BIOCHEMICAL DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS GROWN IN VIVO AND IN VITRO1, 2

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Most of our knowledge concerning the tubercle bacillus stems from studies on bacteria grown under standard laboratory conditions in vitro. Numerous attempts have been made to determine indirectly the relationship between the host environment and the pathogenicity of the tubercle bacillus by a study of the comparative reaction of virulent and avirulent strains to various conditions occurring in the host environment. Although some consistent correlations have been made, the results are open to question since interpretation is based on the assumption that virulent tubercle bacilli grown artificially are identical with bacilli as they multiply in the tissues of an infected animal. This assumption is implicit in most of the work done on the tubercle bacillus. However, there have been isolated reports in the literature indicating a lack of identity between in vitro and in vivo grown tubercle bacilli. Anderson et al. (1943) were unable to detect in tuberculous lung tissue several chemical compounds (phthiocol, tuberculostearic acid, phthioic acid, and specific polysaccharide) that are characteristic constituents of human tubercle bacillus cultivated on artificial medium. These authors raised the question whether tubercle bacilli growing in living tissue produce the same characteristic chemical compounds that are found in bacilli grown in vitro. Sheehan and Whitwell (1949) concluded that a difference exists between the lipid content of artificially cultured tubercle bacilli and that of the organisms in pathological lesions on the basis of their differential method of "sudan black B" staining.

Our understanding of the host-parasite relationship in tuberculosis would be furthered by a study of the tubercle bacillus in its natural parasitic state, with particular reference to any differences that might exist between in vivo and in vitro grown bacilli. Such a study was made possible by the development of a method for separating tubercle bacilli in large numbers directly from the lungs of infected mice.

The method of separating pathogen from its diseased host has in recent years been applied to the study of two other infectious bacteria, Mycobacterium lepraeumurium and Bacillus anthracis. Hanks (1951a) separated the murine leprosy bacillus from testicular tissue of the rat by a method different from that reported here. Hanks (1951b) was able to obtain an estimate of the viability of this noncultivable organism by measurement of hydrogen transfer capacity. A study of the metabolism of separated murine leprosy bacilli was made by Gray (1952) as the only available means for the study of the physiological requirements of this organism. The work of Hanks and Gray served as a valuable guide for the present study. More recently the use of in vivo grown bacteria has been applied by Smith and Keppie (1955) to the anthrax bacillus with highly successful results. Using bacilli separated from exudates and plasma of diseased guinea pigs, they were able to isolate and identify chemical substances involved directly in the pathogenicity of this organism. Their study did not primarily concern itself with establishing differences between B. anthracis grown in vivo and in vitro; however, they did find that the in vivo grown bacilli were rapidly lysed in 0.16 per cent ammonium carbonate solution at 0 C in contrast to in vitro grown bacilli.

In the case of the study reported here on Mycobacterium tuberculosis, the primary objective of examining the comparability of in vivo and in vitro grown bacilli was accomplished by an examination of various biochemical characteris-

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tics. The differences observed are striking and indicate that tubercle bacilli in their natural infectious state are dissimilar to those grown in artificial culture medium.

**EXPERIMENTAL METHODS**

*Method for separation of in vivo grown tubercle bacilli.* The source of *in vivo* grown tubercle bacilli was the lungs of moribund or dead mice 18 to 23 days after infection. Initially mice were infected with a virulent human strain of *M. tuberculosis* (H37Rv), but thereafter with tubercle bacilli separated from infected lungs (LRv). The method for separation of tubercle bacilli from infected lungs is based on the use of isotonic sucrose (0.25 M) for the isolation of particulate cell components by differential centrifugation (Umbreit *et al.*, 1949). Lungs were homogenized in a solution (5 ml per lungs of 1 mouse) of 0.25 M sucrose containing 0.25 per cent bovine albumin. Inclusion of 0.25 per cent albumin in the homogenizing medium was found to stabilize the endogenous respiration of the separated lung bacilli without appreciably affecting the nature and degree of separation. Thorough homogenization was obtained by use of a "virtis" homogenizer run at top speed (29,000 rpm) for 3 min. The entire separation was carried out under sterile conditions and at near-freezing temperatures by means of crushed ice baths. The homogenate was centrifuged in 40-ml round-bottom tubes in an International refrigerated centrifuge at 0 C and at a speed of 2,000 rpm (450 G) for 15 min. The supernatant containing light tissue components was discarded. The sediment was resuspended in albumin-sucrose solution (5 ml per original lungs of 1 mouse), centrifuged for 5 min at 1,500 rpm (250 G), and the supernatant removed to a 40-ml centrifuge tube, which was stored on ice. This latter step was repeated twice, resulting in three separate supernatants. The residual sediment, consisting of heavier tissue components such as nuclei and some tubercle bacilli, was discarded. The three batches of supernatant were then centrifuged at 2,000 rpm (450 G) for 10 min, and the supernatants were discarded to assure complete removal of light tissue components. The three resultant sediments were examined microscopically for purity of separation, using the Ziehl-Neelsen acid-fast stain and prolonged counterstaining with methylene blue. The three sediments were then combined in saline containing 0.25 per cent albumin and centrifuged at 2,000 rpm (450 G) for 10 min; the sediment was resuspended in albumin-saline and centrifuged at 3,000 rpm (1,000 G) for 10 min. The sediment was finally suspended in saline, stained and examined microscopically for purity of separation, and adjusted to standard optical density using a Coleman Universal spectrophotometer.

