STUDIES ON REGENERATION OF BACTERIOPHAGE.

I. The Influence of Partial Anaerobiosis upon Regeneration of a Highly Diluted Lytic Principle.

By GREGORY SHWARTZMAN, M.D.

(From the Laboratory of Bacteriology of the New York Homœopathic Medical College and Flower Hospital, New York.)

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INTRODUCTION.

Quantitative studies on the potency of bacteriophage have established that 1/100,000,000 cc. per cc. of broth or a dilution 10^{-8} of this principle is usually the smallest amount which is able to produce an appreciable lysis. The dilutions are made in a series of tubes containing 4.5 cc. of broth. If larger volumes of broth are used a dilution of bacteriophage still greater than 10^{-8} is able to produce an almost complete lysis. This last observation was made by Gratia and De Kruif (1).

Doerr and Zdansky (2) corroborated Gratia and De Kruif. They pointed out that although it is probable that a certain minimal absolute quantity of lytic principle is necessary for regeneration, as claimed by Gratia and De Kruif, this assumption must be made with some reserve. They also drew attention to the possibility that for a given high dilution 1000, 100, and 10 cc. still contain a few elements of the bacteriophage, while 1 cc. may have none. As is seen these authors did not reach any definite conclusion about the nature of the phenomenon under discussion.

The authors mentioned apparently paid no attention to the possibility that the conditions for the regeneration of a highly diluted bacteriophage may not be identical in the various containers employed on account of differences in the supply of air. Although Gratia and De Kruif did not state the capacity of the containers used it is assumed by the writer that the tubes were those of standard size usually employed for broth cultures in bacteriological laboratories and that

1000 cc. of fluid was put in the "2 liter" Erlenmeyer flasks. In the latter case the access of oxygen[1] to 1000 cc. of fluid in a "2 liter" Erlenmeyer flask and to 10 cc. of broth in a 120 × 22 mm. tube is correspondingly 35 and 10 times more restricted than to 1 cc. of broth in a 120 × 22 mm. tube.

Preliminary experiments have shown that the degree of exposure to air of a highly diluted bacteriophage plays an important rôle in the regeneration of a principle so diluted. In view of this observation the following investigations were planned:

1. To determine whether the results obtained by Gratia and De Kruif depend upon differences in the access of air to lytic cultures contained in vessels of various sizes.

2. To study the relation of partial anaerobiosis to the regeneration of a highly diluted lytic principle.

Methods.

The media employed for this work were always adjusted to pH 7.6 and prepared from Difco meat extract.

The bacteriophage kindly sent to me by Dr. J. Bronfenbrenner was obtained by him from feces of a convalescent from Shiga dysentery by employing d'Hérelle's technique. This principle was then adapted to *B. coli* in this laboratory.

The strength of this phage, as well as that of the ones regenerated under various conditions, was always tested by titration in broth (Appelmans (4)) and its quantitative value expressed by Werthemann's lytic exponents recorded symbolically as E_L (5) (the dilution of the last tube showing lysis).

The count of plaques, as proposed by D'Hérelle for quantitative estimation of the potency of bacteriophage, was never attempted since it had been pointed out by Bronfenbrenner (6) that the number and appearance of these areas depend on many factors, such as concentration of agar, moisture of the surface of the slant, etc., which may considerably interfere with the reliability of results obtained.

Since the degree of lysis and the titer of the bacteriophage may sometimes depend to a certain degree on the size of the *inoculum* a uniform inoculum was used throughout the experiments so far as possible. Unless otherwise stated, about 400,000 bacteria per cc. were usually inoculated. The emulsion was always prepared by washing a 24 hour agar slant culture of *B. coli* and diluting it with normal salt solution to the required density.

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[1] The access of oxygen to a fluid can be measured by the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio (Avery and Morgan (3)).

EXPERIMENTAL.

I.

In repeating Gratia and De Kruif's experiment with the anticolon bacteriophage employed in this laboratory, the following results were obtained for a 10^{-9} dilution of this principle in broth inoculated with 20 million *Bacillus coli* per cc.

1000 cc. – Erlenmeyer flask of "2 liter" capacity—complete lysis.
100 " – " " " 250 cc. " " "
10 " –120 × 22 mm. test-tube—almost complete lysis.
5 " –120 × 22 " " —no lysis.
1 " –120 × 22 " " " "

According to Gratia and De Kruif's explanation of these results, 1 to 5 cc. are the "non-lytic" absolute quantities. Any amount above 5 cc. contains a "lytic" absolute quantity; *i.e.*, an absolute amount sufficiently large to produce lysis.

These preliminary observations were utilized in the following experiments which were planned to determine whether variations in the absolute quantity of an extremely diluted bacteriophage bear a relation to its regeneration.

