OBSERVATIONS ON BACTERIA SENSITIVE TO, RESISTANT TO, AND DEPENDENT UPON STREPTOMYCIN^{1, 2}

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Received for publication May 15, 1948

Streptomycin resistance is usually manifested by growth either in the presence of streptomycin or in its absence. Resistant variants generally are similar, morphologically and biochemically, to the parent sensitive strain (Miller and Bohnhoff, 1946; Murray *et al.*, 1946). Some differences, such as alteration in motility and changes in growth rate and in biochemical reactions, have been reported, but these have been inconsistent (Graessle and Frost, 1946; Klein and Kimmelman, 1946; Murray *et al.*, 1946; Petroff and Lucas, 1946; Seligmann and Wassermann, 1947; Stubblefield, 1947). Streptomycin-resistant organisms have ordinarily been obtained *in vitro* by repeated subculture in increasing concentrations of this antibiotic (Chandler and Schoenbach, 1947; Miller and Bohnhoff, 1946; Murray *et al.*, 1946; Price *et al.*, 1947; Seligmann and Wassermann, 1947), though the occurrence of a few very resistant organisms in a large inoculum of sensitive ones on initial exposure to streptomycin has been described in the case of *Shigella* (Klein, 1947), *Escherichia coli* (Clark and Rantz, 1947; Klein, 1947), *Staphylococcus aureus* (Klein, 1947), *Staphylococcus albus* (Klein, 1947), *Proteus vulgaris* (Klein, 1947), *Hemophilus influenzae* (Alexander and Leidy, 1947), meningococcus (Miller and Bohnhoff, 1947a), and *Mycobacterium tuberculosis* (Vennesland, Ebert, and Bloch, 1947).

In the case of the meningococcus, Miller and Bohnhoff (1947a,b) described another type of resistant variant which apparently required streptomycin for growth. More recently a variant of *Bacillus subtilis* which requires streptomycin for growth has been reported (Kushnick *et al.*, 1947). A possible stimulating effect of streptomycin on growth has been noted in the case of streptomycin-resistant strains of *Brucella* (Hall and Spink, 1947), *Escherichia coli* and *Pseudomonas aeruginosa* (Kushnick *et al.*, 1947), and tubercle bacilli (Vennesland, Ebert, and Bloch, 1947). An increased mortality rate of mice infected with typhoid bacilli and treated with small doses of streptomycin has been reported (Welch, Price, and Randall, 1946). Suitable low concentrations of penicillin and streptomycin stimulated growth of many sporeformers, especially *Bacillus megatherium*, and also *S. aureus* and *Streptococcus agalactiae* (Curran and Evans, 1947).

¹ Aided by a grant from the United States Public Health Service.

² Presented, in part, at the Symposium on Antibiotics, Annual Meeting of the American Association for the Advancement of Science, Chicago, Illinois, December 30, 1947.

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In a preliminary report from this laboratory (Paine and Finland, 1948) the derivation of streptomycin-resistant and streptomycin-dependent variants from several streptomycin-sensitive species of bacteria was noted. Further details of this investigation are given here.

MATERIALS AND METHODS

Definition. The parent strains used to obtain the variants under study were considered to be "sensitive" to streptomycin. Their growth was completely inhibited by concentrations of 100 μ g or less of streptomycin per ml of broth in the usual tube dilution test for sensitivity. "Resistant" strains were those that multiplied equally well in the presence of high concentrations of streptomycin and in its absence. "Dependent" strains were those that multiplied only in the presence of streptomycin; the amount that was required varied with the different organisms.

Media and antibiotics. The broth used throughout these studies was brain-heart infusion broth, pH 7.2 (Difco), and the agar was heart infusion agar (Difco). Various lots of streptomycin hydrochloride (Merck) and streptomycin calcium chloride complex (Merck) as available commercially were used, the amounts being recorded in terms of the streptomycin base. Crystalline penicillin G, potassium salt, was obtained from Commercial Solvents Corporation. The bacitracin was a crystalline preparation provided by Dr. Frank L. Meleney and contained 5,000 units (Johnson, Anker, and Meleney, 1945) per vial. A purified preparation of polymyxin containing 1,800 *E. coli* units (Stansly, Shepherd, and White, 1947) per mg was provided by Dr. Y. SubbaRow of the Lederle Laboratories.

