In Vitro Protein Synthesis Directed by R17 Viral Ribonucleic Acid. I. Initiation of Polypeptide Synthesis in a Purified Cell-Free System*

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ABSTRACT: A purified *Escherichia coli* cell-free system has been used to study the role of magnesium and *N*-formylmethionyl-tRNA in viral ribonucleic acid (RNA) directed synthesis of polypeptides. The stimulation of amino acid incorporation by phage R17 RNA was found to occur at low magnesium concentrations (8–9 mM) only when *N*-formylmethionyl-tRNA was present to initiate protein synthesis by virtue of its ability to bind to the ribosome–phage RNA complex. At magnesium concentrations (15 mM) which allowed the efficient binding of ordinary aminoacyl-tRNA to the ribosome–mRNA complex, protein synthesis was found to proceed in the absence of *N*-formylmethionyl-tRNA.

These observations are interpreted in terms of a simple mechanism for the initiation of protein synthesis based on the ability of *N*-formylmethionyl-tRNA to bind at low magnesium concentrations to the peptidyl-RNA binding site on the ribosome–mRNA complex.

The RNA genome of the related phages R17 and f2 has been shown by several workers to promote protein synthesis in a crude S-30 incorporating system (Nathans *et al.*, 1962; Adams and Capecchi, 1966; Kolakofsky and Nakamoto, 1966), but this stimulatory effect is markedly reduced when more purified cell-free systems are used (Stanley *et al.*, 1966; Clark and Marcker, 1966b). Since several synthetic polynucleotides promote polypeptide synthesis in purified cell-free systems very efficiently (Salas *et al.*, 1965), it has been suggested (Stanley *et al.*, 1966) that translation of natural mRNA cannot be initiated in the absence of some factor(s) present in crude ribosome preparations.

The present report describes a purified cell-free system which is highly efficient in promoting polypeptide synthesis in the presence of a natural mRNA. This system has been used to examine the role of magnesium ions and of *N*-formylmethionyl-tRNA in the initiation of protein synthesis. Phage R17 RNA, as well as a synthetic polynucleotide (poly U) were found to give maximal stimulation of amino acid incorporation into a hot acid-insoluble product at magnesium concentrations of 12–15 mM. When *N*-formylmethionyl-tRNA was added to the system, however, a new optimum for R17 RNA-directed amino acid incorporation appeared at a magnesium concentration of 9 mM, while the pattern of amino acid incorporation in the presence of poly U remained unchanged. Additional studies revealed that whereas the specific binding of aminoacyl-RNA to the ribosome–R17 RNA complex was optimal at high magnesium concentration (12–15 mM), optimal binding of *N*-formylmethionyl-tRNA to the ribosome–RNA complex occurred both at low (9 mM) and high (15 mM) levels of magnesium. These and other observations presented below are discussed in light of recent studies in several laboratories (Sundararajan and Thach, 1966; Nakamoto and Kolakofsky, 1966; Kolakofsky and Nakamoto, 1966; Clark and Marcker, 1966a; Revel and Hiatt, 1965; Bodley and Davie, 1966) concerning the role of magnesium ions and *N*-formylmethionyl-tRNA in the translation of natural mRNA.

Materials and Methods

Cells. Escherichia coli B was used for the preparation of the components for the cell-free protein-synthesizing system. *E. coli* K12, Hfr_1 was used as a host for phage R17 (Paranchych and Graham, 1962). Rat liver, taken from 2-week-old females, was used for the preparation of the enzymes involved in the synthesis of *N*-formyl tetrahydrofolate.

Culture Media. A high salt minimal medium was used for the growth of *E. coli* B. The medium contained per liter: 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of sodium citrate, 5.4 g of KH_2PO_4, 36.6 g of K_2HPO_4, 4.0 g of $(NH_4)_2SO_4$, and 10 ml of glycerol. The basic Tris-maleate medium used to grow *E. coli* K12 was described previously (Paranchych, 1966).

Buffer Solutions. TMKM[1] buffer contained, in

* From the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada. *Received February 9, 1967.* Supported by grants from the Medical Research Council of Canada (MT-1610) and the National Cancer Institute of Canada.

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[1] Abbreivations used: TMKM buffer, Tris-magnesium–potassium–mercaptoethanol buffer; PEP, phosphoenolpyruvic acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; TCA, trichloroacetic acid.

moles per liter: Tris, 0.01; $MgCl_2 \cdot 6H_2O$, 0.01; KCl, 0.06; and β-mercaptoethanol, 0.006 (pH 7.8). The ribosome-wash buffer was identical with the TMKM buffer except that it also contained 0.5 mole/l. of NH_4Cl.

Chemicals. The ^{14}C-labeled amino acid mixture (sp act. 1.5 mc/mg) and the ^{14}C-labeled sodium formate (sp act. 64 μc/mg) were purchased from New England Nuclear Corp. ^{3}H-labeled phenylalanine (sp act. 2.5 c/mmole), ^{3}H-labeled L-methionine (sp act. 1.3 c/mmole), and *E. coli* B RNA were from Schwarz BioResearch, Inc. Poly U (potassium salt) was purchased from Calbiochem. Tetrahydrofolate, deoxyribonuclease, streptomycin sulfate, protamine sulfate, pyruvate kinase, PEP, ATP, and GTP were obtained from Sigma Chemical Co.

