Complex Formation and Energy Transfer from Photoexcited Thiopyronine to Deoxyribonucleic Acid†

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ABSTRACT: Thiopyronine·DNA complexes were investigated with optical and electron paramagnetic resonance techniques. From the dependence of the zero field splitting energies of the thiopyronine triplet state on the molecular environment, it is concluded that there exist two types of complexes. For low DNA phosphate to dye ratios (P:D) the thiopyronine molecules are probably stacked at the surface of the macromolecule. For high P:D ratios thiopyronine behaves like an isolated (intercalated) molecule. The initial energy transfer from the dye to the DNA probably involves the photoinduced radical. Exciting the thiopyronine molecule with visible light shows that there exist excited states involved in electron-hole recombination, delayed fluorescence, and phosphorescence. The variety of possible photochemical pathways seems to explain the efficiency of thiopyronine as photosensitizer for cell damage.

For various organisms extensive investigations have been undertaken to clarify the mutagenic (Brenner *et al.*, 1961; Silver, 1967), carcinogenic (Schmidt, 1958), and photodynamic (Bellin and Grossman, 1965) properties of acridines and structurally related molecules. These heterocyclic molecules may intercalate and/or attach to the surface of the DNA, dependent on the dye:DNA ratio (Lerman, 1961; Gurskii, 1966; Li and Crothers, 1969). The molecular interactions are mediated by van der Waals and coulombic forces (Gersch and Jordan, 1965). Recently relaxation kinetics and circular dichroism experiments have been carried out to study for various polynucleotides (Li and Crothers, 1969) and DNA (I'Haya and Nakamura, 1971) the interaction forces as a function of temperature and salt concentration (Hammes and Hubbard, 1966). In the presence of heterocyclic dyes such as Acridine Orange, Methylene Blue, and thiopyronine, living cells are inactivated by visible light. These molecules act as photosensitizers and in the final stage the killing process consists of lesions induced in certain DNA components (Wacker *et al.*, 1964; Dellweg and Oprée, 1966).

To elucidate the mechanism of energy transfer from the photoexcited DNA to the dye, extensive studies have been carried out, in contrast to the reverse process, *viz.*, from the dye to the DNA. Both singlet as well as triplet energy can be transferred from the DNA to the dye molecule and the type of migration has been discussed on the basis of the exciton model (Galley, 1968; Guéron and Shulman, 1968). Dye-sensitized processes have been investigated for proteins (Spikes and MacKnight, 1970) and DNA (Simon and Van Vunakis, 1964), *e.g.*, guanidine residues in the DNA were photoexcited upon visible light illumination of microorganisms in the presence of Methylene Blue or thiopyronine.

Since thiopyronine proved to be a rather efficient biological photosensitizer, the following work was undertaken to obtain some insight into the mechanism of energy transfer from the photoexcited dye to the DNA molecule. For that purpose the electronic structure of thiopyronine has been investigated spectroscopically in our laboratory (Lalitha and Haug, 1971).

Experimental Section

Samples. Thiopyronine was prepared by the method of Biehringer and Topaloff (1902). The compound was recrystallized several times and further purified on an alumina column. Purity was checked by thin-layer chromatography, high-voltage electrophoresis, and melting point determination. Calf thymus DNA was purchased from Sigma Chemical Co. Both DNA and thiopyronine were dissolved in 0.02 M phosphate buffer (pH 7.6). The concentration of thiopyronine was measured at 564 nm (ϵ 7.4 $\times$ 10^4) (Morita and Kato, 1968) and that of DNA at 260 nm (ϵ 6.6 $\times$ 10^3) (Mahler and Cordes, 1966). Solutions of different DNA phosphate:dye (P:D) ratios were prepared by adding a known concentration of DNA to a known concentration of dye (v/v) followed by thorough mixing. At high concentrations of thiopyronine (>10^{-3} M) a precipitate is formed. Unless otherwise mentioned, sodium chloride was also added to all the solutions to make up a final concentration of 0.1 M. For emission measurements at 77°K redistilled ethylene glycol–phosphate buffer (2:3, v/v) was employed.

