GROWTH AND PHAGE PRODUCTION OF LYSOGENIC B. MEGATHERIUM

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The “lysogenic” strain of B. megatherium, first described by den Dooren de Jong (1931), produces a phage which causes death and lysis of a closely related strain of B. megatherium, but has no apparent affect on the culture which produces it. It seems probable that such lysogenic strains are the source of the bacterial viruses found in nature, since a system in which all infected cells die, as is the case with the most intensely studied cultures, is evidently self-limiting and can only serve as a continuous source of virus under very unusual conditions.

De Jong considered that the culture produced the virus as part of its normal metabolism and concluded that this proved the truth of Bordet’s and Cuica’s hypothesis of the endogenous origin of the bacterial viruses. Subsequent work showed that this conclusion was unjustified since lysogenic strains may be produced by infecting cultures in the laboratory (Gratia, 1936 a).

On the other hand, the fact that lysogenic cultures may be obtained by infecting normal cells, does not prove that all lysogenic cultures arose in this way.

All cells and all spores of the culture produce phage (Bordet and Renaux, 1928; Burnet and McKee, 1929; Lwoff and Gutman, 1950). Spores may be heated to 100°C. without losing the ability to produce phage (den Dooren de Jong, 1931).

The lysogenic strain produces phage in the absence of Ca⁺⁺ (Wahl, 1946), but the sensitive strain requires Ca⁺⁺ (Gratia, 1936 a). Both strains produce a gelatinase under the conditions required for phage production (Northrop, 1939).

The present experiments have confirmed the survival of the virus in heated spores and the production of the virus in the absence of calcium. The course of the reaction is very dependent on the air supply and, in rapidly shaken cultures, no evidence of lysis can be found. If the cultures are allowed to stand, however, lysis commences at once in those cultures which are producing phage. This probably accounts for the conflicting results of others in this connection.

The rate of growth and of phage formation of the lysogenic culture has been studied in a variety of synthetic media in an attempt to find a medium in which phage formation would occur without growth of the culture. No medium
could be found which gave this result, although there was some variation in the ratio of phage production to growth. The cultures which form the most phage also synthesize the most ribose nucleic acid.

In the absence of added Mg\(^{++}\) (or in the presence of more than 0.05 m PO\(_4\)), however, the culture grows quite well, but liberates no phage. Addition of Mg\(^{++}\) to such a culture results in phage production in a short time. Every cell from a culture grown for more than 20 generations in the presence of 0.1 m PO\(_4\) still produces phage as soon as it is grown in the presence of Mg\(^{++}\).

Cultures grown on peptone agar containing glucose do not lose their lysogenic properties as does the lysogenic strain studied by Ehrlich and Watson (1949).

**EXPERIMENTAL RESULTS**

**Cultures.**—The culture of *B. megatherium* 899a used in these experiments was obtained from Professor André Gratia. It is a descendant of the original lysogenic 899a isolated by den Dooren de Jong, and has the characteristics of the smooth strain, described by den Dooren de Jong. Spores were formed after several months on peptone agar slants at 25°C. The culture produced the two types of phage described by Gratia (1936 c).

The phage produced by this culture did not cause lysis when added to a culture of *B. megatherium* 36 sensitive, nor give satisfactory plaques when plated with this strain. It forms good plaques, however, when plated with a *B. megatherium* culture obtained from Professor Krueger (designated as KM).

**Conditions for the Determination of Phage.**—The method of plating described by Gratia (1936 d) was used. The modification of this method, described by Hershey, Kalmanson, and Bronfenbrenner (1943), in which 3.5 ml. of agar is spread on the plate, instead of 1 ml. fails to result in the formation of good plaques, probably because of the reduced air supply.

The number of plaques formed depends on the kind of agar on which the sensitive culture is grown as well as on the type of agar contained in the Petri dish upon which the indicator culture is spread (Table I). The largest number of plaques with filtered phage solutions or suspensions of lysogenic cells is formed on yeast extract-peptone (Y.E.P.) agar plates with an indicator culture also grown on Y.E.P. agar. Lysogenic cells form plaques with the indicator culture on veal infusion broth (Difco) (V.I.B.) agar as well as on Y.E.P. agar but phage solutions do not. Neither the cells nor phage form plaques on tryptose phosphate broth (Difco) (T.P.B.) agar. The failure to form plaques on T.P.B. agar is due to the fact that little or no phage is formed on this medium, although the cells grow quite well (cf. Table VI). The low yield with phage solution on V.I.B. agar is due to the lower absorption of phage by the indicator cells in this medium. The formation of plaques by lysogenic cells is not so dependent upon the absorption since the lysogenic colony produces local high concentrations of
phage which will infect and prevent the growth of the sensitive strain, even though the absorption is very low. The low yield from indicator cultures grown on V.I.B., and then washed and spread on Y.E.P., compared to those grown on Y.E.P., is also probably due to differences in absorption of the phage, since the difference does not appear in the case of lysogenic cells.