Radioactive Measurements. The liquid scintillation fluid used contained 6 g of PPO and 0.5 g of POPOP/l. of toluene. Radioactive counting was carried out in a Beckman LS200 scintillation spectrometer.

Preparation of Ribosomes. E. coli B was grown at 37° for 10 hr in 200 ml of trypticase soy broth (Baltimore Biological Laboratory). The culture was then transferred directly to 20 l. of high salt minimal medium and vigorously aerated at 37° for 10 hr, after which the culture was in mid-log phase and contained about 2.5 g of packed cells/l. The culture was chilled by the addition of 3 kg of ice cubes, and cells were collected by means of a Lourdes continuous-flow centrifuge. Cell extracts were immediately prepared (care being taken not to freeze the cells at this stage) as described by Nirenberg (1963). Before sedimenting the ribosomes, the S-30 fraction was preincubated for 45 min at 37° with the following components in 150 ml of cell extract: 10 mg of *E. coli* B RNA, 0.05 mmole of ATP, 0.005 mmole of PEP, 0.05 ml of pyruvate kinase, and 5 mg of each of 20 amino acids. Following the incubation, the solution was clarified by centrifuging for 20 min at 22,000*g*, and then it was centrifuged for 90 min at 122,000*g* to sediment the ribosomes. After the centrifugation, the top one-third of the supernatant solution was removed, and the remaining supernatant solution was swirled in the centrifuge tube to suspend the light debris overlaying the ribosomal pellet. After discarding this supernatant solution, the centrifuge tube was thoroughly washed with ribosome-wash buffer, after which the ribosomes were suspended in more of the same buffer. Following clarification of the solution by slow-speed centrifugation, the ribosomes were again pelleted by centrifugation for 60 min at 122,000*g*, and again washed vigorously with ribosome-wash buffer. Finally, the ribosomes were resuspended in TMKM buffer and stored in 0.5-ml aliquots under liquid nitrogen. The ribosomes thus prepared were completely free of endogenous amino acid incorporating activity, and they did not bind aminoacyl-tRNA in the absence of added mRNA.

Preparation of Amino Acid Activating and Transformylating Enzymes. Frozen *E. coli* B (50 g) was used to prepare a 100,000*g* supernatant, the S-100 fraction, by the method of Nirenberg (1963). Unless otherwise mentioned, all procedures were carried out at 0–4°. The S-100 fraction was diluted with TMKM buffer to contain 10 mg/ml of protein, and to this solution was added streptomycin sulfate to a final concentration of 0.5% (w/v). The mixture was allowed to stand for 30 min, after which it was clarified by centrifuging for 20 min at 22,000*g*. To the resulting supernatant solution, protamine sulfate was added to a final concentration of 0.05%. The mixture was allowed to stand for 60 min, after which it was clarified by centrifuging for 20 min at 22,000*g*. The resulting supernatant solution was fractionated with ammonium sulfate and the fractions precipitating at 30, 30–50, and 50–60% were collected and labeled PR-I, PR-II, and PR-III, respectively. Each fraction was dissolved in 5 ml of TMKM buffer and dialyzed against the same buffer overnight in the cold. The solutions were finally clarified by low-speed centrifugation and stored at −20°. The major portion of the amino acid activating enzyme activity was recovered in the PR-II fraction. The PR-I fraction was used in the transformylation reaction described later.

Preparation of Rat Liver Enzyme. Freshly excised rat liver (50 g) was pulverized in a mortar at 0°, then suspended in 150 ml of 20% (w/v) sucrose containing 1% (w/v) EDTA (pH 7.3). The mixture was blended at maximum speed for 5 min in a Servall Omni Mixer, after which cell debris was removed by centrifuging for 20 min at 22,000*g*. The supernatant solution was made to contain 5 μg/ml of deoxyribonuclease, and it was centrifuged for 5 hr at 82,000*g*. The resulting supernatant solution was fractionated with ammonium sulfate at 25, 25–50, and 100% saturation, and the precipitates were collected by centrifugation and designated FI, FII, and FIII, respectively. The precipitates were then dissolved in 20 ml of TMKM buffer and dialyzed against the same buffer at 4° overnight. The bulk of the enzyme activity catalyzing synthesis of *N*-formyl tetrahydrofolate was found in fraction FII, which was stored at −20° until used.

