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EARLY HISTORY OF MICROBIOLOGY AND MICROBIOLOGICAL METHODS

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INTRODUCTION

The original title of this chapter was to be “History of Microbiological Methods.” This would seem to indicate that inquisitive minds were consciously attempting to develop new methods for a new field of science. Or perhaps they had a “for profit” motive as if they were working for a company driving them towards new markets or to increase their portfolio of patents from which to draw royalties. This could not be further from the truth. Rather, the history of microbiology is a story of some common folk and other rather peculiar individuals, janitors and hobbyists, amateur lens grinders and chemists, physicians and botanists, housewives and laboratory “slaves” with a quest for discovery. These explorers were sometimes driven by chance or curiosity, and sometimes by a problem for which no one had an answer. And, they were driven sometimes by pride or self-interest. But then there were those altruistic ones driven by the pure compassion for suffering humanity. With the topic being rather broad, and the space being somewhat limited, the scope had to be narrowed so this chapter has mutated into one that attempts to expose the minds behind the discoveries;

discoveries that necessitated the rather simple, though not always obvious, techniques that made revealing the world of microorganisms possible.

The story that unfolds is first a history of events and phenomenon for which there was no understanding or explanation, followed by observations for which there was no obvious application, to the eventual association of cause and effect, on to a full blown field of inquiry. It is quite interesting how much the field of microbiology developed even while the scientific theory of investigation was not a mature approach, or at least did not seem applicable to these studies. The rush to conclusions, often followed by a contest of wills, complete with name-calling and mud-slinging, mark this history, though I suppose that is not so unusual based on the strong personalities involved and the passion of this work. And, yet, embedded within this story are those who withheld important work until more than ample evidence was obtained to substantiate the claims, whereas others would have rushed to make their findings known. The whole story be told, it would be hard to equal the impact of the learning these few noble pioneers have had upon the course of human history and upon the cause and conquest of so much human suffering. As we shall see, it would be difficult to measure their impact on numerous industrial processes, in the field of animal husbandry, and thus, their tremendous impact upon the economies of nations. It was not human suffering that initially motivated investigators, but some rather basic and applied studies into industrial processes that paved the way to the ultimate medical applications.

AN OVERVIEW

In his book, *The Development of Microbiology* (Collard 1976), Patrick Collard described four eras of **Formal Microbiology**:

- 1) Era of Speculation (5000 BC to 1675)
- 2) Era of Observation (1675 to mid 19th century)
- 3) Era of Cultivation (mid 19th century to early 20th century)
- 4) Era of Physiological Study (early 20th century to *present*, 1976)

It was in the 1940s that the nature of bacterial transformation and the role of DNA began to be recognized, but it wasn't until the past three decades that the tools of molecular biology were thoroughly applied to the field. Therefore, I will add a fifth Era to Collard's, the Era of Molecular Study, that overlaps with Collard's Era of Physiological Study and continues to the present. Considering the subject matter of this book, it seems only fitting to refer to yet a sixth Era, the Era of Rapid Methods made possible by discoveries in the previous eras. I trust

that this entire book will give the reader an appreciation of the many advances over a relatively few short years in a relatively young science. This chapter will focus heavily on the period of time encompassing the first three eras as we look closely at the early history of classical microbiology. The period of discovery covered in this chapter is roughly from the mid 17th century to the close of the 19th century, with some brief comments on the 20th century development with which most readers will be familiar.

ERA OF SPECULATION

A Drama in the Making

Ever since there was man (and I mean that in the generic sense of mankind), we know that a myriad of microorganisms interacted with man and his world, in both positive and negative ways. Though oblivious to the fact, his body and his world teemed with these amazing creatures carrying on many important and vital functions. From batch to batch, portions of bread and fermented beverages were retained and added to the next batch. Why? No one knew, except it had to be or the bread would not rise, and the grapes would not ferment. Man made great use of his unknown world.

Now, consider a world where an individual would suddenly turn ill, his temperature would rise, or his appearance would be changed, sometimes in a hideous way, without a clue as to the nature or the cause of his plight. Such was the case of Job in perhaps the earliest of the Biblical accounts. He was covered “with sore boils from the sole of his foot unto his crown” (*Holy Bible*, Job 3:7). The Scriptures do not divulge the precise agent of his condition, though we know today that boils are caused by *Staphylococcus aureus*. Or perhaps these were the pustules of smallpox that, untreated, may cover the entire body.

The account of the plagues of Egypt in the days of Moses provides an interesting scenario. It was around 1500 BC and the nation of Israel was enslaved by the Pharaoh. They cried unto God for hundreds of years, and God lifted up Moses to deliver the nation of Israel from Egypt. He sent plagues among the Egyptians. First, there was death in the rivers and on the land. Carcasses were piled up everywhere: festering, stinking reservoirs of disease. Then came the insect vectors, lice and flies, or more appropriately, flying insects, most likely mosquitoes of the Nile River valley. Then upon man and beast of Egypt came a grievous murrain, or pestilence, boils and blains or malignant pustules, the stuff of various infectious diseases. Livestock and humans alike succumbed to the plagues. Following the last of the plagues, the death of the firstborn throughout Egypt, Pharaoh was humbled and sent Israel away (*Holy Bible*, Exodus 7 – 12).

Fast forward approximately 500 years to 1000 BC and we read that a pestilence came upon Israel from “the morning even to the time appointed (three days): and there died of the people from Dan even to Beersheba seventy thousand men” (*Holy Bible*, II Samuel 24:15). Seventy thousand people died in three days. The precise cause of death is not given, nor the etiological agent revealed, but one may assume it was a severe and rapidly fulminating disease.

Beyond these Biblical accounts, there is much historical documentation of various epidemics and pandemics that have taken great toll on humanity. Hippocrates seems to have been the first observer to document an influenza pandemic in the year 412 BC. Epidemics of plague have been recorded in China since 224 BC. Major plague epidemics occurred in 540 AD in Egypt, reached Constantinople in 542, and spread to Europe and Asia in the following decade; it was called the Plague of Justinian, named after the emperor of the Byzantine Empire from 527 to 565 AD. The most notorious of the plague epidemics consumed 14th century Europe. Beginning in the lower Volga River basin in 1345, it traveled north through Europe, reaching England in 1348, and finally Russia in 1351. When it was all said and done, this scourge, known as *The Great Dying*, *Magna Mortalis*, or *The Black Death*, claimed approximately one-third to one-half the population of Europe and an estimated 40 million worldwide.

