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Structure Stabilization in *Escherichia coli* Transfer Ribonucleic Acid*

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ABSTRACT: Formaldehyde treatment of *Escherichia coli* B transfer ribonucleic acid (tRNA) appreciably changes hydrodynamic and optical properties. The increase in intrinsic viscosity, decrease in sedimentation coefficient coupled with the change in absorbance melting curves, and polarization of fluorescence thermal profiles indicate that (1) Watson–Crick hydrogen bonding is a determinant in the configuration and helix–coil transition of native *E. coli* tRNA and (2) base stacking contributes to the helical rigidity in native tRNA.

Three types of forces are now believed to determine the stability and configuration of polyribonucleotides. In order of chronological recognition they are: hydrogen bonding, base stacking, and the interaction of the C-2 hydroxyl group of ribose with either a phosphate or nucleotide (Spencer *et al.*, 1962; Langridge and Gamatos, 1963; Sato *et al.*, 1966; Ts'o *et al.*, 1966). Since it appears that some polyribonucleotides can exhibit ordered structure in the absence of hydrogen bonding (Fasman *et al.*, 1964, 1965; Poland *et al.*, 1966; Brahms *et al.*, 1966; Leng and Felsenfeld, 1966; Stevens and Rosenfeld, 1966), the contribution of Watson–Crick base pairing to the secondary structure and configuration of ribonucleic acid deserves reassessment.

Formaldehyde treatment reduces or blocks completely polynucleotide interactions which are known to be explicitly dependent upon base pair hydrogen-bond formation (Steiner and Beers, 1959; Grossman *et al.*, 1961; Stollar and Grossman, 1962; Haselkorn and Doty, 1961), by blocking exocyclic amino groups serving as proton donors. Past investigation of the reactivity of tRNA with formaldehyde has shown that agents which promote secondary structure decidedly decelerate the rate of reaction (Penniston and Doty, 1963).

Thus it appears reasonable to assume that by measuring various physical and optical properties of tRNA after reaction with formaldehyde (under reaction conditions in which secondary structure is minimal), at least the qualitative importance of hydrogen bonding in this molecule might be estimated.

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TABLE 1: Effect of Formaldehyde Treatment on the Molecular Weight and Sedimentation Coefficient of *E. coli* tRNA.

Reaction Buffer	Formaldehyde Concn (%)	Temp of Treatment (°C)	Time at Temp	tRNA Concn (mg/ml)	App Mol Wt[a]	S[b]	A^6_{260} after: A_{260} before
TEA (0.01)-KCl (0.3)	None	100	10 min	4.72	45,000	4.20	1.03
TEA (0.01)-KCl (0.3)	None	20	10 min	4.72	25,300	4.01	1.00
TEA (0.01)-KCl (0.3)	2	20	68 hr	7.57	32,200	3.70	
TEA (0.01)-KCl (0.3)	2	20	10 min	4.72	27,900	3.99	1.02
TEA (0.01)	2	50	60 min	3.8	31,500	3.94	1.15
TEA (0.01)-KCl (0.3)	2	100	5 min	6.5	26,000[c]	3.50[c]	1.20
TEA (0.01)-KCl (0.3)	2	100	5 min	6.21	33,600	3.81	1.20
TEA (0.01)-KCl (0.3)	2	100	10 min	4.72		3.78	1.20
TEA (0.01)	4	60	30 min	4.77	37,300		1.18
TEA (0.01)-KCl (0.3)	4	100	2.5 min	6.36	37,700		1.18
TEA (0.01)-KCl (0.3)	4	100	2.5 min	5.2	26,640[c]	3.50[c]	1.20
TEA (0 01)-KCl (0.3)	4	100	3 min	7.5	25,800[c]	3.43[c]	1.21
TEA (0 01)	12	20	72 hr	5.46			1.23
TEA (0.01)	12	80	30 min	6 68	38,000		1.21
TEA (0 01)	12	90	30 min	5.61	38,500		1.24
TEA (0.01)	12	90	30 min	5.72	37,500		1.24

[a] Determined at the indicated concentration. [b] Determined at a concentration of 0.004%. [c] Isolated monomer fraction.

