*TRANSLATION OF THE GENETIC MESSAGE, III. FORMYLMETHIONINE AS INITIATOR OF PROTEINS PROGRAMED BY POLYCISTRONIC MESSENGER RNA**

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Polyacrylamide gel electrophoresis of the virus-specific proteins synthesized *in vivo* upon infection of *E. coli* with phage MS2[1, 2] yields three main fractions designated as proteins I, II, and III, of which protein III is coat protein. Experiments with "amber" mutants indicate that protein I[3] is a viral RNA synthetase, whereas protein II[2, 3] is a "maturation" protein.[4]

Translation of the RNA of coliphages by cell-free systems of *E. coli* gives rise to coat and other virus-specific proteins.[5–8] Two of these proteins have been characterized as RNA synthetase and coat protein.[8] The involvement of formylmethionine in initiation of coat protein synthesis[9, 10] raises the question whether other virus-specific proteins are similarly initiated. There are some indications that this is the case.[9, 11]

The present work was undertaken in order (*a*) to further characterize the proteins formed upon translation of the MS2 genome *in vitro*, and (*b*) to determine whether formylmethionine is the chain initiator of proteins other than coat. For characterization of the proteins synthesized *in vitro*, the C^{14}-labeled proteins formed by a cell-free *E. coli* system with MS2 RNA as messenger were coelectrophoresed with H^3-labeled proteins produced by MS2-infected, actinomycin-treated *E. coli* spheroplasts. The *in vivo* and *in vitro* proteins I and III were qualitatively identical but the *in vitro* system produced protein II to a much lesser extent, if at all. Moreover, the presence of one or more peaks, with a mobility intermediate between that of proteins II and III, suggested that unfinished or degraded peptides had been formed. In order to determine whether formylmethionine is involved in the initiation of both coat protein and protein I, *in vitro* incubations were conducted with H^3-formyltetrahydrofolic acid and C^{14}-methionine, and the proteins isolated by polyacrylamide gel electrophoresis. The two proteins contained H^3 and C^{14} label and both yielded formylmethionine upon digestion with pronase.

Materials and Methods.—Ribosomes, supernatant, and initiation factors: Ribosomes from *E. coli* Q13 (a mutant lacking ribonuclease I and polynucleotide phosphorylase) were purified by chromatography on *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) as in previous work.[12] Supernatant fractions were prepared from *E. coli* Q13 as described earlier,[13] except that only the upper two thirds of the supernatant was used. Chain initiation factors (F_1 and F_2), required for translation of natural messenger when purified ribosomes are used, were prepared from *E. coli* Q13 as previously outlined.[14] They were free of nuclease activity.

Other preparations: RNA from phage MS2 was purified by the method of Strauss and Sinsheimer.[15] Sucrose density gradient centrifugation of the RNA showed a single peak at 28*S*. The preparation was kept frozen for about 3 weeks before use. H^3-FTHF[16] was prepared[17] with H^3-formate using formyltetrahydrofolic acid (FTHF) synthetase kindly provided by Dr. J. C. Rabinowitz, University of California, Berkeley. The H^3-FTHF was purified on a column of Whatman cellulose powder.[18] Formylmethionyl-alanine was prepared by formylation of methionyl-alanine.[19] The reaction mixture was passed through a column of Dowex 50, H^+ form, and the formylmethionyl-alanine was eluted with water.

Amino acid incorporation: (a) *C^{14}-leucine incorporation:* Samples contained the following components in 0.25 ml: Tris-HCl buffer, pH 7.8, 60 mM; ammonium chloride, 50 mM; 2-mercaptoethanol, 16 mM; magnesium acetate, 14 mM; adenosine 5′-triphosphate (ATP), 1.2 mM; guanosine 5′-triphosphate (GTP), 0.3 mM; phosphocreatine, 18 mM; creatine kinase, 9 μg; purified *E. coli* Q13 ribosomes, 14 A_{260} units; *E. coli* Q13 supernatant, 50 μl; C^{14}-leucine, 0.2 mM; each of 19 nonlabeled amino acids, 0.2 mM; *E. coli* W tRNA, 0.25 mg; with or without MS2 RNA, 100 μg; F_1, 6.5 μg of protein; F_2, 13 μg of protein. After 7.5 min at 37°, a 100-fold excess of nonlabeled leucine was added and the incubation continued for 17.5 min. This was done in order to release unfinished, labeled polypeptide chains from the ribosomes. Aliquots (5 μl) were removed at 8, 15, and 25 min to establish the time at which incorporation of radioactivity into protein had ceased. Acid-insoluble radioactivity was determined as previously described.[1]

