INDUCTION OF STREPTOMYCIN RESISTANCE IN SENSITIVE HEMOPHILUS INFLUENZAE BY EXTRACTS CONTAINING DESOXYRIBONUCLEIC ACID FROM RESISTANT HEMOPHILUS INFLUENZAE*

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Knowledge of the control of the inheritance of bacterial cells has stemmed in the main from two approaches, (*a*) the induction of new heritable traits by exposure of growing cells to highly specific desoxyribonucleic acids derived from bacterial cells which possess the trait desired and (*b*) analysis of spontaneously occurring mutants, their patterns of appearance and characteristics. As a result of the first approach important heritable characteristics, type specificity and therefore virulence have been induced with predictable regularity in pneumococci (1), *Hemophilus influenzae* (2), and meningococcus (3). The second approach has encountered a phenomenon in the bacterial world which is of great importance, resistance to antibacterial agents; each species of bacteria tested thus far has been shown to possess a mechanism which suffices to protect it against any clinically effective antibacterial agent tested, provided the population is sufficiently large (4). Mutants resistant to a number of antibacterial agents occur spontaneously at a low rate and they survive and proliferate when the sensitive members of the population are eliminated. The resistant mutants breed true and multiply apparently uninfluenced by the antibiotic, in some cases with their growth even enhanced by it, and eventually a resistant strain of the organism emerges. As pointed out by Demerec and associates (5), the patterns of resistance brought out by all the antibiotics studied fall into two types, the penicillin type and the streptomycin type. Demerec (6) showed that staphylococcus cells resulting from the first spontaneous mutational step leading to resistance to penicillin invariably exhibit but a low degree of resistance; a high degree is attained in nature only by permitting the accumulation of resistance from a succession of mutational steps. In contrast, the first mutational step leading to spontaneous resistance to streptomycin can result in a cell endowed with either a low or a high degree of resistance, greater for example, than that to 1000 μg. per ml. of streptomycin.

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The second approach has assumed even greater importance since the demonstration by Hotchkiss (7) that resistance to penicillin can be induced in penicillin-sensitive populations of pneumococci by exposure to extracts containing desoxyribonucleic acid, obtained from penicillin-resistant pneumococci. Of great interest is the fact that the degree of resistance induced in pneumococci by a single exposure to the specific desoxyribonucleic acid is similar in degree to that which results from a single spontaneous mutational step leading to resistance of staphylococci to penicillin (6). A high degree of resistance to penicillin could be induced artificially in pneumococcus populations only by repeated exposures to the desoxyribonucleic acid, even though the pneumococci from which the desoxyribonucleic acid was obtained exhibited a high degree of resistance. Hotchkiss (7) also reports that resistance to streptomycin can be induced in streptomycin-sensitive pneumococcus populations by exposure to an extract containing desoxyribonucleic acid obtained from streptomycin-resistant pneumococci.

All sensitive *Hemophilus influenzae* (8) populations examined, if large enough, have been shown to contain cells resistant to 1000 μg. of streptomycin per ml.; these highly resistant cells arise by mutation at approximately a constant rate of 10^{-11} per bacterium per bacterial generation. The rapidity of emergence of resistance, both *in vitro* and during therapy, is explained by the fact that a cell may become resistant to 1000 μg. of streptomycin per ml. in a single mutational step. This paper presents evidence that resistance of *H. influenzae* to 1000 μg. of streptomycin per ml. can be induced in normally sensitive *H. influenzae* populations by a single exposure for 10 minutes to desoxyribonucleic acid-containing fractions extracted from highly resistant cells.

Material and Methods

Strains.—Strain Rd of *H. influenzae* was obtained by selection of a single non-iridescent colony which appeared spontaneously in a strain of type d *H. influenzae* (isolated from the respiratory tract of a child who had received no streptomycin therapy) when seeded on the surface of Levinthal agar. The Rd strain was preserved by drying and sealing under vacuum (9). All signs of type d specificity had been lost. Sensitivity tests show that 3 μg. of streptomycin per ml. will prevent the growth of virtually all of 1,000,000 cells. This population shows no growth in 10 μg. per ml.

Strain $Sbsm_{1000}$ is a type b *H. influenzae* originally isolated from spinal fluid withdrawn from a patient 24 hours after the start of streptomycin therapy (40 mg. per kilo daily intramuscularly and 25 mg. intrathecally) (10). No evidence of growth from this spinal fluid specimen was apparent until after 3 days of incubation. Examination of the *H. influenzae* population then present, for its sensitivity to streptomycin, demonstrated that it was 100 per cent resistant to 1000 μg. of streptomycin per ml.

