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MICROBIAL PERSISTENCE**

A number of animal species use the defense tactic of "playing dead" when confronted with a hostile threat. It is the purpose of the present discussion to develop the theme that "playing dead" is not limited to the more organized living creatures. For the adaptive plasticity of microbes to environmental influences is such that, in a sense, they too can "play dead" when menaced by an antimicrobial drug.

When a group of patients with a drug-susceptible infection is treated with the appropriate antimicrobial drug, there is a rapid disappearance of all evidences of illness, and the patients are apparently well. In the majority of the patients this apparent absence of infection is permanent. In others, a small subgroup indistinguishable from the rest, the illness reappears after a treatment-free interval. The microorganisms isolated at the time of this reappearance of illness are not drug-resistant in the usual sense, and retreatment with the drug used originally is generally effective. This capacity of drug-susceptible microorganisms to survive drug attack when subsisting in an animal body may be designated as *microbial persistence.*

In the broad sense the term microbial persistence simply indicates something we all know, namely, that microbes have the ability to survive in this perilous world. In the present discussion the term is used in a more narrow sense, however, to designate the survival of microbes in a certain set of definable circumstances. Employed in this way the term microbial persistence describes a phenomenon whereby microorganisms which are drug-susceptible when tested outside the body are nevertheless capable of surviving within the body despite intensive therapy with the appropriate antimicrobial drug. In clinical practice this phenomenon obviously has to do with the post-treatment "carrier state" and with post-treatment relapse. In short, it is this phenomenon which is responsible for our inability to eradicate an infection from a person or a community by the use of drugs.

The initial mention of this phenomenon as something which can be observed in a "group of patients" was intentional. For the clinician does

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not usually encounter his patients as a group, all with the same infection. Instead, he sees a few patients with one infection and a few with another. Thus, even though the total number of infections he treats in any one year may be quite large, he might never encounter the phenomenon of post-treatment relapse. This is despite the fact that it occurs with regularity among some of the many who are treated for any particular infection.

A number of us have been fascinated by this phenomenon for slightly more than a decade. During that period my co-workers and I have conducted a number of experimental forays into this field without, I must warn you, penetrating very deeply into an understanding of the phenomenon. Indeed, the best it has been possible to do is to skirt the edges, so to speak. It has been possible to demonstrate: the existence of the phenomenon; its wide occurrence among microbial species; its importance to the clinician and the theorists; and above all, its invulnerability to manipulation with drugs. It has not been possible, however, to obtain more than fragments of experimental data which might lead to its understanding.

It should also be noted that during the eleven years in which our own studies of this subject have been in progress, there have been a number of other groups likewise interested in one or another aspect of the phenomenon. I shall make no attempt in this evening's discussion to present a comprehensive review of all the work which has been done in this broad field. Instead, what follows is to be regarded as a presentation of the development of our own thinking in this field and of certain of the experiments which have formed the basis of that thinking.

At the outset it should be noted that recognition that there is a phenomenon of microbial persistence was not possible before the introduction of penicillin. The reason for this is the fact that the early antimicrobial drugs (quinine, the arsphenamines, atabrine, and the sulfonamides) could never be administered in very large doses because of drug toxicity. As a consequence, there was no reason to postulate any adaptive plasticity of the microbes to explain their survival in the tissues despite chemotherapy. Instead, the survival seemed merely the result of our inability to administer a sufficient quantity of the drug. With the introduction of penicillin into syphilotherapy, however, it soon became clear that inadequate drug dosage was not the principal cause of microbial survival.

Our own interest in this subject originated from experience in the treatment of syphilis. It had long been recognized that syphilis had a remarkable capacity to become a latent infection. Moreover, the conviction was widely prevalent that the time antisyphilitic therapy was most effective was when the early infection was actually producing widespread lesions in the form of generalized cutaneous disease. Treatment given *before* that time

was not quite so effective; treatment given *after* that time was also less effective. Indeed, it is not possible to be certain that once true latency has occurred by natural means, the syphilitic infection is influenced at all by chemotherapy.

With the introduction, first, of short-term intensive arsenotherapy and, second, penicillin therapy, it became clear that even the completed treatment of the highly susceptible early (cutaneous) syphilitic infection was never uniformly successful. Instead, the infection invariably reappeared in a certain percentage of patients (5 to 15 per cent). Although some of these "second attacks" of syphilis presumably represented new infections, some were almost certainly relapses. Moreover, the percentage of incidence of these second attacks was remarkably constant with arsenical or penicillin therapy once certain minimal standards of therapy were attained.

Since penicillin, unlike the arsenicals, was effective against other bacteria, an opportunity was afforded to relate the situation with syphilis to that with other infections. Several possibly pertinent observations were available.

In 1942, Hobby, Meyer, and Chaffee[11] reported that 99 per cent of the cells of a culture of hemolytic streptococci were readily killed *in vitro* by appropriate concentrations of penicillin, but that the remaining one per cent were not uniformly eradicated. In 1944, the late Professor Bigger[3] of Dublin likewise reported that in certain circumstances it was not possible to completely sterilize cultures of penicillin-susceptible staphylococci by prolonged exposure to penicillin. He designated these drug-surviving microorganisms "persisters" in order to differentiate them from bacteria which were drug-resistant.

In 1945, in association with Dr. Edith Lincoln, we had been unable to rid a small group of children of the nasopharyngeal carrier state of type 14 pneumococcus even though the strain was highly susceptible to penicillin. At Fort Bragg, Rammelkamp and Kirby[30] found no sustained changes from penicillin therapy in the pneumococci and streptococci in the pharynx of so-called "carriers." We were particularly interested at the time in the fact that certain of the "carriers" in this Fort Bragg study were patients with the lesions of infectious syphilis.[29] For, despite the fact that the microbes in the nasopharnyx were not permanently affected, the treponemes in the inflammatory lesions of the same patients disappeared rapidly under the influence of the drug.

Observations of this type viewed together with the peculiar drug-parasite phenomena in the treatment of syphilis led to the presentation in 1946[22] of the hypothesis that there was something different about parasites when they were actually producing disease which made them more drug-susceptible and that microorganisms which were not drug-resistant in the usual sense were nonetheless capable of surviving drug therapy in the tissues of man, so that antimicrobial therapy was seldom predictably eradica-

tive. In short, it was postulated that microorganisms in tissues had an important degree of adaptive plasticity irrespective of genotype, and that this plasticity affected their response to drugs.

Attention is thus directed to the state of the parasite. If the most drug-susceptible state occurs when it is producing active disease, this directs attention also to the possible role of the inflammatory lesion, a question

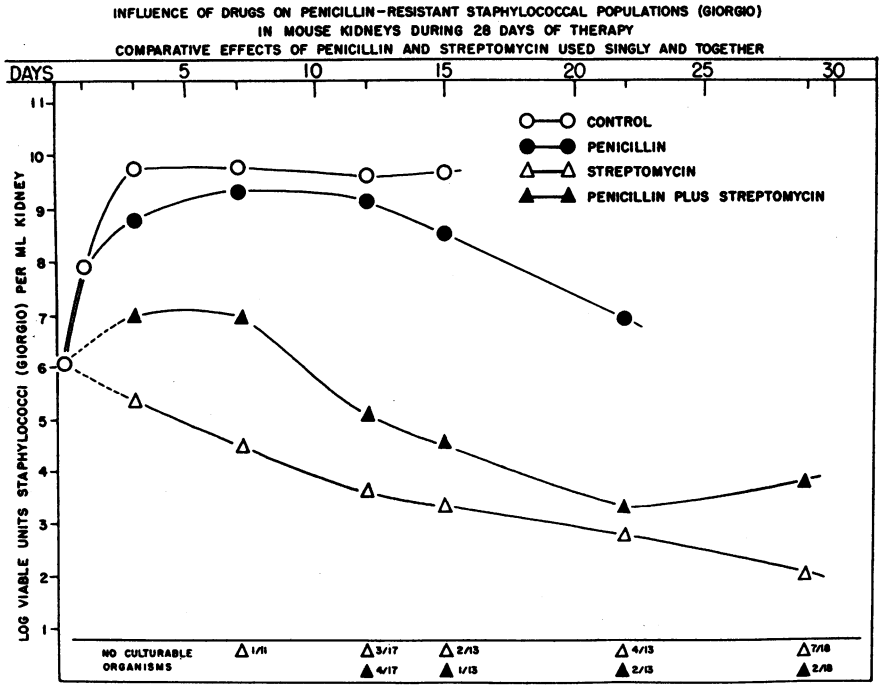

FIG. 1. Reproduced from *Annals of the New York Academy of Sciences*, 1956, *65*, 95.

which will be considered later in this discussion. With introduction of new antimicrobials, additional examples of the failure of a powerful drug to be eradicative in a noninflammatory situation have been observed in clinical practice. The failure of chloramphenicol in typhoid carriers is a case in point, as is the demonstration by Woodward and Smadel[88] that chloramphenicol could be given *too early* in scrub typhus to be completely effective.

