ANALYSIS OF PRODUCTS OF PROTEIN SYNTHESIS DIRECTED BY R17 VIRAL RNA*

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Viral RNA is known to stimulate the incorporation of amino acids into proteins in cell-free extracts of *E. coli*.^{1, 2} Usually analyses of the synthetic products have been confined to the ribosome-free supernatant liquid obtained by sedimenting the incorporation medium. Since the products of protein synthesis may vary in their affinity to ribosomes and in their solubility in the incorporation medium, it is clear that fractionation methods in addition to those of high-speed centrifugation must be developed. The present paper describes methods intended for analysis of essentially all of the products of the cell-free protein synthesis directed by R17 RNA. From these analyses we conclude that, although a product similar to R17 coat protein is made, the major product is a very basic polypeptide resistant to trypsin and soluble in 5 per cent trichloroacetic acid (TCA).

R17 is very similar in its physical and chemical structure and its biological properties to other known bacterial RNA viruses, including f2.³⁻⁶ All of these viruses are very small and spherical. Their RNA is a single molecule of molecular weight about 1 million. The RNA presumably codes for only a few proteins and is therefore one of the simplest polycistronic messengers available at present.

Materials.—Reagents: The dipotassium salt of ATP and the trisodium salt of GTP were obtained from Pabst Laboratories, Milwaukee, Wisconsin; the triethyloxethylammonium salt of phosphoenolpyruvic acid from Calbiochem, Los Angeles, California (the free acid was liberated according to the method of Nirenberg⁷ and was then neutralized with NH₄OH); the uniformly labeled C¹⁴-L-arginine, L-lysine, L-leucine, L-histidine, and algal protein hydrolysate from New England Nuclear Corporation, Boston, Massachusetts (the specific activities ranged from 200 to 240 mc/mM); tritiated sodium acetate with a specific activity of 124 mc/mM from Volk Radiochemical Company, Chicago, Illinois; pyruvate kinase from the Sigma Biochemical Corporation, St. Louis, Missouri; pancreatic DNase I and thrice-crystallized trypsin from Worthington Biochemical Corporation, Freehold, New Jersey; the alumina, Alcoa A-305, from Alumina Company of America, Pittsburgh, Pennsylvania; Sephadex G-25, G-200, and SE-Sephadex C-50 from Pharmacia Fine Chemicals, Inc., New Market, New Jersey; and the Aminex 50-W, X2 (120-325 mesh) from Bio-Rad Laboratories, Richmond, California.

Cell-free extracts: The procedure of Nirenberg,⁷ with some minor modification, was used to prepare cell-free *E. coli* extracts. *E. coli* K12 (Hfr, Methionine⁻) was harvested in the logarithmic phase of growth. The washed frozen cell paste was ground with two parts of alumina and was extracted with 2 vol of the standard buffer. The standard buffer consisted of 0.01 *M* tris-HCl, pH 7.8; 0.01 *M* magnesium acetate, 0.03 *M* NH₄Cl (instead of KCl); and 0.005 *M* 2-mercaptoethanol. The alumina and cell debris were removed by centrifugation at 20,000 *g* for 20 min. Three μ g of DNase were added per ml of the supernatant liquid. The supernate was centrifuged at 20,000 *g* for 30 min. The resulting supernatant liquid was centrifuged at 30,000 *g* for 30 min. The 30,000 *g* supernate (S-30) was incubated at 35°C for 30 min with the components required for *in vitro* protein synthesis: 0.001 *M* ATP; 0.0002 *M* GTP; 0.005 *M* phosphoenol pyruvate (ammonium salt); 20 μ g/ml pyruvate kinase; 0.01 *M* tris-HCl (pH 7.8); 0.01 *M* magnesium acetate; 0.03 *M* NH₄Cl; 0.005 *M* 2-mercaptoethanol. No amino acids were added at this time. One volume of this preincubated mixture was dialyzed at 4°C for 20 hr against 100 vol of the standard buffer with three changes of the buffer. The dialysate (preincubated S-30) was placed in small plastic vials in 1-ml aliquots. They were stored frozen in a liquid-nitrogen refrigerator

for several months without noticeable loss of protein-synthesizing activity. The preparation contained about 20 mg of proteins per ml as estimated by the method of Lowry *et al.*⁸

