A Simple One-Step Method for the Preparation of Highly Purified Formylmethionine Transfer Ribonucleic Acid and Methionine Transfer Ribonucleic Acid from *Escherichia coli**

C. Henes, M. Krauskopf,‡ and J. Ofengand†

ABSTRACT: Phenoxyacetylation of methionyl transfer ribonucleic acid of *Escherichia coli* and fractionation on BD-cellulose at 4° was shown to be a rapid and convenient method for obtaining highly purified formylmethionine transfer ribonucleic acid and methionine ribonucleic acid well separated from each other. In a single run 80% pure formylmethionine transfer ribonucleic acid and 35% pure methionine ribonucleic acid were obtained. If the chromatography was performed at 22°, ribonucleic acids specific for methionine could also be obtained highly purified but the isoaccepting species were not separated from each other. Rechromatography of material from the 4° column resulted in further purification of both ribonucleic acids, approaching 100% for formylmethionine transfer ribonucleic acid and about 70% for methionine ribonucleic acid. The order of elution of the phenoxyacetylmethionyl derivatives of formylmethionine transfer ribonucleic acid and methionine ribonucleic acid was inverted with respect to the two uncharged or aminoacylated ribonucleic acid species. It is suggested that N acylation of the methionine residue has induced a *differential* conformational change in the two ribonucleic acid structures which is detected by altered affinity for the BD-cellulose.

A great deal of interest exists in the methionine RNA species of tRNA because of the existence of two classes of such molecules, $tRNA^{fMet}$ and $tRNA^{Met}$,[1] which when aminoacylated can be distinguished by at least two enzymes, the transformylase of *Escherichia coli* (Kellog *et al.*, 1966; Caskey *et al.*, 1967) and the T enzyme factor of *Bacillus subtilis* (Ono *et al.*, 1968) but not by the homologous aminoacyl-tRNA synthetase (Bruton and Hartley, 1968; Cassio and Waller, 1968). In view of the important role played by fMet-tRNA in initiation of protein synthesis, the conformational differences implicit in these specific recognition reactions have received much attention and led to the development of several methods for the purification of these tRNAs.

Separation of $tRNA^{fMet}$ and $tRNA^{Met}$, identified as such, was first achieved by countercurrent distribution (Kellog *et al.*; 1966; Clark and Marcker, 1966) although extensive purification was not obtained. More recently, two methods suitable for large-scale preparation of pure tRNA have appeared. That of Weiss *et al.* (1968) yielded large amounts of pure $tRNA^{fMet}$ but pure $tRNA^{Met}$ was not obtained by this method. In addition, reversed-phase columns have sometimes proven difficult to operate. The method of Seno *et al.* (1968) utilizes a combination of DEAE-Sephadex and BD-cellulose chromatography to yield extensively purified $tRNA^{fMet}$ and $tRNA^{Met}$. However, separation of the two species was only achieved at the third chromatographic step. Essentially the same method was also used by Dube *et al.* (1968) and Cory *et al.* (1968) to prepare small amounts of pure ^{32}P-labeled $tRNA^{fMet}$ and $tRNA^{Met}$ for sequence determination.

In this laboratory, we undertook the preparation of pure $tRNA^{fMet}$ of *E. coli* by application of the novel methods recently described by Gillam *et al.* (1968) which utilize phenoxyacetylation of aminoacyl-tRNA and chromatography on BD-cellulose. This method does not normally separate isoaccepting tRNA species. However, if the BD-cellulose fractionation is carried out at 4° instead of 22°, our results show that separation and extensive purification of $tRNA^{fMet}$ and $tRNA^{Met}$ can be readily obtained in a single chromatographic run on short columns.

We also find that contrary to all other chromatographic separations of $tRNA^{fMet}$ and $tRNA^{Met}$ in which $tRNA^{fMet}$ always elutes first, the phenoxyacetylmethionyl derivatives elute in the reverse order with the $tRNA^{Met}$ derivative eluting first. We suggest that this effect reflects a differential conformational change in the two tRNA structures which is induced by the presence of the derivative.

Materials and Methods

E. coli tRNA and the phenoxyacetic ester of *N*-hydroxysuccinimide were obtained from Schwarz BioResearch and [^{14}C]methionine was from Amersham–Searle. $N_{5,10}$-[^{14}C]-Methenyltetrahydrofolic acid and purified *E. coli* transformylase (Dickerman *et al.*, 1967) were gifts of Dr. H. Weissbach. A mixed aminoacyl-tRNA synthetase was prepared according to the procedure of Muench and Berg (1966), and BD-cellulose was prepared as described by Gillam *et al.* (1967).

