INCREASE IN BACTERIOPHAGE AND GELATINASE CONCENTRATION IN CULTURES OF BACILLUS MEGATHERIUM

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The increase in phage or virus concentration in the presence of living cells is formally analogous to the formation of enzymes by cells (1–5 and 54) or from their precursors (6), as well as to the growth of cultures of bacteria (40). There is an essential difference between the growth of cells and the formation of enzymes in that cells can use energy to synthesize themselves from simpler compounds whereas, so far as is known, enzymes cannot. It is not yet known whether phage or viruses possess this power of synthesis. Until this knowledge is obtained it does not seem possible to decide as to which of the two analogies has a real physical basis and which is merely formal. In the meantime it appears to the writer that the much more simple analogy with enzyme formation has predicted the facts better than the analogy with cell growth. Thus, some viruses and phages, at least, are proteins (6–9) and may be isolated by the methods of enzyme chemistry; they may be inactivated and reactivated (10–13); they have no measurable respiration (14). Pepsin and trypsin are immunologically distinct from their precursors (15–16), just as phage and virus are immunologically distinct from their host cells.

Pepsin and trypsin are formed by inoculation (28, 45) of solutions of their precursors with the active enzyme, just as phage or virus is formed after inoculation of the host cells with phage or virus. Some cells ("lysogenic" bacteria, virus "carriers") always produce phage or virus just as cells in general always produce "normal" enzymes, and it has been suggested (1, 3, 5) that phage is a normal enzyme of certain cells which is injurious to others. Such lysogenic strains, however, may be produced in the laboratory by growing resistant strains in the presence of phage (22, 40). This result renders less probable the assumption that phage is a normal product of the naturally occurring lysogenic strain. New "adaptive" enzymes may be formed by cells when grown in the presence of different substrates (17–21,

46–48). The problem of the production of new, active, substances is therefore encountered in relation to enzymes as well as to phage or virus.

Both phage and enzymes are usually produced only by growing cells, but under special conditions they may be produced without any cell growth (17–18, 29). Krueger (30–31) has shown that this ability to produce phage without growth is possessed only by cells which have just begun to divide. Hegarty (48) has found that it is precisely these "young" cells which can produce new enzymes. The production of virus in embryonic tissue (49) suggests a similar relation between the condition of the host cell and virus production.

The curves for the rate of formation of phage (23) and of enzymes (24–27) by cells or from their precursors (28, 45) are similar and more or less autocatalytic in character.

It is evident from the foregoing that a formal analogy exists between the production of phage and the formation of enzymes. If this analogy has any real physical significance the simultaneous formation of phage and of an extracellular enzyme in the same culture should follow very similar curves. In order to test this prediction the rate of formation of phage and of a gelatinase by lysogenic and "sensitive" strains of *B. megatherium* has been determined under a variety of conditions. In general the curves for the increase of phage and of gelatinase are very similar. There is a rapid increase in phage and enzyme concentration during the growth stage of the bacteria. The increase in all these quantities is logarithmic (autocatalytic); *i.e.*, the amount formed is proportional to the amount already present. The concentration of phage and gelatinase reaches a maximum and then decreases. The decrease may or may not be accompanied by a decrease in the number of cells depending upon the conditions and strain of bacteria used. The production of phage may be separated from that of gelatinase by varying the calcium concentration. In the presence of high calcium concentration little phage is produced while the gelatinase increases to a measurable extent, but less than it does in low calcium concentration. In the absence of calcium no phage is produced and the production of gelatinase is decreased.

If resting cells are used no increase in phage or gelatinase can be detected without cell growth. If rapidly growing cells are used, however, a narrow range of acidity exists near pH 5.5 in which no further cell growth occurs but a large increase in phage may be obtained. Under proper conditions it is therefore possible to separate phage production from cell growth as may be done with enzyme formation.

EXPERIMENTAL RESULTS

1. Megatherium Culture and Phage.—The culture of *megatherium* (899 T) used was isolated originally by De Jong and further studied by De Jong (5), Gratia (32–35), Wollman (36), and others. The writer is indebted to Professor Gratia for supplying him with the various strains of the bacteria and also with the phage. The organism forms two types of cultures: (1) a smooth strain (899 S) which does not spore and, (2) a spore-forming strain (899 R). Both strains produce a bacteriophage which causes lysis of a sensitive strain (Meg. 36 S) but does not cause lysis of the lysogenic culture 899 which produces it.

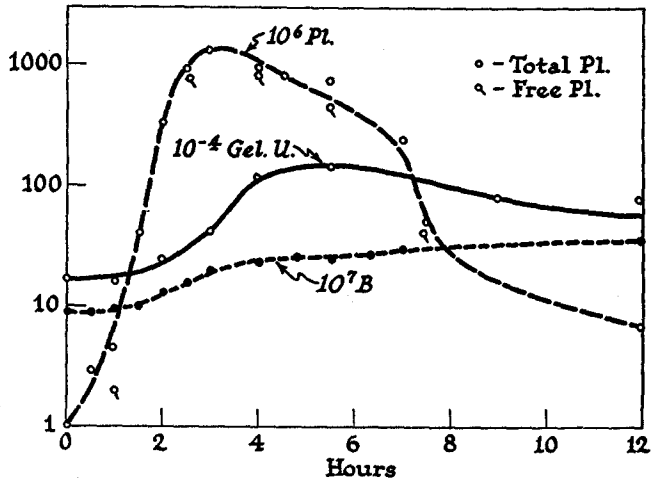

FIG. 1. Increase in plaque count, number of bacteria, and gelatinase concentration during growth of resistant strain, 899 T. Yeast extract media shaken at 35°C.; inoculated from 18 hour 30°C. Blake bottle culture.

Two types of phage are formed (34), one of which causes complete lysis of Meg. 36. This "C" phage was used in the present work. A second type of phage causes only partial lysis of Meg. 36 (Phage T).

