# IN VITRO PRODUCTION OF NEW TYPES OF HEMOPHILUS INFLUENZAE*

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Two genetic traits of *Hemophilus influenzae* populations, type specificity (1, 2) and sensitivity to streptomycin (3), can be changed with predictable regularity by exposure to desoxyribonucleic acid–containing extracts (DNA) isolated from cells of the type desired. The R cell which has lost all signs of type specificity can be changed to the homologous or a heterologous type (1). Fully equipped type-specific populations derived from a single colony, and homogeneous therefore with respect to the type-specific trait, can be changed directly to a new type by the action of DNA extract from the type desired (2). When both substances essential for the reaction are present, the DNA-containing extract and the substrate in cells susceptible to the change—the change in the genetic trait occurs in less than 5 minutes. The data of Zamenhof *et al.* (4) lend support to the premise that the substance responsible for induction of new type-specific traits in *H. influenzae* populations is the DNA itself. The new trait is inherited; therefore, it would seem that the DNA either modifies the gene or plays the dynamic role which has been attributed to genes. In the process of studying the nature of the cell susceptible to the genetic changes just described, genetic substances which control 2 different type-specific traits were found within the same transformed *H. influenzae* cell. This report will present data derived from experiments designed to study the process responsible for the phenomenon; the results are consistent with the premise that the action of DNA on susceptible cells may bring about certain changes heretofore thought to occur only after the fusion of cells in sexual reproduction.

## *Materials and Methods*

*Strains of H. influenzae:*

All strains used were derived originally from a single colony.

*Rb* was derived from type b *H. influenzae* strain $Sb_1$, by selection of a non-iridescent colony which appeared spontaneously; all signs of type specificity are absent.

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$Sb_1$ and $Sb_2$ type b strains were isolated from spinal fluid of patients with *H. influenzae* meningitis.

*Sd*, a type d strain, was isolated from the nasopharynx of a child. *Rd* was derived from *Sd* by selecting a non-iridescent colony which appeared spontaneously on Levinthal agar; all signs of type specificity are absent.

*DNA-Containing Extracts or Transforming Principles:*

The transforming principles used throughout these studies were relatively crude DNA-containing extracts isolated by the method already described (1).

Two types of transformation procedures have been used:—

*1. Transformation in the Presence of Desoxyribonucleic Acid during Growth.*—Levinthal broth, 2 ml. samples containing antiserum against the recipient cell (1:20 dilution for type-specific antiserum, 1:50 dilution for anti-R antiserum) and 0.1 ml. quantities of transforming principle in concentration of 1:10 or 1:20, is seeded with approximately 1,000,000 cells which had grown in Levinthal broth 5½ to 6 hours. After 24 and 48 hour periods of incubation the broth cultures are studied for evidence of capsular swelling by specific antibody of the same type as the cells furnishing the DNA-containing extract. The broth cultures are also seeded on Levinthal agar, incubated for 18 hours, and examined for iridescent colonies which are studied for type-specific characteristics.

*2. Transformation of Initially Susceptible Cells.*—Populations of various sizes were seeded into an environment identical with the one described under (1). After the cells have been exposed for 5 minutes desoxyribonuclease (DNAse) is added to destroy the DNA. The details of the method have been described (1). Under these circumstances newly created cells appearing after growth must have originated from initially susceptible cells, since any new susceptible cells arising during growth could not be transformed after destruction of the DNA activity and the probability of their arising by mutation is almost infinitely remote.

*Diagnostic Antisera:*

The diagnostic antisera used for identifying type-specific cells were produced by methods reported previously (5).

## *Induction of New Hereditary Determinants*

Two new hereditary determinants have been produced; those guiding type specific functions of *Sab* and *Sad H. influenzae* cells. The reactions which created them are outlined in Table I. In each case, cells have evolved which exhibit the capacity to produce within the same cell, the specific polysaccharides of two different types of *H. influenzae* and to transmit the trait to their progeny. *Sab* cells exhibit the capacity to produce the specific polysaccharides of both type a and type b *H. influenzae* within the same cell. *Sad* cells show production of types a- and d-specific polysaccharide within the same cell.

### *Production of Sab Cells:*

*$Sab_1$ Cells.*—*Sab* cells were created by either of 2 reactions. Reaction I produced $Sab_1$ cells during growth for 18 hours of approximately 1,000,000 young type b *H. influenzae* cells (derived from a single colony) in Levinthal broth containing DNA extract of type a cells and type b *H. influenzae* antibody.

