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Glyoxylate Metabolism and Adaptation of *Mycobacterium tuberculosis* to Survival under Anaerobic Conditions

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Tuberculosis is characterized by periods in which the disease may be quiescent or even clinically inapparent, but in which tubercle bacilli persist and retain the potential to reactivate the disease. The present study was carried out in pursuit of an in vitro model which might contribute to the understanding of the physiology of nonreplicating persisters, with oxygen limitation used as the means of inducing this state. When actively growing aerated cultures of *Mycobacterium tuberculosis* were suddenly placed under anaerobic conditions the bacilli died rapidly, with a half-life of 10 h. When the bacilli were grown in liquid medium without agitation, they adapted to the microaerophilic conditions encountered in the sediment; the adapted bacilli in the sediment did not replicate there but were tolerant of anaerobiosis, exhibiting a half-life of 116 h. Among the early events associated with the adaptation were the synthesis of an antigen designated URB, the function of which is not known, and a fourfold increase in isocitrate lyase activity. The bacilli later exhibited a 10-fold increase in synthesis of a glycine dehydrogenase that catalyzes the reductive amination of glyoxylate, concomitantly oxidizing NADH to NAD. Specific activities of other enzymes studied were either not affected or moderately diminished in the sedimented bacilli. It is proposed that the glyoxylate synthesis in this model serves mainly to provide a substrate for the regeneration of NAD that may be required for the orderly completion of the final cycle of bacillary replication before oxygen limitation stops growth completely. This orderly shutdown is essential to continued survival of *M. tuberculosis* in a quiescent form.

When *Mycobacterium tuberculosis* is grown without agitation in medium that contains a detergent, a balance is achieved among the rate of replication, the diffusion of oxygen, and the settling of the bacilli to the bottom of the culture (25). The net result is an apparent arithmetic growth mode which actually reflects continuing logarithmic replication in the upper, oxygen-rich layers, accompanied by a compensating termination of replication of cells after they have settled through the oxygen-poor region. The settling process is associated with an adaptation to survival under anaerobic conditions. These adapted bacilli in the sediment exhibit synchronized replication when resuspended and diluted into oxygen-rich medium (26), and they possess an antigenic component that is not found in continuously aerated cultures (30).

The existence of synchrony in a population of bacilli that has accumulated in the sediment continuously over a period of approximately 2 weeks indicates that the adaptation is directed to dormant survival rather than merely decelerated replication. This idea is further supported by the demonstration, after resuspension of the cells, of a 12-h period of RNA synthesis before DNA synthesis begins (26). This "resting" state may be analogous to the condition of tubercle bacilli lying quiescent in the host, i.e., latent disease in humans or the plateau of bacillary load in late murine infection (24). The study reported here was undertaken to define some of the parameters of adaptation of *M. tuberculosis* to survival under anaerobic incubation.

MATERIALS AND METHODS

Cultivation of *M. tuberculosis*. All experiments were conducted with *M. tuberculosis* $H_{37}Rv$ cultivated in Dubos Tween-albumin broth (DTA) prepared from Dubos broth base (Difco) and Dubos medium albumin (Difco). The complete medium contains (per liter): asparagine, 2 g; casitone (Difco), 0.5 g; Na_2HPO_4, 2.5 g; KH_2PO_4, 1 g; ferric ammonium citrate, 50 mg; $MgSO_4$, 10 mg; $CaCl_2$, 0.5 mg; $ZnSO_4$, 0.1 mg; $CuSO_4$, 0.1 mg; Tween-80, 0.2 g; bovine albumin fraction V, 5 g; and glucose, 7.5 g, at a final pH of 6.6 ± 0.2. The nonreplicating resting bacilli (RB) were prepared as previously described (30) by incubating the cultures for 19 days at 37°C in tall (60 by 160-mm) screw-

capped centrifuge bottles containing 200 ml of DTA per bottle or in screw-capped culture tubes (20 by 125-mm) containing 10 ml of DTA per tube without agitation. The aerated growing (AG) bacilli were grown at 37°C in screw-capped culture tubes (20 by 125 mm) containing 10 ml of DTA per tube, which were rotated at 250 rpm on a G 24 Environmental incubator shaker (New Brunswick Scientific Co.) until the cultures reached mid-logarithmic growth. The AG cells were usually harvested as a pool from 80 tubes. They were grown in tubes rather than flasks to avoid having to supplement the medium with glycerol, as would be required for growth in shake flasks (13, 25).

