Autolysis and Secondary Growth of *Mycobacterium tuberculosis* in Submerged Culture

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*Mycobacterium tuberculosis* has been found to exhibit autolysis and spontaneous secondary growth in modified Sauton medium. The phenomenon is a function of high glycerol concentration of the medium and limitation of air in test tubes. It is not a function of depletion of nutrients and is probably not a manifestation of lysogeny or spheroplast formation. The cycle of growth, autolysis, and secondary growth is related to development of lethal conditions during a period of severe oxygen limitation followed by a metabolic change associated with slower depletion of glycerol.

Autolysis of *Mycobacterium tuberculosis* has been studied under a variety of conditions. Corper and Cohn (2), while examining optimal conditions for tuberculin production, studied the effects of temperature and toxic agents on lysis. White and Knight (12) evaluated the roles of ingredients of Tween-albumin medium in the action of mycobacteriophages. Schaefer et al. (9) and Redmond and Bowman (7) have examined the factors involved in autolysis of tubercle bacilli in nitrogen-deficient media.

During the course of experiments on the growth of *M. tuberculosis* in Sauton medium modified to yield diffuse growth, we observed lysis of the cultures after 2 weeks of growth; approximately 1 week later, the cultures resumed growth and continued to grow for at least 3 weeks or more. The spontaneous resumption of growth after lysis was not a feature of the lytic systems described by the investigators cited above. This is a report on the conditions necessary for autolysis and secondary growth of tubercle bacilli to occur in the modified Sauton medium.

**MATERIALS AND METHODS**

The experiments were performed on cultures of the H37Rv strain of *M. tuberculosis* grown in a modified Sauton medium containing Tween 80 and bovine serum albumin fraction V. The inocula for the experiments were 7- to 10-day-old cultures of the test organism in Dubos Tween-albumin broth. A 0.1-ml amount of such cultures was the usual inoculum for 7 ml of test medium. All experiments were performed in quadruplicate.

The modified Sauton Tween-albumin (MSTA) base was prepared according to the following formula: KH₂PO₄, 0.4 g; MgSO₄·7H₂O, 0.4 g; citric acid, 1.7 g; FeNH₄(SO₄)₂·13H₂O, 0.24 g; asparagine, 3.3 g; Tween 80, 0.2 g; glycerol, 50 g; and distilled and demineralized water to a volume of 900 ml. The pH was adjusted to 7.0 with 6 N KOH.

The MSTA base was dispensed in appropriate small amounts and autoclaved at 15 psi for 15 min. One volume of Dubos Medium Albumin (Difco), which contains 7.5% glucose and 5% albumin fraction V, was added to 9 volumes of the cooled base, and the complete MSTA medium was distributed to sterile tubes or flasks. In some experiments, selected ingredients were omitted, or others were added, as indicated in the presentation of results.

For most experiments, screw-capped tubes (16 by 125 mm) containing 7 ml of MSTA medium were employed. The caps were permitted to remain loose. The depth of the medium in the tubes was approximately 50 mm. In studies of effects of greater aeration, the medium was dispensed in 60-ml amounts to 300-ml Erlenmeyer flasks with fused side arms for optical density determinations (Nepholo-flasks, Belco Glass, Inc., Vineland, N.J.). In these flasks, the medium depth was about 12 mm. Morton closures were used to permit air exchange.

In some comparative experiments, Dubos Tween-albumin liquid medium (DTA) was also used. This was prepared according to the manufacturer's directions, from Dubos Broth Base and Dubos Medium Albumin (Difco).

