MODE OF ACTION OF STREPTOMYCIN ON TYPE b HEMOPHILUS INFLUENZAE

II. Nature of Resistant Variants*

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In a previous publication (1) it was shown that the development of resistance to streptomycin of type b *H. influenzae*, either *in vitro* or during treatment of a patient, is the result of a selective process rather than an adaptive metabolic change; the sensitive organisms are eliminated, permitting the resistant variants, which occur under normal conditions in the parent culture, to declare themselves. All ten strains examined before exposure to streptomycin were shown to contain a very small fraction of members which could thrive in a concentration of streptomycin equal to 1,000 units per cc. Resistance of this degree could be demonstrated with regularity, in sensitive strains, only when large bacterial populations (142 to 522 billion) were cultured; the incidence varied from one resistant colony in 1,100,000,000 to one in 13,800,000,000 organisms. The potentiality of a given strain to emerge resistant either in the patient or *in vitro* was shown to be more dependent upon the size of the bacterial population than upon any other known factor. It was not possible to show a correlation between the initial prevalence of resistant members and the emergence of resistant strains in patients during treatment.

This report presents data from experiments designed to examine the hypothesis that the resistant members, present in all large populations of sensitive strains, represent bacterial mutants. The occurrence of bacterial mutants has been well established. They have been recognized, by a number of characteristics, as different from the rest of the population: by their capacity to form enzymes or pigment, by their ability to synthesize essential growth factors, and by the morphology of their colonies. This subject has been well reviewed and discussed by Dubos (2). More recently Luria and Delbrück (3), Demerec and Fano (4), and Demerec (5) have demonstrated that members of *E. coli* cultures which are resistant to specific bacteriophage are in reality bacterial mutants. Demerec (6) has also shown that resistance to penicillin is the result of occurrence of bacterial mutations.

While knowledge of the genetics of bacteria is very limited, certain char-

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acteristics are accepted as essential criteria for labeling variants as the result of mutation: (1) evidence for the random occurrence of variants showing great variation in prevalence in different independent cultures of the same strain, (2) a low constant rate of occurrence which is consistent with mutation frequency, (3) transmissibility of the variant trait through many generations. Experiments were designed to reexamine the ten strains previously reported (1) for additional evidence of these characteristics.

Materials and Methods

The ten strains used were isolated from patients before institution of streptomycin therapy for clinical infections caused by type b *H. influenzae*. Recovery from the illness was prompt in six patients (strains 5 to 10), but in four patients (strains 1 to 4) streptomycin alone was not adequate in effecting a cure. In three of the unsuccessful cases (strains 1 to 3) therapeutic failure was due to the emergence of organisms resistant to streptomycin.

Initially all ten strains were examined by the following procedure: a dried culture (7), originally desiccated within 5 days of isolation from the patient, was seeded in 20 to 35 cc. of Levinthal broth[1] in a 50 to 125 cc. flask. After 20 to 24 hours' incubation, each of ten Petri dishes containing Levinthal agar[2] was inoculated with 0.5 cc. of the broth culture, rotated to spread the culture on the surface, and incubated for 6 hours. The 6 hour growth was removed by washing each plate with 3.5 cc. of plain nutrient broth and the yield from separate plates pipetted into individual tubes. One cc. of each of the ten suspensions was pipetted into a Petri dish and approximately 15 cc. of Levinthal agar containing 1,000 units per cc. of streptomycin was added. A pool was made by combining equal quantities from each of the individual tubes and a 1 cc. sample of this mixed pool was seeded into each of ten pour plate preparations identical with those used for the individual suspensions. Colony counts were made by culturing appropriate dilutions of the pooled suspension on Levinthal agar in poured plates. The number of colonies growing in streptomycin-containing medium was finally recorded after 72 hours' incubation at 37.5°C.

In order to determine the prevalence of resistant organisms in the broth culture used for seeding the Levinthal agar plates for production of large populations, a 1 cc. quantity of the broth inoculum was inoculated into each of ten Petri dishes and Levinthal agar containing 1,000 units per cc. of streptomycin was added. The pour plates were incubated for 72 hours, and the number of colonies recorded at that time. The total colony count per cubic centimeter on streptomycin-free medium was also determined.

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[1] The broth is a further modification of the Pittman (Pittman, M., *J. Exp. Med.* 1931 **53**, 471) changes in Levinthal broth (Levinthal, W., and Fernbach, H., *Z. Hyg. u. Infektionskrankh.*, 1922, **96**, 456). It is made by combining one part of "Levinthal stock" with three parts of neopeptone broth (Hobby, G., and Lenert, T. F., data to be published). Levinthal stock is prepared as follows: brain heart infusion broth (Difco), made according to directions on bottle, is heated to vigorous boiling and sterile defibrinated horse blood is added to make a final concentration of 10 per cent. The mixture is filtered through Whatman filter paper No. 12 and the clear filtrate is sterilized by passage through Seitz filter.

