TWO STREPTOMYCIN-RESISTANT VARIANTS
OF MENINGOCOCCUS1

C. PHILLIP MILLER AND MARJORIE BOHNHOFF

Department of Medicine, University of Chicago, Chicago, Illinois

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One of the striking differences between penicillin and streptomycin is the
rapidity with which microorganisms develop resistance to the latter. Resistance
to penicillin can be acquired, but always in relatively small increments at each
subcultivation on artificial media or in each passage of the strain through an
experimental animal. A high degree of resistance can be attained in vitro or
in vivo only by repeated exposure to increasing concentrations of the drug.

Miller and Bohnhoff (1947a), for instance, found that 147 transfers onto media
containing increasing concentrations of penicillin raised the resistance of a strain
of meningococcus sufficiently to permit it to grow abundantly on media con-
taining 5,000 units per ml. They have also shown (Miller and Bohnhoff, 1946b,
1947b) that the resistance of a strain of meningococcus could be increased by
serial passage through mice treated with subcurative doses of penicillin. They
used cultures of hearts’ blood as inocula for each succeeding animal passage.
The dose required to protect approximately half of the mice rose from 10 units to
1,700 units in the course of 61 passage inoculations.

Resistance to streptomycin, on the other hand, was found to develop with such
rapidity that two or three transfers onto media containing increasing concentra-
tions sufficed to permit meningococcus or gonococcus to multiply on media
containing 50,000 µg of streptomycin per ml (Miller and Bohnhoff, 1946a). Menin-
gococci which were rendered streptomycin-resistant by this means retained
approximately the virulence of the original parent culture and were resistant to
streptomycin in vivo. Mice inoculated with such resistant meningococci died in
spite of doses of streptomycin which would have protected them against infection
with normal meningococci.

The present communication presents evidence that this rapid development of
streptomycin resistance by meningococcus is due to the selective propagation of
resistant variants which become apparent during growth on streptomycin-
containing media. These variants are presumed to originate from streptomycin-
resistant mutants which are arising regularly in the bacterial population of the
normal parent strain before its exposure to the drug. In the course of these
experiments, a second variant has been encountered which is not only resistant
to streptomycin but is actually dependent on streptomycin for its growth

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in vitro and in vivo. Both variants have developed from each of 18 strains of meningococcus.²

METHODS

Strains of meningococcus. The 18 strains used in these experiments included (a) old stock strains which have been under cultivation in the laboratory for several years, (b) strains recently isolated from cases of epidemic meningitis, and (c) strains isolated from the nasopharynx of healthy carriers. Each of the strains was definitely identified as a member of one of the 3 fixed types: I, II, or II alpha. All of the strains produced colonies typical of meningococcus and fermented only glucose and maltose.

Media. The medium used most commonly throughout these experiments has been described in a previous communication as casein digest agar (Miller and Bohnhoff, 1947a). Several other media were employed at various times for purposes of comparison and to make certain that the results obtained were not dependent on any ingredient of the medium itself. Media thus employed were meat digest cysteine agar (Miller and Bohnhoff, 1947a), Difco nutrient agar, brain heart infusion agar, and proteose peptone no. 3 agar.

The media were usually enriched by the addition of fresh, defibrinated sheep or rabbit blood. A few experiments were conducted with agar containing rabbit serum.

When a liquid medium was required trypticase soy broth³ was used.

Preparation of streptomycin media. A total of 25 preparations of streptomycin⁴ have been used. They were obtained from seven manufacturers and varied widely in streptomycin activity but included some preparations of an especially high degree of purity.

Plates of streptomycin agar were made up as follows and were always used within a few hours of preparation: a saline solution of streptomycin was diluted to convenient concentrations and 1 ml of appropriate dilutions pipetted into each petri dish. Five-tenths ml of fresh, defibrinated blood were then put beside it. Melted agar (cooled to 45 C) was added, and the contents of each plate were thoroughly mixed.

