complement-fixation on the above sera were in close agreement regardless of whether the Fl, B or C antigens were used in the test.

The results of the tests with Sn sera are perhaps more illustrative than the above cited instances. Serum samples were obtained from the same individual at various intervals during and after illness. It may be observed that the Sn serum drawn during the second febrile rise was free from complement-fixing, as well as neutralizing antibodies. On the other hand, serum taken 22 days later showed positive complement-fixation in 1:8 dilution but neutralized only 14 LD_{50} doses of virus in mice. The complement-fixing antibody titer showed no significant difference between the 22nd and 76th days after diagnosis, but the neutralizing index of the serum increased from 14 to 1,000 during the same period. Again, the results of complement-fixation with the 3 samples of Sn serum were the same regardless of whether B or C antigens were employed in performing the test.

Summary and Conclusions. Specific diagnostic complement-fixing antigens for Colorado tick fever have been prepared from infected mouse brains. The benzene-extracted antigens employed gave no false positive reactions in the presence of highly positive human syphilitic sera. Cross fixation tests between Colorado tick fever immune serum and the heterologous antigens of viral and rickettsial origin indicate that Colorado tick fever is a distinct entity and the virus is not related to any of the other infectious agents tested. These results confirm those obtained with the mouse neutralization test previously reported.[13,14] Close correlation was obtained in the complement-fixation and mouse neutralization tests with human convalescent sera. The complement-fixing and neutralizing antibodies apparently appear in the blood of humans at about the 9th to 14th day after diagnosis of illness and may remain demonstrable as long as 34 months later.

The complement-fixation test, as well as the mouse neutralization test, may prove of value in epidemiological studies on the incidence and geographic distribution of Colorado tick fever. Furthermore, if results with animal sera parallel those obtained with human sera, the tests may be applied to study the ecology of Colorado tick fever.

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Studies on Bacterial Resistance to Streptomycin.*

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The rapid development of resistance to streptomycin by various organisms, both *in vitro* and clinically, has recently been reported by a number of workers.[1-4] Miller and Bohnhoff[1] have shown that gonococci and meningococci, originally susceptible to 8-40 units per cc can become resistant to concentrations of streptomycin as high as 75,000 units per cc within 4 to 6 transfers. Klein and Kimmelman[2,3] found that with the 12 strains of Shigella studied, resistance was increased from concentrations of 3-7 units to

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1 Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

2 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **51**, 581.

3 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

4 Alexander, H. E., *J. Pediatrics*, 1946, **29**, 192.

1,000 units of streptomycin per cc, in from 2 to 11 transfers, depending on the particular strain. Clinically, Alexander[4] reported the isolation, from 2 patients, of 2 strains of *H. influenzae* which had the capacity to thrive in the presence of streptomycin in a concentration of 1,000 units per cc. The study reported in the present paper was undertaken to determine: (1) the ease with which resistance to streptomycin could be developed by various organisms; (2) the degree and duration of such resistance; and (3) the ability of resistant strains to grow in freshly defibrinated blood from a normal adult as compared with that of their parent, susceptible strains.

Materials and Methods. The strain of staphylococcus used throughout the tests was a *Staphylococcus aureus* (Merck test strain SM) obtained from Sydenham Hospital.† The strain of *H. influenzae,* type b, was isolated from a patient with influenzal meningitis in Sydenham Hospital. The pneumococcus, strain SVI, was a type I organism originally isolated in 1938 and kept since then on artificial media, with frequent passage through mice to maintain its virulence, until the beginning of the experimental period. The beta hemolytic streptococcus used was a Group A, type 14 strain obtained from Dr. Rebecca Lancefield in 1938. It was received in lyophilized form and was maintained in that state until the beginning of this study. Following initial experiments all cultures were transferred at 3- to 4-week intervals on suitable culture media not containing streptomycin. No attempt was made to maintain the original virulence of the strains during the experimental period.

The susceptibility of the strains to streptomycin was determined by the use of a series of tubes of nutrient broth containing falling concentrations of streptomycin, ranging from 125 to 6.25 μg per cc expressed as pure base.‡ Each tube contained 10% less streptomycin as measured against the initial tube in a regular progression so that the interval 125 to 6.25 represented 15 tubes. Each tube was inoculated with 0.05 cc of an 18-hour broth culture in such dilution as to yield an inoculum of 200,000 to 2 million organisms per tube. The tubes were incubated at 37°C and readings were made at the end of 24 hours. End points by this method were so apparent that plating of the mixtures of organisms and streptomycin after 24 hours incubation was not necessary. When, toward the end of this study, it became necessary to enumerate bacterial colonies, streptomycin dilutions in one cc amounts were added directly to tubes containing 9 cc melted nutrient agar and after thorough mixing were poured into sterile petri dishes. Quantitated inocula of strains to be examined were then streaked on the surfaces of these plates. The number of micrograms of streptomycin in a series of plates varied from 10 to 1000 per cubic centimeter.

