Kinetics of Utilization of Organic Compounds in the Growth of *Mycobacterium tuberculosis*¹

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ABSTRACT

BOWLES, JEAN A. (University of Colorado School of Medicine, Denver), AND WILLIAM SEGAL. Kinetics of utilization of organic compounds in the growth of *Mycobacterium tuberculosis*. J. BACTERIOL. 90:157-163. 1965.—To obtain a workable system for a study of the kinetics of nutrient utilization (based on specific quantitative assay) by *Mycobacterium tuberculosis*, several cultural refinements were introduced: the use of shake culture, a 40-fold increase in the size of inoculum, substitution of glutamate for asparagine as nitrogen source, and elimination of glucose from the medium with glycerol remaining as carbon source. These modifications resulted in reduction to a tenth of the lag phase of glycerol utilization (from 40 to 4 days), and in a greatly increased rate of growth. Both coordinate and sequential patterns of nutrient utilization were in evidence, except in the case of citrate, which was never utilized under a variety of conditions of culture. The coordinate pattern of glucose-glutamate and glucose-glycerol utilization would appear to rule out catabolite repression by glucose. However, elimination of glucose from the medium resulted in elimination of the 4-day lag period before glutamate utilization was initiated, leaving open to question the role of glucose in this system. Evidence is presented for the hypothesis that the sequential pattern of glutamate-glycerol utilization is a function of glutamate repression of glycerol oxidation in the growth of *M. tuberculosis*, although no diauxic effect is apparent. In a determination of which nutrient-utilization systems were regulated by induction, only in the case of glycerol was evidence obtained for an inducible system. The enzymatic mechanisms underlying these patterns of nutrient utilization are presently being investigated.

In earlier studies, Segal and Bloch (1956) suggested that the apparent nonidentity of in vivo and in vitro-grown *Mycobacterium tuberculosis* strain H37Rv was due to a process or processes of environmentally induced adaptation, rather than selection of mutants. More recently, evidence has been obtained (Segal, 1964) demonstrating that reversible phenotypic adaptation is the mechanism involved in the interconversion of the in vivo and in vitro forms of this pathogen. With a view towards investigating the biochemical basis of this adaptive effect, the state of our knowledge of adaptive mechanisms of the in vitro-grown tubercle bacillus was assessed.

A survey of the literature revealed that little has been done in the way of determining patterns of utilization of organic compounds in the process of growth of this organism by quantitative biochemical methods of assay. There does exist

an extensive literature (Long, 1958) on the "utilization" of carbon and nitrogen compounds by the tubercle bacillus, but all of these studies suffer from the serious limitation that the only index of utilization was indirect, namely, growth response to the addition of various nutrients to a complex medium. In a few papers (Dingle and Weinzirl, 1932; Friedmann and Seibert, 1939; Eidus et al., 1961), a direct determination was made of a nutrient in a growth medium, but only initially and at the termination of prolonged periods of growth. Or, the methods of analysis were insensitive and nonspecific, and no relationships were drawn between the utilization of the various nutrients in the medium.

In the present study, time-course analyses were performed by means of specific quantitative determination of the organic nutrients in a defined culture medium throughout the growth period. The results obtained show definite sequential and coordinate patterns of utilization of organic compounds by *M. tuberculosis* in growth, and indicate which substrate systems

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are inducible. In addition, evidence is presented for glutamate repression of glycerol utilization.

MATERIALS AND METHODS

Maintenance of culture and preparation of inocula. A culture of *M. tuberculosis* strain H37Rv, obtained from William Steenken, Jr., Trudeau Foundation, Saranac Lake, N.Y., was grown at 37 C for 6 days in Middlebrook Tween 80 medium (Difco 7H9 broth). Dilutions of this culture were plated on Difco 7H10 agar, and a single colony was picked and grown in 7H9 broth until the culture reached the logarithmic phase of growth [0.4 optical density (OD) in a Bausch and Lomb Spectronic-20 colorimeter at 490 m μ]. A number of serial subcultures were made and allowed to grow for 6 days. The cells were then centrifuged, washed twice in distilled water, suspended to 0.4 OD, and distributed in 1-ml quantities into sterile screw-cap vials. The vials were sealed with pressure tape and frozen at -20 C as suggested by Tarshis (1961).

