Kinetics of Ethidium Bromide Binding as a Probe of Transfer Ribonucleic Acid Structure†

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ABSTRACT: Temperature-jump relaxation methods have been employed to study the kinetics of interaction between unfractionated transfer RNA from yeast and the intercalating dye ethidium bromide. At 25° three relaxation processes can be detected which, on the basis of their concentration dependence, have been ascribed to a bimolecular "outside-binding" followed by an intercalation step and finally a fast isomerization of the intercalated dye–tRNA complex (Tritton, T. R., and Mohr, S. C. (1971), *Biochem. Biophys. Res. Commun. 45*, 1240). The isomerization process vanishes at 10 and 15°, probably because of greater stability of tRNA tertiary structure. Aminoacylation alters the kinetics of ethidium binding, especially in the bimolecular step, indicating that uncharged tRNA and aminoacylated tRNA have different conformations. Addition of 10 mM Mg^{2+}, however, has little effect on ethidium binding kinetics. Rate and equilibrium constants, activation energies, and thermodynamic parameters have been calculated for the elementary steps. Stopped-flow experiments reveal two additional rate processes in the ethidium–tRNA system over a wide temperature range (12–50°) which disappear at low ionic strength. They appear to correspond to dimerization of the dye–tRNA complexes followed (or preceded) by a slower isomerization. The forward rate constant for dimerization is 7.6×10^4 M^{-1} sec^{-1} at 25°.

The principle of using small ligands as spectroscopic probes into structural and functional properties of biological macromolecules is well established (Stryer, 1968; McConnell and McFarland, 1970; Fersht and Requena, 1971; Carey *et al.*, 1972). Numerous acridine and phenanthridine derivatives bind reversibly and noncovalently to nucleic acids (Lerman, 1964; Waring, 1968; Blake and Peacocke, 1968) and their visible absorption spectra and fluorescence properties make them ideal probes into nucleic acid structure. In the case of transfer RNA the phenanthridine ethidium bromide (EB)[1] has further been shown to have a negligible effect on one aspect of the biological activity—aminoacylation (Tao *et al.*, 1970; Lurquin and Buchet-Mahieu, 1971)—at low dye/phosphate ratios. Thus it can be assumed that ethidium does not grossly perturb the conformation of tRNA when it binds under these conditions and that effects observed in its presence most probably also occur in its absence. For these reasons, and also because EB probably binds to a unique site in yeast phenylalanine tRNA (Tao *et al.*, 1970), we have chosen this ligand as a "kinetic probe" of tRNA structure in solution using the method of relaxation spectrometry (Hammes, 1968a,b).

The nature of the interaction between planar heterocyclic dyes and nucleic acids has been increasingly well characterized in recent years by both equilibrium and kinetic methods (Blake and Peacocke, 1968; Crothers, 1971). We have previously reported (Tritton and Mohr, 1971) the relaxation kinetics of the interaction between EB and unfractionated yeast tRNA. Our data best fit the simple mechanism

$$\mathrm{EB} + \mathrm{tRNA} \underset{}{\overset{\tau_1}{\rightleftharpoons}} \mathrm{X_1} \overset{\tau_2}{\rightleftharpoons} \mathrm{X_2} \overset{\tau_3}{\rightleftharpoons} \mathrm{X_3} \qquad (1)$$

where X_1, X_2, and X_3 are dye–tRNA complexes and τ_1, τ_2, and τ_3 are relaxation times associated with the steps indicated. The first two processes resemble those observed in all reported cases of dye–double helical nucleic acid interactions (Li and Crothers, 1969; Schmechel and Crothers, 1971; Ramstein *et al.*, 1972; Steenbergen, C., Jr., and Mohr, S. C., manuscript in preparation). The first step represents an initial, largely electrostatic interaction between the negatively charged nucleic acid phosphate residues and the positively charged dye (at neutral pH). The second step is ascribed to the insertion or intercalation of the planar dye molecule between the base pairs of the double helical structure. The third step in eq 1 is unique to the tRNA class of nucleic acids and we have concluded that it represents a conformational change of the dye–tRNA complex. In this paper we present further evidence that this isomerization is due to a structural rearrangement of tRNA alone with the dye merely acting as a convenient marker. Using this same approach we have also investigated the effects of magnesium ion, aminoacylation, and ionic strength on the EB–tRNA system. We conclude that EB is a useful kinetic probe into structural and functional properties of tRNA.

A previous study of the EB–tRNA system, including some kinetics, has appeared (Bittman, 1969), but since the degree of saturation with ligand was more than ten times what we have used (leading to a much higher percentage of outside-bound dye) the results are not directly comparable to those presented here (*cf.* Tritton and Mohr, 1971).

Materials and Methods

"Soluble" ribonucleic acid from baker's yeast (prepared by the method of Holley *et al.*, 1961) and ethidium bromide were products of the Sigma Chemical Co. The level of residual magnesium in the Sigma RNA was determined by atomic absorption spectrophotometry on a Unicam SP90A instrument. We obtained a maximum value of about 0.25 g-atom of magnesium per mol of tRNA, or one magnesium ion per

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[1] Abbreviations used are: EB, ethidium bromide; tRNA, *unfractionated* transfer ribonucleic acid; aa-tRNA, unfractionated aminoacyl transfer ribonucleic acid.

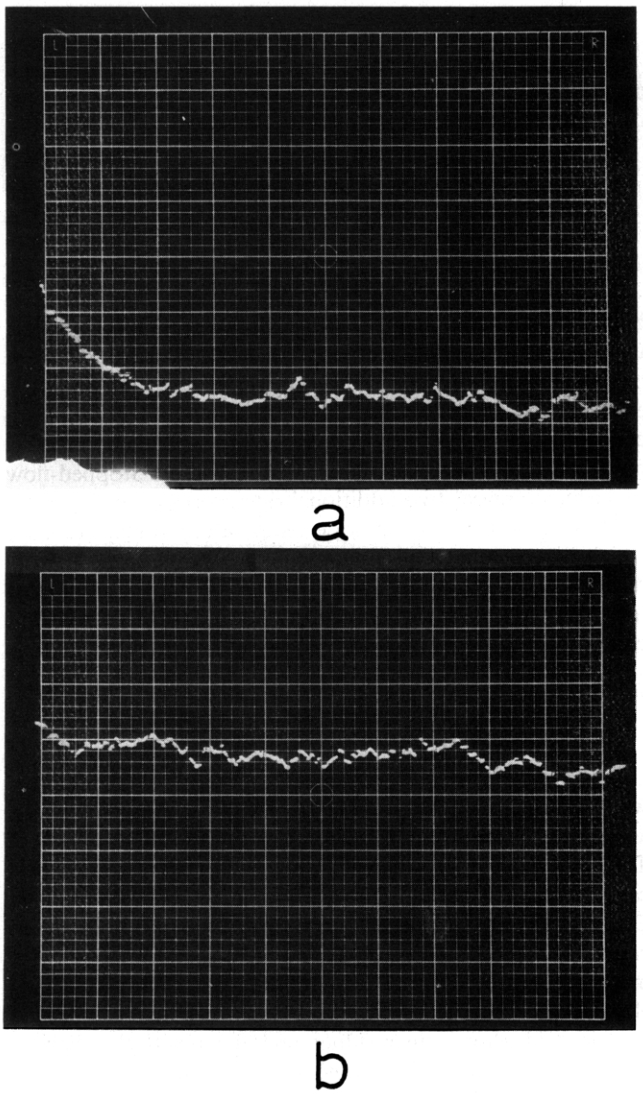

FIGURE 1: Oscilloscope traces of the fastest relaxation process (τ_3) observed after a 7.5° temperature jump: (a) final temperature, 25°; (b) final temperature, 10°. The vertical axis corresponds in arbitrary units to transmittance at 460 nm, the horizontal axis represents time, 20 μsec per major division. tRNA, 4.0×10^{-3} M (as phosphate residues), and EB, 5.0×10^{-5} M, were dissolved in buffer I.

