TABLE I.
Rates of Urea Formation.
(Mg per rat per 4 hr period, corrected to 150 g body weight).

Hr after operation	Rates of urea formation		
	Mock operated controls	Complete nephrectomy	Bilateral ureteral ligation
0– 4	11.7	18.9	15.0
4– 8	5.1	19.5	15.5
8–12	8.1	20.5	15.6
12–16	6.7	20.0	17.1
16–20	3.7	20.5	17.5
20–24	4.3	20.5	18.2
Total for 24 hr	39.6	119.9	98.9

a length of time exceeding the duration of these experiments. Microscopic examination of the kidneys 24 hours after ureteral ligation revealed a little dilatation of some of the tubular lumina and of a few glomerular capsules, but otherwise the kidneys appeared normal.

It was reasoned that if the kidneys inhibit protein catabolism, presumably through the mediation of a humoral agent, this substance would continue to be produced and would continue to be liberated into the blood after bilateral ureteral ligation. The rate of protein catabolism would be less, therefore, in rats subjected to bilateral ureteral ligation than in animals whose kidneys had been completely excised.

A comparison of the rates of urea formation given in Table I, and of the serum urea concentrations shown by Fig. 1, supports this hypothesis. The serum urea concentration and the rate of urea formation of the rats subjected to bilateral ureteral ligation were significantly less than the serum urea concentration and the rate of urea formation of the completely nephrectomized animals. Other factors than the presence of viable renal tissue must be concerned with inhibition of protein catabolism, however, since the rate of urea formation of the rats with ligated ureters was greater than that of mock operated controls(1) studied under the same experimental conditions (Table I.).

Summary. The serum urea concentrations and the rates of urea formation of rats subjected to bilateral ureteral ligation and of completely nephrectomized rats were compared under conditions in which adequate quantities of fluid and calories were provided before and after operation. The serum urea concentration and the rate of urea formation were less in the rats whose ureters were ligated than in the nephrectomized animals. These findings support the hypothesis that the presence of viable renal tissue is a factor concerned with inhibition of the rate of protein catabolism.

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Antibiotic Studies on Beta Hemolytic Streptococci: VI. Acquired *In vitro* and *In vivo* Resistance to Aureomycin.* (17559)

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

It has not yet been reported that beta hemolytic streptococci acquire resistance to aureomycin *in vitro*. Paine, Collins, and Fin-

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land(1) reported that one strain of *Streptococcus mitis* and one strain of *Str. pyogenes* acquired no increase in resistance to aureomycin even after 60 subcultivations on antibiotic-containing medium. Harvey, Mirick, and Schaub(2) demonstrated an 8-fold increase in resistance to aureomycin by a microaerophilic streptococcus and a 16-fold increase in resistance by a *Str. faecalis*. These organisms were isolated from 2 patients during a relapse after several weeks of treatment with aureomycin for subacute bacterial endocarditis. The resistant *Str. faecalis* grew more slowly and formed smaller colonies on blood agar. The resistance of this strain was not permanent since streptococci of the original sensitivity reappeared after several transfers on blood agar.

The present study was undertaken to compare the *in vitro* and *in vivo* development of aureomycin resistance by group A, B, and C beta hemolytic streptococci, and to determine whether or not aureomycin altered the biology of the streptococcus in a manner similar to that produced by penicillin,(3-6) streptomycin,(7) and bacitracin.(8,9)

Materials and methods. Resistance to aureomycin was induced in 23 strains of beta hemolytic streptococci: 8 of group A, 8 of group C, and 7 of group B. Parent strains and their designations are the same as those used in preceding studies.(3-7) Aureomycin sensitivity was determined by the whole plate method and was taken as the maximum concentration of antibiotic that permitted good growth. Bacto heart infusion agar (Difco) containing 5% defibrinated sheep's blood and varying concentrations of aureomycin HCl (Lederle A377, Lot No. 7-8411) were used in 100 mm Petri dishes. Fresh dilutions of the yellow crystalline salt were prepared daily in physiological saline at a pH of 3.1. All antibiotic-containing blood agar plates were poured at a maximum temperature of 45°C, streaked as soon as solidified, and incubated at 37°C for 16 to 18 hours. Growth was recorded without delay after incubation to minimize any variations due to lability of the antibiotic.(1,10) To induce resistance *in vitro* 40 subcultivations were made on media containing aureomycin by the method previously described.(7) Comparison was made of the mouse virulence of the parent strain, a 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 passages in normal mice by an intracerebral inoculation method.(3) To ensure equality of inocula the densities of the Todd-Hewitt broth cultures of the control organism and the variants were adjusted to that of the parent strain on a Coleman junior spectrophotometer, either by the addition of broth or by the subtraction of supernatant fluid after centrifugation. The number of viable streptococci in the adjusted broth was determined by pour plate colony counts.