Strain Rb was derived from a strain of type b *H. influenzae* (isolated from the spinal fluid of a patient who had received no streptomycin) by selecting a non-iridescent colony which appeared spontaneously on cultivation on Levinthal agar. All signs of type b specificity were lacking when tested for by ordinary standards. Sensitivity tests show that 3 μg. per ml. will prevent the growth of virtually all of 1,000,000 cells; 10 μg. per ml. regularly prevents the growth of 100 per cent of the population.

Resistance-Inducing Principle.—$Sbsm_{1000}$ cells were grown in large quantities on Levinthal agar, and by the method already described for *H. influenzae* (2) a desoxyribonucleic acid–containing extract was procured. This relatively crude extract, was shown to contain desoxyribonucleic acid (218 μg. per ml.) by the diphenylamine reaction.

Technic for Inducing Resistance in Sensitive H. influenzae.—Populations of the sensitive cells were grown for approximately 5 hours in Levinthal broth, yielding 1 to 1.5 billion organisms per ml. This suspension was diluted tenfold and 0.6 ml. of this dilution was added to an environment of 4.5 ml. of neopeptone broth and 0.3 ml. of a 1:20 (0.55 μg. desoxyribonucleic acid per ml.) dilution of the undiluted desoxyribonucleic acid–containing extract isolated from $Sbsm_{1000}$ cells. To demonstrate the speed of the resistance-inducing process, after a 10 minute exposure of the cells to the latter environment, crystalline desoxyribonuclease[1] (0.6 ml. of 20 μg. per ml. in 0.03 M magnesium sulfate in neopeptone broth) was added to the treated cell suspension to destroy the desoxyribonucleic acid. The concentration of desoxyribonucleic acid used could be destroyed by the above desoxyribonuclease concentration in 5 minutes.

Examination for Presence in the "Treated Cell Suspensions" of Cells in Which Resistance Has Been Induced.—Tests were carried out in Levinthal broth and Levinthal agar containing 10 or 1000 μg. of streptomycin per ml. When examination of cells for resistance was made in broth, the population sizes indicated in a volume of 0.1 ml. were seeded immediately into Levinthal broth containing streptomycin (10 or 1000 μg. of streptomycin per ml.) and incubated 24 hours before examination was made for turbidity as a sign of growth. The treated suspension was also seeded into Levinthal broth without streptomycin, and incubated 2 hours before the addition of streptomycin to make final concentrations of 10 or 1000 μg. per ml.

In tests with solid media, the immediate exposure of the treated cells to streptomycin was made either on the surface of Levinthal agar containing 10 or 1000 μg. per ml. or in seeded pour plate preparations in Levinthal agar followed by immediate layering with an equal volume of Levinthal agar containing streptomycin (20 or 2000 μg. per ml.). The treated cells to be examined for the resistant trait were first seeded in agar without streptomycin in pour plate preparations, incubated 2 hours at 37°C., and then layered with melted (cooled to 45°C.) Levinthal agar containing 20 or 2000 μg. per ml. of streptomycin. The colonies were counted after 48 hours' incubation.

As controls the same population sizes from the original suspension prior to treatment, were seeded into streptomycin environments: 10 or 1000 μg. per ml. in Levinthal broth and Levinthal agar at 0 hour and after 2 hours' incubation in Levinthal broth and agar without streptomycin. The 0 hour inoculations into streptomycin agar environments were made by 2 different technics: on the surface of Levinthal agar containing the concentrations of streptomycin listed, and into pour plate preparations which were layered as rapidly as possible with melted agar containing streptomycin (20 or 2000 μg. per ml.).

EXPERIMENTAL RESULTS

Populations of Rd *H. influenzae* were treated by the method described for induction of resistance to streptomycin and then examined in the various population sizes for presence of cells resistant to 10 and 1000 μg. of streptomycin per ml., immediately following treatment, and after incubation in absence of streptomycin for 2 hours. The results are shown in Table I. Five samples of each bacterial population were tested in Levinthal broth and 2 in agar. As controls, comparable populations from the initial suspension, which either had not been exposed to desoxyribonucleic acid at all or were exposed to desoxyribonucleic acid to which

[1] Worthington Biochemical Sales Company, Freehold, New Jersey.

desoxyribonuclease was added first to destroy its transforming activity, were tested for their resistance to streptomycin.