A. Complete Regeneration of a Small "Non-Lytic" Quantity of Highly Diluted Bacteriophage under Conditions of Restricted Supply of Air.

The anticolon bacteriophage was diluted up to 10^{-9} in 1012 cc. of broth, inoculated with approximately 20 million *Bacillus coli* per cc.[2] The mixture was shaken and distributed into different containers so that various $\frac{\text{Surface area}}{\text{Total volume}}$ ratios were obtained for "non-lytic" absolute quantities of this principle (1 and 5 cc.). In 24 hours of incubation the degree of lysis was compared macroscopically, then samples from each container were heated to 58° for ½ hour and titrated in broth. Table I represents a summary of results obtained.

As is seen from this table small "non-lytic" absolute quantities (1 and 5 cc.) of 10^{-9} dilution of bacteriophage may produce complete

[2] An inoculum of a similar size was used by Gratia and De Kruif.

lysis and regenerate a lytic principle of the same strength manifested by a large absolute amount, provided that the access of oxygen is restricted to the same degree in both cases.

TABLE I.

Regeneration of a Small, "Non-Lytic" Quantity of Highly Diluted Bacteriophage under Restricted Supply of Air.

Capacity of the container.	Amount of 10^{-9} lytic broth inoculated with *B. coli*.	$\frac{\text{Surface area}}{\text{Total volume}}$ ratio.	Lysis.	E_L of regenerated phage.
	cc.			
Erlenmeyer flask, 2 liter.....	1000	0.1	+	8
Tube 120 × 8 mm.....	5	0.1	+	8
" 120 × 22 ".....	5	0.7	0	0
" 120 × 22 ".....	1	3.5	0	0
" 120 × 8 "	1	0.5	+	8

+ = lysis; 0 = normal growth.

TABLE II.

Absence of Regeneration of a Large, "Lytic" Amount of Highly Diluted Phage under Conditions of Free Access of Air.

Capacity of the container.	Amount of 10^{-9} lytic broth inoculated with *B. coli*.	$\frac{\text{Surface area}}{\text{Total volume}}$ ratio.	Lysis.	E_L of regenerated phage.
	cc.			
Erlenmeyer flask, 2 liter.....	100	1.5	0	0
120 × 22 mm. tube.....	2.34	1.5	0	0
120 × 8 " "	5	0.1	+	8
Erlenmeyer flask, 250 cc.....	100	0.1	+	8

+ = lysis; 0 = normal growth.

B. Absence of Regeneration of a Large, "Lytic" Amount of Highly Diluted Bacteriophage under Conditions of Free Access of Air.

This experiment was made in the same manner as Experiment A, there being a difference, however, in the size of containers and amounts of fluid introduced. Table II presents the results obtained.

As is seen, the regeneration of a large volume of highly diluted bacteriophage depends entirely on the restriction in supply of air usually obtained in such volumes. However, if the supply of air becomes

less restricted, there is no regeneration obtained even in a large volume.

II.

These observations on the influence of restriction in supply of air upon the regeneration of highly diluted bacteriophage have been further supported by a study of this phenomenon in relation to different factors, as in the following four experiments.

TABLE III.

The Relation of $\frac{Surface\ Area}{Total\ Volume}$ Ratio to Regeneration of a Highly Diluted Lytic Principle.

Size of tube.	Amount of 10^{-9} lytic broth inoculated with *B. coli*.	$\frac{\text{Surface area}}{\text{Total volume}}$ ratio.	Lysis.	E_L of regenerated phage.
mm.	*cc.*			
120 × 22	1	3.5	0	0
120 × 22	2	1.75	0	0
120 × 22	3	1.16	0	0
120 × 22	4	0.88	0	0
120 × 22	5	0.7	0	0
120 × 8	1	0.5	+	8
120 × 8	2.5	0.25	+	8
120 × 8	5	0.1	+	8

In the column Lysis, 0 = normal growth; + = lysis.

1. Relation of Various $\frac{Surface\ Area}{Total\ Volume}$ Ratios to Regeneration of a Highly Diluted Bacteriophage.

To determine the restriction of the supply of air necessary for regeneration of a highly diluted bacteriophage, a 10^{-9} dilution of this principle in broth was inoculated with approximately 20 million *Bacillus coli* per cc. Decreasing amounts of this mixture from 5 to 1 cc. were distributed in 120 × 22 mm. and 120 × 8 mm. tubes so that a wide range of $\frac{\text{Surface area}}{\text{Total volume}}$ ratios was obtained. After 24 hours of incubation, readings of the degree of lysis were made macroscopically and

samples from each of the tubes, heated to 58° for ½ hour, were titrated to determine the lytic exponents. Table III gives the details of this experiment and the results obtained.

