Intermediates in the Assembly of ϕX174

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Escherichia coli HF4704 (HCR$^-$) irradiated with ultraviolet light was used as the host in order to study early stages of the assembly process of the ϕX174 phage particle. Two ϕX174 specific precursor particles, 9 and 6 s were found. The 9 s particle is composed solely of the gene product from cistron E and is probably E protein trimer, whereas the 6 s particle is an aggregate of the cistron F proteins, most likely a pentamer. Formation of 9 and 6 s particles do not require the function of ϕX gene products other than E and F, respectively. A particle sedimenting at 20 s and composed of E protein and non-phage proteins was also found. This particle can be formed during cell lysis and subsequent sucrose gradient centrifugation from E protein monomer and non-phage proteins existing in the cell. In contrast, the 9 and 6 s particles are not so formed. The significance of these particles in the early stages of ϕX174 phage assembly is discussed.

1. Introduction

Ultraviolet light irradiation of the host cell *Escherichia coli* HF4704 (HCR$^-$) prior to infection with ϕX174 bacteriophage causes preferential synthesis of phage-specific proteins and DNA. Gelfand & Hayashi (1969) found that subjecting uninfected HF4704 to ultraviolet irradiation reduced radioactive amino acid incorporation by over 99·5% while cells that had been equally irradiated before infection with wild type ϕX174 showed an 8- to 20-fold increase over the level in uninfected, irradiated cells in protein synthesis. Ultraviolet irradiation had similar effects on thymidine incorporation into DNA. The above dosage reduced DNA synthesis in uninfected cells to less than 5% of unirradiated cells. ϕX174 infection of the irradiated cells stimulated DNA synthesis about 8- to 10-fold over the level in uninfected, irradiated cells. Under the condition described above, the burst size of the phage was about 10 to 30. These observations provide a means for analyzing the assembly process of the phage without the complications arising from host cell protein and DNA syntheses.

Proteins corresponding to six of the seven known complementation groups in ϕX174 have been identified by comparing polyacrylamide gel electrophoregrams of the proteins isolated from ultraviolet-irradiated cells infected with either wild type or amber mutants of each cistron (Gelfand & Hayashi, 1969). The seventh gene product (G cistron; lysis function) has not been demonstrated by the above electrophoretic method. However, when a restrictive host is infected by mutants of G group, mature infective particles can be produced with a yield higher than the burst size of the wild type without lysis of the cell (Hutchison & Sinsheimer, 1966). It seems that this gene product is not directly involved in the assembly process of the phage particles.

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The use of conditional lethal mutants, sucrose density-gradient sedimentation, and polyacrylamide gel electrophoresis made it possible to identify various phage precursor particles, their protein components, and some of the assembly process of the phage. In this paper, we describe properties of the precursor particles sedimenting with *S*-values less than 20 s. The analysis of the heavier particle will be presented in a separate paper.

2. Materials and Methods

(a) *Bacteriophage and bacteria*

E. coli HF4704 (HCR$^-$ sus$^-$ thymine$^-$) was given to us by Dr R. L. Sinsheimer. *E. coli* C (BTCC 122) and the wild type ϕX174 are from our laboratory stocks. Amber mutants of ϕX174 were isolated and genetically characterized by Y. Jeng of our laboratory. The mutants used in this paper and their assignment of functions are: N-1 (cistron A) phage protein, H-210 (cistron B) phage protein?, H-90 (cistron C) replicative form synthesis, H-81 (cistron D) function unknown, H-57 (cistron E) capsid protein, H-116 (cistron F) "spike" protein (Sinsheimer, 1968), N-11 (cistron G) lysis. *am3* is a lysis-defective mutant and was given to us by Dr Sinsheimer. Phage stocks were prepared according to the method of Hutchison & Sinsheimer (1966) using *E. coli* CR63.1 (sus$^+$) which was given to us by Dr I. Tessman.

(b) *Media and buffers*

HF medium, HF basal medium, 1×SB and 10×SB buffers have been described in a previous communication from this laboratory (Gelfand & Hayashi, 1969). Tris–EDTA–NaCl is 0·03 M-Tris–HCl, 0·005 M-EDTA, 0·1 M-NaCl (pH 8·0). Tris–EDTA is 0·03 M-Tris–HCl, 0·02 M-EDTA (pH 8·0). Tris–NaCl is 0·03 M-Tris–HCl, 0·1 M-NaCl (pH 8·0).

(c) *Infection and cell lysis*

E. coli HF4704 was grown in HF medium to a concentration of 2 to 3×10^8 cells/ml. The cells were sedimented by centrifugation, washed once with HF basal medium and irradiated with ultraviolet light (2000 ergs/sec/cm^2) for 10 min at a concentration of 5×10^8 cells/ml. in HF basal medium. Irradiated cells were then sedimented, resuspended in the same buffer at 1×10^{10} cells/ml. and infected with a wild type, or a desired mutant of ϕX174 at multiplicity of infection of 5 to 10. The cell–phage mixture was kept at 37°C for 10 min and the infection was started by adding warmed (37°C) HF medium. At a desired time after infection, the culture was quickly cooled to 0°C in a dry ice–methanol bath and the infected cells were sedimented at 4°C in a Sorvall centrifuge. After washing once with 30% sucrose in Tris–EDTA–NaCl buffer, the infected cells were resuspended in Tris–EDTA buffer at a concentration of 2 to 3×10^9 cells/ml., and were lysed according to the method described by Greenlee & Sinsheimer (1968*a*), except that DNase-free pancreatic RNase (50 μg/ml.) was added at the same time as lysozyme. The cell lysates were cleared by centrifuging the thawed preparation in a Sorvall SS34 rotor at 14,000 rev./min for 10 min at 4°C.

(d) *Sucrose gradient centrifugations*

The cleared cell lysates were analyzed by sedimentation through a 5 to 30% linear sucrose density-gradient in Tris–EDTA–NaCl. Either an SW50 or SW41 rotor was centrifuged for the desired time at 18°C in a Spinco model L2 ultracentrifuge. Fractions were collected from the bottom of the centrifuge tube directly onto a piece of Whatman 3MM chromatography paper (2·5 cm × 2 cm), washed with cold 5% trichloroacetic acid and then with 95% ethanol, dried, and the radioactivity was counted in a Beckman scintillation counter. For preparative purposes, fractions of the sucrose density-gradient were collected into a small vial and a sample was processed for radioactivity counting as described above.

(e) *Polyacrylamide gel electrophoresis*

The collected fractions of the sucrose gradient were dialyzed against 10×SB overnight and then two further changes of 1×SB for a period of 12 hr. For the analysis of the total

cell extracts, the samples were prepared as described by Gelfand & Hayashi (1969). The procedures for the gel electrophoresis and the sample preparation for radioactivity counting were as described in the above reference.

(f) *Gel filtration*

Sephadex G200 was suspended in Tris–EDTA–NaCl buffer and allowed to swell at 3°C for one week. A glass column of 28 mm diameter was fitted at the bottom with a porous disk and the gel was poured into the column at about 20 ml./hr at room temperature to a final height of 375 mm. Following the packing, the column was equilibrated in Tris–EDTA–

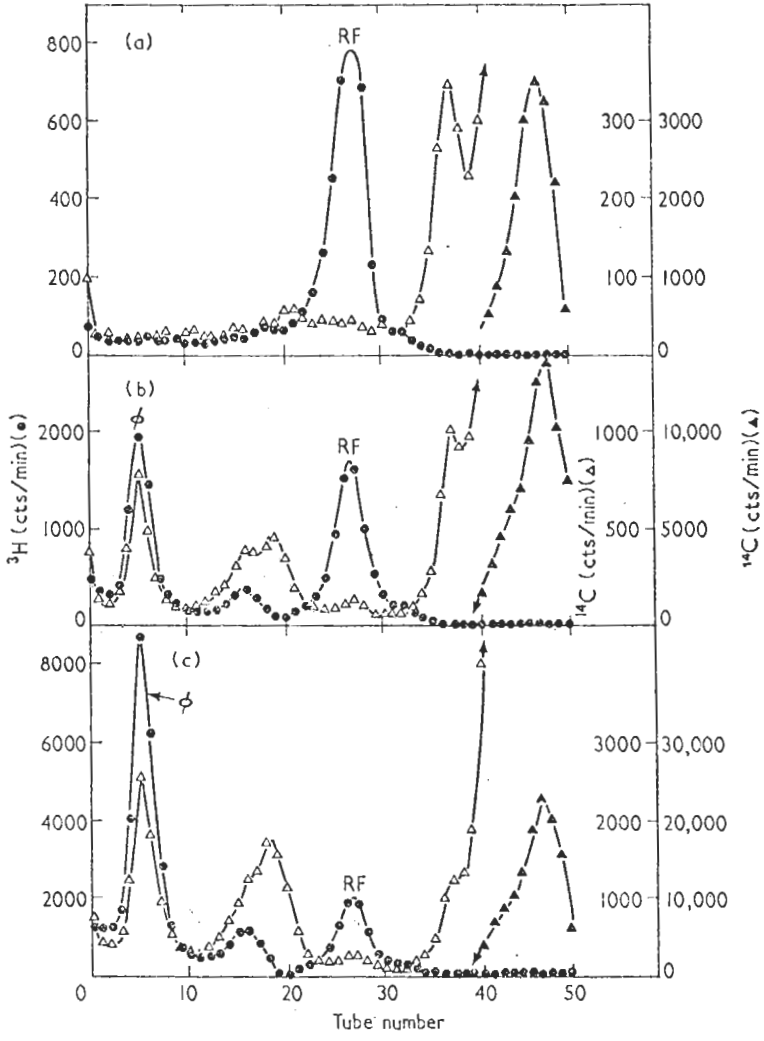


FIG. 1. Sedimentation patterns of DNA and protein in the lysate of ϕX174-infected cells. A 30-ml. culture of u.v.-irradiated *E. coli* HF4704 was infected with wild-type ϕX174 at a multiplicity of 8 ($t = 0$). DNA and proteins were labeled with [^{3}H]thymidine (40 μc/ml.), [^{14}C]alanine (0·76 μc/ml.), [^{14}C]leucine (1·05 μc/ml.), [^{14}C]lysine (0·83 μc/ml.), and [^{14}C]valine (0·68 μc/ml.), (1 μg/ml. each) respectively, from $t = 0$ to $t = 8$ min, 20 min and 30 min. The lysate was prepared from each of the 10-ml. labeled cultures (see Materials and Methods), layered on a 5 to 30% sucrose density-gradient, and centrifuged in an SW41 rotor at 40,000 rev./min, for 150 min. (a) 0 to 8 min; (b) 0 to 20 min; (c) 0 to 30 min. In this Figure and Figs 2, 3, 4, 5 and 9, the right- or left-hand ordinate is increased to facilitate plotting of the data. RF is replicative form; ϕ is phago.

NaCl buffer overnight at 4°C at a controlled flow rate of 5 ml./hr. To 1·20 ml. of a standard protein mixture (catalase, lactoglobulin, ovalbumin, cytochrome *c* and bovine serum albumin, each 1 to 3 mg) in Tris–EDTA–NaCl buffer, a small amount of blue dextran was added. The mixture was then layered carefully under the Tris–EDTA–NaCl buffer. The elution rate (5 ml./hr) was controlled by use of a pump. Cytochrome *c* and catalase were assayed by absorption at 410 mμ; other standard proteins at 280 mμ. ^{3}H-labeled particles, isolated by sucrose gradient centrifugation, were separately run under the identical conditions.

