Table II: Summary of Rate Constants for the Nucleation and Propagation Reactions.

Nucleation	
Formation[a]	
$\tau_{0,2}^{-1} \cong 10^3$ sec^{-1}	
$k_{0,2} \cong 6 \times 10^6$ l. mol^{-1} sec^{-1}	
Dissociation[b]	
$\tau_{2,0}^{-1} = k_{2,0} \cong 6 \times 10^2$ sec^{-1}	
Propagation	
Formation[b]	
$\tau_{2,4}^{-1} = k_{2,4} \gg 10^4$ sec^{-1}	
$\tau_{4,6}^{-1} = k_{4,6} \gg 10^4$ sec^{-1}	

[a] Bimolecular. [b] Unimolecular.

sociated with formation of the AT base pairs ($\gg 10^4$ sec^{-1}) are considerably higher than the dissociation rate of the $(GC)_{central}$ base pairs, $\tau_{2,0}^{-1}$ ($= 6 \times 10^2$ sec^{-1}), a condition required to form a stable nucleus.

The formation of the double helix from separate strands of d-ApTpGpCpApT in aqueous solution can be visualized as the slow formation of a stable nucleus comprising the $(GC)_{central}$ base pairs followed by rapid propagation involving the $(TA)_{internal}$ and $(AT)_{terminal}$ base pairs.

Acknowledgment

The expert technical assistance of Miss L. L. Canuel is acknowledged.

References

Applequist, J., and Damle, V. (1965), *J. Am. Chem. Soc. 87,* 1450.

Bloomfield, V. A., Crothers, D. M., and Tinoco, Jr., I. (1974), Physical Chemistry of Nucleic Acids, New York, N.Y., Harper and Row.

Craig, M. E., Crothers, D. M., and Doty, P. (1971), *J. Mol. Biol. 62,* 383.

Crothers, D. M., Cole, P. E., Hilbers, C. W., and Shulman, R. G., (1975), *J. Mol. Biol.* (in press).

Eigen, M. (1963), *Angew. Chem. 75,* 489.

Patel, D. J. (1974), *Biochemistry 13,* 2396.

Patel, D. J., and Hilbers, C. W. (1975), *Biochemistry,* preceding paper in this issue.

Pohl, F. M. (1974), *Eur. J. Biochem. 42,* 495.

Pörschke, D., and Eigen, M. (1971), *J. Mol. Biol. 62,* 361.

Pörschke, D., Uhlenbeck, O. C., and Martin, F. H. (1973), *Biopolymers 12,* 1313.

Riesner, D., and Römer, R. (1973), in Physico-Chemical Properties of Nucleic Acids, Duchense, J., Ed., New York, N.Y., Academic Press.

Identification of a Unique Ethidium Bromide Binding Site on Yeast $tRNA^{Phe}$ by High Resolution (300 MHz) Nuclear Magnetic Resonance†

Claude R. Jones and David R. Kearns*

ABSTRACT: The binding of ethidium bromide to yeast $tRNA^{Phe}$ has been studied by high resolution (300 MHz) proton nuclear magnetic resonance. Under appropriate experimental conditions one ethidium bromide is bound to each tRNA and two resonances from ring NH protons are shifted upfield. These observations taken in conjunction with the assignments of the low-field spectrum of yeast $tRNA^{Phe}$ show that the unique ethidium bromide binding site is located between base pairs AU_6 and AU_7 of the amino acid acceptor stem. This information should be of value in understanding the way in which ethidium bromide binding alters the biochemical properties of the tRNA molecules.

Ethidium bromide is one of a number of dyes and drugs known to strongly interact with polynucleotides (Waring, 1965; Bauer and Vinograd, 1968) and the physical (Hudson et al., 1969; Bittman, 1969), chemical (Harbers et al., 1972), and biological (Perlman and Mahler, 1971; Lurquin and Buchet-Mahieu, 1971) consequences of this interaction have been the subject of numerous investigations. Antiviral and antibacterial properties have been observed for ethidium bromide (Dickinson et al., 1953) and it is known that ethidium bromide inhibits nucleic acid synthesis (Tomchick and Mandel, 1964), as well as DNA and RNA polymerase activity (Elliott, 1963; Waring, 1964). The observations of in vivo biological activity have raised interesting questions about the way in which ethidium bromide affects the properties of DNA and RNA. It is already known that ethidium bromide binds strongly to double-stranded DNA and RNA (Le Pecq and Paoletti, 1967; Aktipis and Martz, 1974), by intercalating between adjacent base pairs in the double helix (Waring, 1964; Kreishman et al., 1971), but more information about the mechanism of binding and the local site requirements is needed to understand how ethidium

† From the Department of Chemistry, University of California, Riverside, California 92502. *Received September 20, 1974.* The support of the U.S. Public Health Service (Grant GM 21431) is most gratefully acknowledged. This work was also supported in part by a Biomedical Sciences Support Grant (RR 07010) from the National Institutes of Health and a National Science Foundation Grant (GB 41110).

