INFLUENCE OF PHYSICAL AND CHEMICAL FACTORS ON THE HYDROGEN TRANSFER CAPACITY OF MURINE LEPROSY BACILLI

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Another paper (6) summarizes certain implications for leprosy research involved in successful application of metabolic methods to washed suspensions of *M. leprae murium* and by similar studies with cultivable mycobacteria. It must be emphasized that no attempt has been made previously to utilize metabolic methods in such manner that the data obtained could substitute for cultivation or infection data. It should be obvious that premature application of these methods to *M. leprae* has been avoided because the mere compilation of data is futile unless there be also a secure foundation to inform us when metabolic activity can be interpreted in terms of the viability or infectiousness of the microorganisms, and when it cannot.

The preparation of this foundation has depended upon simultaneous study of two fundamental metabolic processes: (*a*) the enzymatic transfer of hydrogen in order to accomplish oxidation of the complex endogenous materials stored in the bacilli, and (*b*) the derivation of useful energy by finally burning this hydrogen in the presence of oxygen. Since neither infection data nor colony counts can be obtained with *M. leprae*, whereas infection data can be obtained with *M. leprae murium*, the latter has been employed during the past four years to represent the noncultivated mycobacteria, and also to measure the infectiousness of suspensions on which metabolic data were obtained. Cultivable mycobacteria have been employed for comparisons, for plate counts, for analysis of principle, or to provide insight as seemed necessary.

Measurement of hydrogen transfer capacity (HTC) permits comprehensive study of many variables with modest numbers of bacilli. This procedure has been used in a search for correlation with results obtained previously when tissue homogenates containing *M. leprae murium* were inoculated into rats after exposure to different experimental conditions *in vitro* (5). Since the HTC method permits adequate repetition of rapid and accurate determinations, our knowledge of the physiological requirements of murine leprosy bacilli has expanded rapidly. Conditions with respect to pH, oxygen, carbon dioxide, and electrolytes required for optimal conduct of endogenous metabolism coincide with those for culti-

[1] With the assistance of Tobey Backerman and Rachel Barrett.

vable mycobacteria. The investigation has also confirmed Gray's conclusion (1) that *M. leprae murium* is unable *in vitro* to derive energy from substrates which support the life of other fastidious microorganisms or tissue cells. A significant finding concerns the extreme toxicity of serum for the murine leprosy bacillus. This additional reason for the intracellular habit of this parasite will be examined in greater detail in subsequent papers.

It is the purpose of this paper to outline the design and the results of experiments on the influence of physical and chemical factors on *M. leprae murium*. The findings constitute the first link in a chain of evidence that metabolic data, obtained under proper conditions, provide significant indication of the viability and infectiousness of noncultivated mycobacteria.

METHODS

The murine leprosy bacilli used have been obtained almost exclusively from testicular lepromas in rats. Bacilli of comparable quality, although in smaller numbers, are obtainable from cutaneous lepromas. Methods of obtaining washed, standardized suspensions of the bacilli and for determining their hydrogen transfer capacity (HTC) have been described (3). The reasons for selecting the metabolic method used, and the principles involved, have been presented in a preceding paper (6). Only an outline of the working conditions of the present inquiry is summarized here.

Mycobacteria preferentially transfer hydrogen to oxygen through their respiratory apparatus. The HTC of mycobacterial suspensions, therefore, must be measured under strictly anaerobic conditions.[2] Tetrazolium violet (TzV) [3] is the only tetrazolium compound which has been found to accept sufficient amounts of hydrogen from *M. leprae murium* during 24 hours of anaerobic color development. Although the suspensions are capable of transferring hydrogen for periods of more than 14 days, the amount of formazan (reduced tetrazolium) extractable in 100 per cent acetone after anaerobic incubation for three or five days was regarded as a measure of the HTC.

The influence of different conditions on the HTC was determined by one or more of the following procedures:

A. To each 0.1 ml aliquot of bacilli, Neph 40 (8), there was added 0.4 ml of buffer solution or experimental variable. There was then added immediately 0.1 ml of TzV 1 per cent. The influence of the variables was determined under continuous anaerobic conditions in the presence of TzV, which is a strong cation.

