

## RELEASE OF GENETIC TRANSFORMING AGENT FROM PNEUMOCOCCAL CULTURES DURING GROWTH AND DISINTEGRATION\*

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Since the appearance, in 1944, of Avery, MacLeod, and McCarty's classic paper (1) on the chemical nature of the transforming principle of *Diplococcus pneumoniae* (pneumococcus) most of the experiments in the field of transformation genetics have been carried out with purified deoxyribonucleate. In 1951 Hotchkiss (2) reported, however, that drug-induced "lysates" of fresh cultures of pneumococci could be used as donor material in transformation experiments. The initial purpose of the present work was to investigate some genetic and physiologic aspects of lysate transformation. In this system penicillin or later streptomycin was used to kill sensitive organisms carrying a suitable genetic marker. If appropriate recipient cells resistant to the drug were mixed with the sensitive population, a fraction of the resistant recipients was transformed by genetic material from the dying population. The lysate transformation method has served principally as a tool for simplified transformation tests. A reinvestigation of lysate transformation is of interest because present knowledge about genetic transformations makes it profitable to study this complex system in which the genetic material may in certain respects resemble that present within living cells.

During the course of experiments on streptomycin-induced lysate transformations, it was found unexpectedly that untreated, presumably normal cultures also released significant amounts of active genetic material into the culture medium (3). This finding broadened the scope of the investigation, and suggested that a biological role for naturally occurring cell-to-cell transformations was not improbable. The present communication deals with the nature of the process of release of genetically active material from streptomycin-treated and from untreated growing pneumococcal cultures.

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### *Materials and Methods*<sup>1</sup>

**Bacterial Strains.**—The experiments to be described were performed with *Diplococcus pneumoniae* strain R6 a single-colony derivative of the rough (unencapsulated) strain R36A (1)—and single or multiple drug-resistant variants of the R6 wild type. The variants were obtained by transformation of the wild type by deoxyribonucleate extracted from cultures of spontaneously arising mutant strains resistant to streptomycin, sulfanilamide, micrococcin, or amethopterin in the following amounts:

**Streptomycin (Sm):** (Streptomycin sulfate-Lilly)-1000  $\mu\text{g}$  per ml. Selection of transformants was usually carried out at 150  $\mu\text{g}$  per ml. The resistant strain will be referred to as RSm.

**Sulfanilamide (SA):** (sulfanilamide-Nutritional Biochemicals Corporation, Cleveland)—800  $\mu\text{g}$  per ml. This mutation, found and analyzed by Hotchkiss and Evans (4), involves a complex locus only parts of which were present in the strains used in this study. These strains and their resistance levels in  $\mu\text{g}$  SA per ml were: RFa (20), RFd (80), and RFad (400). Cells having these genotypes can be selected for respectively at 10, 40, and 150  $\mu\text{g}$  per ml of sulfanilamide.

**Micrococcin (abbreviation, K):** (Kindly supplied by Dr. Norman Heatley, Oxford University)—about 400  $\text{m}\mu\text{g}$  per ml. The selective broth contained 100  $\text{m}\mu\text{g}$  per ml. Resistant strain is designated RK.

**Amethopterin (Am):** (amethopterin-Lederle)—2  $\mu\text{g}$  per ml. Either amethopterin or aminopterin (Lederle) at 0.5  $\mu\text{g}$  per ml could be used for selection of transformants in the presence of wild type cells. Resistant strain is called RAm.

**Preparation and Analysis of Deoxyribonucleate.**—The deoxyribonucleate was prepared by the method described by Hotchkiss (5) and based on the method of McCarty and Avery (6). A measure of the amount of deoxyribonucleate present in a sample of purified material was obtained from the ultraviolet absorption of the sample at 260  $\text{m}\mu$  (Beckman DU spectrophotometer). The native DNA content of the sample was estimated from the absorption increment following digestion of the DNA by pancreatic deoxyribonuclease, taking 33 per cent as the ideal maximum increment for native material (7).

**Growth Media.**—For most of the experiments the bacteria were grown in the casein hydrolysate medium described by Fox (8) based on the medium of Adams and Roe (9). This medium (CH) was modified by the addition of 0.05 per cent neopeptone, 0.05 per cent enzymatic casein hydrolysate (Nutritional Biochemicals Corp.) and bovine serum albumin fraction V (Armour and Company) to reach a final concentration of 0.2 per cent, a concentration which was reported to be optimal (10) for the development of the transformable state in pneumococcal cultures. The yeast extract was adsorbed with Baker and Adamson decolorizing carbon before use. For some experiments, a medium which will be referred to as AGCH was used. The contents of this medium were the same as those of CH except that yeast extract and glucose were omitted and 1 per cent glutamine, 0.5 per cent adenine, and 0.004 per cent sucrose were included. Another medium (AB) was employed which consisted of beef heart infusion broth (0.5 pounds of meat per liter of water), enriched by 1 per cent neopeptone, 0.2 per cent glucose, 0.2 per cent albumin, 0.002 per cent calcium chloride, and 0.05 per cent magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) and buffered by  $\text{m}/80$  dibasic potassium phosphate.

**Transformation Procedure.**—Bacteria to be used as donors were freshly grown on the day of use from an inoculum taken from a culture stored in 10 per cent glycerol at  $-20^\circ\text{C}$  and thawed at  $0^\circ\text{C}$ . The donor populations to be used in streptomycin-induced lysate transformation experiments were streptomycin-sensitive but sulfonamide-resistant.

<sup>1</sup> Abbreviations used (besides those identifying strains, markers, or drugs identified in this section): DNA, deoxyribonucleate; DNase, deoxyribonuclease (either pancreatic or pneumococcal); RNase, ribonuclease, pancreatic.

Bacteria to be used as recipients in transformation experiments were grown in CH medium to a transformable state and then frozen in 10 per cent glycerol at  $-20^{\circ}\text{C}$ . The cultures were thawed at  $0^{\circ}\text{C}$  immediately before use. Such cultures remained transformable for 2 to 3 months of storage (11). The recipients for the streptomycin-induced lysate transformations were streptomycin-resistant but sulfonamide-sensitive.

Transformations were carried out by exposing freshly thawed diluted recipient cultures to donor material for 30 minutes at  $30^{\circ}\text{C}$  except when stated otherwise. For lysate transformations the donor material contained  $250\ \mu\text{g}$  per ml streptomycin, an amount found to produce rapid killing of sensitive cells and effective release of transforming material. Approximately equal volumes (0.5 ml) of donor and recipient were used. The reaction was terminated at the desired time by the addition of DNase (Worthington pancreatic deoxyribonuclease 1X recrystallized)  $1.5\ \mu\text{g}$  per ml and magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) to reach a concentration of 0.02 per cent. The enzyme destroyed any deoxyribonucleate not already taken up by the cells. The cultures were then incubated at  $37^{\circ}\text{C}$  for 30 to 90 minutes to permit expression of the newly acquired traits, after which aliquots of the cultures were diluted into the appropriate scoring medium. The scoring medium (4) was essentially AB medium as described above except that calcium and magnesium were not added and one-sixth the amount of charcoal-adsorbed yeast extract present in CH medium (8) was used. The scoring broth contained 2 to  $5\ \mu\text{g}$  per ml of antibody globulin from horse or rabbit antipneumococcal (anti-R) serum in the presence of which each viable bacterial unit gives rise to a visible colony (4). Total population size and number of transformants were estimated from colony counts after 16 to 20 hours of incubation of the diluted culture at  $37^{\circ}\text{C}$ . In antibody-containing non-selective and selective broth. In some experiments total population size and average chain length also were estimated by microscopic counts in a Petroff-Hausser counting chamber.

*Separation of Cells from Culture Fluid.*—Separation of the cells from culture fluid was accomplished by filtration through Millipore filters of porosity of  $0.45 \pm 0.02\ \mu$  (Millipore Filter Corporation).

*Folic Acid Estimation.*—Estimation of folic acid was done by using the *Streptococcus faecalis* bio-assay. The assay cultures were grown for 48 hours at  $37^{\circ}\text{C}$  in Difco folic acid assay broth according to the directions provided with the medium. The reference substance was pteroylglutamic acid. Optical density readings were taken with a Coleman colorimeter at  $655\ \text{m}\mu$ .