After the initial susceptibility of the various test strains to the action of streptomycin had been determined, each strain was transferred daily to broth tubes containing increasing concentrations of streptomycin. To provide adequate growth factors, 20% rabbit serum was added to the broth in which streptococci and pneumococci were grown, 5% Fildes' peptic digest was added to the broth in which the influenza bacilli were grown, but infusion broth alone proved adequate for the growth of the staphylococci. Control cultures containing no streptomycin were also transferred at daily intervals on all test strains.

The bactericidal power of freshly defibrinated human blood on all strains, both before and after the acquisition of resistance, was determined in parallel each time with the same blood specimen. Five hundredths cc of each 10-fold serial dilution (10^{-1} to 10^{-6}) of an 18-hour broth culture was added to 0.25 cc of freshly defibrinated human blood in each of 6 sterile pyrex tubes. These tubes were sealed, placed in a rotating box and maintained at 37°C for 24 hours. After preliminary observation, incubation without rotation was continued for another 24 hours,

† These strains from Sydenham Hospital were obtained through the courtesy of Dr. Horace L. Hodes and Miss Helen Zepp.

‡ The preparation of streptomycin employed had a potency of 1000 units per milligram of pure base. (Pfizer, lot No. 4613.)

after which the tubes were opened and the contents cultured on blood agar plates. In order to determine the number of organisms added to each tube of blood, count plates were made of the 10^{-5} and 10^{-6} dilutions.

Results. (1) when tested initially, the staphylococcus was inhibited by 8 μg of streptomycin per cc; the *H. influenzae* by 1 μg; the pneumococcus by 37 μg; and the streptococcus by 25 μg per cc. After 6 daily transfers in the presence of streptomycin, the pneumococcus and the streptococcus grew in maximal concentrations of 125 μg per cc. The staphylococcus, originally inhibited by 8 μg, was able to multiply in 1,000 μg per cc after 12 daily transfers. The *H. influenzae,* initially inhibited by 1 μg, grew in 60 μg per cc after 14 daily transfers. Unfortunately, at this time both the parent and resistant strains of *H. influenzae* were lost and further studies could not be carried out.

(2) The susceptibility to streptomycin was retested on all strains (parent, parallel control and resistant) after they had been transferred repeatedly in the absence of streptomycin for 6 months. It was found that the parent and control strains of the streptococcus, pneumococcus and staphylococcus were still inhibited by the same concentrations of the antibiotic. The resistant streptococcus, although not in contact with streptomycin for this period, had not only maintained its ability to grow in 125 μg per cc, but actually showed an increase in resistance in that it could now grow in 500 μg per cc. Growth was inhibited at 1,000 μg per cc. The resistant staphylococcus had also maintained its capacity to grow in 1,000 μg per cc and when tested in even higher concentrations, it was found to grow abundantly in 5,000 and 10,000 μg per cc. Moderate growth was observed in the presence of even 50,000 μg per cc. This strain was not tested at higher concentrations of the drug because the supply of streptomycin was still limited. Unlike the streptococcus and the staphylococcus, the resistant pneumococcus did not maintain its ability to grow in 125 μg of streptomycin per cc. When retested, it was found to be inhibited by concentrations greater than 87 μg per cc.

At the time of reexamination of these strains for their susceptibility or resistance to streptomycin one additional set of experiments was initiated. If resistant strains are mutants thrown off by susceptible strains, it should theoretically be possible to select them much more readily in very young cultures. Accordingly, the susceptible staphylococcus was grown in broth at 37°C for 1 hour, 2 hours and 4 hours, after which a known inoculum of each culture was put into broth to which streptomycin had been added in the following final concentrations: 0, 10, 25, 75, 100, 250, 500, 750, 1000 μg per cc. The tubes were incubated at 37°C for 24 hours and the results were then read. All tests were run in duplicate.

