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The Assay and Isolation of DNA Rings Using an ATP-Dependent Endonuclease†

James R. Hutton and Charles A. Thomas, Jr.*

ABSTRACT: The ATP-dependent endonuclease from *Hemophilus influenzae* is relatively inactive on closed or open DNA rings, yet rapidly hydrolyzes single- or double-chained linear DNA. This enzyme in combination with an exonuclease (exo VII) has been shown to spare various circular DNA molecules including those having single-chain regions of significant length. However, rings containing single-chained regions are broken at a rate depending on the length of these regions. By admixing a linear DNA of alternate radiolabel, a simple assay for DNA rings has been developed. The application of this procedure to the assay of folded rings from *Drosophila* DNA is demonstrated.

While excellent methods are available for the isolation of covalently closed (superhelical) DNA rings (see Freifelder, 1971), the purification and assay of DNA rings that may contain interruptions (nicks) or single-chained regions (gaps) is more difficult especially if the rings are of variable molecular weight. The physical trapping of circular DNA molecules in agar gels is one possible method (Dean et al., 1973).

Here we present another method based on the observation that the ATP-dependent endonuclease from *Hemophilus influenzae* (Rd) does not attack single- or double-chained rings (Friedman and Smith, 1972a–c). Because this nuclease attacks linear single chains at one-tenth the rate observed with linear duplex DNA (Friedman and Smith, 1972c), we add another nuclease, exonuclease VII (Chase and Richardson, 1974), which removes nucleotides from both the 3′ and 5′ ends of single chains. The resulting mixture of nucleases spares duplex rings completely, yet rapidly reduces linear DNA to small oligomers. The spared material can be radioassayed by its Cl_3CCOOH insolubility or by its vastly different sedimentation rate. The fraction of DNA in rings as assayed by this nuclease method agrees with the same fraction determined by electron microscopy. While rings containing gaps are more resistant than linear DNA, they are broken at an observable rate which depends on the length of the single-chain regions. A similar approach has been developed by Mukai et al. (1973) for the isolation of plasmid DNAs employing an ATP-dependent nuclease from *Micrococcus luteus* which spares duplex rings but not single-chain DNA rings. To promote a more complete reaction, they also add an exonuclease, namely exo I.

Materials and Methods

DNAs. Labeled T_7 DNA was prepared by the method of Thomas and Abelson (1966); λ DNA by thermal induction of a stable lysogen (Malamy et al., 1972); SV40 DNA was a gift of George Fareed who prepared it by the method of Gelb et al. (1971). T_7 folded rings were prepared by first resecting the terminals with exonuclease III (Richardson et al., 1964) followed by annealing as described by Ritchie et al. (1967). *Drosophila* DNA was obtained from the stable *D. melanogaster* cell line K established by Echalier and Ohanessian (1970) grown in D22 medium supplemented with 10% heat inactivated fetal calf serum and 20 μCi/ml of [^{3}H]thymine or [^{32}P]phosphate. The confluent cell layers were washed twice with suspension buffer (0.03 *M* Tris–0.01 *M* EDTA (pH 8.3)). The washed cells were scraped from the falcon flask wall and suspended in a small volume of the suspension buffer. An equal volume of lysis buffer consisting of 0.03 *M* Tris, 0.01 *M* EDTA (pH 8.3), 2% sarcosyl, and 500 μg/ml of predigested Pronase was added to the cell suspension. The lysate was rocked gently and incubated at 36° for 12 hr, adjusted to $\rho = 1.45$ g/cm^3 with Cs_2SO_4 and banded by centrifugation at 40,000 rpm for 60 hr in a Beckman Type 65 rotor at 15°. The gradients were fractionated by dripping through a hole punched in the bottom of the tube. The fractions containing DNA were pooled and dialyzed against Tris-EDTA buffer (0.001 *M* EDTA–0.01 *M* Tris (pH 8.0)).

EM. Variations of the conventional Kleinschmidt method were employed (Lee et al., 1970).

