‡ International postdoctoral fellow of the National Institutes of Health, U.S. Public Health Service. Permanent address: Instituto Marañón, Centro de Investigationes Biológicas, C.S.I.C., Madrid, Spain.

§ Postdoctoral fellow of the National Institutes of Health, U.S. Public Health Service (to August 31, 1965).

[1] Salas, M., M. A. Smith, W. M. Stanley, Jr., A. J. Wahba, and S. Ochoa, *J. Biol. Chem.*, **240**, 3988 (1965).

[2] Shorthand writing of polynucleotides and abbreviations for nucleotides, amino acid residues in polypeptide chains, etc., are as recommended by *J. Biol. Chem.* and previously used (ref. 1).

[3] See Khorana, H. G., *Federation Proc.*, in press.

[4] Fiers, W., and H. G. Khorana, *J. Biol. Chem.*, **238**, 2789 (1963).

[5] Rushizky, G. W., C. A. Knight, and H. A. Sober, *J. Biol. Chem.*, **236**, 2732 (1961).

[6] Staehelin, M., *Biochim. Biophys. Acta*, **49**, 11 (1961).

[7] Thach, R. E., and P. Doty, *Science*, **147**, 1310 (1965).

[8] Folk, J. E., R. C. Brannberg, and J. A. Gladner, *Biochim. Biophys. Acta*, **47**, 595 (1961).

[9] Folk, J. E., and J. A. Gladner, *J. Biol. Chem.*, **231**, 379 (1958).

[10] Terzaghi, E., Y. Okada, G. Streisinger, A. Tsugita, M. Inouye, and J. Emrich, *Science*, **150**, 387 (1965).

[11] Thach, R. E., M. A. Cecere, T. A. Sundaranjan, and P. Doty, these PROCEEDINGS, **54**, 1167 (1965).

[12] Nirenberg, M. W., and P. Leder, *Science*, **145**, 1399 (1964).

[13] Matthaei, J. H., *Naturwissenschaften*, in press.

N-FORMYLMETHIONYL-sRNA AS THE INITIATOR OF PROTEIN SYNTHESIS

BY JERRY M. ADAMS* AND MARIO R. CAPECCHI†

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY

Communicated by J. D. Watson, November 22, 1965

The work several years ago of J. P. Waller[1] revealed a bizarre fact about N-terminal groups of bacterial proteins. Instead of a random mixture, Waller found that the great majority of N-terminal groups were either methionine or alanine. This finding suggested that methionine and alanine constituted start signals for the initiation of polypeptide chains. Alternatively, the remaining amino acids were not detected because of acylation of their amino groups. The discovery of N-formylmethionyl-sRNA by Marcker and Sanger[2] provided a means for further study of the problem.

In this compound, the amino group of methionine is formylated, thereby prohibiting its use in polypeptide chain elongation and at the same time making it an attractive candidate for initiation of polypeptide chains. Knowing of Waller's observations, Marcker and Sanger also looked for N-formylalanyl-sRNA. None, however, was detected. If chain initiation required formyl amino acids, the terminal alanine end groups of *E. coli* proteins remained unexplained. There was, of course, the possibility that N-formylmethionine was unrelated to protein synthesis.

The direct way to test involvement of N-formylmethionyl-sRNA in chain initiation is to add this compound to *in vitro* extracts which are carrying out protein synthesis. Here we report experiments in which N-formylmethionyl-sRNA

labeled in the formyl group was used with phage R17 RNA as the messenger RNA template in an *E. coli* extract. Labeled formyl groups were incorporated into at least two, if not all, of the several proteins coded by the R17 RNA. Formylmethionyl-sRNA is thus likely to initiate the synthesis of all the R17 specific proteins.

This is a surprising result since alanine is known to be the N-terminal amino acid of coat protein isolated from intact virus particles.[3] Resolution of the problem came from experiments in which the coat protein made *in vitro* was digested by pronase to see which amino acid was N-formylated. All of the labeled formyl groups were found in N-formylmethionine. Further, these experiments demonstrated that the amino acid adjacent to N-formylmethionine was alanine. We thus suspect that several of the R17 specific proteins, and conceivably a majority of *E. coli* proteins, start with the sequence N-formylmethionylalanine. After synthesis, the terminal formylmethionine residues of certain classes of proteins are enzymatically removed, yielding proteins which have N-terminal alanine.