320 phosphate residues. This agrees well with published values for similarly prepared RNA samples (Wacker and Vallee, 1959). Dialysis of the tRNA against 0.1 mM EDTA, then H_2O, gave no change in ethidium binding kinetics. Uniformly labeled [^{14}C]phenylalanine and Omnifluor were from New England Nuclear. All other chemicals were of reagent grade.

Buffer I is 0.05 M potassium phosphate, pH 7.0, containing 0.15 M KCl. Buffer II is the same plus 10 mM $MgCl_2$.

Absorbance values were measured on a Zeiss PMQ II spectrophotometer and calculations were done by high-speed digital computer (IBM Model 360/50).

All kinetic measurements were performed as described previously (Tritton and Mohr, 1971). The temperature-jump apparatus corresponds closely to that of Faeder (1970) (*cf.* French and Hammes, 1969) and stopped-flow experiments were carried out on a Durrum-Gibson Model D-130 apparatus with a 2-msec dead time. The ratio of bound ethidium to tRNA phosphate residues was maintained in all solutions as $r = 0.013$, corresponding to about one dye molecule bound per tRNA molecule of 80 nucleotide residues. In experiments with Mg^{2+} the amount of Mg^{2+} bound to tRNA was maintained constant in the same manner by applying an association constant of 1.7×10^4 M^{-1} (Sander and Ts'o, 1971). This value, determined with divalent ion-specific electrodes, was confirmed by us using difference spectroscopy.

The purity of EB was checked by thin-layer and paper chromatography in a 4:1:5 (v:v:v) solvent of *n*-butyl alcohol–acetic acid–water. Experiments performed in low light or darkness revealed a single spot, but the presence of strong sunlight caused two distinct spots on the thin-layer plates. For this reason all solutions containing ethidium were protected from light and new stock solutions were prepared frequently.

The extinction coefficient assumed for EB was 5.6×10^3 M^{-1} cm^{-1} at 480 nm (Waring, 1965). tRNA concentrations were estimated by assuming that 1.0 mg/ml (0.1%) had an absorbance at 260 nm of 23 and that the average molecular weight was 2.6×10^4. If tRNA concentration is determined with this method the solutions obey Beer's law within 2–3% over the concentration range of the kinetic experiments (*i.e.*, at an appropriate wavelength log (A/ϵ) *vs.* log concentration yields a slope of 1.0).

The soluble enzyme fraction from baker's yeast containing both the aminoacyl-tRNA synthetases and tRNA nucleotidyl transferase was prepared essentially according to Schmidt *et al.* (1970), except that extensive dialysis replaced the Sephadex step. The aminoacylating fraction was adjusted to 20% glycerol and was stable for several months at −20°.

The aminoacylation reaction was monitored by following [^{14}C]phenylalanine acceptance. The assay mixture of 0.1 ml contained 10 mM ATP, 50 mM $MgCl_2$, 100 mM Tris-Cl, pH 7.5, 0.05 mM [^{14}C]Phe (30 μCi/μmol), 0.2 mM CTP, 25 μl of the enzyme preparation, and 25 μl of the tRNA sample to be assayed. The reaction mixture was incubated at 25° and 20-μl aliquots were spotted on Whatman 3MM filter disks at selected time intervals and the reaction stopped by immersion into ice-cold 5% trichloroacetic acid. The disks were then washed with trichloroacetic acid, ethanol, and ether, dried, and counted in 5 ml of a 4 g/l. Omnifluor–toluene solution on a Picker Nuclear Liquimat 220 scintillation counter.

For preparative aminoacylation the same reaction mixture was scaled up to 5 ml and contained 0.2 mM of 20 [^{12}C]-amino acids and 50 mg of unfractionated tRNA. The optimum time of reaction was determined as above for each enzyme preparation and was usually about 20 min at 25°. The reaction mixture was incubated for the predetermined time and then chilled in ice for 1 min. An equal volume of redistilled, water-saturated phenol was added followed by vigorous shaking at 4° for 10 min. The aqueous layer was then separated by centrifugation and 0.1 vol of 20% KOAc (pH 4.5) and 2 vol of ethanol were added and allowed to stand for 2 hr at −20°. The aminoacyl-tRNA precipitate was recovered by centrifugation, dissolved in cold H_2O, and extensively dialyzed against several changes of 1 mM EDTA and double-distilled H_2O. The preparation was stored frozen at −20° and used within a few days to avoid hydrolytic deacylation. A 0.1-ml mixture containing [^{14}C]Phe and 19 cold amino acids was run in parallel to the preparative aminoacylation to monitor the extent of reaction.

Aminoacyl-tRNA was deacylated by incubation in 1.0 M Tris-Cl, pH 8.5, at 37° for 30 min followed by dialysis and lyophilization to recover the "stripped" tRNA.

We have found that meticulous cleaning of glassware is

TABLE I: Rate and Equilibrium Constants for EB–tRNA Binding.[a]

System	T (°C)	$10^{-5}k_1$ (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	k_2 (sec^{-1})	k_{-2} (sec^{-1})	$10^{-4}k_3$ (sec^{-1})	$10^{-3}k_{-3}$ (sec^{-1})	K_1 (M^{-1})	K_2	K_3	$10^{-3}K_{ap}$ (M^{-1})	$10^{-3}\cdot K_{spec}$[c] (M^{-1})
tRNA[b]	25	5.67[d]	866	468	623	1.57	3.32	655	0.75	4.72	3.5	3.0
tRNA	15	3.1	680	490	71			463	6.9		3.7	
tRNA	10	1.5	220	570	49			334	11.5		4.2	
aa-tRNA	25	2.04	259	413	686	1.49	3.72	788	0.60	4.01	3.17	4.67
tRNA + Mg^{2+}	25	7.04	325	318	373	0.90	4.31	2170	0.85	2.09	7.88	

[a] Values were calculated as described under Results. The buffer conditions in all cases were 0.15 M KCl–0.05 M potassium phosphate, pH 7.0; r = 0.013. [b] From previous work (Tritton and Mohr, 1971). [c] Overall equilibrium constant determined by spectrophotometric titration. [d] Estimated accuracy in rate constants is about ±15% (*cf.* French and Hammes, 1969).

necessary for reproducibility of experiments with tRNA. The procedure used was soaking in sodium dichromate–H_2SO_4 cleaning solution followed by rinsing with distilled water, scrubbing with detergent (Alconox), and finally copious rinsing with double-distilled water to remove remaining traces of chromium ions or detergent.