Preparation of Aminoacyl-tRNA. The reaction mixture contained the following substances in millimoles per milliliter: Tris-HCl (pH 7.2), 0.08; $MgCl_2$, 0.012; KCl, 0.02; β-mercaptoethanol, 0.004; ATP, 0.001; and PEP, 0.0004. In addition, 50 μc of radioactive amino acids, 3 mg of RNA, 10 μg of amino acid activating enzyme protein (PR-II fraction), and 2 μl of pyruvate kinase (containing approximately 20 μg of protein) were added to each milliliter of reaction mixture. After incubating for 20 min at 37°, one-tenth volume of 1 M NaCl was added and the mixture was extracted with two volumes of 80% phenol. After separation of the phenol and aqueous layers, the phenol layer was extracted twice with equal volumes of 0.05 M potassium acetate buffer (pH 5.4). The original aqueous layer was added to the two potassium acetate extracts and the solution was clarified by centrifuging for 20 min at 22,000*g*. Three volumes of absolute methanol was then added, and the solution was allowed to stand at 0° for 2 hr. The precipitate of aminoacyl-tRNA was collected by centrifugation and dissolved in potassium

acetate buffer at a final concentration of 3 mg/ml. When ^{3}H-labeled phenylalanyl- or methionyl-tRNA was prepared in this manner, approximately 80% of the radioactivity was recovered as acyl-tRNA. When the ^{14}C-labeled amino acid mixture was used, the recovery of labeled amino acids in the acyl-tRNA form was approximately 40%.

Preparation of N-Formylmethionyl-tRNA. *N*-Formylmethionyl-tRNA was prepared as described by Adams and Capecchi (1966), except that the pigeon liver fraction was replaced by the FII fraction from rat liver (see section on rat liver enzyme), and the crude *E. coli* supernatant fraction was replaced by the *E. coli* PR-I fraction described earlier in this report. The radioactive *N*-formylmethionyl-tRNA prepared for the studies on the binding of *N*-formylmethionyl-tRNA to the ribosome–mRNA complex contained ^{14}C-labeled formate and ^{3}H-labeled L-methionine. The label in the *N*-[^{14}C]formyl[^{3}H]methionyl-tRNA was found to become 99% soluble in cold 5% TCA on treatment with hot 5% TCA (40 min at 90°), and approximately 95% soluble in cold 5% TCA on treatment with 0.1 M $KHCO_3$ (pH 8) at 37° for 30 min. When the *N*-formylmethionyl-tRNA was exposed only to cold 5% TCA, the label remained completely acid insoluble.

Preparation of Phage R17 RNA. Preparation and purification of phage R17 was carried out as described by Paranchych and Graham (1962), while phage RNA was prepared from purified phage by phenol extraction as described by Strauss and Sinsheimer (1963). The RNA thus prepared was stored in buffer containing, per liter: 0.05 mole of potassium acetate (pH 5.0), 0.1 mole of NaCl, and 10^{-4} mole of EDTA.

Amino Acid Incorporation System. Measurement of the cell-free incorporation of radioactive amino acids into a hot acid-insoluble product was carried out in a 0.5-ml reaction mixture containing per ml: 0.08 mmole of Tris-HCl (pH 7.8), 0.04 mmole of KCl, 0.004 mmole of β-mercaptoethanol, 0.001 mmole of ATP, 0.0004 mmole of PEP, 10^{-7} mmole of GTP, 2 μl of pyruvate kinase, 0.2 mg of RNA, 0.5 μc of ^{14}C-labeled amino acid mixture or 5 μc of ^{3}H-labeled phenylalanine (when the ^{14}C-labeled amino acid mixture was employed, it was supplemented with 5 μg of L-methionine); 10 μg of amino acid activating enzyme (PR-II); mRNA (60 μg, each of either R17 RNA or poly U); 100 μg of purified ribosomes; *N*-formylmethionyl-tRNA (when used) at a concentration equivalent to 6 μμmoles of formate. The final magnesium concentrations used were as described in each individual experiment. The reaction mixture was incubated at 37° for 60 min. At various intervals, 0.1-ml samples were removed and assayed for hot acid-insoluble radioactivity by means of the filter paper disk method of Mans and Novelli (1962).

Transfer Experiment. Measurement of poly U or R17 RNA-directed transfer of amino acids from aminoacyl-tRNA into a hot acid-insoluble product was carried out in a 0.5-ml reaction mixture containing, per milliliter: 0.08 mmole of Tris-HCl (pH 7.2), 0.04 mmole of KCl, 0.004 mmole of β-mercaptoethanol, 10^{-7} mmole of GTP, 100 μg of ribosomes, mRNA (60 μg, if R17 RNA was used, 6 μg if poly U was used), 10 μg of PR-I protein, and 2 μl of $(NH_4)_2SO_4$ saturated at 0°. The final magnesium concentrations and the amount of radioactive aminoacyl-tRNA were as described in each individual experiment. The reaction mixture was incubated at 37° for 20 min and, at various intervals, 0.1-ml samples were removed and assayed for hot acid-insoluble radioactivity by means of the filter paper disk method of Mans and Novelli (1962).

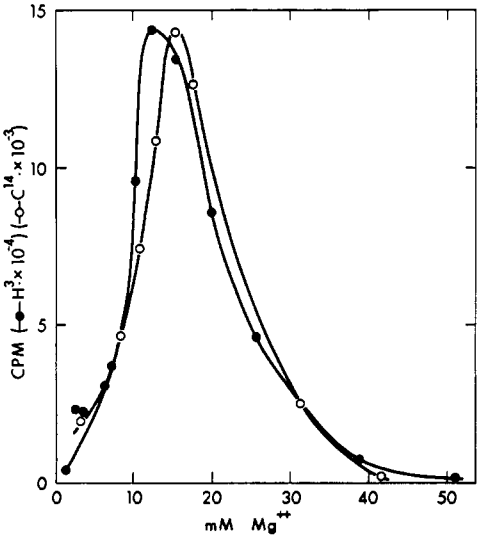

FIGURE 1: Effect of magnesium concentration on poly U and R17 RNA-directed incorporation of amino acids. The reaction mixtures and assay methods were as described in Materials and Methods. (●—●) Poly U directed incorporation of ^{3}H-labeled phenylalanine. (○—○) Phage R17 RNA-directed incorporation of a ^{14}C-labeled amino acid mixture.