B. Exposure of the 0.5 ml systems to the different experimental conditions for

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[2] The original paper (3) failed to state that the evacuated anaerobic bottles are filled with 600 mm. partial pressure of nitrogen; otherwise, there is excessive distillation of water vapor from the tests to the walls of the vessels.

[3] 2-α-naphthyl-3, 5-diphenyl tetrazolium chloride.

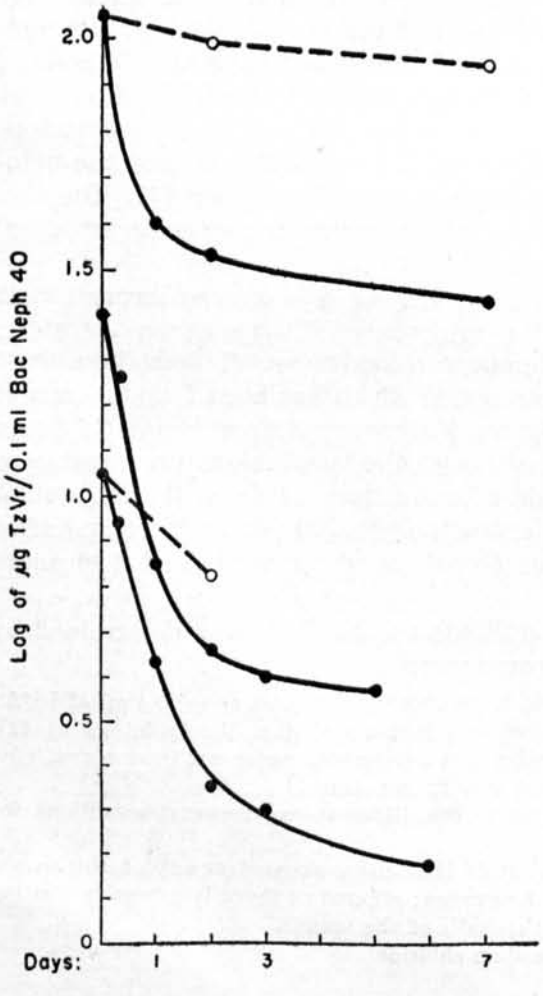
chosen periods of time. This was followed by anaerobic incubation with TzV to measure the residual HTC. In this procedure TzV was present only during anaerobic color development.

C. As B above, except that the experimental aliquots were washed or otherwise brought to a standard condition prior to addition of TzV and determination of their residual HTC.

In the direct procedure A, experimental variables must influence the HTC under anaerobic conditions and in the presence of TzV as a constant modifying factor. Results obtained with this procedure were not accepted unless confirmed by procedure B or C. Ionization of the strongly cationic TzV is diminished in the presence of high concentrations of negatively charged colloid. For this reason, the period of anaerobiosis for color development in the presence of concentrated proteins must be 72 hours or longer.

EXPERIMENTAL RESULTS

Characteristics of the endogenous metabolism.—When subsisting on endogenous metabolism during incubation under optimal conditions, bacilli of average quality retain but 30-40 per cent of their original HTC

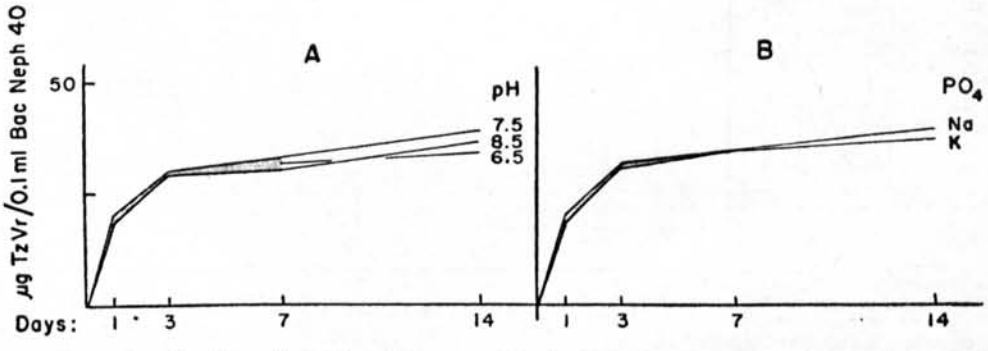


TEXT-FIG. 1. Rates of loss of HTC during refrigeration and incubation of bacilli possessing different initial metabolic activities.

