DETERMINATION OF DEHYDROGENASES IN ATYPICAL STRAINS AND IN OTHER MYCOBACTERIA

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The dehydrogenase activity in mycobacteria is a promising field of research for the following reasons. Youmans et al. (1955, 1957) showed that small particles, containing dehydrogenases and possessing characteristics of mitochondria, stimulate in mice the production of a degree of immunity which is equal to the immunity produced by a 1-mg quantity of whole living cells of H37Ra. On the other hand, Martin et al. (1955) demonstrated that dehydrogenase activity is diminished in the kidney and other organs of guinea pigs in which tuberculosis develops. Therefore, any study of dehydrogenase activity in mycobacteria may contribute to our knowledge of the problem of immunity in tuberculosis. The striking difference in behavior of atypical strains and human strains in the presence of neotetrazolium is certainly another reason to investigate the field of dehydrogenase activity more closely (Gastambide-Odier and Smith, 1958).

Some data on the dehydrogenase activity of mycobacteria can be found in the review by Bönicke (1956/1957) in which the results obtained with 508 mycobacterial strains, using methylene blue as H acceptor, are given. In recent years, other workers used tetrazolium salts for these measurements. Hanks (1951, 1956) showed differences between the hydrogen transfer capacity of Mycobacterium lepraeumurium and Mycobacterium phlei under anaerobic conditions. Vandiviere et al. (1952) established a rapid test of viability based upon triphenyltetrazolium chloride reduction. He worked with R1Rv and BCG strains and used 1.2 to 3.2 mg of bacilli. De Góes et al. (1952–1953) compared the reducing activity of saprophytic strain and human strains. Winling (1953) reviewed many data on tetrazolium salts and described a few applications of these compounds to the study of mycobacteria. Kanai and Yanagisawa (1954) studied in detail the technique of triphenyltetrazolium chloride reduction as a test of viability. They worked with strain H37Rv, whereas Arima et al. (1954) performed the same type of experiments on a BCG strain. The inconvenience of the techniques of the Japanese authors is that they grew the bacilli on Sauton’s medium where only surface growth is obtained. They used very large quantities of bacteria, from 3 to 40 mg, in some instances even more. Koch-Weser and Ebert (1955) studied the reduction of triphenyltetrazolium chloride by the H37Rv strain in Dubos Tween-albumin medium. Segal and Bloch (1956) demonstrated differences in dehydrogenase activity between strains of H37Rv grown in vivo and in vitro. D’Arcy-Hart and Rees (1956) showed differences in dehydrogenase activity between virulent and avirulent strains.

Some observations made by Ling et al. (1957) and by Sourkes and Lagnado (1957) suggest that the mechanism of reduction of methylene blue and tetrazolium salts are different but still incompletely understood. To our knowledge, neotetrazolium has not been used previously in quantitative measurements of dehydrogenase activity in mycobacteria. This substance has numerous advantages over methylene blue. In the reduced state, it is colored purple, the reduction proceeds irreversibly at pH 7, and the reduced acceptor is not sensitive to oxygen. It has also advantages over triphenyltetrazolium chloride. It is much less readily reduced by light (Smith, 1951; Glock and Jensen, 1953; Mustakallio et al., 1955) and the dehydrogenase activity of such small quantities of bacteria as 0.5 to 1 mg can be measured with this compound. If the dehydrogenase level of the strain is high, as it is the case for Myco-

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bacterium fortuitum, even 0.2 mg of bacteria are enough for one measurement.

In this paper, neotetrazolium is used to compare the dehydrogenase activity of atypical tubercle bacilli with the dehydrogenase activity of various other strains of mycobacteria.

METHODS

The method used was mainly taken from the method described by Jardetzky and Glick (1956), but useful information was also found in papers by Glock and Jensen (1953), Mustakallio et al. (1955), Kun and Abood (1949), Bodine and Fitzgerald (1949), and Anderson (1955).

Growth measurements. For each strain to be studied, 18 tubes containing 5 ml of Tween-albumin medium were inoculated with 2 drops of culture at an optical density of 0.05 (640 mµ) and the tubes were then incubated at 37 C. At regular intervals, growth was measured at 640 mµ in a Coleman Junior spectrophotometer, and two tubes were taken for the determination of reducing activity.

