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Chemical modification of the coat protein in bacteriophage fd and orientation of the virion during assembly and disassembly

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The major (gene VIII) coat protein of bacteriophage fd was radiolabelled by treating the virus with methyl[^{3}H]acetimidate without causing any loss of infectivity. Complete amidination of lysine-8 in the amino acid sequence of the protein was achieved but little or no modification of the lysine residues near the C terminus was observed. This supports the assumption that the coat protein is oriented in the viral filament with its N terminus on the outside and its C-terminal region abutting the DNA. *Escherichia coli* was co-infected with radiolabelled bacteriophage and with unlabelled miniphage, a shorter defective form of phage fd. Radiolabel was detected in the progeny miniphage, proving that individual coat protein subunits can be recycled and assembled onto progeny miniphage DNA. About 35% of the coat protein subunits of phage particles infecting *E. coli* were recycled in 1 h. These facts support a model of the assembly and disassembly of the virion at the bacterial membrane in which the end of the particle containing the minor adsorption (gene III) protein, which is presumably the first to disassemble during infection, is the last to assemble during morphogenesis.

Key words: bacteriophage fd/virus assembly/membrane proteins/amidination/topography

Introduction

Bacteriophage fd is a filamentous virus (others are f1 and M13) that infects strains of *Escherichia coli* carrying the F-plasmid (reviewed in Denhardt *et al.*, 1978). The virion consists of a single-stranded loop of DNA, 6408 nucleotides in length (Beck *et al.*, 1978; van Wezenbeek *et al.*, 1980; Beck and Zink, 1981; Hill and Petersen, 1982), enclosed in a tubular array of ~2700 subunits of a major coat protein (Newman *et al.*, 1977). This coat protein (product of viral gene VIII) is largely α-helical and is thought to be oriented on the virus with its basic C-terminal sequence abutting the DNA and its acidic N-terminal sequence exposed at the surface, forming a shingled array (Marvin, 1978; Banner *et al.*, 1981; Caspar and Makowski, 1981). The two ends of the filament are distinguished by the presence of about five copies each of proteins encoded by genes III and VI at one end, and about five copies each of proteins encoded by genes VII and IX at the other (Grant *et al.*, 1981; Simons *et al.*, 1981). The A-protein (product of gene III) is required for adsorption of the virus to the host cell (Goldsmith and Konigsberg, 1977; Woolford *et al.*, 1977). As infecting virus penetrates the cell, the major coat protein is shed and enters the bacterial inner membrane. It is found oriented specifically with its N terminus exposed on the outer face of the membrane (Wickner, 1975). Once infection is established, newly-synthesized coat protein also enters and spans the inner membrane, with the same orientation as the parental protein (Wickner, 1975; Ohkawa and Webster, 1981). The single-stranded viral DNA is sequestered inside the infected cell as a complex with the gene V protein (Gray *et al.*, 1982 and references therein). Virus assembly is thought to occur by a progressive exchange of gene V protein for major coat protein on the DNA and extrusion of the growing filament through the membrane, without lysis of the cell (Marvin and Hohn, 1969). Thus the coat protein apparently adopts a single orientation in the lipid bilayer irrespective of the side of the membrane from which it entered, implying that subunits of protein from parental phage might be re-used and incorporated into progeny virions.

There have been three conflicting investigations of this topic. In all three, the experimental approach was to infect *E. coli* with virus that had been allowed to incorporate radioactive amino acids *in vivo* and to test for the appearance of radiolabel in progeny phage. After the original report (Trenkner *et al.*, 1967), it was argued by Henry and Brinton (1971) that transfer of radiolabel could be due to metabolism of the coat protein and re-use of the radiolabelled amino acids. In addition, intact phage might stick non-specifically to bacterial cells and subsequently be released among the progeny phage. However, Smilowitz (1974) showed that reappearance of label was unlikely to be due to adventitious adsorption and release of whole phage and that coat protein appeared to be used as individual subunits rather than large aggregates.

We have investigated the reaction of bacteriophage fd with methyl[^{3}H]acetimidate (Armstrong *et al.*, 1980) and tested for the appearance of radiolabelled coat protein in progeny miniphage (Griffith and Kornberg, 1974; Hewitt, 1975; Enea *et al.*, 1977) after co-infection of *E. coli* with ^{3}H-amidinated phage fd and unlabelled miniphage. Our results are in accord with the supposed orientation of the major coat protein in the phage particle and demonstrate unequivocally that coat protein subunits can be re-used *in vivo*. Taken in conjunction with other work, they have implications for the mechanisms of assembly and disassembly of filamentous phage at the bacterial membrane, as outlined in a preliminary report (Armstrong *et al.*, 1981a).