Thus the phenomenon of microbial persistence despite appropriate chemotherapy appears to have considerable generality. Moreover, it occurs irrespective of whether the action of a drug, as judged *in vitro*, is bacterio-

static or bactericidal. It is important to note further that at least one and possibly several exceptions exist. Meningococci apparently lack the capacity to persist in the tissues during chemotherapy, and abolition of the "carrier state" with respect to these microbes can be easily accomplished with a sulfonamide. A similar situation probably exists with *Shigella dysenteriae* and the tetracyclines and it may exist with certain antimalarials deemed capable of eliminating the "tissue" phase of *Plasmodium malariae.* Except for these few instances, however, the phenomenon of microbial

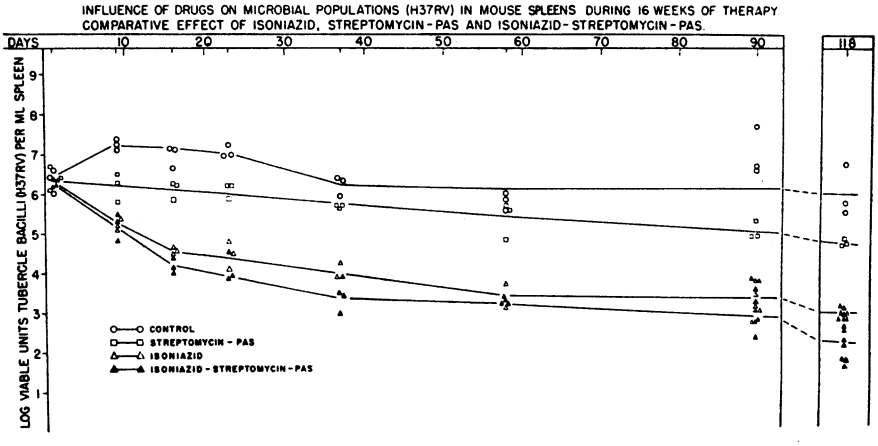

FIG. 2. Reproduced from *American Review of Tuberculosis and Pulmonary Diseases,* 1956, *74,* 102. With the permission of the journal.

persistence seems to occur generally among the microbes which produce infection and disease.

Let us proceed to a demonstration of the phenomenon of microbial persistence in experimental animals. In Figure 1 may be seen the fate of populations of staphylococci in the kidneys of mice with and without the introduction of streptomycin therapy. In the untreated animals, the microbial census rises rapidly until it attains a peak approximating the log of 10. At this point the census stabilizes, and the population remains absolutely constant during the remaining 5 or 6 days until the death of the animal. With the introduction of streptomycin there is no increase in population in this particular organ. Instead, the population slowly falls, but even at the end of the experimental period, in this case 28 days, staphylococci may still be cultured from the kidneys of the animals.

In Figure 2 a similar situation is shown for tubercle bacilli in mice. In this case the spleen is the organ depicted and, as one may see, the census

starts at a level of around 10^6 and slowly rises by more than one logarithmic unit. After approximately 2 weeks at the higher level, the census slowly falls to the original level at which it remains stabilized throughout the experimental period of 118 days. With isoniazid there is an immediate fall in microbial census which then stabilizes at a level of around log 3 and remains constant throughout the remainder of the experimental period. Even at the 118th day it was still possible to culture tubercle bacilli from the splenic tissue of the animals.

What are the possible explanations for this capacity of the microbes to persist in the tissues of the mouse in stable census for many weeks despite presumed exposure to antimicrobial drug? The first thought that comes to mind is the presence of drug resistance of the genotypic form, but this possibility can be very easily excluded. In both of the experiments presented, the culturing of microorganisms in the tissue directly on drug-containing medium showed plainly that the microbes, or rather their immediate descendants, were not resistant to the antimicrobial drugs which had been administered.

A second possibility which deserves only brief mention is the factor of drug-dosage. In similar experiments with isoniazid and tubercle bacilli, for example, a five-fold increase in dosage to 100 milligrams per kilogram per day failed to influence the phenomenon in any way.[20] This is to be compared with a dosage of five milligrams per kilogram which will produce a definite lowering of the microbial population in this experimental system and indeed will produce very marked changes in tuberculous infections in man.

A third possibility which is still frequently stated in the literature is that the drug is confronted by "barriers" in the tissue. These barriers are presumed to exist in the form of "fibrotic walls" of abscesses, large avascular areas, or the "walls" of leukocytes or phagocytes.

The factor of fibrous walls was investigated experimentally by Dr. Robert McCune in our laboratory. Doctor McCune used a technique previously developed whereby triple-layered agar discs about the size of a 25-cent piece are placed in the peritoneal cavity of rabbits or cats.[37] The central layer of the disc can be implanted with microorganisms which in this situation are thus subsisting almost entirely on nutriment provided by the host. In the particular experiment with which we are concerned the discs were not seeded with microorganisms. Instead, Dr. McCune studied the transfer into the center of the disc of a number of commonly used antimicrobial drugs, for example, penicillin and streptomycin. The drug transfer was measured both when the discs had been recently implanted, say, within 12 or 24 hours, and when they had been persisting in the

peritoneal cavities of the animals for a 28-day period. In the latter situation, a 2-millimeter, dense, highly organized wall had formed around the disc. In effect, therefore, each disc represented an area of "dead" tissue, 2.5 centimeters in diameter, surrounded by a living, highly organized, dense fibrous wall.

Dr. McCune was able to demonstrate quite conclusively that the distribution of penicillin and streptomycin and other drugs into the center of the disc occurred rapidly and completely. Moreover, the drug transfer occurred in identical fashion whether the disc had just been implanted in the peritoneal cavity or whether it was surrounded by the fibrous wall at the time the host animal received the drug.*

The question of the barrier provided by the boundary of phagocytes was studied with penicillin and staphylococci by Dr. Ralph Tompsett of the Department of Medicine in our institution.[85] He found that appreciably more staphylococci survived when they were situated within polymorphonuclear leucocytes suspended in plasma *in vitro* than when they were exposed to the penicillin outside the phagocyte. Nevertheless, it is noteworthy that even though the staphylococci survived better within the cells than outside the cells, the populations subsisting inside the cell were reduced by approximately 90 per cent. In other words, there was a great quantitative difference in the results with the intracellular and the extracellular staphylococci, but even the intracellular microbes were influenced by the penicillin to a very large extent.

At the National Institutes of Health, Dr. Harry Eagle[9] has shown that approximately 63 per cent of the penicillin present in an extracellular environment was transferred into Hela cells and mouse fibroblasts. If it can be assumed that human phagocytes behave in the same fashion, very considerable amounts of penicillin should have been present within the phagocytes in Dr. Tompsett's experiments. Nevertheless, in additional experiments he introduced the penicillin in much larger doses, namely 100 units per milliliter. Even in this situation, 5 to 10 per cent of the intracellularly situated staphylococci survived. Thus, the quantitative aspects of the phenomenon were not affected by very considerable increases in the concentrations of penicillin outside the cells.

It appears, therefore, that the ability of a minority of the staphylococci to survive when situated intracellularly is not a consequence of failure in the *delivery* of penicillin to the interior of the cells. On the contrary, the

* A number of observers have demonstrated in caseous lesions in the tuberculous lungs of humans that antimicrobial drugs do indeed penetrate necrotic areas. This has been shown in the case of streptomycin by Canetti[5] and in the case of isoniazid by Dr. Barclay and his associates[1] at the University of Chicago.

persistence of the microbes within the single cell of the phagocyte would seem to be a miniature replica of the situation as it obtains in the host as a whole.

A fourth possibility to explain microbial persistence is that the environment of the inflammatory lesion exerts an antagonistic influence on the action of the antimicrobial drug. This has been a popular concept, and I confess that I have done my share in textbook chapters and similar writings to present it credibly. However, I no longer believe that it can stand up to critical scrutiny as a reasonable explanation.

The case for environmental antagonism of drug activity consists of pointing out that there is a diffusion gradient through nonliving tissue so that, depending on the size of a necrotic area and the drug supply at the periphery, sufficient quantities of drug might not penetrate to the center; that binding to macromolecular protein complexes might easily result in deviation of the drug from its appointed task of reaching the parasite; that changing the environmental pH has a marked effect on the action of certain antimicrobial drugs; and finally, the fact demonstrated in the microbial population studies in mice, that both tubercle bacilli and staphylococci attain considerably higher populations in some organs than in other organs of the same animal. In the case of staphylococci, the organ of maximal census is the kidney; in tubercle bacilli, it is the lung.

Examination of these possibilities for a direct environmental antagonism of drug activity makes it seem unlikely that their role is particularly significant. For example, different drugs presumably have different diffusion gradients through different types of necrotic tissue. But, in the circumstances which obtain in the treatment of humans, it is distinctly improbable that any wide differences materially impede the complete impregnation of the lesion. It will be recalled from the agar-disc experiments, in which only short-term therapy was given, that fairly large-sized areas of nonliving material quickly became full of drug. In the treatment of infections in humans we are not dealing with a period of 12 or 24 hours but with days or months. Throughout these long periods there is a constant delivery of a great excess of antimicrobial drug to the periphery of the necrotic lesion, and, with such a constant excess at the periphery, the drug concentrations in the center should be high.

Essentially the same objection holds against the assumption that drug-effectiveness is seriously impaired by macrocolecular binding. Such protein-binding is an easily reversible phenomenon, and the degree of binding depends upon the concentration of drug in the immediate environment. Hence, even if macromolecular binding were occurring, quantities of drug would also be being released depending on the surrounding concentration.

