ISOLATION AND PROPERTIES OF A DNA-CONTAINING ROD-SHAPED BACTERIOPHAGE

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Summary

A male-specific coliphage, designated AE2, has been isolated from Adelaide sewage. It is a flexible rod, about 8000 Å in length and about 50 Å in diameter, and was shown to be similar in most physical, chemical, and biological properties to those reported for bacteriophages f1, fd, and M13. The phage contains 14·0% by weight of DNA which has a base composition (moles %) of guanine = 21%, adenine = 26%, cytosine = 21% and thymine = 32%, and its sedimentation coefficient at zero concentration, $S^0_{20,w}$, is 43.

Maximum yields of AE2 were obtained under suboptimal conditions for bacterial growth, namely, incubation at 30°C in nutrient broth without added glucose and with gentle agitation. Although the rate of adsorption of phage to host bacteria was very slow, the extracellular phage levels increased on shaking at 30°C to 1–5 × 10^{12} plaque-forming units/ml after incubation for 10 hr using a low initial cell concentration (about 1 × 10^7 viable cells/ml) and either a low (0·001) or higher (1–2) input phage/cell ratio. The rate at which host cells became infected under these conditions was also investigated.

Measurements on cell populations indicated that AE2 was not released from infected bacteria by lysis but that there was a continual production of bacteriophages as the bacteria multiplied. Also, it appeared that infection with phage produced a decrease in the growth rate of the host.

I. INTRODUCTION

Three rod-shaped DNA bacteriophages isolated in the Northern Hemisphere and specific for male strains of *Escherichia coli* have recently been described. These are f1 (Loeb 1960; Zinder *et al.* 1963), fd (Marvin and Hoffmann-Berling 1963*a*, 1963*b*; Hoffmann-Berling, Marvin, and Dürwald 1963; Hoffmann-Berling, Dürwald, and Beulke 1963), and M13 (Hofschneider 1963; Hofschneider and Preuss 1963). They appear in electron micrographs as flexible rods about 8000 Å in length and 50 Å in diameter, and are very similar on the basis of a number of physical and biological criteria (Salivar, Tzagoloff, and Pratt 1964). In contrast to the behaviour of most virulent phages, fd and M13 do not appear to cause lysis of infected cells but allow the host cells to divide at a rate comparable to that of uninfected bacteria while the phage titre in the nutrient medium increases to high levels (Hoffmann-Berling, Dürwald, and Beulke 1963; Hoffmann-Berling and Mazé 1964; Hofschneider and Preuss 1963; Salivar, Tzagoloff, and Pratt 1964). The way in which these long viral particles are released with little apparent damage to the host is not known although, on the basis of electron micrographs, Hofschneider and Preuss (1963) have suggested that they may be secreted in an aggregated form.

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The following paper describes the isolation from Adelaide sewage of another rod-shaped DNA-containing bacteriophage specific for male strains of *E. coli* K-12 and designated AE2. Its biological and chemical properties indicate that it is similar to, but probably not identical with, the three rod-shaped phages already described. Additional properties of this rather unusual type of bacteriophage are also reported.

II. Materials and Methods

(*a*) *Media*

Oxoid Nutrient Broth (CM1) was used for flask cultures and contained per litre: beef extract, 1 g; yeast extract, 2 g; peptone, 5 g; and sodium chloride, 5 g. Bottom layer and top layer agar contained 15 g and 7·5 g agar respectively per litre of nutrient broth.

(*b*) *Bacteria and Bacteriophage*

F+ and F− strains of *Escherichia coli* K-12 (methionine-requiring strain 58–161 Sm^r) were kindly supplied by Dr. P. Reeves, Microbiology Department, University of Adelaide. The bacteriophage AE2, isolated as described below, grows on the F+ but not on the F− strain. Phage were assayed as plaque-forming units (PFU) by the two-layer agar method (Adams 1959).

AE2 was grown as follows. To 500 ml of nutrient broth in a 1-litre conical flask was added 20 ml of a culture of *E. coli* K-12 (F+) (about 5×10^8 viable cells/ml) plus AE2 to give an input phage/cell ratio of about 0·001. The flask and contents were then incubated in a reciprocal shaker at 30°C; extracellular phage levels increased to 1–5 $\times$ 10^{12} PFU/ml after 10 hr under these conditions.

