BACTERIOPHAGE FORMATION WITHOUT BACTERIAL GROWTH

II. The Effect of Niacin and Yeast Extract on Phage Formation and Bacterial Growth in the Presence of Penicillin

By WINSTON H. PRICE

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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In the preceding paper it was found that phage increases in the presence of bacteria whose multiplication was completely inhibited by penicillin. It was also reported that in concentrated bacterial suspensions there was more phage formed in the presence of penicillin than in the absence of the inhibitor. This observation suggested that in the presence of penicillin the bacteria did not utilize a substance which was essential for phage formation.

In this paper it will be shown that bacteria do not use niacin in the presence of penicillin and that niacin is essential for phage production. Experiments will also be presented which show that one or more factors are necessary for phage production besides those that are necessary for bacterial growth.

RESULTS

In broth diluted 1/17 with Locke's solution, the addition of penicillin to bacterial cultures containing 2.5 to 3.5 $\times$ 10^8 cells per ml. permits a good increase in phage. Very little phage is formed without the addition of penicillin (Table I). In bacterial suspensions containing less than 1.5 $\times$ 10^8 cells per ml., there is a large increase in phage without the addition of penicillin (Table II). These experiments suggested that penicillin by preventing the multiplication of bacteria allowed one or more substances to be used for phage formation that were normally utilized by the bacteria. In the presence of a low concentration of cells more of the substance can be utilized in phage formation.

As both niacin and thiamine were utilized for bacterial growth, each of these compounds was added to the dilute broth media containing 2.5 $\times$ 10^8 cells per ml. The addition of niacin alone without the addition of penicillin permitted the formation of phage with no detectable effect on bacterial growth (Fig. 1). Thiamine, pantothentic acid, biotin, pyridoxamine, and riboflavin were without effect on phage formation in the above system.

Subsequent experiments revealed that one or more substances were necessary for phage production besides niacin.

Bacteria were allowed to reach their maximum growth in the dilute broth system and then niacin with or without penicillin was added followed by the addition of phage. There was no increase in phage under these conditions. The addition of more ordinary nutrient broth permitted the formation of phage (Table III). This result suggested that the bacteria had used up one or more substances besides niacin normally present in broth which were essential for phage formation.

TABLE I

Phage Formation in Dilute Broth in the Presence of Concentrated Bacterial Suspensions with or without Penicillin

Each tube contained 5.0 ml. Locke's solution and 0.4 ml. broth and 1 ml. phage diluted in Locke's solution. Bacteria were centrifuged from broth culture, washed once with 5.0 ml. Locke's solution, and then resuspended in Locke's solution. To one tube was added 0.6 ml. of Locke's solution and to the other tube 0.6 ml. of Locke's solution containing 140 γ of penicillin. Total volume 7.0 ml. Sample was taken at the end of 8 hours when tube with penicillin showed complete lysis and tube without penicillin showed no lysis.

Tube No.	Additions	Initial cell count	Maximum cell count	Initial plaque counts	Final plaque counts
				per ml.	*per ml.*
1	None	2.5×10^8	4.3×10^8	4.6×10^5	1.1×10^6
2	20 γ penicillin/ml.	2.5×10^8	2.5×10^8	4.6×10^5	3×10^8

TABLE II

Phage Formation in the Presence of a Low Bacterial Concentration in Dilute Broth

Each tube contained 5.9 ml. of Locke's solution, 0.4 ml. of broth, and 0.7 ml. of phage solution diluted in Locke's solution. Bacteria were centrifuged, washed once with saline, and suspended in the 5.9 ml. of Locke's solution. Samples taken at end of 7 hours when both tubes were completely lysed.

Tube No.	Addition	Initial cell count	Maximum cell count	Initial plaque counts	Final plaque counts
				per ml.	*per ml.*
1	None	9×10^7	1.8×10^8	1.1×10^5	3×10^8
2	20 γ penicillin/ml.	9×10^7	9×10^7	1.1×10^5	4.8×10^7

It was thought of interest to determine whether the utilization of the unknown substance or substances by bacteria was prevented by penicillin. Two tubes were set up containing 1×10^9 cells per ml. in 1.5 ml. of broth. To one tube was added 50 γ of penicillin per ml. The two tubes were then incubated 5 hours at 37° and centrifuged. The effect of the supernatant fluid on phage production was studied by adding it to the synthetic medium of Fildes which does not suffice for phage production. From Table IV it can be seen that the broth in which the bacteria had been suspended no longer caused phage formation when added to the synthetic medium, whether penicillin was present or

