COMPARATIVE PHOSPHORESCENCE QUENCHING OF DNA'S OF DIFFERENT COMPOSITION

I. ISENBERG, R. ROSENBLUTH, *and* S. L. BAIRD, JR.

From the Science Research Institute, Oregon State University, Corvallis, Oregon 97331

ABSTRACT Small amounts of paramagnetic cations quench the phosphorescence of DNA. Although the emission intensity is monotonic with increasing thymine content, the quenching efficiency is not. The cations quench from phosphate sites.

INTRODUCTION

Recent studies have indicated that the low-temperature ultraviolet-induced phosphorescence of DNA may be attributed to thymine-negative ions (1, 2).

Guanine and adenine emit at neutral pH (3, 4). However, adenylic acid emission is quenched at acid pH (5, 6) while thymine, which does not emit at neutral pH, shows a strong emission at high pH (7). The electron spin resonance position of the DNA triplet is close to that of the thymine-negative ion, and no resonance has as yet been found corresponding to an adenine or guanine triplet (2). Both the DNA and the thymine-negative ion emissions have about the same decay constant and appear unstructured, in contrast to those of adenine and guanine which have structure and much longer decay times. Furthermore, GMP emission is quenched by CMP or poly C (2).

All of the evidence is thus consistent with a model in which the guanine emission is quenched by hydrogen bonding to cytosine and, in the excited state, the N_1 proton of thymine is shifted to adenine. With this shift, the adenine no longer emits, and the emission is that due to the resulting thymine-negative ion.

The DNA phosphorescence is quenched by relatively low concentrations of paramagnetic cations (2, 4, 8). The fluorescence is quenched very little, if at all. In order to explain the observed amount of DNA phosphorescence quenched per added paramagnetic cation, it has been postulated (4, 8) that the triplet energy wanders from one base to another, being quenched when the excitation is in the vicinity of a bound paramagnetic cation.

This type of triplet migration has been shown in studies of the emission of crystals (9) and is now well recognized. However, as will be seen, any discussion of triplet migration in polynucleotides must include consideration of the possible occurrence of specific barriers to such migration.

The work reported here involves a comparative study of the phosphorescence

and the quenching of phosphorescence of three double-stranded polynucleotides differing in base composition. It also reports the inhibition of quenching in the presence of large amounts of Mg^{++}.

Rahn, Shulman, and Longworth (2) found that, with minor variations, the phosphorescence of DNA's from varying sources appeared the same, except for the quantum yield which was larger for samples with more A-T content. Our data verify theirs.

This paper presents evidence that the quenching efficiency, and thus presumably the amount of triplet wandering, is not a simple function of A-T content but apparently depends on base sequence. It also shows that the site of a quenching ion is at the polynucleotide phosphate.

MATERIALS AND METHODS

All emissions were measured at 77°K, in 50% glycerol glasses, on an Aminco-Keirs spectrophosphorimeter (American Instrument Co., Silver Spring, Md.) equipped with a Hruska photometer (Hruska Radio Co., Lutherville, Md.). Samples were in quartz capillary tubes of 0.2–0.4 mm diameter. Whenever comparative quantum yields were desired, the same capillary tube and concentrations of DNA giving the same optical density were used. We have observed that samples of *Micrococcus lysodeikticus* DNA, permitted to stand in 50% glycerol for more than several hours at room temperature, showed increased quantum yields and gave irreproducible results. We, therefore, examined all samples in less than ½ hr after mixing, during which time no detectable time variations could be seen.

Sources of the DNA were as follows.

(*a*) salmon sperm DNA. This was obtained commercially and was further purified as previously described (10).

(*b*) *Micrococcus lysodeikticus* DNA. Commercially dried cells (Miles Chemicals Co., Elkhart, Ind.) were extracted, and DNA was purified according to Marmur's procedure (11). The final precipitate was redissolved in 0.0025 M NaCl. The solution (OD = 5.3) was then put on a Sephadex G-25 fine column and eluted with 0.0025 M NaCl. It was then concentrated by flash evaporation at 36°C.

