ATTACHMENT OF f2 BACTERIOPHAGE TO CELLULOSE NITRATE FILTERS

Harvey F. Lodish and Norton D. Zinder
The Rockefeller Institute, New York

Received February 23, 1965

Nirenberg and Leder (1964) have discovered that ribosomes bind to cellulose nitrate (Millipore HA) filters. In this paper we show that, under defined conditions, the RNA containing coliphage f2 also attaches quantitatively to these filters. The adsorption is reversable, as measured both by recovery of isotope in phage and of viable phage. Several applications of this technique to the purification and estimation of phage in crude lysates have been developed.

Methods

Cellulose nitrate filter pads were obtained from Millipore (size HAWP, 0.45 μ pore size, 25 mm diameter). Phage in appropriate solvents (usually 0.15 M NaCl) were filtered under gentle suction through a filter pad clamped in a glass holder.

P^{32} labeled phage was grown and purified as described by Loeb and Zinder (1961) except that Sephadex chromatography was substituted for high speed centrifugation. Tryptone broth was described by Loeb and Zinder (1961).

The following procedure has been developed for the estimation of labeled phage particles in crude lysates. Bacteria were grown in the Vogel Bonner minimal medium (Cooper and Zinder, 1962) supplemented with 0.1% casamino acids (Difco, vitamin free) to a cell density of 2 x 10^8/ml. $CaCl_2$ (0.002 M) and phage (moi = 5) were added. Twenty minutes later uracil C^{14} (New England Nuclear, final concentration of 0.5 μc/ml, 2 μg/ml) was added. After an additional 2 hours of growth, the cells were lysed by the addition of chloroform, lysozyme (10 μg/ml) and EDTA (0.01 M). The cell debris was removed by centrifugation and the supernatant was diluted 1/50 into water. Ribonuclease (50 μg/ml) was added, and after 30 minutes at 25°C, DNase (10 μg/ml) and $MgCl_2$ (0.01 M) were added. After 30 minutes further incubation, the solution was divided into two parts. Total isotope in phage particles was measured by precipitation with TCA to 5% and bovine serum albumin carrier (0.5 mg). The precipitate was collected on glass fiber filter pads (Whatman GF/A) and washed with 5% TCA. Phage particles which adsorb to cellulose nitrate filters was determined by addition of NaCl to 0.15 M and passage through a Millipore filter pad; the filter was washed with 4 x 25 cc of saline.

Dried filter pads were counted with a toluene-based scintillation fluid in a Nuclear Chicago Scintillation counter.

Results and Discussion

The attachment of small amounts of purified P^{32} labeled f2 to Millipore filters is shown in Table 1.

Table 1

Attachment of P^{32} labeled f2 to Millipore filters.

Solvent for phage	cpm adsorbed to Millipore	
0.15 M NaCl	37,972	(96%)
0.015 M NaCl	3,150	(7%)
Broth	478	(1%)
Water	2,298	(5%)
Total cpm added	39,444	

A small aliquot of purified P^{32} phage was added to 10 cc of the above solutions, filtered through the Millipore filter, and washed with 4 x 25 cc of the same solution. After drying, the Millipores were counted directly. The specific activity of the phage is 1 cpm = 10^6 particles = 2 x 10^4 pfu.

Over 95% of the phage particles are retained when filtered in isotonic saline; less than 7% are retained in broth, water, or 0.015 M NaCl.

All stocks of f2 phage contain 5-15% of particles capable of forming plaques (Cooper and Zinder, 1963). Attachment of label, therefore, represents the retention of non-viable phage. Viable phage also attach to Millipore filters and the attachment is reversable (Table 2). P^{32} phage was filtered in 0.15 M NaCl through a Millipore filter; only 3% of the radioactivity and 1% of the

Table 2

Reversible attachment of phage to filters

Sample	cpm P^{32}/0.1 cc		pfu/cc	
Applied	241	(100%)	4.8×10^7	(100%)
Filtrate	8	(3%)	4.2×10^5	(1%)
Eluted from filter into broth	183	(76%)	4.0×10^7	(83%)

2 cc of P^{32} f2 in 0.15 M NaCl was filtered slowly through an HAWP Millipore filter. The filtrate was counted and assayed for viable phage. The Millipore was placed in 2.0 cc of broth for 6 hours. The total counts and the viable phage in this eluate were measured. The specific activity of the P^{32} phage is the same as in Table 1.

viable phage were found in the filtrate. The filter pad was placed in a minimum volume of broth for 6 hours at 25°C; 76% of the isotope and 90% of the plaque forming particles were eluted into the broth.

The mechanism of attachment of the phage to the filters is obscure. Retention of the phage does not involve filtration, for the pore size of the filters (450 mμ) is 20 times the diameter of f2 (about 20 mμ).

