Isolation and Characterization of Two Basic Internal Proteins from the T-Even Bacteriophages¹

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Received for publication 9 June 1970

Two species of basic internal proteins were found in osmotic shock supernatant solutions of bacteriophages T4B, T4D, T2H, T2L, and T6. The major species of protein isolated had a molecular weight of approximately 21,000 daltons, whereas the minor protein molecular weight was near 9,500 daltons. The two protein species exhibited unique isoelectric points and amino acid compositions. The 21,000-dalton protein of T2L showed major electrophoretic and compositional differences from the other 21,000-dalton proteins isolated. Similarities between the 21,000-dalton proteins and phage lysozyme are discussed.

Studies on the role of deoxyribonucleic acid (DNA) in the infective process of the T-even bacteriophages led to the discovery of certain substances which appeared to be associated with the viral nucleic acid and which were detected only after rupture of the head membrane by osmotic shock (23, 24). These substances included: (i) an acid-soluble fraction which derived most of its carbon from arginine and yet yielded no arginine upon acid hydrolysis; (ii) an acid-soluble peptide which yielded predominantly lysine, glutamic acid, and aspartic acid upon acid hydrolysis; and (iii) an acid-insoluble protein fraction which could be distinguished from ghosts and whole phages by immunological means. The first two substances have now been well characterized. Ames and co-workers have demonstrated the presence of the polyamines, putrescine and spermidine, in T4 phage (3), whereas Champe et al. have found two acid-soluble peptides in T2H, T4D, and T6 phage (10, 18, 41). The acid-insoluble fraction, however, has not been as well characterized. Levine and co-workers reported the presence of an internal antigen in T2 and T4 phage (31, 35) which they called the internal protein. Minagawa (34) attempted to correlate this internal antigen with Hershey's (23) acid-insoluble material.

The internal protein has been estimated to account for 3 to 7% of the total phage protein (23, 31, 34) and its function remains unknown. Several laboratories have attempted isolations of the internal proteins from T-even bacteriophages (7, 8, 11, 29, 31, 34; M. L. Coval, V. Møller, and

H. Van Vunakis, Fed. Proc. 19:253, 1960). Coval's group reported a partial amino acid composition in their abstract (M. L. Coval et al., Fed. Proc. 19:253, 1960). The protein from T2 was high in lysine and histidine, and no cysteine was detected. Minagawa (34), however, suggested the presence of more than one internal protein in T2. The recent reports of Bachrach et al. (8) and Kokurina and Tikhonenko (29) would tend to confirm this finding.

The object of the present study was to isolate and characterize the internal proteins. Evidence is presented which demonstrates the presence, in all of the T-even phage examined, of two species of basic internal proteins which can be distinguished by a number of such parameters as molecular weight, electrophoretic characteristics, and amino acid composition.

MATERIALS AND METHODS

Bacteriophage growth and purification. Bacteriophages T4B, T4B01, T4D, T2H, T2L, and T6 were prepared and purified under standard conditions (1). (All of these bacteriophages with the exception of T6 have been used in our laboratory for several years; T6 was obtained from M. Jesaitis.) The mutant phage AmN85 (G48) and AmH21 (G54) were obtained from R. S. Edgar. *Escherichia coli* B was grown in 70-liter volumes of pH 7.3 Casamino Acids-glycerol medium similar to that of Kozloff and Lute (30), which contained per liter: 1.2 g of NH₄Cl, 1.0 g of NaCl, 0.5 g of KCl, 2.4 g of tris(hydroxymethyl)aminomethane, 0.1 g of gelatin, 24 ml of glycerol, 1.0 ml of 37% HCl, 1.0 g of MgSO₄, and 23.5 mg of CaCl₂. Growth of bacteriophage T4B required the addition of 0.2 g of L-tryptophan per liter for adsorption (4, 5). The medium was supplemented with 30 mg of thymine per liter 10 min prior to bacteriophage infection; in some cases, especially

¹ Presented by the senior author in partial fulfillment of the requirements for the Ph.D. degree to the Department of Microbiology, University of Colorado Medical Center.

with T6, better yields of bacteriophage were obtained in the presence of thymine. When the bacterial cell density was 2×10^8 to 3×10^8 cells/ml, the bacteria were infected with phage, prepared 2 days prior to use, at a multiplicity of three phage per bacterium. The phage growth was then allowed to proceed with aeration for another 5 hr.

At the end of the bacteriophage growth period, the phage were concentrated by the polyethylene glycol-dextran two-phase system (2) as modified by S. Ward (*personal communication*). Chloroform (1 ml/liter) and a small quantity of deoxyribonuclease and ribonuclease were added to the lysate. The following materials were then added, in order: 17 g of sodium chloride per liter, 2.3 g of sodium dextran sulfate 500 (Pharmacia Fine Chemicals) per liter, and 71 g of polyethylene glycol 6000 (Carbowax, Union Carbide) per liter. Each compound was dissolved completely before the next was added. The lysate was then placed at 4 C for 1 to 2 days to allow phase separation. The bulk of the upper polyethylene glycol phase was removed by suction and discarded. The lower dextran phase was poured into a 4-liter beaker and allowed to separate again overnight at 4 C. The remaining polyethylene glycol was removed, and the dextran phase was diluted threefold with saline stock solution (0.15 M NaCl, 1 mM $MgSO_4$, and 1 mM PO_4 , pH 7.5). The dextran was then precipitated by the addition of 0.2 volumes of 3 M KCl with stirring. The bacteriophage were purified by two successive cycles of differential centrifugation ($2,000 \times g$ for 10 min, followed by $15,000 \times g$ for 1.5 hr) and finally resuspended in minimal volumes of saline stock solution. Bacteriophage yields were in the order of 10^{15} to 2×10^{15} particles per 70 liters.

