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Terbium as a Fluorescent Probe for DNA and Chromatin†

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ABSTRACT: Terbium reacted with DNA and chromatin to form a complex in which terbium acted as a sensitive fluorescent probe. By measuring the narrow-line emission of Tb^{3+} when DNA is selectively excited, the relative amount of Tb^{3+} bound to the DNA can be calculated. Terbium was bound to DNA until one Tb^{3+} was present for each phosphate group. After this point no more terbium was bound. $TbCl_3$ was bound to chromatin in a linear manner until approximately 0.48 $TbCl_3$ was added for each phosphate group in the chromatin-DNA solution. From these data it appears that 52% of the phosphate groups in chromatin were unavailable for binding. The binding of Tb^{3+} to DNA can be reversed by prolonged dialysis against 0.5 *M* NaCl and chelating agents. The terbium ion is ideal in that it binds DNA tight enough so that completion of the reaction can be assumed but loose enough so that it can be removed by gentle means. Low concentrations of salt (up to 2 m*M* NaCl) enhance the quantum efficiency. Below pH 3 and above pH 7 the DNA-terbium complex will not form. Between pH 3 and pH 7 the quantum efficiency of the DNA terbium complex increases from either pH to a maximum at pH 5.5 to 5.6. Several biochemical uses for Tb^{3+} ion are suggested.

The use of fluorescent probes in the development of methods which yield information about the nature of nucleic acid and chromatin is an important focus in biochemistry today. Organic molecules have been used as fluorescent probes with nucleic acid and chromatin, most notably ethidium bromide and several acridine dyes (Blake and Peacocke, 1968; LePecq and Paoletti, 1967). Several modes of binding can occur with these dyes ("strong and weak", "intercalated or external binding") depending upon the ratio of dye to DNA-P, the ionic strength of the media, and the pH (von Hippel and McGhee, 1972). Several quantitative methods for determining free phosphate in chromatin using Toluidine Blue and azure A have been reported (Klein and Szirmai, 1963; Miura and Ohba, 1967). More recently Clark and Felsenfeld (1971) and Itzhaki (1970) have used polylysine to titrate the "open" portions of chromatin.

Inorganic fluorescent probes, especially the lanthanide ions, have been an area of intense research interest; since Heller and Wasserman (1965) found that selective excitation of the ligands of rare earth chelates yields characteristic line emissions of the lanthanide ions. Crosby (1966) showed that the ligand triplet state must be higher than the resonance level of the lanthanide ion for effective transfer to occur. Filipescu and Mushrush (1968) suggested the use of unchelated lanthanide ions as fluorescent probes for excited triplets in solution. Eisinger and Lamola (1971) published a kinetic model for the transfer of energy from common nucleotides to lanthanide ions in aqueous solution at pH 5.0 or less so that no association with phosphate groups occurred. Luk (1971) had used the lanthanide ions as fluorescent probes in the study of the transferrin molecule conformation. Based on fluorescence, two specific binding sites per transferrin molecule were found.

This paper reports the use of Tb^{3+} as a sensitive fluorescent probe in the solid state with DNA and chromatin. At neutral or slightly acidic pH's terbium will precipitate DNA and chromatin. By measuring the narrow line emission of Tb^{3+} when the DNA is selectively excited, the relative amount of Tb^{3+} bound to the DNA can be calculated.