[2] Levinthal agar is made by adding one part of sterile Levinthal stock to one part of 4 per cent agar (45 gm. proteose No. 3 Difco agar plus 15 gm. Bacto agar (Difco) per liter of water).

EXPERIMENTAL RESULTS

Variation in Prevalence of Resistant Variants in Independent Cultures of the Same Strain.—Table I lists the results obtained with each of the ten strains when the growth produced on Levinthal agar plates was examined for the presence of colonies resistant to 1,000 units of streptomycin per cc. The number of colonies surviving in ten 1 cc. samples of the individual plate suspensions and in ten 1 cc. quantities of a pool from the individual suspensions is listed for each strain. Duplicate tests were performed on different occasions for some strains. It is clear that when the number of resistant organisms is relatively high at the time of the test, the colonies in the plates inoculated from the pool are evenly distributed among the ten pour plates. However, independent cultures from the same test show a greater variation in the number of colonies which grow in 1,000 units of streptomycin per cc. Moreover it is evident that examination of the same strain on other occasions may yield a significantly different prevalence of resistant members.

The significantly different results of test 2 on strain 3 and test 1 on strain 8 have been demonstrated to be due to the seeding of resistant variants already present in the broth inoculum. This subject will be presented in detail later on.

The data resulting from examination of bacterial populations obtained by seeding ten Levinthal agar plates with 5 cc. of broth inoculum (0.5 cc. per plate) proved adequate for statistical analysis only when the prevalence of resistant members was sufficiently high at the time of the tests to provide an even distribution in plates seeded from the pool. However, with two exceptions the variation in the number of resistant colonies was greater in the ten plates seeded from ten different independent cultures than in the ten plates inoculated from the pool. In an effort to provide a prevalence of resistant variants sufficient to allow their even distribution in the ten plates from the pool, suspensions of three strains were produced by inoculating twenty Levinthal agar plates with 10 cc. of the broth culture (0.5 cc. per plate). The 6 hour growth yield from each of two plates was combined and used as one independent culture, so that there were ten independent cultures from the twenty plates. These cultures were then subjected to the same test described above. For comparison, a ten plate source test was performed on the same day using the same broth inoculum. Results are listed in Table II.

It is seen that in three of the four tests in which twice the amount of broth culture was seeded on Levinthal agar plates to produce the suspensions for examination, there is an increase in prevalence of resistant members, a more even distribution of resistant colonies in the plates from the pool, and greater irregularity of distribution of resistant members in populations derived from

TABLE I

Comparison of Prevalence of Resistant Colonies in Populations of Same Size from a Single Pool and Ten Different Independent Cultures of Strain

Strain	Source	Total bacteria cultured	No. of colonies growing in Levinthal agar + 1,000 units of streptomycin per cc.										
			Total resistant colonies	Distribution of colonies among 10 plates									
				1	2	3	4	5	6	7	8	9	10
		billions											
1-2*	Pool	144	22	4	4	3	5	2	1	1	0	1	1
	Individual	144	21	0	4	7	4	0	0	4	1	1	0
2-2	Pool	279	9	1	0	1	2	0	1	1	1	1	1
	Individual	279	3	0	0	0	0	0	0	3	0	0	0
3-1	Pool	301	26	3	4	2	1	3	2	5	1	3	2
	Individual	301	19	3	1	0	1	7	0	0	0	0	7
-2	Pool	252	2,602	270	249	218	315	269	233	258	228	327	235
	Individual	252	2,552	469	57	152	28	60	168	268	384	794	173
4-2	Pool	241	10	0	1	2	0	1	0	2	1	2	1
	Individual	241	9	2	0	4	1	0	1	0	0	1	0
5-1	Pool	253	20	0	2	4	1	2	0	2	3	2	4
	Individual	253	23	0	0	6	0	0	2	11	4	0	0
-2	Pool	196	15	2	3	2	4	0	0	1	3	0	0
	Individual	196	11	0	0	1	2	5	0	0	0	3	0
6-2	Pool	166	15	2	0	0	6	2	1	2	0	1	1
	Individual	166	8	4	0	0	1	0	0	0	1	2	0
-3	Pool	304	53	5	4	6	10	6	4	3	4	7	4
	Individual	304	44	0	0	0	0	1	6	18	1	17	1
7-2	Pool	147	6	2	1	0	0	1	0	1	1	0	0
	Individual	147	3	0	0	1	0	0	0	0	0	2	0
8-1	Pool	256	172	14	22	19	17	17	14	24	14	19	12
	Individual	256	135	0	10	12	66	19	1	1	2	22	2
-2	Pool	293	27	3	1	1	1	6	3	5	1	2	4
	Individual	293	36	4	3	9	8	3	1	2	0	1	5
9-1	Pool	166	12	1	2	2	1	0	0	2	2	1	1
	Individual	166	8	2	0	4	0	0	0	1	0	1	0
-3	Pool	273	18	1	2	1	1	1	2	2	5	3	
	Individual	304	6	0	0	1	0	2	0	0	0	1	2
10-1	Pool	284	37	4	6	4	6	2	2	4	6	1	2
	Individual	284	20	1	3	0	0	8	0	0	3	2	3
-2	Pool	238	12	4	3	3	2						
	Individual	597	39	0	4	1	0	3	1	10	4	8	8