The physiological condition of the indicator culture is of great importance. The largest number of plaques is formed when resting cells are used (cf. Northrop, 1939). Rapidly growing cells form few or no plaques (Table II). This result is related to the fact that the indicator strain used does not undergo

**TABLE I**

*Effect of Various Culture Media on Plaque Count of Lysogenic Cell Suspensions and of Phage Solutions*

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered phage........</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed lysogenic cells</td>
<td>[100]</td>
<td>10</td>
<td>0</td>
<td>[100]</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Plaques/plate as per cent of Y.E.P.-Y.E.P. plate:

| Filtered phage........ | 40     | 50     | 5      | 0      | 0      | 0      | 100    | 100    | 100    | 0      | 0      |        |
| Washed lysogenic cells | 100    | 100    | 100    | 100    | 100    | 100    | 100    | 100    | 100    | 0      |        |

Per cent phage adsorbed 1 hr. -- 35°C., 1 × 10⁶ B/ml.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered phage........</td>
<td>15</td>
<td>10</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>100</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

lysis nor produce much phage when grown in liquid media. This, in turn, is due to the fact that the growing cells do not absorb the phage, whereas resting cells do absorb it (Table III).

*Conditions for Phage Formation with Lysogenic Strain.*—Growth and phage production with aerobic organisms are much more rapid and reproducible if the culture tubes are shaken (Krueger, 1930; Gratia 1936 b; Northrop, 1939).

Failure to agitate the culture, in the case of *B. megatherium* especially, is the reason for the very low phage yields sometimes reported for this culture (Ehrlich and Watson, 1949).

The effect of shaking on growth, phage production, and lysis of the lysogenic strain is shown in Fig. 1. The culture which was shaken continuously increased
from 0.2 to $1.2 \times 10^6 B/ml.$ and from 0 to $1 \times 10^8 P/ml.$ in 7 hours, whereas the culture which was allowed to stand grew only from 0.2 to $0.25 \times 10^6 B/ml.$ in the same period and produced only $0.01 \times 10^8 P/ml.$ The samples removed at various times stop growing at once and begin to clear. The production of phage also decreases, however, at the same time that growth stops, and less phage is formed by the cultures which clear than by those which continue to grow. It appears that the lysogenic cell, when producing phage, requires a very highly aerated medium in order to grow (cf. Lwoff and Gutman, 1950).

### TABLE II

**Effect of the Physiological State of the Test Culture on the Plaque Count**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>B/ml cell suspension A</th>
<th>Plaque count per plate</th>
<th>B/ml cell suspension B</th>
<th>Plaque count per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$10^8$</td>
<td>20</td>
<td>$10^8$</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>130</td>
<td>2.5</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>140</td>
<td>3.6</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>90</td>
<td>5.0</td>
<td>4</td>
</tr>
<tr>
<td>24</td>
<td>20</td>
<td>60</td>
<td>Stand 35°C.</td>
<td>100</td>
</tr>
</tbody>
</table>

### TABLE III

**Adsorption of Phage by Growing or Resting Cells of Indicator Strain**

<table>
<thead>
<tr>
<th>Cells</th>
<th>$10^8$ B/ml</th>
<th>$10^6$ P/ml after 1 hr at 35°C</th>
<th>Adsorption per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In suspension</td>
<td>In supernatant</td>
<td></td>
</tr>
<tr>
<td>Resting cells from 20 hr. Y.E. agar suspended in Y.E.</td>
<td>1.0</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>Cells in logarithmic growth in Y.E.</td>
<td>1.0</td>
<td>80</td>
<td>75</td>
</tr>
</tbody>
</table>

80 X $10^8$ phage/ml added, the suspension shaken 1 hr. at 35°C. Sample centrifuged and the suspension and supernatant plated for phage.
Changes in Turbidity, Cell Count, Colony Count, Phage Content of the Lysogenic Culture Growing in Y.E. (Fig. 2)

The cell concentration as measured by turbidity, chains or clumps by microscopic count, colonies on V.I.B. or T.P.B., and plaques formed by cell suspensions plated with the indicator strain on Y.E. all show the usual S-shaped growth curves and all agree, within the experimental error. The B/ml. by

![Graph showing changes in turbidity, cell count, colony count, and phage content.]

Fig. 1. The effect of shaking on the growth, phage production, and lysis of the lysogenic strain.

Cells from a 20 hour Y.E. agar Petri dish culture washed three times in Y.E. and suspended in Y.E. 100 ml. suspension in 500 ml. Florence flask and shaken at 35°C. Rate of shaking about 20 oscillations per minute through 2 cm. This is just sufficient to produce some foam. 10 ml. samples withdrawn as noted and allowed to stand at 35°C. in 2.0 X 20 cm. test tubes.

