MEASUREMENT OF THE HYDROGEN TRANSFER CAPACITY OF MYCOBACTERIA

JOHN H. HANKS

WITH THE ASSISTANCE OF MISS TOBEY BACKERMAN AND MISS RACHEL BARRETT

Leonard Wood Memorial, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

Received for publication May 21, 1951

Animal experimentation, culture data, and analytical and metabolic studies provide a body of fundamental information concerning the cultivable mycobacteria. There exist no comparable data on human and murine leprosy bacilli. The mysteries which surround the leprosy bacilli are due in part to their failure to grow *in vitro* and in part to their feeble transmissibility. Human leprosy has not been transmitted experimentally to humans or to animals. Though rat leprosy is transmissible in certain rodents, murine leprosy bacilli lose infectiousness very rapidly *in vitro* under the most favorable conditions yet defined. Inordinate periods of time are required for the production of lesions by bacilli which have been incubated under such conditions. Consequently, an intelligent inquiry into factors which may be favorable or unfavorable to *Mycobacterium lepraemurium* could not be completed by this means within the lifetime of any single investigator. A study of metabolism remains the only means by which both cultivable and noncultivable mycobacteria can be compared on common ground and the most practicable means of inquiring into the physiological requirements of the leprosy bacilli.

Prudhomme (1938) showed that *o*-cresol-indo-2-6-dichlorophenol could be reduced by washed suspensions of *M. lepraemurium* after incubation for one or more days. Reduction did not occur if the suspensions had been damaged by heat or chemical agents, or had been incubated for 15 days. Following the introduction of triphenyl tetrazolium chloride into this laboratory by Dr. Clarke T. Gray for cytological purposes, an inquiry into the hydrogen transfer capacity[1] of washed suspensions of murine leprosy bacilli was initiated. It was the purpose of this work to develop a metabolic test requiring but small aliquots of murine leprosy bacilli, and to ascertain whether appropriate measurement of hydrogen transfer capacity could be interpreted in terms of the biological data obtainable with other microorganisms by plate counts or animal inoculation.

The first of the present two papers concerns methods for measuring the hydrogen transfer capacity of mycobacteria, conditions which influence the delicacy and precision of the measurements, and certain differences between the behavior of suspensions of *M. lepraemurium* and *Mycobacterium phlei*.

METHODS

The murine leprosy bacilli were recovered from testicular homogenates, since it is in this organ that the bacilli grow most rapidly and reach the highest final

[1] The term "dehydrogenation" implies knowledge of the substrate which is being oxidized.

concentrations (Hanks and Backerman, 1950). The bacilli were separated from tissue components by being twice centrifuged through dense solutions which tend to support the tissue components, and by a final wash in double distilled water. All reagents and equipment were refrigerated prior to use, and the tubes were kept in crushed ice, except during the 10 minute periods of centrifugation. The hydrogen transfer capacity of the bacterial suspensions was not improved by the use of a refrigerated centrifuge. Sterile solutions and aseptic procedures were employed throughout.

1. The infected organs were disintegrated in 0.2 M sucrose (6.8 per cent) in a Waring blendor for 2 minutes and brought to an even emulsion by further homogenization in a Potter-type homogenizer for 5 minutes.

2. The coarse particles were removed from this 10 per cent tissue homogenate during 5 minutes at 270 xG in the horizontal centrifuge.

3. Twelve ml of the clarified homogenate were stratified above 15 ml of 0.3 M sucrose (10.2 per cent) in a 50 ml centrifuge tube and run for 10 minutes at 4,500 rpm in a type SP Swedish angle centrifuge.

4. The packed sediments were stirred, brought back to 12 ml by the addition of 0.2 M sucrose, stratified on 15 ml of 1.5 M KCl (11 per cent), and again thrown down in the angle centrifuge.

5. The packed sediments from 2 tubes were then suspended in 25 ml of distilled water and again centrifuged for 10 minutes.

