DEVELOPMENT OF INCREASED BACTERIAL RESISTANCE TO ANTIBIOTICS

I. Continuous Spectrum of Resistance to Penicillin, Chloramphenicol, and Streptomycin[1]

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The development of increased bacterial resistance to antibiotics following exposure to the drugs has been demonstrated with every antibiotic and every bacterial species so far studied. In some instances the resistance of the strain gradually returns toward normal levels on repeated subculture in the absence of the drug. In other cases, however, the resistance remains essentially unchanged after hundreds of generations in antibiotic-free media.

Two general mechanisms may be postulated for the development of this heritable change. The resistant organisms may arise as spontaneous mutations which occur in the course of bacterial multiplication. These rare mutants would be present in the original culture, the drug acting merely as a selective factor. Most workers incline to this view, which is strongly supported (Demerec, 1945, 1948, 1949; Bryson and Demerec, 1950) by the results obtained with the variance analysis technic of Luria and Delbrück (1943) and the replicate clone technic of Lederberg and Lederberg (1952). Mutations manifested by increased resistance to penicillin have been estimated to occur approximately once in every 10^8 divisions (Demerec, 1945). In this first step mutation, the resistance of the organisms to penicillin is only slightly increased. In the normal course of events, the first step mutants would presumably be obscured by the overwhelming number of normal bacteria present; but in the presence of suitable concentrations of penicillin, the normal bacteria are killed or inhibited, and only the resistant mutants grow out. In subculture, the resistant variant in turn is considered to undergo mutation, and this second step mutant can in turn be isolated in the presence of suitably higher concentrations of penicillin. By a series of such selective transfers of single step mutants, the resistance of the strain may be significantly increased. The chance of having two simultaneous or successive mutations in the same culture would be extremely small ($P = 10^{-16}$ per cell division); and in the average culture tube, the penicillin-resistant cells would therefore all be first step mutants, presumably at the same level of resistance. In the case of streptomycin, although the mutation rate has been estimated to be on the order of 10^{-9} to 10^{-10} per cell generation, a given culture may contain

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organisms of widely varying resistance; and it has been suggested (Demerec, 1948, 1949; Bryson and Demerec, 1950) that in this case, the first step mutations may involve different genes of varying "potencies", with correspondingly varying phenotypic expression.

If the organisms continued to multiply even after the addition of the drug, a mutation could occur in the presence of the drug, but not determined by it. If the mutant organism grew more rapidly in the presence of the antibiotic than the parent strain, its progeny could eventually replace the original population, and one could end with a uniformly resistant population (Demerec *et al.*, 1950). In this case also, however, one would be dealing with a spontaneous mutation, the drug again acting merely as a selective agent. An analogous situation in the appearance of histidine-requiring mutants of *Escherichia coli* has been carefully studied by Ryan and Schneider (1948).

An alternative possibility is that increased resistance to antibiotics is not the result of a spontaneous mutation, but develops as a direct result of exposure to the drug, and is an adaptive change which persists after its removal. Induced and heritable variations involving a major proportion of the progeny of the treated populations have been described by Sonneborn (1950, 1951) in the killer and serotype characters of paramecia, by Spiegelman, Sussman, and Pinska (1950) in yeast adapting to galactose, by Ephrussi *et al.* (1949 *a*, *b*, *c*) in yeast exposed to acriflavine, and by L'Heritier (1948) in *Drosophila* treated with extracts of a CO_2-resistant strain. In all of these cases, the induced character is cytoplasmically inherited, although in some instances it has been demonstrated that this cytoplasmic inheritance is not autonomous but is in turn genically controlled.

Limited and inconclusive evidence that the development of increased bacterial resistance to antibiotics may involve a similar adaptive and heritable change has been presented by Abraham, Callow, and Gilliver (1946), Eriksen (1949), Linz *et al.* (1949), Linz (1950), Hauduroy and Rosset (1950), Seligmann and Wassermann (1947), and Barer (1951).

The two hypotheses of spontaneous genic mutation and a drug-directed variation as the cause of an increased bacterial resistance to antibiotics are not mutually exclusive. Thus, Gibson and Gibson (1951) have suggested that in the case of *E. coli* and streptomycin, an initial tenfold increase in resistance may be due to adaptation; while highly resistant organisms may arise as the result of a spontaneous mutation. Even if bacteria were conclusively shown to adapt to certain antibiotics, the capacity to adapt might itself be genically controlled, as in the experiments of Sonneborn and of Spiegelman cited before; or the drug might conceivably affect the genic mechanism of the cell directly.

This introductory paper will examine the distribution of resistance in various bacterial populations to penicillin, streptomycin, and chloramphenicol. As will be shown, at threshold concentrations of these antibiotics, a large proportion of the cells is capable of growing out to form resistant colonies. This proportion falls off progressively the higher the concentration of antibiotic in which the organisms are placed. The degree of resistance in the formed colony is related to the concentration of antibiotic at which the organisms had grown out, with no evidence

of a step-wise and discontinuous process. Following papers will consider in greater detail whether the increased resistance in the emergent colonies represents an adaptive change caused by the drug or represents instead the selective multiplication of a spontaneous mutant.