For concentrating phage suspensions, the acid–alcohol precipitation procedure of Hoffmann-Berling, Marvin, and Dürwald (1963) was used. Further purification was achieved by several cycles of centrifugation at 120,000 *g* in the 40·2 rotor of the Spinco model L ultracentrifuge for 2 hr to sediment the phage, dissolving the pellet in water, and then centrifuging at 12,000 *g* for 15 min to precipitate insoluble impurities. Phage preparations made in this way were shown to contain only one component when examined in the Spinco model E analytical ultracentrifuge.

(*c*) *Isolation of Bacteriophage AE2*

Raw sewage, kindly supplied by the Engineering and Water Supply Department, Adelaide, was clarified by centrifugation at 20,000 *g* for 15 min and 300 ml of the supernatant was spun in the 30 rotor of the Spinco model L ultracentrifuge at 65,000 *g* for 3·5 hr to sediment bacteriophage. The pellets were dispersed in 2·0 ml distilled water; 0·2 ml of this suspension was layered onto 4 ml of a 10–40% (w/v) sucrose density gradient, and centrifuged at 80,000 *g* for 30 min in the Spinco SW39 rotor. After the run, 10 fractions were collected and each was assayed for PFU on F+ *E. coli* K-12; the results are given in Figure 1.

To increase the resolution of the coliphages on the basis of sedimentation rate, the top three fractions were pooled and 0·2 ml layered onto another 4 ml sucrose gradient (10–40%, w/v) and centrifuged at 80,000 *g* for 45 min. Five fractions were collected and assayed for PFU on F+ bacteria. Phage samples from 16 plaques

chosen at random from each fraction were plated both on F+ and F− bacteria. Of the 80 plaque samples chosen, 14 grew on the F+ but not on the F− strain; these 14 samples were from the two slowest sedimenting (upper) fractions. The remaining 66 samples grew on both strains. One of the plaques containing male-specific phage was chosen at random from the top fraction and the phage isolated from this plaque was designated AE2.

(*d*) *Physical and Chemical Properties of AE2*

For the determination of the base ratios of AE2 DNA, about 6 mg of dried phage, purified as described above, or about 2 mg of dried DNA prepared from purified phage by the phenol method of Gierer and Schramm (1956) were heated

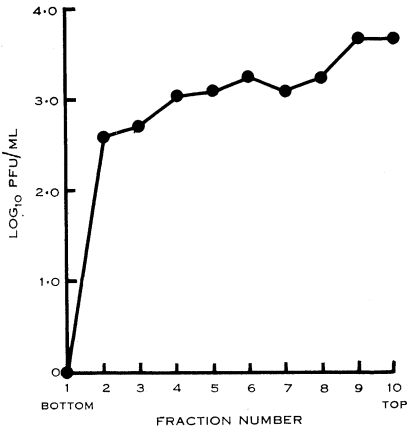


Fig. 1.—Assay of bacteriophages in sucrose density-gradient fractions. Raw sewage was cleared of debris and centrifuged for 3·5 hr at 65,000 *g* to sediment bacteriophages. The pellets were suspended in 2·0 ml distilled water, and 0·2 ml of this suspension was layered onto 4 ml of a 10–40% (w/v) sucrose density gradient, and centrifuged at 80,000 *g* for 30 min. After the run, 10 fractions were collected and plaque-formers assayed on F+ bacteria.

with 0·1 ml 70% perchloric acid at 100°C for 1 hr. The bases were separated and estimated by quantitative paper chromatography (Wyatt 1955). For the estimation of the extinction coefficient and the percentage DNA of intact phage, a purified suspension of AE2 containing about 1×10^{15} PFU/ml was dialysed for 48 hr against distilled water and the optical density at 260 mμ recorded. The DNA content of this phage suspension was determined by the method of Burton (1956) and phosphate by the method of Allen (1940). The dry weight of the concentrated suspension was determined by drying 1·0 ml in an oven at 100°C to constant weight.