Materials and Methods.—(a) *H^3-formyl-tetrahydrofolate:* H^3-formate (specific activity either 31 or 2000 μc/μmole) was activated using a dialyzed $(NH_4)_2SO_4$ fraction of pigeon liver supernatant.[4] Activation was completely dependent on added tetrahydrofolate (THFA) and proceeded to at least 90% of completion. The reaction was stopped by adjusting the solution to 1% PCA, after which all the protein was removed by low-speed centrifugation. The acid converts the product N^{10}-formyl-THFA into N^5, N^{10}-methenyl-THFA.[5] The former is regenerated for use in the transformylation reaction by neutralizing a portion of the solution a few minutes before the reaction. The methenyl form is much more resistant to air oxidation than the N^{10}-formyl compound[5] and in our experience can be stored at −20°C for weeks without significant oxidation.

(b) *Purification of formyl-THFA:* The donor was extensively purified on a column of Whatman cellulose powder.[6] A very large peak of material absorbing strongly at 280 mμ was eluted first, representing mainly THFA and its oxidation products. This peak was well resolved from the 360 mμ-absorbing material (methenyl-THFA). After two passages through this column, the donor had a UV spectrum in acid which agreed closely with the published spectrum of methenyl-THFA.[5]

(c) *Supernatant protein:* The supernatant protein used in the transformylation was prepared by grinding frozen *E. coli* B cells (Grain Processing Co.) with alumina and centrifuging for 5 hr at 78,000 *g*. The supernatant was then dialyzed for 24 hr against 0.01 *M* tris, pH 7.5, at 4°C and stored in small aliquots at −20°C.

(d) *Transformylation reaction:* The reaction for the transformylation of aminoacyl-sRNA contained 10 μmoles tris, pH 7.2, 1.0 μmole $MgCl_2$, 0.5 μmole ATP, 2.0 μg each amino acid, 0.5 mg sRNA (stripped), 2.0 μmoles of mercaptoethanol, 0.03 μmole of H^3-formyl-THFA, and 15 μg of *E. coli* supernatant protein in a total volume of 0.10 ml. The reaction mixture was incubated for 10 min at 37°C and then precipitated with cold 5% TCA on Millipore filters, which were dried and counted in a liquid scintillation counter.

(e) *H^3-formyl-sRNA:* sRNA was isolated from the reaction mixture by phenol extraction, precipitated several times with 66% ethanol, and dialyzed for 24 hr against 0.002 *M* potassium acetate, pH 5.0. The label in the purified sRNA became 96% acid-soluble on addition of RNase or dilute base. About 90% of the label became volatile after treating the product with 0.5 *N* HCl for 15 min at 100°C. Double-labeled sRNA was made similarly using C^{14}-methionine and the other 19 C^{12} amino acids.

(f) *Pronase digestion of R17 coat protein synthesized in vitro:* Fractions from a SW25 sucrose gradient corresponding to the R17 coat protein were pooled and digested with pancreatic RNase (20 μg/ml) in the presence of 0.02 *M* EDTA. The protein was precipitated and washed with 7% TCA. The TCA was removed by washing with ethanol-ether followed by two ether washes. The dried protein was resuspended in 0.05 *M* NH_4HCO_3, pH 7.9, and digested with pronase (0.5 mg/ml) for 15 hr at 37°C. After digestion, the pronase and salt were removed by filtration on a G25 Sephadex column. The samples were lyophilized and resuspended in a small volume of water

(20–30 μl). Aliquots of this material were spotted on Whatman no. 3 MM paper for electrophoretic analysis.

(g) *Electrophoresis and chromatography:* High-voltage electrophoresis was done on a cooled plate (10°C) at 28 v/cm for 3 hr. The electrophoresis buffer contained per liter: 25 ml of glacial acetic acid and 25 ml of pyridine (pH 4.7). Ascending chromatography was done with a pyridine, isobutanol, and H_2O (35:35:30) solvent at 20°C for 24 hr.