(g) *Materials*

Crystalline catalase was purchased from Worthington Biochemical Corp. Lactoglobulin and ovalbumin were given to us by Dr W. Zucker of this department. Cytochrome *c* was purchased from Calbiochem. Bovine serum albumin was from Armour Pharmaceutical Company. Blue dextran was from Pharmacia Fine Chemicals Inc. Electrophoretically pure pancreatic DNase was from General Biochemicals. Pancreatic RNase was from Sigma Chemicals and heat-treated according to Hayashi, Hayashi & Spiegelman (1965). Egg-white lysozyme was purchased from Armour Pharmaceutical Company. [^{3}H]Leucine (44 c/m-mole), [^{3}H]proline (32 c/m-mole), [^{3}H]isoleucine (1·64 c/m-mole), [^{3}H]lysine (2·44 c/m-mole), [^{3}H]thymidine (19·2 c/m-mole), [^{14}C]alanine (136 mc/m-mole), [^{14}C]aspartic acid (156 mc/m-mole), [^{14}C]leucine (263 mc/m-mole), [^{14}C]lysine (247 mc/m-mole), [^{14}C]phenylalanine (410 mc/m-mole) and [^{14}C]valine (200 mc/m-mole) were purchased from New England Nuclear Corp. [^{3}H]Arginine (19 c/m-mole) and [^{3}H]tryptophan (20 c/m-mole) were purchased from Schwartz BioResearch.

3. Results

(a) *φX174 specific particles*

Figure 1 shows the appearance of phage-specific particles during the infection period. Under the conditions used here, the single-stranded phage DNA appears about 10 to 15 minutes after infection, whereas replicative form synthesis starts immediately after infection. Before onset of the single-stranded DNA synthesis, most of the protein synthesized sediments as lighter particles (less than 20 s) (Fig. 1(a)). As infection proceeds, heavier protein particles can be recognized (Fig. 1(b) and (c)). The fastest

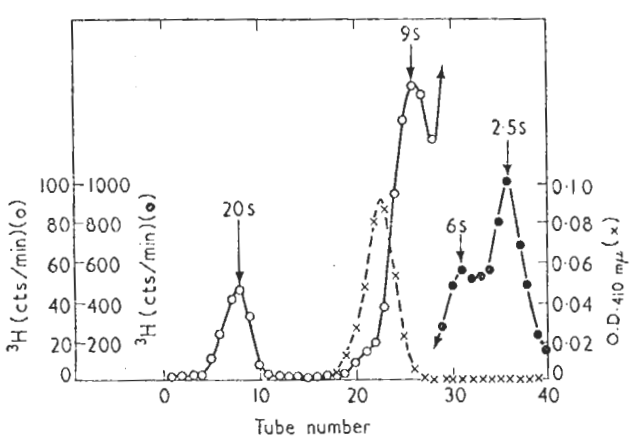


FIG. 2. Determination of the *S*-value of the lighter particles.

A portion of a lysate labeled with [^{3}H]leucine from $t = 12$ to $t = 15$ min after infection was mixed with 0·35 mg of crystalline catalase and layered onto a 5 to 20% sucrose density-gradient in Tris–NaCl buffer, and centrifuged in an SW50 rotor at 49,000 rev./min for 210 min. 10-drop fractions, collected in small vials, were diluted with 0·7 ml. Tris–NaCl buffer in order to measure the optical density at 410 mμ. Radioactivity in the cold 5% trichloroacetic acid-insoluble fraction was then counted.

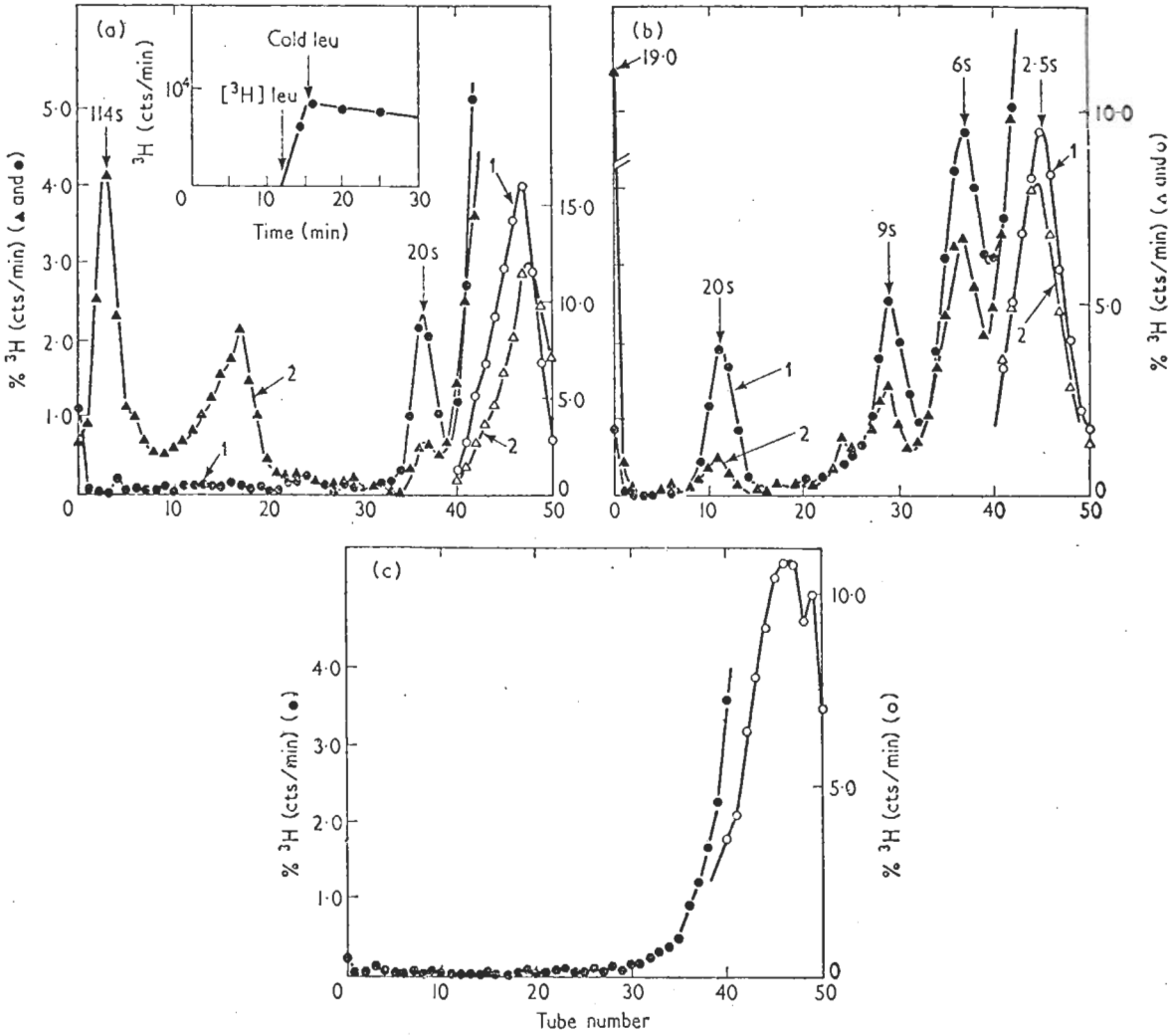


FIG. 3. Amino acid pulse and chase experiment.

A 20-ml. culture of u.v.-irradiated *E. coli* HF4704 was infected with wild-type ϕX174 at a multiplicity of 10 ($t = 0$), and proteins were labeled with [^{3}H]leucine (10 μc/ml., 0·5 μg/ml.) from $t = 12$ to 15 min. At that time a 10-ml. portion (sample 1) was quickly cooled to 0°C in a dry ice–methanol bath and a 200-fold excess of unlabeled leucine was added to the rest of the culture. After 15 min of further aeration at 37°C, the culture (10 ml.) was chilled (sample 2). Lysates were prepared from the two cultures (samples 1 and 2) and a portion equivalent to 8×10^8 cells was layered on a 5 to 30% sucrose density-gradient, and centrifuged in an SW50 rotor at 49,000 rev./min for : (a) 65 min or (b) 290 min. The two profiles (samples 1 and 2) obtained by each centrifugation were superimposed. (c) shows the profile of the lysate prepared from uninfected cells. An ultraviolet-irradiated 10-ml. culture of *E. coli* was labeled with [^{3}H]leucine as sample 1, but without phage infection. The lysate was analyzed by sucrose gradient centrifugation as in (b). The ordinate represents percentage distribution of total counts. In the insert of (a) the kinetics of the incorporation of [^{3}H]leucine into proteins in this experiment is shown. At the indicated time, a 0·1-ml. portion of the culture was removed and incubated with 0·5 ml. of 0·5 N-KOH at 37°C for 6 hr. ^{3}H counts in the alkaline-resistant, cold 5% trichloroacetic acid-precipitable material (collected on a glass filter) were plotted against time after infection.

sedimenting peak is the mature phage particle, as indicated by its infectivity to *E. coli* C (data not shown). The DNA content and protein species in this and other heavier particles (more than 20 s) will be published separately.

The *S*-values of the lighter particles were determined using catalase as a sedimentation marker (Martin & Ames, 1961) (Fig. 2); 20, 9, 6 and 2·5 s particles can be recognized in this preparation. Frequently, 12 s particles also appeared (see Fig. 5).

In order to establish any precursor–product relationship among the particles, an amino acid pulse–chase experiment was performed (Fig. 3(a) and (b)). When the culture was pulsed with [^{3}H]leucine for three minutes at 12 minutes after infection, most of the counts incorporated into the cold trichloroacetic acid-insoluble fraction are found in the lighter particles (curve 1 in Fig. 3(a)). After the chase, the counts initially incorporated into lighter particles appear to be transferred into the heavier particles (curve 2 in Fig. 3(a)). To increase the resolution of the sucrose gradient in the lighter-particle region, the identical samples, pulsed and pulse–chased, were subjected to a longer centrifugation (Fig. 3(b)). It can be seen that the counts initially incorporated into 20, 9 and 6 s decrease after chasing. In Figure 3(c), the count distribution of amino acid incorporated into uninfected cells is also shown. Most of the counts were found near the top of the sucrose gradient ($\sim$2·5 s). It should be noted that the infected cells incorporated approximately eightfold more radioactivity into proteins than uninfected cells under the same condition.

(b) *Lighter particles in amber-mutant infected cells*

In order to identify the gene products involved in the formation of the lighter particles, the syntheses of the particles in restrictive cells infected with each amber mutant were examined in sucrose density-gradients. The results shown in Figure 4 can be summarized as follows. (1) 20 s particles are synthesized by infection with mutants of each of the seven complementation groups. (2) F mutant (H-116) fails to synthesize 6 s particles, whereas 9 s particles are absent in E mutant (H-57) infected cells. (3) Mutation in the D cistron (H-81) causes reduced synthesis of 2·5 s particles.