Incubation (•), M/15 PO_4, pH 7.5, aerobic with 15mm CO_2, 37°C. Refrigeration (○) M/60 PO_4, pH 7.5, 4-6°C.

after 24 hours. The HTC declines more slowly thereafter. The failure of the logs of the residual HTC to fall along the straight lines which should characterize bacterial death rates is shown in Text-fig. 1. Previous work has shown that loss of metabolic activity (4) and of infectiousness (5) does not correspond directly with loss of viability. The data in Text-fig. 1 also reveal that the form of these curves differs in accordance with the metabolic activity of the bacilli. The importance of this point will become evident in the experimental results to be cited.

Bacilli possessing low HTC at the outset tend to deteriorate rapidly irrespective of experimental conditions. Although HTC measurements usually arrange factors in the correct order of excellence (see Text-fig. 2), the differences are not significant. Such suspensions are quite unsuited for measuring the influence of various factors on HTC and infectiousness. Reference to Text-fig. 3 will reveal the greater differences in response by active suspensions even after short periods of exposure.



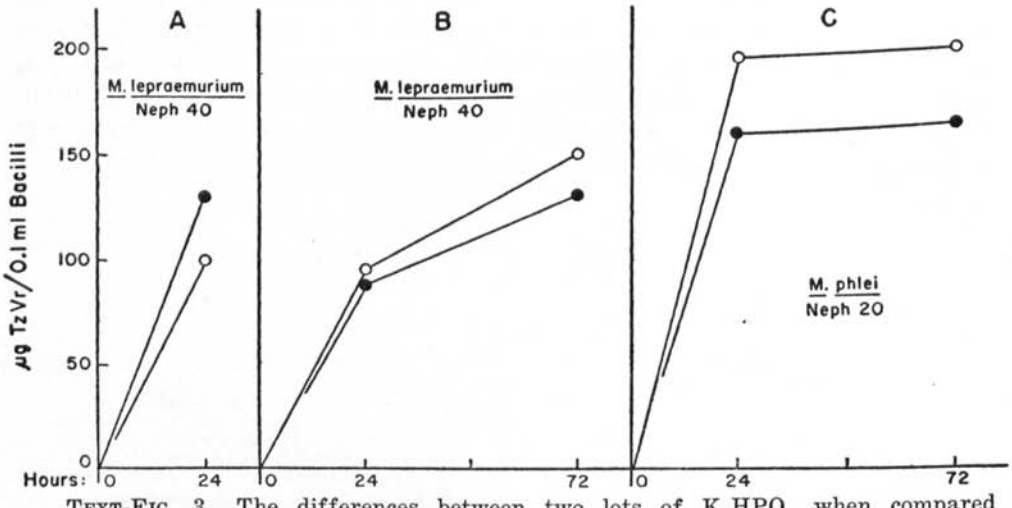
TEXT-FIG. 2. Unsuitability of low quality bacilli for measuring the influence of physical or chemical factors. M/15 PO_4, Procedure A, 37°. A=K_2HPO_4 at different pH values. B=K_2HPO_4 and Na_2HPO_4 combined 5:1 with KH_2PO_4, pH 7.5. Plotted on the usual 2X scale. Width of lines indicates the range in which results fell during the first seven days. The usual order of excellence may be fortuitous rather than significant.

