# Use of Terbium(III) Fluorescence Enhancement to Selectively Monitor DNA and RNA Guanine Residues and Their Alteration by Chemical Modification†

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ABSTRACT: Rat liver DNA and rRNA excited by 290-nm light enhanced terbium(III) ($Tb^{3+}$) fluorescence by 1800-fold and 13 000-fold, respectively. Studies involving the common nucleotide components of DNA and RNA revealed that only the guanine containing nucleotides (Guo-5′-P and dGuo-5′-P) enhanced $Tb^{3+}$ fluorescence significantly. Similarly, comparisons of the homopolymers of the four common ribonucleotides showed only the homopolymer containing guanine, poly(G), to be a strong enhancer of $Tb^{3+}$ fluorescence. In addition, the guanine moiety in the polymer state was found to be increased at least 5- to 6-fold in its ability to enhance $Tb^{3+}$ fluorescence relative to the guanine moiety existing as a free nucleotide. No enhancement was produced either with 7MeGuo-5′-P or Guo-5′-P modified at the 1 and $N^2$ positions by kethoxal and at the 8 position by *N*-acetoxy-2-acetylaminofluorene (NA-AAF). Chemical modification of rat liver DNA and rRNA by in vitro reaction with NA-AAF resulted in 70% and 30% losses, respectively, in their ability to enhance $Tb^{3+}$ fluorescence. It is concluded that $Tb^{3+}$ fluorescence enhancement by nucleic acids is predominantly a result of $Tb^{3+}$ interaction with guanine moieties, and could be useful as a guanine-specific fluorescence probe for studying nucleic acid structure. Furthermore, since chemical alteration of the guanine ring causes loss of its ability to enhance $Tb^{3+}$ fluorescence, this probe may also be useful for monitoring chemical modification of guanine residues in nucleic acids that occur during their biological function or that result from attack by carcinogens.

The direct use of the fluorescence emission properties of nucleic acid and the commonly occurring purines, pyrimidines, and nucleosides in the investigation of their biological properties has been quite limited. This has been due to the fact that, within the physiological pH range of 5–8 and at room temperature, excited electrons of nucleic acids in aqueous solution follow a path of rapid nonradiative decay to their respective ground states rather than emit significant levels of fluorescence (Udenfriend & Zaltzman, 1962; Walaas, 1963; Borresen, 1963). One possible approach to this dilemma utilizes substances which can physically interact with nucleic acids to quench their excited electron states by a mechanism more experimentally useful than the nonradiative decay to ground state. One such class of substances consists of certain cations of the lanthanum series of elements which recently have been shown to demonstrate a striking capacity to undergo enhancement of their own natural fluorescence when interacting with the appropriately irradiated aromatic ring systems of either proteins (Luk, 1971; Axelrod & Klein, 1974; Epstein et al., 1974) or nucleic acids (Lamola & Eisinger, 1971; Formoso, 1973; Kayne & Cohn, 1974). Of the fluorescent lanthanide cations, terbium(III) ($Tb^{3+}$) has received the greatest attention. The principal use of $Tb^{3+}$ fluorescence enhancement in the area of nucleic acid biochemistry has been in the study of tRNA structure (Formoso, 1973; Kayne & Cohn, 1974). In these studies, $Tb^{3+}$'s ability to substitute at $Mg^{2+}$ binding sites allowed it to accept energy from aromatic heterocycles of nucleic acid excited by ultraviolet light. This was useful in assessing the role of the metal cations and their immediate nucleic acid environment. Barela et al. (1975) have demonstrated a similar enhancement of $Tb^{3+}$ fluorescence by rRNA as well as intact ribosomes suggesting its usefulness in an examination of the nature and extent of interaction between rRNA and $Mg^{2+}$. Other studies involving the fluorescence enhancement of terbium salts of DNA and chromatin, examined in the solid state, have indicated a use of $Tb^{3+}$ fluorescence enhancement for the rapid quantitation of DNA and the measurement of DNA phosphodiester backbone "free" to interact with metal cations (Yonuschot & Mushrush, 1975).

In this investigation, DNA and rRNA from rat liver were successfully studied under aqueous conditions at pH 6 and at room temperature with respect to their abilities to enhance $Tb^{3+}$ fluorescence. The results showed that the only moieties of RNA and DNA which significantly enhanced $Tb^{3+}$ fluorescence were Guo-5′-P and dGuo-5′-P. Hence, $Tb^{3+}$ may be useful as a guanine-specific fluorescent probe for RNA and DNA. In addition, since modifications at several different positions on guanine caused loss of $Tb^{3+}$ fluorescence enhancement, the terbium probe may also be useful in detecting guanine modifications.

## Materials and Methods

*Materials.* Ribo- and deoxyribonucleoside mono-, di-, and triphosphates (potassium or sodium salt) were obtained from Sigma Chemical Co., St. Louis, Mo. The homopolymers poly(G), poly(U), poly(A), and poly(C) were from P-L Biochemicals, Inc., Milwaukee, Wis. Terbium oxide and kethoxal were from ICN Pharmaceuticals, Inc., Cleveland, Ohio. *N*-Acetoxy-2-acetylaminofluorene was generously provided by Lionel A. Poirier, Nutrition and Metabolism Section, National Cancer Institute, Bethesda, Md.

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[1] Nonstandard abbreviations used are: 7MeGuo-5′-P, 7-methylguanosine 5′-monophosphate; $N^2$, amino group at the C-2 position of the guanine ring; $\alpha$, $\beta$, and $\gamma$ phosphates, ultimate phosphate groups of nucleoside-5′-mono-, di-, and triphosphates, respectively; AF, 2-aminofluorene; AAF, 2-acetylaminofluorene; NA-AAF, *N*-acetoxy-2-acetylaminofluorene; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane. Cyd, cytidine; dThd.

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*DNA Preparation.* The livers from female Holtzman rats were removed, homogenized in 10 mL/g liver of 0.25 M sucrose, 0.1 M EDTA[1] (pH 8.0) by a motor-driven pestle and then the DNA was extracted according to the procedure of Marmur (1961). Following extraction, DNA was further purified by treatment with α-amylase (Sheehan & Olins, 1974), Pronase (Fujimura & Volkin, 1968) and a final Marmur-type reextraction of DNA. Measurement of DNA concentration was by absorption at 260 nm as described by Kaneko & LePage (1970).

