ON THE CONFORMATION OF TRANSFER RNA

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The determination of base sequences of several transfer ribonucleic acids has led to proposals for their secondary structure. The most popular structural model has had the form of a cloverleaf.[1] There have also been some indications of an additional fixation of the arms of the model in a tertiary structure. So far there is no direct experimental proof for any of these structures, although investigations have included studies of sedimentation, hyperchromicity, viscosity, and kinetics of enzymatic degradation.[2] Recently, methods of nuclear magnetic resonance (NMR),[3, 4] circular dichroism (CD),[5] and optical rotatory dispersion (ORD) have been applied.[6–8]

Attempts have been made to draw conclusions about the secondary and/or tertiary structure from chemical modifications of tRNA. The specific cyanoethylation of pseudouridine[9] in the Gp-Tp-Ψp-Cp-Gp loop occurs only at low ionic strength. Water-soluble carbodiimide reacts preferentially with certain regions in $tRNA^{ala}_{yeast}$, an indication that these are exposed.[10] At elevated temperatures, additional regions become exposed and available to the reagent.

Selective N-oxidation of adenosine to adenosine-1-N-oxide in polynucleotides is possible with monoperphthalic acid and can be followed easily by changes in ultraviolet absorption. The reaction takes place only at the non-hydrogen-bonded N-1-nitrogen of adenosine. Therefore, since this method discriminates between base-paired and non-base-paired adenosines, it can be used to determine the structure of tRNA. The validity of this method has been proved in several investigations.[11]

We now wish to propose a general structure for the conformation of tRNA molecules. This structure is compatible with physicochemical, chemical, and biochemical evidence.[12]

Materials and Methods.—$tRNA^{phe}_{yeast}$ and $tRNA^{ser}_{yeast}$ were obtained from crude tRNA (Boehringer: Mannheim, Germany) by combining extraction[13] with chromatography on benzoylated O-diethylaminoethyl (DEAE) cellulose[14] and DEAE-Sephadex.[15, 16] Brewers' yeast and bakers' yeast were used. Chargeability was 82% with both $tRNA^{phe}_{yeast}$, 58% with $tRNA^{ser}_{br.\ yeast}$, and 89% with $tRNA^{ser}_{ba.\ yeast}$. The tRNA's were pure with respect to 15 other amino acids tested (C^{14} L amino acid, kit B: Schwarz Bio-Research, Orangeburg, N.J.). $tRNA^{bulk}_{E.\ coli}$ was a product of CalBiochem, Los Angeles, Calif. The following enzymes were used: pancreatic RNase, snake venom phosphodiesterase, and alkaline phosphomonesterase from *E. coli* (Boehringer: Mannheim, Germany); T_1RNase (Sankyo: Tokyo, Japan). Spleen phosphodi- and phosphomonoesterase were prepared in our laboratory by Dr. H. Sternbach.

Aminoacyl-tRNA synthetases (E.C.6.1.1.): These were prepared from bakers' yeast (Langemeyer: Mettingen, Germany) by a modification of the procedure of Makman and Cantoni.[17] Seryl- and phenylalanyl-tRNA synthetases were both purified 350-fold.[18] Charging experiments were carried out as described elsewhere.[24]

Hyperchromicity: The measurements were taken on a Gilford type 2000 recording spectrophotometer with a temperature controlled cell-compartment.

N-oxidation with monoperphthalic acid: tRNA (0.06 μmole, 40 OD units) was dissolved in 0.15 ml of water, and this solution was mixed with 0.1 ml of 2 *M* phosphate buffer, pH 7.0. To this was added 0.25 ml of a neutralized aqueous solution containing 20 μmoles of monoperphthalic acid. The solution of monoperphthalic acid was prepared as described earlier.[11] The solution was kept at 40°C for 1 hr, or at 20°C for 6 hr. To recover tRNA, the reaction solution was passed through a 3 × 30 cm Sephadex G25 column, using water as eluant, and then freeze-dried.