For the preparation of inocula, vials were thawed at room temperature and subcultured with constant shaking in the defined medium used in these studies (see below) for a 12-day period. With the exception of the comparative studies of stationary and shake cultures, all cultures were incubated under constant shaking (about 200 rev/min) in a gyrotatory (1 in circle) shaker-incubator. The cells were suspended in fresh medium (see below), ground in a Ten Broeck grinder, and diluted to 0.4 OD; 1-ml quantities were used for inocula. Ten Broeck grinding was necessary for suspension of heavily clumped tubercle bacilli grown in the absence of Tween 80. A 0.4-OD suspension of these cells contained 2×10^8 cells per milliliter, as determined by viable counts. The virulence of the strain and of the originally selected colony was verified by determination of survival time of mice injected intravenously with a suspension of 0.4 OD.

In later experiments, in which a much larger inoculum was required, the entire cell content of a culture flask was used as inoculum by resuspending in fresh medium. Because of the great inaccuracy of obtaining a representative sample of such an inoculum to determine cell quantity, the amount of the entire cell inoculum was recorded by determination of wet weight. Wet weights were determined by centrifuging the cells in tared polypropylene centrifuge tubes, washing the cells twice in distilled water, and weighing. Since wet weight is probably the least accurate method of determining cell quantity, the efficacy of this procedure was checked by comparing a series of wet and dry weights of tubercle bacilli grown in the medium used in these studies. Dry weights of washed cells were determined by drying in tared porcelain crucibles over alumina under vacuum. Drying was continued until constant weight was obtained. Figure 1 shows that a straight line is obtained when the log of dry weight is plotted against the

FIG. 1. Comparison of a series of dry and wet weight determinations as a means of establishing an index of cell quantity of *Mycobacterium tuberculosis* grown in Suter's medium.

log of wet weight, thus justifying the use of wet weight as an index of inoculum quantity.

Media. Determination of the kinetics of utilization of the individual organic constituents in a culture medium requires that the medium be fully defined, that is, composed of chemically pure compounds. For this purpose, the defined medium [Suter's (S) medium] described by Suter et al. (1948) was used in this study unmodified, or with the following modifications. (i) Glutamate (0.5%) was substituted for 0.5% asparagine (SG medium); and (ii) glucose (1.0%) was added to the glutamate medium (SGG). The final composition of the complete medium (SGG) was as follows: $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 3.45 g; KH_2PO_4 , 4.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g; sodium citrate dihydrate, 2.5 g; ferrous ammonium sulfate hexahydrate, 0.01 g; L-glutamate, monosodium salt, 5.0 g; glycerol, 25.0 g; glucose, 10.0 g; and triple-distilled water to 1 liter. The pH of all media used in these studies was 6.8. All media were Millipore-filtered rather than autoclaved to prevent alteration of the initial nutrient concentration by water loss through evaporation.

Quantitative determination of nutrients. Citrate was determined by the method of Saffron and Denstedt (1948). Glycerol was determined by the periodate method of Dyer (1956) or by the enzyme method of Boltralik and Noll (1960), as specified in the text. It was necessary to use the enzymatic method for determining glycerol when glucose was present in the medium, since both constituents are determined by the periodate method. In later

studies, glycerol concentration was routinely determined enzymatically, even in the absence of glucose.

Glutamate was determined by use of glutamic decarboxylase (Sigma Chemical Co., St. Louis, Mo.) and by measuring released CO_2 by the standard Warburg manometric method. Each vessel contained 1.3 ml of triple-distilled water, 0.2 ml of 3 M acetate buffer (pH 5), and 0.5 ml of sample. The enzyme was dissolved in 0.1 M acetate buffer (pH 5), and 0.5 ml was placed in the side arm. The mixture was equilibrated in the Warburg water bath at 37 C, and the reaction was started by tipping enzyme into the main compartment of the vessel.

Glucose was determined by use of glucose oxidase (Sigma Chemical Co.) and by measuring consumed O_2 by the standard Warburg manometric method. Each vessel contained 0.8 ml of triple-distilled water, 0.2 ml of 3 M acetate buffer (pH 5), and 1 ml of sample. The mixture was equilibrated in the Warburg water bath at 37 C, and the reaction was started by tipping enzyme into the vessel. Since the enzyme is contaminated by catalase, calculations of O_2 consumption were corrected for the release of O_2 from H_2O_2 formed, according to the equations:

A standard curve of known concentrations of glucose served to establish the validity of the method of assay.

