

## Studies of the Origin of Bacterial Viruses

### *VI. Effect of manganese on the proportion of phage-producing, terramycin-resistant, streptomycin-resistant, and phage-resistant cells in lysogenic megatherium cultures*

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**ABSTRACT** Exposure of *B. megatherium* 899 to about  $5 \times 10^{-3}$  M  $\text{MnCl}_2$  for 10 minutes at 25°C. results in an increase of about ten times in the proportion of phage-producing cells, terramycin-resistant cells, streptomycin-resistant cells, and phage-resistant cells.

The proportion of phage-producing cells reaches a maximum in about 4 hours and that of the other cells is reached in about 8 hours.

The origin and properties of bacterial viruses may be most simply accounted for by the assumption that they are a specialized type of transforming principle produced as a result of a mutation of the host cell (Northrop, 1958*a*). In confirmation of this mechanism it has been found that ultraviolet light and hydrogen peroxide increase the proportion of phage-producing cells and of terramycin-resistant mutants to about the same extent (Northrop, 1958*a* and *b*).

Demerec and Hanson (1951) and Steinman, Iyer, and Szybalski (1958) found that manganese chloride increased the proportion of all mutants studied (*cf.*, however, Durham and Wyss, 1957); it would be expected, therefore, that  $\text{Mn}^{++}$  would increase the proportion of phage-producing cells also, although negative results with this mutagen have been reported (Jacob, 1954).

In order to test this prediction, lysogenic *B. megatherium* cultures were treated with manganese chloride and the proportion of phage-producing cells, terramycin-resistant cells, streptomycin-resistant cells, and phage-

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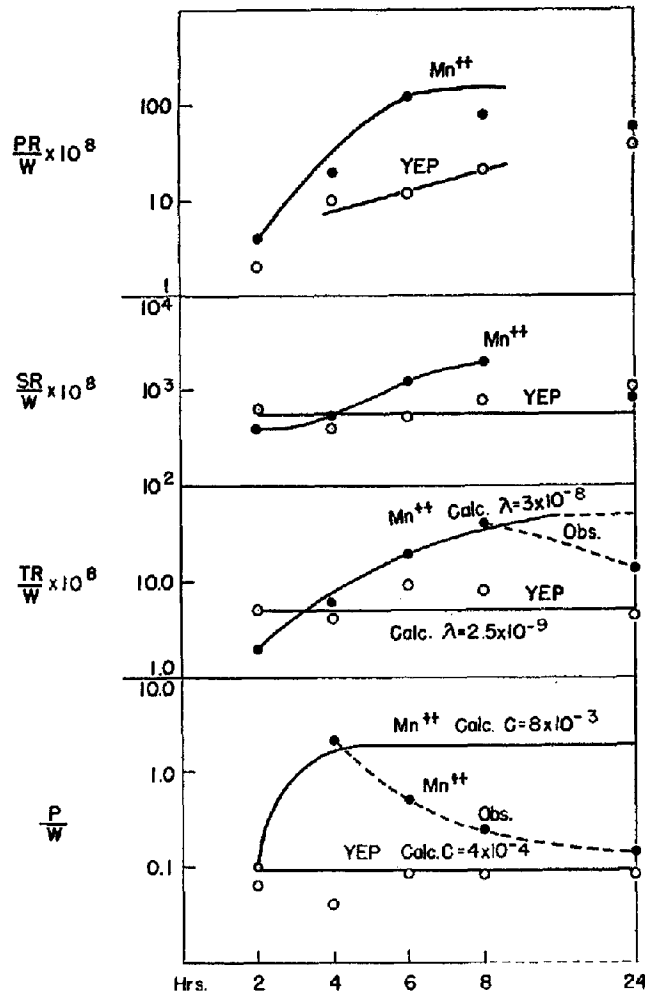


FIGURE 1. Effect of  $Mn^{++}$  on the proportion of phage, terramycin-resistant colonies, streptomycin-resistant colonies, and phage-resistant colonies to wild cells.

One ml. 899 culture ( $1 \times 10^8$  cells) added to 4 ml.  $5 \times 10^{-3}$  M  $MnCl_2$  in yeast extract peptone, four replicate tubes. Ten minutes at  $25^\circ C$ . Centrifuged, washed twice in  
(continued at top of next page)

resistant cells determined. Ten minutes' exposure to  $0.004$  M  $MnCl_2$  results in an increase of about ten times in each type of cell.