(*e*) *β-Galactosidase Assay*

The methods described by Salivar, Tzagoloff, and Pratt (1964) were used for the assay of total and of extracellular enzyme activity in cultures of infected and uninfected cells. *E. coli* was grown in nutrient broth containing 0·2% lactose for induction of β-galactosidase.

(*f*) *Anti-AE2 Serum*

A full-grown rabbit was injected intraperitoneally four times at weekly intervals with 2·5 mg purified AE2. One week after the last injection, blood was taken from the ear vein and serum prepared. The inactivation constant K (Adams 1959) was found to be $2{\cdot}2 \times 10^5$.

(*g*) *Assay of Infected Cells*

Culture samples were diluted in 37°C nutrient broth, anti-AE2 serum was added (0·02 ml undiluted serum/5 ml dilution volume), and the mixtures were incubated at 37°C for 10 min to inactivate extracellular phage. The diluted samples were then assayed both for plaque-formers and for colony-formers and the results compared to determine the proportion of infected cells.

III. Results

(*a*) *Physical and Chemical Properties of AE2*

AE2 particles were seen under the electron microscope as flexible rods about 8000 Å in length (Plate 1, Fig. 1). End-to-end aggregation was observed in the micrographs, and also side-to-side aggregation in which phage particles were bound together somewhat like the fibres in a rope (Plate 1, Fig. 2). These rope-like structures were similar to aggregates of phage M13 which appear to emerge through the wall of the host cell (Hofschneider and Preuss 1963).

Table 1

Some Physical and Chemical Properties of Rod-Shaped Bacteriophages

Phage	$S^0_{20,w}$	$E^{260\ m\mu}_{1\ mg/ml}$	DNA (%)	DNA Base Ratios (moles %)			
				G	A	C	T
fd*	40	3·7	12·2	20	24	22	34
M13†	—	—	—	21	23	20	36
AE2‡	43	3·8	14·0	21	26	21	32

* Hoffmann-Berling, Marvin, and Dürwald (1963).

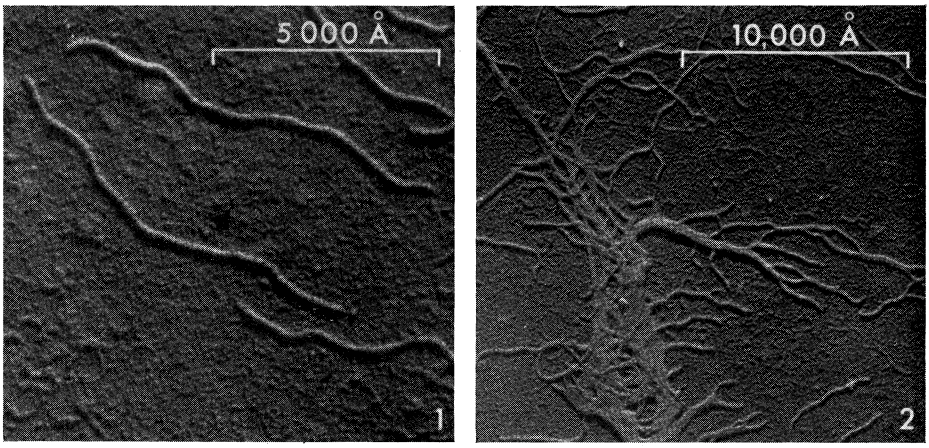
† Salivar, Tzagoloff, and Pratt (1964).

‡ Present work.

As was also shown for the bacteriophages fd and M13 (Marvin and Hoffmann-Berling 1963*b*; Salivar, Tzagoloff, and Pratt 1964), suspensions of AE2 retained high infectivity under such physical stresses as heating and as centrifuging at 120,000 *g*. The plaque count of a suspension of AE2 in nutrient broth, for example, remained unchanged after heating at 80°C for 20 min. However, all infectivity was destroyed after 10 min at 90°C. The phage was reasonably stable on storage at 4°C; a broth stock initially containing $6·5 \times 10^{11}$ PFU/ml still contained $1·1 \times 10^{11}$ PFU/ml after 12 months.