RESULTS

Effect of shake culture on the rate of glycerol utilization. Although S medium had been derived for stationary culture, it was decided to test its adaptability to conditions of aeration possibly to reduce the month-long growth course characteristic of the surface growth of tubercle bacilli on liquid medium, and thereby the concomitant slow rate of nutrient utilization. With use of a gyrotatory shaker-incubator, 125-ml Erlenmeyer flasks containing 50 ml of S medium were inoculated with the standardized suspension of H37Rv and were incubated under constant shaking. As a comparative standard of stationary culture, Roux bottles containing 100 ml of S medium were surface-inoculated with H37Rv and were incubated in the same incubator, but in a stationary position. Glycerol was determined (by the periodate reaction) at various time intervals during the course of growth.

Figure 2 illustrates the greatly increased overall rate of glycerol utilization in shake culture. The low dip in the curve for the stationary culture, representing an increase in the level of initial glycerol content, was due to evaporation of the medium over the long time period of

FIG. 2. Comparative rates of utilization of glycerol by *Mycobacterium tuberculosis* grown in shaken and stationary culture in S medium.

FIG. 3. Effect of substituting glutamate for asparagine on the time of glycerol utilization by *Mycobacterium tuberculosis* in shake culture.

incubation. The significant difference in the two curves is in the greatly extended lag period required before glycerol is utilized in stationary culture. Once glycerol utilization begins, the rates in the two types of culture are comparable.

Effect of substituting glutamate for asparagine on the time of glycerol utilization. In experiments designed to decrease further the lag time in nutrient utilization, glutamate was substituted for asparagine, which is the nitrogen source in S medium. Glycerol was determined by the periodate method throughout the growth period, and the cultures were shaken. Figure 3 shows that, in the glutamate-substituted medium (SG), the lag period in glycerol utilization was effectively reduced by 4 days.

Effect of increased quantity of inoculum on the time of glycerol utilization. Increasing the quantity

of cells in the inoculum could reasonably be expected to result in a decrease in the time period required for total utilization of glycerol. Figure 4 presents the results of such an experiment under conditions of shake culture in SG medium and in which glycerol was determined by the enzymatic method. The increase in inoculum quantity from 0.05 to 1.65 g did significantly decrease (by 2 days) the lag period of time before glycerol utilization was initiated. Further increase in inoculum had little effect in further decreasing the lag, although it resulted in an increased rate of utilization of glycerol.

The effect of increased inoculum in reducing the lag period of glycerol utilization is additive to that produced by shake culture, and to that produced by glutamate substitution for asparagine in the medium. Thus, by combining these three conditions (Fig. 4), the lag period in glycerol utilization of 40 days (Fig. 2) was drastically reduced to 2 days.

Pattern of utilization of organic constituents in the culture medium. By use of the optimal conditions of culture detailed above, the kinetics of utilization of four organic constituents in SGG medium was determined. The curves of rates of utilization of glutamate, glucose, glycerol, and citrate, as well as the growth curve, are presented in Fig. 5. The apparent early growth in the absence of nutrient utilization can be accounted for by the utilization of small quantities of C and N in the organic nutrients, which went undetected by the methods of analysis (assuming a 5% error). After a lag period of 4 days, both glucose and glutamate utilization

FIG. 4. Effect of increasing quantity of inoculum (expressed in grams of wet weight) on the time of glycerol utilization by *Mycobacterium tuberculosis* under shake conditions of culture in a glutamate-containing medium (SG).

FIG. 5. Kinetics of growth and of utilization of glutamate, glucose, glycerol, and citrate by *Mycobacterium tuberculosis* in SGG medium under shake conditions of culture. Growth curve based on inoculum of 0.35 mg (dry weight).

occurred approximately concurrently. It may be of significance that glucose utilization began some 12 hr before glutamate utilization was initiated. Throughout the time-course of growth, the utilization of these two nutrients continued to be concurrent, although their rates differed. In addition, the curve of glucose utilization overlaps that of glycerol utilization. On the other hand, utilization of glycerol was initiated only after a 9-day lag period and appeared to coincide with the disappearance of glutamate from the medium. Lastly, citrate was not utilized at all throughout the extended growth period.