Here again, when dealing with the large excesses of drug given over the long periods that occur in the treatment of an actual infection, it would seem that however active macromolecular binding might be, it still could not possibly "sop up" all the drug with which the lesion is flooded. The factor of environmental acidity is more subtle and will be discussed in connection with the next possibility.

The phenomenon of "organ difference,"* namely, that certain parasites are more susceptible to certain drugs when subsisting in one organ of an animal than in another organ of the same animal, obviously could represent environmental antagonism of drug activity. An important objection to this interpretation stems from the experiments presented previously dealing with the behavior of populations of staphylococci and of tubercle bacilli in the tissues of the mouse. For example, in the case of the mouse spleen and streptomycin we have an organ in which tubercle bacilli actually multiply during drug therapy at first and never fall below the original census. In contrast, with staphylococci in the spleen, even in the absence of drug therapy, the populations fall steadily, and with streptomycin therapy the populations of staphylococci fall to extremely low levels. Thus we have the same drug, streptomycin which in effect is highly "active" in the spleen when it is acting on staphylococci and considerably less "active" in the spleen when it is acting on tubercle bacilli. This makes it most unlikely that the antagonism of the streptomycin action represents an influence of the splenic environment per se on the drug. It is far more credible to infer that there is something about the respective states of staphylococci and tubercle bacilli when they are subsisting in the spleen which causes a difference in their reaction to streptomycin.

In the same way that the same drug in the same organ is highly effective against one parasite and is considerably less effective against another, one can see that the same drug may act differently in different environmental circumstances *in vitro*. For example, it is well recognized that streptomycin loses a considerable portion of its effectiveness in partially anaerobic environments. This can be easily demonstrated for staphylococci and for tubercle bacilli. Nevertheless, in the very same circumstances, streptomycin is active on *E. coli*.[36] Likewise, pyrazinamide is inactive on tubercle bacilli of human origin when tested in environments within the physiologic range

* It should be pointed out in passing that there is a human counterpart for this "organ difference" in that one could say penicillin is an excellent drug for the treatment of pneumococcal infections of the lung but less effective for the treatment of the same infection when it has penetrated the pleural cavity or exists in the central nervous system. The same holds true with streptomycin and tubercle bacilli, namely, the drug is highly effective in the lung, less so in the pleural cavity or in the central nervous system.[23]

of pH. Yet, when the same environment is made acid, the pyrazinamide is highly active on tubercle bacilli of human origin.[25] This change from "physiologic" pH to acidity does not cause tubercle bacilli of bovine origin to become susceptible to pyrazinamide. Thus in two very closely related tubercle bacilli, so closely related that they almost represent mere strain variants, a change in environment produces drug susceptibility in the one but not in the other. This makes it highly likely that the influence of the acidic environment is on the tubercle bacilli and not on the pyrazinamide.

Now to sum up this question of environmental antagonism of drug activity. It is easy to show that environmental changes result in changes in drug effectiveness. By the same token, all of these changes could equally well represent influence of the environment on the parasite instead of on the drug. Some of the observed changes, moreover, presumably reflect influences which could have been exerted *only* on the parasite. In view of these considerations, together with the fact that a large excess of drug should usually be present in the lesion, it appears that environmental antagonism of drug activity may well occur, but that seldom, if ever, should it attain a magnitude that would provide a satisfactory explanation for the phenomenon of microbial persistence.

Let us turn now to consideration of the possibility that insofar as the tissue environment affects drug-susceptibility it does so by an influence exerted primarily on the parasite rather than on the drug. This makes it necessary to presuppose that the parasite is capable of assuming a state in which it is neither permanently incapacitated by the drug nor does it multiply freely in the presence of drug as do the genetically drug-resistant microbes. In short, the parasite may be said to be "indifferent" to the drug and I have taken the liberty of designating such a supposed state as "drug-indifference." Intimately related to this question are the questions of whether "drug-indifference" may also occur essentially independent of environmental influences and whether such a state could be actually *induced* by the presence of antimicrobial drugs in the tissue environment. As it seems easier from the standpoint of the discussion to take up first the question of the relation of tissue environment to "drug-indifference," consideration of these other two questions will be deferred until later.

We are obviously presupposing that the nature of the environment can produce detectable changes in the parasite and that some of these affect its drug-susceptibility. In order to establish these presuppositions, it is necessary to show that a state of microbial "drug-indifference" exists, that it is reversible, and that it may be induced or specially favored by the particular nature of the tissue environment.

It seems to me that highly credible evidence can be adduced in support of all these presuppositions. Examples exist of the fact that the nature of the environment can change the form of the parasite. R. G. Wittler[39] has observed a considerable morphological change in *H. pertussis* during its sojourn in the mouse lung. In more recent experiments, Wittler, Carey, and Lindberg[40] have observed the reversion of a pleuro-pneumonia-like organism (PPLO) to a corynebacterium during residence in Hela cell tissue cultures. Of even greater interest was the fact that the PPLO form showed little if any cytotoxicity for the cells in which it was situated until yeast extract (or staphylococcal filtrate) was added to the extracellular environment. Considerable destruction of tissue ensued, then the PPLO form was converted to the L-form. The L-form was subsequently converted to the orthodox corynebacterium on further cultivation with the aid of mucin. These investigators state: "From our observations, it seems likely that factors which produce changes in host cells may in turn result in changes in the form or in the type of growth of the infecting organism, yet this may not always be readily apparent when the organism is again cultured *in vitro*."

A particularly dramatic example of this same type of phenomenon was recently observed by Dr. Dubos in his studies with *M. fortuitum*.[7] This mycobacterium, like tubercle bacilli and staphylococci, proliferates more readily in certain organs of the mouse than in other organs. For example, the peak census of this microbe is attained in the kidney rather than in the lung. Dr. Dubos has observed that cultures of *M. fortuitum* assume either one of two quite distinct forms. In one form the colonies appear as a hard heaped-up button without a peripheral lacy skirt; in the other form, the colonies are flatter and extend for a considerable distance from the central core forming a peripheral "skirt." The important thing is that the microbial populations cultivated from one organ of the mouse assume one of these colonial patterns, whereas when cultivated *on the same medium* but from another organ they assume the other colonial pattern. There is every reason to believe, therefore, that depending upon certain aspects of the environment in the particular organ, the assumption of one or the other of these colonial patterns becomes predominant. Thus the environment of the organ appears to determine the state of this particular parasite. Dr. Dubos has carried these studies even further and can now demonstrate that a purified substance known to be present in the tissues, when incorporated into the medium, can determine which of the forms is assumed by the parasite.

From these examples chosen—the studies of Wittler and associates and those of Dubos—it may be seen that the first presupposition that environ-

mental factors can determine which of several possible different states will be assumed by a parasite is clearly the case. The presupposition that there should be a state of "drug indifference" and that it should be reversible can very easily be demonstrated by a simple experiment with penicillin and Group A beta streptococci in a favorable medium, maintained first at ice-box temperature and subsequently at incubator temperature. In the cold environment the microbial population remains absolutely unchanged, even though it is afforded a favorable medium and high concentrations of penicillin are present. When this same culture is removed from the cold environment and put in the warm environment, two things happen at the same time. The population rapidly increases in the absence of penicillin and rapidly falls in the presence of penicillin. This simple experiment, which has been performed with a number of microbial species and a number of drugs, clearly shows that there is a state of "drug-indifference," that it is reversible depending on environmental changes, and that such states can be induced or favored by the nature of the environment.

Admittedly, the particular environment chosen from the demonstration cited is a highly artificial one. What is the situation in circumstances more directly related to the circumstances of an infection? First, let us start again with the simple system of Group A beta hemolytic streptococci and penicillin. Virtually all of the published experiments on the dynamics of the action of penicillin or other drugs *in vitro* have employed a relatively standardized system. In this system actively growing microbial populations with a total census of about 10^6 are subjected to the action of the drug, let us say, penicillin. In these circumstances there is a rapid and essentially straightline fall (on a logarithmic scale) of the microbial population to low levels or indeed, in many cases, to zero within a few hours. It is the fact that this does not happen in the animal body that represents the phenomenon of microbial persistence. But why has there been this disparity between the events in the test tube and what happens in the body?

In some unpublished experiments in our laboratory conducted by Dr. Carl Berntsen it was noted that this rapid fall in microbial population under penicillin *did not occur* when the initial populations of streptococci were high, say around 10^8 or 10^9. In the latter circumstances an impressive portion of the population of supposedly penicillin-susceptible streptococci was nevertheless capable of withstanding the action of penicillin for a week or longer. The census slowly fell, but even at the end of the week appreciable numbers of streptococci could be cultured from the medium. In short, penicillin acted dramatically on small microbial populations of the order of 10^6, but its effectiveness was reduced to a very great extent on larger populations of the order of 10^8 or 10^9.

The question immediately arose as to how great are the microbial populations in the lesion of an infection? Dr. Berntsen made a number of studies of this point using vegetations from heart valves of untreated patients dying of bacterial endocarditis (medical examiner's cases), freshly excised tonsillar tissue handled with techniques designed to prevent microbial proliferation of the microbe after excision, and vegetations from experimentally produced endocarditis in rabbits. In addition, there was in progress in the laboratory at the same time a large number of quantitative studies with the microbial enumeration technique of the infections produced in mice by tubercle bacilli and staphylococci.