Methionine acceptance assays were carried out in 0.25-ml reaction mixtures containing 100 mM 3,3-dimethylglutaric acid buffer (pH 6.9), 10 mM MgOAc, 10 mM KCl, 2 mM ATP, 0.02

* From the Department of Biochemistry, University of California Medical Center, San Francisco, California. *Received April 7, 1969.* Supported in part by National Institutes of Health Grant GM 11506 and by a grant from the Hoffman-LaRoche Co.

† Visiting Scientist of the Roche Institute of Molecular Biology.

‡ Permanent address: Instituto de Bioquimica, Universidad Austral, Valdivia, Chile.

[1] $tRNA^{fMet}$ refers to a tRNA species specific for methionine which can be formylated; $tRNA^{Met}$, tRNA specific for methionine which cannot be formylated; BD-cellulose, benzoylated DEAE-cellulose; ODU, absorbance units at 260 nm.

TABLE I: Purification of Methionine tRNA Species on BD-cellulose.[a]

Fraction	At 22°			At 4°		
	% of Column Input		Sp Act. ($\mu\mu$moles of Met/ODU)	% of Column Input		Sp Act. ($\mu\mu$moles of Met/ODU)
	cpm	ODU		cpm	ODU	
0.3 M NaCl	1.5	21.7		0.6	14.6	
0.8 M NaCl	21.1	52.5		16.7	55.7	
Ethanol gradient						
Prepeak	12.5	21.2		8.1	19.6	
Methionine, peak 1						
Total peak	63.1	3.5	850	19.1	1.9	334
As pooled	61.5	2.4	1200	16.2	1.1	460
Methionine, peak 2						
Total peak				32.2	1.6	637
As pooled				26.1	1.0	845
Sum	98.2	98.9		76.7	93.4	

[a] Column chromatography was done as described in Figures 1 and 2. Input specific activity was 53 and 39 $\mu\mu$moles of Met per ODU of RNA for the 22 and 4° columns, respectively.

mM [^{14}C]methionine (53 mCi/mmole), tRNA, and enzyme. The amounts of tRNA and enzyme were adjusted to give complete charging of the tRNA. Incubations were for 30 min at 30°. Reactions were stopped with 3 ml of cold 2 N HCl after addition of 2 mg of carrier RNA. After 10 min at 0°, the precipitates were collected on Whatman GF/C glass filters, washed with cold 2 N HCl, then with cold ethanol, dried for 20 min at 110°, and counted in a toluene-based scintillation fluid at approximately 70% efficiency.

Formylation assays (Dickerman and Weissbach, 1968) were carried out in 50-μl reaction volumes containing 200 mM Tris buffer (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 20 mM mercaptoethanol, 2 mM ATP, 0.2 mM [^{12}C]methionine, activating enzyme, tRNA, 6 μM $N_{5,10}$-[^{14}C]methenyltetrahydrofolic acid (specific activity 67 cpm/$\mu\mu$mole), and purified transformylase. Incubation was for 10 min at 37° in the absence of formyl donor and transformylase in order to allow methionyl-tRNA synthesis to occur, then formyl donor and transformylase were added and incubation was continued for 5 min more. Reactions were stopped with 3 ml of cold 5% trichloroacetic acid. After chilling for 10 min, the usually invisible precipitate was collected on Millipore filters (HA, 0.45 μ) and washed with cold trichloroacetic acid. The filters were dissolved in Bray's (1960) solution for counting at 70% efficiency. In every case, methionine acceptance was also measured in a parallel set of tubes using [^{14}C]methionine and omitting the formyl donor and transformylase. Methionine acceptance as determined by this assay was the same as with the standard assay above. With either assay, prewashing the filters with [^{12}C]-methionine was necessary to reduce the blank.

Large-scale charging of tRNA was done as in the standard assay using 10–20 mM tRNA nucleotide and sufficient enzyme to effect complete reaction. Met-tRNA was isolated either by repeated ethanol precipitation and centrifugation to remove denatured protein (Berg *et al.*, 1961) or by phenol extraction, ethanol precipitation, and Sephadex G-25 gel filtration. Phenoxyacetylation of methionyl-tRNA, reisolation of the derivatized material, and packing and preparing the BD-cellulose columns were done as described by Gillam *et al.* (1968).

