THE REVERSIBLE INACTIVATION OF BACTERIOPHAGE BY BICHLORIDE OF MERCURY

By A. P. KRUEGER and D. M. BALDWIN

(From the Department of Bacteriology, University of California, Berkeley)

(Accepted for publication, November 20, 1933)

We have shown (1) that the inactivation of antistaphylococcus bacteriophage by 1:10,000 $HgCl_2$ (0.01 per cent or M/2720) at 22°C. in infusion broth adjusted to pH 7.6 proceeds according to the equation: $dP/dt = K[HgCl_2]\,[P_o - P_i]$ over the range studied, where P = [phage] in activity units, P_o = initial phage/ml., and P_i = phage/ml. inactivated in time t.

It was further demonstrated in these experiments that if the inactivated suspension were exposed to the action of H_2S when only some 5 per cent of the original phage remained active the initial titre of the phage suspension was restored. Control experiments indicated that the restoration of activity occurring upon precipitation of Hg^{++} ions was due not to any propagation of residual active phage but rather to a reactivation of the phage originally inactivated.

The question arose as to the influence of the time-concentration factor on the inactivation process; *i.e.*, whether prolonged exposure to Hg^{++} ions or the use of high $HgCl_2$ concentrations would irreversibly inactivate phage. The present experiments were conducted with this in mind.

The general procedure was to expose standard phage suspensions containing 1×10^{10} activity units/ml. (2) to the action of various concentrations of $HgCl_2$ for given lengths of time. An aliquot of each suspension was then titrated for residual active phage while the remainder was treated with H_2S to precipitate Hg^{++}. The excess H_2S was removed by thorough aeration and the phage concentration of the reactivated suspension determined. Details of the methods and the experimental controls employed are described in our earlier paper (1).

A. Inactivation

Table I is a summary of eleven experiments. It is evident from the results in Experiments 1–6 that inactivation progressing for long periods of time does not follow the previously recorded inactivation curve which holds for short periods. According to calculations based on the experimentally determined value of K for the equation $k = \frac{1}{t[HgCl_2]} \cdot \ln \frac{P_o}{P_o - P_i}$ when $[HgCl_2] = 1/10{,}000$ (0.01 per cent), all the phage should be inactivated at 4.4 hours. Actually, however, traces of active phage can be detected even after 268 hours. These residual concentrations are beyond the range of titration; for not less than 1×10^4 activity units/ml. can be measured accurately on this scale. It is not possible then to follow the remainder of the reaction quantitatively and it can merely be said that while the inactivation process fits the curve for a pseudomonomolecular reaction until the amount of active phage remaining is less than 0.5 per cent of $[P]_o$, the reaction beyond this point apparently proceeds more slowly than would be anticipated.

B. Reactivation

Reference to Table I shows that 100 per cent yields resulted when reactivation was performed even after exposure of phage to $\frac{1}{2}$ saturated $HgCl_2$ (2.8 per cent) for 216 hours. In certain earlier experiments, not reported here, we were unable to effect a 100 per cent reactivation if $[HgCl_2]$ were very high and found that to accomplish this end it was necessary to dilute the $HgCl_2$-phage mixture sufficiently (*e.g.* 1:1,000) before precipitating the Hg^{++}. When this precaution was followed the entire original quantity of phage could be recovered (*cf.* Experiments 9, 10, and 11).

C. Test for an Activator

It has been suggested that the phenomenon of phage inactivation and reactivation involves some hypothetical activator specific for phage rather than the lytic principle itself. Upon this basis it would be assumed that the reversible inactivation of phage represents a reaction between Hg^{++} and the activator, the phage being altogether unaffected by the bichloride. Against such a postulate are two facts,

first that no one has demonstrated the existence of an activator for phage and, second, that the entire phenomenon of bacteriophagy is explicable as a reaction between a single substance, phage, and the bacterial cell. Accordingly, there would be no necessity at this time to complicate the facts further by picturing phage as consisting of two

TABLE I

Reactivation of Antistaphylococcal Phage after Exposure to $HgCl_2$ by Precipitation of Hg^{++}

All experiments in infusion broth at 22°C. pH 7.6.