All of these observations tended to show the same thing, namely, that in the mouse organ or tissue with the greatest microbial census (or in the other tissues of man mentioned), the value for the microbial population almost always ranged between 10^8 or 10^9 per milliliter of emulsified tissue. A minor exception to this is the fact that in some circumstances in the lung the peak census of tubercle bacilli ranges between 10^7 and 10^8. It is of considerable interest that these values of 10^8 and 10^9 for the peak census of these various microbes are essentially the same as are attained by these three microbial species when they are allowed to grow under favorable conditions in the test tube. Thus with staphylococci, beta hemolytic streptococci, and tubercle bacilli, both in the test tube and in the organ of the mouse in which the highest population occurs, the peak value is essentially the same, namely, 10^8 or 10^9.

Populations of penicillin-susceptible streptococci or penicillin-susceptible staphylococci of this size, 10^8 or 10^9, are not very markedly influenced *in vitro* by the introduction of penicillin. In short, *in vitro* at least, they are "drug indifferent"; they are not "drug resistant." Moreover, in the animal, populations of this size of either streptococci, staphylococci, or tubercle bacilli are considerably less affected by isoniazid or penicillin than populations around 10^6 in the same animal.

We have, therefore, to ask ourselves the question: in what ways other than in size do these large "drug-indifferent" microbial populations differ from the smaller drug-susceptible populations?

With the large populations the microorganisms have been growing in the environment for a longer period in order to attain the high census. This means two things: the large population is an older one, and it has been reacting with its environment for a longer period than is the case with the small populations. Thus there has been a greater opportunity for chemical modification of the environment, either in the culture medium *in vitro* or in the inflammatory lesion in the animals.

In a further series of unpublished experiments by Dr. Carl Berntsen it was shown that when age of the population and the environmental alterations produced by growth are divorced experimentally, large populations of streptococci are as susceptible to penicillin as are small populations. In these experiments, large (10^8 and 10^9), populations of Group A beta hemolytic streptococci were employed in which the majority of the microbes were less than 4 hours old. Such high populations of very young organ-

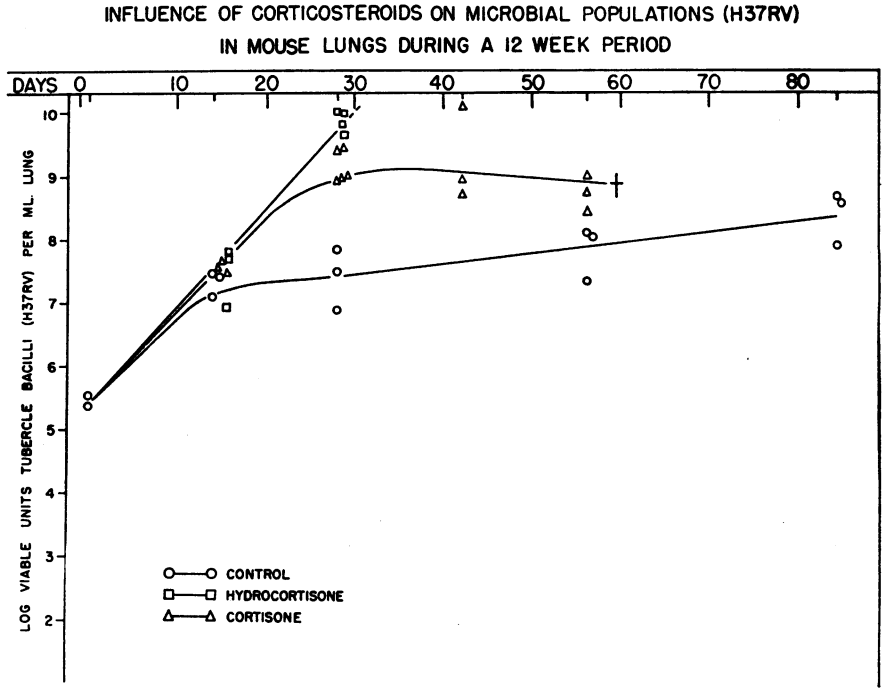

FIG. 3. Reproduced from the *British Journal of Experimental Pathology*, 1957, *38*, 419.

isms were obtained by cultivating the streptococci on a large scale and resuspending them in an appropriate volume of fresh medium. It was possible to demonstrate that when the majority of the cells in such a large population were less than 4 hours old, the population was just as susceptible to penicillin as when the young cells were initially present as a small population. It was also possible to show that this penicillin-susceptibility of the large 4-hour-old populations obtained to the same extent when the populations were implanted in the mouse muscle as when they were inoculated into a test tube. Thus it may be seen that not only were these

large drug-susceptible populations quite young, but in both the mouse and in the test tube they were being tested in an environment which they had had no previous *chance to alter.*

If the relative insusceptibility to penicillin of the large "naturally" grown populations of streptococci were reversible by changing the environment, it would constitute evidence that the "drug-indifference" of the microbes represented an adaptive state to certain environments. Indeed, one aspect of the factor of aging of the population itself conceivably could be altered by environmental changes and regarded as an adaptation, for the proportion of young microbial cells to old cells, i.e., the capacity of the population to form new cells, might vary with the environment.

A related aspect of this question of reversibility of the "drug-indifference" of large populations is the fact that if the peak census attained in the mouse tissues or in the test tube *were not the maximal attainable census* in these situations, it follows that what we designate as the peak census in actuality represents a microbial population under some inhibitory influence. In both the mouse and the test tube it can be shown that this is the case, and that the peak census obtained by the microbes is not the maximal obtainable census if the circumstances are modified.

In the mouse, this can be demonstrated by the administration of cortisone to animals infected with tubercle bacilli, as may be seen in Figure 3. The lowest curve in the figure shows the population of tubercle bacilli in the mouse lung attaining a peak between 10^7 and 10^8 and then stabilizing at essentially that census thereafter. In the animals maintained on hydrocortisone, however, the microbial census was as high as 10^{10}, yet the animals were able to survive at least for a period of a week or so in these circumstances.* In the test tube with staphylococci and penicillin, Dr. David Rogers[81] of our Department of Medicine has shown that a microbial population which has become stable at a census of 10^8 will increase to 10^9 simply by the process of shaking which, of course, also involves aeration.

These two sets of observations are interpreted as showing that the environment constituted by the tissue or the test tube in which microbial populations have grown to 10^8 is exerting an inhibitory influence at that point, for if the cessation of population increase were determined by factors within the parasite and independent of the environment, it should not be regularly influenced by environmental change. Coincidental with this inhibitory influence of the environment on the microbes is their relative indifference to the drugs.

* It should be noted that there is a definite tendency toward stabilization even of these very large microbial populations developing in hormone-influenced animals.

It can easily be shown in the test tube that the inhibitory influence which causes the growing populations to stabilize is not a question of the exhaustion of nutriment from the medium. In Dr. Berntsen's experiments fresh daily inocula of streptococci were repeatedly allowed to grow day after day to the point of stabilization in the same medium from which the bacteria were removed by filtering at the end of each 24-hour period. After 8 or 10 days, the fresh inocula of streptococci in this same medium would still grow logarithmically up to the same peak census and then stabilize. Thus nutriment, in the sense of something generally available through the medium, was not exhausted under these conditions. Likewise, to some extent, the experimental results stand against the concept that toxic substances produced by the microbes are responsible for the cessation of population increase. Certainly the possibility is excluded that a filter-passing substance was present in sufficient quantity to exert an influence throughout the medium as a whole. The possibility remains, however, that minute concentrations of inhibitory substances might be elaborated in the immediate environment of the bacteria or might adhere to the filter. Thus, it appears that there is some factor or factors associated with attainment of the "crowded" state by the microbes which exerts an inhibitory influence on further population growth. This factor may be something intimately associated with the immediate environment of the microbe but does not seem to be something which is detectable throughout the environment as a whole.

To recapitulate, it can be shown that a population of microbes growing either in the tissues or in the test tube becomes subject to some inhibitory influence before it reaches the maximal attainable population in those circumstances. And, at the time that the population becomes subject to the inhibitory stabilizing influence, the microbes also become "drug indifferent."

The important thing from the standpoint of the argument is the fact that when this environment which has now become inhibitory is altered, not only do the populations show themselves capable of further growth, but they also once again become *drug susceptible.* A small aliquot of the large "drug indifferent" microbial population is quite susceptible to penicillin when tested separately in fresh medium. Moreover, Dr. Rogers has clearly shown with the penicillin-staphylococcus system *in vitro* that penicillin-susceptibility reappears coincidentally with the new population upsurge produced by shaking and aeration.

It appears, therefore, that it is not the age of the population per se, but its adaptive changes to the environment it has altered while attaining that age which are associated with its "drug-indifference." These environmental relationships (i.e., the consequences of crowding) are responsible for the failure of the population to continue to increase in the test tube and con-

ceivably are responsible in part for the failure to continue to increase in the tissues. In terms of the population as a whole, age is a factor, but it is a factor which remains responsive to environmental change. When the environment is altered so that the same "old" cells begin to divide into a greater number of freshly born "new" ones (to the extent this occurs), the age of the population has been made more youthful.*

In considering this question of "age," it is intriguing to note that if microbes possessed the capacity to be susceptible to penicillin or isoniazid only when they were quite young, e.g. less than four hours old, certain of the experimental observations presented above or subsequently would be satisfactorily explained.