Some of the properties of AE2 are given in Table 1 together with those of fd and M13 for comparison; the corresponding values for f1 are not available. On centrifugation in the Spinco model E analytical ultracentrifuge, suspensions of AE2 formed supersharp boundaries and the $S_{20,w}$ values showed a marked concentration dependence (Fig. 2) which was greater than that observed for fd by Marvin and Hoffmann-Berling (1963*b*). This behaviour is consistent with the rod-shaped structure of AE2 and the high viscosity of concentrated suspensions of the virus.

A ROD-SHAPED BACTERIOPHAGE



Uranium-shadowed bacteriophage AE2, showing single (Fig. 1) and aggregated (Fig. 2) particles. Magnifications: Fig. 1, ×60,000; Fig. 2, ×30,000.

(*b*) *Growth of Phage and Infected Cells*

The greatest yields of AE2 were obtained under *suboptimal* conditions for bacterial growth, an effect not reported for the other rod-shaped phages. For example, on incubating cells at 30°C instead of 37°C, the yield of phage measured after 10 hr was increased tenfold. Furthermore, if 1% glucose (w/v) was present in the nutrient

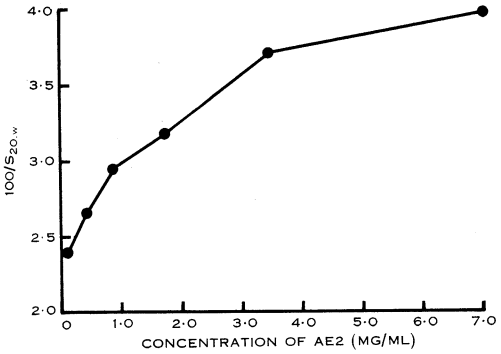


Fig. 2.—Effect of concentration of AE2 on its sedimentation coefficient, as determined from Schlieren patterns obtained with the Spinco model E analytical ultracentrifuge. Solvent: 0·1 M ammonium acetate, 5 mM EDTA, pH 7·0.

broth, only 10% of the yield of PFU grown in broth alone was obtained after 10 hr at 37°C. Vigorous aeration of the growth culture depressed the final PFU count to a similar extent.

TABLE 2

EFFECT OF GROWTH CONDITIONS ON PHAGE YIELDS

Cultures were grown overnight in 500 ml nutrient broth in 1-litre flasks on a reciprocal shaker

Temperature of Incubation	Initial Viable Cells per 1 ml of Culture	10^{-11} × Final PFU/ml for Input Phage/Cell Ratios of:				
		0·001	0·01	0·1	1·0	10
37°C	$1·1 \times 10^7$	1·6	1·0	0·95	1·2	1·2
	17×10^7	0·0015	0·0027	0·023	0·45	0·50
30°C	$1·1 \times 10^7$	15	8·5	12	11	13
	17×10^7	27	18	15	19	15

Table 2 shows the dependence of phage yields on the initial cell concentration, the initial phage/cell ratio, and the temperature of incubation. It can be seen that, at 37°C, there is a marked effect on phage yield of the initial cell concentration whereas there is very little effect at 30°C, at which temperature the phage yields are higher. In addition, variation of the initial phage/cell ratio only affected the phage yield at 37°C with the higher cell concentration tested.

Adsorption of AE2 to host cells, as measured by assays of the decrease in extracellular PFU under the growth conditions described in Section II, was not detectable in the first 5 min of incubation; a very slow rate of adsorption has also been shown for fd (Hoffmann-Berling, Dürwald, and Beulke 1963) and M13 (Tzagoloff

and Pratt 1964). The presence of 5 mM $CaCl_2$ in the nutrient medium did not increase the rate of adsorption in the first 5 min, and the final phage yields were unchanged.

To determine the overall effect of phage production on the cell population, the growth rates of AE2-infected and uninfected cultures were measured by four methods: viable cell counting, cell particle counting in a Petroff–Hausser chamber, turbidity measurements at 650 mμ, and β-galactosidase assays of lactose-induced cells. AE2 phage and F+ bacteria were grown at 30°C, the initial cell concentration being about 3×10^7 viable cells/ml.

