IN VITRO TRANSFORMATION OF PNEUMOCOCCAL TYPES

I. A Technique for Inducing Transformation of Pneumococcal Types in Vitro

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Griffith (1) was the first to show that S forms of pneumococci could be transformed from one specific S type into other specific S types through the intermediate stage of the R form. In recent publications by one of the authors (2, 3), Griffith's observations on the transformation of pneumococcal types were confirmed and extended. The results of these investigations may be summarized as follows:

It was shown that R forms of pneumococci could be converted into S forms of the original type by the subcutaneous injection, in white mice, of small amounts of living R organisms, together with S vaccines of the homologous type. (In this procedure, vaccines prepared from cultures of Type II S and Type III S organisms are equally effective whether heated for 15 minutes at 60°, for 15 minutes at 80° or for 15 minutes at 100°C. Vaccines prepared from Type I S organisms, however, are effective in producing reversion when heated for 15 minutes at 60°C. and for 15 minutes at 80°C., but not when heated for 15 minutes at 100°C.) *In vitro* attempts to effect the R → S reversion by the use of vaccines were uniformly unsuccessful.

It was further shown that R forms of pneumococci, derived from S forms of any specific type, could be transformed into S organisms of other specific types by the following procedure:—The subcutaneous injection, in white mice, of small amounts of living R forms together with vaccines of heterologous S cultures. (The following points were established:—(*a*) S vaccines, heated for 15 minutes at temperatures between 60° and 80°C., are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. (*b*) S vaccines heated for 15 minutes at temperatures between 80° and 100°C. are not effective

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in causing such transformations. (*c*) S vaccines heated for periods as long as 2 hours at 60°C. are effective in inducing transformation of type.) All attempts to induce transformation of type by *in vitro* methods were unsuccessful.

The studies recorded in the present communication are concerned with successful experiments on transformation of pneumococcal types by *in vitro* procedures. Previous unsuccessful attempts to effect similar transformation of type by *in vitro* procedures may be summarized as follows:

R pneumococci were cultured in blood broth to which were added the bacteria from 100 cc. of heterologous S cultures, killed by heating for 15 minutes at 60°. Transfers were continued for fifteen subcultures in this medium without the appearance of S colonies. The addition of anti-R serum (4, 5) to culture media containing S vaccines likewise failed to induce transformation. In other experiments, R forms were grown, under vaseline seal, in blood broth to which was added lymph tissue, muscle tissue and ground-up spleen, as well as large amounts of S vaccines. All such *in vitro* attempts to effect transformation of R forms into S forms of heterologous types yielded negative results. In a further attempt to secure transformation of type by *in vitro* methods the following experiment was done. A series of mice was injected intraperitoneally with large amounts of S vaccine. The animals were sacrificed at varying intervals and the peritoneal contents washed out with sterile saline. The washings were added to plain broth and inoculated with R forms. In no instance was transformation of type effected by this procedure.

In seeking an explanation of the failure to induce transformation of type by the *in vitro* methods previously adopted, two possibilities presented themselves:—either the conditions employed were unsuitable or living tissues were necessary for the transformation process. Before resorting to the latter explanation further *in vitro* studies were undertaken.

Methods

The suspensions of heat-killed organisms were prepared in the same manner as described in previous communications (2, 3). Similar control measures were adopted to establish beyond question the sterility of the vaccines:

1. Cultures were made from all vaccines in blood broth and on blood agar plates. In many experiments this was done in varying dilutions. In no instance was growth obtained.

2. In all experiments mice were injected with large amounts of S vaccine alone. Without exception all such animals survived.

Other controls will be detailed in the description of certain experiments. In

the course of the present and previous investigations pneumococcal vaccines have been employed in several hundred carefully controlled experiments. In no instance has it been possible to demonstrate the persistence of viable bacteria in the vaccines employed.

The R cultures of pneumococci employed were obtained in the usual way by growing S organisms in homologous immune serum. The nature of the R cultures was frequently proven during the course of the experiments in the following manner:—Suitable amounts of the R cultures were injected subcutaneously in white mice. Under these conditions (1, 2) the R forms invariably reverted to the S form of the type from which they had been originally derived.