Spectroscopic Measurements. The absorption spectra were measured with a Cary-15 spectrometer. To determine the binding constant of thiopyronine to DNA at room temperature, a solution of DNA (2 $\times$ 10^{-5} M) was optically titrated (λ 564 nm) by adding small aliquots of thiopyronine.

Moreover, since the dye fluoresces strongly, the fluorescence intensity could also be utilized to obtain information about binding. An Aminco-Bowman spectrofluorimeter was employed to measure the fluorescence spectra at room temperature. For a constant dye concentration (7 $\times$ 10^{-6} M) its fluorescence quenching (λ 590 nm) was determined as a function of DNA concentration.

Using a Gilford 2400 spectrophotometer the melting curves were obtained in the DNA absorption region for various DNA:dye ratios with and without 0.1 M NaCl. Evaporation of the sample was minimized by covering the cuvet with parafilm. The temperature was continuously monitored at a

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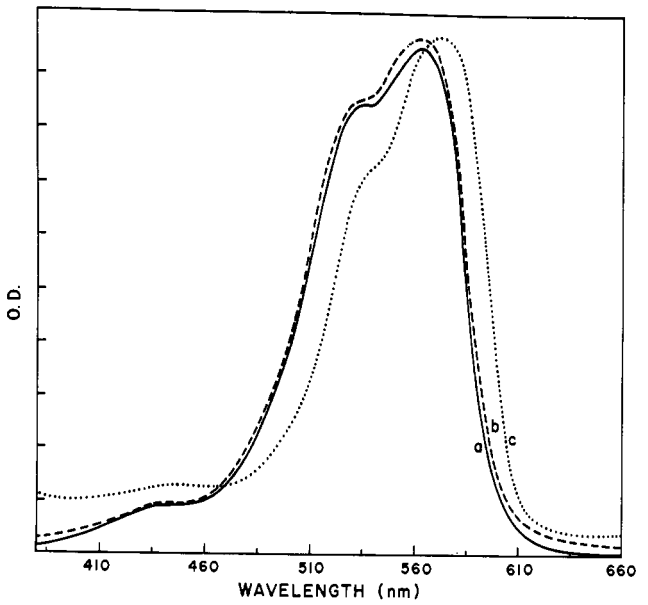

FIGURE 1: Absorption spectra at room temperature in phosphate buffer (pH 7.6). (——) thiopyronine, 2×10^{-5} M; (- - - - - - -) P:D = 0.5; (· · · ·) P:D = 50.

second sample placed in the cell compartment containing a calibrated thermometer.

The phosphorescence spectra and lifetimes were recorded with a phosphoroscope employing appropriate Jarrell-Ash gratings and EMI 6256 and RCA 31000 E photomultipliers, sensitive in the blue and red regions, respectively. A multichannel analyzer, Nuclear Data Model 2200, operated in the signal-averaging mode, was utilized for lifetime measurements. The electron paramagnetic resonance (epr) spectra were obtained with a Varian X-band spectrometer, Model 4502-15, with a Fieldial Mark II magnetic field control unit.

Results

Absorption Spectra of the DNA–Dye Complex. The absorption spectra of Thiopyronine and the DNA–dye complex are shown in Figure 1. With increasing DNA concentration the long-wavelength absorption maximum of thiopyronine shifts toward the red. With thiopyronine as reference the difference spectra of the DNA:dye complex do not show an isosbestic point (Figure 2). The binding constant was determined by absorption and fluorescence measurements and by equilibrium dialysis (Table I). Details of the calculations can be found in the respective articles by Gellert *et al.* (1965), Löber (1968), and Cavalieri and Nemchin (1964).

The melting temperature of DNA increased concomitantly with the dye concentration. In the absence of NaCl the melting temperature of the DNA–dye complex is shown in Figure 3. In 0.1 M NaCl, the melting temperature increased only by 2° upon adding thiopyronine to obtain a final P:D ratio of 30.