(b) *H^3-FTHF and C^{14}-methionine incorporation:* The incubation mixture was as described for (*a*) except for the substitution of nonlabeled for labeled leucine, the substitution of C^{14}-labeled for nonlabeled methionine, and the addition of H^3-FTHF (2.2 mμmoles/0.25 ml). Moreover, the samples contained one fifth the amount of tRNA (i.e., 0.05 mg/0.25 ml), and the final volume was 2.5 ml. After incubation for 7.5 min at 37°, a 100-fold excess of N^5-FTHF and of nonlabeled methionine was added, followed by incubation for 17.5 min. Aliquots (10 μl) were removed at 9, 15, 20, and 25 min as in (*a*). They were diluted with 0.5 ml of water, incubated for 15 min at 37° with 25 μg of RNase, and the acid-insoluble radioactivity was determined as in (*a*).

Polyacrylamide gel electrophoresis of the proteins synthesized in vitro under the direction of MS2 RNA: The incubation mixture was worked up as described for the fractionation of the virus-specific proteins synthesized in *E. coli* spheroplasts infected with MS2 phage.[1] Over 90% of the input radioactivity was recovered from the electrophoretic fractions. In the experiments with C^{14}-leucine the proteins were coelectrophoresed with H^3-leucine-labeled proteins isolated from MS2-infected *E. coli* spheroplasts.[1]

Characterization of formylmethionine in proteins I and III: Four identical polyacrylamide gel columns were loaded with H^3 formate- and C^{14}-methionine-labeled proteins, synthesized *in vitro* with MS2 RNA as messenger, and simultaneously subjected to electrophoresis. One of the columns was sectioned and the radioactivity in the slices determined in order to locate the position of the protein peaks. Proteins I and III were recovered from the other columns by homogenization of the corresponding sections of the gel and extraction, with freezing and thawing,[20] three times in the presence of 0.1 *M* ammonium bicarbonate, 0.1% sodium dodecylsulfate (SDS), 1% 2-mercaptoethanol. The gel particles were removed by centrifugation, and the supernatant was dialyzed exhaustively against 0.01 *M* ammonium bicarbonate for 24 hr. The fractions were concentrated by flash evaporation and treated with pronase (0.6 mg/ml) for 19 hr at 37°. To separate the formylated amino acids and peptides from nonformylated ones, the pronase-treated material was passed through a Dowex 50 column (1 × 16 cm), H^+ form; the formylated amino acids and peptides were eluted with water, concentrated by flash evaporation, and analyzed by high-voltage paper electrophoresis. The electrophoresis was run in a water-cooled plate at 28 v/cm for 3 or 4 hr. The buffer contained 25 ml of glacial acetic acid and 30 ml of pyridine per liter. The paper was dried, cut in sections (1-cm long × 3-cm wide), and the radioactivity determined as described for acid-insoluble radioactivity.[1]

Reagents: H^3-formate (2 c/mmole) was obtained from International Chemical and Nuclear Corp., City of Industry, Calif.; *d, l*-THF from General Biochemicals, Chagrin Falls, Ohio; pronase from Calbiochem, Los Angeles, Calif.; C^{14}-leucine (232 mc/mmole) and C^{14}-methionine (186 mc/mmole) from New England Nuclear Corp., Boston, Mass.; D,L-N-formylmethionine from Sigma Chemical Co., St. Louis, Mo.; L-methionyl-alanine from Mann Research Laboratories, Inc., New York; and N^5-THF (calcium leucovorin) from the Lederle Laboratories Division, Pearl River, N. Y. Other reagents were as in previous work.[1]

Results and Discussion.—In vitro translation of MS2 RNA: A typical coelectrophoretic pattern of the MS2-specific proteins synthesized *in vivo* and *in vitro*, with H^3- and C^{14}-labeled leucine, respectively, is shown in Figure 1. The following features of the *in vitro* pattern may be noted. (1) Peaks I and III are qualitatively normal. The shoulder to the right of peak III appears to be contributed by a

