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Biology of Rickettsial Diseases

Volume I

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Chapter 2

HISTORY OF RICKETTSIOLOGY

Emilio Weiss

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I. INTRODUCTION

Inter armas silent musae (in time of war the muses are silent) — Thus did von Prowazek[1] lament that the outbreak of war between Serbia and Bulgaria was interfering with his investigations on exanthematic typhus.

* * *

A history of rickettsiology is bound to be biased in favor of its early investigators. Asthe number of scientists as well as the number of available technical approaches increases, recognition of individual achievement becomes more difficult. Furthermore, the significance of new discoveries has not been subjected to the test of time. With this limitation in mind, this chapter is devoted to a discussion of three topics:

1. The discovery of the major etiologic agents of rickettsial disease, their vectors, and mechanisms of transmission.
2. The major technological developments that have facilitated the study, control, and treatment of rickettsiae and rickettsial diseases.
3. The evolution of the concept of "rickettsia" from a microbial entity which is neither a typical bacterium nor a typical virus to a well-characterized set of bacteria.

The term "rickettsia" stems from a 1916 publication[2] by da Rocha-Lima, a Brazilian microbiologist who was at the time the chief pathologist at the Tropeninstitut in Hamburg, Germany. He named the etiologic agent of epidemic typhus *Rickettsia prowazeki*, in honor of an American, Howard Taylor Ricketts (Figure 1A), whose work he greatly admired, and an Austrian, Stanislav von Prowazek, a friend and colleague, who like Ricketts had died of typhus while investigating its etiology.

II. ETIOLOGY

A. Rocky Mountain Spotted Fever (RMSF)

When Ricketts arrived in Missoula, Mont. in April 1906, there had been over 200 cases of spotted fever in Montana and twice that number in Idaho. The disease in Montana was quite severe with a mortality rate of 70 to 80%, while the mortality rate in Idaho was 1 to 5%. Local physicians and epidemiologists assigned to study the disease had amassed a great deal of useful information. In a 1904 review of their studies, Wilson and Chowning[3] concluded that there was no evidence of transmission of the disease from person to person or by means of a common water or food supply. Because of the seasonal incidence of the disease, from March to July, transmission by the bite of a tick was the most likely hypothesis. They also hypothesized that a rodent was the natural host. Their contention that the etiologic agent was a protozoan which they named *Pyroplasma* (syn. *Babesia*) *hominis* was quickly refuted.[4] McCalla,[5] an Idaho physician, in association with H. A. Brereton, in 1905 applied a tick obtained from the chest of a man who was very ill with spotted fever to the arm of another patient. The tick remained on the second patient for 48 hr and was then applied to the leg of a woman, where it remained for at least 10 hr. Both came down with relatively mild cases of spotted fever and recovered. The incubation periods were 9 and 3 days, respectively. This experiment was conducted with the full consent of the participants.

Since McCalla[5] did not publish his findings until 1908, Ricketts was not aware of this experiment when he started his work, but when he learned of it, he promptly acknowledged its importance. Ricketts moved very rapidly.[6] He injected the blood of patients into guinea pigs and produced a disease characterized by fever, enormous swelling of the testicles and

A

B

C

D

FIGURE 1 Some of the scientists who greatly advanced our knowledge of rickettsiae, photographed during the approximate time of their major contributions, as described in the text: (A) Howard Taylor Ricketts, (B) Edward H. Dermick, (C) Herald R. Cox, (D) Marianna R. Bovarnick.

scrotum, and in many cases death. Guinea pigs could also be infected with serum or washed cells. Although no bacterium was recovered in bacteriologic media, Ricketts found to his surprise that the infectious agent was retained by a small Berkefeld filter. In collaboration with W. W. King, Ricketts reproduced the disease also in rhesus monkeys. When the route of inoculation was subcutaneous, the syndrome closely resembled the human disease. The next step was to test the hypothesis of Wilson and Chowning[5] that the disease was transmitted by the wood tick. Ricketts transmitted the disease from guinea pig to guinea pig by means of the tick, demonstrated the presence of naturally infected ticks, and showed that the female tick could transmit the agent transovarially.

Ricketts described small diplococcoid bodies or diplobacilli in infected animals, particularly in tick eggs. The association of these bacteria with the disease was indicated by their agglutination by immune but not by normal guinea pig serum. Ricketts was perplexed by the discovery of bacteria of identical morphology and antigenic specificity in eggs of ticks that were not infectious. He explained this phenomenon by assuming that these bacteria were widespread, but varied considerably in virulence.[6] Wolbach,[7] however, deserves the credit for the first detailed description of the etiologic agent in 1919. He clearly recognized it as an intracellular bacterium which was seen most frequently in endothelial cells. He was struck by the fact that in the tick, and also in mammalian cells, the microorganism was intranuclear. The nucleus was often completely filled with minute particles and often was distended. Although Wolbach recognized its similarity to the agent of typhus and tsutsugamushi fever, he did not regard the designation "rickettsia" as appropriate. He proposed the name *Dermacentroxenus rickettsi*. Brumpt felt that the etiologic agent of RMSF, despite some uncertainty about its properties, belonged in the genus *Rickettsia* and in 1922 proposed the name *Rickettsia rickettsi*.[8]

It became apparent in the 1930s that *R. rickettsii* was not confined to the Rocky Mountains and was isolated with increasing frequency in other regions of the U.S. and in South America. It was also obvious that rodents and domestic animals, such as the dog, must play important roles in dissemination and this was supported by serologic surveys. Natural infection among vertebrates was repeatedly demonstrated in Brazil,[9] but recovery from vertebrates in the U.S. has been elusive. The first report was made in 1954 by Gould and Miesse,[10] and the animal involved was a meadow vole (*Microtus pennsylvanicus*), trapped near Alexandria, Va. A decade later several isolations from vertebrates were reported by Burgdorfer et al.[11] in the western U.S. and Bozeman et al.[12] in the eastern U.S.