6. Final sediments were stirred with an inoculating wire, and a small amount of the dense bacterial paste was transferred to a small streak on a glass slide. After Ziehl-Neelsen staining of the heat fixed films, they were differentiated for 3 minutes in 0.1 per cent methylene blue in 95 per cent alcohol to provide a very intense counterstain. The apparent ratio of red microorganisms to blue-staining components was usually greater than 98 to 1.

7. The bacillary sediments were diluted with 10× their volume of M/15 potassium phosphate buffer, pH 7.5. A small aliquot of this suspension was diluted 100 times and its optical density determined in the Coleman Jr. spectrophotometer at a wavelength of 420 mμ. The entire suspension was then diluted to an optical density corresponding to nephelometer 40 or nephelometer 80 on the McFarland scale. Two drops of this suspension were transferred to each of three tubes of Brewer's sterility test medium. The aliquots employed in hydrogen transfer capacity tests have usually been 0.1 ml of suspension equivalent to nephelometer 40. This is one-tenth to one-fifteenth of the numbers of bacilli required for respiration studies in vessels of $KO_2 = 0.5$.

M. phlei (Harvard Medical School strain) was grown in tubes containing 8 ml of heart infusion broth with 1.5 per cent Casamino acid hydrolysate, 2 per cent glucose, and 0.1 per cent sodium acetate, in a roller drum at 37 C for 72 hours. The cultures were freed of clumps by centrifugation at 270 xG for one minute and then by filtration of the supernates through Whatman filter paper no. 5. These suspensions were sedimented in the horizontal centrifuge for 10 minutes at 600 xG and resuspended in M/15 buffer to correspond to nephelometer 10 or 20. Colony counts were obtained in triplicate by adding 0.1 ml aliquots of known dilutions of

M. phlei suspension to 22 by 140 mm screw cap tubes. Upon adding and mixing 3 ml of nutrient agar (56 C) with each sample, the tubes were laid on a horizontal rolling device which distributed the bacteria and medium in a thin film over the entire inner surface of the tube. Such tubes were inclined toward the screw caps and incubated at 37 C for 72 hours. Results are expressed as the number of colonies per 1 ml of nephelometer 1 ($\simeq$ 1 mg moist weight of bacilli).

All solutions were prepared from reagent grade chemicals in double distilled water. The buffer system was provided by the proportion of K_2HPO_4 and KH_2PO_4 appropriate to the pH desired. Unless exceptions are noted, all work was done at pH 7.5. Phenol red 0.001 per cent was present in all solutions except in the sterile stocks of 1 per cent tetrazolium compounds (which flocculate with the indicator). One per cent solutions of the tetrazolium compounds were prepared in water and adjusted to pH 7.5. After autoclaving, the insoluble impurities were removed by centrifugation.

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

A. To each 0.1 ml aliquot of bacilli there were added 0.5 ml of buffer solution or experimental variable and 0.1 ml tetrazolium violet 1 per cent. In this case the influence of the variables was determined under anaerobic conditions in the presence of tetrazolium violet as a modifying factor.

B. Exposure of the aliquots to different conditions in the presence of air prior to anaerobic incubation with tetrazolium violet. In this procedure both the tetrazolium violet and the variables were present while measuring the residual hydrogen transfer capacity of the bacilli.

C. As B above, except that the experimental aliquots were washed or otherwise brought to a standard condition prior to addition of tetrazolium violet to test their residual activity.