Results

Miniphage and methyl[^{3}H]acetimidate-treated bacteriophage fd

Genetic and physical analyses (Griffith and Kornberg, 1974; Hewitt, 1975; Enea *et al.*, 1977) have shown that miniphage are deletion mutants of bacteriophage fd in which only a small region of DNA, including the origin of replication, remains and that this DNA is packaged into a filament that is shorter than the normal phage but is otherwise identical to it. Miniphage can be separated from normal phage by gel electrophoresis (Hewitt, 1975).

After treatment with methyl[^{3}H]acetimidate, normal bacteriophage were indistinguishable from unmodified phage

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Table I. Properties of chymotryptic peptides from coat protein of ^{14}C-amidinated phage fd

Amino acid[a]	Peptide		
	C1	C2	C3
Asp	2.19		
Thr			0.99
Ser			2.06
Glu	1.23		
Pro	0.91		
Gly	1.01		
Ala	3.74		1.03
Phe	0.91	1.07	
Lys	0.27	1.93	0.92
Electrophoretic mobility (pH 6.5)	−0.43	+0.97	+0.40
Specific radioactivity (mol ^{14}C/mol peptide)	0.98	0	0
Dansylation	Dns-Ala	Bis-Dns-Lys, N^6-Dns-Lys	Dns-Thr, N^6-Dns-Lys
Inferred position in sequence	1−11	43−45	46−50

[a]Peptides were hydrolysed for 24 h in 6 M HCl at 105°C before analysis.

by the criteria of SDS-polyacrylamide gel electrophoresis (Armstrong *et al.*, 1980), non-denaturing gel electrophoresis, electron microscopy and u.v. spectroscopy. Plaque assay of the treated phage showed no detectable loss of infectivity.

Topography of the coat protein in bacteriophage fd

Bacteriophage fd was treated with methyl[^{14}C]acetamidate for 15 min at 0°C and pH 10. From the measurements of phage concentration and radioactivity, it was calculated that 1.1 mol of amino group was modified per copy of the major coat protein. The denatured coat protein was digested with chymotrypsin and the peptides were separated by thin-layer electrophoresis and chromatography. Three soluble peptides were observed, whose amino acid compositions and other properties are recorded in Table I. By comparison with the amino acid sequence of the protein (Nakashima and Konigsberg, 1974; Asbeck *et al.*, 1969), it is clear that they represent residues 1−11 (peptide C1), 43−45 (peptide C2) and 46−50 (peptide C3). Residues 12−42 probably form an insoluble peptide that remains at the origin. Autoradiography of the peptide map showed that all the radioactivity was confined to peptide C1.

Peptide C1 was ninhydrin-positive and contained 0.98 mol radiolabel/mol peptide. Dansylation gave alanine as the N-terminal residue but no N^6-Dns-lysine was detected. After 24 h hydrolysis, only 0.3 mol lysine/mol peptide was recovered, consistent with the slow breakdown of N^6-amidinolysine under these conditions (Hale *et al.*, 1979). All these facts point to the existence of an unmodified N terminus and complete modification of lysine-8 in peptide C1. We conclude that under these conditions the principal reaction of methyl acetimidate with gene VIII protein in bacteriophage fd is with lysine-8, there being little or no reaction with the lysine residues at positions 20, 43, 44 and 48 or with the N-terminal alanine residue.

Treatment of bacteriophage fd with methyl[^{14}C]acetimidate for 180 min instead of 15 min led to an increased incorporation of radiolabel (2.5 mol/mol gene VIII protein) corresponding to reaction of further amino groups. Peptide mapping and autoradiography indicated that radioactivity was no longer confined to peptide C1 but could be found in all the other peptides of the coat protein. However, it is clear that even with this prolonged treatment, complete reaction of all six amino groups in the coat protein had not been achieved. We infer that the lysine residues in the C-terminal half of the sequence are substantially protected from reaction in the virus particle whereas the N-terminal region is exposed.

