Proc. Nat. Acad. Sci. USA
Vol. 69, No. 12, pp. 3594–3597, December 1972

Structure and Function of *Escherichia coli* Formylmethionine Transfer RNA: Loss of Methionine Acceptor Activity by Modification of a Specific Guanosine Residue in the Acceptor Stem of Formylmethionine Transfer RNA from *Escherichia coli**

(synthetase recognition/formylation/chemical modification/photooxidation)

LADONNE H. SCHULMAN

Division of Biology, Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461

Communicated by Harry Eagle, September 25, 1972

ABSTRACT **The structural requirements of *E. coli* formylmethionine tRNA for aminoacylation have been examined by chemical modification of the tRNA, followed by separation of the modified molecules into active and inactive components. Photooxidation of $tRNA^{fMet}$ at 50° in the presence of methylene blue results in modification of two guanosine (G) residues in the acceptor stem, at positions no. 2 and no. 71 from the 5′-phosphate terminus. Both of these modifications are present in inactive molecules, but only the G residue at position no. 2 is modified in the acceptor stem of active molecules. Loss of methionine acceptance occurs with first-order kinetics, indicating that inactivation by modification of G residue no. 71 is independent of any other modifications taking place under these conditions. The presence of a modified G residue at position no. 2 in the acceptor stem of active photooxidized molecules shows that disruption of normal base-pairing in this region is not sufficient to inactivate $tRNA^{fMet}$. These data indicate that the inactivating modification at position no. 71 is lethal due to a specific alteration in the nucleotide base, rather than simply as a result of breaking a hydrogen-bonded base pair in the acceptor stem.**

Recent work from this laboratory has been concerned with determination of the specific structural requirements for recognition of *Escherichia coli* formylmethionine tRNA by methionine tRNA synthetase and transformylase. Previous papers in this series have described the effects of chemical modification of pyrimidine and guanosine residues in $tRNA^{fMet}$ on these biological activities (1, 2).

Methylene blue-sensitized photooxidation of $tRNA^{fMet}$ at 20° results in loss of methionine acceptor activity by modification of a G residue in the small loop of the tRNA (2). The present report describes the effects of several new modifications, introduced by photooxidation at 50°, on the ability of $tRNA^{fMet}$ to be acylated and formylated.

METHODS AND RESULTS

E. coli formylmethionine tRNA was isolated from crude *E. coli* K12 tRNA (General Biochemicals), as described by Seno, Kobayashi, and Nishimura (3); it accepted 1.6–1.7 nmol of methionine and formate per A_{260} unit.

Abbreviations: $tRNA^{fMet}$, the methionine tRNA from *E. coli* that can be enzymatically formylated; A_{260} unit, the amount of tRNA in 1 ml that has an absorbance of 1.0 when measured in a 1-cm optical path at a wave length of 260 nm. The numbering of nucleotide residues in $tRNA^{fMet}$ is based on the position of the nucleotide from the 5′-phosphate end of the chain.

* This is paper no. III in a series, "Structure and Function of *E. Coli* Formylmethionine Transfer RNA"; paper II is ref. 2.

Irradiations were performed as described (2), except that the temperature was maintained at 50 ± 2°. After irradiation, methylene blue was removed by gel filtration on Sephadex G-25 in the presence of Mg^{++}. Samples were assayed (2) for methionine and formate acceptance. In the absence of light, $tRNA^{fMet}$ incubated at 50° with methylene blue for 30 min showed no loss of activity.

A sample of $tRNA^{fMet}$ that had been photooxidized to the extent of greater than 90% loss of methionine acceptor activity was examined for chain breaks by passage through a 1 × 100 cm column of Sephadex G-100. A single symmetrical peak was obtained at the elution position corresponding to intact $tRNA^{fMet}$.

The conditions used for large-scale loading, phenoxyacetylation, ribonuclease digestions, and DEAE-cellulose chromatography have been described (2).

