DETERMINATION OF INHERITED TRAITS OF H. INFLUENZAE BY DESOXYRIBONUCLEIC ACID FRACTIONS ISOLATED FROM TYPE-SPECIFIC CELLS*

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Following the discovery of Avery, MacLeod, and McCarty (1), that highly specific desoxyribonucleic acids direct the inherited characteristics of pneumococci, cytochemists have made a number of contributions. One of the most important of these has been reported by Zamenhof and Chargaff (2), who suggest that all cells are equipped with delicately balanced desoxyribonucleic acid systems which act as heritable units or genes. According to this conception these systems include 3 components: (*a*) mixtures of desoxyribonucleic acids, a species-specific one, and many others controlling functions common to most cells, (*b*) specific desoxyribonucleases for depolymerization of the desoxyribonucleic acids, and (*c*) highly specific inhibitors which keep the enzymes in check.

The facts learned from the bacterial world suggest that when cells function abnormally as a result of mutation the character of their desoxyribonucleic acids differs from that of the parent cells, and it is this specific alteration which is responsible for their change in function. The far reaching implications of these statements in the field of genetics, virus, and perhaps of cancer research rest basically upon the proof that highly specific desoxyribonucleic acids (or their complexes) of bacteria function as genes. Convincing data which show that this mechanism operates in pneumococcus populations have been reported (1). Whether there is proof that virulence and type specificity of members of other bacterial families are controlled by desoxyribonucleic acid is a crucial question. Boivin *et al.* (3) in 1945 reported transformation of one strain of *Escherichia coli* by a cell-free filtrate which contained desoxyribonucleic acid. Out of 256 trials Weil and Binder (4) in 1947 reported one transformation of a *Shigella* by a filtrate of unknown composition.

The present investigation presents proof that transformation of R forms of *H. influenzae* into the original or into new types can be directed by a specific desoxyribonucleic acid–containing fraction isolated from the type desired. The R cells susceptible to this action appear to arise by mutation. When susceptible

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cells are present initially in the population exposed to the essential reagent, desoxyribonucleic acid fraction, transformation takes place virtually immediately.

The first example of induced changes in inherited traits of bacteria was demonstrated *in vivo* by Griffith (5) in 1928. Avirulent, non-type–specific cells derived from a virulent type–specific pneumococcus were changed into the type of origin or a new type by exposing them in the subcutaneous tissues of mice to heat-killed type–specific cells of the original or new type. The transformed cells produced local infections or invaded the blood and in some mice caused death. Dawson and Sia (6) and Alloway (7) demonstrated a comparable transformation *in vitro*.

In 1944 Avery, MacLeod, and McCarty (1) reported the isolation, purification, and characterization of the substance responsible for transformation of pneumococci. The substance was identified as a desoxyribonucleic acid and McCarty and Avery (8) subsequently offered direct proof that transformation is induced by desoxyribonucleic acid and not by a trace of some substance intimately associated with it when they demonstrated the complete destruction of the transforming activity of desoxyribonucleic acid by a purified desoxyribonuclease. Analysis by electrophoresis and ultracentrifugation showed a homogeneous solution; a single sharp boundary was seen and the transforming activity moved with it. MacLeod and Krauss (9), Austrian and MacLeod (10), and Taylor (11) demonstrated independently that the specific transforming substance for pneumococcus is a mixture of desoxyribonucleic acids. Different functions are controlled by different desoxyribonucleic acids. Not only is production of the specific polysaccharides of capsular material directed by a specific desoxyribonucleic acid but also production of type–specific proteins of pneumococcus.

Materials and Methods

Preparation of Transforming Principles from Types a, b, c, d, e, and f H. influenzae.—The cell suspensions used for isolation of the transforming principle from each of the 6 types of *H. influenzae* were prepared as follows: A strain showing virtually all encapsulated cells and iridescent colonies on Levinthal agar was grown in Levinthal broth for 16 to 18 hours and seeded in 0.5 cc. volumes on the surface of each of 100 Levinthal agar plates (Petri dishes 4 inches in diameter). Luxuriant growth appeared following incubation at 37°C. for 6 to 7 hours; after storage at 4°C. overnight the growth from each plate was removed by washing with 4 cc. of a solution containing 0.1 M sodium chloride and 0.1 M sodium citrate. The cells were kept cold during the harvesting by immersing the collecting flask in ice water. The cold suspensions were cleared of agar fragments by straining through a double layer of coarse gauze, then centrifuged for 1 hour at 4000 R.P.M. at 4°C., washed twice with citrate-saline solution, leaving a final volume of 10 to 15 cc., and the packed cells were quickly frozen and stored in a dry-ice box. In some other lots of bacteria immediate initiation of the isolation of transforming principle without washing of the cells has yielded active material.