Effect of glucose elimination on the sequence of nutrient utilization. In view of the well established phenomenon of glucose repression of enzyme induction, glucose was eliminated from the culture medium, and the kinetics of utilization of the remaining constituents were determined (Fig. 6). A comparison of the growth curves in Fig. 5 and 6 indicates that there is no appreciable difference in the lag phase or rate of growth, regardless of the presence or absence of glucose. The difference in the two growth curves can be accounted for by the difference in size of inoculum. In the absence of glucose, citrate was again unused. On the other hand, there appears to be a definite repressive effect of glucose on the utilization of glutamate, since in the absence of glucose the 4-day lag period preceding glutamate utilization (Fig. 5) was completely abolished (Fig. 6). Although glucose elimination resulted in diminishing by 5 days the lag of glycerol utilization, this effect is apparently an indirect consequence of the effect of glucose on glutamate utilization. In the absence of glucose, there still remains a lag period of 4 days preceding glycerol utiliza-

FIG. 6. Effect of elimination of glucose from the culture medium on the kinetics of growth and of utilization of glutamate, glycerol, and citrate by *Mycobacterium tuberculosis* in SG medium under shake conditions of culture. Growth curve based on inoculum of 0.27 mg (dry weight).

tion, which corresponds to the first 4 days of incubation during which glutamate is completely utilized. Thus, the sequential pattern of glutamate-glycerol utilization is apparently independent of any repressive effect of glucose. Figure 6 illustrates further that the lag in glycerol utilization is independent of the lag phase of growth.

Inducibility of nutrient utilization. To establish whether or not the utilization of the various organic constituents in the medium was regulated by inducible enzyme systems, utilization rates were determined for each constituent by use of a twin set of cultures differing only in the history of the inoculum. In one case, the inoculum originated from a culture medium containing the constituent (adapted). It was made sure that the cells were in an active state of metabolizing that constituent, that is, by avoiding the de-adapted state known to occur in inducible systems after the substrate has been fully metabolized. In the second case, the inoculum was obtained from a culture medium deficient in the constituent being tested (nonadapted). Glucose was excluded from the medium in all cases, except when its own inducibility was being tested.

For all three organic constituents (glucose, glutamate, and citrate), prior adaptation did not alter the pattern of utilization either in regard to rate, lag, or capability, as in the case of citrate. When tested in the presence of glucose, the 4-day lag period in glutamate utilization was unaltered, regardless of preadaptation. Only in the case of glycerol was evidence obtained for an inducible system. This had not been apparent in preliminary experiments since the cells of the

inoculum had been de-adapted. Figure 7, part 1, shows the results of an experiment in which tubercle bacilli, in an active state of glycerol utilization by having been pregrown for only 6 days in a glycerol medium (adapted), did not exhibit any initial lag period in glycerol utilization during the first day of incubation (20% glycerol utilized). In the case of the nonadapted cells used as inoculum, a lag period of 2 days was evident before utilization of glycerol was initiated.

Glutamate repression of glycerol utilization. As already pointed out regarding the curves in Fig. 5 and 6, glycerol utilization is sequential in relation to preliminary utilization of all the glutamate present in the culture medium. This effect is further corroborated by the data presented in Fig. 7. In part 1 of Fig. 7, it is evident that glycerol was not appreciably utilized by the nonadapted tubercle bacilli until glutamate was largely used up in the first 3 days of incubation. In the case of the preadapted cells, the sequence of utilization is more complex. In the first day of incubation, glycerol was utilized by 20% of initial concentration, concurrently with glutamate utilization. This may be interpreted as being due to residual induced enzymes of glycerol degradation present in the large inoculum of preadapted cells. It also implies that the glutamate effect is not by a mechanism of competitive or feed-back inhibition but rather by catabolite repression of an inducible system. This is further corroborated by the cessation of glycerol utilization for 3 days after the 1st day of initial utilization, which period corresponds exactly to the time at which all the glutamate disappeared from the medium.

FIG. 7. Part 1. Effect of preadaptation of *Mycobacterium tuberculosis* (by pregrowth in glycerol-containing medium) on the initial lag period of glycerol utilization. Part 2. Effect of an additional inclusion of glutamate in the medium at the point when the original glutamate had been fully utilized by *M. tuberculosis*.

To establish the specificity of this effect, glutamate was added to a culture of adapted cells after 4 days of incubation, at which point original glutamate had all been utilized, and glycerol should have started to be actively metabolized. As seen from the results in part 2 of Fig. 7, utilization of glycerol was again delayed for a 2- to 3-day period, corresponding approximately to the period of time required for all of the added glutamate to be utilized. Beyond this point in time, the remaining glycerol in the medium was actively and completely utilized.

DISCUSSION

The results of the present study in which rates of nutrient utilization are established supply several explanations for the previously reported findings of other investigators where only growth rates were followed. Our results also clarify certain misunderstandings in the standard practices of growing *M. tuberculosis*.

