on the El Tor strains (91). We evaluated the potency of the cholera vaccines used in the first field trial of cholera vaccine by CRL (25a). Mosely et al (50) pointed out the interrelations of serological responses in humans and the active mouse protection test to cholera vaccine effectiveness. For several years, Dr. J. C. Feeley participated with the U.S. Cholera Panel of the United States–Japan Cooperative Medical Science Program.

CRL has been superceded by the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh.

Other bacterial products Among other product studies, I developed the guinea pig skin potency assay that was included in the first requirements for tuberculins, and participated with a Public Health Service Committee on the required strength for the skin test dose and in the standardization of the Purified Protein Derivative (PPD-S) prepared by Dr. Florence B. Seibert (106) for the United States and the International Standard.

A diphtheria toxin was selected and standardized for the Shick Test, and the erythema potency assay for the Shick Test dose was developed by Barile et al (8).

Then there were studies on tetanus toxoid with Dr. M. C. Hardegree and associates and on requirements for anthrax vaccine and *Clostridium* gas-gangrene vaccines that have limited use.

General Standards

During the war years, the work load increased in order to meet the needs of the Armed Forces for immunization and treatment products. My participation largely concerned pyrogenicity of plasma and its contamination, and the formula for the sterility test.

PYROGENICITY Intravenous therapy, especially plasma, greatly increased. Occasionally febrile reactions occurred. It was known that microorganisms present in distilled water effected pyrogenicity (124). But there was little information about the pyrexial characteristic of contaminates from biologics: I studied 59 cultures received from eight processing laboratories. Twenty-eight representative strains were selected for evaluation of their pyrexial property in collaboration with Thomas F. Proby (99). Viable and killed bacteria and culture filtrates, all in serial dilutions, were examined using the rabbit pyrogen test as described by the US Pharmacopeia XII, with modifications. All microorganisms were capable of inducing fever but they varied widely both qualitatively and quantitatively. Counts of bacteria did not furnish an index. Gram-negative bacteria were the most pyrogenic: 6000 cfu/ml were pyrogenic. A rabbit pyrogenic test that was applicable to all intravenous fluid products was promulgated.

THE STERILITY TEST The early test to determine the sterility of biologics used infusion broth in the Smith container with a side arm to provide both aerobic and anaerobic conditions. In 1941, fluid thioglycolate medium (FTM) developed by Brewer (17) was adopted. It had two advantages: neutralization of mercurial preservatives and support of the growth of both aerobic and anaerobic bacteria. Shortly thereafter, investigators in two establishments reported the recovery of more contaminants in the infusion broth than in the new FTM. Inhibition was limited by methylene blue, the Eh indicator.

An evaluation of the growth-promoting property of each ingredient in FTM was made. Many consulted persons provided valuable suggestions and assistance. The suggestion to use resazurin for the Eh indicator came from R. H. Breed through H. C. Dunham of Difco. The dairy industry used resazurin to evaluate the degree of contamination in milk. The 26 cultures selected for the study were either from contaminated products or were considered possible contaminants. Each culture was tested to determine the minimum number of bacteria that would grow in each test medium, at different pH and at incubation 36°C and 22–25°C. The selected formula (69) has remained unchanged

except for a reduction of L-cystine from 0.75 to 0.5 mg per liter. This medium is listed in the WHO General Requirements for Sterility of Biological Substances for detection of both aerobic and anaerobic contaminants (122). It has been given in the US Pharmacopeia since 1970.

The use of two temperatures of incubation for the sterility test was not adopted immediately. The use of 37°C to detect the presence of pathogenic contaminants was so ingrained that it took a dramatic event to specify the use of a lower temperature. A patient being treated with plasma developed a febrile shock. This plasma was tested for sterility at 37°C; no growth. At room temperature, growth was obtained from 1.0 ml of a 10^{-5} dilution.

The final study on contaminants was prompted by recurring reports of severe or fatal reactions following administration of bacteriologically contaminated blood. This study provided a better understanding of the problem of bacterial contamination in the handling of blood and blood products. The results were presented in 1952 before the Fifth Annual Meeting of the American Association of Blood Banks (73). Emphasis was placed on the importance of contaminants from the environment, not the donor, and the need to identify the contaminant in order to trace its source. The report also emphasized the need to use two temperatures of incubation for the sterility test. All of the recovered gram-negative bacilli and some gram-positive cocci grew at 10°C, the upper limit of storage of blood. Earlier we had reported (99) that all contaminants tested had the ability to induce a febrile reaction. Furthermore, that of the 13 blood contaminants associated with severe or fatal reactions, 11 grew at 2.5°C. The range of temperatures at which all cultures grew readily was limited to above 25°C and below 30°C. Hence, adjustment of the 37°C temperature of incubation of the sterility test was necessary in order to detect the presence of psychrophilic bacterial contaminants.