$Sab_1$ cells were first recognized when reaction I was carried out in an at-

tempt to induce type a trait in type b cells. Examination of the resultant population for capsular swelling by exposure in separate Neufeld preparations to type a and type b antibody suggested that the same cells showed capsular swelling with each. This characteristic was exhibited by the very long cells occurring singly and possessing large capsules. Most of the unchanged type b cells are removed by agglutination caused by type b antibody used in the environment as a selective agent. Those remaining in the supernatant characteristically occur in small clumps. When such broth populations were seeded on the surface of Levinthal agar and incubated 18 to 20 hours, large opaque mucoid-appearing colonies exhibiting a pinkish iridescence were formed. Study of a number of such colonies by the capsular swelling method for their

TABLE I

*New Types of H. influenzae Produced in Vitro*

| Reaction | Materials used | | New types produced |
|---|---|---|---|
| | Cells | Transforming principles | |
| I | *Sb* | Tpa | $Sab_1$ |
| II | *Rb* | Tpa | $Sab_2$ |
| III | Iab | Tpd | *Sad* |

*S* = type-specific cells; small letter indicates type.

*R* = non-type–specific cells; small letter, type of origin.

I = cells designated as intermediate; type-specific polysaccharide produced in smaller quantities than normal.

Tp = transforming principle; small letter indicates type-specific cells from which it was isolated.

capacity to produce specific polysaccharide showed them to be made up of cells which formed within the capsule of the same cell, polysaccharides of both type a and type b *H. influenzae*. In broth cultures, started presumably from a single cell, these 2 soluble type-specific polysaccharides were elaborated.

$Sab_1$ cells cannot be demonstrated in all populations following reaction I. The frequency with which they have been encountered in a number of experiments is shown in Table II. The induction of type a during reaction I is apparently a rare event as shown in Table II.

*$Sab_2$ Cells.*—$Sab_2$ cells were produced by reaction II during an attempt to induce type a-specific traits in *Rb* cells (R cells derived from type b). Approximately 1,000,000 young *Rb* cells were grown for 24 to 48 hours in Levinthal broth containing a DNA extract isolated from type a *H. influenzae* (Tpa) and anti-R *H. influenzae* antiserum. Some of the colonies which formed on Levinthal agar following this reaction were similar to those found after reac-

tion I and suggested that they might represent *Sab* cells. This suspicion proved correct. However, many attempts to repeat this result with reaction II failed. The production of $Sab_2$ cells with reaction II has been demonstrated in only a small proportion of the trials, as shown in Table II. The results obtained from 11 experiments with reaction II and 7 with reaction I

TABLE II

*Frequency of Production of Sab Cells in Rb and Sb Populations Exposed to Tpa during Growth*

| Cells exposed | Experiment | Samples showing transformation to Type a | Samples showing transformation to Type ab | Total No. samples exposed |
|---|---|---|---|---|
| $Sb_1$ | 1 | 0 | 3 | 3 |
| " | 2 | 0 | 1 | 2 |
| " | 3 | 0 | 4 | 10 |
| " | 4 | 0 | 2 | 10 |
| $Sb_2$ | 1 | 0 | 0 | 2 |
| " | 2 | 0 | 0 | 3 |
| " | 3 | 1 | 3 | 10 |
| *Rb* | 1 | 0 | 0 | 20 |
| " | 2 | 0 | 0 | 15 |
| " | 3 | 0 | 0 | 15 |
| " | 4 | 0 | 1 | 20 |
| " | 5 | 0 | 1 | 10 |
| " | 6 | 0 | 0 | 20 |
| " | 7 | 0 | 0 | 20 |
| " | 8 | 0 | 1 | 20 |
| " | 9 | 0 | 0 | 20 |
| " | 10 | 0 | 0 | 10 |
| " | 11 | 0 | 1 | 20 |

*Sab* = type-specific cells exhibiting types a and b specificity within the same cell.
*Sb* = type b cells.
*Rb* = non-type-specific cells derived from $Sb_1$.
Tpa = transforming Principle isolated from type a cells.

are listed. Two different *Sb* strains and one *Rb* strain were exposed to Tpa during growth of approximately 1,000,000 cells in Levinthal broth containing the corresponding homologous antiserum as a selective agent. The number of samples used in each experiment is listed. It is seen that $Sab_1$ cells were demonstrated in all experiments in which 10 samples of 1,000,000 cells each of *Sb* were treated with Tpa or in 13 of a total of 40 samples. $Sab_2$ cells were demonstrated in only 4 of 190 samples when populations of approximately 1,000,000 cells of *Rb* were exposed to Tpa. However, on rare occasions in experiments with reaction II examination for capsular swelling revealed an

occasional encapsulated cell but no iridescent colonies formed when subcultured on Levinthal agar. Therefore, the possibility that the environment used was not adequate for growth of some transformed cells must be considered.

*Comparison of $Sab_1$ and $Sab_2$ Cells:*

Even though the frequency of the formation of $Sab_1$ and $Sab_2$ has been shown to differ, other differences have not been demonstrated.

*Polysaccharide Production.*—The progeny of $Sab_1$ and $Sab_2$ colonies which were used for the final comparison of their specific polysaccharide production were populations derived from a single *Sab* colony on each of 10 consecutive subcultures. There is, therefore, reason to believe that the same cell produces both type a and type b polysaccharide. The *Sab* trait has been transmitted through many generations and is therefore inherited.