At first glance, this explanation appears to be identical with the familiar one that "penicillin acts only on multiplying bacteria." In crude terms the two explanations are indeed the same; nevertheless, a subtle difference does exist between them. With the familiar explanation it is implicit that microbial cells in the metabolic state which leads directly to cell division are *ipso facto* in a state in which they can be "killed" by penicillin in the environment. At any time a so-called "resting cell" enters into this pre-divisional state it is drug-susceptible. With the presently proposed explanation, multiplication or cell division is only a side aspect of the issue. Instead, it is postulated that as young microbes grow, they lose something or gain something which makes them resistant to drugs.** In other words, an adaptive mechanism becomes activated, and the capacity thus developed might well remain irreversible in terms of the individual cell. When the environment is suitably altered so that more of these "old-adapted" cells give birth to young "unadapted" cells—to the extent this occurs—the population becomes drug-susceptible.

In short, the environment tends to antagonize drug-effectiveness by becoming unfavorable for the birth of drug-susceptible microbes.

Let us turn to consideration of that other aspect of the aging of a parasite-host interaction, namely, the inflammatory changes produced in the environment. Dr. Harry Eagle[8] has studied the influence of penicillin on Group A beta hemolytic streptococci which have been introduced into the gastrocnemius muscle of the intact mouse. He was able to show that when the administration of penicillin was withheld for 18 or 24 hours after the start of the infection, it had far less of a population-reducing effect than

* The concept that bacterial cultures exhibit a period of "physiological youth" with changes in morphology and behavior resembling what occurs in more highly differentiated multicellular organisms has been suggested by several writers (e.g. Sherman and Albus, 1924) and is presented on page 143 of *The Bacterial Cell* by R. J. Dubos (Harvard University Press, 1945).

** Recent work (see Park and Strominger[27]; Lederberg[16]) suggests that penicillin interferes with the synthesis of the cell wall of young microbial cells and that this does not occur in fully developed cells.

when the drug was administered within a few hours. This observation has been repeatedly confirmed in a number of laboratories including our own. It gives rise at once to the question under present consideration, namely, did the inflammatory environment in the older infection exert its influence on the drug or on the parasite?

There are two sets of observations which indicate strongly that the effect of the environment was mediated directly on the parasite and that there were no important environmental effects on the drug. In recent experiments in our laboratory by Drs. McCune and Dineen it was found that when staphylococci (Wolbach strain) have been subsisting in the mouse kidney for 54 hours before penicillin was started, the drug was just as effective in terms of population reduction as when started at the beginning of the infection. (It should be recalled that the kidney of the mouse is the organ in which the most abundant growth of staphylococci occurs, and at 54 hours the populations are at their highest and microscopic abscesses are apparent although no grossly purulent lesions are yet present.)

The difference in penicillin effectiveness in the Eagle experiments and those just cited is more credibly attributable to differences between streptococci and staphylococci in the two sets of circumstances, rather than to environmental effects on the drug.

The second set of observations on this point was made by Dr. Berntsen who produced a sterile purulent-necrotic lesion in the gastrocnemius muscle of the mouse. In control experiments it was demonstrated that this sterile lesion would support the growth of beta hemolytic streptococci to the same extent as occurred in unaltered muscle. When the large populations, i.e., 10^9 of streptococci, most of which were less than four hours old, were introduced into this purulent lesion and the animals were treated with penicillin, there was a rapid fall in microbial census. Indeed, the population-reducing effect of the penicillin in these purulent lesions was exactly the same as it was *in vitro*. When the large "older" populations were introduced, the results were quite different, and the administration of penicillin had only a slow and imperfect effect on the reduction of the microbial census. Thus once again when the state of the parasite was one known to be associated with a high degree of drug susceptibility, the presence of large purulent lesions had no influence on the full penicillin action.

In this same experimental system, populations of streptococci were studied which had been grown *in vitro* for 36 hours. The populations had stabilized under these circumstances and hence were under whatever inhibitory factors make for such stabilization *in vitro*. When these populations were placed in the purulent-necrotic lesion, they were relatively indifferent to the action of the penicillin. Thus, the change from the *in vitro* environment in

which stabilization had occurred to the purulent environment *in vivo* did *not* have the effect of shaking or aeration or cortisone. Instead, the stabilized populations simply maintained their state of relative drug indifference in the necrotic-purulent environment.

Another example of this sort has to do with staphylococci, penicillin, and leukocytes. It will be recalled that Dr. Tompsett, using this system, was able to show that about 10 per cent of the population of staphylococci survived penicillin when they were inside leukocytes, whereas only .0001 per cent survived when they were outside the leukocytes. This could represent either failure of delivery of drug to *all* of the intracellular staphylococci or the possibility that the environment provided by the leukocyte induced or favored the assumption of a state of drug indifference by some of the staphylococci.

In subsequent experiments Dr. Tompsett[35] used disrupted leukocytes in the same numbers as the intact leukocytes used previously. He observed that the same 10 per cent penicillin survival which occurred when the staphylococci were situated in the leukocyte likewise occurred when *disrupted* leukocytes were used instead of intact cells. As he pointed out, the demonstration of the same phenomenon occurring in the two sets of circumstances does not establish the fact that the mechanism is necessarily the same in both instances. Nevertheless, the results are suggestive in themselves and have the additional implication that purulent material per se may represent one of the environments in which microbes assume a state of relative drug indifference.

The last-named point was investigated by Dr. W. Barry Wood[41] in some experiments published last year. He observed that pneumococci inoculated into purulent material did not show a rapid increase in numbers, but instead persisted at the initial census or gradually fell in numbers with the passage of time. Moreover, Canetti[4] and others have shown that the caseous lesion of tuberculosis, when its bronchial communication is not patent, i.e. a "closed" lesion, has a considerable self-sterilizing capacity.

It can easily be shown that the presence of one antimicrobial drug in the environment of a microbial population can influence the susceptibility of the population to another drug. This was originally demonstrated for sulfonamide and penicillin by Hobby and her associates and has been the subject of a series of detailed investigations with a large number of drugs by Dr. Ernest Jawetz.[19] Moreover, in studies with antituberculous drugs in our laboratory,[21] it has been possible to show that the population-reducing effects of isoniazid during the early weeks of its administration could be largely neutralized by the concurrent administration of nicotinamide or its derivative pyrazinamide. Nicotinamide is a weak antituberculous drug,

whereas pyrazinamide is a relatively strong one. It was further shown that tubercle bacilli of human origin exposed *in vivo* to isoniazid (or other antituberculous drugs) had greatly increased susceptibility to the pyrazinamide. The increased susceptibility to pyrazinamide could also be shown when the isoniazid exposure preceded rather than accompanied the exposure to pyrazinamide. Dr. Koch-Weser[18] and his associates have shown that sequential exposure of tubercle bacilli to another pair of antituberculous drugs may affect tubercle bacilli *in vitro* in a manner distinctly different from what happens when the bacilli are exposed to both drugs at the same time.

These examples are cited merely to show that a commonly encountered component of the inflammatory environment, an antimicrobial drug, can exert widely divergent influences on the susceptibility of a microbial population to other antimicrobial drugs. None of these drug-pairings have so far proved to be eradicative, and when the influence of the drug-altered environment happens to be in the opposite direction, namely, toward a lessening of microbial susceptibility, the phenomenon of microbial persistence is thereby favored. Thus the situation appears to be no different with respect to environmental influences in general and environmental influences consisting largely of another antimicrobial drug.

Because the present discussion is primarily concerned with microbial persistence, attention has been focussed on environmental influences and microbial adaptations of a sort that would tend *to lessen* the susceptibility of a microbial susceptibility to certain drugs. In the case of tubercle bacilli, a population which has subsisted in the mouse for 21 days is more susceptible to pyrazinamide, and probably also to streptomycin, than a population freshly introduced into the animal. It will be recalled that the susceptibility of tubercle bacilli of human origin to either of these drugs *in vitro* can be drastically modified by appropriate manipulation of the environment. Moreover, a particular environmental change will modify the pyrazinamide-susceptibility of tubercle bacilli of human origin, whereas it will not affect those of bovine origin. Or a particular change will modify the streptomycin-susceptibility of tubercle bacilli, but not of *E. coli.* As in the case of environmental lessening of drug-susceptibility, the change seems to depend on the adaptive plasticity of the microbe and not on the presence of an environment more favorable for drug "activity."

To sum up this portion of the discussion: it is believed that microbial persistence is the result of the ability of microbial populations to assume a state in which they are relatively insusceptible to an antimicrobial drug. It is believed that the assumption of this state, which is called "drug-indifference," is induced or favored by the influence of the environment on the

microbe. Included in these "environmental influences" are antimicrobial drugs and intermicrobial relationships* as well as the influences of the host cellular and humoral defense reactions and the reactions of inflammation.