Method of inoculation of the streptomycin plates. When heavy seedings of meningococci were to be planted onto a series of plates containing graded concentrations of streptomycin, the following technique was employed because it distributed the inocula evenly and did not break the surface of the agar:

After the agar had set, 5 small glass balls (about 6 mm in diameter), such as

² Our preliminary communication (Miller and Bohnhoff, 1947c) reported that these variants developed from 16 of 18 strains. The 2 strains originally considered failures have been re-examined and found to produce small numbers of both A and B variants.
³ Baltimore Biological Laboratory.
⁴ Preparations of streptomycin were supplied by the Antibiotics Study Section of the National Institute of Health, U. S. Public Health Service; the Division of Penicillin Control and Immunology, Food and Drug Administration; Abbott Laboratories; Commercial Solvents Corporation; Eli Lilly & Company; Merck & Co.; Chas. Pfizer & Company; E. R. Squibb & Son; and Upjohn Company.
are customarily used for defibrinating blood, were placed on the surface of the agar in each plate. It was found to be convenient to have these "beads" distributed in test tubes, 5 to a tube, before sterilization, so that the whole contents of a tube could be rolled out gently onto the agar surface.

The 18-hour growth from an agar culture in an ordinary 16-ounce medicine bottle was harvested in 9.0 ml of gelatin Locke's solution, sedimented by centrifugation, and resuspended in 0.5 ml gelatin Locke's solution. The meningococci were dispersed by drawing the suspension repeatedly into a capillary pipette from which one drop was allowed to fall onto the agar in each plate. These inocula contained approximately $1.0 \times 10^{10}$ microorganisms. The plates were then stacked in a holder and shaken gently in all directions so that the beads rolled back and forth over the surface of the agar and distributed the inocula uniformly. The beads were then discarded. The plates were incubated for 3 days, the first in a candle jar, and then allowed to stand for a few more days at room temperature. They were all examined carefully each day for 5 or 6 days.

Mouse inoculations were made to determine virulence and also streptomycin resistance. A loopful of growth from an 18-hour culture was rubbed up in a few ml of gelatin Locke's solution and the suspension diluted until it reached a density equal to no. 3 of the McFarland series (Kolmer and Boerner, 1945), which experience has shown to contain approximately one billion meningococci per ml. From this standard suspension, 10-fold dilutions were made in 4 per cent mucin and 1 ml quantities injected intraperitoneally into mice weighing 16 to 20 grams (Miller and Castles, 1936).

Mice were treated with streptomycin by the injection of the desired dose in 0.5 ml of saline under the skin of the animal's back.

As many as possible of the mice that died were autopsied, and cultures of hearts' blood were made on casein digest agar and also on the same agar to which 100 $\mu$g of streptomycin per ml had been added.

**EXPERIMENTAL RESULTS**

The two variants described below appeared when meningococci were inoculated onto media containing streptomycin greatly in excess of that which is considered the optimal bactericidal concentration. Identical results were obtained from cultures started with a single isolated colony and from an ordinary transfer of a stock culture. A heavy seeding of an overnight growth of a normal, sensitive strain of meningococcus was planted onto a series of 8 to 12 plates containing graded concentrations of streptomycin. As most of the experiments were performed with one preparation of streptomycin, the concentrations given below are those of that single preparation. The range varied from 10 $\mu$g per ml to 10,000 $\mu$g per ml. The intermediate concentrations were usually 20, 40, 60, 100, 200, 400, 600, 1,000, and 4,000 $\mu$g per ml.

The growth on a series of 6 plates is shown in figure 1.

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4 Locke's solution containing 0.1 per cent gelatin.
4 A preparation marketed for therapeutic use by Eli Lilly & Co.
Fig. 1. Growth of Meningococcus from Equivalent Inocula on Graded Concentrations of Streptomycin
Photographed after 72 hours' incubation

The first plate (10 µg) shows only normal meningococcus colonies. The type A colonies are indicated by arrows. The third (60 µg), fourth (100 µg), and fifth (400 µg) plates show numerous type B colonies. The sixth plate (1,000 µg) shows 5 type B colonies.
After 24 hours' incubation, the plate containing 10 μg per ml showed confluent growth, and the one containing 20 μg per ml a very large number of colonies indistinguishable from normal meningococcus colonies. A few of these normal colonies occasionally appeared on 40 μg per ml, but none on concentrations higher than that.