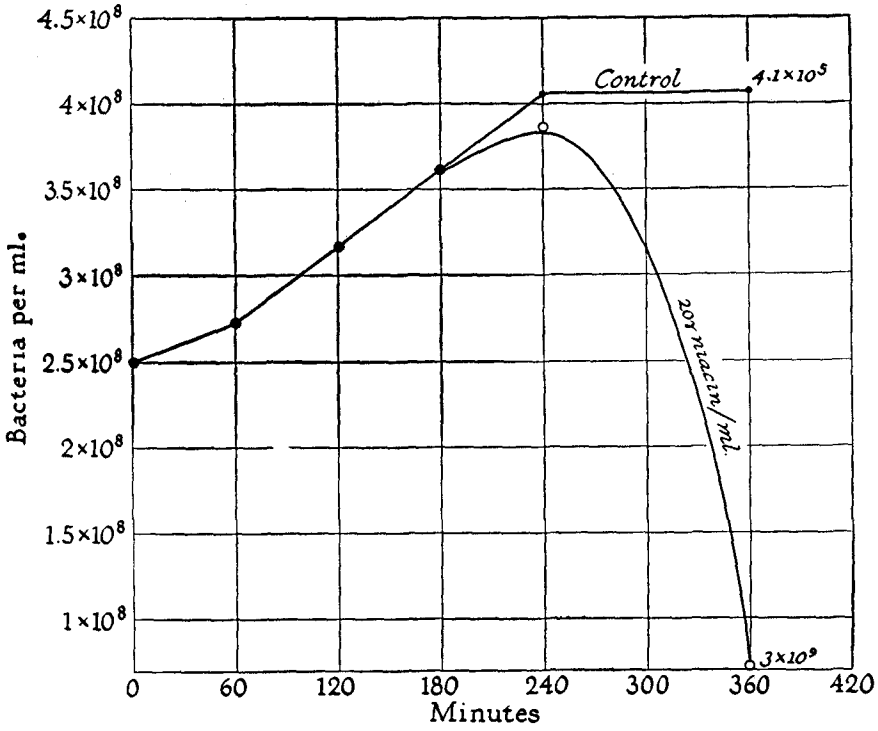

FIG. 1. Effect of niacin on phage formation in a fairly concentrated bacterial suspension. Each tube contained 5.9 ml. of Locke's solution, 0.4 ml. of broth, and 0.7 ml. of phage solution diluted in Locke's solution. Bacteria centrifuged, washed once with saline, and suspended in the 5.9 ml. of Locke's solution. Bacteria incubated 30 minutes before addition of phage and niacin. Initial plaque counts per ml. were 3.7×10^4. Figures at 360 minutes represent final phage plaque count per ml.

TABLE III

Effect of Niacin and Broth on Phage Production after a 5 Hour Multiplying Period of a Bacterial Culture

Bacteria were washed from one agar slant with 15 ml. of nutrient broth and incubated for 2 hours at 37°. The suspension was then centrifuged, and the cells washed once with 15 ml. of saline and then resuspended in 20 ml. of a 1/13 dilution of broth in Locke's solution. 5.4 ml. of this suspension was put in 3 tubes. The initial cell count was 2.9×10^8 which rose to 4.2×10^8 after 5 hours' incubation at 37°. At this time 1.0 ml. of phage diluted 1×10^4 in Locke's solution, penicillin, and niacin was added as shown in Table III. Tubes shaken at 37°. After 8 hours cell count and phage count determined. No lysis in tubes 1 and 2. Complete lysis in tube 3.

Tube No.	Niacin	Penicillin	Locke's	Broth	Cell count		Plaques	
					Initial	Final	Initial	Final
	γ/ml.	γ/ml.	ml.	ml.			per ml.	per ml.
1	20	—	2.0	—	4.2×10^8	4.2×10^8	1.7×10^5	5.5×10^4
2	20	20	2.0	—	4.2×10^8	4.2×10^8	1.7×10^5	3.1×10^4
3	—	—	—	2.0	4.2×10^8	6×10^8	1.7×10^5	2×10^8

not. Evidently, the cells use one or more substances in the broth necessary for phage formation even in the presence of penicillin. This experiment explains the result in Table V. In this experiment it was found that bacterial suspensions over 4.0×10^8 cells per ml. show less phage formation than one would expect from Table I. From the results in Table IV it is obvious that by increasing the bacterial concentration less of the unknown factor or factors are available for phage production.