Experimental Section

Escherichia coli B tRNA (stripped) was purchased from General Biochemical Co. Chagrin Falls, Ohio, and freed of protein and metal ions by procedures described in detail elsewhere (Millar, 1966). The physical properties of the preparation used here (tRNA XIV) are described in detail elsewhere (Millar, 1966). Formaldehyde was used directly without purification as Haselkorn and Doty (1961) have demonstrated that the chief impurity, methanol, does not influence the reaction of formaldehyde with nucleotide amino groups. We should like to point out that in the range of formaldehyde concentrations used by us, the imino group of uracil has probably reacted with formaldehyde at least as judged by the thorough investigations of Lewin (1964) and Aylward (1966) on poly U and the free base. All other chemicals were of reagent grade quality. Triethanolamine (TEA)[1] was used as supporting buffer as it does not react with formaldehyde (Penniston and Doty, 1963). Reaction with formaldehyde was carried out in glass-stoppered vessels and all references to temperature and time of reaction refer to this.

Unless otherwise indicated all molecular weight, sedimentation coefficient, and viscosity determinations were performed in the presence of formaldehyde. Molecular weights were estimated using the multichannel technique described by Yphantis (1960) for tRNA concentrations above 2 mg/ml. At tRNA concentrations below 0.5 mg/ml the Yphantis (1964) meniscus depletion technique was used. Sapphire windows and 7-mm solution columns were routinely employed. Plates were measured on a Nikon Model 6C comparator. In molecular weight and sedimentation coefficient calculations the native $\bar{V}$ was employed (Tissières, 1959) in all cases. It is recognized that this assumption may lead to errors in the calculated molecular weight of formalized tRNA, although they are expected to be slight.

Viscosity measurements were performed employing the methodology outlined elsewhere (Millar and Steiner, 1966; Millar, 1966) except that a Fisher Isotemp bath continuously bucked with a refrigerating coil served to maintain temperature.

The rise in extinction coefficient after reaction with formaldehyde was determined by removing an aliquot from the formalization reaction, diluting it 100-fold into 0.3 M KCl–0.01 M TEA (pH 7.0), reading the absorbance at 260 mμ.

In calculating concentrations for formalized tRNA, the native extinction coefficient of tRNA at 260 mμ (Stephenson and Zamecnik, 1961) was multiplied by the experimentally observed rise in extinction to give an operational extinction coefficient. The accuracy of this procedure was tested by conducting the formalization reaction directly in a Ubbelohde viscometer. The intrinsic viscosity values obtained by computing the concentrations of tRNA as a function of dilution volume and those obtained by absorbance measurements were within a few per cent of each other. An identical experiment followed by dialysis against fresh solvent gave an intrinsic viscosity value within several

[1] Abbreviation used: TEA, triethanolamine.