TABLE I: Binding Constant of Thiopyronine to DNA Determined by Different Methods.

Method	K (l. Mole^{-1})
Absorption	3×10^4
Fluorescence	2×10^4
Equilibrium dialysis	2×10^3

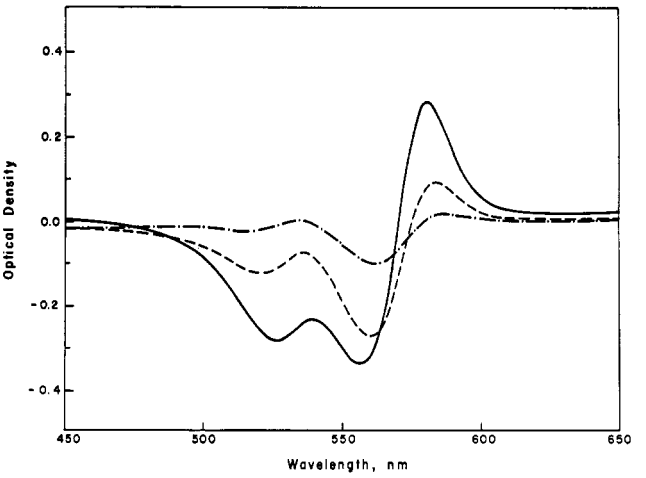

FIGURE 2: Difference spectra at room temperature in phosphate buffer (pH 7.6) with a thiopyronine concentration of 2×10^{-5} M. (—·—·—·) P:D = 1, (- - - - - - -) P:D = 5, and (——) P:D = 50.

Epr Experiments of the DNA:Dye Complex. Keeping the dye concentration constant, the $\Delta m = 2$ position of the lowest triplet state of the DNA–dye complex shifted toward lower magnetic fields (Figure 4). The zero field splitting (zfs) constant D^* was calculated from the following relation, where H_{min} is the field strength at which the transition occurs, ν is the microwave frequency and $g = 2.0023$ (Table II). BM is Bohr's magneton and h is Planck's constant: $D^{*2} = (3/4) \times [(h\nu)^2 - 4(g\mathrm{BM})^2 H_{min}^2]$. Exciting thiopyronine into its main absorption band a radical is generated at 300°K and at 77°K, as determined by epr spectroscopy.

Emission Characteristics of the DNA–Dye Complex. The fluorescence spectra are given in Figure 5. The long-lived emission spectra and lifetimes of the free and bound dye were determined as a function of the P:D ratio. Thiopyronine itself

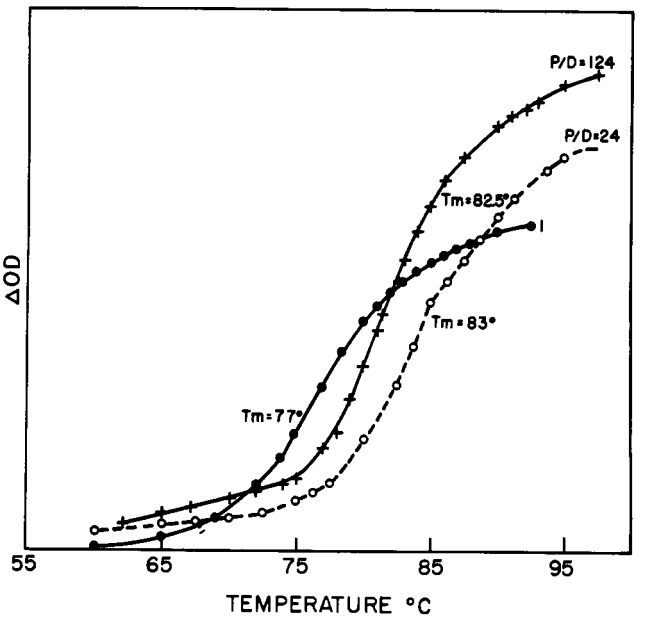

FIGURE 3: Melting curves for a DNA concentration of 5×10^{-5} M in phosphate buffer (pH 7.6) as a function of P:D.

