erately increased resistance were obtained after 20 similar subcultures of a strain of hemolytic streptococcus and one of *Streptococcus viridans*. 4. The strains of increased resistance resulting from repeated subcultures on the erythromycin-containing agar retained that resistance after 10 further subcultures in antibiotic-free broth. 5. Strains of increased resistance to erythromycin derived by repeated subcultures in that antibiotic were almost invariably similar to their respective parent strains in their sensitivity to 8 other antibiotics; however, an erythromycin-resistant strain of type 3 pneumococcus had apparently increased in sensitivity to neomycin following its repeated subcultures in erythromycin. 6. Staphylococcal strains of markedly increased resistance to erythromycin were obtained from blood cultures of 2 patients with endocarditis following 7-10 days of treatment with this antibiotic. 7. The colonial, morphologic and biochemical characteristics of the erythromycin-resistant strains obtained by repeated subcultures in erythromycin-containing media resembled those of the respective parent strains from which they were derived in almost every instance. The strains of *Staphylococcus aureus*, however, lost their ability to produce coagulase and exhibited other changes in their biochemical properties following their repeated subcultures in the presence of erythromycin. Similar changes were not observed in the 2 strains of *Staphylococcus aureus* which had apparently become resistant during erythromycin therapy.

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Received September 2, 1952. P.S.E.B.M., 1952, v81.

Observations on Mode of Action of Erythromycin.* (19817)

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The results of studies carried out in this laboratory on some of the important properties of erythromycin as an antibiotic agent and on the occurrence and development of resistance of bacteria to that agent were presented in the preceding communications(1,2). The original description of the isolation of erythromycin and of its physical, chemical and antimicrobial properties were reported by McGuire *et al.*(3) and additional clinical and laboratory observations were presented by Heilman *et al.*(4). Preliminary results of clinical trials of this agent, chiefly in pneumococcal, streptococcal and staphylococcal infections have also been recorded(3-5). Observations on the mode of action of erythromycin as determined by growth curves of organisms exposed to that agent are described in this paper.

Materials and Methods. The organisms used were: a group A hemolytic streptococcus, No. 98, generally employed in assays of penicillin; a recently isolated strain of a coagulase-positive, hemolytic *Staphylococcus aureus* (No. 194); and the MacLeod strain of *Escherichia coli*. A single, well segregated colony from an agar pour plate of each of these strains was picked into broth and subcultures of these colonies were used for the present studies. The media used were brain-heart infusion broth (Difco) and heart-infusion agar (Difco) each at pH 7.4±. Defibrinated horse blood, 0.5% in broth and 10% in agar, was added for enrichment and as an indicator. Erythromycin in crystalline

* Aided by a grant from the U. S. Public Health Service.

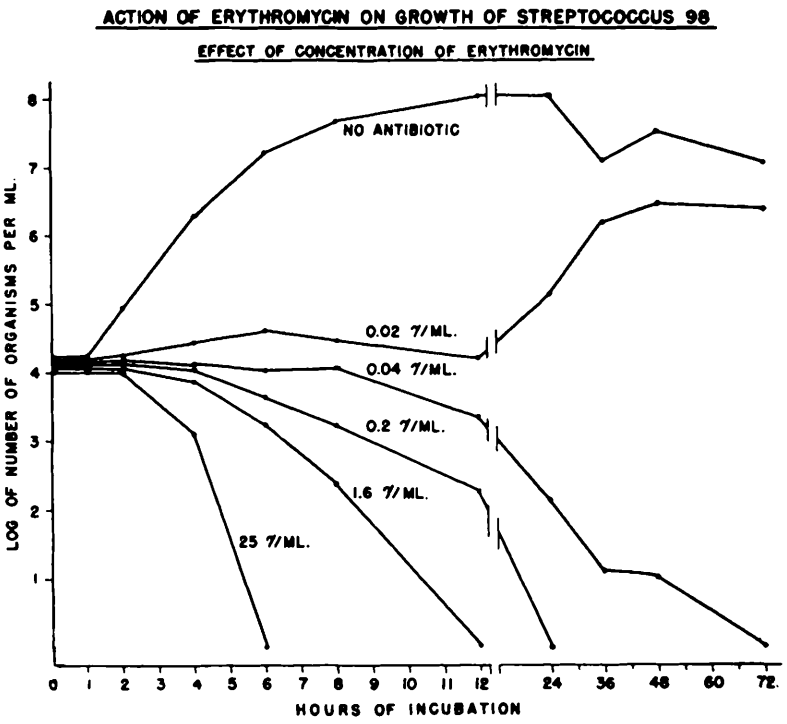

FIG. 1.

