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Terbium Binding to Ribosomes and Ribosomal RNA†

Thomas D. Barela,‡ Stuart Burchett,§ and Donald E. Kizer*

ABSTRACT: Terbium binding to rat liver ribosomes and ribosomal RNA (rRNA) was examined by equilibrium dialysis and fluorescence spectroscopy. Upon binding to ribosomes and rRNA, the enhancement of terbium fluorescence emission at both 488 and 541 nm was dependent only upon the amount of bound terbium and independent of ionic strength. Binding profiles for ribosomes and rRNA suggested that terbium was bound to ribosomes primarily through rRNA interactions. Data suggested that terbium mimicked characteristics previously described for interactions between ribosomes and magnesium. It is proposed, therefore, that fluorescence of terbium bound to ribosomes may prove useful in studies on the nature and extent of interactions between ribosomes and magnesium.

Eucaryotic ribosomes require magnesium to maintain both structure and function (Petermann, 1964; Spirin, 1969; Maden, 1971; Haselkorn and Rothman-Denes, 1973). Ultracentrifugation studies, coupled with bound magnesium quantitation, showed sedimentation properties and protein content of ribosomes to be markedly altered by changes in bound magnesium (Petermann, 1960, 1964). Most magnesium binding involved rRNA (Edelman et al., 1960; Petermann, 1960) probably through interaction with phosphate diester linkages between adjacent ribose moieties (Wiberg and Neuman, 1957; Felsenfeld and Huang, 1959; Edelman et al., 1960). Despite the numerous functions of magnesium in protein synthesis catalyzed by ribosomes, direct studies concerning the role of magnesium have been hampered by a lack of easily examined spectral properties for magnesium.

Lanthanide metal ions recently were used to probe alkaline earth metal binding sites in proteins and nucleic acids (Darnall and Birnbaum, 1970; Luk, 1971; Smolka et al., 1971; Sherry and Cottam, 1973; Starcher and Urry, 1974; Secemski and Lienhard, 1974). The rationale for this approach was based on the abundance of easily examined spectral properties for lanthanides and a paucity of similar properties for alkaline earth metals. Lanthanide–macromolecule complexes were examined by fluorescence (Luk, 1971; Sherry and Cottam, 1973; Formoso, 1973; Kayne and Cohn, 1974), magnetic resonance (Reuben, 1971a,b; Sherry and Cottam, 1973; Valentine and Cottam, 1973; Jones and Kearns, 1974), difference spectroscopy (Birnbaum et al., 1970; Secemski and Lienhard, 1974), and circular dichroism (Smolka et al., 1971; Starcher and Urry, 1974). In several instances, lanthanide ions replaced normal alkaline earth metal ions in macromolecules and the biological function of the macromolecules was preserved. For instance,

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‡ Present address: Medical School, University of New Mexico, Albuquerque, New Mexico 87131.
§ Permanent address: Department of Chemistry, Southwestern Oklahoma State University, Weatherford, Oklahoma 73096.

lanthanide ions substituted for calcium in the conversion of trypsinogen to trypsin, EC 3.4.21.4 (Darnall and Birnbaum, 1970; Gomez et al., 1974), and in the activation of α-amylase, EC 3.2.1.1, from *Bacillus subtilis* (Smolka et al., 1970; Darnall and Birnbaum, 1973) and microbial metalloenzyme, EC 3.4.24.4, from *Bacillus thermoproteolyticus* (Matthews and Weaver, 1974). Whether lanthanide ions successfully replace magnesium or manganese requirements of enzymes seems less certain. Although the magnesium requirement of isoleucyl-tRNA synthetase (EC 6.1.1.5) from *Escherichi coli* was satisfied with lanthanide metals (Kayne and Cohn, 1972), substituting lanthanide ions for manganese resulted in severe inhibition of pyruvate kinase (EC 2.7.1.40) activity (Valentine and Cottam, 1973).

