Amino-terminal Sequence Analysis of Proteins Purified on a Nanomole Scale by Gel Electrophoresis*

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ALAN M. WEINER,† TERRY PLATT,‡ AND KLAUS WEBER
From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

SUMMARY

A simple, rapid, manual technique is described for determining the amino-terminal amino acid sequence of proteins on a nanomole scale. In this modification of the 5-dimethylaminonaphthalene-1-sulfonyl-Edman degradation, inorganic carriers permit convenient manipulation of small amounts of protein, and use of the detergent sodium dodecyl sulfate throughout the procedure maintains protein solubility. Nanomole quantities of pure protein for such sequence analysis are readily isolated from multicomponent systems by analytical scale polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Proteins are recovered quantitatively from the gel by elution. The method is therefore suitable for characterization of the proteins derived from multichain enzymes and viruses.

The decision to determine a protein's amino-terminal sequence depends on availability of the purified species in sufficient quantity. Techniques such as the recently automated Edman degradation (1) have been extensively developed for proteins which can be purified in milligram amounts. Since many proteins can be purified only in microgram quantities, due to limitations in starting material for the purification or lack of convenient milligram scale preparative techniques, amino-terminal sequence analysis often requires a nanomole scale procedure.

The dansyl technique, introduced by Gray (2), is sufficiently sensitive for end group determination at the 0.1 n mole level (3). A combination of this technique with stepwise degradation by the Edman procedure has been successfully used for peptide sequencing by Gray and Smith (4) and by Bruton and Hartley (5). However, microtechniques have not been developed for purifying the amino-terminal peptide derived from nanomole amounts of protein by chemical or enzymatic cleavage.

The dansyl-Edman combination has rarely been applied to intact proteins. Although dansylation has been used to identify the amino-terminal residue of proteins directly, the traditional manual Edman degradation generally has not worked well. One of the main difficulties is that, in contrast to peptides, many proteins become insoluble after one or two cycles of degradation. Since insoluble material does not react completely in subsequent cycles, unambiguous sequence determination is impossible.

We have developed a reliable manual dansyl-Edman procedure which can be used to determine an amino terminal sequence of 5 to 10 residues for any protein with an unblocked amino terminus which can be purified on a nanomole scale. We have overcome protein solubility problems by replacing the aqueous organic solvents of the traditional Edman procedure with an aqueous inorganic buffer containing the detergent sodium dodecyl sulfate. SDS, which is known to denature and to solubilize proteins in aqueous solution (6-8), has also proved useful in the dansylation reaction.

Electrophoresis on analytical polyacrylamide gels in buffers containing SDS or urea is a widely used tool to characterize the individual polypeptide chains of multichain enzymes, viruses, and membrane preparations by charge or molecular weight. The high resolving power of these gels and their simple experimental design make them very attractive as a preparative technique for the study of multicomponent systems on a nanomole scale. We have successfully applied our SDS-dansyl-Edman procedure to nanomole quantities of protein separated on and quantitatively eluted from SDS polyacrylamide gels.

Proteins of both known and unknown amino-terminal sequence have been subjected to the SDS-dansyl-Edman degradation. For proteins of known sequence, our results agree with those found by conventional techniques. For proteins of unknown sequence, such as minor capsid proteins from the coliphages Qβ and R17, our amino-terminal sequence data are compatible with known RNA sequences from the phage genome.

MATERIALS AND METHODS

Chemicals—Reagents for polyacrylamide gel electrophoresis have been described previously (9, 10). Dowex 50W-X8 was obtained from Bio-Rad.

Phenyisothioiocyanate (sequenation grade) was an Eastman product. Sodium dodecyl sulfate, trifluoroacetic acid (both sequanual grade), and dansyl chloride (10%, w/v, in acetone) were purchased from Pierce Chemical Company (Rockford, Illinois). Guanidine hydrochloride was obtained from Schwarz/Mann. A Beckman amino acid calibration mixture (Type I) was used. Cheng-Chin polyamide layer sheets were obtained from Gallard-Schlesinger (Carle Place, New York). Paraflin

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† National Science Foundation Predoctoral Fellow.
‡ The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; SDS, sodium dodecyl sulfate; PTC, phenyylthiocarbamyl.
is an American Can Company product (Marathon Products Division). All chemicals used were reagent grade if not otherwise specified.

Proteins—Aspartate transcarbamylase from Escherichia coli was purified according to the method of Gerhart and Holoubek (11). β-Galactosidase from E. coli was purchased from Boehringer as a crystalline suspension. Egg white lysozyme (two times crystallized) was a Schwarz/Mann product. Lac repressor from E. coli was purified by the procedure of Gilbert and Mueller-Hill (12). Histone III/F was obtained from Worthington. Subunit II of Q8 replicase (gift of Dr. T. Blumenthal) was purified by gel electrophoresis. Trypsophan synthetase A protein was a gift from Dr. C. Yanofsky. Bovine serum albumin (Pentex recrystallized) was obtained from Miles Laboratories. Q8 and R17 bacteriophage were purified as described previously (13).

Equipment—A heated vacuum desiccator (Fisher Scientific) is maintained at 60° and connected to an oil pump through a Dry Ice-acetone cold trap and a trap containing potassium hydroxide pellets to keep water and acids out of the pump oil. A good water aspirator can be substituted for the oil pump, if drying times are increased by about 50%.

A desk top clinical centrifuge (International) is used throughout the procedure, since centrifugation at higher speed is unnecessary. The centrifuge must have swinging buckets, so that precipitates will be packed on the bottom of the tube and supernatants can be cleanly withdrawn with a Pasteur pipette. The reactions are done in either disposable culture tubes (10 mm × 75 mm) or in 15-ml conical centrifuge tubes. The disposable culture tubes (6 mm × 50 mm) which are used for dansylation can be centrifuged inside a larger tube by using a paper towel insert.

Preparative Gel Electrophoresis in Sodium Dodecyl Sulfate

The 10% polyacrylamide SDS gels were prepared according to the method of Weber and Osborn (10). For 5% gels, both acrylamide and methylene bisacrylamide were halved. After polymerization, the gels were aged at room temperature for at least 4 days. They were then removed from the casting tubes by rimming and pre-eluted with stirring at room temperature into three changes of running buffer (0.1 M sodium phosphate, 0.1% SDS, pH 7.2) at 12-hour intervals. Gels were sucked back into the casting tubes by drawing on the opposite end of the tube with a Pasteur pipette. The reactions are done in either disposable culture tubes (10 mm × 75 mm) or in 15-ml conical centrifuge tubes. The disposable culture tubes (6 mm × 50 mm) which are used for dansylation can be centrifuged inside a larger tube by using a paper towel insert.

