Intracellular Infection and the Carrier State

The path of clinical investigation leads from bench to bed and back again.

Joseph E. Smadel

Rickettsiae and viruses have been defined for decades as obligate intracellular parasites. Hence, their role in intracellular infections is well established. However, the idea that pathogenic bacteria are also capable of growing within tissue cells of the infected host has gained ground more slowly. The movement in support of this view began in the late 1930's with Goodpasture's demonstration of Gram-negative bacilli in plasma cells in the intestinal lesions of a fatal case of typhoid fever (*1*). It gained momentum when Goodpasture and his associates demonstrated that *Salmonella typhosa, Pasteurella tularense, Brucella abortus, Mycobacterium tuberculosis avium, Streptococcus viridans*, and *Aerobacter aerogenes* each grows intracellularly in ecto-, meso-, or entodermal cells of the embryonated egg (*2*). These workers found, however, that certain bacteria, such as *Streptococcus haemolyticus, Staphylococcus aureus*, and *Pasteurella pestis*, appeared incapable of intracellular multiplication in chick embryo cells.

Typhoid and Typhus Carriers

In discussing intracellular infections and carrier states, I shall devote particular attention to typhus and typhoid fevers and their etiologic agents. Like most of the great plagues of mankind that have made medical history, epidemic typhus and typhoid fever are now of relatively little importance in the United States. Louse-borne typhus has not occurred here for many years; it is represented by a few cases each year of recrudescent typhus known as Brill-Zinsser disease. Typhoid fever has now been reduced to about 800 cases annually in our country. In contrast to conditions here, both epidemic typhus and typhoid continue to be of importance in many other areas of the world. Indeed, data in the recent National Research Council report on needs and resources in tropical health (*3*) indicate that throughout the tropical regions of the globe the morbidity and mortality rates for typhoid fever average 9.2 and 0.87, respectively, per 100,000 population. The rates for typhus are almost one-quarter those of typhoid. Neither disease stands among the first ten in importance in the survey (from the standpoint of public health), but typhoid ranks 15th and typhus 19th.

A few of the patients who recover from epidemic typhus or from typhoid fever become carriers. "A carrier is a person who harbors a specific infectious agent in the absence of discernible clinical disease and serves as a potential reservoir or source of infection for man" (*4*). About 2 percent of the patients who survive infection with *S. typhosa* become chronic carriers. It has been disappointing to find that chloramphenicol, which is an excellent therapeutic agent for the patient in the acute stages of typhoid fever, does not reduce the incidence of carriers among treated patients to less than that found among untreated individuals (*5, 6*).

There are probably many more carriers among persons who recover from epidemic typhus than among those who recover from typhoid. The data on epidemic typhus are less extensive and less direct than those on typhoid, but they are of help in estimating the percentage of carriers. Thus, it has been shown by Murray and his colleagues in Boston, and by Schaefer and his associates in Chicago, that 20 to 30 percent of the elderly immigrants to this country who were born and spent their early years in typhus areas of Europe still possess specific complement-fixing antibodies against the etiologic agent of epidemic typhus (*7*). This long persistence of antibody in persons who have not been exposed to typhus for a quarter or a half century strongly suggests that these individuals are still harboring the organism and that the occasional release of antigen stimulates and maintains the antibody levels. It has been established that cases of recrudescent typhus are drawn from this immigrant population which maintains an antibody level; the patient with Brill-Zinsser disease displays the clinical manifestations of typhus fever, and *Rickettsia prowazeki* can be recovered from his blood. In contrast, the tissues of healthy immigrants with typhus antibodies only occasionally yield the typhus organism. This was demonstrated by Price, who applied tissue culture techniques to inguinal lymph nodes removed from such persons in connection with abdominal surgery (*8*). Rickettsiae were recovered from two of the 31 patients studied in this manner. Thus, in this small group of selected persons who were considered on serological grounds to be typhus carriers, Price was able to demonstrate that about 7 percent harbored viable rickettsiae in their tissues.

In a recent discussion (*9*) I made some remarks, as follows, which are pertinent here:

> Typhoid and typhus provide contrasting examples of the carrier state. The former represents the type which sheds organisms that can be detected readily by appropriate laboratory techniques. On the other hand, the latter represents the silent type which is recognized with difficulty, if at all, before clinical disease reappears. Both types are relatively common in medical experience. Thus, the shedding carrier is found among persons with parasitic infections such as ascariasis; with protozoal infections such as amoebiasis; with bacterial infections involving the nasopharynx and caused by streptococci, staphylococci, or diphtheriae, or involving the gastrointestinal tract and caused by Shigella; also with viral infections such as cytomegalic inclusion disease in which salivary gland virus may be excreted for months in the urine. Although no example of a shedding carrier is found among the human rickettsial infections, this type does occur in domestic

The author is chief of the Laboratory of Virology and Rickettsiology, Division of Biologics Standards, National Institutes of Health, Bethesda, Md. This article is adapted from a National Institutes of Health Lecture presented 12 December 1962.

animals, namely, in apparently healthy cows which excrete *Rickettsia burneti* [the agent of Q fever] in their milk. The shedding carrier is primarily a public health problem because of his capacity to transmit infection to others. The carrier himself has usually arrived at a stalemate with his organism, but it must be kept in mind that he may develop some chronic lesion as a result of his persistent infection.

The silent carrier's infection may also be caused by agents of diverse classes. Malaria, syphilis, tuberculosis, brucellosis, and herpes simplex represent some of the diseases in which the silent carrier state develops.

The silent typhus carrier is a potential health hazard to the community, in contrast to the typhoid carrier, who constitutes a real and continuing threat. It is only when the former has a recrudescence of typhus with rickettsemia that the potential danger is transformed into a real one, and it is only when the patient and the community are infested with lice that an epidemic of typhus is generated.

My associates and I have been interested for a long time in the typhoid carrier and in the recovered typhus patient, not only because they present a public health hazard but also because they present a fascinating problem in immunity as well as a clinical illustration of the importance of continuing intracellular infections. The term *intracellular infections* is introduced here because the most reasonable explanation for the continued existence of such pathogens as *Salmonella typhosa* and *Rickettsia prowazeki* in the immune person possessing potent serologic and phagocytic cell defense mechanisms is the assumption that the offending organisms have taken sanctuary inside tissue cells where the host's attacking forces cannot reach them. Of course, if one accepts the idea that infecting microbes are thus protected from the defense mechanisms found in circulating blood, he is still faced with the need to explain other aspects of the problem. For instance, how does the intracellular pathogen maintain a successful equilibrium in the cell—that is, the state in which neither the parasite nor the host kills off the other? The subject of talent infections has interested a number of investigators, especially in the field of virology but also in the broader field of microbiology [see the articles of Ginsberg, Henle, Morgan, Shope, Isaacs, and McDermott (*10–12*)]. Here I consider this equilibrium only from the standpoint of pressure by the cell in order to eliminate the intruder.

