

DOE Report Number

DOE/ER/61604--T1

Title

AN EXPLORATION OF SEQUENCE SPECIFIC  
DNA-DUPLEX/PYRENE INTERACTIONS FOR INTERCALATED  
AND SURFACE-ASSOCIATED PYRENE SPECIES

Subtitle

Final Report

Reporting Period

May 1, 1993 - December 31, 1996

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Report Date

March 1997

MASTER

DOE Sponsorship  
DOE Grant Number

PREPARED FOR THE U.S. DEPARTMENT OF ENERGY  
UNDER GRANT NUMBER DE-FG05-93ER61604

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## 2.0 Abstract.

*The broad objective of this DOE sponsored work on photoinduced electron transfer (ET) within covalently modified DNA was to learn about the rates of ET among various DNA bases and commonly used organic electron donor (D) and acceptor (A) molecules.*

Important societal needs that would be impacted by such research include (1) improved knowledge of controlled charge separation for advanced photonics materials, (2) better understanding of biological ET processes, and (3) fundamental advances in the construction of ensembles of reactive species in environments in which diffusion is severely restricted. This hypothesis driven, multidisciplinary project combined skills in modified nucleic acid synthesis and in continuous and time-resolved optical spectroscopies. Covalently modified DNA chemistry as investigated in this program had two specific long term goals. The first was to use experimental and theoretical insights into the mechanisms of electron transfer (ET) reactions to design supramolecular assemblies of redox-active chromophores that function as efficient vectorial ET engines. The second was to construct oligonucleotide probes for real-time monitoring of intracellular processes involving DNA and RNA such as *m*-RNA expression and translocation. This research project laid the groundwork for studying ET reactions within DNA duplexes by examining the photophysics of uridine nucleosides which are covalently labeled at the 5-position with 1-pyrenyl chromophores. Recent work by Geacintov *et al.* and this work by Netzel *et al.* has confirmed the expected rapid ET quenching of pyrene\* by dG and dU nucleosides with covalently attached pyrene labels. Following picosecond laser excitation in the first case, pyrene<sup>-</sup> was identified and in the second pyrene<sup>+</sup> was observed. Additionally, this project's study of pyrene-labeled DNA pentamers established that dG nucleosides are less effective pyrene\* quenchers than either dC or dT nucleosides as predicted by ET quenching free energy estimates. *A surprising result of this study was the observation that dC nucleosides are ca. four times better quenchers of pyrene\* than are dT nucleosides. Thus it appears likely that ET from dT<sup>-</sup> (or dU<sup>-</sup>) to a nearby dC nucleoside is favorable.* Extension experiments based on the results of this program will test this possibility. In keeping with the long terms goals of the project to construct oligonucleotide probes for real-time monitoring of intracellular processes, this project also studied the photophysical properties of monomeric and bichromophoric cyanine dyes bound to duplex DNA.

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**4.0 Introduction and History of Recent Studies of ET in DNA.** *A long range objective of this DOE sponsored work on photoinduced electron transfer (ET) within covalently modified DNA was to learn about the rates of ET among various DNA bases and commonly used organic electron donor (D) and acceptor (A) molecules. The eventual results of this type of research will be significant from several perspectives and to researchers in a variety of fields. For example, since the end of World War II a large body of research has been conducted concerning the health effects of radiation and in particular the effects of ionizing radiation on DNA. Within this field it is important to know the rate at which an initially formed hole or excess electron within DNA will travel to its final trapping site. Indeed it is also important to know what the final trapping sites are, and whether these are kinetically or thermodynamically determined. Other important societal needs that will be impacted by these research results include (1) improved knowledge of controlled charge separation for advanced photonics materials, (2) better understanding of biological ET processes, and (3) fundamental advances in the construction of ensembles of reactive species in environments in which diffusion is severely restricted. This hypothesis driven, multidisciplinary work combined skills in modified nucleic acid synthesis and in continuous and time-resolved optical spectroscopies.*

Three recent studies of DNA-mediated ET in duplexes with fixed D/A positions suggest two differing views of the effectiveness of DNA as an ET facilitator. Two of these studies report that the rates of ET observed are comparable to those found in proteins.<sup>1,2</sup> The other finds an ET rate that is at least a million times faster than expected on the basis of the other two results.<sup>3-5</sup> Recent theoretical calculations support the contention that DNA and proteins are comparable ET mediators.<sup>6-8</sup> However, the electronic coupling that a DNA bridge can provide between a given ET donor and acceptor is inversely proportional to the energy gap between the electronic tunnelling energy of the activated complex and the oxidized and reduced states of the DNA bridge.<sup>6,7</sup> In the three previous studies of DNA mediated ET, this gap is large and little ET coupling enhancement is expected. In this research program, because ET among DNA bases themselves can occur, small tunnelling-energy gaps are present. Thus from a theoretical point of view, enhanced ET rates are possible. The extent of ET rate enhancement will depend, however, on nuclear reorganization energy as well as on electronic coupling effects.<sup>9-14</sup>

The skewed  $\pi$ -stacking of bases in a DNA duplex suggests that there might be enhanced electronic coupling between donors and acceptors embedded within or attached to such a  $\pi$ -stack. As noted above, recent theoretical calculations do not support this hypothesis for systems with large tunnelling-energy gaps, but hold out the possibility that it might occur for ones with small gaps. *Thus carefully constructed and clearly interpretable ET rate measurements in duplexes as functions of D/A separation and base sequence are needed to test theoretical calculations of DNA-bridge electronic couplings.*

The uses of efficient charge-separating supramolecules span diverse fields such as artificial photosynthesis, biomimetic modeling of photoinduced membrane potentials, photocopying, and the development of nonlinear optical devices for information storage and processing. Thus many chemists are interested in developing regiochemically defined supramolecular assemblies for efficient photoinduced charge separation. The DNA double helix provides a unique template for positioning redox active chromophores at desired positions relative to one another for controlled charge separation. However, to use DNA duplexes as templates intelligently, one must know the reorganization energies and electronic coupling properties of covalently modified DNA oligomers and duplexes. *Research under this contract focuses on developing an initial framework for systematic design of redox-active DNA molecules.*

There are also three other perspectives from which this work will be of interest to scientists not directly concerned with photoinduced ET studies themselves. The first of these is found among scientists who are developing antisense therapeutics based on covalent modifications of DNA and RNA oligonucleotides. *The novel nucleoside and phosphoramidite chemistries developed here and the spectroscopic characterizations of the resulting covalently modified DNA oligomers and duplexes carried out in this program will provide important chemical information that is likely to be useful for antisense and antigene drug design.*

The second perspective is found among chemists who are working to understand the kinetics and mechanisms of ribozyme (catalytic RNA) operation. For example, Dr. Douglas Turner (Univ. of Rochester) uses the increase in emission that occurs when a pyrene-labeled RNA substrate binds to a ribozyme to monitor the kinetics of substrate/ribozyme binding and reaction in stopped-flow experiments.<sup>15-20</sup> *This program's work on pyrene labeled DNA nucleosides, oligomers, and duplexes has put Dr. Turner's experiments on firm physical chemical footing.*<sup>21-23</sup> Extension studies will explore additional labeling modifications that are likely to be useful in this type of study.

The third perspective is found among scientists who are trying to understand the mechanisms of carcinogenesis and tumorigenesis for polyaromatic hydrocarbons (PAHs) such as various pyrenes. These widespread environmental carcinogens covalently bind to DNA bases, especially guanine, and in some cases form lethal lesions.<sup>24-30</sup> In most cases the DNA lesions are repaired. *Model DNA oligomers and duplexes incorporating pyrene-uracil lesions can be made from the nucleosides developed in this work and may provide new approaches to studying PAH lesions in DNA.* In particular, it is enormously more economical in time and money to prepare large quantities of synthetic DNA with pyrene-uracil lesions than it is to purify samples of chemically reacted DNA and PAH compounds. Of course, correspondences among the different kinds of lesions in a given experimental setting such as DNA repair will have to be made just as they must be for different kinds of environmentally occurring PAHs.

**4.1 Relation of this DOE Sponsored Research to the Longer Term Goals of the Principal Investigator.** Covalently modified DNA chemistry as investigated in this DOE sponsored program has two long term goals. *The first is to use experimental and theoretical insights into the mechanisms of ET reactions to design supramolecular assemblies of redox-active chromophores that function as efficient vectorial ET engines. The second is to construct oligonucleotide probes for real-time monitoring of intracellular processes involving DNA and RNA such as m-RNA expression and translocation.* Automatic DNA synthesis, combined with the nucleobase modification chemistry being developed in this program and already well developed 2'-O-ribose modification chemistry, offers a practical route for constructing such oligonucleotide assemblies. Commercial applications of covalently modified nucleosides and oligonucleotides could lead to antisense and antigene drugs.

