THE EFFECT OF SUBINHIBITORY CONCENTRATIONS OF CHLOROMYCETIN, AUREOMYCIN, AND DIHYDROSTREPTOMYCIN ON *SALMONELLA CHOLERAESUIS*, *SALMONELLA TYPHIMURIUM*, AND *PASTEURELLA MULTOCIDA* IN VITRO AND IN VIVO

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Charles H. Kupsky

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THESIS

This is to certify that the

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The effects of subinhibitory concentration of chloromycetin, aureomycin and dehydrostreptomycin on Salmonella choleraesuis, Salmonella typhimurium and Pasteurella multocida in Vitro and in Vivo.

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By

CHARLES H. KUPSKY

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Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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To Beverly

and Kathleen

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INTRODUCTION

The field of antibiotics, though relatively new, has progressed at an extremely rapid pace. Since the discovery of penicillin 12 years ago, many new antibiotics have been uncovered from microorganisms that include bacteria, fungi, actinomycetes, and protozoa. These antibiotics have been extensively investigated in vitro and in vivo for their chemotherapeutic values in combating pathogenic organisms. Nevertheless, there are phases which require more complete study to better understand the effect of antibiotics on pathogens; for example: (1) possible immunogenic stimulation by an organism treated with a subminimal concentration of an antibiotic when subsequently inoculated into an animal; (2) morphological and biological variation in these antibiotic-treated organisms.

With these thoughts in mind, this investigation was undertaken to add or confirm some knowledge of the in vitro and in vivo effects produced on pathogenic organisms, subjected to subinhibitory concentrations of an antibiotic.

REVIEW OF LITERATURE

Dihydrostreptomycin

The actinomycetes were recorded possessing lytic action upon certain bacteria as far back as the late part of the nineteenth century. It was not until 1944 that Schatz, Bugie, and Waksman (1944) obtained the antibiotic, streptomycin, from Actinomycetes griseus that possesses clinical value as a chemotherapeutic agent. However, from the very start it was realized that streptomycin was not a good chemotherapeutic agent because of its toxic effects and severe side reactions. Many attempts were made to produce a more nearly pure streptomycin in hopes of reducing its toxicity, but this was to no avail as acute toxic symptoms still accompanied treatment. In 1946, Peck et al. (1946) produced a modified form of streptomycin by catalytically hydrogenating it. This man-made modification was called dihydrostreptomycin. The reduced form of streptomycin differed chemically from streptomycin in that the carbonyl group of the streptobiosamine moiety was reduced to an alcohol, and also differed in that it was less toxic.

Waksman (1947) described streptomycin chemically as a nitrogen-containing glycoside that showed basic properties. He further stated that it could be crystallized by many methods and that it was a thermostable compound, soluble in water and insoluble in ether.

Oswald and Nielsen (1947) determined the stability of streptomycin by holding solutions of 100 micrograms per milliliter and 1,000 micrograms per milliliter at pH 6, pH 7, and pH 8 at a temperature of 10 degrees centigrade. He found that all solutions remained stable for 3 months. Similar studies were made by Erlanson (1951), with concentrations of streptomycin of 100,000 micrograms per milliliter and at a temperature of 5 degrees centigrade. He found that this solution remained stable for 3 months. Wolinsky and Stukin (1946) found that the bacteriostatic effect of streptomycin diminished considerably as the culture neared a pH of 5.5. Bartz et al. (1946) determined from their experiments that dihydrostreptomycin was more stable in an alkaline solution than streptomycin. To confirm this, Donavich and Rake (1947) found dihydrostreptomycin to be more effective when the medium was at pH 9. Pratt and Dufrenoy (1949) revealed that at room temperature, solutions

at pH 6.6 lost about one-third of their activity in 2 weeks. In the dry state dihydrostreptomycin remained stable for 18 months at room temperature.

The studies of Schatz et al. (1944) showed the mode of action of streptomycin to be bacteriostatic, but under certain conditions it possessed strong bactericidal properties like streptothricin. Studies by Garrod (1948) brought forth various factors that determined whether streptomycin was bactericidal. The factors were as follows: (1) concentration of antibiotic; (2) optimum temperature; (3) strain of organism; (4) nature of medium; (5) reaction of medium; (6) size of inoculum. Hamre et al. (1946) found streptomycin to be bactericidal for multiplying and nonmultiplying Klebsiella pneumoniae. The bactericidal action was also confirmed by Erlanson (1951). The postulated theory of the bacteriostatic action of streptomycin by Henry et al. (1948) and Berkman et al. (1948) was that streptomycin had an inhibitory effect on an enzyme or enzymes of a susceptible organism, that were involved in the metabolism of carbohydrates or that streptomycin inhibited their formation. Their experiments revealed that catalase, carbonic anhydrase, urease, carboxylase, cytochrome, succinoxidose, trypsin, or cytochrome

oxidase were not inhibited by high concentrations of streptomycin. Brink and Folkers (1950) found that there was an involvement of the pyruvate-oxalacetate condensation in the action of streptomycin and that the terminal respiration process was inhibited.

Donavich and Rake (1947) studied the toxic effects of dihydrostreptomycin in mice and found the toxic manifestations to be similar to those of streptomycin in mice. Pratt and Dufrenoy (1949) indicated that although streptomycin and dihydrostreptomycin are similar in many respects, they did differ pharmaceutically. Indications were that dihydrostreptomycin was approximately one-third as toxic as streptomycin. Toxic symptoms of streptomycin were of both the chronic and acute types. The work of Brink and Folkers (1950) further confirmed the conclusions of Pratt and Dufrenoy that dihydrostreptomycin was less toxic than streptomycin. The $L.D._{50}$ toxicity level of the dihydrostreptomycin in mice was shown by Baron (1950) to be 225 milligrams per kilogram weight of mouse.

The antimicrobial spectrum of streptomycin was first studied by Schatz, Bugie, and Waksman (1944). Streptomycin was found to be active in vitro against gram-negative pathogens

and gram-positive mycobacteria when compared to streptothricin, which is more specific for gram-positive organisms.

Streptomycin studies on salmonellae were undertaken by Seligmann and Wassermann (1947), using sixty different types and 226 strains of salmonella. The antibacterial range was shown to be between four and eight units of streptomycin. Sensitivity of animal-originated bacteria was investigated by Cole (1948), in which the author had included eleven strains of S. choleraesuis, three strains of S. typhimurium, and seventy-eight strains of P. multocida. The ranges of sensitivities were, respectively, 5.0 to 10.0 micrograms per milliliter of streptomycin for S. choleraesuis, 10.0 micrograms per milliliter of streptomycin for S. typhimurium, and 1.0 to 10.0 micrograms per milliliter of streptomycin for P. multocida.

Mice to be used in a Japanese Bacillus encephalitis vaccine were found to be infected with Salmonella enteritidis by Slanetz (1946). The treatment used consisted of adding streptomycin to the drinking water in concentrations sufficient to give 100 units per mouse per day. This was proved very satisfactory, as only one mouse out of six colonies was S. enteritidis

positive after a short duration. Further work was done by Slanetz on the susceptibility of S. enteritidis and S. typhimurium to streptomycin. The results indicated that S. enteritidis was more susceptible to the antibiotic than S. typhimurium. S. typhimurium and S. choleraesuis experimentally infected mice were studied by Seligmann and Wassermann (1947). The mice were treated with streptomycin by the oral route and by simultaneous oral and subcutaneous routes, with the resulting supression of the organisms. However, after termination of the experiment, the organisms reappeared and the death-rate curve resembled that of the control animals. It was concluded that the only effect of the streptomycin was to prolong the survival time of the mice. Cole (1948) made a similar type investigation of P. multocida, except that sufficiently high concentrations were used to produce 100 percent protection against the organism. Mice, inoculated with 0.1 cubic centimeter of undiluted culture intraperitoneally, received between 0 and 400 micrograms of streptomycin per milliliter of broth and received no protection, whereas mice obtaining 2,000 to 4,000 micrograms per milliliter were protected 100 percent.

Peck et al. (1946) produced the modified streptomycin, dihydrostreptomycin, and compared the activities of both antibiotics. They accomplished this by using the same quantity of each antibiotic and then comparing the activities in units per milligram. It was found that dihydrostreptomycin had an activity of 750 units per milligram, whereas streptomycin had an activity of 800 units per milligram. The antibiotics were further compared by attempting to obtain 50 percent protection of mice against one lethal dose. This comparison was accomplished by Dr. H. Robinson and Mr. O. Graessle of Merck Institute for Therapeutic Research (Peck et al., 1946). The results show that it required 85 units of streptomycin trichloride. A comparison of antibacterial activity in vitro was made by Bartz et al. (1946) in the same year; the activity was found to be almost the same for both antibiotics. For example, Bacillus subtilis required 1.0 microgram per milliliter of streptomycin, and 0.3 microgram per milliliter of dihydrostreptomycin. In 1947, Donavich and Rake (1947) studied some of the biological aspects of dihydrostreptomycin and compared them with those of streptomycin. With the ten organisms used in their antibacterial activity determinations, they found dihydrostreptomycin to be

slightly less active against all the organisms except Mycobacterium tuberculosis. Pratt and Dufrenoy (1949) concluded that streptomycin and dihydrostreptomycin have essentially the same antibacterial activity, since the examples cited revealed that the concentration of the two antibiotics differed by less than a microgram.