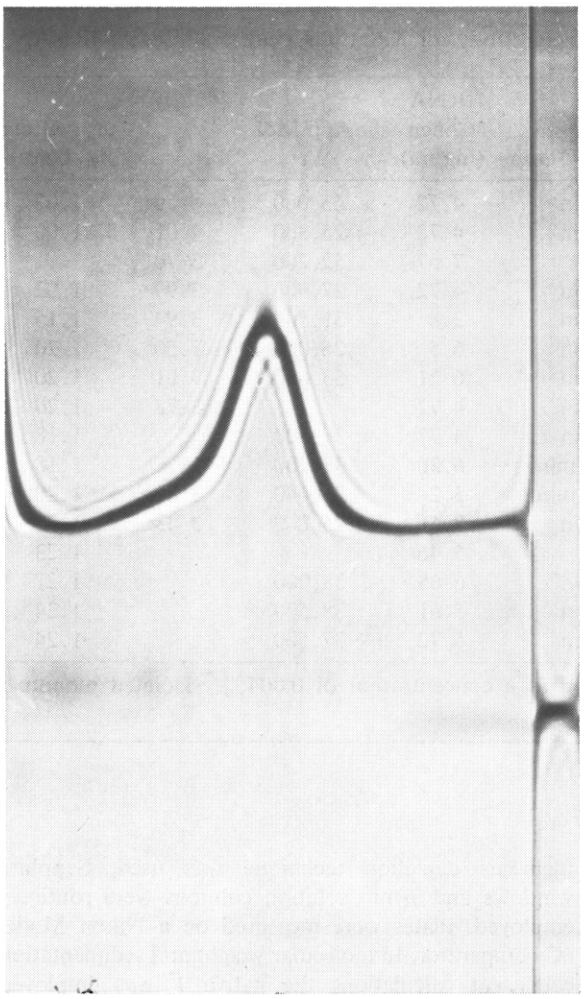

FIGURE 1: Schlieren photo of formalized tRNA (12% formaldehyde, 0.3 M KCl–0.01 M TEA (pH 7.0), 30 min, 90°, concentration 5.7 mg/ml), speed 56,100 rpm. The asymmetry of the centrifugal edge reflects the aggregation.

per cent of the previous experiment. This indicates no change in extinction coefficient occurs upon dialysis.

Absorbance melting curves were performed as described previously (Millar and Steiner, 1966) except that tightly fastened silicone caps sealed the cuvets.

Coupling of the fluorescent dye, acriflavine, to formalized tRNA was accomplished as described previously (Millar and Steiner, 1966) except that prior to periodate oxidation uncombined formaldehyde was removed by three times repeated cold ethanol–KCl precipitation. Measurements of polarization of fluorescence were done as described earlier (Millar and Steiner, 1966).

Results

Effect of the Conditions of Formaldehyde Treatment upon the Molecular Weight and Sedimentation Coefficient of tRNA. Table I illustrates the fact that the solvent conditions and temperatures employed here in blocking polyribonucleotide amino groups with formaldehyde lead to slightly varying degrees of aggregation, although at 100° to a lesser degree than that which occurs in the absence of formaldehyde. In view of the differences in solvents, temperature, and tRNA concentrations, we do not feel that the data in Table I can be uniquely discussed in terms of finding minimal aggregation conditions in the reaction of tRNA with formaldehyde. Figure 1 displays a typical Schlieren photograph of formalized tRNA. Native tRNA at an identical ionic strength shows a completely symmetrical profile (Millar and MacKenzie, 1966).

As estimated by the increase in 260-mμ absorbance most of the conditions employed lead to a similar degree of reaction. By comparison with the data of Penniston and Doty (1963) who achieved complete reaction of yeast tRNA in 0.3% formaldehyde in 75–100 hr at room temperature, we may estimate that nearly complete reaction occurs in the bulk of conditions used here. Our results are also compatible with the data of Furano *et al.* (1966) who found complete reaction of *E. coli* tRNA in 20 hr in 5% formaldehyde with a rise in extinction coefficient of 25%.

Chromatography of Formalized tRNA. Several molecular weight concentration dependency tests were performed on formalized tRNA samples. The results

TABLE II: Effect of Formaldehyde Treatment upon the Intrinsic Viscosity of tRNA.[a]

Reaction Buffer (M)	Formaldehyde Concn (%)	Temp of Treatment (°C)	Time of Treatment (min)	Intrinsic Viscosity (dl/g)
TEA (0.01)	2	50	60	0.090
KCl (0.3)–TEA (0.01)	2	100	5	0.090[b]
TEA (0.01)	4	60	30	0.10
TEA (0.01)	4	60	30	0.091
KCl (0.3)–TEA (0.01)	4	100	3	0.095[b]
KCl (0.3)–TEA (0.01)	4	100	3	0.10[b]
KCl (0.3)–TEA (0.01)	4[c]	100	5	0.090[c]

[a] All viscosity determinations performed in 0.3 M KCl–0.01 M TEA (pH 7.0) plus (unless otherwise indicated) the appropriate formaldehyde concentration and measured at 20°. [b] The monomeric fraction obtained by column chromatography as described in the text. [c] In this experiment, unreacted formaldehyde was removed by ethanolic–KCl precipitation followed by 18-hr dialysis *vs.* two changes of 0.3 M KCl–0.01 M TEA (pH 7.0).