*rRNA Preparation.* Total cytoplasmic ribosomes were prepared from female Holtzman rat livers (Venkatesan & Steele, 1972). Ribosomal RNA was then extracted from ribosomes as described by Lanclos and Bresnick (1972) with the exception that EDTA was omitted from the extraction buffer. Isolated rRNA was concentrated by precipitation with cold ethanol (Kedes & Gross, 1969) and resuspended in 20 mM cacodylic acid (pH 6.0) to give a final concentration of approximately 1.5 mg/mL as determined by orcinol reaction (Volkin & Cohn, 1954). Polyacrylamide electrophoretic analysis of rRNA (Loening, 1967) gave banding characteristics of 28S, 18S, and 5S rRNAs as well as trace levels of tRNA.

*Terbium(III) Stock Solution.* Terbium(III) stock solutions were prepared from terbium oxide (Smolka et al., 1971) and $Tb^{3+}$ concentration determined by Lyle & Rahman's (1963) method of titration with EDTA.

*Measurement of Terbium Fluorescence.* Terbium(III) displays an inherent fluorescence emission spectrum with characteristic maxima at 488 and 540 nm when excited at appropriate wavelengths and when present in sufficient concentration. Shown in Figure 1 is a typical excitation, Figure 1a, and emission spectrum, Figure 1b, of a 100 mM $Tb^{3+}$ solution. Also demonstrated is the drop in fluorescence intensity to a point just below the threshold of instrumental detectability that accompanies a lowering of $Tb^{3+}$ to a concentration of 100 μM. Enhancement of $Tb^{3+}$ fluorescence can thus be conveniently studied at or below 100 μM $Tb^{3+}$ without contributions of fluorescence from unbound $Tb^{3+}$ to the fluorescence intensity. Nucleic acid enhancement of $Tb^{3+}$ fluorescence was determined by excitation of nucleic acid·$Tb^{3+}$ complexes at 290 nm. Unless stated otherwise, all emission experiments were conducted at pH 6 in a 20 mM cacodylic acid buffer to avoid the formation of insoluble terbium hydroxides (Prados et al., 1974). An ionic strength of 50 mM was maintained in the samples with appropriate amounts of KCl. All samples were allowed 10 min to equilibrate at room temperature before making fluorescence measurements. Since polynucleotide chains have been reported to be cleaved in the presence of lanthanide ions (Eichhorn & Butzow, 1965; Rordorf & Kearns, 1976), prolonged exposure of $Tb^{3+}$ to DNA and rRNA was specifically avoided by preparing such samples at 4 °C then warming to room temperature just prior to measurements of fluorescence. Fluorescence measurements were made with an Aminco-Bowman recording spectrophotofluorometer equipped with an ellipsoidal condensing system, R106 photomultiplier tube, xenon lamp, and an Aminco 4-8912 ratio photometer. A 1-cm light path cuvette was used along with 2.0-nm entrance slits and 0.5-nm exit slits. All fluorescence enhancement readings are given relative to controls containing $Tb^{3+}$ but lacking nucleic acid. The fluorescence readings are reported from spectra uncorrected for instrument characteristics and are given in units of relative fluorescence, i.e., the product of the spectrophotometer sensitivity setting and fluorescence intensity meter reading.

*Kethoxal Reaction.* The conditions used for reaction of the guanine-specific reagent, kethoxal (β-ethoxy-α-ketobutyraldehyde), with Guo-5′-P and dGuo-5′-P were essentially as described by Noller & Chaires (1972). A typical reaction consisted of a 1.5 mL final volume containing 100 mM cacodylic acid (pH 6.0), 25 to 225 μg/mL kethoxal, and 0.25 mM Guo-5′-P or dGuo-5′-P. Reaction mixtures were incubated at 37 °C for 75 min. The extent of Guo-5′-P modification was followed spectrophotometrically (Staehelin, 1959; Shapiro & Hachmann, 1966). For this purpose, a 0.15-mL reaction mixture aliquot was diluted to 1.0 mL and scanned from 220 to 310 nm by a Gilford 250 spectrophotometer equipped with a 2530 wavelength scanner. To ensure that changes in Guo-5′-P's enhancement of $Tb^{3+}$ fluorescence was not influenced by hydrolytic loss of phosphate from Guo-5′-P, 0.025-mL aliquots of reaction mixtures were routinely analyzed for the presence of guanosine. This was accomplished by high-pressure liquid chromatography using a Waters Associates Model 440 liquid chromatograph equipped with a Waters Associates 4 mm i.d. × 30 cm μBondapak $NH_2$ weak anion exchange column. Resolution of guanylic acid, guanosine, and guanine was obtained by elution with 0.75 M ammonium phosphate (pH 4.2). In spectrophotofluorometric determinations, a 1.0-mL aliquot of incubated reaction mixture was made 100 μM $Tb^{3+}$, excited at 290 nm and relative fluorescence at 488 nm recorded. In these experiments, enhancement of $Tb^{3+}$ fluorescence intensity is regarded as the difference between experimental samples undergoing reaction as described above and control samples representing zero reaction time. The control samples contained no kethoxal but were incubated along with the experimental samples. Following incubation, appropriate amounts of kethoxal were then added to the control samples just prior to spectrophotofluorometric measurements. The controls allow monitoring of changes in fluorescence intensity resulting from such factors as interference by kethoxal and hydrolytic loss of phosphate from nucleotides.

*NA-AAF Reaction with Guo-5′-P, dGuo-5′-P, RNA, and DNA.* In a final volume of 2.0 mL, 0.25 mM Guo-5′-P or dGuo-5′-P and 0.5 mg/mL NA-AAF in 100 mM cacodylic acid (pH 6.0) were reacted during a 37 °C incubation for 3 h in the dark and under a nitrogen atmosphere. Following incubation, the reaction mixture was extracted six times with 1-volume portions of ethyl ether. Controls demonstrated that ethyl ether extraction of samples removes all detectable amounts of AAF and unreacted NA-AAF. The ultraviolet optical properties of a 0.15-mL aliquot of the aqueous phase diluted to 1.0 mL were then analyzed directly and after alkali treatment for spectral changes shown by Kapuler & Michelson (1971) to be characteristic of *N*-(5′-phosphoguanosin-8-yl)-AAF and *N*-(5′-phosphoguanosin-8-yl)AF formation. High-pressure liquid chromatographic monitoring of nucleotides and

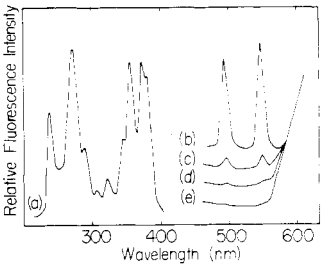


FIGURE 1: Excitation and emission spectra of $Tb^{3+}$ fluorescence. In the presence of 20 mM cacodylic acid (pH 6.0) and 10 mM KCl are shown the excitation spectrum ($\lambda_{em}$ 540 nm) of 100 mM $Tb^{3+}$ (a) and the emission spectra ($\lambda_{ex}$ 290 nm) of $Tb^{3+}$ at 100 mM (b); 10 mM (c); 1 mM (d); and 0.1 mM (e).