Results

Temperature Dependence of the EB–tRNA Relaxation Spectrum. We have investigated the effect of temperature on the interaction of EB with tRNA over the range 10–25°. The single most striking observation is the disappearance of the fast relaxation effect τ_3 at low temperature. The amplitude of this effect which is clearly visible at 25° is negligible or zero at 15 and 10° (Figure 1). Effects corresponding to both τ_1 and τ_2 (eq 1) are present at reduced temperature, however, and the concentration dependencies fit the simple mechanism

$$EB + tRNA \underset{}{\overset{\tau_1}{\rightleftharpoons}} X_1 \overset{\tau_2}{\rightleftharpoons} X_2 \qquad (2)$$

Standard analysis where $1/\tau_1 \gg 1/\tau_2$ yields

$$1/\tau_1 = k_1(\overline{tRNA(P)} + \overline{EB}) + k_{-1} \qquad (3)$$

$$1/\tau_2 = \frac{k_2}{1 + 1/K(\overline{tRNA(P)} + \overline{EB})} + k_{-2} \qquad (4)$$

where k_1,k_{-1}, and k_2,k_{-2} are the forward and reverse rate constants respectively, for the two steps, $K = k_1/k_{-1}$, and $\overline{tRNA(P)}$ and $\overline{EB}$ represent the concentrations of uncomplexed tRNA phosphate residues and free ethidium bromide. The time scales of τ_1 and τ_2 at low temperature are similar to the corresponding processes at 25° (0.1–0.4 msec and 1–4 msec, respectively). The corresponding rate and equilibrium constants have been calculated at each temperature studied and are collected in Table I.

The forward rate constants are plotted logarithmically *vs.* reciprocal temperature in Figure 2. k_2 increases with decreasing temperature and therefore does not correspond to a simple elementary step (see Discussion). By assuming it to be independent of temperature we can calculate the energy of activation ($E^{\pm}$) for each mechanistic step i by means of the Arrhenius equation

$$\frac{d(\ln k_i)}{d(1/T)} = -\frac{E^{\pm}_i}{R} \qquad (5)$$

where R is the gas constant and T is absolute temperature. Activation and thermodynamic parameters are given in Table II. In comparison with published values for proflavine–nucleic acid interactions (Li and Crothers, 1969; Schmechel and Crothers, 1971) the enthalpy and entropy changes for ethidium binding to tRNA are substantially greater.

Effect of Aminoacylation on the Relaxation Spectrum. The apparent equilibrium constant for the interaction between EB and aa-tRNA was determined spectrophotometrically according to the method of Li and Crothers (1969). The data are plotted in Figure 3 and extrapolation to infinite tRNA concentration (*i.e.*, r = 0) yields a value of $K_{ap} = 4.7 \times 10^3$ M^{-1}. This value is substantially different from that determined for uncharged tRNA and EB by the same method (3.0×10^3 M^{-1}).

In order to demonstrate reproducibility and to rule out the presence of artifacts we have conducted kinetic experiments on several different preparations of aminoacyl-tRNA. Three distinct relaxations which correspond approximately to those observed with unacylated tRNA at 25° were observed in all cases. The mechanism is thus qualitatively (but not quantitatively, *vide infra*) the same in both cases and conforms to eq 1. For $1/\tau_3 > 1/\tau_1 > 1/\tau_2$ the concentration de-

TABLE II: Interaction Energies[a] between tRNA and Ethidium Bromide (kcal/mol).

$E^{\pm}_1$	$E^{\pm}_{-1}$	$E^{\pm}_2$	$E^{\pm}_{-2}$	ΔH°_1	ΔH°_2	$T\Delta S^{\circ}_1$[b]	$T\Delta S^{\circ}_2$[b]
14.3	13.8	−2.0	29.5	7.3	−31.5	11.2	−31.6

[a] $E^{\pm}_i$ is the Arrhenius activation energy for the ith mechanistic step. ΔH°_i and ΔS°_i are the corresponding thermodynamic enthalpy and entropy changes. Conditions were 0.15 M KCl–0.05 M potassium phosphate, pH 7.0, temperature, 10–25°, r = 0.013. Parameters were evaluated by the method of least squares. [b] Calculated at 25°.

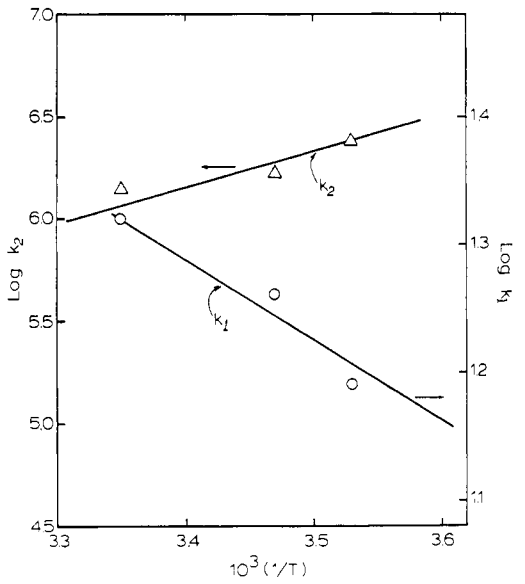

FIGURE 2: Arrhenius plots for k_1 and k_2 (*cf.* Table I). The lines are arbitrary.

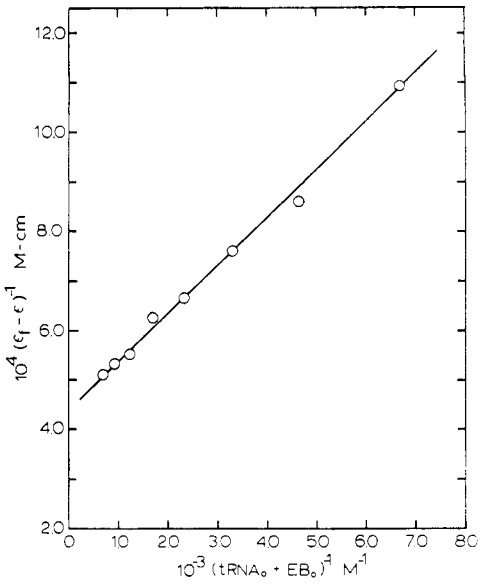

FIGURE 3: Double reciprocal plot of extinction coefficient measurements at 480 nm against concentration for aminoacyl-tRNA–ethidium bromide solutions in buffer I.

