STUDIES ON THE NATURE OF HEMOPHILUS INFLUENZAE CELLS SUSCEPTIBLE TO HERITABLE CHANGES BY DESOXYRIBONUCLEIC ACIDS*

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There is now convincing evidence that highly specific desoxyribonucleic acids (DNA's) can direct some inherited traits of members of 3 different bacterial species, *Diplicoccus pneumoniae* (1), *Hemophilus influenzae* (2, 3), and *Neisseria meningitidis* (4), and suggestive evidence that heritable traits of certain viruses, rabbit fibroma (5), and bacterial viruses (6), may be influenced by the action of DNA. The new virus particles produced by Hirst (7) through the interaction of 2 different antigenic types of influenza A viruses may result from a process comparable to those processes producing allogenic transformation of pneumococci by Ephrussi-Taylor (8) and type S*ab H. influenzae* cells (9) produced by the interaction of type *a H. influenzae* DNA on type *b H. influenzae* cells.

Earlier findings (2, 9, 10) have demonstrated 8 different "heredity determinants" in DNA-containing preparations, which can induce highly specific heritable changes with predictable regularity in *H. influenzae* populations (Table I). DNA has been shown to be an essential component of each; desoxyribonuclease (DNAse) destroys the activity which induces heritable changes. Six of the 8 heredity determinants control the type-specific traits, *a*, *b*, *c*, *d*, *e*, and *f* (2). One controls streptomycin (SM) resistance (10) and one induces the S*ab* trait (9). Two different purification procedures (3, 11), applied to crude DNA extracts of type *b* and type *c H. influenzae* by Zamenhof *et al.*, have yielded DNA fractions of a high degree of purity, biologically active in a concentration as low as 3×10^{-5} μg. per ml.

The action of the DNA which is essential for inducing a heritable change is virtually immediate if transformable cells are present (2). A medium which supports growth is not needed for the action of DNA. The reaction can occur in a simple environment, such as unenriched broth containing no x and v factors which are required for growth of *H. influenzae*. These characteristic features of the DNA action could be demonstrated whether the "recipient population" (population exposed to the DNA) is made up of "R" (non-encapsulated)

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H. influenzae cells derived originally from S or encapsulated, type-specific cells but devoid of all type-specific traits or whether it is a fully equipped type-specific population among which R cells are difficult or impossible to demonstrate (12).

While all evidence is consistent with the hypothesis that DNA is the dynamic component of the heredity determinant of *H. influenzae*, the expression of its action, induction of a new heritable trait, requires some substance or property found only in a small proportion of the total population exposed. This paper will present the results of experiments designed to explore the nature of the cells making up this small fraction of the population. These cells will be referred to as "susceptible cells." The data suggest that the susceptible cells differ

TABLE I

Heritable Changes Induced in H. influenzae by Exposure to DNA's

Recipient cells	Changes induced by DNA's							
	Type-specific DNA's							DNA_{SM1000}
	a	*b*	*ab*	*c*	*d*	*e*	*f*	
R*a*	*a**	—	*a*	—	—	—	—	SM1000
R*b*	*ab*	*b*	*b, ab*	*c*	*d*	—	—	"
S*b*	*a, ab*	N.T.	N.T.	*c*	*d*	—	N.T.	"
R*c*	—	—	N.T.	—	—	—	—	—
R*d*	*a*	*b*	*a, b, ab*	*c*	*d*	*e*	*f*	SM1000
S*d*	*a*	*b*	*a, b, ab*	*c*	N.T.	*e*	N.T.	"
R*e*	—	—	N.T.	—	—	*e*	—	"
R*f*	—	—	N.T.	—	—	—	—	—

N.T. = not tested.
— = no evidence of transformation.
SM1000 = resistance to 1000 μg. per ml. of SM.
* Letter indicates type specificity induced.

in their properties from the other members and may therefore represent specialized cells (13).

MATERIALS AND METHODS

DNA Preparations.—All DNA extracts, unless stated otherwise, were relatively crude. The method of preparation has been described previously (2).

Technique Used for Inducing Change in Type Specificity.—The technique for inducing changes and the criteria used for their recognition have already been described in detail (2).

Technique Used for Inducing Streptomycin Resistance.—In all experiments, unless otherwise indicated, the same procedure was used. The designated populations in 1.7 ml. of Levinthal or neopeptone broth are exposed to the crude DNA extract from cells resistant to 1000 μg. per ml. of streptomycin (SM) by adding 0.1 ml. of a 1:10 dilution of the DNA extract, corresponding to approximately 2 μg. of DNA, to the suspension of recipient cells. After a 10 to 30 minute exposure period, 0.2 ml. of 20 μg. per ml. of desoxyribonuclease (DNAse), in 0.03 M $MgSO_4$ neopeptone broth, is added. The enzyme destroys the activity of the DNA in the concentrations used in less than 5 minutes. The treated populations are then seeded

in appropriate population sizes (over a range known to provide a number of colonies which can be counted accurately) into pour plate preparations of Levinthal agar and incubated at 37°C. for 2 hours. Melted and cooled Levinthal agar containing 2000 μg. per ml. of SM is then layered in an equal volume over the seeded agar and the preparations incubated for 48 hours before the final colony count is recorded. All colonies formed in this concentration of SM agar are found resistant to more than 1000 μg. SM per ml. The 2 hour period of incubation prior to addition of SM agar permits completion of the essential change that occurs almost immediately, thus allowing maximal expression of induced resistance.

EXPERIMENTAL RESULTS

The experiments carried out to explore the nature of the susceptible cell have used 2 different varieties of heredity determinants to produce heritable changes: (1) the type-specific DNA's and (2) the DNA inducing resistance to SM. These investigations are designed for 2 purposes: (I) to investigate the factors which influence the size of the small proportion of cells in a population which are susceptible to heritable change and (II) to determine whether all heritable changes induced in a given population of *H. influenzae* take place in the same cells.

I. Factors Which Influence the Size of the Small Proportion of Population Susceptible to Heritable Changes

A. Influence of Specific Type from Which Recipient Cell Populations Are Derived:

1. Use of Type-Specific DNA as a Heritable Marker.—The induction of type specificity using DNA-containing preparations extracted from type-specific cells does not permit an accurate measure of frequency or rate of occurrence of susceptible cells. However, an index of frequency of induced type-specific cells, hereafter referred to as "frequency pattern," can be established by determining the smallest population of recipient cells in which transformation can be demonstrated. Non-encapsulated, non-iridescent "R" *H. influenzae* strains derived from different type-specific strains have been examined in this way for their frequency pattern of induced type-specific cells under 2 circumstances: (*a*) exposure of varying population sizes of "R" cells to a single dose of homologous DNA's during growth and (*b*) exposure of varying population sizes of recipient cells to DNA's for a limited period to detect the frequency of initially susceptible cells.

(*a*) *Determination of the Frequency Pattern of Induced Type-Specific Cells, Characteristic of Each Recipient Type When Exposed to DNA during Growth.*—

Twenty-five independent spontaneously occurring R colonies selected after various subcultures of each of 2 strains of types *a, b, d,* and *e* were studied. The population grown from each R colony was examined to determine the influence of the initial population size on the degree of uniformity with which transformation can be demonstrated during growth for 48 hours in the presence of the homologous type-specific DNA. R strains were exposed to homologous DNA since induction of heterologous type specificity in R*a* and R*e* populations

could not be demonstrated. All R strains were first examined for reversion to type by growing them in Levinthal broth containing anti-R *H. influenzae* antiserum. Only those strains, in which it was impossible to demonstrate spontaneous reversion to type, were used.

In these experiments (Table II), Levinthal broth cultures of R cell populations, 5½ to 6 hours of age, were diluted in neopeptone broth 10^{-1} through 10^{-6} (corresponding to approximately 10 million and 100 cells). All R cell strains were examined in the following population sizes: 1×10^6, 1×10^4, and 1×10^2. Three samples of each population size listed were seeded into 2 ml. Levinthal broth, containing anti-R *H. influenzae* antiserum at a final dilution of 1:50, and 0.1 ml. of 1:10 dilution of crude DNA extract of homologous type-specific cells. After a period of growth for 18 to 24 hours the treated populations were examined for type-

TABLE II

Characteristic Patterns of Induced Type Specificity Found in R Strains from 4 Types of H. influenzae Grown for 48 Hours in Presence of Homologous DNA

R strains	Induction of homologous type specificity in majority of samples of varying population sizes				
	Frequency patterns found			Per cent of 25 R stains showing each pattern	
	Initial population size				
	1×10^6	1×10^4	1×10^2	S_1*	S_2*
R*a*	0	0	0	76	88
	+	0	0	8	4
	0	+	+	8	0
	+	+	+	8	8
R*b*	0	0	0	0	8
	+	0	0	88	8
	+	+	0	8	28
	+	+	+	4	56
R*d*	+	+	+	100	100
R*e*	+	0	0	8	36
	+	+	0	52	64
	+	+	+	40	0

+ = transformation demonstrated in 2 or 3 samples of triplicate series.
0 = transformation not demonstrated in any or present in only one of three samples.
Letters after R strain refer to type of origin.
* S indicates type-specific strain from which 25 R colonies isolated.

specific cells by examining for capsular swelling by the type-specific antibody corresponding to the DNA type. At this time a 2 mm. loopful of the population was also seeded on the surface of Levinthal agar and examined for iridescent colonies after an incubation period of 18 hours. Samples which showed no type-specific cells at 24 hours were examined again after 48 hours. The results in Table II are based on the 48 hour reading. Since the point of interest was to determine the influence of initial population size on uniformity of transformation the + symbol designates induction of heritable change in at least 2 of the 3 samples treated. The 0 reading represents either failure to demonstrate type-specific cells in all samples or presence in only one of the three.

