Specificity of Polyribosomes in the Synthesis of T4 Bacteriophage Head Protein*

Joseph D. Padayatty and Ronald Rolfe†

ABSTRACT: A method for the preparation of polyribosomes from spheroplasts of *Escherichia coli* after T4 bacteriophage infection has been developed. Nascent polypeptides on polyribosomes were labeled with high levels of radioactivity by growing phage in a synthetic medium containing $^{35}SO_4^{2-}$ as the sole source of sulfur or by pulse labeling with radioactive amino acids. Three ^{35}S-labeled peptides were characterized as constituents of phage head protein by high-voltage electrophoresis of the oxidized chymotryptic digests of polyribosomal polypeptides. When *amber* B17, a mutant in the gene that specifies the phage head protein, was grown on a nonpermissive host, *E. coli* B, it did not make detectable head protein peptides.

However, when grown on a permissive host of *E. coli*, it produced all these peptides. Using these peptides as markers for T4 phage head protein, it was found that the synthesis of head protein takes place predominantly on polyribosomes estimated to contain approximately 11–12 ribosomes.

Polyribosomes are the site of protein synthesis in animal (Wettstein *et al.*, 1963; Warner *et al.*, 1962; Penman *et al.*, 1963; Gierer, 1963) and bacterial systems (Watson, 1963; Gilbert, 1963; Kiho and Rich, 1964). Polyribosomes from spheroplasts of *Escherichia coli* infected with T4 bacteriophage reflect the changing pattern of protein synthesis which is a consequence of the sequential expression of phage genes. At early times, the polyribosomes of infected cells are markedly heterogeneous with respect to size in keeping with the heterogeneity of the proteins being synthesized to carry out early functions (Rolfe and Rich, 1965). Later, the polyribosome distribution becomes more monodisperse, reflecting the increasing specialization of infected cells for the synthesis of large amounts of a few structural proteins, especially phage head protein. The appearance of phage head protein on "late polysomes" was investigated by comparing the electrophoretic patterns of chymotryptic digests of nascent polypeptides from different sizes of polyribosomes to digests of phage head protein.

Materials and Methods

Synthetic Medium. This medium contained per liter: 5.4 g of NaCl, 3.0 g of KCl, 1.1 g of NH_4Cl, 0.011 g of $CaCl_2 \cdot 2H_20$, 0.203 g of $MgCl_2 \cdot 6H_2O$, 0.027 g of Na_2SO_4, 3.78 g of Na_2HPO_4, 1.62 g of KH_2PO_4, 5 g of glucose, 0.01 g of $FeCl_3$, and 12.1 g of Tris-HCl (pH 7.4).

Luria Adsorption Medium (LAM). This medium contained per liter: 1.47 g of KCl, 4.68 g of NaCl, 1.07 g of NH_4Cl, 1.0 g of $MgCl_2 \cdot 6H_2O$, 0.029 g of $CaCl_2 \cdot 2H_2O$, 0.001 g of $FeCl_3$, 0.1 g of gelatin, 1.05 g of Na_2HPO_4, 0.45 g of KH_2PO_4, and 1.5 ml of glycerol. LAM-I contained 20 mg of 5-methyl-DL-tryptophan and LAM-II 100 mg of L-tryptophan.

Spheroplast Minimal Medium. This medium contained per liter: 5.4 g of NaCl, 3 g of KCl, 1.1 g of NH_4Cl, 0.011 g of $CaCl2 \cdot 2H_2O$, 92.54 g of sucrose, 5 g of glucose, 0.263 g of Na_2HPO_4, 0.113 g of KH_2PO_4, 4.2 g of $MgCl_2 \cdot 2H_2O$, 10 mg of ribonucleosides, 0.01 g of $FeCl_3$, and 72.5 mg of L-tryptophan.

Lysis Medium. This medium contained deoxyribonuclease (free from ribonuclease): 100 μg in 10 ml of 0.005 M Tris-HCl (pH 7.4), 0.05 M KCl, 0.05 M KCl, 0.01 M $MgCl_2$, and 0.05 g of polyoxyethylene-20-cetyl ether (Brij-58), Atlas Chemical Industries.

Modified M9 Medium. M9 (Adams, 1959) contains 0.1235 g of $MgSO_4 \cdot 7H_2O$ and 0.01 g of gelatin per l.

Sucrose Gradient. Gradients were made by mixing 13.7 ml of 30% and 13.5 ml of 15% sucrose in 0.05 M KCl, 0.005 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, and 0.01 M NaN_3. Sucrose, free from ribonuclease (Merck), was used.

Enzymes. Enzymes used were purchased from Worthington Biochemical Corp.

Radioactive Material. Carrier-free $H_2{}^{35}SO_4$ and labeled L-amino acids were purchased from New England Nuclear Corp.