An environmental change of a particular sort may make the same parasite display widely different behavior to different drugs or may make different parasites display less than their maximal susceptibility to the same drug. For these reasons it is believed that it is the adaptive plasticity of the microbe which is the important factor in the influence of environment on drug effectiveness *and not a physical or chemical antagonism exerted directly by the environment on the drug.*

The environmental factors which can influence microbial populations toward lessened or enhanced susceptibility to drugs include the orthodox host-immune mechanisms, such as phagocytes and antibody, and the previous or concurrent presence of another drug. Probably of even greater importance are forces as yet undefined but nonetheless visible in the form of effects, among them those, neutralized by steroid hormones, which prevent the microbial populations from rising in certain organs to the peak attained in other organs of the same animal; the consequences of tissue alteration, i.e. inflammation and necrosis; and the consequences of microbial crowding or "the territoriality phenomenon" which can be demonstrated *in vitro* and can be reversed by environmental manipulation.

Although instances of drug-enhancement from environmental adaptation of the microbes occur, thus far they have not been observed to lead to total eradication of a microbial population. Consequently, the net over-all effect of the various environmental influences on drug effectiveness is in the direction of providing situations which favor microbial persistence.

The question arises as to the relation of microbial persistence to the production of tissue changes and the calling forth of immune mechanisms. There is some reason to believe that stabilization of a microbial population per se, as may happen, for example, at a high census in an untreated infection, does not necessarily coincide with a cessation of progressive changes in the tissues. With respect to "persisters," however, we are considering a population which is present at a very low census. The possibility cannot be excluded that such small numbers of apparently "inactivated" microbes do not give rise to tissue reactions. It can be said, however, that if such changes occur, they are subtle in nature, for the characteristic tissue

* With respect to the purely intermicrobial aspects of this situation, the concept is, of course, a broadening of the concept of the viral "interference phenomenon" and the application to microbes of the biological concept of "territoriality" as it applies to animals of the same and different species (see *The prevalence of people* by Marston Bates[2]).

changes produced by tubercle bacilli or staphylococci are not seen in the animals harboring "drug-persisters." With respect to persisters and the evocation of immune mechanisms, it can be said, as will be discussed below, that in certain circumstances at least, microbial persistence can occur without evoking the host-immune response characteristic of that particular infection.

Reflection on the question of the relation of persisters to tissue changes or immunity leads naturally to speculation concerning the form in which microbial persisters exist during their periods of hibernation. Until quite recently there was no convincing demonstration of a correlation between a particular microbial morphology and a physiological state of drug-indifference. One had to be content with analogies drawn from the phenomenon of bacterial sporulation[24] or advance the dead-end argument that nonvisible, drug-indifferent forms existed for microbial species not known to have multiform life cycles. Within the past few years, however, there have been a number of studies dealing with the L-forms or protoplasts of certain microbes that appear to have a definite bearing on the action of penicillin.

Before considering these recent penicillin studies, it should be noted that Schnitzner and his associates[32] observed the occurrence of small colony variants, the so-called gonidial or G-forms, in cultures of staphylococci exposed to penicillin. Wise and Spink have made an extensive study of this subject.[38] In their studies with staphylococci the colonies were so minute as to be barely visible macroscopically and the microbes appeared to be unaffected, or at least they survived, concentrations of penicillin which were usually inhibitory. On subsequent cultivation in the absence of penicillin, the minute colonial forms gave rise to staphylococci of orthodox colonial morphology and in this state the microbes were susceptible to penicillin. Moreover, they found evidence of the presence (but not the reversion) of these G-forms *in vivo*. A somewhat similar type of demonstration was made by C. H. Lack[14] with tubercle bacilli. Using phase contrast microscopy and a hot box, he observed that tubercle bacilli subjected to starvation or antimicrobial drugs (streptomycin, PAS, penicillin, isoniazid) became progressively smaller until the cells were coccal rather than bacillary. When nutrition was restored, some of the coccal forms grew back to bacillary forms. Although Lack believed that these coccal forms probably represented a stage associated with drug survival, he did not consider the point as having been established.

The results of these various studies suggested the existence of morphologic counterparts to certain physiologic states involved in drug susceptibility, yet none were presented as constituting clean cut evidence on that point. Within the past year, however, several studies have been reported

dealing with the L-forms or protoplasts of bacteria which seem to have a definite bearing on the subject.

When the cell wall of a bacterial cell is lysed under appropriate conditions, the bacterial cytoplasm and its limiting membrane may continue to exist as the so-called protoplast (Weibull[36]). This process is regarded by several groups of investigators (Hahn and Ciak,[10] Park and Strominger,[27] Lederberg,[16] Pease[28]) as being identical with what happens in the first stage of the formation of the L-form from the normal vegetative form of certain bacteria. The protoplast is quite fragile and is especially responsive to the osmolarity of the environment. Nevertheless, protoplasts can survive *in vitro* with appropriate manipulations of the environment and if protoplasts and L-forms are identical microbial forms, protoplasts obviously survive *in vivo,* where the osmotic homeostatis might be expected to be more protective. The environmentally induced conversion of L-forms to a vegetative corynebacterium in Hela cells and a similar type of change with *H. pertussis* in the mouse lung by Wittler and her associates have been previously mentioned.

The importance of these studies of protoplasts with respect to microbial persistence lies in the fact that several investigators have shown (Lederberg,[16] Liebermeister and Kellenberger,[19] Hahn and Ciak,[10] Pease[28]) with two microbial species (*E. coli; Proteus vulgaris*) that protoplasts can be regularly *induced* by appropriate exposure to penicillin. Moreover, the protoplasts so induced *are not destroyed by penicillin and when the penicillin is removed from the environment, the protoplasts revert to the vegetative (and penicillin-susceptible)* form of the microbe. In addition to these two microbial species in which penicillin-induced protoplast formation has been shown, Park and Strominger have presented highly suggestive evidence with staphylococci that it is the synthesis of the bacterial cell wall which is interfered with by penicillin.

Thus there now exist experimentally demonstrated *biological precedents* for every step of the argument that microbes possess an adaptive plasticity in relation to their environment which permits them to persist in the animal body despite exposure to the appropriate antimicrobial drug.

It is important to realize that what has been demonstrated has to do with *precedents* or *examples* and not with the actual mechanics of the phenomenon of microbial persistence per se. Protoplast induction has been demonstrated only for penicillin (of the commonly used antimicrobial drugs) and only for two microbial species, neither of which produces what is conventionally regarded as a penicillin-susceptible infection in man. At the moment, therefore, protoplast formation might well be regarded more as an explanation of why penicillin fails to be effective in certain Gram-negative

bacillary infections rather than why it fails to eradicate the Gram-positive microbes from animal tissues. Nevertheless, these recent studies clearly show the existence of a drug-induced microbial morphology which is correlated with the physiological state of "drug-indifference" and both the morphological expression and the physiological state are reversible by appropriate manipulation of the environment of the microbes.

In the "persisting" states which have just been conjectured, the microbes are not only physiologically unreceptive to the drug, but have been morphologically altered as well. There is some reason to suspect that this concept represents too crude an oversimplification of what actually happens, or at least that it represents only one type of a phenomenon which may occur in several forms. The principal reason for this suspicion is the fact that, in certain circumstances, microbes which are genetically resistant to a particular drug may nevertheless "persist" in animals treated with that drug without showing any tendency to increase in numbers. If microbial persistence were entirely dependent on a morphological change in the microbe, for example, protoplast formation with the loss of the cell wall, it is difficult to visualize how this could occur if the individual microbial cell were genetically resistant to the drug in question.

When a strain of tubercle bacilli or staphylococci that will grow freely in the presence of a particular drug *in vitro* is injected into a mouse and the corresponding drug is administered, the microbial population in the mouse will increase in essentially the same way as in untreated animals. The microbes cultured from the tissues at any time during the course of therapy, will show the same drug-resistance *in vitro* (considering the strain as a whole) as was the case with the pre-inoculation strain. In contrast, as we all know, when the infecting strain is drug-susceptible and the drug is administered, the initially high census of culturable microbial cells falls to a greater or lesser degree and persists apparently unchanged thereafter. The persisting cells (or rather their descendants) are almost invariably drug-susceptible when subsequently tested *in vitro;* when drug-resistant variants emerge, the microbial population usually shows a rapid upsurge despite the chemotherapy.

The point for consideration, however, is the fact that this upsurge of a small drug-resistant population does not invariably occur. Sometimes, with both tubercle bacilli and staphylococci, these drug-resistant microbes just persist, showing no tendency to increase in census. Whether the continued administration of the drug is a factor in this situation, despite the drug-resistance of the persisting strain, cannot be stated from our own studies because thus far we have not made observations on such populations after the cessation of therapy.

At the semantic level, if not otherwise, it is difficult to visualize exactly how genetically drug-resistant cells would have the opportunity to assume physiological states of "drug-indifference." This suggests, therefore, that there are aspects and implications of the physiological states of drug-indifference which have nothing to do with the drugs. This is to say that the microbe is capable of assuming states in which it is sufficiently in balance with its host environment so that it neither proliferates nor is destroyed and can persist in such states for long periods. Obviously, this is but another way of saying something we all know, namely, that microbial infections (including the drug-resistant cells therein) can become dormant or truly latent in an animal host by natural processes and not only as a consequence of antimicrobial therapy. In short, it is probable that the mechanisms of microbial persistence despite drug therapy are very much like the mechanisms which permit infections to become dormant or latent in the absence of therapy.