* The number of the experiment from which data were obtained corresponds with the numbers of Table IV.

different independent cultures. In the other test this influence is not seen. Apparently two factors operate to make the twenty plate test more satisfactory for assessing the frequency and distribution of resistant members of a strain: the population is usually larger and therefore the influence of chance is reduced; the use of a larger total quantity of broth inoculum, as well as a greater number

TABLE II

Comparison of Prevalence and Distribution of Resistant Variants in Populations Grown on Ten Levinthal Agar Plates with Those Grown on Twenty Plates

Strain	Source	Population from twenty plates			Population from ten plates		
		Total bacteria cultured	Resistant colonies / Total / Prevalence	Distribution resistant colonies	Total bacteria cultured	Resistant colonies / Total / Prevalence	Distribution resistant colonies
		billions			*billions*		
1	Pool	230	58 / $1:4.0 \times 10^9$	6-9-3-7-3-12-8-1-5-4	144	22 / $1:6.5 \times 10^9$	4-4-3-5-2-1-1-0-1-1
	Individual	460	82 / $1:5.6 \times 10^9$	5-5-3-27-14-11-5-6-2-4	144	21 / $1:6.8 \times 10^9$	0-4-7-4-0-0-4-1-1-0
6	Pool	304	45 / $1:6.4 \times 10^9$	3-3-6-6-3-7-6-3-7-1	294	53 / $1:5.8 \times 10^9$	5-4-6-10-6-4-3-4-7-4
	Individual	304	35 / $1:6.8 \times 10^9$	6-1-1-4-6-0-1-9-7-0	294	44 / $1:7.4 \times 10^9$	6-18-1-17-1-0-0-0-0-1
10	Pool	567	114 / $1:4.9 \times 10^9$	11-10-6-11-14-15-9-13-14-11	238	12 / $1:19.9 \times 10^9$	4-3-3-2*
	Individual	567	93 / $1:6.1 \times 10^9$	2-2-0-21-3-21-8-25-7-4	597	39 / $1:15.3 \times 10^9$	0-4-1-0-3-1-10-4-8-8
10	Pool	428	104 / $1:4.1 \times 10^9$	8-15-6-13-11-12-10-9-9-11	284	37 / $1:7.7 \times 10^9$	4-6-4-6-2-2-4-6-1-2
	Individual	428	112 / $1:3.8 \times 10^9$	8-5-4-37-8-0-14-3-7-26	284	20 / $1:14.2 \times 10^9$	1-3-0-0-8-0-0-3-2-3

* Only four culture plates studied due to accident.

of independent culture sources on Levinthal agar, increases the likelihood of irregularity of distribution in the independent cultures.

In order to obtain additional confirmation of the variation in the prevalence of resistant organisms in independent cultures of the same strain, a comparison was made of the proportion of resistant members found in a genetically heterogeneous and a genetically homogeneous population using strain 1.

The fifth subculture of the broth inoculum used for test 2 of strain 1 in Table I was used to seed the broth inoculum for the first of the three tests performed on 3 consecutive days to

determine the variability in the prevalence of resistant colonies when a heterogeneous population was employed. The culture source of the broth inoculum for the other two tests was a subculture of the one used for seeding the broth inoculum for the test of the previous day. The broth inoculum used for each test was examined for prevalence of resistant variants by inoculating 2 cc. of it into each of twenty-five pour plate preparations of Levinthal agar containing 1,000 units of streptomycin per cc. The homogeneous culture was derived from a single colony. The broth inoculum used for strain 1, test 2, in Table I was so seeded on Levinthal agar that discrete colonies formed after 24 hours' incubation. One of these was transferred to Levinthal broth in which it was subcultured. On four occasions during a 10 day period the procedure was repeated and a single colony was selected for subculture in Levinthal broth. This procedure can be expected to produce a relatively homogeneous population so far as its genetic characteristics are concerned. The progeny of the last isolated single colony was examined on 3 consecutive days by the same test used for the heterogeneous cultures.