As will be seen, the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.5 supplies the minimal degree of anaerobiosis required for regeneration of the highly diluted bacteriophage. An increase in the ratio above this point completely prevents the regeneration of bacteriophage.

2. The Rate of Regeneration of Highly Diluted Bacteriophage under Conditions of Partial Anaerobiosis, and the Relation of This Regeneration to the Rate of Bacterial Growth.

To investigate the rate of regeneration of bacteriophage under a restricted supply of air and to establish the relation of this regeneration to bacterial growth, it was necessary to make a quantitative study. This was done as follows:

Bacteriophage was diluted up to 10^{-9} in 10 cc. of broth previously inoculated with *B. coli.* 5 cc. of this mixture was then placed into two tubes measuring 120 × 22 mm. and 120 × 8 mm. respectively with result that $\frac{\text{Surface area}}{\text{Total volume}}$ ratios of 0.7 and 0.1 were obtained. Before incubation and every 30 minutes during the following 6½ hours of incubation, samples were taken from these tubes for counts of viable organisms. At the same intervals of time 0.1 cc. was taken from each tube, added to 9.9 cc. of broth, and heated at 58° for ½ hour. These mixtures were later titrated in broth for determination of their lytic exponents.

Charts 1 and 2 represent the results obtained.

As is seen, the regeneration of a highly diluted lytic principle occurs only under conditions of partial anaerobiosis, begins distinctly at the 3rd hour of incubation, and is completed in about 6 hours. When the growth curves of Charts 1 and 2 are compared, very little difference can be noted in the rate of growth of *Bacillus coli* during the first 3 to 4 hours of incubation. It is suggested, therefore, that partial anaerobiosis does not favor the regeneration of bacteriophage by modifying the rate of bacterial growth, but has some direct relation to the regeneration of this principle. Further investigation, however, is necessary to establish the point definitely.

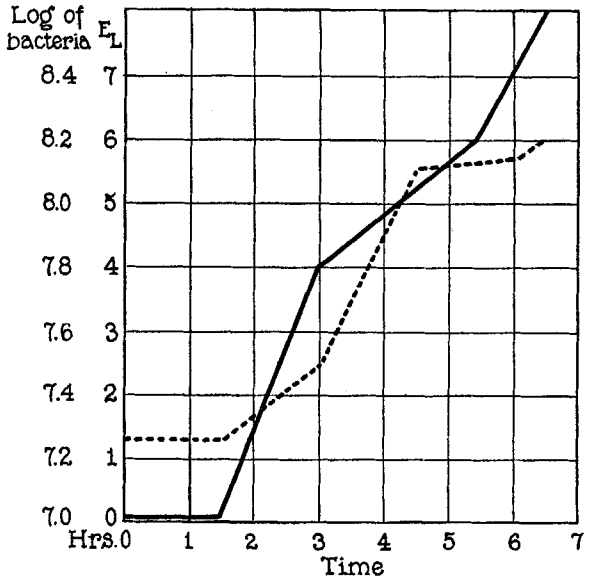

CHART 1.

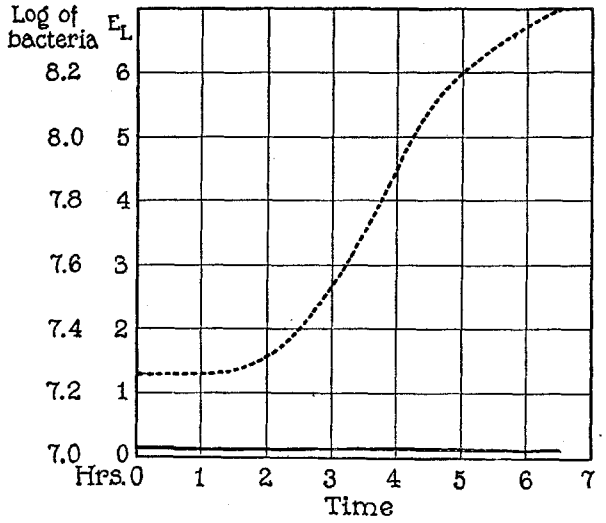

CHART 2.

CHART 1. The relation of regeneration of bacteriophage under partial anaerobiosis to the bacterial growth curve. The dotted line represents the rate of bacterial growth at $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1. The solid line represents the regeneration of bacteriophage at this ratio.

CHART 2. Absence of regeneration of a highly diluted bacteriophage at $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.7. The dotted line represents the bacterial growth at the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.7. The solid line represents the regeneration of a highly diluted phage at this ratio. Through an error in drawing this chart, the curve of bacterial growth was made in free hand.

3. Minimal Time of Restricted Air Supply Required for Regeneration of Highly Diluted Bacteriophage.

The object of this experiment was to find out the shortest time during which the culture with a highly diluted bacteriophage must be kept under conditions of partial anaerobiosis to obtain regeneration.