*Deoxyribonucleoside Estimation.*—Deoxyribonucleosides were assayed by the method of Travaglini and Schultz as described by Miller (12). The bio-assay organism, *Lactobacillus acidophilus* R-26, and the constituents of the assay broth were kindly provided by Dr. Travaglini. The samples to be assayed were incubated for 4 hours at  $37^{\circ}\text{C}$  with pancreatic DNase and snake venom at a concentration of  $10\ \mu\text{g}$  per ml each. As a control for the digestion procedure a known sample of high molecular weight DNA was also used in each experiment. The digested filtrates were assayed directly. To measure intracellular deoxyribonucleosides, prior to enzymatic digestion the cultures were treated with deoxycholate, 0.005 per cent, for 30 minutes at  $37^{\circ}\text{C}$ , a procedure which lysed the cells but did not interfere with the assay system if the lysate was diluted at least 1:10 in the final assay tube. The growth medium used for these experiments was AGCH without neopeptone. Media containing neopeptone or yeast extract could not be used because they were found to produce very high blanks. *Crotalus adamanteus* venom was purchased from the Ross Allen Reptile Institute, Silver Springs, Florida. Optical density readings were taken with a Coleman colorimeter at  $655\ \text{m}\mu$ . The lactobacillus strain R-26 used in this method is independent of vitamin  $\text{B}_{12}$ , cannot utilize the free bases or deoxyribose, but depends entirely on the presence of deoxyribonucleosides and deoxyribonucleotides for growth (12). It was found from control studies with polymerized pneumococcal DNA that both the DNase and the venom phosphatase were required for maximal growth-promoting activity to be released from the DNA. It was also found that a

sample of digested DNA which was boiled at pH 2 for 5 minutes could promote only one-half the amount of growth of the lactobacillus as could a control sample. This procedure should destroy the growth-promoting effects of purine deoxyribonucleosides and deoxyribonucleotides and therefore should reduce the growth effect of a DNA digest to about one-half. These results indicate that the materials being measured in this bio-assay were deoxyribonucleosides and deoxyribonucleotides.

*Viscosity Measurements.*—Viscosity measurements were made with Ostwald viscometry pipettes with a flow time for water at 20°C in the range of 80 to 100 seconds. The diluted DNA sample was mixed with an equal volume of culture filtrate or sterile medium containing  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  to reach a final concentration of 0.01 per cent which is suitable for pancreatic DNase activity (13). 5 ml of the mixture was placed in each viscometry pipette, after which 0.05 ml toluene, reagent grade, was added to the lower bulb of each pipette to prevent contamination. Care was taken not to draw any toluene into the capillary. Flow times were measured at 37°C.

The thymus deoxyribonucleate for these tests was kindly provided by Dr. Muriel Roger Beckmann of this laboratory.

## RESULTS AND DISCUSSION

### 1. *Experiments on Streptomycin-Induced Lysate Transformations*

*Nature of the Streptomycin-Induced Lysate Transformation System.*—In confirmation of previous findings on penicillin-induced lysate transformation (2), the transforming activity of streptomycin lysates was completely destroyed by pancreatic DNase. Therefore DNA was specifically responsible for the activity, and in this respect, the streptomycin lysate system also resembles transformation by purified DNA.

*Kinetics of Release of Transforming Material from Streptomycin-Treated Sensitive Cultures.*—The results of the experiment illustrated in Fig. 1 are typical of those obtained when young cultures were exposed to streptomycin for varying lengths of time. Although under these conditions at least 80 per cent of the cells lost viability within the first 15 minutes of drug treatment, release of DNA-containing material continued for at least an hour. This observation agrees qualitatively with the work of Roth *et al.* (14), and Anand *et al.* (15), on purine nucleotide excretion by streptomycin-treated *Escherichia coli*. According to their hypothesis, streptomycin acts primarily by damaging the permeability of the cell membrane, subsequent to which loss of material from the cells occurs. From microscopic observations it was noted that streptomycin-treated pneumococcal cells remained morphologically intact for a period of a few hours so that one would not expect a sudden release of total cell contents into the medium as occurs following treatment with an agent such as deoxycholate which ruptures sensitive cells rapidly. From these experiments, it was evident that there was a slow, progressive release of transforming material from streptomycin-treated cells which was a direct result of the action of streptomycin.

*Donor and Recipient Dosage Relationship and Transformation Yield.*—In

order to analyze more complex situations in lysate transformations, it was necessary to determine what concentrations of both donors and recipients would give optimal yields. For this purpose both donor and recipient cultures were diluted or concentrated (by Millipore filtration); five concentrations of donor and six of recipient were then used in 30 different combinations. The results, in terms of absolute yield, are plotted in Fig. 2. Where possible, a relative yield was calculated and indicated. Maximal yields were observed at

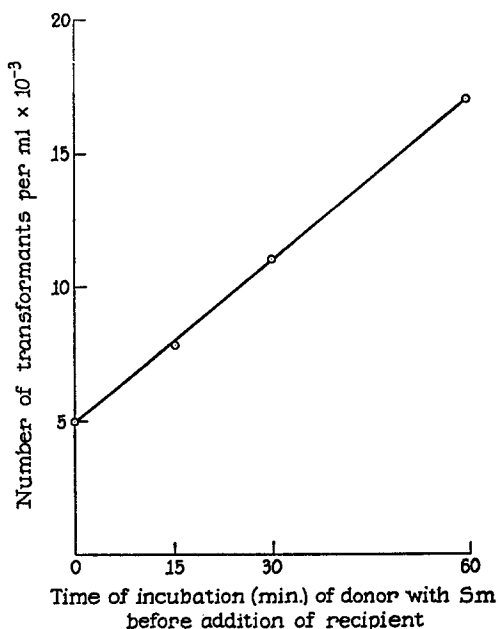


FIG. 1. Effect of increasing exposure of a donor culture to streptomycin before addition of recipient on number of transformants produced. Recipient: RSm,  $6 \times 10^6$  colony-forming units per ml (0.4 ml); Donor: RFad,  $5 \times 10^7$  colony forming units per ml (0.4 ml); Growth medium: AGCH; Exposure of recipient to donor: 45 minutes at  $30^\circ\text{C}$ .

a donor density of about  $6 \times 10^6$  colony-forming units per ml. Relative yields were highest at a low recipient level, whereas maximal absolute yields were obtained with a recipient density between  $6 \times 10^6$  and  $6 \times 10^7$ . Higher recipient levels could not be used because the selective action of sulfanilamide was insufficient to reveal a small number of transformants on a very large background of sensitive cells. It appeared, then, that the extent of transformation depends in considerable part on the concentration of both donor and recipient cells.

*Quantitative Comparison of Transformation Yields Using Streptomycin Lysates and Purified DNA as Donor Material.*—The DNA content of from 1 to 10

pneumococcal cells (16, 17), or  $2$  to  $20 \times 10^{-9}$   $\mu\text{g}$  DNA, under special conditions of high cell to DNA ratios was sufficient to produce one transformant. Under the present experimental conditions the purified DNA recovered from 250 cells gave one transformant when the total yield was optimal ( $10^{-1}$   $\mu\text{g}$  DNA giving  $4 \times 10^5$  transformants). This calculation allows for a probable 50 per cent recovery of the total DNA in the purified product. With penicillin-induced

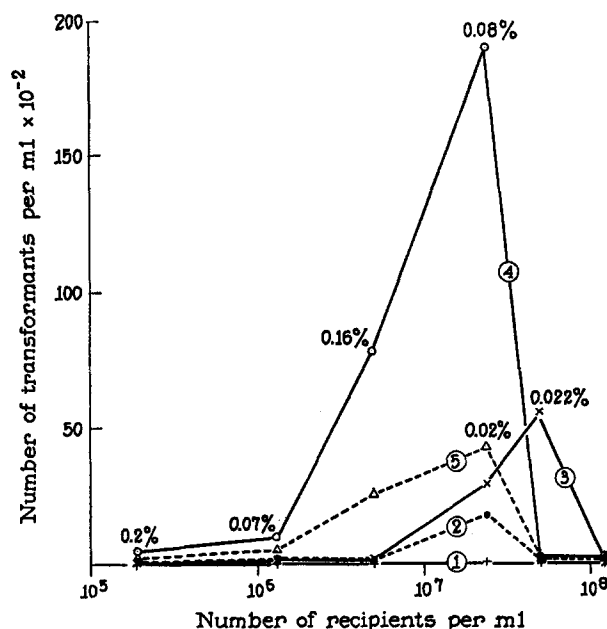


FIG. 2. Effect of varying donor and recipient concentrations on transformation yield. Recipient: RSm; Donor: RFad grown to  $1.2 \times 10^7$  colony-forming units in AGCH was exposed to Sm 250  $\mu\text{g}$  per ml for 30 minutes at  $37^\circ\text{C}$  before use in a transformation experiment. Donor concentration in colony-forming units per ml. Curves: 1,  $3 \times 10^4$ ; 2,  $3 \times 10^5$ ; 3,  $6 \times 10^6$ ; 4,  $6 \times 10^7$ ; 5,  $6 \times 10^7$ . The relative yield as percentage of recipients transformed is indicated on the curves.

lysates it had been estimated that the ratio of donors to transformants could be as low as 300 to 1. In the present work with streptomycin-induced lysates, this ratio was frequently 100 to 1.