Table III lists the results of these experiments. The high prevalence of resistant colonies in test 1 of the heterogeneous culture is explained by the large number of resistant organisms in the broth inoculum. The total inoculum seeded, 10 cc., contained twenty-four resistant colonies. It is seen that the variation in prevalence of resistant variants in independent cultures of a genetically homogeneous population is not significantly different from that demonstrated in genetically heterogeneous populations.

In Table IV are recorded the data from all tests designed to explore the factors influencing prevalence and distribution of resistant variants. The numbers designating strains and tests correspond to those in Table I. The figure for prevalence of resistant colonies in the 6 hour Levinthal agar cultures was obtained by averaging the results from both the pool and independent samples. The results show the relationship between the prevalence of resistant members in large populations grown on Levinthal agar and the number of resistant colonies in the broth cultures used for seeding. It is clear that examination of 10 cc. of broth inoculum is inadequate for a quantitative determination of this effect but provides a fair estimate of the influence since this volume is twice that used for production of the suspensions examined. When a resistant variant occurs during an early generation in the broth inoculum its reproduction results in a high prevalence of resistant members, which then enhances the likelihood of their transfer to the Levinthal agar seeded for production of large populations; this event is responsible for the most marked variation in independent cultures as shown in Table I, strain 3, test 2, and Table III, test 2, on the heterogeneous culture. The number of resistant organisms in the broth cultures used for seeding the Levinthal agar plates is so small in most tests that transfer of these variants is an unlikely source of resistant colonies. The irregular occurrence of new resistant variants in repeated subcultures is consistent with the mutation theory. It is apparent that the rate of occurrence rather than prevalence must be used to compare the results of tests on the same and on different strains in order to demonstrate

fundamental differences in their potentialities for developing resistance to streptomycin of the order of 1,000 units per cc.

TABLE III

Comparison of Prevalence and Distribution of Resistant Variants of Strain 1 from Genetically Heterogeneous and Homogeneous Sources

Test	Source	Total bacteria examined	Resistant colonies / Total / Prevalence	Distribution of resistant colonies	Total bacteria examined	Prevalence resistant colonies
	Culture origin genetically heterogeneous					
	Levinthal agar populations				Broth inoculum	
		billions			*billions*	
1	Pool	228	322 / 1:0.69 × 10^9	28-41-35-37-25-33-36-38-24-25	53.0	1:0.87 × 10^9
	Individual	456	578 / 1:0.77 × 10^9	34-126-1-4-7-1-21-38-7-3-48-1-25-2-66-3-2-19-169-1		
2	Pool	266	20 / 1:13.3 × 10^9	3-3-5-1-0-0-2-1-3-2	67.5	1:7.5 × 10^9
	Individual	532	49 / 1:10.8 × 10^9	2-5-1-2-0-2-3-2-1-0-1-13-1-1-2-4-3-2-3-1		
3	Pool	258	42 / 1:6.1 × 10^9	6-3-5-4-6-5-2-3-3-5	48.0	1:48 × 10^9
	Individual	516	94 / 1:5.5 × 10^9	1-4-0-2-0-4-2-1-3-0-0-6-1-3-0-0-5-29-0-33		
	Culture origin genetically homogeneous					
1	Pool	150	25 / 1:6.0 × 10^9	2-5-0-2-2-4-1-1-6-2	75.0	1:18.7 × 10^9
	Individual	300	29 / 1:10.3 × 10^9	6-1-2-0-0-0-1-1-1-0-5-3-1-0-6-0-0-2-0-0		
2	Pool	163	27 / 1:6.0 × 10^9	3-2-3-0-3-4-1-6-1-4	43.0	1:8.6 × 10^9
	Individual	326	104 / 1:3.1 × 10^9	7-3-6-8-21-0-1-54-0-0-0-1-3-0-0-0-0-0-0-0		
3	Pool	246	30 / 1:8.2 × 10^9	6-1-3-4-2-4-5-2-3-0	118	0:118 × 10^9
	Individual	492	41 / 1:12 × 10^9	3-1-5-1-4-4-8-0-0-2-6-0-2-0-3-0-1-0-1-0		

The Rate of Occurrence of the Variants Is Consistent with the Mutation Hypothesis.—The mutation rate for the 6 hour growth period on Levinthal agar could have been determined by direct calculation if accurate measure had been

