A comparative study of the interaction of 5,10,15,20-tetrakis (N-methylpyridinium-4-yl)porphyrin and its zinc complex with DNA using fluorescence spectroscopy and topoisomerisation

John M.Kelly and Martin J.Murphy

Department of Chemistry, University of Dublin, Trinity College, Dublin 2, Ireland, and

David J.McConnell and Colm OhUigin

Department of Genetics, University of Dublin, Trinity College, Dublin 2, Ireland

ABSTRACT

Binding of 5,10,15,20-tetrakis(N-methylpyridinium-4-yl)porphyrin (H_2TMPyP^{4+}) and its zinc complex ($ZnTMPyP^{4+}$) to DNA is demonstrated by their coelectrophoresis and by absorption and fluorescence spectroscopic methods. Topoisomerisation of pBR322 DNA shows that H_2TMPyP^{4+} unwinds DNA as efficiently as ethidium bromide showing that it intercalates at many sites. $ZnTMPyP^{4+}$ may cause limited unwinding. Marked changes in the fluorescence spectra of the porphyrins are found in the presence of DNA. The fluorescence intensity of either H_2TMPyP^{4+} or $ZnTMPyP^{4+}$ is enhanced in the presence of poly (d(A-T)), whereas in the presence of poly (d(G-C)) the fluorescence intensity of $ZnTMPyP^{4+}$ is only slightly affected and that of H_2TMPyP^{4+} markedly reduced. Both the porphyrins photosensitise the cleavage of DNA in aerated solution upon visible light irradiation.

INTRODUCTION

Although meso-substituted porphyrins are much larger than well-known intercalators such as acridine or phenanthridine dyes, it is now apparent from the work of Fiel, Pasternack and their coworkers (1-11) that certain cationic water-soluble porphyrins can interact strongly with DNA in solution. It is clear that there is more than one mode of binding and the nature of this interaction depends on the structure of the porphyrin, on the relative concentrations of the porphyrin and the DNA base pairs, and on the base composition of the DNA. Some porphyrins appear to be able to intercalate. The best evidence for this is the effect of the porphyrin on the electrophoretic mobility of DNA (2) and on the viscosity of DNA solutions (1,9) and from circular dichroism studies (1,6,7,8,10). Porphyrins which do not intercalate are presumed to bind to the outside of the DNA, probably interacting both with the bases and the phosphate groups (11). Generally it has been noted that porphyrins which intercalate do not have axial ligands and have meso-groups which can easily rotate into the plane of the tetrapyrrole ring (8,10,11).

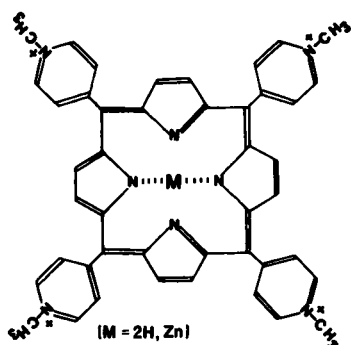The base specificity of this interaction has been studied by both abs-

Figure 1. Structural formulae of
5,10,15,20-tetrakis(N-methylpyrid-
inium-4-yl)porphyrin and its zinc
complex.

orption spectroscopy (10) and circular dichroism (8,10). Porphyrins which
are believed to intercalate (e.g. H_2TMPyP^{4+} - see Figure 1 - or its CU(II)
or Ni(II) derivative) appear to interact strongly with poly(d(G-C)). In
contrast the spectroscopic properties of non-intercalating porphyrins are
hardly changed by poly(d(G-C)) but are markedly altered by poly(d(A-T)).
This suggests that intercalation occurs predominantly at residues. The
kinetics of association and dissociation of porphyrins and DNA or synthetic
polynucleotides has been treated in detail by Pasternack et al. in a recent
paper (11).

We have recently started to study how the photoproperties, including the
non-linear optical properties, of organic molecules and metal complexes are
affected by binding to DNA. The known photochemical and photophysical
properties of porphyrins (12) make them particularly attractive as probes
for the behaviour of DNA and its complexes in solution and also as sensitis-
ers for the photocleavage (possibly base-specific) of polynucleotides.
Initially we have chosen to study two water-soluble porphyrins (H_2TMPyP^{4+}
and $ZnTMPyP^{4+}$) which fluoresce strongly in solution. As indicated above,
evidence suggests that H_2TMPyP^{4+} intercalates into DNA, but $ZnTMPyP^{4+}$ does
not. We have investigated this matter by carrying out a sensitive assay
for intercalation, namely the topoisomerisation of supercoiled covalently
closed circular (ccc) DNA in the presence of the porphyrins. We demonstrate
that the fluorescence spectrum and intensity of the porphyrins are changed
substantially in the presence of DNA and that the photophysical properties
of the porphyrins are sensitive to the base composition of the polynucleo-
tide. It is shown that both porphyrins are equally effective in causing
photo-cleavage of the polynucleotide in aerated solutions (4).

MATERIALS AND METHODS

Porphyrins.   5,10,15,20-tetrakis(4-pyridyl)porphyrin (H_2TPyP) was
prepared by refluxing 4-pyridylcarboxaldehyde and pyrrole in a 70:1 propionic
acid/acetic acid mixture (13). The purified H_2TPyP was then refluxed with
methyl-p-toluene sulphonate in dimethyl formamide (DMF) to yield H_2TMPyP^{4+}
as the tetratosylate salt (14). Extinction coefficient at 423 nm (2.2×10^5)
is in agreement with the literature (10). ZnTMPyP^{4+}. 4Cl$^-$ was obtained in
three ways: (i) by refluxing H_2TMPyP^{4+} with a 300-fold excess of ZnCl$_2$ in
DMF (15,16), (ii) by refluxing H_2TMPyP^{4+} with a 500-fold excess of ZnCl$_2$
in water (10), and (iii) commercially manufactured by Midcentury Ltd. The
absorption spectra for ZnTMPyP^{4+} solutions from the above three sources
were identical and the extinction coefficient at 437 nm was taken to be
2.3×10^5 (17,18).