It is not the purpose of the present discussion to consider the host mechanisms possibly operative in maintaining an infection in the dormant or truly latent state.* Obviously, both the known and probably certain as yet unknown host reactions play a most important role in this process. The point for emphasis in the present discussion is that perhaps we have tended to be too one-sided in our appraisal of this situation and, in quite properly focusing attention on the host, we have neglected the possible adaptive plasticity of the parasite. I am thus suggesting that there has been a tendency to regard the parasite in a dormant or latent infection as being very much like the same parasite when it is actively producing disease, and hence to regard the difference between the latent and active stages of a particular infection as depending almost entirely on the momentary status of the bodily defenses. It is not so much that this viewpoint might be untrue as that it is almost certainly only one part of a larger picture.

To be sure, in many instances, a change in the tissue environment provided by the host for the microbe will result in an evocation of an infection previously dormant. The observations of the Danish medical students imprisoned in the German concentration camps during the latter days of World War II represent a case in point.[15] The diseases that appeared were all produced by microbial species almost certainly a part of the personal flora of those afflicted. The extremes of the deprivation of the host were

* In this discussion, the word *dormant* is used for infections persisting at a low but detectable level. The word *latent* is reserved for situations in which the presence of the infection cannot be demonstrated by any of the available methods and the fact that it *is* present can only be detected in retrospect by the appearance of relapse.

such that the tissue environment of the microbes was almost certainly altered substantially. Hence, there is no particular reason to assume that any more marked changes occurred in the microbe than regularly occur in the change from a "resting cell" to one in the stage of logarithmic growth.

But there are instances, in which the situation is less clear, for example, Brill-Zinsser disease or latent syphilis. Within a relatively short period (probably two years) after a syphilitic infection is first established, it becomes latent in the majority of those infected. By this is meant that the presence of the infection can be detected by serological tests, but there is no way of demonstrating *where* the infection is persisting in the living patient nor indeed on post-mortem examination of those dead from other causes. Once this state of latency is established, it is highly unlikely that spirochetemia ever occurs thereafter. It should be noted, moreover, that the major late complications of syphilis, cardiovascular or neurosyphilis, almost certainly do not represent evocations of latent syphilis with invasion of new areas, but rather the emergence to clinical recognition of disease processes which have been slowly progressive ever since the initial infection. Indeed, the only evidence that the syphilitic infection is still present in the stage of latency are the facts that the tissues react to re-inoculation in a markedly altered fashion and in a small number of those infected cutaneous or skeletal gummata eventually develop.

It is of considerable interest, in this connection, that despite the many decades of observation of persons infected with syphilis and subjected to severe privations, such as occurred in a concentration camp, no one has ever reported anything remotely resembling the evocation of latent syphilis. When the latent infection is evoked, as it is in the formation of exterior gummas, the lesions almost invariably occur at a site of continued trauma. Since the stage of spirochetemia in such cases was presumably over two or three decades previously, it is reasonable to assume that *T. pallidum* must have been implanted many years before in the area of skin or bone which gives rise to the gumma. Because of the peculiar predeliction of such lesions for the sites of continued trauma, however, one must either attribute an unusual prescience to *T. pallidum,* or assume that the initial invasion of these tissues by the spirochete, and its continued presence thereafter, is something which occurs on quite a widespread basis. If this is so, it is all the more remarkable that no amount of deprivation of the host as a whole appears to do what is apparently done by the continued trauma to a part.

These observations suggest to me that *T. pallidum* is widely distributed throughout the tissues initially and exists thereafter in a form quite different from the one we have learned to recognize. The fact that *T. pallidum* can be demonstrated to be present in the aortic and neural lesions, but not

usually in gummas, is in keeping with this view. With full recognition of the highly speculative nature of these "reflections on syphilis," it does seem as if evocation from the latent state by a particular microbial species might require some rather considerable adaptive changes on the part of the parasite and not just merely a breakdown of some control mechanisms of the host. Indeed, it is not too inconceivable to suggest that the difference between a dormant infection such as a tuberculous pulmonary lesion and a truly latent one such as syphilis, might rest right at this very point, namely, that for evocation the dormant infection requires only alterations in the host, whereas the latent infection requires changes in the parasite itself. It is possibly relevant in this connection that a truly latent bacterial infection in rats and mice which can be evoked by cortisone[18] is produced by one species of the genus Corynebacterium, another member of which has been recently shown to progress through a complicated life cycle in mammalian tissue.[40]

So much for this highly speculative consideration of microbial states during latency. A question far more germane to the present discussion is whether the continued administration of an antimicrobial drug exerts any influence at all in the direction of maintaining an infection in the dormant or latent state. In other words, are microbes that are not producing disease, actually drug-susceptible? As most of the information bearing on this point also has to do with the question of whether microbial persistence just "happens" or is actively induced by drugs, these two questions will be considered together.

There is some reason to believe that when a microbial population first infects a new host, some members of the population are in (or quickly assume) a state of "drug-indifference" and can be kept in that state for so long as appropriate antimicrobial therapy is administered. The evidence on this point consists of a series of clinical observations; indeed the earlier observations are the ones which originally provoked our interest in the subject of microbial persistence. In essence, the observations are of two sorts: recognition of our customary inability to render humans predictably and uniformly free from the carrier state of otherwise drug-susceptible microbes, and recognition that microbial persistence usually occurs when infections are treated immediately after their inception.

The question of carrier states has been considered earlier in this discussion as was the observation in syphilis that if therapy were started very early, the results in all the large series of treated patients were never quite so good as when treatment had been started after clinical evidences of generalized infection had become apparent in the so-called secondary stage

of syphilis. In the pre-penicillin days, these differences in therapeutic results were never marked but they were consistent and appeared to be definite. Many an experienced syphilologist would tell one privately that *if he acquired syphilis* he would attempt to keep that fact a secret until the infection had progressed to the secondary phase with a generalized cutaneous infection, before he would permit the start of arsphenamine therapy. In short, a drug which was effective when the infection had progressed to a certain point was somewhat less effective when started prior to that point.

A similar phenomenon has been observed in the chemotherapy of four other infectious diseases: malaria, scrub typhus, Q-fever, and tuberculosis. In the first three infections, there is evidence that antimicrobial therapy exerts its maximal influence only after the infection has evolved to a certain point—with an apparently static microbial persistence occurring if treatment is started earlier. With tuberculosis, the evolution to a more satisfactory stage from the standpoint of chemotherapy has not yet been shown but the apparently static stage of microbial persistence following very early chemotherapy has been observed.

The evidence from malaria consists of the observation of Coggeshall[6] and his associates that the post-treatment relapse rate in tertian malaria was appreciably less when chemotherapy was started after the patient had had several individual paroxysms than when the same chemotherapy in the same time-dose relationships was started after the initial paroxysm of the disease. The observations in tuberculosis were made by Lelong and his associates[17] in Paris. They had the opportunity of starting isoniazid therapy in four infants born of mothers with pulmonary tuberculosis in an infectious stage. The antimicrobial therapy was started shortly after birth and the infants were immediately removed from contact with their mothers or other tuberculous persons and maintained in a protected environment. At the start of isoniazid treatment, the infants showed no evidence of tuberculous infection and their cutaneous reactions to tuberculin were negative. The isoniazid was administered daily for a 3- to 4-month period during which the infants developed normally and continued to remain nonreactors to tuberculin. Within four to six weeks of cessation of the isoniazid, however, and without any known renewed exposure to tuberculosis, all four infants developed positive cutaneous reactions to tuberculosis. It appears, therefore, that the isoniazid was able to hold this very early tuberculous infection suppressed at a level so low that cutaneous sensitivity to tuberculin failed to develop. Yet even in what might be considered a most advantageous situation from the standpoint of drug effectiveness, the isoniazid was unable to eliminate the infection.

The observations in the rickettsial infections, scrub typhus and Q-fever are the most clean-cut as they were made in the form of actual experiments with the use of human volunteers.

In Malaya, Drs. Smadel, Woodward, and their associates[33] showed that the initiation of chloramphenicol within a matter of hours after heavy exposure of volunteers to the rickettsia of scrub typhus prevented any clinical manifestations of illness so long as the chloramphenicol administrations were continued. Once the drug therapy was discontinued, however, even after so long a treatment period as 40 days, the infection would emerge to become the complete clinical illness of scrub typhus. It should be recalled that only 36 to 48 hours of chloramphenicol therapy are necessary for the successful treatment of the clinically apparent scrub typhus. Thus, in the presence of a fully established infection, 36 to 48 hours of drug therapy would produce an effect which was not producible *when 40 days of therapy with the same drug were given at an earlier stage of infection.*

This general phenomenon has been further defined in studies with Q-fever which were reported last May by Drs. Tigertt and Benenson[34] before the Association of American Physicians. These investigators were able to show that 5 days of tetracycline therapy represented perfectly satisfactory therapy for the treatment of established Q-fever. They also showed that the incubation period of experimentally induced Q-fever in man or in the guinea pig could be shortened or lengthened by appropriate manipulation of the dosage of infective particles. Thus the incubation period could be quite precisely predicted. In this standardized experimental situation, they found that 5 days of tetracycline therapy started immediately after an infection with a predicted incubation period of 12 or 14 days had no real effect on the subsequent course of events. To be sure, the incubation period might be extended for a few days beyond that predicted, but in all cases the subjects developed the complete clinical illness of Q-fever. The Tigertt-Benenson observations up to this point, therefore, were the same as the Smadel-Woodward observations on scrub typhus, and the old clinical inferences from the behavior of infectious syphilis. The additional information of considerable significance provided by the Tigertt-Benenson experiment, however, came from the fact that they found that if the 5 days of tetracycline were given late in the incubation period, immediately before the anticipated outbreak of the clinical illness, no clinical illness developed.

