THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 246, No. 4, Issue of February 25, pp. 1055–1061, 1971
Printed in U.S.A.

Polypeptide Chain Termination

PURIFICATION OF THE RELEASE FACTORS, R_1 AND R_2, FROM *ESCHERICHIA COLI**

(Received for publication, September 2, 1970)

HERBERT A. KLEIN‡ AND MARIO R. CAPECCHI§
From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

We have extensively purified the release factors R_1 and R_2 from *Escherichia coli*. These proteins can each mediate polypeptide chain termination. The physiological substrate for this reaction is a completed polypeptide chain in a peptidyl-transfer RNA-messenger RNA-ribosome complex. The reaction consists of recognition of a chain-terminating signal in the mRNA and hydrolysis of the peptidyl-tRNA ester bond, releasing the polypeptide chain. For the purification we were guided by two kinds of assay, the hexapeptide release assay and the formylmethionine release assay, each named according to the molecule released in a model reaction analogous to physiological chain termination. The two factors have different codon specificities, R_1 acting in response to UAG or UAA, and R_2 in response to UGA or UAA.

Both factors were purified from a 1-kg batch of frozen *E. coli* MRE600 by a scheme which carried the material through four steps before reaching a branch point at the fifth step, when R_1- and R_2-rich fractions were produced. The two fractions were then treated similarly but separately through three more steps.

The products were studied by polyacrylamide gel electrophoresis using both routine and sodium dodecyl sulfate techniques. For the 6.4 mg of purified R_1, we estimate 85% purity after 2,000-fold purification. For the 9.4 mg of purified R_2, we estimate 99% purity after 1,500-fold purification. Our results indicate that each release factor consists of a single polypeptide chain with a molecular weight of 44,000 for R_1 and 47,000 for R_2. We calculate that there are about 500 molecules of R_1 and 700 molecules of R_2 per *E. coli* cell.

When all the elongation steps are completed in the biosynthesis of a polypeptide chain, the chain retains an ester linkage to the transfer RNA of the last (carboxyl-terminal) amino acid residue and, by virtue of this bond, is part of a complex with the messenger RNA and ribosome. The ribosome, having traversed a series of amino acid-designating codons in the mRNA, has reached a chain-terminating signal. When this signal is recognized, hydrolysis of the final peptidyl-tRNA ester linkage occurs, releasing the polypeptide chain. The recognition and hydrolysis events constitute the polypeptide chain termination reaction. The release factors from *Escherichia coli*, R_1 and R_2, are proteins each of which can mediate this kind of reaction (1, 2).

Whereas the physiological reaction terminates completed polypeptide chains, the release factors were originally shown by their mediation of two analogous reactions, with shorter released products. The analogous reactions form the bases of the hexapeptide release assay (3) and the formylmethionine release assay (4), each named according to the molecule released. They make use of the trinucleotide sequences, UAA,[1] UAG, and UGA, known to be able to code for termination. Relying on these assays, we have extensively purified R_1 and R_2 by a scheme which is summarized in Table I.

The hexapeptide release assay uses mRNA from an amber mutant of the RNA coliphage R17 (amB_2), in which the seventh codon in the viral coat protein gene, CAG, has mutated to the chain terminating codon, UAG. By virtue of its altered coding properties, this RNA can be used in a protein-synthesizing scheme *in vitro* to construct a relatively simple substrate for release factor action. The substrate consists of the amino-terminal hexapeptide of the coat protein (fMET–Ala–Ser–Asn–Phe–Thr) complexed with tRNA, ribosome, and mRNA, with UAG in position to be read. One can assay for the activity of R_1 by incubating this complex with protein fractions and measuring the amount of hexapeptide released (and also the amount remaining bound).

In the formylmethionine release assay developed by Caskey *et al.* (4), the mRNA function is performed by two trinucleotides used in succession to signal initiation and termination. The substrate is formed by first making a complex of ribosome, AUG (the initiator triplet), and fMET-tRNA. Then a release triplet and test protein are added, and release of free formylmethionine is measured. The different codon specificities of the two release factors are reflected in this assay, as shown by Scolnick *et al.* (5). Thus, R_1 mediates the reaction in response to UAG or UAA, and R_2 in response to UGA or UAA. This explains why

* This work was supported by Research Grant GM 16280-02 from the National Institutes of Health.

‡ Recipient of National Institutes of Health Special Research Fellowship 5-F03-GM-23,345-02. Present address, Department of Biochemistry, New York University School of Medicine, New York, New York 10016.

§ Established Investigator of the American Heart Association.

[1] An abbreviation of the type UAA signifies either a trinucleoside diphosphate of the type UpApA or the same sequence occurring within mRNA. The other abbreviation used is: fMet, formylmethionyl.

the hexapeptide release assay detects only R_1, for it depends on the codon UAG in the mutated RNA. The formylmethionine release assay can be used to detect R_1, R_2, or the sum of their activities, depending on the choice of the terminator triplet (UAG, UGA, or UAA, respectively).

Recent studies of release factor action with both wild type and mutant viral messengers to direct protein synthesis confirmed the terminator triplet specificities of R_1 and R_2 (1) and, further, showed that the factors mediate normal, physiological terminations (1, 2). The analogous reactions remain useful for routine assay purposes.