* To whom correspondence should be addressed. Present address: Department of Chemistry, University of California—San Diego, La Jolla, California 92037.

bromide alters biological function. Several previous investigations have attempted to provide some of this information by studying changes in nuclear magnetic resonance (NMR) and optical properties of ethidium bromide when it binds. One result of particular interest here is that the binding of ethidium bromide to tRNA molecules is very dependent on the concentration of positive ions (Bittman, 1969). Under most salt conditions studied each tRNA binds several ethidium bromide molecules. At lower Na^+ concentrations and at room temperature, yeast tRNA^{Phe} binds up to six ethidium bromide per tRNA. However, in the presence of a small additional amount of Mg^{2+} and at low temperature, only one ethidium bromide binds per tRNA^{Phe} (Urbanke et al., 1973; Tao et al., 1970). The fact that it is possible to find conditions where only one ethidium bromide binds per tRNA should improve the usefulness of ethidium bromide as a biological and structural probe, even though there is no information about the local site requirements that gave rise to unique site binding.

One difficulty in obtaining information about site requirements for ethidium bromide binding is that in most (but not all) binding studies, changes in the spectral properties of ethidium bromide were monitored (Bittman, 1969; Aktipis and Martz, 1974; Kreishman et al., 1971). In contrast to most previous investigations, we have studied ethidium bromide binding via its effect on the low-field NMR spectrum of tRNA^{Phe}. The effect which ethidium bromide binding has on the spectral properties of tRNA, further supports the conclusion that ethidium bromide binds by intercalation. Furthermore, since the low-field spectrum of tRNA^{Phe} has been previously assigned (Lightfoot et al., 1973) we are able, for the first time, to identify the unique binding site on this tRNA.

Experimental Section

An ethanol precipitate of yeast tRNA^{Phe}, which was reported to be at least 70% chargeable, was a gift from Professor Simon Chang. It was dissolved initially in 85 m*M* Tris-HCl (pH 7.5), 35 m*M* Cl_2, and 1.6 *M* NaCl and an insoluble precipitate was discarded. This solution was then reassayed in our laboratory and found to be 85% chargeable. This material gave an NMR spectrum which was identical with material which was 100% chargeable and showed no detectable resonances from any other tRNA species. The fact that this solution was less than 100% chargeable may be due to the deacylation which was noted during the assay, or some of the tRNA^{Phe} may have a damaged or missing CCA end. Changes in, or loss of, these three bases would affect charging but not the NMR spectrum. Samples of tRNA^{Phe} (25 mg/ml) were prepared in three different buffers. Sample 1 contained 375 m*M* NaCl, $MgCl_2$ (by dialysis free Mg^{2+} = 8 m*M*), 400 m*M* $(NH_4)_2SO_4$, and 20 m*M* Tris-HCl at pH 7.5. Sample 2 contained 375 m*M* NaCl, 8 m*M* free Mg, and 20 m*M* Tris-HCl at pH 7.5 and sample 3 contained 100 m*M* NaCl, 10 m*M* free $MgCl_2$, 10 m*M* cacodylic acid at pH 6.0, and 18 m*M* EDTA to reduce the Mg^{2+} concentration. Ethidium bromide from Calbiochem was dissolved in water and added directly to specially constructed Wilmad NMR micro cells containing the samples of tRNA^{Phe}. NMR spectra were obtained with a Varian HR 300 NMR spectrometer and the temperature was controlled to ±1°. The spectra were signal averaged for up to 3 hr using a Nicolet 1020A computer. Resonance positions are in parts per million (ppm) downfield from the usual standard sodium 2,2-dimethylsilapentane-5-sulfonate.

