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Interactions of new water soluble porphyrins with nucleic acids

Xu, Zhengjiu, Ph.D.

City University of New York, 1990

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Ann Arbor, MI 48106**

A

**INTERACTIONS OF NEW WATER SOLUBLE
PORPHYRINS WITH NUCLEIC ACIDS**

by

Zhengjiu Xu

**A dissertation submitted to the Graduate Faculty in
Chemistry in partial fulfillment of the requirements for the
degree of Doctor of Philosophy, the City University of
new York.**

1990

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This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

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date

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Abstract

INTERACTIONS OF NEW WATER SOLUBLE POYPHYRINS WITH NUCLEIC ACIDS

by

Zhengjiu Xu

Adviser: Professor David K. Lavalley

Tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and its N-methyl derivative N-MTAMPP were synthesized because (1) The peripheral amino groups of both porphyrins are predicted to be protonated at neutral pH, which is an important property for studies of interactions of porphyrins with double stranded nucleic acid, and (2) N-methylated porphyrins may be used for selective alkylation of DNA studies. In the synthetic scheme we have used, it is possible to use an uncharged precursor porphyrin in which the amino groups are protected during the alkylation step. It was found that the meso aryl substituents of N-MTAMPP rotate slower than the NMR time scale. The effect is consistent with the peripheral meso-aryl groups spending more time perpendicular to the porphyrin ring at room temperature than porphyrins whose aryl groups rotate more rapidly. This result would cause stronger basicity of the amino groups of N-MTAMPP ($pK_a = 7.8$) than those of TAMPP ($pK_a = 6.2$), at ionic strength $[I] = 0.12$ M.

Four methods have been used to study the binding of TAMPP and N-MTAMPP with calf thymus DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC):

(1) Induced circular dichroism (CD) spectra of porphyrin-nucleic acid systems indicate directly that the two porphyrins do interact and bind to the three nucleic acids;

(2) Changes in the fluorescence spectra of porphyrin on binding to nucleic acids disclose that there are different binding modes between porphyrins and nucleic acids.

Intercalation into and electrostatic attraction with nucleic acids result in hypochromic character. Insertion into groove of nucleic acid results in hyperchromic character;

(3) The hypochromic and bathochromic characteristics of visible spectra of porphyrins on binding to nucleic acids is used to deduct modes of binding;

(4) A new method of competitive-binding data treatment makes it straight forward to determine the binding constant of a porphyrin with nucleic acids using the method of competitive binding against a fluorescence probe, ethidium bromide (EB).

The information uncovered by above four different methods are consistent. It was found that : (1) Both TAMPP and N-MTAMPP bind mostly at A-T rich regions of DNA; (2) Both TAMPP and N-MTAMPP bind on the outside of DNA. The TAMPP may bridge two strands of DNA at the narrow groove, interacting by electrostatic attraction with two adjacent negatively charged phosphate groups. In contrast, N-MTAMPP may insert into alternate major grooves of DNA and cause the two adjacent grooves to be deformed; (3) Both TAMPP and N-MTAMPP can intercalate into Poly(dG-dC) • Poly(dG-dC); (4) All of the porphyrin — nucleic acid systems have relative high affinity constant s (10^6 or greater).

Metalloporphyrins, Mn(III)•TAMPP and Co(II)•N-MTAMPP, were synthesized. Their interaction with nucleic acids was studied by circular dichroism , visible absorption and binding affinity. The results demonstrate that the two metalloporphyrins have the higher binding affinities (10^7) to DNA than the metal free forms.

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Table of Contents

Chapter 1	Introduction	1
	Bibliography	8
Chapter 2	Synthesis of Tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and N-methyl tetrakis(p-aminomethylphenyl) porphyrin	10
2-1	Introduction	10
2-2	Materials and Instruments	11
2-3	Results and Discussion	12
2-4	Experimental Section	67
	Bibliography	84
Chapter 3	Acidity Constant Studies of TAMPP and N-MTAMPP	86
3-1	Introduction	86
3-2	Materials and Instruments	89
3-3	Results	90
3-3.1	The acidity constants of porphyrin pyrroleninic ring — spectrophotometric method	90
3-3.1a	TAMPP	90
3-3.1b	N-MTAMPP	96
3-3.2	The acidity constants of porphyrin pyrroleninic ring — potentiometric method	108
3-3.2a	TAMPP	108
3-3.2b	N-MTAMPP	121
3-3.3	The acidity constants of amino group on outside of porphyrin ring — potentiometric titration method	122
3-4	Discussion	127
	Bibliography	134
Chapter 4	Circular Dichroism, Absorption and Fluorescence Spectra of Porphyrin — Nucleic Acid System in the Visible Region	135

4-1	Introduction	135
4-2	Materials and Instruments	140
4-3	Results	141
4-3.1	Circular dichroism of porphyrin — nucleic acid	142
4-3.2	Absorption spectra of porphyrin — nucleic acid	147
4-3.3	Fluorescence spectra of porphyrin — nucleic acid	157
4-4	Discussion	164
	Bibliography	176
Chapter 5 The Affinity of Tetrakis(4-aminomethylphenyl)porphyrin and its N-methyl Derivative for Poly- Nucleic Acid		
5-1	Introduction	178
5-2	Materials and Instruments	180
5-3	Analytical Method.....	182
5-3.1	Derivation of general equation for competitive binding of a molecule to nucleic acid	182
5-3.2	Specific equation for this work	186
5-3.3	Examination of the extent of energy transfer from ethidium bromide to porphyrin	189
5-3.4	Spectrofluometric determination of the bound concentration of ethidium bromide	203
5-4	Results and Experimental Approach	206
5-5	Discussion	218
	Bibliography	228
Chapter 6 The Synthesis and Interaction Studies of Mn•TAMPP and Co•N-MTAMPP with Nucleic Acids		
6-1	Introduction	230
6-2	Materials and Method	231
6-2.1	Materials	232
6-2.2	Synthesis of metalloporphyrin	233
6-2.2a	Synthesis of Mn•TAMPP	233
6-2.2b	Synthesis of Co•N-MTAMPP	238
6-2.3	Instruments	245
6-3	Results	245

6-3.1	Circular dichroism spectra of metalloporphyrin	
	— nucleic acid system	245
6-3.2	Visible absorption spectra of metalloporphyrin	
	— nucleic acid system	250
6-3.3	Affinity constant studies of metalloporphyrin	
	— CT.DNA systems	255
6-4	Discussion	255
	Bibliography	259
Appendix 1	The experimental data Of spectrophotometric titration to TAMPP.....	260
Appendix 2	The experimental data of potentiometric titration to TAMPP.....	264
Appendix 3	The experimental data of spectrophotometric titration to N-MTAMPP.....	270
Appendix 4	The experimental data of potentiometric titration to N-MTAMPP.....	274
Appendix 5	The equation for the competitive binding of two different drugs.....	282
Appendix 6	Experimental data for affinity constant studies of TAMPP—DNA and N-MTAMPP—DNA.....	286
Appendix 7	Experimental data for affinity constant studies of TAMPP—Poly(dA-dT) and N-MTAMPP—Poly(dA-dT)	295
Appendix 8	Experimental data for affinity constant studies of TAMPP—Poly(dG-dC) and N-MTAMPP—Poly(dG-dC)	307
Appendix 9	Experimental data for affinity constant studies of Mn•TAMPP—DNA and Co•N-MTAMPP—DNA	318

List of Tables

Chapter 3

Table 3-1.	129
Table 3-2.	130
Table 3-3.	131

Chapter 4

Table 4-1. Summary of circular dichroism, absorbance and fluorescence results in the visible region	166

Chapter 5

Table 5-1. Data for affinity constant study of EB — CT.DNA	207
Table 5-2. Data for affinity constant study of TAMPP — CT.DNA	212
Table 5-3. Affinity constants of TAMPP—nucleic acids	216
Table 5-4. Affinity constants of N-MTAMPP—nucleic acids	217
Table 5-5. Affinity constants of porphyrin—nucleic acids	219
Table 5-6. Number of nucleic acid base pairs per bound drug	219

Chapter 6

Table 6-1. affinity constants of Mn•TAMPP — CT.DNA and Co•N-MTAMPP — DNA complexes	256

List of Figures

Chapter 1.

Figure 1-1. The molecular structure of TMPyP-4 2

Chapter 2

Figure 2-1. The NMR spectrum of α -bromo-tolunitrile 14

Figure 2-2. The NMP spectrum of 4-formylbenzylbromide 16

Figure 2-3. The NMP spectrum of 4-formylbenzylphthalimide 18

Figure 2-4. The NMP spectrum of tetrakis(p-phthalimidomethylphenyl) porphyrin 20

**Figure 2-5. The NMP spectrum of N-methyl
tetrakis(p-phthalimidomethylphenyl)porphyrin 24**

Figure 2-6. The NMP spectrum of
tetrakis(p-phthalamidomethylphenyl)porphyrin 28

Figure 2-7 The molecular structure of
tetrakis(p-phthalamidomethylphenyl)porphyrin 30

Figure 2-8. The NMP spectrum of TAMPP 32

Figure 2-9. The NMP spectrum of N-MTAMPP (room temp.) 35

Figure 2-10. The molecular structure of N-MTAMPP 37

Figure 2-11. The NMP spectrum after decoupling D₁ of N-MTAMPP 39

Figure 2-12. The NMP spectrum after decoupling D₂ of N-MTAMPP 41

Figure 2-13. The NMP spectrum after decoupling D₆ of N-MTAMPP 43

Figure 2-14. The NMP spectrum after decoupling D₃ of N-MTAMPP 46

Figure 2-15. The NMP spectrum after decoupling D₄ of N-MTAMPP 48

Figure 2-16. The NMP spectrum after decoupling D₅ of N-MTAMPP 50

Figure 2-17. The NMP spectrum after decoupling D_c of N-MTAMPP 53

Figure 2-18. The NMR spectrum of N-MTAMPP (at 50 °C) 55

Figure 2-19. The NMR spectrum of N-MTAMPP
(at 30 °C, 50 °C, 70 °C and 90 °C) 58

Figure 2-20. The NMP spectrum after decoupling D_c of
N-MTAMPP at 90 °C 60

Figure 2-21. The NMP spectrum after decoupling D_a of N-MTAMPP at 90 °C 62

Figure 2-22. The NMR spectrum of TAMPP

(at 30 °C, 50 °C, 70 °C and 90 °C)	64
Figure 2-23. The NMP spectra of TAMPP and TMPyP-4	73
Figure 2-24. The visible spectrum of TAMPP	76
Figure 2-25. The NMP spectra of N-MTAMPP and TMPyP-4	78
Figure 2-26. The visible spectrum of N-MTAMPP	80

Chapter 3

Figure 3-1. The visible spectra of TAMPP in its neutral and diprotonated form (I=0.12 M).....	91
Figure 3-2. The spectrophotometric titration curve of TAMPP: A_{648}/A_{516} vs. pH (I=0.12 M)	93
Figure 3-3. The visible spectra of TAMPP in its neutral and diprotonated form (I<0.01 M).....	97
Figure 3-4. The spectrophotometric titration curve of TAMPP: A_{648}/A_{516} vs. pH (I<0.01 M)	99
Figure 3-5. The visible spectra of N-MTAMPP in its neutral mono- and diprotonated form (I=0.12 M).....	101
Figure 3-6. The spectrophotometric titration curve of N-MTAMPP: A_{666}/A_{618} vs. pH (I=0.12 M)	104
Figure 3-7. The spectrophotometric titration curve of N-MTAMPP: A_{666}/A_{588} vs. pH (I=0.12 M)	106
Figure 3-8. The visible spectra of N-MTAMPP in its neutral mono- and diprotonated form (I<0.01 M).....	109
Figure 3-9. The spectrophotometric titration curve of N-MTAMPP: A_{666}/A_{618} vs. pH (I<0.01 M)	111
Figure 3-10. The spectrophotometric titration curve of N-MTAMPP: A_{666}/A_{588} vs. pH (I<0.01 M)	113
Figure 3-11. The potentiometric titration curve of TAMPP (I=0.12 M)	117
Figure 3-12. The potentiometric titration curve of TAMPP (I<0.01 M)	119
Figure 3-13. The potentiometric titration curve of N-MTAMPP (I=0.12 M)	123
Figure 3-14. The potentiometric titration curve of N-MTAMPP (I<0.01 M)	125

Chapter 4

Figure 4-1. The comparison between absorption and circular dichroism spectroscopy	137
Figure 4-2. The circular dichroism spectra of TAMPP with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	143
Figure 4-3. The circular dichroism spectra of N-MTAMPP with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	145
Figure 4-4. The visible spectra of TAMPP with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	148
Figure 4-5. The visible spectra of TAMPP in pH 1.5 to 4.1	150
Figure 4-6. The visible spectra of N-MTAMPP with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	153
Figure 4-7. The visible spectra of TMPyP-4 with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	157
Figure 4-8. The fluorescence spectra of TAMPP with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	158
Figure 4-9. The fluorescence spectra of N-MTAMPP with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	160
Figure 4-10. The fluorescence spectra of TMPyP-4 with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC)	162
Figure 4-11. The molecular structure of fluorene and biphenyl	172

Chapter 5

Figure 5-1. The molecular structure of ethidium bromide	187
Figure 5-2. The fluorescence spectra of EB, EB+DNA+TAMPP, EB+DNA, TAMPP and TAMPP+DNA	190
Figure 5-3. The fluorescence spectra of EB, EB+Poly(dA-dT)+TAMPP, EB+Poly(dA-dT), TAMPP and TAMPP+Poly(dA-dT)	192
Figure 5-4. The fluorescence spectra of EB, EB+Poly(dG-dC)+TAMPP, EB+Poly(dG-dC), TAMPP and TAMPP+Poly(dG-dC)	194
Figure 5-5. The fluorescence spectra of EB, EB+DNA+N-MTAMPP, EB+DNA, N-MTAMPP and N-MTAMPP+DNA	196
Figure 5-6. The fluorescence spectra of EB, EB+Poly(dA-dT) +N-MTAMPP, EB+Poly(dA-dT), N-MTAMPP and N-MTAMPP+Poly(dA-dT)	198

Figure 5-7. The fluorescence spectra of EB, EB+Poly(dG-dC) +N-MTAMPP, EB+Poly(dG-dC), N-MTAMPP and N-MTAMPP+Poly(dG-dC)	200
Figure 5-8. The scatchard's plot of EB+DNA system	208
Figure 5-9. The plot of table (5-2) for EB+DNA+TAMPP system	213
Figure 5-10. The possible binding manner of N-MTAMPP on DNA	224
Figure 5-11. The possible binding manner of TAMPP on DNA	226

Chapter 6

Figure 6-1. The visible absorption spectra of TAMPP and Mn•TAMPP	234
Figure 6-2. The fluorescence absorption spectra of TAMPP and Mn•TAMPP	236
Figure 6-3. The visible spectra of N-MTAMPP and Co•N-MTAMPP in ph 5.5	239
Figure 6-4. The fluorescence absorption spectra of N-MTAMPP and Co•N-MTAMPP	241
Figure 6-5. The visible spectra of N-MTAMPP and Co•N-MTAMPP in pH 7.4	243
Figure 6-6. The circular dichroism spectra of Mn•TAMPP with DNA, Poly(dA-dT) and Poly(dG-dC)	246
Figure 6-7. The circular dichroism spectra of Co•N-MTAMPP with DNA, Poly(dA-dT) and Poly(dG-dC)	248
Figure 6-8. The visible absorption spectra of Mn•TAMPP with DNA, Poly(dA-dT) and Poly(dG-dC)	251
Figure 6-9. The visible absorption spectra of Co•N-MTAMPP with DNA, Poly(dA-dT) and Poly(dG-dC)	253

Chapter 1

Introduction

There have been extensive studies of the interactions between water soluble meso-substituted porphyrins bearing four or less than four positive charges on their peripheral groups and natural DNA (calf thymus) or synthetic homopolymers (Poly (dG-dC) • Poly (dG-dC) and Poly (dA-dT) • Poly (dA-dT)).

In 1979, Fiel and coworkers ¹ first reported that the water soluble, cationic tetrakis(4-N-methylpyridyl)porphine (TMPyP-4, figure 1-1) can intercalate in calf thymus DNA. Since then, the interaction of porphyrins with nucleic acids has been studied a great deal by using a variety of experimental techniques and different TMPyP-4 derivatives or analogues.

The porphyrins that have been studied can be subdivided into two groups: (1) those which are planar and have no axial ligands when they bind to nucleic acids, including both free base porphyrins as well as metalloporphyrins without axial ligands (for example Ni(II), and Cu(II)-TMPyP-4 derivatives) and (2) those nonplanar metalloporphyrins which have axial ligands. Examples of the later type of metalloporphyrins are Mn(III), Fe(III), Co(III) and Zn(II)-TMPyP-4 derivatives. It has been shown that the planar group of TMPyP-4 derivatives can intercalate into DNA in the GC-rich regions and the nonplanar metal complexes of TMPyP-4 cannot intercalate. The latter can bind on the outside of the AT-rich regions of DNA ¹⁻⁵.

The intercalation of a variety of porphyrins containing positively charged peripheries into DNA or a polynucleic acid were studied by Carvlin et al ^{6,7}. They employed two positional isomers (2-N-methylpyridyl and 3-N-methylpyridyl groups in place of the 4-N-methylpyridyl groups) and one analogue (p-trimethylanilinium groups

Figure 1-1. The molecular Structure of tetrakis(4-N-methylpyridyl) porphyrin (TMPyP-4)



in place of the 4-N-methylpyridyl groups of meso-tetrakis(4-N-methylpyridyl)porphine) as probes to identify and characterize ligand-induced alteration of DNA and polynucleic acid structures. They found that the molecular conformation of the porphyrin ligand, that is the location and orientation of the positively charged substituents, alters the intercalation profile of a meso-substituted porphine and determines whether a given derivation can be incorporated into the DNA helix.

Sari and coworkers ⁸ used various meso-tetrakis(4-N-methylpyridyl)porphine derivatives bearing 1 to 4 positive charges on their meso-substituents to investigate the importance of the number and relative position of the positive charges of cationic porphyrins on their interaction with DNA. Their results show that porphyrins bearing 3 or only 2 positive charges are intercalated into calf-thymus DNA and therefore they suggest that the presence of the polyaromatic tetrapyrrol ring is more important than a great number of positive charges for the intercalation to take place. They derived high affinity constants (between 10^6 - 10^7) that depends on the net charge carried in some extent and the binding pattern between porphyrin and DNA.

However, until now the interaction of N-alkylated TMPyP-4 analogue derivatives with nucleic acids has not been reported. The reason is that the synthesis of these compounds is quite difficult. The synthesis of N-substituted tris- or tetrakis(4-methylpyridyl)porphines was attempted in this laboratory using existing methods several years ago without success. There was no detectable alkylated product from direct alkylation with neat methyl-fluorosulfonate at 100 °C for three weeks, from the treatment of the Zn(II) complexes with ethyldiazoacetate and subsequent work-up under several different sets of reaction conditions ⁹, from the treatment with diphenylsulfonium salts at high temperature ¹⁰, by Fe (III) migration reactions ¹¹, or even by the acid-assisted oxidative migration methods using cobalt ¹² that provided nearly quantitative methylation with most porphyrins.

The N-substituent of metalloporphyrins can sometimes be removed very easily ¹³⁻¹⁶ since the driving force of the formation of the much more stable planar metalloporphyrin leads to the ready release of the N-substituent under certain conditions, for example, in the presence of a strong nucleophile such as an amine. Therefore, it could be very nice if we could make some kind of N-alkylated porphyrin with a positively charged periphery which can intercalate into DNA and then pass its alkyl group to a specific base pair of DNA. From previous studies ¹³⁻¹⁶, the nitrogenous atom of nucleotide should behave as an especially active nucleophile. It would be interesting to see if there is evidence of intercalation or outside binding and if the association constants of the N-alkyl porphine derivatives with nucleic acids are high.

Due to the bulk of the N-substituent, N-alkylated porphyrins cannot maintain a planar structure: instead they assume a folded structure ^{17, 18}. Pasternack's results ³ show that nonplanar metal complexes of TMPyP-4 associate strongly but do not actually intercalate into nucleic acids. We also expect that the nonplanar N-alkylporphyrins may have difficulty intercalating.

It would also be interesting to bind a known DNA intercalator like 9-amino acridine to one of the peripheral groups of a porphyrin with positively charged peripheral groups. The purpose of the peripheral intercalator is to provide an "anchor" for the bound porphyrin, while the other three positively charged peripheral groups would help attract the N-substituent metalloporphyrin to the negatively charged surface of DNA. This combination might provide the best possibility for site-specific alkylation of DNA. In this way, the selective alkylation of DNA bases may be approached since the selectivity of an N-alkylporphyrin complex could lead to site specific selective alkylation of nitrogenous bases of DNA if the porphyrin has been anchored.

Due to the above reasoning, we are interested in synthesizing new non-substituted and N-substituent porphyrin pairs having positive charges on their peripheral

groups, which can be derived in acceptable yields. We decided to synthesize tetrakis-(p-aminomethylphenyl)porphine and its N-methylated derivative.

In this synthesis, we decided to begin with α -bromo-p-tolunitrile, a commercially available compound. Our plan was to reduce the tolunitrile group to an aldehyde and substitute the 2-bromo group with a phthalimide group. After the corresponding porphyrin had been synthesized by the standard Adler method ¹⁹, alkylation of pyridine nitrogen should be possible because the amino groups on the peripheral porphyrin would be protected from alkylation by phthalimido groups. Normally, phthalimido groups can be readily hydrolyzed by a hydrazine and alkaline or acidic media. The positive charge on the periphery of the porphyrin can be achieved by simple protonation by water at neutral pH to form NH_3^+ groups. Each type of reaction in this synthetic scheme can be found in the existing literature and we expected they could be modified to suit the properties of our specific compounds.

Eventual applications of DNA alkylation by N-substituted porphyrins as cytotoxic agents would require that the reagent have a lifetime sufficiently long enough to be reactive after location of the porphyrin in its correct position. The lifetime of an N-substituted metalloporphyrin in an aqueous solvent system and at a particular temperature depends not only on the nucleophile (in this case, the nitrogen atom of nucleotide), but also on the metal ion and the N-substituent ^{14, 15}. The most suitable metal ion for further selective alkylation studied may be Co(II) because its half-life for the loss of the N-substituent (from N-methyl or other simple N-alkyl group) is a few hours at 37 °C.

Non-N-methylated metalloporphyrins are the most extensively studied. Fiel, Pasternack, Kelly, Dabrowiak and their coworkers ²⁰⁻²³, have shown that the metal complexes of TMPyP-4 bind DNA with two modes of binding, outside binding and intercalation, depending on the nature of the bound metal ion and the porphyrin

structure. It has also been shown^{23, 24} that in the presence of various activators, such as potassium superoxide, Mn^{3+} , Co^{3+} and Fe^{3+} of TMPyP-4 can cleave DNA. This cleavage can be very useful in identifying the interaction sites of DNA and in uncovering the sequence dependent changes in DNA structure. Therefore we would like to synthesize the non-N-alkylated Mn(III)-TAMPP metalloporphyrin and study its specific properties when it binds with nucleic acids.

This work will begin with a discussion of the synthesis of tetrakis (p-aminomethylphenyl) porphine (TAMPP) and its N-methyl derivative (N-MTAMPP). Then the determination of the acid dissociation constants which are necessary to decide the pH which is to be used for studies of the interaction between the porphyrins and nucleic acids, will be discussed. Next, the binding characteristics of newly synthesized porphyrins with DNA, Poly(dA-dT)•Poly(dA-dT) and Poly(dG-dC)•Poly(dG-dC) will be investigated and the determination of affinity constants of the porphyrins with the three nucleic acids using a new data analysis method will be explained. We also synthesized the corresponding metalloporphyrins, Mn(III)•TAMPP and Co(II)•N-MTAMPP, and investigated their binding features and affinity constants with nucleic acids.

The above results discussed in this work should form the basis for two types of further studies: (1) cutting of DNA using the Mn(III) complex of the non-N-alkylated derivatives and (2) studies of the degree of opening of double stranded DNA using the transfer of ^{14}C labeled alkyl group from the N-alkyl derivatives.

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Chapter 2

Synthesis of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and N-methyl tetrakis(p-aminomethylphenyl)porphyrin.

2.1 Introduction

As previously stated, our object is to study the interaction between an N-substituted porphyrin containing positive charges on its peripheral groups and DNA. In previous work in this laboratory, numerous methods ¹⁻⁴ were used in attempting to synthesize N-substituted tetrakis(4-methylpyridyl)porphine (TMPyP-4) without success. Thus, we need to try a new approach in order to synthesize a new non-N-substituted and N-substituted porphyrin pair which have positive charges on their peripheral groups and can be obtained in acceptable yield.

The synthesis of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and its N-methylated derivative (N-MTAMPP) are reported in this work. There are several reasons to choose these porphyrins for our studies. First, the amino groups of TAMPP and N-MTAMPP are predicted to be protonated at neutral pH ($K_b = 4.2 \times 10^{-10}$ for aniline, while $K_b = 0.2$ for benzylamine). This is a useful property for studies of double stranded nucleic acids, because previous work by the groups of Fiel ⁵, Pasternack ⁶ and Le Pecq ⁷ have shown that porphyrins with positively charged peripheries have relatively high affinity constants for binding to DNA (for example, the apparent association constant found for the tetracationic porphyrin TMPyP-4 with calf thymus DNA is 10^7 M^{-1}). Second, the alkylation reaction can be accomplished by using an uncharged precursor porphyrin in which the amino groups are protected by phthalimide groups, a standard method, and then deprotonated afterward by hydrolysis

of phthalimide group using hydrazine or an alkaline medium. Thirdly, it was possible to construct a synthetic scheme for those compounds that appeared feasible using published methods. From literature precedents, we designed the synthetic scheme as follows. The initial step would begin with α -bromo-p-tolunitrile, a commercially available compound, by reduction of the tolunitrile ⁸, and then the bromine atom would be replaced with a phthalimide group. The derived compound should react with pyrrole to produce a tetrakis(p-phthalimidomethylphenyl)porphyrin ⁹. A pyrroleninic nitrogen atom of this porphyrin can then be methylated by methyltrifluoromethane ¹⁰. After this step, the phthalimido groups can be removed by hydrolysis using hydrazine ^{12,13,14}, or alkaline ¹¹, or acidic media ¹¹. Although each step of the potential synthetic scheme might require some changes from published procedures for analogous compounds (because the molecules we synthesize are different from those in previously published work), the known synthetic methods published for relevant functional groups provided us with an approach to our final product.

2.2 Materials and Instruments

The following starting materials: α -bromo-p-tolunitrile, calcium hydride, diisobutyl aluminum hydride as a 1 M solution in methylene chloride, pyrrole, and barium oxide were purchased from Aldrich Chemical Company, Inc. Barium hydroxide, methylene chloride, as well as other organic solvents were purchased from Fisher Scientific Company.

Nuclear Magnetic Resonance spectra were run on a GE QE-300 Spectrometer, equipped with the decoupling unit and a variable-temperature probe. Spectra were obtained by locking on to the specific solvent resonances for different solvents using the frequency lock system of the instrument.

To determine the apparent molecular weight of synthesized porphyrin, tetrakis(1-methyl-4-pyridyl)porphine as the tetra-p-tosylate salt (TMPyP-4) purchased from Aldrich Chemical Company, Inc. was used as a standard.

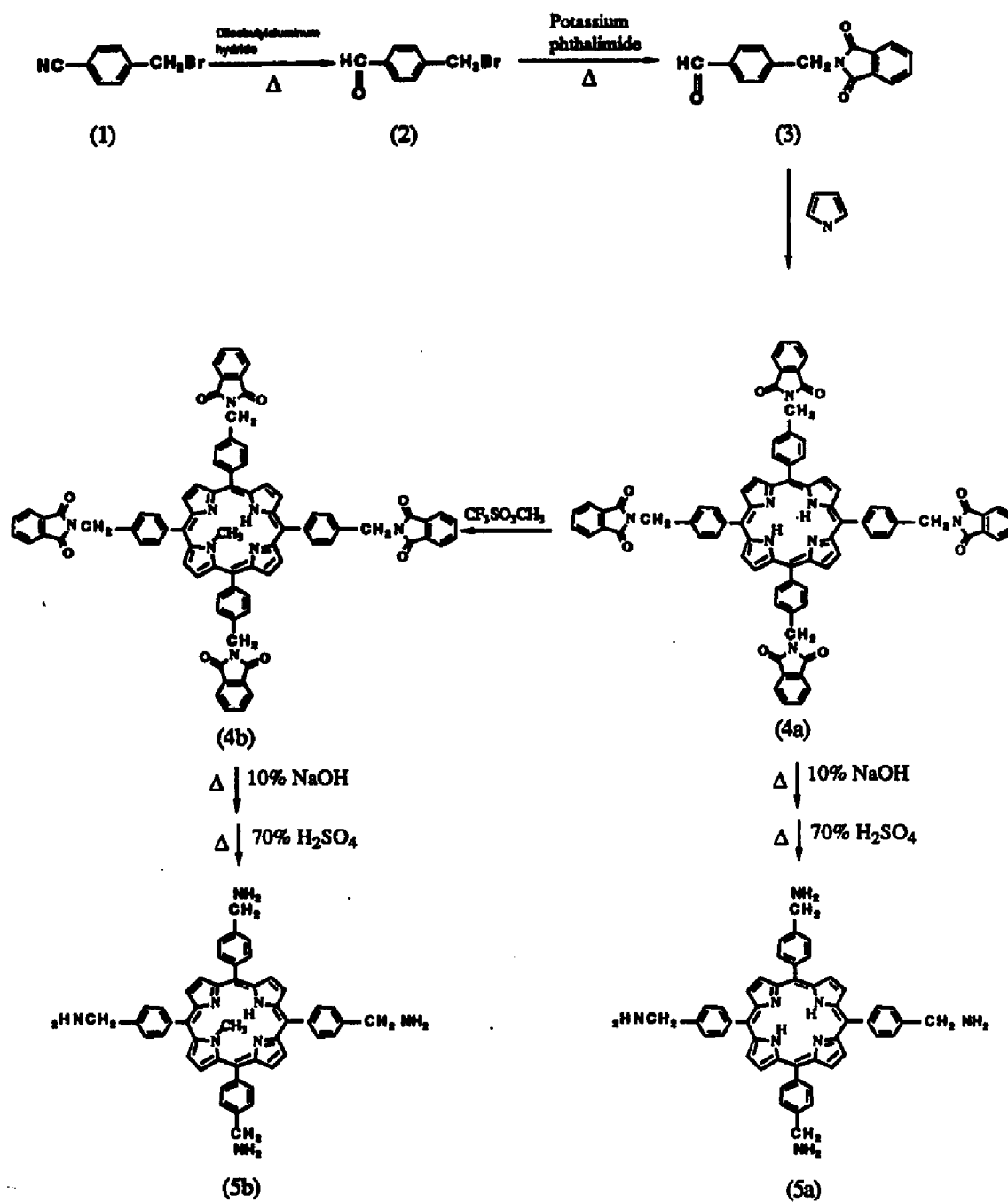
Visible absorption spectra were taken on the Beckman Du-8 spectrophotometer. Mass spectrometry experiments were run by Mass Spectrometric Biotechnology Resource of Rockefeller University. The elemental analysis experiments were conducted by Analytical Laboratories GMBH in Engelskirchen, Germany.

2.3 Results and Discussion

The synthetic process is shown in scheme I. It begins with the reduction of α -bromo-p-tolunitrile (1) with diisobutylaluminum hydride (Dibal), a very vigorous reaction which should be conducted under extremely dry conditions. Figure 2-2 shows the ^1H NMR spectra of synthesized 4-formylbenzylbromide (2) in which an extra new singlet ^1H peak appears. Comparing the NMR spectra of (1) (figure 2-1), and figure 2-2, a new singlet peak appears at position about 10.1 ppm. This position is typically that of an aldehyde group.

Next the phthalimido group was introduced to replace the bromine atom, to form 4-formylbenzylphthalimide (3). The ^1H peak of phthalimido group can be seen in figure 2-3 (two 2H quadruplets at the position of 7.78 and 7.92 ppm). One of the doublet peaks of the benzyl group mixes into one of the phthalimido quadruplets at the position about 7.9 and the other benzyl doublet is located at 7.62 ppm.

