TETRAZOLIUM REDUCTION TEST AS A MEASURE TO EXAMINE THE TOTAL VIABILITY OF SUSPENSIONS OF TUBERCLE BACILLI

I. STUDIES ON THE TECHNIQUES OF THE TEST

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INTRODUCTION

In 1941, Kuhn and Jerchel reported, citing the pioneering observations of several authors including those of themselves, that tetrazolium salt, a colorless compound, is reduced to the red formazan by the enzymatic activity of living cells. Here is a very rare example in organic chemistry that the reduced state of a dye is colored and the oxidized one is colorless. In addition, this red substance has convenient properties that it is stable against oxygen, water insoluble, but soluble in organic solvents such as aceton, ether and so on. Later studies on this phenomenon presented an additional support for the applicability of tetrazolium salts for many kinds of experimental purposes in biology and medicine.

Black and his co-workers have engaged in their cancer research employing 2, 3, 5 triphenyltetrazolium chloride (TTC). Mac Kenzie and Fuller, and Schuermann also used this substance as an indicator for malignant tumors. Some others attached their interest to the study of cell metabolism by the tetrazolium reduction phenomenon. These investigations are based in principle upon the dehydrogenase activity of cells measured by the amount of formazan produced by them. On the other hand, the experiments by Mudd et al, and Burns and Militzer suggested that the localization of TTC reaction in bacteria may be the mitochondoria. Now, the recent paper of Mac Vandiviere showed that the total viability of a suspension of tubercle bacilli could be estimated by photometric measurement of the optical density of aceton solution of the reduced TTC produced by living tubercle bacilli. This report stimulated us considerably and led to the attempt to confirm and extend his observation.

The present paper deals chiefly with the fundamental aspects of the techniques of the TTC reduction test against tubercle bacilli, presenting the data obtained in the following experiments which were made for the purpose of detecting the most suitable condition for the test.

MATERIALS AND METHODS

1. The strain H37Rv was employed through these experiments except one case. The strain was kept subcultured on Sauton synthetic liquid media dis-
pensed into 100 ml Erlenmeyer flasks in the amount of 30 ml. These cultures served for the occasional preparation of bacillary suspension used in the experiments. The procedure was as follows: The bacillary mass of growth on the media was harvested on filter paper attached to a funnel and washed with distilled water. Then, it was transferred into layered folds of absorbent filter paper to remove the moisture. An appropriate amount of this partially dried material was weighed on a balance and put into a 500 ml round hard glass flask containing 70 crystal balls of 7 mm in diameter. These steps were conducted aseptically with sterile equipments and materials. Then, the bacillary mass was ground by rotating the flask by hand for three minutes, during which sterile 1/15 M phosphate buffer (pH 7.0) was pipetted into it little by little up to 3 ml. And finally, a needed amount of the buffer was further added to make the concentration of the homogeneous bacillary suspension as desired.

2. 2, 3, 5 triphenyltetrazolium chloride (TTC) was used in the form of 1/15 M phosphate buffer solution of pH 7.0.

3. The tetrazolium reduction test was performed in Wassermann type tubes, arranging at least two tubes to each kind of reaction systems which consisted of bacillary suspension, TTC solution and substrate. The bacillary suspension prepared as above was dispensed in the amount of 0.5 ml to the tubes and 0.2 ml of a TTC solution was mixed with each of them. When substrates were employed, 0.3 ml of a 1/100 M aqueous solution of them was further added to the tubes, then they were placed in an incubator maintained at 37°C. In these conditions the colorless TTC is reduced by enzymatic activity to a red water insoluble formazan. After a lapse of definite time, the tubes were taken from the incubator and 0.2 ml of 10 per cent neutral formalin was added to stop the reaction. Then, 2 ml of aceton was added to them and they were agitated vigorously to extract the dye bound in the bacillary bodies. When the color intensity of aceton solution of the dye was too strong, more aceton was added in order to bring it within the proper range of the colorimeter scale. The extraction of the dye was made easier when the tubes were immersed into a water-bath at 50°C for several seconds. The extraction being complete, the tubes were centrifuged at 4,000 R.P.M. for ten minutes.

