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Antibiotic Studies on Beta Hemolytic Streptococci. I. Penicillin Resistance Acquired by Group A Organisms.

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There are many reports on the development *in vitro* of resistance to penicillin by staphylococcus, pneumococcus, and meningococcus, but few on beta hemolytic streptococcus. Two groups of investigators studied a single strain of streptococcus (C203, a Group A, type 1 organism) and reported somewhat different rates of development of resistance. Rake and his co-workers[1] found a 30-fold increase in resistance after 58 transfers in penicillin broth, while Todd *et al.*[2] noted only a 5-fold increase after 35 subcultures and a 4-fold increase after 78 subcultures in penicillin broth. No references to induced resistance for other strains of Group A streptococci and none for Group B or C organisms were found.

The present study was made to look for differences among strains within the A Group. Those reported in the succeeding two communications were undertaken to compare these with the behavior of Group B and C organisms respectively. Variations in penicillin sensitivity have been shown by organisms of these 3 serological groups.[3-8] The similarities and differences reported by these investigators were observed by us in a screening test of 203 recently isolated strains of Group A, 16 strains of Group B, and 24 strains of Group C streptococci, using the Fleming[9] ditch plate method.

Materials and Methods. All the strains of beta hemolytic streptococci were isolated from cultures of the nose or throat obtained from patients with acute upper respiratory disease. None had received penicillin therapy. The organisms were grouped by the Lancefield technic. Bacto heart infusion agar (Difco), containing 5% defibrinated sheep's blood, was used as the solid medium. Veal infusion broth, containing 5% defibrinated sheep's blood, served as the liquid medium. In the earlier series, designated R and A, commercial, amorphous sodium penicillin (Lederle) was used, while in the later series, S, T, and U, crystalline sodium penicillin G (Lilly) was employed. Standardization of these penicillins and the final sensitivity tests were performed with standard sodium penicillin G* with a potency of 1667 units per mg.

The ditch plates used to determine the penicillin sensitivity of streptococci were prepared by removing a central trough from a blood agar plate and replacing it with melted agar containing 5% sheep's blood and varying concentrations of standard penicillin G. When this central strip solidified, heavy inocula of organisms were streaked in rows directly across the plate. The character of the growth on both penicillin and penicillin-free media was observed on these plates. The absolute penicillin sensitivity, however, could not be measured by this technic since penicillin diffused into the agar adjacent to the trough.

1 a. McKee, C. M., and Houck, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33; b. Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., *J. Immunol.*, 1944, **48**, 271.

2 Todd, E. W., Turner, G. S., and Drew, L. G. W., *Brit. Med. J.*, 1945, **2**, 603.

3 Watson, R. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 65.

4 Selbie, F. R., Simon, R. D., and McIntosh, J., *J. Path. and Bact.*, 1945, **57**, 47.

5 Springarn, C. L., and King, A. N., *U. S. Nav. Med. Bull.*, 1946, **46**, 239.

6 Simmons, R. T., and Christie, R., *M. J. Australia*, 1946, **1**, 349.

7 Rantz, L. A., Randall, E., Spink, W. W., and Boisvert, P. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 54.

8 Rutherford, R. H., Marquardt, G. H., and Van Ravenswaay, A. C., *J. Missouri State Med. Assn.*, 1946, 535.

9 Fleming, A., *Proc. Roy. Soc. Med.*, 1941, **34**, 342.

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TABLE I.
Penicillin Resistance Induced in Group A Beta Hemolytic Streptococci by Subculture on Penicillin Medium.

Strain	Number of transfers	Initial penicillin Sensitivity,* u/ml	Final penicillin Sensitivity,* u/ml	Fold change
S_1	60	.01	.04	4
S_2	60	.02	.04	2
S_3	60	.01	.17	17
S_4	60	.01	.01	0
S_5	60	.01	.045	4.5
S_6	60	.01	.04	4
U_5	60	.01	.06	6
U_6	60	.01	.17	17
A_1	60	.01	.04	4
A_2	60	.01	.04	4
R_2	56	<.01	.015	>1.5
R_4	56	<.01	.02	>2
R_5	56	<.01	.03	>3

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

Penicillin sensitivity is defined as the maximum concentration of penicillin on which bacterial growth occurs on the medium in the trough.