The result of a single experiment in which the proportion of the various mutants was determined at intervals after 10 minutes' exposure to  $0.004$  M manganese chloride is shown in Fig. 1.

The figure shows that 4 hours after treatment with manganese all the mutant cells had increased, more or less, compared to the controls. The ratio of phage/cells then decreased, while the other mutants continued to increase for at least 8 hours. At the end of 24 hours the culture had returned to its

FIGURE 1 (continued from opposite page)

yeast extract peptone, precipitates combined and suspended in 40 ml. yeast extract peptone, 10 ml. in each of four tubes. Four control tubes (no  $Mn^{++}$ ) treated in the same way. Shaken at 25° to  $1 \times 10^8$  B/ml. Mixed sample plated for various mutants, and diluted  $1/10$  in yeast extract peptone. Shaken at 25° to  $1 \times 10^8$  B/ml. and repeated.

This experiment is not accurately reproducible. It represents an average result. The increase in phage-resistant cells in the control tube is unusual.

The curve for the terramycin-resistant mutants was calculated from

$$\frac{M}{W} = \frac{2\lambda A}{B - A} [e^{(B-A)t} - 1] + \frac{M_0 e^{(B-A)t}}{W_0} \quad (\text{Northrop and Kunitz, 1957})$$

	In YEP	YEP + $Mn^{++}$
$\lambda$	$2.5 \times 10^{-8}$	$3 \times 10^{-8}$
$A$	1.0	1.0
$B - A$	-0.10	-0.10
$\frac{M_0}{W_0}$	$5.0 \times 10^{-8}$	$2 \times 10^{-8}$

The curve for the  $P/W$  ratios was calculated by means of the equation

$$\frac{P}{W} = \frac{IC[e^{(C-A)t} - 1]}{C - A} + \frac{P_0}{W_0} [e^{(C-A)t}] \quad (\text{Northrop, 1958b})$$

	in YEP	YEP + $Mn$
$C$	$4 \times 10^{-4}$	$8 \times 10^{-4}$
$(\lambda)$	$2 \times 10^{-4}$	$4 \times 10^{-4}$
$A$	1.0	1.0
$I$	250	250
$\frac{P_0}{W_0}$	0.10	0.10

$TR$ , terramycin-resistant colonies  
 $SR$ , streptomycin-resistant colonies  
 $PR$ , phage-resistant colonies  
 $W$ , wild cells  
 $P$ , phage

original composition. This result confirms the conclusion, indicated by earlier experiments (Northrop, 1958*b*, 1958*c*), that the change from normal to phage-producing cells occurs without cell division. Changes in genetic character caused by the transforming principle also occur without cell division (Fox, 1959). Those cells which are changed to virus producers are killed in the process and, therefore, the cycle ends. The cells which produce the other mutants, however, survive and continue to produce mutants at a higher rate than untreated cells for some time. The exact determination of the mutation frequency rate constants under these conditions is not possible

without more information. In the first place, the growth rate of the mutants and of the wild cells before and after exposure to  $Mn^{++}$  must be known. If a mutant grows at the same speed as the wild (or faster), it must of necessity overgrow the culture. This does not happen with any of the present mutants and hence, they must grow more slowly than the wild, and this is confirmed by direct determination.

If a culture of wild cells ( $W$ ) growing at the rate  $A$  produces mutants ( $M$ ) growing at the rate  $B$  ( $B < A$  and the mutation rate  $\lambda, \ll 1$ ), then the ratio of  $M/W$  approaches the value  $2\lambda A/(A - B)$ , and will require  $\ln 2/(A - B)$  hours to reach the half-way point (Northrop and Kunitz, 1957). At equilibrium

$$\lambda = \left(\frac{M}{W}\right)_e \frac{A - B}{2A} \quad (1)$$

Since the growth rates of the terramycin-resistant, streptomycin-resistant, and phage-resistant mutants are from 0.05 to 0.1  $hr.^{-1}$  less than that of the normal cells, it should require 7 to 14 hours to reach half-equilibrium.