Aureomycin

After the discovery of streptomycin and its modification, dihydrostreptomycin, screening programs to discover new antibiotics were greatly intensified, with the result that Duggar (1948) discovered aureomycin. While studying many strains and species of microorganisms in his particular screening program, he discovered an actinomycete, Streptomyces aurefaciens, which synthesized aureomycin.

Aureomycin was described by Duggar (1948) and Baron (1950) as a crystallizable antibiotic which contained lemon-yellow rhomboid crystals. Chemically, aureomycin was a weak base that contained nitrogen and nonionic chlorine, as reported by Broschard et al. (1949), and readily formed the hydrochloride salt. Baron (1950) further revealed that it had a molecular

weight of 508, decomposed at 210 degrees centigrade, and had a solubility of 14 milligrams per milliliter of water at 25 degrees centigrade.

The stability of the antibiotic was investigated by Bliss and Chandler (1948) and Chandler and Bliss (1948), and also reported by Baron (1950). The results indicated that room temperature caused rapid deterioration of the antibiotic, as there was a 50-percent loss of activity in 24 hours, 60 percent in 18 hours at 37 degrees centigrade, and 75 percent in 4 hours at 56 degrees centigrade. Price, Randall, and Welch (1948) used various media at room temperature, but found that even in distilled water there was rapid deterioration. Paine et al. (1948) found aureomycin to be stable in solution at high concentration, 200 milligrams per milliliter at 4 degrees centigrade, for 2 weeks. Erlanson (1951) reported aureomycin stable for 3 months at 5 degrees centigrade at a concentration of 1,000 micrograms per milliliter. Paine et al. also found aureomycin to be most active and stable in an acid pH range, and much less active and stable in the alkaline range.

The mode of action of aureomycin was found to be bacteriostatic by Chandler and Bliss (1948), as the growth curves

of their experiments demonstrated a steady decline of growth of organisms for 24 hours, and then a steady increased growth thereafter. During the same year, Bryer et al. (1948) found aureomycin to be bactericidal in high concentrations. Lomis (1950) studied the mechanism of this action and postulated that aureomycin depressed phosphorylation without inhibiting respiration of the organism. Van Meter and Oleson (1951) studied the action of aureomycin on the respiration of normal rat-liver homogenates, and through their work and data, suggested that aureomycin could possibly block some part of the Krebs cycle.

The toxicity of aureomycin was investigated by the determination of the $L.D._{50}$ in mice for various routes of inoculations. The $L.D._{50}$ was 50 to 100 milligrams per kilogram for the intravenous route, and was 3,000 to 4,000 milligrams per kilogram for the subcutaneous route. The work was done by Harned et al. (1948), Bryer et al. (1948), and Goldin and Stern (1951).

The antibacterial spectrum of aureomycin was shown by Duggar (1948) to include both gram-positive and gram-negative organisms. This work was confirmed by the investigations of Paine, Collins, and Finland (1948), Bliss and Chandler (1948),

Price, Randall, and Welch (1948), Broschard et al. (1949), and Neter (1950). The activity of aureomycin against gram-positive organisms was shown to be of a lower order than that of penicillin by Bliss and Chandler (1948), and polymyxin was more active against gram-negative organisms than aureomycin. However, Paine, Collins, and Finland (1948) studied the effect of aureomycin on penicillin-resistant strains, streptomycin-resistant strains, and streptomycin-dependent strains, and indicated that the resistance developed by these strains to a particular antibiotic (penicillin or streptomycin) had no effect on the activity of aureomycin.

Included among the organisms Duggar used in his initial work were fifty species of Salmonella pullorum and Eberthella typhosa, which he indicated were readily susceptible to aureomycin. Paine, Collins, and Finland (1948) obtained sensitivities of many organisms in vitro, including S. choleraesuis. Their data showed S. choleraesuis to be susceptible to 6.3 micrograms per milliliter of aureomycin, whereas 250 micrograms per milliliter of streptomycin was required to obtain the end point. They also studied the effect of aureomycin on large 10^{-1} dilution and small 10^{-4} dilution of members of Staphylococcus

pyogenes, and found greatest activity of aureomycin on the small inoculum, which had displayed the more vigorous growth. Price et al. (1948) ran sensitivity tests in vitro on various gram-positive and gram-negative organisms, among which were twelve strains of salmonellae. One strain of S. typhimurium was inhibited by 1.56 micrograms of aureomycin per milliliter in 24 hours. Clinical studies of aureomycin on five typhoid-fever patients and three patients with salmonella infections were reported by Collins, Paine, and Finland (1948). Success was indicated in the cases of typhoid fever, as all patients recovered; however, two of the three salmonella-infected patients died from a severe bacteremia.

Neter and Gorzynski (1950) investigated the susceptibility of eight different strains of P. multocida to aureomycin in vitro. They used a large inoculum of bacteria containing one million to one hundred million organisms per milliliter, and a small inoculum containing one hundred to one thousand organisms per milliliter. The sensitivity range of the small inoculum of P. multocida was 0.01 to 0.3 microgram per milliliter, whereas the large inoculum required from 0.1 to 3 micrograms per milliliter for inhibition of growth. Little (1948) experimentally

infected one-day-old chicks with P. multocida, and then gave doses of aureomycin by the following routes: intra-abdominally, intramuscularly, and orally. Only the intra-abdominal route gave 100-percent protection, and this required 10 milligrams per kilogram weight of the chick. Neter, Gorzynski, and Cass (1951) infected mice intraperitoneally with a 10^{-4} dilution of P. multocida, and then gave aureomycin subcutaneously in doses ranging from 0.2 milligram to 2 milligrams to different groups of mice. They concluded from their data that aureomycin was effective in delaying death and lowering the fatality rate over a 15-day period. The clinical usefulness of aureomycin was investigated by Neter, DeKleine, and Egan (1951). The case reported was that of a 15-year-old boy who had been kicked in the face by a horse in 1944. Loss of the nasal bones required plastic repair with the implantation of preserved human cartilage in the summer of 1949. Inflamation and a purulent discharge from the nose instigated a bacteriological investigation, revealing a pure culture of P. multocida. Ten months later, treatment with aureomycin was begun and continued for 3 weeks, resulting in marked clinical improvement.

Chloromycetin (chloramphenical)

The soils in the vicinity of Caracas, Venezuela, contain a genus of actinomycetes (streptomyces) that is of great significance to the field of antibiotics. This genus is found in many other soils throughout the world; however, it was from a soil sample from this particular area that Dr. Paul R. Burkholder (Ehrlich et al., 1947) first isolated Streptomyces venezuelae. Agar streak cultures of this organism by Ehrlich et al. demonstrated its antibacterial activity upon Bacillus subtilis, Streptococcus pyogenes, Salmonella schottmuelleri, and various other organisms. Growing Streptomyces venezuelae in submerged aerated culture with various types of media, it was discovered that the organism produced chloromycetin, a new antibiotic. Since this initial discovery, a similar, if not identical, culture of this streptomyces species has been isolated from a compost pile in Illinois (Pratt and Dufrenoy, 1949). Parke, Davis and Company (Neter, 1950) has furthered the importance of chloromycetin by synthesizing it in its research laboratories. Chloramphenical, the laboratory-synthesized compound in question, is the only one of four possible isomers that possessed the same

biologic, chemical, and physical properties as the biosynthesized chloromycetin.

Crystallization of the antibiotic was accomplished by Ehrlich et al. (1947), while going through the procedures of purification and concentration of chloromycetin. The crystals are colorless needles of elongated plates. The crystallized antibiotic is a neutral compound that contains nitrogen and non-ionic chlorine within its structure. The name "chloromycetin" was originated because of the large amount of chlorine in the molecule. The solubility of the compound in water was found to be about 2.5 milligrams per milliliter at 25 degrees centigrade, but was freely soluble in methanol, ethanol, buranol, acetone, and propylene glycol.

