INITIATION OF PROTEIN SYNTHESIS, I. EFFECT OF FORMYLATION OF METHIONYL-TRNA ON CODON RECOGNITION

By Philip Leder and Hela Bursztyn

BIOCHEMISTRY SECTION, WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL

Communicated by Ephraim Katchalski, September 20, 1966

Since the discovery of N-formylmethionyl-tRNA by Marcker and Sanger,[1] a number of investigations[2-8] have suggested that this compound is involved in the initiation of protein synthesis. These studies, largely in bacterial systems, indicate that only one of two Met-tRNA species may be formylated[4, 9-11] and incorporated in the N-terminal position of several *in vitro* synthesized proteins[2-4, 8] and polypeptides.[4] A preferential phase of translation also appears to be promoted by the methionine codon in which triplet sequences overlapping ApUpG are not translated.[7]

We have examined the function of formyl-accepting Met-tRNA, both formylated and nonformylated, in the initial binding step of protein synthesis. In this communication we wish to present evidence that sufficient information is contained in formyl-accepting Met-tRNA, prior to its formylation, to permit efficient ribosomal binding and phase selection as measured in the presence of the methionine codon ApUpG and that formylation affects these functions adversely or not at all. In addition, evidence is presented that formylation of Met-tRNA may take place *subsequent* to codon recognition on the ribosome and that formylation results in the alteration of certain physical properties of Met-tRNA. An investigation of the role of formylation of Met-tRNA in a subsequent step in protein synthesis, formation of the initial peptide bond, is described in a forthcoming paper.[12]

Materials and Methods.—Preparation of supernatant fraction and ribosomes: Supernatant and ribosomes were prepared from frozen *E. coli* MRE-600 cells as previously described by Nirenberg.[13] In addition, ribosomes were suspended in a wash buffer (0.01 *M* Tris-Cl, pH 7.2, 0.05 *M* KCl, 0.014 *M* $MgCl_2$) containing 0.5 *M* NH_4Cl at 0° for 2 hr and centrifuged at 100,000 × *g* for 2 hr. They were then resuspended in the wash buffer, minus NH_4Cl, recentrifuged, resuspended in the same solution, and stored under liquid air. A precipitate was obtained by addition of NH_4SO_4 to 80% saturation. It was redissolved in wash buffer and stored frozen at −18°C.

Preparation and analysis of aminoacyl-tRNA and formylmethionyl-tRNA: E. coli B tRNA was obtained from General Biochemicals, Inc. Acylation was carried out at 37° in 5-ml reaction mixtures containing 0.01 *M* potassium cacodylate, pH 7.5; 0.006 *M* KCl; 0.01 *M* magnesium acetate; 0.005 *M* ATP (potassium salt); 2×10^{-4} *M* reduced glutathione; 5–100 × 10^{-6} *M* C^{14}-amino acid (New England Nuclear Corp.); 4×10^{-5} *M* each of 19 other C^{12}-amino acids (or all 20 C^{12}-amino acids for unlabeled material); 25 mg tRNA and 1 mg supernatant containing aminoacyl-tRNA synthetases and methionyl-tRNA transformylase. Reaction mixtures in which the synthesis of N-formyl-Met-tRNA was desired contained 1 mg of a mixture of N^5- and N^{10}-formyl-folate-H_4 (referred to in text as N^{10}-formyl-folate-H_4) prepared according to Huennekens *et al.*[14] Analysis of N-formyl-C^{14}-methionyl-tRNA was carried out by hydrolysis at 37° in 0.1 *N* NH_4OH for 60 min, lyophilization, application of hydrolysate to Whatman 3 MM paper, and electrophoresis in 0.05 *M* sodium acetate buffer, pH 3.5, at 60 v/cm for 1 hr.[4] One-cm strips were cut out for determination of radioactivity in a liquid scintillation counter. Peaks of radioactivity were compared to authentic samples of methionine and N-formyl-methionine, the latter prepared according to Sheehan and Yang.[15] Another useful method for determination of N-formyl-C^{14}-Met-tRNA synthesis involved hydrolysis as above, rapid acidification to pH 1.0 with a small aliquot of 10 *N* HCl, rapid extraction with 1.5 vol ethyl acetate, and determination of radioactivity of a 1.0-ml aliquot of the ethyl acetate extract added to 10 ml Bray's solution[16] in a