B. Epidemic and Endemic Typhus Fevers

Because of their world-wide distribution, these diseases were investigated in many countries. Credit for the first significant observations on the epidemiology of epidemic typhus goes to Nicolle,[13] director of the Pasteur Institute in Tunis. He was struck by the fact that typhus patients were highly infectious to close contacts until they were admitted to the hospital. Once their clothing had been removed and the patients had been shaved and washed, they were no longer infectious. To Nicolle this strongly suggested that the culprit responsible for transmission was the louse. To prove his point, in 1909 Nicolle and associates[13,14] transmitted the disease by means of the blood of a typhus patient to a chimpanzee and from the chimpanzee to the macacus monkey. The disease was then transmitted from macacus to macacus by means of the louse. This accomplishment became a powerful impetus for the establishment of delousing procedures which played an important role in the control of epidemic typhus in the city of Tunis and shortly thereafter in World War I. In 1911 Nicolle et al.[15] transmitted the infection to the guinea pig. Although fever was the only sign of disease, the guinea pig remained for many decades the most useful experimental animal. Nicolle was awarded the Nobel Prize in 1928 for his studies on exanthematic typhus. In 1959 a symposium on rickettsiae was held in Tunis in commemoration of the 50th anniversary of Nicolle's major discoveries.

In December 1909, Ricketts and Wilder went to Mexico City to investigate epidemic typhus.[6] Within a few months (Ricketts died in May 1910), they confirmed the results of Nicolle. They regarded the agent as somewhat similar to the agent of RMSF: it was not filterable and small diplobacilli could be seen in Giemsa-stained blood smears from patients at the height of infection. However, the two agents were distinct since cross-immunity or cross-agglutination could not be demonstrated.

The next step in the investigation of typhus took place in Serbia in 1913 and a Russian prisoner-of-war camp in eastern Germany in 1915. von Prowazek[7] and da Rocha-Lima[8] confirmed the observations of previous workers, reinforced the view of the bacterial nature of the etiologic agent, cast doubt on the supposition that the rickettsia is transmitted trans-ovarially in the louse, and emphasized the importance of louse feces in transmission.[16] The definitive work on the etiology of typhus and the intermediate role of the louse is generally attributed to the painstaking experiments carried out in 1920 in Poland by the Typhus Research Commission of the International Red Cross, headed by Wolbach et al.[17]

During the first two decades of the 20th century, numerous attempts were made to cultivate bacteria from the blood, urine, and stools of typhus patients. Two such attempts are worth mentioning. In 1909 and 1910, Wilson[18] reported from Belfast that he had occasionally isolated from stools and rarely isolated from urine a coliform bacterium which he designated *Bacillus* U. He did not isolate it from blood and did not regard it as the etiologic agent of typhus. He demonstrated, however, that the bacterium was agglutinated by sera of typhus patients to a titer three to ten times greater than by sera from normal individuals or patients with other diseases. His observations received virtually no recognition. Comparable results, possibly identifying typhus immune sera more clearly, were obtained in 1916 in Berlin by Weil and Felix[19] with a *Proteus* isolate from patients' urine. At that time the world desperately needed a diagnostic test for typhus and their results were immediately applied. The Weil-Felix test remained for many decades the chief tool for the serological diagnosis of the typhus fevers.

Two problems remained to be investigated with regard to the natural history of typhus. Is there just one or more than one type of typhus fever? If the louse is only an intermediate host, what is the reservoir of typhus during interepidemic periods? The two questions were intertwined because recrudescent (epidemic) typhus and endemic (or murine) typhus were not always differentiated and both were considered to be mild forms of typhus.

Ricketts and several of the early workers wondered if Mexican and European typhus were the same. It had been clearly established by previous workers that fever was the only sign of disease in guinea pigs inoculated with European typhus. In 1917, however, Neill[20] noted that male guinea pigs inoculated with the blood of typhus patients in south Texas often developed a scrotal reaction, somewhat similar to the lesions elicited by *R. rickettsii*, but milder. His results were fully confirmed by Mooser,[21] who studied the pathology and etiology of tabardillo (Mexican typhus) in great detail. Mooser[22] concluded that the American and European varieties can be clearly differentiated by their reactions in the guinea pig. Mooser probably did not recognize at the time that in Mexico epidemic and endemic typhus coexisted. In the late 1920s and early 1930s, through the efforts of many investigators, it was recognized that the agent of endemic typhus is more virulent for the rat than *R. prowazekii:* rats are naturally infected and the flea is the principal vector. The designation "murine" typhus was adopted since it was established that the agent was well entrenched in the rat population and its flea throughout the world. This information is detailed in an excellent recent review.[23]