Binding Experiment. Studies on the binding of aminoacyl-tRNA to the ribosome–mRNA complex were carried out essentially as described by Nirenberg and Leder (1964), except that Millipore DA 0.65-μ filters were used instead of HA 0.45-μ filters. The total volume of each reaction mixture was 0.05 ml. The reactions were allowed to proceed for 20 min at 24°, after which the mixtures were diluted 100-fold into a buffer of the same ionic composition and pH as that of the reaction medium. The diluted mixture was then passed through a membrane filter (presoaked with the same buffer solution), and the filter was washed three times with 5 ml of the same buffer. After drying the membrane filters at 100° for 2 min, they were placed in vials containing 5 ml of scintillation fluid and counted in a liquid scintillation spectrometer.

Results

Effect of Magnesium Concentration on R17 RNA- and Poly U Directed Incorporation of Amino Acids.

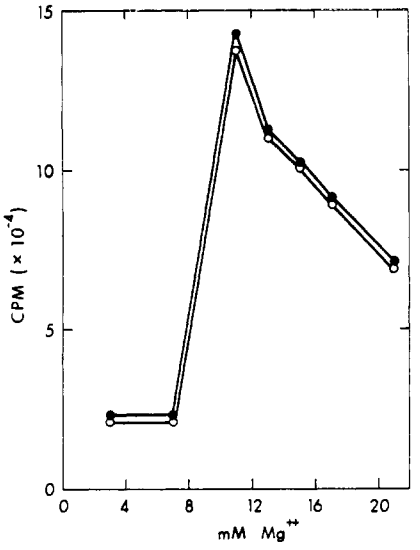

FIGURE 2: Effect of *N*-formylmethionyl-tRNA on poly U directed incorporation of ^{3}H-labeled phenylalanine at various concentrations of magnesium. The reaction mixtures and assay methods were as described in Materials and Methods. (●—●) Minus *N*-formylmethionyl-tRNA. (○—○) Plus *N*-formylmethionyl-tRNA.

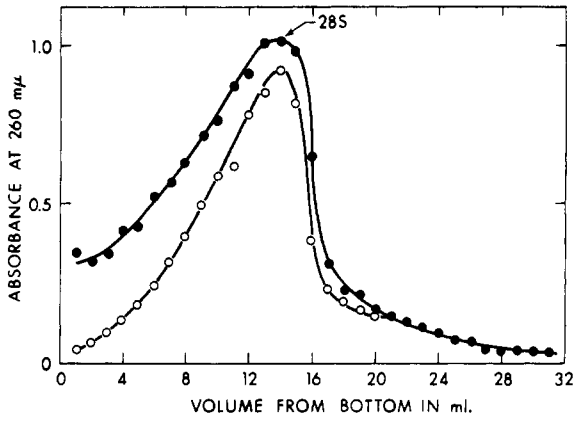

FIGURE 4: Sucrose gradient analysis of phage R17 RNA. The phage RNA samples were mixed to give a total of 1.5 mg of phage RNA/ml. The RNA solution (1 ml) was layered on top of a 30-ml sucrose density gradient (5–20%) and centrifuged in the SW25 rotor at 22,500 rpm for 19 hr (0°). After centrifugation, 1-ml fractions were collected through a puncture in the bottom of the tube and the absorbance at 260 mμ of each fraction was determined. (●—●) Freshly prepared R17 RNA. (○—○) R17 RNA after 3-months storage at −20°.

Incorporation of radioactive amino acids into hot acid-insoluble material was tested in the purified system described in Materials and Methods, in which the magnesium concentration was varied from 2 to 50 mM. At all magnesium concentrations used, no endogenous incorporation occurred when the purified cell-free system was incubated in the absence of R17 RNA or poly U.

It may be seen in Figure 1 that very efficient stimulation of amino acid incorporation was obtained with both phage RNA and poly U. Moreover, it is clear that the magnesium requirement in both systems was very critical, optimal stimulation for phage RNA and poly U occurring at 15 and 12 mM, respectively. It is worth noting that the slight difference in magnesium optima observed for the two types of polynucleotides was highly reproducible.