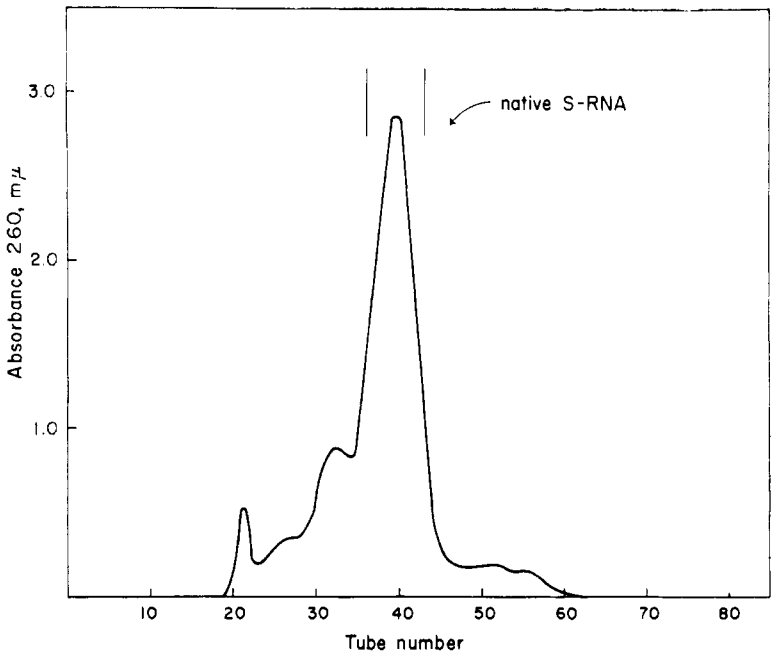

FIGURE 2: G-75 Sephadex chromatography of formalized tRNA (4% formaldehyde, 5 min, 100°) at 20°. The eluting solvent in the case shown is 0.3 M KCl–0.01 M TEA, 4% formaldehyde, pH 7.0. Column dimensions 81 × 2.9 cm. Approximately 3-ml fractions were collected. Native tRNA on the same column but in the absence of formaldehyde elutes as a single peak at the point indicated by the arrow.

indicated only a slight concentration dependency, thus suggesting that the aggregates consisted of tightly bound monomer units. Since high-temperature treatment resulted in aggregation in the absence of formaldehyde (Table I), it is likely that the polymers of tRNA resulted from intermolecular hydrogen bonds formed as the solutions cooled. These results confirm the temperature-induced aggregation previously reported by Brown and Zubay (1960). Clearly, the presence of aggregates renders the interpretation of both hydrodynamic and optical evidence ambiguous. Separation of the aggregates from the monomers was achieved by chromatography on G-75 Sephadex. Figure 2 shows a typical elution diagram. The main peak was isolated as indicated by the vertical bars, then pervaporated down to a suitable volume and dialyzed against 0.3 M KCl–0.01 M TEA and the appropriate formaldehyde concentration. Molecular weight and sedimentation coefficient estimations for several such preparations are shown in Table I. Figure 3 shows typical molecular weight data. The linearity of the ln Y *vs.* r^2 plot confirms the expected homogeneity of the column isolate. Sedimentation velocity experiments also demonstrated the homogeneity of the isolated column peak. The molecular weights of the isolated fractions show them to be monomeric tRNA. As Table I shows, the $s^0_{20,w}$ of the monomer is considerably decreased compared to that of native tRNA.

The Intrinsic Viscosity of Formalized tRNA. Table II shows the intrinsic viscosity of formalized tRNA. Data obtained with aggregate-containing solutions and column-isolated monomer are presented. It may be noted that there is not a great difference between the intrinsic viscosity of aggregate-containing solutions and monomer solutions. It is possible that this arises because of shape factors in the aggregates which compensate for the expected increase in viscosity due to the higher average molecular weight of the solution.

In any event, formaldehyde concentrations up to 4% seem to exhibit trivial denaturing effects on formalized tRNA since the viscosity of formalized tRNA measured in the absence of formaldehyde is the same as that value obtained in the presence of formaldehyde. The intrinsic viscosity of formalized tRNA may be compared with the value of 0.059 dl/g (25°) for native tRNA measured at an identical ionic strength (Millar and Steiner, 1966). Clearly, treatment of tRNA with formaldehyde increases the effective hydrodynamic volume of tRNA.