A standard strain of beta hemolytic streptococcus (C203 MV), a group A type 1 organism, was passed serially 25 times in mice and 30 times in embryonated hens' eggs protected with varying concentrations of aureomycin in an effort to induce aureomycin resistance *in vivo*. The method employed was essentially the same as that used to induce penicillin resistance(6) but with the following modifications: 1) neopeptone Todd-Hewitt broth cultures were adjusted to comparable densities spectrophotometrically for each passage in both the mouse and egg series; 2) the streptococci were given intraperitoneally in mice in 0.2 ml amounts; 3) mouse virulence was de-

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

μg/ml	Group A strains No.	%	Group B strains No.	%	Group C strains No.	%
.02	17	8.3				
.05	105	51.5	5	22.7	22	45.8
.1	79	38.7	16	72.7	21	43.7
.2	3	1.5	1	4.6	3	6.3
.3					1	2.1
.5					1	2.1
Total	204	100.0	22	100.0	48	100.0

TABLE II.
Aureomycin Resistance Induced in Group A, B, and C Beta Hemolytic Streptococci by Subcultivation 40 Times on Aureomycin Medium.

Group	Strain	Initial aureomycin sensitivity,* μg/ml	Final aureomycin sensitivity,* μg/ml	Fold change
A	S1	.5	1.	2
	S2	.5	3.	6
	S3	.5	3.	6
	S4	.5	3.	6
	S5	.5	2.5	5
	S6	.5	3.	6
	U5	.5	4.	8
	U6	.3	3.	10
B	T5	.08	1.	12
	T7	.08	1.	12
	T8	.08	2.	25
	T9	.08	2.	25
	T10	.08	2.	25
	T11	.08	1.	12
	T12	.08	1.	12
C	U1	.1	1.	10
	U2	1.0	60.	60
	U3	1.0	20.	20
	U7	.1	4.	40
	U8	.1	3.	30
	U9	.1	4.	40
	U10	.5	5.	10
	U11	.1	2.	20

* Aureomycin sensitivity is defined as the maximum concentration of aureomycin in the medium permitting good colonial growth.

termined separately for each mouse passage by intraperitoneal modification of the previously described technic; 4) the dosages of aureomycin in mice were 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg; in eggs they were from 0.1 to 2.0 mg per egg. *In vitro* sensitivities were performed at intervals by the whole plate technic.

Results. IN VITRO. A. *Initial Aureomycin Sensitivity.* Table I lists the aureomycin sensitivity of 274 recently isolated beta hemolytic streptococci of groups A, B, and C. The sensitivity varied from 0.02-0.5 μg/ml. The range of sensitivity within groups widened in the order C greater than A greater than B.

B. *Acquired Resistance Aureomycin-containing medium.* Table II summarizes the aureomycin resistance induced in 23 strains of group A, B, and C beta hemolytic streptococci after 40 serial subcultivations on aureomycin medium. The minimum increase in resistance was 2-fold in a group A organism, and maximum increase was 60-fold in a group C organism. The group B strains were the

TABLE III.
In vitro Sensitivities of Aureomycin-Resistant Beta Hemolytic Streptococci After Transfer on Control Medium or Passage Through Mice.

Group	Strain	Parent, μg/ml	Resistant variant, μg/ml	Sensitivity after transfer on control medium, μg/ml				Sensitivity after 12 normal mouse passages, μg/ml
				25 × s	50	75	100	
A	S1	.5	1	1	1	1	1	
	S2	.5	3	3	3	3	1	1.8
	S3	.5	4	3	1	1	1	4.0
	S4	.5	3					3.0
B	T5	.08	1	1	1	1	1	1.5
	T7	.08	1					1.0
	T8	.08	2					2.0
	T10	.08	2	1	1	1	1	
	T11	.08	1	1	1	1	1	
C	U1	.1	1	1	1	1	1	0.5
	U2	1.0	60	50	7	7	7	3.0
	U3	1.0	20	30	3	3	3	8.0

most uniform in their rate of development of resistance. By group, the rate of development of resistance was A less than B less than C.

C. *Acquired Resistance on Control Medium.* All 23 parent organisms were subcultured serially on control medium (aureomycin-free blood agar). Dissociation was minimal and no changes in hemolysis were observed. After 40 transfers, no significant increase in aureomycin resistance was discernible.

