A METHOD FOR THE DETERMINATION OF THE CULTURE CYCLE AND THE GROWTH RATE OF VIRULENT HUMAN TYPE TUBERCLE BACILLI[1]

GUY P. YOUMANS

Department of Bacteriology, Northwestern University Medical School, Chicago, Illinois

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Accurate determinations of the normal culture cycle and the growth rate of *Mycobacterium tuberculosis* var. *hominis*, to the knowledge of the author, have never been reported. Until recent years this organism has routinely been cultivated in the form of a surface pellicle on solid or liquid media. Under these conditions tubercle bacilli grow in relatively dry coherent masses, and neither direct counts nor plate counts can be used for an estimation of the number of organisms present. Attempts have been made to measure the amount and rate of growth of tubercle bacilli by drying and weighing the total surface pellicle growth from liquid media after varying intervals of time. The technical difficulties and the inaccuracies of this method for the determination of bacterial mass have been pointed out by Mueller (1935). Furthermore, when tubercle bacilli are employed, this method is even more inaccurate since a uniform inoculum cannot be used and the rate and type of growth may vary from flask to flask. In addition, since relatively large amounts of growth are necessary for the determinations, the earlier periods of the growth cycle might well be missed.

Accurate determination of the growth rate would be of value for the determination of the effect of physical or chemical agents on the growth of virulent tubercle bacilli. Previously such information either has been obtained subjectively, with a consequent large and unpredictable degree of error, or has been determined by the difference in total mass of culture after an arbitrary growth period. The latter method, however, frequently gives values that bear no relation to the rate of growth.

The demonstration by Drea (1940, 1942) and Youmans (1944*a*, 1944*b*) that fine suspensions of virulent human type tubercle bacilli will grow readily beneath the surface of synthetic media permits the use of a uniform homogeneous inoculum and leaves only the problem of measuring accurately the subsequent growth. Since nitrogen is a relatively uniform constituent of living cells, Mueller (1935) recommended the use of nitrogen determinations on cultures of bacteria for determining the amount of growth. Hershey (1939) has shown that nitrogen determinations on growing cultures of *Escherichia coli* give values that are proportional to the total mass of growth, although not always proportional to the number of organisms present.

The present paper details the use of micro-Kjeldahl nitrogen determinations for the purpose of determining the normal culture cycle, the growth rate, and the generation time of the H37Rv strain of virulent human type tubercle bacilli.

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METHODS

The synthetic medium employed contained asparagine, 0.5 per cent; monopotassium phosphate, 0.5 per cent; magnesium citrate, 0.15 per cent; potassium sulfate, 0.05 per cent; and glycerol, 2.0 per cent; dissolved in water redistilled from glass. The pH was adjusted to 7.0 with sodium hydroxide, using a Coleman electrometer, and the medium was sterilized in the autoclave at 10 pounds' pressure for 20 minutes, although higher temperatures can be used without ill effect.

Pyrex test tubes, 200 by 25 mm, were cleaned by standing overnight in concentrated sulfuric acid. They were then thoroughly rinsed with tap water and distilled water and allowed to dry. When dry they were plugged with cotton and sterilized in the autoclave at 20 pounds' pressure for 20 minutes. Following sterilization, 10.0 ml of sterile synthetic medium were introduced aseptically into each tube using an accurately calibrated volumetric pipette.

The entire surface pellicle growth of a 21-day-old flask culture of the H37Rv strain of *M. tuberculosis* was washed by centrifugation three times with 20- to 30-ml portions of sterile 0.01 molar phosphate buffer solution, pH 7.0. The washed tubercle bacilli were transferred to a sterile mortar and ground with a sterile pestle, with the gradual addition of 0.01 molar phosphate buffer solution until a relatively homogeneous suspension resulted. Following transfer to a sterile tube the suspension was allowed to stand for 30 minutes to permit the larger clumps to settle out. The fine supernatant suspension was transferred to a sterile tube and standardized by micro-Kjeldahl determinations on suitable aliquot portions. Before the nitrogen determinations were done it was necessary to centrifuge and wash the aliquot portions in the manner to be described below, because in suspensions prepared in this fashion 30 to 40 per cent of the nitrogen was found to be in solution, possibly due to the rupture of many organisms during grinding.

Following standardization, the suspension was diluted with 0.01 molar phosphate buffer solution to the desired nitrogen concentration, and each tube of medium was inoculated with 1.0 ml of this diluted suspension, using a volumetric pipette. The tubes were shaken, capped with waxed paper, and incubated at 37 C.

