BACTERIOLOGIC STUDIES ON AUREOMYCIN[1]

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Unpublished data made available to us suggested that a new antibiotic, aureomycin,[3] was of low toxicity, was highly effective against a variety of experimental infections in animals, and, therefore, warranted a clinical trial. The bacteriological studies which were conducted as part of the clinical evaluation of this agent are presented in this paper.

SENSITIVITY OF VARIOUS BACTERIA

Aureomycin hydrochloride was found to have considerable antibacterial action *in vitro* against a wide range of gram-positive and gram-negative bacteria. The sensitivities to aureomycin of 186 strains of organisms recently isolated from patients in the Boston City Hospital are presented in table 1. Among the strains of the various organisms tested, only those of *Proteus vulgaris* and of *Pseudomonas aeruginosa* were regularly found to be relatively resistant to aureomycin.

COMPARATIVE SENSITIVITY TO THREE ANTIBIOTICS

The sensitivity to aureomycin, penicillin, and streptomycin of a selected group of microorganisms is presented in table 2. Weight for weight, aureomycin was found to be less effective than penicillin, but more effective than streptomycin, in the case of most coccal organisms. Aureomycin was about as effective as streptomycin against most gram-negative bacilli. Aureomycin possesses equal antibacterial activity against penicillin-sensitive and penicillin-resistant staphylococci as well as against streptomycin-sensitive and streptomycin-resistant bacteria. It is also effective against streptomycin-dependent organisms.

FACTORS INFLUENCING ANTIBACTERIAL ACTIVITY OF AUREOMYCIN

The concentration of antibiotic which is necessary to inhibit any bacterium depends upon many factors, some of which are concerned chiefly with the antibiotic and others of which have largely to do with the microorganisms. Those

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[3] Work on the method of isolation of aureomycin, its pharmacology, and the determination of its bacteriological, antiviral, and antirickettsial activity was carried out by investigators of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. Their findings, some of which were furnished in personal communications, were presented at the New York Academy of Sciences, July 21, 1948.

TABLE 1

Aureomycin sensitivity of bacteria recently isolated from cases of human infections

ORGANISM	NUMBER OF STRAINS TESTED	INHIBITING CONCENTRATION μG/ML (COMPLETE)
Aerobacter aerogenes	10	12.5–50
Diplococcus pneumoniae	13	0.1–1.0
Escherichia coli	31	3.1–100*
Hemophilus hemolyticus	1	0.8
Hemophilus influenzae, type ?	5	1.0–2.0
" " , type b	1	2.0
Klebsiella pneumoniae	4	6.3–50
Neisseria catarrhalis	2	2.0
" *gonorrhoeae*	37	0.25–1.0
" *meningitidis*	1	0.5
Pleuropneumonialike	1	0.25
Proteus morganii	1	4.0
" *vulgaris*	13	125–250
Pseudomonas aeruginosa	7	100–250
Salmonella, sp.	6	3.1–25
" *typhosa*	6	3.1–25
Staphylococcus albus	2	1.0–2
Staphylococcus aureus	27	1.0–12.5†
Streptococcus faecalis	2	6.3
" *mitis*	4	0.8–6.3
" *pyogenes*	12	0.5–1.0

Some of these tests were made by a tube dilution method and others by a surface streak method.

* Twenty-two strains inhibited by 12.5 or 25 μg per ml.

† Twenty-four of these inhibited by 1 to 2 μg per ml.

TABLE 2

Sensitivity of selected strains to three antibiotics

ORGANISM	AUREOMYCIN	PENICILLIN	STREPTOMYCIN
D. pneumoniae	0.5	0.02	25
E. coli S	50	24	6.3
" R	50	24	>50,000
" D	12.5	24	Dependent
P. morganii S	50	750	100
" R	25	750	>50,000
" D	12.5	375	Dependent
S. cholerae-suis	6.3	60	250
S. typhosa	6.3	6	100
S. aureus	2	60	25
" S	6.3	0.08	25
" R	6.3	0.08	50,000
" D	3.1	0.02	Dependent
S. faecalis	6.3	6	3.1
S. mitis	1	0.16	6.3
S. pyogenes	0.5	0.02	25

All sensitivities given in μg per ml for complete inhibition.

