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An In Vitro Model for Sequential Study of Shiftdown of *Mycobacterium tuberculosis* through Two Stages of Nonreplicating Persistence

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It was demonstrated previously that abrupt transfer of vigorously aerated cultures of *Mycobacterium tuberculosis* to anaerobic conditions resulted in their rapid death, but gradual depletion of available O_2 permitted expression of increased tolerance to anaerobiosis. Those studies used a model based on adaptation of unagitated bacilli as they settled through a self-generated O_2 gradient, but the model did not permit examination of homogeneous populations of bacilli during discrete stages in that adaptation. The present report describes a model based on culture of tubercle bacilli in deep liquid medium with very gentle stirring that keeps them in uniform dispersion while controlling the rate at which O_2 is depleted. In this model, at least two stages of nonreplicating persistence were seen. The shift into first stage, designated NRP stage 1, occurred abruptly at a point when the declining dissolved O_2 level approached 1% saturation. This microaerophilic stage was characterized by a slow rate of increase in turbidity without a corresponding increase in numbers of CFU or synthesis of DNA. However, a high rate of production of glycine dehydrogenase was initiated and sustained while the bacilli were in this state, and a steady ATP concentration was maintained. When the dissolved O_2 content of the culture dropped below about 0.06% saturation, the bacilli shifted down abruptly to an anaerobic stage, designated NRP stage 2, in which no further increase in turbidity was seen and the concentration of glycine dehydrogenase declined markedly. The ability of bacilli in NRP stage 2 to survive anaerobically was dependent in part on having spent sufficient transit time in NRP stage 1. The effects of four antimicrobial agents on the bacilli depended on which of the different physiologic stages the bacilli occupied at a given time and reflected the recognized modes of action of these agents. It is suggested that the ability to shift down into one or both of the two nonreplicating stages, corresponding to microaerophilic and anaerobic persistence, is responsible for the ability of tubercle bacilli to lie dormant in the host for long periods of time, with the capacity to revive and activate disease at a later time. The model described here holds promise as a tool to help clarify events at the molecular level that permit the bacilli to persist under adverse conditions and to resume growth when conditions become favorable. The culture model presented here is also useful for screening drugs for the ability to kill tubercle bacilli in their different stages of nonreplicating persistence.

The recent dramatic rise in incidence of tuberculosis after several decades of decline calls attention anew to the importance of latency in the natural history of tuberculosis. *Mycobacterium tuberculosis* can lie quiescent in the host for months or years without producing overt disease and then revive and initiate the production of lesions and progressive tuberculosis (11). Furthermore, dormant tubercle bacilli may resist the bactericidal action of antimicrobial agents that are lethal to replicating bacilli. Appreciation of the factors that affect latency and activation of this disease will require clarification of the mechanisms by which *M. tuberculosis* shifts down to the dormant state and back up to active growth. We have previously described an in vitro model in which tubercle bacilli that settled through a self-generated oxygen depletion gradient in undisturbed liquid culture shifted down in an orderly metabolic process; the bacilli in the sediment were in a nonreplicating state and were tolerant to the usually lethal effects of anaerobiosis (13). These dormant bacilli exhibited a modified enzyme composition, including a shift into the glyoxylate cycle, with a glyoxylate-to-glycine shunt providing a mechanism for regenerating NAD (13). Upon subsequent aeration, the dormant bacilli exhibited synchronized replication (10). The dormant bacilli were moderately to highly resistant to the bactericidal action of rifampin or isoniazid but susceptible to the anaerobic bactericidal action of metronidazole, which has no effect on aerobic cultures of *M. tuberculosis* (15).

Although the model that depends on the settling of cells through a self-generated oxygen gradient has been useful for the in vitro study of dormancy of *M. tuberculosis*, it has some significant limitations. First, this model may yield erratic results; a slight change in growth or settling rate may upset the equilibrium between replication and settling that is required to maintain the oxygen gradient and thus support the orderly shift down to dormancy (9, 10). Second, and perhaps most important, it is not possible to isolate and study the sequential events that characterize the process of shiftdown to dormancy in the heterogeneous population of settling bacilli. We have recently developed a model system that is based on controlled agitation of sealed liquid cultures exposed to limited headspace volumes of air. This model, which is based on adaptation to a temporal rather than a spatial O_2 gradient, yields nonreplicating bacilli that are comparable to those produced in the settling system

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but permits sampling and characterization of populations of cells in at least two stages in the shiftdown process.

MATERIALS AND METHODS

Strain of *M. tuberculosis*. All experiments were conducted with *M. tuberculosis* H37Rv from the culture collection maintained in this laboratory. Small aliquots of seed stocks were maintained at −70°C and subcultured once in liquid medium before inoculation to an experimental culture.

Cultivation in liquid medium. All liquid culture experiments were conducted in Dubos Tween-albumin broth prepared according to the manufacturer's instructions from Dubos broth base (Difco, Detroit, Mich.) and Dubos medium albumin (Difco) at a final pH of 6.6 ± 0.2. The medium was aseptically dispensed in appropriate amounts to sterile tubes or culture flasks.