2. Increase in Number of Bacteria, Bacteriophage, and Gelatinase in

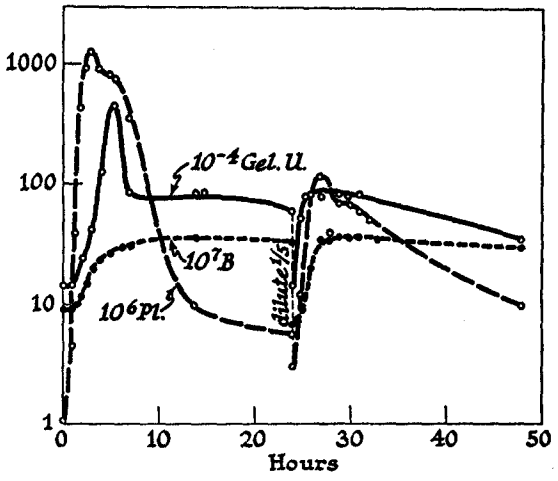

FIG. 2. Increase in plaque count, number of bacteria, and gelatinase concentration during growth of resistant strain 899 T. After 24 hours 10 cc. of culture added to 40 cc. fresh yeast extract media, shaken at 35°C.

Growing Lysogenic Cultures of B. megatherium (899 T) in Yeast Extract Media at 35°C.—The results of an experiment in which the bacteria concentration, the plaque count, and the gelatinase activity were determined are shown in Fig. 1.

There is a lag period of about ½ hour in which no change is detectable. The cell concentration then increases continuously while the phage concentration and gelatinase concentration increase much more rapidly than the cells at first, reach a maximum, and then decrease. The curves are all logarithmic during the stage of rapid increase. The cell concentration remains constant after about 14 hours. There is no demonstrable lysis. The maximum number of plaques per ml. is about 1000×10^6 or about 2–3 times that of the maximum bacteria concentration 40×10^7. The plaque count in the filtrate is nearly the same as that of the suspension. The maximum gelatinase concentration is $100\text{–}200 \times 10^{-4}$ gelatin units per ml. The relation of gelatinase production to cell growth is the same as that found by Kocholaty, Weil, and Smith (25) with *Cl. histolyticum.*

3. Increase in Phage, Bacteria, and Gelatinase in Successive Subcultures.—The results of an experiment in which 10 ml. of culture was added to 40 ml. of fresh yeast extract after 24 hours are shown in Fig. 2. The general slope and relation of the curves remain the same although there is considerable variation in the maximum gelatinase concentration and the maximum plaque count obtained. This experiment was carried through five subcultures without any significant change in the results. Repetition of the experiment gave the same general result. In some cases the maximum gelatinase concentration was much higher and was reached later than in the experiment reported. The decrease in the phage and gelatinase is caused by the growth of the culture and is not due to simple inactivation since filtrates retain their activity under the same conditions.

Increase in Phage, Bacteria, and Gelatinase in Cultures of the Sensitive Strain (B. megatherium 36)

1. Lower Phage Concentration.—The changes in phage and cell concentration of a culture of the susceptible strain inoculated with phage depend upon the relative initial concentration of cells and phage. If cultures containing more than 10^7 bacteria per ml. of *megatherium* 36 are inoculated with lower concentration of phage so as to give 10^3 or less plaques per ml. the results are very similar to those obtained with the resistant strain (Fig. 3). The phage concentration reaches a maximum during the rapid growth of the bacteria and then decreases. The relative concentration of phage to bacteria at this point is below the critical value

and therefore no lysis occurs. The maximum gelatinase concentration in this experiment was reached later. The oxygen consumption per minute was determined in a Warburg respirometer. It increases at first in proportion to the increase in bacteria and then decreases at about the time the plaque count decreases. A similar relation between oxygen consumption and virus production was found by Zinsser and Schoenback (37).

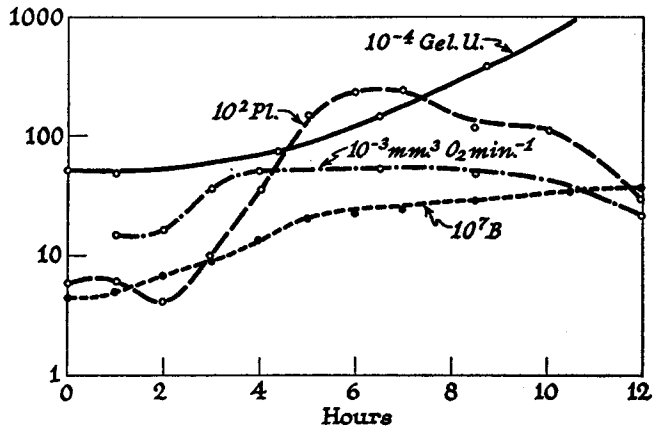

FIG. 3. Increase in plaque count, number of bacteria, and gelatinase concentration and oxygen consumption per minute of susceptible strain, *megatherium* 36 sensitive. Inoculated with low concentration phage "C;" yeast extract media, shaken at 35°C.

2. High Phage Concentration.—If cultures of the sensitive strain containing less than 10^8 bacteria per ml. are inoculated with high phage concentration so that the initial plaque count is 10^5 per ml. or more lysis occurs. Under these conditions the results are more irregular. If the ratio of phage to bacteria is high the critical ratio necessary for lysis is soon reached and lysis occurs rapidly and is nearly complete. As the ratio of phage to bacteria is decreased lysis is delayed and is less complete and a resistant strain of the organism develops in the culture (Fig. 4). This resistant strain also produces phage but does not undergo lysis. Occasionally a second increase in growth rate occurs and is accompanied by a second increase in phage

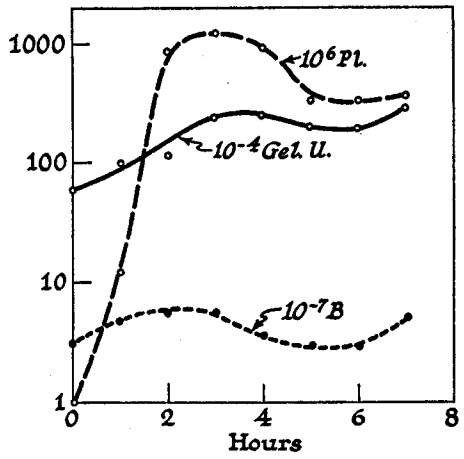

FIG. 4. Increase in plaque count, number of bacteria, and gelatinase concentration of susceptible strain, *megatherium* 36 sensitive. Inoculated with high concentration of phage "C;" yeast extract media, shaken at 35°C.

