TRANSFORMATION OF *NEISSERIA MENINGITIDIS* BY DEOXYRIBONUCLEATES FROM CELLS AND FROM CULTURE SLIME[1]

B. WESLEY CATLIN

Department of Microbiology and Immunology, School of Medicine, Marquette University, Milwaukee, Wisconsin

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Highly polymerized deoxyribonucleate (DNA) is a component of some slimes that accumulate in ordinary broth cultures of a variety of bacteria (Smithies and Gibbons, 1955; Catlin, 1956*a*). Such DNA, designated as extracellular, may be distinguished from intracellular DNA of the same culture by its accessibility to crystalline pancreatic deoxyribonuclease. Small quantities of this enzyme destroy the sticky consistency of the DNA-containing slime. DNA within intact cells, however, is not affected by added deoxyribonuclease and, subsequent to enzyme action, may be isolated in a highly polymerized form by disrupting the washed cells in a medium that inhibits deoxyribonuclease.

The origin of extracellular DNA may be obscured, in some cases, by cryptic multiplication. Investigation of *Micrococcus halodenitrificans* using radioactive phosphorus-labeled cells has shown that the DNA in culture slime was of intracellular origin (Takahashi and Gibbons, 1957). When this halophilic organism was cultivated in suboptimal concentrations of sodium chloride, some cells of the population apparently ruptured, releasing nucleic acid; much of the DNA adhered to the cells, whereas the ribonucleate diffused into the medium. Cellular lysis, also, can account for the DNA present extracellularly in cultures of *Pseudomonas fluorescens* (Catlin, *unpublished data*) and *Neisseria meningitidis*. In other cases the source remains to be determined.

Extracellular accumulation of highly polymerized DNA can occur only in the absence of deoxyribonuclease activity. *Staphylococcus aureus*, *P. fluorescens*, and *Alcaligenes faecalis* produce enzymes which are different, but which have in common the capacity to depolymerize certain DNA preparations. Under the proper circumstances these enzymes apparently were inactive, and extracellular DNA accumulated in cultures (Catlin, 1956*b*; Catlin and Cunningham, 1958). It was not certain that such DNA had escaped damage entirely. If structurally intact, however, extracellular DNA might possess genetic information. This possibility, which is important from the standpoint of population dynamics, has been explored using *N. meningitidis*.

Selection of the meningococcus for this study was based on its formation of slime in cultures without experimental intervention, and its capacity for DNA-conferred genetic change (transformation). Alexander and Redman (1953) demonstrated transformation of type IIa meningococci (either R or S) to type I under the influence of DNA-containing extracts from type I cells. Their method of detecting transformation did not lend itself readily to a quantitative approach. Therefore, antibiotic resistance transformations were investigated as a basis for developing a quantitative method (Hotchkiss, 1957), which could be used to compare the genetic activities of meningococcus DNA preparations.

Quantitative transformation tests involving antibiotic resistance markers are carried out in four main steps. (1) Cells are cultivated in an environment and for a period of time designed to bring the greatest proportion of the population to a state of competence, i. e., capacity to become transformed. (2) These cells are mixed under suitable conditions with a known concentration of DNA. After a brief exposure period, crystalline deoxyribonuclease is added to destroy the activity of DNA that has not been irreversibly bound by the cells. A sample of the population is assayed at this time to determine the number of exposed cells per ml (*E*). (3) The population is allowed to carry out metabolic activities for a

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period long enough to assure development of the transformant phenotype by each of the modified cells. (4) Before the transformed cells undergo division, the population is brought into a selective environment which will inhibit reproduction of the untransformed cells, but will allow colony formation by each of the transformants (number of transformants per ml = T). The transformation ratio (T:E) may be calculated from these data.

Investigations of these steps, which were required for developing and for evaluating the method, will be summarized in Part I in the Results section. Part II will present results of comparative studies of meningococcus DNA preparations from intracellular and from extracellular locations.

MATERIALS AND METHODS

Strains and antibiotics. N. meningitidis strains 10 and 15 from the departmental culture collection, and variants derived from them by mutation and by transformation, were used exclusively. Both strains were isolated from spinal fluid from cases of fatal (nonepidemic) meningitis, and after a minimal number of transfers were retained frozen (at −60 C). They are typical gram-negative diplococci, which are oxidase positive, and ferment glucose and maltose (lactose, mannitol, and sucrose tests are negative). Aerobic incubation at 37 C of cultures on moist blood agar or infusion media results within 2 days in colonies having a diameter of 4 mm. Isolated colonies are visible within 10 hr. A waterjacketed (National) incubator was used to avoid growth restriction associated with drying of agar surfaces.

Both strains were highly sensitive to all antibiotics tested. Antibiotic resistant strains used in the experiments reported were derived by the following methods. Strain 15 *ery-r*, resistant to 3 μg per ml erythromycin (Erythrocin lactobionate, Abbott), and strain 15 *car-r*, resistant to 50 μg per ml carbomycin (Magnamycin, generously provided by Chas. Pfizer and Co., Inc.) were obtained by repeated exposure to increasing concentrations of each antibiotic, using the gradient plate technique (Szybalski, 1952).

