were 201, 205, and 193, respectively. The nearly equal specific activities indicate that Hb III is synthesized in the duckling and that its presence does not represent simply a carry-over from late embryonic life.

The chronology of cell hemoglobin values is shown in Table 1. The times of the activation and deactivation of hemoglobin synthesis have not yet been determined. Cell hemoglobin content is maximum in the 7- to 9-day embryo. The subsequent decrease at 14 days is conincident with the disappearance of the so-called "primitive" cells (*8*). The reciprocal change in cell content of Hb's I and II stabilizes in the adult, while Hb III decreases and ultimately disappears.

We suggest that the appearance of Hb III in the duck embryo, its presence in the duckling, and its disappearance in the adult are similar to characteristics of fetal hemoglobin in the human. According to current epigenetic concepts, the inactivation of genes for duck embryonic hemoglobins occurs at widely separate times, that for Hb IV early in embryonic life and that for Hb III after hatching.

THOMAS A. BORGESE
JOHN F. BERTLES
Arthur H. Bunker Hematology Research Laboratory, St. Luke's Hospital and *Institute of Nutrition Sciences, Columbia University, New York, New York*

References and Notes

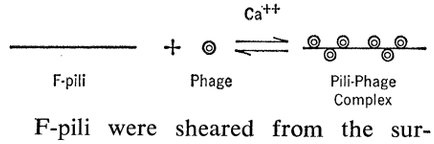
1. R. Cabannes and G. Serain, *Comp. Rend. Soc. Biol.* **149**, 1193 (1955).
2. A. Saha, R. Dutta, J. Ghosh, *Science* **125**, 447 (1957).
3. J. M. Fine, J. Uriel, J. Faure, *Bull. Soc. Chim. Biol.* **38**, 649 (1956).
4. A. Saha, *Biochim. Biophys. Acta* **32**, 258 (1959).
5. C. Manwell, C. M. Ann Baker, J. D. Roslansky, M. Foght, *Proc. Nat. Acad. Sci. U.S.* **49**, 496 (1963).
6. V. D'Amelio and A. M. Salvo, *Acta Embryol. Morphol. Exp.* **4**, 250 (1961).
7. We thank Dr. Charles Christian for this determination.
8. A. Romanoff, *The Avian Embryo* (Macmillan, New York, 1960), p. 1305; A. M. Lucas and C. Jamroz, *Atlas of Avian Hematology* (Agricultural Monograph 25, U.S. Dept. Agr., Washington, D.C., 1961), pp. 115–128; R. C. Fraser, *Exp. Cell Res.* **25**, 418 (1961).
9. S. Raymond and Y. J. Wang, *Anal. Biochem.* **1**, 391 (1962).
10. J. R. Warner, A. Rich, C. E. Hall, *Science* **138**, 1399 (1962).
11. T. G. Ferris, R. E. Easterling, R. E. Budd, *Blood* **19**, 479 (1962).
12. T. H. Huisman and A. M. Dozy, *J. Chromatogr.* **7**, 180 (1962).
13. W. H. Crosby, J. I. Munn, F. W. Furth, *U.S. Armed Forces Med. J.* **5**, 693 (1954).
14. H. G. Kunkel and G. Wallenius, *Science* **122**, 288 (1957).
15. Supported by NIH research grant AM 08154 and Health Research Council (HRC) of the City of New York research grant U-1291. One of us (J.F.B.) holds a career scientist award from the HRC.

4 February 1965

Complexes of F-Pili and RNA Bacteriophage

Abstract. *Cell-free filtrates of F+ strains of* Escherichia coli *"inactivate" RNA phages. The active agents have been identified in the electron microscope as thin appendages called F-pili. Complexes of F-pili and phage may be easily assayed because of their retention by membrane filter pads. Calcium ion is required for formation of the complex.*