From these results we conclude that: (1) formation of 9 s and 6 s particles require wild-type E and F gene products, respectively; (2) since mutants in B (H-210), C (H-90) and D (H-81) cistrons cannot synthesize single-stranded DNA (Siegel & Hayashi, 1969) and since 20, 9 and 6 s particles can be formed in the cells infected by either of these mutants, single-stranded DNA synthesis is not required for the formation of these particles.

Treatment of the extract made from the wild type phage-infected cells with 10 μg/ml. of pancreatic DNase for 10 minutes at 25°C in the presence of 0·01 M-$MgCl_2$ did not change the sedimentation pattern of the particles (data not shown).

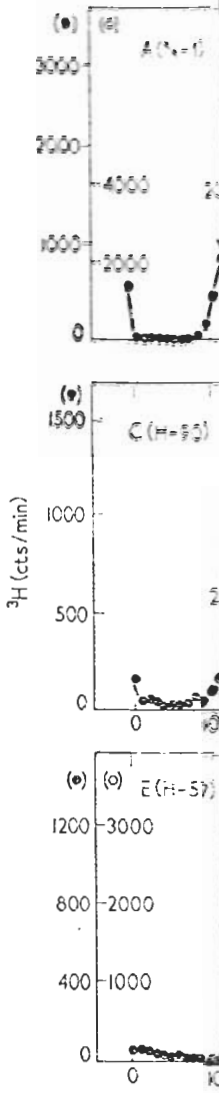
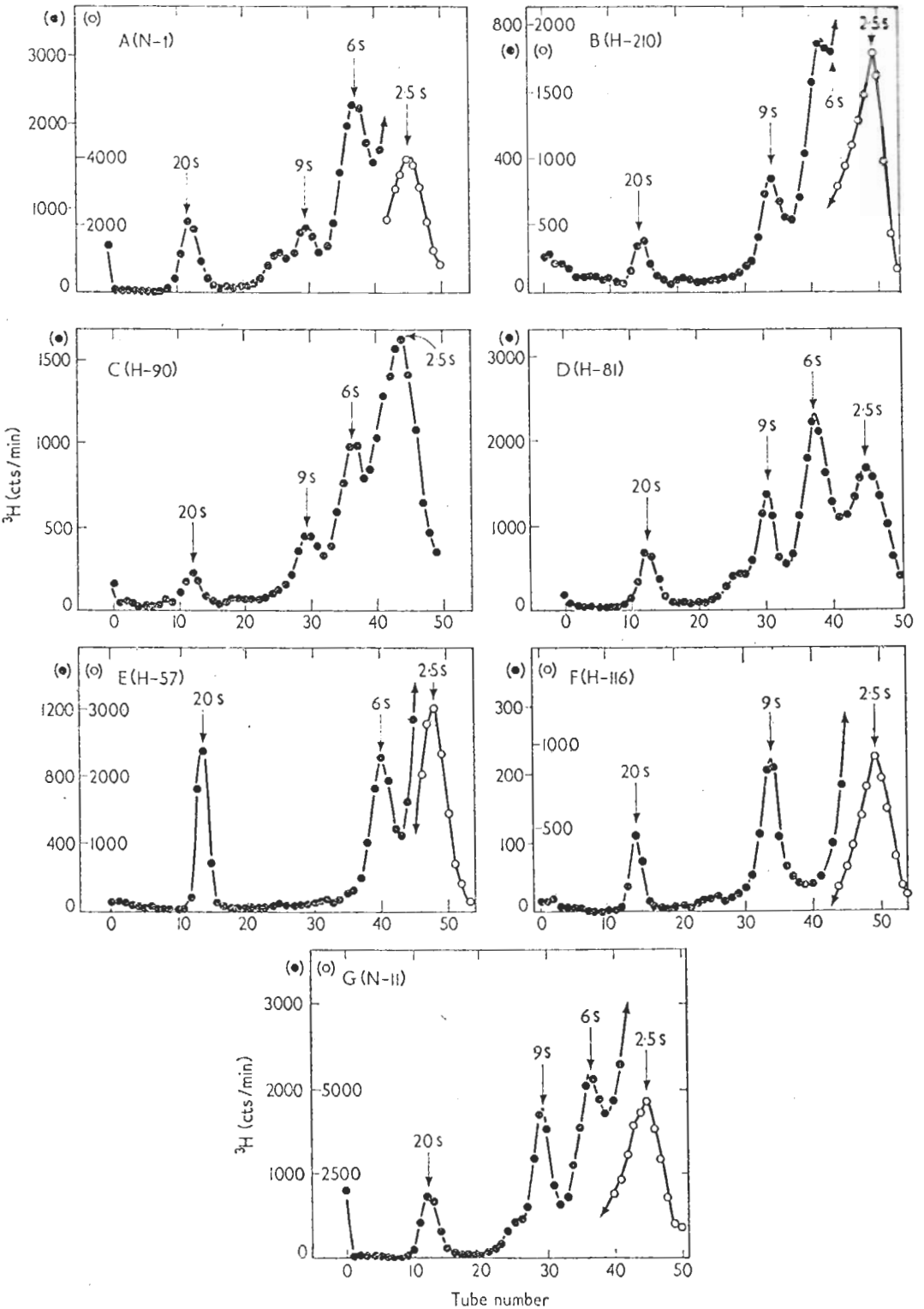
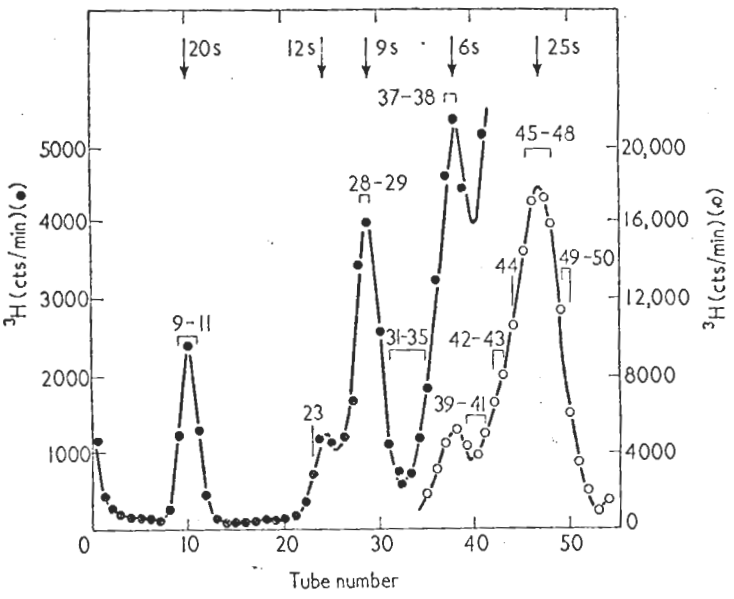


FIG. 4. Sedimentation patterns of proteins in the lysates of ϕX174 amber mutant-infected cells. A 5- or 10-ml. u.v.-irradiated culture of *E. coli* HF4704 was infected with an amber mutant representing each of the seven complementation groups at an input multiplicity of 5 to 10 (t = 0), and the proteins were labeled with either [^{3}H]leucine (10 μc/ml., 0·5 μg/ml.) from t = 12 min to 15·5 min in case of mutants N-1 (cistron A), H-90 (C), H-81 (D) and N-11 (G) or by a mixture of four [^{3}H]amino acids ([^{3}H]arginine, 10 μc/ml., 5·8 μg/ml.; [^{3}H]isoleucine, 10 μc/ml., 0·8 μg/ml.; [^{3}H]lysine, 10 μc/ml., 2·4 μg/ml.; [^{3}H]tryptophan 10 μc/ml., 0·7 μg/ml.) from t = 13 min to 17 min in case of mutants H-210 (B), H-57 (E) and H-116 (F). The lysate prepared from each labeled culture was layered on a 5 to 30% sucrose density-gradient and centrifuged either in an SW50 rotor at 49,000 rev./min for 270 min (N-1, H-90, H-81 and N-11) or in an SW41 rotor at 40,000 rev./min for 480 min (H-210, H-57 and H-116). The absolute numbers of counts are not comparable from one sample to another because conditions of infection and radioisotope labeling varied.


A (N-1)
B (H-210)
C (H-90)
D (H-81)
E (H-57)
F (H-116)
G (N-11)
20 s
9 s
6 s
2·5 s
(•)
(o)
^{3}H (cts/min)
Tube number



Fraction / φX Protein	9-11	23	28-29	31-35	37-38	39-41	42-43	44	45-48	49-50
A/B	*	0	0	0	0	2	2	≃0	0	0
C	*	≃0	0	≃0	0	10	8	3	≃0	0
D	*	≃0	0	≃0	≃0	6	30	61	76	64
E	18	57	97	13	0	15	32	7	≃0	0
F	*	43	3	85	98	52	3	≃2	≃6	≃2
Others	82	≃0	0	2	2	15	25	27	18	34

FIG. 5. Protein composition of the lighter particles.

A 30-ml. culture of u.v.-irradiated *E. coli* HF4704 was infected with wild-type φX174 at a multiplicity of 10 ($t = 0$), and proteins were labeled with a mixture of 6 [^{3}H]amino acids ([^{3}H]arginine, 10 μc/ml., 5·8 μg/ml.; [^{3}H]isoleucine 20 μc/ml., 1·6 μg/ml.; [^{3}H]leucine, 20 μc/ml., 0·24 μg/ml.; [^{3}H]lysine 10 μc/ml., 2·4 μg/ml.; [^{3}H]proline, 30 μc/ml., 0·6 μg/ml.; [^{3}H]tryptophan, 8 μc/ml., 0·5 μg/ml.) from $t = 13·5$ min to 17 min. 0·75 ml. lysate prepared from 15 ml. of culture (approximately 3×10^9 infected cells) was layered onto a 5 to 30% sucrose density-gradient and centrifuged in an SW41 rotor at 40,000 rev./min for 570 min. After centrifugation, fractions were collected into small vials and 5 μl. of each fraction was used for radioactivity counting, the numbered fractions were pooled and subjected to gel electrophoresis. The percentage composition of protein species of each of the pooled fractions indicated in the sucrose gradient profile was calculated from the gel electrophoregrams shown in Fig. 7 and summarized in the lower part of Fig. 5.* Those proteins which do not migrate with any of the identified φX174 proteins (A/B, C, D, E and F proteins) are grouped and designated "others." Among all the proteins of the 20 s particle (fractions 9 to 11), only one (the E protein) appears to co-electrophorese with the marker proteins from wild-type φX174 infected cells. The remaining proteins are tentatively assigned to others since a reasonable estimation of their designation is impossible.

(c) *Protein species in the particles*

In order to determine the proteins in the particles, each region of the sucrose gradient from the wild type phage-infected lysate was subjected to a polyacrylamide gel electrophoresis. Figure 5 represents a sucrose gradient profile of the lysate and some of the electrophoregrams of the fractions are shown in Figure 7. Positions of the six ϕX174-specific proteins (A to F) in the polyacrylamide gel electrophoresis have previously been reported (Gelfand & Hayashi, 1969) and are shown again in Figure 7.

