

THE ACCELERATING EFFECT OF MANGANOUS IONS ON PHAGE ACTION*

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In previous papers dealing with the properties of an antistaphylococcus phage and its mode of action on a strain of *Staphylococcus aureus* (1-9) it has been shown that:

1. In a mixture of phage and growing bacteria, phage in or on the cells is in equilibrium with the phage free in the medium.
2. Phage formation is intimately related to bacterial growth and as far as can be determined does not occur in its absence. The rate of phage production is considerably greater than the rate of bacterial reproduction; as a consequence the ratio of phage to bacteria is constantly increasing. Upon accumulation of a certain high ratio of phage to bacteria (lytic threshold) lytic destruction of bacteria begins and proceeds rapidly to completion.
3. Phage can be completely inactivated by high concentrations of HgCl_2 and subsequently can be reactivated by removal of the Hg^{++} ions. Similarly inactivation with KCN can be reversed by conversion of the CN^- into $\text{Ag}(\text{CN})_2^-$.

None of the experimental evidence so far elicited indicates clearly the nature of phage; it may be animate or inanimate—either concept will fit the few known facts. Additional data about phage and its mode of action upon bacteria constitute an essential requirement before any serious attempt to define its nature can be made.

To continue the study of one particular phage and bacterium, the antistaphylococcus phage and *Staphylococcus aureus* above mentioned, systematic experiments on the effect of electrolytes upon the phage-bacterium reaction were undertaken. It was found, as would be

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anticipated, that a variety of electrolytes in appropriate concentrations served to inhibit the reaction. Of greater interest, however, was the acceleration noted in some instances, particularly in the case of Mn^{++} salts. The Mn^{++} ion in extremely low concentrations lessens the time required for lysis to begin in a given mixture of phage and bacteria, diminishes the final yield of phage obtained, and shifts the equilibrium between intracellular and extracellular phage in favor of the latter fraction.

The Acceleration Effect

Cultures of *Staphylococcus aureus* were grown in infusion broth containing 0.00016 molar $MnCl_2$ at 36°C. When $[B]^1 = 1 \times 10^8$ 1.0 ml. of the suspension was added to 4.0 ml. amounts of various concentrations of phage containing 0.00016 molar $MnCl_2$. These tubes were shaken in the 36°C. water bath together with identical controls containing no manganese. The results are recorded in Table I. For any given phage concentration the manganese-containing cultures lysed 0.5 hour ahead of the controls. To determine whether this result was due to the anion or cation, experiments were run with equivalent solutions of NaCl and $MnSO_4$. There was no accelerating action due to NaCl in the concentration used while the $MnSO_4$ produced the same effect as the $MnCl_2$. The action was therefore attributed to the Mn^{++} ion.

Analysis of the Mn^{++} Effect

In considering the mechanism underlying the accelerating effect of Mn^{++} ions three general possibilities suggested themselves; first, that the ion stimulates bacterial growth, increasing the rate of phage production and hastening attainment of the lytic threshold; second, that phage formation is somehow enhanced independently of bacterial growth; third, that the Mn^{++} ion reduces the lytic threshold and renders the cells more susceptible to phage action.

In order to test out the first of these hypotheses, that bacterial growth stimulation primarily was involved, a series of experiments was run following in each case the bacterial growth curve in phage-bacteria mixtures and in similar mixtures to which had been added manganese.

¹ $[P]$ = phage concentration in activity units/ml. $[B]$ = concentration of bacteria or staphylococci/ml. P.U. = phage activity unit.

TABLE I
Acceleration of Phage Action by Mn^{++}

	1×10^8 P.U./ml.	1×10^7 P.U./ml.	1×10^6 P.U./ml.	1×10^5 P.U./ml.
Initial phage concentration	1×10^8 P.U./ml.	1×10^7 P.U./ml.	1×10^6 P.U./ml.	1×10^5 P.U./ml.
Initial bacterial concentration	2.5×10^7 B/ml.	2.5×10^7 B/ml.	2.5×10^7 B/ml.	2.5×10^7 B/ml.
Time of onset of lysis: with Mn^{++}	0.8	1.5	2.15	2.9
Time of onset of lysis: without Mn^{++} ..	1.3	2.0	2.6	3.4

Temperature = 36°C . pH = 7.2.