Anaerobic conditions were established in 1-liter mason jars (Wheaton) by the copper-activated iron wool method described by Parker (19). Samples of cultures in 20 by 125-mm tubes, loosely capped, were placed in the jars, and approximately 3 g of freshly activated iron wool was placed in each jar immediately before it was sealed. An indicator tube of AG culture containing methylene blue (2.5 μg/ml) was also placed in each jar; marked decolorization was evident within 2 h.

Estimation of survival. The viability of bacilli in suspensions subjected to various treatments was determined by performing serial 10-fold dilutions in DTA and plating 0.1-ml samples of each dilution to triplicate wide-mouthed, screw-capped jars (40 by 40 mm) containing 8 to 10 ml of Dubos oleic albumin agar per jar. The medium was prepared from Dubos oleic agar base (Difco) and Dubos oleic albumin complex (Difco). The jars were incubated at 37°C until colony counts remained constant for 1 week. Viability was expressed as colony-forming units per milliliter.

Preparation of extracts. Bacilli were harvested and sonically disrupted as described by Wayne and Sramek (30). In some cases the sonic extracts were clarified by adsorption of the protein onto and elution from DEAE (30) (DEAE-clarified products). In other instances when this treatment led to inactivation of enzymes, the extract was clarified by centrifugation at 7,500 × *g* for 1 h at 5°C, and the supernatant was filtered through a 0.45-μm membrane filter (Millipore Corp.) (centrifuge-clarified products). Specific modifications in the suspension fluids for individual enzyme experiments will be presented below. The protein concentration in these extracts was determined by the Coomassie brilliant blue dye-binding technique with the Bio-Rad protein assay reagents and protocol (Bio-Rad Laboratories).

Immunological methods. Line-and-rocket immunoelectrophoresis was performed as described by Wayne and Sramek (30). The reference antiserum was specific for the URB antigen that had been reported to be unique to and characteristic of tubercle bacilli in the RB phase (30). A unit of URB activity is that amount of antigen which causes a 1-mm peak deflection of the reference URB line in the reference line-and-rocket immunoelectrophoresis system. Specific activity is expressed as units per milligram of protein.

The identity of catalase from RB to that from AG bacilli was confirmed by determining immunological distance by the seroprecipitation dilution method described by Wayne and Diaz (28).

Enzyme assays. Catalase (EC 1.11.1.6) was assayed in DEAE-clarified products by the method of Diaz and Wayne (3). One unit of enzyme activity represents the amount necessary to bring about a 90% destruction of substrate during the 2-min incubation period. Specific activity is expressed as units per milligram of protein.

Superoxide dismutase (EC 1.15.1.1) was assayed in DEAE-clarified products by the method of McCord and Fridovich (14). One unit represents the amount necessary to cause a 50% decrease in the rate of reduction of the ferricytochrome *c* in the assay system, and specific activity is expressed as units per milligram of protein.

NAD glycohydrolase (EC 3.2.2.5) in DEAE-clarified products was estimated by monitoring the destruction of NAD by the cyanide addition method of Zatman et al. (32). The buffer in which the bacilli were sonicated and the DEAE elution buffer were supplemented with 3 mM mercaptoethanol and 3 mM AMP, since the same preparations were used for the threonine dehydratase assay. The NAD glycohydrolase in the extracts was activated by rapidly diluting samples in 0.1 M phosphate buffer, pH 6.5, which had been preheated to 85°C (9). After 90 s, the samples were chilled rapidly in an ice bath. One unit of activity causes destruction of 1 μmol of NAD per h. Specific activity is expressed as units per milligram of protein.