B/ml. determined by turbidity on suspension.
P/ml. determined by plating supernatant.

turbidity is higher than the other values since this measurement is standardized against cell count rather than chain count. The chains contain an average of about 3 cells at the beginning and 2 cells at the end. This result shows that every chain is viable and can give rise to a colony on V.I.B. or T.P.B. agar and also that every chain produces phage since the number of plaques formed by the cell suspension is equal to the number of colonies. Toward the end of the experiment, nearly half the “chains” consist of one cell so that many of the colonies must come from one cell and hence, infected cells must be able to grow.

The phage concentration increases in a similar, but more rapid way, so that, as usual, the more rapid the growth of the culture, the more rapid the rate of
PHAGE PRODUCTION OF LYSOGENIC B. MEGATHERIUM

Fig. 2. Changes in turbidity, cell count, phage concentration, and colony count on various media, in a lysogenic culture growing on Y.E.

20 hour culture of 899α from Y.E. agar, washed three times in Y.E., suspended in 100 ml. Y.E., and shaken at 35°C. Sample removed as noted and analyzed as follows:—

B/ml. by turbidity, read at once, and after standing 24 hours in test tube.

Chains of cells/ml. by microscopic count.

Colonies/ml. Diluted 1/10⁶ and plated on Y.E., agar, V.I.B. agar, and T.P.B. agar.

P/ml. supernatant. Centrifuged, supernatant diluted 1/10⁶, and plated for plaques on Y.E.P. agar. This is the free phage. Precipitate suspended in original volume Y.E., suspension diluted 1/10⁶, and plated as above. This count is the lysogenic cell count.

phage formation. Over the logarithmic part of the curve, the results may be expressed by the equation derived for S. aureus by Krueger and Northrop: \[
\log P = K \log B + C
\]

\[
\frac{dP}{Pdt} = K \frac{dB}{Bdt}
\]

K is the ratio of the growth rate of phage to cells and in this case has a value
of about 3.1 This equation is identical with that found by Huxley (1932) to
relate the rate of growth of various organs of the body (Northrop, 1939 b).

There is no indication of lysis, or of any injury to the host cells in any of the
above determinations. If, however, the culture is plated for colonies on Y.E.
agar, the colony count decreases very rapidly, just as the parent culture begins
to grow and produce phage. A cell which grows rapidly in concentrated sus-
pension in liquid Y.E., therefore, will not form a colony on Y.E. agar, but will
form colonies on V.I.B. agar or T.P.B. agar. The failure to grow on Y.E.
agar is due to a change in the cells themselves and not to any substance present
in the culture medium, since such growing cells will not form colonies on Y.E.
agar, even after repeated washings, while resting cells added to the supernatant
of the growing cells, give rise to the same number of colonies on Y.E. agar as
on T.P.B. agar (Table IV).

The failure of cells to grow in Y.E. agar, at the time when rapid phage pro-
duction occurs, shows that these cells are more sensitive to cultural conditions
then cells which are not in this state. The failure to grow in the Y.E. agar
might be due to an inadequate oxygen supply, since cells in this state die and
undergo lysis, if the parent tube is not shaken (cf. Fig. 2).

The failure may also be due to the fact that Y.E. is not as suitable a medium
for growth as V.I.B. or T.P.B. either because it contains some inhibitory sub-
stance, or because it lacks some necessary growth accessory. The question may
be answered by determining the minimum number of cells which are required
to cause growth in liquid Y.E., liquid V.I.B., or in the filtrate from a growing
culture in Y.E. The results of such an experiment are shown in Fig. 3. The
number of cells required to cause growth in Y.E. increases rapidly at the time
when the number of colonies on Y.E. plates decreases, and then decreases

1 D’Herelle (1926) found, under certain conditions, that the virus concentration
increased in steps and considered the steps to represent rapid lysis of some cells with
the sudden liberation of virus. This observation was confirmed and extended by Burnet
(1929) and Ellis and Delbrück (1938). Delbrück and Luria (1942) were able
to standardize conditions so that the step-like curves were obtained with great reg-
ularity.

Under other conditions (Doerr and Grüninger, 1923; Krueger and Northrop, 1930)
smooth logarithmic curves are obtained. These different results are not contradictory
since overlapping of many single steps will give a smooth curve, just as the curve of
growth of a bacterial culture is a smooth curve, while the curve representing the in-
crease of a single cell would be a series of steps, each step representing a cell division.
The hydrolysis of cane sugar would also occur in steps, if a single molecule is considered,
but statistically a smooth curve is obtained. There is, however, an essential difference
between the curve of cell division and that of a single chemical reaction in that the
time of division of a cell into two depends on its past history, whereas the splitting of
a molecule is a matter of probability only.
again as the number of colonies increases. The number of cells required to cause growth in V.I.B. or Y.E. filtrate from a growing culture is much less than that required in untreated Y.E. and remains constant over the entire period.