For the Mn^{++} series the bacteria were grown in broth containing 0.00016 M MnCl_2 .
The phage dilutions used contained the same concentration of $MnCl}_2$.

The growth curves were found to be identical as is indicated in Fig. 1, a record of one experiment.

To test for the possible action of Mn^{++} on the phage-forming mechanism, mixtures of phage and growing bacteria with and without manganese were incubated at $36^{\circ}C.$ and samples were taken at intervals for titration of phage. Employing the procedures outlined below for quantitative determination of phage and bacteria, it was found that the rate of phage formation was not increased by manganese.

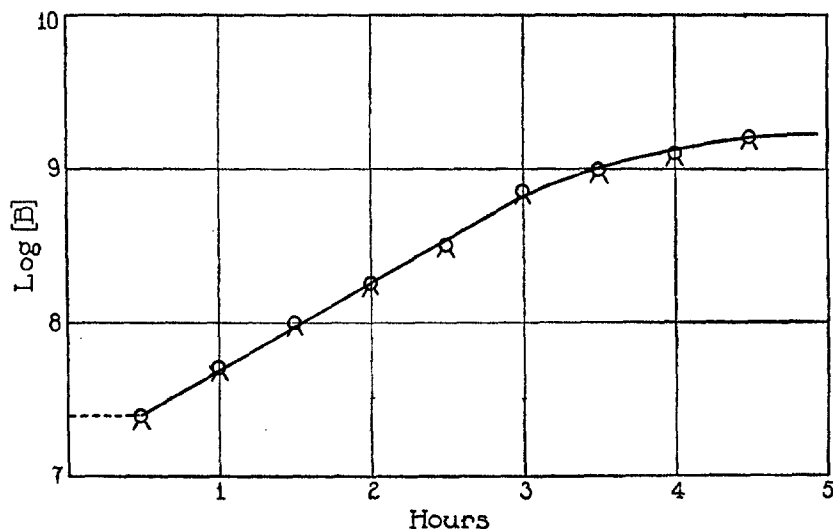


FIG. 1. Bacterial growth curves with and without $MnCl_2$ as measured by turbidity method (see text). $36^{\circ}C.$ pH = 7.2. O = initial [phage] = 1×10^4 [bacteria] = 2.5×10^7 , $MnCl_2$ 0.00016 M. Q = initial [phage] = 1×10^4 [bacteria] = 2.5×10^7 , no manganese.

However, there was a decided difference in the distribution of phage between the medium and the cells. Manganese increases the extra-cellular phage fraction to about four times the ordinary free phage concentration found in the absence of manganese. This is shown in Fig. 2. Distribution of phage evidently is of the simple type previously described (4) both in the presence of and in the absence of manganese. The average value of the partition coefficient K calculated from the equation $K = \frac{Pb/B \cdot 25 \times 10^{10}}{P_E}$ (where Pb = phage in bac-

teria/ml., B = bacteria/ml., and P_E = phage free in medium/ml.) was 15×10^3 in the absence of manganese. With manganese present the value for a number of experiments was 4×10^3 .

