group specificity in some strains after serial transfers on penicillin medium remain unexplained.

Summary. 1. Ten strains of Group B streptococci were transferred serially on media containing increasing quantities of penicillin in an effort to produce resistance. All 10 organisms showed a significant increase in resistance varying from 11 to 190-fold over the parent strains.

2. No increased resistance was induced by serial transfers on control medium.

3. In contrast to Group A organisms, Group B streptococci did not lose resistance on subculture on blood agar or on serial passage through mice.

4. Virulence decreased in all resistant strains of streptococci. The pathogenicity was partially restored in one organism but remained unchanged in two after passage in normal mice.

5. In only 2 resistant strains was the group specificity lost.

6. Transient alterations in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were demonstrated by all strains when grown on maximal concentrations of penicillin. These colonies reverted to the parent type when subcultured on blood agar.

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Antibiotic Studies on Beta Hemolytic Streptococci: III. Penicillin Resistance Acquired by Group C Organisms.

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Streptococci of Lancefield Group A or B present group differences as well as strain variations within a group, when transferred serially on media containing penicillin. These characteristics are described in the preceding reports.[1,2] In general, Group A differ from Group B streptococci in having a lower initial penicillin sensitivity. They show less tendency to acquire penicillin resistance *in vitro,* lose acquired resistance more rapidly on passage in mice or plain medium, and develop colonial and hemolytic changes less quickly when growing on penicillin medium.

The initial penicillin sensitivity of Group C organisms is reported[3-5] to vary from 0.0078 to 0.0313 units of penicillin per ml of medium. We have studied 24 recently isolated strains by the Fleming[6] ditch-plate method and have found their sensitivity range to be 0.007 to 0.03 units of penicillin per ml of medium.

The present study was undertaken to determine the similarities and differences between streptococci of Groups A, B, and C as regards their ability to acquire penicillin resistance.

Results. Acquired Resistance on Medium Containing Penicillin. Five strains of Group C streptococci underwent serial transfer on blood agar containing penicillin. Three of the 5 organisms after 60 subcultures demonstrated a moderate increase in penicillin resistance varying from 11- to 16-fold. Two forms, U_3 and R_3, after 60 and 56 transfers respectively, developed only a slightly increased resistance. The results are given in Table I.

Acquired Resistance on Control Medium. Four strains of streptococci subcultured from

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TABLE I.
Induced Penicillin Resistance in Group C Beta Hemolytic Streptococci.

Strain	Number of passages	Initial penicillin sensitivity,* u/ml	Final penicillin sensitivity,* u/ml	Fold change
U_1	60	.01	.16	16
U_2	60	.01	.14	14
U_3	60	.03	.14	4
U_4	60	.02	.33	16
R_3	56	<.01	.02	> 2

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

TABLE II.
Comparison of the Rate 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
U_1	.033	.020
U_2	.030	.020
U_3	.033	.016
U_4	.033	.034

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

the original parent organisms were passed on control (*i.e.*, penicillin-free) blood agar. After 60 transfers these bacteria were retested for penicillin sensitivity by the Fleming ditch-plate method. For purposes of comparison alternate lines on plates containing varying concentrations of penicillin were inoculated with the paired resistant and control organisms. No increased penicillin resistance was demonstrated by these control organisms.

In an effort to discover whether adaptation to growth on artificial medium would influence the development of penicillin resistance, the control organisms were carried on penicillin medium for the 61st to 75th passages. The mean values for the first 15 transfers of the parent and the control strains on penicillin medium are given in Table II. Again as was found with both Group A and B streptococci, the repeated subcultivation on plain blood agar does not facilitate the acquisition of resistance to penicillin.

Loss of Acquired Resistance. Only one strain, R_3, was grown serially on penicillin-free medium and two strains, U_1 and R_3, passed in untreated mice in an effort to restore the penicillin sensitivity. After 25 transfers on plain blood agar and 12 passages intracerebrally in mice no decrease in the acquired resistance of the organism had occurred.

D. *Change in Virulence.* Using the method described in the first report of this series,[1] mouse virulence studies were performed on all 5 strains. The results appear in Table III. As was observed previously with Group A and B streptococci, all organisms suffered a marked loss of mouse virulence after repeated subcultivation on penicillin-containing medium and relatively little change after a like number of transfers on control medium.

Two resistant strains were passed 12 times

TABLE III.
Mouse Virulence of Group C 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
U_1	.00015 ml	.000096 ml	>.03 ml	.006 ml
U_2	.0018	.0015	>.03	
U_3	.0000048	.000030	>.03	
U_4	.000060	.00048	>.03	
R_3	.000096		>.03	.00096

* 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				
	U_1	U_2	U_3	U_4	R_3
Parent culture	C	C	C	C	C
Final control culture	C	C	C	C	—
Final penicillin resistant culture	C	N	N	N	C

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

in mice in trying to restore the virulence. The LD_{50} values are given in Table III. In both instances only a partial restoration was accomplished.