liquid scintillation counter. Methionine is essentially insoluble in ethyl acetate at pH 1.0 in contrast to N-formylmethionine. N-formyl-C^{14}-Met-tRNA contained 73% N-formylmethionine and 27% free methionine. At least 73% of the C^{14}-methionine-labeled tRNA was therefore the formyl-accepting species. Conditions under which C^{14}-Met-tRNA was synthesized permitted less than 5% Met-tRNA formylation. N-formyl-C^{14}-Met- and C^{14}-Met-tRNA contained 62 and 50 $\mu\mu$moles C^{14}-methionine per mμmole tRNA, respectively. C^{14}-Val- and C^{14}-Phe-tRNA contained 47 $\mu\mu$moles C^{14}-valine and 5.4 $\mu\mu$moles C^{14}-phenylalanine per mμmole tRNA, respectively.

Preparation and analysis of oligonucleotides: Synthesis, purification, and characterization of all oligonucleotides were carried out, with minor modifications,[17] by use of the methods described by Leder, Singer, and Brimacombe.[18] Oligonucleotides of the ApUpGpU... series were first digested with T1 ribonuclease[19] for characterization, and fragments separated and analyzed as above. The purity of each compound was >98%.

C^{14}-AA-tRNA binding assay: The assay has been described previously.[20] Each 50-μl reaction mixture contained 0.1 *M* Tris-acetate, 0.05 *M* KCl, 0.01 *M* magnesium acetate (or as otherwise indicated) and ribosomes, C^{14}-AA-tRNA, oligo- or polynucleotide as indicated. Reaction mixtures were incubated for 20 min at 23°. Radioactivity of ribosomal-bound C^{14}-AA-tRNA on nitrocellulose filters was determined in a liquid scintillation counter at an efficiency of 73%. All determinations were performed in duplicate.

Methylated albumin silicic acid (MAS) column chromatography: MAS was prepared as described by Okamoto and Kawade.[21] Chromatography was carried out according to Stern and Littauer.[22] Acylated tRNA (3 mg) was applied to the column at 15° in 0.05 *M* sodium acetate pH 5.5, 0.8 *M* NaCl solution. Elution was with 400 ml linear gradient between 0.8 and 1.15 *M* NaCl in 0.05 *M* sodium acetate, pH 5.5. Three-ml fractions were collected. The A^{260} of each was determined directly in the Zeiss spectrophotometer. Radioactivity of the C^{14}-AA-tRNA was determined by TCA precipitation of 0.1-ml aliquots of fractions.

Results.—Relative affinity of formylated and nonformylated Met-tRNA for ribosomal binding sites(s): As may be noted in the initial portion of the curve in Figure 1, at excess ribosome concentration, approximately equivalent amounts of C^{14}-Met-tRNA and N-formyl-C^{14}-Met-tRNA are bound to ribosomes. However, when ribosome concentration becomes limiting, as in the later portion of the same curve, a quantitative difference in the ApUpG-induced binding of C^{14}-Met-tRNA and N-formyl-C^{14}-Met-tRNA is evident. In this experiment, approximately twice as much nonformylated material is bound, both preparations having been acylated to approximately the same extent.

The data in Table 1 show the diluting effect of the addition of equivalent amounts of nonformylated C^{12}-Met-tRNA and tRNA on the ApUpG-induced binding of C^{14}-Met- and N-formyl-C^{14}-Met-tRNA to ribosomes. ApUpG is present in excess.