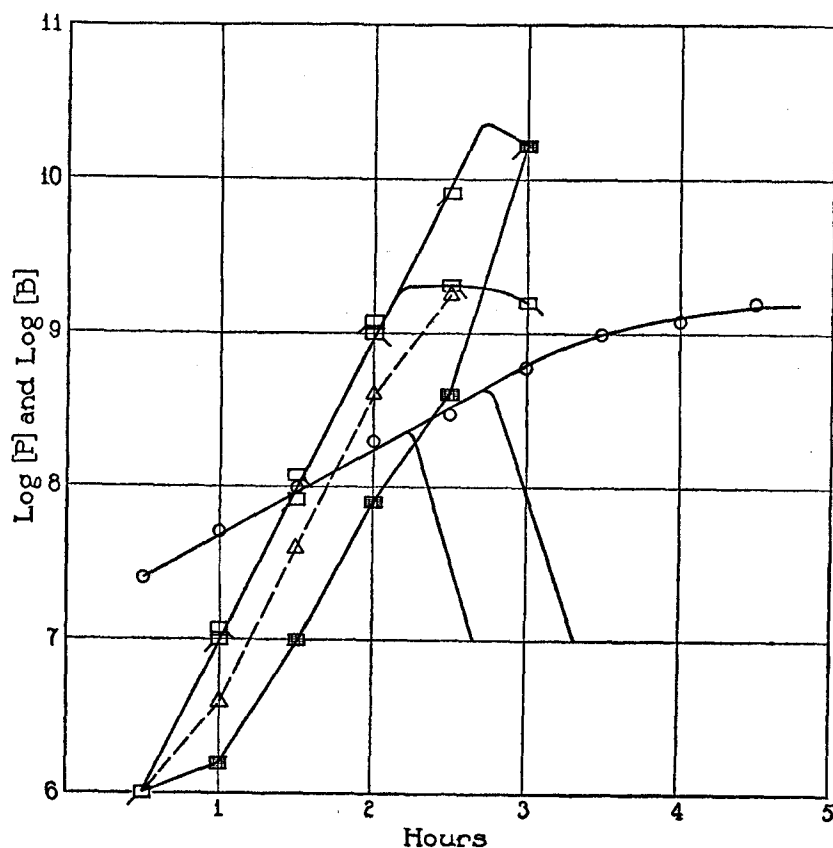


FIG. 2. The distribution of phage between bacteria and medium during growth and phage production in plain broth and in $MnCl_2$ broth. $36^\circ C$. $pH = 7.2$. \circ = bacterial growth curve; \square = total phage/ml. in absence of Mn^{++} ; \blacksquare = extracellular phage/ml. in absence of Mn^{++} ; \square = total phage/ml. in presence of Mn^{++} ; \triangle = extracellular phage/ml. in presence of Mn^{++} .

It is clear from Fig. 2 that lysis requires a lower ratio of phage to bacteria in the case of the manganese mixtures. As determined in current experiments, the usual ratio of phage to bacteria at the time of

lysis in the absence of manganese is 54 phage units/cell. In the presence of manganese this is reduced to a figure of about 12 (average of several experiments).

TABLE II

The Effect of MnCl₂ on the Lytic Threshold

Standard phage 1×10^{10} P.U./ml.	8.0	7.0	6.0	5.0	4.0	3.0	2.0
Broth, ml.....	1.0	2.0	3.0	4.0	5.0	6.0	7.0
Bacteria (8×10^8 /ml.).....	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lytic curves followed by means of turbidity measurements $B = \text{Cells} \times 10^7/\text{ml.}$							
Time	[B]	[B]	[B]	[B]	[B]	[B]	[B]
	With Mn	Without Mn	With Mn	Without Mn	With Mn	Without Mn	Without Mn
<i>hrs.</i>							
0.2	8	8	8	8	8	8	8
0.4	6	6	6	6	8	8	8
0.6	3	4	4	4	10	6	10
0.8	0	0	0	0	10	4	12
1.0					6	0	12
1.2					4		12
1.4					0		8
1.6							12
1.8							10
2.0							6
2.2							0
2.4							8

Determination of critical phage/bacteria ratio for lysis in absence of bacterial growth.

Staphylococcus-phage mixtures containing: (a) 0.00016 molal MnCl₂; (b) no MnCl₂.

[Bacteria] constant and [phage] varying. Young bacterial cultures used and growth inhibited by preliminary icing. Tubes transferred to 36°C. bath and turbidity measurements made every 0.2 hour. The critical phage/bacteria ratio for lysis with Mn⁺⁺ present = 38 and with no Mn⁺⁺ = 88.

If the lytic threshold in growing mixtures is lowered by manganese it would be expected that similar results could be obtained with static (non-growing) mixtures of phage and bacteria. To test this point

bacteria were grown in the presence of manganese and were then iced to inhibit growth. A unit volume of bacteria was added to varying concentrations of phage, the mixtures were iced to avoid bacterial growth and phage formation during the time required for establishment of equilibrium between intracellular and extracellular phage, and they were then shaken in the 36°C. water bath. Turbidity measurements were taken every 0.1 hour. Table II shows the results obtained in the presence and in the absence of manganese. Evidently the lytic threshold in static mixtures of phage and bacteria is lowered as in the case of the actively growing mixtures although in the absence of growth higher phage/bacteria ratios are required for lysis.