pendencies can be shown by standard methods (Czerlinski, 1966) to be

$$1/\tau_1 = k_1(\overline{\text{tRNA(P)}} + \overline{\text{EB}}) + k_{-1} \tag{6}$$

$$1/\tau_2 = \frac{k_2}{1 + 1/K_1(\overline{\text{tRNA(P)}} + \overline{\text{EB}})} + \frac{k_{-2}}{1 + K_3} \tag{7}$$

$$1/\tau_3 = k_3 + k_{-3} \tag{8}$$

where we define

$$K_1 = k_1/k_{-1}$$

$$K_2 = k_2/k_{-2} \tag{9}$$

$$K_3 = k_3/k_{-3}$$

The overall equilibrium constant K_{ap} is related to the kinetic constants by

$$K_{ap} = K_1[1 + K_2(1 + K_3)] \tag{10}$$

As we have pointed out previously (Tritton and Mohr, 1971) there is no unique analytical solution to these equations. However, Hammes and Schimmel (1970) have shown that the number of invariant properties of the secular determinant which defines the relaxation spectrum is in general equal to the number of relaxation times. For the case in hand the determinant is $|\mathbf{A} - (1/\tau)\mathbf{I}|$ where $\mathbf{I}$ is the unit matrix and

$$\mathbf{A} = \begin{bmatrix} k_1(\overline{\text{tRNA(P)}} + \overline{\text{EB}}) + k_{-1} & -k_1 & 0 \\ -k_2 & k_2 + k_{-2} & -k_{-2} \\ 0 & -k_3 & k_3 + k_{-3} \end{bmatrix} \tag{11}$$

This 3×3 matrix $\mathbf{A}$ with elements a_{ij} has the following invariant properties (eq 12–14) (Bellman, 1970).

$$\sum_{i=1}^{3} 1/\tau_i = \sum_{i=1}^{3} a_{ii} = \text{tr}\,(\mathbf{A}) \tag{12}$$

$$\prod_{i=1}^{3} 1/\tau_i = \det(\mathbf{A}) \tag{13}$$

$$\sum_{i=i+1}^{3}\sum_{i=1}^{2} (1/\tau_i)(1/\tau_j) = \sum_{i=i+1}^{3}\sum_{i=1}^{2} a_{ii}a_{jj} - \sum_{j=i+1}^{3}\sum_{i=1}^{2} a_{ij}a_{ji} \quad (i \neq j) \tag{14}$$

(Equation 14 simply says that the sum of the products of all pairs of eigenvalues equals the sum of the products of all pairs of diagonal elements minus the sum of the products of all off-diagonal elements of the type $a_{ij}a_{ji}$ where $i \neq j$.) These equations (12–14) then yield the following experimentally useful relationships (eq 15–17) (*cf.* Hammes and Haslam, 1968).

$$1/\tau_1 + 1/\tau_2 + 1/\tau_3 = k_1(\overline{\text{tRNA(P)}} + \overline{\text{EB}}) + k_{-1} + k_2 + k_{-2} + k_3 + k_{-3} \tag{15}$$

$$1/\tau_1\tau_2\tau_3 = k_1(\overline{\text{tRNA(P)}} + \overline{\text{EB}})(k_2k_3 + k_2k_{-3} + k_{-2}k_{-3}) + k_{-1}k_{-2}k_{-3} \tag{16}$$

$$1/\tau_1\tau_2 + 1/\tau_2\tau_3 + 1/\tau_1\tau_3 = k_1(\overline{\text{tRNA(P)}} + \overline{\text{EB}})(k_2 + k_{-2} + k_3 + k_{-3}) + k_{-1}k_{-2} + k_{-2}k_{-3} + (k_{-1} + k_2)(k_3 + k_{-3}) \tag{17}$$

The left-hand side of each equation is plotted as a function of $(\overline{\text{tRNA(P)}} + \overline{\text{EB}})$ in Figure 4 and three independent sets of

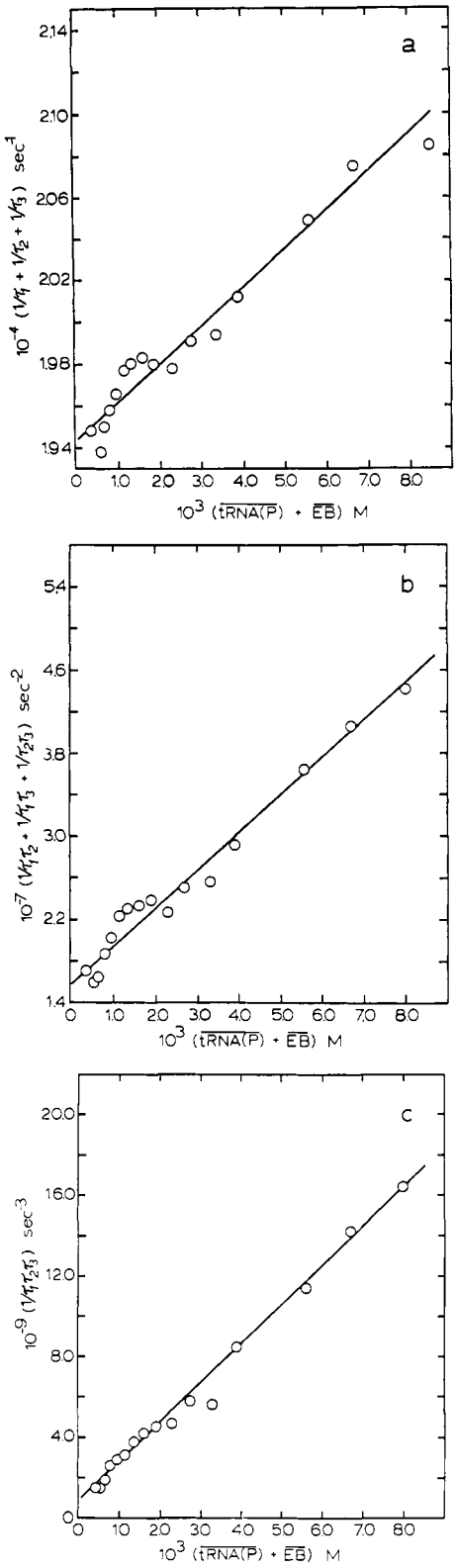

FIGURE 4: Secondary plots of kinetic data according to eq 15 (a), 16 (b), and 17 (c). See text for details.

slopes and intercepts are obtained. All six rate constants and the corresponding equilibrium relations may be calculated. These rate constants are then used to solve the secular determinant for each individual relaxation time as a function of concentration. This procedure has been carried out over the error range of the data and the best calculated fit is shown

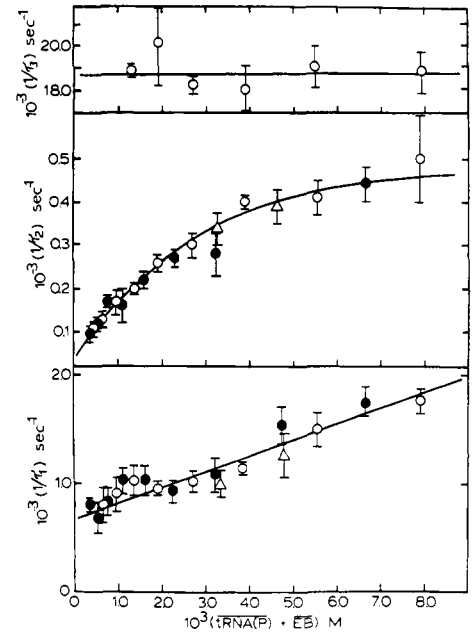

FIGURE 5: Concentration dependence of reciprocal relaxation times for ethidium binding to aminoacyl-tRNA in buffer I, 25°. Different symbols correspond to different aa-tRNA preparations. Error bars give the standard deviations of $1/\tau_i$ for the four–eight traces analyzed. The solid lines were calculated as described under Results, assuming the mechanism given in eq 1.

as the solid lines in Figure 5 and agrees well with the experimental data.