Sample Preparation—Samples are heated to 95° for 2 min in loading buffer (0.01 M sodium phosphate, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.001% bromphenol blue, pH 7.2) supplemented with sufficient SDS to maintain a 2-fold weight excess over protein, then covered with Parafilm and completely reduced for 4 hours at 37°. When there is reason to suspect that the sample is contaminated with proteases, special precautions may be taken to avoid proteolysis during sample preparation as discussed by Pringle (14).

Determining Maximal Loading—On SDS gels, an overloaded protein band “trails” severely, although the leading edge of the protein band remains sharp with a slightly increased mobility. A single gel which is overloaded with respect to one or more proteins may nonetheless give narrow symmetrical bands for other proteins present in lower concentration. This situation often arises when intact virions are subjected to preparative electrophoresis. Minor viral components can be clearly resolved despite smearing of the major capsid proteins, as can be seen in Fig. 2. Maximal loading depends on how well the protein of interest is separated from adjacent protein bands. In the simplest case, a protein which cannot be resolved from adjacent bands on 6-cm gels may be cleanly separated on gels of 12- or 18-cm length. This is possible because electrophoresis is much faster than diffusion of the protein bands. If proteins with similar mobilities are also present in much greater quantity than the protein of interest, trailing will be more of a problem. In this case, even longer gels may not give adequate separation and loading must be reduced. In the worst possible case, a protein which cannot be purified in a single step must be subjected to a second preparative run. After preparative electrophoresis and elution of the protein bands, a small aliquot of each protein is always checked for purity by analytical polyacrylamide gel electrophoresis in SDS.

Removing Nucleic Acid—Intact viral DNAs and RNA's are excluded from 5% and 10% polyacrylamide gels. At current densities exceeding 6 ma cm−2 of gel cross-section, such highly charged polyanions may cause the top of the gel to collapse. For this reason, when intact virions are disrupted for preparative gel electrophoresis by heating with SDS and 2-mercaptoethanol, the gels are run at a current density not exceeding 6 ma cm−2 until the tracking dye has moved at least 1.5 cm into the gel. The nucleic acid is then flushed from the top of the gel and removed with a Pasteur pipette, taking care not to touch the gel surface. After this, the current is raised to its normal level of 25 ma cm−2.

Locating Protein Bands in Preparative Gels—We have used the following three techniques.

a. A lightly loaded analytical gel is run in parallel with each batch of preparative gels. Since protein mobilities vary slightly, depending on the precise conditions of polymerization (for a review see Reference 15), the analytical gel must be poured at the same time and in the same diameter tube as the preparative gels. After electrophoresis, the middle of the tracking dye band, as judged by eye, is marked by inserting across the gel diameter a 26 gauge hypodermic needle which has been dipped in India ink (16). We have found that this procedure permits more accurate determination of mobilities than slicing through the dye band with a razor blade. The analytical gel is stained in Coomassie brilliant blue (10) for 2 to 12 hours and destained by diffusion at 37° on a roller in a culture tube which contains destaining solution and sufficient Dowex 501-X8 to absorb unbound stain irreversibly (17). A 4-ml gel requires approximately 2 g of Dowex. Gels destained in this fashion are clear and free of the artifacts sometimes produced by electrophoretic destaining. Since both mobilities and band width increase slightly with heavy loading, analytical gels should be used only as a guide to determine an approximate mobility range for each protein band.

b. After electrophoresis, a single longitudinal guidestrip is cut from the edge of the preparative gel with a modified version of the gel slicer described by Fairbanks, Levinthal, and Reeder (18). The bulk of the gel is kept at 4° while the guidestrip is stained for 15 min and destained as in a. Because guidestrips cut from 10% polyacrylamide gels may stretch as much as 5% in length, this method is less accurate than the first, but prefer-
able for preparative gels with diameter of 1.0 cm or larger. The method should not be used for 5% polyacrylamide gels, since 5% polyacrylamide guide strips do not stretch uniformly.

c. When adjacent protein bands are too poorly resolved for mobilities to be a reliable guide, the entire preparative gel should be frozen and sliced transversely into 1-mm sections. These sections are then eluted individually into sufficient 5 mm NaHCO₃ containing 0.05% SDS to cover small analytical gels which are stained as usual to localize the protein bands. Eluates containing a single protein species are pooled, and those containing a mixture are rerun on preparative gels.

Eluting Protein Bands—Gel sections no thicker than 2 mm are eluted with agitation at 37° into 10 volumes (up to 5 ml) of 5 mm NaHCO₃ containing 0.05% SDS. After 12 hours, acrylamide fragments are spun out in a clinical centrifuge and the supernatant is lyophilized. The lyophilized residue can then be dissolved directly in coupling buffer as described below.

**Stepwise Sodium Dodecyl Sulfate-Dansyl-Edman Procedure**

Each cycle of SDS-dansyl-Edman degradation removes the NH₂-terminal amino acid and exposes a new amino-terminal residue, which is identified by dansylating an aliquot of the protein. A complete cycle consists of four steps, as shown in Fig. 1: coupling, precipitation, cyclization and cleavage, and finally removal of an aliquot of protein for dansylation before the cycle is repeated. Allow enough starting material so that 0.25 to 0.5 nmole of protein can be withdrawn after each cycle for the desired number of cycles.

Before attempting to sequence an unknown, practice is strongly recommended with several cycles of SDS-dansyl-Edman degradation on 5 nmole of a protein of known sequence such as lysozyme. To avoid difficulty during chromatography, 1 nmole aliquots should be withdrawn at each step for dansylation.

**Coupling**—The sample is dissolved in 200 µl of coupling buffer (0.5 x NaHCO₃ adjusted to pH 9.8 with NaOH) and no less than 20 µl of 10% SDS solution (w/v). For efficient detergent action, SDS must be present in at least 1.4-fold weight excess over protein (6–8). Then 10 µl of phenylisothiocyanate are added to the tube, which is immediately warmed to 50° in a water bath, flushed with nitrogen, and sealed with several layers of Parafilm. Most of the phenylisothiocyanate will remain as a separate organic phase at the bottom of the tube. The reaction proceeds for 30 min at 50°, with occasional vortexing every 5 or 10 min to keep the coupling buffer saturated with phenylisothiocyanate.