Thus there exist five examples of infectious diseases in humans, in which it has been possible to observe the effectiveness of antimicrobial therapy started very soon after the inception of infection. In all five examples (syphilis, malaria, scrub typhus, tuberculosis, Q-fever) the therapy is not

eradicative, but simply holds the situation "frozen," so to speak, for as long as its administration is continued. In addition, except possibly for tuberculosis, there is evidence from these various diseases that there exists a stage to which the host-parasite reaction must mature before the infection is fully drug-susceptible as measured by post-treatment relapse. The Tiggert observations with Q-fever show that this particular stage of maturity is not necessarily so old as the stage of evolution to the full clinical illness.

Aside from the possibility of eradication during therapy, the prevention of post-treatment relapse reflects the extent of the mobilization of certain host mechanisms that require both the passage of time and a sufficient "antigenic" stimulus to become fully operative, or the development of certain evolutionary changes in the parasite. When an increase in numbers of the infecting population is artificially prevented by administration of an appropriate drug, any evolutionary changes which might have occurred in the parasite and the production of a sufficient "antigenic" stimulus are both hindered. It is important to realize that in these circumstances the infecting population as a whole cannot be said to be *uninfluenced* by the drug. For, the evolution of the infection to overt disease apparently *can* be arrested for an indefinite period despite the absence of a sufficient host reaction to prevent post-treatment relapse. Thus the microbial population may be regarded as paradoxically being both "drug-influenced" and "drug-indifferent" at one and the same time.

Presumably what is happening is that a portion of the population is and remains drug-indifferent. When members of this drug-indifferent group assume a state of greater drug-susceptibility or are new-born into such a state, they are presumably immediately incapacitated by the antimicrobial drug present in the environment. The microbial population as a whole may, therefore, be said to be in a state of *"physiological imprisonment."* In this situation the individual microbes are not eradicated, neither can they evolve to the point of permitting a host-parasite reaction to develop which would be susceptible to drug treatment with appreciably less risk of post-treatment relapse.

In the clinical examples cited this situation apparently obtained from the moment of infection or soon thereafter. This suggests either that some of the infecting microbes were drug-indifferent at the time of infection or became so very shortly thereafter, and that microbial persistence may have its beginning in the very earliest stages of an infection. If the concept presented earlier should have validity, namely, that within a few hours of birth microbial cells may have matured beyond the point of penicillin-susceptibility, every penicillin-susceptible infection would presumably contain microbes already "drug-indifferent" at the moment of infection. By the same

token, such phenomena as the relative drug-indifference of microbes held intracellularly, or the induction of protoplast formation by penicillin exposure, suggest that if microbes are capable of assuming a state of drug-indifference as an adaptation to their environment, they could do so within a few moments of infection especially perhaps in the presence of chemoprophylaxis.

In actuality, there is no evidence at all on this question of whether certain cells in a new infection are drug-indifferent right from the beginning or whether they promptly become so as a response to the new environment including the antimicrobial drugs therein. As may be inferred from the foregoing discussion, I *believe* that both phenomena obtain. The point is not necessarily an important one, however, because in either case the net result would be the same and is something which may be of considerable importance in considerations of chemoprophylaxis.

The actual physical transfer of microbes to a new host is presumably not always followed by their successful colonization there. In such circumstances, the additional inhospitable factor of an antimicrobial drug in the new environment might well make the difference between the success or the failure of the implantation. The point for emphasis, however, is the strong possibility indicated by the considerations presented above, that *even if the appropriate antimicrobial drug is present in the tissue environment at the moment of implantation, it does not necessarily follow that no microbes will survive there as persisters.* Indeed, from experience in the penicillin prophylaxis of gonorrhea, when the subject was simultaneously exposed to syphilis, there is some evidence that *T. pallidum* implanted in the tissues of a host where extracellular fluid contains penicillin can nevertheless survive and eventually give rise to syphilitic infection. The acquisition of gonorrhea is, to be sure, prevented by the penicillin. But, as mentioned previously, Neisseria represent one of the two microbial groups that apparently lack the capacity to exhibit microbial persistence.

To sum up this aspect of the discussion, one must beg the question as to whether microbial persisters are actively drug-induced or arise only as an evolutionary adaptation to their environment. What can be reasonably inferred, however, from the five clinical examples cited and the experience with "healthy carriers" is: that microbial persistence can occur right from the early stages of an infection before environmental changes as a consequence of microbial-host interaction would be too advanced, and that in the examples cited it is clear that the over-all effectiveness of an antimicrobial drug is at its maximum when the infection is actually producing disease.

What are the prospects that antimicrobial drugs will be developed which will abolish microbial persistence—drugs which will be predictably eradicative instead of just being suppressive as is the case with our present drugs? Thus far, the prospects do not look hopeful. In the first place, the two microbial groups (Neisseria and B. dysenteriae) which appear to lack the capacity to persist are apparently eradicated from the tissues by drugs such as sulfanilamide (Neisseria) or the tetracyclines (B. dysenteriae) neither among the most powerful of our antimicrobial drugs. In other words, the eradicative action of these drugs in these infections seems to represent more the essential fragility of the parasites rather than any qualitatively different types of drug action. Tubercle bacilli were studied from this standpoint using the nicotinamide derivative, pyrazinamide. When pyrazinamide and any one of several antituberculous drugs are administered in appropriate time-dose relationships to tuberculous mice, the tubercle bacilli completely vanish from the tissues of the mice and the infection is rendered truly latent.[21] By this is meant that it is not possible to detect the presence of the tubercle bacilli by microscopy, by elaborate culture techniques, or by subinoculation of guinea pigs. To this extent, therefore, the therapy is eradicative. It is easily demonstrated that the infection has not really been eradicated, however, by observing the animals for a 90-day treatment-free interval. When this is done, tubercle bacilli in small numbers can be cultured from the tissues of approximately 40 per cent of the mice. Moreover, these microbes are true persisters for as a general rule, they are susceptible to the drugs involved when subsequently tested *in vitro*.

In short, microbial persistence may occur at different levels of detectability, yet the phenomenon still endures. Moreover, thus far in the microbial species studied experimentally the phenomenon has remained invulnerable despite concerted attacks with all of the available antimicrobial drugs used singly or in various multiple-drug regimens.

SUMMARY

From certain inferences derived from clinical phenomena and experimental studies in animals, the phenomenon of microbial persistence can be demonstrated to occur broadly throughout the microbial world. The phenomenon occurs in the absence of antimicrobial therapy, but it is its occurrence despite antimicrobial therapy that makes understanding of the phenomenon of such importance in considering treatment failures, post-treatment relapse, and the chemoprophylaxis of either infection or its subsequent individual manifestations as disease.

As to how the phenomenon of microbial persistence occurs, there is little if any truly direct evidence. From indirect evidence interpreted specu-

latively, it appears that microbial persistence is environmentally induced and is one reflection of a high degree of adaptive plasticity of individual microbes. It is believed that the range of such types of microbial adaptation *in vivo* is substantial and that it is this capacity of individual microbes to express individuality which determines in large measure the success or the failure of antimicrobial therapy. An important role of the host defenses in this process would be to participate in providing "proper" environments for the adaptive capabilities of the microbe. As the environments represent the interaction of the host and the parasite, neither can be dismissed from consideration. It is suggested, however, that perhaps we have been taking too one-sided a viewpoint in our preoccupation with host mechanisms and should focus equal attention on this probable wide range of reversible changes on the part of the microbe.

As host reactions are *ad hoc* in nature and not necessarily beneficial to the host as a total organism, the particular environment provided by a host reaction may or may not lead to a microbial adaptation which favors maximal drug-susceptibility. Thus the environment of an actively progressing infection may be ideal for the maximal drug-susceptibility of the microbe, whereas necrotic areas or the interior of phagocytes in the same host may favor microbial adaptation to assume a state of "drug-indifference." Viewed in this way, the influence of the inflammatory environment is probably not exerted by impeding, neutralizing, or enhancing the activity of a drug, but rather by providing an environment favorable or unfavorable for the assumption of certain states necessary for drug-susceptibility of the parasite.

In other words, it is suggested that the nature of the lesion does not directly influence antimicrobial drug activity. Instead, it is the adaptive response of the microbe to a particular lesion or a particular host environment which determines the direction an individual drug-microbe collision will take. And, it is one manifestation of this adaptive plasticity of the microbe—the ability to "play dead" so to speak—that determines microbial persistence and thus provides a means for the survival of microbial species despite the development of powerful antimicrobial therapies.

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