That the accelerating effect of manganese is due to depression of the lytic threshold is evidenced also by the reduction in final titre of phage when Mn^{++} is present. Experimentally, for any given initial phage concentration the end titre is uniformly about 1/10th of that developed in the absence of manganese. This would be anticipated if a lower ratio of phage to bacteria is required to institute lysis. (See Fig. 2.)

Methods

A. Materials and Titration Technique.—The broth used in our work was beef infusion containing in each liter 10 gm. Fairchild's peptone and 5 gm. NaCl. The hydrogen ion concentration was adjusted to between pH 7.2 and pH 7.4.

The stock manganese solutions contained 0.08 molal $MnCl_2$ or $MnSO_4$ and were diluted in physiological saline solution to 0.0016 molal strength before each run. Final 1:10 dilutions were made in broth so that the latter contained 0.00016 molal $MnCl_2$ or $MnSO_4$.

18 hour cultures of *Staphylococcus aureus* grown in Blake flasks were suspended in saline solution for each day's experiments. In order to have a uniform cell suspension the bacteria were seeded into manganese broth and plain broth and were grown at 36°C. until the bacterial concentration was sufficient for use. Measurements of bacterial concentrations were done by a simple turbidity comparison method as described by Krueger (1).

The bacteriophage was the antistaphylococcus phage used in previous experimental work (1-9). The standard phage solution contained 1×10^{10} activity units/ml.

Quantitative determinations of phage were routinely done by the method previously described by Krueger (1). In this method varying dilutions of unknowns and of standard phage are mixed with a unit concentration of susceptible bacterial cells. The time of lysis, *i.e.* the length of time required to reduce the

growing suspension to a particular turbidity end-point, is determined under controlled conditions. The smaller the amount of phage present the longer it takes for the suspension to lyse and the initial phage concentration of an unknown is read directly from a graph in which the time of lysis of the standard controls is plotted against the logarithms of phage units present. The phage unit is an arbitrary one and represents the minimum quantity producing complete clearing of 1.25×10^8 cells in 5.0 ml. pH 7.6 broth at 36°C.

For determining phage free in solution in mixtures of phage and bacteria, the bacterial cells were centrifuged down and the supernatants diluted and titrated as mentioned above. In the presence of Mn^{++} ions such dilutions do not yield true titration values because of the accelerating action of manganese on the titration mixture; the values are always too large. For this reason it was found necessary to add some substance which would inhibit the manganese effect on the titration set-up; 0.00016 Na_2SiO_3 in broth was found satisfactory for this purpose. In practice manganese-containing phage unknowns were diluted for titration in the silicate broth. Manganese-containing controls were diluted with plain broth and with silicate broth to make sure that the manganese effect was successfully blocked by the silicate.

To determine total phage/ml., *i.e.* intracellular phage + extracellular phage, various dilutions of the phage-bacteria mixtures were titrated just as in the case of the supernatants. It was found that Na_2SiO_3 would not inhibit the manganese effect in such mixtures and consequently they titrated too high. It was therefore necessary to include control sets with each titration consisting of a number of bacterial concentrations representing points on the logarithmic growth curve to which had been added varying amounts of phage. The mixtures were iced to allow the intracellular and extracellular phage fractions to come to equilibrium and were then diluted. The usual aliquot of bacterial suspension for phage titration was added to each tube and the time of lysis was determined in the routine way. By means of this additional series of controls it was possible to determine the amount of phage in the given manganese-containing mixture providing the concentration of bacteria originally present was known.

B. The Accelerating Effect of Mn^{++} on Phage Action.—Phage dilutions in plain broth and in manganese broth were prepared using 4.0 ml. amounts of 1×10^8 , 1×10^7 , 1×10^6 , 1×10^5 , and 1×10^4 phage units/ml. To the plain broth-phage dilutions were added 1.0 ml. of a growing staphylococcus suspension containing 12.5×10^7 bacteria/ml. The manganese-phage mixtures were similarly seeded employing a staphylococcus culture growing in manganese broth. All the tubes were shaken in the 36°C. water bath and turbidity measurements were made at intervals as described in a previous paper (1).