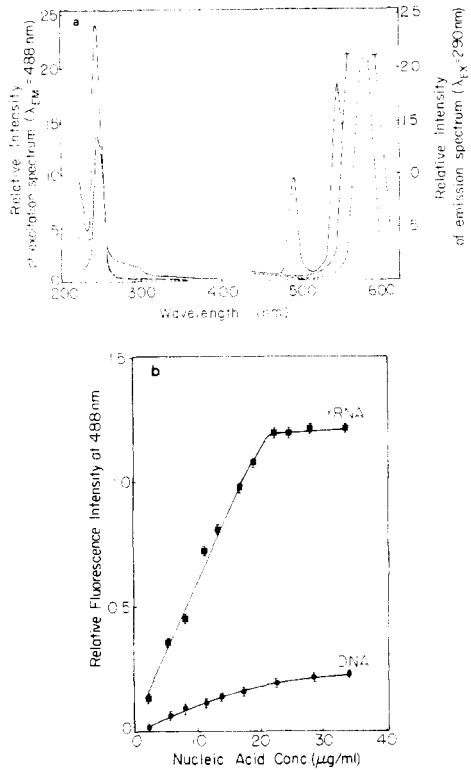


FIGURE 2: Enhancement of $Tb^{3+}$ fluorescence by rat liver rRNA and DNA. (a) A typical excitation ($\lambda_{em}$ 488 nm) and emission ($\lambda_{ex}$ 290 nm) spectrum of 16 μM $Tb^{3+}$ (• • •), 12 μg/mL rRNA or DNA (- - -), and 16 μM $Tb^{3+}$ plus 12 μg/mL rRNA or DNA (—). (b) Enhancement of $Tb^{3+}$ fluorescence at 488 nm ($\lambda_{ex}$ 290 nm) for 16 μM $Tb^{3+}$ by rRNA and DNA at concentrations given above.

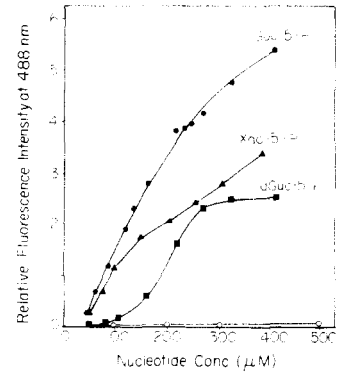


FIGURE 3: Enhancement of $Tb^{3+}$ fluorescence by nucleotides. Measurements were made over nucleotide concentrations from 50 μM to 500 μM Guo-5′-P (●—●), Xao-5′-P (▲—▲), dGuo-5′-P (■—■), and Cyd-5′-P, dCyd-5′-P, Ado-5′-P, dAdo-5′-P, Urd-5′-P, dThd-5′-P, and Ino-5′-P (○—○). Terbium(III) concentration was 100 μM and excitation at 290 nm.

the spectrophotofluorometric determinations were performed as described for the kethoxal reactions.

Reaction of rat liver rRNA and DNA with NA-AAF was performed as described for reaction with nucleotides except that 17 μg/mL of either DNA or rRNA replaced the nucleotide. Controls for these experiments consisted of rRNA and DNA samples incubated under the same conditions as reaction mixtures but without NA-AAF and then processed through ethyl ether extraction and fluorescence measurements in the same manner as reaction mixtures. Spectrophotofluorometric analysis of the nucleic acid adducts was done as described for the nucleotide adducts except that 10 μM $Tb^{3+}$ was used instead of 100 μM $Tb^{3+}$.

## Results

*Rat Liver rRNA and DNA Enhancement of $Tb^{3+}$ Fluorescence.* The ability of $Tb^{3+}$ to display enhanced fluorescence upon addition of rat liver rRNA and DNA is illustrated in Figure 2. Both rRNA and DNA were able to enhance $Tb^{3+}$ fluorescence from a level of no detectable fluorescence to a readily observable level. Figure 2a depicts a typical fluorescence excitation and emission spectrum of 16 μM $Tb^{3+}$ before and after the addition of 12 μg/mL rRNA or DNA. Also shown, under the same conditions, are spectra typical for rRNA and DNA in the absence of $Tb^{3+}$. Characteristic of nucleic acid enhancement of $Tb^{3+}$ fluorescence emission at 488 nm and 540 nm is a concomitant increase in the level of excitation in the 250-nm and 300-nm region of the excitation spectrum. This wavelength region is coincident with regions of absorption in the purine and pyrimidine ultraviolet absorption spectra. Also shown in these spectra are two peaks that are not a result of $Tb^{3+}$ fluorescence enhancement: (1) the peak in the 580-nm region of the emission spectrum, and (2) the peak in the 248-nm region of the excitation spectrum. These two peaks were present regardless of whether conditions necessary for $Tb^{3+}$ fluorescence enhancement had been achieved (see Figure 2a). It is likely that the 580- and 248-nm peaks are simply diffraction beam artifacts, arising from the spectrophotofluorometer's excitation grating monochromator and Rayleigh light scattering by sample molecules, that occur when emission spectra are recorded for 290-nm excitation light and when excitation spectra are observed at the emission wavelength of 488 nm. As indicated in Figure 2b, rRNA enhanced $Tb^{3+}$ fluorescence more than did a comparable amount of DNA. This difference in rRNA and DNA enhancements is portrayed as the increase in $Tb^{3+}$ fluorescence monitored at the 488-nm emission maximum over a nucleic acid concentration range of 1 to 34 μg/mL. It is clear that rRNA enhanced to a greater net extent than DNA and it did so by a means which produced greater fluorescence intensity per μg of nucleic acid, i.e., slopes of rRNA and DNA lines in Figure 2b between 1 and 17 μg/mL nucleic acid indicated that the rate of change in fluorescence intensity for rRNA was approximately five times greater than that of DNA. At concentrations of rRNA or DNA exceeding 20 μg/mL, colloid formation occurred in the samples and fluorescence enhancement leveled off despite increasing rRNA and DNA concentrations. The level of enhancement of $Tb^{3+}$ fluorescence by rRNA and DNA at 17 μg/mL is 13 000-fold and 1875-fold, respectively, when compared with the amount of fluorescence that would be generated by $Tb^{3+}$ alone at 10 μM.

