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Chemical Modification of the Fluorescent Base in Phenylalanine Transfer Ribonucleic Acid*

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ABSTRACT: The tRNA^{Phe} of wheat germ, tRNA$_1^{Phe}$, exhibits fluorescence in solution due to the presence of a fluorescent base, Y_w^1, adjacent to the 3′ end of the anticodon. When this tRNA was exposed to ammonium carbonate at pH 9, it was converted into tRNA$_2^{Phe}$ which exhibits the same fluorescence but is chromatographically distinct from tRNA$_1^{Phe}$. The conversion was due to the modification of Y_w^1 to a new fluorescent base, Y_w^2, which has a free acidic group (pK_a = about 4) not present in Y_w^1. Thus at around neutrality tRNA$_2^{Phe}$ has an acidic group with a net negative charge on the base adjacent to the 3′ end of the anticodon; in every other respect it is identical with tRNA$_1^{Phe}$. The specific modification had no effect upon the rate at which the tRNA was esterified by the Phe-tRNA synthetase, but it reduced the rate of poly(U)-directed polyphenylalanine synthesis. The free base Y_w^2 can be further degraded by alkaline hydrolysis to Y_w^3 and then to Y_w^4 without any change in the spectrum of the fluorescent chromophore.

In the conversion of Y_w^3 into Y_w^4 a blocking group is removed from an aliphatic amino group on a side chain. These results indicate that Y_w^1 is similar to the Y base of yeast in having a blocked amino acid side chain on the characteristic Y base chromophore. This paper presents evidence that Y_w^1 differs from the Y base of yeast in the structure of the distal portion of the side chain. The Y base of beef tRNA^{Phe} is indistinguishable from that of wheat germ. A simple procedure was found for purifying wheat germ and yeast tRNA^{Phe} employing two benzoylated DEAE-cellulose columns, one run in the presence of unbuffered $MgCl_2$ and one in the presence of EDTA buffered at pH 4.5.

The major phenylalanine tRNAs (tRNA^{Phe}s) isolated so far from eukaryotic organisms have all been distinguished by the presence of an unusually hydrophobic and highly fluorescent base, the Y-type base. A base of this type was first detected in the tRNA^{Phe} of yeast by RajBhandary *et al.* (1967) and was called Y. It was found to be located contiguous with the 3′ end of the anticodon. A related Y base was found in the same position in the structure of the tRNA^{Phe} of wheat germ by Dudock *et al.* (1969). A Y-type base is present, presumably in the same location, in the tRNA^{Phe}s from rat liver (Fink *et al.*, 1968), beef liver (Yoshikami *et al.*, 1968), and peas (G. A. Everett, personal communication). The Y base has not been detected in any other species of tRNA other than tRNA^{Phe} (Yoshikami *et al.*, 1968). The Y base thus appears to have a role unique to the function of the tRNA^{Phe}s of eukaryotes, yet it is not an essential feature of tRNA^{Phe} in general since it is not present in the tRNA^{Phe} of *Escherichia coli* (Barrell and Sanger, 1969).

Other tRNAs exhibit, in the same locus adjacent to the 3′ end of the anticodon, a wide variety of hypermodified residues (Schweizer *et al.*, 1969) such as 1-methylinosine (Holley *et al.*, 1965), N^6-isopentenyladenosine (Biemann *et al.*, 1966), N^6-isopentenyl-2-methylthioadenosine (Burrows *et al.*, 1968), and *N*-(purin-6-ylcarbamoyl)threonine ribonucleoside (Schweizer *et al.*, 1969). It has been found that most of the tRNAs which have an A as the 3′ base of the anticodon possess a hypermodified residue such as N^6-isopentenyladenosine or N^6-

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isopentenyl-2-methylthioadenosine (Nishimura *et al.*, 1969; Peterkofsky and Jesensky, 1969; Rosenberg and Gefter, 1969).

To investigate the role of the hypermodified base adjacent to the anticodon, we undertook a study of the $tRNA^{Phe}$ of wheat, beef, and yeast, all of which contain a Y base in this position. In the course of the study of the $tRNA^{Phe}$ of wheat germ it was found that the Y base can be chemically modified *in situ* under conditions sufficiently mild that other bases in the tRNA remain unaffected. This paper presents the conditions for this specific modification, evidence for the nature of the modification, and a study of the effect of this modification upon the functional activity of the tRNA.

Materials

Commercially processed raw wheat germ (*Triticum durum*) was purchased from Shiloh Farms, Sherman, N. Y., and was stored at 4° in plastic bags. Active tRNA, enzymes, and ribosomes were obtained from this material even after storage for over 2 years. tRNA was obtained from the wheat germ by the procedure described by Dudock *et al.* (1969). Yeast tRNA was isolated from baker's yeast (Fleischmann, *Saccharomyces cerevisiae*) by the procedure of Holley (1964). Beef liver tRNA was prepared according to Brunngraber (1962). The procedures for preparing purified soluble enzymes from wheat germ, yeast, and beef liver and for preparing wheat germ ribosomes are given by Yoshikami (1970) and are only slightly modified from the procedures used by Leis and Keller (1971).

DEAE-cellulose, type No. 70, capacity 0.9 mequiv/g, was purchased from Carl Schleicher and Schuell Co., Keene, N. H. Benzoylated DEAE-cellulose (BD-cellulose),[1] prepared by the method of Gillam *et al.* (1967), was a gift from Dr. B. S. Dudock. Sephadex G-100 and G-25 were from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Cellulose thin-layer chromatographic plates (nonfluorescent) were purchased from Analtech, Inc., Wilmington, Del., and Brinkman Instruments, Westbury, N. Y.

RNase T_1 (EC 2.7.7.26) prepared by Sankyo, Ltd., Tokyo, was purchased from Calbiochem, Los Angeles, Calif. [^{14}C]-Phenylalanine was obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. Poly(U) with a sedimentation coefficient of 8.4 S or a number-average molecular weight of about 500,000 (Moore, 1966) was purchased from Miles Chemical Co., Elkhart, Ind.

Methods

Ultraviolet Spectroscopic Analysis. RNA concentrations were measured by absorbance at 260 nm (A_{260}) with a 1-cm light path in neutral solutions containing 10 mM $MgCl_2$. Amounts of RNA are expressed in A_{260} units: 1 A_{260} unit of RNA in 1 ml of solution gives an A_{260} reading of 1. For pure $tRNA^{Phe}$, 1 A_{260} unit was taken to be equal to 1.83 nmoles of $tRNA^{Phe}$ (Wimmer *et al.*, 1968).

