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Isolation and Characterization of the Four Major Proteins in the Virion of Bacteriophage ϕX174†

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ABSTRACT: A preparative method is described for the isolation of the major protein species from the virion of bacteriophage ϕX174. Two proteins, the cistron G and H products, are located in the virion spikes. After removal of the spikes, the capsid contains the cistron F product as well as a small protein which is the product of cistron J and the majority of the DNA. During the removal of the spikes, a precipitate containing the F and G proteins is formed. The proteins from the spike, capsid, or precipitate can be isolated on the basis of size by gel-filtration chromatography. The cistron G protein has an amino-terminal methionine, while the small J protein has an amino-terminal serine. Amino acid compositions as well as peptide maps indicate each species is unique and that, in sum, they account for over half the coding capacity of the viral genome.

The number of proteins detected in the virion of bacteriophage ϕX174 (ϕX) has increased from one to six with increasing sophistication in molecular isolation techniques (Carusi and Sinsheimer, 1961; Zuccarelli et al., 1972). The recent report of an intracellular ϕX precursor with an extra protein not present in the mature phage may explain one of the minor proteins (Weisbeek and Sinsheimer, 1974). There is no genetic evidence that the two minor proteins are viral coded. There does appear to be general agreement that four of the virion proteins are viral coded (Burgess and Denhardt, 1969; Gelfand and Hayashi, 1969; Mayol and Sinsheimer, 1970; Godson, 1971; Benbow et al., 1972; Freymeyer et al., 1977).

The four major proteins in the virion are the 48 000 molecular weight cistron F protein, the 36 000 molecular weight cistron H protein, the 19 000 molecular weight cistron G protein, and the small cistron J protein of 4050 molecular weight (Burgess and Denhardt, 1969; Benbow et al., 1972; Freymeyer et al., 1977). Since the proteins are all separable on the basis of size in $NaDodSO_4$[1]-polyacrylamide gels, a column-chromatography procedure was devised to isolate the proteins on the basis of size and is described here.

Column chromatography under denaturing conditions has been used previously to purify the J, the G, and the F proteins (Suruda and Poljak, 1971). The F and H proteins proved difficult to separate due to the similarity of their molecular weights and, in fact, the H protein was not recovered (Suruda and Poljak, 1971) by these methods. The fivefold molar excess of the larger protein further complicated the F–H separation (Burgess, 1969).

Edgell et al. (1969) reported a method for removal of the spikes from the ϕX capsid. The capsid, after spike removal, was shown to be a structure containing the DNA and the F protein. All the other major virion proteins were found in the spike structure (Edgell et al., 1969; Burgess, 1969). We reasoned therefore that it should be possible to separate the F protein from the H protein by first removing the spikes from the capsid. All the proteins should then be separable on the basis of size using gel-filtration chromatography under denaturing conditions. This report describes a preparative isolation procedure for each protein. In addition, the locations of the four major protein species within the virion are characterized. The amino acid composition, tryptic peptide map, and detectable amino terminus of each protein are presented.

Materials and Methods

Virus Growth. The virus used in this study was the lysis mutant ϕX am3. Am3 has been shown to be in cistron E (Hutchison and Sinsheimer, 1966) which is not known to code for any virion protein. *Escherichia coli* strain C was grown with vigorous aeration in 15-L carboy flasks in minimal medium supplemented with casein hydrolysate. The medium contained per 15 L: NH_4Cl, 15 g; Na_2HPO_4, 90 g; KH_2PO_4, 45 g; NaCl, 45 g; casamino acids (Difco Certified), 45 g; 10% $MgSO_4$, 15 mL; glucose, 15 g; and $CaCl_2$, 0.83 g. Routinely, eight carboys were grown at 37 °C to a titer of 2×10^8 cells/mL. The cells were then infected at a multiplicity of 10 pfu/cell. After 2 h, the cells were spun down in a Sharples centrifuge. The average yield from 120 L was 100 g wet weight of cells. Cells were stored at −20 °C for up to 6 months without loss of virus titer.