Brutto base analysis was done by hydrolysis with 0.3 *M* KOH, and the bases were estimated by the method of Katz and Comb.[19]

Enzymatic digests of $tRNA^{phe}_{yeast}$ *after N-oxidation:* For the evaluation of oxidizable adenosines, the $tRNA^{phe}_{yeast}$ was split after oxidation with pancreatic RNase alone or combined with T_1 RNase. Usually 400 optical density (OD) units were digested in 6 ml of 0.02 *M* phosphate buffer, pH 7.2, at 37° for 7–9 hr with 300 units of T_1 and/or 500 μg of pancreatic RNase.

Separation of oligonucleotides was achieved by chromatography on 1.5 × 45 cm DEAE-cellulose columns (CO_3^{--} form). Elution was carried out with the use of two gradients. To work up the combined digestion with T_1 and pancreatic RNase, the first gradient was prepared from 300 ml each of 0.01 *M*, 0.08 *M*, and 0.17 *M* $(NH_4)_2CO_3$. The second gradient was prepared from 300 ml each of 0.15 *M* and 0.55 *M* $(NH_4)_2CO_3$.

For the separation of the digestion products of pancreatic RNase, the first gradient was the same as in the combined digest. The second gradient was prepared from 750 ml each of 0.15 *M* and 0.8 *M* $(NH_4)_2CO_3$. After separation, the A-1-N-oxide containing oligonucleotides could easily be detected by their characteristic UV spectrum. These were freed from ammonium carbonate and water by freeze-drying and analyzed as described.[21]

Results.—N-oxidation of tRNA: When bulk tRNA is oxidized at room temperature in 0.4 *M* phosphate buffer, a plateau is reached which is altered neither by prolonged reaction time nor by the addition of a larger excess of reagent. In $tRNA^{bulk}_{yeast}$ 27 per cent and in $tRNA^{bulk}_{E.\ coli}$ 36 per cent of the adenosine residues are oxidized. Assuming a random distribution of all four bases, one calculates an average per molecule of 28.1 base pairs in yeast tRNA (19.8% A, average chain length 77) and 23.5 base pairs in *E. coli* tRNA (20.5% A, average chain length 73). The reaction was also carried out at 20° and 40° with $tRNA^{bulk}_{yeast}$ and $tRNA^{phe}_{yeast}$ (Table 1).

TABLE 1. *Oxidation of yeast tRNA's at 20°C and 40°C.*

tRNA	Temperature (°C)	Per cent adenosine oxidized	Number of A-residues oxidized
Bulk	20	27	4.1 (average)
	40	39	6.0 (average)
Phe	20	22	4.0
	45	22	4.0

These results indicate that in bulk tRNA, two adenosine residues are involved in a structure which is lost prior to 40°C. Since tRNA contains, on the average, 20 per cent adenosine, ten bases (five base pairs) may be involved in this folding. In pure $tRNA^{phe}_{yeast}$, in contrast, no additional A residues become available at 40°C or even 45°C.

Hyperchromicity and chemical reactivity: Figure 1 shows the melting curve for $tRNA^{ser}_{yeast}$. The T_m of the first transition is 39°C. The second is 63°C. A two-step melting profile also is exhibited by other purified tRNA's, e.g., $tRNA^{leu}_{yeast}$,[2] $tRNA^{tyr}_{yeast}$,[20] $tRNA^{ala}_{yeast}$,[20] and $tRNA^{val}_{yeast}$.[20] However, $tRNA^{phe}_{yeast}$ exhibits a sharp

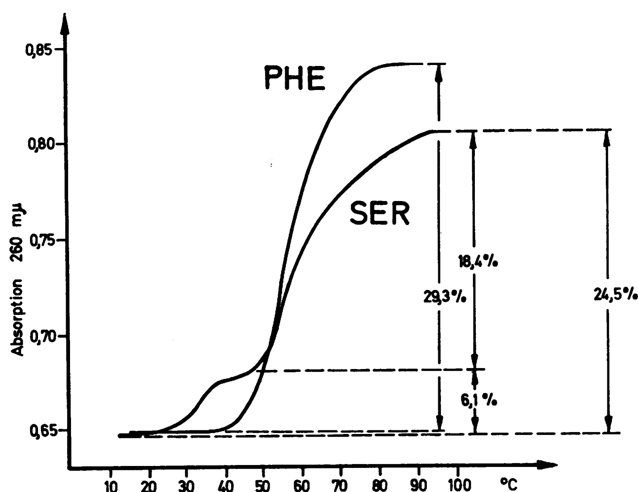

FIG. 1.—Melting curve of $tRNA^{ser}_{yeast}$ and $tRNA^{phe}_{yeast}$ in 0.4 *M* phosphate buffer at pH 7.0.

