is shown a representative kymograph tracing from the series of experiments illustrating the contrast between the two preparations. In Table I is presented a summary of the several experiments.

The average apnic dose for a dog is approximately 20 μ while 200 μ will produce apnea in about 80% of normal humans. Thus, the dose levels of Intocostrin producing significant effects in the dog heart-lung preparation are, in terms of units per mass of tissue, considerably in excess of the amounts required for the desired paralyzing action in intact animals or man.

Conclusion. A highly purified curare preparation (Intocostrin) produces a positive inotropic effect in the isolated dog heart. This effect is not due to the *d*-tubocurarine content of the preparation or to the preservative and, therefore, must be due to some unidentified component.

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Antibiotic Studies on Beta Hemolytic Streptococci.* V. Streptomycin Resistance Acquired by Group A, B, and C Organisms.†

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The development of *in vitro* resistance to streptomycin has been reported for a number of organisms including staphylococci, meningococci, gram negative enteric bacilli, chromogenic bacteria, Klebsiella, and *Hemophilus influenzae*. Miller and Bohnhoff,[1] for example, demonstrated approximately a 2000 fold rise in resistance in four strains of gonococcus and nine of meningococcus after 4 to 6 daily transfers on streptomycin medium. Seligmann and Wassermann[2] described a 50,000 fold increase in streptomycin resistance by a strain of *Staphylococcus aureus* after 7 transfers on antibiotic medium and a change in excess of 50,000-fold in one strain of *B. prodigiosus* after 9 transfers.

Resistance by streptococci has not been thoroughly studied. In a group A, type 14 organism reported by Chandler and Schoenbach,[3] a 5-fold rise in resistance after 6 daily transfers on streptomycin medium was observed. No other reference to streptomycin resistance of beta hemolytic streptococci has been found.

The present study compares the rate at which resistance develops among strains of Lancefield group A, B, and C streptococci. In addition we have attempted to compare the hemolytic, antigenic and virulence behavior of streptomycin resistant strains with that of penicillin resistant strains described in preceding communications.[4,5,6]

The streptomycin sensitivity range in our laboratory[7] for recently isolated strains of beta hemolytic streptococci has been found to be 10 to 50 μg/ml for 275 strains of group A, 30 to 400 μg/ml for 20 strains of group B, and 10 to 50 μg/ml for 47 strains of group C

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† This report was presented in part at the meeting of the American Association for the Advancement of Science in Chicago, December 30, 1947.

1 Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1946, **130**, 485.

2 Seligman, E., and Wassermann, M., *J. Immunol.*, 1947, **57**, 351.

3 Chandler, C. A., and Schoenbach, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 208.

4 Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 208.

5 Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 212.

6 Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 215.

7 Unpublished data.

TABLE I.
Streptomycin Resistance Induced in Groups A, B, and C Hemolytic Streptococci by Subcultivation on Streptomycin Medium.

Group	Strain	No. of transfers	Initial streptomycin sensitivity,* μg/ml	Final streptomycin sensitivity,* μg/ml	Fold change
A	S_1	40	25	3,500	140
A	S_2	40	50	3,500	70
A	S_3	40	10	1,400	140
A	S_4	40	25	3,500	140
A	S_5	40	25	6,000	240
A	S_6	40	25	4,500	180
A	U_5	40	50	3,500	70
A	U_6	40	10	1,200	120
B	T_5	24	75	30,000	400
B	T_7	40	75	30,000	400
B	T_8	40	100	4,000	40
B	T_9	40	75	7,000	93
B	T_{10}	40	75	12,000	160
B	T_{11}	40	75	7,000	93
B	T_{12}	24	100	30,000	300
C	U_1	38	10	30,000	3,000
C	U_2	40	25	15,000	600
C	U_3	40	25	5,000	200
C	U_7	40	50	12,000	240
C	U_8	40	25	12,000	480
C	U_9	40	25	5,000	200
C	U_{10}	40	10	2,000	250
C	U_{11}	40	25	6,000	240

* Streptomycin sensitivity is defined as the highest concentration of streptomycin in the medium which permits colonies to grow.

organisms. The Fleming[8] ditch plate method was employed for these determinations. Sensitivities falling within these ranges were reported by Hobby and Lenert[9] for two strains of group A and one each of groups B and C streptococci.

