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Quantitative Measurement of Growth of *Mycobacterium tuberculosis*. Effect of Streptomycin.*†

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Quantitative estimation of the growth of *Mycobacterium tuberculosis* has been seriously handicapped by lack of simple, accurate, and safe methods of measurement. For this reason, the determination of the normal culture cycle of this organism, the rate of growth, and the *in vitro* effects of growth-promoting and growth-inhibiting substances has been difficult. Visual approximation of growth and weighing of pellicles with all the concomitant danger due to handling of the culture have been the only available, though unsatisfactory, methods. These were dangerous, or they involved technical difficulties and inaccuracies, as pointed out by Mueller.[1]

Recently, Youmans[2] measured the normal culture cycle, growth rate, and generation time of the H37Rv strain of the virulent human type tubercle bacilli by means of micro-Kjeldahl nitrogen determinations. He suggested the use of this procedure in the evaluation of the efficacy of chemotherapeutic agents upon the organism of tuberculosis. Because of the complexity of this test, its use for mass determinations may be questioned.

Dubois[3] suggested the possibility of making use of nephelometric measurements in establishing the value of the oil of chaulmoogra and its derivatives on the growth of avian bacilli in Kirchner's medium.[4] Homogeneous growth in this medium, contrary to the usual result obtained with cultures of mycobacteria, was reported.

Dubos,[5] using a modification of the Kirchner formula, developed a medium in which both the pathogenic and the saprophytic varieties of mycobacteria produce a diffuse type of growth. At times, human bacilli form microscopic clumps of loosely packed cells, which can be readily dispersed by moderate shaking to form a uniformly turbid suspension. Such uniformly diffused growth facilitates accurate turbidimetric measurements.

Since none of the existing procedures was satisfactory for safe and accurate determination of growth of mycobacteria, advantage was taken of the uniform turbidity produced in Dubos' medium to develop a suitable method for estimating the growth of these organisms. This method found special application in the study of the effect of streptomycin upon the growth of this organism.

Procedure. Stock cultures of *M. tuberculosis avium* (ATCC No. 7992), an avirulent strain of *M. tuberculosis var. hominis* No. 607, a virulent strain of *M. tuberculosis var. hominis* H37Rv, and a streptomycin-resistant strain of *M. tuberculosis var. hominis* H37Rv obtained from Dr. G. P. Youmans were maintained on glycerol-nutrient agar slants and in Dubos' medium.[5] For the growth of virulent human varieties, 0.2% bovine serum albumin (fraction V) was added to the medium. The ages of the cultures varied depending on their rate of growth, namely, 7 days for *M. avium* and *M. tuberculosis* No. 607 and 3 to 5 weeks for *M. tuberculosis* H37Rv and H37Rv streptomycin-resistant.

For purposes of inoculation, a homogeneous suspension of cells was made in a simplified modification of Dubos' medium, which was incapable of supporting significant growth and consisted only of the salt mixture plus

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1 Mueller, J. H., *J. Bact.*, 1935, **29**, 383.