What makes this and the Biblical accounts so eerily familiar is that humanity was helpless and hopeless, having absolutely no understanding of either the cause or the cure. The only thing that remained was the burying of the dead. Oh, we have a few rather charming children’s songs that came from these plagues like, “Ring around the rosies (believed to refer to rosary beads, or the reddish spots with white centers that appeared on the afflicted), A pocket full of posies (flowers carried to mask the smell emanating from the diseased), Ashes, ashes! (the corpses were burned and only ashes remained), We all fall down (i.e., die).” In light, or rather darkness, of the ignorance of the day, according to *History of Epidemics and Plagues* (Author Unknown 2001), the proposed remedies were interesting: burning incense, dipping handkerchiefs in aromatic oils, ringing church bells and firing cannons, wearing talismans (charms or magical figures), bathing in human urine, placing ‘stinks’ (dead animals) in their dwellings, bleeding via leeches and bloodletting, drinking the pus extracted from a suppurated bubo (my favorite), applying dried toads to relieve the pain of the buboes by absorbing the “poisons,” drinking liquid gold or powdered emeralds (at least for the well-to-do), and joining groups of flagellants (that’s another story). It was during this time that the concept of **quarantine** (from the Italian *quarentina*, meaning forty, supposedly based on the number of days that Christ spent in the wilderness) was instituted. Though infected individuals were kept isolated from others, rats (the reservoir) and

the rat fleas (the vector) were allowed to roam freely. Other outbreaks of *The Plague* have been recorded for centuries with the last major pandemic occurring from 1855-1896, mostly in China and India, where more than 12 million died. It wasn't until 1894, that Yersin and Kitasato described the causative agent, now known as *Yersinia pestis*. And, it wasn't until 1897 that the mode of transmission was conclusively identified.

Other notable scourges include syphilis that spread throughout Europe in the 1490s. It was on this particular ailment that Paul Ehrlich, in the first decade of the 20th century, focused his search for the "magic bullet," more on this later. Cholera seems to be a more contemporary attack on humanity. There have been seven major pandemics occurring from 1817 through 1970 and some consider an eighth underway over the last decade. Though not deadly on the same scale as *The Black Death*, each pandemic has left tens of thousands of dead in their aftermath. Of the viral pandemics, we can speak with great awe of the horrendous scope of the infamous poxvirus, variola, or smallpox, and of influenza. During its hay-day in the centuries prior to 1725, smallpox is estimated to have killed more than 100 million people worldwide. Here remains one of the great chapters in microbiology. This deadly disease that eluded the early microbe hunters, that once consumed humanity, was declared by the World Health Assembly in 1980 to be eradicated. In the late 18th century, the work by Edward Jenner, who developed a vaccine from the similar virus of cowpox, paved the way for this tremendous feat. The term vaccine was born from the Latin word for cow, *vacca*, based on Jenner's work.

If we consider *The Black Death* the granddaddy of bacterial pandemics, we would have to consider the influenza outbreak of 1918-1919 its fraternal twin brother of viral origin. No one single agent, in such a short time, has devastated so vast an area as during this pandemic. The "Spanish" Influenza pandemic, as it is called, killed 40 – 50 million people, about 2 – 3% of the world's population. The flu typically attacks the very young and the very old, taking advantage of the frail immune system of both. But this particular epidemic set its sights on the most robust of the population, the 19 – 34 age group. It swept across five continents: Asia, Africa, Europe, and North and South America. The U.S. death toll was about 700,000. Big cities were hardest hit with mortality rates of 10 – 15% in several major cities. Its effect on the military during the First World War turned the course of several battles. Though much progress had been made in the field of bacteriology, advances in the field of virology would have to wait the development of the electron microscope, for which we can thank the Belgian physicist Marton (who built the first electron microscope in 1934).

More can be told of the various plagues, pestilences, epidemics, and pandemics that have changed the course of history and the lives of untold

millions of people, of the impact on native populations during the age of exploration when the “New World” was being discovered, and the subsequent impact on the growth and development of nations. For now, it is sufficient to say that when we did not know what we were dealing with, these microorganisms were our masters, and cruel they were. Since the late 17th century, humanity’s understanding of this heretofore unseen and unknown world took a turn. A slow and methodical pursuit began that has seen many turns that have given us some handle on all of this. Yet, even with the advances made, and the arsenal of weapons that have been accumulated, much remains to be learned to put us continually in the command position. According to the World Health Organization, in 2003, more than eleven million people died of infectious diseases including AIDS, tuberculosis, and malaria, half of them in Africa. The work of the men and women described below may serve as both a guide to further exploration and an encouragement to persevere. If they could learn so much, with so little, what can be accomplished in this age?

ERA OF OBSERVATION

The Script is Drafted

Virus! That’s what they called it; the unknown, transmissible substance that caused disease for which there was no real explanation. The word *virus*, Latin for poison, had been in use for hundreds of years. Without suitable tools, there was little that man could do to unravel the observations and piece together the puzzle that plagued humanity. Man did not know that he was looking for “invisible” creatures. This was during the time that man believed the smallest of living creatures was the cheese mite, a gigantic 0.4 mm in size. An understanding into the world of creatures less than one-thousandth of the size of the “smallest of creatures” known to man would await the fortuitous creation of suitable tools and the persistence of investigators with an insatiable appetite for scientific study and curiosity. Into the 17th century and beyond were born such men and woman called *Microbe Hunters* by Paul De Kruif (De Kruif 1954) in the book by the same title.

EARLY MICROBIOLOGISTS

Anton van Leeuwenhoek (1632 – 1723)

The First Man Ever to See Bacteria

By the end of the 16th century, lenses were available that could magnify objects a few hundred-fold, not nearly the magnification needed to see bacteria. They also suffered from chromatic and spherical aberrations that obscured these tiny objects. In 1632, Anton van Leeuwenhoek was born in Delft, Holland. This Delft janitor and draper learned lens grinding as a hobby, and prepared lenses that he meticulously ground himself, better than any others of his day. Leeuwenhoek was not driven to find sources of life that had never been found before, but he had a great curiosity to see small things bigger. He wanted to view the finer stuff of life. He turned his lenses on many objects, basically any little thing he could get his hands on: insects, fabric, dust, and the like. It was the year 1675. Why he chose to look at rainwater is anyone's guess. Every one knew rainwater was clear and would have nothing to interest such a curious observer. Then it happened... "There are little animals in this rain water!" or "wretched beasties" as he came to call them. "He gaped at their enormous littleness" (De Kruif 1954). What to make of these beasties was not Leeuwenhoek's concern; it was enough that he had seen what no one else had seen before. It would be almost 200 years before it would be demonstrated that bacteria caused disease.

The sharp taste of pepper interested Leeuwenhoek. He thought it must have spikes or needles that would stick into the tongue. So he ground some pepper in water and examined it over several days. With each passing day, he found the numbers and types of beasties increasing. In 1676, Leeuwenhoek wrote a letter to the Royal Society of England describing his observations. Robert Hooke and Nehemiah Grew were commissioned by the Royal Society of England to build the best microscopes and brew pepper water. In 1677, they confirmed Leeuwenhoek's observations. Leeuwenhoek's investigations might have led a more prepared mind to conceive of the association of disease and specific bacteria. Take, for example, the unique organism he spied from a wet prep of plaque from deteriorated teeth when compared to his own healthy teeth with its amazing variety of microbes. Nor did he associate the creatures with disease from his own intestines when he had a bout with "the runs."

