THE APPLICATION OF METABOLIC STUDIES TO LEPROSY RESEARCH [1]

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The failure of both human and murine leprosy bacilli to propagate in ordinary bacteriological media or in association with living tissue cells *in vitro* imposes severe limitations on studies of their physiological requirements. Fortunately, however, there are methods by which the basal (i.e., endogenous) metabolism of such microorganisms can be studied. Since these methods permit measurement of both losses and gains in activity, it is not necessary that the bacterial suspensions grow or synthesize. There is the further advantage that the effect of single substances or conditions can be tested, in the absence of the necessary complexity of biological systems.

A continuous transfer of energy is essential to life, and all biological exchange of energy is dependent on two things, namely, the burning of substrate hydrogen as a fuel, and the generation of high-energy phosphate bonds. It follows, therefore, that appropriate measurement of the amount of hydrogen transfer or of the amount of energy-rich phosphate present will provide fundamental information concerning the biological state and the biochemical activity of any type of living cell. Since methods for determining hydrogen transfer are more highly developed than those for measuring high-energy phosphate, and they are more readily utilized for a variety of biochemical analyses, the former have been used in the present work.[2]

Mycobacteria and other strict aerobes derive energy by a final transfer of metabolized hydrogen through the respiratory (cytochrome, heme-containing) system and by burning it with oxygen to form water. The rate of oxygen consumption indicates therefore, the rate at which hydrogen is being removed from the substrate and burned. The most versatile and widely used instrument for such determination is the Warburg respirometer. A sensitive modification of this apparatus has been used by one of us to study factors which influence the respiration of a broad spectrum of cultivable mycobacteria (7), and of murine leprosy bacilli (8).

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[1] In an abbreviated form this paper was read at the VI International Congress of Leprology, Madrid, October 1953.

[2] Existing metabolism procedures were developed solely for the determination of enzyme reaction rates, and are not designed to provide data of direct biological significance. Previous work (14) and the present summary show that, in order to replace biological data, metabolism determinations must be made under conditions similar to those used in biological experimentation.

An important purpose of this present study has been to analyze the rate of utilization of the carbon and nitrogen compounds known to be essential for the production of energy and for synthesis. Since the Warburg apparatus permits the simultaneous analysis of only a small number of variables, and since each determination requires enormous numbers of microorganisms, an additional method of measuring hydrogen transfer has been developed (13).

If oxygen is excluded from the environment, the oxidation of hydrogen is accomplished by transfer to (and reduction of) suitable artificial acceptors.[3] Since the diversion of hydrogen occurs just before it enters the respiratory (cytochrome) system, such methods measure the earlier steps in the transfer of hydrogen even when the cytochrome system has been destroyed.

Colorless hydrogen acceptors have great advantages over dyes formerly used as acceptors, particularly because a suitable excess may be added and either rates of reduction or total hydrogen transfer capacity (hereinafter usually to be referred to as HTC) can be measured. Colorless tetrazolium compounds are reduced to brilliantly colored, water-insoluble pigments (formazans) which are relatively stable in the presence of oxygen. When extracted in suitable solvents the clear solutions are ideal for spectrophotometric determinations.[4]

As a result of this study, it has become evident that the intracellular trend among the pathogenic mycobacteria is to be explained by increasing limitations in oxidative capacity. These deficiencies are expressed in part by an increasing sensitivity to inhibition in extracellular environment. Utilizing *M. leprae murium* as a major point of reference, it is now pro-

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[3] The term "acceptor" signifies a substance which "accepts" hydrogen in such systems. Artificial acceptors are exemplified by substances to be mentioned shortly. The process is probably carried out by means of the flavoprotein "diaphorase."

[4] The dye-reduction studies of Prudhomme (23) and of Marchoux and Prudhomme (20) represent earlier determinations of the influence of several factors on the *in vitro* metabolic activity of murine and human leprosy bacilli. Our experience with murine type bacilli leads us to believe that their observations and conclusions were valid.

Our preference for tetrazolium compounds rests upon the following considerations: (*a*) Reduced dyes are auto-oxidizable, and the degree of reduction must be measured in individual anaerobic tubes in the presence of dense bacterial suspensions; (*b*) an unknown portion of the dye is adsorbed, even by dead microorganisms, and observation is terminated when 50-90% of the dye appears to have been reduced; (*c*) since the limiting factor is depletion of the dye system rather than of the donor system, the situation in dye reduction is comparable to conducting respiration studies in the presence of a rapidly depleted supply of oxygen.