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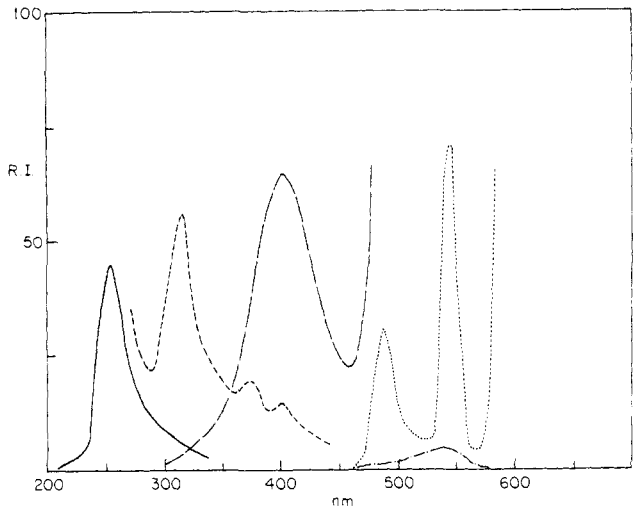

FIGURE 1: Fluorescence spectra of DNA-Tb complex. (- - -) Excitation spectrum for emission at 545 nm; (—) excitation spectrum for emission at 400 nm; (· · ·) emission spectrum for excitation at 310 nm; (— —) emission spectrum for excitation at 248 nm; (· - · -) Tb^{3+} emission spectrum in absence of DNA excitation at 310 nm. All spectra were run in the solid state sample accessory on an American Instrument spectrophotofluorometer.

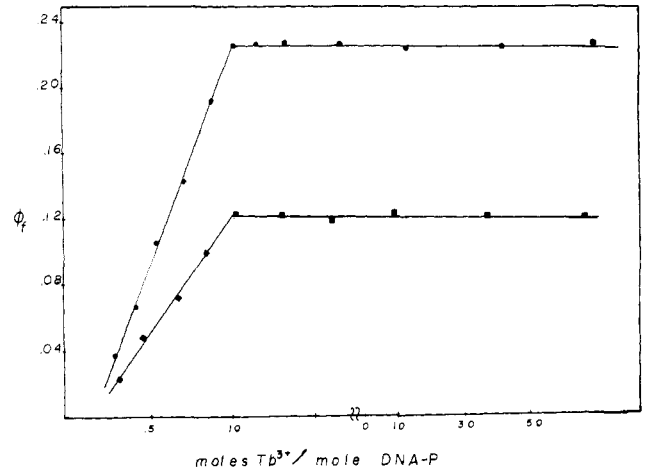

FIGURE 2: Quantum efficiency of Tb^{3+} fluorescence as a function of the Tb^{3+} to DNA ratio with calf thymus DNA. Excitation wavelength 310 nm. (■) DNA-Tb complex formed without NaCl; (●) the DNA-Tb complex formed in 20 m*M* NaCl.

The mechanism of energy transfer probably involves absorption by the bases and then by an intramolecular process, the electronic excitation energy is transferred to Tb^{3+} with concomitant fluorescence. Terbium binds to DNA-phosphate and chromatin phosphate stoichiometrically. The relative amount of free phosphate and bound phosphate in chromatin was determined. The effects of salts, pH, and chelating agents on this binding were studied. Other uses of Tb^{3+} as a sensitive probe are also proposed.

Materials and Methods

Nuclei were isolated by a method modified from Chauveau et al., (1956). Livers were removed from Spraque Drawley albino rats of mixed sex weighing approximately 250 g and perfused with cold 0.15 *M* NaCl. The tissue was minced, mixed with 10 volumes of 0.25 *M* sucrose-5 m*M* $CaCl_2$ and homogenized in a glass homogenizer with a motor-driven Teflon pestle. The slurry was centrifuged at 1000*g* for 15 min. The supernatant was discarded. The pellet was suspended in 2.3 *M* sucrose and centrifuged in a Beckman 30B rotor at 23,000 rpm for 1 hr. The pellet of nuclei was used to prepare chromatin.