(Fig. 5). If subcultures are made after 24 hour intervals the curves are similar but the maximum phage and bacteria concentration obtained vary depending upon the relative amounts present at the time the subculture was made (Fig. 6).

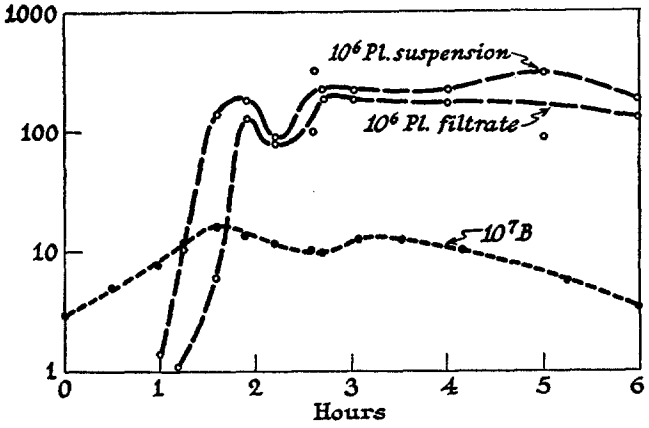

FIG. 5. Increase in plaque count and bacteria concentration of *megatherium* 36 sensitive in yeast extract media shaken at 35°C.

If the original culture is shaken for 48 hours the resistant strain grows during the second day and this is accompanied by a second increase in phage and gelatinase (Fig. 7).

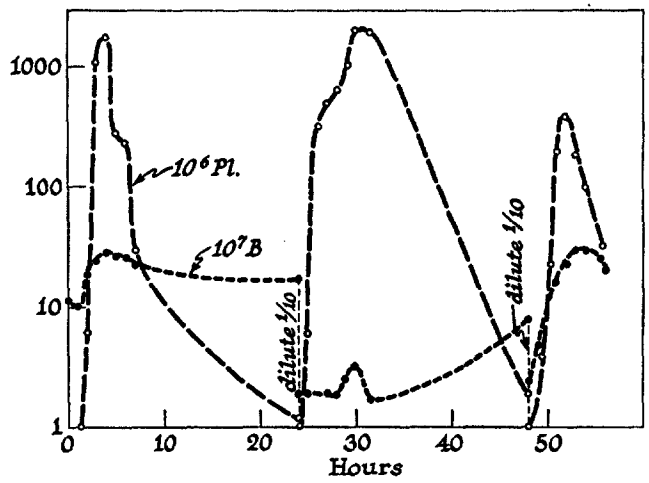

FIG. 6. Increase in plaque count and bacteria concentration in successive cultures of *megatherium* 36 sensitive in yeast extract shaken at 35°C. Subculture made at 24 hour intervals by adding 10 cc. of old culture to 100 cc. of fresh yeast extract.

If a subculture is made from the resistant culture phage and gelatinase are again produced during the growth of the culture but no lysis occurs. The culture now behaves (35) like the original lysogenic strain 899 T. It produces phage but does not undergo lysis. The phage and gelatinase concentration were unusually high in this experiment and it may be noted that nearly as much phage was formed by the resistant subculture as in the original sensitive culture.

Free and Total Phage and Gelatinase.—The increase in free and total

phage during growth of the culture is shown in Fig. 8. 70–80 per cent of the phage is free as Gratia states (33). This ratio remains nearly constant during the growth of the culture. In a few experiments (Fig. 5)

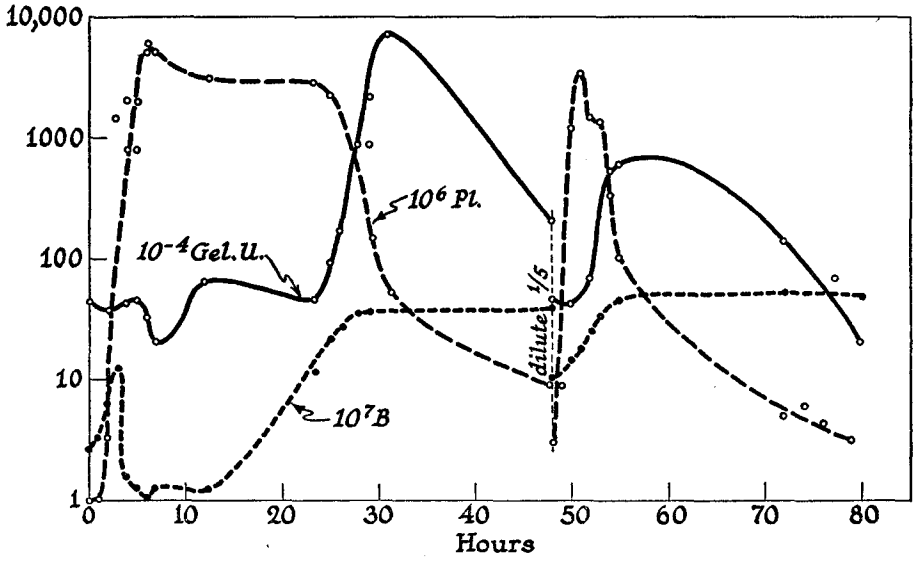

FIG. 7. Increase in plaque count, bacteria concentration, and gelatinase concentration of culture *megatherium* 36 sensitive in yeast extract media shaken at 35°C. Subculture made after 48 hours when resistant strain was developed.

