JCI The Journal of Clinical Investigation

EXPERIMENTAL STUDIES ON THE ACTION OF STREPTOMYCIN, AUREOMYCIN, AND CHLOROMYCETIN ON BRUCELLA

Ellard M. Yow, Wesley W. Spink

J Clin Invest. 1949;28(5):871-885. https://doi.org/10.1172/JCI102171.

Research Article

EXPERIMENTAL STUDIES ON THE ACTION OF STREPTOMYCIN, AUREOMYCIN, AND CHLOROMYCETIN ON BRUCELLA [1, 2]

By ELLARD M. YOW and WESLEY W. SPINK

(From the Division of Internal Medicine, University of Minnesota Medical School, Minneapolis)

The use of streptomycin in the treatment of brucellosis revealed that most of the patients failed to respond satisfactorily to its administration, and among those who did respond, relapses almost always followed (1). It was subsequently observed that when sulfadiazine was given simultaneously with streptomycin, the result was much more encouraging (2–4). However, this combination was also followed by relapses (5). Brucella could be recovered after treatment from the blood of approximately 25 per cent of the patients with infections due to *Br. abortus* and 45 per cent of those with disease due to *Br. melitensis*.

In this clinic the failure of streptomycin and sulfadiazine to eradicate Brucella consistently from the tissues prompted the study of why such a therapeutic combination failed, and stimulated the search for more effective antibrucella agents. The problem was approached by *in vitro* studies, animal experiments, and, finally, by the clinical trial of newer antibiotic agents. This report is concerned primarily with the results of the *in vitro* experiments.

I. STREPTOMYCIN AND DIHYDROSTREPTOMYCIN

In vitro *Sensitivity of Brucella*

Forty-one strains of Brucella recently isolated from patients were tested for sensitivity to streptomycin and dihydrostreptomycin.

Method: Solutions of streptomycin and dihydrostreptomycin were prepared by adding 10 ml. of physiologic saline solution to 1.0 gram of base of the drugs. The stock solution was stored at 4° C. and was diluted to a concentration of 1000 or 100 μg. per ml. immediately before the sensitivity tests were performed. To each of 12 test tubes (12 × 100 mm.), except the first, was added 0.5 ml. of tryptose phosphate broth. The first two tubes were inoculated with 0.5 ml. each of the test solution of streptomycin or dihydrostreptomycin. Two-fold dilutions of the drug were made by transferring 0.5 ml. of the mixture in the second tube to the third tube, and so on, through the 11th tube. The 12th tube contained only broth and organisms. Each of the 12 tubes was then inoculated with 1.5 ml. of a 1:100 dilution of a 24-hour culture in tryptose phosphate broth. Thus, the first tube contained a 1:4 dilution of the test solution of the antibiotic; the second, 1:8; third, 1:16, through the 11th, which contained a 1:4096 dilution. The cultures of *Br. abortus* were incubated for 48 hours at 37° C. in an environment containing a 10 per cent concentration of carbon dioxide, while the strains of *Br. suis* and *Br. melitensis* were subjected to normal atmospheric conditions. At the end of this time, the tubes were examined for gross turbidity and the contents of each tube streaked on tryptose agar. The sensitivity of the organism was reported as the minimum concentration of streptomycin or dihydrostreptomycin required to kill all the organisms.

Results: Thirteen strains of *Br. abortus,* ten of *Br. melitensis,* and one of *Br. Suis,* all isolated before being exposed to streptomycin, were found to be sensitive to concentrations between 1.0 and 2.5 μg. of streptomycin and dihydrostreptomycin per ml. The results are presented in Tables I and II.

The Bactericidal Action of Streptomycin on Brucella

The effect of streptomycin on the multiplication and viability of Brucella was studied in the following manner.

Methods: A saline suspension of organisms, equal in turbidity to a barium sulfate No. 1 standard, was prepared from a 48-hour agar culture of *Br. abortus.* Three tubes containing 10 ml. of tryptose phosphate broth were inoculated with 0.05 ml. of the bacterial suspension so that the final concentration of organisms was approximately 5,000,000 bacteria per ml. Sufficient streptomycin was added to each of two tubes to make a concentration of

[1] Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11–12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

[2] This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, and by a grant from the Lederle Laboratories. The aureomycin was supplied by the Lederle Laboratories; the dihydrostreptomycin, by E. R. Squibb Company; polymyxin, by the Stamford Research Laboratories, American Cyanamid Company; bacitracin and Q-19, by the Upjohn Company; and chloromycetin, by Parke, Davis and Company.

TABLE I

Sensitivity of Brucella abortus *to streptomycin and dihydrostreptomycin*

Strain	Streptomycin (μg./ml.)		Dihydrostreptomycin (μg./ml.)	
	Minimum conc. inhibiting growth	Maximum conc. permitting growth	Minimum conc. inhibiting growth	Maximum conc. permitting growth
Lynch	1.2	0.6	1.6	0.8
Kreutz	2.0	1.0	1.2	0.6
Kreutz*	1.2	0.6	1.2	0.6
Rivera	1.2	0.6	1.2	0.6
Rivera*	2.0	1.0	1.2	0.6
Archer*	1.2	0.6		
Archer*	1.2	0.6	1.2	0.6
Steinbrecker	1.2	0.6	1.2	0.6
Steinbrecker*	1.2	0.6	1.2	0.6
Wimmer	1.2	0.6	1.2	0.6
Wimmer*	1.2	0.6		
Garrity	2.0	1.0		
Garrity*	2.0	1.0		
Shaeffer	1.0	0.5	1.0	0.5
Hamilton	2.0	1.0	1.6	0.8
Elsted	1.0	0.5	1.6	0.8
Elsted†	10,000	7,500	10,000	7,500
Guck	1.6	0.8	1.6	0.8
Nelson	1.6	0.8	1.6	0.8
Johnson	1.6	0.8	1.6	0.8
Schweim	1.6	0.8	1.6	0.8

* Strain isolated after streptomycin-sulfadiazine therapy
† Isolated after streptomycin therapy

10 μg. per ml. in one tube and 20 μg. per ml. in the other. The third tube was used as a control. The same procedure was carried out using cultures of *Br. melitensis* and *Br. suis*. The tubes were incubated under the proper tension of carbon dioxide at 37° C., and a standard loopful of the broth from each tube was subcultured on streptomycin-free agar at 15-minute intervals during the first hour, at three and six hours after inoculation, and then daily for 14 days. The subcultures were incubated for 96 hours and examined for growth. The broth cultures were inspected for evidence of growth turbidity at the time of each subculture.

Results: No evidence of bacterial multiplication appeared in the broth cultures containing streptomycin, while in the control tubes there was a turbid growth at the end of the first 24 hours of incubation. No viable Brucella could be recovered from the tubes containing streptomycin after three hours of incubation, whereas the control cultures contained living organisms throughout the 14 days of observation (see Tables III and IV).