form (890 μg of active base per mg) was furnished by Dr. J. W. Smith of Lilly Research Laboratories. Solutions of the antibiotic, usually 2000 μg of active base per ml, were made first in distilled water, using a few drops of ethanol to facilitate solution. Further dilutions of antibiotic and all those of the cultures were made in broth. Growth curves were made by starting with 10 ml of culture containing the desired inoculum of organisms and concentration of erythromycin and then making pour plate counts with 0.1 ml of the culture removed at appropriate intervals.

Growth in Varying Concentrations of Erythromycin. A series of 6 cultures of *Strep.* 98 were made in broth using the same inoculum (0.01% of a fully grown culture) in every instance; one of these contained no antibiotic while the others contained graded concentrations of erythromycin from 0.02 to 25 μg per ml. Pour-plate counts were then made at appropriate intervals during 72 hours of incubation at 37°C. The resulting growth curves are shown in Fig. 1. It is seen that with increasing concentrations of erythromycin there was roughly a proportionate decrease in the period during which the numbers of viable organisms remained stationary; after this period of bacteriostasis, the cultures containing 0.04 μg or more of erythromycin per ml all showed a steady decline in the number of viable organisms, the rate of this decline being roughly proportional to the concentration of the antibiotic. The time required to render these cultures entirely free of viable organisms was, therefore, inversely proportional to the concentration of erythromycin, within the range of concentrations employed. In 0.02 μg/ml, a 24 hour period of bacteriostasis (during which there was essentially a stationary number of viable organisms) was followed by multiplication over a period of another 24 hours during which the number of viable organisms almost reached that of the control culture grown without antibiotic. Qualitatively similar growth curves were obtained in the same manner with *Staph.* 194, as shown in Fig. 2. Greater concentrations of erythromycin, however, were required to achieve effects comparable to those obtained with *Strep.* 98. With this staphylococcus, there was only a small additional effect

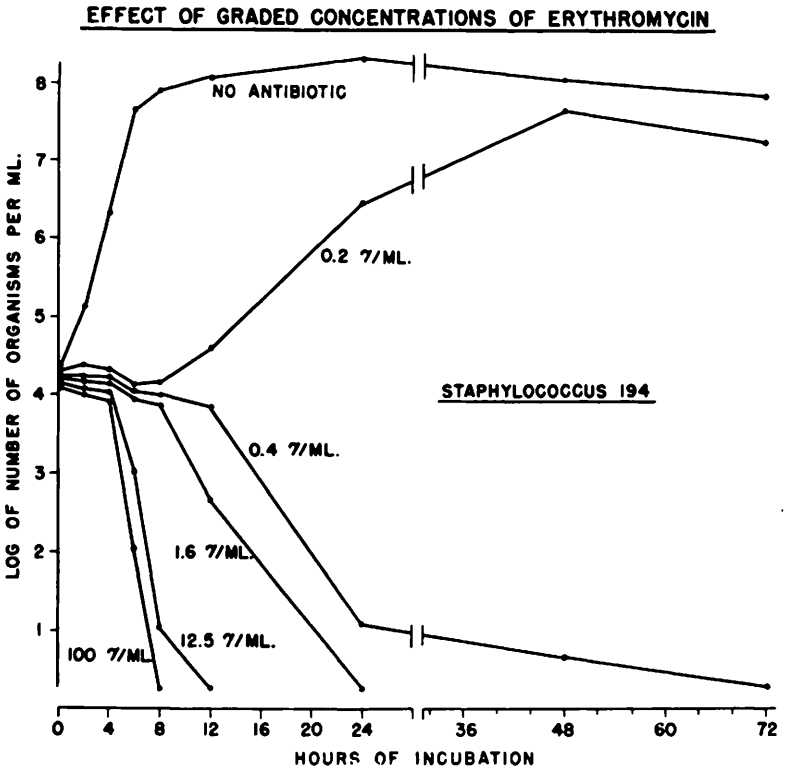

FIG. 2.

from 100 μg per ml of erythromycin over that exerted on the growth of this organism by a concentration of 12.5 μg per ml.