METHODS AND MATERIALS

Organisms studied. Six bacterial species[3] were studied, a type 3 group A β-hemolytic streptococcus (strain C-203), a group B β-hemolytic streptococcus, *Streptococcus faecalis*, *Diplococcus pneumoniae*, type III, *Micrococcus pyogenes* var. *aureus*, and *Escherichia coli* (strain K-12). Single colony isolations were repeated 3 to 4 times before the culture was used for the present studies. However, this precaution to obtain a culture deriving from one or two cells proved unnecessary: with each of the six species here studied, the distribution of resistance within such single colony cultures was the same as in the original stock culture.

Antibiotics used. The sodium penicillin G and streptomycin sulfate used in these studies were commercial samples. The courtesy of the Lederle Laboratories in supplying aureomycin hydrochloride, and of the Parke Davis Company in supplying chloramphenicol (synthetic "chloromycetin") is gratefully acknowledged. The studies here described could not be carried out with aureomycin because of its rapid deterioration under the conditions of the test.

Technic of tests for resistance. Cultures were grown in beef heart infusion broth, in some cases enriched with 2 per cent horse blood. When the number of organisms had reached 50 to 500 $\times$ 10^6 per ml (as determined by microscopic count, with a simultaneous estimation of the number of organisms per clump), the culture was diluted to contain 5 $\times$ 10^7 organisms per ml. Three serial 40-fold dilutions in blood broth were prepared from this stock suspension and immediately placed in ice water until used. Two-tenths ml of each dilution was added to a tube containing 8.6 ml of beef heart infusion glucose agar, 0.5 ml of horse blood, and varying amounts of antibiotic; and the total was adjusted to 10 ml with broth. (In later experiments, the total volume was increased to 12 and to 15 ml.) The blood agar was poured on petri plates after thorough admixture. Unless otherwise specified, all the plates were poured in duplicate, and the average value has been used in plotting the figures and in the tables. After incubation at 37 C for 5 to 7 days, the total colony count on each plate was determined with the aid of a dissecting microscope at 9$\times$ magnification, and referred to that which grew out in control plates containing no antibiotic as 100.

Sources of error: a. *Association of bacteria in chains or clumps.* At the higher concentrations of antibiotic, at which most of the organisms were nonviable, each of the few colonies which developed would, with a high degree of probability, be the clone of a single surviving organism if the bacteria originally associated with it in short chains or small clumps had been killed by the antibiotic. In estimating the proportion of resistant cells, the number of colonies would then have to be

[3] The strains of *Streptococcus faecalis*, *Diplococcus pneumoniae*, type III, *Micrococcus pyogenes* var. *aureus*, and *Escherichia coli* here used were obtained through the courtesy of Drs. Justina Hill, Colin MacLeod, W. F. Verwey, and J. Lederberg, respectively.

referred to the total number of bacteria inoculated, rather than to the number of clumps microscopically visible in the original culture, or to the number of colonies formed on the control plates. This would assume that all the microscopically visible organisms were in fact viable, and would in addition be subject to the large error in the method of microscopic enumeration when conducted rapidly on the scale necessary for these experiments. On the other hand, if all the organisms in a single clump or chain were identical in their reaction to the antibiotic, the number of colonies would have to be referred to the number of viable clumps in the dilution actually used for plating. It would then be necessary to assume that the average number of organisms per surviving clump was equal to the average number per clump in the original culture.

The latter method of calculation has been used in this and following papers, not because it is necessarily the more correct, but solely for reasons of technical simplicity. A variable and sometimes significant error is thereby introduced if the organisms in each clump were not in fact identical in their reaction to the antibiotic.

b. *Volume of agar in tubes.* The volume of agar medium in a tube decreases materially in the course of sterilization and storage. Because a slight difference in the concentration of antibiotic sometimes has a striking effect on the number of surviving cells, it was found necessary to adjust the volume of the agar in appropriately etched tubes prior to the addition of measured volumes of the antibiotic and the inoculum.

c. *pH.* As shown by Abraham and Duthie (1946), the pH of the medium profoundly modifies the bactericidal activity of the penicillin and streptomycin. As will be shown elsewhere, a relatively small difference in pH may change the number of surviving organisms several hundredfold (Eagle, to be published). In most of the experiments here described, the initial pH of the broth and agar medium was 7.15 to 7.25.

d. *Temporary exposure of bacteria to high concentrations of antibiotic.* When a solution of streptomycin was added to melted agar at 52 C, it sometimes formed a more or less concentrated layer of drug on top of the agar. When an inoculum was then introduced, unless care was taken to shake the contents of the tube immediately and completely, the temporary exposure of the bacteria to the high concentration of antibiotic in the top of the tube in some instances materially reduced the number of colonies which eventually grew out when the contents of the tube were plated.