After these steps, the standard porphyrin synthesis method of Adler⁹ was used to derive tetrakis(p-phthalimidomethylphenyl) porphyrin (4a). The NMR spectrum of this porphyrin is shown in figure 2-4 with the singlet located at 8.81 ppm (at the typical position for pyrolleninic β -hydrogen resonance¹⁵) assigned as 8 protons, the relative integration for the other resonances (a singlet 2H peak from central nitrogenous



Scheme (I)

Figure. 2-1. The 300 MHz ^1H NMR spectrum of a solution of α -bromo - p - tolunitrile (substance 1 in scheme I) in CDCl_3 , room temperature, (The peak located at 7.3 ppm is contributed by trace of CHCl_3 in solvent)

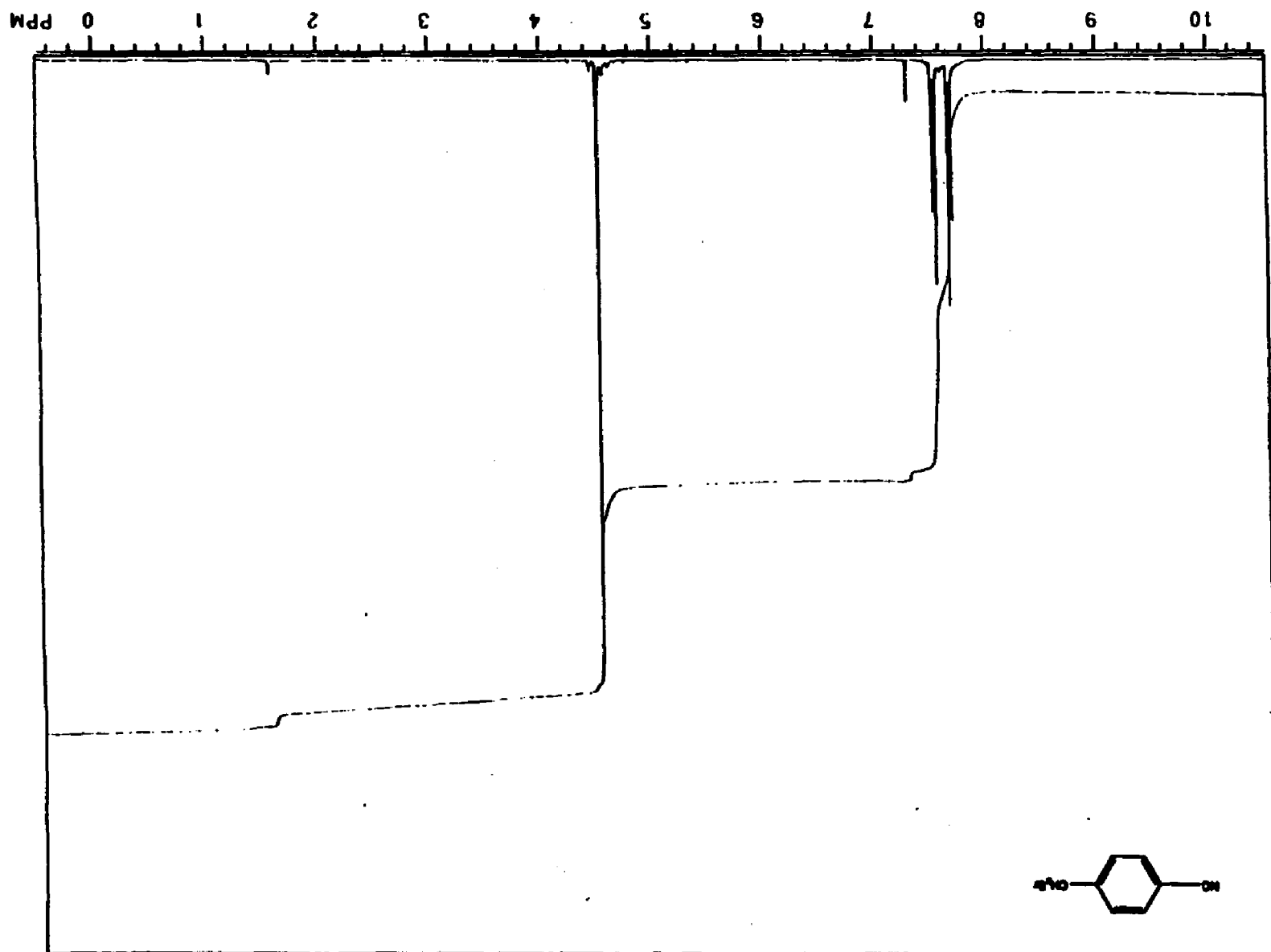


Figure. 2-2. The 300 MHz ^1H NMR spectrum of a solution of 4-formylbenzylbromide (substance 2 in scheme I) in CDCl_3 , room temperature, (The peak located at 7.3 ppm is contributed by trace of CHCl_3 in solvent)

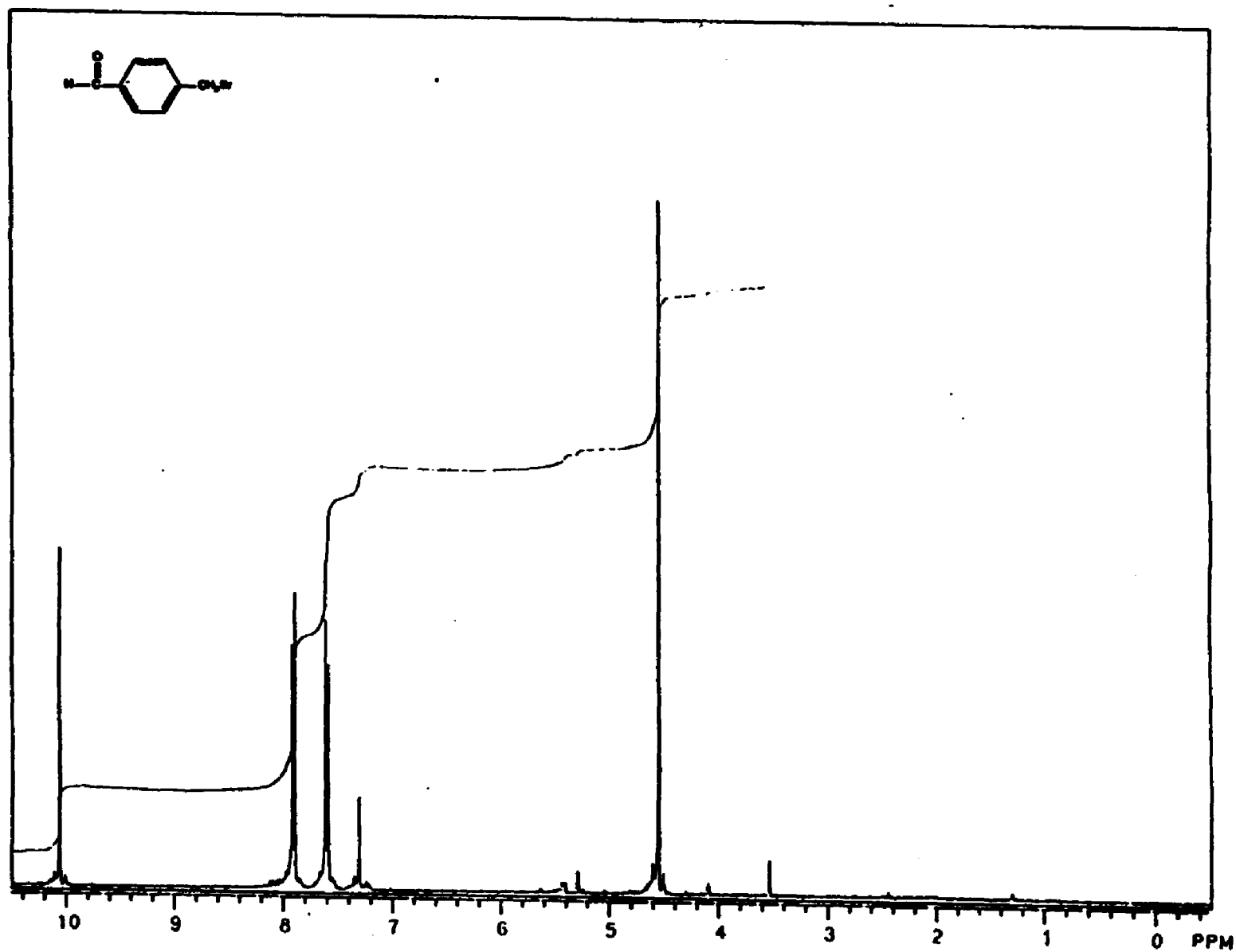


Figure. 2-3. The 300 MHz ^1H NMR spectrum of a solution of 4-formylbenzylphthalimide (substance **3** in scheme I) in CDCl_3 , room temperature, (The peak located at 7.3 ppm is contributed by trace of CHCl_3 in solvent)

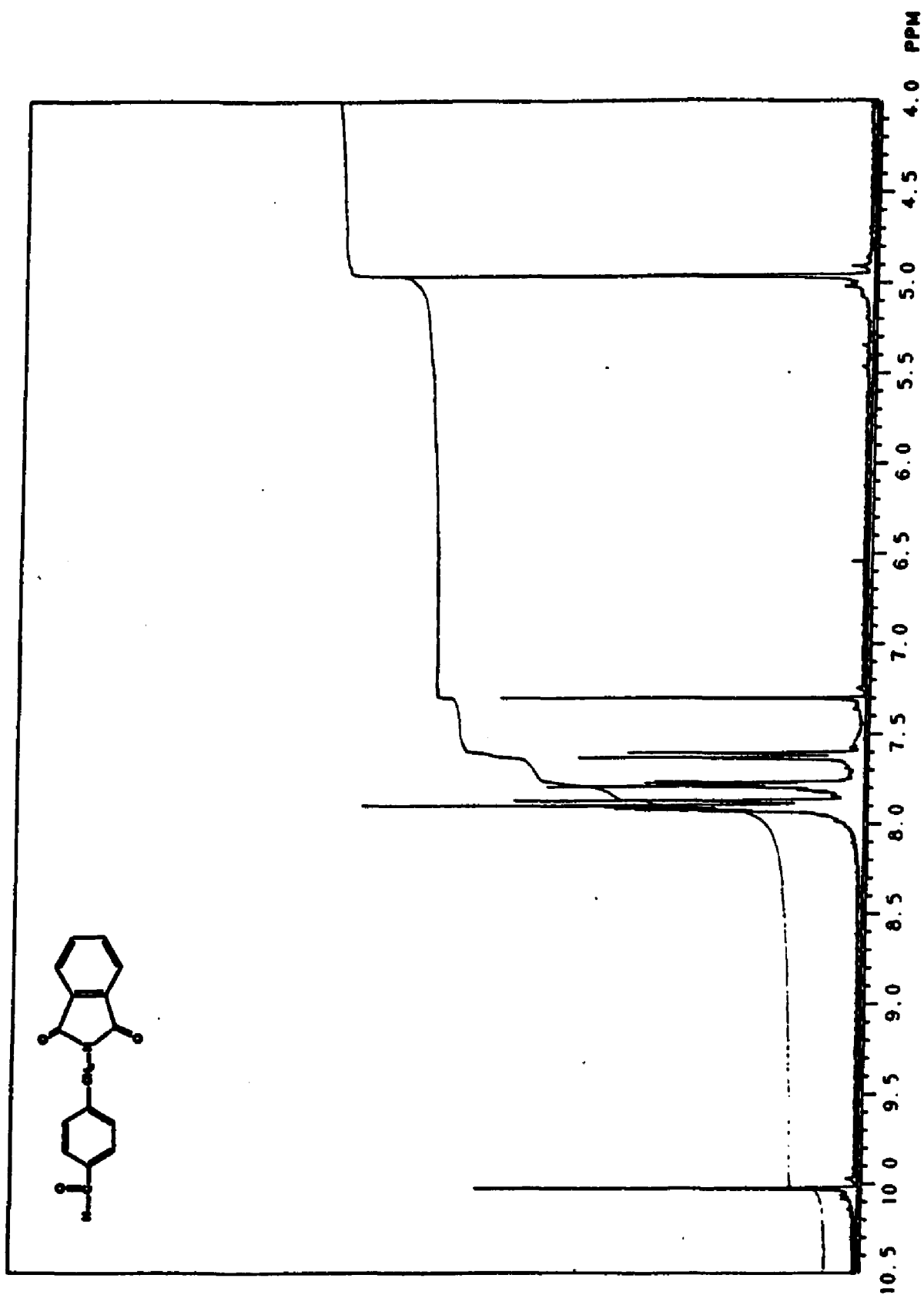
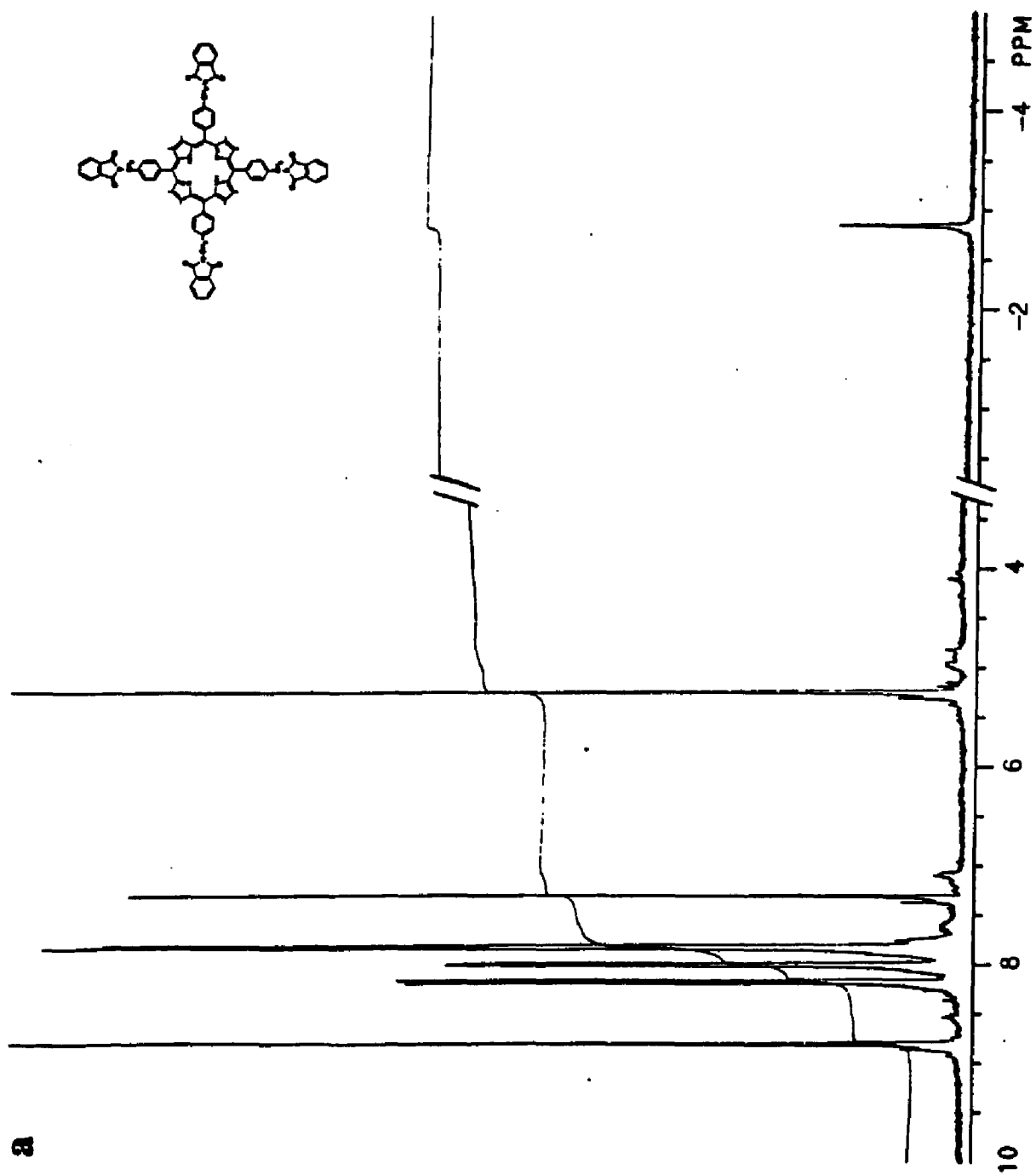
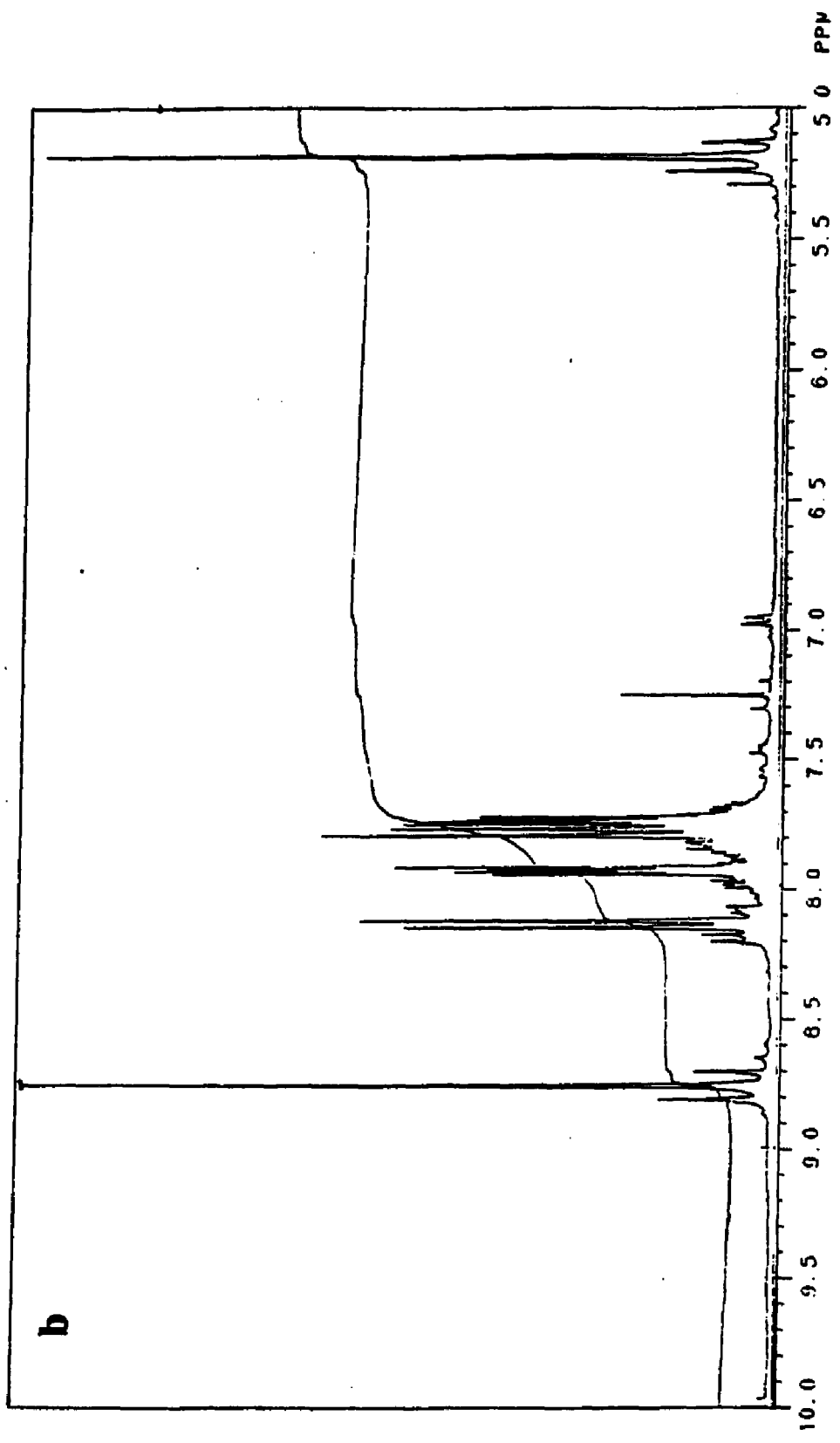


Figure. 2-4. (a) The overall , and (b) the expand 300 MHz ^1H NMR spectrum of a solution of tetrakis(p-phthalimidomethylphenyl) porphyrin (substance 4a in scheme I) in CDCl_3 , room temperature, (The peak located at 7.3 ppm is contributed by trace of CHCl_3 in solvent)





hydrogen atoms located at the upfield position -2.8 ppm, a singlet 8H from β -pyrrolenine hydrogen atoms of porphyrin ring located at 8.8 ppm, a singlet 8H from four methylene groups located at 5.2 ppm, two doublet 8H pairs from benzyl group and two quadruplet 8H pairs from the phthalimido groups are located closely at about 8.20, 7.8, 8.0 and 7.8 ppm) indicates the formation of the porphyrin (4a).

The phthalimido porphyrin (4a) is a key intermediate. In this compound, the aminomethylphenyl nitrogen atoms are protected during the step in which the central nitrogen of the porphyrin is alkylated. Figure 2-5a shows the overall ^1H NMR spectrum of N-methyl tetrakis(p-phthalimidomethylphenyl)porphyrin (4b). It is a much more complicated spectrum than that shown in figure 2-4. The singlet located at strong upfield at -4.2 ppm is contributed by N-methyl hydrogens ¹⁶ and the 8H singlet from four methylene hydrogens is still located at about 5.2 ppm. The region between 9.5 and 7.0 ppm of figure (2-5a) is expanded in figure (2-5b). It can be seen that the original 8H singlet located at 8.8 ppm assigned to the β -pyrrolenine hydrogens of porphyrin ring is split into two 2H singlets, S_1 and S_2 (located at 8.78 and 7.40 ppm) and two 2H doublets, D_1 and D_2 (located at 8.59 and 8.41 ppm). This change is caused by dissymmetry introduced by the methyl group of one of the central nitrogen atoms. The other peaks are contributed by the benzyl and phthalimido hydrogens and can be better explained after later discussion.

The phthalimido groups of porphyrins (4a) and (4b), cannot be quantitatively removed by the general method using an alkaline medium and hydrazine, but can be removed in a two-step hydrolysis process. Base first converts the imide functionality into an amide group. Then 70% sulfuric acid converts the amide to the amine ¹⁷. Figure 2-6 shows the ^1H NMR spectra of the first hydrolysis product, the non-methylated p-amidomethylphenylporphyrin. It can be seen easily from figure 2-6 that the compound still contains eight phthalimido hydrogen atoms. However, their

Figure. 2-5a. The overall 300 MHz ^1H NMR spectrum of a solution of N-methyl tetrakis(p-phthalimidomethylphenyl)porphyrin (substance 4b in scheme I) in CDCl_3 , room temperature.

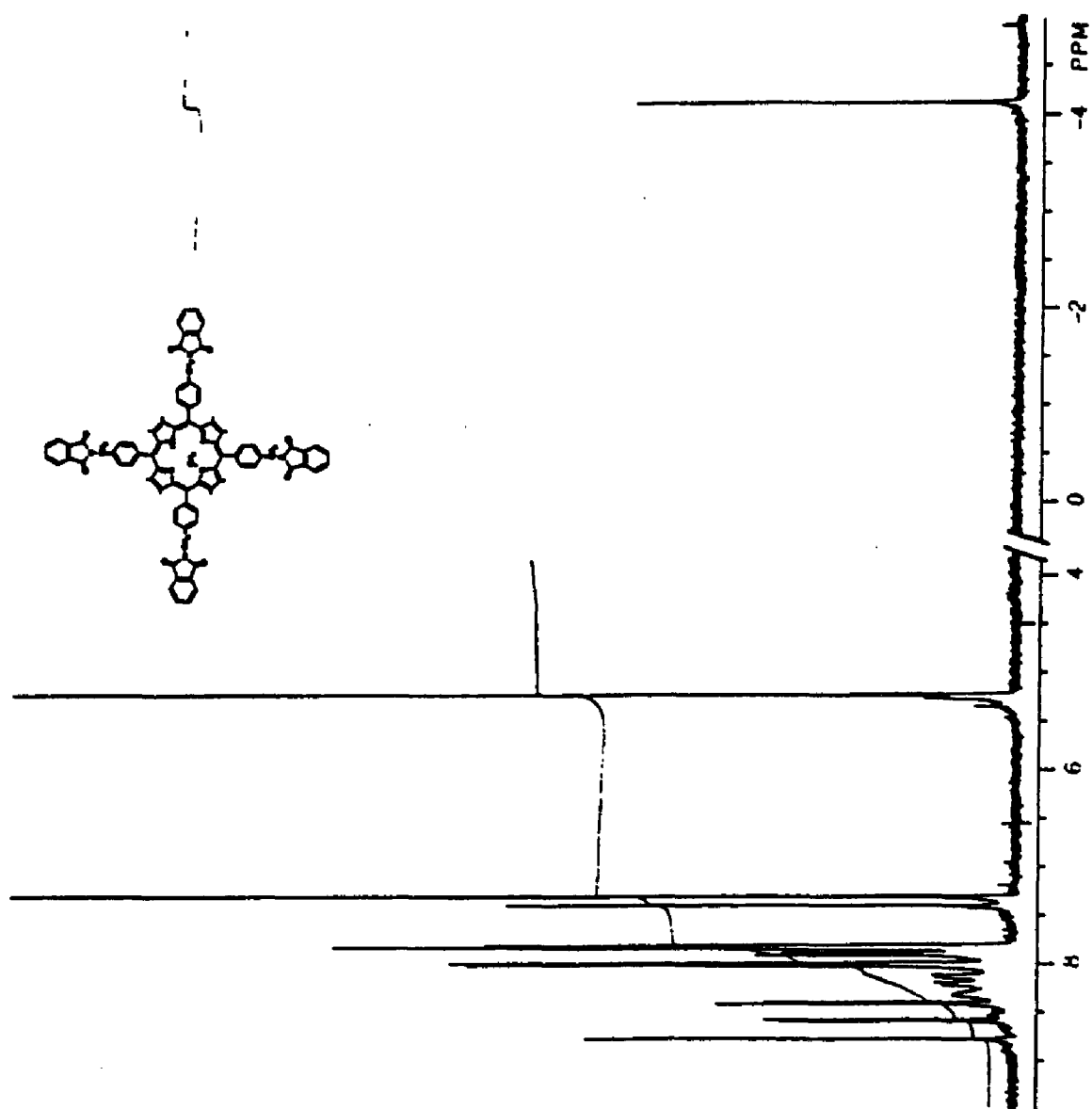


Figure. 2-5b. The expanded 300 MHz ^1H NMR spectrum (2-5a) between 9.50 and 7.00 ppm of a solution of N-methyl tetrakis(p-phthalimidomethylphenyl)porphyrin (substance 4b in scheme I) in CDCl_3 , room temperature, (The peak located at 7.3 ppm is contributed by trace of CHCl_3 in solvent)

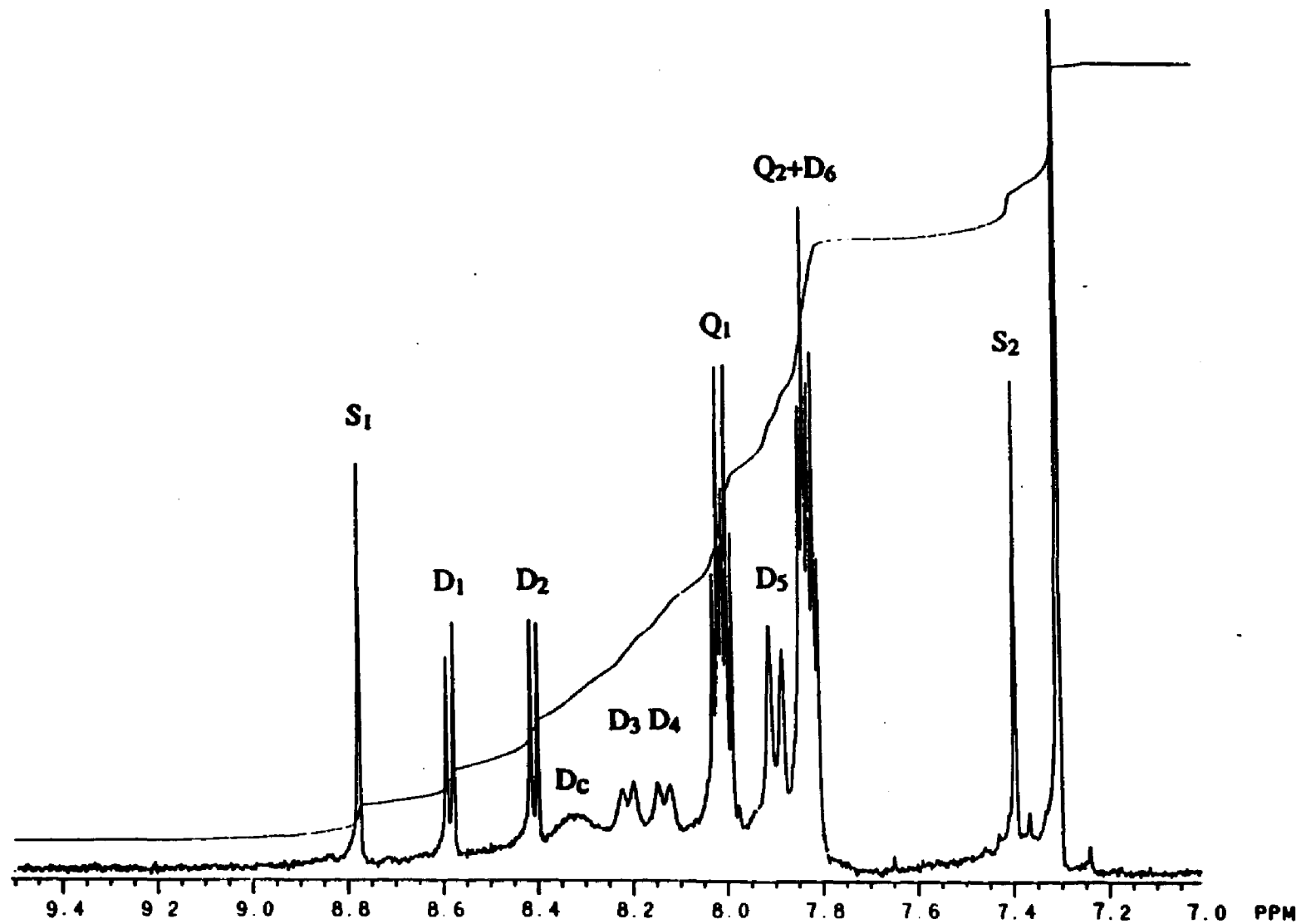


Figure. 2-6. The 300 MHz ^1H NMR spectrum of a solution of non-N-methylated tetrakis(p-phthalamidomethylphenyl)porphyrin (refer figure 2-7 for its structure) in CD_3OD , room temperature.