(h) *Chemicals:* d,l-Tetrahydrofolic acid (sealed under nitrogen) and *E. coli* sRNA were obtained from General Biochemicals; N-formylmethionine, N-formylalanine, Cyclo Chemical Corp.; methionylalanine, Mann Research; pronase, Calbiochem; H^3-formate, (2 c/mmole), Tracer Lab; C^{14}-alanine (123 mc/mmole), New England Nuclear; C^{14}-methionine (200 mc/mmole), Schwarz BioResearch.

Results.—Identification of an active formyl donor: Marcker and Sanger[2] first observed that methionyl-sRNA could be partially formylated by *E. coli* extracts. Formate itself was not the formyl donor since addition of labeled formate did not result in incorporation of radioactivity into aminoacyl-sRNA. This is not surprising, since most biological transformylations use N^{10}-formyl-tetrahydrofolic acid (N^{10}-formyl-THFA) as the immediate donor.[5] To test whether this compound might be responsible for the formylation of methionyl-sRNA, H^3-labeled N^{10}-formyl-THFA was prepared and incubated with an *E. coli* supernatant fraction supplemented with uncharged sRNA and the 20 amino acids (see *Materials and Methods*). Table 1 shows excellent transfer of the labeled formyl groups to material identified as aminoacyl-sRNA by its sensitivity to pancreatic RNase and by its sedimentation constant (i.e., 4*S*) on a sucrose gradient. The transformylation proceeded in a linear fashion until the reaction was about three quarters complete. The final level varied somewhat with different batches of sRNA but typically was about 0.6 mμmole of formate per mg of sRNA. The same sRNA preparations could accept about 1.0 mμmole of methionine per mg of sRNA.

If the purified donor is subjected to air oxidation for 1 hr, 85 per cent of its donor capacity is lost. Thus the donor cannot be N^5-formyl-THFA formed in small

TABLE 1

CONDITIONS FOR THE TRANSFER OF FORMATE FROM FORMYL-THFA TO AMINOACYL-sRNA

Conditions	Cpm incorporated
Complete system (see *Materials and Methods*)	1202
	1213
No supernatant	31
	38
Boiled supernatant	55
	41
Incubation at 0° for 5 min	448
	468
+ RNase (10 μg/ml)	55
	47
Incubation of the complete system, then hydrolysis at pH 9.5 for 30 min at 37°	27

The transformylation reaction was done as described in *Materials and Methods*, except that in this case the supernatant protein was 3 mg/ml and each sample was extracted with phenol prior to precipitation with cold TCA. The H^3-formyl-THFA used had a specific activity of 31 μc/μmole.

TABLE 2

DEMONSTRATION THAT FORMATE IS TRANSFERRED ONLY TO METHIONYL-sRNA

Addition of amino acids	Cpm incorporated	% of incorporation with all amino acids added
No added amino acids	1013	14
	909	12
Each amino acid separately except methionine	754 to 1110	11 to 15
All amino acids except methionine	950	13
	932	13
Methionine only	7300	99
	8200	111
All amino acids	8100	110
	6640	90

The transformylation reaction was done as described in *Materials and Methods*, except that amino acids were added only as indicated in the table. The amount of sRNA was 0.25 mg per tube, and the specific activity of the formate 500 μc/μmole.

amounts on neutralization of methenyl-THFA[5] (see *Materials and Methods*) because the N^5-formyl derivatives are stable to air oxidation. Therefore, the donor of our system is most likely N^{10}-formyl-THFA or possibly methenyl-THFA. Conceivably the natural donor may be one of the poly-γ-glutamyl derivatives of N^{10}-formyl-THFA.[5] That N^{10}-formyl-THFA functions as a donor has been found independently by Marcker.[7]

Evidence that the H^3-formyl groups are attached to methionyl-sRNA: Transfer of H^3-formyl groups from formyl-THFA to aminoacyl-sRNA is strongly dependent upon the presence of methionine in the transformylation reaction mixture. Table 2 shows that the final level of incorporation in the absence of any amino acid supplementation or in the presence of all the amino acids except for methionine is only 13 per cent of that found when methionine is present. Particularly important is the lack of stimulation by alanine. The much lower level of incorporation in the absence of added methionine most likely reflects traces of methionine not removed by dialysis.