Since calcium and magnesium bind to ribosomes with equal affinity (Wiberg and Neuman, 1957; Banerjee and Perkins, 1962), we examined the possibility that lanthanides could substitute effectively for magnesium in ribosomes from rat liver. The lanthanide, terbium, was chosen for these studies due to its low intrinsic fluorescence in buffer and large fluorescence enhancement upon binding to protein and RNA (Sherry and Cottam, 1973; Formoso, 1973; Luk, 1971; Kayne and Cohn, 1974).

Methods

Ribosome and rRNA Isolation. Female Holtzman rats, 8 to 12 weeks old, were housed in a room with a 7:00 a.m. to 7:00 p.m. light cycle and were fed (Teklad Mouse and Rat Diet, Mogul Corporation, Winfield, Iowa) ad libitum. They were fasted 24 hr before sacrifice by cervical fracture at 8:00 a.m. Hepatic membrane-associated ribosomes were isolated from post-mitochondrial supernatants essentially by the procedure of Hallinan and Munro (1965). The mixture of monosomes and polysomes was incubated in a medium for in vitro protein synthesis (Tsukada et al., 1968) which contained 1 m*M* puromycin (Williamson and Schweet, 1965). Ribosomes were recovered by centrifugation at $6.9 \times 10^7 g$ av-min through a discontinuous sucrose gradient (0.5 to over 1 *M*) containing 10 m*M* Tris (pH 7.6), 500 m*M* KCl, and 2 m*M* $MgCl_2$. They were stored at −80° C. Since terbium forms insoluble hydroxides, ribosomes were resolubilized in 20 m*M* cacodylate (pH 6.0), containing either 10 m*M* KCl or 750 m*M* KCl. Peak displacements during centrifugation in a Beckman L2-75B centrifuge equipped with a schlieren optic accessory permitted computation of sedimentation coefficients (Schachman, 1957); these were 41 and 56 S in low KCl buffer, and 48 and 69 S in high KCl buffer. Ultraviolet spectrophotometry revealed an A_{260}:A_{235} ratio of 1.75–1.90. Failure to observe absorbance at 320 nm suggested that the ribosomes were essentially free of ferritin contamination (Wilson and Hoagland, 1965). Washed ribosomes were active in in vitro protein synthesis using a poly(uracil) message.

For isolation of rRNA,[1] frozen ribosomes were suspended in Tris–sodium dodecyl sulfate buffer (Lanclos and Bresnick, 1972) and protein was extracted with a mixture of phenol, chloroform, and isoamyl alcohol; rRNA was precipitated in the cold after addition of ethanol (Kedes and Gross, 1969). Following electrophoresis (Loening, 1967) and staining (Stenram et al., 1969), rRNA preparations were free of detectable amounts of mRNA but trace amounts of tRNA were present.

The concentration of ribosomes was determined from absorbance at 260 nm using $E_{1\%}^{1cm} = 132$ (von der Decken et al., 1970) and a molecular weight of 4.7×10^6 (Spirin, 1969). RNA concentration was determined at 260 nm using $E_{1\%}^{1cm} = 200$ (Wolfe et al., 1968) and a molecular weight of 2.4×10^6 (Spirin, 1969).

Terbium. Terbium oxide of 99.9% purity was donated by the Molybdenum Corporation of America. Stock terbium solutions were prepared as described by Smolka et al. (1971). Terbium concentration was determined by titration with EDTA[1] (Lyle and Rahman, 1963). The EDTA had previously been titrated with $CaCO_3$.

Fluorescence. Fluorescence measurements were made with an Aminco-Bowman SPF recording spectrofluorometer equipped with a ratio attachment. Entrance slits were 2.0 nm and exit slits were 0.5 nm when recording emission spectra, and were reversed for excitation spectra. Relative fluorescence is used throughout this paper. A fluorescence unit was arbitrarily defined as the product of sensitivity setting and meter reading; other instrument parameters were unaltered throughout this study. Fluorescence was always relative to a blank; in studies involving equilibrium dialysis, the blank was the external solution.