The degree of anaerobiosis during the reduction tests was standardized by assembling the cotton stoppered 13 mm tubes in 500 ml ground glass stoppered bottles with an evacuating port drilled through the stopper and the neck of the bottle as in a Thunberg tube. To each bottle there was also added a control tube containing 1 ml of the methylene blue anaerobic indicator of Ulrich and Larsen (1948). Provision for oxygen cleanup (Mueller and Miller, 1941) was made by adding 60 mg of chromium metal at one margin of the bottom of each bottle. One ml of 15 per cent sulfuric acid was then added at the opposite margin of the tilted bottle. After the bottle had been evacuated for 3 minutes, i.e., until the liquids were degassed and the manometer was within 10 mm of the prevailing barometric pressure, the sulfuric acid was tipped onto the chromium. The per cent reduction in the methylene blue control tube was recorded at the end of the incubating period. Data obtained at 90 to 95 per cent reduction were corrected by the addition of the appropriate formazan (reduced tetrazolium) values to each test, while those obtained under less complete anaerobiosis were discarded.

After 24 or more hours of anaerobic incubation the formazan content of each test was determined. Formazan colors fade slowly in the refrigerator or at room temperature. If determinations could not be made immediately, the color was

stabilized by adding one drop of formalin 30 per cent to each tube. Such samples may be refrigerated for a week or more without loss of color. Prior to color extraction the bacteria and formazan were washed by filling each tube with formalin 2 per cent and by centrifugation. The supernatants were discarded carefully and the buttons shaken with a carefully measured volume of 80 per cent acetone or of isoamyl alcohol to extract the color. Extraction was repeated if the repacked buttons were appreciably colored. The extract was diluted appropriately and the concentration of formazan determined photometrically, using a Coleman Jr. spectrophotometer no. 6 at a wavelength of 480 mμ. The readings were converted to micrograms of tetrazolium compound reduced (μg TzVr) by reference to standard curves established in the presence of known amounts of reduced compounds.

EXPERIMENTAL RESULTS

Optimal conditions for measuring the hydrogen transfer capacity. Due to the feeble endogenous metabolism of murine leprosy bacilli, insufficient formazan was produced in the presence of 0.2 per cent triphenyl tetrazolium chloride,[2] neotetrazolium phosphate 2B,[3] neotetrazolium chloride,[3] blue tetrazolium,[4] or 2,3,diphenyl-5-methyl tetrazolium chloride[5] during 24 hours' incubation under anaerobic conditions. Potassium tellurite was not reduced. Tetrazolium violet[5] was the only compound which produced a workable intensity of color. Although extracellular crystallization of the formazan makes tetrazolium violet unsuitable for cytological work, it does not invalidate the usefulness of this compound as a hydrogen acceptor for suspensions.

M. phlei suspensions, on the other hand, produce much lower potentials and transfer adequate amounts of hydrogen to the other tetrazolium compounds.

Suspensions of *M. lepraemurium* are much less sensitive than those of *M. phlei* to the concentration of tetrazolium violet. They tolerate at least twice the concentration used without decreased hydrogen transfer capacity. *M. phlei* cannot transfer persistently in the concentration routinely employed (0.15 per cent); doubled concentrations reduce the reported yields by 50 per cent. Reference to figure 1 will reveal that 24 hours at pH 8.5 or 72 hours at pH 7.5 permits maximal color development by *M. phlei* under anaerobic conditions. *M. lepraemurium* suspensions, on the other hand, produce formazan during 7 days at pH 8.5 and for 14 or more days at pH 7.5. The data with *M. phlei* have been collected after 24 or 72 hours of anaerobic incubation with tetrazolium violet, depending on the pH employed for color development. Those with *M. lepraemurium* have been collected after 72 hours at pH 7.5.

Duplicate aliquots of *M. lepraemurium* usually agree within 10 per cent. Due to the greater tendency of *M. phlei* suspensions to creep and become dehydrated on the walls of the tubes, duplicate aliquots often disagree by more than 10 per cent and triplicates are necessary.

[2] Purchased from Schwarz Laboratories, Incorporated, New York.
[3] Purchased from Montclair Research Corporation, Montclair, New Jersey.
[4] Purchased from Monomer-Polymer, Incorporated, Chicago, Illinois.
[5] Purchased from The Synthetical Laboratories, Chicago, Illinois.