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[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; CM, carboxymethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Tos-PheCH_2Cl, (1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

Virus Purification. In a standard preparation, 200 g of cells was suspended in a volume of 625 mL of 0.1 M sodium borate. Egg-white lysozyme (Worthington three times crystallized) was added to a final concentration of 0.6 mg/mL. After incubation at room temperature for 10 min, 75 mL of 4% EDTA was added and the incubation continued for 1–2 h until the solution was very viscous. The lysate was then sonicated to reduce the viscosity and clarified at 13 000*g* for 30 min at 5 °C. The pellet was reextracted by mixing with 250 mL of 0.1 M sodium borate in an omnimixer (Sorvall) and clarified again. The supernatant fractions were pooled and made to 0.3 M NaCl. One-quarter volume of ice-cold 30% poly(ethylene glycol) (Carbowax 6000, Union Carbide) was added and the mixture was placed at 5 °C for at least 2 h. The precipitate was spun down at 13 000*g* for 10 min at 5 °C. Less than 0.1% of the viable phage remained in the supernatant. The pellet was resuspended in 150 mL of 0.1 M sodium borate overnight at 5 °C with gentle stirring. The suspension was spun down at 13 000*g* for 10 min at 5 °C. The pellet was reextracted with 100 mL of 0.1 M sodium borate and stirred overnight at 5 °C. After centrifugation, the supernatants were pooled and made up to 1.35 g/cm^3 with solid CsCl. The virus was centrifuged to equilibrium in the 60 Ti rotor (Beckman) at 30 000 rpm for 24 h at 5 °C. The viral band at 1.45 g/cm^3 was collected and dialyzed against 0.05 M sodium borate at 5 °C. The virus was then layered on a 5–20% sucrose gradient in 0.05 M sodium borate with a CsCl pad of 1.65 g/cm^3. The gradients were centrifuged for 12 h at 22 000 rpm, 5 °C, in the SW 27 rotor (Beckman). The viral band was collected off the CsCl pad, dialyzed into 0.05 M sodium borate, and stored at 5 °C. The stocks have an average of 12 mg/mL of virus with a total yield of 1.4 g from 200 g of cells. The virus was free of contamination as assayed by $NaDodSO_4$–polyacrylamide gel electrophoresis of disrupted virions.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. A continuous gel system similar to that described by Summers et al. (1965) was used. The buffer was 0.075 M sodium phosphate, pH 7.2. Gels containing 14% acrylamide (Eastman electrophoresis grade), 0.14% bisacrylamide, 0.075 M sodium phosphate, pH 7.2, 0.1% $NaDodSO_4$, 0.04% *N,N,N′,N′*-tetramethylethylenediamine, and 0.05% ammonium persulfate were cast in 100 × 6 mm acid-cleaned glass tubing. Samples for electrophoresis were placed in shell vials with 50 mg of urea (Schwarz/Mann Ultra Pure), 0.1% $NaDodSO_4$, and 5% 2-mercaptoethanol in a total volume of 50 μL. Samples were disrupted by heating to 80 °C for 10 min. After cooling, the samples were made to 0.001% bromophenol blue and layered beneath the upper electrode buffer. Electrophoresis was carried out toward the anode at 2.5 mA/gel for 30 min. The current was then raised to 5 mA/gel and electrophoresis continued for 5 h. The gels were then removed, stained with Coomassie brilliant blue R250 and destained by diffusion.

Spike Removal. Spikes were removed from the virion by a slight modification of the method of Edgell et al. (1969). Virus stocks at 1–10 mg/mL were mixed with an equal volume of freshly deionized 8 M urea in 0.05 M sodium borate, 0.02 M glycine. Deionized urea and glycine were used to prevent carbamoylation of proteins during isolation. One-tenth volume of 4% EDTA was added and the solution stirred at 30 °C for 3 h. The sample was then dialyzed at 5 °C against 0.05 M sodium borate overnight. The precipitate which forms was spun down at 12 000*g* for 10 min and saved. The supernatant was layered onto a 5–20% sucrose gradient in 0.05 M sodium borate with a 4-mL pad of CsCl (1.65 g/cm^3). A 14-mL sample was put onto the gradient and centrifugation was carried out at 22 000 rpm, 4 °C, in the SW 27 rotor for 15 h. The upper 20 mL of the gradient was collected from above as spike material. The capsid fraction was collected off the CsCl pad. After extensive dialysis against water, the samples were lyophilized.