Type A variant. On plates containing 40 μg per ml, a second type of colony was visible at the end of 24 hours' incubation and continued to grow for the next 48 hours, reaching a size of 3 to 5 mm in diameter; i.e., considerably larger than normal meningococcus colonies. It differed from normal colonies in color as well as size, for it acquired a distinctly yellowish tinge which became more marked during the second and third day of incubation and after another day or two at room temperature. This variant, which developed from each of the 18 strains, has been designated type A.

Except on plates containing 10 to 20 μg per ml which were so crowded with normal colonies that they could not be distinguished, type A variants developed in about equal numbers from any given strain on all concentrations of the drug. This number, however, varied from strain to strain. Most strains produced 2 to 5 colonies per plate, an average incidence of approximately 1 to 3 in 10^10 of original bacterial population. Figure 2 presents the results of 32 experiments performed with one strain (113) and illustrates the uniformity of incidence of type A colonies. One strain, however, developed greater numbers of type A colonies, 5 to 30 per plate.

The type A variants had the following properties: They were highly resistant to streptomycin as they were able to grow on concentrations of the drug as high as 10,000 μg per ml. They were also able to multiply on streptomycin-free media. They retained all of the following properties of the parent strains from which they arose: morphology, staining characteristics, sugar fermentation, virulence for mice, and type specificity as determined by agglutination and by mouse protection tests. Their streptomycin resistance was demonstrated in vivo by inoculating mice with mucin suspensions and treating the animals with 15,000 μg in 3 subcutaneous injections of 5,000 μg each at 1, 3, and 5 hours after inoculation. The mice regularly died of meningococcal sepsis, and type A variants were cultured from their hearts' blood.

No loss of streptomycin resistance has been detected in the type A variants either during passage through mice or during subcultivation on streptomycin-free media. Two strains have been transplanted every 5 to 7 days for one year. Type A variants were found to be slightly more sensitive to penicillin than the parent strain from which they arose.

Type B variants. After 48 hours of incubation a second type of variant appeared on all concentrations of streptomycin above 40 μg per ml. After another 24 hours' incubation, additional colonies of this type developed on concentrations of 60 and 100 μg per ml, but no new colonies appeared after 72 hours. The size and color of these colonies varied with the concentration of streptomycin on which they grew. On plates containing 60 to 100 μg per ml, they were very small and light gray; on concentrations above this range, they
were larger and had a distinctly yellowish tinge. On concentrations greater than 400 μg per ml, they resembled the type A colonies in size and pigmentation. The identification of doubtful colonies was made by subcultivation onto streptomycin-free and streptomycin-containing agar.

The number of type B colonies which developed from one type I strain (113) are plotted in figure 3, which shows (a) that the actual numbers varied considerably in different experiments and (b) that they were always most numerous between concentrations of 100 and 400 μg per ml. Curves of the numbers of colonies in individual experiments differed in height but almost always had the shape of the curve of the mean shown in figure 3.

The meningococci composing these type B colonies had the following properties: They were resistant to streptomycin, for they were able to grow on concentrations as high as 5,000 μg per ml. They were dependent on streptomycin for growth; that is, they would grow abundantly from small inocula on concentrations between 100 and 400 μg per ml and would also grow from large inocula on concentrations as low as 5 μg per ml, but they could not be subcultured on media containing less than that minimum of streptomycin. They were nonvirulent for mice unless the mice were treated with streptomycin as described below. They were gram-negative and fermented glucose and maltose when the test media contained 100 μg of streptomycin per ml. They retained the type specificity of the parent strain from which they arose. Rabbit sera prepared against the parent strain conferred protection against experimental infection with these variants in mice treated with streptomycin.