C. The Effect of Manganese on Phage Production and on Phage Distribution.—To 35 ml. of broth there was added 5.0 ml. of phage solution containing the proper concentration of phage units/ml. and 10.0 ml. of a broth culture of growing staphylococci containing 12.5×10^7 bacteria/ml. An identical set-up was prepared at the same time using manganese broth, phage diluted in manganese broth, and

bacteria growing in manganese broth. Samples for the determination of total phage/ml. and extracellular phage/ml. were taken at half-hour intervals. The bacterial growth curves and lytic curves were followed by the turbidity method mentioned above. All dilutions for phage titration were kept in ice water until completion of the experiment when all were titrated at once. An experiment of this sort is shown in Fig. 2. In this particular case 5.0 ml. of phage suspension containing 1×10^7 phage units/ml. was added to 35.0 ml. of broth together with 10.0 ml. of bacterial suspension. The final phage concentration therefore was 1×10^6 phage units/ml.

D. Determination of Lytic Threshold.—The lytic threshold in growing mixtures was determined from the accumulated experimental data of the distribution experiments. For the threshold in the absence of bacterial growth, bacteria were grown in plain broth and manganese broth. When $[B] = 8 \times 10^8$ the suspension was iced for 15 minutes to stop growth. Mixtures with various dilutions of plain phage and similar dilutions of phage in manganese broth were then made and the tubes were iced 0.5 hour. The mixtures were transferred to the 36°C. water bath and turbidity measurements were made every 0.1 hour.

DISCUSSION

It has long been known that dilute solutions of certain electrolytes will stimulate the action of enzymes. Falk (10) showed that dilute solutions of $MnSO_4$, $MnCl_2$, $MgSO_4$, $CaCl_2$, and $BaCl_2$ increased the activity of castor bean lipase toward ethyl butyrate. Calcium and magnesium salts accelerate tryptic digestion and the neutral salts of strong monobasic acids enhance the activity of salivary amylase (11). Aluminum sulfate and monophosphates in dilute solution have been found to stimulate enzyme activity (12); potassium bromate in low concentrations stimulates the digestion of casein by trypsin (13). In some cases the mechanism of the stimulating action of electrolytes on enzymes has been worked out. For example, the chlorides of the alkaline earths stimulate the hydrolytic action of pancreatic lipase and Pekelharing (14) has shown that the action probably consists in saponification of the fatty acids thereby removing them from the system and allowing hydrolysis to proceed. Not only is it possible to stimulate the activity of enzymes with salts but their presence is also capable of increasing enzyme production by microorganisms (15).

The experiments performed in connection with the accelerating effect of manganous salts on phage action have shown that it is not due to a stimulation of bacterial growth nor to an enhancement of

phage formation. There is a clear-cut lowering of the lytic threshold and also a change in the distribution of phage between the bacterial cell and its environment. The Mn^{++} ion increases the extracellular fraction at the expense of the phage fraction associated with the cell. In previous work it was not possible to determine which of the two