Throughout the period of investigation of the two types of typhus agents, there was considerable discussion as to whether they were variants of the same species or two different species. The latter view eventually prevailed. In 1920, on the basis of meager data, Wolbach and Todd[24] proposed the designation *D. typhi* for the Mexican variety of typhus. In time *D. typhi* was changed to *R. typhi*, although some rickettsiologists still prefer the designation *R. mooseri*.[25]

Knowledge of the interepidemic survival of *R. prowazekii* stems from the study of Brill's disease. As early as 1898, Brill[26] had reported on a disease that had some clinical resemblance to typhoid fever, but did not elicit a Widal reaction. As the number of cases of this disease accumulated and the majority appeared to occur among immigrants from eastern Europe, the suspicion arose that it was a mild form of typhus fever. In 1912 Anderson and Goldberger[27] infected rhesus monkeys with the blood from cases of Brill's disease and showed that monkeys that had recovered from the infection were subsequently immune to Mexican typhus fever and vice versa. In 1933, Zinsser and Castaneda[28] isolated strains of *R. prowazekii* from cases of Brill's disease, and through careful analysis of epidemiological data, Zinsser[29] came to the conclusion, fully supported by subsequent events, that recrudescent typhus cases (or patients with Brill's disease) served to maintain endemic prevalence of *R. prowazekii* by bridging breaks in the chain of man-louse-man propagation. This chain was often reestablished by human stupidity and brutality.[30]

C. Trench Fever

When trench fever made its explosive appearance in late 1915 and early 1916, it was a disease of such enormous military importance that numerous teams were established to investigate the mechanism of its transmission and etiology. The investigators were hampered by the fact that the louse was the vector of both trench fever and typhus, and it was not always known that human volunteers (used in transmission experiments or to feed uninfected experimental lice) had not been previously infected and were not carriers of one infection or the other. It is not easy, therefore, to identify the first unequivocal demonstration that a rickettsia is the etiological agent of trench fever. It was seen in lice obtained from trench fever patients as early as 1916 and the designation *R. quintana* was introduced in 1917.[31] *R. pediculi*, described by some as a harmless extracellular bacterial parasite of the louse,[32] was undoubtedly the same organism. In 1918, Strong, head of the Medical Research Committee of the American Red Cross, and co-workers published a detailed study of the mechanism of transmission of trench fever.[33] However, they came to the conclusion that the etiologic agent was a virus and not a rickettsia. This was quickly refuted by Arkwright et al.,[34] who concluded that there was a remarkable association between a rickettsia and trench fever, which suggested a causal relationship. This view was reinforced by Bacot,[35] an entomologist who was feeding clean lice on himself for the epidemic typhus experiments of Wolbach et al.[17] While visiting a bath house in Warsaw, he contracted trench fever and watched his stock of clean lice gradually become infected with rickettsiae. He remained infectious for the louse at least 3 months after recovery and there is good evidence from other work that patients remain infectious for the louse for over a year. Bacot[35] provided dramatic evidence that there is no cross-immunity between trench fever and typhus. While working with epidemic typhus in Egypt, a few years later, he succumbed to the disease.

For decades the cultivation of *R. quintana* remained elusive. It does not infect common laboratory animals and it grows only to a limited extent in eggs. A disease comparable to that seen in man was reproduced in baboons by Codeleoncini[36] and an even milder disease in rhesus monkeys by Mooser and Weyer.[37] The first in vitro cultivation of *R. quintana* was probably accomplished in 1921 by Sikora,[32] who reported the appearance of tiny colonies on blood agar after 20 days. Unfortunately, she thought that she was cultivating the harmless *R. pediculi* instead of the trench fever agent and discontinued her studies. Vinson and Fuller[38] deserve the credit for the first well-documented cultivation of *R. quintana*, published in 1961. Fuller infected himself with lice obtained from Mooser. Mooser had obtained infected lice at the conclusion of World War II in a German prisoner-of-war camp near Osijek, Yugoslavia.[39] He infected himself and maintained the strain in lice fed on human volunteers, who often became infected. Fuller came down, as did Mooser and others before him, with a typical case of trench fever. At the height of infection, he furnished blood to Vinson, who

grew the agent on blood agar. In later experiments, Vinson et al.[40] fulfilled Koch's postulates by inducing typical clinical trench fever in volunteers infected with microorganisms passaged exclusively on blood agar. Their accomplishments prompted the recognition of a new genus[41] for the agent of trench fever (*Rochalimaea quintana*) and paved the way for the methodical study of its biological properties.

D. Scrub Typhus

The early history of the investigations on scrub typhus was reviewed in detail by Blake et al.[42] in 1945. Scrub typhus is often called tsutsugamushi disease, which means "mite disease". As an illness limited to certain parts of Japan, affecting farmers in July and August in the overflow lands of river valleys, it was described well before the turn of the century and attributed to the bite of a mite, usually the red mite or "akamushi". Investigations on the etiology of the disease were initiated in 1893 by Kitasato,[42] who studied under Robert Koch and is better known for the isolation of the plague bacillus. As early as 1908 a similarity was noted by Japanese workers between the symptomatology and mechanism of transmission of scrub typhus and RMSF, and in 1918 Kitashima and Miyajima[43] concluded that the etiologic agent of scrub typhus belonged to the same group as the agents of RMSF and typhus.