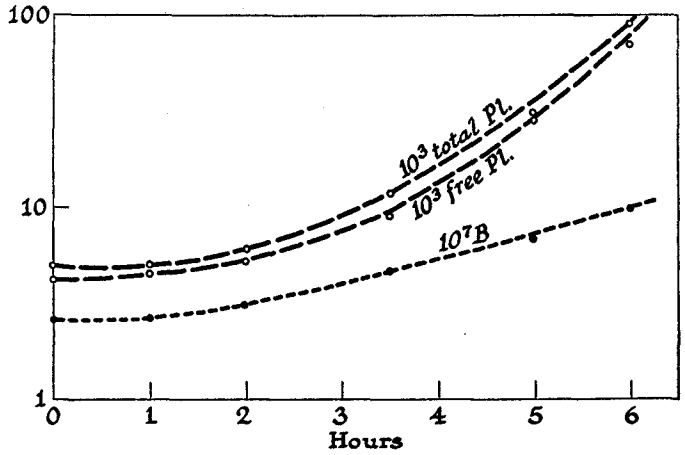

FIG. 8. Increase in plaque count in suspension (total) and in filtrate (free) in the presence of growing cultures of *megatherium* 36 sensitive. Yeast extract media shaken at 26°C.

the percentage of free phage was lower during the first part of the curve. There is some indication that the per cent of free phage increases at about the time lysis commences but the concentrations are changing very rapidly with time at this stage of the reaction and it is difficult to be sure that the free and total phage counts on a sample are really comparable owing to the necessary difference in handling the sample. It seems probable that this difficulty accounts for the discrepancy between Wollman's (36) and Gratia's (33) results. No further change in the amount of free phage occurs on standing. Most of the phage combined with the cells may be removed by repeated washing but about one-quarter of the amount remains fixed. It must be noted that these figures are all "infective units" and may not represent actual quantity of phage. It is quite possible that much larger quantities of phage are combined with the cells than these figures indicate since one infected cell will show only one plaque no matter how much phage is in or on the cell.

The gelatinase activity of the filtrate is the same as that of the whole suspension. Washed cells have no demonstrable gelatinase activity. Similar results have been obtained by Kocholaty, Weil, and Smith (25) with *Cl. histolyticum*.

Increase in Total or in Combined Phage.—The preceding experiment shows that most of the phage in these cultures is free. This fact renders it possible to determine whether the increase in phage depends on the total phage concentration or on the quantity of combined phage. With most cultures such as *Staphylococcus* this experiment cannot be done since practically all the phage is combined with the cells.

In order to determine whether the increase in phage is proportional to the total or to the combined phage a culture was inoculated with phage and shaken until the cell and phage count was beginning to increase. 5 ml. aliquots of the culture were then centrifuged and one set of aliquots stirred again and shaken at 35°C. The supernatant solution containing the free phage was removed from the other aliquots and the supernatant of a similar culture but which contained no phage, added to the precipitate. The precipitate was then stirred and the suspension shaken. The results of this experiment are shown in Table I. The figures are the average of four tubes. They show that the entire suspension contained 1300 plaques per ml. while a suspension of the precipitate contained only 300 plaques per ml. After 2 hours shaking both suspensions contained about 18,000 plaques per ml. Thus the same amount of phage was produced by the precipitate alone as by the whole suspension although the latter contained originally 4 times as many infective units. The quantity of phage produced is there-

fore determined by the combined phage only. As in the previous experiment there is the possibility that the actual quantity of phage in the precipitate is much larger than that indicated by the plaque count in which case this experiment is inconclusive.

Effect of Phosphate, Oxalate, or Excess Calcium on Phage or Gelatinase Production.—Bordet (4) and Gratia (35) have found that calcium is necessary for the production of phage. Merrill and Clark (38) found that

TABLE I

Formation of Phage by Whole Culture and by Precipitate Alone

100 ml. peptone plus bacteria from 18 hr. Blake bottle plus 0.01 ml. filtered phage. Shake at 25°C.			100 ml. peptone plus bacteria from 18 hr. suspension. No phage. Shake at 25°C.
I			II
Hrs. at 25°C.	Bacteria per ml.	Plaques per ml.	Bacteria per ml.
0	0.37×10^8	200	0.40×10^8
1.5	0.52		0.55
3.0	0.80		0.83
4.0	0.92	1,600	0.95

Eight 5.0 ml. aliquots of No. I centrifuged 10 minutes

Aliquots No. 1, 2, 3, 4 resuspended in supernatant. Transferred to flasks and shaken at 25°C.			Aliquots No. 5, 6, 7, 8, supernatant removed and replaced by supernatant from No. II. Precipitate stirred and suspension shaken at 25°C.	
Hrs. shaken	Bacteria per ml.	Plaques per ml.	Bacteria per ml.	Plaques per ml.
	Average of 4 tubes		Average of 4 tubes	
0	1.06 ± 0.05	1,300 ± 100	1.08 ± 0.05	300 ± 50
1	1.34 ± 0.05	700 ± 100	1.40 ± 0.05	200 ± 50
2	1.84 ± 0.05	17,300 ± 300	1.70 ± 0.10	19,600 ± 300
Increase in plaques per ml.		16,000		19,300
Per cent increase in plaques per ml.		1,000		6,000

proteus would not produce proteinase without calcium. Similar results were obtained by Haines (38*a*). Tables II and III show that no phage and very little gelatinase is produced in the presence of excess phosphate or oxalate ions which remove the calcium ions. After several subcultures in such solutions the culture no longer produces phage when returned to the control solution without phosphate or oxalate but the gelatinase is formed again under these conditions. Excess calcium also prevents phage formation but does not decrease gelatinase formation markedly.

Increase in Phage without Cell Growth.—Gratia and others early pointed out that increase in phage occurred only when the host cells were increasing (39). Krueger and Northrop (23) found that this relation held quite accurately but that the ratio of the increase in phage to that of bacteria varied somewhat under different conditions indicating that it might be possible

TABLE II

Effect of Phosphate or Oxalate on Phage or Gelatinase Production

Sodium oxalate or sodium phosphate added to 100 ml. peptone. pH adjusted to 7.6. Autoclaved. Inoculated with 1 × 10^8 bacteria per ml. and 100 plaques per ml. Shaken at 35°C.

Time	0			M/100 sodium oxalate			M/50 sodium phosphate		
	10^8 bacteria per ml.	Plaques per ml.	Gel. U. per ml.	10^8 bacteria per ml.	Plaques per ml.	Gel. U. per ml.	10^8 bacteria per ml.	Plaques per ml.	Gel. U. per ml.
hrs.									
0	1.0	10^2	0.004	1.0	10^2	0.004	1.0	10^2	0.004
3	2.3	10^5	0.006	2.3	20	0.002	2.4	0	0.005
5	4.5	2.7 × 10^3	0.40	3.5	0	0.05	2.7	0	0.02

TABLE III

Effect of Addition of Calcium Chloride

100 ml. yeast extract plus calcium chloride, pH adjusted to 7.6. Boiled and filtered. 50 ml. filtrate plus 1 × 10^8 bacteria per ml. plus 2.6 × 10^2 plaques per ml. Shaken 35°C.