Resistance of Brucella to the Antibacterial Action of Streptomycin and Dihydrostreptomycin

The rapid development of resistance of gram-negative bacilli to streptomycin has been a frequent

TABLE II

Sensitivity of Brucella melitensis *and* Brucella suis *to streptomycin and dihydrostreptomycin*

Strain	Streptomycin (μg./ml.)		Dihydrostreptomycin (μg./ml.)	
	Minimum conc. inhibiting growth	Maximum conc. permitting growth	Minimum conc. inhibiting growth	Maximum conc. permitting growth
Br. melitensis				
3752	2.5	1.2	1.2	0.6
3752*	2.5	1.2	2.5	1.2
3925	2.5	1.2	1.2	0.6
3925*	>50,000	>50,000	>50,000	>50,000
2716	2.5	1.2	1.2	0.6
2716*	2.5	1.2	2.5	1.2
3777	2.5	1.2	2.5	1.2
3777*	2.5	1.2	2.5	1.2
3988	2.5	1.2	1.2	0.6
3988*	2.5	1.2	1.2	0.6
4103	2.5	1.2	1.2	0.6
4103*	2.5	1.2	2.5	1.2
3858	2.5	1.2	1.2	0.6
3858*	2.5	1.2	2.5	1.2
4241	1.5	1.0		
4241†	1.5	1.0	1.2	0.6
4185	1.5	1.0	1.2	0.6
4185†	1.5	1.0		
4290	1.5	1.0		
4290†	1.5	1.0		
Br. suis				
374	1.2	0.6	1.2	0.6

* Isolated after streptomycin-sulfadiazine therapy
† Isolated after aureomycin therapy

cause of the failure of the antibiotic in the treatment of human infections. The most acceptable theory of the origin of streptomycin-resistant strains of bacteria is that they arise from innately resistant organisms normally present in small numbers in every large population of a sensitive strain (6). The presence of naturally occurring streptomycin-resistant organisms in sensitive strains of Brucella was demonstrated by exposing

TABLE III

Bactericidal action of streptomycin on Brucella abortus

Time interval	Control		Streptomycin (10 μg./ml.)		Streptomycin (20 μg./ml.)	
	Broth turbidity	Subculture	Broth turbidity	SM-free subculture	Broth turbidity	SM-free subculture
5 min.	−	++	−	++	−	++
15 min.	−	++	−	++	−	++
30 min.	−	++	−	++	−	++
1 hour	−	++	−	+	−	+
3 hours	−	++	−	−	−	−
6 hours	−	++	−	−	−	−
24 hours	+	+++	−	−	−	−
2 days	++	++++	−	−	−	−
3 days	+++	++++	−	−	−	−
4 days	++++	++++	−	−	−	−

TABLE IV

Bactericidal action of streptomycin on Brucella melitensis *and* Brucella suis

Time interval	*Brucella melitensis*				*Brucella suis*			
	Control		Streptomycin (20 μg./ml.)		Control		Streptomycin (20 μg./ml.)	
	Broth turbidity	Subculture	Broth turbidity	SM-free subculture	Broth turbidity	Subculture	Broth turbidity	SM-free subculture
5 min.	−	++	−	++	−	++	−	++
15 min.	−	++	−	++	−	++	−	++
30 min.	−	++	−	++	−	++	−	++
1 hour	−	++	−	+	−	++	−	+
3 hours	−	++	−	−	−	++	−	−
6 hours	−	++	−	−	−	++	−	−
24 hours	++	+++	−	−	++	+++	−	−
2 days	+++	++++	−	−	+++	++++	−	−
3 days	++++	++++	−	−	++++	++++	−	−
4 days	++++	++++	−	−	++++	++++	−	−

large bacterial populations to varying concentrations of streptomycin.

Method: A concentrated suspension of organisms was prepared by heavily inoculating a large tryptose agar slant with a culture of Brucella. After 48 hours of incubation, the bacteria were washed from the slant with 1.0 ml. of tryptose phosphate broth. The suspension of organisms was spread evenly over the agar surface of bottles, prepared by adding 60 ml. of melted agar to 16

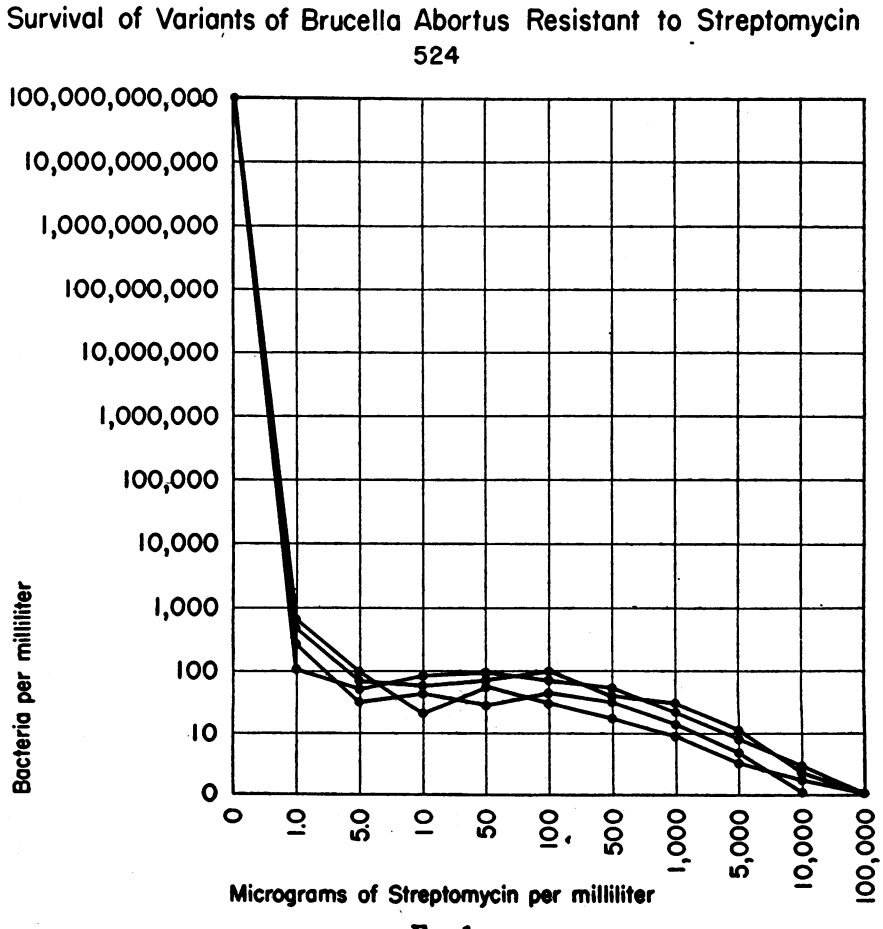

FIG. 1

oz. oval prescription bottles with screw caps. The bottles were incubated with the inoculated agar surface down for 48 hours, and then the bacterial growth was resuspended in 5 ml. of broth. Bacterial counts on the suspension of organisms were performed with a photoelectric colorimeter by comparing the turbidity of a 10^{-3} dilution to a barium sulfate standard No. 1. The concentration of bacteria was adjusted to approximately 100 billion organisms per ml. and then exposed to varying concentrations of streptomycin. Eleven pour plates were prepared, using 1.0 ml. of the bacterial suspension, 9.0 ml. of tryptose agar, and sufficient concentrations of streptomycin to make final concentrations of 1.0, 2.5, 5.0, 10, 50, 100, 500, 1,000, 5,000, 10,000, and 100,000 μg. per ml. Each experiment was performed in triplicate and the plates were incubated in jars at 37° C. Colony counts were performed weekly for a month.

Results: The number of the surviving streptomycin-resistant variants decreased very rapidly as the concentration of streptomycin increased from 1.0 to 5.0 μg. per ml., then more gradually to a concentration of 10,000 μg. per ml., after which there were no surviving variants. Streptomycin survival curves for *Br. abortus* are shown in Figure 1, and for *Br. suis,* in Figure 2.