The first attempt at naming the organism was made in 1920 by Hayashi,[44] who described it as a protozoan with a developmental cycle and called it *Theileria tsutsugamushi*. The organism was described as a rickettsia and named *Rickettsia orientalis* by Nagayo et al.[45] in 1930. Hayashi and others agreed soon thereafter that the microorganism was a rickettsia. A heated debate ensued as to whether or not Hayashi in his 1920 paper had actually described a rickettsia and thus his designation should simply be amended to *R. tsutsugamushi*. The sixth edition of *Bergey's Manual of Determinative Bacteriology*,[25] published in 1948, the first one to describe rickettsiae, adopted the designation *R. tsutsugamushi*, but Blake et al.,[42] Tamiya,[46] and others felt that Nagayo had priority and that it would be more appropriate to call this bacterium *R. orientalis*.

The establishment of a useful diagnostic test for scrub typhus was due, as it happens sometimes in science, to a mislabeled tube.[47] Kingsbury brought to Malaya in 1924 a sample of *Proteus* X 19 from the British national collection of type cultures. This bacterium was agglutinated by the sera of scrub typhus patients, but not by the sera of patients with "shop" typhus (probably murine typhus). It turned out that the tube brought from England contained a different strain of *Proteus* and the antigen derived from it was named OX K. The fact that rickettsiae from the three major groups reacted with one or another *Proteus* antigen (OX 19, OX 2, and OX K) has given rise to considerable speculation on the relationship of rickettsiae to each other and of rickettsiae to *Proteus*. It is known now that the Weil-Felix reaction is not encountered in all animal species and is not generally elicited anamnestically. It is most likely due to carbohydrate moieties and the evolutionary significance of this reaction remains unknown.

As in the case of other rickettsiae, some of the initial experiments involved the inoculation of monkeys, and guinea pigs have been used extensively for the isolation, growth, and maintenance of scrub typhus rickettsiae. However, it became apparent as early as 1933 that the white mouse was the experimental animal of choice.[48] Although it has long been suspected that strains of scrub typhus rickettsiae vary in antigenic specificity, this was demonstrated in the late 1940s. Bengston[49] observed serological heterogeneity among strains in complement fixation tests. Bennett et al.[50] demonstrated differences more clearly by cross-neutralization tests. The possible impact of these differences on a prospective vaccine development program was quickly recognized. Pronounced differences in virulence for the experimental mouse were also recognized. Recently, Groves et al.[51] showed that resistance of the mouse to the Gilliam strain of scrub typhus was due to a single autosomal, dominant gene, whose position on the chromosome was identified.

During World War II and in the decades thereafter, it became apparent that the ecology of scrub typhus involved a wide area of the Far East. The rickettsiae survive in "ecological islands" ranging from semideserts to alpine reaches and from sandy beaches to dense but disturbed forests. Essential for survival are adequate chigger and rural rat populations, sustained by transitional types of vegetation. The emergence of these concepts was described in detail by Audy[47] and by Traub and Wisseman.[52]

E. Q Fever

In Australia in the fourth decade of this century investigators were well acquainted with the three major groups of pathogenic rickettsiae. To accept a rickettsia as the etiologic agent of a new disease was no longer difficult, but to trace the natural history of such a rickettsia was still challenging. The new disease, for want of a better name, was called "Q fever."[53] In August 1935, and possibly earlier, it affected a number of workers in a meat-packing plant in Brisbane. The disease was characterized by a sudden onset of fever, lasting 7 to 24 days, and severe headache. None of the cases in the Brisbane outbreak was fatal.

Edward H. Derrick (Figure 1B) was asked to investigate this disease, and the paper which describes his results is considered to be one of the classics in infectious diseases.[53] He reproduced the fever in guinea pigs by the injection of patients' blood or urine and maintained the agent by serial passage in the guinea pig. Since one attack conferred immunity, he established a specific diagnostic procedure based on the simultaneous inoculation of a susceptible and an immune guinea pig. His studies led him to the conclusion that the agent was not one of the previously recognized pathogens. It differed from the known rickettsiae by not eliciting a skin rash in the patient or a positive Weil-Felix reaction. Since his attempts at cultivation of the agent on bacteriologic media had been uniformly negative, he enlisted the help of Burnet. Burnet and Freeman[54] examined sections and smears of infected mouse livers and spleens and demonstrated relatively large numbers of rickettsiae as intracytoplasmic microcolonies. The rickettsiae were filtered with difficulty through 0.7-μm gradacol membranes. Suspensions of rickettsiae prepared from the infected tissues were agglutinated by patients' sera. On the basis of these findings, Derrick[55] in 1939 generously attributed the discovery of the agent to Burnet and proposed the name *R. burneti*.

