The Bacteriophages of *Pseudomonas pyocyanea*

2. Bacteriophage Reproduction in an Iridescent Strain

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SUMMARY: Two new compounds inhibited the increase of phage when two phages of *Pseudomonas pyocyanea* were grown on an indicator strain. These compounds also inhibited the appearance of iridescence in a lysogenic strain of this organism.

One iridescent lysogenic strain showed a rise in phage titre at 1½-2 hr., which corresponded to the latent period of the phage when it acted on an indicator strain. Both these increases were inhibited by the compounds. This lysogenic strain also showed a rise in phage titre at 4 hr. but this was unaffected by the compounds. The final phage titre of the lysogenic culture was not affected significantly, unlike the complete and permanent suppression of phage increase when free phage acted on an indicator strain. Cultures and filtrates of three lysogenic strains of *Ps. pyocyanea* showed a great increase in phage titre when assay plates (against an indicator strain) were incubated for a further period after the initial reading at 18 hr.

Many workers have described iridescent strains of *Pseudomonas pyocyanea*. Warner (1950) gave a detailed description and concluded that the iridescent clearing was not caused by bacteriophage action but was a distinct lytic phenomenon, probably the result of bacterial variation. Don & van den Ende (1950) studied thirty strains of *Ps. pyocyanea* with particular reference to the metallic lytic effect and bacteriophage action but the relationship, if any, between these two effects remained obscure. Following an investigation of drug action on the phages of *Ps. pyocyanea* (Dickinson, 1948), an explanation was sought for the lack of action of drugs on phages associated with iridescent cultures of this organism. It was decided to investigate the reproduction of phage in one of these cultures. The present paper reports this work and includes observations which correlate iridescence with bacteriophage action in this particular strain.

The following abbreviations and definitions are used in this paper. C1-C10 are cultures of *Ps. pyocyanea*; Pa, Pb, Px are bacteriophages of *Ps. pyocyanea*; F1-F10 are the filtrates of cultures after filtration through Ford's Sterimats (Seitz type). *Free phage* is phage present in an extracellular form. *Lysogenic bacteria* are bacteria which can perpetuate bacteriophage without the intervention of free phage. *Indicator strain*, for example C10, is a strain sensitive to the phage of a lysogenic strain. Only phages acting on C10 were assayed in this work. Phage giving rise to plaques on C10 in an 18 hr. assay is called 18 hr. phage and phage which only gives clear plaques after 48 hr. is termed 48 hr. phage. *Latent period* is the time interval between infection of a culture by bacteriophage and liberation of phage by that culture. The finely adjusted system of Ellis & Delbrück (1939) with only one phage particle/bacterium/tube was unnecessary for this work since the 'burst size' was not required.

EXPERIMENTAL

Strains. The same strains of *Ps. pyocyanea* were used as by Dickinson (1948), particularly the iridescent cultures C1 and C7X and the non-iridescent cultures C7 and C10. Strain C7X was originally obtained from the spleen of a mouse which had been inoculated intraperitoneally with a mixture of C7 and the filtrate of C1. Strain C1 was later found to yield two bacteriophages, named Pa and Pb. C10 was the indicator strain used to assay Pa, Pb and also Px, the phage in filtrates of C7X.

Media. The chemically-defined medium consisted of: lactic acid, 2.4 g.; NaCl, 5 g.; $(\text{NH}_4)_2\text{HPO}_4$, 1 g.; KH_2PO_4 , 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; distilled water 1 l., pH 7.0 (Dickinson, 1948). Better growth was obtained when 1 % casein hydrolysate (vitamin free) was added.

Titration of bacteriophage. One ml. volumes of serial dilutions of the preparations in Ringer's solution were added to 0.5 ml. of a 24 hr. nutrient broth culture of C10; 3 ml. melted nutrient agar were added and the mixture 'sloped' and incubated for 18 hr. *only* (this time is particularly important in view of later work). Plaques were then counted.

Investigations into bacteriophage reproduction

Latent period of phages Pb and Px on C10. Preparations of Pb or Px containing about 10^6 phage particles/ml. were mixed in broth with a suspension of C10 containing about 10^8 viable bacteria/ml. This solution was diluted so that the final dilution bottle contained 10–20 phage particles/ml. and 1 ml.