Preparation of Polyribosomes. A culture of *E. coli* B, grown in the 500-ml medium of Fraser and Jerrel (1953) at 30° with aeration to 5×10^8 cells per ml, was centrifuged at 3300*g* in a prewarmed (37°) head for 5 min. The cells were suspended in 12 ml of LAM-I at 30°. Freshly prepared T4 phage, suspended in 12 ml of LAM-I (multiplicity of 3), was added to the bacterial suspension and shaken for 2 min. Infected cells were converted into spheroplasts by adding 12 ml of 1.5 M sucrose, 3.6 ml of 30% bovine serum albumin, 2.4 ml of lysozyme (2 mg/ml) in 0.25 M Tris-HCl (pH 8), and 4.8 ml of EDTA

* From the Department of Microbiology, St. Louis University School of Medicine, St. Louis, Missouri 63104. *Received December 5, 1967.* This investigation was supported by Public Health Service Research Grant GM-14145-01A1.

† Deceased, Sept 7, 1967.

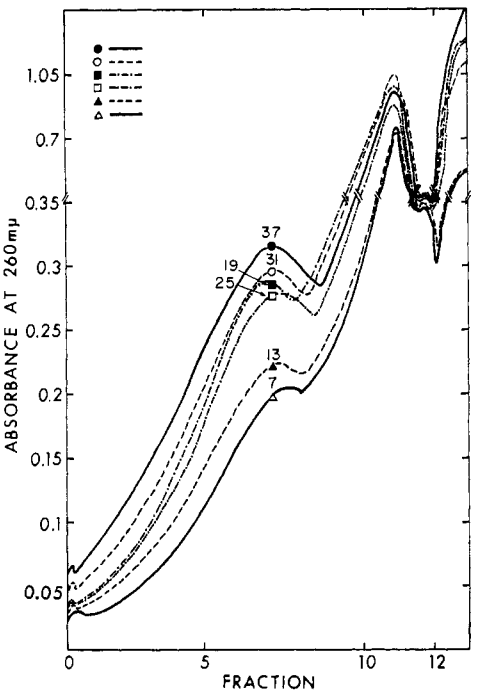

FIGURE 1: Distribution of polyribosomes at different periods after infection of *E. coli* B with T4 phage. *E. coli* B was grown to 5×10^8 cells per ml in the medium of Fraser and Jerrel, harvested, infected with T4 phage, and spheroplasts were prepared and incubated in spheroplast minimal medium containing amino acids and Na_2SO_4 at 30° as described under Methods and Materials. The spheroplasts were harvested, lysed, and 0.5-ml portions of the lysate almost free from cell membrane debris were centrifuged through 15–30% sucrose at 22,600 rpm for 180 min in a SW25.3 rotor of the Spinco Model L-2 ultracentrifuge. The tube was punctured, the solution was pumped at a uniform rate (10 drops/fraction), and the absorbance at 260 mμ was monitored.

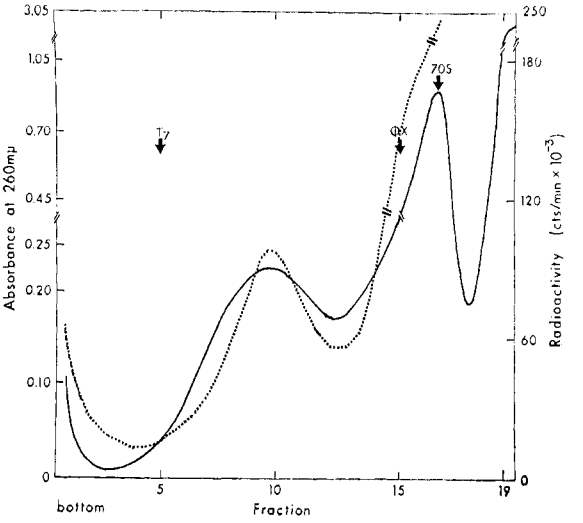

FIGURE 2: Distribution of polyribosomes and radioactivity of polyribosomal nascent polypeptides. *E. coli* B was grown to 5×10^8 cells per ml in a synthetic medium, harvested, infected with T4 phage, and spheroplasts were prepared and grown in a spheroplast minimal medium for 36 min at 30° as described under Methods and Materials. The nascent polypeptides were labeled with ^{35}S by adding $H_2{}^{35}SO_4$. The spheroplasts were harvested, lysed, and 1-ml portions of the lysate almost free from cell membrane debris were centrifuged through 15–30% sucrose at 25,000 rpm for 2 hr. Fractions of 20 drops were collected and the absorbance at 260 mμ was monitored, φX174, T7, and 70S monosome (internal) were used as sedimentation markers. Aliquots of 10 μl from each fraction were disolved in 100 μl of H_2O, and 5 ml of aqueous scintillator was added and counted for radioactivity. Absorbancy at 260 mμ (——); counts per minute (-----).