No.	[$HgCl_2$]	Reactivated after	Initial [phage]	Residual [phage] after inactivation	[Phage] after reactivation	Phage recovered
		hrs.				*per cent*
1	1/10,000 0.01 per cent	24	5×10^9	5×10^7	4×10^9	80
2	1/10,000 0.01 per cent	48	5×10^9	3×10^5	5×10^9	100
3	1/10,000 0.01 per cent	48	5×10^5	$1 \times 10_5$	4.5×10^9	90
4	1/10,000 0.01 per cent	72	5×10^9	3×10^4	5×10^9	100
5	1/10,000 0.01 per cent	168	5×10^9	Trace	5×10^9	100
6	1/10,000 0.01 per cent	268	5×10^9	Trace	5×10^9	100
7	1/2,000 0.05 per cent	48	5×10^9	Trace	4.8×10^9	96
8	1/2,000 0.05 per cent	120	5×10^9	Trace	5.2×10^9	104
9	1/36 2.8 per cent	50	5×10^9	None	4.5×10^9	90
10	1/36 2.8 per cent	50	5×10^9	None	5×10^9	100
11	1/36 2.8 per cent	216	5×10^9	None	4.7×10^9	94

components. Certainly the tendency to analyze biological phenomena by invoking different reactants for each stage of a reaction has not produced happy results in the past.

To seriously rule out the existence of an activator for phage is a formidable experimental task and for the present it seemed adequate

merely to attempt its detection by certain simple obvious tests. These have been conducted by assuming that the hypothetical activator has a particle size different from that of phage or that it is more heat-resistant than phage.

Experimental.—Phage was filtered through an acetic collodion membrane (3) of the minimal pore size permitting it to pass without reduction in titre. If the activator particles were larger than phage one would expect them to be retained by the membrane. The primary filtration was followed by filtration with five separate 10 cc. amounts of broth and the membrane was then triturated in broth to put in suspension retained activator particles. This fraction was called No. 1.

Similarly phage was filtered through an acetic collodion membrane (3) of maximal pore size retaining phage completely. Should the activator be smaller than phage the filtrate (fraction 2) should contain it.

It has been shown (4) that a temperature of 60°C. acting for 1 hour will completely inactivate phage. Were the activator more resistant to temperature than phage it should survive this treatment. Accordingly phage was heated to 60°C. for 1 hour under the conditions specified in the earlier paper (4) and after cooling, it was designated fraction 3.

To test for the presence of an activator in the above three fractions phage was partially inactivated by exposing it to 0.01 per cent $HgCl_2$ for 0.4 hour. Samples for titration were diluted 1:1,000 and 1:10,000 with broth, in order to determine the residual concentration of "activated" phage. Identical samples were diluted 1:1,000 with each of the three fractions to be tested for the presence of the activator. If such an activator were present it would be expected to replace that destroyed by $HgCl_2$. However, the titration figures were identical in each case indicating the absence of an activator under the conditions obtaining in these tests.

DISCUSSION

The accepted mechanism of action of bichloride upon bacterial cells involves a twofold effect: first, a preliminary stunning of the growth function, and, second, with increasing time-concentration values, a profound lethal action. It has been demonstrated that in certain instances the primary toxic effect can be reversed by precipitation of

the Hg ions (5). The second phase of the reaction, however, cannot be reversed.

This sequence of events apparently does not take place when $HgCl_2$ acts upon phage. That is, within comparable limits of concentrations and time the bichloride-phage reaction is completely reversible while the bichloride-bacterium reaction is only partially reversible. Unfortunately one difficulty presents itself in comparing available data concerning the two reactions; the reports on reversible disinfection of bacteria employ a qualitative criterion of survival in judging the efficacy of reversal procedures, and reversal is said to have occurred if any fraction of the treated bacterial suspension, no matter how small, is capable of growth. There is nothing to suggest in such end-point experiments that reactivation has taken place uniformly throughout the bacterial population, whereas in our phage experiments we not only have demonstrated the qualitative phenomenon of reversal but have shown that *all* the inactivated phage could be reactivated.