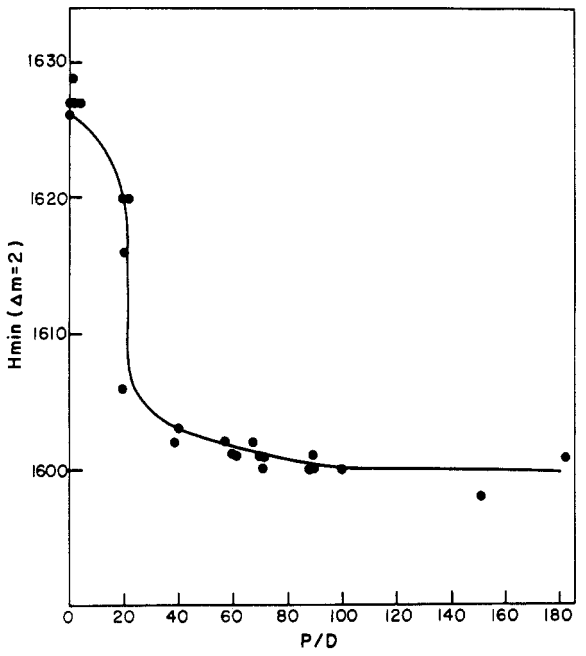

FIGURE 4: Variation of H_{min} with P:D for a thiopyronine concentration of 3×10^{-5} M in phosphate buffer-glycol (pH 7.6) and a microwave frequency of 9239 MHz.

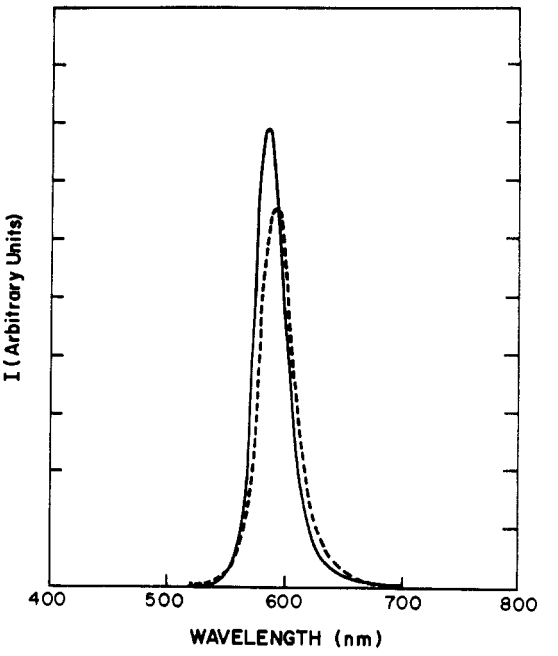

FIGURE 5: Fluorescence spectra at room temperature. (——) 2×10^{-5} M thiopyronine and (-------) P:D = 150.

exhibits three long-lived emissions at 77°K (S. Lalitha, D. Graber, and A. Haug, 1971, unpublished results). For the sake of clarity, the characteristics of these emissions of thiopyronine are outlined briefly. The emission maximum of the phosphorescent triplet state of thiopyronine shifts toward shorter wavelengths as the concentration decreases with a concomitant increase in lifetime (Lalitha and Haug, 1971). Another long-lived emission (590 nm) of thiopyronine has been assigned to delayed fluorescence. A blue emission has been attributed to electron-hole recombination luminescence. Such a luminescence has been observed in the case of acridine dyes dissolved in a rigid matrix of ethanol. The primary process involves the emission from excited singlet states generated from recombination of radical ions and electrons initially produced by one-electron photoionization of the dye molecules (Ewald and Durocher, 1971). For a constant dye concentration the phosphorescence maximum of thiopyronine undergoes a blue shift and the lifetime increases as the DNA concentration is enhanced (Table III). This phosphorescence emission can be induced by exciting the DNA–dye complex with light of any wavelength shorter or equal to 564 nm, which is the long-wavelength absorption band of the dye molecule. Varying the DNA concentration, the position of the peak and the lifetime of the blue emission do not alter measurably (Figure 6). The excitation spectrum of the blue emission of the DNA–dye complex is similar to that of thiopyronine having a strong peak at 280 nm and a small one at about 350 nm. At 77°K, DNA has a triplet state with a peak at 470 nm which decays with a half-life of 0.3 sec.