Glycerol osmotic shock procedure. Osmotic shock of the phage particles by using high salt concentrations (22) was found to liberate moderate amounts of phage structural proteins. Consequently, osmotic shock was induced by rapid dilution of phage in glycerol. Purified phage, resuspended from pellets in minimal volumes of saline stock solution at 3×10^{13} to 5×10^{13} phage per ml were mixed with 0.43 volumes of glycerol (4 M final concentration). After 1 hr of equilibration in the presence of a small amount of deoxyribonuclease, the phage were osmotically ruptured by rapid dilution with 18 volumes of water at 25 C containing the deoxyribonuclease medium of Cummings (12). After 2 hr of deoxyribonuclease digestion, the phage capsids [or "ghosts" (22)] were removed by sedimentation at $40,000 \times g$ for 3 hr. The supernatant solution was again centrifuged at $40,000 \times g$ for 15 hr to remove any remaining phage substructures (14). This final supernatant solution, which contained the internal proteins, was then analyzed by chromatography on carboxymethyl cellulose (CM-cellulose).

Chromatography on CM-cellulose. CM-cellulose (CM23, Whatman) was equilibrated with ammonium acetate buffer (0.1 M acetate, pH 5.0) and dried by suction. The supernatant solution containing the internal proteins was adjusted to pH 4.8 with acetic acid and mixed with sufficient CM-cellulose to make a column 2.5 by 10 cm (39). The mixture was gently

stirred for 30 min and poured into the column. The column was washed with starting buffer (0.1 M acetate, pH 5.0) until all digested DNA and unbound materials were eluted. All buffers were 0.1 M in acetate and adjusted to the desired pH with concentrated ammonium hydroxide. Two linear gradients were then used in sequence to elute basic proteins (buffer at pH 5 to buffer at pH 10; followed by buffer at pH 10 to the same buffer containing 0.2 M Na_2CO_3). The flow rate was 2.5 ml/min, and 5 ml fractions were collected. Protein peaks were monitored by fluorescence with an Aminco-Bowman Spectrophotofluorometer (excitation, 280 nm; emission, 340 nm). Fractions were pooled in each peak and precipitated with 5% trichloroacetic acid (final concentration). The pH 5 to 10 gradient was very steep across the pH 6 to 8 region since it buffered very poorly in this pH range.

Chromatography on 6% agarose. Molecular weights were determined from the partition coefficients of the proteins on 6% agarose (100 to 200 mesh, control no. 6470, Bio-Rad Laboratories) as described by Davison (16). Protein samples were dissolved in purified 6 M guanidine (36) containing 3 mM Cleland's reagent (dithiothreitol, A grade, Calbiochem). The column (2 by 95 cm) was equilibrated with 5 M guanidine (Sigma Chemical Co.), 0.05 M LiCl, 0.01 M ethylenediaminetetraacetic acid, and 3 mM Cleland's reagent. This guanidine solution was prepared as follows. The ingredients, less Cleland's reagent, were mixed, heated to 45 to 50 C for solution, cooled overnight at 4 C, filtered, and stored at room temperature. Cleland's reagent was added to the guanidine solution, at the time it was used, for column elution. The flow rate was approximately 8 ml/hr, and 4-ml samples were collected. Protein elution was monitored by fluorescence in an Aminco-Bowman Spectrophotofluorometer (excitation, 280 nm; emission, 340 nm). Dextran blue and dinitrophenyl-alanine were used as void volume and internal volume markers, respectively. The following molecular weight (MW) standards (40) were used to calibrate the column: pepsin (2X crystallized; Sigma Chemical Co., MW = 35,500); trypsin (type III, Sigma Chemical Co., MW = 23,800); horse hemoglobin (Pentex, MW = 16,000); egg white lysozyme (isoelectric enzyme, Schwarz BioResearch Inc., MW = 14,400); insulin (Sigma Chemical Co., MW = 5,773). As shown in Fig. 1, a linear relationship exists between the partition coefficient [$K = (V_e - V_o)/(V_i - V_o)$ where V_e = elution volume of the protein sample, V_i = internal volume, and V_o = void volume] and the log MW.

Isoelectrofocusing in polyacrylamide gels. Isoelectrofocusing was performed in a disc electrophoresis unit (DE102, Hoefer Scientific Instruments, San Francisco, Calif.) by a method modified from that of Dale and Latner (15). Gels (7 cm) were poured in glass tubes (0.4 by 12 cm). The gel solution contained 8.0% acrylamide (w/v, Eastman Organic Chemicals), 0.2% *N,N'*-methylenebisacrylamide (w/v, Eastman Organic Chemicals), 0.2% *N,N,N',N'*-tetramethylethylenediamine (w/v, Eastman Organic Chemicals), 8 M urea, 0.01 M Cleland's reagent, and 1.5% ampholine, pH ranges 3 to 10 (LKB Instruments). Acrylamide was recrystallized from chloroform by the

FIG. 1. Chromatography of MW standards on a 6% agarose column. Pepsin (35,500 daltons), trypsin (23,800 daltons), horse hemoglobin (16,000 daltons), egg white lysozyme (14,400 daltons), and insulin (5,773 daltons) (40) were dissolved in 6 M guanidine hydrochloride containing 3 mM Cleland's reagent and were chromatographed on a 6% agarose column.

procedure of Bishop et al. (9). No difference in the electrophoretic profile of the proteins was observed by using purified acrylamide. Protein samples dissolved in not more than 0.05 ml of 10 M urea plus 0.1 M Cleland's reagent were placed in the bottom of gel tubes. Ammonium persulfate (E-C Apparatus Corp.) was added at 1.4 mg/10 ml of gel solution as catalyst. The gels were quickly poured with thorough mixing of the protein sample throughout the gel. Usually six tubes could be poured with 10 ml of gel before polymerization began. The gels were given 15 min to set properly and were placed in the disc electrophoresis unit so that the 7-cm gels were completely immersed in the lower water-jacketed chamber. The bottom cathode solution was 1% ethylenediamine in water, and the upper anode solution was 1.4% orthophosphoric acid. Electrophoresis was for 6 hr with an initial current of 5 mamp per tube.