Table 1. *Latent periods of phage Pb on Ps. pyocyanea strain C10 in nutrient broth and in Ringer's solution*

Time (min.)	Phage count/tube (triplicate readings)					
	In broth			In Ringer's solution		
0	6	9	12	1	6	3
15	7	13	7	6	9	0
30	3	6	10	18	4	3
45	10	16	5	31	23	19
60	13	8	7	9	11	10
75	7	8	22	6	8	14
90	—	18	23	10	10	7
105	66	82	80	17	8	15
135	86	60	122	14	22	22
180	82	116	109	10	10	17

volumes were then distributed in a series of tubes, which were incubated in a water-bath at 37°. At intervals, duplicate or triplicate sample tubes were removed and assayed for phage (against C10, as above) in the sample tube. When high counts were expected the 1 ml. volumes were diluted before the addition of the indicator culture. A typical result to show the variance encountered on counts, before and after the rise in titre, is shown in Table 1. Using the same technique, it was found that T2 phage of *Escherichia coli*

gave a value for the latent period of 20–25 min., compared to 21 min. quoted by Delbrück (1946).

Culture C7X. A 24 hr. culture of strain C7X was diluted so that the required number of 18 hr. phage particles (i.e. phage particles revealed by the 18 hr. assay described above) were in the sample tubes in 1 ml. volumes. Assays against C10 were made at intervals; the assay tubes therefore contained a small number of C7X host cells.

Bacterial counts were made by the pour-plate method, using nutrient agar; colonies were counted after 2 days' incubation.

Testing of drugs. The methods of Dickinson (1948) were employed when testing compounds for the inhibition of iridescence on nutrient agar plates. Certain compounds were also tested for their effect on the growth of bacteriophage; the methods used were as above, the compounds having been previously incorporated in the final dilution bottle at a concentration known to permit good host growth.

RESULTS

Description of strain C7X of Pseudomonas pyocyanea and its phage, Px

There is evidence that *Ps. pyocyanea* strain C7X was produced *in vivo* from C7 and F1 (the filtrate from the iridescent C1 strain containing at least two distinct phages, Pa and Pb). Filtrates from C7 did not act on any of our

Table 2. *Neutralization of Pa, Pb and Px phages by homologous and heterologous antisera.*

Sera	Number of phage particles neutralized by 1 ml. serum		
	Pa	Pb	Px
Anti-Pa	1,000–10,000	< 10	1,000
Anti-Pb	< 10	1,000,000	< 10
Anti-Px	10,000–100,000	< 10	10,000– 1,000,000
Normal serum	< 10	< 10	< 10

Equal volumes of inactivated sera dilution 1/10 and serial tenfold dilutions of the phage preparations were incubated for 30 min. at 37° and then assayed for phage. Only approximate counts were made, i.e. < 10, 10–100 (hence the values in the tables).

strains of *Ps. pyocyanea* (Dickinson, 1948). Serologically Px (the phage in filtrates of C7X) appeared very similar to Pa but quite unrelated to Pb in neutralization tests. Thus, rabbit antiserum against Pb phage did not neutralize Pa or Px phages, whereas antisera against Px or Pa phage were equally effective against either of these two phages, but were inactive against Pb phage (Table 2). Plaques of Px and Pa on C10 were very similar in appearance. Neither Px, Pa nor Pb gave rise to an iridescent culture when grown on C10. Unfortunately, C7 was a very slowly growing strain and gradually died out. In these circumstances one can dispute the origin of C7X but it was quite distinct from any of our other strains. It produced a green, but not a blue-green, pigment and all colonies examined gave rise to the metallic sheen; typical colonies are shown in Pl. 1, fig. 1.