Fluorescence Assays. All fluorescence measurements were conducted at room temperature in an Aminco-Bowman spectrophotofluorimeter fitted with a xenon arc lamp and an RCA IP-21 photodetector. Standard quartz 1 × 1 cm path-length cuvets were used. Excitation at 310 nm was used for all measurements in order to minimize inner filter effects due to the RNA. A minimum number of collimating slits was used, one 1.55-mm entrance slit and one 0.8-mm exit slit, thus sacrificing spectral resolution for sensitivity. To further maximize sensitivity, two stainless steel mirrors were inserted behind the cuvet. The fluorescence intensity reading obtained under these conditions at an emission wavelength of 440 nm is called F_{440}. The F_{440} values reported here were corrected for solvent blanks.

Column Chromatography. The procedures for chromatography of tRNAs on BD-cellulose columns were adaptations of Gillam *et al.* (1967). Oligonucleotides were fractionated on DEAE-cellulose columns in the presence of 7 M urea (Tomlinson and Tener, 1962). The columns (0.4 × 60 cm) were equilibrated with 7 M urea–20 mM Tris·HCl (pH 8 or 8.5). The sample and developing solutions were pumped through the column with a peristaltic pump at a rate of about 0.5 ml/min. The A_{260} of the effluent was monitored on a Gilford recorder Model 2000, Gilford Instrument Inc., Oberlin, Ohio.

Cellulose Thin-Layer Chromatography. Chromatography was carried out at room temperature. Solvents used were as follows (all ratios are expressed in volumes): I, isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2); II, isobutyric acid–concentrated NH_4OH–H_2O (50:2:28); and III, 1-butanol–concentrated formic acid–H_2O (7:1:2).

Electrophoresis. Flat-bed paper electrophoresis was carried out on Whatman No. 1 paper. Samples were wet spotted along with standards and subjected to a field of about 40 V/cm for about 30 min at 15 to 20°. Final mobilities were calculated relative to the mobility of cytidine at pH 1–3 after corrections for electroendosmosis. Solutions used were: distilled water adjusted to pH 1 with HCl; 10% acetic acid adjusted to pH 2 with formic acid; 0.5% NH_4OH adjusted to pH 3 with formic acid; 0.4% NH_4OH adjusted to pH 4 with formic acid; 0.4% NH_4OH adjusted to pH 5 with acetic acid; 0.5% acetic acid adjusted to pH 6 with pyridine; 0.2% acetic acid adjusted to pH 6.5 with pyridine; 0.05 M $NaHCO_3$, pH 8.5; 0.05 M Na_2CO_3 adjusted to pH 10 with 0.05 M $NaHCO_3$; and 0.05 M Na_2CO_3, pH 11.1.

Conversion of $tRNA_1^{Phe}$ into $tRNA_2^{Phe}$ by Treatment with Ammonium Carbonate. Each sample (less than 300 A_{260} units) of $tRNA_1^{Phe}$ was dissolved in 1.5 ml of water. To this was added 1.5 ml of 1 M ammonium carbonate. The final pH was 9. About 20 μl of $CHCl_3$ was added to the solution to prevent bacterial growth, and the mixture was incubated at 42° for the desired time. The reaction was terminated by precipitating the tRNA with 6 ml of absolute ethanol. The precipitate was dissolved in 3 ml of water, 0.5 ml of 2 M NaCl was added, and the tRNA was precipitated with 6 ml of absolute ethanol and desiccated.

Excision of the Y Base. Thiebe and Zachau (1968) were the first to demonstrate the acid catalyzed excision of the Y base from yeast $tRNA^{Phe}$. We used a procedure slightly different from theirs. Dry $tRNA^{Phe}$ or oligonucleotide containing the Y base was taken up either in water adjusted to pH 2.7 with formic acid or HCl, or in 0.1 M ammonium formate (pH 2.7), and incubated in a sealed glass capillary tube at 60° for 30–60 min. The hydrolysate was then spotted directly on a thin-layer plate for chromatography.

Acceptor Assay for $tRNA^{Phe}$. The assay procedure is given by Yoshikami (1970) and is only slightly modified from the procedure used by Leis and Keller (1971).

Poly(U)-Directed Polyphenylalanine Synthesis. The standard 1-ml reaction mixture consisted of the stated amount of $tRNA^{Phe}$, 10 μM [^{14}C]phenylalanine (10 μCi/μmole), 75 μg

[1] Abbreviations that are not listed in *Biochemistry* 5, 1445 (1966), are: Y_w, Y_y, and Y_b, the fluorescent bases from wheat germ, yeast, and beef liver $tRNA^{Phe}$s, respectively; BD-cellulose, benzoylated DEAE-cellulose; A_{260}, absorbance at 260 nm with a cell path of 1 cm; F_{440}, fluorescence intensity at 440 nm upon excitation at 310 nm.

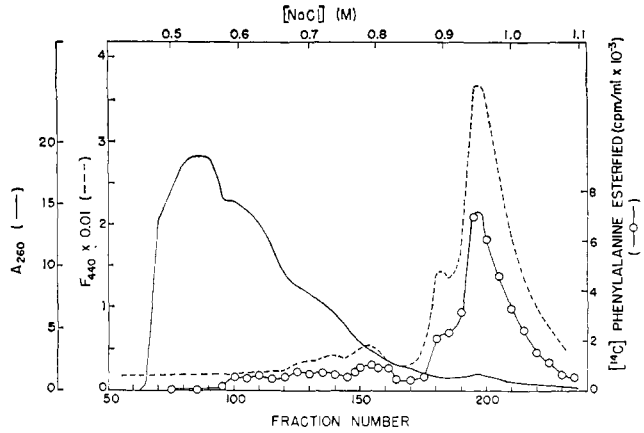

FIGURE 1: BD-cellulose column chromatography of bulk wheat germ tRNA. Bulk wheat germ tRNA (about 300 mg, previously purified by gel filtration on a Sephadex G-100 column) was dissolved in 30 ml of start solution and applied to a BD-cellulose column (1.23 × 90 cm). The column was then eluted with a linear gradient from 0.3 to 1.2 M NaCl containing 10 mM $MgCl_2$ (1.2-l. total volume). Fractions of 5 ml were collected at a flow rate of 0.5 ml/min.