Additional evidence that the product of the transformylation reaction is N-formylmethionyl-sRNA comes from treatment of H^3-labeled aminoacyl-sRNA with mild alkali. This releases N-formyl methionine as shown by coelectrophoresis of this material with a formyl S^{35}-methionine standard, prepared by formylation of the amino acid.[8] As a control the same H^3-labeled material was also run with formyl-C^{14}-alanine. The formyl-alanine ran sufficiently ahead of the H^3 peak to allow us to conclude that at most 2 per cent of the H^3-formyl groups could be in formylalanine.

Stability of H^3-formyl-C^{14}-methionyl-sRNA: Further evidence that the reaction product is N-formylmethionyl-sRNA comes from experiments using sRNA which has been incubated with C^{14}-methionine as well as the H^3-formyl donor. Figure 1 indicates the rate of release of the two labels in 0.1 M tris, pH 8.6, from sRNA. The methionine is clearly present in two forms, which we interpret as methionyl-sRNA and formylmethionyl-sRNA. Since there is considerable variation in the rate constants for the hydrolysis of the various aminoacyl-sRNA's,[9] the fact that the H^3-formate-labeled sRNA decays to 5 per cent of its original value with a single rate constant favors the belief that only a single amino acid is formylated. The half life for the alkaline hydrolysis of formylmethionyl-sRNA under these conditions is 9.5 min, and of methionyl-sRNA about 2.1 min. An increase in stability at high pH on blocking the amino group of aminoacyl-sRNA has also been found with polyphenylalanyl-sRNA.[10] The decay rate constant for formyl-

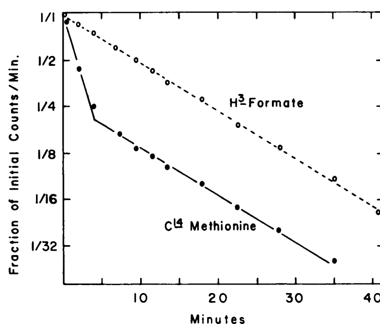

FIG. 1.—Alkaline hydrolysis of H^3-formyl-C^{14}-methionyl-sRNA. Double-labeled sRNA containing 60,000 cpm of H^3-formate and 40,000 cpm of C^{14}-methionine was adjusted to 0.1 M tris pH 8.6 and incubated at 37°C. At various times, aliquots were removed precipitated with 5% cold TCA onto Millipore filters which were counted on a Packard liquid scintillation counter.

methionyl-sRNA supports the conclusion that the formylmethionine is bound to sRNA by the normal 2′ or 3′ ester bond. If it were bound to the N^6 of adenine, as suggested by Hall in analogy with compounds of this type found in yeast,[11] it would be much more stable. For example, the N^6-amide bond is reported to be stable for 24 hr at pH 8.5 and 37°C.[12]

Incorporation of H^3-formyl groups into protein: Incubation of a preincubated S-30 extract with R17 RNA[13] and H^3-formylmethionyl-sRNA results in the incorporation of H^3-formyl groups into an alkaline-resistant, TCA-precipitable product. Alkali was used to destroy the aminoacyl-sRNA rather than hot acid because of the lability of the formyl bond in hot acid. The kinetics of this incorporation in the presence and absence of R17 RNA is shown in Figure 2. One obtains very similar kinetics of R17 RNA-directed incorporation of H^3-formyl groups if one adds to the incubation mixture the formyl donor, labeled formyl-THFA, in place of the H^3-formylmethionyl-sRNA.

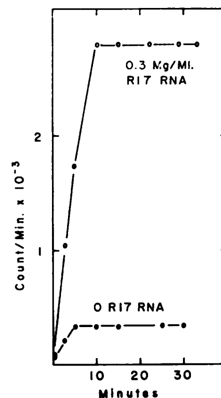

FIG. 2.—Kinetics of incorporation of H^3-formylmethionine in the presence and absence of R17 RNA. The 0.5-ml reaction mixtures contained 125 µl of preincubated S-30 and 62,000 cpm of H^3-formylmethionyl-sRNA (170,000 cpm/mg sRNA). At the designated times 50-µl aliquots were removed, incubated at pH 12.0 for 15 min at 0°C, and precipitated with cold 7% TCA onto Millipore filters.