At almost exactly the same time period in Montana, Davis and Cox[56] isolated from *Dermacentor andersoni* a rickettsia-like organism that produced fever in guinea pigs. Cox[57] emphasized that the agent passed through filters, albeit with difficulty, that retained other rickettsiae. He named it *R. diaporica*. Burnet and Dyer of the Rocky Mountain Laboratory exchanged strains and became convinced that the Australian and American strains were indistinguishable.[58,59] One plague affecting the laboratories on both sides of the Pacific was the high frequency of laboratory infection. As Derrick put it in his 1953 review,[60] "Q fever hits back at those who would attack it. About 200 laboratory infections have been recorded."

In the early Montana studies, Parker and Davis[61] showed that the Q fever agent was transmitted by the tick to the next generation. Derrick[62] studied the epidemiology of Q fever extensively and in 1944 drew a preliminary scheme of its natural history. There is a basic cycle involving the bandicoot and other bush animals and *Hemaphysalis* and *Ixodes* ticks. The latter may occasionally infect a bush worker and quite frequently cattle, which starts a secondary cycle involving *Hemaphysalis* ticks. The ticks on the cattle are the source of human infection, not through their bite, but through the inhalation of tick feces. During the next few years it became apparent that Q fever, often mistaken for atypical pneumonia, occurred in most countries of the world. Through the efforts of Jellison in Montana, Lennette in California, and many others world-wide,[63] it also became apparent that the agent achieves enormous titers in the placenta and birth fluids of infected domestic animals and the time of parturition presents the greatest danger to associated workers. Since the agent is stable under conditions that inactivate most nonsporogenic bacteria, the threat of aerosol infection is quite persistent in a contaminated environment.

The difference between *R. burneti* and the other rickettsiae became sufficiently apparent in the 1940s to justify greater taxonomic differentiation. The designation *Coxiella burnetii* was adopted in the 1948 edition of *Bergey's Manual of Determinative Bacteriology*.[25] In 1956 it was clearly shown by Stoker and Fiset[64] that *C. burnetii* differs in yet another major respect from other rickettsiae. It undergoes phase variation, which is somewhat similar to the smooth to rough variation seen in other bacteria. The importance of paying attention to phase in vaccine development efforts was elucidated by Ormsbee et al. in 1964.[65]

F. Rickettsialpox

Rickettsialpox was first recognized in 1946 as a new disease affecting the residents of a New York City housing development. This benign and self-limited illness presented a remarkably uniform symptomatology, characterized by an initial skin lesion followed by fluctuating fever and a vesiculopapular eruption, somewhat similar to that of chickenpox. The etiology and mechanism of transmission were promptly identified by a Public Health Department team lead by Huebner.[66-68] They were aided in their work by Sussman[69] and Shankman,[70] who treated the first cases and many of the subsequent ones, recognized the disease to be a new one, and in their oral and written discussion linked it to RMSF. A press release stimulated Pomerantz, a self-taught entomologist and exterminator, to search, with Shankman's permission, for mites in the cracks of the basements of the housing development. He took specimens of mites to the Department of Agriculture in Washington, D.C. where they were identified as *Allodermanyssus sanguineus*. Shortly thereafter, Huebner asked him to collaborate with him and Jellison and to collect additional mites and mite-infested mice from the same housing development. Within a few months identical agents, with properties typical of rickettsiae, were isolated from a patient (M. K.), mites, and a naturally infected house mouse. The rickettsia was named most appropriately *R. akari*.[67] A popular account of these events was written by Roueché.[71]

Within a few years rickettsialpox was recognized in several other cities of the U.S. In 1949 and 1950, Russian investigators described a disease and an agent in their country indistinguishable from rickettsialpox and *R. akari*.[72] The ubiquity of this rickettsia was further illustrated by Jackson et al.,[73] who in 1957 isolated *R. akari* from a Korean vole.

Other rickettsial species, too many to be described here, have been discovered. Some infect humans; others are believed to be harmless.[72-74]

III. TECHNOLOGY

A. Staining Rickettsiae

Since rickettsiae stain poorly with the Gram stain, most of the early observations were made on smears and sections stained with Giemsa. Giemsa staining, however, was not always satisfactory, in part because the rickettsiae are not well differentiated from the surrounding cytoplasm, since both stain with azur II. For *R. tsutsugamushi*, however, Giemsa staining is quite satisfactory when preceded by fixation with Carnoy's, which is a powerful fat solvent.[75] In 1930 Castaneda[76] substituted, to some advantage, the azur II and eosin of Giemsa's with methylene blue and safranin.

More successful was the procedure introduced in 1937 by Macchiavello, described by Zinsser et al.,[77] which was based on the assumption that rickettsiae are slightly acid-fast. The slides are stained with basic fuchsin and the rickettsiae retain the red stain following decolorization with citric acid and counterstaining with methylene blue. In 1964 Giménez[78] recognized that the counterstain, rather than citric acid, was responsible for the differentiation and that malachite green was a better counterstain than methylene blue. This procedure has gained wide acceptance. It is possible that even the Giménez procedure will soon be relegated to history, as many are now staining rickettsiae with acridine orange.

B. Preantibiotic Chemotherapy

Preantibiotic chemotherapy of rickettsial diseases came to a close in 1947 when broad-range antibiotics proved to be highly effective. However, a quick look at early chemotherapy may provide information on some of the biologic properties of rickettsiae.