Two types of containers were used for liquid cultures: screw-cap test tubes, 20 by 125 mm, with a total fluid capacity of 25.5 ml, and screw-cap Nephelo (Wheaton, Millville, N.J.) (sidearm) conical flasks, of nominal 500-ml capacity and actual total fluid capacity of 600 ml. Depending on the conditions of aeration desired, solid caps with latex liners were either loosely or tightly screwed down; in some instances, tightly sealed caps with rubber septa were used to permit addition of reagents by needle without opening the container. For some experiments, tubed cultures were incubated on a model G24 rotary shaker-incubator (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37°C and 250 rpm; in others, stirring was achieved with 8-mm Teflon-coated magnetic stirring bars in the tubes (120 rpm) and 50-mm bars in flasks (70 rpm) on a Biostir 4 magnetic stirrer (Wheaton, Millville, N.J.). The mode and vigor of agitation of the sealed containers were critical factors in the rate of depletion of O_2 and the attendant adaptation of the bacilli to microaerophilic and anaerobic conditions. Growth was monitored by measuring the A_{580} in a Coleman Jr. IIA spectrophotometer (Coleman Instruments, Maywood, Ill.). Linear corrections for particle interference at an A_{580} above 0.200 were made as previously described (12).

Colony counting on solid medium. In preparation for counting of colonies on agar, dilutions of liquid culture were made in Dubos Tween-albumin broth. Dubos oleic-albumin agar was prepared from Dubos oleic agar base (Difco) supplemented with Dubos oleic albumin complex to final concentrations of 50 μg of oleate and 5 mg of albumin per ml and dispensed in 3-ml amounts to each of the 12 wells of sterile Falcon no. 3043 tissue culture plates (Becton Dickinson, Lincoln Park, N.J.). The agar surfaces were inoculated in triplicate with 20 μl of selected dilutions of test culture, and the plates were incubated at 37°C. Colonies were counted twice weekly by inverting the closed plate on a dissecting microscope at a magnification of ×10, using transmitted light. The readings were discontinued after the counts were stable for a week or all wells showed diminishing counts due to spreading and fusion of colonies.

Estimation of oxygen consumption. Two methods were used to assess the rates of consumption of O_2 in sealed cultures. A volumetric method was used to monitor the headspace O_2 in tubed cultures. A 3-ml syringe barrel was connected through a Luhr fitted stopcock to a 25-gauge needle and loaded with a measured volume of 0.1 M NaOH. At selected times, tubed cultures with tightly sealed septum caps were placed upright in a clamp and the syringe needle was inserted through the septum. The stopcock was opened, and the volume of liquid that was sucked into the tube, which corresponded to the O_2 that had been consumed, was recorded.

Dissolved O_2 was monitored in sealed flask cultures with a 12-mm autoclavable polarigraphic O_2 sensor and model 170 O_2 amplifier (Mettler-Toledo Process Analytical, Inc., Wilmington, Mass.).

Additionally, in some tubed cultures, a sterile solution of methylene blue (500 μg/ml) was diluted in the medium to yield a dye concentration of 1.5 μg/ml. Reduction and decolorization of this dye served as a visual indication of oxygen depletion.

Assay of protein. Protein concentration of extracts was determined by the Coomassie brilliant blue dye-binding technique with the Bio-Rad protein assay reagents and protocol (Bio-Rad Laboratories, Hercules, Calif.).

Assay of bacillary ATP. Our protocol for assay of ATP is a modification of the method of Dhople and Hanks (2) that provides improved reproducibility in our hands. Exactly 1.0 ml of the culture to be assayed is transferred to a 1.5-ml microcentrifuge tube and spun at approximately 10,000 × *g* for 5 min. The supernatant is discarded, and the tube is spun again to permit removal of the last droplets of supernatant that may have adhered to the walls. The cells are then resuspended in 1.0 ml of 0.025 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.75 (HB), supplemented with 0.02% Tween 80. Freshly prepared dilutions of an 80-ng/ml ATP standard stock solution are similarly treated to prepare standard curves for conversion of light units to actual concentrations of ATP. Appropriate volumes of sample and of plain HB, i.e., without Tween 80, to make a total volume of 100 μl are transferred to glass tubes (13 by 100 mm), and 30 μl of chloroform is added to each. The open tubes are placed in an 80°C aluminum heating block in a fume hood for exactly 20 min. The depth of the holes in the block is 45 mm, leaving the upper 55 mm of the tubes exposed to the flowing air of the hood. Under these conditions, the chloroform evaporates away and water vapor condenses on the upper wall of the tube; the samples themselves do not dry out. Each sample is then quickly diluted with 4.9 ml of plain HB and mixed on a vortex stirrer. This dilution eliminates any inhibitory action of traces of residual Tween 80 or chloroform on the luciferase indicator system. Exactly 100 μl of treated sample and 50 μl of plain HB are mixed gently in a luminometer tube, which is placed in a Turner model TD-20e luminometer (Turner Designs, Sunnyvale, Calif.), 50 μl of Turner luciferin-luciferase reagent is added, and the light unit reading is recorded.

Assay of GDH activity. Sonic extracts of the bacilli to be assayed were prepared as previously described (13, 14) and assayed by the method of Goldman and Wagner (5). The assay is based on optical measurement of the rate of oxidation of NADH to NAD that accompanies the reductive amination of glyoxylate to glycine by glycine dehydrogenase (GDH). Briefly, the enzyme is assayed in a UV transparent 1-ml microcuvette containing, in a final 1.0-ml volume, 0.04 M phosphate buffer (pH 6.4), 0.4 M ammonium sulfate, 0.05 M sodium glyoxylate, and 8×10^{-5} M NADH. The reaction is measured as the rate of decrease of A_{340} on a model 640 DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Specific activity of GDH is expressed as micromoles of NADH oxidized per hour per milligram of protein.