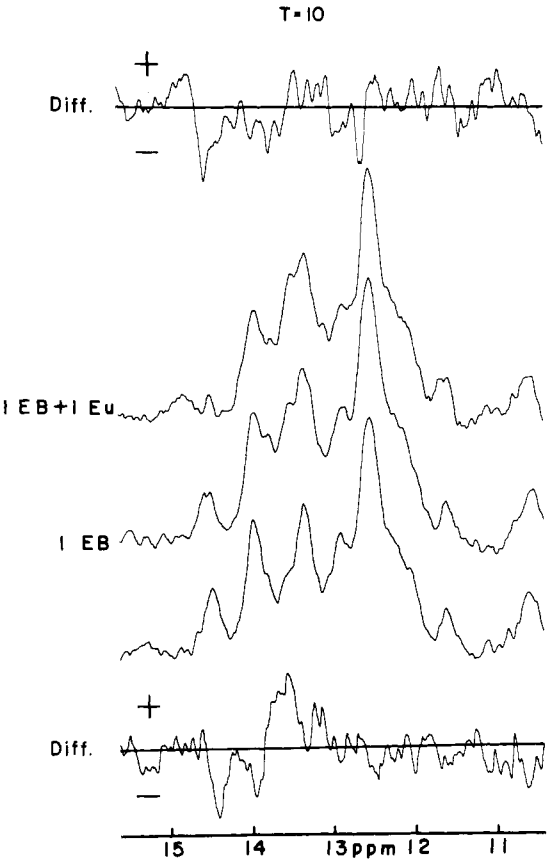

FIGURE 1: The 300-MHz proton NMR spectrum at 10° of tRNA^{Phe} sample 1 which contained 375 m*M* NaCl, 8 m*M* free Mg^{2+}, 400 m*M* $(NH_4)_2SO_4$, and 20 m*M* Tris-HCl at pH 7.5. Three NMR spectra are shown in the middle of the figure and two difference spectra are also shown, one at the top and one at the bottom of the figure. In the bottom NMR spectrum the sample contained no ethidium bromide and no Eu^{3+} ions. The middle NMR spectrum is a spectrum of sample 1 to which one ethidium bromide (EB) molecule per tRNA molecule had been added. The difference spectrum at the bottom of the figure was obtained by subtracting the bottom NMR spectrum from the middle NMR spectrum and shows the losses (−) and gains (+) in intensity produced by the addition of ethidium bromide. In order to emphasize these differences the difference spectrum was recorded at a sensitivity which was two times greater than that used for the NMR spectra. The top NMR spectrum is due to sample 1 to which one Eu^{3+} ion plus one ethidium bromide molecule per tRNA molecule had been added. The top difference spectrum was obtained by subtracting the middle NMR spectrum from the top one (at two times sensitivity).

Results

The binding of ethidium bromide to tRNA^{Phe} was studied under a variety of temperatures and salt conditions in order to determine which sites, if any, in the molecule were preferred binding sites for ethidium bromide. Since low temperatures favor binding at a reduced number of sites (Urbanke et al., 1973), ethidium bromide was added to sample 1 at 10° and the results of this experiment are shown in Figure 1. Comparing the spectrum before and after addition it can be seen that the effect on the two lowest field peaks (14.3 and 13.9 ppm) is quite pronounced (the small loss in resolution compared to spectra obtained at higher temperature is due to slower tumbling of the tRNA molecules). The difference spectrum at the bottom of Figure 1 shows that both of these peaks lose intensity and correspondingly intensity is gained in the region from 13.3 to

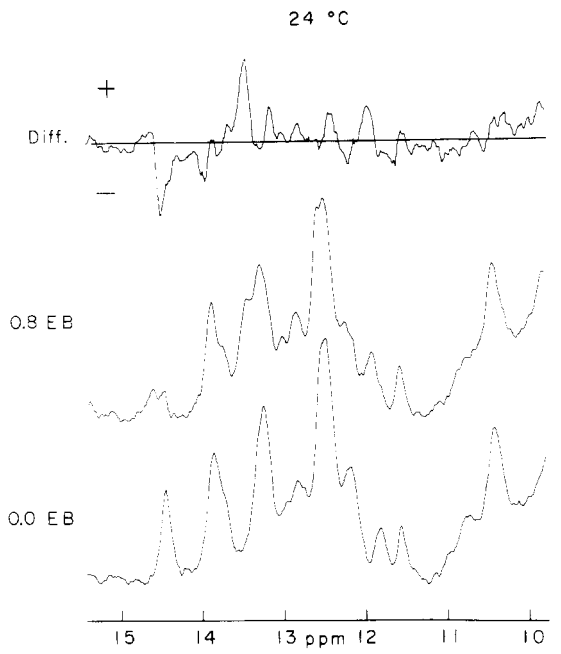

FIGURE 2: The 300-MHz proton spectrum at 24° of $tRNA^{Phe}$ sample 2 which contained 375 m*M* NaCl, 8 m*M* Mg^{2+}, and 20 m*M* Tris-HCl at pH 7.5. The bottom spectrum is sample 2 with no ethidium bromide and the next spectrum is sample 2 to which 0.8 ethidium bromide molecule had been added per tRNA molecule. The top spectrum is a difference spectrum obtained by subtracting the bottom spectrum from the one above it.