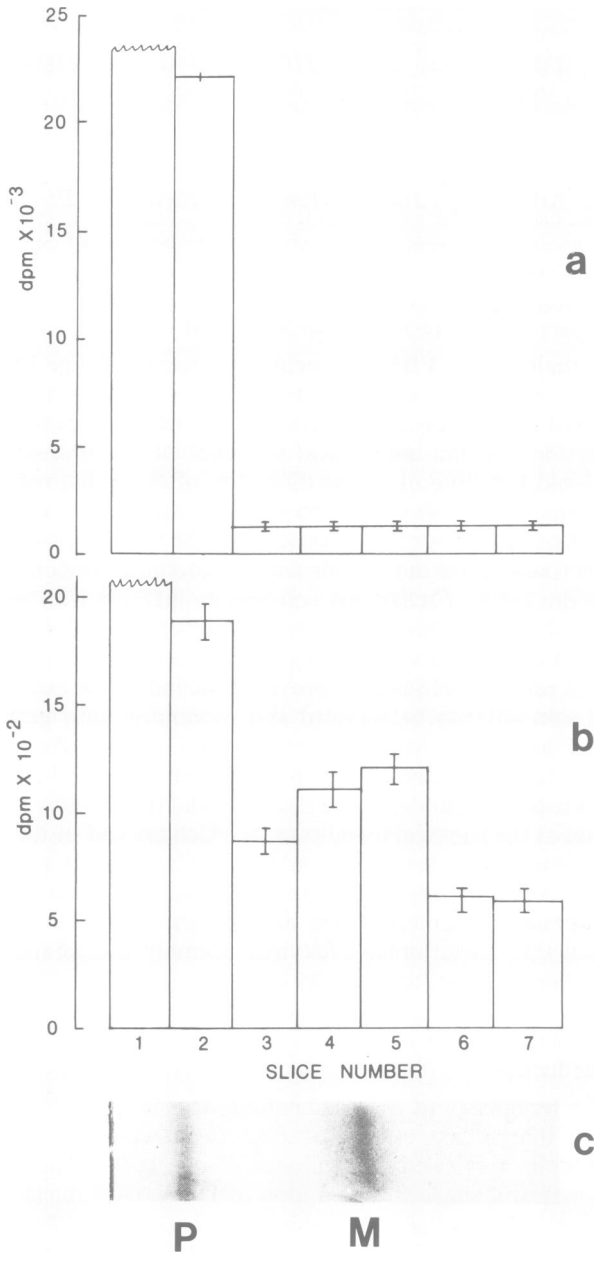

Fig. 1. Electrophoretic separation of bacteriophage fd and miniphage. Electrophoresis was carried out in 0.5% agarose/3% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue and then sectioned. The sections were counted for radioactivity, as described in Materials and methods. **(a)** Electrophoresis of 1.5 mg of miniphage stock and 4 μg of ^{3}H-amidinated normal-size phage. **(b)** Electrophoresis of progeny virus particles from *E. coli* cells co-infected with miniphage and ^{3}H-amidinated normal-size phage. **(c)** Electrophoresis of miniphage and normal size phage from *E. coli* cells co-infected with both types of particle as in **(b)**. The gel was stained with Coomassie Brilliant Blue. P, normal phage; M, miniphage.

Recycling of phage fd coat protein

In a control experiment, 1.5 mg of miniphage stock was mixed with 4 μg of ^{3}H-amidinated normal size phage and the mixture was analysed by electrophoresis in non-denaturing agarose-acrylamide gels (Figure 1a). The gel was sectioned and counted for radioactivity as described in Materials and methods. No peak of radioactivity was found associated with the miniphage band, indicating that no breakdown of normal-size phage in the gel or spontaneous exchange of subunits between phage and miniphage could be detected.

E. coli 5274 was infected with miniphage and ^{3}H-amidinated normal-size phage and the virus was purified after 1 h of growth. When it was analysed by electrophoresis in a non-denaturing gel as above, densitometry of a Coomassie-stained gel indicated that miniphage comprised 40% of the particles by weight (Figure 1c) and a small peak of radioactivity was found associated with the miniphage band (Figure 1b). Some of the radioactivity between the bands of phage and miniphage could be due to the known heterogeneity of the miniphage component, which is not so readily revealed by Coomassie staining but is detected by the more sensitive counting of radioactivity. The radioactivity at the top of the gels in Figures 1a and b must be attributed to phage and miniphage particles that did not penetrate the matrix.