Production of specific polysaccharides by *Sab* cells was studied by the capsular swelling phenomenon and the precipitin test on populations grown in Levinthal broth. The incubation time used for growth of *Sab* cells in Levinthal broth cultures before examination for capsular swelling has a marked influence on the demonstration of the type b polysaccharide in the capsule. Cultures of *Sab* grown for 6 hours usually show at most a suggestion of capsular swelling with type b serum, while capsular swelling with type a antiserum is striking. If on the other hand cultures are examined after 2 hours of incubation, capsular swelling with type b antiserum usually is clearly demonstrable and the capsular swelling with type a antiserum is just as striking as at 6 hours. Capsular swelling of single type a cells with type a antibody is usually much more prominent than in type b cells exposed to type b antibody. In keeping with the demonstration by capsular swelling of apparently a reduced amount of type b polysaccharide in the capsule, the precipitin test showed a normal amount of type a polysaccharide with less than usual type b.

*Morphology of Individual Sab Cells.*—The morphology of *Sab* cells differs from that of type a and type b. Differential staining technics and quantitative measurements will be necessary before these differences can be defined. However, when compared to the morphology of both types a and b *H. influenzae* in young Levinthal broth cultures exposed to the homologous antibody and methylene blue stain, most of the *Sab* cells are at least 3 times the length of the monotype cells, many contain terminal, clear, cyst-like areas, and the body of the cell which takes the stain is terminal instead of being in the center. The periphery of the large swollen capsule seen after exposure to type a antibody often exhibits a wavy outline suggesting some shrinkage.

*$CO_2$ Requirements.*—Study of a number of $Sab_1$ and $Sab_2$ colonies for evidence of enhancement of growth by $CO_2$ demonstrated that individual members differ in their reaction to $CO_2$ environment. Some require $CO_2$ for growth on the

surface of Levinthal agar but the need of others for this gas could not be demonstrated. Examination of type a and type b cells for influence of $CO_2$ failed to demonstrate a comparable effect.

*Source of the Genetic Substances in Sab:*

Even though the quantity of type b polysaccharide was by a crude measure smaller than the amount of type a, the evidence for its presence in the same cell with type a and in the progeny of *Sab* cells seems conclusive. Hereditary determinants responsible for types a and b polysaccharide production may therefore be considered to exist in the same cell.

There is evidence that a highly purified DNA directs type-specific polysaccharide production in both pneumococcus and *H. influenzae* systems. A purified *H. influenzae*-transforming agent which contains 96 per cent DNA lost none of its original activity as the purification process virtually eliminated proteins, polysaccharides, and ribonucleic acids (4). Though the experiments reported in this paper were not carried out with purified DNA extracts, DNA has been shown to be essential for the induction of *Sab*. Destruction of the DNA by crystalline DNAse prevents the induction of $Sab_1$ trait. The results presented therefore suggest that a highly specific DNA controlling type a *H. influenzae* and one controlling type b are present in the same cell.

The most likely explanation of the process responsible for production of $Sab_1$ would seem to be the introduction of the genetic substance controlling type a polysaccharide into a cell already equipped with the type b hereditary determinant. $Sab_1$ cells have been demonstrated with greater frequency than *Sa* cells after reaction I. Our result (Table II) would suggest that observation of the latter is a rare event. If this difference is not explained by failure of reproduction of the induced monotype a because of inadequate environment, it is suggested that some interaction of the type a and type b hereditary determinants is needed for production of the type a component of $Sab_1$.

The origin of the hereditary component controlling type b specificity in $Sab_2$ cells is more difficult to explain. The crude Tpa contained no type b–specific polysaccharide, therefore, presumably no normally functioning type b genetic substance, the *Rb* cells showed no traits of type b cells, and precipitin tests on supernatants of young and old broth cultures failed to produce precipitation characteristic of type b cells with type b antibody. Furthermore, $Sab_2$ cells were created from *Rb* populations by the action of Tpa isolated from 2 different *Sa* strains. Three sources for the type b genetic determinant would seem possible. (*a*) A minute proportion of *Rb* cells contain the type b genetic substance. (*b*) All the cells contain the type b determinant but the expression of the trait is prevented by some inhibitory mechanisms which the Tpa counteracts. (*c*) The molecular structure of the hereditary determinants of type a and type b may permit their interaction so that part of the molecule of type a may

react with the molecule controlling the *Rb* trait and in this way complete the type b genetic component of $Sab_2$ cells. In favor of the first and second possibilities is the fact that in saline washings of *Rb* cells after freezing and thawing or in filtrates of lysed cells from large quantities of packed *Rb* cells grown on Levinthal agar, a soluble-specific substance indistinguishable from type b polysaccharide can be demonstrated by its precipitation with type b antibody.