TABLE 1

EFFECT OF tRNA AND AMINOACYL-tRNA ON THE SPECIFIC BINDING OF N-FORMYL-C^{14}-MET-tRNA AND C^{14}-MET-tRNA TO RIBOSOMES

	Oligonucleotide			
	None	ApUpG	None	ApUpG
Additions	C^{14}-Met-tRNA bound ($\mu\mu$moles)		N-formyl-C^{14}-Met-tRNA bound ($\mu\mu$moles)	
None	0.18	3.88	0.02	0.78
C^{12}-Met-rRNA (one equivalent)	0.13	2.49	0.04	0.37
tRNA (one equivalent)	0.26	2.10	0.01	0.39

Reaction mixtures contained components described under *Materials and Methods;* 0.15 A^{260} units of ApUpG, where indicated, and 1.6 A^{260} *E. coli* MRE-600 ribosomes were used in reaction mixtures. 0.30 A^{260} units C^{14}-Met-tRNA and 0.13 A^{260} units N-formyl-C^{14}-Met-tRNA were added where indicated. Also where indicated, an equivalent amount in A^{260} units of unlabeled Met-tRNA and tRNA were added to reaction mixtures simultaneously with the labeled material.

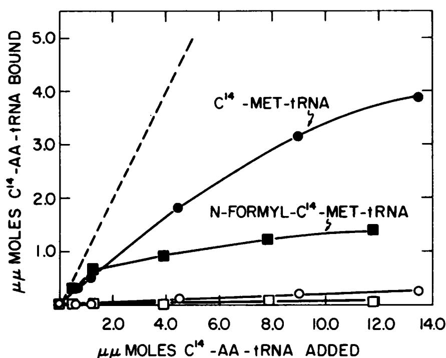

FIG. 1.—Specific binding of C^{14}-Met-tRNA and N-formyl-C^{14}-Met-tRNA to ribosomes as a function of AA-tRNA concentration. Reaction mixtures contained components described under *Materials and Methods.* Each reaction mixture also contained 2.0 A^{260} units *E. coli* MRE-600 ribosomes and 0.15 A^{260} units ApUpG. Symbols represent ribosomal binding of the following: (●——●), C^{14}-Met-tRNA plus ApUpG; (■——■), N-formyl-C^{14}-Met-tRNA plus ApUpG; (○——○), C^{14}-Met-tRNA minus oligonucleotides; (□——□), N-formyl-C^{14}-Met-tRNA minus oligonucleotides; (- - -), theoretical binding of 100% of added C^{14}-AA-tRNA.

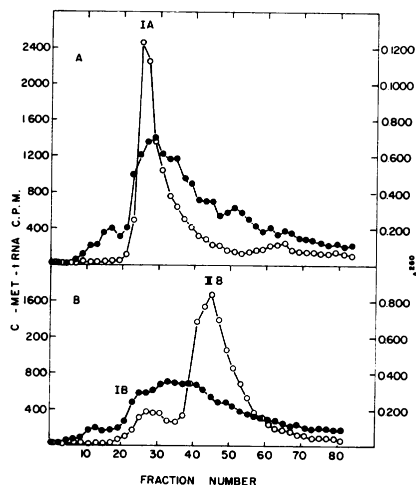

FIG. 2.—MAS column chromatography of C^{14}-Met- and N-formyl-C^{14}-Met-tRNA. Preparation of C^{14}-Met-tRNA and N-formyl-C^{14}-Met-tRNA and operation of the MAS column are described under *Materials. and Methods* (*A*) Elution pattern of C^{14}-Met-tRNA; (*B*) N-formyl-C^{14}-Met-tRNA. Symbols represent the following: (●——●), absorbency of solution of 260 mμ; (○——○), TCA-precipitable cpm.

Again, more extensive binding is associated with the absence of the formyl group. The binding of N-formyl- and C^{14}-Met-tRNA is reduced approximately 40–50 per cent by both C^{12}-Met-tRNA and tRNA, as would be expected on the basis of dilution alone. This result suggests that the site(s) to which formylated compound is bound under these conditions may be occupied by both nonformylated and non-acylated tRNA molecules.