Microscopically, the type B organisms varied somewhat with the concentrations of streptomycin on which they had developed. Preparations made from the small gray colonies grown on 60 or 100 μg per ml showed them to be slightly larger than normal meningococci. Type B organisms growing on higher concentrations in larger pigmented colonies were indistinguishable from normal meningococci. This difference may well be related to the stimulating action of streptomycin mentioned below.

Although the colonial development and microscopic appearance of type B variants differed according to the concentration of streptomycin on which they grew, the identity of all members of this variant was indicated by the following observations: When a type B variant was taken from any concentration and
STREPTOMYCIN-RESISTANT MENINGOCOCUS subcultured onto another concentration, it always grew in colonies of the type regularly produced on that particular concentration. In other words, small gray colonies always developed on concentrations of 60 to 100 μg per ml and large colonies tinged with yellow on concentrations above 200 μg per ml, regardless of the concentration from which the inocula were taken.

![Graph showing the relationship between concentrations of streptomycin and the number of colonies developing from heavy seedings on graded concentrations of streptomycin.](http://jb.asm.org/)

**Fig. 3. Numbers of Colonies of Type B (Streptomycin-dependent) Variants Developing from Heavy Seedings on Graded Concentrations of Streptomycin**

Results of 35 experiments with meningococcus 113. The individual inocula contained approximately 1.0 to 2.0 × 10¹⁰.

The dependence of type B variants on adequate concentrations of streptomycin for growth was also demonstrated by subculturing them into broth containing graded concentrations of the drug (see figure 4). It will be seen that no growth occurred in the broth containing the low and high concentrations of streptomycin. The optimum range for multiplication in liquid media, therefore, approximated that for solid media.

When a series of plates containing graded concentrations of streptomycin was planted with a pure culture of the B variant in small but equal inocula, the numbers of colonies which developed bore exactly the same relationship to
concentrations of the drug as did the B variants developing from the original inoculations with heavy seedings of the normal, parent strain. These experiments were made as follows: A suspension of B variant was prepared and diluted to a density equal to no. 3 in the McFarland series, which experience has shown to contain about one billion meningococci per ml. This suspension was further diluted a millionfold and a drop (containing 35 to 50 meningococci) planted, by

![Fig. 4. Growth of Type B Variants of Meningococcus in Broth Containing Graded Concentrations of Streptomycin](image)

**Left to right:** Tube 1, control. Tubes 2 to 7 contain streptomycin—10, 40, 100, 400, 1,000, 4,000 μg per ml. The tubes were slanted sufficiently to afford a maximum increase in the surface of the broth and incubated for 24 hours.

**TABLE 1**

*Number and appearance of colonies developing from small, equal inocula of pure culture of type B variant*

<table>
<thead>
<tr>
<th>STREPTOMYCIN (μg per ml)</th>
<th>NUMBER OF COLONIES</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>40</td>
<td>3</td>
<td>small, gray</td>
</tr>
<tr>
<td>60</td>
<td>8</td>
<td>medium, gray</td>
</tr>
<tr>
<td>100</td>
<td>33</td>
<td>medium to large, gray to slightly yellowish</td>
</tr>
<tr>
<td>200</td>
<td>35</td>
<td>large, slightly yellowish</td>
</tr>
<tr>
<td>400</td>
<td>30</td>
<td>large, yellowish</td>
</tr>
<tr>
<td>1,000</td>
<td>25</td>
<td>large, yellowish</td>
</tr>
<tr>
<td>4,000</td>
<td>6</td>
<td>small medium, yellowish</td>
</tr>
<tr>
<td>10,000</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

the method described above, onto a series of plates containing varying concentrations of streptomycin. A portion of the inocula undoubtedly adhered to the beads and was removed with them. The results of a typical experiment are presented in table 1. It shows that the number and appearance of colonies developing on each concentration resemble the number and appearance of type B variants which developed on those concentrations from the heavy seedings made originally with the parent strain. The homogeneity of the culture was estab-
lished by the fact that a number of colonies from each plate transferred onto streptomycin-free and streptomycin-containing agar grew only on the latter.