Rate of photooxidation at 20° and 50°

Irradiation of GpA-OH with visible light in the presence of oxygen and methylene blue resulted in loss of guanosine, with a half-life of 43 min at 20° and 22 min at 50° (Fig. 1, *left*). Irradiation of $tRNA^{fMet}$ resulted in loss of methionine acceptor activity at a rate equal to the rate of modification of GpA-OH at 20° (Fig. 1, *right*). This inactivation has been shown to be mainly due to modification of the G residue at position 46 in the small loop of $tRNA^{fMet}$ (2). When irradiation was done at 50°, methionine acceptor activity was lost at a rate more than double that observed for modification of GpA-OH under the same conditions. The ability of the tRNA to accept formate decreased at the same rate as its ability to accept methionine. These results suggested that at least

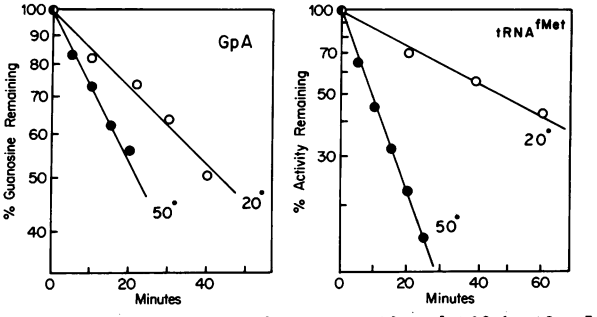

FIG. 1. Rates of photooxidation at 20° and 50° in 10 mM $MgCl_2$. The concentration of GpA-OH was 0.4 mM, $tRNA^{fMet}$ was 10 A_{260} units/ml (0.4 mM guanosine residues), and methylene blue was 40 μM. Loss of guanosine and methionine acceptor activity were followed as described (2).

one modification in addition to that of residue G_{46} was contributing to the observed inactivation at 50°.

Separation of active and inactive molecules of tRNA^{fMet} photooxidized at 50°

In order to determine the modification sites responsible for inactivation of tRNA^{fMet} at 50°, 100 A_{260} units of purified tRNA was photooxidized to an extent that resulted in the loss of 70% of its biological activity. The remaining active molecules were enzymatically esterified, and the resulting [^{14}C]methionyl-tRNA^{fMet} was derivatized with the *N*-hydroxysuccinimide ester of phenoxyacetic acid as described by Gillam *et al.* (4). The mixture was chromatographed on benzoylated DEAE-cellulose (Fig. 2). Peak I contained inactive photooxidized tRNA^{fMet}, plus a small amount of active modified tRNA^{fMet} that failed to react with the phenoxyacetic acid ester. Peak II contained a mixture of active and inactive modified tRNA^{fMet}. Peak III contained only *N*-phenoxyacetyl-[^{14}C]methionyl-tRNA^{fMet}.

Sites of modified guanosine residues in tRNA^{fMet} photooxidized at 50°

Sites of modified guanosine residues are determined by measurement of absorbancy changes in oligonucleotides isolated after digestion of tRNA^{fMet} with RNase and chromatography on DEAE-cellulose in the presence of 7 M urea (2). Fig. 3*a* and *b* shows the oligonucleotide profiles obtained from active modified tRNA^{fMet} (peak III, Fig. 2) after digestion with RNase T_1 and pancreatic RNase. Identification of modified residues at positions 2, 8, 9, 10, and 12 of active molecules after photooxidation at 20° has been described (2). These modifications are also present in active molecules photooxidized at 50°. The only significant difference in the active fractions isolated after photooxidation at 20° and 50° is an increase in the extent of modification of G residues no. 19 and 20 in the dihydrouridine loop of the tRNA. Reaction of these residues after photooxidation at 50° is indicated by the large loss of G_{19}–G_{20}–Dp (peak **8**, Fig. 3*b*) from the oligonucleotide profile obtained after pancreatic RNase digestion of active modified molecules. The comparatively small loss of C–C–U–G_{19}p from the T_1 RNase profile (peak **7**, Fig. 3*a*) indicates that G_{19} is modified to only a small extent, and that the large loss of G_{19}–G_{20}–Dp is due to extensive modification of G_{20}.

Fig. 4*a* and *b* shows the oligonucleotide profiles obtained from the inactive fraction of tRNA^{fMet} photooxidized at 50°