Final concentration calcium chloride added ..	0			0.012			0.025			0.05			0.10		
Time	10^8 b. per ml.	10^2 pl. per ml.	Gel. U. per ml.	10^8 b. per ml.	10^2 pl. per ml.	Gel. U. per ml.	10^8 b. per ml.	10^2 pl. per ml.	Gel. U. per ml.	10^8 b. per ml.	10^2 pl. per ml.	Gel. U. per ml.	10^8 b. per ml.	10^2 pl. per ml.	Gel. U. per ml.
hrs.															
0	1.0	2.5	0.004	1.0	2.6	0.003	1.0	3.0	0.004	1.0	2.7	0.004	1.0	2.6	0.004
3	2.5	10,000		2.4	10,000		1.6	10,000		1.8	8,000		1.7	2,000	
24			0.07			2.0						1.0			0.7

to separate phage increase from cell growth. Such a result is to be expected from the analogy between phage and enzyme production since microorganisms may produce enzymes (17–18, 29) under certain conditions without cell division. Pepsin and trypsin may be formed from their precursors *in vitro* (28). Thus, if any real relation exists between phage produc-

tion and enzyme formation it should be possible to obtain increase in phage without cell growth and probably even in cell free extracts. Krueger and his co-workers (30–31) was subsequently able to separate the two variables and obtain increase in phage without any increase in cells and also in cell free extracts (50). The increase in phage without cell growth can only be obtained when "young" cells are used. This result is in striking agreement with the results of Hegarty (48) who found that only young cells could produce new enzymes in the presence of new substrates. Krueger's experiments (30–31) were done with *Staphylococcus* phage and have been repeated and confirmed in this laboratory. A large number of unsuccessful attempts to repeat these results with *B. megatherium* were made under a variety of conditions. Eventually, however, it was found that such an increase could be obtained by adjusting rapidly growing cultures to a very narrow range of pH near 5.5. In this narrow range there is rapid increase in phage with a slight decrease in cells. In very slightly more acid solution both cells and phage decrease while in slightly more alkaline solution both cells and phage increase.

The results of such an experiment are shown in Fig. 9. The number of bacteria decreases slightly at pH 5.43, 5.52, and 5.58. The plaque count decreases at 5.43 but increases more than 50 times in an hour at pH 5.52 and 5.58. At 5.64 there is some increase in cells. Control experiments without phage give the same figure for the changes in bacteria concentration as in the cultures which contained phage.

Phage Production during Growth or Lysis.—d'Herelle (39, 40) found that the plaque count in growing cultures increased in steps or "bursts" and this observation has recently been confirmed by Ellis and Delbrück (41). d'Herelle considered that the phage particle multiplied in the bacteria cells and that the cell then burst liberating a large number of phage particles. These particles attached themselves to other bacteria and the process was then repeated.

Burnet (42) tested this hypothesis by determining the increase in plaque count in small samples containing originally one or two plaques but the results are not very convincing owing to the small number of counts made. A more serious difficulty lies in the fact that the percentage of samples showing positive results increased from 40 to 100. This result shows either that the method used will not detect one particle or else that the phage increases, not from 1 to 40 particles but from 0 to 40. If the method cannot detect a single particle the results indicate that a certain minimum concentration of phage is needed to give positive results and the sudden increase in plaque count occurs when this minimum is overstepped.

In any case there are no data to show that the sudden increase occurs during lysis as the number of bacteria was not determined.

Doerr and Grüninger (53), Bronfenbrenner (39), and Krueger and Northrop (23) found that the principal increase in phage occurs during growth of the bacteria and very little after lysis starts. Similar results were obtained by Clifton and Morrow (52). Krueger and Northrop considered that lysis occurred when the phage per bacteria ratio reached a certain critical value. In confirmation of this idea they found that the

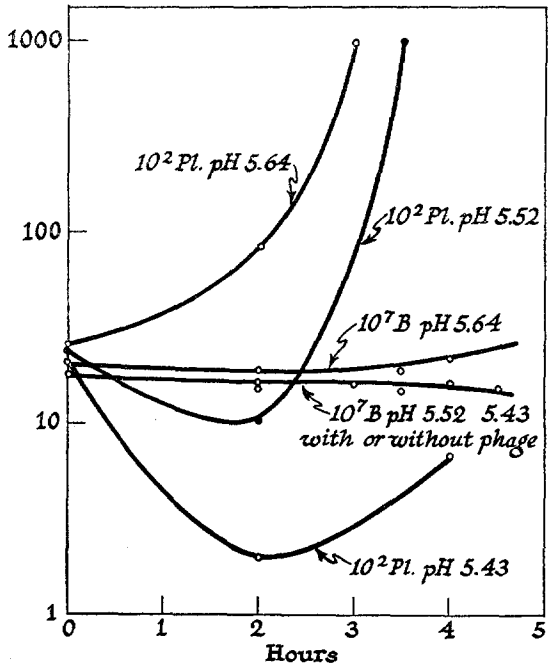

FIG. 9. Increase in plaque count without cell growth at pH 5.52. 100 ml. yeast extract inoculated with 10×10^7 bacteria per ml. from 18 hour 30°C. Blake bottle. Suspension shaken at 35°C. for 2 hours. 20 ml. aliquots titrated to pH 5.43, 5.52, and 5.64. 25×10^2 plaques per ml. added and suspension shaken at 35°C.

addition of very concentrated phage to growing cultures caused immediate lysis and that under these conditions no increase in phage occurred. This experiment has been repeated by the writer with the result shown in Fig. 10. The results show that lysis starts immediately after the addition of the concentrated phage. The curve is a typical probability integral and when plotted in the differential form gives the usual probability curve. This is the result usually obtained when a culture of bacteria is killed. If lysis is caused by the increase of the phage particle inside the cell there should be

a lag period of 20–40 minutes before any lysis occurs and the phage concentration should increase. Actually there was a slight decrease in phage instead of an increase.