*Examination of Nucleic Acid Enhancement of $Tb^{3+}$ Fluorescence at the Nucleotide Level.* To understand the basis of the enhancement of $Tb^{3+}$ fluorescence by RNA and DNA, various ribose and deoxyribose nucleotides were examined with respect to enhancement of $Tb^{3+}$ fluorescence (see Figure 3). Of these, the only nucleotides producing significant fluorescence enhancement were Guo-5′-P, Xao-5′-P, and dGuo-5′-P. Comparisons of their fluorescence intensities with the intensity of an identical amount of $Tb^{3+}$ alone gave relative enhancement factors of $4.85 \times 10^3$ for Guo-5′-P, $2.96 \times 10^3$ for Xao-5′-P and $2.62 \times 10^3$ for dGuo-5′-P. Under identical conditions, Cyd-5′-P, dCyd-5′-P, Ado-5′-P, dAdo-5′-P, Urd-5′-P, and Ino-5′-P failed to produce significant fluorescence. dThd-5′-P was observed to give a barely detectable fluorescence corresponding to a relative enhancement factor of 90. Comparison of enhancement abilities of a nucleotide basis reveals the strong enhancers, i.e., Guo-5′-P, Xao-5′-P, and dGuo-5′-P, are at least

TABLE I: Summary of the Effect of Phosphate Position upon Nucleoside Monophosphate Enhancement of Terbium(III) Fluorescence.[a]

| nucleotide (0.25 mM) | rel fluorescence intensity at 488 nm[b] 5′ | 2′ | 3′ |
|---|---|---|---|
| Guo-P | 3.95 | 0.15 | 0.65 |
| dGuo-P | 2.71 | | 1.30 |
| Cyd-P | 0.00 | 0.21 | 0.00 |
| dCyd-P | 0.00 | | 0.00 |
| Ado-P | 0.00 | 0.00 | 0.00 |
| dAdo-P | 0.00 | | 0.00 |
| Urd-P | 0.00 | 0.00 | 0.00 |
| dThd-P | 0.00 | | 0.00 |

[a] Terbium(III) concentration was 100 μM. [b] Position of nucleotide phosphate.

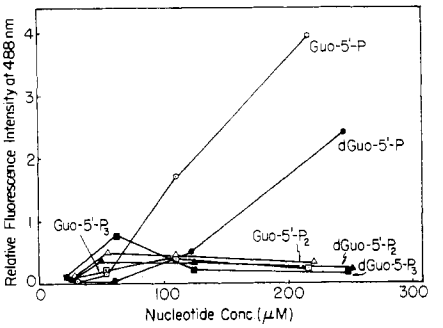


FIGURE 4: Enhancement of $Tb^{3+}$ fluorescence by ribose and deoxyribose guanosine mono-, di-, and triphosphates. Nucleotides were excited at 290 nm and examined over a concentration range of 50–250 μM in the presence of 100 μM $Tb^{3+}$. Guo-5′-P (○—○), Guo-5′-$P_2$ (□—□), Guo-5′-$P_3$ (△—△), dGuo-5′-P (●—●), dGuo-5′-$P_2$ (▲—▲), and dGuo-5′-$P_3$ (■—■).

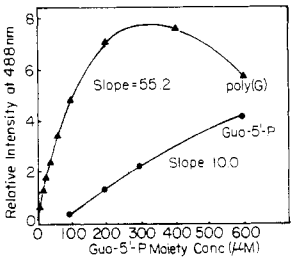


FIGURE 5: Comparison on an equal molar Guo-5′-P moiety basis of the enhancement of $Tb^{3+}$ fluorescence by Guo-5′-P and poly(G). Guo-5′-P (●—●) was examined over a 100–600 μM concentration range and poly(G) (▲—▲), based on molar concentration of polymer phosphate, was examined over a Guo-5′-P moiety molar concentration range of 10–600 μM. Both were analyzed for their ability to enhance the fluorescence of 100 μM $Tb^{3+}$ upon excitation by 290-nm light.

200- to 300-fold higher (limited by instrumental sensitivity) than the nonenhancing nucleotides. Unlike the ribose nucleotides, Guo-5′-P and Xao-5′-P, dGuo-5′-P enhancement appears to level off at dGuo-5′-P concentrations at and above 0.3 mM. An opalescence also appeared at and above 0.3 mM dGuo-5′-P. Since $Tb^{3+}$ forms insoluble salts with nucleic acids (Yonuschot & Mushrush, 1975), the opalescence may be a colloidal suspension of $Tb^{3+}$·dGuo-5′-P salt. The leveling of fluorescence may then be a consequence of either having reached a saturation level of fluorescing $Tb^{3+}$·dGuo-5′-P complexes, or may be a result of filtering and/or reabsorption of fluorescence by the colloidal suspension. Ribose nucleotides displayed similar patterns of fluorescence leveling and occurrence of opalescence at concentrations above 0.5 mM.

The uniqueness of the Guo-5′-P and dGuo-5′-P structures for $Tb^{3+}$ fluorescence enhancement was next probed with respect to the role of their phosphates. Since we (data not shown) and others (Formoso, 1973) had observed that neither guanosine nor deoxyguanosine enhanced $Tb^{3+}$ fluorescence, it was considered worthwhile to investigate the influence of the position of the phosphate in the nucleotide on nucleotide ability to enhance $Tb^{3+}$ fluorescence (see Table I). It was found that Guo-2′-P, Guo-3′-P, and dGuo-3′-P enhanced $Tb^{3+}$ fluorescence but at significantly reduced levels, i.e., less than 25% of the 5′ isomers. The various phosphate isomers of the remaining nonenhancing nucleotides, with the exception of Cyd-2′-P, continued to show no enhancement of $Tb^{3+}$ fluorescence. The ability of Cyd-2′-P to enhance fluorescence was low with respect to Guo-5′-P and dGuo-5′-P (less than 10%), but well within the range of Guo-2′-P, Guo-3′-P, and dGuo-3′-P. The relationship illustrated in Table I was unchanged when nucleotides were examined over a concentration range of 0.02–0.25 mM.