4. The supernatants were transferred into special cuvettes of 10 mm diameter and presented for the measurement of the optical density by a Coleman spectrophotometer. The uncolored supernatant from the tube in which killed bacilli had been used instead of living bacilli served as a blank of the measurement as the optical density O.

**EXPERIMENTAL**

1. *Absorption curve of the aceton solution of reduced TTC* In the first place, the wave length suitable for the spectrophotometric measurement of the
TETRAZOLIUM REDUCTION TEST

1. Color intensity of the aceton solution of reduced TTC was determined experimentally. Mac Vandiviere\textsuperscript{11} used a 550\textmu m filter, and Black\textsuperscript{2} worked using a Coleman spectrophotometer set at 470\textmu m. 0.2 ml of a 0.25 per cent solution of TTC was reduced to formazan by the action of 6 mg of the H37Rv bacilli suspended in 0.5 ml of the buffer solution for three hours. Then, according to the method already described, the color intensity of formazan solved in aceton was measured using various kinds of wave length from 400\textmu m to 700\textmu m with 25\textmu m interval. Plotting the readings of the optical density to each wave length, an absorption curve was formed as shown in Fig. 1. The maximum absorption was proved to occur at 475\textmu m. This means that 475\textmu m is suitable for the test. The similar experiment was repeated with 7 mg of another strain of the acid fast bacillus (Smegma). This strain had a property to produce yellow pigment abundantly into Sauton medium, but this did not interfere with the nature of the color of TTC reduced by them, as shown in Fig. 1. This shows that the maximum absorption occurs in 475\textmu m also in this strain. The reading of the TTC reactions was made in one hour in the latter case.

2. Toxicity of TTC against tubercle bacilli It is generally known that dyes have a bacteriostatic activity more or less. Therefore, it is quite necessary to know the toxicity of TTC against tubercle bacilli. For this purpose, the sensitivity test of tubercle bacilli against TTC was carried out in both Ogawa solid and Kirchner liquid media, in which TTC had been added in various concentrations. To Ogawa media the bacilli were inoculated in three different
amounts (10^{-1}, 10^{-3} and 10^{-6} mg) and to Kirchner media only in 10^{-1} mg. Table 1 is the result obtained after four weeks incubation at 37°C. The table indicates evidently that the toxicity of TTC is largely influenced by the amount of the bacilli which are brought into contact with TTC. It will be easily supposed from this that a large amount of the bacilli can covert the toxic water soluble TTC into the form of innocuous water insoluble formazan so rapidly that the concentration of TTC decreases to sublethal degree in a short time. At this time, a mention should be made of the fact that the colonies grown on the media containing TTC presented the red color, more deeply stained in the higher concentrations.

Table 1 The growth inhibitory effect of 2, 3, 5 triphenyltetrazolium chloride (TTC) against tubercle bacilli in solid and liquid media

<table>
<thead>
<tr>
<th>Amount of bacilli inoculated</th>
<th>TTC Concentration</th>
<th>Ogawa solid medium</th>
<th>Kirchner liquid medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>10^{-1}mg</td>
<td>###</td>
<td>###</td>
<td>###</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>#</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>13col.</td>
<td>12col.</td>
<td>12col.</td>
</tr>
</tbody>
</table>

Note: Growth graded macroscopically from ± to ### except the case of solitary colonies.

3. The relation between the concentration of TTC and the intensity of the TTC reduction It was made clear, in the above experiment, that TTC has a certain degree of toxicity against tubercle bacilli. In an enzymatic reaction like the TTC reduction test, it is desirable that the reaction is produced in the physiologic state of the bacilli. The excessive use of the dye will disturb the observation of the true state of the activity because of the toxic action of the dye to the bacilli. In this connection, the relation between the concentration of TTC and the intensity of the reaction was studied. The culture of nine days old was used being suspended in the concentration of 12 mg per ml. As a substrate, a 1/100 M lactate solution was used, and at the same time, distilled water for the measurement of the endogeneous dehydrogenase activity. The TTC solutions were prepared to range from 0.05 to 5.0 per cent consisting of six different concentrations. The reduction test was conducted in the manner already described arranging three tubes for each kind of the TTC concentrations, and the reading was made after three hours incubation at 37°C. The
results were demonstrated graphically in Fig. 2. In this figure, straight line