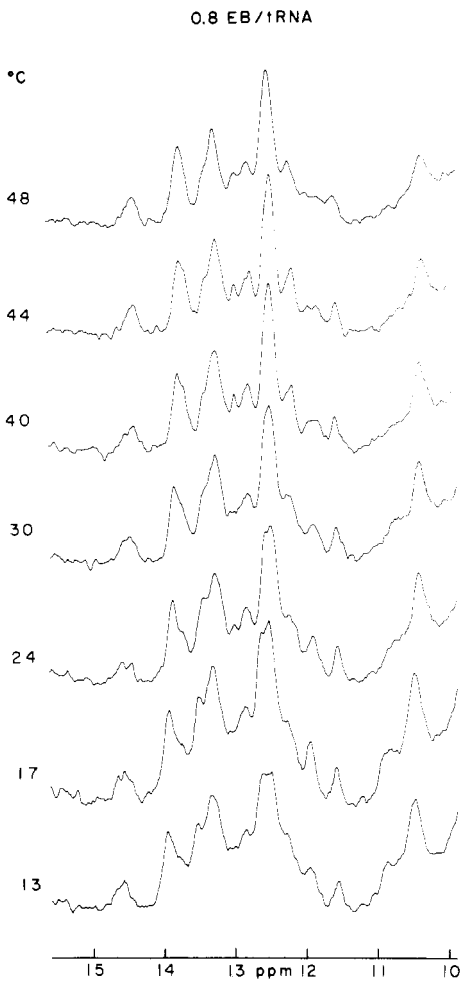

FIGURE 3: The 300-MHz proton spectra of $tRNA^{Phe}$ sample 2 to which 0.8 ethidium bromide molecules per tRNA had been added at various temperatures.

13.7 ppm. The top spectrum in Figure 1 shows the effect of adding 1 paramagnetic Eu^{3+} ion per ethidium bromide–tRNA complex. The peak at 14.4 ppm is most affected by Eu^{3+} ions. This same peak was strongly affected by the addition of one Eu^{3+} ion per $tRNA^{Phe}$ molecule in samples that did not contain ethidium bromide, but the observed shift was in the opposite direction (Jones and Kearns, 1974).

To check the possibility that these experiments might be influenced by the large amount of NH_4^+ ion and to investigate the temperature dependence of the binding, an additional set of experiments was carried out. Figure 2 shows the effect of adding 0.8 ethidium bromide per $tRNA^{Phe}$ on the low-field NMR spectrum of sample 2 at 24°. The temperature dependence of the spectrum of the ethidium bromide–tRNA complex is shown in Figure 3. By comparing the spectra with and without ethidium bromide in Figure 2 it can be seen that once again the two lowest field resonances lose intensity and new intensity appears in the region from 13.7 to 13.3 ppm. This is especially clear in the difference spectrum (top of Figure 2) which shows that the greatest changes in the spectrum are the losses at 13.8- and 14.4-ppm region and a corresponding increase around 13.4 ppm. Some small changes at 12.4 and 12.1 are also noted. The temperature dependence of ethidium bromide–tRNA spectrum in Figure 3 shows that the effect on the strong peak at 12.4 is more pronounced at 13°, so clearly the high concentration of NH_4^+ ions does influence the way that ethidium bromide binding affects this peak. Figure 3 also shows that the ethidium bromide induced shifts persist up to 24°, but above this temperature the effects begin to diminish. Sample 3 was used for a preliminary experiment (Figure 4) to study the effects of higher amounts of ethidium bromide on the $tRNA^{Phe}$ NMR spectrum. The spectrum without ethidium bromide shown at the bottom of Figure 3 was actually taken with a different sample, but at these salt conditions different $tRNA^{Phe}$ samples that we have examined show only slight variability in their NMR spectrum. Two obvious differences between this spectrum and those obtained with higher Mg^{2+} concentrations are in the peaks around 12.2 and 12.8 ppm. Each of these peaks has an intensity corresponding to two resonances per tRNA (see Table I) and in the low Mg^{2+} spectra these two resonances are at the same spectral position which gives rise to a single sharp, intense peak. In the high Mg^{2+} spectra the two resonances in each peak are at slightly different spectral positions and this gives a noticeably broader peak at both 12.2 and 12.8 ppm. A separate study has shown that these differences are due solely to the difference in Mg^{2+} concentration and that a single intense peak in each of these regions is characteristic of Mg^{2+} deficient $tRNA^{Phe}$ (C. R. Jones and D. R. Kearns, unpublished results). In favorable cases it has been possible to clearly resolve the splitting at both 12.2 and 12.8 ppm. An examination of the assignments in Table I shows that both of these changes could be due to a change in the orientation of the dimethyl G at position 26 in the sequence.