DNA and polynucleotides. The plasmid pBR322 was isolated from
E.coli K-12 C600 by standard procedures (19), using chloramphenicol
amplification. The plasmid DNA was further purified on CsCl/ethidium
bromide density gradients. The ccc DNA was extracted by isopropanol four
times, dialysed against TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4), and
stored at 4°C. High molecular weight calf thymus DNA was obtained from
Sigma (Cat. No. D4764). It was dissolved in water (100 mg in 50 ml of
doubly distilled water), made to 1% sodium dodecyl sulphate (SDS) and 1 M
NaCl before extraction with an equal volume of a phenol:chloroform:isoamyl
alcohol mixture (25:24:1) containing 0.5% w/v of 8-hydroxyquinoline. This
solvent mixture had been saturated by standing over aqueous 100 mM Tris
HCl buffer pH 7.5. This extraction was repeated once, and the DNA
spooled out on a glass rod while adding 2 volumes of ice cold ethanol.
The DNA was dissolved in TE buffer to a final concentration of approximat-
ely 2.8×10^{-3} M of phosphate and dialysed extensively against TE buffer.
Storage was over a few drops of chloroform at 4°C. Polynucleotides
poly[d(G-C)] and poly[d(A-T)] were obtained from PL Biochemicals (Cat. No.
7910 and 7870 respectively).

Co-electrophoresis of DNA-porphyrin complexes: DNA-porphyrin com-
plexes were prepared in 10 µℓ reaction mixtures containing 0.2 µg of
pBR322 DNA and porphyrin, as specified, in TBE buffer (0.089 M Tris base,
0.089 M boric acid, 0.002 M EDTA). The reaction mixtures were allowed
to stand for 10 minutes at room temperature before being electrophoresed
for 3 hours in a horizontal agarose (0.9% w/v) gel (20 cm x 10 cm x 0.4 cm)
at a constant 200 volts. The reservoir and gel buffers were TBE. The

gel was photographed using transmitted light to detect the porphyrin.   It
was then stained for 30 minutes in ethidium bromide (0.5 µg/ml) before
photographing using a long wave ultra violet light source to induce
fluorescence of the ethidium bromide.   Finally the gel was allowed
to stand for 48 hours at room temperature in ethidium bromide solution be-
fore photography as before.   This last procedure was found necessary to
allow porphyrin bound to DNA to dissociate and ethidium bromide to bind.

    Topoisomerisation of DNA and DNA-porphyrin complexes.   The enzyme
topoisomerase I (Calf thymus, Bethesda Research Laboratories;   Cat. No.
8042 SA/SB) was used to convert supercoiled ccc pBR322 DNA to topoisomers,
by the procedure of Keller (20).   Samples of pBR322 DNA (in excess of 80%
ccc DNA) containing ca. 1.6µg DNA in 50µl of the recommended reaction
buffer (50mM Tris, 50mM KCl, 10mM $MgCl_2$, 0.5mM DDT, 0.1mM EDTA, 30µg/ml
bovine serum albumin), topoisomerase and porphyrin or ethidium at the speci-
fied concentration (see Figure 3) were incubated at $37^{\circ}C$ for 4 hours.   The
reaction was stopped by the addition of an equal volume of the phenol:
chloroform:isoamyl alcohol:8-hydroxyquinoline mixture and the aqueous layer
recovered after shaking and centrifugation.   This extraction is known to
remove ethidium bromide and also removes the porphyrins used in these ex-
periments.   The DNA was recovered by ethanol precipitation after the aqueous
solution had been made 0.3M in sodium acetate.   After drying, the precipi-
tate was redissolved in 10µl of TBE buffer and electrophoresed on submerged
horizontal agarose gels (0.9% w/v, measuring 20cm by 22cm by 0.4cm) for 12
hours on TBE buffer at room temperature.   The gel was stained for 30
minutes in ethidium bromide (0.5µg/ml) and excess ethidium bromide was re-
moved by standing in magnesium sulphate (1mM) for 20 minutes before photo-
graphing under ultra violet light as before.   ε at 460 nm for ethidium
bromide was taken to be 4220 (20).

    Absorption and Fluorescence Spectra.   Absorption spectra were obtained
either on a Pye-Unicam SP8200 UV/visible spectrometer or on a Cary 219 UV/
visible spectrometer interfaced to a PDP 11/34 minicomputer for data analy-
sis.   Fluorescence measurements were recorded on a Perkin Elmer MPF-44B
fluorimeter and the spectra were not corrected for the wavelength response
of the (R928) photomultiplier.   The excitation wavelengths were 423 nm for
all H_2TMPyP^{4+} solutions, and 437nm for all $ZnTMPyP^{4+}$ solutions (unless
otherwise stated).   Fluorescence spectra were carried out in a 10mm path-
length quartz cuvette at room temperature in a phosphate buffer containing
0.025M K_2HPO_4 and 0.025M KH_2PO_4 (pH = 6.9).   Optical densities at the excitat-

ion wavelength were 0.5 or less. As H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ tend to stick to surfaces including glass (21), procedures were used to minimise the effect of this on quantitative fluorescence or absorption spectra. For example, for fluorescence measurements the clean cuvette was first flushed with a portion of the prophyrin sample and then with distilled water.   A further aliquot (1.0ml) of the porphyrin was added to the cuvette, shaken, and the spectrum taken.   The required amount of DNA was then added to this and the spectrum taken.   Sodium chloride, when required, was added as solid.   Porphyrin-sticking is also reduced by treatment of glassware with Me_2SiCl_2.   Results obtained by such procedures were reproducible.

Photolysis: Solutions of DNA and porphyrin were prepared in the dark by adding 25µℓ of a 10µM porphyrin solution to 235µℓ of a solution containing 4µg of pBR322 DNA in a 6mm diameter glass tube.   Sodium azide (100mM) was present when specified.   The solution was mixed gently by pipetting and irradiated with 436 nm light isolated from a medium pressure mercury lamp(Thorn MED 250W) using a high radiance monochromator.   Sample tubes were clamped in pairs 3cm distant from the monochromator, aliquots (40µℓ) were removed at specified times and added to 50µℓ of phenol (buffered by storage over 1M Tris HCl pH 7.5) before vigorous mixing to extract the porphyrin from the aqueous phase.   The aqueous phase was reduced by lyophilisation to about 20µℓ for electrophoresis over-night on a agarose gel (0.8 w/v) at 4 volts per cm.   The gels were stained with ethidium bromide and photographed as before.