Furthermore, in the case of pathogenic mycobacteria data on *rates* of dye reduction are not simple to interpret. Bloch has emphasized the failure of young cultures of highly virulent tubercle bacilli to reduce methylene blue in his standard test, whereas older cultures, which possess lower viability and infectiousness, reduce methylene blue. Less virulent strains and saprophytes reduce it at greater rates (1). Thus, the results tend to be inversely related to virulence or infectiousness, and they may at times be inversely related to viability.

posed to summarize certain observations made by means of these metabolic methods and to discuss their implications with regard to the microbiology of leprosy. Details concerning experimental design and supporting data are being prepared for later publication.

METHODS

Because of the availability of a susceptible animal in which the interpretation of metabolic data can be checked, *M. leprae murium* was chosen for study rather than *M. leprae*. Comparative studies have also been made with certain cultivable mycobacteria: *M. phlei* (HMS, ATCC 354 and 355), *M. smegmatis* (ATCC 101), *M. ranae* (ATCC 110), *M. avium* (Kirschberg) and *M. enteritidis* (Johne's bacillus, Hagan).

Methods of obtaining washed, standardized suspensions of the murine leprosy bacillus [5] from testicular lesions and for measuring their respiration and hydrogen transfer capacity have been described ([8], [13]). The Warburg apparatus used in these experiments is two to three times more sensitive than standard equipment, and requires 0.6 ml of suspension per vessel; 0.1 ml suffices for HTC tests in duplicate.[6]

OBSERVATIONS AND IMPLICATIONS

A. BIOLOGICAL SIGNIFICANCE OF METABOLIC OBSERVATIONS

Viability.—The relationship between the HTC and viability (plate counts) of *M. phlei* has been investigated under a variety of circumstances (14). It was concluded that the HTC of washed suspensions of a standard age provides an estimate of the number of living cells. This relationship is also valid in damaged suspensions, provided the bacilli are re-washed prior to testing HTC. The HTC of suspensions incubated without substrate, however, declines more rapidly than viability, and provides estimates of the relative rather than the true viability. The HTC of suspensions of *M. leprae murium* was shown to be influenced similarly under each of the conditions studied.

Infectiousness.—In the case of noncultivated mycobacteria the eventual usefulness of metabolic data is dependent upon correlations with infectiousness. This relationship, therefore, has been subjected to critical investigation by the use of *M. leprae murium*. Conditions included: incubation in the presence and absence of oxygen, serum, albumin and yeast supplement, and also refrigeration in glycerol, sucrose, phosphates, albumin and yeast supplement. Experimentation with refrigerated bacilli is

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[5] Microscopic spreads made directly from densely packed buttons, after intense counterstaining, show only traces of stainable tissue components, and are rated as 98-99% purified bacilli. The suspensions are devoid of succinic oxidase, the most stable oxidative system of tissue origin ([8]).

[6] No method is inherently more sensitive than measurement of gas exchange. Smaller aliquots can be used for measurement of HTC because the determinations are made after longer periods of incubation. Due to the ease with which 30 or 40 variables may be studied simultaneously, this method has been useful for a broad survey of factors which influence the endogenous metabolism of the bacilli. Respiration is our method of choice for measuring rates and for determining the status of the cytochrome system.

particularly interesting, since the biochemical content of the bacilli is modified very slowly,[7] while significant differences in metabolic activity and infectiousness are induced readily. Both HTC and infectiousness, for example, were enhanced by exposure of bacilli to albumin and yeast supplement at 5°C for periods thought to be too brief to permit alteration in the lipid content of the bacilli. When, on the other hand, metabolism is depressed by anaerobiosis or by serum inhibition there is a loss of infectiousness. Other evidence of this relationship will be cited repeatedly. The reasons for the essential dependence of infectiousness on metabolic integrity will be discussed in a later publication.