Some of the streptomycin-resistant colonies were found to be largely dependent upon streptomycin for growth. The dependency of the colonies upon streptomycin was determined by adding suspensions of the colonies to pour plates with and without the addition of 100 μg. of streptomycin per ml. The resistant colonies grew equally well in the presence or absence of streptomycin, whereas the dependent colonies multiplied only in the plates containing the antibiotic. These dependent strains would grow if streaked heavily on streptomycin-free agar, but not as luxuriously as non-dependent strains. Streptomycin-dependent strains of Brucella grew equally well in the presence of dihydrostreptomycin, and strains dependent upon dihydrostreptomycin multiplied in media containing streptomycin. Media containing

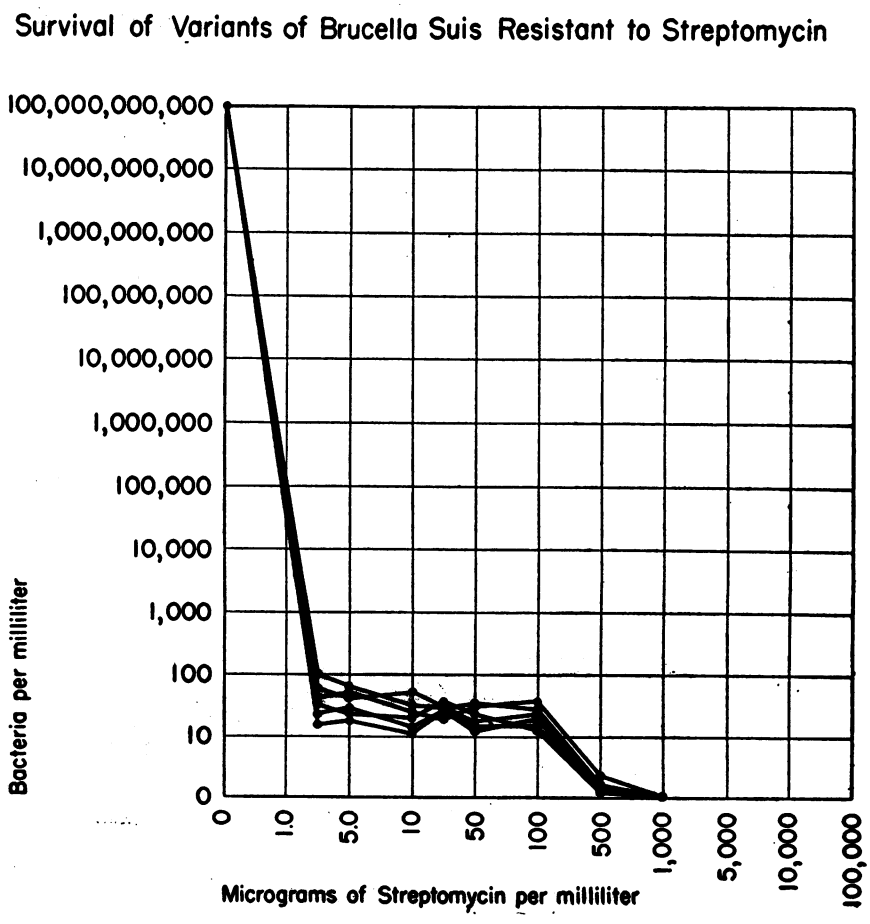

FIG. 2

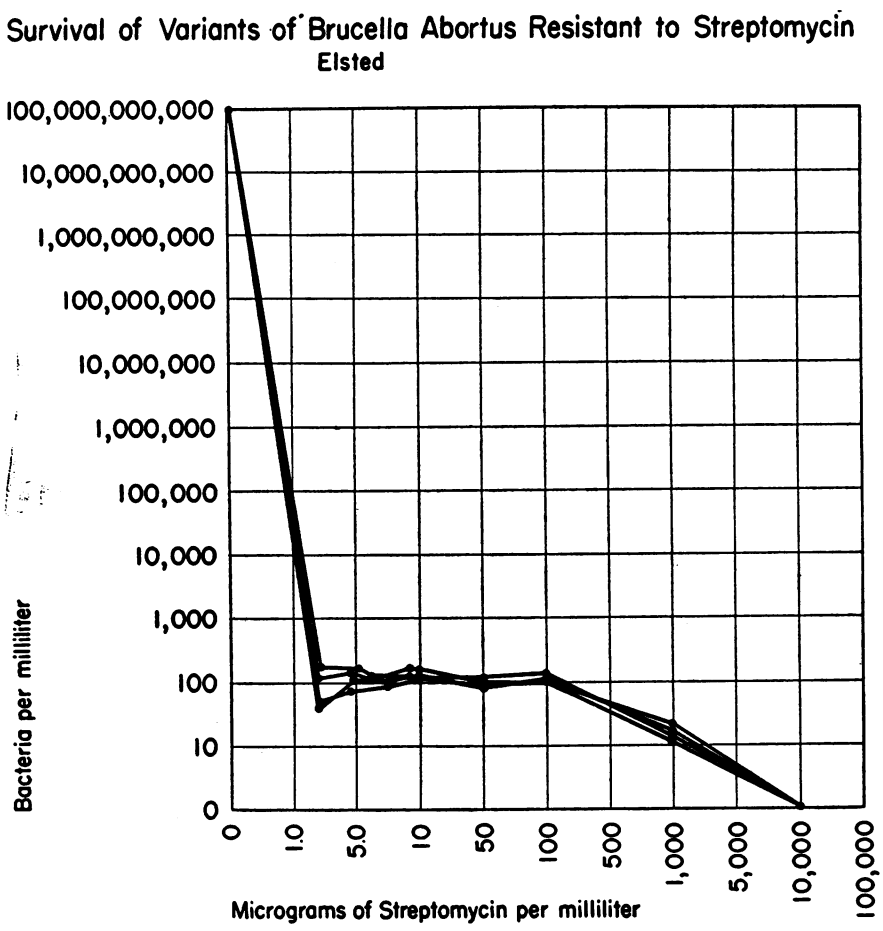

FIG. 3

aureomycin or chloromycetin did not support the growth of streptomycin-dependent strains.

Sensitivity of Strains of Brucella Isolated after Streptomycin Therapy

Streptomycin resistance as a cause of therapeutic failure in cases of brucellosis was investigated, first, by determining the sensitivity of the strains isolated after therapy as compared to the sensitivity of the strains isolated from the same patient before therapy; and, secondly, by comparing the distribution and number of the resistant variants occurring in these strains of Brucella.

Fifteen strains of Brucella isolated after streptomycin therapy were tested for sensitivity to streptomycin. Seven strains of *Br. abortus* and six strains of *Br. melitensis* cultured after combined sulfadiazine and streptomycin therapy showed no change in sensitivity to streptomycin. One strain of *Br. abortus* obtained after treatment with only streptomycin was studied by Hall and Spink (7) and was found to have increased in resistance from 1.0 μg. per ml. to 10,000 μg. per ml. One strain of *Br. melitensis,* isolated after combined streptomycin and sulfadiazine therapy, changed in sensitivity from 2.5 μg. to 50,000 μg. of streptomycin per ml.

The two strains of Brucella which developed resistance to streptomycin were studied for evidence of streptomycin dependency. The strain of *Br. melitensis* did not depend upon the presence of streptomycin for growth, but the resistant strain of *Br. abortus* contained predominantly colonies that were smaller than normal and grew on streptomycin-free agar only when a heavy inoculum was used. The growth of the small colonies was greatly enhanced by the addition of from 50 to 1,000 μg. of streptomycin per ml. (7).