(4%) (pH 8). LAM-I (12 ml) was added slowly followed by the addition of 60 ml of 0.025 M Tris-HCl buffer (pH 7.4), 0.3 M sucrose, and 0.02 M $MgCl_2$, prewarmed to 30°. All these operations were done at room temperature. The spheroplast suspension was shaken at low speed on a gyrotory shaker at 30°. At the end of 10 min, 400 ml of spheroplast minimal medium containing amino acids (20 μg/ml) and Na_2SO_4 (10 μg/ml) at 30° was added slowly. The suspension was divided into six 500-ml flasks (75 ml in each flask) and incubated for different periods of time at 30° with shaking at low speed. Another 20-ml fraction was kept for measuring the increase in phage titer. Aliquots were taken every 7–10 min for 2 hr and the phage titers were measured. The titer increased more than 100-fold during the incubation period. The growth was stopped with the addition of 2.5 ml of 1 M NaN_3 and the suspension was cooled for 5 min in an ice bath. All subsequent operations were performed at 0–4°. The spheroplasts were centrifuged at 2445*g* for 15 min and lysed by grinding by a glass rod in 3 ml of lysis medium. The tubes were rinsed with 0.75 ml of the lysis medium, and the lysate was centrifuged at 9750*g* for 10 min. The supernatant was layered on 13.6 ml of a 15–30% sucrose gradient and centrifuged at 7° for 180 min at 22,600 rpm in a SW25.3 rotor of the Spinco Model L2 ultracentrifuge. The tube was punctured, solution was pumped at a uniform rate (Sigma motor pump), and the absorption at 260 mμ was recorded in a Gilford spectrophotometer.

In order to detect polypeptide chains on polyribosomes, spheroplasts of T4 phage-infected *E. coli* B, previously grown in a synthetic medium, were prepared. They were grown in the spheroplast minimal medium (150 ml) containing 350 μCi of $H_2{}^{35}SO_4$ at 30° with shaking at low speed for 36 min. Harvesting and lysing of the spheroplasts were identical with that described above. The supernatant portion (1 ml) of the lysate almost free from cell debris was centrifuged through 27.6 ml of 15–30% sucrose at 7° for 120 min at 25,000 rpm in a SW25.1 rotor. Fractions of 20 drops were collected starting from the bottom of each gradient and absorbance at 260 mμ was recorded.

To calibrate sedimentation rates in gradients, phages T7 (487 S; Davison and Friefelder, 1962) and φX174 (114 S; Sinsheimer, 1959) were used as markers. A total of 100 μl, containing 10^7 of each phage, was added to the sample before it was layered on the gradient. A 100-μl sample of each gradient fraction was then diluted and plaques were counted on *E. coli* B and C to detect peaks of T7 (Adams, 1959) and φX174 (Sinsheimer, 1959) differentially. In a number of runs, the fraction of the distance moved (distance from the top of the

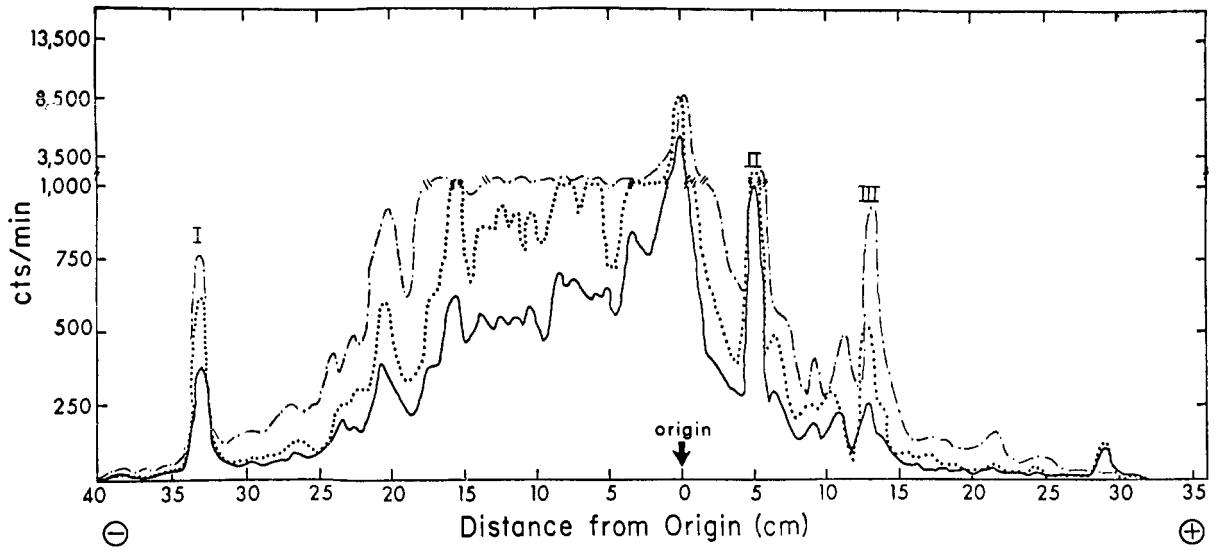

FIGURE 3: Patterns of ^{35}S-labeled peptides on electrophoresis of oxidized, chymotryptic digests of polyribosomal polypeptides, and T4 phage protein at pH 3.5. ^{35}S-Labeled polyribosomal polypeptides from sucrose density gradient fractions were oxidized, digested by chymotrypsin, and subjected to high-voltage electrophoresis (50 V/cm) at pH 3.5 on Whatman No. 3MM paper for 90 min. Paper strips of 2.6 × 0.5 cm were cut and counted for radioactivity. Profile of radioactivity from fraction 9 (——) fraction 10 (·····), and control (T4 phage protein) (—·—).

gradient to the peak divided by the distance from top to bottom on the chart paper) was found to be: monosome peak (70 S), 0.17; φX174 (114 S), 0.25; polyribosome peak, 0.51; and T7 phage (480 S), 0.78, indicating a linear relationship between the fractions and the S values of the markers.