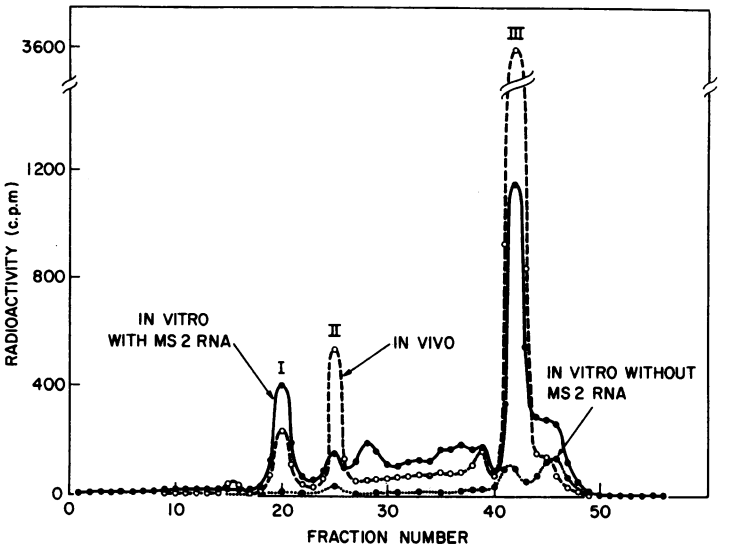

FIG. 1.—Electrophoretic pattern of proteins synthesized *in vitro* and *in vivo*. Proteins from *in vitro* (C^{14}-leucine label) and *in vivo* (H^3-leucine label) incubations were mixed and subjected to polyacrylamide gel electrophoresis. The radioactivity of the fractions was measured simultaneously in two channels of the Packard liquid scintillation spectrometer. Electrophoresis was for 4 hr at 7 v/cm. The anode is to the right in this and subsequent patterns. *Solid line and circles* (-●—●—●-), *in vitro* incubation with MS2 RNA; *dotted line and solid circles* (·●···●···●·), *in vitro* incubation without MS2 RNA; *dashed line and open circles* (-O- -O- -O-), *in vivo* incubation (MS2-infected spheroplasts).

nonspecific protein present in the blank, i.e., the sample incubated without added MS2 RNA. (2) There is but a small peak of radioactivity in the position of protein II. Its size varies in different incubations and occasionally it is barely discernible. (3) An additional small peak and some other material, possibly unfinished peptides and/or degradation products of completed proteins, are seen between peaks II and III. (4) Although the *in vitro* system, like that *in vivo*, synthesizes more coat protein (peak III) than protein I, relatively much more of the latter is produced *in vitro*. Apparently the mechanisms operating *in vivo* to curb the synthesis of proteins I and II relatively early in the replication cycle[1] fail to function in the *in vitro* system used here. *In vitro* synthesis of proteins I and III has recently been reported by Nathans *et al.*[2]

In vitro translation of MU9 RNA, having an "amber" mutation in the coat cistron, gives rise to an electrophoretic pattern virtually identical to that obtained upon translation of the wild-type RNA, except for the lack of the coat protein peak.

Formylation of proteins I and III: Formylmethionine residues at the amino-terminal end of biologically synthesized proteins arise from formylmethionyl~tRNA (formylmet~$tRNA_f$) formed by transfer of formate from formyltetrahydrofolic acid to met~$tRNA_f$.[21] In order to determine whether the *in vitro* translation of MS2 RNA leads to the incorporation of formylmethionine not only in the coat protein but also in protein I, incubations were conducted in the presence of H^3-FTHF and C^{14}-methionine, along with 19 nonlabeled amino acids, and the labeled

proteins isolated as before by polyacrylamide gel electrophoresis. A typical electrophoretic pattern is shown in Figure 2.

The following features may be noted. (1) Peaks of H^3 and C^{14} radioactivity overlap throughout the pattern; hence, formate is incorporated into all the proteins that incorporate methionine. (2) Protein peaks I and III are again qualitatively normal but peak II is lacking altogether. (3) Four distinct peaks of radioactivity are seen in positions intermediate between that corresponding to peak II and peak III. Since the electrophoretic mobility of SDS-protein complexes in polyacrylamide gels is inversely related to their molecular weight,[22] these peaks may be assumed to correspond to peptides smaller than protein II. These peptides must have arisen either by translation of partially degraded messenger chains, by degradation of some of the finished proteins, or both. For reasons that will be discussed at another time we believe that these results are probably due in the main to messenger degradation. The findings reported here suggest that the degradative processes affect mainly, if not exclusively, protein II.