Nucleases. Smith's ATP-dependent *Hemophilus* endonuclease (SAHN) was a gift from H. O. Smith and later prepared by the method described in Friedman and Smith

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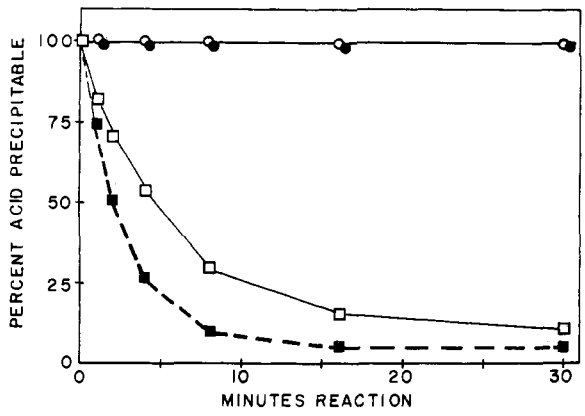

FIGURE 1: The resistance of SV40 DNA to SAHN + exo VII. Two experiments are shown. ^{32}P-labeled SV40 DNA, form I and form II (12,000 cpm, 0.02 μg), was mixed with intact linear ^{3}H-labeled T_7 DNA (75,000 cpm, 1.0 μg) with 8 units of SAHN and 4 units of exo VII in the reaction buffer (final volume 110 μl), incubated at 36° and 10-μl aliquots removed for the Cl_3CCOOH solubility assay. The fractions of the input labels found in the supernatant are plotted. (○) ^{32}P-labeled SV40; (□) ^{3}H-labeled T_7 DNA. The second experiment contained ^{3}H-labeled SV40 DNA (19,000 cpm, 0.4 μg) and ^{32}P-labeled λ DNA that was sheared to approximately 10,000 base pairs (8000 cpm, 0.2 μg). The acid-soluble fractions are shown for (●) ^{3}H-labeled SV40; (■) ^{32}P-labeled sheared λ DNA. Notice that the circular SV40 is totally preserved, while the linear molecules are rapidly solubilized.

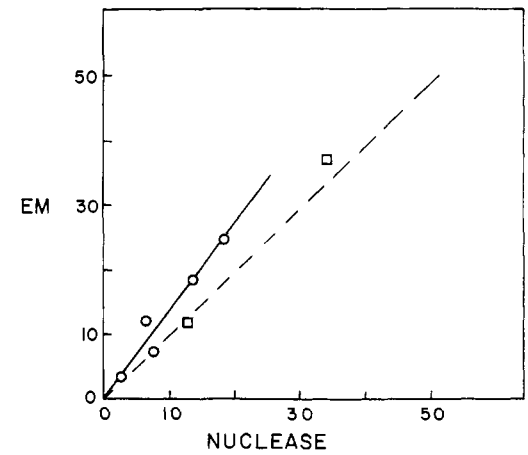

FIGURE 2: The percentage of T_7 DNA in folded rings as determined by EM and by SAHN-exo VII resistance. An artificial mixture of ^{3}H-labeled T_7 folded rings and linears was prepared in which the ring frequency was determined by electron microscopy (ordinate). This was mixed with an arbitrary amount of ^{32}P-labeled T_7 DNA. After 30 (□) and 45 (○) min of reaction with SAHN-exo VII, the Cl_3CCOOH supernatant was collected and the $^{3}H/^{32}P$ ratio measured. The fraction of resistant DNA, r (rings), was calculated from eq 1 and plotted on the abcissa. Dotted line is 45°.

(1972a). One unit of activity is defined as the amount of enzyme required to Cl_3CCOOH solubilize 1 nmol of DNA in 20 min at 37°. Exo VII was a gift of J. Chase (Chase and Richardson, 1974a,b). A unit is the activity required to solubilize 1 nmol of [^{3}H]thymidine in 30 min.

The Reaction Mixture. Linear and circular DNAs in alternate radiolabel were mixed and treated with a mixture of SAHN and exo VII. A typical reaction mixture of 0.225 ml contained <1.5 μg of DNA, 9 units of SAHN and 6 units of exo VII, 2.25 μmol of $MgCl_2$, 2.25 μmol of Tris-HCl (pH 8.0), 11.25 μmol of NaCl, and 0.675 μmol of ATP. Incubation proceeded at 36°. Aliquots were removed for assay.