curves were manifested between 0.01 per cent and 0.05 per cent of TTC. In the
higher concentrations up to 0.2 per cent, the intensity of the reaction in-
creased gradually but not in proportion to the concentration of the dye. The
excessive use of the dye appears to inhibit the reaction. These tendencies were
not altered by the presence of lactate. Similar experiment was repeated to
confirm and analyze of this fact in more detail. This time, the culture of eleven
days growth was used being suspended in the buffer solution in three different
concentrations (16, 8 and 4 mg/ml). The lactate served again as a substrate.
The reading of the reactions in three hours was presented in Fig. 3. The
figure demonstrated quite the same tendency as the previous experiment. The
straight line curves were also formed between 0.01 per cent and 0.04 per cent
of TTC, regardless of the amount of the bacilli. Attention, however, should
be directed to the fact that when a larger amount of the bacilli was employed,
the maximum intensity of the reaction occurred in the higher concentration of
TTC, for instance in 0.3 per cent for 8 mg of the bacilli, in 0.15 per cent for
4 mg and in 0.08 per cent for 2 mg. This observation appears to coincide well.
with the results of the TTC sensitivity test of the bacilli in culture media as described previously. The next point of which a consideration must be made is that the concentration of TTC in the reaction system is not constant but decreases in the course of reaction as the result of the transformation of TTC into formazan. Therefore, it will be easily supposed that the adequate concentration of TTC will be kept only in the limited range of time. In this connection, an attempt was made to analyze the relation between the initial concentration of TTC and the development of the reaction. The experimental materials were as follows; a 12 mg per ml buffer suspension of tubercle bacilli made from 15 days old culture, a 1/100 M lactate solution, and four kinds of TTC solution (0.4, 0.2, 0.1 and 0.05 per cent). Of each concentration of TTC, the reactions were recorded in 1, 1.5, 2, 3, 4 and 6 hours. The results were shown graphically in Fig. 4. As the curves indicate, it is clear that 6 mg of the bacilli can produce more formazan in proportion to the reaction time in the first several hours, possibly until the concentration of TTC in the system decreases below a definite level proper to each amount of the bacilli.
4. Development of TTC reducing reaction by different amounts of the bacilli

Being based upon the experiences in the above three experiments, the use of a 0.25 per cent buffer solution of TTC was decided in the experiments conducted thereafter. The use of the 0.25 per cent solution means that TTC is contained in 0.05 per cent being diluted in the reaction system in which the bacillary suspension, the substrate solution and the TTC solution are mixed in the amount of 0.5, 0.3 and 0.2 ml respectively. Now, in this concentration of TTC, an experiment was undertaken in an attempt to observe the development of the TTC reducing reaction using different amount of the bacilli in the presence of lactate (a 1/100 M lactate) as a substrate. The reactions were conducted by 24, 12, 6 and 3 mg of the bacilli suspended in 0.5 ml of the buffer solution and the development of the reactions were pursued in the course of time. The readings of the reactions were graphically summarized in Fig. 5. In the first place, we know from the figure that TTC was given enough even for 24 mg of the bacilli and that the formazan production can increase strictly in proportion to the reaction time, although in the limited range of time different in each amount of the bacilli respectively, as the part of straight line in each curve indicates evidently. For instance, the amount of formazan production by 12 mg of the bacilli increased proportionally to the reaction time between one and five
hours. At the same time, it is obvious that a certain amount of the bacilli can reduce only a limited amount of TTC into formazan. These state of affairs must be taken into our considerations when we work with the bacillary suspensions of unknown viability. Concerning this point, a discussion will be repeated elsewhere.