Portions (10 μl) of each gradient fraction were dissolved in 100 μl of water in vials, 5 ml of aqueous scintillator (Kinard, 1957) was added, and the samples were counted for radioactivity in a liquid scintillation spectrometer (Nuclear-Chicago Mark I or Packard series 3375).

Preparation of ^{35}S-Labeled Phage. Bacteria were grown in 500 ml of synthetic medium to 5×10^8 cells per ml and cells were harvested and washed with 80 ml of LAM-II. The cells were suspended in 4 ml of LAM-II and infected with 4 ml of a suspension of the phage or phage mutants in LAM-II (multiplicity of 3) and shaken for 3 min. Infected cells (2 ml) were transferred into 80 ml of the spheroplast minimal medium. Carrier-free $H_2{}^{35}SO_4$ (100 μCi) and Na_2SO_4 (400 μg) were added and incubated at 37° with aeration for 4 hr. The cells were lysed with $CHCl_3$ and ^{35}S-labeled phage and phage components were obtained by low- and high-speed centrifugation in modified M9 medium (Stretton and Brenner, 1965). ^{35}S-Labeled intact phages were prepared by growing T4 on *E. coli* B and amber B17 (gene 23) and amber B7 (gene 29) on the permissive host *E. coli* CR63. ^{35}S-Labeled phage components were prepared by growing the amber B17 and amber B7 on the nonpermissive host *E. coli* B. (Amber mutants were supplied by Dr. R. S. Edgar, California Institute of Technology.)

Oxidation, Digestion, and Electrophoresis of ^{35}S-Labeled Phage Protein and Polyribosomal Polypeptides. Fractions from the sucrose gradients were dialyzed against three changes of 1.2 l. of sterilized cold water for 12 hr. The dialyzed gradient samples and aliquots of labeled phage T4, amber B7, and amber B17 were lyophilized and then oxidized by performic acid at 0° according to the method of Hirs (1956). The oxidation was stopped by the addition of water and the acid was removed by repeated lyophilization. The residue was dissolved in 0.25 ml of water, heated over a boiling-water bath for 10 min, cooled to 37°, and RNase (5 μg) was added. After the sample was incubated at 37° for 2 hr, 10% NH_4HCO_3 was added to give a 2% solution. Chymotrypsin (5 μg) was then added and the sample was stirred at 37° along with a drop of toluene to prevent bacterial growth. More chymotrypsin (5 μg) was added at the end of 3 and 8 hr. The total digestion time was 18 hr. The reaction was stopped by the addition of acetic acid, and the samples were relyophilized. The lyophilized peptides were dissolved in 0.1 ml of pyridine acetate buffer (pH 3.5) (10 ml of pyridine and 100 ml of glacial acetic acid per l.) and applied on Whatman No. 3MM paper. The electrophoresis was done at pH 3.5 (50 V/cm) for 90 min in a Gilson immersion-type high-voltage electrophoresis apparatus, according to the method of Katz *et al.* (1959). The dried paper was cut into 2.6-cm strips along the direction of current flow and further cut into horizontal segments of 0.5 cm each; radioactivity of each segment was measured in 5 ml of scintillator containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene.

The peaks of radioactivity were eluted with water, lyophilized, and dissolved in 0.1 ml of pyridine acetate buffer (pH 6.4) (100 ml of pyridine and 4 ml of glacial acetic acid per l. They were then repurified on Whatman No. 3MM paper by electrophoresis at pH 6.4 (40 V/cm) for 120 min. Paper strips (2.6 × 0.5 cm) were again cut and the radioactivity was determined.

