APPEARANCE OF NEW PHAGE TYPES AND NEW LYSOGENIC STRAINS AFTER ADAPTATION OF LYSOGENIC B. MEGATHERIUM TO AMMONIUM SULFATE CULTURE MEDIUM

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The production of enzymes (Monod and Audureau, 1946; Tatum and Lederberg, 1947; Klein and Doudoroff, 1950; Lederberg *et al.*, 1951) or of transforming principle (Hotchkiss, 1953–54) is a genetic property of the bacterial cell. If bacteriophages are products of metabolism of the cell, like enzymes (Bordet, 1931; Krueger, 1936; Northrop, 1938; Bawden, 1953) or the transforming principle (Gratia, 1936 *c*) or genes (Wollman, 1925), then it would be expected that the production of phage would also be a genetic property of the bacterial cell. The recent work of Lederberg and Lederberg (1953), Wollman (1953), and Fredericq (1953 *a*, 1954 *b*) confirms this prediction.

It would also be expected, from this point of view, that a change in character of the bacterial cell (by mutation or otherwise) might be accompanied by changes in the virus produced by the cell.

One way to change the character of the bacterial cell is to adapt it to different culture media. The present experiments show that adaptation of lysogenic *Bacillus megatherium* to a minimal synthetic medium results in the appearance of new cell types which produce new phage types.

EXPERIMENTAL RESULTS

Attempts to adapt *B. megatherium* 899*a* to a medium containing asparagine, glucose, PO_4, Mg, and Fe by the usual repeated transfer technique failed to show any adaptation and also failed to cause any changes in phage types (Murphy, 1954). The results did show, however, that infection of a sensitive cell is not a necessary step in the production of the various phage types, since sensitive cells, even if present, cannot be infected in asparagine culture medium.

Attempts to adapt the cultures to a medium containing ammonium sulfate, sodium citrate, phosphates, and iron by repeated transfer also failed.

The organism was then grown in a minimal medium (ASCM) containing ammonium sulfate, glucose, sodium and potassium phosphate, magnesium

sulfate, and iron in the steady state apparatus (Table I). The culture grew slowly (growth rate, 0.5/hour) for nearly 2 months, during which time it produced about 1 per cent as high phage concentration as in peptone and less

TABLE I

Growth Rate and Phage Production of B. megatherium 899a in Various Culture Media in Steady State Apparatus

Date	Growth		Culture medium, colony type, and procedure	Phage type			Gel U
	Rate	Type		Total/ml.	Cloudy (*T, br, vbr, m*)	Clear (*s,c*)	
					per cent	*per cent*	*U/ml.*
1952–53		Diffuse	899*a* transfer every 24 hrs. in either asparagine or 0.35 per cent peptone (Murphy, 1954)	10^{6}–10^{7}	>99	0.02	<10
5/1/1952 to 4/28/1953	0.4	Diffuse	899*a* transfer every 24 hrs. in ASCM	10^{8}	>99	0.01	<10
5/19 to 7/28	0.5	Diffuse	899*a*—grow in ASCM in steady state apparatus—large, smooth, dense	10^{6}	>99		<10
	1.0						
7/31 to 8/20	1.0–1.5	Granular mucoid		10^{6}	20–80	20–80	
				10^{6}	20–80	20–80	
			Colony AC8 isolated				
					t		
5/24/54			ASCM inoculated with AC8				
5/25	0.4	Diffuse	Rough, striated	10^{5}	30–60	30–60	<10
5/27 to 7/29	1.5–2.0	Mucoid	Smooth, translucent, wrinkled	10^{8}	0–40	60–100	
7/30	1.0		Change to 2.5 per cent peptone				
7/31 to 9/23	2.0		Rough	10^{6}	60	30	300–600
	2.0–2.5	Mucoid	Rough	10^{7}–	30–60	30–60	400–600
			Change to 5 per cent peptone	10^{8}			
9/25 to 10/14	2.0–2.5	Mucoid		10^{8}	30–60	30–60	
8/11/54			Colony AC82 isolated from culture AC8				
10/23 to			ASCM inoculated + AC82				
11/16 to	0.5	Diffuse	½ smooth	10^{5}	40–60	40–60	<10
12/27	1.0–1.5	Mucoid	" "	10^{6}	1–10	90–100	
12/28			Change to 2 per cent peptone				
9 a.m.	2.0			10^{4}	1–10	90–100	
12 to 4 p.m.	0.5		Colonies mostly striated and translucent	10^{8}		100	500
12–29 to	1.5–		10^{9} T phage/ml. added		1–10	30–60	
1/20/55	2.0			10^{8}	20–40	20–40	

In the present paper, the group of phages classed as "turbid" includes T (wild type), br, and vbr and similar types; the clear class includes *s,c, ss*, and similar types. (Murphy, 1954). These classes may be separated by plating on yeast extract agar (see Experimental Procedure). After the culture has been adapted to ASCM, *t* phage is produced in place of T. The *t* phage forms smaller plaques than T, the center colonies are denser and the halo narrower.

than 1 per cent as much gelatinase. The phage produced was more than 99 per cent T (or wild type), with small numbers of other types, as in peptone (Murphy, 1954). At the end of this time, the culture changed slowly to a

mucoid type. The growth rate increased (in about 10 days) from 0.5 to 1.5 to 2.0 and clear plaques of various types increased from about 1/2000 turbid to 1/10 or more.