To investigate whether recycling of label was due to metabolism of parental coat protein and re-use of N^6-amidinolysine in protein synthesis, a trichloroacetic acid precipitate of proteins was prepared from bacteria that had been co-infected with miniphage and ^{3}H-amidinated phage, as described in Materials and methods. These proteins were separated by SDS-polyacrylamide gel electrophoresis and the gel was sectioned for scintillation counting (Figure 2). All the detectable radioactivity above background co-migrated with phage coat protein; in particular, no peak was observed at the position of the phage gene V protein, which is synthesized in large amounts in phage-infected cells. Thus it is unlikely that N^6-amidinolysine has been metabolized and incorporated into newly-synthesized protein.

Mixed infection of *E. coli* 5274 with miniphage and ^{3}H-amidinated normal-size phage results in the appearance of radiolabelled coat protein in progeny miniphage. This is not due to adventitious release or mechanical breakage of the normal-size phage nor can it be attributed to direct coat protein exchange or to re-use of the labelled amino acid from the parental protein. The obvious explanation is that intact coat protein subunits have been recycled from infecting normal-size phage to progeny miniphage.

Measurement of number of F-pili per cell and efficiency of coat protein re-use

Two 90 ml cultures of *E. coli* strains 5274 (F^+) and JM173 (F^-) at identical cell densities were co-infected with miniphage and ^{3}H-amidinated phage as before but incubated for only 20 min, by which time phage adsorption to F-pili should be complete (Pratt *et al.*, 1969). The cells were centrifuged down and the radioactivity associated with each pellet was determined by scintillation counting. The difference (11 600 d.p.m., 83% of the radioactivity associated with F^+ cells) was taken as due to specific adsorption of phage to pili. The number of cells per ml of culture immediately before addition of phage, measured by plating out on nutrient agar, was 4.0×10^8. From the specific radioactivity of the ^{3}H-amidinated phage (7900 d.p.m./μg) and assuming a phage particle mol. wt. of 16.4×10^6 (Newman *et al.*, 1977), we could calculate that ~1.5 radiolabelled phage particles were adsorbed per cell. This value is in good agreement with the number of F-pili per cell observed in the electron microscope (Jacobson, 1972; Yamamoto *et al.*, 1980).

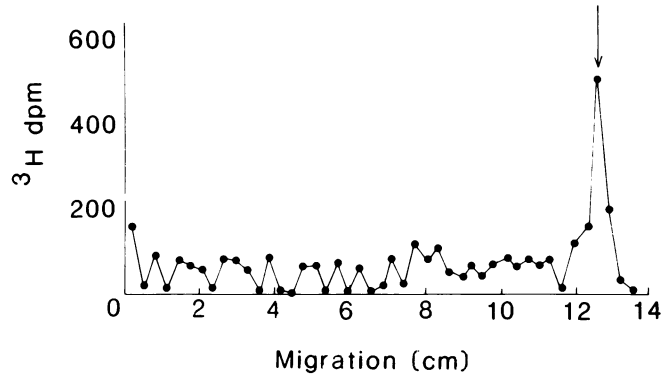

Fig. 2. Electrophoretic separation of proteins from *E. coli* infected with ^{3}H-amidinated phage fd. *E. coli* cells were co-infected with miniphage and ^{3}H-amidinated normal-size phage. After 1 h, bacterial proteins were precipitated with 10% trichloroacetic acid and separated by SDS-polyacrylamide gel electrophoresis. The gel was sliced and the sections were counted for radioactivity, as described in Materials and methods. The arrow marks the position of migration of ^{3}H-amidinated phage coat protein.

In other experiments, it was found that ~20% of the radioactivity in ^{3}H-amidinated phage applied to a non-denaturing gel was recovered after electrophoresis. If this correction was applied to the radioactivity detected in miniphage separated by gel electrophoresis after co-infection of *E. coli* 5274 with miniphage and ^{3}H-amidinated phage, it could be calculated that ~35% of the coat protein subunits of ^{3}H-amidinated phage that had infected a cell were recycled into progeny phage particles in 1 h. This calculation makes the reasonable assumption that ^{3}H-labelled coat protein monomers are randomly incorporated into full-size bacteriophage fd and miniphage particles alike, and is at best approximate. However, it shows that there is significant re-use of parental coat protein subunits in the assembly of progeny virions.