The gels were stained with bromphenol blue by the method of Awdeh (6). Minor bands were more evident if the gels were placed in distilled water for a few hours after destaining by the Awdeh method. Also, the bands were more stable when stored in water than in the destaining solution.

Numerous attempts were made to calibrate the isoelectrofocusing gels for accurate determination of isoelectric points. This was not possible for two reasons. First, the ampholine carrier ampholytes did not give a perfectly linear gradient and there were minor variations between different batches of ampholine. Second, there were not enough proteins available with defined isoelectric points to span the gradient. At best, only an approximation could be made for isoelectric pH values by using the following proteins as standards: lysozyme ($pI \approx 10.5$ to 11.0), chymo-

trypsinogen A ($pI \approx 9.2$), trypsin ($pI \approx 10.0$), bovine serum albumin ($pI \approx 5.1$), and horse hemoglobin ($pI \approx 6.9$). The pH gradient was calibrated by a linear plot of pH versus the ratio of the position to which a protein species migrated from the anode end of the gel over the total length of the gel. This ratio for a given protein was reproducible, but the isoelectric points of the standard proteins were not defined under the conditions used here. Thus, the accuracy of the estimated pH gradient with these standards was probably no greater than ± 0.5 pH units.

Isoelectrofocusing in a sucrose gradient. The LKB electrofocusing column (no. 8101) was used for measurement of the isoelectric points of the basic internal proteins. Protein samples were dissolved in 10 M urea and applied in the middle of the sucrose step gradient of the column. Ampholine carrier ampholytes with a pH range of 7 to 10 were used. The experiments were performed in the normal manner with the exception that 8 M urea was used in the suspending medium. Electrophoresis was for 24 hr at 400 v with the cathode as the lower electrode. At the completion of the electrofocusing experiment, 2-ml fractions were collected and analyzed for pH and for fluorescence in an Aminco-Bowman Spectrophotofluorometer (excitation, 280 nm; emission, 340 nm).

^{125}I labeling of proteins. Proteins were labeled with ^{125}I by the method of McConahey and Dixon (33). Amounts (mg) of the protein samples were diluted in 1 ml of 0.05 M phosphate buffer (pH 7.0) in a 10-ml beaker and stirred gently in an ice bath; 0.05 mCi of ^{125}I in 0.05 M phosphate buffer was added. Labeling was then initiated by the addition of 50 μg of chloramine T in phosphate buffer. The reaction was allowed to progress for 10 min and was then stopped by the addition of 50 μg of sodium metabisulfite in phosphate buffer. Guanidine (1.0 g/ml) and a small amount of Cleland's reagent were used to dissolve the labeled proteins, which were then fractionated by gel filtration on the 6% agarose column. The free ^{125}I was eluted with the internal volume (V_i) of the column. Portions (1 ml) of eluted fractions were counted in an Automatic Well Gamma Counter (Nuclear-Chicago Corp.).

Amino acid analyses. Amino acid analyses were performed in a Beckman-Spinco model 120 Amino Acid Analyzer equipped with sensitive cuvettes and a model CRS-110A Automatic Digital Integrator (Infotronics Corp., Houston).

Quantities (mg) of the protein samples were hydrolyzed in vacuo at 110 C for 24 hr in 2-ml volumes of three times distilled 6 N HCl containing 2-mercaptoethanol and phenol (10 ml of 6 N HCl plus 5 μl of each of 2-mercaptoethanol and liquified phenol) to retard destruction of tryptophan (J. M. Stewart, *personal communication*). By using egg-white lysozyme as a standard, approximately 75 to 85% recovery of tryptophan was achieved by this method. Half-cystine was determined as cysteic acid by the performic oxidation method of Hirs (25).

RESULTS

Isolation of the basic internal proteins. The non-sedimentable proteins released from the phage by

osmotic shock were fractionated on carboxymethyl cellulose. Elution profiles for these proteins from phages T4, T2H, T6, and T2L are shown in Fig. 2. All phage supernatant solutions examined, with the exception of T2L, resulted in two peaks of proteins which differed in pH and ionic strength required for elution from this resin. Bacteriophage T2L contained both components found in the other viruses, but they eluted too closely together to be separated by the method used here (Fig. 2D). The material from the T2L peak was further fractionated by gel filtration on 6% agarose.

Fractions across peaks 1 and 2 were separately pooled, and the proteins were precipitated with 5% trichloroacetic acid. The proteins in these peaks appeared to contain all of the internal proteins released by osmotic shock. No other trichloroacetic acid-insoluble material was detected across the elution profile other than occa-

sional trailing from one of these peaks. In all cases examined, the material from peak trails was identical to that of the preceding peak. No further trichloroacetic acid-insoluble material was released from the CM-cellulose upon the addition of 0.5 N NaOH solution containing 0.5 M NaCl. The void volume of the column contained only minor amounts of phage structural proteins and small amounts of peak 1 material. Thus, no acid-insoluble acidic internal proteins were found in any of the phage examined.

As will become evident in the electrophoretic studies, the CM-cellulose column did not always fractionate the peaks cleanly. As a result, small amounts of the peak 2 proteins could be demonstrated in peak 1 and very minor amounts of the peak 1 material were recovered from the void volume. Somewhat better separations occurred by using step gradients, but splitting of each of the two peaks was observed with this method.

