A QUANTITATIVE ANALYSIS OF THE RESISTANCE OF MYCOBACTERIA TO STREPTOMYCIN[1]

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The rapid rate at which bacteria develop resistance to streptomycin has been demonstrated *in vitro* and *in vivo*. Quantitative studies have been made of certain species of non-acid-fast organisms which give rise to streptomycin-resistant variants (Klein, 1947; Alexander and Leidy, 1947; Klein and Kimmelman, 1946). These investigations show that small numbers of resistant cells are present in susceptible populations *independent* of the presence of the drug, and it is now generally agreed that these arise by a process of spontaneous mutation. Naturally resistant forms of tubercle bacilli have been isolated by Pyle (1947) directly from the sputa of patients who have not been treated with streptomycin, and the presence of naturally resistant variants in stock cultures of H37RV has been demonstrated by Vennesland, Ebert, and Bloch (1947).

This is a report of studies made of the orderly manner in which spontaneous variants occur in cultures of *Mycobacterium tuberculosis* and *Mycobacterium ranae*. The quantitative data were obtained from the many tests required to establish figures large enough to bear statistical analysis. These data seem to provide a reasonable explanation for the variation in resistance encountered in the cultures of patients who have been treated with identical therapeutic regimens. In addition, it will be shown that the number of variants in a given susceptible population is directly affected by the concentration of the drug present in the medium. Similarly, the incidence of variants is increased as the size of the population increases, provided the concentration of the drug remains constant. These and other findings will be discussed in this report.

The quantitative study was made by growing the microorganisms in liquid tween albumin medium (Dubos and Davis, 1946). Following this, known numbers of cells were plated on solid medium containing specific concentrations of streptomycin. The progeny of organisms growing in the presence of the drug were further analyzed to determine their resistance to various concentrations of streptomycin. The details of the experimental procedures are given under Materials and Methods.

MATERIALS AND METHODS

Tubercle bacilli used in this study were strain H37RV and two strains, WS and WR,[2] isolated from patients' sputa. The strains were obtained from patients with advanced pulmonary tuberculosis prior to the institution of treatment with streptomycin. Although both patients received $\frac{1}{6}$ gram of streptomycin every

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4 hours for 120 days, organisms from the sputa of WS were resistant to 1,000 micrograms per ml of streptomycin after 30 days of treatment, whereas organisms from the sputa of WR remained sensitive to 1.0 to 2.5 micrograms per ml of streptomycin after the 120 days of treatment were completed. In addition, *M. ranae* was used. Each of the strains was grown on Hohn's medium, and these cultures served as the source of organisms for subsequent experiments.

The media used in this investigation are liquid tween albumin and a solid tween albumin medium prepared as follows:

I. Concentrated mineral base:

KH_2PO_4	1 g
Na_2HPO_4 $12H_2O$	6.25 g
Na-citrate	1.5 g
Ferric ammonium citrate	0.1 g

Dissolve in 50 ml of distilled water.

Dissolve 0.6 grams $MgSO_4 \cdot 7H_2O$ in 50 ml distilled H_2O. Mix the two solutions.

II. To 100 ml of concentrated mineral base add 900 ml H_2O.

Add asparagine	4 g
"Tween 80" (10 per cent in H_2O)	5 ml

pH adjusted to 7.0

Add agar	15 g

Autoclave, cool to 60 C, and add 100 ml of bovine albumin (5 per cent in 1.5 per cent NaCl, Seitz filtered and heated at 55 C for 30 minutes). Add sterile 25 per cent glucose solution, 20 ml. Add streptomycin as needed.

The preparation is shaken well, and after streptomycin is added plates are poured containing about 25 ml each of the medium.

A flask of liquid medium inoculated from a stock culture is incubated, with occasional shaking, for from 7 to 10 days until a growth of the desired turbidity is obtained. The flask is then shaken well for at least one-half hour to obtain an even suspension of cells. Following this, the number of cells per ml is determined by diluting by the usual methods and then plating on the surface of the solid medium. One ml of a liquid culture to which streptomycin has been added until the desired concentration of the drug is obtained is placed on a series of plates containing the same concentration of streptomycin. After being tilted to ensure uniform spread of the inoculum, the plates are placed in the incubator until the excess moisture has evaporated and then are sealed with wide rubber bands. The final reading of colonies growing on plates is made after approximately 35 days' incubation.

To analyze further the resistance of a colony growing in a known concentration of streptomycin, the colony is inoculated into liquid medium containing no streptomycin. When the desired growth is obtained, the cells are plated on solid medium and on plates containing the same and higher concentrations of streptomycin. The plates are incubated as in the procedure mentioned above and the results tabulated.