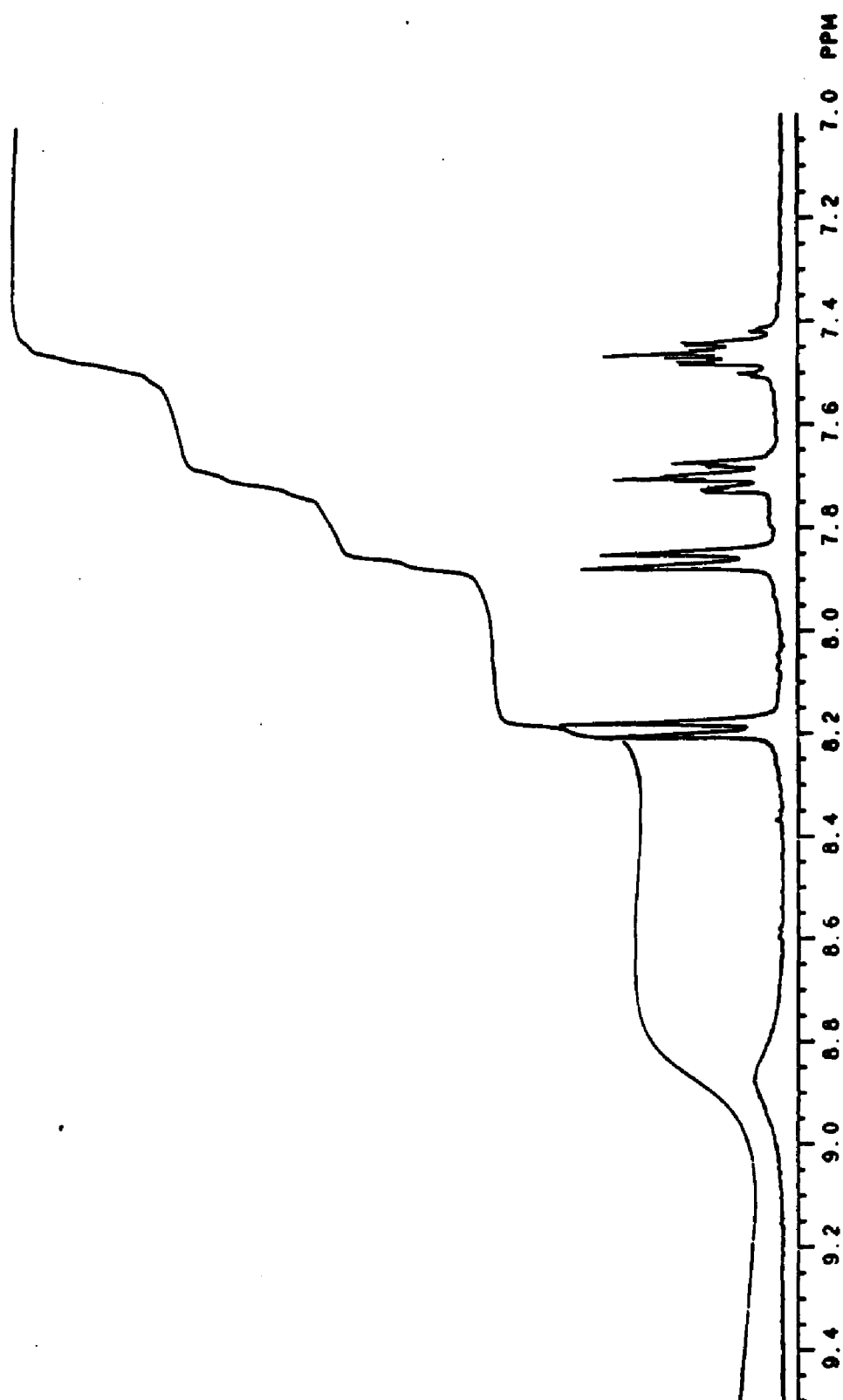


Figure. 2-7. The molecular structure of tetrakis(p-phthalamidomethylphenyl)porphyrin

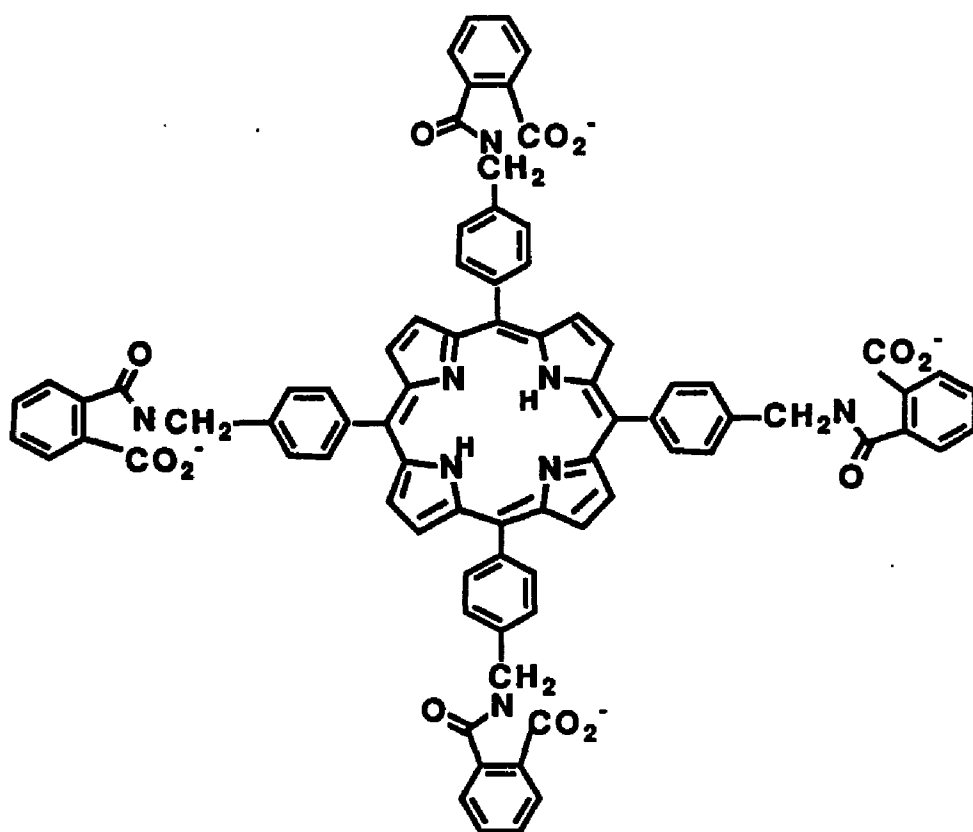
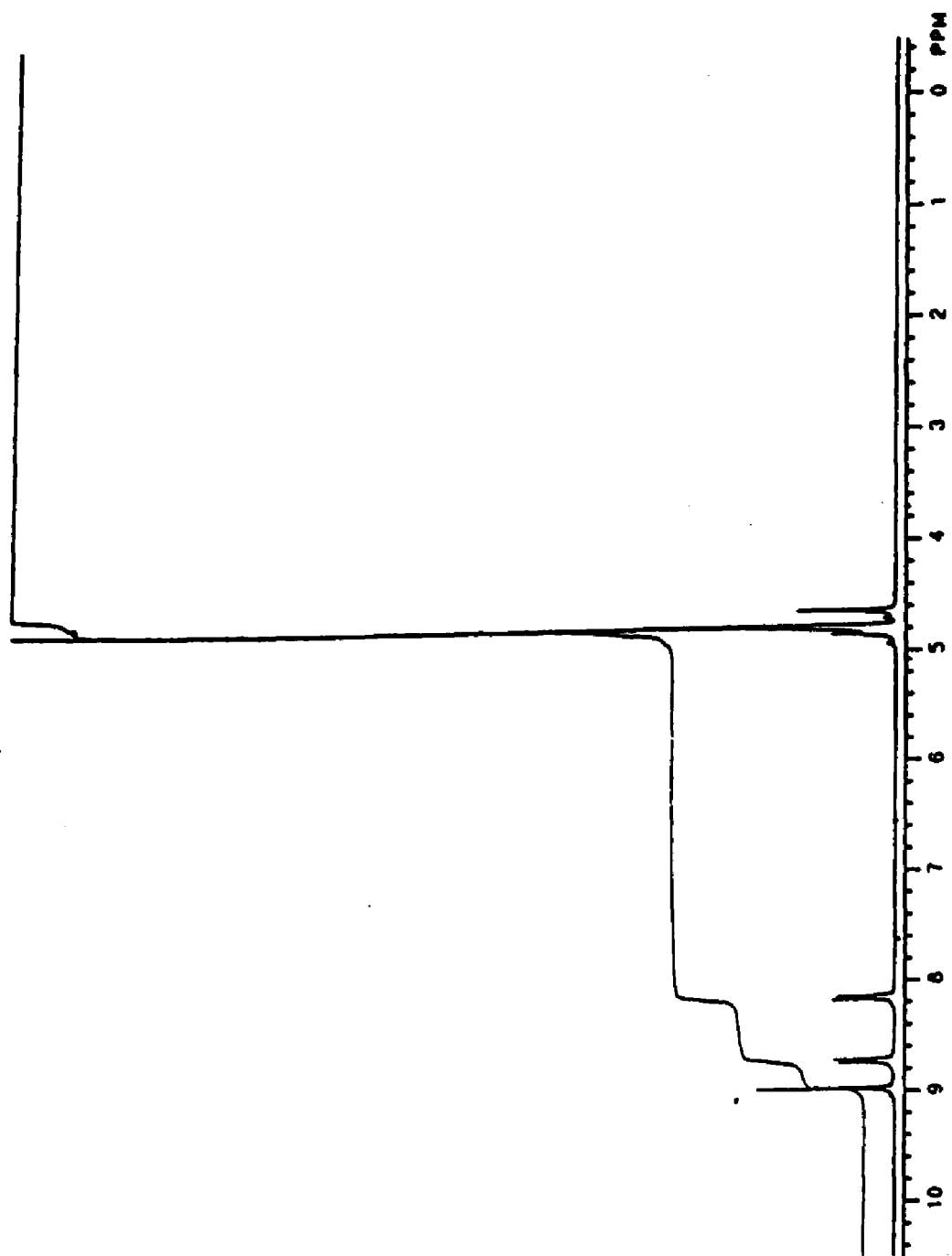


Figure. 2-8. The 300 MHz ^1H NMR spectrum of a solution of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) (substance 5a in scheme I) in D_2O . (The big peak located at 4.8 ppm is contributed by H_2O in solvent)



chemical shifts change substantially, indicating that a chemical change has occurred. The most probable explanation is that a p-phthalamidomethylphenylporphyrin was formed (its structure is shown in figure 2-7). After the intermediate p-amidomethylphenylporphyrin was hydrolyzed under severe reaction conditions (70% H_2SO_4), the p-aminomethylphenyl porphyrins, (5a) and (5b), are formed. Figure 2-8 shows the ^1H NMR spectrum of (5a) after the second hydrolysis step. It is clear that the phthalimido group is removed completely at this time. The presence of an 8H singlet located at 9.00 ppm contributed by β -pyrroline hydrogen atoms, two 8H doublets located at 8.75 and 8.18 ppm contributed by the four benzyl groups on the exterior of the porphyrin ring, and a singlet 8H peak located at 4.6 ppm contributed by four methylene groups, confirm the synthesis of tetrakis(p-aminomethylphenyl)porphine (TAMPP).

The ^1H NMR spectrum of (5b) is shown in figure 2-9. It is a much more complicated spectrum than was expected. For a better understanding of figure 2-9, a series of ^1H NMR decoupling experiments were conducted. Because of the N-methyl group, the porphyrin ring does not remain planar.^{18,19} The nonplanarity causes the chemical shifts of the system to move to upfield (smaller δ) since the aromatic ring current decreases. Because of the N-methyl group, the symmetry of porphyrin ring is reduced from D_{2d} to C_2 and the original 8H singlet will be replaced by two singlets, S_1 (possibly from hydrogen (4) of figure 2-10 because it is far away from the N-methyl group and its chemical shift will be less affected) and S_2 (possibly from hydrogen (1) because it is closer to N-methyl group and its chemical shift will be affected more, moving it substantially upfield), as well as two doublets, D_1 (possibly from hydrogen (3)) and D_2 (possibly from hydrogen (2) because it is closer to the N-methyl group than hydrogen (3) and its chemical shift will be affected more than that of hydrogen (2)). These S_1 , S_2 , D_1 and D_2 peaks are located at 8.72 ppm, 7.92 ppm, 9.05 ppm and 8.88

Figure. 2-9. The 300 MHz ^1H NMR spectrum of a solution of N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) (substance 5h in scheme I) in D_2O , room temperature.

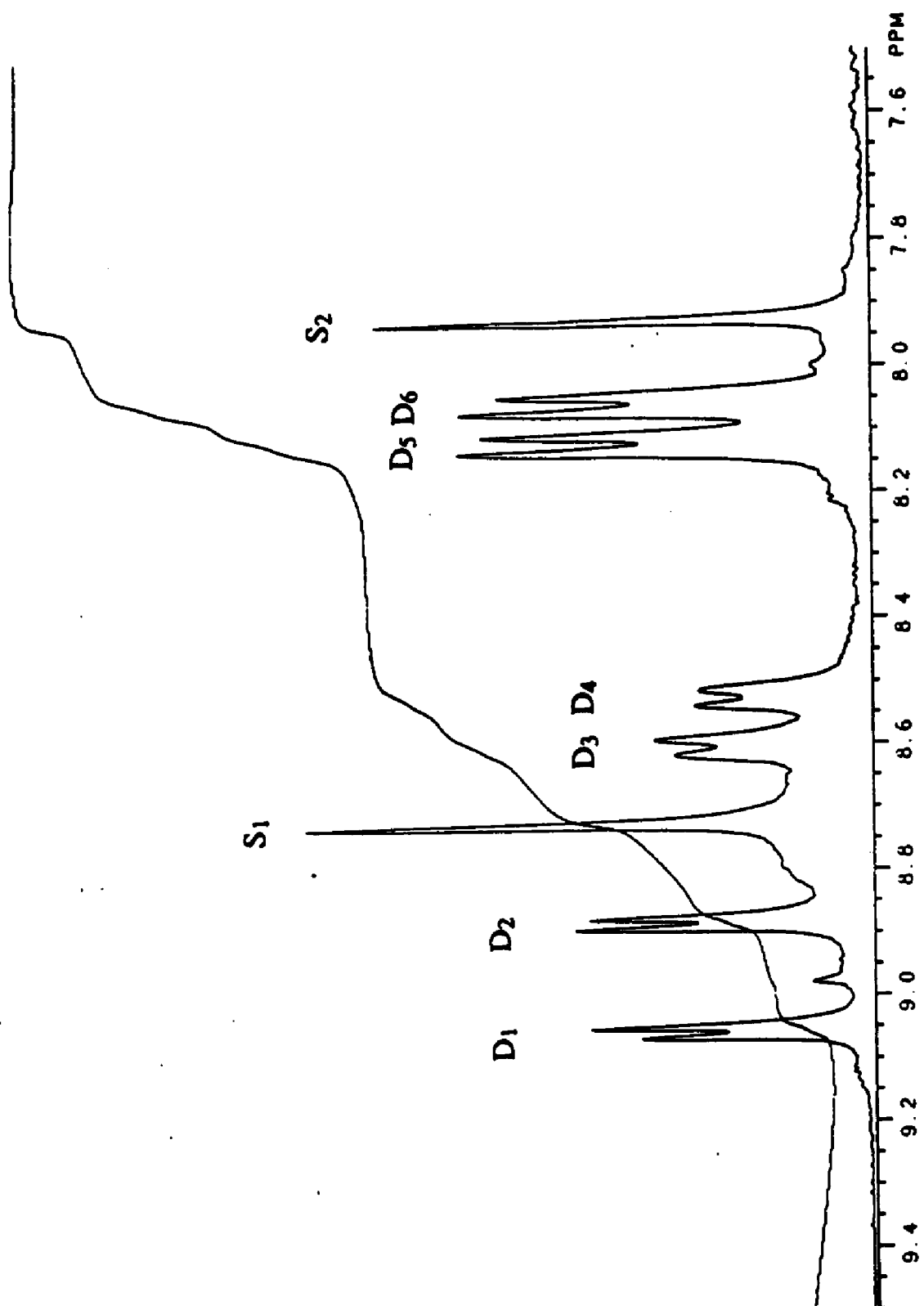


Figure. 2-10. The molecular structure of N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP)

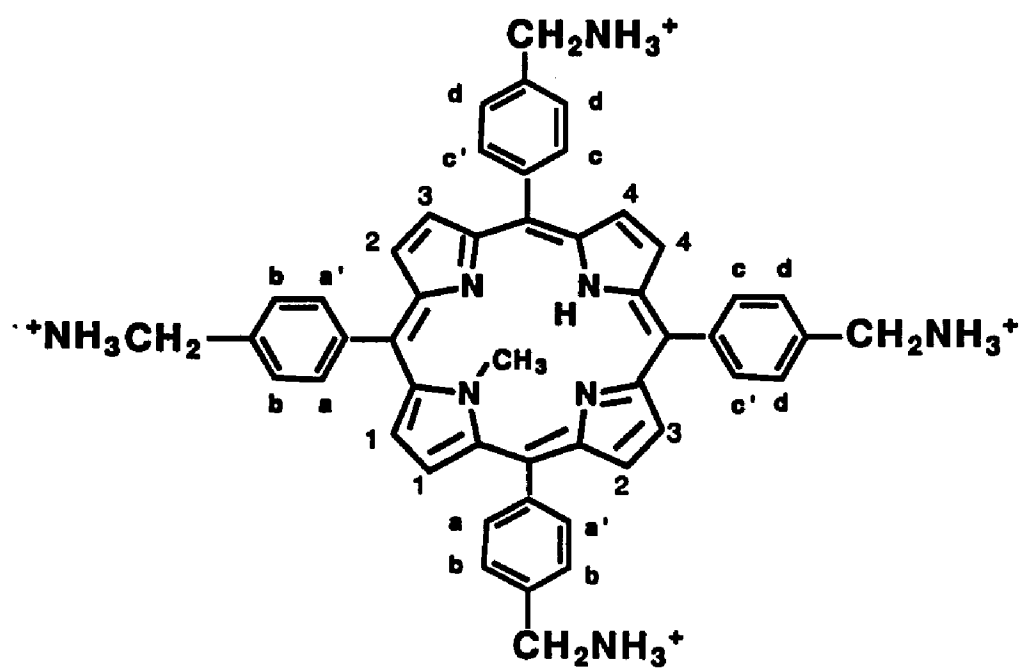


Figure. 2-11. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_1 of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (assignments are shown in figure 2-9) was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 80, NA: 80, L_2 : 2830, D_2O , room temperature)

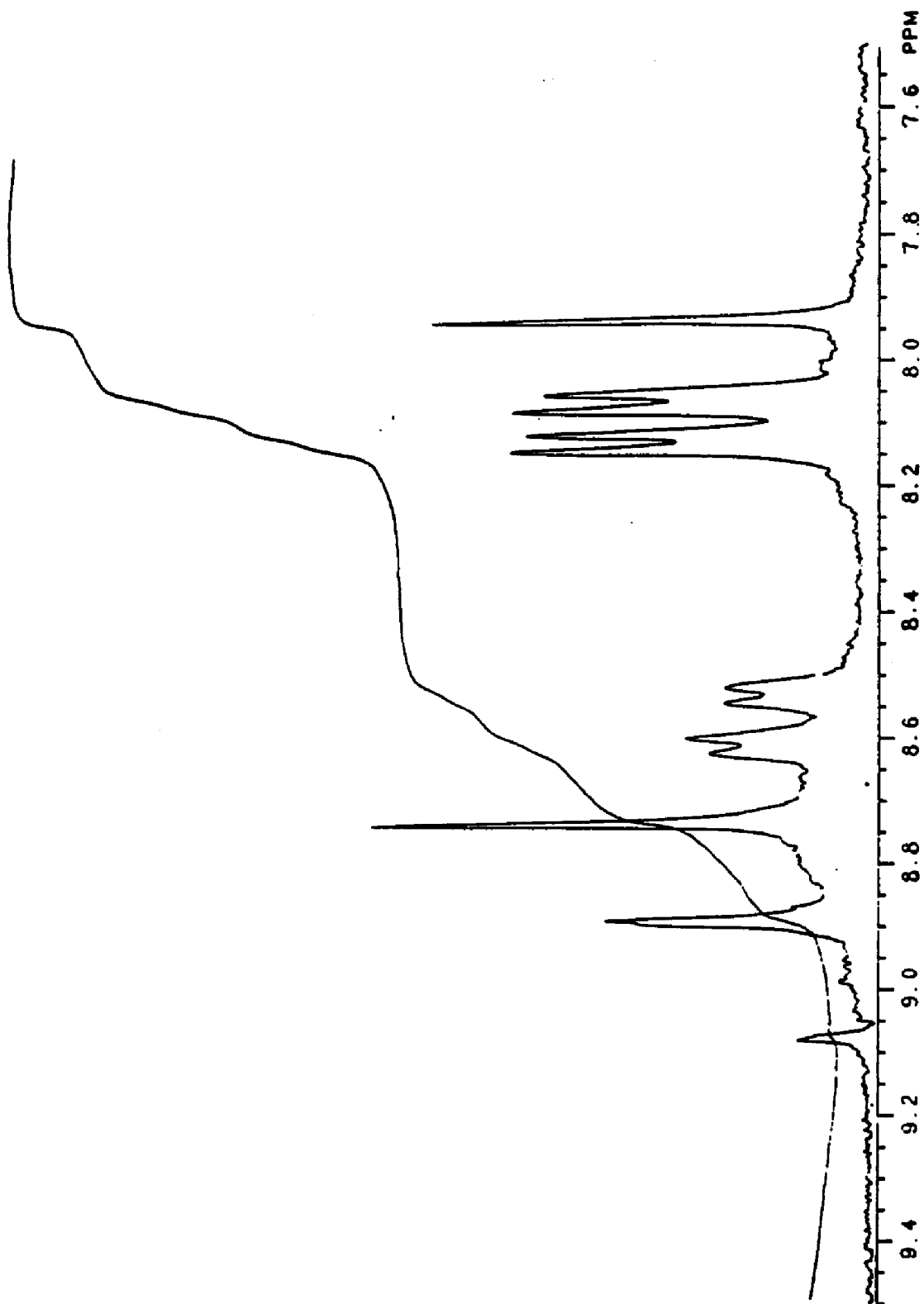


Figure. 2-12. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_2 of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (assignments are shown in figure 2-9) was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 80, NA: 80, L_2 : 2830, D_2O , room temperature)

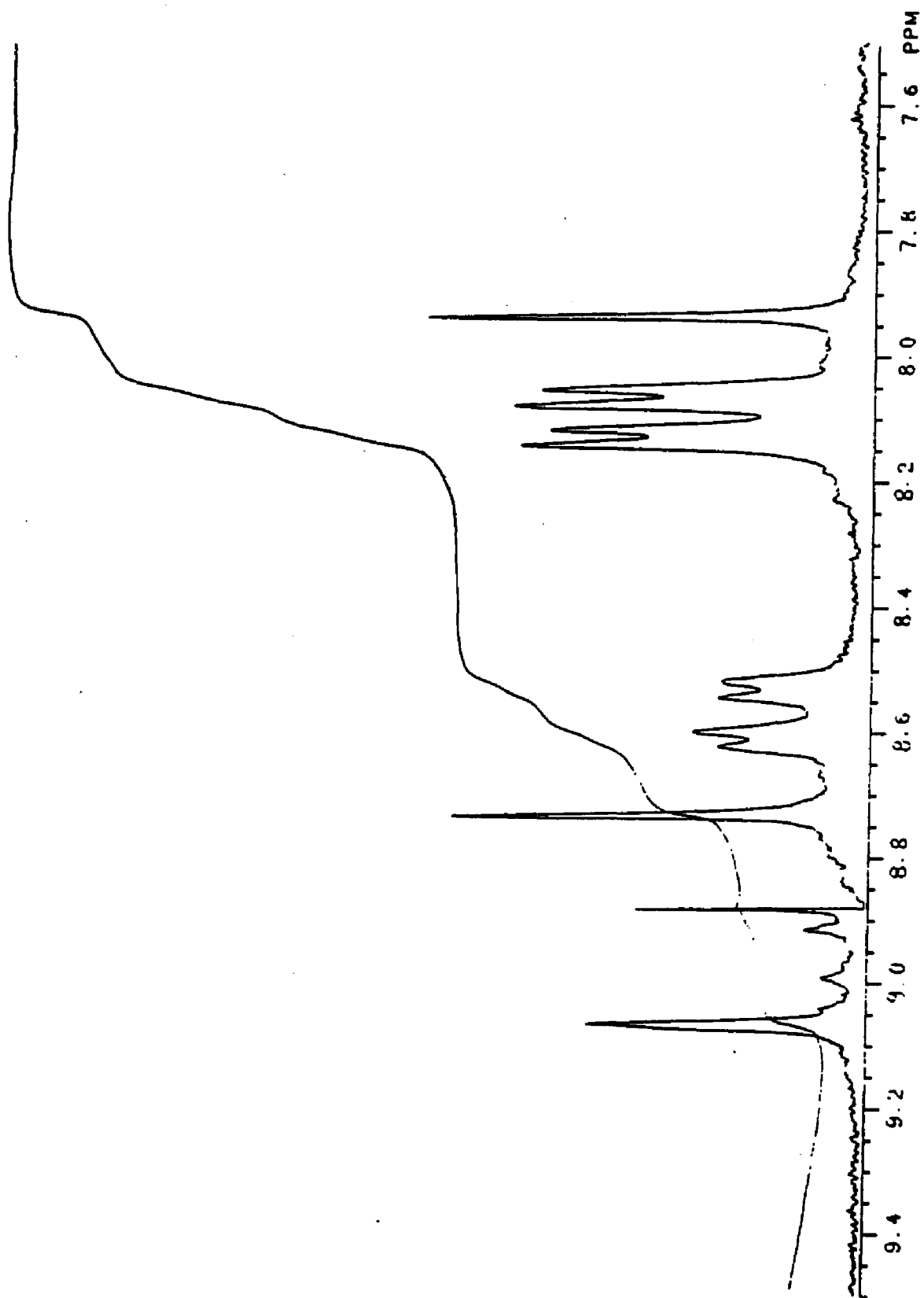
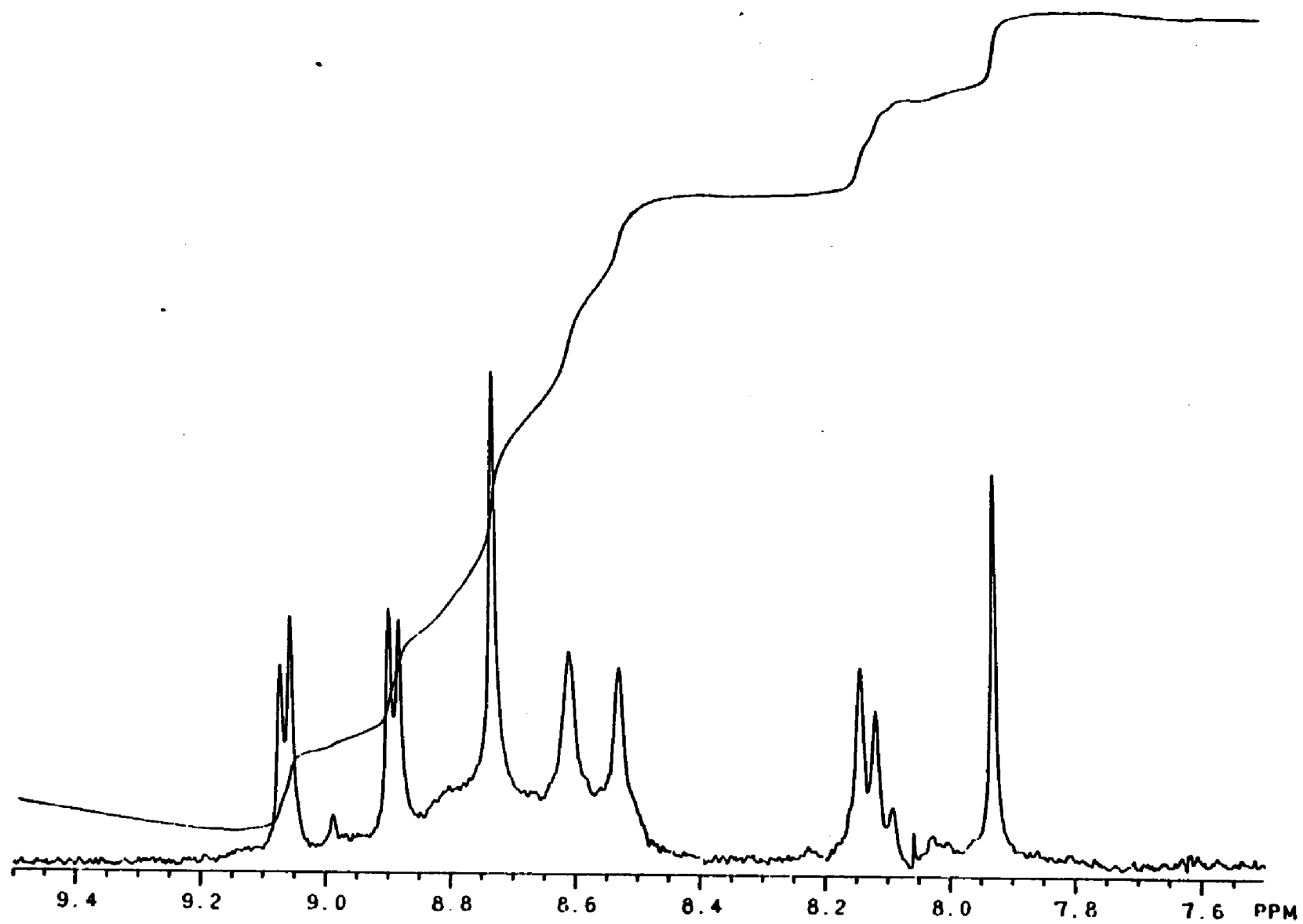


Figure. 2-13. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_6 of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (assignments are shown in figure 2-9) was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 80, NA: 80, L_2 : 2750, D_2O , room temperature)



ppm respectively at room temperature based on above most likely assignment (figure 2-9).

Figure 2-11 and 12 display the decoupling spectra after doublet peaks D_1 and D_2 were separately decoupled. It is obvious that D_1 and D_2 are related to each other. Figure 2-13 shows the decoupling spectra after the doublet D_6 was decoupled. The interesting phenomenon is that both doublets D_3 and D_4 change to singlets. If we decouple D_3 and D_4 , D_6 becomes an incomplete doublet or imperfect singlet (figure 2-14 and 15). When greater decoupling power was used for decoupling the D_4 or D_3 , the intensity of D_3 or D_4 was reduced. This is because the chemical shifts of D_3 and D_4 are too close to each other and either of them is easily reduced by decoupling power applied to the other. The results shown by figures 2-13, 14 and 15 imply that both D_3 and D_4 are related directly to D_6 . But when D_5 was decoupled, there is no obvious change in the spectrum (figure 2-16) except for some decrease of the height of peak D_6 attributed to the location of D_6 close to D_5 (it is easily affected by the decoupling power applied to decouple D_5).