The method used by Krueger and Northrop (23) determined total phage and not infective units as does the plaque count method. No sudden in-

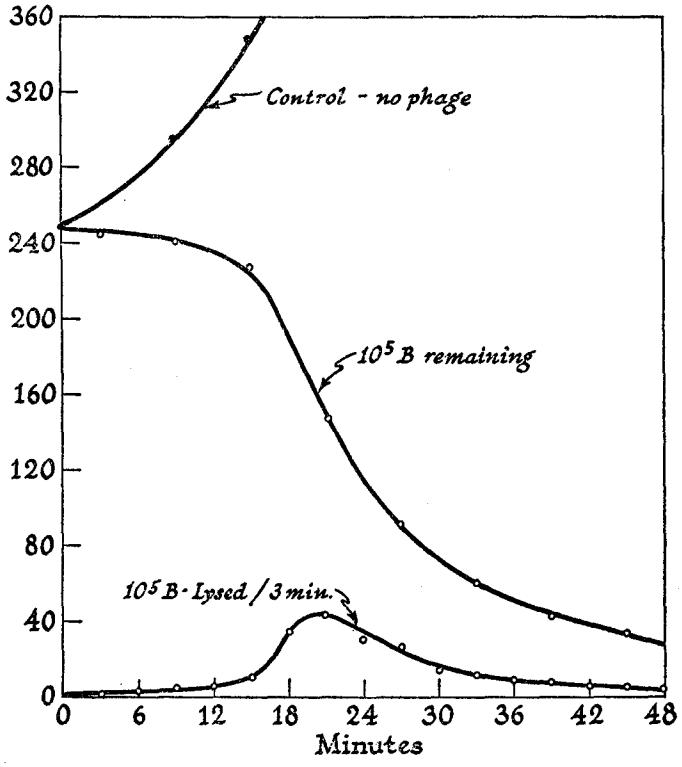

FIG. 10. Lysis of suspension of *Staphylococcus muscae* upon addition of concentrated phage solution. 50 cc. yeast extract containing 100 × 10^5 bacteria per ml. shaken at 35°C. for 2 hours. 1 cc. purified concentrated bacteriophage (6) solution containing 10^{12} plaques per ml. added. Decrease in bacteria concentration determined by turbidity method. Initial plaque count per ml. suspension 2 × 10^{10}, final plaque count 1 × 10^{10}.

crease at lysis would be predicted by d'Herelle's hypothesis when the total quantity is determined since it is assumed that there is merely a redistribution of phage at that time. Also no lysis could be detected during the upward part of the growth curve when most of the phage is formed. Ellis and Delbrück account for this by assuming that the percentage of bacteria lysed at this time is so small as to escape detection.

With the resistant strain of *B. megatherium* used in the first two experiments reported no lysis can be observed at any time yet the plaque count reaches a height of more than 10^9 per ml. This figure is 3-4 times that of the total cell concentration. If the phage particles have been liberated by undetected lysis of cells it must be supposed that each cell liberates large numbers of phage particles.

It is difficult, from this point of view, to account for the increase in both bacteria and phage from one infected organism. If the phage increases only when the organism undergoes lysis then one infected organism could give rise to either more phage or more cells but not both. If the cell lyses more phage will be liberated but no cells will be present. If the cell does not lyse there will be more cells but no increase in phage. However, single cells of this lysogenic strain give rise to colonies as well as to more phage (55).

If the percentage of organisms lysed during the growth period is very small the percentage of phage formed during this period must also be very small if it is assumed that each lysed cell liberates a constant amount of phage. According to this idea the principal increase in plaque count must occur after visible lysis starts and the increase in plaque count must be roughly proportional to the decrease in the number of cells. If, on the other hand, the phage is produced on or in the growing cells then the principal increase in phage will occur during growth instead of during lysis. This relation is best shown by plotting phage concentration *versus* bacteria concentration. If phage is liberated during lysis such a curve should rise only a few per cent as bacteria increase and the principal increase in phage should appear as bacteria decrease. This will give a curve of type L in Fig. 11. If, on the other hand, phage is formed and liberated continuously by the growing bacteria then the phage count should increase rapidly as bacteria increase and slowly or not at all as lysis starts and bacteria decrease. This will give a curve of the type G in Fig. 11. The results of ten experiments are plotted in Fig 11. All the curves but two (27 and 93) are of the G form. In the two L curves the amount of phage formed per bacteria lysed is small and the form of the curve is determined by only one point. These curves are similar to those found by Krueger and Northrop with *Staphylococcus* phage.

The decrease in phage during lysis cannot be ascribed to an inhibiting substance since filtrates from growing cultures or from cultures which have been dissolved by lysozyme do not cause a decrease in plaque count. If anything there is an increase in the presence of such filtrates.

In the case of *megatherium* and of *Staphylococcus*, therefore, it cannot be assumed that phage is liberated during lysis unless some secondary hy-

pothesis is added. In the case of some strains of *B. coli*, however, there is an increase in phage during lysis and the curves are of the L type (personal communication from Dr. Ellis).