A 10^{-9} dilution of lytic principle in broth was inoculated with approximately 20 millions *B. coli* per cc. and distributed in 5 cc. quantities into series of 120 × 8 mm. tubes $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 0.1\right)$ and into one control tube 120 × 22 mm. in size $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 0.7\right)$. These tubes were placed in the incubator and kept there for various periods of time from 10 minutes up to 2 hours. Every 10 minutes one 120 × 8 mm. tube was taken from the incubator and its contents poured into a 120 × 22 mm. tube which was further incubated up to 24 hours. In this manner a series of cultures kept under the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1 for different intervals of time was obtained. The degree of lysis in these tubes in 24 hours was estimated macroscopically and their lytic exponents determined by titration in broth.

It was found that 1 hour of incubation at the ratio 0.1 was sufficient for complete lysis and full regeneration ($E_L = 8$) of a highly diluted bacteriophage.

4. The Influence of Partial Anaerobiosis upon Regeneration of Highly Diluted Bacteriophage at Different Points on the Bacterial Growth Curve.

In the previous experiment, as was seen, the supply of air restricted to a certain degree for 1 hour at the beginning of the growth curve was sufficient for regeneration of a highly diluted bacteriophage. The object of this experiment was to find out whether any regeneration of such a principle would occur if the conditions of partial anaerobiosis were created at phases of growth following the 1st hour.

In order to investigate this point 5 cc. of *Bacillus coli* cultures containing a 10^{-9} dilution of the bacteriophage was incubated in 120 × 22 mm. tubes $\left(\frac{\text{Surface area}}{\text{Total volume}} \text{ ratio} = 0.7\right)$ for various intervals of time

and then transferred into 120 × 8 mm. tubes $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1) and incubated for 1 hour. At the end of this hour the fluid was poured back into 120 × 22 mm. tubes and incubated to bring the total time of incubation up to 24 hours. A series of counts of viable organisms in each tube was made every 30 minutes for a period of 6 hours. After 24 hours of incubation the degree of lysis in all the tubes was determined macroscopically and their lytic exponents found by titration in broth. Since the counts showed that the growth of bacteria was not interfered with by transferring the culture from 120 ×

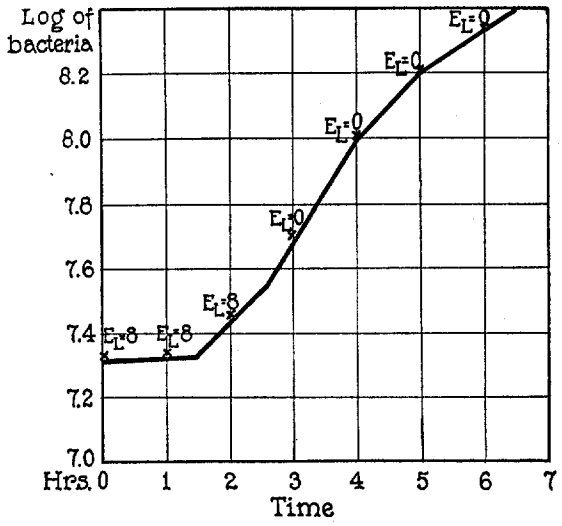

CHART 3. The influence of partial anaerobiosis upon regeneration of a highly diluted bacteriophage at different points of the growth curve.

22 mm. into 120 × 8 mm. tubes for 1 hour, only one growth curve is presented in Chart 3. Crosses at different parts of this curve indicate the points at which the $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.1 was created for 1 hour. The lytic exponent of the bacteriophage regenerated under corresponding conditions is placed above each cross.

As will be seen from the chart, if one is to obtain regeneration of a highly diluted lytic principle it is necessary to restrict the access of air at the 1st, 2nd, or 3rd hour of growth. At the later phases of

growth no regeneration of a principle so diluted occurs even under restriction of the supply of air.

SUMMARY.

The regeneration of an extreme dilution of bacteriophage in a large volume of broth, as described by Gratia and De Kruif, is due to the influence of partial anaerobiosis upon the regeneration of the principle.

The influence of partial anaerobiosis upon regeneration of an extremely diluted bacteriophage was investigated in relation to different factors with the following results.

1. The $\frac{\text{Surface area}}{\text{Total volume}}$ ratio = 0.5 expresses the degree of anaerobiosis necessary for regeneration of an extreme dilution of the bacteriophage. An increase in this ratio interferes with regeneration.

2. The regeneration of the highly diluted bacteriophage under partial anaerobiosis begins definitely at the 3rd hour of bacterial growth and is completed after about the 6th hour.

3. To obtain the full regeneration of an extreme dilution of bacteriophage it is sufficient to restrict for one hour the supply of air to a culture containing it, providing that this restriction be produced within the first 3 hours of the bacterial growth.

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