sented in Fig. 4. A similar pattern of surface striations was observed on each of the 4 strains of *Euglena gracilis* studied.

Unfortunately, no intact flagella were observed and, although replicas of flagella were occasionally encountered, no evidence of structural differentiation was seen.

Summary. A regular pattern of groove-like striations was observed on the pellicle of *Euglena gracilis*.

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In vitro Sensitivity of *Brucella* to Streptomycin: Development of Resistance During Streptomycin Treatment.*

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Like many other species of bacteria brucella are remarkably sensitive *in vitro* to the action of streptomycin.[1-5] Experimental brucella infections in animals[1,3] have also been favorably influenced by the administration of streptomycin. However, the use of streptomycin in human brucella infections has not been attended by consistently favorable results.[4-8] The cause of this has not been elicited and there have been no reports in the literature of the development of resistance during the treatment of human brucella infections with streptomycin. The present report summarizes the results of numerous tests of the *in vitro* sensitivity of the 3 varieties of brucella to streptomycin. Studies are reported concerning a strain of *Br. abortus* which developed marked resistance to the action of streptomycin during the treatment of a patient having subacute bacterial endocarditis due to this organism.

Methods and Materials. The method of testing the *in vitro* sensitivity of 40 strains of brucella was as follows: a loopful of a 24-hour culture grown on a Bacto tryptose phosphate agar† slant was transferred to 10 ml of sterile tryptose phosphate broth so as to give a suspension with a turbidity equal to that of a barium sulfate No. 1 standard. This suspension was then serially diluted to 10^{-3} with tryptose phosphate broth. Pour plate colony counts indicated that this diluted suspension contained 30,000 to 300,000 viable cells per ml. Each of a series of 10 test tubes was inoculated with 4.4 ml of tryptose phosphate broth. To each of 9 tubes was added 0.1 ml of the 10^{-3} dilution of brucella. The 10th tube was used as a sterility control. To all but the sterility control was added 0.5 ml of streptomycin dissolved in sterile physiological saline, the amount of streptomycin added being sufficient to give a final concentration of 0.5 to 10 μg of streptomycin base per ml. The streptomycin solution

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† Difco Laboratories, Detroit.

was preserved in the frozen state in a dry ice freezing cabinet and showed no decline in activity as a result of storage for several weeks. Commercial streptomycin in the form of the sulfate or hydrochloride from several different producers was used. Each lot of streptomycin was checked for potency against a sensitive strain of *Br. abortus*. After the addition of the streptomycin each tube was mixed thoroughly by rotation. The tubes were then incubated at 37°C. Unless otherwise stated the strains of *Br. abortus* were incubated under 10% CO_2. After 24 hours incubation each tube was mixed and one standard loopful was subcultured on a tryptose phosphate agar plate. The plates were incubated at 37°C (*Br. abortus* under 10% CO_2) and examined for colonies of brucella with a hand lens after 96 hours.

The majority of the brucella cultures were tested for streptomycin sensitivity within a few days after they had been isolated from the blood of patients. In some cases the cultures had been suspended in 10% horse serum and stored in the frozen state for several months. After a few transfers on tryptose phosphate agar each culture was tested to ascertain that it was smooth and had the biochemical characteristics of one of the 3 varieties of brucella. We had the opportunity of testing the sensitivity to streptomycin of cultures of *Br. abortus* isolated from the blood of 3 patients before, during and/or after streptomycin therapy.

Results. In Table I are given the concentrations of streptomycin which completely inhibited the growth of 26 cultures of *Br. abortus*, 13 cultures of *Br. suis*, one stock culture of *Br. melitensis* (of unknown origin) and one stock rough culture of *Br. abortus*. None of the patients had received streptomycin therapy prior to the isolation of the organisms. Inspection of Table I reveals that all the strains of brucella were very sensitive to the action of streptomycin *in vitro* with the exception of the rough stock culture of *Br. abortus*. At first glance strains of *Br. suis* appear to be more sensitive than most strains of *Br. abortus*. The difference lies in the fact that incubation under an atmosphere containing 10% carbon dioxide reduces the sensitivity of brucella to streptomycin. Strains of *Br. abortus* which had been adapted to aerobic growth increased in sensitivity to streptomycin when the test was carried out aerobically. When strains of *Br. suis* were tested for streptomycin sensitivity in an atmosphere containing 10% carbon dioxide streptomycin proved to be less effective in inhibiting their growth. The magnitude of the difference is shown in Table II.