The number of the resistant variants present in the strains isolated after therapy was compared

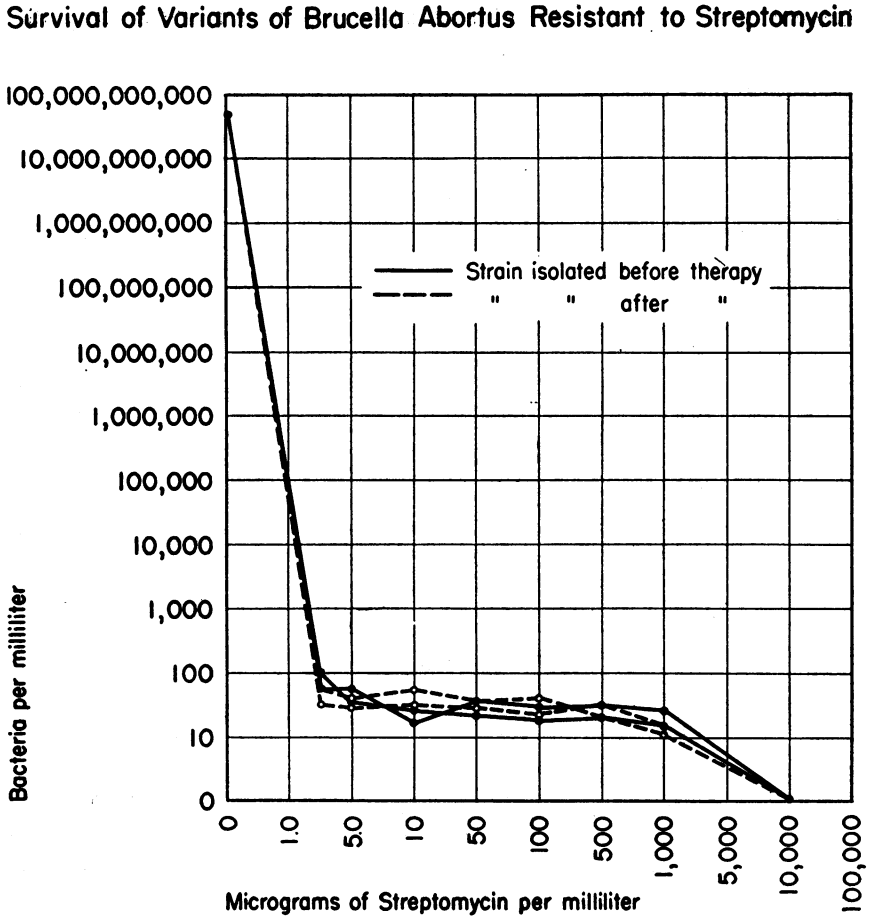

FIG. 4

TABLE V

Survival of variants of Brucella abortus *in a bacterial population of 100 billion organisms resistant to 100 μg. per ml. of streptomycin*

	Rivera	Kreutz	Steinbrecker	Wimmer	Garrity	Archer	Elsted	Lynch
Strain isolated before therapy	74	52	61	46	56	—	63	52
	96	35	51	57	48		91	63
	84	44	54	60	63		96	48
	31	96	48	73	71		82	59
	70	72	38	82	52		79	79
	81	83	48	53	49		46	82
	91	56	52	75	47		62	49
	42	65	46	41	64		68	67
	68	76	39	87	59		85	63
	72	81	45	69	56		83	85
Strain isolated after therapy	39	95	40	47	45	64	Innumer-	—
	48	77	30	94	51	78	able	
	69	75	25	96	67	62		
	41	58	30	79	45	75		
	86	91	62	91	51	79		
	68	62	37	82	62	68		
	59	73	27	58	55	56		
	62	85	48	62	49	63		
	72	64	57	82	57	84		
	68	59	46	79	60	77		

TABLE VI

Survival of variants of Brucella melitensis *in a bacterial population of 100 billion organisms resistant to 100 μg. per ml. of streptomycin*

	2716	3988	4103	3925	472
Strains isolated before therapy	240 230 236 239 233	230 228 234 230 241	240 229 230 221 224	280 271 279 286 266	246 200 266 232 244
Strains isolated after therapy	200 263 220 210 208	234 226 220 239 230	269 241 226 230 237	Innumerable	—

to the number present in strains isolated from the same patients before therapy and to strains isolated from patients who responded satisfactorily to treatment. The streptomycin survival curve of a strain of *Br. abortus* (Elsted) which subsequently became resistant to streptomycin is shown in Figure 3. There is no significant difference between this curve and that of the control strain (Lynch) which responded to therapy (Figure 1), and, as seen in Figure 4, the curve of the surviving resistant variants of a strain isolated after therapy is identical to the curve of the strain cultured from the same patient before therapy.

In order to obtain data on the number of resistant organisms present in a large number of strains of Brucella, these strains were exposed to an arbitrary concentration of 100 μg. of streptomycin per ml. These studies revealed that the number of variants present in strains obtained after therapy did not vary significantly from the number of survivors present in strains isolated before therapy. The number of resistant survivors found in the cultures of *Br. melitensis* was greater than the number of survivors present in strains of *Br. abortus* (Tables V and VI).

Other properties of the strains of Brucella recovered from patients after the completion of streptomycin therapy were studied. There were no changes in the gross colony or growth characteristics, or were changes in cell morphology noted. There were no differences in the susceptibility of these strains to the bactericidal effect of normal human serum or convalescent brucellosis serum, nor in their sensitivity to other antibiotics.

TABLE VII

Sensitivity of Brucella abortus *to aureomycin*

Strain	Minimum conc. inhibiting growth μg./ml.	Maximum conc. permitting growth μg./ml.	Sterilizing concentration μg./ml.
Lynch	1.0	0.8	125
Kreutz	1.2	0.6	62.5
Kreutz*	1.2	0.6	
Rivera	1.2	0.6	
Rivera*	1.2	0.6	62.5
Archer*	1.2	0.6	
Archer*	1.2	0.6	
Steinbrecker	1.2	0.6	62.5
Steinbrecker*	1.2	0.6	62.5
Wimmer	1.2	0.6	
Wimmer*	1.2	0.6	
Garrity	1.2	0.6	
Garrity*	1.2	0.6	
Shaeffer	1.0	0.5	62.5
Hamilton	1.2	0.6	
Elsted	1.0	0.5	
Elsted†	1.0	0.5	
Guck	1.2	0.5	
Nelson	1.2	0.6	
Johnson	1.2	0.6	
Schweim	1.2	0.6	
Schweim‡	1.2	0.6	

* Isolated after streptomycin-sulfadiazine therapy
† Streptomycin resistant
‡ Isolated after aureomycin therapy

TABLE VIII

Sensitivity of Brucella melitensis *and* Brucella suis *to aureomycin*

Strain	Minimum conc. inhibiting growth μg./ml.	Maximum conc. permitting growth μg./ml.	Sterilizing concentration μg./ml.
Br. melitensis			
3752	0.8	0.6	62.5
3752*	1.4	1.2	
3925	0.8	0.6	
3925†	0.8	0.6	
2716	0.8	0.6	
2716*	1.4	1.2	
3777	1.4	1.2	
3777*	1.4	1.2	
3988	0.8	0.6	62.5
3988*	1.2	1.0	
4103	0.8	0.6	
4103*	1.4	1.2	125
3858	0.8	0.6	
3858*	0.8	0.6	
4241	1.5	1.0	
4241‡	1.5	1.0	
4185	1.5	1.0	
4185‡	1.5	1.0	
4290	1.5	1.0	
4290‡	1.5	1.0	
Br. suis			
374	1.2	0.6	125