2 Youmans, G. P., *J. Bact.*, 1946, **51**, 703.

3 Dubois, A., *Ann. Soc. belge de Med. Trop.*, 1944, **24**, 1.

4 Kirchner, O., *Zentral. f. Bakteriologie*, 1932, I Orig., **124**, 403.

5 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

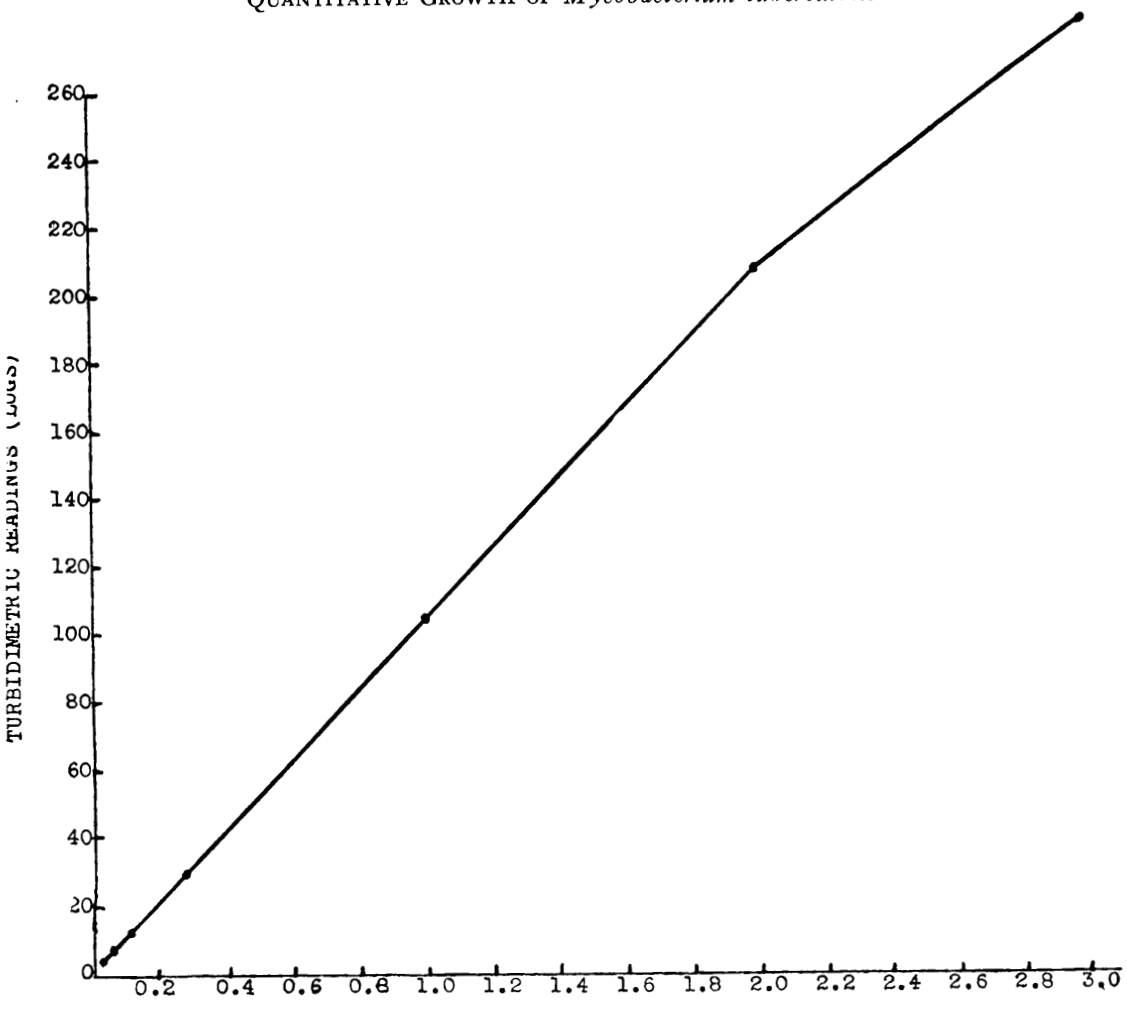

FIG. 1.
Turbidimetric Measurement of Suspensions of *Mycobacterium tuberculosis* No. 607.

Tween 80.‡ The purpose of this Tween-salt solution was to avoid, as much as possible, the introduction of nutrients when inoculating nutritionally-deficient media.

The suspension was prepared as follows: Growth from an agar slant was added to a small amount of the Tween-salt mixture and shaken with sterile glass beads. This resulted in a homogeneous suspension which could be diluted to the desired density. If cultures grown in Dubos' medium were used, they were diluted or were used undiluted, depending upon their density. Turbidimetric readings of suspensions containing weighed amounts of cells have shown that 1 mg of cells (wet weight) per milliliter of fluid formed a suspension which gave turbidimetric readings of 100-105 on a Klett-Summerson colorimeter. A certain amount of growth of the organism was suspended in the medium and adjusted to give a reading of 100. Further dilution was made with the Tween-salt solution until the required concentration of cells was obtained.

The experiment was now set up by seeding the media with 4.5 ml portions of a suspension of bacterial cells in test tubes. For controls, 5-ml amounts were used; for the study of the effect of streptomycin, 0.5-ml portions of the various concentrations of drug

‡ Tween 80 is a polyethylene derivative of sorbitan monooleate and acts as a detergent in this medium.