Column Chromatography. Sephadex G-150 (Pharmacia Fine Chemicals) was equilibrated with 7.5 M deionized urea, 0.05 M sodium phosphate, pH 6.5, 0.01 M glycine, 0.1% 2-mercaptoethanol. A 2.5 × 100 cm column was packed and run at 5 °C. The absorbance was monitored with an ultraviolet monitor (Isco UA-2). Protein samples were dissociated in 6 M guanidine hydrochloride, 0.075 M Tris, pH 8.6, 1% 2-mercaptoethanol at a concentration of 10–20 mg/mL. Samples of 5–10 mL were applied to the column directly in the dissociation buffer. Purification of the small J protein was performed on a 2.5 × 80 cm Sephadex G-100 column equilibrated with 0.5% formic acid at room temperature. Fractions from the columns were analyzed on $NaDodSO_4$–polyacrylamide gels to determine their protein content prior to pooling.

Tryptic Peptide Maps. A 1–2-mg sample of protein was oxidized with performic acid by the method of Hirs (1967). The oxidized protein was suspended in 0.1 M ammonium bicarbonate at 5–10 mg/mL and treated with 1% (w/w) Tos-PheCH_2Cl-trypsin (Worthington) for 2 h at 37 °C. A second equal volume of Tos-PheCH_2Cl-trypsin was added and the incubation continued for 1 h. The sample was then frozen and immediately lyophilized. After redissolving in 0.1 M ammonium bicarbonate, the insoluble material was removed by low-speed centrifugation. The mapping was performed on Whatman 3MM paper by a modification of the method of Katz et al. (1959). Descending chromatography was performed in a chamber equilibrated with *n*-butyl alcohol–acetic acid–water (3.3:1:5). Electrophoresis in the orthogonal direction was in 10% acetic acid, 1% redistilled pyridine, pH 3.5, for 45 min at 3000 V, 20 °C, in a Gilson Model DW electrophorator. The maps were then stained with ninhydrin–collidine.

N-Terminal Amino Acid Analysis. N-terminal amino acids were determined by three techniques: dansylation and thin-layer chromatographic identification of the dansyl derivatives (Gray, 1972), manual Edman degradation with gas–liquid chromatographic identification of the phenylthiohydantoins, and by automatic sequence analysis in the Beckman 890 Sequinator with gas–liquid chromatographic identification of the phenylthiohydantoins as described by Niall (1973).

Amino Acid Analysis. Samples for amino acid analysis were hydrolyzed in vacuo for 18 h at 110 °C in 6 N redistilled hydrochloric acid. Amino acid analyses were performed on a Technicon TSM amino acid analyzer using a two-column 90-min program. Correction for destructive losses of amino acids were not made.

Results

Location of Major Capsid Proteins. Edgell et al. (1969) reported that treatment of the φX virion with 4 M urea removed the spikes from a central capsid. Electron microscopic studies confirmed that the 12 spikes were removed from the DNA containing central capsid. The gel system employed at that time did not allow complete resolution of all the virion proteins. It did appear, however, that the capsid was composed of a single major protein component. It was shown that the cistron G protein and at least one other were components of the spike. Various lines of evidence suggest the H protein must be associated with the spike. The virion has been shown to attach to the bacterial cell via its spikes (Brown et al., 1971). Mutants in cistron H fail to attach to the host cell or attach at altered rates (Siegel and Hayashi, 1969; Benbow et al., 1972). Recently, Jazwinski et al. (1975) have shown that the H pro-