It is evident from these estimates that both the penicillin- and streptomycin-induced lysate transformation systems are in the same range of efficiency as transformation processes mediated by purified DNA. The potential efficiency of the lysate systems may well be higher, since probably only a minor fraction of the total DNA content of the donor cells becomes available for transformation.

When used at the highest feasible concentrations, streptomycin-induced lysates can transform from 0.1 to 1 per cent of a competent recipient population. Since under standard conditions in this range the transformation yield is still dependent upon the concentration of lysate added, this yield compares quite favorably with those of 6 to 10 per cent which ordinarily are the maximum that purified DNA at any concentration can produce.

It can be concluded that lysate transformation processes are as efficient as transformation by purified DNA whether yields are expressed in relation to donor or to recipient cells.

TABLE I  
*Effect of the Age of a Culture on Its Ability to Release Active Transforming Material After Treatment with Streptomycin*

	Hrs. of incubation at 37°C	No. of colony-forming units per ml	No. of transformants per ml to sulfonamide resistance	Per cent transformation
Experiment 1	1	$5.1 \times 10^4$	$2.1 \times 10^3$	0.07
	2	$1.7 \times 10^5$	$2.8 \times 10^3$	0.08
	4.5	$1.4 \times 10^7$	$6.3 \times 10^3$	0.25
	5.7	$1.6 \times 10^7$	$4.1 \times 10^3$	0.13
		Purified DNA	$6.6 \times 10^4$	2.0
Experiment 2	1	$2.1 \times 10^6$	$3.0 \times 10^2$	0.06
	1.5	$2.8 \times 10^6$	$3.2 \times 10^2$	0.07
	2	$1.8 \times 10^7$	$8.0 \times 10^2$	0.17
	2.5	$3.9 \times 10^7$	$1.0 \times 10^3$	0.21
	3	$5.7 \times 10^7$	$4.3 \times 10^2$	0.09
	4	$6.4 \times 10^7$	$1.8 \times 10^2$	0.03
	4.5	$9.0 \times 10^7$	$<10^2$	$<0.02$

*Pattern of Release of Active Transforming Material with Culture Growth.*—A culture, as it ages, changes in many of its responses to various environmental challenges, such as susceptibility to the deleterious effects of a variety of drugs and other noxious agents (18). The age of a culture might therefore also influence its ability to release active transforming material.

To test this possibility, aliquots were removed at different times during the growth of a culture, treated with streptomycin for 10 minutes at 37°C, and used as donors in transformation experiments. The results of two typical experiments are presented in Table I.

The data in Table I demonstrate that as pneumococcal cultures continued to grow, their ability to act as donors of genetic material increased, and then, during the latter part of the logarithmic phase of growth, it decreased. The decrease was not due to increasing resistance to the action of streptomycin

with age, because under the conditions used streptomycin was found to kill rapidly and completely. It was necessary, however, to separate the effects of age from the effects of crowding a larger number of cells within the same volume of culture. Samples removed at different times from a growing culture were therefore diluted or concentrated by filtration and resuspension before treat-

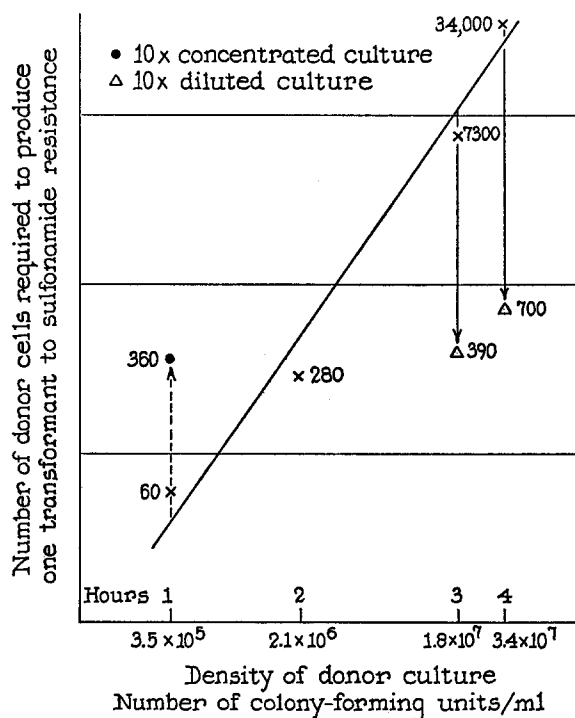


FIG. 3. Efficiency of streptomycin lysates from cultures of different ages and in different concentrations as donors of genetic material. Recipient: RSm; Donor: RFad grown in AGCH. Aliquots of the culture were taken at different times during growth, diluted or concentrated as indicated, treated with Sm and used in a transformation experiment. The number of donors required to produce one transformant is indicated for each point in growth, and these values are plotted on a logarithmic scale.

ment with streptomycin. Fig. 3 illustrates the results from one of four such experiments. It is evident that both the age of the donor culture and the population density of cells at a given age influence the ability to release transforming material. The results can be explained either by the production during growth of a substance interfering with transformation or of a substance suppressing the release of transforming agent. In the light of later experiments, the first possibility proved to be the more satisfactory explanation.

*Localization of the Active Material in the Lysate.*—A further step in the analy-



sis of the nature of streptomycin-induced lysate transformations was localization of the source of transforming activity in the streptomycin lysate; that is, to discover whether the active material was released in soluble form or remained attached to cell bodies. Millipore membrane filters were used to separate cells from culture fluid. It was found that active transforming material was released

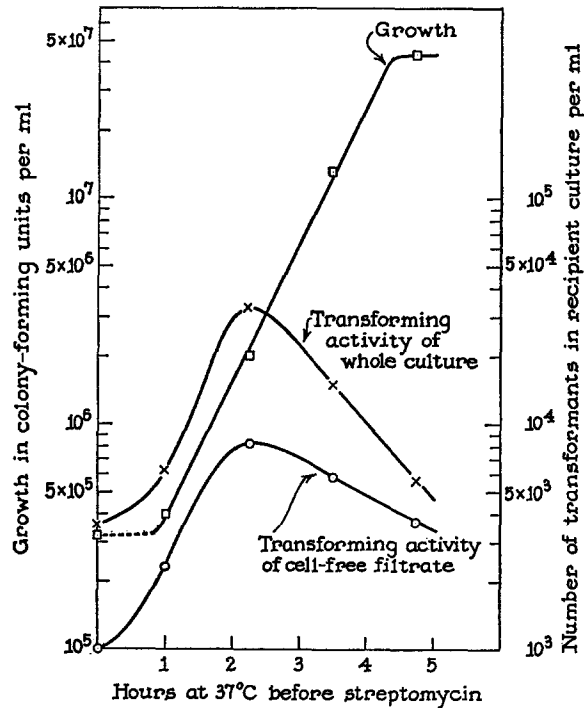


FIG. 4. Transforming activity of whole lysates and of cell-free filtrates of lysates from a culture at different times during growth. Recipient: RSm; Donor: R<sub>F</sub>ad grown in AGCH. At the times indicated, aliquots of the culture were treated with Sm for 30 minutes at 37°C, and then portions were filtered. The cell-free filtrates and the whole lysates were used as donor material in transformation experiments.

in soluble form but that considerably more remained in the cell fraction. The pattern of release of soluble active material was demonstrated in seven experiments one of which is illustrated in Fig. 4. As described above, there was a rise and then a fall in the liberation of transforming agent from streptomycin-treated cells as the culture grew. The release of soluble transforming agent followed the same pattern as the appearance of total active material, but the soluble material accounted for only about one-fifth of the total activity.