To induce resistance, 5 to 7 strains of streptococci were inoculated in single lines on each blood agar plate of a series. Between 3 and 8 concentrations of penicillin were used in a set for each serial transfer. In the R series, subcultures were made alternately on plain blood infusion broth and blood agar medium containing penicillin for a total of 56 serial transfers on each. In the A, S, T, and U series the confluent bacterial growth on the blood agar plate was emulsified in saline and subcultured directly to a new set of plates; a total of 60 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 penicillin permitting growth. The penicillin sensitivity for any transfer was considered to be the highest concentration of penicillin that allowed good growth.

Mouse virulence titrations were made as follows: The organisms were incubated at 37°C for 18 hours in tryptose phosphate broth (Difco) containing 5% normal horse serum. The culture was centrifuged at 2000 RPM for 5 minutes, the supernatant fluid discarded, and the sediment resuspended in a sufficient quantity of normal saline to give a final density equal to the McFarland Standard No. 1. The average plate colony count with this concentration of organisms was approximately 2×10^9 organisms per ml of the saline suspension. Four- to five-week-old white mice (Germantown strain) were inoculated intracerebrally in pairs with 0.03 ml of the 10^0 to 10^4 dilution of the organisms. For this procedure the mice were lightly anesthetized with ether, the skin and hair sterilized with iodine, and the injection made midway between the right ear and eye at a depth of approximately 4 mm. The infected mice were observed for a period of five days. The LD_{50} was calculated by the Reed and Muench formula.

A. *Resistance Acquired on Media Containing Penicillin.* Thirteen strains of Group A streptococci were subcultivated serially in an attempt to induce resistance to penicillin. A seventeen-fold rise in penicillin resistance was the maximum change obtained. In only two strains did an increase of this magnitude occur. Eleven organisms showed either no change or only a slightly increased resistance after 56 to 60 transfers onto media containing penicillin. Table I summarizes these results.

B. *Resistance Acquired on Control Media.* Eight sensitive parent strains of Group A streptococci were subcultured serially on penicillin-free blood agar medium. After 60 transfers no appreciable increase in penicillin resistance was found when tested by the penicillin whole plate method or by the ditch

TABLE II.
Comparison of the Rates of Development of Penicillin Resistance of Parent and Control Strains of Streptococci.

Strain	Mean of first 15 transfers of parent* strain on penicillin medium u/ml	Mean of first 15 transfers of control† strain on penicillin medium u/ml
S_1	.013	.026
S_2	.015	.012
S_3	.011	.013
S_4	.012	.017
S_5	.015	.009
S_6	.011	.011
U_5	.016	.008
U_6	.015	.019

* Parent strain is a freshly isolated organism.
† Control strain is the parent strain after 60 transfers on control medium.

plate method.

Because preliminary results suggested that adaptation of the streptococci to growth on artificial medium influenced the development of penicillin resistance, the organisms after 60 subcultures on control medium were changed to penicillin medium for 15 transfers. The mean value for the first 15 subcultures of the parent and the control strains on penicillin medium are given in Table II. From these values it is evident that repeated subculturing on plain blood agar does not facilitate the acquisition of resistance to penicillin for most strains of Group A streptococci.

C. *Loss of Acquired Resistance.* Five strains of streptococci after 56 to 60 transfers on penicillin agar, were subcultured serially on penicillin-free medium in an attempt to restore the original sensitivity. The three strains from the R series (R2, 4, and 5) were transferred daily 25 times and the 2 strains from the A series (A1 and 2), 50 times. At the end of 25 subcultures 4 of the 5 strains had returned to their original level of penicillin sensitivity. After 50 transfers the fifth strain had returned to its original level. Todd and his co-workers[2] observed this same behavior with a resistant culture of the C203 strain after 76 transfers in plain broth.