FIG. 2. Chromatography of the internal proteins on carboxymethyl cellulose. Supernatant solutions from osmotic shock of the bacteriophages were equilibrated with sufficient carboxymethyl cellulose to pour a column 2.5 by 10 cm. The column was washed with starting buffer (0.1 M acetate, pH 5.0) until all unbound materials and digested DNA were eluted. Separation was then achieved with two linear gradients. The first gradient (buffer at pH 5 to buffer at pH 10) covered approximately fractions 1 to 60, followed by the second gradient (buffer at pH 10 to the same buffer containing 0.2 M Na_2CO_3). Variations occurred in the gradient volumes, resulting in displacement of peaks in different experiments.

The mass ratio of the trichloroacetic acid-insoluble proteins of phages T4B, T4D, and T2H recovered by trichloroacetic acid-precipitation from the CM-cellulose peaks was about 5:1 (peaks 2/1). Phage T6, on the other hand, gave a ratio of 1:2 (peaks 2/1). The reason for this difference was not clear, but it may have reflected preferential losses of the peak 2 material during purification of the T6 proteins. Losses of this material were often observed during dialysis, when performed; some loss is attributed to adherence to glassware. The best protein yields were obtained when their concentrations were kept high. The growth of phage T6 was reproducibly poor, and, consequently, less starting material was available. Since the same number of pieces of glassware was used in each experiment, it would appear that the material seen in peak 2 of the CM-cellulose profile is that amount of protein remaining after losses on the glassware. Further work with isotopically labeled protein is necessary before this difficulty can be resolved.

Molecular weight determinations. Samples of the trichloroacetic acid-precipitated proteins from peaks 1 and 2 of CM-cellulose were washed in 0.05 M phosphate buffer (pH 7.0) and labeled with ^{125}I . Purified guanidine (1.0 g/ml) and a small amount of Cleland's reagent were added to dissolve the labeled protein suspension, and this was combined with unlabeled molecular weight standards (Fig. 1) also dissolved in 6 M guanidine containing 3 mM Cleland's reagent. Molecular weights of the internal proteins were then determined by gel filtration on the 6% agarose column (Fig. 3). CM-cellulose peak 1 material from phage T4 eluted from the agarose column as a single

peak (Fig. 3A) with a molecular weight of approximately 9,500 daltons. The peak 2 material from phage T4 also showed a single peak (Fig. 3B) on the agarose column with an average molecular weight of about 21,000 daltons.

Both 21,000- and 9,500-dalton proteins were found in each of the T-even bacteriophages examined. The amount of these proteins recovered varied somewhat with the phage studied. High yields of T4B, T4D, and T2H permitted recovery of 1 to 2 mg of the 9,500-dalton protein and 5 to 15 mg of the 21,000-dalton protein per 2×10^{15} phage. The trichloroacetic acid-insoluble material from CM-cellulose peak 1 of T2L phage gave both a 21,000- and a 9,500-dalton protein when further fractionated on a 6% agarose column. Detection of these proteins was by trichloroacetic acid precipitation of the fractions across the elution profile since these proteins yielded only low levels of fluorescence (Fig. 2), especially in the presence of the high background of 5 M guanidine. Only minute amounts of the 9,500-dalton proteins were recovered from T2L phage supernatant solutions. The isolation procedure for the proteins from this phage involved more steps, and the reduced recovery may have been a reflection of losses incurred by the extra steps. T6 phage yielded only small quantities of the 21,000-dalton protein, as mentioned earlier, which may have been the result of preferential loss on glassware.

Electrophoretic studies. Electrofocusing of the isolated proteins on polyacrylamide gels demonstrated the presence of two, or at most three, species of basic internal proteins (Fig. 4). The 21,000-dalton proteins from T4B (Fig. 4, d)

FIG. 3. MW determination of the internal proteins of phage T4 by chromatography on 6% agarose. ^{125}I -labeled internal proteins dissolved in 6 M guanidine and 3 mM Cleland's reagent were combined with the unlabeled MW standards pepsin (a), trypsin (b), and lysozyme (c) and fractionated on 6% agarose. The arrows designate the order of elution of the three MW standards along with the void volume (V_0) and internal volume (V_i) of the column. (A) Chromatography of peak 1 from the carboxymethyl cellulose column. (B) Chromatography of peak 2 from the carboxymethyl cellulose column.

FIG. 4. *Electrofocusing of the internal proteins on polyacrylamide gels. Electrophoresis of the protein samples mixed throughout the gels was for 6 hr at 400 v with pH 3 to 10 range ampholine. Cathode (—) was at the top. Gels of the 21,000-dalton proteins are (a) T2H, (b) T2L, (c) T4B01, (d) T4B, and (e) T4 lysozyme. Gels of the 9,500-dalton proteins read (f) T2H, (g) T6, and (h) T4D. Variations in gel lengths occurred resulting in displacement of some minor bands. The gels were aligned by isoelectric points of the major bands.*

and T2H (Fig. 4, a) gave single bands by this method at approximately pH 9.5. The 21,000-dalton protein from T2L phage (Fig. 4, b), however, exhibited a minor band at this pH and a major band at about pH 9.0. As will be discussed later, major differences in the amino acid composition were also noted between the 21,000-dalton protein of T2L and those of the other phages. The 21,000-dalton protein of T6 phage was not examined on gels since the small amount isolated was used for amino acid analysis.

Included in Fig. 4 is a gel of the 21,000-dalton protein from phage T4B01 (Fig. 4, c). This isolate was prepared by direct trichloroacetic acid precipitation of the phage supernatant solution after sedimentation of the phage capsids. It was thus contaminated by small amounts of the more acidic 18,000-dalton structural proteins of the phage head (21; G. Forrest and D. Cummings, *unpublished data*), illustrating the necessity for identification of other minor proteins released by osmotic shock.

Finally, an electrofocusing gel of T4 lysozyme (lot 802025, Calbiochem) has been included (Fig. 4, e). It is of interest that this protein with a molecular weight similar to the 21,000-dalton protein should also have the same isoelectric point. This similarity will be considered in greater detail later.