Crystalline dihydrostreptomycin sulfate (Squibb) was used for all studies involving streptomycin-resistant (*str-r*) and streptomycin-dependent (*str-d*) strains (Miller and Bohnhoff, 1947). Strain 10 *str-r* and strain 15 *str-d* arose during cultivation of the respective *str*-sensitive (*str-s*) strains in shaken flasks of brain heart infusion; after a primary incubation period of 18 hr, a quantity of *str* sufficient to give 500 μg per ml was added. The flasks were reincubated for 20 hr to allow replication of the mutants, which were recovered upon subsequent inoculation of an agar medium (HI-1, see below) containing 500 or 1000 μg *str* per ml. A single isolated colony (per original flask) was used to establish a strain. Strain 15 *str-r* 51 was obtained by transformation, following treatment of strain 15 *str-s* with DNA extracted from strain 10 *str-r*. Strain 15 *str-d* differed from the *str-r* strains in its requirement for streptomycin (15 μg per ml or more) for unlimited multiplication.

Media. Brain heart infusion (Difco) and heart infusion broth (Difco) supported growth of these meningococci when relatively large inocula were used. Heart infusion broth was more satisfactory when supplemented with ribonucleic acid (Nutritional Biochemicals Corporation), 250 μg per ml; sodium glutamate, 0.00005 M; and calcium chloride, 0.0005 M (separately added sterile solutions). In this medium, designated HI-1, growth of strain 15 was initiated using fewer than 10^5 cells per ml, and generation times were 31 to 33 min (29 min with addition of 0.2 per cent glucose).

HI-1 with agar (Difco) added made a suitable solid medium for meningococcal growth. Concentrations of agar employed were 1.4 per cent (hard agar) or 0.7 per cent (soft agar). HI-1 agar with addition (before sterilization) of yeast extract (Difco), 0.3 per cent (by weight), was the plating medium routinely employed in transformation tests (HIY-1 agar); as before, the three supplements were added aseptically after sterilization.

Another fluid medium, HI-2, used in some experiments was heart infusion broth supplemented with 10 per cent (by volume) fresh yeast extract, 10 per cent standard salt mixture, and 0.2 per cent glucose. The salt mixture was composed of NH_4Cl, 2 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 3 g; Mg (as $MgCl_2$), 10 mg; S (as Na_2SO_4), 25 mg; distilled water 900 ml. Yeast extract was prepared from fresh filter press yeast (a gift from Red Star Yeast and Products Company, Milwaukee). The yeast cells, suspended in distilled water, were disrupted with

borosilicate beads in a mechanical shaker; the extract was washed from the beads, centrifuged, and sterilized by Seitz filtration.

DNA preparations. Extracellular DNA was obtained from the supernatant broths of slimy cultures ranging in age from 2 to 6 days (see Part II). The slime was dispersed by hand shaking, and the culture was centrifuged (3000 $\times$ G, 1 hr, 8 C). The supernatant broth was decanted into a beaker, which was rotated vigorously by hand in a horizontal plane while 95 per cent ethanol (2 volumes) was added slowly. Ideally, the resulting fibrous precipitate could be lifted out as an intact web to separate it from the fluid in which a flocculent precipitate was forming; occasionally, it was necessary to collect the precipitate by centrifugation. The fibers, washed in 75 per cent ethanol and freed of excess fluid, were dissolved in 1 M NaCl with overnight stirring at 3 C. The resulting solution was centrifuged (32,000 $\times$ G, 1 hr, 3 C) and, from the supernatant fluid, a fibrous precipitate was obtained by addition of 1 volume of ethanol. The fibers were lifted, washed in 75 per cent ethanol, and dissolved in a solution containing NaCl (0.14 M) and sodium citrate (0.015 M; "standard buffer" of Zamenhof *et al.*, 1953). Solutions of crude extracellular DNA were subjected to deproteinization with sodium dodecyl sulfate, as previously described (Catlin and Cunningham, 1958).

Intracellular DNA was obtained by lysing cellular suspensions with detergent. The cells were obtained by centrifugation of 16-hr HI-1 broth cultures or, more commonly and in higher yield, by washing off the surface growth from cultures on HI-1 agar (2.0 per cent agar) in large dishes (incubation for 16 to 18 hr at 37 C, with humidity increased to 70 per cent). To the cells, suspended in minimal amounts of standard buffer solution, finely powdered recrystallized sodium dodecyl sulfate was slowly added to a final concentration of 1 to 5 per cent; the fluid, at room temperature, was subjected to slow mechanical stirring. When lysis was essentially complete (60 to 120 min), microscopic inspection revealed only a few intact cells per oil immersion field. The massive fibrous precipitate, which formed upon addition (see procedure, above) of 2 volumes of 95 per cent ethanol, was lifted or removed by centrifugation if necessary, and was dissolved in 1 M NaCl solution with overnight stirring at 3 C. Further procedures were similar to those described for extracellular DNA. Two transforming preparations were subjected to more extensive purification, involving cetyltrimethylammonium bromide and calcium chloride steps (Catlin and Cunningham, 1958). Care was taken at all times to avoid dehydration and exposure to low ionic concentration, which reduce transforming activity (Zamenhof *et al.*, 1953).