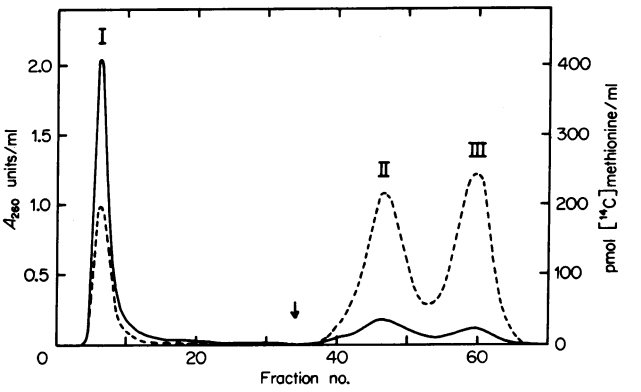

FIG. 2. Separation of active and inactive molecules of tRNA^{fMet} after photooxidation at 50°. 80 A_{260} units of tRNA^{fMet} in 5 ml of a solution containing 0.3 M NaCl–10 mM $MgCl_2$–10 mM sodium acetate (pH 4.5) were added to a 2.2 × 13 cm column of benzoylated DEAE-cellulose. The column was washed with 10 ml of the same buffer, then eluted with a solution containing 0.8 M NaCl–10 mM $MgCl_2$–10 mM sodium acetate (pH 4.5). The flow rate was 1 ml/min and 11-ml fractions were collected. At the position marked by an *arrow*, elution was started with solutions containing 1 M NaCl–10 mM $MgCl_2$–10 mM sodium acetate (pH 4.5) with a linear gradient from 0 to 30% ethanol over 750 ml. ——, A_{260} units/ml; - - -, pmol of [^{14}C]-methionine per ml.

(peak I, Fig. 2) after digestion with RNase T_1 and pancreatic RNase. All of the G residues that are modified in the active molecules are also modified in the inactive molecules. Examination of the RNase T_1 profile shows that peak **6** is substantially modified in the inactive fraction and unmodified in the active fraction (Fig. 4*a*). This peak contains A–A–Gp and C–A–A–C–C–A–OH. Separation of the two components shows that the loss of absorbance of peak **6** after photooxidation at 50° is due to loss of both oligonucleotides (Fig. 5).

Loss of A–A–G_{46}p due to modification of residue G_{46} corresponds to the inactivating modification observed after photooxidation at 20° (2). Loss of C–A–A–C–C–A–OH from the T_1 RNase profile of inactive molecules is due to modification of residue G_{71} in the acceptor stem. Modified G residues are not cleaved by RNase T_1 (2, 5, 6); thus, modification of residue G_{71} also results in loss of the adjacent oligonucleotide C–C–C–C–C–G_{71}p from the RNase T_1 profile of the inactive fraction (peak **9**, Fig. 4*a*). In addition, modification of residue

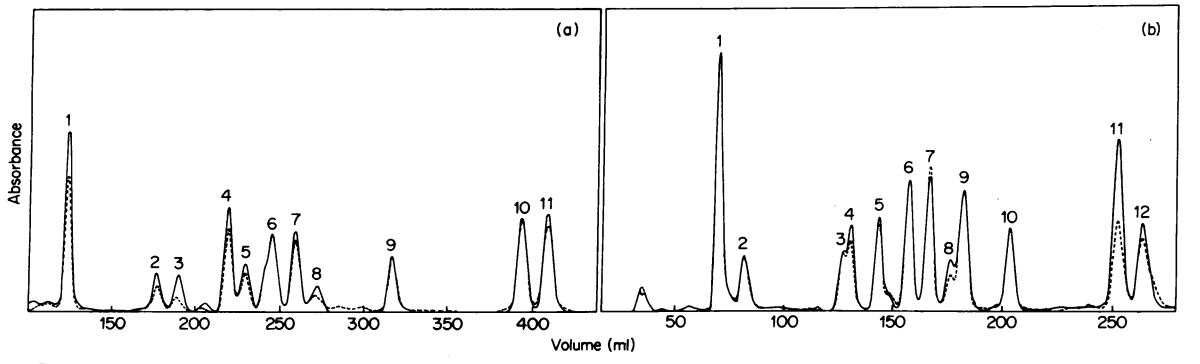

FIG. 3. Fractionation of oligonucleotides obtained from RNase digests of active tRNA^{fMet} isolated after photooxidation at 50°. Chromatography was performed on DEAE-cellulose in 7 M urea (2). (*a*) RNase T_1 digest; (*b*) pancreatic RNase digest. ——, unmodified tRNA^{fMet}; - - -active tRNA^{fMet} (peak III, Fig. 2) photooxidized at 50°.