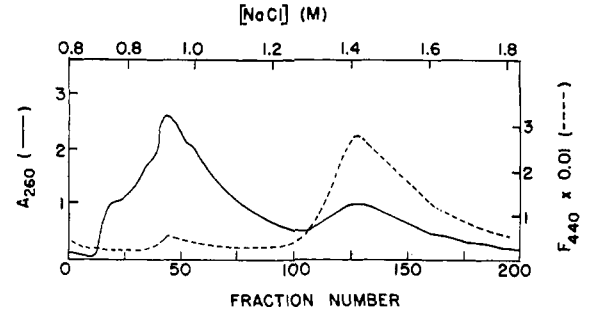

FIGURE 2: Chromatography of partially purified $tRNA_1^{Phe}$ on BD-cellulose at pH 4.5 in the presence of EDTA. Partially purified wheat germ $tRNA_1^{Phe}$ (864 A_{260} units, from chromatography as in Figure 1 of bulk wheat germ soluble RNA not treated by gel filtration) was applied to a BD-cellulose column (1 × 90 cm) and eluted with a linear gradient from 0.8 to 2 M NaCl containing 1 mM EDTA and 10 mM sodium acetate (pH 4.5) (800-ml total volume). Fractions of 4 ml were collected at a flow rate of 1 ml/min. Each tube in the fraction collector contained 0.2 ml of 0.2 M $MgCl_2$–1 M Tris·HCl (pH 7.5) in order to raise the Mg^{2+} concentration and the pH of the effluent as it emerged from the column.

of poly(U), 50 mM Tris·HCl (pH 7.6), 84 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.6 mM GTP, 1 mM ATP, 5 mM phosphoenolpyruvate, 7 A_{260} units of wheat germ ribosomes, and about 1.2 mg of wheat germ soluble fraction enzyme preparation. The mixture was incubated at 37° for the desired time. The reaction was terminated by raising the pH to 11–12 with 0.1 ml of 1 M NaOH, and the mixture was incubated at 37° for 15 min in order to hydrolyze all esterified tRNAs. The solution was neutralized with 0.1 ml of 1 M HCl and then 2.5 ml of 0.4% $NaWO_4$–8% sodium trichloroacetate (pH 1.7) was added to precipitate the polyphenylalanine. The precipitate was collected and rinsed on a Millipore filter and assayed for ^{14}C as in the acceptor assay. All data were corrected for blank controls which were treated identically as above except no poly(U) was added.

Results

Fractionation of $tRNA^{Phe}$s from Wheat Germ. Bulk tRNA from wheat germ was first fractionated on a BD-cellulose column (Gillam *et al.*, 1967) using a linear gradient of NaCl concentration in the presence of 10 mM $MgCl_2$ (Figure 1). Good resolution was obtained by the use of a slow flow rate and a shallow gradient extending up to 1.2 M NaCl. The column fractions were assayed for fluorescence at 440 nm upon excitation at 310 nm (F_{440}) as well as for phenylalanine-acceptor activity. As shown previously (Yoshikami *et al.*, 1968), the F_{440} pattern coincides almost exactly with the elution pattern of the $tRNA^{Phe}$s. The pattern in Figure 1 shows a number of minor $tRNA^{Phe}$s in addition to the major species, $tRNA_1^{Phe}$, which has its peak at tube 197. The small peak in tubes 145–165 is the $tRNA_2^{Phe}$ which was previously detected (Yoshikami *et al.*, 1968) in variable amounts in different bulk wheat germ tRNA preparations.

A hitherto unreported peak, $tRNA_3^{Phe}$, appears in variable amount on the leading edge of the major peak. $tRNA_3^{Phe}$ was found to be monomeric by gel filtration on Sephadex G-100. The fluorescent Y base obtained by mild acid hydrolysis of this tRNA was chromatographically identical with that from $tRNA_1^{Phe}$. $tRNA_3^{Phe}$ cochromatographs with $tRNA_1^{Phe}$ on BD-cellulose after it has been heated. It therefore appears to be a conformer of $tRNA_1^{Phe}$.

Purification of $tRNA_1^{Phe}$ from Wheat Germ. The major species, $tRNA_1^{Phe}$, is obtained from the BD-cellulose column described above at a purity of about 30%. Chromatography of this material on a second BD-cellulose column, this time using a NaCl gradient containing EDTA at pH 4.5, yields $tRNA_1^{Phe}$ that is at least 85% pure as judged by acceptor activity (Figure 2). These two successive column fractionations on BD-cellulose provide a rapid method for obtaining highly purified wheat germ $tRNA^{Phe}$. This procedure can also be used for obtaining purified $rRNA^{Phe}$ from yeast. A precaution must be observed in using a BD-cellulose column at pH 4.5 with $tRNA^{Phe}$s which have a Y-type base. As shown by Thiebe and Zachau (1969), the Y base can be excised from the tRNA at low pH. We have noted a very slow excision of Y during chromatography at pH 4.5 at room temperature. To minimize this, a relatively fast flow rate was maintained, and aliquots of Tris buffer at pH 7.5 were placed in the receiving tubes to raise the pH of the effluent (see Figure 2).

Nature of $tRNA_2^{Phe}$ from Wheat Germ. This tRNA has the same fluorescence emission spectrum as $tRNA_1^{Phe}$ (Yoshikami *et al.*, 1968) showing that a Y-type is present. The amount of $tRNA_2^{Phe}$ was variable in different preparations of bulk tRNA suggesting that it was derived from $tRNA_1^{Phe}$ during the isolation of the bulk tRNA. $tRNA_2^{Phe}$ was not simply a conformer of $tRNA_1^{Phe}$ since it was not converted to the latter on heating in solution at 80°. Since $tRNA_2^{Phe}$ had a lower affinity for BD-cellulose than $tRNA_1^{Phe}$, and since the Y base is responsible for the high affinity of $tRNA_1^{Phe}$ for this resin (*cf.* Thiebe and Zachau, 1969), it was suspected that $tRNA_2^{Phe}$ had been formed by a chemical modification of the Y base.