MATERIALS AND METHODS

Commercial Materials—Bio-Solv was purchased from Beckman-Spinco; ATP, GTP, bovine serum albumin (crystalline, A grade), dithiothreitol (A grade), P-enolpyruvate, and pyruvate kinase from Calbiochem; Casamino acids from Difco Laboratories, Inc.; acrylamide (recrystallized), *N*,*N*′-methylenebisacrylamide, and glycine (ammonia-free) from Eastman; isopropyl alcohol (Spectranalyzed) from Fisher; calcium leucovorin from Lederle Laboratories; and $(NH_4)_2SO_4$ (special enzyme grade), alanine, asparagine, methionine, serine, and threonine from Mann. Glycerol (Spectroquality) was a product of Matheson Coleman and Bell, East Rutherford, New Jersey, as was sodium dodecyl sulfate which we recrystallized out of acetone. AUG, UAA, UAG, and UGA were obtained from Miles Labs, Elkhart, Indiana; levigated alumina from Norton Company, Worcester, Massachusetts; DEAE-Sephadex A-50, Sephadex G-25, Sephadex G-100, and Dextran T 500 from Pharmacia; DEAE-cellulose (Whatman, microgranular, DE-52) from Reeve Angel Company, New York; ^{14}C-phenylalanine (300 mCi per mmole) and ^{3}H-methionine (3 Ci per mmole) from Schwarz Bio-Research; Tris base and Tris-HCl from Sigma; and polyethyleneglycol (PEG) (Carbowax 6000) from Union Carbide and Carbon Corporation, New York.

Hexapeptide Release Assay—To synthesize the substrate for assaying 30 samples, a 500-μl reaction mixture was prepared containing 50 mM Tris-HCl, pH 7.8; 11 mM $MgCl_2$; 50 mM NH_4Cl; 4 mM dithiothreitol; 2.7 mM ATP; 0.14 mM GTP; 0.75 mg of P-enolpyruvate; 20 μg of pyruvate kinase; 20 nmoles each of methionine, alanine, serine, and asparagine; 1.5 μCi of ^{14}C-phenylalanine; 0.5 mg of tRNA; 60 μg of calcium leucovorin; 0.9 mg of *E. coli* S26 supernatant proteins; 1.5 mg of *E. coli* S26 ribosomes; and 0.15 mg of amB_2 RNA. After incubation for 15 min at 35°, the mixture was cooled in ice, combined with 300 μl of Buffer A (50 mM Tris-HCl, pH 7.6; 60 mM KCl; 10 mM $MgCl_2$; 0.5 mM EDTA; 2 mM dithiothreitol), layered on a 12-ml linear sucrose gradient (10% to 30% with 50 mM Tris-HCl, pH 7.5; 60 mM KCl; 10 mM $MgCl_2$; 0.5 mM EDTA; and 1 mM dithiothreitol), and centrifuged with the International SB283 rotor at 40,000 rpm for 185 min at 4°. The sucrose gradient centrifugation is necessary to eliminate supernatant release factors before supplying the sixth amino acid of the hexapeptide. Fractions were collected from the bottom, and all material sedimenting faster than about 70 S (judged from the ribosomal absorbance pattern) was pooled.

This material, containing a pentapeptidyl-tRNA-ribosome-mRNA complex, was combined with 0.4 mg of threonyl-tRNA, 1.8 μmoles of GTP, and 0.3 mg of T-factor to allow incorporation of the carboxyl-terminal amino acid of the hexapeptide fragment and completion of the substrate. The mixture was made up to 7.75 ml with Buffer A. Portions (250 μl) were added to protein samples of 5- to 20-μl volume, followed by incubation at 35° for 12 min. The samples were cooled in ice, combined with 500 μl of 5% trichloracetic acid, and further processed to determine quantitatively released and bound hexapeptide as previously described (6).

Because synthesis of a stable substrate requires withholding the threonyl residue until after sucrose gradient centrifugation, the supernatant proteins must be free of amino acids and aminoacyl-tRNA. For such a protein preparation, we ground 15 g of frozen *E. coli* S26 in a mortar with 30 g of alumina and then worked up the paste in 50 ml of Buffer B (50 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol; 20% glycerol). Debris was centrifuged out in two successive centrifugations, 12,000 × *g* for 30 min and 30,000 × *g* for 30 min. The supernatant fluid was made up to 50 ml with Buffer B. Polymer solutions and salt were added with stirring over the course of 45 min as follows: 20% dextran, 5.8 ml; 30% polyethyleneglycol, 15.5 ml; NaCl, 16.5 g (7). After another hour of stirring, two aqueous phases were separated by centrifugation at 4,300 × *g* for 5 min. The upper phase was dialyzed against 4 liters of Buffer C (50 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol; 5% glycerol) for 2 hours. Then $(NH_4)_2SO_4$ (19.2 g) was added to the 98-ml sample (for 33% saturation). After 30 min of stirring, two phases were separated by centrifugation at 7,700 × *g* for 10 min. The lower phase, 93.8 ml, was transferred by siphoning with polyethylene tubing and treated by addition of 16.6 g of $(NH_4)_2SO_4$ (for 60% saturation). After 45 min of stirring, the precipitated proteins were collected by centrifugation at 12,000 × *g* for 30 min and dissolved in 10 ml of Buffer D (50 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol; 50% glycerol). The supernatant proteins were finally dialyzed against Buffer D for 2 hours, with 600 ml in two portions. The product was stored in a −20° freezer.

E. coli S26 ribosomes were prepared as described previously (3) from a 30,000 × *g* supernatant fraction by sedimentation through a solution containing 50 mM Tris-HCl, pH 7.6; 10 mM $MgCl_2$; 0.10 M NH_4Cl; 1 mM EDTA; and 0.5 mM dithiothreitol.

E. coli S26 tRNA was prepared as described previously (8).

The mutant phage amB_2 was grown and purified according to the procedure of Gesteland and Boedtker (9) with the permissive *E. coli* strain S26R1E. The RNA was purified as described previously (8) by phenol extraction and ethanol precipitation.