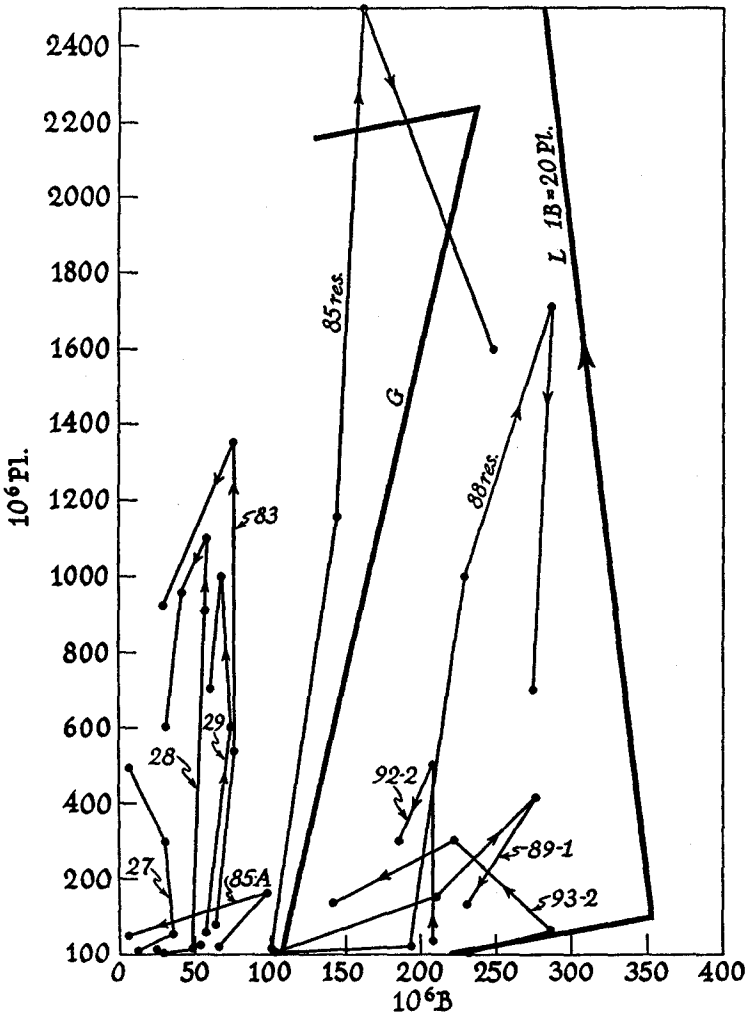

FIG. 11. Changes in bacteria and phage concentration during growth and lysis of various cultures. The number of bacteria per ml. has been plotted against the plaque count per ml. If the plaque count increases during growth of the organism the curve must rise sharply with a positive slope and a sharp break at the time the bacteria concentration decreases. The general shape will be that of curve G. If the plaque count increases during lysis the curve will have a small positive slope during growth of the cells and the principal increase in plaque count must occur when the cell concentration decreases. Most of the curve, therefore, must have a negative slope as in curve L.

Several curves have been obtained which show the step-like form described by d'Herelle and Ellis and Delbrück but there have always been slight increases in cell concentration corresponding to the sudden increase in phage concentration (Figs. 5 and 6). Since changes in bacterial growth rate are greatly exaggerated in phage increase rates a very small change in bacteria may make a very large change in phage.

These step-like curves are found only in cultures which contain resistant forms and not in cultures like *Staphylococcus* where lysis is complete and no resistant form appears. It is suggested that the step-like curves are due to the presence of two or more strains of bacteria or two or more phages of different rates of increase.

It was noted by Burnet that some phage solutions gave a longer lag period than others and this observation has been confirmed in the course of this work. Obviously the presence of these two types of phage will give step-like curves.

Experimental Methods

Bacterial Concentration.—The light absorption of a series of standard suspensions of known bacterial content was compared with that of a solution of M/25 copper sulfate in M/10 sulfuric acid in a Klett photoelectric colorimeter. The ratios of the colorimeter readings were then plotted against the cell content of the standard suspension. The bacterial concentration of the unknown sample was determined by comparing the unknown with the copper sulfate and reading off the equivalent bacterial concentration from the copper sulfate-standard suspension curve (Fig. 12).

Preparation of Standard Suspension.—A rapidly growing suspension in 2 per cent peptone was analyzed for total cell count by direct microscopic count, number of colonies by plating on agar, and total dry weight by washing and drying to constant weight at 100°C. The suspension contained 1×10^8 cells per ml., usually in clusters of 4–8 cells; 0.2×10^8 colonies and 0.2 mg. dry weight.

The dry weight content of different suspensions may be determined within a few per cent by this method but the cell count per milligram dry weight varies quite widely owing to the difference in the size of the bacteria. The results have been expressed in terms of number of cells in a standard suspension having the same light absorption in order to show the approximate relation between the actual number of cells and the number of phage plaques. They are accurate to a few per cent for the total quantity of bacteria expressed in milligrams but there may be a considerable error in the number of cells. The method does not distinguish between living and dead cells.

Example of a Determination.—1 ml. unknown suspension added to 19 ml. one per cent formalin and read against M/25 copper sulfate in M/10 sulfuric acid in Klett photoelectric colorimeter. Copper sulfate reading = 32, suspension = 20, ratio $\frac{\text{copper sulfate}}{\text{suspension}}$ = 1.6. From Fig. 1 this is equivalent to 0.02 mg. dry weight per ml., or 1×10^7 bacteria per ml. This is the concentration of bacteria per milliliter of the dilution in formalin. This is 1/20 the concentration of the original suspension so that the original

suspension contained $20 \times 0.02 = 0.4$ mg. $\doteq 20 \times 10^7$ bacteria per ml. If the culture media is colored or if low dilutions are used it is necessary to correct for the color by making a determination on the culture media alone.

