Influence of Age on Metabolic Activity of *Mycobacterium tuberculosis*. (24253)

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Metabolic studies of *Mycobacterium tuberculosis* have been carried out in the main using bacterial cells obtained from surface growth on a variety of media incubated for 3-5 weeks (1-3). This method has the advantage of harvesting relatively large numbers of cells from small amounts of media, but it has the disadvantage of employing tubercle bacilli which have passed the logarithmic phase of growth. With the introduction of technics allowing growth of tubercle bacilli in a submerged-dispersed condition and the availability of high-speed refrigerated centrifuges it is no longer a serious problem to harvest bacterial cells from large quantities of media. Furthermore, the more rapid rate of growth under these conditions permits use of younger bacterial cells which may not only be more active metabolically than their older counterparts grown on surface cultures but may actually differ in their metabolic pathways. Although comparatively little work has been reported relating metabolic activity of the tubercle bacillus with the age of the culture, Loebel, Shorr, and Richardson(4) demonstrated that younger cells metabolize more actively than older cells. It would appear therefore that many investigators have sacrificed metabolic activity per cell in order to obtain improved yields of cells.

The present study was initiated to investigate the metabolic activity of *M. tuberculosis* grown dispersed in liquid media and to relate the influence of age on this activity. The system chosen was oxidation of lactic acid. Intact cells were employed in order to gain a better physiological picture being fully cognizant of the fact that disrupted cells may be of value in future investigations.

Materials and methods. *M. tuberculosis* strain H37Ra* was used throughout the study and was cultured in a submerged-dispersed condition employing the medium of Sauton (5). Although a wetting agent is ordinarily not included in Sauton's medium, Tween 80† (final concentration 0.02%) was incorporated in the present study to permit dispersed growth of the tubercle bacillus. The media were dispensed in liter quantities into 2000 ml Erlenmeyer flasks and inoculated with 20 ml of a 7 day culture of H37Ra grown in the respective medium. The cultures were incubated under stationary conditions at 37°C except that the flasks were rotated briefly 3 times a day throughout the incubation period for purposes of aeration.

The bacterial cells were harvested by centrifugation, washed twice with distilled water and resuspended in the same menstruum to give a final concentration of approximately 10-12 mg dry weight of tubercle bacilli per ml. The dry weight was determined either by direct weighing of an aliquot of cell suspension after drying in an oven at 100°C for 24 hrs or by an indirect method. This consisted of interpolation from a previously constructed straight-line graph relating mg dry weight of bacterial cells per ml of washed cell suspension to % optical transmission at a wave length of 600 mμ using the Coleman Junior Spectrophotometer. Experimental results revealed that the greatest accuracy was obtained when the washed cell suspension was diluted to a point where the optical transmission fell within the range of 40-60%.

The Warburg constant volume respirometer was used to measure oxygen uptake of the resting cell suspensions. The temperature of the bath was adjusted to 37°C and the shaking rate to 140 strokes per min. The main compartment of the Warburg vessel contained phosphate buffer, bacterial cell suspension and

* Obtained from the U. S. Public Health Service, Communicable Disease Center, Chamblee, Ga.

† Polyoxyethylene sorbitan monooleate produced by the Atlas Powder Co., Wilmington, Del.

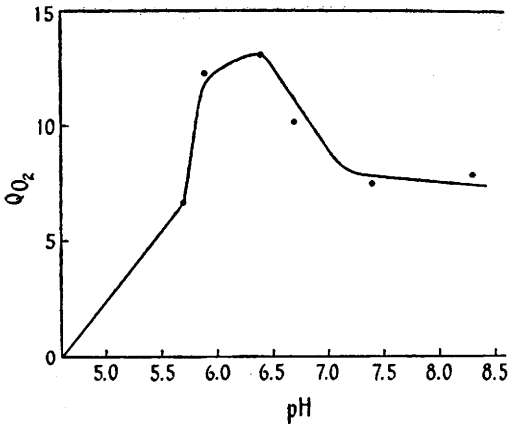

FIG. 1. Effect of pH on oxidation of lactic acid by washed cell suspensions of *M. tuberculosis* strain H37Ra. 0.066 M phosphate buffer; 0.022 M lactate; age of culture, 7 days; Sauton's medium; 3.6 mg dry wt bacterial cells/cup.

sufficient distilled water to bring the volume to 3 ml; the inner well contained 0.15 ml of 20% KOH; the side arm housed the substrate which was tipped after temperature equilibration. Readings were made at suitable intervals for a period of 5 hrs and the results are expressed in terms of Q_{O_2} *i.e.*, μl O_2 consumed per mg dry weight of cells per hr.

Results. Prior to the investigation of the influence of age of culture on metabolic activity of *M. tuberculosis* it was necessary to learn the optimal pH and the substrate concentration for such studies. The results presented in Fig. 1 relate the rate of lactate oxidation with pH. A final concentration of 0.066 *M* phosphate buffer was employed in order to achieve ample buffering capacity over the wide range of pH studied. It is quite clear from the data presented that the optimum range of pH was approximately 5.9-6.6 with maximum activity at pH 6.4. It is also manifest that the rate of lactate oxidation fell off quite markedly on either side of the optimal range.

With respect to substrate concentration the data in Fig. 2 reveal that rate of lactate oxidation increased markedly at substrate levels between 1.66 and 2.32 x 10^{-2} *M*. Concentrations of lactic acid in excess of the latter figure caused no further increase in metabolic activity.