Cultures were incubated upright at 37 C, and were shaken briefly three times a week, for optical density determinations. For some studies, small Teflon-coated magnetic stirring bars were dropped in the tubes, which were placed in a Tri-R magnetic stirrer inside the incubator. Optical densities of cultures were determined at 580 µm in a Lumetron colorimeter or a Coleman Junior spectrophotometer. The Lumetron colorimeter readings were not linear at optical densities above 0.50. Therefore, a curve was established, by use of barium sulfate standards, to correct all readings in excess of 0.500 to a linear scale. Because some experiments lasted as long as 6 weeks,
it became necessary to correct the observed optical densities for evaporation. This was done by noting rates of evaporation in the different culture containers, and at different temperatures, and adjusting actual readings back to the original volume of fluid in the containers. Undoubtedly evaporation had some effect on actual growth rates, but the adjusted readings appeared to permit more meaningful analysis of the curves. In experiments on utilization of substrates, both corrected and uncorrected curves were plotted. The corrected data were necessary to plot actual amounts of substrates consumed; the uncorrected data indicated existing concentrations of substrates at a given time.

Counts of viable units of bacilli in liquid cultures were determined by plating appropriate dilutions to screw-capped jars (55-mm diameter) containing 20 ml of Dubos Oleic agar. This was prepared according to the manufacturer's directions from Dubos Oleic Agar Base and Dubos Oleic Albumin Complex (Difco).

Analysis for glycerol was performed by the method of Greenwalt and Steane (4) to eliminate difficulties due to the citrate in the medium. In the concentrations employed, glucose did not interfere with this analysis. Glucose was estimated as described by Kornberg and Horecker (6), by use of glucose Calsuls from Calbiochem (Los Angeles, Calif.). Changes in pH of cultures were determined on a Beckman model H2 pH meter.

Total dehydrogenase activities of cultures were estimated by their ability to reduce triphenyl tetrazolium chloride (TTC). The analysis was performed by adding 2.8 ml of culture to 0.2 ml of a 2 mg/ml sterile solution of TTC (Nutritional Biochemicals Corp., Cleveland, Ohio) in 0.067 M phosphate buffer of pH 7.4. The preparations were incubated at 37 C for 24 hr, after which the reaction was stopped by the addition of 0.1 ml of Formalin. The red formazan of reduced TTC was then extracted into 5.0 ml of ethyl acetate on a Vortex mixer. The suspensions were shielded from light while they were allowed to settle, and 1-ml portions of the supernatant ethyl acetate were mixed with 3 ml of ethyl acetate in 13-mm screw-capped tubes. The optical densities were determined in a Coleman Junior spectrophotometer at 490 mu, against an ethyl acetate blank.

RESULTS

When M. tuberculosis was grown in MSTA medium, the initial stages of the growth curves were comparable to those seen in DTA. After 12 to 14 days, however, when the optical densities reached values around 0.200, the MSTA cultures began to exhibit marked declines in optical density. These declines continued for 5 to 7 days, after which growth resumed and continued for at least 3 more weeks, when most experiments were terminated. This phenomenon had not been seen in DTA, and a comparison of the two media was made to clarify the conditions necessary for the peculiar growth curves to occur.

Cations. The two media contained comparable amounts of ferric and magnesium ions. DTA contains 0.5 μg of CaCl₂ and 0.1 μg each of ZnSO₄ and CuSO₄ per ml; MSTA does not contain these cations. To assess the possible role of a cation deficiency in the peculiar growth cycle seen in MSTA, batches of this medium were prepared separately with each of the following supplementary metal ions, supplied as the sulfate or chloride: Ca++, Cd++, Co++, Cr++, Cu++, Mn++, Ni++, and Zn++. Each cation was tested as two concentrations, 4 × 10⁻⁴ and 8 × 10⁻⁴ M. The Ca++ was also tested at 5 × 10⁻⁵ M. None of these cations modified the characteristic growth-lysis-secondary growth pattern.

Nitrogen source. The MSTA medium contains 0.025 M asparagine. In Redmond and Bowman's study (7), 0.018 M NH₄Cl was sufficient to prevent nitrogen deficiency autolysis. When we substituted 0.025 M NH₄Cl for the asparagine in the MSTA medium, the growth was about 1 week slower, but typical autolysis and secondary growth occurred. Substitution of glutamate for asparagine did not change the curve. There was no evidence that the pattern was related to nitrogen deficiency or a specific nitrogen source.