Apart from the Mg^{2+} dependent changes at 12.2 and 12.8 ppm, the other changes in the spectrum at 0.8 ethidium bromide/tRNA are that the three features below 13.0 ppm are broader than is usually the case in Mg^{2+} deficient spectra in the absence of ethidium bromide. When the ethi-

Table I: The Assignments of the Low-Field Resonances in Yeast tRNA^{Phe}.

Intensity	Position (ppm)	Assignment
1.7	14.4	AU 5, 6
3	13.8 13.7	AU 7, 12, 52
3	13.3	AU 29, 50 Aψ 31
1	13.0	GC 11
2	12.8	GC 2, 10
6	12.5 ± 2	GC 1, 3, 30 49, 51, 53
2	12.2	GC 27, 28
1	11.6	GC 13

dium bromide/tRNA ratio is increased to 2:1 and 3:1 the lowest field feature at 14.4 ppm and possibly the feature at 13.8 ppm lose intensity relative to the rest of the spectrum. Simultaneously, the region from 13.7 to 13.3 ppm gains intensity relative to the rest of the spectrum. The features between 12.0 and 13.0 ppm, however, seem unchanged in appearance. There is a gradual loss of overall intensity in the spectrum up to 3:1, by which time the total intensity has dropped by 20%. Upon increasing the ethidium bromide/tRNA ratio from 3:1 to 4:1 the appearance of the spectrum changes abruptly and there is an overall loss of 30% of the intensity. While no precipitate was noticed in the sample, any material which formed large suspended aggregates probably would not be observable in the NMR experiment because of dipolar broadening. Clearly, not all of the tRNA could be aggregated since some reasonably sharp features are still seen in the spectrum.

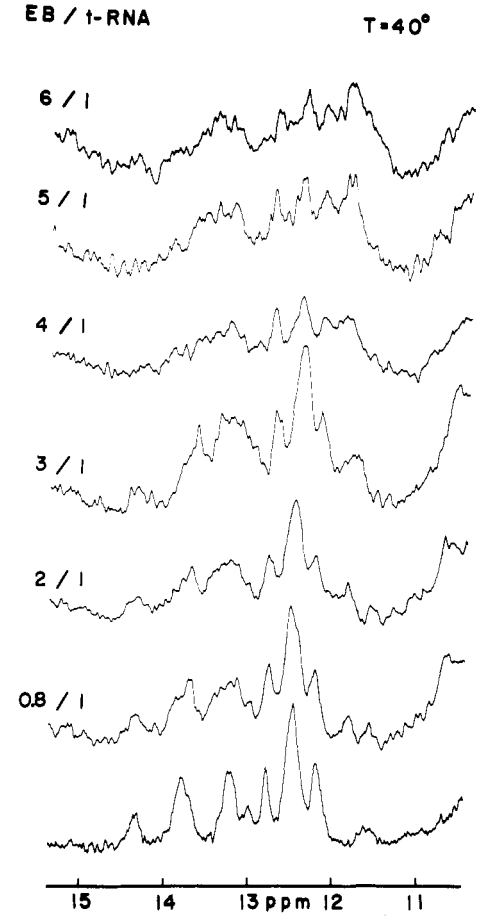

FIGURE 4: The 300-MHz proton spectra of tRNA^{Phe} sample 3 with increasing amounts of ethidium bromide. The solvent contained 100 m*M* NaCl, 10 m*M* free Mg^{2+}, 18 m*M* EDTA, and 10 m*M* cacodylic acid at pH 6.0. The top four spectra were measured with an instrument sensitivity that was two times more sensitive than was the case for the bottom three spectra.

Discussion

Evidence for Intercalation. The clearest examples of the effects of ethidium bromide binding on the low-field NMR spectrum of tRNA^{Phe} are provided by Figures 1 and 2. Both show that addition of ethidium bromide produced a loss of intensity in the two lowest field peaks, those at 14.4 and 13.8 ppm, and the appearance of new intensity in the region from 13.3 to 13.7 ppm. In the difference spectrum the loss at 14.4 appears greater than the loss at 13.8 ppm which may indicate that there is some overlap in the regions gaining and losing intensity. It is well known that aromatic molecules such as ethidium bromide give rise to large ring current shifts (Johnson and Bovey, 1958; Pople et al., 1959; Waugh and Fessenden, 1957) and therefore it was expected that the binding of these molecules to tRNA would give rise to large upfield shifts, providing the binding was by intercalation (Waring, 1965). The observation of these upfield shifts in the NMR spectrum is therefore consistent with and further supports the conclusion that the strong binding observed between ethidium bromide and tRNAs is due to intercalation.