To test hypothesis I and II a crude DNA-containing extract was isolated from large quantities of *Rb* cells and examined for its capacity to induce type b specificity in *Rd* and *Rb* cells. To date all attempts have failed as shown in Table III. It has been reported earlier (1) that type b cells are easily demonstrable when very small *Rd* populations, 10 cells, are exposed to Tbp during

TABLE III

*Comparison of TpRb and Tpb in Induction of Type b Trait during Growth of Rb and Rd Cells*

| R cell culture exposed | Experiment | Result of exposure of multiple samples of 1,000,000* cells to transforming principles | |
|---|---|---|---|
| | | TpRb | Tpb |
| *Rb* | I | 000000000 | ++ |
| | II | 0000000000 | +++++ |
| *Rd* | I | 0000000000 | ++ |
| | II | 0000000000 | +++++ |

\+ = transformation to type b.
0 = no evidence of transformation to type b.
TpRb = transforming principle from Rb cells.
Tpb = transforming principle from Sb cells.
* Approximate number of viable cells.

growth. It is seen that at least a million times as many cells, when exposed under optimal conditions to the Tp from *Rb* cells have failed to reveal any sign of transformation to type b. Larger populations of *Rb* cells are required in order to induce type b trait with predictable uniformity by Tpb but at least 200 times the population needed for transformation with Tpb have been exposed to Tp*Rb* without demonstrable evidence of the type b trait.

*Production of Sad Cells:*

A population derived from an Iab colony formed during subculture of $Sab_2$ produced by reaction II and possessing characteristics of an intermediate between R and S was used for production of *Sad* cells by reaction III (Table I). Approximately 1,000,000 young Iab cells were grown for 18 to 24 hours in Levinthal broth containing the transforming principle isolated from type d *H. influenzae* and anti-R *H. influenzae* antiserum. The population which

resulted from this procedure was seeded on Levinthal agar and examined after 18 hours' growth for iridescent colonies, for evidence of double type-specific genetic traits by capsular swelling and precipitation of the soluble-specific polysaccharide with types a, b, and d antibody. The results showed the presence of both type a and type d polysaccharide within the same cell. The population derived from one of these colonies has been subcultured over 20 times and appears homogeneous with respect to the *Sad* trait; single colonies were used for the first two subcultures. In *Sad* cells type d polysaccharide was produced in larger quantities than type a. Type b polysaccharide could not be demonstrated.

TABLE IV

*Action of DNA Sab on Rd Cells after 5 Minutes Exposure*

| Experiment | Types induced in population sizes of Rd cells exposed to DNA *Sab* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1,000,000 | | | 100,000 | | | 10,000 | | |
| | *Sa* | *Sb* | *Sab* | *Sa* | *Sb* | *Sab* | *Sa* | *Sb* | *Sab* |
| I | | | | 9/15 | 3/15 | 1/15 | 1/10 | 0/10 | 1/10 |
| II | | | | 6/10 | 4/10 | 5/10 | | | |
| III | 5/5 | 4/5 | 2/5 | 3/5 | 0/5 | 1/5 | 1/5 | 0/5 | 0/5 |
| IV | 5/5 | 5/5 | 5/5 | 2/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |

DNA Sab = transforming principle (DNA-containing extract) isolated from $Sab_1$ Cells.
*Rd* = non-type-specific cells derived from type d.
*S* = encapsulated type-specific cells—small letters indicate the type.
Right-hand number = total number of samples examined.
Left-hand number = number of samples showing specific type at head of column.

### *Significance of Induction of $Sab_1$ Trait*

The significance of induction of hereditary determinants controlling type-specific traits of 2 types of *H. influenzae* depends to a great extent upon whether it merely represents within the same cell the presence of the 2 separate genetic substances controlling type a and type b *H. influenzae* or whether the reaction between them has resulted in a new genetic substance with a new function.

*Action of DNA Extracts of $Sab_1$ Cells:*

To explore this point crude DNA-containing extracts (Tpab) were isolated from $Sab_1$ cells derived in all probability from a single cell by single colony isolation on repeated subculture followed by the dilution technic.

Table IV presents the results obtained when *Rd* populations of the varying sizes listed were exposed to Tpab for 5 minutes before the addition of DNAse, which destroys its transforming activity. The experiments therefore consisted of an examination of these populations for the presence of initially susceptible cells. The *Sab*-transforming principle exhibits all the characteristics of those

isolated from cells with a single type-specific trait when they induce single new genetic traits. The heritable $Sab_1$ trait is induced within 5 minutes and only a minute proportion of the cells exposed are susceptible. The frequency of cells susceptible to change by Tpab is not significantly different; 1,000,000 cells must be exposed to make sure of the presence of susceptible cells. The varieties of types induced and data for estimating the frequency of each are listed. Not only are *Sab* cells formed but also *S* cells showing single type-specific traits, a and b.

It is seen that not only is the *Sab* trait passed on to the progeny of the original *Sab* cells formed, but DNA extracts isolated from $Sab_1$ cells derived in all probability from a single cell can induce the new trait in suitable cells with predictable uniformity.