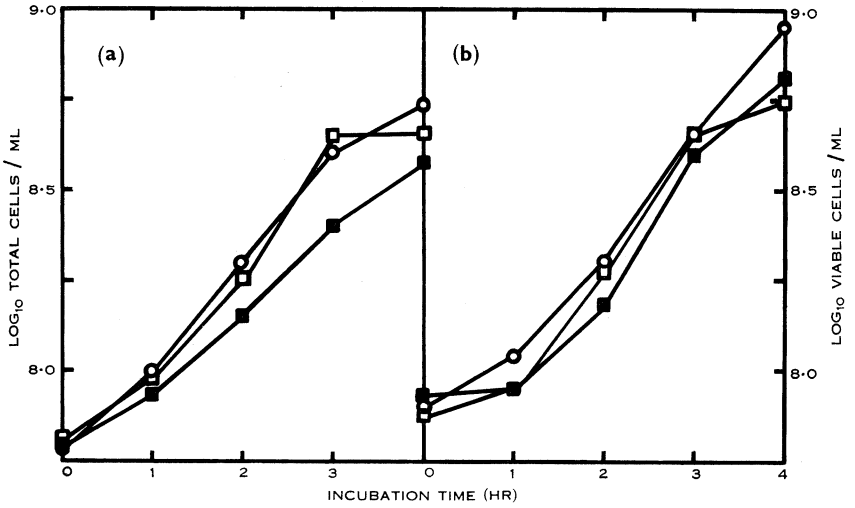


Fig. 3.—Total cell counts (*a*) and viable cell counts (*b*) on infected and uninfected cultures. AE2 was added at the input phage/cell ratios of 0·001 (□) and 1 (■) to F+ cultures containing about 3×10^7 viable cells/ml. Incubation was carried out at 30°C in a reciprocal shaker. ○ Uninfected culture.

(i) *Viable Cell Counts*

Hourly samples were taken for viable cell counts over 4 hr from cultures infected with phage at two input phage/cell ratios, 0·001 and 1, and from an uninfected culture [Fig. 3(*b*)]. Although the growth rate of infected cultures was comparable with that of uninfected cultures, some decrease in growth rate resulted on infection, an effect also observed by Salivar, Tzagoloff, and Pratt (1964) and Hoffmann-Berling and Mazé (1964). This decreased growth rate was more noticeable in the culture with the higher initial phage/cell ratio.

Longer incubation of infected cultures was carried out in order to follow the effects of virus infection. For example, an infected culture of input phage/cell ratio of 0·001 and an uninfected culture were incubated at 37°C for 16 hr and viable cell counts taken at hourly intervals. After 10 hr, the ratio of viable cells in the infected to uninfected culture was 0·06 to 1. However, between 10 and 16 hr, the concentration of viable cells in the infected culture increased so that, at 16 hr, the two cultures contained approximately the same number of viable cells. By contrast with bacteria from 2-hr colonies, 16-hr bacteria could not support phage growth, which

indicated that a new phage-resistant strain had arisen. The development of phage-resistant bacteria in infected cultures has also been reported by Hoffmann-Berling, Dürwal, and Beulke (1963) and Hoffmann-Berling and Mazé (1964) for fd, and Zinder *et al.* (1963) for f1.

(ii) *Total Particle Counts and Turbidity Measurements*

Both the total cell particle counts [Fig. 3(*a*)] and the turbidity measurements (650 mμ) of infected and uninfected cultures up to 4 hr incubation at 30°C were consistent with the viable cell counts of Figure 3(*b*). In each case the growth rate of the infected cultures was comparable to, but less than, that of the uninfected cultures.

(iii) *β-Galactosidase Assays*

The proportion of total β-galactosidase released into the medium by infected and uninfected lactose-induced bacteria was used as a measure of the effect of phage infection and release on the leakage or lysis (or both) of the bacteria. The production of phage was not affected by the presence of lactose in the growth medium.