**Precipitation**—Nine volumes of acetone are added while vortexing the reaction tube, and the flocculent white precipitate is spun down for 2 min in the clinical centrifuge. The yellowish supernatant is withdrawn with a Pasteur pipette and discarded; the precipitate is washed with 2 ml of 100% acetone, spun down, and the colorless supernatant again discarded. The precipitate is spread around the walls of the tube by vortexing, and the tube is set nearly horizontal in the heated vacuum desiccator where the damp slurry is dried under vacuum for 20 min. The slurry must remain on the walls of the tube when it is set in the desiccator. Rapid evaporation of acetone under reduced pressure will cause lumps on the bottom to explode and be blown out the mouth of the tube.

**Cyclization and Cleavage**—Tubes are allowed to cool to room temperature, and 0.2 ml of trifluoroacetic acid is added to each. If the resulting solution is not clear, the precipitate from the coupling step was not completely dry. The degradation is continued in any case, but drying times should be increased by 5 to 10 min in subsequent cycles. The tubes are warmed to 50°, flushed with nitrogen, sealed with Parafilm, and incubated for 5 min at 50°. The Parafilm is removed, a small crystal of dry SDS (about 1 mg) is added, and while still warm the tube is vortexed to dissolve the SDS. The trifluoroacetic acid is blown away in the hood under a stream of nitrogen, so that a residue forms on the walls of the tube. The tube is then set nearly horizontal in the heated vacuum desiccator and dried in vacuo for 20 min.

The residue from trifluoroacetic acid treatment is taken up immediately in 0.2 ml of coupling buffer and at least 20 µl of 10% SDS solution. The sample foams as bicarbonate neutralizes any remaining trifluoroacetic acid. Heating to 50° may be required to bring all the SDS into solution, if too large a crystal was added after cyclization and cleavage. Clumps of partially insolubilized protein are divided as finely as possible by triturating with the tip of a Pasteur pipette. The pH is checked with pH paper which has been standardized in a buffer of known pH containing SDS. Indicator dyes have higher pK values when dissolved in SDS micelles. An aliquot (0.25 to 0.5 nmole in 10 to 100 µl, including a small portion of any insolubilized material) is withdrawn for dansylation into a disposable culture tube (6 mm x 50 mm). The degradation may be continued by adding 10 µl of phenylisothiocyanate to the remaining solution, and proceeding as described under “Coupling.”

**Dansylation**—The stock solution of dansyl chloride (10%, w/v, in acetone) is diluted 1:20 into acetone to give a few milliliters of working solution at 5 mg per ml. Small aliquots of stock solution are withdrawn from the “Hypovial” using a hypodermic syringe. At 4° in the dark, the stock solution may be used for at least 1 year, but the working solution is replaced weekly.

One-half volume of the working dansyl chloride solution is added to the protein aliquot withdrawn for dansylation, making it 33% in acetone. A precipitate may form, which will not interfere with dansylation. The tube is covered with Parafilm and incubated at 37°. Prewarming the tube is unnecessary and may even cause evaporation of the acetone, which is needed to solubilize the dansyl chloride. The reaction is terminated after 20 min by acid precipitation, following the suggestion of Gros and Labouesse (19). At protein concentrations greater than 50
µg per ml (for example, 5 µg/100 µl), the dansylated protein can be precipitated directly by cautious addition of ice-cold 20% trichloroacetic acid. The trichloroacetic acid solution must be added dropwise and with constant vortexing, or evolving carbon dioxide will cause uncontrollable foaming in the detergent solution. Below 30 µg per ml, 5 µg of succinylated carrier proteina are added before trichloroacetic acid precipitation to assure quantitative recovery.

In either case, the precipitate is spun down and the supernatant withdrawn with the finely drawn tip of a Pasteur pipette and discarded. The precipitate is washed once with 0.2 ml of 1 N HCl to remove dansylic acid. After centrifugation the supernatant is withdrawn, and 60 µl of 6 N HCl (reagent grade concentrated HCl diluted 1:1 with distilled water) are added. A neck is drawn on the tube using a fine oxygen torch, and the tube is sealed under water aspirator vacuum (oil pump unnecessary). After hydrolysis for 4 to 6 hours at 105°, as recommended by Gros and Labrousse (19), the tubes are opened and the hydrolysates dried down under vacuum in the heated vacuum desiccator. NH₂-terminal isoleucine, leucine, and valine occasionally require longer hydrolysis, as discussed under “Chromatography.”

Chromatography—Chromatography is performed essentially according to the method of Hartley (3) as modified by Neuhold (cited in Reference 3). Polyamide plates coated on both sides of the plate, registry of the spots should be sufficiently accurate to distinguish dansyl-isoleucine from dansyl-leucine after differentiation. Before several trial cycles of SDS-dansyl-Edman are performed according to the method of Woods and Wang (21) as modified by Hartley (3). Solvents I, II, III, and IV are run in the same direction perpendicular to Solvent I.

Solvent I 1.5% formic acid in water
Solvent II benzene-acetic acid, 9:1
Solvent III ethyl acetate-acetic acid-methanol, 29:11:1
Solvent IV 0.05 M NaPO₄ in 25% aqueous ethanol

The four solvents can be made in advance and stored in tightly capped bottles at room temperature. Solvents should be kept covered at all times and never used for longer than 3 hours, since differential evaporation affects their chromatographic properties. Solvents should be discarded whenever they cease to resolve the standard dansyl-amino acid mixture adequately.

Altogether, chromatography requires less than an hour: 5 min each for Solvents I, II, and III; 10 min in Solvent IV; at least 10 min of drying after Dimension I and 5 min each after Dimensions II and III. Dimension IV requires only brief drying before examination under ultraviolet light. Solvents II, III, and IV need a solvent-saturated atmosphere, and can be run in 150-ml beakers covered with a stretched sheet of Parafilm. The solvent should just cover the bottom of the beaker evenly. Plates are placed in beakers and withdrawn using a tweezers. The resulting abrasion marks are not harmful, however, since the solvent front is never allowed to run to the edge. Plates are kept as vertical as possible during chromatography. The lower edges of the plate must not touch the walls of the beaker or capillarity may distort the solvent front.