It was also found that the size of the

TABLE I.
Sensitivity of Brucella to Streptomycin *in vitro*.

Variety of brucella, and strain	Max. conc. permitting growth, μg/ml	Min. conc. completely inhibiting growth, μg/ml
Brucella abortus		
Stock	0.5	1
83	1	2
165	—	0.5
94	2	3
98	1	2
104	2	3
110	2	3
132	1	2
372	1	2
419	1	2
424	1	2
439	1	2
449	1	2
467	2	3
483	1	1.5
484	3	4
488	0.5	2
490	2	3
495	1	2
524	0.5	2
552	0.5	1
607	1	2
Grono (Rough)	50	100
Rowe 11-9	0.5	1
Shadick 11-5	1	1.5
Elsted 9-28	0.5	1
Brucella suis		
Stock	1	2
67	0.5	1
156	1	2
263	1	2
303	0.5	1
306	0.5	1
346	0.5	1
456	0.5	1
37	2	3
80	1	2
88	1	2
145	0.5	1
616	0.5	1
Brucella melitensis		
Stock	1	2

TABLE II.
Effect of Carbon Dioxide on *in vitro* Sensitivity of Brucella to Streptomycin.

Variety of brucella and strain	Minimum concentration of streptomycin completely inhibiting growth, μg/ml	
	Aerobic conditions	10% CO_2 added
Brucella abortus		
439	1	2
552	0.5	1
Brucella suis		
37	2	3
80	2	3
88	2	3
145	1	3
616	1	3

inoculum affected the concentration of streptomycin necessary to completely inhibit growth *in vitro*. The growth of strain 524 of *Br. abortus* was completely inhibited by 1 μg per ml when an inoculum of 30,000 cells was used but 4 μg per ml was necessary when the inoculum was 30,000,000 cells. The concentration of streptomycin necessary to completely inhibit the growth of brucella is also dependent upon the time factor. One loop subcultures taken at 24-hour intervals revealed that after 48 hours exposure to streptomycin 15 of 20 strains of *Br. abortus* were inhibited by a concentration of streptomycin equal to 50% of that necessary to inhibit their growth after only 24 hours exposure. Subcultures at the end of 3 and 7 days revealed no further apparent increase in sensitivity. Similar observations were made with 3 of 7 strains of *Br. suis* and one strain of *Br. melitensis*. Furthermore there was no evidence that prolonged incubation of brucella in contact with streptomycin promoted the growth of streptomycin-resistant variants.

It is of interest that in 2 patients with chronic brucellosis and bacteremia who failed to benefit from streptomycin therapy there was no evidence that this failure was due to the development of streptomycin-resistant organisms. Nor was there any evidence that the presence of human serum reduced the *in vitro* sensitivity of these strains of *Br. abortus* to streptomycin. The first of these patients (P.L.) received 19 g of streptomycin intramuscularly in 9 days. Strain 424, *Br. abortus*, was isolated from her blood stream before treatment and proved to be sensitive to 2 μg of streptomycin per ml. Strain 524 was isolated from her blood after 8 days of streptomycin therapy and was equally sensitive. The second patient (A.S.) was given 27.6 g of streptomycin intramuscularly in 9 days. Strain 483, *Br. abortus*, was recovered from his blood before streptomycin therapy and was inhibited by 1.5 μg streptomycin per ml. Four months later strain 11-5 was isolated from his blood, and it showed no decrease in streptomycin sensitivity.

However, in a third patient (K.E.) streptomycin resistance did develop during streptomycin therapy. This patient was a young farmer who developed acute brucellosis in 1944. His symptoms remitted with sulfonamide and vaccine therapy. However, his symptoms recurred 2 years later and were accompanied by signs of subacute bacterial endocarditis. *Br. abortus* strain 9-28 was isolated from his blood culture; it proved to be sensitive to 1.0 μg streptomycin per ml. He was then given 118 g of streptomycin intramuscularly over a period of 31 days. Strain 11-6 was isolated from his blood on the 29th day of streptomycin therapy. This strain grew in the presence of 7,500 μg of streptomycin per ml but its growth was inhibited in the presence of 10,000 μg per ml. *Br. abortus* was isolated from his blood 4 and again 5 days later; these cultures were equally resistant to streptomycin *in vitro*. One loop subcultures revealed the presence of a few viable brucella when these resistant cultures were exposed for 24 hours to concentrations as high as 50,000 μg of streptomycin per ml *in vitro*. The patient was given large doses of sulfadiazine orally for 2½ months at the conclusion of his streptomycin therapy and at the present time his infection is apparently arrested.