It is apparent from Figure 5 that different preparations of aa-tRNA give substantially the same results and are quantitatively different from the "stripped" tRNA case (Tritton and Mohr, 1971; see also Table I). In order to check the reversibility of the changes one of the preparations was chemically deacylated as described under Materials and Methods. The results of charged *vs.* uncharged tRNA kinetic studies are shown superimposed in Figure 6. As before the lines represent the calculated best fit to the data assuming the mechanism of eq 1. It is clear that the difference—primarily in τ_1—observed upon aminoacylation is completely reversible; the deacylated sample (points with error bars in Figure 6) shows the same kinetic behavior as control tRNA which was never aminoacylated. The small changes in τ_2 and τ_3 upon aminoacylation lie within experimental error. Appropriate rate and equilibrium constants are presented in Table I.

Effect of Magnesium Ion on the Relaxation Spectrum. Magnesium ions are known to exert an effect on the conformation and conformational stability of tRNA. In order to relate our findings to more physiological conditions we have investigated the kinetics of EB binding to tRNA in the presence of *ca.* 0.01 M Mg^{2+} at 25°. The relaxation spectrum is qualitatively the same as in the absence of Mg^{2+} but again quantitative differences are observed. The data were treated as described above and the resulting calculated curves are presented in Figure 6. For illustrative clarity the individual data points are not shown, but calculated and experimental results are in good agreement. Table I contains the elementary step kinetic constants for reaction in the presence of Mg^{2+} ion.

Stopped-Flow Kinetics in the Presence and Absence of Magnesium Ion. In addition to the three temperature-jump relaxation effects, we also observe two stopped-flow effects for the EB–tRNA system both in the presence and absence

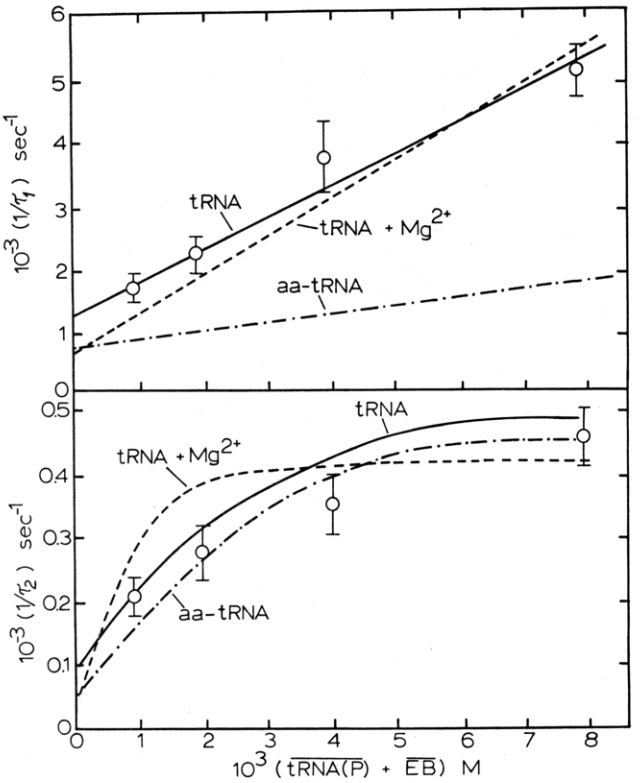

FIGURE 6: Comparison of the relaxation kinetics for the first two steps in ethidium binding to tRNA and aa-tRNA in the absence of Mg^{2+} (buffer I) and to tRNA in the presence of 0.01 M Mg^{2+} (buffer II). The temperature in all cases was 25°. Points with error bars correspond to aa-tRNA samples which were deacylated as described in Materials and Methods prior to the kinetic run. Other data points have been omitted for clarity (*cf.* Figure 5 and Figures 4–6 of Tritton and Mohr, 1971).

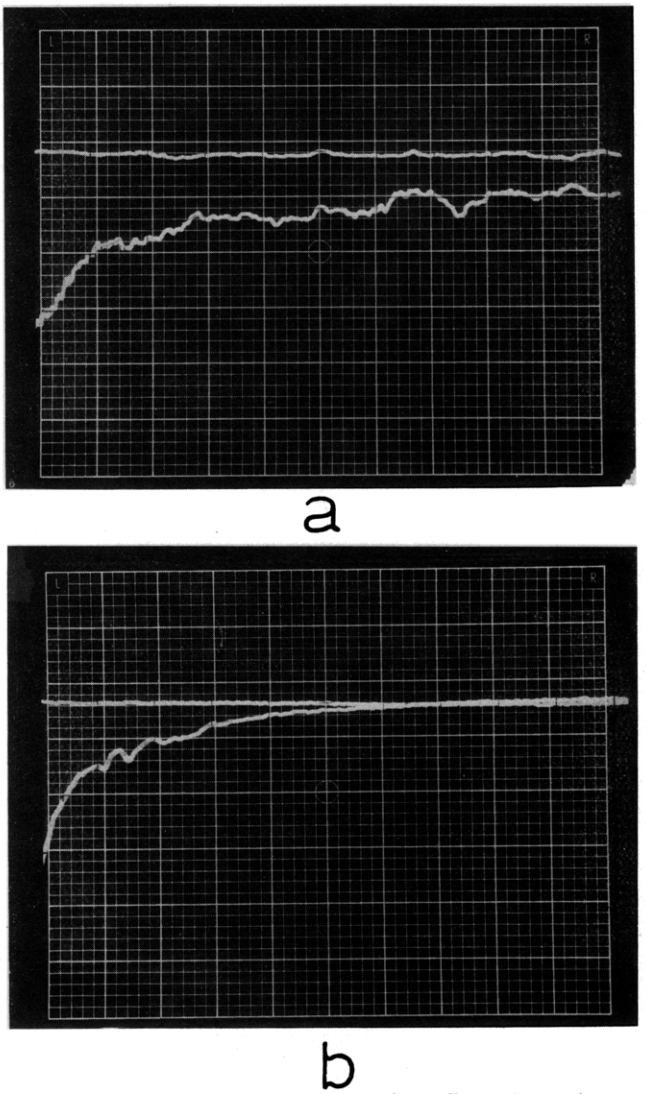

FIGURE 7: Oscilloscope traces of stopped-flow effects observed upon mixing 3.7×10^{-5} M EB and 2.7×10^{-3} M tRNA(P) in buffer I, 25°. The vertical axis corresponds to transmittance at 460 nm (1% T per major division) and the horizontal axis represents time, 10 msec per major division in (a) and 100 msec per major division in (b). The horizontal trace is the final equilibrium transmittance (≈10 sec after mixing).