Isolation of the transforming principle was carried out by the method of McCarty and Avery (12). The yield from 100 plates was treated as if it were ⅓ to ¼ the yield of pneumococci obtained by McCarty from 50 liters of broth. The frozen cells of a type-specific *H. influenzae* were thawed at 4°C. and added to 125 to 170 cc. of 0.1 M citrate-saline solution; 10 per cent sodium desoxycholate, 1.2 to 1.7 cc., was then added and the cells allowed to lyse at room temperature for 30 minutes. Gram stains of the cells before and after disruption with the bile salt indicate that all 6 types were well lysed. Three consecutive deproteinizations

were carried out as follows: ⅛ volume of chloroform and $^1/_{10}$ volume of amyl alcohol were added to the lysate and the mixture shaken for 30 minutes on a wrist action shaking machine. The mixture was then centrifuged for 15 minutes at 1500 R.P.M. at 0°C. and the supernatant removed from the chloroform gel with a pipette. The gels resulting from each extraction were pooled and extracted once with 50 to 100 cc. of the citrate-saline solution; this supernatant was combined with that of the third extraction. 2 volumes of absolute alcohol was added to the supernatants. The precipitates formed, both fibrous and fine, were allowed to stand in the alcohol solution overnight at 4°C. The alcohol was then decanted, the precipitates washed in absolute alcohol and dissolved in 75 to 100 cc. of 0.85 per cent sodium chloride. Two to four additional deproteinizations were carried out and 2 volumes of absolute alcohol added to the final supernatant. The fine precipitate was decanted from the fibrous and the latter washed with absolute alcohol and dissolved in 20 to 30 cc. of saline. 5 volumes of alcohol was then added for sterilization; if a significant amount of the fine precipitate was formed it was decanted, the fibrous was washed in alcohol and redissolved in 20 to 30 cc. of saline. This process was repeated and the fibrous precipitate was left in the alcohol solution (5 volumes) overnight at 4°C.; the alcohol was decanted, the flask or tube drained, and 20 to 30 cc. sterile saline (made slightly alkaline with sodium hydroxide) added. The final products or unpurified transforming principles will be designated Tp followed by the letter of type of *H. influenzae* from which each is isolated; Tpa, Tpb, Tpc, Tpd. and Tpe have been shown to contain desoxyribonucleic acid, as identified by the diphenylamine reaction of Dische (13); and the transforming activity of each of the 6 Tp's can be destroyed by a purified[1] or crystalline[2] desoxyribonuclease. However, these solutions also contain the specific polysaccharides and ribonucleic acids. Tpb was further purified by the McCarty and Avery (12) method (calcium chloride and alcohol) and will be labeled DNAb; the resultant product was free of specific polysaccharide but was shown still to contain desoxyribonucleic acid by means of the diphenylamine reaction of Dische (13) and the Stumpf (14) reaction. Transformation was induced uniformly in concentrations as low as 0.005 μg. per cc. The transforming activity was completely destroyed by a crystalline desoxyribonuclease. Attempts were made to purify Tpc and Tpa without success; the transforming activity of each was lost in the process. At times this method failed in isolating from type b *H. influenzae* a desoxyribonucleic acid–containing substance with transforming activity.

R Strains Used for Transformation—R colonies Ra, Rb, Rc, Rd, Re, and Rf were isolated in 1943 and 1944 from the corresponding type-specific strains of *H. influenzae* by selection of a spontaneously appearing, non-iridescent, non-opaque colony from among the iridescent, opaque ones characteristic of S cells after growth on Levinthal agar for 18 hours. They have been preserved in the interval by a method of Brown (15) which desiccates and seals under vacuum. Our experience with this method suggests that the characteristics of bacterial populations of many species are kept stationary. During the past year the experiments reported were performed on cultures maintained under cultivation for periods of less than 1 week. Subculture and isolation of characteristic R colonies have been repeated many times to make certain of stability of the culture. At no time has evidence of type specificity been detectable by study of colony type, capsular swelling tests, or precipitin reactions on supernatants of broth cultures with the exception of one example of reversion to type of origin in Rc and Re out of many tests. We have not been successful in transforming either of these 2 R strains. Twenty passages of the Rb strain through mice (in mucin suspensions) failed to demonstrate presence of type-specific organisms.

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[1] Supplied by Dr. Maclyn McCarty.