It is seen in Table I that when the treated cells were seeded immediately into media containing streptomycin at a concentration of 10 μg. per ml., re-

TABLE I

Examination of Rd Populations for Cells in Which Resistance to Streptomycin Has Been Induced by a 10 Minute Exposure to a Desoxyribonucleic Acid-Containing Extract from $Sbsm_{1000}$ Cells

Initial population size	Incubation time between treatment and test for SM resistance							
	0				2 hrs.			
	10 μg./ml. SM		1000 μg./ml. SM		10 μg./ml. SM		1000 μg./ml. SM	
Treated cells	Broth	Agar (surface)	Broth	Agar (surface)	Broth	Agar (pour plate)	Broth	Agar (pour plate)
8.6×10^5	+++++	146*	00000	0	+++++	490‡	+++++	470‡
8.6×10^4	+++++	12	00000	0	+++++	47	+++++	32
8.6×10^3	0 + 000	1	00000	0	+++++	5	+++++	2
8.6×10^2	00000	0	00000	0	0 + 0 + 0	0	000 + 0	0
Controls Untreated cells								
8.6×10^5	00000	0	00000	0	00000	1§	00000	0
8.6×10^4	00000		00000		00000		00000	
Controls Cells exposed to DNA destroyed by enzyme								
8.6×10^5	00000	0	00000	0	00000	7§	00000	0

+ = presence of turbidity after 24 hours' incubation.

DNA $Sbsm_{1000}$ = desoxyribonucleic acid–containing extract from $Sbsm_{1000}$ cells.

SM = streptomycin.

* Figures represent number of resistant colonies—average of duplicate samples.

‡ Approximate.

§ Colonies not resistant to 1000 μg./ml. streptomycin.

sistance to this concentration had already been induced. The resistant cells demonstrated their presence within a 24 hour period by growth in streptomycin Levinthal broth. The maximal number of colonies were found in 48 hours in Levinthal agar containing 10 or 1000 μg. per ml. Only a portion of the total cells examined in streptomycin agar, about one-fourth of those in which resistance had been induced, could resist 10 μg. per ml. immediately; but if allowed to grow in the absence of streptomycin for at least 2 hours, 4 times as many colonies were formed. The process necessary for the acquisition of this

degree of resistance apparently requires time for completion in most cells. In the broth environment also, the number of cells which show their resistance on immediate exposure to streptomycin (10 μg. per ml.) is less than after further incubation in streptomycin-free media; again, a time requirement is demonstrated. The colonies which formed in the plate containing 10 μg. streptomycin per ml. showed normal growth when exposed to a higher concentration, 1000 μg. per ml. When media containing 1000 μg. of streptomycin per ml. were used immediately as the selective environment for the cells in which resistance had been induced, no colonies formed on the agar and no growth occurred in the broth seeded with any of the population sizes used. However, if the treated cells were allowed to incubate 2 hours in Levinthal broth or agar before exposure to streptomycin the results were quite comparable with those found in a 10 μg. per ml. environment when a comparable period of 2 hours was allowed for incubation in the absence of the antibiotic. The proportion of cells in which resistance was induced was at least one cell per 8,600 cells treated. This frequency is contrasted with the much lower frequency with which highly resistant cells might arise through random spontaneous mutation.

These results suggest that a high degree of streptomycin resistance (1000 μg. per ml.) can be induced in sensitive populations of Rd *H. influenzae* as a result of exposure for 10 minutes to desoxyribonucleic acid–containing extracts isolated from type b *H. influenzae* cells resistant to 1000 μg. streptomycin per ml. The capacity of the desoxyribonucleic acid extracts to induce resistance can be destroyed by exposure to the crystalline enzyme, desoxyribonuclease. Therefore the process which induces the resistant trait in this experiment in some instances requires less than 10 minutes. The pattern of resistance brought out in a population by artificial means is similar to the pattern displayed in sensitive populations when streptomycin selects out the spontaneously occurring mutants. In each case a single spontaneous mutational step leading to resistance to streptomycin, or a single exposure of sensitive cells to DNA procured from a mutant *H. influenzae* resistant to 1000 μg. of streptomycin per ml., can result in a high degree of resistance.