of Mg^{2+}. These new effects are separated on the time scale from each other and from the temperature-jump effects by about an order of magnitude. Previously we reported a complete absence of stopped-flow effects at $r = 0.013$ (Tritton and Mohr, 1971), but this applies only to a narrow wavelength range ($\lambda \cong$ 500–510 nm). When all wavelengths are investigated the presence of slower effects is evident. They are only observed in situation A of Table III, however, and thus cannot represent the time course of a reaction caused by the interaction of either EB or Mg^{2+} with tRNA. These spectral changes rather are due to a dilution or concentration jump effect on the EB–tRNA complex. Representative oscilloscope traces of the two stopped-flow effects are shown in Figure 7. The faster one is independent of concentration, but the slower exhibits a dependence on tRNA concentration which would be anticipated for a bimolecular step (Figure 8). We believe these results are best explained by the combination of a dimerization and an isomerization step, that is

$$2\text{tRNA} \rightleftharpoons (\text{tRNA})_2 \rightleftharpoons (\text{tRNA})_2^* \qquad (18)$$

This point will be expanded upon in the Discussion. The mathematical description of this mechanism (when the second step equilibrates much more rapidly than the first) is

$$1/\tau_{S_1} = 4k_1'(\overline{\text{tRNA}}) + k_{-1}'/(1 + K_2') \qquad (19)$$

$$1/\tau_{S_2} = k_2' + k_{-2}' \qquad (20)$$

TABLE III: Experimental Set-Ups for Stopped-Flow Measurements.[a]

Situation	Syringe No. 1	Syringe No. 2[b]
A	tRNA	EB
B	tRNA + Mg^{2+}	tRNA + Mg^{2+} + EB
C	tRNA + EB	tRNA + EB + Mg^{2+}

[a] See text for details. [b] When the same substance was present in syringe no. 2 as in no. 1 the concentrations were identical.

where $K_2' = k_2'/k_{-2}'$ and $(\overline{tRNA})$ represents the total concentration of monomeric tRNA species. Only k_1' is extractable from the equations; at 25° in buffer I, $k_1' = 7.6 \times 10^4$ M^{-1} sec^{-1}. It is the same within experimental error in buffer II (see Figure 8).[2]

Effects of Temperature and Salt on the Stopped-Flow Reactions. When the ionic strength of the solutions is reduced to that which is due to EB and tRNA alone (*i.e.*, no added salts) the amplitude of both stopped-flow effects falls to zero. We have also observed that both stopped-flow relaxations are present at low temperature (12°) and high temperature (40, 50, and 59°). The amplitude of both effects increases with increasing temperature up to 50° and then at 59° falls to zero for the slow effect and near zero (but still detectable) for the faster effect. We have not investigated the concentration dependence of the effects in detail at these other temperatures.

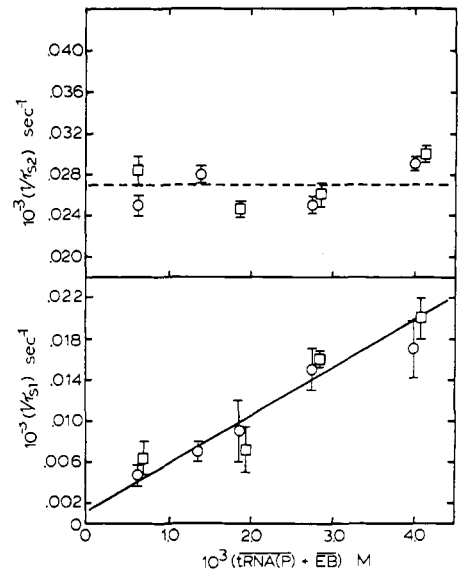

FIGURE 8: Concentration dependence of the two stopped-flow processes observed upon mixing EB with tRNA: top, the faster, concentration-independent effect (τ_{S_2}); the dashed line is arbitrary; bottom; the slower effect (τ_{S_1}); the solid line is a least-squares fit to the data. Circles and squares correspond to the absence and presence of 0.01 M Mg^{2+}, respectively. All data were obtained at 25°.

Discussion

Mechanism of Ligand Binding. We have observed (at 25°) *five* distinct rate processes in the ethidium bromide–unfractionated yeast transfer RNA system, all at a low value of r (ratio of bound dye to tRNA phosphate residues) where on the average a single ethidium moiety is bound to each tRNA molecule. Simultaneous solution of all the rate equations for this system would be quite complicated. It is possible, however, mathematically to treat the two slowest processes (observed by stopped-flow measurements) as separate and uncoupled from the three faster effects (observed by temperature-jump measurements) since they are sufficiently separated on the time scale (Hammes and Schimmel, 1966).

The positive slope in the Arrhenius plot for k_2 (Figure 2) means that eq 1 does not completely describe the mechanism of ethidium binding. Any process which corresponds to a single elementary step must have a positive activation energy. Other systems displaying such "anti-Arrhenius" behavior include the recombination reaction between complementary oligoribonucleotides (Pörschke and Eigen, 1971; Craig *et al.*, 1971) and the dimerization of *Escherichia coli* $tRNA_1^{Tyr}$ at 59° (Yang *et al.*, 1972). In each instance the complex behavior has been attributed to preequilibria which are shifted in the direction of dissociation by an increase in temperature. Such a dissociation cannot explain the behavior of k_2 since it formally corresponds to a unimolecular process. Nevertheless, intervention of an additional spectroscopically undetectable step between the outside-binding reaction and true intercalation might be postulated. It would have to have a negative enthalpy of reaction in the forward direction and could correspond either to a reorientation of the outside-bound ethidium prior to intercalation or to a rearrangement of tRNA structure. In this regard it is of interest that the rate constants for the proflavine–DNA (Li and Crothers, 1969) and proflavine–poly(A)·poly(U) (Schmechel and Crothers, 1971) systems exhibit normal temperature dependence.

The mechanisms given by eq 1 and 18 represent simplifications of the actual chemical processes occurring for the further reason that in all likelihood any two of the tRNA species may dimerize, *i.e.*, reactions such as

$$\begin{aligned} X_1 + X_3 &\rightleftharpoons X_1 \cdot X_3 \\ X_1 + tRNA &\rightleftharpoons X_1 \cdot tRNA \end{aligned} \qquad (21)$$

probably occur (X_1 and X_3 have the same meaning here as in eq 1).

We have argued previously (Tritton and Mohr, 1971) that the conformational transition in the EB–tRNA complex which gives rise to τ_3 may have a counterpart in free tRNA. This implies that eq 1 should be replaced by

$$\begin{array}{ccccc} EB + tRNA & \rightleftharpoons & X_1 & \rightleftharpoons & X_2 \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ EB + tRNA^* & \rightleftharpoons & X_1^* & \rightleftharpoons & X_3 \end{array} \qquad (22)$$

where the starred species correspond to tRNA in a conformation analogous to that of X_3. The hypothetical isomerization of the uncomplexed tRNA cannot be observed without the ethidium probe, however, and the absence of additional relaxation effects prevents drawing any conclusions about the reactions emanating from X_1^*. Equation 1 therefore remains adequate.