Identification of the Unique Binding Site. The assignment of the features in the low field NMR spectrum to individual Watson-Crick base pairs given in Table I (Lightfoot et al., 1973) shows that all the resonances in the 14.5–13.3-ppm region are due to AU or Aψ base pairs. The most strongly affected peak at 14.4 ppm is assigned to AU 5 and 6 so the loss of intensity in this peak clearly shows that the site of intercalation is at the base of the amino acid acceptor stem. The peak at 13.8 ppm is also affected by the binding of ethidium bromide and this peak is assigned to resonances from AU_7, AU_{12}, and AU_{52}. Since the intercalation of ethidium bromide should give an upfield shift to resonances from base pairs above and below the ethidium bromide binding site, the changes in the peaks at 14.4 and 13.8 ppm indicate that the exact binding site is between AU_6 and AU_7.

The identification of the strongest site for ethidium bromide binding raises the question as to what aspect of the AU_6–AU_7 site is special. One possibility is that the ethidium bromide binding is sequence specific and the AU_6–AU_7 site in tRNA^{Phe} is unique because it is the only place in the secondary structure of the molecule where there are two adjacent A's on the same side of the helix. In this regard, the ethidium bromide binding would be reminiscent of the actinomycin D binding to DNA where sequence determines the binding site. Alternatively, AU_6–AU_7 site may be unique because of special constraints imposed by the tertiary structure of the molecule. This could arise if the binding at other potential sites disrupted the tertiary structure, whereas binding at the 6–7 site does not disrupt tertiary structure, or else produces a slightly more stable structure. If this latter alternative turns out to be correct, then we might expect to find the binding between base pairs 6 and 7 unique in other tRNA molecules (e.g., class I tRNA) provided of course they have the same structure as tRNA^{Phe}.

Comparison of the low-temperature spectra with (Figure

1) and without (Figures 2 and 3) NH_4^+ ions shows that the same spectral changes are seen in the 14.4–13.0-ppm region. In the sample without NH_4^+ ions there is an additional change in the appearance in the 11.5–12.5-ppm region of the spectrum but the difference spectrum (Figure 2) shows that the changes in this region are less important than the changes in the 13.0–14.5-ppm region. The fact that the changes in the 11.5–12.5-ppm region seen in one sample but not in the other indicates that changes in these two regions arise in different ways. One way that the 11.5–12.5-ppm region of the spectrum could be affected is by a small amount of ethidium bromide binding at a second site. A quantitative measurement of the amount of intensity change in the peak at 14.4 ppm could not be made with sufficient accuracy to exclude the possibility that as much as 15% ethidium bromide was bound at another site. However, the fact that unique site binding was found in optical studies in the absence of high concentrations of NH_4^+ ions (Urbanke et al., 1973) argues against but does not exclude a small amount of binding at a second site in these experiments. An alternative explanation of the changes in the 11.5–12.5-ppm region is that they result from an indirect effect due to binding at the site between AU_6 and AU_7. The intercalation of ethidium bromide requires an extension and unwinding of the helix at the binding site and this certainly could change tertiary structural interactions between that portion of the amino acid acceptor stem which is above the ethidium bromide binding site and the rest of the tRNA molecule. The position in the spectrum of resonances from base pairs at the termini of helices (such as GC_{13} and GC_{27} in this case) are sensitive to tertiary structural interactions and could thus be affected by ethidium bromide binding to the strong binding site at AU_6–AU_7.