e. *Dehydration of plate and deterioration of antibiotic.* With each of the three antibiotics and all of the bacterial species here studied, at concentrations of antibiotic which sufficed to kill some but not all the organisms, the surviving bacteria grew out slowly, the colonies becoming visible at 9× magnification only after 2 to 5 days at 37 C. As a routine procedure, the colonies were therefore counted after incubation at 37 C for 5 days. By this time, significant amounts of the various antibiotics might have deteriorated; and with those antibiotics which were not particularly labile, the effective concentration in the agar might actually have increased because of the interim dehydration of the plates. Experiments

were therefore conducted to determine the effect of incubation at 37 C on the antibiotic activity of chloramphenicol, streptomycin, and penicillin in blood agar plates and on the rate of dehydration. The results are summarized in table 1. [The technic of assay used for all three antibiotics was a serial dilution technic similar to that previously described for penicillin assay (Eagle and Newman, 1947), using five amounts of the antibiotic solution for each 2-fold difference in concentration (0.8, 0.72, 0.6, 0.48, and 0.4 ml).] Under the conditions of the present experiments and with the media here used, the agar decreased in weight by an average of 7, 20, and 34 per cent after 1, 3, and 5 days, respectively, at 37 C. The penicillin activity of the agar after 1, 3, and 5 days averaged 80, 74, and 51 per cent, respectively, of that originally placed in the plate; the chloramphenicol activities were 103, 124, and 119 per cent, and the streptomycin activities were 81, 104, and 99 per cent. Penicillin was thus the most susceptible to deterioration, and that deterioration was counteracted only in part by the concentration of the agar due to its evaporation. Chloramphenicol deteriorated only slightly, with the result that its effective concentration in the plate actually increased; and in the case of streptomycin, the two processes proceeded at approximately the same rate. The slight increase in the concentration of chloramphenicol proved a serious source of error after the resistance of the strains had been built up by serial selection and transfer. In such resistant cultures there was often only a slight difference between the most susceptible and the most resistant organisms in a given culture; and in such instances, an increase in concentration of only 20 per cent often meant that a large proportion of the organisms which would otherwise have grown out to form a visible colony was prevented from multiplying.

TABLE 1

Effect of incubation at 37 C on weight of blood agar in poured plates and on its antibiotic activity

DURATION OF INCUBATION AT 37 C	0	1 DAY	3 DAYS	5 DAYS
Weight loss of agar, per cent		6.9 ± 1.5* (12)†	20 ± 4.1 (30)	34 ± 6.9 (30)
Penicillin activity,‡ per cent	92 ± 2* (5)†	80 ± 9 (5)	74 ± 16 (10)	51 ± 11 (10)
Chloramphenicol activity,‡ per cent	91 ± 18 (4)	103 ± 9 (5)	124 ± 31 (10)	119 ± 19 (10)
Streptomycin activity,‡ per cent	82 ± 4 (5)	81 ± 7 (5)	104 ± 22 (10)	99 ± 20 (10)

* Standard deviation calculated as $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$

† Number of plates tested shown in parentheses.

‡ Original concentrations in agar were: penicillin, 0.2 μg/ml; chloramphenicol, 4 μg/ml; streptomycin, 20 μg/ml.

Since most of the colonies which developed in 5 days on plates containing penicillin or streptomycin were already visible on the fourth day of incubation, and the organisms had therefore presumably been multiplying for a period of at least 24 to 48 hours, it follows that with these two antibiotics most of the organisms eventually counted as colonies had begun to grow out in the presence of a concentration of antibiotic not significantly different from that originally placed in the medium.

EXPERIMENTAL RESULTS

It is well known that if a bacterial culture is exposed in an agar base medium to varying concentrations of an antibiotic (penicillin, streptomycin, chloramphenicol), there is a lower limiting concentration at which all the organisms grow out to form colonies, and an upper limit at which no colonies appear. Between these two values is a range, sometimes extremely narrow, sometimes quite broad, in which the number of organisms which survive and multiply to form visible colonies decreases with increasing concentrations of the drug.[4] The magnitude of the differences in the sensitivity of the individual organisms varies with the particular strain, and varies also with the antibiotic used. This is evident on comparing the left hand curves in the several sections of figures 1, 2, and 3. Qualitatively similar results were obtained with the other three bacterial species studied. Comparing the 6 bacterial species here studied, the individual staphylococci in a single colony culture varied to a considerably greater degree in their resistance to penicillin or streptomycin than any of the other species tested, but not in their resistance to chloramphenicol. Comparing the three antibiotics, all the bacterial species tested showed a much greater variability when tested with streptomycin than with either chloramphenicol or penicillin. Thus, in the case of the group A streptococcus in penicillin or the type III pneumococcus in chloramphenicol, as little as a 1.3-fold difference in antibiotic concentration made the difference between 10 per cent[5] and 0.0001 per cent survival; whereas

[4] The first portion of the curves relating the proportion of survivors to the concentration of antibiotic (down to 0.001 to 0.01 per cent survivors) often conforms to a "normal" distribution, in that the survivors are linearly related to the log of antibiotic concentration. However, the last portion of the curve often deviates markedly from that relationship, and a simple log-log plot has therefore been used in all the figures in the present paper.