The rationale for developing oligonucleotide probes of biomolecular processes is readily apparent. However what advantages does DNA chemistry offer for constructing supramolecular ET assemblies? One major advantage is that automatic solid-phase synthesizers offer a versatile way of constructing multicomponent arrays of redox active chromophores. A second advantage over most other approaches to making multicomponent assemblies is that moderate length duplexes of DNA can have well defined double-helical structures. Detailed 2D-NMR and x-ray experiments on selected assemblies will be needed to assign absolute configurations and validate molecular modeling parameters. However, simple optical studies such as circular dichroism (CD) and duplex-melting experiments can verify the construction of targeted duplex configurations. These

two advantages of DNA chemistry afford great control over the relative positions of the individual chromophores: in short they provide *versatility* and *generalizability*. Additional benefits are: (1) the number of atoms in these assemblies is small enough that they can be modeled with sophisticated molecular mechanics and dynamics calculations and even semi-empirical Hartree-Fock CNDO/S calculations and (2) the charge distribution along the DNA assembly can also be controlled because the internucleotide phosphate groups can be modified to be either negative, positive, or neutral. Extension of this work will actively pursue both *ab initio* and semi-empirical calculations of the electronic properties of covalently substituted nucleosides and specific sequences of DNA bases stacked in B-form geometry.

**4.2 An Overview of ET Theory.** ET theories can be classical, quantum mechanical, or semi-classical.<sup>31-36</sup> In the high temperature limit for weak electronic coupling, there is no difference between the semiclassical and quantum mechanical rate expressions.<sup>31,35</sup> Solvent vibrational modes at room temperature are universally treated in the high temperature limit. The same is usually done for the D/A vibrational modes, but it is unlikely that this assumption is valid. The semi-classical rate expression described below explicitly makes both of these assumptions. However, it is not necessary to use only semi-classical theory.<sup>5,31,35,37-40</sup> Fortunately, the general form of the rate-versus-driving-force function for the semi-classical and fully quantum mechanical theories is much the same for moderate driving forces.<sup>5</sup> For large driving forces, a quantum mechanical treatment must be used.<sup>37-40</sup> For the purposes of defining a small number of terms for discussion here, semi-classical ET theory will be outlined briefly.<sup>6-8,12,34-36,41-52</sup> In terms of this theory, the electron transfer rate ( $k_{ET}$ ) is given in eq 1.

$$k_{ET} = (4\pi^2/h) |H_{ab}|^2 (FC) \quad (1)$$

where  $h$  = Planck's constant,  $H_{ab}$  = the electronic coupling between the donor and acceptor, and FC = a nuclear Franck-Condon factor which is given in eq 2.

$$FC = (4\pi \lambda k_B T)^{-1/2} \exp[-(\Delta G^0 + \lambda)^2 / (4 \lambda k_B T)] \quad (2)$$

where  $\lambda = \lambda_{in} + \lambda_{out}$ ,  $\lambda_{in}$  = the D/A nuclear reorganization energy,  $\lambda_{out}$  = the solvent nuclear reorganization energy,  $k_B$  = Boltzmann's constant,  $T$  is the temperature, and  $\Delta G^0$  is the reaction driving force (free energy). In terms of eqs 1 and 2, small to moderate driving forces correspond to  $\Delta G^0 \geq -\lambda$ , and activationless ET processes have  $\Delta G^0 = -\lambda$ .

As D/A separation  $r$  increases,  $\lambda$  increases.<sup>5,12,13,35,51,53-56</sup> For moderate driving forces, this will decrease  $k_{ET}$ . However a much larger decrease in  $k_{ET}$  with increasing  $r$  is produced by the approximately exponential dependence of  $H_{ab}$  on separation distance as shown in eq 3.

$$|H_{ab}|^2 = |H_{ab}^0|^2 \exp[-\beta(r - r_0)] \quad (3)$$

where  $r$  is the center-to-center donor acceptor distance,  $r = r_0$  when the donor and acceptor are in contact, and  $H_{ab}^0$  is the electronic coupling between donor and acceptor when  $r = r_0$ . Eq 4 is a widely used, but coarse-grained approximation that is taken from the distance dependence of an electron tunnelling through a one dimensional square barrier.<sup>5-8,57</sup> Note that eq 3 is a convenient way of summarizing results from many different experiments. There are, however, semi-empirical Hartree-Fock methods for explicitly calculating  $H_{ab}$  for any D/A configuration in a DNA or protein environment.<sup>6,7,56,58,59</sup>

**4.3 Recent Studies of ET in DNA.** Measurements of ET reaction rates for D/A groups bound to DNA are still sparse. One reason is that the synthetic chemistry involved is more complex than that necessary for studying ET processes between D/A groups bound to proteins. Also ET studies in substituted proteins began to be reported in 1982,<sup>60</sup> while the first report of ET in a DNA duplex involving bound D/A groups appeared in 1992 by Brun and Harriman.<sup>1</sup> Since then two more studies have been reported, one by Barton and

Turro *et al.*<sup>3</sup> in 1993 and the other by Meade and Kayyem<sup>2</sup> in 1995. Brun and Harriman studied ET between the excited-state donors ethidium (EB<sup>+</sup>) or protonated acridinium (AOH<sup>+</sup>) and the acceptor *N,N'*-dimethyl-2,7-diazapyrenium (DAP<sup>2+</sup>) with each D/A pair intercalated into CT-DNA.<sup>1,61</sup> Because the donors and acceptors were not covalently attached to specific sites, there was uncertainty both about which bases and about how many bases separated them. Using indirect arguments about the number of separating bases, they determined  $\beta$  in their experiments to be 0.9 Å<sup>-1</sup>. The appropriate value of  $\lambda$  for these ET processes is unknown. Brun and Harriman suggest 0.2 eV. This is very much smaller than the 0.8-1.2 eV values found in protein studies that used ruthenium complexes and iron porphyrins as donors and acceptors.<sup>49-51,54-56,62-66</sup> The strength of this study is that three different ET rates were measured presumably corresponding to three different D/A separation distances. From the three sets of distances and rates,  $\beta$  could be determined without knowing  $\lambda$ . Theoretical calculations of electronic coupling between donors and acceptors imbedded in proteins found the distance decay parameter  $\beta$  to range from 0.9 to 1.5 Å<sup>-1</sup>.<sup>45,57,67-70</sup>

The most thoroughly characterized ET study in ds DNA was reported by Meade and Kayyem.<sup>2</sup> There complementary octaoligonucleotides each had either a ruthenium(II) donor or a ruthenium(III) acceptor attached to their 5'-ends. The approximate edge-to-edge D/A separation in this system was 12.5 Å [corresponding to a metal-to-metal distance of 20.5 Å],<sup>71</sup> and neither ruthenium complex was intercalated into the duplex. Importantly in this study (and in the one by Brun and Harriman above), the production of a charge transfer product was directly observed. In this experiment only one ET rate was measured. Meade and Kayyem point out that use of a semiclassical model (see eq 1) with  $\Delta G^0 = -0.7$  eV,  $\lambda = 0.9$  eV, and their measured ET rate allows calculation of the maximum ET rate ( $k_{ET}^{max}$ ) for this reaction,  $2.5 \times 10^6$  s<sup>-1</sup> (obtained when  $\Delta G^0 = -\lambda$ ). Interestingly this maximum rate is comparable to those in cytochrome *c* with histidines 33 or 39 modified by ruthenium substitution. There the maximum ET rates and edge-to-edge distances for histidines 33 and 39 (with  $\lambda = 0.8$  eV) are, respectively,  $3.3 \times 10^6$  s<sup>-1</sup> at 12.3 Å and  $2.7 \times 10^6$  s<sup>-1</sup> at 11.1 Å.<sup>56</sup> Thus  $k_{ET}^{max}$  along the length of a DNA duplex, even with its unique base stacking, is much the same as that found at comparable distances in protein systems, albeit ones with highly efficient ET processes. *This conclusion accords with reports that the presence of aromatic groups between donors and acceptors does not of necessity produce faster ET processes than would an entirely aliphatic intervening medium.*<sup>56,72</sup> A recent semi-empirical CNDO/S calculation of  $H_{AB}$  by Beratan *et al.*<sup>6,7</sup> for this same 8-base pair duplex combined with  $\lambda = 0.9$  eV in eqs 1 and 2 yields  $7.1 \times 10^6$  s<sup>-1</sup> for the  $k_{ET}^{max}$  rate and agrees very well with the experimentally determined maximum rate of  $2.5 \times 10^6$  s<sup>-1</sup>.