**Sensitivity of type B colonies to penicillin.** When type B colonies were tested for their sensitivity to penicillin, their growth was inhibited by approximately the same concentrations that inhibited the growth of the normal parent strain from which each variant arose. They appeared, therefore, to be as sensitive as normal meningococci to penicillin. It should be pointed out, however, that the tests could not be made on the same media because of the necessity of providing sufficient streptomycin for the development of type B variants in amounts which were bacteriostatic for the normal strain.

**Reversion of type B variants.** The type B variants continued to exhibit all the characteristics described during repeated subcultivation on streptomycin agar. Their dependence on the drug has been complete except for four instances in which a single colony has developed on streptomycin-free agar. The four exceptions were the only ones to occur among many subcultivations onto streptomycin-free agar. In each instance the colony grew out slowly, but thereafter multiplied readily on streptomycin-free media. They retained all of the properties of meningococci and are regarded as mutations back toward normal. Their reversion to normal was not quite complete, however, for three of them developed no type B variants when planted onto graded concentrations of streptomycin, but only type A. The other reverted strain was able to develop both type A and type B colonies, but the numbers of the former were greater than those produced by its original parent strain. It is clear, therefore, that none of these reverted mutants had regained all of the potentialities of the parent strain from which they were originally derived.

**Effect of inactivated streptomycin.** The type B variants were unable to grow on media containing streptomycin inactivated by hydroxylamine hydrochloride according to the method of Donovick, Rake, and Fried (1946) or by cysteine hydrochloride according to the method of Denkelwater, Cook, and Tishler (1945).

**Experimental infection with type B variants.** The dependence of the type B variants on streptomycin for their multiplication could be demonstrated in vivo as well as in vitro. When mice were inoculated with mucin suspensions of type B variants, the mice usually survived unless they were treated with streptomycin. An occasional mouse died if very large inocula were used, but meningococci were rarely recovered from its heart’s blood, and then only on streptomycin-containing agar.

On the other hand, mice treated with adequate doses of streptomycin usually succumbed to meningococcal sepsis, and type B variants were regularly recovered from cultures of their hearts’ blood on streptomycin-containing agar. Although hearts’ blood was always planted onto streptomycin-containing and streptomycin-free media, no meningococci ever grew out on the latter.

In table 2 are presented the results of a typical experiment in which mice were inoculated with $10^8$ or $10^7$ type B variants. Streptomycin was administered subcutaneously 3 hours after infection in doses of 5, 50, 500, 5,000, and 10,000
μg, the last given in 2 doses of 5,000 each, the second dose \( \frac{1}{2} \) hour after the first. It will be seen that all of the untreated controls survived and that the mortality rose as the dose of streptomycin increased up to the largest dose. One or more

**TABLE 2**

*Effect of streptomycin treatment on infection with type B variants*

<table>
<thead>
<tr>
<th>STREPTOMYCIN TREATMENT 3 HR AFTER INFECTION</th>
<th>APPROXIMATE NUMBERS OF MENINGOCOCCI INOCULATED</th>
<th>100,000,000</th>
<th>10,000,000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Result</td>
<td>Blood cultures</td>
<td>Result</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strep.-free media</td>
<td>Strep.* media</td>
</tr>
<tr>
<td>None</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>21†</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>50</td>
<td>21†</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>500</td>
<td>21†</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>5,000</td>
<td>21†</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>10,000 (2 doses of 5,000 ea)</td>
<td>21†</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = positive for meningococci; 0 = negative for meningococci; — = not cultured; S = survived.

* Streptomycin media = media containing 100 micrograms of streptomycin per ml.
† Figures = hours of death.

mice in each group were autopsied and cultures of their hearts’ blood made on streptomycin-free and streptomycin-containing agar. In every case meningococci were recovered on the latter but not on the former. The meningococci recovered from the hearts’ blood cultures had all of the characteristics of B
variants; that is, they retained their type specificity and their ability to ferment glucose and maltose but required streptomycin for growth.

Unusually large inocula were used in the experiment just described. It has not as yet been possible, however, to produce fatal meningococcal sepsis regularly in mice with inocula smaller than $10^4$ meningococci even though the animals received multiple injections of streptomycin. The virulence of the type B variants appears, therefore, to be less than that of the type A variants.

