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Molecular rulers for measuring RNA structure: Sites of crosslinking in chlorambucilyl-phenylalanyl-tRNA^{Phe} (yeast) and chlorambucilyl-pentadecaprolyl-phenylalanyl-tRNA^{Phe} (yeast) intramolecularly crosslinked in aqueous solution*

(tRNA solution structure/alkylation/gel sequence determinations)

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ABSTRACT **Intramolecular crosslinking of yeast phenylalanine tRNA in aqueous solution with rigid, variable-length crosslinking reagents, which we call "molecular rulers," has given results in reasonable agreement with the crystal structure. Chlorambucilyl-[^{3}H]phenylalanyl-tRNA^{Phe} crosslinked intramolecularly at G-71 and A-73, whereas chlorambucilyl-pentadecaprolyl-[^{3}H]phenylalanyl-tRNA^{Phe} crosslinked at G-20 and Y-37. The pentadecaprolyl reagent was predicted to be 62 Å long, including chlorambucil and phenylalanine; the sites that it reached are 60 Å distant from the 3′ OH (in the case of G-20) or 80 Å distant (in the case of Y-37) in the crystal structure of tRNA^{Phe}. The close agreement between the length of the reagent and the distance of G-20 from the 3′ OH in the crystal structure illustrates the rigidity of the tRNA^{Phe} molecule in the dihydrouridine loop region at the corner of the molecule. The apparent ability of the 62-Å-long reagent to crosslink to a site, Y-37, that is 80 Å distant from the 3′ OH in the crystal structure appears to illustrate the flexibility of both the 3′ A-C-C-A terminus and the anticodon stem and loop, with respect to the tRNA molecule. These observations demonstrate the utility of oligoproline-based crosslinking reagents as rigid, variable-length molecular rulers for biological macromolecules in solution.**

Remarkable agreement has been observed between the crystal structure of yeast phenylalanine tRNA and its biological function and solution chemistry (1, 2). The generality of that structure for the function of all tRNA molecules has been noted (3). On the other hand, substantial questions remain as to the existence and significance of other conformations of tRNA in solution, aminoacylated or unacylated, bound to synthetase, or bound to a ribosome (4–6).

Rigid, variable-length oligoproline crosslinking reagents, which we call "molecular rulers" (7) are a potentially powerful tool for probing the solution structures of complex biological macromolecules or assemblies of macromolecules. The usefulness of known-length oligoprolines as molecular rulers was demonstrated by Stryer and Haugland (8) in a series of singlet-singlet fluorescence energy-transfer experiments. Poly(L-proline) has a *trans* conformation in solid state and aqueous solution known as poly(L-proline) II (9). This conformation is particularly stiff, and the dimensions of poly(L-proline) II in solution approximate the dimensions found in the crystalline state (3.12 Å per residue) (9) up to 50 or more residues long (10, 11).

We have chosen to test the feasibility of molecular rulers on a well-studied model system, yeast phenylalanine tRNA (1, 2), before applying them to less well-understood structures. The resolution offered by oligoproline molecular rulers as a technique for distance measurement in solution also may provide useful data to help answer the questions mentioned above concerning the solution structures of tRNA. Our approach, which is similar to that of Grachev and Rivkin (12) in the case of zero proline residues, is shown schematically in Fig. 1. High purity oligoprolines of defined length have been synthesized with 5, 11, and 15 residues (14), modified with chlorambucil at the NH_2 terminus, activated at the COOH terminus with *N*-hydroxysuccinimide, and then allowed to react with phenylalanyl-tRNA^{Phe} (7). Modifications of tRNA were carried out in 80% dimethyl sulfoxide (Me_2SO) or 40% Me_2SO and 40% $CHCl_3$, conditions under which tRNA deacylation and alkylation were minimal (7, 15). Phenylalanyl-tRNA^{Phe} modified with chlorambucilyl-oligoproline molecular rulers was then redissolved in aqueous buffer to activate the chlorambucil and to allow intramolecular crosslinking at sufficient dilution (≤ 0.1 μM) so