(*c*) poly dAT. Synthetic poly dAT was a gift of Dr. Thomas M. Jovin, Stanford University. It was dialysed extensively against 0.0025 M NaCl. In three batches, a total of 0.882 ml of concentration 4.96×10^{-4} M in phosphates was evaporated in a weighed 0.5 ml polyethylene centrifuge tube to a final weight of 40.9 mg dAT solution. Evaporation was performed below the dAT melting point. The concentrations of dAT were calculated using $\epsilon = 6650$ liter $-$ cm^{-1} $-$ $moles^{-1}$, based on phosphate, at 262 mμ (12).

Preparation of Samples

Samples used for emission contained 0.03 M NaCl in 50% glycerol-water mixtures. The divalent cations were added generally as chloride salts, but controls run with perchlorate salts yielded the same results. The pH of the samples was 5.5 at room temperature in 50% glycerol.

The actual volume of sample required in the tubes used for emission studies was only about 1 μl. However, samples of at least 0.1 ml had to be prepared in order to have confidence in the accuracy of their composition. For salmon sperm and *M. lysodeikticus* DNA separate samples were mixed for each emission measurement. However, only small amounts of dAT

were available, and special micromixing techniques were employed to obtain quenching curves on single 0.1 ml samples. The technique was checked by obtaining identical quenching curves for salmon sperm DNA using both ordinary and micro methods. For the micro technique, $MnCl_2$ was added in increments to one sample, and the phosphorescence was measured on an aliquot after each addition. Changes in the sample size and concentration due to addition of $MnCl_2$ and evaporation during mixing were monitored by weighing and were adjusted when necessary. At the end of the experiment the OD at 262 mμ was measured and compared with the value expected from all monitoring weighings. The two values differed by only 10%. Two independent series of measurements yielded the same quenching curve. After running a

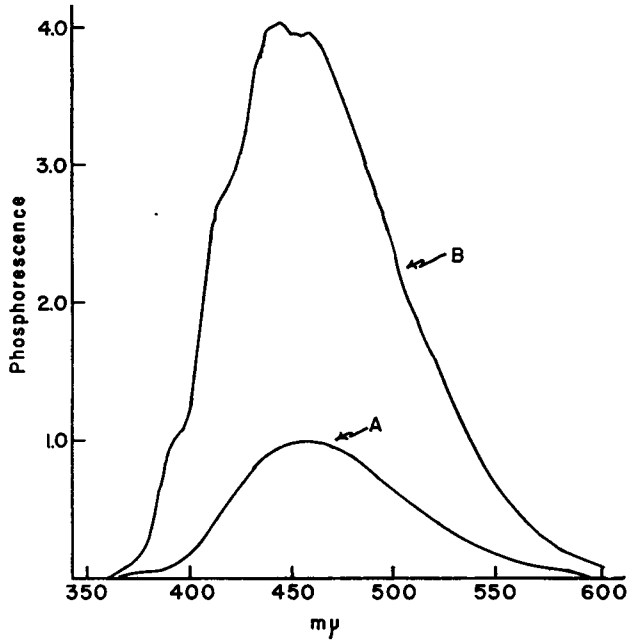

FIGURE 1 Phosphorescence of native and denatured DNA: salmon sperm, 4×10^{-3} M DNA-P. Curve *A*, native DNA; curve *B*, denatured DNA: the same sample as *A* after heating for 30 min at 90°C followed by quick chilling; ordinate: phosphorescence, relative units.

quenching curve, a melting point curve of the sample was run, still in 50% glycerol. It was found to melt at 37°C with a hypochromism of 42%. This, together with the absence of adenine emission, indicated that spectra had been run on hydrogen-bonded dAT.

In general, an internal check on the nativeness of the DNA's used is obtained by observation of the phosphorescence (see results below).

RESULTS

Fig. 1, curve *A*, shows the phosphorescence spectrum for native salmon sperm DNA. Curve *B* is a typical spectrum obtained from heat-denatured DNA. Denaturation brings with it a much more structured spectrum and an increase in signal. The increase in quantum yield upon denaturation is variable, ranging from about 2 to

about 8 depending upon the concentration of DNA and the extent of heating. Similar changes have been reported by Rahn et al. (2). The increased structure is presumably due to the phosphorescence of purines which are now no longer hydrogen bonded.

