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Mode of Action of Primaquine: Preferential Inhibition of Protein Biosynthesis in *Bacillus megaterium*

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The growth of a strain of *Bacillus megaterium* was prevented by a minimal inhibitory concentration of primaquine of 52 μg/ml or 2×10^{-4} M. When exponentially growing cultures received the drug at 6×10^{-4} M, the rate of growth was drastically reduced and no further growth occurred after 15 min of exposure. At this concentration, primaquine was bactericidal, causing a 50% reduction in the viable population after one doubling time of 45 min. Supplying primaquine to cultures 30 min after adding radioactive-labeled phenylalanine, thymidine, uracil, or diaminopimelic acid produced an immediate and complete inhibition of protein biosynthesis but no inhibition of deoxyribonucleic acid biosynthesis for at least 15 min, and caused the formation of ribonucleic acid and cell wall polymer to proceed linearly at rates similar to those established prior to the addition of drug. This pattern of inhibition of macromolecular biosyntheses suggests that the major in vivo action of primaquine in *B. megaterium* is to block protein synthesis.

The major drugs employed in the chemotherapy of malaria may be classified into two groups according to their mode of action. In one group are the so-called antifolics, comprised of such compounds as proguanil, pyrimethamine, and the sulfonamides, which act on the *p*-aminobenzoic acid-folic-folinic acid pathway of reactions (11). The second is the quinoline-acridine group, which may be subdivided into (i) the cinchona alkaloids, the acridines, and the 4-aminoquinolines, all of which appear to act by inhibiting the biosynthesis of nucleic acids (10), and (ii) the 8-aminoquinolines, whose antimalarial action has yet to be defined.

Of the 8-aminoquinolines, primaquine is the most extensively used in the treatment of malaria. Primaquine structurally (Fig. 1) consists of a quinoline nucleus with a methoxy substituent at the 6-position and a 4-amino-1-methylbutylamino side chain at position 8. In spite of the clinical importance of primaquine, very little is known about its mode of action. One reason for this stems from the widely held belief that the antimalarial properties of primaquine and of other 8-aminoquinolines reside in intermediates formed during metabolic transformations of these drugs in the host (11). The general in vitro insensitivity of test microorganisms to primaquine has tended to support this belief.

Nevertheless, we have found a strain of *Bacillus megaterium* whose growth was inhibited by primaquine at a minimal inhibitory concentration of 52 μg/ml or 2×10^{-4} M. Reported here are studies concerned with the mode of action of primaquine on this bacterium, the results of which show the most pronounced effect to be an immediate and complete inhibition of protein biosynthesis.

MATERIALS AND METHODS

Chemicals. Uracil-*2*-^{14}C, thymidine-*2*-^{14}C, and ^{14}C-L-phenylalanine were purchased from New England Nuclear Corp., Boston, Mass.; diaminopimelic acid-*2,5*-^{3}H was purchased from Amersham/Searle, Des Plaines, Ill. Primaquine diphosphate was supplied by the Division of Medicinal Chemistry of the Walter Reed Army Institute of Research.

Cultural conditions. Methods for cultivating and for determining the viability of cells of the test bacterium, *B. megaterium* ATCC 14581, have been described (9). Drug exposure and primaquine-free control experiments were performed on 20-ml portions of a mass culture grown to 10^7 cells/ml.

Synthesis of macromolecules. The biosynthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, and cell wall polymer was followed by measuring the incorporation of radioactive-labeled precursors by use of a previously outlined membrane filtration technique (9). Thymidine-*2*-^{14}C (5 mCi/mmole), ^{14}C-phenylalanine (8 mCi/mmole), and diaminopimelic acid-*2,5*-^{3}H (47 mCi/mmole) were

added to a final concentration of 5 μg/ml of culture; uracil-2-^{14}C (0.5 mCi/mmole), to 10 μg/ml. To promote the incorporation of ^{14}C-thymidine into DNA, deoxyadenosine was added to a concentration of 200 μg/ml of culture medium (2, 3). At 30 min after the addition of radioactive-labeled isotopes, primaquine was added to a concentration of 6×10^{-4} M.