Fig. 5 Development of TTC reducing reaction by different amount of tubercle bacilli

5. The effect of substrates on the intensity of the TTC reduction Of several kinds of substrates, the effect on the intensity of the TTC reaction was studied using 6 mg of the tubercle bacilli of fourteen days culture, and a 0.25 per cent solution of TTC. Substrates were Sauton medium and the 1/100 M aqueous solutions of citrate, pyruvate, glucose, malate, glutamate and lactate. Distilled water was used for the measurement of the dehydrogenase activity for endogeneous substrates as a control. The composition of the reaction system was the same as usual. The results were graphically demonstrated in Fig. 6. This shows that lactate is outstandingly successful as a substrate to enhance
TETRAZOLIUM REDUCTION TEST

Fig. 6 Effect of substrates on TTC reducing reaction by tubercle bacilli

The reduction of TTC and other substrates act only weakly to intensify the reaction. In the same way as before, this time, the concentration of substrates was examined of its relation to the reaction. Two separate experiments were conducted; one on lactate and malate using 6 mg of the bacilli of 12 days culture, and the other on pyruvate and glucose using 6 mg of 13 days culture. The reading of the reactions was made after three hours incubation at 37°C. Fig. 7 is the results demonstrated graphically. It is clear that these substrates should be employed in the concentration below 1/20 M, and the excessive use of the substrates will exert some inhibitory effect to the reaction.

6. Comparison of the intensity of the TTC reducing reactions in aerobic and anaerobic conditions

As has been pointed out by many investigators, the red formazan can not be changed by oxygen, and therefore we can work with TTC in aerobic condition. In this connection, an attempt was undertaken to compare the intensity of the TTC reactions in aerobic and anaerobic condition. Three Thunberg tubes were dispensed with 10 mg of the bacilli suspended in 0.5 ml of the buffer solution to the main chamber, and with 0.2 ml of the 0.25 per cent solution of TTC to the side chamber. After being evacuated to 5 mm Hg vacuum, the contents of both chambers were mixed and the tubes were placed in an incubator at 37°C for one hour. The same procedures were done simultaneously in aerobic condition also using three Thunberg tubes. The re-
actions were read as usual. The results were summarized in Table 2, which indicated almost the same intensity of those reactions in aerobic and anaerobic conditions.

![Graph showing TTC reducing reaction by tubercle bacilli in the presence of different concentrations of substrates](image)

**Fig. 7** TTC reducing reaction by tubercle bacilli in the presence of different concentrations of substrates

<table>
<thead>
<tr>
<th>Substrate concentrations</th>
<th>Optical density of acetone solution of reduced TTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.725</td>
</tr>
<tr>
<td>0.15</td>
<td>0.83</td>
</tr>
<tr>
<td>0.20</td>
<td>0.90</td>
</tr>
<tr>
<td>0.30</td>
<td>0.98</td>
</tr>
<tr>
<td>0.40</td>
<td>1.10</td>
</tr>
<tr>
<td>0.50</td>
<td>1.20</td>
</tr>
<tr>
<td>0.60</td>
<td>1.30</td>
</tr>
<tr>
<td>0.70</td>
<td>1.40</td>
</tr>
<tr>
<td>0.80</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Table 2 TTC reducing reaction in aerobic and anaerobic condition

<table>
<thead>
<tr>
<th>Test tubes</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.45</td>
<td>0.46</td>
</tr>
<tr>
<td>2</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>3</td>
<td>0.42</td>
<td>0.46</td>
</tr>
</tbody>
</table>

7. **Effect of pH on the intensity of the TTC reduction by tubercle bacilli**

It is well known that enzymatic reactions have their own optimum pH. In this connection, an attempt was made to determine the relation between pH and the intensity of the TTC reducing reaction by tubercle bacilli, using experi-
mental materials as follows; a 12 mg per ml suspensions of the bacilli of 12 days old culture, a 0.25 per cent buffer solutions of TTC and distilled water or 1/100 M lactate as a substrate. The bacillary suspensions and the TTC solutions were prepared in the 1/15 M phosphate buffers of different pH. The reactions were demonstrated in Table 3, which indicates that the reactions in problem occure more actively with the advancement of pH to alkaline side in the range from 4.49 to 9.18. This is particularly remarkable in the presence of lactate. Ascorbic acid, cystein and glutathion which are capable of producing formazan only in alkaline reaction may have worked to intensify the TTC reduction.