One more discovery made by Leeuwenhoek bears mention in this chapter. He found that by placing bacteria in water and heating it, he could kill the bacteria. The idea for these studies came from an observation he made after drinking his morning coffee, which he liked very hot. Upon examining the bacteria from his teeth, he found dead bacteria rather than the usual swarming masses that he was accustomed to seeing. From Leeuwenhoek's drawings, cocci, bacilli, and spirochetes were clearly discernible. In 1723, Leeuwenhoek breathed his last breath, but his life and findings were the very first step in a long journey that would be traveled by others who would uncover the mysteries of these microbes and eventually gain an understanding of their significance in industrial processes and human health.

Lazzaro Spallanzani 1729 – 1799

While Leeuwenhoek was busy preparing the world for a new field of science, a debate was raging among the philosophers of the *Middle Ages*. It was generally well accepted that spontaneous generation was commonplace, even in regard to whole animals. The work of Francesco Redi in the mid 17th century to prove that maggots were not produced by rotting meat (but rather only appeared if the meal was available to adult flies), went a long way to bring some science and sense into this debate. However, the microscopic observations of Leeuwenhoek gave new spark to the debate. Additional fuel was added to the fire in the mid 18th century when Joseph Needham showed microbes generated in samples of broth that had been boiled and then sealed with corks.

A priest turned scientist, Lazzaro Spallanzani challenged the work of Needham. He first needed to learn how to grow bacteria or "animalcules," as he chose to call them. His recipes were simple. He prepared mixtures of water and seeds or peas or almonds. His experiments were simple yet profound. He prepared duplicate sets of several flasks. One set of flasks he sealed with heat, the other set were the "Needham controls," i.e., corked flasks. These he boiled for various lengths of time, from only a few minutes to an hour. After a suitable storage time, he found that sealed flasks boiled for a few minutes had living animalcules, but not those he boiled for an hour. In comparison, the corked flasks, even those that had been boiled for one hour were contaminated. From this work, he concluded that the corks were not a suitable seal and that the animalcules had come from the air. Further, he disproved spontaneous generation, though there were still those that held to the belief, and it would have to be disproved again (by Pasteur in the 1860s). Thus, the concept of steam under pressure as a sterilizing agent was born.

Further studies by Spallanzani demonstrated that some animalcules could live in the absence of air. Thus was the first observation of anaerobic microorganisms. During his microscopic observations, he often noted two organisms stuck together. The natural question would be, have two come together to breed? This is what nature would tell you. At the suggestion of deSaussure, who offered the opposing view that what he was seeing was one animalcule dividing into two, Spallanzani performed a rather ingenious experiment to isolate a single animalcule and test the concept. He placed a fine drop of culture on a sterile glass slide. A short distance away on the same slide he placed a drop of sterile medium and then formed a narrow liquid bridge between the two drops. He watched the channel under the microscope until he saw a single bacterium enter the channel and make its way to the sterile drop. He then wiped away the liquid bridge. As he observed the single cell for some time, it began to elongate and, ultimately, divide in two just as deSaussure had postulated (Umbreit 1962).

While this work was going on, advances were being made in the field of microscopy. In the early 19th century, the Italian astronomer Giovanni Battista Amici developed achromatic objective lenses and magnifications were now possible up to 600 diameters. While the equipment was improving, techniques were still primitive and problematic. No stains were being used so only the outlines of microorganisms were clearly visible. Only wet preps were used, so Brownian movement was a problem. The 19th century witnessed many further advances in the physics of microscopy. The problem of chromatic aberration had been suitably addressed in the 18th century by John Dollond by using two lenses of different refractive indices. Building on this and aided by the talent of such men as Carl Zeiss and the physicist Ernst Abbe, the issue of spherical aberration was resolved by the use of a concave lens added to the convex objective lens to “flatten out” the focal points across the field of observation. It was in 1878 that Abbe introduced the oil immersion lens.

With some of the basic tools and concepts in place, the fledgling field of microbiology was about to emerge from a period of simple observation, to what Collard called the “Era of Cultivation.” Onto a stage prepared by the masterful set designers above would step some of the greatest actors in the industry. What follows is not an exhaustive list of the many investigators who advanced the science and art of microbiology, but rather a brief look into the lives of a few key individuals who gave us the basic understanding and tools of the trade of microbiology.

ERA OF CULTIVATION

The Drama Unfolds

Louis Pasteur 1822 – 1895 and Robert Koch 1843 – 1910

Despite the observations and discoveries made over more than 150 years, the cause of infectious diseases was still completely unknown. The story that immediately follows is that of two contemporary pioneers, giants in the search for understanding. The first, Louis Pasteur, earned his doctorate in Chemistry at the Collège de France, Paris. The second, Robert Koch, was educated in medicine, but was in love with research, though not in bacteriology since it was not taught at that time.

Advances in microbial metabolism were not so much a pure search for an understanding into this strange new world of the 'invisible' neighbors all around us. Rather, it was born out of the applied studies into the processes of fermentation that had been used for millennia, but that had gone badly from time to time. So, in 1857, Pasteur, the chemist with a microscope, enlisted to resolve the woes of the industry, delved into the secrets of the process that turned beet sugar into alcohol, but also sometimes acid. He examined the contents of the acidic vats under his microscope and compared them with the contents of good vats that produced alcohol. The good vats contained budding yeasts, and the bad vats contained "tiny rod-like things" that produced lactic acid. The study of this phenomenon was somewhat hindered by the complex "medium" of the beet-pulp from the vats. Pasteur turned his attention to the development of a clear medium to aid his investigation. Pasteur eventually succeeded by using a filtered extract of boiled yeast, combined with sugar, and chalk to neutralize the mixture. The art of the preparation of culture medium was born and yeast extract, as we now call it (Pasteur called it "yeast soup"), has been used ever since. The early media were liquid and the problem of obtaining pure cultures was immense. The lack of a suitable means to obtain pure cultures was a major impediment to the development of the "Germ Theory of Disease."

Pasteur proceeded to inoculate the medium from the bad vat. Following incubation in an oven, he examined the culture microscopically and found that the rods had multiplied. The relevance of the work referred to here is that it marked a turn in Pasteur's life, from that of an organic chemist, studying racemic mixtures and crystal structure, to the field of fermentation. It also marked a turn in science, for if anyone was going to put a stake in the ground to nail down the beginning of the science of microbiology, this may well be the place to have at it with a hammer. What Pasteur deduced from these studies was far

more marvelous than the direct benefit to the alcohol business. Up to this time, it was believed that fermentation was a purely chemical degradation process. Pasteur was perhaps the first to associate the growth of specific microbes with the production of specific fermentation products. Having made this association would have been remarkable in its own right, and many others may have gone no further. It was P.T. Barnum of the famed Barnum and Bailey's Circus who said, "There is a sucker born every minute." I have not quite seen the data to substantiate that claim, but what is certain is that pure genius is not so prevalent. The ability to see beyond the obvious is a gift and Pasteur saw the bigger picture. He deduced, "Why, no one can tell that other kinds of small beings did a thousand other gigantic and useful and perhaps dangerous things in the world" (De Kruif 1954). His paper, 'Mémoire sur la fermentation appelée lactiqué,' published in 1857, which distilled his thoughts on this matter, is one of the classic papers on applied microbiology and opened a new field of study (Brock 1961).