TABLE IV

Effect of Penicillin on the Utilization of the Unknown Phage Factors by Bacteria

Two tubes, A and B, contained 1.5 ml. broth plus 1×10^9 cells per ml. Tube B received 50 γ of penicillin per ml. Tubes then incubated 5 hours at 37° and centrifuged. 1.0 ml. of the supernatant fluid from samples A and B was respectively added to 2 tubes each containing 8.0 ml. of the synthetic medium and having 1.5×10^8 cells per ml. All tubes then incubated. Phage assay at end of 8 hours. Tube 1 completely lysed at this time. Tubes 2 and 3 were not lysed.

Tube No.	Additions	Initial plaque counts	Final plaque counts
		per ml.	*per ml.*
1	1.0 ml. normal broth	2.1×10^5	1.2×10^9
2	1.0 ml. from tube A	2.1×10^5	3.1×10^4
3	1.0 ml. from tube B	2.1×10^5	1.2×10^4

TABLE V

Effect of Increasing Bacterial Concentrations on Phage Production in Dilute Broth in the Presence of Penicillin

Each tube contained 5.2 ml. of Locke's solution, 0.4 ml. of broth, 0.7 ml. of Locke's solution containing 350 γ of penicillin, and 0.7 ml. of phage diluted in Locke's solution. Bacteria were centrifuged, washed once with saline, and suspended in the 5.2 ml. of Locke's solution. Phage assay taken at end of 8 hours.

Tube No.	Cell count per ml.	Initial phage plaque counts	Final phage plaque counts
		per ml.	*per ml.*
1	1.0×10^8	6.6×10^4	1.7×10^7
2	2.5×10^8	6.6×10^4	1×10^9
3	4.5×10^8	6.6×10^4	1.1×10^7
4	7.0×10^8	6.6×10^4	2.2×10^6

That one or more compounds besides niacin were necessary for phage formation was also indicated by experiments in which phage formation was studied in the synthetic medium of Fildes[1] (2). This medium permitted good bacterial

[1] This medium contains the following amino acids; alanine, valine, leucine, cystine, glycine, proline, oxyproline, aspartic acid, glutamic acid, methionine, phenylalanine, tyrosine, tryptophane, arginine, histidine, and lysine; also glucose, ferrous ammonium sulfate, niacin, thiamin, phosphate, sodium nitrate, and magnesium.

growth but did not form phage with or without penicillin unless broth or yeast extract was added (Fig. 2). A solution containing biotin, guanine, adenine, uracil, xanthine, and thymine, β-alanine, riboflavin, pyridoxamine, guanylic acid, adenylic acid, yeast ribonucleic acid, choline, a flavin component from liver, ribose, inositol, *p*-aminophenyl alanine, pantothentic acid, *p*-aminobenzoic acid, and a strepogenin concentrate could not replace broth or yeast extract in causing an increase of phage in the synthetic medium. This solution did not inhibit phage production when added to the normal broth media.

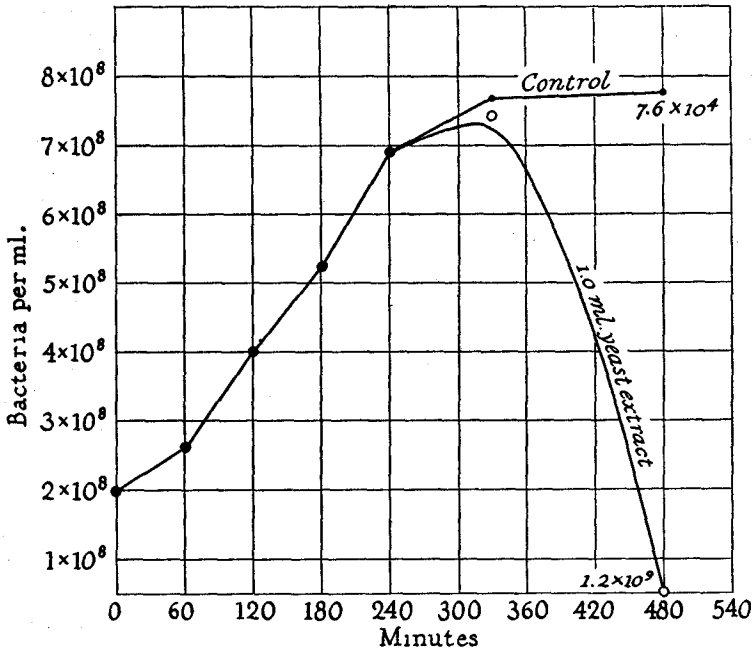

FIG. 2. Effect of yeast extract on phage formation in the synthetic medium. Each tube contained 8.15 ml. of the synthetic medium, 0.15 ml. 0.01 M $CaCl_2$, and 0.9 ml. of phage solution diluted in the synthetic medium. Bacteria were centrifuged, washed once with saline, and suspended in the 8.15 ml. of the synthetic medium. Initial phage plaque counts per ml. were 5.5×10^5. Figures at 480 minutes represent final phage plaque counts per ml.

