A METHOD FOR THE DETERMINATION OF THE RATE OF GROWTH OF TUBERCLE BACILLI BY THE USE OF SMALL INOCULA

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Older observations on the nutritive requirements and metabolism of virulent tubercle bacilli do not have quantitative significance because of the lack of accurate methods for the estimation of the rate of growth. The recognition in recent years that these organisms grow just as well when submersed in liquid media as on the surface of liquid or solid media has eliminated some of the difficulties inherent in measuring the amount of growth of tubercle bacilli. This type of culture eliminates many technical problems and permits the use of uniform homogeneous inocula. Employing subsurface growth, two methods have been described for the quantitative estimation of the amount and rate of growth of virulent tubercle bacilli. The first involves the use of micro-Kjeldahl nitrogen determinations (Youmans, 1946; Sattler and Youmans, 1948). Though accurate, this method suffers from being somewhat laborious and requiring relatively large numbers of organisms. The second employs photometric turbidity readings on cultures grown in Dubos' medium (Wolinsky and Steenken, 1947; Smith, 1947). The disadvantage of the second method resides in the composition of the medium that must be employed. Dubos' medium contains polyoxyethylene sorbitan monooleate, a surface-active agent that promotes a more diffuse type of growth and permits turbidimetric measurements (Dubos and Davis, 1946). Unfortunately, this substance has been shown to have a retarding effect on the growth of virulent tubercle bacilli and, furthermore, may markedly affect the action of substances present in the medium on the multiplication of tubercle bacilli (Fisher, 1948; Wong, Hambly, and Anderson, 1947; Forrest, Hart, and Walker, 1947; Kirby and Dubos, 1947; Youmans and Youmans, 1948).

Because of the importance, for purposes of diagnosis, of the isolation of small numbers of tubercle bacilli from pathologic material, very small numbers of tubercle bacilli have been used as inocula to determine the suitability of various media for the initiation of growth. This method has also been used to investigate the various nutritive requirements of the organism. However, because of the manner in which it has been employed, only approximate indications of the rate of growth have been obtained. Use of very small inocula for such purposes has suffered also from the lack of appreciation on the part of many workers of the sampling error involved (Drea, 1942). In using this method to determine the suitability of various media for the growth of tubercle bacilli, the usual procedure has been to inoculate tubes of media with one or more quantities of suspensions of tubercle bacilli which varied from $10^{-1}$ to $10^{-10}$ mg. Following incubation at 37 C for some arbitrary period of time, the tubes have been examined to determin-

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mine the presence of growth, and when it is present, rough estimate is frequently made of the amount. It is obvious that although such a method will tell which medium will support growth of the smallest number of tubercle bacilli, no accurate determination can be made of the actual speed of multiplication on any given medium or under any set of conditions. The lack of quantitative data from such experiments probably accounts in part for the lack of unanimity of opinion regarding the most suitable media for the isolation and growth of virulent tubercle bacilli. A simple, relatively accurate method for the determination of the rate of growth of virulent tubercle bacilli is one of the greatest needs in the field today.

The use of inocula containing different quantities of tubercle bacilli actually provides a solution to the problem, provided the rate of growth of the tubercle bacilli is the same regardless of the number of bacilli used to inoculate the tubes and provided a method can be devised for determining the time at which each inoculum grows up to a certain standard mass. The latter can be accomplished by recording the time at which growth with each inoculum first becomes visible. If the rates of growth of the inocula employed are the same, a linear relationship should be obtained when the time of first appearance of growth of each inoculum is plotted against the logarithm of the amount of inoculum employed. The slope of the straight line so obtained will represent the rate of growth of the tubercle bacilli and can be used for the calculation of the growth rate and, if desired, the generation time. Thus, relatively accurate comparisons could be made of the rate of growth of tubercle bacilli in different media and under different conditions. The present paper details the successful application of the foregoing principles to the estimation of the growth rate of virulent human type tubercle bacilli.