Absorbance Melting Curve of Formalized tRNA. Figure 4 displays the absorbance melting curves of formalized tRNA (monomer) in the presence and absence of magnesium ion. Clearly, the cooperative melting behavior shown in neutral salt (Millar and MacKenzie, 1966a) and in 0.02 M Mg^{2+} (Millar and Steiner, 1966) is abolished. Instead, an essentially linear variation of absorbance with temperature occurs. Fasman *et al.* (1965) has reported a similar phenomenon with the optical rotatory dispersion (ORD) melting curve of formalized tRNA in 0.01 M Mg^{2+}. The absorbance change does not appear to be complete at the highest temperature attained.

The absorbance thermal profile of formalized tRNA

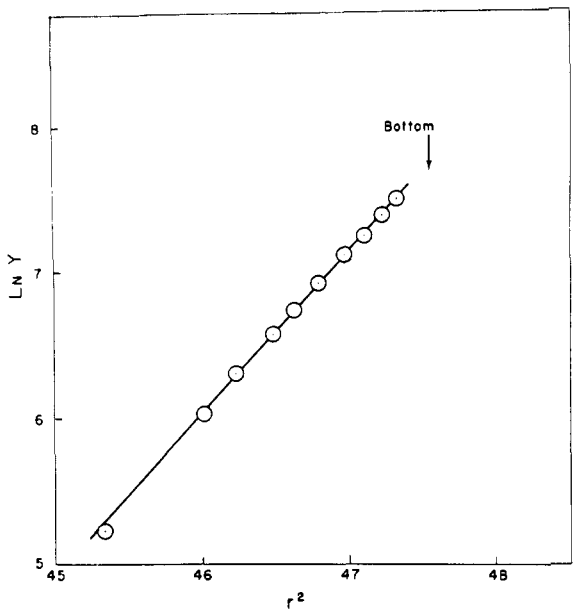

FIGURE 3: Plot of ln Y *vs.* r^2 of a typical column isolated monomer. Initial concentration was 0.3 mg/ml. Solvent, 0.3 M Tris–0.01 M TEA, 2% formaldehyde, pH 7.0. Speed 17,980 rpm.

reveals another interesting fact. With native tRNA magnesium ion displaces the thermal profile to higher temperatures and sharpens the melting curve. Figure 4 shows that there is no major effect of magnesium ion on the melting curve of formalized tRNA.

Polarization of Fluorescence of Acriflavine Conjugates of Formalized tRNA. Clearly, the absorbance thermal profiles of formalized tRNA indicate the presence of some sort of organized structure. To gain further information a sample of tRNA was formalized (5′, 100°, 4% formaldehyde) and the monomer isolated by column chromatography and coupled to the fluorescent dye acriflavine by the procedures described in detail elsewhere (Millar and Steiner, 1966).

Figure 5 shows the results. For comparison, the calculated thermal dependence of polarization for a rigid sphere of mol wt 26,000 and a volume of 30,000 A^3 is included in the figure. Details of the calculation are shown elsewhere (Millar and Steiner, 1965). Since formaldehyde is present in the experiment, precise calculation of relaxation times is hazardous due to possible alteration of the dye's lifetime. *Assuming*, however, that the dye's lifetime is not greatly altered, the continuous upward curvature suggests a progressive increase in internal flexibility and is consistent with the monotonic, uncooperative transition evidenced in the absorbance thermal profile. Both phenomena are different from those shown by native tRNA in which the loss in absorbance is cooperative in character and the appearance of internal flexibility is more abrupt (Millar and Steiner, 1966; Millar and MacKenzie, 1966a). However, the fact that there is internal rigidity (that is, lack of total freedom of rotation between bases along the ribose phosphate backbone) definitely shows the presence of rotationally restraining internucleotide forces. Since fluorescent conjugates of the purine homopolymer, poly A, also show a restricted internal rotation (Millar and Steiner, 1965), the idea