The amount of radioactivity retained by the filter membranes (Millipore Corp.; 0.45 μm) was measured as described in a previous publication (9).

RESULTS

Effects on growth and viability. Primaquine, when added to exponentially growing cultures of *B. megaterium* in Trypticase Soy Broth (BBL) to a concentration of 5×10^{-4} M, produced an immediate decrease in growth rate which became more pronounced after 30 min. After 30 min of exposure to 6×10^{-4} M primaquine, growth was completely inhibited. Concentrations of 7×10^{-4} M or higher caused a progressive decline in the turbidities of the experimental cultures.

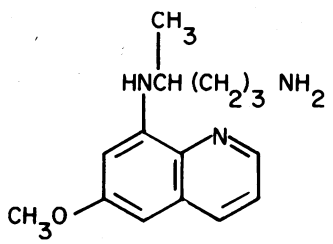

FIG. 1. *Chemical structure of primaquine.*

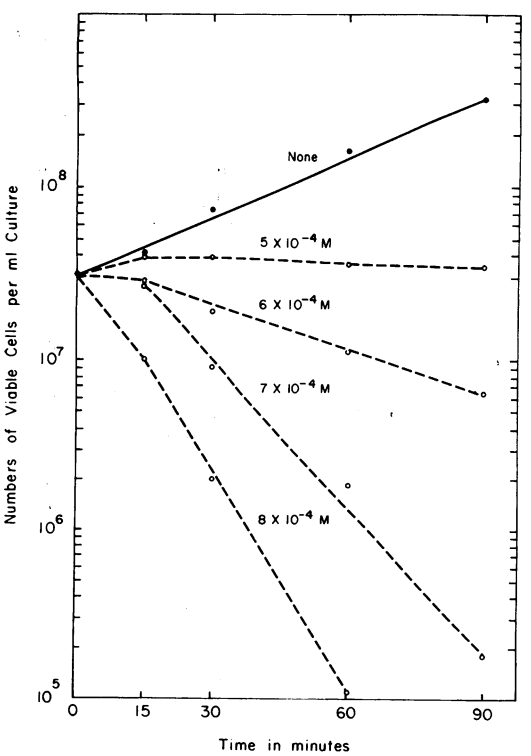

FIG. 2. *Effect of graded concentrations of primaquine on the viability of Bacillus megaterium.*

The inhibition of growth at concentrations of 6×10^{-4} M or higher was accompanied by an exponential decline in the numbers of viable cells (Fig. 2). When samples from cultures exposed to 6×10^{-4} M primaquine were subjected to serial dilution plate counting, it was found that the numbers of colony-forming bacteria decreased by 50% after 45 min, the approximate duplication time of *B. megaterium* under the cultural conditions employed. At 7×10^{-4} and 8×10^{-4} M, only 10 and 1%, respectively, of the treated cells survived exposure. At 5×10^{-4} M, however, the action of primaquine was primarily bacteriostatic.

Effects on the biosynthesis of macromolecules: protein. The progressive incorporation of ^{14}C-labeled phenylalanine into hot trichloroacetic acid-insoluble material was used as a measure of protein biosynthesis. Radioactive isotope was added at zero time (Fig. 3) to a culture of *B. megaterium* growing exponentially in Trypticase Soy Broth. After the incorporation had been monitored for 30 min, the culture was divided into