Although the greater part of the transforming agent seemed to be cell-bound, repeated filtration and resuspension of the same streptomycin-treated

cells was found to release much of the bound material into soluble form. Similar results were obtained when centrifugation was used to separate cells from culture fluid.

The foregoing results can be explained by assuming that on treatment with streptomycin, sensitive cells release transforming principle and in addition either a substance counteracting the transforming principle or a substance preventing further release. Interaction of these opposing processes at different times in growth and under different conditions could lead to the observations described.

With knowledge of optimal conditions for high yields of lysate transformation it became possible by using sterile culture filtrates to test whether "normal," living, untreated cultures also released transforming agent. This possibility was examined by Sia and Dawson (19) with negative results. In the present studies, it was indeed found that filtrates of untreated cultures were able to cause transformation. This observation led to the experiments presented in the next section.

## *2. Experiments on Transformations by Spontaneously Released Transforming Agent*

*Nature of the Recombination Process.*—During the course of experiments on streptomycin-induced lysate transformations, it was discovered that small volumes of filtrates of growing pneumococcal cultures are able to transmit genetic traits of these cultures to appropriate recipient cells. This property is relatively widespread, inasmuch as the culture filtrates of nine different laboratory stocks of pneumococci had genetic activity, and six different drug resistance markers could be transferred in this manner (streptomycin, micrococcin, two levels of sulfanilamide, amethopterin, and *p*-nitrobenzoic acid resistance). In various experiments five different recipient strains were transformed by culture material.

It was demonstrated that the genetic activity of culture filtrates is completely destroyed by DNase but is unaffected by RNase or trypsin (Table II). The sensitivity of five different transforming agents to DNase is illustrated in Table III.

Transformation by culture lysates or filtrates parallels the usual transformations mediated by purified DNA also in other respects. Of various cultures tested, only those which were competent in DNA-mediated transformations, were susceptible to genetic modification by culture filtrates. The ability of a population to be transformed by filtrate material depended on the development of the physiologic state which was also prerequisite for transformation by purified DNA. Thus the number of transformants which could be produced by a given filtrate was predictable on the basis of the degree of receptivity of the cells for transformation by purified DNA. The similarity of the two systems

extended also to the frequency of transfer of linked markers and to the efficiency of transformations by culture fluids and by purified DNA, as will be discussed later. Thus it can be concluded that the recombination process in question is DNA-mediated transformation.

*Quantitative Comparison of Transformations by Culture Filtrates with Transformations by Purified DNA.*—Under typical experimental conditions, the filtrate of 1 ml of culture containing  $2 \times 10^6$  freshly grown cells could produce

TABLE II  
*Effect of DNase, RNase, and Trypsin on Transformations of the R6 Wild Type by Filtrates of Sulfonamide-Resistant Cultures and by Purified DNA*

Enzyme (20 min. at 37°C)	No. of transformants per ml from 0.4 ml filtrate	No. of transformants per ml from 0.03 µg per ml of purified DNA
None.....	$1.1 \times 10^4$	$2.6 \times 10^4$
DNase 1.5 µg per ml.....	0	0
RNase 2.0 µg per ml.....	$1.1 \times 10^4$	$2.5 \times 10^4$
Trypsin 10 µg per ml.....	$1.8 \times 10^4$	$2.7 \times 10^4$

TABLE III  
*Effect of DNase on Transformation of Different Drug Resistance Factors by Filtrate of a Culture (RFadKAm) Simultaneously Resistant to Sulfonamide, Micrococcin, and Amethopterin*

Enzyme (30 min. at 30°C)	Number of transformants per ml from 0.4 ml filtrate					
	Marker.....	Fa	Fd	Fad	K	Am
None.....		$6.8 \times 10^3$	$1 \times 10^3$	$1 \times 10^3$	$1.3 \times 10^3$	$1.8 \times 10^3$
DNase 1.5 µg per ml...		0	0	0	0	0

$1.3 \times 10^4$  transformants per ml. The yields correspond to 0.3 to 1 per cent of the recipient population. Therefore, under these conditions of relatively low efficiency the filtrate of about 150 cells was able to produce one transformant to a single marker. As pointed out above, an amount of purified DNA sufficient to accomplish this same result is normally recovered from about 250 cells. As was found for the streptomycin-released material, spontaneously released transforming principle was in the same range of efficiency, on a weight basis, as purified DNA. Since it is even less likely than in the case of the streptomycin-treated cultures that the total DNA content of growing cells was released into the medium, spontaneously released transforming agent must be at least as efficient as purified DNA and probably is more efficient.

*Frequency of Linked Transformations by Culture Filtrates, by Streptomycin Lysates, and by Purified DNA.*—One criterion of the intactness of genetic material is the frequency of simultaneous transfer of markers known to be "linked" (20, 4, 21, 22). Certain markers are introduced together by purified DNA with characteristically greater frequency than expected on the basis of chance. When this occurs, the markers are inferred to be in the same region of the bacterial chromosome. The higher the frequency of simultaneous transfer, the closer the markers are thought to be.

TABLE IV  
*Frequency of Linked Transformations by DNA from Cultures and by Purified DNA*

Donor material	Genotype		Per cent of linked transformations*	No. of experiments
	Donor	Recipient		
Purified DNA.....	F + d	Fa +	32 ± 3	16
Streptomycin-treated culture.....	F + d	Fa +	28 ± 3	21
Purified DNA.....	Fad	++	55 ± 4	5
Streptomycin-treated culture.....	Fad	++	50 ± 2	100
Filtrate of streptomycin-treated culture..	Fad	++	38 ± 3	33
Filtrate of untreated culture.....	Fad	++	42 ± 2	30
Purified DNA.....	FaSm	++	1.1 ± 0.1	8
Filtrate of untreated culture.....	FaSm	++	1.9 ± 0.1	7

The symbol + represents the wild type alternative form of the respective drug resistance markers Fa, Fd, or Sm.

\* Indicated standard error is based upon the total number of colonies counted in all experiments.

If the linkage frequency obtained from spontaneously released transforming agent were greater than that from purified DNA, this would suggest that DNA existed in a form more highly organized than that obtained from the purification procedures. To investigate this question, the transfer of Fa, a low sulfonamide resistance marker, was measured in conjunction with that of Fd (intermediate sulfonamide resistance) to which it is strongly linked, and with that of Sm (streptomycin resistance) to which it is weakly linked (4). From the results presented in Table IV, it is evident that the percentage of linked transformations produced by the whole streptomycin-treated culture and by purified DNA were the same.<sup>2</sup> The transfer of the weakly linked Fa and Sm markers by filtrates of very young cultures (less than  $5 \times 10^6$  colony-forming units per ml) was the same as, or slightly greater than, the transfer of these markers by

<sup>2</sup> For either lysate or DNA, the frequency of linked transfers is greater for *ad* than for *+d*. However, the *a* region of the DNA molecule need not be identical with its + counterpart. The non-reciprocity of transformation reactions is currently being investigated by one of us.

purified DNA. For the closely linked pair Fa-Fd, filtrates of either a streptomycin-treated or an untreated culture (about  $10^7$  colony-forming units per ml) gave a somewhat lower frequency of double transformations than did purified DNA.

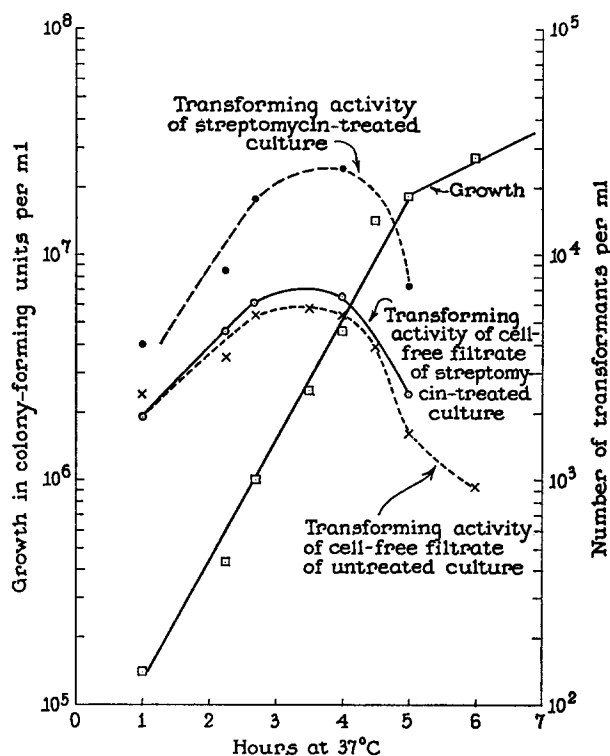


FIG. 5. Transforming activity of untreated culture and of culture treated with Sm for 30 minutes at 37°C. Recipient: RSm; Donor: RFad grown in AGCH. Aliquots of the donor culture were removed at different times during growth and filtered with and without Sm treatment. Filtrates and lysates were used as donor material in a transformation experiment.