RESULTS

Co-electrophoresis of DNA and porphyrins.   The porphyrins H_2TMPyP^{4+} and $ZnTMPyP^{4+}$, being positively charged, migrate to the cathode when subjected to electrophoresis in TBE buffer at pH 8.3.   DNA, being negatively charged, moves in the opposite direction.   If these porphyrins bind strongly to DNA they are expected to co-electrophorese with the DNA, and under the conditions of low ionic strength they should reduce the electrophoretic mobility of the DNA.   These effects were observed with both H_2TMPyP^{4+} and $ZnTMPyP^{4+}$.   Solutions containing pBR322 DNA were prepared with increasing concentrations of porphyrins (0µM, 3µM, 7µM, 50µM) and electrophoresed. Porphyrins when alone migrated towards the cathode as a smear and this was observed easily from its yellow-green colour. When DNA was mixed with a low concentration of porphyrin, no porphyrin was observed moving to the cathode, but instead two narrow yellow-green bands were observed moving to the anode

corresponding to ccc DNA and oc DNA.   If the concentration of porphyrin was increased to 50μM, (P/D = 1.2) three coloured bands were observed, one a smear towards the cathode typical of free porphyrin and the other two towards the anode apparently corresponding to ccc and oc DNA. The positions of the ccc and oc DNA were confirmed by staining with ethidium bromide.   After staining the gel for 30 minutes in ethidium bromide, DNA complexed with a high concentration of porphyrin was not detected or showed only as faint bands, but after staining for 48 hours these bands were easily detected. This indicated that either the binding of ethidium bromide to DNA is reduced if the DNA is already complexed with H_2TMPyP^{4+} or $ZnTMPyP^{4+}$, or that the fluorescence of ethidium bromide-DNA complexes is quenched by the porphyrins. The gels showed that the DNA-porphyrin complexes which are formed at higher concentrations of H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ have a lower electrophoretic mobility as expected for complexes between the polyanionic DNA and the cat-ionic porphyrins.

Topoisomerization of pBR322 and pBR322-porphyrin complexes.   The enzyme topoisomerase I catalyses the nicking and closing of both positively and negatively supercoiled ccc DNA molecules, producing a series of topoiso-mers, i.e. DNA molecules which differ in winding number and therefore in the number of supercoils. A population of topoisomers shows a nearly normal distribution of molecules which differ in the number of superhelical turns by unit amounts distributed about a mean which is affected by the conditions of the reaction – temperature, salt concentration etc. – at the time of ring closure by topoisomerase I. Keller showed that agents which were known to unwind DNA, such as ethidium bromide, could be studied through their effect on the distribution of topoisomers formed by topoisomerase I (20).   His method provides an elegant way of demonstrating unwinding and quantifying the efficiency of the effect of an unwinding agent. We have used this method to determine whether H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ unwind DNA, using the known inter-calator ethidium bromide as a standard.

The addition of increasing quantities of the porphyrin H_2TMPyP^{4+} to the topoisomerization reaction shifts the centre of the distribution of topoisomers towards that of native negatively-supercoiled cccDNA (Figure 2). Note that the temperature and ionic strength conditions of electrophoresis and topoisomerisation differ so that the distribution of topoisomers formed in the absence of unwinding agent is centred at ca. +3 supercoils. Addition of unwinding agent, either H_2TMPyP^{4+} or ethidium bromide brings the centre of the distribution initially through 0 supercoils. This explains the apparent
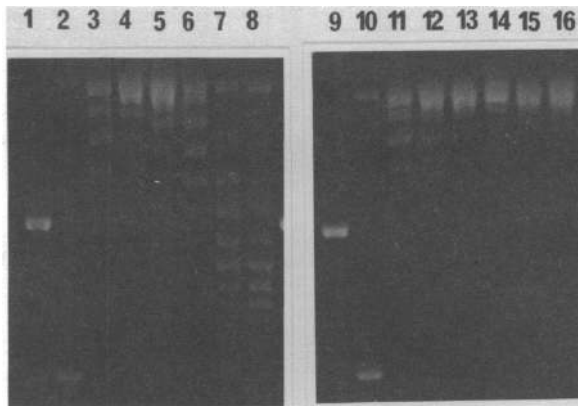
Figure 2. Unwinding of DNA by H_2TMPyP^{4+}. Samples of pBR322 plasmid DNA were
treated with topoisomerase I in the presence and absence of H_2TMPyP^{4+} and
ZnTMPyP^{4+} as described in Materials and Methods. The reactions were stopped,
the samples phenol-extracted and electrophoresed. pBR322 DNA not treated
with topoisomerase I consists of oc and ccc DNA only (lanes 2 and 10) pBR322
DNA cut with the enzyme Hind111, for which there is a single site on the
plasmid, shows one band of DNA only (lanes 1 and 9). pBR322 DNA treated
with topoisomerase I in the absence of either porphyrin (lanes 3 and 11)
shows bands corresponding to topoisomers with a mean of +3 supercoils.
pBR322 DNA treated with topoisomerase I in the presence of increasing amounts
of H_2TMPyP^{4+} (lanes 4 to 8) shows topoisomer bands with increasing numbers of
negative supercoils. ZnTMPyP^{4+} (lanes 12-16) has a much smaller effect
than H_2TMPyP^{4+}.

decrease in the mean of the distribution at low concentrations of unwinding

agent. The positively supercoiled molecules (lanes 3,4 and 11) migrate more

slowly than negatively supercoiled molecules (lanes 5 to 8). The effects on

the distributions caused by H_2TMPyP^{4+} and ethidium bromide were quantified

by estimating the means (centres) of the distributions and plotting the

change in the means against the concentration of unwinding agent (Figure 3).

The least squares lines give the change in the number of supercoils per uM

concentration of unwinding agent of 5 ± 1 for ethidium bromide (c.f. 4.9 in

reference 20) and 6 ± 1 for H_2TMPyP^{4+}.   ZnTMPyP^{4+} (lanes 12 to 16) has a

much smaller effect. It causes a maximum negative change of 2 - 3 supercoils.

The limit is not due to inhibition of the topoisomerase. ZnTMPyP^{4+} added at

a P/D = 20 does not inhibit the effect of ethidium bromide (P/D = 20) on

topoisomerisation. The limit suggests that ZnTMPyP^{4+} unwinds DNA through

interactions at a small number of sites on the DNA. Alternatively it is

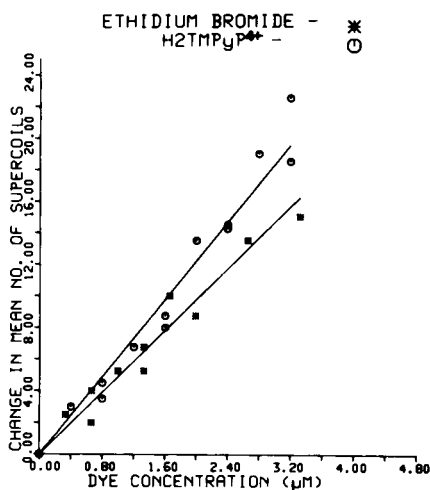possible the effect of ZnTMPyP^{4+} is due to a derivative of ZnTMPyP^{4+} formed

Figure 3. Change in supercoiling induced by H_2TMPyP^{4+} and ethidium bromide. Change in the mean number of supercoils at the centre of the topoisomer distribution plotted against concentration of intercalator required to induce that change. The lines through the points are derived from a least squares analysis. pBR322 at a concentration of 105 μM nucleotide.

by reaction with a limited trace amount of impurity in the topoisomerisation reaction.