The presence of small molecular materials such as glucose, serum dialysate, synthetic solutions for cell maintenance or miscellaneous microbiological nutriments did not modify the usual deterioration of the HTC during incubation. All data, therefore, are measures of endogenous metabolism.

Influence of pH, O_2 and CO_2.—Earlier data, obtained by procedure A, indicated that pH 8.5 provides the greatest rate of hydrogen transfer during periods of 24 hours, after which the rate declines. At pH's 7.5 and 6.5 the hydrogen transfer was persistent for periods of at least 14 days; pH 6.0 was unfavorable (4). Since the most persistent rate and greatest production of formazan occurred at pH 7.5, this value was accepted provisionally as optimal for washed bacilli suspended in potassium phosphate buffer.

In the present experiments the mutual interdependence of pH, O_2 and CO_2 requirements has been studied by rotating bacilli at 37° both aerobically and anaerobically in M/15 potassium phosphate at different pH values, in the presence and in the absence of 15 mm. CO_2. After exposure to these conditions for intervals of 2 to 7 days, the residual HTC of the bacilli was determined by means of procedure C. The conditions were standardized during this portion of the tests by adjusting the pH of all experimental aliquots to 7.5 and by anaerobic incubation. The results of four experiments are summarized below.

Irrespective of O_2 or CO_2 tensions, bacilli which had been incubated for more than 24 hours at pH 8.5 suffered the greatest loss of HTC.



TEXT-FIG. 3. The differences between two lots of K_2HPO_4 when compared against a constant source of Na_2HPO_4. M/15 PO_4, Procedure A, 37°. A=K_2HPO_4 of first lot (solid) vs Na_2HPO_4 (open), pH 8.5. B & C=K_2HPO_4 of second lot combined 5:1 with KH_2PO_4 (solid) and Na_2HPO_4 combined 5:1 with KH_2PO_4 (open), pH 7.5 in each case.

Those at pH 7.5 always retained the greatest HTC. Those incubated for from 72 to 144 hours at pH 6.5 possessed about 80 per cent of the HTC observed at pH 7.5.

The HTC of suspensions which had been incubated in the presence of 80 per cent oxygen was comparable to that of those incubated in air. The addition of CO_2 to the atmospheres contributed to preservation of HTC. At all pH values, in the presence of air, the beneficial effect of CO_2 was reasonably constant and averaged 40 per cent. Oxygen tensions modified the influence of CO_2 somewhat. In the presence of 80 per cent oxygen the enhancement was only 10 per cent. In the presence of air it was 40 per cent, and anaerobically it was 60 per cent.

Influence of anaerobiosis.—Anaerobiosis was 60 per cent more favorable than aerobic conditions for preservation of the HTC of incubated suspensions. Gray, however, has found that the respiration of *M. leprae murium* is damaged by anaerobiosis (1).

Results of one of the subsequent experiments done in collaboration

with Gray are given in Table 1. The HTC of bacilli incubated anaerobically for 72 hours was 64 per cent greater, while the respiration was 75 per cent less, than that of bacilli which had been rotated in the presence of air. The metabolism of both aerobically and anaerobically incubated bacilli was stimulated about four times by the presence of albumin and yeast supplement. The effects of anaerobic damage, therefore, are not alleviated by a combination of the two most beneficial agents known.

Negligible influence of electrolytes.—Since a previous study of the loss of infectiousness of murine leprosy bacilli during refrigeration and incubation (5) had suggested that intracellular ratios of NaCl:KCl exerted favorable effects on the bacilli, this question was investigated in detail. Six carefully controlled experiments, including 143 determinations in

TABLE 1.—*Influence of anaerobiosis on the HTC and respiration of* M. leprae murium.