*Comparison of the Action of Tpab (DNA Extracts from $Sab_1$ Cells) with Tpa Plus Tpb:*

The induction of type a- and type b-specific cells in addition to *Sab* cells by the action of Tpab on *Rd* cells as shown in Table IV raises the question whether the hereditary determinant of the ab trait merely represents the additive effects of Tpa and Tpb. The results of 2 experiments designed to answer this question are presented in Table V. In each experiment three different population sizes were exposed (5 samples of each) to 4 different DNA-containing extracts. In Experiment I the action of a DNA-containing extract isolated from $Sab_1$ cells, $Tpab_1$, is compared with that of DNA-containing extracts from type a and type b cells separately and in conjunction. It is seen that the reactivity of $Tpab_1$ is greater than any of the 3 other Tp's on the same suspension of *Rd* cells. All 5 samples of the $10^6$ population showed *Sa*, *Sb*, and *Sab* cells when exposed to $Tpab_1$ but only 1 of 5 exposed to type a extract and 1 of 4 to type b extract showed induction of the corresponding type. In addition the $Tpab_1$ induced type a in the $10^5$ population size. The combined action of transforming principles isolated from naturally occurring type a and b cells resulted in induction of type a in 4 of 5 samples and of type b in 1 of 5; *Sab* cells could not be demonstrated. The latter part of the experiment has been repeated a number of times and in each, no *Sab* cells could be demonstrated.

Experiment II (Table V) compared the action of DNA-containing extract from $Sab_1$ cells with the DNA-containing extracts isolated from the type a ($a_{rd}$) and type b ($b_{rd}$) cells, created by exposing *Rd* populations to $Tpab_1$. The separate and combined influence of $Tpa_{rd}$ and $Tpb_{rd}$ have been studied. The data demonstrate that $Tpa_{rd}$ and $Tpb_{rd}$ possess a greater capacity than Tpa and Tpb to induce type a and type b traits in *Rd* cells. Their reactivity was even greater than Tpab. All 5 samples of the 100,000 population show induction of the corresponding single type and at least one of the 5 samples of the 10,000 population. When exposed to the influence of equal concentrations

of $Tpa_{rd}$ and $Tpb_{rd}$ the reactivity with *Rd* cells is not significantly different in the production of single types a and b traits. However, again no *Sab* cells could be demonstrated.

These data suggest that the $Sab_1$ genetic trait is not determined by the presence of naturally occurring type a and type b hereditary determinants within the same cell. It has been demonstrated that the hereditary determinant of $Sab_1$ possesses a function (induction of $Sab_{rd}$ cells) which the Tpa and Tpb in conjunction have not been shown to exhibit. The data also show that the single

TABLE V

*Comparison of the Effect on Rd Populations of DNA Extracts of $Sab_1$ Cells with DNA Extracts from Type-Specific Cells a and b Separately and in Conjunction*

| Experiment | DNA-containing extract* | μg. of DNA per ml. | Frequency of types induced in populations of | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $1.0 \times 10^6$ | | | $1.0 \times 10^5$ | | | $1.0 \times 10^4$ | | |
| | | | a | b | ab | a | b | ab | a | b | ab |
| I | $ab_1$ | 0.24 | 5/5 | 5/5 | 5/5 | 2/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| | a | 0.20 | 1/5 | | | 1/5 | | | 0/5 | | |
| | b | 0.13 | | 1/4 | | | 0/5 | | | 0/5 | |
| | a + b | 0.33 | 4/5 | 1/5 | | 0/5 | 0/5 | | 0/5 | 0/5 | |
| II | $ab_1$ | 0.24 | 5/5 | 4/5 | 2/5 | 3/5 | 0/5 | 1/5 | 1/5 | 0/5 | 0/5 |
| | $a_{rd}$ | 0.28 | 5/5 | | | 5/5 | | | 1/5 | | |
| | $b_{rd}$ | 0.27 | | 5/5 | | | 5/5 | | | 3/5 | |
| | $a_{rd} + b_{rd}$ | 0.55 | 5/5 | 5/5 | | 4/5 | 4/5 | | 2/5 | 1/5 | |

DNA = desoxyribonucleic acid–containing extract. Small letters indicate type-specific cells from which isolated.

$a_{rd}$ and $b_{rd}$ DNA extracts isolated from type a and type b cells induced by DNA Sab.

Right-hand number = total samples studied.

Left-hand number = number of samples showing induced type.

* Type from which isolated indicated by small letters.

type-specific cells, $a_{rd}$ and $b_{rd}$ which are induced along with the $Sab_{rd}$ type when *Rd* cells are exposed to $Tpab_1$, are controlled by genetic substances which have quantitative effects different from those which control naturally occurring types a and b. Additional data suggest that the action of $Tpa_{rd}$ on *Rb* cells differs qualitatively from the action of the hereditary determinant of naturally occurring type a cells (Tpa). As has been pointed out earlier, induction of type a in *Rb* cells by exposure to DNA-containing extract from type a must be a rare event, if it occurs at all. In contrast, when *Rb* is exposed to $Tpa_{rd}$, type a cells can be induced with predictable uniformity as shown in Table VI. In Table VI are listed the results when the same suspensions of *Rb* cells were exposed simultaneously to the separate action of Tpa and $Tpa_{rd}$. The comparable action of DNA extracts isolated from 2 different strains of $Sa_{rd}$ is also shown

(Table VI). The difference in the action of these 2 inducing agents on the same suspension is apparent. The negative results of many experiments attempting to induce type a in *Rb* cells, but carried out at other times, are shown in Table II. Even though $Tpa_{rd}$ induces type a with predictable regularity no *Sab* cells have been isolated.