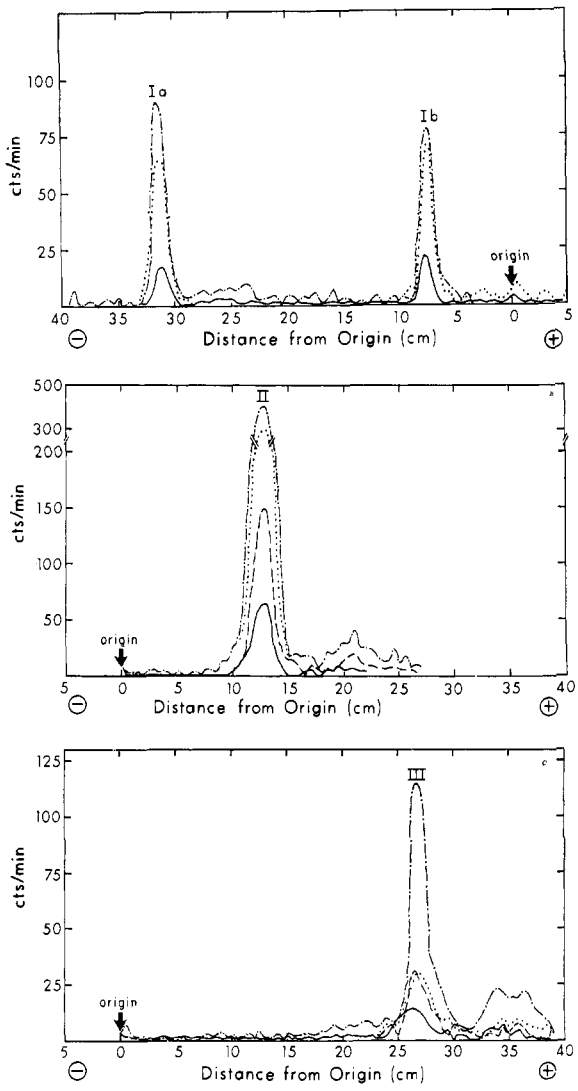

FIGURE 4: Peptide pattern on electrophoresis of ^{35}S-labeled peptides I–III at pH 6.4. The peptides, I (basic), II, and III (acidic), from fractions 7–12 (Figure 3) of the sucrose gradient were subjected to high-voltage electrophoresis (40 V/cm) at pH 6.4 on Whatman No. 3MM paper for 2 hr. Paper strips of 2.6 × 0.5 cm were cut and counted for radioactivity. (A) Resolution of peptide I from fraction 9 (——), fraction 10 (·····), and control (T4 phage protein) (· — ·). (B) Resolution of peptide II from fraction 8 (——), fraction 9 (— —), fraction 10 (·····), and control (· — ·). (C) Resolution of peptide III from fraction 8 (——), fraction 9 (— —), fraction 10 (·····), and control (· — ·).

TABLE I: Sedimentation Coefficient and Number of Ribosomes per Polysome in each Fraction of Sucrose Gradient.

Fraction	S Values[a]	Ribosomes/ Polyribosome
17–14	<150	<4
13	150–205	4–6
12	205–243	6–9
11	243–275	9–11
10	275–292	11–12
9	292–340	12–15
8	340–380	15–18
8–1	>380	>18

[a] S values were determined according to the method of Martin and Ames (1961). Number of ribosomes per polysome were calculated from the relationship that S values are directly proportional to 0.58 power of their molecular weights (Pfuderer *et al.*, 1965).

Results

Distribution of Polyribosomes and Label in Sucrose Gradient. Seven minutes after infection of the spheroplasts of *E. coli* B with T4 phage, a polyribosome peak appeared, which grew in size with time and reached a maximum after 25 min (Figure 1). The distribution of polyribosomes and the label 36 min after infection is shown in Figure 2. The polyribosome peak and the radioactivity peaks were found in the tenth fraction of the sucrose gradient. Similar patterns were observed when infected cells were labeled for 3 min with L-[^{3}H]-arginine and L-[^{14}C]- or [^{3}H]tyrosine. Polyribosomes were completely sensitive to RNase and the label was moved to 70S and lighter fractions.

The sedimentation coefficients of the polyribosomes in each fraction of the gradient were determined. Assuming that monosomes of *E. coli* have a sedimentation coefficient of 70 S, and polyribosomes of increasing size behave like increasingly larger members of the same polymer series, the number of ribosomes corresponding to the S values of the polyribosomes was calculated (Table I).

Characterization of Polyribosomal Polypeptides as Constituents of Phage Head Protein. Typical electrophoretic profiles of ^{35}S-labeled chymotryptic peptides from the polyribosomes peak, from a heavier fraction, and from isolated T4 phage protein, are presented in Figure 3. Three well-defined peptides, one basic (I) and two acidic (II and III), were obtained from gradient fractions 7–12. These peptides were present in very small quantities in gradient fractions 1–6 and were not clearly separated in electrophoretic profiles of fractions 13–15.

The three radioactive peaks, I–III, from fractions 7–12 were subjected to high-voltage electrophoresis at pH 6.4 along with the peptides from the phage protein. The basic peptide, I, was separated into two fragments (Figure 4A) which were identical with the two fragments (Ia and b) obtained from the basic peptide of phage protein. The two acidic peptides, II and III, moved toward the anode and were identical with those obtained from phage protein (Figure 4B,C). The acidic peptide, II, appears to be the cysteic acid peptide identified, characterized, and used by Sarabhai (1963) as a marker for phage head protein. The three peptides may be characteristic of the phage head protein, since the phage head protein constitutes 90% of the phage protein and contamination by other proteins appears to be negligible.