Effect of formylation of Met-tRNA on preferential recognition of the ApUpG codon at low Mg^{++} concentration: Sundararajan and Thach[7] noted a valine codon, GpUpU, overlapping the methionine codon, ApUpG, in an oligonucleotide of the sequence $ApUpGpUpUpU(pU)_n$ fails to induce the binding of C^{14}-Val-tRNA to ribosomes. The adjacent UpUpUp...U sequence is recognized, but the effect, termed "phasing," depends specifically upon the presence of the ApUpG sequence and requires that the binding reaction be carried at relatively low Mg^{++} concentrations. A similar experiment, shown in Table 2, testing the recognition of codons adjacent to and overlapping the sequence ApUpG was carried out using small oligonucleotides of the series ApUpGpU..., through the hexanucleotide containing two nonoverlapping codons, *ApUpGpUpUpU.* The use of such short oligonucleotides overcomes differences in (Mg^{++}) optima observed between oligo- and polynucleotide-induced ribosomal binding, poly U being more effective at lower (Mg^{++}) concentration than UpUpU.[20] The optimal Mg^{++} concentration required for the binding of formyl-accepting Met-tRNA to ribosomes appears to be lower than that of other AA-tRNA's, approximately 0.01 *M*.[4,7]

Binding of C^{14}-Met- and N-formyl-C^{14}-Met-tRNA is three- to fivefold more ex-

TABLE 2

EFFECT OF (Mg^{++}) ON CODON SELECTION IN OLIGONUCLEOTIDES CONTAINING THE ApUpG SEQUENCE

	C^{14}-Met-tRNA		C^{14}-N-formyl-Met-tRNA		C^{14}-Val-tRNA		C^{14}-Val-tRNA*		C^{14}-Phe-tRNA	
	(Mg^{++}) Molarity									
	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02
Addition	C^{14}-AA-tRNA or N-Formyl-C^{14}-Met-tRNA Bound to Ribosomes ($\mu\mu$moles)									
None	0.34	0.39	0.07	0.26	0.30	0.26	0.18	0.22	0.10	0.18
ApUpG	7.00	2.80	3.15	0.68	0.23	0.22	0.16	0.19	0.10	0.13
ApUpGpU	4.90	1.37	2.44	0.50	0.25	0.26	0.18	0.19	0.10	0.12
ApUpGpUpU	4.34	1.15	1.91	0.39	0.27	0.43	0.19	0.45	0.09	0.17
ApUpGpUpUpU	4.50	1.10	2.07	0.37	0.38	0.38	0.28	0.39	0.10	0.37
GpUpU	—	—	—	—	1.22	1.52	0.77	1.22	—	—
UpUpU	—	—	—	—	—	—	—	—	0.10	0.50
Poly U	—	—	—	—	—	—	—	—	0.82	1.13

Reaction mixtures contained components described under *Materials and Methods;* Mg^{++} concentration is indicated in the table; 0.25 A^{260} units of each oligonucleotide and polymer, 2.0 A^{260} units of *E. coli* MRE-600 ribosomes, and 0.20 A^{260} units C^{14}-AA-tRNA were added to reaction mixtures.
* Contains C^{12}-N-formyl-Met-tRNA.