Absorption and Fluorescence Spectra. The effect on the visible absorption spectra of adding a large excess of CT-DNA to a solution of either H_2TMPyP^{4+} or ZnTMPyP^{4+} in 0.05M (pH = 6.9) potassium phosphate buffer with no added sodium chloride is shown in Figure 4. In close agreement with the data of Pasternack et al. (10), we find that for H_2TMPyP^{4+} there is a substantial shift in the Soret band peak position (from 423 to 437 nm) and a pronounced hypochromicity. In contrast, for ZnTMPyP^{4+} there is only a much smaller shift (from 437 to 439 nm) and the extinction coefficient is only slightly affected. The wavelengths of the Q-bands of the porphyrins are also altered by DNA-interaction, those for H_2TMPyP^{4+} being red-shifted, while those for ZnTMPyP^{4+} move to the blue. When sodium chloride is added to these porphyrin-DNA solutions (to a final concentration of NaCl of 1 M) all these shifts are largely reversed (Figure 4b).

The influence of DNA base composition on these spectral shifts was examined by studying the synthetic polynucleotides poly(d(G_C)) and poly(d(A-T)). For H_2TMPyP^{4+} the peak position of most bands are substantially red-shifted for poly (d-(G-C)) but less so for poly(d(A-T)). The Soret band at 423 nm shifts to 445 nm in the presence of poly(d(G-C)) and to 431 nm with poly (d(A-T)) in u = 0.2 M buffer. On the other hand, for ZnTMPyP^{4+} there are only small red shifts in the Soret band position for either poly(d(G-C)) or poly(d(A-T)) and a small blue shift of the Q-bands with poly(d(A-T)). As expected CT-DNA (42% GC) shows intermediate behaviour.
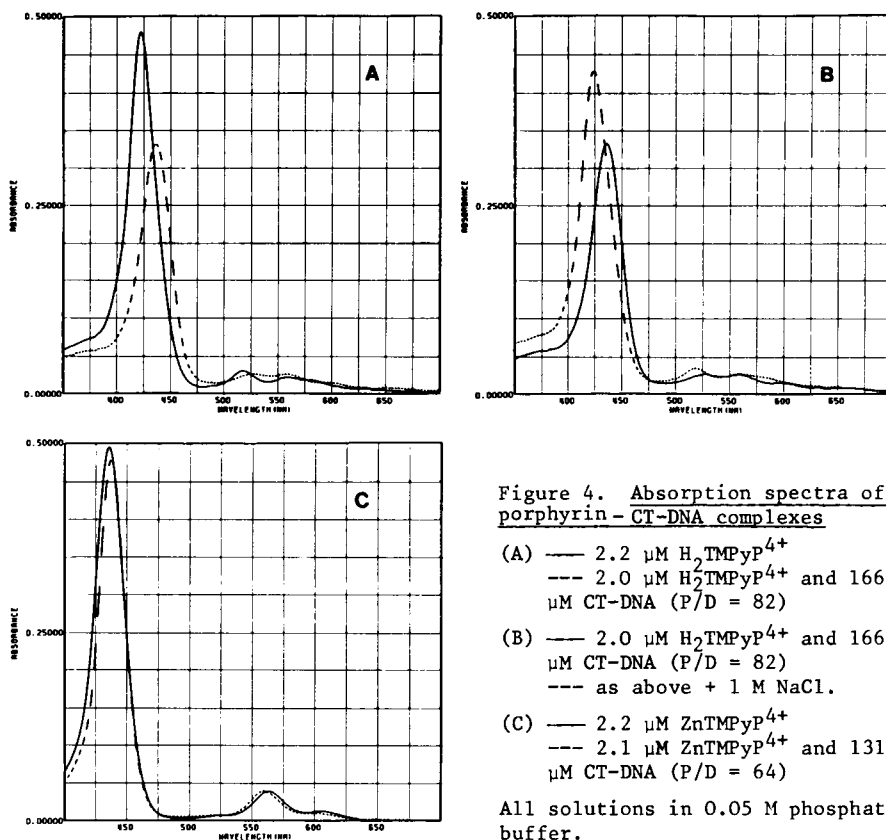
Figure 4. Absorption spectra of porphyrin – CT-DNA complexes

(A) —— 2.2 μM H_2TMPyP^{4+}
--- 2.0 μM H_2TMPyP^{4+} and 166 μM CT-DNA (P/D = 82)

(B) —— 2.0 μM H_2TMPyP^{4+} and 166 μM CT-DNA (P/D = 82)
--- as above + 1 M NaCl.

(C) —— 2.2 μM $ZnTMPyP^{4+}$
--- 2.1 μM $ZnTMPyP^{4+}$ and 131 μM CT-DNA (P/D = 64)

All solutions in 0.05 M phosphate buffer.