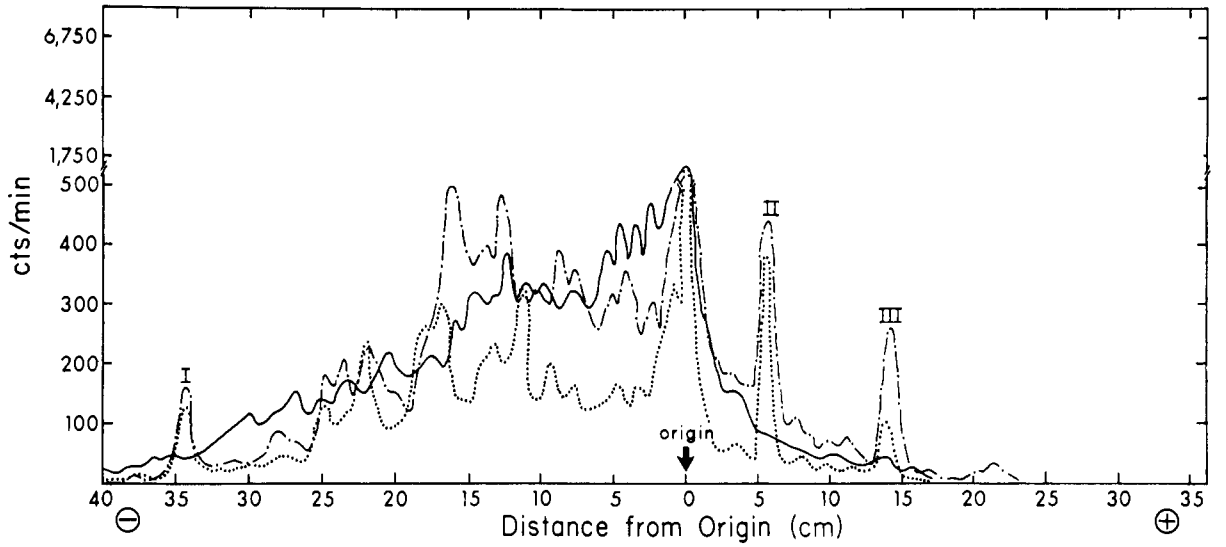

FIGURE 5: Distribution of ^{35}S-labeled peptides on electrophoresis of oxidized chymotryptic digests of amber B17 and T4 phage. Amber B17 and phage components were labeled with ^{35}S by growing on nonpermissive host *E. coli* B and permissive host *E. coli* CR63. The ^{35}S-labeled phage proteins were oxidized, digested by chymotrypsin, and subjected to high-voltage electrophoresis (50 V/cm) at pH 3.5 on Whatman No. 3MM paper for 90 min. The paper was cut into 2.6 × 0.5 cm strips and the radioactivity was counted. ^{35}S peptide pattern from B17 grown on *E. coli* B (—), from B17 grown on *E. coli* CR63 (·····), and control (T4 phage protein) (· — ·).

T4 amber mutant B17 (gene 23) produces all proteins except the head protein when grown on a nonpermissive host, *E. coli* B, and produces whole phage particles when grown on permissive host *E. coli* CR63 (Edgar and Wood, 1966). The ^{35}S-labeled phage proteins, from amber B17 grown on *E. coli* B and *E. coli* CR63, were oxidized, digested, and subjected to high-voltage electrophoresis at pH 3.5. The peptides (I–III) from amber B17 grown on *E. coli* CR63 and from T4 on *E. coli* B were identical (Figure 5). These peptides were absent in the case of amber B17 grown on *E. coli* B. Sarabhai *et al.* (1964) have reported that there were no ^{35}S-containing head protein peptides obtained from amber B17 grown on *E. coli* B in a medium containing $H_2{}^{35}SO_4$. This supports the view that the three peptides (I–III) are characteristic of the phage head protein. Amber B7 (gene 29) produces T4 heads when grown on nonpermissive host, *E. coli* B, and whole phage when grown on permissive host, *E. coli* CR63 (Edgar and Wood, 1966). The oxidized chymotryptic digests of ^{35}S-labeled proteins of amber B7 grown on *E. coli* B and CR63 on electrophoresis at pH 3.5, produced the three peptides, I–III, which were identical with the peptides obtained from ^{35}S-labeled T4 phage protein.

Discussion

At "late times" after infection of *E. coli* B by T4 phage the polyribosome-rich lysates we have studied consistently showed a major 280S peak, containing about 50% of the total ribosomes in polyribosomes. The reproducibility of the pattern, coupled with the specific changes of profile in response to specific amber mutations (Rolfe, 1965), suggested that the lysates preserve a specific polyribosome peak that exists in the infected cells.

Since the incorporation of label should be a direct measure of the amount of protein synthesized, polyribosomes in the tenth fraction of the sucrose gradient were most active in protein synthesis. There were large amounts of incorporated label in the monomer region of the gradient (Figure 2), but the specific activity of the monomers in protein synthesis was very low. They may represent, for the most part, a form of inactive ribosome accumulated in the spheroplasts, since protein synthesis is not proceeding as fast in infected spheroplasts as it is in uninfected cells. Alternatively, the monomers may represent, like many of the polyribosomes, a specifically inactivated fraction. For example, large amounts of early function messengers persist at late times, when early proteins are no longer being made. Completely or partially degraded messengers may remain attached to monomers or even in polyribosomes, and even direct amino acid incorporation, but cannot function to make complete chains (compare the stuck proteins of Ganoza and Nakamoto, 1966; Mangiarotti and Schlessinger, 1966).