Three 300-ml cultures were grown to *c.* 3×10^7 cells/ml and two of the cultures were infected with AE2 at the input phage/cell ratios of approximately 0·001 and 1. The percentage of total β-galactosidase released into the medium by the infected and uninfected cultures was less than 2% after 4 hr incubation at 30°C, indicating, as for fd (Hoffmann-Berling, Dürwald, and Beulke 1963) and M13 (Salivar, Tzagoloff, and Pratt 1964), that infected cells did not undergo significant lysis. Further, AE2-infected cultures synthesized β-galactosidase at a comparable rate to uninfected cultures.

(*c*) *Proportion of Infected Cells*

Measurements of the growth rates of cultures as described above suggested that AE2-infected cells were capable of self-reproduction while actively producing phage. These results, however, did not eliminate the possibility that only a few cells in the population were infected at any time while the remaining uninfected bacteria grew normally. A direct assay of infected cells was therefore made and results calculated as the percentage of infected to total viable cells (see Section II).

The percentages of infected cells in two cultures of the approximately input phage/cell ratios of 0·001 and 1, together with extracellular phage assays, are shown in Figure 4 which represents an incubation period of 4 hr at 30°C. As can be seen, there was a marked dependence of the rate of infection on the input phage/cell ratio; after 4 hr, practically all the cells in the culture of higher input ratio [Fig. 4(*a*)] had become infected and were producing 100–200 PFU/infected cell/cell generation, an estimate to be compared with that of 300 obtained for fd by Hoffmann-Berling, Dürwald, and Beulke (1963). Hence, although most of the cells were infected and producing phage, they were still growing at more or less the normal rate.

In the culture of lower input phage/cell ratio [Fig. 4(*b*)], however, only about 20% of the total cells had become infected after 4 hr but phage production per cell was occurring at a greater rate. Nevertheless, despite these differences between the two types of cultures, further incubation of both cultures produced a similar concentration of extracellular phage (cf. also Table 2).

IV. Discussion

The results presented indicate that AE2 is similar in many ways to the other two well-characterized rod-shaped bacteriophages, fd and M13. Although particles of AE2 are approximately the same size as those of fd and M13 and have a percentage DNA close to that of fd, AE2 is probably not identical with either of these two phages as, for example, is shown by a difference in the DNA base ratios (Table 1).

Perhaps the most unusual property of this type of phage is its ability to multiply in and be released from the host bacteria while the cells are dividing. Like M13-infected cells (Salivar, Tzagoloff, and Pratt 1964), AE2-infected cells formed smaller

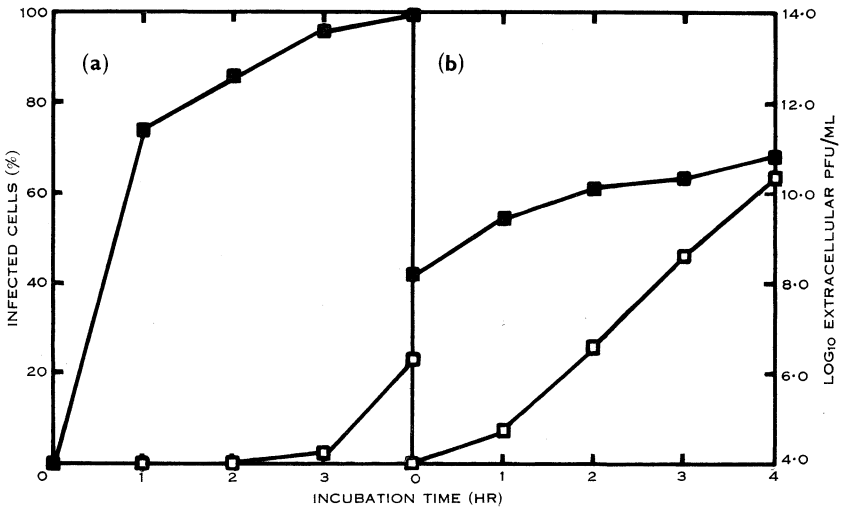


Fig. 4.—Rate of infection of bacterial cells as a percentage of total viable cells (*a*) and the production of extracellular phage (*b*). Cultures containing *c.* 3×10^7 cells/ml were infected with AE2 at the input phage/cell ratios of 0·001 (□) and 1 (■) and incubated at 30°C in a reciprocal shaker. Samples were removed every hour and the proportion of infected cells determined as described in Section II. Extracellular PFU/ml was measured after removal of bacteria by centrifugation.