Hydrolysis of the phenoxyacetylmethionine residue from the tRNA was accomplished with 1 M Tris buffer (pH 9.0) (Sarin and Zamecnik, 1964). At 23°, the half-life was 3 min, and 20 min was routinely used for stripping. Discharged tRNA was recovered by ethanol precipitation. Absorbance units were determined at 260 nm in 10 mM Mg–3 mM Tris (pH 7.4), and salt and ethanol concentrations were measured by conductivity.

Results and Discussion

The experiments of Figures 1 and 2 illustrate the separation achieved when the phenoxyacetyl derivative of Met-tRNA is

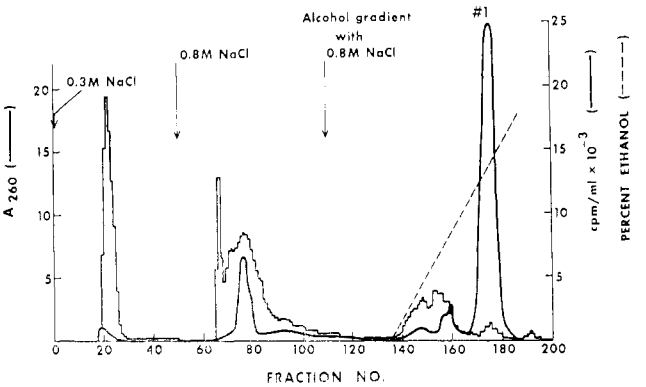

FIGURE 1: Chromatography of tRNA containing phenoxyacetylmethionyl-tRNA on BD-cellulose at 22°. Labeled tRNA (1770 ODU) in 11 ml was loaded on a 2.5 × 23 cm column of BD-cellulose equilibrated with 0.3 M NaCl, 0.01 M NaOAc (pH 5.5), 0.01 M $MgSO_4$, and 0.01 M mercaptoethanol. Fractions (7 ml) were collected at a flow rate of 105 ml/hr. Elution was stepwise as indicated with a final gradient of ethanol in the 0.8 M NaCl buffer.