Latent periods of phages Pb and Px on strain C10

Phage Pb, originally obtained from C1, had been used as a test virus in chemotherapy work so that much information was available regarding its growth on C10. Table 1 shows that, in nutrient broth, there was a marked rise in phage titre between $1\frac{1}{2}$ and $1\frac{3}{4}$ hr. There was no increase in phage when the test was carried out in Ringer's solution. In lactate medium, with or without 1% casein hydrolysate, the latent period was also about $1\frac{1}{2}$ –2 hr. Phages Pa and Px behaved similarly to Pb; results for Px are given in Fig. 1. Their long latent periods are possibly explained by a slow rate of adsorption since, under the same conditions in broth, the quickly adsorbed T2 phage of *Esch. coli*. gave a latent period of 20–25 min. whereas the slowly adsorbed T1 and T5 phages took much longer than the times quoted by Delbrück (1946). Attempts to find the adsorption time by centrifugation and the use of homologous anti-sera were not successful.

Bacteriophage reproduction in strain C7X

Pa, Pb and Px phages when grown on strain C10 in either nutrient broth or defined medium regularly gave 10^9 to 10^{10} phage particles/ml. However, C7X never yielded phage preparations with as many as 10^8 particles/ml. when counted, filtered or unfiltered, by an 18 hr. assay against C10; the host colony count was of the order of 10^9 /ml. Strain C7X was therefore investigated: (i) in the presence of initial 18 hr. phage, and (ii) at a dilution which eliminated 18 hr. phage but not host cells. The results of one experiment are given in Fig. 1 and show that when there was 18 hr. phage initially in the tube, there was a marked rise in phage titre at $1\frac{1}{2}$ –2 hr. This period of about $1\frac{3}{4}$ hr. is particularly interesting because this is the duration of the latent period under similar conditions, of Pa, Pb and Px phages acting on C10.

When there was no initial 18 hr. phage and about 10 host cells/ml., there was no increase of phage at $1\frac{1}{2}$ –2 hr., but phage appeared at 4–5 hr. On statistical grounds a single 18 hr. phage particle would be present in approximately 1 tube in 10 so the possibility exists that this small amount was not detected in the assays until after the second cycle or after cell division. However, out of 95 tubes read from 0–4 hr. all were negative or contained 1 or 2 phage particles only, whereas 7/8 read after 4 hr. had titres greater than 25/tube; the negative sample was a 4 hr. one.

Action of various compounds on phage production

A number of compounds have been found to suppress the growth of Pb and Px phages at concentrations not affecting the growth of C10. However, none of these had any effect on the 24 hr. phage titre of unfiltered cultures of strain C7X. Proflavine was first investigated, but the assay tubes were so difficult to read that it was useless for this work. Attention was therefore concentrated on the following two compounds (p. 6):

Fig. 1. Production of bacteriophage in cultures of *Ps. pyocyanea* strain C7X and of phage Px on *Ps. pyocyanea* strain C10, with and without compounds A or B.

Curve symbol	Test	Initial 18 hr. phage particles/ml.	Initial bacterial colony count/ml.
x - - - x	Phage Px on strain C10	20	1000
x — x	Phage Px on C10 (+ compound B)	20	1000
● — ●	Phage C7X (with initial 18 hr. phage)	17	900
● - - - ●	Phage C7X (with initial 18 hr. phage + compound A)	17	900
○ — ○	Phage C7X (with initial 18 hr. phage + compound B)	17	900
▲ — ▲	Phage C7X (no initial 18 hr. phage)	0	9

Compound A—4:4-Bis-(2-dihydroglyoxaliny) stilbene dihydrochloride, monohydrate (Oxley & Short, 1947):

Compound B—2:7-Bis-(2-dihydroglyoxaliny)-9-phenyl-phenanthridine trihydrochloride, dihydrate:

Either of these compounds, incorporated in lactate medium or broth, inhibited the increase of phage shown by control cultures of C10 inoculated with phages Pb or Px. Cultures originally containing an inoculum of approximately 20 phage particles/ml. and 10^5 bacteria/ml. normally contain, after 24 hr. incubation, 10^8 phage particles/ml.; one loop of such cultures when 'spotted' on a nutrient agar plate seeded with C10 lyses the area on which it has been spotted. Chemicals can be incorporated in the medium, and when the phage does not multiply an area of 'no lysis' on the spot is observed. Provided that C10 itself has grown in the tube (observed visually) this indicates that the compound is active in some way. Preliminary tests showed that compound A inhibited the increase of phage at 0.1 mg./ml.; it permitted host growth equal to that of a control tube at 1 mg./ml. Compound B inhibited phage growth at 0.01 mg./ml.; it permitted host growth equal to the control at 1 mg./mg. Furthermore, when these drug-treated cultures were incubated for periods up to 7 days there was no increase of phage. After 48 hr., when the bacterial count in all tubes was between 10^8 and 10^9 organisms/ml., phage titres/ml. were as follows: control, 28×10^8 /ml.; with compound A (1 mg./ml.), 0/ml.; with compound B (1 mg./ml.), 0/ml.

Growth curves for strain C10, with and without phage in the presence and absence of compounds A and B. In assessing the significance of the action of a compound on virus infections the effect of the compound on the host is obviously important. The action of compounds A and B on the growth rate of culture C10 was therefore investigated in the presence and absence of phage Pb. After the initial addition of phage to host in broth, the solution was diluted and inoculated into 50 ml. volumes of casein hydrolysate medium containing: (a) 0.1 mg./ml. of compound A; (b) 0.1 mg./ml. compound B; (c) control bottle without compound. The initial host colony count was 10^4 /ml. and the initial phage count was 120/ml. In a similar test carried out at the same time the effects of the compounds on C10, grown in the absence of phage, were investigated. Colony counts and phage counts were made at intervals by the plate methods. The results showed (Figs. 2, 3) that compounds

A and B at a concentration of 0.1 mg./ml. had no effect on the growth rate of C10. Both compounds inhibited the phage increase when phage Pb was grown on C10, the phage count falling to zero after several hours' incubation. There was sufficient phage left viable, however, to have given an increase at 1½–2 hr., when the control count was rising rapidly. The host colony counts

Fig. 2

Fig. 3

Fig. 2. Growth curves for phage Pb and host strain C10 of *Ps. pyocyanea* when grown together, with and without compounds A and B (0.1 mg./ml.).

Fig. 3. Growth curve for host strain C10 of *Ps. pyocyanea*, with and without compounds A and B (0.1 mg./ml.).

at 12 and 24 hr. were similar whether phage had multiplied or not, although the cultures containing compound A or B looked more turbid (about twice) than the control. Complete lysis of C10 has never been observed during these investigations; whether this is due to the growth of phage-resistant strains has not been determined. The unexpected rise in the host colony count between 0 and 3 hr. followed by a fall, which did not occur when the host cell alone was under test, has not been explained.

Contact test of compounds A and B on phage Px. In order to see whether the compounds were simply inactivating free phage, Px phage suspensions containing (a) 24×10^4 and (b) 24 phage particles/ml. were left in contact with the compounds for 24 hr. at 37° in casein hydrolysate medium.

Results appear in Table 3 and show that the fall in titre was not sufficient to account for the action of the compounds A and B by a contact action alone. The treated phage was in all cases capable of infecting untreated host cells and giving high phage titres. When the initial phage titre in the contact tests was high (a) and dilutions were necessary for the phage count, then the compounds were so diluted that they could not be acting by preventing the

adsorption of phage on the host cells in the assay, since they were diluted to 1/100th of their effective concentrations. Similar results were obtained with Pb phage. These experiments suggested, but did not prove, that compounds

Table 3. *Contact action of compounds A and B on phage Px of Ps. pyocyanea at 37° in casein hydrolysate medium*

Medium		Phage titre/ml. after 24 hr.	
Compound	(mg./ml.)	Initial titre = 10^6	Initial titre = 100
Control		48×10^4	50
A	1.0	1×10^3	8
A	0.1	5×10^3	10
B	1.0	77×10^2	3
B	0.1	67×10^2	2

A and B inhibited phage multiplication within the cell. Further work is in hand on this point, but the data reported here enabled the compounds to be used against strain C7X with the knowledge that they prevented phage action without apparent damage to the host cell.