E. coli threonyl-tRNA was partially purified by the method of Weiss, Kelmers, and Stullberg (10) with reverse phase chromatography. It was then charged with threonine by supernatant proteins, deproteinized with phenol, and chromatographed on Sephadex G-25.

The transfer factor, T, was obtained from DEAE-Sephadex chromatography of supernatant proteins and identified by complementation of G-factor in a poly-U-dependent polyphenylalanine-synthesizing system, and by binding of GTP.

Formylmethionine Release Assay—This assay was done by the method of Caskey *et al.* (4). The fMet-tRNA-AUG-ribosome complex was formed by incubating, for 15 min at 25°, a 100-μl reaction mixture containing: 50 mM Tris-HCl, pH 7.2; 11 mM $MgCl_2$; 0.10 M NH_4Cl; 1 mg of *E. coli* MRE600 ribosomes (washed with 0.5 M NH_4Cl); 10 pmoles of f-^{3}H-met-tRNA^{fMet}; and 4 nmoles of AUG. For each release reaction, 4.5 μl of the complex were incubated for 20 min at 25° in a 45-μl reaction

mixture containing: 50 mM Tris-HCl, pH 7.2; 30 mM $MgCl_2$; 0.10 M NH_4Cl; 1 mM dithiothreitol; 1 nmole of UAG, UGA, or UAA; and protein sample. The reaction was stopped by cooling and addition of 250 μl of 0.1 N HCl. The released formyl-^{3}H-methionine was partitioned into 1.4 ml of ethyl acetate, and a 1.0-ml sample was counted in toluene-fluor with Bio-Solv. Unlike the hexapeptide release assay, the formylmethionine release assay was performed with no transfer factors or GTP and with only one species of tRNA present.

$tRNA^{fMet}$ was prepared by Oak Ridge National Laboratories under Atomic Energy Commission-National Institute of General Medical Sciences NIGMS interregional agreement and distributed by National Institute of General Medical Sciences.

Comments on Assays—By way of comparing the two kinds of assay, the hexapeptide release assay, with a viral mRNA, can be considered to approximate better the physiological termination reaction. On the other hand, the formylmethionine release assay is more rapid and convenient. Both kinds of assay were useful for evolving the details of the purification scheme.

When we used the assays to evaluate protein fractions obtained at various stages of purification, the extent of reaction in a standard time interval was routinely determined as a function of varying amounts of the protein sample. For purified release factors, this function increased linearly up to extents of reaction in which the substrate was no longer saturating. However, for relatively impure fractions, with either kind of assay, this function showed downward curvature, attributable to inhibitors or interfering enzymes. Any deformylase or peptidase which left the labeled part of the release product with a free amino group could explain this effect, for the counts would be lost in the separation schemes which lead to final quantitation in each assay. Ability to obtain a linear region of protein dependence was our criterion for reliability of the assay for a given protein fraction. Judging in this way, we found the hexapeptide release assay to be reliable for Fraction III and all purer fractions of R_1; the formylmethionine release assay was reliable for Fractions VIa and VIb and the purer fractions of both release factors. (Tabulated data obtained from assay of the less pure fractions are approximations based on the lowest amounts of protein assayed.)

The assay data presented in any column of Table I compare samples assayed simultaneously and represent a reproducible relation among the specific activities of different samples. The results are not so meaningful in absolute terms, for they varied from assay to assay especially if different sets of components were used.

Calcium Phosphate Gel—Calcium phosphate gel was prepared by a method based on that of Keilin and Hartree (11). The mixture, 0.6 M $CaCl_2 \cdot H_2O$ (150 ml), was made up to 1600 ml with distilled water, and 0.4 M $Na_3PO_4 \cdot H_2O$ (150 ml) was added dropwise with stirring. The mixture was brought to pH 7.4 with 10% acetic acid. We washed the precipitate many times with distilled water, letting it settle and decanting. Finally, the gel was sedimented at 4300 $\times$ *g* for 15 min in 50-ml centrifuge tubes and then homogenized with Buffer E (50 mM Tris-HCl, pH 7.0; 0.1 M KCl; 1 mM dithiothreitol) with a Teflon pestle which fit the tubes with 1-mm clearance. In this manner, the gel was washed three times with Buffer E, with 253 ml per wash. Finally, one batch of sedimented gel weighed 65 g. On this wet weight basis, 30.3 g of gel were used per gram of protein to be adsorbed.

Growth of Bacteria—*E. coli* MRE600, a B strain lacking RNase I, was used. The medium was 20 mM KH_2PO_4; 80 mM K_2HPO_4; 1 mM $MgCl_2$; 15 mM $(NH_4)_2SO_4$; Casamino acids, 4 g per liter; and glucose, 10 g per liter. The cells were grown to late log phase in 100 liters in a Fermacell fermentor (New Brunswick Scientific Company, Inc., New Brunswick, New Jersey) at 35° with aeration. They were collected with a Sharples centrifuge and washed twice by suspension in Buffer F (50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$) and centrifugation at 5,000 $\times$ *g* for 25 min. About 900 g of cells, wet weight, were obtained from each such session of growth, and they were stored at −70°.

Protein Determination—Protein concentrations were determined from optical density at 280 mμ and 260 mμ by the method of Warburg and Christian (12). For low concentrations, the method of Lowry *et al.* (13) was used, with crystalline bovine serum albumin as standard. For Fraction I, determination according to the method of Lowry was performed after treating the sample with DNase and RNase, precipitating with 5% trichloracetic acid, and dissolving in 1 N NaOH.