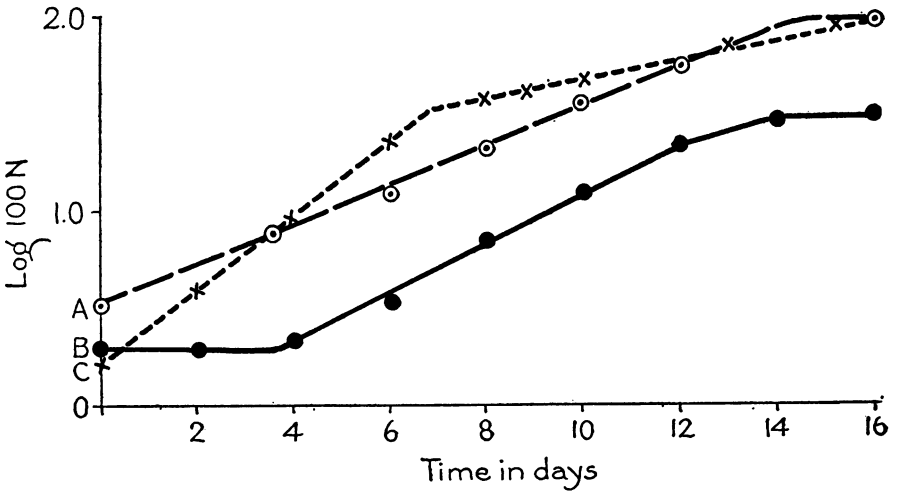
At intervals following inoculation, usually every 2 days, a number of tubes were removed from the incubator and heated in a boiling water bath for 10 minutes; to each tube was then added, at room temperature, 10.0 ml of a saturated solution of potassium lauryl sulfate, and 1.0 ml of a 2 per cent suspension of super-cel. The tubes were centrifuged at about 3,000 rpm for 5 minutes, and the supernatant fluid was removed from the sediment by decantation or aspiration. The organisms were then washed twice with 20.0-ml portions of the potassium lauryl sulfate solution. After the final washing, digestion and nitrogen determinations of the entire contents of each tube were made according to the method of Ma and Zuazaga (1942).

Numerous experiments have shown that the preliminary heating in this procedure did not alter the nitrogen content. The potassium lauryl sulfate solution served as a wetting agent and prevented the fine growth of tubercle bacilli from climbing the sides of the tubes or from floating on the surface of the liquid. The

super-cel greatly facilitated centrifugation, giving a more firmly packed sediment, and greatly reduced the centrifugation time.

RESULTS

Three types of growth curve were obtained with the virulent human type tubercle bacillus, strain H37Rv, and are illustrated in graph 1. Curve B is similar to those obtained with other bacteria when the plate count method is employed: a lag phase followed by a period of logarithmic growth and finally by a period of decreasing growth. Curves A and C, however, show that tubercle bacilli may begin immediately to grow at a constant rate. According to Hershey (1939) and Winslow and Walker (1939) in experiments with *E. coli*, when determinations of cell mass were made using a favorable medium for growth of the culture, there



GRAPH 1. NORMAL CULTURE CYCLES OF M. TUBERCULOSIS (H37Rv)

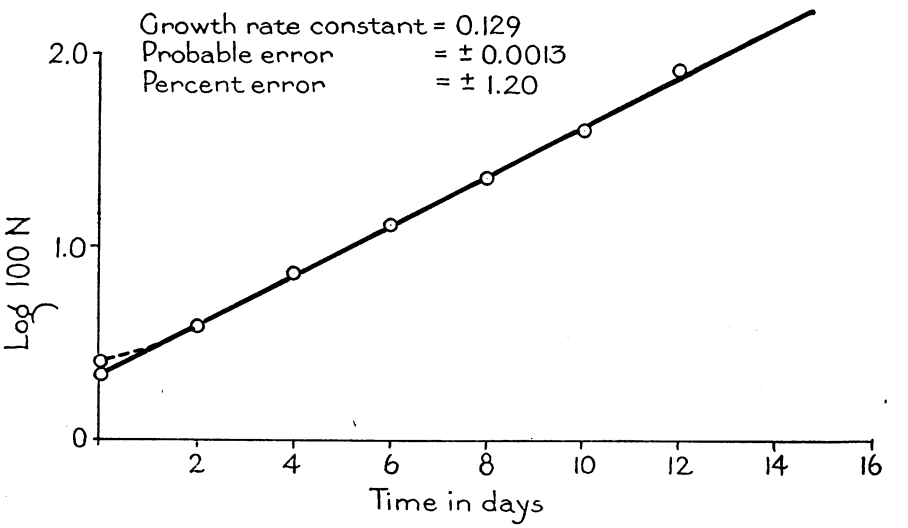
was no lag phase regardless of the age of the culture used for inoculation. The logarithmic growth phase started immediately because of the fact that the total mass of the culture increased at a constant rate even though cell division was retarded. The synthetic medium used for the tubercle bacilli would theoretically be a relatively unfavorable one; therefore, it was of interest that occasionally no evidence of a lag phase was obtained. It is possible, of course, that the results in graph 1 might have been due to the fact that the organisms used as an inoculum had, in the case of curve B, already passed the period of logarithmic growth, whereas those used in curves A and C were in the logarithmic growth phase at the time of inoculation. Curve C is also interesting since we have on several occasions noted this very rapid rate of growth for a period of 6 to 8 days, followed by a sudden decrease in rate, which then remained constant for 6 to 8 days. No obvious explanation for this phenomenon presents itself, since all known physical and chemical factors were carefully controlled.