The initial work on chloromycetin by Ehrlich et al. (1947) included studies performed to determine the stability of the antibiotic in solution. The results of the work indicated that chloromycetin remains stable for more than 24 hours at room temperature within the range of pH 2 to pH 9. When suspended in distilled water, boiling for 5 hours did not affect the antibiotic. The reports of Bartz (1948) and Baron (1950) confirmed this pH range at which chloromycetin was stable at room

temperature. Further work by Bartz (1948) revealed that at pH 10.82 the antibiotic was only 13-percent active. He also found that the antibiotic in distilled water remained stable for a month at 37 degrees centigrade. Erlanson (1951) stored chloromycetin at 5 degrees centigrade at a concentration of 1,000 micrograms per milliliter with no loss of activity. In the dry state, Pratt and Dufrenoy (1949) found the antibiotic to be thermostable for a longer period of time.

The type of action by chloromycetin was investigated by Seligmann and Wassermann (1949), both in vitro and in vivo. Bacteriocidal action was found to prevail in the in vitro work. Sensitivity tests were run on twenty-three salmonellae and the concentration of antibiotic required for growth supression of these organisms did not increase over a 24-hour incubation period. However, the course of the disease in treated animals following termination of treatment was almost identical with the course in control animals. Their conclusion was that chloromycetin reaction in animals was purely bacteriostatic. Erlanson's (1951) work confirmed the bacteriostatic action of this antibiotic. Smith et al. (1949) observed a possible clue to the bacteriostatic action of chloramphenical in their

investigations upon the correlation between the esterase activity and the growth of Escherichia coli. There was a close agreement in the final results, since a low concentration of the antibiotic has no effect on its growth or esterase activity. However, with increased concentration of the antibiotic, there was a decrease in the growth of the organism, and also a decrease in esterase activity. Finally, at a therapeutic concentration of chloramphenicol, there was neither growth of the organism nor esterase activity.

Toxic manifestations of chloromycetin were first investigated by Ehrlich et al. (1947); its $L.D._{50}$ in mice was determined. For a 20-gram mouse, the intravenous $L.D._{50}$ was 3.0 milligrams. The following year, Smith et al. (1948) studied specific toxic effects on small animals and found chloromycetin to be only slightly toxic. The $L.D._{50}$ in mice, for the intravenous route, was reported as 245 milligrams of antibiotic per kilogram weight of the mouse. Mice tolerated 1 gram per kilogram orally and 400 milligrams per kilogram subcutaneously. Baron (1950) determined the mouse $L.D._{50}$ for all the different routes of inoculation. Intravenously, the $L.D._{50}$ was 109.5 to 202.6 milligrams per kilogram; intraperitoneally it was 1,320

milligrams per kilogram; orally, 2,640 milligrams per kilogram; and subcutaneously, 100 milligrams per kilogram.

The antibacterial spectrum of chloromycetin (chloramphenical) was first uncovered by Ehrlich et al. (1947), who showed that the antibiotic was more active against gram-negative organisms than against acid-fast and gram-positive organisms. The gram-negative Salmonella schottmuelleri required 0.33 microgram of chloromycetin per milliliter of broth, while the gram-positive M. tuberculosis required 12.5 micrograms of antibiotic per milliliter of broth. Co-workers of Ehrlich, Smith et al. (1948), confirmed the specific activity of chloromycetin against gram-negative organisms by determining the activity of the antibiotic on many more organisms, both gram-negative and gram-positive. The activity of chloromycetin was also compared to penicillin and streptomycin, with the final results indicating that chloromycetin was more active against gram-negative organisms than either penicillin or streptomycin. McLean et al. (1949) did a very thorough study of the susceptibility of various bacteria (including gram-negative and gram-positive) and viruses. Although many salmonellae were assayed, only three strains of S. typhimurium were included. The inoculum was a one to ten

million dilution, and was found to be inhibited between 2.5 micrograms per milliliter and 5.0 micrograms per milliliter. Specific studies on the effect of chloromycetin on salmonellae were accomplished by Seligmann and Wassermann (1949). Twenty-three salmonellae were studied, including S. typhimurium and S. choleraesuis. The sensitivities indicated that nineteen salmonellae were susceptible to 2 micrograms per milliliter, whereas the other four were inhibited by 4 micrograms per milliliter. In the in vivo experiments which followed, mice were inoculated either orally or intraperitoneally with 0.1 cubic centimeter of an 18-hour broth culture of S. typhimurium, 1908, after which they were treated either orally or subcutaneously with 2,000 to 2,500 micrograms of chloromycetin each day. At the end of the treatment, the eighth day, the mice began to die at a more rapid rate, and at the end of 21 days only a very few remained alive. Seligmann and Wassermann concluded that death could be delayed, but not prevented. Ross et al. (1949) reported the effects of chloromycetin in five clinical cases of salmonellosis. Two cases were initially treated with streptomycin and aureomycin for 4 months with no appreciable results. However, treatment with chloromycetin rendered the stool free

of salmonella without a later reappearance of the organism. The third case had a positive test for salmonella in the blood. This was rapidly cleared with the treatment of chloromycetin. The fourth case showed persistently positive stool samples, and, at the time of publication, had not been affected by the antibiotic. The last patient had a severe bacteriemia and died 12 hours after the first treatment. Successful treatment of clinical cases of typhoid fever with chloromycetin was reported by Woodward et al. (1948). For the first 24 hours, 40 to 100 gamma per cubic centimeter was given orally. The next 72 hours, 20 gamma per cubic centimeter was administered orally. Relapses were noticed in only two out of the ten cases. These two cases were further treated with the same concentration of chloromycetin as initially, since no increase in resistance of the organism was indicated in the in vitro assay. The patients showed steady progress following the second treatment, and recovered in the same manner as the other eight. A later publication by Woodward et al. (1950) included twenty-two cases of typhoid fever, which received an average chloromycetin total of 23.4 grams over an average period of 9.2 days. Four relapses were reported. However, with further treatment these

cases recovered rapidly. Similar studies by Neter (1950) brought forth the spectacular effects of chloromycetin in typhoid fever in four cases at Buffalo Children's Hospital. Salmonella or paratyphoid infections were found to require a much higher dosage of antibiotic than typhoid-fever infection.

Neter and Gorzynski (1950) determined the minimal growth inhibitory concentrations of eight different antibiotics, among which chloromycetin was included, on eight different strains of P. multocida. The range of inhibitory concentrations for all eight strains of the organism was 0.01 microgram per milliliter to 10 micrograms per milliliter, although it must be pointed out that only one strain required 10 micrograms per milliliter, whereas the other seven strains were inhibited by 1.0 microgram per milliliter. Two assays were run on each strain; one in which one million to one hundred million organisms per milliliter were inoculated, and the other in which one hundred to one thousand organisms were inoculated. The smaller number of bacteria required much less chloromycetin. The sensitivities of three strains of Pasteurella avicida were determined by McLean et al. (1949) in their broad study of various organisms. An inoculum of one to two thousand organisms was

found to be effectively inhibited by 0.5 microgram per milliliter to 0.25 microgram per milliliter. These experiments were further followed by Gorzynski and Neter (1951), in which the efficacy of chloromycetin in vivo was determined. The mice were infected with 10^4 to 5 x 10^4 MLD of P. multocida (strain No. 200) and series of experiments were carried out in which the antibiotic was administered in single and divided doses. The data show a total concentration of 2.0 to 2.4 milligrams of chloromycetin to be more effective when given in multiple doses, while a total concentration of 0.125 to 0.3 milligram was not effective in either single or divided doses.

METHODS AND MATERIALS

Sensitivity Determinations

The fourteen strains of S. typhimurium, thirteen strains of S. choleraesuis, and sixteen strains of P. multocida used in this experiment were obtained from various sources. The strains and numbers of the organisms are as follows:

S. typhimurium:

SF-366, SF-361, SF-360, SF-368, 13-4, 13-5, 13-6, 13-7, 13-8, 4-7d, 73-6, O.S.U.-1a, N.Y.S.-3a, and L-169.

S. choleraesuis:

15-1, 15-2, 15-3, 15-6, 15-9, 15-10, 15-12, 14-8, 8-A1, 19-8, O.S.U.-1, N.Y.S.-3, and L-2.

P. multocida:

49-50-800, 50-51-939, 651, 49-50-812, 50-51-111, 49-50-638, 1184, 49-50-829, 49-50-618, 49-50-681, 49-50-557, 50-51-110, O.S.U.-1b, N.Y.S.-3b, and L-169.

A stock culture of each organism was maintained in a semisolid medium that required transferring the organism every 3 to 4 weeks. The salmonellae were kept viable in the following medium at 4 degrees centigrade:

Peptone	-	1 gram
Sodium chloride	-	0.5 gram
Agar	-	0.3 gram
Distilled water	-	100 milliliters

Pasteurellae required a different type stock medium at 25 degrees centigrade, which was made up as follows:

Brain heart infusion (dehydrated)	-	3.7 grams
Agar	-	0.3 gram
Distilled water	-	100 milliliters

Sensitivities of the above-mentioned organisms were determined by use of the tube-dilution method. Aureomycin hydrochloride,[1] chloromycetin,[2] and dihydrostreptomycin sulfate[3] were

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[1] Aureomycin hydrochloride was supplied by Lederle Laboratories.