Polyacrylamide Gel Electrophoresis—Routine runs, essentially following the method of Davis (14), were performed with 7.5% polyacrylamide gels (6 $\times$ 56 mm) which were buffered with 0.375 M Tris-HCl (pH 8.9) and topped with 13-mm high spacer gels containing 2.5% polyacrylamide, 29 mM Tris base and 16 mM H_3PO_4 (pH 7.1). Samples were diluted into 24 mM Tris base, 13 mM H_3PO_4, 10% glycerol (pH 7.1), layered on to the gels, and subjected to electrophoresis at 4° with a reservoir buffer of 9.9 mM Tris base, 77 mM glycine (pH 8.4). The gels were run at 2.5 ma per gel, with migration toward the anode, for 135 min and stained with Coomassie brilliant blue.

Sodium dodecyl sulfate gels followed the method of Shapiro, Viñuela, and Maizel (15). The gels, 6 $\times$ 58 mm, contained 7.5% polyacrylamide, 0.1 M sodium phosphate, 0.1% sodium dodecyl sulfate (pH 7.3). Protein samples were diluted into 13 mM sodium phosphate, 0.14% sodium dodecyl sulfate, 1.3% 2-mercaptoethanol (pH 7.4) and incubated for 2 hours at 37°. Each sample was then made 20% in glycerol and had 1 μg of bromphenol blue dye added before application to the top of the gel. Electrophoresis was at room temperature (25°) with a reservoir buffer of 75 mM sodium phosphate, 0.075% sodium dodecyl sulfate (pH 7.2). The gels were run at 8 ma per gel with migration toward the anode until the dye was near the bottom at about 265 min. They were stained with Coomassie brilliant blue.

RESULTS

Both release factors were purified from a batch of *E. coli* by a scheme (Table I) which carried the material through four steps before reaching a branch point at the fifth step, when R_1- and R_2-rich fractions were produced. They were treated similarly but separately through the rest of the purification. Procedures were performed in a 4° cold room. Volumes and pH values for the preparation of solutions are room temperature values (25°). Double distilled water was used.

Purification of R_1

Step 1: Crude Extract—Frozen *E. coli* MRE600 (1 kg) was homogenized in a Waring Blendor with 1 liter of Buffer G (50 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol; 15% glycerol). When the homogenate had warmed to −3°, it became much less viscous, and the cells were then broken

TABLE I

Scheme for purification of release factors, R_1 and R_2, from Escherichia coli

Purification of R_1 and R_2 from 1 kg of frozen *E. coli*. The calcium phosphate gel step is the branch point in the scheme, yielding two fractions enriched for R_1 and R_2, respectively, which are treated similarly but separately through the rest of the purification. The hexapeptide release assay was used to determine the activity of R_1 fractions (III through VIIIa). The formylmethionine release assay was also applied to R_1 fractions, with UAG and UAA, as indicated, as the terminator trinucleotides. The latter assay was applied to R_2 fractions (III through VIIIb) with UGA and UAA, as indicated. The activities of Fraction VIIIa with UGA and Fraction VIIIb with UAG were negligible. Fractions I and II could not be reliably assayed. Since only approximate data could be obtained by the formylmethionine release assay for the next three stages, these are given in parentheses. Specific activities of the release factors are given as the number of picomoles of product released per min per mg of protein assayed. Total activities given for the release factors are based, for R_1, on the results of the hexapeptide release assay and, for R_2, on the results of the formylmethionine release assay using UGA. We estimate an over-all degree of purification (relative to Fraction I) of 2000-fold for R_1 and 1500-fold for R_2.

Step	Fraction produced		Total protein		Specific activity (hexapeptide) R_1	Specific activity (formylmethionine)				Total activity	
	R_1	R_2	R_1	R_2		R_1 UAG	R_1 UAA	R_2 UGA	R_2 UAA	R_1	R_2
			mg		*pmole/min/mg*	*pmole/min/mg*				*pmole/min*	
Crude extract	I		77,500								
Aqueous polymers	II										
DEAE-cellulose	III		5,770		10.4	(0.75)	—	(1.48)	—	60,000	(8,550)
$(NH_4)_2SO_4$	IV		2,540		19.8	(1.17)	—	(2.91)	—	50,300	(7,400)
Calcium phosphate gel	Va	Vb	665	552	44.7	(2.71)	—	(7.8)	—	29,700	(4,310)
Isopropyl alcohol	VIa	VIb	238	330	102	8.3	9.4	10.9	14.6	24,300	3,600
Sephadex G-100	VIIa	VIIb	33	59	561	46.2	58.0	46.6	48.0	18,500	2,750
DEAE-Sephadex	VIIIa	VIIIb	6.4	9.4	1,660	145	187	172	208	10,600	1,620

by two passes through a Gaulin Mill under 8,000 p.s.i., followed by a 200-ml rinse of the mill with Buffer G. This process raised the temperature to 20°. Debris was removed by centrifugation at 10,000 × *g* for 50 min.

Step 2: Aqueous Polymer Partitioning—The supernatant fluid (Fraction I, containing 77.5 g of protein) was treated by partitioning between dextran and polyethyleneglycol in the manner of Albertsson (7) to remove ribosomes, the bulk of the free nucleic acid, and a portion of protein. For this purpose, the crude extract (1,600 ml) was stirred with gradual addition of the polymers and salt to the desired concentration *i.e.*, 20% dextran, 168 ml; 30% polyethyleneglycol, 462 ml; NaCl, 326 g. After a total of 55 min of stirring, the phases were separated by centrifugation at 10,000 × *g* for 15 min. The lower, dextran-rich, phase was discarded.