Although several chemical compounds were shown to inhibit rickettsiae, *p*-aminobenzoic acid (PABA) emerged as the only one that was clinically effective. There is good evidence that PABA has saved lives of epidemic typhus patients, including liberated inmates of the Dachau concentration camp, and a few RMSF patients. It was also successfully used in scrub typhus, provided the dose was increased and the drug was well buffered to prevent acidosis and kidney damage.

The antirickettsial action of PABA was discovered independently in 1942 and 1944 by two teams who were testing two different hypotheses. Snyder[79] and others were motivated by the fact that the sulfonamides were not only ineffective, but detrimental. They tried therefore the sulfonamide antagonist, PABA. However, in later studies no evidence was obtained that the sulfonamides enhance the growth of rickettsiae. Greiff et al.[80] tested PABA to enhance the limited inhibitory activity of penicillin. Since penicillin inhibits only actively growing cells, with some bacteria PABA has a synergistic effect on the bacteriostatic action of penicillin. To their surprise, Greiff et al. found that PABA inhibited rickettsial growth in the presence or absence of penicillin.

An explanation of the mode of action of PABA on rickettsiae was provided in 1951 by Davis[81] and Snyder and Davis.[82] Davis showed that a strain of *Escherichia coli* that is inhibited by PABA requires *p*-hydroxybenzoic acid (POB). Snyder and Davis showed that the same was probably true in rickettsiae, since in both cases POB acted as a competitive inhibitor of PABA. Weiss et al.[83] cultivated the E strain of *R. prowazekii* in the presence of increasing amounts of PABA and isolated PABA-resistant mutants. The resistant strains had increased susceptibility to acetylsalicylic acid (aspirin). Inhibition by aspirin was competitively reversed by PABA. When POB was added to the mixture of aspirin and PABA, the POB-PABA competition reappeared, and aspirin was once again inhibitory. These studies have not been pursued further, but they do suggest that an investigation of the role of POB in rickettsial biosynthesis may have merit.

C. Early Vaccines

The FDA had not yet set its rigid rules for the production and testing of vaccines and field trials had little resemblance to present-day "double blind" studies. High risks were sometimes taken and interpretation of data was in many cases open to question.[84-86] An example was the injection of virulent rickettsiae, sometimes mixed with antisera in low doses and by unusual routes. Such attempts were terminated as the results proved to be unpredictable and hazardous.

One of the earliest more successful and safer vaccines (safer for the user, but not for the nonimmune producer) was developed in 1924 for RMSF by Spencer and Parker.[87] The procedure, improved over the 15 years of its use, can be described as follows. Laboratory-reared larval wood ticks were fed on infected guinea pigs for 5 to 7 days, collected, and stored in the refrigerator until processed. Surface bacteria were eliminated by treatment with merthiolate, and the ticks were homogenized in a solution of phenol or formalin. The suspension was then diluted with saline and centrifuged lightly and gross tick particles were discarded. The usual dosage was two injections, each the product of 2 1/2 ticks, repeated each year. In his 1941 summary, Parker[87] indicated that this vaccine protected laboratory workers, greatly reduced infection following exposure to the milder Idaho strains, and reduced severity and fatality following exposure to the virulent strains of Western Montana. It is not certain that vaccines against RMSF developed more recently do better.[88,89] Weigl's typhus vaccine is another example of one derived from the vector.

To the uninitiated, Weigl's method appears to be enormously difficult. However, at the height of production enough vaccine was obtained to immunize 2000 individuals per month, and it is estimated that a total of 120,000 people were given the vaccine from 1929 to 1938. The procedure can be described as follows: lice were inoculated under the microscope intrarectally with a fine pipette and allowed to feed twice a day on typhus-immune individuals for 10 days. Of course, human louse feeders could not be kept on the job indefinitely as they developed considerable anemia. The intestines of the typhus-engorged lice were dissected out, triturated in phenolized saline, and used as the vaccine. About 90 lice provided the three doses of the vaccine. There is considerable evidence that the vaccine caused only minor reactions and was protective.[84,85]

A so-called "attenuated" vaccine against typhus, developed in the 1930s, was used on a much wider scale. It was produced first in guinea pigs, later in fleas, and eventually in mice infected with murine typhus rickettsiae. It depended on the cross-reaction between murine and epidemic typhus and on the "attenuation" of the agent in animal tissues or flea feces by bile salts and in later experiments by yolk or olive oil. The mechanism of action of bile salts on rickettsiae is not known; therefore, further research in this area is desirable. According to the authors, the rickettsiae were not killed, just coated in such a way that adsorption was retarded. The first dose presumably caused premunition (or prevented superinfection), while the second dose produced true immunity. Apparently the vaccine very effectively stopped typhus epidemics among native Moroccans, but, quite frequently, produced severe febrile reactions among Europeans. Severe reactions also occurred in a field trial in Chile. Why the results were so different in different populations is not known. One possibility is that vaccine preparation required meticulous attention to detail and this was not always achieved. Another possibility is that Moroccans, but not Europeans and Chileans, were protected from the vaccine by a low level of antibody elicited by a previous infection.[84,85]

Two additional attempts at killed vaccine production, one in laboratory animals and one in tissue cultures, deserve to be mentioned, as they provide information on the biology of rickettsiae. The first one was based on observations made by Zinsser and Castaneda[90] and others[84,85] that the yield of rickettsiae from laboratory animals could be greatly increased by maintaining guinea pigs on a vitamin-deficient diet or by subjecting rats to a preliminary lethal dose of X-rays. Furthermore, it was shown that mice, rats, and rabbits were highly susceptible to infection when the inoculum was instilled intranasally and large yields could be collected from their lungs. Of course, this last procedure was highly hazardous.