The fluorescence spectra of each of the porphyrins were taken in the presence of a large excess of CT-DNA, poly[d(G-C)] and poly[d(A-T)], (see figures 5 and 6); μ = 0.2 M buffer solutions and identical excitation conditions (423 nm for H_2TMPyP^{4+} and 437 nm for $ZnTMPyP^{4+}$) were used for each series. For H_2TMPyP^{4+}, the interaction with CT-DNA causes splitting of the broad fluorescence band (centred at about 675 nm in the absence of DNA) into two distinct bands with peaks at 654 and 714 nm. (A further very weak band may also be discerned at about 615 nm. This feature is more pronounced if solutions of H_2TMPyP^{4+} were not freshly prepared and might be caused in part by an impurity). As is also shown in Figure 5, the base composition of the polynucleotide exerts a strong influence on the emission intensity from H_2TMPyP^{4+}. With poly[d(A-T)] present, two bands are noted at 655 and 715 nm, the intensity being greatly enhanced compared to that of H_2TMPyP^{4+} alone. By contrast, in the presence of poly[d(G-C)] the emiss-
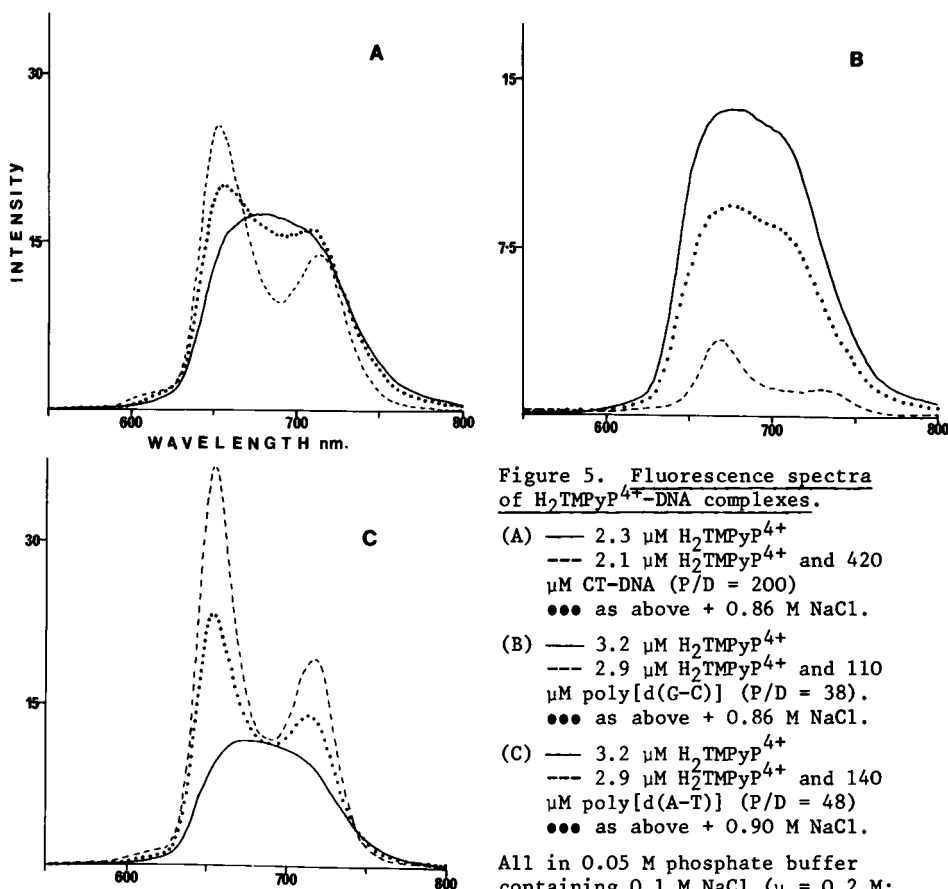
Figure 5. Fluorescence spectra of H_2TMPyP^{4+}-DNA complexes.

(A) —— 2.3 μM H_2TMPyP^{4+}
--- 2.1 μM H_2TMPyP^{4+} and 420 μM CT-DNA (P/D = 200)
••• as above + 0.86 M NaCl.

(B) —— 3.2 μM H_2TMPyP^{4+}
--- 2.9 μM H_2TMPyP^{4+} and 110 μM poly[d(G-C)] (P/D = 38).
••• as above + 0.86 M NaCl.

(C) —— 3.2 μM H_2TMPyP^{4+}
--- 2.9 μM H_2TMPyP^{4+} and 140 μM poly[d(A-T)] (P/D = 48)
••• as above + 0.90 M NaCl.

All in 0.05 M phosphate buffer containing 0.1 M NaCl (μ = 0.2 M; pH = 6.8). λ_{exc} = 423 nm.

ion intensity of H_2TMPyP^{4+} is markedly quenched and the peaks are also shifted to longer wavelengths (669 and 730 nm) compared to those found for poly[d(A-T)]. The reduced emission intensity in this case is mainly a consequence of less absorption of the excitation due to shifting of the absorption maximum of H_2TMPyP^{4+} from 423 to 445 nm, but it is however also partly due to a reduced quantum yield of fluorescence for H_2TMPyP^{4+} when bound to poly[d(G-C)]. Making the solution 1 M in NaCl reduces the observed effects, but in each case, and especially for poly[d(A-T)], there is clear evidence for binding of a substantial fraction of the H_2TMPyP^{4+} to the polynucleotide even under these high salt conditions. (In the absence of DNA, 1 M NaCl has no effect on the emission spectrum of H_2TMPyP^{4+}).
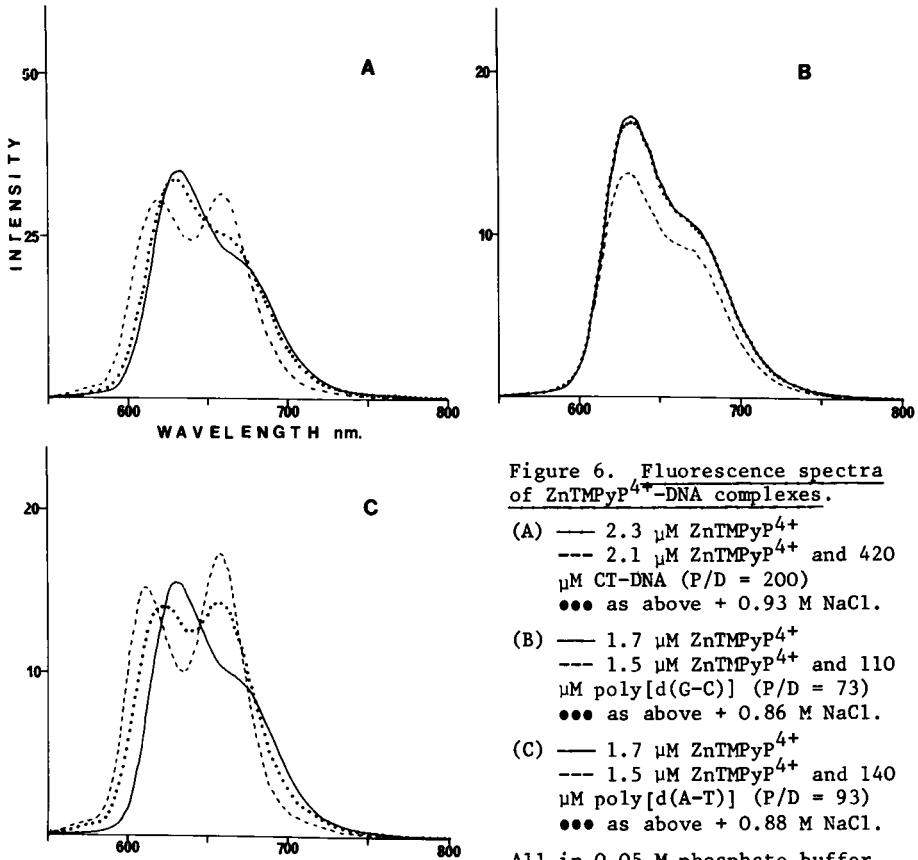
Figure 6. Fluorescence spectra of ZnTMPyP^{4+}-DNA complexes.