In an effort to find other biological forms of guanine and deoxyguanine nucleotides which might also enhance $Tb^{3+}$ fluorescence, the di- and triphosphate forms were examined (see Figure 4). The presence of additional phosphates at the 5′ position was found to cause an 80–90% lowering of fluorescence intensities for nucleotides at concentrations above 100 μM. Below 100 μM $Tb^{3+}$ there was significantly larger $Tb^{3+}$ fluorescence enhancement by the nucleoside di- and triphosphates relative to their respective nucleoside monophosphates. This may be the result of the greater net charge of the nucleoside di- and triphosphates. They may hold $Tb^{3+}$ in fluorescent complexes at $Tb^{3+}$ concentrations too low to produce a sufficient concentration of fluorescent $Tb^{3+}$·nucleoside monophosphate complex(es). In each case, once the number of nucleoside di- or triphosphates exceeded the number of $Tb^{3+}$s in solution, 100 μM, the fluorescence enhancements diminished to a lower level. Another class of nucleotides with biological significance which were examined for their fluorescence enhancement abilities were the cyclic nucleotides, Guo-3′:5′-P, Guo-2′:3′-P, and Ado-3′:5′-P. All three were devoid of ability to enhance the fluorescence of 100 μM $Tb^{3+}$ at the nucleotide concentrations ranging from 0.1 to 1.0 mM (data not shown).

*The Influence of the Nucleic Acid State of Polymerization on the Capacity of Guo-5′-P and Other Ribonucleotides to Enhance $Tb^{3+}$ Fluorescence.* In the course of examining the nature of DNA and RNA enhancement of $Tb^{3+}$ fluorescence with nucleotides, it was found necessary to use elevated concentrations of $Tb^{3+}$ (100 μM) relative to those suitable for DNA or RNA (10 μM) in order to obtain detectable fluorescence emission intensities. This suggested that perhaps the efficiency with which dGuo-5′-P and Guo-5′-P moieties enhanced $Tb^{3+}$ fluorescence might be increased by its inclusion into the polymeric form of nucleic acids. To examine this possibility, the abilities of poly(G) and Guo-5′-P to enhance $Tb^{3+}$ fluorescence were examined at 100 μM $Tb^{3+}$. As indicated in Figure 5, when compared on an equal Guo-5′-P-moiety molar basis, the Guo-5′-P moiety of poly(G) was more efficient than Guo-5′-P in its ability to enhance $Tb^{3+}$ fluorescence. A comparison of the slopes resulting from a linear regression analysis indicated that the Guo-5′-P moiety in the polymer was at least five to six times more efficient than the nucleotide. The decreased fluorescence at higher poly(G) concentrations was accompanied by sample opalescence analogous to that of the aforementioned $Tb^{3+}$·dGuo-5′-P colloidal suspensions and likely reflects the effects of filtering

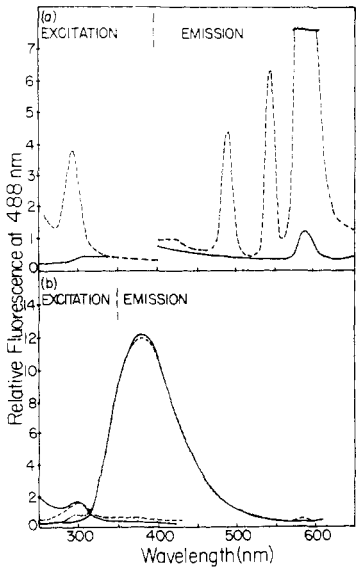


FIGURE 6: Fluorescence spectra for the enhancement of $Tb^{3+}$ fluorescence by 7MeGuo-5′-P and Guo-5′-P. All spectra were of 100 $\mu$M $Tb^{3+}$ in the presence of 250 $\mu$M nucleotide. (a) Excitation ($\lambda_{em}$ 488 nm) and emission ($\lambda_{ex}$ 290 nm) spectra of Guo-5′-P in the presence (- - -) and in the absence (—) of $Tb^{3+}$. (b) Excitation ($\lambda_{em}$ 488 nm) and emission ($\lambda_{ex}$ 290 nm) spectra of 7MeGuo-5′-P in the presence (- - -) and in the absence (—) of $Tb^{3+}$.

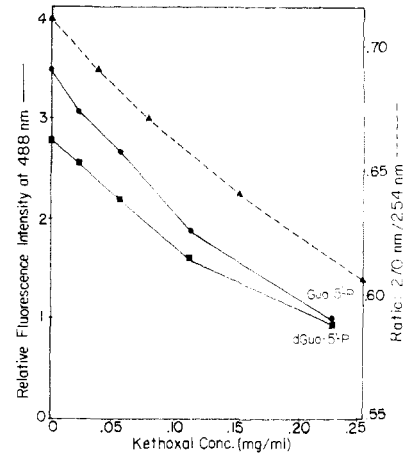


FIGURE 7: Enhancements of $Tb^{3+}$ fluorescence by Guo-5′-P and dGuo-5′-P following modification by kethoxal. Reaction between 0.025–0.25 mg/mL kethoxal and 0.25 mM nucleotide was performed and product formation monitored by the change in nucleotide 270 nm/254 nm absorbance ratios (▲ - - ▲) as described in Materials and Methods. Following reaction, Guo-5′-P (●—●) and dGuo-5′-P (■—■) were monitored for their enhancement ($\lambda_{ex}$ 290 nm) of 100 $\mu$M $Tb^{3+}$ fluorescence.

and/or reabsorption of emitted light. When the $Tb^{3+}$ concentration was lowered to $10^{-5}$ M, conditions in which 250 $\mu$M Guo-5′-P no longer produces detectable enhancement, fluorescence enhancement with homopolymeric Guo-5′-P moieties was still readily observable, i.e., 1.7 relative intensity units at 25 $\mu$M nucleotide content, and nearly linear over a range of 5 to 50 $\mu$M nucleotide content. Studies, in which poly(U, A, G) was found to display enhancement levels comparable with that of poly(G), indicated that the polymeric Guo-5′-P moiety did not require a homologous sequence to demonstrate greater ability to enhance than the nucleotide, Guo-5′-P. In other experiments conducted at $10^{-5}$M $Tb^{3+}$, we examined the $Tb^{3+}$ fluorescence enhancing abilities of the homopolymers, poly(U), poly(A), and poly(C), to determine whether the inclusion of "nonenhancing" nucleotides in polymer form would significantly increase their fluorescence enhancement abilities. It was found that polymerization of these previously "nonenhancing" nucleotides raised their fluorescence from background to low but detectable levels of fluorescence, i.e., less than 8% that of comparable amounts of poly(G).