Isolation of resistant and dependent variants. Each organism, previously unexposed to streptomycin, was grown overnight in broth; 10 ml of the culture were then centrifuged at 2,000 rpm for 30 minutes, the supernatant was decanted, and the sediment was suspended in 0.6 ml of saline. One-tenth ml of this suspension was used for bacterial counts in agar pour plates, and the rest was spread evenly over the surface of an agar plate containing 1,000 μ g of streptomycin per ml of agar. The plate was incubated, without inverting, at 37 C for 3 to 5 days, and any colonies which appeared in that time were subcultured on plain agar plates and on plates containing graded concentrations of streptomycin.

Growth curves. A series of tubes containing graded concentrations of streptomycin in 10-ml amounts of broth were each inoculated with 0.1 ml of a 10^{-4} dilution of an overnight broth culture. The dependent variant was always grown in broth containing 1,000 μ g of streptomycin per ml prior to its inoculation into the growth curve tubes. The tubes were incubated at 37 C, and aliquots were removed at varying intervals for agar pour plate counts of viable bacteria. Counts of the dependent variants were made both in streptomycin-free agar and in agar containing 1,000 μ g of streptomycin per ml.

Sensitivity determinations. The sensitivity of these bacteria to antibiotics was tested by a tube dilution method (Finland *et al.*, 1946) utilizing 0.5-ml amounts of serial broth dilutions of the antibiotic to which were added similar volumes of

a 10^{-4} dilution of an overnight broth culture of the test organism (usually containing 50,000 to 100,000 organisms). The sensitivity, in each instance, was defined as the minimum concentration in which no growth occurred after 24 to 48 hours' incubation at 37 C in the antibiotic-containing broth and after another subculture for 24 hours on antibiotic-free agar. Tests with the dependent variants were always conducted in media containing 1,000 μ g of streptomycin per ml.

Biochemical reactions. Fermentation tests on various sugars and other biochemical tests were made on all strains. For the dependent variants, 1,000 μ g of streptomycin per ml were included in all the test materials.

RESULTS

Isolation of variants. Streptomycin-resistant and streptomycin-dependent variants were isolated from sensitive strains of *Staphylococcus aureus*, *Escherichia coli*, *Proteus morgani*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The incidence of resistant and dependent variants in these bacterial populations which had not been previously exposed to streptomycin ranged from 1 in 2 billion to 1 in 400 billion organisms. The readiness with which these variants appeared differed considerably; they were obtained quite regularly from 2 strains each of *S. aureus*, *P. morgani*, and *K. pneumoniae*, but only from 1 of 4 strains of *E. coli* and from 1 of 3 strains of *P. aeruginosa*. A statistically valid assay of the incidence of resistant and dependent variants is not possible on the basis of the number of experiments conducted thus far.

Following an initial exposure of an apparently sensitive strain to 1,000 μ g of streptomycin per ml of agar, some colonies appeared which on subculture proved to consist entirely of highly resistant organisms. These organisms remained resistant on subsequent subcultures both in the presence and in the absence of streptomycin.

Evolution of the dependent variants occurred in a different manner. The "dependent" potentialities of a single colony developing from the initial exposure of a sensitive strain to a high concentration of streptomycin was determined by subculturing a saline suspension of the entire colony on a series of agar plates containing graded concentrations of streptomycin. If the colony contained dependent organisms, there was a predominance of growth on the agar plates with the higher concentrations of streptomycin. However, there was usually slight growth in the lower concentrations of streptomycin or even in its absence. After one or more subcultures from the plates containing the higher concentrations of streptomycin in which growth had been most vigorous, it was possible to obtain strains that would not grow in the absence of streptomycin, that is, "pure" dependent strains.