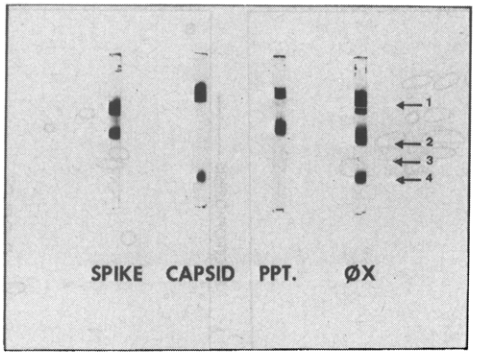

FIGURE 1: Products of the ϕX spike-removal procedure. Samples of the spike, capsid, and precipitate fraction from the ϕX spike removal were disassociated and run on $NaDodSO_4$–polyacrylamide gels. The gels were stained with Coomassie brilliant blue. The gels were slightly overloaded to show the purity of the various fractions. The reported molecular weights of the ϕX proteins are 48 000, 36 000, 19 000, and 5000 for the F, H, G, and J proteins, respectively (Burgess and Denhardt, 1969). The positions of molecular weight markers ovalbumin (45 000), myoglobin (17 200), cytochrome *c* (12 300), and insulin B chain (3400) are indicated by the arrows numbered 1–4, respectively.

TABLE I: Yields of Purified Proteins.[a]

	F	H	G	J
Spike	0	4 mg/30%	3.5 mg/10%	0
Capsid	37 mg/40%	0	0	0.8 mg/10%
Precipitate	6 mg/5%	0	10 mg/30%	0
Total	43 mg/45%	4 mg/30%	13.5 mg/40%	0.8 mg/10%

[a] Spikes were removed from a 200-mg sample of virus and the various fractions separated on Sephadex G-150. The yields were calculated assuming the composition of the virus proposed by Burgess (1969). Purified proteins were measured by amino acid analysis.

tein is the phage adsorption protein recognizing the bacterial lipopolysaccharide receptor. If the H protein is a component of the spike it would be possible to separate it from the F protein by removal of the spikes.

When the ϕX virion was treated at high concentrations with 4 M urea for 3 h at 30 °C and dialyzed at 5 °C against 0.05 M sodium borate, a precipitate formed. The precipitate was removed by low-speed centrifugation and the spike and capsid fractions were prepared as described. Analysis of each sample on $NaDodSO_4$–polyacrylamide gel electrophoresis revealed that each sample contained two proteins. The precipitate contained the F and G proteins. The spike contained the G and H proteins. The capsid contained the F and J proteins. Figure 1 shows the proteins contained in the spike, capsid, and precipitate fractions.

Isolation of the Major Capsid Proteins. The protein fractions prepared by removal of the spikes all proved amenable to resolution into individual protein species by gel-filtration chromatography under denaturing conditions. The separation of the spike, capsid, and precipitate fractions on Sephadex G-150 is illustrated in Figure 2. The proteins were pooled as indicated, extensively dialyzed against water in acetylated dialysis tubing, and lyophilized. The yield of each protein can be estimated if one assumes the virion is composed of 60 molecules of the F, G, and J proteins and 12 molecules of the H protein as proposed by Burgess (1969). Table I summarizes the yields of the virion proteins from the Sephadex G-150 column.

Amino Acid Compositions. Samples of the isolated proteins were oxidized with performic acid and hydrolyzed, and the