Equilibrium Dialysis. Cellulose dialysis tubing was boiled 1 hr in 1% Na_2CO_3 containing 100 μM EDTA. Tubing was carefully rinsed and then was boiled in distilled water for 30 min. Dialysis bags were filled with 1.7–1.9 ml of ribosomes or rRNA and placed in Nalgene 25-ml graduated cylinders containing terbium in 20 m*M* cacodylate buffer (pH 6.0) and KCl as indicated in the Results section. After dialysis at room temperature for 5 hr, contents within the bags were monitored for fluorescence enhancement and total terbium concentration. Here, the terbium concentration was determined by chelation with EDHHA[1] (Taketatsu and Yoshida, 1972). Specifically, the method consisted of mixing 0.05–2.0 ml of ribosome or rRNA-containing solution, 1.0 ml of 2.5 m*M* EDHHA, 0.4 ml of 5% triethanolamine (v:v), and 0.02 ml of 5 *N* HCl. These solutions were then diluted to 5 ml with water or buffer. Fluorescence excitation was at 295 nm and emission was recorded at 541 nm. Reproducibility was critically dependent upon adjusting the final pH value to 7.7. EDHHA was purchased from K & K Laboratories, Plainview, N.Y.

Glassware and Chemicals. Glassware was cleaned by the procedure of Smolka et al. (1971). Nalgene plasticware was cleaned by a similar treatment except that the aqua regia step was omitted. All solutions were prepared with glass-distilled–deionized water. Magnesium content of this water was less than 10 n*M* as determined using a Perkin-Elmer 403 atomic absorption spectrophotometer equipped with an HGA graphite furnace. Unless otherwise specified, chemicals used were reagent grade.

Results

Fluorescence Studies. Fluorescence excitation and emission spectra for 122.3 m*M* Tb^{3+} in 20 m*M* cacodylate buffer (pH 6.0) are shown in Figure 1. Emission spectra were characterized by two maxima at 488 and 541 nm. The position and relative intensities of these peaks were consistent with prior studies (Sherry and Cottam, 1973; Kayne and Cohn, 1974). At 12.23 μM Tb^{3+} in cacodylate buffer no emission was apparent at the most sensitive instrument settings. Upon addition of approximately 80 n*M* ribosomes or

[1] Abbreviations used are: rRNA, ribosomal ribonucleic acid; FU, fluorescence unit (defined in Methods); EDTA, ethylenediaminetetraacetic acid; EDHHA, α,α'-ethylenediiminodi(*o*-hydroxyphenylacetic acid); mRNA, messenger RNA; tRNA, transfer RNA.

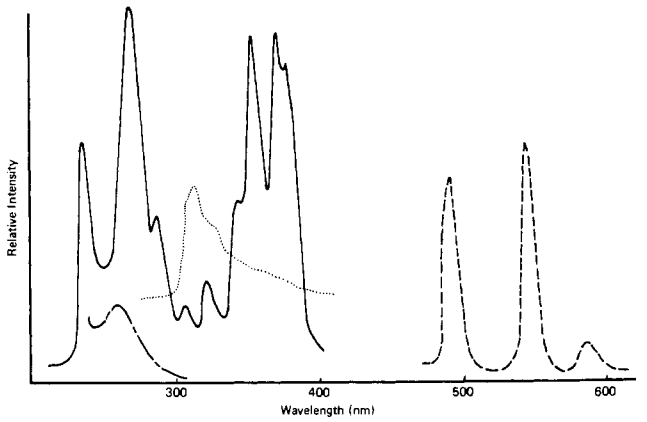

FIGURE 1: Terbium excitation and emission spectra in the presence and absence of ribosomes. Solutions were prepared in 20 mM cacodylate–10 mM KCl buffer (pH 6.0; 23°C): (—) excitation spectrum, 122.3 mM Tb^{3+}; (---) absorption spectrum, 0.09 μM ribosomes; (···) ultraviolet emission spectrum, 0.09 μM ribosome or rRNA alone or with 10 μM Tb^{3+}; (- - -) visible emission spectrum, 122.3 mM Tb^{3+} or 10 μM Tb^{3+} + 0.09 μM ribosomes. Relative intensities between spectra are not represented quantitatively.