Intracellular Infections in Laboratory Models

Experiments on tissue cultures inoculated with *Rickettsia tsutsugamushi,* the agent of scrub typhus, or with *Salmonella typhosa* have helped to develop ideas on intracellular infections and their cure by therapeutic procedures. When *R. tsutsugamushi* is added to a culture of mouse lymphoblasts there is a progressive increase in the number of intracellular rickettsiae during the next week or so. Indeed, careful quantitative studies with this model revealed that the rickettsiae approximately tripled every 24 hours (*13*). This is an amazingly slow rate of multiplication as compared with that of *S. typhosa,* which divides at intervals of about 30 minutes, whether in the intracellular position or in cell-free bacteriologic media (*14*).

The data of Fig. 1 illustrate the gradual decrease in *Rickettsia tsutsugamushi* in tissue culture cells maintained in growth media in which chloramphenicol is present at a concentration of 5 micrograms per milliliter (*15*). Even after 2 weeks a few of the cells still contain rickettsiae that are recognizable on microscopic examination, and the culture contains an appreciable number of infectious rickettsiae. However, after the third week the culture is cured—that is, there is no microscopic evidence of rickettsiae in the treated culture, or in subcultures maintained without chloramphenicol, and none when the culture is inoculated into susceptible mice. It may be noted in passing that higher concentrations of chloramphenicol result in a more rapid initial reduction of rickettsiae in the infected tissue cultures. However, such cultures are not cured after 2 weeks of treatment.

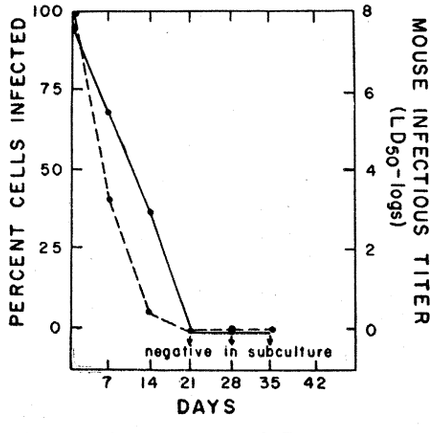


Fig. 1. Disappearance of *R. tsutsugamushi* (Karp strain) from infected L929 cells after prolonged treatment of cultures with chloramphenicol (5 μg/ml). (Dashed line) Percentage of infected cells; (solid line) mouse infectious titer. [From H. E. Hopps *et al. (15)*]

These findings on the persistence of *Rickettsia tsutsugamushi* in treated tissue cultures might, at first glance, appear to be inconsistent with our initial observations on the response of patients treated with chloramphenicol; these observations had shown that fever disappeared within 24 hours and the patient went on to an uneventful recovery (*16*). On the other hand, the findings are consistent with the results of chemoprophylactic studies made some years ago in man. Figure 2 summarizes an experiment in which volunteers were inoculated intradermally with about 100 infectious doses of a strain of *R. tsutsugamushi* (*17*). The volunteers in the control group (Fig. 2, top section) developed scrub typhus 10 to 12 days later, and their disease was promptly terminated through treatment with one of the broad-spectrum antibiotics. As the solid bars which appear on the graph for this group 15 to 18 days after inoculation indicate, four of the members of the control group suffered relapses; they again responded to therapy and remained well thereafter. The important portion of Fig. 2 for the present discussion is the middle section (AA-2). This portrays the results obtained in six volunteers who were given 1 gram of chloramphenicol daily for a period of 4 weeks after the intradermal inoculation of living rickettsiae. These persons remained afebrile during the 4-week period; furthermore, as indicated by the open triangles on the chart, none of the patients had rickettsemia. Four days after the last chemoprophylactic dose of chloramphenicol—that is, on day 32 after inoculation—the initial evidences of clinical scrub typhus infection appeared in five of the six members of this group. These evidences were mild headache and slight elevation of temperature; a fever of 100°F or over did not occur for another 2 days. At the time these forewarnings of disease were first noted, one of the three volunteers tested had rickettsemia; this is indicated on the chart by the solid triangle on day 32. At about this point all six members of the group were hospitalized and were given a course of therapy.

Thus, the small number of rickettsiae which were injected into these men survived 4 weeks of continuous daily chemotherapy, and when the antibiotic

was withdrawn, the rickettsiae were still able to induce clinical disease in these individuals.

The bottom section of Fig. 2 shows that other volunteers who were inoculated in the same way and then given intermittent chemoprophylaxis with 3-gram doses of chloramphenicol had a suppressed infection which was evidenced by rickettsemia. At no time during the course of chemoprophylaxis did they appear sick. Furthermore, when prophylaxis was continued for 4 weeks, none of the volunteers developed disease after the antibiotic was withdrawn. Data not included in Fig. 2 revealed that when the intermittent chemoprophylactic regimen was shorter than 3 weeks, some of the volunteers displayed symptoms of clinical scrub typhus a few days after the last dose of antibiotic had been administered; in other words, they had not yet had time to develop immunity as a result of the subclinical infection.

It may be noted in passing that the data in Fig. 2 provided the basis for a complex procedure for immunizing people against scrub typhus. Because of its complexity, the procedure has had only limited usefulness. Nevertheless, the principle is sound: to infect the individual with a partially attenuated organism and, subsequently, to maintain the infection at a subclinical level by means of antibiotic until the individual develops his immunological responses (*17*).

Can Infected Cells Cure Themselves?