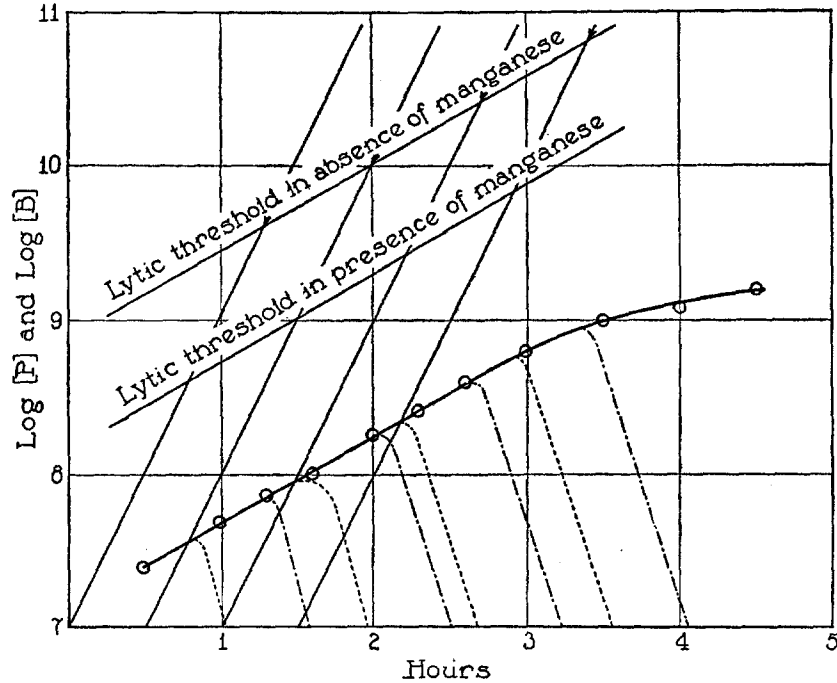


FIG. 3. Graphic representation of bacterial growth, phage production, and lytic ratios with and without $MnCl_2$. Originating in the bacterial growth curve (○) are the curves of bacterial lysis (-----) with $MnCl_2$ and adjacent broken lines (-----) the identical mixtures without $MnCl_2$. Above are two lines paralleling the logarithmic phase of the growth curve; both are crossed by a series of steep curves representing phage formation for various initial phage concentrations. The intercepts of these latter lines with the two parallel lines indicate attainment of the lytic phage/bacteria ratios requisite for lysis. At corresponding time intervals on the curve for bacterial growth the curves of lytic destruction of bacteria begin. With Mn^{++} present the total phage concentrations at lysis are constantly one-tenth of those prevailing in its absence, predicting that for any given initial [phage] in a growing mixture of phage and bacteria, the presence of Mn^{++} will reduce the end titre to one-tenth that of a control mixture.

fractions, intracellular or extracellular phage, is requisite for lysis. The conclusion was reached that the significant condition for the occurrence of lysis is attainment of either a particular concentration of phage inside each bacterium or a certain concentration of phage in each ml. of surrounding solution; these two quantities are always in constant ratio to each other and it was not possible to invoke either fraction alone under equilibrium conditions.

In the manganese mixtures lysis occurs when total phage/bacterium = 12 as compared with a total phage to bacteria ratio of 54 in controls. In the present experiments the intracellular phage/bacteria ratios and extracellular phage/bacteria ratios at lysis for various mixtures of phage and bacteria were not constant. The essential ratio for initiation of lysis seems to be total phage/bacteria, that is, intracellular + extracellular phage per bacterium.

The essential features of manganese acceleration are graphically shown in Fig. 3. In this plot the bacterial growth rate is seen to be identical with and without manganese. Similarly the rate of phage production is not changed by Mn^{++} nor is the rate of bacterial destruction altered once lysis begins. The reduced threshold for lysis is represented by a line paralleling the logarithmic portion of the growth curve. Intercepts of the phage curves with this line indicate points at which the critical ratio of phage to bacteria requisite for lysis is attained and the bacterial lytic curves take their origin from the growth curve at these points. In the absence of manganese, bacterial growth and phage production continue until about ten times more phage has been produced, when lysis ensues. The lytic threshold in the absence of manganese is represented by a line paralleling the growth curve and connecting points on the phage production curves which represent the critical ratios of phage to bacteria essential for lysis. From this plot it is apparent that for any given initial concentration of phage the end titre in the manganese-containing mixture after lysis will be one-tenth that developed in the control.

SUMMARY AND CONCLUSIONS

Dilute solutions of $MnCl_2$ or $MnSO_4$ accelerate the lytic effect of phage upon susceptible staphylococci. Under the conditions of our

experiments the manganese-containing mixtures lysed regularly 0.5 hour sooner than the controls.

The effect is shown to be due to a lowering of the lytic threshold, *i.e.* the quantity of phage/bacterium requisite for lysis; Mn^{++} reduces the ratio from 54 to about 12. In the presence of Mn^{++} phage distribution is altered and in growing phage-bacteria mixtures the extracellular phage concentration is increased by manganese to approximately 4 times that occurring in the absence of manganese. There appears to be no enhancement of phage formation nor any affect on the rate of bacterial growth. As would be anticipated, for any given initial phage concentration the end titre after completion of lysis is less in the presence of manganese than in its absence. This is due to the reduced lytic threshold produced by Mn^{++} , there consequently being less phage needed to bring about lytic destruction of the bacteria.

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