Four strains were passed serially by intracerebral inoculation in white mice. All of these streptococci had returned to their original level of penicillin sensitivity by the 12th passage.

D. *Change in Virulence.* Mouse virulence titrations were performed on 10 of the 13 parent strains, on subcultures of the same strains after 60 transfers on control medium, and on all strains after 56 to 60 transfers on penicillin medium. The results are summarized in Table III. All strains showed a marked loss of mouse virulence after serial transfers on penicillin medium. Most of the strains demonstrated relatively little loss of mouse virulence after serial transfers on control medium.

In an attempt to restore the virulence lost by growth on penicillin medium, five strains were passed intracerebrally 12 times through mice. An increase in virulence occurred in 3 of 5 strains.

E. *Antigenic, Hemolytic, and Colonial Changes.* The serological behavior of the streptococci after 56 to 60 serial transfers on penicillin or penicillin-free medium is given in Table IV. In 6 resistant and 2 control strains (*i. e.,* after repeated transfers on control medium) we were unable to demonstrate the presence of the group specific precipitinogen by the Lancefield technic.

A change in the hemolytic behavior of the streptococci when growing on penicillin medium was observed in 10 of the 13 strains studied. Transient conversion from beta to alpha or gamma type of colony appeared when the organisms were growing on maximal concentrations of penicillin. The same strains showed the normal beta hemolysis on lower concentrations of penicillin or on plain blood agar. In some instances a mixture of alpha, beta, and gamma types of colonies was evident in a given culture on the same concentration of penicillin. When single colonies of any of these types were picked under a dissecting microscope and subcultured on lower concentrations of penicillin or on plain blood agar, they always showed beta hemolysis. The possibility of contamination was eliminated by regrouping isolated alpha and gamma colonies.

The colonial appearance of resistant strains of streptococci did not differ appreciably from the parent or the control strains. How-

TABLE III.
Mouse Virulence of Group A Streptococci with Acquired Penicillin Resistance.

Strain	Initial LD_{50}*	LD_{50}* after 60 transfers on control medium	LD_{50}* after 60 transfers on penicillin medium	LD_{50}* of resistant strain after 12 mouse passages
S_1	.0000096 ml	.00012 ml	.0030 ml	.000048 ml
S_2	.00012	.00048	.015	.00096
S_3	.00096	.000039	>.03	
S_4	.000030	.00075	>.03	
S_5	.000096	.00012	.015	
S_6	.000030	.000096	>.03	
U_5	.00048	.00019	>.03	
U_6	.0012	.00096	>.03	
A_1	.00096	.0096	.0060	
A_2	.000030	.0096	.0096	
R_2			.030	.0096
R_4			.030	>.03
R_5			.030	>.03

* 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 2×10^9 organisms per ml.

TABLE IV.
Group Specificity of Streptococci After 56 to 60 Serial Transfers on Penicillin and Penicillin-free Media.

	Strain												
	S_1	S_2	S_3	S_4	S_5	S_6	U_5	U_6	A_1	A_2	R_2	R_4	R_5
Parent culture	A	A	A	A	A	A	A	A	A	A	A	A	A
Final control culture	A	A	A	A	A	A	N	A	N	A	—	—	—
Final penicillin-resistant culture	A	A	A	A	A	A	N	N	A	N	N	N	N

A = group A.
N = not groups A, B, or C.
— = grouping not performed.

ever, transient changes were noted during the process of serial transfers when organisms were growing on maximal concentrations of penicillin. On these plates the colonies frequently were tiny with narrow zones of alpha or beta hemolysis or with gamma hemolysis. No permanent dissociation from mucoid to matt or matt to glossy types was observed. When heavy inocula were streaked on plates with a concentration of penicillin too high to permit visible colonies, hemolysis in the medium and a disappearance of the surface luster were commonly found. Subcultures from these plates failed to grow.