If the mutant is produced without cell division and does not grow (*i.e.*,  $B = 0$  and  $C \ll A$ ), the proportion of mutants approaches  $C/A$  in which  $C$  is the mutation time rate constant (Northrop and Kunitz, 1957), and the time to reach the half-point will be  $\ln 2/A$ . Since  $A$ , in the experiments, is about 1, the phage-producing cells should reach half the equilibrium value in about 1 hour provided there is no change in the value of  $C$ .

The figure shows that both predictions are approximately correct. The terramycin- and streptomycin-resistant mutants require about 6 to 10 hours to reach the half-point, while the phage-producing cells require 2 to 3 hours, equivalent to two to three cell divisions.<sup>2</sup> There is a lag period of 0.5 to 1.0 hour before the cells reach logarithmic growth, and this is not allowed for in the calculation of the equilibrium time.

The ratios then decrease, instead of continuing to increase to the equilibrium value. This is no doubt due to a decrease in the mutation rate which slowly reverts to its original value.

<sup>1</sup> This dependence of the equilibrium value of the ratio of  $M/W$  on the growth rate may account for the effect of changing the temperature or culture medium on the proportion of mutants (*cf.*, for instance, Doudney and Haas, 1958). If the growth rate of either the mutants or wild cells changes even slightly, the difference between the two and hence, the equilibrium value of  $M/W$ , may be changed very markedly.

<sup>2</sup> The growth rate  $A$  is defined as fractional increase per unit of time  $A = dB/Bdt$ . The generation time (cell division time) is  $1/A$ , the generation number is  $At$  (*cf.* Novick and Szilard, 1950), and the time for the number of organisms to double at constant volume is  $\ln 2/A$ . This doubling time is frequently defined as the "generation" time, but this definition leads to the paradox that the generation time of an organism growing in a constant volume will be  $\frac{1}{2} \frac{1}{A}$  that of the same organism having the same rate of cell division but growing at constant cell concentration, as in the steady state apparatus or chemostat.

The curve for the ratio of terramycin-resistant mutants to wild cells is calculated on the assumption that the mutation frequency rate constant,  $\lambda$ , increases from  $2.5 \times 10^{-9}$  to  $3 \times 10^{-8}$  during the first 2 hours after treatment with  $Mn^{++}$  and then remains constant. This curve fits the observed values for about 8 hours, but then continues to rise to the equilibrium value (for that value of  $\lambda$ ) of  $60 \times 10^{-8}$  while the observed value drops to about  $10 \times 10^{-8}$ .

The curve for the proportion of phage to cells is calculated on the assumption that the change from wild to phage-producing cells occurs without cell division and that the value of the mutation time rate constant,  $C$ , increases from  $5 \times 10^{-4}$  to  $4 \times 10^{-3}$  sometime during the first 2 hours and then remains constant. The observed points agree with the calculated curve for about 2 hours, but then decrease, while the calculated ratio remains at the maximum value expected for the value of  $C$ .

It may be noted that, although both equations contain five constants, only one of these ( $\lambda$  or  $C$ ) is determined by the experiment itself. The other four are determined by independent measurement.

This behavior makes the calculation of the mutation rate constants from the  $M/W$  ratios very uncertain. The value for the ratio of mutants to wild cells is probably not the true equilibrium value, corresponding to the mutation rate after  $Mn^{++}$  treatment, but is smaller than that value. Also, it is difficult to determine whether or not the  $Mn^{++}$  treatment changes the growth rate of either the wild or mutant cells, since this determination requires 6 to 8 hours (Northrop, 1957) and in the meantime the effect of the  $Mn^{++}$  may have worn off. No effect of the treatment with  $Mn^{++}$  on the growth rate of either wild cells, terramycin-resistant, streptomycin-resistant, or phage-resistant mutants could be detected, but this result is not conclusive owing to the length of time required for the determination.

The mutation time rate constant of the phage-producing cells is given by the equation:

$$C = \frac{P_e A}{W_e l} \quad (\text{Northrop, 1958b}) \quad (2)$$

in which  $P_e/W_e$  is the ratio of phage/cells at equilibrium and  $l$  is the number of phage particles released by 1 phage-producing cell. The growth rate does not enter this equation, but the burst size does, and so it is necessary to be sure that the increase in  $P/W$  is not due merely to an increase in  $l$ , the burst size. In the present experiments the burst size at  $25^\circ$  in yeast extract peptone was  $250 \pm 30$ , with or without previous treatment with  $Mn^{++}$ .