* Isolated after streptomycin-sulfadiazine therapy
† Streptomycin resistant
‡ Isolated after aureomycin therapy

TABLE IX

The action of aureomycin on Brucella abortus

Time interval	Control		Aureomycin (1 μg./ml.)		Aureomycin (10 μg./ml.)		Aureomycin (100 μg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	AM-free subculture	Broth turbidity	AM-free subculture	Broth turbidity	AM-free subculture
30 min.	−	++	−	++	−	++	−	++
1 hour	−	++	−	++	−	++	−	+
6 hours	−	++	−	+	−	++	−	+
24 hours	+	+++	−	+	−	+	−	−
2 days	++	++++	+	++	−	+	−	−
3 days	+++	++++	++	+++	−	+	−	−
4 days	++++	++++	+++	++++	+	++	−	−
6 days	++++	++++	++++	++++	++	+++	−	−
10 days	++++	++++	++++	++++	++++	++++	−	−
14 days	++++	++++	++++	++++	++++	++++	−	−

II. AUREOMYCIN

Studies with aureomycin were approached, first, by investigating the *in vitro* activity of the antibiotic against the three species of Brucella; secondly, by an evaluation of its action against experimentally produced infections in the chick embryo, mouse, and guinea pig; and finally, by its clinical trial in the treatment of patients with brucellosis (8, 9). The results of the *in vitro* experiments will be summarized in this report.

In vitro *Sensitivity*

Forty-four strains of Brucella, including 23 strains of *Br. abortus,* 20 strains of *Br. melitensis,* and one strain of *Br. suis,* were tested for sensitivity to aureomycin.

Method: The broth dilution method was used and varied slightly from that described for the determination of streptomycin sensitivity. The stock solution of aureomycin was prepared by adding 20 ml. of a sterile saline solution to a vial containing 20 mg. of aureomycin hydrochloride. The stock solution was stored at 4° C. for as long as a month and diluted to a concentration of 100 μg. per ml. the day the sensitivity tests were performed. In contrast to the values expressed for streptomycin and dihydrostreptomycin, the sensitivity of Brucella to aureomycin was reported as that concentration of the antibiotic required to prevent the multiplication of the bacteria, as measured by broth turbidity. The concentration actually sterilizing the cultures was determined in some of the tests by subculturing the broth from each tube on agar, and was reported as the minimal concentration of the drug from which no live organisms could be recovered.

Results: The sensitivity of the strains of Brucella to aureomycin varied from 0.6 to 1.5 μg. per ml., though concentrations as high as 62.5 to 125 μg. of aureomycin per ml. were required to sterilize the cultures (see Tables VII and VIII). Two strains of Brucella resistant to streptomycin showed no comparable increased resistance to aureomycin.

The Action of Aureomycin on Brucella

The technique of the experiment was identical to that described already for streptomycin, except that the concentrations of aureomycin employed were 1.0, 10, and 100 μg. per ml.

Results: The broth containing 1.0 μg. of aureomycin per ml. became turbid on the second day of incubation, one day later than the control. Broth turbidity developed in the tube containing 10 μg. per ml. on the fourth day, but there was no evidence of multiplication of Brucella in the broth containing 100 μg. of aureomycin per ml. Organisms were consistently recovered from the tubes containing 1.0 and 10 μg. per ml., but no Brucella were cultured from the aureomycin concentration

TABLE X

The action of aureomycin on Brucella suis

Time interval	Control		Aureomycin (10 μg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	AM-free subculture
30 min.	−	+++	−	+++
1 hour	−	+++	−	+++
6 hours	−	+++	−	++
24 hours	++	++++	−	+++
2 days	+++	++++	−	++++
3 days	++++	++++	−	++++
4 days	++++	++++	+	++++
6 days	++++	++++	+++	++++
10 days	++++	++++	++++	++++
14 days	++++	++++	++++	++++

TABLE XI

The action of aureomycin on Brucella melitensis

Time interval	Control		Aureomycin (10 μg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	AM-free subculture
30 min.	−	+++	−	+++
1 hour	−	+++	−	+++
6 hours	−	+++	−	++
24 hours	+	++++	−	++++
2 days	+++	++++	−	++++
3 days	++++	++++	−	++++
4 days	++++	++++	+	++++
6 days	++++	++++	+++	++++
10 days	++++	++++	++++	++++
14 days	++++	++++	++++	++++

of 100 μg. per ml. afer the first 24 hours of incubation (see Tables IX, X and XI).

The Relation Between the Antibrucella Effect of Aureomycin and the Bactericidal Power of Human Serum

Clinical experience with aureomycin in the treatment of brucellosis reveals that the antibiotic is apparently more effective against Brucella in human beings than it is *in vitro* or in experimentally infected animals (9). In an effort to investigate further the mechanism of action of aureomycin and its effect on certain immune mechanisms, the relationship between the bactericidal power of human serum and the antibrucella effect of aureomycin was studied.

Method: Seventy test tubes (12 × 100 mm.) were arranged in 10 series, with seven tubes in each series. To each of the 70 tubes was added 0.25 ml. of serum. Each tube in the first series was inoculated with 0.1 ml. of a solution of aureomycin containing 500 μg. per ml.; the tubes in the second series, with 0.1 ml. of a solution containing 250 μg. per ml.; and so on, decreasing the concentration by halves through the ninth series. No aureomycin was added to the tubes in the tenth series, which served as a control for determining the action of the serum alone against Brucella. The first tube in each series was then inoculated with 0.15 ml. of a suspension of a 48-hour culture of *Br. abortus,* equal in turbidity to a barium sulfate standard No. 1; 0.15 ml. of a 10^{-1} dilution was added to the second tube in each series; 0.15 of a 10^{-2} dilution, to the third tube; and so on, through the seventh tube in each series, to which was added 0.15 ml. of a 10^{-6} dilution of the suspension of Brucella. Thus, the total volume in each tube was 0.5 ml., and the final concentration of aureomycin in the first series of tubes was 100 μg. per ml., through 0.39 μg. per ml. in the ninth series. After incubating for 24 hours under 10 per cent carbon dioxide at 37° C., the contents of each tube were subcultured on tryptose agar, using a 4 mm. wire loop. The subcultures were incubated for 96 hours and the growth recorded from + to ++++.

The experiment was performed using the serum from three normal individuals, serum from the same donors heated at 56° C. for 30 minutes, and from two patients with high agglutination titers for brucellosis. A control was performed with each experiment in which broth was substituted for the serum.

Results: In series one and two, which contained the highest concentrations of aureomycin, serum interfered with the activity of aureomycin. The bactericidal power of serum, on the other hand, was enhanced by the addition of aureomycin in proportion to the concentration of the latter. The inactivated serum had no effect in increasing the antibrucella activity of aureomycin when compared to the broth control, and convalescent brucellosis serum increased only slightly the effect of the aureomycin. The most striking antibrucella

TABLE XII

The relationship between the antibrucella activity of aureomycin and the bactericidal power of serum. I. Normal human serum

No. of bacteria										
150,000,000	+	++	++	++	++	++	++	++	++	++++
15,000,000	−	+	+	+	+	+	+	+	+	++++
1,500,000	−	−	−	−	−	−	−	+	+	+++
150,000	−	−	−	−	−	−	−	−	−	+
15,000	−	−	−	−	−	−	−	−	−	−
1,500	−	−	−	−	−	−	−	−	−	−
150	−	−	−	−	−	−	−	−	−	−
	100	50	25	125	6.25	3.12	1.56	0.78	0.39	0 μg./ml.