Figure 4 also shows representative electrofocusing gels of the 9,500-dalton internal pro-

teins. It is clear that in many of the cases examined, the 9,500-dalton proteins exhibited both a major and several minor bands. The major band of all of the 9,500-dalton proteins (Fig. 4; f, g, h) was at approximately pH 9.0, whereas the minor bands of T2H and T6 were in the region of the 21,000-dalton protein. By ^{125}I labeling and gel filtration on 6% agarose, these minor bands have been shown to be the result of contamination of the CM-cellulose peak 1 with the 21,000-dalton material from peak 2. The 21,000-dalton protein of T2L (Fig. 4, b), on the other hand, was at the same position as the 9,500-dalton proteins. This would be expected from its behavior on the CM-cellulose column.

The electrofocusing gel of the 9,500-dalton protein from T6 phage (Fig. 4, g) illustrates again how minor contaminants can be identified by this method. In this case, several of the structural capsid proteins eluted in CM-cellulose peak 1 (13, 14, 21; G. Forrest and D. Cummings, *unpublished data*). Only the head structural 11,000-dalton protein had an isoelectric point near the internal proteins (21; G. Forrest and D. Cummings, *unpublished data*). It gave an isoelectric point of approximately pH 7, near the middle of the gel.

Structural proteins isolated from the heads of the T4 amber mutants AmN85 (G48) and AmH21 (G54) were also examined on isoelectrofocusing gels. Both of these mutant phages produced free heads and tail plates when grown under restrictive conditions (R. S. Edgar, *personal communication*). Free heads devoid of DNA were separated from tail plates and isolated as described previously (13, 14, 21) and were examined without osmotic shock treatment. Both the 9,500- and 21,000-dalton internal proteins were present in these heads. It is of interest that these empty heads contained the internal proteins and may indicate either that the presence of DNA is not necessary for their occurrence or that the heads originally contained DNA which was digested by deoxyribonuclease treatment during purification. The major point to be made is that these internal proteins could not have originated from tail substructures released during osmotic shock.

The internal proteins were also electrofocusing in sucrose gradients by using the LKB electrofocusing column to determine more precisely their isoelectric points. This instrument allowed direct measurement of the pH across the ampholine gradient, whereas the pH gradient of the gels was estimated from positions to which proteins of known isoelectric points migrated. The results obtained for the 21,000- and 9,500-dalton proteins of T4B from the electrofocusing column by using pH 7 to 10 range ampholine are presented

in Fig. 5. Figure 5A demonstrates that the 21,000-dalton protein gave a single peak at pH 9.2, corresponding closely with the isoelectric point of pH 9.5 estimated for this protein from the electrofocusing gels.

The isoelectric point for the 9,500-dalton protein, by this method, was pH 8.86 which corresponds closely with the gel results which gave pH 9.0. This isolate of the 9,500-dalton protein was contaminated with the heavier 21,000-dalton protein, resulting in the second peak. This experiment clearly demonstrated the distinctive isoelec-

tric characteristics of the two proteins and the reproducibility of the method.

Amino acid analysis. Amino acid analyses of the internal proteins verified the basic nature of these proteins. The basic amino acid content (lysine, arginine, and histidine) of all of the 21,000-dalton proteins examined varied from 18.2 to 19.4% (Table 1). The compositions of the 9,500-dalton proteins of T4B and T4D (Table 2) yielded 17.5 and 17.1%, respectively, for the same amino acids. Although the 9,500-dalton proteins of T2H, T2L, and T6 are not presented here, the preliminary amino acid compositions of these proteins indicated that they also were composed of relatively high amounts of the basic amino acids.

The amino acid compositions of the 21,000-dalton proteins of T4B, T4D, T2H, and T6 were in close agreement. All were high in alanine and tryptophan, but none had detectable half-cystine. These proteins also contained approximately three times more phenylalanine than tyrosine. The 21,000-dalton protein of T2L phage, on the other hand, was different from the other 21,000-dalton proteins in a number of amino acids. The T2L protein had neither tryptophan nor proline and nearly equal amounts of tyrosine and phenylalanine. It was high in glutamic acid-glutamine and low in alanine.

The 9,500-dalton proteins of the T-even bacteriophages have not been as well characterized as the 21,000-dalton proteins. Only those of T4B and T4D have as yet been obtained free of contaminating 21,000-dalton proteins (Fig. 4). The 9,500-dalton proteins of these phages, however, exhibited an amino acid composition distinct from that of the 21,000-dalton proteins (Table 2). The 9,500-dalton proteins had only about half the aspartic acid-asparagine content of the 21,000-dalton proteins. The tyrosine-phenylalanine ratio was near unity in the 9,500-dalton proteins, whereas the 21,000-dalton proteins contained about three times more phenylalanine than tyrosine. The 9,500-dalton proteins were high in histidine and low in tryptophan. Amino acid analyses of the 9,500-dalton proteins of T2H, T2L, and T6, which were similar to those proteins isolated from T4B and T4D, have been omitted from Table 2 since the degree of contamination from the 21,000-dalton proteins has not yet been ascertained.

DISCUSSION

Evidence has been presented which indicates that there are two basic internal proteins in the T-even bacteriophages. The major internal proteins of T4B, T4D, T2H, and T2L were found to have a molecular weight of 21,000 daltons. Phage

FIG. 5. Electrofocusing of the internal proteins of phage T4 in sucrose gradients. Protein samples dissolved in 10 M urea were applied at the center of the sucrose step gradient. Ampholine (pH 7 to 10) was used; electrophoresis was for 24 hr at 400 v. Fractions (2 ml) were collected and analyzed for pH (broken line) and fluorescence (solid line). (A) The 9,500-dalton internal protein, contaminated by small amounts of the 21,000-dalton protein. (B) The 21,000-dalton internal protein.