There is ample proof that spontaneous mutants resistant to 1000 μg. of streptomycin per ml. are arising at a practically constant slow rate, 10^{-11} per bacterium per bacterial generation, in *H. influenzae* strains sensitive to streptomycin; in order to demonstrate their presence very large populations of the order of 10 billion must be used. This degree of resistance has been demonstrated in treated populations of about 10,000. Therefore, the possibility that the majority of the highly resistant cells in the treated population which have formed colonies in Levinthal agar (or exhibited growth in Levinthal broth) containing 1000 μg. of streptomycin per ml., represent spontaneous mutants can be excluded. The controls showed no cells resistant to 1000 μg. of streptomycin per ml. and the proportion of the treated populations forming colonies

in streptomycin (1000 μg. per ml.) agar is over 1,000,000 times greater than could be expected on the basis of appearance of spontaneously occurring resistant mutants. The agar pour plate technic used to provide the selective streptomycin environment prevents the reproduction of the originally resistant cells, either spontaneous mutants or those artificially induced, from contributing significantly to the greater number of resistant cells found after 2 hours' incubation. Study of sensitivity of colonies selected out from the treated suspensions shows them to be homogeneous with respect to resistance to 1000 μg. of streptomycin per ml. Since the cells from the treated populations which grow in 10 μg. of streptomycin per ml., even when exposed at 0 hour, exhibit

TABLE II

Influence of Population Size and Incubation Time on Expression of Resistance after Its Induction by Exposure for 15 Minutes to DNA $Sbsm_{1000}$

Initial population size	Treated cells exposed to 1000 μg./ml. SM after incubation period indicated					Controls exposed to 1000 μg./ml. SM	
	0	15 min.	30 min.	1 hr.	2 hrs.	No treatment	Treatment with DNA $Sbsm_{1000}$ first exposed to DNAse
1.5×10^7	000	000	+ 00	+++	+++	00	00
1.5×10^6	000	000	000	000	+++	00	00
1.5×10^5	000	000	000	000	+++	00	00
1.5×10^4	000	000	000	000	00 +	00	00
1.5×10^3	000	000	000	000	000	00	00

+ = Presence of turbidity after 24 hours' incubation. Subcultures grew in 1000 μg. SM per ml.

DNA $Sbsm_{1000}$ = desoxyribonucleic acid–containing extract from $Sbsm_{1000}$ cells.

DNAse = desoxyribonuclease.

SM = streptomycin.

resistance to 1000 μg. of streptomycin per ml., the possibility is raised that each of the concentrations used, 10 or 1000 μg. per ml., selects out cells in which the same degree of resistance has been induced; about one-fourth of them could survive if exposed immediately to 10 μg. per ml. but there are no survivors in these population sizes when the treated cells are seeded in an environment of 1000 μg. of streptomycin per ml.

Table II presents the results of an experiment which sought more information on the time required for completion of the trait leading to resistance to 1000 μg. per ml. of streptomycin when different population sizes of the treated cells were exposed to that concentration of streptomycin after incubation in Levinthal broth for various time intervals. In this experiment Rd cells, grown for about 5½ hours in Levinthal broth, were exposed for 15 minutes to DNA extracted from $Sbsm_{1000}$ cells before DNAse was added, as already described. The results again show that only a small proportion of the cells in which re-

sistance is induced can express that trait if exposed immediately to a high concentration of streptomycin (1000 μg. per ml.); time or growth appears to be needed for a process which has already been started to be completed. A significant number of cells can withstand this action after incubation for 1 hour, and a rare one after 30 minutes when the largest population of treated cells is examined. The influence of population size is clear, suggesting that the resistant trait is induced in only a small fraction of the total population exposed

TABLE III

Comparison of the No. of Colonies of Resistant Cells Selected by 10 and 1000 μg. of SM per Ml. in Pour Plates from Suspensions of Rd Populations Treated with DNA $Sbsm_{1000}$

Environment	Initial population size	Exposure to SM agar (approximate)	Period of incubation before exposure to SM						
			0	15 min.	30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.
		μg./ml.							
Cells exposed to DNA $Sbsm_{1000}$ for 10 min.	1.0×10^7	10	Inn.	Inn.	Inn.	Inn.	Inn.	Inn.	Inn.
		1000	125	244	Many	Inn.	Inn.	Inn.	Inn.
	1.0×10^6	10	500*	408	424	514	426	452	484
		1000	8–8‡	27	73	243	429	461	460
	1.0×10^5	10	47–37	51	36	44	54	49	43
		1000	0	1	2	30	42	39–32	50
Control									
Cells not exposed to DNA $Sbsm_{1000}$	1.0×10^7	10	1			8–8	8	32	37
		1000	0			0	0	0	0

Inn. = innumerable.

SM = streptomycin.

DNA $Sbsm_{1000}$ = Desoxyribonucleic acid–containing extract from $Sbsm_{1000}$ cells.