B. BIOCHEMICAL AND BIOLOGICAL CHARACTERISTICS OF *M. LEPRAE MURIUM*

Position in the mycobacterial spectrum.—A comparative study of oxidative metabolism among the mycobacteria by classical procedures reveals that as one passes from the saprophytes toward the more parasitic species, a step-wise limitation of oxidative activity predicts a complete failure of the more parasitic mycobacteria to exhibit enhanced respiration in the presence of substrates *in vitro*. Thus, the respiration of *M. phlei* is enhanced by succinate, acetate and long- and intermediate-chain fatty acids; *M. smegmatis* and *M. ranae* fail on succinate; and the tubercle bacilli on both succinate and acetate. Johne's bacillus, the most fastidious of the cultivable species, possesses the ability to enhance oxidation rates only in the presence of octanoic acid.[8] *M. leprae murium* fulfills the prediction by failing to oxidize any of some 50 single substrates and complexes utilized by bacteria, plants and animals. Even in the presence of the protective and stimulating influences of serum albumin and yeast extract (see below), and with observation extended to 50 hours, combinations of these individual substrates fail to enhance respiration (8).

A biological modification of the classical metabolic procedures is provided if the bacilli are incubated in the presence of presumably utilizable

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[7] Bloch has demonstrated an interesting relationship between the infectiousness of tubercle bacilli and the amount of lipid known as "cord factor" (2, 3). These lipids are among those burned or lost during incubation of mycobacterial suspensions. We wished, therefore, to inquire whether the rapid decline of infectiousness and metabolism of *M. leprae murium* should be considered primarily in terms of lipids burned, if it is more directly referable to declining metabolic activity. It will be seen from data to be presented that conditions permitting the most rapid and continuous oxidations (burning of lipids) serve best to preserve infectiousness. Anaerobiosis, which most effectively prevents the burning of lipids, destroys infectiousness. Although components similar to "cord factor" may play a role in the pathogenesis of murine leprosy, we have concluded that the types of information needed should be obtained by metabolic measurements and not in terms of chemical content.

[8] Dr. Marcus S. Brooke participated in the experiments on Johne's bacillus.

sources of nitrogen and carbon for longer periods, e.g., 3 to 7 days.[9] During such intervals, serum dialysates, synthetic solutions useful for cell maintenance, and other materials permitting growth of certain other fastidious microorganisms, fail to modify the rapid decline in infectiousness (15) and in HTC (16) of *M. leprae murium*. For these reasons, all data available are measures of the endogenous metabolism.

Endogenous (basal) metabolism.—Different lots of freshly washed suspensions of *M. leprae murium* exhibit widely different abilities to conduct respiration and hydrogen transfer. Those showing high activity are not stimulated by purified serum albumin, while those of low activity show a remarkable enhancement of metabolic response (8, 16). These observations suggest that the bacilli in different preparations may possess a fairly uniform activity, but may be inhibited in varying degrees in the animal lesions or during their recovery by washing. The probable cause of this inhibition will be suggested by data on serum toxicity.

M. leprae murium, like *M. tuberculosis*, exhibits very slow rates of hydrogen transfer. This process, however, is much more persistent than that of the saprophytes (14), and reveals a considerable *capacity*. The endogenous metabolism of *M. leprae murium* declines more precipitously than that of other pathogens, and (as noted) is attended by rapid loss of infectiousness. This phenomenon may be due in part to the inhibitions just discussed.

Optimal conditions for sustaining the endogenous metabolism during aerobic incubation resemble those for cultivable mycobacteria and are provided by small amounts of CO_2 in air (16) and by adequately buffered solutions at pH 7.5 (8, 16). The change in respiratory quotient from 0.9 to 0.7 during the first few hours of incubation is characteristic of all mycobacteria (7), and indicates a shift from the oxidation of amino acids and carbohydrates to the oxidation of lipids. Respiration is resistant to inhibition by azide and fluoride. Like that of other pathogenic species, it is sensitive to anaerobic damage. This organism, in short, displays a typical mycobacterial endogenous metabolism. It is for this reason that cultivable species are legitimate controls for certain types of experimental analyses.