Antigenic and Hemolytic Changes. The results of the serological grouping of the streptococci before and after repeated transfers on penicillin and control media are given in Table IV. In the 4 control organisms studied no loss of the group specific antigen

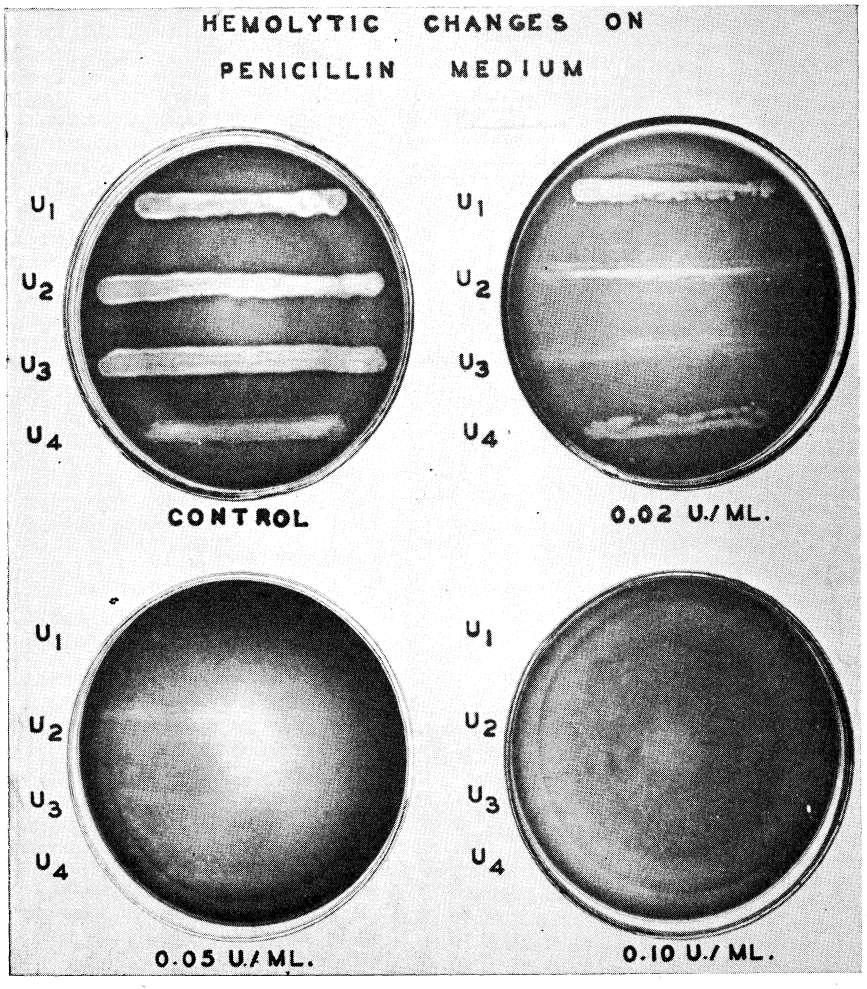

FIG. 1.