Obviously much more elaborate mechanisms for the binding of EB to tRNA could be devised. The treatment presented here, however, delineates the main features of the interaction and permits us to make deductions about tRNA structure based on the dye-binding kinetics. Comparison with simpler systems such as proflavine–DNA (Li and Crothers, 1969; Ramstein *et al.*, 1972), proflavine–poly(A)·poly(U) (Schmechel and Crothers, 1971), and proflavine–poly(I)·poly(C) (Steenbergen, C., Jr., and Mohr, S. C., manuscript in preparation) leaves little doubt that we have correctly identified and analyzed the processes corresponding to τ_1 and τ_2 as outside binding and intercalation, respectively.

[2] To calculate these values we have assumed that the concentration of dimers was negligible compared to the total tRNA concentration (see Discussion). Note also that while the data in Figure 8 are presented in terms of $\overline{tRNA(P)} + \overline{EB}$ to facilitate comparison with the other figures, the calculation of k_1' is based upon $\overline{tRNA}$. To a good approximation $[\overline{tRNA(P)} + \overline{EB}] = 80(\overline{tRNA})$.

The fact that we have employed unfractionated tRNA in these experiments means that our results represent the average behavior of a heterogeneous population of macromolecules. Considering this heterogeneity the observation of discrete relaxation effects for ethidium binding must be taken as further evidence for the great degree of structural homology among tRNA species (*cf.* Blake *et al.* (1970); Tao *et al.* (1970)). Extensive preliminary experiments have shown that the kinetics of ethidium binding to purified yeast $tRNA^{Phe}$ are qualitatively identical with whose reported here for unfractionated yeast tRNA (Tritton, T. R., Kotsiopoulos, P. and Mohr, S. C., unpublished results).

Conformational Flexibility of tRNA. More than other types of nucleic acid, transfer RNAs resemble globular proteins (Fresco *et al.*, 1966). They are soluble, compact macromolecules whose primary sequence of nucleotide residues gives rise to a defined secondary and tertiary structure essential for physiological function. In some cases they can be converted to "denatured," biologically inactive forms which are stable at room temperature, somewhat analogous to denatured proteins (Lindahl *et al.*, 1966; Gartland and Sueoka, 1966). These observations imply a certain conformational flexibility.

Equilibrium studies (Fresco *et al.*, 1966; Millar and Steiner, 1966) have strongly indicated the possibility of conformation changes occurring in tRNA in the range of 20–40° at moderate ionic strength, *i.e.*, well below the true melting transition. The difficulty long experienced in crystallizing tRNA might also be explained by a multiplicity of conformational states. Yarus (1972) has recently postulated two conformational states of $tRNA^{Ile}$ (*E. coli*) to explain his results in studies of the binding of this molecule to isoleucyl-tRNA synthetase.

Our results indicate conformational flexibility of tRNA in two ways. First the existence of the process which gives rise to τ_3 implies that at least in the EB–tRNA complex the tRNA can undergo a rapid structural transition. tRNA by itself very likely possesses a related transition. The second and more definitive evidence for conformational flexibility in tRNA is the behavior of the kinetic parameters k_1 and k_{-1} for EB binding upon aminoacylation. As described under Results above these parameters *clearly differ* for aa-tRNA and the differences are reversible.

Origin of τ_3. From the kinetics we have demonstrated that the fastest relaxation most probably corresponds to an intramolecular process subsequent to intercalation. The fact that it virtually disappears at 10 and 15°, coupled with other evidence for a thermally induced conformational change in tRNA structure in the 20–40° range (Henley *et al.*, 1966; Millar and Steiner, 1966; Pilz *et al.*, 1970) strongly suggests that this process involves a rearrangement of tRNA tertiary structure. The absence of such a process in all dye–double helical nucleic acid systems examined—where tertiary structure is likewise absent—strongly supports this interpretation. tRNAs from *E. coli* and from rat liver, however, do exhibit τ_3 upon interaction with ethidium bromide (Tritton, T. R., and Mohr, S. C., unpublished experiments). Further work, preferably with a single, purified tRNA species must be done to identify the principal ethidium binding site before speculations as to the detailed molecular processes giving rise to τ_3 are warranted.

Effect of Aminoacylation. Comparison of the kinetic parameters for EB binding to tRNA and aa-tRNA shows that significant differences appear in k_1 and k_{-1}, both of which are approximately one-third as great in the case of aa-tRNA as in the case of unacylated tRNA. The other rate constants are virtually the same within experimental error. It is possible that this difference in the kinetics of the outside-binding step reflects electrostatic repulsion between the ethidium cation and the protonated α-amino group of the amino acid esterified to the tRNA. This seems unlikely, however, since such an effect would be expected to *diminish* k_1 but *if anything to increase* k_{-1}.

A more plausible interpretation is that aminoacylation leads to a conformation change such that the binding site for ethidium becomes less accessible. This could by simple steric effects lead to a decrease in both k_1 and k_{-1}. It should be noted here that the outside-binding reaction is several orders of magnitude slower than that expected on the basis of diffusion control (*ca.* 10^9 M^{-1} sec^{-1}), implying that orientation effects are important in this process. This agrees with the results for the interaction of proflavine with double-helical nucleic acids (Li and Crothers, 1969; Schmechel and Crothers, 1971; Ramstein *et al.*, 1972; Steenbergen, C., Jr., X., and Mohr, S. C., manuscript in preparation). The extensive hydration shell of nucleic acids (Kuntz *et al.*, 1969; Tao *et al.*, 1970) may slow the outside-binding process.

We conclude that tRNA and aminoacyl-tRNA have significant structural differences which are reflected by the kinetics of ethidium binding. Contradictory findings with regard to this question have appeared for a number of years. Recently Hänggi and Zachau (1971) have reported no change in sensitivity to nuclease digestion in going from aminoacylated tRNA to stripped tRNA, while Chin and Kidson (1971) have performed equilibrium dialysis studies on the binding of various hormonal steroids to tRNA with the result that the binding is quite dependent upon having the tRNA in the aminoacylated form, presumably because this form exists in a unique conformation. Using circular dichroism and optically monitored thermal denaturation Watanabe and Imahori (1971) claimed to detect differences in the conformation of aminoacylated and stripped $tRNA_f^{Met}$. Wickstrom (1971), however, also using circular dichroism, reported exactly the opposite result. His data show no significant differences in the circular dichroism of fMet-$tRNA_f^{Met}$, Met-$tRNA_f^{Met}$, and unacylated $tRNA_f^{Met}$. He concluded that any structural differences which may exist are no more extensive than one or two base pairs and one or two stacking interactions. Hydrogen–tritium exchange studies also reveal no differences between tRNA and aa-tRNA (Englander *et al.*, 1972), but mesurement of the enhancement factor for longitudinal relaxation of solvent H_2O protons by bound Mn^{2+} has shown a distinct, reproducible change upon going from unacylated tRNA to aminoacyl-tRNA and back (Cohn *et al.*, 1969). Thus, although considerable evidence, including the present study, favors a conformational change upon aminoacylation of tRNA, the change must be a subtle one, detectable by some techniques, but not by others.