Before leaving the rickettsia-tissue-culture model I will mention an observation that has been of continuing interest to us. Figure 3 summarizes information on the growth of *Rickettsia tsutsugamushi* in L cells suspended in two types of media (*18*). The section at the left portrays the ordinary rate of growth in cells nurtured by a medium containing Gey's balanced salt solution, chick embryo extract, and horse serum, plus a few other ingredients, including colchicine to inhibit multiplication of the tissue culture cells. In this culture the rickettsiae increased progressively over the 3 days of observation. By contrast, when aliquots of the same infected L cells were placed in balanced salt solution, the organisms initially present decreased progressively in number over a period of 72 hours, while the tissue culture cells remained in a healthy state but did not multiply. It may be noted that nutritionally deficient infected cultures, when given complete media, displayed a resurgence of growth of both cells and rickettsiae.

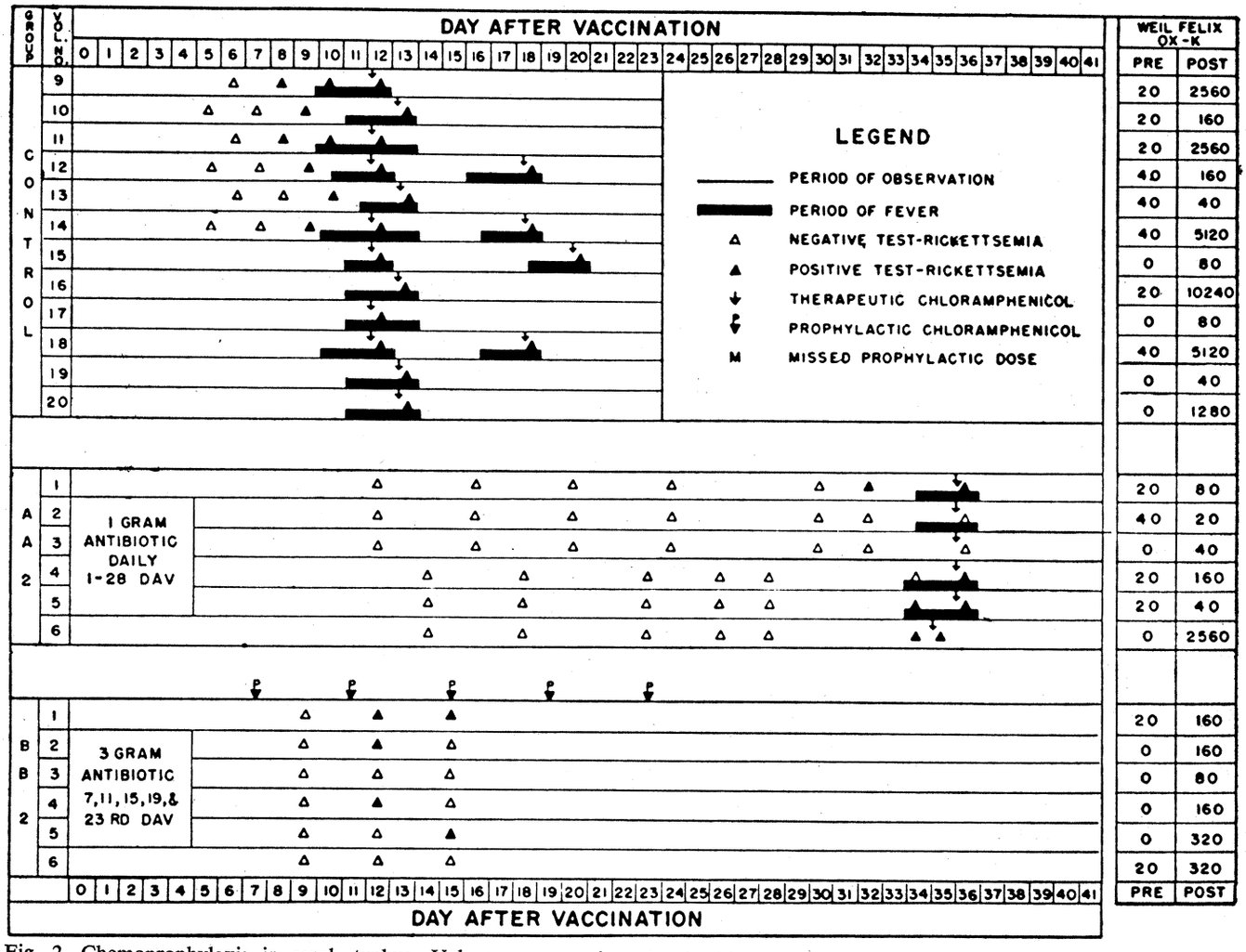


Fig. 2. Chemoprophylaxis in scrub typhus. Volunteers were inoculated with 100 infectious doses of the Karp strain of *R. tsutsugamushi*. Members of group AA-2, who received daily doses of chloramphenicol, were without evidence of infection until the drug was discontinued but then developed scrub typhus. Thus, rickettsiae persisted during 4 weeks of chemotherapy and then caused disease in nonimmune persons. Members of group BB-2, who received intermittent chemoprophylaxis, had subclinical infection and were immune when the regimen was terminated. [After H. L. Ley, Jr., *et al.* (*17*)]

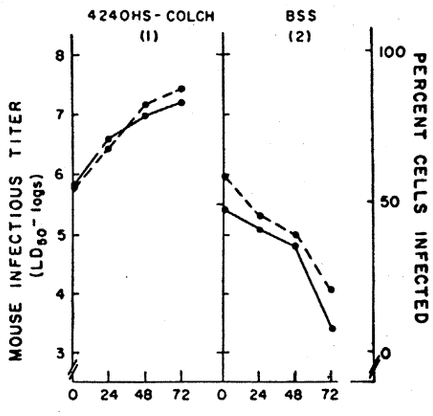


Fig. 3. Graph indicating death of *R. tsutsugamushi* in viable but nonmultiplying L cells suspended in simple tissue culture medium (balanced salt solution). (Left) Graph illustrating normal growth of rickettsiae in L cells in protein-rich tissue culture medium. Here colchicine has been added to prevent multiplication of L cells. (Dashed line) Percentage of cells infected; (solid line) infectious titer. [After H. E. Hopps *et al.* (*18*)]