TABLE IV

Comparison of Prevalence of Resistant Members in Large Populations of a Strain on Different Occasions Showing Influence of Number of Resistant Variants in Broth Inoculum

Strain	Broth inoculum			Levinthal agar growth		
	Prevalence resistant colonies*	Bacteria cultured	Amount cultured	Prevalence resistant colonies*	Bacteria cultured	No. of culture plates
		billions	*cc.*		*billions*	
1-1‡				1:6.7 × 10^9	254	10—(3.8)§
-2	1:21.0 × 10^9	42.3	30	1:6.7 " "	288	10—(2.2)
-2	1:21.0 " "	42.3	30	1:4.9 " "	690	20—(4.6)
-3	1:0.88 " "	53.0	50	1:0.75 " "	684	20—(30.2)
-4	1:7.5 " "	67.5	50	1:11.6 " "	798	20—(2.3)
-5	1:48.0 " "	48.0	50	1:5.7 " "	774	20—(4.5)
2-1				1:6.2 " "	142	10—(2.3)
-2	1:19.7 " "	19.7	10	1:46.5 " "	558	10—(0.6)
3-1	0:12.7 " "	12.7	10	1:13.4 " "	602	10—(2.2)
-2	1:0.08 " "	16.4	10	1:0.10 " "	504	10—(257.8)
4-1				1:14.6 " "	292	10—(2.0)
-2	1:22.0 " "	22.0	10	1:25.4 " "	482	10—(0.9)
5-1	0:11.0 " "	11.0	10	1:11.8 " "	506	10—(2.1)
-2	1:3.5 " "	3.5	10	1:15.1 " "	392	10—(1.3)
6-1				1:1.1 " "	348	10—(32.7)
-2	0:15.0 " "	15.0	10	1:14.4 " "	332	10—(1.1)
-3				1:6.3 " "	608	10—(4.8)
-3				1:7.3 " "	588	20—(4.0)
7-1				1:10.4 " "	188	10—(1.8)
-2	0:8.6 " "	8.6	9	1:32.7 " "	294	10—(0.4)
-3	1:19.8 " "	19.8	20	1:25.6 " "	692	20—(1.3)
8-1	1:8.2 " "	8.2	10	1:1.7 " "	512	10—(15.3)
-2	1:16.0 " "	16.0	8	1:9.3 " "	586	10—(3.1)
9-1	0:9.9 " "	9.9	10	1:16.6 " "	332	10—(1.0)
-2	0:12.7 " "	12.7	10	1:14.6 " "	204	9—(1.5)
-3	0:25.6 " "	25.6	20	1:24.0 " "	577	19—(1.2)
10-1	0:17.8 " "	17.8	10	1:9.9 " "	568	10—(2.8)
-1	0:17.8 " "	17.8	10	1:3.9 " "	856	20—(10.8)
-2	1:7.5 " "	15.2	10	1:16.4 " "	835	10—(3.6)
-2	1:7.5 " "	15.2	10	1:5.5 " "	1134	20—(10.3)

* Ratio between resistant colonies and total population.

‡ The number of the experiment from which the data were obtained.

§ Average number of resistant colonies per plate.

made of (1) the prevalence of resistant variants in the broth inoculum used for each test, (2) the total population obtained from the seeding, and (3) the total number of resistant colonies developing in streptomycin-containing Levinthal agar. However, an accurate estimate of the prevalence of mutants in the broth inoculum was obtained in only a few tests; the quantity examined was too small in the other tests. Moreover the total number of organisms grown on the surface of Levinthal agar after seeding from the broth could only be estimated since only about three-fourths of the total was cultured.

In order to arrive at an evaluation of mutation frequency from the data available, it was suggested to us by Demerec (8) that we apply the method reported by Luria and Delbrück (3). We have carried out these calculations, using the data listed for each strain in Tables V and VI.

In this report (3) an equation is derived:
$r = aN_t \ln (N_t Ca)$, in which
r = average number of mutants per culture,
a = mutation rate, or the chance of mutation per bacterium in a single division cycle,
N_t = total number of bacteria per sample,
C = number of cultures used in experiment.
In a graph r is plotted as a function of aN_t for selected values of C. With r and C known (Table V), the corresponding value aN_t may be derived from the graph by projection. Values for a are then obtained by simple calculation.
Example: strain 1, Table V:
$r = 2.2$ (Table IV)
$C = 20$
$N_t = 1.4 \times 10^{10}$
$aN_t = 0.8$

$$a = \frac{0.8}{1.4 \times 10^{10}} = 5.7 \times 10^{-11}$$

The mutation rate for each of the ten strains studied is listed in the last column of Table V. Data were used only from those tests in which the results showed no evidence of transfer of mutants already present in the broth inoculum. It is obvious that the mutation rates of the ten strains do not differ significantly. Therefore the development of resistance of strains 1, 2, and 3 during streptomycin treatment of the patients cannot be explained on the basis of higher mutation rates.