R17 RNA and coat protein: R17 RNA was prepared by deproteinization of the virus suspension with phenol in the presence of sodium dodecyl sulfate and bentonite and subsequent precipitation,⁹ R17 coat protein and *E. coli* ribosomal proteins by the method of Fraenkel-Conrat.^{6, 10, 11}

Methods.—Amino acid incorporation: The standard incorporation mixture contained the following in μ M/ml: 10 tris-HCl, pH 7.8; 10 magnesium acetate; 30 NH_4Cl ; 5 2-mercaptoethanol; 1 ATP, potassium salt; 0.2 GTP, sodium salt; 5 phosphoenol pyruvic acid, ammonium salt; 20 μ g pyruvate kinase; 0.025 each of 20 L-amino acids minus radioactive amino acids; 2 μ Ci of radioactive amino acids; 3–6 mg as protein of preincubated S-30; 1–2 mg of R17 RNA. The mixtures were incubated at 35°C. Many of the experiments were carried out with C^{14} lysine plus arginine to facilitate analysis of tryptic digests of the synthesized products.

Solubilization and dissociation of products: One volume of 0.02 M disodium EDTA in 10 M urea was mixed with one volume of a reaction mixture in order to dissolve the products and dissociate ribosome-product complexes.¹² A minute amount of insoluble materials remained.

Gel filtration: Two to 3 ml of the products in the EDTA-urea solvent were passed through a G-25 Sephadex gel column (2 $\times$ 13 cm, equivalent to 10 gm of dry gel) which was equilibrated with 0.01 M EDTA in 5 M urea at pH 5.3. Filtration was at room temperature and was completed within 1 hr. Products having molecular weight higher than that which corresponds to the exclusion limit of the gel (approximately 5,000) were expected to be excluded from the column, while free amino acids and other low-molecular-weight materials were expected to be retarded. The fractions were stored frozen until used. Gel filtration of alkali-treated products was carried out in 0.05 M NaOH in 5 M urea.

SE-Sephadex chromatography: SE-Sephadex (C-50; medium; sodium form) was suspended and swollen in a large excess of solvent for more than 10 hr. The solvent consisted of 0.025 M citric acid, 0.05 M Na_2HPO_4 , 0.075 M H_3BO_3 in 5 M urea, giving a pH of 5.2. Fine particles were removed by repeated decantation in the solvent. The gel in a bed 2 cm in diameter by 6 cm in height was equilibrated with the solvent. The sample was applied to the column, the column was washed with the solvent, and pH-gradient elution was carried out with 50 ml of the solvent in a constant-volume mixing chamber to which 0.1 M NaOH in 5 M urea was continuously supplied as the elution proceeded. The second solvent was freshly prepared just before the elution was started. The flow rate was maintained at 24 ml/hr. Approximately 0.7-ml fractions were collected. The elution was stopped when the pH of the eluate reached 12.

One-tenth-ml aliquots of the fractions were pipetted onto Whatman 3 MM filter paper circles and radioactivity was measured in toluene-based scintillator in a Packard scintillation counter.

Tryptic digestion: To the chromatographic fractions still in 5 M urea was added as a carrier R17 coat protein equal to about 5 times the protein content of the fractions. The mixtures were diluted with water to a final concentration of 2 M urea and the pH of the solutions was adjusted to 8 with NaOH or HCl. Trypsin was added to give a substrate-to-enzyme ratio of about 50 (w/w). The solutions were incubated at 25°C for 2 hr. Fresh trypsin of the same quantity as above was added and the digestion was continued for an additional 3 hr. The pH of the mixture changed less than 0.2 pH unit during this 5-hr digestion period. Digestion was terminated by acidifying the mixtures to pH 2.5 with HCl. The peptides were adsorbed to Aminex 50-W, $\times 2(\text{H}^+$ form, 120–325 mesh, about 10 ml bed volume). Urea was washed from the column with 300 ml of water. The peptides were eluted with 50 ml of 4 N NH_4OH or in some instances with 4 N HCl. The eluates were quickly evaporated to dryness by means of a flash evaporator at 40°C. They were repeatedly dissolved in small amounts of water and dried to remove traces of the eluents. Finally the materials were dissolved in a known volume of water and stored frozen.