Step 3: DEAE-cellulose Chromatography—For application to the ion exchange column, the upper, polyethyleneglycol-rich and protein-rich phase (Fraction II, 1940 ml) was dialyzed in bags of 21-mm diameter against Buffer H (25 mM Tris-HCl, pH 7.6; 0.5 mM EDTA; 0.5 mM dithiothreitol). A total of 29 liters of Buffer H were used in three portions over 6 hours. The conductivity of the protein solution was finally checked to make sure that it was lower than that of Buffer I (25 mM Tris-HCl, pH 7.6; 0.1 M KCl; 0.5 mM EDTA; 1 mM dithiothreitol; 5% glycerol). A small amount of precipitated polyethylene glycol was removed by centrifugation at 10,000 × *g* for 45 min. The supernatant fluid (3 liters) was fed on to the column over 18 hours. The column, which had been equilibrated with Buffer I, had a bed of microgranular DEAE-cellulose, 9.5 cm high and 8.7 cm in diameter.

The polyethyleneglycol and some protein passed through the column during the application and a wash with Buffer I, 800 ml. The protein remaining adsorbed was eluted in two steps, carried out with a flow rate of 7.2 ml per min and collecting 20.8-ml fractions. First, elution with 980 ml of Buffer J (25 mM Tris-HCl, pH 7.6; 0.145 M KCl; 0.5 mM EDTA; 1 mM dithiothreitol; 5% glycerol) yielded fractions containing 8840 mg of protein, which were discarded. Then, elution with 980 ml of Buffer K (50 mM Tris-HCl, pH 7.6; 0.26 M KCl; 0.5 mM EDTA; 0.5 mM dithiothreitol; 5% glycerol) yielded fractions containing 5770 mg of protein. The elution with Buffer K was followed by elution with Buffer L (25 mM Tris-HCl, pH 7.6; 0.6 M KCl; 0.5 mM EDTA) in order to complete the recovery of the Buffer K. The material was pooled (Fraction III) for further processing.

Step 4: $(NH_4)_2SO_4$ Fractionation—Fraction III was precipitated by gradually adding 446 g of solid $(NH_4)_2SO_4$ (for 70% saturation) to the 959-ml eluate, with stirring. After an additional 45 min of stirring, the precipitate was collected by centrifugation at 25,000 × *g* for 40 min, and a protein fraction containing the release factors was extracted from it with $(NH_4)_2SO_4$ solutions. These had been prepared by adding $(NH_4)_2SO_4$ to measured volumes of Buffer M (50 mM Tris-HCl, pH 7.3; 1 mM dithiothreitol). First, the sediment was worked up in 940 ml of Buffer N (277 g of $(NH_4)_2SO_4$ per liter of Buffer M, for 45% saturation), followed by stirring for 45 min and centrifugation at 25,000 × *g* for 40 min. The supernatant fluid was set aside, and the sediment was then worked up, with the aid of a Dounce homogenizer, in 455 ml of Buffer O (256 g of $(NH_4)_2SO_4$ per liter of Buffer M, for 42% saturation), followed by stirring and centrifugation as before. The two supernatant fluids were pooled, giving a volume of 1492 ml (including additional amounts of Buffers N and O which were required to fill the centrifuge bottles). Solid $(NH_4)_2SO_4$, 95.6 g, was added slowly to give about 55% saturation to precipitate Fraction IV, which was collected, after 45 min of additional stirring, by centrifugation at 25,000 × *g* for 40 min.

Step 5: Calcium Phosphate Gel Fractionation—Fraction IV was dissolved in 39 ml of Buffer E and dialyzed for 2 hours with

3,500 ml of Buffer E in two portions. The fraction contained 2,540 mg of protein in 75 ml, and on this basis 77 g of calcium phosphate gel were homogenized with 193 ml of Buffer E and slowly added to the protein solution for adsorption. Protein fractions were later eluted from the gel with 268-ml portions of buffers of increasing phosphate concentration. The gel was homogenized with Buffer E and with the elution buffers in 15 50-ml centrifuge tubes with a Teflon pestle just as in the last steps of preparing the gel (see above). At each step (adsorption and elution), the homogenate was stirred for 45 min and centrifuged at 4,300 × *g* for 15 min to pellet the gel. Elution was performed twice with Buffer P (10 mM potassium phosphate, pH 7.0; 1 mM dithiothreitol), twice with Buffer Q (45 mM potassium phosphate, pH 7.0; 1 mM dithiothreitol), and once each with Buffer R (80 mM potassium phosphate, pH 7.1; 1 mM dithiothreitol) and Buffer S (0.12 M potassium phosphate, pH 7.1; 1 mM dithiothreitol). The fractions obtained with Buffer Q were pooled to give Fraction Va, which was rich in R_1; the fractions obtained with Buffers R and S were pooled to give Fraction Vb, which was rich in R_2. Tris-HCl (1 M, pH 8.0, 27.3 ml) was added to Fraction Va (519 ml), and the protein was precipitated by gradual addition of 258 g of solid $(NH_4)_2SO_4$ (for 70% saturation). After 45 min of stirring, the protein was sedimented at 25,000 × *g* for 40 min. Fraction Vb was treated in the same manner.

Step 6: Isopropyl Alcohol Fractionation—Fraction Va (665 mg) was taken up in 13.9 ml of Buffer T (50 mM Tris-HCl, pH 6.9; 50 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol and dialyzed against the same buffer for 2 hours, with 1,200 ml in two portions. A precipitate was removed by centrifugation at 12,000 × *g* for 20 min. The solution was brought to 60.8 ml with Buffer T, and 17.2 ml of isopropyl alcohol was added over 25 min with stirring (for 22% concentration). The solution was in a steel beaker held at 0° while receiving the first 6.8 ml of isopropyl alcohol (10%), at −6° in a refrigerated bath for the remainder. The lower temperature was maintained during another 10 min of stirring and during centrifugation at 29,000 × *g* for 20 min.