*The Effect of Guanine Ring Modification on the Ability of Guo-5′-P and dGuo-5′-P to Enhance $Tb^{3+}$ Fluorescence.* The specificity of $Tb^{3+}$ fluorescence enhancement for guanine nucleotides may be a property of the electronic character of the guanine heterocycle. To test this possibility, the electronic state of the guanine heterocycle was perturbed by chemical modification. A readily available Guo-5′-P derivative of common biochemical occurrence, 7MeGuo-5′-P, was investigated with respect to its ability to enhance $Tb^{3+}$ fluorescence. The excitation and emission spectra of 250 $\mu$M 7MeGuo-5′-P both in the absence and in the presence of 100 $\mu$M $Tb^{3+}$ are compared with those of 250 $\mu$M Guo-5′-P in Figures 6a and 6b. They indicate that, relative to Guo-5′-P, 7MeGuo-5′-P had no apparent ability to enhance $Tb^{3+}$ fluorescence. The 488- and 540-nm emission peaks seen with the $Tb^{3+}$·Guo-5′-P complex were totally absent with 7MeGuo-5′-P (Figure 6b). Furthermore, methylation of Guo-5′-P at the N-7 position resulted in the appearance of a new, broad, highly fluorescent emission peak at 375 nm (Figure 6b). The 7MeGuo-5′-P excitation spectrum also failed to demonstrate characteristics associated with enhancement of $Tb^{3+}$ fluorescence. While Guo-5′-P displayed increased excitation in the 300-nm region upon the addition of $Tb^{3+}$ (Figure 6a), the excitation spectrum of 7MeGuo-5′-P showed essentially no change (Figure 6b). The inability of 7MeGuo-5′-P to enhance $Tb^{3+}$ fluorescence remained unchanged when examined at other concentrations ranging from 0.1 to 1.6 mM.

The guanine heterocycle also was modified by reaction with kethoxal and NA-AAF. Kethoxal reacts with the guanine heterocycle by cycling with the 1 and $N^2$ positions (Staehelin, 1959; Shapiro & Hachmann, 1966). The effect of kethoxal modification of Guo-5′-P and dGuo-5′-P on their ability to enhance $Tb^{3+}$ fluorescence is described in Figure 7. Over a range of kethoxal concentrations from 0 to 0.25 mg/mL, the decrease in the 270 nm/254 nm absorbance ratios, which indicates the extent of kethoxal adduct formation (Staehelin, 1959; Shapiro & Hachmann, 1966), is paralleled by a concomitant loss in their ability to enhance $Tb^{3+}$ fluorescence. Since the results were based on controls for phosphate hydrolysis and quenching by kethoxal reaction components (see Materials and Methods), it would appear that the decreased $Tb^{3+}$ fluorescence enhancement was due to modification of Guo-5′-P and dGuo-5′-P by chemical alteration at the guanine 1 and $N^2$ positions.

The second reagent employed to modify Guo-5′-P and dGuo-5′-P was NA-AAF, a reagent found to react principally at the 8 position of the guanine ring (Kriek et al., 1967). The result of NA-AAF modification of Guo-5′-P and dGuo-5′-P is shown in Figure 8. As can be seen, with increasing concentrations of NA-AAF in the reaction mixtures, there was a dramatic decrease in the ability of the nucleotides to enhance $Tb^{3+}$ fluorescence. Observations on appropriate controls (see Materials and Methods) support the conclusion that loss of fluorescence enhancement was the result of chemical modification of the guanine ring.

*Reaction of NA-AAF with Rat Liver DNA and rRNA and Its Influence on Their Abilities to Enhance $Tb^{3+}$ Fluorescence.* Based on the ability of guanine nucleotide modification to result in a corresponding loss of fluorescence enhancement, NA-AAF was reacted with DNA and RNA. In these experi-

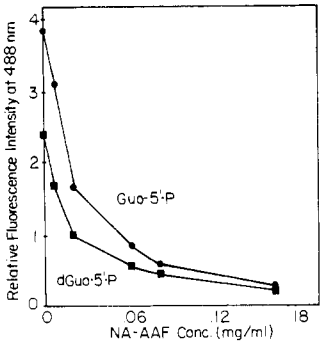


FIGURE 8: Enhancement of $Tb^{3+}$ fluorescence by Guo-5′-P and dGuo-5′-P following modification by NA-AAF. Reaction between 0.008–0.16 mg/mL NA-AAF and 0.25 mM nucleotide was performed as described in Materials and Methods. Guo-5′-P (●—●) and dGuo-5′-P (■—■) reaction products were examined for their ability to enhance the fluorescence of 100 μM $Tb^{3+}$ upon excitation by 290-nm light.

TABLE II: Influence of NA-AAF Reaction with Rat Liver DNA and rRNA on Their Abilities to Enhance Terbium(III) Fluorescence.[a]

| samples | rel fluorescence intensity at 488 nm | % of control fluorescence lost upon reaction with NA-AAF |
|---|---|---|
| DNA control | 0.095 ± 0.005 | |
| DNA + NA-AAF | 0.029 ± 0.001 | 69 |
| RNA control | 0.53 ± 0.04 | |
| RNA + NA-AAF | 0.37 ± 0.01 | 30 |

[a] Preparation of rat liver DNA and rRNA and conditions for reaction with NA-AAF are described in Materials and Methods.

ments, 17 μg/mL of rat liver DNA and rRNA were reacted with NA-AAF under essentially the same conditions as employed for reaction with Guo-5′-P and dGuo-5′-P. The NA-AAF modified DNA and rRNA were then compared with identically processed but unreacted control samples of DNA and RNA (see Materials and Methods) for their ability to enhance 10 μM $Tb^{3+}$ fluorescence. As indicated in Table II, the modified DNA and rRNA displayed 70% and 30% losses, respectively, in their ability to enhance $Tb^{3+}$ fluorescence.

## Discussion

The data support two general conclusions concerning $Tb^{3+}$ fluorescence enhancement. This phenomenon appears to be a reliable probe for: (a) detecting guanine residues in nucleic acids; and (b) detecting the extent to which guanine moieties have been modified by reaction with carcinogens.