The total extract prepared from cells infected with each amber mutant used in this paper completely lacks the material at the peak position corresponding to the assigned cistron of the gel pattern of the wild-type ϕX174 protein (Gelfand & Hayashi, 1969); thus, any material comigrating with the wild-type ϕX174 protein is considered to be the protein from the corresponding ϕX174 cistron. Conversely, material co-migrating with none of the known ϕX174 gene products is tentatively considered to be non-phage-specific proteins. In Table 1, distribution of each ϕX174 specific protein

TABLE 1

Percentage distribution of ϕX174 proteins among sucrose gradient fractions

ϕX protein	Tube number 8-12 (20 s)	22-25 (12 s)	26-30 (9 s)	31-35 (9-6 s)	36-38 (6 s)	39-41 (6-4 s)	42-43 (4 s)	44 (4-2·5 s)	45-48 (2·5 s)	49-52 <(2·5 s)
A/B	0	0	0	0	0	55	45	0	0	0
						100				
C	0	0	0	0	0	47	41	12	0	0
						100				
D	0	0	0	0	0	1	6	8	63	22
						100				
E	4	9	51	3	0	9	20	4	0	0
	4	9	54			33				
F	0	5	1	15	40	22	2	1	12	2
		5	80					15		

Those sucrose gradient fractions of Fig. 5 of which gel electrophoregrams are not shown in Fig. 7 were separately analyzed in the same gel electrophretic system. From these results and those in Fig. 7, the distribution of each ϕX protein along the whole gradient was calculated. Fractions in tubes 1 to 7 and 13 to 21 were not analyzed because they did not contain significant numbers of counts.

along the sedimentation profile was calculated from Figure 5 and electrophoregrams of the fractions of Figure 5. Results from these experiments are summarized as follows. (1) A and/or B protein are detected between 6 s and 4 s region. (2) C protein sediments between 6 and 2·5 s region. (3) D protein is rich in 2·5 s region as expected from the mutant study (see section (b) of Results). (4) E protein exists in 20, 12 and 9 s regions, but is absent in 6 and 2·5 s regions. It also exists in fractions between 6 and 2·5 s (4 s). (5) F protein is found in the 12, 6 and 2·5 s regions. The small peak at the position of F protein in the electrophoregram shown in Figure 7(c) in 9 s region is considered to be due to contamination from 6 s region. (6) Non-phage-specific proteins

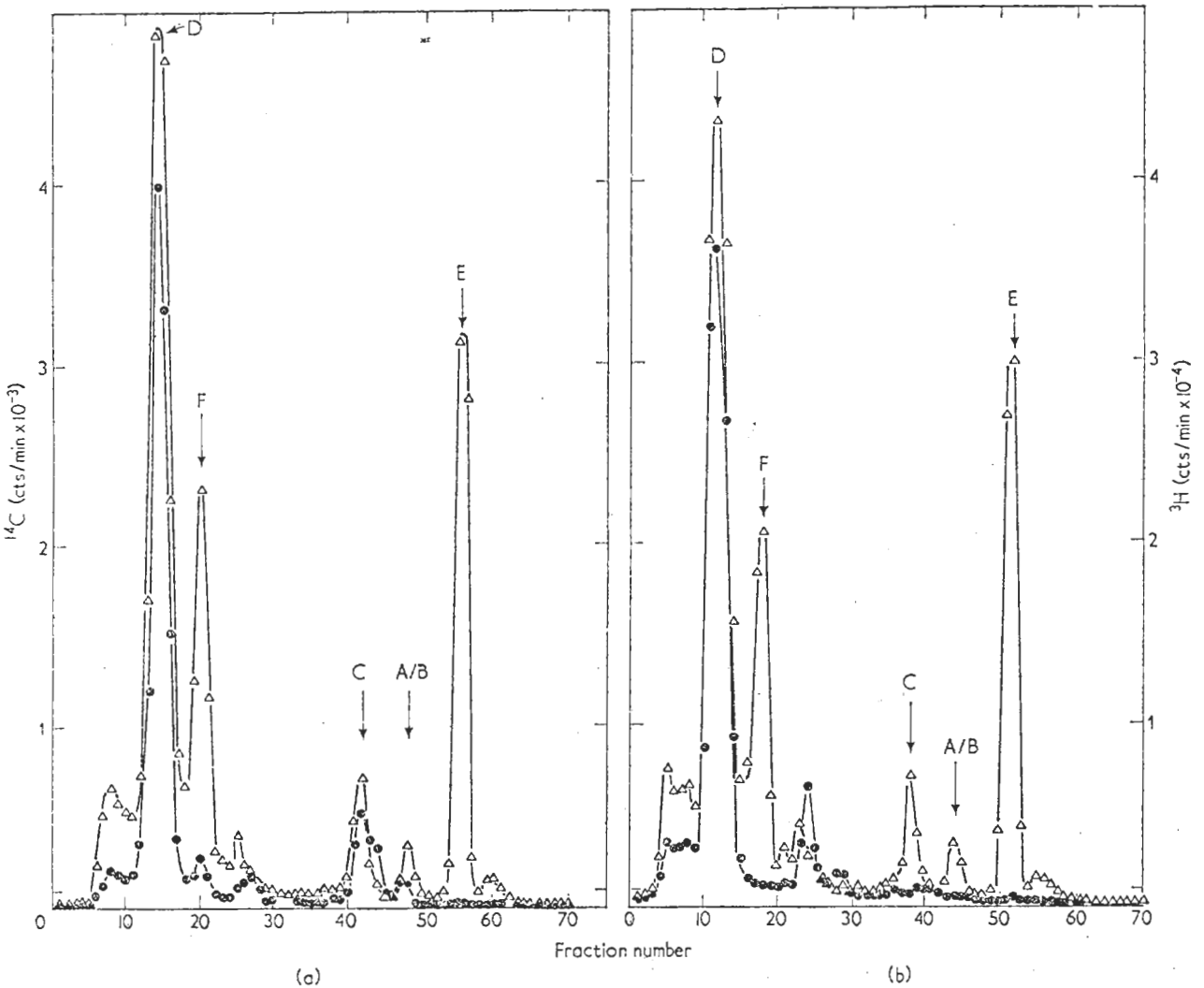


FIG. 6

are found in 20 s region and near the top of the gradient, but not in 12, 9 and 6 s regions (see Fig. 5). (7) The existence of E and F proteins in 4 s and 2·5 s regions respectively was confirmed by the absence of materials at the corresponding positions of the gel electrophoresis patterns when E or F mutant-infected cells were lysed and 4 s region of the E mutant lysate or 2·5 s region of the F mutant lysate was examined by gel electrophoresis (Fig. 6(a) and (b)).

We conclude from these results that: (1) 20 s particles are composed of E protein and non-phage specific proteins; (2) 9 and 6 s particles are exclusively composed of E and F proteins, respectively; (3) apart from the non-phage specific proteins, those proteins sedimenting slower than 6 s consist of C, A/B, E, F and D.

(d) *20 s particles*

The electrophoretic analysis of the wild type 20 s particles shows that the particles contain E protein (Fig. 7(a)). This apparently contradicts the results obtained from the mutant study; in the E mutant extract, greater than normal amounts of 20 s particles are found (Fig. 4(E)). However, Gelfand & Hayashi (1969) found that the E mutant (H-57) produces an amber protein with a molecular weight of approximately two-thirds of the wild-type E protein. Thus the apparent contradiction can be explained if the E amber protein can replace the wild type E protein for the 20 s particles to be formed. This was shown to be the case by demonstrating the existence of the E amber protein in the E mutant 20 s particles (Fig. 8). The *species* of the non-phage specific proteins in the 20 s particles of both the wild type and the E mutant seems to be similar (compare Fig. 7(a) and Fig. 8). However, the relative *amounts* of the non-phage-specific proteins in the 20 s particles to E or amber E protein are very different. It would be fortuitous that the wild-type 20 s particles and the amber E 20 s particles sediment with a similar S-value. It would be expected that the other E amber mutants producing amber E protein with different molecular weight would produce either different "20 s aggregate" or none at all.

These observations lead us to speculate that the 20 s particle formed in the wild type infected cells may not be a precursor particle of the assembly process. Rather, it may be an artifact produced during the preparation of the lysate. This will be shown to be the case in the following section.

(e) *Interconversion of the particles during cell lysis*

In order to examine any possible protein aggregation or disaggregation which might occur during the preparation of the cell lysate and subsequent sucrose density gradient centrifugation, reconstruction experiments were performed.

FIG. 6. Gel electrophoregram of the 2·5 to 4 s region of the lysates from amber E- or F-infected cells.

H-57 (E)- or H-116 (F)-infected cell lysate was prepared as described in Fig. 4. Proteins were labeled with [^{3}H]leucine, [^{3}H]proline, [^{3}H]phenylalanine and [^{3}H]alanine (80 μc/ml., 2 μg/ml.) from t = 13·5 min to 17·5 min after infection. The lysate prepared from each labeled culture was layered on a 5 to 30% sucrose density-gradient and centrifuged in an SW41 rotor at 40,000 rev./min for 480 min. After collecting fractions, samples were taken and the radioactivity of each fraction was determined. Data are not shown but are very similar to the profiles shown in Fig. 4 (E) and 4 (F). Regions corresponding to fractions 42 to 48 of Fig. 4 (E) (H-57 infected cell lysate) and fractions 47 to 49 of Fig. 4 (F) (H-116 infected cell lysate) were pooled and analyzed by a gel electrophoresis. ^{14}C-labeled wild-type infected cell protein was used as a reference.

(a) 4 to 2·5 s region of amber E-infected cell lysate

(b) 2·5 s region of amber F-infected cell lysate; —●—●—, ^{3}H radioactivity; —△—△— ^{14}C radioactivity.