For comparison with the results in all ten strains in Table V, Table VI lists similar data obtained from four tests on the same strain 1; two of the cultures of this strain were grown from genetically heterogeneous sources and two from genetically homogeneous sources. The variation in mutation rates is not significantly different from that seen among the ten different strains.

Transmissibility of the Variant Trait.—From the ten strains listed, a total of 440 of the colonies which grew in Levinthal agar containing 1,000 units of streptomycin per cc. were retested for sensitivity to this concentration.

Single resistant colonies were seeded in Levinthal broth containing no streptomycin and incubated 18 to 24 hours; a 2 mm. loopful was then inoculated on the surface of Levinthal agar

TABLE V

Application of Method of Luria and Delbrück (3) to Data from Examination of Ten Strains for Determination of Mutation Rate per Bacterial Generation per Bacterium

Strains	Test	Average resistant colonies per culture	No. of cultures used	Organisms per culture	aN_t*	a‡
				billions		
1	2	2.2	20	14.4	0.80	5.7×10^{-11}
2	1	2.3	10	14.2	1.00	7.0×10^{-11}
3	1	2.2	20	30.1	0.80	2.6×10^{-11}
4	1	2.0	10	29.2	0.95	3.2×10^{-11}
5	1	2.1	20	25.3	0.78	3.1×10^{-11}
6	2	1.1	20	16.6	0.50	3.1×10^{-11}
7	1	1.8	10	18.1	0.86	4.7×10^{-11}
8	2	3.1	20	29.3	1.05	3.6×10^{-11}
9	1	1.0	20	16.6	0.48	3.0×10^{-11}
10	1	2.8	20	28.4	0.97	3.4×10^{-11}

* aN_t = values read on abscissa from graph.

‡ a = mutation rate per bacterial generation per bacterium.

TABLE VI

Application of Method of Luria and Delbrück (3) for Mutation Rate per Bacterium per Bacterial Generation to Results Obtained from Multiple Tests on the Same Strain No. 1

Genetically heterogeneous source*	Average No. of resistant variants per culture	No. of cultures	Bacterial population per sample	aN_t‡	a§
			billions		
Test 2	2.3	30	26.6	0.76	2.9×10^{-11}
" 3	4.5	30	25.8	1.30	5.2×10^{-11}
Genetically homogeneous source					
Test 1	1.8	30	15.0	0.64	4.2×10^{-11}
" 3	2.3	30	24.6	0.76	3.1×10^{-11}

* Data used were obtained from results of tests listed in Table III.

‡ aN_t = Values read on abscissa from graph.

§ a = Mutation rate per bacterial generation per bacterium.

containing no streptomycin and on Levinthal agar containing 1,000 units of streptomycin per cc. All cultures grew well on the streptomycin Levinthal agar within 48 hours. Seventy or 16 per cent of the cultures either did not grow on Levinthal agar containing no streptomycin, or grew poorly.

The results suggest the occurrence of two types of resistant variants: those which grow well in both the presence and absence of a high concentration of streptomycin and those which are favored by a streptomycin-containing medium.

From each of the ten strains studied, a sample colony was selected from the survivors of the large population which 48 hours before had been seeded in pour plate preparations of Levinthal agar containing 1,000 units of streptomycin per cc. Subcultures made in Levinthal broth and incubated overnight were studied for transmissibility of the resistant trait by the following procedure. A subculture was made in Levinthal broth and incubated 6 hours. Dilutions 10^{-7} of the 6 hour broth cultures were made in nutrient broth and 0.5 cc. quantities were seeded in pour plate preparations of Levinthal agar and 0.5 cc. into the same medium containing 1,000 units of streptomycin per cc. Colony counts were compared after 72 hours' incubation. The initial proportion of the population resistant to 1,000 units of streptomycin per cc. was thus determined. The above procedure was repeated after five and ten subcultures in Levinthal broth in the absence of streptomycin. Results are listed in Table VII.

TABLE VII

Transmissibility of the Resistant Trait in Ten Variants Subcultured in the Absence of Streptomycin

Colonies per cubic centimeter in 10^{-7} dilution of 6 hour Levinthal broth culture.