S, R, and D represent streptomycin-sensitive, -resistant, and -dependent variants, respectively, of the same strain (Paine and Finland, 1948).

which primarily concern the antibiotic may be grouped under two headings: (1) stability of the antibiotic and (2) conditions or substances which inhibit or augment the action of the antibiotic, such as the hydrogen ion concentration and the presence of salts.

The factors which are related particularly to the organisms and which may influence the action of the antibiotic are: (1) the innate susceptibility or resistance of the organism to the antibiotic, (2) the number and growth phase of the microorganisms involved in the test, (3) the presence of conditions or substances which interfere with or promote growth of the organism, such as pH, type of media, etc., (4) the elaboration of substances by the bacteria which may interfere

TABLE 3

Stability of aureomycin in solution

Diluent	broth	H_2O	plasma	broth	H_2O	BAP	plasma
Concentration (μg/ml) of solution stored	8*	2,000†	8*	8*	2,000†	200‡	8*
Temperature C	4	4	4	37	37	37	37
Day 0	0.5§	0.5	0.5	1.0	0.5	1.6	0.5
1	—	—	1.0	4.0	0.5	6.2	2.0
3	0.5	0.5	4.0	>4.0	0.5	12.5	>4.0
7	0.5	0.5			0.5		
14	2.0	0.5			0.5		
21	2.0	1.0			0.5		
28	>4.0	1.0					
35	—	2.0					

* pH 7.2 ±. †pH 4.

‡ Series of agar plates containing 10 per cent horse blood and graded concentrations of aureomycin from 200 to 0.019 μg per ml.

§ Minimum concentration of aureomycin for complete inhibition of *Streptococcus* no. 98.

with the activity of the antibiotic, and (5) the development of resistance by the microorganism.

Stability of aureomycin: effect of temperature and diluent. Aureomycin, in the form of the crystalline hydrochloride, as made available to us, was a stable preparation and maintained its potency at ±22 C in dried form for at least 6 months. It was also stable in high concentrations in distilled water, both in the refrigerator and in the incubator at 37 C, for periods up to 2 or 3 weeks. The pH of the preparations in distilled water was 4.0 to 4.5. However, when the solutions were kept in low concentrations, either in broth, in pooled human plasma, or incorporated in 10 per cent horse blood agar, there was a rapid loss of activity on incubation at 37 C, and a less rapid loss of activity on refrigeration. Under these circumstances, the pH of the materials was about neutral. The data are shown in table 3.

Natural resistance. Of the organisms studied in this laboratory, only strains of *Proteus vulgaris* and *Pseudomonas aeruginosa* have shown any considerable

degree of innate resistance to aureomycin. These organisms are not completely resistant to aureomycin, however, and can be inhibited *in vitro*, provided that sufficient antibiotic is present. The relative resistance of these two species of gram-negative bacilli may reflect the degree to which these microorganisms will be susceptible to aureomycin therapy, and it might be expected that they would be difficult to eradicate from certain sites where high concentrations of the antibiotic cannot be obtained. This has already been confirmed in preliminary clinical trials.

Effect of inoculum size and growth phase. The number of organisms present was found to influence the concentration of aureomycin required for complete inhibition of bacterial growth in brain heart infusion broth (Difco). However, in sensitivity determinations made by streaking from broth cultures of bacteria directly to the surface of agar plates containing graded concentrations of aureomycin, the number of organisms present in the inoculum had little influence on the concentration of this antibiotic required to inhibit growth. Table 4 shows the influence of the size of the inoculum on inhibition end points obtained with aureomycin when the tests were made by the tube dilution and the surface streak methods with *Streptococcus pyogenes*, strain 98. Similar results were also obtained with a strain of *Klebsiella pneumoniae* and a subtilislike gram-positive bacillus (*Bacillus* no. 5, obtained from Dr. B. M. Duggar).