Incubation/*a* 72 hours M/60 PO_4 pH 7.5	Residual metabolism of bacilli tested in the presence of:	HTC/*b*			Respiration/*c*		
		μg TzVr /0.1 ml neph 80	Per cent	Response to Alb_6YS_{17}	μl O^2/ 1.0 ml neph 80	Per cent	Response to Alb_6YS_{17}
Aerobic	M/90 PO_4, pH 7.5	58	100	1.0	6.1	100	1.0
15 mm. CO^2 rotated	Alb_6YS_{17} in above	275	474	4.7	22.2	364	3.6
Anaerobic	M/90 PO_4, pH 7.5	95	164	1.0	1.5	25	1.0
15 mm. CO^2	Alb_6YS_{17} in above	320	550	3.4	5.6	92	3.7

a Two aliquots (1 ml of bacilli at neph 80) were incubated aerobically and two were incubated anaerobically for 72 hours, at 37°C. To the first tube of each pair there was added 0.5 ml water. To the second tube of each pair 0.25 ml of 35% sterile albumin solution (Armour) (making Alb_6) and 0.25 ml Bacto Yeast Supplement B (YS_{17}). Respiration was tested immediately. Tests of HTC were deferred 7 hours, during which all four aliquots were rotated in the presence of air and CO_2. This precaution ensured that low potentials produced during the preceding anaerobiosis were not the cause of the high HTC values.

b HTC measured over a period of 72 hours. The heading μg TzVr signifies "micrograms of TzV reduced."

c Average rate over a period of three hours.

duplicate or triplicate, revealed that the previous inoculation data could not be explained by a consistent influence of electrolytes on HTC of the bacilli. Data on individual salts at different concentrations in the absence of buffer were not consistent because the pH values could not be stabilized even by daily readjustment. The differences between the individual salts in poorly buffered systems were attributed to variations in pH rather than to the influence of the salts tested. If, on the other hand, PO_4 and/or CO_3 were used as buffers it was impossible to state the extent to which cations were ionized, adsorbed to the buffer or adsorbed to the bacilli.

From this study, however, there emerged three equally favorable solutions: (*a*) M/7 (isotonic) or M/15 phosphate buffers at pH 7.5 with 15 mm. CO_2, (*b*) the balanced salt solution with the sodium bicarbonate-CO_2 (30 mm.) buffer system used for cell cultures, and (*c*) the mineral

base [4] found most satisfactory for rapid growth of *M. phlei* when a suitable carbon source is added. These solutions on the average were superior to a balanced salt solution which duplicated an intracellular assortment of ions. They were always superior to any solutions of low buffer content. Thus, only the hydrogen ion was identified as significant.

Furthermore, slight differences may occur between different lots of phosphate buffer, due probably to impurities. The left panel in Text-fig. 3 presents data obtained in the earliest comparisons (2) of potassium and sodium phosphates, while the second and third panels show that a subsequent lot of K_2HPO_4 was measurably inferior to the Na_2HPO_4. It may be seen that the distinctions between the salts were comparable whether *M. leprae murium* or *M. phlei* were used as test organisms.[5]

Influence of serum and of serum albumin.—Previous data obtained in rats suggested that purified bovine serum albumin was favorable for preservation of the infectiousness of incubated bacilli and that rat serum was unfavorable (5). These findings were readily substantiated by measurements of the HTC of bacilli which were incubated in the presence of these proteins under a variety of conditions.

TABLE 2.—*Favorable influence of purified bovine serum albumin and adverse effect of rat serum on the HTC of low-quality murine leprosy bacilli.*

Protein concentration /[a]	HTC	
	μg TzVr/ 0.1 ml neph 40	Per cent
5% Bovine serum albumin	22	550
1% Bovine serum albumin	6	150
0% IcBSS control	4	100
1% Rat serum 14.5%	3	75
5% Rat serum 71.5%	2	50

a Incubation 6 days aerobic prior to testing HTC during 72 hours anaerobic, Procedure B, pH 7.5; CO_2 40 mm.; diluent intracellular balanced salt solution (IcBBS).

The dramatic effect of albumin [6] on HTC was first observed during experiments designed to test the value of reproducing intracellular electrolyte and protein concentrations (Table 2). Low-quality bacilli were incubated for six days in intracellular electrolyte solution containing 1 per cent and 5 per cent of protein as albumin and rat serum, respectively.