Materials and Methods. Twenty-three strains of beta hemolytic streptococci, 8 each of group A and C and 7 of group B, were isolated from cultures of the nose or throat obtained from patients with acute upper respiratory disease. None had received streptomycin therapy. The organisms were grouped by the Lancefield technic. The strain designations as well as many of the parent strains are the same as those used in a preceding communication.[4]

Bacto heart infusion agar (Difco) containing 5% defibrinated sheep's blood and varying concentrations of streptomycin was used in 100 mm Petri dishes. All plates were poured at a maximal temperature of 50°C. Commercial streptomycin sulfate (Lilly), lot No. 6329-451634 with an expiration date of November 30, 1948, was employed for the entire study. Fresh stock solutions were made in normal saline on alternate days and refrigerated at 4°C. The potency of the streptomycin solutions was controlled by standardization against *Staphylococcus aureus SM.*

The ditch plates used to determine streptomycin sensitivity have been described previously.[4]

To induce resistance 7 or 8 strains of streptococci were inoculated in single lines on each blood agar plate. The group A, B, and C organisms were each considered as a separate series. Between 4 and 8 concentrations of streptomycin were used in a set for each serial transfer. The confluent bacterial growth on the blood agar plate was emulsified in saline and subcultured directly onto a new set of plates; a total of 40 such subcultivations was made in each series. Transfers to the next set of plates were made from colonies on medium with the highest concentration of

[8] Fleming, A., *Proc. Roy. Soc. Med.*, 1941, **34**, 342.

[9] Hobby, G. L., and Lenert, T. F., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 242.

TABLE II.
Reduction in Streptomycin Resistance After Passage Through Normal Mice.

Group	Strain	No. of transfers on streptomycin medium	*In vitro* streptomycin sensitivity, μg/ml	No. of passages in normal mice	Final streptomycin sensitivity after serial passages in normal mice, μg/ml
A	S_1	40	3,500	15	1,000
A	U_5	40	3,500	15	1,000
B	T_5	24	30,000	15	4,000
B	T_9	40	7,000	15	1,000
C	U_1	38	30,000	15	20,000
C	U_2	40	15,000	15	15,000

streptomycin permitting growth. The streptomycin sensitivity for each was considered to be the highest quantity of the antibiotic that allowed good growth. Serial transfers were discontinued when a strain had acquired a resistance of 30,000 μg of streptomycin per ml of medium.

The method for determining virulence by intracerebral inoculation of mice has been described in a preceding report.[4] Streptolysin O and S production were determined by the methods given by Todd.[10] Streptokinase (fibrinolysin) content was measured by the method outlined by Boisvert.[11]

Results. A. Resistance Acquired on Medium Containing Streptomycin. Eight strains each of group A and C streptococci and 7 of group B were subcultivated serially in an attempt to induce resistance to streptomycin. Table I summarizes these results. The minimum increase in resistance was 40-fold in a group B organism and the maximum, 3000-fold in a group C organism, after 40 and 38 transfers respectively. In general the group A, B, and C organisms acted similarly, most strains showing from a 100- to 400-fold rise in resistance after 40 transfers on increasing concentrations of streptomycin. When the strains within a group are compared, the group C organisms are the most uniform in their rate of development of resistance and the group B the most variable.

B. Resistance Acquired on Control Medium. All 23 organisms were subcultured serially on control medium, *i.e.*, streptomycin-free blood agar. No increase in streptomycin resistance was found after 40 transfers, when the organisms were tested by the streptomycin whole-plate technic or by the ditch plate method. No changes in hemolysis or in the colonial form were observed; a minimum of dissociation from mucoid to matt or matt to glossy was seen.