Male albino mice of the CFI strain (Carworth Farms, New City, New York) were injected intravenously with 0.1 ml of saline suspensions of LRv adjusted to 40 per cent transmittance, approximately equal to 0.02 mg dry weight or 5 million bacilli as determined by plate counts. Plate counts were made of colonies grown on oleic acid-albumin agar (Fenner, 1951).

*Source of in vitro grown tubercle bacilli.* For comparative purposes a 10-day "tween"-albumin submerged culture (Dubos and Middlebrook, 1947) of the virulent human strain (H37Rv) of *M. tuberculosis* was used. The cells were separated from the culture medium by centrifugation, washed twice in a salt solution containing 0.05 per cent "tween 80," and adjusted to standard density. It is of interest to note that the presence of tween 80 is essential to obtain an even suspension of H37Rv whereas the separated lung bacilli LRv suspend evenly in saline or distilled water in the absence of tween 80.

*Quantitative comparison of in vivo and in vitro grown tubercle bacilli.* Table 1 summarizes data on the quantitative comparability of suspensions of equal optical density of *in vivo* and *in vitro* grown tubercle bacilli, as well as indicating the source, age, and yield. In order to compare the viable numbers of LRv and H37Rv by plate counts, it was necessary to eliminate the cords and large clumps of bacilli that occur in cultures of H37Rv. This was accomplished by centrifuging for 2 min at 1,000 rpm (125 G) and discarding the sediment. The supernatant was found to contain only single bacilli and small aggregates of less than 30 bacilli, similar to those resulting in the separation of lung bacilli. Quantitative estimation was obtained by adjustment of optical density and determination of dry weight.

As seen in table 1, the yield of tubercle bacilli from infected lungs was considerable, supplying adequate material for use in Warburg studies. Suspensions of H37Rv and LRv adjusted to the same optical density were found to contain comparable amounts of bacilli as determined by dry.

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weight and plate counts on oleic acid-albumin agar. These results confirmed the purity of the suspensions of lung bacilli and established a basis for quantitative biochemical comparison of tween-grown and lung-grown tubercle bacilli.

**Biochemical methods.** An index of general metabolic activity was obtained by testing the hydrogen transfer capacity of bacilli in the presence of substrate. This was done by measuring in the Coleman Universal spectrophotometer the reduction of 2,3,5-triphenyl tetrazolium chloride after 2 hr of incubation in the presence of tween-albumin medium containing 0.5 per cent glucose (Vandiviere et al., 1952).

The specific biochemical test used throughout this study was the comparative response of H37Rv and Lrv to various substrates, as measured by oxygen consumption using standard Warburg manometry. The gas phase was air, the temperature was 37.8 C, CO2 was absorbed by 0.2 ml of 20 per cent KOH in the center well, and the total volume of liquid in the Warburg vessels was 2.2 ml. The oxygen uptake of 5 to 6 mg dry weights of bacilli was determined over 2- or 3-hr periods and from these values the QO, was determined. Endogenous respiration was determined on bacilli suspended in m/15 phosphate buffer, pH 6.8, which also was the final pH of the various substrates in the Warburg vessels. Comparative substrate response is expressed as per cent increase in the QO, value over that of the endogenous respiration.