The only other reported study of long-range ET in ds DNA between selectively anchored D/A groups was performed by Barton and Turro *et al.*<sup>3</sup> In this work the excited-state donor, a ruthenium(II) complex, and the acceptor, a rhodium(III) complex, were each covalently bound to opposite ends of a 15-base pair DNA duplex. Each complex was also partially intercalated into the duplex. Because the tethers between the metal complexes and the DNA stands were 16 atoms long, there was  $\pm 1$  base pair ( $\pm 3.4$  Å) uncertainty about the location of each donor and the acceptor in this system. However, the smallest distance separating *the intercalated groups* on the two metals was 37.4 Å (11 base pairs), and the corresponding edge-to-edge distance of separation was 26 Å.<sup>71</sup> The basic result is that Ru(II)\* emission quenching is interpreted as signaling photoinduced ET from Ru(II) to Rh(III) at a rate  $\geq 3 \times 10^9$  s<sup>-1</sup> over a minimum edge-to-edge D/A distance of 26 Å with a driving force ( $\Delta G^0$ ) equal to -0.75 eV.<sup>3</sup> This result can be compared to that of Meade and Kayyem for a tethered Ru/Ru-DNA duplex. In this latter study the driving force is similar

(-0.7 eV), but the D/A distance is much shorter, only 12.5 Å. In spite of the appreciably smaller D/A separation in the Ru/Ru-DNA duplex, the observed ET rate is more than 1,900-fold slower,  $1.6 \times 10^6 \text{ s}^{-1}$ , than that found in the Ru/Rh-DNA duplex.

The ET rate for the tethered Ru/Rh-DNA duplex can be compared to rates found by Brun and Harriman for intercalated donors and acceptors. The driving force for the AOH<sup>+</sup>/DAP<sup>2+</sup> system is similar, -0.67 eV, and a comparable ET rate,  $4.3 \times 10^9 \text{ s}^{-1}$ , is found but at a D/A separation distance of 10.2 Å. For these two experiments to make sense,  $H_{AB}$  would have to remain constant as the D/A separation in a duplex increases from 10.2 to 26 Å. This violates the expectation of eq 3 (electron tunnelling through a one dimensional square barrier) that  $H_{AB}$  should decay exponentially with distance. It was earlier pointed out that in the Brun and Harriman experiments  $\beta$  for intercalated donors and acceptors in a DNA duplex had a decay value of  $0.9 \text{ Å}^{-1}$ . According to this value of  $\beta$ , the ET rate in the tethered Ru/Rh-DNA duplex (with 15.8 Å greater D/A separation) should be  $1.5 \times 10^6$ -fold smaller than is inferred from the rate of Ru(II)\* emission quenching. Assuming that photoinduced ET in the Ru/Rh-DNA duplex is nearly activationless, a  $\beta$ -value of  $\leq 0.31 \text{ Å}^{-1}$  is calculated using the semi-classical ET model and eq 3 with  $k_{ET}^{max}$  equal to  $10^{13} \text{ s}^{-1}$  and  $k_{ET} \geq 3 \times 10^9 \text{ s}^{-1}$  for a D/A separation of 26 Å. This value of  $\beta$  disagrees fundamentally with the one measured by Brun and Harriman. Also, it is dramatically smaller than  $\beta$ -values typically found in proteins,  $0.9\text{-}1.4 \text{ Å}^{-1}$ .<sup>41,55,56,62,63,72</sup>

Comparison of  $H_{AB}$  from semi-empirical CNDO/S calculations with the ET rate found in the Ru/Rh-DNA experiment depends in this case only to a minor extent on the choice of  $\lambda$ .

Barton and Turro *et al.* suggest 0.4 eV should be used.<sup>3</sup> Using this value of  $\lambda$ , the experimental free energy (-0.75 eV), and the calculated  $H_{AB}$  value in eqs 1 and 2, yields  $k_{ET}^{max} = 2.6 \times 10^2 \text{ s}^{-1}$  versus an experimental rate of  $\geq 3 \times 10^9 \text{ s}^{-1}$ .<sup>6,7</sup> *This discrepancy of  $10^7$  between the calculated and experimental ET rates demonstrates that important aspects of ET in this D/A-DNA system are not understood.*

None of the above studies of DNA-mediated ET between specifically positioned D/A groups directly involve the oxidation or reduction of DNA bases. Also the energy differences between their tunnelling energy (the average of the electronic energies of the D/A localized states in an activated complex) and the energies of the oxidized and reduced states of the DNA bridges are large (by one estimate, as large as several eV).<sup>6</sup> Thus enhanced electronic couplings and ET rates are not expected in these three studies.

However, a significant increase in electronic coupling and therefore of ET rate is predicted by theory as the tunnelling energy approaches the energies of the redox-active virtual states of the bridge.<sup>6-8</sup> Eq 2 shows that the corresponding increase in ET rate will be proportional to the square of the increase in electronic coupling. *The extension ET studies of covalently modified DNA that will be based on the results of this DOE research program will directly involve oxidation and reduction of DNA bases and of necessity therefore probe electronic coupling effects for small tunnelling gaps, i.e. small differences between the electronic energies of the activated complex and the redox-active states of a DNA bridge.*

**4.4 D/A Redox Data.** In recent years a number of key redox potentials have been better defined for DNA nucleosides in water.<sup>21-23,27,73-79</sup> This body of work shows that dG is the easiest nucleoside to oxidize and that dC and dT are the easiest nucleosides to reduce. dA is the least redox-active nucleoside except for inosine (dI), and dU reduces at the approximately same potential as dT. In synthetic oligomers and duplexes, dI substitution can test the redox-inertness of dA nucleotides. Table 1 lists the relevant reduction potentials of these 2'-deoxyribonucleosides in water as well as those for dimethylaniline (DMA), methyl viologen (MV<sup>2+</sup>), and pyrene in organic solvents such as dimethylformamide (DMF) and acetonitrile (MeCN).<sup>27,79-82</sup>



**Table 1. Reduction Potentials of Electron Donors and Acceptors.**

Oxidized/Reduced Couple	Reduction Potential, V (versus SCE)
pyrene <sup>+</sup> /pyrene	+1.28
dI <sup>+</sup> /dI	+1.16
dA <sup>+</sup> /dA	+1.08
dG <sup>+</sup> /dG	+0.83
DMA <sup>+</sup> /DMA	+0.76
MV <sup>2+</sup> /MV <sup>+</sup>	-0.70
dC/dC <sup>-</sup>	-1.45
dT/dT <sup>-</sup>	-1.45
pyrene/pyrene <sup>-</sup>	-2.09

The above electrochemical data can be combined with the energy of the first excited  $\pi,\pi^*$  state of pyrene,  $E_{0,0}(\text{pyrene}^*) = 3.25 \text{ eV}$ , to estimate free energies for excited-state electron-transfer quenching of pyrene\* within pyrene-labeled DNA molecules according to eq 4.<sup>83,84</sup>

$$\Delta G^0 = e[E^0(\text{D}^+/\text{D}) - E^0(\text{A}/\text{A}^{\cdot-})] - E_{0,0}(\text{pyrene}^*) + w(r) \quad (4)$$

where  $E^0$  is a reduction potential, D is an electron donor, A is an electron acceptor, and  $w(r)$  is a coulombic interaction term between oxidized donor and reduced acceptor which represents free energy due to separating the products a distance  $r$  relative to each other,  $w(\infty) = 0$ .<sup>12,22,53</sup>

### 5.0 New Results From Dr. Netzel's DOE Sponsored Research Program.

*This research project laid the groundwork for studying ET reactions within DNA duplexes by examining the photophysics of uridine nucleosides which are covalently labeled at the 5-position with 1-pyrenyl chromophores.* Pyrenyl-uridine analogs with well defined direct attachment to the 5-position of uridine had not been reported previously requiring the development of an appropriate synthetic methodology. Previous methods resulted in a mixture of pyrene regioisomers,<sup>85</sup> which were not suitable for unambiguous photophysical studies. Photoredox-active labels attached directly to uridine are most desirable because they have a limited number of conformations making both NMR and molecular modeling studies more tractable.<sup>86-91</sup>

Few studies of covalently substituted bases in well defined nucleic acid environments have been published. In contrast, there are many studies of the photophysical properties of polyaromatic hydrocarbon/DNA and other chromophore/nucleic acid complexes. However, these latter studies are of systems which contain a large number of chromophore microenvironments and thus are difficult to interpret. This work begins to establish the photophysical and photochemical properties of some well defined chromophore/nucleic acid complexes.

**5.1 Photophysics of Pyrene-Uridine Nucleosides.** Pyrene-uridine conjugates have been used previously because pyrene is a stable chromophore with a reasonably long fluorescence lifetime (*ca.* 200-400 ns) depending upon the type of substitution and solvent.<sup>92-96</sup> Its long emission lifetime is a consequence of the fact that  $\pi,\pi^*$  absorption to its lowest-energy electronic excited state ( $S_1$ ) is spin-allowed but orbitally forbidden.<sup>95,97</sup> Since it is conveniently derivatized at the 1-position, it has frequently been employed as a fluorescent label especially in biological studies.<sup>24,25,28,29,93,96,98-102</sup> It is also true that pyrene can be reversibly oxidized and reduced in both its ground and lowest-energy excited states.<sup>27,103-110</sup> Recently its photophysics has been extensively studied as a carcinogenic and mutagenic benzo[*a*]pyrenediol epoxide (BPDE) derivative bound to the exocyclic amino group of dG nucleosides in native DNA.<sup>24,25,27,29,105,111</sup> In polar organic solvents dG

quenches the emission of photoexcited BPDE and yields pyrenyl radical anions. However in the same solvents, the covalent adduct of BPDE and dG does not show radical products on time scales  $>10$  ns, but it does show enhanced triplet formation most likely as a result of intramolecular ET excited-state quenching followed by rapid back ET. A recent picosecond kinetics study was the first to provide unambiguous evidence of ET between photoexcited pyrene (pyrene\*) and a covalently attached nucleic acid base.<sup>105</sup> However, in general it is possible for other processes to be important in the excited state deactivation of pyrene\* by nucleic acid bases.