tensive at 0.01 *M* than at 0.02 *M* Mg^{++} throughout the series of oligonucleotides tested. Again, the presence of the N-formyl group on Met-tRNA reduces the extent of its binding by 0.5- to 0.3-fold at both Mg^{++} concentrations and with each oligonucleotide. ApUpG is more effective in inducing binding on Met- and N-formyl-Met-tRNA than the longer oligonucleotides in the series. When the penta- and hexanucleotides containing a GpUpU sequence overlapping ApUpG (*ApUpGpUpU*) are tested at 0.01 *M* Mg^{++} for their ability to induce the binding of C^{14}-Val-tRNA to ribosomes, little or no binding is observed as compared to the triplet GpUpU used alone, though a minimal induction is seen with ApUpGpUpUpU. At 0.02 *M* Mg^{++} C^{14}-Val-tRNA binding induced by the overlapping GpUpU sequence increased, but less than one third observed in the presence of the triplet. The effect is uninfluenced by formylation of Met-tRNA. The hexanucleotide, ApUpGpUpUpU, containing the nonoverlapping UpUpU sequence fails, as does the triplet, UpUpU, to induce the binding of C^{14}-Phe-tRNA to ribosomes at 0.01 *M* Mg^{++}. Poly U, in contrast, is effective. At 0.02 *M* Mg^{++} the nonoverlapping UpUpU sequence in the hexanucleotide, UpUpU, as well as poly U are effective.

Effect of formylation of Met-tRNA on its mobility in MAS column chromatography: Since formylation of Met-tRNA reduces its efficiency in codon recognition, a difference in physical properties, likely reflecting an alteration of secondary structure, was sought. A MAS column elution pattern of nonformylated C^{14}-Met-tRNA is shown in Figure 2*A*. A single readioactive peak is observed which, as may be seen from analytic data shown in Table 3, consists almost entirely of C^{14}-Met-tRNA. The elution pattern of C^{14}-Met-tRNA which has been formylated in the presence of N^{10}-formyl-folate-H_4 and transformylase is shown in Figure 2*B*. Two peaks are

TABLE 3

DETERMINATION OF N-FORMYL-C^{14}-MET-tRNA IN AA-tRNA FRACTIONS OBTAINED BY MAS COLUMN CHROMATOGRAPHY

Addition of N^{10}-formyl-folate-H_4 during AA-tRNA syn thesis	Fraction no.	Total analyzed (cpm)	Recovered as N-formyl-C^{14}-Met (cpm)	Per cent recovered as N-formyl-Met-tRNA
None	Peak IA-25	5200	213	4.1
0.5 μM/ml	Peak IB-26	720	49	6.8
	Peak IIB-46	4460	3921	88.0

Method of analysis, involving alkaline hydrolysis and ethyl acetate extraction, is described under *Materials and Methods.*

TABLE 4

FORMYLATION OF FREE AND RIBOSOMAL-BOUND C^{14}-MET-tRNA

Addition	Soluble Transformylase Plus	Minus
	N-Formyl-C^{14}-Methionine ($\mu\mu$moles)	
None	0.20	0.20
N^{10}-formyl-folate-H_4	0.85	0.19
N^{10}-formyl-folate-H_4 ribosomes	0.93	0.74
N^{10}-formyl-folate-H_4 ribosomes, ApUpG	1.03	0.72

Reactions were carried out in two stages. Each reaction mixture was first incubated and contained the components of the binding assay as described under *Materials and Methods*. Reaction mixtures contained 1.88 $\mu\mu$moles C^{14}-Met-tRNA and 3.0 A^{260} units *E. coli* MRE-600 ribosomes and 0.35 A^{260} units ApUpG, where indicated. After the binding incubation, 5 μg N^{14}-formyl-folate-H_4 and 40 μg *E. coli* MRE-600 supernatant containing transformylase were added, where indicated, to reaction mixtures and, as a second stage, incubation was continued for 10 min at 37°. N-formyl-C^{14}-Met-tRNA was determined by alkaline hydrolysis of reaction mixtures, acidification, and ethyl acetate extraction as noted under *Materials and Methods*.