We have been intrigued by the observation that infected cells, when placed in simple media, begin to clear themselves of rickettsiae with an efficiency and speed comparable to that displayed by infected cells bathed in specific antibiotic of low therapeutic dosage. One wonders whether intracellular rickettsiae gradually lose components essential to their integrity by leakage into the surrounding host cytoplasm. This phenomenon has been demonstrated in vitro by Bovarnick and Allen, who showed that loss of rickettsial infectivity can be prevented by maintaining suspensions of purified organisms in fluid with an adequate level of nicotinamide adenine dinucleotide (NAD) and glutamate. Furthermore, an appreciable proportion of rickettsiae rendered noninfectious by storage in inadequate media can be reactivated by subsequent incubation in a solution containing glutamate and excess NAD (*19*). More recently, Cohn *et al.* reported leakage of nucleic acids when purified preparations of *Rickettsia mooseri* are held in an isotonic solution containing glutamate (*20*). If this leaching of substances from the rickettsiae goes on intracellularly when conditions are such that the host cell receives an inadequate supply of nutrients, the concentration of materials essential for maintaining viability of the organisms may fall below the critical level in a relatively short period. Thus, one wonders whether the obligate intracellular parasitism of rickettsiae may be dependent, in part at least, on the high degree of permeability of the cell wall of the organisms, which would make them more susceptible to environmental changes than free-living bacteria are. In view of the varying susceptibility to osmotic influences that is displayed by the different stages of bacteria—from L forms, through protoplasts and bacteria, to spores—it might be postulated that the rickettsiae occupy a position between the protoplast and the ordinary bacterial organism (*21*).

Whether or not such ideas regarding rickettsial permeability and the inactivation of intracellular rickettsiae through lack of essential nutrients in viable but starving cells are applicable in the model system, they are unlikely to be applicable to the infected cell in the mammalian host. Hence, we have explored other ideas which might account for the curing of infected cells by natural processes.

The concept that cells which have been damaged by multiplication of viruses or rickettsiae can recover through their own natural defense mechanism has been considered but has not been clearly established. Thus, Bodian, in considering the sequence of cytopathologic changes in motor neurons of the spinal cord of the monkey during the course of destruction by poliovirus, and also the changes which occurred in cells which presumably were infected but which recovered, stated (*22*), "Although it has been my opinion that in the spared cells there are morphological evidences of viral effects . . . there is still no conclusive evidence that these neurons were ca-

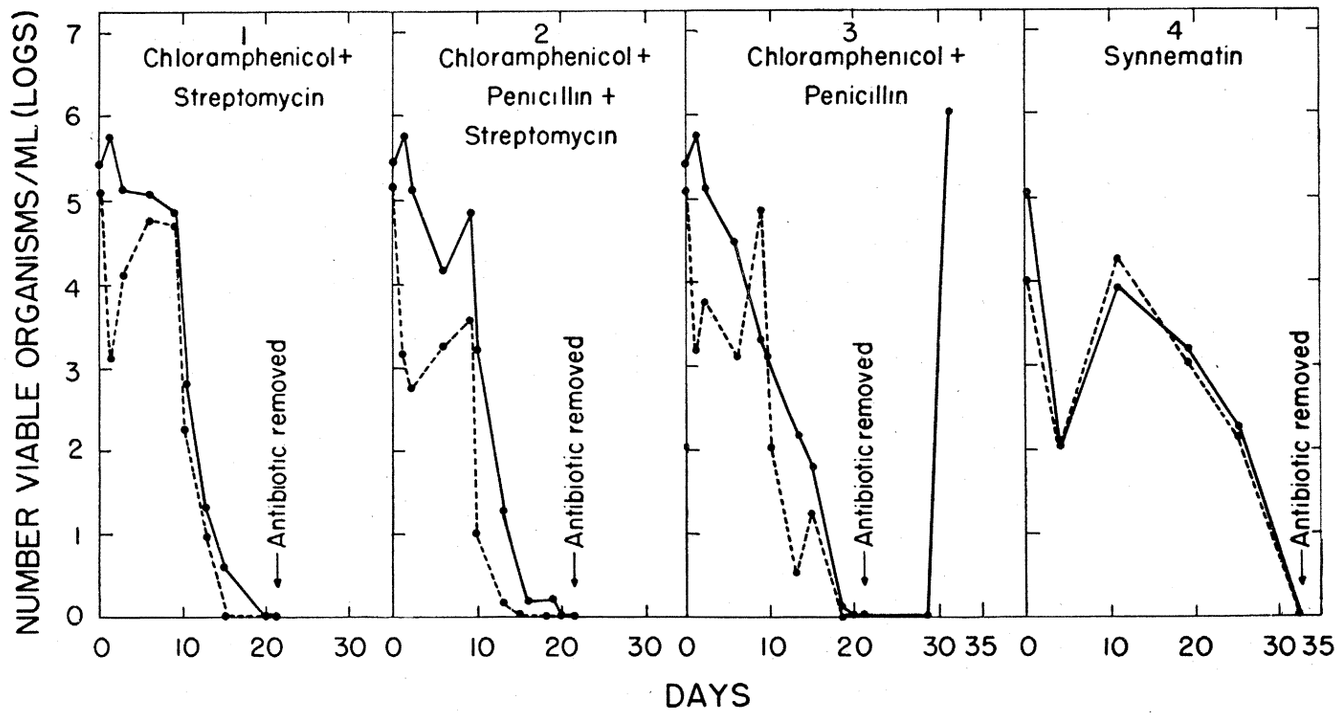


Fig. 4. Response of *S. typhosa* in infected tissue cultures to treatment with various antibiotics. (Dashed line) Supernatant fluid; (solid line) homogenized cells. [From H. E. Hopps *et al.* (*27*)]

pable of supporting virus growth without being destroyed . . . ."

The observations of Kundin on the pathogenesis of West Nile virus infection in experimentally inoculated chicks studied by means of fluorescent antibody techniques are pertinent (*23*). Kundin found that young chicks inoculated by parenteral routes with a few hundred mouse LD_{50}'s of West Nile virus developed viremia but at no time showed overt signs of disease. Using immunofluorescence techniques he found appreciable amounts of West Nile virus antigen in smooth muscle cells of the gizzard of chicks killed a few days after inoculation, when the silent infection was at its height. The gizzards of infected birds that were observed for a longer period before sacrifice showed a rapid decrease in both fluorescent viral antigen and infectious virus, and the West Nile agent was no longer detectable by either method 14 days after inoculation. It appears that Kundin's model provides an excellent lead for further studies on the recovery of tissue cells which have supported the multiplication of virus.

If it is postulated that cells infected with viruses and rickettsiae do indeed recover through their individual cellular defense mechanisms, then what are these mechanisms? And how can they be stimulated and be made to assist in the clinical recovery of the patient and in the curing of the infected tissue cell?