one-step melting profile with T_m 57° (Fig. 1). One obtains a linear plot by charting the chemically determined extent of double-strandedness of bulk tRNA's versus the total hyperchromicity (Fig. 2). Extrapolation to 100 per cent double-strandedness leads to the hyperchromicity value characteristic for a fully base-paired DNA molecule. Because of this relation, one can deduce the extent of the double-stranded regions in tRNA molecules by measuring hyperchromicity.

Splitting pattern of $tRNA^{phe}_{yeast}$: The oligonucleotide pattern exhibited by the pancreatic RNase digest of oxidized $tRNA^{phe}_{yeast}$ was, with one exception, identical to that of the nonoxidized molecule.[21] The hexanucleotide 2′OMeG A A Y A ψ did not appear, but in its place was found a tetranucleotide, 2′OMeG Aox Aox Y′, and a dinucleotide. This is in agreement with the fact that the unknown base Y on chemical treatment is converted to Y′, which, in a pancreatic RNase digestion, behaves like a pyrimidine.[21] Furthermore, there is no nucleoside A from 3′ end but only the N-oxidized nucleoside A-1-N-oxide. From this, it follows that only the A residues in or adjacent to the anticodon (A35, A36, A38) and the 3′-terminal A (A76) are oxidized; the 14 other A's remain unchanged.

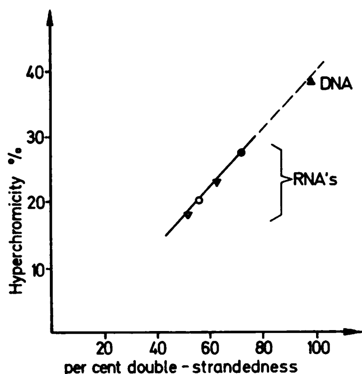

FIG. 2.—Relation between hyperchromicity and double-strandedness as determined by N-oxidation: $tRNA^{bulk}_{E. coli}$ native (▽) and thermally denatured (100°, 2 days) (▼); $tRNA^{bulk}_{yeast}$ native (○) and thermally denatured (100°, 2 days) (●).[28]

Charging experiments at different temperatures: Charging of $tRNA^{ser}_{yeast}$ and $tRNA^{phe}_{yeast}$ was done in the temperature range between 15° and 45°. The rate of aminoacylation is plotted versus the reaction temperature in Figure 3. For $tRNA^{ser}_{yeast}$ the rate drops be-

tween 37° and 40° to a lower plateau. The corresponding melting curves are also given in Figure 3. As can be seen, the melting curves in each case are reciprocal to the charging curves. $tRNA^{phe}_{yeast}$, which has a high melting point and no low-temperature step in the melting profile, does not exhibit the two-step temperature/rate profile. In separate experiments it could be shown that, up to 43°, the ser enzyme was fully stable under the conditions of the reaction; the phe enzyme is stable up to 38.5°. The preceding aminoacyl adenylate formation, as measured by pyrophosphate formation,[18] shows a normal linear temperature/rate profile up to 45° (ser) and 50° (phe).