Reaction Assays. Two assays were employed: Cl_3CCOOH solubility and sedimentation. For the Cl_3CCOOH assay, small aliquots (10 μl) were removed from the reaction mixture, mixed with 63 μg of carrier DNA and Cl_3CCOOH (5% final), chilled, and centrifuged and the supernatant was counted in a Triton-based fluor (15.0 g of 2,5-diphenyloxazole, 0.325 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 1.9 l. of toluene, 950 ml of Triton X-100, and 200 ml of H_2O).

The sedimentation assay consisted of removing an aliquot (60 μl) from the reaction mixture, adding it to 100 μl of carrier salmon sperm DNA (1.25 mg/ml) to stop the reaction, and layering 100 μl of this on a CsCl step gradient that was prepared by depositing 0.5-ml volumes of CsCl in Tris-EDTA buffer into a 5-ml polypropylene centrifuge tube in the following order: ρ = 1.90, 1.70, and 1.50 g/ml followed by 1.5 ml of ρ = 1.30 g/ml. After 3 hr of centrifugation in an SW50.1 rotor at 42 krpm at 18° fractions were collected from the bottom. Under these conditions DNA longer than approximately 1500 base pairs will appear in the zone containing undigested DNA.

Results

As can be seen in Figure 1, both superhelical and nicked forms of SV40 rings are not digested by a mixture of SAHN and exo VII, while linear DNA present in alternate radiolabel was rapidly solubilized.

These results suggested a simple assay for the amount of DNA in rings by simultaneously counting both labels in the Cl_3CCOOH supernatant. For example, suppose one has a mixture of ^{32}P-labeled DNA that is known to be linear, and ^{3}H-labeled DNA containing an unknown weight fraction r in rings and the remainder in linear pieces. We assume that the linear DNA of both radiolabels is solubilized at exactly the same rate described by a function of time, $f(t)$, and that the ring DNA is totally resistant. As the reaction proceeds the acid-soluble radioactivity of both types is given by

$$H_t = H_0(1 - r)f(t)$$
$$P_t = P_0 f(t)$$

where H_t and P_t are the total soluble radiolabels at time t, and H_0 and P_0 are the total input ^{3}H and ^{32}P. Taking the ratios, one has $(H/P)_t = (H/P)_0(1 - r)$, and rearranging

$$r = 1 - [(H/P)t/(H/P)_0] \quad (1)$$

We have tested the applicability of eq 1 in the following way. ^{3}H-labeled T_7 DNA was partly resected with exo III and annealed to form folded rings. The frequency of rings was determined by EM to be 34%. To this preparation, various amounts of linear ^{3}H-labeled T_7 DNA was added to give the fraction of rings shown on the ordinate of Figure 2. To this mixture, ^{32}P-labeled T_7 linear DNA was added and the mixture digested with SAHN-exo VII. At 30 and 45 min of digestion the Cl_3CCOOH soluble portion was counted and the $(H/P)_t$ calculated. The ratio $(H/P)_0$ was obtained by counting a corresponding aliquot that had been hydrolyzed in the same Cl_3CCOOH by heating at 95° for 10 min. From these two ratios, r was calculated from eq 1 and plotted on the abscissa of Figure 2. If this assay were in perfect agreement with the ring frequency determined by EM, the points should fall along the 45° diagonal. Within the error of both methods, this appears to be the case for 30 min of digestion. However, at 45 min the points drift somewhat above the diagonal which means that r underestimates the fraction of ring DNA probably because some rings are eventually broken and destroyed by the nuclease mixture. Since the major difference between the T_7 rings and SV40