C. Loss of Acquired Resistance. Three strains each of group A, B, and C streptococci after acquiring streptomycin resistance were subcultured serially 100 times on streptomycin-free medium in an attempt to restore the original sensitivity. The organisms were tested on the 25th, 50th, and 100th transfer by the whole-plate method. No change in resistance outside the limit of experimental error was observed.

Two strains each of groups A, B, and C streptococci after acquiring streptomycin resistance were passed intracerebrally 15 times in normal mice. The results are given in Table II. Both strains of group A and both of group B demonstrated a significant loss of streptomycin resistance. One strain of group C (U_2) showed no change, and one a 33% reduction in resistance. None, however, returned to the original level of sensitivity.

D. Change in Virulence. Mouse virulence titrations were performed on parent strains, on subcultures of the same organisms after 40 transfers on control medium, and on the strain after 40 transfers on streptomycin medium. A total of 9 strains, 3 each of groups A, B, and C, were studied. The results are summarized in Table III. All of the group A and B streptococci showed a marked loss of mouse virulence after serial transfers on streptomycin medium. Two strains of group C showed a similar loss while one (U_2) remained unchanged. Most of the micro-

[10] Todd, E. W., *J. Path. and Bact.*, 1938, **47**, 423.
[11] Boisvert, P. L., *J. Clin. Invest.*, 1940, **19**, 65.

TABLE III.
Mouse Virulence of Groups A, B, and C Streptococci with Acquired Streptomycin Resistance.

Group	Strain	Initial LD_{50}*	LD_{50}* after 40 transfers		LD_{50}* of resistant strains after 15 mouse passages
			Control medium	Streptomycin medium	
A	S_1	.000024	.00060	>.03	.00030
A	S_3	.00077	.00048	.0095	
A	U_5	.000095	.00017	.0017	.00053
B	T_5	<.0000030	.0000012	.030†	.00030
B	T_8	<.0000030	.0000060	.0095	
B	T_9	.00030	.00011	.0053	.0095
C	U_1	.000095	.000060	.030‡	.0095
C	U_2	.00060	.0017	.00053	.00095
C	U_3	.00018	.000060	.0030	

* LD_{50} is stated as the amount of undiluted bacterial suspension in normal saline that will kill 50% of the mice injected. This suspension has a density equal to McFarland No. 1 standard and contains approximately 2×10^8 organisms per ml.
† After 24 transfers.
‡ After 38 transfers.

organisms demonstrated relatively little loss of mouse virulence after serial transfers on control medium.

In an attempt to restore the virulence lost by growth on streptomycin medium, six strains were passed intracerebrally 15 times in normal mice. The virulence was only partially restored in 5 strains, and was unchanged in one (U_2) in which the original virulence had not been lost.

Since mouse virulence of beta hemolytic streptococci is probably related to at least two factors, M substance and hyaluronic acid, an effort is being made to demonstrate changes in these substances. Most investigators agree that hyaluronic acid is contained in the capsule of groups A and C streptococci. Using the India ink wet preparations described by Hirst and Lancefield,[12] we have looked for the presence or absence of a demonstrable capsule in 4-hour subcultures of parent and resistant organisms. There appears to be a loss of the capsule in both group A and C resistant bacteria. Confirmation of these observations is being sought through chemical studies.

E. Antigenic, Hemolytic, and Colonial Changes. All 23 organisms were again grouped by the Lancfield technic after acquiring streptomycin resistance. All remained group specific.

Studies are in progress on changes in streptokinase, streptolysin O, and streptolysin S production of representative parent and resistant streptococci. These will be reported in detail in the near future. A brief summary of the preliminary findings follows. None of the group B strains produced streptokinase or streptolysin O or S. All of the parent group A and C organisms and all except 2 of the resistant strains lysed a normal human fibrin clot in less than one hour. Two group A resistant strains failed to lyse the clot in 24 hours. Quantitative studies using purified human fibrinogen and human thrombin are being carried out.

Qualitative differences between parent and resistant organisms in streptolysin S production were found in the 2 group A and 3 group C streptococci studied. This difference will be quantitatively titrated against standard anti-streptolysin rabbit serum.