From a different vantage point, studies of radiation damage in biological systems are concerned with the identity of products of DNA oxidation and reduction, their rates of formation and decay, and their states of protonation.<sup>78,112-124</sup> While numerous studies of DNA bases, nucleosides, and monophosphate nucleotides have been conducted, it is generally difficult to investigate oxidation and reduction processes in DNA oligomers and duplexes at specific base sites.

The above discussed excited state and redox characteristics of pyrene and uridine imply that following photoexcitation pyrene\* can directly inject an electron onto uridine. The fate of the reduced uracil radical anion in both oligomers and duplexes with a variety of nearest neighbor bases may therefore be able to be examined. In related work in this research program (see below), we have shown that dC nucleotides are better ET quenchers of pyrene\* emission than are dT nucleotides.<sup>21</sup> This nucleotide reactivity ranking supports conclusions drawn from gamma-irradiation studies of DNA.<sup>125</sup> In this latter work, initially formed dT<sup>-</sup> was shown upon warming to form the reversibly protonated ET product, dC(N3)H. *These results imply that it is possible for a reduced uridine (or thymidine) to transfer an electron to a nearby cytidine nucleotide. How effectively such a secondary, internucleotide ET can compete in oligomers or in duplexes with charge recombination within a covalently labeled uridine nucleoside is a significant question that extension studies will address.*<sup>3,8,75,113,119,126-130</sup>

One paper<sup>22</sup> published as a result of this research contract reports syntheses, electronic absorbance and emission spectra, and emission kinetics results for two types of pyrene-substituted uridine nucleosides as part of ongoing work which is examining the photophysical and photochemical behaviors of these same nucleosides embedded in DNA oligomers and duplexes. The two labels are 1-pyrenyl itself and 1-carboxypyrenyl (1-pyrenoyl) which are each joined directly to the 5-position of 2'-deoxyuridine (dU). These direct attachments significantly restrict the range of conformations available to the pyrene label when it is attached to a DNA oligomer or duplex.  $\pi,\pi^*$  emission is absent for 5-(1-pyrenyl)-dU, **1**, in methanol (MeOH) but present in tetrahydrofuran (THF). For continuous excitation of **1** in MeOH, broad charge-transfer (CT) emission is present with a maximum at 470 nm and a quantum yield of 0.027; for **1** in THF,  $\pi,\pi^*$  emission is present with a maximum at 395 nm and quantum yield of 0.42. *Thermodynamic considerations suggest that the CT photoproduct of **1**, which emits in MeOH, is pyrene<sup>-</sup>/dU<sup>+</sup>.* The emission kinetics of **1** in MeOH are triexponential, but the wavelength variation of the relative amplitudes of the different decay lifetimes indicates that the CT-state relaxations are biexponential with lifetimes of  $\leq 50$  ps and 0.9 ns. Similarly, the  $\pi,\pi^*$  state of **1** in MeOH (observed only in time resolved experiments) also has two ET quenching lifetimes of  $\leq 50$  ps and 2-3 ns. The continuous-excitation emission spectrum of **1** in MeOH shows that emission from the few long-lived  $\pi,\pi^*$  states is dominated at all wavelengths by the 0.9-ns lived CT emission.

In contrast, only  $\pi,\pi^*$  emission is observed for 5-(1-pyrenoyl)-dU, **2**, in both MeOH and THF with emission quantum yields of 0.002 and 0.028, respectively. The emission kinetics for **2** in THF are at least quadruply exponential having a longest emission lifetime of ca. 95-ns. However, only about 10% of the relative emission amplitude decays with lifetimes >10 ns. Approximately 90% of the emission amplitude decays on the same time scale as for **2** in MeOH ( $\leq 10$  ns). *The emission kinetics data as a function of wavelength for nucleosides 1 and 2 support the conclusion that each has multiple conformers in solution.* Additionally, the  $\pi,\pi^*$  ET-quenching times for a single pyrene-labeled nucleoside vary from  $\leq 30$  ps (for **2** in MeOH) to  $\geq 95$  ns (for **2** in THF where subnanosecond quenching times are also present), a 3000-fold difference. For **1** in MeOH,  $\pi,\pi^*$  ET-quenching times vary from  $\leq 50$  ps to 3 ns, 60-fold, and the charge recombination times vary from  $\leq 50$  ps to 0.9 ns, 20-fold. *These results suggest that the relative orientation of the pyrene and uridine  $\pi$ -systems (and thus their degree of electronic coupling) play crucial roles in determining the rates both of ET quenching of pyrene\* and of charge-recombination within the photoproduct formed from this quenching.*

### 5.3 Direct Observation of Photoinduced ET in Pyrene-Uridine

**Nucleosides.** A follow-up paper<sup>23</sup> also published as a result of this research contract examines the ET photophysics of the same two pyrene-labeled nucleosides in several different solvents with both transient absorbance and emission spectroscopies. For **2** in MeOH, a maximal change-in-absorbance ( $\Delta A$ ) increase at 460 nm characteristic of pyrene<sup>+</sup> occurs during the time of photoexcitation ( $\leq 30$  ps). (A control experiment insures that pyrene<sup>+</sup> is formed from the singlet excited state of pyrene and not as result of multiphoton ionization of pyrene by the laser pulse.) The pyrene<sup>+</sup> signal decays in 20-70 ps slightly more slowly than the  $S_1$  state's positive  $\Delta A$  signal at 510 nm. *These results prove that the  $S_1$  state of pyrene is quenched due to intramolecular ET.* Similar results are also obtained for **2** in MeCN where the pyrene<sup>+</sup> absorbance at 460 nm decays in ca. 100 ps.

For **1** and **2** changing solvent from MeOH to MeCN increases both emission lifetimes and quantum yields. For **1** the emission yield increases 13-fold, while for **2** it increases 3-fold. Since the dielectric constants of MeOH and MeCN are similar, respectively, 33.6 and 37.5, the large emission yield increase and accompanying striking change in the emission spectrum for **1** on switching from MeOH to MeCN is consistent with lessened ET quenching due to raising the free energy of the pyrene<sup>+</sup>/dU<sup>-</sup> CT product relative to the  $\pi,\pi^*$  state of pyrene. Since dU(H)<sup>+</sup> should require less energy to form than dU<sup>-</sup>, ET quenching should be more favorable in MeOH where dU(H)<sup>+</sup> can be formed than in MeCN where only dU<sup>-</sup> can be produced. *If this model is correct, the time of protonation of dU<sup>-</sup> in MeOH is  $\leq 30$  ps based on transient absorbance measurements of the appearance of pyrene<sup>+</sup> for **2**.*

### 5.4 Relative ET Quenching Efficiencies of dA, dG, dC, and dT

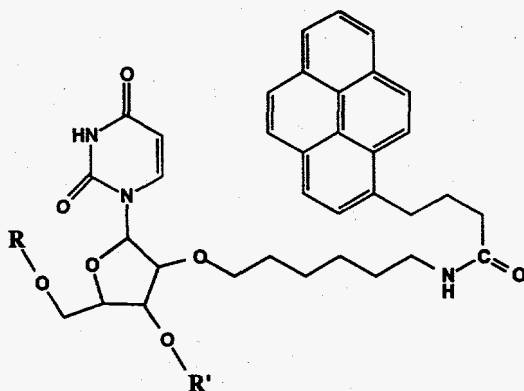
**Nucleosides Toward Pyrene\*.** In general it is possible for a number of processes, including but not limited to ET quenching, to be important in the excited state deactivation of pyrene\* by nucleic acid bases. To gain insight into broader aspects of such quenching processes, Geacintov *et. al.*<sup>27</sup> added six 2'-deoxyribonucleosides individually to solutions of photoexcited tetrahydroxytetrahydrobenzo[*a*]pyrene (BPT) and their static and dynamic quenching properties were measured. dG, dT, dC, and dU were all found to be strong dynamic quenchers of pyrene\* emission, but their quenching rates were so close to the diffusion controlled limit that there was no dependence of ET quenching rate upon the free energy for the reaction as would be expected if ET quenching were occurring.<sup>10,46,51,131-134</sup> In contrast, dA and dI were weak dynamic quenchers of pyrene\* emission. This latter result agrees with  $\Delta G^0$  estimates for these reactions as being less favorable than for the more reactive nucleosides.<sup>27,75,77,78</sup> Importantly, a recent nanosecond time scale transient



absorbance study has shown that BPT<sup>++</sup> radical cations are formed with very small quantum yields when pyrene\* is quenched in 0.1 M aqueous dC and dT solutions.<sup>135</sup>