Preparation of Chromatin. Chromatin was prepared from purified nuclei essentially by the method of Huang and Huang (969). The nuclei were homogenized in 5 volumes of 75 m*M* NaCl-24 m*M* EDTA (pH 7) with a Dounce homogenizer. The homogenate was centrifuged for 10 min at 3500*g*. The pellet was washed again in the same buffer and three times in 50 m*M* Tris (pH 7.5) with centrifugation for 15 min at 3500*g*. The pellet was then washed twice in 1m*M* Tris (pH 7.5) with centrifugation for 15 min at 3500*g*. Finally, the chromatin was dissolved in 10 volumes of distilled water by stirring overnight at 4°. The DNA content of the chromatin was determined by the absorption at 260 nm using a molar extinction coefficient of 7600 for chromatin and 6800 for DNA as determined by Tuan and Bonner (1969).

All experiments were run using the calf thymus DNA purchased from Worthington Biochemical Corporation, Freehold, N.J. Terbium chloride of at least 99.9% purity was purchased from American Potash and Chemical Corporation. All solvents were spectroquality, available from Matheson Coleman and Bell.

Formation of the DNA-Terbium (DNA-Tb) Complex. A typical experiment was done as follows. One milliliter of an aqueous terbium chloride solution was added to 3 ml of solution containing 1 mg of calf thymus DNA and any other acids, bases, salts, or buffers required for the particular experiment. Under certain conditions specified in this paper, a precipitate (DNA-Tb) formed. The solution was stirred intermittently for 10 min to ensure thorough mixing and complete precipitation. Since it is an aqueous $TbCl_3$ solution the actual species (by hydrolysis) that are responsible for the precipitation are $Tb(OH)^{2+}$ etc. This prevents a charge density from accumulating. The precipitate was centrifuged in a Fisher Safety centrifuge with a clinical head at maximum speed 400*g*. The precipitate was extracted with absolute alcohol. The alcohol was removed by decantation after centrifugation as above. The extraction step was repeated. Finally, the precipitate was extracted with ether. The ether was removed by centrifugation as above and the DNA-Tb precipitate was dried. This procedure was found to remove all nonchelated terbium. This was confirmed by the absence of terbium fluorescence in the supernatant.

Fluorescence Spectra of the DNA-Terbium Complex. Fluorescence spectra were run on an Aminco-Bowman spectrophotofluorometer. The solid dry DNA-Tb precipitate was pressed into a pellet and run in the solid state front surface sample holder manufactured by Aminco. The samples were excited at 307 nm and the emission was scanned in the 450-600-nm range. The apparent Tb^{3+} emission spectra were corrected for the photomultiplier response and the change with frequency of the monochromator bandwidth. The integrated area under the corrected emission curves was compared with the integrated area from a quantum efficiency standard at the same excitation wavelength, absorbance, and geometry. The resulting ratio of areas is termed quantum efficiency (ϕ_f). The secondary quantum efficiency standard used was terbium tris(threonyltrifluoroacetonate)phenanthroline.

Results

The DNA sensitized Tb^{3+} emission is illustrated in Figure 1. Two of the three normal emission bands of Tb^{3+} are

shown at 491 and 545 nm. These bands were used to calculate the quantum efficiency of DNA-Tb^{3+}. The third band is normally seen at 580 nm. However, since the emission spectrum tails off with scattered radiation above 570 nm, this band is not shown for all runs. The figure also shows that Tb^{3+} in the absence of DNA does not fluoresce appreciably by itself at 491 and 545 nm when irradiated at 307 nm. The band at 400 nm represents the emission of the DNA when it is excited at 248 nm. The excitation spectra of DNA was obtained by scanning the excitation monochromator when the emission monochromator was held at 400 nm for DNA. The excitation spectra of DNA-Tb was run at the emission wavelengths (491 and 545 nm) of terbium. Both excitation spectra of the DNA-Tb were the same with respect to band position.

The relative concentration of DNA was measured by its emission at 400 nm. From run to run, the DNA varied slightly in concentration. During the course of over 100 runs, this emission did not vary by more than approximately 1%. Thus, the fluorescence emission measured at 491 and 545 nm was always relative to a fixed absorption of DNA at 248 nm.