Effect of N-Formylmethionyl-tRNA on R17 RNA- and Poly U Directed Incorporation of Amino Acids at Various Magnesium Concentrations. It is to be noted in the above experiment that the magnesium concentrations required for optimal incorporation of amino acids were relatively high. Nakamoto and Kolakofsky (1966), as well as several others (Revel and Hiatt, 1965; Sundararajan and Thach, 1966), have also observed a requirement for high levels of magnesium when synthetic messengers are used in an *in vitro* protein-synthesizing system. This situation contrasts with the fact that viral messenger ribonucleates (Adams and Capecchi, 1966; Kolakofsky and Nakamoto, 1966), as well as certain synthetic messengers (poly AGU; Nakamoto and Kolakofsky, 1966), are capable of promoting polypeptide synthesis at lower magnesium concentrations when *N*-formylmethionyl-tRNA is present in the system. In view of the recent suggestions (Clark and Marcker, 1966a; Adams and Capecchi, 1966) that *N*-formylmethionyl-tRNA may

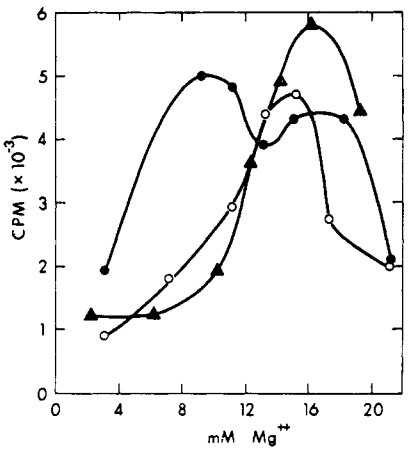

FIGURE 3: Effect of *N*-formylmethionyl-tRNA on R17 RNA-directed incorporation of ^{14}C-labeled amino acids at various concentrations of magnesium. The reaction mixtures and assay methods were as described in Materials and Methods. (●—●) Plus *N*-formyl methionyl-tRNA; R17 RNA freshly prepared. (○—○) Minus *N*-formylmethionyl-tRNA; R17 RNA freshly prepared. (▲—▲) Plus *N*-formylmethionyl-tRNA; R17 RNA stored at −20° for 3 months.

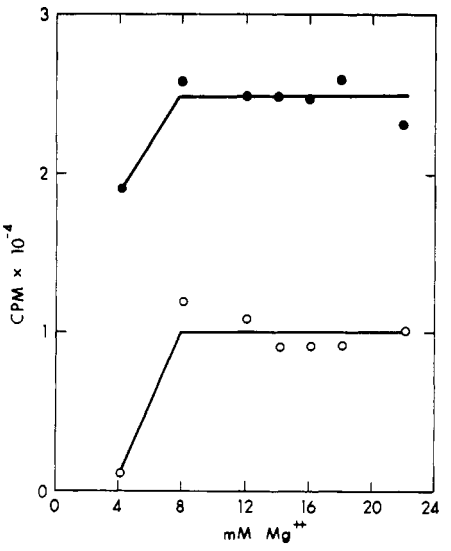

FIGURE 5: Effect of magnesium concentration on the formation of aminoacyl-tRNA. The reaction mixtures and assay methods were as described in Materials and Methods except that the ribosomes, mRNA, and GTP were omitted, and the incubation time was only 15 min. The specific activity of ^{3}H-labeled phenylalanine is 2.5 c/mmole. The specific activity of ^{14}C-labeled amino acid mixture is 1.5 mc/mg. (●) Formation of ^{3}H-labeled phenylalanyl-tRNA. (○) Formation of ^{14}C-labeled aminoacyl-tRNA.

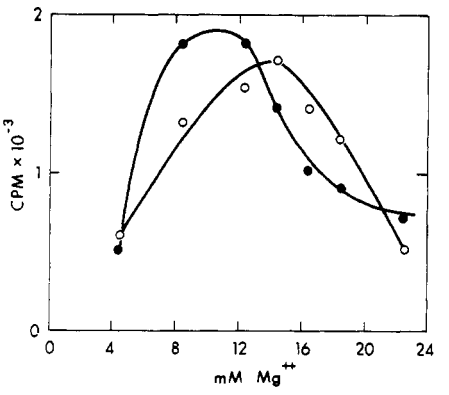

FIGURE 6: Effect of magnesium concentration on amino acid transfer from aminoacyl-tRNA. The conditions of the transfer experiment were as described in Materials and Methods. The reaction mixtures contained 2×10^4 cpm of ^{3}H-labeled phenylalanyl-tRNA or 3.2×10^4 cpm of ^{14}C-labeled aminoacyl-tRNA. (●) Poly U directed transfer of ^{3}H-labeled phenylalanine. (○) R17 RNA-directed transfer of a ^{14}C-labeled mixture of amino acids.

be involved in the initiation of protein synthesis, the effect of this RNA derivative on polypeptide synthesis was examined with the purified cell-free systems used in our laboratory.

Figure 2 illustrates the effect of *N*-formylmethionyl-tRNA on poly U directed incorporation of ^{3}H-labeled phenylalanine at various concentrations of magnesium. It is evident that the addition of *N*-formylmethionyl-tRNA to the incorporating system had no effect whatever on the capacity of poly U to direct polypeptide synthesis at any of the magnesium concentrations tested. This result is not unexpected, however, since *N*-formylmethionyl-tRNA apparently does not bind to a ribosome–poly U complex (Clark and Marcker, 1966a).