The term "anti-R" serum, as employed in the present communication, requires some explanation. Avery and Heidelberger (6) showed that type-specific, anti-pneumococcus sera contain not only type-specific (anti-S) antibodies, but also antibodies reacting with the protein substance, which is common to all pneumococci. Reimann (7) subsequently showed that sera prepared with R forms are immunologically similar to sera prepared with the protein of pneumococcus. In the experiments to be reported anti-S serum of heterologous type was therefore used as a convenient source of anti-R antibodies.

The following procedure was adopted in identifying the organisms obtained in transformation experiments.

The cultures were streaked on blood agar plates which were allowed to incubate at 37°C. for 18 to 24 hours. At the end of this period the colonies were identified under a Zeiss colony microscope. Frequently it was found convenient to allow the plates to remain at room temperature for a further period of 24 hours. In no instance, however, was colony morphology alone used as the sole criterion of the nature of the organisms composing the colonies. In all transformation experiments the results were confirmed by agglutination tests carried out on cultures obtained from individual colonies.

EXPERIMENTAL

As a preliminary step *in vitro* experiments were arranged in an attempt to effect reversion of R forms to S forms of the original type by the use of homologous S vaccines.

A series of mice was injected intraperitoneally with III S vaccine. The animals were killed after intervals of 2, 4 and 6 hours. The peritoneal contents were washed out with sterile saline and the recovered material was seeded with a 3 R culture. The technique adopted varied in two particulars from that which had previously been employed: (1) very small seedings, representing 1 drop of a 10^{-6} dilution of the the R culture, were used; (2) the cultures were allowed to incubate for several days at 37°C. and blood agar plates were streaked at 24 hour

intervals. Serial transplants were not carried out. These two variations in technique were subsequently found to be of great importance.

In this preliminary experiment the R → S reversion was effected in a few instances by a single transfer of the R forms under these conditions. The experiment was then repeated using six mice and sacrificing the animals after a period of 8 hours. The peritoneal contents of each mouse were washed out with sterile saline and inoculated with 1 drop of a 10^{-6} dilution of the 3 R culture. The cultures were incubated at 37°C. and blood agar plates streaked at daily intervals for 4 days. In one instance typical III S colonies were recovered after 96 hours incubation. All previous plates streaked from this culture yielded only R colonies.

An attempt was then made to induce transformation of R forms into S forms of heterologous type by similar procedures.

Six mice were injected with III S vaccine, each animal receiving the equivalent of 100 cc. of culture. The animals were sacrificed after 6 hours and the peritoneal contents washed out with sterile saline. The recovered material was inoculated with 1 drop of a 10^{-6} dilution of a 2 R culture.

Typical III S colonies were observed in the plate streaked from one culture after 24 hours growth. This constituted the first successful attempt to secure *in vitro* transformation of type and stimulated further investigation.

In the next experiment six mice were injected intraperitoneally with III S vaccine, each mouse again receiving the equivalent of 100 cc. of culture. The animals were sacrificed after 6 hours and the peritoneum of each mouse was washed out with 1 cc. of plain broth. The peritoneal washings were transferred to six sterile agglutination tubes. To three of the six tubes was added 0.1 cc. of anti-R serum (4, 5). All six tubes were inoculated with 1 drop of a 10^{-6} dilution of a 2 R culture. The cultures were incubated at 37°C. and blood agar plates were streaked at 24 hour intervals for 4 days. Typical III S colonies were recovered from all the cultures to which anti-R serum had been added. In one instance the III S colonies appeared after 24 hours incubation and in the other two instances after 48 hours incubation. III S colonies were recovered after 24 hours incubation from one of the three cultures to which anti-R serum had not been added. The remaining two cultures constantly yielded only R colonies.

This experiment demonstrated that R forms, derived from a II S culture, could be transformed into III S organisms by growth in media

containing the peritoneal washings of mice previously injected with III S vaccine.

An attempt was next made to effect transformation of type by employing the filtrate of peritoneal washings similarly prepared.

As in the preceding experiment, six mice were injected with III S vaccine. The animals were sacrificed after 6 hours and the peritoneal contents washed out with plain broth. The material obtained from the six mice was pooled and filtered through a Berkefeld filter. The resulting filtrate was divided into four portions of 1 cc. each. 0.1 cc. of anti-R serum was added to two of the four samples. All four samples were inoculated with 1 drop of a 10^{-6} dilution of a 2 R culture. The cultures were incubated at 37°C. and blood agar plates streaked at daily intervals for 4 days. In no instance were III S colonies observed.