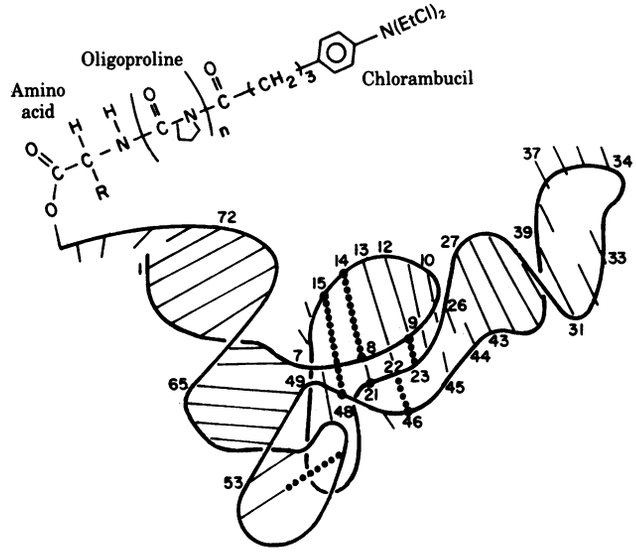

FIG. 1. A molecular ruler, chlorambucilyl oligoproline, linked by a peptide bond to an aminoacyl-tRNA. When this species is dissolved in aqueous buffer, intramolecular alkylation may occur. The tRNA structure is that of yeast tRNA^{Phe}, adapted from Robertus *et al.* (13).

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that no intermolecular crosslinking was detected (unpublished data).

In this paper we report the sites of intramolecular crosslinking observed when molecular rulers with 0 and 15 proline residues were applied to yeast phenylalanine tRNA and discuss the significance of these results for the solution structure of that tRNA and the general applicability of the molecular ruler technique.

MATERIALS AND METHODS

Yeast phenylalanine tRNA (10 μM; Boehringer Mannheim) was alkylated bimolecularly with 1.0 mM chlorambucil (Burroughs Wellcome, Research Triangle Park, NC) in 1.0 mM $Mg(OAc)_2$/10 mM $(EtOH)_3N{\cdot}HCl$, pH 8.0, for 1 hr at 37°C, as described (16). Chlorambucilyl-(prolyl)$_n$-[^{3}H]phenylalanyl-tRNA^{Phe} with n = 0 and 15 were synthesized in 80% Me_2SO (for n = 0) or in 40% Me_2SO and 40% $CHCl_3$, (for n = 15) to minimize intramolecular crosslinking during the modification reaction (7) and to maximize the yield (15). Intramolecular crosslinking was carried out in 1.0 mM $Mg(OAc)_2$/10 mM NH_4OAc, pH 6.0, for 4 hr at 37°C, as described (7). Alkylated or crosslinked nucleosides were determined by the rapid gel sequence-determination technique of Peattie (17), in which the chlorambucil alkylation step substituted for the dimethyl sulfate step. Labeling of the 3′ OH of crosslinked tRNA molecules with [5′-^{32}P]cytidine-