Following Pasteur's studies in sugar beet fermentation and his studies dealing with the manufacture of vinegar in the early 1860s, Pasteur became the man you called if your fermentation process went bad. When the beloved wine industry of France ran into problems, Napoleon III sought Pasteur for a remedy. The story is pretty much a carbon copy of the sugar beet problem, right microorganisms = right product, wrong organisms = wrong product. An outcome of this work was the modification of the Spanish method of slow heating of wine to alter the color. In order to prevent secondary fermentation products, Pasteur determined that by gently heating the wine at 68°C for 10 minutes, followed by rapid cooling, he could destroy the organisms that caused spoilage. A by-product of this process of "Pasteurization" was that wines could be shipped over long distances. Viva la France! This process would later be applied to prevent the spoilage of milk and other heat-labile substances.

The concept of contagion and contagious diseases was not new. Girolamo Fracastoro in 1546 described the knowledge of the time and the process as best he could without the benefit of anyone ever seeing a microorganism. In 1840, J. Henle expounded upon the concepts bolstered by the more recent findings. It was not until the year 1865 that the "Germ Theory of Disease" was truly inaugurated. The silkworm industry in France was almost as important as the wine industry. In the South of France, a disease was killing silkworms, and by 1865, the production of cocoons was a meager 4 million kilograms, down from the peak of 26 million just a decade earlier. The disease was manifested by black spots on the worms, thus the name given, pébrine (pepper) disease. Duma, the famous chemist and senator, was enlisted in this battle. He immediately drafted Pasteur, the well-known trouble-shooter of fermentation processes. By

Pasteur's own admission, he was ill prepared for this work. Nor did he relish being distracted from the work he had been doing in his new-found field of applied microbiology. Together with his understudies, Gernez and Maillot, they found that little globules or corpuscles in the worms came from outside the worms, and were not only a sign of the disease, but its cause. "These globules are alive" (De Kruif 1954). Pasteur noted that adults without these bodies never gave rise to diseased eggs, though having the globules did not assure that the eggs would be contaminated. Having deduced that the disease was passed on from the adult female to her offspring, Pasteur advocated examining the eggs microscopically for corpuscles and destroying those offspring that so contained them. The successful trouble-shooter of fermentations was now the victorious trouble-shooter of worms. And, the wheels were turning. Pasteur was beginning to see more clearly than perhaps anyone had ever seen what all of this meant. "If I can be permitted this antithesis, the role of infinitely small beings appeared to me infinitely large, either as cause of various diseases, especially contagious diseases, or as contributors to the decomposition and to the return to the atmosphere of everything which has lived," so wrote Pasteur (Lechevalier and Solotorovsky 1965).

The ongoing debate of spontaneous generation influenced Pasteur's work and theories. He had given some attention to this matter. He believed that microbes did not arise spontaneously and that prior work that was meant to demonstrate the opposite suffered from a major flaw, exposure to air. He postulated that specks of dust carried microbes and, aided by the physicist Tyndall, demonstrated that this was so. It was during this time that Pasteur performed his famous swan-neck flask experiments. A note on Tyndall: it was he that later determined that bacteria had both a heat-labile and a heat-stable form based on the variable time that it took to effect sterilization by boiling. Ferdinand Cohn in 1877 described the spores of *Bacillus subtilis* and demonstrated their heat-resisting properties. The joint efforts of Pasteur, Tyndall, and Cohn finally killed the chimera of spontaneous generation (Lechevalier and Solotorovsky 1965). As Pasteur began to put all the pieces together he waxed prophetic, "It is in the power of man to make parasitic maladies disappear from the face of the globe, if the doctrine of spontaneous generation is wrong, as I am sure it is." Whether or not his prophecy is ever realized, two things are certain, that the stories below show remarkable progress towards this lofty goal, and also that we have a long way to go. With the exception of one last endeavor in the field of industrial microbiology, to understand the role of microorganisms in the brewing and spoilage of beer, Pasteur's future was to turn to the study of infectious diseases. I pause here to reintroduce Dr. Robert Koch before returning to Louis Pasteur and his impact on the understanding and control of contagious diseases.

Robert Koch 1843 – 1910

As we have watched Louis Pasteur masterfully perform his art on the stage prepared for him by the likes of van Leeuwenhoek and Spallanzani, Act II in this drama belonged to a hesitant physician from Germany. He was not fond of being a run-of-the-mill physician without hope of curing the many ailments he would encounter. With an interest awakened by his professors, his eye was on research. It was Henle, one of Koch's instructors, whose theories paved the way for the eventual codification of Koch's postulates. In the late 1860s, the surgeon Dr. Joseph Lister was initiating the practices of antiseptics to prevent microbial contamination and disease during wound healing, surgery, and childbirth. While studying lactic fermentation, Lister realized the connection of the souring of milk and human disease. During his studies, in order that he might obtain pure cultures of bacteria, he used dilution to obtain single cells and described what is the basis of the most probable number method used today (Brock 1961). Ferdinand Cohn was the preeminent German botanist and microbiologist at the time. His interests were in morphology and life cycles. Cohn recognized the importance of Pasteur's work and was aware of the paradigm shift that was occurring. It was Cohn that introduced Robert Koch to the scientific world (Lechevalier and Solotorovsky 1965) and who served as his advisor and reviewer.

Koch's beginning as a microbe detective occurred early in his professional life while appointed to the General Hospital of Hamburg. A cholera epidemic captured Koch's attention. Armed with a microscope, he observed the slightly curved cells with the wavy appendages from intestinal samples. He was later to identify these bacteria as *cholera vibrio*. Seemingly and hopelessly stuck in the era of observation, there was little more that could be done than to report his findings. The early 1870s found Koch practicing medicine in Poland. To foster his passion for investigation, he improvised a laboratory. It was here in 1873 that Koch began his studies on anthrax, a disease that was prevalent in his location. He began looking at the blood of animals that died of anthrax under the microscope and saw rod-shaped bodies. But, were they alive? The answer to this question followed the injection into mice of blood from sheep containing the rods. The mice soon succumbed and when he examined the blood of the diseased, deceased rodents, there were the rods.

To study the life cycle of the rod-shaped bodies Koch sought a way to grow and view them *in-vitro*. What follows is a simple, yet profound, invention. His materials included a heat sterilized glass slide, a thick piece of glass with a concave well dug in it (both sterilized by heat), petrolatum gel, a tiny bit of spleen from an infected mouse, and his trusted microscope. One more thing

was needed, a clear growth medium. For this, he chose the aqueous humor from the eye of an ox. A drop of this fluid was placed on the glass slide, and the fragment of spleen was added to the drop. The concave well of the thick glass, rimmed with petrolatum, was placed over the drop and onto the surface of the glass slide, with the petrolatum being used to adhere the two together. The whole device was inverted to prepare the first ever hanging-drop slide. As he watched under the microscope over a period of hours, the rods began to grow. The vitality of the rods thus being confirmed, Koch deduced that the growth of these rods was the cause of the animals dying. What he did next represents a turning point in science, in the field of microbiology, and in the "Germ Theory of Disease."