TABLE I.
Influence of Inoculum on Rate of Growth of *Mycobacterium tuberculosis* No. 607.*

Time of incubation, hr	Concentration of cells, mg/ml				
	10^{-3}	10^{-5}	10^{-7}	10^{-9}	10^{-10}
	Turbidimetric readings (in logs)				
24	9	7	0	0	0
48	26	7	0	0	0
72	44	34	8	0	0
96	60	54	31	8	0
184	106	100	83	72	0
330	150	159	138	153	0

* Dubos' medium without enrichment with dextrose, vegex, and albumin.

were added to 4.5 ml of the seeded medium. The cultures were incubated at 37°C. and turbidimetric readings were made at frequent intervals. In media with extremely low nutritive value, some firm clumps of cells were formed after 10 to 21 days of incubation, depending on the rate of growth of the culture. No clumping was observed in Dubos' medium even after 6 weeks' incubation.

The tubercle bacilli can be grown on fairly simple media. Dubos' medium, consisting of KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, $MgSO_4 \cdot 7H_2O$, sodium citrate, Tween 80, an enzymatic digest of casein, dextrose, vegex (a vegetable extract), and bovine serum-albumin, is rather complex and supports abundant growth of the human pathogenic strains of mycobacteria as well as of the saprophytic forms. However, considerable growth can still be obtained if all complex organic materials are omitted and ammonium salt is used as a source of nitrogen with the citrate as the sole source of carbon. Thus the above medium can be simplified so as to be made synthetically, and still remain adequate for growth of the organism. By the use of this or similar modifications, it was possible to study the physiology of the tubercle bacilli and to investigate the effect of streptomycin on the metabolism of the organism.

In preliminary experiments, bacterial suspensions were prepared for purposes of inoculation using weighed amounts of cells. The uniform turbidity of these suspensions made possible the construction of a standard curve for the determination of the weight of the cell material in terms of turbidity readings. This is shown in Fig. 1, where the weight of the cells ranging from 0.01 mg/ml to 2.0 mg/ml was plotted against turbidimetric readings as given on the scale of the Klett-Summerson colorimeter. Readings above 2.0 mg/ml diverged from a straight line, whereas those below 0.01 mg/ml could not be accurately measured. Two advantages of such a procedure are obvious, namely, the ease of correlating turbidity with the weight of cell material, and the elimination of undue handling of the culture.

If a standard curve is made based on the plate counts of viable bacteria in a suspension, the value of the turbidimetric readings

TABLE II.
Influence of Nitrogenous Materials on the Rate of Growth of *Mycobacterium tuberculosis var. hominis* H37Rv.

Concentration of cells in mg/ml, 5×10^{-3}				
Constituent of medium	NH_4 citrate, 0.2%	Asparagine, 0.2%	Tryptophane, 0.1%	Dubos' medium (albumin)
Time of incubation, hr	Turbidimetric readings (in logs)			
24	0	0	2	0
72	0	2	8	3
120	0	4	13	16
244	9	16	20	56
334	11	19	26	85
507	11	13	34	244

TABLE III.
Influence of Nitrogenous Materials on the Bacteriostatic Action of Streptomycin. *Mycobacterium tuberculosis* No. 607*.

Source of nitrogen	Streptomycin, μg/ml	Incubation in hr: 24	48	72	187	312	Final pH
		Turbidimetric readings (in logs)					
NH_4 citrate, 0.2%	0.4	0	17	27	27	67	6.20
	0.2	0	11	27	25	67	6.20
	0.1	1	16	27	30	72	6.20
	0	1	14	29	30	60	6.20
Asparagine, 0.2%	0.4	0	0	0	0	5	7.88
	0.2	0	0	0	30	45	7.92
	0.1	0	16	37	35	31	8.05
	0	2	25	38	37	85	8.06
Casein digest, 0.2%	0.4	0	0	0	0	0	8.20
	0.2	2	2	3	6	6	8.18
	0.1	2	16	34	83	108	8.30
	0	3	13	43	113	154	8.40
Beef extract, 0.3%	0.4	0	0	0	52	155	7.88
	0.2	1	10	25	96	157	8.38
	0.1	4	27	48	150	165	8.48
	0	5	31	55	165	150	8.45

* The media were seeded with 10^{-3} mg cells/ml.