Rates of nucleic acid synthesis. Rates of synthesis of RNA and DNA were monitored by measuring the rate of uptake of [5,6-^{3}H]uracil as described previously (10). DNA was estimated from the amount of bound label remaining after alkali treatment of the bacilli to destroy the RNA; the RNA was estimated as the difference between total bound label and that remaining after alkali treatment of the bacilli. It must be noted that specific radioactivity of the DNA would be approximately one-half that of the RNA as a result of displacement of the tritium in the 5 position of the uracil in its conversion to thymine.

Replication of experiments. Most experiments were repeated between two and five times. In those cases in which the experiments were exact replicates of one another (with the exception that some of the readings or samplings may have been made at slightly different times), the figures were prepared from composite data. In other cases, either inoculum size or some other factor differed somewhat between replicates. In these cases, data from only one of the experiments were used to prepare figures as long as all experiments gave curves of the same shape and led to the same conclusions.

RESULTS

A preliminary experiment was conducted to establish limiting oxygen conditions in agitated liquid cultures of *M. tuberculosis*. Inoculated Dubos broth was distributed to screw-cap test tubes in different amounts calculated to yield ratios of headspace air volume to liquid volume ranging from 0.25 to 1.0; these proportions are hereafter referred to as headspace ratios (HSRs). One set of tightly sealed tubes was incubated at 37°C on the rotary shaker at 250 rpm (rapid rotary stir), a speed that produced definite agitation of the surface and promoted rapid and continuous equilibration between headspace gas and medium. The sealed tubes in a second set contained 8-mm stirring bars and were placed on a magnetic stirrer rotating at 120 rpm (slow magnetic stir). Under these conditions, the bacilli remained uniformly distributed throughout the medium, but the surface of the medium was not disturbed; the rate of diffusion of headspace gas into the medium in these tubes was probably no greater than that in unagitated cultures. The vigorously agitated cultures exhibited abrupt terminations of growth and entry into turbidimetric plateau after 95 to 140 h of incubation, at optical densities that were proportional (correlation coefficient, $r = 0.98$) to HSR over the range studied (Fig. 1A). In contrast, although a deflection in the rapid growth curves of the slowly stirred cultures was seen after less than 72 h, when the initially dissolved O_2 was depleted, slow logarithmic increases in A_{580} were seen for additional periods in excess of 100 h before the curves reached plateau levels (Fig. 1B). Although the plateau levels of A_{580} of the slowly stirred cultures were also proportional to HSR ($r = 0.95$), they averaged only 55% of those of the corresponding rapidly agitated cultures.

Further experiments were limited to tubes or flasks that contained one-half volume of initial headspace air per volume of medium (0.5 HSR); this comprised a volume of 17.0 ml of Dubos broth and 8.5 ml of headspace (surface-to-volume ratio = 0.14 cm^2/ml) in the 20- by 125-mm tubes and 400 ml of broth with 200 ml of headspace (surface-to-volume ratio = 0.08 cm^2/ml) in the Nephelo flasks. In one set of experiments,

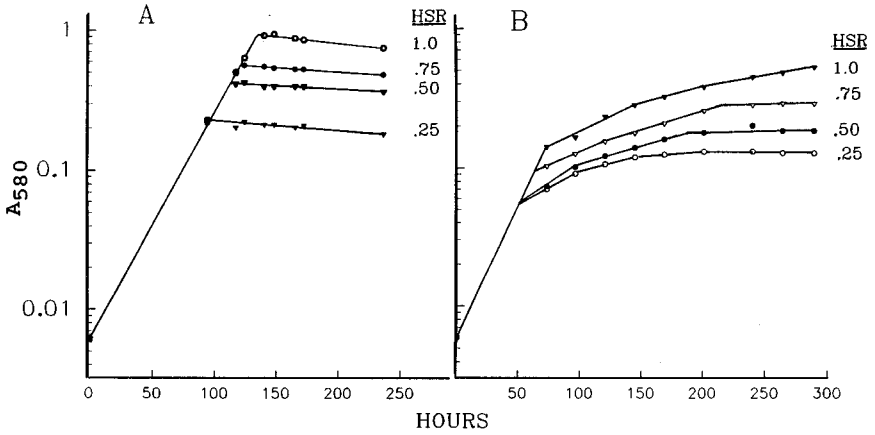

FIG. 1. Logarithmic growth curves of vigorously agitated (A) and slowly stirred (B) sealed tubed cultures of *M. tuberculosis* at different HSRs. Growth is expressed as log of turbidity (A_{580}).

inoculated 0.5-HSR tubes were capped with tightly sealed septum caps; some were incubated under conditions of rapid rotary mixing, and others were subjected to slow magnetic stir, as in the experiments described above. At intervals, tubes were removed for estimation of the depletion of O_2 from the headspace. The rate of headspace O_2 depletion in the rapidly rotated cultures paralleled that of the increase in A_{580}, which terminated abruptly at an A_{580} of 0.345, when approximately 1.5 ml (84%) of the originally available O_2 had been consumed (Fig. 2A). Further depletion of the remaining headspace O_2 continued in these tubes at a slower rate and was complete after about 35 h of further incubation, during which the culture turbidity remained at plateau. In the slowly stirred tubes, a deflection in the rapid rise in A_{580} occurred when only 28% of the headspace O_2 had been depleted, and the balance of O_2 was consumed slowly as the A_{580} increased at a greatly reduced rate (Fig. 2B).