No further change occurred for nearly a month. The culture was now streaked on peptone agar plates. All colonies were large, smooth, and dense, very similar to the original 899*a* colonies. Twenty colonies were streaked on a sensitized plate (*cf.* p. 620). Four colonies, which gave clear halos, were tested for phage production and growth in peptone. Two colonies gave rapid, diffuse growth and produced T phage; two colonies grew slowly in large clumps and produced about equal numbers of clear and turbid phage. One of these latter colonies was replated twice on peptone agar and then transferred once a week on 2 per cent peptone agar and also on ASCM agar (strain AC8).

This strain was then grown in ASCM in the steady state apparatus six times. The results were similar in each case. The last experiment is shown in Table I, 5/24/54.

The growth was slow (about 0.4) and diffuse for 1 to 2 days, the colonies on peptone agar were mostly rough, the phage production low (1×10^5/ml.) and the phage types were 30 to 70 per cent turbid and 30 to 70 per cent clear. These turbid plaques (*t*) were smaller and had denser centers than the original T plaques. This new type replaced the original T phage, and continued to appear in all subsequent tests. After 2 to 3 days the growth rate increased to 1.5 to 2.0, the culture became mucoid, the colonies became smooth, translucent, and wrinkled, and the phage titer increased to 10^6. No gelatinase could be detected. No further change occurred in the next 2 months.

The culture medium was then changed to 2.5 per cent peptone. The colonies now changed back to rough, the phage titer increased to 1×10^6 to 1×10^7 and the phage types remained about the same. The gelatinase increased to about 500 units/ml.

The *t* and miscellaneous clear phages remained the principal types. Detailed analysis of the plaque types, however, showed the presence of nine distinct types (Fig. 1). At least two of these (small *s* and *t*) have never been observed in any 899*a* cultures. Five of the nine phages were recombinants as shown by the result of crossing them with T phage. Since these phages came from a peptone culture, in which infection may occur, it is possible that they arose as the result of double infection of a sensitive cell with two different phage types. Such an occurrence is extremely unlikely in view of the very small number of sensitive cells present in such a culture. It is more likely that these recombinants arose as the result of a cross between two lysogenic strains.

The culture which was used at the beginning of this experiment had been transferred for some time on agar slants. It seemed advisable therefore to repeat the experiment with a freshly isolated, pure culture.

Stock culture AC8 was streaked on peptone agar and one colony, which

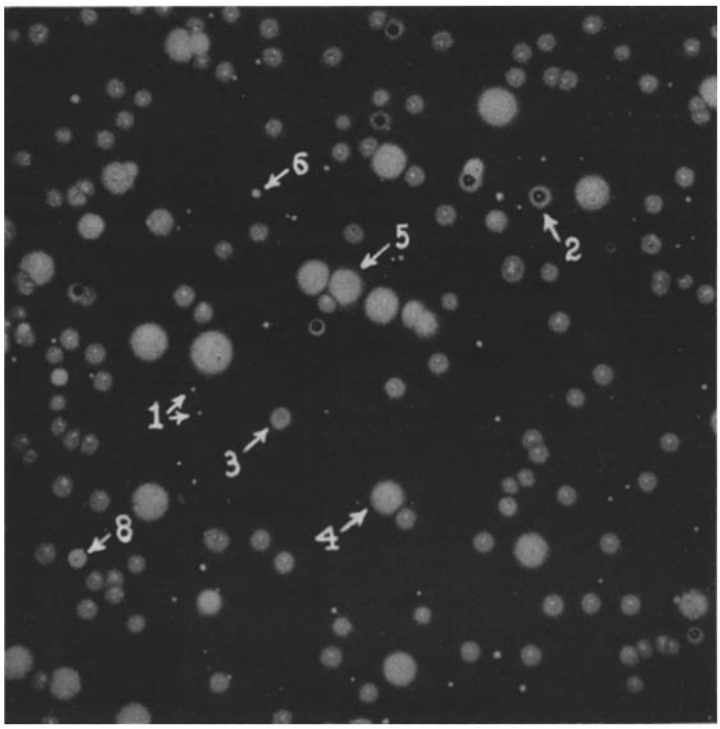

FIG. 1. Phage types produced in peptone by lysogenic *B. megatherium* (strain AC8) after adaptation to ASCM.

Sample from steady state cell filtered, diluted 1×10^{-7}, and plated with sensitive strain KM.

No.	Per cent total	
1	40+	Very small *c*
2	2	*t*
3	40	Small *s*
4	10	*c*
5	2	*c* variant
6	5	Small *c*
7	0.5	Small *s* variant (not shown)
8	0.5	Medium small *c*
9	0.5	Bright *s* (not shown)

produced a clear halo on a sensitive plate, was grown up, streaked, and the process repeated 3 times, one colony was transferred to an ASCM agar slant, transferred once a week and named AC82.