[4] M/15 K_2HPO_4, M/250 $MgSO_4$, M/29000 $FeSO_4$ and M/45 $(NH_4)_2$.H citrate.

[5] Dr. Eric Ball, of the Department of Biochemistry, Harvard Medical School, informs me that traces of aluminum in phosphates are frequently the contaminant of biological significance.

[6] Kindly supplied by Dr. L. L. Lachat, of Armour and Co., Union Stockyard, Chicago 9, Illinois.

Preservation of HTC was improved by increasing albumin concentration, but affected adversely by increasing concentrations of rat serum.

Serums from all animal species tested were toxic. The magnitude of the effects produced by albumin and by serum was dependent on the metabolic activity of the bacilli used. Highly active suspensions exhibited no early enhancement of respiration in the presence of albumin, but rates did not decline as rapidly as in control suspensions (1). After 24 hours of rotation in albumin 4 per cent at 37°C the residual HTC was two or three times greater than that of control suspensions. These highly active bacilli, however, were extremely susceptible to the toxicity of serum. After 24 hours in rat or human serum 40 per cent, the HTC was usually only 5-10 per cent of that possessed by control suspensions in balanced salt solution and only 2-3 per cent of the original values. Suspensions with low activity, on the contrary, showed great stimulation by albumin and minimal inhibition by serum.

DISCUSSION

Data in this paper provide important elements in support of earlier conclusions (1, 2, 6) regarding the physiological requirements of *M. leprae murium*. Although this organism is unable *in vitro* to utilize single or complex substrates or to tolerate the toxicity of serum, its endogenous requirements with respect to oxygen, pH, electrolytes, etc., resemble those of the cultivable mycobacteria. One is assured, therefore, that failure of growth *in vitro* has not been due to unanticipated toxicity of the ionic or nutritional ingredients which are more or less universally employed for the cultivation of microorganisms or tissue cells.

The rapid loss of metabolic activity by *M. leprae murium* during incubation under optimal conditions is thought to explain the remarkable decrease in infectiousness reported earlier (5). The failure of any small molecular materials to modify this rapid loss affords also an explanation for the observation that such compounds do not prevent a precipitous decline in infectiousness (5). Gray's data on respiratory activity were presented primarily in terms of the failure of such substances to enhance respiration rates (1). The plotted curves illustrate comparable deterioration of metabolic activity *in vitro*. The improvement of HTC and respiration by purified serum albumin and metabolic inhibition by serum coincide also with the effects demonstrated in animal experiments (5). Thus, the major conclusions, based on results of animal inoculation are consistent with, and elucidated by, the observations which can be made by metabolic study.

The present data do not confirm earlier observations in rats suggesting a distinct preference of *M. leprae murium* for inverted Na:K ratios or other features of intracellular electrolyte solutions (5). While the distinctions between the infectiousness of bacilli recovered from the individual storage tubes were doubtless correct, more extensive experience has

shown that reproducible data cannot be obtained in the presence of the buffer levels employed at that time. Definite effects on metabolic activity are produced by different concentrations of hydrogen ions, and by differences in toxicity of the salts available from time to time. Our present working conditions are based on this evidence that suitable buffer and electrolyte are provided by M/7 or M/15 phosphates, by the balanced salt solution for cell cultivation, or by a mineral base employed for other mycobacteria.

The optimal pH for conserving HTC and respiration of *M. leprae murium* during incubation is pH 7.5, with pH 6.5 as second choice. Prudhomme (9) investigated the influence of pH on the infectiousness of murine leprosy bacilli in tissue homogenates added to bouillon and incubated for 14 days. Infectiousness persisted in the range of pH 6.1 to 7.2. Bacilli incubated at pH values of 7.3 or above and at values below 6.0 failed to produce lesions. When tubes of medium stand erect at 37°C, the bacilli and tissue components settle to the bottom of the tubes. The majority of the bacilli, therefore, were maintained under anaerobic conditions. In this circumstance pH 6.5, which minimizes oxygen demand but is otherwise not too unfavorable to metabolism, would be expected to be optimal. Thus, it appears there is an essential agreement between Prudhomme's observations and these metabolic data.