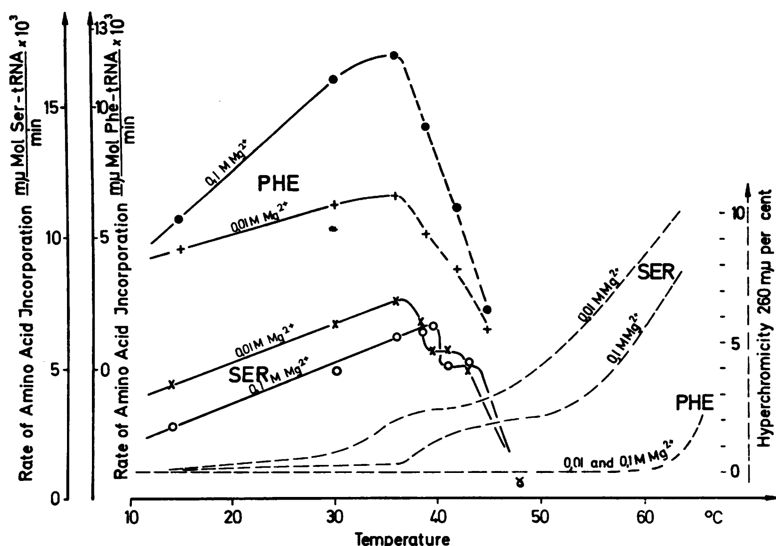

Fig. 3.—Temperature dependence of the rate of amino acylation and of hyperchromicity in $tRNA^{phe}_{yeast}$ and $tRNA^{ser}_{yeast}$, charging procedure as described.[24] Hyperchromicity: 0.15 *M* Tris-HCl, pH 7.4, 0.01 *M* Mg^{++} or 0.1 *M* Mg^{++} (- - -); $tRNA^{ser}_{yeast}$ T_m, 69.5° (0.01 *M* Mg), 74.5° (0.1 *M* Mg); $tRNA^{phe}_{yeast}$ T_m, 75.0° (0.01 *M* Mg), 78.0° (0.1 *M* Mg).

Discussion.—Henley *et al.*[22] and Sarin *et al.*[7] were able to show that several physical properties of tRNA are altered between 20° and 40°. The changes are mainly in hydrodynamical properties, whereas the changes in ultraviolet extinction in this region are relatively small. These results suggest that a breakdown of tertiary structure occurs during the thermal denaturation of most tRNA's between 20° and 40°. Above 40°, secondary structures will melt (e.g., $tRNA^{ser}_{yeast}$) or tertiary and secondary structures will melt cooperatively (e.g., $tRNA^{phe}_{yeast}$). The loss of the secondary structure is accompanied by a strong increase in optical density. Because of the relationship between the extent of N-oxidation and hyperchromicity (Fig. 2), it is possible to interpret the melting curve of $tRNA^{ser}_{yeast}$ (Fig. 1) quantitatively. $tRNA^{ser}_{yeast}$ has 24 base pairs in its cloverleaf. Assuming that the 18.4 per cent hyperchromicity of the second step in the melting curve corresponds to the melting of the 24 base pairs, then the 6.1 per cent hyperchromicity of the first step would correspond to 8 base pairs, or base-pair equivalents, for the tertiary structure of $tRNA^{ser}_{yeast}$. In bulk tRNA, two A residues be-

come exposed at 40°, corresponding on the average to a melting of five base pairs. Moreover, $tRNA^{phe}_{yeast}$ exhibits a sharp one-step melting above 50° (Figs. 1 and 3), and shows no difference between 20° and 40° in the amount of oxidizable A residues (= 4). Thus $tRNA^{phe}_{yeast}$ has a rather stable tertiary structure that melts, in a cooperative manner, together with the secondary structure.

How can a tertiary structure be formed from the cloverleaf using the numbers of base pairs estimated above? In all tRNA's known so far, it is possible to fold together the arms of the cloverleaf by hydrogen-bonding the ΨC in the TΨ-loop with the AG in the dihydro-U-loop, as well as the GG in the dihydro-U-loop with the CC at the CCA end (Fig. 4). In $tRNA^{ser}_{yeast}$ there is an additional possibility of folding the UU of the extra loop to the AA in the dihydro-U-loop. The results of the N-oxidation are in agreement with these proposals for the secondary and tertiary structures.