The magnesium ion dependence of the H^3-formyl group incorporation was measured and observed to be a typical magnesium profile for R17 RNA-directed protein synthesis with a sharp maximum at 11 mM Mg. This indicated that the formyl groups were being incorporated into newly synthesized polypeptides. In order to determine which phage-specific proteins contained the labeled formyl groups, the reaction mixture was analyzed on a sucrose gradient.

Sedimentation properties of the in vitro product: Sucrose gradient analysis of the newly synthesized polypeptides in the reaction mixture, after incubation with R17 RNA and C^{14}-amino acids, reveals radioactivity peaks with sedimentation constants of 30*S* and 20*S*, in addition to the nascent chains bound to ribosomes and the much slower-sedimenting material (2–4*S*) at the top of the gradient. In an earlier paper it was shown that the protein which sediments in the 30*S* region consists of complete R17 coat protein molecules bound to R17 RNA and that the second R17 RNA-coded protein, sedimenting in the 20*S* region, was not coat protein.[13]

Figure 3 shows a sucrose gradient analysis of the alkaline-resistant, TCA-precipitable formyl counts after 15 min of incubation with R17 RNA and H^3-formylmethionyl-sRNA. The bottom curve designated with x's shows the incorporation in the absence of R17 RNA. This experiment demonstrates that a large proportion of the formyl counts are incorporated into the *in vitro* synthesized coat protein (i.e., the 30*S* protein). Identical sucrose gradient profiles are obtained, whether the source of formyl groups is N-formylmethionyl-sRNA or N-formyl-THFA.

Two sources produce the shoulder in the 20*S* region: (1) incorporation of formyl groups into the 20*S* protein, and (2) partial degradation by endogenous RNase of the R17 RNA to which coat protein molecules are bound. This was shown by giving the reaction mixture a mild RNase treatment (1 µg/ml, 10 min at 0°C) prior to layering it onto a sucrose gradient. The material in the 30*S* peak was shifted to

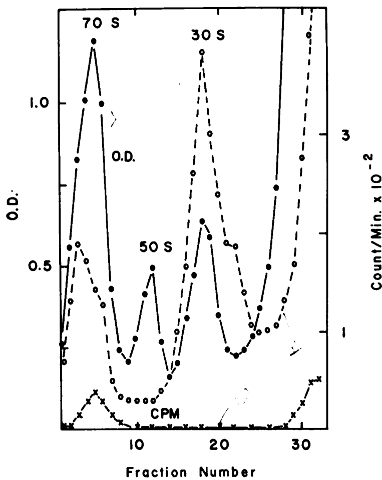

FIG. 3.—Sucrose gradient analysis of the total amino acid-incorporating system after 15 min incubation with H^3-formylmethionyl-sRNA. The 300-μl reaction mixture contained 75 μl of preincubated *E. coli* S-30, 60,000 cpm of H^3-formylmethionyl-sRNA, and either 0.3 mg/ml (O. .O) or 0.0 mg/ml (x—x) R17 RNA. A 150-μl portion was layered onto a 5-ml (5–20% sucrose) gradient and centrifuged for 2.5 hr at 38,000 rev/min at 4°C. Each 0.16-ml fraction was first assayed for optical density at 260 mμ and then incubated at pH 12.0 for 15 min at 0°C to prepare samples for radioactive analysis. The optical density profile for the reaction mixture, which did not contain R17 RNA is not shown on the above figure. The 70*S* ribosomal O.D. peak, and the 50*S* and 30*S* subunit peaks were at the same position in the two gradients.

the top of the gradient revealing a small peak of radioactivity in the 20*S* region. The relatively small peak in the 20*S* region may reflect both the larger monomeric molecular weight of the 20*S* protein and the higher frequency of reading of the coat protein cistron.

The N-formyl bond is more sensitive than the peptide bond to mild acid hydrolysis. When double-labeled protein, containing the H^3-formyl group and C^{14}-amino acids, was heated at 100°C for 10 min in 1 *N* HCl, the H^3-formyl counts were reduced by 95% while the C^{14} counts decreased less than 5 per cent. We conclude that the formyl group is incorporated into the phage protein as a N-formyl amino acid directly from the aminoacyl-sRNA.