In considering possible means by which the mammalian cell might, through its own mechanisms, suppress growth of the rickettsial intruder for a long time until the organism eventually dies of inanition or senescence (I suspect that this is the means by which the infected tissue culture cell treated with antibiotics is eventually freed of its rickettsiae), one thinks, of course, of interferon (*11*). We have begun to study the effect of this substance in a rickettsiae-tissue-culture system. However, this is only one of a number of substances that may become active inside the cell against the invading microbe. The recent reviews of Skarnes and Watson and of Hirsch give detailed information on the known antimicrobial tissue factors, including lysozyme and other basic proteins, basic polypeptides, lipid materials, and phagocytin, a globulin substance (see *24*).

The possibility that antibody may play a role in the recovery process in individual infected cells should not be neglected. I have in mind antibody generated within the reasonably healthy infected tissue cell, not circulating antibody which may gain entrance into a moribund cell of abnormal permeability. Although the plasma cell is regarded as the producer par excellence of antibodies, many tissue cells may have a primordial capacity to produce classical antibodies, albeit in such small amounts that their detection has not yet been accomplished. To state it another way, it is possible that the protein-synthesizing mechanism of the mammalian cell scans the invading organism and produces antibody-like substances, or enzymes, which eventually contribute to the death and destruction of the organism.

One mechanism which can play a role in curing the infected tissue cell is the extrusion of intracellular rickettsiae from the living cell. This event has been observed on several occasions by means of phase microscopy: A rickettsial body was trapped in a microfibril as it protruded from the surface of the cell and was subsequently left on the glass slide, outside the cell, when the microfibril receded some minutes later (*25*). This mechanism of removal of microbes from cells by extrusion probably is operative in several viral infections (see *25*) and certainly operates in our typhoid-tissue-culture model; however, it probably plays only a minor role in the series of events which may lead to survival of the infected cell.

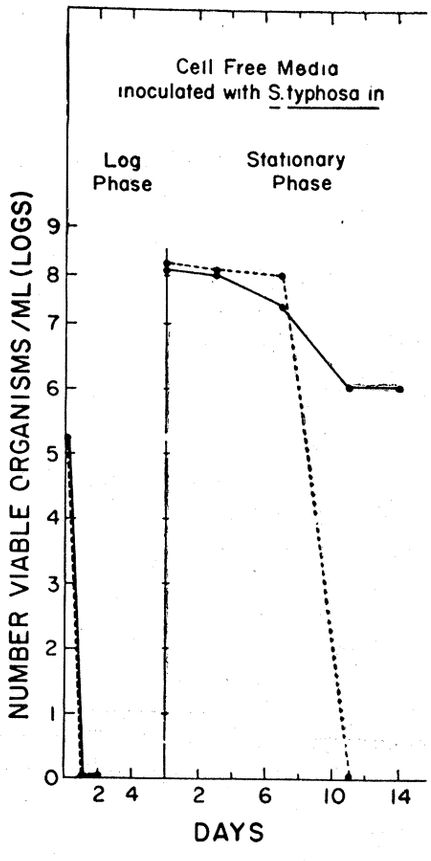


Fig. 5. Comparative inactivation rates for *S. typhosa* treated in vitro with synnematin in various culture media. (Dashed line) In nutrient broth; (solid line) in culture medium. [After H. E. Hopps *et al.* (*27*)]

Typhoid Carriers and Models

In turning from scrub typhus to typhoid fever, I would like to quote several paragraphs from a manuscript that Woodward and I recently prepared (*6*).

Despite the effectiveness of chloramphenicol in the treatment of patients with typhoid fever, our early work with the drug in Malaya suggested that (1) the chronic carrier state did develop with about its usual frequency in patients who had received specific therapy and (2) chloramphenicol was essentially ineffective in inducing permanent cures among chronic typhoid carriers [see *26*]. These early observations have been borne out by many investigations since 1948.

The importance of the chronic carrier in public health and the need for an effective means for curing him require no emphasis. Consequently, numerous workers have tried combinations of chloramphenicol therapy with other antibiotics or with cholecystectomy. Although various combinations employed by different authors have been reported to be useful, no particular regimen has gained wide acceptance.

Our interest in the problem has resulted in certain approaches which we have found stimulating although they have not provided the ultimate solution. Laboratory observations have indicated that *S. typhosa* in the test tube in ordinary bacterial media is inhibited though not killed by chloramphenicol but is killed under such circumstances by penicillin [*27*]. At the University of Maryland a group of patients was placed on frequent, large doses of penicillin, i.e., sufficient with the aid of benemid to maintain blood levels of the order of 60 units/ml, and kept on such treatment for about two weeks. When this work was reported, four of the six carriers under study were apparently cured [*28*]; subsequent observations indicated that these four were indeed permanently cured, i.e., they did not shed organisms during the one to two-year period of followup. Jersild and his colleagues obtained similar results [*29*] but Nicol in the United Kingdom was less successful [*30*].

Another type of approach was followed by our group at the National Institutes of Health. A laboratory model which we believe may have some points in common with the typhoid carrier was developed in tissue cultures infected with *S. typhosa* [*27*].

This typhoid-tissue-culture model stemmed directly from the rickettsiae-tissue-culture system discussed earlier. However, unlike the latter, the former required the continual presence of antibiotics to suppress the extracellular multiplication of *Salmonella typhosa* when the system was used in studies that extended over an appreciable period. Work with this model was begun several years ago in an attempt to understand why it is so difficult to cure the chronic typhoid carrier with antibiotics. The problem of successful treatment of chronic microbial infection is, of course, much broader than that of the typhoid carrier and has been examined by many investigators. As a result of their observations and ideas, a number of hypotheses had been developed to account for the apparent failure of antibiotics to act against bacteria inside cells in the same manner that they acted in test tubes. For the most part, these hypotheses centered around the idea that the antibiotic, if it did indeed get into the cell, was ineffective because it was degraded in some way, or, contrariwise, that the intracellular environment protected the antibiotic-damaged organism by supplying the bacterium with metabolites or with physical conditions necessary for survival and growth (*9, 12*).