TABLE 1. *Amino acid composition of the 21,000-dalton internal proteins^a*

Amino acid	T4B	T4D	T2H	T2L	T6
Aspartic acid + asparagine.....	13.4 ± 0.3	13.5 ± 0.5	13.0 ± 0.5	12.9 ± 0.5	13.0 ± 0.5
Threonine.....	3.2 ± 0.1	3.0 ± 0.1	3.5 ± 0.3	6.6 ± 0.1	3.2 ± 0.1
Serine.....	4.7 ± 0.3	4.8 ± 0.2	4.9 ± 0.3	6.6 ± 0.3	5.0 ± 0.9
Glutamic acid + glutamine.....	6.3 ± 0.2	6.5 ± 0.2	6.5 ± 0.2	9.8 ± 0.4	6.7 ± 0.1
Proline.....	2.0 ± 0.5	2.1 ± 0.3	2.3 ± 0.4	0	2.1 ± 0.5
Glycine.....	7.3 ± 0.2	7.4 ± 0.3	7.3 ± 0.3	8.2 ± 0.2	8.0 ± 0.3
Alanine.....	14.6 ± 0.3	14.5 ± 0.5	14.0 ± 0.5	9.7 ± 0.3	14.6 ± 0.2
Valine.....	5.5 ± 0.3	6.0 ± 0.5	6.0 ± 0.3	6.2 ± 0.2	5.1 ± 0.7
Half-cystine ^b	0	0	0	0	ND ^c
Methionine.....	1.0 ± 0.1	1.2 ± 0.2	1.4 ± 0.3	2.2 ± 0.2	1.3 ± 0.1
Isoleucine.....	3.7 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	5.8 ± 0.2	3.8 ± 0.1
Leucine.....	6.1 ± 0.2	6.3 ± 0.2	6.2 ± 0.2	4.2 ± 0.2	6.4 ± 0.1
Tyrosine.....	2.0 ± 0.2	2.1 ± 0.2	2.3 ± 0.2	3.9 ± 0.1	2.0 ± 0.1
Phenylalanine.....	7.0 ± 0.3	7.1 ± 0.2	6.7 ± 0.3	5.4 ± 0.3	6.9 ± 0.1
Lysine.....	13.5 ± 0.5	13.7 ± 0.3	13.2 ± 0.5	13.0 ± 0.7	13.8 ± 0.3
Histidine.....	1.0 ± 0.2	1.2 ± 0.4	1.2 ± 0.2	1.7 ± 0.1	1.1 ± 0.1
Tryptophan.....	4.1 ± 0.3	3.0 ± 0.5	1.3 ± 0.1	0	2.5 ± 0.4
Arginine.....	4.2 ± 0.1	4.3 ± 0.3	4.5 ± 0.1	3.5 ± 0.2	4.5 ± 0.2

^a Values given in micromole per cent.^b Determined as cysteic acid.^c Not determined.TABLE 2. *Amino acid composition of the 9,500-dalton internal proteins^a*

Amino acid	T4B	T4D
Aspartic acid + asparagine.....	6.9 ± 0.4	7.0 ± 0.2
Threonine.....	6.3 ± 0.2	5.9 ± 0.1
Serine.....	6.1 ± 0.2	5.8 ± 0.3
Glutamic acid + glutamine.....	8.8 ± 0.3	8.7 ± 0.3
Proline.....	2.3 ± 0.3	2.7 ± 0.2
Glycine.....	9.5 ± 0.4	9.0 ± 0.2
Alanine.....	13.5 ± 0.5	13.0 ± 0.4
Valine.....	4.6 ± 0.3	5.5 ± 0.4
Half-cystine ^b	0	0
Methionine.....	1.2 ± 0.2	1.4 ± 0.2
Isoleucine.....	9.5 ± 0.4	9.6 ± 0.4
Leucine.....	9.5 ± 0.2	9.5 ± 0.4
Tyrosine.....	1.2 ± 0.2	1.4 ± 0.1
Phenylalanine.....	1.1 ± 0.1	1.5 ± 0.3
Lysine.....	13.6 ± 0.4	12.9 ± 0.5
Histidine.....	2.5 ± 0.3	2.5 ± 0.1
Tryptophan.....	1.0 ± 0.4	0.8 ± 0.2
Arginine.....	1.4 ± 0.1	1.7 ± 0

^a Values given in micromole per cent.^b Determined as cysteic acid.

T6 may have contained the 21,000-dalton protein in major amounts also, but losses in handling perhaps reduced the final yield. The 21,000-dalton proteins of T4B, T4D, and T2H were shown to have an isoelectric point of about pH 9.2, whereas

that of T2L was slightly lower. The minor internal proteins of T4B, T4D, T2H, T2L, and T6 phage were found to have molecular weights in the range of 9,500 daltons. The isoelectric points of the 9,500-dalton proteins of T4B, T4D, T2H, and T6 were found to be near pH 8.86, as was the 21,000-dalton protein of T2L phage.

The procedures used in the present study to isolate the internal proteins resulted in good recovery of the 21,000-dalton proteins in chromatographically and electrophoretically pure form. The 9,500-dalton proteins, however, were usually contaminated by small amounts of other phage proteins, including the 21,000-dalton internal proteins. The 9,500-dalton proteins, therefore, will necessarily have to be further fractionated by gel filtration before they can be characterized in more detail. Since undetermined losses of each of the proteins occurred during isolation, no attempt has been made to calculate the amount of each species of internal protein per phage particle.

Amino acid analyses of the two species of internal proteins supported the basic nature of the proteins exhibited by the electrofocusing gels. The proteins were composed of nearly 20% basic amino acids (lysine, arginine, and histidine). Major differences between the 21,000- and 9,500-dalton proteins were observed in several amino acids which added support to the premise that these two protein species were unique.