Consistent growth of murine typhus rickettsiae in tissue cultures was first obtained in 1930 by Nigg and Landsteiner,[91] who used Maitland cultures, consisting of Tyrode solution, serum, and minced guinea pig tunica tissue. This method was extended to epidemic typhus by Kligler and Aschner,[92] who demonstrated that such cultures yielded sufficient antigen to immunize experimental animals. Zinsser and co-workers showed that greater yields of rickettsiae could be obtained on agar cultures with the same constituents. In later experiments,[93] they used Kolle flasks for the mass cultivation of rickettsiae from minced tissues obtained from mouse and chick embryos. In connection with these studies, Zinsser and Schoenbach[94] in 1937 defined the physiologic conditions of the cultivated cells that best supported the growth of viruses and rickettsiae. They observed that equine encephalomyelitis virus multiplication was greatest during the period of maximal O_2 consumption, while typhus rickettsiae continued to multiply after O_2 consumption had sharply declined. These results were used by many investigators as a criterion to differentiate the interaction of viruses and of rickettsiae with their host cells. It is known today that rickettsiae not only depend on host cells for growth, but also compete with them for essential metabolites.

D. The Yolk Sac Revolution

The frantic quest for better ways to grow rickettsiae to produce more effective vaccines

came to a halt in 1938 when Herald R. Cox (Figure 1C)[95] demonstrated that rickettsiae could be cultivated most satisfactorily in the yolk sacs of chick embryos. Cox's discovery paved the way for a new generation of vaccines and diagnostic reagents and greatly facilitated studies of rickettsial susceptibility to chemotherapeutic agents and rickettsial physiology. It was extended to the growth of chlamydiae and many viruses, in the latter case as a simple means of infecting the embryos. Details of optimal inoculation with the various rickettsial species and procedures of harvesting and processing for various purposes are too much a part of current technology to be discussed here. A pertinent question is how this discovery came about.

Egg yolk has played an important role as a bacterial nutrient and bacteria have on occasion been inoculated into the yolk sac of embryonated eggs as a convenient culture medium. However, agents that require host cells have generally been placed in close proximity to membranes, such as the chorioallantois. Zia[96] and Bengston and Dyer[97] had successfully cultivated typhus and RMSF rickettsiae, respectively, on such membrane. The function of the yolk sac is to transfer the nutritive constituents of the yolk to the embryo. The yolk is such a good rickettsial stabilizer that it more than makes up for any delay in rickettsia-host cell contact.

At a recent meeting, Cox[98] was asked to reminisce about the early days of yolk sac inoculation. His discovery stems from an experiment with the Montana strain of Q fever rickettsiae which was to be grown in a Maitland type of culture with minced tissues from chick embryos and their chorioallantoic membranes. A demanding protocol left him little choice but to add some minced yolk sac. To his amazement, he found that growth was far better in the yolk sac than in the other tissues. During a sleepless night, he realized that it was not necessary to take out the yolk sac, mince it, and put it in an Erlenmeyer flask. It was much simpler to leave it in place and inoculate it via the air sac with a long needle.

In his 1941 review of the work of the preceding 2 1/2 years, Cox[99] discussed changes in virulence that might occur in rickettsiae passaged repeatedly in yolk sacs. A strain of *R. rickettsii* that had been transferred serially 240 times in eggs had lost its virulence for guinea pigs and rhesus monkeys, but these animals became solidly immune to challenge with virulent *R. rickettsiii*. Cox suggested the possibility of using such a strain as a vaccine for humans, but this work was not pursued further. On the other hand, comparable work done with *R. prowazekii*, first by Clavero and Perez Gallardo and later by Fox[100] and associates, has led to the development of the attenuated vaccine strain E. It originated from a 1941 isolate from a typhus case in Madrid. The isolate was passed routinely in eggs and reduced virulence for guinea pigs was first noted during the 11th passage. Extensive field trials were conducted in Peru and Burundi[101] with strains passed in eggs at least 265 times. Both the merit and possible objections to the use of strain E as a human vaccine have been discussed by Wisseman.[101]

In conclusion, the yolk sac method of growing rickettsiae has had an enormous impact on rickettsial technology and as a general procedure for obtaining large pools of rickettsiae is not likely to become obsolete soon. However, the Cox-type vaccines still in use and the strain E vaccine may before long be replaced by vaccines generated through recombinant DNA technology.