* = approximate number for 3 digits above 200.

‡ = first figure—total number of colonies; second figure—number of colonies tested for resistance to SM.

to the inducing DNA. The larger the population, the larger the number of cells in which the trait is induced and the greater the chances that there will be some cells initially in which the process is sufficiently complete to withstand the rapid lethal action of streptomycin in a concentration of 1000 μg. per ml.

The results of an experiment designed to test the hypothesis that both environments, 10 and 1000 μg. per ml., select out cells of the same degree of resistance are shown in Table III.

The technic was the same as in the 2 preceding experiments. Approximately 10^8 cells per ml. were exposed for 10 minutes to DNA-containing extract isolated from $Sbsm_{1000}$ cells. DNAse was then added to destroy the biological acivity of the DNA. After allowing 10

minutes for action of the DNAse, the various population sizes of the treated cells were then seeded in 0.1 ml. volumes in Levinthal agar pour plate preparations. Untreated cells from the original suspension were exposed to streptomycin in agar after the same intervals of incubation to serve as controls.

It is seen that the largest treated populations, 10^6 and 10^7 cells, when exposed to streptomycin (1000 μg. per ml.) at 0 hour, show the survival of a small fraction of the cells in which ultimately, after 2 hours' incubation, a high degree of resistance has been shown to have been induced. In this experiment the number of treated cells which form colonies when exposed to 10 μg. of streptomycin per ml. at 0 hour is similar to the number found after 2 hours' incubation without streptomycin before exposure to this concentration. The greater lapse of time between the completion of treatment of cells and the exposure to streptomycin at 0 hour may be responsible for the difference between these results and those listed in Table I. In the experiment in Table I the 0 hour test was made on the surface of streptomycin-containing agar. The pour plate technic required a longer period between treatment of cells and the test of their sensitivity to streptomycin than did the surface seeding of cultures, and therefore may have permitted completion of the change to streptomycin resistance in a higher proportion of cells before exposure to streptomycin. Of great significance is the fact that at 2 hours the number of resistant colonies selected out by each of the 2 concentrations is essentially the same. 37 of the 47 colonies selected out by 10 μg. per ml. at 0 hour from the 1×10^5 population were tested: all 37 grew normally on media containing 1000 μg. of streptomycin per ml., just as did those colonies selected out by 1000 μg. per ml. at 0 hour from the 10^6 population or from the 10^5 population when the streptomycin was added after 3 hours of incubation. Failure of increase in incubation time (prior to exposure to streptomycin) beyond 2 hours to increase the number of colonies which form, suggests that the maximum amount of expression of induced resistance has occurred within this period.

The control populations, initially 10^7 cells which had not been exposed to the DNA extract, showed 8 colonies in the pour plates containing 10 μg. per ml. after 1 and 2 hours' incubation. All 8 colonies, when transplanted onto fresh media containing 10 μg. streptomycin per ml. failed to grow, and the 3 which were tested failed also to grow on 1000 μg. per ml. These colonies therefore represent relatively sensitive cells, the survival of which in the original 10 μg. per ml. environment suggests that the streptomycin did not diffuse rapidly enough to prevent the survival of mutants of a low degree of resistance. The increase in the population sizes after growth for 3 and 4 hours readily reflects the observed increase in the number of colonies which are of a lower order of resistance than 10 μg. per ml.

Tables IV and V present the results of experiments which were designed to examine the suggestion that a period of 2 hours' incubation after exposure to

DNA, before allowing streptomycin to exert its selective influence, is adequate for expression of resistance in all the cells in which the resistant trait has been induced.

Table IV lists results from an experiment which explored the influence of incubation over a 6 hour period on the proportion of cells expressing resistance to 1000 μg. of streptomycin per ml.

TABLE IV

Influence of Incubation Time after Induction of Resistance on Proportion of Cells Expressing Resistance in a Selective Environment of Approximately 1000 μg. Streptomycin per Ml.

Environment	Initial population size	Average No. of colonies forming in SM agar				
		Incubation time between treatment and test for SM Resistance				
		0		2 hrs.	4 hrs.	6 hrs.
		Seeding technic				
		Surface	Pour plate	Pour plate	Pour plate	Pour plate
Cells exposed to DNA $Sbsm_{1000}$ for 10 min.	1.2×10^6	0	2–4*	170	181	178
	1.2×10^5	0	1–1	19–37	18	20–40
Cells not exposed to DNA $Sbsm_{1000}$	1.2×10^7		0	0	3–5	1–1
	1.2×10^6		0	0	0	0
Cells exposed to DNA $Sbsm_{1000}$ after action of DNAse	1.2×10^6		0	0	0	0

SM = streptomycin.