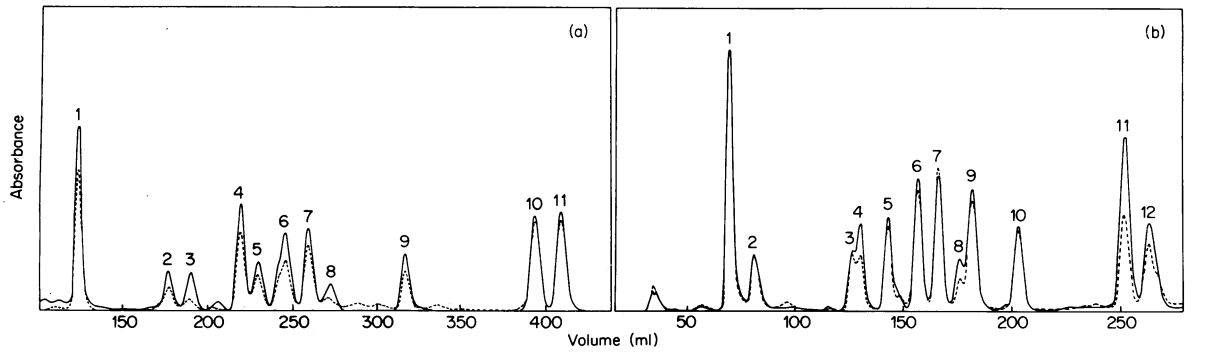

FIG. 4. Fractionation of oligonucleotides obtained from RNase digests of inactive tRNA^{fMet} isolated after photooxidation at 50°. Chromatography was performed as in Fig. 3. (*a*) RNase T_1 digest; (*b*) pancreatic RNase digest. ——, unmodified tRNA^{fMet}; - - -, inactive tRNA^{fMet} (peak I, Fig. 2) photooxidized at 50°.

G_{71} is seen as a greater loss of G-Cp (peak **4**, Figs. 3*b* and 4*b*) from the pancreatic RNase profile of inactive, compared to active, modified tRNA^{fMet}. Loss of G_2–Cp is observed in both active and inactive molecules (2), while loss of G_{71}–Cp is observed only in the pancreatic RNase profile of the inactive fraction.

The extent of modification of residue G_{46} is not sufficient to account for the observed loss of methionine acceptor activity, but the sum of modifications at residues G_{46} and G_{71} is sufficient to account for inactivation. Therefore, it is concluded that modification of *either* the G residue in the small loop *or* the G residue on the 3′ side of the acceptor stem is a lethal event.

The sites of modified G residues in active and inactive molecules after photooxidation at 50° are summarized in Fig. 6.

DISCUSSION

Previous studies from this laboratory have shown that modification of nucleotides in the double-stranded stem adjacent to the dihydrouridine loop of tRNA^{fMet} does not prevent aminoacylation or formylation of the tRNA (1, 2). The present data indicate that two of the G residues in the adjoining loop are also not required for amino-acid or formate acceptance. This result is in agreement with the previous report of Seno *et al.* (7) that nucleotides in the dihydrouridine loop of tRNA^{fMet} can be removed by limited nuclease digestion without elimination of these biological activities.

The most interesting result to be obtained from the data in the present report is that tRNA^{fMet} can be inactivated with respect to methionine acceptance by modification of a specific G residue on the 3′-hydroxyl side of the acceptor stem. This result is made more significant by the fact that the same type of chemical modification on the opposite side of the acceptor stem does not inactivate the tRNA. Thus, loss of base pairing in this region is not sufficient to prevent recognition of tRNA^{fMet} by methionine tRNA synthetase.

Seno *et al.* (8) have shown that removal of the two 5′-terminal nucleotides from tRNA^{fMet} by limited nuclease digestion also does not prevent attachment of methionine; however, the resulting modified tRNA has a K_m for acylation that is 10-fold higher than that found for intact tRNA^{fMet}. I have also shown that tRNA^{fMet} molecules that are photo-

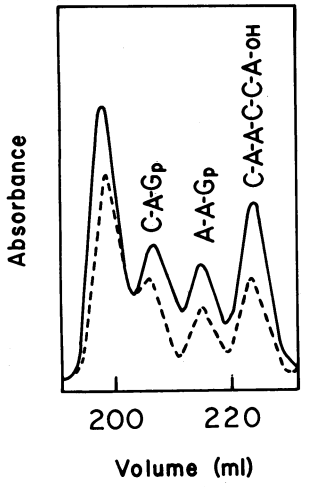

FIG. 5. Fractionation of oligonucleotides obtained from RNase T_1 digestion of inactive tRNA^{fMet} isolated after photooxidation at 50°. Elution was done as in Fig. 4(*a*), but with a column prepared from a different batch of DEAE-cellulose. ——, unmodified tRNA^{fMet}; - - -, inactive tRNA^{fMet} (peak I, Fig. 2) photooxidized at 50°.