Lederberg and his associates have offered evidence convincing to geneticists that some cells in *Escherichia coli* populations multiply by a process which leads to events indistinguishable from those of sexual reproduction. The acceptance is based on demonstration of two phenomena: (*a*) combination

TABLE VI

*Comparison of the Action of Tpa and $Tpa_{rd}$ on 1,000,000 Rb Cells in Inducing Type a Trait*

| Experiment | Induction of type a trait in *Rb* cells | | | |
|---|---|---|---|---|
| | By Tpa from 2 strains of type a | | By $Tpa_{rd}$ from 2 strains of type $a_{rd}$ | |
| | 1 | 2 | 1 | 2 |
| I | | | +++++ +++++ | |
| II | 00000 | | +++++ | |
| III | | 00000 | | +++ |
| IV | 00000 | 00000 | +++++ | +++++ |

\+ = transformation to type a.
0 = no transformation demonstrable.
Tp = transforming principle; small letter indicates type of origin.
Type a = naturally occurring type a cells.
Type $a_{rd}$ = cells with type a trait produced by action of $Tpab_1$ on *Rd* cells.

within the same cell of genetic units from each of two genetically different parents, resulting in a cell with traits of each but different from either parent, and (*b*) segregation of the traits in subsequent progeny (6). The data presented in this paper suggest that the formation of $Sab_1$ might represent the first phenomenon. A new hereditary determinant, $Sab_1$, has resulted from the interaction of DNA-containing extract of type a cells with the genetic substances in type b cells. Genetic material in the living cells *Sb*, which may be considered one parent and DNA-containing extract from genetically different cells, *Sa*, the other parent, have reacted to form an individual, $Sab_1$, with at least one trait of each parent but also differing from each.

Whether the data presented can be interpreted as showing that $Sab_1$ production represents a phenomenon comparable to that which occurs following fusion of cells in sexual reproduction will depend upon whether true segregation of

type a and type b can be demonstrated. Preliminary experiments have explored the time of appearance of single type cells. An $Sab_1$ population derived in all probability from a single cell was subcultured at 24 hour intervals in Levinthal broth. At each 24 hour interval, a 2 mm. loopful (approximately 1,000,000 cells) was also seeded into each of 2 tubes of Levinthal broth, one

TABLE VII

*Analysis of $Sab_1$ Populations on Levinthal Agar at Each 24 Hour Subculture in Levinthal Broth with and without Selective Environment*

| Subculture | Media seeded | Approximate per cent of each type found | | | |
|---|---|---|---|---|---|
| | | *Sab* | *SaIb* | *Sb* | *R* |
| 1 | Levinthal broth | 99.9 | <0.1 | | |
| | " + a antibody | 95 | | 2 to 5 | <1 |
| | " + b antibody | 99.9 | <0.1 | | |
| 2 | Levinthal broth | 99.9 | | | <0.1 |
| | " + a antibody | 30 | | 50 | 20 |
| | " + b antibody | 95 | | | <5 |
| 3 | Levinthal broth | 99.9 | <0.1 | | <1 |
| | " + a antibody | 30 | | 50 | 20 |
| | " + b antibody | 60 | <1 | | 40 |
| 4 | Levinthal—7 hr. growth | 99.9 | <0.1 | | <1 |
| | " + a antibody | 40 | | 60 | <1 |
| | " + b antibody | 50 | 1 to 2 | | 48 |

*Sab* = type-specific cells containing type a and type b genetic units within the same cell.

*SaIb* = cells exhibiting normal capsular swelling and precipitation of polysaccharide by type a antibody but which showed a reduced amount of type b polysaccharide by precipitin test and no capsular swelling.

*Sb* = type-specific b cells.