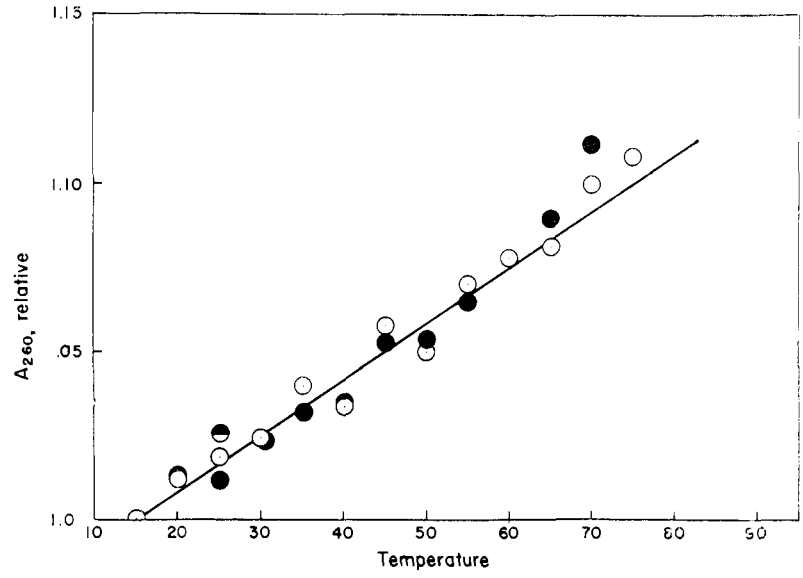

FIGURE 4: Absorbance melting curves of formalized (2% formaldehyde, 5 min, 100°) monomer tRNA. (○) 0.3 M KCl, 0.01 M TEA, and 2% formaldehyde. (●) 0.01 M TEA, 0.02 M Mg^{2+}, and 2% formaldehyde. ◒ indicates the values obtained when the solution is cooled. Similar melting curves are seen for unfractionated formalized tRNA. Formaldehyde concentrations up to 12% produce only slightly altered thermal profiles. tRNA treated the same way but in the absence of formaldehyde shows a sigmoidal melting curve with a T_m of approximately 59°.

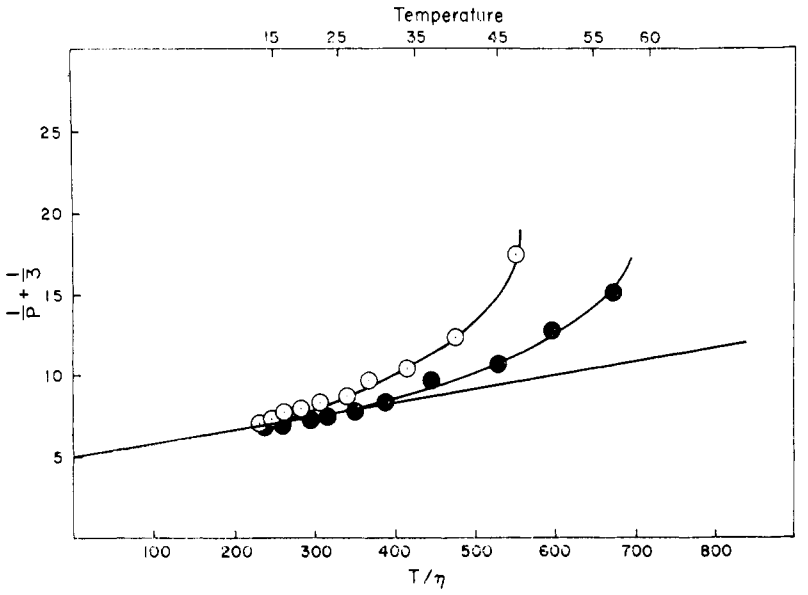

FIGURE 5: Polarization of fluorescence thermal profiles of formalized monomer tRNA. Here P is polarization and T the temperature in degrees Kelvin; η is solvent viscosity. (○) 0.01 M TEA, 0.3 M KCl, and 4% formaldehyde. (●) 0.01 M TEA, 0.02 M Mg^{2+}, and 4% formaldehyde. λ activation 436 mμ, λ emission 510 mμ. The polarization of fluorescence thermal profile is not corrected for the viscosity of formaldehyde. Inclusion of this correction would displace the curves to the left by about 10%. Some stabilization by magnesium ion over that of neutral salt is apparent in this experiment.

that base stacking and possibly the phosphate–ribose–base interactions mentioned previously confer the rotational rigidity observed in formalized tRNA is persuasive. This notion receives support from the observation that fluorescent conjugates of poly U show a very high degree of rotational flexibility above 20° where the helix–coil transition is complete (as judged by absorbance thermal profiles) and hence base stacking should be largely minimized (Millar and Steiner, 1965).