Chromatography is not difficult but the manipulations require practice. Before several trial cycles of SDS-dansyl-Edman are attempted on a protein of known sequence, reliable chromatographic identification of the dansyl derivative must be possible. When the standard dansyl-amino acid mixture is applied to both sides of the plate, registry of the spots should be sufficiently accurate to distinguish dansyl-isoleucine from dansyl-leucine after Dimension II; dansyl-alanine from dansyl-amide, dansyl-threonine from dansyl-serine, dansyl-glutamic acid from dansyl aspartic acid, α-dansyl-histidineb from ε-dansyl-lysine after Dimension III.

We have found that α-dansyl-histidine cannot be resolved from ε-dansyl-lysine in Dimension III unless (a) the polyamide thin layers are presoaked for at least 10 min in Solvent I and (b) the dansylated carrier protein is prepared according to the method of Habebeb et al. (20). Protein was dissolved at 10 mg per ml in guanidine hydrochloride buffer (pH 7.0) with 1 M NaClO₄ and a 40-fold molar excess of solid succinic anhydride over amino groups was added in three equal aliquots at 1-hour intervals. The solution was dialyzed extensively to 0.05 M NaHCO₃ and stored in the cold. Succinylated carrier protein should be dansyl-negative in the following test. An aliquot of 0.05 ml sodium bicarbonate solution containing about 5 µg of succinylated carrier protein is added to 50 µl of coupling buffer made 1% in SDS. The succinylated carrier protein is dansylated, precipitated, hydrolyzed, and chromatographed as usual. No dansyl derivatives other than a faint ε-dansyl-lysine should be detectable even on overloaded polyamide plates. Any protein may be used as carrier (for example, bovine serum albumin or lysozyme). Protein purity is irrelevant since reactive amino groups are reversibly succinylated, and succinylation assures solubility even in the absence of detergent or denaturing agents (20).

ä Capillary Micropipettes—Kimax melting point capillaries and a microburner without flame-spreader are used. The center of the capillary is rotated just above the flame, taking care to heat short a length of tubing as possible. When the glass has softened, the capillary is removed from the flame and immediately drawn out. If the capillary is drawn in the flame, it will melt in two. With the capillary resting on a clean towel, a single scratch is made at the midpoint with a diamond pencil. When the capillary is bent slowly into a “U” with the scratch on the convex side, the resulting break is even. Finely drawn Pasteur pipettes are made similarly.
This problem does not arise when polyamide thin layers are reused after regeneration according to the method of Wang and according to Gray (a), but the dansylation reaction is terminated by adding excess formic acid. In this way the bicarbonate buffer is not applied to the polyamide sheet, where it may cause smearing in the first dimension by neutralizing the 1.5% formic acid solvent.

A 100-μl aliquot of an amino acid standard mix (Beckman Instruments amino acid calibration mixture, 2.5 μm in each amino acid in 0.01 M HCl) is dried down in the heated vacuum desiccator, redissolved in 0.5 ml of 0.1 M NaHCO₃ and dansylated by adding 0.25 ml of fresh working dansyl chloride solution at 0.35 mg per ml in acetone. The tube is covered with Parafilm and incubated at 37° for 30 min. Reaction is terminated by adding 25 μl of 88% formic acid. The dansylated standard mixture is used directly for spotting (0.1 μl contains 0.025 nmole of each amino acid) and stored in the cold. This dansyl standard mixture contains didansyl-histidine, which is converted to α-dansyl-histidine by acid hydrolysis. To obtain α-dansyl-histidine, an aliquot of the standard mix may be dried down and hydrolyzed, or alternatively a stock solution of histidine can be dansylated with a 2-fold excess of amino acid over dansyl chloride as described by Gray (2).

**RESULTS**

**Sequence Results**—Amino-terminal sequence results obtained with the SDS-dansyl-Edman microtechnique are presented in Table I. Proteins ranging in molecular weight from 14,000 (Q8 coat protein) to 135,000 (β-galactosidase) have been studied. Those purified by gel electrophoresis in the presence of SDS are indicated in the table with a superscript c. No more than 0.25 nmole of protein per step was needed for amino-termi-

### Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol wt</th>
<th>Amino-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>From E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>14,000</td>
<td>Thr-Met-Ile-Thr-Asx-Ser-Leu-Ala*</td>
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<tr>
<td>lac repressor</td>
<td>38,000</td>
<td>Met-Lys-Pro-Val-Thr-Leu-Thr-Asx-Val-Ala-Glx-Tyr-Ala-Gly-Val-Ser*</td>
</tr>
<tr>
<td>Aspartate transcarbamylase catalytic subunit</td>
<td>38,000</td>
<td>Met-Arg-Ala-Phe-Ser-Ala-Leu-Asx*</td>
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<tr>
<td>Tryptophan synthetase A protein</td>
<td>39,000</td>
<td>Ala-Asx-Pro-Leu-Tyr-Glx-Lys-Ile-Ile</td>
</tr>
<tr>
<td>Minor capsid proteins from RNA coliphage</td>
<td>30,000</td>
<td>Ala-Lys-Val-Gly-Val-Thr-Val-Thr-Leu</td>
</tr>
<tr>
<td>R17 maturation protein*</td>
<td>36,000</td>
<td>Ala-Ser-Asx-Phe-Thr-Glx-Phe-Val-Leu-Leu</td>
</tr>
<tr>
<td>Q3 read-through protein (IIb)*</td>
<td>14,000</td>
<td>Ala-Lys-Leu-Gly-Thr-Val-Thr-Leu</td>
</tr>
<tr>
<td>Major capsid proteins from RNA coliphage</td>
<td>14,000</td>
<td>Ser-Lys-Thr-Ala*</td>
</tr>
<tr>
<td>R17 coat protein*</td>
<td>14,000</td>
<td>Lys-Val-Phe-Gly*</td>
</tr>
<tr>
<td>Q3 coat protein*</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>Other proteins</td>
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<td></td>
</tr>
<tr>
<td>Q9 repressor subunit II*</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>Hen egg white lysozyme</td>
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<td></td>
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<tr>
<td>α-protein purified by gel electrophoresis</td>
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<td></td>
</tr>
<tr>
<td>Protein purified with conventional techniques</td>
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<td></td>
</tr>
<tr>
<td>a Amino-terminal sequence agrees with that determined with conventional techniques by I. Zabin and A. V. Fowler (manuscript in preparation).</td>
<td></td>
<td></td>
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<tr>
<td>b Protein purified with conventional techniques by Platt et al.</td>
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<td></td>
</tr>
<tr>
<td>c Protein purified with conventional techniques by Billeter et al.</td>
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<tr>
<td>d Amino-terminal sequence agrees with that determined with conventional techniques by Guest et al. (23).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e Major capsid proteins from RNA coliphage</td>
<td>14,000</td>
<td></td>
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<tr>
<td>f Amino-terminal sequence agrees with that determined with conventional techniques by Moore et al. (26).</td>
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<tr>
<td>g Amino-terminal sequence agrees with that determined with conventional techniques by Konigsberg et al. (28).</td>
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<td></td>
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<tr>
<td>h Amino-terminal sequence agrees with that determined with conventional techniques by Canfield (30).</td>
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<tr>
<td>i Sequence agrees with that determined with conventional techniques by Dr. D. J. Koons.</td>
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<tr>
<td>j Sequence agrees with that determined with conventional techniques by Guest et al. (23).</td>
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<tr>
<td>k Sequence agrees with that determined with conventional techniques by Moore et al. (26).</td>
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<tr>
<td>l Sequence agrees with that determined with conventional techniques by Konigsberg et al. (28).</td>
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<tr>
<td>m Sequence agrees with that determined with conventional techniques by Canfield (30).</td>
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though dried in a hot air stream before applying the sample. This problem does not arise (3, 5) when polyamide thin layers are reused after regeneration according to the method of Wang and Wu (22).
nal sequence analysis, although more was used in several instances when sufficient starting material was available.