Work is in progress on the identification of the substance or substances in yeast extract necessary for phage formation.

DISCUSSION

Bacterial concentrations of 2.5 to 3.5×10^8 cells per ml. in broth diluted 1/17 in Locke's solution form phage in the presence of penicillin but form very little phage in the absence of penicillin. The addition of niacin to the above

bacterial concentrations permitted great increases of phage in the absence of penicillin. This result can be interpreted by assuming that penicillin prevents the utilization of niacin by bacteria thus preventing their growth and allowing the phage to utilize niacin. This accounts also for the action of penicillin on bacteria. However, the non-utilization of niacin could also be accounted for by assuming that penicillin prevents some other reaction (3, 4) which inhibited the growth of the organism, and that non-multiplying bacteria did not use niacin. Further work is necessary to decide between these two hypotheses.

Bacteria will grow in the synthetic medium of Fildes but will not form phage unless yeast extract or broth is added. This observation may be extremely important if it could be shown to apply to other viruses.

The results in this paper indicate that there is a continual competition between the bacteria and the phage for essential building elements. Anything which upsets the equilibrium will of course greatly increase the formation of one of the components at the expense of the other. By limiting the amount of a compound essential for virus multiplication, but not necessary for the host, the growth of viruses could be controlled. Since it is well known that the nutritional state of the animal greatly influences the resistance of the host to infection (5–8) further work along the lines outlined above may prove helpful in the control of infectious disease.

By the use of the penicillin system described in this paper, it should be possible to gain a further insight into phage formation by the use of inhibitory metabolic analogues. Such a study will be reported at a later date.

SUMMARY

1. The addition of penicillin greatly increases the production of phage in bacterial suspensions containing 2.5 to 3.5 $\times$ 10^8 cells in 0.4 ml. broth plus 6.6 ml. Locke's solution.

2. Addition of niacin also greatly increases the formation of phage in the above system without the addition of penicillin.

3. The results indicate that niacin is necessary for phage production and that bacteria cannot utilize niacin in the presence of penicillin.

4. *Staphylococcus muscae* will grow in the synthetic medium of Fildes but do not form phage unless broth or yeast extract is added.

5. Phage formation requires the presence of one or more factors, besides niacin, present in broth and yeast extract which are not essential for bacterial growth. Penicillin does not prevent the utilization of the unknown substance or substances by the bacteria.

6. A solution containing biotin, guanine, adenine, β-alanine, riboflavin, uracil, pyridoxamine, guanylic acid, adenylic acid, yeast nucleic acid, choline, *p*-aminobenzoic acid, a flavin component from liver, ribose, thymine, xanthine, folic acid, inositol, *p*-aminophenyl alanine, pantothentic acid and a strepogenin

concentrate cannot replace broth or yeast extraction in increasing phage formation in the synthetic medium of Fildes.

7. The results indicate there is a continual competition between the bacteria and phage for certain essential building elements.

8. The results are discussed in relation to possible methods of control of virus diseases.

Experimental Methods

All methods for measuring and preparing the bacteria and phage are described in the preceding paper (1).

The yeast extract used in this paper was prepared according to Northrop (9).

Addendum.—Experiments concluded after this paper was sent in for publication showed that neither niacin nor the unknown factor is needed for the adsorption of the phage to the bacteria, but that both substances are necessary for the actual multiplication of the phage. A detailed analysis of the effect of the two compounds on phage formation is in progress and will be presented in a later paper of this series.

BIBLIOGRAPHY

1. Price, W. H., *J. Gen. Physiol.*, 1947, **31,** 119.
2. Fildes, P., and Richardson, G. M., *Brit. J. Exp. Path.*, 1937, **18,** 292.
3. Gale, E. F., and Taylor, E. S., *Nature*, 1946, **158,** 676.
4. Krampitz, L. O., and Werkman, C. H., *Arch. Biochem.*, 1946, **12,** 57.
5. Hitchings, G. H., and Falco, E. A., *Science*, 1946, **104,** 568.
6. Gowen, J. W., *Ann. Missouri Bot. Gardens*, 1945, **32,** 184.
7. Rous, P., *J. Exp. Med.*, 1911, **13,** 397.
8. Schneider, H. A., *J. Exp. Med.*, 1946, **84,** 305.
9. Northrop, J. H., *J. Gen. Physiol.*, 1938, **21,** 335.