In table 1 are shown the growth rate constants and the generation times of ten separate growth curve determinations obtained by using 21-day-old surface cultures of H37Rv as inocula. The values in the table were obtained by plotting the logarithms of the nitrogen values against the time in days and drawing the

TABLE 1

Growth rate constants and generation times of M. tuberculosis (H37Rv)

	AMT. OF INOCULUM IN MG OF N	GROWTH RATE CONSTANTS	GENERATION TIME IN DAYS
1	0.04	0.08	3.70
2	0.035	0.10	3.01
3	0.025	0.11	2.73
4	0.02	0.125	2.48
5	0.03	0.13	2.31
6	0.015	0.13	2.31
7	0.035	0.14	2.13
8	0.02	0.15	2.00
9	0.03	0.185	1.62
10	0.015	0.195	1.54

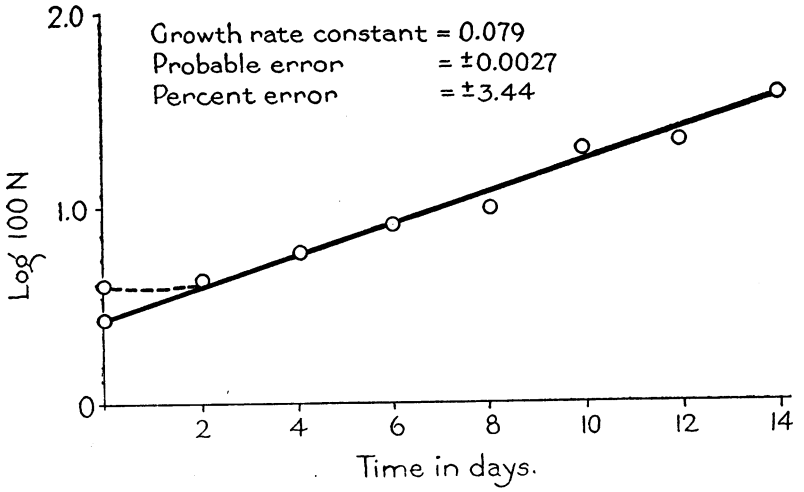


GRAPH 2

curves that best fitted the data. The growth curve constants and the generation times were calculated from the straight-line portions of the curves, the logarithmic growth phase. In most cases the slope of the line was determined by inspection, but two curves were selected which seemed to represent, respectively, experiments in which the points deviated least markedly and most markedly from a straight line, and the rate of growth and the probable error were calculated by the method of least squares. The probable errors were, respectively, plus or

minus 1.2 and 3.4 per cent. These are illustrated in graphs 2 and 3. In graph 2 six tubes of growth were used to determine each point on the curve, and in graph 3 three tubes were used. Therefore it would appear that, depending on the number of tubes of growth analyzed and the amount of care exercised in the determinations, the growth rate determinations have an accuracy of between a plus or minus 5 to 15 per cent.

As shown by the data in table 1, the growth rates, and therefore the generation times, varied markedly regardless of the amount of inoculum or the age of the culture used for inoculation. The chemical composition of the medium and the physical growth conditions were constant; consequently, these results would seem to represent differences in the physiological state of the cultures used as inocula.



