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THE ACTION OF 2:7-BIS(2'-DIHYDROGLYOXALINYL)-9-PHENYLPHENANTHRIDINE ON A BACTERIOPHAGE OF *PSEUDOMONAS PYOCYANEA*

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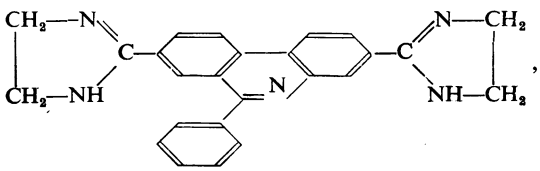
During investigations into virus chemotherapy, a number of phenanthridines were found active against bacteriophage Pb of *Pseudomonas pyocyanea* (Dickinson, Chantrill, Inkley, and Thompson, 1953). One of these, No. 1367, 2 : 7-bis(2'-dihydroglyoxalinyl)-9-phenylphenanthridine trihydrochloride dihydrate,



, $3HCl,2H_2O$

was chosen for more detailed investigation. Some preliminary results were reported by Dickinson and Codd (1952). They found that 1367 prevented multiplication of phage Pb at 0.01 mg./ml., but allowed host growth at 1 mg./ml. They considered the activity to be too great to be due to the slight action of 1367 on free phage Pb. The work reported here extends their study to: (i) the action of 1367 on the adsorption of phage Pb on its host, (ii) the effect on intracellular phage when No. 1367 is added at different times during the latent period, and (iii) antagonists of the drug.

METHODS

Strain C10 of *Ps. pyocyanea* (Dickinson, 1948) was used as host for phage Pb. It was maintained by daily subcultures in nutrient broth. Monthly subcultures were made from nutrient agar slopes stored at 5° C.

The stock of Pb phage was prepared by inoculating 100 ml. nutrient broth with 1 ml. 24 hr. broth culture of strain C10 and 10^4 phage particles. After incubating 24 hr. at 37° C. it was filtered through a gradacol membrane of porosity 1 μ. The final titre was 4×10^9/ml. For use it was diluted 10^{-2} in Ringer's solution and stored at 5° C.

The medium of Dickinson (1948) was used; it was termed medium B. It consisted of NaCl, 5 g.; $(NH_4)_2HPO_4$, 1 g.; KH_2PO_4, 1 g.; $MgSO_4.7H_2O$, 0.2 g.; lactic acid, 2.4 g.; casein hydrolysate (vitamin free), 1 g.; water, 1 litre. The *p*H was adjusted to 7.4 with NaOH and the medium sterilized by autoclaving at 15 lb./sq. in. pressure for 15 min. The final *p*H was 7.2.

Titration of Bacteriophage.—1 ml. of a 24 hr. broth culture of strain C10 was added to 1 ml. phage in a tube, followed by 3 ml. nutrient agar. After mixing the contents the tubes were "sloped" and incubated at 37° C. Counts were made after 18 hr. They were always approximately one-quarter of those made on plates. The reduction was probably due to the greater depth of agar in the tubes, as plaques were only observed on the agar surface.

Phage Multiplication.—The method for investigating phage multiplication was essentially that of Dickinson and Codd (1952). A 24 hr. culture of strain C10 on medium B was mixed with phage to give 10^8 viable bacteria and 10^6 phage particles/ml. After time for adsorption (5 min. when adsorption was in medium B, 10 min. when in Ringer's solution), the mixture was diluted in medium B and distributed in tubes to give 20–40 plaque-forming particles/tube. With this number, sufficient adsorbed phage was present to give fairly uniform counts in different tubes after a burst had occurred. This reduced the number of replicate tubes needed to give significant results. The action of No. 1367 could be investigated by adding it to the adsorption medium or to the tubes after adsorption and dilution.

This method was originally designed for latent period experiments and had not the sensitivity of the classical system of 1 phage/1 bacterium/tube of Ellis and Delbrück (1939). In particular it could not distinguish between reduction in phage yield due to reduced burst size and that due to fewer bursts. Such distinction, though, was not important in this work, and the disadvantage was greatly outweighed by economy in apparatus and labour.