Fibers obtained from the final ethanol precipitation were soaked in 75 per cent ethanol at room temperature for 2 to 3 hr. Using sterile glassware and aseptic precautions, excess alcohol was pressed out by rolling a stirring rod to flatten the fibers against the side of a beaker. The fibers dissolved in sterile solutions of 0.14 M NaCl or of standard buffer were stored at 3 C or were frozen at -20 C. Solutions of all DNA preparations proved to be sterile. DNA concentration was determined by the diphenylamine reaction (Dische, 1955).

Deoxyribonuclease. Crystalline pancreatic deoxyribonuclease (sterile, Worthington) was dissolved, 10 mg per ml., in sterile 2 per cent gelatin solution, and was stored at 3 C. Immediately before use, a mixture was prepared in the proportion 1 part deoxyribonuclease to 4 parts 2 M $MgCl_2$ to 5 parts 2 per cent gelatin. A quantity of mixture sufficient to give 5 to 10 μg enzyme per ml destroyed transforming activity in less than 1 min.

Recipient cells. Strain 15, and mutants derived from it, were the recipients in all transformation tests reported. Except where competence was being investigated specifically, cells to be exposed to transforming DNA were taken from cultures on HI-1 agar or blood agar which had been incubated 11 to 17 hr at 37 C. A small amount of growth from a number of punctiform colonies was dispersed in warm HI-1 broth or other exposure medium. Suspensions having a slight visible turbidity contained about 10^7 colony-forming units per ml. Microscopic examination of wet mounts showed 25 to 50 per cent paired cells, and not more than 1 to 2 per cent of larger aggregates, whereas the remainder apparently were single cells. The suspension (further diluted 1:10 to 1:100 in warm medium, as required) was mixed immediately with DNA at 36 C; deoxyribonuclease commonly was added 15 min later.

Assays. Total number of colony-forming units (= viable "cells") was determined by adding

measured volumes of an appropriate dilution of cells to 4-ml amounts of HIY-1 soft agar (liquefied and retained in a water bath at 44 C). Inoculated agar was poured without delay on top of supporting layers of solidified HIY-1 hard agar. Counts of numbers of colonies on triplicate plates, incubated 2 to 3 days at 37 C, agreed within 10 per cent. In contrast, spreading of the inoculum on agar surfaces yielded erratic colony counts, attributed to deleterious effects of relatively dry culture medium.

In the standard transformation test, *str-r* transformants were plated from 30 to 90 min after initial cellular exposure to DNA; platings were completed before the transformed cells initiated division. A sample (1.0 to 0.1 ml) was mixed with 40 ml of *str*-free HIY-1 soft agar at 44 C, and 4-ml aliquots immediately were pipetted on supporting layers (20 ± 0.5 ml) of 10 HIY-1 hard agar plates (poured several days before and held at room temperature to dry). The overlay agar solidified within 5 min, and plates promptly were placed at 37 C (without stacking); surface moisture occasionally developed, which was allowed to evaporate by judicious partial opening of the inverted plates. Five hr after the time of initial exposure of cells to transforming DNA, plates were removed to room temperature, and were overlaid with a 4-ml top layer of HIY-1 soft agar containing *str* in a quantity sufficient to give 500 μg per ml after diffusion of the *str* through the underlying agar. Plates were incubated at 37 C for 3 to 4 days before colonies were counted.

Strain 15 *str-d*, used as recipient for transformation to *str-r* or *str-s*, was cultivated on HI-1 agar containing 500 μg *str* per ml. Nondependent transformants were plated in HIY-1 *str*-free soft agar, as described; top layers were not added.

Tests of transformant colonies. Several hundred transformants of each class (*str-r, str-s, str-d*) were identified by suspending a small amount of colonial growth in a drop of sterile broth and streaking corresponding areas of two kinds of HI-1 agar plates, one *str*-free and the second containing 500 μg *str* per ml. Growth on both plates indicated that the transformant was *str-r*. *Str-d* produced visible growth only on the *str*-containing agar, and *str-s* only on *str*-free agar. Without exception, transformants corresponded to the type of cell from which the transforming DNA had been extracted.

The oxidase reaction was employed as a rapid method for detecting possible contaminant colonies. Most contaminants fail to react, whereas colonies of neisseria rapidly darken in the presence of a 1 per cent solution of dimethyl-*p*-phenylenediamine hydrochloride (*p*-aminodimethylaniline monohydrochloride, Difco). The reagent readily penetrated to deep colonies when these were stabbed with a sterile needle.

Controls. All transformation tests were accompanied by one or both of two controls: (1) transforming DNA which had been inactivated by addition of crystalline deoxyribonuclease 5 min before adding recipient cells, (2) DNA preparation extracted from the same strain which was to serve as recipient. In other respects these control tests were similar to the transformation tests. Therefore, together, they served to exclude error which might arise from: mutations (spontaneous or, conceivably, induced); selective action on the recipient population of DNA-split products (Firshein and Braun, 1958), or of intact DNA (which lacks the specific genetic marker being investigated); failure of deoxyribonuclease to inactivate transforming DNA; the presence of contamination in any of the solutions. Controls were uniformly negative in the standard test; mutants were encountered in two special cases, as will be indicated.