Step 7: Sephadex G-100 Chromatography—The sedimented protein (Fraction VIa) was taken up in 2.7 ml of Buffer U (50 mM Tris-HCl, pH 7.4; 0.15 M KCl; 1 mM EDTA; 2 mM dithiothreitol) and dialyzed against the same buffer for 2 hours, with 500 ml in two portions. Insoluble material was removed by centrifugation at 12,000 × *g* for 30 min. The supernatant fluid (238 mg in 4.5 ml) was applied to a column of Sephadex G-100, 2.5 × 200 cm, and eluted with Buffer U at 0.35 ml per min. Fractions of 3.5 ml were collected and assayed for R_1 by the formylmethionine release assay. Active fractions were pooled as Fraction VIIa.

Step 8: DEAE-Sephadex Chromatography—Fraction VIIa (33.2 mg in 60 ml) was applied over the course of 150 min to a column of DEAE-Sephadex, 1.5 × 20 cm, which had been equilibrated with Buffer U. A linear gradient, progressing from Buffer U to Buffer V (50 mM Tris-HCl, pH 7.6; 0.50 M KCl; 1 mM EDTA; 2 mM dithiothreitol) in a total volume of 500 ml, was applied to elute the proteins. A flow rate of 0.4 ml per min was maintained. Fractions of 3.0 ml were collected and assayed. The pooled, active fractions, which centered at 0.28 M KCl, constituted Fraction VIIIa (6.42 mg in 35.7 ml), the most highly purified fraction of R_1. This represents a 160-fold purification and 17.8% recovery in relation to Fraction III, the earliest fraction reliably assayed by the hexapeptide release method. Since Fraction III had a 13.4-fold reduction of the amount of protein in the crude extract (Fraction I), we estimate an over-all purification of about 2,000-fold.

In order to store R_1 in concentrated form, we added 5 mg of bovine serum albumin and then 15.3 g of $(NH_4)_2SO_4$ (for 65% saturation) to Fraction VIIIa. After 30 min of stirring, the precipitate was sedimented at 29,000 × *g* for 30 min, dissolved in 1.6 ml of Buffer W (50 mM Tris-HCl, pH 7.5; 0.15 M KCl; 10 mM $MgCl_2$; 1 mM EDTA; 2 mM dithiothreitol), and dialyzed against Buffer W, 1,000 ml in two portions, for 2 hours.

Purification of R_2

Steps 1 to 5 of the purification of R_1 supplied Fraction Vb (552 mg), which was further processed to purify R_2.

Step 6: Isopropyl Alcohol Fractionation—This step was performed exactly as for Fraction Va.

Step 7: Sephadex G-100 Chromatography—The sedimented protein (Fraction VIb) was taken up in Buffer U, 7.6 ml, and dialyzed against the same buffer for 2 hours, with 500 ml in two portions. Insoluble material was removed by centrifugation at 12,000 × *g* for 30 min. The supernatant fluid was concentrated in a dialysis bag immersed in dry Sephadex G-200 to a volume of 5.3 ml. Precipitated protein was removed by centrifugation at 12,000 × *g* for 30 min. The supernatant fluid (330 mg) was applied to a column of Sephadex G-100, 2.5 × 200 cm, and eluted with Buffer U at 0.35 ml per min. Fractions of 3.5 ml were collected and assayed for R_2 by the formylmethionine release assay. Active fractions were pooled as Fraction VIIb.

Step 8: DEAE-Sephadex Chromatography—Fraction VIIb (58.5 mg in 65 ml) was applied over the course of 160 min to a column of DEAE-Sephadex, 1.5 × 20 cm, which had been equilibrated with Buffer U. A linear gradient progressing from Buffer U to Buffer X (50 mM Tris-HCl, pH 7.6; 0.60 M KCl; 1 mM EDTA; 2 mM dithiothreitol) in a total volume of 500 ml was applied to elute the proteins. A flow rate of 0.4 ml per min was maintained. Fractions of 3.0 ml were collected and assayed. The pooled, active fractions, which centered at 0.32 M KCl, constituted Fraction VIIIb (9.42 mg in 34.9 ml), the most highly purified fraction of R_2. We estimate a 116-fold purification and 19.0% recovery in relation to Fraction III, based on the formylmethionine release assay with UGA. Reasoning as for R_1, this represents an approximate over-all purification of 1,500-fold over the crude extract (Fraction I). We added 5 mg of bovine serum albumin and then 15.0 g of $(NH_4)_2SO_4$ (for 65% saturation) to Fraction VIIIb. After 30 min of stirring, the precipitate was sedimented at 29,000 × *g* for 30 min, dissolved in 1.7 ml of Buffer W, and dialyzed against Buffer W, 1,000 ml in two portions, for 2 hours.

Characterization of Release Factors

R_1—On routine polyacrylamide gel electrophoresis, purified R_1 (Fraction VIIIa) gave rise to a single major band and several minor bands (Fig. 1). The major band was estimated to account for 85% of the staining material. This band was identified as R_1 by the correlation of its intensity with R_1 activity of analyzed fractions. Electrophoretic analysis of purified R_1 by the sodium dodecyl sulfate method also showed a major band and minor bands, the former again accounting for about 85% of the total and taken, therefore, to represent R_1 (Fig. 2).