The results of this test show that R*d* cells exhibit the highest frequency of induced type-specific cells; all initial populations of 100 cells provide suscep-

tible cells during the 48 hour period of growth. R*a* cells show the lowest frequency; in other experiments initial populations of 10 million cells were required for demonstration of induction of type *a* in a majority of samples. Populations of R*b* and R*e* show intermediate frequency patterns. Induction of homologous type specificity has not been demonstrated in any populations of R*c* or R*f* tested.

A comparison of the frequency patterns of induced homologous type-specific cells in R*b* and R*d* populations is shown in more detail in Table III. The populations from 10 different R*b* and R*d* colonies are compared to show the influence of length of

TABLE III

Influence of Period of Growth on Population Size Needed for Demonstration of Induction of Homologous Type Specificity in Rb and Rd Populations

Population No.	Induction of homologous type specificity during 24 and 48 hr. growth periods											
	R*b* No. of cells seeded						R*d* No. of cells seeded					
	1×10^6		1×10^4		1×10^2		1×10^6		1×10^4		1×10^2	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1*	+++		000	000	000	000	+++		+++		+++	
2	+++		000	000	000	000	+++		+++		+++	
3	+++		000	000	000	000	+++		+++		+++	
4	+++		++0	++0	000	00+	+++		+++		+++	
5	+++		00+	0++	000	000	+++		+++		+++	
6	+++		000	+++	000	+++	+++		+++		+++	
7	+++		00+	+++	000	+++	+++		+++		+++	
8	+++		000	++0	000	0+0	+++		+++		+++	
9	+++		000	000	000	0+0	+++		+++		+++	
10	+++		0++	0++	00+	0++	+++		+++		+++	

\+ = transformation to homologous type.
0 = no evidence of transformation.
* Population derived from single colony.

growth period of the seeding population on the demonstration of induction of type specificity in a majority of samples. Uniform induction of type *b* in R*b* cells is demonstrated within a 24 hour period only in populations of 10^6 whereas induction of type *d* is demonstrated uniformly within this time with a seeding population of 100 cells. Some of the smaller populations of R*b* show uniform induction of type *b* during a 48 hour period of growth.

The frequency pattern characteristic of the specific type of origin of a cell as it occurs in nature is maintained through many generations and therefore can be looked on as an inherited trait. The results of experiments designed to determine whether induction of a new type changes the original frequency pattern are shown in Table IV.

The young cultures (5 to 6 hour growth) of R*b* and R*d* populations were exposed separately during growth to DNA*b*, DNA*c*, and DNA*d*, and pure cultures of each of the induced type-specific cells were prepared from single colonies. From the induced types *b*, *c*, and *d* cultures,

TABLE IV

Inheritance of Frequency Pattern of Specific Type of Origin

Specific type of origin	Induced type	Recipient cells	Type of DNA	Colony No.	Induction during growth of type specificity corresponding to DNA type in varying population sizes					
					1×10^6		1×10^4		1×10^2	
					24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
R*b*	*b*	R*b*	DNA*b*	1	+++		++0	+++	00+	+++
				2	+++		+++		000	0++
				3	+++		+++		000	+0+
				4	+++		000	000	000	000
				5	+++		0+0	0+0	000	000
	c	R*c*	DNA*c*	1	+++		000	000	000	000
				2	+++		000	000	000	000
				3	+++		000	000		000
				4	+0+	+0+	000	000		000
				5	+++			000		000
	d	R*d*	DNA*d*	1	+++		000	000	000	000
				2	+++		000	000	000	000
				3	+++		000	+++	000	+++
				4	+++		0++	0++	000	000
				5	+00	++0	000	000	000	000
R*d*	*b*	R*b*	DNA*b*	1	+++		+++		+++	
				2	+++		+++		+++	
				3	+++		+++		+++	
				4	+++		+++		+++	
				5	+++		+++		+++	
	c	R*c*	DNA*c*	1	+++		+++		+++	
				2	+++		+++		+++	
				3	+++		+++		+++	
				4	+++		+++		+++	
				5	+++		+++		+++	
	d	R*d*	DNA*d*	1	+++		+++		+++	
				2	+++		+++		+++	
				3	+++		+++		+++	
				4	+++		+++		+++	
				5	+++		+++		+++	

+ = transformation to type of DNA.
0 = no evidence of transformation.

R cells were isolated by selecting non-iridescent colonies which appear spontaneously when type-specific *H. influenzae* cultures are subcultured a number of times in Levinthal broth.

Three population sizes of these R strains, approximately 1×10^6, 1×10^4, and 1×10^2 cells, were exposed during growth to the DNA corresponding to the induced type from which they had been derived and were then examined for induction of type specificity.

It is seen in Table IV that R populations derived from an induced heterologous or homologous type tend to maintain the frequency pattern characteristic

of the natural type of origin (Table III). R*b* and R*d* cells continue to exhibit the frequency pattern characteristic of the type of origin when they are repeatedly transformed to type *c* just as they do when the homologous type is induced.

(*b*) *Frequency Pattern of Initially Susceptible Cells.*—The frequency patterns of initially susceptible cells characteristic of R*a*, R*b*, and R*d* are shown in Table V.

For this purpose the undiluted cultures grown for 4½ hours were exposed to the listed DNA's in concentrations of 0.5 to 1.0 μg. per ml. for 15 minutes. DNAse was then added to destroy the transforming activity of the DNA and after a 5 minute period to insure the destruction, the treated population was diluted in Levinthal broth containing anti-R antiserum, to make 5 samples of each of the population sizes listed, and incubated for 24 hours. Examination was then made for presence of type-specific cells corresponding to the type

TABLE V

Influence of Type of Origin of Recipient Cell on Frequency Pattern of Cells Initially Susceptible to Induction of Homologous and Heterologous Type Specificity

Recipient cells	Type of DNA	Induction of type specificity corresponding to type of origin of DNA in varying population sizes			
		$0.5 - 1.2 \times 10^7$	$0.5 - 1.2 \times 10^6$	$0.5 - 1.2 \times 10^5$	$0.5 - 1.2 \times 10^4$
R*b*	*b*	+++++	+++++	+000+	00000
	c	+++++	+++++	0000+	00000
R*d*	*b*		+++++	0++++	00000
	c		+++++	++0++	0+000
R*a*	*a*	+0000	++000	00000	
	a	+0000	00000		

\+ = transformation to type of DNA.
0 = no evidence of transformation.
R*b* and R*d* compared at the same time for each DNA.

of DNA used. No type-specific cells were demonstrated in recipient R cell populations grown in anti-R antiserum broth without DNA.

The frequency of susceptible R*d* cells is again the highest, 100,000 cells containing susceptible cells in almost all samples. A slightly lower frequency of cells susceptible to transformation is seen in the experiments with R*b* populations. R*a* populations show the lowest frequency. Even 10 million cells do not uniformly contain cells susceptible to type *a* DNA. It is of great interest that, in both R*b* and R*d* populations, the frequency of the cells susceptible to DNA of type *b* is similar to the frequency of cells susceptible to DNA of type *c*.

Even though type specificity can be induced in smaller population sizes when the DNA is present during growth, a frequency pattern, characteristic of a type, has been demonstrated when the exposure is prolonged or limited to 15 minutes. In a given population the pattern of frequency of cells susceptible to 2 different DNA's has not been demonstrated to differ.

2. Use of DNA Controlling SM Resistance as a Marker.—The SM resistance DNA is a much more efficient marker than type-specific DNA for quantitating the frequency of cells susceptible to induction of heritable changes. Induced SM resistance is detected by demonstrating the capacity of the cells to form colonies in pour plate preparations containing SM (1000 μg. per ml.). The high degree of selective action of the SM permits the detection of all cells in which resistance has been induced.