At 77°K, the DNA–dye complex (P:D > 100) exhibits delayed fluorescence with a peak at 590 nm. For the phosphoroscope speeds available (25 msec/rev) the emission can be characterized by a lifetime of 100 msec, and the intensity depends linearly on that of the incident exciting light.

Discussion

The shift of the absorption peak to longer wavelengths at higher values of P:D indicates that thiopyronine is bound to the DNA. The changes observed are probably due to alteration of the absorption band of the dye due to binding with the DNA. The difference spectra as well as the absorption spectra do not reveal a single isosbestic point, thereby indicating that a single type of binding does not occur. The negative peak (550 nm) in the difference spectrum increases at higher salt concentration. With increasing dye concentration, the change in melting temperature and the difference spectra of the DNA–dye complex are sensitive to ionic

TABLE II: The Zfs Parameter D^* for Various DNA:Dye Ratios Determined at 77°K in Phosphate Buffer–Ethylene Glycol (pH 7.6) for a Thiopyronine Concentration of 3×10^{-5} M.

P:D	D^* (cm^{-1})
3	0.0467
10	0.0486
20	0.0528
60	0.0636
88	0.0643

TABLE III: Phosphorescence Maximum and Lifetime of Various DNA:Dye Ratios, for a Concentration of Thiopyronine of 4×10^{-5} M in Phosphate Buffer–Glycol (pH 7.6).

P:D	λ_{max}	Lifetime (msec)
0.5	7400	91
5.0	7300	93
100	7000	142

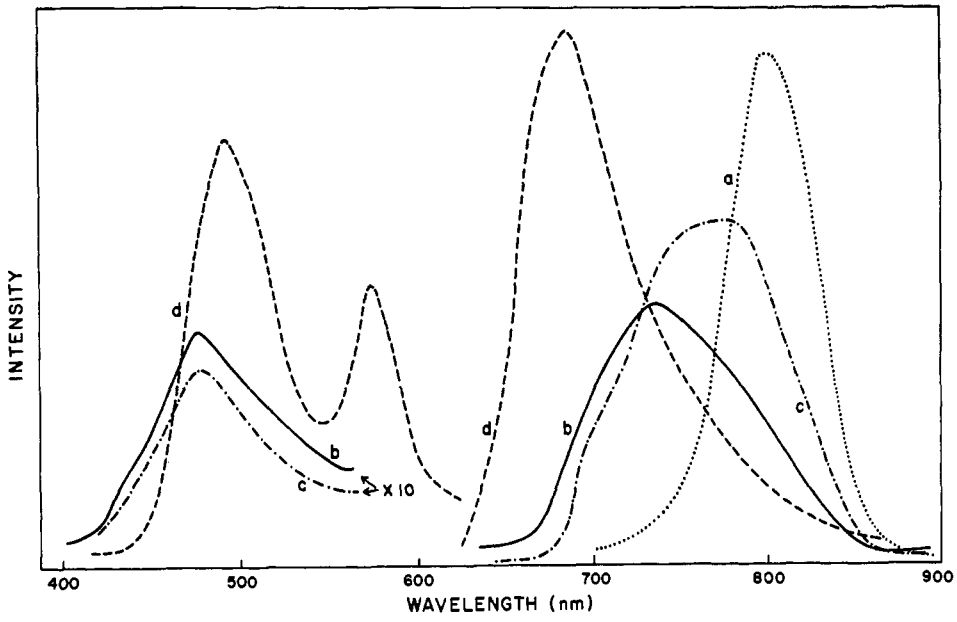

FIGURE 6: Phosphorescence spectra in phosphate buffer–glycol at 77°K. (a) (· · · ·) Thiopyronine, 10^{-2} M; (b) (——) thiopyronine, 10^{-5} M; (c) (—·—·—) P:D = 24; (d) (--------) P:D = 124. In the blue spectral region, the intensity of the curves b and c is multiplied by a factor of 10 for illustration purposes. All emission peaks plotted can be excited with wavelengths shorter than about 350 nm. The phosphorescence emission in the red spectral region can be also excited *via* absorption in the long-wavelength absorption band (564 nm).