In the tube dilution method there was little evidence of antibacterial activity when large numbers of organisms were used in the inocula, even in the presence of high concentrations of aureomycin. With fewer organisms in the inocula, the visible end points of growth were not greatly altered in the tube dilution method by changes in the size of the inocula. The surface streak method, however, showed a sharp inhibition end point, which was essentially the same over a wide range of inoculum size. It has been suggested that this difference in inhibition end points observed in the tube dilution and in the surface streak methods, when large inocula are used, may simply reflect the lack of vigorous growth from the large inocula in the tube dilution method, the organisms having already attained nearly their maximum growth with insufficient fresh media provided to allow vigorous multiplication. In the case of the surface streak method, there is not such a limiting factor, so far as the media are concerned, since there probably is vigorous growth both from the large and the small inocula used. These observations suggest that the antibacterial effect of aureomycin is exerted largely on multiplying organisms.

The effect of inoculum size on inhibition of bacterial cultures by aureomycin was also apparent from a study of growth curves, starting with different numbers of bacteria. In figure 1 are shown the growth curves of a beta hemolytic streptococcus in graded concentrations of aureomycin. Progressive inhibition of the rate of growth by increasing concentrations of the antibiotic is apparent and the streptococcus failed to multiply in the presence of 2 μg of aureomycin per ml. In this test a relatively small number of organisms was inoculated and the opportunity for initiating vigorous growth was present. Under these conditions the presence of a relatively small amount of aureomycin, 2 μg per ml, was sufficient to inhibit growth of this organism. On the other hand, when large numbers of

TABLE 4

Effect of size of inoculum on the antibacterial action of aureomycin

INOCULUM*	METHOD†	INCUBATION	FINAL CONCENTRATION OF AUREOMYCIN, μG/ML OF MEDIUM									
			100	50	25	12.5	6.3	3.1	1.6	0.8	0.4	0
		hrs										
10^{-1}	A	18	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	B	24	3	+	++	+++	+++	+++	+++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	+++	+++	+++
10^{-2}	A	18	0	0	0	0	0	0	0	+	++	+++
	B	24	0	0	0	0	1	+++	+++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	+++	+++	+++
10^{-3}	A	18	0	0	0	0	0	0	0	0	+	+++
	B	24	0	0	0	0	0	1	+++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	++	++	++
10^{-4}	A	18	0	0	0	0	0	0	0	0	+	+++
	B	24	0	0	0	0	0	0	++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	+	+	+

Organism: *S. pyogenes*, no. 98. Media: beef heart infusion broth (Difco), pH 7.2 ±, and heart infusion agar (Difco) to which 10 per cent defibrinated horse blood has been added.

+, ++, and +++ = estimation of growth; 0 = no visible growth; numerals indicate number of discrete colonies observed.

When the inoculum was an undiluted culture (1:2 final dilution in A) there was vigorous growth in all dilutions in A and B and the results in C were identical to those obtained with the 10^{-1} inoculum.

* Dilution of fully grown culture: 0.5 ml added to each tube containing 0.5 ml of diluted aureomycin in broth; and a 2-mm loopful streaked on the surface of the blood agar plates containing antibiotic.

† A = tube dilution method, reading visible growth; B = subcultures from A to blood agar plates without antibiotic; C = surface streak method on agar containing aureomycin.