Since mycobacteria are strict aerobes, measurement of their capacity to transfer hydrogen to an artificial acceptor system is possible only under strictly anaerobic conditions (see table 1). Calculations rather than actual data are used to illustrate this relationship. A standard formula permitting corrections for the presence of traces of oxygen has not yet been developed.

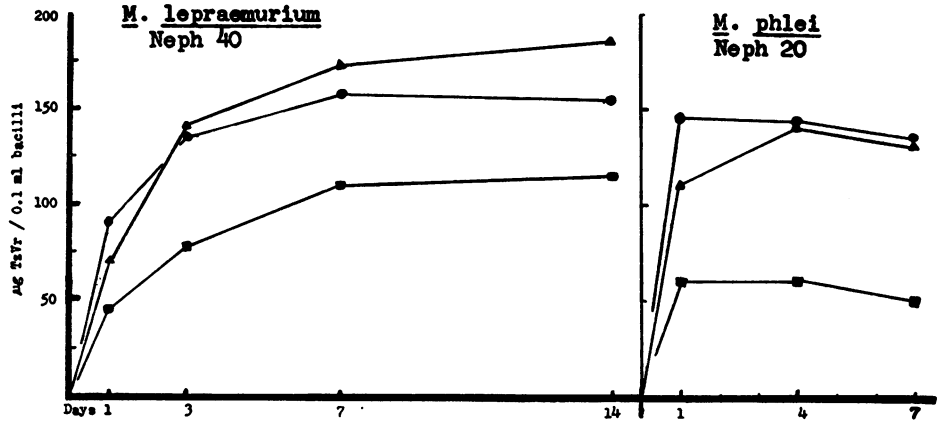

Figure 1. The influence of pH on the hydrogen transfer capacity of *Mycobacterium lepraemurium* and *Mycobacterium phlei* suspensions. Procedure A, 37 C, M/15 buffer, anaerobic, in presence of tetrazolium violet 1,000 μg @ 0.15 per cent. pH 8.5 ●, pH 7.5 ▲, pH 6.5 ■.

TABLE 1

The importance of complete anaerobiosis in hydrogen transfer capacity determinations

	ANAEROBIC HYDROGEN TRANSFER CAPACITY OF STANDARD DOSE OF BACILLI/24 HR (μg TzVr)*	ASSUME H TRANSFERRED TO O IS EQUIVALENT TO (μg TzVr)*	ACTUAL AMOUNT OF TzVr (μg)	CONCLUSION REACHED
a	200	0	200	a is 10 × superior to b
b	20	0	20	b is 10 × superior to c
c	2	0	2	c has minimal activity
a	200	2	198	a is 11 × superior to b
b	20	2	18	b is infinitely superior to c
c	2	2	0	c has no activity
a	200	20	180	a is infinitely superior to b and to c
b	20	20	0	
c	2	20	0	

* Micrograms tetrazolium violet reduced.

Experience has revealed certain advantages and limitations in each of the experimental conditions employed. In procedure A the immediate combination of fresh organisms with the experimental variables and with tetrazolium violet results in the greatest yields of formazan. Although this direct procedure is the pre-

ferred method of exploring certain questions, it is open to several objections: (a) the modifying circumstances must influence the hydrogen transfer capacity of the bacilli under anaerobic conditions and in the presence of tetrazolium violet as a very active cation and (b) the availability of tetrazolium violet is diminished in the presence of high concentrations of negatively charged colloids. Results obtained by procedure A have not been accepted unless they could be confirmed by procedure B or C. Procedure C is a logical method for determining the influence of different gaseous environments, since these conditions are automatically standardized during anaerobic incubation with tetrazolium violet. After several days of incubation, final washing of murine leprosy bacilli to standardize a variable chemical background lowers the hydrogen transfer capacity to undesirably low levels. It will be shown in the next paper that final washing, procedure C, is the most reliable method for suspensions which have been exposed briefly to adverse circumstances and for incubated suspensions of mycobacteria which are capable of responding to added substrate.