These findings indicate that the procedures used for the isolation of DNA do not bring about degradation, for freshly released untreated material did not seem to possess greater intactness. Filtrates of streptomycin-treated or of living cultures past a certain age contained transforming agent which seemed to be even slightly more degraded than a standard preparation of purified DNA, since though still efficient in single transformations, they did not produce as many double transformations.

*Pattern of Release of Transforming Material during Growth of the Culture.*—It was regularly observed that the transforming activity of the culture fluid

increased with growth, and then decreased, as was observed for streptomycin-treated cultures (Fig. 5). As in previous experiments the maximum activity of the whole streptomycin lysate was about four times as high as that for the filtrate from the lysate. The latter gave the same transforming activities, qualitatively and quantitatively, as the filtrates of the untreated culture.

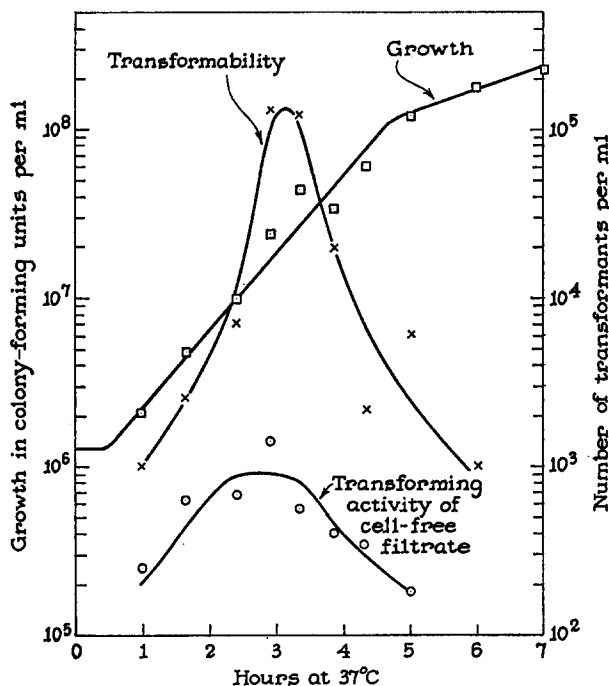


FIG. 6. Development of transformability and release of transforming material by a growing untreated culture. Recipient: RSm; Donor: RFad grown in CH and filtered at different times in growth. Filtrates were used as donor material to transform RSm. Transformability of RFad was tested by adding purified DNA (1.5  $\mu$ g per ml) from a Sm-resistant culture to aliquots of RFad at different times in growth. The cells were exposed to DNA for 30 minutes at 30°C, and the reaction was terminated by DNase.

Filterable active material did not increase markedly with time of exposure to streptomycin, suggesting that the streptomycin-released transforming material remains loosely bound to the cell bodies, whereas the filtrate activity in streptomycin-treated cultures represents spontaneously released transforming agent.

*Relationship of Release of Transforming Agent to the Development of Transformability.*—The pattern of release of transforming material with growth is reminiscent of the development by a growing pneumococcal culture of competence, *i.e.*, the ability to incorporate exogenous DNA genetically (23). A series of experiments was carried out, therefore, in which the abilities of a given

culture both to release and to accept genetic material were tested at various times during growth. The results of one such experiment are represented in Fig. 6. The time at which a culture is most receptive to exogenous DNA is also the time at which the culture filtrate exhibits maximum transforming activity.

The possibility that the development of competence by a culture was causally related to its ability to be a donor of transforming principle was considered. It is conceivable that conditions leading to instability of the genetic material and to increased permeability of the cell membrane would permit exchange of DNA in both directions to occur more readily. Such conditions might have

TABLE V  
*Separation of Abilities to Accept and Release Transforming DNA in Growing Cultures*

Culture and conditions	Transformability (No. of transformants per ml)	No. of transformants produced by 0.4 ml of culture filtrate
1. Standard medium (CH)		
RA-7—"untransformable".....	<100	$8.0 \times 10^3$
RB-1—"untransformable".....	<100	$1.1 \times 10^3$
R6—transformable .....	$1.1 \times 10^5$	$1.5 \times 10^4$
2. CH medium lacking albumin		
R6—transformable .....	<100	$6.0 \times 10^3$
3. Maleate buffered CH medium*		
R6—transformable.....	<100	$8.1 \times 10^3$

\* Maleate-buffered medium differed from the modified Adams and Roe medium (8) by not containing tryptophane or cysteine; but by including 0.02 per cent sucrose, 0.025 per cent sodium pyruvate, 0.002 per cent glutamine, 0.38 per cent neopeptone, 0.1 M sodium maleate, and only 0.0005 M dibasic potassium phosphate. Cells grown in this medium do not ordinarily develop competence.

maximal effect during the time when a culture is growing at its greatest rate and therefore metabolizing rapidly. It is during a late stage of exponential growth that both transformability and ability to release DNA reach their peak.

Even though the circumstances most propitious for both processes are similar, experimental conditions under which they can be separated were readily found, and are summarized in Table V. Strains RA-7 and RB-1 are not transformable under the conditions used, whereas strain R6 is readily transformed in suitable growth medium. Both transformable and untransformable strains, however, release active genetic material. Other experiments showed that an untransformable strain releases transforming DNA in the same pattern as a transformable one and in comparable amounts. In addition, a transformable culture grown under conditions in which it will not develop receptivity, can still release active transforming agent. Therefore, even if the

processes of release and receptivity are related, receptivity can be suppressed or offset without affecting the ability to release transforming DNA.

*Attempts to Modify the Pattern of Release of Transforming Agent by Growing Pneumococcal Cultures.*—The release of genetic material by several strains growing under different conditions indicated that liberation of transforming principle is a normal accompaniment of pneumococcal growth. The question remained whether DNA-containing material is secreted by living cells or is released from a fraction of the population which is dying. In view of the efficiency of transformation by culture filtrates evidence of massive death in growing cultures was sought. In most experiments the donor cultures passed from a frozen to a thawed state in 10 per cent glycerol and then were diluted into an essentially glycerol-free medium at the start of growth. These environmental changes are not responsible for the subsequent appearance of transforming agent since cultures maintained continuously at 37°C for six passages either in glycerol-free AB or CH medium still liberate the agent during growth. It was also found that gradual dilution from 10 per cent to less than 0.1 per cent glycerol in several steps instead of in one step affected neither viable counts nor DNA release. Although it was recently reported that the production of extracellular DNA material by *Micrococcus sodonensis* was medium-dependent (24), for pneumococcal cultures variations in the growth medium had little effect on the release of transforming DNA. For example, varying from 0 to 0.4 per cent the content of bovine serum albumin which might be osmotically protective to fragile cells had no influence on the release of transforming agent. Variations in the amount of carbohydrate (glucose or sucrose) or the substitution of phosphate buffer by maleate buffer also were without effect on the release of genetic material. The same was true for growth factors contained in yeast since cultures grown in AGCH medium (without yeast extract) and CH medium (with yeast extract) produced active filtrates in the same pattern (Figs. 5 and 6).

The relatively defined casein hydrolysate media used might be more conducive to death of cells than complex meat infusion broth. The results using meat infusion-neopeptone broth (AB) were the same as those for casein hydrolysate media, but in AB medium the peaks of transformability and release of transforming DNA occurred earlier in growth.

It is unlikely that the filtration process itself causes cell rupture and release of transforming agent, because unfiltered, living cultures can transform other cultures. A streptomycin-resistant recipient culture was briefly exposed to a freshly grown sulfonamide-resistant, streptomycin-sensitive culture. At the end of the exposure time, DNase was added to prevent subsequent transfer of transforming DNA. Any transformants thus far produced were scored by dilution into broth containing sulfanilamide, streptomycin, DNase, and specific antibody. The streptomycin destroyed the donor population, DNase was



included to prevent streptomycin-induced lysate transformation from taking place in the scoring tubes, and sulfanilamide was used to select for transformant colonies. It is evident from Table VI that the filtrate of an untreated culture or the whole culture itself can transform equivalent numbers of recipient cells. For comparative purposes, data for the same donor culture treated for 10 minutes at 37°C with 250  $\mu$ g per ml of streptomycin are included. Since the activities of culture and filtrate were so similar, there is no sign that the filtration procedure itself caused release of active transforming agent. Additional evidence for this point comes from the cross-transformation of two cultures grown together and not filtered at any time, as described below. As previously noted, streptomycin treatment does not cause early release of additional soluble transforming agent.