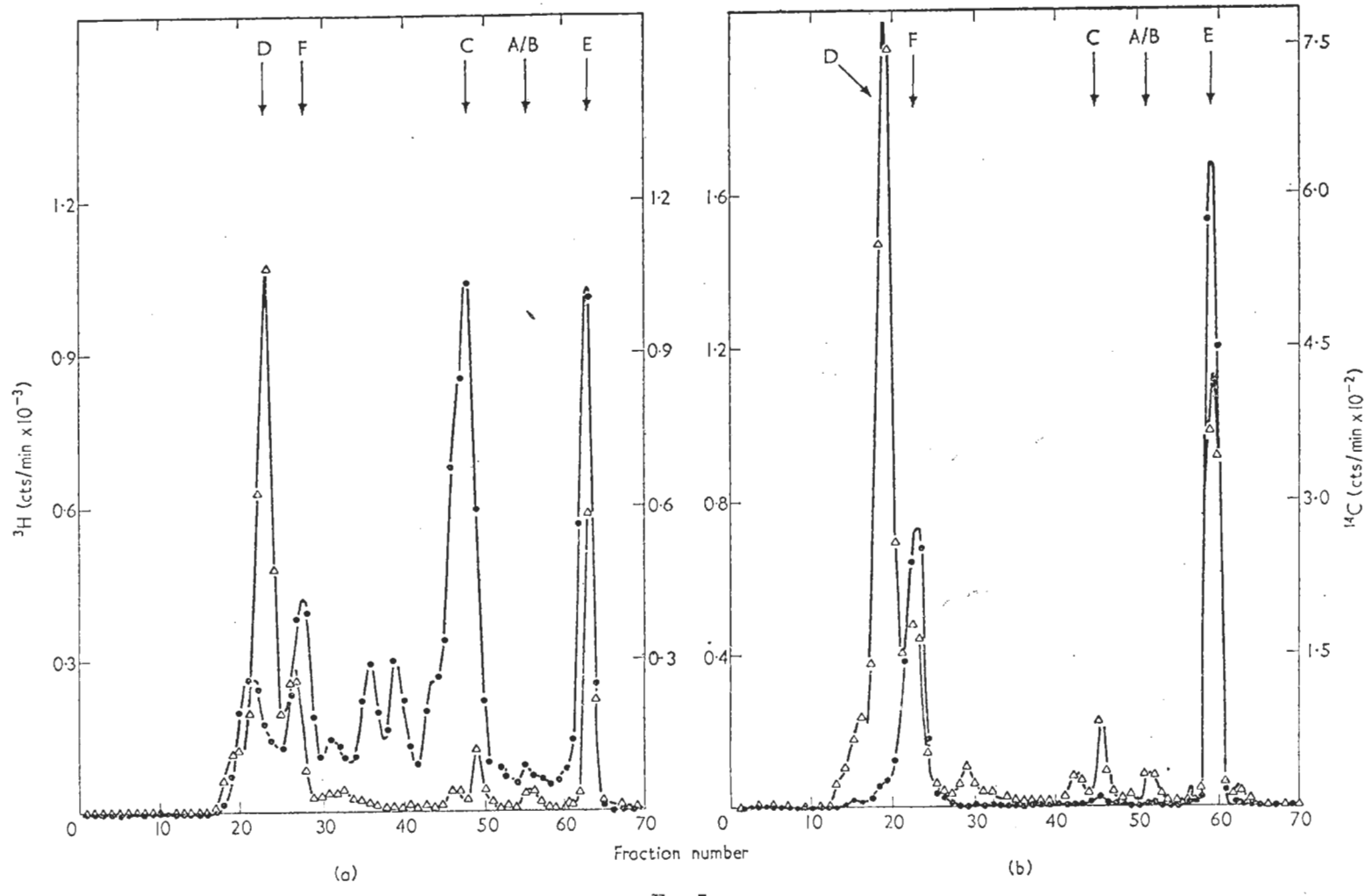


FIG. 7.