RESULTS

Part I. Factors influencing transformation ratios. (1) Phenotypic expression:—When streptomycin-sensitive cells are exposed to transforming DNA extracted from a *str-r* culture and, shortly thereafter, are challenged with *str*, few or no transformants resistant to 500 μg *str* per ml are obtained. Transformation is demonstrated, however, if the same cells are incubated for a period sufficient to allow several divisions in *str*-free medium before exposure to the antibiotic. This delay in phenotypic expression, which has been recognized for all the transformation systems, creates a methodological problem. On the one hand, each cell which receives a *str-r* marker must be retained in a suitable *str*-free environment until resistance has developed. On the other hand, these cells must be placed in solitary confinement before any one of them undergoes division; only thus will each colony indicate an independent genetic event. Under the conditions defined for the pneumococcus system (Hotchkiss,

TABLE 1

Time required for phenotypic expression of streptomycin-resistance by Neisseria meningitidis strain 15 str-s

A. In Streptomycin-free Broth at 37C[a]

Time sampled	Total viable cells plated/ml	*Str-r* cells/ml[b]	*Str-r* cells/ one million viable cells
min			
5	4.1×10^5	0	—
30	6.1×10^5	0	—
80	1.4×10^6	1	0.7
110	2.4×10^6	182	76
140	4.3×10^6	572	133
165	7.8×10^6	1182	152
193	1.3×10^7	2186	168

B. In Streptomycin-free Agar at 37 C[c]

Total incubation before addition of *str*	Transformants/ml
min	
270	472
300	547
330	546

[a] Cells taken from 17-hr HI-1 agar, suspended in HI-1 broth +0.2 per cent glucose; DNA from strain 15 *str-r* 51, 5 μg/ml, added at time 0; deoxyribonuclease added at 15 min.

[b] Assayed 1.0 ml in 39 ml HIY-1 soft agar + 500 μg *str*/ml, overlaid 4 ml/plate on 10 supporting layers HIY-1 hard agar + 500 μg *str*/ml.

[c] Culture (*A*, above) sampled at 45 min, 3 ml mixed with 117 ml HIY-1 soft agar, overlaid 4 ml/plate on 30 supporting layers HIY-1 hard agar; at intervals, groups of 10 plates were top-layered with HIY-1 soft agar + *str* to give 500 μg *str*/ml after diffusion.

1957), all of the *str-r* transformants develop resistance in broth during a limited time beginning about 30 min after exposure to DNA, but their division is delayed for another 1 to 2 hr. During this interval, the population may be sampled directly into a *str*-containing medium.

Phenotypic expression was investigated in six experiments with meningococci. Table 1 (*A* and *B*) shows results of one of these experiments; comparable results were obtained in others, which tested cell suspensions prepared from 15-hr blood agar cultures, and several modifications of the exposure medium (HI-2 broth, with separate omissions of the glucose and the fresh yeast extract). With the media employed, individual cells of a population varied with respect to the period of metabolic activity required before resistance to 500 μg *str* per ml was developed. A few cells became resistant 80 to 90 min after initial exposure to the DNA; no *str-r* transformants were detected in earlier samples. In the six experiments, expression times for the remaining transformants were distributed over the ensuing 60- to 90-min period (continuous incubation at 37 C); this is reflected (table 1*A*) in the rapid increase of the number of resistant cells per one million total viable cells plated during the 80- to 140-min period. Thereafter, increase of *str-r* presumably was due largely to division of cells whose resistance was expressed earlier, though a small part of the increase may have been due to longer delay of phenotypic expression.

Since a period of incubation in broth was not found which would provide 100 per cent phenotypic expression without also allowing division of some transformants, the procedure of plating the DNA-treated cells 30 min after application of crystalline deoxyribonuclease was investigated in two of the experiments mentioned above. Table 1*B* shows results of a test in which phenotypic expression and subsequent division of transformants occurred after the cells were plated in *str*-free HI-1 soft agar. Top layers of medium containing *str* were added at intervals. One-hundred per cent phenotypic expression was obtained with an incubation period of 5 hr elapsing between time of initial exposure of cells to DNA and time of addition of streptomycin. In this test, one transformant was detected for every 750 cells exposed to DNA. This plating procedure was employed for subsequent transformation tests.

(2) Cellular competence:—Meningococcus populations exhibited the capacity to become transformed when sampled at intervals during an entire 24-hr period of cultivation in broth at 37 C (table 2). During the lag and logarithmic growth phases, a relatively large proportion of the cells was competent, in comparison with cultures of *Haemophilus influenzae* (Alexander *et al.*, 1954) and pneumococcus (Hotchkiss, 1954) at correspondingly early hours.

In three experiments with *N. meningitidis* strain 15 *str-s*, transformation tests were carried out periodically with samples taken from broth

TABLE 2

Relation between culture age and cellular competence of Neisseria meningitidis strain 15 str-s

Culture[a]		Transformation Test[b]		
Time sampled	Total viable cells/ml	Total viable cells/ml exposed to DNA (*E*)	*Str-r* transformants/ml (*T*)	$(T/E) \times 10^5$
hr				
0	1.5×10^6	1.5×10^6	1,673	112
1	2.9×10^6	2.9×10^6	4,950	171
2	7.2×10^6	7.2×10^6	12,240	170
3	2.0×10^7	2.0×10^7	39,700	199
4	6.9×10^7	6.9×10^7	—[c]	—
5	2.0×10^8	2.0×10^6	4,365	218
6	3.9×10^8	3.9×10^6	8,400	215
7	6.2×10^8	6.2×10^5	1,309	211
8	1.1×10^9	1.1×10^6	1,323	120
24.5	2.8×10^9	2.8×10^9	2,137	0.076
		2.8×10^6	815	29
Controls:				
0	1.5×10^6	1.5×10^6	0	
24.5	2.8×10^9	2.8×10^9	787[d]	
		2.8×10^6	0	

[a] HI-2 broth inoculated at 0 hr with cellular suspension from 13-hr HI-1 agar culture; incubated at 37 C without aeration.