The general procedure when *M. ranae* is employed is the same as the foregoing procedure outlined for *M. tuberculosis* with two exceptions: plain glycerol agar is used in place of solid tween albumin medium; and the incubation period before counting the colonies is from 5 to 7 days.

TABLE 1

Mycobacterium tuberculosis H37RV

DISTRIBUTION OF COLONIES AMONG PLATES

Number of bacteria per inoculum	Number of colonies growing on each plate containing 1 microgram of streptomycin per ml of medium										Total number of resistant colonies
208 billion	10	15	6	16	11	13	15	18	10	19	
	17	8	5	12	14	14	17	19	9	10	
	11	12	14	15	5	3	13	12	15	17	375
	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
298 billion	2	1	1	0	0	1	2	4	1	2	
	3	4	2	0	1	2	1	1	1	2	
	1	1	1	2	0	3	3	2	4	2	
	1	0									51
149 billion	1	0	0	0	2	2	0	1	1	0	
	1	0	1	0	0	1	0	1	1	2	
	0										14
10 billion	0	0	0	0	0	0	0	0	0	0	0
	Number of colonies growing on each plate containing 1,000 micrograms of streptomycin per ml of medium										
208 billion	1	1	0	0	2	0	2	2	0	0	
	1	1	1	0	0	2	0	1	0	1	
	1	2	1	0	2	0	1	1	0	1	24

RESULTS

The results of the experiments are arranged in the four tables. In table 1 showing the results obtained with H37RV, it is to be noted that as the size of the sample of organisms increases, the number of variants resistant to 100 micrograms per ml of streptomycin is significantly increased. With samples of the same size there is great variation in the number of resistant colonies appearing on any one plate. Several plates contain no colonies. As the size of the sample is reduced, the number of plates showing no growth is increased; and when the sample size is reduced still further, the chances of growth on a plate become negligible. When the concentration of the drug in the medium is reduced to 1 microgram per ml, the number of resistant colonies growing is increased and every plate contains some colonies; but when the concentration of the drug is increased

to 1,000 micrograms per ml, there is no significant change in the number of naturally resistant colonies from the number growing in 100 micrograms per ml. The samples listed in table 1 were obtained from the same liquid culture. When other cultures of the same strain are used at different times, the figures obtained will vary but the fundamental relations between sample size and concentration of drug will hold.

TABLE 2

Mycobacterium tuberculosis strains WR and WS

Number of bacteria per inoculum	DISTRIBUTION OF COLONIES AMONG PLATES										Total number of resistant colonies
	Strain WR										
499 billion	0	1	3	2	0	2	1	2	1	1	
	3	2	1	4	2	0	2	3	2	1	
	4	3	1	2	0	3	3	2	2	1	54
	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
998 billion	0	2	1	1	0	0	0	0	0	0	
	3	0	1	0	0	1	1	1	3	0	
	1	0	0	1	0	0	2	0	1	0	19
	Strain WS										
	Number of colonies growing on each plate containing 1 microgram of streptomycin per ml of medium										
200 billion	4	5	3	2	5	1	4	6	0	1	
	1	5	3	1	6	4	5	4	8	2	70
178 billion	4	3	12	12	15	10	7	5	4	13	
	5	4	3	2	16	15	1	20	18	10	179
	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
400 billion	2	1	1	2	3	2	3	3	0	1	
	2	0	0	2	1	1	6	2	3	0	35
178 billion	1	2	2	1	0	0	3	1	2	4	
	3	2	4	4	3	0	3	6	5	1	47

A comparison of the results shown in table 2, obtained with strains WS and WR, confirm the prediction that more resistant variants would be found in strain WS, the strain which developed resistance to streptomycin after 30 days of treatment of the patient. Because of population changes due to subculturing, these results may not always be predicted (see Discussion). When samples from different subcultures of strain WS were used, the number of resistant forms, as noted in table 2, varied greatly in number. Although the results in the table show the