**DISCUSSION**

In these experiments two variants have arisen from cultures of meningococcus planted in heavy seedings onto a series of plates containing graded concentrations of streptomycin. It should be emphasized that each experiment was begun with a culture which had never been exposed to streptomycin and that inoculation onto the various concentrations of streptomycin was made at one time. Both variants developed from all of the 18 strains of meningococcus studied. They were gram-negative diplococci which retained the characteristic sugar fermentation and type specificity of the parent strains from which they arose. Both variants were highly resistant to streptomycin.

One variant, designated type A, grew in large, yellowish colonies which appeared in approximately equal numbers on all concentrations of the drug although the numbers varied considerably from strain to strain. Its resistance to streptomycin was demonstrated *in vivo* as well as *in vitro*, for it produced infection in mice which proved uniformly fatal in spite of the administration of maximal doses of streptomycin tolerated by the mice.

The incidence of the other variant, designated type B, as well as the size and color of its colonies depended on the concentrations of streptomycin onto which the original seedings were planted. Nevertheless, all of the B variants derived from any strain were found to be genetically alike.

The striking characteristic of this variant was its dependence on streptomycin for multiplication on solid and in liquid media and in the body of an animal host. The animal experiments indicated that this variant was nonvirulent for mice unless the animals were treated with adequate doses of streptomycin and that the dependence on streptomycin for growth persisted during and after multiplication within the body of the infected animal.

It is impossible at the present time to be certain whether the substance required for the growth of the type B variant is streptomycin itself or some impurity which has been present in all of the preparations we have used. These numbered 25 and were obtained from seven manufacturers. Two of the preparations were described as being of an especially high degree of purity. It should be noted that streptomycin inactivated by hydroxylamine or by cysteine failed to support growth of the type B variants. This aspect of the problem is under investigation.

The origin of these variants is difficult to explain unless one assumes that they both arise by current mutation; i.e., from mutants which are constantly appearing in the original bacterial population of the parent strain. The type A
variants developed from any given strain with about equal frequency on all concentrations of streptomycin, although the frequency varied from strain to strain.

The incidence of the type A variants from most strains was estimated to average 1 to 3 in 10⁷ of original bacterial population. One strain produced about 3 to 30 in 10¹².

The maximum incidence of the type B variants varied from 2 to 15 per billion meningococci in the parent culture.

When the type B variants were first observed, they were thought to arise by mutation which was induced by streptomycin. Subsequent observations have failed to support this hypothesis and have tended instead to indicate that, like the A variants, they, too, originated from mutations which were occurring regularly in the parent bacterial population.

The fact that they appeared only on the high concentrations and only in greatest numbers within a certain range was explained by the demonstration that this range of concentrations was optimal for their development. Pure cultures of the B variants developed colonies on each concentration of streptomycin in the same relative numbers as did heavy seedings of the parent strain from which they arose. The greater proportion of them were able to reproduce only on certain concentrations; above and below that optimal range few or none developed. In other words, the type B variants developed approximately the same number of colonies on each of a series of concentrations whether they were planted in pure culture or together with myriads of normal, streptomycin-sensitive meningococci. This observation seems to indicate that the streptomycin requirement of the type B variants for their multiplication is quantitative as well as qualitative.

The variation in size and color of colony of the B mutants can only be attributed to the direct effect of streptomycin on the physiology of the microorganisms. Benham (1947) found that streptomycin increased the oxygen uptake of a normal strain of typhoid bacilli but not of a resistant one unless high concentrations of the drug were used.

Several studies on the development of streptomycin resistance have appeared: Chandler and Schoenbach (1947) for staphylococcus, streptococcus, and pneumococcus; Hamre, Rake, and Donovick (1946) for Klebsiella; and Klein and Kimmelman (1946a, 1946b). Alexander and Leidy (1947), using a technique similar to ours, isolated streptomycin-resistant variants from Hemophilus influenzae and estimated their incidence as 1 in 1.1 billion to 1 in 13.8 billion members of the original bacterial population. As none of these authors mentions dependence on streptomycin as a characteristic of the resistant strains, one must conclude that they were dealing with resistant variants analogous to the type A variants herein described. It is quite certain that the streptomycin-resistant gonococci and meningococci reported earlier by Miller and Bohnhoff (1946a) were type A variants.