colonies than uninfected cells on nutrient agar. By contrast with M13, however, AE2 infection did not cause a reduction in colony-forming efficiency so that assays of viable cells and total cells could be compared. Since there was no observable increase in total cells over viable cells in an infected culture (Fig. 3), and since the proportion of infected cells approached 100% after 3 hr [Fig. 4(*a*)], it is very likely that the difference in cell counts between infected and uninfected cultures in the first 4 hr of incubation was due to a decreased rate of division of each infected cell.

It is hoped that a study of the metabolism of infected bacteria will provide information on the way this unusual type of bacteriophage can multiply in, and be released from, bacteria which are dividing at almost the normal rate.

V. Acknowledgments

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VI. References

Adams, M. H. (1959).—"Bacteriophages." (Interscience Publishers Inc.: New York.)

Allen, R. J. L. (1940).—The estimation of phosphorus. *Biochem. J.* **34**: 858–65.

Burton, K. A. (1956).—A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**: 315–23.

Gierer, A., and Schramm, G. (1956).—Die Infektiosität der Nucleinsäure aus Tabakmosaikvirus. *Z. Naturf.* **11b**: 138–42.

Hoffmann-Berling, H., Marvin, D. A., and Dürwald, H. (1963).—Ein fädiger DNS-Phage (fd) und ein sphärischer RNS-Phage (fr) wirtsspezifisch für männliche Stämme von *E. coli*. I. Präparation und chemische Eigenschaften von fd und fr. *Z. Naturf.* **18b**: 876–83.

Hoffmann-Berling, H., Dürwald, H., and Beulke, I. (1963).—Ein fädiger DNS-Phage (fd) und ein sphärischer RNS-Phage (fr) wirtsspezifisch für männliche Stämme von *E. coli*. III. Biologisches Verhalten von fd und fr. *Z. Naturf.* **18b**: 893–8.

Hoffmann-Berling, H., and Mazé, R. (1964).—Release of male-specific bacteriophages from surviving host bacteria. *Virology* **22**: 305–13.

Hofschneider, P. H. (1963).—Untersuchungen über "kleine" *E. coli* K12 Bakteriophagen. I. Die Isolierung und einige Eigenschaften der "kleinen" Bakteriophagen M12, M13, and M20. *Z. Naturf.* **18b**: 203–5.

Hofschneider, P. H., and Preuss, A. (1963).—M13 bacteriophage liberation from intact bacteria as revealed by electron microscopy. *J. Molec. Biol.* **7**: 450–1.

Loeb, T. (1960).—Isolation of a bacteriophage specific for the F+ and Hfr mating types of *E. coli* K-12. *Science* **131**: 932–3.

Marvin, D. A., and Hoffmann-Berling, H. (1963*a*).—Physical and chemical properties of two new small bacteriophages. *Nature, Lond.* **197**: 517–8.

Marvin, D. A., and Hoffmann-Berling, H. (1963*b*).—A fibrous DNA phage (fd) and a spherical RNA phage (fr) specific for male strains of *E. coli*. II. Physical characteristics. *Z. Naturf.* **18b**: 884–93.

Salivar, W. O., Tzagoloff, H., and Pratt, D. (1964).—Some physical–chemical and biological properties of the rod-shaped coliphage M13. *Virology* **24**: 359–71.

Tzagoloff, H., and Pratt, D. (1964).—The initial steps in infection with coliphage M13. *Virology* **24**: 372–80.

Wyatt, G. R. (1955).—Separation of nucleic acid components by chromatography on filter paper. In "The Nucleic Acids". Vol. 1. pp. 243–65. (Eds. E. R. Chargaff and J. N. Davidson.) (Academic Press, Inc.: New York.)

Zinder, N. D., Valentine, R. C., Roger, M., and Stoeckenius, W. (1963).—f1, a rod-shaped male-specific bacteriophage that contains DNA. *Virology* **20**: 638–40.