The quantum yield of DNA phosphorescence depends on the DNA examined. Fig. 2 shows the relative emissions of *M. lysodeikticus* (28% A-T), salmon sperm DNA (58% A-T), and poly dAT. It may be seen that the size of the emission increases with an increasing amount of A-T base pairs. The emissions are roughly in

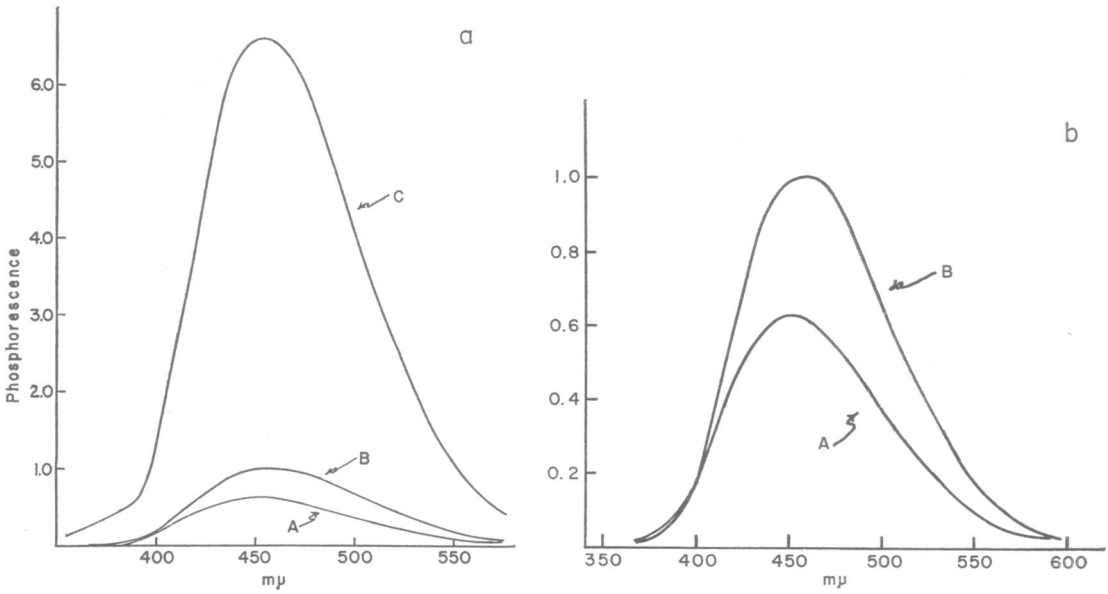

FIGURE 2, a–b. Phosphorescence of three different DNA's excited at 284 mμ. All samples had same absorbance at 284 mμ. Curve *A*, *Micrococcus lysodeikticus* DNA; curve *B*, salmon sperm DNA; curve *C*, poly dAT; ordinate: phosphorescence, relative units.

the ratio of 1:1.6:10. Rahn et al. (2) have also reported that dAT has a larger emission than calf thymus DNA.

Upon turning off the exciting light, the time decays for the emissions were found to be the same, having a time constant of approximately 0.3 sec. This, together with the similarity in shape, suggests that the source of the emission is the same in all cases.

Let R = moles of cation added per mole of DNA phosphate. Then, for small amounts of cation added, compared to nucleic acid phosphate present, the phosphorescence intensity, S, is given by:

$$S = S_0 (1 - \beta R).$$

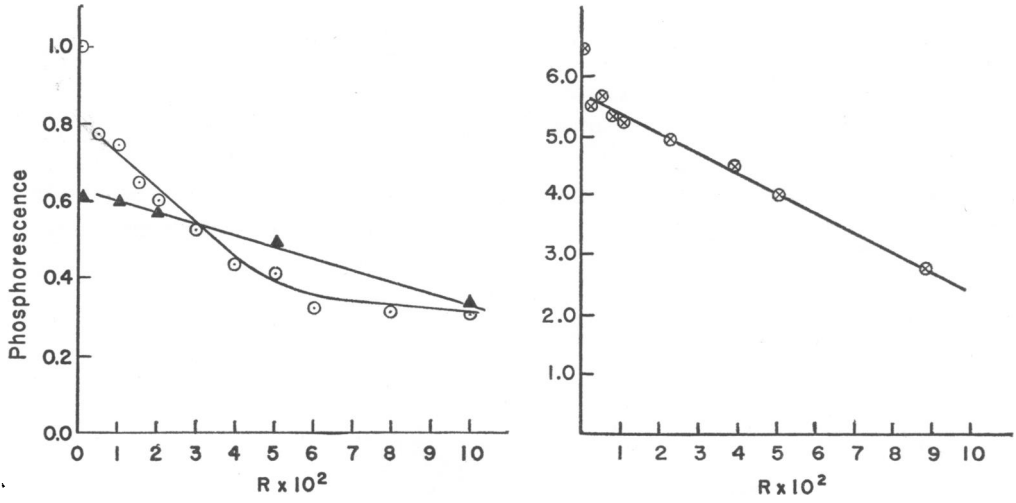

FIGURE 3. Quenching of phosphorescence by $MnCl_2$; comparison of three different DNA's: salmon sperm DNA ⊙⊙⊙, *Micrococcus lysodeikticus* DNA ▲▲▲, poly dAT ⊗⊗⊗. *R* is the molar ratio of $MnCl_2$ to DNA phosphates. Ordinate: amplitude of phosphorescence, same relative units used.

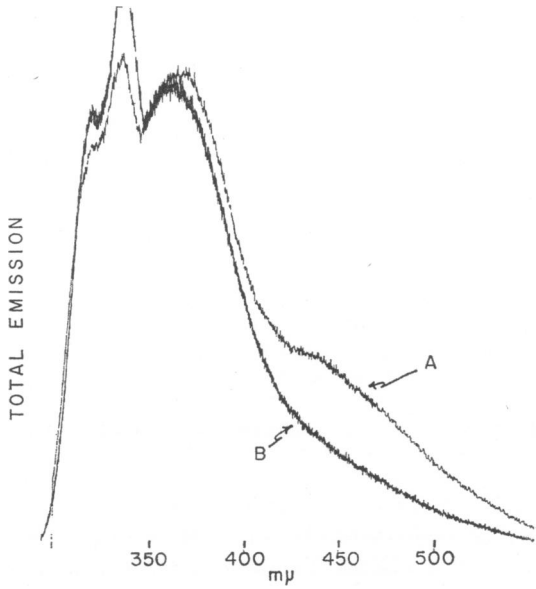

FIGURE 4 Total emission, salmon sperm DNA. Curve *A*, no added Cu^{++}; curve *B*, 0.1 moles of Cu^{++} per mole of DNA phosphate. Note that there is little or no change in fluorescence in the vicinity of 365 mμ, but phosphorescence at 450 mμ is severely quenched. [Compare with Mn^{++} quenching, Fig. 6 of Rahn et al. (2).]

β is a measure of the quenching efficiency. It is the average region of DNA, as measured in DNA phosphates, that is quenched by one added cation. Fig. 3 shows the comparative quenching of *M. lysodeikticus* DNA, salmon sperm DNA, and poly dAT by Mn^{++}. These have β values of 5, 11, and 6, respectively.

As previously reported (8), quenching also occurs upon the addition of Co^{++},

Ni^{++}, or Cu^{++}, and β appears to show an increase, within a factor of 2, as one progresses along the series Mn^{++}, Co^{++}, Ni^{++}, Cu^{++}. This quenching occurs with little or no change in the fluorescence (Fig. 4).

In the presence of Mg^{++}, the quenching due to the paramagnetic cations diminishes (Fig. 5).

If the concentration of Mg^{++} is high enough, added Mn^{++}, Co^{++}, and Ni^{++} will no longer quench. It may be noted, however, that the amount of Mg^{++} that must