DNA $Sbsm_{1000}$ = desoxyribonucleic acid–containing extract from $Sbsm_{1000}$ cells.

* First figure = average number colonies—duplicate samples; second figure = number colonies tested for resistance to SM. Each grew well on surface of Levinthal agar containing 1000 μg. SM per ml.

Agar containing 1000 μg. of streptomycin per ml. was used to detect the presence of resistant cells.

After exposure of 1.2×10^8 cells per ml. to desoxyribonucleic acid from cells $Sbsm_{1000}$ and destruction of the DNA 10 minutes later, the treated suspension of cells was diluted and 0.1 ml. quantities of the dilution seeded on the surface of Levinthal agar containing 1000 μg. of streptomycin per ml. at 0 hour and also in pour plate preparations layered with 2000 μg. of streptomycin per ml. after hardening. Treated cells in the same population sizes were seeded in pour plate preparations in the absence of streptomycin, incubated for 2, 4, and 6 hours, and melted Levinthal agar containing 2000 μg. streptomycin per ml. was then layered on the seeded pour plates. Incubation continued for 48 hours before count of the colonies. The same populations of the original untreated Rd cell suspensions were seeded in a similar manner in pour plate preparations and layered with melted Levinthal agar containing 2000 μg. of streptomycin per ml. as soon as the agar solidified and after the listed incubation periods. All tests were carried out in duplicate.

TABLE V

Influence of Incubation Time after Induction of Resistance on Proportion of Cells Expressing Resistance in Selective Environments of Approximately 10 or 1000 μg. Streptomycin per Ml.

Environment	Initial population size	μg. per ml. SM for selection after incubation	Average No. colonies forming in SM agar			
			Period of incubation before layering SM agar			
			0	1 hr.	2 hrs.	4 hrs.
Cells exposed to DNA $Sbsm_{1000}$ for 10 min.	1.3×10^6	10	178	172	201	199
		1000	10–19*	96	186	186
	1.3×10^5	10	17–32	20	16	23–45
		1000	2–4	17	16	16–26
Controls Cells not exposed to DNA $Sbsm_{1000}$	1.3×10^6	10	1–2	1–1	3–5	63
		1000	0	0	0	0
	1.3×10^5	10	0	1	1–2	4–8
		1000	0	0	0	0
Controls Cells exposed to DNA $Sbsm_{1000}$ after action of DNAse	1.3×10^6	10	1–2	1–2	9–18	56
		1000	0	0	0	0
	1.3×10^5	10	1	0	3–6	12–23
		1000	0	0	0	0

SM = streptomycin.

DNA $Sbsm_{1000}$ = desoxyribonucleic acid–containing extract from $Sbsm_{1000}$ cells.

* Second figure = number colonies tested for resistance to SM.

Study of SM Sensitivity of Sample Colonies Selected

Source of colonies	Total colonies selected	Growth on SM agar					
		10 μg./ml.				1000 μg./ml.	
		No growth	Few colonies	Reduced	Normal	No growth	Normal
Treated cells							
10 μg. series	77	0	2	2	73	0	73
1000 μg. series	54					0	54
Control cells							
10 μg. series	69	5	44	20	0	69	0

Again there is no significant increase in the number of colonies formed when the treated populations are incubated in Levinthal agar pour plates for longer than 2 hours before the selective action of streptomycin is introduced. No colo-

nies formed in comparable populations in the controls. As in other comparable experiments, the difference in the 0 hour results, depending upon whether the treated cells are seeded on the surface of streptomycin agar or in pour plate preparation, is believed to be a function of the longer time which elapses between treatment of cells and exposure to the selective action of streptomycin in the case of the pour plate method.

Table V shows data from a comparable experiment to study the effect of incubation for 1, 2, and 4 hours before the treated cells were exposed to the selective action of 2 different concentrations of streptomycin, 10 and 1000 μg. per ml. The results are similar to those in Tables III and IV. There is no increase after 2 hours in the proportion of cells which can express resistance to either 10 or 1000 μg. of streptomycin per ml.; apparently the process is completed by 2 hours in all cells in which the change needed for resistance has been induced. Again the results show that most or all of the cells can express resistance at 0 hour in the 10 μg. streptomycin per ml. pour plate preparation environment, while in that containing 1000 μg. per ml. only a small proportion can express the trait. For expression of resistance in the latter environment almost all of the cells in which this trait has been induced require incubation for approximately 2 hours. As in the experiment shown in Table III the expression of induced resistance of all or most cells in 10 μg. of streptomycin per ml. at 0 hour is believed to be explained by technical factors related to diffusion of streptomycin.