[2] Prepared by Dr. M. Kunitz or purchased from The Worthington Biochemical Laboratories, Freehold, N. J.

Production of Polyvalent Anti-R H. influenzae Antiserum—R cells derived from each of the 6 specific types of *H. influenzae* a, b, c, d, e, and f were grown for 6 hours on the surface of Levinthal agar plates. The growth was harvested in 5 cc. of saline containing 0.2 per cent of formalin and the suspension diluted to a turbidity corresponding to approximately 100 on the Klett-Summerson photoelectric colorimeter. Colony counts on suspensions in broth showed from 1 to 1.5 billion live organisms per cc. Equal quantities of suspensions of R cells derived from each of the 6 types were combined. Rabbits were given an initial dose of 0.1 cc., and this was increased daily by 0.1 cc. for 4 days of each week until 1.0 cc. daily was reached. Serum of rabbits injected for a period of 1 month has provided an environment suitable for demonstration of uniform transformation when used in a 1:50 dilution in Levinthal broth in the presence of desoxyribonucleic acid under circumstances of the experiments described. The antiserum was heated at 65°C. for 30 minutes.

EXPERIMENTAL RESULTS

Influence of Transforming Principles Isolated from Each of 6 Types of H. influenzae on R Cells Derived from Each of These Types.—Evidence of change in the inherited characteristics of *H. influenzae* by desoxyribonucleic acid–containing fractions is clearly defined and unmistakable. The R colonies on Levinthal agar are non-iridescent and translucent; the cells are not encapsulated and they fail to produce the soluble specific carbohydrate in Levinthal broth. The S forms or type-specific cells into which the R cells have been transformed form opaque iridescent colonies on Levinthal agar, and young Levinthal broth cultures show swelling of the capsules of the organisms and precipitation of the specific polysaccharide in the supernatant in the presence of type-specific homologous antibody.

The procedure used to detect change in inherited traits of R *H. influenzae* cells was carried out as follows:—

The transforming principle was added in quantities of 0.1 cc. of 1:10 dilution to 2 cc. of Levinthal broth containing polyvalent R *H. influenzae* rabbit antiserum in a concentration of 1:50. The constituents of the environment which have been found adequate for transformation of *H. influenzae* have been reported (16). Each of 5 samples of this complete reaction system and each of 2 samples without desoxyribonucleic acid was seeded with a 2 mm. loopful (approximately 1,000,000 cells) of a 5 to 6 hour old Levinthal broth culture of the R strain to be transformed, and the tubes incubated 24 to 48 hours. Each sample was then examined for capsular swelling by the specific antiserum against the type from which the Tp being tested was isolated. In addition a 2 mm. loopful was seeded on the surface of a Levinthal agar plate; after 18 hours' incubation the plates were examined for iridescent colonies.

When the prevalence of transformed cells is high the change to type-specific cells in the broth culture after 18 hours' incubation can easily be demonstrated by capsular swelling but when it is low the change can be seen only by the appearance of iridescent colonies on the plate; cells fished from these iridescent colonies show capsular swelling, and usually by the time they can be identified the broth cultures, then 40 hours old, also show capsular swelling. The induced type specificity is inherited by all subsequent generations. Table I shows the

influence of the transforming principles isolated from each of the 6 types of *H. influenzae* on an R strain derived from each of these 6 types; the letter after each, Tp or R, designates the specific type of *H. influenzae* from which it was isolated. It is seen that each one of the unpurified transforming principles and the purified Tpb has brought about transformation of at least one of the R strains. In none of the anti-R antiserum controls were homologous type-specific cells found. The greater versatility of the Rb and Rd strains may be an index to the prevalence of cells susceptible to the action of desoxyribonucleic acid or it may reflect differences in molecular structure of the somatic antigen. This question will be discussed later.

Mode of Action.—McCarty, Taylor, and Avery (17) have reported observations on certain phases of the mode of action of transformation in pneumococci. They have defined certain elements of the environment as essential for uniform transformation: a medium which provides rapid growth, an anti-R antiserum, susceptible cells, and the specific desoxyribonucleic acid from S cells of the type desired. This environment proved adequate for transformation of *H. influenzae* and has been used for all the experiments to be described unless otherwise stated. The anti-R antiserum could be replaced by a combination of 3 separate constituents in both the pneumococcus and *H. influenzae* reaction system:—

1. An agglutinin for R cells which may contribute by providing local reducing conditions. This is supplied by the gamma globulin fraction of the anti-R serum.
2. A protein which may be supplied by bovine albumin fraction V[3] (11).
3. A dialyzable constituent found in anti-R antiserum apparently in loose linkage with the enzyme-like protein believed to be needed for a closely associated enzyme action. This property can be restored after dialysis by the action of sodium pyrophosphate.