The retention of larger amounts of purified phage by Millipore filters is shown in Table 3. 1.25 mg of f2 was diluted into 0.15 M NaCl and filtered; the optical density of the filtrate was determined. Over 70% of the phage was retained, and no further material adsorbed upon repeated passage through fresh filters. From Table

Table 3

Attachment of purified f2 to Millipore filters

Sample	O.D.$_{260}$mμ	O.D.$_{230}$mμ	O.D.$_{230}$/O.D.$_{260}$
Initial	1.72	1.90	1.10
After one filtration	1.20	1.45	1.20
After two filtrations	0.66	0.82	1.24
After three filtrations	0.59	0.74	1.24

5 cc purified f2 at the indicated concentration (145 μg f2 = 1 O.D.260) was filtered through a Millipore filter and the optical density of the effluent measured. This effluent was passed twice more through new filters. The initial sample and the third effluent were banded in CsCl (Figures 2 and 3).

---

3 one can calculate that a single Millipore filter can retain about 380 μg of phage. Attachment of phage as a function of salt concentration is shown in Figure 1. No phage is adsorbed at salt concentrations less than 0.01 M and maximum retention is reached at 0.1 M.

It is to be noted that usually 10%, and often as much as 30% of f2 phage particles, do not bind to Millipore filters, even after repeated passage through fresh filters or upon increasing the salt concentration. Phage purified from large batches (40 liters) of infected cells frequently adsorbed poorest (Table 3). The material which does not adsorb has a significantly

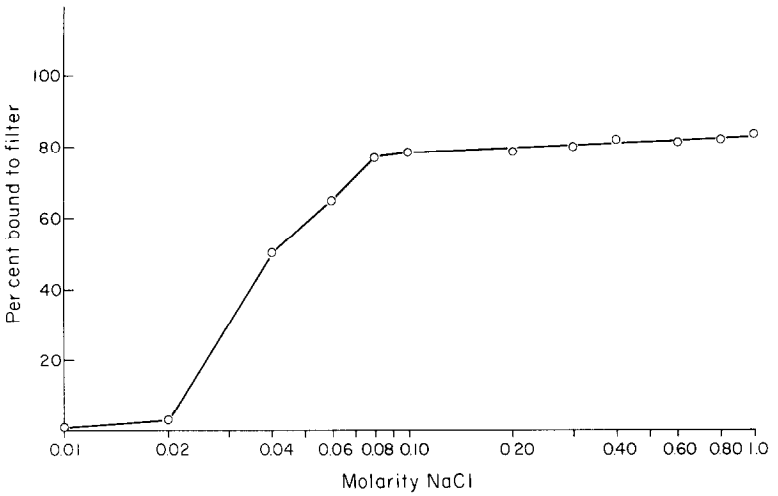

Figure 1. Retention of phage as a function of salt concentration. 0.5 cc of phage (0.5 O.D./ml) in the indicated NaCl solutions was passed through a Millipore filter and the $O.D._{260}$ of the filtrate measured.

higher optical density ratio ($O.D._{230}/O.D._{260}$) than the material which does, indicating a higher ratio of protein to RNA.

Pure f2 (same stock as used above, Table 3) was banded in CsCl (Figure 2) with a trace amount of P^{32} labeled f2 as a density marker. The P^{32} phage was the same preparation as that described in Table 1 and adsorbs completely to Millipore filters. Note the large lighter density peak in the optical density profile which is absent from the P^{32} profile. After three passages through Millipore filters (Table 3) the filtrate was mixed with the same P^{32} phage and banded in a separate CsCl density gradient. It can be seen that

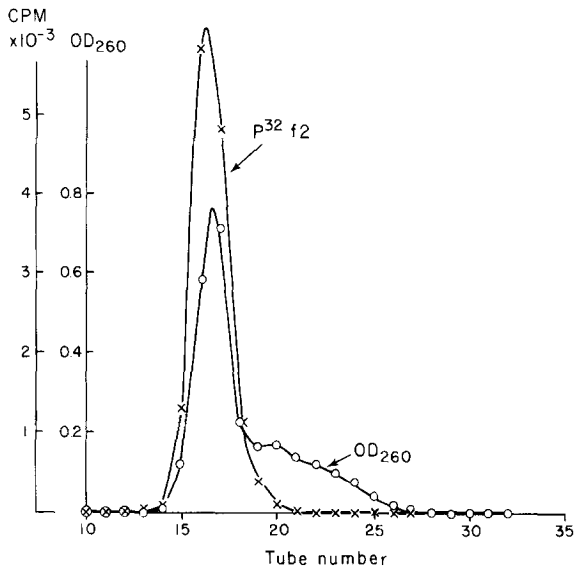

Figure 2. CsCl density gradient of f2 phage. 3 cc pure f2 (same stock as used in Table 3 above) was mixed with 20 µl of a solution of P^{32} f2 (same as used in Tables 1 and 2) and 1.95 g CsCl was added. The solution was centrifuged in the Sw 39 rotor of the Spinco Model L Ultracentrifuge at 37,000 rpm for 24 hours. Two drop samples were collected from a hole punched in the bottom of the tube; the samples were diluted and the $O.D._{260}$ read. The P^{32} counts were determined after TCA precipitation and collection of the precipitate on glass filter pads.