Aureomycin concentration

effect was seen in the combination of aureomycin and normal serum having good bactericidal power (see Tables XII–XV).

The Resistance of Brucella to Aureomycin

Several unsuccessful attempts were made to isolate aureomycin-resistant variants of Brucella

TABLE XIII

The relationship between the antibrucella activity of aureomycin and the bactericidal power of serum. II. Broth control

No. of bacteria										
150,000,000	−	−	+++	++++	++++	++++	++++	++++	++++	++++
15,000,000	−	−	+	++	++	+++	+++	+++	+++	++++
1,500,000	−	−	++	++	++	++	+++	+++	+++	++++
150,000	−	−	+	+	++	++	++	++	++	+++
15,000	−	−	−	−	+	+	+	+	+	++
1,500	−	−	−	−	−	−	+	+	+	+
150	−	−	−	−	−	−	−	−	−	+
	100	50	25	125	6.25	3.12	1.56	0.78	0.39	0

μg./ml.

Aureomycin concentration

TABLE XIV

The relationship between the antibrucella activity of aureomycin and the bactericidal power of serum. III. Inactivated serum

No. of bacteria										
150,000,000	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
15,000,000	+++	+++	+++	+++	+++	+++	+++	+++	++++	++++
1,500,000	++	++	+++	+++	+++	+++	+++	+++	+++	+++
150,000	++	++	++	++	++	++	+++	+++	+++	+++
15,000	−	−	−	−	+	+	+	+	+	++
1,500	−	−	−	−	+	+	+	+	+	+
150	−	−	−	−	−	−	+	+	+	+
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0

μg./ml

Aureomycin concentration

TABLE XV

The relationship between the antibrucella activity of aureomycin and the bactericidal power of serum. IV. Convalescent brucellosis serum

No. of bacteria										
150,000,000	++	+++	+++	+++	+++	++++	++++	++++	++++	++++
15,000,000	+	+	++	++	++	++	+++	+++	++++	++++
1,500,000	+	+	+	+	++	++	++	++	+++	+++
150,000	+	+	+	+	+	+	+	+	+	++
15,000	−	−	−	+	+	+	+	+	+	+
1,500	−	−	−	−	−	−	−	−	−	−
150	−	−	−	−	−	−	−	−	−	−
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0

μg./ml.

Aureomycin concentration

present in a large bacterial population, using the method described for the isolation of streptomycin-resistant variants. A further attempt was made to develop aureomycin-resistant strains of Brucella by exposing the organisms to increasing concentrations of aureomycin.

Method: A 1:100 dilution in broth of a 24-hour culture of *Br. abortus* was exposed to aureomycin in concentrations from 250 to 0.25 μg. per ml. in the manner described for the broth dilution method of determining the sensitivity of Brucella to aureomycin. After incubating for 72 hours, the broth-aureomycin mixture in each tube was examined for turbidity and at the same time a loopful was streaked on tryptose agar. The turbid broth containing the highest concentration of aureomycin was diluted 1:100 and reexposed to aureomycin in the same manner. This procedure was repeated at 72-hour intervals, using as the inoculum in each test the broth from the preceding test containing the highest concentration of aureomycin permitting the multiplication of the bacteria.

Results: After 16 exposures of the strain of *Br. abortus* to aureomycin, there was no significant increase in resistance, as measured by the antibiotic necessary to inhibit growth, or by that amount necessary to kill the bacteria (see Figure 5).

Strains of Brucella Isolated after Aureomycin Therapy

One strain of *Br. abortus* and three strains of *Br. melitensis* isolated after aureomycin therapy were tested for sensitivity to the antibiotic. There were no changes in the sensitivity to aureomycin when compared to the sensitivity of the strain isolated from the same patient before therapy.

III. CHLOROMYCETIN

The observation by Ehrlich (10) and Gottlieb (11) that chloromycetin inhibited the growth of gram-negative organisms prompted the investigation of the action of his antibiotic against Brucella.

In vitro *Sensitivity of Brucella to Chloromycetin*

Twenty-five strains of Brucella, including 14 strains of *Br abortus,* 10 strains of *Br. melitensis,* and one strain of *Br. suis,* were tested for sensitivity to chloromycetin (lots No. Rx X3146 and