The nature of the organisms in the predominantly dependent colonies which grew, though sparsely, in the lower concentrations or in the absence of streptomycin was of interest. On subculture, these organisms behaved as predominantly sensitive bacteria. Thus, isolation of "pure" dependent strains involved selective subculture using the higher streptomycin concentrations in which growth had occurred, in order to eliminate the sensitive organisms with which

the dependent variants were associated. A protocol showing the derivation of *S. aureus* variants is given in table 1.

TABLE 1
Derivation of variants of Staphylococcus aureus

COLONY	CONCENTRATION OF STREPTOMYCIN, μ G PER ML OF AGAR						
	1,000	100	25	6.3	3.1	1.5	0
Initial subculture							
S1	+++ ^a	+++	+++	+++	+++	+++	+++ ^b
S2	+++ ^a	+++	+++	+	+	6	3 ^{b, c}
S3	+++ ^a	+++	+++	+	6	4	3 ^b
Second subculture							
S1a	+++	+++	+++	+++	+++	+++	+++
S1b	+++	+++	+++	+++	+++	+++	+++
S2a	+++	+++	+++	+	0	0	0
S2b	3 ^d	+	++	+++	+++	+++	+++ ^e
S2c	1 ^f	+	++	+++	+++	+++	+++ ^g
S3a	+++	+++	+++	+	0	0	0
S3b	0	5 ^e	+	+++	+++	+++	+++ ^d
Third subculture							
S2d	+++	+++	+++	1	0	0	0
S2e	1 ^h	5	++	+++	+++	+++	+++ ^j
S2f	+++	+++	+++	++	++	+	0
S2g	0	0	+	+++	+++	+++	+++
S3c	0	+	++	++	+++	+++	+++
S3d	0	3	+	++	+++	+++	+++ ^f
Fourth subculture							
S2h	+++	+++	+++	+++	+++	+++	+++
S2j	0	5	++	+++	+++	+++	+++
S3f	0	0	6	+++	+++	+++	+++

Each plate was inoculated with a 2-mm loopful of an emulsion of a single colony in 0.2 ml of saline.

The colonies S1, S2, and S3 were picked from an agar plate containing 1,000 μ g of streptomycin per ml of agar on which the parent sensitive strain was exposed to streptomycin for the first time.

The letters indicate colonies picked from the different plates.

+++ , ++ , + , and 0 indicate amount of growth on the surface. When only a few isolated colonies appeared, their numbers are indicated.

Dependence upon streptomycin was not nearly so permanent a characteristic as that of resistance to the antibiotic. When grown in suboptimal concentrations of streptomycin, organisms rapidly reverted from the "dependent" to the sensitive and sometimes to the resistant state.

Colony characteristics and morphology. When grown under optimal conditions,

there were usually no differences in the colonial appearance of the sensitive, resistant, and dependent strains of the same organism. In the case of *Proteus*, however, the resistant and dependent variants showed less tendency to swarm than did the parent sensitive strains.

Microscopically, the sensitive strains showed only slight alteration from the normal appearance when grown in sublethal amounts of streptomycin. This was also true of the resistant variants grown either in the presence or in the absence of the antibiotic. On the other hand, the dependent variants showed marked pleomorphism when cultured in suboptimal concentrations of streptomycin. The dependent *Staphylococcus*, under these circumstances, exhibited great variation in the size of the cocci, some appearing greatly enlarged. Dependent

Figure 1. Representative growth curves of a sensitive strain of *P. morgani* and its resistant and dependent variants in broth containing graded concentrations of streptomycin.

variants of gram-negative bacilli grown in suboptimal amounts of streptomycin showed long filamentous structures. This was particularly striking in the case of the dependent *Proteus*, which also showed the presence of bulbous, fusiform, and densely stained swellings along the shafts of some of the filaments. No such forms were noted when any of the dependent variants were grown in optimal concentrations of streptomycin.