Isolation of the N-formyl amino acid from in vitro synthesized coat protein: These results presented a paradox because the amino terminal amino acid of R17 coat protein is alanine.[3] A rather surprising solution was found by examining the nature of the *in vitro* coat protein. Double-labeled *in vitro* product was made by incubating the R17 RNA-dependent amino acid incorporating system with either C^{14}-alanine and H^3-formyl-THFA or C^{14}-methionine and H^3-formyl-THFA. The *in vitro* synthesized coat protein was separated from other newly synthesized polypeptides by sucrose gradient centrifugation. The coat protein was then digested with pronase and the hydrolysate analyzed by electrophoresis. The results with the C^{14}-alanine-H^3-formyl and the C^{14}-methionine-H^3-formyl-labeled products are shown in Figures 4 and 5, respectively. We observe that there are two peaks labeled with H^3-formyl groups. Peak 1 contains C^{14}-methionine as well as H^3-formyl groups, whereas peak 2 contains H^3-formyl counts, C^{14}-methionine, and C^{14}-alanine. The simplest interpretation of these results is that peak 1 is N-formyl methionine, and peak 2 is the dipeptide N-formylmethionylalanine. This interpretation is consistent with the markers *a*, *b*, and *c*, indicated in Figures 4 and 5. These standards are (*a*) N-formylalanine, (*b*) N-formylmethionine, and (*c*) N-formylmethionylalanine (prepared by formylation of the methionylalanine dipeptide[8]). Further evidence that peak 1 is N-formylmethionine is obtained by eluting peak 1 from the C^{14}-alanine-H^3-formyl-labeled product (which contains only tritium counts) and adding to the eluted material a S^{35}-formyl methionine standard. On electro-

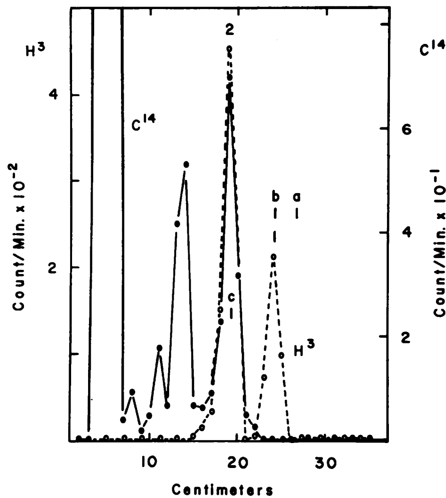

FIG. 4.—Electrophoretic analysis of the pronase digest of *in vitro* synthesized R17 coat protein labeled with H^3-formyl groups and C^{14}-alanine. The 1.0-ml reaction mixture contained 6 $\times$ 10^6 cpm of H^3-formyl-THFA (82 cpm/$\mu\mu$mole) and 8.8 $\times$ 10^5 cpm of C^{14}-alanine (18 cpm/$\mu\mu$mole). After 15 min incubation at 37°C with R17 RNA, the reaction mixture was put on a sucrose gradient to isolate the *in vitro* synthesized coat protein. The conditions for pronase digestion and electrophoretic separation of the protein hydrolyzate are described in *Materials and Methods*. The origin was at 6 cm. The letters *a, b,* and *c* designate the positions of the markers N-formylalanine, N-formylmethionine, and N-formylmethionylalanine, respectively.

phoresis and chromatography the S^{35} and H^3 counts superimposed exactly. During this process it was observed that a large proportion of the methionine auto-oxidized to methionine sulfoxide. To facilitate identification of the different peaks, both the samples and markers were oxidized with performic acid.[14] Similarly, it was found that the oxidized peak 2's of both the C^{14}-alanine-H^3-formyl and the C^{14}-methionine-H^3-formyl-labeled products ran in parallel on chromatography. Both peaks still contained C^{14} and H^3 counts in the expected ratios and ran with the oxidized N-formylmethionylalanine marker.

Discussion.—We have shown that in an *in vitro* amino acid incorporating system programed with R17 RNA, N-formylmethionine is incorporated into R17 coat protein as well as into the phage-specific 20S protein. We have also observed that the pronase digest of the *in vitro* synthesized coat protein contains the dipeptide N-formylmethionylalanine. From these observations we propose that the *in vitro* sequence of the phage coat protein is:

N-formyl met ala ser aspNH_2 phe thr...

in contrast to the expected *in vivo* amino terminal sequence:

ala ser aspNH_2 phe thr...