Effect of compounds A and B on the latent period of phages Pb and Px on C10. Compounds A and B at 0.1 mg./ml. prevented the rise in titre at $1\frac{1}{2}$ –2 hr. in experiments on the effect of these compounds on the latent period. Results for the action of compound B on phage Px appear in Fig. 1.

Effect of compounds A and B on bacteriophage reproduction in strain C7X. Compounds A and B at concentrations which did not affect the growth rate of the host cells had no significant effect on the bacteriophage titres after 24 hr. incubation of strain C7X. In a growth curve experiment using compound B at 0.1 mg./ml. the curves for control and treated cultures ran parallel to each other, both for host cell and 18 hr. phage counts. The compounds (at 0.1 mg./ml.) were also tested in the presence of a low initial amount of 18 hr. phage (17 particles/ml.); the results are shown in Fig. 1. With compound B there was no rise in titre at $1\frac{1}{2}$ –2 hr. such as occurred in the control C7X tube; with compound B the rise was very small and also delayed. However, with both compounds there was a marked rise in titre at 4 hr. and the final phage titre approached that of the control. This rise in titre at 4 hr. corresponded to the rise which occurred with C7X cultures when the initial inoculum contained host cells but no 18 hr. phage, as well as to the second rise occurring in tubes which did contain initial 18 hr. phage.

Attempts were made to see whether filtered cultures of strain C7X contained phage after growth in the presence of compounds A and B. Results showed a considerable fall (phage titres were about 100/ml., compared to 10^6 /ml. for untreated control filtered cultures). However, compounds A and B also lowered the phage titres in C7X filtrates when added immediately before filtration of the culture so part, if not all, of the fall in titre, could have been due to losses during filtration.

Increase of phage titre by incubating assay tubes for 48 hr.

The results of the experiments on the phage reproduction in strain C7X presented several problems. In particular, it was difficult to explain the fact that, when the host cells produced phage at 4–5 hr., the initial phage counts for these tubes should not have given rise to plaques in the assays. As the host cells only appeared to liberate phage after a certain delay, the assay

Table 4. *Px phage titres in 24 hr. broth cultures assayed at 18 and 48 hr. against Ps. pyocyanea strain C10*

(a, b and c indicate separate tests. 18 and 48 hr. assay carried out on same plate.)

Material assayed	Derived from	Log dilution	18 hr.	48 hr.	Host cell colony (count/ ml.)
			assay (Phage plaques/ml.)	assay (Phage plaques/ml.)	
Culture C7X (a)	24 hr. broth cultures	–6	34	125	140
(b)	24 hr. broth cultures	–6	2	119	816
(c)	24 hr. broth cultures	–6	64	156	—
(c)	24 hr. broth cultures	–7	0	12	63
(c)	24 hr. broth cultures	–8	1	1	9
Filtrate of C7X (= F7X)	24 hr. broth cultures	–5	45	100	0
		–6	3	9	0
Culture (C10 + F7X)	24 hr. broth culture of C10 + F7X, grown together	–6	336	336	—
		–8	6	6	—
Filtrate (C10 + F7X)	24 hr. broth cultures of C10 + F7X, grown together	–6	585	590	0
		–8	6	6	0
Cultures (48 hr. F7X (a)	48 hr. plaque from assay	–8	20	20	—
plaques + C10) (b)	of filtrate from C7X	–8	5	5	—
Culture (18 hr. F7X	18 hr. plaque from assay	–6	189	196	—
plaque + C10)	of filtrate from C7X	–7	16	16	—

tubes were incubated for a further period after the first reading at 18 hr. The titre, as assayed at 48 hr., was then found to be as much as 100 times that read at 18 hr. This phenomenon was better observed when the assays were made by the agar layer technique where, instead of ‘sloping’ the tubes, the contents were poured on to a nutrient agar plate. In every plate tested there was an increase (Table 4). These figures showed that the phage counts at 48 hr. were almost as high as the host cell counts made from the same dilution as the phage counts (both counts being made by the plate method). These results explained many puzzling facts, in particular the occasional hazy backgrounds in the assay tubes of C7X cultures and the variable appearance of plaques in such assays.