[2] Chloromycetin was supplied by Merck Laboratories.

[3] Dihydrostreptomycin sulfate was supplied by E. R. Squibb and Sons.

the three antibiotics employed in both the in vitro and in vivo experiments. The procedure followed was the serial-dilution method, in which twofold dilutions were utilized. This method was modified to resemble closely the method used by Dorbush (1949) for the determination of sensitivities of organisms to aureomycin. Joslyn et al. (1949) described a similar method for the determination of sensitivities of organisms to chloromycetin. The method specified the use of 0.5 milliliter of diluted antibiotic added to 1.5 milliliters of a one to one hundred dilution of an 18-hour-old organism in Wasserman tubes, and incubation for 4 hours. The usual serial dilution was made with 9 milliliters of a seeded broth, plus 1 milliliter of diluted antibiotic and was incubated for 16 to 24 hours (Waksman, 1947).

Preparation of the stock solution of chloromycetin was accomplished by dissolving the exact amount of antibiotic in sterile distilled water in concentrations of 200 milligrams per 100 milliliters of solution. The desired concentrations of aureomycin hydrochloride and dihydrostreptomycin sulfate were obtained by the addition of an exact amount of sterile distilled water to the dry powder contained within a sterile vial. Subsequent dilutions in distilled water yielded the stock solution containing 200 milligrams per 100 milliliters of solution.

All stock solutions not used during the day of preparation were put in sterile stoppered flasks and placed in the freezer, which maintained a temperature below 10 degrees centigrade. Stock solutions held for more than 1 month in the frozen state were not used.

Desired concentrations of the antibiotics were obtained by diluting the stock solutions in sterile distilled water to provide an initial concentration of 200 micrograms per milliliter of solution. This solution was then serially diluted by halves with broth, through an additional eight dilutions. This was so calculated that 0.5 milliliter of the diluted solutions, when added to 1.5 milliliters of the seeded broth, gave the required concentrations of antibiotic.

Brain heart infusion broth was employed in the sensitivity determination of aureomycin hydrochloride and chloromycetin. Mycin assay broth was used solely for dihydrostreptomycin sulfate.

The sensitivity of each organism to each antibiotic was tested in duplicate in all cases. Three controls were employed with every assay of antibiotic. The first control consisted of adding 0.5 milliliter of each concentration of antibiotic to 1.5

milliliters of uninoculated broth. A second control consisted of one tube, which contained 2.0 milliliters of inoculated broth. A third control also consisted of one tube that contained only unseeded broth.

A culture known to be sensitive was also included in each test for comparison. Bacillus cereus No. 5, known to be sensitive, was replaced by S. typhimurium strain 13-4, which was found to be sensitive to all three antibiotics.

The tubes were incubated at 37 degrees centigrade, examined for visible turbidity at the end of a 4-hour period, and again at the end of 18 hours. The lowest concentrations of each antibiotic inhibiting growth at the end of 4 hours and 18 hours were taken as the end-point readings. Any degree of turbidity contained in a tube was considered positive, and the end point was the last clear tube, which was usually easily distinguished.

The subinhibitory concentration of the antibiotic used in all following experiments was that which did not totally supress the growth of the organism.

In Vitro Experiments

Media. The media utilized were produced by the Difco Laboratories. Preparation and sterilization of each individual medium was accomplished according to directions.

The media used were:

1. Liquids: glucose, maltose, xylose, sorbital, lactose, arabinose, dextrose, mannitol, motility medium, indol medium, urea medium, brain heart infusion broth, and mycin assay broth.
2. Solids: MacConkey's agar, S. S. agar, blood agar, gelatin, and Kligler's iron agar.

Ten milliliters of rabbit blood were added to 100 milliliters of agar base.

All the liquid media, except brain heart infusion broth and mycin assay broth, were dispensed in sterile Wasserman tubes and then sterilized according to specification of the Difco Manual. Sterile Petri plates were used for dispensing the sterilized solid media, except Kligler's iron agar. Brain heart infusion broth, mycin assay broth, and Kligler's iron agar were dispensed in regular-sized sterile test tubes, and then sterilized.

Sample tubes and Petri dishes containing the various media were incubated at 37 degrees centigrade for 24 hours to check for sterility.

Storage of the media was not required in most instances, since it was most desirable to use freshly prepared media. However, when the media were not used immediately, they were placed in a refrigerator, at a temperature of 4 degrees centigrade. Media stored at 4 degrees centigrade for 2 weeks or longer were discarded.

Inoculation of media. Each organism was transferred from its stock culture medium to brain heart infusion broth and mycin assay broth, and then incubated for 4 hours and 18 hours at 37 degrees centigrade. At the end of each period, a loopful of the inoculated broths was transferred to specific media, depending upon the organism.

Salmonella cultures were inoculated into the following media:

1. Glucose, maltose, xylose, sorbital, lactose, arabinose, dextrose, mannitol, motility medium, indol medium, and urea medium.

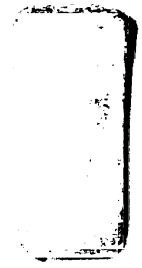
2. MacConkey's agar, S. S. agar, gelatin, and Kligler's iron agar.

Pasteurellae were inoculated into the following media:

1. Glucose, maltose, xylose, sorbital, lactose, arabinose, dextrose, mannitol, motility medium, indol medium, and urea medium.
2. Gelatin tubes and blood-agar plates.

All inoculated media were incubated at 37 degrees centigrade for 24 hours. The various reactions of the organisms on the media were recorded.

The next phase required the organisms to be treated with a subinhibitory quantity of antibiotic before being inoculated in the above media, and then run in parallel with one of the sensitivity determinations. The sensitivity determinations were run as previously discussed. Aureomycin hydrochloride, dihydrostreptomycin sulfate, and chloromycetin were used. At the end of 4 hours, a loopful of the last tube containing growth was inoculated in the various media, and then further incubated at 37 degrees centigrade for 24 to 48 hours. This procedure was also used at the end of 18 hours. After 24 to 48 hours of



further incubation, the reactions obtained upon the media were tabulated.

Morphologic observations. Slides were made of normal or untreated organisms, and also of organisms treated with subinhibitory quantities of aureomycin hydrochloride, dihydrostreptomycin, and chloromycetin.

The organisms were transferred from the stock cultures into brain heart infusion broth and mycin assay broth, and incubated at 37 degrees centigrade for 4 hours and 18 hours. At the termination of these two periods, each tube was agitated, and a loopful of each culture was placed on a clean slide. The material was allowed to air dry, and was then warmed over the flame of a Bunson burner. The slide was stained by the Gram stain. For the pasteurellae, an additional slide was prepared and then stained with methylene blue. This procedure was for the untreated organisms.

At the time of a sensitivity determination, slides, stained as described above, were made of the organisms which were treated individually with the antibiotic. Microscopic examination of each set of slides followed the staining procedure.

In Vivo Experiments

Mice were selected for the in vivo experiments. The animals were of mixed breeds and came from different colonies. The weight of each animal used was approximately 20 grams.

Number 10 tin cans were used to house the mice used in the experiment. Inasmuch as all infected mice were kept in the same room, these cans provided a means of isolation. Each can was cleaned every other day to reduce the residual bacteria contained in the fecal material. Upon completion of an experiment, the can and its accessories (watering bottle and screen top) were autoclaved and then thoroughly washed in a detergent before being used in further experiments.

The organisms used in the in vivo experiments were S. typhimurium strain SF-368 and strain 13-5; S. choleraesuis strain 15-9 and strain 15-6, and P. multocida strain 49-50-638. These organisms were subjected to in vitro sensitivity tests, described in detail in the section on "Sensitivity Determinations." These tests served a dual purpose in that they were a check on the previous sensitivity determinations, and the organism that was partially inhibited by the antibiotic served as the inoculum for the mice.

S. typhimurium strain SF-368 was employed in the experiment with aureomycin, and also with chloromycetin. At 4 hours, 1.56 micrograms per milliliter of aureomycin were required, while 6.25 micrograms per milliliter were necessary at 18 hours for partial inhibition. The subinhibitory concentration of chloromycetin for S. typhimurium strain SF-368 was a constant 1.56 micrograms per milliliter for 4 hours and 18 hours. S. typhimurium strain 13-5 was subjected to dihydrostrptomycin. The subinhibitory concentrations for 4 hours and 18 hours were, respectively, 3.13 and 25.0 micrograms per milliliter.