As an illustration of the SDS-dansyl-Edman procedure as applied to proteins purified on a nanomole scale by gel electrophoresis, we describe here a model experiment which yielded the amino-terminal sequences of the two minor capsid proteins of bacteriophage Q\(\phi\). The major coat protein species accounts for 95% of the capsid protein, and the two minor protein species IIb and IIa for 4% and less than 1%, respectively (31). In the model experiment, 6 mg of purified Q\(\phi\) virions were disrupted for 2 min at 95° with a 2-fold weight excess of SDS and 1% 2-mercaptoethanol. After reduction for 4 hours at 37°, equal aliquots of the denatured virus were layered onto six “pre-eluted” 10% polyacrylamide SDS gels (0.8 cm × 12 cm). About 5% of the sample was run in parallel on a fifth gel for use as a guide during elution. After electrophoresis at 3 mA each for 1 hour, viral RNA which had been excluded from the gel was removed with a Pasteur pipette. The current could then be raised to 8 mA each, and the gels run for 16 hours. The preparative gels were frozen at -20° while the guide gel was stained and destained as usual. Using mobilities determined from the guide gel, the IIa and IIb bands were cut out separately from each preparative gel and eluted into 4 ml of 0.05% SDS buffered with 5 mM NH\(_4\)-HCO\(_3\) for 12 hours at 37° with agitation. Elution under these conditions permitted quantitative recovery of the minor phage proteins after gel electrophoresis (see Table II). To determine purity, 5% of each eluate was rerun on analytical SDS polyacrylamide gels. Fig. 2 demonstrates that after elution, each minor phage protein is completely free of the other and of the major coat protein species. The eluates were then lyophilized, redissolved in coupling buffer, and subjected to three cycles of SDS-dansyl-Edman degradation. Using 40 \(\mu\)g of IIa protein we obtained the amino-terminal sequence Pro-Lys-Leu-Pro, and using 100 \(\mu\)g of IIb protein the sequence Ala-Lys-Leu-Glx-Thr-Thr-Thr-Leu (Table I). The biological implications of these sequence data are considered in the “Discussion.”

**Preparative Gel Electrophoresis**—Proteins for SDS-dansyl-Edman sequence analysis can be quantitatively recovered from “pre-eluted” gels. In the model experiment described in Table II, greater than 99% recovery was observed for Q\(\phi\) coat protein (molecular weight 14,000) run on 10% polyacrylamide SDS gels and for \(\beta\)-galactosidase (molecular weight 135,000) run on 5% gels. These gels combine experimental convenience, extraordinary sensitivity (less than 0.5 \(\mu\)g of protein is easily visible after staining), and high resolution, although they separate polypeptide chains almost entirely on the basis of molecular weight (10, 33, 34).

When we attempted to apply our SDS-dansyl-Edman technique to microgram quantities of protein eluted from such gels, the first results were disappointing. Gel electrophoresis and subsequent elution of the protein reduced the sensitivity of the sequencing technique by 5-fold, so that 2.5 nmoles of protein instead of 0.25 nmoles were required per step. Since we had expected quantitative recovery of protein, we attributed the decrease in sensitivity to either (a) chemical modification of the NH\(_2\)-terminus of the protein during electrophoresis, or (b) direct interference with the SDS-dansyl-Edman degradation by material eluted together with the protein from the polyacrylamide. Pre-electrophoresis with running buffer or with methyl green (34) to eliminate persulfate artifacts (for review see Reference 15) did not increase sensitivity, nor did pre-running the gels.

### Table II

<table>
<thead>
<tr>
<th></th>
<th>Q(\phi) coat</th>
<th>(\beta)-Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counts per min in uneluted control gel...</td>
<td>79,400</td>
<td>10,400</td>
</tr>
<tr>
<td>Counts per min in eluted gel...</td>
<td>600</td>
<td>100</td>
</tr>
<tr>
<td>Efficiency of elution after 10 hours</td>
<td>95%</td>
<td>95%</td>
</tr>
<tr>
<td>Efficiency of elution after 16 hours</td>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>

**Fig. 2.** Purification of minor capsid proteins from bacteriophage Q\(\phi\) by polyacrylamide gel electrophoresis in the presence of SDS. This model experiment is described under “Sequence Results.” A, complete Q\(\phi\) virion; B, purified IIa maturation protein; C, purified IIb read-through protein.
with a low molecular weight protein (9). Because the interfering material seemed to be electrophoretically neutral, we resorted to diffusion as suggested by Weber and Kuter (9). In a model experiment, 100 μg of lac repressor were recovered from such a pre-eluted gel (see “Preparative Gel Electrophoresis” under “Materials and Methods”), and four steps of SDS-dansyl-Edman degradation were successfully completed by withdrawing 10-μg (0.25 nmole) aliquots at each step for Dansylation. We do not know whether the interfering substance is residual acrylamide monomer, polyacrylamide, or polyacrylic acid, a contaminant in one of the reagents, or by a product of the polymerization reaction (for a review see Reference 15); however, it appears to be removable by diffusion.