Carbon source. Both DTA and MSTA media contain 0.75% glucose, 0.5% bovine serum albumin fraction V, and 0.02% Tween 80. The DTA contains a mixture of amino acids, as opposed to a single amino acid in MSTA. The MSTA contains approximately 0.009 M citrate, and the DTA has only a trace of this anion, as the ferric ammonium salt. When citrate was omitted from MSTA, it was necessary to reduce the concentration of ferric ammonium sulfate to 0.024 mg/ml, to minimize the turbidity due to iron precipitates. Omission of citrate from the medium caused no change in the growth pattern, i.e., citrate is not a significant factor in the phenomenon.

Only the MSTA medium contains glycerol. When glycerol was omitted from MSTA, the optical density curves of the cultures leveled off after 14 days, but no significant degree of lysis occurred, and little secondary resumption of growth was seen (Fig. 1). The presence of glycerol in the medium appeared to be responsible for the peculiar pattern of growth. This was confirmed by cultivating the bacilli in tubes of DTA medium enriched with 5% glycerol. In this medium also, a pattern of growth, lysis, and secondary growth was seen.

Analysis for glycerol at weekly intervals in the complete MSTAcultures indicated that the concentration of this substrate is decreased in a linear manner during the first 21 days of incubation; the actual concentration dropped from 50 to 31 mg/ml in that time (not corrected for
evaporation). Optically evident lysis of the bacilli terminated by the 21st day, when growth recommenced. The glycerol concentration did not change between the 21st and 28th days of the experiment, but, between the 28th day and the 42nd day, further depletion occurred, to a final level of 26 mg/ml. When corrected for evaporation, this represented 20 mg/ml of original culture, i.e., after 6 weeks the bacilli had used 60% of the glycerol supplied in the medium. Some autolysis and secondary growth was found to occur when the bacilli were grown in MSTA medium initially prepared to contain as little as 10 mg of glycerol per ml. This demonstrates that cessation of autolysis and resumption of growth is not contingent on depletion of glycerol, because this was found to occur when actual concentration of glycerol was 31 mg/ml. The depletion of glycerol during the first phase of growth and of lysis occurred at a rate over twice as fast as was seen during secondary growth.

The specificity of the role of glycerol was tested by substituting a 5% glucose supplement for the 5% glycerol in MSTA. (The glucose was sterilized before addition to previously autoclaved base.) Cultures grown in the glucose-rich medium showed less autolysis than those grown with glycerol, but did exhibit secondary growth (Fig. 1). The culture medium, after 35 days of incubation, had been depleted by only 2.4 mg of glucose per ml (corrected to original volume). Thus, only about 4% of the available glucose was utilized under these growth conditions, compared to 54% of the glycerol utilized in a comparable time, in glycerol-rich medium (Table 1).

Aeration. All of the experiments described up to this point were performed in culture tubes (16 by 125 mm) with loose caps. When experiments were performed with complete MSTA medium in loosely capped 300-ml Nephlo-flasks, autolysis did not occur. When similar experiments were performed with MSTA from which glycerol had been omitted, autolysis did occur, but there was no secondary growth. When 50 mg of glucose per ml was substituted for the glycerol, autolysis did not occur (Fig. 2). After 5 weeks of incubation, the cultures which originally contained 7.5 mg of glucose per ml had utilized 1.1 mg of glucose per ml; those which originally contained 57.5 mg/ml had utilized 3.0 mg/ml (Table 1). Thus, under conditions of moderate aeration in 5.75% glucose medium in flasks, glucose was not utilized much more extensively than it was under the limitations of aeration that existed in the tubes. The autolysis which occurred in the flasks with no glycerol and only 7.5 mg/ml of glucose was not followed by secondary growth, and so was different from the growth pattern which occurred when glycerol was used under conditions of limited aeration in tubes.