In studies undertaken with several of my colleagues, it was possible to demonstrate directly that antibiotics promptly penetrate mammalian cells and are as effective in inhibiting the growth of intracellular bateria as they are in inhibiting the growth of extracellular bacteria (*14*). These studies were carried out with *Salmonella typhosa* in a mouse fibroblast tissue culture system which was observed by phase microscopy over an extended period. By this means, bacteria inside a mammalian cell were observed to undergo multiplication at intervals of about 30 minutes. With a relatively simple modification of this model we were able to perfuse a culture with a selected antibiotic while a given intracellular bacterium was under direct and continuous microscopic observation. Under such circumstances, bacterial multiplication ceased, both inside and outside the cell, within a few minutes after the addition of chloramphenicol, penicillin, or synnematin and within 40 to 50 minutes after perfusion with streptomycin. Although indirect evidence of various kinds had indicated that antibiotics were effective against intracellular bacteria and rickettsiae (see *14*), the experiments just mentioned were the first to prove the point conclusively through direct observation.

Despite the prompt inhibition of growth of intracellular *Salmonella typhosa* by antibiotics, the number of viable organisms in an infected treated tissue culture remained elevated for a week or so. Figure 4 summarizes four quantitative experiments in which the numbers of culturable *S. typhosa* in the extracellular medium and in the homogenate of tissue cells were determined at intervals after treatment (*27*). It is evident from Fig. 4, section 1, that after 10 days of exposure to chloramphenicol and streptomycin, the number of viable bacteria both in the cell brei and in the cell-free fluid had been reduced by only about 10 percent. However, during the next 10 days the number of organisms decreased rapidly, and none were detectable by day 20. Comparable results were obtained in the experiment summarized in Fig. 4, section 3. However, in this instance, when chloramphenicol and penicillin were removed from the culture, *S. typhosa* reappeared, multiplied, and eventually destroyed the tissue culture. In brief, the data of Fig. 4 represent three instances of cure in the test-tube model of the typhoid carrier and one instance of relapse after an apparent cure. The important point is the long duration of treatment required to obtain a cure —3 to 4 weeks.

In trying to learn why the cure of infected tissue-culture cells requires such a time-consuming process, we reexamined the effect of antibiotics on *Salmonella typhosa* in the log phase, or phase of rapid growth, and in the nonmultiplying, stationary phase; in addition, we studied the effect of the suspending medium on the reaction.

An experiment of this kind, in which synnematin was present at a concentration of 20 micrograms per milliliter in each of the suspending media, is summarized in Fig. 5 (*27*). The section at left shows that all of the several

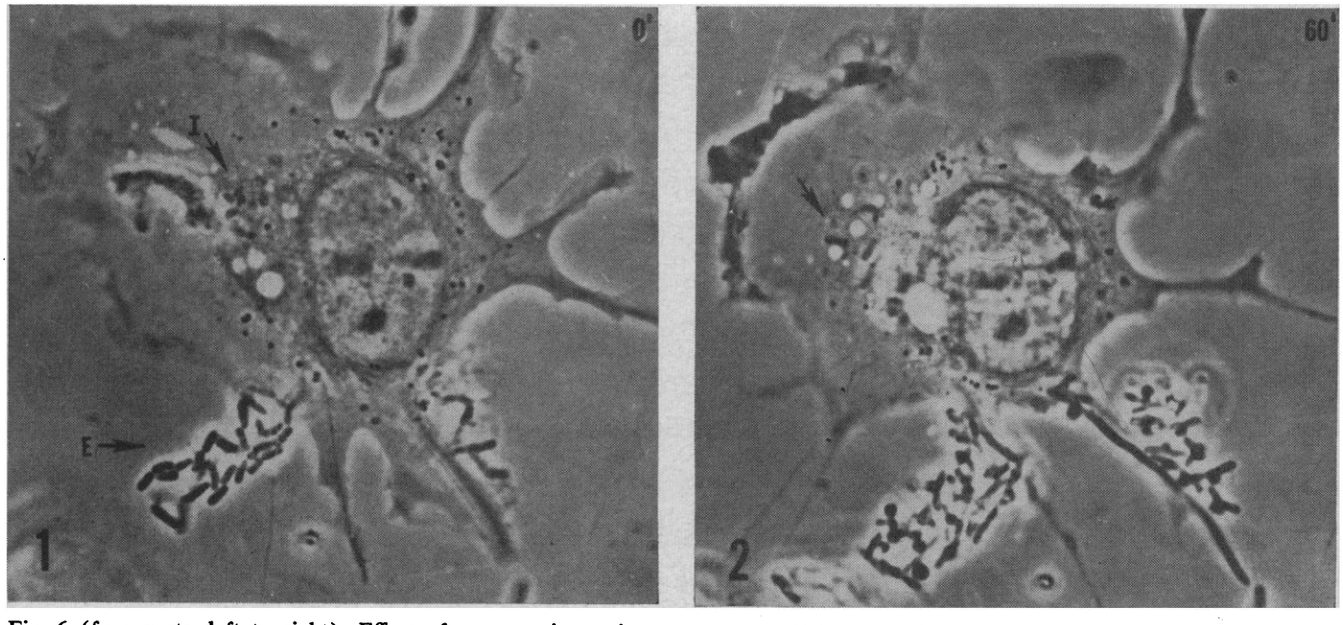


Fig. 6 (four parts, left to right). Effect of synnematin on intra- and extracellular *S. typhosa* in L cells in tissue cultures. (1) An infected cell immediately after the addition of synnematin; *E* and *I* denote extra- and intracellular organisms. (2) Spheroplast formation, 1 hour later, by the extracellular bacilli. (3) Photomicrograph which shows that practically all the extracellular organisms have

hundred thousand *Salmonella typhosa* in the log phase of activity were rendered nonviable within 48 hours, whether they were suspended in ordinary bacterial culture medium containing synnematin or in enriched tissue culture medium containing synnematin. By contrast, at least one in ten organisms in the stationary phase survived for a week under similar conditions. Indeed, in the complex, protein-rich tissue culture medium containing synnematin there were still a million viable bacteria per milliliter at the end of 2 weeks, although by this time all of the organisms in the nutrient broth containing the antibiotic were dead. The effects of penicillin, streptomycin, and kanamycin were similar, but chloramphenicol and chlortetracycline were less lethal for *S. typhosa,* in both bacterial and tissue culture medium. Other workers had shown previously that antibiotics were more rapidly lethal for bacteria in the logarithmic phase than for those in the stationary phase (*31*). However, what had not been emphasized before was the prolonged survival of the stationary-phase organism in enriched acellular medium containing antibiotic. Such findings undoubtedly have a bearing on the protracted therapy required to cure an infected tissue culture, but whether they are of primary importance remains to be determined.