Beneficial effects of serum-albumin and yeast extracts.—The favorable influence of purified bovine albumin on *M. leprae murium* was first observed in animal experiments (15), readily substantiated by HTC data (16) and studied quantitatively by the respiration method (8). Failure of this substance to stimulate the respiration of the most active suspen-

[9] The failure of cultivable mycobacteria to exhibit significant increase in oxidation rates within the usual period of 4 to 12 hours may be overcome by this means. Suspensions shaken for several days in the presence of compounds providing utilizable sources of nitrogen and carbon are readily revealed to have enhanced the original capacity for respiration and hydrogen transfer. As noted, such procedures do not support the metabolism of *M. leprae murium*.

sions, and its dramatic effects on the metabolism of those showing poor rates, have been mentioned. Since we can obtain no chemical evidence that the albumin molecules (or accompanying traces of fatty acids) are modified or utilized by the bacilli, we must assign to serum albumin a role as protector against inhibitory materials, which is its only known role in mycobacterial metabolism (4). Further consideration of its action will appear in connection with the inhibitory properties of serum.

Yeast and liver extracts also enhance the endogenous metabolism of *M. leprae murium*. A product designated "yeast supplement," [10] at a final concentration of 16 per cent, enhances immediately the respiration of all suspensions by approximately 50 per cent, irrespective of original metabolic activity and of the presence of albumin (8). After incubation for 72 hours in 4 per cent solutions, the HTC is usually three times that of control suspensions. Because of the uniformity of stimulation in suspensions of differing original activities, the benefits of these materials are not due primarily to protective effects. It has not been possible to show that there is a true substrate response. These materials may relieve a deficit which ordinarily limits the endogenous activity, and/or replace small molecular components which have been removed by washing of the bacilli.

The active components of yeast supplement are less effective than serum albumin in neutralizing the toxic effects of serum or body fluids. Their value in supporting metabolism and infectiousness under several circumstances will become evident as further details are published.

C. PHYSIOLOGICAL FACTORS WHICH DAMAGE THE INTEGRITY OF ENDOGENOUS METABOLISM *IN VIVO*

The susceptibility of *M. leprae murium* to anaerobic damage is a liability shared by all pathogenic mycobacteria which have been studied. The restricted oxidative capacity of this more intracellular species, moreover, is expressed in part by extreme susceptibility to inhibition by components of serum and body fluids. This susceptibility, therefore, is an additional reason for the dependence of *M. leprae murium* upon the intracellular location. It will be seen that in certain circumstances its chances for metabolism and proliferation are indeed limited.

Anaerobic damage to respiration.—Although strict anaerobiosis does not interfere with the viability and respiratory capacity of *M. phlei* and other saprophytes (8, 18), it damages severely the respiration (8) and infectiousness of *M. leprae murium* and of the tubercle bacilli (18). Since

[10] Bacto (yeast) Supplement B (an autolysate) from Difco Laboratories, Detroit, Michigan.

the severe impairment of respiration and infectiousness is associated with enhancement of the HTC,[11] the anaerobic damage is confined to some sensitive link in the cytochrome system and does not involve earlier steps in the oxidation of hydrogen. It is also evident that the infectiousness of *M. leprae murium* is strictly dependent upon its own respiratory activity and that it does not rely on tissue cells for this purpose. Whether anaerobic damage may be an important factor in the killing of mycobacterial pathogens by immune cells is a subject which remains unexplored.

Inhibition by serum and body fluids.—Laboratory-adapted strains of tubercle bacilli exhibit enhanced respiration and growth in the presence of blood serum of diverse origins. Wild strains—i.e., primary isolates—are inhibited by serum from other than naturally susceptible hosts, but show a distinct stimulation of primary outgrowth in the presence of serum from the natural host species (17).

In the case of *M. leprae murium,* both infectiousness (15) and HTC (16) are sharply inhibited by serums from all animal species tested, including that from the natural host. Suspensions possessing the highest degrees of metabolic activity are more severely damaged than those of lower activity.

In the presence of serum in 20 per cent concentration, respiration declines measurably in less than two hours and becomes negligible in six hours. Suspensions exhibiting fairly high activity, when incubated in 40 per cent serum for 24 hours, retain but 9 per cent of the HTC of control suspensions in balanced salt solution and only 3 per cent of the original HTC. Comparable damage to the endogenous metabolism of cultivable mycobacteria is attended by a serious decline in viability.

The crude lipids of serum, and the major proteins (the albumin and the globulins), are much more favorable to HTC than are inorganic control solutions. The toxicity of native serum is referable to two classes of protein complexes—the lipoproteins and the mucoproteins. Other body fluids are less inhibitory, in accordance with their decreased lipo- and mucoprotein content. Since lipo- and mucoproteins also inhibit the stability and infectiousness of certain viruses, some of these data will be presented elsewhere.