Since the gaseous atmospheres in the present experiments were maintained in 500 ml bottles, it should be noted that the control suspensions (no added CO_2) were not deliberately deprived of atmospheric CO_2, and that during incubation respiratory CO_2 accumulated. The consistently beneficial effects of the added CO_2 represent, therefore, a requirement of measurable significance.

The significance of anaerobic damage to *M. leprae murium* and other pathogens (7) has been discussed (6). The enhancement of HTC as described in this paper occurs because, under anaerobic conditions, neither oxygen nor TzV is available as a hydrogen acceptor. In consequence, unburned hydrogen (unoxidized substrates) accumulates. Since the damage does not influence the early steps in energy transfer, and since the ability to transfer hydrogen to TzV is unimpaired, the HTC is enhanced. It is the simultaneous deterioration of ability to transfer hydrogen to oxygen which indicates that the damage is confined to a cytochrome linkage (i.e., terminal respiratory system).[7]

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[7] A simple modification of the HTC method permits measurement of damage to respiration without use of the Warburg respirometer. The procedure requires simply the preparation of duplicate "anaerobic bottles" from which not all the oxygen is removed. Since mycobacteria preferentially transfer hydrogen to oxygen, those aliquots with normal respiratory capacity show a greatly diminished yield of formazan. If, on the other hand, respiration has been impaired, oxygen does not cause a comparable decrease in formazan yields. Careful exploitation of this principle would permit HTC measurements to assume the same significance as standard methods for measuring respiration, and would lack only the advantage of early determination rates.

The forms of the deterioration curves of *M. leprae murium* emphasize again that the residual activity of incubated mycobacterial suspensions existing endogenously, whether measured as HTC or as infectiousness, is not a measure of viability (4, 5). Investigators attempting to demonstrate correlations between the endogenous reduction rates and the viability of BCG have been disappointed, both because of the technical error of conducting such procedures aerobically and because direct correlations should not be anticipated under the conditions used. In the case of incubated suspensions *M. phlei,* correspondence of HTC and viability have been demonstrated only when the bacilli were permitted to respond to complete medium for several hours before testing HTC (4). In the case of refrigerated *M. leprae murium,* it will be demonstrated that the closest correlations with infectiousness are obtained when HTC is determined under conditions permitting the maximal activity which this organism can exhibit *in vitro.*

The present data provided our earliest basis for the conclusion that *M. leprae murium* is recovered from tissue homogenates in an inhibited state (6). The following observations necessitate this conclusion. The metabolic activity of washed suspensions of *M. leprae murium* is far from uniform. Suspensions exhibiting moderate and low levels of activity deteriorate more rapidly than highly active ones. The addition of serum albumin to the less active suspensions results in a dramatic apparent enhancement of metabolic activity (see also reference 1), which must be interpreted as a release from inhibition rather than actual utilization of substrate from albumin. Exposure to serum inhibitors is one of the inevitable consequences of liberating intracellular agents from cells into tissue homogenates. Subsequent papers will provide further insight into the importance of inhibition by serum and body fluids with respect to the transmission and pathogenesis of this and other diseases caused by intracellular agents.

Since a major explanation of low *in vitro* activity of *M. leprae murium* is the degree of inhibition, it should be evident why suspensions exhibiting very low activity are not useful for investigating the influence of experimental conditions. It is desirable, therefore, to be assured of suitable metabolic activity in the bacilli before engaging in expensive and time-consuming animal experiments.[8] A significant influence of variables may

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[8] A minimal useful safeguard is provided as follows: A small slice of infected, fresh tissue is dropped into 0.5 ml of 0.2% TzV in M/15 PO_4, pH 7.5, and allowed to stand for 15 minutes at room temperature. Tissues containing bacilli suitable for metabolic and infection experiments develop a uniform deep purplish red, while inferior tissues develop weaker or irregular colors. The latter should be discarded. This test is simply one for the physiological state of tissue cells. The fact that *M. leprae murium* loses metabolic activity and infectiousness *in vivo* when not enclosed in healthy cells affords strong support to evidence now being assembled with respect to inhibition of this microorganism in the extracellular environment.

be observed when active bacilli are employed, while the results of comparable experiments with bacilli of very low activity may suggest these experimental factors to be unimportant.