strength. This supports the idea that dye molecules are externally bound to the polymer under certain conditions (Li and Crothers, 1969).

The binding constants (K) determined by different techniques agree within error of measurements. The equilibrium dialysis method consistently gives lower values for K since some of the dye molecules (about 15%) are absorbed on the dialysis bag. It is noted that the binding constant of Thiopyronine to the DNA is comparable to that of acridine derivatives (Löber, 1968).

Regarding the nature of the DNA–dye complex our epr experiments indicate that there are at least two types of complexes (Figure 4). Depending on the dye concentration and ionic strength the dye molecules can intercalate and/or bind to the phosphate groups of the DNA (Lerman, 1963; Yamabe, 1969). For low P:D ratios, the zfs parameter is lower than that for high P:D ratios (Table II). For lower DNA concentration, the zfs parameter decreases thus demonstrating delocalization of the electrons contributing to the triplet state. Such a delocalization becomes possible when the triplet electrons can be distributed over neighboring molecules. The adjacent molecules are probably thiopyronine molecules, because thiopyronine can form aggregates as evidenced by the behavior of its lowest triplet state (Lalitha and Haug, 1971). It has to be pointed out that this triplet state is that of the DNA–dye complex as demonstrated by other spectroscopic measurements. For high P:D ratios one may tentatively assume that the dye molecule intercalates between the base pairs, resulting in isolated thiopyronine molecules as characterized by larger D^* values. Figure 4 shows that the H_{min} changes sharply at a critical P:D ratio. Going from a high P:D ratio to a low one, the thiopyronine molecules probably stack up externally along the macromolecular axis. The apparent transition may indicate that the stacking of additional thiopyronine molecules is possibly a cooperative process accompanied by conformational change of the macromolecule (Klotz *et al.*, 1969; Akinrimisi *et al.*, 1965; Blears and Danyluk, 1967).

The information obtained from epr experiments is further confirmed by the phosphorescence data. For a low P:D ratio, the phosphorescence maximum approaches that of the dye at that concentration. For a high P:D ratio, the lifetime of the photoexcited triplet state is longer than that at a low P:D ratio, indicating that for low DNA concentrations, dye stacking on the phosphates takes place, favoring quenching processes. These measurements demonstrate that quenching due to the interaction between dye–DNA base pairs is appreciably smaller as compared to that of dye–dye complexes, as evidenced by the concentration dependence. Increasing the dye concentration leads to self-quenching as reflected by changes in the phosphorescence lifetime and intensity (Lalitha and Haug, 1971).

Delayed fluorescence could be observed only at a high concentrations of DNA. As the delayed fluorescence was only measurable for times longer than several msec it cannot result from singlet–singlet annihilation. It may be generated from triplet–triplet annihilation or recombination processes (Parker, 1967). Delayed fluorescence has been reported also for other dye–DNA complexes (Kubota *et al.*, 1969).

The origin of the blue emission from thiopyronine is not obvious. It is clear however, that it cannot be attributed to delayed fluorescence since experiments with thiopyronine incorporated into poly(vinyl alcohol) films exhibit a delayed emission with a peak at 590 nm which coincides with the fluorescence peak. Furthermore, photodynamically related molecules such as proflavine (Ewald, 1969), Crystal Violet ion (Lewis *et al.*, 1942), Eosin (Ewald and Durocher, 1971), also show long-lived emissions in the blue spectral region, besides an emission from the lowest phosphorescent triplet state. Since phosphorescence from a higher triplet state is improbable, one may tentatively assume that the blue emission of thiopyronine results from electron-hole recombination luminescence (Ewald and Durocher, 1971). The intensity of the blue emission of thiopyronine is proportional to to that of the exciting light and depends on the concentration of the molecules. Epr investigation of the lowest triplet