Hall and Spink (1947) describe a strain of Brucella which became highly resistant to streptomycin. This strain was recovered from the blood stream of a
patient with Brucella endocarditis and had apparently developed a considerable degree of resistance in vivo. After it had become highly resistant, it produced two types of colonies, a large one, which grew rapidly, and a small one, which grew slowly. Although this latter variant was able to grow on streptomycin-free agar, it grew better on media containing 50 to 100 μg of streptomycin per ml. It is possible that this second type of colony is similar to our B variant.

It should be noted that Welch, Price, and Randall (1946) were able to demonstrate large numbers of viable typhoid bacilli in broth cultures containing streptomycin in concentrations greater than the minimum which inhibited growth. They also found that the mortality rate of mice infected with typhoid bacilli was increased by treatment with small doses of streptomycin (0.05 to 1.0 μg). The mortality rate, however, was decreased when larger doses were administered.

For a comprehensive discussion of the general problem of bacterial mutation, the reader is referred to the recent review by Luria (1947).

Studies on the growth requirements of mutants isolated from cultures of Escherichia coli after treatment with bacteriophage (Anderson, 1944; Luria and Delbrück, 1943; Luria, 1945) or X-ray (Tatum, 1945; Gray and Tatum, 1944) have demonstrated a variety of deficiencies in their metabolic processes. Similar observations have been made on mutants induced in Neurospora by X-ray (Beadle, 1945).

Emerson (1944) has described a mutant of Neurospora which required sulfanilamide for growth and for which para-aminobenzoic acid was toxic. This variant appeared in his cultures only once. The type B variants of meningococcus, on the other hand, developed regularly from all of 18 strains which included types I, II, and II alpha, some of which strains had recently been isolated from cases of epidemic meningitis and some from carriers, but others were old stock strains which had been under cultivation in the laboratory for many years.

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SUMMARY

Two streptomycin-resistant variants developed from each of 18 strains of meningococcus, including types I, II, and II alpha, when heavy seedings were planted onto a series of plates containing streptomycin in concentrations varying from 40 to 10,000 μg per ml. One variant, designated type A, appeared in small and approximately equal numbers on all concentrations. It grew in large yellowish colonies on streptomycin-free and streptomycin-containing media. It retained the original virulence for mice possessed by its parent strain.

The other variant, designated type B, appeared in greatest numbers on concentrations between 100 and 400 μg per ml, the concentrations optimal for its

* Personal communication to authors.
multiplication. Its colonies varied in size and color depending upon the concentrations of streptomycin on which they developed. They were small and gray on concentrations of less than 100 μg per ml and larger and slightly yellowish on concentrations of 200 μg or more per ml. Nevertheless, the type B variants from any strain were found to be genetically identical and the differences in their colonial appearance to be determined by the concentration of streptomycin on which they grew.

The type B variants were dependent on streptomycin for multiplication in vitro and in vivo. They were nonviable on media containing concentrations of less than 5 μg per ml and grew best on 100 to 400 μg per ml. They were nonvirulent for mice, unless the mice received streptomycin. In mice treated with streptomycin, they produced a fatal meningococcal sepsis and were recovered from the hearts' blood provided the cultures were made on streptomycin-containing media.

Both variants retained the characteristic sugar fermentations of meningococci and the type specificity of the parent strains from which they arose. Both variants are presumed to arise from mutants which are constantly appearing in the bacterial population of the parent strain.

Whether the substance required by the B variants for their multiplication is streptomycin itself or some impurity has not yet been determined. These variants developed on all of 25 preparations of streptomycin obtained from 7 manufacturers. They failed to develop on streptomycin inactivated by hydroxylamine or by cysteine.

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