This experiment suggested that the factor responsible for transformation of type was either not filtrable or that it was destroyed during the process of filtration.

Attention has already been called to the fact that the technique adopted in the foregoing experiments varied in certain particulars from that which had been employed in previous unsuccessful experiments to effect transformation of type by *in vitro* methods. The question then naturally presented itself, could transformation of type be effected by this new technique without subjecting the vaccines to preliminary intraperitoneal treatment? The following experiment was therefore arranged.

Two 0.5 cc. samples of III S vaccine, each representing the bacteria from 100 cc. of culture, were inoculated with 1 drop of a 10^{-6} dilution of a 2 R culture. 0.5 cc. of blood broth was added to each. In addition one sample received 0.1 cc. of anti-R serum. Both cultures were incubated at 37°C. and blood agar plates were streaked at daily intervals. In both instances typical III S colonies were recovered from the plates after 48 hours incubation. This experiment was repeated many times and similar results were constantly obtained.

These results indicated that a technique had been established for effecting transformation of type entirely by *in vitro* methods. Experiments were accordingly undertaken to analyze the conditions responsible for transformation as induced by this procedure. A preliminary report on these studies has recently been presented (8).

Experiments to Determine the Effect of Seeding Varying Amounts of an R Culture in Media Containing Heterologous S Vaccine

It has been pointed out that the technique employed in the foregoing experiments varied in certain particulars from that which had been adopted in previous unsuccessful *in vitro* experiments. One of these variations consisted in the use of very small seedings of the R culture. It was therefore of interest to determine the effect of inoculating media containing S vaccine with various amounts of an R culture. Media containing samples of III S vaccine were seeded with 1 drop of varying dilutions of a 2 R culture. The details of the experiment appear in Table I.

TABLE I

The Effect of Inoculating Media Containing S Vaccines with Varying Amounts of Living R Forms Derived from Heterologous S Types

2 R culture, 1 drop in dilutions as detailed; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 20 cc. of original culture.

Dilutions of 2 R culture	Colonies on plates streaked		
	24 hours	48 hours	72 hours
Whole culture	R only	R only	R only
10^{-1} dilution	" "	" "	" "
10^{-2} "	" "	Several III S	Nearly all III S
10^{-3} "	" "	Numerous " "	" " " "
10^{-4} "	" "	" " "	" " " "
10^{-5} "	" "	" " "	" " " "
10^{-6} "	" "	" " "	" " " "

Controls: III S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice, injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

The results presented in Table I show that transformation of type was obtained only when relatively small seedings of the R culture were used. The experiment was repeated and similar results were obtained.

On this occasion numerous III S colonies were again recovered in all instances in which the seeding of the R culture was less than 1 drop of a 10^{-1} dilution. One III S colony was recovered after 24 hours from the 10^{-1} seeding; likewise

one III S colony was recovered after 96 hours from the whole culture seeding. All the remaining colonies obtained from the whole culture seeding and the 10^{-1} dilution seeding were of the R variety.

These experiments demonstrated the importance of employing small amounts of the R culture and afforded an explanation for the failure of many previous attempts to secure transformation of type by *in vitro* methods.

Experiments to Determine the Amount of an S Vaccine Necessary to Effect Transformation of Type in Vitro

Both for theoretical and practical reasons it was of importance to determine the amount of S vaccine necessary to effect transformation by the *in vitro* procedure.

Six sterile agglutination tubes were set up, in duplicate, containing varying dilutions of a III S vaccine, amounts representing the bacteria from 100, 50, 25, 10, 5 and 1 cc. of the original culture. To each tube were added 0.5 cc. blood broth and 0.1 cc. anti-R serum. Six of the tubes were inoculated with 1 drop of a 10^{-6} dilution of a 2 R culture. The remaining six were kept as controls. For further control purposes two mice were injected intraperitoneally with 0.5 cc. of the vaccine, representing 100 cc. of culture. The control mice were alive and well when sacrificed after a period of 10 days. All the tubes were incubated at 37°C. and blood agar plates were streaked at 24 hour intervals for 5 days. The results of the experiment appear in Table II.