The latter sequence was determined by Konigsberg[15] for the very closely related bacteriophage f2. Comparison of the amino acid sequences of R17

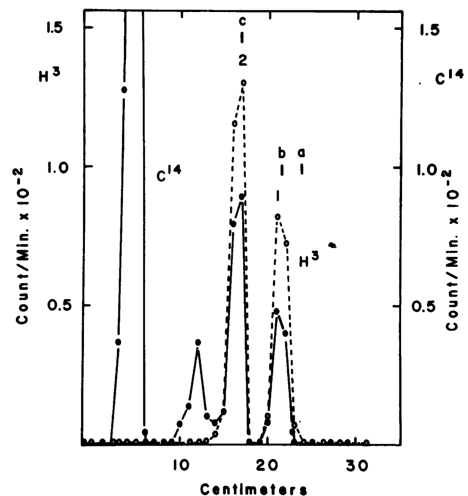

FIG. 5.—Electrophoretic analysis of the pronase digest of *in vitro* synthesized R17 coat protein labeled with H^3-formyl groups and C^{14}-methionine. The reaction mixture contained 6 $\times$ 10^6 cpm of H^3-formyl-THFA (82 cpm/$\mu\mu$mole) and 1.9 $\times$ 10^6 cpm at C^{14}-methionine-(50 cpm/$\mu\mu$mole). The experimental conditions are described in Fig. 4.

and f2 coat proteins to date indicates greater than 90 per cent homology.[16]

We are currently attempting to isolate the amino-terminal chymotryptic peptide of the coat protein synthesized *in vitro*. In an accompanying paper, Zinder and his coworkers describe the isolation of the incomplete polypeptide chain synthesized in a cell-free system directed by RNA with an amber mutation in the coat protein cistron. Surprisingly, this small amino terminal peptide (6 amino acids) was found to contain one mole of formylmethionine per mole of phenylalanine.[17] From the consistency of these two unexpected observations we would like to propose the following model for polypeptide initiation:

(1) There exists an initiation signal for protein synthesis. This signal may be a codon for N-formylmethionyl-sRNA (for instance, AUG or UUG[18]) or be a longer sequence of six or nine bases containing an alanine or even a serine codon. Pertinent to this is the recent demonstration by Marcker that only one of the two methionyl-sRNA's can be formylated.[7]

(2) *In vivo*, after completion of the polypeptide chain an enzyme removes the N-formylmethionine from the nascent R17 or f2 coat proteins.

For *E. coli* proteins, the same enzyme may cleave the formyl group or after methionine, etc.,

which would account for the unexpectedly high values for methionine, alanine, and even serine as the amino terminal residues of *E. coli* proteins (about 45, 30, and 15%, respectively).[1] The position of enzymatic cleavage could be controlled by the proteins' three-dimensional conformation. Experiments are now in progress to test for this hypothetical peptidase activity.

Several reasons for employing N-formylated aminoacyl-sRNA as a polypeptide chain initiator can be envisioned. The N-formylaminoacyl-sRNA may inherently define a direction for polypeptide growth on the ribosomal surface. This could be accomplished, for example, if the N-formyl bond mimicked the peptide bond and thus aided in the selection of one of the two proposed sRNA binding sites on the ribosome.[19] Also, blocking the amino group of the first amino acid may facilitate the formation of the initial peptide bond by eliminating the positive charge on the amino group.

The authors are grateful to Prof. J. D. Watson for his guidance and inspiration while pursuing this study and for his valuable criticism during the preparation of this manuscript. We are also happy to acknowledge the expert technical assistance of Mrs. Nancy Capecchi. This work was supported by grants from the National Institutes of Health RG-9541.

* Predoctoral trainee of the National Institutes of Health.

† Predoctoral fellow of the National Science Foundation.

[1] Waller, J. P., *J. Mol. Biol.*, **7**, 483 (1963); Waller, J. P., and J. I. Harris, these PROCEEDINGS, **47**, 18 (1961).