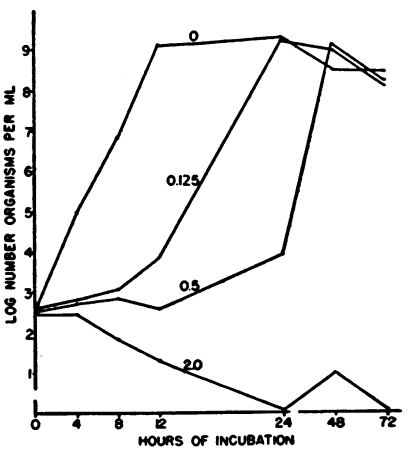

Figure 1. Effect of aureomycin on growth of *Streptococcus* 98. Each tube contained 10 ml of broth with aureomycin in the final concentrations, in μg per ml, noted on the curves and was inoculated at "0" hour with 0.1 ml of a 10^{-4} dilution of a 6-hour broth culture of the organism. The numbers of viable organisms were estimated at intervals by pour plates in aureomycin-free blood agar.

this same organism in 24-hour cultures were inoculated in various concentrations of aureomycin, little evidence of antibacterial activity was manifested, even in the presence of 100 μg of the antibiotic per ml, as shown in figure 2. It would thus seem that the opportunity for vigorous multiplication must be present in order for aureomycin to exert its antibacterial activity, though the influence of the concentration of bacteria may have been a determining factor.

End points in sensitivity tests. In reading the bacterial sensitivity to aureomycin in the tube dilution test, the usual experience has been that the visual end point of growth in the tubes is lower than the end point observed when subcultures were made from the tubes to antibiotic-free agar and incubated for an additional 24 hours. Table 4 shows this difference in end points. It may be that some organisms, possibly those in the resting state, survive the 18 hours of incubation in the broth containing lethal concentrations of antibiotic, and their

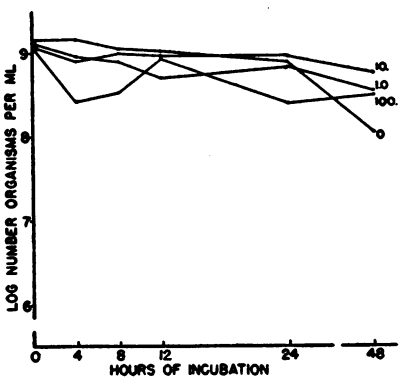

Figure 2. Effect of aureomycin on fully grown culture of *Streptococcus* 98. Each tube contained 9 ml of a 24-hour broth culture of the streptococcus, and at "0" hour was added 1 ml of broth containing sufficient aureomycin to give the indicated final concentrations in μg per ml. The numbers of viable organisms were then estimated at intervals by pour plates in aureomycin-free blood agar.

presence becomes apparent on subculture to antibiotic-free agar where growth can occur. This phenomenon may also be related to the rapid loss of antibacterial activity of aureomycin on incubation in broth.

Effect of pH. Alteration of the pH from the acid to the alkaline range resulted in a considerable decrease in the antibacterial activity of aureomycin. This effect is shown in table 5 in the case of *Bacillus* no. 5; similar results were obtained with other organisms. The decrease in activity of aureomycin in the basic pH range is in sharp contrast to the decrease in activity of streptomycin in the acid pH range (Abraham and Duthie, 1946).

Effect of added substances. The presence of a number of substances in the concentrations listed in table 6 had little or no effect on the antibacterial activity of aureomycin when they were incorporated into the broth making up the media for a tube dilution assay of the sensitivity of a test organism.

The type of solid medium employed for the surface streak sensitivity method may influence the inhibition end points obtained with aureomycin. The end

TABLE 5

Effect of pH on the antibacterial action of aureomycin

pH	READING*	CONCENTRATION OF AUREOMYCIN, μG/ML								
		0.78	0.39	0.19	0.095	0.048	0.024	0.012	0.006	0
6.1	A	0	0	0	0	0	0	0	+	+++
	B	0	0	0	0	0	0	0	+++	+++
6.6	A	0	0	0	0	0	0	+	+++	+++
	B	0	0	0	0	0	2	+++	+++	+++
7.0	A	0	0	0	0	0	0	+++	+++	+++
	B	0	0	0	0	0	+++	+++	+++	+++
7.5	A	0	0	0	+	+++	+++	+++	+++	+++
	B	0	0	0	+++	+++	+++	+++	+++	+++
8.0	A	0	0	+++	+++	+++	+++	+++	+++	+++
	B	0	+++	+++	+++	+++	+++	+++	+++	+++

Organism: *Bacillus* no. 5. Inoculum: 20,000 per ml.