Chloromycetin and dihydrostreptomycin were used for S. choleraesuis strain 15-6 experiments. The concentration of chloromycetin required for subinhibition was 1.56 micrograms per milliliter for the 4-hour and 18-hour cultures. The 4-hour incubation of S. choleraesuis strain 15-6 required 0.78 microgram per milliliter of dihydrostreptomycin, whereas the organism required 3.13 micrograms per milliliter at 18 hours for subinhibition. S. choleraesuis strain 15-9 was treated with a subinhibitory amount of aureomycin which was 1.56 micrograms per

milliliter for 4 hours, and 6.25 micrograms per milliliter for 18 hours.

The subinhibitory concentration of aureomycin required for P. multocida strain 49-50-638 was 0.39 microgram per milliliter for both the 4- and 18-hour cultures. P. multocida strain 49-50-638 was also subjected to subinhibitory amounts of dihydrostreptomycin. These concentrations were 0.78 and 1.56 micrograms per milliliter, respectively, at 4 and 18 hours of incubation. P. multocida strain 49-50-638 required 0.78 microgram of chloromycetin per milliliter for subinhibition at both 4- and 18-hour incubations.

The subinhibitory concentration of each antibiotic for each organism for the 4- and 18-hour periods was inoculated into nine series of mice. No further antibiotic treatment of the organisms followed the initial exposure to in vitro subinhibitory concentrations. There were three mice in each series in which chloromycetin and dihydrostreptomycin were employed, whereas six mice were used in each series subjected to aureomycin treatment.

Inoculations were made with sterile 1.0 cubic centimeter Tuberculin syringes and 27-gauge needles. The inoculum was

thoroughly agitated before being drawn into the syringe. The mice were injected with 0.1 cubic centimeter of the inoculum intraperitoneally.

The inoculated animals were observed twice a day throughout the entire time of the experiment.

Control experiments were run in conjunction with the above-mentioned experiments. Two series of mice were inoculated with brain heart infusion broth and mycin assay broth. A control series of mice was run on a broth suspension of each organism, diluted one to one hundred, and incubated for 4 hours and 18 hours. A third control group of mice was inoculated with 1.0 milligram per milliliter of each antibiotic, which served as a toxicity control. All control mice were inoculated intraperitoneally with 0.1 cubic centimeter of specified solutions or suspensions (see Tables IV and V).

All mice which survived, following an injection with an organism subjected to a subinhibitory concentration of one of the antibiotics, were challenged with a normal culture of the same organism to determine if some degree of immunity had developed.

One-tenth cubic centimeter of an untreated, diluted, 18-hour broth suspension of a specified strain of the organism was inoculated intraperitoneally with a Tuberculin syringe using a 27-gauge needle. All syringes and needles were sterilized before use. The quantity used in the case of each organism was found to be a lethal dose for normal mice.

A brain heart infusion broth suspension of S. choleraesuis strain 15-6 was incubated for 18 hours at 37 degrees centigrade. This suspension was diluted so that the inoculum used was 0.1 cubic centimeter of a 10^{-4} dilution of the original 18-hour culture. One-tenth cubic centimeter of this 10^{-4} dilution of S. choleraesuis was found to kill normal mice within six days. Mice that had recovered from an initial injection of S. choleraesuis strain 15-6 which had been treated with a subinhibitory concentration of chloromycetin for 4 hours and 18 hours were inoculated with 0.1 cubic centimeter of a 10^{-4} dilution. Also, mice recovering from infection with the organism treated with dihydrostreptomycin for 4 hours and 18 hours were challenged.

A similar procedure was followed with S. typhimurium strain 13-5. The organism was inoculated into brain heart infusion broth and incubated at 37 degrees centigrade for 18 hours.

Mice recovering from injection with S. typhimurium strain 13-5 which had been subjected to minimal concentrations of dihydrostreptomycin for 4 hours or 18 hours were inoculated with 0.1 cubic centimeter of a 10^{-4} dilution of the untreated 18-hour broth suspension of the organism.

A brain heart infusion broth suspension was made of P. multocida strain 49-50-638 and incubated for 18 hours at 37 degrees centigrade. This suspension was diluted so that the inoculum was a 10^{-3} dilution of the original. Mice recovering from a P. multocida strain 49-50-638 suspension which had been treated with a subinhibitory concentration of aureomycin for 4 hours or 18 hours were inoculated with the 0.1 cubic centimeter of a 10^{-3} dilution of the normal culture. Mice recovering from infection with P. multocida strain 49-50-638 which had been subjected to a subinhibitory concentration of chloromycetin for 4 hours or 18 hours were also inoculated with the 10^{-3} dilution of the normal culture, as were the mice recovering from P. multocida strain 49-50-638 which had been treated with the minimal concentration of dihydrostreptomycin for 4 hours and 18 hours.

Care was exercised in removing dead mice from the cans containing the challenged mice, and also in removing fecal material.

RESULTS AND DISCUSSION

Sensitivity Determinations

The validity of determinations of sensitive microorganisms to antibiotics is based upon the ability to duplicate the initial results. This applies to the tube-dilution method, or any other method by which the sensitivity of an organism to an antibiotic is determined.

Certain physical factors must be kept constant through several assays to eliminate the variables which could produce results entirely different from those of the initial determination.

Most significant is the sensitivity of an organism to the antibiotic. Trials with test organisms must be run simultaneously with tests on organisms of unknown sensitivities. The end point of the test organism and organism to be tested is compared, and in this way the degree of sensitivity of the organism tested is determined. Also, a test organism, whose sensitivity is known, can be utilized in determining the

concentration of antibiotic solutions, the approximate concentrations of which are not known.

Secondly, the organism must be of the same strain and growth phase in each and every assay. Pratt and Dufrenoy (1949) indicated that different strains of an organism that produced pulmonary tuberculosis showed a thirtyfold difference in their sensitivities to streptomycin. The growth phase of the organism is important because some antibiotics are more active on actively metabolizing organisms than on less active, older organisms.

Thirdly, it is important that the incubation temperatures, as well as the length of incubation time, be identical in every assay. If the incubation temperatures are increased, the incubation period should be decreased, as thermolable antibiotics are significantly affected by the temperature increase over a specific period of time. This was found to be true of aureomycin. This results in a variable end point, as the activity of the antibiotic is destroyed or decreased.

A constant number of organisms per assay is essential for some antibiotics. Increased numbers of organisms result in a greater concentration of the antibiotic to produce inhibition.

Lastly, the medium in which the assay is performed should remain constant. Waksman (1947) indicated that streptomycin could be inactivated or antagonized when certain sugars, cysteine, or ketone reagents were added to medium used in assaying the antibiotic. This resulted in a deviation of the antibacterial activity of streptomycin.

The consistency of the pH of the medium is also essential. Streptomycin is most effective in an acid medium. Chloromycetin is effective in either an acid or alkaline medium, since its effectiveness lies in the pH range from 2 to 9.

There are uncontrollable factors that cause variation in sensitivity tests, though the factors mentioned have been eliminated. The human element is one uncontrollable factor, influencing accuracy of laboratory procedures and interpretation of results. Another uncontrollable factor is the lack of constant homogeneity within a bacterial population. Different degrees of resistance or possibly antibiotic dependence of individual organisms within a bacterial population could cause a fluctuation of the end point. The growth phase of the organism at the time of the sensitivity determination would also cause a fluctuation of the end point. It is logical to assume that all

bacteria within a population are not at exactly the same stage of growth at any specific time. Some bacteria are in the logarithmic growth phase, while others are in a lag phase. Generally, the organisms in the lag phase are least susceptible, whereas those in the logarithmic phase are most susceptible, depending, of course, upon the antibiotic used.

Waksman (1948) was of the opinion that the antibacterial action of each antibiotic was distinct from that of all other antibiotics. The difference was determined by the site and type of action upon the organism. Importance was attached to the variability of reactions on various substrates, serum, glucose, or salt.

Jackson et al. (1950) concluded from their results that the enteric organisms as a group vary greatly in their sensitivity to different antibiotics. However, the authors indicated that the results obtained were valid only for the conditions under which those tests were maintained, since there were many factors which could influence the sensitivity determinations, and those factors varied from one antibiotic to another.

Maintaining all factors as constant as possible, the sensitivities of all organisms were determined and the results were tabulated (see Tables I, II, and III).

The susceptibility of the organisms generally varied from antibiotic to antibiotic, and also among species of the bacteria within the group. There was also a noticeable change of susceptibility of the organisms to the antibiotics in the 4-hour and 18-hour results, with one exception. Chloromycetin maintained steady end points for 18 hours' incubation, as compared to the 4-hour sensitivities. All salmonellae and pasteurellae tested were readily susceptible to chloromycetin.