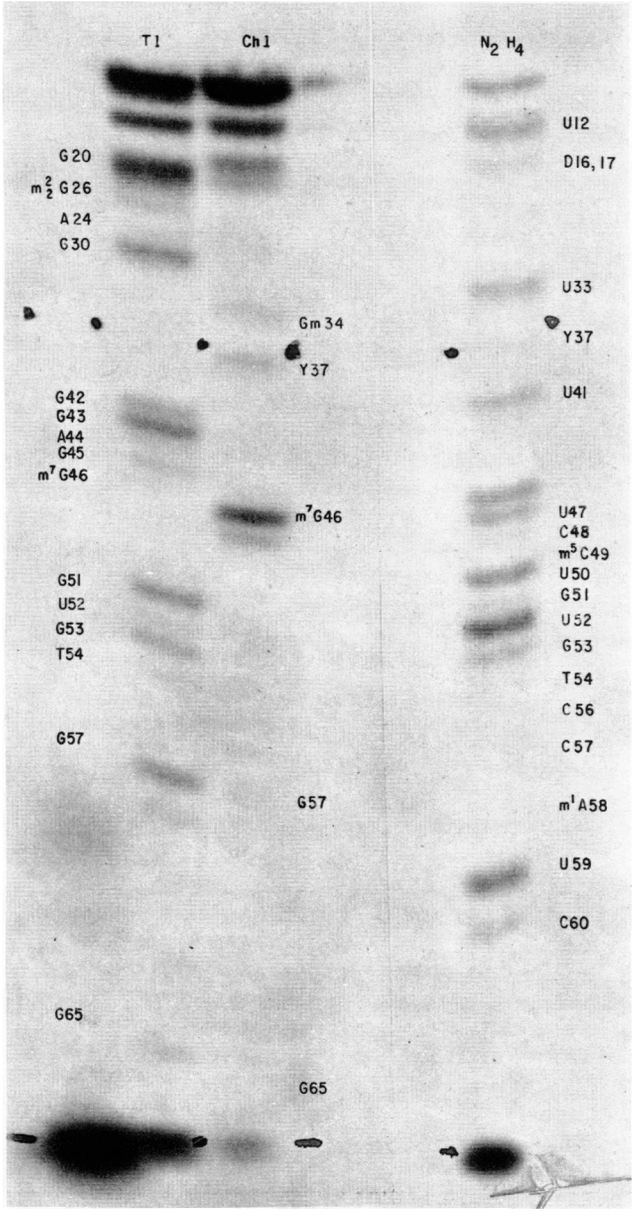

FIG. 2. Autoradiogram of yeast tRNA^{Phe} alkylated bimolecularly with chlorambucil and then analyzed by rapid gel sequencing (17). Lanes: T1 and N_2 H_4, calibration ladders from treating nonalkylated tRNA with ribonuclease T1 and hydrazine, respectively, after end labeling; Chl, yeast tRNA^{Phe} alkylated with chlorambucil before end labeling and treatment with $NaBH_4$ and aniline.