As methods to obtain pure cultures of organisms were deficient, Koch performed successive subcultures of the hanging drop in the same fashion as the original. He succeeded in obtaining and maintaining a pure culture of the anthrax bacillus free from animal tissue. To demonstrate that this species alone was all that would be necessary to cause the disease, he inoculated the rods subcutaneously into a mouse. The mouse developed the usual symptoms of anthrax and died, following which Koch observed the anthrax bacillus in spleen preparations. Definitive cause and effect! This was the first time that it was proven that a specific microorganism could cause a specific disease in animals. His experiments were successfully reproduced in several species of animals.

The technique thus utilized was far too laborious for routine use and there was no assurance that a pure culture was obtained. Much confusion had been generated as to the bacterial causes of disease owing to the problem of mixed cultures. This led to the development of "Koch's Postulates." In order to relate a given organism as the cause of a specific disease, one must prove four points:

1. Find the organism present in all cases of the disease and not present in healthy individuals.
2. Isolate and cultivate the organism in pure culture, demonstrating that it alone is present.
3. Induce the disease in a healthy individual (animal, if possible) with this pure culture.
4. Demonstrate that the organism is present in the induced disease, and that on reisolation in pure culture, it is the same organism that was used to induce the disease (Umbreit 1962).

However, the art of obtaining pure cultures was not a pretty picture at this time since broth cultures were uniformly used. Serial dilutions were performed and the entire series was incubated. The highest dilution showing no growth

would, in effect, be considered sterile. The previous dilution would be taken to consist of growth from a single cell. This was obviously not fool-proof. It may have been Ferdinand Cohn who recommended to Robert Koch the use of slices of potatoes to grow bacteria. But it is more generally believed that the discovery was by Koch and quite by accident. It is believed that he was the first to use a solid surface to grow microorganisms. As the story goes, he noticed different colored specks growing on his half-boiled potato that was exposed to air in the lab. As he examined the specks, he found that each was composed of a single bacterial type. He reasoned, "In liquid culture, germs of different sorts get all mixed up and swim among each other, when they fell from the air onto the solid surface of the potato, each one has to stay where it falls and there it multiplies into millions of its own kind" (De Kruif 1954).

Following this observation, Loeffler and Gaffky, working in Koch's lab, devised the streak method whereby they would use a fine wire or needle to streak a potato with the organism. A second idea was to dilute the organism suspension down to a few cells, distribute them in a liquid, and then suddenly solidify the liquid to prevent the remixing of the bacteria. Koch, being an amateur photographer, and the first to produce photomicrographs while working on anthrax, used gelatin for this purpose. This seemed suitable at the time, but several major drawbacks became obvious. The first was that gelatin is not typically sterile and could not be effectively heat sterilized due to degradation. Secondly, gelatin melted at temperatures above 15°C and therefore could not be incubated at the optimal temperatures for growth of many of the organisms they were trying to study. Thirdly, gelatin could be liquefied by many organisms.

The final solution came in 1882, when Frau Angelina Hesse, the wife of a graduate student in Koch's lab, suggested agar-agar, which she often used as a thickener to prepare jellies. At 1 – 1.5%, its properties were well-suited for use in microbiology, having a melting point of 100°C and a setting point of less than 45°C. Since the agar would not remelt at less than 100°C, the cultures could then be incubated at any temperature desired below this point. In addition, agar had neither nutritional value nor inhibitory properties. And such was the perfect solution to a nagging problem from the kitchen of the wife of a graduate student, what's his name? Here it began, and it has never been abandoned since. A modification of this method was developed where the liquid agar-containing culture, once inoculated, was poured onto glass plates and solidified. The plates were then placed under a bell-jar to minimize contamination from dust. The glass plates and the bell-jar were bulky and cumbersome, and presented a contamination risk, especially each time the bell-jar was removed to examine the culture. It would be another five years before a suitable replacement was devised. We take for granted the use of solid media today, but then, at the verge

of discovery of the causative agents of many diseases, this was a godsend. This technique revolutionized cultural bacteriology and enabled the great advancement of the subject during the last two decades of the 19th century.

Before leaving the work of Koch and associates on anthrax, remember that Dr. Koch was first a physician, or more appropriately, a researcher who happened to be a physician. But here, the physician in him awakened. He was not satisfied in finding a cause for a disease. What he wanted, what drove him into research in the first place, was being able to use the knowledge gained to alleviate suffering. And since he had no knowledge of how to treat the disease, he focused his attention on prevention. Through his studies, he determined that the heat-resistant spores were the agents that could survive for long periods in the field and were the means of infecting healthy animals. He proposed two methods of properly disposing of diseased animals upon death. The first was to burn them as soon as they died, otherwise to bury them deep enough so that the spores would not germinate under the cold soil. "So Koch changed the whole business of doctors from a foolish hocus pocus with pills, elixirs and leaches into an intelligent fight where science instead of superstition was the weapon" (De Kruif 1954).

Koch, having developed, or rather invented, the skills needed to determine the cause of disease; turned his attention with all of his meager weapons upon the suffering of man. In his day, consumption (tuberculosis, TB) was responsible for one out of every seven deaths in the developed world. Its contagious nature was known. The year was 1882 and methods for the staining of bacteria were in their infancy. Koch prepared stained preparations of tubercles from a recently claimed victim using heat to penetrate the waxy outer envelope. The small bacilli were obvious and plentiful. He infected lab animals with the infectious tubercles and, after several weeks, they began to die. He subsequently recovered organisms of the same morphology from the deceased animals. This he repeated in other animals, but until he was able to show the same effect from a pure culture of the organism, he was not to be convinced (Koch's Postulates 2 and 3). This was not an easy task. After many unsuccessful attempts to cultivate the tubercle bacillus, he conceived of preparing them a meal much like that they were used to eating. He prepared slants of blood-serum jelly that was heated to coagulate the serum proteins, inoculated them with freshly crushed tubercles from a deceased guinea pig, and incubated them at 37 – 39°C, the normal temperature of the animal. Most organisms would have been obvious in a matter of days, but two weeks had passed and there was no obvious sign of growth. Many others would have given up too soon, but his knowledge of the slow progression of the disease caused him to tarry long enough to have his patience rewarded.

Upon examination of the tardy colonies, he observed the same rods he had seen from the tubercles, and only these tiny crooked rods. Before moving on to Postulate 3, infecting healthy animals with the pure culture, he performed several subcultures to confirm the purity. He then exposed many animals of various types and all died of consumption, and from them he recovered the same tiny rods from the murderous tubercles. With confidence, he shared his findings with the medical establishment of Europe. The cause of consumption had been proven. It was the medical “shot heard around the world.”

During intermission from this scene in the drama, Koch was summoned to India to address the endemic cholera problem. He found the same slightly curved rod in every victim of the disease, but never in healthy individuals. Koch, convinced of the fallacy of spontaneous generation, began a search for the source of the organism. He did not have to look far, no further than the polluted water around the huts where he was working. The “Germ Theory of Disease” was advanced brilliantly as Koch demonstrated the association between specific microbes and specific diseases, that there was an external source of the germs, and to cause disease, the germs must gain entrance into the victim. This was clearly time for Dr. Robert Koch to take a bow.