**RESULTS**

No significant differences were found to exist when comparing in vivo and in vitro grown tubercle bacilli with respect to the following characteristics: morphological appearance, staining properties, colonial characteristics and the rate of growth on oleic acid-albumin agar plates, and pathogenicity for mice. It was, however, upon examination of their biochemical state that striking differences were revealed.

A comparison of the general metabolic activity of H37Rv and Lrv, as measured by hydrogen transfer capacity, established a large difference between them (table 2). The reading of 80 per cent transmittance shown by the lung bacilli represents a very low state of metabolic activity particularly in comparison to the reading of 29 per cent for H37Rv. This result is of added interest in the light of the correlation previously demonstrated (Segal and Bloch, 1955) between virulence of three strains of *M. tuberculosis* grown in vitro and degree of hydrogen transfer capacity. An avirulent strain (H37Ra) exhibited the highest capacity of hydrogen transfer (10 per cent T), an attenuated strain (BCG) was intermediate (20 per cent T), and the virulent strain (H37Rv) showed the least activity (28 per cent T). This decreasing scale of metabolic activity as correlated with increase in virulence of the strain would indicate a high state of virulence for the activity shown by tubercle bacilli separated from infected lungs, although this has not as yet been verified by direct animal test.

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**Table 1**

Quantitative comparison of suspensions of in vitro and in vivo grown tubercle bacilli

<table>
<thead>
<tr>
<th>Type</th>
<th>Source and Age</th>
<th>Avg Yield</th>
<th>Dry Wt*</th>
<th>Plate Count*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>10-day Tween culture</td>
<td>8 per flask of 30 ml</td>
<td>5.6</td>
<td>3.2 \times 10^9</td>
</tr>
<tr>
<td>Lrv</td>
<td>Lungs of moribund or dead mice</td>
<td>4 per lungs of 1 mouse</td>
<td>5.3</td>
<td>1.9 \times 10^9</td>
</tr>
</tbody>
</table>

* Both suspensions of H37Rv and Lrv were adjusted to 10 per cent transmittance in the Coleman Universal Spectrophotometer.
† Initially mice were infected with H37Rv, but thereafter with Lrv.

**Table 2**

Comparison of metabolic activity of H37Rv and Lrv

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dry wt</th>
<th>Hydrogen Transfer Capacity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>H37Rv</td>
<td>2.8</td>
<td>29</td>
</tr>
<tr>
<td>Lrv</td>
<td>2.6</td>
<td>80</td>
</tr>
</tbody>
</table>

* Substrate was complex, made up of tween-albumin medium with 0.5 per cent glucose.
† Per cent transmittance of reduced 2,3,5-triphenyl tetrazolium chloride measured photometrically after 2-hr incubation.
TABLE 3

<table>
<thead>
<tr>
<th>Substrate (Final Conc in Vessel)</th>
<th>H37Rv</th>
<th>LRv</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Qo₂*</td>
<td>Increase over endogenous</td>
</tr>
<tr>
<td>Complex media:</td>
<td>Substrate</td>
<td>Endogenous</td>
</tr>
<tr>
<td>0.5 per cent tween-albumin medium</td>
<td>17.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Infected lung filtrate†</td>
<td>7.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Normal lung filtrate†</td>
<td>4.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Yeast extract (Difco) (1 per cent)</td>
<td>7.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Carbohydrate and intermediates:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (0.2 m)</td>
<td>7.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Glycerol (0.2 m)</td>
<td>10.1</td>
<td>5.0</td>
</tr>
<tr>
<td>n-Lactic acid (0.0038 m)</td>
<td>7.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Sodium acetate (0.0122 m)</td>
<td>8.2</td>
<td>5.4</td>
</tr>
<tr>
<td>Sodium pyruvate (0.01 m)</td>
<td>7.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Benzoate and related compounds:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium benzoate (0.0035 m)</td>
<td>8.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Benzoaldehyde (0.0047 m)</td>
<td>8.0</td>
<td>5.0</td>
</tr>
<tr>
<td>m-Hydroxybenzoic acid (0.0018 m)</td>
<td>4.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Sodium salicylate (0.0016 m)</td>
<td>9.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Fatty acids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Heptanoic acid (m/300)</td>
<td>8.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Octanoic acid (m/300)</td>
<td>8.4</td>
<td>5.7</td>
</tr>
<tr>
<td>Oleic acid (m/1000)</td>
<td>10.2</td>
<td>5.6</td>
</tr>
</tbody>
</table>

* Each value represents the mean of between 3 and 7 parallel experiments. Statistical analysis applied to replicate values for each substrate response and its corresponding replicate values of endogenous Qo₂ established significance for all positive responses above the endogenous Qo₂ with the highest values of P being <0.05.