It was found that with an inoculum of 200 million organisms per tube the one-hour culture was able to grow in a concentration up to 25 μg of streptomycin. The 4-hour culture, in an inoculum of 11 million organisms per tube (the culture having been diluted to 10^{-2}, because growth was so heavy at 4 hours) grew out in 10 μg of streptomycin per cc but not at higher concentrations. Growth was observed with the 2-hour culture with an inoculum of 400 million bacteria per tube at 10, 25, 50, 100, 500, 750 and 1000 μg of streptomycin per cc. No growth was apparent in those tubes containing 75 μg per cc. Thus, at a very early stage of growth it was possible, by chance selection, to isolate highly resistant mutants. When these resistant mutants were subcultured to broth containing streptomycin it was found further that they bred true in that they grew in the same concentrations or higher. Therefore with a single transfer, resistance to streptomycin in a concentration as high as 1,000 μg per cc was obtained.

An attempt was made to explain this last observation. A series of agar plates containing 0, 10, 25, 50, 75, 100, 250, 500, 750 and 1,000 μg of streptomycin were inoculated with 0.1 cc amounts of a 10^{-5} dilution of each of the following: (1) the original staphylococcus which grew initially in not more than 8 μg of streptomycin; (2) the one-

TABLE I.

Proportion of Organisms in Various Staphylococcus Cultures Growing at Indicated Streptomycin Concentrations.

Test inoculum of staphylococci	Concentration of streptomycin in medium (μg per cc)																			
	0		10		25		50		75		100		250		500		750		1000	
	Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Control culture	720	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Culture with growth at 25 μg streptomycin per cc	640	100	—	—	200	31.3	2	0.3	2	0.3	4	0.6	2	0.3	0	0	0	0	0	0
Same at 100 μg	1200	100	—	—	—	—	—	—	—	—	800	66.5	920	76.6	800	66.6	760	63.2	720	60
Same at 500 μg	700	100	—	—	—	—	—	—	—	—	—	—	—	—	700	100	600	85.7	600	85.7

hour culture which grew in not more than 25 μg; (3) the 2-hour culture which grew at 100 μg, and (4) the 2-hour culture which was capable of growth in the presence of 500 μg. After 36 hours incubation at 37°C, colonies were observed with the original staphylococcus on the plate containing no streptomycin, but none were present on those plates containing 10 to 1,000 μg. The one-hour culture which grew initially in 25 μg showed abundant growth on plates containing streptomycin up to this level. On each of the plates containing 50, 75, 100 and 250 μg, 2 to 4 colonies grew out, indicating that approximately 1% of the inoculated organisms were resistant to higher concentrations of streptomycin while 99% were inhibited by more than 25 μg per cc. The 2-hour culture, resistant to 100 μg in broth, grew well on all plates. Colony counts indicated that most of the organisms able to grow in 100 μg could also grow in all 4 higher concentrations. Likewise, with the 2-hour culture originally resistant to 500 μg, almost all the organisms were found to grow in concentrations of 500, 750 and 1,000 μg. These results are summarized in Table I.

(3) A comparison of the ability of parent and resistant strains to grow in fresh human blood as in a bactericidal test was the third objective of this study. During the initial period of these experiments, a comparison of the ability of parent, parallel control and resistant strains to grow in normal human blood revealed no difference with the 3 organisms tested—the staphylococcus, the pneumococcus and the streptococcus. As measured by this method, the virulence or potential invasiveness of all 3 resistant strains was unchanged with respect to the virulence of the homologous parent strains.

When retested 6 months later, it was found that the virulence of the parent, control and resistant strains of the pneumococcus had diminished markedly and to the same extent. All 3 strains, parent, control and resistant, of staphylococci had, on the other hand, maintained their original virulence. Finally, in the case of the streptococcus, whereas the parent and control strains showed marked

TABLE II.
Summary of Bactericidal Studies on Streptomycin Susceptible and Resistant Strains.

Organism	Strain examined	Streptomycin resistance, μg per cc	Min. No. of organisms required to initiate growth per cc of blood	
			Initial period	Six-month re-test
Pneumococcus	*Parent	37	160	100,000
	Resistant	125	340	100,000
Staphylococcus	*Parent	8	320	500
	Resistant	1000-50,000	200	150
Streptococcus	*Parent	25	32	120,000
	Resistant	125-500	36	160

* Since the parallel control strains gave essentially identical results as the parent strains in every instance, they were not recorded in this table.

diminutions in virulence, the resistant strain had retained its original virulence. This observation was repeated and no evidence of a decrease in titre was found. These results are summarized in Table II.