**Stepwise Sodium Dodecyl Sulfate-Dansyl-Edman Degradation**

A complete cycle of SDS-dansyl-Edman degradation consists of four steps as shown in Fig. 1.

**Coupling Reaction**—In this step the protein is coupled with the water-insoluble Edman reagent, phenylisothiocyanate, to form a phenylthiocarbamyl derivative. The manual Edman degradation has usually been restricted to peptides, since many proteins are insoluble in aqueous organic solvents such as 50% pyridine which have been used to dissolve the Edman reagent. Other proteins become insoluble after one or two cycles of degradation.

The detergent SDS is known to solubilize and denature proteins in aqueous solution (6–8). Preliminary attempts to avoid solubility problems by adding SDS directly to the aqueous organic solvents of the traditional manual Edman procedure were unsuccessful. Model experiments revealed that the detergent action of SDS was greatly reduced in 50% pyridine. However, above its critical micellar concentration (7) SDS has been shown to bring not only proteins but also water-insoluble organic dyes into aqueous solution (for a review see Reference 6). We therefore used SDS in an aqueous inorganic coupling buffer to solubilize both the protein and the water-insoluble Edman reagent. When excess phenylisothiocyanate is present and forms a two-phase system, SDS mixtures presumably transport molecules of phenylisothiocyanate from the denser organic phase to SDS-protein complexes in the aqueous phase.

**Precipitation**—It is necessary to remove excess phenylisothiocyanate and nonvolatile by-products of the coupling reaction (4) before cyclization of the PTC-protein in anhydrous acid (1). Incomplete removal can lead to elevated backgrounds during identification of the dansylated NH₂-terminal amino acid SDS in the aqueous coupling buffer precludes repeated extractions with an immiscible organic solvent to remove phenylthiocarbamoyl groups and by-products, since (a) the detergent action of amphiphilic SDS prevents formation of a sharp interface, and (b) PTC-protein might partition into the organic phase in the presence of high concentrations of detergent. We therefore chose precipitation with nine parts acetone since SDS, phenylisothiocyanate, and the by-products of the coupling reaction are all soluble in aqueous acetone.

When a solution of protein in aqueous SDS is buffered with an organic compound, the protein precipitates from 90% acetone as a gummy residue while the buffer remains soluble. In model experiments with lysozyme and R17 coat protein, the PTC-protein was precipitated with nine parts acetone from aqueous SDS buffered with dimethylallylamine-trifluoroacetic acid (35). The precipitates were thoroughly washed with acetone and dried under vacuum at 60°. In neither case would the dry residue dissolve completely in anhydrous trifluoroacetic acid, even after prolonged vortexing at 50°. Preliminary performic acid oxidation of the proteins (36) did not affect these results.

Use of a strong 0.5 M sodium bicarbonate-carbonate buffer in the coupling reaction solved this problem, since the inorganic salt NaHCO₃ gives a flocculent precipitate in aqueous acetone. When PTC-protein and NaHCO₃ co-precipitate from 90% acetone, the flocculent inorganic precipitate acts as a carrier to keep the insoluble PTC-residue finely dispersed. This co-precipitate, when thoroughly washed with acetone and dried briefly in vacuo at 60°, dissolves immediately in anhydrous trifluoroacetic acid. Moreover, the acetone precipitation extracts sodium trifluoroacetate formed in the previous cycle of degradation by neutralization of NaHCO₃ and NaCO₃. Since sodium trifluoroacetate is readily soluble to at least 1 m in trifluoroacetic acid and to at least 4 m in 90% acetone, there is no accumulation of salt during the degradation.

**Cyclization and Cleavage**—Incubation with trifluoroacetic acid catalyzes cyclization and cleavage of the PTC-protein to produce the 2-anilino-5-thiazolinone derivative of the NH₂-terminal amino acid and to expose a new NH₂ terminus. Edman (for ref...
After cyclization and cleavage in trifluoroacetic acid is identified, the residue from trifluoroacetic acid treatment should always be redissolved as quickly as possible in coupling buffer. Reducing the cumulative exposure to strong acid in this way will (a) reduce chromatographic backgrounds due to nonspecific acid hydrolysis of the polypeptide chain, and (b) minimize the danger of blocking the SDS-dansyl-Edman degradation by acid-catalyzed cyclization of a newly exposed NH2-terminal glutamine to pyrrolidone carboxylic acid (41). Internal glutamine residues which have become NH2-terminal in the course of degradation can be identified by dansylation. The thiazolinone derivative of the previous amino-terminal residue, resulting from trifluoroacetic acid treatment, may be ignored when the Edman procedure is used in conjunction with dansylation (4). Presumably, thiazolinone derivatives of the amino acids do not react with dansyl chloride, or if they do, yield products which move with the solvent front during the second chromatographic dimension.

Excessive dansylic acid (the hydrolysis product of dansyl chloride) interferes with thin layer chromatography. The dansylated protein is therefore freed of dansylic acid by precipitation with trichloroacetic acid according to the method of Gros and Labouesse (19), which simultaneously extracts SDS and inorganic salts. At concentrations exceeding 0.005 mg per ml (10 μg/200 μl), the dansylated protein can be precipitated directly with 20% trichloroacetic acid. At lower concentrations, 0.05 mg per ml of succinylated carrier protein should be added before trichloroacetic acid precipitation to assure quantitative recovery of the dilute dansylated protein. Succinylated carrier protein is dansyl-negative and introduces no spurious chromatographic background, since all reactive amino groups have been irreversibly succinylated.