This culture was used to inoculate ASCM in the steady state apparatus, and the cycle, growth in ASCM→ peptone, repeated. It grew diffuse and slowly

for a few days and then became mucoid and grew at a rate of 1.5 to 2 per hour. The phages formed were nearly all clear types. No gelatinase could be detected. No changes in the culture occurred for over a month. At the end of this time, the culture medium was changed to 2 per cent peptone.

After 2 to 3 hours, the growth rate decreased from 2 to about 0.5, the culture became full of fragments of cells, the phage titer increased from 10^4 to 10^8 (nearly all *s* and *c*) and the gelatinase increased to 500 gel units/ml.

This change probably represents lysis which occurs when the strain adapted to ASCM is transferred to peptone. The same result was obtained in two other experiments.

In the course of the next 12 hours, the growth rate returned to 1.5 to 2, the gelatinase remained the same. A large percentage of very small plaques appeared on the plates, although the phage remained mostly clear types. 1×10^9 T phage (wild type) /ml. was then added to the culture in order to see whether it would cause the appearance of new phage types. The T phage added disappeared at about the expected washout rate, and the culture returned to its former behavior.

This culture was now streaked on peptone plates and the colonies tested for halo formation, growth rate, phage production, and gelatinase production in peptone or ASCM. Several different colony types were present although the original inoculation came from a single colony.

The appearance of the colonies was not correlated with either growth rate or phage production.[1] The type of halo found, however, indicated qualitatively the principal types of phage produced. Those colonies which produced cloudy halos produced mostly *t* phage, while the colonies which formed clear halos produced a large proportion of clear phage. Colony 12 produced nearly pure *c* phage. These colonies all grew in large clumps in liquid medium, so that the rate of growth could not be determined. Colony 12 was then streaked again and the daughter colonies tested as above. These colonies all gave clear halo's and produced a large proportion of clear plaques, like the parent No. 12.

Strain AC82 from which the preceding culture was derived was then tested in the same way. This culture had been transferred once a week on ASCM agar slants for 6 months. In this case also, only 2 colonies of 100 gave clear halos and these produced principally clear phages, like the original AC82 colony. The other colonies tested produced mostly *t* phage. These results show that the culture after adaptation in ASCM disassociates (De Kruif, 1921) on repeated transfer on either peptone or ASCM agar slants to produce several different cell types which produce different phage types. None of these produces T (wild type) phage and therefore they differ from the original 899*a*.

The original strain 899*a* was now tested in the same way, starting from the

[1] This conclusion is confirmed by the result of experiments in which lysogenic strains for several different phages were produced by infecting sensitive cells. The colonies of the lysogenic strains obtained in this way were indistinguishable.

spore tube which was used to inoculate the original steady state cultures in ASCM. In this case, 1 colony out of 100 tested formed a partly clear halo, but formed only T (wild type) phage. All the other colonies were indistinguishable. This culture therefore had not changed during the course of the experiments, and no colonies were found which formed a large proportion of clear phages. The number of plaques examined was too small to detect the low percentage of phages (other than T) normally produced by this culture (Murphy, 1954).

No sensitive (phage-free) colonies have ever been found in this culture and, in fact, it is very difficult to understand how a phage-sensitive cell could exist in cultures of the lysogenic strain, growing in peptone, which contain $>10^8$ P/ml., since such a cell would be infected almost at once, and would then either lyse, or become lysogenic itself.[2]

Sensitive cells have occasionally been found in the culture growing in ASCM in which infection does not occur. These sensitive cells, in view of the recent results concerning the genetics of phage production, are presumably phage-free mutants of the lysogenic strain or recombinants resulting from the crossing of two lysogenic strains. The production of phage-free cells by the latter method has been reported by Fredericq (1953 *a*).

Changes in Growth Rate of the Culture in Various Media

The original lysogenic strain 899*a* has a growth rate of about 1.7/hour in peptone when grown in test tubes shaken rapidly at 35° (Northrop, 1953 *a*), but grows at a rate of 2.5 in the steady state apparatus. This difference is probably due to the fact that under the usual condition of varying *B* concentration the bacteria do not have time to become completely adapted to the culture medium, since the composition of the latter changes constantly during the growth of the culture. This is indicated by the fact that the log part of the growth curve is quite short, and extends over a range of only about 20 to 200 $\times$ 10^6 *B*/ml. In the steady state apparatus, on the other hand, the composition of the medium in which the cells are growing remains constant and the cells gradually adapt to this medium. They then grow at the rate 2.5/hour. This corresponds to a generation time of about 24 minutes.