Mapping of tryptic digests: Tryptic peptides were mapped by a two-dimensional combination of paper electrophoresis and paper chromatography.¹³ The map was radioautographed on clinical X-ray film (Anso safety film 3) for more than 1 month.

Results.—Stimulation of incorporation by R17 RNA: Table 1 shows the incorporation of several amino acids in the absence and presence of R17 RNA. Both the amount of amino acid incorporated and the ratios of amounts of amino acids incorporated in the presence of the added RNA to that in the absence of the RNA

TABLE 1

EFFECT OF R17 RNA UPON INCORPORATION OF AMINO ACIDS INTO PROTEINS

C ¹⁴ amino acid	Cpm Incorporated		Ratio +RNA/-RNA
	-RNA	+RNA	
Leucine	13,600	219,380	16
Histidine	1,960	66,540	34
Arginine plus lysine	4,400	1,066,800	240

Each 1-ml reaction mixture contained about 6 mg preincubated S-30 protein, 2 mg of R17 RNA for +RNA, 2 μ c of each C¹⁴-amino acid, and all other components given in *Methods*. The reaction mixtures were incubated at 35°C for 60 min. The cpm listed are those in excess of the zero-time blank.

TABLE 2

DISTRIBUTION OF INCORPORATED C¹⁴-ARGININE PLUS LYSINE SEPARATED ON A SE-SEPHADEX COLUMN

Fraction	Percentage in	
	Excluded materials (100%)	Amino acids incorporated (100%)
Peak I		
Aminoacyl-sRNA	18	—
Peptidyl-sRNA	9	11
Broad band	14	17
Peak II	7	8.5
Peak III	7	8.5
Peak IV	45	55
Sum	100	100

differed with labeled amino acids added to the reaction mixture. R17 RNA stimulated the incorporation of histidine which is lacking in virus coat protein. With C¹⁴-lysine and C¹⁴-lysine plus arginine labeling, usually 10–15 per cent of the added radioactive amino acids were incorporated during 60 min incubation.

Figure 1 shows the time course of leucine incorporation at two R17 RNA concentrations. Both the rate and the extent of the incorporation were directly dependent on the RNA concentrations. The incorporation leveled off after about 60 min.

Figure 2 shows the effect of concentration of R17 RNA on leucine incorporation. The response was approximately linear between RNA concentrations, 0.4 and 2.4 mg/ml. Saturation was not achieved at the concentrations used.

Sedimentation of products: Upon fractionating the incubation mixture by sedimentation in the manner of Nathans *et al.*¹ and Aach *et al.*,² the majority of the incorporated radioactivity sedimented with the ribosomes. For example, with C¹⁴-leucine, 60 min incorporation, 77 per cent of the incorporated counts were sedimented in 2 hr at 105,000 *g*. Since we wished to study all synthetic products, we sought alternative analytical methods.

Solubilization and dissociation of products: The following kinds of radioactive materials will be expected in a reaction mixture: (1) radioactive amino acids either in the free form or in the form of aminoacyl-sRNA not yet incorporated into polypeptides; (2) intermediates of protein synthesis in the form of peptidyl-sRNA (incomplete proteins); (3) finished products (complete proteins); (4) free peptides which may occur, e.g., due to proteolytic activity in the extracts. Some aminoacyl-sRNA's and almost all peptidyl-sRNA's will be attached to the ribosomes. We wished to eliminate these associations before fractionation and also to require that all products remain soluble.

More than 99 per cent of the incorporated radioactivity remained in solution in the urea-EDTA solvent at pH 6. Product-ribosome complexes presumably were dissociated. It was essential that the pH not be below 5 or alkaline. At pH lower than 5, up to one third of the TCA-insoluble radioactivity precipitated. At alkaline pH's, the ester bonds of the sRNA derivatives would become increasingly labile.