TABLE VI  
*Transforming Activity of Whole Cultures and of Culture Filtrates*

Donor material (Sulfonamide-resistant culture $10^7$ colony-forming units per ml)	No. of transformants from 0.4 ml donor
Untreated whole culture.....	$1.7 \times 10^8$
Filtrate of untreated culture.....	$1.5 \times 10^8$
Streptomycin lysate (10 min. exposure to drug).....	$2.8 \times 10^8$
Filtrate of streptomycin lysate.....	$1.4 \times 10^8$

In summary, the appearance of genetic material in growing pneumococcal cultures could not be attributed to the effects of osmotic or thermal shock, to ingredients of a particular medium, or to mechanical stress of filtration procedures.

*Determination of Other cell Substances Released by Growing Cultures.*—Under the conditions of the experiments described, the increase in viable count proceeded in the expected logarithmic fashion, and there was no evidence of massive death. The source of transforming material, however, could be death (and turnover of certain cell constituents) of a fraction of the population too small to be detectable by ordinary counting methods. To investigate this possibility, evidence of release of other cell components during growth was sought.

Since pneumococci do not utilize exogenous folic acid compounds (unpublished results of this laboratory) but do synthesize them intracellularly, the accumulation of these compounds was measured by *Streptococcus faecalis* bio-assay. The pattern of liberation of folic acid compounds provided a measure of release of intracellular substance independent of the measure of transforming agent. It was considered that comparison of the two patterns might give some insight into the growth processes which lead to release of active genetic material.

The data in Fig. 7 demonstrate that extracellular folic acid compounds continue to increase with growth and follow a pattern different from that of active transforming agent. If the release of the transforming agent and of folic acid were due to death of a fraction of the population, the transforming activity would have continued to increase with growth as did the extracellular folic acid compounds. Direct measurement of the deoxyribonucleoside content of culture filtrates was undertaken to determine whether the release of DNA-containing material continued with growth. This might be accompanied by

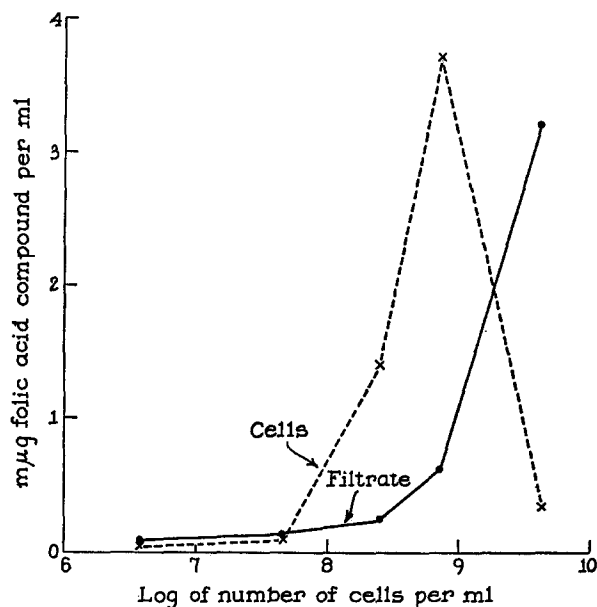


FIG. 7. Release of folic acid compounds by a growing culture. *Streptococcus faecalis* growth assay of folic acid compounds in filtrates and in cells from a culture of RSm growing in AGCH without neopeptone.

simultaneous release of a nuclease, for example, which would explain the decline in transforming activity in the later filtrates. The experiments on streptomycin-induced lysates had also pointed to this possibility.

The amounts of total deoxyribonucleoside (including deoxyribonucleotides) in filtrates of a streptomycin-resistant culture at different stages of growth, and in a sodium deoxycholate lysate of the whole culture were determined. The DNA equivalents of the deoxyribonucleoside content of filtrates and cells (whole culture minus filtrate) were calculated (1 gm mole deoxyribonucleoside = 310 gm DNA) and are presented in Fig. 8. Samples of filtrate were also used to transform recipient cells to streptomycin resistance. As shown in Fig. 8 the amount of DNA material in the cells increased progressively during growth,

whereas in the filtrate it began to be detectable only late in growth. Filtrates of still older cultures contained larger amounts of DNA material. Thus as the deoxyribonucleotide content of the filtrates increased, their transforming activity decreased. This series of experiments gave no evidence of a rise and fall in the DNA content of the filtrates to parallel the rise and fall of their transforming activity. The amount of deoxyribonucleotides present may be

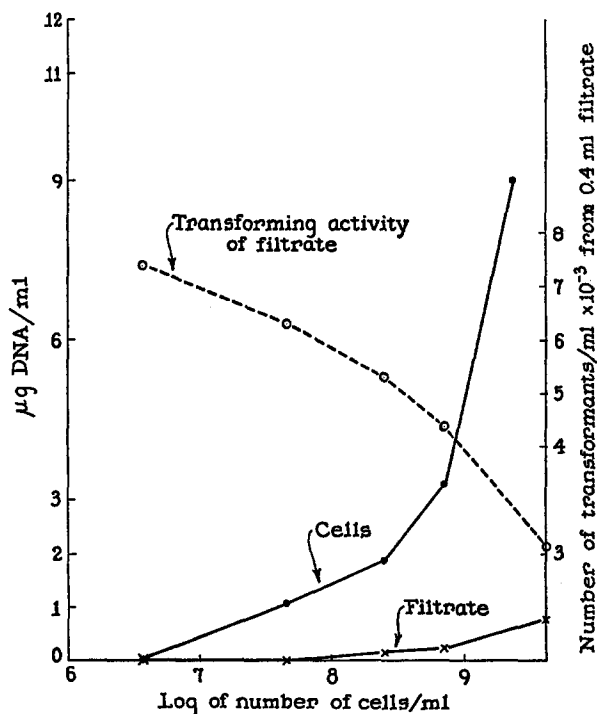


FIG. 8. DNA content of cells and filtrates of a growing culture. Recipient: RFD; Donor (same culture as in Figure 7): RSm grown in AGCH without neopeptone; DNA by deoxyribonucleoside bio-assay.

too small to detect by the method used, although as little as 0.1  $\mu\text{g}$  of total deoxyribonucleoside per ml can be detected. Since this amount of fully active purified DNA would still account for the activity detected, in the main one can only conclude that the genetically active DNA which is released is not accompanied by rapidly increasing amounts of degraded DNA rising to more than 0.1  $\mu\text{g}$  per ml. It can be further concluded that the fall in transforming activity of culture filtrates with continued growth is not due to a removal, perhaps by increased cellular uptake, of deoxyribonucleotide material from the medium since this material was observed to increase late in growth. The

decreased transforming activity of filtrates of older cultures, therefore, is most likely caused by inactivation of previously released transforming agent.

*The Release of Deoxyribonuclease by Pneumococcal Cultures.*—Release of DNase from autolyzing pneumococcal cultures has been known for many years (1). To our knowledge, the presence of small amounts of DNase in filtrates of young pneumococcal cultures, however, has not been previously reported. DNase in filtrates was demonstrated through its effect on the relative viscosity of a solution of calf thymus DNA. Fig. 9 illustrates the fall in relative viscosity of thymus DNA as it was incubated with filtrates of a culture nearing the end

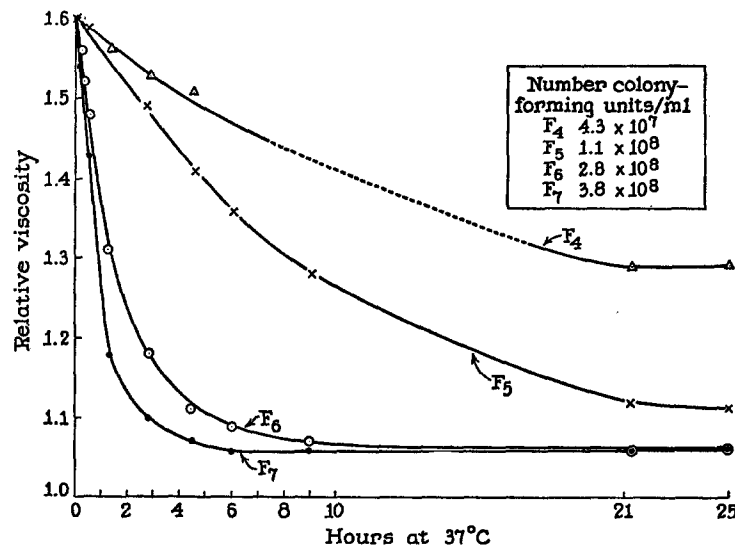


FIG. 9. Effect of culture filtrates on relative viscosity of calf thymus DNA (270  $\mu$ g per ml).

of logarithmic growth. It can be seen that the late culture filtrates produced a marked drop in the relative viscosity of thymus DNA. The filtrate nuclease was active in the presence of magnesium ions, and like pancreatic DNase, was rendered inactive in the presence of versene.