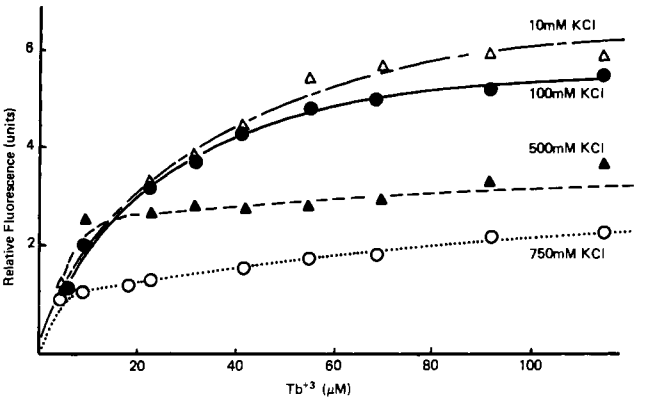

FIGURE 2: Influence of terbium concentration and ionic strength upon fluorescent enhancement of ribosomes. For each curve, ribosome pellets were dissolved in the appropriate ionic strength buffer, and 2.0-ml aliquots were adjusted with terbium and buffer to give the indicated concentration. The final concentration of ribosomes was 0.11 μM. Fluorescence enhancement was measured as the 488-nm peak height, 15–45 min after mixing; λ_{ex} = 290 nm.

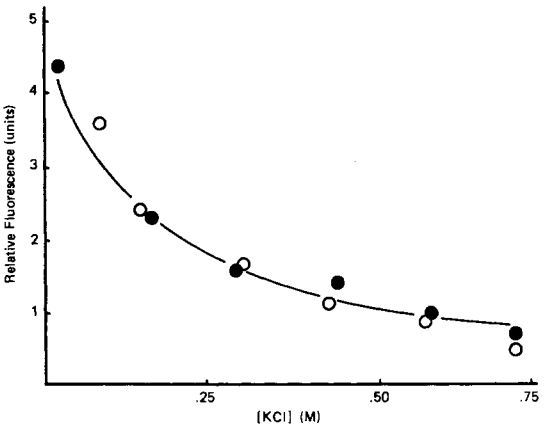

FIGURE 3: Comparison of the influence of ionic strength upon fluorescent enhancement of ribosomes or rRNA. Ribosomes and rRNA were dissolved in 20 mM cacodylate–10 mM KCl buffer (pH 6.0). Ionic strength was adjusted with 20 mM cacodylate–4.5 M KCl buffer (pH 6.0) and 10 mM KCl buffer such that the indicated values were obtained after terbium addition. Final concentrations were: (●) 0.0208 μM RNA, 85.1 μM Tb^{3+}; (○) 0.0184 μM ribosomes, 74.1 μM Tb^{3+}; λ_{ex} = 290 nm; λ_{em} = 488 nm.

rRNA to 12.23 μM Tb^{3+}, fluorescence emission peaks were apparent. Thus, the emission spectrum was "enhanced" several thousandfold by the addition of ribosomes or rRNA. Enhancement was very rapid; it reached maximum intensity within 5 min and remained constant for the ensuing 48 hr. Terbium emission spectra at either high terbium concentrations in buffer or low terbium concentrations in the presence of ribosomes or rRNA were identical with respect to emission maxima and relative peak intensities at 488 and 541 nm. In data that follow, relationships are derived for fluorescence emission at 488 nm; however, all figures have identical counterparts for emission at 541 nm.

Fluorescence enhancement of ribosome–terbium mixtures was dependent upon terbium concentration and ionic strength as illustrated in Figure 2. When ribosome concentration and ionic strength were held constant, addition of increasing amounts of terbium resulted in concomitant increases in fluorescence intensity at both 488 and 541 nm (only 488-nm emission is depicted). At higher concentrations of terbium, all curves leveled off in a manner suggesting saturation. Fluorescence intensity at "saturation" decreased with increasing concentrations of KCl. Similar curves were seen when ribosomes were replaced by rRNA. This latter observation, coupled with previous observations by others (e.g., Kayne and Cohn, 1974), led us to investigate whether terbium binding to ribosomes was mediated by rRNA interactions. As shown in Figure 3, ionic strength affected fluorescent enhancement of both ribosomes and rRNA. Since losses of fluorescence intensity were similar for both ribosomes and rRNA, it seemed likely that terbium interacted principally with rRNA.