Serum inhibitors as barriers to experimental transmission.—Since impairment of metabolism by serum is greater than that exerted by other body fluids and is of rapid onset, one might anticipate that any circumstance which exposes the bacilli to serous exudates would measurably reduce their infectiousness. In human leprosy such conditions may occur during lepra reaction, and during certain types of chemotherapy (see below). In transmission by inoculation, the bacilli lie in serous exudates

[11] This discordance between results by the two metabolic methods as employed will occur in every circumstance where terminal respiration is damaged. A subsequent paper will provide an explanation, and show that a simple modification of the HTC method permits measurement of damage to respiration (16).

along the needle track for some hours prior to their complete ingestion by cells. Since this circumstance has been shown to interfere with experimental transmission of murine leprosy, it may be added to other factors previously analyzed in connection with the problem of experimental transmission of leprosy (12).

Serum inhibition of human and murine bacilli in cell cultures.—In spite of the ease with which other pathogenic agents of intracellular habit usually propagate within cultured cells, all attempts to cultivate human leprosy bacilli within human cells maintained in serum-containing nutrients have failed (11). Murine-type bacilli were later employed, because one may use bacilli recovered from cell culture experiments for inoculations, to explore the causes of failure. In the optimal cell systems, after one month in 30-40 per cent serum, from 80 per cent to 130 per cent of the original numbers of bacilli were recovered; these were highly infectious for moderately resistant rats. Because of the destructive action of cells on mycobacteria, recovery of approximately original numbers was dependent on some slight degree of intracellular multiplication. In systems managed in identical manner but lacking cells, the bacilli became noninfectious in less than five days. When it became evident that the favorable nature of the intracellular system was outweighed by toxicity of the extracellular fluids, cell culture work was discontinued until some means might be found to analyze the inhibitory properties of these fluids. As a result of metabolic study, it has now been shown that the inhibition was due to the serum, and not to the inorganic or small molecular components of the media.

These conclusions are supported by other evidence that the extracellular inhibitors [12] can prevent the propagation of a readily cultivable intracellular parasite. Illuminating experiments by the Duran-Reynals (5) show that the multiplication of the vaccinia virus in cell cultures is inhibited by the addition of hyaluronic acid from ground substance. Observations on the infectiousness of intracellular virus and the inactivity of virus in supernatant fluids were similar to those just described for murine leprosy bacilli.

D. MECHANISM OF ACTION OF CHEMOTHERAPEUTIC AGENTS

Although knowledge of the mode of action of chemotherapeutic compounds is important chiefly because of practical considerations, such information should also throw light on the kinetics of mycobacterial metabolism and growth. Isoniazid, for example, exhibits a remarkable specificity for mycobacteria; the processes with which it interferes are patently more fundamental to mycobacteria than to other microorganisms.

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[12] This term is used at times to avoid repetition of the longer explanatory phrase, "inhibitions in serum and body fluids, and also to call attention to the fact that the infectiousness of intracellular agents may be impaired by other natural body components." "Serum inhibitors" signifies those present in the serum.

Since pathogenic species of mycobacteria exhibit low oxidation rates, while saprophytes or isolates from cold-blooded animals permit observation of response to substrate, and of degrees of inhibition, the latter have been used to study mechanisms of drug action.

In concentrations required for chemotherapeutic effect in mycobacterial infections, the compounds studied thus far in this laboratory do not impair the endogenous metabolism of the mycobacterial suspensions used. They, therefore, appear to be incapable of diminishing the infectiousness of bacilli which are existing *in vivo* on stored intracellular materials. Since the drugs which interfere with metabolism, such as streptomycin and isoniazid, are active only when the organisms are utilizing energy from exogenous sources, their action is quite different from the destruction of endogenous metabolism by anaerobiosis and by extracellular inhibitors.

Sites of metabolic inhibition by streptomycin and isoniazid.—Streptomycin restricts in particular the enhancement of oxidation which is normally produced in *M. phlei* by malic acid, and also more slowly that produced by long-chain fatty acids (10). Enhanced oxidation of long-chain fatty acids by tubercle bacilli is also inhibited (22). Since there is no interference with the transport of hydrogen to artificial acceptors, action is confined to the terminal respiratory system (10). As a result of these restrictions, the normal energy production and the assimilation of carbon from these important sources is inhibited.