The results of these experiments lead to the following conclusions. The 10 and 1000 μg. streptomycin per ml. environments select out cells in which a comparable degree of resistance has been induced by DNA $Sbsm_{1000}$. Only a small proportion of the total population exposed is transformed to resistant cells, roughly 1 in 10,000. In each case the change which enables bacterial cells to express resistance has been initiated within a 10 minute period, since contact with the DNAse at the end of that time interval has destroyed the biologic activity of the specific DNA. When cells so treated are exposed to streptomycin immediately, the results suggest that the change which induces resistance to 1000 μg. per ml. is not sufficiently complete to permit them to grow in this concentration. In sensitive populations of *H. influenzae* this concentration has been shown to be more rapidly lethal than 10 μg. streptomycin per ml. The results suggest that the process which equips a cell to grow in 10 μg. per ml. has been completed in a high proportion of the induced population by the time they can be exposed in pour plate preparations. When seeded immediately on the surface of Levinthal agar containing streptomycin, the proportion equipped to grow in 10 μg. per ml. is much smaller. The proportion of treated cells which can grow in pour plate preparations containing 1000 μg. per ml. at 0 hour is minute; only very large populations show any such resistant colonies.

It is clear that the development of resistance in all the cells in which the process has been initiated required 2 hours between treatment and test for resistance, whether 10 or 1000 μg. per ml. was used as the selective environment. Intervals longer than 2 hours between treatment and test for resistance have not yielded a larger number of resistant colonies. Whether temperature plays an important role is not known, since incubation at 37°C. was used for all time intervals. Whether the completion of the process which leads to genetically determined resistance requires some steps necessary for reproduction

TABLE VI

Examination of Rb Populations for Cells in Which Resistance to Streptomycin Has Been Induced by a 10 Minute Exposure to DNA $Sbsm_{1000}$

Environment	Initial population size	Period of incubation before exposure to SM			
		0		2 hrs.	
		μg. SM used for selection after incubation			
		10 μg./ml.	1000 μg./ml.	10 μg./ml.	1000 μg./ml.
Cells exposed to DNA $Sbsm_{1000}$ for 10 min.	1.2×10^7	00000	00000	+++++*	+++++
	1.2×10^6	00000	00000	+++++	+++++
	1.2×10^5	00000	00000	+ 000 +	0++++
	1.2×10^4	00000	00000	0000+	00000
Cells not exposed to DNA $Sbsm_{1000}$	1.2×10^7	000	000	000	000
	1.2×10^6	000	000	000	000
	1.2×10^5	000	000	000	000
Cells exposed to DNA $Sbsm_{1000}$ after action of DNAse	1.2×10^7	000	000	000	000
	1.2×10^6	000	000	000	000
	1.2×10^5	000	000	000	000

SM = streptomycin.

DNA $Sbsm_{1000}$ = desoxyribonucleic acid–containing extract from $Sbsm_{1000}$ cells.

* + = presence of turbidity after 24 hours' incubation.

is not known. In an interval of 2 hours, under these conditions 1 or 2 generations could be produced. Yet the marked difference in expression of resistance to 10 μg. per ml. resulting from the short time difference between the pour plate and surface tests of resistance suggests that growth is not required for resistance to 10 μg. streptomycin per ml. to be expressed.

All the evidence gained supports the conclusion that the increase in the number of resistant colonies during time up to 2 hours represents completion of a step in a process which is necessary for expression of resistance, rather than for reproduction of those cells which were resistant initially. In each cell which starts one of the resistant colonies the essential action of the DNA had

got under way during the 10 minute exposure. This inference is supported by 2 lines of evidence.

(*a*) The nature of the procedure used to demonstrate resistant cells in the treated suspensions—the pour plate preparation and surface spreading for discrete colonies—makes it possible to detect the number of cells in which resistance was induced initially by their reproduction to form colonies. Study of subcultures of the population of cells forming a resistant colony on Levinthal agar with and without streptomycin shows them to be homogeneous with respect to the resistant trait.

(*b*) The failure to demonstrate an increase in resistant colonies after 2 hours is proof that if micro clones have formed during the 2 hour interval, they are not scattered by the subsequent layering of agar.