McCarty, Taylor, and Avery (17) conclude from their experiments that all of these environmental influences are needed for a 4 hour period to prepare the pneumococcus cell for the action of the desoxyribonucleic acid. Our studies fail to establish comparable requirements for *H. influenzae.*

Prevalence of Susceptible Cells.—In *H. influenzae* cultures the vital action of the desoxyribonucleic acid fraction responsible for transformation takes place almost immediately if susceptible cells are present. Our data suggest that susceptible cells vary greatly in their prevalence in different R strains of *H. influenzae.* This is shown in Table II by demonstrating the influence of population size on uniformity of transformation in 2 different strains Rb and Rd.

[3] Prepared by Armour and Company according to technique developed in the laboratory of Dr. E. J. Cohn.

Five samples of 2 cc. volumes of Levinthal broth containing 1:10 dilution of the transforming principle indicated and 1:50 dilution of polyvalent anti-R antiserum were seeded with each of the population sizes indicated; the organisms had been grown in Levinthal broth for 5½ to 6 hours. After incubation for 18 to 40 hours at 37°C. the bacteria were examined for swelling of the capsules by the specific antiserum of the type corresponding to the type of origin of the Tp. Organisms were also seeded on the surface of Levinthal agar at these periods and the colonies examined for iridescence after overnight incubation. The plus marks indicate transformation into type corresponding to the type of origin of the Tp, and the zero, failure to demonstrate transformation.

It is seen that one can anticipate irregularity of transformation of Rb strain into type b when relatively small populations are used; at least 1,000,000 cells

TABLE I

Influence of Transforming Principles from Each of 6 Types of H. influenzae on R Cells Derived from Each of These Types

R cells* exposed	Results of exposure to transforming principles					
	Tpa	DNAb	Tpc	Tpd	Tpe	Tpf
Ra	Sa	0	0	0	0	0
Rb	Sa	Sb	Sc	Sd	0	0
Rc	0	0	0	0	0	0
Rd	Sa	Sb	Sc	Sd	Se	Sf
Re	0	0	0	0	0	0
Rf	0	0	0	0	0	0

* Letters a through f refer to type from which R cells originated.

S = type-specific cells; type indicated by small letter.

0 = R cells only.

Tp = unpurified transforming principle—type of *H. influenzae* from which isolated indicated by letters a through f.

DNAb = purified transforming principle isolated from type b *H. influenzae*.

of this strain must be seeded to obtain transformation with uniformity. Much smaller seeding populations of the Rd strain, 10 cells, show regularity of transformation to type d. The difference between these 2 strains Rb and Rd is seen not only when transformation to the homologous type is brought about but also when they are transformed to heterologous types. Large populations of Rb must be used in order to demonstrate transformation to types a, c, and d; but in the case of Rd all 5 heterologous types can be produced with regularity when as few as 10 cells are seeded into a complete reaction system. Therefore the same cells may be susceptible to transformation by all transforming principles or, if they are different cells, their prevalence is the same. These data suggest either that the prevalence of susceptible cells is much greater in Rd initially or that the rate of occurrence of new susceptible cells during growth is higher. It should be pointed out that these experiments fail to show whether

TABLE II

Influence of Population Size on Uniformity of Transformation in 2 Different R Strains of H. influenzae

R strain used	New type induced by Tp	Transformation results: Total organisms seeded in each sample						
		1,000,000	100,000	10,000	1,000	100	10	1
Rb	a	00000 00000 +00 +0000	00000					
	b	+++++ +++++ ++++ ++++	+++++ +++++ +000 +000	++++00 +0000 0000 0000	0000 00000 0000 0000			
	c	+++++	+++++	00000	+0000			
	d	+++++	+++++	+0000	+000+			
Rd	a	+++++	+++++	+++++	+++++	+++++	++++0	++++−
	b	+++++	+++++	+++++	+++++	+++++	+++++	+++0−
	c	+++++	+++++	+++++	+++++	+++++	+++++	++++−
	d	+++++ +++++ +++	+++++	+++++	+++++	+++ +++ +++	+++ +++ +++	++− +−− 0++
	e	+++++	+++++	+++++	+++++	+++++	+++++	−+−0
	f	+++++	+++++	+++++	+++++	+++++	+++++	−−00+

Tp = transforming principle.
0 = no evidence of transformation.
+ = encapsulated cells of type corresponding to origin of Tp.
− = no growth.

the cells which are transformed within 18 hours are present initially or arise during reproduction of the R cells.