Relationship between Bound Terbium and Fluorescence. Ionic strength could have affected the extent of terbium binding or the fluorescence intensity per bound terbium molecule. To distinguish between these possibilities, mixtures of terbium and ribosomes or rRNA were subjected to equilibrium dialysis. Ribosomes or rRNA were dissolved in cacodylate buffer containing KCl at the desired ionic strength. These solutions were dialyzed against buffer of the same ionic strength containing terbium. After dialysis, fluorescence enhancement was measured relative to the external solution. Internal and external terbium concentrations were determined to estimate bound terbium. As shown in Figure 4, both fluorescence intensity and the extent of terbium binding concomitantly decreased with increasing ionic strength. Furthermore, when figures were drawn which depicted the ratios μM Tb^{3+} bound/relative fluorescence units (FU) (on the ordinate) vs. increasing ionic strength (on the abscissa), all points fitted a horizontal line that intersected the ordinate at 4.55 ± 0.34 μM Tb^{3+} bound/FU for ribosomes, and 5.13 ± 0.60 μM Tb^{3+} bound/FU for rRNA. These plots reaffirmed a contention that fluorescence intensity per molecule of bound terbium was not appreciably altered by changes in ionic strength.

Since it also was possible that fluorescence intensity was a function of bound terbium, the binding of terbium and fluorescence intensity at constant ionic strength was determined. Concentrations of 10 and 750 mM KCl were chosen for these studies because they represented concentrations where intact monosomes and subunits, respectively, were commonly studied. The experimental design was analogous to that previously described. Figures 5A and 5B show the

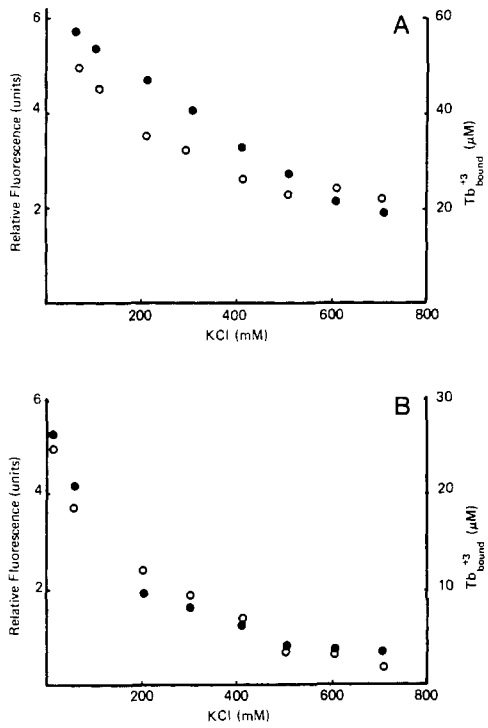

FIGURE 4: The influence of ionic strength upon terbium binding and fluorescent enhancement of ribosomes (A) and rRNA (B). In Figure 4A ribosomes, 0.056 μM in 20 mM cacodylate buffer (pH 6.0) containing 10 mM KCl, were adjusted to ionic strengths indicated with 20 mM cacodylate buffer (pH 6.0) containing 4.5 M KCl. Ribosomes (2.0 ml) were dialyzed against 25 ml of 81.5 μM Tb^{3+} in buffer of the same KCl concentration. Dialysis proceeded 5 hr at 23°C. Fluorescence enhancement and bound terbium were determined as described under Methods: (●) relative fluorescence; (○) Tb^{3+}_{bound}. (B) rRNA solutions were prepared in the manner described above; 0.136 μM rRNA was dialyzed against 34.8 μM Tb^{3+}: (●) relative fluorescence; (○) Tb^{3+}_{bound}; λ_{ex} = 290 nm; λ_{em} = 488 nm.