Effect of Temperature on Unique Site Binding. The temperature dependence of spectra of the ethidium bromide–tRNA complex (Figure 3) shows that the spectral changes induced by the ethidium bromide are not appreciably altered when the temperature is increased from 13 to 24°, but they diminish at higher temperatures. This indicates that the percentage of ethidium bromide bound decreases as the temperature is increased above 24° although there still appears to be some small effects of ethidium bromide binding even at 48°. This decrease in ethidium bromide binding beginning at 30° is consistent with a low enthalpy of binding (Urbanke et al., 1973). These conclusions are further supported by the results of the titration of $tRNA^{Phe}$ with ethidium bromide at 40° (Figure 4). In this set of experiments the region 13.0–14.5 ppm once again was most affected by the addition of ethidium bromide, but the effects were less pronounced than was the case in the low temperature experiments discussed above. Furthermore, as the ethidium bromide concentration is increased the effects in the 13.0–14.5-ppm region of the spectrum increase indicating that the same strong site continues to bind ethidium bromide until the amount of added ethidium bromide is three times the tRNA concentration. Consequently, we conclude that all the results except those at ethidium bromide/tRNA ratios above 4:1 in Figure 4 are consistent with ethidium bromide binding at the same strong site, although the percentage of the total amount of ethidium bromide which is actually intercalated at this site depends on the temperature. The changes observed above four ethidium bromide/tRNA indicate an abrupt transition from single site binding to multiple site binding, but because of the loss of intensity above three ethidium bromide/tRNA conclusions drawn from these results must be taken with caution.

Effect of Metal Ions on Unique Site Binding. Most previous studies of ethidium bromide binding to tRNA have been done at low concentrations of Na^+ (typically less than 30 m*M*) and with no Mg^{2+} (Bittman, 1969; Lurquin and Buchet-Mahieu, 1971; Urbanke et al., 1973; Tao et al., 1970). Under these conditions several ethidium bromide bind to tRNA and in the particular case of $tRNA^{Phe}$ six strong binding sites are observed (Urbanke et al., 1973). However, increasing the metal ion concentration decreases the number of binding sites (Urbanke et al., 1973) and the magnitude of the binding constants (Bittman, 1969). Consequently, it is not surprising that with the high concentration of metal ions used in our studies (particularly Mg^{2+}) only single site binding is observed (Urbanke et al., 1973; Tao et al., 1970). Our results also support the conclusion of Tao et al. (1970) that the unique binding site in the presence of magnesium is located far away from the anticodon loop.

The decrease in binding with increasing metal ion concentration indicates that ethidium bromide binding and stabilization of the native structure of tRNA, which is favored by increased metal ion concentrations, are in competition. The NMR study using Eu^{3+} ions as a probe of the structure (Figure 1) is an example of how ethidium bromide binding and the stabilization of structure by polyvalent metal ions could come into conflict. In previous studies where we used Eu^{3+} ions as probes of the structure of $tRNA^{Phe}$ shifts were seen in the same region of the NMR spectrum as are seen in Figure 1 and it was shown that the first Eu^{3+} binding site shifts resonances from base pairs AU_6 and GC_{11} (Jones and Kearns, 1974). Even though the same regions of the spectrum are affected by the binding of one Eu^{3+} ion per ethidium bromide–tRNA complex, the shifts are in the opposite direction to those observed in the absence of ethidium bromide. This indicates that the metal binding site is in the same general region of the molecule, but the way in which the metal binds is altered by the ethidium bromide.

The results which we have presented here should considerably enhance the use of ethidium bromide as a probe of tRNA structure (Tao et al., 1970; Urbanke et al., 1973). In future studies we hope to be able to correlate information about specific sites of ethidium bromide binding with changes in the biological activity of tRNA (Lurquin and Buchet-Mahieu, 1971). We also plan to study the binding of ethidium bromide to other tRNA molecules to determine whether it is the base sequence which gives rise to the strong binding site, or whether it is the special location in tRNA secondary structure which is important. Regardless of the outcome, studies of the binding of ethidium bromide and other intercalating dyes to tRNA should prove to be interesting.

Acknowledgment

Some preliminary ethidium bromide binding experiments were initiated by Dr. Yeng P. Wong and we thank him for helpful discussions.

References

Aktipis, S., and Martz, W. W. (1974), *Biochemistry 13,* 112–118.

Bauer, W., and Vinograd, J. (1968), *J. Mol. Biol. 33,* 141–171.

Bittman, R. (1969), *J. Mol. Biol. 46,* 251–265.