One other thing that trips to India taught Koch was that lugging around suitcases of glass plates and bell-jars was going to shorten his brilliant career. Fortunately for him, back at the “dressing room,” one of his assistants was revising the script. In 1887, Julius Petri made modifications to the plating procedure by using flat double dishes in which the upper dish served as a lid. To eliminate condensation and contamination, he would incubate the plates upside down. Eventually, the glass was substituted with clear plastic, the basic design of which has not been modified since. The only other innovations made were the addition of grids to aid in counting, and the addition of various compartments, bi-plates or quad-plates, following the invention of selective and differential media. It was Chantemesse and Widal in the same year that prepared the first differential medium using glucose and lactose peptone water to differentiate *E. coli* from *Salmonella typhi*. Soon after, the Japanese bacteriologist Kitasato developed a test for indole production to further aid in the differentiation of these species. In 1892, Wurtz of Paris introduced the use of indicators in the medium to detect acid production. Many developments in selective media were advanced during the 20th century based on clues provided by studies in microbial physiology.

Louis Pasteur was still busy while Koch was receiving his acclaim and praise. During the 1880s, Pasteur, the father of microbiology, was about to lead the world around another corner. Having isolated what he believed was the causative agent of chicken cholera, he began inoculating healthy animals

with the invader. The normal course of the disease followed by death was the typical outcome. He made successive broth cultures from previous ones and noted that, depending on the length of time between subcultures, the virulence of the organism could be altered as evidenced by a reduced death rate. He also observed that when the surviving animals were challenged with more virulent microbes, they were now immune. This significance of this process of attenuation, as Pasteur referred to it, was not lost upon him. He likened these findings to those of Edward Jenner and his smallpox vaccine almost a century earlier with one notable difference, as Pasteur reported, that Jenner was not able to recover the virus. The uniqueness of Pasteur's vaccine can be found in this, here was the first demonstration of the alteration of virulence and the development of a vaccine produced by laboratory manipulation. A corner had been turned, and the curtain went up on the field of preventative medicine.

Pasteur and his colleagues, Emile Roux and Chamberland, had further successes using attenuated anthrax bacilli. Pasteur's final bow (and here is where Koch's Postulates may falter) was when he was unable to isolate an organism from cases of rabies, and he developed a vaccine by successive transfer of infected tissue in rabbit brains. He found that he could attenuate the virulence of tissue taken from the rabid rabbits by altering the time and conditions under which the tissue was dried, until eventually no virulence would remain. By a series of injections, each one being more virulent than the previous, he rendered a number of dogs immune to the disease. Because of the high fatality rate from the bite of rabid animals, it was not difficult to find human cases for trial. His initial trials on children that were severely bitten by rabid dogs were successful. Pasteur's final curtain call was at the near misfortune of nineteen Russians that were bitten by rabid animals. Only four would have survived typically, but Pasteur and his series of inoculations spared the life of all but three. For this, Pasteur was honored by the Tsar of Russia and was granted money to start the building of the l'Institut Pasteur. Pasteur died soon thereafter in 1895 at the age of 73.

ON THE STAINING OF BACTERIA AND STAINED PREPARATIONS

Before drawing the curtain closed on the 19th century, a remarkable century of discovery and applied microbiology, a brief history of the use of dyes deserves mention. Ferdinand Cohn was the first to stain histological sections using vegetable dyes in 1849. Perkin of Manchester synthesized the first aniline dyes in 1856. In 1875, Weigert used methylene blue to stain bacteria, though he stained them in suspension and examined them in the wet state. In 1877, Robert Koch

was the first to prepare dried films of bacteria and to stain them with methylene blue. Films were dried in air and fixed with alcohol, a technique developed by Paul Ehrlich for blood films. The fixed and stained films were protected by coverslips and became permanent preparations. Using this technique, Koch made the first photomicrographs of bacteria. Paul Ehrlich published further developments in the use of methylene blue for the staining of bacteria in 1881. The following year, Koch stained tubercle bacilli with alkaline methylene blue, using heat to penetrate the waxy envelope.

Ehrlich liked to “paint” organ sections with dyes and painted a sick liver from a consumption (TB) patient and saw little colored rods, which he thought were crystals. But after Koch’s discovery of the bacteria of consumption, Ehrlich knew that he was seeing microbes, the tubercle bacilli. Ehrlich went to Koch to show him a novel way to stain the tubercle microbe. He used methyl violet or alcoholic fuchsin in aniline water and acid as a decolorizing agent. Ziehl introduced phenol with methyl violet. In 1883, Neelsen modified Ziehl’s method in which the cells were stained with hot carbol fuchsin and used 15% sulphuric acid as a decolorizing agent. Cells retained the red dye while the tissue cells were decolorized and counter-stained with methylene blue or malachite green, a technique now referred to as the Ziehl-Neelsen stain. Staining of tissues was difficult with using methylene blue since both the background and organisms were stained blue. Decolorization of tissue sections was tried but the tissue and sometimes the bacteria were decolorized. Hans Christian Gram, the Danish bacteriologist, was aware of the Koch-Ehrlich method of differentially staining tubercle bacilli (acid-fast stain). Gram had been working to differentially stain pneumococci in tissue sections. In 1884, he introduced the technique of counter-staining after decolorization. Tissue sections that were treated with absolute alcohol were immersed in Ehrlich’s aniline gentian violet (crystal violet) for 1 – 3 minutes. The stained slides were transferred without rinsing to an iodine-potassium iodide solution for 1 – 3 minutes. The sections were then treated with absolute alcohol until completely decolorized and clarified in cedar oil. Bacteria were stained intensely blue and the background tissue was light yellow. Gram went on to suggest a counterstain (Bismarck brown) to stain the background tissue. The technique became of greater value in dividing bacteria into two classes based on the ability of the bacteria to retain the dye when applying the decolorizing agent. Those that retained the violet dye were referred to as Gram-positive and those that were decolorized and stained by the counter stain, as Gram-negative. Frederick Loeffler, in 1890, first stained and demonstrated flagella to answer the question of why some bacteria were motile and others were not. Cell walls were not stained until 1894 by Fischer using a technique of plasmolysis to separate the cell membrane from the cell wall.

ADVANCES IN IMMUNOLOGY

Aside from the work cited earlier on the discovery and production of vaccines, immunology was advancing on other fronts as well. In the mid 1880s, Elias Metchnikoff, a Russian born Jew with a doctorate in invertebrate biology, was busy advancing his theory of the cellular basis of immunity. He set out to demonstrate that it was the white cells in blood that protected us from invading microbes. It was his colleague, Professor Claus, who coined the term *phagocytes*, from the Greek word for devouring cells. For many years, the scientific battle raged over the cellular (phagocytic) and humoral (serum antibodies) basis for immunity. Metchnikoff clung tenaciously to his theory of gobbling cells; being so blinded by his pet theory that he could not consider the evidence for the other. Nor was there any room in his mind for the possibility that both may be true.

Metchnikoff, together with Roux, began to study syphilis, born out of Metchnikoff's new found interest in aging and death, as he himself was aging, and the finding by the Scandinavian scientist Edgren that syphilis led to hardening of the arteries. This, he concluded, was one of the causes of death. Metchnikoff learned that calomel ointment, if applied to the site of inoculation with the syphilis organism, would prevent the spread of the disease.