Direct determination of the mutation rate by the null fraction method also is complicated by the fact that the rate must be changing with the time after the  $Mn^{++}$  has been added and so the value obtained will depend on

the time required to make the determination; this is different, depending on the mutant.

In view of these considerations it seems preferable to express the results in terms of the experimentally determined ratios rather than as mutation rates. If the ratios represent equilibrium values, which is doubtful, then the

TABLE I  
EFFECT OF VARIOUS CONCENTRATIONS OF  $Mn^{++}$  ON THE  
INCIDENCE OF PHAGE-PRODUCING, AND OF TERRAMYCIN-RESISTANT,  
STREPTOMYCIN-RESISTANT, AND PHAGE-RESISTANT CELLS  
IN *B. MEGATHERIUM* CULTURES

One ml. of yeast extract peptone culture of 899 containing  $1 \times 10^8$ B/ml. added to four replicate tubes containing 4 ml. of  $Mn^{++}$  noted. Stand 10 minutes at  $25^\circ$ . Centrifuge and wash twice in yeast extract peptone. Precipitates from replicate tubes combined, added to 40 ml. yeast extract peptone. The suspension divided among four tubes and shaken at  $25^\circ$  until  $1 \times 10^8$ B/ml. Duplicate determinations made on mixed sample from each four replicate tubes. Experiment repeated on 5 successive days, using the same spore tube and the same  $Mn^{++}$  solutions. The figures given are the means, with their average deviations, of the series of determinations.

$Mn^{++}$	$\frac{\text{Col.} + Mn}{\text{Col. YEP}}$	$P/W$	$Cp \times 10^4$	$TR/W \times 10^8$	$\lambda_{TR} \times 10^8$	$SR/W \times 10^8$	$\lambda_{SR} \times 10^8$	$PR/W \times 10^8$	$\lambda_{PR} \times 10^8$
<i>mol/liter</i>									
0	1.0	$0.2 \pm 0.02$	8	$6.6 \pm 0.6$	0.3	$1.4 \pm 0.16$	0.07	$5.2 \pm 1.4$	0.1
$2 \times 10^{-3}$	$0.9 \pm 0.12$	$2.5 \pm 0.2$	100	$18.0 \pm 4$	0.9	$1.8 \pm 0.3$	0.1	$9.4 \pm 3$	0.18
$4 \times 10^{-3}$	$0.75 \pm 0.02$	$2.0 \pm 0.3$	80	$8.0 \pm 3$	0.4	$2.2 \pm 0.4$	0.13	$25.0 \pm 6$	0.5
$6 \times 10^{-3}$	$0.005 - 0.3$	$1.6 \pm 0.04$	64	$30.0 \pm 6$	1.5	$34 \pm 7$	1.4	$80.0 \pm 16$	1.6
From direct measurement	$A$ $A - B$ $l$	1.0 1.0 250		1.0 0.1		1.0 0.12		1.0 0.05	

$$C_p = \frac{P_c A}{W_c l} \quad \lambda_M = \frac{M(A-B)}{W \cdot 2A}$$

$P/W$ —ratio of phage to cells.

$TR/W$ —ratio of terramycin-resistant colonies to cells.

$SR/W$ —ratio of streptomycin-resistant colonies to cells.

$PR/W$ —ratio of phage-resistant colonies to cells.

corresponding mutation frequency rate constant,  $\lambda$ , may be determined from (1) and the mutation time rate constant  $C$  from (2). This has been done in Table I.

The table shows that the ratios of phage-producing, terramycin-resistant, streptomycin-resistant, and phage-resistant cells to wild cells all increase from 2 to 20 times after 10 minutes' exposure to from 2 to  $6 \times 10^{-3}$  M  $MnCl_2$ .<sup>3</sup>

<sup>3</sup> The proportion of phage-producing cells present is larger than that of the other mutants, but much lower than that found for some mutant types, especially the smooth and rough colony mutants first described by DeKruif (1921).