A number of previous studies involved pyrene linked covalently to oligodeoxyribonucleotides and to oligoribonucleotides.<sup>19,30,101,136-140</sup> In general these studies found that the amount of pyrene emission from the linked assemblies was very sensitive to the environment surrounding the pyrene label. One such study concluded that 5'-linked pyrene labels were ideal for probing the binding and dynamics of RNA substrates.<sup>19</sup> A frequent finding in these studies was that the pyrene labeled duplexes emitted more strongly than did the corresponding labeled oligomers. One interesting study constructed a tripartite duplex with short 3' and 5' end-labeled oligomers, both complexed to a common 30-nucleotide-long complimentary strand such that the two pyrene labels could contact each other in the middle of the duplex. However, pyrene-excimer emission was not seen.<sup>139</sup> In contrast, pyrene-excimer emission has been observed in DNA duplexes with multiple covalent adducts of BPDE.<sup>24,141-143</sup>

In spite of a significant number of studies involving the quenching of pyrene\* emission by nucleic acid bases, nucleosides, and nucleotides, a clear picture of relative reactivity of the different nucleic acids had not emerged.<sup>24,25,27,98,104,105,143-149</sup> Similarly, a number of oligomers and duplexes had been constructed with covalently attached pyrene labels, yet little systematic work had been done comparing how flanking bases affect the emission properties of these pyrene labels.<sup>19,20,24,25,30,101,136-139,150</sup> Finally, a number of labeling studies relied solely on continuous emission measurements and did not time-resolve the pyrene label's emission kinetics.



U(12)\*OH (3): R=H, R'=H

U(12)\*ODMT (4): R=dimethoxytrityl (DMT), R'=H

U(12)\*-oligomer: R=R'=phosphodiester link

**Figure 1.** Structural drawing of two pyrene-labeled nucleosides, 3 and 4, and of a pyrene-labeled uridine positioned at an internal substitution site, U(12)\*, in a polynucleotide strand.

In another paper published as a result of this research contract,<sup>21</sup> we report both continuous and time-resolved emission properties of pyrene labels covalently attached to three types of nucleic acid systems. The first type consists of simple ribonucleosides with uridine joined at the 2'-oxygen to a 1-pyrene propyl carbonyl label, U(12)\*OH (3) and U(12)\*ODMT (4) (see Figure 1). The second type consists of a set of four pentameric oligonucleotides with a central U(12)\*-nucleotide flanked symmetrically by pairs of nucleotides, dX<sub>2</sub>U(12)\*dX<sub>2</sub>, where dX is dA, dG, dT, or dC. The third type consists of complementary duplexes each

of which is 18 base pairs (bp) long and incorporates the above symmetrical pentamers as central units with flanking dA nucleotides, 5'-...dAdX<sub>2</sub>U(12)\*dX<sub>2</sub>dA...-3'. The other 11 nucleotides in each strand are either dA or dT, added to produce duplexes with sufficiently high melting temperatures (T<sub>m</sub>) that ca. 99% of the oligomers would be double stranded at room temperature.<sup>151,152</sup>

The goals of this work are two fold. *First, we want to establish a clearer picture of the relative reactivities of the various DNA bases toward ET quenching of pyrene\* emission. Second, we want to establish a better understanding of the differences in ET quenching efficiencies of pyrene\* labels in DNA oligomers versus duplexes. This information will facilitate rational design of pyrene labeled oligonucleotide systems.* For example, one will be better able to design either longer-lived or very much shorter-lived pyrene\* labels based on the type of nucleoside attachment site and DNA or RNA base sequence surrounding the label. Also, it may be possible to design systems in which there is enhanced differential emission between ss and ds conformations.<sup>19,20</sup> Finally, tuning pyrene\*-label ET emission-quenching can maximize the production of either oxidized or reduced nucleotide bases. Such specifically produced oligonucleotide ionizations will provide starting points for studies of electron motion in DNA oligomers and duplexes<sup>1-3,8,75,118,126-129,153,154</sup> and of the mechanisms of radiation damage in DNA and RNA.<sup>75,116,122,123,155-157</sup>

This paper reports both continuous and time-resolved spectroscopic studies of the emission properties of photoexcited pyrene labels covalently attached to uridine nucleosides and oligonucleotides. The 400-nm emission kinetics for the four U(12)\*-labeled pentamers establish the following order of pyrene\*-quenching reactivities by flanking DNA bases: dA < dG < dT < dC. This ordering of pyrene\*-quenching reactivities is generally consistent with estimates of the free energies of pyrene\*-quenching by ET to or from DNA bases. In the case of dG nucleosides, pyrene\* is expected to be reduced; in the case of dT, dU, and dC nucleosides, pyrene\* is expected to be oxidized. The shortened emission lifetimes in MeOH for the U(12)\*OH (3) and U(12)\*ODMT (4) nucleosides (30-40 ns) compared to that found for PBA in the same solvent (230 ns) are also consistent with ET quenching of pyrene\* by the covalently attached uridine to form pyrene\*/U(12)<sup>-</sup>. *Finally, emission spectra and lifetimes in the 495-nm region for both U(12)\*-labeled pentamers and duplexes provide direct evidence for the formation and decay of the pyrene\*/U(12)<sup>-</sup> CT product.* In general only ca. 20% of this CT-emission decays in the 1-7 ns time range with ca. 70-80% of it decaying in ≤0.2 ns.

*Emission kinetics results for U(12)\*-labeled duplexes show that pyrene\*-quenching by pairs of flanking dC and dT nucleotides is equally effective both with respect to type of nucleotide and with respect to whether these nucleotides are located on the same-strand as the U(12)\*-label or on the opposite strand. Additionally, the longest π,π\* emission-lifetimes in duplexes exceed those in the corresponding pentamers.* This likely reflects restricted access by pyrene\* to the base-paired nucleotides in DNA duplexes compared to access to them in ss oligomers. *A measure of duplex-induced restricted access to bases in ds versus ss DNA can be obtained by noting that the average π,π\* emission-lifetimes (>1-ns lifetime components) lengthen 3-fold on going from the dT<sub>2</sub>U(12)\*dT<sub>2</sub> pentamer to the corresponding 18-bp U(12)\* duplex and 9-fold on going from the dC<sub>2</sub>U(12)\*dC<sub>2</sub> pentamer to the corresponding 18-bp U(12)\* duplex.*

The dC<sub>2</sub>U(12)\*dC<sub>2</sub> pentamer has a uniquely short emission decay with its longest emission-lifetime component lasting only 5.6 ns. Also, the average emission lifetimes for the dC<sub>2</sub>U(12)\*dC<sub>2</sub> and dT<sub>2</sub>U(12)\*dT<sub>2</sub> pentamers are respectively, 1.9 and 5.8 ns. These data show that flanking dC-nucleotides are more reactive toward pyrene\* quenching than are flanking T-nucleotides. This result is surprising because the free energies of ET quenching for both dC and dT nucleotides are similar, ca. -0.52 eV. However, it does not

conflict with this fact, because other factors such as the sizes of the corresponding nuclear reorganization energies or the details of how proton transfer processes may be coupled to ET quenching in these two cases could account for the modestly higher quenching rate of flanking-dC over flanking-dT nucleotides.

**5.5 Summary of Recent Results on ET Quenching of Photoexcited Pyrene in Covalently Labeled Nucleosides.** Recent work by Geacintov *et al.* and in this research program and has confirmed the expected rapid ET quenching of pyrene\* by dG and dU nucleosides with covalently attached pyrene labels.<sup>21-23,27,103,105,135,149,158</sup> Following picosecond laser excitation in the first case, pyrene<sup>-</sup> was identified and in the second pyrene<sup>+</sup> was observed. Additionally, this project's study of pyrene-labeled DNA pentamers established that dG nucleosides are less effective pyrene\* quenchers than either dC or dT nucleosides as predicted by the above presented ET quenching free energy estimates.<sup>21</sup> *A surprising result of this same work is that dC nucleosides are ca. four times better quenchers of pyrene\* than are dT nucleosides. Thus it is likely that ET from dT (or dU) to a nearby dC nucleoside is favorable.* Extension experiments based on the results of this DOE sponsored program will test this possibility.