Study of the prevalence of susceptible cells in 3 other Rb and Rd strains shows similar patterns and suggests that the prevalence of transformable cells differs with the specific type of origin of the R cells.

Time Required for Action of Desoxyribonucleic Acid.—The results of experiments designed to determine the time required for action of Tpd on Rd cells to transform them to type d *H. influenzae* are shown in Table III. They also provide information on the initial prevalence of susceptible cells. The principles of this test have been used earlier by McCarty (17). To test the time required for transformation of Rd cells to type-specific ones the action of the Tp was stopped at the various intervals indicated after being in contact with R cells, by destruction with a purified or crystalline desoxyribonuclease. The time needed for complete destruction of the transforming activity by the enzyme

TABLE III

Time Required for the Action of Tpd on Rd Cells

Time of addition of enzyme in relation to inoculum	Desoxyribonuclease added Transformation in 3 samples			Controls No desoxyribonuclease Transformation in 2 samples		
	Inoculum size			Inoculum size		
	1,000,000	1,000	100	1,000,000	1,000	100
30 min. before	000	000	000	++	++	++
15 " "	000	000	000	++	++	++
5 " "	000	000	000	++	++	++
3 " "	000	000	000	++	++	++
3 " after	+++	000	000	++	++	++
15 " "	+++	+0+	000	++	++	+
30 " "	+++	+0+	000	++	++	++
1 hr. "	+++	0++	000	++	++	++
2 hrs. "	+++	00+	0+0	++	++	++
3 " "	+++	+++	00+	++	++	++
4 " "	+++	++0	000	++	++	++

Tpd = transforming principle isolated from type d *H. influenzae.*
Rd = variants lacking type-specific traits selected from type d *H. influenzae.*

was explored by adding the enzyme at the intervals indicated before the inoculation of R cells.

Levinthal broth containing 1:50 concentration of polyvalent R antiserum was tubed in 1.8 cc. quantities in test tubes 100 × 15 mm. Tpd diluted 1:50 in slightly alkaline (nitrazine paper) 0.85 per cent sodium chloride was added in 0.1 cc. quantities to each tube. For each time interval studied there were 3 tubes for the test and 2 for controls. A culture of Rd cells after 5½ to 6 hours' growth in Levinthal broth was diluted in neopeptone broth 10^{-1} through 10^{-7}; 0.1 cc. of 10^{-2} contained approximately 1,000,000 cells, 10^{-5}, 1,000 cells, and 10^{-6}, 100 cells. The 10^{-7} dilution was seeded into pour-plates to determine the number of viable cells present in the inocula used.

Desoxyribonuclease in a concentration of 20 μg. per cc. in neopeptone broth containing 0.03 M magnesium sulfate was added in 0.2 cc. quantities (4 μg.) to each tube in which the Tp was to be destroyed, and thoroughly mixed. The enzyme solution was prepared just before use. Each control tube received 0.2 cc. of 0.03 M magnesium sulfate in neopeptone broth.

The cultures were incubated at 37°C. and examined after 24 and 48 hours for presence of capsules and seeded on Levinthal agar plates to examine for iridescence of colonies which form after 18 hours' incubation. The tests designed to study the effect of short periods of exposure to Tp were left at room temperature. Three different population sizes were studied: 1,000,000, 1,000, and 100 cells. The second column of table III shows the results when 1,000,000 cells are seeded into these environments and incubated for 18 to 24 hours. Previous experiments had shown that susceptible cells are present initially in this population.

Since no transformation of Rd to type d occurred when the enzyme was added 3, 5, 15, and 30 minutes before the addition of organisms, it can be concluded that desoxyribonucleic acid is completely destroyed by the enzyme within 3 minutes. The controls which contained no enzyme showed transformation. When the enzyme was added 3 minutes after the organisms had been in contact with the desoxyribonucleic acid the vital action had already occurred, for transformation was demonstrated after incubation overnight. Therefore the desoxyribonucleic acid must be taken up by the cells within approximately 3 minutes. It has been shown repeatedly that the vital action of desoxyribonucleic acid has already taken place, or at least it is inaccessible to the action of the enzyme, when the desoxyribonuclease is added 15 minutes after the organisms. The results are the same for longer intervals.