GRAPH 3

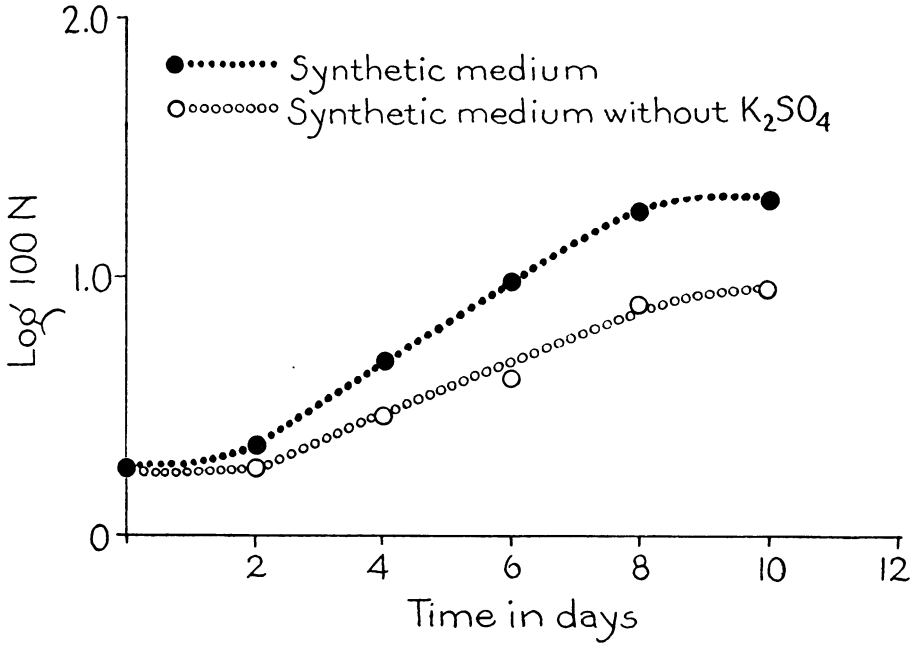
Similar divergent results have been obtained in a few experiments using 14-day-old cultures as inocula.

It should also be pointed out that in these experiments measurements are first made of the amount of subsurface growth and secondly of combined subsurface and surface growth since, depending on the amount of inoculum and the rate of growth, a surface pellicle will begin to form in the culture tubes in from 4 to 8 days.

Graph 4 shows the effect on the rate and amount of growth of the H37Rv strain when the potassium sulfate was omitted from the synthetic medium. The sulfur requirements of the tubercle bacillus are not well known, but the results show that although growth occurs in its absence, except for such small amounts as may be present as impurities in the other constituents of the medium, it is considerably reduced in rate and amount.

The usefulness of the method for the determination of the degree of inhibition of growth produced by bacteriostatic substances is illustrated in graphs 5 and 6. The substance used, 4,4′ diamino diphenyl sulfone, exerts a suppressive effect on

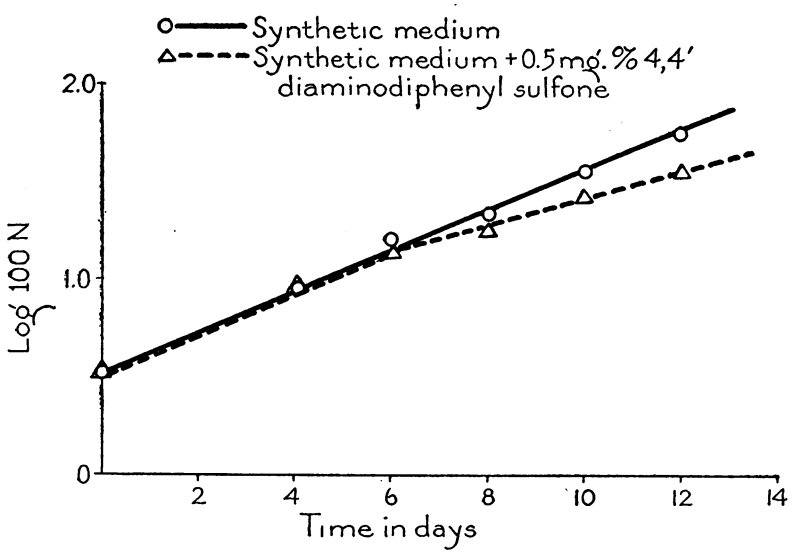
experimental tuberculosis of guinea pigs (Feldman, Hinshaw, and Moses , 1943), and derivatives of this compound have had clinical trial as chemotherapeutic agents for tuberculosis of humans. This compound has also been shown to be bacteriostatic for tubercle bacilli *in vitro* (Steenken and Heise, 1943; Youmans, 1944*b*). When 0.05 mg per cent of 4,4′ diamino diphenyl sulfone was present in the medium, the rate of growth was inhibited approximately 28 per cent (graph 5). With a concentration of 1.0 mg per cent, the inhibition of the rate