Before the end of the decade, Emile Roux discovered a strange poison seeping from the bacillus of diphtheria. About the same time, Emil Behring, a student of Robert Koch, observed a unique property in the blood of guinea pigs that could make the diphtheria toxin completely harmless. Together, with the aid of Frederick Loeffler, they discovered the diphtheria antitoxin. Based on Loeffler's finding that the diphtheria bacillus remained localized in relatively few numbers, he deduced that the organism must make a poison by which it was able to kill the host. Loeffler's hunch was proven correct by Roux who grew the bacilli in flasks and separated the cells from the broth using a fine porcelain filter. Large doses of the cell-free broth were injected into rabbits resulting in death. Meanwhile, Behring was evaluating various chemicals to protect mice from diphtheria. He found that tri-chloride of iodine protected some of the animals. When he subsequently injected the surviving animals with diphtheria toxin, the animals lived. He reasoned that there was something in their blood that was protecting them. He later saved the lives of guinea pigs infected with the diphtheria bacillus by injecting them with antitoxin he had produced in sheep. Roux and Behring began producing diphtheria antitoxin in horses and used the serum to inject babies suffering with the illness. The cure rate was as high as 75% for a disease that heretofore was nearly uniformly fatal.

HUNTERS OF INSECT-BORN DISEASES

Having highlighted the many important discoveries and advancements in microbiology during the 19th century, this chapter would be incomplete without at least a brief mention of the work that led to the understanding of insect vector-born diseases. Over the last decade and a half of this remarkable century, spurred on by the foundations prepared by the early masters of microbiology, came a new breed of microbiologists.

These were men like Theobald Smith who earned his medical degree from Albany Medical School at a time when there was no course in bacteriology in any medical school in America. He devoured everything he could that was written about Robert Koch. While employed by the Bureau of Animal Industry in Washington, he learned the art of microbiology. Here, he learned to make a new kind of vaccine from the remaining proteins obtained from filtered microbial preparations, the first organism-free vaccines. But perhaps his most important work was also perhaps the least glamorous, unless you consider picking thousands of ticks off of cattle to be glamorous. This he did to try a theory of Texas herders as to the cause of "Texas Fever," a rather troublesome and fatal disease that afflicted northern cows that came in contact with southern cows. In 1888, Smith found pear-shaped living creatures in the blood from infected cows. Through pain-staking experiments over several tick-growing seasons, he demonstrated conclusively that the ticks, and more specifically, the offspring of mature female ticks, were responsible for the transmission of the disease. This was a novel concept at the time, and not well received by the medical establishment. It was finally in 1893 that Theobald Smith sat down to summarize the findings of four years of investigation and, for the first time, mankind became aware that disease could be carried by insects and that, by using insect control strategies, you could control the spread of the disease. Theobald Smith also developed the first apparatus (a side arm to collect gas) for the visual observation of gas production (an important characteristic to distinguish pathogenic enterobacteria) from fermentation of carbohydrates. And it was in 1898 that Durham, a British bacteriologist, published the use of a simple glass tube that could be placed in the test tube containing the fermentable carbohydrate. Over one hundred years later, these "Durham tubes" are still in use.

In the late 1890s, on the heels of Theobald Smith, David Bruce, a young doctor in the British Army Medical Service was sent to Malta where he would study the cause of Malta fever. He set up a lab and spent weeks learning how to make culture medium out of beef broth and agar-agar to grow the unknown germ of Malta fever. He and his new bride worked tirelessly and did discover

the germ responsible. Yet, he was ordered away from Malta and sent to Egypt, and then back to England. Later in Africa (Zululand), Bruce was the first to set his eyes on the trypanosomes that caused nagana, a fever of animals. He showed that these trypanosomes were transmitted by tsetse flies. He found similar organisms as the causative agent of sleeping sickness in Uganda and determined that the disease was transmitted by the Kivu, the Ugandan name for the tsetse fly. The Kivu lived only by the water and so he proposed a way to eradicate the disease by moving all people away from the Lake Victoria Nyanza. The disease seemed to disappear for a time but came back. Bruce postulated that there must be a "reservoir" of the organism in wild animals. He later proved this so in the antelope. He later returned to the problem of Malta fever and succeeded in showing that the bacillus of Malta fever was transmitted in goat milk.

In 1898, a contemporary of Theobald Smith, and encouraged by the work of both Smith and Bruce, Ronald Ross, demonstrated that the parasite responsible for malaria was transmitted by the bite of the mosquito. At the same time, and quite unaware of the work of Ronald Ross in India, Giovanni Battista Grassi was traipsing the low hot places of Italy to catalog the types of mosquitoes found where malaria was prevalent, hoping to determine, where it was not, as he supposed, that only certain types of mosquitoes were the carriers. And he found that the *Anopheles claviger* always was present where there was malaria. Grassi later became aware of and read Ross' work and found the life cycle of avian malaria in mosquitoes to be the same for the human species. Grassi demonstrated that it was not the mosquito offspring, as was the case with Smith's Texas fever ticks that transmitted the disease, but the adult.

The work of Dr. Walter Reed and his associates at the turn of the century was a true work of love. American soldiers in Cuba were dying of yellow fever. Sanitary measures that were instituted to combat other diseases had no impact on the transmission rate of yellow fever. Since no animal model for yellow fever existed, they needed human subjects and used themselves. To prove that yellow fever was transmitted by mosquitoes, they were to become bait for mosquitoes that had bitten several yellow fever patients. The death rate of this malady was greater than 20%. Dr. James Carroll and Private William Dean provided the first meals and both caught yellow fever. However, since both had been in dangerous yellow fever zones before, the investigators were not convinced. Among them, during these experiments, Dr. Jesse Lazear was bitten and subsequently died of yellow fever.

Walter Reed asked for volunteers and so came Private Kessenger and civilian John J. Moran. Following quarantine to ensure they did not acquire yellow fever naturally, they were submitted to the bites of infected mosquitoes. Kessenger came down with a non-fatal case of yellow fever. The experiment

was repeated on five Spanish immigrants with a “success” rate of four confirmed cases. Further testing was done to prove that clothing and bedding from yellow fever victims would not transmit the disease as was thought at the time. Additional tests were performed in carefully contained areas where specific contaminated and non-contaminated mosquitoes were given access to brave volunteers. Definitive proof was obtained. Having proven the vector, the causative agent still eluded them. Reed and Carroll theorized that an ultra-microbe, too small to be seen by the best microscope, was responsible. Carroll filtered yellow fever victim blood through a porcelain filter so fine that no “visible” microbe could pass through, and inoculated three healthy volunteers with the filtrate; all three developed yellow fever. Based on this work, yellow fever was ultimately controlled by eradicating the *Stegomyia* mosquitoes, the specific vector responsible for transmission. (The field of virology may be considered to have started with Jenner and his smallpox vaccine. Further study commenced with the work of Iwanowski in 1892 that showed that tobacco mosaic disease was caused by a filter-passing ultra-microscopic organism. Six years later, Loeffler and Frosch showed that foot-and-mouth disease was caused by a similar organism. Pasteur’s work on rabies preceded this work, but did not lead to a concept of filter-passing ultra-microscopic organisms.)