If this explanation is correct, it would be expected that the cells would grow at the 2.5 rate even in test tubes, if the time for adaptation were increased. This may be done by starting the culture with very low cell concentrations. Under these conditions the bacteria grew for several hours before reaching the concentration at which they are held in the steady state apparatus and without any change in the composition of the medium. The results of such an

[2] If sensitive colonies are found, it is probable that the sensitive cell appeared (as a mutant) after the culture was diluted for plating.

experiment are shown in Fig. 2. Those cultures which grew in the peptone for 3 hours or more before reaching the concentration range (40 to 60 × 10^6) set in the steady state apparatus grow at the same rate as in the steady state cell, *i.e.*, 2.5/hour, while those which started with a higher cell concentration and reached log growth sooner, grew more slowly. This result is not due to selection since a culture from the steady state apparatus or from a fast growing

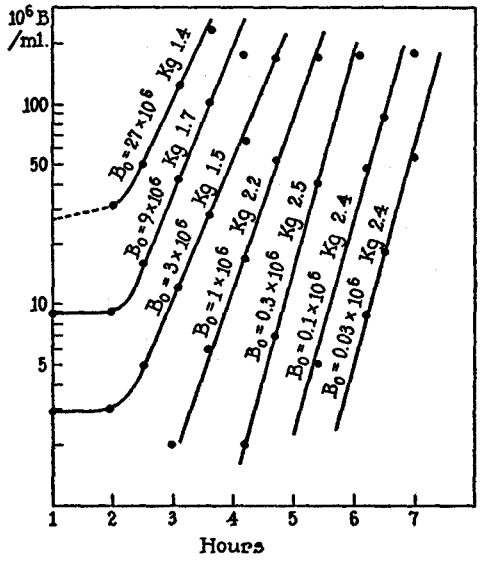

FIG. 2. Growth rate of *B. megatherium* 899*a* in 5 per cent peptone shaken at 35°, with varying initial *B* concentration.

Cells from 48 hours, 5 per cent peptone agar slant washed off in 5 per cent peptone and diluted in 5 per cent peptone to concentration noted. 10 ml. in 20 mm. test tubes. Shake 400/minute, in water bath at 35°. Cell concentration determined by turbidity in Klett photoelectric colorimeter.

tube diluted to a concentration of 10 to 20 × 10^6 in peptone grows at the usual 1.7 rate.

The growth rate of the original culture 899*a* in ASCM in test tubes is 0.4 and about 0.8 to 1.0 in the steady state apparatus. This difference may be decreased by the same process as that described above, but the tube cultures never grow as rapidly as those in the steady state apparatus. When the culture becomes adapted to ASCM, it grows at a rate of 1.5 to 2.0 in the steady state apparatus, but the rate drops at once to about 0.8 as soon as the culture is removed from the steady state apparatus and grown in test tubes, and this rate cannot be increased by changing the inoculation or by repeated transfer. This rapidly growing strain is very mucoid and grows in large clumps in test

tubes, even if violently agitated. In the steady state cell, however, growth is diffuse, owing probably to the mechanical stirring action of the wiper rings. This probably accounts for the increased growth rate.

The growth rate of the culture in ASCM changed from 0.4 to 2.0 at the same time that the culture became mucoid and the new colony and phage types appeared. The growth rate varies, however, depending on conditions of growth, whereas the types of phage produced remain nearly the same and only the concentration changes. This is clearly shown, for instance, by strain AC8.

This colony was obtained from a culture growing at the rate of 2.0 in ASCM in the steady state apparatus. It grew in large flocs in ASCM in the test tubes at a rate of about 0.4, and produced about 5×10^4 *s* and 5×10^4 *t* phages/ml. during log growth.

The colony was transferred to ASCM agar slants, and transferred once a week. After 3 weeks it was grown in ASCM in the steady state apparatus again. The growth was now diffuse, and the growth rate 0.5/hour, about the same as the original culture. The types of phage produced, however, remained about the same as before the culture was transferred to the agar slants. The growth rate increased slowly and after 2 days reached the same fast rate as that observed when the culture was first adapted to ASCM. This experiment was repeated six times after increasing numbers of transfers had been made on the ASCM-agar slants.

The time required for the growth rate to increase from 0.4 to 2.0 was longer, the longer the culture had been grown on agar, and after a year, nearly 2 weeks were required to reach the rapid growth rate. The phage types produced did not vary significantly but the concentration increased with the growth rate, as was to be expected. (*cf.* Krueger and Northrop, 1930). The increase in the growth rate is gradual, and not in steps. If the change from slow (0.5/hour) growth to 2.0/hour were due to the appearance of a fast growing mutant then the rate in the steady state apparatus would change from 0.5 to 2.0 in about 3 hours. (Northrop, 1954 *b*) This sudden change occurred once, but on all other occasions several days were required, at least. If a fast growing culture is added to a slow growing one, the growth rate changes from 0.5 to 2.0 in about 4 hours, which is a little longer than the calculated expectation, and hence the growth rates are nearly independent. The changes in growth rate during adaptation to the ASCM, therefore, are not in accord with predictions based on the occurrence of a mutation, unless a number of secondary hypotheses are invoked. The change is gradual and not in steps. It is not permanent, but returns rapidly to its original value as soon as the culture is removed from its fast growing environment.

These results are much more readily explained as due to changes in the enzymatic character of the bacteria when growing in different media, than they are by assuming the appearance of mutants whose enzymatic composition is rigidly fixed. This is in agreement with Hinshelwood's conclusions (1946).

The appearance of new lysogenic strains which produce the new phage types, however, may be due to a mutation. The type of phage produced does not change, depending on the media, although the growth rate and the ratio P/B does.