To test this hypothesis, the Y base from each of the two $tRNA^{Phe}$s was excised by mild acid treatment (Thiebe and Zachau, 1969) and the chromatographic and electrophoretic mobilities of the two bases were compared. The Y base excised from $tRNA_2^{Phe}$ was found to differ from the base excised from $tRNA_1^{Phe}$. The former will therefore be designated Y_w^2 and the latter Y_w^1. In cellulose thin-layer chromatography using organic solvent mixtures, Y_w^2 has a lower R_f than Y_w^1 (Table I).

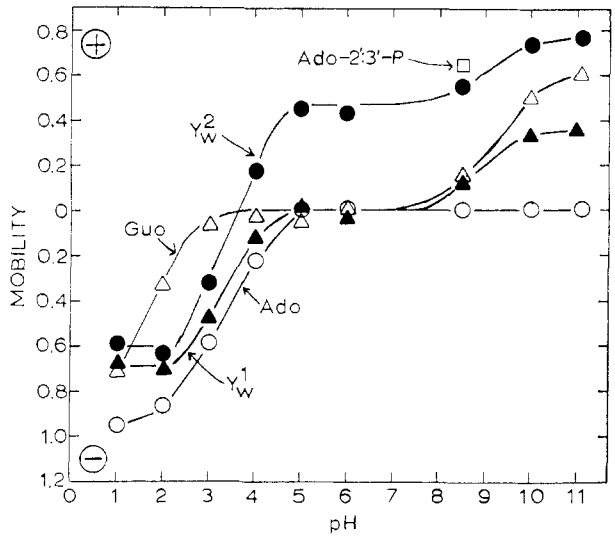

FIGURE 3: Electrophoretic titration of Y^1_w and Y^2_w. Y^1_w and Y^2_w were obtained by mild acid hydrolysis of wheat germ $tRNA_1^{Phe}$ and $tRNA_2^{Phe}$, respectively, and subsequent thin-layer chromatography in solvent II. Electrophoresis was performed at the various pH's as detailed in Methods.

TABLE I: Chromatographic and Electrophoretic Mobilities of the Y Bases.

Base	R_F in Different Solvents[a] I	II	III	μ[b] at Different pH's 2.7	3.5	6.5	10
Wheat							
Y^1_w	0.67	0.90		+0.5	+0.3	0	0
Y^2_w	0.30	0.75	0.31	+0.4	+0.2	−0.5	−0.5
Y^3_w	0.40	0.55		−0.1[c]	−0.1[c]	−0.5	−0.5[c]
Y^4_w	0.22	0.65		+0.8	+0.4[c]	0	0[c]
Beef							
Y^1_b	0.67	0.90			+0.3	0	
Y^2_b	0.30	0.75	0.31	+0.4		−0.5	
Yeast							
Y^1_y	0.84	0.93			+0.3	0	
Y^2_y	0.39	0.77	0.50			−0.5	

[a] Cellulose thin-layer chromatography. [b] μ is the electrophoretic mobility taking the mobility of cytidine at pH 1.0, where it has a net charge of −1, as +1.0. [c] Interpolated values from Figure 9.

More information about the difference between Y^1_w and Y^2_w was obtained by measuring the electrophoretic mobilities of the two over a wide range of pH values (Figure 3). The electrophoretic titration curves of the two bases are quite different. By comparison to standards, Y^1_w appears to have a single protonatable group with a pK_a of about 3.3 and a weak acid group with a pK_a near 9. The curve for Y^1_w is similar to that of guanosine except that the first pK_a of Y^1_w is higher. A comparison of the curve for Y^2_w to that of Y^1_w shows that the latter has an additional titratable group with pK_a = about 4 so that in the neutral pH range (5–7.5) it has a net negative charge. (Y^1_w has a zero charge in the neutral region.) The results suggest that Y^2_w has a free carboxyl group on a side chain on the fluorescent chromophore. The net negative charge on Y^2_w at neutral pH could contribute to the decreased hydrophobicity exhibited by $tRNA_2^{Phe}$ on BD-cellulose columns.

The variable conversion of Y^1_w into Y^2_w occurred presumably at some stage during the isolation of the bulk tRNA, possibly during DEAE-cellulose chromatography. The condition of this chromatography may have been inadvertently too alkaline in some cases. RajBhandary *et al.* (1968) and Katz and Dudock (1969) had found that the Y base in oligonucleotides was labile in ammoniacal solvents. An experiment was therefore performed to see if the free base Y^1_w could be converted to Y^2_w under mild alkaline conditions. It was found that some conversion did occur when Y^1_w was incubated in 5 N NH_4OH (pH = about 12) for 12 hr at room temperature. It seems probable that Y^1_w has an ester group which is hydrolyzed by mild alkaline conditions to yield the free carboxyl group in Y^2_w.

No large molecular weight change is evident in the conversion of Y^1_w to Y^2_w since the electrophoretic mobilities of the two bases are essentially the same at pH 1 where they are both fully protonated (Figure 3). Thus the base-catalyzed conversion could open a lactone ring or release a low molecular weight alcohol.

Conversion of $tRNA_1^{Phe}$ into $tRNA_2^{Phe}$. If these two RNAs differ from each other only in the Y base, they would be useful in studying the effect of a specific modification in the hyperon on the function of a tRNA. Conditions were therefore sought to convert Y^1_w to Y^2_w *in situ* in the tRNA by base catalysis where no other changes would occur in the tRNA. There are three other modified residues in $tRNA^{Phe}$ which are alkali labile, 7-methylguanosine, 1-methyladenosine, and dihydrouridine. Basic conditions can cause the opening of the imidazole ring of the 7-methylguanosine (Lawley and Brookes, 1963), a rearrangement of 1-methyladenosine to N^6-methyladenosine (Brookes and Lawley, 1960; Macon and Wolfenden, 1968), and a ring opening of dihydrouridine (Green and Cohen, 1957). In each reaction, as is the case for base-catalyzed modification of Y^1_w, the product has a different charge from the parent compound at neutral pH. These residues appear in different oligonucleotides when the tRNA is digested by RNase T_1. Thus the extent of conversion of each base can be determined by examining the RNase T_1 digest products which have been fractionated on a DEAE-cellulose column in 7 M urea.

It was found that specific modification of the Y base *in situ* could be brought about by treating the $tRNA_1^{Phe}$ with 0.5 M ammonium carbonate (pH 9) at 42° for 12 hr. The conversion to $tRNA_2^{Phe}$ under these conditions was about 50%, as is shown in Figure 4.