THE DAWNING OF THE ‘MODERN’ AGE OF CHEMOTHERAPY

In 1676, Leeuwenhoek first published his observation of microbes. Over 200 years later, Paul Ehrlich said, “We must learn to shoot microbes with magic bullets.” This was not a truly novel concept for as early as 500 BC, the Chinese had used plant extracts to treat disease, and mercury was used for the treatment of syphilis in the latter end of the 16th century and for the treatment of malaria by Jesuit missionaries in the early 17th century. Ehrlich was not a microbe hunter in the pure sense, but rather a chemist with a fascination with dyes. To his amazement, he found that after injecting methylene blue into a rabbit ear vein, the dye selectively stained the nerve endings. So what? Ehrlich began to dream, “Here is a dye that stained only one tissue out of all the tissues in an animal’s body...there must be one to hit no tissue of men, but to stain and kill microbes that attack men” (De Kruif 1954). Here is that gift to see beyond the obvious that we observed in Louis Pasteur. It wasn’t until fifteen years later that he was able to try his theory. His search for the magic bullet lasted eight years. In 1902, he tried 500 dyes to protect mice from a trypanosomal disease of horses. His approach was trial and error, all the mice died. He tried the dye benzo-purpurin and once again failed. Or, like Thomas Edison, it may be said of

these attempts that Ehrlich had discovered 501 ways not to cure the disease. He then added sulfo groups to this compound and a mouse lived. In typical Paul Ehrlich fashion, he jumped to the conclusion, "I have a dye to cure a mouse – I shall find one to save a million men," so he dreamed.

A later preparation, number 606 of Atoxyl, cured mice of the trypanosomes. Ehrlich's assistant Sahachiro Hata took a rabbit infected with *spirochete pallida* (so named by Fritz Schaudinn), the agent of syphilis, and he viewed the spirochetes under a dark-field microscope. Following one injection with preparation 606, the spirochetes were killed and the rabbit's ulcers were healed. Following subsequent successes in rabbits, it was time to test the drug on humans. These early trials were a huge success, resulting in immediate reversal in the disease state. The magic bullet salvarsan had been invented. In 1910, 65,000 doses of salvarsan were injected into humans with much success, but also with some side effects and occasional deaths. The cause was never isolated. But Ehrlich and Hata turned a corner in microbiology. The answer to disease was not only in the serums and vaccines of Koch and Pasteur, not only in the insect eradication programs of Smith, Bruce, Grassi, and Reed, but also in "magic bullets." The modern age of antimicrobial chemotherapy had begun.

A BRIEF LOOK AT THE 20TH CENTURY

With the groundwork suitably laid in the 19th century, the harvest forward was sure to be rich and prosperous. The tree of microbiology had deep roots and had blossomed nicely. The investigators of the 20th century were now ready to pick some fairly low hanging fruit, though there would be some new trees to plant. What follows is a brief catalog of some milestones in the advancement of microbiology in the 20th century.

The 1900s witnessed tremendous advances on several fronts: microbial metabolism, microbial genetics and molecular biology, antimicrobial therapies, development of selective and differential culture media, and virology, just to name a few. Besides the basic understanding gained in the field of biochemistry, studies in bacterial metabolism made possible the development of the many diagnostic methods to select for or differentiate between microorganisms. Secondly, the art of microbial taxonomy moved from a purely morphological basis to one based on metabolic characteristics. The reclassification of species and sometimes genera resulted much in the same way that we are seeing with molecular methods that allow grouping of organisms according to their genetic makeup.

The role and nature of DNA was born out of studies aimed at understanding bacterial variation and antibiotic resistance. The processes of bacterial

transformation and mutation were the basis for determining that all genetic information of cells is coded for by DNA. The belief that DNA is the essential genetic material was derived from the demonstration that new traits were acquired upon the transfer of DNA from one bacterial cell to another. On the heels of these discoveries, Watson and Crick published the double helix model for DNA in 1953, and later unraveled the genetic code. In 1983, DNA became a useful tool when Dr. Kary Mullis invented the technique of polymerase chain reaction, which made it possible to rapidly amplify DNA thus making many copies available for study and for diagnostic and therapeutic endeavors. The impact of these discoveries is only beginning to be realized.

The development of antibiotics, though of great interest to the clinical microbiologist, has little relevance to the subject of methods in microbiology with the exception of the microbial fermentation industry that it spawned and the subsequent advancements in the necessary technology. The year 1928 saw the fortuitous discovery of penicillin by Alexander Fleming. His work describing an extracellular substance that inhibited several species of bacteria was limited by the lack of facilities and technology to purify the substance. As a result, limited trials for wound healing were minimally successful. It would be a full dozen years later when Howard Florey and associates, driven by the intense needs of the Second World War, picked up where Fleming left off. By 1940, penicillin was purified. The issues related to commercial production were quickly disposed of and, within one year, penicillin was available commercially. With such a success, the hunt was on for other natural antimicrobial weapons produced by soil microorganisms. Later, streptomycin was discovered and other broad-spectrum antibiotics followed by the development of semi-synthetic antibiotics.

The "magic bullet" of Ehrlich paved the way for other developments in useful antimicrobial chemotherapeutic agents. Work on additional dyes, and subsequent elucidation of the active moiety of prontosil red, led to the discovery of sulphonamide. The study of the effect of dyes on bacterial growth also led to their use in a variety of selective media, especially to aid in the recovery of enteric pathogens. The 1920s – 1940s witnessed the development of many of the selective media used today, as well as techniques for the isolation of anaerobic organisms. With the advent of the London County Council Laboratory Services in the 1930s, with its numerous labs, a central medium kitchen was established. It was this lab service that led to the commercial development of dehydrated media in 1950.

Advances in virology were made possible by: 1) the use of fertile hens' eggs as a method for the cultivation of a virus, 2) the development of the ultra-violet microscope by Barnard to give the first view of the elementary bodies

of a number of viruses, 3) the development of tissue culture techniques in the 1920s, and 4) the development of the electron microscope by Marton in 1934. In 1954, the development of the monolayer technique and the technique of using cytopathic effect for viral detection led to the availability of live virus vaccines. The development of antiviral chemotherapies has been a critically important field ever since.

THE FIRST RAPID METHOD

It seems fitting to conclude this chapter with a description of what may be one of the earliest attempts at developing rapid microbiological techniques, and more specifically, a rapid plate count technique. It appears that one Professor Frost, who must have been an impatient man, devised such a means. Frost prepared serial dilutions of organisms in melted agar medium cooled to 45°C, and spread 0.5 mL of each dilution on sterile glass slides. He incubated these for 6 to 8 hours, heated the slides to fix them, stained them, and counted the miniature colonies under the microscope. Though simple, practical and easy, this method has never caught on (Umbreit 1962). What is hoped for is that the many rapid methods described in this volume do indeed catch on, receive wide acceptance, and become the norm in the microbiology lab.

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