Uptake and Binding of $^{14}$C-Ethambutol by Tubercle Bacilli and the Relation of Binding to Growth Inhibition

WILLIAM H. BEGGS AND NANCY E. AURAN

Bacteriology Research Laboratory, Veterans Administration Hospital, Minneapolis, Minnesota 55417

Received for publication 20 September 1972

Studies were designed to characterize ethambutol uptake by Mycobacterium tuberculosis (H37Ra) and to relate uptake to the time-dependent, concentration-independent nature of growth inhibition by ethambutol. When cells grown aerated at 37°C in Sauton medium were exposed for 7 hr to 0.2, 0.5, 1.0, 2.5, and 5.0 μg of $^{14}$C-ethambutol per ml, uptake increased with time and was a linear function of concentration. The process was inhibited at 22°C. Studies with chloramphenicol, sodium azide, and 2,4-dinitrophenol indicated that uptake is independent of requirements for protein synthesis and energy. The organism did not accumulate ethambutol against a concentration gradient. It can be concluded that ethambutol enters the cells in a passive manner. Kinetic studies of $^{14}$C loss from tubercle bacilli pretreated with labeled drug suggested the existence of two ethambutol fractions within the cell: a highly variable labile pool and a second fraction that is small and quite firmly bound. Levels of cell-bound drug may be independent of total uptake, but this possibility was not established unequivocally. Definitive evidence showing identity in the concentrations of bound drug regardless of total uptake could explain the apparent discrepancy between concentration-dependent uptake and concentration-independent growth inhibition.

Sophisticated new approaches to tuberculosis chemotherapy have raised numerous questions in the area of drug-microbe interactions. A need for more knowledge regarding the effects of pulsed drug exposures on tubercle bacilli has been intensified by recent interest in intermittent chemotherapy. Thus, in vitro studies have been designed in this laboratory to relate drug concentration and exposure time to uptake of drug and the degree of growth inhibition after single pulses with various single antituberculous drugs (2, 4, 5). We previously reported that inhibited growth responses of Mycobacterium tuberculosis subsequent to 3- to 16-hr pulsed ethambutol exposures were transient and reversible in nature, were a direct function of exposure time, and were relatively independent of drug concentration (4). The recent preparation of $^{14}$C-labeled ethambutol at this hospital, permitted an examination of drug uptake by the organism in relation to growth inhibition. Several years ago, Forbes et al. (6) reported that uptake of $^{14}$C-ethambutol by nonproliferating mycobacteria suspended in phosphate buffer was concentration-dependent. An intracellular to extracellular ratio of about 2:1 was achieved for each concentration of labeled drug tested. They suggested that ethambutol enters the cells by diffusion and is bound. In the studies presented here, the direct proportionality between concentration and uptake was extended to growing cells of M. tuberculosis, and several new characteristics of the uptake process were determined. Additional experiments were designed to resolve the apparent discrepancy between concentration-dependent uptake and the concentration-independent nature of growth inhibition by ethambutol (4).

MATERIALS AND METHODS

Organism. The H37Ra strain of M. tuberculosis used in these studies was grown in 500-ml Erlenmeyer flasks containing 200-ml volumes of modified Sauton synthetic liquid medium supplemented with 0.02% Tween 80 (1). Cultures were incubated at 37°C with aeration (rotary shaking at 150 rev/min). The minimal inhibitory concentration of ethambutol under these conditions was approximately 0.2 μg per ml. $^{14}$C-ethambutol. $^{14}$C-labeled ethambutol [dextro 2,2′-(ethylene-di-$^{14}$C-diimino)-di-l-butanol dihydrochloride] was prepared by the method of Wilkinson et al. (7). The synthetic compound was chromatographically pure. Only one spot was visible in an iodine
chamber after the material was developed for 3.5 hr on a Silica-Gel GF-254 thin-layer chromatography plate with an n-propanol-ammonia (70:30) solvent system. A scan of the chromatogram revealed one radioactive peak that coincided with the spot seen in the iodine chamber. The synthetic $^{14}$C-ethambutol showed biological activity identical to that of unlabeled drug, and it had a specific activity of 5.12 $\mu$Ci per mg (6.92 $\mu$Ci per mg of free base). All concentrations are expressed in terms of micrograms or nanograms of the free base drug.