Strain	No. of subcultures in absence of streptomycin											
	1		5		10		12		23		39	
	Media*		Media		Media		Media		Media		Media	
	Control	Streptomycin‡	Control	Streptomycin‡	Control	Streptomycin‡	Control	Streptomycin‡	Control	Streptomycin‡	Control	Streptomycin‡
1	118	116	142	126	96	86						
2	212	164	116	92	70	80						
3	0	116	0	86	42	14	128	4	82	90	124	129
4	96	110	130	164	50	94						
5	204	184	118	128	122	108						
6	188	190	144	178	60	64						
7	2	128	0	36	0	6	30	0	127	150	112	128
8	158	140	96	128	180	152						
9	154	166	124	158	84	90						
10	80§	156	120	50	116	18	166	0	117	56	113	105

* Levinthal agar.

‡ Concentration, 1,000 units per cc.

§ No colonies apparent until 48 hours.

It is seen that after one subculture in the absence of streptomycin seven of the ten strains show no significant difference between the number of colonies growing in Levinthal agar without streptomycin and the number growing in the same medium containing 1,000 units of streptomycin per cc. The other

three strains, 3, 7, and 10, grew poorly or not at all in the control Levinthal agar whereas growth was normal in the streptomycin-containing medium.

The results obtained on reexamination after five and again after ten subcultures in the absence of streptomycin show no change in the degree of resistance to streptomycin exhibited by the seven strains which originally grew as well in control Levinthal agar as in the presence of 1,000 units of streptomycin per cc. One of these strains, No. 1, has shown no change in degree of resistance after being subcultured at 24 or 48 hour intervals for a period of 6 months.

TABLE VIII

Comparison of Prevalence of Colonies of Resistant Variants Which Grow in Levinthal Agar Containing 100 Units of Streptomycin per Cc. with That Obtained in a Concentration of 1,000 Units of Streptomycin per Cc.

Strain	Concentration of streptomycin			
	100 units per cc.		1,000 units per cc.	
	Total bacteria cultured	Total resistant colonies	Total bacteria cultured	Total resistant colonies
	billions		*billions*	
2–2*	139.5	5	139.5	4
3–1	150.5	17	150.5	13
–2	126.0	1,281	126.0	1,321
4–2	120.5	6	120.5	4
5–2	98.0	4	98.0	11
6–2	83.0	5	83.0	10
7–2	73.5	2	73.5	4
8–2	146.5	15	146.5	12
9–3	113.5	3	113.5	8

* Test from which data were obtained.

It seems clear that the resistant trait of these strains is inherited since it is transmitted unchanged in degree through many generations.

The behavior of strains 3, 7, and 10 deserves comment.

Strains 7 and 10 showed a decrease in the number of colonies growing in streptomycin agar after five subcultures in the absence of streptomycin, and strain 10 grew normally in control Levinthal agar. After ten subcultures all three strains showed a decreased capacity to grow in the presence of streptomycin and an increase in growth in the control medium. Because of these changes these three strains were studied for a longer period. After twelve subcultures, when these strains failed to grow in pour plate preparations of streptomycin Levinthal agar and grew well in control Levinthal agar, a larger inoculum (2 mm. loopful of a 6 hour Levinthal broth culture without dilution) on the surface of Levinthal agar containing 1,000 units of streptomycin per cc., yielded a satisfactory growth; the growth on the surface of normal Levinthal agar was better however. After twenty-three subcultures the growth of strains 3 and 7 was normal both in control and streptomycin-containing Levinthal agar, whether the cultures were seeded on the surface or into pour plate preparations. Strain 10

showed twice as many colonies in the control medium; however after the thirty-ninth subculture the number of colonies in the control medium and streptomycin Levinthal agar did not differ significantly.

Whether the change in behavior of these strains is caused by a change in the resistant trait or is the result of a second variant trait which changes their growth requirements, cannot be answered at present. The results suggest that these three strains represent bacterial mutants which differ from the parent culture in at least two respects: in their resistance to streptomycin, and their nutritional requirements. The relationship of these two traits is under investigation.

DISCUSSION

Large populations of all sensitive strains of type b *H. influenzae* contain organisms of widely varying degrees of sensitivity to streptomycin. All but a small fraction are eliminated by 10 units per cc., a still smaller fraction survives in concentrations between 10 and 100 units per cc., and the numbers which thrive in concentration of 100 units per cc. do not differ significantly from those in 1,000 units per cc.; they constitute a minute fraction of the whole, varying from one in 1,000,000,000 to one in 46,000,000,000 organisms. The results of experiments designed to study the influence of concentrations of streptomycin, 100 units per cc. compared with 1,000 units per cc., are shown in Table VIII; these data were furnished by experiments which yielded the results listed in Table I. Preliminary experiments had shown no significant difference between the number of colonies which appeared when a given suspension was seeded in media in the presence of 100 and 1,000 units per cc. Therefore five of the ten samples of the pool from nine experiments indicated in Table VIII were seeded in 100 units of streptomycin per cc.