The role of severe limitation of oxygen in causing the autolysis was confirmed in experiments employing tubed cultures containing small magnetic stirring bars. Tubed cultures in MSTA were incubated in a stationary manner, with manual agitation three times a week. After 10 days, before autolysis had started, the tubes were

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**TABLE 1. Utilization of glycerol and glucose by Mycobacterium tuberculosis after 35 days of cultivation in tubes and flasks**

<table>
<thead>
<tr>
<th>Cultures in</th>
<th>Initial concn (mg/ml)</th>
<th>Amount utilized (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycero</td>
<td>Glucose</td>
</tr>
<tr>
<td>Tubes</td>
<td>50</td>
<td>7.5</td>
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<tr>
<td>Tubes</td>
<td>0</td>
<td>7.5</td>
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<tr>
<td>Tubes</td>
<td>0</td>
<td>57.5</td>
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<tr>
<td>Flasks</td>
<td>50</td>
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<tr>
<td>Flasks</td>
<td>0</td>
<td>7.5</td>
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<tr>
<td>Flasks</td>
<td>0</td>
<td>57.5</td>
</tr>
</tbody>
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* Not determined.
Growth is recorded as logarithm of optical density.

When magnetic stirring resulted in stationary conditions, lysis stopped and growth rates under these conditions, as opposed to the linear rates usually seen in stationary submerged cultures of M. tuberculosis (3), attained optical densities in excess of 0.900 within 8 days of continuous agitation. When tubes were removed from the magnetic stirrer after only 5 days of incubation, i.e., before a critical cell concentration was reached, and were permitted to continue incubation under stationary conditions, the growth dropped to a linear rate and lysis commenced after 5 more days. Lysis was followed by secondary growth (Fig. 3). When continuous stirring was maintained for periods in excess of 5 days, a critical cell concentration was passed, and termination of the magnetic stirring resulted in immediate initiation of lysis. This was not followed by resumption of secondary growth.

Timing. The effect of glycerol as a function of time of exposure to this substrate was determined by growing the bacilli in tubed MSTA prepared without glycerol. At selected intervals, 0.5 ml of 75% glycerol was added to quadruplicate sets of tubes containing 7 ml of culture. If the glycerol was added at the time of inoculation or after 7 to 10 days of incubation, autolysis was induced, and secondary growth followed. If the glycerol was added after 14 or more days of incubation, a slight, immediate, and temporary depression in optical density was seen, in excess of that caused by simple dilution effect; secondary growth was not seen (Fig. 4).

The results of the preceding experiment indicated that glycerol per se did not cause lysis, but rather that some metabolic product or process initiated the phenomenon. Experiments were conducted to determine the roles of preconditioning of the bacilli, and changes in the medium itself. Tubercle bacilli were grown in the complete MSTA medium, and, at selected intervals, sets of five tubes were removed and centrifuged, and the supernatant medium was pooled, sterilized by membrane filtration (Millipore Filter Corp., Bedford, Mass.), and redistributed in 7-ml

Fig. 2. Growth curves of Mycobacterium tuberculosis in flasks of MSTA medium containing different carbon sources. A: 5% glycerol and 0.75% glucose. B: 0.75% glucose. C: 5.75% glucose.

Fig. 3. Effect of termination of agitation of Mycobacterium tuberculosis growing in tubed MSTA medium on a magnetic stirrer. A: Magnetic stirring terminated after 5 days. B: Magnetic stirring terminated after 6 days. C: Magnetic stirring terminated after 8 days. Growth is recorded as logarithm of optical density.
old cultures supported the same type of curve as an uncentrifuged control, i.e., a pattern of growth, lysis, and secondary growth with no change in timing of these phenomena. The 14-day supernatant fluid gave essentially a straight-line growth curve throughout the 35 days of incubation, only slightly greater in rate than the usual secondary phase curve (Fig. 5). The 21- and 28-day preparations gave slightly greater early growth rates (but markedly slower than the typical first, or prelytic, phase curve), which slowly leveled off to parallel a secondary phase curve. A pool of the staled MSTA did not always give these consistent results. Sometimes experiments with staled media did yield some autolysis. The reasons for the variability in the phenomenon are not yet known.