In order to establish that the sole difference between these two tRNAs resides in the Y base, an RNase T_1 hydrolysate of each of the tRNAs isolated from the column in Figure 4 was fractionated on a DEAE-cellulose column in the presence of 7 M urea (Figure 5). The elution pattern of the digest of the $tRNA_1^{Phe}$ recovered after the ammonium carbonate treatment (Figure 5A) is virtually identical with that of untreated $tRNA_1^{Phe}$ (Katz and Dudock, 1969). The fluorescence at 440 nm indicates the presence of the Y base in the dodecanucleotide peak 17. The elution pattern of the digest of the $tRNA_2^{Phe}$ (Figure 5B) is identical with that of $tRNA_1^{Phe}$ with the sole exception that the fluorescent dodecanucleotide is shifted to peak 18. Katz and Dudock (1969) had previously detected a small amount of peak 18 and showed that it

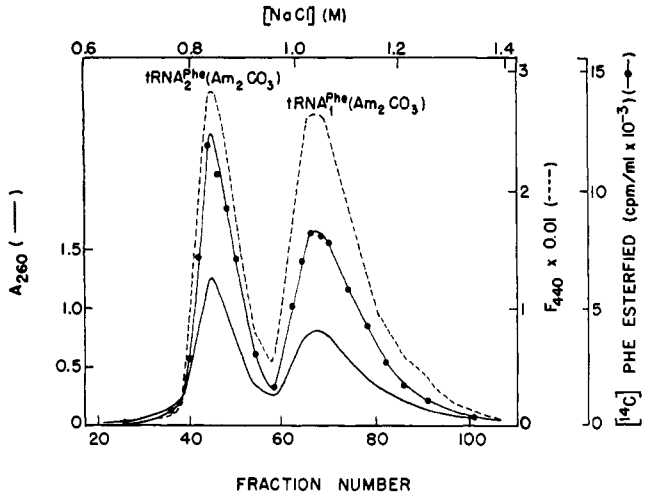

FIGURE 4: BD-cellulose column chromatography of ammonium carbonate treated wheat germ tRNA$_1^{Phe}$. tRNA$_1^{Phe}$ (98 A_{260} units, 85% pure) from fractions 120–145 in Figure 2 was treated with ammonium carbonate (pH 9) for 12 hr (see Methods) and applied in 3 ml of start solution to a BD-cellulose column (0.5 × 108 cm). The column was then eluted with a 500-ml linear gradient from 0.5 to 2.0 M NaCl containing 10 mM $MgCl_2$ (500-ml total volume). Fractions of 3 ml were collected at a flow rate of 0.6 ml/min.

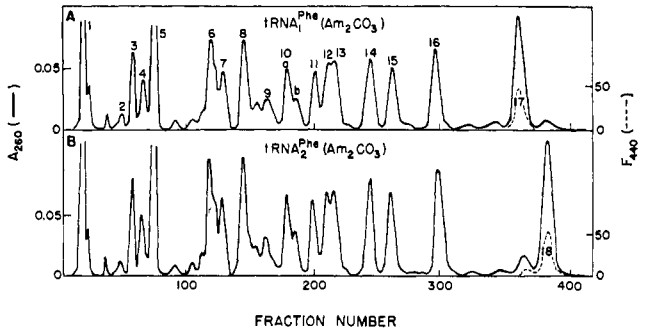

FIGURE 5: Chromatography of RNase T_1 digests of wheat germ tRNA$_1^{Phe}$ and tRNA$_2^{Phe}$ on DEAE-cellulose. (A) tRNA$_1^{Phe}$ (9 A_{260} units, Am_2CO_3 treated) from the second peak in Figure 4, and (B) tRNA$_2^{Phe}$ (10 A_{260} units, Am_2CO_3 treated) from the first peak in Figure 4 were each digested with 500 units of RNase T_1 in 0.6 ml of 50 mM Tris·HCl (pH 7.7) for 3 hr at 37°. Each digest was then made 7 M in urea, applied to a DEAE-cellulose column (0.4 × 60 cm), and eluted with linear gradient from 0 to 0.3 M NaCl containing 20 mM Tris·HCl (pH 8.5) and 7 M urea (600-ml total volume). Fractions of 1.3 ml were collected at a flow rate of 0.4 ml/min. The peaks are numbered according to Katz and Dudock, 1969.

differed from peak 17 only in the fluorescent residue. The Y base excised from peak 18 was found to be Y_w^2. The later elution of the dodecanucleotide presumably reflects, in part, the negative charge on Y_w^2. There was no detectable destruction of dihydrouridine or 7-methylguanosine by the alkaline treatment since there was no perturbation of peaks 8 or 10b, the oligonucleotides containing these residues.

The DEAE-cellulose columns in Figure 5 were run at pH 8.5. At this pH there is no charge difference between 1-methyladenosine and N^6-methyladenosine, so it was necessary to repeat the chromatographic analysis at pH 8. When this was done, peak 15 was resolved into 15a and b (Katz and Dudock, 1969). Peak 15a contains the oligonucleotide with 1-methyladenosine. It could then be estimated that less than 10% of the 1-methyladenosine in the tRNA^{Phe} had been converted into N^6-methyladenosine by the ammonium carbonate treatment. The analysis at pH 8 was also repeated on tRNA^{Phe} treated with ammonium carbonate for 50 hr. Under this condition a significant amount of modification of 1-methyladenosine and 7-methylguanosine could be detected in addition to some hydrolysis of phosphodiester linkages.

Functional Activity of tRNA$_2^{Phe}$ from Wheat Germ. The phenylalanine-acceptor activity of tRNA$_2^{Phe}$ was demonstrated when it was first detected (Yoshikami *et al.*, 1968). To see what effect the conversion of Y_w^1 into Y_w^2 has on the interaction of the tRNA with its synthetase, a study was made of the rate of aminoacylation of the two tRNA^{Phe}s (Figure 6). No detectable difference in rate was found. In line with this, Igo-Kemenes and Zachau (1969) have been able to reduce the Y base of yeast tRNA^{Phe} with $NaBH_4$ with no observable change either in the K_m for the tRNA or the V_{max} of the charging reaction.