Except for latent period measurements counts were made at only two times—once during the latent period and once after the rise period had ended. All times were measured from the moment when phage started to grow on the host, i.e., from mixing phage and host when adsorption was in medium B, and from dilution into medium B when adsorption was in Ringer's solution.

RESULTS

Action on the Host.—Dickinson and Codd (1952) could find no action of No. 1367 on the growth rate of young (<12 hr.) cultures of strain C10 when tested at a concentration of 0.1 mg./ml. No attempt was made to repeat this work in detail.

Action on Phage

Active Concentration of No. 1367.—A preliminary test was made by the serial dilution method of Chantrill, Coulthard, Dickinson, Inkley, Morris, and Pyle (1952). No. 1367 allowed host growth at 1 mg./ml. and stopped growth of phage Pb at 0.005 mg./ml. in synthetic medium. A latent period experiment was carried out with No. 1367 present throughout at concentrations from 0.2 mg./ml. to 0.025 mg./ml. At 0.1 mg./ml. there was neither phage multiplication nor much phage loss (Fig. 1).

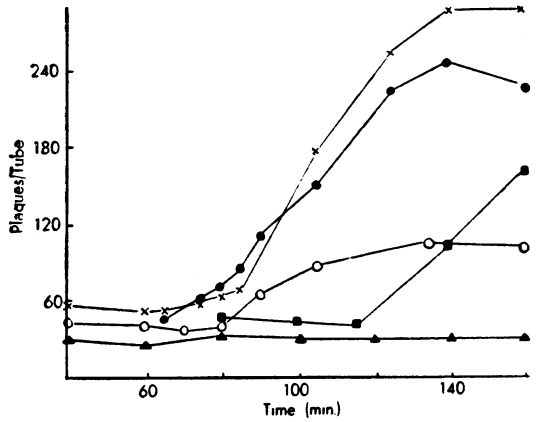


FIG. 1.—Effect of No. 1367 on the latent period of phage Pb. ×——× In absence of No. 1367. ▲——▲ No. 1367 present throughout. ■——■ No. 1367 initially present, diluted out at 60 min. O——O No. 1367 added at 7 min. ●——● No. 1367 added at 60 min.

For this concentration the fivefold dilution in nutrient broth and agar when sloping the tubes was sufficient to allow plaques to appear. This concentration was used for the rest of the work reported here.

Action on Free Phage.—An exposure of 2½ hr. to 0.1 mg./ml. of No. 1367 reduced the phage count to about two-thirds the initial level (Table I). This agreed with the results of Dickinson and Codd (1952) when allowance was made for the 24 hr. exposure used by them. If No. 1367 was absent the phage count increased fivefold in 2½ hr. in a latent period experiment (Fig. 1), so the total suppression of multiplication could not be due solely to action on free phage.

TABLE I

ACTION OF NO. 1367 AND GUANOSINE ON FREE Pb PHAGE

Compound	Concentration (mg./ml.)	Phage Count at 0 hr.	Phage Count at 2½ hr.
—	—	134, 154, 169	133, 114, 162
Guanosine ..	0·5	205, 221, 229	173, 173, 173
No. 1367 ..	0·1	116, 139, 159	79, 79, 95

Action on Adsorption (Table II).—0.1 mg./ml. of No. 1367 was present during adsorption. After adsorption it was diluted to one-thousandth of the minimum active concentration, before distribution in tubes, to prevent any further action on the phage. The phage yield at 2½ hr. was reduced by this treatment, but a burst still occurred. Consequently, though the reduction might be due to a direct action on adsorption, there must have been some action on intracellular phage. The control, in which the drug was present only after dilution, gave very variable results in different experiments. Its behaviour is discussed in detail later.

TABLE II

ACTION OF NO. 1367 ON THE ADSORPTION OF Pb PHAGE ON TO ITS HOST

Concentration of No. 1367, 0·1 mg./ml.

No. 1367 During Adsorption	No. 1367 After Adsorption	Count at 20 min.	Count at 2½ hr.
—	—	37, 37, 29	286, 290, 237
+	—	24, 32, 27	191, 187, 183
+	+	22, 22, 27	11, 13, 9
—	+	27, 33, 20	109, 130, 123

Attempts at Antagonism.—The following nucleic acid derivatives were used in attempts to antagonize No. 1367 in one-step growth experiments:

Adenine, guanine, thymine, uracil;
adenosine, guanosine, cytidine;
adenylic acid, guanylic acid, cytidilic acid;
yeast and thymus nucleic acids (B.D.H.).