When proteins are analyzed by the latter method, they run as dissociated polypeptide chains. The mobilities of the chains are

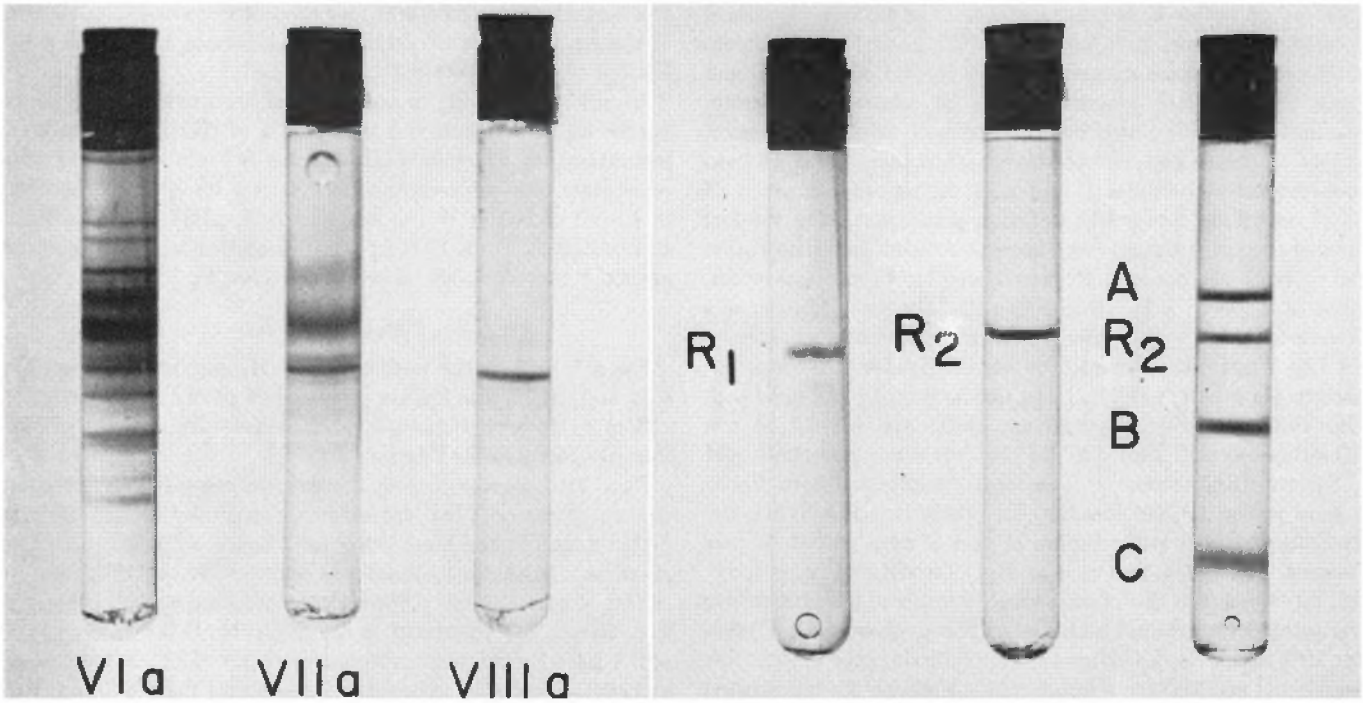

FIG. 1 (*left*). R_1 in the last three stages of purification analyzed by routine polyacrylamide gel electrophoresis. Migration was toward the bottom (anode). The amounts of protein applied were: VIa, 44 μg; VIIa, 14 μg; VIIIa, 6.3 μg.

FIG. 2 (*right*). The purest fraction of each release factor analyzed by polyacrylamide gel electrophoresis using the sodium dodecyl sulfate technique. Migration was toward the bottom (anode). R_1 (Fraction VIIIa), 4.5 μg, was applied to the first gel; R_2 (Fraction VIIIb), 4.1 μg, to the second. The third gel shows R_2 subjected to coelectrophoresis with three of the markers used for molecular weight calibration (see Fig. 4). The markers and their single chain molecular weights are: *A*, catalase, 60,000; *B*, carbonic anhydrase, 29,000; and *C*, R17 coat protein, 13,750.

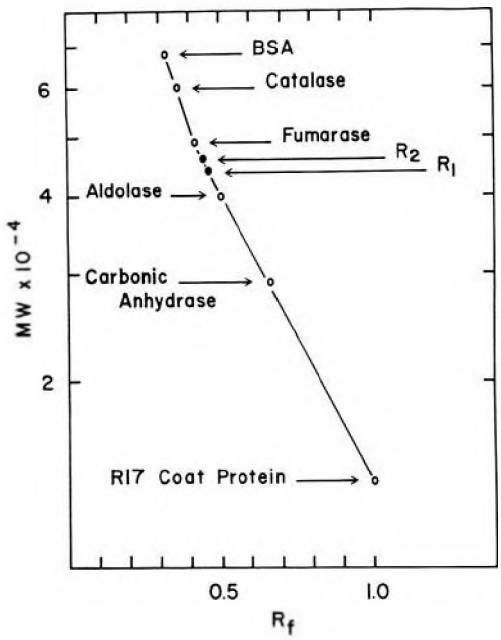

FIG. 3. Calibration curve from the polyacrylamide gel electrophoresis of protein standards by the sodium dodecyl sulfate method. The molecular weights of dissociated polypeptide chains are plotted on a log scale as a function of mobility relative to that of R17 coat protein. The interpolated mobilities of R_1 and R_2 indicate molecular weights of 44,000 and 47,000, respectively.