A change in the hemolytic behavior of streptococci when growing on streptomycin medium was observed one or more times in all 23 strains. Transient conversion from beta to alpha or gamma type of colonies appeared when the organisms were growing on maximal concentrations of streptomycin. The same strains showed the normal beta hemolysis on lower concentrations of streptomycin or on plain blood agar. In some instances a mixture of alpha, beta, and gamma types of colonies were evident on the same concentra-

[12] Hirst, G. K., and Lancefield, R. C., *J. Exp. Med.*, 1939, **69**, 425.

tion of streptomycin. Group C organisms showed this hemolytic change earliest and most consistently. None, however, demonstrated an irreversible change.

Minute colonies resembling glossy variants were observed with all strains when growing on maximal concentrations of streptomycin. These were not true glossy variants, however, since the usual matt form was seen in the next subcultivation on lower concentrations of streptomycin.

Discussion. Streptococci of groups A, B, and C develop streptomycin resistance at about the same rate and to the same degree. The relative rate of development of resistance to penicillin, in which group A organisms acquired resistance slowly and to a slight degree, group C intermediately, and group B most rapidly, has been previously described.[4,5,6] When compared with resistance in meningococci,[1] or staphylococci,[2] streptococci acquire resistance relatively slowly.

The loss of virulence found in both penicillin- and streptomycin-resistant streptococci is of considerable interest. A simple explanation could be the concurrent loss of capsular substance with its content of hyaluronic acid. It has been fairly well demonstrated that this is at least one of the factors responsible for the virulence of the streptococcus. This is probably only a partial explanation. Further studies are being made on the type-specific M substance to determine whether a similar alteration occurs in this antigen, which seems to play an important role in the virulence of these organisms.

Another fundamental change in their bacterial metabolism is found in penicillin-resistant,[4,5,6] streptomycin-resistant, and bacitracin-resistant[13] streptococci; with all 3 antibiotics transient reversible changes in hemolysis from beta to alpha or gamma types of colonies were observed. After observing this phenomenon in cultures in our laboratory, Dr. Edgar W. Todd suggested that the change probably was in streptolysin S production of resistant organisms, since streptolysin S is largely responsible for surface hemolysis. We have studied only 5 strains of group A and C streptococci to date but in all 5 the parent strains produced streptolysin S in large amounts and the resistant members produced little or none. Final proof of this depends, however, on titration of these lysins against a known anti-streptolysin S serum. This is being carried out with rabbit antiserum.

Loss of the group specificity, which was previously observed in members of all three groups on acquiring penicillin resistance, was not seen after induced streptomycin resistance.

Summary. 1. Twenty-three strains of beta hemolytic streptococci of groups A, B, and C were subcultured serially on media containing streptomycin in an effort to induce resistance. The minimum increase in resistance after 40 transfers was 40-fold and the maximum 3000-fold. Most organisms, however, of all 3 groups showed increases of 100- to 400-fold.

2. The streptomycin sensitivity range for recently isolated strains of beta hemolytic streptococci is 10 to 50 μg/ml for 275 strains of group A, 30 to 400 μg/ml for 20 strains of group B, and 10 to 50 μg/ml for 47 strains of group C organisms.

3. Organisms which had acquired streptomycin resistance maintained it throughout subcultivations on blood agar medium.

4. Partial loss of resistance was observed in 5 of 6 strains on serial passages through mice.

5. Mouse virulence was decreased in 8 of 9 resistant organisms studied. Virulence was partially restored by passage through mice.

6. Group specificity was maintained by all resistant organisms.

7. Reduction in streptokinase production occurred in 2 of 23 resistant organisms and streptolysin S in 5 of 5 group A and C strains.

8. Transient changes in the colonial appearance and changes in hemolysis from beta to alpha or gamma types of colonies were demonstrated by all strains when growing on maximal concentrations of streptomycin. These colonies reverted to their original type when subcultured onto blood agar.

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[13] Unpublished data.