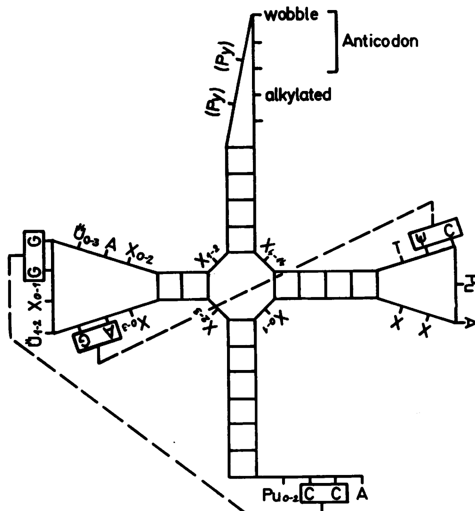

FIG. 4.—Schematic general cloverleaf structure of the known yeast tRNA's. The anticodon loop is drawn according to the model of Fuller and Hodgson.[23] The broken lines connect the eight bases which are proposed to form base pairs in the basic tertiary structure.
(*X* = any nucleoside, *Pu* = purine nucleoside, *Py* = pyrimidine nucleoside, *A* = adenosine, *G* = guanosine, *C* = cytidine, *U* = uridine, *T* = thymidine, $\overset{H}{U}$ = dihydrouridine, and *ψ* = pseudouridine.)

The results of the N-oxidation of $tRNA^{phe}_{yeast}$—showing that only the four A residues [A35, A36, A38, and A76 (3′-terminus)] are unpaired—lead to a model with the highly ordered structure depicted in Figure 5(*a* and *b*). The anticodon arm is directed away from the other three arms of the cloverleaf, which are folded tightly together. In this model additional AU pairs are formed between A9 and U47, A14 and Ψ55, A21 and U59, A44 and U8, and A73 and T54. Additional GC pairs are formed between G10 and C25, G15 and C56, G18 and C75, G19 and C74, G45 and C48, and Ġ57 and C60. Such a structure was built in a 2.0 cm/Å scale (Cambridge Repetition Engineers Ltd., wire models). In this, all the above base pairs are possible and at the same time a maximum of stacking is achieved. A simplified model, though correct in scale (0.5 cm/Å), can be obtained by a combination of LEGO®-toy building blocks (Fig. 5*b*).

It is still not known how the aminoacyl-tRNA synthetase recognizes its specific tRNA. Probably the enzyme cannot recognize the anticodon and relate

it to the CCA end.[25] No specific sequence in any part of the molecule has been found that would be typical for amino acid recognition. Since in the proposed model the tertiary structure for each tRNA is unique, one is led to the hypothesis that recognition may be related to tertiary structure.