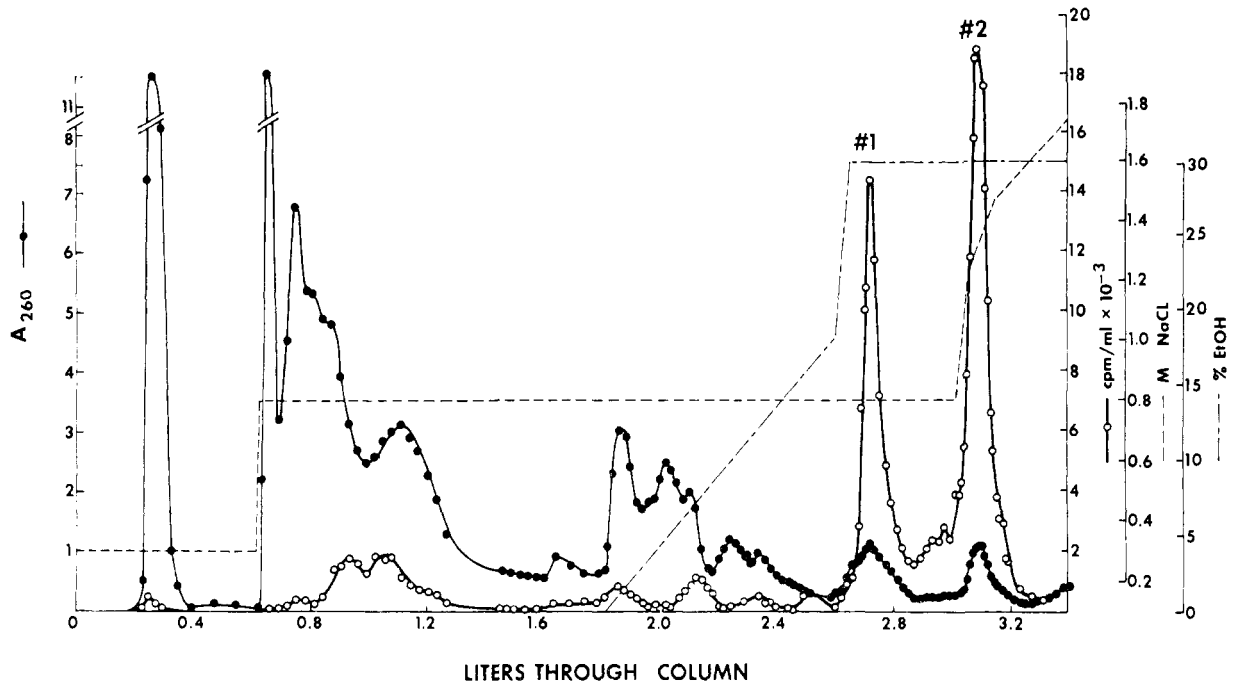

FIGURE 2: Chromatography of tRNA containing phenoxyacetylmethionyl-tRNA on BD-cellulose at 4°. Labeled tRNA (5350 ODU) in 21 ml was loaded on a 4.0 × 23 cm column equilibrated as in Figure 1 but at 4°. Elution took place at approximately 120 ml/hr with the salt and ethanol solutions as indicated.

TABLE II: Methionine and Formate Accepting Activity of tRNA Fractions Isolated by BD-cellulose Chromatography.[a]

tRNA Fraction	Methionine (A) ($\mu\mu$moles/ODU)	Formate (B) ($\mu\mu$moles/ODU)	B/A
22° column (Figure 1), peak 1	555	275	0.50
4° column (Figure 2), peak 1	495	64	0.13
peak 2	1065	1210	1.15
Rechromatographed peak 1 (Figure 3)			
Tube 35	62	<11	<0.18
Tube 47	915	<11	<0.01
Tube 65	463	<11	<0.02
Rechromatographed peak 2 (Figure 4)			
Tube 62	1415	1320	0.94
Tube 64	1485	1380	0.93

[a] The pooled tRNA peaks of Figures 1 and 2 were first stripped of their derivatized amino acid by Tris treatment and then reassayed for methionine and formate acceptance as described in Methods. The peak tubes (see arrows) of Figures 3 and 4 were assayed directly using 10–150-μl aliquots in suitably scaled-up standard assay mixtures.

chromatographed at 22 and 4°. The result obtained at 22° (Figure 1) is as expected based on the work of Gillam *et al.* (1968). The use of an ethanol gradient (also described by Litt, 1968) instead of a fixed ethanol concentration markedly improved the purification. Indeed Table I shows that this peak is about 85% pure Met-tRNA although a mixture of $tRNA^{fMet}$ and $tRNA^{Met}$. The small peak of radioactivity eluting with salt alone is presumably underivatized Met-tRNA. In other experiments, this peak of radioactivity was absent.

By contrast, chromatography at 4° (Figure 2) separates the peak of activity into two fractions, subsequently identified as $tRNA^{Met}$ and $tRNA^{fMet}$. The over-all yields and purity of the peaks as isolated are given in Table I. Other experiments have shown that the ethanol gradient used here is unnecessary and can be replaced by an 0.8 M NaCl–15% ethanol solution which removes virtually all the contaminating absorbance as a sharp peak. However, this solution should be changed to 0.8 M NaCl–30% ethanol as soon as the absorbance peak has decreased as

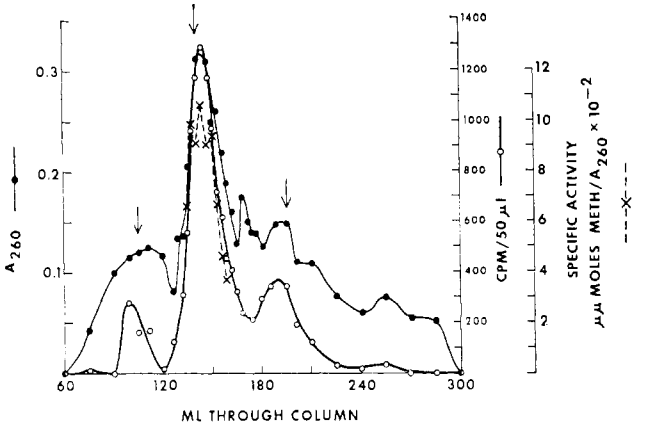

FIGURE 3: Rechromatography of partially purified $tRNA^{Met}$ on BD-cellulose. Peak 1 of Figure 2 was first stripped of its derivatized amino acid by Tris treatment and then applied (29 ODU in 1 ml) to a BD-cellulose column (0.9 × 24 cm) equilibrated at 4° in the same buffer as in Figure 1. A linear salt gradient of 2 mmoles/ml was used for elution at a flow rate of 18 ml/hr. Methionine acceptance assays were performed directly on 50-μl aliquots as described in Methods; 78% of the input acceptance activity was recovered.