Several investigators (Long, 1958; Lenert, Stasko, and Hobby, 1958; Weiss, 1959) reported that the growth of different strains of the tubercle bacillus is enhanced in the logarithmic phase and by greatly increased growth yields when cultures are aerated directly or by mechanical agitation. None of these studies specifically tested the effect of shake culture on the utilization of any of the constituents in the medium. The results of our study point up the probable mechanism whereby shake culture enhances growth of *M. tuberculosis* by decreasing the lag period required before utilization of a nutrient is initiated. This would apply to those medium constituents which involve inducible enzyme systems in their oxidation, e.g., glycerol, in the case studied here. For constitutive systems, the increased O₂ tension may also have an enhancing effect on the rate of oxidation of such nutrients. Our results further suggest that glycerol induction may well be an O₂-dependent process.

The widely accepted notion among investigators is that glycerol is the preferred carbon source for the growth of mycobacteria (Long, 1958). Our results clearly illustrate that glycerol is not utilized during the early stages of growth. Rather, the preferred nutrient for growth initiation is glutamate. This finding corroborates the report of Ginsburg and Dunn (1957) that, in their growth studies of mycobacteria, glutamic acid is a highly preferred nitrogen source. Although glucose utilization is initiated coordinately with glutamate, its presence had a marked repressive effect by causing a 4-day lag period preceding glutamate utilization. This result also clarifies the observations on growth rates of

strains H37Rv and H37Ra reported by Lyon, Lichstein, and Hall (1961): that under conditions of aeration, glucose caused an inhibition of growth in contrast to growth stimulation when glycerol was substituted for glucose. (The nitrogen source in their experiments was asparagine.) Our results, furthermore, illustrate that this glucose effect persists in the presence of glycerol.

Another unexpected finding is that citrate, a common constituent of media designed for growth of *M. tuberculosis*, is never utilized under the variety of conditions of culture employed in this study, including the elimination of glucose from the medium. Furthermore, elimination of citrate from the culture medium had no effect on the growth curve or the utilization of the other nutrients. The possibility that the deficiency in the utilization of citrate lies in the absence of a citrate-permease system is being tested directly by uptake experiments of bacilli exposed to C¹⁴-labelled citrate.

Thus, the combined conditions for reducing (to one-tenth) the lag phase in time of glycerol utilization and for greatly increasing the overall rate of growth of *M. tuberculosis* were the use of shake culture, a 40-fold increase in the size of inoculum, substitution of glutamate for asparagine as nitrogen source, and elimination of glucose from the medium, with glycerol remaining as the carbon source. These operational refinements in culture afford a *modus operandi* for conducting quantitative studies of the growth and metabolism of *M. tuberculosis*.

The enzymatic mechanisms underlying the patterns of nutrient utilization elucidated in this report are being presently investigated by use of resting cell suspensions. However, certain preliminary conclusions can be drawn from the present results based on rates of nutrient utilization by growing cells of tubercle bacilli. The sequential pattern of glutamate-glycerol utilization, independent of any glucose effect, is clearly a function of glutamate repression of glycerol utilization. Whether this glutamate effect is truly "repression," according to the Jacob and Monod (1961) model, remains to be established. Preliminary results (Bowles, *unpublished data*) of enzymatic analysis of resting cells indicate that glutamate does inhibit synthesis of one or more of the inducible enzymes of glycerol oxidation. Possible repression by glutamate of a glycerol-permease system has been excluded as part of the repressive mechanism by means of kinetic studies of uptake of C¹⁴-labeled glycerol in the presence and absence of glutamate (Segal, *unpublished data*).

The conclusion that glutamate represses glycerol oxidation is also consistent with the finding that, of the four nutrients studied, only glycerol utilization is under the control of an inducible system. Thus, one would expect it to be susceptible to repressive effects. In the case of glutamate repression, this apparently represents a case of "catabolite repression" (Magasanik, 1961) and is an exceptional case of this phenomenon, to the best of our knowledge, in the sense that the repression of glycerol oxidation is by an amino acid. Furthermore, the glutamate repression in evidence in this study does not appear to exhibit the properties of a "diauxie" effect (Monod, 1942), since there is no evidence of a lag phase in growth separating the two exponential phases of the growth curve during which glutamate and glycerol are sequentially utilized (Fig. 6).

The coordinate patterns of glucose-glutamate and of glucose-glycerol utilization would appear to rule out catabolite repression by glucose. However, elimination of glucose from the culture medium results in the elimination of the 4-day lag period before glutamate utilization is initiated, even though it has no effect on the sequential pattern of glutamate-glycerol utilization, and no apparent effect on the growth curve. This leaves open to question and further investigation the role of glucose in this system. Experiments are now in progress to determine whether glucose represses a glutamate-permease system.

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