0, +, ++, and +++ represent extent of growth (numbers represent number of discrete colonies).

* A = visible growth in tubes after incubation for 18 hours; B = growth on subculture of 18-hour broth culture to aureomycin-free blood agar.

TABLE 6

Effect of various substances on antibacterial action of aureomycin

SUBSTANCE	AMOUNT ADDED*	M.I.C.†	AMOUNT ADDED	M.I.C.	AMOUNT ADDED	M.I.C.	AMOUNT ADDED	M.I.C.
Sodium chloride	0	0.5	M/10	0.5	1M	—‡		
Glucose	0	0.5	M/10	0.5	1M	0.5		
Sodium thioglycolate	0	1.0	M/1,000	0.25	M/100	1.0	M/10	2.0
Cysteine hydrochloride	0	1.0	M/1,000	1.0	M/100	1.0	M/10	2.0‡
Semicarbazide	0	1.0	M/1,000	1.0	M/100	1.0	M/10	—‡
Urea	0	0.5	M/2,000	0.5	M/200	0.5	M/20	0.5
Glutamic acid	0	0.5	M/2,000	0.5	M/200	0.5	M/20	0.5
p-Aminobenzoic acid	0	0.5	M/800	0.5	M/200	0.5	M/80	0.25
Pteroylglutamic acid	0	0.5	M/2,000	0.5	M/200	0.5	M/40	0.5
Human plasma, pooled	0	0.6	12.5%	0.6	25%	0.3	50%	0.3

Tube dilution method; brain heart infusion broth pH 7.2 ± (Difco).

Organism: *Streptococcus* no. 98. Inoculum: ±100,000 per ml.

* Final concentration in addition to content of media.

† Minimum concentration of aureomycin for complete inhibition.

‡ Poor growth or no growth of test organism with and without antibiotic.

points observed on heart infusion agar (Difco) and on the same medium containing 10 per cent horse blood were the same; whereas the end points of aureomycin inhibition observed in the case of the same organisms on eosin methylene

blue agar were lower. This difference may possibly be related to poorer growth on the eosin methylene blue agar.

Effect of aerobiosis. There was no indication that sensitivity determinations made on surface streak agar plates in a decreased oxygen tension, with approximately 3.5 per cent carbon dioxide as obtained in a candle jar, altered the inhibition end points observed with the same organisms under strictly aerobic incubation. Preliminary observations, however, suggest that greater anaerobiosis may interfere with the antibiotic action of aureomycin.

"Aureomycinase." No evidence could be obtained that any aureomycin-inhibiting substance (similar to penicillinase) was present in filtrates of broth cultures of *Streptococcus* 98 and *Proteus vulgaris* filtered through sintered glass. These same organisms did not yield any apparent aureomycin-inhibiting material when the bacterial cells were disrupted by repeated freezing and thawing.

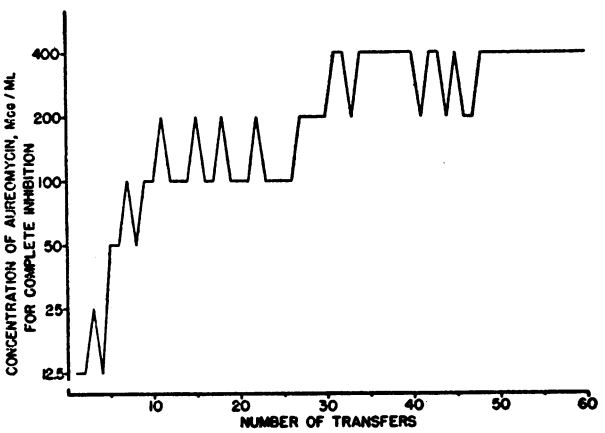

Figure 3. Development of resistance to aureomycin *in vitro*. *Aerobacter aerogenes*, strain J.