TABLE 5

STABILITY OF C^{14}-MET-tRNA-APUPG-RIBOSOME COMPLEX DURING TRANSFORMYLATION

Additions	C^{12}-Aminoacyl-tRNA Minus	Plus
	C^{14}-Met-tRNA Bound ($\mu\mu$moles)	
None	1.05	0.96
N^{10}-formyl-folate-H_4	0.83	0.77
Soluble transformylase	0.88	0.87
N^{10}-formyl-folate-H_4, soluble transformylase	0.88	0.88

Reactions were carried out and contained components as noted in the legend to Table 4. Prior to the addition of the N^{10}-formyl-folate-H_4 and transformylation enzyme, at the conclusion of the binding incubation, C^{12}-AA-tRNA, equivalent in A^{260} units to the C^{14}-Met-tRNA already present, was added to the indicated reaction mixtures and incubations were continued as noted in the legend to Table 4. The binding assay is described under *Materials and Methods*.

observed. The analytical data in Table 3 indicate that the peak with the new mobility, *IIB*, contains N-formyl-C^{14}-Met-tRNA, while *IB* contains nonformylated C^{14}-Met-tRNA.

Formylation of ribosomal-bound Met-tRNA: Since nonformylated Met-tRNA is more efficiently bound to the ribosome, the possibility of formylation occurring subsequent to the codon recognition reaction was considered. Using conditions under which 89 per cent of the added C^{14}-Met-tRNA is bound to the ApUpG-ribosome complex, the extent of its formylation was measured. Results are shown in Table 4. Ribosomal binding of C^{14}-Met-tRNA was allowed to proceed to completion prior to the addition of formyl donor and soluble transformylase. Formylation carried out after formation of the complex was as extensive as that with free C^{14}-Met-tRNA. There also appears to be sufficient transformylase activity associated with the NH_4Cl-washed ribosomes to effect formylation. Controls indicating that the C^{14}-Met-tRNA-ApUpG-ribosome complex does not dissociate during the formylation reaction are shown in Table 5. After binding of C^{14}-Met-tRNA has occurred, the addition of C^{12}-AA-tRNA and the various formylation components fail to reduce the amount of C^{14}-Met-tRNA bound, indicating that there is no exchange of bound and free Met-tRNA or N-formyl-Met-tRNA during the formylation reaction.

Discussion.—Among factors which might primarily influence the specific interaction between a unique species of Met-tRNA and an initiation codon, two may be considered. Either sufficient information is contained in the primary structure of the initiator Met-tRNA[4] to permit preferential selection of a unique codon among an array, or formylation of this Met-tRNA species confers such specificity.[5, 6] Clark and Marcker[4] observe N-terminal incorporation of formylatable Met-tRNA under conditions which do not permit formylation. On the other hand, Nakamoto and Kolakofsky[5] report a dependence of protein synthesis under specified conditions upon formylation of Met-tRNA or addition of peptidyl-tRNA. The latter suggest a more stable type of binding interaction between N-formyl-Met-tRNA or peptidyl-tRNA, their respective codons, and the ribosome.

The results we have presented are consistent with an initiation model involving recognition of the initiator codon by formyl-accepting Met-tRNA, prior to its formylation. This species of Met-tRNA appears to have access to the same binding site(s) on the ribosome whether formylated or not. It is also clear, however, that formylation of Met-tRNA reduces the efficiency with which it is bound to ribosomes. In addition, the ability of Met-tRNA to select...ApUpG...from an array of overlapping sequences which are excluded from translation does not depend upon the presence of the formyl group. A change in mobility of Met-tRNA on a MAS column which occurs following formylation suggests that this effect may reflect an alteration of its secondary structure. Similar alteration in mobility of tRNA upon acylation has been noted by Stern and Littauer.[22] Formylation may occur, therefore, following the initial codon recognition step. Such a notion is supported by the observed formylation of Met-tRNA while in complex with ribosome and messenger, though free Met-tRNA is readily formylated.