Figure 3 shows the results of studies carried out to examine the effect of *N*-formylmethionyl-tRNA on R17 RNA-directed incorporation of amino acids. It may be seen that optimal stimulation of amino acid incorporation in the absence of *N*-formylmethionyl-tRNA occurred, as before (Figure 1), at a magnesium concentration of 15 mM. Upon the addition of *N*-formylmethionyl-tRNA to the system, however, efficient stimulation of amino acid incorporation was found to occur at low, as well as high levels of magnesium, the new peak of incorporating activity appearing at 9 mM magnesium. Also shown in Figure 3, is the effect of *N*-formylmethionyl-tRNA on amino acid incorporation directed by a sample of R17 RNA which had been stored for 3 months at −20°. Much to our surprise, this sample of RNA no longer possessed the capacity to respond to *N*-formylmethionyl-tRNA by stimulating polypeptide synthesis at the lower magnesium concentrations. Our initial reaction to this observation was to suspect that the RNA had become degraded during the period of storage. However, upon examination of the sedimentation properties of the sample in a sucrose density gradient (Figure 4), it was found to have the same sedimentation properties as the freshly prepared phage RNA. The two RNA preparations were also found to have identical sedimentation properties following a 1-min treatment at 95°, and when sedimented through sucrose gradients under conditions of low ionic strength (0.02 M potassium acetate, no divalent cations). These observations were taken to be an indication that the stored phage RNA probably contained no "hidden breaks." We were thus led to believe that very subtle changes in the physical or chemical properties of phage RNA, such as those caused by the cleavage of a small number of nucleotide residues from either the 5′ or 3′ end of the molecule, may be sufficient to prevent a normal response to *N*-formylmethionyl-tRNA in an *in vitro* protein-synthesizing system.

In an attempt to gain a better understanding of the results illustrated in Figure 3, an examination was carried out on the effect of *N*-formylmethionyl-tRNA and magnesium on some of the isolated steps which go to make up the over-all protein-synthesizing system. These studies are described in what follows.

Magnesium Requirement for Formation of Aminoacyl-tRNA. To determine the magnesium requirement for the formation of aminoacyl-tRNA, studies were carried out on the incorporation of ^{3}H-labeled phenylalanine or ^{14}C-labeled amino acids into a cold acid-insoluble

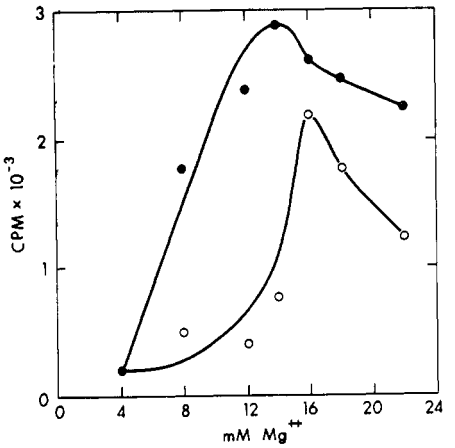

FIGURE 7: Effect of magnesium on the specific binding of aminoacyl-tRNA to the ribosome–mRNA complex. Each reaction mixture contained 6×10^3 cpm of radioactive aminoacyl-RNA, either 10 μg of poly U or 100 μg of R17 RNA, 10 μg of ribosomes, and various amounts of magnesium in 0.05 ml of TMKM buffer (pH 7.2). The mixtures were allowed to stand for 15 min at 24°, after which they were filtered, washed, and assayed for radioactivity as described in Materials and Methods. (●) Binding of ^{3}H-labeled phenylalanyl-tRNA to poly U ribosomes. (○) Binding of ^{14}C-labeled aminoacyl-tRNA to R17 RNA–ribosomes.

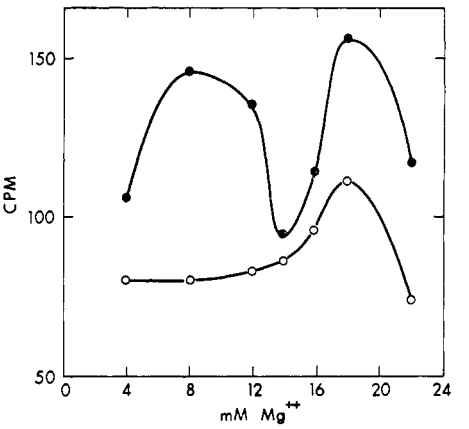

FIGURE 8: Effect of magnesium on the specific binding of *N*-formylmethionyl-tRNA to the ribosome–R17 RNA complex. The reaction mixtures contained 500 cpm of [^{14}C]formate-labeled *N*-formylmethionyl-tRNA, 100 μg of R17 RNA, 10 μg of ribosomes, and various concentrations of magnesium in 0.05 ml of TMKM buffer (pH 7.2). The mixtures were allowed to stand for 15 min at 24°, after which they were filtered, washed, and assayed for radioactivity as described in Materials and Methods. (●) Freshly prepared R17 RNA. (○) R17 RNA after 3-months storage at −20°.

product. The reaction medium was identical with that used for measuring amino acid incorporation into polypeptides, except that ribosomes and mRNA were absent from the mixture. Figure 5 shows that the formation of aminoacyl-tRNA was maximal at magnesium concentrations of 6 mM or greater. For reasons which are unclear, the synthesis of phenylalanyl-tRNA was more efficient than were the syntheses of aminoacyl-tRNAs from a mixture of labeled amino acids.