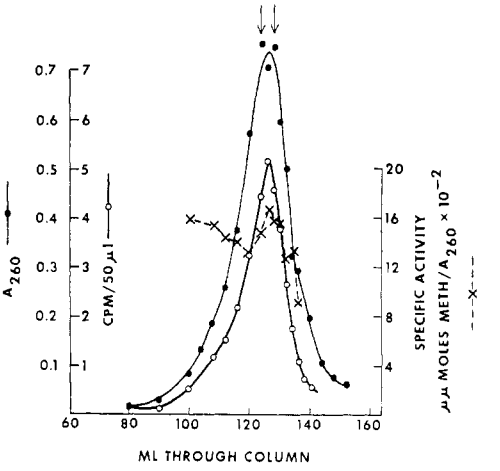

FIGURE 4: Rechromatography of $tRNA^{fMet}$ peak on BD-cellulose. Peak 2 of Figure 2 was stripped of amino acid and chromatographed as in Figure 3; 25 ODU in 1 ml was applied. Recovery of methionine acceptance activity was 84%.

the 15% ethanol solution will eventually elute $tRNA^{Met}$ as a broad shallow peak. The 30% ethanol solution elutes $tRNA^{Met}$ sharply. In turn it should be changed to 1.5 M NaCl–30% ethanol as soon as the $tRNA^{Met}$ peak has decreased in order to elute the $tRNA^{fMet}$ as a sharp peak. Continuing the previous solution will also separate $tRNA^{fMet}$ from $tRNA^{Met}$ but then the $tRNA^{fMet}$ elutes as a broad shallow peak. With either elution schedule, similar yields and purity were obtained.

The relative heights of peaks 1 and 2 in Figure 2 suggested that peak 2 was $tRNA^{fMet}$ despite the fact that in all chromatographic systems so far studied including-reversed-phase chromatography (Weiss *et al.*, 1968), DEAE-Sephadex (Takeishi *et al.*, 1968), and BD-cellulose (Roy and Söll, 1968; Seno *et al.*, 1968; Takeishi *et al.*, 1968), $tRNA^{fMet}$ elutes *before* $tRNA^{Met}$. This identification was confirmed by direct assay after stripping the derivatized amino acid from each of the isolated peaks (Table II). Clearly peak 2 can accept formate to the same extent as methionine while peak 1 cannot. Moreover, peak 2 appears to be about 80% pure as isolated when compared with the repurified material. Peak 1 is less pure. This table (line 1) confirms that the single peak from the 22° column is a mixture of the two tRNA species since only approximately 50% of the tRNA chains which can accept methionine can also accept formate. The low specific activity is probably due to partial inactivation during the process of stripping and recovery of the tRNA in this particular preparation.

Further purification of each of these tRNA fractions was accomplished by chromatography of the stripped tRNA on small columns of BD-cellulose as described by Gillam *et al.* (1968). In the case of $tRNA^{Met}$ (Figure 3) substantial further purification was obtained to approximately 950 μμmoles of Met/ODU for the peak fractions. This compares favorably with the purity achieved by Seno *et al.* (1968). It was confirmed that this material lacked formyl acceptor capacity (Table II). The two minor peaks of activity are not contaminating $tRNA^{fMet}$ since they cannot be formylated either. They may be related to the multiple peaks of $tRNA^{Met}$ reported by Seno *et al.* (1968) and Cory *et al.* (1968) or may be artifacts of the procedure.

$tRNA^{fMet}$ was already quite pure and rechromatography demonstrated this fact (Figure 4). The peak fractions, however, did show an increased purity to approximately 1450 μμmoles of Met/ODU. This value should be compared with that of 1200 μμmoles/ODU reported by Weiss *et al.* (1968) for an essentially pure $tRNA^{fMet}$. Furthermore, analysis of the peak fractions showed that this material had full formate acceptor capacity (Table II) confirming the identification of peak 2 as $tRNA^{fMet}$.