**Limitations of Sodium Dodecyl Sulfate Dansyl-Edman Procedure**—Edman (42) notes that during manual degradation the repetitive yield is only 90 to 95%, which he attributes primarily to oxidative desulfurization of the phenylthiocarbamyl group by small amounts of oxygen that are difficult to exclude in a manual technique. After desulfurization, the resulting phenylthiocarbamyl protein cannot cyclize and cleave in the trichloroacetic acid treatment, and is effectively blocked from further degradation. Fortunately, as Hartley (3) has emphasized, peptide chains which have been blocked in this way do not react with dansyl chloride, so that a clean NH2-terminal identification can be made at each step, albeit in decreased yield. Oxidative desulfurization may be a major limitation on the sensitivity of the manual SDS-dansyl-Edman procedure, since we have occasionally been unable to proceed beyond four cycles of degradation despite adequate starting material.

In the course of sequence analysis on *E. coli lac* repressor, Platt et al. (43) have found that the dansyl-Edman technique of Bruton and Hartley (5) for peptides will proceed through an internal tryptophan residue, although no dansyl derivative of tryptophan could be identified. We have not sequenced an internal tryptophan using our SDS-dansyl-Edman technique for proteins, but the two procedures are sufficiently similar so that no difficulties are anticipated.

Dansyl-proline background may rise prohibitively after several cycles of degradation with proteins rich in aspartyl- and asparaginyl-proline bonds, if the trifluoroacetic acid step is prolonged (see "Cyclization and Cleavage" under "Results").

Small polyamide sheets are very sensitive to overloading. Since chromatography on polyamide thin layers can detect 0.01 nmole of dansyl-l-lysine acid (8), SDS-dansyl-Edman sequencing on this scale should in principle present no problem. However, even 1 μg of total hydrolysis residue on an application spot of 1-mm diameter will cause dansyl-arginine, dansyl-histidine, and ε-dansyl-lysine to smear. The reason for this is that the first

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19 A 5-μl aliquot of Qβ bacteriophage at 17 mg per ml in buffer (0.1 M Tris-HCl-10 mM MgCl2·0.1 M NaCl, pH 7.2) was added to 0.2 ml of anhydrous trifluoroacetic acid, flushed with nitrogen, and incubated at 50°C. After 15 min a small crystal (about 1 mg) of dry SDS was added to the solution and the sample was dried down, taken up in coupling buffer, dansylated, hydrolyzed, and chromatographed as usual. In the control experiment, 5 μl of the Qβ solution were extensively dried in the heated vacuum desiccator, 0.2 ml of anhydrous trifluoroacetic acid, flushed with nitrogen, and incubated for 15 min at 50°C in trifluoroacetic acid containing 2.5% water revealed no significant hydrolysis of internal peptide bonds.

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20 A 5-μl aliquot of Qβ bacteriophage at 17 mg per ml in buffer (0.1 M Tris-HCl-10 mM MgCl2·0.1 M NaCl, pH 7.2) was added to 0.2 ml of anhydrous trifluoroacetic acid, flushed with nitrogen, and incubated at 50°C. After 15 min a small crystal (about 1 mg) of dry SDS was added to the solution and the sample was dried down, taken up in coupling buffer, dansylated, hydrolyzed, and chromatographed as usual. In the control experiment, 5 μl of the Qβ solution were extensively dried in the heated vacuum desiccator before addition of the trifluoroacetic acid. In both the model experiment containing 2.5% water and in the anhydrous control, incubation for 15 min at 50°C revealed less than 1% NH2-terminal proline relative to NH2-terminal alanine as judged by visual dilution analysis. A 10-fold dilution of the sample into 4% pyridine yields a dansyl-alanine spot of intensity comparable to the dansyl-proline contaminant in the undiluted sample. The NH2-terminal proline can be attributed entirely to the Qβ maturation protein (see “Sequence Results”) which constitutes less than 1% of the total capsid protein. This confirms earlier work by Smyth, Stark, and Koningsberg (cited in reference 30) in which no new amino groups could be detected by the cyanoate technique (40) after incubation for 24 hours at 25°C in moist trifluoroacetic acid.
amino-terminal sequence of 5 to 10 residues can be obtained for any protein with an unblocked aminoterminus which is available from membrane preparations also remains very near the origin in Dimensions I, II, and III (Fig. 3).

amidation chromatographic solvent must extract these dansyl derivatives from the total hydrolysis residue applied to the origin. Since smearing is proportional to the product of the mobility of a species and the time required to extract it, species which move with the solvent front will trail most severely. Consequently, loading must be increased about 5-fold for NH₄-terminal arginine and histidine, despite the fact that overloaded polyamide plates resolve all other dansyl-amino acids well. Thus 0.25 nmole of protein per step becomes the realistic maximum sensitivity for the SDS-dansyl-Edman degradation.

Chromatography presents other difficulties which may reduce the sensitivity of the technique. The dansyl derivatives of aspartic and glutamic acid are occasionally obscured by an O-dansyl-tirosine smear resulting from dansylation at pH 9.8 instead of pH 8.1 as recommended by Gros and Labouesse (19). Use of a coupling buffer at pH 9.0 instead of 9.8 would probably reduce the O-dansyl-tirosine smear without interfering with the degradation.

Dansyl-methionine may appear as both the oxidized derivative or as the sulfone, since the thioether is susceptible to air oxidation (44). This can cause problems when working at the limits of the technique's sensitivity, by reducing the intensity of both spots (see Fig. 3).

When an internal lysine becomes NH₄-terminal in the course of degradation, it reacts with dansiyl chloride to form an α-dansyl-ε-PTC-lysine derivative. Because this dissubstituted thioether is unstable to acid hydrolysis (39), two spots may appear during chromatography. Nonetheless, the chromatogram can be interpreted easily, since one spot moves with leucine in Dimension II and with methionine in Dimension III, while the other spot remains very near the origin in Dimensions I, II, and III (Fig. 3).