Development of resistance in vitro. Most of the bacteria studied did not readily become resistant to aureomycin *in vitro*. Several strains of bacteria recently isolated from patients were subcultured at frequent intervals from the surface of 10 per cent horse blood agar containing graded concentrations of the antibiotic, to similar series of plates, using large inocula from the plates containing the highest concentration of aureomycin on which each strain had previously grown. The plates were freshly prepared and were used promptly each time. Only one strain of *Aerobacter aerogenes*, among several organisms that were studied, showed a significant increase in resistance to aureomycin, as shown in figure 3. The same strain of *A. aerogenes*, subcultured in the same manner but in the absence of the antibiotic for a total of 60 times, showed no increase in resistance to aureomycin. A strain of *Klebsiella pneumoniae*, type A, showed an increase in resistance to aureomycin, from 6.2 to 25 μg per ml for complete inhibition, after 42 subcultures in the presence of the antibiotic. The latter strain subcultured for 60 times in the absence of the antibiotic showed no increased resistance to aureomycin. Two strains of *Staphylococcus aureus*, 1 strain of *Strepto-*

coccus mitis, and 1 strain of *Streptococcus pyogenes* showed no increase in resisttance to aureomycin after 60 subcultures in the presence of the antibiotic.

Another strain of *Streptococcus mitis* apparently increased in resistance from 1.5 to 12.5 μg per ml after 21 subcultures in the presence of the antibiotic; however, the same strain transferred in the absence of aureomycin for a total of 30 times showed a similar increase in resistance, suggesting that in this case the slight augmentation in resistance was probably the result of some cultural alteration from continued subculture on the laboratory medium and not necessarily related to the exposure to the antibiotic.

In an attempt to demonstrate the possible presence of a few very resistant organisms in large populations of sensitive strains of bacteria, broth cultures of *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* were centrifuged and the sediments from 10 ml were inoculated on the surfaces of agar containing 200 to 1,000 μg of aureomycin per ml. No aureomycin-resistant or aureomycin-dependent organisms could be obtained in this manner.

The difficulty of making microorganisms resistant to aureomycin *in vitro* suggests that the development of resistance to this antibiotic *in vivo* should not be an important problem. The clinical observations made to date seem to bear this out.

ACKNOWLEDGMENTS

The aureomycin hydrochloride was supplied by the Lederle Laboratories as a dry crystalline powder in sterile sealed ampoules containing 20 mg each and in 50-mg capsules. These studies were conducted with the technical assistance of Clare Wilcox, Janice M. Bryan, and Paul F. Frank. Most of the strains of organisms from the clinical cases were isolated and identified by Marion E. Lamb and A. Kathleen Daly in the Bacteriological Laboratory of the Mallory Institute of Pathology. The strains of gonococcus were isolated by Helen Trousdale.

SUMMARY

Aureomycin has been shown to be active *in vitro* against many bacteria, including certain penicillin-resistant, streptomycin-resistant, and streptomycin-dependent microorganisms. The organisms studied have shown no marked tendency to become resistant *in vitro* on repeated exposure to aureomycin, though a moderate increase in resistance occurred in some strains. The concentration of the antibiotic required for complete inhibition of bacteria is influenced by the number of organisms present, their phase of growth, and the pH of the test media.

Aureomycin is a stable preparation as a dry powder in sealed ampoules and also in high concentrations in solution in distilled water at 4 C and 37 C. It loses potency rapidly if stored in low concentrations in broth, plasma, or in blood agar at 37 C, though it is somewhat more stable in these media at 4 C.

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