TABLE IV

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Sample No.</th>
<th>B/ml turbidity</th>
<th>Chains/ml.</th>
<th>Colonies/ml on Y.E.</th>
<th>Colony on V.I.B.</th>
<th>Plaques/ml on Y.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 ml. sample centrifuged and precipitate suspended in 2 ml. Y.E.</td>
<td>$0.15 \times 10^9$</td>
<td>$0.04 \times 10^9$</td>
<td>$0.03 \times 10^6$</td>
<td>$0.035 \times 10^6$</td>
<td>$0.04 \times 10^6$</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 ml. sample centrifuged and precipitate suspended in 2 ml. Y.E.</td>
<td>0.60</td>
<td>0.15</td>
<td>0.004</td>
<td>0.30</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Suspension 1 centrifuged and cells added to supernatant from Sample B</td>
<td>0.65</td>
<td>0.10</td>
<td>0.15</td>
<td>0.09</td>
<td>0.20</td>
</tr>
</tbody>
</table>

The decrease in colony count in Y.E. plates, therefore, is due to the fact that Y.E. is not entirely suitable for cell growth until it has been in contact with the cells for some time.

Resting cells are able to survive and grow in this solution, but rapidly growing cells which are producing phage cannot, unless the cell concentration is high ($>10^8$/ml). Usually, rapidly growing cells are better for inoculating a new culture than resting cells, so that the failure of these cells to grow is probably due to the fact that they are producing phage at this time. This explanation is
FIG. 3. Changes in cell concentration, phage concentration, colonies on Y.E. agar, and minimum number of cells to grow in 1 ml. of Y.E., V.I.B., or filtrate from 899a in Y.E.

Growth from 21 hour Y.E.P. plate washed three times in Y.E., suspended in Y.E., and shaken at 35°C.
P/ml. determined by plating 1/10⁶ ml. with sensitive strain.
B/ml. by turbidity.
Chains/ml. by microscopic count.
Colonies on Y.E. Dilute in Y.E. and 1/10⁶ ml. plated on Y.E. agar.
Minimum number of cells to grow in Y.E., V.I.B., or Y.E. filtrate: Suspension diluted 10⁻², 10⁻³, ..., 10⁻⁶ in media noted. 1 ml. of each dilution shaken at 35°C. for 48 hours and tubes which showed growth at that time taken as the end-point. No growth occurred in the tubes which were clear at 48 hours on further incubation.
Preparation of Y.E. filtrate: 1 × 10⁸B/ml. suspended in Y.E. and shaken for 2 hours at 35°C., cooled to 0°C., and filtered through Seitz filter paid. The filtrate may be boiled without changing the result. Concentrated suspensions of cells grow slightly faster in the filtrate than in Y.E.

borne out by the results of an experiment in which cells were grown in tryptose phosphate broth and plated on Y.E. agar and on V.I.B. agar, as in the preceding experiments. In this case, the cells form colonies on Y.E., just as well as on
Fig. 4. Growth of 899a in tryptose phosphate broth. Changes in turbidity, chains/ml., phage/ml., and colonies formed on yeast extract-peptone agar or veal infusion agar.

**TABLE V**

Phage Production from Spores after Heating at 100°C.

<table>
<thead>
<tr>
<th>Time at 100°C., min.</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonies/plate</td>
<td>1300</td>
<td>45</td>
<td>15</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

Colonies from 4 minute sample tested for phage by streaking on KM—Y.E. agar plates. All formed plaques.

V.I.B., although the rate of growth of the culture in T.P.B. is very nearly the same as in Y.E. (Fig. 4). Very little phage is produced in T.P.B., however. It appears that lysogenic *megatherium* cells can grow and produce phage under favorable conditions, but that the cell metabolism is disturbed so that in less suitable media or under less favorable conditions, the cells die. Under these
unfavorable conditions, such as lack of sufficient oxygen or incomplete or injurious culture medium, the *Megasrerium* cells behave in the same way as infected *B. coli* or staphylococcus cells.

**Phage Production by Spores after Heating to 100°C. (Table V)**

The results of these experiments confirm those of den Dooren de Jong. They show that every colony formed from surviving spores of the lysogenic strain is lysogenic. Some of these colonies, at least, came from single spores, and hence it follows that, in this case, infected cells are able to multiply. It is also evident that if the cells contain active phage, the phage is now as resistant, or more resistant to heat than the most heat-resistant spores. The temperature coefficient of the heat inactivation rate of phage in solution is very high (Krueger, 1932; Adams, 1949) and the reaction proceeds rapidly at 60°C. Survival of active phage at 100°C for 10 minutes therefore requires that the inactivation rate of phage in the spores be less than 1/10^9 as fast as would be expected from the results at 50–60°C.

On the other hand, when in cells which are more sensitive to heat than the virus, the virus is more rapidly inactivated than when in solution (Gratia, 1932).

**Growth and Phage Production on Various Media**

The results of a series of experiments in which the increase in the number of cells and the increase in phage were determined after 6 hours' growth in a number of different culture media are shown in Table VI.