Sets of sealed vigorously agitated and slowly stirred 0.5-HSR tubed cultures were removed at intervals for sampling to assess bacillary ATP concentrations and CFU. In tubes subject to rapid rotary mixing, the A_{580} readings paralleled the ATP concentration and CFU per milliliter during the initial growth period (Fig. 3A). In parallel cultures that were supplemented with 1.5 μg of methylene blue per ml, visual inspection indicated that complete decolorization occurred fairly abruptly about 24 h after the growth stopped. As soon as A_{580} stopped rising, the ATP concentration of the cells dropped abruptly, declining by about 50% over the next 30 h. The viable count did not start to decline notably until about 44 h after the optical increase stopped and then underwent a 50% decline over the next 54 h. Thereafter, both the ATP concentration and CFU per milliliter declined at a parallel rate, with a half-life of 100 to 110 h. We had previously noted an approximately 11-fold increase in specific activity of GDH when bacilli shifted from active aerobic growth down to anaerobic dormancy by settling through a self-generated O_2 gradient (13). In rapidly agitated sealed 0.5-HSR cultures, GDH rose somewhat as growth stopped, reaching a maximum specific activity of only 0.52 μmol/mg of protein per h, i.e., slightly over twice as high as that seen during active aerobic growth, and quickly declined to baseline levels on further incubation (Fig. 3C).

With the slowly stirred 0.5-HSR tube system, in which the cells remained suspended but the surface was undisturbed and O_2 diffusion was slow, the rapid replication associated with initially dissolved O_2 lasted about 58 h. The baseline ATP and GDH activities during this growth period were the same as

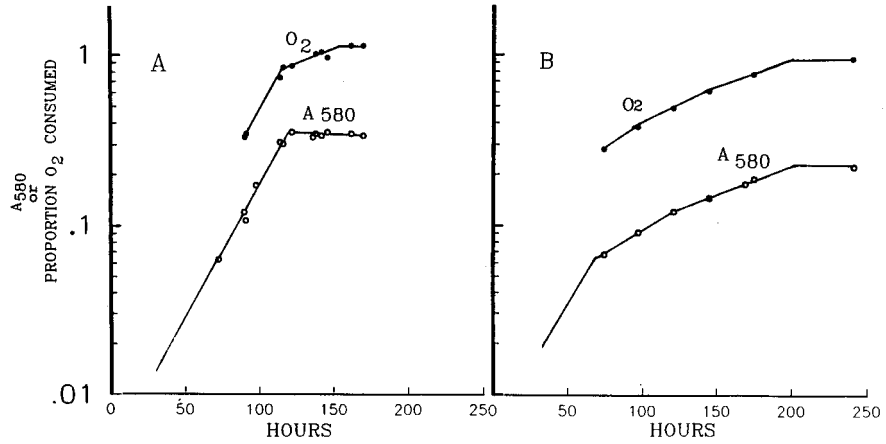

FIG. 2. Relationship of rates of headspace oxygen depletion and growth rates of vigorously agitated (A) and slowly stirred (B) tubed cultures of *M. tuberculosis* at 0.5 HSR. Growth is expressed as log of turbidity (A_{580}), and O_2 depletion is expressed as log of the proportion of initially available headspace gas consumed. Panel A is based on composite data from two independent experiments.