When only 1,000 cells are seeded into the reaction system the time relationships are much the same. Failure to demonstrate transformation in triplicate series when desoxyribonculease is added 3 minutes after the inoculum suggests that by chance susceptible cells fail to be present initially among the 1,000 R cells. After 15 minutes' exposure transformation is seen in 2 of 3 samples, the same incidence as after later periods. Since this time interval would not permit a reproductive cycle it can be assumed either that mutants started earlier come to completion or that the phase of cell growth which is sensitive to desoxyribonucleic acid must be reached within 15 minutes. In populations of 100 the irregularity of transformation is so great that it is impossible to study the time relationship. These results would be expected if each sample of 1,000 to 2,000 cells includes approximately one susceptible cell. It has already been shown in Table II that if 10 Rd cells reproduce in the presence of a complete reaction system for a 24 to 48 hour period transformation will occur regularly; in the controls 100 cell populations showed transformation. These data suggest that susceptible mutants appear during growth. The results are consistent with the hypothesis that the rate of occurrence of new mutants is higher in the Rd than in the Rb strain. More data are needed for proof.

A duplicate experiment yielded comparable results, as shown in Table IV, when 1,000,000 cell populations were used; the results with populations of 1,000 were comparable but show that the prevalence of susceptible cells in the original population varies in independent cultures of the same strain and population size. Approximately 1 out of 3 samples showed transformation in this experiment whereas 2 out of 3 samples in the first experiment (Table III)

TABLE IV

Time Required for the Action of Tpd on Rd Cells, Duplicate Test

Time of addition of enzyme in relation to inoculum	Desoxyribonuclease added Transformation in 3 samples		Controls No desoxyribonuclease Transformation in 2 samples	
	Inoculum size		Inoculum size	
	1,000,000	1,000	1,000,000	1,000
15 min. before	000	000	++	++
5 " "	000	000	++	++
3 " "	0+0	000	++	++
3 " after	+++	000	++	++
15 " "	+++	000	++	++
30 " "	+++	000	++	++
1 hr. "	+++	0+0	++	++
2 hrs. "	+++	+0+	++	++
3 " "	+++	+00	++	++
4 " "	+++	0+0	++	++

Tpd = transforming principle isolated from type d *H. influenzae.*
Rd = variants lacking type-specific traits selected from type d *H. influenzae.*

TABLE V

Time Required for the Action of Tpc on Rd Cells

Time of addition of enzyme in relation to inoculum	Desoxyribonuclease added Transformation in 3 samples		Controls No desoxyribonuclease Transformation in 2 samples	
	Inoculum size		Inoculum size	
	1,000,000	1,000	1,000,000	1,000
30 min. before	000	000	++	++
5 " "	000	000	++	++
3 " "	000	000	++	++
3 " after	+++	000	++	++
15 " "	+++	000	++	++
30 " "	+++	00+	++	++
1 hr. "	+++	00+	++	++
2 hrs. "	+++	000	++	++
3 " "	+++	000	++	++
4 " "	+++	+00	++	++

Tpc = transforming principle isolated from type c *H. influenzae.*
Rd = variants lacking type-specific traits selected from type d *H. influenzae.*

showed type d cells. Table V lists the results of a comparable experiment which tested the action of Tpc on 1,000,000 and 1,000 Rd cells. The results are similar. Destruction of this transforming principle by desoxyribonuclease also oc-

curs within 3 minutes and the change by the desoxyribonucleic acid fraction, essential for transformation, also occurs within 3 minutes when susceptible cells are present initially. Analysis of results with approximately 1,000 cells suggests that the initial prevalence of cells susceptible to Tpc is not unlike

TABLE VI

Time Required for the Action of Tpa on Rd Cells

Time of addition of enzyme in relation to inoculum	Desoxyribonuclease added Transformation in 3 samples		Controls No desoxyribonuclease Transformation in 2 samples	
	Inoculum size		Inoculum size	
	1,000,000	1,000	1,000,000	1,000
15 min. before	000	000	++	++
5 " "	000	000	++	++
5 " after	0+0	000	++	++
15 " "	0++	000	++	++
30 " "	0++	000	++	++
1 hr. "	+0+	000	++	++
2 hrs. "	0++	000	++	++

Tpa = transforming principle isolated from type a *H. influenzae.*
Rd = variants lacking type-specific traits selected from type d *H. influenzae.*

TABLE VII

Variation in Initial Prevalence of Cells Susceptible to Tpc in Genetically Different Cultures of Same Population Size