These interesting phenomena can only be explained by the limited rotation of the benzyl groups on the exterior of the porphyrin ring. That is, the ortho hydrogen atoms of benzyl group are not the same if one is on the side of porphyrin ring where the methyl group is located and the other is on the side without the methyl group. The four meta hydrogen atoms are less affected by the difference due to the N-substitution because they are further from the porphyrin ring. Comparing the structure of N-methylated porphyrin (5b) (figure 2-10) to figure (2-9), it is very possible that (a) and (a') hydrogen atoms shown in figure (2-10) are responsible for the signal of the D_3 and D_4 pair while (b) hydrogen atoms are responsible for the signal of the D_6 peak in figure 2-9. This assumption is consistent with the phenomena shown in figures 2-13, 14, and 15, that is, when D_3 or D_4 was decoupled (removing the spin-spin split effect of hydrogen atoms

Figure. 2-14. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_3 of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (assignments are shown in figure 2-9) was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 80, NA: 80, L_2 : 2830, D_2O , room temperature)

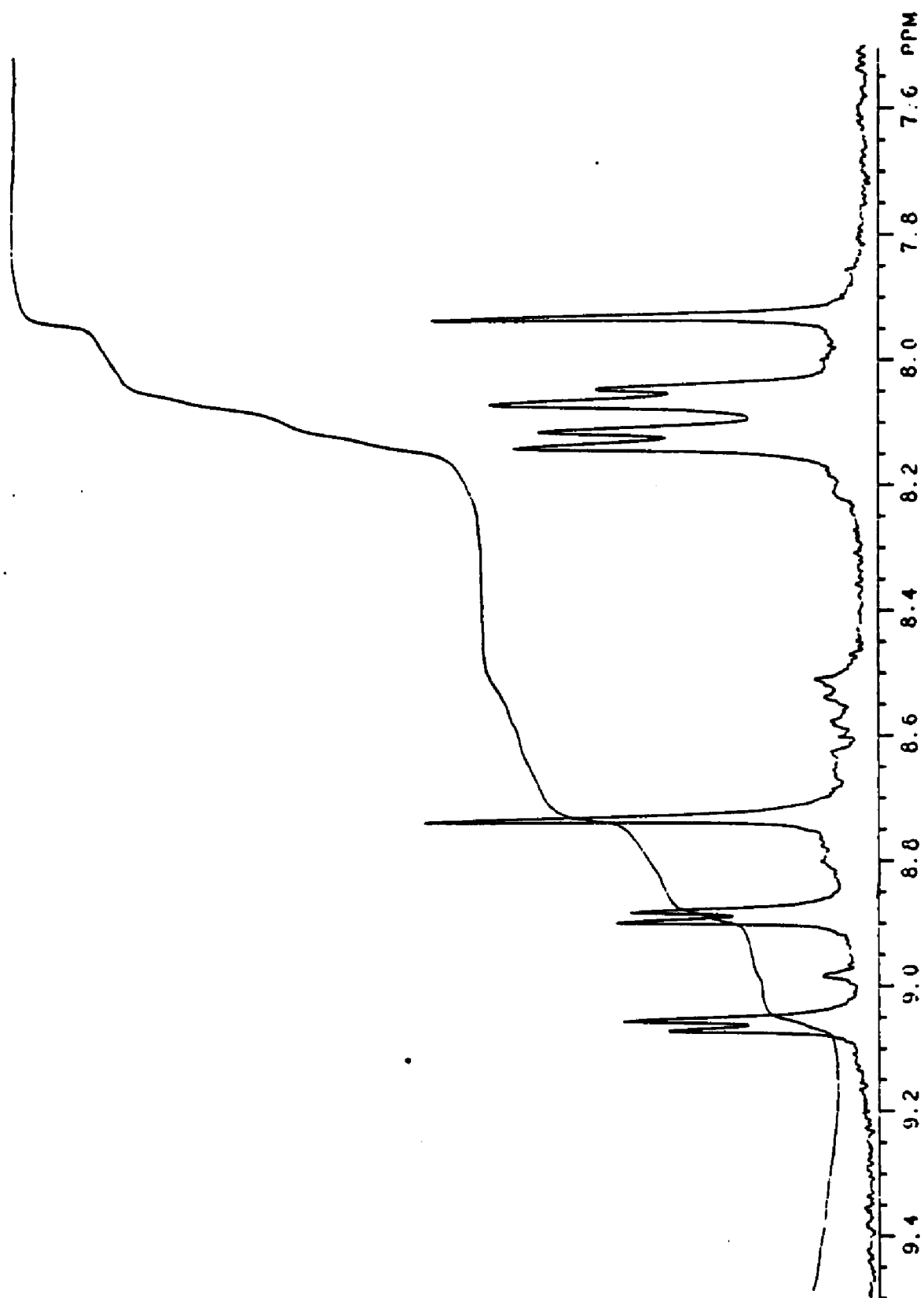


Figure. 2-15. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_4 of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (assignments are shown in figure 2-9) was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 80, NA: 80, L_2 : 2830, D_2O , room temperature)

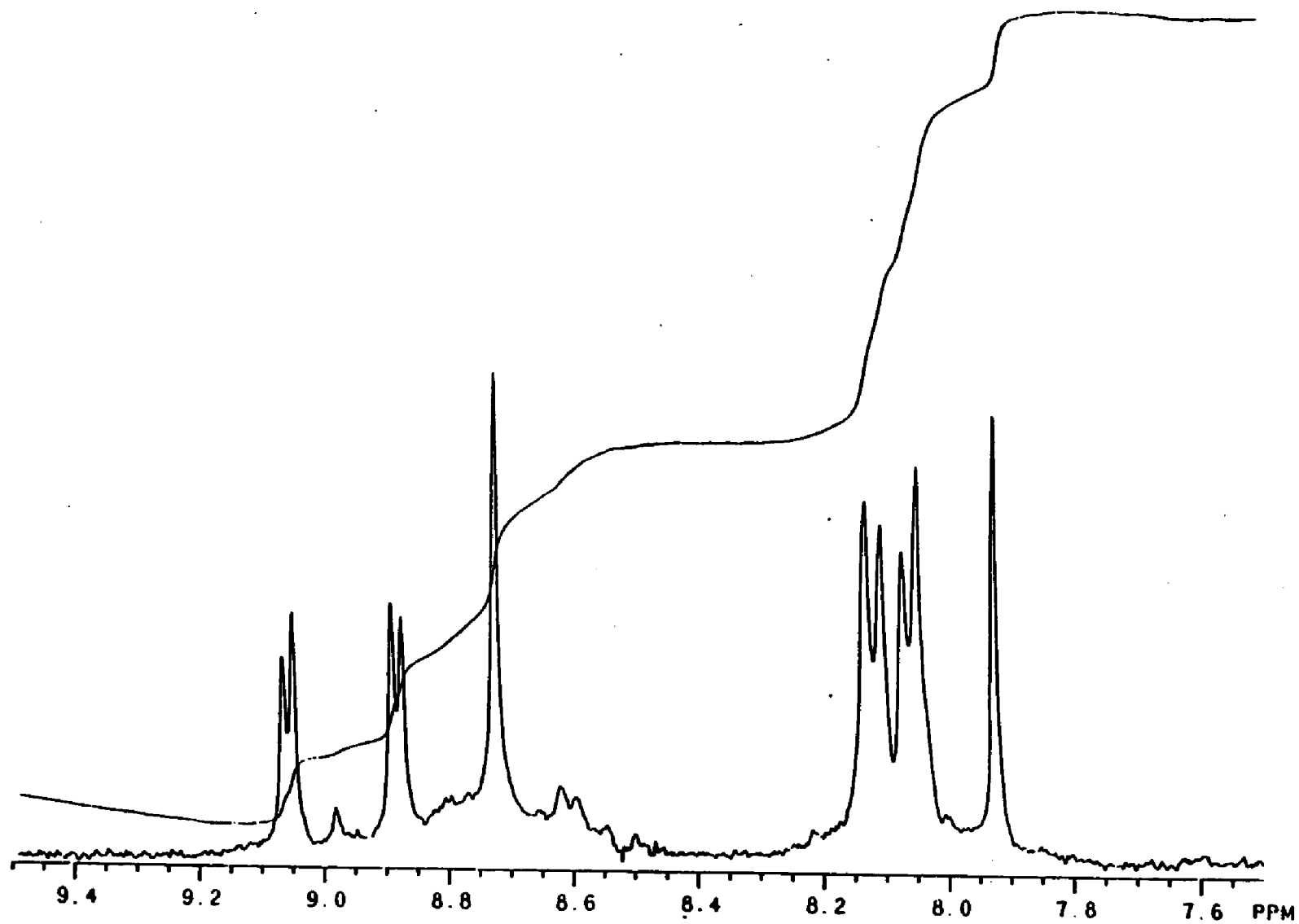
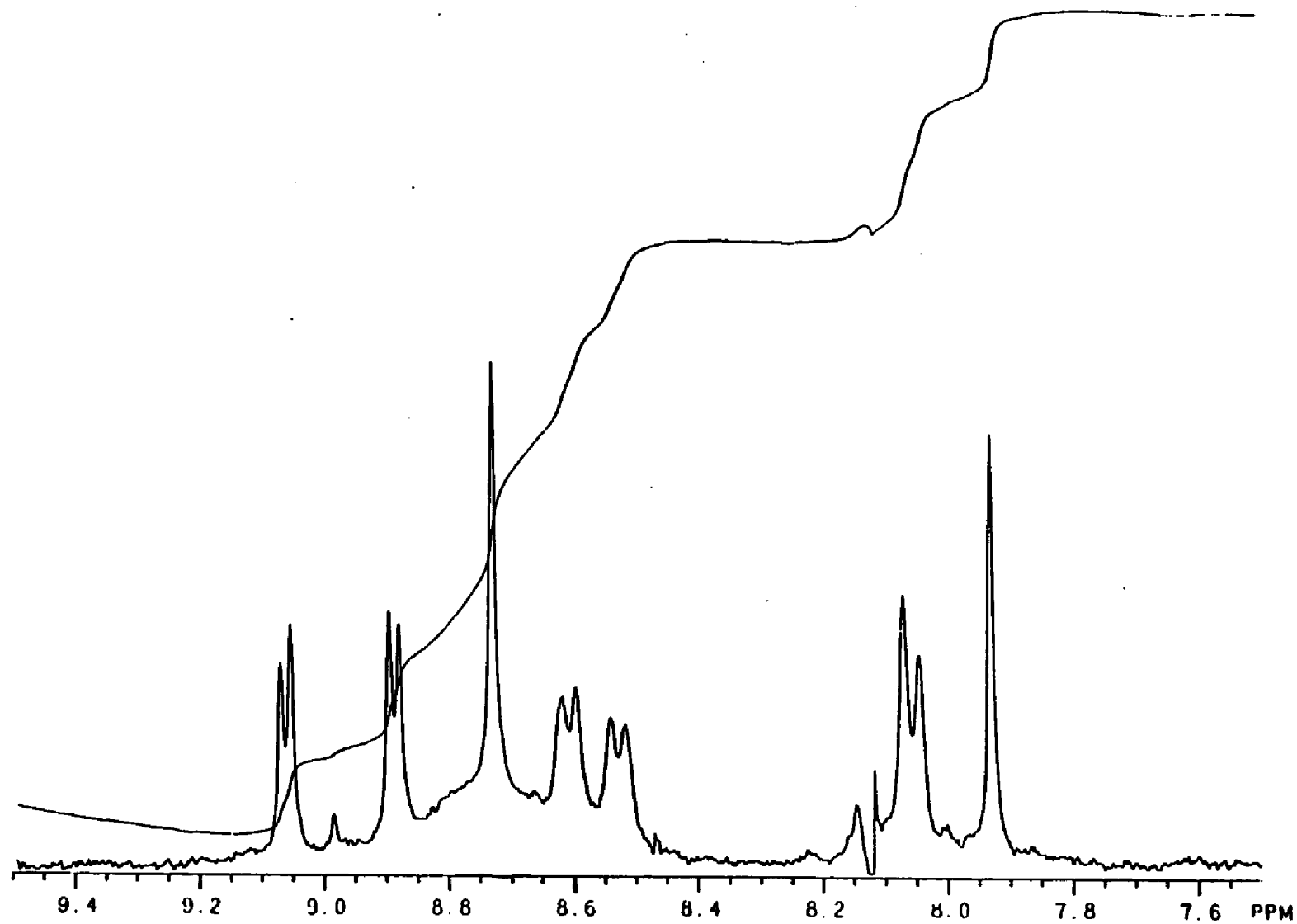


Figure. 2-16. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_5 of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (assignments are shown in figure 2-9) was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 80, NA: 80, L_2 : 2830, D_2O , room temperature)



(a) or (a') to hydrogen atoms (b)), the doublet peak D_6 was only partly affected (only half of (b) hydrogen atoms felt the difference after hydrogen atoms (a) or (a') was decoupled) and when D_6 was decoupled (removing the spin-spin split effect of hydrogen atoms (b) to hydrogen atoms (a) or (a')), both D_3 and D_4 changed to singlets (both (a) and (a') hydrogen atoms felt the difference after the resonance of hydrogen atoms (b) were removed).

One more problem has not been solved, however. If the hydrogen atoms (d) give rise to D_5 on figure (2-9), then where is the peak due to hydrogen atoms (c) and (c')? Are hydrogen atoms (c) and (c') the same or are they different? To answer these questions, let us compare figure (2-5) and figure (2-9). Ignoring the two quintuplets Q_1 and Q_2 contributed by the phthalic acid group, then the differences between figures 2-5 and 2-9 are that (1) all of peaks except S_1 shift downfield; (2) there is an extra, broad peak D_c in figure 2-5 not present in figure 2-9 and (3) there is a higher background baseline in the range of 8.5 to 8.9 ppm in figure 2-9 than in figure 2-5. The points (2) and (3) suggest that if D_3 , D_4 , D_5 and D_6 in figure 2-5 correspond to the peaks of D_3 , D_4 , D_5 and D_6 in figure 2-9, then D_c in figure 2-5 is assumed to be related to D_5 and is contributed by hydrogens of (c) and (c'). The broadness of peak D_c implies that the two benzyl rings which are far away from the N-methyl group can rotate, but do so more slowly than is common for porphyrins (which typically have magnetically equivalent hydrogen atoms on the meso phenyl rings due to rotation²⁰). Therefore hydrogen atom (c) is different from hydrogen atom (c') but this difference is much smaller than that of hydrogen atoms (a) and (a'). The two very close doublets (contributed by hydrogens (c) and (c')) make a broader peak D_c , while this D_c is hidden under the peaks D_2 , S_1 and D_3 in figure 2-9 and cause a higher background baseline in that region of the spectrum. To find out the true relationship between D_c and D_5 , D_c in figure 2-5 was decoupled by

Figure. 2-17. The 300 MHz ^1H NMR decoupling spectrum after broad peak D_c of N-methyl tetrakis(p-phthalimidomethylphenyl) porphyrin in figure 2-5 was decoupled. (LB: 0, Tip angle: 32, Scan number: 160, NA: 160, L_2 : 2730, CDCl_3 , room temperature)

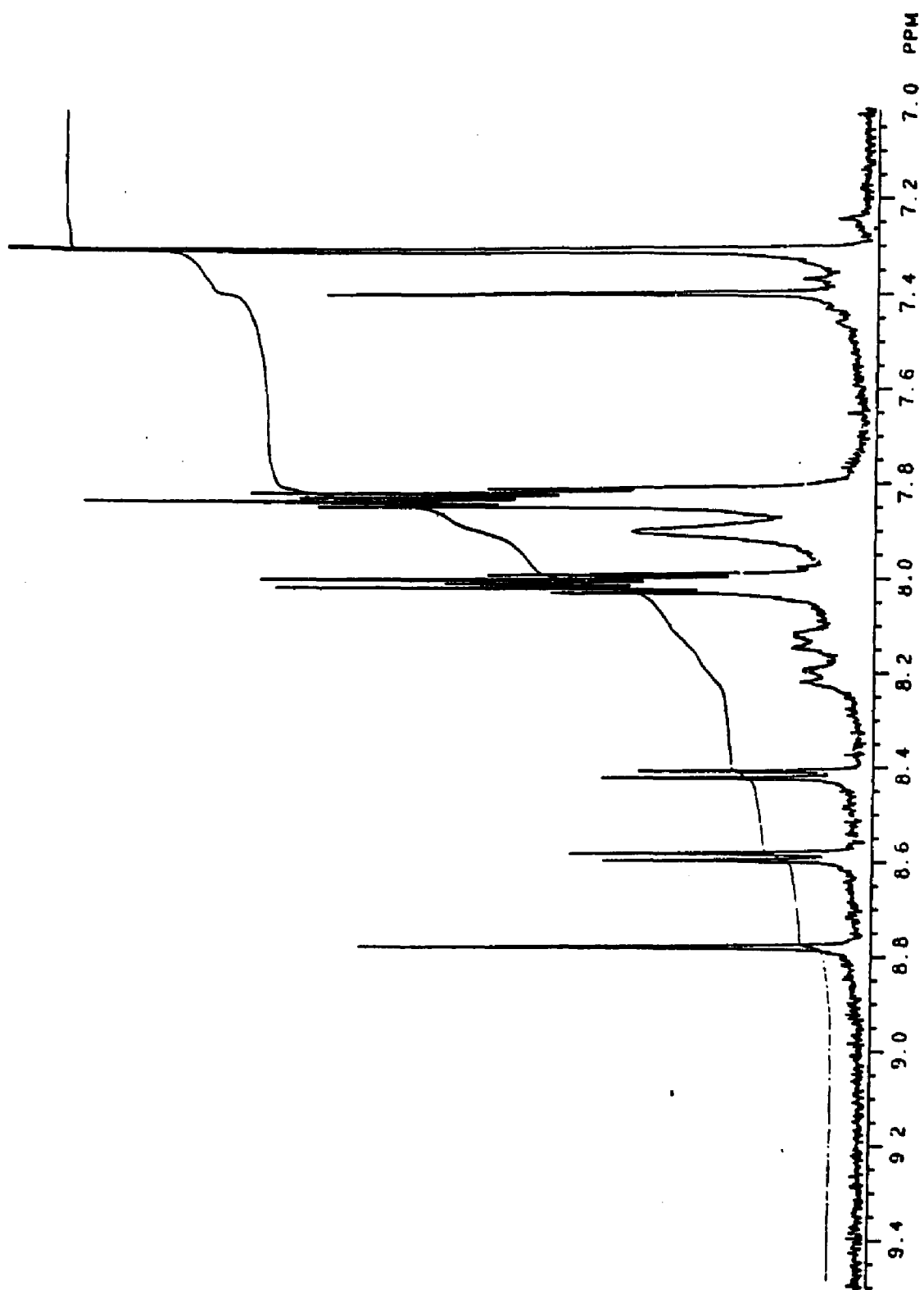
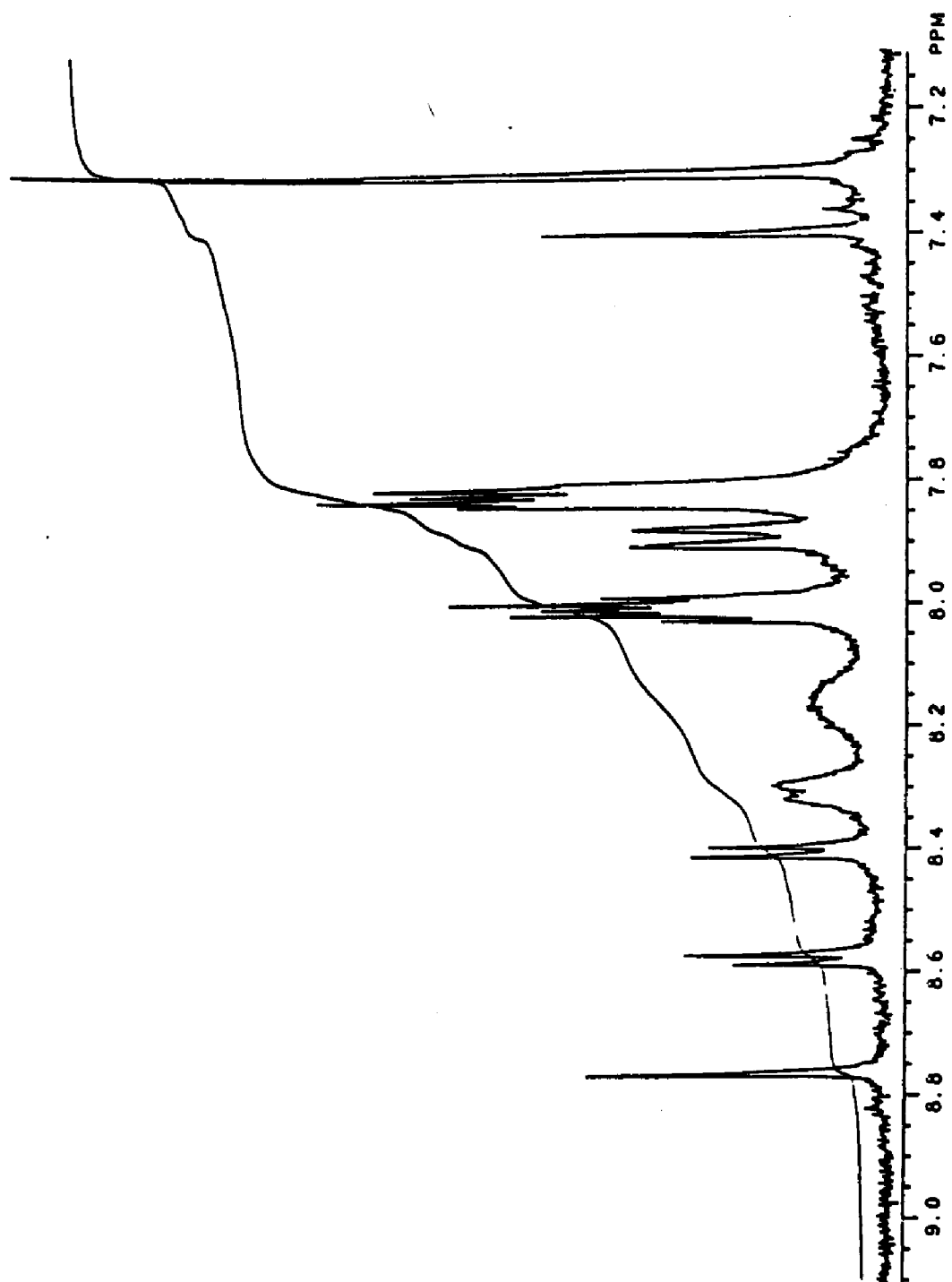


Figure. 2-18. The 300 MHz ^1H NMR spectrum of N-methyl tetrakis(p-phthalimidomethylphenyl) porphyrin at 50 °C of CDCl_3 .



higher decoupling power. The expected results were obtained: D_5 changes to a singlet (figure 2-17). This experiment proves positively that D_c is related to D_5 .

In order to demonstrate if the broad peak D_c is caused by relatively slow rotation for two of the meso-benzyl groups and if the D_3 and D_4 are caused by the more difficult rotation of the other two meso-benzyl groups at a rate corresponding to the NMR time scale, high temperature ^1H NMR experiments were conducted. Figure 2-18 shows the ^1H NMR spectra of compound (4b) at 50 °C of CDCl_3 . Compared with figure 2-5, D_c changes to a doublet, while D_3 and D_4 combine into a broad peak. This result implies that D_c and D_3 , D_4 are truly caused by restricted rotation. The benzyl groups which give rise to D_c change from slow rotation at room temperature (a broad D_c peak in figure 2-5) to a faster rotation at higher temperature (a "doublet" D_c peak in figure 2-18). While the benzyl groups giving rise to D_3 and D_4 change from rotation that is slower than the NMR time scale at room temperature (two doublet peaks in figure 2-5) to rotation at the rate corresponding to the NMR time scale at higher temperature (a broad peak D_a in figure 2-18). More obvious results are shown in figure 2-19: a series of ^1H NMR spectra of D_2O solutions of N-MTAMPP (5b) were recorded at temperatures from 30 °C to 90 °C. Compared with figure 2-9 which shows the spectrum taken at room temperature, the D_c resonance which had been hidden previously by D_2 , S_1 is now quite separate and occurs as a nicely resolved doublet, while D_3 and D_4 gradually converges and at last combines to a doublet peak too! The coupling relationship between D_c and D_5 , D_a and D_6 pairs are confirmed more positively by high temperature decoupling experiments shown in figures 2-20 and 2-21. That is, when D_c was decoupled, D_5 changes to a singlet (figure 2-20) and when D_a was decoupled, D_6 changed to a singlet (figure 2-21).

As temperature is increased, all the ^1H chemical shifts of porphyrin N-MTAMPP (5b) move gradually downfield. This phenomenon implies that the benzyl groups of

Figure. 2-19. The 300 MHz ^1H NMR spectra of N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) at 30, 50, 70 and 90 °C of D_2O .

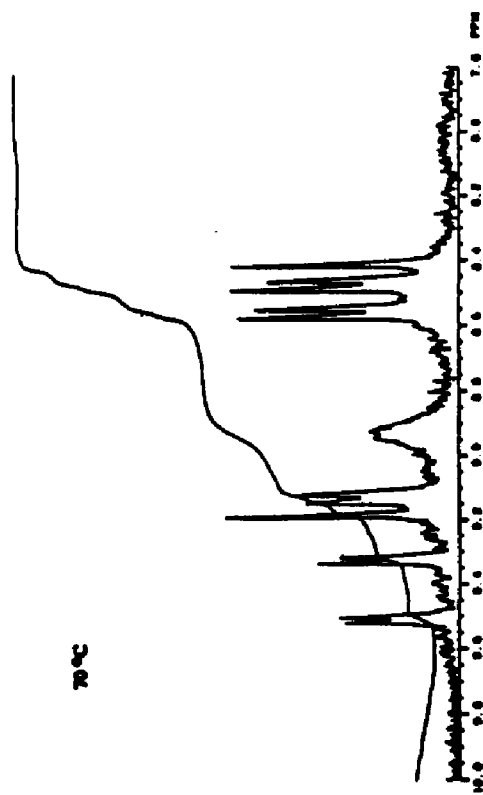
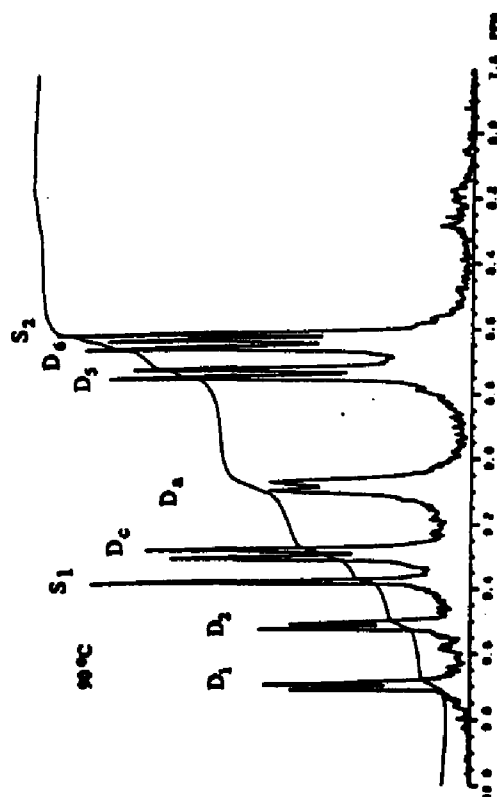
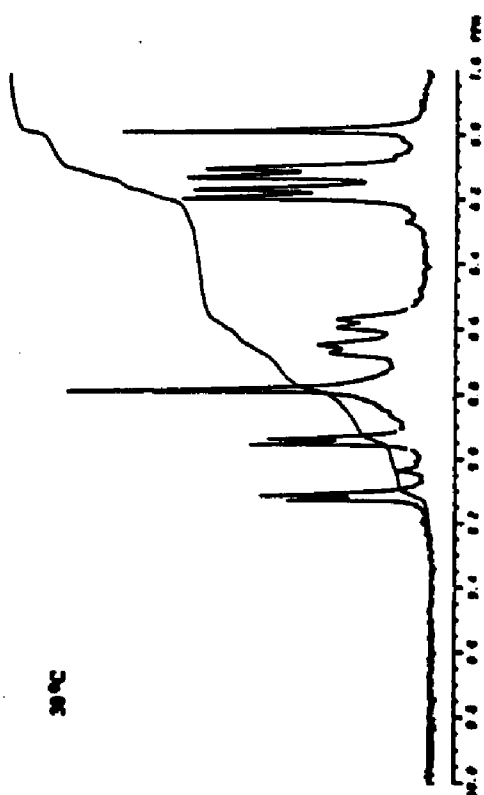
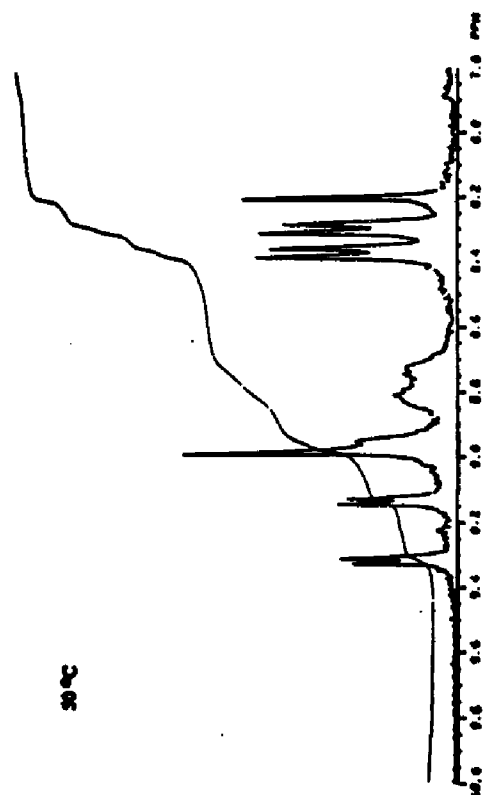


Figure. 2-20. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_c of N-methyl tetrakis(p-phthalimidomethylphenyl) porphyrin in figure 2-19 was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 16, NA: 16, L_2 : 2800, D_2O , 90 °C).

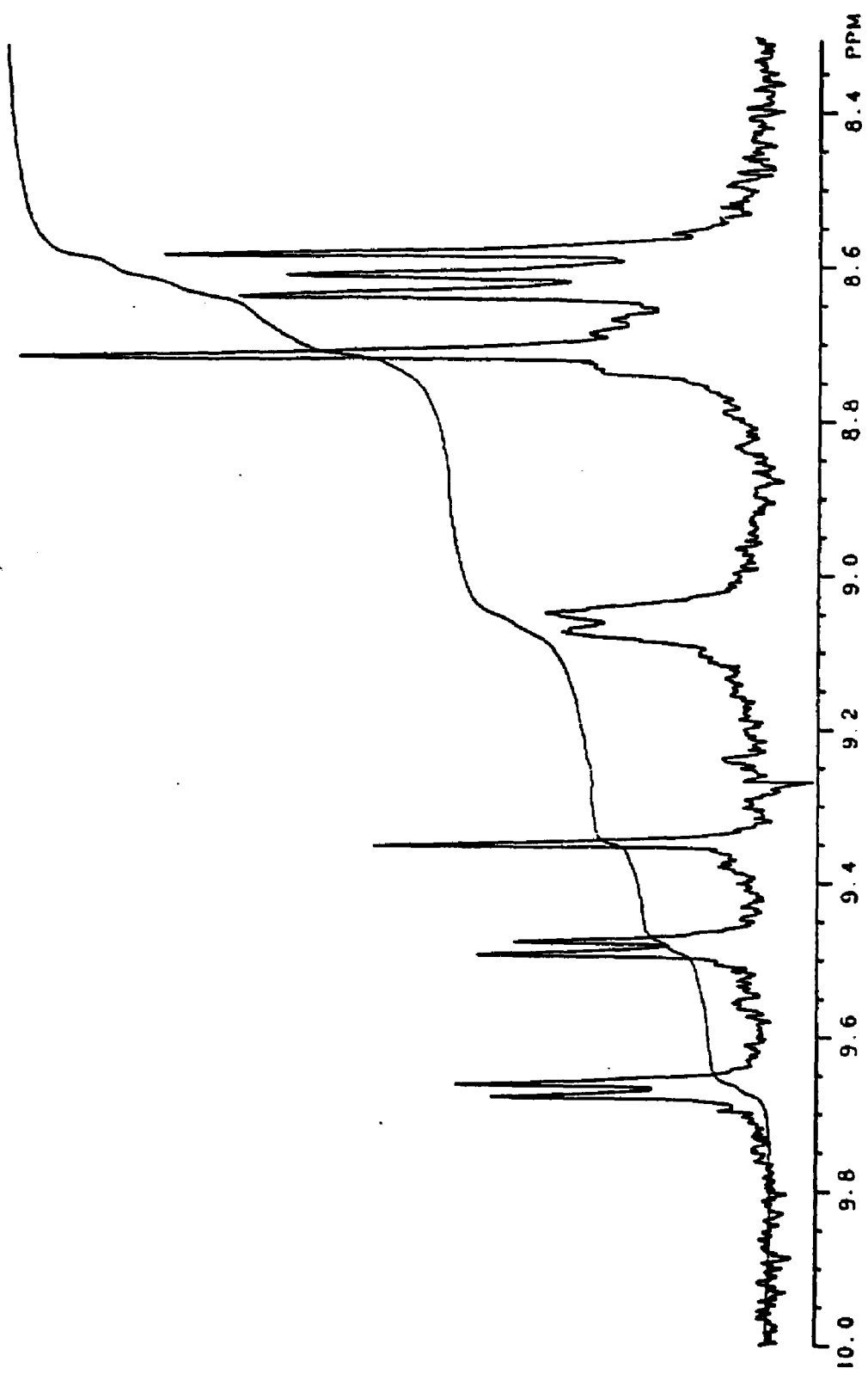


Figure. 2-21. The 300 MHz ^1H NMR decoupling spectrum after doublet peak D_a of N-methyl tetrakis(p-phthalimidomethylphenyl) porphyrin in figure 2-19 was decoupled. (LB: 0.5, Tip angle: 60, Scan number: 16, NA: 16, L_2 : 2800, D_2O , 90 °C)