[2] Marcker, K., and F. Sanger, *J. Mol. Biol.*, **8**, 835 (1964).

[3] Guidotti, G., unpublished results (1965). Dr. Guidotti, employing the cyanate procedure of Stark and Smyth for determining the amino terminal residue, found 0.89 mole of alanine, 0.10 mole of glycine, and less than 0.01 mole of the other amino acids per mole of coat protein. Alanine also has been shown to be the amino terminal residue for the coat proteins of the very closely re-

lated bacteriophages f2 and MS2. Personal communications from W. Konigsberg and H. Fraenkel-Conrat.
[4] Goldthwait, D. A., and G. R. Greenberg, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1955), vol. 2, p. 516.
[5] Rabinowitz, J. C., in *The Enzymes*, ed. P. D. Boyer, H. Lardy, and K. Myrbäck (New York: Academic Press, 1960), vol. 2, p. 185.
[6] Huennekens, F. M., P. P. K. Ho, and K. G. Scrimgeour, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), vol. 6, p. 809.
[7] Marcker, K., *J. Mol. Biol.*, in press.
[8] Sheehan, J. C., and D. H. Yang, *J. Am. Chem. Soc.*, **80**, 1154 (1958).
[9] Sarin, P. S., and P. C. Zamecnik, *Biochim. Biophys. Acta*, **91**, 653 (1964).
[10] Gilbert, W., *J. Mol. Biol.*, **6**, 389 (1963).
[11] Hall, R. H., and G. B. Chheda, *J. Biol. Chem.*, **240**, PC 2754 (1965).
[12] Hall, R. H., *Biochemistry*, **3**, 769 (1964).
[13] Capecchi, M. R., and G. Gussin, *Science*, **149**, 417 (1965).
[14] Nathans, D., *J. Mol. Biol.*, **13**, 521 (1965).
[15] Konigsberg, W., personal communications (1965).
[16] Weber, K., and W. Konigsberg, personal communications (1965).
[17] Webster, W., D. Engelhardt, and N. Zinder, these PROCEEDINGS, **55**, 155 (1966).
[18] Clark, B. F. C., and K. A. Marcker, *Nature*, **207**, 1038 (1965).
[19] Watson, J. D., *Bull. Soc. Chim. Biol.*, **46**, 1399 (1965).

---

*IN VITRO PROTEIN SYNTHESIS: CHAIN INITIATION**

BY ROBERT E. WEBSTER,† DEAN L. ENGELHARDT, AND NORTON D. ZINDER

THE ROCKEFELLER UNIVERSITY

Communicated by E. L. Tatum, November 22, 1965

Nonsense mutations, when not suppressed, result in the premature termination of growing protein chains.[1] In a previous publication[2] we described the appearance of a suppressible protein fragment when RNA from a phage mutant was added to protein-synthesizing extracts of *Escherichia coli.* The fragment was thought to be the amino terminal portion of the phage coat protein.

In this communication, we shall present evidence that this fragment is indeed derived from the amino terminal portion of the coat protein. In addition, we shall show that, although protein isolated from phage particles has alanine with a free amino group at its amino terminus,[3] both the fragment and the whole coat protein molecules when synthesized *in vitro* have masked amino groups. The masking group is N-formylmethionine. A similar finding is reported by Adams and Capecchi.[4]

Materials and Methods.—Bacteria and phage: S26, a nonsuppressing (Su −) strain of *E. coli* K12;[5] f2, wild-type phage; sus4A, a mutant of f2 with a nonsense mutation in the gene specifying the coat protein.[6]

In vitro incorporation: The S-30 system described by Schwartz[7] was employed, but 0.03 M NH_4^+ was used in place of K^+. When alanine was to be incorporated, 10 mM creatine phosphate and 30 μg/ml of creatine phosphate kinase was added instead of phosphoenolpyruvate and pyruvate kinase. Uniformly C^{14}- and H^3-labeled L-amino acids were obtained from New England Nuclear. H^3 L-phenylalanine was also obtained from Schwarz BioResearch. The specific activities used in all calculations were those specified by the suppliers. The concentration of radioactive label in the reaction mixture was approximately 10^{-5} M. The amount of phage RNA