Apparently the type of phage produced depends on the genetic character of the bacterial cell, and cannot be changed by changing conditions, whereas the growth rate of the culture is not a genetic property and may be changed over a wide range, depending on the environment.

The preceding experiments show that the adaptation of *B. megatherium* to a minimal synthetic medium results in the appearance of new cell types which produce many new phage types.

This result may be accounted for by several different hypotheses.

1. The strains were present in the original culture and appear as the result of selection. The original culture, however, came from a series of single colony transfers, in none of which were any colonies observed similar to those appearing after adaptation occurred. Accidental contamination is extremely unlikely since no strains resembling the new ones had ever been seen in the laboratory.

2. The new lysogenic strains are the result of infection of an old lysogenic strain, or a sensitive cell (phage-free mutant) (Fredericq, 1953 *a*) with phage types present in the original culture (*cf.* Hewitt, 1954).

Murphy (1954) has shown that the incidence of the various phage types in the parent strain is the same in peptone in which infection can occur as in asparagine medium, in which infection cannot occur. Infection does not occur in the ASCM either, so that any hypothesis involving infection of a cell is improbable. In order to test this possibility further, however, several control experiments were carried out.

(*a*) Growth of a sensitive cell in the filtrate from the new lysogenic culture.

The overflow from the steady state apparatus in which the lysogenic culture was growing (in ASCM) was filtered through a Seitz filter, placed in a steady state apparatus, and inoculated with a culture of sensitive cells isolated from the lysogenic culture itself. These cells grew rapidly for 2 days in the filtrate from the lysogenic culture, which contained about 10^5 *t* and 10^5 clear phage types. After that time the lysogenic filtrate was replaced by ASCM. The cells continued to grow, while the phage titer dropped about in proportion to the dilution and after 48 hours, no phage could be detected, nor could any lysogenic cells be found. This experiment was repeated with the sensitive strain of *B. megatherium* (KM) used as indicator; the result was the same as in the first experiment and no lysogenic strain appeared.

(*b*) The original lysogenic strain 899*a* was grown in the ASCM in the steady state apparatus. Before adaptation occurred, a culture of *B. megatherium* sensitive was added. The sensitive cells could be detected for 10 to 12 hours, but were soon washed out (since they do not grow as rapidly as the lysogenic in

this medium). No new lysogenic strains, or new phage types appeared as a result of this experiment.

(*c*) Sensitive *B. megatherium* in 5 per cent peptone was infected with the filtrate from lysogenic strain AC82, which is about ½ *t* and ½ clear phage types. The culture lysed and an overgrowth appeared which was lysogenic, formed colonies similar to AC82, and produced clear and *t* phages. On repeated transfer in peptone, however, the phage titer (and also the ratio of clear/turbid) fell until, after the 13th transfer, the phage titer was about 1×10^4 and the ratio of clear/turbid about 1/1000. The lysogenic strain isolated from the adapted culture, under these conditions, continued to produce 1×10^8 *P*/ml. with a ratio of clear/turbid of about 1/1. Even if a sensitive cell is infected with the phage from the lysogenic culture, therefore, the new lysogenic culture is not the same as the one which appears as the result of adaptation of the lysogenic *B. megatherium*.

The remaining hypothesis assumes that the production of phage is a genetic property of the lysogenic cell, just as is the production of an enzyme, and hence, when new cell types appear, either by mutation or otherwise, they may differ in the phage types they produce, just as in any other cell character.

DISCUSSION

The experiments described in this paper show that if new strains of lysogenic *B. megatherium* 899*a* are derived by adapting the organism to different culture media, new phage types appear. Similar results have been reported by Hewitt (1954). This is in agreement with the work of Lederberg and Lederberg (1953), Wollman (1953), and Fredericq (1953 *a*, 1954 *b*), who have shown that the ability to produce phage is a genetic character of the bacterial cell.

These results are expected and are indeed corollaries of the assumption that the phage is a product of the metabolism of the bacterial cell (Bordet, 1931; Krueger, 1936; Northrop, 1938; Bawden, 1953) similar, possibly, to the transforming principle (Gratia, 1936 *c*), or the genes (Wollman, 1925).

These experiments, therefore, add another to the rapidly growing list of observations which are predictable if the virus is a product of metabolism of the bacterial cell, but which are unexpected and difficult to explain, if the virus is considered to be an independent organism.

A partial list of the more important of these observations is as follows:—

1. The particles have no respiration (Bronfenbrenner, 1926; Schüler, 1935).

2. They have the chemical composition of a nucleoprotein (Northrop, 1938; Cohen and Anderson, 1946; Taylor, 1946; Herriott and Barlow, 1954).

3. They may be readily inactivated and reactivated (Krueger and Baldwin, 1933; Krueger and Elberg, 1934; Krueger and Baldwin, 1935; Krueger and Mundell, 1936; Northrop, 1954 *a*; 1955).

They are inactivated by trypsin, chymotrypsin, and desoxyribonuclease

(Northrop, 1938, 1955). They are not inactivated by chloroform (Fredericq, 1950 *a*; Schultz, 1928) or toluene.