Discussion

The values of $s^0_{20,w}$ and $[\eta]$ obtained for formalized tRNA monomer are 3.5 and 0.09 dl/g. Similar values are seen in the thermal denaturation of yeast tRNA at about 50° (Henley *et al.*, 1966). At this temperature about half the secondary structure is gone as judged by absorbance data. As the T_m of native *E. coli* tRNA in 0.3 M KCl is about 57–58° (Millar and MacKenzie, 1966a) and other physical parameters (Millar and Steiner, 1966; Millar, 1966; Millar and MacKenzie, 1966b) are similar to those of yeast tRNA (Lindahl *et al.*, 1965; Henley *et al.*, 1966), a comparison of properties between the two tRNA's is justified. Since our values of s and $[\eta]$ are determined at 20°, the reaction of formaldehyde with *E. coli* tRNA clearly produces a highly denatured molecule, probably unfolded.

It is pertinent that formalization of poly A and poly C results in only minor changes in the absorbance melting curve (Stevens and Rosenfeld, 1966; Fasman *et al.*, 1964) and that only a 4% change in intrinsic viscosity occurs for poly A (Cartwright and Larcom, cited by Stevens and Rosenfeld, 1966). It thus appears that for a polynucleotide in which internucleotide interactions are restricted only to base stacking forces, formalization has only little effect on secondary structure or on hydrodynamic properties. Clearly, the data presented in this report demonstrate that formalization produces significant alterations in both the optical and hydrodynamic properties of tRNA. Thus it seems reasonable to conclude that the presence of intact amino groups (*i.e.*, hydrogen-bond donor groups) is instrumental in maintaining the solution *configuration* and *helical properties* of native tRNA.

The difference in shape of the absorbance melting curve of formalized tRNA as compared to native tRNA may imply that the native hydrogen-bonded structure in some manner moderates the way in which base stacking forces are thermally disrupted. In this connection, it is probably more than coincidental that the absorbance melting curves and polarization thermal profiles of neutral (single stranded) and acid (double stranded, hydrogen bonded) poly A show distinct differences. The former show diffuse, gradual transitions while in the latter the order–disorder transition is compressed and both absorbance and polarization profiles are congruent (Millar and Steiner, 1965).

Since the stabilizing effect of magnesium ion is largely reduced in formalized tRNA, it may be that it is the presence of a particular conformation of native tRNA or the ability to assume such a conforma-

tion which is involved in the magnesium ion effect. This suggestion has been advanced on different grounds (Millar and Byrne, 1967).

It is important to point out, however, that after formalization some sort of organized structure is present as is evidenced by both absorbance melting curves and the polarization profiles. Fasman *et al.* (1965) have previously observed this by ORD measurements on formalized tRNA. Since most base pair hydrogen-bonding ability is probably gone in formalized tRNA, this result implies the stabilizing importance of base stacking in native tRNA. Thus the data presented in this report are consistent with a role for both hydrogen bonding and base stacking in stabilizing tRNA structure and indeed suggest that the two forces are not independent of each other. In this connection it may be mentioned that Vournakis and Scheraga (1966) have recently suggested that alanine and tyrosine tRNA are both stabilized by hydrogen bonding and base stacking. It may be noted that the conclusions reported in this paper do not support the view that the molecular architecture and secondary structure of tRNA are derived chiefly from base stacking (Kay and Oikawa, 1966; Brahms *et al.*, 1966; Fasman *et al.*, 1965). It is more likely that structure stabilization in tRNA is a result of many factors, only a few of which are presently appreciated.

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