ated derivatives of estradiol. These latter two reports dealt with substances which had little or no estrogenic activity. The estrone sulfate used in these experiments had marked estrogenic potency but still did not show any marked localization in the estrogenic susceptable tissues, the highest concentration encountered was in the kidney which is one of the routes of excretion. This is borne out by the high amount found in the urine. That some of this hormone is excreted by way of the gastrointestinal tract is demonstrated by the presence of radioactive estrone sulfate in the small intestine and feces. This observation substantiates the finding of Levin(5) who reported large amounts of estrogen in the feces of pregnant cows.

Summary and conclusions. 1. Radioactive estrone sulfate shows no definite localization in any of the target organs following intravenous or subcutaneous injection into mature female rats in spite of marked estrogenic response. 2. Estrone sulfate is rapidly hydrolyzed in the body. 3. Estrone sulfate is excreted not only by way of the urinary tract but considerable amounts find their way into feces.

We wish to thank Dr. Edward C. Reifenstein, Jr., and Ayerst, McKenna and Harrison for the labeled and non-labeled estrone sulfate which they generously supplied.

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Antibiotic Studies on Beta Hemolytic Streptococci: VII. Acquired *in vitro* Resistance to Bacitracin* (17954)

HORACE M. GEZON, DORCAS M. FASAN, AND GEORGE R. COLLINS
(Introduced by C. Phillip Miller)
From the Department of Pediatrics, University of Chicago.

Acquisition of bacitracin resistance by beta hemolytic streptococci has not been reported to date. Meleney(1) found no increase in resistance to bacitracin in 5 strains of group A beta hemolytic streptococci after 26 serial transfers on bacitracin agar. Stone(2) studied 4 strains of *Staphylococcus aureus* and reported an increase in resistance of a maximum of 125-fold in one strain after 62 exposures to bacitracin and a minimum of 3-fold in another strain after 35 exposures. The resistance decreased rapidly on serial transfers in bacitracin-free broth but was stable on storage in a refrigerator for a period up to 29 days.

The present study was undertaken (1) to determine whether or not streptococci of groups A, B and C would develop bacitracin resistance in the manner similar to that observed for other antibiotics and (2) to determine whether or not bacitracin altered the biology of streptococci in a manner similar to that produced by penicillin(3-6), streptomycin(7), and aureomycin(8).

Materials and methods. The 23 strains of beta hemolytic streptococci, 8 of group A, 8

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Bacitracin was supplied by the Commercial Solvents Corporation, through the Antibiotics Study Section of the National Institute of Health.

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TABLE I.
Bacitracin Sensitivity of Group A, B, and C Beta Hemolytic Streptococci Isolated in the Period from December 7, 1948 to March 22, 1949.

Units per ml	Group A strains		Group B strains		Group C strains	
	No.	%	No.	%	No.	%
.003	116	57				
.005	66	32	4	18	27	54
.01	22	11	6	27	17	34
.1			7	32	5	10
.5			3	14	1	2
1			2	9		
Total	204		22		50	

of group C, and 7 of group B, previously described(3-8) were again employed. Bacitracin sensitivity was determined by the Fleming(9) ditch plate method. All of the bacitracin used for the induction of resistance was from a single lot. Some of the subsequent studies were performed with bacitracin of two other lots that had been standardized against the original material. The antibiotic was made up at 3 to 5 day intervals in physiological saline at a pH of 6.8 and stock solutions stored in the refrigerator. To induce resistance *in vitro*, 40 subcultivations were made on blood agar containing graded concentrations of bacitracin by the method previously described(3,8). Comparison was made of the mouse virulence of the parent strain, the control strain (parent strain after 40 serial transfers on blood agar plates containing no antibiotic), the induced resistant strain and the resistant variant after 12 to 14 passages in normal mice by an intracerebral inoculation method(3,8). Serial dilutions were prepared in broth and pour plate colony counts determined on each strain. The methods used for the determination of streptolysin "S", streptokinase, proteinase, and ribonuclease activity have been described previously(10). The desoxyribonuclease activity of the streptococci was measured by the turbidimetric method described by McCarty(11).

Results. A. *Initial bacitracin sensitivity.* Table I shows the bacitracin sensitivity of 276 recently isolated beta hemolytic streptococci of groups A, B, and C. The sensitivity varied from 0.003 to 1 unit per ml. The range of sensitivity within groups increased in the order B greater than C greater than A.

B. *Acquired resistance on bacitracin media.* The minimum increase in resistance was 8-fold in a group A organism; the maximum increase was 3,750-fold in a group B organism. In general there were wide differences in the rate of development of resistance within all 3 groups but the extreme variations were most evident in group B. This is clearly demonstrated by comparing the mean and median fold increase in resistance by groups. There appears to be no characteristic pattern in the group behavior. The results are summarized in Table II.

C. *Acquired resistance on control media.* The 23 parent strains were subcultured serially 40 times on bacitracin-free blood agar. No change in bacitracin resistance was evident at the end of these serial subcultivations and no colonial or hemolytic changes were noted.