Gel filtration: The filtration patterns for a C¹⁴ lysine plus arginine incorporation system are shown in Figure 3. Clearly, the stimulated products contained a much larger proportion of materials excludable in the gel than did the endogenous control. The difference in peak positions is not significant. The excluded peak contains not only polypeptides, but also the sRNA derivatives of small oligopeptides and

amino acids. To see how much radioactivity corresponding to that peak was due to polypeptides of high molecular weight, the reaction mixture in the EDTA-urea solvent before gel filtration was adjusted to pH 11.5 with 5 *N* NaOH and was incubated at 35°C for 1 hr. This alkali treatment hydrolyzes the ester bonds of the sRNA derivatives. The incubated mixture was then gel-filtered on Sephadex G-25 run in 0.05 *M* NaOH in 5 *M* urea (Fig. 4). About 16 per cent of the radioactivity input in the stimulated products was incorporated into polypeptides excludable in the G-25 gel, while practically no radioactivity was found in the corresponding peak region of the unstimulated products, thus indicating the synthesis of high-molecular-weight polypeptides under the direction of R17 RNA.

SE-Sephadex chromatography: The elution pattern of the materials excluded from the G-25 gel, chromatographed on SE-Sephadex, is shown in Figure 5. About 27 per cent of the radioactive materials applied to the column were unadsorbed (peak I). As the pH of the effluent increased from 5.2 to 8.14 per cent of the radioactivity was eluted as a broadly distributed band. Between pH 8 and 11, the remaining radioactivity was eluted as three distinct peaks (peaks II, III, and IV). The pH of the fractions at peaks II, III, and IV were 9.2, 10.1, and 10.7.

To demonstrate that peak I contained both aminoacyl-sRNA and peptidyl-sRNA, the peak fraction was adjusted to pH 10.5 with 5 *M* NaOH and incubated at 30°C for 1 hr in order to cleave the ester bonds of these sRNA derivatives. The alkali-treated products in peak I were then filtered on Sephadex G-25 run in 0.05 *M* EDTA-5 *M* urea, pH 5.3. Before alkali treatment, all materials were excludable from the gel; after alkali treatment, about two thirds of the materials became retarded and the remainder were excluded. This suggests that about two thirds of the radioactivity of peak I was due to aminoacyl-sRNA and the remainder probably to peptidyl-sRNA. Before alkali treatment none of the materials in peak I was adsorbed to SE-Sephadex; after alkali treatment about 80 per cent of the materials excluded on the G-25 column were adsorbed. We conclude that peak I consisted of aminoacyl-sRNA and peptidyl-sRNA, intermediates of protein synthesis. The above results are summarized in Table 2.

It is clear that peak IV corresponds to the major synthetic product. When the reaction products labeled with C¹⁴-histidine or with a C¹⁴-amino acid mixture (algal protein hydrolysate) were chromatographed on the SE-Sephadex column, they

FIG. 1.—Time course of leucine incorporation at two concentrations of R17 RNA. The incubation mixtures were as described under *Methods* and contained, per ml, 2 μ c of C¹⁴-leucine, 3 mg of preincubated S-30 proteins, and R17 RNA in the following amounts: (O) 1.12 mg, (Δ) 0.28 mg, (---) no RNA. Each point represents the TCA-insoluble counts in 1.0 ml of reaction mixture.

FIG. 2.—Effect of concentration of R17 RNA on leucine incorporation. The incubation was as described under *Methods* and contained, per ml, 2 μ c of C¹⁴-leucine, 3.5 mg of preincubated S-30 proteins, and R17 RNA in the amount indicated in the figure. The mixtures were incubated at 35° for 60 min. Each point represents the TCA-insoluble counts in 1.0 ml of reaction mixture.

FIG. 3.—Gel filtration patterns of C^{14} -arginine plus lysine-labeled products. The columns were prepared by suspending 10 gm of Sephadex G-25 in 0.01 *M* EDTA and 5 *M* urea, pH 5.3. The filtrations were carried out in the same solvent. The average size of the fractions was approximately 0.7 ml per tube; 0.05-ml samples from the fractions were put on Whatman 3 MM filter paper circles; the circles were dried, placed in scintillation vials, and counted in a toluene-based scintillator. The full line represents the RNA-stimulated product; the dashed line represents the endogenous product. The figure shows the composite of the two filtration patterns.