For estimation of NAD glycohydrolatase inhibitor activity, samples of unheated extract were added to NAD glycohydrolase assay mixtures containing 0.17 U of heat-activated enzyme. A unit of the inhibitor inhibits one unit of NAD glycohydrolase.

Threonine dehydratase (EC 4.2.1.16) was assayed by the continuous spectrophotometric method of Phillips and Wood (21), but dithiothreitol was substituted for glutathione (20). The buffer in which the bacilli were sonicated and the DEAE elution buffer were supplemented with 3 mM mercaptoethanol and 3 mM AMP. A unit of activity represents 1 μmol of α-ketobutyrate produced per h, and specific activity is expressed as units per milligram of protein. For distinguishing between biosynthetic and biodegradative threonine dehydrase, some samples were assayed in the presence of 1 mM L-isoleucine (12).

Acetyl-CoA carboxylase (EC 6.4.1.2) was assayed by the $^{14}CO_2$ method described by Erfle (7). The sonic extract of bacilli was prepared in the same manner as for the isocitrate lyase assay. Specific activity is expressed as μmoles of $^{14}CO_2$ fixed per hour per milligram of protein.

Isocitrate lyase (EC 4.1.3.1) was assayed by the method of Roche et al. (22), with 9.6 mM sodium isocitrate used as the substrate. The bacilli were suspended in phosphate-buffered saline (pH 7.5) containing 1 mM dithiothreitol and 5 mM $MgCl_2$ before sonication, and the extract was clarified by centrifugation and filtration. The specific activity is expressed as μmoles of glyoxylate formed per hour per milligram of protein.

Malate synthase (EC 4.1.3.2) was assayed by the method described by O'Connell and Paznokas (18). The same extracts were used as for the isocitrate lyase assay. However, these extracts had to be desalted against phosphate-buffered saline on a Sephadex G-25 column (Pharmacia Fine Chemicals) to remove the dithiothreitol, which interferes with optical measurement of free coenzyme A (CoA). The specific activity is expressed as μmoles of CoA released from acetyl-CoA per milligram of protein per hour.

The oxidation of NADH during reductive amination

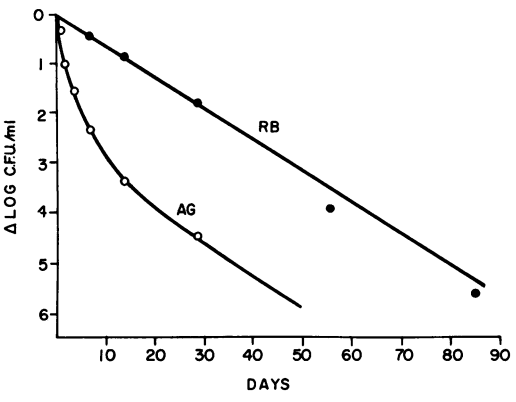

FIG. 1. Rates of decline in viability of AG and RB cells of *M. tuberculosis* under anaerobic incubation.

of glyoxylate by an enzyme referred to as glycine dehydrogenase by Goldman and Wagner (8) was monitored by the method described by them. The same extracts were used as for the isocitrate lyase assay. Specific activity is expressed as μmoles of NADH oxidized per milligram of protein per hour.

RESULTS

Survival under anaerobic conditions. In earlier studies (25, 26) the viability of bacilli in the anaerobic sediments of RB cultures was monitored for about 3 weeks. Thereafter pellicles frequently formed, making it impossible to assess the state of the bacilli in the sediments with assurance. These studies have now been extended by transfer of the cultures to anaerobic jars, which prevent pellicle formation. Tubed samples of resuspended RB culture sediments and of logarithmic-phase agitated AG cultures were distributed to anaerobic jars and incubated at 37°C. At selected intervals samples were diluted and plated, and the bacillary survival was calculated in terms of the viability counts of samples of the original suspensions.