Discussion

Amidination is a benign chemical modification in the study of topography, structure and function in proteins [for a review, see Perham *et al.* (1980)]. When bacteriophage fd was treated with methyl acetimidate, the lysine residue at position 8 in the major coat protein reacted freely whereas the amino groups of lysine residues towards the C-terminal end of the molecule were slow to react, even on prolonged incubation with the imidoester. This is consistent with the proposal that the coat protein subunit is oriented in the viral filament with its N terminus exposed on the outside and its more basic C-terminal regions abutting the encapsidated DNA (Marvin, 1978). The lack of modification of the N-terminal alanine residue under our conditions of brief incubation with imidoester can be attributed to the weaker nucleophilicity of the N terminus compared with an N^6-amino group. Similar chemical modification studies coupled with X-ray diffraction analysis indicate that the same orientation of coat protein subunits is found in a different but related phage Pf1 (Nave *et al.*, 1981).

Simple proteins such as enzymes and immunoglobulins frequently retain biological activity even after extensive amidination (Perham *et al.*, 1980). It is clear that bacteriophage fd has likewise retained full infectivity after treatment with methyl acetimidate, a treatment that certainly modifies its gene VIII and gene III proteins (Armstrong *et al.*, 1980) and probably its other minor coat proteins (genes VI, VII and IX) also. The initial binding of ^{3}H-amidinated phage to *E. coli* 5274 (F^+) cells was consistent with previous estimates from electron microscopy of an average of 1.1 F-pili per cell (Jacobson, 1972; Yamamoto *et al.*, 1980). The attachment of the phage to the pilus, as well as its subsequent infection and replication, is therefore unimpaired by the chemical modification of its attachment (gene III) protein.

Our experiments rebut the arguments of Henry and Brinton (1971) and support the conclusion of Smilowitz (1974) that a significant proportion of the coat protein subunits from a particle of infecting phage fd can be re-used in the assembly of progeny virions. It is unlikely that this property is of any direct biological significance since newly synthesized coat protein subunits will greatly outnumber those from parental phage and any metabolic advantage gained by recycling coat protein will be trivial. It is more obviously a consequence of the fact that coat protein discarded by infecting phage and newly synthesized coat protein intended for virion assembly are both oriented in the bacterial membrane with their N-terminal residue outside the cell (Wickner, 1975; Ohkawa and Webster, 1981).

It is believed that the viral filament attaches to the F-pilus by means of the adsorption (gene III) protein located at one end of the particle (Henry and Pratt, 1969; Woolford *et al.*, 1977; Goldsmith and Konigsberg, 1977). This presumably would be the end of the virus at which disassembly begins and it has hitherto been widely supposed that this same end of the virus is the first to assemble and emerge from the infected cell [for reviews, see Denhardt *et al.* (1978) and Kornberg (1980)]. However, if the coat protein in the bacterial membrane has the same orientation during viral disassembly and assembly, there would then have to be a mechanism for inverting the coat protein orientation at the membrane during assembly so that correct orientation of protein with respect to the DNA could be maintained. This is an awkward mechanism topologically and runs counter to current views on transmembrane proteins. It follows that the orientation of the viral filament itself at the bacterial membrane is more likely to be the same for disassembly and assembly, with the end of the filament that contains the adsorption (A, gene III) protein being the last to assemble during morphogenesis (Chang *et al.*, 1979; Armstrong *et al.*, 1981a, 1981b). More direct evidence in favour of the proposed direction of extrusion has recently been obtained by Lopez and Webster (1983).

The DNA in the virion has a fixed orientation in the viral filament, with the hairpin loop normally found near the origin of replication located at one end of the particle (Ikoku and Hearst, 1981; Webster *et al.*, 1981). This hairpin loop resides at the end of the virion opposite from that carrying the A (gene III) protein (Webster *et al.*, 1981) and has obvious attractions as a defined feature of secondary structure in the DNA that could be intimately involved with initiating filamentous phage assembly (Ikoku and Hearst, 1981; Webster *et al.*, 1981; Dotto *et al.*, 1981). The A-protein comprises two structural domains (Gray *et al.*, 1981), a large N-terminal one that appears to have the property of binding