The frequency of cells in R populations in which SM resistance can be induced has also been shown to be dependent on their type of origin. The influence of each type on the frequency pattern is of the same order of magnitude as that shown when the type-specific DNA's were used as markers. Table VI shows the size of the proportion of cells in which SM resistance can be induced in populations derived from 3 different types.

Young populations (5 hours old) of R*a*, R*b*, and R*d*, of approximately the same size, 6.5×10^8 to 7.3×10^8 cells per ml., were exposed for 15 minutes to SM resistance DNA

TABLE VI

Influence of Type of Origin on Proportion of Cells Susceptible to Induction of SM Resistance

Recipient cells	Population per ml. exposed to $DNAR_{a_{SM_{1000}}}$	Total Colonies per ml. of treated cells forming on SM agar	Proportion of treated cells with SM resistance
R*a*	7.3×10^8	5.0×10^0	$1:1.5 \times 10^8$
R*b*	6.8×10^8	1.6×10^3	$1:4.3 \times 10^5$
R*d*	6.5×10^8	4.6×10^3	$1:1.4 \times 10^5$

isolated from R*a* cells resistant to 1000 μg. SM per ml. The particular DNA controlling SM resistance, isolated from R*a* cells resistant to 1000 μg. of SM per ml., was chosen in order to include in the study transformation of R*a* cells in which susceptible cells are of lowest frequency; the selection of DNA made from resistant R*a* cells represented an attempt through the use of a homologous DNA to reduce to some degree the phenomenon of chance in transformation of R*a* populations in which the frequency of susceptible cells is so low. DNAse was then added to inactivate the DNA and the treated populations were examined for presence of cells which could form colonies in SM agar (1000 μg. per ml.). A number of population sizes prepared by diluting the treated cell suspensions in order to obtain an accurate count of SM-resistant colonies, were seeded into Levinthal agar, incubated 2 hours after hardening, and then exposed to the action of SM.

R*a* populations exhibit the lowest frequency, about 1 in 100 million; R*b*, 1 in 400,000; and R*d*, 1 in 100,000.

A more detailed comparison of young R*b* and R*d* cells (4 to 5½ hours) for their frequency of cells susceptible to induction of SM resistance is shown in Table VII. In this experiment each population size examined was exposed separately to SM resistance DNA isolated from type *b* cells resistant to 1000 μg. SM per ml. and the proportion of susceptible cells in each was studied. R*d* cells were exposed for 15 min-

utes to the DNA and R*b* cells for 30 minutes. In both R*b* and R*d* cultures the proportion of susceptible cells is the same for all populations studied except for the smallest one where chance plays a greater role. A higher frequency of cells susceptible to SM resistance is seen in all population sizes of R*d* even though the period of exposure to the DNA was shorter. However, this proportion is only a small part of the total cells exposed and the number of colonies forming in SM agar shows a high correlation with the size of the population exposed.

B. Influence of Concentration of DNA:

1. Type-Specific DNA's as Marker.—To explore the suggestion that all cells are equally sensitive if a sufficiently high concentration of DNA is present, the effect of varying population sizes and DNA concentrations in the same experiment was studied. The influence of these factors on the frequency

TABLE VII

Proportion of Varying Population Sizes of Rb and Rd Cells Susceptible to Induction of SM Resistance (1000 μg. per ml.)

Recipient cells	Population per ml. exposed to $DNAb_{SM1000}$ 1.2 μg. per ml.	Total colonies forming on SM agar 1000 μg. per ml. from 1 ml. treated cells	Proportion of treated cells resistant to SM
R*d*	2.1×10^{10}	2.7×10^{6}	$1:7.8 \times 10^{3}$
	7.2×10^{8}	1.1×10^{6}	$1:6.5 \times 10^{3}$
	8.5×10^{7}	9.8×10^{3}	$1:8.7 \times 10^{3}$
	8.5×10^{6}	7.2×10^{2}	$1:1.2 \times 10^{4}$
R*b*	4.5×10^{8}	3.1×10^{4}	$1:1.5 \times 10^{4}$
	5.3×10^{7}	1.9×10^{3}	$1:2.8 \times 10^{4}$
	5.3×10^{6}	1.7×10^{2}	$1:3.1 \times 10^{4}$
	5.3×10^{5}	1.0×10^{1}	$1:5.3 \times 10^{4}$

pattern was examined during growth and on the proportion of susceptible cells present initially, using induction of type-specificity as a tool.

Table VIII presents the results of varying population sizes of R*d* cells exposed to varying concentrations of DNA*d* and DNA*b* in the same experiment during growth. It is seen that the frequency pattern of induced type-specific cells does not differ with exposure to homologous and heterologous DNA. Within the limits of the conditions of the experiments the larger the initial population the smaller the concentration of DNA which induces the type specificity. The influence of incubation time on the demonstration of transformed cells is seen when the small seeding population sizes are exposed to low concentrations of DNA. In Table IX comparable results are shown when R*b* cells are exposed to DNA*b* and DNA*d*. These data suggest that a new type specificity can be induced in 10^6 per ml. populations with lower concentrations of DNA than are found effective in the smaller populations; this is probably made possible by an enhancement of opportunity for contact between the small number of DNA molecules and the larger number of susceptible cells.

TABLE VIII

Influence of Concentration of DNA on Smallest Population Size of Rd Showing Transformation to Types b and d during Growth

Transforming principle Type	DNA	Population size prior to DNA exposure during growth					
		1.0×10^6		1.0×10^4		1.0×10^2	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
	μg./ml.						
DNA*b*	1.3	+++		+++		+++	
	0.13	+++		+++		+++	
	0.013	+++		+++		000	+++
	0.0013	+++		000	++0	000	0++
	0.00013	+00		000	000		
DNA*d*	0.8	+++		+++		+++	
	0.08	+++		+++		+++	
	0.008	+++		+++		+++	
	0.0008	+++		+0+	+++	+00	+++
	0.00008	+++		000	000	000	000
	0.000008	+++		000	000		

Tests performed on same day.
Same DNA's as those used for R*b* cells (Table IX).
+ = transformation to type of DNA.
0 = no evidence of transformation.

TABLE IX

Influence of Concentration of DNA on Smallest Population Size of Rb Showing Transformation to Types b and d during Growth

Transforming principle Type	DNA	Population size prior to DNA exposure during growth					
		1.0×10^6		1.0×10^4		1.0×10^2	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
	μg./ml.						
DNA*b*	1.3	+++		+++		000	+++
	0.13	+++		++0	++0	000	+0+
	0.013	+++		+00	+00	000	0+0
	0.0013	00+	00+	000	000	000	000
DNA*d*	0.8	+++		+00	+++	000	+00
	0.08	+++		+0+	+0+	000	++0
	0.008	+++		000	000	000	000
	0.0008	000	000	000	000	000	000

Tests performed on same day.
Same DNA's as those used for R*d* cells (Table VIII).
+ = transformation to type of DNA.
0 = no evidence of transformation.

Experiments which examined the influence of concentration of DNA*c* on the proportion of cells in R*d* populations initially susceptible to change to type *c* are summarized in Table X.

Experiment I examines the influence of a 1,000-fold difference in concentration of DNA on the frequency of demonstrable transformation, as an index of the proportion of R*d* cells

susceptible to DNA*c* after only 15 minutes' exposure. The lower concentration used, 0.02 μg. per ml., represents 1000 times the minimal effective concentration of this product, therefore, 20 μg. per ml. represents 1 million times this value. Two equal samples of a 10^{-1} dilution of a 5½ hour culture of R*d* were exposed to the 2 concentrations of DNA*c* for 15 minutes. DNAse was then added, allowed 5 minutes to destroy the DNA and dilutions were made in 10-fold steps in anti-R serum broth to provide the populations listed.

From the data, it is seen that a 1000-fold increase in concentration of DNA*c* did not increase the proportion of cells in which type *c* was induced.

In Experiment II (Table X), equal samples of a 10^{-1} dilution of a 4½ hour culture of R*d* cells were exposed to each one of the 3 concentrations of DNA listed for 15 minutes before addition of DNAse which was allowed 5 minutes to destroy the DNA. The treated suspen-

TABLE X

Influence of Concentration of DNAc on Proportion of Rd Cells Susceptible to Induction of Type c during 15 Minute Exposure

Experiment No.	DNA	Organisms per ml. treated	Induction of type *c* in varying populations of treated cells			
			2.8×10^8	2.8×10^8 to 6.6×10^8	2.8×10^4 to 6.6×10^4	2.8×10^3 to 6.6×10^3
	μg./ml.					
I	20.0	1.4×10^7	+++++	+++++	+++++	00+00
	0.02	1.4×10^7	+++++	+++++	+++++	000+0
II	12.9	6.6×10^7		+++++	+++++	000+0
	0.13	6.6×10^7		+++++	+++++	00+00
	0.0013	6.6×10^7		+++++	0+00+	00000

\+ = transformation to type *c*.
0 = no evidence of transformation.

sions were diluted in anti-R serum broth to attain the population sizes which were examined for presence of type *c*. The results are comparable to those found during growth.