The first such experiment examined long-term survival. The organisms in RB phase exhibited a slow logarithmic decay over 12 weeks with a half-life of 116 h (Fig. 1). The AG organisms, on the other hand, exhibited a rapid initial decline, with a half-life of only 10 h; thereafter the death rate decreased, and after 2 weeks of anaerobiosis the rate of decline of the AG preparation paralleled that of the RB. The displacement of the later stages of the decay curves suggests that about 0.2% of the AG bacilli were either already in RB phase or adapted to that phase before anaerobiosis was complete.

In the next experiments AG cells were preconditioned by terminating agitation for various periods and RB cells were preconditioned by agitating them continuously for various times immediately before they were placed in anaerobic jars and incubated there for 7 days. The RB cells showed little loss of tolerance to anaerobiosis after 8 h of aerobic preconditioning, but after 24 h of aeration they exhibited a 10-fold decline in anaerobic survival (Fig. 2B). Conversely, simple termination of agitation without abrupt total deprivation of O_2 permitted the AG bacilli to initiate an adaptation as they settled through a self-generated O_2 gradient. This adaptation was initiated by the fourth hour of settling and progressed through 24 h. However, even though both classes of cells would have had time to go through a cycle of division within 24 h, the RB did not reach the level of susceptibility to an-

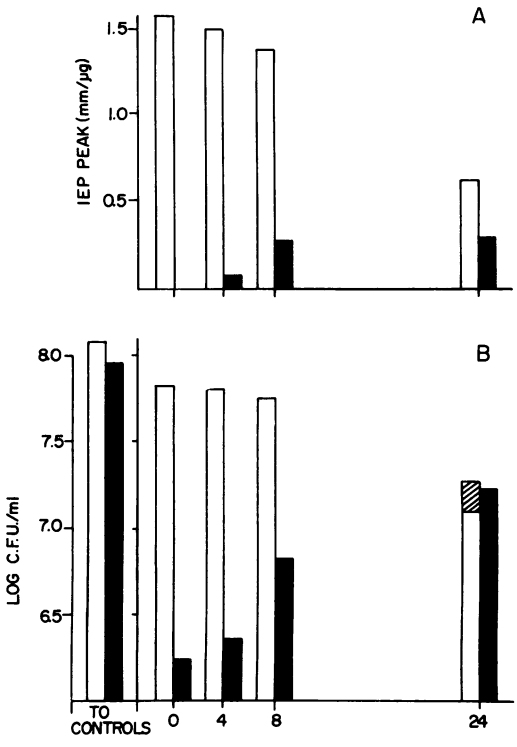

FIG. 2. URB antigen synthesis by and anaerobic survival of AG- and RB-phase cultures of *M. tuberculosis* as a function of preconditioning. Open bars: RB cultures preconditioned by continuous aerobic shaking for indicated periods of time before being tested. Solid bars: AG cultures preconditioned by terminating aeration and allowing them to settle for the indicated periods of time before being tested. (A) URB antigen synthesis expressed as specific activity (peak height in millimeters per microgram of protein) of bacillary extracts. (B) Survival after 7 days of anaerobic incubation. The control bars at the left represent RB and AG cultures that were sampled and plated without anaerobic treatment before the initiation of preconditioning. The shaded portion at the top of the 24-h RB bar represents the growth increment (log = 0.18) contributed by the 24 h of preconditioning aeration.

TABLE 1. Comparison of enzyme activities in extracts of AG and RB cells of *M. tuberculosis*

Enzyme	Sp act (U/mg of protein)[a] in extracts of:	
	AG	RB
Catalase	3.35	1.68
Superoxide dismutase	15.7	5.1
Acetyl-CoA carboxylase	0.107	0.073
Threonine dehydratase	0.234	0.228
NAD glycohydrolase	0.113	0.115
NAD glycohydrolase inhibitor	2.23	1.86

[a] Units of each enzyme are defined in the text.

aerobiosis characteristic of an AG culture; similarly, that period of preconditioning did not permit the AG cells to reach the full level of resistance of an untreated RB culture.