may be extended to correlate turbidity measurements with the number of viable cells. A series of 10 determinations were made with 7-day-old cultures of *M. tuberculosis* No. 607 and of *M. avium* on glycerol-nutrient agar. Consistently reproducible counts of viable cells were obtained, the numbers of viable organisms being 50.6 million cells for *M. tuberculosis* and 59.5 millions for *M. avium* per milligram, with a turbidity reading of 101, with 1 mg of cell material per milliliter. When determining numbers of viable organisms, certain limitations must be kept in mind. First, at least on solid medium, it is often difficult to initiate growth with small numbers of tubercle bacilli; wide variation in results may, therefore, occur. Secondly, optical densities are proportional to the numbers of bacteria only where organisms of the same size or size distributions occur.[6] When grown in the presence of antibiotic agents, the organisms frequently assume elongated shapes, increasing in volume rather than dividing into separate components. In comparing cultures grown in the presence and in the absence of antibiotic agents, the optical density is a measure of the relative volumes or masses rather than of the numbers of bacteria. Even with these limitations, turbidimetric readings were found to permit satisfactory interpretation of results once the relationship between turbidity, weight of cells, and numbers of viable organisms had been established.

Experimental Results. The application of the turbidimetric method for the quantitative estimation of the growth of tubercle bacilli is demonstrated in several experiments. Table I shows the influence of inoculum on the rate of growth of *M. tuberculosis* No. 607 in a modification of Dubos' medium. During the first 96 to 184 hours, the rate of growth was dependent upon the size of the inoculum; then growth appeared to proceed at a more or less uniform rate regardless of the initial concentration of the cells, as indicated by the readings at 330 hours. Similar results were obtained with *M. avium* and with the streptomycin-resistant and -sensitive strains of *M. tuberculosis* H37Rv. In this case, as in all other media used for the growth of tubercle bacilli, the strains pathogenic to man grew at a much slower rate than the saprophytes. The data also agree with the plate counts of viable bacteria, growth taking place

[6] Treffers, H. P., *Yale J. of Biol. and Med.*, 1946, **18**, 609.

with 10^{-9} mg of cell material per milliliter but not with 10^{-10}. According to the actual count, 10^{-7} mg of cell material should contain 6 viable cells; it is, of course, possible that one or more cells would be found even in the higher dilutions which would account for the growth recorded for the 10^{-9} mg ml suspension.

When a medium is inoculated with increasing concentrations of cells, there is a corresponding decrease in the lag period of growth, but all of the resulting cultures finally reach the same degree of density. On the other hand, if the inoculum is held constant and the nutrient value of the medium is varied, quite different results are obtained. An increase in the amount of growth is found to parallel enrichment of the medium. This is accompanied by a shortening of the lag period.

The effect of nitrogenous materials on the rate of growth of *M. tuberculosis var. hominis* H37Rv is presented in Table II. The incubation time required for the initiation of growth in a medium containing ammonium citrate was 10 times that required in one containing casein, a vegetable extract and serum-albumin. The difference in the amounts of growth was even more striking, the more complex medium giving 20 times more abundant growth. The rate and amount of growth are dependent not only on the number and concentration of nutrients but on the nature of the nutritive material. Tryptophane in a concentration of 0.1% is a better nutrient than 0.2% asparagine for all of the strains of mycobacteria.

Having established the normal rate of growth of mycobacteria, it was now possible to evaluate the effect of streptomycin upon the growth of mycobacteria in the presence of different sources of energy. Regardless of the source of nitrogen or of the amount of growth supported by the medium, little difference was found in the amount of streptomycin required for bacteriostasis, as shown in Table III. The only exception is found in the ammonium citrate medium; limited effectiveness of streptomycin is possibly due to the high acidity of this medium resulting from the growth of the organism.[7] Similar results were obtained with *M. avium* and the human pathogenic varieties.

Summary. A simple, safe, and accurate method is described for the quantitative estimation of the growth of *Mycobacterium tuberculosis* based upon turbidimetric measurements in Dubos' medium.

This procedure can be applied to measuring the rate and amount of growth of *M. tuberculosis* in various media, as affected by the presence of streptomycin.

[7] Waksman, S. A., Bugie, E., and Schatz, A., *Proc. Staff Meetings of Mayo Clinic*, 1944, **19**, 537.

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Preparation of an Anti-Ulcer Factor from Human Urine.*

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The preparation of crude extracts from urine (Urogastrone) and hog intestinal mucosa (Enterogastrone) which possess antigastric secretory and antiulcer activity in dogs, such as the Mann-Williamson and the Pavlov preparation has been reported in the literature. Two methods have been applied to urine for the preparation of Urogastrone or Urogastrone-like substances. Necheles[1] has prepared an inhibitor of gastric secretion and motility by treating normal human urine with ammonium sulphate and fractionating the resulting precipitate between vary-

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[1] Necheles, H., Hanke, M. E., and Fantl, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 618.