The endogenous behavior of M. lepraemurium and M. phlei. In order to ascertain the suitability of *M. phlei* as a test object permitting interpretation of the hydrogen transfer capacity data obtained with *M. lepraemurium*, a series of experiments was conducted to compare the endogenous hydrogen transfer capacity of both microorganisms. A few of the data are presented to illustrate the forms of hydrogen transfer capacity curves produced by suspensions of each organism during incubation under the conditions of procedure A and of procedure C.

To determine the influence of pH by procedure A, duplicate and triplicate aliquots of *M. lepraemurium* and *M. phlei*, respectively, were added to the required number of tubes containing 0.5 ml buffer at the chosen pH values; 0.1 ml tetrazolium violet 1 per cent was added to each tube; the tubes were divided into bottles to be opened on the several sampling dates; and anaerobiosis was established. Typical results obtained at pH 8.5, 7.5, and 6.5 are shown in figure 1.

The more rapid reduction rates at pH 8.5 are not due solely to the greater reduction potentials attained at this pH. Both organisms also respire more rapidly at pH 8.5 for several hours, after which the rates decline sharply (Gray, 1950). Since the activity at pH 7.5 is of equal or greater capacity and is more sustained, this range of hydrogen ion concentration may be defined as optimal. Both microorganisms find pH 6.0 promptly unfavorable. The conclusions with respect to optimal pH were confirmed for *M. lepraemurium* by procedure C, and for *M. phlei* are consistent with cultivation data.

The pathogen differs from the saprophyte in two respects: (a) in functioning persistently at pH 6.5 under all procedures employed and (b) in the capacity for sustained hydrogen transfer under the conditions of procedure A. The downward slope of the *M. phlei* curves at 4 and 7 days indicates that this organism has completely expended its hydrogen transfer capacity under these conditions. The rate of hydrogen transfer does not keep pace with the fading or destruction of formazan.

Similar forms of curves, with the characteristic differences in the rate and persistence of the hydrogen transfer capacity of the two species, resulted from a

study of the influence of electrolytes by procedure A. The two species showed comparable behavior with respect to electrolyte, and it was not possible to select any electrolyte mixture which was consistently more favorable than M/15 potassium phosphate.

Similar studies on pH, electrolytes, and gaseous environment by methods B and C also showed that during incubation *M. phlei* exhausts its endogenous hydrogen transfer capacity more rapidly than does *M. lepraemurium*. Optimal conditions for preserving the endogenous hydrogen transfer capacity of both microorganisms during slow rotation of the suspensions in the presence of air at 37 C were provided by M/15 potassium phosphate and the presence of carbon dioxide at 15 mm Hg. The addition of Mg, Ca, Fe, NH_4, or citrate ions was not found to be necessary. In the absence of the inhibitory effects of tetrazolium violet