Growth curves. These were conducted with all of the organisms and their variants. There were several characteristics common to the variants of all of the different organisms studied. The curves for the *Proteus* variants depicted in figures 1 and 2 will serve as examples.

Sensitive strains grown in sublethal amounts of streptomycin showed a progressive increase in the lag period as the concentration of the antibiotic was increased. Though not shown in figure 1, this progression was demonstrable

with graded concentrations below 12.5 μg per ml of streptomycin in the case of the sensitive *Proteus*.

The resistant variants grew equally well in plain broth and in concentrations of streptomycin up to 1,000 μg per ml. In concentrations of 10,000 to 50,000 μg per ml there was usually some inhibition of the rate of growth of the resistant strains.

Dependent variants, except in the case of *E. coli*, apparently did not multiply in the absence of streptomycin or in very low concentrations of the drug. The dependent coli in broth containing no streptomycin showed slight initial multi-

Figure 2. Growth curves of the dependent strain of *P. morgani*. Each pair of curves represents the counts from the same tube of broth poured simultaneously in agar with and without streptomycin.

plication, but growth was not maintained and the organisms soon disappeared in a fashion similar to the dependent *Proteus* shown in curve A, figure 1. All of the dependent strains showed a stepwise increase in the rate of growth, or shortening of the lag period, in increasing concentrations of streptomycin. Maximum rates of growth were achieved in the range to 500 to 1,000 μg per ml. Some inhibition of the rate of growth of the dependent variants occurred in very high concentrations of the antibiotic (curve H in figure 1). The dependent *Staphylococcus* occasionally failed to grow in a concentration of 20,000 μg of streptomycin per ml. The maximum rates of growth of both the resistant and dependent variants were usually less rapid than the maximum rate of growth of the corresponding parent sensitive strains.

Certain other properties of the dependent variants are illustrated by the curves shown in figure 2. In this instance the solid lines represent the colony counts in agar containing 1,000 μg of streptomycin per ml, and the broken lines depict colony counts from the same tube of broth poured simultaneously in agar containing no antibiotic. In each tube in which vigorous growth of the dependent organism took place organisms appeared in the pour plates both with and without streptomycin. Colonies appearing in the plain agar plates probably did not represent dependent organisms growing in the small amount of streptomycin carried over from the broth tubes, since these concentrations were inadequate for that purpose.

From the tubes containing the higher concentrations of streptomycin the colonies which appeared in the plain agar pour plates never approached in numbers those appearing simultaneously in the streptomycin agar pour plates. On the other hand, in the lower concentrations (6.2 to 50 μg per ml) the colonies which appeared in the plain agar plates usually approached in numbers those appearing at the same time in the streptomycin agar plates and sometimes exceeded them.

Studies with single colonies from plates poured after 25 days' incubation of the broth tubes showed that the organisms from the 6.2- and 25- μg -per-ml tubes were now largely sensitive bacteria; the colonies from the 50- μg -per-ml tube consisted largely of resistant organisms; whereas those from the tubes containing 100 and 1,000 μg per ml were still predominantly dependent. Thus, in the case of the dependent *Proteus* reversion from the dependent to the sensitive state occurred after prolonged incubation in the presence of 6.2 or 25 μg of streptomycin per ml, and resistant organisms emerged in the presence of 50 μg per ml.

It seems likely that vigorous growth of the dependent strains was accompanied by the appearance of sensitive variants. In general, when this occurred in the presence of high concentrations of streptomycin, further growth of the sensitive variants was suppressed; but when this took place in low concentrations of the antibiotic, multiplication of the sensitive variants was possible and either sensitive or resistant strains seemed to emerge. When vigorous growth of the dependent strains did not take place, as in the very low concentrations of streptomycin, neither sensitive nor resistant variants appeared.