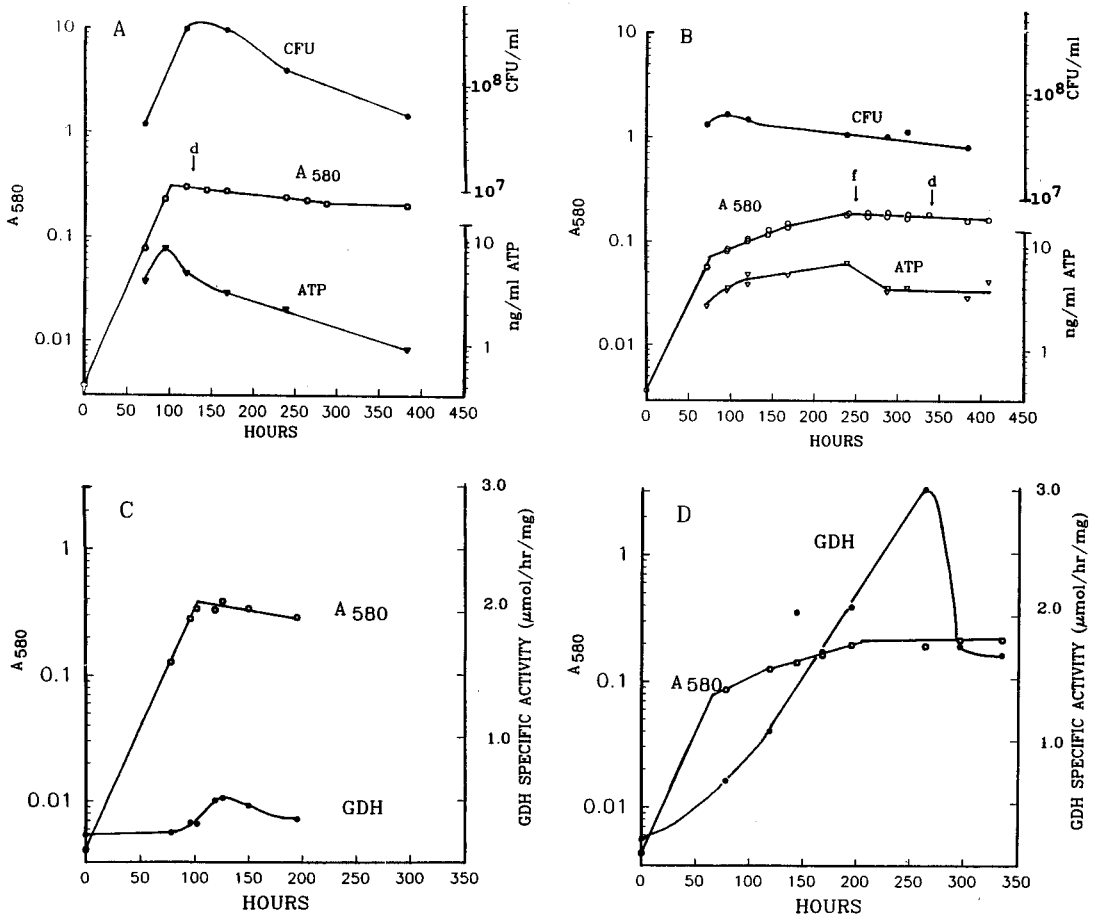

FIG. 3. Growth, survival, and physiologic state of tubed cultures of *M. tuberculosis* grown under 0.5-HSR conditions. (A) Turbidimetric measurement (A_{580}), CFU, and ATP content of cultures grown with vigorous rotary agitation; (B) the corresponding measurements of cultures grown with slow stirring; (C) turbidimetric measurement and GDH content of cultures grown with vigorous rotary agitation; (D) the corresponding measurements of cultures grown with slow stirring. A_{580}, CFU per milliliter, and nanograms of ATP per milliliter are displayed on a logarithmic scale, and specific activity of GDH (micromoles of NADH oxidized per hour per milligram of protein) is displayed on a linear scale. The times when an indicator culture tube containing 0.5 μg of methylene blue per ml exhibited noticeable fading (f in panel B) and complete decolorization of the dye (d in panels A and B) are indicated. Panel B is based on composite data from two independent experiments.

those seen in the rapid-stir system (Fig. 3B and D). Over the next 150 h, the turbidity increased more slowly while the CFU count leveled off and ATP increased slightly; i.e., the slow increase in A_{580} during this stage was not reflected in an increase in CFU count (Fig. 3B). However, when a calibrated ocular micrometer was used to estimate the size of the acid-fast stained bacilli, it was seen that the length of the bacilli increased by 32%, from a median length of 2.23 μm during active aerobic growth to 2.94 μm during the period of O_2 depletion, which is consistent with the increase in turbidity in the absence of replication. During this first nonreplicating stage characterized by slowly increasing turbidity, which was designated NRP stage 1, the GDH-specific activity increased rapidly to a level of 3.01 μmol/mg of protein per h, about 10-fold greater than the baseline level seen during cell replication. As the turbidity readings approached the plateau value of the second nonreplicating stage, designated NRP stage 2, a small decline in ATP concentration occurred; the GDH specific activity started to decline about 30 h after entry of the culture into the NRP stage 2 plateau until it reached a level about one-half that of the peak value. Fading of methylene blue in slowly stirred indicator tubes did not start until turbidity (A_{580}) reached its plateau, and complete decolorization took about an extra 100 h. Throughout the NRP stage 2 period, the viable count (CFU per milliliter) dropped very slowly, with a half-life of 360 h.

The slow logarithmic increase in turbidity during NRP stage 1 in slowly stirred tubes was not reflected in an increase in CFU per milliliter (Fig. 3B). The incorporation of ^3H derived from uracil into DNA also stopped when the first deflection of the turbidity curve occurred (Fig. 4). Although generation of labeled RNA also appeared to stop at this point, the crude methods of assessing incorporation of label into RNA would not have permitted reliable detection of continuing synthesis of RNA, especially mRNA, coincident with ongoing turnover of previously synthesized RNA. However, subsequent pulse-labeling experiments with bacilli in NRP stages 1 and 2 have provided evidence of continuing RNA synthesis during both of these stages.

The experiments illustrated in Fig. 2 reflected depletion of O_2 in the headspace over the cultures but did not measure the actual concentrations of dissolved O_2 in the medium itself. To measure this effect, it was necessary to conduct experiments in sidearm flasks that were large enough to accommodate an O_2 sensor. These experiments were limited to study of the slow-stir system, which was characterized by the three discrete stages of growth and shiftdown. The deflection from rapid

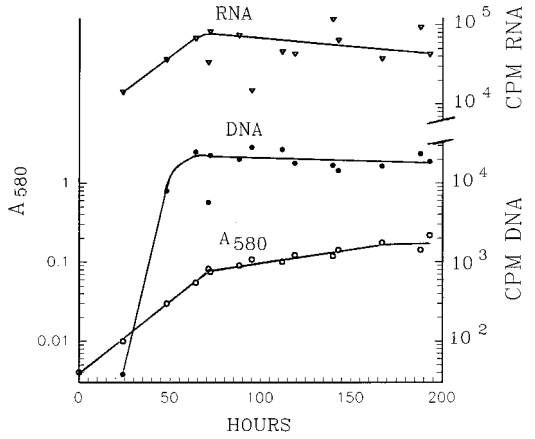

FIG. 4. Uptake of radiolabel from [^{3}H]uracil into total RNA and DNA during growth and shiftdown of *M. tuberculosis* in slowly stirred 0.5-HSR tubed cultures. This figure is based on composite data from two independent experiments.