It seemed possible that a modification of the base adjacent to the anticodon could influence the interaction of the tRNA with its codon on the ribosome in the course of the transfer reaction. Accordingly a comparison of the transfer function of tRNA^{Phe}s 1 and 2 was made. The two tRNAs were tested for their ability to catalyze the poly(U)-dependent synthesis of polyphenylalanine with ribosomes and crude enzymes from wheat germ (Figure 7). Under the conditions of the assay, the rate of polymerization was linearly dependent upon the concentration of added tRNA^{Phe}. The optimum pH, temperature, and $MgCl_2$ concentration for this reaction was found to be the same for both tRNA^{Phe}s. The rate of polymerization catalyzed by tRNA$_2^{Phe}$ was found to be about 70% of that by tRNA$_1^{Phe}$. This difference in activity was maintained at Mg^{2+} concentrations from 8 to 15 mM, at pH 6–8.5 and at temperatures from 20 to 45°. Thus, the presence of a free-acid group with a negative charge in the hyperon does not abolish the capacity of the tRNA to participate in the transfer reaction, but it does measureably slow the rate at which it functions.

Other Alkaline Degradation Products from Y_w^1. Y_w^1 may be hydrolyzed under alkaline conditions to at least two other forms, Y_w^3 and Y_w^4, in addition to Y_w^2 (Figure 8). All these forms are readily separated by thin-layer chromatography (Table I). On extended hydrolysis, the Y_w^2 first formed is converted to Y_w^3, and finally the latter is converted into Y_w^4. All

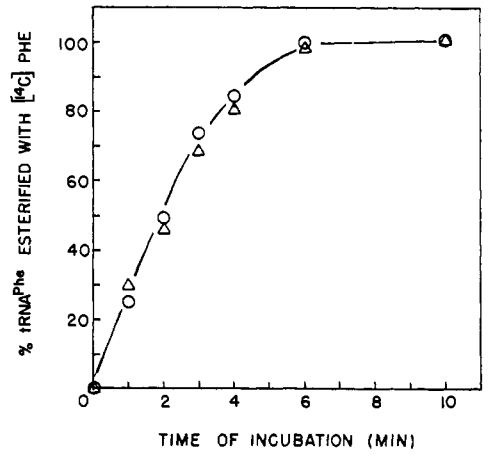

FIGURE 6: Rate of aminoacylation of tRNA$_1^{Phe}$ and tRNA$_2^{Phe}$. The tRNAs (85% pure, Am_2CO_3 treated from Figure 4) were compared in the acceptor assay (see Methods). Each assay contained 34 μg of wheat germ soluble fraction enzyme preparation in a volume of 0.2 ml. (Δ) tRNA$_1^{Phe}$ (Am_2CO_3), 25 pmole/assay; (○) tRNA$_2^{Phe}$ (Am_2CO_3), 27 pmole/assay.

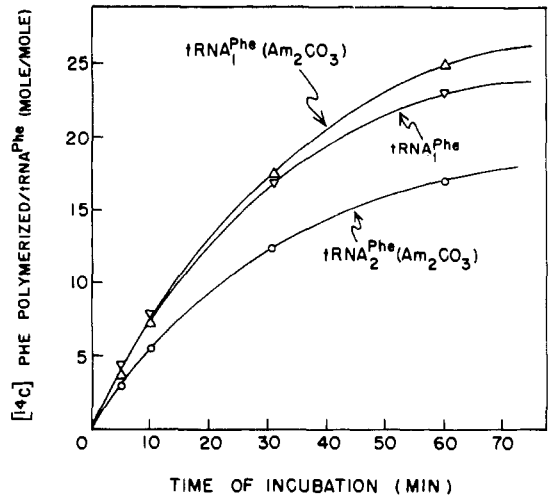

FIGURE 7: Rate of polyphenylalanine synthesis catalyzed by $tRNA_1^{Phe}$ and $tRNA_2^{Phe}$. The two treated $tRNA_s^{Phe}$ (Figure 4) were compared to untreated $tRNA_1^{Phe}$ (Figure 2) in the wheat germ system for polyphenylalanine synthesis described in Methods. Each tube contained 90 pmoles of $tRNA^{Phe}$ as determined by acceptor assay.

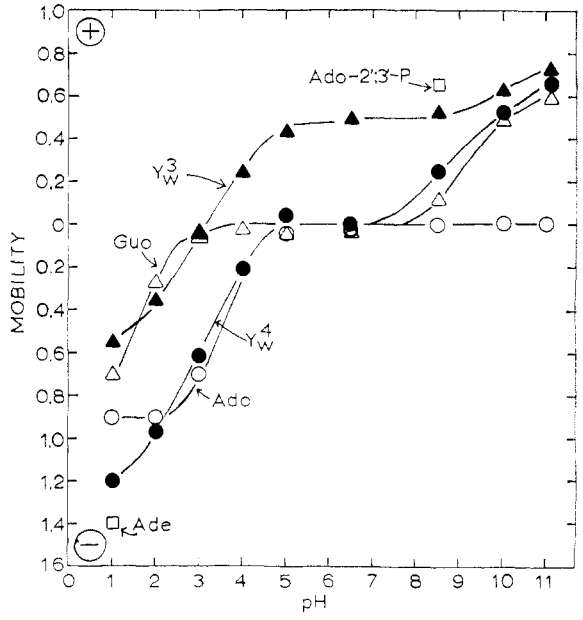

FIGURE 9: Electrophoretic titration of Y_w^3 and Y_w^4. These bases were obtained by KOH hydrolysis of Y_w^2 (see Figure 8) and subsequent thin-layer chromatography in absolute methanol and then in solvents I and II. Electrophoresis was performed as in Methods.

three alkaline degradation products fluoresce and have ultraviolet absorption spectra similar to that of Y_w^1 (*cf.* Katz and Dudock, 1968). From this it can be inferred that this alkaline hydrolysis has acted on the structure of side chains and not on the fluorescent chromophore of the Y base itself. When the products from an even more extensive alkaline hydrolysis are chromatographed, a number of new ultraviolet-absorbing compounds can be detected. These have different absorption spectra and have lost the characteristic fluorescence of the Y type base.

The electrophoretic mobility of Y_w^3 over a wide pH range is similar to that of Y_w^2 (Figure 9). There is no detectable molecular weight change in the conversion of Y_w^2 into Y_w^3, and the hydrolysis or rearrangement does not yield any new titratable group. There is, however, a change in chromatographic properties (Table I).