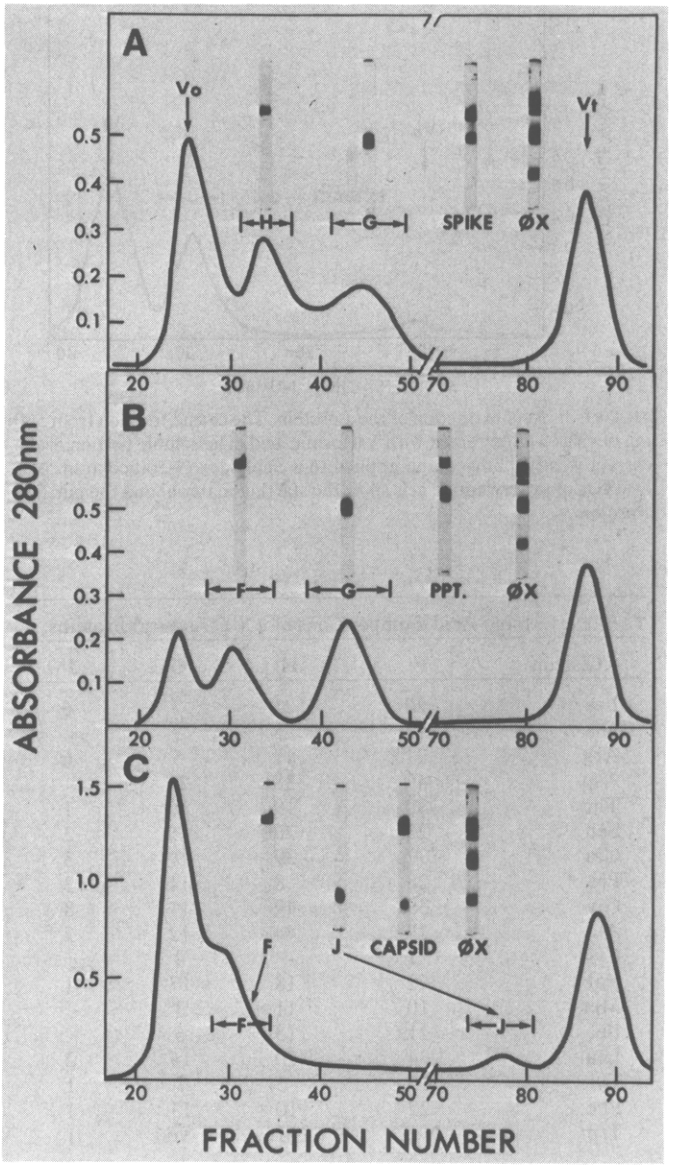

FIGURE 2: Separation of ϕX proteins by gel filtration chromatography. Samples of the purified capsid, spike, and precipitate fractions were dissociated in 6 M guanidine hydrochloride and applied to a 2.5 × 100 cm Sephadex G-150 column. Samples of the starting material and each pool were run on $NaDodSO_4$–polyacrylamide gels. The samples applied to the column were not phenol extracted; therefore, there is a DNA peak eluting at the void volume (V_0). The DNA peak is very large in the capsid fraction, since most of the DNA is retained within the capsid. The peak eluting at the total volume (V_t) is due to the 1% 2-mercaptoethanol in the dissociating buffer. (A) Spike fraction from 500 mg of ϕX; (B) precipitate from 200 mg of ϕX; (C) capsid fraction from 200 mg of ϕX.

amino acid compositions shown in Table II were determined. In general, the compositions of the F and G proteins agree with those published by Suruda and Poljak (1971). The composition of the J protein, which differs significantly from previous reports (Poljak, 1968; Suruda and Poljak, 1971), has been confirmed both by isolation of tryptic peptides and amino acid sequence analysis (Freymeyer et al., 1977).

Acid Extraction of the J Protein. From its amino acid composition, it was clear that the J protein was quite basic. It seemed possible that this molecule could be selectively extracted into acid. The capsid fraction from 500 mg of ϕX was suspended at 10 mg/mL in 5% formic acid for 2 h at room temperature. The insoluble material was removed by centrifugation at 9000 rpm (Sorvall HB-4 rotor) for 10 min at 5 °C.

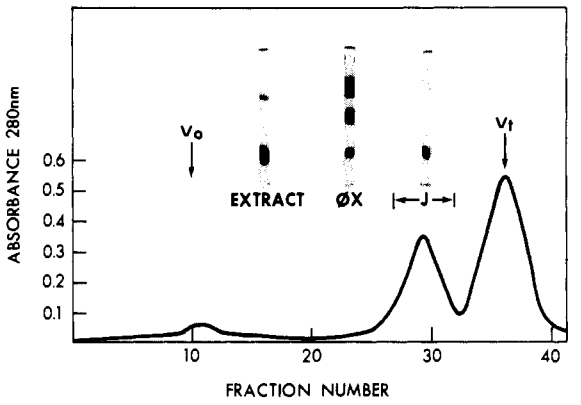

FIGURE 3: Acid extraction of the J protein. The capsid fraction from 500 mg of virus was extracted with 5% formic acid. The soluble portion of the extract was lyophilized and applied to a Sephadex G-100 column. Na-$DodSO_4$-polyacrylamide gels show the starting material and the purified J protein.

TABLE II: Amino Acid Compositions of ϕX Structural Proteins.