Table 3 Effect of pH on TTC reducing reaction by tubercle bacilli

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.49</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.022</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>0.075</td>
</tr>
</tbody>
</table>

8. Effect of temperature on the intensity of the TTC reduction by tubercle bacilli The effect of temperature on the TTC reduction was studied. The experiments were conducted as usual using a 12 mg per ml buffer suspension of tubercle bacilli of 16 days growth, a 0.25 per cent buffer solution of TTC and no substrate. Each 2 tubes were placed in a refrigerator or incubators maintained at 0°C, 27°C, 37°C, 45°C and 56°C, and the reactions were read in three hours. The results were as demonstrated in Table 4. It may be stated that so far as the enzyme is not damaged, the higher the temperature becomes the more intensely the reaction occurs.

Table 4 Effect of temperature on TTC reducing reaction by tubercle bacilli

<table>
<thead>
<tr>
<th>Test tubes</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5°C</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Hitherto, the study on the dehydrogenase activity of bacteria has been usually made using Thunberg tubes in anaerobic condition. And the measurement of the activity in this method is based upon the reduction time of methyl-
enblue or other dyes. Therefore, the strictly quantitative measurement has been rather difficult. Contrary to this, the tetrazolium reduction test can be conducted in aerobic condition, and the dehydrogenase activity of various materials can be known very precisely by the spectrophotometric measurement of the reduced dye. Because of the simplicity of the equipment and procedure, the tetrazolium reduction test has a wide sphere of application in biology and medical science. The present paper has presented many evidences that this substance has many good points for the quantitative measurement of the dehydrogenase activity of tubercle bacilli. On the other hand, however, it was also shown that 2,3,5 triphenyltetrazolium chloride (TTC) must be used in appropriate conditions, particularly paying attentions to the concentration of TTC, the amount of the bacilli, substrates, reaction time, pH and the composition of the reaction system. Concerning the concentrations of TTC, it will be said from the observation obtained that those between 0.01 and 0.04 per cent can be used safely for any amount of the bacilli, but for the purpose of obtaining the intense reaction, the optimal concentration are present for each amount of the bacilli. In addition to this, it is clear that the reading of the reactions should be made after two or three hours incubation at 37°C, in which the intensity of the reaction increases in proportion to the reaction time, comparatively regardless of the change of the bacillary amount and the TTC concentration as demonstrated in Fig. 4 and Fig. 5.

Among substrates so far examined, lactate was only one substance that has the property to enhance the TTC reduction remarkably. Other substances acted only very weakly in this way. It has been often reported that tetrazolium salts can penetrate into cells and be reduced to formazan within them possibly in the mitochondoria. Therefore, it is quite natural that although no substrate is added, the TTC reduction can occur in the presence of one or more dehydrogenase systems with favourable redox potentials in the cells. The final object of our investigation is to find the relation between the viability of tubercle bacilli and their dehydrogenase activity. In this connection, although the reduction may occurs more actively in the alkaline (pH 9.18) environment and in the temperature of 45°C, yet it will be desirable that the reduction test is conducted in the physiologically favourable condition of the bacilli as pH 7.0 and 37°C. And the addition of substrate will be not always necessary.

**SUMMARY**

As a preliminary report of the study on the tetrazolium reduction test as a measure to examine the total viability of suspensions of tubercle bacilli, the fundamental aspects of the techniques was analyzed in detail in this paper. The following are the findings obtained.

1. 2,3,5 triphenyltetrazolium chloride has a certain degree of toxicity against tubercle bacilli, therefore the TTC reduction test should be conducted
in the appropriate concentrations of TTC (0.01–0.04 per cent) in order to observe the TTC reducing activity of the bacilli in their physiologic condition.

2. The addition of lactate as a substrate enhanced greatly the TTC reduction, but substrates are not always necessary to bring about the TTC reduction by tubercle bacilli.

3. The TTC reduction occurs in almost the same intensity in aerobic and reduction by tubercle bacilli.

4. The reading of the reaction should be made in two or three hours, during which the formazan production increases in proportion to the reaction time.

5. The amount of reduced TTC (formazan) can be measured quantitatively by Coleman spectrophotometer set at 475 mλ, after being dissolve in aceton.

REFERENCES