The thin filaments growing out from the surface of male (F+ and Hfr) strains of *Escherichia coli* and related organisms are called F-pili (*1, 2*). While several types of morphologically similar pili (also called fimbriae, *2*) are synthesized by Gram-negative bacteria, Crawford and Gesteland (*1*) observed in the electron microscope that a male-specific bacteriophage, R-17, was adsorbed only to pili of an Hfr and F+ strain of *E. coli* but not to pili of an F− strain. Brinton, Gemski, and Carnahan (*2*) obtained similar results with the RNA phage M12, and found that usually only one or two F-pili were present on each male cell along with large numbers of more common pili, Type I pili, which were unrelated to maleness. The F-pili were easily distinguished in electron micrographs since at high concentration the phage almost covered the surface of the F-pili without interacting with other pili on the same bacterium (*2*). The F-pili were genetically controlled by the fertility factor of *E. coli* (*2*). Brinton *et al.* (*2*) have proposed the name F-pili for this kind of appendage and suggested the interesting idea that F-pili may serve as hollow tubes through which nucleic acid may be transferred. An assay in vitro for complexes of bacteriophage and F-pili would have obvious advantages. We have devised a simple and rapid filtration assay which has enabled us to study RNA phage adsorption to F-pili in vitro. Evidence will be presented for the following reaction:



F-pili were sheared from the surface of *E. coli* cells by treatment in the Waring blendor (*3*). In a typical experiment, 20 g of cell paste of an F+ strain of *E. coli* (K 12) grown to late log phase in tryptone–yeast extract broth (*4*) with gentle aeration were suspended in 200 ml of water and blended for 2 minutes. After centrifugation the cell-free supernatant containing the F-pili fragments was dialyzed and stored at 0°C. This "F-pili concentrate," which also contained Type I pili and other impurities, was sufficient for several thousand assays. Radioactive virus used for the F-pili assay were prepared by infecting 20 ml of cell culture containing radioactive phosphorus with coliphage f2 (*5*). Approximately 3×10^{12} phages (plaque-forming units) containing P^{32} (1.6×10^7 count/min) were obtained after purifi-

Table 1. Adsorption of P^{32}-labeled phage to F+ cells (*E. coli*, K 12). A culture containing 4×10^8 cells/ml was infected with about 3×10^7 f2 per ml (1300 count/min; P^{32}-labeled). After 10 minutes (for adsorption), the cells were centrifuged to remove unattached phage, precipitated with 5 percent cold trichloroacetic acid, and the precipitate was collected on glass filter pads for counting. The cell supernatant was assayed for viable phage, and the number of adsorbed phage was calculated by difference. In the blending experiment cells were treated for 2 minutes in the Waring blendor. Incubation was at 37°C.

Conditions	Adsorbed phage	
	Radioactivity (count/min per 10 ml)	No. (plaques/ml)
Complete	156	2.2×10^7
Minus cells	6	0
F− cells	9	1×10^6
Complete (100 μg/ml ribonuclease)	14	
Complete (cells blended before adsorption)	83	
Complete (su-1 defective added instead of f2, 1600 count/min)	5	
Complete (3 min incubation instead of 10)	89	1.6×10^7
Complete (cells blended after 3 min adsorption)	11	4×10^6

Table 2. "Inactivation" of phage by F-pili. In this experiment 5 ml of F-pili were added to a suspension of 7×10^7 phage per milliliter. After incubation (37°C) with F-pili, the remaining viable phage was assayed by plaque count. Blending of the complexes was carried out for 2 minutes.

Conditions	$10^7 \times$ Viable phage remaining (plaques/ml)
Control (0 time sample)	6.5
Complete (10 min incubation)	3.6
Complete (complexes blended)	6.0
Complete (complexes blended; reincubated 40 min)	3.2

Table 3. Filtration assay for F-pili–bacteriophage complexes. Conditions as in text. Type I pili from F⁻ cells were prepared in the same manner as the F-pili concentrate; boiled pili were heated at 100°C for 2 minutes. Approximately an equal number of radioactive su-1 (defective) particles were used as for f2. Acid-treated pili were incubated at *p*H 1.9 for 30 minutes at 25°C and neutralized with KOH to *p*H 7.1. Alkali-treated pili were incubated at *p*H 11 for 30 minutes and neutralized.