Again it would seem that when the element of chance for contact between susceptible cell and DNA molecule is greatly reduced by using large enough populations to provide enough susceptible cells, the frequency pattern of R*d* cells susceptible to DNA*c* has not been significantly increased by a 10,000-fold increase in the concentration of DNA*c*.

2. DNA Inducing SM Resistance as a Marker.—Induction of SM resistance was used to measure only the proportion of initially susceptible cells. For this purpose a comparison has been made of the influence of varying concentrations of DNA controlling SM resistance on the size of the initial proportion of a constant R*d* population in which SM resistance can be induced.

Equal samples of a single R*d* population, 10^8 cells per ml., were exposed to concentrations of DNAb_{SM1000} varying from 12 to 1.2×10^{-7} μg. per ml. for 15 minutes. The action was terminated by DNAse and the treated populations, appropriately diluted, were examined by

the method already described, for the proportion of the total population in which resistance was induced.

It is seen in Table XI that the greatest influence of concentration of DNA occurred in the low ranges. The increase in proportion of susceptible cells with the 10-fold increases in concentrations of DNA is believed to result from a greater opportunity for contact between the susceptible cells and the larger number of molecules of the DNA during the exposure time permitted. In the higher concentrations, 1.2×10^{1} to 1.2×10^{-1} μg. per ml., increase in concentration does not increase the proportion of cells in which resistance is induced. In the region of low DNA concentrations the increase in the proportion of cells in which SM resistance is induced by an approximately 10-fold degree as the concentration of DNA is increased 10 times, suggests that in this range the number of molecules of DNA is not adequate for interaction with all susceptible cells.

TABLE XI

Influence of Concentration of $DNAb_{SM1000}$ on Proportion of Sensitive Rd Cells in Which Resistance to 1000 μg. per ml. SM Is Induced

Concentration of DNA	No. of resistant colonies formed per 1.2×10^{8} sensitive cells	Proportion of total cells exposed in which resistance is induced
μg. per ml.		
1.2×10^{1}	124,000	1:1,000
1.2×10^{0}	118,000	1:1,050
1.2×10^{-1}	79,000	1:1,600
1.2×10^{-2}	42,000	1:3,000
1.2×10^{-3}	5,700	1:22,000
1.2×10^{-4}	720	1:170,000
1.2×10^{-5}	60	1:2,000,000
1.2×10^{-6}	5	1:25,000,000
1.2×10^{-7}	0	

The data suggest that when the number of molecules is sufficient to react with all susceptible cells, as would seem to be the case in the concentration 1.2×10^{-1} μg. per ml., higher concentrations do not significantly increase the size of the proportion of cells in which resistance is induced. Fig. 1 is a graphic representation of the data. The curve is linear only in the region of the lower concentrations.

The evidence offered by experiments which used the type-specific DNA's as heredity determinants and by those which used SM resistance DNA to induce heritable changes suggests that the small size of the proportion of cells susceptible to heritable change is not the result of insufficient concentrations of DNA. The concentrations used in all experiments which explored the factors controlling the size of the proportion of susceptible cells are well above those beyond which no increase in size of the proportion is obtained. Even when the DNA is increased 100 times the concentration at which the curve in Fig. 1 shows no significant additional rise, the size of the proportion of susceptible cells is still small, no greater than 1:1000.

C. Influence of Phase of Growth Cycle of Populations at Time of Exposure to DNA:

1. Use of Type-Specific DNA as Marker.—To explore this question the influence of growth of the total population on the frequency pattern of R*d* cells susceptible to DNA*c* was studied. Two experiments were carried out. In Table XII a 6 hour period of growth was studied and in Table XIII a 24 hour period.

In each experiment a 16 hour culture in Levinthal broth was diluted to obtain an 80 ml. volume of the initial populations per ml. listed. In the first experiment, Table XII, 2 popula-

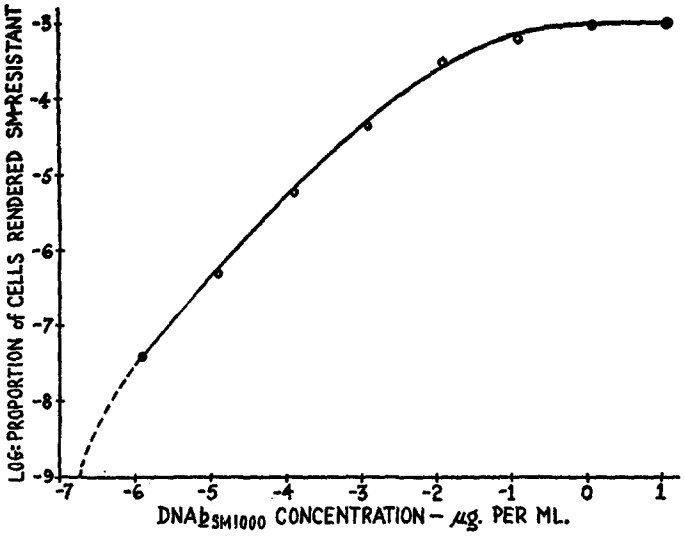

FIG. 1. Influence of concentration of $DNAb_{SM1000}$ on the proportion of R*d* cells in which streptomycin resistance (1000 μg. per ml.) can be induced.

tion sizes were followed for changes in frequency pattern of susceptible cells at intervals during growth for 6 hours. In the second experiment (Table XIII) the effect of growth of one population size in 2 different environments was examined: in Levinthal broth with anti-R *H. influenzae* antiserum and in Levinthal broth without antiserum.

In each experiment the initial populations were incubated and at 0 hours and at the intervals listed, samples were removed, diluted when necessary, and treated in the same way: 5 samples of each population size in Levinthal broth with anti-R antiserum were exposed to DNA*c* for 5 minutes at which time the DNA was destroyed by DNAse. The samples of treated populations were then incubated and examined for type *c* cells at 24 and 48 hours.

In Table XII, in the larger of the 2 population sizes, it is seen that the frequency of initially susceptible cells before incubation is greater than 1 in 100,000. After 4 hours of growth when the total population has increased 100-fold the frequency of susceptible cells is less than one in 10 million; instead of increasing the susceptible

TABLE XII

Frequency of Cells Initially Susceptible to DNAc in Two Rd Population Sizes during Growth for 6 Hours

Period of growth	9.3×10^5 per ml.			9.3×10^3 per ml.		
	Total population size per ml.	Population per ml. treated	Type *c* cells found	Total population size per ml.	Population per ml. treated	Type *c* cells found
hrs.						
0	9.3×10^5	9.3×10^5	+++++	9.3×10^3	9.3×10^3	00+00
2	2.1×10^6	2.1×10^6	+++++	2.8×10^4	2.8×10^4	00000
4	1.6×10^7	1.6×10^7	++0+0			
		1.6×10^6	+0000	2.9×10^5	2.9×10^5	00000
		1.6×10^5	00000			
6	1.2×10^8	1.2×10^6	+++++		7.4×10^6	+0000
		1.2×10^5	+++0+	7.4×10^6	7.4×10^5	00000
		1.2×10^4	00000			

\+ = transformation to Type *c*.
0 = no evidence of transformation.

TABLE XIII

Influence of Phase of Growth Cycle of Rd Cells at Time of Exposure to DNAc

Frequency of cells initially susceptible to DNAc at intervals during a 24 hour growth cycle in presence and absence of R antibody.