*R* = cells which lack the usual traits of type-specific cells. Sample colonies studied proved to be intermediates through the 3rd subculture. A few typical R colonies were present in population of 4th subculture.

containing type a and the other type b antibody to serve as selective agents in order to detect the presence of type a or type b as soon after their appearance as possible. At each interval a 2 mm. loopful of undiluted culture in each of the 3 Levinthal broth cultures was seeded on Levinthal agar and after 18 hours' incubation the population was studied to estimate the proportion of each of the colony types formed. The results of study of the first 4 subcultures are shown in Table VII. The listed estimate of the proportion of each colony type as assessed by gross inspection was checked by examination of a sample of colonies of each variety for presence of type-specific polysaccharides; the precipitin test and

the capsular swelling were used for this purpose. The limitations of this method for recognition of type a cells should be emphasized. Early recognition depends upon colony characteristics which permit *Sab* colonies to be distinguished from

TABLE VIII

*Analysis on Levinthal Agar of $Sab_1$ Populations at Each 24 Hour Subculture in Levinthal Broth by Proportion of Each Colony Type* Listed*

| Subculture | Results obtained in 3 independent cultures of same $Sab_1$ strain | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | | | 2 | | | | 3 | | | |
| | *Sab* | *SaIb* | *Sb* | *R* | *Sab* | *SaIb* | *Sb* | *R* | *Sab* | *SaIb* | *Sb* | *R* |
| 1 | 100 | | | | 100 | | | | 100 | | | |
| 2 | 100 | | | | >99 | <0.1 | | | >99 | <0.1 | | <0.1 |
| 3 | 100 | | | | 100 | | | | 99 | <0.1 | | 1 |
| 4 | >99 | | <0.1 | | | | | | 93 | 1 | | 6 |
| 5 | | | | | >99 | <0.1 | <0.2 | 0.4 | 54 | 1 | | 45 |
| 6 | 75 | | | 25 | >99 | <0.2 | <0.2 | 0.5 | 15 | <0.1 | | 85 |
| 7 | 65 | | <0.1 | 35 | 85 | 3 | 6 | 9 | <0.1 | <0.1 | | >99 |
| 8 | <50 | <0.1 | <0.1 | >50 | 55 | | 8 | 37 | <0.1 | <0.1 | | |
| 9 | 25 | | | 75 | | | | | <0.1 | <0.1 | | >99 |
| 10 | 4 | | | 96 | 9 | <0.1 | 1 | 90 | <0.1 | <0.1 | | >99 |
| 12 | <1 | | | 99 | 20 | | <0.1 | 80 | | | | |
| 13 | <1 | | | >99 | 15 | | | 85 | | | | |
| 14 | 1 | <0.1 | | >99 | | | | | | | | |
| 15 | 5 | <0.1 | | 95 | | | | | | | | |
| 16 | 20 | | | 80 | | | | | | | | |
| 18 | | | | 100 | | | | | | | | |
| Total colonies studied..... | 454 | | | | 281 | | | | 320 | | | |

*SaIb* = normal capsular swelling and precipitation of soluble specific polysaccharide with type a antibody. No capsular swelling with type b antibody; smaller amount of type b polysaccharide by precipitin test. Differs from *Sab* in morphology.

*Sab*, *Sb* = type-specific cells; small letter indicates specific type.

*R* = cells lacking in the usual signs of type specificity.

* Estimate checked by study of sample colonies for presence of type-specific genetic units.

type b colonies by their gross appearance. Type a colonies on the other hand may have the same gross appearance as *Sab* colonies. It is seen that the *Sab* trait is transmitted unchanged through many generations in 99.9 per cent of the population in a non-selective environment. It is also apparent that type b cells indistinguishable at present from the naturally occurring type b are present after several generations (in the first subculture studied) if the selective action of type a *H. influenzae* antiserum is present to sediment *Sa* and *Sab*

cells so that their presence can be detected. Under the latter circumstances, in the second subculture or after approximately 14 generations, about 50 per cent of the population in the suspension is composed of type b cells. The early appearance of type b cells makes segregation one of the possible explanations. However, extensive quantitative studies will be needed for proof.

Explanations of the early appearance of type b cells other than segregation are equally plausible. The type b cell may represent the absence of type a genetic substance as a result of mutation to R form. The ab hereditary determinant may represent a loose linkage of type a and type b genetic substances which is easily broken even though under the influence of the combination it has a function different from a plus b genetic forces.

When the selective action of antiserum is not used it is not possible to detect the type b cells in the population following the first subculture; their presence is masked by the cells which make up the majority of the population. This is well shown by the data listed in Table VIII. Three $Sab_1$ populations which were propagated from the same suspension, derived originally by a process which in all probability provided a single cell, have been subcultured daily in Levinthal broth without a selective agent and the resultant populations, 10 to 18 subcultures, have been studied as described in experiments shown in Table VII. The marked differences found under this non-selective environment demonstrate the need for a selective agent to detect small numbers of cells of a specific type. The changes in later subcultures suggest that the *Sab* trait may be relatively unstable. However, the disappearance of *Sab* cells may merely represent the need of *Sab* cells for special growth requirements. The resurgence of *Sab* cells in populations of the 15th and 16th subcultures of strain I is of interest but the forces responsible are unknown. What influence other than that of a selective agent, the presence of type a antibody has on the appearance of type b, intermediates, and R cells, is not known.