Several years ago, McDermott mentioned "drug indifference" as a likely explanation of the observation that "microbes within cells are less drug susceptible than microbes outside cells. . ." (*12*). He considered the protoplast induced by antibiotics which interfere with cell-wall synthesis to be an example of "drug indifference." Such protoplasts are capable, under proper conditions, of multiplying in the presence of the antibiotic (*32*). Of interest in this connection is the finding that in our *Salmonella typhosa* tissue culture system, spheroplast formation occurs in extracellular organisms when penicillin or synnematin is present.

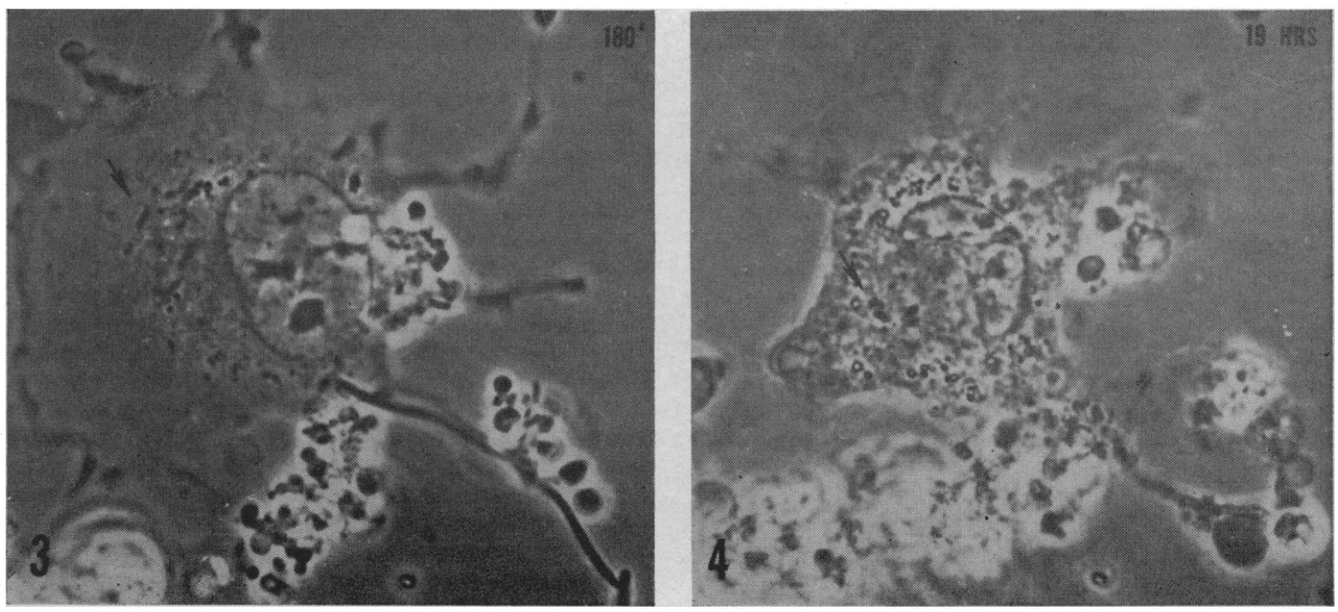
Table 1. Inhibition of multiplication of intracellular *S. typhosa* by antibiotics.

Antibiotics	No bacterial division after time indicated (min)	Formation of extracellular spheroplasts
Streptomycin	40–50*	No
Penicillin	Few	Yes
Chloramphenicol	Few	No
Synnematin	Few	Yes

*Some resistant organisms continued to divide at intervals of about 30 minutes.

The photomicrographs, taken by phase microscopy, which are reproduced in Fig. 6 clearly show that spheroplast formation was well under way 1 hour after synnematin had been added to the culture and was essentially complete in the *extracellular* bacteria by hour 3. However, the important point is that the *intracellular* bacteria do not form spheroplasts under these conditions. Bacillary organisms are clearly discernible in the cytoplasm of the L cell of Fig. 6 at 3 hours, and also at 19 hours when the L cell was moribund. Streptomycin and chloramphenicol, unlike penicillin and synnematin, fail to induce spheroplast formation in extracellular *Salmonella typhosa* and, of course, do not induce it in the intracellular organisms. Hence, it seems unlikely that the long survival but eventual death of nonmultiplying *S. typhosa* in antibiotic-treated tissue culture cells can be specifically related to the spheroplast stage of the organism. The effects of four antibiotics on intracellular multiplication of *S. typhosa* and on spheroplast formation among extracellular organisms in tissue cultures are summarized in Table 1.

Earlier in this article I mentioned the clearing of microbes from infected cells by egestion. Extrusion of this type was observed in the *Salmonella typhosa* tissue culture system on a number of occasions, but in no instance did the activity clear the cell of all the recognizable bacteria. While this mechanism does provide the invaded cell with a means of curing itself, the procedure appears to be relatively ineffective even when antibiotic is present in quantities sufficient to prevent growth of the microbe. Otherwise, one would anticipate that the antibiotic-treated cell would rid itself of the pathogen in less than the 3 or 4 weeks required in the rickettsiae and typhoid tissue culture models.



formed spheroplasts whereas organisms within the cell have retained their rodlike morphology. (4) Photomicrograph after 19 hours. The cell is dead, but a few intracellular bacillary forms can be seen. [After J. L. Showacre *et al.* (*14*)]

Conclusions

The evidence summarized in this article is sufficient to support conclusions as follows.

1) Antibiotics penetrate rapidly into mammalian cells and inhibit multiplication of susceptible microbes within the cell as promptly and effectively as they inhibit multiplication of such microbes outside the cell. This is consistent with clinical experience.

2) Death of individual organisms inside mammalian cells bathed in medium containing antibiotics continues over several weeks but may not proceed *pari passu* with time. This observation supports the as yet meager clinical data, which suggest that eradication of the typhoid carrier state may be accomplished through antibiotic therapy, provided (i) the proper antibiotic is selected; (ii) it is administered in amounts sufficient to obtain continuous suppression of growth of intracellular organisms; and (iii) the regimen is maintained for 2 or 3 weeks.