Period of growth	Levinthal broth			Levinthal broth + R antibody		
	Total population size per ml.	Population per ml. treated	Type *c* cells found	Total population size per ml.	Population per ml. treated	Type *c* cells found
hrs.						
0	6.0×10^5	6.0×10^5	+++++			+++++
		6.0×10^4	+0+0+	6.0×10^5		+0+0+
		6.0×10^3	0000+			0000+
2	5.1×10^6	5.1×10^6	000+0		3.7×10^6	00000
		5.1×10^5	00000	3.7×10^6	3.7×10^5	00000
		5.1×10^4	00000		3.7×10^4	00000
4	6.7×10^7	6.7×10^7	0+000		6.3×10^7	00000
		6.7×10^6	00000	6.3×10^7	6.3×10^6	00000
		6.7×10^5	00000		6.3×10^5	00000
6	8.0×10^8	8.0×10^6	+++++		9.1×10^6	+++++
		8.0×10^5	+++++	9.1×10^8	9.1×10^5	+++++
		8.0×10^4	+++++		9.1×10^4	+++++
8	8.3×10^8	8.3×10^5	+++++		7.5×10^5	+++++
		8.3×10^4	+++++	7.5×10^8	7.5×10^4	+++++
		8.3×10^3	0+0+0		7.5×10^3	000+0
		8.3×10^2	0+000		7.5×10^2	00+00
11	1.0×10^9	1.0×10^6	+0+++		7.2×10^5	+++++
		1.0×10^5	+++++	7.2×10^8	7.2×10^4	+++++
		1.0×10^4	+0++0		7.2×10^3	0+000
24	9.5×10^8	9.5×10^6	+++++			
		9.5×10^5	0000+			
		9.5×10^4	00000			

\+ = transformation to type *c*.
0 = no evidence of transformation.

cells appear to decrease. After growth for 6 hours, however, the initial frequency is again found. Before incubation of the smaller population, 9.3×10^3 organisms per ml., demonstration of any susceptible cells is a chance phenomenon. After 2 and 4 hours of growth their presence could not be demonstrated and at 6 hours, when the population has reached 7.4×10^6 organisms per ml., only a rare cell is susceptible. A comparable population size, 10^6 cells per ml. of the culture started from the larger population, 9.3×10^5 cells per ml., contains susceptible cells in all samples after growth for 6 hours. However, the larger population, after a comparable growth period, 6 hours, is apparently in the late logarithmic phase and has reached a density of 10^8 cells per ml. The smaller population size culture is apparently still in the most active logarithmic phase after 6 hours of growth.

When the effect of longer periods of growth of a population comparable to the larger of the 2 populations, is studied (Table XIII) both in the presence and absence of anti-R antiserum, increase in frequency of initially susceptible cells could not be demonstrated. The decrease in the frequency seen after 2 and 4 hours of growth is greater than can be accounted for by their dilution through increase in size of the population as a whole. Again within a 2 hour period following their low point there is an emergence of apparently new susceptible cells. There is no evidence of increase in frequency thereafter but rather there appears to be a slow decrease.

The data suggest that the combined influence of 2 factors condition the proportion of cells in which heritable changes can be induced; the phase of the growth cycle at the time of exposure to the DNA and the population density. It would seem that susceptible cells emerge late in the logarithmic phase when the population density is at least 10^8 cells per ml.; the highest frequency of susceptible cells is found in the late logarithmic phase or early stationary period. In the most rapid growth period when the population as a whole is reproducing at the highest rate the lowest frequency of susceptible cells is found.

The presence of "R" *H. influenzae* antibody has not been shown to influence the frequency of cells susceptible to induction of type *c*.

2. SM Resistance DNA as Genetic Marker—Experiments using SM resistance DNA as a heredity determinant for exploring the influence of population changes during growth, on the frequency of susceptible cells, have yielded results which parallel those obtained from the use of a type-specific DNA as a marker. Because of the efficiency of SM as a selective tool detection of all cells in which SM resistance is induced becomes possible and quantitative data are made available.

The influence of population changes during growth of R*d* cells on the proportion of cells susceptible to induction of streptomycin resistance is shown in Table XIV.

From a single 15 hour old culture of actively growing cells the 2 population sizes listed were seeded in Levinthal broth. At 0 hours and after each specified interval during growth at 37°C., samples were removed from the original cultures and the following procedures were carried out. Each sample was exposed for 15 minutes to a constant concentration (1.2

μg. per ml.) of DNA controlling streptomycin resistance; subsequently DNAse was added. The treated suspension was then examined for cells which could form colonies in pour plate preparations containing 1000 μg. SM per ml. after an incubation period of 48 hours.

The proportion of cells in which resistance was induced is listed in Table XIV for each interval; this represents the ratio of the number of colonies which form in streptomycin agar to the population per milliliter seeded. From these ratios it is seen that the susceptible cells present initially do not increase in number during the early growth period; instead they decrease 1000-fold when the total population has increased 100-fold. In the larger population susceptible cells emerge in large numbers at the 3 hour interval. Their peak incidence is reached within 1 hour; this same proportion remains relatively stationary during the next 4 hours but by 24 hours has declined again.

TABLE XIV

Proportion of SM-Resistant Cells in Samples of Rd Populations Treated for 15 Minutes with $DNAb_{SM1000}$ at Intervals Following Initiation of Growth

Period of growth	Initial population size			
	6.1×10^6		6.1×10^8	
	Cells per ml. treated	Proportion SM-resistant cells	Cells per ml. treated	Proportion SM-resistant cells
hrs.				
0	6.1×10^6	$1:4.7 \times 10^3$	6.1×10^5	$1:4.4 \times 10^3$
1	2.6×10^7	$1:1.0 \times 10^4$	2.6×10^6	$1:5.7 \times 10^4$
2	1.3×10^8	$1:1.6 \times 10^6$	1.4×10^7	—
3	4.1×10^8	$1:3.4 \times 10^3$	5.4×10^7	$1:4.5 \times 10^6$
4	9.3×10^8	$1:1.3 \times 10^3$	1.6×10^8	$1:3.1 \times 10^4$
5	1.1×10^9	$1:2.1 \times 10^3$	4.9×10^8	$1:2.0 \times 10^3$
6	1.3×10^9	$1:3.1 \times 10^3$	1.1×10^9	$1:1.4 \times 10^3$
7	1.3×10^9	$1:3.3 \times 10^3$	1.3×10^9	$1:2.6 \times 10^3$
8	1.7×10^9	$1:4.0 \times 10^3$	1.4×10^9	$1:4.4 \times 10^3$
24	3.6×10^8	$1:1.8 \times 10^6$	6.0×10^8	$1:1.5 \times 10^6$

— = no resistant colonies formed.

The highest frequency is seen after 4 hours' growth when the population appears to be in the early stationary phase. In the smaller population susceptible cells are difficult to demonstrate after 2 hours' growth; and the top frequency is reached more slowly.

When R*b* populations are studied in this same way comparable results are found (Table XV). The frequency of susceptible cells is lower than in R*d* at all periods of the growth cycle, as is to be expected. In both populations studied the period of apparent disappearance is longer than in R*d* populations; emergence of susceptible cells occurred between 4 and 5 hours in the larger one and between 6 and 7 in the smaller population. In the smaller population it was not possible to demonstrate susceptible cells during the period from 0 hour through 6 hours. Again the range 2 to 4×10^8 cells is reached in these 2 populations of R*b* cells in the active growth phase at the time when susceptible cells appear.

From the results of these experiments it is seen that when the initial culture is so

prepared that the population has reached a density greater than 2 to 4 × 10^8 cells per ml. in the late logarithmic phase, induction of the heritable change can be regularly induced in about 1 cell of each 1000 R*d* cells treated and in about 1 cell in each 10,000 R*b* cells treated. Examination of these cultures during growth shows that during the most active logarithmic phase the proportion of cells in which SM resistance can be induced decreases 1000-fold as the population as a whole increases 100 times. After this apparent disappearance susceptible cells emerge as the end of the logarithmic period and a population density of 2 to 4 × 10^8 cells per ml. are reached. The peak frequency of cells in which SM resistance can be induced occurs when the population reaches the early stationary phase. The proportion of susceptible cells then remains virtually unchanged for a few hours but after growth for 24 hours it decreases by at least a 1000-fold.

TABLE XV

Proportion of SM-Resistant Cells in Samples of Rb Populations Treated for 15 Minutes with $DNAb_{SM1000}$ at Intervals Following Initiation of Growth

Period of growth	Initial population size			
	1.6 × 10^6		1.6 × 10^5	
	Cells per ml. treated	Proportion SM-resistant cells	Cells per ml. treated	Proportion SM-resistant cells
hrs.				
0	1.6 × 10^6	1:5.5 × 10^4	1.6 × 10^5	—
1	4.7 × 10^6	—	3.9 × 10^5	—
2	1.5 × 10^7	—	1.4 × 10^6	—
3	3.5 × 10^7	—	5.3 × 10^6	—
4	1.6 × 10^8	—	1.8 × 10^7	—
5	3.3 × 10^8	1:1.3 × 10^8	4.1 × 10^7	—
6	7.0 × 10^8	1:1.6 × 10^4	1.6 × 10^8	—
7	9.3 × 10^8	1:1.8 × 10^4	4.3 × 10^8	1:2.1 × 10^5
8	1.2 × 10^9	1:3.5 × 10^4	1.6 × 10^9	1:2.9 × 10^4
24	1.1 × 10^9	—	1.5 × 10^9	—

— = no resistant colonies formed.

The data suggest that the susceptible cells either fail to reproduce their kind or die more rapidly than the rest of the population unless they are changed by the stimulus of an appropriate DNA.