Conditions	Counts retained on filter pad (cpm)
Complete	120
Minus F-pili	4
Boiled F-pili	6
F-pili, 70°C, 2 min	54
Acid-treated F-pili	144
Alkali-treated F-pili	113
Su-1 (defective) phage added instead of f2	6
Complete, plus 100 μg ribonuclease and 20 μg deoxyribonuclease	114
Pili from F⁻ cells	4
Complete (incubated at 0°C)	38

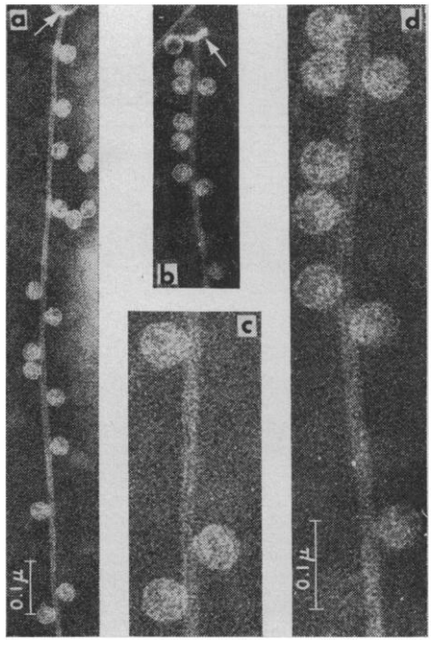


Fig. 1. Electron micrographs of F-pili–RNA bacteriophage complexes formed in vitro. Bacteriophage appear as small spheres (diameter, ~ 250 Å) attached to the surface of the long filaments (F-pilus). The preparation was freed of unattached phage by several cycles of sedimentation and washings. Complexes were negatively (phosphotungstic acid) stained (*8*). *a* and *b*, × 70,000; *c* and *d*, × 110,000.

cation (*5*), a quantity sufficient for several thousand assays. A radioactive lysate of a host-dependent mutant su-1 (defective) was prepared as above and assayed as described by Zinder and Cooper (*6*).

About 10 to 15 percent of the total radioactivity of the f2 phage preparation was adsorbed to susceptible bacteria, whereas 80 to 90 percent of the viable phages were adsorbed (Table 1). Thus only viable radioactive phage attaches to bacteria, with noninfective particles being excluded. In support of this, noninfective phage-like particles produced by an f2 mutant (su-1) did not adsorb (Table 1). Furthermore, of the total radioactivity (from f2) adsorbed, as much as 50 percent was present shortly after infection in the "replicative form" described by Kelly and Sinsheimer (*7*), demonstrating that a large proportion of the adsorbed particles took part in the infective process. As expected, no P^{32} was adsorbed by female strains. These results suggest that the aforementioned assay with P^{32}-labeled phage may be considered valid for measuring the attachment of f2 phage to male bacteria.

When F-pili were mixed with RNA bacteriophages, the phages adsorb along the surface of the pilus (Fig. 1) as seen in the electron microscope. Crawford and Gesteland (*1*) and Brinton *et al.* (*2*) also observed that almost the entire surface of the F-pilus could become covered with virus particles. The fragments of F-pili observed in the electron micrographs were of varying lengths, the longest being in the order of 1 μ. The number of attached phage particles per F-pilus fragment varied and depended on the phage titer and the method of preparation. The spherical bodies marked by arrows in the micrograph are apparently not phage particles but may represent a membranal base or root of the F-pili. When present, these particles are observed only at the tips of pili fragments. In Fig. 1*b* there is a separate pilus with a single attached phage lying near the basal body. There is also an attached cluster of three phage particles in Fig. 1*a*, as well as a free phage particle. The more heavily stained center region of the pilus in Fig. 1*d* suggests a tubular structure with an axial hole as seen by Brinton *et al.* (*2*).

The electron micrographs helped to explain another curious finding. In a number of experiments it was observed that addition of F-pili to a phage lysate reduced the viable phage titer as much

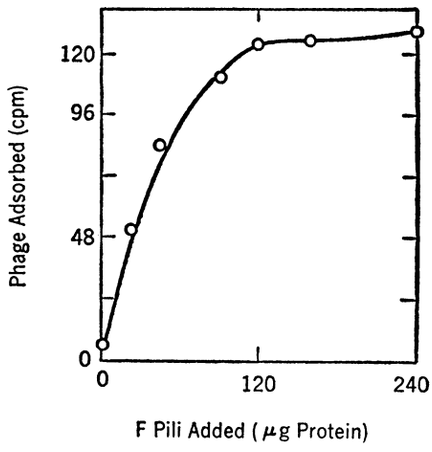


Fig. 2. Phage adsorption as a function of F-pili concentration. Standard assay as in text.