[5] Ten per cent survival is used as the base point rather than 100 per cent for several reasons. (a) The plate-counting technic is not sufficiently accurate to distinguish with certainty between 100 and, e.g. 80, per cent survival except by doing a prohibitive number of replicates, and is therefore inadequate to spot with reasonable accuracy the concentration which first effects a reduction in the number of surviving organisms. (b) When more than 10 per cent of the organisms grow out on the plate, a complication is introduced by the fact that bacteria in culture are associated in chains or clumps. Thus, if 10 per cent of the bacteria in the original inoculum were associated in pairs, 30 per cent were in clumps of 4, 40 per cent in clumps of 6, 20 per cent in clumps of 8, and 10 per cent in clumps of more than 8, and if bacteria were killed in the same proportions no matter how they were associated, then an actual mortality of 50 per cent would be reflected in a decrease in the plate of only $\frac{10}{4}+\frac{20}{16}+\frac{40}{64}+\frac{20}{128}+\frac{10}{128}+\ldots = 5$ per cent. Similarly, an actual mortality of 80 per cent

in the case of *Micrococcus pyogenes* var. *aureus* in streptomycin (figure 2), it required ten times as much antibiotic to reduce the survivors to 0.0001 per cent as sufficed to kill off 90 per cent of the population.

If these differences in the susceptibility of individual organisms reflected a noninherited variability ("normal" distribution), then subcultures of colonies which had grown out at different concentrations of antibiotic would show essen-

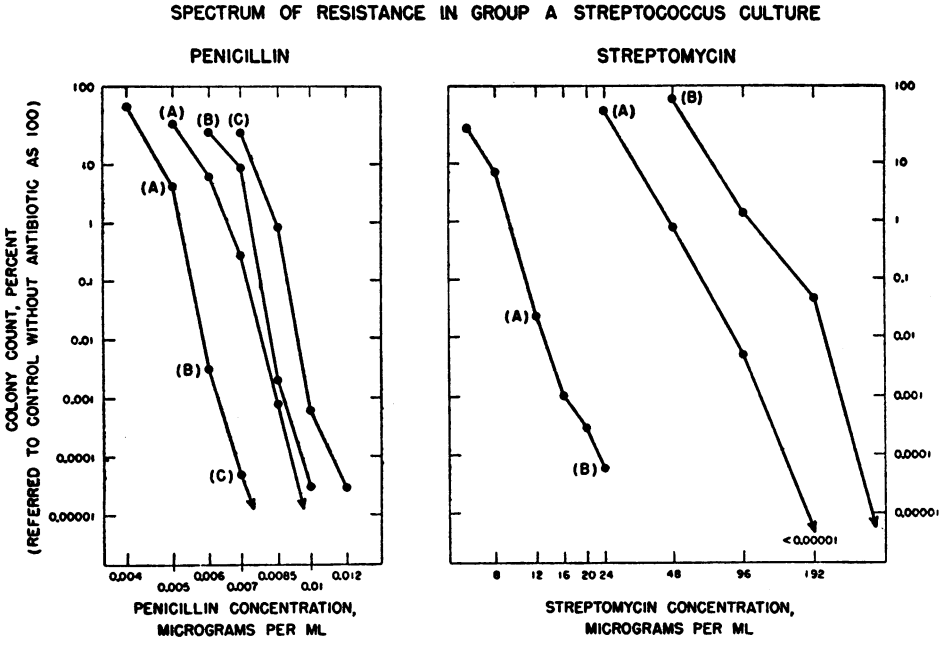

Figure 1. The spectrum of resistance in normal single colony cultures of a group A streptococcus (strain C-203), and in the subcultures of colonies which had grown out at various concentrations of antibiotic.

The left hand curve in each section shows the spectrum of resistance in the normal culture. The letters (A), (B), and (C) along that left hand curve indicate colonies which were fished and subcultured in the absence of antibiotic. The results obtained with those subcultures are indicated in the curves headed by the corresponding letter. A constant inoculum of 10^7 organisms, determined by microscopic count, was used throughout.

tially the same distribution of resistance as the parent culture. This did not prove to be the case. Instead, organisms growing out at different concentrations of antibiotic regularly gave rise to cultures the resistance of which was related to the concentration from which they had been isolated. In a typical experiment with group A streptococcus in penicillin (table 2), the percentage of organisms growing

would give a plate mortality of $(10 \times 0.8^2) + (20 \times 0.8^4) + (40 \times 0.8^6) + (20 \times 0.8^8) + \ldots = 28$; and only at mortalities well in excess of 90 per cent would the decrease in plate count begin to correspond to the actual mortality. The difficulty in using the initial microscopic count (number of bacteria) as the basis of reference rather than the initial plate count (number of clumps) has been previously discussed.