One of the major problems encountered in the present study has been identification of the internal proteins. This is evident in Fig. 4, which shows that the isolates of these proteins can be contaminated by a variety of phage structural proteins. Simultaneous investigations of other phage structures in this laboratory (13, 14, 21) have been invaluable in this respect. Thus, contamination of the internal protein preparations by 18,000- and 11,000-dalton head structural proteins were easily monitored on the electrofocusing gels. Three procedures employed in the present study have greatly reduced the level of contamination with other phage proteins. Osmotic shock with glycerol, instead of high salt, greatly decreased the amount of head structural proteins released. At the same time, osmotic shock induced by the glycerol method resulted in ionic conditions suitable for chromatography of the proteins on the carboxymethyl cellulose. Use of this resin allowed recovery of the 21,000-dalton protein virtually free of contamination from the more acidic 18,000-dalton head proteins. Finally, centrifugation of the osmotic shock supernatant solution for 15 hr after removal of the capsids resulted in quantitative removal of free tail components and unsedimented capsids which would have accounted for a significant proportion of the proteins isolated.

Additional support for the belief that the two proteins isolated were indeed internal proteins of the phage head came from preliminary studies of proteins isolated from two amber mutants of T4. Electrofocusing gels of proteins isolated from purified heads of AmN85 (G48) and AmH21 (G54) clearly demonstrated the presence of the two internal proteins. It should also be pointed out that Black (L. Black, *personal communication*) found that there were two, and possibly three, internal proteins in T4D, having properties similar to those properties reported here. Also, Champe (S. Champe, *personal communication*) has indicated that these internal proteins were phage specific and accumulated during the time-course of infection.

Correlation of the previous findings with those of other laboratories has been difficult since it is not known if the other groups isolated both of the internal proteins together or only one of the two proteins. Levine et al. (31) reported isolation of the internal antigen in an electrophoretically, chromatographically, and immunochemically pure form. Coval et al. (Fed. Proc. 19:253, 1960) stated that this protein had an amino acid composition which was high in lysine and histidine and contained no cysteine. Both the 9,500-dalton protein isolated in the present study and the 11,000-dalton head structural protein (G. Forrest

and D. Cummings, *unpublished data*) would fit this brief description. The composition of the 21,000-dalton internal protein, however, was clearly not high in histidine.

Four studies reported the possible existence of multiple internal proteins. Levine (32) reported that the ^{35}S -labeled nonsedimentable proteins released by osmotic shock were separated by chromatography and yielded label in several fractions, only one of which possessed antigenic activity. Minagawa (34) also found that 20% of the ^{35}S -labeled nonsedimentable proteins of T2 were not antigenically active against antibody produced against ruptured phage. Recently Bachrach et al. (8) found one major and two minor proteins could be isolated with phage DNA released by Sarkosyl rupture of the phage head membrane. Kokurina and Tikhonenko (29) fractionated ruptured phage on Sephadex G-200 and found four fractions which reacted to antibody prepared against ruptured phage. Only two of these four fractions lacked cross-reactivity to capsid proteins.

Earlier in this report, the close similarities of the 21,000-dalton internal protein and T4 lysozyme were noted. The two proteins possess similar molecular weights and identical isoelectric points. The amino acid compositions of the two proteins are quite different, however (27, 42). Several earlier reports suggested the presence of a lysozyme-like activity in phage lysates and internal protein preparations (26, 32, 34, 37, 38). Panijel (26, 37, 38) found a "prolysine" activity, capable of lysing acetone powders of bacteria, which was released from purified phage by osmotic shock. Levine (32), however, reported that the lytic activity of Panijel and the internal antigen were separable by chromatography. Minagawa (34) found that lysozyme activity was present in internal protein preparations prepared by osmotic shock with high salt, but were absent when glycerol was substituted. In the latter case, the lysozyme activity was found associated with the capsids. Emrich and Streisinger (20) recently found low levels of a new lytic activity in purified phage carrying deletions of the *e* gene. The phage used were *es* mutants [*s* = spackle; the spackle mutation allowed *e* gene mutants to lyse, releasing free phage (19)]. The low level of lytic activity observed was not blocked by antibody to the *e* gene lysozyme, suggesting the presence of a second species of lysozyme in purified phage.

Several other possible functions for the internal proteins have been proposed; these include a role in organization of the phage DNA during maturation (28), possible involvement in regulation of transcription of the phage DNA (11), and restoration of the membrane function after phage infec-

tion (17). Several of these theories, and other possibilities, will be the subject of future study by using the two proteins isolated here.

ACKNOWLEDGMENTS

We are indebted to Selina Janion for translation of the Kokurina and Tikhonenko paper.

This investigation was supported by Public Health Service grants AI-06472 and AI-08265 from the National Institute of Allergy and Infectious Diseases and GM-01379 from the National Institute of General Medical Sciences.