Effect of Magnesium Concentration on the Transfer Step of Protein Synthesis. The poly U and R17 RNA-directed transfer of amino acids from aminoacyl-tRNA into hot acid-insoluble products was measured at various concentrations of magnesium in order to determine whether polypeptide synthesis in the absence of aminoacyl-tRNA formation would show a different magnesium requirement than that observed for the over-all incorporation system (Figure 1). It may be seen in Figure 6 that amino acid incorporation was similar to that found in Figure 1, except that optimal levels of incorporation occurred over somewhat greater ranges of magnesium concentration. It should be noted that optimal poly U directed incorporation was again at a slightly lower magnesium concentration than that found for the R17 RNA system.

Specific Binding of Aminoacyl-tRNA to the Ribosome–mRNA Complex. Studies on the specific binding of phenylalanyl-tRNA to a ribosome–poly U complex, and a mixture of aminoacyl-tRNAs to a ribosome–R17 RNA complex were carried out in the manner described in Materials and Methods. It should be mentioned that, in the system described, no binding of aminoacyl-tRNA to ribosomes occurred in the absence of added poly U or R17 RNA and/or in the presence of heat-denatured ribosomes. The results are illustrated in Figure 7, where it may be seen that optimal binding of aminoacyl-tRNA to the ribosome–R17 RNA complex occurred at a slightly higher magnesium concentration (16 mM) than did binding of phenylalanyl-tRNA to the ribosome–poly U complex (14 mM). On the basis of the striking resemblance of the curves in Figure 7 to those shown in Figures 1 and 6, it is tempting to speculate that the marked dependence on magnesium of the cell-free amino acid incorporation system may arise at the level of the aminoacyl-tRNA binding to the ribosome–mRNA complex.

Specific Binding of N-Formylmethionyl-tRNA to the Ribosome–mRNA Complex. The specific binding of *N*-formylmethionyl-tRNA to the ribosome–mRNA complex was examined in a similar manner to that used in the previous section. Since there was no detectable binding of this RNA derivative to the ribosome–poly U complex, these results have not been shown. Figure 8 shows that when freshly prepared R17 RNA was used, binding of *N*-formylmethionyl-tRNA to the ribosome–RNA complex was optimal at both 8 and 16 mM magnesium. When aged R17 RNA was used in this system, however, optimal binding of *N*-formylmethionyl-tRNA occurred only at 16 mM magnesium. Upon comparing the results shown in Figures 7 and 8, it becomes evident that ordinary aminoacyl-tRNAs are capable of binding to the ribosome–R17 complex only

at high levels of magnesium. Since the same level of magnesium is required for polypeptide synthesis in the absence of *N*-formylmethionyl-tRNA, it is probable that the initiation of protein synthesis is dependent simply on the ability of aminoacyl-tRNAs to attach to the ribosome–mRNA complex at a particular binding site. *N*-Formylmethionyl-tRNA apparently is able to bind to this site at a lower concentration of magnesium, providing that the mRNA contains the appropriate nucleotide sequence in the correct position on the ribosome.

Discussion

The results presented in this communication demonstrate that highly efficient polypeptide synthesis can be achieved with a purified cell-free system when viral RNA is used as the natural mRNA. Although phage RNA has previously been shown to promote protein synthesis in a purified system (Clark and Marcker, 1966b; Stanley *et al.*, 1966), the level of protein synthesis achieved in the present system is of the same order as that obtained with synthetic polynucleotides. This observation was particularly evident when approximately equal amounts of aminoacyl-tRNA were provided to the poly U and phage RNA-directed systems (Figure 6) rather than equal amounts of free amino acids and RNA (Figure 1). This by-passing of the aminoacyl-tRNA-synthesizing reaction was necessary to compensate for the observation that phenylalanyl-tRNA synthesis was more efficient than the synthesis of aminoacyl-tRNAs from a mixture of labeled amino acids (Figure 4). It should also be noted that the amount, by weight, of phage RNA used in the transfer experiment exceeded the amount of poly U by a factor of 10. This was necessary to correct for the fact that the molecular weight of phage RNA (1.1×10^6) was approximately ten times greater than that of the poly U (mol wt $\simeq 10^5$).

Several recent studies (Clark and Marcker, 1966a; Nakamoto and Kolakofsky, 1966; Sundararajan and Thach, 1966) have indicated that the initiation of protein synthesis is dictated by a unique triplet (AUG) which directs the binding of *N*-formylmethionyl-tRNA to the ribosome–mRNA complex. These observations have led several workers (Nakamoto and Kolakofsky, 1966; Bretscher and Marcker, 1966) to propose a mechanism for the initiation of protein synthesis based on the ability, at low magnesium concentration, of *N*-formylmethionyl-tRNA to bind to the ribosomal site which is specific for peptidyl-RNA.