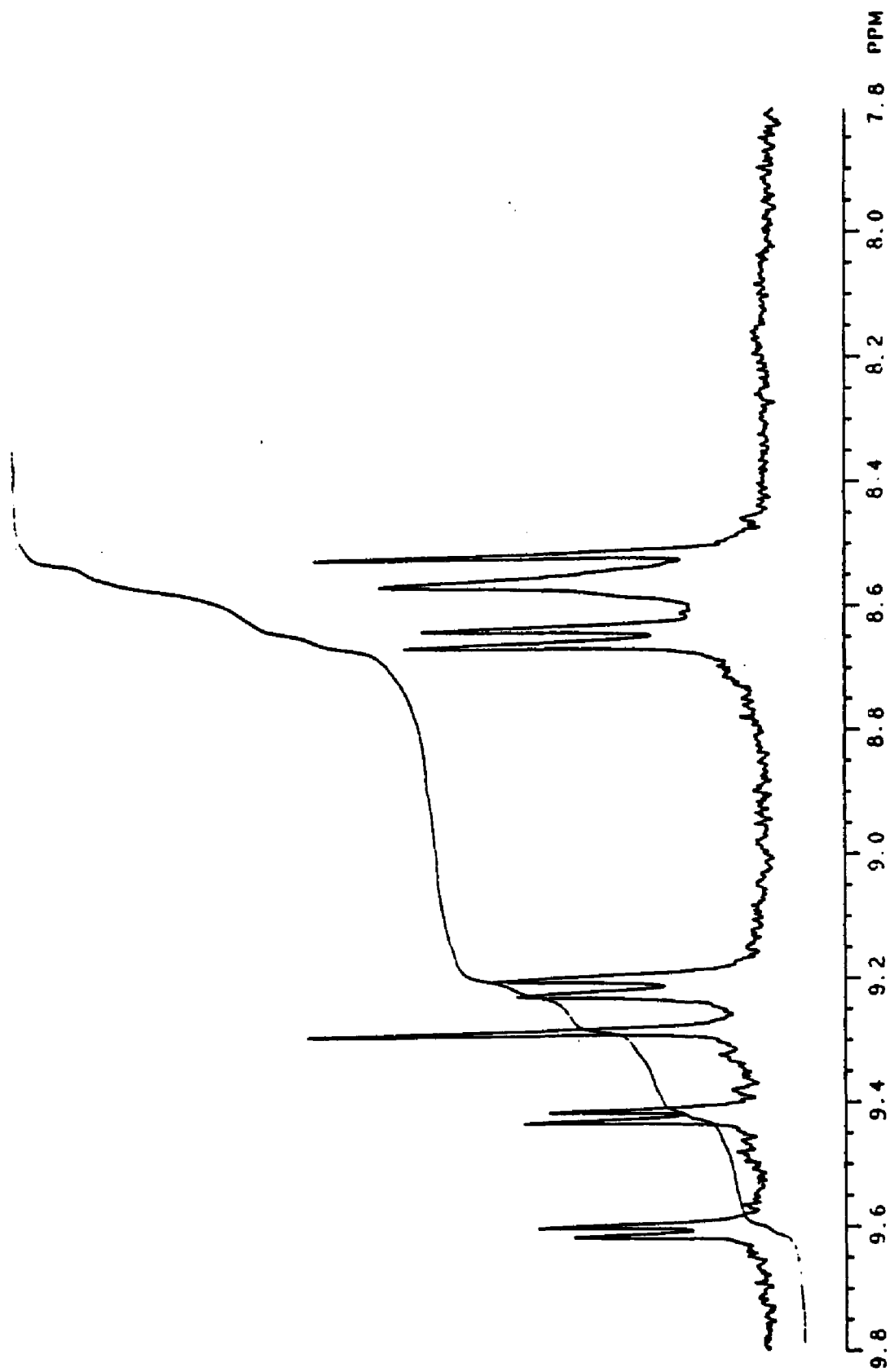
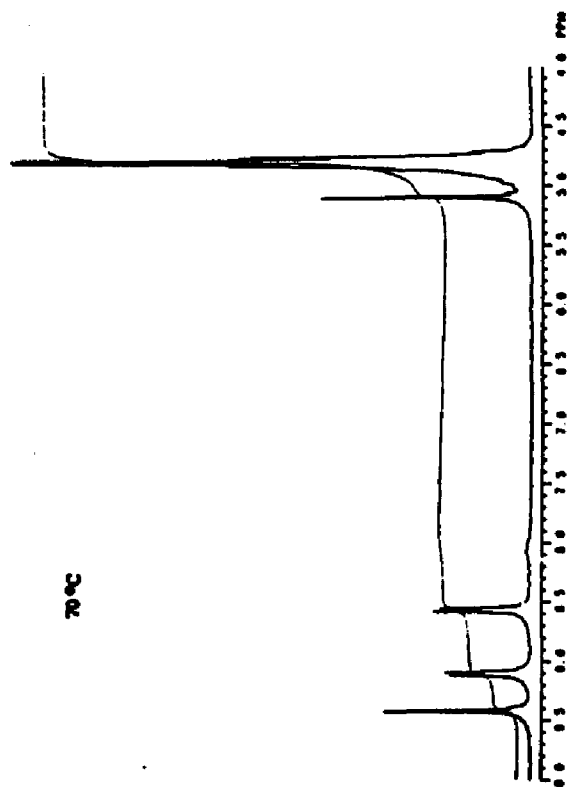
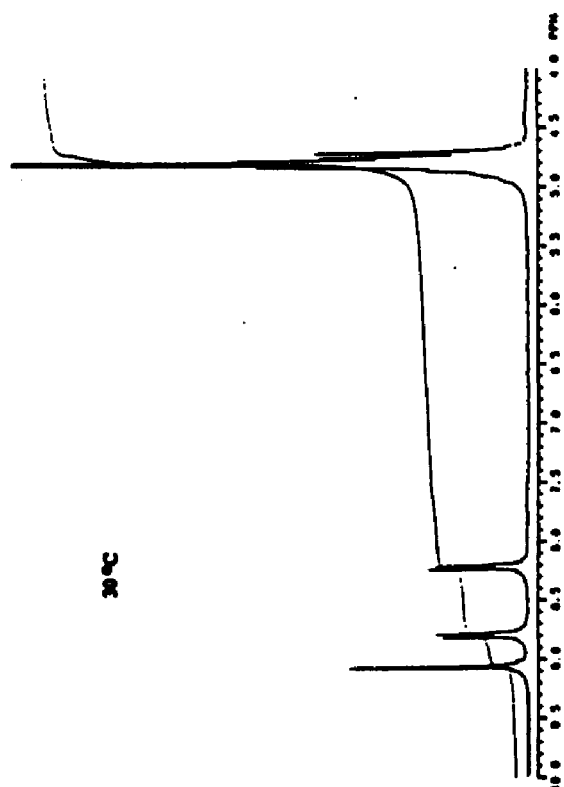
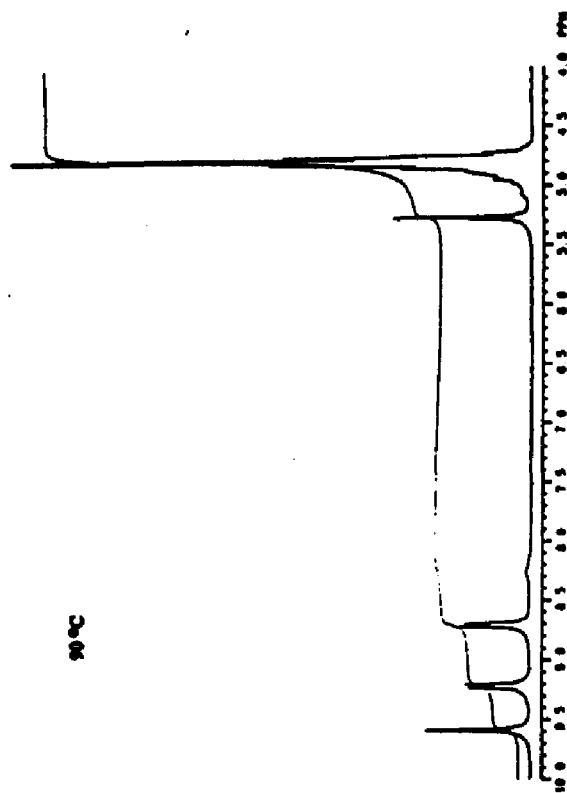
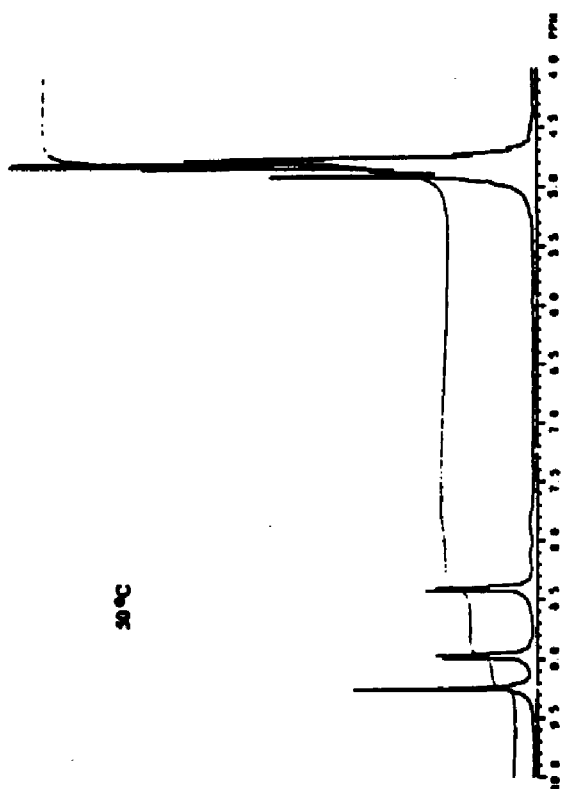


Figure. 2-22. The 300 MHz ^1H NMR spectra of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) at 30, 50, 70 and 90 °C of D_2O .



(5b) are, on the average, closer to perpendicular to the porphyrin ring at room temperature than at higher temperature. Therefore, as the temperature is increased, the rotational tendency of the benzyl ring will increase and the benzyl ring will spend more time in or near the same plane as porphyrin ring. This will cause the chemical shift of protons located both on benzyl and porphyrin ring to move downfield because of the deshielding effect of aromatic protons brought about by ring current. Figure 2-22 shows the series ^1H NMR spectra of a D_2O solution of TAMPP (5a) in a temperature range of 30 °C to 90 °C. Similarly, as the temperature is increased, the chemical shifts move downfield, consistent with the benzyl groups of (5a) preferentially remaining nearly perpendicular to the porphyrin ring at room temperature too. The simple ^1H NMR spectrum of TAMPP at any temperature results from its high symmetry. All of the ortho hydrogen atoms of the benzyl groups remain the same whether or not the benzyl groups rotate.

The decoupling and high temperature ^1H NMR experiments discussed above lead to the conclusions that at room temperature: (1) of the four benzyl groups of N-MTAMPP, two (probably those closest to the N-methyl group) either do not rotate or rotate slower than the NMR time scale; (2) of the four benzyl groups of N-MTAMPP, two (probably those farther away from the N-methyl group) rotate slowly on the NMR time scale; (3) the benzyl rings of TAMPP and N-MTAMPP prefer a position more nearly perpendicular to the porphyrin ring at room temperature than at higher temperature.

Because of the N-methyl group, the porphyrin ring of N-MTAMPP can not be planar^{18,19}, as a result, there is larger steric hindrance between hydrogen atoms on positions (a) or (a') of benzyl groups and β -pyrroline hydrogen atoms at positions (1) or (2). In contrast to TAMPP, which has a planar porphyrin ring has less steric hindrance to rotation and the benzyl groups can be more nearly coplanar with the

porphyrin ring. Therefore, it seems that it is more likely that the benzyl groups of TAMPP will be more nearly coplanar with its porphyrin ring than will be the case for the benzyl groups of N-MTAMPP. This consideration is also consistent with the results of studies of the basicity of the amino groups on the outside of the porphyrin ring discussed in chapter (3).

2.4 Experimental Section

Synthesis of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP)

The detailed procedures for the synthesis of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and N-methyl tetrakis(p-aminomethylphenyl)porphyrin (NM-TAMPP) are shown as in scheme I.

Synthesis of 4-formylbenzylbromide (2) from α -bromo-p-tolunitrile (1): α -bromo-p-tolunitrile was dissolved in fresh distilled methylene chloride (dried overnight with CaH_2) as the ratio of 1 g / 3 ml (for example, 50 grams of α -bromo-p-tolunitrile in 150 ml methylene chloride). After N_2 was bubbled into the stirred solution for 30 mins, an equimolar amount of diisobutylaluminum hydride which is 1.0 M in methylene chloride solution (255 ml) was added slowly using a syringe. The solution was cooled in an ice bath at the time of mixing. The reaction solution was warmed at 45 °C for 4 hrs. The reaction mixture was cooled and the remaining DiBAL would be decomposed soon by acidifying the solution very carefully with 10% H_2SO_4 to a pH less than 4 (stop foaming). The mixture was stirred overnight at room temperature and the aldehyde that had been formed was extracted with CH_2Cl_2 several times. The organic mixtures were combined and dried over MgSO_4 , filtered, and concentrated under reduced pressure. This crude product was recrystallized with hexane. The purified yield is about 60%.

The resulting 4-formylbenzylbromide (2) gives white needle crystals and its melting point is 98 - 99 °C. Its ^1H NMR spectrum is shown in figure 2-2 and the peaks are assigned as follows: one hydrogen (1H) singlet located at about 10.06 ppm as an aldehyde hydrogen atom, two 2H doublets located at 7.92 and 7.61 ppm as benzyl hydrogen atoms and a 2H singlet located at 4.56 ppm as methylene hydrogen atoms. The calculated elemental composition for $\text{C}_8\text{H}_7\text{OBr}$ is: C, 48.3; H, 3.5; O, 8.0 and Br, 40.2%. The found elemental analysis result is: C, 48.3; H, 3.4; O, 7.9 and Br, 40.1%.

Synthesis of 4-formylbenzylphthalimide (3) from 4-formylbenzylbromide (2): Equimolar amounts of 4-formylbenzylbromide (5 grams) and potassium phthalimide (4.6 grams) were dissolved completely in a minimum amount of N,N-dimethyl formamide (DMF) (about 200 ml) that was dried overnight (by BaO) and freshly distilled. The system was refluxed for 4 hrs and then cooled to room temperature. Because the product is soluble in DMF as well as in CH_2Cl_2 , (but not water), water (about same amount of volume as DMF) was added and the product extracted with CH_2Cl_2 several times. After most of CH_2Cl_2 was removed by rotorevaporation, the CH_2Cl_2 layer was extracted reversely with water several times to remove the remaining DMF. Then, the product was dried and recrystallized with a mixture of hexane and methylene chloride in a 9 to 1 ratio. The yield after purification is about 90%.

The product 4-formylbenzylphthalimide (3) gives a melting point at 132-133 °C and its ^1H NMR spectrum is shown in figure 2-3. The typical peaks from specific groups are one 1H singlet still located at 10.06 ppm from aldehyde atom, one 2H singlet which shifts to 4.97 ppm from methylene hydrogen atoms, two 2H doublets located at 7.90 and 7.63 ppm from benzyl hydrogen groups and two 2H quadruplets located at 7.91 and 7.80 ppm from phthalimido hydrogen atoms. The calculated elemental

composition for $C_{16}H_{11}O_3N$ (3) is: C, 72.4; H, 4.2; N, 5.3 and O, 18.1%. The element analysis results for this compound are: C, 72.4; H, 4.2; N, 5.4 and O, 18.0%.

Synthesis of *tetrakis(p-phthalimidomethylphenyl)porphyrin* (4a) from 4-formylbenzylphthalimide (3): The standard method of porphyrin synthesis ⁹ was used. The crude porphyrin was purified by aluminum gel column chromatography (1 gram of crude porphyrin required about 0.5 liters of aluminum gel) slowly eluted with CH_2Cl_2 solution. The final yield was 26%.

The molecular weight of *tetrakis(p-phthalimidomethylphenyl)porphyrin* (4a) determined from mass spectrometry is 1251.7 which agrees well with the molecular weight (1251.3) calculated from its structure. The ^1H NMR spectrum of porphyrin (4a) is shown in figure 2-4. The typical peak assignments are: a 8H singlet located at 8.81 ppm from β -pyrrolenine hydrogen atoms and one 2H singlet located at upfield -2.85 ppm contributed from hydrogen atoms of pyrrolenine central nitrogen atoms. The extinction coefficients of product (4a) are $\epsilon_{647.3} = 4.58 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{590.1} = 4.66 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{549.8} = 7.53 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{515.6} = 1.54 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The calculated elemental composition for $C_{80}H_{50}N_8O_8$ (4a) is: C, 76.8; H, 4.0; N, 9.0 and O, 10.2%. The elemental analysis results for the compound (4a) are: C, 76.5; H, 4.2; N, 8.8 and O, 10.4%.

Synthesis of *N-methyl tetrakis(p-phthalimidomethylphenyl)porphyrin* (4b) from *tetrakis(p-phthalimidomethylphenyl)porphyrin* (4a): The porphyrin (1 gram) was dissolved completely in CH_2Cl_2 (about 500 ml). Then 15% molar excess of methyltrifluoromethane ($CF_3SO_3CH_3$) in CH_2Cl_2 (0.1 ml $CF_3SO_3CH_3$ in 5 ml CH_2Cl_2) was added slowly drop by drop while the porphyrin solution was stirred. The mixture was refluxed for about 8 hrs. After that time it was cooled and rotorevaporated.

The best way to separate non-, mono- and di-methylated porphyrin was to run two different columns. First, a silica gel column was used (1 gram crude product to 150 ml silica gel slurry). The non-methylated porphyrin was eluted first using 2% acetone/98% methylene chloride solution. After the first band (unreacted porphyrin) was removed from column, the percentage of acetone was increased to 5% and then 10% which gives mostly mono- and some diprotonated porphyrin. Second, this semicrude mono-N-methylated porphyrin obtained from silica gel was dissolved in CH_2Cl_2 containing about 5 ml of 2,6 lutidine and was eluted from an aluminum gel column. The semicrude product was eluted first with pure methylene chloride to remove any remaining lutidine and second with a mixture of acetone and methylene chloride solution in a 1 to 19 ratio. Under these conditions, the neutral mono-methylated porphyrin was eluted first. The yield was about 30% (300 mg of N-methylated porphyrin obtained).

The ^1H NMR spectrum of synthesized tetrakis(p-phthalimidomethylphenyl) porphyrin is shown in figure 2-5a and 5b. The peak assignments are: two 2H singlets locate at 7.40 ppm and 8.78 ppm from the position "1" and "4" β -pyrroline hydrogen atoms, two doublets located at 8.41 ppm and 8.59 ppm from the position "2" and "3" β -pyrroline hydrogen atoms (figure 2-10: these assignments have been discussed in section 5.3), one 8H singlet located at 5.20 ppm from four methylene hydrogen atoms and one 3H singlet located at strong upfield -4.20 ppm from N-methyl hydrogen atom.

The molecular weight of (4b) calculated from its structure is 1265.3 and the one determined from mass spectrometry is 1265.2. The extinction coefficients at wavelength 677.3, 576.4 and 534.8 nm are 5.32×10^3 , 1.45×10^4 and $8.35 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.

The elemental composition calculated for $\text{C}_{81}\text{H}_{52}\text{N}_8\text{O}_8$ (4b) is: C, 76.9; H, 4.1; N, 8.9 and O, 10.1%. The elemental analysis results found for the compound (4b) (scheme I) are: C, 76.1; H, 4.4; N, 8.6 and O, 11.0%.

Synthesis of *tetrakis(p-aminomethylphenyl)porphyrin* (TAMPP) (5a) from its corresponding phthalimido protected porphyrin (4a): The phthalimido groups in compound (4a) was removed in two steps. In the first hydrolysis step: the phthalimido porphyrin (4a) (500 mg) was dissolved in dioxane (in 1mg/ml ratio, 500 ml dioxane was used). Then a 1:1 mixture of methanol and water was added (1/3 of the volume of dioxane). The solution was stirred and heated to about 50 °C and all solid material completely dissolved. Then, 5 N NaOH solution (74 ml) was added dropwise into the reaction system until the final concentration of NaOH is 0.5 N. The system was refluxed for 15-30 min and then cooled. About half the total volume of water (370 ml) was added. Then the unreacted original porphyrin and dioxane were removed by extracting the solution with methylene chloride several times. The water layer was evaporated with a rotary evaporator until no more liquid remained. The condensed crude phthalimide porphyrin-residue (its structure shown in figure 2-7) can be directly used for the next step.

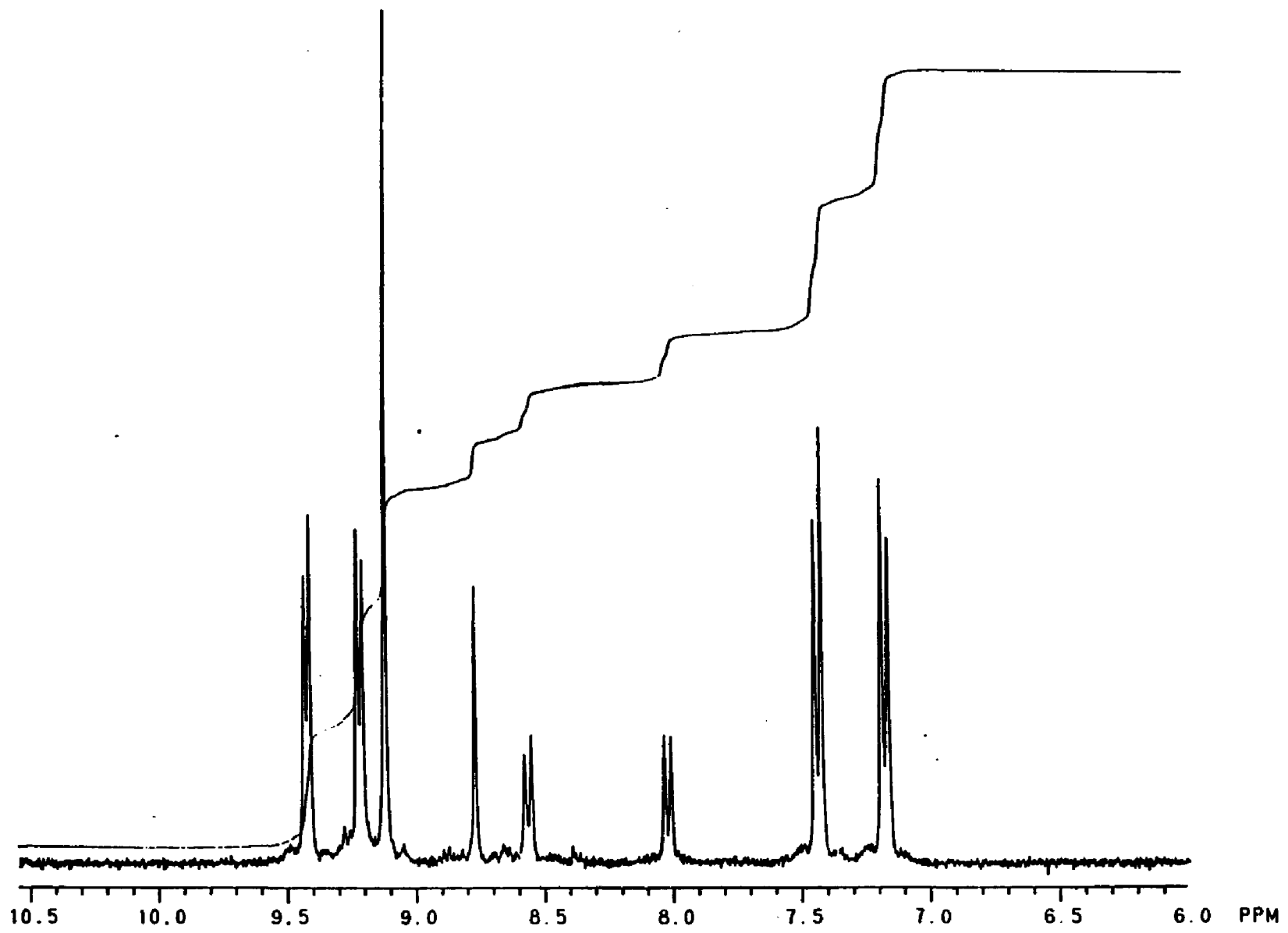
In the second hydrolysis step, the above crude phthalimide porphyrin was dissolved in a minimum amount (not more than 50 ml) of 70% H₂SO₄ and refluxed for 2 to 3 hrs. The system was cooled to room temperature and neutralized by adding saturated barium hydroxide solution slowly. During this process, the mixture was immersed in an ice bath and constantly stirred, until the solution reaches neutrality (this removes the sulfate ions as precipitated barium sulfate). The system was acidified with 5% HCl solution and the color of the upper solution gradually changed from colorless (the porphyrin was originally adsorbed on the barium sulfate) to green (as the porphyrin dissolved into protonated form). The supernatant liquid was decanted and large volume of precipitate washed several times with 5% HCl solution until the water layer shown only a slight green coloration. All of acidic aqueous washes were collected and the water

removed using a rotary evaporator. The paste was dissolved with 5% HCl solution and filtered through a fine mesh sintered glass funnel. The precipitate was discarded. The filtrate was made basic by adding concentrated aqueous ammonia. This basic solution was extracted with CH_2Cl_2 several times until the water layer was almost colorless. After rotorevaporation, the collected CH_2Cl_2 layer was reduced to a small volume (about 50 ml) and washed with distilled water several times to remove all salts and dissolved ammonia. The CH_2Cl_2 layer was reduced to dryness using a rotary evaporator and acidified with a 10% aqueous HCl solution. The acidic water solution was washed with CH_2Cl_2 again to make sure that any possible mixed phthalic acid, unhydrolyzed porphyrin, and any other organic material were removed. Then the water layer was reduced to a small volume (about 2ml) and lyophilized to dryness. The tetrakis(p-aminomethylphenyl) porphyrin (TAMPP) obtained by above purification procedure is in its diprotonated salt. The neutral porphyrin is obtained by exchanging the order of the above purification steps, i.e. washing the acidic aqueous porphyrin aqueous solution first with CH_2Cl_2 and then washing the methylene chloride porphyrin solution with water. The yield after purification is about 70%.

The ^1H NMR spectrum of TAMPP at 30 °C and pH = 2.8 is shown in figure 2-22. The assignment for the peaks observed at 30 °C are: one 8H singlet located at 9.05 ppm from β -pyrroline hydrogen atoms, a pair 8H doublets located at 8.79 ppm and 8.21 ppm from the ortho- and meta- hydrogen atoms of the benzyl groups.

The product TAMPP is obtained in its fully protonated state (with 6HCl) and some water of crystallization as well. In order to determine the extinction coefficient, the apparent molecular weight was determined by an ^1H NMR spectral method using tetrakis(4-N-methylpyridyl)porphine as the tetra-p-tosylate salt (TMPyP-4), a well defined compound, as the standard. TMPyP-4 and TAMPP were weighed separately in the same beaker and then D_2O and two drops of DCl were added. After all of the solid

Figure. 2-23. The 300 MHz ^1H NMR spectra of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and tetrakis(4-N-methylpyridyl)porphine (TMPyP-4) in D_2O .+ 2 drops DCl, room temperature (the experiment method is described on page 72)



materials were dissolved and mixed very well, the mixture solution was transferred into an NMR tube and the spectrum was taken. The mole ratio of TMPyP-4 to the porphyrin of unknown mass in the tube will be equal to the ratio of the relative integral height per TMPyP-4 hydrogen to the relative integral height per TAMPP hydrogen in the ^1H NMR spectrum. The apparent molecular weight of TAMPP can be easily derived, since the molecular weight of TMPyP-4 and the weight used to obtain ^1H NMR for TMPyP-4 and TAMPP are known. The molecular weight of TAMPP $\cdot 6\text{HCl}\cdot x\text{H}_2\text{O}$ determined from the ^1H NMR spectrum as described above (figure 2-23) is 1217.9. Then the extinction coefficients of visible absorption for TAMPP was determined from its visible spectrum (figure 2-24). At pH = 1, $\epsilon_{648} = 3.64 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for TAMPP.

The molecular weight of neutral TAMPP (crystallized from methylene chloride) calculated from its structure is 730.9 while the one determined from mass spectrometry is 730.4.

The elemental composition of fully protonated porphyrin TAMPP (crystallized from hydrochloric acid aqueous solution) calculated for 5a ($\text{C}_{48}\text{H}_{48}\text{N}_8\text{Cl}_6$) is: C, 60.8; H, 5.1; N, 10.1 and Cl, 22.5%. The elemental analysis results found for the compound (5a) (scheme I) are: C, 43.0; H, 4.7; N, 9.9 and Cl, 25.4%.

Synthesis of *N*-methyltetrakis(*p*-aminomethylphenyl) porphyrin (N-MTAMPP) (5b) from its corresponding phthalimido-protected porphyrin (4b): The same two hydrolysis steps and purification procedures were used to derive N-MTAMPP. In this work, the N-MTAMPP is obtained also in its diprotonated salt. The yield of this hydrolysis step is about 60%.

The ^1H NMR spectrum of N-MTAMPP at 30 °C is shown in figure 2-19a or at 90 °C in figure 2-19d at pH = 2.8 (the peak positions change when the temperature is changed). The assignment for the typical peaks at 90 °C are: two 2H singlets located at

Figure 2-24 The visible spectrum of tetrakis(p-aminomethylphenyl) porphyrin (TAMPP) at pH=1, H₂O, room temperature.

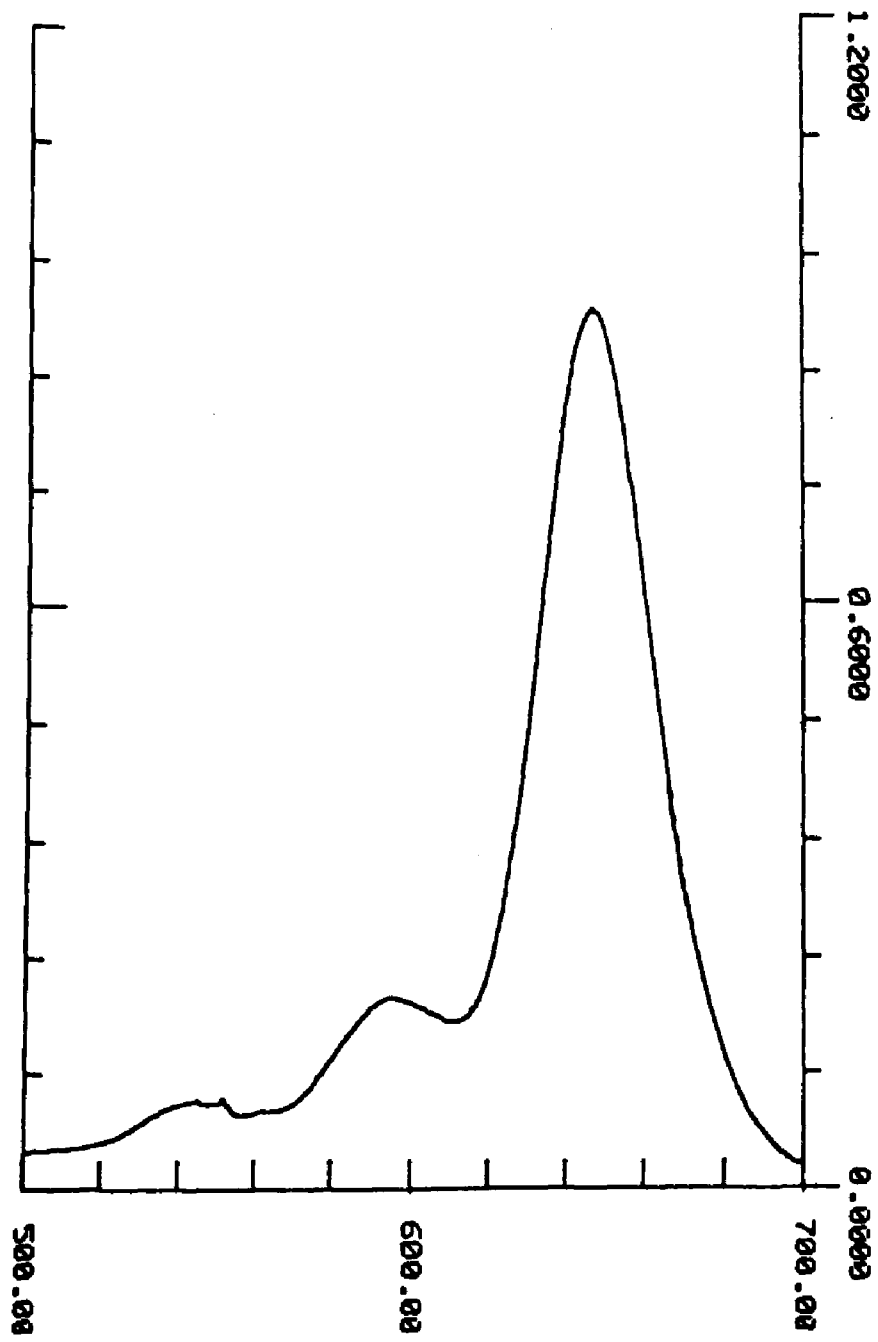


Figure. 2-25. The 300 MHz ^1H NMR spectrum of N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) and tetrakis(4-N-methylpyridyl)porphine (TMPyP-4) in D_2O , room temperature (the experimental method is described on page 72).