as 50 to 75 percent (Table 2). This "inactivation" was reversed if the F-pili–phage complex was blended immediately prior to phage assay. On further standing "inactivation" again took place; this suggests a re-formation of the complexes (Table 2). Such "inactivation" may now be explained by a reversible attachment to the F-pili. A single pili–phage complex containing many individual phage particles would score as a single plaque, thus accounting for a reduced titer. Violent disruption of such complexes would account for the subsequent increase in the number of plaque-forming units to the original number. We conclude that most of the f2 phage particles cannot become inactivated by simple attachment to free F-pili.

We observed that F-pili–phage (P^{32}-labeled) complexes, but not unattached phage particles, were retained by membrane filter pads; this permitted a rapid and quantitative assay. Portions (10 ml) of reaction mixture containing 0.05 tris buffer (*p*H 7.1) and 0.0032*M* $CaCl_2$ were added to test tubes. F-pili were added and the reaction was initiated by addition of 0.1 ml of radioactive phage (approximately 3×10^8 phage particles). Tubes were incubated at 25°C for 5 minutes and chilled to 0°C before filtering (Schleicher and Schull, B-6, 2.4 cm). Each pad was washed with 20 ml of water and the radioactivity was determined with a thin window gas-flow counter (Table 3). Experiments in which sedimentation (40 minutes at 30,000*g* replaced filtration gave comparable results. Some fluctuation in the amount of radiation in the background was observed with different lots of filters.

Low concentrations of F-pili were effective for adsorption (Fig. 2). Saturation was reached when about 120 μg protein of the F-pili preparation was added to the 10-ml reaction tube. In our most purified preparations as little as 1 to 2 μg gave a positive reaction. Whereas the protein nature of F-pili has not actually been established, thus far we have followed the purification of F-pili as a function of the protein concentration. Electron micrographs also show that F-pili vary greatly in length (even on the same bacterium), with sizes varying from about 0.1 μ to 8 μ. It is difficult to obtain an accurate absolute count or "titer" of F-pili since even the small fragments appear to adsorb phage. For the micrographs of Fig. 1 the phage concentration was several times higher than for the standard assay (Fig. 2).

Preparations dialyzed overnight adsorbed phage very poorly. A number of divalent metal ions including Ca^{++}, Mg^{++}, and Mn^{++} restored activity (Fig. 3). The role of the metal ion in the attachment process is not known, but is consistent with an earlier finding that Ca^{++} is required for phage development (*4*).

The filtration assay is a convenient tool for studying certain general properties of F-pili. F-pili are remarkably resistant. They retain full activity after precipitation with 0.1*N* HCl, and are unaffected by lyophilization, freezing and thawing, and incubation at *p*H 11. Upon exposure to *p*H 1.9 for 30 minutes followed by neutralization, an increase in activity was observed (Table 3).

On the other hand, heating at 70°C (Table 3) for 2 minutes resulted in about 50-percent loss of activity, whereas higher temperatures or treatment with high-frequency sound completely destroyed their ability to adsorb phage. They were sedimented by centrifugation at 100,000*g* for 30 minutes and were readily precipitated with $(NH_4)_2SO_4$ (up to 30 percent, wt to vol). By these criteria, F-pili are somewhat similar to other types of pili (*9*), although only F-pili adsorb phage.

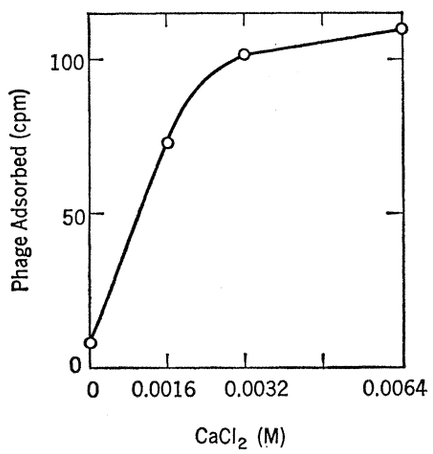


Fig. 3. Calcium-ion requirements for phage adsorption to F-pili. Incubated 5 minutes at 25°C; 0.3 ml of F-pili concentrate was used. Note optimum at 0.0032*M* $CaCl_2$.