DISCUSSION

We have presented a simple, manual SDS-dansyl-Edman procedure using nanomole quantities of protein. Generally, an amino-terminal sequence of 5 to 10 residues can be obtained for any protein with an unblocked aminoterminus which is available on a nanomole scale. Nanomole quantities of protein for sequencing, all from the family of single-stranded RKA coliphages such as QB (29) and R17 (24). In general, ribosome binding sites can be assigned unambiguously to specific phage cistrons only by using the genetic code to correlate the RNA sequence with the corresponding amino-terminal sequence of the cistron's protein product. For instance, the amino-terminal sequence of the phage-specific subunit II of Qβ replicase, which is presented in Table I, enabled Hindley and Staples (29) to assign their ribosome binding sequence to the βα replicase cistron. In a similar case, the amino-terminal sequence of the R1 maturation protein product (Table I) not only confirmed the identity of the maturation cistron initiation site (24) but also extended the RNA sequence at the initiation site.

b. Amino-terminal sequence analysis can demonstrate relationships between viral proteins. For instance, sequence data obtained in the same model experiment described under “Sequence Results” and presented in Table I reveal that the major coat protein (molecular weight 14,000) of bacteriophage Qβ and the minor capsid component IIb (molecular weight 30,000) have identical amino-terminal sequences for at least 8 residues. This extraordinary fact led us to postulate that the IIb protein is a read-through product resulting from polypeptide chain elongation past an inefficient UGA stop signal at the natural termination site of the Q8 coat protein cistron. Growth of wild type Q8 on a high level UGA suppressor confirmed this hypothesis by

with SDS solution, and subjected directly to amino-terminal sequence analysis without removing the detergent.

Recent work in our laboratory employing the SDS-dansyl-Edman microtechnique for amino-terminal sequence analysis suggests the generality of this procedure and the range of its potential application. Three examples are considered below.

a. RNA sequencing of several single-stranded RNA coliphages is well under way (see for example References 24, 25, and 29) and similar work can be anticipated with other systems and with purified messenger RNA species. Amino-terminal sequence data for the corresponding proteins can be used in conjunction with ribonucleotide sequence data to identify which of many potential initiation sites for protein synthesis actually function in vivo. The SDS-dansyl-Edman microtechnique must be used when the protein of interest is available only in nanomole quantities. For instance, in the sequence of Billeter et al. (25) there are three AUG and two GUG codons within the first 175 nucleotides from the 5'-end of bacteriophage Q8 RNA, any one of which could potentially function as an initiation site (for review see Reference 46). The amino-terminal sequence of the Q8 maturation protein obtained in the model experiment described under “Sequence Results” and presented in Table I, enabled us to identify one of these codons (the AUG codon beginning at position 62) as the true initiation site and incidentally to place the maturation protein cistron at the 5'-end of the RNA genome (31).

RNA sequences coding for the initiation of protein biosynthesis can also be identified as ribosome binding sites, which are oligonucleotide tracts of messenger RNA protected from mild RNAse digestion by the initiation complex for protein synthesis. In this complex the ribosome is specifically bound to an mRNA molecule in the presence of initiation factors, fMet-tRNA, and GTP (46). Currently, 6 ribosome binding sites have been sequenced, all from the family of single-stranded RNA coliphages such as Q8 (29) and R17 (24). In general, ribosome binding sites can be assigned unambiguously to specific phage cistrons by using the genetic code to correlate the RNA sequence with the corresponding amino-terminal sequence of the cistron's protein product. For instance, the amino-terminal sequence of the phage-specific subunit II of Q8 replicase, which is presented in Table I, enabled Hindley and Staples (29) to assign their ribosome binding sequence to the Q8 replicase cistron. In a similar case, the amino-terminal sequence of the K11 maturation protein (Table I) not only confirmed the identity of the maturation cistron initiation site (24) but also extended the RNA sequence at the initiation site.

b. Amino-terminal sequence analysis can demonstrate relationships between viral proteins. For instance, sequence data obtained in the same model experiment described under “Sequence Results” and presented in Table I reveal that the major coat protein (molecular weight 14,000) of bacteriophage Q8 and the minor capsid component IIb (molecular weight 30,000) have identical amino-terminal sequences for at least 8 residues. This extraordinary fact led us to postulate that the IIb protein is a read-through product resulting from polypeptide chain elongation past an inefficient UGA stop signal at the natural termination site of the Q8 coat protein cistron. Growth of wild type Q8 on a high level UGA suppressor confirmed this hypothesis by

Moore et al. (26) reached a similar conclusion independently, using conventional sequencing techniques with 100 times as much starting material.
increasing the molar fraction of 11b protein in purified phage from 2 to 7% (31).

Precursor-product relationships between distinct capsid proteins have already been established by other techniques for bacteriophage T4 (47) and poliovirus (48). In the future we can expect that characterization of viral proteins by amino-terminal sequence will yield similar results.

c. SDS-dansyl-Edman sequence analysis can also demonstrate relationships between mutant and wild-type proteins. Work in progress on E. coli lac repressor has shown that protein biosynthesis can be identified by dansylation.14 Moreover, these same proteins can also be characterized by enzymatic (9, 15) and immunological activity (53),13 since Weber and Kuter (9) have found that residues 43 through 46 of the wild-type sequence correspond to the amino-terminal sequence of the reinitiated fragment. This indicates that an AUA triplet which codes for the internal residue methionine in wild-type lac repressor can be translated as alanine by E. coli ribosomes. The reinitiated fragment of lac repressor was first isolated from a crude cellular extract by precipitation with antibody prepared against wild-type repressor (49),14 and then separated from the antibody polypeptide chains by electrophoresis on 10% polyacrylamide SDS gels. Because the cross-reacting repressor fragment constitutes only 0.02% of a crude cellular extract, antibody precipitation yielded very small amounts of material which could be sequenced only by the SDS-dansyl-Edman microtechnique. The results are presented in Table 1. When this sequence was compared with that of wild-type lac repressor which had been purified and partially sequenced by conventional means,1 it was found that residues 43 through 46 of the wild-type sequence correspond to the amino-terminal sequence of the reinitiated fragment. This indicates that an AUG triplet which codes for the internal residue methionine 42 in wild-type lac repressor can function as a initiation site for protein synthesis when activated by an early amber mutation in the gene. Stewart et al. (50) have drawn similar conclusions in their work with a eukaryotic yeast, Candida albicans, and with a phage, bacteriophage 14 (47) and poliovirus (48).

The results are presented in Table 1. When this sequence was compared with that of wild-type lac repressor which had been purified and partially sequenced by conventional means, it was found that residues 43 through 46 of the wild-type sequence correspond to the amino-terminal sequence of the reinitiated fragment. This indicates that an AUG triplet which codes for the internal residue methionine 42 in wild-type lac repressor can function as an initiation site for protein synthesis when activated by an early amber mutation in the gene. Stewart et al. (50) have drawn similar conclusions in their work with a eukaryotic yeast, Candida albicans, and with a phage, bacteriophage 14 (47) and poliovirus (48).

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