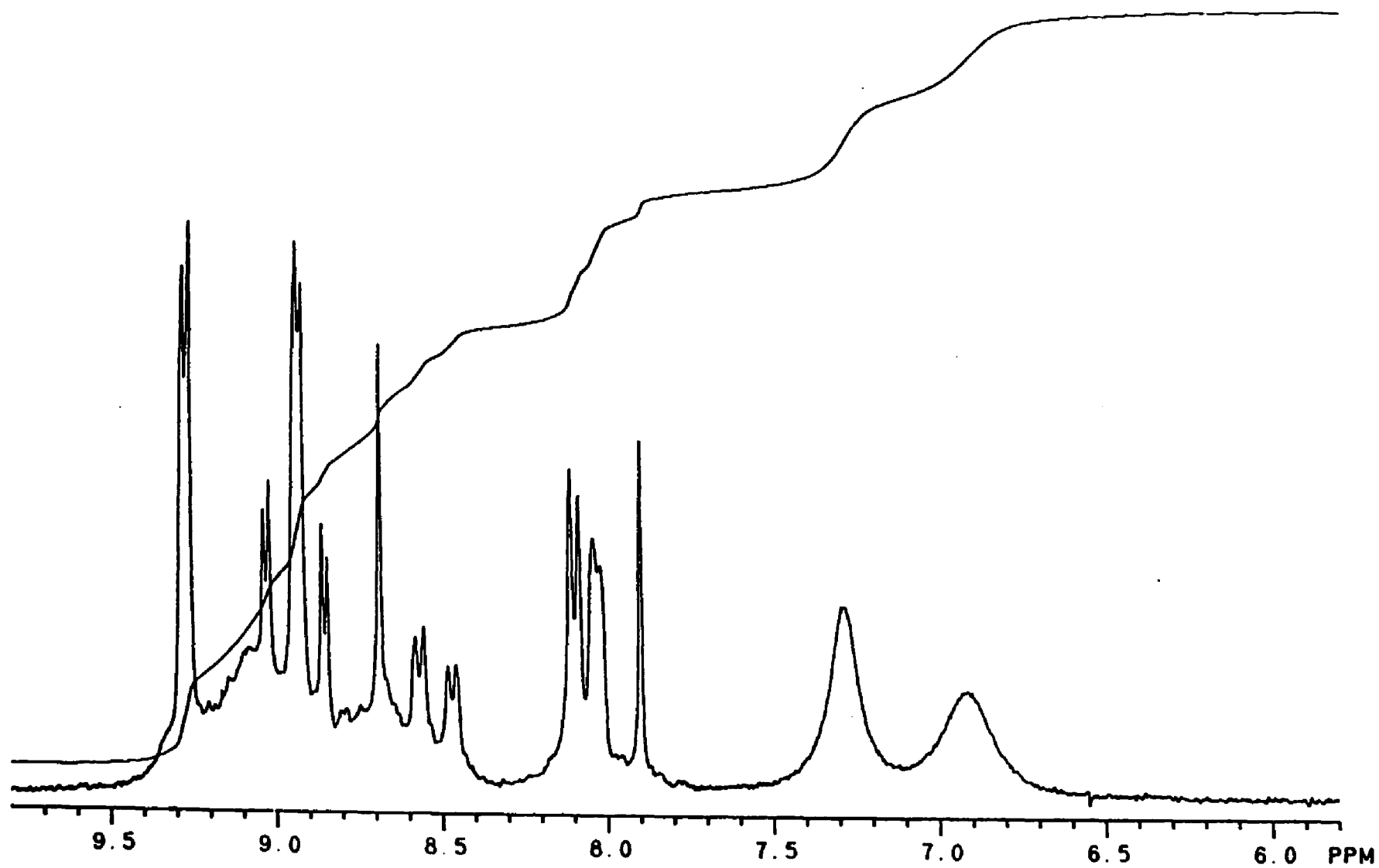
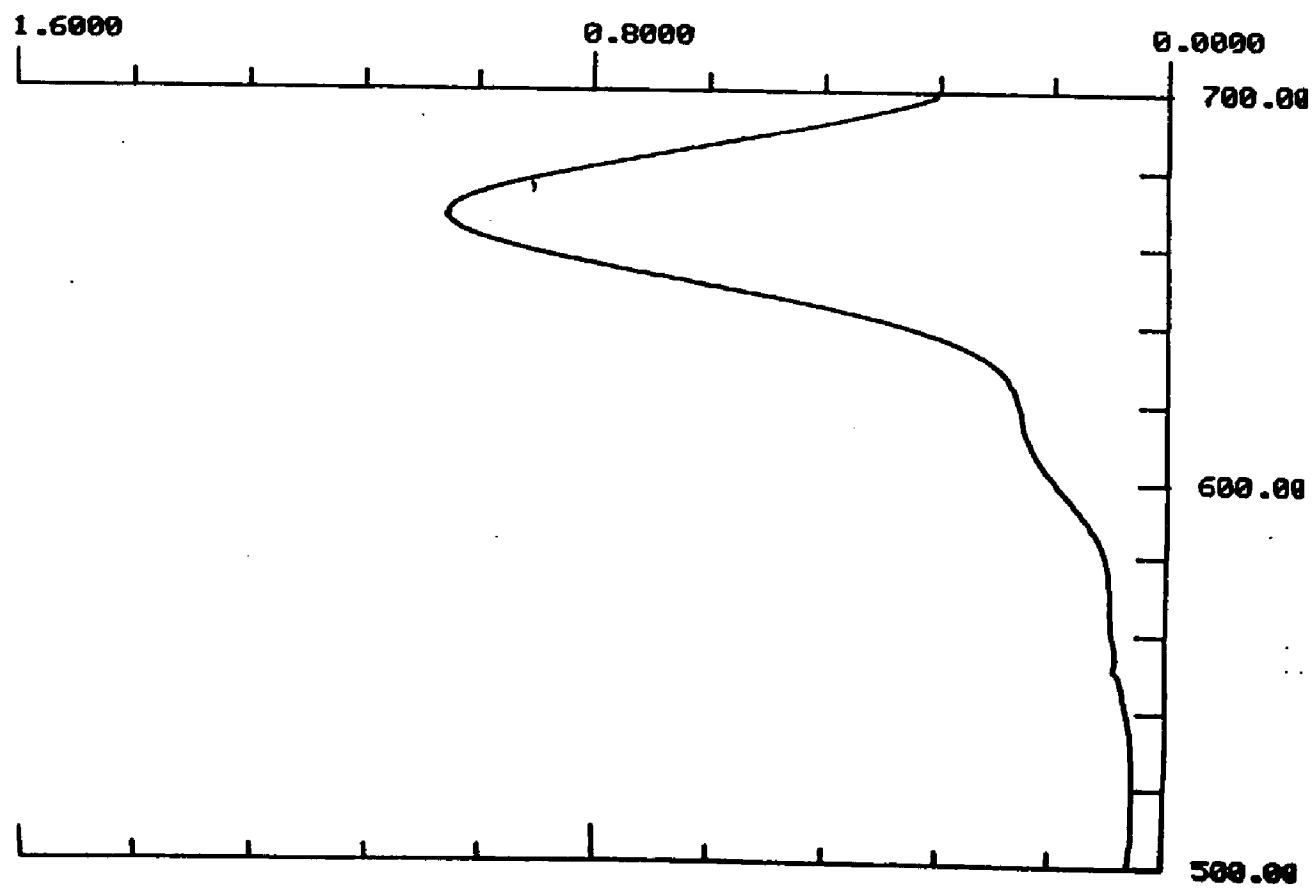


Figure. 2-26. The visible spectrum of N-methylated tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) at pH=1, H₂O, room temperature.



8.61 ppm and 9.37 ppm from the positions "1" and "4" of β -pyrrolenine hydrogen atoms, a pair 2H doublets located at 9.51 ppm and 9.69 ppm from the positions "2" and "3" of β -pyrrolenine hydrogen atoms, respectively, a pair 2H doublets located at 8.64 ppm and 9.08 ppm from the position "b" and "a", a" (a' and a are equivalent at the high temperature) of benzyl groups hydrogen atoms and a pair 2H doublets located at 8.73 ppm and 9.29 ppm from the position "d" and "c", c" (c' and c are equivalent at the high temperature) of outside benzyl group hydrogen atoms.

The N-MTAMPP is also obtained in its fully protonated salt form (with 6HCl) with some amount of water of crystalization (N-MTAMPP·6HCl·yH₂O). The method used to determine the apparent molecular weight of N-MTAMPP was similar to that used for TAMPP except that DCl is not added in the mixture of N-MTAMPP and TMPyP-4 in deuterium oxide solution. The apparent molecular weight of N-MTAMPP determined from its ¹H NMR spectrum based on method described on page 72 (figure 2-25) is 1254.8. Then the extinction coefficient of visible absorption for N-MTAMPP was determined from its visible spectrum (figure 2-26). At pH = 1, $\epsilon_{669} = 4.47 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for N-MTAMPP.

The molecular weight of neutral N-MTAMPP (crystallized from methylene chloride) calculated from its structure is 744.9 while the one determined from mass spectrometry is 744.5.

The elemental composition of fully protonated N-MTAMPP (crystallized from hydrochloric acid aqueous solution) calculated for 5b (C₄₉H₅₀N₈Cl₆) is: C, 61.2; H, 5.2; N, 10.0 and Cl, 22.1%. The elemental analysis results found for the compound (5b) (scheme I) are: C, 53.4; H, 5.3; N, 9.7 and Cl, 18.4%.

The elemental analysis of water soluble porphyrins, TAMPP and N-MTAMPP, does not appear to be useful for evaluating the molecular weight. It is commonly

observed that solvents co-crystallize with porphyrins and the amount of solvent is difficult to determine unambiguously without performing crystallographic analysis ²¹.

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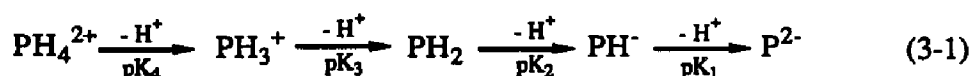
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Chapter 3

Acidity Constant studies of TAMPP and NMTAMPP

3.1 Introduction

In general the acidity constants of porphyrins are described by pK values as follows:



where PH_2 represents a neutral or free base porphyrin and PH_3^+ and PH_4^{2+} represent mono- and diprotonated porphyrins, respectively. Forms of porphyrin in which hydrogen atoms have been removed from central nitrogen atoms of the free base to form anions, PH^- and P^{2-} , are such strong bases that they are seldom formed in aqueous solution. Therefore, the acidity constants of porphyrins that are typically reported are pK_3 and pK_4 , which describe the properties of mono- and diprotonated porphyrins, respectively.

The protonation constants are usually determined from the changes in the characteristic absorption spectra of porphyrin species upon titration with acids and bases¹.

In this work, spectrophotometric and potentiometric titration methods were both conducted. Based on these results, an approximate calculation of apparent pK_3 and pK_4 values can be made. These values are necessary to establish the nature of the species present in the binding studies with polynucleic acids reported in this dissertation.

In a spectrophotometric titration, the inflection point of the plot of absorbance values vs pH does not provide information about the pK directly. The extinction

coefficients of the species before and after protonation must be considered. Thus, when only one reaction:



characterized by the equilibrium constant

$$\frac{[\text{PH}_2][\text{H}^+]}{[\text{PH}_3^+]} = K_3 \quad (3-3)$$

predominates in the solution, the most useful relation used in the spectrophotometric method of determination the acidity constant is the ratio:

$$\frac{A_{\lambda_1}}{A_{\lambda_2}} = \frac{\epsilon_{\lambda_1}^{\text{PH}_2} [\text{PH}_2] + \epsilon_{\lambda_1}^{\text{PH}_3^+} [\text{PH}_3^+]}{\epsilon_{\lambda_2}^{\text{PH}_2} [\text{PH}_2] + \epsilon_{\lambda_2}^{\text{PH}_3^+} [\text{PH}_3^+]} \quad (3-4)$$

For best results equation (3-4) should be used to treat data at the wavelengths where the largest absorbance changes occurs during the titration, that is:

$$\frac{A_{\lambda_{\text{max}}, \text{PH}_3^+}}{A_{\lambda_{\text{max}}, \text{PH}_2}} = \frac{\epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_2} [\text{PH}_2] + \epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_3^+} [\text{PH}_3^+]}{\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2} [\text{PH}_2] + \epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_3^+} [\text{PH}_3^+]} \quad (3-5)$$

where $A_{\lambda_{\text{max}}, \text{PH}_3^+}$ and $A_{\lambda_{\text{max}}, \text{PH}_2}$ are the absorbancies at the wavelengths of the maximum absorbance of the monoprotinated and free base porphyrin, $\epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_2}$ is the extinction coefficient of PH_2 at the wavelength of maximum absorbance of PH_3^+ , $\epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_3^+}$ is the extinction coefficient of PH_3^+ at the wavelength of maximum

absorbance of PH_3^+ , $\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2}$ is the extinction coefficient of PH_2 at the wavelength of maximum absorbance of PH_2 , and $\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_3^+}$ is the extinction coefficient of PH_3^+ at the wavelength of maximum absorbance of PH_2 . At the mid point of titration, i.e. when $[\text{PH}_3^+] = [\text{PH}_2]$, equation 3-3 and 3-5 will typically be

$$K_3 = [\text{H}^+]_{\text{mid}}$$

or

$$\text{p}K_3 = [\text{pH}]_{\text{mid}} \quad (3-6)$$

and

$$\left(\frac{A_{\lambda_{\text{max}}, \text{PH}_3^+}}{A_{\lambda_{\text{max}}, \text{PH}_2}} \right)_{\text{mid}} = \frac{\epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_2} + \epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_3^+}}{\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2} + \epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_3^+}} \quad (3-7)$$

To use this equation, $A_{\lambda_{\text{max}}, \text{PH}_3^+}$, $A_{\lambda_{\text{max}}, \text{PH}_2}$ and pH were obtained from each titration and then plotted as $\frac{A_{\lambda_{\text{max}}, \text{PH}_3^+}}{A_{\lambda_{\text{max}}, \text{PH}_2}}$ versus pH. This diagram can be used to estimate $\text{p}K_3$ provided we can calculate $\left(\frac{A_{\lambda_{\text{max}}, \text{PH}_3^+}}{A_{\lambda_{\text{max}}, \text{PH}_2}} \right)_{\text{mid}}$ using the equation(3-7). Similarly, $\left(\frac{A_{\lambda_{\text{max}}, \text{PH}_4^{2+}}}{A_{\lambda_{\text{max}}, \text{PH}_3^+}} \right)_{\text{mid}}$ is calculated using equation:

$$\left(\frac{A_{\lambda_{\text{max}}, \text{PH}_4^{2+}}}{A_{\lambda_{\text{max}}, \text{PH}_3^+}} \right)_{\text{mid}} = \frac{\epsilon_{\lambda_{\text{max}}, \text{PH}_4^{2+}}^{\text{PH}_3^+} + \epsilon_{\lambda_{\text{max}}, \text{PH}_4^{2+}}^{\text{PH}_4^{2+}}}{\epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_3^+} + \epsilon_{\lambda_{\text{max}}, \text{PH}_3^+}^{\text{PH}_4^{2+}}} \quad (3-8)$$

and $\text{p}K_4$ is estimated from the plot of $\left(\frac{A_{\lambda_{\text{max}}, \text{PH}_4^{2+}}}{A_{\lambda_{\text{max}}, \text{PH}_3^+}} \right)$ versus pH.

Potentiometric titrations were used as another method to check the dependability of spectrophotometric methods and also to determine $\text{p}K_a$, the acidity constant of amino groups outside of the porphyrin ring. Protonation of these peripheral groups does not

result in useful spectroscopic changes in the uv-visible region. The basic approach can be summarized as follows.

Plots of pH vs V_{NaOH} and $\Delta\text{pH}/\Delta V_{\text{NaOH}}$ vs. V'_{NaOH} are made from the experimental data. V'_{NaOH} is the modified volume of titrated NaOH and can be derived by

$$V'_{\text{NaOH}} = V_{\text{NaOH}}(a) + \frac{V_{\text{NaOH}}(b) - V_{\text{NaOH}}(a)}{2} \quad (3-9)$$

The inflection point can be obtained easily from the plot of $\Delta\text{pH}/\Delta V_{\text{NaOH}}$ vs. V'_{NaOH} . The end point pH(end) and V'_{NaOH} (end), the volume of NaOH solution added at the end point, are then determined from the plot of pH vs. V_{NaOH} . The relationship between pK with the pH(end) and V'_{NaOH} (end) is easily determined from the equilibrium expressions for amphoteric systems.

3.2 Experimental and instruments

Two ionic strength conditions were examined for all of pK determinations. One of them is $[I] = 0.12 \text{ M}$ (adjusted by NaCl) which is in the same ionic strength as was used in the binding characteristic studies of porphyrins with nucleic acids discussed in chapter (4) and (5). The other is $[I] < 0.01 \text{ M}$. The low ionic strength was used to allow comparison with the acidity constants of other porphyrins and to investigate the sensitivity of the acidity constants to changes in ionic strength. The spectrophotometric titration and potentiometric titration were each done using 4 ml cuvettes. Titrations were either carried out beginning at about pH 3 and adding 0.092 M sodium hydroxide to raise the pH or adding 0.01 M nitric acid to reduce the pH.

$\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2}$, $\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2}$, and $\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2^+}$, $\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2^+}$, $\epsilon_{\lambda_{\text{max}}, \text{PH}_2^+}^{\text{PH}_2^+}$ were calculated from visible spectra of each corresponding porphyrin species by very careful

pH adjustment. $\epsilon_{\lambda_{\text{max}}, \text{PH}_2^+}^{\text{PH}_2^+}$ and $\epsilon_{\lambda_{\text{max}}, \text{PH}_2^+}^{\text{PH}_2^+}$ were calculated from corresponding absorbance readings by adding larger amounts of nitric acid to pH = 1.

Absorbancies at different wavelengths were read from HEWLETT PACKARD 8452A DIODE ARRAY spectrophotometer and visible spectra were taken from BECKMAN DU-8 spectrophotometer. The pH value of solutions at different titration points were detected by a RADIOMETER COPENHAGEN PHM84 RESEARCH pH meter.

3.3 Results

One of the series of trials conducted in this work is discussed in detail in this section in order to specify the way that acidity constants pK_3 , pK_4 and pK_a were derived for the porphyrins TAMPP and N-MTAMPP at two different ionic strengths using spectrophotometric and potentiometric methods.

3.3.1 The acidity constants of porphyrin pyrroleninic ring (pK_3 and pK_4) — spectrophotometric method

3.3.1a TAMPP

Figures 3-1c and 3-3c show the major parts of the titration process for TAMPP in $[\text{I}] = 0.12 \text{ M}$ and $[\text{I}] < 0.01 \text{ M}$, respectively. The apparent "isosbestic point" (which does not coincide exactly due to slightly changes in volume) shown for each graph implies that little of the monoprotonated porphyrin accumulated as the diprotonated porphyrin dissociated into the free base porphyrin. The existence of the monoprotonated porphyrin must be limited to a very narrow range of acidity. The dissociation of diprotonated TAMPP in solution then can be considered simply as follows (where H^+ is used to represent the hydronium ion, H_3O^+ , in aqueous solutions):

Figure.3-1. The visible spectra of tetrakis(p-aminomethylphenyl) porphyrin (TAMPP) in its (a). neutral form, PH_2 (pH=6.0); (b). diprotonated form, PH_4^{2+} (pH=1) and (c). mixture of neutral and diprotonated form, titration process (pH of solution was changed gradually from pH 2 to 5.5), 0.12M NaCl, room temperature.

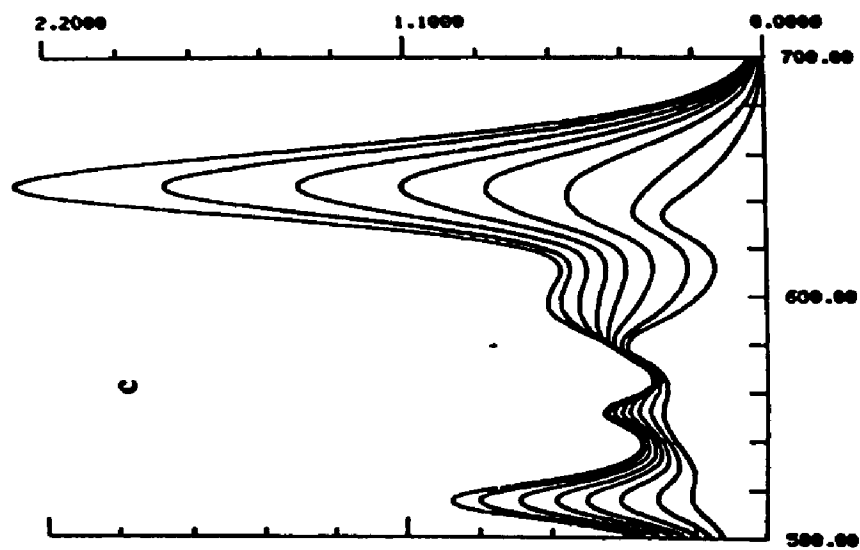
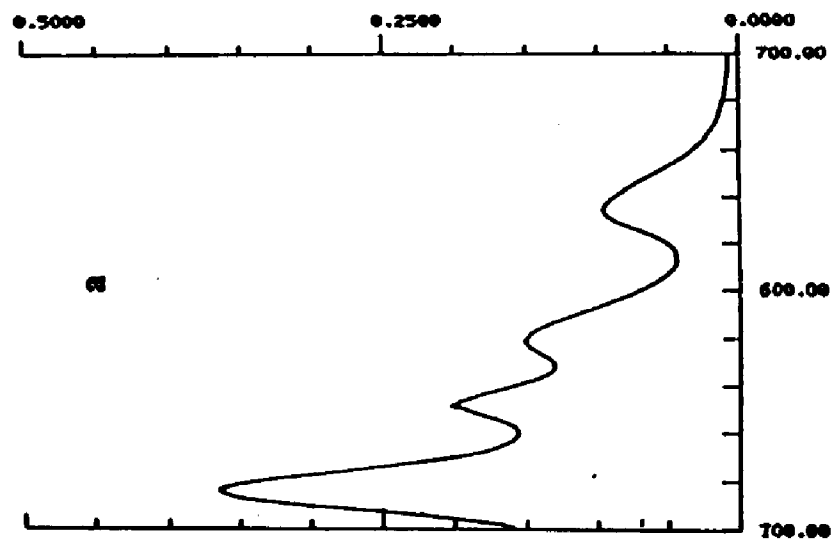
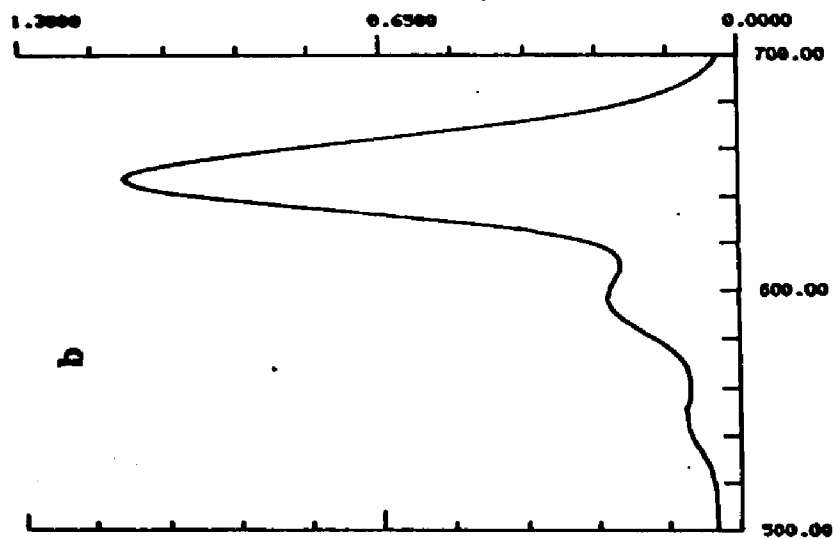
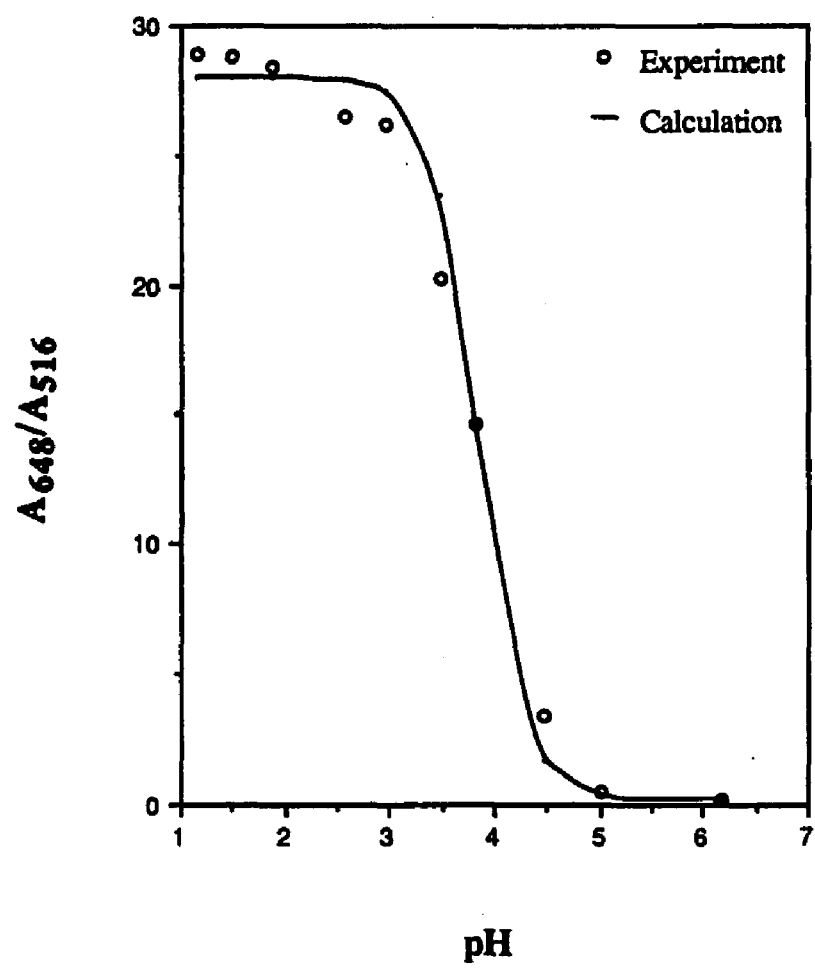


Figure.3-2. The spectrophotometric titration curve: A_{648}/A_{516} versus pH of tetrakis(p-aminomethylphenyl) porphyrin (TAMPP) (0.12M NaCl, room temperature), (•) the experimental data which is given in appendix 1-1 and (—) the best fit result which gives $pK_3 = pK_4 = 4.4$.





which gives:

$$K_{\text{app}} = \frac{[\text{PH}_2] [\text{H}^+]^2}{[\text{PH}_4^{2+}]} \quad (3-11)$$

At the titration mid point, i.e. $[\text{PH}_2] = [\text{PH}_4^{2+}]$, we have

$$\text{p}K_{\text{app}} = 2 \cdot \text{pH}_{\text{mid}} \quad (3-12)$$

where $K_{\text{app}} = K_3 \cdot K_4$ and $K_3 \approx K_4$. Equation (3-7) and (3-8) will be composed as:

$$\left(\frac{A_{\lambda_{\text{max}}, \text{PH}_4^{2+}}}{A_{\lambda_{\text{max}}, \text{PH}_2}} \right)_{\text{mid}} = \frac{\epsilon_{\lambda_{\text{max}}, \text{PH}_4^{2+}}^{\text{PH}_2} + \epsilon_{\lambda_{\text{max}}, \text{PH}_4^{2+}}^{\text{PH}_4^{2+}}}{\epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_2} + \epsilon_{\lambda_{\text{max}}, \text{PH}_2}^{\text{PH}_4^{2+}}} \quad (3-13)$$

Figures 3-1a, and 3-1b show the spectra of neutral and diprotonated porphyrin at $[\text{I}] = 0.12 \text{ M}$. The specific extinction coefficients at wavelengths 516 and 648 nm can be calculated as: $\epsilon_{516}^{\text{PH}_2} = 1.08 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{648}^{\text{PH}_2} = 1.96 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{516}^{\text{PH}_4^{2+}} = 1.28 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{648}^{\text{PH}_4^{2+}} = 3.58 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, which give:

$$\begin{aligned} \left(\frac{A_{648}}{A_{516}} \right)_{\text{mid}} &= \frac{\epsilon_{648}^{\text{PH}_2} + \epsilon_{648}^{\text{PH}_4^{2+}}}{\epsilon_{516}^{\text{PH}_2} + \epsilon_{516}^{\text{PH}_4^{2+}}} \\ &= \frac{1.96 \times 10^3 + 3.58 \times 10^4}{1.08 \times 10^4 + 1.28 \times 10^3} = 3.13 \end{aligned}$$

Using figure 3-2, the best fitting results are: $pK_3 = pK_4 = 4.4$ and thus $pK_{app} = 8.8$ at $[I] = 0.12 \text{ M}$.

Figures 3-3a and 3-3b show the spectra of neutral and diprotonated porphyrin at $[I] < 0.01 \text{ M}$. The specific extinction coefficients can be calculated as: $\epsilon_{516}^{PH_2} = 1.37 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{648}^{PH_2} = 3.25 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{516}^{PH_2^+} = 1.61 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{648}^{PH_2^+} = 3.54 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, from which $(A_{648}/A_{516})_{mid}$ was calculated equal to 2.52 using this $(A_{648}/A_{516})_{mid}$ value, we will have $pK_3 = pK_4 = 3.6$ and $pK_{app} = 7.2$ for $[I] < 0.01 \text{ M}$ from figure 3-4. More experimental data are given in appendix (1).

3.3.1b NM-TAMPP

The dissociation of diprotonated NM-TAMPP is a two step process:



which is evident from the fact that two "isosbestic points" appear during dissociation of the diprotonated porphyrin to form the free porphyrin (see figures 3-5 and 3-8). Figures 3-5e and 3-8e show the parts of spectra in (3-14) titration process, i.e. the change of porphyrin from the diprotonated to monoprotonated state, which gives an isosbestic point at about 636 nm for both ionic strengths. Figures 3-5d and 3-8d show parts of spectra in the titration that involves the process shown in equation (3-15), i.e. the change of the porphyrin from the monoprotonated state to free base. In this series of spectra there is an "isosbestic point" at about 596 or 593 nm for titration conditions with $[I] = 0.12 \text{ M}$ and $[I] < 0.01 \text{ M}$, respectively.

Figures 3-5a, 3-5b and 3-5c are the spectra of neutral, monoprotonated and diprotonated forms of N-MTAMPP, respectively, in aqueous solution at an ionic

Figure.3-3. The visible spectra of tetrakis(p-aminomethylphenyl) porphyrin (TAMPP) in its (a). neutral form, PH_2 (pH=6.0); (b). diprotonated form, PH_4^{2+} (pH=1) and (c). mixture of neutral and diprotonated form during titration process (pH of solution was changed gradually from pH 2 to 5.5), ionic strength ≤ 0.01 M, room temperature.

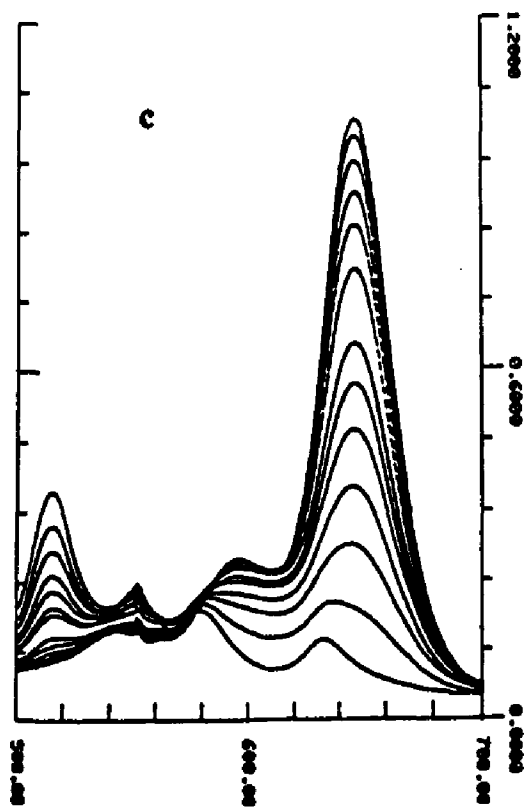
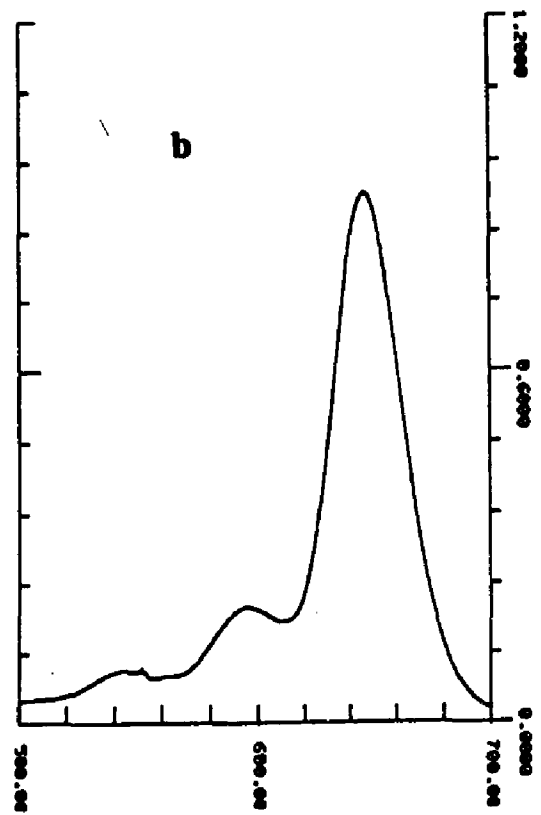
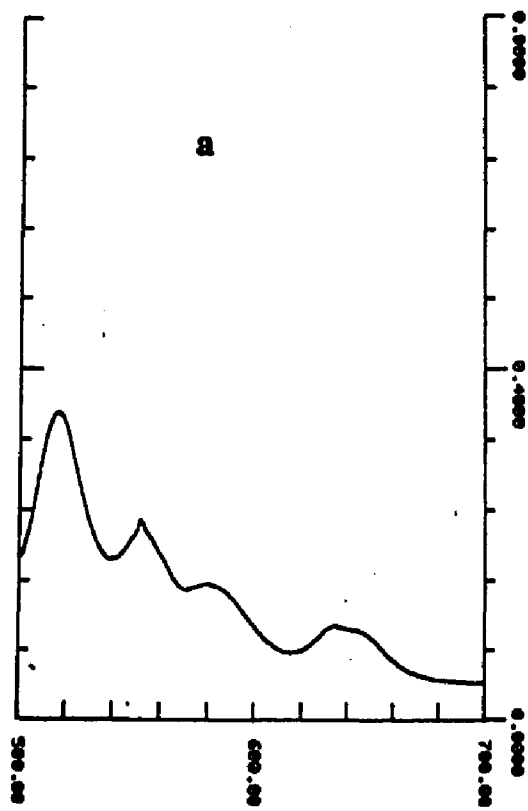


Figure.3-4. The spectrophotometric titration curve of tetrakis(p-aminomethylphenyl) porphyrin (TAMPP) (ionic strength ≤ 0.01 M, room temperature) , ($\bullet$) the experimental data which is given in appendix 1-2 and (—) the best fit result which gives $pK_3 = pK_4 = 3.6$.