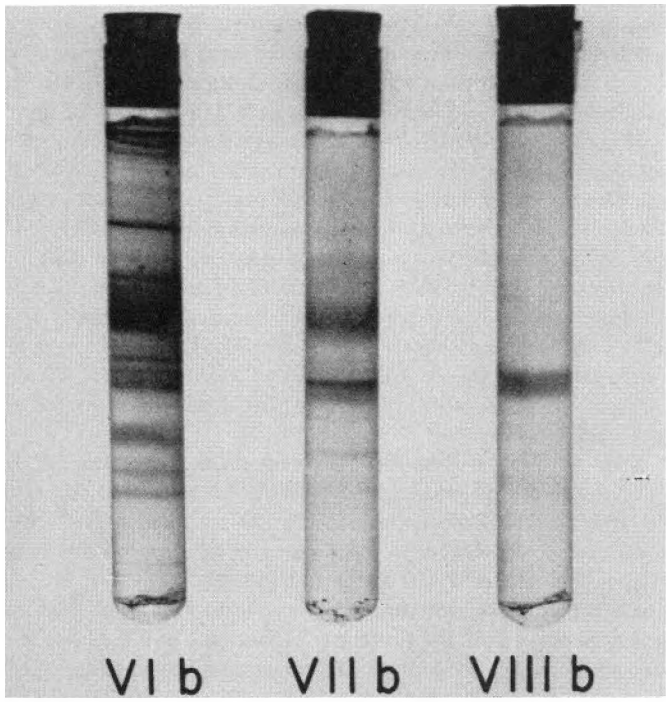

FIG. 4. R_2 in the last three stages of purification analyzed by routine polyacrylamide gel electrophoresis. Migration was toward the bottom (anode). The amounts of protein applied were: VIb, 44 μg; VIIb, 14 μg; VIIIb, 5.4 μg.

inversely related to the logarithms of their molecular weights (15, 16). The results for R_1 allowed us to infer that it consists of a single species of polypeptide chain with molecular weight of 44,000. The molecular weight estimate was obtained by running R_1 with a series of standard proteins of known dissociated chain molecular weights and measuring the distances migrated relative to that of the fastest standard, the R17 coat protein. A smooth, nearly linear curve of dependence on the logarithm of molecular weight was obtained (Fig. 3), allowing interpolation for the release factor. The behavior of R_1 on a calibrated sucrose gradient (3) and on the Sephadex G-100 column is consistent with the assignment of the molecular weight of the single chain to the native protein.

R_2—Purified R_2 (Fraction VIIIb) gave rise, on both routine (Fig. 4) and sodium dodecyl sulfate electrophoresis (Fig. 2), to a very predominant major band accounting for an estimated 99% of the staining material. On the routine gels, the band had a diffuse quality, possibly due to aggregation, but the band obtained by the sodium dodecyl sulfate technique was sharp. In the same manner as for R_1, the single chain molecular weight was estimated at 47,000. Again, from our observation of the behavior of R_2 on Sephadex G-100, we conclude that the native protein is a monomer of the same molecular weight.

DISCUSSION

Purification of the release factors enabled us to increase our understanding of their nature and their role in protein synthesis.

The efficacy of the purified factors as antigens permitted studies clarifying their physiological function (1). After challenging rabbits with them, we obtained highly specific antisera to each of the factors. We then found that the antisera could be used to control the release of the two detectable products of protein synthesis *in vitro* programmed with RNA from the coliphage R17, the coat protein and RNA synthetase. The addition of both antisera was necessary and sufficient to inhibit release of each phage protein, and either R_1 alone or R_2 alone, when added back in excess of the antisera, could overcome the inhibition and release the proteins. This result showed that the factors mediate normal terminations as well as the model reactions and was consistent with UAA signaling release at the termination loci for both the coat protein and the RNA synthetase, for it is the only codon to which both factors respond. UAA had been found, in fact, to occur at the terminus of the coat protein cistron in the RNA sequence determined by Nichols (17).

In the present studies we observed that the behavior of both release factors through the fractionation scheme and electrophoretic analysis is entirely typical of proteins. Evidence against the presence of a nucleic acid portion includes the demonstration of resistance to RNase digestion (3, 4) and, in the case of R_1, a negative analysis for phosphorus (18). Establishing the chemical nature of the factors was important since they are probably the molecules which recognize the sequences coding for termination in mRNA (18, 19).

One can envision a site in the ribosomal complex for codon recognition and another for reactions (peptide bond formation or hydrolysis) involving the carboxyl end of a growing or completed polypeptide chain. When the distance (not necessarily invariant) between these sites becomes known, it will be pertinent to compare it to the dimensions of R_1 and R_2 because of the involvement of the release factors in both recognition and hydrolysis. Any molecule (such as aminoacyl-tRNA) which functions simultaneously at both sites must be long enough to span the distance. If the release factors are spheres of the stated molecular weights, we estimate a diameter of 47 A for each, with 1.4 g cm^3 for their density. There is some question as to whether the release factors directly catalyze hydrolysis of the peptidyl-tRNA ester bond. The catalysis may be by the ribosomal peptidyl transferase (the enzyme which forms the peptide bonds), with the release factors acting only to trigger this function (18, 20, 21).

From our purification data we now estimate that there are 500 molecules of R_1 and 700 molecules of R_2 per *E. coli* cell. The calculations used our purity estimates for Fractions VIIIa (85%) and VIIIb (99%), the molecular weights (44,000 for R_1 and 47,000 for R_2), the assumption of 85% recovery between Steps I and III for both factors, and the figure of 10^{15} cells per kg of frozen *E. coli*. The result may be compared with the ribosome content of 30,000 per cell which we have found in cells grown under the same conditions. Data of Leder, Skogerson, and Nau (22) indicate that G factor molecules, which participate in each amino acid addition to a growing polypeptide chain, have an abundance exceeding that of ribosomes. A great excess of G factor over release factor molecules is consistent with the fact that the elongation step occurs many times and the release step only once in the synthesis of a polypeptide chain.

Acknowledgment—We are greatly indebted to Mrs. Nancy E. Capecchi for expert technical assistance.

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