4. No evidence for the existence of the particle can be found in certain lysogenic cells (Gratia, 1936 *d*) even by sensitive immunological methods (Miller and Goebel, 1954) or in the infected cell (at certain times) (Anderson and Doermann, 1952).

5. The rate of formation of the phage is related to the rate of growth of the cells by exactly the same relation as is the rate of production of an enzyme, gelatinase, and is influenced in the same way as the production of gelatinase by changing the composition of the media (Northrop, 1938, and the present paper).

6. Production of phage is increased by the same yeast fraction which stimulates adaptive enzymes in yeast (Price, 1949).

7. Cell-free preparations of *B. megatherium*, which can synthesize protein and adaptive enzymes, also produce phage (McQuillan, 1955; Brenner and Stent, 1955).

8. The ability to cause an infected cell to produce phage is a property of the DNA and does not require the protein part of the phage particle (Hershey, 1953 *a*; *cf.* Northrop, 1951).

9. The DNA of the bacterium is the precursor of phage-DNA (Hershey, 1953). This confirms and explains the results of Krueger and Mundell (1938) and Krueger and Scribner (1939) that rapidly growing cells produce more phage than resting cells, since they contain more NA, and also that the phage per cell is roughly proportional to the nucleic acid content of the cells (Hedén, 1951; Northrop, 1953).

10. There is no qualitative difference between infected sensitive cells and lysogenic cells. One may be changed to the other by changing the medium (Northrop, 1952) or by exposure to ultraviolet light (Lwoff, Siminovitch, and Kjeldgaard, 1950).

11. Lysogenicity (the ability to produce phage) is a genetic character of the host cell and is in some cases linked to the production of an enzyme, galactase (Lederberg and Lederberg, 1953; Wollman, 1953; Fredericq, 1953 *a*). This is true of either the natural lysogenic strains or of strains produced in the laboratory (Fredericq, 1954 *a*).

This fact together with the observation that infected cells do not differ qualitatively from lysogenic cells, offers a simple explanation for the production of phage recombinants by the infection of a single cell with two or more phages (Delbrück and Bailey, 1946; Hershey, 1946). This experiment is essentially the same as crossing two separate lysogenic cells as Fredericq has done. The result, in either case, is a single cell containing both types of phage, which then gives rise to recombinants in the same way as though it were the result of fusion of two separate cells. The results, therefore, should be referred to the

bacterial chromosome, and not to the phage. This is clearly shown by the observation of Lederberg and Lederberg (1953) that lysogenicity in certain strains is linked to galactase formation, which is surely a genetic character of the bacteria (*cf.* also Demerec, Blomstrand, and Demerec, 1955).

12. The character of the host cell is changed by the phage, just as by the transforming principle (Zinder and Lederberg, 1952; Groman, 1953; Hewitt, 1954).

13. The properties of the phage are changed by passage through different hosts (Fredericq, 1950 *b*; Ralston and Krueger, 1952, 1954; Bertani and Weigle, 1953) and this effect cannot be due to selection or adaptation (Ralston and Krueger, 1952, 1954).

14. Irradiation of the host cell, before infection, results in the appearance of new phage types. Irradiation of the phage alone does not (Hershey, Garen, Fraser, Hudis, 1954).

15. Phage "mutants" appear in groups, from single cells, as they should if the cell is responsible for the mutation (Gratia, 1921; Fredericq, 1950), and not in a Poisson distribution as they should if the mutants arise as chance variation in the particles (Adams, 1953; Hershey *et al.*, 1954).

These facts are all in agreement with Bordet's suggestion that the virus is a product of cell metabolism. They are quite unexpected if the virus is an independent organism, and in fact, are difficult to account for on this basis.

If the virus is a genetic character of the host, and is a product of the host metabolism, then it is evidently unnecessary to assume the existence of a pro-phage, other than the gene, as Wollman (1953) has remarked.

Experimental Procedure

Samples were taken from the steady state apparatus once or twice a day or oftener, if any change occurred. They were analyzed for colony types, phage types, and gelatinase. The growth rate was calculated from the flow rate of the culture media as recorded by the kymograph (Northrop, 1954 *b*).

Steady State Apparatus.—The culture cell described previously allowed the bacteria to collect on the shoulder of the cell and these organisms became mixed with those in suspension, when a sample was taken. The cell shown in Fig. 3 avoids this difficulty, since any organisms on the cell shoulder or in the overflow tube are killed and washed out by running 50 per cent ethyl alcohol through the top of the cell, before a sample is taken. The overflow tube was changed so as to prevent changes in the rate of flow of the culture medium, depending on the volume of overflow (Fig. 4).

It was found that four rubber segments moving through 2.5 cm. kept the cell cleaner than the earlier arrangement.