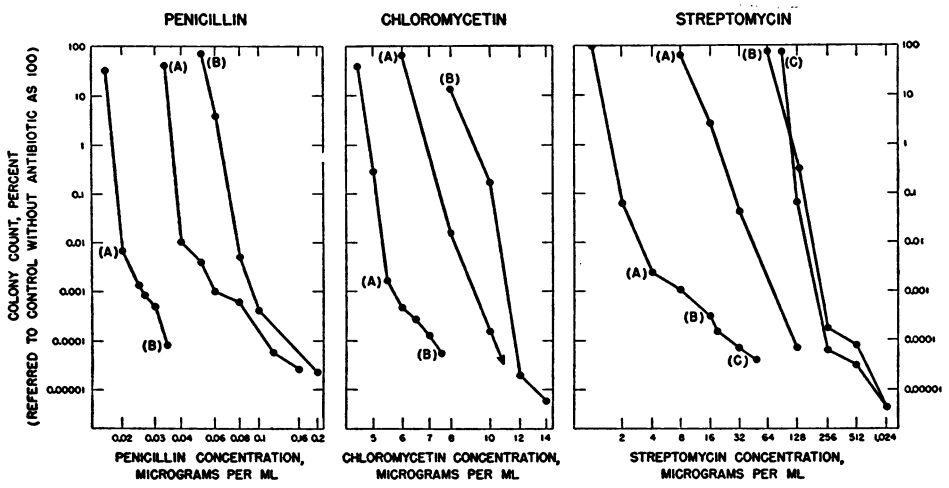

Figure 2. The spectrum of resistance in normal single colony cultures of *Micrococcu pyrogenes* var. *aureus* and in the subcultures of colonies which had grown out at various concentrations of antibiotic.

The left hand curve in each section shows the spectrum of resistance in the normal culture. The letters (A), (B), and (C) along that left hand curve indicate colonies which were fished and subcultured in the absence of antibiotic. The results obtained with those subcultures are indicated in the curves headed by the corresponding letter. A constant inoculum of 10^7 organisms, determined by microscopic count, was used throughout.

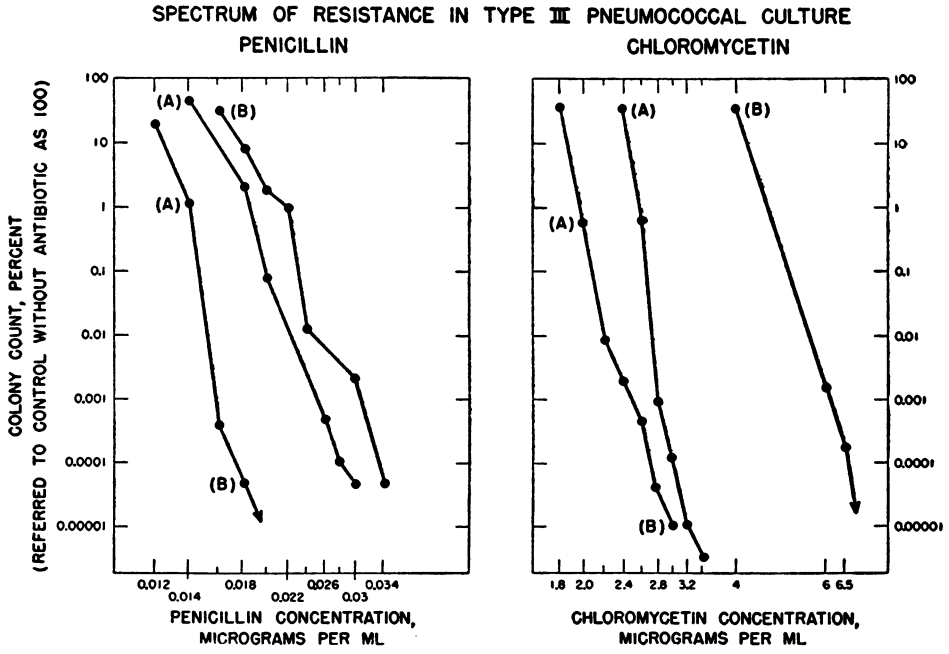

Figure 3. The spectrum of resistance in normal single colony cultures of *Diplococcus pneumoniae*, type III, and in the subcultures of colonies which had grown out at various concentrations of antibiotic.

The left hand curve in each section shows the spectrum of resistance in the normal culture. The letters (A), and (B), along that left hand curve indicate colonies which were fished and subcultured in the absence of antibiotic. The results obtained with those subcultures are indicated in the curves headed by the corresponding letter. A constant inoculum of 10^7 organisms, determined by microscopic count, was used throughout.

out at concentrations of 0.004, 0.005, 0.006, 0.007, and 0.0085 micrograms per ml was 86, 52, 0.25, 0.003, and 0.00003, respectively. When a colony isolated from 0.006 micrograms per ml was subcultured once in the absence of antibiotic and retested, it now gave 37 per cent survivors at that concentration, instead of 0.25. A subculture from a colony which had grown out at 0.007 micrograms per ml gave 36 per cent survivors when retested at that same concentration, 10,000 times the "normal" value of 0.003 per cent. In each instance, the progeny of organisms which had grown out at a given concentration of penicillin was largely resistant to that concentration and was killed to a significant degree only at a slightly higher concentration of drug.

Figures 1, 2, and 3 illustrate qualitatively similar relationships observed in many experiments with each of the six bacterial species here studied, and with

TABLE 2

The spectrum of resistance in group A streptococcal cultures as a function of the concentration of penicillin at which the parent colony had grown out

Single colonies which had grown out in varying concentrations of penicillin (top horizontal line in table) were subcultured in antibiotic-free medium and retested to determine the altered spectrum of resistance.