At low concentrations the phage-producing cells increase more than the others, and this effect was also noted with ultraviolet light (Northrop, 1958*b*).

The results show that large increases in the phage-producing cells are caused by low concentrations of  $Mn^{++}$ , which kill very few of the cells. Similar mutagenic effects, with little injury, were noted by Demerec and Hanson. In the present experiments, however, the other mutants did not increase markedly until most of the cells were killed by the  $Mn^{++}$ . There is great variation in the fraction of cells killed (as is usual) and this is the principal source of variation in the experiments reported here. If the experiments are grouped in classes defined by the fraction of cells which survive the  $Mn^{++}$ , the average deviation of the mean is smaller than when the results are classed by  $Mn^{++}$  concentration, as in Table I.

### *Experimental Results*

Preliminary experiments gave irregular and non-reproducible results, as Demerec and others reported. Some experiments gave complete lysis, some were entirely negative. The effect of the manganese was found to depend on the type of culture medium, the concentration of bacteria, the physiological state of the bacteria, the concentration of manganese, the temperature, the time of exposure, and the order of mixing the components. Addition of cells to a solution of  $MnCl_2$  in yeast extract peptone caused much more effect than if the  $MnCl_2$  was added to a suspension of bacteria in yeast extract peptone. In addition, it was found that the concentration of manganese which increased the mutation rate decreased the colony count in the presence of the antibiotics, especially streptomycin and bacitracin. The effect was so marked with the latter that it could not be used. A further difficulty arose from the fact that phage production increased and the growth rate decreased, for an hour or so, after the addition of manganese, but both phage production and growth rate then returned to their original values. The proportion of other mutants, on the other hand, continued to increase. This behavior prevents any quantitative comparison between the effect of manganese on the phage-producing and on the other cells, under these conditions, since the results vary depending upon the time at which the culture was analyzed.

In order to avoid these difficulties, the culture was treated with manganese for a short time and the cells then centrifuged and washed and allowed to grow up in yeast extract peptone at 25°C.

Repeated efforts to improve the reproducibility of results led to the following conditions:

Manganese chloride solutions were prepared in yeast extract peptone and allowed to stand at 25°C. for 24 hours or more. The supernatant from these solutions was used. A precipitate forms in the mixture and hence, the exact concentration of  $Mn^{++}$  is uncertain.

A spore culture of *megatherium* 899 was prepared as described by Grelet (1951) and kept at 20°.

A loopful of this culture (representing about  $5 \times 10^7$  spores) was transferred to

10 ml. yeast extract peptone in a  $22 \times 175$  mm. test tube and the culture allowed to stand at  $25^\circ$  for 21 hours. It was then shaken (250 strokes/min.) at  $25^\circ$  for 3 hours, at which time there were 1 to  $1.2 \times 10^8$  B/ml. One ml. of this culture was then added to each of four tubes containing 4 ml. of the  $\text{MnCl}_2$  yeast extract peptone and the suspension allowed to stand for (usually) 10 minutes at  $25^\circ$ . The cultures were centrifuged, washed twice with yeast extract peptone, and the precipitates combined and suspended in 40 ml. yeast extract peptone. Ten ml. of the suspension were placed in each of four tubes and the tubes shaken at  $25^\circ$  until there were about  $1 \times 10^8$  B/ml. The four  $\text{Mn}^{++}$  tubes were then combined and a sample plated for the various mutants. The combined sample was then diluted  $\frac{1}{10}$  and again divided into four tubes, which were grown up to  $1 \times 10^8$  B/ml. and plated and diluted as before. Four control tubes, containing no  $\text{Mn}^{++}$ , were treated in the same way.

This procedure averages out the large differences which occur between replicate tubes without increasing the number of plates. The plating error is much smaller than the difference between replicate tubes containing  $\text{Mn}^{++}$ .

Even under these conditions, however, there are occasional marked changes in the effect of the  $\text{Mn}^{++}$  or in the proportion of the various mutants present in the culture.

The greatest variation occurs in the proportion of surviving colonies which may vary five to ten times in replicate tubes, in the presence of injurious quantities of  $\text{Mn}^{++}$ .

Attempts to cause increases in phage-producing or any of the other cells, in synthetic media, failed, probably owing to the presence of  $\text{Mg}^{++}$  ions which counteract the mutagenic action of  $\text{Mn}^{++}$  (Demerec and Hanson, 1951).