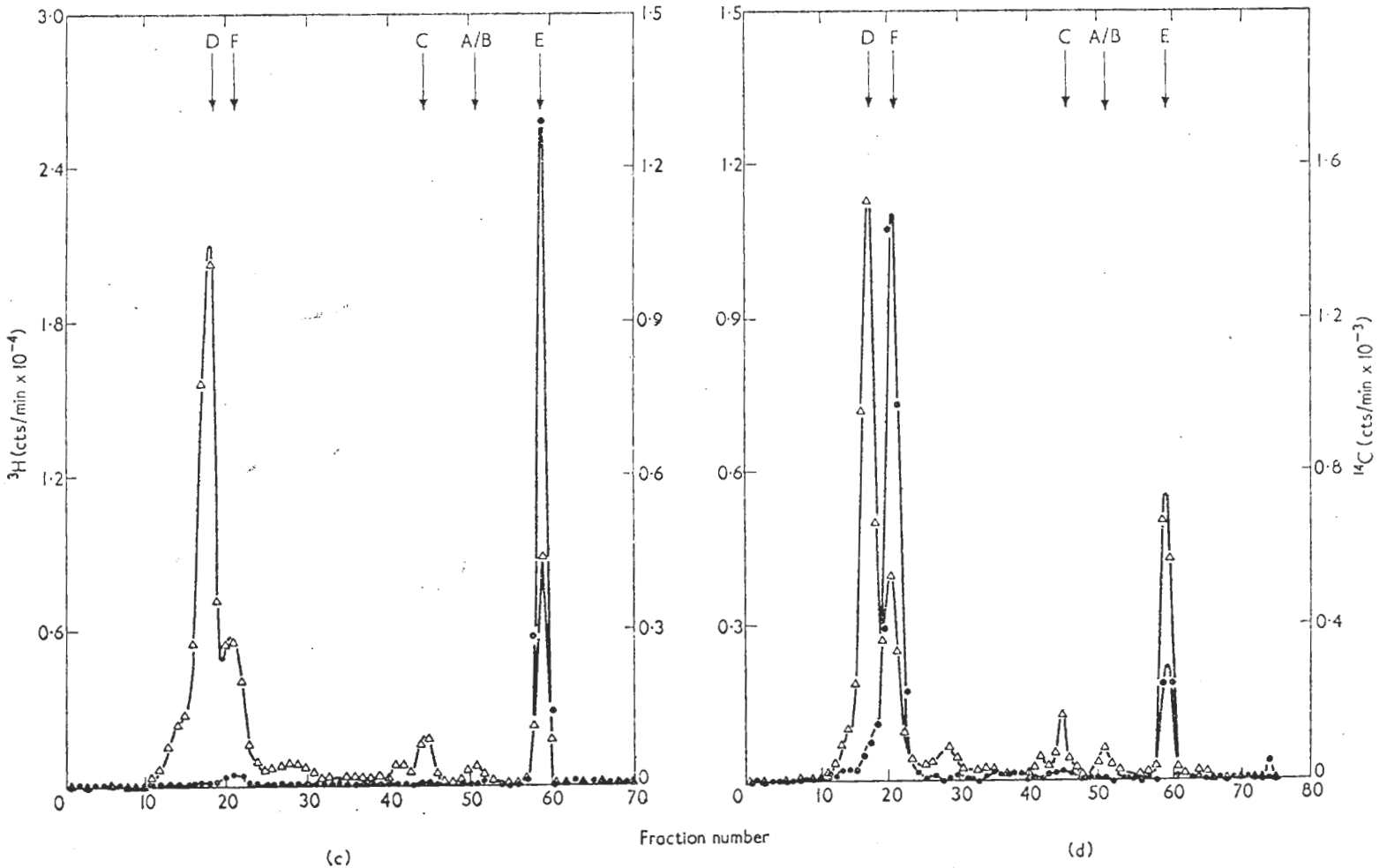


FIG. 7—*continued.*

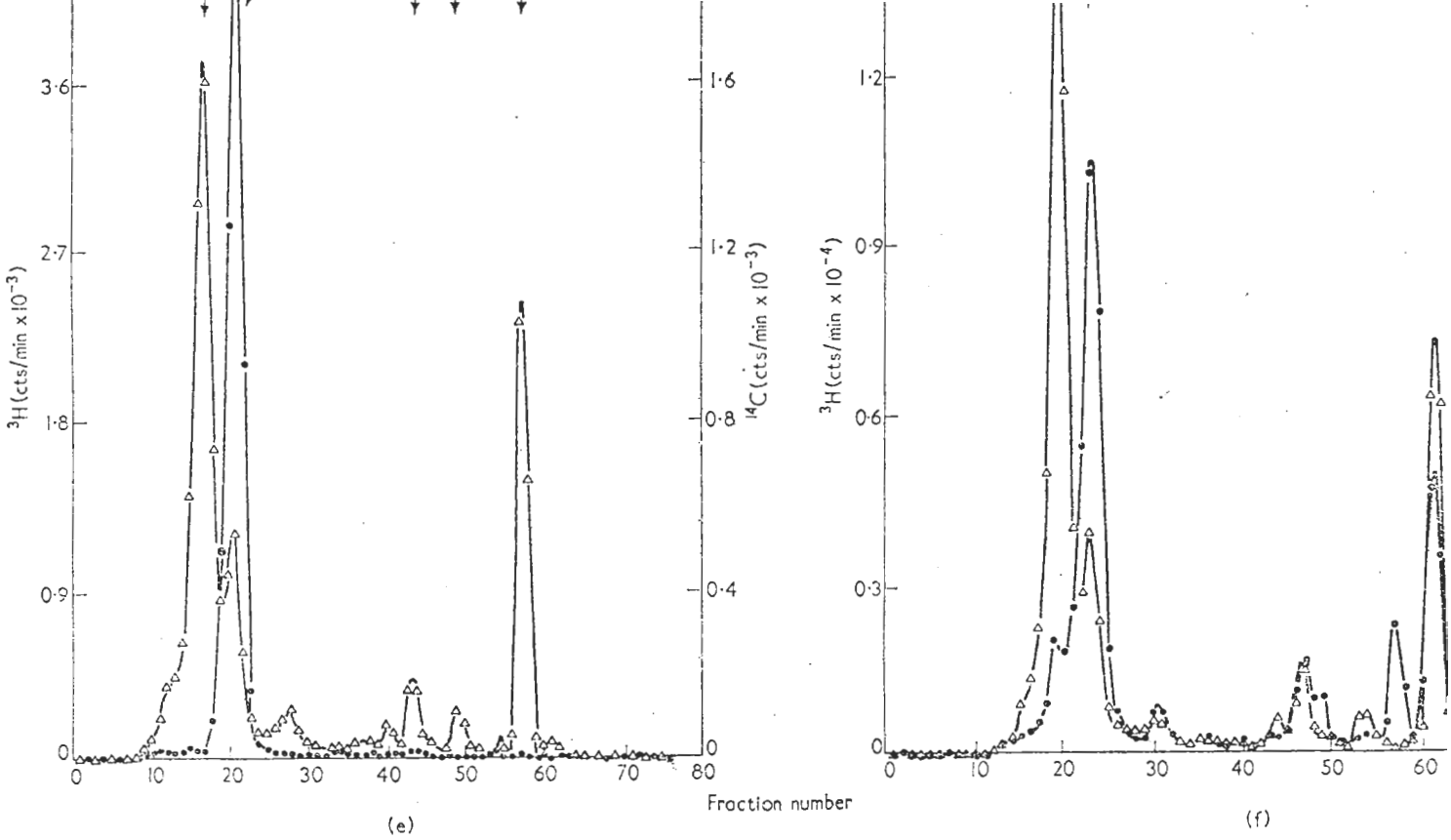


FIG. 7—*continued.*

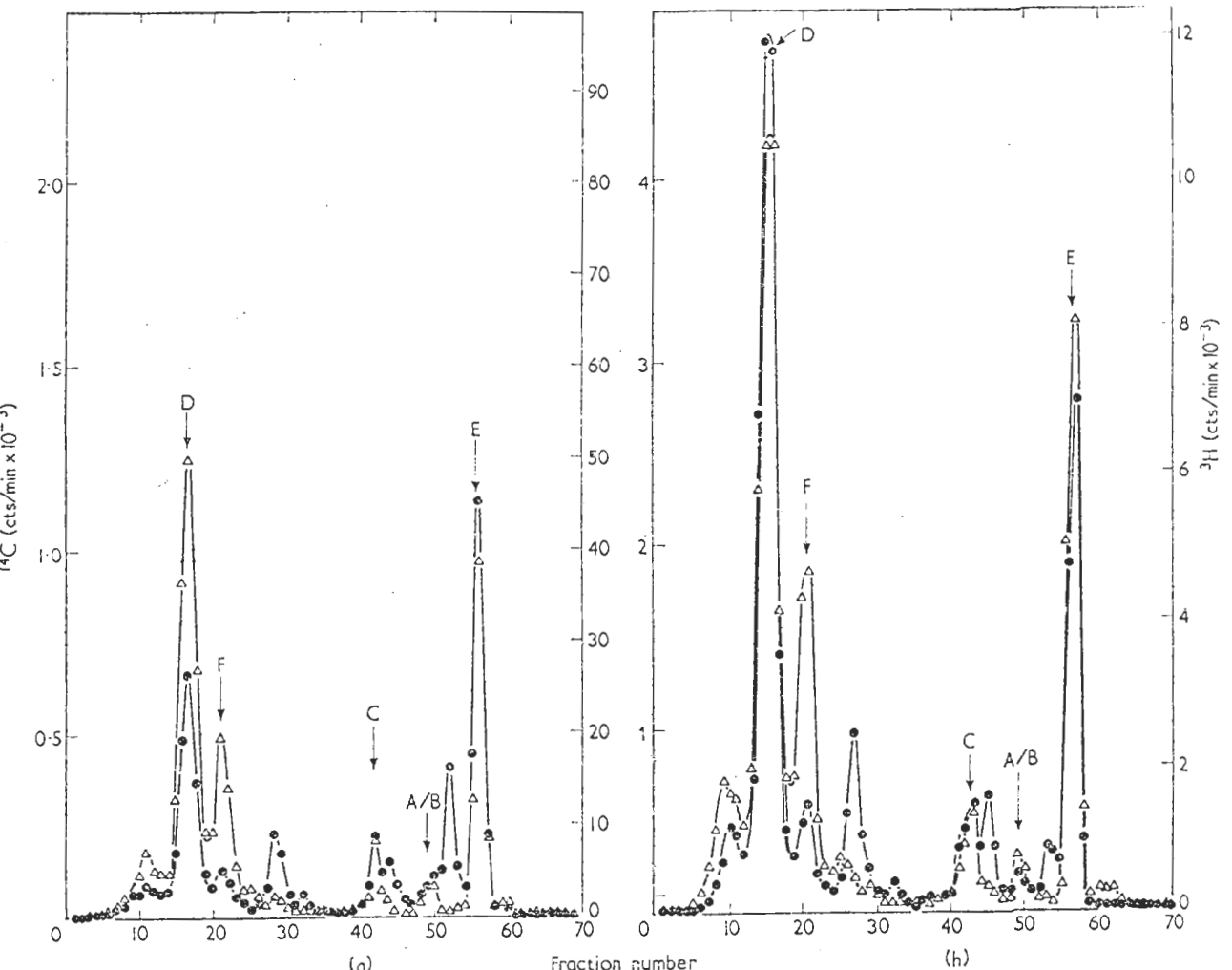


FIG. 7—*continued.*

FIG. 7—*continued.*

FIG. 8. Gel elect
tive host
(—●—●—) ^{3}H

FIG. 7. Gel elect
cells. Appropriato
was added to a fin
a marker. The sam
gels were run from
prepared from ϕX
(a) (—●—●—
(—●—●—) ^{3}H f
^{3}H fraction 28-29
31-35; (—△—△-
(—△—△—) ^{14}C
wild type total pro
proteins; (h) (—●
(—●—●—) ^{3}H fr
^{3}H fraction 49-50;

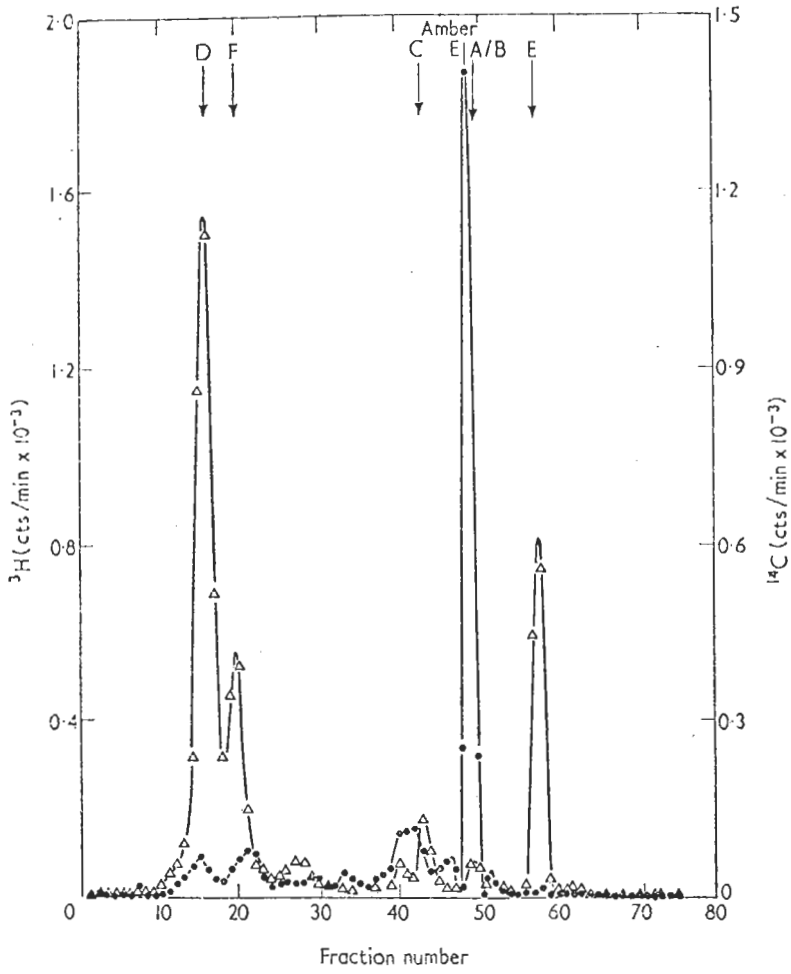


FIG. 8. Gel electrophoregram of the 20 s particle isolated from H-57 (cistron E)-infected restrictive host

(—●—●—) ^{3}H 20 s E particle; (—△—△—) ^{14}C wild-type proteins.

FIG. 7. Gel electrophoregrams of the lighter particles prepared from wild-type ϕX174-infected cells. Appropriate ^{3}H- and ^{14}C-labeled samples were mixed in a final volume of 0·15 ml., glycerol was added to a final concentration of 7·5% and a small amount of bromophenol blue was added as a marker. The samples were heated at 60°C for 30 min, cooled, and layered on top of the gel. The gels were run from cathode to anode at 180 v for about 5·5 hr. ^{14}C-labeled marker proteins were prepared from ϕX174 wild-type infected cells according to Gelfand & Hayashi (1969).

(a) (—●—●—). ^{3}H fraction 9-11 (20 s); (—△—△—) ^{14}C wild type total proteins; (b) (—●—●—) ^{3}H fraction 23 (12 s); (—△—△—) ^{14}C wild type total proteins; (c) (—●—●—) ^{3}H fraction 28-29 (9 s); (—△—△—) ^{14}C wild type total proteins; (d) (—●—●—) ^{3}H fraction 31-35; (—△—△—) ^{14}C wild type total proteins; (e) (—●—●—) ^{3}H fraction 37-38 (6 s); (—△—△—) ^{14}C wild type total proteins; (f) (—●—●—) ^{3}H fraction 39-41; (—△—△—) ^{14}C wild type total proteins; (g) (—●—●—) ^{3}H fraction 42-43; (—△—△—) ^{14}C wild type total proteins; (h) (—●—●—) ^{3}H fraction 44; (—△—△—) ^{14}C wild type total proteins; (i) (—●—●—) ^{3}H fraction 45-48 (2·5 s); (—△—△—) ^{14}C wild type total proteins; (j) (—●—●—) ^{3}H fraction 49-50; (—△—△—) ^{14}C wild type total proteins.

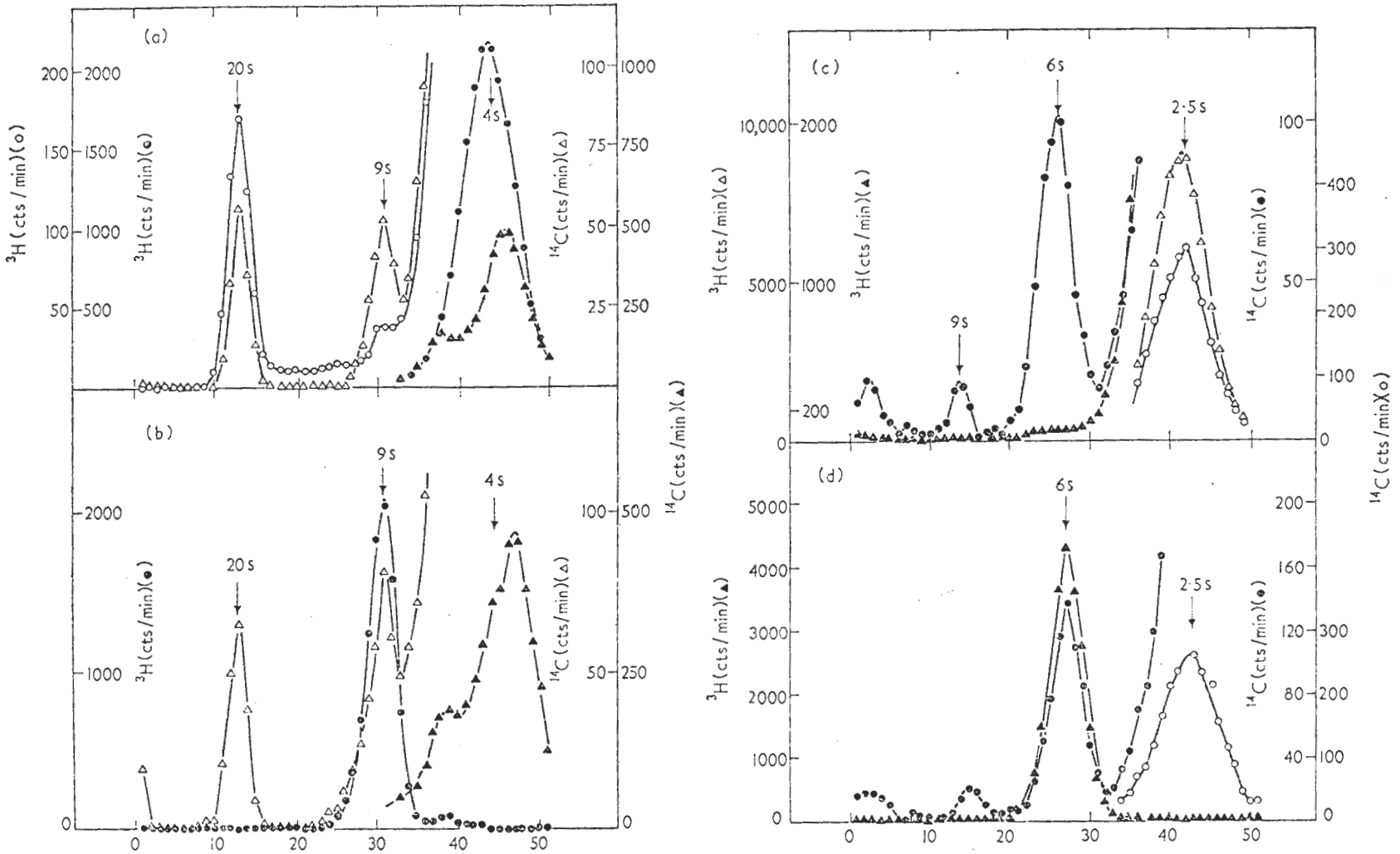

(a)
20s
9s
4s
^3H (cts / min)(o)
^3H (cts / min)(●)
^{14}C (cts/min)(△)
(b)
9s
4s
20s
^3H (cts/min)(●)
^{14}C (cts/min)(△)
^{14}C (cts/min)(▲)
(c)
6s
2·5s
9s
^3H (cts/min)(△)
^3H (cts/min)(▲)
^{14}C (cts/min)(●)
(d)
6s
2·5s
^3H (cts/min)(▲)
^{14}C (cts/min)(●)
^{14}C (cts/min)(o)
Tube number

FIG. 9.