CONC OF PENICILLIN AT WHICH COLONY HAD GROWN OUT, μG/ML	PERCENTAGE OF ORGANISMS VIABLE AT						
	0.004	0.005	0.006	0.007	0.0085	0.01	0.012 μG/ML
0 (Normal colony grown in antibiotic-free medium)	86	52	0.25	0.003	0.00003	0	
0.005		42	10	0.3	0.008	0	
0.006			37	8.6	0.002	0.00003	
0.007				36	0.8	0.0006	0.00003

each of the three antibiotics. In each section of those figures, the left hand curve shows the spectrum of resistance in the original single colony culture. The symbols (A), (B), and (C) along those curves indicate the concentrations of antibiotic from which daughter colonies were fished and subcultured for retest; and the curves headed (A), (B), and (C) show the results obtained on that retest.

As shown by the similarity in the shape of the curves in these figures, the individual organisms in the resistant cultures showed the same variation in resistance as the parent strain but at uniformly higher concentrations of antibiotic. In most cases, the colony subcultures were wholly resistant to approximately that concentration of antibiotic to which the parent cell had originally been exposed; a significant fraction of the organisms was killed only at slightly higher concentrations (table 2). As is seen in the figures, this relationship was not quantitative. Not infrequently a colony which had grown out in a given concentration of antibiotic was resistant to significantly higher concentrations than that to which it had been exposed. In general, however, the higher the exposure concentra-

tion, the greater was the resistance of the emergent colony. A similar relationship between the resistance of a bacterial strain and the concentration of drug to which it had previously been exposed has been pointed out for *E. coli* in sulfonamide by Kirby and Rantz (1943), for *E. coli* in chloramphenicol by

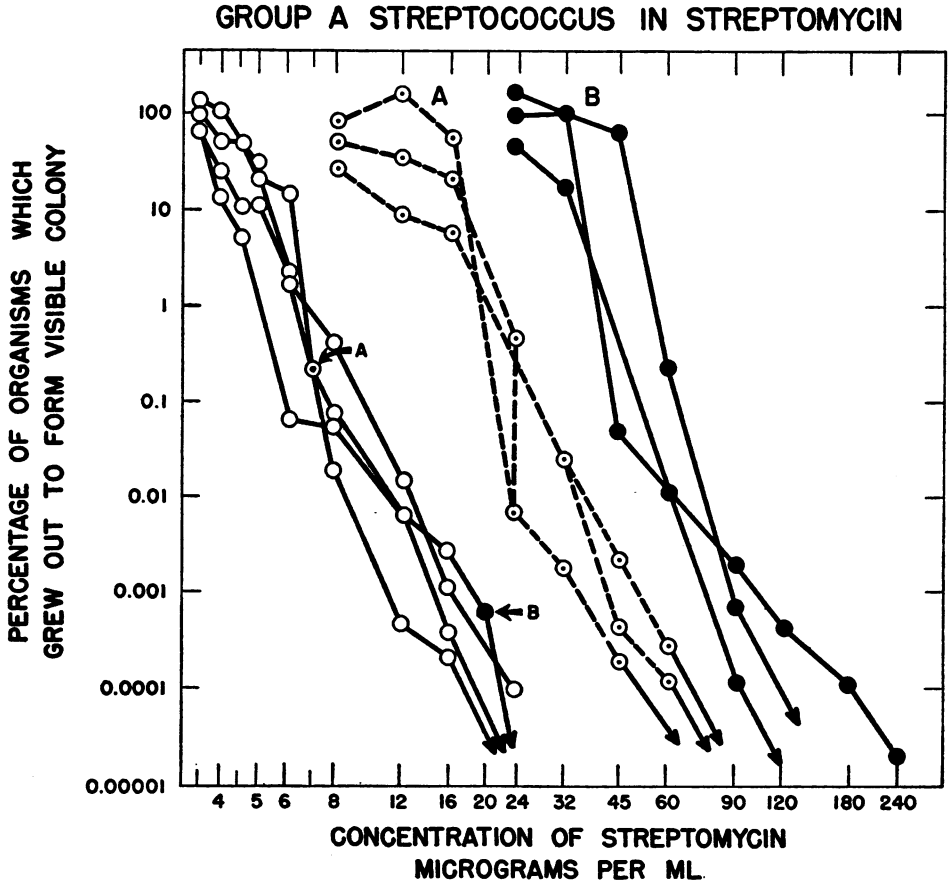

Figure 4. The resistance to streptomycin in subcultures of group A streptococcus colonies.

○——○ = normal colonies.
(A) ⊙- - - -⊙ = colonies which had grown out in 7 μg/ml streptomycin.
(B) ●——● = colonies which had grown out in 24 μg/ml streptomycin.

Cavalli and Maccacaro (1950), and for *Bacterium lactis aerogenes* in streptomycin by Barer (1951).