Similar preconditioning experiments were done with 200-ml batches of AG and RB cultures. These cultures were sonically disrupted, and the extracts were examined by line-and-rocket immunoelectrophoresis for quantitative determination of the URB antigen (30) (Fig. 2A). The RB cells exhibited a decline in URB antigen after aeration, dropping in 24 h to a level that corresponded to a 50% loss in specific activity of the reference extract. Since this also corresponded to a doubling of the number of bacilli in the aerated suspension, the decrease in specific activity of this antigen probably represents simple dilution with freshly synthesized protein rather than destruction of the antigen. The AG cell extracts, in which URB antigen could not be detected before preconditioning of the bacilli, displayed traces of the antigen after 4 h of settling and reached a plateau after the cells had settled for 8 h, which extended for 24 h. The specific activity of the URB antigen in these AG preparations reached a level corresponding to only 18% of that of an untreated RB preparation. This indicates that URB synthesis is induced by partial anaerobiosis but cannot be supported after the O_2 concentration falls below a critical level.

The specific activities of catalase and of superoxide dismutase were markedly lower in RB than in AG preparations (Table 1), but both products had readily demonstrable amounts of both enzymes. When the catalases in the AG and RB extracts were compared for immunological distance from one another (28), they were seen to be identical.

Similar activities of acetyl-CoA carboxylase were found in extracts of AG and RB cells (Table 1), although the activity in AG cells was slightly higher. No fixation of $^{14}CO_2$ could be demonstrated in the absence of acetyl-CoA.

When *Escherichia coli* is subjected to anaerobiosis, synthesis of a biodegradative threonine dehydratase is induced (6) which functions in anaerobic energy production. A second type of threonine dehydratase, which is not dependent on anaerobiosis for its induction, serves a biosynthetic function in isoleucine synthesis, and only this type is susceptible to inhibition by L-isoleucine. AG- and RB-phase extracts of *M. tuberculosis* exhibited equal specific activities for threonine dehydratase (Table 1). However, 1 mM isoleucine completely inhibited this activity in both preparations; therefore, the enzyme is not of the anaerobically induced biodegradative type. This enzyme also deaminated L-serine but at a 10-fold lower rate than L-threonine.

NAD glycohydrolase was found in equal amounts in AG- and RB-phase extracts (Table 1), as was the enzyme inhibitor, which was present in approximately 15- to 20-fold excess to the enzyme itself. These results suggest that NAD glycohydrolase and its inhibitor do not play a major role in modulation of the activity of NAD-dependent enzymes during the transition of *M. tuberculosis* between the AG and RB phases.

Among those enzyme systems listed in Table 1, the RB extracts exhibited activities only equal to or lower than the AG extracts. In contrast, isocitrate lyase activity, which initiates the glyoxylate bypass in the tricarboxylic acid cycle, was markedly elevated in RB preparations (Table 2). When AG cultures were preconditioned by allowing them to settle without shaking at 37°C, the isocitrate lyase increased with time, but, as was the case with induction of the URB antigen and tolerance to anaerobiosis, it did not reach the level seen in an RB culture (Table 2).

The enzyme involved in the reentry of the glyoxylate bypass products into the tricarboxylic acid cycle, malate synthase, catalyzes the condensation of glyoxylate with the acetate from acetyl-CoA. Malate synthase was detected in comparably high amounts in both the AG and RB extracts (Table 2). On the other hand, Goldman and Wagner (8) described another pathway for utilization of glyoxylate by *M. tuberculosis*, which depends on reductive amination of glyoxylate by a glycine dehydrogenase with the concomitant oxidation of NADH to NAD. In the present study we found a striking increase in reductive amination of glyoxylate in RB compared with that seen in AG extracts. However, in this case the preconditioning settling of the AG cultures was associated with only slight and erratic changes in the enzyme concentration. If induction of an increase in the glycine dehydrogenase depends on the product resulting from the earlier induction of isocitrate lyase, then the

TABLE 2. Specific activities of isocitrate lyase, malate synthase, and glycine dehydrogenase from shake-aerated cultures subsequently preconditioned by nonaerated settling before harvest and from the sediment of a culture not aerated during growth