Once the DNA was precipitated by the $TbCl_3$, it was very stable. Prolonged periods of irradiation did not appreciably change the DNA or DNA-Tb emission spectra. The excitation wavelength 307 nm was selected on the basis of the excitation spectrum in Figure 1. At wavelengths below 300 nm, the instrumental problem of "scatter radiation" interferes with the Tb^{3+} emission at 545 nm. Thus the relative quantity of Tb^{3+} complexed to a certain amount of DNA can be measured by the ϕ_f of fluorescence at 491 and 545 nm.

The DNA-Tb complexes which were formed in the presence of NaCl and shown in Figure 2 were prepared as follows. Solutions containing 1 mg of calf thymus DNA and 80 μmol of NaCl in 3 ml were combined with varying amounts of $TbCl_3$ in 1 ml so that the final volume was 4 ml. The amount of $TbCl_3$ used varied from 0.5 to 200 μmol. The precipitate which formed was shaken intermittently for 10 min and centrifuged (400g) for 5 min. The precipitate was then extracted twice with absolute alcohol to remove excess terbium chloride. Finally, the DNA-Tb complex was extracted with ether and dried. The ϕ_f values ran from a low of 0.037 to a high of 0.225 as the Tb^{3+} concentration was varied.

As shown, the reaction of $TbCl_3$ with DNA was linear until approximately one Tb^{3+} ion was added for each phosphate group in the DNA. After this point no excess terbium was bound. Therefore, terbium fluorescence can be used as a sensitive probe to measure the amount of phosphate groups available for binding in DNA. Furthermore, when less than one Tb^{3+} ion was present for each available phosphate, then no terbium fluorescence was detected in the supernatant after centrifugation. This indicated that reacting terbium is very tightly bound.

At very high Tb^{3+} concentration, well above those shown, the ϕ_f values are slightly higher than 0.225. This is most likely due to the excess Tb^{3+} being incompletely extracted by our procedures. The curve not passing through the origin is due primarily to the error in ϕ_f values and other operable quenching processes that occur significantly when the Tb^{3+} concentration is very low. Also, when the ratio of moles of terbium ion to moles of DNA-P in DNA or chromatin was 0.15 or below no precipitation occurred. Precipitation did occur when this ratio was increased to 0.30.

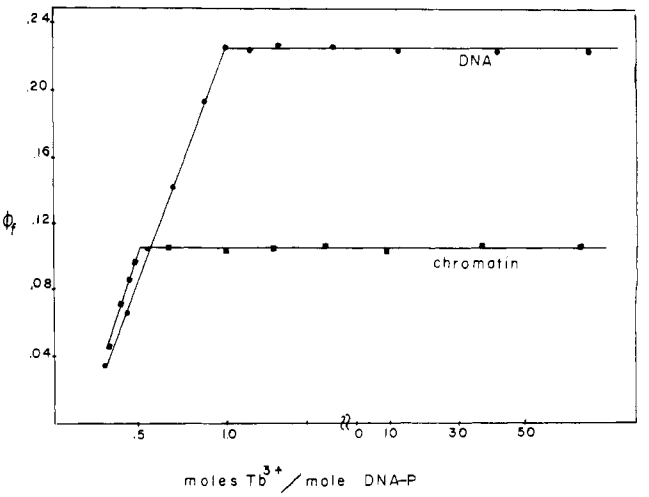

FIGURE 3: Qrantum efficiency of terbium fluorescence as a function of the Tb^{3+} to DNA ratio in the DNA-Tb complex (●) and rat liver chromatin-Tb complex (■). Both complexes were formed in the presence of 10 mM sodium acetate buffer at pH 5.5

The figure also shows the titration of DNA when sodium chloride is left out of the reaction. As seen, the stoichiometry of the reaction is the same, that is: the reaction between $TbCl_3$ and DNA was still linear until one Tb^{3+} had been added for each phosphate group available in the DNA. However, in the absence of NaCl the quantum efficiencies of the DNA-Tb complexes were lower than in the presence of NaCl.