The data presented in Table II show that transformation of type can be effected *in vitro* by the use of small amounts of S vaccine, amounts representing the bacteria from as little as 1 cc. of the original cultures. This experiment was repeated many times and similar results were obtained. The smallest amount of vaccine proving effective in any experiment was that representing the bacteria from 0.1 cc. of the original culture.

Determination of Culture Media Necessary to Effect Transformation of Type in Vitro

In the experiments carried out up to this point blood broth and anti-R serum were added to the broth suspensions of S vaccines as culture media for the growth of R forms. Experiments were next

arranged to determine whether the presence of these additional substances was necessary for the transformation process.

The following variations in culture media were employed: plain broth alone, plain broth and normal rabbit serum, plain broth and anti-R serum; blood broth alone, blood broth and normal rabbit serum, blood broth and anti-R serum. The results of the experiment appear in Table III.

TABLE II

The Amount of Heat-Killed Suspensions of S Forms Necessary to Induce Transformation of Type in Vitro

2 R culture, 1 drop 10^{-6} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, amounts as detailed.

III S vaccine (in cc. of original culture)	Colonies on plates streaked	
	24 hours	48 hours
100	Nearly one-half III S	Nearly all III S
50	" " " "	" " " "
25	Numerous " "	" " " "
10	20–30 " "	About one-half " "
5	6–10 " "	" " " "
1	A few " "	Numerous " "
Controls: 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine		
100	No growth	No growth
50	" "	" "
25	" "	" "
10	" "	" "
5	" "	" "
1	" "	" "

Two mice, injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 14 days.

The results presented in Table III show that transformation of type was effected in all instances except those in which plain broth alone was used as the culture medium. Type III S colonies appeared more abundantly in those instances in which both blood broth and serum were employed. Normal rabbit serum, however, appeared to be somewhat less effective than anti-R serum. The experiment was repeated on two occasions and comparable results were obtained. In

subsequent experiments both blood broth and anti-R serum were therefore used as culture media in transformation experiments.

TABLE III

Culture Media Necessary to Induce Transformation of Type in Vitro

2 R culture, 1 drop 10^{-6} dilution; III S vaccine, equivalent of 10 cc. of original culture; culture media, as detailed.

Culture media		Colonies on plates streaked			
		24 hours	48 hours	72 hours	96 hours
0.6 cc. plain broth	1	R only	R only	R only	R only
	2	" "	" "	" "	" "
0.5 cc. plain broth and 0.1 cc. anti-R serum	3	" "	" "	Several III S	Numerous III S
	4	" "	" "	R only	R only
0.5 cc. plain broth and 0.1 cc. normal rabbit serum	5	" "	One III S	A few III S	" "
	6	" "	R only	R only	" "
0.6 cc. blood broth	7	" "	Several III S	Numerous III S	Nearly all III S
	8		R only	R only	R only
0.5 cc. blood broth and 0.1 cc. anti-R serum	9	" "	One III S	Numerous III S	Nearly all III S
	10	" "	Numerous III S	Nearly all III S	" "
0.5 cc. blood broth and 0.1 cc. normal rabbit serum	11	" "	A few III S	Several III S	Numerous III S
	12	" "	R only	R only	R only

Controls: III S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice, injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

Thermal Differentiation

It was previously pointed out (3) in the *in vivo* experiments on transformation of type that the temperature at which an S vaccine is heated materially affects the results obtained. S vaccines heated for

15 minutes at 80°C. proved effective in inducing transformation of type. However, S vaccines heated for a like period at 90°C. and 100°C. were ineffective. It was a matter of considerable interest to determine whether similar results would be obtained in the *in vitro* procedure.

The bacteria from 240 cc. of a III S culture were suspended in 1.2 cc. of plain broth and divided into two equal portions of 0.6 cc. each. One sample was heated for 15 minutes at 80°C. and the other for 15 minutes at 100°C. The two lots of vaccine were used in transformation experiments as detailed in Table IV.

TABLE IV

The Effect of the Temperature at Which an S Vaccine Is Heated upon Its Efficacy in Inducing Transformation of Type

2 R culture, 1 drop 10^{-6} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 30 cc. of original culture, heated for 15 minuts at temperatures indicated.