**EXPERIMENTAL RESULTS**

The practicability of the method for the estimation of the rate of growth of tubercle bacilli outlined in the introduction was tested in the following manner: Suspensions of 14- to 21-day-old cultures of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* were prepared and standardized as previously described (Youmans and Karlson, 1947). From this suspension dilutions were made with 0.01 M phosphate buffer solution, pH 7.0, so that the inocula to be employed contained, respectively, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, and $10^{-8}$ mg tubercle bacilli wet weight. Dilutions can also be prepared directly from cultures grown in "tween" albumin medium. Ten tubes, each containing 5.0 ml of the modified Proskauer and Beck basal synthetic medium (Youmans, 1946), were inoculated with each of the 8 quantities of H37Rv, making a total of 80 tubes in all. In addition, two modifications of the basal medium, one containing 0.2 per cent crystalline bovine albumin and the other 10.0 per cent sterile beef serum, were inoculated in the same manner. All tubes were then incubated at 37 C and examined daily for the presence of the typical granular growth. The time of the first appearance of such growth for each inoculum in each medium was recorded and then plotted against the logarithm of the inoculum. The results are shown in figure 1. The actual quantities of tubercle bacilli may be used along the ordinate since they are proportional to the logarithms.
An examination of figure 1 reveals that a linear relationship does exist between the time of the first appearance of growth and the logarithms of the number of organisms used as inocula. This indicates that the rate of growth is the same regardless of the amount of inoculum employed. Furthermore, the slopes of the lines in figure 1 represent the actual rates of growth. These two conclusions can be demonstrated mathematically or can be illustrated as in figure 2. In figure 2 lines have been drawn, representing an arbitrary similar rate of growth for each amount of inoculum, until they intersect the abscissa. When the time in days, represented by the point of each intersection, is replotted against the logarithm of the respective inoculum, a straight line is obtained (line A), the slope of which is the same as the rate of growth of each inoculum. In such experiments the lack of a linear relationship between the time of appearance of growth and the logarithm of the inoculum would indicate either technical errors or actual differences in the rates of growth of the various inocula.

It should be borne clearly in mind that, when estimations are made of the time at which growth of each inoculum is first visible, we are in effect detecting in

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**Figure 1.** The rate of growth of *M. tuberculosis* (H37Rv) in three types of media.

**Figure 2.** Diagrammatic representation of the estimation of the rate of growth of tubercle bacilli when using several small inocula.
each case approximately the same number of tubercle bacilli, i.e., the least number of bacilli that are visible. Therefore, in figure 1 an extension of the line should intersect the ordinate at a point that will be equivalent to the least number of tubercle bacilli that we can actually detect visually. When this is done with the data in figure 1, we obtain a figure of 1.0 mg wet weight of tubercle bacilli per 5 ml of medium. Using suspensions of tubercle bacilli of known concentration,

![Figure 3.](image)

*Figure 3.* Diagrammatic representation of the effect of a lag phase of constant length on the estimation of the rate of growth of tubercle bacilli when using several small inocula.

![Figure 4.](image)

*Figure 4.* Diagrammatic representation of the effect of a lag phase the length of which varies inversely with the amount of inoculum on the estimation of the rate of growth of tubercle bacilli.

we have found that the least number of tubercle bacilli that are visible by the method used is equivalent to approximately 0.5 mg per 5.0 ml of medium. The difference between this value and the one obtained from the point of intersection on the ordinate in figure 1 may be due to either experimental error or to the presence of a lag phase of 1 or 2 days.