Infected cells labeled with [^{14}C]amino acid were mixed, before lysis, with ^{3}H-labeled particles which had been isolated by a separate sucrose density-gradient centrifugation. If any aggregation or disaggregation occurs during these procedures, ^{3}H counts will be shifted to new positions in a sucrose density-gradient. This type of experiment is shown in Figure 9. Cells infected with wild type ϕX174 were labeled with [^{14}C]amino acids and lysed in the presence of ^{3}H-labeled 4 s particles (Fig. 5, fractions 42 and 43, 32% of counts in the fractions are E protein (Fig. 7(g)). Some of the ^{3}H counts are shifted to the 20 s region (Fig. 9(a)). The 20 s region of Figure 9(a) was analyzed by a gel electrophoresis (Fig. 10). Figure 10 shows that 30% of ^{3}H counts were found at the position of E protein. Therefore, during the lysis procedure 4 s E protein is transferred to E component of 20 s particles in association with non-phage specific proteins existing in infected cells. Small amounts (30 cts/min) of the ^{3}H counts can be seen in the 9 s region of Figure 9(a). The 4 s particle added during the lysis procedure contained 6000 cts/min of E protein (32% of the input 18,770 cts/min). Subtracting ^{3}H E protein counts shifted to the 20 s and 9 s regions from 4 s E protein (220 cts/min: 30% of ^{3}H counts at the 20 s region of Fig. 9(a) and 30 cts/min at the 9 s region) 5750 cts/min remain in the 4 s region. The ratio of 9 s [^{3}H]protein counts to the 4 s ^{3}H-labeled E protein counts is 0·5 to 99·5. On the other hand, the distribution of the ^{14}C-labeled E protein between 9 s and 4 s region in Figure 9(a) would be similar to the results shown in Table 1 (54 *versus* 33). Therefore, most of the 9 s particles would exist in the cell before lysis. When ^{3}H-labeled 9 s particles (composed of E protein only, Fig. 5, fractions 28 and 29) are mixed with ^{14}C-labeled infected cells, the ^{3}H counts are not shifted to any other particle (Fig. 9(b)). It seems that 9 s E protein, unlike 4 s E protein is specifically prevented from combining with the cellular proteins to form 20 s particles.

FIG. 9. Sedimentation patterns of lysates prepared from wild-type ϕX174-infected cells mixed with isolated E or F protein particles.

(a) A 3-ml. culture of u.v.-irradiated *E. coli* HF4704 (6×10^{8} cells) was infected with wild type ϕX174 at a multiplicity of 10 ($t = 0$), and proteins were labeled with a mixture of 6 amino acids ([^{14}C]alanine, 0·76 μc/ml., 0·5 μg/ml.; [^{14}C]asparatic acid, 0·32 μc/ml., 0·5 μg/ml.; [^{14}C]leucine 1·05 μc/ml., 0·5 μg/ml.; [^{14}C]lysine, 0·83 μc/ml., 0·5 μg/ml.; [^{14}C]phenylalanine, 2·03 μc/ml., 0·5 μg/ml. and [^{14}C]valine, 0·68 μc/ml., 0·5 μg/ml.) from $t = 13$ min to 17 min. At $t = 17$ min, the culture was quickly chilled to 0°C in a dry ice–methanol bath. The infected cells were sedimented by centrifugation, washed in 3 ml. of 30% sucrose in Tris–EDTA–NaCl buffer, and resuspended in 0·3 ml. Tris–EDTA buffer. The cell suspension was incubated with 100 μg of lysozyme and 17 μg of pancreatic RNase at 0°C for 20 min. At the end of the incubation, 0·1 ml. of the fractions 42 and 43 of Fig. 5 (^{3}H-labeled) was added to the cell suspension. 0·1 ml. of this fraction corresponds to approximately 1·2 ml. of the infected cell culture with respect to 4 s E protein. The mixture was frozen in dry ice–methanol bath and stored in a −20°C freezer for 12 hr, and then thawed in a 37°C water bath for 3 min. The thawed lysate was cleared by a low-speed centrifugation and the whole supernatant fraction (0·3 ml.) was layered onto a 5 to 30% sucrose density-gradient in Tris–EDTA–NaCl buffer and centrifuged in an SW50 rotor at 49,000 rev./min for 270 min.

(b) 0·1 ml. of ^{3}H-labeled fractions 26 to 30 of Fig. 5 was mixed with ^{14}C-labeled, infected cells equivalent to 3 ml. of original culture in a final volume of 0·4 ml. The fraction, tubes 26-30, is 97% pure in 9 s E protein in terms of ^{3}H counts (see Fig. 5). 0·1 ml. of this fraction corresponds to approximately 1·1 ml. of infected cell culture with respect to 9 s E protein. The rest of the experimental procedures are as described in (a).

(c) 0·1 ml. of ^{3}H-labeled fractions 45 to 48 (equivalent to 1·2 ml. of the original, infected cell culture) of Fig. 5 was mixed with ^{14}C-labeled, infected cells, lysed and processed as described in (a) except that the centrifugation was for 540 min.

(d) 0·1 ml. of ^{3}H-labeled fractions 36 to 38 (equivalent to 1·2 ml. of the original, infected cell culture) of Fig. 5 was mixed with ^{14}C-labeled, infected cells, lysed, and processed as in (c).

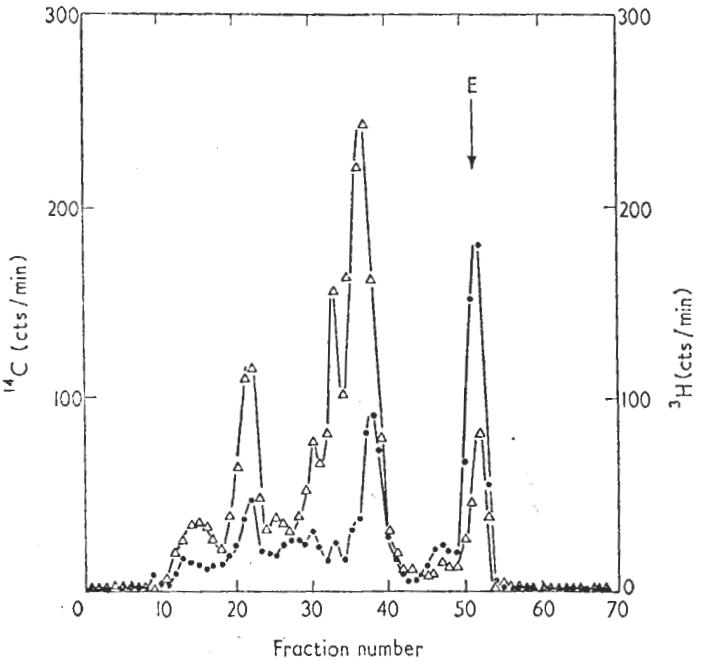


FIG. 10. Gel electrophoregram of the reconstructed 20 s particles.
Samples of 20 s particles in Fig. 9(a) were subjected to gel electrophoresis. The position of E protein was determined by a simultaneous run of the standard protein (not shown).
(—●—●—) ^{3}H radioactivity; (—△—△—) ^{14}C radioactivity.

The same type of experiment was performed by mixing F protein-containing particles with infected cell extract. ^{3}H-labeled 2·5 s protein (fractions 45 to 48 of Fig. 5) was mixed with ^{14}C-labeled infected cells, lysed and analyzed by sucrose density-gradient sedimentation. Total ^{3}H input count was 75,000 cts/min, of which 6% were F protein (Fig. 5). Small amounts of ^{3}H counts (about 140 cts/min) appeared at the 6 s region (Fig. 9(c)). The distribution of [^{14}C]protein between the 6 and 2·5 s regions can be estimated as 80 to 15 from Table 1. When ^{3}H-labeled 6 s particles (fractions 37 and 38 of Fig. 5) were mixed with ^{14}C-labeled infected cells, lysed and analyzed as described above (Fig. 9(d)), all of the ^{3}H counts sedimented at 6 s and no counts were found in the 2·5 s region. These experiments show that the 6 s F particle must exist before lysis and the intracellularly formed 6 s F particle cannot be converted to 2·5 s F protein during lysis of the cell and the subsequent sucrose density-gradient.

(f) *Protein composition of the φX174 particle*

In order to find any possible correlation between protein subunits of the mature phage particle and the particles described in the preceeding sections, the protein composition of φX174 was estimated. Figure 11 shows the gel electrophoregram of the proteins isolated from the purified φX174 particle labeled with [^{14}C]alanine, [^{14}C]isoleucine, [^{14}C]leucine, [^{14}C]phenylalanine and [^{14}C]valine. Besides the A and/or B, E and F proteins, which have been previously shown (Gelfand & Hayashi, 1969) to be the phage structural proteins, a fourth peak (X) is observed. This component migrated faster than D protein. In Table 2 the calculated protein composition of three