LITERATURE CITED

- Adams, M. H. 1959. Bacteriophages. Interscience Publishers Inc., New York.
- Albertsson, P.-A. 1960. Partition of cell particles and macromolecules. John Wiley and Sons, New York.
- Ames, B. N., D. T. Dubin, and S. M. Rosenthal. 1958. Presence of polyamines in certain bacterial viruses. *Science* (Washington) 127:814-816.
- Anderson, T. F. 1945. The role of tryptophan in the adsorption of two bacterial viruses on their host, *E. coli*. *J. Cell. Comp. Physiol.* 25:17-26.
- Anderson, T. F. 1948. The activation of the bacterial virus T4 by L-tryptophan. *J. Bacteriol.* 55:637-649.
- Awdeh, Z. L. 1969. Staining method for proteins after isoelectric focusing in polyacrylamide gel. *Sci. Tools* 16:42-43.
- Bachrach, U., and A. Friedmann. 1967. Purification and some possible functions of internal proteins from coliphage T2. *Biochem. Biophys. Res. Commun.* 26:596-601.
- Bachrach, U., R. Levin, and A. Friedmann. 1970. Studies on phage internal proteins: isolation of protein-DNA complexes from T2 phages and from phage-infected bacteria. *Virology* 40:882-892.
- Bishop, D. H. L., J. R. Claybrook, and S. Spiegelman. 1967. Electrophoretic separation of viral nucleic acids on polyacrylamide gels. *J. Mol. Biol.* 26:373-387.
- Champe, S. P., and H. L. Eddleman. 1967. Polypeptides associated with morphogenic defects in bacteriophage T4, p. 55-70. *In* J. S. Colter and W. Paranchych (ed.), *The molecular biology of viruses*. Academic Press Inc., New York.
- Chaproniere-Rickenberg, D. M., H. R. Mahler, and D. Fraser. 1964. The interaction of DNA and internal protein from coliphage T2. *Virology* 23:96-102.
- Cummings, D. J. 1963. Subunit basis of head configurational changes in T2 bacteriophage. *Biochim. Biophys. Acta* 68:472-480.
- Cummings, D. J., A. R. Kusy, V. A. Chapman, S. S. DeLong, and K. R. Stone. 1970. Characterization of T-even bacteriophage substructures. I. Tail fibers and tail tubes. *J. Virol.* 6:534-544.
- Cummings, D. J., V. A. Chapman, S. S. DeLong, A. R. Kusy, and K. R. Stone. 1970. Characterization of T-even bacteriophage substructures. II. Tail plates. *J. Virol.* 6:545-554.
- Dale, G., and A. L. Latner. 1968. Isoelectric focusing in polyacrylamide gels. *Lancet* 1:847-848.
- Davison, P. F. 1968. Proteins in denaturing solvents: gel exclusion studies. *Science* 161:906-907.
- Duckworth, D. H. 1970. The metabolism of T4 phage ghost-infected cells. I. Macromolecular synthesis and transport of nucleic acid and protein precursors. *Virology* 40:673-684.
- Eddleman, H. L., and S. P. Champe. 1966. Components in T4-infected cells associated with phage assembly. *Virology* 30:471-481.
- Emrich, J. 1968. Lysis of T4-infected bacteria in the absence of lysozyme. *Virology* 35:158-165.
- Emrich, J., and G. Streisinger. 1968. The role of phage lysozyme in the life cycle of phage T4. *Virology* 36:387-391.
- Forrest, G. L., and D. J. Cummings. 1970. Head proteins from T-even bacteriophage. I. Molecular weight characterization. *J. Virol.* 5:398-405.
- Herriott, R. M., and J. L. Barlow. 1957. The protein coats or 'ghosts' of coliphage T2. *J. Gen. Physiol.* 40:809-825.
- Hershey, A. D. 1955. An upper limit to the protein content of the germinal substance of bacteriophage T2. *Virology* 1:108-127.
- Hershey, A. D. 1957. Some minor components of bacteriophage T2 particles. *Virology* 4:237-264.
- Hirs, C. H. W. 1956. The oxidation of ribonuclease with performic acid. *J. Biol. Chem.* 219:611-621.
- Huppert, J., and J. Panijel. 1956. Recherches sur les prolysines. II. Le cas général de la synthèse des prolysines. *Ann. Inst. Pasteur* 90:711-727.
- Inouye, M., and A. Tsugita. 1968. Amino acid sequence of T2 phage lysozyme. *J. Mol. Biol.* 37:213-223.
- Kellenberger, E. 1961. Vegetative bacteriophage and the maturation of the virus particles. *Advan. Virus Res.* 8:1-61.
- Kokurina, N. K., and T. I. Tikhonenko. 1969. Isolation and fractionation of inner proteins of T2 and D₂VI bacteriophages. *Vop. Virusol.* 2:224-228.
- Kozloff, L. M., and M. Lute. 1960. Calcium content of bacteriophage T2. *Biochim. Biophys. Acta* 37:420-424.
- Levine, L., J. L. Barlow, and H. Van Vunakis. 1958. An internal protein in T2 and T4 bacteriophages. *Virology* 6:702-717.
- Levine, L. 1961. Immunochemical nature of the internal material in the T-even coliphages, p. 171-182. *In* M. Heidelberger and O. J. Plescia (ed.), *Immunochemical approaches to problems in microbiology*. The Rutgers University Press, New Brunswick.
- McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy* 29:185-189.
- Minagawa, T. 1961. Some characteristics of the internal protein phage T2. *Virology* 13:515-527.
- Murakami, W. T., H. Van Vunakis, and L. Levine. 1959. Synthesis of T2 internal protein in infected *Escherichia coli*, strain B. *Virology* 9:624-635.
- Nozaki, Y., and C. Tanford. 1967. Acid-base titrations in concentrated guanidine hydrochloride. Dissociation constants of the guanidinium ion and of some amino acids. *J. Amer. Chem. Soc.* 89:736-742.
- Panijel, J., and J. Huppert. 1956. Recherches sur les prolysines. I. La prolysine du phage Fc₂. *Ann. Inst. Pasteur* 90:619-636.
- Panijel, J. 1959. Les activités enzymatiques liées aux phages et la synthèse phagique. *Ann. Inst. Pasteur* 97:198-217.
- Rhodes, M. B., P. R. Azari, and R. E. Feeney. 1958. Analysis, fractionation, and purification of egg white proteins with cellulose cation exchanger. *J. Biol. Chem.* 230:399-408.
- Sober, H. A. 1968. Handbook of biochemistry. The Chemical Rubber Co., Cleveland.
- Sternberg, N., and S. P. Champe. 1969. Genetic determinant of an internal peptide of bacteriophage T4. *J. Mol. Biol.* 46:377-392.
- Tsugita, A., and M. Inouye. 1968. Complete primary structure of phage lysozyme from *Escherichia coli* T4. *J. Mol. Biol.* 37:201-212.