Viability. Plate counts were performed on MSTA cultures at intervals, to confirm the fact that optical-density changes during apparent autolysis were indeed reflections of loss of viability of the bacilli. Dilutions were plated from cultures immediately after inoculation and after 7, 10, 12, 14, 21, 28, and 35 days of incubation. A logarithmic decay in numbers of viable units was seen from the 10th through the 21st day, after which the counts rose (Fig. 6). Examination of Ziehl-Neelsen stained smears of these prepara-

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Fig. 6. Viable-unit counts and TTC reducing ability of MSTA grown cultures of Mycobacterium tuberculosis. Solid line: the viability counts are expressed as viable units per ml. Broken line: the TTC reducing ability is expressed as the optical densities of the dilute ethyl acetate extracts of the formazan produced by the cultures.

The experiments showed the average degree of aggregation to range from 1.6 to 2.3 bacilli per clump during the period of initial growth and apparent lysis. This proved that the 90% decline in apparent viability count between the 10th and 21st days was not merely a consequence of excessive aggregation of the bacilli.

Koch-Weser and Ebert (5) stated that the amount of TTC reduced by tubercle bacilli in DTA depends “only on the number of organisms in the medium, and is not related to the phase of growth nor the age of the medium.” A similar relationship was seen when MSTA cultures were incubated with TTC; however, the diminution in TTC reducing activity did not occur as soon, nor was it as great, as the loss in viability associated with lysis. The optical density due to extracted TTC formazan produced in 24 hr of incubation increased to the 14th day, declined over the following week, and then increased during the ensuing 14 days of the experiment (Fig. 6). The apparent increase in TTC reduction during secondary growth exceeded the actual increase in viable units.

Physicochemical effects. Changes in pH of the MSTA cultures did not appear to be causative factors in the pattern. Experiments were conducted at initial pH values of 6.4 and 7.2, and both sets yielded typical curves. Cultures in the complete MSTA and in MSTA from which glycerol had been omitted were tested for pH changes during incubation. The initial pH of the two batches of medium in this experiment was 6.4. The pH of the medium which contained glycerol rose to 7.1 after 3 weeks, at which time the glycerol-free preparation, in which no lysis had occurred, had a pH of 7.4. Between the 4th and 6th week, the pH of the complete medium dropped back to 6.6, and that of the glycerol-free cultures to 6.8.

Another experiment was performed to test the possibility that depletion of the surface-active agent Tween 80 could be a factor in resumption of growth after the lytic period. Sets of 7-ml MSTA cultures were grown in the usual manner, and 0.1 ml of sterile 1.4% Tween 80 was added to different sets of four tubes at weekly intervals. Addition of Tween 80 did not have any effect on the growth patterns.

Sucrose, which is not metabolized by M. tuberculosis, has been used to stabilize mycobacterial spheroplasts (11). Batches of MSTA medium were enriched with 5, 10, 15, and 20% sucrose to see whether this substance would interfere with autolysis. At levels up to 10% sucrose caused some lag in the growth curves, but did not interfere with lysis or secondary growth. The 15% level of sucrose permitted late lysis, and a very limited resumption of growth. First-phase growth was retarded over 50% by 20% sucrose, and lysis was slightly diminished.

Other factors. M. tuberculosis was cultured through four serial passages in the complete MSTA medium. The inoculum for each passage was 0.1 ml of the 28-day-old (secondary growth phase) preceding culture. The cultures continued to exhibit a characteristic pattern of growth, lysis, and secondary growth through all four passages.

A possible role of lysogeny in the cycle was examined by spotting a drop of MSTA cultures weekly to overlay plates inoculated with M. tuberculosis H37Rv and with Mycobacterium sp. 607, as described by Russell et al. (8). The Mycobacterium sp. 607 culture has a broad range of mycobacteriophage susceptibility. Plaques were not produced on either host. A strain of M. tuberculosis H37Rv lysogenized with D32 phage by Russell et al. (8) was similarly tested, and plaques were produced on Mycobacterium sp. 607, but not on the M. tuberculosis plates, at each sampling from the 7th through the 28th day of incubation. Thus, the MSTA medium itself did not mask the phage released from a known lysogenic system.

An experiment was performed to determine the effect of the chelating agent ethylenediaminetetraacetic acid (EDTA) on the pattern of growth in MSTA medium. Tubes of MSTA were inoculated, and, at selected intervals, 0.1 ml of a
sterile 35 mg/ml solution of the sodium salt of EDTA was added to sets of four tubes.