The proportion of available phosphate groups in chromatin was determined by titration with Tb^{3+}; 4 ml solutions of 5.25 mM chromatin-P-10 mM sodium acetate (pH 5.5) were combined with a series of Tb^{3+} solutions (Figure 3). At the same time, DNA solutions were treated with the same series of Tb^{3+} solution and used as a standard. As is seen in the figure, when one Tb^{3+} ion has been added for each phosphate group, then no more Tb^{3+} binds. However, only 0.48 of the Tb^{3+} ions were bound for each phosphate group in the chromatin. We interpret this as meaning that only 48% of the phosphate groups in chromatin were available for binding.

The probability exists that a small amount of Tb^{3+} in the chromatin-Tb^{3+} complex is bound to the phosphoprotein. Tb^{3+} does not form a precipitate with bovine serum albumin, insulin, or isolated histones. However, when a protein such as casein which contains bound phosphate is reacted with Tb^{3+} a copius precipitate results. This precipitate is probably due to the binding of Tb^{3+} to protein phosphate. This protein-Tb^{3+} complex is not excited by light at 307 nm. Protein bound Tb^{3+} in chromatin would probably not be excited either unless it was close enough to the nitrogeneous bases for energy transfer to occur. We are therefore assuming that protein bound Tb^{3+} does not interfere with the estimation of the available phosphate groups in chromatin.

The enhancement and inhibition of the terbium-DNA complex by salt are illustrated in Figure 4. Solutions containing 5 μmol of $TbCl_3$, 4 μmol of DNA-P, and a varying concentration of NaCl in 4 ml were prepared. The solutions were left in the refrigerator at 4° for 2 days with occasional stirring to ensure that equilibrium was reached. The quantum yield of the DNA-Tb complex was enhanced upon the addition of salt. The enhancement reached a maximum at 2 mM NaCl and remained constant up to a concentration of

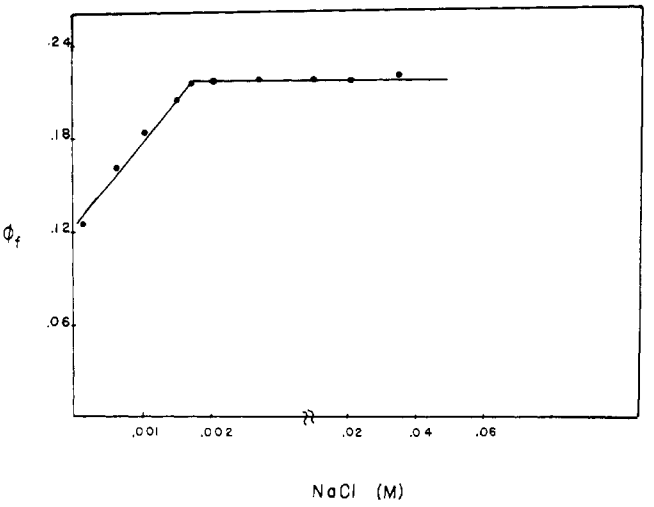

FIGURE 4: Quantum efficiency of Tb^{3+} fluorescence in the Tb-DNA complex as a function of the NaCl concentrate in the reaction mixture. The concentration was 4 mM DNA and 5 mM $TbCl_3$ for all reactions.