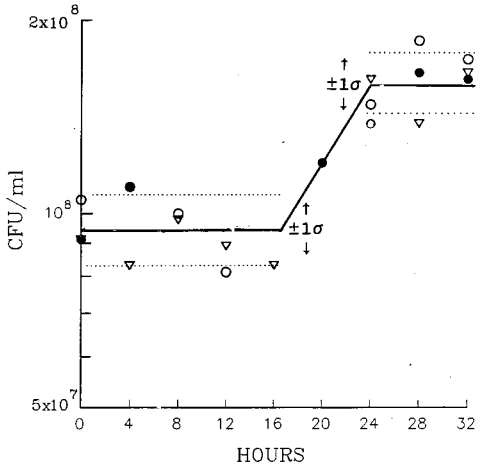

FIG. 6. Synchronous replication of *M. tuberculosis* after dilution of NRP stage 2 cultures into fresh, oxygen-rich medium. The solid lines between 0 and 16 h and between 24 and 32 h represent the respective mean counts (n = 11 and 9 samples, respectively) during those periods of shiftup; the dotted lines represent 1 standard deviation (σ) above and below these means. This figure is based on composite data from two independent experiments.

first-stage growth into NRP stage 1 appeared to start about the time that the O_2 concentration of the medium approached 1% of the original saturation level (Fig. 5); at that point, the O_2 concentration dropped so precipitously that it was not possible to define the precise degree of O_2 depletion that was associated with the shift from active growth to NRP stage 1. The culture remained in NRP stage 1 until the O_2 concentration dropped below a level corresponding to approximately 0.06% saturation, when a further deflection occurred as the culture entered NRP stage 2 plateau.

In a number of experiments in which flasks at 0.5 HSR were used instead of test tubes, it was noted that the NRP stage 1 lasted until the A_{580} reached values between 0.40 and 0.45 before shifting into NRP stage 2; this is about twice the highest level seen in tubes. As noted above, the surface-to-volume ratio in the tubes was 0.14 cm^2/ml and that in the flasks was only 0.08 cm^2/ml; therefore, that factor cannot account for the longer duration of NRP stage 1 in the flasks. However, there is a more prominent meniscus in tubes, which suggests that surface tension effects in the tubes may account for slower diffusion of residual headspace O_2 into the medium, resulting in earlier termination of NRP stage 1 metabolism than occurs in flasks. This difference is of special interest, since GDH specific activity in slowly stirred 0.5-HSR flasks reached levels of 6 to 7 μmol/mg of protein per h, which is approximately twofold higher than that seen in tubes. Observations of further interest in this regard were made when tubes and flasks with 0.5-HSR configurations were incubated with slow stirring, but with their caps loose instead of tightly sealed. The loosely capped cultures shifted from active aerobic growth to NRP stage 1 at the same time as did sealed cultures. However, in contrast to what was seen in sealed systems, the bacilli remained in NRP stage 1, with the A_{580} continuing to rise to values in excess of 1.0 in the flasks without shifting into NRP stage 2. The total concentration and the specific activity of GDH continued to rise in parallel with turbidity, suggesting continued protein synthesis as long as the bacilli remained in NRP stage 1. In loosely capped flasks with O_2 sensors, the dissolved O_2 content did not drop below 0.06% saturation, whereas in sealed flasks the shift to NRP stage 2 accompanied a drop in dissolved O_2 content below detectable levels, i.e., about 0.02% saturation.

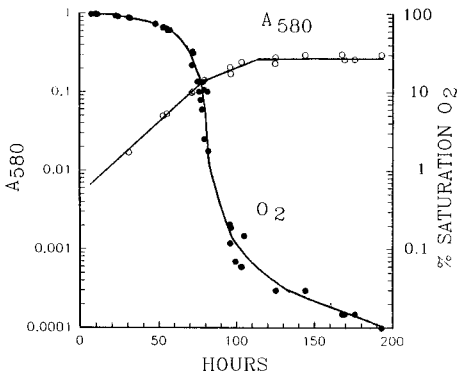

FIG. 5. Depletion of dissolved O_2 in slowly stirred 0.5-HSR flask cultures of *M. tuberculosis* during growth and shiftdown. The O_2 concentration is expressed as percent saturation of the medium with O_2 in terms of medium initially equilibrated with ambient air. This figure is based on composite data from two independent experiments.

Suspensions of bacilli in NRP stage 2 were diluted 1:100 in fresh, oxygen-rich medium, incubated, and sampled at intervals for further dilution and plating to agar medium. A plateau of nonreplication was recorded, with a mean viable count (composite data from three independent experiments) of (0.99 ± 0.11) × 10^8 CFU/ml, over the period between the time of initial dilution through 16 h postdilution (Fig. 6). An abrupt approximate doubling then occurred, and a second plateau of nonreplication was sustained, with a mean viable count of (1.67 ± 0.16) × 10^8 CFU/ml from h 24 through 32 postdilution, thus demonstrating synchronous replication. The replication of bacilli from NRP stage 1 cultures was not synchronous on dilution and incubation.