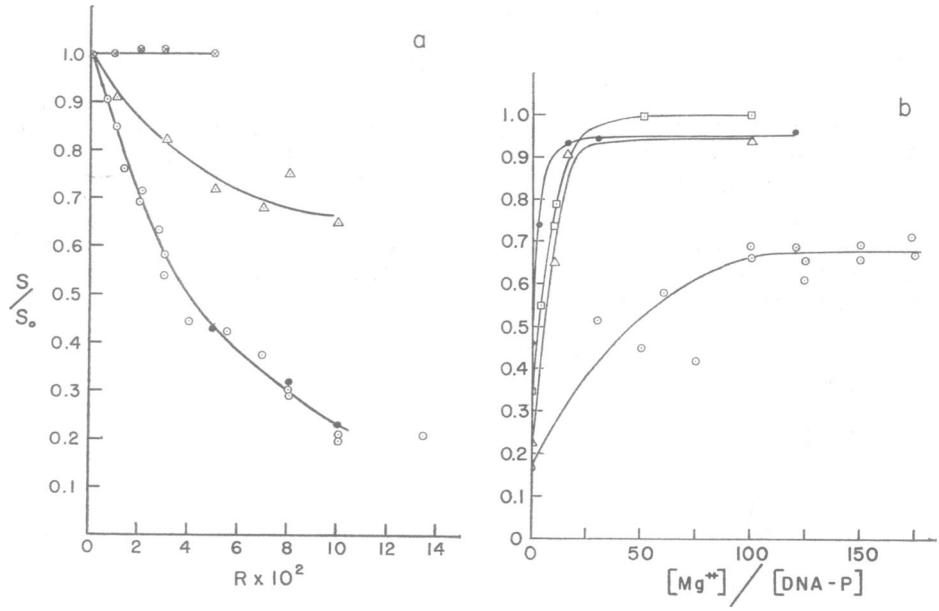

FIGURE 5 Inhibition of phosphorescence quenching by $MgCl_2$. S is the amplitude of phosphorescence; S_0 is the amplitude in the absence of paramagnetic cation.
5 a: quenching by Ni^{++} at various $MgCl_2$ concentrations. R is the molar ratio of Ni^{++} to DNA phosphates. ⊙ = no added Mg^{++}; ● = 1 Mg^{++} per DNA-P; △ = 10 Mg^{++} per DNA-P; ⊗ = 40 Mg^{++} per DNA-P.
5 b: comparison of different paramagnetic ions. All samples contained 0.1 paramagnetic cations per DNA phosphate. ● = Mn^{++}, ⊡ = Co^{++}, △ = Ni^{++}, ⊙ = Cu^{++}.

be added to inhibit the quenching is large compared to DNA phosphate. For complete inhibition, in the presence of 3×10^{-3} M (DNA-P), about 0.045 M Mg^{++} is needed. As may be seen in Fig. 5, for a given concentration of Cu^{++}, the emission increases with added Mg^{++} reaching a plateau at about 0.3 M Mg^{++}. The signal is then 70% of its value in the absence of Cu^{++}. We regard the value of 70%, instead of 100%, as an artifact. In the presence of high Mg^{++}, Cu^{++} precipitates DNA. Before samples were examined for emission, they were routinely centrifuged, and aliquots from the supernate were used. These showed a decrease in optical density

which could account for the dimunition of phosphorescence from that observed without Cu^{++}. Furthermore, such curves varied appreciably from run to run. The 70% plateau of Fig. 5 b, therefore, does not demonstrate that the Cu^{++} binding site is different from that of the other cations.

It should be noted that Mg^{++} alone does not alter the emission of DNA even when added in large concentrations. This has been checked up to a ratio of 130 Mg^{++} ions per DNA phosphate.

DISCUSSION

As noted in the introduction, the accumulated evidence indicates that the phosphorescence of DNA is from the thymines. The results reported here are consistent with this interpretation. The similarity between the emissions of the three polymers, of widely different base ratios, indicates that the emission is due to the same source. However, the emission does not appear to be simply proportional to the amount of thymine present, although the intensity is larger for higher A-T content.