relationship between fluorescence intensity and terbium binding for ribosomes in 10 and 750 mM KCl, respectively, while Figures 5C and 5D show similar relationships for rRNA. In each instance, least-squares lines were drawn and each represented correlation coefficients greater than 0.99. Slopes of the lines represent the concentration of bound terbium per unit of fluorescence. Values at low and high ionic strengths were 4.88 ± 0.20 and 4.99 ± 0.70 μM Tb^{3+}/FU for ribosomes, and 3.91 ± 0.38 and 5.01 ± 0.85 μM Tb^{3+}/FU for rRNA, respectively. As shown in Figure 5B, at high ionic strength, about 9 μM terbium was bound to ribosomes before fluorescence was detectable. Experimental errors about binding determinations were larger for studies with high ionic strength buffers. With ribosomes, the range of terbium concentrations studied was narrower because they precipitated at higher terbium concentrations.

Discussion

Terbium binding to rat liver ribosomes was accompanied by terbium fluorescence enhancement similar to that observed for other proteins (Luk, 1971; Sherry and Cottam, 1973) and nucleic acids (Formoso, 1973; Kayne and Cohn, 1974). When terbium and ribosome or rRNA solutions were mixed fluorescence emission was fully manifested within 5 min and remained constant for the ensuing 48 hr. Increases in fluorescence intensity were observed for both 488 and 541-nm emission peaks. It appeared that molecular transitions involved in the two emission peaks were responding to experimental parameters in the same manner but with different intensities. Although it has been suggested that ionic characteristics of proposed binding sites affect both the probability of binding (Silber, 1974) and the shape of resulting fluorescence spectra (Luk, 1971), fluorescence excitation and emission spectra patterns of both ribosomes and rRNA were unaltered by the parameters of our studies.

Intact ribosomes and rRNA exhibited similar ionic strength dependent decreases in terbium binding, suggesting that rRNA may be the primary site of Tb^{3+} binding to ribosomes. Fluorescence intensity was dependent only upon the concentration of bound terbium, and additional studies at constant ionic strength (Figure 5) re-affirmed this conclusion. Although others (Formoso, 1973; Kayne and Cohn, 1974) by indirect or assumptive evidence assumed that fluorescence intensity was quantitatively related to the amount of bound terbium, the present study appears to be the first in which direct evidence is presented. Studies by others, however, suggest that this may not be a general occurrence (Luk, 1971; Teuwissen et al., 1972), but, rather, may depend upon the nature of the binding site. Although we observed similar characteristics for the binding of terbium to ribosomes or rRNA, it still is possible that some Tb^{3+} was bound to protein moieties with different fluorescence characteristics. If such interactions occurred, they may have been masked by the intense binding to rRNA.

An objective of this research was to study magnesium interactions through terbium probes. Characteristics described herein for terbium interactions with ribosomes are similar to those described earlier for magnesium binding to ribosomes (Wiberg and Neuman, 1957; Edelman et al., 1960; Petermann, 1960). Magnesium binding to ribosomes was similar to that observed for binding to rRNA except that removal of protein increased the number of available magnesium sites by 30% (Edelman et al., 1960). This is consistent with observations that, at moderate ionic strength, the conformation of free rRNA is identical with rRNA remaining in the protein complex (Cotter et al., 1967; Wolfe and Kay, 1969). Increases in ionic strength decreased the mole ratio of magnesium bound, ostensibly by competition of monovalent cations for the same sites (Goldberg, 1966; Choi and Carr, 1967). Terbium binding followed similar patterns except that terbium saturation was approached (Figure 2) at Tb^{3+} bound/ribosome (or rRNA) values near 0.1 saturation as computed from constants determined earlier, viz., 4.55 ± 0.34 μM Tb^{3+} bound/FU for ribosomes and 5.13 ± 0.60 μM Tb^{3+} bound/FU for rRNA, respectively, and assuming 6500 possible binding sites (Petermann, 1960). At higher saturation values, ribosome and rRNA precipitation occurred. In view of this, possibly the influence of bound terbium upon structure is additive to the influence exerted by the magnesium bound to ribosomes. Since ribosome structure is severely altered in the absence of magnesium (Petermann, 1960), magnesium-free ribosomes were not examined. Because of its greater positive charge, it is possible that terbium exerted a more pronounced influence upon structure. Data supporting this possibility were obtained from studies on the effects of lanthanides on the conversion of trypsin to trypsinogen (Darnall and Birnbaum, 1970; Gomez et al., 1974). Additional data will be required to distinguish between these possibilities.