Reducing activity measurements. A given volume of culture was taken for the measurement of reducing activity and was transferred to thick-walled Hopkins type tubes. These tubes were centrifuged at 3600 rpm for 10 min, the supernatants discarded, and the sediment washed 3 times with phosphate buffer (pH 7) containing Tween 80 (1.25 ml in 500 ml buffer). After the third washing, 1 ml of 1:10,000 neotetrazolium (in buffer) was added to each tube. The tubes

Since neotetrazolium is irreversibly reduced when it is transformed into the purple diformazan, its presence creates abnormal physiological conditions. Therefore, in dehydrogenase determinations, the problem is to use a concentration which is not too harmful for the bacteria but at the same time concentrated enough so as to measure the total quantity of dehydrogenase present. Because of its high sensitivity to the inhibitory action of neotetrazolium (Gastambide-Odier and Smith, 1958), Mycobacterium phlei was used in experiments designed to determine this concentration. A 1:10,000 concentration of neotetrazolium was found to contain about 4 times the quantity required for the measurement of dehydrogenase activity and to give the maximal amount of reduced dye (diformazan). After overnight contact with neotetrazolium at 1:10,000 in phosphate buffer, all strains except the human strain and Mycobacterium smegmatis, were still capable of multiplying at a normal rate if they were subcultivated into Dubos Tween-albumin medium.

<table>
<thead>
<tr>
<th>Strain of Bacilli</th>
<th>Time (Days)</th>
<th>Reducing Activity (Optical Density 530 mµ)</th>
<th>Growth (Optical Density 640 mµ)</th>
<th>Ratio: Reducing Activity/Amount of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (H37Rv)</td>
<td>1</td>
<td>0.010</td>
<td>0.003</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.169 × 2</td>
<td>0.029</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.490 × 2</td>
<td>0.089</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.328 × 2.5</td>
<td>0.138</td>
<td>5.9</td>
</tr>
<tr>
<td>Bovine (virulent)</td>
<td>2</td>
<td>0.034</td>
<td>0.007</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.303 × 2</td>
<td>0.055</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.311 × 2.5</td>
<td>0.128</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.398 × 2.5</td>
<td>0.173</td>
<td>4.9</td>
</tr>
<tr>
<td>Avian (virulent)</td>
<td>1</td>
<td>0.027</td>
<td>0.005</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.102</td>
<td>0.020</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.214</td>
<td>0.028</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.230 × 2</td>
<td>0.037</td>
<td>12.4</td>
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<tr>
<td></td>
<td>12</td>
<td>0.339 × 2</td>
<td>0.050</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.379 × 2</td>
<td>0.060</td>
<td>12.6</td>
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<tr>
<td>Mycobacterium</td>
<td>1</td>
<td>0.055</td>
<td>0.019</td>
<td>2.9</td>
</tr>
<tr>
<td>phlei</td>
<td>2</td>
<td>0.333 × 2</td>
<td>0.062</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.352 × 2.5</td>
<td>0.104</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.229 × 2.5</td>
<td>0.141</td>
<td>4.1</td>
</tr>
<tr>
<td>Atypical† acid-</td>
<td>1</td>
<td>0.016</td>
<td>0.012</td>
<td>1.3</td>
</tr>
<tr>
<td>fast bacillus</td>
<td>3</td>
<td>0.367</td>
<td>0.031</td>
<td>11.9</td>
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<tr>
<td>no. 4 (photo-</td>
<td>5</td>
<td>0.259 × 2</td>
<td>0.038</td>
<td>13.6</td>
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<tr>
<td>chromogen)</td>
<td>7</td>
<td>0.394 × 2.5</td>
<td>0.057</td>
<td>17.3</td>
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<tr>
<td></td>
<td>9</td>
<td>0.510 × 2.5</td>
<td>0.052</td>
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<td></td>
<td>13</td>
<td>0.179 × 5</td>
<td>0.080</td>
<td>11.2</td>
</tr>
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<td></td>
<td>16</td>
<td>0.173 × 5</td>
<td>0.115</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>0.178 × 5</td>
<td>0.128</td>
<td>7.0</td>
</tr>
<tr>
<td>Atypical† acid-</td>
<td>1</td>
<td>0.114</td>
<td>0.014</td>
<td>8.1</td>
</tr>
<tr>
<td>fast bacillus</td>
<td>3</td>
<td>0.049 × 2</td>
<td>0.025</td>
<td>3.9</td>
</tr>
<tr>
<td>no. 7 (nonpho-</td>
<td>6</td>
<td>0.120 × 2</td>
<td>0.035</td>
<td>6.9</td>
</tr>
<tr>
<td>tochromogen)</td>
<td>9</td>
<td>0.435 × 2</td>
<td>0.058</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.254 × 2.5</td>
<td>0.087</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* As an indication of the error in these measurements, the standard deviation for the growth measurement of this culture at 3 days (8 separate tubes) was 0.006. The standard deviation for 8 separate determinations of reducing activity was 0.072 and the standard deviation for the ratio reducing activity to amount of growth was 0.6.