Cistron	F	H	G	J
Lys	20	25	7	6
His	13	5	3	-
Arg	31	12	5	6
Asp	47	38	21	-
Thr	30	19	16	1
Ser	28	26	16	2
Glu	37	39	9	3
Pro	28	8	11	3
Gly	39	49	11	8
Ala	27	44	12	2
Cys	1	-	4	-
Val	22	18	17	1
Met	10	11	3	-
Ile	21	15	6	-
Leu	34	20	14	2
Tyr	19	5	7	1
Phe	22	10	13	1
Trp	Nd	Nd	Nd	1
Total	429	344	176	37

[a] Nd, not done; -, not detected. Values for the F, H, and G proteins represent the average of three analyses normalized to molecular weights of 48 000, 36 000, and 19 000. The composition of the J proteins was normalized to six Lys in accord with its amino acid sequence. Trp was determined by Freymeyer et al. (1977).

The soluble fraction was lyophilized and analyzed on Na-$DodSO_4$-polyacrylamide gels (Figure 3). The minor contamination of the acid extraction with the F protein could be removed by chromatography on Sephadex G 100 as shown in Figure 3. The J protein could be extracted in this manner from ϕX virions, total ϕX protein produced by phenol extraction or purified ϕX capsid. Acid extraction of the J protein led to higher yields, since there was no exposure to urea and, therefore, no dialysis. The yield of the J protein by acid extraction was approximately 70% of the theoretical yield.

Occasionally, particularly with old capsid preparations, the region pooled as the J protein from the Sephadex G-100 column had a contaminating species containing aspartic acid (Freymeyer et al., 1977). This contaminant is believed to be a breakdown product of the F protein. It is possible to separate the contaminant from the J protein on CM-Sephadex. The sample is applied to the column in 0.1 N sodium acetate, pH 6.5. The column is developed with a linear gradient of 0–1 M sodium chloride in 0.1 M sodium acetate. The contaminant elutes at 0.25 M sodium chloride, while the J protein elutes at 0.5 M salt.

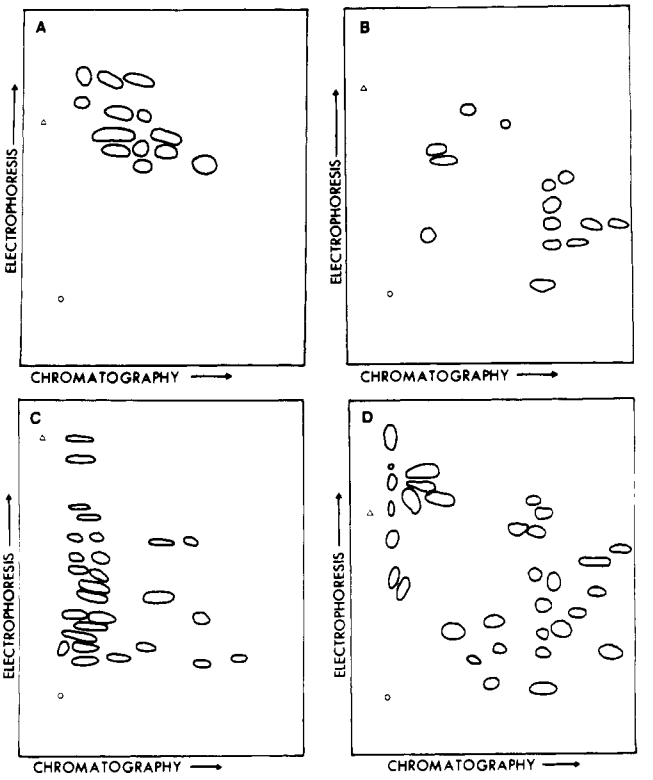

FIGURE 4: Tryptic peptide maps of ϕX structural proteins. Performic acid oxidized ϕX proteins were digested with Tos-PheCH_2Cl treated trypsin and tryptic peptide maps were made: (A) 1 mg of small protein; (B) 2 mg of G protein; (C) 2 mg of H protein; (D) 2 mg of F protein. The origin (○) and an arginine marker for the electrophoresis dimension are indicated (△).

N-Terminal Amino Acids. The N-terminal amino acids present in whole ϕX protein were reported to be methionine and serine by Poljak (1968). We have detected methionine as the N-terminal of the G protein by dansylation and by analysis of the phenylthiohydantoins from both manual and automated Edman degradations as others have previously reported (Suruda and Poljak, 1971; Air and Bridgen, 1973). The N terminal of the J protein was determined to be serine by dansylation and automated Edman degradation. No free N terminals were detected for the F and H proteins by dansylation or manual Edman degradation. It is possible that the N terminals were blocked during isolation. Air (1976) has reported serine to be the N terminal of the F protein.