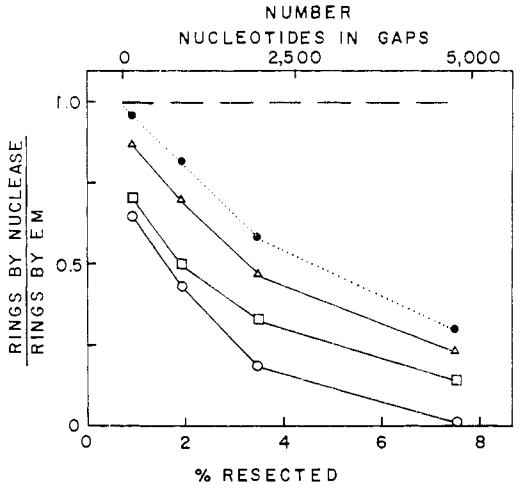

FIGURE 3: The nuclease resistance method increasingly underestimates ring frequency when rings contain very long single-chain regions. The nuclease resistant fraction, r, defined by eq 1 was determined on four different preparations of ^{3}H-labeled T_7 DNA folded rings that had been resected by exo III to various extents shown on the abcissa. After annealing, 55–60% (111/200, 121/200, 111/200, 113/200, respectively) of the molecules were rings. The total length of the single-chain regions in the rings was calculated as follows: 2 × (fraction Cl_3CCOOH solubilized × 37,500 − 260). Control experiments without resection gave nuclease resistant fractions, r, of 0 ± 2%. The measured values of r were divided by the percentage of rings determined by electron microscopy and plotted on the ordinate. The nuclease method underestimates the ring frequency by an amount depending on both time of digestion and the length of single-chain regions. (△) 15 min, (□) 30 min, (○) 60 min of digestion with SAHN-exo VII. (●···●) Values obtained by extrapolating the above values of r to zero time assuming a linear time dependence; (— —) the response expected if the single-chain gaps were totally resistant to the enzyme treatment.

is the presence of single-chain regions on either side of the double-helical portion corresponding to the terminal repetition, we hypothesized that these single-chain regions were susceptible to some contaminating activity in our preparations.

In order to test the hypothesis that single-chain regions were more sensitive to contaminating endonucleases, the experiment shown in Figure 2 was repeated with T_7 rings that were formed after various extents of resection. If this hypothesis were correct, rings with small gaps would be expected to be more resistant than rings with larger gaps. Since the terminal repetition in T_7 DNA is only 260 nucleotides (Ritchie et al., 1967) any resection in excess of this will leave single-chain gaps of increasing length. Figure 3 shows the ratio of the rings determined by nuclease resistance to that determined by electron microscopy for T_7 rings that had been overresected to various extents. Both the percentage of nucleotides released during resection and the corresponding lengths of single-chain regions exposed are shown on the abcissa. Since the results depend on the time of digestion with SAHN-exo VII, three different reaction times are shown. As expected, Figure 3 shows that the underestimation of ring frequency by nuclease resistance depends on the extent of overresection. When the single-chain regions are 2000 nucleotides long the resistant fraction is about 50% of that expected by EM. When the single-chain gaps approach 4000 nucleotides, the resistant fraction is less than 25% that expected from EM. It should be emphasized that these rings contain single-chained regions of considerable extent, and this residual activity does not invalidate the method. These experiments were devised to explore the limits of its useful application. Clearly, when rings contain substantial single-chained regions, shorter reaction times are desirable to avoid underestimating the fraction of ring DNA.