The uniformity in the resistance spectrum of normal colonies, of the slightly resistant colonies which emerge at low concentrations of antibiotic, and of the more resistant colonies which grow out at higher concentrations, is illustrated in the experiment of figure 4 with a group A streptococcus in streptomycin. With

this antibiotic, however, one occasionally encounters colonies in which some of the cells are resistant to enormously higher concentrations of antibiotic than that at which the colony had grown out (Demerec, 1945; Klein and Kimmelman, 1946*a*,*b*; Murray *et al.*, 1946; Meads and Haslam, 1949; Newcombe and Hawirko, 1949. This phenomenon will be considered in a following paper. It is not observed with penicillin (Miller and Bohnhoff, 1945) and has not yet been observed with chloramphenicol or terramycin.

When colonies which had grown out in antibiotic were suspended and tested directly, they showed essentially the same degree of enhanced resistance as subcultures from those colonies in antibiotic-free media. This has several important implications. It shows that the colonies themselves consist almost wholly of resistant cells. The increased resistance of their subcultures is therefore not due to the presence in the colony of a few resistant mutants which then overgrow the normal population on antibiotic-free subculture. It indicates also that the enhanced resistance persists on subculture even when the parent colony has grown out in a threshold concentration of antibiotic.

Experiments are now in progress to determine the time for which the increased resistance persists, as a function of the concentration of antibiotic to which the cell had been exposed and the duration of that exposure. As will be shown in a following paper, even when organisms grow out in a threshold concentration of antibiotic, the individual organisms in the emergent (slightly) resistant colonies may retain that enhanced resistance for varying periods of subculture in antibiotic-free media. Some revert to normal levels of resistance within one subculture (less than 25 generations); while with others, the increased resistance may persist for more than 200 generations.

DISCUSSION

It seems clear that bacterial cultures do not consist of a preponderant number of normal organisms and a few one-step antibiotic-resistant mutants. There is instead a continuous spectrum of resistance, even within single colony cultures, to penicillin, chloramphenicol, and streptomycin. Further, when an organism grows out in the presence of antibiotic to form a colony and that colony is then subcultured in antibiotic-free media, the resistance of the progeny is generally related to the concentration at which the parent organism had originally grown out. Thus, at threshold concentrations of penicillin, chloramphenicol, or streptomycin, from 10 to 90 per cent of the cells inoculated may grow out to form colonies which in antibiotic-free subculture regularly prove slightly but significantly more resistant than the parent strain. At the other extreme, at high concentrations of antibiotic only one cell in 10^7 or 10^8 may grow out to form a colony. Every organism in that colony may then be resistant to a concentration of antibiotic at which originally only one cell in 10^7 or 10^8 had survived.

It follows that the "resistance" of a given culture can be defined only in terms of a complete distribution curve, i.e., the proportion of organisms capable of growing out at each of a series of test concentrations. Apparent mutation rates

based on the number of (resistant) organisms which grow out in the presence of antibiotic have no absolute significance but must be defined in terms of the concentration of antibiotic used. Further, the size of the inoculum, the conditions of incubation, and in particular, the pH of the medium may profoundly modify the results obtained in such a test. Even at a fixed concentration of penicillin, streptomycin, or chloramphenicol, the apparent number of resistant variants may be increased as much as 100-fold by an appropriate and minor change in the pH of the medium (Eagle, to be published).

The present experiments offer no evidence of step-wise and discontinuous differences in the resistance of bacteria to penicillin, streptomycin, or chloramphenicol. If there are such discontinuous differences, the steps are either too small to be detected by the methods here used or each single step may include differences of such widely varying magnitude as to give the effect of a continuous variation.

It has been shown here that the resistance of a colony which grows out in antibiotic is generally related to the concentration to which the organisms had been exposed. This enhanced resistance persists, at least temporarily, on subculture in antibiotic-free media. It follows that the rate at which the resistance of a bacterial strain can be stepped up by repeated selective transfer of the most resistant fraction of the population will be determined in large part by the shape of the distribution curve, i.e., by the degree to which the individual organisms vary in their capacity to grow out in the presence of antibiotic. An exception may, however, be noted in the case of streptomycin, with which organisms may suddenly appear which are resistant to concentrations greatly exceeding those to which the strain had previously been exposed.

Some consideration may be given to the bearing of the present observations on the mechanism whereby bacteria develop increased resistance to antibiotics. Three possible mechanisms have been suggested: (a) resistant cells which are present in low frequency as spontaneous mutants in the original culture grow out selectively in the presence of antibiotic; (b) a rare spontaneous mutation toward increased resistance occurs during the period of exposure to the drug and is followed by the selective multiplication of the resistant mutant; and (c) an adaptive change occurs in the presence of the drug as the result of which organisms give rise to progeny uniformly more resistant than the original normal culture. It has been shown here that at low (threshold) concentrations of antibiotic, a large proportion of the organisms in a single colony culture may grow out to form slightly resistant clones. That slight increase in resistance is definite, reproducible, and may persist for more than 200 generations on subculture in antibiotic-free media. It appears unlikely that in a single colony culture consisting of 10^6 to 10^8 cells, as many as 10 to 90 per cent of the organisms could have been preformed resistant mutants. This would imply an improbably high mutation rate. If bacterial cultures contained so large a proportion of preformed mutants, then individual colonies grown in antibiotic-free medium, each deriving from one or two cells, should differ frequently and significantly in their resistance spectrum. This has not been found to be the case. Normal colonies grown in antibiotic-free media, when suspended and tested at varying concentrations of