II. Different Heritable Traits Are Induced through the Same Cells of a Population

The earlier suggestion of similar frequency patterns of induced type-specific cells in R*d* and R*b* populations exposed to different type-specific DNA's and the demonstration of predictable fluctuations in their frequency during growth of the total populations, stimulated a study to determine whether all heritable changes induced in a population by DNA's take place in the same cells.

Two groups of experiments were designed for this purpose. The first compared the frequency patterns of cells susceptible to different type-specific

DNA's, and the frequency of cells in which type specificity can be induced with those in which SM resistance can be induced. The second examined for evidence of competition between type-specific DNA's and exclusion of one DNA by another.

A. Comparison of Frequency Pattern of Cells Susceptible to Different DNA's:

Comparison of Frequency of Cells Susceptible to Different Type-Specific DNA's.—

In a given population the cells susceptible to each of the heterologous DNA's exhibit comparable frequency patterns. In Table XVI are shown the results of an experiment in

TABLE XVI

Comparison of Rb and Rd Populations for Frequency Patterns of Cells Susceptible to Induction of Types b, c, and d

R*b* and R*d* cells exposed to DNA*b*, DNA*c*, and DNA*d* for 30 min.

Recipient cells	Organisms per ml. treated	DNA type	Induction of type specificity corresponding to DNA type in population listed			
			4.4×10^6	4.4×10^5 to 5.3×10^5	4.4×10^4 to 5.3×10^4	4.4×10^3 to 5.3×10^3
R*b*	4.4×10^8	*b*	+++++	+++++	0++++	0000+
		c	+++++	+++++	+0+00	00000
		d	+++++	+++++	+++++	00000
R*d*	5.3×10^8	*b*		+++++	+++++	00000
		c		+++++	+++++	0+00+
		d		+++++	+++++	+0+++

\+ = transformation to type corresponding to DNA type.
0 = no transformation demonstrated.

which equal populations of R*b* and R*d* cells were exposed simultaneously to 3 different DNA's in the same concentration (0.6 to 0.7 μg. per ml.) for 30 minutes before their destruction by DNAse. The treated suspensions were diluted to the population sizes listed and examined for the smallest number of organisms in which uniform induction of type specificity occurs. It is seen that in both R*d* and R*b* populations the cells susceptible to change to new types by DNA*b*, DNA*c*, and DNA*d* exhibit the same frequency pattern.

These data raise the possibility that in R*b* and R*d* populations the same cell is susceptible to each of the 3 type-specific DNA's since cells susceptible to induction of each type specificity exhibit similar frequency patterns.

Comparison of Frequency Patterns of Cells Susceptible to Type-Specific DNA's and to DNA_{SM1000}.—The results of 5 experiments which explored this question are shown in Table XVII.

In Experiments I and II, samples of 5-hour-old R*d* cultures were exposed separately to DNA*d* (1.4 μg. per ml.) or $DNAR_{dSM1000}$ (1.4 μg. per ml.) for 15 minutes. In Experiments

III, IV, and V, R*b* and R*d* populations 4½ to 5 hours old, were exposed to DNAb_{SM1000} (1.2 μg. per ml.) for 15 minutes. In these experiments (III, IV, and V) the cells were exposed to a single DNA extract, DNAb_{SM1000}, and then examined for frequency of cells in which the 2 different heritable changes had been induced, the type *b* trait and SM resistance. Following inactivation of the DNA by DNAse the treated populations were appropriately diluted and 5 samples of each population size listed examined for induction of appropriate type specificity, resistance to SM, or both. For demonstration of induction of SM resistance the treated populations were seeded in 2 ml. of Levinthal broth, incubated 2 hours, and then SM added to give a concentration of 1000 μg. per ml. Treated populations examined for induction of type-specific traits were grown in Levinthal broth containing anti-R antiserum.

TABLE XVII

Comparison of Frequency Patterns of Cells Susceptible to Induction of Type Specificity and SM Resistance

Experiment	Recipient cells	DNA used for treatment	Heritable induced change	Frequency of induced change in varying populations of treated cells				
				0.5 to 1.0 × 10^7	0.5 to 1.0 × 10^6	0.5 to 1.0 × 10^5	0.5 to 1.0 × 10^4	0.5 to 1.0 × 10^3
I	R*d*	S*d*	*d**		+++++	+++++	00+++	000++
		Rd_{SM1000}	SM1000		+++++	+++++	0++0+	00000
II	R*d*	S*d*	*d*		+++++	+++++	+++++	0000+
		Rd_{SM1000}	SM1000		+++++	+++++	+++0+	+0000
III	R*d*	Sb_{SM1000}	*b*		+++++	+++++	++00+	00000
			SM1000		+++++	+++++	+++++	00000
IV	R*d*	Sb_{SM1000}	*b*		+++++	0++++	00000	00000
			SM1000		+++++	+++++	+++++	+000+
	R*b*	Sb_{SM1000}	*b*	+++++	+++++	+000+	00000	
			SM1000	+++++	+++++	+++++	0+00+	
V	R*d*	Sb_{SM1000}	*b*		+++++	+++++	++0++	00+00
			SM1000		+++++	+++++	+++++	00++0
	R*b*	Sb_{SM1000}	*b*	+++++	000+0	00000		
			SM1000	+++++	+++++	00000		

SM1000 = resistance to 1000 μg. streptomycin per ml.

* Small letter refers to type specificity induced.

In each experiment the data are interpreted as showing no real difference in the frequency pattern of cells susceptible to induction of type-specificity and streptomycin resistance.

B. Competition between Type-Specific DNA's:

To explore this question, mixtures of different type-specific DNA's in varying proportions of the total DNA were examined for evidence of competition for the same susceptible cells in R*d* populations. The results are shown in Table XVIII.

Cultures after 5 to 6 hours' growth in Levinthal broth were seeded in populations of 1×10^6 (known to contain susceptible cells) into Levinthal broth containing the 2 DNA's in the

different proportions listed. After the populations had grown in these environments for 18 to 24 hours they were examined for the proportion of cells of each of the 2 induced types. This proportion, which is only an approximation, was estimated by 2 methods. (1) A 2 mm. loopful of the resulting culture was exposed in separate Neufeld preparations to each of the type-specific antibodies, corresponding to the types of origin of the 2 DNA's used as genetic markers, and examined for capsular swelling. (2) A 2 mm. loopful of the culture was seeded on the surface of Levinthal agar—in a manner to obtain isolated colonies; after 18 hours' incubation a number of the iridescent colonies were selected for study of type.

The data show that the proportion of each induced type correlates well with the proportion of the corresponding DNA type. In each case the majority of type-specific cells exhibit the type specificity of the DNA which contributes the major portion of the total DNA. When DNA*b* and DNA*c* are present in equal quantities, an approximately equal proportion of each of the 2 induced types is found. However, when DNA*a* and DNA*b* are mixed in equal quantities the exposed population shows type *b* in excess. The reason for the dominance of DNA*b* is unknown.

TABLE XVIII

Rd Cells (1.0×10^6) Exposed Simultaneously during Growth to Mixture of Two Type-Specific DNA's of Varying Proportions

Experiment	Transforming agents			Transformed type-specific cells		
	Type and per cent DNA used			Approximate per cent of type observed		
	a	*b*	*c*	*a*	*b*	*c*
I	75	25		80	20	
	50	50		16	84	
	25	75			100	
II	98	2		90–99	1–10	
	8	92			100	
III		75	25		82	18
		50	50		48	52
		25	75		20	80
IV		96	4		>99	<1
		5	95		<1	>99

These data, together with the results of experiments which show that in a given population the cells susceptible to different heterologous DNA's exhibit the same frequency pattern, suggest that DNA's *a*, *b*, and *c* compete for the same cell.

C. Exclusion of One DNA by Another:

Exclusion of Action of One Type-Specific DNA by a Different Type-Specific DNA.—A second approach to the question of whether the induction of each type specificity occurs through the same cell, examined the capacity of DNA's *d* and *c* and DNA's *b* and *c* to exclude the action of each

other. Both R*b* and R*d* populations were used for this purpose. The results are summarized in Table XIX.