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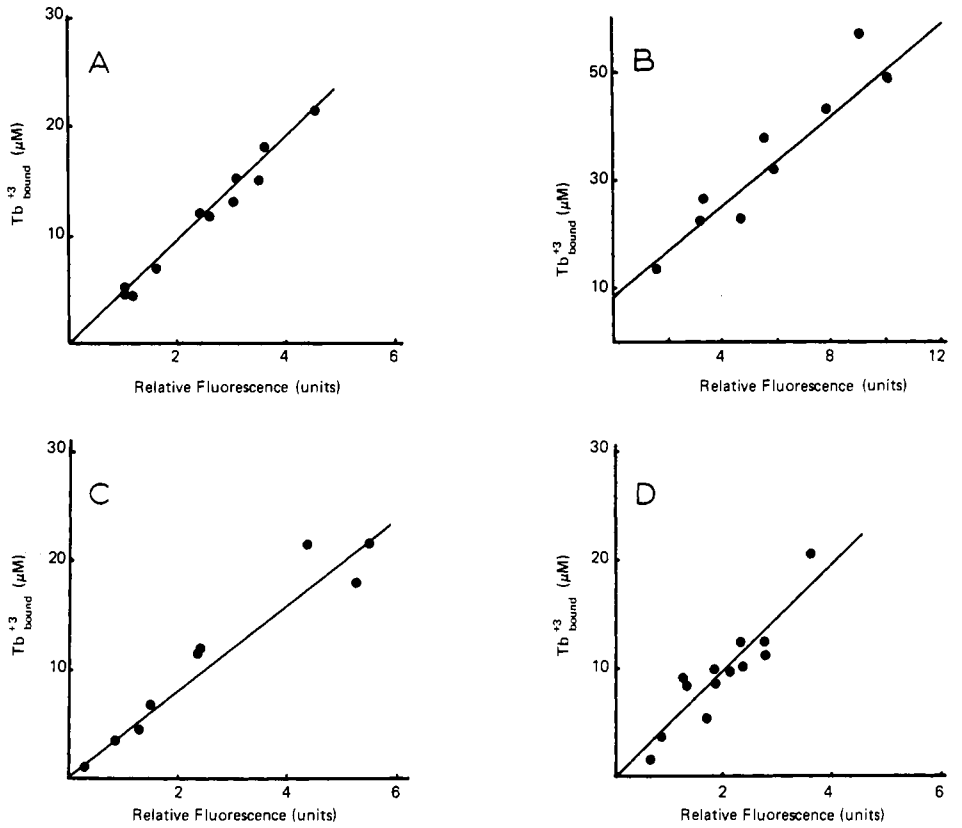

FIGURE 5: The relationship between extent of terbium binding and fluorescent enhancement for ribosomes (A,B) and rRNA (C,D) at low and high ionic strengths. For each graph, ribosomes or rRNA were dissolved in 20 m*M* cacodylate buffer containing the indicated concentrations of KCl and dialyzed against the same buffer containing variable concentrations of terbium. Dialysis proceeded 5-7 hr at 23°C, after which fluorescence enhancement and terbium were determined as described under Methods. Each graph depicts co-plotted data from at least two experiments. The concentrations of ribosomes and rRNA were: (A) 0.034 μ*M*, 0.042 μ*M* ribosomes; (B) 0.088 μ*M*, 0.078 μ*M* ribosomes; (C) 0.009 μ*M*, 0.052 μ*M*, 0.075 μ*M* rRNA; (D) 0.028 μ*M*, 0.040 μ*M* rRNA; λ_{ex} = 290 nm; λ_{em} = 488.