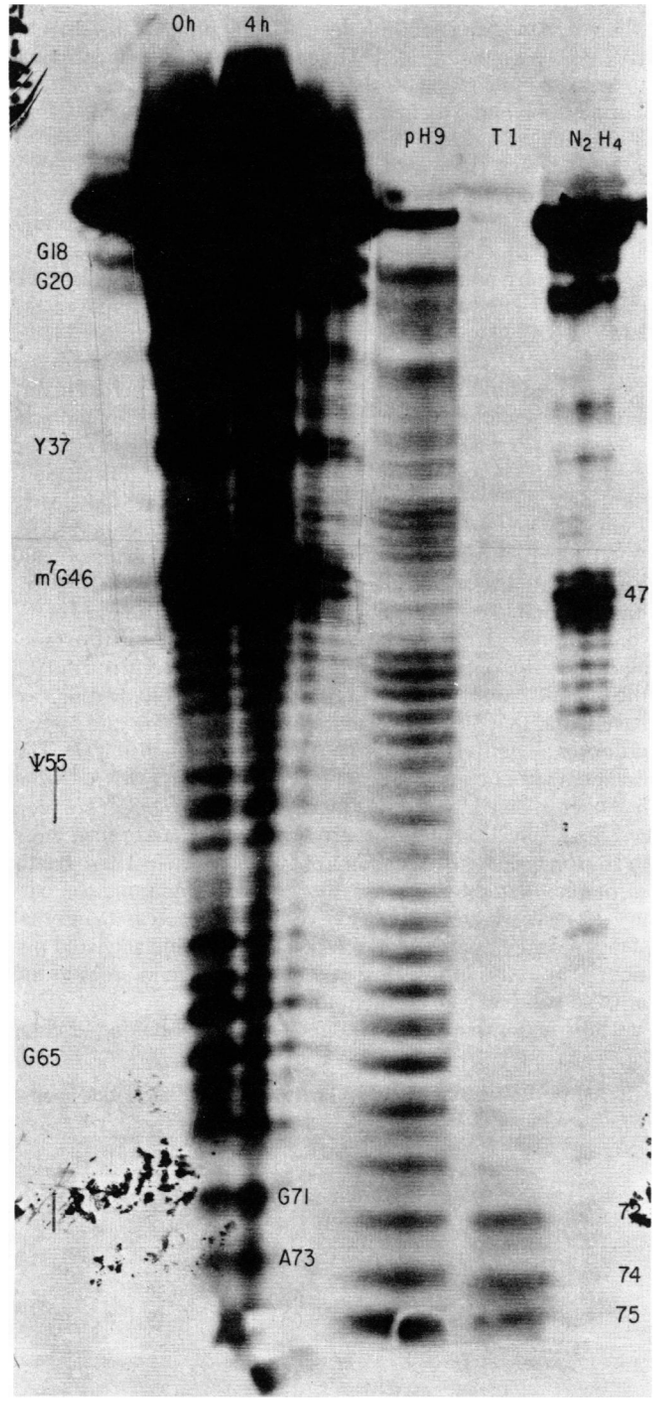

FIG. 3. Autoradiogram of chlorambucilyl-[^{3}H]phenylalanyl-tRNA^{Phe}, analyzed by rapid gel sequence determination as in Fig. 2. The sample in lane 0h was not allowed to crosslink in aqueous buffer, whereas the sample in lane 4h was allowed to crosslink for 4 hr.

3′,5′-bisphosphate (New England Nuclear) (18) was allowed to proceed for 48 hr to allow deacylation to occur in the labeling reaction mixture. This eliminated the need for a separate deacylation step. Gels were autoradiographed with Kodak X-Omat RP film backed by a pair of DuPont Lightning-Plus screens at −70°C. Densities of bands on autoradiograms were quantitated by scanning the films at 580 nm with an ISCO UA-5 detector equipped with a gel scanning accessory on line to a Columbia Scientific CSI-38 integrator.

RESULTS

The reaction pattern of bimolecular chlorambucil alkylation of $tRNA^{Phe}$ is shown in Fig. 2. The more prominent bands appear to be led by "ghost" bands that are one residue shorter, perhaps caused by an impurity in the $tRNA^{Phe}$ preparation that lacks the 3′ terminal adenosine. Within the limits of resolution of the gel autoradiogram, the most reactive nucleotides (Fig. 2, lane Chl) appear to include G-18, G-20, mG-34, and Y-37. Gel autoradiograms run for shorter times also showed reactive nucleotides at G-71, A-73, and A-76.

The pattern of intramolecular crosslinking of chlorambucilyl-[^{3}H]phenylalanyl-$tRNA^{Phe}$ is seen in Fig. 3. A sample of the latter tRNA, which had been modified but had not experienced intramolecular crosslinking in aqueous buffer, was analyzed (Fig. 3, lane 0h) to observe the effects of whatever crosslinking and bimolecular alkylation had occurred during the modification step. [Previously we had observed that about 10% of the modified tRNA appeared to have been crosslinked during modification (7)]. The pattern of reactivity (Fig. 3, lane 0h) closely resembles that for bimolecular chlorambucil alkylation (Fig. 2, lane Chl). Because liquid chromatography on Sephadex G-100 had indicated a lack of intermolecularly crosslinked tRNA dimers (7), and because none were seen on these autoradiograms, some bimolecular alkylation of $tRNA^{Phe}$ by the *N*-hydroxysuccinimide ester of chlorambucil appears to have occurred during the modification reaction. This is not unreasonable, because we have found that 80% Me_2SO inhibits the alkylation rate by two orders of magnitude but does not completely abolish it (7). The fragments from chlorambucilyl-[^{3}H]phenylalanyl-$tRNA^{Phe}$ that had been allowed to crosslink for 4 hr in aqueous buffer are seen in Fig. 3, lane 4h. Bands that are significantly darker than those in lane 0h occur at the positions of G-71 and A-73. These bands were quantitated by scanning the autoradiograms and comparing the peak areas with that of m^7G-46, which acts as an internal standard in Peattie's technique (17) for detecting alkylated purines. The ratios of the alkylated nucleotide band areas to the m^7G-46 band area are presented in Table 1.