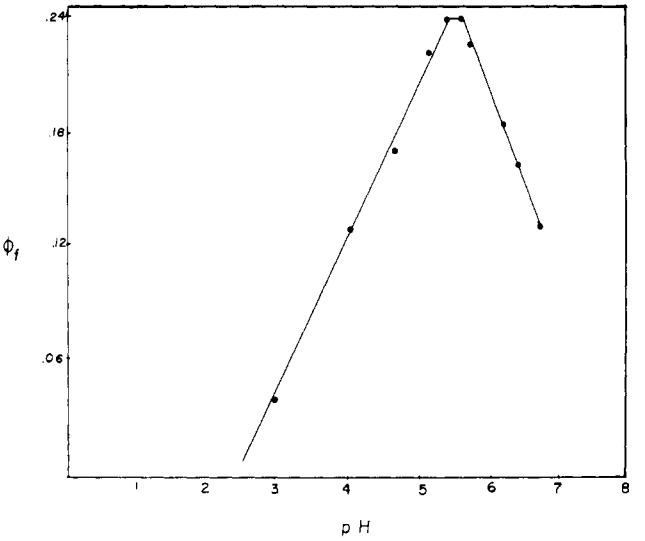

FIGURE 5: Quantum efficiency of Tb^{3+} fluorescence as a function of pH. The pH was maintained by a sodium acetate buffer (10 mM final concentration). The DNA-Tb complex was formed by adding 1 ml containing 4 μmol of DNA-P to 3 ml containing 5 μmol of Tb^{3+}.

30 mM. At 50 mM NaCl concentration and above the formation of the DNA-Tb complex was inhibited in a nonlinear fashion and at 0.5 M NaCl the DNA-Tb complex would not form at all. Not only does 0.5 M NaCl inhibit the formation of the DNA-Tb complex but it will dissociate a DNA-Tb complex which has already formed. DNA from the DNA-Tb complex can be redissolved by dialysis against several changes of 0.5 M NaCl solution. At high concentrations, the sodium ion probably competes with Tb^{3+} for the phosphate group.

The effect of pH on the DNA-Tb complex is shown in Figure 5. One milliliter containing 4 μmol of DNA-P was added to 3 ml containing 5 μmol of Tb^{3+} at varying pH's. The solutions having values pH 1.0, 2.0, and 3.0 were adjusted with HCl. Those solutions having values pH 4.0–6.7 were buffered with sodium acetate so that the final concentration was 10 mM. The DNA-Tb complex does not form in stoichiometric amounts at pH 7.0 and above. This is due to the removal of Tb^{3+} from solution as the hydroxide. Also, the terbium-DNA complex does not form in stoichiometric amounts below pH 3. Between pH 3 and pH 7 the quantum yield varies dramatically reaching a narrow plateau between 5.5 and 5.6. It is obvious from this figure that the pH, if not regulated, could be a source of error in an experiment.

In addition, it was found that both 5% and 10% Cl_3CCOOH would decrease the quantum efficiencies of a preformed DNA-Tb complex. The DNA complexes were made by combining 5 mM $TbCl_3$ and 3 mM DNA-P. The quantum yields of the original DNA-Tb complex and DNA-Tb complex after each of three successive extractions with 5 ml of 5% and 10% Cl_3CCOOH were measured and are shown in Table I.

The DNA-Tb complex does not form in the presence of chelating agents. The formation of a precipitate in a 4-ml solution containing 4 μmol of DNA-P and 5 μmol of $TbCl_3$ was inhibited completely in the presence of 0.1 M sodium citrate (pH 4.1, 5.0, 6.0, 6.9), 0.1 M EDTA (pH 4.7), and 0.1 M sodium tartrate (pH 6.0). A nonstoichiometric precipitate did form in the presence of 0.1 M sodium citrate at pH 3.1. Dialysis against these chelating agents removed Tb^{3+} from the DNA-Tb complex and allowed for the resolution of the DNA.

Discussion

Terbium reacted in a stoichiometric manner with DNA and chromatin forming a stable bond and precipitate. The amount of terbium bound to DNA was measured after excitation at 307 nm by fluorescence emission at 491 and 545 nm.