The action of isoniazid is expressed by interference with enhanced substrate oxidation, especially acetate and succinate.[13] This action depends primarily on combination with iron porphyrins (heme compounds), some of which are essential to respiration. Dosage requirements in different mycobacterial species are modified by their respiratory activity. The greater sensitivity of pathogenic species is in some way associated with the smaller quantities of heme catalysts which they contain (9). The property most important to students of leprosy is the fact that, within a given species, dosage requirements are directly proportional to bacterial numbers.

As a result of the findings with respect to metabolic patterns and the action of isoniazid, the following generalization appears justified: Limited quantities of components essential to respiration are found in all the mycobacteria. There appear to be no useful alternate pathways. It is the severe restriction of respiratory systems in the pathogens which accounts for their poorer capacity to oxidize acetate and succinate, their greater tendency to reduce acetate to lipids, and their greater sensitivity to isoniazid (9). These limitations in oxidative capacity explain also the difficulties which they encounter in serum or body fluids.

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[13] The first of these compounds is important in tissues and mycobacteria for lipid synthesis, and the second facilitates the acquisition of energy and readily assimilated carbon.

Action of antimetabolism drugs in vivo.—Many important considerations bearing on the action of chemotherapeutic agents in intracellular mycobacterial disease were reviewed recently by Suter (25). Since the two types of leprosy bacilli are thought capable of achieving an enhancement of metabolism only within the intracellular environment, we also feel that drugs or antibiotics possessing a high ratio of intracellular effectiveness are of prime interest in the chemotherapy of leprosy. At the same time, however, we have slowly come to realize that there may be two limitations to the decisive action of such compounds in human leprosy.

The first of these is probably widely recognized. The fact that there are on the order of five billion bacilli per gram of lepromatous tissue affords a strong possibility that drug resistant bacilli may exist in the tissues even before the initiation of treatment. It is also known that continuous presence of drugs such as streptomycin and isoniazid induces in the cultivable and transmissible species a fairly rapid emergence of resistant forms. For these reasons, it seems to us that continuous treatment with such compounds may, in the long run, prove to be less desirable than intermittent therapy.

A second limitation lies in the fact that drugs exerting antimetabolism effects produce dramatic inhibitions only in those species or in those physiological states where the rate of substrate utilization is fairly high. When mycobacteria are existing on endogenous (basal) metabolism, there is no action; when they make but slight use of exogenous substrate, the action is not appreciable.

A brief consideration of the isoniazid problem suffices to illustrate certain possible impediments to continuous or effective action of this type of compound in human leprosy. The great numbers of bacilli afford the possibility of rapid emergence of drug resistance, with the danger that early beneficial results may be obscured. Because of globi and the dense masses of bacilli, very high local concentrations of drug may be required. Furthermore, the slow growth and accumulation of *M. leprae,* even in the most susceptible persons, suggests that its rate of energy turnover may not permit demonstration of any dramatic effect by present methods of assessing drug action.

Action of natural inhibitory factors in vivo.—Insofar as may be judged from study of tubercle bacilli and *M. leprae murium,* the unfavorable action of anaerobiosis and of natural inhibitors occurs at a much more fundamental level. These natural factors disrupt the endogenous metabolism on which persistence of viability and infectiousness depends.

Anaerobic damage kills the cultivable species and decreases the infectiousness of *M. leprae murium.* We may be assured, therefore, that if appropriate exposure to this circumstance occurs *in vivo,* it is of serious consequence to a mycobacterium. The influence of intracellular anaerobiosis in infected tissues, however, is difficult to assess for several reasons: (*a*) The intracellular trend in the mycobacteria lowers oxygen demand,

but may also permit continued existence at very low oxygen potentials; (*b*) a major reduction in respiration or infectiousness of *M. leprae murium* in our experiments has required anaerobiosis for 48 or 72 hours; (*c*) we do not know whether cell metabolism ever restricts oxygen supply below critical levels in lesions endowed with the usual capillary supply. On the other hand, high local concentrations of both cells and bacilli characterize lepromatous lesions, while in tuberculoid lesions there is the added feature of cell clusters. At the moment it can only be said that the possibility of creating an oxygen deficit in certain stages of disease, or of increasing such deficits as may exist, should not be overlooked in further study of pathogenesis and chemotherapy.