One of the ^{35}S-labeled chymotrypsin peptides analyzed is identical in its electrophoretic properties with that reported for chymotryptic digests of isolated T4 head protein (Sarabhai, 1963). This peptide, as well as two additional ^{35}S-labeled peptides, is shown here to be present in chymotryptic digests of intact T4 bacteriophage but is missing in extracts of *E. coli* infected with T4 amber B17, a mutant in the gene specifying the phage head protein. This T4 mutant is unable to form T4 head protein in *E. coli* B, but can synthesize T4 head protein in the permissive host, *E. coli* CR63. When grown on CR63, the T4 mutant was found to contain the three characteristic ^{35}S-labeled chymotryptic peptides. Amber B7, a mutant in the later gene, produced all these peptides when grown on permissive and nonpermissive

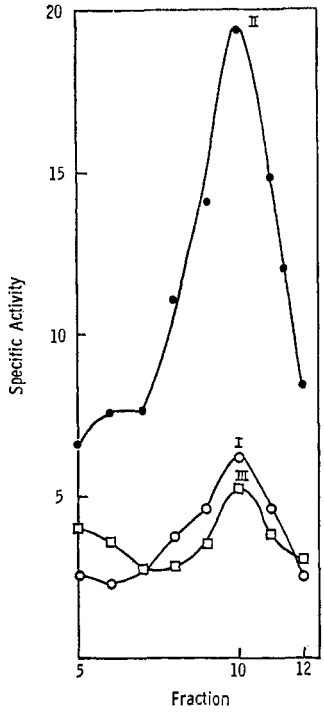

FIGURE 6: Specific activities of polyribosomes in T4 phage head protein synthesis. Specific activities: the amounts of peptides I–III (Figure 3) per unit absorbance of polyribosomes (Figure 1) with respect to peptide I (○——○), peptide II (●——●), and peptide III (□——□).

hosts of *E. coli*. These results argue that the isolated peptides examined here are, in fact, parts of the T4 phage head protein polypeptide chain.

Since ^{35}S-labeled phage protein and polyribosomal polypeptides were treated under identical conditions, and due precautions were taken to transfer samples quantitatively, the amounts of peptides I–III should be proportional to the areas of the peaks of radioactivity. Amounts of these peptides are then a measure of the quantity of the head protein synthesized on polyribosomes. Specific activities of peptides I–III were maximum in the tenth fraction of the sucrose gradient (Figure 6). The yields of peptides I–III from digested polypeptides of the peak polyribosome fraction were 3, 9, and 2.5%, respectively. Polyribosome fractions heavier and lighter than the peak fraction yielded lower amounts of these peptides. The percentages of peptides I–III from the peak polyribosome fraction were comparable with the yields of these peptides from digested phage protein. These results show that maximum synthesis of the phage head protein was in the tenth fraction of the sucrose gradient on polyribosomes sedimenting at 275–292 S. According to the helical polysome model of Pfuderer *et al.* (1965), such fractions probably contain 11–12 ribosomes/polyribosome. We conclude that phage head protein is synthesized on a sharply defined class of polyribosomes each containing approximately 11–12 ribosomes.

Cummings (1963) showed that the T2 phage head protein can be dissociated into homogeneous protein subunits having a molecular weight of 42,000 daltons. It was demonstrated that the T4 and T2 head protein finger prints were identical except for a very few peptides (cited as personal communication, Sarabhai, 1963). Hence T4 phage head protein subunit may have a molecular weight of 42,000 daltons. Polyribosomes involved in the synthesis of hemoglobin of mol wt 17,000 daltons contain five ribosomes per polysome (Warner *et al.*, 1962). Assignment of polyribosomes containing about 11–12 ribosomes for the synthesis of the phage head protein having a molecular weight of 42,000 daltons, thus appears to be reasonable, and is consistent with a comparable loading of mRNA molecules with ribosomes per unit length. The result suggests that the head protein gene is transcribed alone rather than as part of a polycistronic mRNA.

Acknowledgment

I am grateful to Drs. Milton Schlesinger and David Schlessinger for valuable discussions and suggestions.

References

Adams, M. H. (1959), Bacteriophages, New York, N. Y., Interscience, p 447.

Cummings, D. J. (1963), *Biochim. Biophys. Acta 68*, 472.

Davison, P. F., and Freifelder, D. (1962), *J. Mol. Biol. 5*, 635.

Edgar, R. S., and Wood, W. B. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 498.

Fraser, D., and Jerrel, E. A. (1953), *J. Biol. Chem. 205*, 291.

Ganoza, M., and Nakamoto, T. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 162.

Gierer, A. (1963), *J. Mol. Biol. 6*, 148.

Gilbert, W. (1963), *J. Mol. Biol. 6*, 374.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.

Kiho, Y., and Rich, A. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 111.

Kinard, F. E. (1957), *Rev. Sci. Instr. 28*, 293.

Mangiarotti, G., and Schlessinger, D. (1966), *J. Mol. Biol. 20*, 123.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.