† Filtrates of infected and normal lungs were prepared by homogenization of the lungs of 3 mice in 10 ml of phosphate buffer, pH 6.8, centrifugation at 3,000 rpm for 15 min, and Seitz filtration of the supernatant.

The comparative metabolic activity of lung and artificially cultured tubercle bacilli was further tested by measuring respiratory response to specific substrates in the Warburg. It was found that both H37Rv and LRv have a similar endogenous rate of respiration in M/15 phosphate buffer at pH 6.8, with Qo₂ values ranging from 4.1 to 6.1 for H37Rv and 4.1 to 5.6 for LRv. These values apply to short range runs of 2 or 3 hr. There is some evidence that endogenous reserves are greater for lung bacilli which show higher levels of endogenous respiration over a 20-hr period. For each experiment to be reported, the endogenous Qo₂ was determined routinely in order to determine specific substrate response. In addition, the constancy of this value also served as an excellent criterion for purity and intactness of the separated lung bacilli.

Mean Qo₂ values of substrate response and of corresponding endogenous oxygen uptake obtained from the results of between 3 to 7 repeated experiments are presented in table 3. Statistical analysis applied to replicate values for each substrate response and its corresponding replicate values of endogenous Qo₂ established significance for all positive responses above the endogenous Qo₂ with the highest values of P being <0.05. These values represent two types of experiments: one in which a group of related substrates was tested using aliquots of the same batch of cells; the other in which different batches of bacilli were used to test the same substrate. The use of bacilli harvested at different times from cultures of H37Rv and from infected lungs largely accounts for their variation in endogenous respiration. However, this variation did not appreciably alter the net per cent increase in substrate response. The limited variations that did occur were in the

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*Footnotes and references have been omitted for brevity.*

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opposite direction to what might be expected mathematically, that is, a lower endogenous respiration generally resulted in a somewhat lowered substrate response on a percentage basis. When aliquots of the same batch of bacilli were used, little variation occurred. This particularly applied to suspensions of LRv which contain small aggregates of 1 to 30 bacilli and which do not clump in water, saline, or phosphate solutions. Equal aliquots of suspensions of H37Rv were obtained by the inclusion of 0.02 per cent tween 80 in the salt solution in which H37Rv was adjusted and from which aliquots were taken before final saline washing and suspension in phosphate buffer for use in the Warburg vessel.

Several complex media were used as substrates in an attempt to see whether the respiratory response of lung bacilli could be activated as known for H37Rv grown in vitro, and to obtain some clue to the kind of materials to which it might respond. The results are presented in table 3. Paralleling the low hydrogen transfer capacity shown previously for LRv, 0.5 per cent tween-albumin medium gave only a limited mean response of 59.1 per cent increase over the endogenous respiration as compared with the large increase of over 300 per cent for H37Rv. Infected lung filtrate gave a definite response indicating the absence of toxic activity for both LRv and H37Rv. Normal lung filtrate and yeast extract (Difco) failed to stimulate the respiration of lung bacilli, whereas H37Rv responded to yeast extract (Difco) with a mean increase in respiration of 75.0 per cent over the endogenous.

On the basis of these results, the response to specific substrates which have been reported in the literature to stimulate the oxygen uptake of H37Rv was measured for both H37Rv and LRv. The concentrations of the different substrates tested are based on those reported in the literature.

The comparative effect of carbohydrate and several intermediates of glycolysis showed a striking difference in the biochemical activity of H37Rv and LRv as seen in table 3. The respiration response of H37Rv to glucose, glycerol, sodium lactate (Loebel, Shorr, and Richardson, 1933), sodium acetate, and sodium pyruvate (Cutinelli, 1940) was found to be consistent with that reported in the literature, while tubercle bacilli separated from lung tissue gave no response above that of the endogenous to any of these substrates.