**5.6 Photophysical Properties of Monomeric and Bichromophoric DNA Stains.** In keeping with the second of Dr. Netzel's long terms goals, to construct oligonucleotide probes for real-time monitoring of intracellular processes involving DNA and RNA such as *m*-RNA expression and translocation as well as with his long time interests in DNA ET chemistry, another paper resulting from this project reports the results of studying the photophysical properties of monomeric and bichromophoric cyanine dyes bound to duplex DNA.<sup>159</sup>

Cyanine dyes have a rich history in the photographic industry<sup>160</sup> and have recently become important in the staining of biological samples. A particularly important application in the latter area involves the staining of duplex DNA.<sup>161</sup> Cationic cyanine dyes exhibit very large degrees of fluorescence enhancement on binding to nucleic acids.<sup>162,163</sup> In addition covalent linkage of two cyanine dyes to form a bichromophore increases the nucleic acid binding affinity by approximately two orders of magnitude, as previously found in the case of phenanthridium dyes (ethidium bromide).<sup>164</sup> These characteristics of fluorescence enhancement and high binding affinity are crucial for high sensitivity nucleic acid detection applications.<sup>165-168</sup> *In this paper we investigate the mechanisms underlying the fluorescence enhancement, addressing the questions of whether these processes are sensitive to the base content of the nucleic acid target and whether the photophysical properties of cyanine nucleic acid stains are significantly modified upon coupling to form bichromophores in order to obtain higher binding affinity.*<sup>159</sup>

In parallel with the development of biological applications of cyanine dyes,<sup>162,163,165-172</sup> numerous physical chemical investigations have been carried out to understand the nonradiative decay mechanisms which control the excited state lifetimes of these dyes.<sup>173-181</sup> The generally accepted view is that the nonradiative decay of photoexcited cyanine dyes is controlled by the rate of rotation or torsion about the central methine bridge which links the heterocycles at either end.<sup>174,177,181</sup> Kemnitz *et al.*<sup>182</sup> studied the fluorescence lifetimes of monomeric 5,5'-dichloro-3,3'-disulfopropyl-9-ethylthiacarbocyanine in methanol and adsorbed on SiO<sub>2</sub> and quartz. Presumably because of restricted motion for the adsorbed carbocyanine dye molecules, the 70-ps fluorescence lifetime of this dye in methanol lengthened 40-fold when adsorbed on silica and quartz. Serpone *et al.*<sup>183</sup> studied the photophysics of two dithiacarbocyanine dyes in dichloromethane and found that while both intersystem crossing and internal conversion occur at comparable rates, torsional motion of the polymethine chain is prerequisite to both processes. Additionally, there is evidence that the rate of isomerization of cyanine dyes increases with decreasing length of the central

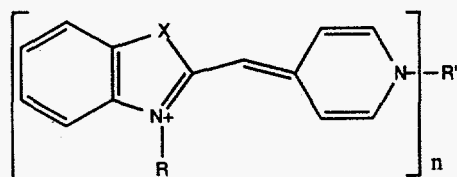
polymethine chain.<sup>184</sup> A particularly important study in this area showed the rate of radiationless decay rate to be inversely proportional to the solvent's viscosity.<sup>174</sup> However, more recently Sauerwein *et. al.*<sup>173</sup> demonstrated a very strong correlation of nonradiative lifetime with the molecular weight of the solvent in four solvent series. In this work solvent viscosity was not sufficient to account for the dynamics of cyanine dye torsion among the large number of solvents surveyed. Rather, solvents of high molecular weight transferred less rotational momentum per collision to the cyanine dyes than solvents of low molecular weight and thus less readily deactivated their excited states. Finally, there does not appear to be an intrinsic electronic barrier to torsion about the central methine bridge in the lowest energy, singlet excited state of these dyes.<sup>173,180</sup>

This paper reports fluorescence quantum yield and fluorescence lifetime measurements on ten different cyanine dyes each complexed to calf thymus DNA (CT-DNA) and to (dAdT)<sub>10</sub> and (dGdC)<sub>6</sub> self-complementary duplexes. Applications of these dyes to stain ds DNA had found both no dependence of emission enhancement upon DNA duplex base content<sup>163,166-168,170</sup> and moderate dependence on DNA base content.<sup>172,185</sup> To the extent the emission enhancements are independent of DNA base content, they can be used quantitatively to measure total ds DNA content in analytical protocols.

The monomeric dicationic and bichromophoric quadruply-cationic cyanine dyes bind to ds DNA more strongly than monocationic cyanines such as thiazole orange (TO).<sup>162,164,167,170</sup> Order of magnitude estimates for the dyes studied here of the ds DNA binding constants for the monomeric and bichromophoric forms, respectively, are  $10^6 \text{ M}^{-1}$  and  $10^8 \text{ M}^{-1}$ .<sup>186</sup> Lee *et. al.*<sup>162</sup> postulated that the fluorescence enhancement upon nucleic acid binding of dyes such as TO (which exhibits approximately 3000-fold enhancement on RNA) is due to a change in the relative orientation of the benzothiazole and quinolinium rings from skewed to coplanar. TO is nearly identical to the dye TO-PRO-1 studied here with the difference that R' is a methyl group for TO (see Figure 2). A recent study by Carlsson *et. al.*<sup>187</sup> agrees with these ideas and shows for YO-PRO-1 in glycerol that the decrease in emission quantum yield with increasing temperature has a very similar activation energy, 53 kJ/mol, to the activation energy for viscous flow, 63 kJ/mol. Because of this result, they conclude that the increase in emission quantum yield for YO-PRO-1 upon binding to ds DNA is due to restricted rotation about the benzoxazole/quinoline bridge in the bound state relative to the free state. These authors also make the important point that there is only one electronic transition in the low-energy  $S_0 \rightarrow S_1$  absorbance region for YO-PRO-1 bound to ds DNA.

In keeping with this, the radiative (or natural) lifetime ( $\tau_{\text{rad}}$ ) calculated from the absorbance spectrum, 5 ns, agrees well with the value obtained from emission lifetime ( $\tau_0$ ) and quantum yield ( $\Phi_{\text{em}}$ ) measurements, 6 ns.<sup>187</sup> Each of the other nine dyes studied here is structurally similar to YO-PRO-1, and therefore each of them is also likely to have only one low-energy electronic transition.

## Monomethine Cyanine Dyes



POPO-1: X = O, R = Me, n = 2,

R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3-$

PO-PRO-1: X = O, R = Me, n = 1, R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2-$

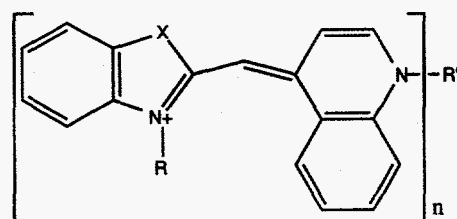
Analog 1: X = O, R = Et, n = 1, R' = Et

BOBO-1: X = S, R = Me, n = 2,

R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3-$

BO-PRO-1: X = S, R = Me, n = 1, R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2-$

Analog 2: X = S, R = Et, n = 1, R' = Et



YOYO-1: X = O, R = Me, n = 2,

R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3-$

YO-PRO-1: X = O, R = Me, n = 1, R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2-$

Analog 3: X = O, R = Et, n = 1, R' = Et

TOTO-1: X = S, R = Me, n = 2,

R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2(\text{CH}_2)_3-$

TO-PRO-1: X = S, R = Me, n = 1, R' =  $-(\text{CH}_2)_3\text{N}^+(\text{CH}_3)_2-$

Analog 4: X = S, R = Et, n = 1, R' = Et

**Figure 2.** Structural drawing of four monomethine cyanine dyes as (1) dicationic monomers, (2) quadruply cationic bichromophores, and (3) monocationic analogs of the DNA-staining dyes.

*A priori* two processes come to mind which could cause base content to produce differential emission enhancements for cyanine dyes complexed to ds DNA. The first is that dAdT-sequences could in principle produce different types of binding sites than dGdC-sequences. If one type of site provided greater immobilization toward torsion about the central methine bridge, it could thereby produce a greater emission enhancement. The second process which could differentially alter emission enhancements for cyanine dyes bound to DNA duplexes is excited-state ET quenching by DNA nucleosides.<sup>182,188</sup> As discussed above guanosine is the easiest nucleoside to oxidize, while thymidine and cytidine are the easiest nucleosides to reduce. Adenosine is both ca. 100 mV harder to oxidize than guanosine and the most difficult nucleoside to reduce.<sup>75-77</sup> This study also reports electrochemical measurements of the oxidation and reduction potentials of six cyanine dyes which model all ten of the DNA stains studied in this program. These redox potentials are used to estimate



the free energy for both reductive dye\* quenching by dG,  $\Delta G^0$  (dG<sup>+</sup>/dye<sup>\*</sup>), and oxidative dye\* quenching by dT and dC,  $\Delta G^0$ (dye<sup>\*</sup>/dT<sup>\*</sup>) and  $\Delta G^0$  (dye<sup>\*</sup>/dC<sup>\*</sup>), respectively.