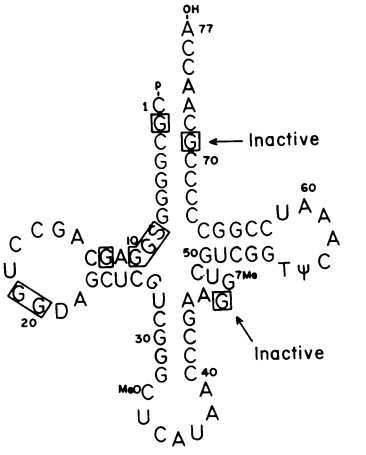

FIG. 6. Sites of modified nucleotides after photooxidation of tRNA^{fMet} at 50°. The primary structure of tRNA^{fMet} is taken from Dube *et al.* (16). *Boxes* indicate modified residues. The two guanosine modifications that result in loss of methionine acceptor activity are indicated as *'inactive'* on the figure. The other modifications resulting from photooxidation at 50° do not prevent aminoacylation or formylation of tRNA^{fMet}. *7MeG*, 7-methylguanosine; *OMeC*, 2′-*O*-methylcytidine; *D*, dihydrouridine; *S*, 4-thiouridine; *ψ*, pseudouridine.

oxidized at residue G_2 have an increased K_m for acylation (2). These data indicate that loss or modification of residue G_2 results in a decreased binding affinity of the tRNA for the synthetase. In view of this result, it becomes necessary to determine whether the inactivation resulting from photooxidation of residue G_{71} is due to alteration of this specific nucleotide, or whether it is the cumulative effect of modifications at both residues G_2 and G_{71}. These two possibilities can be distinguished by examination of the rate of inactivation of methionine acceptance by photooxidation at 50° (Fig. 1). If successive alteration of two nucleotides or disruption of two base-pairs in the acceptor stem were required for inactivation, a distinct lag in the rate of loss of activity would be observed at early times in the photooxidation. The finding of a linear, first-order inactivation rate indicates that loss of activity by modification of residue G_{71} is not dependent on any other reaction, including modification of residue G_2, and the inactivation resulting from this alteration is a more specific effect than simply loss of ordered structure in the acceptor stem.

We have recently converted the 5′-terminal C residue in $tRNA^{fMet}$ to a U residue, using sodium bisulfite (9). Introduction of a U residue into the first position of the acceptor stem of $tRNA^{fMet}$ actually leads to the formation of one *additional* U–A hydrogen-bonded base pair in this region. This modification had no effect on amino-acylation of the tRNA, the K_m being the same, within 10%, as that obtained for the unmodified tRNA. Thus, three nucleotides in the acceptor stem of $tRNA^{fMet}$ have now been modified or removed, with loss or gain of base pairs in this region. Only the alteration of residue G_{71} has inactivated the molecule, indicating that this specific nucleotide is required for attachment of methionine to $tRNA^{fMet}$.

E. coli $tRNA^{fMet}$ is the second example of a tRNA that can be inactivated with respect to aminoacid acceptance by chemical modification of nucleotides on the 3′-hydroxyl side of the acceptor stem. Schulman and Chambers (10) observed loss of alanine acceptor activity after modification of pyrimidine residues in the first three base-pairs of the acceptor stem of yeast $tRNA^{Ala}$ by irradiation with ultraviolet light.

The importance of a nucleotide near the 3′ terminus in synthetase recognition of another *E. coli* tRNA has recently been indicated by the findings of Hooper *et al.* (11) and Shimura *et al.* (12). A single A → G base change in the fourth nucleotide from the acceptor end of *E. coli* tyrosine suppressor tRNA markedly inhibits tyrosine acceptor activity.

In contrast to these results, Lagerkvist and Rymo (13) have reported data that indicate that nucleotides near the 3′ terminus of yeast $tRNA^{Val}$ may not be required for synthetase recognition of that tRNA. Modified $tRNA^{Val}$, which is missing seven nucleotides from the 3′-hydroxy terminus, formed a stable complex with yeast valine tRNA synthetase. The complex could be formed under conditions that were specific for $tRNA^{Val}$, and the binding constant was the same as that found for the unmodified tRNA within a factor of 2.