The reaction pattern of intramolecular crosslinking of chlorambucilyl-pentadecaprolyl-[^{3}H]phenylalanyl-$tRNA^{Phe}$ is shown in Fig. 4, in which a sample of the modified tRNA that had not been exposed to aqueous buffer after modification (lane 0h) can be compared with a sample that had been allowed to crosslink for 4 hr in aqueous buffer (lane 4h). Bands in lane 4h that are significantly darker than those in lane 0h occur at the positions of G-20 and Y-37. The quantitation of these bands is shown in Table 1.

Table 1. Ratios of intramolecularly crosslinked nucleotide band areas to the m^7G-46 band area

	Band area relative to that of m^7G-46			
	0 prolines*		15 prolines*	
Nucleotide	0 hr†	4 hr†	0 hr†	4 hr†
G18	0.54	0.45	0.40	0.49
G20	0.53	0.49	0.50	0.87
mG34	0.06	0.08	0.19	0.18
Y37	0.24	0.29	0.83	2.25
G71	0.06	0.38	<0.01	<0.01
A73	0.06	0.21	<0.01	<0.01
A76	0.03	0.04	<0.01	<0.01

* Chlorambucilyl-$(prolyl)_n$-[^{3}H]phenylalanyl-$tRNA^{Phe}$ was synthesized with 0 (n = 0) and 15 (n = 15) prolines.

† Length of time of intramolecular crosslinking in aqueous buffer.