This work compares  $\Phi_{em}$ ,  $\tau_0$ , and  $\tau_{rad}$  data for ten different cyanine dyes in the presence of three different types of ds DNA and  $\Phi_{em}$  data for the same dyes in the absence of ds DNA to learn some of the answers to the following questions. (1) What kinds of emission decay kinetics are present for these different dyes on different types of ds DNA? (2) How do the emission enhancements (or emission quantum yields) vary for the different dyes on different types of ds DNA? (3) Is there evidence that any of the DNA-bound dyes have their emission lifetimes shortened due to ET quenching by DNA bases? (4) Does changing from one dye to another when studying ds DNA mean that very different emission characteristics and binding modes will occur for the different dyes? (5) Are there important differences in the emission properties of these dyes when they are bound to long segments of ds DNA, as found for samples of CT-DNA, versus when they are bound to much shorter duplexes such as (dAdT)<sub>10</sub> and (dGdC)<sub>6</sub>? (6) Are there major differences between monomeric and bichromophoric forms of these dyes when they are bound to different types of ds DNA? (7) How invariant is  $\tau_{rad}$  among these different dyes? (8) What free (or unbound) dye lifetimes are consistent with the measured emission enhancements and calculated  $\tau_{rad}$  values?

All of the ten monomeric and bichromophoric cyanine dyes investigated under this contract exhibit either bi- or triexponential emission decay kinetics reflecting different dye/ds DNA modes of binding. Complicated emission decay patterns are reasonable for these dye/ds DNA complexes, because of the simple excited-state lifetime control mechanism operative in cyanine dyes. In particular, restriction of torsion about the central methine bridge in these dyes produces both longer excited state lifetimes and increased emission quantum yields.<sup>174,183,187</sup> Thus it is likely that cyanine dyes can bind to DNA duplexes in a variety of ways so as to produce complexes with a variety of degrees of torsional restriction. *In keeping with this interpretation, the average radiative lifetime for 16 bichromophore/ds DNA systems is measured to be  $5.1 \pm 0.8$  ns (99% confidence level) which agrees very well with previously reported radiative lifetimes for a monomeric cyanine dye, YO-PRO-1, bound to CT-DNA: 5 ns (theoretical) and 6 ns (experimental).<sup>187</sup> Also in agreement with these results is the average radiative lifetime of 5.6 ns found in this study for the monomeric cyanine dyes bound to CT-DNA duplexes. Assuming that free monomeric dyes have the same radiative lifetimes as when they are bound to ds DNA, the emission enhancements measured here imply that free monomeric dyes have emission lifetimes of 1-5 ps in aqueous buffer solutions. At the other extreme, the longest emission lifetimes for YOYO-1 and YO-PRO-1, which are known to intercalate into CT-DNA duplexes,<sup>189</sup> are found to be 3-5 ns in this work. *These data and observations demonstrate that a wide range of emission lifetimes (from picoseconds to nanoseconds) is possible for cyanine dye/ds DNA complexes consistent with varying degrees of restricted torsion about their central methine bridge.**

Although binding-induced torsional restriction is responsible for the large emission enhancements of these dyes (up to 1800-fold in this work), it is also possible that there could be competing ET quenching processes. In this case, significant base-content sensitivity of the emission enhancements might be produced. *Careful scrutiny of the lengths of average emission lifetime for ten cyanine dyes on (dAdT)<sub>10</sub> and (dGdC)<sub>6</sub> duplexes finds that they do not vary as expected if ET emission quenching were an important excited state deactivation process.* Predictions of the relative rates of ET quenching of excited dye emission by the four DNA nucleosides are based on estimates of

the free energy of such reactions using redox data for cyanine dye analogs of the DNA-staining dyes.

The pattern of longer average emission lifetimes for oxazole dyes than for thiazole dyes for both monomeric and bichromophoric forms is reflected in oxazole dyes having a greater emission quantum yields than thiazole dyes with no exceptions on all three kinds of ds DNA. *In spite of the lower emission yields of the thiazole dyes, however, they always produce larger emission enhancements on CT-DNA.* These observations suggest that the thiazole to oxazole switch (which is structurally minor, one atom in monomers and two atoms in bichromophores) changes fundamental photophysical properties of the dye, but is benign in terms of the different types of ds DNA. Diagnosics users of these dyes can thus choose an oxazole if they need maximum fluorescence from a DNA stain or a thiazole if they require minimum background emission from unbound stain.

There are also differences in emission quantum yield between the pyridinium and quinolinium dyes when bound to (dAdT)<sub>10</sub> and (dGdC)<sub>6</sub> duplexes. These differences are very distinct for the monomeric dyes where pyridinium dyes have 4-fold greater emission yields on (dAdT)<sub>10</sub> duplexes and quinolinium dyes have 2-fold greater emission yields on (dGdC)<sub>6</sub> duplexes. A 2-fold quantum yield increase on switching from (dAdT)<sub>10</sub> to (dGdC)<sub>6</sub> duplexes is also present for the quinolinium bichromophore, TOTO-1. Very importantly, for this bichromophore and the four monomers the emission quantum yield on CT-DNA matches very well the higher of the short duplex quantum yields. *This suggests, but does not prove, that the pyridinium and quinolinium cyanine dyes selectively associate with AT- and GC-rich regions, respectively, when bound to CT-DNA.*

For the other five bichromophores studied, there is little difference in emission quantum yield between the two types of short duplexes. Additionally, the emission yields of these bichromophores when bound to CT-DNA do not clearly correspond to either of the short duplex emission yields. Thus, for these dyes there is no clear evidence that they exhibit base-content selectivity with respect to emission yield.

*Base-content binding selectivity and hence emission yield selectivity for the monomeric dyes and the general absence of base-content emission yield selectivity for the bichromophoric dyes is consistent with a lower ds DNA association constant ( $\sim 10^6 M^{-1}$ ) for the monomers than for the bichromophores ( $\sim 10^8 M^{-1}$ ).<sup>186</sup> Because of their reduced binding strengths, monomers are more likely to be more sensitive to the structure of ds DNA than bichromophores. Surrounding media might also affect the binding selectivity of cyanine dyes. In particular, high salt conditions weaken the binding of cationic dyes to ds DNA and thus might enhance their base-content selectivities for binding and emission yield. This is mostly likely to occur for cyanine monomers. In contrast, low salt conditions strengthen the binding of cationic dyes to ds DNA and thus might further reduce the already low level of base-content emission yield selectivity found here for the bichromophoric dyes.*

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## 7.0 Lists of Scientific Papers, Meeting Abstracts, and Research Seminars.

### 7.1 Papers Published.

1. T.L. Netzel Technical Progress Report, Year 1, Contract ID No. DE-FG05-93ER61604, United States Department of Energy, Project Title: "An Exploration of Sequence Specific DNA-duplex/Pyrene Interactions for Intercalated and Surface-Associated Pyrene Species." Period: 5/15/93 to 04/30/94.
2. T.L. Netzel Technical Progress Report, Year 2, Contract ID No. DE-FG05-93ER61604, United States Department of Energy, Project Title: "An Exploration of Sequence Specific DNA-duplex/Pyrene Interactions for Intercalated and Surface-Associated Pyrene Species." Period: 5/01/94 to 04/30/95.
3. An Exploration of Sequence Specific DNA-Duplex/Pyrene Interactions for Intercalated and Surface-Associated Pyrene Species. T.L. Netzel, DOE/ER-0645 Summaries of Fiscal Year 1994 Projects in Medical Applications and Biophysical Research, U. S. Dept. of Energy, Office of Health and Environmental Research, Med. Applications and Biophys. Res. Div., April, 31-32 (1995). Avail. U. S. Dept. of Commerce, Technology Admin., Nat. Technical Infor. Ser., Springfield, VA.