Four antimycobacterial agents of known mechanism were evaluated for activity against tubercle bacilli in each of the stages under study here. Aliquots of appropriate concentrated stock solutions of isoniazid, rifampin, ciprofloxacin, and metronidazole were added to the tubed cultures to yield final concentrations of 0.4, 0.1, 1.0, and 12 μg/ml, respectively. One set of loosely capped actively growing aerated cultures received the first three of these drugs by pipette when they were in mid-log stage and 96 h old; metronidazole was not tested in aerated cultures. A set of slowly stirred 0.5-HSR cultures that

TABLE 1. Survival of *M. tuberculosis* after exposure to isoniazid (0.4 μg/ml), rifampin (0.1 μg/ml), ciprofloxacin (1.0 μg/ml), and metronidazole (12 μg/ml) during active growth, during NRP stage 1, and at various intervals after entering NRP stage 2[a]

Age of culture when sampled (h)	Growth stage when sampled	% of bacilli surviving exposure to:			
		Isoniazid	Rifampin	Ciprofloxacin	Metronidazole
192 (aerated)	Active growth	2.0	4.2	0.026	ND[b]
192 (0.5 HSR)	NRP stage 1	60.0	26.0	102.000	141.0
336 (0.5 HSR)	NRP stage 2	98.0	17.0	87.000	50.0
432 (0.5 HSR)	NRP stage 2	ND	17.0	ND	12.0
528 (0.5 HSR)	NRP stage 2	ND	12.0	ND	2.5

[a] For the actively growing set, percent survival is expressed in terms of the count in the drug-free control at the time drugs were added. For the sets in NRP stages 1 and 2, percent survival is expressed in terms of the drug-free control at the time samples were taken to compensate for microaerophilic or anaerobic attrition in those controls. All drugs except metronidazole were added to the NRP cultures through rubber septa 96 h before the indicated samples were taken; metronidazole was added at the beginning of the experiment.

[b] ND, not determined.

were sealed with septum caps received isoniazid, rifampin, or ciprofloxacin by syringe and needle when they were in NRP stage 1 and at different times during NRP stage 2. Metronidazole was added to a set of 0.5-HSR cultures immediately after inoculation, since this drug has no effect on actively growing cultures of *M. tuberculosis* (15). Samples were taken for dilution and determination of viability counts (CFU per milliliter) 96 h after each addition of isoniazid, rifampin, and ciprofloxacin; drug-free controls and cultures that contained metronidazole were sampled at the same times as were cultures containing the other three drugs. The counts (CFU per milliliter) of drug medium were compared with those of corresponding control cultures that had not received drugs, and the results were expressed as percent survival compared with these controls. For the actively growing preparations, the reference control count represented the culture on the day drug was added to the other tubes, since growth continued to occur in the control. For the nonreplicating preparations, the reference count represented the control culture on the day that the drug-containing tubes were sampled, to correct for the small extent of attrition that occurred in those drug-free controls.

Isoniazid, rifampin, and ciprofloxacin killed over 95% of the actively growing aerated bacilli within 96 h of exposure (Table 1). However, isoniazid and ciprofloxacin had negligible effects on bacilli in either NRP stage 1 or NRP stage 2, with over 50% survival in each case; rifampin exhibited some definite but reduced bactericidal activity against bacilli throughout both nonreplicating stages. Metronidazole did not kill tubercle bacilli in NRP stage 1 and had a negligible effect early in NRP stage 2. However, increasing killing was seen later in NRP stage 2; we had previously observed action of this drug against tubercle bacilli only under anaerobic conditions (15). It is noteworthy that rifampin continued to exhibit some bactericidal effect even after redox conditions had dropped low enough for the antimicrobial activity of metronidazole to be expressed.

DISCUSSION

In our earlier model of in vitro dormancy of *M. tuberculosis*, the shiftdown to anaerobic dormancy was effected by the slow settling of bacilli through a self-generated spatial gradient of O_2 depletion (9, 10). The existence of an intermediate physiologic state in which enzyme inductions occurred that permitted adaptation from aerobic growth to anaerobic dormancy was inferred (13), but bacilli in the intermediate state could not be isolated from those cultures for study. The newer model described here, in which the shiftdown occurs in a temporal O_2 depletion gradient achieved by slow controlled mixing of cultures under a restricted air column, does permit the study of the events associated with the shiftdown from active growth to dormancy by allowing harvest of cells during the induction process. There is a growing body of knowledge about the different mechanisms which have evolved among nonsporulating bacteria that enable them to adapt to survival under inhospitable conditions (6a). It will be important to determine which of these mechanisms are shared by *M. tuberculosis.*

The nature of the response of an actively growing liquid culture of *M. tuberculosis* to O_2 depletion is a function of the rate at which that depletion occurs. When a vigorously aerated culture is abruptly subjected to anaerobic conditions, there is no opportunity for adaptation to occur and unbalanced metabolism leads to rapid death, with a half-life of approximately 10 h (13). However, as shown in the present study, it is possible to regulate the rate of O_2 depletion in a manner that permits the occurrence of differential adaptive responses that allow the bacilli to survive longer under microaerophilic and/or anaerobic conditions. The pattern of O_2 depletion in a sealed system is determined by both the initial volumetric ratio of air to medium and the rate of equilibration between gas and liquid phases (Fig. 1). In rapidly agitated tubes on a rotary mixer, equilibration between gas and liquid phases is so efficient that no deflection in the growth curve is seen until the headspace O_2 has been about 84% depleted. However, depletion of the balance of the O_2 thereafter is so rapid that growth terminates abruptly (Fig. 2A), and only a minimal adaptation to anaerobic conditions occurs; a decline in ATP content of the cells, followed shortly by a parallel decline in CFU, is seen, with a half-life of about 54 h (Fig. 3A). Further declines in ATP and CFU are seen with a half-life of around 100 h, suggesting partial adaptation of the survivors to a greater tolerance to O_2-limited conditions. Minimal production of GDH was detected in the rapid-agitation system (Fig. 3C).