Growth from Inocula of Varying Size. A series of 8 broth cultures were seeded in pairs with 100-fold dilutions of a fully grown culture of *Strep.* 98, beginning with a 1% inoculum. One culture of each pair contained erythromycin in a concentration of 1.6 μg per ml while the other served as a control of growth without antibiotic. The resulting growth curves are shown in Fig. 3. The control cultures all grew in normal fashion and achieved the same maximum numbers in about 12 hours. The cultures grown in erythromycin, 1.6 μg per ml all showed a similar period of bacteriostasis, namely about 2 hours, after which the numbers of organisms began to decline at about the same rate in each of the cultures. The time required until no viable organisms could be demonstrated was, therefore, roughly proportional to the number of organisms per ml of the starting culture.

Incubation of Fully Grown Culture with Erythromycin. In this experiment a culture of *Strep.* 98 was grown for 24 hours after which 10 ml aliquots were measured into 4 culture tubes. Erythromycin in a volume of 0.1 ml of broth was then added to 3 of the tubes so as to make final concentrations of 0.4, 1.6 and 6.3 μg per ml, respectively, and 0.1 ml of antibiotic-free broth was added to the fourth tube. All 4 tubes were then incubated at 37°C and pour plate counts were made from each after various intervals. The results are shown in Figure 4. There was no significant difference in the numbers of viable organisms found in each of the 4 cultures throughout the period of observation.

Effect of the Phase of Growth. A series of 8 tubes of broth were inoculated at the same time and in an identical manner so that each contained a 10^{-6} dilution of a fully grown culture of *Strep.* 98 (approximately 100 colonies per ml in pour plate counts). One of these cultures served as a control, while to each of the others, in succession erythromycin

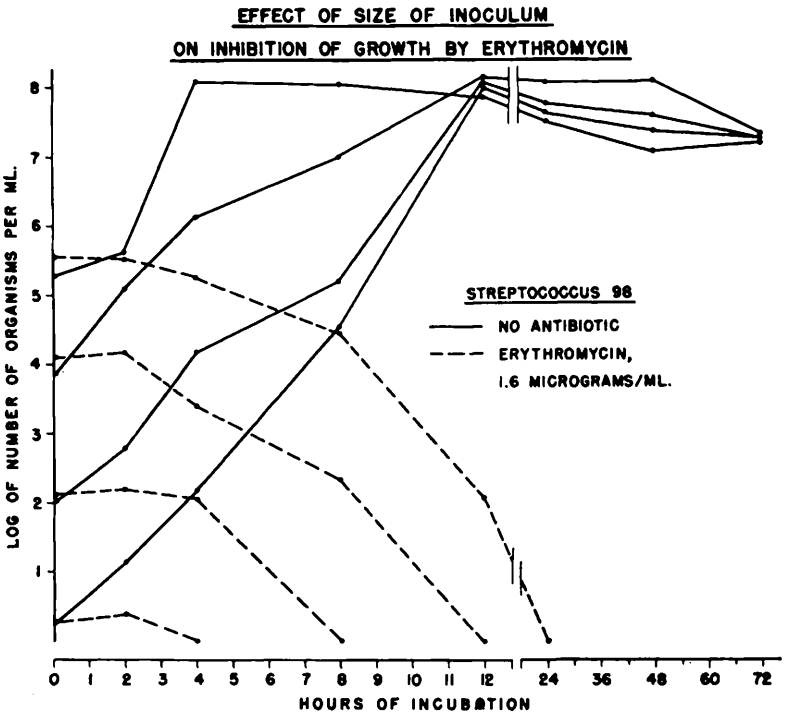

FIG. 3.