The importance of nucleotides on the 5′-phosphate side of the acceptor stem of yeast $tRNA^{Val}$ has been indicated by the recent results of Mirzabekov *et al.* (14). Removal of two nucleotides from the 5′ terminus resulted in complete loss of valine acceptor activity. The 5′-terminal nucleotides of *E. coli* $tRNA^{fMet}$ (2, 8) and yeast $tRNA^{Ser}$ (15), on the other hand, are not required for aminoacid attachment. Thus, it appears that different tRNAs have different structural requirements within the acceptor stem region for recognition by their cognate synthetases. It is not clear whether all tRNAs contain essential structural elements within this region.

Mutants of *E. coli* Su^+_{III} $tRNA^{Tyr}$ containing single base changes in the fourth nucleotide from the 3′ terminus or in the first two base-pairs of the acceptor stem are mischarged with foreign amino acids *in vivo* (11, 12). In several cases, the mutant tRNAs retain their ability to be acylated with tyrosine (11).

We have examined the ability of our modified $tRNA^{fMet}$ species to accept new amino acids *in vitro* using a crude *E. coli* tRNA synthetase mixture, and have detected no new activities. Attempts to observe the altered specificity of several of the Su^+_{III} $tRNA^{Tyr}$ mutants *in vitro* have also been unsuccessful (11). The *in vivo* results of Smith and coworkers indicate, however, that at least in the case of Su^+_{III} $tRNA^{Tyr}$, structural integrity of the acceptor stem region is important for maintaining the required *specificity* of aminoacylation, even though certain nucleotides in this region can be modified without loss of tyrosine acceptor ability (11).

From other studies on *E. coli* $tRNA^{fMet}$, it is clear that structural regions outside of the acceptor stem are also required for acylation of this tRNA. A base change of C → U in the anticodon of $tRNA^{fMet}$ completely inactivates the molecule with respect to methionine acceptance (9). In addition, chemical modification of the G residue in the small loop causes loss of activity (2). The overall data are best interpreted as suggesting that the aminoacyl-tRNA synthetase interacts with several different parts of the tRNA structure simultaneously, and that very specific nucleotides within each of these structural regions are required for acylation of $tRNA^{fMet}$.

I am indebted to Mrs. Heike Pelka for expert technical assistance. This work was supported by research grants from the National Institutes of Health, USPHS (GM 16995) and the American Cancer Society (NP-19). L. H. S. is a Research Career Development Awardee (Grant GM 25161 from the National Institute of General Medical Sciences).

1. Schulman, L. H. (1970) *Proc. Nat. Acad. Sci. USA* **66,** 507–514.
2. Schulman, L. H. (1971) *J. Mol. Biol.* **58,** 117–131.
3. Seno, T., Kobayashi, M. & Nishimura, S. (1968) *Biochim. Biophys. Acta* **169,** 80–94.
4. Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M. & Tener, G. M. (1968) *Biochemistry* **7,** 3459–3468.
5. Simon, M. I., Grossman, L. & Van Vunakis, H. (1965) *J. Mol. Biol.* **12,** 50–59.
6. Kuwano, M., Hayashi, Y., Hayashi, H. & Miura, K. (1968) *J. Mol. Biol.* **32,** 659–671.
7. Seno, T., Kobayashi, I., Fukuhara, M. & Nishimura, S. (1970) *FEBS Lett.* **7,** 343–346.
8. Seno, T., Sano, K. & Katsura, T. (1971) *FEBS Lett.* **12,** 137–140.
9. Schulman, L. H. & Goddard, J., *J. Biol. Chem.*, in press.
10. Schulman, L. H. & Chambers, R. W. (1968) *Proc. Nat. Acad. Sci. USA* **61,** 308–315.
11. Hooper, M. C., Russell, R. L. & Smith, J. D. (1972) *FEBS Lett.* **22,** 149–155.
12. Shimura, Y., Aono, H., Ozeki, H., Sarabhai, A., Lamfrom, H. & Abelson, J. (1972) *FEBS Lett.* **22,** 144–148.
13. Lagerkvist, U. & Rymo, L. (1970) *J. Biol. Chem.* **245,** 435–438.
14. Mirzabekov, A. D., Lastity, D., Leirma, E. S. & Bayev, A. A. (1971) *Nature New Biol.* **229,** 21–22.
15. Philippsen, P. & Zachau, H. G. (1971) *FEBS Lett.* **15,** 69–74.
16. Dube, S. K., Marcker, K. A., Clark, B. F. C. & Cory, S. (1968) *Nature* **218,** 232–233.