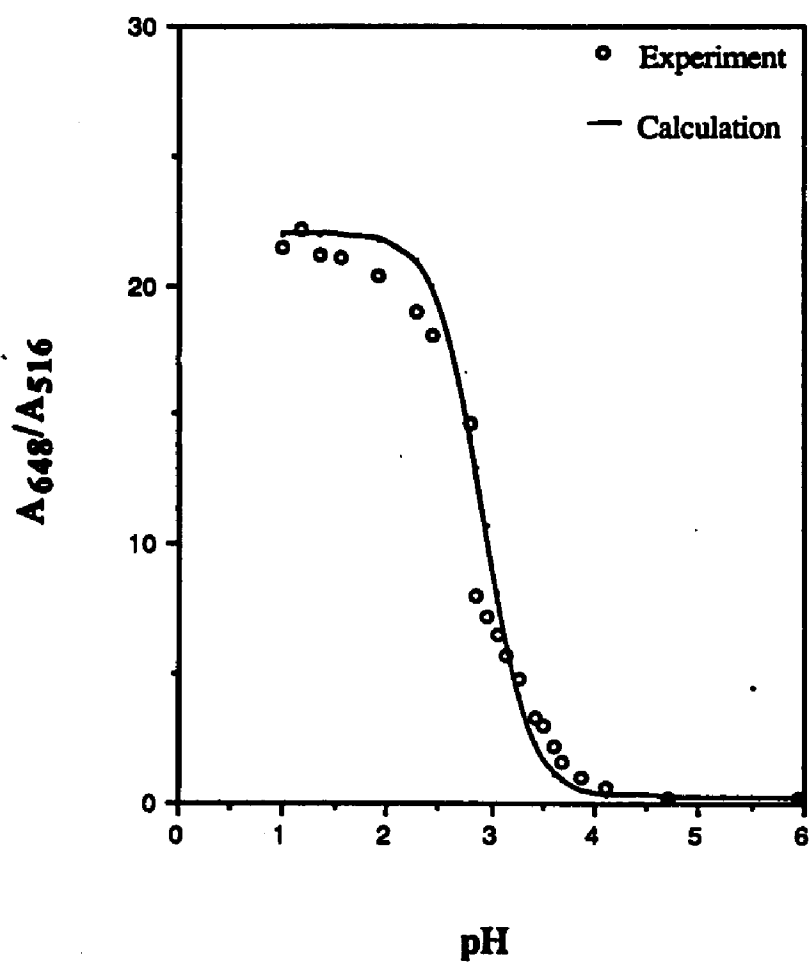
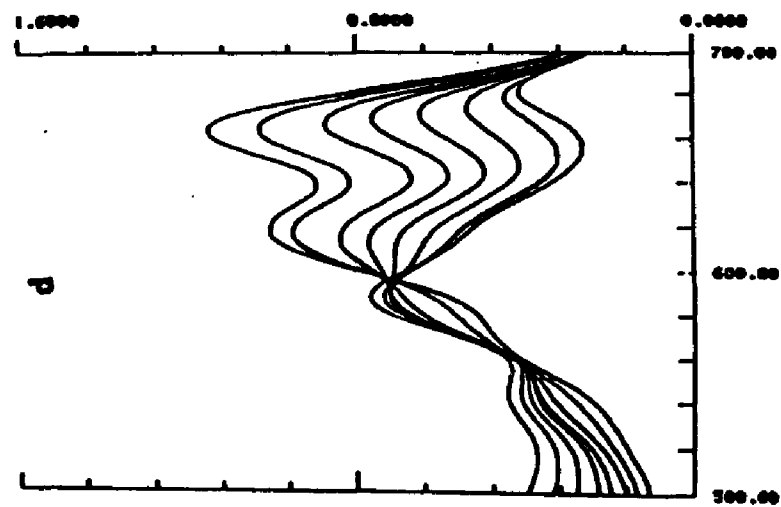
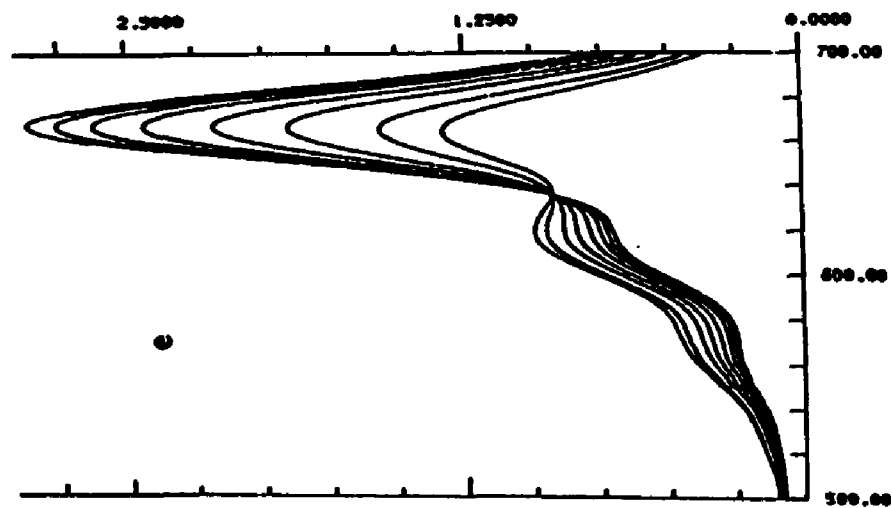
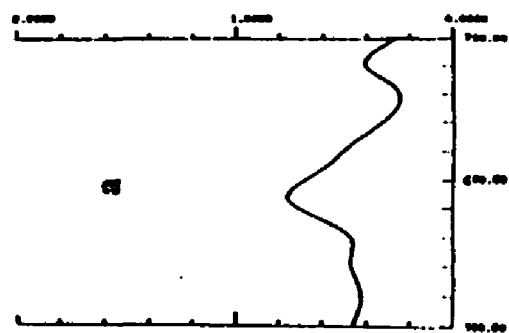
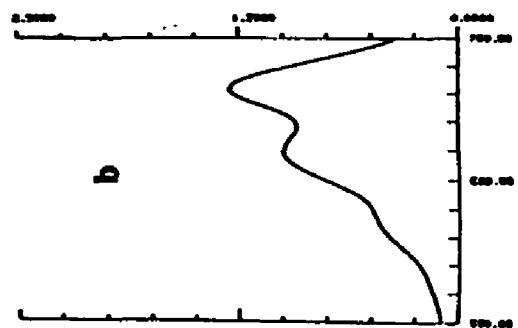
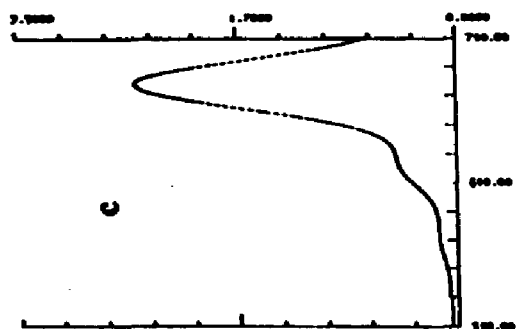


Figure.3-5. The visible spectra of N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) in its (a). neutral form, PH_2 (pH = 8.5); (b). monoprotonated form, PH_3^+ (pH = 5.6), (c) diprotonated form, PH_4^{2+} (pH = 1), (d). mixture of neutral and monoprotonated form: titration process (3-15) (pH of solution was changed gradually from pH 5.8 to 8.4) and (e). mixture of monoprotonated and diprotonated form: titration process (3-14) (pH of solution was changed gradually from pH 2 to 5.0), 0.12M NaCl, room temperature.



strength of 0.12 M. The extinction coefficients under these condition are: $\epsilon_{666}^{\text{PH}_2} = 7.64 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{618}^{\text{PH}_2} = 1.02 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{588}^{\text{PH}_2} = 1.09 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{666}^{\text{PH}_3^+} = 1.67 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{618}^{\text{PH}_3^+} = 1.50 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{588}^{\text{PH}_3^+} = 8.57 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{666}^{\text{PH}_2^+} = 4.48 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{618}^{\text{PH}_2^+} = 9.06 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$, and $\epsilon_{588}^{\text{PH}_2^+} = 3.95 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. For the process (3-14), we have

$$\begin{aligned} \left(\frac{A_{666}}{A_{618}} \right)_{\text{mid}} &= \frac{\epsilon_{666}^{\text{PH}_2^+} + \epsilon_{666}^{\text{PH}_3^+}}{\epsilon_{618}^{\text{PH}_2^+} + \epsilon_{618}^{\text{PH}_3^+}} \\ &= \frac{4.48 \times 10^4 + 1.67 \times 10^4}{9.06 \times 10^3 + 1.50 \times 10^4} = 2.56 \end{aligned}$$

and

$$\begin{aligned} \left(\frac{A_{666}}{A_{588}} \right)_{\text{mid}} &= \frac{\epsilon_{666}^{\text{PH}_2^+} + \epsilon_{666}^{\text{PH}_3^+}}{\epsilon_{588}^{\text{PH}_2^+} + \epsilon_{588}^{\text{PH}_3^+}} \\ &= \frac{4.48 \times 10^4 + 1.67 \times 10^4}{3.95 \times 10^3 + 8.57 \times 10^3} = 4.91 \end{aligned}$$

which leads to $\text{pK}_4 = 3.1$ using figures 3-6 and 3-7, respectively. Similarly, for the process (3-15), we can have $\text{pK}_3 = 8.1$ from its related

$$\left(\frac{A_{666}}{A_{618}} \right)_{\text{mid}} = \frac{\epsilon_{666}^{\text{PH}_3^+} + \epsilon_{666}^{\text{PH}_2}}{\epsilon_{618}^{\text{PH}_3^+} + \epsilon_{618}^{\text{PH}_2}} = 0.96$$

and

$$\left(\frac{A_{666}}{A_{588}} \right)_{\text{mid}} = \frac{\epsilon_{666}^{\text{PH}_3^+} + \epsilon_{666}^{\text{PH}_2}}{\epsilon_{588}^{\text{PH}_3^+} + \epsilon_{588}^{\text{PH}_2}} = 1.25$$

Figure.3-6. The spectrophotometric titration curve: A_{666}/A_{618} versus pH of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (0.12M NaCl, room temperature), (•) the experimental data which is given in appendix 3-1 and (—) the best fit result which gives $pK_3 = 8.1$ and $pK_4 = 3.1$.

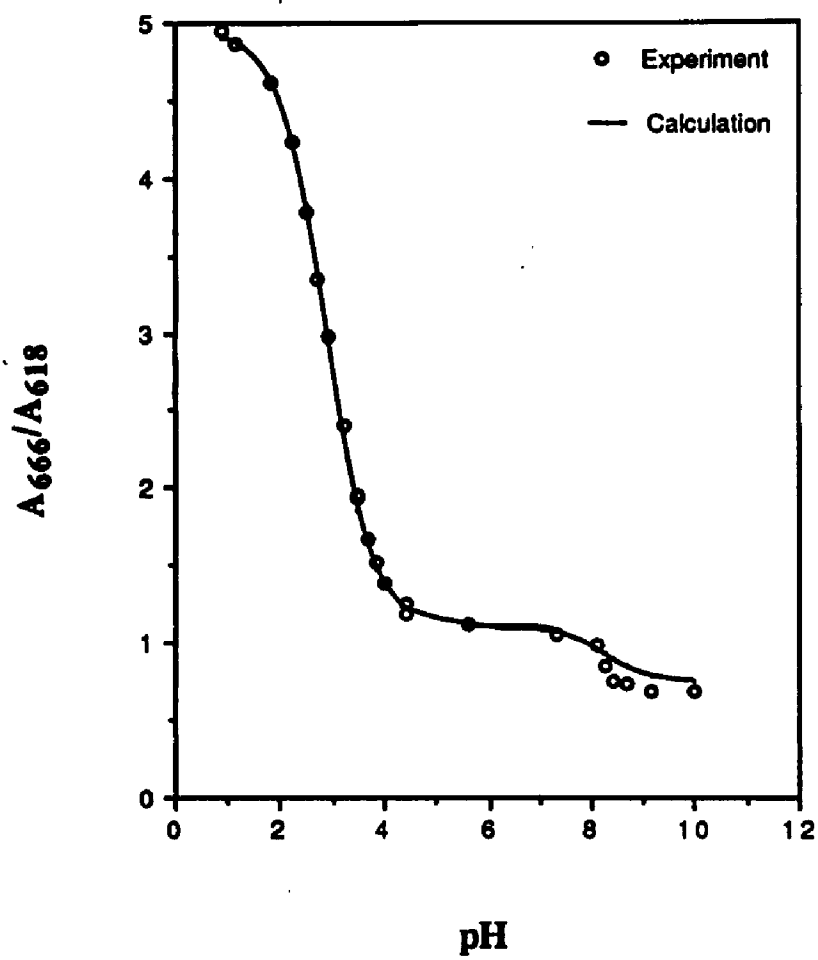
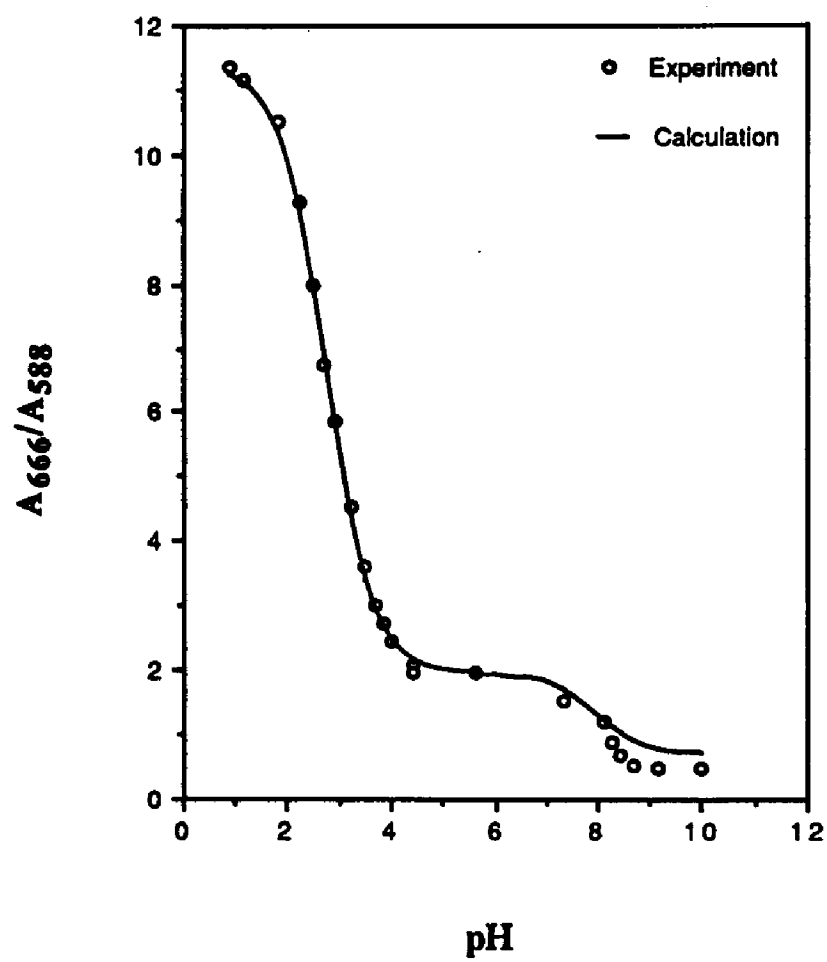


Figure.3-7. The spectrophotometric titration curve: A_{666}/A_{588} versus pH of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (0.12M NaCl, room temperature), (•) the experimental data which is given in appendix 3-1 and (—) the best fit result which gives $pK_3 = 8.1$ and $pK_4 = 3.1$.



values.

Figures. 3-8a, 3-8b and 3-8c show the spectra of aqueous solution of neutral, monoprotonated and diprotonated forms of N-MTAMPP respectively at $[I] < 0.01$ M. The extinction coefficients for these species at specific wavelengths and $[I] < 0.01$ M are: $\epsilon_{666}^{PH_2} = 4.72 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{618}^{PH_2} = 7.58 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{588}^{PH_2} = 9.62 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{666}^{PH_3^+} = 1.64 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{618}^{PH_3^+} = 1.37 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{588}^{PH_3^+} = 7.91 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{666}^{PH_4^{2+}} = 4.21 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{618}^{PH_4^{2+}} = 8.86 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{588}^{PH_4^{2+}} = 4.32 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. Therefore, for the process (3-14) we have $(A_{666}/A_{618})_{mid} = 2.59$ and $(A_{666}/A_{588})_{mid} = 4.78$ which gives pK_4 values of 2.4 using both figures 3-9 and 3-10. Similarly, for the process (3-15), we have pK_3 equal to 7.4 from both of the related $(A_{666}/A_{618})_{mid}$ and $(A_{666}/A_{588})_{mid}$ values.

3.3.2 The acidity constant of the porphyrin pyrroleninic ring (pK_3 and pK_4) — potentiometric method

3.3.2a TAMPP

At the end of the titration, all of the PH_4^{2+} is changed to PH_2 . The pH of the solution is determined by the hydrolysis of PH_2 :



and the dissociation of water. The appropriate equilibrium expression is:

$$\frac{K_w^2}{K_{app}} = \frac{[PH_4^{2+}] [OH^-]^2_{End}}{[PH_2]} \quad (3-17)$$

Figure.3-8. The visible spectra of N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) in its (a). neutral form, PH_2 (pH = 8.3); (b). monoprotonated form, PH_3^+ (pH = 3.5), (c) diprotonated form, PH_4^{2+} (pH = 1), (d). mixture of neutral and monoprotonated form: titration process (3-15) (pH of solution was changed gradually from pH 4.0 to 8.0) and (e). mixture of monoprotonated and diprotonated form: titration process (3-14) (pH of solution was changed gradually from pH 1.5 to 3.0), ionic strength ≤ 0.01 M, room temperature.

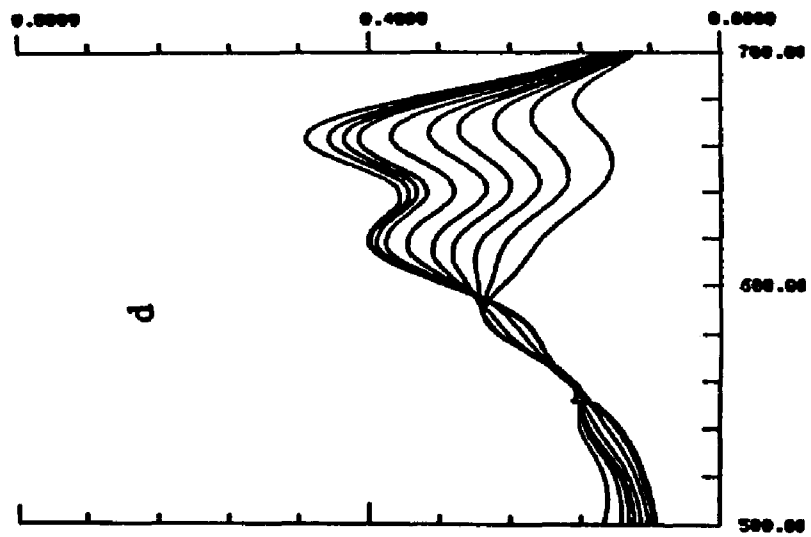
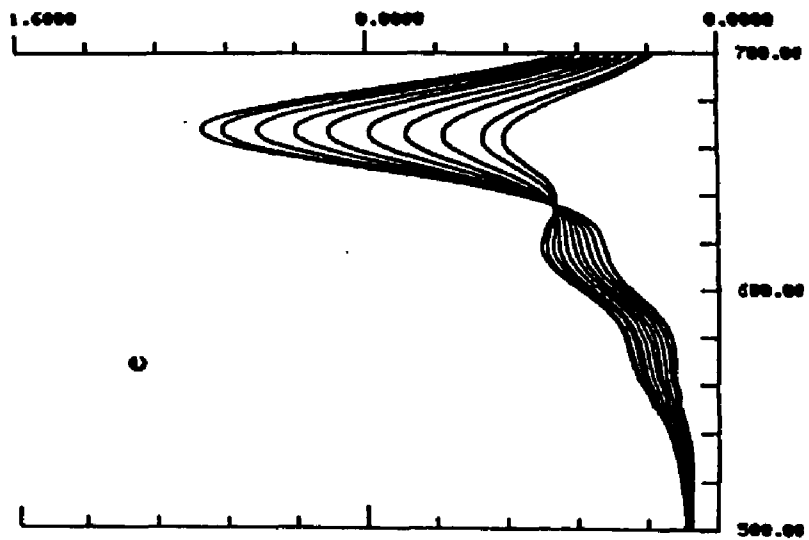
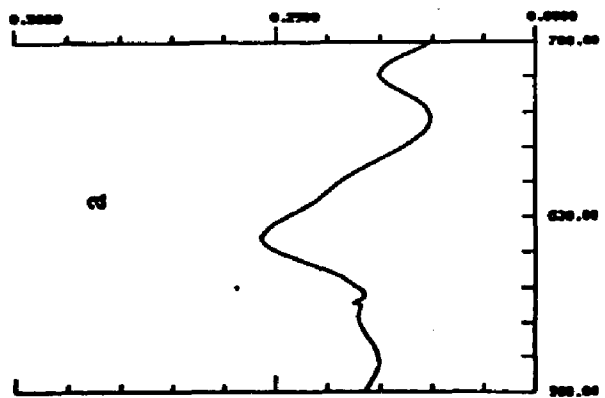
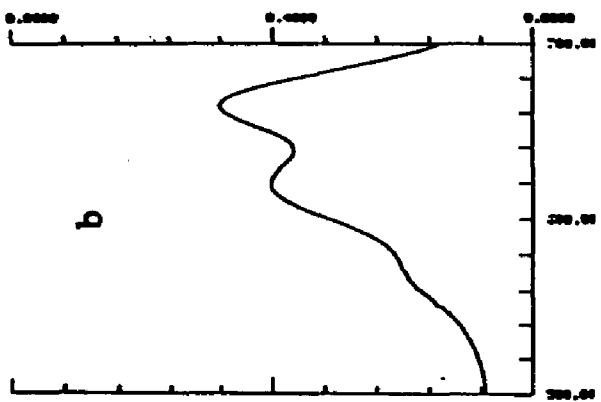
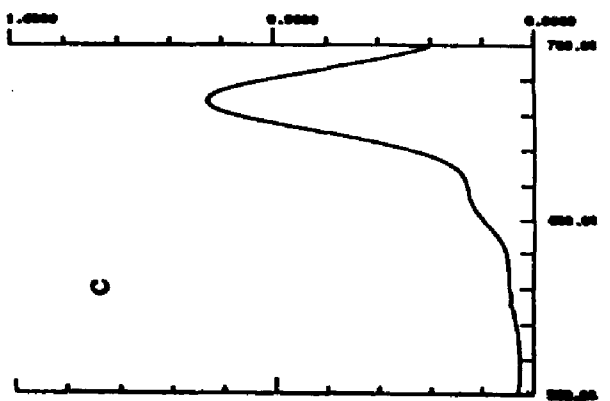


Figure.3-9. The spectrophotometric titration curve: A_{666}/A_{618} versus pH of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (ionic strength ≤ 0.01 M, room temperature) , ($\cdot$) the experimental data which is given in appendix 3-2 and (—) the best fit result which gives $pK_3 = 7.4$ and $pK_4 = 2.4$.

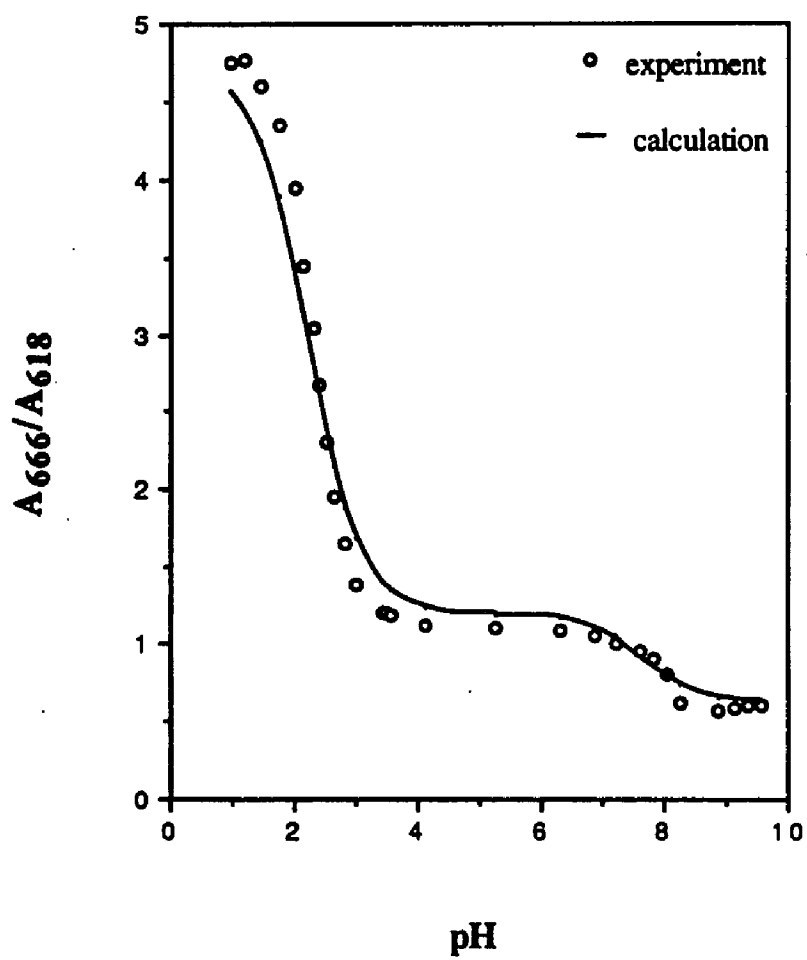
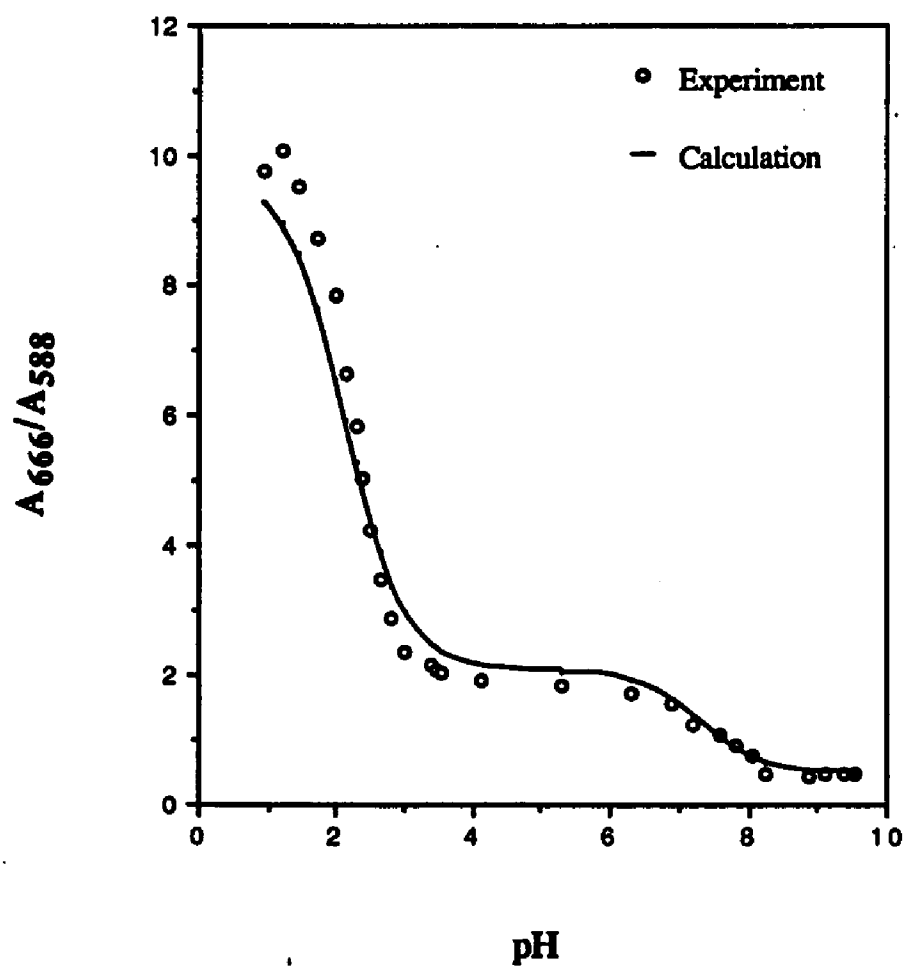


Figure.3-10. The spectrophotometric titration curve: A_{666}/A_{588} versus pH of N-methyl tetrakis(p-aminomethylphenyl) porphyrin (N-MTAMPP) (ionic strength ≤ 0.01 M, room temperature), (•) the experimental data which is given in appendix 3-2 and (—) the best fit result which gives $pK_3 = 7.4$ and $pK_4 = 2.4$.



where K_w is the dissociation constant of water, K_{app} is the apparent dissociation constant of equation (3-10), which is related to K_3 and K_4 as $K_{app} = K_3 K_4$. The concentration of OH^- at the end point is represented by $[OH^-]_{End}$. The equilibrium concentrations of neutral, diprotonated porphyrins at the end point are $[PH_2]$ and $[PH_4^{2+}]$. For the dilute solutions of a weak base, the hydroxide ion concentration is given by

$$[OH^-]_{End} = 2 \cdot [PH_4^{2+}] + [H^+]_{hyd} \quad (3-18)$$

where the term " $2 \times [PH_4^{2+}]$ " provides a measure of concentration of hydroxide ions contributed by the dissociation of weak base, PH_2 , described by process (3-16) and $[H^+]_{hyd}$ is a measure of the concentration of hydroxide ions due to the dissociation of water. $[PH_4^{2+}]$ and $[H^+]_{hyd}$ can be expressed by equation (3-17) and the relationship of $[H^+]$, $[OH^-]$ with K_w , respectively. Substitution of these two expressions in equation (3-17) gives

$$[OH^-]_{End} = 2 \frac{K_w^2}{K_{app}} \cdot \frac{[PH_2]}{[OH^-]_{End}^2} + \frac{K_w}{[OH^-]_{End}} \quad (3-19)$$

Reorganization of equation (3-19) gives the general equation which relates the apparent equilibrium constant of process (3-16) with the concentration of hydroxide ions at the end titration point $[OH^-]_{End}$ as:

$$K_{app} = 2 K_w^2 [PH_2] \cdot \left(\frac{1}{[OH^-]_{End}^3} - \frac{1}{K_w [OH^-]_{End}} \right) \quad (3-20)$$

Where $[PH_2]$ is the concentration of neutral TAMPP at the titration end point and can be calculated from:

$$[PH_2] = C_o'(TAMPP) - [PH_4^{2+}] \quad (3-21)$$

by considering the equilibrium (3-16). In equation (3-21), $[PH_4^{2+}]$ at the end point is always less than $1/2 \times [OH^-]$ since the concentration of hydrogen ions at end point includes contribution from process (3-16) and also the dissociation of water. Therefore, $[PH_4^{2+}]$ can usually be neglected from equation (3-21) because all of the potentiometric titration curves in this work (figures 3-11 and 3-12) show that $[OH^-]_{End}$ is less than 10^{-7} M which is much less than the value of $C_o'(TAMPP)$. $C_o'(TAMPP)$ means the modified original porphyrin concentration and is obtained by

$$C_o'(TAMPP) = \frac{C_o(TAMPP) \times V_s}{V_s + V_{NaOH}} \quad (3-22)$$

Here $C_o(TAMPP)$ is the stock concentration of TAMPP at pH about 3, V_s is the starting titration volume, which is 3 ml in this work, and V_{NaOH} is defined in equation (3-9).