Growth condition	Preconditioning (h of settling)	Sp act (μmol/h per mg of protein)		
		Isocitrate lyase	Malate synthase	Glycine dehydrogenase
Shaken	0	0.11	7.5	0.11
Shaken	4	0.12	5.6	0.14
Shaken	8	0.17	5.0	0.13
Shaken	24	0.25	6.5	0.17
Stationary		0.42	5.7	1.23

rapid self-induced anaerobiosis of the unagitated, high-density AG culture may have limited the late expression of this induction by curtailing biosynthetic activity. Lineweaver-Burke reciprocal plots for each of the substrates in the reductive amination of glyoxylate by a crude DEAE extract of RB cells yielded apparent K_m values of 5.4×10^{-3} M glyoxylate, 1.2×10^{-1} M ammonium ion, and 8.7×10^{-6} M NADH. The apparent K_m for glyoxylate for a similar AG extract was 4.3×10^{-3} M. The products of the reductive amination of glyoxylate (i.e., glycine and NAD) were titrated in the assay system against our RB extract. A 400:1 molar ratio of glycine to glyoxylate was required to effect a 50% decline in the initial rate of NADH reduction, whereas a ratio of NAD to NADH of only 60:1 was needed to achieve a 50% decline.

Although supernatant medium from RB cultures was not analyzed for metabolic products, it should be noted that the pH of the medium from a 30-day-old RB culture was 6.8, indicating little or no accumulation of highly acidic products. When AG cultures were grown in DTA supplemented with 0.05 M glycine, no change in growth rate was seen compared with the glycine-free control. Higher concentrations (0.15 and 0.2 M) caused some retardation of growth but that was due to osmotic effects, since sucrose, which is not metabolized by tubercle bacilli, gave comparable effects. RB cultures supplemented with 0.05 M glycine grew at a rate about 40% lower than that of the controls for the first 7 days and about 70% lower for the next 7 days, suggesting some competition for NAD as oxygen became limiting. When RB bacilli were suspended in DTA with 0.25 M glycine and incubated anaerobically for 7 days, their half-life was only 15% lower than that of glycine-free controls. Fumarate, 0.04 M in DTA adjusted to pH 7.0, had no effect on the rate of growth of AG or RB cultures as compared with fumarate-free controls.

DISCUSSION

The classical example and most extensively studied model of an organism that is capable of persisting for long periods without replication is the bacterial spore. However, only a very small proportion of the known pathogenic bacteria are spore formers. There remains, therefore, a need to understand the mechanisms by which a nonsporulating pathogen can persist in indolent lesions with little, if any, replication, yet be capable of proliferating and producing overt disease after months or years of quiescence.

There is ample evidence that *M. tuberculosis* can persist in quiescent or even occult form in tissues. Sever and Youmans (24) demonstrated that tubercle bacilli exhibit logarithmic growth in the organs of mice for 2 to 3 weeks after intravenous inoculation. Thereafter the numbers of viable bacilli per organ (lung, liver, or spleen) stabilize at a concentration that is proportional to the original inoculum size. The termination of growth is thus not the result of a limitation in nutrients provided by the host organ but is related to a change in environment associated with the development of cell-mediated immunity and necrosis of tissue. Among the environmental changes that can affect survival and replication of tubercle bacilli in the presence of toxic necrotic products is the depletion of oxygen (4, 5). Even after extensive and apparently successful chemotherapy of experimentally infected mice, some tubercle bacilli may persist in the tissues and resume multiplication long after cessation of therapy (10, 15, 16). In human surgical specimens also, viable tubercle bacilli have been recovered from blocked lesions with no patent bronchial access to air from patients whose sputum had been negative for bacilli for 9 months or more (29). Furthermore, Corper and Cohn have demonstrated the viability of tubercle bacilli in cultures that were sealed with paraffin and incubated at 37°C for 12 years (2).