Biochemical reactions. Study of the fermentation of various carbohydrates, the production of indole, ammonia, hydrogen sulfide, and acetoin, nitrate reduction, citrate utilization, liquefaction of gelatin, hemolytic and coagulase activity, pigment production, and motility disclosed no differences between the sensitive bacteria and their derived resistant and dependent variants in most instances. Two sensitive strains of *K. pneumoniae* fermented dulcitol with the production of gas, whereas their resistant and dependent variants did not ferment dulcitol. A sensitive strain of *K. pneumoniae*, type C, uniformly showed capsular swelling and clumping with homologous rabbit antiserum; its resistant variant showed capsule swelling and clumping of only some of the organisms, whereas the dependent variant exhibited neither swelling of the capsules nor clumping in the presence of the antiserum.

Sensitivity to other antibiotics. In table 2, are shown the results of sensitivity determinations conducted with the 3 variants of each of the organisms against penicillin, bacitracin, and polymyxin. There were only minor differences in the sensitivity of the three variants of each organism to these antibiotics.

Effect of streptomycin inhibitors. Cysteine hydrochloride and semicarbazide, two substances which inhibit the action of streptomycin on susceptible bacteria, also interfered with the utilization of this antibiotic by the dependent variants.

Critical concentration of streptomycin. As previously noted (Paine and Finland, 1948), growth ceased in the case of both the sensitive and dependent strains

TABLE 2
Sensitivity to other antibiotics of variants sensitive to, resistant to, and dependent upon streptomycin

ORGANISM	VARIANT	MINIMUM CONCENTRATION FOR COMPLETE INHIBITION, UNITS PER ML		
		Penicillin	Bacitracin	Polymyxin
<i>Staphylococcus aureus</i>	Sensitive	0.125	1.0	>450
	Resistant	0.125	1.0	>450
	Dependent	0.031	0.125	225
<i>Escherichia coli</i>	Sensitive	39	500	0.016
	Resistant	39	500	0.016
	Dependent	39	500	0.031
<i>Proteus morgani</i>	Sensitive	1,250	500	>450
	Resistant	1,250	500	>450
	Dependent	625	500	50
<i>Pseudomonas aeruginosa</i>	Sensitive	>10,000	>500	1.0
	Resistant	>10,000	>500	2.0
	Dependent	5,000	>500	1.0
<i>Klebsiella pneumoniae</i>	Sensitive	78	200	2.0
	Resistant	39	50	0.125
	Dependent	39	100	0.25

of the same organism at about the same concentration of streptomycin. This was true for *S. aureus*, *E. coli*, *P. aeruginosa*, and *P. morgani*. The same was true also of *K. pneumoniae*; growth of its parent sensitive strain was inhibited by 3.1 μ g of streptomycin per ml, and this was the minimum concentration supporting growth of its dependent variant.

DISCUSSION

The most widely accepted explanation for the ability of microorganisms to survive in unfavorable concentrations of chemotherapeutic or antibiotic agents is that it results from the selection of chance mutants which can survive in that environment (Alexander and Leidy, 1947; Demerec, 1945; Klein and Kimmel-

man, 1946; Luria, 1947; Miller and Bohnhoff, 1947*a,b*). The present observations, like those of Miller and Bohnhoff (1947*a,b*) with the meningococcus, show that the ability of certain bacteria to survive exposure to an ordinarily lethal concentration of streptomycin is due to the appearance of variants which possess either of two characteristics: (1) independence of streptomycin activity, more commonly known as resistance to streptomycin, or (2) dependence upon streptomycin for growth. The first trait seems to be a relatively permanent one and apparently involves the entire progeny of the resistant organism once it appears. It thus more nearly fulfills the usual criteria of a mutant. This resistance, however, is not completely permanent in every instance since reversion to a sensitive status has been recorded in the case of certain streptomycin-resistant organisms when they were subcultured many times in the absence of the antibiotic (Murray, Wilcox, and Finland, 1947).