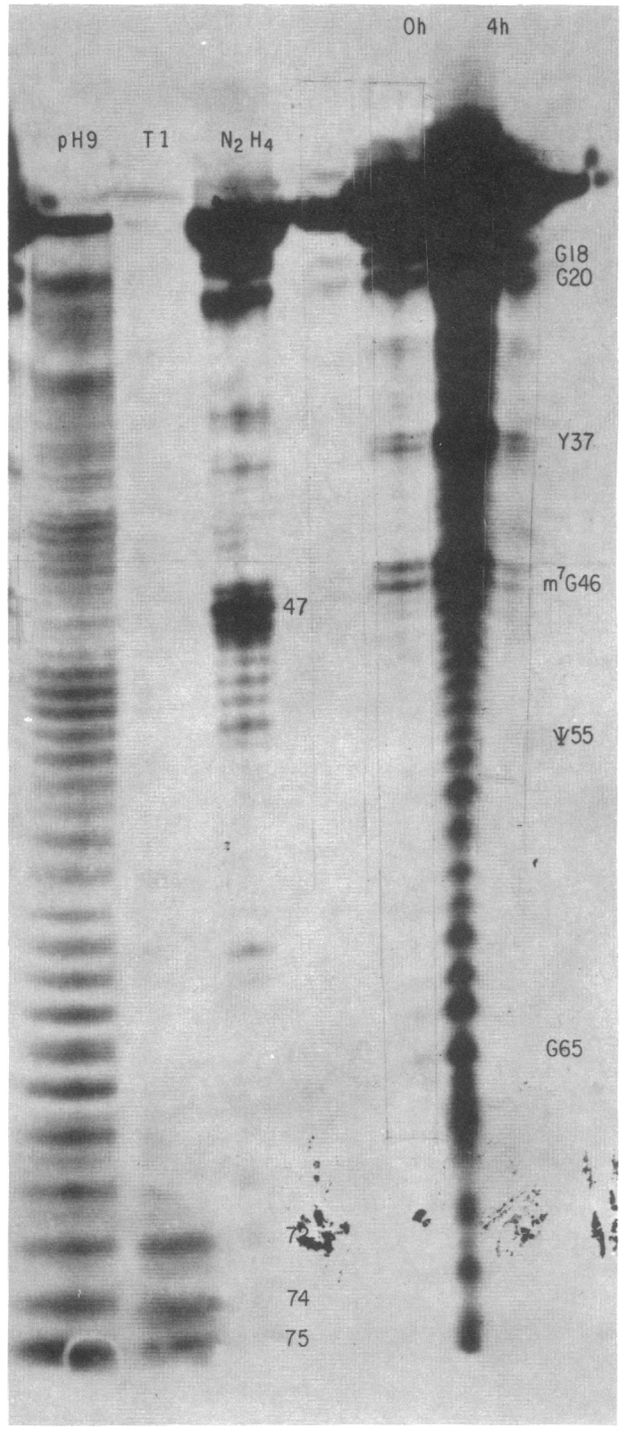

FIG. 4. Autoradiogram of chlorambucilyl-pentadecaprolyl-[^{3}H]-phenylalanyl-$tRNA^{Phe}$, analyzed by rapid gel sequence determination as in Fig. 2. The sample in lane 0h was not allowed to crosslink in aqueous buffer, whereas the sample in lane 4h was allowed to crosslink for 4 hr.

DISCUSSION

The sites of reactivity in yeast $tRNA^{Phe}$ for alkylation by chlorambucil agree reasonably well with the results of other chemical modification experiments (19, 20). Our observation of intramolecular crosslinking to G-71 and A-73 by the molecular ruler with no proline residues is consistent with similar work by Grachev and Rivkin (12) and with the 15-Å length of chlorambucilyl phenylalanine.

Model building and the structure of poly(L-proline) II (9) predict that chlorambucilyl-pentadecaprolyl-phenylalanine should be 62 Å long. It is of interest to ascertain whether our observation of crosslinking to G-20 and Y-37 with the latter molecular ruler is consistent with the observed crystal structure of yeast $tRNA^{Phe}$ (1, 2). Examination of the atomic coordinates in the crystal structure (A. Rich, personal communication) indicates that the N-7 of G-20 is 60.1 Å distant from the 3′ OH, and that the N-12 of Y-37 is 80.7 Å distant.

At first glance, it appears that we have excellent agreement in the case of G-20 but poor agreement for Y-37. However, for tRNA in solution, the dihydrouridine loop at the corner of the L form is relatively rigid because of tertiary structure interactions (2), whereas the anticodon loop is relatively flexible and the aminoacyl A-C-C-A terminus is very flexible. The crystal-structure distance from C-72 to the 3′ OH is 19.1 Å; flexibility of this single-stranded region and the relative flexibility of the anticodon stem and loop with respect to the rest of the tRNA molecule may account for the ability of the 15-proline ruler to reach a site in the solution structure of $tRNA^{Phe}$ that is too far away in the crystal structure. From the point of view of resolution in molecular-ruler distance measurements, it is significant that mG-34 (near Y-37 in the anticodon loop) and G-18 (near G-20 in the dihydrouridine loop) were not significantly crosslinked.

These results may be viewed from two perspectives. If one assumes the oligoproline spacers to exist in the poly(L-proline) II conformation (9), then crosslinking of the 62-Å-long chlorambucilyl-pentadecaprolyl-phenylalanine from the 3′ OH to G-20 and Y-37 confirms that the solution structure of yeast $tRNA^{Phe}$ is reasonably similar to the crystal structure (1, 2). If one assumes in the first place that the crystal structure and solution structure of yeast $tRNA^{Phe}$ are similar (3), then the crosslinking results establish the accuracy of oligoproline molecular rulers for distance measurements.