In nearly every case the rate of production of the phage is nearly proportional to the rate of growth of the cells and no medium tested would allow phage production without cell growth. The rate of growth and of phage formation is best in Y.E., but almost as rapid in Fildes' synthetic medium. Aspartic acid and, or asparagine are the best nitrogen sources of any one compound tested. Price (1950) has found that aspartic acid is essential for phage production with *S. muscae*.

T.P.B. medium or Fildes' synthetic medium (cf. Fildes and Richardson, 1937) with no added Mg++ allows cell growth but no phage formation. The effect of varying the concentration of various ions on phage production and cell growth is shown in Fig. 5.

Sr, Mn, Ca, and Mg stimulate growth and phage production in the order named. Ba, Zn, and Co do not. Phage production requires higher salt concentrations than does cell growth and hence, it is possible to find conditions under which the culture will grow, but no phage will be formed.

Mg and Mn are known to be activators of several enzyme systems and it is possible that these particular enzymes are essential for phage production, but not for cell growth.
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The Effects of Varying the Concentrations of PO₄ Ions Added to Fildes' Amino Acid Mixture Containing Fe, Mg, and Glucose (Fig. 6)

In this case, the phage production is stimulated by lower concentrations of PO₄ than is cell growth and the optimum concentration for phage production

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Y.E.</th>
<th>Y.E.</th>
<th>V.I.B.</th>
<th>T.P.B.</th>
<th>Fildes' synthetic + glucose</th>
<th>All A.A. FePO₄</th>
<th>All A.A. MgPO₄</th>
<th>All A.A. MgGlucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁶ P/ml sus</td>
<td>7</td>
<td>7</td>
<td>0.2</td>
<td>0</td>
<td>5</td>
<td>5.4</td>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>10⁷ P/ml</td>
<td>1.6</td>
<td>2</td>
<td>1.8</td>
<td>1.5</td>
<td>1</td>
<td>2.2</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>P/B</td>
<td>4.3</td>
<td>3.5</td>
<td>0.1</td>
<td>0</td>
<td>5</td>
<td>2.4</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

FIG. 5. Effect of various salts on cell growth and phage production. 899a grown on Y.E. agar 20 hours at 35°C. Washed three times in water and suspended in medium containing 2 mg. aspartic acid, 2 mg. arginine, 20 mg FeSO₄, and 4 mg glucose per ml. 0.01 M PO₄ pH 7.5. Various salts added and the suspension shaken 6 hours at 35°C. B₀ = 0.2 X 10⁶, P₀ = 0. B determined by turbidity and P by plating supernatant.
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is at about 0.01 M PO₄ whereas for cell growth the optimum is near 0.1 M. At 0.1 M PO₄ no phage can be detected in the supernatant or precipitate from growing cultures.

The inhibiting effect of high PO₄ on phage formation is probably due to the precipitation of Mg and Ca salts.

LE VI

action in Various Culture Media

a noted. 0.2 × 10⁹ B/ml. shaken at 35°C. 6 hours and increase in B determined by turbidity. Increase in P by Fildes and Richardson (1937). The results in the tables are the averages of 6 to 10 experiments each.

<table>
<thead>
<tr>
<th>All A.A.</th>
<th>All A.A.</th>
<th>0.02 M PO₄, Mg, Fe, glucose +</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0</td>
<td>1.0 0.7 0.5 0.3 0.4 0.1 0.14 0 0 0 0 0 0 1.4</td>
<td></td>
</tr>
<tr>
<td>0.8 1.0</td>
<td>0.5 0.4 0.5 0.4 0.4 0.6 0.5 0 0.1 0.2 0 0 0.6</td>
<td></td>
</tr>
<tr>
<td>0 0</td>
<td>2 1.8 1.0 0.9 1.0 0.16 0.3 0 0 0 0 2.3</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. Effect of phosphate concentration on cell growth and phage production. Experimental conditions the same as described under Fig. 3 except that all tubes contained 10⁻² M/liter MgSO₄. Various concentrations of pH 7.5 PO₄ added.

Continued Growth of the Culture without Phage Formation

The preceding experiments show that the lysogenic culture grows fairly well in an amino acid mixture containing Fe and glucose and 0.1 M PO₄ but forms no phage. The culture may be transferred repeatedly under these conditions and no phage can be detected at any time. If the cells are plated in Y.E., however, they give rise to plaques, and the number of plaques agrees approximately with the number of chains present. Each cell, therefore, retains its potential ability to produce phage and can do so almost immediately as soon as the proper culture medium is present. The results of an experiment in which the
Fig. 7. Effect of the addition of Mg\textsuperscript{2+} on phage production of lysogenic \textit{B. megatherium}.