In contrast to the rapid depletion of O_2 in vigorously rotated cultures, the cultures subjected to slow magnetic stirring with no detectable perturbation of the surface of the medium had a much slower rate of equilibration with the air, and the headspace exhibited a much slower rate of O_2 depletion. When the O_2 content of the headspace air had been reduced by only 28%, an abrupt deflection in the turbidity curves occurred, and the rate of turbidity increase and headspace O_2 depletion paralleled one another until the O_2 was gone and the exponential increase in turbidity ceased (Fig. 2B). Unexpectedly, this stage of slow increase in turbidity was not reflected in an increase in CFU (Fig. 3B). This period during which turbidity increased slowly without significant change in CFU counts or ATP content is perhaps the most interesting as a model for studying the

ability of tubercle bacilli to survive under conditions that do not support replication, since it probably resembles conditions encountered in inflammatory or necrotic tissue (11). At the point where oxygen depletion exerted a demonstrable negative effect on replication, production of GDH began to rise, reaching a peak over 10-fold higher than baseline and then declining to about half that level (Fig. 3D). We had previously speculated that this enzyme provides a mechanism for regenerating essential NAD as an adaptation to hypoxic conditions (11, 13). The termination of DNA synthesis (Fig. 4) coincided with the first deflection in the turbidity curve and the cessation in increase in CFU counts, confirming that replication had indeed terminated rather than reflecting a balance between replication and death of cells.

While the plateau in the incorporation of label into the DNA of the cultures reflected the termination in cell replication, some continuing RNA synthesis was demonstrated by pulse-labeling during the nonreplicating cell stages. The marked increase in GDH that was induced shortly after the first deflection in the turbidity curve of the slowly stirred cultures strongly suggests that mRNA synthesis occurred at least during NRP stage 1.

Further insight into the special nature of the bacilli in NRP stage 2 under complete O_2 depletion is offered by the fact that the bacilli exhibited synchronous replication upon resumption of aeration. There must be a well-defined induction of a highly specialized array of regulatory products which govern synthesis of some enzymes that are involved in maintenance metabolism, while repressing the production or activity of others that are involved in cell replication as O_2 is depleted, and a reversal of these functions when aeration is resumed. Studies of these mechanisms are presently under investigation in this laboratory.

The experiments involving the use of antimicrobial agents provide additional insights into the phenomenon of nonreplicating persistence (Table 1). Isoniazid is believed to exert its bactericidal effect by interfering with synthesis of mycolic acids that are needed in the cell walls (8); there is evidence that this effect depends on interaction of the drug with mycobacterial peroxidase and H_2O_2 to produce reactive oxygen radicals (7). Our observations that enlargement of bacilli occurs without replication during NRP stage 1 suggest that some continuing cell wall synthesis occurs. The failure of isoniazid to exert pronounced bactericidal effects under these conditions probably reflects the suppressed production of H_2O_2 during the microaerophilic metabolism; we had previously demonstrated only a minor decline in peroxidase content of the dormant bacilli (13). Ciprofloxacin inhibits the gyrase A subunit of DNA gyrase (1), which is essential for DNA synthesis, and the failure of this drug to exert bactericidal effects on tubercle bacilli that are not replicating or producing DNA is consistent with that mechanism. Rifampin, on the other hand, inhibits DNA-dependent RNA synthesis (6) and continued to exhibit some bactericidal action even after O_2 depletion occurred. The demonstration of some continuing action of rifampin on bacilli in these stages, even after the redox potential has dropped sufficiently for metronidazole to initiate its bactericidal action, supports the inference that the enzymes that may be induced during microaerophilic, or even subsequent anaerobic, metabolism are essential to survival. Metronidazole exerts its bactericidal action through an intermediate reduction product that is produced only at a reduction potential below -430 mV and which kills by nicking DNA (4). The identity of an enzyme system in *M. tuberculosis* that is capable of producing this low a reduction potential remains to be established (15).

A slow depletion of O_2 appears to permit the occurrence of adaptations that favor long-term nonreplicating persistence of tubercle bacilli under microaerophilic conditions and also enhance the ability of the bacilli to survive anaerobic conditions. This versatility could account for long-term latency of tuberculosis in the mammalian host. Much effort today is directed toward characterizing the interaction of tubercle bacilli with various components of the host's cellular defense mechanisms, and this is critical to the understanding of the process of early infection and induction of disease. However, bacilli also persist in necrotic areas of host tissue which may be acellular and/or avascular and are thus exposed to a very wide range of physiologic conditions (11). The model presented here should be useful for identifying the molecular events and products that are responsible for the versatility of *M. tuberculosis* in surviving under that range of conditions, as well as those associated with reactivation of these bacilli.

The rationale for routine use of drug combinations in the treatment of tuberculosis has traditionally been to avoid the risk of emergence of drug-resistant strains under monotherapy. However, as suggested in 1981 by Dickinson and Mitchison (3), drug combinations may also serve to attack mixed populations of growing and of semidormant tubercle bacilli. The results of studies presented here support this second function of multidrug therapy, the need to attack all of the members of a physiologically heterogeneous population of the tubercle bacilli in whichever metabolic stage they happen to occupy. Efforts to eradicate tuberculosis must not rely solely on attacking the actively growing tubercle bacilli but must also include selected and possibly different antimicrobial agents for killing those in both microaerophilic and anaerobic nonreplicating stages. The culture model presented here should be useful for screening existing and new drugs for the ability to kill nonreplicating tubercle bacilli.

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