Temperature at which S vaccine was heated		Colonies on plates streaked		
		24 hours	48 hours	72 hours
°C.				
80	1	Few III S	Several III S	All III S
	2	No " "	Few " "	" " "
100	3	" " "	No " "	No " "
	4	" " "	" " "	" " "

Controls: III S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

The data presented in Table IV show that an S vaccine heated for 15 minutes at 80°C. was effective in inducing transformation of type. An S vaccine heated for 15 minutes at 100°C., however, failed to induce the change.

In other experiments the vaccine was heated for a period of 15 minutes at temperatures of 60°, 70°, 80°, 90° and 100°. The results obtained showed slight variations with different lots of vaccine. Generally speaking, however, S vaccines heated for 15 minutes at

temperatures up to and including 80°C. proved effective in inducing transformation of type, while those heated for a similar period at 90° and 100° were uniformly ineffective.

The effect of heating an S vaccine for successive periods at various temperatures was next determined.

Aliquot portions of a III S vaccine were heated for 15 minutes for three successive occasions at temperatures of 60°, 70°, 80°, 90° and 100°C. The results of the experiment appear in Table V.

The results detailed in Table V show that a III S vaccine, heated for 15 minutes on three successive occasions at 60°C., retained its effectiveness in inducing transformation of type. Likewise a III S vaccine heated for 15 minutes on two successive occasions at 70°C. and for one period of 15 minutes at 80° was similarly effective in bringing about the change. However when heated for three successive periods of 15 minutes at 70°, and for two periods of 15 minutes at 80°, the vaccine was apparently rendered ineffective. Vaccines heated for as short a period as 15 minutes at 90° and 100°C. uniformly failed to induce transformation.

Duration of Heating

The following experiment was done to determine the effect of heating on S vaccine for a prolonged period at 60°C.

A suspension of III S organisms was heated at 60°C. At hourly intervals for 4 hours samples of the vaccine were withdrawn and used in transformation experiments. The results appear in Table VI.

The data presented in Table VI indicate that a III S vaccine, heated for 4 hours at 60°C., still retained its effectiveness in inducing transformation of type. The results suggest, however, that the samples of vaccine heated for periods of 3 hours and 4 hours at 60°C. were somewhat less effective than those lots which were heated for periods of 1 hour and 2 hours at the same temperature.

Transformation of a 2 R Culture into Type I S Organisms

In all *in vitro* experiments carried out up to this point a 2 R culture and III S vaccine were employed. In previous *in vivo* experiments

TABLE V

Effect of Heating an S Vaccine for Successive Periods of 15 Minutes at Various Temperatures between 60° and 100°C.

2 R culture, 1 drop 10^{-6} dilution; 0.7 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 20 cc. of original culture, heated as indicated.

Temperature at which vaccine was heated	No. successive 15 minute periods	Colonies on plates streaked					
		24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
°C.							
60	One 1	No III S	No III S	Several III S	Nearly all III S	All III S	All III S
	One 2	" " "	" " "	Numerous " "	" " " "	" " "	" " "
	Two 3	" " "	" " "	No " "	No " "	Several " "	Several " "
	Two 4	" " "	" " "	Numerous " "	Numerous " "	Numerous " "	Numerous " "
	Three 5	" " "	" " "	" " "	" " "	" " "	" " "
	Three 6	" " "	" " "	" " "	" " "	" " "	" " "
70	One 7	" " "	" " "	Several " "	" " "	Nearly all " "	Nearly all " "
	One 8	" " "	" " "	Numerous " "	" " "	" " " "	" " " "
	Two 9	" " "	" " "	No " "	Several " "	Numerous " "	Numerous " "
	Two 10	" " "	" " "	" " "	No " "	Several " "	" " "
	Three 11	" " "	" " "	" " "	" " "	No " "	No " "
	Three 12	" " "	" " "	" " "	" " "	" " "	" " "
80	One 13	" " "	" " "	Numerous " "	Nearly all " "	Nearly all " "	Nearly all " "
	One 14	" " "	" " "	No " "	Several " "	Numerous " "	Numerous " "
	Two 15	" " "	" " "	" " "	No " "	No " "	No " "
	Two 16	" " "	" " "	" " "	" " "	" " "	" " "
	Three 17	" " "	" " "	" " "	" " "	" " "	" " "
	Three 18	" " "	" " "	" " "	" " "	" " "	" " "

90	One	19	No	III	S	No	III	S	No	III	S	No	III	S	No	III	S	"	III	S		
		20	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
	Two	21	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
		22	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
	Three	23	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
		24	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
100	One	25	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
		26	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
	Two	27	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
		28	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
	Three	29	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		
		30	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"		

Controls: III S vaccine, heated for 15 minutes at 60°C., 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

(3) it had been demonstrated that an R culture, derived from S forms of any type, could be transformed into S forms of any other type by the use of S vaccines of the appropriate type. It therefore seemed of interest to determine whether the 2 R culture that had been transformed into III S organisms by the *in vitro* procedure could be similarly transformed into I S organisms by the use of a Type I S vaccine.