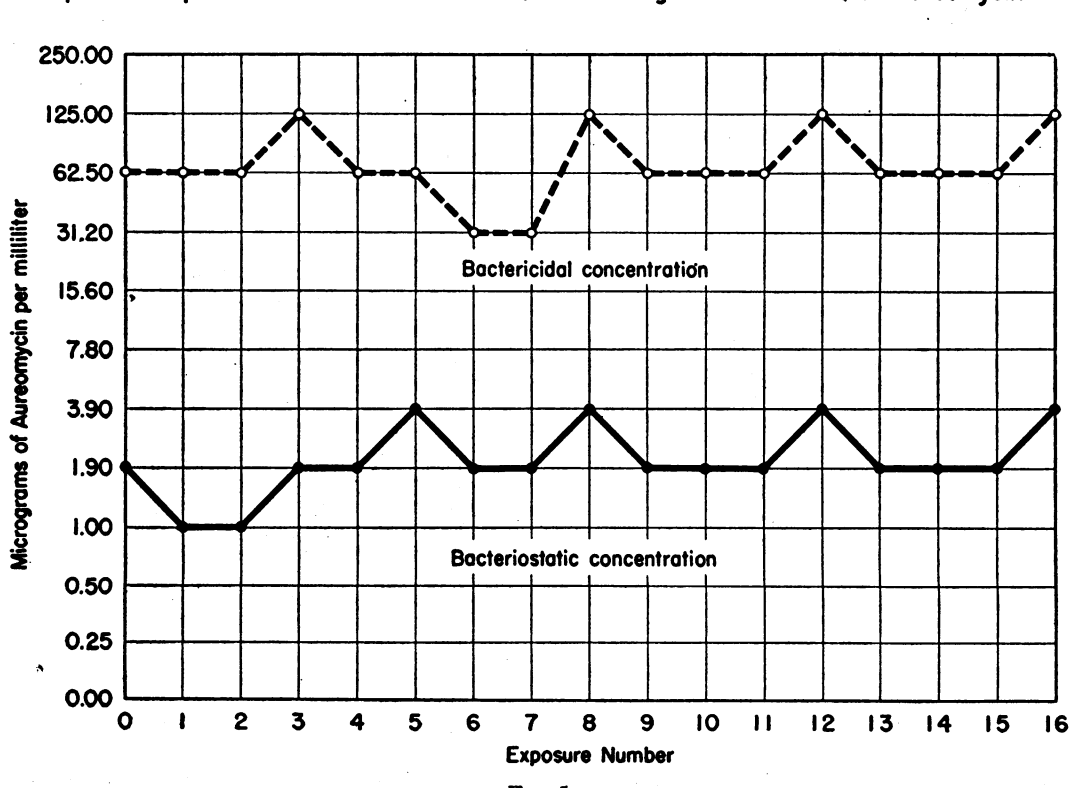

FIG. 5

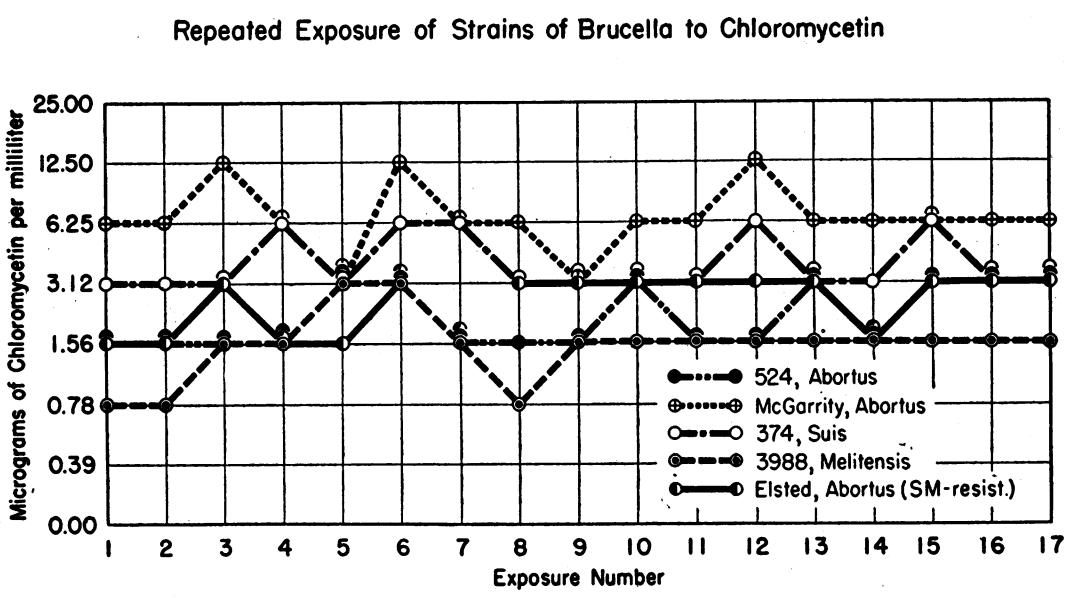

FIG. 6

120021). The method used was that described for the determination of the sensitivity of Brucella to aureomycin. The concentration of chloromycetin required to inhibit the multiplication of the strains of Brucella tested varied from 1.56 to 6.25 μg. per ml., while the concentration required to kill the organisms was from 15.6 to 125 μg. per ml. (see Tables XVI and XVII).

The Action of Chloromycetin on Brucella

The method used to study the action of chloromycetin on Brucella was similar to that described for streptomycin. The concentrations of chloromycetin used were 5.0, 20, and 100 μg. per ml. The tests revealed that there was no evidence of multiplication of *Br. abortus* when exposed to chloromycetin. Viable organisms could be recovered after 48 hours from the chloromycetin-broth mixture containing 5 μg. per ml. for five days, from the mixture containing 20 μg. per ml. for three days, and from the tube containing 100 μg. per ml. for two days. The results with strains of *Br. melitensis* and *Br. suis* varied from

TABLE XVI

Sensitivity of brucella abortus *to chloromycetin*

Strain	Lowest conc. inhibiting growth μg./ml.	Highest conc. permitting growth μg./ml.	Conc. sterilizing culture at 48 hrs. μg./ml.
Lynch	1.56	0.78	15.6
Kreutz	3.12	1.56	
Archer*	3.12	1.56	
Steinbrecker	3.12	1.56	
Steinbrecker*	3.12	1.56	
Wimmer	6.25	3.12	
Wimmer*	6.25	3.12	
Garrity	3.12	1.56	
Garrity*	3.12	1.56	>125
Elsted†	3.12	1.56	
Johnson	3.12	1.56	
Nelson	3.12	1.56	
Guck	3.12	1.56	
Schweim	3.12	1.56	

* Isolated with streptomycin-sulfadiazine therapy
† Streptomycin resistant

TABLE XVII

Sensitivity of Brucella melitensis *and* Brucella suis *to chloromycetin*

Strains of *Brucella melitensis*	Minimum conc. inhibiting growth μg./ml.	Maximum conc. permitting growth μg./ml.	Conc. sterilizing culture at 48 hrs. μg./ml.
3752	3.12	1.56	
3925	1.56	0.78	
3925*†	1.56	0.78	
2716	3.12	1.56	
3777	3.12	1.56	
3988	1.56	0.78	125
3988*	1.25	0.62	
4103	1.56	0.78	
4103*	3.12	1.56	
4858	1.56	0.78	
Strain of *Brucella suis*			
374	3.12	1.56	7.8

* Isolated after streptomycin-sulfadiazine therapy
† Streptomycin resistant

those obtained with strains of *Br. abortus* in that the multiplication of the former occurred in 5 μg. of chloromycetin per ml., though the appearance of the turbidity was delayed when compared to the broth control (see Tables XVIII–XX).

The Development of Resistance of Brucella to Chloromycetin

Preliminary attempts to produce chloromycetin-resistant variants of Brucella have been unsuc-

TABLE XVIII

The action of chloromycetin on Brucella abortus

Time interval	Control		Chloromycetin (5 μg./ml.)		Chloromycetin (20 μg./ml.)		Chloromycetin (100 μg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture
30 min.	−	++	−	++	−	++	−	++
1 hour	−	++	−	++	−	++	−	++
3 hours	−	++	−	++	−	++	−	++
24 hours	+	+++	−	++	−	+	−	+
2 days	++	++++	−	++	−	+	−	+
3 days	+++	++++	−	+	−	+	−	−
4 days	++++	++++	−	+	−	−	−	−
5 days	++++	++++	−	+	−	−	−	−
6 days	++++	++++	−	−	−	−	−	−
7 days	++++	++++	−	−	−	−	−	−

TABLE XIX

The action of chloromycetin on Brucella suis

Time interval	Control		Chloromycetin (5 μg./ml.)		Chloromycetin (20 μg./ml.)		Chloromycetin (100 μg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture
30 min.	−	+++	−	+++	−	+++	−	+++
1 hour	−	+++	−	+++	−	+++	−	+++
3 hours	−	+++	−	+++	−	+++	−	+++
24 hours	++	++++	−	+++	−	++	−	++
2 days	+++	++++	−	++	−	+	−	+
3 days	++++	++++	−	++	−	+	−	−
4 days	++++	++++	+	+++	−	−	−	−
5 days	++++	++++	+	+++	−	−	−	−
6 days	++++	++++	+	+++	−	−	−	−
7 days	++++	++++	+	+++	−	−	−	−

TABLE XX

The action of chloromycetin on Brucella melitensis

Time interval	Control		Chloromycetin (5 μg./ml.)		Chloromycetin (20 μg./ml.)		Chloromycetin (100 μg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture
30 min.	−	+++	−	+++	−	+++	−	+++
1 hour	−	+++	−	+++	−	+++	−	+++
3 hours	−	+++	−	+++	−	+++	−	+++
24 hours	++	++++	+	++++	−	+++	−	++
2 days	+++	++++	+	++++	−	++	−	+
3 days	++++	++++	+	++++	−	++	−	+
4 days	++++	++++	++	++++	−	+	−	−
5 days	++++	++++	+++	++++	−	−	−	−
6 days	++++	++++	+++	++++	−	−	−	−
7 days	++++	++++	+++	++++	−	−	−	−

cessful. The methods used included the exposure of a large bacterial population to concentrations of 10, 50 and 100 μg. of chloromycetin per ml., as described for streptomycin, and the repeated exposure of four strains of Brucella to increasing concentrations of chloromycetin (see Figure 6).

IV. OTHER ANTIBIOTICS

Eight strains of *Br. abortus* and one strain of *Br. suis* were tested for sensitivity to amorphous penicillin and to crystalline penicillin G. The broth dilution method was used. The concentration of amorphous penicillin required to prevent the multiplication of Brucella was found to vary from 5 to 31 units per ml., while the concentration required to sterilize the cultures was from 16 to 62 units per ml. The concentration of crystalline penicillin G inhibiting growth varied from 1.0 to 62 units per ml., whereas the sterilizing concentration varied from 12.5 to 250 units per ml.