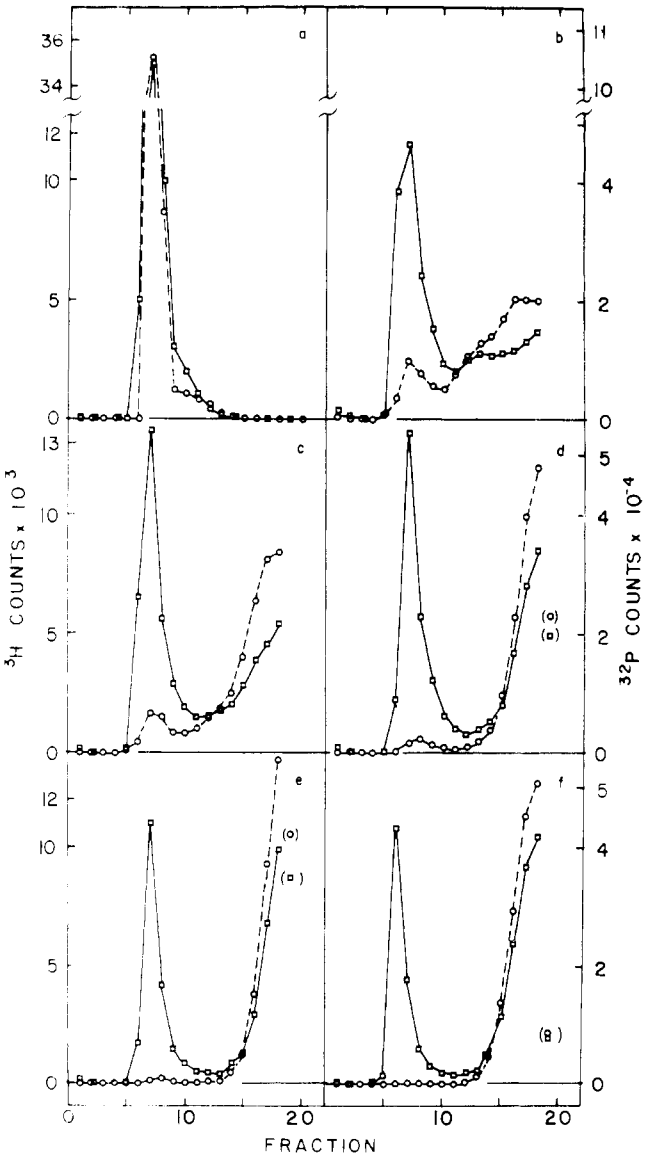

FIGURE 4: The isolation and separation of SAHN-exo VII resistant DNA (rings) from admixed linear DNA. ^{3}H-labeled T_7 DNA which had been resected 0.87% with exo III and annealed to yield 56% circular DNA (167/300) was mixed with linear ^{32}P-labeled T_7 DNA and digested by SAHN + exo VII followed by CsCl step gradient centrifugation as described under Materials and Methods. Figure 4a–f is for 0-, 1-, 2-, 4-, 6-, and 8-min reaction times, respectively. The size of the last fraction varies from one gradient to another and in the gradients where it is significantly less than the usual 10-drop fraction size, these data points are contained in parentheses. The ordinate shows measured counts per 10 min; the abcissa, fraction number. (○) ^{32}P; (□) ^{3}H.

From the foregoing it is clear that short reaction times are desired, yet the Cl_3CCOOH solubility assay requires substantial endonucleolytic degradation because oligomers must be reduced to smaller than about 18 in order to be completely solubilized (Cleaver and Boyer, 1972). Therefore, we turned to a sedimentation assay that did not require such extensive degradation to allow separation of the resistant DNA. The scheme, based on CsCl step gradients (see Materials and Methods), is shown in Figure 4. In these experiments an aliquot of the reaction mixture is placed on

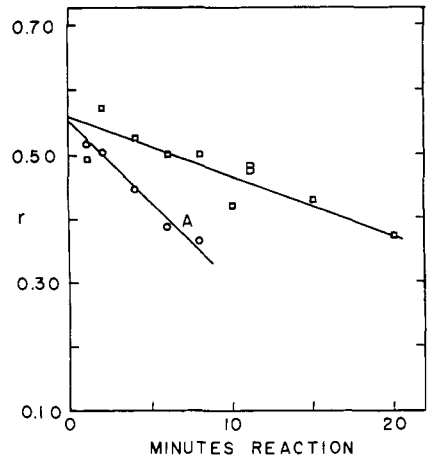

FIGURE 5: The fraction of nuclease-resistant DNA, *r*, as determined by sedimentation and Cl_3CCOOH assays. A mixture of T_7 rings and linears was digested by SAHN-exo VII and assayed in two ways. Curve A (○) shows *r* calculated from the step gradient assay (Figure 4). In this case the fraction of radiolabel sedimenting slower than fraction 12 was taken to be "soluble". Curve B (□) shows *r* calculated from the Cl_3CCOOH soluble assay.