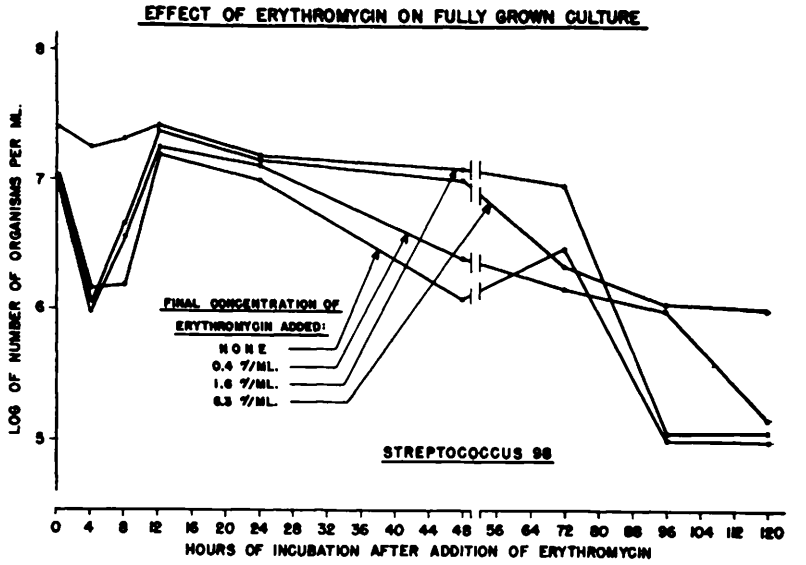

FIG. 4.

(in 0.1 ml of broth) was added after various periods of incubation up to 48 hours. The final concentration of erythromycin in each of the 7 cultures was 1.6 μg per ml. Pour plate counts were made from each of these cultures before and at intervals after the erythromycin was added and from the control culture throughout the experiment. The resulting series of growth curves is shown in Fig. 5. As might be expected from the previous ex-

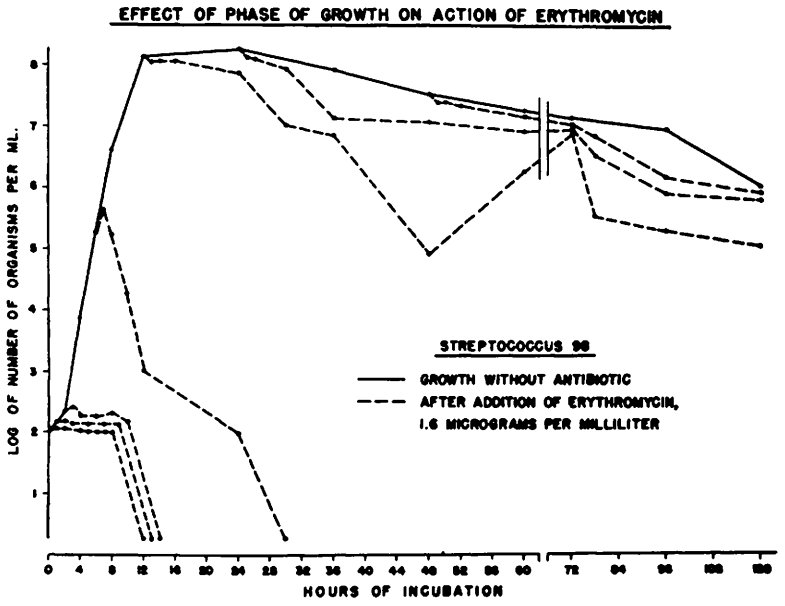

FIG. 5.

periments, as shown in Figures 3 and 4, when the antibiotic was added at 0, 1 or 2 hours and the numbers of organisms at the time of this addition was about the same, the resulting growth curves were very similar. When the erythromycin was added during the most active phase of growth, namely after 6 hours, there was a brief period of bacteriostasis, after which the number of organisms declined at a rapid rate. The culture that had just achieved full growth when the erythromycin was added showed a significant decline in the number of viable organisms at first with some subsequent growth. The addition of erythromycin after the stationary phase of growth had already been attained for several hours resulted in no significant decrease in the number of viable organisms as compared with the control culture to which antibiotic was not added.