The results reported in the present communication are also in accord with this hypothesis. Phage RNA-directed polypeptide synthesis was found to occur at low magnesium concentrations only if *N*-formylmethionyl-tRNA was present in the system. This was found to be due to the fact that ordinary aminoacyl-tRNAs were unable to bind tot he ribosome–mRNA complex at this magnesium concentration (9 mM), whereas *N*-formylmethionyl-tRNA was able to do so. At magnesium concentrations (15 mM) which allowed the efficient binding of ordinary aminoacyl-tRNAs to the ribosome–mRNA complex, protein synthesis was found to proceed in the absence of *N*-formylmethionyl-tRNA. The fact that stored phage RNA did not respond to *N*-formylmethionyl-tRNA at low magnesium concentrations would suggest that the coding triplet for *N*-formylmethionyl-tRNA may have been removed from the phage RNA as a result of limited degradation during storage. Since the amount of this degradation was sufficiently small that it could not be detected by sucrose gradient analysis, it is tempting to postulate that the *N*-formylmethionyl-tRNA codon occurs only once in the phage RNA genome, presumably at or near the 5′ (OH) chain end. It is also possible, however, that the *N*-formylmethionyl-tRNA codon occurs more than once in the viral genome, but that the secondary structure of the phage RNA molecule restricts interaction of the "inner" AUG codons with the ribosomes.

It is of interest that Clark and Marcker (1966a) have recently shown that methionyl-$tRNA_F$ (the species of methionyl-tRNA which becomes formylated to give *N*-formylmethionyl-tRNA) is capable of initiating polypeptide synthesis both in the formylated and nonformylated states. These results contrast with our own, as well as those of Kolakofsky and Nakamoto (1966), which show a strong requirement by the phage RNA-directed protein-synthesizing system for the formylated RNA derivative. We are presently unable to provide a satisfactory explanation for these conflicting observations. Leder and Bursztyn (1966) have recently proposed that the reaction sequence may involve formylation of the methionyl-tRNA after it has become attached to the ribosome–mRNA complex. These and other possible explanations are presently being tested in our laboratory with a view to gaining a clearer understanding of the mechanism of the initiation of protein synthesis.

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Antibiotic Glycosides. VI. Definition of the 50S Ribosomal Subunit of *Bacillus subtilis* 168 as a Major Determinant of Sensitivity to Erythromycin A*

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ABSTRACT: Two strains of *Bacillus subtilis* have been used for the preparation of ribosomes or subribosomal particles and a partially purified enzyme fraction, which together with synthetic polynucleotides, amino acids, and an energy source will synthesize polypeptides. One bacterial strain is sensitive to the medium-ring macrolide antibiotic erythromycin A, and the other strain is a mutant which is less sensitive. The effect of erythromycin A on polypeptide synthesis has been measured as a function of the source of the ribosomes, subribosomal particles, and the enzyme fraction. The concentration of erythromycin A that is required for maximal inhibition of polypeptide formation depends markedly on both the source of the 50S subribosomal particle and the nature of the polymer being synthesized. In particular, the 50S ribosomal subunits differ. Reconstituted systems with 50S subunits from erythromycin-sensitive *B. subtilis* show a 15-fold greater sensitivity to the antibiotic than those which employ 50S subunits from the resistant strain of bacteria. The bacterial mutation which has led to a reduced sensitivity to erythromycin A probably has produced some change in the structure of the 50S subribosomal particle, and this affects the interaction of the antibiotic with the reactions of protein synthesis.

Brock and Brock (1959) were the first to suggest that erythromycin A (EaDC)[1] acts as a selective inhibitor of protein synthesis. This conclusion was based on the observation that both protein synthesis and growth of an *Escherichia coli* strain ceased in the presence of 1000 μg/ml of erythromycin A, while nucleic acid synthesis continued. Taubman *et al.* (1963) continued the investigation of the mechanism of bacterial sensitivity to this macrolide antibiotic with two strains of *Bacillus subtilis* 168, one which is sensitive to less than 1 μg/ml of erythromycin A and the other which is relatively resistant (about 100-fold less sensitive) as the result of a mutation. It was shown that erythromycin A, at concentrations which are of physiological significance, blocked protein biosynthesis in the bacteria which are sensitive, and that these same concentrations had little effect on the strain which had acquired resistance. The studies were extended (Taubman *et al.*, 1963, 1964), employing a cell-free system derived from the antibiotic-sensitive strain of *B. subtilis*, and erythromycin A was found to inhibit the incorporation of amino acids into polypeptides. Wolfe and Hahn (1964), who studied an *E. coli* strain similar in sensitivity to that used by Brock and Brock (1959), have reported a similar finding.

Taubman *et al.* (1964) have defined the action of erythromycin A as an inhibition of the ribosome-dependent transfer of activated amino acids (aminoacyl-

* From the Department of Biochemistry, Western Reserve University, School of Medicine, Cleveland, Ohio. *Received December 28, 1966.* Presented in preliminary form at the 6th Interscience Conference on Antimicrobial Agents and Chemotherapy, Philadelphia, Pa., Oct 26–28, 1966, p 43. For the previous paper in this series see Taubman *et al.* (1963). Supported by research grants from Abbott Laboratories, The American Heart Association, Inc. (65 G 126), and the U. S. Public Health Service (AI-06758, 5-T1-GM-35, and GM-AM- 13971-01).

† Data taken from a thesis which will be presented to the Graduate School of Western Reserve University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ Career Development Awardee 5-K3-GM-2545 of the U. S. Public Health Service.

[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; EaDC, erythromycin A; TCA, trichloroacetic acid.