### *Experimental Procedure*

Phage determinations were made as described previously (Northrop, 1951).

Terramycin-resistant colonies were determined as described previously (Northrop, 1957).

Streptomycin-resistant colonies were determined by plating  $1 \times 10^8$  cells on yeast extract peptone agar containing  $2 \gamma$  streptomycin/ml. The colonies retained their resistance to  $2 \gamma$  through repeated transfer, but were not resistant to more than  $10 \gamma$ . The increase in resistance is, therefore, much smaller than is usual with this antibiotic. The streptomycin-resistant mutant is not resistant to terramycin, nor is the terramycin-resistant mutant resistant to streptomycin. Attempts to isolate the streptomycin-resistant mutant by Lederberg's replica technique were not successful owing to the high incidence of this mutant. As a result, nearly all colonies contain resistant cells, when they have attained visible size.

**PHAGE-RESISTANT CELLS** The phage used was obtained from *megatherium* 20  $\Delta$ , isolated by Dr. James S. Murphy from a deep water sample from San Francisco Bay, July 10, 1952 (personal communication). This *megatherium* produces phage which forms large clear plaques on both 899 and KM *megatherium*. It also produces high titer phage when added to 899 or KM, but there is no massive lysis of 899. *Megatherium* 20  $\Delta$  is lysed by T and C phage of 899.

Subcultures from the phage-resistant colonies or the overgrowth from 899 in the



presence of 20  $\Delta$  phage are completely resistant to the phage. They produce a phage (or phages) which forms turbid plaques on KM and clear plaques on 899. The phage-resistant cells, therefore, are probably lysogenic for the 20  $\Delta$  phage. It is possible, in fact, that all phage-resistant mutants are lysogenic, since the character of a bacterial virus may be changed after passage through a different host (Fredericq, 1950; Ralston and Krueger, 1952). Hence, the fact that a phage-resistant culture does not produce the same phage as that to which it is resistant does not prove that it is not lysogenic.

On the other hand, the ratio of *P/B* used in the determination is so high ( $> 100/1$ ) that the sensitive cells are probably destroyed by non-productive lysis (Krueger and Northrop, 1930), lysis from without (Delbrück, 1940) caused by a virolysin (Ralston, Lieberman, Baer, and Krueger, 1957), rather than by reproductive lysis.

If lower *P/B* ratios are used, many more colonies appear, but most of them are translucent and disappear again after 24 hours. If the phage-cell mixtures are allowed to grow up in yeast extract peptone instead of plated on yeast extract peptone agar, many more lysogenic cells survive. If, for instance, a suspension of  $1 \times 10^{10}$  phage particles/ml. and  $10^6$  *B*/ml. is prepared and 1 ml. samples are plated, no colonies appear, but if 1 ml. samples are shaken at 25°, all grow. The phage is, therefore, virulent if tested by plating for colonies, but temperate if tested in liquid culture.

Goffart-Roskam (1957) has shown that lysogenic colonies are formed from mutants present in the sensitive strain.

**PREPARATION OF 20  $\Delta$  PHAGE** A spore tube of *Megatherium 20  $\Delta$*  was prepared according to Grelet. Ten ml. of yeast extract peptone were inoculated with the spores, grown up at 25°C. to  $1 \times 10^8$  *B*/ml. and filtered through a Seitz filter. The filtrate contained about  $2 \times 10^8$  phage/ml.

One ml. of this phage was added to 10 ml. yeast extract peptone containing about  $1 \times 10^6$  KM/ml. and the tube shaken at 25°, until lysis occurred. Two ml. toluene were added, and the tube allowed to stand *in vacuo* until the toluene evaporated. This phage solution was sterile and contained about  $1 \times 10^{10}$  phage/ml.

**DETERMINATION OF PHAGE-RESISTANT COLONIES** One-tenth ml. culture containing about  $1 \times 10^7$  cells was added to 1 ml. 20  $\Delta$  phage containing about  $1 \times 10^{10}$  particles, 0.1 ml. 1 per cent agar added, and the mixture spread on a yeast extract peptone plate, and incubated at 35° for 24 hours.

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