A reaction sequence is suggested in which free Met-tRNA, the formyl-accepting species, selects the initiator codon, is formylated, and subsequently interacts with the penultimate AA-tRNA to form the initial peptide bond. Experiments examining the role of N-formyl-Met-tRNA in formation of the initial peptide bond are presented in a forthcoming paper.[12]

Summary.—The role of N-formyl-Met-tRNA in the initial step of protein synthesis has been examined. The formyl-accepting species of Met-tRNA, prior to its formylation, efficiently recognizes the methionine codon ApUpG in appropriate phase. Formylation reduces the efficiency of this interaction, perhaps through an alteration of secondary structure of Met-tRNA, for it also alters the mobility of Met-tRNA on a MAS column. Formylation of Met-tRNA following binding of this compound to the messenger-ribosome complex has also been demonstrated. A reaction sequence is suggested in which free Met-tRNA selects the initiator codon in complex with the ribosome, is formylated and subsequently interacts with the penultimate AA-tRNA to form the initial peptide bond.

It is a pleasure to acknowledge the advice of Drs. J. Ramachandran and M. Ruttenberg in all chemical syntheses and that of Drs. R. Stern and U. Z. Littauer in MAS column chromatography. One of us (P. L.) was supported by grant PF 245 of the American Cancer Society. Funds for this work were provided under agreement no. 675133 between the USPHS National Institutes of Health and the Weizmann Institute of Science under terms of Public Law 480.

The following abbreviations are used: tRNA, transfer RNA; AA-tRNA, aminoacyl-tRNA; Met-, N-formyl-Met-, Val-, Phe-tRNA, methionyl-, N-formylmethionyl-, valyl-, phenylalanyl-tRNA, respectively; A, adenosine; U, uridine; C, cytidine; G, guanosine; poly U, polyuridylic acid; TCA, trichloroacetic acid; MAS, methylated albumin-silicic acid. For oligonucleotides of specific structure, internal phosphodiesters (3′-5′) between nucleosides are indicated as in ApA for di-adenosine monophosphate.

[1] Marcker, K. A., and F. Sanger, *J. Mol. Biol.*, **8**, 835 (1964).
[2] Adams, J. M., and M. R. Capecchi, these PROCEEDINGS, **55**, 147 (1966).
[3] Capecchi, M. R., these PROCEEDINGS, **55**, 1517 (1966).
[4] Clark, B. F. C., and K. A. Marcker, *J. Mol. Biol.*, **17**, 394 (1966).
[5] Nakamoto, T., and D. Kolakofsky, these PROCEEDINGS, **55**, 606 (1966).
[6] Noll, H., *Science*, **151**, 1241 (1966).
[7] Sundararajan, T., and R. Thach, *J. Mol. Biol.*, **19**, 74 (1966).
[8] Webster, R. E., D. L. Engelhardt, and N. D. Zinder, these PROCEEDINGS, **55**, 155 (1966).

[9] Clark, B. F. C., and K. A. Marcker, *Nature*, **207**, 1038 (1965).
[10] Marcker, K. A., *J. Mol. Biol.*, **14**, 63 (1965).
[11] Kellogg, D., B. Doctor, J. Loebel, and M. Nirenberg, these PROCEEDINGS, **55**, 912 (1966).
[12] Zamir, A., P. Leder, and D. Elson, these PROCEEDINGS, in press.
[13] Nirenberg, M. W., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), pp. 17–23.
[14] 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), pp. 806–809.
[15] Sheehan, J. C., and D. H. Yang, *J. Am. Chem. Soc.*, **80**, 1154 (1958).
[16] Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
[17] Leder, P., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan, in press.
[18] Leder, P., M. F. Singer, and R. L. C. Brimacombe, *Biochemistry*, **4**, 1561 (1965).
[19] Leder, P., and M. Nirenberg, these PROCEEDINGS, **52**, 420 (1964).
[20] Nirenberg, M., and P. Leder, *Science*, **145**, 1399 (1964).
[21] Okamoto, T., and Y. Kawade, *Biochem. Biophys. Res. Commun.*, **13**, 324 (1963).
[22] Stern, R., and U. Z. Littauer, personal communication.