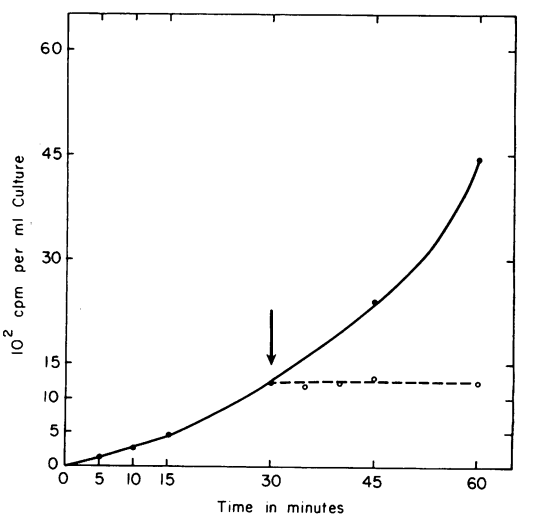

FIG. 3. *Effect of primaquine on the incorporation of ^{14}C-phenylalanine into the protein of Bacillus megaterium. ^{14}C-L-phenylalanine (8 mCi/mmole) was added to a concentration of 5 μg/ml of culture at zero time. The incorporation was monitored for 30 min, and then the culture was divided into two portions: to one (dashed line), primaquine was added to a final concentration of 6×10^{-4} M (time of addition shown by the arrow); the other (solid line) continued to serve as the primaquine-free control culture.*

two equal portions: to one, primaquine was added to a final concentration of 6×10^{-4} M; the other continued to serve as the primaquine-free control culture. Addition of primaquine produced an immediate and complete cessation of protein biosynthesis. Identical results were obtained with ^{14}C-leucine.

Nucleic acids. In marked contrast to the effect on protein biosynthesis, the biosynthesis of DNA (Fig. 4), as measured by the deoxyadenosine-facilitated incorporation of ^{14}C-labeled thymidine, was not affected until 15 min after the addition of primaquine.

The incorporation of ^{14}C-uracil into global RNA (Fig. 5), although decreased by primaquine, continued at approximately the rate established prior to the addition of drug.

Cell wall. The effect of primaquine on cell wall biosynthesis was followed by measuring the incorporation of ^{3}H-diaminopimelic acid into cold trichloroacetic acid-precipitable material by cells growing in lysine-supplemented medium. The lysine represses the synthesis and inhibits the activity of diaminopimelic acid decarboxylase (7), thus minimizing the conversion of labeled diaminopimelate to lysine and maximizing the selective incorporation into cell wall glycopeptide. Addition of primaquine permitted the continued incorporation of diaminopimelate (Fig. 6), although at a diminished rate.

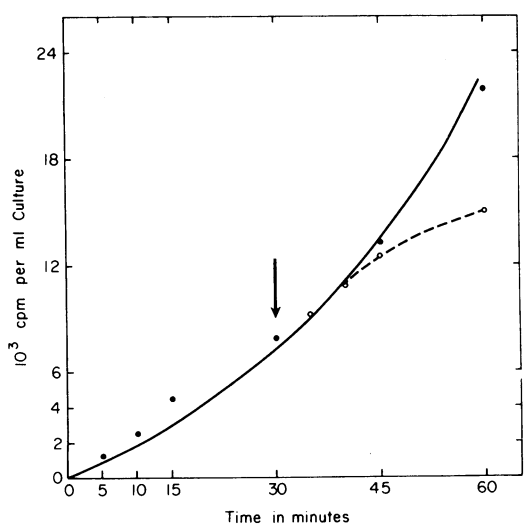

FIG. 4. *Effect of primaquine on the incorporation of ^{14}C-thymidine into the DNA of cultures of Bacillus megaterium supplemented with 200 μg of deoxyadenosine per ml. Thymidine-2-^{14}C (5 mCi/mmole) was added to a final concentration of 5 μg/ml of culture. Other conditions were the same as for Fig. 3.*