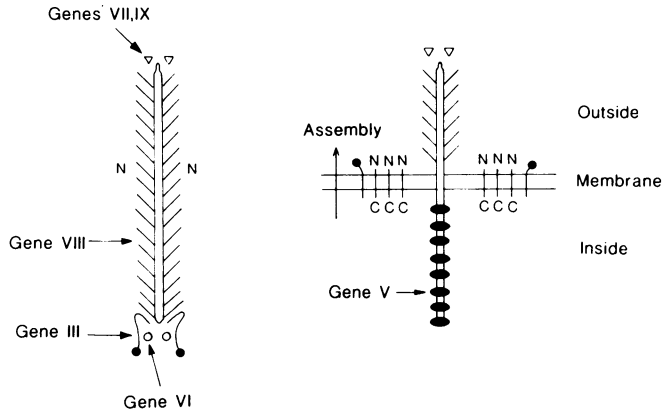

Fig. 3. Schematic model of bacteriophage fd and its orientation at the bacterial cell membrane during assembly and disassembly. It should not be taken to imply any particular interactions between minor coat proteins and the DNA. The minor coat proteins are labelled according to the genes that encode them.

to the F-pilus and a smaller C-terminal region that attaches the A-protein to the viral filament by means of a hydrophobic stretch of amino acids (Armstrong *et al.*, 1981b). The A-protein is synthesized *in vivo* with a leader sequence at its N terminus and it has been proposed that the A-protein is inserted into the bacterial membrane where it is anchored by the hydrophobic stretch of amino acids close to its C terminus to await attachment to the emerging viral filament (Armstrong *et al.*, 1981b). This proposal has been elegantly supported by the observation that removal of that part of the gene coding for the C-terminal region of the A-protein causes the protein to be secreted from the cell without being arrested in the membrane (Boeke and Model, 1982).

Taking account of all these lines of evidence, we favour a model of the assembly and disassembly of the virion at the membrane shown in Figure 3. It appears to us likely that the gene V protein-DNA complex from which the virus is assembled at the membrane will have the same orientation of DNA as the mature virion. The part, if any, played by other minor proteins in this process is obscure but is now open to investigation.

Materials and methods

Bacteriophage and bacteria

A sample of phage fd was kindly provided by I.Molineux, Imperial Cancer Research Fund, London. Miniphage stocks (containing approximately equal numbers of normal-size and miniphage particles) and the host bacterium, *E. coli* 5274, were described by Hewitt (1975). The *E. coli* strain, JM173, F^- *ΔlacX74 thyA*, was kindly made available by M.Jones-Mortimer, Department of Biochemistry, University of Cambridge. Phage were grown on *E. coli* 5274 and purified by polyethylene glycol precipitation and CsCl density centrifugation (Yamamoto *et al.*, 1970).

Radioamidination of phage and peptide mapping

Methyl[^{3}H]acetimidate (13 Ci/mol) (Armstrong *et al.*, 1980) and methyl-[^{14}C]acetimidate (0.23 Ci/mol) (Bates *et al.*, 1975) were synthesised as previously described. Phage fd (5.3 mg/ml), in 0.1 M sodium borate buffer, pH 10.0, was treated with 0.1 M methyl[^{3}H]acetimidate (high specific radioactivity for biological experiments) or 0.1 M methyl[^{14}C]acetimidate (for ease of autoradiography in peptide mapping experiments) at 0°C for 15 min at (Armstrong *et al.*, 1980) and then dialysed against 50 mM Tris-HCl, 1 mM EDTA, pH 7.5. The specific radioactivity of the ^{3}H-modified phage was calculated to be 7900 d.p.m./μg.

A sample (5 mg) of ^{14}C-amidinated phage in 1 ml of ammonium bicarbonate (5 g/l) was made 10 g/l in SDS and heated to 100°C for 2 min. The solution was then dialysed against ammonium bicarbonate (5 g/l) for 24 h at

room temperature. Chymotrypsin (1:50 by weight) was added and the mixture was incubated at 37°C for 4 h before being freeze-dried. Techniques of peptide mapping, amino acid and N-terminal analysis (Perham, 1978) were used to characterize the products.

Electron microscopy

Phage samples were spread, applied to copper grids coated with Celloidin (E.M.Scope, London) and stained with uranyl acetate according to Davis *et al.* (1971). The spreading solutions contained 10 mM Tris-HCl, pH 7.5, 15% formamide in the lower phase, and 10 mM Tris-HCl, pH 7.5, 50% formamide, 0.05 mg/ml cytochrome c, 0.4 μg/ml phage in the upper phase. Grids were rotary-shadowed with platinum in an AEI Metravac high-vacuum apparatus, and examined at a magnification of 10 000 x in an AEI EM801 electron microscope.