Preliminary studies on the nature of the two types of plaques have indicated that the picture is very complex, but the following experiments have been carried out and confirmed. As stated above, when cultures of C7X were assayed against C10, both 18 and 48 hr. plaques were found. The same phenomenon occurred with strains C1 and with the non-iridescent strain

C1R, which was isolated from C1 in 1948. *Filtrates* of all these cultures also gave both types of plaques. When filtrates of C7X were sown on C10 and the resulting cultures or filtrates assayed, only 18 hr. plaques were produced. The obvious explanation was that the 48 hr. plaques were derived from host cells, but the results with filtrates do not support this theory. Filtrates of the cultures C7X, C1 and C1R were tested for sterility by adding 1 ml. volumes of undiluted filtrates to 9 ml. nutrient broth and incubating for 7 days. No growth of bacteria occurred. Filtrates of these strains have been kept at +5° for 18 months and are still bacteriologically sterile.

It should be noted that in assays of C7X, the 48 hr. plaques first appeared as hazy areas (almost as a background to the sharply defined 18 hr. type plaques), when the assay plates had been incubated for about 24 hr. They gradually became more distinct although, even at 48 hr., they were never as clear as the 18 hr. type.

A 48 hr. plaque from an assay of a filtrate of culture C7X was picked off with accompanying C10 cells, and incubated in broth. The resulting culture was assayed against C10, when it yielded the 18 hr. plaques, with no increase on further incubation. This, of course, agrees with the fact that C7X filtrates when grown on C10, did not show the increase. An 18 hr. plaque, picked off and treated in the same manner, gave rise only to 18 hr. plaques (Table 4).

Failure to isolate a sensitive variant from strains C7X, C1 and C1R

Since phage liberation occurred at 1½–2 hr. in cultures of C7X, i.e. at the same time as when free Px phage acted on strain C10, and because of the apparent suppression of this mechanism by the action of compounds A and B, attempts were made to isolate a sensitive variant from strain C7X. This would be expected to be difficult since, being sensitive, such a variant would normally be lysed. Culture C7X was subcultured six times in medium containing 0.25 mg. compound B/ml. to suppress lysis of the assumed variant. The culture was then diluted in Ringer's solution containing hydrogen peroxide (a 1/10⁵ dilution of an approximately 40 % solution was the maximum concentration which did not affect host growth) and the dilutions held at room temperature for 3 hr. This treatment was known to eliminate most of the free phage (Dickinson, 1948). Volumes (0.25 ml.) of treated C7X culture were then spread on the surface of nutrient agar plates by a wire spreader, and similar volumes were spread on the surface of agar plates containing 1 mg. compound B/ml. to favour the selection of a variant if present. Similar counts were also made after growing strain C7X in the presence of antiserum to Px phage at a dilution of 1/10. Many thousands of colonies were examined, in particular those appearing to be non-iridescent, and about 300 colonies were subcultured into broth and tested, but all were insensitive to filtrates of C7X and were iridescent on subculture. Strain C1 and its non-iridescent variant C1R were also examined; all colonies produced phage in broth and were insensitive to filtrates of C1 and C1R.

Iridescence

Dickinson (1948) noted that iridescence was not apparent when cultures were grown on nutrient agar plates incorporating phenol, hexylresorcinol, agaric acid or proflavine; only proflavine inhibited the growth of bacteriophage. Warner (1950) reported that saponin, hexylresorcinol and other surface-active agents suppressed iridescence but had no effect on phage action. All the above-mentioned compounds, with the exception of proflavine, might be expected to disperse the iridescent material which Warner found to be a waxy substance. When these surface-active agents were incorporated in our liquid media and 24 hr. cultures plated on nutrient agar, iridescence was very marked. Proflavine at 1.0 and 0.1 mg./ml. nutrient agar inhibited iridescence of all five of our iridescent strains and, at the same concentrations, inhibited lysis of C10 by Pa, Pb and Px phages on the same plates. Proflavine was not a very satisfactory substance to use, as already explained, and compounds A and B were therefore tested. These also inhibited both the lysis of C10 and iridescence of C7X and other cultures. After eight subcultures in media containing 0.25 mg. compound A or B/ml. the cultures still produced iridescence when grown on nutrient agar, and also produced phage. Pl. 1, fig. 2, shows the colony appearance of C7X on agar containing 1 mg. compound B/ml.