state emitting in the red region, demonstrates delocalization of the triplet electrons contributing to the triplet state upon increasing the concentration (Lalitha and Haug, 1971). Because the blue emission is also concentration dependent, it is thus conceivable that at certain concentrations solute aggregates favor the transfer of a photoinduced electron to an adjacent molecule followed by recombination luminescence in the blue region, whereas at high concentrations ($>10^{-2}$ M) quenching occurs. For dyes, the minimum energy necessary to generate a free electron in an aggregate has been estimated to be of the order of 3.1 eV (Ewald and Durocher, 1971). This minimum energy is consistent with our findings that the blue emission of thiopyronine can only be observed with excitation light of at least 360 nm (3.4 eV).

Considering our low-temperature experiments, the following possibilities exist for the photochemical degradation of DNA in the presence of thiopyronine exposed to visible light. First, DNA has been shown to have a broad phosphorescence peak at 470 nm and its triplet state can be characterized by a lifetime of 0.3 sec. The onset of thymine phosphorescence is around 380 nm (Eisinger and Shulman, 1968). Therefore, there is no overlap between the absorption spectrum of the acceptor (nucleic acid) and the emission spectrum of the donor molecule, *viz.*, thiopyronine with its blue emission. Thus energy transfer from the dye to DNA cannot occur *via* a resonance process. However, because the primary photoprocess leading to the blue emission seems to be explained by a short-range electron phototransfer (Ewald and Durocher, 1971) in molecular aggregates, it is conceivable that those photoinduced electrons may be transferred to nucleic acid bases of the dye–DNA complex, thus inducing photochemical lesions. On the other hand, electrons from the DNA may be transferred to the photoexcited dye. In this context it is worth while to mention that an epr radical signal can be detected upon illumination of Methylene Blue in dimethyl sulfoxide. This radical seems to be produced by the electron transferred from the solvent to the dye and which is presumably located at the sulfur atom of the dye (Lohmann and Fowler, 1966).

Secondly, since illumination of thiopyronine with visible light generates a radical in the presence or absence of DNA, it is probable that this radical is involved in the photoprocesses occurring in the DNA–dye complex. This assumption is supported by the following facts. Free radicals can be induced by exciting thiopyronine in its main absorption band only, which is consistent with the action spectrum for photosensitisation of *E. coli* in the presence of thiopyronine (Dellweg and Oprée, 1966). Moreover, illuminating acridine derivatives in a rigid matrix up to the longest wavelength absorption band, the dye molecules become initially photoionized. The radical ions and electrons, thus generated, may recombine (Lim *et al.*, 1965; Balazs and Young, 1968) or may become involved in photochemical alterations of the DNA–dye system. The radical may be induced *via* the phosphorescent triplet state which can be highly populated through the main absorption band of thiopyronine. Energetically it is improbable that the phosphorescent triplet state is directly involved in the photosensitization of DNA. Since the formation of the dye–DNA complex is concentration dependent, this may also affect the efficiency of energy or electron transfer. Carrying out our experiments at 77°K, no oxygen effect could be detected. At room temperature, however, where diffusional encounters between photoexcited thiopyronine and metastable singlet oxygen molecules are more probable, dye-sensitized oxidation of guanine residues may play an important role in killing microorganisms in the presence of oxygen and a photosensitizing dye (Simon and Van Vunakis, 1964; Spikes and MacKnight, 1970; Nanaie, 1967).

To summarize, the variety of possible photochemical pathways seems to explain the efficiency of thiopyronine as a photosensitizer for cell damage. To elucidate further the early stages of photosensitization, study of complexes between thiopyronine and synthetic polynucleotides may be helpful.