[b] Three and nine-tenths ml of culture (undiluted, or diluted with fresh HI-2 broth) mixed at 37 C with 0.1 ml DNA (strain 10 *str-r*), final concentration 2 μg/ml; deoxyribonuclease added after 12 min; samples for assay of *T* plated at 45 min.

[c] Colonies too numerous to count.

[d] Spontaneous *str-r* mutants developing upon background growth; correction factor used to obtain value of *T* for corresponding culture.

cultures. In the first experiment (table 2), the number of transformants per 10^5 treated cells ($(T/E) \times 10^5$) ranged from 112 to 218 during the first 8 hr, and the value was 29 at the end of 24.5 hr. Undiluted samples of the culture were tested at all times and, beginning with the 5-hr sample, a dilution of the culture in fresh broth also was tested. Although uncountable, the estimated numbers of colonies on transformant plates prepared from the undiluted 5-hr to 8-hr samples were roughly in agreement with results obtained with the corresponding diluted samples. In contrast, a countable number of colonies developed, together with a haze of background growth, on plates from the transformation test carried out with the undiluted 24.5 hr culture; the corresponding test, carried out with a 1:1000 dilution of the same culture sample, resulted in a 380-fold increase in recovery of *str-r* transformants (in terms of *T* for the undiluted culture). This resembles the dilution effect shown by Schaeffer (1956) with younger (3 to 4 hr) cultures of *H. influenzae*.

The early growth phases were reexamined in two experiments. The cultures, in HI-1 broth, were sampled at 30 min intervals for 5 hr, and uniformly were diluted 0.5 ml in 2.0 ml of fresh broth containing the transforming DNA preparation. For one culture, the number of transformants per 10^5 treated cells varied only within the range 125 to 178; the second culture, however, gave fluctuating values: 74 at 0 hr, increasing to 1241 at 3 hr, and thereafter decreasing to about 700. During exponential growth, generation times were 31 and 33 min for the cultures in HI-1 broth, and 36 min for the culture in HI-2 broth.

TABLE 3

Effects of concentration of DNA and time of exposure on transformation of Neisseria meningitidis to streptomycin-resistance[a]

Deoxyribonucleate		Transformants/ml (*T*)	$(T/E) \times 10^5$
Conc	Exposure time[b]		
μg/ml			
5.0	15	479	399
2.0	15	410	342
1.0	15	330	275
0.5	15	306	255
0.1	15	198	165
0.01	15	36	30
0.001	15	7	6
2.0	10	246	205
2.0	5	131	109
2.0	2	44	37
2.0	1	30	25
2.0	+	1301	1083

[a] Strain 15 *str-s*, from 16-hr culture on HI-1 agar, suspended in HI-2 broth; 1.2×10^5 cells/ml (*E*) were exposed at 36 C to DNA (from strain 10 *str-r*).

[b] Exposure to effective DNA terminated by deoxyribonuclease, except for "+" where cells were plated, after 50-min exposure, without added enzyme.

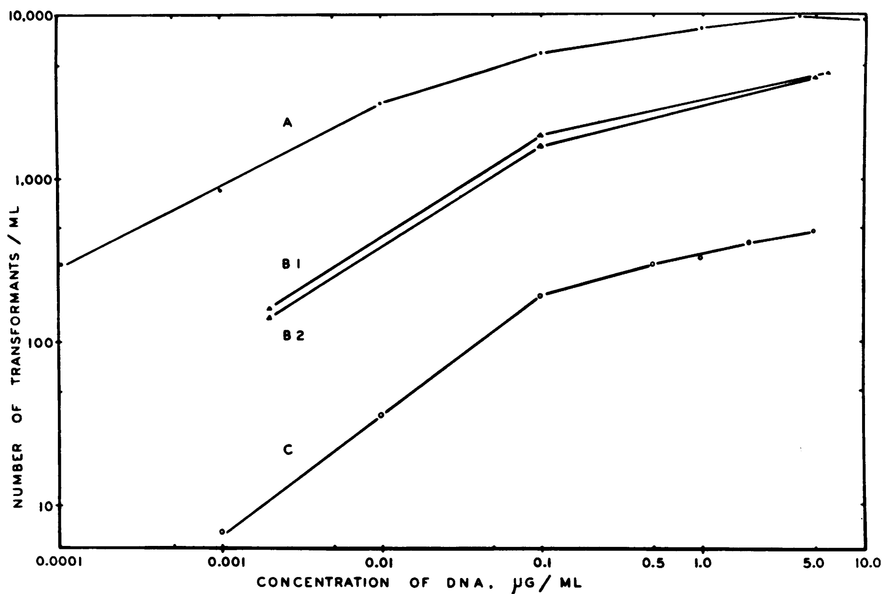

Figure 1. Concentration-response curves of *Neisseria meningitidis str-r* transformants plotted against concentration of transforming DNA preparation (both logarithmic). *Curve A:* transformation of strain 15 *str-d* (4.0×10^6 viable "cells"/ml) by DNA from strain 15 *str-r* 51. *Curves B1* and *B2:* transformation of strain 15 *car-r str-s* by DNA preparations (intracellular, B1; extracellular, B2) from strain 15 *str-r* 51 (table 5). *Curve C:* transformation of strain 15 *str-s* by DNA from strain 10 *str-r* (table 3).