Five-hour-old populations (0.2 ml. of R*b* or R*d*) were seeded into 1.5 ml. of neopeptone broth containing the appropriate concentration of the first DNA; 15 minutes later the cells were exposed to the second DNA for 15 minutes. DNAse (0.2 ml. of 20 μg. per ml.) was then added, allowing 10 minutes for inactivation of the DNA's. The concentrations of the second DNA's were so chosen that they represented 3 different ratios between the first and second DNA, 10:1, 100:1, and 1000:1. The suspensions so treated were seeded in 0.1 ml. quantities

TABLE XIX

Exclusion of DNA of One Type by Prior Exposure for 15 Minutes to High Concentration of DNA of Another Type

Recipient cells	Treatment of R cells		Approximate per cent of induced types		
	DNA's First / Second	DNA	*b*	*c*	*d*
		μg./ml.			
R*b*	*d*	**8.8**			
	c	0.85		<1	>99
		0.085		0	100
		0.008		0	100
	c	**12.8**			
	d	1.2		>99	<1
		0.12		100	0
		0.012		100	0
R*d*	*b*	**6.3**			
	c	0.65	94	6	
		0.065	>99	<1	
		0.006	100	0	
	c	**12.8**			
	b	1.2	5	95	
		0.12	1	99	
		0.012	0	100	

in duplicate into Levinthal broth containing anti-R *H. influenzae* antibody and in addition into the same environment plus type-specific antibody corresponding to the type of DNA to which the cultures were first exposed. The latter environment aimed to remove by agglutination the cells changed by the first DNA in order to detect more easily any cells changed by the second DNA. After 18 to 24 hours of growth each treated population was examined for capsular swelling by type-specific antibodies corresponding to the types of origin of both the 1st and 2nd DNA to which it had been exposed. In addition a representative population was seeded on Levinthal agar and after growth for 18 hours 50 iridescent colonies were selected and their type specificity was determined. The treated population was also subcultured in an environment containing type-specific antibody corresponding to the first DNA in order to enhance the detection of type-specific cells induced by the second DNA.

It is of great interest that exposure of either R*d* or R*b* populations to one type-specific DNA in a concentration 1000 times greater than the second type of DNA,

added 15 minutes later, can completely prevent the induction of heritable changes by the second. The first type of DNA in a concentration 10 times greater than the second does not completely prevent the action of the second DNA; approximately 5 to 6 per cent of the types induced in R*d* populations are of the type of the second DNA introduced. In R*b* populations under comparable circumstances less than 1 per cent of the induced types exhibit the type specificity of the second type DNA. When used alone each of the concentrations of the second DNA is sufficient to induce its type specificity with predictable uniformity in populations equal to those used here. Whether this exclusion phenomenon is merely a blocking action preventing the interaction of the second DNA with the specific cell component, or whether the interference with the action of the second DNA represents a specific competition for the same portion of a molecule, cannot be conclusively answered from these data. However, the importance of the size of the ratio between the 1st and 2nd DNA in order to completely exclude the 2nd DNA suggests that this exclusion phenomenon results from utilization of attachment sites by the first DNA which are also needed for the second DNA. Since in a given population frequency pattern of cells susceptible to different DNA's is not significantly different, these data suggest that in R*d* populations induction of types *c* and *d* and in R*b* populations induction of types *b* and *c* occur through changes in the same cells.

The findings indicate that under appropriate circumstances susceptible cells can be made insusceptible to a second DNA.

Exclusion of DNARd$_{SM1000}$ by Prior Exposure to a Type-Specific DNA.—Type-specific DNA's under appropriate circumstances can completely prevent the induction of SM resistance. This more quantitative tool confirms the results of experiments which suggest that one type-specific DNA can exclude the action of a different type-specific DNA which otherwise can induce a new heritable type-specific change.

In Table XX are summarized the results obtained in 3 different experiments in which R*d* populations (10^8 cells of a 4 to 5 hour old culture) were exposed first to either the homologous or a heterologous type-specific DNA and after a 15 to 150 minute interval to an equal concentration (0.5 to 1.0 μg. per ml.) of the DNA controlling streptomycin resistance, DNAR*d*$_{SM1000}$. Exposure to the latter continued for 15 minutes in Experiments I and II, and in Experiment III for 30 minutes, before inactivation of the DNA's by DNAse. The treated populations were diluted in broth so that pour plate preparations contained the populations listed. After 2 hours' incubation of the preparation to permit maximal expression of the induced trait, agar containing SM (2000 μg. per ml.) was layered in approximately equal volume. The pour plate preparations were then incubated for 48 hours and examined for the number of colonies formed in the presence of 1000 μg. of SM per ml.

When compared with the same population of the control, cells exposed only to streptomycin resistance DNA, a significant reduction of the number of streptomycin-resistant colonies has resulted from previous exposure to a type-specific DNA. The cells in which streptomycin resistance has been induced have been reduced to as little as one-sixth of those in the controls. Exposure

to the first DNA for a period as long as 150 minutes did not increase the degree of exclusion produced by a type-specific DNA in a concentration equal to the streptomycin resistance DNA.

Experiments using R*b* populations as the recipient cells yielded comparable results as shown in Table XXI.

To determine whether it is possible to exclude completely the action of the streptomycin resistance DNA by the prior action of type-specific DNA the effect of increasing the concentration of the type-specific DNA to 10, 100, and 1000 times that of the DNARd_{SM1000} was studied. The results of these experiments are shown in Table XXII.

TABLE XX

Reduction of Proportion of Rd Cells Susceptible to DNA Rd$_{SM1000}$ by Prior Exposure to DNAd or DNAb in Equal Concentrations

Experiment No.	Treatment of cells			Examination of treated cells for resistance to 1000 μg. per ml. SM		
	DNA's First	DNA's Second	Control single DNA	Period of exposure	Population size examined	No. resistant colonies	Reduction by first DNA
				min.			*per cent*
I	*d*			30			
	Rd$_{SM1000}$			15	7.3×10^5	96	68
			Rd$_{SM1000}$	15		299	
II	*b*			30			
	Rd$_{SM1000}$			15	4.3×10^5	14	83
			Rd$_{SM1000}$	15		83	
III	*d*			150			
	Rd$_{SM1000}$			30	1.2×10^6	7	74
			Rd$_{SM1000}$	30		27	

It is seen that in 2 different strains, R*d* and R*b*, of population sizes sure to contain susceptible cells, induction of SM resistance by DNARd_{SM1000} was not demonstrated if the cells had been exposed previously for only 15 minutes to either DNA*d* or DNA*c* in a 1000-times greater concentration. In a population of comparable size but not previously exposed to DNA, the lowest concentration of DNARd_{SM1000} used, judging from the controls, would have been expected to induce streptomycin resistance in approximately 2000 cells.

The competition among type-specific DNA's for the same cells, the virtually complete exclusion of one DNA by another and the demonstration that in a given population, the cells susceptible to each DNA exhibit the same frequency pattern are interpreted as evidence that the same cells in the *H. influenzae* sys-

TABLE XXI

Reduction of Proportion of Rb Cells Susceptible to DNARd_{SM1000} by Prior Exposure to DNAd or DNAb in Equal Concentrations

Experiment No.	Treatment of cells			Examination of treated cells for resistance to 1000 μg. per ml. SM		
	DNA's First Second	Control single DNA	Period of exposure	Population size examined	No. resistant colonies	Reduction by First DNA
			min.			*per cent*
I	*b*		90			
	Rd_{SM1000}		30	4.4×10^8	6	85
		Rd_{SM1000}	30		41	
II	*d*		30			
	Rd_{SM1000}		15	4.7×10^8	9	74
		Rd_{SM1000}	15		35	
III	*b*		150			
	Rd_{SM1000}		30	8.5×10^8	10	81
		Rd_{SM1000}	30		54	

TABLE XXII

Prevention of Induction of SM Resistance by DNARd_{SM1000} by Previous Treatment with Type-Specific DNA's for 15 Minutes

R cells	Experiment No.	Treatment of R cells		No. of SM-resistant colonies in 5×10^7 treated cells		Reduction by first DNA
		DNA's First Second	DNA	DNARd_{SM1000} only	Type-specific DNA prior to DNARd_{SM1000}	
			μg./ml.			*per cent*
R*d*	I	*d*	**8.0**			
		Rd_{SM1000}	0.012	13,700	16	99.9
R*d*	II	*c*	**12.9**			
		Rd_{SM1000}	1.2	5,200	80	98.5
			0.12	5,100	20	99.6
			0.012	2,300	0	100
R*b*	III	*c*	**12.9**			
		Rd_{SM1000}	1.2	2,830	40	98.6
			0.12	2,310	10	99.6
			0.012	2,100	0	100

tem are susceptible to each of the DNA's and suggest that these cells have a special role.

To determine to what extent interference or blocking of the action of DNARd_{SM1000} by a previous exposure to a type-specific DNA represents a

non-specific DNA phenomenon, calf thymus DNA (TNA)[1] was examined for its blocking effect. The results of 3 experiments designed for this purpose are listed in Table XXIII.