The influence of the lag phase on the accuracy of the method is illustrated in figures 3 and 4. There are two possibilities to consider if a lag phase is present, (1) that the length of the lag phase is constant regardless of the amount of inoc-
ulum, (2) that the length of the lag phase is inversely proportional to the logarithm of the amount of inoculum. The first possibility is illustrated diagrammatically in figure 3. In figure 3 an arbitrary lag phase of 10 days has been assumed for each inoculum. It is apparent that this does not affect the estimation of the rate of growth (line A). It affects only the point at which the line that represents the rate of growth intersects the abscissa. Furthermore, the time in days represented by this intersect gives the length of the lag phase. In figure 4 it has been assumed that the lag phase, if present, is inversely proportional to the logarithm of the amount of inoculum, and, in this case, an arbitrary increase of one day in the lag with each smaller inoculum has been assumed. When these data are treated as in figure 2, it can be seen that although a straight line is still obtained (line A) the slope of this line is less than the slope of the lines that represent the actual rate of growth after the lag phase has ended. The degree of divergence of the slope of the line from the true rate of growth will depend upon the degree to which the length of the lag phase is influenced by the number of organisms present. This, however, will not affect the accuracy of the estimations seriously unless this effect is marked. There is no information available on the influence of the number of tubercle bacilli on the length of the lag phase, but with other species of bacteria it is generally assumed that the lag phase, when present, is inversely proportional to the amount of inoculum (Winslow and Walker, 1939; Topley and Wilson, 1946; Porter, 1946).

In referring to the lag phase, care must be taken, however, to differentiate between increase in cell numbers and increase in cell mass. Hershey (1939) and Winslow and Walker (1939) have shown that when bacterial cells are inoculated into a favorable medium, the increase in cell mass is independent of the age of the culture and proceeds from the beginning at a constant rate, whereas the rate of cell division as measured by plate counts may indicate the presence of a lag phase. The method presented in this paper is essentially an estimation of the rate of growth by a measurement of bacterial mass. Therefore, the presence or absence of a lag in multiplication may possibly be of little significance.

Ingraham (1933) employed this same method in a study of the effect of gentian violet on the growth of 24 species of microorganisms other than mycobacteria. This author, however, thought that slopes of the straight lines so obtained were a function of the length of the lag phase. Herrington (1934) later, using the data reported by Ingraham, recognized that the slope of the line should be only a function of the rate of growth, but because of the fact that Ingraham stated that gentian violet affected growth only during the lag phase, he was forced to the conclusion that the length of the lag phase was affected by the amount of inoculum. Examination of the graphs given by Ingraham indicates that little or no lag phase was actually present. Unfortunately, Ingraham's conclusions that gentian violet affected *Escherichia coli* only during the lag phase were based on an enumeration of the number of cells by plate counts and direct counts, whereas measurement of the degree of effect of gentian violet on *E. coli* was made by the method herein described which, as has been pointed out previously, is a measure of the total mass of cells.
Kohn and Harris (1941), in their studies on the mode of action of sulfonamides, used a method for the estimation of the rate of growth of *E. coli* that was in principle similar to the one we have employed. These authors, using turbidimetric determinations, measured the time required for *E. coli* to multiply until a certain standard number of cells were present per ml of medium. They used the relation between these times to calculate the rate of growth. In the present work young actively growing cultures of tubercle bacilli were employed. It is well recognized (Winslow and Walker, 1939; Topley and Wilson, 1946; Porter, 1946) that, when actively growing cultures are transferred to a new medium, multiplication continues at the same rate.

From the data in figure 1 the slopes of the lines and therefore the growth rates were calculated by use of the following formula:

\[ K = \frac{\log a - \log b}{t} \]

Where \( K \) = growth rate constant

\( a \) = largest inoculum employed (in mg)
\( b \) = smallest inoculum employed (in mg)
\( t \) = time in days or hours

The generation time (time for one cell division) was calculated in the following manner:

\[ G = \frac{\log 2}{K} \]

Where \( G \) = generation time
\( K \) = growth rate constant

A total of six experiments similar to the one represented by figure 1 were done at different times. Table 1 gives the findings in detail of these six experiments, and figure 1 shows graphically the results obtained in one such experiment. The actual growth rate constants and generation times obtained in each experiment are given in table 2. The high degree of reproducibility of results using this method is apparent and the high degree of significance of the difference in growth rate between the basal medium and that medium containing crystalline bovine albumin indicates its usefulness. The tubercle bacilli actually grew on an average 13.0 per cent faster in the medium containing the bovine albumin. The marked stimulating effect of bovine plasma on the growth of tubercle bacilli is obvious, the average increase in rate over the basal medium being 27.6 per cent.