Dependence upon streptomycin appears to be a much more labile trait than resistance to this antibiotic. In this respect the dependent variants studied in this laboratory varied considerably from the dependent strains of meningococci described by Miller and Bohnhoff (1947*b*), in which there apparently was little tendency for a change from the dependent state. All of the dependent variants studied here were found, during growth in broth, to give rise to organisms which would grow in the absence of streptomycin. The evidence suggests that the dependent variants give rise only to streptomycin-sensitive organisms. The latter, in turn, if given the opportunity to multiply in sublethal amounts of streptomycin, may then give rise to resistant strains. Reversion from the dependent state took place only during multiplication of the organisms. Mutations directly from the dependent to resistant strains or the reverse may occur, but the present observations do not favor such possibilities.

The present findings suggest that dependence upon and sensitivity to streptomycin may be closely related phenomena. (1) Both sensitive and dependent organisms were demonstrated in the same "predominantly dependent" colony which appeared following the initial exposure of the sensitive strain to streptomycin. (2) The dependent variants seem to give rise only to sensitive organisms under appropriate conditions. (3) The same concentration of streptomycin which completely inhibited the growth of the parent sensitive strain seemed necessary for growth of its dependent variant. No such relationships could be demonstrated in the case of the resistant variants.

The exact mechanism by which streptomycin exerts its antibacterial effect is not known. Assuming that it is effective against sensitive organisms by blocking some essential metabolite or enzyme system, it seems to be more than mere coincidence that approximately the same concentration of streptomycin which inhibits growth of the sensitive strain becomes necessary for growth of the dependent variant of the same strain. Is the original essential metabolite now replaced by some moiety of the streptomycin molecule which can be utilized in its place? In the case of the resistant variants, they grow independently of the presence or absence of streptomycin. It would seem, therefore, that an alternative mechanism or system exists in the resistant variant which functions

in the place of the metabolite or enzyme system that is blocked by streptomycin in the sensitive strain.

Another change which appears in both the dependent and resistant variants is that they grow at a slower rate than their parent sensitive strain. This is an interesting mutational character similar to the slower growth rates described in the case of certain phage-resistant variants (Luria, 1946). This slower growth rate of the resistant variant may explain why it is not normally encountered until the more rapidly growing sensitive organisms are inhibited by streptomycin.

Consistent biochemical differences between the sensitive, dependent, and resistant variants of the same organisms were not encountered except in the case of *K. pneumoniae*. Similar changes were previously noted in resistant strains of this organism (Murray *et al.*, 1946). The differences in sensitivity to other antibiotics of some of the three variants are not explained.

The pleomorphic forms of the streptomycin-dependent variants grown in suboptimal amounts of streptomycin were similar to those observed by many workers when organisms were grown under unfavorable conditions (Dienes, 1947; Gardner, 1925, 1945; Gay and Clark, 1937; Shanahan, Eisenstark, and Tanner, 1947; Tunnicliff, 1939; Walker and Murray, 1904; Wyckoff, 1933, 1934).

Miller and Bohnhoff (1947b) have pointed out that one cannot be certain that their dependent variants were actually dependent on streptomycin itself and not on some impurity. However, other bacterial and fungal mutants have been described that have undergone marked transformations in their growth requirements. For instance, Kohn and Harris (1942) were able to derive a methionine-requiring variant of *E. coli* by subculturing the parent strain in a medium containing sulfanilamide and certain amino acids. A strain of *Neurospora* requiring sulfanilamide for growth has been described (Emerson, 1947) and stimulation of growth of *P. aeruginosa* and *Alcaligenes faecalis* by sulfanilamide has been observed (Lamanna, 1942).

SUMMARY AND CONCLUSIONS

Streptomycin-resistant and streptomycin-dependent variants have been obtained from streptomycin-sensitive strains of *Staphylococcus aureus*, *Escherichia coli*, *Proteus morgani*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

The growth characteristics, morphologic features, and certain biochemical reactions of these organisms and of their derived variants have been described.

The evidence suggests that resistance to streptomycin is a relatively permanent trait, whereas dependence upon streptomycin is a readily reversible phenomenon.

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