These experiments were duplicates of those outlined in Table XXII with the exceptions that R*d* populations were used in all 3 examinations and TNA was substituted for the type-specific DNA's. It is seen that a 1000-fold excess concentration of TNA did not completely exclude the action of DNARd_{SM1000}. Moreover, when the TNA concentration was only 10 times that of the DNA inducing SM resistance no interference was demonstrated. If real interference has been produced by TNA when

TABLE XXIII

Influence of Calf Thymus DNA (TNA) on Induction of SM Resistance in Rd Populations by DNA Rd_{SM1000}

Experiment No.	Treatment of R*d* cells		No. SM-resistant colonies per 9.0×10^7 cells		Reduction by TNA
	DNA's First Second	DNA	DNARd_{SM1000} only	TNA prior to DNARd_{SM1000}	
		μg./ml.			*per cent*
I	TNA	12.0			
	Rd_{SM1000}	1.2	11,300	14,700	0
		0.012	2,100	1,000	52.4
II	TNA	14.4			
	Rd_{SM1000}	1.4	6,200	6,000	0
		0.14	4,400	2,700	38.6
		0.014	910	460	49.5
III	TNA	14.4			
	Rd_{SM1000}	1.4	9,450	11,600	0
		0.14	7,300	6,350	0*
		0.014	2,250	800	64.5

* Difference known to be within experimental error.

the ratio to the second DNA is 1000:1, it is only about one-half the degree which has been demonstrated for type-specific DNA's.

DISCUSSION

Induction of heritable changes has been demonstrated in only a small proportion of the total population exposed to the action of DNA's. The crucial question is whether the other cells are insusceptible or whether the reaction with them fails to occur for some reason.

In an attempt to explore this question an effort was made to induce heritable changes in the majority of the cells exposed by employing a number of factors

[1] TNA prepared by Dr. Stephen Zamenhof by a process used previously for *H. influenzae* DNA (11).

which might influence the size of the proportion of susceptible cells. Increase in the concentration of DNA seemed one of the most promising. When comparable bacterial populations are exposed to concentrations of DNA ranging from 10^{-6} μg. per ml. to 12 μg. per ml. in 10-fold increments, the proportion of cells in which changes can be induced becomes larger with increase in DNA concentration until a concentration of 10^{-1} μg. per ml. is reached. The curve is linear in the lower concentration range. However, a 100-fold increase beyond 10^{-1} μg. per ml. does not yield a significantly higher proportion of susceptible cells. This raises the question whether 10^{-1} μg. per ml. represents the concentration which contains enough molecules of the DNA employed to react with all of the susceptible cells under the conditions of the experiment (11).

The specific type of origin of *H. influenzae* as it occurs in nature endows a population with some trait which determines the frequency of susceptible cells; cells derived from type *d* show the highest incidence, 1 in 1000, and those from type *a* the lowest, 1 in 10 million; this trait is an inherited one and appears to be uninfluenced by repeated changes to heterologous type.

The phase of the growth cycle of a recipient population together with the density reached exert an important influence on the size of the proportion of a population which is susceptible to heritable change. During the most active logarithmic period, when the population as a whole has increased 100 times there is a marked decrease in frequency of susceptible cells. At the end of the logarithmic phase when the population density reaches 2 to 4 $\times$ 10^8 cells per ml., apparently new susceptible cells emerge. The peak is reached in the early stationary phase; thereafter there is a gradual decrease from 1:1000 to 1:1,000,000 after 24 hours of growth.

Additional evidence in favor of susceptibility of only a small proportion is the influence of population size on the predictable uniformity with which heritable changes can be induced. It has been shown repeatedly that a comparably small proportion is susceptible whether a single large population is exposed to DNA and diluted to obtain the populations to be examined or whether each fractional population size is exposed individually to the DNA. The data in Tables VIII and IX show that in the induction of type specificity, the larger the population size exposed, the lower the concentration of DNA which induced type specificity, and the higher the concentration of DNA the smaller the population size in which a new type was induced. These results suggest that opportunity for interaction between susceptible cells and the DNA molecules may play an important role in determining whether transformation occurs.

Preliminary results of experiments with the strains studied have failed to demonstrate the liberation of DNAse that might act as a limiting factor on the proportion of cells in which heritable changes can be induced (11).

It is conceivable that failure to change more than a small proportion may

represent interference by other DNA's present. Any DNA solution isolated from cells can be supposed to be made up of many heredity determinants; the genetic marker used in any transformation experiment is only a small part of the total and therefore may be interfered with by the rest of the DNA's. In this case a larger proportion of cells susceptible to a given genetic change can be anticipated in the absence of such interfering DNA's.

The evidence presented suggests that a number of different type-specific DNA's and the DNA controlling SM resistance induce their changes in the same cells.

In a given population the cells susceptible to different type-specific DNA's and the DNA inducing SM resistance show similar frequencies. The same factors influence the size of the small proportion of susceptible cells and to a comparable degree. Support of these suggestions that the different heritable changes take place in the same cells is provided by evidence that different type-specific DNA's compete for the same cell and that type-specific DNA's in appropriate concentrations can prevent the action of a second DNA added 15 minutes later, either a different type-specific DNA or the DNA controlling SM resistance. The data presented indicate that complete prevention of the action of the second DNA appears to be possible only when the ratio of the concentrations of the first and second DNA is of the order of 1000:1. However, the degree of exclusion approaches 100 per cent when the ratio is 100:1 and is over 90 per cent when the ratio is 10:1.

Examination of a totally unrelated DNA, thymus nucleic acid (TNA), for its capacity to interfere with induction of SM resistance by DNARd_{SM1000} failed to demonstrate complete exclusion even on exposure to a 1000-fold excess concentration of TNA. The degree of interference was approximately one-half of that produced by exposure to type-specific DNA's. Moreover, in contrast to the exclusion by specific DNA's which can induce heritable traits no exclusion was demonstrated when the TNA concentration is only 10 times greater than that of the second DNA. The results suggest that the TNA interferes through a mechanism which differs from the process by which complete excluclusion is accomplished when a population is exposed first to a DNA which can induce heritable traits.

Several sources of evidence lend support to the hypothesis that the cells which are susceptible to induction of heritable change are specialized cells. The same cells appear to be susceptible to all DNA's studied, different type-specific ones and the DNA inducing SM resistance. The size of the proportion of susceptible cells is controlled by some property characteristic of the type of origin and is inherited unchanged after repeated heterologous transformations. Predictable fluctuations in frequency occur during phases of the growth cycle but there is no evidence that susceptible cells reproduce their kind in an environment which is optimal for other members of the population.

While in a given population it has not been possible to induce heritable changes in more than 1 per 1000 cells at any one time, each cell of that population appears to produce a clone containing relatively the same proportion of susceptible cells.

SUMMARY

In *H. influenzae* the highly specific desoxyribonucleic acids (DNA's) which play the role of heredity determinants of type specificity and SM resistance, have induced these traits in only a small proportion of the population exposed to their action. The evidence suggests that this small proportion, "the susceptible cells" possess a property or substance needed by the DNA in order to induce an heritable change.

The size of the small proportion of susceptible cells can be influenced significantly by a number of factors; when all the factors now to be listed are operating the frequency has not exceeded 1:1000.

The Type of Origin of Recipient Cells.—Type *a* exhibits the lowest frequency, about 1:10,000,000, and type *d* the highest, approximately 1 per 1000 cells exposed. This type-specific property which controls the frequency of susceptible cells is an inherited trait; repeated change to a heterologous type shows no influence on the incidence of these cells.

Concentration of DNA.—Within certain limits increase in the concentration of DNA controlling streptomycin resistance can increase the size of the proportion of cells in which streptomycin resistance can be induced. However, increases in concentrations greater than 10^{-1} μg. per ml. have not induced streptomycin resistance in a higher proportion of cells.

Phase of Growth Cycle.—Predictable fluctuations in frequency of induced heritable changes have been demonstrated in both R*b* and R*d* populations during growth. There is no evidence that susceptible cells reproduce their kind; they emerge in all experiments when the population reaches the end of the logarithmic period and a density of 2 to 4 $\times$ 10^8 cells per ml. In the early logarithmic phase it is difficult to demonstrate the presence of susceptible cells. The peak frequency of susceptible cells occurs in the early stationary phase of the growth cycle. Thereafter, the decline in frequency is a gradual one.

The data suggest that in a given population the same cells are susceptible to a number of different type-specific DNA's and the DNA controlling SM resistance.

Comparison of Frequency of Cells Susceptible to Different DNA's.—In a given population the frequency of cells susceptible to different type-specific DNA's and the DNA controlling streptomycin resistance is not significantly different.

Competition between Type-Specific DNA's.—The data suggest that DNA's of types *a*, *b*, and *c* compete for the same cells in R*d* populations. When R*d*

populations are exposed simultaneously to 2 of these 3 DNA's in different concentrations the proportion which each type contributes to the total type-specific cells induced is closely correlated with the concentration of the corresponding DNA.

Exclusion of DNA's.—Induction of one type specificity or streptomycin resistance can be completely prevented in a population containing susceptible cells by previous exposure for 15 minutes to a 1000-fold higher concentration of another type-specific DNA.

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