TABLE 3

Mycobacterium ranae

Number of bacteria per inoculum	DISTRIBUTION OF COLONIES AMONG PLATES										Total number of resistant colonies
	Number of colonies growing on each plate containing 1 microgram of streptomycin per ml of medium										
12 billion	1	0	2	1	0	0	0	1	2	0	
	0	1	1	0	0	1	0	2	0	1	
	1	1	2	0	1	1					19
210 billion	5	13	8	5	5	14	7	3	6	11	
	10	10	8	6	9	10	7	11	9	10	
	15	7	8	12	5	7					221
	Number of colonies growing on each plate containing 10 micrograms of streptomycin per ml of medium										
88 billion	1	5	0	1	1	0	0	1	3	1	
	1	0	1	2	2	2	1	0	2	4	
	1	0	5	1	1	2					38
120 billion	5	3	2	0	1	0	3	2	1	0	
	0	4	0	3	3	0	1	1	3	4	
	1	0	5	1	1	2					46
42 billion	0	0	1	2	0	1	0	0	1	2	
	0	0	0	0	1	1	0	2	0	0	
	1	1	0	0	0	0					13
	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
14 billion	0	0	0	0	0	0	0	0	0	0	
	1	0	0	0	0	0	0	0	0	0	
	0	0	0	0	0	0	0				1
168 billion	0	0	1	0	2	0	0	0	1	0	
	1	1	0	0	0	1	1	0	0	0	
	0	0	1	0	0	1	0	0			10
115 billion	2	0	0	1	2	0	0	0	0	1	
	1	0	0	0	0	0	1	2	0	0	
	0	0	1	0	2	0	0	0			13
453 billion	1	2	4	0	1	3	0	0	4	3	
	2	4	1	0	3	1	1	0	0	0	
	1	2	0	0	1	2	0	0			36

number of resistant variants to be increased when strain WS was subcultured, other samples of subcultures of the same strain have been shown to contain fewer variants.

In table 3 are recorded the results when *M. ranae* was used; and it can be readily

TABLE 4

Analysis of the resistance to streptomycin of colonies growing in the presence of 1 unit of streptomycin per ml of medium

CONCENTRATION OF STREPTOMYCIN IN MICROGRAMS PER ML OF MEDIUM		NUMBER OF BACTERIA
I. *M. tuberculosis* strain H37RV		
A.	0	210 billion
	1	226 billion
	10	198 billion
	100	2,000
	1,000	200 (to 2,000)
B.	0	1.2 million
	1	1.4 million
	10	980,000
	100	0
	1,000	0
II. *M. tuberculosis* strain WR		
	0	78 billion
	1	81 billion
	10	67 billion
	100	305
	1,000	125
III. *M. tuberculosis* strain WS		
	0	128 billion
	1	109 billion
	10	101 billion
	100	684
	1,000	278
IV. *M. ranae*		
	0	1.9 billion
	1	1.6 billion
	10	1.8 billion
	100	400
	1,000	10

Analysis of the resistance to streptomycin of colonies growing in the presence of 100 units of streptomycin per ml of medium

I. *M. tuberculosis* strain H37RV		
A.	0	26 million
	100	26 million
	1,000	25 million
B.	0	228 billion
	100	225 billion
	1,000	210 billion
II. *M. ranae*		
	0	31 million
	100	30 million
	1,000	32 million

seen that the general relationships between the number of organisms in a sample concentration of drug and the number of resistant colonies hold for this organism. The data obtained with *M. ranae* are probably more accurate than those with *M. tuberculosis* because the size of the sample is more accurately determined for *M. ranae*. With growth of this organism in liquid media cells show no clumping, whereas with *M. tuberculosis* microscopic clumping is occasionally seen even after thorough shaking.

In analysis of the resistance of colonies growing on plates containing 1 microgram per ml of streptomycin, table 4 shows that all the organisms are resistant to 1 microgram per ml of streptomycin. It is of interest that the number of organisms resistant to higher concentrations of streptomycin is greater among these organisms resistant to 1 microgram per ml of streptomycin than it is among the original parent population. (See tables 1, 2, 3 for comparison.)

DISCUSSION AND SUMMARY

From the foregoing observations it seems evident that the nature of resistance of mycobacteria to streptomycin is that of selection of normally occurring hereditary variants that are present in the original cell population. That the addition of streptomycin to the medium renders it selective for the growth of resistant variants cannot be disputed. The specific induction of such forms by the drug can be seriously questioned since the number of variants present in an original population is so small. Furthermore, it was shown previously by Yegian, Budd, and Middlebrook (1946) that tubercle bacilli exposed to sulfonamides under conditions precluding multiplication fail to develop resistance. Additional studies using quantitative methods with streptomycin showed that no increase in the number of resistant variants occurred under these conditions. The facts that the resistant variants are genetically stable and that they are present in small numbers in a random population support the contention that they are produced by a process of mutation regardless of the presence of the drug.

It is evident from an examination of the data that an increase in the population of one parent strain is accompanied by an increase in the number of variants resistant to a specific concentration of the drug. It is to be noted, however, that the populations in different cultures show a great variation in the number of resistant variants present. Even the samples taken from the same culture show a great variation in the number of resistant forms. These findings are readily explained on the basis of chance selection.

The data presented show clearly that the number of resistant forms is directly affected by the concentration of the drug in the media. A marked decrease in the number of colonies occurs when the drug concentrations are increased from 1 microgram per ml to 10 micrograms per ml and then to 100 micrograms per ml; however, when certain high concentrations of the drug are used, such as 100 micrograms per ml and 1,000 micrograms per ml, no significant difference in the number of resistant colonies is observed.