$^{14}$C-ethambutol uptake experiments. Rapidly growing 5- to 6-day-old cultures with an optical density in the range of 0.24 to 0.28 were diluted with fresh Sauton medium to an optical density of 0.14 (equivalent to 0.25 mg [dry weight] of cells per ml). Optical densities were measured on homogenized samples of cell suspensions in tubes (18 by 150 mm) at 600 nm with a Coleman Junior spectrophotometer. After this adjustment, 80-ml volumes of suspension were distributed into the required number of 500-ml Erlenmeyer flasks and equilibrated briefly at 37 C with rotary shaking. $^{14}$C-ethambutol was added to each flask at the desired concentration, and incubation was continued. At time intervals, which varied from 15 min to 7 hr, the cells contained in 8.0-ml samples were collected on GA-4 Metrical filter membranes (diameter, 2.54 cm; pore size, 0.8 $\mu$m; Gelman Instrument Co., Ann Arbor, Mich.) under negative pressure. The cell-containing filter membranes were quickly washed three times with 2-ml volumes of Sauton medium, glued to stainless-steel planchetts, and placed in a desiccator until the membranes appeared to be dry. The samples were then heated in a drying oven at 90 C for 30 min, cooled, and assayed for $^{14}$C activity in a Nuclear-Chicago automatic gas-flow planchet counter. All samples were counted for 120 min and corrected for background. Based on our determination that 20.0 ng of $^{14}$C-ethambutol yielded 5,500 counts per 120 min, corrected for background, counts per milligram (dry weight) of cells were converted to nanogram equivalents of $^{14}$C-ethambutol.

Since the GA-4 filter membranes themselves bound $^{14}$C-ethambutol, a standard curve was constructed to permit a proper correction of counts observed in the experimental samples (Fig. 1). $^{14}$C-ethambutol was added to portions of Sauton medium at final concentrations of 0.2, 0.5, 1.0, 2.0, and 5.0 $\mu$g per ml. Volumes of 8.0 ml were then passed through GA-4 filter membranes. An 8.0-ml volume of M. tuberculosis cell suspension containing 0.25 mg (dry weight) per ml without labeled drug was then filtered over each membrane. The samples were washed twice with 2 ml of Sauton medium, glued to planchetts, dried, and assayed for $^{14}$C activity as previously described.

Stability of $^{14}$C-ethambutol binding by tubercle bacilli. Two methods were used to measure the kinetics of $^{14}$C loss by cells receiving a prior 3- to 5-hr pulse with labeled drug. In method A, the cells in 50 ml of $^{14}$C-ethambutol-treated culture were collected on a bacteriological filter membrane and washed with Sauton medium as described above, except that the cell mass was kept moist. The wet cell-containing filter membrane was transferred to 25 ml of Sauton medium contained in a large tissue homogenizer and subjected to Vortex mixing. After the cells were dislodged, the filter membrane was removed and clumps of cells were dispersed with a Teflon tissue grinder. The resulting suspension was adjusted to the original cell density with Sauton medium in a 125-ml Erlenmeyer flask. Cell-bound radioactivity was determined immediately on an 8.0-ml sample. At time intervals during aerated incubation at 37 C, additional samples were taken. In method B, 70 ml of $^{14}$C-ethambutol-treated culture was poured into a large filter apparatus fitted with a membrane that had a pore size of 0.45 $\mu$m (Millipore Corp., Bedford, Mass.). Gentle negative pressure was applied, and the suspension was stirred during filtration. When the volume reached 2 to 3 ml, it was adjusted to 70 ml with fresh medium and filtration was repeated. The cell suspension was adjusted to 70 ml and transferred to a 500-ml Erlenmeyer flask. From this point, incubations and samplings were as described for method A. Once $^{14}$C-ethambutol-treated cells were suspended in drug-free medium, cell-bound $^{14}$C activity determinations did not have to be corrected for filter-membrane adsorption.