Discussion and Conclusions. Penicillin resistance in strains of Group A streptococci would be of considerable clinical importance, but there is little evidence that such strains actually produce human infections. No proven instance has been found in a review of the literature. Experimentally, resistance to penicillin can be induced in most strains by *in vitro* technics. However, the increase in resistance, as compared with that of strains of staphylococci, is relatively slight with a 17-fold rise as the maximum change obtained. Using *in vivo* methods in embryonated eggs and in mice,[10] no alteration in penicillin sensitivity was observed. Furthermore, when resistance is induced, animal virulence falls. A similar loss of animal virulence has been described by Miller and Bohnhoff[11] for meningococci and McKee and Houck[1a,1b] for the C203 strain of Group A streptococcus, type 3 pneumococcus and staphylococcus.

The change in hemolysis seen in streptococci that are growing on medium containing penicillin may be the result of an alteration by penicillin in the metabolism of the organism. A change in streptolysin O or S must

[10] Unpublished data.

[11] Miller, C. P., and Bohnhoff, M., *J. Inf. Dis.*, 1947, **81**, 147.

also be considered. Further study is necessary to elucidate the nature of this interesting phenomenon.

Summary. 1. Thirteen strains of Group A streptococci were transferred serially on medium containing penicillin in an effort to induce resistance. Two strains developed a 17-fold increase in resistance. Eleven strains either remained unchanged or showed an increase up to only 6-fold at the end of 60 subcultures.

2. No increased resistance was induced in the same organism by serial transfers on control medium.

3. Strains which had acquired resistance were restored to their original level of penicillin sensitivity by serial subculture on penicillin-free medium or by serial intracerebral passages in mice.

4. A marked loss of virulence for mice was shown by all resistant strains of streptococci. The virulence was restored in 2 strains by passage in normal mice.

5. In about one-half of the resistant strains the group specific antigen could not be demonstrated.

6. Transient changes in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were shown by most strains when growing on maximal concentrations of penicillin.

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Antibiotic Studies on Beta Hemolytic Streptococci: II. Penicillin Resistance Acquired by Group B Organisms.

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Most strains of group B beta hemolytic streptococci possess a higher degree of initial penicillin resistance than group A organisms. The range reported by 3 groups of investigators[1,2,3] was 0.0625 to 0.25 units of penicillin per ml of medium. We have found the upper and lower limits of sensitivity to extend from 0.01 to 0.10 unit of penicillin per ml of medium by the Fleming ditch-plate method[4] for 16 freshly isolated strains of group B streptococci.

The present study was carried out in an effort to determine the rate of development of penicillin resistance *in vitro*. In addition we were interested in learning whether the antigenic, hemolytic, colonial, and mouse virulence changes found in resistant group A organisms[5] occurred in group B streptococci.

Results. A. *Acquired Resistance on Medium Containing Penicillin.* Ten strains of group B streptococci were transferred on increasing concentrations of penicillin. The results are given in Table I. A straight line curve results when the mean of fifteen transfers is plotted on semi-logarithmic paper. The graph of the daily values shows the same general trend but with many irregularities in the curve. These variations can be explained by slight differences in the amount of medium per plate, daily loss of potency of penicillin in stock solution, and errors in judgment in estimating the proper range of penicillin concentrations needed for each transfer.

The maximum change occurred in the R_1

1 Watson, R. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 65.

2 Simmons, R. T., and Christie, R., *M. J. Australia*, 1946, **1**, 349.

3 Rutherford, R. H., Marquardt, G. A., and Van Ravenswaay, A. C., *J. Missouri State Med. Assn.*, 1946, 535.

4 Fleming, A., *Proc. Roy. Soc. Med.*, 1941, **34**, 342.

5 Gezon, H. M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 207.