The ability of $Tb^{3+}$ to display enhancement of its natural fluorescence when in the presence of 290 nm irradiated rat liver DNA or rRNA was examined. Both types of nucleic acid were found to give significant enhancement of $Tb^{3+}$ fluorescence in aqueous solution (pH 6.0) and at room temperature. Although RNA and DNA from different sources have been examined under a number of conditions (Formoso, 1973; Kayne & Cohn, 1974; Yonuschot & Mushrush, 1975; Barela et al., 1975), no previous report has compared the abilities of DNA and RNA from the same source to enhance $Tb^{3+}$ fluorescence under one set of experimental conditions. When compared on an equal concentration basis (μg/mL), rRNA was found to enhance $Tb^{3+}$ fluorescence to a considerably greater degree than DNA, viz., 13 000- and 1875-fold, respectively. This difference may be associated with differences (a) in the physicochemistry of ribose vs. deoxyribose in polymer backbones or (b) in the amount of a single and double-stranded helix (Horer et al., 1977). Nevertheless, these levels of $Tb^{3+}$ fluorescence enhancement by RNA and DNA impart to $Tb^{3+}$ great sensitivity as a probe for investigating nucleic acid macromolecules.

The usefulness of $Tb^{3+}$ fluorescence enhancement as a tool for the detection and investigation of nucleic acid structure and function, in part, depends upon an adequate understanding of those features of nucleic acid which permit such enhancement. Toward this end, several aspects of DNA and RNA enhancement of $Tb^{3+}$ fluorescence were revealed by a study of the phenomenon at a nucleotide level. When the common ribo- and deoxyribonucleotide constituents of RNA and DNA were studied, the major $Tb^{3+}$ fluorescence enhancers were the guanine nucleotides Guo-5′-P and dGuo-5′-P. Of the ribonucleotides, Guo-5′-P was found to possess an enhancement capacity superior, by 300-fold or more, to that of Ado-5′-P, Urd-5′-P, and Cyd-5′-P. This result is in qualitative agreement with the results of Formoso (1973) which indicated that Guo-5′-P was on the order of 15- to 50-fold better able to enhance $Tb^{3+}$ fluorescence than Ado-5′-P, Urd-5′-P, and Cyd-5′-P. However, on a quantitative basis, the relative enhancement capacity of Guo-5′-P in our studies is approximately one order of magnitude greater than that reported by Formoso (1973). This probably reflects the fact that in our studies all ribonucleotides were excited at 290 nm, the wavelength corresponding to the Guo-5′-P excitation maximum for enhancing $Tb^{3+}$ fluorescence. Formoso (1973) studied enhancements by ribonucleotides at their individual excitation maxima for enhancing $Tb^{3+}$ fluorescence. By excitation at 290 nm, fluorescence enhancement by Guo-5′-P is optimized, while fluorescence enhancement by the other common ribonucleotides is essentially eliminated. Investigations of the previously unexamined common deoxyribonucleotides, dGuo-5′-P, dCyd-5′-P, dAdo-5′-P, and dThd-5′-P, revealed essentially the same pattern of $Tb^{3+}$ fluorescence enhancement capacities. With the exception of a weakly, fluorescence-enhancing dThd-5′-P, i.e., approximately 2% that of a comparable amount of dGuo-5′-P, the guanine nucleotide again displayed a greater than 100-fold ability than the other deoxyribonucleotides to enhance $Tb^{3+}$ fluorescence. A comparison of Guo-5′-P and dGuo-5′-P enhancement abilities reveals that the ribonucleotide is about twice that of the deoxyribonucleotide. This difference at the nucleotide level may partially account for the previously noted greater fluorescence enhancement displayed by RNA relative to that displayed by DNA.

An examination of the nucleotide heterocyclic structures reveals that those displaying greater than 1000-fold $Tb^{3+}$ fluorescence enhancement, viz., Guo-5′-P, dGuo-5′-P, and Xao-5′-P, had two structural characteristics in common which set them apart from all other nucleotides studied. Each of these strong enhancers possesses a purine ring, and each of the rings was substituted at both the 2 and 6 positions. It would appear that this structural combination is required for strong enhancement. The substituents at the 2 and 6 positions of the strong enhancers were electronegative and capable of donating electrons to the heterocycle electron resonance systems; however, it is not known whether this characteristic is required for strong enhancement.

While there is a clear and an absolute requirement for the presence of the phosphate in nucleotides for production of

detectable $Tb^{3+}$ fluorescence enhancement, the effect of phosphate position in the nucleotide, viz., sugar 2′, 3′, or 5′ positions, is somewhat variable. Although the classification of the nucleotide as an enhancer or a nonenhancer is generally not affected by phosphate position (exception, Cyd-2′-P), the extent of fluorescence enhancement by phosphate isomers of a specific enhancer nucleotide is affected. For instance, with guanosine monophosphate, the 5′-phosphate isomer is a better enhancer than either the 2′ or 3′ isomer, and the $\alpha$-phosphate of Guo-5′-P is better than either the $\beta$- or $\gamma$-phosphates of Guo-5′-$P_2$ of Guo-5′-$P_3$, respectively.

The enhancing capacity of Guo-5′-P moiety in poly(G) was fivefold greater than that of Guo-5′-P, the free nucleotide. The increased enhancement capacity of polymeric Guo-5′-P may reflect an influence of (a) the increased proximity of Guo-5′-P moieties to one another, (b) the availability of two phosphate binding sites (5′ and 3′) for each guanosine moiety, or (c) a favorable restriction imposed by the polymeric state on nucleotide rotational freedom. Furthermore, since DNA and RNA, in a manner similar to poly(G), display appreciable $Tb^{3+}$ fluorescence enhancement at 10 $\mu$M, while nucleotide forms require 100 $\mu$M $Tb^{3+}$, one should be able to detect guanine moieties in polymeric nucleic acids with little interference from guanine nucleotides. The increase of Guo-5′-P's enhancement capacity upon inclusion in the polymer state does not appear to be unique for Guo-5′-P moieties. Examination of the homopolymers of Ado-5′-P, Cyd-5′-P, and Urd-5′-P reveals that previously nonenhancing nucleotides are able to give low but detectable enhancement of $Tb^{3+}$ fluorescence when in the polymer state. Nevertheless, poly(G) was still clearly the strong enhancer, whereas homopolymers of Ado-5′-P, Cyd-5′-P, and Urd-5′-P were either weakly or nonenhancing.

Modifications at the 1, $N^2$, 7, or 8 positions of guanine nucleotides resulted in loss of the $Tb^{3+}$ fluorescence enhancement. Among the modified guanine moieties, only 7MeGuo-5′-P was studied in the pure form. This modification causes both a significant alteration in ring electronic state as indicated by the appearance of a broad fluorescence peak at 375 nm, and, according to Hickey et al. (1977), a positive charge at the 7 position which electrostatically interacts with the 5′-phosphate to produce a new, conformational rigidity in the backbone portion of the nucleotide. Either of these changes may contribute to the loss of fluorescence enhancement. Furthermore, our data indicate that modifications of guanine moieties in RNA and DNA also result in loss of $Tb^{3+}$ fluorescence enhancement. Therefore, terbium may be useful in the detection of guanine modifications in nucleic acid.