The rate of the wipers should be as rapid as possible, without causing foam to collect in the cell. This is about 100 to 140 strokes a minute. For this reason a D.C. variable speed motor is necessary. This arrangement keeps the cell bottom and walls entirely free of bacteria, even with a very mucoid culture. Extremely mucoid cultures (like the lysogenic *B. megatherium* adapted to ASCM) adhere to the rubber wipers and

to the steel wire. This may be avoided by increasing the rate of the motor just enough to form foam in the cell, for 10 to 15 seconds every half hour. This can be accomplished automatically by a cam on the kymograph axle which opens a microswitch.[3]

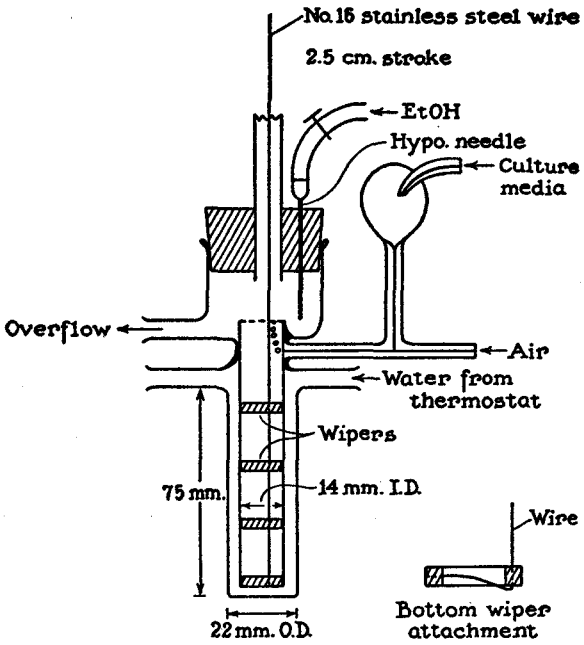

FIG. 3. Steady state cell.

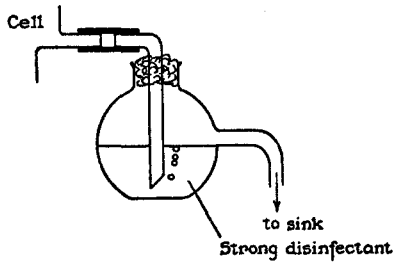

FIG. 4. Overflow from steady state cell.

Some cultures foam so violently that the overflow from the cell is nearly all foam even at slow stirring speeds. The foam contains less bacteria per milliliter than does the liquid inside the cell.

[3] The apparatus was constructed by Hallikainen Instruments, Berkeley.

The relation between the growth rate and the change in volume, $K_g = \frac{\Delta v}{V_0 \Delta t}$, is based on the assumption that the concentration of bacteria is the same in the overflow as in the suspension inside the cell (Northrop, 1954 *b*). If this is not true, it is necessary to correct the growth constant calculated from the volume change. The corrected growth rate is

$$K_g = \frac{\Delta V \; B/\text{ml. outflow}}{V_c \Delta t \; B/\text{ml. in cell}}$$

V_c = volume of cell
ΔV = volume of overflow in the time Δt
This correction may amount to 50 per cent in extreme cases.

Colony Analysis:

The culture was streaked on 5 or 6 peptone agar plates. After 2 days at 35°, the colonies were inspected and about 100 (including any which differed from the majority) were numbered, touched with a wire, and streaked on a sensitive plate. The sensitive colony plates were incubated for 2 days at 35° and the type of halo surrounding the various colony streaks noted. Most colonies form turbid halos. Some of these together with any that formed clear halos were now fished from the original colony plates and inoculated into 1 ml. of ASCM. The tubes were allowed to stand at 25° for 24 hours and then 10 ml. ASCM added. 1 ml. of this suspension was then added to 9 ml. of 5 per cent peptone and the tubes all shaken rapidly at 35°. (Krueger and Northrop, 1930). The cell concentration was measured by turbidity and the growth rate calculated from the growth curves. Samples for phage count and gelatinase were taken during the period of log growth.

Plaque counts and plaque identification were carried out as described by Murphy (1952).

The two classes of phage types, clear and turbid, may be separated by plating on yeast extract agar, using a *B. megatherium* sensitive strain which has been grown on yeast extract agar, as indicator. The turbid phages do not form plaques under these conditions, since the *B. megatherium* sensitive, when grown in yeast extract medium, becomes lysogenic after infection with turbid phages and so does not lyse (Northrop, 1952). These phages are therefore virulent in peptone, but temperate in yeast extract, whereas the clear phages are always virulent.

Preparation of "Sensitized" Plates:

One ml. of a suspension of about 2×10^8 *B. megatherium* sensitive cells in 5 per cent peptone, ½ per cent agar was layered on a 5 per cent peptone, 2 per cent agar Petri dish. After about 1 hour at 25°, the plate was streaked with the colonies to be tested, and incubated at 35°. Lysogenic colonies develop a halo. The halo of those producing *s* or *c* phage is larger and clearer than that of T-producing colonies. Colonies which form no halo by this method sometimes form phage when grown in liquid peptone.

Phage Determination:

The phage content of some cultures in synthetic media is so low that the phage cannot be plated from the centrifuged supernatant, because there are too many cells present, nor from the ultrafiltrates, because too much phage is lost in the filtration. The cells are killed by toluene, however, while the phage and gelatinase are not affected.