The observation that one added paramagnetic cation quenches a section of salmon sperm DNA of 10–20 phosphates led (4, 8) to a model in which the triplet excitation wandered from one position to another, being quenched only when in the vicinity of a paramagnetic cation. However, if triplet wandering does occur, the results reported here indicate that such migration is not a simple function of the amount of thymine present. The wandering must also depend on the order of the bases, at least in a statistical sense. If the delocalization were only a function of the average thymine content, perhaps being greatest for polynucleotides with highest thymine content, one would expect the emission from poly dAT to be quenched most efficiently. Instead, the quenching efficiency, using poly dAT, in which an A regularly alternates with a T along the chain, is smaller than that of salmon sperm DNA. In fact, it is about the same as that for *M. lysodeikticus* which has only 28% A-T. Therefore, the extent of triplet wandering is not dependent alone on thymine content, but probably depends on the sequence of bases along the chain. In salmon sperm DNA, one might expect a larger number of thymines close to one another than in either of the other two DNA's. It may be expected that a high efficiency of transfer from one thymine to another will occur if the thymines are adjacent to one another. Such a high efficiency of transfer has been observed in single-stranded poly A where β has been found to be large, of the order of 100 (20). Our results suggest that even one adenine between two thymines serves to sharply reduce the efficiency of transfer of excitation energy between the thymines.

The low quenching efficiency of Mn^{++} when added to *M. lysodeikticus* can be understood on the same model. The low thymine content makes it likely that between most nearest pairs of thymines, there will be a number of nonthymine bases serving as barriers to the transfer of triplet excitation.

This model predicts that the quenching efficiency should be large when using

natural DNA's of high A-T content, but not having a regular alternation of bases as in poly dAT.

A complete characterization of barriers to triplet migration must await further experimental information. An understanding of the range of migration not only involves the question of what constitutes an effective barrier but also depends on an understanding of the quenching range of a paramagnetic cation. Any ultimate theory must also explain the observations (2, 20) that the lifetime of the residual luminescence of calf thymus DNA or of the neutral pH form of poly A is independent of the degree of quenching. These observations suggest that absolute barriers to migration exist, and if a quencher is complexed between such barriers, the luminescence of the region is quenched completely, perhaps because migration between barriers is very rapid.

A related question involves the site of binding of the quenching cations. Even though a wide variety of evidence has accumulated over the past 10 years that Mg^{++}, Mn^{++}, Co^{++}, Ni^{++} site bind to the phosphates of polynucleotides, (13–18), studies of triplet quenching in liquid media at room temperature have indicated that for quenching to occur, the quenching cation must complex directly with the emitter. Furthermore, theoretical discussions of such quenching have invoked mechanisms involving wave function overlap between the emitting species and the quenching cation.

The experimental demonstrations of site binding at phosphates all have sufficient experimental errors, so that one could raise the possibility that a minority species of cation existed, bound directly to bases, this species alone being responsible for quenching, while the majority of bound cations at phosphate sites do not quench (4). We believe that the experiments reported here rule out the existence of such a presumed minority component.

The strongest argument in favor of cations bound to phosphates being the quenchers lies with the general shape of the quenching curve. If extremely small amounts of cations are added to DNA, a presumed minority fraction should become negligibly small, since the binding energy to phosphates would certainly be much larger than that to the presumed base sites. If cations bound to phosphates did not quench, the initial portion of the quenching curve would therefore have a small slope. Experimentally one finds that this region has the largest slope.

The inhibition of quenching by Mg^{++} may also be interpreted most simply as indicating that it is the cations bound to phosphates that are the quenchers. Any presumed base binding should certainly be weak. Consequently, the electrostatic potential attributed to the over-all charge of the DNA would contribute a major fraction of the binding energy. Effects of this type, in which the charge on one site effects the binding to another have been amply demonstrated by Felsenfeld and Huang (19). Therefore, if one were to have quenching only by a presumed fraction of cations bound directly to bases, the quenching would be quite sensitive to small

amounts of Mg^{++}. We do not find this. On the contrary, only rather large quantities of Mg^{++} inhibit quenching. Such inhibition may be most simply attributed to competition for phosphate sites with both paramagnetic cations and Mg^{++} binding to phosphate. The quenching inhibition is in the sequence Mn^{++}, Co^{++}, Ni^{++}, Cu^{++} (Fig. 5). This is the order commonly found for increasing binding energy of these cations for ligands (21, 22).

Since all evidence indicates that quenching is due to phosphate-bound paramagnetic cations, the mechanism of quenching must be regarded as unknown. However, space-filling models of DNA show that the location of a cation bound to a phosphate site may actually be fairly close to the bases, depending, in large part, on the exact orientation of the phosphate oxygens and the cation.

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