D. *Loss of acquired resistance.* Three bacitracin-resistant strains of group A, 3 of group B, and 3 of group C were transferred serially 100 times on bacitracin-free blood agar plates and simultaneously 12 to 14 times intracerebrally in normal mice. The organisms were retested by the whole plate method on the 50th and 100th serial plate transfers and at the end of 12 to 14 serial mouse passages. In addition, these strains were tested after 20 months storage in blood infusion broth at 4°C. During this period of 20 months, 2 subcultivations had been made into fresh blood

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TABLE II.
Bacitracin Resistance Induced in Group A, B, and C Beta Hemolytic Streptococci After 40 Subcultivations on Bacitracin Medium.

Group	No. strains	Range fold incr.	Mean fold incr.	Median fold incr.
A	8	8- 100	43	40*
B	7	15-3,750	596	50
C	8	12- 300	99	50*

* Approx. values.

TABLE III.
In vitro Sensitivities of Bacitracin-Resistant Beta Hemolytic Streptococci After Serial Transfers on Control Medium, Storage in Broth at 4°C or Passage Through Mice.

		Mean sensitivity u/ml				
Group	No. strains	Parent	Resistant	Plate transferred 100 χ s	Stored at 4°C	Mouse-passed, 12 χ s
A	5	.01	.5	.01	.02	.01
B	5	.7	32	5	4	2
C	3	.03	7	2	1	1

TABLE IV.
Mouse Virulence of Beta Hemolytic Streptococci with Acquired Bacitracin Resistance.

			LD_{50}* after 40 transfers		LD_{50}* of resistant strain after 12 mouse passages
Group	Strain	Initial LD_{50}*	Control medium	Bacitracin medium	
A	S2	1.6×10^2	1.3×10^4	2.7×10^3	1.8
	4	3.7×10^2	3.8×10^3	3.4×10^4	1.5×10^4†
B	T5	1.9×10^4	1.2×10^2	$>1.2 \times 10^7$	2.2×10^5
	8	7.8×10^2	7	4.9×10^4	1.6×10^3
C	U1	2.1×10^5	4.9×10^4	5.1×10^4	2.2×10^4†
	2	9.3×10	2.5×10^3	1.1×10^6	2.8×10^5

* The LD_{50} is expressed in terms of the number of viable streptococci injected intracerebrally.
† Virulence determined after 14 mouse passages.

broth. These results are recorded in Table III. It is evident that acquired bacitracin resistance is a fairly labile characteristic of streptococci and that cold storage as well as normal plate or mouse passage permits a decrease in resistance. Three of 3 resistant group A strains returned to the level of the parent sensitivity by the 100th serial transfer on control media. Two of 5 lost all while the remaining 3 lost part of the acquired resistance on storage for a period of 20 months. Three of 3 lost all acquired resistance on serial passages through normal animals. The group B and C resistant organisms were easily resensitized by any of the 3 methods but this was to a lesser degree than those of group A.

E. *Change in virulence.* The mouse virulence of 9 organisms was studied before and after induced resistance. The results in representative strains are given in Table IV. Decreased virulence accompanied acquired resistance in 7 of 9 strains. The change varied from approximately 10-fold in 2 organisms to approximately 10,000-fold in 1 strain. Twelve to 14 serial passages in normal mice partially or completely restored the virulence in 6 of 9 strains.

F. *Antigenic and hemolytic changes.* The 23 beta hemolytic streptococci were regrouped by the Lancefield technic after acquiring bacitracin resistance. All group A and C strains remained group specific. Three of the 7 group B strains could not be grouped after induced resistance. The parents of the S_6 and U_6 strains were the only group A strains which could be typed by the precipitin technic.†

† These results were confirmed by Naval Medical Research Unit No. 4.

TABLE V.
Streptolysin "S" Production by Bacitracin-Resistant Beta Hemolytic Streptococci.

Group	Strain	Parent		Control‡		Bacitracin-resistant		Resensitized by 50 or 100 plate transfers		Resensitized by 16 mouse passages	
		Units*	Density†	Units	Density	Units	Density	Units	Density	Units	Density
A	S2	100	69	100	66	20	79	100	67	100	67
	3	100	66	100	66	20	75	100	67	65	67
	4	65	69	50	71	65	68	—	—	65	59

* Hemolytic units are expressed as the reciprocal of dilution of lysin where 50% hemolysis of red blood cells was observed after 30 min. incubation at 37°C.

† Density of growth is expressed as % light transmission at λ 600 γ on a Coleman, Jr., spectrophotometer.

‡ Control organism are parent strains after 60 transfers on plain blood agar.

The S_6 strain was a type 43 and the U_6 a type 18 organism. At the end of 40 transfers on bacitracin medium the resistant variant of the S_6 strain was no longer typable while the U_6 resistant organism remained type-specific. On repeating this the S_6 strain has remained typable through 31 transfers of the parent organism on bacitracin medium. This work is continuing. Transient conversion from beta to alpha hemolysis occurred on sub-inhibitory concentrations of bacitracin in most of the 23 strains. Minute colonies also appeared when inhibitory concentrations of bacitracin were approached.