Use of chemically-defined media

Ps. pyocyanea grows well in a simple chemically-defined medium consisting of lactate and salts; potent phage preparations can readily be obtained by the use of glycerol or heat (to inactivate the host cells) but not by filtration (Dickinson, 1948). Lysis on this medium solidified with agar was, however, very variable. When Px, Pb and Pa phages were spotted on C10 grown on this solid medium, with or without 1 % casein hydrolysate, there was often no apparent lysis. Organisms from the spotted area, however, were resistant to phage action when tested on nutrient agar, whereas controls from other areas on the plate were still sensitive. Don & van den Ende (1950) noted similar difficulties with certain batches of solid medium. The same batch of casein hydrolysate medium solidified with agar was used to test the action of T1 phage on *Esch. coli*; the lysis here was clear-cut and discrete plaques were quite distinct. It is possible that resistant strains grew up very rapidly on this medium as the same medium without the agar gave high phage titres.

The iridescent strains did not exhibit their iridescence on chemically-defined medium solidified with agar, but iridescence reappeared when the cultures were grown on nutrient agar. Strain C7X produced the same amount of phage in liquid defined medium as in broth. These cultures possessed an iridescent pellicle, often with a plaque appearance, and gave iridescent colonies when plated on nutrient agar. Since normal phage action was so difficult to assess on defined medium agar, the suppression of iridescence may be related to this problem.

DISCUSSION

Many hypotheses have been suggested to account for the behaviour of iridescent cultures of *Ps. pyocyanea*. Warner (1950) considered that the iridescent material was a distinct lytic entity, but it has not been proved that this material can in fact cause lysis. The evidence for Warner's view lies mainly in the action of surface-active agents and the effects of different media. Don & van den Ende (1950) noted that iridescence was suppressed on MacConkey's and Bacto S.S. media. It is possible that this suppression is due to the presence of surface-active bile salts in the media. The delayed and progressive appearance of iridescence compared to the production of plaques at 18 hr., has been previously taken as evidence for dissociating phage activity from the appearance of iridescence. However, with cultures C7X and C1, plaques increase in size and number after 18 hr. There is, therefore, no convincing evidence that iridescence is a distinct lytic entity. All these phenomena, as well as the effect of compounds which inhibit phage multiplication and the appearance of the plaque-like iridescent areas in iridescent cultures (see Pl. 1), could equally well be explained by the hypothesis that the iridescent substance is produced during the breakdown of cells lysed by phage action. As non-iridescent lysogenic strains, e.g. C1R, occur and since phage lysis can occur without iridescence, e.g. phage Px acting on strain C10, then, on this hypothesis, the iridescent material must only be produced during the destruction of certain strains of *Ps. pyocyanea*.

The mechanism of phage action in strain C7X is of interest in relation to the problem of lysogenic bacteria. Lwoff & Gutman (1950) found that apparently every cell in their strain of *Bacillus megatherium* 'carried' phage and that lysis, which produced free phage in their cultures, was precipitated by some environmental factor. A further paper by Lwoff, Siminovitch & Kjeldgaard (1950), appearing since the present work was submitted, supports this view. Boyd (1950) mentioned that in certain circumstances, symbiotic phages of *Salmonella typhimurium* split off variants which had lost their adaptive properties and were lytic in nature.

This case of *Ps. pyocyanea* appears to be more complex than these. Don & van den Ende (1950) found that their L strain 'carried a phage or phages which were shown in several tests to be active against its own variants'. Filtrates from the metallic (i.e. iridescent) variant derived from the L strain also lysed variants. A non-lysogenic variant gave rise to lysogenic variants, and the action of phage on a non-metallic variant produced a metallic variant (this may be similar to the origin of C7X from C7 and F1).

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Fig. 1

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. 48 hr. colonies of C7X. Magnification, $\times 2$.

Fig. 2. Colony appearance of C7X on agar containing compound B. Magnification, $\times 2$.

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