The conversion of Y_w^3 into Y_w^4 results in a marked difference in the electrophoretic titration curve (Figure 9). The alkaline hydrolysis of Y_w^3 to Y_w^4 has released a basic amino group with a pK_a near 9 not present in the other forms of the Y base. This amino group could be blocked by formaldehyde; when 4% formaldehyde was present in the electrophoresis buffer the mobility of Y_w^4 at pH 8.5 was increased to -0.5. The negative charge indicates that the carboxyl group present in Y_w^3 is still present in Y_w^4. The mobilities of Y_w^1, Y_w^2, and Y_w^3 were not affected by formaldehyde at pH 8.5. In the absence of formaldehyde, Y_w^4 has a zero mobility at pH's 5–7 where it is a zwitterion. The high mobility of Y_w^4 at pH 1 indicates that it has two positive charges at this pH; one of these charges can be accounted for by the weakly basic group (pK_a = near 3) present also in the other forms of the Y base, the other by the strongly basic group released by hydrolysis. Since the spectrum of the fluorescent chromophore of Y_w^4 is unchanged, the latter group must be on a side chain.

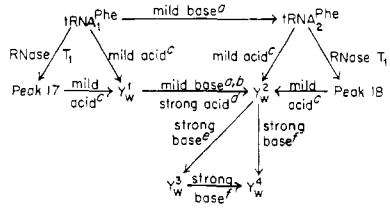

FIGURE 8: Degradation scheme of the Y base of wheat germ $tRNA_1^{Phe}$. Reaction conditions were as follows: [a] 0.5 M ammonium carbonate (pH 9), 42°, 12 hr; [b] 5 M NH_4OH, 23° 12 hr; [c] 0.1 M ammonium formate (pH 3.0) or dilute HCl (pH 3), 60°, 30 min; [d] 0.1 M HCl, 100°, 30 min; [e] 0.5 M KOH, 60°, 30 min; [f] 0.5 M KOH, 60°, 2 hr. Peaks 17 and 18 are T_1 digest fragments (see Figure 5).

Y Base in tRNA^{Phe} from Beef Liver. When bulk beef liver tRNA is analyzed on a BD-cellulose column as in Figure 1, the pattern of phenylalanine-acceptor activity and F_{440} is almost identical with that for wheat germ (*cf.* Yoshikami *et al.*, 1968). There is a shoulder of $tRNA_3^{Phe}$ on the leading edge of the major $tRNA_1^{Phe}$ peak, and this is preceded by a small peak of $tRNA_2^{Phe}$. The Y bases excised from these tRNAs, Y_b^1 and Y_b^2, appear to be identical with those from the corresponding tRNAs of wheat germ since their respective spectroscopic, chromatographic, and electrophoretic properties are all identical (Table I). When $tRNA_1^{Phe}$ from beef liver (Figure 10A) was incubated in 0.5 M ammonium carbonate (pH 9) for 20 hr at 42° and rechromatographed, about 75% conversion of Y_b^1 into Y_b^2 *in situ* was obtained (Figure 10B). This is about the same amount of conversion that is found with wheat germ $tRNA_1^{Phe}$ under the same conditions.

Y Base in tRNA^{Phe} from Yeast. When bulk yeast tRNA is analyzed on a BD-cellulose column as in Figure 1, the pattern of phenylalanine acceptor activity and F_{440} which is obtained differs from that of wheat germ. In no instance has a peak corresponding to wheat germ $tRNA_3^{Phe}$ been observed. Instead, there is a shoulder of F_{440} and phenylalanine acceptor activity following the major $tRNA_1^{Phe}$ which, like $tRNA_3^{Phe}$ from wheat, may represent a stable conformer of $tRNA_1^{Phe}$. There is a $tRNA_2^{Phe}$ peak in some preparations, but it is usually quite small. When partially purified $tRNA_1^{Phe}$ (Figure 11A) was incubated with 0.5 M ammonium carbonate (pH 9) for 20 hr at 42° there was 50% conversion into $tRNA_2^{Phe}$

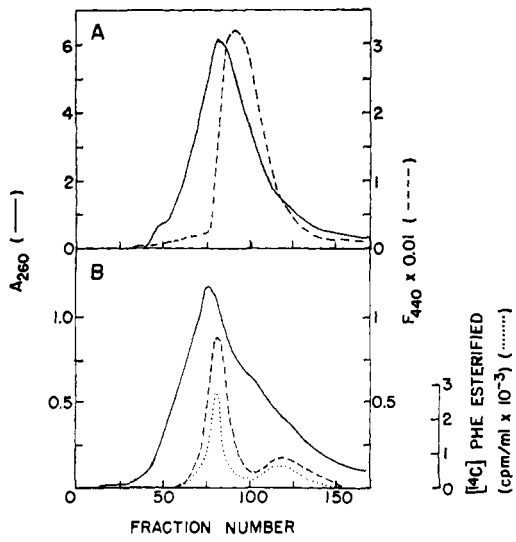

FIGURE 10: BD-cellulose column chromatography of beef liver tRNA^{Phe} before and after treatment with ammonium carbonate. (A) Partially purified beef liver tRNA^{Phe} (950 A_{260} units, from a BD-cellulose column as in Figure 1) was dissolved in 10 ml of start solution, applied to a BD-cellulose column (1 × 45 cm), and eluted with a linear gradient from 0.5 to 2.4 M NaCl containing 10 mM $MgCl_2$ (500-ml total volume). Fractions of 3 ml were collected at a flow rate of 1 ml/min. (B) Half of the RNA recovered from fractions 90 to 116 in part A was treated with ammonium carbonate for 20 hr (see Methods) and chromatographed as in part A.