On the other hand, Wayne and Diaz (27) have shown that abrupt termination of aeration of actively replicating tubercle bacilli in a dense culture causes rapid death of most of the bacilli. The present study has been directed toward finding the mechanism by which all or part of a population can adapt, under a suitably gradual change in conditions, to tolerance of, if not necessarily growth in, an oxygen-depleted environment. The model selected for this study was the unagitated culture, in which the bacilli settle through a self-generated oxygen gradient, adapting to a nonreplicating stage as they accumulate in the oxygen-poor sediment. It was reported previously that extracts of these RB contain an antigen, designated URB, which could not be demonstrated in extracts of AG-phase organisms (30), and that the RB exhibited synchro-

nous replication 8 h after resuspension and aeration but initiated DNA synthesis only after completion of the first cycle of division (26). We have now shown that transfer of AG cultures to an anaerobic jar resulted in an initial death rate more than 10-fold greater than that of RB cultures similarly treated (Fig. 1). After the initial 10-day decline in viability of the AG cells, the survivors, corresponding to about 0.2% of the original population, died at a rate comparable to that of RB cells, suggesting a conversion of that small proportion of the bacilli to the RB state. Furthermore, when the AG cultures were permitted to create their own microaerophilic environment simply by terminating agitation of a mid-log-phase culture, a partial adaptation to anaerobic survival was demonstrated over a period of 24 h (Fig. 2). They did not, however, attain a level of tolerance to anaerobiosis comparable to that seen with RB which had been growing in their self-generated oxygen gradient. The speed with which the oxygen was consumed by the dense AG cultures appeared to inhibit the biosyntheses that were associated with a high level of tolerance. That is, the adaptation appears to be initiated by a partial reduction in available oxygen, but it cannot be completed anaerobically. The synthesis of the URB antigen, the actual function of which is still not known, paralleled the increased anaerobic tolerance in the adapting culture. However, aerobic preconditioning of RB cultures for 24 h before placement in an anaerobic jar led to a 90% increase in susceptibility to the lethal effect of anaerobiosis but only a 50% decline in the URB antigen (probably simply by dilution with freshly synthesized protein) (Fig. 2). Furthermore, URB synthesis terminated 8 h after stationary preconditioning of the AG cultures, but their tolerance to anaerobiosis continued to increase for an additional 16 h. Although this antigen is a good indicator of RB status, it is not necessarily a determinant of anaerobic survival.

The adaptation to a resting state is not associated with a generalized nonspecific depletion of enzymes (Table 1). Catalase and superoxide dismutase, which are most closely associated with disposition of terminal products of oxidative metabolism, exhibited a two- to threefold decrease in activity. Two enzymes involved in biosynthetic activities, acetyl-CoA carboxylase and the biosynthetic type of threonine dehydratase, exhibited comparable activity in both RB and AG extracts.

Murthy and colleagues (17) demonstrated the presence of enzymes of the citric acid cycle and of the glyoxylate bypass in extracts of *M. tuberculosis* grown as pellicles on synthetic medium and harvested after 10, 14, 21, or 28 days of incubation. Three of the five citric acid cycle enzymes studied, isocitric dehydrogenase, α-ketoglutaric dehydrogenase, and malic dehydrogenase, as well as pyruvate dehydrogenase, which feeds the cycle, exhibited maximum specific activity in the 14-day-old cultures, and by day 28 of culture had declined by anywhere from 53 to 86%. Similarly, two other enzymes in the cycle, aconitase and fumarase, showed maximum activity at 10 days and declined by 58 and 48%, respectively, by day 28. In contrast, the glyoxylate pathway enzyme, isocitrate lyase, increased steadily from day 10 to day 28, with an overall fivefold increase in specific activity. Our own studies showed a similar difference in isocitrate lyase activity of extracts of AG (0.11 μmol/h per mg of protein) and of RB (0.42 μmol/h per mg of protein) cells which were grown in a rich and complex medium. This suggests that the shift into the glyoxylate pathway seen by Murthy et al. was related to the limited accessibility of air to bacilli in the old thick pellicles rather than to an increased demand for utilization of fatty acids, as they suggested (17). We found no comparable increase in malate synthase, the second enzyme involved in the glyoxylate bypass. However, Goldman and Wagner's description (8) of a novel glycine dehydrogenase, which catalyzes the reductive amination of glyoxylate by *M. tuberculosis* $H_{37}Ra$, prompted us to investigate the occurrence of that enzyme in our model system. RB cells produced this enzyme in approximately 10-fold greater concentration than did the AG bacilli (Table 2). The reaction of this enzyme oxidizes 1 mol of NADH to NAD for each mole of glycine produced, and it required much less NAD than glycine to affect a decrease in the rate of the reaction. Therefore, of the two products of reductive amination of glyoxylate, the NAD is the one that has the major effect on the rate, suggesting that the function of the enzyme in this system is to replenish NAD rather than to produce glycine.