3 ml. of the suspension (in peptone) were covered with 1 ml. toluene and allowed to stand 24 to 48 hours at room temperature. It was then plated for phage as usual. Control experiments (Table II) showed no change in the phage or gelatinase concentration of a filtered preparation under these conditions. The colony count and

TABLE II

Effect of Toluene on Colony Count, Plaque Count, and Gelatinase Concentration of B. Megatherium Cultures in Peptone

Sample of *B. megatherium* AC82 from steady state cell growing in ASCM, diluted 1/10 in 5 per cent peptone. Centrifuge 5 ml. Filter 10 ml. (Seitz).

Suspension, supernatant, and filtrate assayed for colonies, plaques and gelatinase, 3 ml. in 1.5 cm. test tubes covered with 1 ml. toluene, shake, and stand at 25°. Assay for plaques, colonies, and gelatinase as noted. (3 tubes in each set.)

Time at 25° under toluene	*B*/ml. turbidity	Suspension			Supernatant			Filtrate		
		Col./ml.	Pl./ml.	Gel U/ml.	Col./ml.	Pl./ml.	Gel U/ml.	Col./ml.	Pl./ml.	Gel U/ml.
		10^4	10^4		10^4	10^4		10^4	10^4	
0 hr.	5×10^7	100	100	120	5.0	20	110	0	1.0	130
0.25 "		0	6	105	0.1	8	120	0		
24.0 hrs.	1×10^7	0	4	100	0	3	130	0	0.8	125
48.0 "		0	6	120	0	5	125	0	1.2	100
7.0 days		0	5			6			1.1	120

also the plaque count of a suspension, however, drop off rapidly until the cells have been killed, and then remain constant.

Gelatinase Determination:

Gelatin Solution.—25 gm. isoelectric gelatin added to 500 ml. H_2O + 0.4 gm. ammonium acetate. Heat to 90° for 5 minutes, add 5 ml. toluene, shake, and pour 10 ml. in test tubes; keep stoppered at 0° until needed.

Assay.—10 ml. gelatin tubes heated to 90° for 1 minute, cool to 35°, and 1.0 ml. test solution (diluted if necessary) added. Stand 24 hours at 35° and the viscosity determined in a capillary viscosimeter. Control tubes contain 1 ml. *boiled* test solution. One gelatinase unit = quantity of gelatinase which will cause 1 per cent change in viscosity of 10 ml. of a standard gelatin solution in 24 hours at 35°.

A curve showing the relation between the change in viscosity and the enzymatic activity (gel U) added is shown in Fig. 5. 1000 gel U as defined by this method are

equivalent to about 1 gamma trypsin. This unit is about 1/1000 of that used previously (Northrop, 1938). The enzyme hydrolyzes casein, but does not activate chymotrypsinogen or trypsinogen.

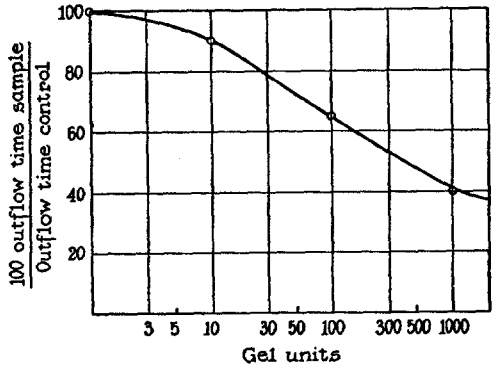

FIG. 5. Per cent change in viscosity of 5 per cent gelatin solution containing various amounts of gelatinase after 24 hours at 35°.

Ammonium sulfate culture medium (ASCM).

Substance	Quantity/liter H_2O
Ammonium sulfate	2 gm.
Dextrose	2 "
KH_2PO_4	3 "
$MgSO_4$	0.2 gm.
Ferrous ammonium sulfate 0.04 M in 1 M HCl	0.1 ml.

Autoclave 1 hour; adjust pH to 7.0–7.4 (about 3 ml. 5 M NaOH).

SUMMARY

1. Lysogenic *B. megatherium* 899*a* was adapted to growth in a minimal ammonium sulfate medium (ASCM).

2. Adaptation took place slowly and the following changes in the culture occurred:

(*a*) The growth rate increased from 0.5 to 1.5–2.0/hr.

(*b*) The culture changed from diffuse to mucoid.

(*c*) The total phage titer, and the gelatinase concentration decreased to 1/100 or less.

(*d*) The types of phage produced changed from >99 per cent T (wild type) to 30 to 60 per cent miscellaneous clear types. The original T phage was replaced by a different smaller *t*, never observed in the original 899*a* culture.

(*e*) Several new colony types also appeared, but the colony morphology

was not correlated with the phage types produced. None of the colony types was stable on repeated transfer either in peptone or ASCM, but continued to disassociate into different colony types (*cf.* Ivánovics, 1955).

3. Control experiments showed that these changes in phage production and colony types could not be brought about by growing sensitive *B. megatherium* in the presence of the various new phages, in ASCM. It is therefore unlikely that the changes observed in adapted culture were due to infection of a sensitive cell with phage.

4. Continued growth of the ASCM-adapted strain in peptone resulted in increasing the total phage titer, and also the gelatinase concentration. The growth rate returned to its original value and the ability to grow rapidly in ASCM was soon lost. The phage types, however, remained the same as in the ASCM.

5. An improved cell for steady state growth is described.

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