Conformational differences between aminoacylated and uncharged tRNA would be in accord with the biochemical functions of these molecules. A conformational change seems one likely means for the protein synthesizing machinery to discriminate between tRNA and aa-tRNA. It is known that both deacylated tRNA and N-acylated aa-tRNA are unable to form a complex with the protein synthesis elongation factor T_u and GTP (Ravel *et al.*, 1967; Gordon, 1968; Skoultchi *et al.*, 1968; Ertel *et al.*, 1968; Jerez *et al.*, 1969). Similarly in the case of the repression of the histidine operon of *Salmonella typhimurium* charged His-$tRNA^{His}$ acts as corepressor while stripped $tRNA^{His}$ has no regulatory effect

(Lewis and Ames, 1972). Binding to the repressor might depend upon a conformation change in $tRNA^{His}$ upon aminoacylation.

Effect of Magnesium Ion. Divalent cations have long been recognized as playing an essential role in the structure and function of tRNA (Penniston and Doty, 1963; Fresco *et al.*, 1966; Millar and Steiner, 1966; Reeves *et al.*, 1970; Robison and Zimmerman, 1971). Nevertheless, recent studies demonstrate that in some instances they have little effect (Yarus and Rashbaum, 1972; Eldred and Schimmel, 1972).

Our results show that with respect to ethidium binding the *average* structure of unfractionated yeast tRNA in 0.15 M KCl is not greatly affected by the addition of 10 mM Mg^{2+}. We cannot rule out the possibility that the low levels of divalent metal ions contained in our samples exerted an influence upon tRNA structure. Such contamination, however, amounted to less than 10^{-5} M. Addition of Mg^{2+} to levels one thousand times this only slightly altered the kinetics of ethidium binding (Table I and Figure 6). k_{-1} and k_{-2} are significantly *lower* in the presence of Mg^{2+} and this in turn is reflected by a larger value of K_{ap} which means that at the low degree of saturation which we have employed (r = 0.013) the dye is somewhat more tightly bound in the presence of Mg^{2+} than in its absence.

Dimerization of tRNA. The reactions which we have observed by stopped-flow spectrometry occur only when the tRNA concentration changes and added salt is present. These facts plus the concentration dependence of $1/\tau_{S_1}$ (Figure 8) support the notion that under these conditions the tRNA can dimerize with the bound ethidium functioning as an indicator. The concentration-independent process characterized by τ_{S_2} must be some sort of further isomerization coupled to the formation of dimers. Whether the first-order process follows dimerization as we have indicated in eq 18 or precedes it cannot be determined from these data since the bimolecular process is the slower step.

Only a small fraction of uncomplexed tRNA can exist as dimeric species since molecular weight determinations yield values close to that for the monomer (Lindahl *et al.*, 1965). Likewise, the fact that the A_{260} of our tRNA samples obeyed Beer's law over a wide concentration range indicates either that very little aggregation occurred or that aggregation does not produce an absorbance change. Bound ethidium could, of course, promote formation of dimers and higher aggregates. The dimerization process which we have observed may involve only some of the 50-odd species present.

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Synthesis of Ribonucleic Acid Containing 6-Thioguanylic Acid Residues†

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ABSTRACT: 6-Thioguanosine was labeled with ^{35}S and then phosphorylated under conditions which avoid oxidation and release of the labile thioketone sulfur to give 6-[^{35}S]thioguanosine triphosphate. s^6GTP substituted only for GTP during RNA synthesis by DNA-dependent RNA polymerase. With calf thymus DNA as template and with s^6GTP, RNA synthesis amounted to 25–30% of that with GTP. s^6GTP initiated only a limited number of RNA chains while it had no effect on RNA chain initiation by ATP. The process of RNA elongation seemed unaffected by incorporation of s^6GMP residues. With poly(C) as template, RNA polymerase synthesized poly(s^6G), but polymerization ceased always after 30 min. Like s^6GMP, poly(s^6G) has two absorption maxima, one at 342 nm, specific of the thioketone, and one at 250 nm. However sulfur of the thioketone moiety became very labile once s^6GMP was polymerized. This fact prevented isolation of pure poly(s^6G). When polynucleotide phosphorylase activity was determined in the presence of s^6GDP an irreversible inhibition of the enzyme was observed.

The nucleoside 6-thioguanosine was first synthesized by Fox *et al.* (1958) and was found to be an inhibitor of tumor growth. As pointed out by Guschlbauer (1972), the behavior of guanosine is often qualitatively quite different from that of the other three normal nucleosides. Thus, studies of guanosine analogs are of particular interest (Darlix *et al.*, 1971). We have studied the utilization of phosphorylated derivatives of 6-thioguanosine by polynucleotide phosphorylase and by DNA-dependent RNA polymerase of *Escherichia coli*, since spectral and chemical properties of 6-thioguanosine are particularly favorable for a variety of physical studies and for possible formation of chemical derivatives of polynucleotides.

In this paper, we report the preparation of [^{35}S]s^6GTP[1] under conditions which avoid oxidation and release of the labile thioketone sulfur (Doerr *et al.*, 1961), in contrast with previously published methods (Roy *et al.*, 1961; Naruse and Fujimoto, 1966). It is shown that s^6GTP substitutes only for GTP during DNA-directed RNA synthesis and that s^6GTP is polymerized by RNA polymerase with poly(C) as template.

Material and Methods

RNA polymerase was prepared according to a previously published method (Darlix *et al.*, 1969). Polynucleotide phosphorylase was a gift of Dr. M. Grunberg-Manago (Paris).

Enzymatic Assay. The concentrations of DNA and enzyme in the reaction mixtures are given in the individual legends. The ionic conditions are: 5×10^{-2} M Tris-HCl (pH 8), 5×10^{-3} M $MgCl_2$, 1×10^{-3} M $MnCl_2$, and 8×10^{-3} β-mercaptoethanol.

The syntheses were followed by precipitation of the RNA with 5% trichloroacetic acid and filtration on HAWP 025 Millipore membranes; direct filtration technique was also used (Sentenac *et al.*, 1968). In both cases radioactivity was determined using a Nuclear-Chicago scintillation counter.

Analysis of RNA synthetized *in vitro* was performed by MAK column chromatography as already described (Mandell and Hershey, 1960; Darlix *et al.*, 1968), in the presence of total nucleic acids extracted from *Escherichia coli* B, as markers.

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[1] Abbreviations used are: s^6GMP, s^6GDP, and s^6GTP, the mono-, di-, and triphosphates of 6-thioguanosine; 8-azaGTP, the di- and triphosphates of 8-azaguanosine; formycin-P_2 and -P_3, the di- and triphosphates of formycin; poly(s^6G), the homopolymer of s^6GMP; MAK, column made of methylated serum albumin adsorbed on kieselghur.