Independent cultures of Rd cells	Transformation results—5 samples				
	No. of Rd cells exposed to Tpc for 5 min.				
	10,000,000	1,000,000	100,000	10,000	1,000
Rd_1	+++++	0 ++++	0+000	00000	00000
Rd_2		+++++	0++00	00000	00000
Rd_3		++0++	00000	00000	00000
Rd_4		+0+++	00000	00000	
Rd_5	++0++	00000	00000		
Rd_6		+++++	00+00	0000	00000
Rd_7		+++++	++000	00000	00000

+ = transformation to type c.
0 = no transformation evident.

that of cells susceptible to Tpd. Table VI shows the results of a similar experiment using Rd cells and Tpa; again the results are comparable. However, the prevalence of susceptible cells in the original populations, both the 1,000,000 and 1,000 groups, is significantly lower. This difference could be explained by

the random occurrence of mutants in different phases of the growth cycle; when they arise early the prevalence is high and when they appear late, and therefore before they have time for reproduction, the prevalence is lowest.

The results of experiments designed to show the variation in prevalence of initially susceptible cells in different independent cultures of the same R strain, Rd, and population size are shown in Table VII.

Rd cell populations varying from 1,000 to 10,000,000 in 2 cc. of either the complete reaction system, or Levinthal broth, or neopeptone broth, were exposed to Tpc, 0.1 cc. of 1:20 dilution, for 5 minutes. Desoxyribonuclease (4 μg.) was then added to each tube to stop the action of desoxyribonucleic acid. 5 minutes later anti-R serum was added to the environment used in test on Rd_6 and Rd_7; in addition Levinthal stock was added to neopeptone broth used

TABLE VIII

Results of Experiments Showing Completion of Transformation of Rd Cells to Type c H. influenzae within 15 Minutes

Experiment	Approximate No. of R cells seeded per plate	Total plates seeded	Approximate total of R colonies examined	No. of type c colonies formed	Approximate control colonies examined; no type c formed
1	10,000	10	100,000	0	100,000
2	540	50	27,000	0	27,000
3	750	100	75,000	1	
4	25,000	20	500,000	3	
5	60,000	75	4,500,000	0	4,500,000
6	10,000	100	1,000,000	0	
7	59,000	150	8,850,000	7	

in test on Rd_7. After incubation for 24 and 48 hours the cultures were examined for presence of capsules by capsular swelling with type c *H. influenzae* antiserum; they were also seeded on Levinthal agar at these intervals and the colonies forming after 18 hours' incubation were examined for iridescence. With exception of the examination of cultures Rd_6 and Rd_7, which was carried out on the same day, tests on all others were performed on different days. Cultures Rd_1 through Rd_5 were exposed to Tpc in the complete reaction system, Rd_6 in Levinthal broth, and Rd_7 in neopeptone broth.

The results show that the prevalence of initially susceptible cells in different independent cultures of Rd can vary from as low as less than one in 1,000,000 cells to one in 250,000. It is clear that prevalence of susceptible cells was as great in Levinthal broth and neopeptone broth as in the complete reaction system.

To determine whether the desoxyribonucleic acid fraction actually completes transformation in a short time interval (3 minutes) or whether it merely starts a process which then requires the subsequent influence of environmental constituents, the following experiments were carried out.

Samples of Rd cells varying in number from 1.5 to 680 million in 2 cc. of neopeptone broth were exposed to Tpc, 0.1 cc. of 1:20 dilution for 15 minutes. Desoxyribonuclease 4 μg. per

tube (0.2 cc. of 20 μg. per cc. in 0.03 M magnesium sulfate broth) was then added and after a 5 minute period to allow destruction of desoxyribonucleic acid at room temperature, the populations shown in Experiments 1 through 7 (Table VIII) were seeded on the surface of a series of Levinthal agar plates. After incubation for 18 hours the colonies were examined for iridescence.

The results of these experiments (Table VIII) show that when susceptible R cells are present the action of desoxyribonucleic acid which makes the altered cell capable of forming a colony of type-specific cells is completed within 15 minutes. The change occurs when the R cells are suspended in neopeptone broth and is therefore not dependent upon any of the environmental constituents reported to be needed for transformation of pneumococci. Moreover, reproduction is apparently unnecessary since this medium will not permit cell multiplication. The data also show the size of the population of R cells which must be used in order to have susceptible cells present initially and suggest that this varies from time to time, presumably depending upon their random occurrence. The prevalence has varied from one in 75,000 to less than one in 4,500,000.