Gel electrophoresis

Intact phage and miniphage were separated by electrophoresis in non-denaturing slab gels containing 0.5% agarose, 3% acrylamide, 0.1% N,N′-methylene bis-acrylamide (Peacock and Dingman, 1968). Buffers for the gel, electrode wells and sample were those of Laemmli (1970) but without SDS. Electrophoresis was for 7 h at 150 V. Gels were fixed in methanol:acetic acid:water (5:1:5 by volume), stained in the same solution containing 1 g/l Coomassie Brilliant Blue, and destained in 7% acetic acid, 5% methanol (by volume).

SDS-polyacrylamide gel electrophoresis of proteins was performed by the method of Laemmli (1970), with 5% acrylamide in the stacking gel and 15% acrylamide in the resolving gel. Fixing, staining and destaining were as for non-denaturing gel electrophoresis. Stained gels were scanned with a Transdyne 2955 densitometer.

Recycling of phage fd coat protein

E. coli 5274 was grown in two 700 ml cultures for 4 h by which time A_{595} was 0.52. To each flask were added 192 μg methyl[^{3}H]acetimidate-treated phage fd, and 158 μg miniphage; this constituted approximately equal numbers of labelled and unlabelled phage particles, and a total multiplicity of ~50 phage per cell. The incubation was continued for 1 h, and the virus was then purified.

One quarter of the purified virus, in 1.5 ml, was dialysed against 0.1 M Tris-HCl, pH 6.8, and 75 μl glycerol were added. The sample was submitted to non-denaturing electrophoresis in a gel 0.5 cm thick and 16 cm wide. After staining, a vertical strip 2 cm wide was cut from the side of the gel for densitometry. The top 7 cm of the remainder of the gel was cut into horizontal strips 1 cm deep x 14 cm wide and these strips were further divided into pieces of 0.5 x 0.05 x 1 cm. The pieces from each horizontal strip were divided into four 20 ml scintillation vials and dried overnight on an oil pump. To each vial was added a mixture comprising 13.5 ml 5 g/l PPO in toluene, 1.5 ml 'Lumasolv' (Lumac Ltd.) and 0.3 ml glass-distilled water. The vials were capped, shaken vigorously and left for 24 h at room temperature. Radioactivity in the vials was determined in an LKB Beta scintillation counter, with automatic quench correction by the external standard-channels ratio method. The radioactivities in the four vials of each set were then summed.

Possible metabolism of ^{3}H-amidimated phage protein

E. coli 5274 was grown in a 100 ml culture until A_{595} was 0.8. To the culture were added 19 μg miniphage stock and 35 μg methyl[^{3}H]acetimidate-treated phage fd, and the incubation was continued for 1 h. The bacteria were collected by centrifugation and resuspended in 2 ml glass-distilled water. A portion of 0.4 ml was removed and mixed with 2 ml 10% trichloroacetic acid at 0°C. After 5 min, the precipitated protein was collected by centrifugation, and the pellet was washed twice with 0.4 ml acetone. The pellet was mixed with 0.4 ml dissolving buffer, containing 2% SDS, 0.08 M Tris-HCl, pH 6.8, 12 mM dithiothreitol and 10% glycerol. For comparison, 18 μg methyl[^{3}H]-acetimidate-treated phage was mixed with 0.4 ml dissolving buffer. Both samples were then analysed by SDS-polyacrylamide gel electrophoresis in a gel 0.5 cm thick. The tracks were excised vertically, frozen and cut horizontally into 1 mm slices. Groups of three successive slices were placed in scintillation vials and soaked in 0.4 ml 1% SDS at 37°C for 48 h, before being counted for radioactivity as above.

Attachment of phage particles to F-pili

The amount of radiolabelled phage that associates with bacterial cells under the conditions of the recycling experiment was estimated for *E. coli* strain 5274 (F^+) and for strain JM173 (F^-). The difference was taken as a measure of functional phage attachment to F-pili.

Cultures (90 ml) of both strains were grown until A_{595} was 0.52. To each were added 12.3 μg methyl[^{3}H]acetimidate-treated phage and 10.2 μg miniphage, and the incubations were continued for 20 min, by which time phage adsorption should be complete (Pratt *et al.*, 1969). The cells were collected by centrifugation and resuspended in 2 ml glass-distilled water. A sample (0.8 ml) of each suspension was mixed with 0.2 ml 10% SDS, heated at 100°C for 5 min, and 2 portions of 0.4 ml were then taken for scintillation counting.

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