Note Added in Proof. Since submitting this manuscript, we have found that chlorambucilyl-pentaprolyl-[^{3}H]phenylalanyl-$tRNA^{Phe}$ crosslinks intramolecularly to G-45 (unpublished data). Crosslinking of *Escherichia coli* MRE600 ribosomes with chlorambucilyl-[^{3}H]phenylalanyl-$tRNA^{Phe}$ directed by poly(U) resulted in labeling of 50S ribosomal proteins L4, L18–20, and L26–27 (21) and single-strand RNA but not 23S RNA (unpublished data).

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1. Rich, A. (1978) *Acc. Chem. Res.* **10,** 388–396.
2. Kim, S. H. (1979) in *Transfer RNA: Structure, Properties, and Recognition,* eds. Schimmel, P. R., Söll, D. & Abelson, J. N. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 83–100.
3. Clark, B. F. C. (1978) in *Transfer RNA,* ed. Altman, S. (MIT Press, Cambridge, MA), pp. 14–47.
4. Crothers, D. M. & Cole, P. E. (1978) in *Transfer RNA,* ed. Altman, S. (MIT Press, Cambridge, MA), pp. 196–247.
5. Ofengand, J. (1977) in *Molecular Mechanisms of Protein Biosynthesis,* eds. Weissbach, H. & Pestka, S. (Academic, New York), pp. 8–79.
6. Reid, B. R. (1977) in *Nucleic Acid-Protein Recognition,* ed. Vogel, H. J. (Academic, New York), pp. 375–390.
7. Reuben, M. A., Ainpour, P. R., Hester, H. L., Neveln, V. L. & Wickstrom, E. (1981) *Biochim. Biophys. Acta,* in press.
8. Stryer, L. & Haugland, R. P. (1967) *Proc. Natl. Acad. Sci. USA* **58,** 719–726.
9. Cowan, P. M. & McGavin, S. (1955) *Nature (London)* **176,** 501–503.
10. Steinberg, I. Z., Harrington, W. F., Berger, A., Sela, M. & Katchalski, E. (1960) *J. Am. Chem. Soc.* **82,** 5263–5279.
11. Schimmel, P. R. & Flory, P. J. (1967) *Proc. Natl. Acad. Sci. USA* **58,** 52–59.
12. Grachev, M. A. & Rivkin, M. I. (1975) *Nucleic Acids Res.* **2,** 1237–1260.
13. Robertus, J. D., Ladner, J. E., Rhodes, D., Brown, R. S., Clark, B. F. C. & Klug, A. (1974) *Nature (London)* **250,** 546–551.
14. Ainpour, P. R. & Wickstrom, E. (1980) *Int. J. Pept. Protein Res.* **15,** 225–235.
15. Reuben, M. A., Kusnezov, I. J. & Wickstrom, E. (1979) *Biochim. Biophys. Acta* **565,** 219–223.
16. Wickstrom, E. & Yarus, M. J. (1975) *Abstracts of the 170th National Meeting of the American Chemical Society,* Biological Chemistry Division (Am. Chem. Soc., Washington, DC), Abstr. 127.
17. Peattie, D. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1760–1764.
18. Bruce, A. G. & Uhlenbeck, O. C. (1978) *Nucleic Acids Res.* **5,** 3665–3677.
19. Robertus, J. C., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. F. C. & Klug, A. (1974) *Nucleic Acids Res.* **1,** 927–932.
20. Peattie, D. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4679–4682.
21. Wickstrom, E., Parker, K. K., Hursh, D. A. & Newton, R. L. (1981) *FEBS Lett.,* in press.