The ability of organisms to develop resistance to chloromycetin was indicated by Coffey et al. (1950). After twenty transfers, the organisms had gradually developed an increased resistance to the antibiotic in vitro. Woodward et al. (1948) noted lack of development of resistance of Salmonella typhosa in vivo. Patients treated with chloromycetin had relapses, and the organism was reisolated from the patients to check the sensitivity of this organism. The results of the sensitivity test were identical with the initial tests, indicating the lack of developed resistance.

TABLE I

RESULTS OF IN VITRO TESTS FOR SENSITIVITY OF FOURTEEN STRAINS OF SALMONELLA TYPHIMURIUM TO AUREOMYCIN, CHLOROMYCETIN, AND DIHYDROSTREPTOMYCIN[1]

Culture Number	Dihydrostreptomycin		Aureomycin		Chloromycetin	
	4 hrs.[2]	18 hrs.	4 hrs.	18 hrs.	4 hrs.	18 hrs.
SF-360	6.25	50.0	6.25	25.0	6.25	6.25
SF-361	12.5	50.0	12.5	25.0	12.5	12.5
SF-366	6.25	50.0	6.25	25.0	6.25	6.25
SF-368	6.25	50.0	3.13	12.5	3.13	3.13
13-5	6.25	25.0	12.5	25.0	6.25	6.25
13-6	6.25	50.0	3.13	12.5	6.25	6.25
13-7	3.13	50.0	3.13	25.0	6.25	6.25
13-8b	12.5	50.0	12.5	12.5	3.13	3.13
4-7d	6.25	50.0	1.56	6.25	3.13	3.13
73-6	6.25	50.0	6,25	12.5	6.25	6.25
L-88	12.5	50.0	1.56	12.5	6.25	6.25
OSU-1a	6.25	50.0	12.5	25.0	12.5	12.5
13-4[3]	1.56	3.13	1.56	3.13	1.56	1.56
NYS-3a	25.0	50.0	1.56	12.5	6.25	6.25

[1] Micrograms per milliliter inhibiting growth of organisms.

[2] Hours of exposure to antibiotic.

[3] Assay organism.

TABLE II

RESULTS OF IN VITRO TESTS FOR SENSITIVITY OF THIRTEEN STRAINS OF SALMONELLA CHOLERAESUIS TO AUREOMYCIN, CHLOROMYCETIN, AND DIHYDROSTREPTOMYCIN[1]

Culture Number	Dihydrostreptomycin		Aureomycin		Chloromycetin	
	4 hrs.[2]	18 hrs.	4 hrs.	18 hrs.	4 hrs.	18 hrs.
14-8	25.0	25.0	12.5	25.0	12.5	12.5
15-1	25.0	50.0	3.13	25.0	12.5	12.5
15-2	6.25	25.0	12.5	25.0	6.25	6.25
15-3	12.5	50.0	6.25	12.5	3.13	3.13
15-6	1.56	6.25	12.5	25.0	3.13	3.13
15-9	25.0	50.0	3.13	6.25	12.5	12.5
15-10	25.0	50.0	12.5	12.5	12.5	12.5
15-12	25.0	50.0	6.25	25.0	12.5	12.5
19-8	25.0	50.0	12.5	50.0	6.25	6.25
8A-1	0.19	0.78	1.56	3.13	12.5	12.5
OSU-1	6.25	50.0	12.5	12.5	6.25	6.25
NYS-3	25.0	50.0	12.5	25.0	12.5	12.5
L-2	12.5	50.0	6.25	12.5	6.25	6.25

[1] Micrograms per milliliter inhibiting growth of organisms.

[2] Hours of exposure to antibiotic.

TABLE III

RESULTS OF IN VITRO TESTS FOR SENSITIVITY OF SIXTEEN STRAINS OF PASTEURELLA MULTOCIDA TO AUREOMYCIN, CHLOROMYCETIN, AND DIHYDROSTREPTOMYCIN[1]

Culture Number	Dihydrostreptomycin		Aureomycin		Chloromycetin	
	4 hrs.[2]	18 hrs.	4 hrs.	18 hrs.	4 hrs.	18 hrs.
5-11a	1.56	6.25	0.19	0.39	0.39	0.39
5-11b	3.13	6.25	0.78	1.56	0.78	0.78
49-50-800	1.56	3.13	0.78	1.56	3.13	3.13
50-51-110	3.13	6.25	0.78	1.56	0.78	0.78
50-51-111	3.13	6.25	3.13	3.13	1.56	1.56
651	1.56	3.13	0.39	0.78	0.39	0.39
49-50-812	3.13	3.13	0.39	0.78	0.78	0.78
49-50-638	1.56	3.13	0.78	0.78	1.56	1.56
1184	0.39	50.0	0.39	3.13	6.25	6.25
49-50-829	3.13	6.25	3.13	3.13	3.13	3.13
49-50-618	1.56	6.25	0.78	0.78	1.56	1.56
49-50-681	1.56	3.13	0.19	0.39	0.39	0.39
49-50-557	0.78	3.13	0.39	0.78	0.39	0.39
OSU-1b	6.25	50.0	6.25	12.5	0.78	0.78
NYS-3b	0.19	0.78	0.39	0.78	0.39	0.39
L-169	50.0	50.0	0.39	3.13	6.25	6.25

[1] Micrograms per milliliter inhibiting growth of organisms.

[2] Hours of exposure to antibiotic.

Similar effectiveness was noted for aureomycin against S. choleraesuis and S. typhimurium at the 4-hour incubation. However, there was a decided variation in the sensitivities of the salmonellae at the end of 18 hours' incubation. There was a one- to twofold difference between the time periods needed to sensitize most of the organisms. P. multocida was very susceptible to aureomycin at the end of 4 hours of incubation, and showed less than a onefold average variation after 18 hours' incubation. These fluctuating end points are quite conceivable when one considers the instability of aureomycin in solution at room temperature. Bliss and Chandler (1948) and Price et al. (1948) found that low concentrations of aureomycin deteriorated rapidly even in distilled water at 25 degrees centigrade. Chandler and Bliss (1948) studied growth curves of bacteria subjected to low concentrations of aureomycin and found the curves declined rapidly for 24 hours. After this period of time, the growth steadily increased.

Dihydrostreptomycin sulfate is less effective, generally, against S. choleraesuis than either chloromycetin or aureomycin at both 4- and 18-hour incubations. However, dihydrostreptomycin was as effective as chloromycetin and aureomycin against S.

typhimurium at the 4-hour incubation. The 18-hour incubation indicated a great fluctuation of the end point, inasmuch as there was an average threefold difference from the 4-hour results. Dihydrostreptomycin was more effective against P. multocida strains than against the salmonella strains. At 4 hours' incubation, all the pasteurella strains were susceptible, except strain L-169. This organism appeared to be a highly resistant strain, as 50.0 micrograms per milliliter of dihydrostreptomycin did not suppress its growth even slightly. The growth of three strains of P. multocida, L-169, O.S.U.-1b, and 1184, similarly was not suppressed by 50.0 micrograms per milliliter after 18 hours of incubation. Sensitivity tests on the other pasteurellae indicated an average onefold variation between the two time intervals.

The variation of the sensitivities of the organism at 18 hours was due to the development of a streptomycin-resistant variant within the bacterial population, and not to deterioration of the antibiotic, for it had been found to be relatively stable. Miller (1948) stated that susceptible strains of bacteria easily developed resistance to streptomycin, noting that they tolerated up to 50,000 micrograms per milliliter when transferred twice

to streptomycin medium. He further revealed that bacterial mutation initiated the development of these variant strains, which consisted of streptomycin-resistant and streptomycin-dependent organisms. The organism used in this experiment appeared to be a resistant strain, as no organisms required streptomycin to remain viable, which is an essential criterion for the streptomycin-dependent strains.

Morphology

All antibiotic-treated organisms, stained with Gram and methylene-blue stains, were observed under a binocular microscope and were found to be morphologically the same as the untreated organisms. Miller (1948) and Paine and Finland (1948) observed no morphological variation in organisms that were either resistant to or dependent upon streptomycin.

Biochemical Reactions

The results obtained for both periods of incubation with the normal organisms and organisms subjected to the antibiotics were identical. In the cases of aureomycin and chloromycetin subinhibitory concentrations, the antibiotic evidently was not in

sufficient amounts to inhibit all of the organisms present. This probability was suggested by the work of various workers showing the difficulty in producing an aureomycin- or chloromycetin-resistant organism.

However, dihydrostreptomycin is one of the antibiotics to which organisms can easily develop resistance if not in sufficient quantities to be bactericidal. Assuming that the organisms have developed resistance to a sublethal dose of the antibiotic, it is easy to understand why the biochemical reactions are the same as those of untreated organisms. Miller (1948) and Paine and Finland (1948) studied the effects of streptomycin-dependent and streptomycin-resistant strains on the various biochemical reactions. Both papers concluded that dependent and resistant strains reacted biochemically in a manner identical to that of normal organisms. Berkman et al. (1948) stated that streptomycin does not interfere with the carbohydrate metabolism of dependent or resistant strains, even though the resistant strains adsorb streptomycin onto their cell surface, as do the susceptible strains.