4. Direct Observation of Ultrafast C-C Bond Fragmentation in a Diamine Radical Cation. L.A. Lucia, Y. Wang, K. Nafisi, T.L. Netzel, K.S. Schanze, *J. Phys. Chem.* **99**, 11801-11804 (1995).
5. Early-Time Dynamics and Reactivity of Polyoxometalate Excited States. Identification of a Short-Lived LMCT Excited State and a Reactive Long-Lived Charge Transfer Intermediate Following Picosecond Flash Excitation of  $[W_{10}O_{32}]^{4-}$  in Acetonitrile. D.C. Duncan, T.L. Netzel, and C.L. Hill, *Inorg. Chem.* **34**, 4640-4646 (1995).
6. Photophysics of 2'-Deoxyuridine (dU) Nucleosides Covalently Substituted with Either 1-Pyrenyl or 1-Pyrenoyl: Observation of Pyrene-to-Nucleoside Charge-Transfer Emission in 5-(1-Pyrenyl)-dU. T.L. Netzel, M. Zhao, K. Nafisi, J. Headrick, M.S. Sigman, and B.E. Eaton, *J. Am. Chem. Soc.* **117**, 9119-9128 (1995).
7. Measuring Electron Transfer in DNA. N.B. Thornton, D.W. Dixon, and T.L. Netzel, *Inter-Amer. Photochem. Soc. Newslett.* **18**, 14-19 (1995).
8. Base-Content Dependence of Emission Enhancements, Quantum Yields, and Lifetimes for Cyanine Dyes Complexed to Double-Strand DNA: Photophysical Properties of Monomeric and Bichromophoric Types of DNA Stains. T.L. Netzel, K. Nafisi, M. Zhao, J.R. Lenhard, and I. Johnson, *J. Phys. Chem.* **99**, 17936-17947 (1995).
9. Base-Sequence Dependence of Emission Lifetimes for DNA Oligomers and Duplexes Covalently Labeled with Pyrene: Relative Electron-Transfer Quenching Efficiencies of A, G, C, and T Nucleosides Toward Pyrene\*. M. Manoharan, K. Tivel, M. Zhao, K. Nafisi, and T.L. Netzel, *J. Phys. Chem.* **99**, 17461-17472 (1995).
10. Direct Observation of Photoinduced Electron Transfer in Pyrene-Labeled dU Nucleosides and Evidence for Protonated 2'-Deoxyuridine Anion, dU(H)<sup>•-</sup>, as a Primary ET Product. T.L. Netzel, K. Nafisi, J. Headrick, and B.E. Eaton, *J. Phys. Chem.* **99**, 17948-17955 (1995).
11. Dynamic Behavior in the Light and Dark Phases of Alkane Photochemical Functionalization by  $[W_{10}O_{32}]^{4-}$ . D. C. Duncan, B. S. Jaynes, T. L. Netzel, and C. L. Hill, *Proc. Indian Acad. Sci. (Chem. Sci.)* **107**, 729-733 (1995).
12. Electron Transfer Reactions in DNA. T. L. Netzel, *J. Chem. Ed.* an invited review in press (1997).

## 7.2 Abstracts Published at Scientific Meetings.

Sequence-specific Effects on Electron Transfer Quenching of Photo-excited Pyrene in DNA Oligomers and Duplexes: Injecting an Excess Electron into DNA. T. L. Netzel, U. S. DOE Radiological and Chemical Physics Contractor's Meeting, New Orleans, LA, June 7-9, 1994.

Sequence-specific Effects on Electron Transfer Quenching of Photo-excited Pyrene in DNA Oligomers and Duplexes: Injecting an Excess Electron into DNA. T. L. Netzel, M. Manoharan, B. E. Eaton, K. Tivel, J. Headrick, M. Zhao, and K. Nafisi, 1994 American Crystallographic Association Annual Meeting, Atlanta, GA, June 26 - July 2, 1994.

Sequence-specific Effects on Cyanine Dye Emission Enhancements and Lifetimes Upon Binding to DNA Duplexes. T. L. Netzel, I. Johnson, J. Lenhard, M. Zhao, and K. Nafisi, American Chemical Society Meeting, Washington, D. C., August 21-26, 1994.

Sequence-specific Quenching of Pyrene\* Emission in Covalently Labeled DNA Oligomers and Duplexes. T. L. Netzel, M. Manoharan, B. E. Eaton, K. Tivel, J. Headrick, M. Zhao, and K. Nafisi, American Chemical Society Meeting, Washington, D. C., August 21-26, 1994.

Mechanism of Alkane Activation by the Photocatalyst  $W_{10}O_{32}^{4-}$  Deduced from Picosecond Transient Absorption Kinetics Measurements. D. Duncan, T. L. Netzel and C. L. Hill, American Chemical Society Meeting, Washington, D. C., August 21-26, 1994.

Base-sequence Dependence of Electron-transfer Quenching of Covalent Pyrene\*-labels by Nucleotide Bases in DNA Oligomers and Duplexes. T. L. Netzel, Seventh Inter-American Photochemical Society Winter Conference, Clearwater Beach, FL, January 1-5, 1995.

Dynamic Behavior in the Light and Dark Phases of Alkane Photochemical Functionalization by  $[W_{10}O_{32}]^{4-}$ . C. L. Hill, D. C. Duncan, B. S. Jaynes, and T. L. Netzel, 3<sup>rd</sup> International Conference on Solar and Applied Photochemistry, Cairo, Egypt, January 8-14, 1995.

Photophysical Studies of 2'-Deoxyuridine (dU) Nucleosides Covalently Substituted with Either 1-Pyrenyl or 1-Pyrenoyl: Observation of Pyrene-to-Nucleoside Charge-Transfer Emission. T. L. Netzel, Fifth Annual University System of Georgia Research Symposium, "Genes - The Cure and Cause of Disease," Medical College of Georgia, Augusta, GA, May 21-22, 1995.

Direct Observation of Photoinduced Electron Transfer in Deoxyuridine Nucleosides with Covalent Pyrene Adducts. T. L. Netzel, J. Headrick, K. Nafisi, and B. E. Eaton, American Chemical Society Meeting, Chicago, IL, August 20-25, 1995.

Intramolecular Electron Transfer Reactions Within D-A Complexes Bound to Double Stranded DNA. N. Thornton, H. Wojtowicz, T. L. Netzel, and D. Dixon, American Chemical Society Meeting, Chicago, IL, August 20-25, 1995.

Electron Transfer Reactions in DNA. T. L. Netzel, American Chemical Society Meeting, Orlando, FL, August 25-29, 1996.

Studies of the Effect of DNA Binding on Intramolecular Electron-Transfer Reactions within Ruthenium-Naphthalene Diimide Donor-Acceptor Complexes. N. B. Thornton, V. Kiselyova, D. Ebner, D. W. Dixon, and T. L. Netzel, 48<sup>th</sup> ACS Southeast Regional Meeting, Greenville, S. C., November 10-13, 1996.

Ruthenium-Naphthalene Diimide Donor-Acceptor Complexes: DNA Binding and Intramolecular Electron Transfer Reactions. D. W. Dixon, V. Kiselyova, N. B. Thornton, and T. L. Netzel, Metals in Biology Gordon Conference, Ventura, CA, January 19-24, 1997.

### 7.3 Research Seminars Presented.

Washington State University, Pullman, WA, March 17, 1994.  
Molecular Science Research Center, Pacific Northwest Laboratory, Richland, WA, March 22, 1994.  
University of Florida, Gainesville, FL, April 14, 1994.  
University of Georgia, Athens, GA, April 25, 1994.  
University of Pittsburgh, Pittsburgh, PA, May 23, 1994.  
Department of Energy Radiological and Chemical Physics Contractor's Meeting, New Orleans, LA, June 7, 1994.  
American Crystallographic Society Meeting, Atlanta, GA, July 1, 1994.  
New York University, New York, NY, November 17, 1994.  
Rutgers University, New Brunswick, NJ, November 18, 1994.  
7<sup>th</sup>-Inter-American Photochemical Society Winter Conference, Clearwater Beach, FL, January 4, 1995.  
Northwestern University, Evanston, IL, February 23, 1995.  
University of Rochester, Rochester, NY, March 23, 1995.  
American Chemical Society National Meeting, Chicago, IL, August 24, 1995.  
Boston University, Boston, MA, September 18, 1995.  
Brandeis University, Boston MA, September 19, 1995.  
California Institute of Technology, Pasadena, CA, March 11, 1996.  
University of California at Riverside, Riverside, CA, March 12, 1996.  
University of California at Santa Barbara, Santa Barbara, CA, March 13, 1996.  
Isis Pharmaceuticals, Inc., Carlsbad, CA, March 18, 1996.  
University of California at Irvine, Irvine, CA, March 19, 1996.  
American Society for Photobiology National Meeting, Atlanta, GA, June 18, 1996.  
American Chemical Society National Meeting, Orlando, FL, August 27, 1996.

**8.0 Acknowledgments.** This work was supported by the United States Department of Energy, Office of Health and Environment, Radiological and Chemical Physics Research Division (Grant No. DE-FG05-03ER61604). I acknowledge helpful conversations with Drs. David Wilson, Alex Siemiarczuk, David Beratan, Thomas Meade, Dabney White-Dixon, Nicholas Geacintov, Jerome Lenhard, and Ian Johnson. I thank my collaborators Drs. Bruce Eaton, Muthiah Manoharan, and Kohinoor Begum who made much of this research possible by synthesizing families of new DNA macromolecules. I also acknowledge the scientific literature research and proofreading assistance of Marla Netzel. Since this is a final project report, I especially thank the Department of Energy and my contract's technical manager, Dr. Matesh Varma. This research contract allowed me to begin an exciting area of research that is now poised to make significant contributions to cellular biology and to our understanding and controlled use of photoinduced ET in supramolecular systems. The new research initiatives in these areas that I am now pursuing rest entirely on the deep and solid foundation that this research grant allowed me and my research collaborators to establish.



**9.0 Signature of the Principal Investigator.**

This Final Performance Report was prepared by

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**10.0 List of Attached Documents.**

**10.1 DOE F 1332.15. Recommendations for Announcement and Distribution of DOE Scientific and Technical Information.**

**10.2 DOE F 2050.11. Patent Certification.**

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