(A) —— 2.3 μM ZnTMPyP^{4+}
    --- 2.1 μM ZnTMPyP^{4+} and 420 μM CT-DNA (P/D = 200)
    ●●● as above + 0.93 M NaCl.

(B) —— 1.7 μM ZnTMPyP^{4+}
    --- 1.5 μM ZnTMPyP^{4+} and 110 μM poly[d(G-C)] (P/D = 73)
    ●●● as above + 0.86 M NaCl.

(C) —— 1.7 μM ZnTMPyP^{4+}
    --- 1.5 μM ZnTMPyP^{4+} and 140 μM poly[d(A-T)] (P/D = 93)
    ●●● as above + 0.88 M NaCl.

All in 0.05 M phosphate buffer containing 0.1 M NaCl (μ = 0.2 M; pH = 6.8). λ_{exc} = 437 nm.

The effect of polynucleotides on the fluorescence spectrum of ZnTMPyP^{4+} is shown in Figure 6. This spectrum (λ_{max} at 632 nm, shoulder at ca.665 nm), is hardly affected by poly[d(G-C)], and an almost complete reversal of the small reduction in intensity observed is caused by making the solution 1 M in NaCl. With poly[d(A-T)], however, two peaks are observed in the spectrum which are blue shifted (at 612 and 658 nm) compared to that of uncomplexed ZnTMPyP^{4+}. 1 M NaCl causes a shifting of these peaks to 624 and 658 nm, but it is clear that much of the ZnTMPyP^{4+} remains bound to the polynucleotide. With CT-DNA pronounced splitting of the bands is also observed the peaks being at 618 and 657 nm.

As addition of polynucleotide causes substantial changes in the inten-

sity of porphyrin fluorescence at certain wavelengths, fluorimetry may be
used to assay the extent of binding of the porphyrin and hence to calculate
binding constants.   In optically dilute samples the intensity of emission
at a particular wavelength will be controlled by the absorption of the
excitation by the bound and unbound porphyrin (i.e. by their relative
concentrations and extinction coefficients at λ_{exc}) and by their relative
fluorescence yield at the emission wavelength (λ_{em}).   The intensity of
emission of the bound porphyrin may be estimated by extrapolation of the
data at high P/D values.   From this and the intensity data at various P/D
ratios, the binding isotherm r/m versus r may be obtained (r = number of
moles of bound porphyrin per mole of base pairs of polynucleotide;
m = molar concentration of free porphyrin) (22).   Preliminary experiments
of this kind have been carried out for H_2TMPyP^{4+} (2.4 x 10^{-6} M) in
poly[d(G-C)] (λ_{exc} = 423 nm; λ_{em} = 668 nm), for H_2TMPyP^{4+} (2.4 x 10^{-6} M)
in poly[d(A-T)] (λ_{exc} = 423 nm; λ_{em} = 655 nm) and for $ZnTMPyP^{4+}$ (1.6 x 10^{-6}M)
in poly[d(A-T)] (λ_{exc} = 437 nm; λ_{em} = 660 nm) all in 0.05 M KH_2PO_4/K_2HPO_4:
0.1 M NaCl (μ = 0.2 M) buffer and with polynucleotides at concentrations so
that the P/D varied from 0 to 25 or greater.   Analysis of these data
(corrected for the optically non-dilute conditions used) employing the
McGhee-von Hippel method (10,22,23) yields similar binding constants
(K_{app}) of 1 - 2 x 10^6 M^{-1} for H_2TMPyP^{4+} in poly[d(G-C)], or in poly[d(A-T)]
and for $ZnTMPyP^{4+}$ in poly[d(A-T)].   These K_{app} values may be compared to
literature values obtained from absorption spectral data for H_2TMPyP^{4+} in
poly[d(G-C)] of 1.1 x 10^7 M^{-1} (from Scatchard analysis) (1) and 7.7 x 10^5 M^{-1}
(from McGhee-von Hippel analysis) (10).

   Photolysis of DNA.   Although DNA is not affected by low energy visible
light it has been reported that phosphodiester bonds may be photolysed in
the presence of porphyrins including H_2TMPyP^{4+} (4).   The effect of $ZnTMPyP^{4+}$
has not been reported.   We have studied the effects of both compounds on
the photolysis of DNA by visible light by following the conversion of ccc
DNA to oc DNA to ℓ DNA using pBR322 at a P/D ratio of ca. 50   (Figure 7).
A single break in the phosphodiester bonds of the sugar phosphate backbone
of pBR322 ccc DNA will convert it to oc DNA, and if two breaks occur in
opposite strands less than 5-10 base pairs from each other then oc DNA will
be converted to ℓ DNA.   The wavelength (436 nm) of the irradiation used was
close to the Soret maxima of the H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ in DNA.   No photo-
lysis of the DNA backbone occurred in the absence of porphyrin, and both
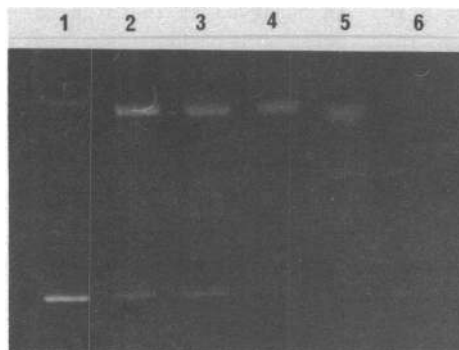porphyrins sensitised photolysis.   As shown in Figure 7 during the first

Figure 7. H_2TMPyP^{4+}-induced photolysis of plasmid DNA. pBR322 DNA irradiated for varying lengths of time in the presence of H_2TMPyP^{4+} using light of 436 nm wavelength at a P/D of ca.50. Lanes 1 to 6, left to right, 0, 5, 10, 15, 30 and 100 minutes exposure time.

15 minutes of irradiation the ccc DNA band disappeared with a corresponding increase in the oc DNA band. After longer periods of irradiation a discrete band corresponding to ℓ DNA of unit size appeared, while some DNA migrated faster than ℓ DNA in a continuous distribution, presumably smaller DNA which had accumulated a large number of breaks.