The mechanisms responsible for resistance of this degree are therefore clearly defined, and so far as has been determined the resistance is complete. When colonies grow in the presence of 100 to 1,000 units per cc., 100 per cent of the population exhibits this degree of resistance.

Only when very large populations, 150,000,000,000 and over, are examined is it possible to demonstrate with regularity in all sensitive strains the presence of organisms resistant to 1,000 units per cc. Therapeutic experience shows that persistence of organisms with this degree of resistance in patients receiving adequate treatment with streptomycin, occurs only in severe infections. On the other hand, resistance of a lesser degree may develop when the dose of streptomycin is inadequate or when, in patients with *H. influenzae* meninigitis, streptomycin is not administered intrathecally, even though the infection is not severe. We have seen both of these mechanisms operate with resultant failure of streptomycin therapy.

The variants showing virtually complete resistance to streptomycin have

been demonstrated to possess characteristics of bacterial mutants. Their prevalence has been shown to differ widely in different cultures of the same strain even when all conditions of the experiments are kept as constant as possible; this is true whether the tests are carried out on the same day with the same broth inoculum, on different days, or whether the inoculum is so prepared by single colony isolation that the progeny are genetically homogeneous. The rate of occurrence is consistent with mutation. Finally the resistant trait has been transmitted through many generations.

The procedure described offers a simple method for study of bacterial mutation. Since streptomycin in a concentration of 100 to 1,000 units per cc. eliminates all *H. influenzae* except approximately one organism in each several billion, enormously large bacterial populations can be examined. Only under such conditions is it possible to determine their prevalence because of the low incidence of the resistant variants. When the bacterial population cultured is large enough to encompass at least a few mutants, their prevalence is found to vary widely from test to test and in different independent cultures of the same strain. The factor which exerts the greatest influence on variation in prevalence is the number of variants in the broth inoculum used for production of large populations on Levinthal agar; this number depends in turn upon the point in the growth cycle at which mutation occurs, that is, whether in early or in late generations. When mutants appear early, their reproduction is responsible for their occurrence in large numbers in the broth cultures used for plating out; and because of the relatively small bacterial populations involved in seeding these cultures on Levinthal agar plates by conventional technics there results the observed irregularity in prevalence of resistant colonies in different independent cultures of the same test.

When this factor is eliminated by calculating the rate of occurrence of mutations, it is seen that the three strains 1, 2, and 3, isolated from cases in which streptomycin failed because of emergence of resistance, are not significantly different from the six strains cultivated from patients who recovered promptly following streptomycin alone.

It is of great significance therapeutically that organisms exhibiting complete resistance to streptomycin are sensitive to sulfadiazine. When streptomycin is administered in recommended dosage (9) the number of resistant organisms present, even when the infection is severe and the bacterial population large, can be expected to be relatively small and therefore can be eliminated by the addition of sulfadiazine. This agent is greatly limited when used alone in severe infections. On the other hand, when it is used for a period prior to or along with streptomycin it enhances the action of this antibiotic: in the first instance it reduces the population of *H. influenzae* in the biologic fluids, reducing the likelihood of the presence of a significant number of resistant variants;

and when used simultaneously in severe infections, it can act upon the small population which is resistant to streptomycin.

SUMMARY

In all of ten strains of *H. influenzae* examined prior to exposure to streptomycin a very small fraction of the bacteria formed colonies in the presence of 1,000 units of streptomycin per cc. These variant organisms possess the characteristics of bacterial mutants.

1. Different independent cultures of the same strain on different occasions, and even cultures seeded in a single test with the same inoculum, show marked variation in prevalence of resistant variants in populations of comparable size. This variation is just as great in genetically homogeneous culture sources as in heterogeneous sources. Evidence is presented for the continuous random occurrence of these resistant organisms.

2. The rate of occurrence of the resistant members is not significantly different for the ten strains studied; it varies from 2.6×10^{-11} to 7.0×10^{-11} per bacterium per bacterial generation. The results of five different tests on the same strain show comparable variation, 2.9×10^{-11} to 5.3×10^{-11}. This low rate contributes evidence consistent with the mutation hypothesis.

3. Variants exhibiting resistance to 1,000 units of streptomycin per cc. transmit this trait unchanged in degree through many generations. The results obtained after twelve or more subcultures in streptomycin-free media suggest that in a portion of the colonies resistant to streptomycin an additional trait, differing from those exhibited by the parent culture, is associated; the nutritional requirements of these cultures are different.

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