## GENERAL DISCUSSION

Even though the exact chemical nature of the gene, the unit of hereditary determinants, is a controversial subject, the known facts are consistent with the view that the dynamic role of the gene in some bacteria and in bacterial viruses is carried on by a highly specific form of DNA. The nature of the substrate necessary for the expression of a genetic trait is unknown. Purified DNA's of *Diplococcus pneumoniae* and *H. influenzae* have been shown to lose no activity when impurities, polysaccharide, ribonucleic acid, and protein are removed. The capacity of these purified products, containing 96 per cent of DNA to induce a new hereditary determinant can be completely destroyed by crystalline DNAse. For the above reasons, it would seem justifiable at least tentatively to interpret a change in a gene or hereditary determinant in *H. influenzae* as a change in a specific DNA.

The action of DNA-containing extract of type a *H. influenzae* on type b cells has produced a new type of cell, $Sab_1$. One explanation which would seem to be supported by the data presented is that the DNA molecule of type a cells has combined with the DNA molecule of type b cells with the formation of a new DNA molecule, DNA ab. $Sab_1$ cells exhibit a change in colony type, morphology of individual cells, reaction to $CO_2$ of some of the cells and contain a new hereditary determinant for directing the type-specific polysaccharide production. Immunologic methods have demonstrated that the polysaccharides of both type a and type b are produced by the same cell and that this trait is inherited. Type a polysaccharide is produced in quantities characteristic of type a cells but type b polysaccharide production is less than usual by type b cells.

The new hereditary determinant present in the DNA-containing extract of $Sab_1$ cells has induced a heritable trait *Sab* in *Rd* cells with predictable uniformity. In addition to $Sab_{rd}$ cells, type a ($a_{rd}$) and type b ($b_{rd}$) cells are produced by this reaction. Whether induction of $Sa_{rd}$ and $Sb_{rd}$ represents the result of a break in a loose linkage between the 2 latter genetic substances or whether a single genetic substance can induce $Sab_{rd}$, $Sa_{rd}$, or $Sb_{rd}$, depending upon which part of the molecule reacts with the substrate in the *Rd* cells, cannot be answered.

The data presented suggests that the $Sab_1$ DNA does not merely represent the presence of the DNA of type a and the DNA of type b within the same cell. The evidence raises the possibility that as a result of the reaction between the 2 DNA's, a new molecular configuration may have occurred and may be responsible for the new hereditary determinant with a new function, DNA ab. The $Sab_1$ DNA is more reactive than either DNAa or DNAb. Moreover, it has not been possible to produce *Sab* cells by the action of DNAa plus DNAb on *Rd* cells. It is of great interest that the DNA's from $Sa_{rd}$ and $Sb_{rd}$ induce the corresponding types in *Rd* cells with a greater frequency than is characteristic for the DNA's of naturally occurring types a and b.

The results suggest that either the DNA extracts of $a_{rd}$ and $b_{rd}$ cells are more reactive with the same substrate in susceptible cells or their DNA's differ in molecular configuration from those of type a and b cells, and, therefore need a different substrate for expression. Of greater importance is the fact that a qualitative difference has been demonstrated between the action of the transforming principles of naturally occurring types a *H. influenzae* and the transforming principle of type $a_{rd}$. The DNA extract of $Sa_{rd}$ cells induces type a trait in *Rb* cells with predictable uniformity whereas this must be a very rare event, if it occurs at all, as a result of the action of Tpa from naturally occurring type a.

The data therefore suggest that the hereditary determinant of type a present in the DNA extract from natural type a strains has reacted with the genetic substance in type b cells resulting in a new hereditary determinant capable of a

new function. It would seem that a genetic component of one parent cell as a result of reaction with an extracted genetic substance of another genetically different parent cell has produced an individual different from each parent but with a trait of each. Since monotype b cells are found after several generations in the progeny of *Sab* cells, presumably derived from a single cell, segregation is one of the possible explanations.

It would seem therefore that the action of a DNA extract under appropriate circumstances on genetically different cells may produce some of the changes which have been thought to occur only after the fusion of cells.

## SUMMARY

Two new types of *Hemophilus influenzae*, *Sab* and *Sad* have been produced *in vitro*. Each exhibits the presence of the type specific polysaccharides of 2 types of *H. influenzae* within the same cell. In *Sab* the polysaccharides of types a and b have been demonstrated and in *Sad* those which characterize types a and d. The *Sab* and *Sad* traits are inherited.

*Sab* was produced by the action of DNA-containing extract isolated from type a on either type b cells or *Rb* cells (non-encapsulated non-type–specific cells derived from type b). *Sad* cells were formed as a result of the action of the DNA-containing extract isolated from type d on cells intermediate between *Rab* and *Sab* cells.

DNA-containing extracts isolated from *Sab* cells have induced the *Sab* trait in *Rd* cells with predictable regularity.

Evidence has been presented that the hereditary determinant of *Sab* cells is a new genetic substance with new functions. Therefore, the interaction of the DNA-containing substance from cells of one genetic type with living cells of a genetically different type has produced what appears to be a new individual which differs from each of the cells contributing the differing genetic traits but has at least one trait in common with each.

*Sab* cells derived presumably from a single cell show the appearance of type b cells sometime during the first 7 generations.

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