Fiel et al. have noted that sodium azide (58 mM), which is known to be a quencher of singlet oxygen, completely inhibits the effects of three porphyrins (HPD, TCPP and TSPP) on the photolysis of DNA and partially inhibits the effect of H_2TMPyP^{4+} [4]. In our hands sodium azide (at 100 mM) also partially blocked the effect of both H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ (data not shown), confirming that singlet oxygen probably has a role in photolysis of H_2TMPyP^{4+} and that this is also probably the case for $ZnTMPyP^{4+}$

DISCUSSION

The experiments reported here corroborate and extend the evidence that both H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ interact with DNA. This is, for example, demonstrated by their coelectrophoresis with DNA. The difference in the nature of this binding, however, is revealed by the experiment using topoisomerase I. $ZnTMPyP^{4+}$ may unwind DNA to a very limited extent: H_2TMPyP^{4+} does with an efficiency similar to that of ethidium bromide, a known intercalator [20]. This is an explicit demonstration of intercalation for H_2TMPyP^{4+}, and confirms the conclusions of earlier electrophoresis [2], viscosity [1,9] and circular dichroism [1,6,7,8,10] experiments. If we assume that all H_2TMPyP^{4+} molecules are bound during topoisomerisation we may calculate the unwinding angle per molecule of bound DNA. The measured change in supercoils per uM of H_2TMPyP^{4+} added was 6 (Figure 3). This is equivalent to an unwinding angle of 26° per residue, similar to that determined for ethidium bromide [24]. No

diminution in the unwinding ability of H_2TMPyP^{4+} is observed at the highest concentrations tested (P/D = 32) so we conclude that not all intercalative/ unwinding sites have been filled at this ratio. This suggests there are more than 300 intercalative sites per pBR322 molecule (4362 base pairs) for H_2TMPyP^{4+}. As yet there are insufficient data on site specificity. The binding constants for H_2TMPyP^{4+} with poly(d(G-C)) and poly(d(A-T)) estimated from fluorescence show that H_2TMPyP^{4+} binds about equally to G-C and A-T sites. In agreement with this the fluorescence spectrum of H_2TMPyP^{4+} in CT-DNA with 42% G-C is intermediate between that of poly(d(A-T)) and poly (d(G-C)).

The effect of $ZnTMPyP^{4+}$ on topoisomerisation is much less than that of H_2TMPyP^{4+} (Figure 2) even though both compounds bind about equally tightly. At the lowest concentration (0.4 μM $ZnTMPyP^{4+}$) equivalent to a P/D ratio of 200 there is a change of 2 - 3 negative supercoils, but this is the maximum effect observed with no further change up to 3.2 μM $ZnTMPyP^{4+}$. It is poss- ible that $ZnTMPyP^{4+}$ may unwind through interactions at a number of specific sites which are saturated at P/D = 200 or more. This would be a particularly interesting interpretation if true, and further experiments are being carried out to test it. It is clear at this stage that the interaction of $ZnTMPyP^{4+}$ with DNA differs quantitatively with respect to unwinding from that of H_2TMPyP^{4+}.

As illustrated in Figures 5 and 6 interaction with DNA leads to sub- stantial changes in the intensity and spectra of the fluorescence of the porphyrins. Furthermore these changes depend on the base composition of the polynucleotide. Upon interaction with poly(d(A-T)), for example, the broad fluorescence band of H_2TMPyP^{4+} splits into two main bands with an overall shift to higher energy and a very considerable increase in intensity. As Kano et al. have recently pointed out the broad featureless fluorescence band of H_2TMPyP^{4+} is atypical of most monomeric porphyrins and these authors have suggested that in aqueous solution this porphyrin may exist as an aggregate even at concentrations as low as 10^{-7} M (26). Most other evidence, however, points to H_2TMPyP^{4+} being monomeric under these condit- ions (27 and references therein). If H_2TMPyP^{4+} were to be present as a dimer or other oligomer then the change of the fluorescence spectrum upon interaction with DNA could be viewed, at least in part, as a consequence of deaggregation. It should be noted however that enhancement of intensity upon interaction of other dyes, such as acridines or phenanthridines, with poly(d(A-T)) is well established (28,29) and this behaviour has been

ascribed largely to the removal of water from the solvation shell of the
dye. Indeed in the case of ethidium it has been argued that the excited
state is deactivated via proton transfer to water (30). It is striking
that the fluorescence spectrum of H_2TMPyP^{4+} in poly(d(A-T)) very closely
resembles that in methanol (26). This may be attributed to the H_2TMPyP^{4+}
in this polynucleotide being in an environment similar to that of a polar
organic solvent and in particular to a reduction of water in its immediate
vicinity. By contrast in poly(d(G-C)) the emission quantum yield of
H_2TMPyP^{4+} is somewhat reduced and the emission bands are at about 300 cm^{-1}
to lower energy than in poly(d(A-T)). Quenching of the fluorescence of
dyes intercalated into poly(d(G-C)) is commonly found (28,29) and is consid-
ered to be a consequence of a redox interaction between the excited state
of the dye and guanine (29,31). A similar explanation is probably appro-
priate here as the singlet excited state of H_2TMPyP^{4+} is expected to be a
strong reductant. $(E(P^*/P^-)$ may be calculated as +1.60 V using the data
in reference 27).

The fluorescence spectra of $ZnTMPyP^{4+}$ are markedly different in
poly(d(G-C)) and poly(d(A-T)). The spectrum in poly(d(G-C)) is very similar
to that in buffer, although there is a small ($\sim 20\%$) decrease in emission
intensity. This may be taken to indicate that any interaction of the por-
phyrin with the polynucleotide does not appreciably perturb its energy
levels. With poly (d(A-T)), however, a very marked change in the emission
spectrum is observed. This modification of the fluorescence may be con-
trasted with the rather slight changes observed in the absorption spectrum
of this porphyrin upon addition of the polynucleotide. The splitting of
the fluorescence into two bands and the overall shifting to higher energy
indicates that there must be a substantial modification of the environment
of the porphyrin molecule upon interaction. That this is not merely a weak
electrostatic binding is supported by the fact that making the solution 1 M
in NaCl only partly reduces the binding (whereas the spectrum of $ZnTMPyP^{4+}$
in a solution containing poly(d(G-C)) and 1 M NaCl is closely similar to
that of the porphyrin by itself in buffer). Interestingly, unlike the
behaviour observed for H_2TMPyP^{4+} a splitting of the broadened fluorescence
band is not observed in methanol indicating that merely removing most of the
water does not appreciably alter this compound's photophysical properties.
Indeed we have been unable to find a report of conditions in which $ZnTMPyP^{4+}$
exhibits the type of spectrum found upon interaction with poly(d(A-T)). One
possibility is that when bound to the polynucleotide the molecule is held

more rigidly, leading to a sharpening of its features. This matter requires further investigation.