antibiotic, have consistently given uniform resistance curves. One must conclude that in this instance, mechanism (a), previously mentioned, probably does not apply, and that the large scale emergence at low concentrations of antibiotic of slightly resistant colonies is due to either mechanism (b) or (c); there is either an initial multiplication in the presence of the antibiotic, with the appearance of a resistant cell as a spontaneous mutant which then grows out selectively (Demerec *et al.*, 1950), or there is an adaptive change to the drug.

A quite different situation obtains at high concentrations of antibiotic in which only a rare organism grows out to form a highly resistant colony. At these high concentrations there is usually only a negligible degree of initial multiplication, and the bactericidal action of both penicillin and streptomycin proceeds rapidly. In this case, the resistant organisms could not have developed as spontaneous mutations arising during a period of multiplication in the presence of the drug. Mechanism (b) is thus improbable; the highly resistant organisms either were present in the original inoculum, presumably as rare spontaneous mutants, or developed as an adaptive change in the course of their exposure to the drug.

The possibilities with respect to the development of increased resistance may therefore be tabulated as follows:

At low (threshold) concentrations of antibiotic	*At high (substerilizing) concentrations of antibiotic*
A large proportion of the organisms inoculated grows out to form colonies slightly but significantly more resistant than normal.	Rare organisms grow out to form highly resistant colonies.
The resistant organisms may be	The resistant organisms may be
(1) rare spontaneous mutants which appear in the course of multiplication in the presence of the antibiotic, and then rapidly overgrow the normal cells.	(1) spontaneous mutants present in the original inoculum, and which grow out selectively in the presence of the antibiotic.
(2) the result of an adaptive process in the presence of the drug.	(2) the result of an adaptive process in the presence of the drug.

The evidence that the highly resistant organisms in bacterial cultures represent spontaneous mutants seems reasonably conclusive. The mechanism responsible for the emergence of low level resistance at threshold concentrations of antibiotic will be considered in the following papers of this series. The fact that the number of organisms capable of growing out in the presence of an antibiotic falls off smoothly with increasing concentrations of the drug, and that the resistance of the emergent colony is related to the concentration to which the organisms had been exposed, suggests the possible involvement of an adaptive process. Such data do not, however, exclude spontaneous mutations as the basis of the observed variation. A single mutation may result in only a minute increase in resistance; but given a sufficiently high mutation rate (e.g., one in

10^2 to 10^3 cell divisions), a single culture could contain bacteria which had undergone varying numbers of successive mutations, or of simultaneous mutations at multiple loci. (Alternatively, one can assume with Demerec that although a single colony culture of, e.g. 10^8, bacteria per ml contains only first step mutants, those first step mutations have occurred at different loci which vary so widely in their phenotypic expression as to give the effect of a continuous variation.) In either case, because of the steepness of the survival concentration curve, organisms growing out at a given concentration of antibiotic would consist preponderantly of bacteria barely resistant to the test concentration. The resistance of most of the formed colonies would thus be related to the concentration of antibiotic in which they had developed. The rate at which resistant bacteria revert to normal on subculture in antibiotic-free medium is of obvious importance in relation to the nature of the change responsible for the enhanced resistance, and is under present study. The possibility suggested by Gibson and Gibson (1951) that the slightly enhanced resistance which follows exposure to relatively low concentrations of antibiotic develops by a process of adaptation, while the few highly resistant organisms in a bacterial population represent spontaneous mutations, deserves serious consideration.

SUMMARY

In single colony cultures of *Micrococcus pyogenes* var. *aureus*, *Streptococcus pyogenes* (group A), *Streptococcus agalactiae* (group B), *Streptococcus faecalis*, *Escherichia coli*, and *Diplococcus pneumoniae* (type III), tested with penicillin, chloramphenicol, or streptomycin, the number of organisms capable of growing out to form visible colonies decreases with the concentration of the drug. The emergent colonies and subcultures of those colonies are regularly more resistant than the parent culture, and in general, their resistance is related to the concentration at which the colony had grown out. At low threshold concentrations of antibiotic, 10 to 90 per cent of the organisms inoculated may survive to form colonies slightly but significantly more resistant than the parent culture. At high concentrations, just below the sterilizing level, only a few organisms grow out to form highly resistant colonies. Between these two extremes there is a smooth gradation with no indication of discontinuous stepwise differences. A similar "spectrum" of resistance is observed in subcultures of the resistant colonies which grow out in the presence of antibiotic when they are re-tested at appropriately higher concentrations of the drug.

The mechanism whereby bacteria develop increased resistance to antibiotics, and the dual possibility of (a) spontaneous mutations which grow out selectively in the presence of the antibiotic, or (b) a drug-directed adaptive process, have been discussed in the light of the present observations.

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