TABLE VI

The Effect of the Duration of Heating upon the Efficacy of an S Vaccine in Inducing Transformation of Type

2 R culture, 1 drop 10^{-6} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; III S vaccine, equivalent of 10 cc. of original culture, heated at 60° for periods indicated.

Duration of heating at 60°C.		Colonies on plates streaked			
		24 hours	48 hours	72 hours	96 hours
hrs.					
1	1	R only	Numerous III S	Over half III S	Nearly all III S
	2	" "	" " "	" " " "	" " " "
2	3	" "	" " "	" " " "	" " " "
	4	" "	" " "	" " " "	" " " "
3	5	" "	Suggestive III S only	Suggestive III S only	Suggestive III S only
	6	" "	One III S	A few III S	Several III S
4	7	" "	A few " "	Several " "	" " "
	8	" "	Suggestive III S only	Suggestive III S only	Suggestive III S only

Controls: III S vaccine, 1 loopful and 1 drop cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice injected intraperitoneally with III S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

A Type I S vaccine was prepared in the usual way from a virulent Type I S culture. A sample of the I S vaccine was seeded with a 2 R culture under the conditions outlined in Table VII.

Considerable difficulty was encountered in identifying the colonies which appeared on plates streaked from the cultures after 48 hours incubation. Several apparently smooth colonies were picked from the plates and transferred to blood broth. Agglutination tests on the

resulting cultures gave a typical Type I agglutination in all instances. The virulence of these cultures was determined by mouse inoculation. They were found to be of maximal virulence and cultures from the hearts' blood of the infected animals gave a typical Type I S agglutination. It therefore appeared that a 2 R culture could be transformed with equal ease into III S organisms or into I S organisms, according to the type of S vaccine employed.

Further experiments on transformation of pneumococcal types by *in vitro* procedures are described in the following paper.

TABLE VII

Transformation of R Forms Derived from a II S Culture into S Forms of Type I

2 R culture, 1 drop 10^{-6} dilution; 0.5 cc. blood broth; 0.1 cc. anti-R serum; I S vaccine, equivalent of 40 cc. of original culture.

	Colonies on plates streaked			
	24 hours	48 hours	72 hours	96 hours
1	R only	R only ?	Several I S ?	Several I S ?
2	" "	" " ?	" " " ?	" " " ?

Several suggestive I S colonies were picked and cultured in blood broth. Resulting growth gave Type I agglutination in all instances.

Controls: I S vaccine, 1 loopful and 1 drop, cultured in blood broth and on blood agar plates:—No growth in 72 hours.

Two mice injected intraperitoneally with I S vaccine in amounts equivalent to 100 cc. of original culture:—Survived 10 days.

DISCUSSION

The discussion of the results presented in the foregoing communication will be confined to a consideration of the conditions under which transformation of pneumococcal types may be effected by *in vitro* procedures.

In previous studies (2, 3) it had been shown that S pneumococci could be transformed from one specific S type into other specific S types through the intermediate stage of the R form. In this transformation process *in vivo* procedures were employed. In the present communication it has been demonstrated that similar transformations of type may be effected entirely by *in vitro* methods. The *in vitro* method

consists in growing small amounts of an R culture in suitable culture media to which has been added a vaccine of heterologous S type. Under these conditions the R forms may be transformed into S forms of the same type as the vaccine.

In considering this phenomenon the first possibility which suggests itself is that the transformation may be apparent rather than real, and may be due to the survival of S organisms in the vaccines employed. This possibility has received full consideration in previous communications (2, 3) on transformation of type by *in vivo* procedures. In the course of the present and previous investigations pneumococcal vaccines have been employed in several hundred carefully controlled experiments. In no instance has it been possible to demonstrate the occurrence of viable organisms in the vaccines employed. The conclusion must therefore be drawn that, if the transformation is due to the persistence of living bacteria in the vaccines, the surviving cells do not conform to any recognized form of bacterial life.