The inclusion of the medium to support the recovery of fungal contaminants was based on studies by Pittman & Feeley (90) and Cox et al (21).

Cooperative Studies

It is imperative that biological standards be uniform within a nation and throughout the world, and best accomplished through cooperation.

After World War I, the international cooperation of NIH began. Dr. George W. McCoy, Director of NIH served as an invited unofficial member of the Health Organization, League of Nations Committee at Copenhagen, and NIH furnished gas-gangrene antitoxins for International Standards (cf. 83).

Dr. Roderick Murray, Director, DBS, NIH, was one of the first to serve on the WHO Expert Committee on Biological Standardization of the United Nations, which succeeded the League of Nations Committee. I was involved as Consultant of the WHO Secretariat in the preparation of the requirements for the bacterial vaccines and the general requirements for the Sterility of Biological Substances and their revision in 1973 (122).

A number of WHO collaborative studies related to the International Pertussis Vaccine Standard, the Cholera Vaccine, the Opacity Standard, and the variables that influence the mouse potency test of pertussis vaccine (51), as well as the relation of mouse toxicity to human toxicity (61, cf. 81).

The studies relating to typhoid vaccine, cholera vaccine, and epidemic acute conjunctivitis were discussed earlier.

The US Pharmacopeia Panel on Sterility Tests and the Panel on Biological Indicators (to control effectiveness of steam and dry heat) contributed to ensuring the sterility of biologics and other products. I was a consecutive member of the Panels during 1967–73.

As an associate member of the Commission on Immunization of the Armed Forces Epidemiological Board, I kept abreast of the immunological program in the Armed Forces (1969–1970).

Participation at workshops, symposiums, national and international scientific society meetings, especially the meetings of the International Association of Biological Standardization and its journal, were also helpful.

The most extensive study was on "Immunization against neonatal tetanus in New Guinea" with Dr. F. D. Schofield and Dr. R. MacLennon (7, 31, 32, 44, 95). Besides the success in preventing neonatal tetanus with tetanus toxoid, there was some untoward reactivity with the use of tetanus toxoid containing mineral oil adjuvant. At that time, there was much interest in the use of mineral oil in allergens and other products. DBS initiated a series of studies under contract to evaluate safety. The final DBS report indicated that the use of mineral oil adjuvants in the human population may be hazardous and should not be recommended for general use in humans (54).

AFTER RETIREMENT, 1971–1990

To paraphrase the title of the book *Life Begins at Forty*, I might use the title *Life Continues at Seventy*. The years have been full, challenging, and gratifying.

It has been an unusual privilege to be a Guest Worker (without payment) with an office and a parking space on the NIH campus. Without official responsibility, I was able to clear my desk of pending manuscripts. Now two reviews are pending—early work on *H. influenzae* and on the Koch-Weeks bacillus. In addition, I have been invited to give lectures, participate in workshops, and write articles for journals and books.

Serving as consultant or Guest Scientist in nine countries has been stimulating and pleasant. With WHO, I completed the revision of requirements for the sterility of biological substances, and consulted on cholera vaccine in Cairo, Egypt and Madrid, Spain for three months each. Three months at the Razi Institute, Teheran, Iran were very interesting and informative, historically and politically. The most productive work was at the University of Glasgow.

There were four visits. A study started in the Netherlands was completed on the pathophysiological response of mice to *B. pertussis* respiratory infections (92). The participating pharmacologist, Dr. Furman, has continued the work on insulin and glucose metabolism. There is some evidence that hypoglycemia may play an important role in pertussis encephalopathy. I was at the University of Glasgow when the hypothesis was conceived that pertussis toxin was the cause of the harmful effects and prolonged immunity of whooping cough (80). More pieces are yet to be fit into the jigsaw puzzle of pertussis. Pieces are still missing in the jigsaw puzzle of epidemic acute conjunctivitis as well as other infectious diseases.

A statement made by Sir J. Willard in a letter to the Academy of Arts and Sciences in January 1986 has been a helpful guide in doing and directing research. It reads, "one of the principal objects of theoretical research is to find the point of view from which the subject appears in its greatest simplicity." There is a truism to be remembered. "That whatsoever is known has always seemed indeductable to the known, and equally whatever is alien has seemed fanciful and arbitrary." We may see but not perceive if the mind is not prepared.

With home training and inspiratory education, my life's journey led to biological products. Many scientific and social friends and church activities provided inspiring vistas. Life has brought satisfaction and gratitude for many honors. Lacking has been the enhancement of the inborn spirit of youth. Hence, I have helped to initiate and am promoting the Urban Ministry Program at Wesley Theological Seminary.

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