Effect of Erythromycin at 4° C. Two series of broth cultures of *Strep.* 98, one undiluted and the other diluted 1:1000 from a fully grown culture, were kept in a refrigerator at approximately 4° C. One culture of each series contained no antibiotic while graded concentrations of erythromycin were added to the others at the time they were stored. Pour plate counts were then made of each culture at intervals up to 5 days. The exposure to erythromycin in the concentrations used at this temperature had no significant effect on the numbers of viable organisms found at each interval, in either series, as compared with those found in the corresponding cultures in the antibiotic-free broth.

Action of Erythromycin on Coliform Organisms. Cultures of *E. coli,* MacLeod, using 0.01% inoculum from a fully grown culture, were made in antibiotic-free broth and in broth containing erythromycin concentrations ranging from 1.6–400 μg/ml. Growth curves of these cultures indicated that in the lower concentration of erythromycin there was a period of bacteriostasis with some decline in the number of viable organisms and this was followed by multiplication until the numbers approximated those of the control group without antibiotics. In concentrations of 25–400 μg/ml of erythromycin there was a slow and steady depletion of the cultures but viable organisms were still demonstrated after long periods of incubation. Further studies with these organisms are in progress.

Conclusions. Erythromycin may be either bacteriostatic or bactericidal depending on the sensitivity of the organism and on the concentration of the antibiotic. Erythromy-

cin exerts its effect only against multiplying bacteria.

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Received September 2, 1952. P.S.E.B.M., 1952, v81.

Distribution of Amethopterin in Normal Mouse Tissues Following Intravenous Injection.* (19818)

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The antifolic activity of amethopterin (4-amino-N^{10}-methyl pteroyl-glutamic acid) has been well established, bacteriologically(1) and by animal experiments(2-3). It has been shown to be effective in prolonging the survival time of leukemic mice(4) presumably by an induced relative deficiency of pteroylglutamic acid (PGA) and citrovorum factor (CF) which retards the development of the leukemic process(5). In a similar manner it causes remissions in a significant percentage of acute leukemias in childhood(6-9).

Probably as a result of the lack of a satisfactory simple method for assaying amethopterin directly, little has been reported on the fate of this compound following its administration either to human subjects or to the experimental animal. Swendseid(10) using a back titration modification of the standard method for microbiological assay of PGA, has reported on the excretion of aminopterin (4-amino-pteroylglutamic acid) during treatment of leukemic patients. The recent development(11) of a simple disc method for determining the concentration of amethopterin in blood and urine led us to broaden the scope of this technic to assist in the study of the metabolism of this compound in the normal mouse. As a preliminary to these studies, the distribution of amethopterin in the tissues of the normal mouse following intravenous injection was determined.

Materials and Method. Young mice of the inbred Akm stock, weighing approximately 20 g each, were used. All the mice were maintained on a standard diet of Purina Laboratory Chow prior to and during the experiment. Water was allowed *ad libitum* throughout the experiment. Each mouse was given a single injection of amethopterin at a dose of 0.5 mg/kg by the intravenous route. This was considered an optimum dose, producing readily assayable amounts in the tissues and yet it was well below the maximum tolerated daily dose, and approximately 1/200th of the acute LD_{50} dose(2). Mice were sacrificed at 15, 30, 60, 120, 180, and 240 minutes after injection. Under ether anesthesia, blood was collected in a heparinized syringe by severing the axillary vessels, the animal being finally killed by decapitation. In those mice injected 1, 2, and 4 hours previously the liver, spleen, kidneys and lungs were then removed

* This investigation was supported (in part) by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service, in part by an institutional grant from the American Cancer Society, and, in part, by funds from the Damon Runyon Memorial Fund for Cancer Research, the Lasker Foundation and the Grant Foundation.

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