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Fluorescent Probes of Acetylcholinesterase†

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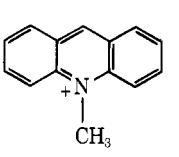
ABSTRACT: *N*-Methylacridinium ion and bis(3-aminopyridinium)-1,10-decane are two potent cationic inhibitors of acetylcholinesterase from *Electrophorus electricus* which should be useful as fluorescent probes of the various types of binding interactions in which the enzyme can participate. The fluorescence spectra of both inhibitors are strongly quenched upon binding to the enzyme, and one molecule of each inhibitor binds to the enzyme per catalytic site titratable by 7-dimethylcarbamyl-*N*-methylquinolinium iodide. The dissociation constant for each inhibitor determined by fluorometric titration agrees closely with the inhibition constants obtained from steady-state kinetic studies. For *N*-methylacridinium ion, the dissociation constant is $2.32 \pm 0.75 \times 10^{-7}$ M while for bis(3-aminopyridinium)-1,10-decane the dissociation constant is $5.16 \pm 1.41 \times 10^{-8}$ M. All the available data are consistent with both probes binding in a region which overlaps the acetylcholine specific site at the active site. In addition, one aminopyridinium moiety of bis(3-aminopyridinium)-1,10-decane appears to interact at a cationic specific binding site remote from the active site. By studying the competitive displacement of both probes from the enzyme, it should be possible to examine the binding specificity of both cationic binding sites toward various acetylcholine antagonists. The use of 7-dimethylcarbamyl-*N*-methylquinolinium as an active-site titrant has permitted the precise measurement of the turnover number per active site of a commercial enzyme preparation before and after purification by affinity chromatography. Using this value, the purified enzyme appears to have 4 catalytic subunits/260,000 molecular weight.

Recent studies on the ligand binding properties of acetylcholinesterase (AChE)[1] have indicated that the enzyme may possess binding sites for acetylcholine (ACh) and acetylcholine antagonists at loci remote from the active site (Changeux, 1966; Meunier and Changeux, 1969; Kato *et al.*, 1970; Kitz *et al.*, 1970). Kinetic studies have constituted the main experimental approach to suggest the existence of these peripheral sites although recently nuclear magnetic resonance (nmr) experiments have supported the existence of a specific binding site for atropine which is distinct from the binding site at the active site (Kato *et al.*, 1970).

An additional and potentially very useful approach for the study of the ligand binding properties of AChE would be to devise specific fluorescent probes of the different binding sites on AChE and use them as indicators of the types of interactions in which acetylcholinesterase can participate. Two properties of fluorescent probes make them well suited for the study of AChE. First, the sensitivity of fluorescence measurements permits the use of relatively low concentrations of the enzyme; and secondly, fluorescence spectra of ligands bound to proteins are frequently sensitive to the microenvironment in which they are located.

In the present communication, we wish to report that N-methylacridinium chloride (I) and bis(3-aminopyridinium)-1,10-decane diiodide (II), two highly fluorescent compounds



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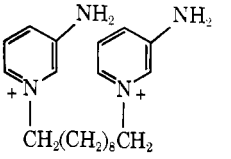

II

bind very tightly to AChE. They are suitable as fluorescent probes of the enzyme since the fluorescence spectra of both of them are strongly quenched upon binding to the enzyme. The fluorescence changes which occur as a consequence of the formation of the enzyme–inhibitor complex can be readily used to quantitate their interactions with the enzyme as well as those of the ligands which affect the binding of I and II. They can also be used to determine the binding site normality of a solution of electric eel AChE.

The enzyme normalities determined with I and II agree with those measured using 7-dimethylcarbamyl-*N*-methylquinolinium iodide (III) as an active-site titrant. The titrimetric method employing III is a single turnover assay which is based on the intense fluorescence of 7-hydroxy-*N*-methylquinolinium (IV) (Prince, 1966b) in its zwitterionic form (V) and the inhibition of AChE by III (Kitz *et al.*, 1967). This procedure has been reported by Rosenberry (1970) and by

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[1] Abbreviations used are: AChE, acetylchloinesterase; ACh, acetylcholine.