D. *Loss of Acquired Resistance.* Three aureomycin-resistant strains of group A, 3 of group B, and 3 of group C streptococci were subcultured serially 100 times on aureomycin-free blood agar plates in an attempt to restore their original sensitivity. The organisms were tested on the 25th, 50th, 75th, and 100th transfer by the whole plate method. Only the 2 most resistant group C strains (U_2 and U_3) lost 5/6ths of the acquired resistance after the 50th passage on plain blood agar plates. Neither, however, returned to its original level of sensitivity. These values, recorded in Table III, remained unchanged over an 8-month period of storage in broth at 4°C. Three strains each of group A, B, and C streptococci after acquiring aureomycin resistance were passed intracerebrally 12 times in normal mice and their *in vitro* sensitivities determined by the whole plate technic. Only the 2 most resistant group C strains (U_2 and U_3) showed a significant decrease in resistance (Table III).

E. *Change in Virulence.* Only 3 strains of streptococci, the U_2 and U_3 group C and the S_2 group A strains, showed an appreciable loss of mouse virulence after acquiring aureomycin resistance. In general, the behavior of the control strains was similar to that of the parent organisms. In 5 of 6 group A, B, and C strains, the virulence of the resistant variant was increased over that of either the parent or resistant organism by 12 intracerebral passages in normal mice. These results are given in Table IV. The number of viable streptococci in cultures of the parent and resistant strains was essentially the same.

F. *Antigenic and Hemolytic Changes.* The 23 beta hemolytic streptococci were regrouped by the Lancefield technic after acquiring aureomycin resistance. In only 1 resistant group B organism, the T_5 strain, were we unable to demonstrate the group-specific precipitinogen. Transient conversion from beta to alpha hemolysis occurred on concentrations of aureomycin which limited growth in several group A, B, or C organisms. These strains, when growing on control medium, had the normal beta type of hemolysis. Two group C organisms which produced an area of greening beyond the clear zone, showed an exaggeration of this color when growing on low concentrations of aureomycin. This greenish hemolytic aura completely disappeared leaving only a clear beta zone as the inhibiting concentration of aureomycin was reached.

G. *Enzymatic Alterations.* Early results of comparisons of antigenic and enzymatic alterations produced in the same beta hemolytic streptococci by the separate induction of penicillin-, streptomycin-, bacitracin-, and aureomycin-resistance have been reported from this laboratory.(9) Aureomycin-resistant organisms, in general, showed a less altered metabolism, as measured by their streptolysin-S, proteinase, streptokinase and ribonuclease production, than did either penicillin- or streptomycin-resistant streptococci. No significant change in streptolysin S activity accompanied induced aureomycin resistance in 5 group A and 3 group C organisms surveyed. The 2 group A strains that produced proteinase showed unaltered enzyme activity after induced aureomycin resistance. However, reduced streptokinase production was demonstrated in 1 of 3 group A and 1 of 2 group C resistant variants when compared with their controls. Ribonuclease activity was lessened in 2 of 7 group A streptococci by transfer on aureomycin medium.

IN VIVO. A. *Acquired Resistance in Protected Mice or Embryonated Eggs.* The C203 MV strain of beta hemolytic streptococcus developed no *in vivo* aureomycin resistance after 25 serial mouse passages, nor was there a change in the *in vitro* sensitivity at the end of these passages. The PD_{50} for aureomycin and LD_{50} for the 1st and 25th passages respectively were essentially the same. The sensitivities of the parent, intermediate and final strains as determined by the whole plate technic were identical. Similarly, the C203 MV strain showed no acquisition of resistance to aureomycin through 30 egg passages, as measured by the successive amounts of aureomycin needed to sterilize the eggs and by the change in *in vitro* sensitivity of the passaged organism.

Discussion. Resistance to aureomycin may be induced in beta hemolytic streptococci by a technic similar to that used for penicillin or streptomycin. Many subcultivations are necessary on sub-inhibitory concentrations of the drug to produce a detectable change in the sensitivity of any of the streptococci employed. The magnitude and rate of the changes are quite comparable to those previously seen for penicillin.(3,5) Failure to demonstrate the development of aureomycin resistance *in vivo* in a group A strain was obtained also with penicillin.(6) Both the *in vivo* and *in vitro* results suggest that the development of aureomycin resistance in beta hemolytic streptococci will not be of clinical importance if an analogy between laboratory studies and clinical results for penicillin and aureomycin is valid. To date, penicillin resistance by beta hemolytic streptococci has not been of clinical importance. This had been predicted by several laboratory studies(11,12,3,6) on induced resistance. Aureomycin appears to produce alterations in the biology of streptococci less frequently than does streptomycin or penicillin. The mouse virulence is only infrequently decreased, the group antigenicity is unchanged, and the enzyme systems are only occasionally inhibited.