20 hour Y.E. agar culture of 899a washed twice in
aspartic acid, arginine,
\[5 \text{ mg./ml. 0.01 M pH 7.5 PO}_{4} + \text{ Fe and glucose.}\]

Suspend in the same media and shake two tubes at 35°C. B by turbidity on suspension. P in supernatant by plating on Y.E. agar. \(10^{-8}\) mole MgSO\textsubscript{4} added to tube 2 after 1 hour.

\begin{footnotesize}
\centering
\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
Subculture No. & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\
\hline
\hline
\(10^9\) B/ml. after 6 hrs. (turbidity) & 1.2 & 1.0 & 0.8 & 0.8 & 1.0 & 0.8 & 0.9 & 0.8 & 0.7 \\
\hline
\(10^6\) B/ml. after 6 hrs. by colony count on Y.E. & 0.3 & 0.15 & 0.1 \\
\hline
\(10^3\) P/ml. after 6 hrs. in suspension & 0.08 & 0.04 & 0.1 & 0.08 & 0.04 & 0.08 & 0.1 & 0.1 & 0.08 \\
\(10^3\) P/ml. lysogenic cells. & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\hline
\end{tabular}
\caption{Repeated Transfer in 0.1 M PO\textsubscript{4}}
\end{table}
\end{footnotesize}

20 colonies from culture 10 tested for phage production— all positive.
culture was carried through ten successive subcultures (equivalent to at least 200 generations) are shown in Table VII.

The addition of Mg to a culture growing in 0.1 M PO₄ causes the formation of phage in about 1 hour (Fig. 7).

**Nucleic acid Content of Cells Grown on Various Media**

Price (1949) has found that phage production in *S. muscae* is related to the RNA content of the cells. Cells in the logarithmic growth stage contain more RNA than resting cells and produce more phage.

**TABLE VIII**

*RNA and DNA from Cells Grown in Various Media*

Average of 6 to 8 experiments. Nucleic acid determination as described by Price (*J. Gen. Physiol.*, 1949, **33**, 17) except that the cells were not ground, since grinding the *B. megatherium* cells did not change the results.

<table>
<thead>
<tr>
<th>Condition of cells</th>
<th>Media</th>
<th>Y.E. agar</th>
<th>Y.E. liquid</th>
<th>All A.A. 0.02 M PO₄</th>
<th>Mg Glucose</th>
<th>Fe Glucose</th>
<th>All A.A. 0.02 M PO₄</th>
<th>Mg Glucose</th>
<th>Fe Glucose</th>
<th>Pb. Al. Prolene 0.06 M PO₄</th>
<th>Mg Glucose</th>
<th>Fe Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg. RNA in 10¹⁰ cells</td>
<td></td>
<td>2.1 ± 0.1</td>
<td>6.1 ± 0.12</td>
<td>4.2 ± 0.2</td>
<td>3.7 ± 0.15</td>
<td>3.3 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg. DNA in 10¹⁰ cells</td>
<td></td>
<td>1.7 ± 0.1</td>
<td>2.5 ± 0.15</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase in RNA</td>
<td></td>
<td>4.0</td>
<td>2.1</td>
<td>1.6</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td></td>
<td>0.8</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10¹⁰ P/10¹⁰ B</td>
<td></td>
<td>4.3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg. RNA per 10¹⁰ cells in solution</td>
<td></td>
<td>0.6</td>
<td>0.4</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg. DNA per 10¹⁰ cells in solution</td>
<td></td>
<td>1.2</td>
<td>1.3</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results of analyses of lysogenic *B. megatherium* grown in various media are shown in Table VIII. As in the case of *S. muscae*, the resting cells contain the smallest amount of nucleic acids and the logarithmic growth cells, the most. Also the cells which produce the most virus synthesize the most RNA, as in the case of *S. muscae*, although cells grown in the presence of 0.1 M PO₄, which produce no virus, contain more RNA than resting cells.

**Effect of Lysozyme on Cell and Virus Content of Suspension of Lysogenic Culture**

*B. megatherium* is rapidly cleared by low concentrations of lysozyme and Wollman and Wollman (1935) reported that phage was liberated from lysogenic cells, under these conditions. Gratia (1936 e), however, was unable to detect any phage, provided the lysogenic cells were washed before the addition
of the lysozyme, so as to remove any free phage. The results of such an experiment are shown in Table IX. No phage could be detected in the cleared culture, as Gratia stated.

Similar results have been obtained in this laboratory with infected S. muscae and S. aureus cells. No phage has been recovered from such cells by any method of disintegration so far tried. Killed cells absorb phage but this phage cannot be found after the cells have been destroyed, either. The failure to find active phage in living cell suspensions cleared with lysozyme, therefore, cannot be considered proof that the phage particle disintegrates in the living cell. Gratia

TABLE IX

Effect of Lysozyme on Viable Cells, Lysogenic Cells, and Phage Content of Lysogenic Strains

20 hour culture of 899a on Y.E. agar washed twice and suspended in

\[
\begin{align*}
\text{Aspartic acid} & \quad 5 \text{ mg./ml.} \\
\text{Arginine} & \quad 5 \text{ mg./ml.} \\
\text{PO}_4, \text{ Mg, Fe, glucose, pH 7.5}
\end{align*}
\]

Shaken at 35°C. for 4 hours. Centrifuged, washed, and suspended in same medium but with no Mg++. 0.01 mg. lysozyme/ml. added and samples analyzed for B by turbidity, chain count, viable cells (plated on V.I.B.), lysogenic cells (plated on V.I.B. + sensitive strain), and lysogenic cells + phage (plated on Y.E. + sensitive strain).