Resistance has also been induced in Rb strains of sensitive *H. influenzae* by exposure to the DNA-containing extract isolated from $Sbsm_{1000}$ cells. The plan and technics of the experiments which demonstrated this change are those employed for induction of resistance in Rd populations, and the results are shown in Table VI. The data suggest that a high degree of resistance is induced in a single step. Exposure to DNA extracts from $Sbsm_{1000}$ for only 10 minutes induces heritable resistance to 1000 μg. of streptomycin per ml. Only a minute proportion of the total population exposed is made resistant. The true rate of occurrence has not been measured, but the results suggest that the frequency of cells susceptible to this induced resistance is lower than in Rd populations.

DISCUSSION

The demonstration that a number of genetic traits in bacteria are controlled by highly specific DNA's has raised the question whether all genetic traits are directed by such a factor. There are many examples of spontaneous genetic changes resulting in resistance to antibacterial agents.

A genetic change, resistance to streptomycin, indistinguishable from the one which occurs spontaneously, has been induced in sensitive *H. influenzae* by exposure to DNA-containing extracts isolated from type b *H. influenzae* cells which have become resistant to 1000 μg. of streptomycin per ml. as a result of spontaneous mutation. Resistance to 1000 μg. of streptomycin per ml. can be induced with predictable regularity in a relatively small population, about 10,000 cells. The capacity of the DNA-containing extract to induce resistance can be destroyed by crystalline DNAse; and by using the enzyme to put an abrupt stop to the biologic action of the DNA at the end of a measured time interval it has been possible to demonstrate that the reaction between the DNA and susceptible cells is sometimes completed, more frequently initiated, within 10 minutes. Only a very small proportion of the total population exposed is susceptible to induction of resistance, about 1 in 10,000

in Rd and 1 in 100,000 to 1 in 1,000,000 in Rb populations. It is of great interest that a similar difference in frequency of cells susceptible to change has been demonstrated in Rd and Rb cells when a new type specificity is induced. However, both in Rb and Rd populations the frequency of cells susceptible to change in type is lower than the frequency of cells susceptible to induction of resistance.

Of significance is the fact that the data offer evidence that the trait, resistance to 1000 μg. of streptomycin per ml., is induced in a single step. In other words, the pattern of induced resistance is comparable to that which is brought out when spontaneously occurring mutants are merely selected out by streptomycin. The rate of occurrence of spontaneously occurring *H. influenzae* cells resistant to 1000 μg. per ml. has been found to be 10^{-11} per bacterium per bacterial generation. At least 10 billion organisms are needed in order to predict the growth of one colony in the selective environment of Levinthal agar containing 1000 μg. per ml. When sensitive *H. influenzae* populations are exposed to DNA $Sbsm_{1000}$, one cell in approximately each 10,000 will form a colony in the presence of 1000 μg. of streptomycin per ml. The frequency of the induced resistant cell is more than 1,000,000 times that of the spontaneously occurring resistant mutant.

Resistance induced by DNA-containing extracts is an inherited trait. Populations derived from each of 2 colonies selected out by 10 μg. per ml. and from each of 2 colonies which formed in 1000 μg. per ml. were subcultured 11 times in Levinthal broth without streptomycin at 24 or 48 hour intervals and then retested for their sensitivity to streptomycin. There was no significant difference in the number of colonies formed when equal numbers of cells (100 to 200) of each of the 4 populations were seeded on Levinthal agar and Levinthal agar containing 1000 μg. per ml. of streptomycin.

SUMMARY

Resistance to streptomycin, of a degree exceeding 1000 μg. per ml., has been induced in sensitive strains of *Hemophilus influenzae* by exposure for 10 minutes to desoxyribonucleic acid–containing extracts isolated from a strain of type b *Hemophilus influenzae* which had emerged resistant to 1000 μg. of streptomycin per ml. DNA is essential for the process which brings out this change; the reaction can be prevented by destruction of the DNA with crystalline desoxyribonuclease. The resistant trait which is created in this way is heritable.

The nature of the process which induces resistance is similar in all respects to the reaction which induces heritable changes in type specificity of *H. influenzae.*

These results offer another example of the gene-like action of highly specific DNA's.

The pattern of resistance brought out in a bacterial population exposed to the DNA-containing, resistance-inducing extract, is similar to that which occurs when emergence of resistance of *H. influenzae* to streptomycin follows the selection by streptomycin of spontaneously occurring resistant mutants. The change in a bacterial cell from average susceptibility to streptomycin to resistance to 1000 μg. of streptomycin per ml. can occur in a single step.

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