IV. RICKETTSIAE AS ORGANISMS

A. Rickettsial Physiology

In the late 1940s, a professor asked a student to define the difference between a rickettsia and a virus. After some hesitation the student replied that he knew what the difference was, but, at the moment, he had forgotten. "It's most unfortunate," exclaimed the professor, "the only person who ever knew the difference between a rickettsia and a virus has forgotten

it." The professor had expressed the view commonly held by investigators during the first four decades of research on rickettsiae that the difference could not be defined. Of course, no true virus could be seen by light microscopy or was retained by a Berkefeld filter, and Zinsser and Schoenbach[94] had shown that rickettsiae, unlike viruses, continued to multiply well after the respiration of the host cells had greatly diminished. Differences, however, more or less ended there. Methods of studying rickettsiae were identical to those used for viruses. Rickettsiae were generally referred to as "viruses", although investigators often used the word "virus" in the extended Latin sense, meaning living poison or infectious agent.

It is not possible to attribute the change of view that a rickettsia is not a virus, but a prokaryote, to a single experiment. It came about gradually as a result of advances both in virology and rickettsiology. Experiments in rickettsial physiology, antibiotic chemotherapy, and electron microscopy have greatly contributed to the emphasis that rickettsiae are to be regarded as organisms.[102] Only some of the early experiments on rickettsial physiology will be summarized here.

In 1949, Marianna R. Bovarnick (Figure 1D) and Snyder[103] purified pools of three strains of typhus rickettsiae by a relatively new method of removing most of the yolk sac constituents by adsorption to Celite® (a product generally used for the clarification of beer). The rickettsiae thus purified consumed O_2 in the presence of casein hydrolysate and, of the individual amino acids tested, only glutamate stimulated respiration as well or better. There was no O_2 uptake with glucose or lactate. Besides its theoretical significance, this finding led to a practical application, namely, the establishment of sucrose-phosphate-glutamate as the buffer of choice for the preservation of rickettsial activity.[104] Glutamate was included because of the correct assumption that rickettsiae, as most bacteria, are stabilized by a metabolizable substrate. Phosphate was buffered to pH 7.0 with K^+ rather than Na^+, because, as correctly expected, this intracellular bacterium was adapted to an environment rich in K^+ rather than Na^+. The next step was to determine the pathway of a glutamate metabolism. Bovarnick and Miller[105] found that glutamate was transaminated and oxidized with a strong indication that these reactions led to the citric acid cycle. These results were confirmed and expanded by Wisseman et al.,[106,107] who obtained excellent evidence that at least the dicarboxylic portion of the cycle was operative.

Do rickettsiae derive adenosine triphosphate (ATP) from the oxidation of glutamate? This is the next question that Bovarnick[108] answered in the affirmative in a curious way. She incubated rickettsiae with glutamate plus glucose, adenosine diphosphate (ADP), and hexokinase as the indicator system for ATP production. She demonstrated that glucose-6-phosphate was produced. The rickettsiae were most accommodating since, as recently shown by Winkler,[109] rickettsiae, unlike most other bacteria, exchange ADP and ATP with the environment. In a subsequent study, Bovarnick and Allen[110] confirmed that rickettsiae produce ATP from ADP by the firefly luminescence method.

Encouraged by these results, Bovarnick attempted to grow rickettsiae in a host cell-free medium. Frustrated in her attempts, she turned her attention to protein synthesis.[111] She demonstrated that rickettsiae are capable of some protein synthesis when suspended in a medium of the appropriate ion composition, containing all amino acids, a source of energy, such as glutamate, as well as exogenous ATP. These results were recently confirmed and expanded by Dasch et al.,[112] who also showed that, under conditions similar to those used by Bovarnick, radiolabeled methionine was incorporated into all major rickettsial proteins and that some RNA synthesis also took place. In vitro DNA synthesis has not yet been demonstrated.

B. What is a Rickettsia?

This question has been asked for almost 80 years. A definition that has persisted for a

long time is that a rickettsia is any small intracellular organism of the general appearance of a bacterium that cannot be grown on bacteriologic media and that is associated with arthropods and sometimes with other invertebrates and protozoa. The 1922 classification by Brumpt[8] lists eight species in the genus *Rickettsia*, three human pathogens and five presumably harmless endosymbionts. As knowledge of rickettsiae progressed more rapidly than that of endosymbionts, endosymbionts were gradually weeded out. In the 1948 edition of *Bergey's Manual of Determinative Bacteriology*,[25] the human pathogens were well separated from endosymbionts and animal pathogens such as *Cowdria*. Subsequent editions[74] have taken cognizance of advances in knowledge of the biology of rickettsiae and of some of the animal pathogens, such as *Ehrlichia*. Classification has been based on the principle that what is known and what is not known must be defined and a useful guide must be provided to the bench scientist. Perhaps *Bergey's Manual of Determinative Bacteriology* has gone too far in the separation of human pathogens from animal pathogens and nonpathogens and has lost some of its drive to probe into the phylogeny of rickettsiae.

As the base composition of the DNA was added as a criterion for the classification of bacteria, work on rickettsiae has not lagged far behind. The base ratio of the DNA of *Coxiella burnetii* was established in 1951 by Smith and Stoker[113] and of *R. prowazekii* in 1952 by Wyatt and Cohen.[114] Work on DNA has progressed since, but has by no means been completed. Many of the questions of the phylogeny of rickettsiae will eventually by answered by an analysis of the nucleotide sequence of the DNA segment that codes for ribosomal RNA.[115]

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