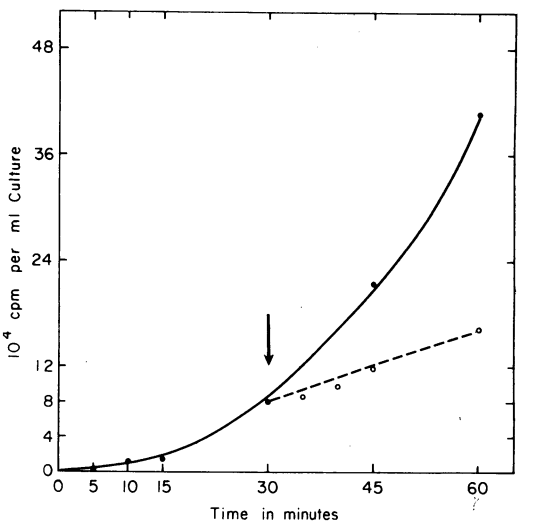

FIG. 5. *Effect of primaquine on the incorporation of ^{14}C-uracil into the RNA of Bacillus megaterium. Uracil-2-^{14}C (0.5 mCi/mmole) was added to a concentration of 10 μg/ml of culture. Conditions were the same as noted in Fig. 3.*

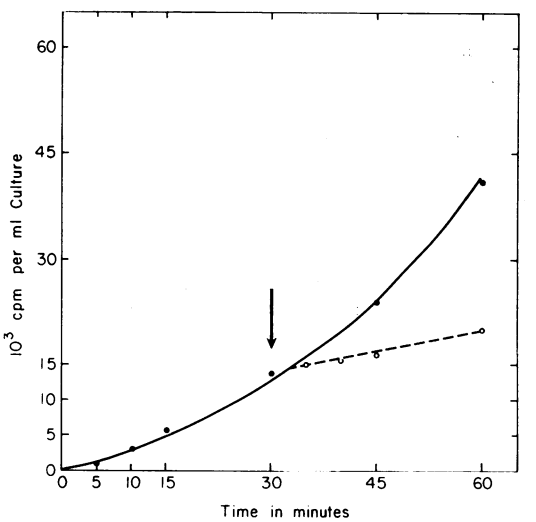

FIG. 6. *Effect of primaquine on the incorporation of ^{3}H-diaminopimelic acid into cell wall glycopeptide of Bacillus megaterium. ^{3}H-diaminopimelic acid (47 mCi/mmole) was added to a concentration of 5 μg/ml of cultures supplemented with 100 μg of lysine per ml. Other conditions were the same as described in Fig. 3.*

DISCUSSION

The present results show that the earliest and most pronounced effect of primaquine on in vivo macromolecular synthesis was the immediate and complete inhibition of protein biosynthesis; DNA

biosynthesis proceeded, presumably, through one cycle of replication, and the exponential incorporation of radioactive-labeled substrates into RNA and into cell wall polymer were converted to linear rates.

Others (R. Roskoski, Jr., and S. R. Jaskunas, Fed. Proc. **28**:893, 1969) have stated that, in a cell-free system from mammalian liver, primaquine inhibited the polyuridylic acid- and poly AGU-directed incorporation of phenylalanine, provided these polynucleotides were preincubated with the drug. In contrast, the endogenous amino acid incorporation, mediated by residual natural messenger RNA attached to liver ribosomes, was not affected by primaquine. This suggests that the inhibitions of polycondensation of phenylalanine were produced by complexing of synthetic model messengers with the drug. Morris and his associates have studied the binding of primaquine to various polynucleotides and have speculated that protein synthesis as well as the actions of nucleic acid polymerases may be affected by such drug-binding reactions (8).

The progressive linear incorporation of ^{14}C-uracil in primaquine-treated *B. megaterium* showed that the action of preexisting RNA polymerase was not influenced by primaquine but that no further active enzyme was synthesized in the presence of the drug.

Primaquine has been shown by Aikawa and Beaudoin (1) to be localized and to cause morphological changes in the mitochondria of exoerythrocytic forms of avian malarial parasites. Mitochondria contain a protein-synthesizing system which differs from its cytoplasmic counterpart but resembles the protein-synthesizing machinery of bacteria (5). In view of our present results, we speculate that the antimalarial action of primaquine may be due to a specific inhibition of mitochondrial protein synthesis in plasmodia.

Since *B. megaterium* has been shown in this and other mode-of-action studies to be uniquely sensitive to a variety of antimalarials (4, 9), cultures of this bacterium might serve as a useful primary screen for new potential antimalarial drugs.

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