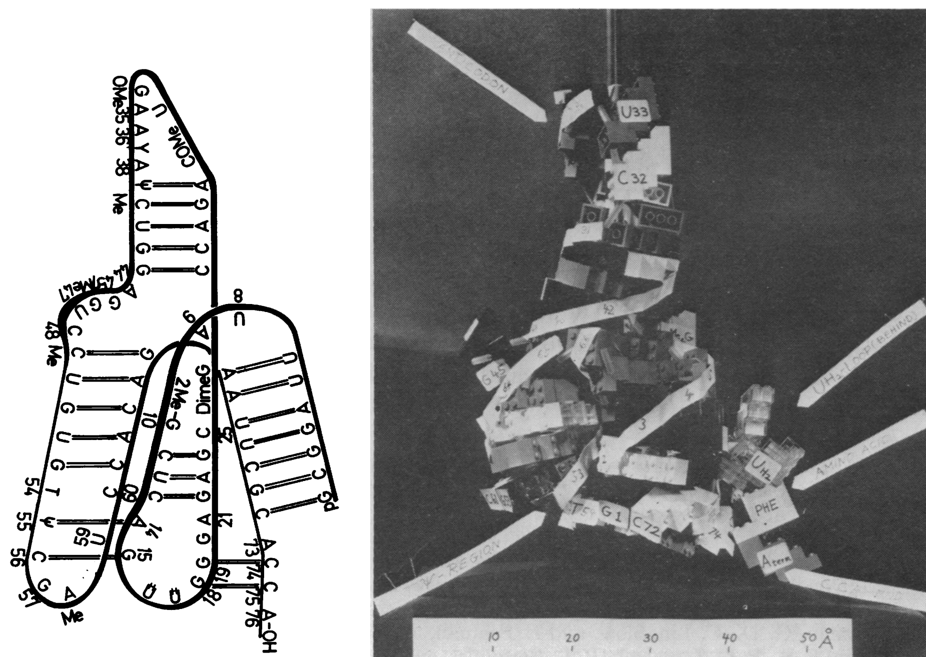

FIG. 5.—(*a*) Two-dimensional projection of the proposed tertiary structure of $\mathrm{tRNA}^{\mathrm{phe}}_{\mathrm{yeast}}$. The over-all length is about 70 Å if the anticodon arm is extended. The width is approximately 44 Å. *Y* = unknown nucleoside, *MeA* = N^1-methyl-adenosine, *7MeG* = N^7-methyl-guanosine, *2MeG* = N^2-methyl-guanosine, *DimeG* = N^2-dimethyl-guanosine, *OMeG* = $O^{2'}$-methyl-guanosine, *MeC* = 5-methylcytosine, and *OMeC* = $O^{2'}$-methylcytosine.[29]

(*b*) Three-dimensional model of the proposed tertiary structure of $\mathrm{tRNA}^{\mathrm{phe}}_{\mathrm{yeast}}$ made from LEGO-blocks (registered trademark).

In comparing the different tRNA's, one finds that the possible variable elements (with exception of the anticodon) are situated only in the central part of the molecule and in the dihydro-U-loop. In the proposed model, the constant double-helical regions form a trigonal prism from which the anticodon loop extends in the opposite direction (Fig. 5). The CCA end, to be charged, is fixed in a specific geometry and held rigidly in place. The total geometry of the molecule in the tertiary structure, then, is determined by the size of the dihydro-U-loop and the central part. In order to establish the relationship of tertiary structure to recognition, we studied the dependence on temperature of the rates of charging the various tRNA's with their amino acids.[18] These experiments were carried out in the region of the lower melting step of the tRNA (Fig. 3).

The charging of $\mathrm{tRNA}^{\mathrm{ser}}_{\mathrm{yeast}}$ shows a two-step temperature profile with a T_m of 39° in 0.01 *M* Mg^{++}, whereas the hyperchromic melting indicates a T_m of 35°.

Upon an increase in the Mg^{++} concentration from 0.01 to 0.1, both the T_m of charging and the T_m of melting are raised. The effect cannot be due to the aminoacyl-adenylate formation, since the rate of pyrophosphate formation shows a linear temperature dependence up to 45°.[18] The final decrease in the rate of amino acid incorporation is due to the inactivation of aminoacyl synthetase which takes place above 43° for serine and above 38.5° for phenylalanine.

The behavior of $tRNA^{ser}_{yeast}$ with respect to the aminoacyl synthetase is reminiscent of the temperature dependence of the phosphorolysis of $tRNA^{ser}_{yeast}$.[26]

From the column and sedimentation behavior, one can conclude that uncharged tRNA is most compact, aminoacyl-tRNA slightly unfolded, and peptidyl-tRNA even more unfolded.[27] The flexible geometry of tRNA tertiary structure seems to meet functional requirements. As discussed above, rigid geometry in tertiary structure is probably required for recognition. In peptidization, on the other hand, an exposed CCA amino acid end might be necessary to transfer the peptide. The anticodon should, at the same time, be bound to the messenger. Other parts of the molecule might be bound to particular ribosomal sites. Many of these functional configurations can be derived from the proposed general conformation of tRNA.

[1] For a summarizing article see: Zachau, H. G., D. Dütting, H. Feldmann, F. Melchers, and W. Karau, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966) p. 417.

[2] For reference see: Fresco, J. R., A. Adams, R. Ascione, D. D. Henley, and T. Lindahl, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966) p. 527.

[3] McTague, J. P., V. Ross, and J. H. Gibbs, *Biopolymers*, **2**, 163 (1964).

[4] McDonald, C. C., W. D. Phillips, and J. Penswick, *Biopolymers*, **3**, 609 (1965).