(3) DNA concentration:—The relation between yield of transformants and DNA concentration has been examined for each of the recipient strains, and for the various DNA preparations. Results obtained with a preparation of intracellular DNA from strain 10 *str-r* are shown in table 3. A suspension containing 1.2×10^5 colony-forming units per ml of strain 15 *str-s* provided the recipient cells. Use of a denser suspension, in other experiments, demonstrated transforming activity in lower concentrations of this same DNA preparation, and the shapes of the concentration-response curves were the same.

For purposes of comparison with other concentration-response curves, data of table 3 (15-min exposures) are plotted in figure 1 (*C*). Curves *B1*, *B2*, and *C*, which delineate transformation from *str-s* to *str-r*, are essentially parallel in spite of differences relating both to the DNA-donor and to the number and kind of recipient cells. A linear relation in the range below 0.1 μg DNA per ml was found, as expected on the basis of results with other transformation systems (Alexander *et al.*, 1954; Hotchkiss, 1957; Spizizen, 1958).

Cellular capacity for response to DNA is "saturated" when concentrations of DNA between 5 to 10 μg per ml are present for 15 min before addition of deoxyribonuclease. Higher concentrations of DNA usually did not increase the yield of *str-r* transformants in samples of a given population. This is shown in figure 1 (*A*) for transformation of the streptomycin-dependent strain to nondependence (*str-r*), where 4.0 μg DNA per ml elicited 248 transformants per 10^5 exposed cells. DNA concentrations of 10.0 and 1.0 μg per ml both elicited slightly fewer transformants.

(4) Time of exposure to DNA:—Transforming DNA is taken up by competent cells and bound in some manner which protects it from destruction by added crystalline deoxyribonuclease (Lerman and Tolmach, 1957). This process was completed in less than 1 min by some cells of an exposed meningococcus population, as shown in table 3. The number of transformants increased linearly with time of exposure to transforming DNA during the initial 15 min. When deoxyribonuclease was not added, and the cells were plated after an exposure period of 50 min, over 1 per cent of the population became transformed.

A standard exposure time of 15 min before

addition of deoxyribonuclease was arbitrarily chosen (Hotchkiss, 1957) for most of the experiments described in Part II, although a longer time would have increased the yield of transformants.

Part II. Transformation by extracellular DNA. Early investigators recognized the rapid loss of viability characteristic of meningococcus cultures, and the necessity for frequent subcultures. Flexner (1907) observed a progressive appearance of swollen forms, loss of staining, and disintegration of meningococci during a 2- to 3-day period of incubation. These changes, which were attributed to the action of an autolytic enzyme, were shown to be accelerated by desiccation of the surface of agar media, and to be delayed by addition of calcium to an otherwise suitable medium.

Strain 15 employed in the present investigation, likewise, is subject to rapid loss of viability, both on agar and in broth. The composition of the medium and degree of aeration were important, as would be expected, in determining both the inoculum size required for initiating growth and the characteristics of subsequent growth phases. A population size of over 10^9 colony-forming units per ml commonly was maintained well beyond 24 hr in an appropriately supplemented heart infusion broth (table 2). However, with heart infusion broth supplemented only with 0.2 per cent glucose, or with brain heart infusion, an inoculum of 10^6 viable cells per ml, and aeration, were required for initiation of growth; moreover, loss of viability of some cultures exceeded 3 logarithmic decades within 24 hr (table 4). When such cultures were removed from the shaker at 16 hr, but were retained at 37 C, a slimy sediment gradually accumulated without experimental intervention. The time of appearance and extent of accumulation of slime varied somewhat in cultures of the same strain, possibly owing to differences in rate of decline of culture viability. Commonly, however, slime could be detected within 24 hr as a film adherent to the glass; 5 to 10 ml of thick, tenacious material often could be aspirated with a pipette from the bottom of a 300-ml culture within 2 to 4 days.

Extracellular DNA preparations from cultures of strain 15 *str-r*, which had been incubated at 37 C for 2 to 4 days, were found in preliminary experiments using strain 15 *str-s* and *str-d* as recipients to have transforming activities comparable to those of intracellular DNA preparations. This raised the question whether the crude slime has similar transforming activity.

To eliminate viable *str-r* slime-donor cells without affecting the slime which was the source of transforming DNA, an antibiotic (either erythromycin or carbomycin) was employed which inhibited the growth of donor cells, but not of recipient cells. This antibiotic was incorporated in all culture media used in the transformation test after exposure of recipient cells to slime. Thus, cells to be transformed were in contact with the slime in the usual antibiotic-free medium. The presence of the extra antibiotic did not interfere with the selective action of streptomycin, added subsequently. The competence of recipient strains (resistant to 3 μg *ery* per ml or to 50 μg *car* per ml) was somewhat reduced in comparison with that of the sensitive parent strain 15 under the same conditions. Because of its higher level of resistance, the *car-r* strain was used where the slime was expected to contain numerous viable cells. Each dilution of slime (in heart infusion broth) tested for transforming activity was examined also in a control test (crystalline deoxyribonuclease added to the slime 5 min prior to contact with recipient cells). Control plates were negative in all tests, except one (table 4, B, 3) where the slime had contained enough viable cells to provide the opportunity for mutation to erythromycin (1 μg per ml) resistance (T was corrected, accordingly).