The nucleosides and nucleotides were ribose derivatives. The compounds were prepared as 1:200 solutions or suspensions in medium B, *p*H 7. Each was tested at a concentration of 0.5 mg./ml. by addition to adsorption medium, medium after dilution, or both, in presence and absence of No. 1367. The nucleic acids were also tested at a concentration of 2 mg./ml., as they (but not the other compounds) antagonized No. 1367 in a serial dilution test at this concentration (Dickinson, personal communication).

Only yeast and thymus nucleic acids at 2 mg./ml. antagonized the phenanthridine ($p<0.05$), and then

only when nucleic acid and phenanthridine were present together. Thymus nucleic acid (Table III) was much more effective than yeast nucleic acid. Under these conditions yeast nucleic acid gave a precipitate with No. 1367. None was observed with thymus nucleic acid, but this may have been due to masking by the initially turbid solution. In absence of No. 1367 thymus nucleic acid reduced phage yield slightly (Table III). No further work has been done on this.

TABLE III

EFFECT OF NUCLEIC ACIDS AND NO. 1367 ON THE YIELD OF Pb PHAGE

Concentration of No. 1367, 0·1 mg./ml.; of nucleic acid, 2 mg./ml.

Additions to Adsorption Medium	Additions to Dilution Medium	Phage Count at	
		20 min.	2½ hr.
—	—	37, 36, 38	148, 158, 161
—	Thymus nucleic acid	39, 32, 24	128, —. 135
No. 1367 ..	—	25, 22, 25	171, 132, 121
,, ,, ..	Thymus nucleic acid	18, 17, 19	97, 98, 108
,, ,, ..	No. 1367	26, 20, 27	17, 21, 22
,, ,, ..	No. 1367 and thymus nucleic acid	37, 35, 20	112, 97, 106
—	—	37, 41, 47	314, 325, 333
—	Yeast nucleic acid	37, 31, 33	295, 292, 248
No. 1367 ..	—	42, 52, 51	228, 248, 218
,, ,, ..	Yeast nucleic acid	45, 38, 41	190, 213, 245
,, ,, ..	No. 1367	21, 37, 18	23, 27, 18
,, ,, ..	No. 1367 and yeast nucleic acid	29, 32, 21	65, 66, 48

Guanosine and adenosine both reduced phage yield when present after dilution if No. 1367 was absent, guanosine showing the effect more clearly. The effect was more obvious after adsorption in No. 1367, but also occurred after adsorption in its absence (Table IV). Under the conditions used guanosine had no effect on free phage Pb (Table I). The action of adenosine was not investigated.

TABLE IV

EFFECT OF GUANOSINE AND NO. 1367 ON THE YIELD OF Pb PHAGE

Concentration of No. 1367, 0·1 mg./ml.; of guanosine, 0·5 mg./ml.

Additions to Adsorption Medium	Additions to Dilution Medium	Count at	
		20 min.	2½ hr.
—	—	49, 46, 37	384, 347, 307
—	Guanosine	56, 49, 38	323, 305, 356
No. 1367 ..	—	49, 49, 50	213, 241, 226
,, ,, ..	Guanosine	41, 48, 47	180, 188, 178
,, ,, ..	No. 1367	25, 25, 31	13, 23, 14
,, ,, ..	No. 1367 and guanosine	25, 32, 26	22, 17, 18

Action on Intracellular Phage.—In the above work medium B had been used as the adsorption medium. Phage particles adsorbed at the beginning of a 5 min. adsorption period would have undergone 5 min. development before No. 1367 was added after dilution; this would mask any action it had on the early stages of multiplication. To overcome this, adsorption was carried out with starved host cells suspended in Ringer's solution. (Benzer (1952) had previously shown that T_2 phage adsorbed on starved cells of *Escherichia coli B* underwent very little development.) To increase sensitivity a further modification was introduced. After adsorption the host-phage mixture was diluted 10^{-3} into medium B, giving ~4,000 particles/ml. No. 1367 was added to this. The mixture was diluted to 20–200 particles/ml. immediately before sloping. This treatment gave many more bursts/tube and so reduced variation due to differences in burst size and distribution of bursts among the tubes.