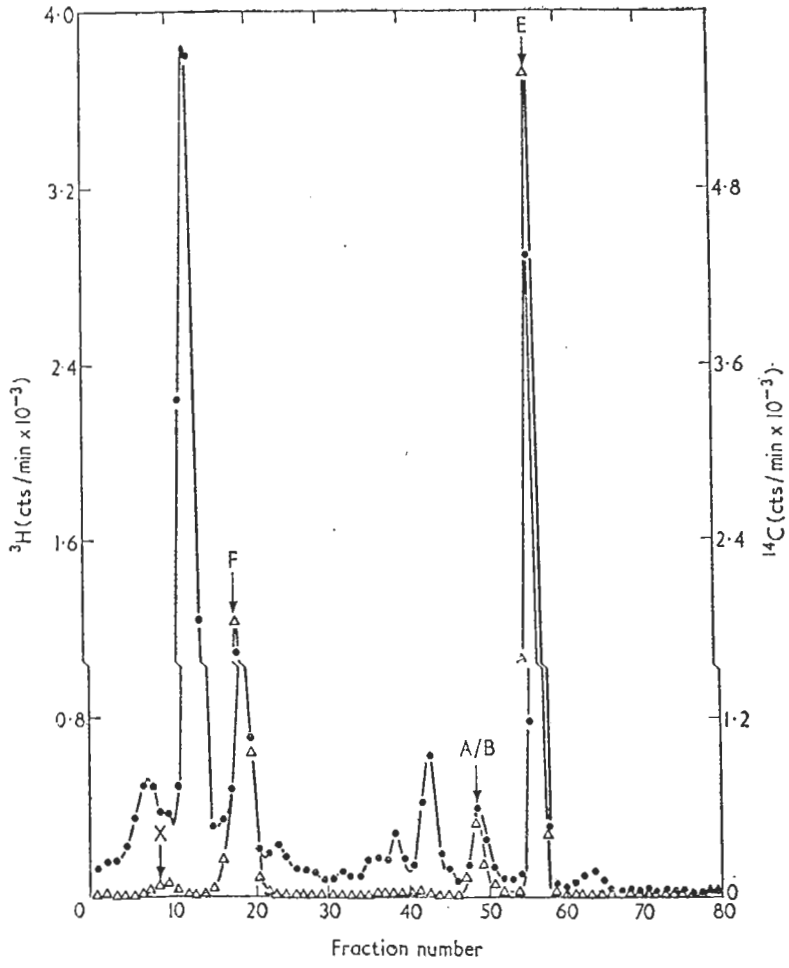


FIG. 11. Gel electrophoregrams of proteins purified from wild-type ϕX174 mature phage particle. A 20-ml. culture of *E. coli* HF4704 was infected with *cm3* at a multiplicity of 5 ($t = 0$), and proteins were labeled with a mixture of five [^{14}C]amino acids ([^{14}C]alanine, 2·5 μc/ml., 1·64 μg/ml.; [^{14}C]isoleucine, 2·5 μc/ml., 1·21 μg/ml.; [^{14}C]leucine, 2·5 μc/ml., 1·26 μg/ml.; [^{14}C]phenylalanine, 2·5 μc/ml., 1·00 μg/ml.; [^{14}C]valine, 2·5 μc/ml., 1·47 μg/ml.) from $t = 0$ min to 120 min. Phage particles were released from the cell by lysozyme and freezing–thawing and purified by sucrose gradient centrifugation and CsCl equilibrium centrifugation. Proteins of the purified phage were dissociated by dialysis against 10 × SB, then against 1 × SB, each for 12 hr. ^{3}H-labeled wild type standard protein mixture (—●—●—) and ^{14}C-labeled phage proteins (—△—△—).

TABLE 2

Protein composition of the ϕX174 particle

Experiment	Isotope	ϕX174 structural proteins			
		A/B	E	F	X
1	[^{3}H]leucine	6·4	64·2	25·7	3·3
2	[^{3}H]alanine, [^{3}H]leucine [^{3}H]valine, [^{3}H]asparatic acid	6·4	64·8	26·8	1·7
3	[^{14}C]alanine, [^{14}C]isoleucine, [^{14}C]phenylalanine, [^{14}C]valine	6·7	63·4	27·3	2·6

In each experiment phage was labeled with the indicated amino acids at a similar specific activity, purified by sucrose gradient centrifugation and equilibrium centrifugation in CsCl. The distribution of the counts among the four protein components is expressed by percentage.

preparations of ϕX174 particles, each labeled by a different combination of amino acids, are listed. The calculated compositions of the three major proteins (A and/or B, E and F) fell in a narrow range of variations regardless of the kinds of radioactive amino acids used: A and/or B protein between 6·4 and 6·7%, E protein between 63·4 and 64·8%, and F protein between 25·7 and 27·3%. The composition of the fourth protein (X) seems to vary somewhat depending upon the isotopes used (1·7 to 3·3%).

4. Discussion

(a) *Small molecular weight particles*

Greenlee & Sinsheimer (1968*b*) first observed in ϕX174-infected cells the formation of 6 s particles (subunit material) which are precipitable with anti-ϕX174 serum. They also showed that the subunit material can be chased into the mature phage and non-infective 70 s particles. In this paper, we found ϕX174 specific particles sedimenting at 6 s. We have also demonstrated that the 6 s particle contains only F protein by showing that the F mutant (but not other mutants) fails to produce the particle (Fig. 4), and by analyzing the protein component of the wild-type 6 s particles (Fig. 7(e)). Since our F mutants cannot complement with Sinsheimer's mutants in cistron III (Jeng & Hayashi, unpublished observation) which have been shown to be responsible for serum blocking power (Sinsheimer, 1968), it is considered very likely that our 6 s particles and their 6 s subunit material are identical. We have also shown that the formation of the 6 s particle does not require the function of ϕX174 gene products other than F protein.

We found a new precursor particle which is composed of E protein only and sediments at 9 s. The E protein is also found in 20 s particles and in a region of approximately 4 s. The 20 s particles are aggregates of E protein and non-phage specific proteins which are synthesized in the infected cells. The frequently observed 12 s particle contains F and E proteins.

(b) *The assembly process*

Based on the observations recorded here, we will attempt to explain the assembly of these particles.

First, we exclude the 20 s particle from the main pathway of the process because the major portion of this particle, if not all, can be explained as arising during preparation and sucrose gradient sedimentation of the cell lysate. Second, we assume the process proceeds from a lighter particle to a heavier particle.

The E protein, when released from the protein-synthesizing apparatus of the cell, appears as a 4 s molecule. The molecular weight of E protein was estimated from acrylamide gel electrophoregrams to be 52,000 (Gelfand, personal communication). This number does not agree with the molecular weight obtained by Carusi & Sinsheimer (1963). Their estimation is 25,000. Treating the proteins isolated from mature phage with iodoacetamide and mercaptoethanol did not change the electrophoregram. Our preparation of E protein is larger than ovalbumin (mol. wt 45,000) (Warner, 1954) in a sodium dodecyl sulfate–acrylamide gel system (Gelfand, personal communication). The *S*-value of a globular protein can be obtained from its molecular weight (Martin & Ames, 1961). On the assumption that the E protein is not atypical with respect to shape, we estimate it to be 4 s. Thus, the E protein we find in the 4 s region would be a monomer.

As a second step, the 4 s E protein aggregates to form a 9 s particle. This aggregation process does not require the function of any other ϕX174 gene products. Again, under the assumption that the 9 s E protein is globular, it would have a molecular weight of 170,000. Therefore, the 9 s particles would be a trimer of E protein.

The molecular weight of ϕX174 phage is $6{\cdot}2 \times 10^6$ daltons (Sinsheimer, 1959*a*). Subtracting the DNA component of the phage ($1{\cdot}7 \times 10^6$) (Sinsheimer, 1959*b*), the total molecular weight of the protein fraction is $4{\cdot}5 \times 10^6$ daltons. From acrylamide gel electrophoresis of the mature phage (Fig. 11 and Table 2), we estimate that about 64% of the phage protein components is E protein, which is $2{\cdot}9 \times 10^6$ daltons. Dividing by the molecular weight of E protein (52,000), there would be about 60 E-protein molecules per phage. The particles of ϕX174 are icosahedral with knobs or spikes at the 12 apices of 5-fold symmetry (Sinsheimer, 1968). Sinsheimer also reported that the spike can be removed from the particle by treatment with 4 M-urea, leaving a core of nucleic acid surrounded by a protein jacket (capsid). The capsid consisted of E protein (Edgell, Hutchison & Sinsheimer, 1969). In order to construct an icosahedral shell (capsid), the smallest number of subunits required is 60 (Casper & Klug, 1962). In this case, one face contains three structural units. The calculation showing that the 9 s E particle is a trimer of E protein may imply that the particle would be a face of the icosahedron. The molecular weight estimation of the 9 s E particles was also attempted by gel filtration through Sephadex G200. However, it was found that the 9 s particles were eluted heterogeneously between the positions of catalase and bovine serum albumin, with considerable tailing toward the lower molecular weight region under the conditions used. This may be due to the partial dissociation of the particles to a smaller aggregate and/or adsorption to the Sephadex particles.

The F protein (the molecular weight by acrylamide gel electrophoresis ~20,000 (Gelfand, personal communication)) appears as 6 s particles. A globular 6 s protein would have a molecular weight of 95,000. The molecular weight estimation by Sephadex column chromatography also supports this estimation (Fig. 12). Therefore, the the 6 s F particle would contain five molecules of F protein.

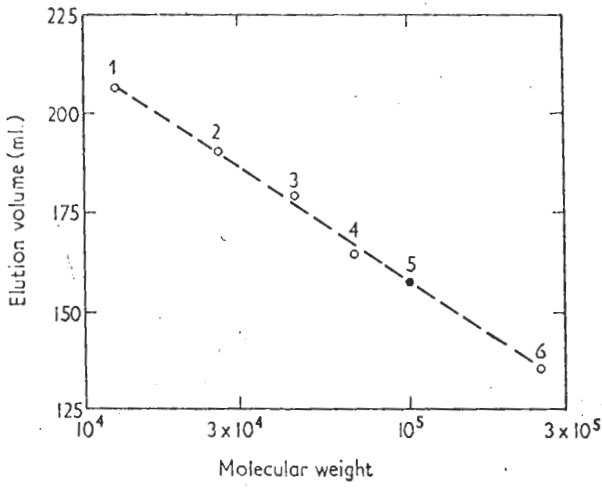


FIG. 12. Determination of molecular weight of 6 s particle by gel filtration.
(1) Cytochrome c; (2) lactoglobulin; (3) ovalbumin; (4) bovine serum albumin; (5) 6 s particle; (6) catalase.

By acrylamide gel electrophoresis of whole phage protein, 26% has been shown to be F protein (Table 2). Therefore, total F protein per phage would be $1{\cdot}2 \times 10^6$ daltons. There are 12 spikes per phage at each apex of an icosahedron. If these 12 spikes are identical, in each spike there must be 1×10^5 dalton equivalents of F protein, that is, it must contain F pentamer.

At the present time, we do not know whether or not the morphologically recognizable spike is composed of F protein solely. It is known that A and/or B protein are involved some way in the phage structure (Jeng & Hayashi, unpublished observation). However, it is extremely interesting to note that the 6 s F protein pentamer is an already mature spike component as far as F protein is concerned. If A and/or B proteins are required for the final assembly to a spike, these proteins must attach to an F pentamer. The fact that the 6 s particle is a pentamer raises another interesting consideration. At each apex of an icosahedron, there is 5-fold symmetry. If a spike attaches to an apex in a symmetrical way, then the spike would also have a 5-fold symmetry, and so would the 6 s F-protein pentamer.

It is a tempting speculation that each subcomponent of the mature phage, that is, faces of the capsid icosahedron and spike component, have been finished at 9 s E trimer and at 6 s F pentamer, respectively.

Frequently observed 12 s particles have not been studied extensively. Here we describe some properties of this particle. It contains 57% of E and 43% of F proteins; therefore, it could be an $((F)_2E)n$ complex. It is easily converted to ~9 s particles during storage at 0°C in Tris–EDTA buffer. Whether or not this particle is a component in the assembly process is not known.

We thank Drs W. Zucker and M. Simon for help in preparing this manuscript.

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Note added in proof: Burgess & Denhardt ((1969) *J. Mol. Biol.* 44, 377) have estimated the molecular weight of phage proteins using a slightly different gel electrophoresis system. Their estimates of the molecular weight of cistron VII (our cistron E) protein, and cistron III (our cistron F) protein are 48,000 and 19,000, respectively.

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