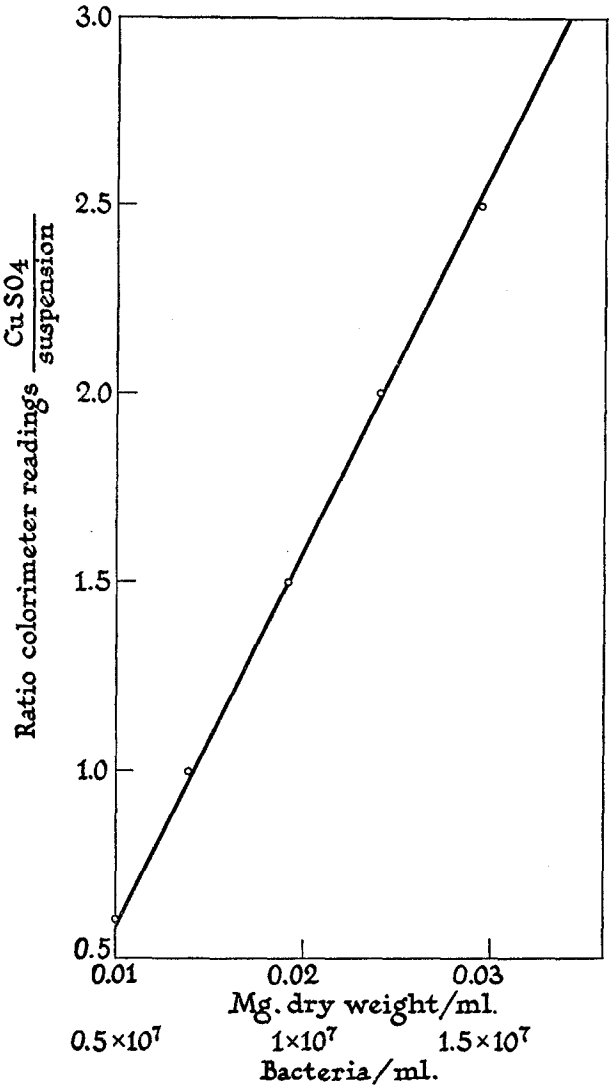

FIG. 12. Comparison of the light absorption of M/10 copper sulfate and M/25 sulfuric acid against suspension of washed bacteria in 10 per cent formalin. Ratio of colorimeter readings is plotted against the milligrams dry weight per ml. 0.01 mg. dry weight per ml. is equivalent by direct microscopic count to about 0.5×10^7 bacteria per ml. The results on a dry weight basis are accurately reproducible. The cell count in different suspensions may vary considerably owing to difference in the size of the cells.

Phage Determination.—The time of lysis method described by Krueger (43) cannot be used with *B. megatherium* since lysis is too slow and incomplete to allow accurate determination of the time required for lysis. The plaque count method was therefore used following the elegant technique described by Gratia (32). This method gives very accurate and reproducible plaque counts. The most accurate counts are obtained with 200–500 plaques per plate. Under these conditions the probable error of the average of 5 plates is less than 10 per cent. The results by this method are complicated by the fact that what is determined is the number of infected cells rather than the concentration of phage itself. Thus a cell which is infected will give rise to one plaque no matter how much or little phage it contains. Since the *megatherium* cell grows in clumps a further complication arises because a clump containing one or several infected cells will give one plaque in either case. The results also vary somewhat depending upon the agar concentration, as Bronfenbrenner and Korb (44) found.

Indicator Culture.—The sensitive strain of *megatherium* 36 was used in making plaque counts. The culture was kept on broth agar slants, incubated 24 hours at 30°C., and stored at 6°C. Transplants were made once a week. If transferred every day the culture may become resistant. The growth was washed from the slant with 5 ml. sterile yeast extract and broth agar Blake bottles were inoculated with 1 ml. of the suspension and incubated at 30°C. for 18 hours. The growth was washed off in 10 ml. of yeast extract and diluted to 0.1 mg. bacteria per ml. (5×10^7 bacteria) and shaken 1 hour at 35°C. in Florence flasks. It was then allowed to stand at 20°C. and 3.5 ml. used to prepare the plate as described by Gratia. Control experiments showed that the count varies with the condition of the culture. Thus, if the suspension is used immediately after washing off the Blake bottle the count is about one-half that which is obtained after 1 hour shaking. After this time the culture gives constant counts for 15–20 hours and then gives lower results again.

Culture Media. 1. Peptone.—2 per cent Fairchild's peptone plus 1 per cent sodium chloride pH 7.6, no phosphate. No phage is formed in some preparations of this peptone although good growth is obtained. Addition of 0.01–0.02 M calcium chloride may cause phage production but the results are irregular.

2. Yeast Extract Media.—1 ml. glacial acetic acid added to 1 liter distilled water and boiled. 40 gm. Fleischmann's dried yeast added and suspension boiled ½ hour. Filtered. Filtrate titrated to pH 7.8 and boiled 15 minutes. Cooled to 10°C. for 24 hours. Filtered and autoclaved. This media gives luxuriant growth of *Staphylococcus*, typhoid, *coli*, *megatherium*, and many other bacteria.

Cultural Conditions.—Krueger and Northrop (23) found that much more regular results were obtained with *Staphylococcus* culture and phage if the culture were shaken. Gratia (32) has noted the same result with *megatherium* and this result has been confirmed in the present work. Merrill and Clark (38) found greater production of gelatinase in aerated cultures. The culture was grown in 250 ml. Florence flasks containing 100 ml. or less culture media. They were rocked in a water bath at the desired temperature so as to cause violent agitation. Under these conditions the bacteria concentration doubles in less than 1 hour and a maximum concentration of $4–6 \times 10^8$ bacteria per ml. equivalent to 1 mg. per ml. dry weight is reached. If the culture is not shaken growth is very much slower and never reaches such high values.

Gelatinase Determination. Gelatin Solution.—25 gm. air dry isoelectric gelatin added to 500 ml. $\left\{\begin{matrix}0.015\ \text{M ammonium acetate}\\0.01\ \text{M sodium hydroxide}\end{matrix}\right.$. Heat to 55°C., adjust to pH 7.6. Filter and store at 0°C. For use warm to 80°C. for 5 minutes, pipette 5 ml. in test tube, cool to 35°C. for 10 minutes or longer. 1 ml. culture added to 5 ml. gelatin, solution poured into Ostwald viscosimeter and viscosity determined at intervals. The time of outflow is plotted against the elapsed time and the per cent change in the relative viscosity per minute interpolated from the curve. One gelatin unit (gel. u.) is defined as a change in the specific viscosity of the gelatin solution at the rate of 1 per cent per minute (51).

Most of the experimental work reported in this paper was done by Miss Elizabeth Shears and Mr. J. F. Gettemans.

SUMMARY

1. The increase in bacteria, phage concentration, and gelatinase concentration in cultures of *B. megatherium* has been determined.

2. With lysogenic cultures the phage concentration, gelatinase concentration, and bacteria concentration increase logarithmically at first. The phage and gelatinase concentration then decrease while the bacteria concentration increases to a maximum.

3. The results are the same with sensitive cultures if the ratio of phage to bacteria is small. If the ratio of phage to bacteria is large phage, gelatinase, and bacteria concentration all increase at first and then decrease. The maximum rate of increase coincides approximately with the maximum rate of oxygen consumption of the culture.

4. 60–90 per cent of the phage is free from the cells.

5. The amount of phage produced is determined by the combined phage and not by the total phage.

6. Phage is produced during growth of the cells and not during lysis.

7. In a very narrow range of pH near 5.55 no increase in bacteria occurs but large increases in phage may be obtained.

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