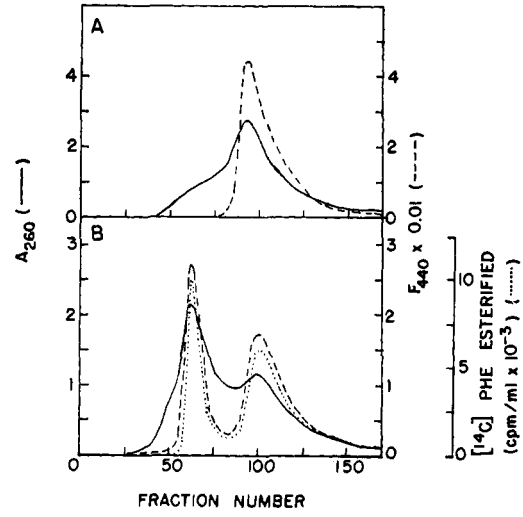

FIGURE 11: BD-cellulose column chromatography of yeast tRNA^{Phe} before and after ammonium carbonate treatment. (A) Partially purified yeast tRNA^{Phe} (330 A_{260} units, from a BD-cellulose column as in Figure 1) was chromatographed on BD-cellulose as in Figure 10A. (B) Another portion of the same tRNA (270 A_{260} units) was incubated with ammonium carbonate for 20 hr (see Methods) and chromatographed in the same way.

(Figure 11B). Acid excision of the Y bases from tRNA$_1^{Phe}$ and tRNA$_2^{Phe}$ yielded Y_y^1 and Y_y^2, respectively (Table I). This conversion was slower than in the case of beef and wheat germ. Furthermore, the base Y_y^1 is itself more resistant to base-catalyzed conversion into a Y_y^2 base than is Y_w^1 or Y_b^1. However, for all Y bases, the conversion to a Y^2 base exposes a free acidic group (Table I). It appears then that the susceptible linkage in Y_y^1 is more stable to alkali than that in Y_w^1 or Y_b^1. Both Y_y^1 and Y_y^2 are chromatographically distinguishable from their counterparts in wheat and beef. This indicates that some substituent on the chromophores of these bases differ though the fluorescent chromophores themselves may be identical as judged by their similar spectroscopic characteristics (RajBhandary *et al.*, 1968; Katz and Dudock, 1969; Yoshikami *et al.*, 1968).

Discussion

To understand the role of the hypermodified base adjacent to the anticodon of different tRNAs, we chose to study the tRNA^{Phe}s which have the fluorescent Y-type base in this locus. We were able to isolate three products of the wheat germ Y base which retain the fluorescent chromophore and represent progressive stages of alkaline hydrolysis. The first stage of hydrolysis, the conversion of Y_w^1 into Y_w^2, can be effected *in situ* under a very mild alkaline condition which has no effect on any other part of the tRNA^{Phe}. This was used to generate a tRNA^{Phe} specifically modified in the anticodon-adjacent base, namely tRNA$_2^{Phe}$. Our analysis indicates that the modification produces a net negative charge on the Y base.

The acceptor activity of wheat germ tRNA$_2^{Phe}$ was comparable to that of the naturally occurring tRNA$_1^{Phe}$. On the other hand, tRNA$_2^{Phe}$ was less efficient than tRNA$_1^{Phe}$ in supporting the polymerization of phenylalanine coded for by poly(U). tRNA$_2^{Phe}$ catalyzed this reaction at 70% the rate of tRNA$_1^{Phe}$. Thus, although the modification of the tRNA^{Phe} does not affect the acceptor activity of the tRNA, it does significantly alter its efficiency in the transfer reaction when tested *in vitro* with poly(U). Further analysis would be required to determine the efficiency of the transfer reaction with a natural mRNA and whether the modification affects the fidelity of translation.

These results are congruent with those obtained by a number of other workers who have demonstrated that the structural integrity of the anticodon-adjacent base is necessary for optimum functioning of the tRNA in the transfer reaction, but that modification of this base does not critically affect acceptor activity (Fittler and Hall, 1966; Thiebe and Zachau, 1968; Gefter and Russell, 1969; Ghosh and Ghosh, 1970; Furuichi *et al.*, 1970).

Nakanishi *et al.* (1970) have recently proposed a structure for the Y base from yeast tRNA^{Phe} (Y_y^1). In this structure the fluorescent chromophore bears an α-amino acid side chain. The amino group is blocked with a carbomethoxy moiety, and the carboxyl group is present as a methyl ester. The carboxyl group released in the conversion of Y_y^1 into Y_y^2 is undoubtedly the latter carboxyl group. The release of a carboxyl group in forming Y_w^2 and of a free amino group in forming Y_w^4 indicates that the wheat germ Y base has a blocked α-amino acid side chain similar to that of yeast Y.

There is a difference, however, both in chromatographic behavior and rate of hydrolysis between the yeast Y and wheat germ Y (and the apparently identical beef liver Y). Furthermore, there appears to be some structural feature in wheat germ Y not present in the proposed structure of Y_y^1 which is responsible for the formation of the additional intermediate, Y_w^3. That the difference occurs in the distal portion of the side chain is indicated by a low-resolution mass spectral analysis of Y_w^1 (Yoshikami, 1970). Prominent peaks at *m/e* 216 and 230 were observed just as in the analysis of Y_y^1 by Nakanishi *et al.* (1970). The latter authors showed that these two peaks represented the fluorescent chromophore with one and two carbons of the side chain, respectively. The identity of these

mass spectral peaks supports the spectroscopic evidence that the various Y bases have identical fluorescent chromophores.

Acknowledgments

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On the Conformation of Lysozyme and α-Lactalbumin in Solution*

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ABSTRACT: Lysozyme and α-lactalbumin have highly homologous primary sequences but different biological functions. W. R. Krigbaum and F. R. Kügler (1970, *Biochemistry 9*, 1216) have recently reported small-angle X-ray-scattering studies from both proteins in aqueous solution, and conclude that lysozyme and α-lactalbumin have quite different conformations in solution. They also present evidence for the presence of α-lactalbumin dimer in solution. We demonstrate that all of the observed differences in small-angle X-ray scattering from α-lactalbumin and lysozyme in solution can be rigorously accounted for by such dimerization. Thus the experiments of Krigbaum and Kügler strongly suggest that these two proteins have quite similar conformations in solution.

Lysozymes cause cell wall lysis of gram-positive bacteria by a mechanism which is now fairly well understood (Phillips, 1966), while α-lactalbumin has been implicated in the lactose synthetase system (Ebner *et al.*, 1966). The primary sequences of hen egg white lysozyme and bovine α-lactalbumin have been shown to be strikingly similar, with regard to both residue identity (49 residues out of 123–129) and the positions of the disulfide bridges (Brew *et al.*, 1967, 1970). This homology is somewhat surprising in view of the differences in function, but does provide grounds for anticipating homology in tertiary

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