In Vivo Results

Thus far, it has been observed that organisms subjected in vitro to a subinhibitory concentration of an antibiotic showed no observable effect morphologically or metabolically. Observation has shown suppression without inhibition of growth. In vivo investigations were carried out in an attempt to find out if antibiotic-affected organisms were more susceptible to the host's defenses than normal untreated organisms.

Organisms that were found to be highly sensitive to the antibiotic were selected for the experiment. S. typhimurium strain SF-368 was utilized for chloromycetin and aureomycin experiments because it was highly sensitive and was found to be pathogenic for mice. One-tenth of the culture diluted 10^{-4}, injected intraperitoneally, killed the mice in six days. An undiluted culture (0.1 cc) was also inoculated intraperitoneally into mice. All mice succumbed within a 36-hour period. S. typhimurium strain SF-368 was sensitive to dihydrostreptomycin after 4 hours' incubation, but after 18 hours' incubation the culture had become resistant to 50 microgramps per milliliter. Therefore, S. typhimurium strain 13-5 was selected for the tests with

dihydrostreptomycin, for the organism was more susceptible at the termination of the 18-hour period. This organism, undiluted, was found to kill mice in 36 hours. All animals were injected intraperitoneally with 0.1 cubic centimeter of the culture.

S. choleraesuis was selected for this part of the study because it is pathogenic for mice. S. choleraesuis strain 15-6 was selected for the study on chloromycetin and dihydrostreptomycin, while S. choleraesuis strain 15-9 was found to be suitable for tests with aureomycin. These organisms, undiluted, killed inoculated mice in 84 hours (see Table IV).

One strain of pasteurellae was used for the experiments with all three antibiotics. P. multocida strain 49-50-638 was sensitive to all the antibiotics and killed control mice in 24 hours.

Chloromycetin. Chloromycetin, in subinhibitory concentrations, was 100 percent effective against S. choleraesuis strain 15-6 and P. multocida strain 49-50-638 for both the 4-hour and 18-hour periods (see Table V). However, against S. typhimurium strain SF-368 chloromycetin only seemed to delay death of the mice. The first death occurred in the 18-hour series at the

TABLE IV

BROTH, ORGANISM, AND ANTIBIOTIC CONTROLS

No. of Mice	Organism	Culture Number	Vehicle	Antibiotic
3	S. typhimurium	SF-368	B.H.I.*	
3	S. typhimurium	SF-368	B.H.I.	
3	S. typhimurium	13-5	B.H.I.	
3	S. typhimurium	13-5	B.H.I.	
3	S. choleraesuis	15-6	B.H.I.	
3	S. choleraesuis	15-6	B.H.I.	
3	S. choleraesuis	15-9	B.H.I.	
3	S. choleraesuis	15-9	B.H.I.	
3	P. multocida	49-50-638	B.H.I.	
3	P. multocida	49-50-638	B.H.I.	
3			B.H.I.	
3			B.H.I.	
3			M.A.B.**	
3			M.A.B.	
3			B.H.I.	Chloromycetin
3			M.A.B.	Dihydro-streptomycin
3			B.H.I.	Aureomycin

* Brain Heart Infusion Broth.
** Mycin Assay Broth.

TABLE IV (Continued)

Hours Incubation In Vitro	Concentration of Antibiotic (ug/ml)	No. Injected / No. Survived	Percent Survived
4		3 / 0	0
18		3 / 0	0
4		3 / 0	0
18		3 / 0	0
4		3 / 0	0
18		3 / 0	0
4		3 / 0	0
18		3 / 0	0
4		3 / 0	0
18		3 / 0	0
4		3 / 3	100
18		3 / 3	100
4		3 / 3	100
18		3 / 3	100
	100	3 / 3	100
	100	3 / 3	100
	100	3 / 3	100

TABLE V

EFFECT OF SUBINHIBITORY CONCENTRATIONS OF DIHYDROSTREPTOMYCIN SULFATE, AUREOMYCIN, HYDROCHLORIDE, AND CHLOROMYCETIN UPON *SALMONELLA TYPHIMURIUM*, *SALMONELLA CHOLERAESUIS*, AND *PASTEURELLA MULTOCIDA* INOCULATED INTO MICE

No. of Mice	Organism	Culture Number	Antibiotic
4	*S. typhimurium*	SF-368	Chloromycetin
4	*S. typhimurium*	SF-368	Chloromycetin
4	*S. choleraesuis*	15-6	Chloromycetin
4	*S. choleraesuis*	15-6	Chloromycetin
4	*P. multocida*	49-50-638	Chloromycetin
4	*P. multocida*	49-50-638	Chloromycetin
4	*S. typhimurium*	13-5	Dihydrostreptomycin
4	*S. typhimurium*	13-5	Dihydrostreptomycin
4	*S. choleraesuis*	15-6	Dihydrostreptomycin
4	*S. choleraesuis*	15-6	Dihydrostreptomycin
4	*P. multocida*	49-50-638	Dihydrostreptomycin
4	*P. multocida*	49-50-638	Dihydrostreptomycin
6	*S. typhimurium*	SF-368	Aureomycin
6	*S. typhimurium*	SF-368	Aureomycin
6	*S. choleraesuis*	15-9	Aureomycin
6	*S. choleraesuis*	15-9	Aureomycin
6	*P. multocida*	49-50-638	Aureomycin
6	*P. multocida*	49-50-638	Aureomycin

TABLE V (Continued)

Hours Incubation In Vitro	Subinhibitory Concentration (ug/ml)	No. Injected / No. Survived	Percent Survived
4	1.56	4 / 0	0
18	1.56	4 / 0	0
4	1.56	4 / 4	100
18	1.56	4 / 4	100
4	0.78	4 / 4	100
18	0.78	4 / 4	100
4	3.13	4 / 4	100
18	12.5	4 / 4	100
4	0.78	4 / 4	100
18	3.13	4 / 4	100
4	0.78	4 / 1	25
18	1.56	4 / 4	100
4	1.56	6 / 0	0
18	6.25	6 / 0	0
4	1.56	6 / 2	33
18	3.13	6 / 0	0
4	0.39	6 / 3	50
18	0.39	6 / 3	50

end of the third day, and the last mouse died on the tenth day. In the 4-hour series, the first mouse died on the ninth day, and the last mouse died on the eleventh day. Salmonella was isolated from the heart blood agar culture plates.

Studies by Eagle et al. (1950) on host reaction to penicillin-treated organisms might well be applied to the different reactions obtained by S. typhimurium strain SF-368 as compared to S. choleraesuis strain 15-6 and P. multocida strain 49-50-638. Group A and Group G hemolytic streptococci, and also Type I and Type III pneumococci were used by Eagle et al. Mice showed little or no protection against these organisms. They investigated the length of time it took the four bacterial species in vitro and in vivo to recover from the effect of penicillin, and to begin multiplication. The bacteria were found to recover more rapidly in vitro than in vivo. Group A and Group B hemolytic streptococci required 6 to 8 hours to recover and resume multiplication in the host. During this recovery period, the viable organisms decreased progressively. It was noted that 98.6 to 99.6 percent of the viable organisms were apparently killed by the host, indicating that organisms damaged by penicillin were more susceptible to the host than were the

untreated bacteria. These organisms showed a decrease in virulence in later experiments. The reactions of Type I and Type II pneumococci differed from the streptococci. The pneumococci required 4 hours to recover in vivo before resuming multiplication, and the decrease in number of viable organisms ranged from 45 to 65 percent. There was no appreciable evidence of enhanced susceptibility of organisms damaged by penicillin to the host, as these organisms demonstrated only a slight decrease in virulence.

The bactericidal properties of chloromycetin (Seligmann and Wassermann, 1949) exerted on S. typhimurium strain SF-368, S. choleraesuis strain 15-6, and P. multocida strain 49-50-638 might well have affected these organisms like it did those studied by Eagle et al. (1950). The chloromycetin-affected S. typhimurium strain SF-368 organisms resembled the Type I and Type III pneumococci used by Eagle et al., in that the organisms were more susceptible to the host reactions than were normal organisms. Though the organism was more susceptible to the host, the degree of susceptibility was not sufficient to enable the host to combat the remaining viable organisms, with the result that the mice began to die on the

third day. The last mouse of this series died on the eleventh day.