In summary it may be seen from the absorption and fluorescence spectra that poly(d(G-C)) strongly perturbs the electronic properties of H_2TMPyP^{4+} but has only a minor influence on those of $ZnTMPyP^{4+}$. This is consistent with intercalation at G-C base pairs occurring with the free base prophyrin but not with its zinc complex. It is probable that $ZnTMPyP^{4+}$ is only bound electrostatically to the outside of poly (d(G-C)). This differing behaviour is probably a consequence of hindrance due to the axial ligand attached to the zinc, as has been discussed by Pasternack et al. (10). On the other hand, there are obvious and apparently similar changes in the emission spectra of both H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ when they interact with poly(d(A-T)). The effect of salt shows that this binding cannot be merely electrostatic and there is almost certainly substantial interaction with the bases. As the topoisomerase experiment indicates that little unwinding occurs for the $ZnTMPyP^{4+}$, it is likely that this interaction with poly(d(A-T)) is non-intercalative for this compound.

The conversion of pBR322 from its closed circular to open circular form upon visible light irradiation in the presence of H_2TMPyP^{4+} or $ZnTMPyP^{4+}$ indicates that this process causes single strand nicking. That both porphyrins show similar reactivity suggests that intercalation is not a prerequisite for photocleavage, as has already been pointed out by Fiel and co-workers for H_2TMPyP^{4+} and other porphyrins (4). With both H_2TMPyP^{4+} and $ZnTMPyP^{4+}$ the photosensitised cleavage is partially inhibited by 0.1 M sodium azide, a known scavenger for singlet oxygen. The singlet oxygen is presumably formed by reaction of the triplet state of the porphyrin with ground state oxygen. In this connection it may be noted that the yield of the triplet state is substantially reduced when H_2TMPyP^{4+} intercalates into DNA (32), implying that the yield of $^1O_2$ should also be lower in this system. It is interesting to speculate whether the base specificity of intercalation, the influence of the bases on the photophysical properties of the porphyrins and the effects of salt or quenchers on the photochemical reaction may be used to induce base selective cleavage reactions. These matters are under investigation at present.

ships for MJM and COhU, and the Radiation Laboratory University of Notre Dame for a visiting fellowship for MJM during which some of this work and related studies were carried out.

Abbreviations:

P/D : Molar concentration of DNA phosphate to dye (e.g. porphyrin).

ccc : covalently closed circular. ℓ : linear.   oc : Open circular.

H_2TMPyP^{4+} and $ZnTMPyP^{4+}$: see Figure 1.   CT-DNA : Calf thymus DNA.

DTT = dithiothreitol.

REFERENCES
1.   Fiel, R.J., Howard, J.C., Mark, E.H. and Datta-Gupta, N. (1979) Nucleic Acids Res. 6, 3093-3118.
2.   Fiel, R.J. and Munson, B.R. (1980) Nucleic Acids Res. 8, 2835-2842.
3.   Musser, D.A., Datta-Gupta, N. and Fiel, R.J. (1980) Biochem. Biophys. Res. Commun. 97, 918-925.
4.   Fiel, R.J., Datta-Gupta, N., Mark, E.H. and Howard, J.C. (1981) Cancer Res. 41, 3543-3545.
5.   Fiel, R.J., Beerman, T.A., Mark, E.H. and Datta-Gupta, N. (1982) Biochem. Biophys. Res. Commun. 107, 1067-1074.
6.   Carvlin, M.J., Datta-Gupta, N. and Fiel, R.J. (1982) Biochem. Biophys. Res. Commun. 108, 66-73.
7.   Carvlin, M.J. and Fiel, R.J. (1983) Nucleic Acids Res. 11, 6121-6139.
8.   Carvlin, M.J., Mark, E., Fiel, R.J. and Howard, J.C. (1983) Nucleic Acids Res. 11, 6141-6154.
9.   Banville, D.L., Marzilli, L.G. and Wilson, W.D. (1983) Biochem. Biophys Res. Commun. 113, 148-154.
10. Pasternack, R.F., Gibbs, E.J. and Villafranca, J.J. (1983) Biochemistry 22, 2406-2414.
11. Pasternack, R.F., Gibbs, E.J. and Villafranca, J.J. (1983) Biochemistry 22, 5409-5417.
12. (a) Gouterman, M. (1978) in: The Porphyrins. 3, 1-65, ed. by Dolphin, D., Academic Press, New York.
     (b) Darwent, J.R., Douglas, P., Harriman, A., Porter, G. and Richoux, M.C. (1982) Coord. Chem. Rev. 44, 83-126.
13. Little, R.G., Anton, J.A., Loach, P.A. and Ibers, J.A. (1975) J. Hetero-cyc. Chem. 12, 343-349.
14. Pasternack,R.F., Huber, P.R., Boyd, P., Engasser, G., Francesconi, L., Gibbs, E., Fasella, P., Cerio, V.G. and Hinds, L. (1972) J. Am. Chem. Soc. 94, 4511-4517.
15. Quimby, D.J. and Longo, F.R. (1975) J. Am. Chem. Soc. 97, 5111-5117.
16. Kalyanasundaram, K. and Neumann-Spallart, M. (1982) J. Phys. Chem. 86, 5163-5169.
17. Harriman, A., Porter, G. and Richoux, M.C. (1982) J. Chem. Soc. Faraday Trans. II, 78, 1955-1970.
18. Neta, P. (1981) J. Phys. Chem. 85, 3678-3684.
19. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. a Laboratory Manual, Cold Spring Harbour Laboratory.
20. Keller, W. (1975) Proc. Natl. Acad. Sci. USA 72, 4876-4880.
21. Ishii, H., Satoh, K., Satoh, Y. and Koh, H. (1982) Talanta 29, 545-550.
22. Dougherty, G. and Pigram, W.J. (1982) C.R.C. Crit. Rev. Biochem. 103-131.

23. McGhee, J.D. and von Hippel, P.H. (1974) J. Mol. Biol. 86, 469-489.
24. Wang, J.C. (1974) J. Mol. Biol. 89, 783-801.
25. (a) Dougherty, G. and Waring, M.J. (1982) Biophys. Chem. 15, 27.
    (b) Dougherty, G. (1982) Comments Mol. Cell Biophys. 1, 337-354.
26. Kano, K., Miyake, T Yomoto, K., Sato, T., Ogawa, T. and Hashimoto, S. (1983) Chem. Lett. 1867-1870.
27. Kalyanasundaram, K. and Neumann-Spallart, M. (1982) J. Phys. Chem. 86, 5163-5169.
28. Schreiber, J.P. and Daune, M.P. (1974) J. Mol. Biol. 83, 487-501.
29. Löber, G. (1981) J. Lumin. 22, 221.265.
30. Olmsted, J. and Kearns, D.R. (1977) Biochemistry 16, 3647-3654.
31. Löber, G. and Kittler, L. (1973) Stud. Biophys. (Berlin) 73, 25-30.
32. Murphy, M.J. (1984) Unpublished results.