We feel much more confident that a significant role is played in leprosy by natural extracellular inhibitors. A review of virus literature indicates that difficulties in experimental transmission of intracellular disease are usually due to such inhibitors. Wild strains of tubercle bacilli (primary isolates) are inhibited by serums from other than the natural host species, and *M. leprae murium* is inhibited by serums from all species tested. Since lowered oxidative capacity and susceptibility to such inhibition are associated trends in the mycobacteria, the slow growth rate of *M. leprae* and the failures of experimental transmission suggest that this organism is even more sensitive than *M. leprae murium.* Since it is not known whether the presumed sensitivity of *M. leprae* to extracellular inhibitors is sufficient to impede its proliferation in cells bathed in body fluids as dilute as lymph, it is not possible to assess the role which might be played by such factors during insidious or quiescent phases of the disease. Further consideration, therefore, is confined to circumstances which appear likely to cause increased exposure to extracellular environment.

Suter (25) has suggested that in the tuberculoid cells of the more resistant patients there may be alterations in cell permeability which cause more exposure of the bacilli to extracellular factors than in lepromatous leprosy. Increased exposure may also occur during the reactive episodes of lepromatous leprosy, and during treatment with sulfone drugs. The observations by Muir (21) and particularly those by Rath de Souza and de Souza Lima (24) led those writers to conclude that the chemotherapeutic action of the sulfones is exerted by means of some normal mechanism which operates also in untreated patients. There exists, therefore, the intriguing possibility that the inhibition induced by sulfone administration is due in part to natural antibiotics in the leprosy patient.

Although the natural physiological factors interfere with mycobacterial metabolism at a more fundamental level than the antimetabolism drugs, their action in turn appears to be limited by the same law of diminishing returns. For example, if the sulfones mediate changes primarily in heavily parasitized cells, their action will become less effective as the number of bacilli per cell decline. Furthermore, when mycobac-

terial metabolism is depressed to very low levels, the action of extracellular inhibitors also becomes less significant. It has been pointed out that the most dramatic effects on *M. leprae murium* occur in suspensions exhibiting high metabolic activity, while lesser effects are exerted in suspensions of low activity. In consequence, we have found no conditions of exposure which obliterate metabolic activity.

GENERAL DISCUSSION AND CONCLUSIONS

The application of metabolic methods to the study of *M. leprae murium* has revealed its essential relationship in the orderly arrangement of the cultivated members of the mycobacterial family. This species possesses endogenous metabolic processes typical of those in cultivable pathogens. It differs principally in its inability to survive in extracellular fluids or to benefit from substrate *in vitro*. Since *M. leprae* (i.e., the human pathogen) propagates more slowly in tissues, and human leprosy is not experimentally transmissible, this organism is believed to be metabolically even less active and more severely inhibited than *M. leprae murium*.

Until the problem of the inhibited state of such bacilli has been solved, and utilization of exogenous substrate *in vitro* has been demonstrated, it would appear that direct bacteriological attempts to cultivate human and murine leprosy bacilli will continue to be unproductive.

It is also evident that *M. leprae murium* is not equipped for extracellular survival or growth within susceptible hosts. Since survival of the bacilli *in vitro* is dependent upon the cell, further biological studies on cultivation should be directed toward the maintenance of cell cultures in solutions which are not fatal to the bacilli.

Murine leprosy bacilli do multiply in animal tissues, where cells are in close contiguity and are bathed in body fluids, and they fail to grow in tissue cultures where the cells are dispersed on a plane and are exposed to higher concentration of serum inhibitors. It is also known that intracellular viruses are not neutralized by antibody if they are first acquired by cells *in vivo*, but they are neutralized in cell cultures *in vitro*. These observations warn us that extracellular inhibitors may be of little effect during quiescent phases of leprotic infections. They may, however, be of consequence under special conditions.