the top of the preformed gradient and centrifuged for about 3 hr. Under these conditions DNA fragments (single or double chained) shorter than about 1500 base pairs remain in the top half of the gradient, while resistant material larger than 3000 base pairs is recovered in the zone shown in Figure 4. The analogous "soluble limit" is near 1500 rather than 18. Therefore, the extent of degradation required to produce the same "soluble fraction" is substantially less: in principle 1500/18 or 83-fold less (see eq A18; Thomas and Dancis, 1973). As can be seen in Figure 4d,e only 4–6 min of reaction are required to separate circular T_7 from admixed linear DNA. This assay has the added advantage that the DNA rings are available for further studies.

A comparison of the sedimentation and Cl_3CCOOH-soluble assays is shown in Figure 5. The experiment shown in Figures 4 and 5 was performed on a ^3H-labeled T_7 preparation of folded rings having 167/300 or 56% rings. The total activity in the gradients of both sorts was divided into two parts corresponding to the large and small molecules and the resistant fraction, *r*, calculated. Aliquots were also taken from the quenched reaction samples for Cl_3CCOOH assay and *r* again calculated. As shown in Figure 5, both values decrease with increasing reaction time but both extrapolate to 0.55–0.57, the fraction of rings observed in the EM. Theoretically, both Cl_3CCOOH and sedimentation assays should yield the same results: curves A and B should superimpose. However, this is not the case. It is likely that this difference is the result of the finite time required for a newly linearized molecule to be scored as "soluble" in the Cl_3CCOOH assay.

We now turn to the assay of *Drosophila* DNA folded rings by the nuclease resistance method. It is the characterization of these rings that was the motive for developing this assay. The results published separately (Hutton and Thomas, in preparation) show that the majority of the rings in this preparation that are recovered by this method are composed of satellite DNA that is recognizable in CsCl and actinomycin D–CsCl gradients. In the experiments presented here, DNA was extracted from the nuclei of a stable cell line K of *Drosophila melanogaster* (Echalier and Ohanessian, 1970) that was labeled with [^3H]thymidine or [^{32}P]orthophosphate. Both DNAs were sheared to produce fragments 3000–6000 base pairs long, a portion of the [^3H]DNA was resected and annealed, and the frequency of rings was scored in the EM. About 10% (102/1000) of all molecules seen were circular. This was added to ^{32}P-labeled linear DNA, the mixture was treated with SAHN-exo VII, and the resistant fraction *r* was determined by counting the Cl_3CCOOH supernatant. When the values of *r* obtained (0.09, 0.08, 0.07, and 0.01) were graphed as a function of the SAHN-exo VII reaction time (5, 10, 15, and 30 min, respectively) and extrapolated to zero time digestion, a value of 0.11 ± 0.02 was obtained for *r* in good agreement with EM value. When sheared (uncyclized ^3H- and uncyclized ^{32}P-labeled DNA) DNA was used in the above experiment as a control, values of *r* of 0.02, −0.02, 0.01, and 0.01 were obtained at times 5, 10, 15, and 30 min, respectively, to give a zero time intercept of approximately 0.01 ± 0.01 for *r*.

So it appears that the weight fraction of both T_7 and *Drosophila* satellite rings can be estimated by the nuclease resistance method. However, it is clear that the reactions must be assayed at several reaction times and a correction to zero digestion time made.

Discussion

The method developed here should be useful in purifying DNA rings. It is limited by the sensitivity of single-chained regions (gaps) within the ring, but this is not serious if the gaps are small, say a few hundred nucleotides. It is not known whether this sensitivity of single-chained regions is a consequence of some contaminating nuclease, or whether the single-chained regions are intrinsically susceptible to SAHN. Friedman and Smith (1972) found that under certain reaction conditions SAHN demonstrated an ATP-dependent endonucleolytic activity on single-strand phage fd circular DNA which could be effectively competed out by the presence of the preferred substrates single- or double-stranded linear DNA suggesting an intrinsic activity. We have made a similar observation in that circular T_7 DNA molecules with large gaps are opened much less rapidly when linear DNA is present than when it is not. We have made some efforts to test which of the components of our reaction mixtures are responsible for endonucleolytic cleavage of single chains. It appears that both SAHN and exo VII have some limited ability to break single-chained regions even in the absence of ATP. Therefore, we favor the view that further purification of the enzymes may lower this activity and thus extend the useful application of this method.