The technique as conducted in the experiments mentioned above is still laborious, involving as it does the preparation of 80 tubes of medium and the repeated handling of large numbers of tubes. This can be simplified considerably by employing fewer inocula. Actually, only two inocula would be necessary in order to establish the slope of the line and permit a calculation of the growth rate. For the sake of accuracy we prefer three; either \( 10^{-3}, 10^{-4}, \) and \( 10^{-4} \), or \( 10^{-2}, 10^{-4}, \) and \( 10^{-4} \). The number of tubes inoculated with each quantity of tubercle bacilli can be reduced, since sampling errors are not significant with
amounts of $10^{-4}$ mg or more. We now routinely use 5 tubes, since this number usually prevents technical errors or contamination from invalidating an experiment. This reduces the number of tubes for a growth rate estimation from 80 to only 15. The tubes, however, should be examined every day, or at the least

every other day; if readings are made farther apart than this, gross errors are introduced.

The technique of examining the tubes for the presence of growth is important. Tubes of uninoculated medium should be used for comparative purposes. A constant source of light and a dark background are essential. We have found the
use of a Quebec colony counter ideal. This not only provides a constant light source and a dark background, but a small amount of magnification which facilitates the readings. With practice, the error is not more than plus or minus 1 day. Some objective method for the detection of a standard number of tubercle bacilli would be highly desirable, but, at present, because of the flocculent type of growth, this is not practical.

DISCUSSION

The method described herein for the estimation of the rate of growth of virulent tubercle bacilli is simple and relatively accurate. Although the method for the estimation of the presence of growth is subjective, the error is slight and the data can be plotted so that they have quantitative significance. The method is applicable to solid as well as liquid media and permits an accurate expression of the relative efficacy of different media for the growth of tubercle bacilli. This would tend to eliminate controversy regarding the relative merits of this or that medium, either for the growth of cultures of tubercle bacilli or for primary isolation. In the latter connection, it should be possible to dilute heavily positive sputum samples after concentration and estimate the rate at which bacilli from a human source will grow on any given medium at the time of isolation.

The usefulness of the method for the study of the nutritive requirements and metabolism of the tubercle bacillus is obvious. Subsequent papers will give in detail results of the application of the method to such studies.

Attention should be called to the discrepancy between the growth rates obtained with this method and those previously reported that were obtained by micro-Kjeldahl nitrogen determinations (Youmans, 1946; Sattler and Youmans, 1948). With the same basal medium, the shortest generation time obtained by the latter method was 38.0 hours, approximately twice as long as those obtained with the method reported herein. Although it is technically impossible to compare the two methods directly, it would appear that conditions are more favorable for the growth of minute inocula (10^{-1} to 10^{-8} mg) than for very large inocula (2 to 5 mg).

SUMMARY

Decimal dilutions of virulent human type tubercle bacilli were prepared which varied from 10^{-1} to 10^{-8} mg moist weight. Tubes of three types of liquid media were inoculated with each concentration of tubercle bacilli. These were incubated at 37 C and examined daily to determine the time at which visible subsurface growth of each inoculum first appeared. The determination of this point represented a measurement of approximately the same number of organisms in each set of tubes.

By plotting the logarithms of the inocula employed against the time at which visible growth of each inoculum first appeared, a linear relationship was found. From the slope of the straight line so obtained, the growth rate and the generation time could be calculated. This method can be used to determine with greater accuracy the effect of various substances or physical conditions on the rate of growth of tubercle bacilli.
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