Penman, S., Sherrer, K., Becker, Y., and Darnell, J. E. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 654.

Pfuderer, P., Cammarano, P., Holladay, D. R., and Novelli, G. D. (1965), *Biochim. Biophys. Acta 109*, 595.

Rolfe, R. (1965), Abstracts, 9th Biophysical Society Meeting, p FF-3.

Rolfe, R., and Rich, A. (1965), Abstracts, 9th Biophysical Society Meeting, p FF-2.

Sarabhai, A. S. (1963), Ph.D. Thesis, University of Cambridge, England.

Sarabhai, A. S., Stretton, A. O. W., Brenner, S., and Bolle, A. (1964), *Nature 201*, 13.

Sinsheimer, R. L. (1959), *J. Mol. Biol. 1*, 37.

Stretton, A. O. W., and Brenner, S. (1965), *J. Mol. Biol.*

12, 456.
Warner, J. R., Rich, A., and Hall, C. E. (1962), *Science 138*, 1399.
Watson, J. D. (1963), *Science 140*, 17.
Wettstein, F. O., Staehelin, T., and Noll, H. (1963), *Nature 197*, 430.

Direct Incorporation of Hydroxyproline into *Avena* Coleoptile Proteins*

Robert Cleland and Alfred C. Olson

ABSTRACT: The *cis* isomer of L-hydroxyproline (allohydroxyproline), when present at a growth-inhibitory level (0.5 mM), is directly incorporated into *Avena* coleoptile proteins at a rate that is about three times greater than the normal rate of formation of *trans*-hydroxyproline. This incorporation is predominately into cytoplasmic proteins, is linear with time, and is only slightly affected by 0.15 mM α,α-dipyridyl. The ability of proline to prevent this incorporation suggests that the *cis*-hydroxyproline is being incorporated into protein in place of proline. Label from [^{3}H]*trans*-hydroxyproline also appears in protein-bound hydroxyproline, but the bulk of this incorporation appears to be indirect (*i.e.*, through proline) since it can be severely inhibited by dipyridyl. Both isomers of hydroxyproline can be converted to proline by *Avena* coleoptile tissues, but the *trans* isomer is more efficiently utilized in this reaction. The lack of correlation between the incorporation of hydroxyproline isomers into protein and their ability to inhibit cell elongation indicates that while the direct incorporation of hydroxyproline into protein may contribute to the inhibition of auxin-induced growth, it is unlikely to be its principal cause.

The widespread occurrence of protein-bound hydroxyproline in plants is well established (Vanetten *et al.*, 1963; Lamport, 1965). As in animal systems (Stetten, 1949), proline rather than hydroxyproline is the normal precursor of the bound hydroxyproline (Steward and Pollard, 1958; Olson, 1964; Lamport, 1965). The possibility that some direct incorporation of hydroxyproline can occur in plants was suggested, however, by the finding that free hydroxyproline is an effective inhibitor of plant growth (Steward *et al.*, 1958; Cleland, 1963).

Studies on the direct incorporation of hydroxyproline have been hampered by the indirect incorporation of hydroxyproline, *i.e.*, incorporation into protein only after prior conversion into proline. Thus label in protein-bound hydroxyproline can arise from labeled free hydroxyproline either by direct incorporation or indirectly by conversion into proline followed by reconversion into hydroxyproline. For instance, Cleland and Olson (1967) showed with *Avena* coleoptiles that when free hydroxyproline is at a noninhibitory level, the transfer of label from free to protein-bound hydroxyproline is due to the indirect incorporation pathway. The best evidence, to date, for direct incorporation of hydroxyproline into plant proteins is the finding of Holleman (1967) that α,α-dipyridyl interferes to a greater extent with the conversion of proline into hydroxyproline (Hurych and Chvapil, 1965; Hutton *et al.*, 1967) than with the transfer of label from free to protein-bound hydroxyproline.

Advantage has been taken in this study of the fact that the *cis* isomer of L-hydroxy-4-proline (*cis*-Hypro[1]), which is more effective than the normal *trans* isomer (*trans*-Hypro) as a growth inhibitor (Cleland, 1967a), is not normally found in *Avena* coleoptile proteins and is not formed from proline. The appearance of any *cis*-Hypro in protein following incubation with free *cis*-Hypro can only be due to direct incorporation of this isomer. It is shown here that direct incorporation of *cis*-Hypro into *Avena* coleoptile proteins does occur.

Experimental Section

Materials

The plant material consisted of 14-mm sections cut from 25–32-mm coleoptiles of *Avena sativa*, var. Vic-

* From the Department of Botany, University of Washington, Seattle, Washington, and the Western Regional Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, California. *Received December 6, 1967.* This work was supported, in part, by Grant GM-12881 from the U. S. Public Health Service and Grant GB-5385 from the National Science Foundation.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: *cis*-Hypro, *cis*-hydroxy-4-proline (allohydroxyproline); *trans*-Hypro, *trans*-hydroxy-4-proline; IAA, indoleacetic acid; CHA, cycloheximide; DiP, α,α-dipyridyl.