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Polynucleotide Analogs: Acrylic Acid and Maleic Acid Copolymers of 1-Vinyluracil and 9-Vinyladenine†

Paul E. Olson, Phillip Fraher, Sophia Boguslawski, and Mathias P. Mertes*

ABSTRACT: Radical-induced copolymerization of 1-vinyluracil and maleic anhydride gave, after hydrolysis, a polymer containing a 1:1 monomer ratio of 1-vinyluracil–maleic acid. γ-Ray-induced copolymerization of 1-vinyluracil with acrylic acid gave a polymer with a ratio of 1:1.7. Similar treatment of 9-vinyladenine and acrylic acid resulted in a polymer with a 1:3.2 ratio. These three compounds are potent stimulants of poly(uridylic acid) coded polyphenylalanine synthesis in an in vitro cell free system purified from *Escherichia coli* MRE 600. The double-stranded polymer, poly(inosinic acid)-poly(cytidylic acid), also stimulates polyphenylalanine synthesis in this assay.

A novel approach to the control of disease would be a class of agents that do not act by affecting the rate of catalysis of an enzyme but, rather, exert their action by controlling the level of enzymes. Such agents, to be effective, must act selectively to either decrease or increase the formation of the target enzyme. If the primary lesion in a particular disease is identified as an excess of an enzyme, the ideal agent would be one acting specifically to decrease the formation of that enzyme. To achieve this selectivity such an agent must alter genetic expression at the level of transcription or translation.

While this approach is premature with the current state of knowledge in molecular biology, the enormous effort directed to elucidating the control mechanisms of gene expression implies a great deal of confidence, among molecular biologists, that the goal is attainable. As one example, the sequence of bases in the *Escherichia coli lac* operator is known (Gilbert and Maxam, 1973; Maizels, 1973).

Assuming the primary information for the control of gene expression ultimately resides in unique sequences of bases in nucleic acids, therapeutic agents that compliment and have high affinity for a control sequence should inhibit the expression of that gene product—a particular enzyme. In contrast, a therapeutic agent that mimics the control sequence also should have affinity for the natural repressor. The result would be derepression of the gene and formation of that particular enzyme.

Preliminary studies leading to the distant goal of medicinal agents acting by the control of gene expression have been directed to identifying structural requirements in analogs of nucleic acids that have affinity for or mimic natural nucleic acids. To this end copolymers of 1-vinyluracil with maleic anhydride or acrylic acid and the copolymer of 9-vinyladenine with acrylic acid were prepared. These agents stimulate, in vitro, the poly(uridylic acid) coded synthesis of polyphenylalanine using *Escherichia coli* MRE 600. The double-stranded polynucleotide, poly(I)-poly(C)[1], a potent interferon inducer (Field et al., 1967), also has been found to stimulate protein synthesis in this system.

Experimental Section

Poly(U), poly(A), and poly(I)-poly(C) were purchased from Miles Laboratories. [^{14}C]- and [^{3}H]Phenylalanine were purchased from Schwarz/Mann. *Escherichia coli* MRE 600 cells were purchased as frozen packed 3/4 log cells from General Biochemicals; *Escherichia coli* tRNA was from Plenum Scientific. Poly(acrylic acid) was obtained from Aldrich Chemicals. ATP, GTP, DNase, and other common reagents were products of Sigma Chemicals.

Copolymerization of 1-Vinyluracil and Maleic Anhydride. A solution of 1-vinyluracil (Ueda et al., 1968) (250 mg, 1.8 mmol), maleic anhydride (355 mg, 3.6 mmol), and

† From the Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, Kansas 66045. *Received June 23, 1975.* Supported by Grants CA 16423 from the National Cancer Institute and GM 1341 from the Institute of General Medical Sciences, National Institutes of Health.

[1] Abbreviations used are: poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); poly(I)-poly(C), poly(inosinic acid)-poly(cytidylic acid); poly(vU-MA), poly(vinyluracil–maleic acid); poly(vU-AA), poly(vinyluracil–acrylic acid); poly(vA-AA), poly(vinyladenine–acrylic acid).