Admitting this incongruity of data, there is still sufficient analogy to make comparison interesting. For example, Gegenbauer (6) found that *S. aureus* cultures exposed to 1:2,000 $HgCl_2$ (0.05 per cent) contained viable survivors after 1.3 hours but were altogether nonviable after 2.3 hours. When exposure was followed by treatment with H_2S, survivors were present after 72 hours in $HgCl_2$ but not after 101 hours. Chick's results with *B. paratyphosus* (5) are of the same general order. With phage 100 per cent of the original lytic principle can be recovered after 120 hours exposure to the identical concentration of bichloride.

Even when the very resistant spores of *B. anthracis* are employed as the test organism and the same end-point technique is followed, bacterial protoplasm is found to be irreversibly damaged by high concentrations of Hg^{++}. Thus Müller (7) reported the following experimental data:

Anthrax Spores Treated with $HgCl_2$ at 37°C.

Concentration $HgCl_2$	Growth when antidote was added after	No growth when antidote was added after
per cent	*days*	*days*
0.1	9	12
1.0	7	9
2.0	7	9
3.0	6	7

In contrast phage can be reactivated with a 100 per cent yield after 9 days in 2.8 per cent of $HgCl_2$.

The experimental evidence then sets phage apart from bacterial protoplasm whether the latter is a vegetative cell or a resistant spore form, in that a single reagent, bichloride, acting upon these two substances appears to evoke fundamentally different reactions. It might be said, of course, that just as anthrax spores are more resistant to the final lethal action of $HgCl_2$ than vegetative cells in general, so phage is simply a still more resistant form of the same elementary protoplasm and that analogy is incomplete merely because we have not examined all existing forms of bacterial life from the standpoint of inactivation and reactivation. This conclusion is theoretically as tenable as another which could be drawn from the experimental data; namely, that the difference between the bichloride-bacterium reaction and the bichloride-phage reaction speaks for a significant difference between bacterial protoplasm and the substance we call phage. Here analogy would place phage with the enzymes, for there are recorded numerous instances of *complete* reactivation of enzymes after inactivation with a variety of ions (8–11).

In the light of present knowledge one cannot conclusively select one hypothesis to the absolute exclusion of the other. It can merely be said that if phage consists of small corpuscles of protoplasm, as some believe, it is a unique form of this substance, infinitely less susceptible to the toxic effects of $HgCl_2$ than the most durable forms so far investigated.

CONCLUSIONS

1. The complete inactivation of antistaphylococcal phage by $HgCl_2$ (2.8 per cent for 216 hours) can be reversed by precipitation of Hg^{++} with restoration of the phage to its original titre.

2. This behavior seems more compatible with the known properties of certain enzymes than with those of living protoplasm.

BIBLIOGRAPHY

1. Krueger, A. P., and Baldwin, D. M., *J. Gen. Physiol.*, 1933–34, **17**, 129.
2. Krueger, A. P., *J. Gen. Physiol.*, 1929–30, **13**, 557.
3. Krueger, A. P., and Ritter, R. C., *J. Gen. Physiol.*, 1929-30, **13**, 409.
4. Krueger, A. P., *J. Gen. Physiol.*, 1931–32, **15**, 363.

5. Chick, H., *J. Hyg.*, 1908, **8**, 92.
6. Gegenbauer, V., *Arch. Hyg.*, 1921, **90**, 23.
7. Müller, A., *Arch. Hyg.*, 1920, **89**, 363.
8. Marston, H. R., *Biochem. J.*, London, 1923, **17**, 851.
9. Euler, H. V., and Svanberg, O., *Fermentforschung*, 1919–20, **3**, 330; 1920–21, **4**, 29, 90, 142.
10. Hata, S., *Biochem. Z.*, Berlin, 1909, **17**, 156.
11. Kehoe, R. A., *J. Lab. and Clin. Med.*, 1922, **7**, 736.