When 0.1 mg./ml. of No. 1367 was added less than 3 min. after the end of adsorption, there was no phage multiplication. When it was added between 3 min. and 12 min. phage yield increased rapidly with time of addition of the phenanthridine. From 12 min. to 80 min. the yield was fairly constant and considerably less than that of controls (Fig. 2).

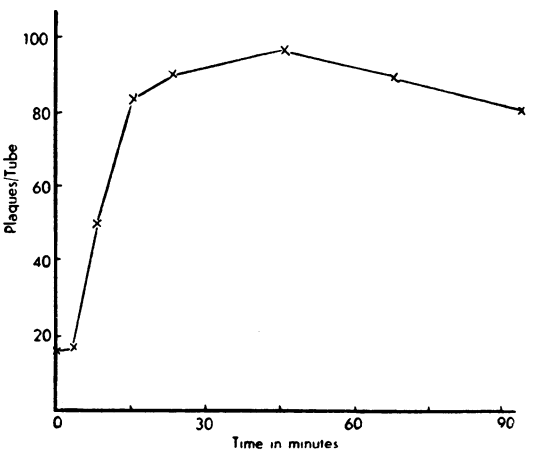


FIG. 2.—Variation in yield of Pb phage at 150 min. with time of addition of No. 1367. Initial count was 23/ml. Count at 150 min. in absence of No. 1367 was 120/ml.

Action on Latent Period.—When 0.1 mg./ml. of No. 1367 was added at 7 min. and then the latent period measured, no difference from controls was found (Fig. 1), so the effect of No. 1367 under these conditions was directly on phage multiplication. For addition between 12 min. and 80 min. this seemed unlikely. Premature lysis of infected cells, as had been found by Hotchin (1951) for some acridines on staphylococcus phage K, appeared more probable. This was confirmed (Fig. 1) for addition of No. 1367 at 60 min.

Effect of Diluting Out.—Adsorption was carried out in presence of 0.1 mg./ml. of No. 1367 and the host-phage mixture diluted into medium B + No. 1367. At 60 min. the drug was diluted out and then the latent period measured. It was about 50 min. longer than that of a control omitting the drug (Fig. 1).

Discussion

The principal action of No. 1367 clearly is exerted in the first three minutes after infection, though there is a less complete effect on later stages of development. Similar results (unpublished work) have also been found for T_1 and T_2 phages of *Esch. coli B*. Its action is very different from that of proflavine on the T phages of *Esch. coli B* (Foster, 1948), where only a very late stage in phage synthesis is inhibited. The results allow no definite conclusions on the mechanism of action of No. 1367. They suggest that it interferes with reorganization of the infecting phage rather than with the synthesis of new phage material.

The antagonism of No. 1367 by yeast and thymus nucleic acids is not considered to imply that No. 1367 acts by preventing nucleic acid synthesis. It seems more likely that they combine with the phenanthridine and lower its effective concentration below the limit of activity.

The reduction in yield of phage Pb by guanosine was unexpected, and no explanation is offered for it. It is perhaps interesting, though, that guanosine has recently been found to act on *Esch. coli B* as an antimutagen (Novick and Szilard, 1952).

Summary

1. 2:7-Bis(2′-dihydroglyoxalinyl)-9-phenylphenanthridine trihydrochloride (No. 1367) had little action on free phage Pb or on the adsorption of phage Pb on the host bacterium.

2. It prevented multiplication of phage Pb if added during the first three minutes of phage growth.

3. If added when the phage had been growing more than 3 min. the phage yield varied with time of addition.

4. The phenanthridine did not affect the latent period of phage Pb when added at 7 min. It reduced it slightly when added at 60 min.

5. When the phenanthridine was initially present and later diluted out, the latent period of phage Pb was increased.

6. Yeast and thymus nucleic acids antagonized the action of the phenanthridine in one-step growth experiments. The action of thymus nucleic acid was much the stronger. They acted only when nucleic acid and phenanthridine were together. Nucleotides, nucleosides, purines, and pyrimidines tested failed to antagonize it.

7. Guanosine and adenosine reduced the yield of phage Pb in one-step growth experiments. Guanosine had little action on free phage Pb.

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