The comparative effect of sodium benzoate and related compounds is summarized in table 3. Consistent with earlier observations (Bernheim, 1941), benzoate, benzaldehyde, and salicylate (o-hydroxybenzoate) all produced considerable increase in oxygen uptake of H37Rv over the endogenous, whereas m-hydroxybenzoate failed to stimulate respiration. On the other hand, the lung bacilli failed to respond to benzoate and benzaldehyde as well as to m-hydroxybenzoate, but for the first time showed a very marked response to salicylate, consistently above that shown for H37Rv. The singular response of in vivo grown M. tuberculosis to salicylate is especially noteworthy in view of the significance that has been attached to respiratory response to this substance as correlated with pathogenicity of mycobacteria by Bernheim (1942) and Lehmann (1946), who reported a negative response for nonpathogenic strains. More recently Pope (1954) has reported that this correlation does not apply when a large number of virulent, avirulent, and attenuated strains are tested. We have also found that the avirulent strain H37Ra does respond to salicylate (0.0016 M) to the extent of 45.5 per cent increase of oxygen uptake over the endogenous respiration. This result is in partial agreement with the observations of Bernheim and of Lehmann in that the avirulent strain, H37Ra, showed a considerably lower response to the same concentration of salicylate than that of the virulent strain H37Rv (table 3: 89.6 per cent).

The response of H37Rv and LRv to several fatty acids was determined. Loebel et al. (1933) showed that 0.1 per cent oleic acid increased the respiration of H37Rv, and Cutinelli (1940) observed marked respiratory stimulation of a human pathogenic strain by normal saturated fatty acids of chain length C12 to C18. As mentioned previously, sodium acetate (listed under the heading “Carbohydrate and intermediates” in table 3) did not stimulate the oxygen uptake of LRv in contrast to the mean increase of 51.8 per cent observed for H37Rv. However, the other three fatty acids tested significantly stimulated respiration of LRv to degrees similar to those observed for H37Rv. The significance of this more generalized response of lung bacilli to fatty acids will be discussed later in this paper.

In order to examine the stability of the ob-
served biochemical characteristics of LRv, separated lung bacilli were inoculated into tween-albumin medium (Dubos and Middlebrook, 1947). The rate of growth was slow and in some cases no growth resulted. However, sufficient growth occurred in the first passage to supply bacilli for testing in the Warburg. As seen from the results presented in table 4, the lung bacilli after one passage through tween-albumin medium (LTRv) behaved just as H37Rv, with full response to glycerol and acetate in contrast to LRv. Also the response to salicylate was lower than that obtained for LRv. These results indicate reversion of the lung bacilli to the original H37Rv strain after primary growth in tween-albumin medium.

A series of control experiments was conducted in order to eliminate the possible operation of other factors in the observed effects of differential substrate response by H37Rv and LRv. The use of moribund or dead mice, and the mode of killing mice (ether or bleeding) was found to have no effect on the respiration of bacilli separated from their lungs. It was important to use mice with heavily infected lungs in order to obtain adequate yields and purity of separation. The possible damaging effect of the separation procedure as an explanation of the limited respiratory response of LRv was discounted by applying the entire procedure to H37Rv homogenised with mildly infected lungs in isotonic sucrose containing 0.25 per cent albumin. It was also necessary to add 0.1 per cent tween 80 to the homogenizing medium in order to prevent flocculation of H37Rv—an effect which does not occur with lung bacilli which suspend readily in water, saline, or sugar solutions. As seen in table 5, the endogenous Qo was not significantly affected by the separation procedure. Nor was respiratory response to two specific substrates, glycerol and salicylate, appreciably altered.

Further indication of the absence of inhibitory substances in infected lung homogenate was obtained by including diluted homogenate of mildly infected lungs in the vessel with H37Rv, with and without substrate (table 5). Without substrate there was a limited increase over the endogenous respiration. With lactate the presence of diluted homogenate had no inhibitory effect on oxygen uptake by H37Rv.

The possibility that tissue enzymes carried over in the separation of LRv might account for substrate response was eliminated. Normal and infected lung filtrates resulted in no uptake of oxygen when lactate or salicylate was included.
in the Warburg vessels in the absence of bacilli. Furthermore, even when a diluted homogenate from a mildly infected lung was included with several substrates, two of which had stimulated oxygen uptake of LRv (salicylate and oleic acid), no enhancement of response was obtained.

The inclusion of 0.5 per cent tween 80 or 2.5 per cent albumin alone or in combination was shown to have no effect on the negative response of LRv to glycerol. In the case of H37Rv the presence of 0.5 per cent tween 80 resulted in only a partial increase in response to glycerol.