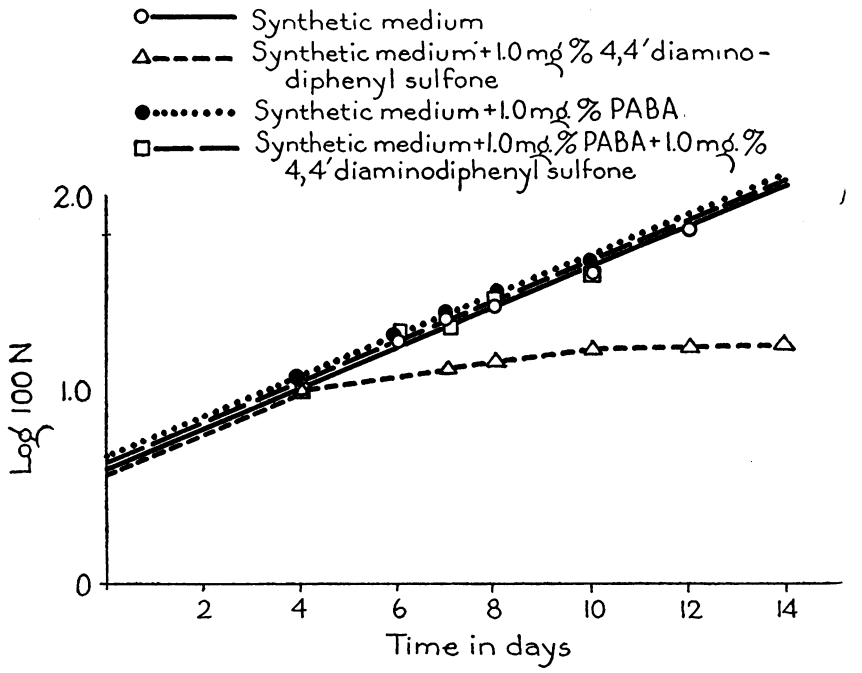
GRAPH 4. EFFECT OF OMISSION OF K_2SO_4 FROM SYNTHETIC MEDIUM ON GROWTH OF M. TUBERCULOSIS (H37Rv)

of growth was approximately 62 per cent. Furthermore, 1.0 mg per cent *para*-aminobenzoic acid completely reversed the bacteriostatic action of 4,4′ diamino diphenyl sulfone and did not itself exert any stimulating or inhibitory effect on the growth of the tubercle bacilli (graph 6). Of further interest is the fact that the method clearly shows a lag in the inhibitory action of this sulfone on the tubercle bacillus similar to the lag in the action of the sulfonamides that has been observed with other bacteria (Henry, 1943).

The validity of this method for the determination of the amount and rate of growth of tubercle bacilli is, of course, based on the assumption that the increase in bacterial nitrogen is proportional to the increase in the total mass of the bacterial population. This has been shown to be true of *E. coli* (Hershey, 1939), and presumably would also be true of the tubercle bacillus. However, the possibility should be kept in mind that under conditions different from those used in the present work the ratio of nitrogen to the total mass might be different.



GRAPH 5. THE EFFECT OF 0.5 MG PER CENT 4,4' DIAMINO DIPHENYL SULFONE ON GROWTH OF M. TUBERCULOSIS (H37Rv)



GRAPH 6. THE EFFECT OF 1.0 MG PER CENT 4,4' DIAMINO DIPHENYL SULFONE AND PABA ON GROWTH OF M. TUBERCULOSIS (H37Rv)

SUMMARY

By the use of micro-Kjeldahl nitrogen determinations, the culture cycle of the virulent human type tubercle bacillus, strain H37Rv, was determined. From the logarithmic portion of the growth curve, the growth rates and generation times were calculated. Under the conditions of the experiment the generation times were found to vary between approximately $1\frac{1}{2}$ and $3\frac{1}{2}$ days. The usefulness of the method for accurately determining the amount of growth of tubercle bacilli and the effect on the rate of growth of alteration of the composition of the medium and of the presence of growth-inhibiting substances is illustrated.

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