Culture filtrates also show a pronounced effect upon the biological activity of DNA. In Fig. 10 are illustrated the effects of six filtrates taken at different times during growth on the transforming activity of a limited amount of purified DNA. All filtrates except the first caused inactivation; the degree of inactivation increased sharply with the age of the culture. It should be emphasized that in these tests the filtrate was given an opportunity to act upon the DNA for a long period (30 minutes) before transformable cells were added. As the ability of later filtrates to inactivate added DNA increased (under

these favorable conditions), the transforming activity of the filtrates declined together with the ability of the culture itself to be transformed (Fig. 11).

For reasons of economy, viscosimetric measure of nuclease was carried out with thymus DNA. It has been reported that meningococcal DNase has limited

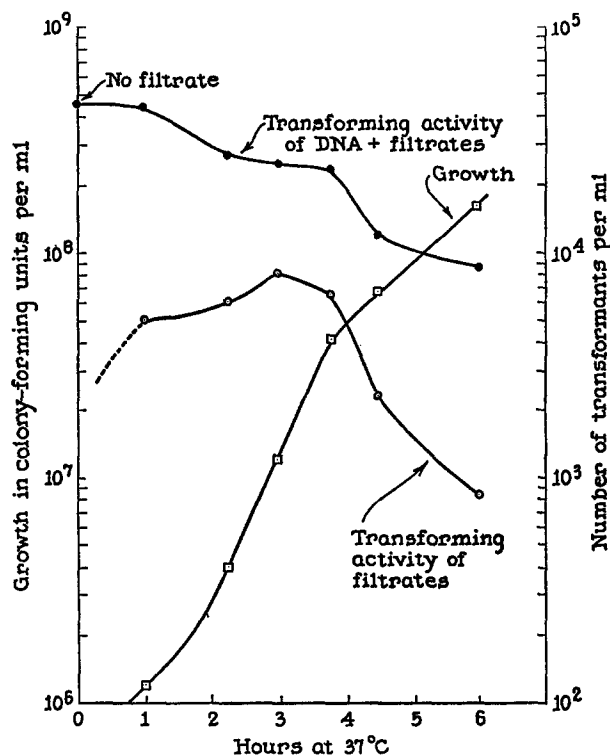


FIG. 10. Effect of culture filtrates on transformation by purified DNA. Recipient: RFD; Donor: DNA from Sm-resistant culture and filtrates from R6 (wild type). DNA (0.025  $\mu\text{g}$  per ml) and filtrates were incubated together for 30 minutes at 37° C before use in a transformation experiment. The transformants were detected by increase in their resistance (from 1 to 20  $\mu\text{g}$ ) toward *p*-nitrobenzoic acid (25).

ability to depolymerize DNA from the same strain (26). In addition to destruction of the biological activity of pneumococcal DNA, we have found that the filtrates also reduced the viscosity of pneumococcal DNA.

On the basis of these data it is concluded that the observed decline in the transforming activity of filtrates of cultures in the latter part of logarithmic growth is due to the inactivation of transforming agent by DNase as it is released. The liberation of DNase by growing pneumococci could also explain the disappearance of active transforming agent from streptomycin-treated

cultures. Endogenous DNase probably causes the mild degradation of freshly released transforming agent which was suggested by the results of the linkage studies described. Finally, the production of small quantities of DNase may contribute to the loss of ability to accomplish transformation by added DNA in an aging culture.

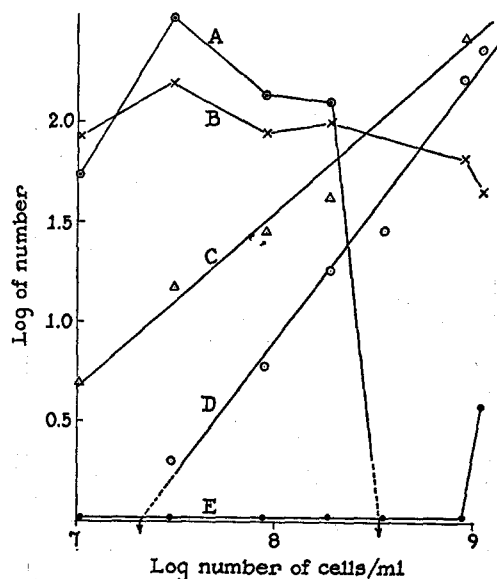


FIG. 11. Processes accompanying growth in pneumococcal cultures. The culture was R6 (wild type) grown in AGCH. The logarithms of the numbers below are plotted on the ordinate. *A*: Transformability, number of transformants to Sm resistance in  $10^{-8}$  ml of culture. Purified DNA was the donor material. *B*: Transforming activity of filtrates, number of transformants from R6 to wild type produced by  $4 \times 10^{-8}$  ml of filtrate. See legend, Fig. 10. *C*: Release of folic acid compounds,  $\mu\text{g}$  folic acid compounds per  $10^8$  ml filtrate. *D*: DNase,  $\mu\text{g}$  per 10 ml filtrate. *E*: DNA,  $\mu\text{g}$  DNA equivalents per 10 ml filtrate, from total deoxyribonucleosides.

*Summary of Studies on Processes Accompanying Growth in Pneumococcal Cultures.*—In order to relate the different processes studied to each other, total cell number, transformability, transforming activity of the filtrates, release of folic acid compounds, DNase activity, and total deoxyribonucleosides were measured at different times during growth of the same culture (Fig. 11). Growth in four different cultures was followed in this manner. A logarithmic plot is used in order to provide a direct comparison of the five parameters, especially for the low values at low culture densities. Estimations of the amount of DNase present in culture filtrates were made from a plot of the initial rates of decline of the relative viscosity of thymus DNA incubated with several known concentrations of pancreatic DNase. The initial slopes of these curves were directly proportional to the concentration of DNase used (13).

Fig. 11 demonstrates that DNase and folic acid release are roughly proportional to the increase in cell population, since the slopes of the two logarithmic curves are near 1 (0.9 for folic acid and 1.2 for DNase). The curves of transformability and of release of transforming agent have a different pattern, showing the rise and fall already discussed. The release of DNA and of its breakdown products as measured by lactobacillus assay follows a third pattern, being negligible for most of the growth period and rising late in growth. It may be that the release of total DNA components is also logarithmic but in amounts too small to be measured by the growth bio-assay. If, however, the release of transforming agent by a culture (see curve *B*) were taken as an index of the rate of release of DNA, and that release assumed to continue at the early rate, then measurable quantities of deoxyribonucleosides should have appeared well before they did appear. These experiments support the following conclusions:

(*a*) Folic acid and DNase release are directly related to growth. (*b*) There is no evidence of massive death early in growth since the total and viable counts are the same, growth continues exponentially and no substantial accumulation of DNA components can be detected. (*c*) Therefore, it appears possible that DNase and folic acid are excreted by living cells. (*d*) The spontaneously appearing transforming agent must come from leakage of genetic material from living cells, or otherwise from death and lysis of small numbers of cells.<sup>3</sup> (*e*) The decline in transforming activity of filtrates and quite possibly the decline in transformability of the culture itself are due to inactivation of the transforming DNA by DNase from the cells. (*f*) The appearance of deoxyribonucleotide material in culture filtrates in amounts detectable by the bio-assay method is probably related to the beginning of true autolysis of the culture.