The endogenous respiration and substrate response of LRv was determined at three levels of pH—6.0, 6.8, and 7.6—to test for the possible effect of pH. No significant increase in the negative response of LRv to glucose, glycerol, and lactate was observed. The endogenous respiration of LRv also remained unaffected by changes in pH.

**DISCUSSION**

The development of a method for the separation of tubercle bacilli from lungs of infected mice has enabled a comparison of these bacteria with those of the same strain grown in artificial culture medium. On the basis of the differences reported in this paper in respect to substrate stimulation of respiration, it is quite possible that an intensive study will reveal basic differences in the metabolism of in vivo grown bacilli as compared to those grown in vitro. The implications of these findings are far-reaching. The results presented disprove the commonly held assumption that virulent tubercle bacilli grown artificially are identical to bacilli in the infected animal. Although this assumption has not been explicitly stated, it is implicit in most of the work on the tubercle bacillus, particularly that which has attempted to relate effects obtained with the organism grown in vitro to the pathogenicity of the organism in vivo. The most common attempt to do this has been to apply the method of comparative biochemistry to a study of virulent, attenuated, and avirulent strains. Differential responses to various test conditions that could be correlated with virulence of strain have been considered to reflect the nature of the host-parasite relationship and the nature of pathogenicity.

From the limited results presented in this paper the biochemical differences that have been shown in the literature to exist between avirulent and virulent strains of *M. tuberculosis* are relatively minor compared to the differences shown here between a virulent strain grown in vitro and the same strain separated from growth in vivo. The differences are sufficient in scope to justify classification of H37Rv and LRv as strain variants. This is not meant to imply that there is an absolute difference between them. On the basis of the reversion of LRv to H37Rv after growth in artificial culture medium (table 4), a more likely explanation of the observed biochemical differences would seem to be adaptation to widely diverse environments. Thus LRv might be expected to respond, upon prolonged incubation, to substrates to which its respiratory response has been negative within a 3-hr period. To date, extension of the test period to as long as 20 hr has failed to enhance the capacity for increased respiratory response to substrates. It is possible that a more complex supply of nutrients and a still longer period of incubation are necessary before LRv can adapt to the oxidation of foreign substrates in a Warburg vessel. That it does so eventually is clearly evident from its reversion to H37Rv after primary growth in artificial culture medium. The reverse process of conversion of H37Rv to LRv that evidently takes place in vivo should theoretically be possible in vitro, assuming an adaptive mechanism for the changeover. This could be accomplished by duplication in vitro of the essential environmental conditions in infected tissue that determine the dissimilar characteristics of LRv. Such determinants will be reflected by a comparative study of in vivo and in vitro grown tubercle bacilli.

Since the observed differences are biochemical in nature, it is likely that considerable differences in chemical composition exist. The ease with which LRv suspends in water, saline, or sucrose solutions in contrast to H37Rv, which requires the presence of a surface active agent such as tween 80, indicates an important difference in surface properties which is probably due to a difference in chemical structure. The two reports cited earlier in this paper (Anderson et al., 1943; Sheehan and Whitwell, 1949) also point to a chemical difference between bacilli grown in artificial culture and those growing in infected tissue. In turn, differences in immunogenic properties might also be found to occur. Preliminary results have been obtained which show marked differ-
ences between the immunogenic properties of phenol-killed suspensions of LRV and H37Rv.

The lack of differences in growth characteristics of submerged cultures as well as of colonies on agar plates is not surprising in view of the findings shown in table 4, where after one growth-passage through tween-albumin medium, LRV has reverted to H37Rv in respect to biochemical characteristics. Differences in growth properties would be expected to occur as a function of this adaptive cycle ending in reversion. The delay of several days in the growth of LRV transferred to artificial culture medium indicates the adaptive nature of its change to H37Rv.

Other questions arise in attempting to explain the differences between LRV and H37Rv. The factor of permeability might be operative, but this would not readily explain the specific differential response to salicylate. In addition, the presence of tween 80 had no effect on negative responses of LRV to glycerol. The explanation that LRV does not react due to an over supply of endogenous food reserves is relevant. Although evidence to date indicates that endogenous respiration is more stable over a 20-hr period for lung bacilli, this need not necessarily result in negative substrate response. Again the differential responses to salicylate and the fatty acids as well as response to infected lung filtrate suggest that high endogenous reserves are not a factor. A few trials have been made to test response after preliminary starvation but with no success. However, neither of these possible explanations has been ruled out and further studies using the starvation technique and cell-free extracts have been initiated toward this end.