In addition to the usefulness of this procedure for the rapid preparation of relatively pure isoaccepting $tRNA^{Met}$ species, the interesting fact that has emerged is that derivatization of $tRNA^{Met}$ and $tRNA^{fMet}$ with exactly the same chemical material, namely, phenoxyacetylmethionine, has had a differential effect on the elution position for the two tRNAs. Other workers (Roy and Söll, 1968; Seno *et al.*, 1968; Takeishi *et al.*, 1968) have shown that uncharged $tRNA^{fMet}$ elutes earlier than $tRNA^{Met}$ from BD-cellulose although the studies with *E. coli* tRNA done at 4° were in the absence of Mg (Roy and Söll, 1968; Seno *et al.*, 1968). We have confirmed that the same order applies with our preparation of *E. coli* tRNA at 4° in the presence of Mg (M. Krauskopf, C. Henes, and J. Ofengand, unpublished results). Consequently, the inversion of order seen here with derivatized tRNA suggests that some part of the derivatization process has changed the conformation of $tRNA^{fMet}$ relative to $tRNA^{Met}$.

Phenoxyacetylation of some minor base in $tRNA^{fMet}$ which is absent in $tRNA^{Met}$ could have caused the increased column retention, but this seems unlikely since the only unusual minor nucleotides in $tRNA^{fMet}$, 4-thiouridylate, and 7-methylguanylate are also present in $tRNA^{Met}$ (Dube *et al.*, 1968; Cory *et al.*, 1968). Moreover, the presence of two phenoxyacetyl residues instead of one should have led to a much stronger retention than was observed, judged by the various observations of Gillam *et al.* (1967, 1968).

Aminoacylation alone is not sufficient to invert the elution order since Met-RNA^{fMet} and Met-RNA^{Met} elute in that se-

quence when cochromatographed on BD-cellulose (M. Krauskopf, C. Henes, and J. Ofengand, unpublished results). The remaining possibility, namely, that N acylation of the methionine residue is responsible for the *differential* conformational change in the two methionine tRNA species is intriguing in view of the special role played by fMet-tRNA in protein synthesis. We are currently testing this hypothesis.

References

Berg, P., Bergmann, F. H., Ofengand, E. J., and Dieckmann, M. (1961), *J. Biol. Chem. 236*, 1726.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Bruton, C. J., and Hartley, B. S. (1968), *Biochem. J. 108*, 281.

Caskey, C. T., Redfield, B., and Weissbach, H. (1967), *Arch. Biochem. Biophys. 120*, 119.

Cassio, D., and Waller, J. P. (1968), *European J. Biochem. 5*, 33.

Clark, B. F. C., and Marcker, K. A. (1966), *J. Mol. Biol. 17*, 394.

Cory, S., Marcker, K. A., Dube, S. K., and Clark, B. F. C. (1968) *Nature 220*, 1039.

Dickerman, H. W., Steers, Jr., E., Redfield, B. G., and Weissbach, H. (1967), *J. Biol. Chem. 242*, 1522.

Dickerman, H. W., and Weissbach, H. (1968), *Methods Enzymol. 12*, 681.

Dube, S. K., Marcker, K. A., Clark, B. F. C., and Cory, S. (1968), *Nature 218*, 232.

Gillam, I., Blew, D., Warrington, R. C., v. Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7*, 3459.

Gillam, I., Millward, S., Blew, D., v. Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3043.

Kellog, D. A., Doctor, B. P., Loebel, J. E., and Nirenberg, M. W. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 912.

Litt, M. (1968), *Biochem. Biophys. Res. Commun. 32*, 507.

Muench, K., and Berg, P. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D., Ed., New York, N. Y., Harper & Row, p 375.

Ono, Y., Skoultchi, A., Klein, A., and Lengyel, P. (1968), *Nature 220*, 1304.

Roy, K., and Söll, D. (1968), *Biochim. Biophys. Acta 161*, 572.

Sarin, P. S., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91*, 653.

Seno, T., Kobayashi, M., and Nishimura, S. (1968), *Biochim. Biophys. Acta 169*, 80.

Takeishi, K., Ukita, T., and Nishimura, S. (1968), *J. Biol. Chem. 243*, 5761.

Weiss, J. F., Pearson, R. L., and Kelmers, A. D. (1968), *Biochemistry 7*, 3479.