*Spontaneous Occurrence of Genetic Recombination in Growing Pneumococcal Cultures.*—The foregoing data show that transforming agent is present in pneumococcal cultures in peak amounts at the time when the cells are most transformable. It seemed desirable, therefore, to determine whether genetic exchange can take place in growing cultures without experimental intervention. To this end, two cultures, one resistant to sulfonamide and amethopterin and the other resistant to streptomycin and micrococcin were inoculated into the same culture tube and incubated for 3 hours at 37°C. None of the genetic markers employed shows linkage to any of the others in the usual transformation experiments, so that virtually all transformations would be to single traits. Scoring in medium containing the appropriate combinations of three drugs could thus be used to detect the acquisition by cells of either doubly marked strain of single markers from the other strain. As indicated in Table VII each of the two strains used was able to donate singly both of its markers to the other strain, as well as to be separately transformed for both drug resistance

<sup>3</sup> Studies on *Escherichia coli* (27) also seem to indicate that cell death is rare during active growth of the population.

factors derived from the other strain. Transformation led to the emergence of new genotypes in the mixed culture  $10^4$  to  $10^5$  times as frequently as could have been expected by spontaneous mutation.

TABLE VII  
*Transformation in Mixed Growing Populations*

Type	Markers	Cells present	Origin of type
Parental types:			
P <sub>1</sub> .....	F, Am	$4 \times 10^7$	(Inoculum $5 \times 10^5$ )
P <sub>2</sub> .....	Sm, K	$10^7$	(Inoculum $5 \times 10^5$ )
Transformants:			
T <sub>1</sub> .....	F, Am, Sm	$1.7 \times 10^3$	Transformation of P <sub>1</sub> to Sm
T <sub>2</sub> .....	F, Am, K	$1.4 \times 10^4$	Transformation of P <sub>1</sub> to K
T <sub>3</sub> .....	Sm, K, F	$2.7 \times 10^4$	Transformation of P <sub>2</sub> to F
T <sub>4</sub> .....	Sm, K, Am	$1.3 \times 10^4$	Transformation of P <sub>2</sub> to Am

Populations sampled after 3 hours growth, given in colony-forming units per ml.

Drug resistance markers: F, sulfonamide; Am, amethopterin; Sm, streptomycin; K, micrococcin.

#### GENERAL DISCUSSION

The experiments presented show that extracellular transforming DNA is present in growing pneumococcal cultures. Several authors have reported the presence of DNA in the culture fluids of different bacteria, including *Brucella* (28), *Staphylococcus* (29), *Alcaligenes* (30), *Pseudomonas* (30), *Flavobacterium* (30), *Neisseria* (31), and some halophiles (32). Takahashi and Gibbons (33) as well as Catlin (31) have considered this extracellular DNA to result from death and lysis of a fraction of the population in the presence of DNase inhibitor (30). The DNase inhibitor (perhaps a ribonucleic acid) would permit the accumulation of polymerized DNA in the culture. In most of the cases mentioned, DNA accumulated only under unfavorable conditions or very late in growth but then it was amassed in such amounts as sometimes to make the culture visibly slimy. In pneumococcal cultures transforming DNA is detectable early in growth, and under presumably physiologically favorable conditions when other cell constituents studied had not accumulated significantly. Since DNase activity also appears, high molecular DNA does not accumulate upon prolonged incubation.

It may be emphasized that in pneumococcal cultures genetically active DNA is present at the time of maximal transformability. Though extracellular DNA harvested from cultures of *Neisseria* was also found to be genetically active (31), the occurrence of recombination in such cultures was not reported.



Takahashi has recently mentioned that he has observed spontaneously occurring DNA-mediated transformation during certain recombination experiments with *Bacillus subtilis* (34). The presence of material with genetic potential in bacterial cultures is thus not unique to pneumococcus, so that spontaneously occurring transformations may well be of more general occurrence, not only in the laboratory, but also perhaps in the natural habitat of some microbial species.

Naturally occurring transformations would be of biological significance since they could provide a means for the rapid evolution of a highly pathogenic microbial species from recombination between two less pathogenic types. Such a situation was analyzed by conventional transformation experiments with the capsular system in pneumococcus (35, 36). Interspecific transformations have been carried out in the laboratory (37-41). Transformation of properties between two different species residing in close proximity to each other, as for example, pneumococcus and *Streptococcus*, might also lead to the sudden emergence of a new virulent microorganism. Although the present experiments were carried out *in vitro*, there is ample evidence from the work of Griffith (42), and others (43-46) that under artificial conditions genetic transformations can occur *in vivo* as well. It can hardly be doubted therefore that the sudden juxtaposition by transformation processes of two or more properties such as virulence and drug resistance, or two biochemical capacities within one strain, might appear to give almost *de novo* production of a new pathogenic species, capable of invading effectively a new host or medium.<sup>4</sup>

For the microorganism, transformation could thus provide a mechanism for the efficient accumulation of favorable traits. If these occurred on a large scale, most favored strains surviving in nature would be expected to be transformable. It is by no means apparent that transformability (or capability of conjugation or susceptibility to transduction) is widespread in strains of bacteria occurring in nature. It is possible, therefore, that these recombination processes do not now account for an important part of the equilibration or distribution of mutations. At some time in the past for any given species, however, such processes might have accounted for the rapid accumulation and equilibration of valuable traits. After many efficient bacterial lines had developed, such mechanisms of exchange might disappear since they also demand some cellular instability. Species still benefitting from rapid evolution would tend to preserve these mechanisms, particularly those exposed to an environment itself adaptable or variable. It may be noted that it is principally among the parasitic bacteria (pneumococcus (42), meningococcus (48), *Hemophilus* (49), *Strepto-*

<sup>4</sup> Recent *in vitro* experiments indicate that pneumococcus can be transformed by streptococcal culture filtrates. Also it has been reported that interspecific genetic recombination (presumably *via* conjugation) can occur *in vivo* (47).

*coccus* (38), *Xanthomonas* (50), enteric bacteria, and perhaps the viruses<sup>5</sup>) that transformation has been detected. While these microorganisms may be among those most investigated in this respect, it may also be worth considering that in these organisms, propagating chiefly in living creatures, there would still be considerable value derived from a prompt response to the defense mechanisms of the host. To this end, a rapid and efficient sharing, through intercellular transformation, of genetic experiences otherwise too rare to be accessible would continue to be advantageous for the parasite.

#### SUMMARY

Genetic transformations of pneumococcus mediated by streptomycin-induced lysates were studied to gain some insight into the nature of freshly released transforming principle, and the influence of the physiologic state of the donor population on the transformation process. It was found that streptomycin could make the DNA of sensitive cells available for transformation of other cells.

Living cultures of pneumococcus growing exponentially in ordinary media were also found to discharge significant quantities of genetically active DNA. Such cultures, not treated with any drug, showed no evidence of concomitant cell disintegration or death.

Both single markers and small linkage groups could be transferred in transformations mediated by drug-induced lysates and by filtrates of living cultures. The quantity of DNA liberated is small (less than 0.1  $\mu\text{g}$  per ml), but these transformations are at least as efficient as transformations mediated by purified DNA, when compared on the basis of total DNA available. Up to 1 per cent of the cells in an average recipient culture can be transformed by a small quantity of culture fluid.

Both in drug-treated and in untreated cultures the amount of transforming activity increased and then decreased during growth of the culture. Although the source of transforming DNA in growing cultures could not be established, the decline in the transforming activity of aging drug-treated or untreated cultures was attributed to the presence of deoxyribonuclease. The release of this nuclease by pneumococcal cultures midway in exponential growth is sufficient to result in a mild degradation of the low concentration of freshly released transforming agent present.

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<sup>5</sup> The case of *Bacillus subtilis*, a transformable organism (51) which is not parasitic or notably fastidious in its growth requirements, might seem to be inconsistent with these speculations. Evolution in bacteria need not, however, be unidirectional. A given species can at any time be evolving either towards heterotrophy or towards autotrophy. *Bacillus subtilis*, by present criteria a close relative of the pathogen *Bacillus anthracis*, might therefore be an example of an organism which is evolving towards the autotrophic state, but has not yet discarded its mechanisms of recombination.

Maximal release of active transforming agent by a living culture coincided in time with the development of maximal receptivity to exogenous DNA by that culture. As a result, recombinants could be recovered from appropriately genetically marked strains growing in each other's presence.

In view of these results, it seems possible that DNA-mediated transformations might provide, or might have provided, a mechanism of genetic recombination in nature for some bacterial species in which sexual mechanisms may not be available.

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