The possible operation of such factors as damage, inhibition, or stimulation in determining respiratory response of LRV to substrates as a result of the method of separation or due to the possible presence of traces of tissue material, has been eliminated by the various control experiments described earlier in this paper. Lastly, pH was also shown not to be a determining factor.

The nature of the positive responses of LRV to salicylate and higher fatty acids remains to be determined. The question whether the stimulation of oxygen uptake by these compounds is only indirect or whether these substances are utilized and actively metabolized is primary. Fitzgerald and Bernheim (1948) concluded that salicylic acid is not oxidized by pathogenic tubercle bacilli (H37Rv), and attributed its stimulating effect on oxygen uptake to catalysis of endogenous reactions. A more extensive testing of compounds of biological importance might offer more clues regarding the nature of the specific responses thus far observed.

Related to the question of high endogenous food reserves is the impression given by the results that lung bacilli present in infected tissues exist in a reduced state of activity and upon separation appear sluggish and nonreactive. However, the very marked responses to fatty acids and particularly salicylate contradict this. This apparent limited range of activity is quite consistent with the well-established correlation that bacteria are increasingly fastidious in their nutritional requirements when they are parasitic in their natural environment and as their parasitism is more obligatory. The mycobacteria fit the pattern well. Franke and Schillinger (1944) have indicated that the range of substrates oxidized by mycobacteria decreases sharply as acid fastness and pathogenicity increase. Hanks and Gray (1954) have classified the mycobacteria according to their metabolic properties, illustrating that enhancement of respiratory response becomes more and more limited in a scale extending from the various saprophytes through the tubercle bacillus to M. Johnei, M. leprae, and M. leprae, the most parasitic of all.

The negative response of LRV to carbohydrates and its positive response to fatty acids places it high in the scale of pathogenicity according to this scheme of Hanks and Gray, actually beyond the level of listing of the tubercle bacillus, which has been based on studies using in vitro grown bacilli.

Whatever the eventual elucidation of mechanisms involved in producing these differences of in vivo and in vitro grown tubercle bacillus, their existence is definite. By means of this approach a truer picture of the tubercle bacillus in its natural parasitic state can now be obtained.

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SUMMARY

The development of a method for the separation of Mycobacterium tuberculosis in large numbers directly from the lungs of infected mice has
enabled a comparative biochemical study of in vivo and in vitro grown bacilli of the same strain.

Saline suspensions of tubercle bacilli separated from infected lungs (LRv) were shown to be quantitatively comparable, as to dry weight and plate counts, to equally dense suspensions of virulent tubercle bacilli of the same strain grown on artificial culture medium (H37Rv). This finding confirmed the purity of separation of bacilli from tissue, as determined by microscopic examination, and established a quantitative basis for biochemical comparison of LRv and H37Rv.

A large difference in hydrogen transfer capacity was shown to exist between LRv and H37Rv, with the lung bacilli exhibiting a much lower state of metabolic activity in the presence of substrate. Paralleling the low hydrogen transfer capacity of LRv, complex culture media had little or no stimulating effect on its endogenous respiration, whereas they produced a large increase in oxygen uptake over the endogenous respiration of H37Rv. The endogenous rate of respiration was found to be similar for both types of bacilli.

Striking differences in the biochemical activity of H37Rv and LRv were observed with respect to substrate response. The positive respiratory responses of H37Rv to glucose, glycerol, lactic acid, sodium acetate, sodium pyruvate, sodium benzoate, benzaldehyde, sodium salicylate, 3-heptanoin, octanoic, and oleic acids were found to be consistent with reports in the literature. In contrast, only sodium salicylate and the latter three fatty acids were found capable of stimulating the endogenous respiration of LRv.

The adaptive nature of the observed biochemical differences was indicated by reversion of LRv to H37Rv with respect to response to glycerol and sodium acetate after primary growth in artificial culture medium.

These results indicate that tubercle bacilli grown artificially are dissimilar to bacilli of the same strain as they multiply in the lungs of an infected animal.

**Addendum**

More recently other biological differences have been found to exist between in vivo and in vitro grown tubercle bacilli. LRv exhibits a greater degree of pathogenicity for mice, but a lesser immunizing capacity than H37Rv. These results will be published in the near future.

**REFERENCES**


BIOCHEMICAL DIFFERENTIATION OF M. TUBERCULOSIS