The quantum efficiency of the DNA-Tb complex increased upon the addition of low concentrations of NaCl to the reaction mixture. Since the stoichiometry of DNA-P to Tb^{3+} in the complex was 1:1 with or without 10 mM NaCl (see Figure 2), then the increase in quantum efficiency of the DNA-Tb complex was not due to a difference in the amount of Tb^{3+} bound. Also since the quantum efficiency of DNA at 400 nm remained constant as the NaCl concentration was varied between 0 and 0.06 M, it is doubtful that the increased quantum efficiency of the DNA-Tb complex was due to an increased absorption of light at 307 nm. It is most probable that the NaCl enhanced the intramolecular energy transfer and thus made the assay more sensitive. Whether this enhancement was due to the NaCl present in the solid DNA-Tb^{3+} complex or due to the influence of salt on the nature of the DNA-Tb^{3+} complex is not known. The decrease in the quantum efficiency of Tb^{3+} due to salt concentrations higher than 50 mM is probably the result of dissociation of Tb^{3+} from the DNA since repeated washing with solutions containing high concentrations of salt will redissolve the DNA.

Tb^{3+} ion is ideal in that it binds DNA tight enough so that completion of the reaction can be assumed but loose enough so that it can be removed by gentle means. Since chelating agents facilitate the removal of terbium from the DNA-Tb complex and since Tb^{3+} is precipitated above pH 7 as an insoluble hydroxide, then treatment with EDTA at pH's greater than 7 might facilitate the removal of Tb^{3+} and the resolution of chromatin.

If several quantum efficiency values are determined within a short space of time, errors due to changes in geometry, lamp output, etc., are not great and instrumental accuracy is well within 1%. Thus, small changes in the available phosphate groups on DNA should be measurable by this

Table I: Effect of Cl_3CCOOH on the Quantum Efficiency of the DNA–Tb Complex.

DNA–Tb Complex	Quantum Efficiency[a]
Standard	0.22
Washed once with 10% Cl_3CCOOH	0.18
Washed twice with 10% Cl_3CCOOH	0.13
Washed three times with 10% Cl_3CCOOH	0.10
Washed once with 5% Cl_3CCOOH	0.20
Washed twice with 5% Cl_3CCOOH	0.18
Washed three times with 5% Cl_3CCOOH	0.14

[a] One milliliter aliquot containing 4 μmol of DNA-P was added to a 0.5-ml aliquot containing 5 μmol of $TbCl_3$. One precipitate was used as a standard. The other precipitates were washed one, two or three times with 5 or 10% Cl_3CCOOH solutions.

method. It should be emphasized that water quenches the fluorescence at 490 and 545 nm. For this reason the DNA–Tb complex must be thoroughly extracted or the quantum efficiencies will be low. Also, samples will absorb water from the air during storage. Samples used in these experiments were stored at 4° under ether in stoppered test tubes.

Since Tb^{3+} does not precipitate chromatin or DNA until enough is added to complex 15–30% of the phosphate groups, perhaps one can use Tb^{3+} to selectively separate chromatin with many available phosphate groups from chromatin without as many available phosphates. If it turns out that terbium inhibits certain nuclease, then it could be used with protease to isolate the available regions of chromatin after other portions of the chromatin are digested with protease and nuclease.

Not only does Tb^{3+} function as a probe for DNA and RNA, but it is also possible that Tb^{3+} could be used as a probe with any phosphoester such as those existing on ribosomes or membranes. Terbium will precipitate phosphoprotein but does not precipitate other proteins. Therefore, Tb^{3+} could be used as a probe for phosphate groups on proteins or for the separation of phosphoproteins from other proteins.

Azure A has been used to titrate available phosphate groups in chromatin. This method showed that approximately one-half of the phosphate groups were available. (Klein and Szirmai, 1963). Titration using Toluidine Blue showed that either 0.48 or 0.28 of the phosphate in calf thymus chromatin was available (Miura and Ohba, 1967). Two authors have used polylysine to determine the percentage of chromatin available for titration. Clark and Felsenfeld (1971) found that about half of the DNA was open. Itzhaki (1970) found that 38% of the DNA of rat thymus chromatin was available for titration. Our value for the available phosphate groups in chromatin was 48%.

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