The streptomycin-resistant strain differed from strain 9-28 in several particulars. It grew much more slowly on tryptose phosphate agar and in tryptose phosphate broth. Its growth was particularly slow in the depth of tryptose phosphate agar pour plates, colonies not being visible until the end of 8 to 11

days of incubation. Two distinct colony forms were noted: (1) A large colony type growing relatively rapidly, producing colonies 2 to 3 mm in diameter in 48 hours. These colonies had a smooth surface and were very sensitive to the bactericidal action of human serum but grew in as much as 50,000 μg of streptomycin per ml. Gram stains revealed typical coccobacilli of uniform size and eosinophilic staining properties. They did not ferment the usual sugars and were agglutinated to a high titer by antibrucella rabbit serum. This colony type formed only a small percentage of the total bacterial population. (2) A small colony form was much more numerous. Minute colonies 0.1 mm in diameter appeared in large numbers 4 to 5 days after the resistant strain was streaked on the surface of a tryptose phosphate agar slant. They died quickly unless the inoculum was heavy. They were relatively resistant to the bactericidal action of human serum but would not grow *in vitro* in the presence of more than 7,500 μg of streptomycin per ml. Gram stains revealed a mixture of bizarre, tiny, amorphous, coccoid organisms staining only faintly red and large, dark red, coccoid forms. The small colony variant also had all the serological and biochemical characteristics of *Br. abortus*. Both colony types were smooth and grew only in the presence of added carbon dioxide.

The resistance of strain 11-6 to streptomycin has not changed over a period of 3 months; during this period it has been transferred frequently on tryptose phosphate agar slants. This strain was streaked across the surface of a tryptose phosphate agar plate containing a heavy inoculum of sensitive *Br. abortus,* strain 524 and 3 μg streptomycin per ml. No satellite colonies of strain 524 appeared adjacent to the growth of the streptomycin-resistant strain. It was concluded that the resistant strain did not produce an extracellular, soluble, diffusible streptomycin inhibitor. The apparent resistance of this strain *in vitro* decreased as the length of exposure to streptomycin was increased. Thus subcultures at the end of 7 days incubation revealed that the large colony form was inhibited by 2,500 μg per ml but the small colony variant remained resistant to 7,500 μg per ml. It is of interest that the *in vitro* growth of the resistant strain was stimulated by the addition of sublethal concentrations of commercial streptomycin. The stimulation of growth was particularly noticeable with concentrations of 50 to 1,000 μg of streptomycin per ml. This phenomenon was observed in tryptose phosphate broth, meat infusion broth, tryptose phosphate agar and human serum. The growth of the small colony form appeared to be stimulated more than that of the large colony form. No conclusive evidence was obtained indicating that any similar phenomenon occurs when sensitive strains of *Br. abortus* were exposed to sublethal concentrations of streptomycin.

Discussion. With the exception of one rough strain, all the brucella cultures tested proved to be very sensitive *in vitro* to streptomycin. Brucella appear to develop streptomycin resistance *in vivo* only after prolonged streptomycin treatment. The situation is analogous to that which obtains with *M. tuberculosis* and is presumably due to the slow rate of growth of brucella. It does not appear that the development of resistance to streptomycin by brucella can be the explanation for the frequent therapeutic failures which have been observed after relatively short periods of streptomycin therapy in human brucellosis. While better therapeutic results might follow more extended periods of streptomycin therapy, the possibility of the ensuing development of streptomycin-resistant strains of brucella must also be considered. It is reassuring however that one such streptomycin-resistant strain of *Br. abortus* was less refractory to the bactericidal action of human blood than its sensitive progenitor and remained sensitive to sulfadiazine.

Summary. 1. A method for the *in vitro* testing of sensitivity of brucella to streptomycin is described. 2. The streptomycin sensitivity of 40 cultures of brucella isolated from humans is given. 3. Studies are reported concerning a strain of *Br. abortus* which developed marked resistance to streptomycin during the treatment of a patient having subacute bacterial endocarditis.