<table>
<thead>
<tr>
<th>Time at 35° (min.)</th>
<th>B turbidity</th>
<th>Chains</th>
<th>Colonies on V.I.B. = viable cells</th>
<th>Plaques on V.I.B. = lysogenic cells</th>
<th>Plaques on Y.E. = lysogenic cells + phage</th>
<th>Control, 0.1 × 10⁹ P/ml. added to lysogenic cells, Plaques on Y.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8 × 10⁸</td>
<td>0.2 × 10⁸</td>
<td>0.1 × 10⁸</td>
<td>0.1 × 10⁸</td>
<td>0.1 × 10⁸</td>
<td>0.2 × 10⁸</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>0</td>
<td>0.004</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1 × 10⁸</td>
</tr>
</tbody>
</table>

found that lysogenic cells gave rise to a weak anti-phage serum, whereas uninfected B. megatherium cells did not (1937).

Most of the experiments reported in this paper were carried out by Miss Marie King.

DISCUSSION

The results of these experiments confirm those of earlier workers in showing that lysogenic cultures grow normally, as far as can be determined, and produce large amounts of phage, under certain conditions.

The results of varying the composition of the media, in general, indicate that phage production requires a higher concentration of essential compounds, than does cell growth. The same effect was noted by Price in connection with the S. muscae system. Both the casein factor (Price, 1948 a) and the niacin concentration (Price, 1947) required for maximum cell growth are lower than those required for maximum phage production.

Very similar results were reported by Bellamy and Gunsalus (1944) for the
production of an adaptive enzyme (tyrosin decarboxylase) by streptococci cultures. In this case, also, higher concentrations of nicotinic acid are required for enzyme production than for cell growth.

The relationship between adaptive enzyme formation and phage formation is further indicated by the experiments of Monod and Wollman (1947), who found that infected cells which were producing phage, were unable to produce adaptive enzymes.

A nucleic acid fraction from yeast which was found to stimulate adaptive enzyme formation (Reiner and Spiegelman, 1948) was also found to stimulate phage production (Price, 1948 b).

These results confirm the striking similarity between adaptive enzyme production and virus production (cf. Northrop, 1939 a; Price, 1948 b).

The phage-producing property is carried by every cell and by all spores and has the same or more heat resistance than the spores. No phage is produced in culture media lacking in Mg or containing more than 0.06 m PO₄. The culture may be transferred indefinitely in such media, and will produce phage in about an hour after the Mg is added. Similar results were obtained by Gratia (1932) with a lysogenic culture of B. coli which produced no phage in media containing glucose.

It seems quite clear, that, in this culture, infected cells can grow, and that the potential ability to produce phage is passed along through cell division. This confirms the results of Bordet and Renaux (1928), and Burnet and McKee (1929), and Lwoff and Gutman (1950). The ability to produce phage varies with conditions, therefore, just as does the ability to secrete enzymes and toxins. There is no convincing proof that the cells contain phage particles and the direct experimental evidence indicates that they do not. Evidence is accumulating from many different results that the phage particle loses its identity when in the growing cell.

1. The heat resistance of phage in infected cells or spores is the same as that of the host cells and may be either much greater (den Dooren de Jong, 1931), or much less (Gratia, 1932) than that of phage in solution. The pH inactivation range is also that of the host cells (Gratia, 1932).

2. The rate of inactivation by x-rays (Latarjet, 1947) or ultraviolet light (Luria and Latarjet, 1947) of free phage is different from that of phage contained in cells.

3. No phage can be detected in infected cells by extraction or clearing by lysozyme (Gratia, 1936 e).

4. Cells infected with phage containing radioactive P liberate most of the P³² into the medium (Kotzloff and Putnam, 1949).

5. Multiply infected cells can produce phage in culture media in which singly infected cells do not. The necessary substrate for phage production is supplied by the excess phage (Price, 1950).
6. Phage inactivated by ultraviolet light may be reactivated if more than one particle is absorbed per host cell (Luria and Dulbecco, 1949).

7. If the phage particle retains its identity, then it must be assumed that the particle multiplies in exact synchronism with cell multiplication under all conditions. If the phage particle multiplied more rapidly than the cells, sooner or later the cells would be killed; while, if it multiplied more slowly, phage-free cells would appear. Neither of these results is observed.