---

the phage material which does not adsorb to Millipore filters has a lighter density in CsCl, consistent with a higher protein/RNA ratio. This material appears the same as normal phage in the electron microscope (Stockenius, unpublished). It may be analogous to the non-viable phage particles produced by a host dependent mutant (su-1) of f2 when grown in non-permissive hosts (Zinder and Cooper, 1964; Lodish, et al., 1965).

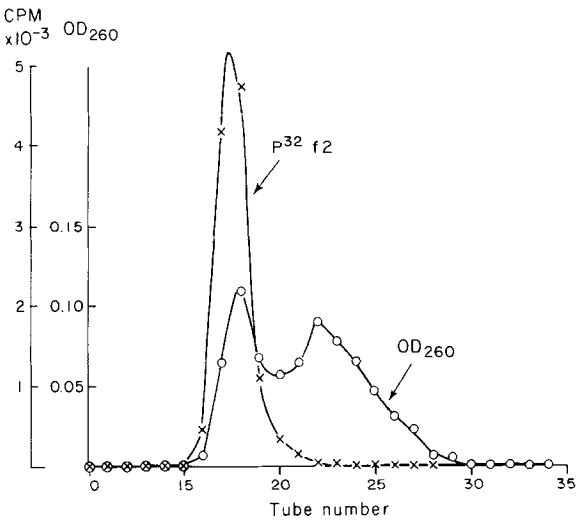

Figure 3. CsCl density gradient of f2 phage after passage through a Millipore filter. 3 cc of f2 which had been passed 3 times through a Millipore filter (Table 3) was mixed with 20 μl of P^{32} phage. The material was banded and the samples analyzed as in Figure 2.

The Millipore filtration technique is an aid in the rapid enumeration and recovery of phage particles in crude lysates (see Methods). Uracil C^{14} was chosen as a label for the phage RNA, as P^{32} is also incorporated into (lipoidal) material not removed by nuclease treatment. It is to be noted that RNase digestion is carried out in very dilute salt solutions, such that the double-stranded phage RNA formed after infection would be degraded (Weissman, et. al., 1963, 1964). Following nuclease digestion, the number of phage particles in a crude lysate is measured both by precipitation with acid and by attachment to Millipore

Table 4

Estimation of Uracil C^{14} phage in crude lysates

	Phage infected	cpm on Millipore	cpm TCA ppt
Expt. 1	None	80	68
	f2	3463	3679
Expt. 2	None	53	45
	f2	3205	3620

Phage were grown and labeled as in Methods. 0.4 cc clarified lysate was added to 20 cc water and nucleases added as in Methods. 10 cc was made isotonic in NaCl and filtered on Millipores; 10 cc was precipitated with TCA and counted.

filters. The two procedures give comparable results for wild type phage (Table 4). About 95% of the (acid precipitable) total phage adsorbs to Millipore filters. Less than two per cent of material from similarly treated uninfected cells was precipitated by TCA or retained by Millipores. In other experiments uracil C^{14} labeled phage in crude lysates was mixed with a known amount of carrier phage to monitor any losses during purification (Cooper and Zinder, 1963). Phage was purified by the standard procedure. In all cases the corrected amount of isotope in the purified phage was identical to that measured by the simpler Millipore filtration technique.

The filtration procedure also has some applica-

tions in the rapid purification and concentration of small amounts of phage. Phage in crude lysates following nuclease treatment is simply attached to a Millipore filter and then eluted into broth. This procedure is especially useful for the rapid purification of high-specific activity labeled phage.

A modification of the filtration technique enables the rapid measurement of adsorption of phage to bacteria. Phage-bacterial complexes are diluted two fold into broth and filtered on Millipore filter pads. Adsorption of labeled phage to bacteria can be measured by counting the filters directly since under these conditions the bacteria are filtered onto the pads, while free phage itself does not attach to the filter.

References

Cooper, S. and N.D. Zinder. (1962). Virology 18: 405.

Cooper, S. and N.D. Zinder. (1962). Virology 20: 605.

Lodish, H., K. Horiuchi and N.D. Zinder. (1965). Virology (in preparation).

Loeb, T. and N.D. Zinder. (1961). Proc. Nat. Acad. Sci. U.S. 48: 1121.

Nirenberg, M. and P. Leder. (1964). Science 147: 1399.

Weissman, C. and P. Borst. (1963). Science 142: 1188.

Weissman, C., P. Borst, R.H. Burdon, M.A. Billeter and S. Ochoa. (1964). Proc. Nat. Acad. Sci. U.S. 51: 682, 890.

Zinder, N.D. and S. Cooper. (1963). Virology 23: 152.