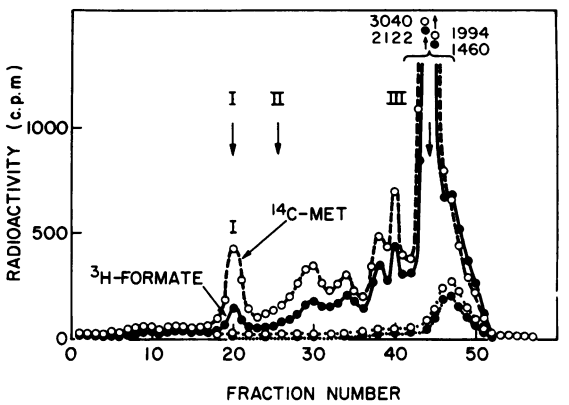

FIG. 2.—Electrophoretic pattern of proteins synthesized *in vitro* with H^3-TFHF and C^{14}-methionine. Conditions of electrophoresis as in Fig. 1. H^3 radioactivity (-●—●—●-) with MS2 RNA, and (·●··●··●·) without MS2 RNA. C^{14} radioactivity (-O- -O- -O-) with MS2 RNA, and (·O··O··O···) without MS2 RNA.

The labeled proteins from sections of the gels corresponding to peaks I and III were extracted, digested with pronase, and subjected to high-voltage paper electrophoresis, as described under *Materials and Methods*, along with markers of formylmethionine and formylmethionyl-alanine. Whereas enough coat protein was obtained for this purpose from one column, the pooled fractions from 12 individual columns were used in the case of protein I which is synthesized in much smaller amounts. Figure 3*A* shows, in agreement with previous results,[9, 10] the presence of formylmethionine and formylmethionyl-alanine in the coat protein digest. In the case of protein I (Fig. 3*B*) pronase digestion yielded mainly formylmethionine together with small amounts of formylmethionine-containing peptides.

Since the formyl residues are present only at amino-terminal ends, the ratio H^3 radioactivity in coat protein/H^3 radioactivity in protein I should be equal to the ratio number of coat peptide chains/number of protein I peptide chains present at

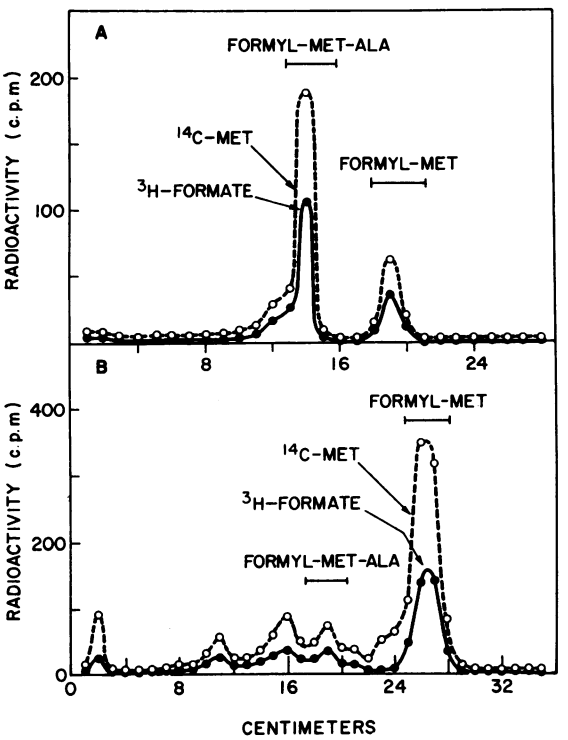

FIG. 3.—High-voltage paper electrophoresis of pronase digests of proteins III (*A*) and I (*B*) synthesized *in vitro* with H^3-FTHF (83 × 10^6 cpm/μmole) and C^{14}-methionine (144 × 10^6 cpm/μmole), in the presence of MS2 RNA. Digests and markers of formylmethionine and formylmethionyl-alanine were electrophoresed on Whatman 3 MM paper for 3 (*A*) or 4 (*B*) hr at 28 v/cm. Origin at 1 cm. The markers were spotted by the procedure of Knight and Young.[23] Their position on the electropherograms is indicated. H^3 and C^{14} radioactivity were measured simultaneously in two channels of the Packard liquid scintillation spectrometer.

the end of incubation, provided there is no alteration by deformylation. In the experiment of Figure 2 this ratio was 15. Thus, many more molecules of coat protein are made than of protein I. As noted in the preceding section, this excess would be considerably greater *in vivo*.

The experimental results presented here support the view that formylmethionine is involved in initiation of each of the polypeptide chains programed by the polycistronic messenger RNA's of *E. coli* phages.

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[16] Abbreviations: THF, tetrahydrofolic acid; FTHF, formyltetrahydrofolic acid; SDS, sodium dodecylsulfate. Other abbreviations are as in previous papers (refs. 12–14).

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