None of these results is conclusive evidence that the virus actually disintegrates, since all may be interpreted in other ways.

It is known, for instance, that the rate of inactivation by heat, light, or x-rays (Gates, 1934; Hussey and Thompson, 1922; Homes, 1950) depends upon the conditions. The change of form of the x-ray curves noticed by Latarjet could be interpreted as a change from a true logarithmic curve, when the virus is in solution and therefore homogeneous in character, to probability curves, where the virus is present in cells, which are not a homogeneous population and hence, give rise to probability curves, instead of true logarithmic curves. Heat inactivation curves of the virus alone are accurately logarithmic (Krueger, 1932; Adams, 1949), but heat inactivation curves of bacteria invariably show a more or less pronounced "lag" period when plotted as logarithmic curves and are better considered as probability curves (cf. Loeb and Northrop, 1917).

A change in the ultraviolet "inactivation spectrum" of the virus, when absorbed by the cell would be more convincing evidence, but such experiments have not been carried out.

The results of the experiments on the release of Pr and of reactivation, or utilization of amino acids present in the phage particle may be explained by simple exchange of the amino acids or other constituents of the particle without actual disintegration. Borsook (1949) has shown, for instance, that proteins in tissue homogenates take up single amino acids quite rapidly. Other evidence for an equilibrium of some kind between proteins and their constituents has been demonstrated by Schoenheimer (1942) and by Madden and Whipple (1940).

In spite of these possible alternate explanations, the assumption that the phage particle loses its identity when in the host cell appears to be the simplest explanation of the observed results. If this is the case, then any hypothesis concerning the method of multiplication of the virus, which depends upon the presence of an intact virus particle, must be abandoned. Hypotheses analogous to cell division, or a synthesis directed by some sort of template mechanism or simple autocatalytic reactions are, therefore, ruled out. The nucleic acid may be the essential, autocatalytic part of the molecule, as in the case of the transforming principle of the pneumococcus (Avery, MacLeod, and McCarty, 1944), and the protein portion may be necessary only to allow entrance to the host cell.
**Experimental Procedure**

**Stock Cultures.**—Lysogenic strain: Grown on 2 per cent peptone agar slants. Transferred once a week. Incubated at 35°C. for 18 hours and then stored at 5°C.

Sensitive strain: Grown on 2 per cent peptone agar slant and transferred daily.

**Experimental Cultures.**—

Lysogenic strain: Petri dish containing Y.E. agar inoculated with 1 ml. suspension of stock culture and incubated 20 hours at 35°C.

Sensitive strain:

Plating: Y.E.P. Petri dish inoculated from 24 hour 2 per cent peptone slant of KM. Incubated 18 hours at 35°C., washed off in 5 ml. H2O. Kept in test tube at 25°C. Enough of the suspension (3 to 5 drops) added to 3.5 ml. Y.E. in test tube to give a turbidity reading of 50 to 100 × 106B/ml. 0.5 ml. sample + 1 ml. hot (100°C.) agar added, mixed, and 1 ml. spread on Y.E. Petri dish. Allowed to stand at 25°C. 1 or 2 hours. Incubated at 35°C. for 18 hours.

Cell counts by turbidity measurements were made as described by Northrop (1939) except that a Klett-Summerson photoelectric colorimeter was used.

Yeast extract: Prepared as described by Northrop (1939).

Yeast extract-peptone: 250 ml. yeast extract and 100 gm. Difco bacto-peptone added to 1 liter of water. pH adjusted to 7.6. This mixture gives slightly better plaques than Y.E. alone.

**SUMMARY**

Cell multiplication and phage formation of lysogenic *B. megatherium* cultures have been determined under various conditions and in various culture media.

1. In general, the more rapid the growth of the culture, the more phage is produced. No conditions or culture media could be found which resulted in phage production without cell growth.

2. Cultures which produce phage grow normally, provided they are shaken. If they are allowed to stand, those which are producing phage undergo lysis. Less phage is produced by these cultures than by the ones which continue to grow.

3. Cells plated from such phage-producing cultures in liquid yeast extract medium grow normally on veal infusion broth agar or tryptose phosphate broth agar, which does not support phage formation, but will not grow on yeast extract agar.

4. Any amino acid except glycine, tyrosine, valine, leucine, and lysine can serve as a nitrogen source. Aspartic acid gives the most rapid cell growth.

5. The ribose nucleic acid content is higher in those cells which produce phage.

6. The organism requires higher concentrations of Mg, Ca, Sr, or Mn to produce phage than for growth.

7. The lysogenic culture can be grown indefinitely in media containing high phosphate concentrations. No phage is produced under these conditions, but
the cells produce phage again in a short time after the addition of Mg. The potential ability to produce phage, therefore, is transmitted through cell division.

8. Colonies developed from spores which have been heated to 100°C. for 5 minutes produce phage and hence, infected cells must divide.

9. No phage can be detected after lysis of the cells by lysozyme.

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