S. choleraesuis strain 15-6 and P. multocida strain 49-50-638 appeared to be the type of organisms that become highly susceptible to host reactions after being subjected to chloromycetin. The mice were well and alive at the end of the 30-day period. These mice were further used to detect evidence of resistance, on the part of the host, to subsequent inoculations of untreated organisms of known pathogenicity.

Dihydrostreptomycin. The general reaction of dihydrostreptomycin appeared to increase the susceptibility of S. typhimurium strain 13-5, S. choleraesuis strain 15-6, and P. multocida strain 49-50-638 to the body defenses of the host. There was 100-percent survival of the mice inoculated with dihydrostreptomycin-treated S. typhimurium strain 13-5 and S. choleraesuis strain 15-6 organisms. The same results were obtained with 18-hour P. multocida strain 49-50-638, but the organisms incubated for 4 hours apparently were little affected by the action of dihydrostreptomycin.

The mice which were protected 100 percent from infections with the various organisms were evidently aided by the

damaging effect of dihydrostreptomycin upon those organisms. As in the chloromycetin-affected organisms, the dihydrostreptomycin-treated organisms were more susceptible to the host defense. It is also quite logical to assume that there was a decrease in virulence of the organism, as was shown by the Group I and Group II hemolytic streptococci used in the experiments by Eagle et al. (1950). Another possibility is that the organisms might have become dependent upon dihydrostreptomycin, and that without the antibiotic the organisms could not remain viable.

Aureomycin. Aureomycin hydrochloride demonstrated less effect upon the organisms tested in vivo than did the other two antibiotics. Organisms treated with S. typhimurium strain SF-368 destroyed their respective hosts within 15 days from the day of inoculation. The first death was observed on the fourth day, and the last death was on the fifteenth day. Treated S. choleraesuis strain 15-9 followed a similar course in destroying its hosts. The noticeable difference between the two salmonellae was that S. choleraesuis strain 15-9 did not kill its hosts as rapidly as the antibiotic-treated S. typhimurium strain SF-368. The first death was observed on the seventh

day, and the last death was recorded on the thirty-second day. This prolonged survival time of the infected mice seemed to indicate that, although aureomycin deteriorates rapidly in vitro, the organisms were affected sufficiently to enable the defense of the host to respond effectively. Another reason for the prolonged survival time could have been a decrease in virulence of the organism for the host resulting from the action of the antibiotic before deteriorating.

Aureomycin-treated P. multocida strain 49-50-638 was only 50-percent effective in killing its hosts (Table IV). The organisms that killed their respective hosts did so within a 2-day period. The first death was recorded on the second day after inoculation, and the last death occurred on the fourth day. All mice surviving the fourth day remained alive and well until the termination of this experiment. These results could have been due to one of the many uncontrollable variables in the in vivo experiments, including individual susceptibility of the host.

All mice surviving injection with antibiotic-treated organisms were challenged with a lethal dose of the untreated organism. All mice survived a 10-day test period. This

indicates that there might have been an effective immunological response by the host to the antibiotic-treated organism. Normal control mice received a subsequent inoculation of the same lethal dose given to the recovery mice. All mice in the control group receiving S. choleraesuis strain 15-6 were dead by the end of the sixth day, as were the mice receiving S. typhimurium strain 13-5. The mice in the third series, receiving 0.1 cubic centimeter of a 10^{-3} dilution of P. multocida strain 49-50-638 died on the fifth day (see Table VI).

Unfortunately, the in vivo data are not sufficient to justify definite conclusions as to the effect of a subinhibitory concentration of an antibiotic on an organism. Many more mice would have to be used. Antibody production in response to treated organisms would have to be checked by serological tests to determine the titer of the antibodies produced. This would necessitate serological tests at different time intervals, following the inoculation of the antibiotic-treated organism. The results would demonstrate that the antibiotic-treated organisms retain their ability to stimulate antibody production.

This would suggest that organisms so treated could serve as effective immunizing agents.

TABLE VI

IMMUNITY ESTABLISHED BY ORGANISMS TREATED WITH SUBINHIBITORY CONCENTRATIONS OF THREE ANTIBIOTICS

No. of Mice	Initial Inoculum		
	Culture Number	Antibiotic	Hours Incubation In Vitro
4	15-6	Chloromycetin	4
4	15-6	Chloromycetin	18
4	49-50-638	Chloromycetin	4
4	15-6	Dihydrostreptomycin	4
4	15-6	Dihydrostreptomycin	18
3	13-5	Dihydrostreptomycin	4
4	13-5	Dihydrostreptomycin	18
1	49-50-638	Dihydrostreptomycin	4
4	49-50-638	Dihydrostreptomycin	18
3	49-50-638	Aureomycin	4
3	49-50-638	Aureomycin	18
6	Control		
6	Control		
6	Control		

TABLE VI (Continued)

Challenge Culture	Dilution	No. Injected / No. Survived	Percent Survived
15-6	10^{-4}	4 / 4	100
15-6	10^{-4}	4 / 4	100
49-50-638	10^{-3}	4 / 4	100
15-6	10^{-4}	4 / 4	100
15-6	10^{-4}	4 / 4	100
13-5	10^{-4}	3 / 3	100
13-5	10^{-4}	4 / 4	100
49-50-638	10^{-3}	1 / 1	100
49-50-638	10^{-3}	4 / 4	100
49-50-638	10^{-3}	3 / 3	100
49-50-638	10^{-3}	3 / 3	100
15-6	10^{-4}	6 / 0	0
13-5	10^{-4}	6 / 0	0
49-50-638	10^{-3}	6 / 0	0

Further investigation along these lines might prove profitable.

SUMMARY

The in vitro sensitivity of fourteen strains of Salmonella typhimurium, thirteen strains of Salmonella choleraesuis, and sixteen strains of Pasteurella multocida to aureomycin, chloromycetin, and dihydrostreptomycin was tested.

There was a slight variation in the sensitivity of the strains within the same species to the same antibiotic, and to all three antibiotics at the end of 4 hours.

The sensitivities of all organisms to chloromycetin was constant at the termination of 18 hours. There was a decided fluctuation in the sensitivities of all the organisms to aureomycin and dihydrostreptomycin. Also, decided variation of sensitivities between strains of the same species to the same antibiotic was noted at 18 hours.

The order of activity of the three antibiotics determined from the sensitivity tests was: (1) chloromycetin, (2) aureomycin, and (3) dihydrostreptomycin. This order was generally valid for all three species of organisms.

A comparison of the biochemical reactions of normal cultures of all the organisms and those organisms subjected

separately with subinhibitory concentrations of each of the three antibiotics showed no observable difference.

Morphological studies were made on all organisms separately subjected to a subinhibitory concentration of each antibiotic. There was no observable difference between the treated and normal organisms.

A few selected strains of S. typhimurium and S. choleraesuis, and one selected strain of P. multocida were subjected to subinhibitory concentrations of an antibiotic and then inoculated into mice.

All mice inoculated with S. choleraesuis strain 15-6 and P. multocida strain 49-50-638, both of which had been treated with chloromycetin for 4 and 18 hours, survived. S. typhimurium strain SF-368, subjected to the same antibiotic, killed all mice at both time intervals.

S. choleraesuis strain 15-6 and S. typhimurium strain 13-5 which had been treated with dihydrostreptomycin for 4 and 18 hours and the 18-hour-treated P. multocida strain 49-50-638 did not kill mice. P. multocida strain 49-50-638 which had been treated with dihydrostreptomycin for 4 hours killed 75 percent of the mice in the series.

Aureomycin-treated S. choleraesuis strain 15-9 and S. typhimurium strain SF-368 treated for 4 and 18 hours killed mice. Fifty percent of the two series of mice inoculated with P. multocida strain 49-50-638 for the two time periods survived.

Immunity responses of the mice surviving injections with the antibiotic-treated organisms were checked by challenging the mice with a lethal dose of the normal cultures.

Mice surviving an injection with S. choleraesuis strain 15-6 treated with chloromycetin received an 0.1 cubic centimeter of the untreated culture, which was diluted 10^{-4}. All mice were alive and well at the end of 10 days. Mice surviving injections with P. multocida strain 49-50-638 treated with chloromycetin, survived a challenge of the normal organism diluted 10^{-3}.

Mice inoculated with dihydrostreptomycin-treated S. typhimurium strain 13-5 survived 0.1 cubic centimeter of a 10^{-4} dilution of the untreated organism. Similarly, mice receiving S. choleraesuis strain 15-6 subjected to dihydrostreptomycin survived 0.1 cubic centimeter of a 10^{-4} dilution of the normal organism. Mice initially inoculated with dihydrostreptomycin-treated P. multocida strain 49-50-638 survived 0.1 cubic centimeter of a 10^{-3} dilution of the organism.

Mice inoculated with aureomycin-treated P. multocida strain 49-50-638 also recovered when challenged with a 10^{-3} dilution of the normal organism.

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