† The classification is that proposed by Timpe and Runyon (1954) and corresponds to the strains in the collection made available by the Laboratory Subcommittee of the American Trudeau Society.
were placed overnight at 37 C. On the following morning, the tubes were again centrifuged, the uncolored supernatants were discarded, and the diformazan was extracted from the cells with 2.5 ml of alcohol-tetrachlorethylene (1:1) overnight at 37 C. The tubes were then centrifuged and the clear supernatant was transferred to calibrated nephelometric tubes (10 by 75 mm). The optical density of the extracted diformazan was measured at 520 m in a Coleman Junior spectrophotometer. By comparing readings obtained with the data of a preestablished calibration curve, the quantity of diformazan could easily be determined. In the range of optical density from 0.06 to 0.7, the quantities of diformazan were proportional to the optical density. All necessary precautions were taken to make reducing activity measurements only in this zone: at the beginning of growth, the measurements were made with 5 ml of culture, whereas at the end of the growth period they were made with 1 or 2.5 ml of culture. In blanks, made by autoclaving the cells after washing and before the 1:10,000 neotetrazolium was added, none of the strains reduced this substance.

**RESULTS**

Growth and reducing activity measurements as a function of time were performed on a total of 7 atypical strains and on 9 known mycobacterial strains. Duplicate samples were used for each measurement. In table 1 are given the details of variation of reducing activity with age of culture for selected strains from each type studied. The maximal ratio of reducing activity to amount of growth is shown in italics for each culture. In table 2, the maximal ratio of reducing activity to amount of growth for additional strains is presented.

**DISCUSSION**

It can be seen from an examination of tables 1 and 2 that *M. fortuitum* has an extremely high reducing activity. It is also obvious that the group of atypical acid-fast bacilli is not a homogeneous group with respect to dehydrogenase activity.
activity. The two photochromogenic strains and two nonphotochromogenic strains have a very high dehydrogenase activity, whereas one nonphotochromogenic and two scotochromogenic strains have lower dehydrogenase activities which do not differ much from the dehydrogenase activity of Mycobacterium avium. Froman et al. (1957), using phage typing techniques (Will et al., 1957), classifies these strains (no. 5, 6, and 7) with strain 3 among the atypical strains of the avian type. The data obtained here show that strains 3 (high reducing activity) and 5 (the highest ratio of reducing activity to amount of growth is reached in 3 days) are different from strains 6 and 7 (for these strains as well as for M. avium, the highest ratio of reducing activity to amount of growth is reached in 9 to 12 days). Mycobacterium smegmatis can be differentiated from M. phlei on the basis of its very low dehydrogenase activity. There is also a suggestion that the reducing activity for a recently isolated culture of the human variety of tubercle bacillus is less than for standard laboratory strains of the same type of organism (H37Rv and H37Ra).

It is known that the photochromogens are more microaerophilic than the scotochromogens, whereas the human strains are aerobic (Koch et al., 1958). The differences in reducing activity observed here might be related to the microaerophilic character of the atypical strains.

Strains of M. fortuitum that have a high dehydrogenase activity may be sensitive to the inhibitory action of neotetrazolium, whereas strains which are only weakly inhibited by neotetrazolium may have a low level of dehydrogenase (strain 6) (Gastambide-Odier and Smith, 1958). Therefore, the dehydrogenase systems are probably not the only systems involved when neotetrazolium acts as an inhibitor.

Concerning the classification of mycobacteria, the results obtained with the present neotetrazolium technique are quite different from those obtained with the methylene blue technique. With neotetrazolium, no important difference can be observed between saprophytic, human, and bovine strains, whereas with methylene blue, it is possible to differentiate saprophytic strains from human and bovine strains (Bloch, 1950).

**SUMMARY**

The method of determination of dehydrogenase activity by means of neotetrazolium is applied to mycobacteria and technical details concerning these measurements are given.

*Mycobacterium fortuitum* has the highest level of dehydrogenase activity, whereas a recently isolated human strain and *Mycobacterium smegmatis* have a very low dehydrogenase activity. The atypical strains are not a homogeneous group. Four strains have a very high dehydrogenase activity and can easily be differentiated from other mycobacteria, although this differentiation is not possible for three other atypical strains.

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