From analyses of populations resistant to 1 microgram per ml of streptomycin, it is evident, as expected, that all the organisms are resistant to 1 microgram per

ml of streptomycin, and that there are more highly resistant variants in the populations resistant to 1 microgram per ml than in the original parent populations. The elimination of all organisms susceptible to 1 microgram per ml of drug selects a population all of whose members are resistant to streptomycin to some degree; this would appear to facilitate not only the multiplication of the (relatively few) highly resistant forms already present but possibly the production by mutation of more highly resistant forms. From his studies of resistance of bacteria to penicillin Demerec (1945) concluded that the degree of resistance to the drug increases by definite increments as the naturally resistant forms multiply. A similar process may be occurring during our analyses of resistant populations. Also, it is observed from the data presented that variants resistant to 100 micrograms per ml of streptomycin are equally resistant to much higher concentrations of the drug. Apparently streptomycin in any concentration has no effect on forms resistant to a certain minimum concentration of the drug.

The clinical significance of the findings presented must be emphasized, for often data concerning the sensitivity of tubercle bacilli to streptomycin are either inadequate or improperly interpreted. It has been shown that, when the sample of bacteria used is sufficiently large and when the time allowed for growth is prolonged, the chances of finding resistant forms are increased. As the sample size decreases, the chances of resistant forms being observed are greatly decreased. When only one sample is used, the conclusions drawn must be very limited, for we have noted great variations among samples from the same parent population. When sensitivity studies are made in the usual manner, a time limit is used for allowing growth of resistant forms. When the time limit is extended, a few resistant forms may be allowed to multiply adequately and the results may be given a quite different interpretation. In addition, the collection of sputa specimens from which the original stock cultures are grown must be considered inadequate in certain instances. All the variations in resistance of the bacterial population in the diseased areas of the patient are probably never represented in any single or even in several sputa samples.

It is known that multiplication of cells is essential to increase the number of resistant forms. The reproduction rate of tubercle bacilli within patients under treatment with streptomycin may vary greatly in different patients; in addition, the reproduction rate of the stock cultures and subcultures varies greatly. Of great importance is the character of the individual population. The mutation of resistant forms is very dependent upon this factor, which is difficult to analyze.

When tubercle bacilli strains WS and WR were studied prior to streptomycin treatment of the patient (table 2), the predication was verified that more resistant variants would be found in strain WS, the strain which later developed resistance to streptomycin after 30 days of treatment of the patient. Numerous subcultures of the strains were made, and because of population changes the results might have been different. Distinct variations in the number of resistant forms found in strain WS were noted when different subcultures of the same strain were used. Some samples showed definite decreases in the number of variants present.

It is conceivable that all the bacteria within a patient are not exposed to the same concentration of streptomycin. Because of a low concentration of streptomycin in certain of the diseased areas, bacteria which may be readily susceptible to streptomycin are allowed to exist during the treatment period. The presence of these organisms in the sputum at a later date may go undetected during routine sensitivity studies because the resistant forms outnumber these remaining sensitive organisms and because the procedure is designed to "select out" only the resistant forms. It is conceivable, also, that an extension of disease in the patient after the treatment period is concluded could be caused by these streptomycin-susceptible organisms. It is essential that complete studies be made of tubercle obtained from many different areas in patients who have undergone treatment with streptomycin and subsequently died.

It is apparent that many factors can influence the results obtained in the usual sensitivity studies. It must be borne in mind constantly that chance and chance alone may account for the findings obtained. Sample variations, the multiplication of the cells, the time allowed for growth, and the characteristics of an individual population must always be considered.

The demonstration of the resistant variants in a random population, as has been shown, is a relatively simple procedure. It is known that resistance is specific and permanent in nature. Is it not possible that the resistant variants may occasionally give rise to mutants resembling the parent strain in their susceptibility to streptomycin? Study of this problem is rendered difficult because there is no selective medium that prevents growth of the resistant strain and allows growth of the streptomycin-susceptible strain that may have mutated from the resistant strain. The problem resolves itself into isolating from a resistant strain still another variant that requires streptomycin for its growth. Isolation of such a variant of *M. ranae* has been accomplished. By isolating this variant we have obtained an organism for which a selective medium exists (Yegian and Budd, 1948). From studies of large populations of this variant strain that requires streptomycin for its growth, a few colonies have been isolated that resemble the parent strain in their susceptibility to streptomycin and sulfonamides, in their biochemical reactions, and in their morphological and cultural characteristics. These findings are similar to those obtained by Miller and Bohnhoff (1947) in their studies of meningococcus.

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