In a more specific sense, terbium may be important in the detection of carcinogen interactions with RNA and DNA. A key concept in the induction of cancer invokes as a necessary step the covalent linkage of the carcinogen, or an activated metabolite, to functional intracellular macromolecules (Kriek, 1974). In investigations in which specific carcinogens have been traced to their sites of covalent binding to nucleic acids, linkage predominantly has been found to be to guanine residues (Heidelberger, 1975). The *N*-(guanosin-8-yl)- and *N*-(deoxyguanosin-8-yl)-AAF adducts formed in vivo during hepatocarcinogenesis with AAF (DeBaun et al., 1967; Kriek, 1969, 1970) were also formed in vitro by reaction of RNA and DNA with NA-AAF (Miller et al., 1966; Kriek et al., 1967). Since adduct formation with either Guo-5′-P or dGuo-5′-P or guanosine moieties in polymeric nucleic acids resulted in losses of $Tb^{3+}$ fluorescence enhancement, it may be possible to use terbium as a probe for the extent of in vivo binding of carcinogens to RNA and DNA.

Acknowledgments

The authors are grateful to Dr. Lionel A. Poirier of the National Cancer Institute for supplying *N*-acetoxy-2-acetylaminofluorene and to Dr. Mark G. Rockley for stimulating discussions.

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# Stimulation of Eukaryotic Transcription by Glycerol and Polyhydroxylic Compounds†

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ABSTRACT: In the presence of glycerol, ethylene glycol, glucose, or sucrose there is a marked increase in the transcriptive activity of crude or partially purified rat thymic RNA polymerase II on calf thymus DNA. Transcription stimulation is concentration dependent and is pronounced at concentrations as low as 1–2% (v/v). Glycerol-mediated transcription stimulation is temperature dependent. On a molar basis, transcription stimulation appears to be correlated with the number of hydroxyl substituents (sucrose ≫ glucose > glycerol). From evidence obtained using glycerol and the antibiotic AF/013, we propose that glycerol and similar polyhydroxylic compounds (1) stabilize the association of the RNA polymerase and the DNA template and (2) permit RNA polymerase to transcribe through template stop signals or permit reinitiation to occur. These effects may be due to the capacity of polyhydroxylic compounds to weaken hydrophobic bonding and thereby alter the structure of the DNA template and RNA polymerase during interaction at promoter regions and during transcription. When glycerol is added to thymic nuclear homogenates, endogenous transcription is inhibited. Dimethyl sulfoxide stimulates transcription in reconstituted eukaryotic systems at concentrations up to 10% and then progressively inhibits transcription. These data suggest that polyhydroxylic compounds may be involved in the regulation of eukaryotic transcription. The uniformity of glycerol stimulation in reconstituted eukaryotic and prokaryotic transcriptive systems suggests that the mechanisms regulating transcription are similar. Virtually all eukaryotic RNA polymerase isolation and purification procedures utilize glycerol and/or sucrose, and assays for RNA polymerase contain glycerol as an ingredient of the assay medium or through the addition of RNA polymerase stored in high concentrations of glycerol. These observations suggest, therefore, that studies of eukaryotic transcription inadvertently contain altered transcription kinetics and overestimate RNA polymerase activity.

Glycerol is a component of cellular triacylglycerols and phosphoglycerides and enters the glycolytic pathway after conversion to dihydroxyacetone phosphate (Stryer, 1975). Glycerol is added to cell and enzyme preparations to prevent protein denaturation during freezing and thawing (Ashwood-Smith & Warby, 1972; Derrick et al., 1972; McKee & McCarty, 1973; Fansler & Loeb, 1974; Valeri, 1975). Glycerol is added to muscle preparations to alter surface membranes and permit the direct access of added components to muscle fibers (Yamaguchi & Fujino, 1972; Marston, 1973; Nakajima et al., 1973) and has been injected intravenously following cerebral infarction to reduce cerebral edema (Meyer et al., 1975). Glycerol has been used as a reaction medium, e.g., to facilitate acetylation and carboxymethylation (Bradbury & Jakoby, 1972; Siegel & Awad, 1973; Grove & Levy, 1975). While glycerol has been used extensively in protein isolation procedures and to provide a protective medium during ultracentrifugation, this use has been predicated upon the assumption that glycerol acts as an enzyme stabilizer. We demonstrate in the following paper that glycerol and other polyhydroxylic compounds may act to stimulate RNA polymerase activity in reconstituted eukaryotic transcriptive systems.

## Materials and Methods

### *Materials*

Unlabeled ribonucleoside triphosphates, calf thymus DNA, and crystalline bovine serum albumin were obtained from P-L Biochemicals. Anhydrous glycerol was Baker Analyzed Reagent grade. Sucrose, dithiothreitol, and other chemicals were of the highest grade available from Sigma Chemical Co. DEAE[1]-Sephadex A-25 was obtained from Pharmacia Fine Chemicals, Inc., and $\alpha$-amanitin from Boehringer Mannheim Biochemicals. [5-$^3$H]Uridine 5′-triphosphate (24 Ci/mmol) was ordered from Amersham/Searle Corp. Scintanalyzed toluene was obtained from Fisher Scientific Co., and fluors and tissue solubilizer were from Packard Instrument Co. AF/013 was the generous gift of Dr. Renato Cricchio, Research Laboratories, Gruppo Lepitit, Milan, Italy.

### *Methods*

*Isolation of Crude RNA Polymerase from Rat Thymus Glands.* Sprague-Dawley rats (breeding stock obtained from Simonsen Laboratories, Gilroy, Calif.) were decapitated and their thymus glands excised, blotted, and minced in cold buffer. Ten minced glands were homogenized in 12 mL of 2.0 M sucrose containing 0.5 M Tris-HCl, 0.025 M KCl, and 0.005 M $MgCl_2$ (2.0 M sucrose–TKM) with five and ten strokes, re-

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[1] Abbreviations used are: BSA, bovine serum albumin; RNAP (in figures), RNA polymerase; DEAE, diethylaminoethyl; DTT, dithiothreitol; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

0006-2960/78/0417-4825$01.00/0 