Summary. 1. The aureomycin sensitivity range of 274 recently isolated group A, B, and C beta hemolytic streptococci was 0.02-0.5 μg/ml.

2. Aureomycin resistance was induced in 23 strains of group A, B, and C beta hemolytic streptococci by 40 serial subcultivations on aureomycin medium. The minimum increase in resistance was 2-fold in a group A organism, and maximum increase was 60-fold in a group C organism.

3. Serial transfer on control medium or passage through normal mice partially restored aureomycin sensitivity to resistant variants.

4. Mouse virulence was decreased in only the 2 group C organisms which had shown the highest degree of aureomycin resistance.

5. In only 1 group B resistant variant was the group specificity lost after the development of aureomycin resistance.

6. Growth on maximum concentrations of aureomycin stimulated transient conversion from clear to green hemolysis in organisms normally producing only a clear beta zone. Conversely, group C organisms with normal

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TABLE IV.
Mouse Virulence of Group A, B, and C Beta Hemolytic Streptococci with Acquired Aureomycin Resistance.

Group	Strain	Initial LD_{50}		LD_{50} after 40 transfers on			LD_{50} of resistant strains after 12 mouse passages
				Control medium	Aureomycin medium		
		Dilution*	No. organisms†	Dilution*	Dilution*	No. organisms†	Dilution*
A	S2	$10^{-4.8}$	1.6×10^2	$10^{-2.8}$	$10^{-1.5}$	3.0×10^5	
	S3	$10^{-4.5}$	2.0×10^2	$10^{-3.7}$	$10^{-3.0}$	8.4×10^3	
	S4	$10^{-4.3}$	3.7×10^2	$10^{-3.2}$	$10^{-3.0}$	4.5×10^3	$10^{-6.2}$
B	T5	$10^{-3.0}$	1.9×10^4	$10^{-4.8}$	$10^{-2.5}$	4.3×10^4	$10^{-7.0}$
	T7	$10^{-4.0}$	6.3×10^2	$10^{-5.3}$	$10^{-4.0}$	5.9×10^2	$10^{-3.2}$
	T8	$10^{-4.0}$	7.8×10^2	$10^{-6.3}$	$10^{-4.5}$	8.5×10^2	$10^{-7.0}$
C	U1	$10^{-1.5}$	2.1×10^5	$10^{-2.5}$	$10^{-1.3}$	1.0×10^6	$10^{-3.6}$
	U2	$10^{-6.0}$	9.3×10^1	$10^{-4.0}$	10^{-0}	2.2×10^7	
	U3	$10^{-2.5}$	5.8×10^4	$10^{-2.8}$	$10^{-1.2}$	2.0×10^6	$10^{-5.7}$

* The LD_{50} is expressed in terms of the dilution of the whole broth culture after it had been adjusted in density to that of its corresponding parent strain.
† The LD_{50} is expressed in terms of the number of viable streptococci inoculated.

green hemolytic characteristics lost their green hemolytic zones when grown on maximum aureomycin concentrations.

8. Reduction in streptokinase production occurred in 2 of 5 group A and C resistant strains and diminished ribonuclease activity in 2 of 7 group A resistant variants. No change in streptolysin S or proteinase activity was observed.

9. The C203 MV strain of group A streptococcus failed to acquire aureomycin resistance after repeated exposures to sublethal amounts of the antibiotic either in mice or embryonated hens' eggs.

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VII. Vitamin B_{12} and the Intrinsic Factor. (17560)

DONALD E. WOLF, THOMAS R. WOOD, JOHN VALIANT, AND KARL FOLKERS
(Introduced by Gladys A. Emerson)
From the Research Laboratories of Merck and Co., Inc., Rahway, N. J.

Soon after the discovery by Minot and Murphy(1) of the effectiveness of liver in the dietary treatment of pernicious anemia, it was shown that foods such as beef muscle contain a heat-stable substance, designated the extrinsic factor,(2) which produces little or no response when ingested by a pernicious anemia patient unless normal gastric juice is also administered with it. The activity of gastric juice has been ascribed to the so-called intrinsic factor; gastric juice alone has no oral hematopoietic power.(3) Over a period of years the relationship between the extrinsic and intrinsic factors has been the subject of considerable clinical experimentation by Castle and his associates, and other investigators. Recent findings on this subject are as follows: Vitamin B_{12}, active parenterally at the microgram level, has been found to be essentially inactive when administered orally

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