FIG. 4.—Gel filtration patterns of alkali-treated products labeled with C^{14} -arginine plus lysine. The columns were prepared by suspending 10 gm of Sephadex G-25 in 0.05 *M* NaOH and 5 *M* urea. The filtrations were carried out in the same solvent. Each tube fraction contained approximately 0.9-ml samples. The radioactivity of 0.1-ml samples from the fractions was counted in the same method as described in Fig. 3 legend. The full line represents the RNA-stimulated product; the dashed line represents the endogenous product. The figure shows the composite of the two filtration runs. Free arginine and lysine were separated in this gel at an alkaline pH.

yielded the same major peak as was obtained with C^{14} -lysine plus arginine. When tritium-labeled authentic R17 coat protein was co-chromatographed with C^{14} -labeled synthetic products, it was eluted in the same region as peak II (as shown in Fig. 5). The major synthetic product in peak IV is thus much more basic than authentic coat protein. *E. coli* ribosomal proteins are known to be a mixture of basic proteins with isoelectric points above pH 9.¹⁴ When tritium-labeled ribosomal proteins were chromatographed on SE-Sephadex, they gave several minor peaks and one major peak which was the most basic among them. The major ribosomal protein peak was eluted at pH 9.6 (indicated as an arrow in Fig. 5) and thus also is less basic than the major synthetic product.

The products from peaks I, II, III, and IV were individually digested with trypsin together with unlabeled authentic coat proteins added as a carrier. After the digestion, urea and salt were removed on the Aminex-50 column. When the adsorbed materials were eluted with 4 *N* NH_4OH , the recovery of radioactivity from the trypsin-treated peak IV material, i.e., the major peak, was only 5 per cent, while almost quantitative recovery was obtained with the tryptic digests of authentic coat protein and with materials from peaks I, II, and III. Another peptide

FIG. 5.—SE-Sephadex column chromatography of products excluded from a Sephadex G-25 column. Details of chromatographic procedures are given under *Methods*. Each tube fraction contained approximately 0.7 ml. The radioactivity of 0.1-ml samples from the fractions was counted as described in the legend of Fig. 3. Window settings for double counting were such that cross-channel contamination was 27% in the direction of C^{14} to tritium, and 0.8% in the reverse direction. Full line represents C^{14} -labeled synthetic product; dashed line represents tritium-labeled authentic coat protein; arrow indicates position of peak of major ribosomal proteins.

eluent, pyridine-acetic acid-water (30:4:66, v/v), also failed to recover any of the remaining radioactivity of the trypsin-treated peak IV. A different desalting procedure was therefore used. After removing urea from the digestion mixture, the Aminex-50 column was washed with 0.5 *N* HCl to remove Na^+ and then with 4 *N* HCl to elute out the material. The eluate was evaporated to dryness by means of flash evaporator, and was chromatographed on SE-Sephadex. The trypsin-treated material of peak IV was eluted in the same place as the untreated material, indicating that it had not been digested by trypsin. Carrier coat protein added to the same digestion mixture was completely digested. This probably explains the fact that this material could not be recovered from the Aminex-50 column with ordinary peptide eluents, because unfolded proteins are known to be very tightly adsorbed to Aminex-50, $\times 2$.

Mapping of tryptic digests: Radioautograms of the tryptic peptides of the products in peaks I, II, and III were qualitatively similar to each other and to the map of authentic R17 protein. Figure 6 shows a diagram of the radioautogram of the tryptic map of peak III. The positions of the ninhydrin-stained spots of the tryptic digests of carrier R17 coat protein are shown also. A total of 12 ninhydrin spots was evident. Three of them (which migrated toward the anode) correspond to peptides which contain neither arginine nor lysine. The other nine peptides contain lysine or arginine.⁶ Six of the radioactive spots were exactly congruent with ninhydrin spots. Two radioactive spots partially overlapped two ninhydrin spots. The radioautogram also contained about ten other relatively faint spots which did

FIG. 6.—Composite map of two-dimensionally separated ninhydrin-stained tryptic peptides of R17 coat protein and radioautogram of tryptic peptides of C^{14} -arginine plus lysine-labeled products of peak III. The circled areas are ninhydrin spots containing no appreciable amount of radioactivity; the hatched areas are radioactive spots having no ninhydrin stain; the cross-hatched areas are radioactive spots congruent with ninhydrin spots. The equivalent patterns obtained from tryptic digest of peaks I and II were qualitatively the same as the above pattern.