Results of three experiments employing the *ery-r* strain as recipient are compiled in table 4. Transforming capacity of crude slime, in final dilutions of 1:5 to 1:25, was equal to or slightly greater than that of a partially purified preparation of extracellular DNA, which was tested in each experiment at a concentration of 5 μg per ml (and, also, in lower concentrations in experiment C), as shown by the ratio T (slime) to T (DNA, 5 μg per ml). Thus, procedures for extracting and deproteinizing the DNA were not required for demonstrating transforming activity of culture slime.

To determine whether transforming activity of slime would decline with storage at room temperature, undiluted slime retained in a tightly capped sterile tube was retested (table 4, C, 4) 7 days after the initial test (B, 4). Transforming activity of the 1:25 dilutions examined in the

TABLE 4

Transforming activity of slime DNA accumulating in meningococcus cultures of various ages

Expt	Culture (strain 15 *str-r ery-s*)[a]			Transformation Test[b]			
	No.	Incubation time	Total viable cells/ml	Culture slime (final dilution)	T	$(T/E) \times 10^5$	T (slime) / T (DNA, 5 μg/ml)
		hr					
A	1	72	5.0×10^1	1:5	1678[c]	258	4.44
	2	9	5.1×10^8	—			
	2	24	3.2×10^5	—			
	2	47	1.5×10^2	1:8	380	58	1.01
B	3	24	1.4×10^7	—			
	3	45	3.0×10^5	1:5	886	74	1.01
	3	45	3.0×10^5	1:25	830	69	0.95
	4	144	0	1:25	1068	89	1.22
C	4	144[d]	0	1:25	554	71	1.09
	5	92	0	1:5	597	77	1.18
	5	92	0	1:25	540	69	1.07
	5	92[e]	0	1:5	620	80	1.23
	DNA	—	—	DNA, μg/ml			
				5.0	506	65	—
				0.5	386	50	—
				0.05	161	21	—
				0.005	60	8	—

[a] Cultures 1 to 4 in brain heart infusion; 5 in heart infusion with 0.2 per cent added glucose; incubated at 37 C, on shaker for original 18 hr. DNA = extracellular DNA harvested at 76 hr from supernatant broth of culture 1 (experiment A), and deproteinized.

[b] Recipient cells, strain 15 *str-s ery-r*, from 15-hr cultures on HI-1 agar + 1.0 μg *ery*/ml, were suspended in heart infusion broth; 2.0 ml was mixed at 36 C with 0.5 ml of culture slime (or DNA); E (number of recipient cells/ml exposed to DNA) 6.5×10^5 for experiment A, 1.2×10^6 for B, 7.8×10^6 for C. Deoxyribonuclease added after 15 min, except in experiment A, 1. T (number of *str-r* transformants/ml) assayed in HIY-1 soft agar + 1.0 μg *ery*/ml; top layer, same + *str* (to give 500 μg *str*/ml after diffusion).

[c] Sample plated after 50 min exposure without added deoxyribonuclease.

[d] Slime aspirated aseptically from 144-hr culture (B, 4), stored at room temp for 7 days, and retested.

[e] Undiluted slime heated at 80 C (±0.1 C) for 30 min, immediately cooled, and tested.

two tests exceeded that of the DNA preparation, used at 5 μg per ml. Thus, a deoxyribonuclease highly active against slime DNA was not released under these conditions.

Several lines of reasoning suggest the necessity for investigating possible deoxyribonuclease activity in meningococcus cultures: (1) When bacteria release DNA, they might also release intracellular deoxyribonuclease. (2) Extracts of sonically disintegrated meningococci contain a deoxyribonuclease which, although highly active against several DNA preparations, exhibited little activity in viscometric tests against meningococcus DNA preparations (Catlin, 1959). This or another deoxyribonuclease might become active under certain special conditions. (3) Cultures of meningococci grown under apparently similar conditions do not become equally slimy. Consequently, the activity of slime accumulating in unusually small amounts was investigated.

Table 5 shows the transforming activity of slime from a culture in brain heart infusion (300 ml in 1000-ml flask) incubated at 37 C for 116 hr (with shaking for the first 19 hr). The turbid broth was decanted from a slight film of slime clinging to the bottom of a flask. The slime was

TABLE 5

Transforming activity of culture slime and DNA preparations from Neisseria meningitidis strain 15 str-r 51[a]

Material Tested	Transformants/ml
Crude slime, 116-hr culture:	
1:5 dilution	4120
1:5 dilution (control)[b]	0
1:100 dilution	1612
1:100 dilution (control)[b]	0
Extracellular DNA solution, μg/ml:	
5.0	4237
0.1	1611
0.002	142
Intracellular DNA solution, μg/ml:	
6.0	4513
0.1	1900
0.002	162
6.0 (control)[b]	0

[a] DNA solution or culture slime, 0.5 ml, was mixed at 36 C with 2.0 ml suspension in HI-1 broth of recipient cells (strain 15 *car-r str-s*, 6.1 × 10^6 viable cells/ml exposed); deoxyribonuclease added after 15 min; transformants plated in HIY-1 soft agar + 20 μg *car*/ml; top layer of same medium + *str* (to give 500 μg *str*/ml after diffusion) added at 5 hr.