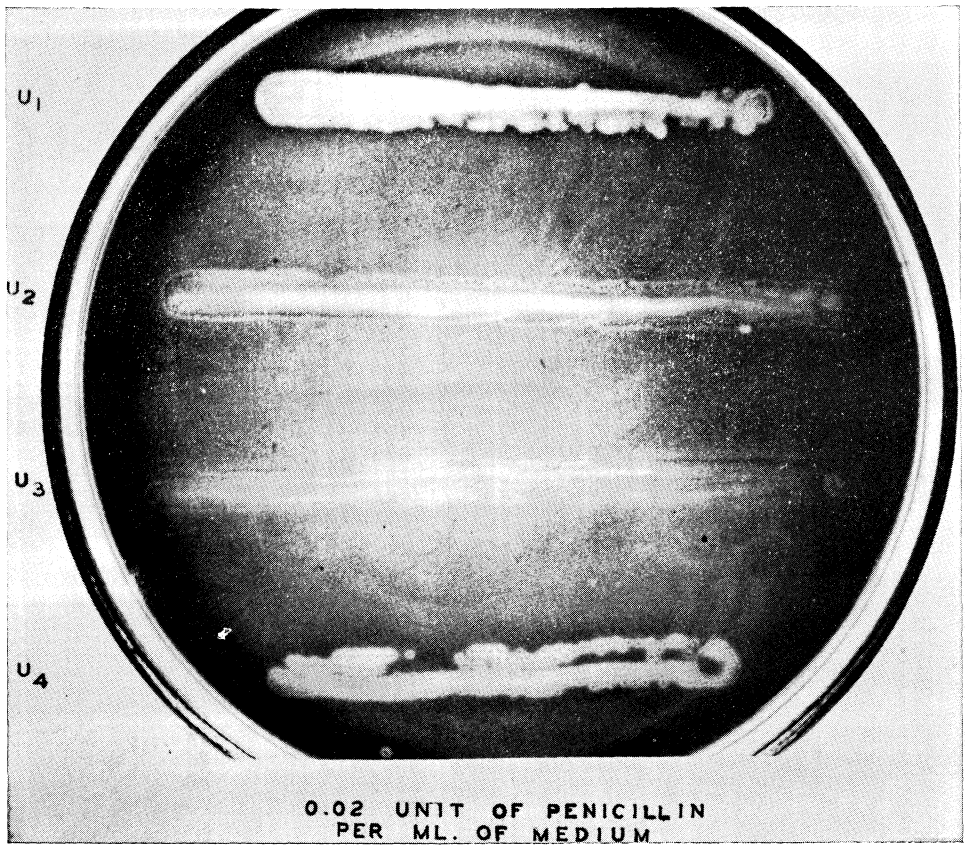

FIG. 2.

occurred. After acquiring penicillin resistance, only 2 of 5 strains were classifiable by the Lancefield technic.

The change in the character of the hemolysis described for Group A and B organisms appeared as well in all 5 strains of Group C streptococci when the organisms were grown on medium containing penicillin. An example of this hemolysis is given in Fig. 1. A close-up view of the 0.02 unit per ml plate of Fig. 1 is shown in Fig. 2. Here the hemolytic changes are best demonstrated by the U_2 strain with both green and clear hemolysis present in the same growth. Frequently the transformation to alpha or gamma types of colonies was present in the maximum concentration of penicillin and also at levels below that which would permit growth. Mixtures of alpha, beta, and gamma hemolytic colonies were seen on plates containing a single concentration of penicillin. Some strains of Group C streptococci normally show a zone of greenish hemolysis outside the wide clear zone. Forms with this characteristic display an exaggeration of this color when grown on minimal concentrations of penicillin.

Discussion and Conclusions. The behavior of Group C streptococci on penicillin medium resembles in many respects that of Group A and B organisms.[1,2] The same drop in mouse virulence, transient hemolytic changes, and colonial changes were demonstrated by nearly all strains of the 3 groups. In general, Group B streptococci develop a much higher degree of *in vitro* resistance at a faster rate than either Group A or C organisms. The acquired resistance appears to be a more permanent characteristic of Group B and C organisms than of Group A. Only in the latter did passage in normal mice or on penicillin-free medium restore the sensitivity.

If these *in vitro* studies could lead to the

conclusion that resistance to penicillin in Group A and C streptococci is not likely to develop, it would be of great clinical importance. However, it should be emphasized that these observations on the group and strain behavior of artificially resistant streptococci, while interesting and valid, must not be assumed to apply by analogy to natural resistance.

Summary. 1. Five strains of Group C streptococci were subcultured serially on medium containing penicillin in an effort to induce resistance. Three of the 5 strains developed a 14- to 16-fold increase after 60 transfers. Two strains demonstrated only a 2- to 4-fold increase after similar subcultures.

2. No increased resistance was induced by serial transfers on control medium.

3. Acquired resistant organisms maintained their resistance on serial subcultures on blood agar medium or on serial passages through mice.

4. Decreased mouse virulence was shown by all resistant strains of streptococci. Relatively little change appeared in the control organisms. Lost virulence was partially restored in 2 strains by passage in normal mice.

5. The group specific precipitinogen was demonstrable in only 2 of the 5 resistant strains, but was present in 4 control organisms.

6. Transient changes in the colonial appearance, and changes in hemolysis from beta to alpha or gamma were demonstrated by all strains when grown on maximal concentrations of penicillin. These colonies reverted to the parent type when subcultured on blood agar.

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Propagation of the Mammary Tumor Milk Agent in Tumors from C_{57} Black Mice.*

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If females of the C_{57} black strain obtained the mammary tumor milk agent by nursing females of cancerous strains, mice of some sublines may, when they are maintained as breeders, show incidences of mammary cancer in excess of 50%[1-4] although mice of this strain are generally regarded to have a "low susceptibility" for spontaneous mammary cancer. In 2 studies it was determined that the incidence may be approximately as high,[4] or even higher,[2] in the progeny than in the mice of the fostered generation, demonstrating that the females may propagate and transmit the milk agent. Fostered females and their descendants of a line of the C_{57} black strain which gave a low incidence of spontaneous tumors have also been found to transmit the milk agent.[5]

The milk agent, one of the 3 causative factors for mammary cancer in mice,[6] may be

* Assisted by grants from the Citizens Aid Society of Minneapolis, the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, the Cancer Research Fund of the University of Minnesota Graduate School, the Minnesota Cancer Society, and the Elsa U. Pardee Foundation.

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