The malic acid that is produced in the malate synthase step of the glyoxylate bypass in the citric acid cycle is normally oxidized to oxaloacetate by a step that requires NAD. Under conditions of oxygen depletion, greater demands are placed on the NAD pool to meet the needs of compensating dehydrogenase reactions. In these circumstances diversion of some of the glyoxylate into the reductive amination pathway can serve to regenerate NAD from its reduced form, providing energy for further, as yet unidentified, steps in the shift-down of *M. tuberculosis*. This function is thus analogous to the anaerobic threonine dehydratase induction in *E. coli* (20), which also regenerates NAD. The difference is that the threonine dehydratase system in *E. coli* probably serves as an interim energy source during adaptation to anaerobic growth, whereas

the reductive amination of glyoxylate by glycine dehydrogenase appears to provide an energy source for *M. tuberculosis* to shift down to a stable, nonreplicating form that merely tolerates anaerobiosis. The energy demands for a process that permits the orderly anaerobic termination of synthetic activities should be markedly less than they are for one that is required to synthesize a set of alternate enzymes to support sustained anaerobic biosynthesis.

The natural history of tuberculosis dictates the adaptation of *M. tuberculosis* to at least three kinds of environmental conditions. Immediately after implantation in the alveoli, the bacilli are in a highly aerobic environment. Later, as the disease progresses and inflammation and necrosis define the environment, any further replication that occurs must do so under conditions of reduced availability of oxygen. Finally, bacilli may persist in blocked lesions without replicating in the presence of little or no oxygen. Adaptation to these changing conditions of aeration requires mechanisms that permit modulation of the supply of necessary dinucleotide cofactors such as NAD. However, at least two impediments to the maintenance of NAD pools in *M. tuberculosis* have been described. Konno et al. (11) have demonstrated a defect in the niacin scavenging mechanism for replenishment of NAD in *M. tuberculosis* by virtue of a failure to convert niacin to niacin ribonucleotide. Furthermore, tissues of infected mice and guinea pigs produce elevated levels of NAD glycohydrolase (1, 31), thus reducing the availability of exogenous NAD in infected animals. The tubercle bacilli themselves also produce an NAD glycohydrolase, but its activity in bacillary extracts is completely repressed by an excess of a tightly associated heat-labile specific enzyme inhibitor (9). We could detect no significant differences in the relative amounts of this enzyme and of its inhibitor in AG and RB cells, so there is presently no evidence for a role for this complex in modulating the NAD pool in this model system.

The low levels of isocitrate lyase and glycine dehydrogenase and the high constant concentration of malate synthase in actively growing cultures may play a role in modulating the metabolic response of the tubercle bacilli to minor transient fluctuations in oxygen. However, a prolonged period of oxygen depletion leads to increased synthesis of isocitrate lyase. The marked increase in synthesis of glycine dehydrogenase is a later occurrence and may be the mechanism for providing NAD, and thus ATP, to support the completion of a final cycle of DNA synthesis before shutdown of replication. The actual mechanism for shutting down that synthesis remains to be elucidated. However, the apparent dependence of resumed DNA synthesis on the completion of one synchronized division of the bacilli 8 to 12 h after the restoration of oxygen (26) suggests that the final stages of replication shutdown may be controlled by interference with the synthesis of or the masking of a DNA initiation site on the cell membrane. Interference with cell wall synthesis has been shown to prevent DNA initiation in *Bacillus subtilis* (23). The events of these late stages in *M. tuberculosis* are now under study.

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