Although RNA phages readily form complexes with F-pili, the natural role of these thin filaments in phage infection is open to some question. Phages may be detached from F-pili without loss of viability, and this indicates that their RNA was not injected into the core of the pilus. Furthermore, treatment of F-pili–phage complexes with ribonuclease neither destroyed the viability of the phage nor disrupted the complex (Table 3). Similar treatment of cell-phage complexes resulted in complete hydrolysis of the infecting RNA (Table 1). Adsorption of phage must therefore be clearly differentiated from the process of RNA injection.

RAYMOND C. VALENTINE
METTE STRAND
Biochemistry Department,
University of California, Berkeley

References and Notes

1. E. M. Crawford and R. F. Gesteland, *Virology* **22**, 165 (1964).
2. C. C. Brinton, P. Gemski, J. Carnahan, *Proc. Nat. Acad. Sci. U.S.* **52**, 776 (1964); the term F-pili is derived from "F," referring to the fertility or F factor of *E. coli*, and pili, meaning hairlike filaments.
3. C. C. Brinton, A. Buzzell, M. A. Lauffer, *Biochim. Biophys. Acta* **15**, 452 (1954).
4. T. Loeb and N. D. Zinder, *Proc. Nat. Acad. Sci. U.S.* **48**, 1424 (1961).
5. J. E. Davis and R. L. Sinsheimer, *J. Mol. Biol.* **6**, 203 (1963).
6. N. D. Zinder and S. Cooper, *Virology* **23**, 152 (1964).
7. R. Kelly and R. L. Sinsheimer, *J. Mol. Biol.* **8**, 602 (1964).
8. T. F. Anderson, in *The Interpretation of Ultrastructure*, R. J. C. Harris, Ed. (Academic Press, New York, 1962), vol. 1, p. 251.
9. C. C. Brinton, *Nature* **183**, 782 (1959).
10. We are especially indebted to Dr. C. Willson for helpful discussions and Dr. G. Cohen-Bazire Stanier for the electron micrographs. Supported by NIH grant.

23 February 1965

Glucose-6-Phosphatase: Reexamination of the RNA-Induced Activity in Mouse Ascites Tumor Cells

Abstract. *The glucostat method for the measurement of glucose released by glucose-6-phosphatase has been reexamined; it is accurate and sensitive enough to measure glucose-6-phosphatase activity in mouse ascites tumor cells. In seven different experiments, treatment with RNA from liver led to increases in enzyme activity varying between 125 to 200 percent. The activity of induced enzyme is optimum from* p*H 6 to 6.4.*

Ribonucleic acid is capable of inducing protein (enzyme) biosynthesis. Apparently the protein produced is related to the tissue source of the RNA (*1*). If mouse ascites tumor cells are treated with liver RNA, the cells synthesize serum albumin (*2*), glucose-6-phosphatase, tryptophan pyrrolase (*3*), catalase, and cysteine desulfurase (*4*). The acquisition of newly synthesized glucose-6-phosphatase was first shown by an increased enzyme activity of the homogenates as measured by the release of phosphate determined by a slightly modified method of Cori and Cori (*5*). This procedure was later replaced by the measurement of glucose with an improved glucostat method (reagents purchased from Worthington Company).

Imsande and Ephrussi (*6*) reported that under their conditions the glucostat method was inadequate for the assay of glucose-6-phosphatase activity in Ehrlich ascites tumor cells. Their conclusion was based on: (i) the insensitivity of the method to low concentrations of glucose; (ii) the presence of peroxidase inhibitor in ascites cell homogenates which interferes with the assay. They also suggested that the activity of ascites cell homogenates might be due to an acid phosphatase rather than to glucose phosphatase.

In view of the report of Imsande and Ephrussi, I have reexamined the adequacy of my technique for measurement of glucose-6-phosphatase activity. First the sensitivity of the glucostat method was tested with respect to the recovery of glucose added to ascites cell homogenates; this was accomplished by adding different concentrations of glucose to Nelson ascites tumor cell homogenates and then measuring the recovery of the added glucose in the incubated mixture. The optical density at 400 mμ (Table 1) of the reaction mixture (column 4) is proportional to the amount of sugar added