G. *Enzymatic*‡ *changes.* The enzyme changes observed in streptococci after acquired bacitracin resistance have been published(10). The same strains, S_2, S_3 and S_4, after storage for approximately 20 months at 4°C, were transferred an additional 20 times on bacitracin medium to restore the lost resistance. These organisms were then assayed for (a) proteinase-streptokinase activity, (b) streptolysin "S" titre and (c) ribo- and desoxyribonuclease activity. No differences were observed when comparisons were made of enzymatic activity of resistant strains grown in the test broth with and without sub-inhibitory concentrations of bacitracin. The streptolysin "S" titre was lowered in 2 of 3 bacitracin-resistant streptococci assayed after 60 antibiotic transfers (Table V). However, these two strains also showed decreased density of growth measured spectrophotometrically, as compared with their controls. The relationship between titre and density of growth has not been evaluated. Equal or higher enzyme activity has been produced by variants resistant to other antibiotics when their growth was less than that in corresponding controls(12). At the 60th transfer, the production of ribo- and desoxyribonucleases by the S_2, S_3 and S_4 bacitracin-resistant variants as well as their proteinase-streptokinase ratios remained unchanged.

Discussion. Resistance to bacitracin can be induced in beta hemolytic streptococci with relative ease as compared with penicillin or aureomycin. The technics are comparable. Large single-step increases in resistance such as were seen with streptomycin do not occur. The induced bacitracin resistance of streptococci is the most labile antibiotic resistance we have studied. The alterations in the biology of the streptococcus parallel those with the other antibiotics. Mouse virulence is decreased consistently and the changes from beta to alpha hemolysis were seen in almost all of the strains studied. The changes in the enzyme systems for the most part were minimal.

Summary. 1. The bacitracin sensitivity range of 276 strains of groups A, B and C hemolytic streptococci was 0.003 to 1 unit/ml. 2. Bacitracin resistance was induced in 23 strains of groups A, B, and C streptococci by 40 serial transfers on bacitracin-agar. The increase in resistance ranged from 8-fold in a group A to 3,750-fold in a group B organism. The median fold increase in resistance was comparable for all 3 groups. 3. Serial trans-

‡ Streptolysin "S" is included for simplicity of discussion although this is an unproved enzyme system.

12. Unpublished data.

fers on control media, passages through normal mice and storage at 4°C partially or completely restored the bacitracin sensitivity to all resistant variants. 4. Mouse virulence was decreased in 7 of 9 strains. It was restored in 6 of the 7 strains by subsequent passages in normal mice. 5. Group specificity was lost in 3 of 7 group B strains after induced resistance. 6. Growth on sub-inhibitory concentrations of bacitracin stimulated transient conversion from beta to alpha hemolysis in most strains studied. 7. The streptolysin "S" titre was decreased in 2 of 3 resistant variants. Ribo- and desoxyribonuclease activity and the proteinase-streptokinase ratios remained unchanged after acquired resistance.

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The Cultivation of Coxsackie Virus.*† (17955)

EBEN A. SLATER‡ AND JEROME T. SYVERTON
From the Department of Bacteriology, University of Minnesota, Minneapolis, Minn.

Recent laboratory studies(1-6) have established a newly discovered(1) group of viruses, the Coxsackie or "C" group, as the etiological agents responsible for human disease diagnosed clinically as nonparalytic poliomyelitis (1-4,7), "summer grippe"(5), aseptic meningitis(3) and epidemic myalgia(4). The viruses have been recovered from feces and throat swabs of patients and from sewage and flies(3). This group of viruses is characterized by its pathogenicity for newborn mice and hamsters with resultant myositis, paralysis and death.

In the experiments reported herein evidence is presented for the successful propagation *in vitro* in the presence of living tissue cells of a member of the Coxsackie group of viruses, type 4, Minnesota 1 strain.§

Materials and Methods. Virus. The strain of virus was isolated from a fecal specimen which had been obtained on about the 10th day following the onset of the illness of a patient whose diagnosis, both on admission and discharge from the University of Minnesota Hospitals, was nonparalytic poliomyelitis. The feces were made up in 10% suspension, homogenized for 5 minutes in a Waring blender and centrifuged at 10,000 r.p.m. for 20 minutes. The supernatant fluid was removed, filtered through a Seitz-EK sterilizing filter pad and the filtrate was employed for injection intraperitoneally, 0.05 ml, into each member of a litter of Rockland Swiss albino mice within 24-36 hours of their birth. When these infant mice were ill or dead, they were frozen, triturated in a mortar in 0.85% saline to yield a 10% suspension, filtered as described above, and the filtrate, 0.03 ml, was used as the inoculum for passage to infant mice. Following 10 mouse passages in series, 0.03 ml of filtrate similarly prepared was used to initiate each tissue culture passage series.

Tissue Cells. Infant mice killed within about 48 hours before or after birth provided the tissues which were employed to

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