When one is dealing with an unfamiliar DNA, resistance to SAHN-exo VII does not necessarily mean circularity. It is quite possible that linear DNA having blocked ends would be resistant to these enzymes. Therefore evidence confirming circularity would be required.

Acknowledgments

We thank Dr. H. O. Smith for his gift of SAHN, Dr. Jack Chase for his gift of exo VII, and both for their helpful advice. We thank Dr. Maxime Brunfaut for generously providing the cultured *Drosophila* cells.

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Europium as a Fluorescent Probe of Transfer RNA Structure†

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ABSTRACT: The binding of europium(III) to *Escherichia coli* $tRNA^{fMet,Glu}$ and to unfractionated *E. coli* tRNA has been investigated by using the 4-thiouridine sensitization of europium $^5D_0 \rightarrow {}^7F_1$ emission and changes in the lifetime of the $^5D_0$ state of europium reported earlier (J. M. Wolfson and D. R. Kearns (1974), *J. Am. Chem. Soc. 96,* 3653). Binding of the first 3–4 europium ions is independent and sequential, approximately 600 times stronger than the magnesium binding, and the binding sites are located near the 4-thiouridine residue found at position 8 in a number of *E. coli* tRNA. Competition experiments suggest the strong binding sites are the same for magnesium and europium. The europium binding properties of both unfractionated *E. coli* tRNA and purified $tRNA^{fMet}$ are quite similar, indicating that the location of the strong binding sites and their binding constants are nearly the same for a large group of tRNA. The europium binding properties of native and denatured tRNA are quite different, however.

There are a number of experiments which demonstrate that divalent metal ions stabilize tRNA in biologically active conformations (Fresco et al., 1966).

Magnesium fulfills the divalent cation requirement, but other divalent ions (Mn^{2+}, Zn^{2+}) and even trivalent rare earth ions can substitute for Mg^{2+} in the amino acylation of tRNA molecules (Igarashi et al., 1971; Kayne and Cohn, 1972). The rare earth ions are especially well suited for use in studying the metal binding properties of polynucleotides in solution since their optical properties (relative intensity of bands in the absorption and emission spectra, band positions, polarization, lifetime, radiative and nonradiative quantum yields) are sensitive to the nature of attached ligand groups (Gallagher, 1964, 1965). There is additional interest in the rare earth ions since they are currently being used in X-ray diffraction studies of tRNA crystals (Kim et al., 1974; Suddath et al., 1974; Ladner et al., 1972; Pasek et al., 1973; Robertus et al., 1974).

We have already published a preliminary account of optical and nuclear magnetic resonance (NMR) studies of the binding of Eu^{3+} to tRNA molecules in solution (Wolfson and Kearns, 1974; Jones and Kearns, 1974), and Formoso (1973) has reported preliminary studies of the binding of Tb^{3+} to RNA. We now present the results of a comprehensive study of the binding of Eu^{3+} to a number of different tRNA. As we shall show, the binding of Eu^{3+} occurs independently and sequentially under our conditions (0.1 *M* NaCl, pH 7, no Mg^{2+}) rather than cooperatively, as has been reported for Mn^{2+} ions under different experimental conditions (Danchin, 1972; Danchin and Gueron, 1970), and that most *E. coli* tRNA exhibit a similar pattern of metal binding.

The general picture which emerges from these studies is that most tRNA exhibit nearly identical metal binding properties insofar as the strong metal binding sites are concerned, and this result supports the notion that the majority of the tRNA have similar conformations.

Materials and Methods

Biochemicals. Three samples of yeast $tRNA^{Phe}$ (purified by Robert Wang, K. Lim Wong, Donald Lightfoot, and Simon Chang) were used, all having 95% amino acid accep-

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