Dickinson, L., Chantril, D. H., Inkley, G. W., and Thomp-

son, M. J. (1953), *Br. J. Pharmacol, 8,* 562.
Elliott, W. H. (1963), *Biochem. J. 86,* 562–567.
Harbers, K., Thiebe, R., and Zachau, H. G. (1972), *Eur. J. Biochem. 26,* 132–143.
Hudson, B., Upholt, W. B., Devinny, J., and Vinograd, J. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 813–820.
Johnson, C. E., and Bovey, F. A (1958), *J. Chem. Phys. 29,* 1012–1014.
Jones, C. R., and Kearns, D. R. (1974), *J. Am. Chem. Soc. 96,* 3651–3653.
Jones, C. R., and Kearns, D. R. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4237–4240.
Kreishman, G. P., Chan, S. I., and Bauer, W. (1971), *J. Mol. Biol. 61,* 45–58.
Le Pecq, J. B., and Paoletti, C. (1967), *J. Mol. Biol. 27,* 87–106.
Lightfoot, D. R., Wong, K. L., Kearns, D. R., Reid, B. R., and Shulman, R. G. (1973), *J. Mol. Biol. 78,* 71–89.
Lurquin, P., and Buchet-Mahieu, J. (1971), *FEBS Lett. 12,* 244–248.
Perlman, P. S., and Mahler, H. R. (1971), *Nature (London), New Biol. 231,* 12–16.
Pople, J., Schneider, W. G., and Bernstein, H. J. (1959), in High Resolution Nuclear Magnetic Resonance, New York, N.Y., McGraw-Hill, p 403.
Tao, T., Nelson, J. H., and Cantor, C. R. (1970), *Biochemistry 9,* 3514–3524.
Tomchick, R., and Mandel, H. G. (1964), *J. Gen. Microbiol. 36,* 225–235.
Urbanke, C., Romer, R., and Maass, G. (1973), *Eur. J. Biochem. 33,* 511–516.
Waring, M. J. (1964), *Biochim. Biophys. Acta 87,* 358–361.
Waring, M. J. (1965), *J. Mol. Biol. 13,* 269–282.
Waugh, J. S., and Fessenden, R. W. (1957), *J. Am. Chem. Soc. 79,* 846–849.
Wells, R. D., and Larson, J. E. (1970), *J. Mol. Biol. 49,* 319–342.

Preparation of Membrane-Free Chromatin Bodies from Avian Erythroid Cells and Analysis of Chromatin Acidic Proteins†

Roger Harlow and Julian R. E. Wells*

ABSTRACT: A primary objective, realized in this study, was the preparation from avian erythroid cells of chromatin free of contaminating membrane, as a prerequisiste to the study of chromatin acidic proteins from cells throughout the maturation pathway. Cells are lysed in saponin (S), washed in Nonidet-P40 (N), and plasma membrane removed by high-speed rotating knives (K). Purified SNK nuclear bodies are recovered free of membrane after centrifugation through 2.35 *M* sucrose. The chromatin acidic proteins from such preparations of the three major avian erythroid cell types were studied. Reticulocyte SNK chromatin was compared with reticulocyte chromatin derived from saponin lysis of cells and subsequent dispersion in EDTA solutions (Harlow et al. (1972), *Cell Differ. 2,* 341). The dispersed preparation has a lower acidic protein/DNA ratio, but the pattern of these proteins is more complex, presumably due to the contaminating membrane. In examining SNK acidic proteins throughout the maturation pathway it is clear that there are quantitative and qualitative differences. In the dividing erythroblast, the pattern of proteins is complex and the amount relative to DNA is 1.25:1.0. For nondividing, but transcriptionally active reticulocytes and also for transcriptionally inactive erythrocytes, the pattern is very much simpler, being dominated by three bands visible on sodium dodecyl sulfate polyacrylamide gels. The ratios of chromatin acidic proteins in these preparations relative to DNA are 0.69:1.0 and 0.36:1.0, respectively. These results, obtained with purified populations of cells from a single cell line, indicate a strong positive correlation between the transcriptional activity of the cell and both the amount and complexity of non-histone proteins associated with chromatin. The correlation does not indicate whether the proteins are the cause or result of increased transcription.

Current interest in the acidic proteins found in isolated eukaryote chromatin stems from the search for factors within the chromatin that may control gene-specific transcription. Histones are unlikely to possess enough specificity for this task, and chromosomal RNA as defined by published preparative procedures (Dahmus and McConnell, 1969; Mayfield and Bonner, 1971) is a product of RNA degradation (Artman and Roth, 1971; Tolstoshev and Wells, 1974).

Cytochemical staining of fixed preparations suggests that there are acidic proteins associated with the genetic material of eukaryotes in vivo (Swift, 1964; Zirkin, 1973), but it is by no means certain that these are adequately represented by the acidic protein populations found in isolated chromatin. The term chromatin is a very loose one, since isolation methods vary widely, and nonspecific loss or addition of components can occur. Some preparations consist essentially of whole nuclei, hypotonically disrupted (Paoletti and Huang, 1969; Sadgopal and Bonner, 1970), whereas others

† From the Department of Biochemisty, University of Adelaide, Adelaide, South Australia, 5001. *Received October 18, 1974.* This work was supported by the Australian Research Grants Commission D65/15758.