RESULTS

Time studies of $^{14}$C-ethambutol uptake by tubercle bacilli exposed to five different concentrations of labeled drug over a 25-fold range are shown in Fig. 2A. These data indicated that drug uptake by viable cells in Sauton medium is a direct function of both time and concentration. At any particular time between 15 min and 7 hr, the relationship between concentration and uptake appeared to be linear (Fig. 2B).
Various metabolic inhibitors were tested for a possible effect on uptake. When cell suspensions were treated with 1.0 mM sodium azide, 0.1 mM 2,4-dinitrophenol, or 50 µg of chloramphenicol per ml for 15 to 30 min before the addition of 14C-ethambutol (5.0 µg per ml), the resulting uptake curves were not significantly different from that obtained with 14C-ethambutol only. Uptake was, however, retarded at low temperature. After 3 hr of exposure to 14C-ethambutol at the submaximal growth temperature of 22°C, cell-bound 14C activity varied from 65 to 80% less than in the controls at 37°C.

Consistent with the inhibitor studies, tubercle bacilli apparently cannot concentrate 14C-ethambutol from the external environment. Based on methods and results described in an earlier report (3), it was determined that 0.25 mg (dry weight) of tubercle bacilli per ml of cell suspension is equivalent to about 3.4 µliters of cell volume per ml. From these figures, the internal to external concentration ratios of 14C-ethambutol shown in Fig. 2A were calculated. Since intracellular water space is obviously something less than total cell volume, an error was introduced which intended to lower the concentration ratios from their true values. It appears likely, therefore, that 5-hr concentrations of internal and external drug were roughly equal. These results seem to agree quite well with those of Forbes et al. (6), who reported a concentration ratio of only 2:1.

We next attempted to demonstrate that, of the total 14C-ethambutol taken up by cells exposed to relatively high concentrations of drug, only a small fraction is firmly bound. Cells pulsed with 1.0 and 5.0 µg of 14C-ethambutol per ml for 3 to 5 hr quickly lost most of their 14C activity during incubation in drug-free medium at initial rates that depended on the time-zero levels (Fig. 3). After the first 30 to 60 min of rapid loss, the rates...
shifted and were drastically reduced. Although the levels of cell-bound $^{14}$C-ethambutol existing at 90 min all appeared to be something less than 3 ng per mg of dry cells, the assay system was not of sufficient sensitivity in this range to establish identity.

**DISCUSSION**

Results from our studies with *M. tuberculosis* H37Ra indicated that the total uptake of $^{14}$C-labeled ethambutol by growing cells is both time- and concentration-dependent, that it is independent of protein synthesis and of an energy requirement, and that drug is not accumulated against a concentration gradient. These data have extended earlier work reported by Forbes et al. (6), and we can conclude that ethambutol is taken up by proliferating organisms in a purely passive manner.

Data presented in Fig. 3 indicated that the time- and concentration-dependent nature of $^{14}$C-ethambutol uptake shown in Fig. 2 reflects the existence of both a highly variable labile pool fraction of cellular ethambutol and a second fraction that is very small and quite firmly bound. We were not able to demonstrate unequivocally that cell-bound levels of the latter fraction are the same regardless of total uptake. However, it is tempting from an examination of Fig. 3 to speculate that such is the case. This possibility could provide the basis for a plausible explanation of the apparent discrepancy between concentration-dependent drug uptake shown in Fig. 2 and concentration-independent growth inhibition reported earlier (4). It would suggest that tubercle bacilli contain a small and relatively fixed number of ethambutol-binding sites that are saturated in the presence of drug levels equal to or greater than a minimal inhibitory concentration. Additional drug taken up would enter a pool and could not directly participate in inhibitory activity, other than to ensure that the ethambutol-binding sites remained saturated.
ACKNOWLEDGMENTS
Herbert T. Nagasawa and Frances N. Shirota prepared the synthetic 14C-labeled ethambutol used in these studies. We are most grateful for their cooperation and for the time and effort they devoted to this task.

LITERATURE CITED