Mycobacteria such as *M. leprae murium* survive and grow in cells under precarious circumstances. Two general types of antibacterial action seem capable of limiting pathogenesis, but incapable of causing sterilization of infected tissues. These are: (*a*) the destruction of endogenous (basal) metabolism by anaerobiosis and by inhibitors in serum and body fluids, and (*b*) interference with the utilization of external sources of energy by means of chemotherapeutic agents. The present examination of the metabolic properties of the mycobacteria and of these two classes of antibacterial action lead us to the conclusion that the development of a successful treatment of leprosy will be dependent on intensification of

investigation in three directions: (1) regarding the properties and mode of action of new chemotherapeutic agents, (2) regarding the utilization of natural inhibitory factors, and (3) regarding the physiological mechanisms which enable cells to destroy the intracellular mycobacteria, a matter which deserves more active interest.

Perhaps the most significant result of the series of investigations here summarized has been the demonstration that useful biological information can be obtained from noncultivated mycobacteria by appropriate application of biochemical methods. The development of procedures which permit direct application of these methods to unwashed bacilli and to bacilli in tissues, should permit further and more direct contributions to our knowledge of tuberculosis and leprosy.

SUMMARY

1. Utilization of metabolic methods has permitted an analysis of the position of *M. leprae murium* in the spectrum of mycobacterial species and a discussion of the properties of *M. leprae*.
2. Like other pathogenic mycobacteria, *M. leprae murium* possesses a slow but persistent endogenous metabolism; both respiration and infectiousness are impaired by anaerobiosis.
3. It differs from cultivable pathogens in two important respects: (*a*) an inability to acquire energy *in vitro* and (*b*) an extreme susceptibility to inhibition by serum and body fluids. Since slow growth, lowered oxidative capacity, sensitivity to extracellular inhibitors and difficulty of transmission are associated trends in the mycobacteria, *M. leprae* is believed to be even more severely handicapped.
4. The inhibited state of *M. leprae murium* in tissue homogenates and the energy problem account for its inability to grow in bacteriological media. Susceptibility to serum inhibition accounts for the failure to multiply in cell cultures and is an impediment to experimental transmission. Circumstances permitting growth in tissues are more precarious than those for tubercle bacilli.
5. Aside from immunological mechanisms (unstudied), two general types of antibacterial action seem capable of limiting pathogenesis: (*a*) interference with the utilization of external sources of energy by means of certain chemotherapeutic agents which are effective within cells, and (*b*) the more drastic action of anaerobiosis and of extracellular inhibitors.
6. Consideration of the mode of action of these two general mechanisms *in vivo* indicates that when mycobacterial metabolism is depressed there are serious limitations to their effective action either separately or in combination.

RESÚMEN

La aplicación de métodos metabólicos ha permitido analizar la ubicación del *M. leprae murium* en el espectro de las especies micobacteriales y sugiere una discusión respecto a las propiedades del *M. leprae.*

Como cualquier otro micobacterio patógeno, *M. leprae murium* dispone de un metabolismo endógeno lento pero constante; tanto la respiración como la capacidad infecciosa son disminuidos por la anaerobiósis.

Difiere de los patógenos cultivables en dos hechos importantes: a) inhabilidad para adquirir energía *in vitro* y b) una extrema susceptibilidad a la inhibicion por la acción del suero y secreciones del cuerpo. Desde el momento que, un crecimento lento y una capacidad oxidativa disminuida, conjuntamente con la sensibilidad hacia los inhibidores estracelulares y dificultades en la transmisión marchan asociadamente en las especies micobacterianas, se cree que el *M. leprae* sea mas severamente afectado aún.

El estado de inhibición del *M. leprae murium* en los tejidos emulsionados y el problema relacionado con la energía influyen en su inhabilidad para crecer en un medio bacteriológico. La susceptibilidad para inhibirse en presencia de suero esplica su incapacidad para multiplicarse en cultivos celulares y es un obstáculo para la trasmision esperimental. Las circunstancias que facilitan el desarrollo en los tejidos son mas precarias que las que encontramos para el bacilo de la tuberculósis.

Además de los mecanismos immunológicos, (sin estudiar) existen 2 tipos generales de acción antibacterial que parecen capaces de limitar su patogénesis: a) el hecho de privar a los bacilos de fuentes de energía externa por intermedio de ciertos agentes químicos terapéuticos que son efectivos dentro de las células y b) la acción aun mas drástica por anaerobiósis y por los inhibidores extracelulares.

Consideraciones respecto al modo de actuar en general de estos dos mecanismos *in vivo*, indican que cuando el metabolismo micobacterial esta disminuido, existen serias limitaciones con respecto a su acción efectiva ya sea separadamente o combinada.

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