SUMMARY AND CONCLUSIONS

1. Washed suspensions of *M. leprae murium* subsisting on endogenous reserves during incubation lose hydrogen transfer capacity (HTC) rapidly. This deterioration is not due directly to loss of viability. The decrease in HTC like that of infectiousness, is not modified by the presence of glucose, serum dialysates or other small molecular nutriments utilized by other forms of life.

2. Optimal conditions for sustaining the endogenous HTC during aerobic incubation resemble those for other mycobacteria. They are provided by small amounts of CO_2 and by adequately buffered solutions at pH 7.5. There is no choice between three electrolyte solutions suitable, respectively, for metabolic studies, cell cultivation, or mycobacterial growth.

3. Under anaerobic conditions *M. leprae murium*, like other pathogenic mycobacteria, suffers damage to its terminal respiratory system; i.e., there is enhancement of HTC and impairment of oxygen consumption.

4. *M. leprae murium* differs from cultivable pathogenic mycobacteria with respect to: (*a*) inability to utilize substrate *in vitro* and (*b*) extreme susceptibility to natural inhibitors in serum.

5. The metabolic activity of *M. leprae murium* recovered from lesions is variable, due in part to an inhibited state of the bacilli in tissue homogenates. The improvement of metabolism and infectiousness by purified serum albumin is due to partial alleviation of this inhibition. Bacilli exhibiting low activity are not suitable for investigating the influence of experimental conditions *in vitro* by means of metabolic study or animal inoculation.

6. This study provides evidence that metabolic activity, properly measured, affords an index of the infectiousness of murine leprosy bacilli after incubation *in vitro*.

RESÚMEN Y CONCLUSIONES

1. Suspensiones lavadas de *M. leprae murium* que subsisten por las reservas endógeñas durante la incubación pierden rápidamente la capacidad de transferir hidrógeno (HTC); ésto no es debido directamente a la pérdida de capacidad vital.

Esta disminución como la de infecciosidad, no se modifica por la presencia de glucosa, dializados de suero u otras pequeñas moléculas nutritivas utilizados por otros organismos vivientes.

2. Condiciones óptimas para mantener la HTC endógena durante la incubación aeróbica se parece a la de los otros micobacterios. Estan provistas de pequeñas cantidades de CO_2 y de soluciones con capacidad "buffer" adecuada a un pH 7.5. No hay preferencia entre las tres soluciones electrólitos adaptadas respectivamente para estudios metabólicos, cultivo celular o crecimiento micobacterial.

3. Bajo condiciones anaeróbicas, *M. leprae murium*, como otros micobacterios patógenos sufren trastornos en su sistema respiratorio terminal, como por ejemplo: aumento de la HTC y disminución del consumo de oxígeno.

4. *M. leprae murium* difiere de los micobacterios patógenos cultivables con respecto a: *a*) incapacidad para utilizar substrato *in vitro* y *b*) extrema susceptibilidad para los inhibidores naturales en el suero.

5. La actividad metabólica del *M. leprae murium* recuperada de las lesiones es variable, debido en parte a un estado de inhibición en los tejidos emulsionados. El mejoramiento del metabolismo y la infecciosidad por medio, de la albumina del suero purificada es debido a una disminución parcial de esta inhibición. Los bacilos que denotar una actividad limitada no son adecuados para investigar la influencia de condiciones esperimentales *in vitro* por medio de estudio metabólico o inoculación animal.

6. Este estudio demuestra que la actividad metabólica, medida apropiadamente, constituye un índice de la infecciosidad del bacilo de la lepra murina después de la incubación *in vitro*.

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