DISCUSSION

The method of McCarty and Avery (12) which yielded unpurified pneumococcus-transforming principles along with serologically reactive substances and ribonucleic acid, has proved applicable to all 6 types of *H. influenzae*. The unpurified Tp's from types a, b, c, d, and e have been shown to contain significant quantities of desoxyribonucleic acid. Uniform transformation occurred in the presence of a small fraction of a microgram of each of these Tp's. These products are crude and contain a variety of other substances. However, that the desoxyribonucleic acid fraction is responsible for the transformation process has been demonstrated by the complete and rapid (less than 5 minutes) destruction of the transforming activity by purified or crystalline desoxyribonuclease. The data presented therefore suggest that either a highly specific desoxyribonucleic acid, or a complex containing it, directs the inherited traits of *H. influenzae*. Purification of Tpb by the method of McCarty and Avery (12) resulted in a product free of type-specific polysaccharide as judged by ordinary methods; a quantity as small as 0.01 μg. brought about uniform transformation of *H. influenzae*. This transforming activity was rapidly destroyed by a crystalline desoxyribonuclease.

The uniform predictability with which the specific desoxyribonucleic acids can change the inherited traits of both pneumococcus and *H. influenzae* R cell populations containing susceptible cells makes each of these reaction systems a suitable tool for exploring the mode of action of desoxyribonucleic acid. Knowledge of this mechanism may prove of value in investigating whether a similar control of inheritance applies to cells of higher species.

The change in heritable traits of R *H. influenzae* cells can be induced *in vitro* in a predictable fashion during a growth period of less than 24 hours by

the action of the desoxyribonucleic acid–containing fraction. These results suggest therefore that transformation in the nasopharynx is a real possibility under the influence of suitable stimuli. But more important is the demonstration that desoxyribonucleic acid fractions of two different bacterial families function as genes; they control type specificity and virulence in both penumococci and *H. influenzae*. The probability that the inheritance of all bacterial species may be directed by a similar mechanism adds weight to the suggestion of the cytochemists, that the function and structure of all cells are controlled by delicately balanced desoxyribonucleic acid systems. This in turn supports speculation that some abnormal functions of cells of higher species, which presumably depend upon an abnormal desoxyribonucleic acid, may be changed by altering the desoxyribonucleic acid system. An understanding of the mechanism of transformation of bacteria can be expected to contribute in reaching this goal.

The reaction which effectuates transformation of *H. influenzae* has been shown to be complete within 5 minutes if populations large enough to contain susceptible cells are exposed to active transforming principles. In contrast to the findings in the pneumococcus reaction system change in the inherited traits of *H. influenzae* R cells has been demonstrated without the presence of anti-R antiserum, the combined effect of the 3 constituents which can replace it, or factors in the environment which are necessary for growth of *H. influenzae*. The experimental data show that anti-R serum is not essential for *H. influenzae* transformation. However, it is a necessary reagent for study of some phases of this problem; for example, study of the rapidity of action of desoxyribonucleic acid fractions, prevalence of susceptible R cells, and in some strains even for demonstration that transformation has occurred. It is apparent that demonstration of change in inherited characteristics without the presence of anti-R serum or its 3 important constituents and without an environment which supports growth, is possible only in R strains such as Rd which have a high prevalence of susceptible cells.

The *H. influenzae* transformation process therefore behaves as if the change is brought about by a highly specific chemical reaction between special molecules of the somatic antigen of R *H. influenzae* cells and the type-specific desoxyribonucleic acid fraction. The complex environment which Avery and McCarty considered essential may play a role in enhancing growth, with resulting occurrence of new susceptible cells which may arise through mutations. All the facts are consistent with this hypothesis. The special molecules of the somatic antigen occur only in a small proportion of the total population; when they are present the chemical reaction occurs virtually immediately.

SUMMARY

Change of non-typable (R) strains of *H. influenzae* into the specific type of origin or new types (S) has been effected *in vitro* in a predictable fashion within

a single 24 hour growth period, by a desoxyribonucleic acid–containing fraction isolated from type–specific cells of the type desired.

Only a small proportion of the population of R cells are susceptible to the change induced in inherited characteristics by the desoxyribonucleic acid fraction. The data suggest that the number of susceptible cells present in any given population size varies with the specific type of origin of the R cells; a lesser degree of variation in different independent cultures of the same strain and population size has been demonstrated. The results suggest that the R *H. influenzae* cells which are susceptible to transformation arise as the result of mutation.

It has been demonstrated that the reaction necessary for transformation takes place virtually immediately if susceptible cells are present. Furthermore it has been shown that the change which enables an R cell to form a colony of type–specific organisms has been completed within 15 minutes in an environment which does not permit cell multiplication.

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