Discussion. Streptomycin resistance can readily be induced *in vitro*. In these studies, the relative ease was found to vary with the particular organism used. With the streptococcus, pneumococcus, and *H. influenzae*, the initial resistance could be enhanced from 4 to 20 times by serial passage in streptomycin containing media. The strain of staphylococcus, originally completely inhibited by 8 μg was observed to acquire the ability to grow in the presence of more than 50,000 μg of streptomycin.

Although repeated serial passage appeared necessary to select out the resistant variants, further study with the staphylococcus revealed that in the early logarithmic stage of growth it was possible, by chance selection, to isolate highly resistant mutants in a single transfer.

The proportion of resistant organisms present in an inoculum could be estimated by seeding streptomycin containing plates. The original strain was completely inhibited by 10 μg. A strain found to be resistant to 25 μg per cc contained 33% resistant colonies at this drug level as compared with the control plate. This same strain contained 0.2% to 0.4% colonies resistant to 50, 75, 100 and 250 μg of streptomycin per cc. The proportion of colonies resistant to higher concentrations up to 1,000 μg of streptomycin was exceedingly high (60 to 100%) when the inoculum was derived from strains found resistant to 100 μg or greater. This distribution of resistant colonies was present when serial transfer or random selection from young cultures was used as the screening procedure. In the usual method for determining streptomycin resistance, the small number of resistant organisms present is probably insufficient to initiate growth and, therefore, it may not contribute to the observed end point. The rate at which resistance is apparently acquired is a function of the proportion of resistant organisms present at each successive level. This phenomenon may also explain erratic results sometimes noted. The growth which appears at a high level of streptomycin despite the absence of growth at lower levels, may be attributed to the random selection of resistant variants.

Repeated transfer by subculture on non-streptomycin containing media over a period of 6 months did not result in a loss of streptomycin resistance, with the possible exception of one strain, the pneumococcus.

The bactericidal studies were performed as an index of virulence. No loss in potential invasiveness accompanied the acquisition of streptomycin resistance. After serial transfer of parent, parallel control and resistant strains for a period of 6 months, the ability of the pneumococci to survive in whole blood was greatly diminished while that of the staphylococci was unchanged. With the susceptible parent and parallel control strains of streptococci a marked diminution was noted, but the resistant streptococcus retained its potential invasiveness.

The general experience has been that strains which become resistant to penicillin lose their invasive properties as indicated by growth in whole blood.[5,6] Strains which have developed resistance to sulfonamides, however, retain this capacity unchanged.[7] From the present observations it appears that, like the latter, organisms resistant to streptomycin may also retain their virulence. In addition, such strains may preserve the ability to maintain their invasiveness upon prolonged artificial cultivation to a greater extent than the parent, nonresistant culture. The clinical implications of these observations that invasiveness may be maintained and retained are evident. The development of streptomycin resistance should be reduced to a minimum through the use of a dosage schedule which will ensure early, adequate blood levels. In the light of these studies, an adequate level must be defined in terms of the specific susceptibility of the organism plus its potential ability to acquire resistance. The level of tolerance to streptomycin as well as the rate at which this tolerance is acquired are important factors and a large margin of safety is necessary.

Summary. 1. Resistance to streptomycin can be induced by repeated transfer of various organisms in streptomycin containing media. 2. In the early logarithmic stage of growth, highly resistant mutants can be isolated, by chance selection, in a single transfer. 3. In most instances, acquired resistance to streptomycin is maintained. 4. Organisms resistant to streptomycin may retain their original virulence as measured by the bactericidal test. 5. The acquisition of resistance to streptomycin with the maintenance of virulence may have certain therapeutic implications.

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Effect of Atabrine on Auricular Fibrillation in the Dog.

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At present there are no available data concerning the action of atabrine on cardiac muscle. However, the analogous physiological effects of quinine and atabrine in diminishing the contractibility and increasing the refractory period of striated and smooth musculature,[1] lead to a reasonable assumption that atabrine will produce similar actions to those of quinine on cardiac muscle.

Quinine and especially its more powerful isomer, quinidine, have been used for many years as a routine practice in the treatment of auricular fibrillation presumably because of their ability to prolong the refractory period in cardiac muscle and its nodal tissues as well as to decrease myocardial excitability.[2] Since recent evidence has been advanced[1,3] which clearly indicates that atabrine possesses more potent physiological and pharmacological properties when compared with quinine, it seemed most interesting to see whether atabrine would also control auricular fibrillation. Experiments in the dog were, therefore, devised to test the validity of this concept.

Methods. Five dogs were anesthetized

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