A series of experiments were performed next, designed to relate physiological activity (using lactate oxidation as a parameter) with age of the bacillus. Aliquots of culture were removed at daily intervals and turbidimetric measurements made of the unconcentrated culture and of the culture concentrated 5 times. The latter procedure was considered advisable because of the sparse growth of the organism especially during the first week of incubation. Concentration was achieved by centrifugation of a 10 ml aliquot of culture for 30 min at 12,500 x gravity followed by resuspension of the sedimented cells into 2 ml of the supernatant fluid. Resuspension was facilitated by making use of a Teflon homogenizer. The results so obtained were employed for construction of the growth curves presented in Fig. 3. At intervals from the 4th through the 20th day of incubation, respiration studies were performed employing washed bacterial cell suspensions on the day of harvesting in order to avoid varying reductions in Q_{O_2} following refrigeration of such suspensions. The results of such studies (Fig. 3) revealed maximum lactate oxidation between the 10th and the 12th day of incubation which approximated the mid-portion of the logarithmic phase of growth.

Discussion. The results herein presented make known that the metabolic activity of *M. tuberculosis*, as revealed by a study of lactate oxidation, is influenced markedly by

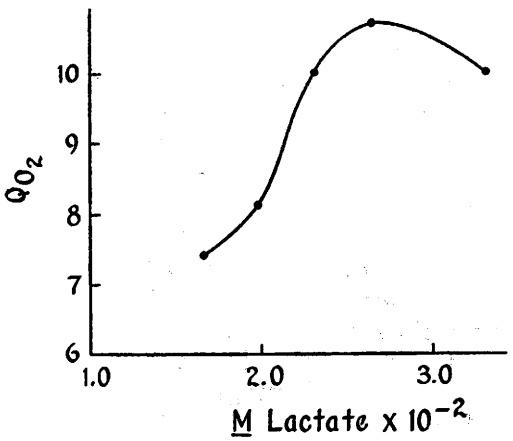

FIG. 2. Effect of substrate concentration on rate of oxidation by washed cell suspensions of *M. tuberculosis* strain H37Ra. 0.066 M phosphate buffer, pH 6.4; 7.3 mg dry wt bacterial cells/cup; age of culture, 7 days; Sauton's medium.

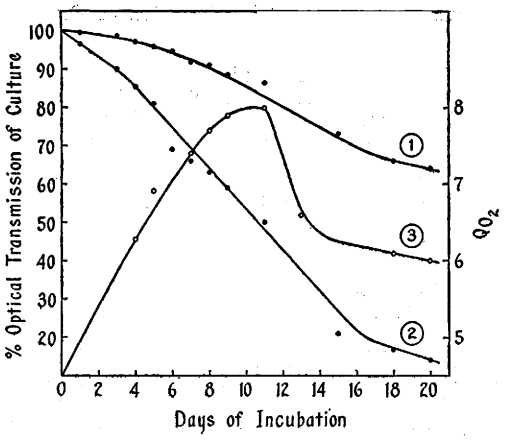

FIG. 3. Effect of age of culture on rate of lactate oxidation by washed cell suspensions of *M. tuberculosis* strain H37Ra. Curve 1 = % optical transmission of culture. Curve 2 = optical transmission of culture concentrated 5 times. Curve 3 = Q_{O_2}. 0.066 M phosphate buffer, pH 6.4; 0.022 M lactate; 6-9 mg dry wt bacterial cells/cup; Sauton's medium.

age of the bacterial cells. It is manifest from the data given that rate of lactate oxidation by washed cell suspensions increases with age of the cells, reaching a maximum between the 10th and the 12th day of incubation which coincides with the middle of the logarithmic phase of growth. Upon continued incubation, rate of lactate oxidation falls rapidly reaching a minimum during the stationary phase of the bacterial growth cycle. Thus, it is evident that the tubercle bacillus responds in this respect in a similar manner to other micro-organisms(6) and suggests that young cells derived from dispersed cultures might properly be more widely used in studies of the physiology of this organism. Although admittedly these data were obtained using only one strain of the tubercle bacillus and inspecting only one enzyme system, the results suggest further study along these lines. Furthermore, since it has been established that the lipid content of tubercle bacilli increases with age(7), which may well influence the permeability of the older intact cells, it would appear desirable to utilize the younger cells for investigative purposes.

Summary. A study of lactate oxidation by washed cells of *M. tuberculosis* strain H37Ra revealed a marked influence of the age of the cells. Cells obtained during the logarithmic phase of growth were the most active metabolically.

1. Geronimus, L. H., Ph.D. Dissertation, Ohio State Univ., Columbus, 1949.
2. Goldman, D. S., *J. Bact.*, 1956, v71, 732.
3. Millman, I., Youmans, G., *ibid.*, 1954, v68, 411.
4. Loebel, R. O., Shorr, E., Richardson, H. B., *ibid.*, 1933, v26, 139.
5. Willis, H. S., Cummings, M. M., Charles C. Thomas, Springfield, Ill., *Diagnostic and Experimental Methods in Tuberculosis* (2nd ed.), 1952, 126.
6. Oginsky, E. L., Umbreit, W. W., W. H. Freeman and Co., San Francisco, Calif., *An Introduction to Bacterial Physiology*, 1954, 59.
7. Frouin, A., Guillaumie, M., *Ann. Inst. Pasteur*, 1928, v42, 667.

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Biliary Excretion of Co^{60} Labeled Vitamin B_{12} in Dogs.* (24254)

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Previous reports indicate that radioactivity is present in bile and feces after injection of labeled B_{12}, but observation periods were short and data not critically analyzed(1,2,3). The following study was instituted to evaluate the rate of Co^{60} excretion in bile and its role as a source of fecal radioactivity after intravenous

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