[b] Deoxyribonuclease added 5 min before mixing with cells.

dislodged by vigorous mixing with 2 ml of sterile heart infusion broth. An assay of this slime mixture revealed 1.7 × 10^7 colony-forming units per ml. As in other cultures, extracellular DNA was present at a concentration sufficient to elicit approximately the same number of transformants as were obtained with transforming DNA preparations from extracellular and from intracellular locations, tested in the same experiment at concentrations of 5.0 and 6.0 μg DNA per ml. Results obtained with these two DNA preparations are plotted in figure 1 (*B1*, *B2*) to show their similarity to curves obtained using different strains as recipients and as DNA-donors.

DISCUSSION

The work which has been described was initiated to investigate the structural integrity of extracellular DNA. DNA separated by alcohol precipitation from the supernatant broth of centrifuged slimy meningococcus cultures, and subsequently purified, was genetically active. That such DNA actually was extracellular, rather than merely being liberated during experimental manipulation of the culture, was verified by the capacity of crude culture slime to elicit transformation as a result of less than 15 min contact with recipient meningococci. The similarity of transforming activities of DNA preparations from intracellular and from extracellular locations indicates that DNA may remain in the extracellular environment for some time with little or no destruction of the information-bearing molecular structure.

The possibility that the genetic change elicited by crude culture slime is the result of a process of conjugation or of virus-mediated transduction, rather than of transformation, appears unlikely in view of the susceptibility of the agent to the action of deoxyribonuclease, and the high frequency of the genetic event. Moreover, transforming activity was retained by crude slime which had been heated for 30 min at 80 C (table 4, C, 5); this finding was not unexpected, in view of the heat stability of purified preparations of *H. influenzae* transforming DNA (Zamenhof *et al.*, 1953).

Hotchkiss (1951) suggested that conditions for transformation with lysates might arise in nature. Transformation was obtained in a mixture of two strains of pneumococci; one was penicillin-sensitive and streptomycin-resistant, the other was *pen-r* and *str-s*. Addition of penicillin resulted in lysis of *pen-s* cells, whose DNA thereupon transformed the *pen-r* cells to *str-r*.

Likewise, transformation of meningococci by the DNA of slime which forms spontaneously may occur in nature. This would require the simultaneous presence of genetically active extracellular DNA, competent cells, and conditions for uptake of the DNA. Although the optimum conditions for the development of cellular competence may not be the same as those required for uptake of transforming DNA, they are not mutually exclusive. Thus, capacity to become transformed was maintained by a considerable fraction of meningococci during an entire 24-hr period of cultivation (table 2). Competence was demonstrated not only when the aging cells were diluted into a fresh medium containing transforming DNA, but also when a small amount of DNA preparation was added to the

8-hr or 24-hr culture. These findings provide a basis for the idea that natural variation of meningococci, and perhaps other bacteria, may be enhanced through the agency of transformation. Some meningococci which differ by one or more genes from other members of a heterogeneous population may survive in an environment in which slime DNA is accumulating. Competent cells taking up extracellular DNA at random may construct new genetic combinations, thereby increasing the range of adaptibility of the total population.

The transformation method described for meningococcus is reproducible, and satisfies minimal requirements for a quantitative system (Hotchkiss, 1957). The factors which influence the yield of transformants have not been investigated sufficiently, however, to justify comparisons with other transformation systems, beyond those previously indicated. Experiments with defined media, which are in progress, may elucidate factors contributing to competence of meningococci, and thereby provide a basis for increasing transformation ratios.

SUMMARY

Highly polymerized deoxyribonucleate (DNA) is a component of the slime which may accumulate in cultures of *Neisseria meningitidis* as cellular viability declines. To investigate possible genetic activity of such extracellular DNA, a quantitative transformation method was developed. Various factors were studied which influence transformation of streptomycin-sensitive meningococci to streptomycin resistance: cellular competence, time of exposure and concentration of transforming DNA, and phenotypic expression. Transformation of a streptomycin-dependent meningococcus strain to nondependence, also, was studied.

Extracellular DNA was separated by ethanol precipitation from the supernatant broth of centrifuged slimy cultures (streptomycin-resistant strain cultivated in brain heart infusion broth at 37 C for 2 to 4 days). Intracellular DNA was extracted from detergent-lysed cells. Solutions of partially purified DNA preparations obtained from the two locations exhibited similar transforming activities. Moreover, crude culture slime, in dilutions as high as 1:25, elicited about the same number of transformants as were obtained using transforming DNA preparations, tested at a concentration of 5 μg DNA per ml. Thus, procedures for extracting and purifying the DNA were not required for demonstrating transforming activity of extracellular DNA. The possibility was discussed that natural variation of meningococci, and perhaps of other bacteria, may be enhanced through the agency of transformation by slime layer DNA.

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