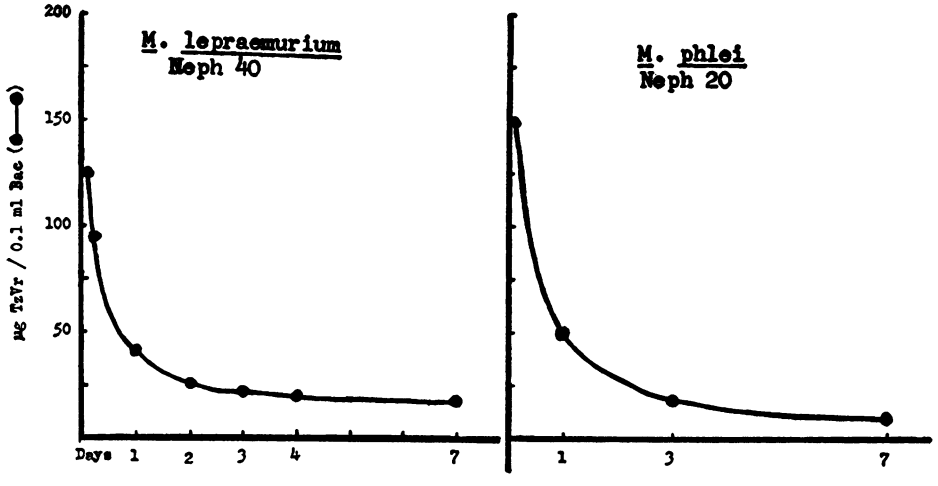

Figure 2. The residual hydrogen transfer capacity of *Mycobacterium lepraemurium* and *Mycobacterium phlei* suspensions following slow rotation of 37 C in M/15 buffer, pH 7.5 in the presence of air and 15 mm CO_2.

on *M. phlei* the forms of the curves for the residual hydrogen transfer capacity of the two types of suspensions are closely comparable (see figure 2).

DISCUSSION

The availability of colorless hydrogen acceptors which form brilliantly colored and relatively stable formazans permits measurement of hydrogen transfer to serve a purpose which is not met when dyes are employed as hydrogen acceptors. In the dye systems one-half or more of the acceptor must be reduced to obtain data. Although these systems permit measurement of rates, depletion of the acceptor prevents obtaining data on the actual hydrogen transfer capacity of the donor system. In the case of tetrazolium compounds only a small portion of the total acceptor system (e.g., 4 to 100 μg out of 1,000) needs to be reduced to provide accurately determinable results. The total capacity of the donor system can,

therefore, be measured. Evidence will be presented in a subsequent paper that the measurement of capacity rather than of rate permits important inferences concerning the biological state of the uncultivated murine leprosy bacilli.

The present data reveal certain characteristic differences between the washed suspensions of *M. lepraemurium* and those of the rapidly growing *M. phlei*. With respect to the reduction potentials produced and the rates of hydrogen transfer *M. lepraemurium* is very feeble. According to Gray (1950) its respiration cannot be enhanced by simple exogenous sources of energy. Nevertheless, this organism possesses a considerable capacity to transfer hydrogen. This ability is remarkably persistent and is more stable than that of *M. phlei*. *M. phlei* suspensions can transfer hydrogen at a much higher rate. They respond to substrate under both aerobic and anaerobic conditions. They are badly handicapped at the pH levels existing in organs. Under the conditions of procedure A, they are much more sensitive to the presence of tetrazolium violet.

Identical conditions appear to be optimal for preserving the hydrogen transfer capacity of the two microorganisms when surviving on endogenous reserves during incubation. It is under these circumstances that they may most reliably be compared.

ACKNOWLEDGMENT

I am indebted to Dr. Clarke T. Gray for many helpful discussions during the course of this investigation.

SUMMARY AND CONCLUSIONS

A metabolic method (based on the reduction of tetrazolium violet) for measuring the hydrogen transfer capacity of murine leprosy bacilli and other biological donor systems has been described. The procedures developed for the study of the hydrogen transfer capacity of mycobacteria have been summarized. The results obtainable have been illustrated by comparing the behavior of *Mycobacterium lepraemurium* and *Mycobacterium phlei* suspensions in the absence of added substrate.

REFERENCES

GRAY, CLARKE T. 1950 *Personal communication.*

HANKS, J. H., AND BACKERMAN, T. 1950 The tissue sites most favorable for the development of murine leprosy in rats and mice. Intern. J. Leprosy, **18**, 185–207.

MUELLER, J. H., AND MILLER, P. A. 1941 A modification of Rosenthal's chromium-sulfuric acid method for anaerobic cultures. J. Bact., **41**, 301–303.

PRUDHOMME, R. 1938 Moyen de reconnaitre *in vitro* si le bacille de Stefansky est mort ou vivant. Ann. inst. Pasteur, **61**, 512–518.

ULRICH, J. A., AND LARSEN, A. M. 1948 A single solution indicator for anaerobiosis. J. Bact., **56**, 373–374.