3) The actual cause of death and destruction of intracellular microbes in treated cells remains an enigma. To attribute this to inanition or senescence of the organisms, without describing mechanisms, is to avoid the issue. The hypotheses dealing with inactivation of intracellular microbes by intracellular antimicrobial substances and antibodies deserve to be explored.

4) Evidence from the ward and laboratory suggests that infected cells can clear themselves of invading pathogens and recover. Although antibiotics have played the major role in demonstrating this in the laboratory, it is possible that they do nothing more than hold the intruders in abeyance while natural defense mechanisms of the cell gain the upper hand. Certainly such cellular mechanisms must provide a potent force for survival; otherwise, why would 98 percent of typhoid patients fail to become chronic carriers?

5) The continued study of infections, including the carrier state, with a view to understanding the abnormalities created by the multiplying intracellular microbe and the means by which the cell corrects these and eliminates the intruder, should open new vistas in chemotherapy and immunology.

References and Notes

1. E. W. Goodpasture, *Am. J. Pathol.* **13**, 175 (1937).
2. ——— and K. Anderson, *ibid.*, p. 149; G. J. Buddingh and F. C. Womack Jr., *J. Exptl. Med.* **74**, 213 (1941).
3. "Tropical Health—Summary Report on a Study of Needs and Resources" (National Research Council, Washington, D.C., 1962), pp. 1–121.
4. *Control of Communicable Diseases* (American Public Health Association, New York, ed. 9, 1960), p. 11.
5. M. E. O'Connor, *Public Health Rept. U.S.* **73**, 1039 (1958).
6. T. E. Woodward and J. E. Smadel, in *Science, Technology and Developments* (Government Printing Office, Washington, D.C., 1963), vol. 6, *Health and Nutrition.*
7. E. S. Murray, S. Cohen, J. Jampol, A. Ofstrock, J. C. Snyder, *New Engl. J. Med.* **246**, 355 (1952); G. L. Schaefer, M. Friedman, C. Lewis, *Ann. Internal Med.* **42**, 979 (1955).
8. W. H. Price, H. Emerson, H. Nagel, R. Blumberg, S. Talmadge, *Am. J. Hyg.* **67**, 154 (1958).
9. J. E. Smadel, *J. Immunol.* **84**, 1 (1960).
10. H. S. Ginsberg, *Progr. Med. Virol.* **1**, 36 (1958); G. Henle, F. Deinhardt, V. V. Bergs, W. Henle, *J. Exptl. Med.* **108**, 537 (1958); W. Henle, G. Henle, F. Deinhardt, V. V. Bergs, *ibid.* **110**, 525 (1959); H. R. Morgan, in *Symposium on Latency and Masking in Viral and Rickettsial Infections* (Burgess, Minneapolis, 1958), pp. 112–117; R. E. Shope, *J. Gen. Physiol.* **45**, suppl., 143 (1962).
11. A. Isaacs, in *Perspectives in Virology*, M. Pollard, Ed. (Burgess, Minneapolis, 1961), vol. 2, pp. 117–125.
12. W. McDermott, *Public Health Rept. U.S.* **74**, 485 (1959).
13. F. M. Bozeman, H. E. Hopps, J. X. Danauskas, E. B. Jackson, J. E. Smadel, *J. Immunol.* **76**, 475 (1956).
14. J. L. Showacre, H. E. Hopps, H. G. DuBuy, J. E. Smadel, *ibid.* **87**, 153 (1961).
15. H. E. Hopps, E. B. Jackson, J. X. Danauskas, J. E. Smadel, *ibid.* **82**, 172 (1959).
16. J. E. Smadel, T. E. Woodward, H. L. Ley, Jr., R. Lewthwaite, *J. Clin. Invest.* **28**, 1196 (1949).
17. H. L. Ley, Jr., F. H. Diercks, P. Y. Paterson, J. E. Smadel, C. L. Wisseman, Jr., R. Traub, *Am. J. Hyg.* **56**, 303 (1952).
18. H. E. Hopps, E. B. Jackson, J. X. Danauskas, J. E. Smadel, *J. Immunol.* **82**, 161 (1959).
19. M. R. Bovarnick and E. G. Allen, *J. Gen. Physiol.* **38**, 169 (1954); *J. Bacteriol.* **73**, 56 (1957).
20. Z. A. Cohn, F. E. Hahn, W. Ceglowski, F. M. Bozeman, *Science* **127**, 282 (1958).
21. E. Landman, R. A. Alternbern, H. E. Ginoza, *J. Bacteriol.* **75**, 567 (1958); J. F. Wilkinson, *Bacteriol. Revs.* **22**, 46 (1958).
22. D. Bodian, in "Cellular Biology, Nucleic Acids and Viruses," *N.Y. Acad. Sci. Spec. Publ. No. 5* (1957), pp. 57–75.
23. W. D. Kundin, *Arch. Ges. Virusforsch.*, in press.
24. R. C. Skarnes and D. W. Watson, *Bacteriol. Revs.* **21**, 273 (1957); J. G. Hirsch, *ibid.* **24**, 133 (1960).
25. M. Schaechter, F. M. Bozeman, J. E. Smadel, *Virology* **3**, 160 (1957).
26. T. E. Woodward, J. E. Smadel, H. L. Ley, Jr., *J. Clin. Invest.* **29**, 87 (1950).
27. H. E. Hopps, J. E. Smadel, B. C. Bernheim, J. X. Danauskas, E. B. Jackson, *J. Immunol.* **87**, 162 (1961).
28. R. T. Parker, J. C. Fitzgerald, H. D. Perry, T. E. Woodward, *J. Clin. Invest.* **32**, 593 (1953).
29. T. Jersild, E. Neukirch, J. Raun, K. Ritwerts-Eriksen, S. Tulinius, *Antibiot. Med. Clin. Therapy* **6**, 292 (1959).
30. C. G. Nicol, *World Med.* **30**, 310 (1961).
31. G. L. Hobby and M. H. Dawson, *Proc. Soc. Exptl. Biol. Med.* **56**, 181 (1944); G. L. Hobby, T. F. Lenert, D. Pikula, M. Kiseluk, M. E. Hudders, *Ann. N.Y. Acad. Sci.* **53**, 266 (1950); G. L. Hobby and T. F. Lenert, *Am. Rev. Tuberc. Pulmonary Diseases* **76**, 1031 (1957).
32. E. H. Freimer, R. M. Krause, M. McCarty, *J. Exptl. Med.* **110**, 853 (1959).