Addition of the EDTA to the medium before inoculation permitted only a trace of growth to occur, and lysis occurred after 9 days (Fig. 7). Addition of the EDTA at 4, 7, 11, and 14 days after inoculation resulted in initiation of lysis when the cultures were 14 days old, i.e., at the same time lysis began without EDTA. There was some reduction in peak prelytic growth when EDTA was added to the 4-day-old culture, but cultures receiving EDTA between the 7th and 14th days all achieved the same prelytic peak optical density as the untreated controls.

The most notable effect of addition of the chelating agent before lysis had begun was the failure of the cultures to commence secondary growth a week after lysis started; instead, lysis continued for another week. Addition of EDTA to 21-day-old cultures permitted initiation of secondary growth, which stopped abruptly 1 week later, when the lytic pattern was resumed. Addition of the EDTA to the cultures which were well into their secondary growth resulted in a gradual tapering off of growth and, finally, a slight lysis.

**DISCUSSION**

Several general interpretations of the phenomenon of spontaneous autolysis and secondary growth may be examined. The induction of spheroplasts which immediately lyse is unlikely, inasmuch as sucrose does not prevent autolysis in the presence of magnesium ion. Activation of phage in a lysogenic system is also unlikely, because one would expect the secondary growth to consist of bacilli which are resistant to the phenomenon; this was not found to be the case. Attempts to detect phage on two possible indicator hosts were unsuccessful, although a known lysogenic strain of *M. tuberculosis* released detectable phage in this system. In view of the lack of resistance to lysis of the serial passage cultures, it was not considered worthwhile to extend attempts to detect phage on a large range of possible indicator host organisms.

The autolysis of tubercle bacilli in the glycerol-rich tubed medium is not a simple physical function of the presence of glycerol itself. When the glycerol is not added until the 14th day, the bacilli appear to have passed the stage in which the phenomenon can occur, and neither autolysis nor secondary growth occurs. On the other hand, when bacilli that have just started to lyse in the complete MSTA medium are centrifuged, and the cells are resuspended in fresh complete MSTA, the lysis proceeds through its course and is followed by secondary growth. This indicates that, after a period of initial growth in the complete MSTA, they become "committed" to a metabolic course leading to lysis in that medium.

Bowles and Segal (1) have demonstrated that glycerol utilization by *M. tuberculosis* is an induced function which is stimulated by oxygen. Furthermore, glycerol is markedly stimulatory to oxygen consumption by these organisms (10). The phenomenon of lysis and secondary growth in MSTA medium is a function of glycerol and limited aeration. In the tubed stationary cultures, the concentration of cells, which are actively consuming glycerol, reaches a critical level, at which point oxygen is not supplied as rapidly as it is consumed, resulting in a severely unbalanced metabolism which leads to autolysis. During a period of approximately 1 week, in which the autolysis continues, glycerol continues to be consumed. The termination of autolysis and resumption of growth coincide with a cessation of glycerol utilization. At 1 week after growth has resumed, a slow consumption of glycerol commences, at somewhat less than half of its prior rate. Thus, under these conditions of diminished glycerol metabolism, the rate of oxygen depletion...
is balanced by oxygen solution produced by the thrice weekly shaking of the tubes. The likelihood that a feedback mechanism is involved is supported by the observation that stalled filtered medium supports growth of fresh inocula at a rate comparable to those seen with cultures at the stages of growth from which the stalled media were derived. It is noteworthy that the count of viable units in these tubes during secondary growth does not reach the prelytic level, indicating that, for the configuration of these tubes, a critical maximal concentration of metabolizing cells exists. However, the lower rate of multiplication and glycerol utilization during secondary growth appears to prevent these bacilli from achieving the unbalanced conditions which led to the prior lysis. Vigorous aeration at any time during the period of cultivation either prevents or terminates autolysis, and leads to very high growth rates. Abrupt termination of this vigorous aeration results in the unbalanced metabolism and a very rapid initiation of lysis.

LITERATURE CITED