The sensitivity of six strains of *Br. melitensis* to bacitracin was determined. Concentrations greater than 10 units per ml. were required to prevent multiplication of the bacteria. Two strains of *Br. abortus,* one of *Br. melitensis,* and one of *Br. suis* were tested for sensitivity to the antibiotic Q–19 (Research No. 8836–1). The concentration which inhibited growth of *Br. abortus* was 62.3 units per ml., though concentrations greater than 125 units per ml. were required to prevent the growth of the strains of *Br. melitensis* and *Br. suis.*

DISCUSSION

No naturally occurring strains of Brucella resistant to streptomycin, dihydrostreptomycin, aureomycin, or chloromycetin were found. On a weight basis, aureomycin was the most active agent against Brucella, streptomycin and dihydrostreptomycin were slightly less active, and chloromycetin was the least active antibrucella agent. There was very little strain or species variation in the sensitivity of Brucella to streptomycin, dihydrostreptomycin, or aureomycin, but there was considerable variation from strain to strain in chloromycetin sensitivity.

Streptomycin and dihydrostreptomycin were rapidly bactericidal in their action against Brucella. Aureomycin and chloromycetin, on the other hand, acted primarily by preventing the multiplication of the bacteria, and concentrations much higher than have been reported in the blood were necessary to kill Brucella.

Strains of Brucella resistant to streptomycin and dihydrostreptomycin could be readily produced *in vitro* by culturing a large bacterial population in the presence of the antibiotics, suppressing the sensitive organisms in the population and allowing the normally present resistant variants to multiply. Similar attempts to produce strains resistant to aureomycin and chloromycetin were unsuccessful. Attempts to adapt strains of Brucella to growth in the presence of high concentrations of aureomycin and chloromycetin by repeated exposure of the organisms to the antibiotics were also largely unsuccessful. One would be tempted to predict from these results that the development of resistance of Brucella in human infections to streptomycin and dihydrostreptomycin might occur rapidly and frequently, but resistance to aureomycin and chloromycetin would occur infrequently and progress very slowly.

The problem of the development of resistance of Brucella as a cause of failure in the therapy of human infections was investigated by determining the sensitivity of strains of Brucella recovered from patients after therapy. Of approximately 26 patients with bacteriologic proved brucellosis due to *Br. abortus* treated at the University of Minnesota Hospitals, there have been six relapses following combined sulfadiazine and streptomycin therapy, while approximately 30 of 65 patients treated in Mexico with infections due to *Br. melitensis* subsequently had proven bacteremia. There were two relapses out of 12 patients with infections due to *Br. abortus* following aureomycin therapy, while Brucella were recovered after treatment from five out of 26 patients with infections due to *Br. melitensis.* The clinical use of chloromycetin in brucellosis is now being studied.

Of the 13 cultures of Brucella isolated after combined streptomycin and sulfadiazine therapy, one strain of *Br. melitensis* showed increased resistance to streptomycin. Another strain of *Br. abortus,* recovered after therapy with streptomycin alone, had marked resistance to the drug. There was no evidence of an increase in the normally present streptomycin-resistant variants in the strains which failed to respond to therapy.

Three strains of *Br. melitensis* and one strain of *Br. abortus,* cultured from human subjects after aureomycin therapy, showed no changes in sensitivity to aureomycin.

One may conclude from these studies that the relapse of brucellosis following combined streptomycin and sulfadiazine or aureomycin therapy is rarely due to a change in the sensitivity of the bacteria to streptomycin or aureomycin. The evidence suggests that the strains cultured following therapy, with the exception of the two streptomycin-resistant strains, are identical to the strains isolated from the same patients before therapy. The most likely explanation for these therapeutic failures is that the antibiotics do not actually come into direct contact with all the Brucella harbored by the host within tissue cells, within granulomata present in the reticuloendothelial system, or within other walled-off foci (12). These and other possibilities are now being investigated.

SUMMARY

1. Strains of *Br. abortus, Br. suis,* and *Br. melitensis,* isolated from patients before antibiotic therapy, were sensitive *in vitro* to concentrations of streptomycin and dihydrostreptomycin of 1.0 to 2.5 μg. per ml., to aureomycin in concentrations of 0.6 to 1.5 μg. per ml., and to concentrations of chloromycetin of 1.56 to 6.25 μg. per ml.

2. The action of streptomycin against Brucella was bactericidal, while aureomycin and chloromycetin were bacteriostatic in their action.

3. Resistance of Brucella to streptomycin and dihydrostreptomycin could be developed readily *in vitro,* but increased resistance to aureomycin and chloromycetin was not demonstrated.

4. The study of 13 strains of Brucella isolated after combined streptomycin and sulfadiazine therapy revealed no change in their sensitivity to streptomycin with the exception of one strain of *Br. melitensis,* which increased in resistance from 2.5 to 50,000 μg. of streptomycin per ml. One strain of *Br. abortus* isolated after a course of streptomycin alone increased in resistance from 1.0 to 10,000 μg. of streptomycin per ml. Three strains of *Br. melitensis* and one strain of *Br. abortus* recovered from patients after aureomycin therapy showed no change in sensitivity to aureomycin.

BIBLIOGRAPHY

1. Keefer, C. S., and Hewitt, W. L., The Therapeutic Value of Streptomycin. J. W. Edwards, 1948.
2. Spink, W. W., Hall, W. H., Shaffer, J. M., and Braude, A. I., Human brucellosis: its specific treatment with a combination of streptomycin and sulfadiazine. J. A. M. A., 1948, **136**, 382.
3. Pulaski, E. J., and Amspacher, W. H., Streptomycin in brucellosis. Bull. U. S. Army, M. Dept., 1947, **7**, 221.
4. Eisele, C. W., and McCullough, N. B., Combined streptomycin and sulfadiazine treatment in brucellosis. J. A. M. A., 1947, **135**, 1053.
5. Spink, W. W., Hall, W. H., Shaffer, J. M., and Braude, A. I., Treatment of brucellosis with streptomycin and a sulfonamide drug. J. A. M. A., 1949, **139**, 352.
6. Demerec, M., Origin of bacterial resistance to antibiotics. J. Bact., 1948, **56**, 63.
7. Hall, W. H., and Spink, W. W., In vitro sensitivity of brucella to streptomycin: development of resistance during streptomycin treatment. Proc. Soc. Exper. Biol. and Med., 1947, **64**, 403.
8. Magoffin, R., Anderson, D., and Spink, W. W., Therapy of experimental brucella infection in the developing chick embryo. IV. Therapy with aureomycin. J. Immunol. (In press).
9. Spink, W. W., Braude, A. I., Castaneda, M. R., and Goytia, R. S., Aureomycin therapy in human brucellosis due to brucella melitensis. J. A. M. A., 1948, **138**, 1145.
10. Ehrlich, J., Bantz, Q. R., Smith, R. M., and Joslyn, D. A., Chloromycetin, a new antibiotic from a soil actinomycete. Science, 1947, **106**, 417.
11 Gottlieb, D., Bhattacharyya, P. K., Anderson, H. W., and Carter, H. E., Some properties of an antibiotic obtained from a species of streptomyces. J. Bact., 1948, **55**, 409.
12. Spink, W. W., Pathogenesis of human brucellosis with respect to prevention and treatment. Ann. Int. Med., 1948, **29**, 238.