FIG. 7.—Composite map of two-dimensionally separated ninhydrin-stained tryptic peptides of R17 coat protein and radioautogram of trypsin-treated product of peak IV. The circled areas are ninhydrin spots containing no appreciable amount of radioactivity; the hatched area is a radioactive region having no ninhydrin stain.

not correspond to any of the ninhydrin spots. These results imply that peaks I, II, and III contain materials similar to coat protein and also other materials.

The fact that the maps of peaks II and III were very similar raises some questions. It is possible that the products in peak II are a mixture of peptides secondarily derived from the finished products, for example, by proteolytic activity of the *E. coli* extract. In spite of their difference in adsorption on the SE-Sephadex column, the products of peak I also gave a map with spots congruent with some of the authentic coat protein spots. This is consistent with the suggestion that peak I contains intermediates of

protein synthesis including coat protein-like peptide regions.

The radioautogram of the trypsin-treated peak IV was somewhat striking (Fig. 7). It consisted of a straight streak directed sharply toward the cathode. No movement of radioactive materials was observed in chromatography. No other spots were found in addition to this streak. The streak was uniformly blackened, indicating that this material was adsorbed during the electrophoretic movement. The tip of the streak in the electrophoretic direction outran authentic lysine. This behavior again confirms that the product of peak IV is basic and was not affected by trypsin under conditions which digest R17 coat protein effectively.

Other properties of the major synthetic product: Other investigators who analyzed the products of protein synthesis directed by bacterial virus RNA did not report detection of the synthetic product we have described. Since we did not use TCA precipitation as a fractionation procedure, we suspected that our major synthetic product might be soluble in 5 per cent TCA. To test this, the peak IV fraction was returned to an incubation mixture containing all ingredients required for protein synthesis except radioactive amino acids. The TCA-insoluble radioactivity of such a mixture was assayed by the filter paper circle method of Mans and Novelli.¹⁵ All radioactivity due to the peak IV fraction was extracted with hot TCA (5% TCA at 90° for 15 min) and no radioactivity remained with the precipitate. Lysine-rich histones are known to be soluble in 5 per cent TCA.¹⁶ Amino acid analysis of C¹⁴-arginine plus lysine-labeled major synthetic product showed that it contained more lysine than arginine residues. When the major synthetic product was dialyzed with vigorous stirring against 0.1 M NaOH in 5 M urea at 4°C for 18 hr using Visking dialysis tubing 20/32, no radioactivity was lost to the outside of the sack.

Discussion.—The major product synthesized under our conditions is more basic than R17 coat protein and *E. coli* ribosomal proteins. It was resistant to trypsin under conditions which permit the complete digestion of other synthetic products and authentic coat protein. Like known trypsin inhibitors, it probably forms a complex with trypsin and other proteolytic enzymes and becomes resistant to their action. Possibly, because of this resistance, it survived as the major product.

Ribosomal RNA synthesis in *E. coli* is rapidly inhibited at an early stage of R17

virus infection.¹⁷ The striking early suppression of DNA-dependent RNA synthesis has been observed following the infection of the host cells by a number of different small RNA-containing animal viruses.¹⁸⁻²¹ Holoubek and Rueckert²² have found that the arginine labeling rate of the acid-soluble nuclear protein fraction of Ehrlich ascites cells markedly increases shortly after infection with Maus-Elberfeld virus with a concomitant sharp decrease of the labeling rate of RNA by uridine. They have postulated that the characteristic suppression of nuclear RNA synthesis induced by the infection of these RNA animal viruses is the result of the blockade of host DNA by a histone-like material which is synthesized shortly after virus infection. The major product synthesized *in vitro* by R17 virus RNA may well correspond to such histone-like material.

Summary.—RNA isolated from R17 bacterial virus stimulates the incorporation of amino acids into polypeptides in cell-free extracts of *Escherichia coli*. The synthetic products were fractionated by gel filtration and column chromatography, both in the presence of urea. The final fractions were characterized by tryptic digestion followed by two-dimensional peptide mapping. They consist of nascent polypeptide chains (intermediates of protein synthesis), substances similar to R17 coat protein, and some other unidentified products. The major product (accounting for more than half of the arginine- and lysine-labeled product) is a very basic polypeptide. This product is resistant to trypsin under the conditions which permit complete digestion of all other products and authentic coat protein. It is similar to lysine-rich histones in that it is soluble in 5 per cent trichloroacetic acid and contains more lysine than arginine residues.

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