[5] Brahms, J., W. H. F. M. Mommaerts, and Ch. Sadron, *Compt. Rend.*, **258**, 2203 (1964).

[6] Fasman, G. D., C. Lindblow, and E. Seaman, *J. Mol. Biol.*, **12**, 630 (1965).

[7] Sarin, P. S., P. C. Zamecnik, P. L. Bergquist, and J. F. Scott, these PROCEEDINGS, **55**, 579 (1966).

[8] Cantor, C. R., S. R. Jaskunas, and I. Tinoco, Jr., *J. Mol. Biol.*, **20**, 39 (1966).

[9] Yoshida, M., and T. Ukita, *Biochim. Biophys. Acta*, **123**, 214 (1966).

[10] Brostoff, S. W., and V. M. Ingram, *Science*, **158**, 666 (1967).

[11] (*a*) Seidel, H., and F. Cramer, *Biochim. Biophys. Acta*, **108**, 367 (1965); (*b*) Doepner, H., H. Seidel, and F. Cramer, *Angew. Chem.*, **78**, 601 and 682 (1966); *Angew. Chem. Intern. Ed. Engl.*, **5**, 591 and 671 (1966); and (*c*) Cramer, F., and V. A. Erdmann, *Nature*, **218**, 92 (1968).

[12] Cf. Cramer, F., *Angew. Chem.*, **79**, 653 (1967); *Angew. Chem. Intern. Ed. Engl.*, **6**, 642 (1967) reported at the 7th International Congress of Biochemistry in Tokyo, August 1967; Doepner, H., H. Seidel, F. v.d. Haar, E. Schlimme, and F. Cramer, results presented at the Fourth Meeting, Federation of European Biochemical Societies, Oslo, July 1967.

[13] Khym, J. X., *Biochemistry*, **2**, 401 (1963); Khym, J. X., *J. Biol. Chem.*, **240**, PC 1488 (1965).

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

[15] Cherayil, J. D., and R. M. Bock, *Biochemistry*, **4**, 1174 (1965).

[16] v.d. Haar, F., unpublished results.

[17] Makman, M. H., and G. L. Cantoni, *Biochemistry*, **4**, 1434 (1965).

[18] Schlimme, E., F. v. d. Haar, and F. Cramer, in preparation.

[19] Katz, S., and D. G. Comb, *J. Biol. Chem.*, **238**, 3065 (1963).

[20] Fresco, J. R., L. C. Klotz, and E. G. Richards, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 83.

[21] RajBhandary, U. L., R. D. Faulkner, and A. Stuart, *J. Biol. Chem.*, **243**, 575 (1968).

[22] Henley, D. D., T. Lindahl, and J. R. Fresco, these PROCEEDINGS, **55**, 191 (1966).

[23] Fuller, W., and A. Hodgson, *Nature*, **215**, 817 (1967).

[24] Neuhoff, V., F. v. d. Haar, E. Schlimme, and M. Weise, *Hoppe-Seylers Z. Physiol. Chem.*, in press.

[25] Nishimura, S., and G. D. Novelli, these PROCEEDINGS, **53**, 178 (1965); Venkstern, T. V., A. A. Bajev, A. D. Mirsabekov, A. I. Krutilina, L. Li, and V. D. Axelrod, results presented at the Fourth Meeting, Federation of European Biochemical Societies, Oslo, July 1967.
[26] Thang, M. N., W. Guschlbauer, H. G. Zachau, and M. Grunberg-Manago, *J. Mol. Biol.*, **26**, 403 (1967).
[27] Littauer, U. Z., and R. Stern, results presented at the Fourth Meeting, Federation of European Biochemical Societies, Oslo, July 1967; Kaji, H., and Y. Tanaka, *Biochim. Biophys. Acta*, **138**, 642 (1967).
[28] Lindahl, T., *J. Biol. Chem.*, **242**, 1970 (1967).
[29] RajBhandary, U. L., S. H. Chang, A. Stuart, R. D. Faulkner, R. M. Hoskinson, and H. G. Khorana, these PROCEEDINGS, **57**, 751 (1967).