The suggestion that the transformation may only be the result of a temporary acquisition, by the R form, of the attributes of the S cell, has also received full consideration in previous communications. It was shown (3) that the newly formed S organisms not only possessed all the characteristics of type-specific S cells but that they continued, when subcultured, to reproduce S forms apparently indefinitely. The results of the experiments therefore indicate that, under the conditions provided, the R cell acquires the capacity of elaborating a specific polysaccharide of the same type as that of the vaccine in which it has been grown.

The conditions under which transformation of type may be effected merit some consideration. It was shown that relatively small inocula of R cells provide the most suitable conditions. Large inocula usually resulted in the growth of R forms only. The growth of large numbers of R pneumococci apparently created conditions unfavorable for transformation. No adequate explanation of this finding is offered. The suggestion is made, however, that the unsuitable conditions may have been produced by the elaboration of bacterial peroxide by the large amounts of R inocula. Further experiments are required to establish this point.

In the experiments reported very small quantities of vaccine, quantities representing the bacteria from as little as 0.1 cc. of the original culture, were effective in inducing transformation of type. Smaller quantities of vaccine, representing the bacteria from less than 0.1 cc. of the original culture, proved ineffective. Attempts to extract the essential factor responsible for transformation are described in the following paper.

In effecting transformation of type by *in vitro* procedures the nature of the culture media employed appears to be a matter of considerable importance. In all successful experiments either serum or red blood cells were added to the culture media. When plain broth alone was used transformation failed to occur. The suggestion is offered that the addition of the blood and serum may have afforded a convenient source of catalase and peroxidase. In the absence of these substances sufficiently reduced conditions may not have been present. Further experiments relating to this point are described in the following paper.

In previous communications Dawson and Avery (4) and Dawson (5) showed that the growth of R pneumococci in media containing anti-R serum frequently resulted in the conversion of R forms into S forms of the original type. For this reason anti-R serum was employed in the majority of the transformation experiments reported in this study. During the course of the investigation it was found that, although the use of anti-R serum appeared to facilitate transformation, the change could frequently be effected in the absence of anti-R antibodies.

The observation that the property of an S vaccine responsible for transformation of type is thermolabile is of considerable interest. Transformation was effected with S vaccines which had been heated for a period of 4 hours at 60°C. The change was also induced with vaccines heated for a period of 15 minutes at 70° and for 15 minutes at 80°C. However, vaccines heated for a similar period at 90° and at 100°C. were not effective. Furthermore, transformation was induced with vaccines heated for 15 minutes on three successive occasions at 60°C., for the same period on two successive occasions at 70°C. and for one period of 15 minutes at 80°C. Vaccines heated for a period of 15 minutes on three successive occasions at 70°C. and for a similar period on two successive occasions at 80°C. were not effective. Refer-

ence will be made to the significance of these observations in the following paper.

In vitro experiments have been described in which R forms of pneumococcus, derived from a Type II S culture, have been transformed into S forms of Type I and into S forms of Type III, according to the type of vaccine employed. This observation supports previously described *in vivo* transformation experiments in which it was shown that R forms, derived from one specific type, may be transformed into S forms of any other specific type (3).

The nature of the mechanism by which transformation of type may be brought about by *in vitro* procedures and the significance of these findings in the field of bacteriology and epidemiology will be discussed in the accompanying paper.

SUMMARY AND CONCLUSIONS

1. Type-specific S pneumococci may be transformed from one specific S type into other specific S types entirely by *in vitro* methods.

2. R forms of pneumococci, derived from S forms of one specific type, may be transformed into S forms of other specific types by the following *in vitro* procedure:—the growth of small inocula of R forms in media containing vaccines prepared from heterologous S cultures.

3. Transformation of type may be effected in this procedure by the use of small quantities of S vaccine,—quantities representing the bacteria from as little as 0.1 cc. of the original culture.

4. Transformation of type, as induced by this procedure, is most readily effected by employing anti-R serum in the culture medium. Transformation of type may be effected, however, in media which do not contain anti-R antibodies.

5. Previous findings on the thermal characteristics of the property of S vaccines responsible for transformation of type have been confirmed and extended.

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