Tryptic Peptide Maps. To further characterize the isolated proteins, tryptic peptide maps were made. Samples of the proteins were oxidized with performic acid and digested with trypsin. Tryptic peptide maps of the proteins are shown in Figure 4. In general, the number of peptide spots detected agrees well with the amino acid composition presented. Each peptide map is unique. It, therefore, seems unlikely that the smaller proteins represent cleavage products of the larger proteins.

Discussion

Four major proteins were isolated from the ϕX capsid by gel filtration chromatography of the spike, capsid, and precipitate fractions generated by removal of the spikes. The spike contains the H and G proteins.

The capsid after spike removal contains the majority of

DNA, the F protein, and the J protein. Edgell et al. (1969) concluded that the capsid fraction was composed of a single protein species. Although we have observed some effect of concentration on the partition of these proteins during spike removal, we have not attempted to resolve this difference. We have observed a precipitate which forms during the removal of the spikes which contains the F and G proteins. This precipitate is of similar composition to the intracellular precursor isolated by Tonegawa and Hayashi (1970) and characterized by Siden and Hayashi (1974).

The J protein is quite small, has a molecular weight of 4050 as determined by sequence analysis, and is highly basic, 32% arginine and lysine. With the localization of this molecule in the capsid rather than the spike, it is attractive to postulate a functional interaction with the DNA. It is, therefore, very interesting to note the observation of Siden and Hayashi (1974) that when normal virion assembly is prevented by mutation in ϕX B gene a DNA–protein complex is formed in which 70% of the protein is the J gene product.

The four major ϕX virion proteins can easily be purified in yields ranging from 30 to 70% by the following procedure: (1) The virus is treated with 4 M urea, and the spike, capsid, and precipitate fractions are prepared. (2) The spike is fractionated on Sephadex G-150 to isolate the H and G proteins. (3) The precipitate is fractionated on Sephadex G-150 to isolate the F and G proteins. (4) The capsid is extracted with 5% formic acid and the soluble portion fractionated on Sephadex G-100 to isolate the J protein. The insoluble portion is solubilized in urea and then fractionated on Sephadex G-150 to isolate the F and G proteins which contaminated the capsid and any J protein not extracted into the formic acid. The four proteins were pure as judged by polyacrylamide gel electrophoresis, N-terminal analysis, and tryptic peptide maps.

The N-terminal serine and methionine in the virion proteins come from the J and G proteins, respectively. No free N-terminal amino acids were detected on the major capsid protein F or the minor spike protein H.

Tryptic peptide maps as well as amino acid compositions indicate that the four proteins represent distinct molecular species and are not cleavage products of each other. These four structural proteins comprise 985 amino acids (excluding tryptophan) and therefore require nearly 3000 nucleotides of coding capacity or approximately 55% of the coding capacity of the ϕX genome. The average molecular weight per residue is 108. Assuming the distribution of amino acids in the remaining 45% of the genome is similar, we can estimate the total coding capacity of the ϕX genome (1800 codons $\times$ 108 daltons) as 194 000 daltons of proteins. This estimate is 15% smaller than the estimate of Benbow et al. (1972) based on the incorrect calculation of 124 daltons per residue. The total molecular weight of the "ϕX-coded" proteins listed by Benbow et al. (1972) is 295 000. It is clear therefore that some of these proteins would have to be coded for by unusual mechanisms to all be generated by the ϕX genome.

Using DNA-sequencing techniques coupled with partial amino acid sequence data, Sanger et al. (1977) have proposed a complete nucleotide sequence and gene placement for the ϕX genome. The nucleotide sequence confirms the observation that the structural proteins are all coded for by distinct regions of the genome and is in good correlation with the amino acid compositions presented here. In addition, the nucleotide sequence revealed a novel form of information transfer whereby one nucleotide sequence codes for two proteins in different reading frames (Barrell et al., 1976) thereby increasing the coding capacity of the genome.

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