When the contribution from dissociation of water is smaller than that of equilibrium (3-16) by one unit or when $[OH^-]_{End} \leq 10^{-8}$, Equation (3-20) can be simplified to:

$$K_{app} = \frac{2K_w^2 C_o'(TAMPP)}{[OH^-]_{End}^3} \quad (3-23)$$

This expression can also be derived directly from equation (3-16) and (3-17).

Figure.3-11. (a). The potentiometric titration curve of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP), pH versus V_{NaOH} , and (b). differential curve of (a), $\Delta\text{pH} / \Delta V_{\text{NaOH}}$ versus V_{NaOH} , (0.12M NaCl, room temperature). The experimental data is given in appendix 2-1.1.

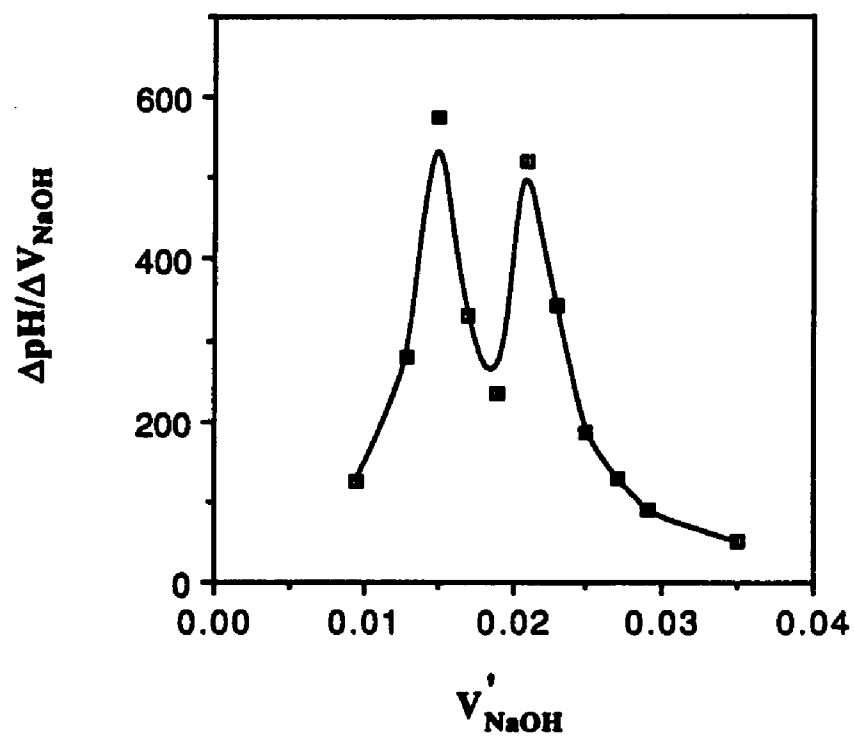
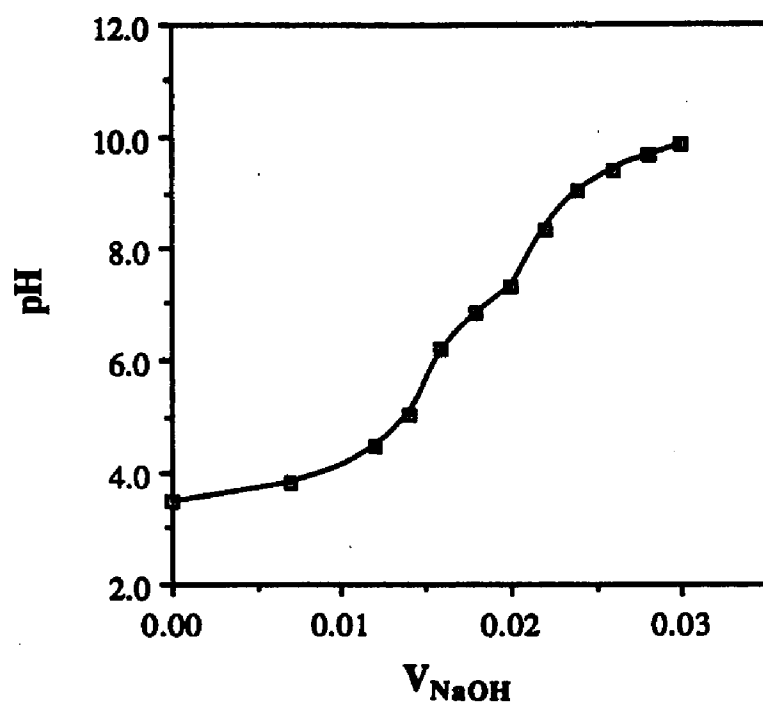
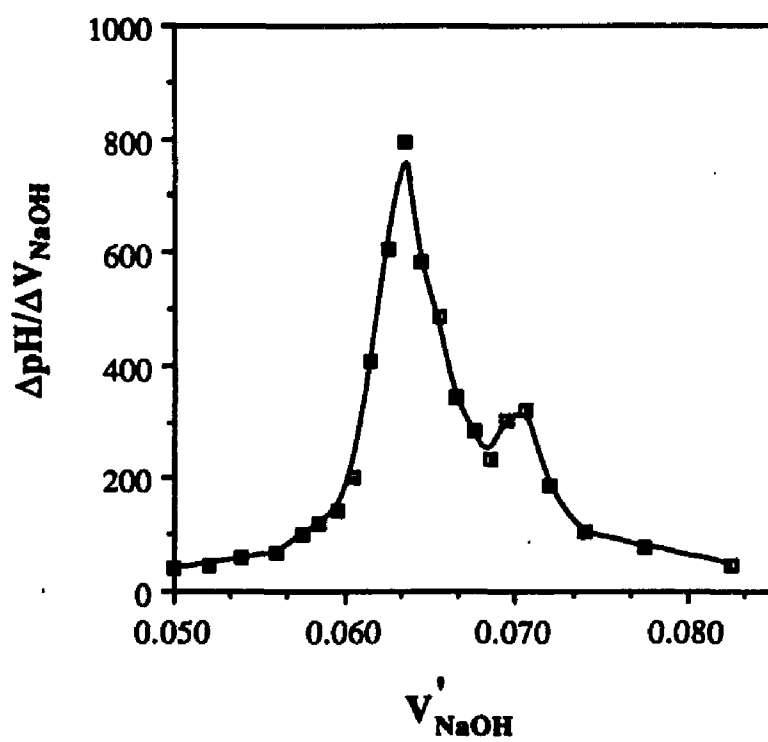
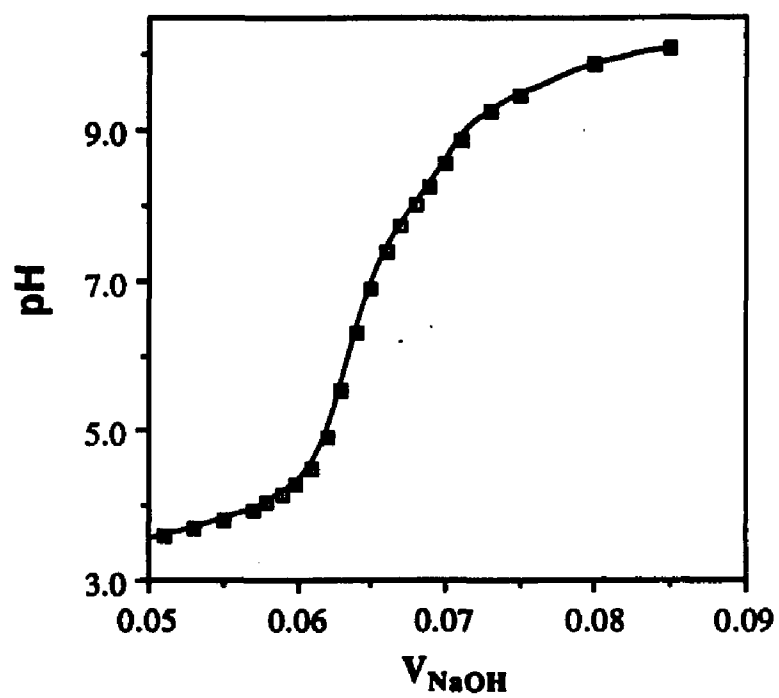


Figure.3-12. (a). The potentiometric titration curve of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP), pH versus V_{NaOH} , and (b). differential curve of (a), $\Delta\text{pH} / \Delta V_{\text{NaOH}}$ versus V'_{NaOH} , (ionic strength ≤ 0.01 M, room temperature). The experimental data is given in appendix 2-2.1.



In this work, the results reported for the trials of $[\text{OH}^-]_{\text{End}} > 10^{-8} \text{ M}$ are based on the general equation (3-20) and for the trials of $[\text{OH}^-]_{\text{End}} \leq 10^{-8} \text{ M}$, the results are reported based on equation (3-23). The values obtained by this method are $\text{pK}_{\text{app}} = 9.4$ and $\text{pK}_3 = \text{pK}_4 = 4.7$ for an ionic strength of 0.12 M and $\text{K}_{\text{app}} = 7.5$, $\text{pK}_3 = \text{pK}_4 = 3.7$ for an ionic strength less than 0.01 M. These value are in excellent agreement with the pK_3 or pK_4 values obtained by the spectrophotometric method.

3.3.2b NM-TAMPP

At the first end point of the titration, the hydronium ion concentration in the solution is contributed by the following dissociation and hydrolysis process of amphiprotic ion PH_3^+ :



The hydronium ion concentration at the first titration end point will satisfy the first approximate equation for amphiprotic substances ²:

$$[\text{H}^+] = \sqrt{\frac{\text{K}_3 \text{C}'_0(\text{N-MTAMPP}) + \text{K}_w}{1 + \text{C}'_0(\text{N-MTAMPP})/\text{K}_4}} \quad (3-26)$$

where K_3 and K_4 are defined in equations (3-14) and (3-15), $\text{C}'_0(\text{N-MTAMPP})$ has a similar

$$\begin{aligned} \text{C}'_0(\text{N-MTAMPP}) &= \frac{\text{C}_0(\text{N-MTAMPP}) \times V_s}{V_s + V_{\text{NaOH}}} \\ \text{C}'_0(\text{N-MTAMPP}) &= \frac{\text{C}_0(\text{N-MTAMPP}) \times V_s}{V_s + V_{\text{NaOH}}} \end{aligned} \quad (3-27)$$

but replacing $C_0(\text{TAMPP})$ by $C_0(\text{N-MTAMPP})$, the stock concentration of N-MTAMPP at about pH 3. It is impossible to solve K_3 and K_4 separately from one equation since the second titration process involving a central nitrogen atom of the porphyrin ring is not clearly separate from the protonation of peripheral amino group nitrogen atoms. However equation (3-26) can be used to calculate the hydronium ion concentration at the end point from the K_3 and K_4 values which were obtained by the spectrophotometric method. The calculated pH_{cal} based on the data obtained from the spectrophotometric experiment is 6.2 and the experimental pH_{exp} derived directly from the potentiometric method shown in figure 3-13 is 6.1 for $[\text{I}] = 0.12 \text{ M}$. The pH_{cal} by spectrophotometric method is 6.0 and pH_{exp} from potentiometric method shown in figure 3-14 is also 6.0 for $[\text{I}] < 0.01 \text{ M}$. The two different methods give results in good agreement with each other.

3.3.3 The acidity constant of amino group on outside of porphyrin ring (pK_a) — Potentiometric titration method

The acidity constant (K_a) which describes the dissociation of protonated peripheral amino group



can be determined by the potentiometric titration method but not by the spectrophotometric method. This is because the nonprotonated amino porphyrin is much less soluble and also there is little difference in the visible spectra of porphyrins that differ only by protonation or nonprotonation of the peripheral amino groups.

The sequential deprotonation of each of the four $-\text{NH}_3^+$ groups on the porphyrin periphery are not distinguishable from the data we obtained. Therefore, we

Figure.3-13. (a). The potentiometric titration curve of N-methylated tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP), pH versus V_{NaOH} , and (b). differential curve of (a), $\Delta\text{pH} / \Delta V_{\text{NaOH}}$ versus V_{NaOH} , (0.12M NaCl, room temperature). The experimental data is given in appendix 4-1.1.

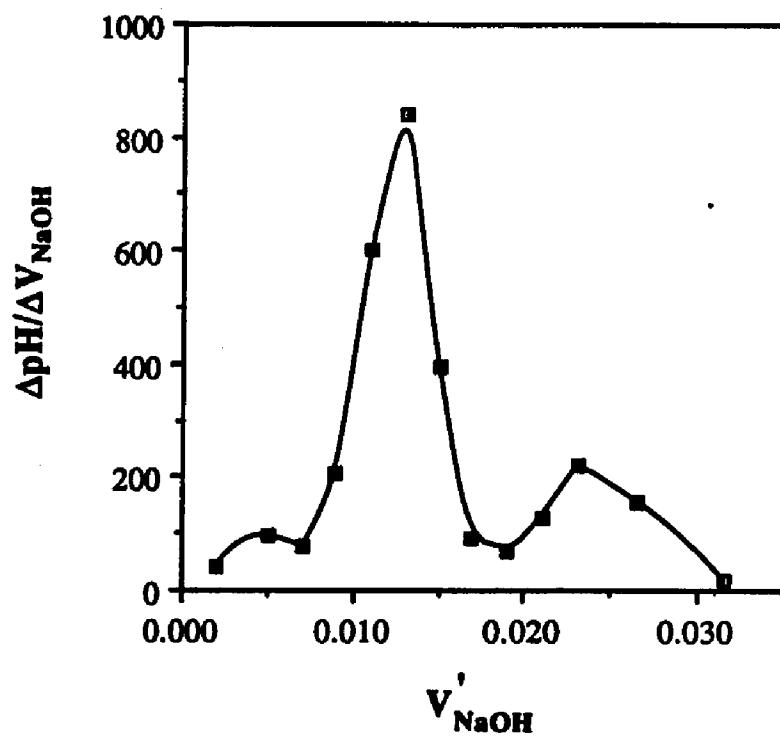
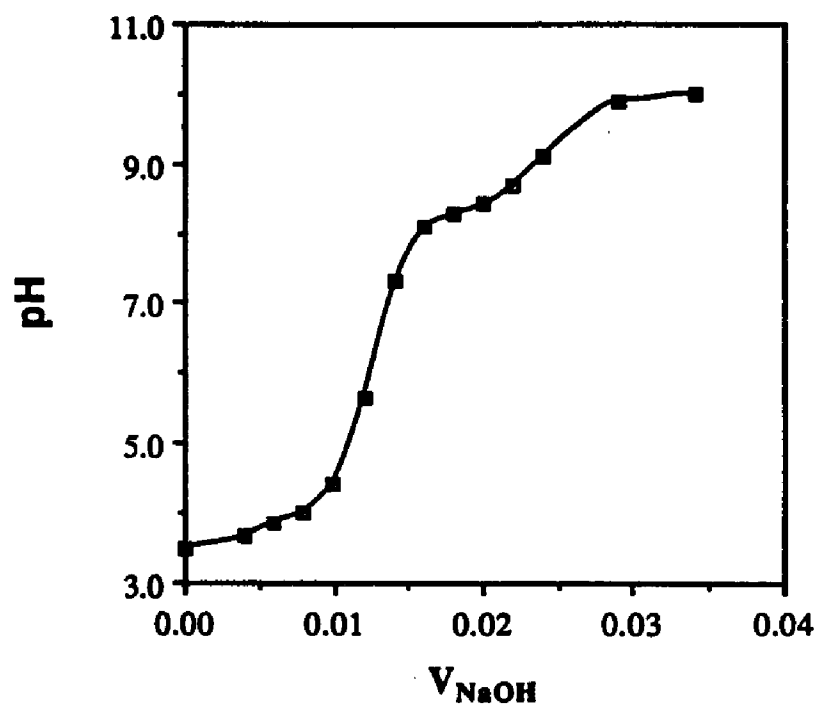
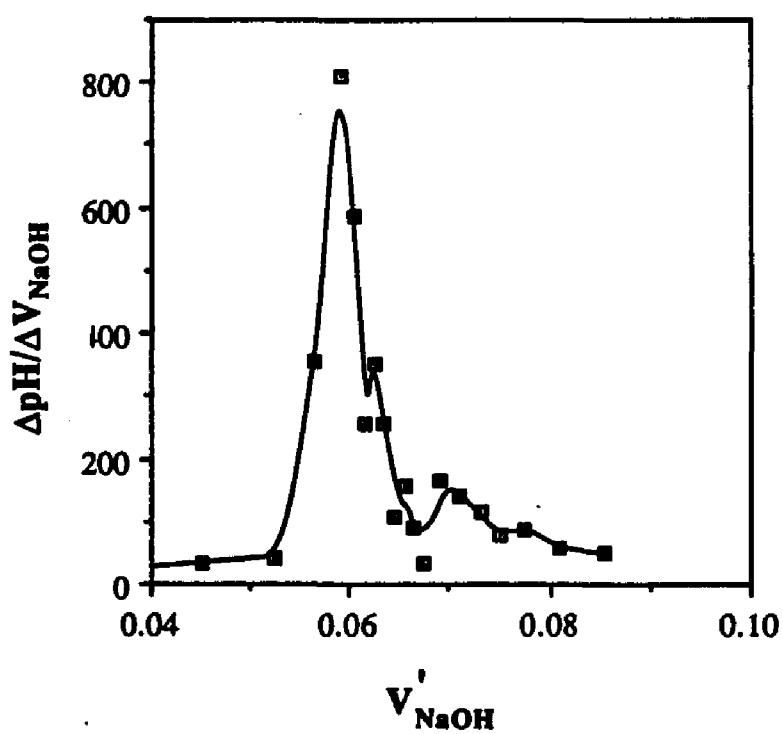
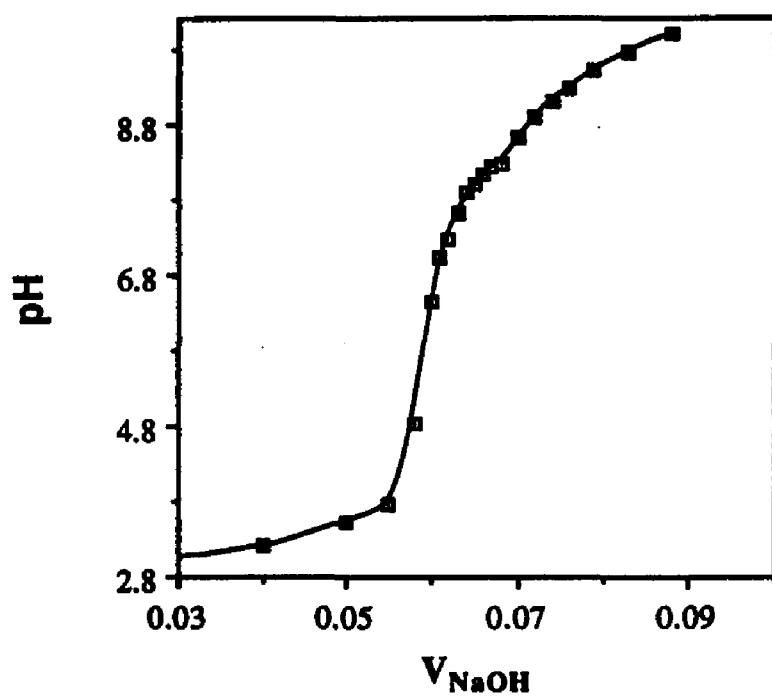
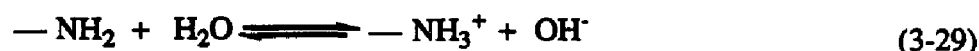


Figure.3-14. (a). The potentiometric titration curve of N-methylated tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP), pH versus V_{NaOH} , and (b). differential curve of (a), $\Delta\text{pH} / \Delta V_{\text{NaOH}}$ versus V_{NaOH} , (ionic strength ≤ 0.01 M, room temperature). The experimental data is given in appendix 4-2.1.



consider dissociation of four —NH_3^+ groups at the outside of the porphyrin ring as the simple dissociation of monoprotic acid where the concentration of amino groups is considered to be four times larger than the corresponding apparent concentration of porphyrin. The average acidity constant is then obtained by analyzing the titration curves shown in figures 3-11, 12, 13 and 14. At the end point of the titration, the pH of the solution is determined by the hydrolysis of amino group:



which gives

$$\frac{K_w}{K_a} = \frac{[\text{OH}^-]_{\text{End}}^2}{C_o' - [\text{OH}^-]_{\text{End}}} \quad (3-30)$$

where the term C_o' is the modified original porphyrin concentration and can be derived by equation (3-22) for TAMPP sample and by equation (3-27) for N-MTAMPP sample but make both of them four times larger in this case, $[\text{OH}^-]_{\text{End}}$ is the concentration of hydroxide ions at the second end point of titration which can be determined easily from the figures 3-11, 12, 13 and 14. K_a is the acidity constant of dissociation of —NH_3^+ to —NH_2 described in equation (3-28).

Then at $[\text{I}] = 0.12 \text{ M}$, we have $\text{p}K_a = 6.5$ for the amino group of TAMPP and $\text{p}K_a = 7.8$ for the amino group of N-MTAMPP.

At $[\text{I}] < 0.01 \text{ M}$, we have $\text{p}K_a = 6.6$ for the amino group of TAMPP and $\text{p}K_a = 7.2$ for the amino group of N-MTAMPP.

3.4 Discussion

Tables (3-1) and (3-2) list all of the experimental data for TAMPP and N-MTAMPP obtained in this work for the conditions of $[I] = 0.12 \text{ M}$ and $[I] < 0.01 \text{ M}$. It is clear that the experimental results are satisfactorily reproducible and the two titration methods, spectrophotometric and potentiometric, are coincident with each other.

The average pK_3 , pK_4 and pK_a values for TAMPP and N-MTAMPP at ionic strength 0.12 M and less than 0.01 M based on the spectrophotometric and potentiometric methods are summarized in Table (3-3). The numbers shown after " $\pm$ " are the corresponding uncertainties which are specified as the average deviation from the mean for the various acidity constants. The obtained pK_3 and pK_a values of N-MTAMPP may not be as accurate because the processes (3-24) and (3-28) interfere with each other. But these data are sufficient for us to identify the nature of species present in the studies of binding with nucleic acids.

A very interesting characteristic shown in Table (3-3) is that the average pK_a for the peripheral amino groups of N-MTAMPP is one unit larger than the pK_a of TAMPP. This fact can be explained by the specific structural features of N-MTAMPP which were discussed in chapter (2). Because of the N-methyl group, the porphyrin ring of N-MTAMPP is not flat like TAMPP, but folded. This structural change causes the rotational energy barriers of the meso aryl groups of the N-MTAMPP ring to be larger than those of the meso aryl groups of TAMPP. Thus, at ambient temperature, the meso aryl groups of TAMPP are more likely to exhibit configuration in which the phenyl ring is nearly coplanar with the aromatic porphyrin ring than is the case for N-MTAMPP. Therefore, the lone pair electrons on the nitrogens of the amino groups can conjugate with TAMPP porphyrin ring more easily than the amino groups of the N-MTAMPP porphyrin ring. This causes the weaker basicity for free amino group of TAMPP or stronger acidity (smaller pK_a) for its corresponding conjugate acid and the stronger

Table (3-1)

Acidity Constants K_4 , K_3 and K_a for TAMPP

Ionic Strength		[I] = 0.12 M				[I] < 0.01 M			
Exper. Methods		spectrophotometric	potentiometric			spectrophotometric	potentiometric		
pK_4									
$PH_4^{2+} \longrightarrow PH_3^+$		4.5	4.2	4.7	4.6	3.7	3.6	3.1	3.8 3.7
pK_3									
$PH_3^+ \longrightarrow PH_2$		4.5	4.2	4.7	4.6	3.7	3.6	3.1	3.8 3.7
pK_a									
$\cdot NH_3^+ \longrightarrow \cdot NH_2$		—	6.0	6.5	6.2	—		5.7	6.9 6.6

Table (3-2)

Acidity Constants K_4 , K_3 and K_a for N-MTAMPP

Ionic Strength	[I] = 0.12 M					[I] < 0.01 M				
	spectrophotometric		potentiometric			spectrophotometric		potentiometric		
pK_4 $PH_4^{2+} \longrightarrow PH_3^+$	3.1	3.1	—			2.4	2.4	—		
pK_3 $PH_3^+ \longrightarrow PH_2$	8.1	8.1	—			7.4	7.4	—		
1 st end point pH^*	6.2	6.2	6.1	6.0	6.0	6.0	6.0	6.0	6.0	6.0
pK_a $-NH_3^+ \longrightarrow -NH_2$	—		7.8	7.9	7.8	—		7.3	7.5	7.2

* For the spectrophotometric titration method: the first end point is calculated from the corresponding pK_4 and pK_3 by equation :

$$[H^+] = \sqrt{\frac{K_3 C_o' + K_w}{1 + C_o' / K_4}}$$

For the potentiometric titration method: the first titration end point is read from experimental curves of pH vs. V_{NaOH} and $\Delta pH / \Delta V_{NaOH}$ vs. V_{NaOH}

Table (3-3)Average Acidity Constants K_4 , K_3 and K_a for TAMPP and N-MTAMPP

Porphyrin	TAMPP		N-MTAMPP	
Ionic Strength	[I] = 0.12 M	[I] < 0.01 M	[I] = 0.12 M	[I] < 0.01 M
pK_4 $PH_4^{2+} \longrightarrow PH_3^+$	4.5(± 0.2)	3.6(± 0.2)	3.1(± 0.1)	2.4(± 0.1)
pK_3 $PH_3^+ \longrightarrow PH_2$	4.5(± 0.2)	3.6(± 0.2)	8.1(± 0.1)	7.4(± 0.1)
pK_a $-NH_3^+ \longrightarrow -NH_2$	6.2(± 0.2)	6.4(± 0.5)	7.8(± 0.1)	7.3(± 0.1)

basicity for the free base amino group of N-MTAMPP or weaker acidity (larger pK_a) for its corresponding conjugate acid. These are the results we found in this part of work.

Another characteristic shown by Table (3-1) and (3-2) is that there is a much larger difference between pK_3 and pK_4 of N-MTAMPP ($\Delta pK = 5$) than between pK_3 and pK_4 of TAMPP ($pK < 0.01$). Also, neutral N-MTAMPP is much stronger base ($pK_3(\text{N-MTAMPP}) = 8.1$ at $[I] = 0.12$ M and $pK_3(\text{N-MTAMPP}) = 7.4$ at $[I] < 0.01$ M) by almost four units than the non-N-alkylated species, TAMPP, in the same solution conditions ($pK_3(\text{TAMPP}) = 4.5$ at $[I] = 0.12$ M and $pK_3(\text{TAMPP}) = 3.6$ at $[I] < 0.01$ M). Similar phenomena were observed before. Conant et al.³ observed only a single inflection in the titration curve of five non-N-methylated porphyrins studied. They assumed that pK_3 and pK_4 values are equal and reported average values for them. On the otherhand, N-methylated porphyrins usually give a wider separation of pK values than the corresponding non-N-methylated porphyrin. Neuberger and Scott⁴ studied the non-N-methylated and N-methylated coproporphyrin I pair in aqueous solutions of ionic strength 0.1 M and found that $pK_3=7.2$, $pK_4=4.2$, for the non-N-methylated $H_2(\text{copro-III})(-4)$ and $pK_3=11.3$, $pK_4=0.7$ for N-methylated $H(\text{N-Mcopro-III})(-4)$. The similar phenomenon was observed also for non-N-methylated and N-methylated tetraphenylporphine pair in nitrobenzene ($pK_3=4.38$, $pK_4=3.85$ for non-N-methylated TPP and $pK_3=5.64$, $pK_4=3.85$ for N-methylated N-MTPP) by Aronoff⁵ and Lavallee and Gebala⁶. N-methylated substituents can cause an appreciable increase in basicity, which were confirmed by the coproporphyrin pair⁴, tetraphenylporphyrin pair^{5,6} and the octaethylporphyrin pair⁷ ($pK_3=0$ for non-N-methylated octaethylporphyrin and $pK_3=4.1$ for its N-methylated derivative). The stronger basicity for N-methylated porphyrin occurs because the bulky central N-methyl groups force the porphyrin ring to distort from planarity. Consequently, several of the central nitrogen atoms of N-methylated porphyrins have more basic character than non-N-methylated porphyrins⁸.

Crystal structure studies show that the diprotonated forms of $H_2(TPP)^9$, $H_2(TpyP)^9$ and the monocation of $H_2(OEP)^{10}$ are rather distorted porphyrins. One explanation⁹ is that upon adding a proton to the relatively planar PH_2 of non-N-methylated porphyrin, the formed non-planar monocation PH_3^+ has its pyrrole rings tilted alternatively upwards and downwards with respect to the mean porphyrin plan. Such deformation facilitates protonation of the remaining lone pair, thus limiting the existence of the monoprotonated porphyrin. Therefore it displays closer pK_3 and pK_4 values for non-N-methylated porphyrin. On the other hand, the addition of the hydrogen ion to N-methylated porphyrins causes less structural change. The methylated nitrogen atom does not typically get protonated and the positive charge introduced by the first proton would tend to repel a second proton approaching to the molecule. Thus, it is much more difficult to convert the monoprotonated N-methyl porphyrin to its diprotonated form compared with the corresponding free base porphyrin, which is reflected in the more widely separate pK_3 and pK_4 values for N-methylated porphyrins.

The third characteristic shown by Tables (3-1) and (3-2) is that the pK_3 , pK_4 values of TAMPP and N-MTAMPP are about one unit less when the ionic strength of solution is less than 0.01 M than when the ionic strength of solution is equal to 0.12 M. The reason for the higher basicity of the neutral porphyrin at larger ionic strength may be because the more greater ionic atmosphere would stabilize the protonated porphyrin since negatively charged chloride ions would be more readily available to neutralize the positive charge. The difference in the average acidity constants for the peripheral amino groups because of the change in ionic strength is within the range of reproducibility of measurement at either ionic strength conditions. Hence no sizable variation is evident.

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Chapter 4

Circular Dichroism, Absorption and Fluorescence Spectra of Porphyrin — Nucleic Acid Systems in the Visible Region

4.1 Introduction

Although meso-substituted porphyrins are much larger than well-known intercalators such as ethidium bromide or acridine, it is apparent from the work of Fiel, Pasternack and coworkers¹⁻¹¹ that some cationic water-soluble porphyrins can intercalate strongly into DNA and other nucleic acids in solution. In addition, it is clear that there is more than one type of binding or interaction between porphyrins and nucleic acids, depending on the detailed structural features of the porphyrin, on the relative concentrations of porphyrin and nucleic acid base pairs, and on the composition of neighboring base pairs of nucleic acids.

Porphyrins experience two broad types of interaction with DNA: electrostatic association with the phosphate groups (outside binding)^{7,8} and intercalation into DNA with a high affinity for the G-C sequence^{7-10,12}. The outside binding has been further subdivided into two modes: a non-selective type of general electrostatic association with phosphate groups and a structurally specific type in A-T regions (perhaps in a groove) originally suggested by Pasternack⁹ and confirmed by Bromley¹³. Marzilli and coworkers^{12,14} have given further compelling evidence using ¹H and ³¹P-NMR and flow circular dichroism experimental techniques. The combined experimental results from circular dichroism, absorption spectral and viscosity studies can give rise to a several types of useful information to distinguish the interaction character between the cationic porphyrin with nucleic acids.

The circular dichroism technique (CD) is a particular kind of absorption spectroscopy that uses circularly polarized light rather than unpolarized light. It can be applied to molecules that are asymmetric, that is, molecules that contain no plane or center of symmetry.

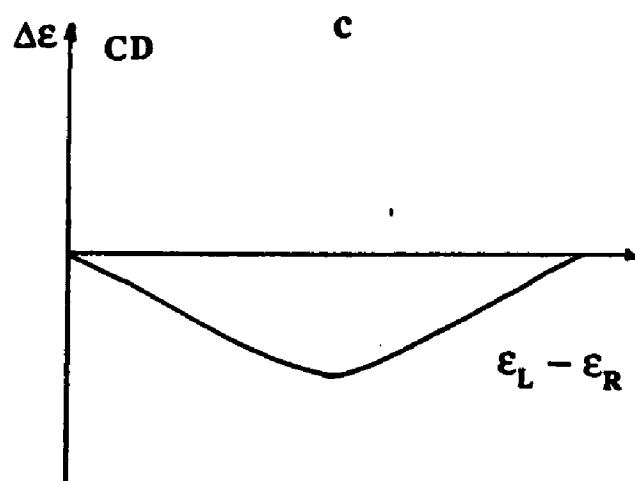
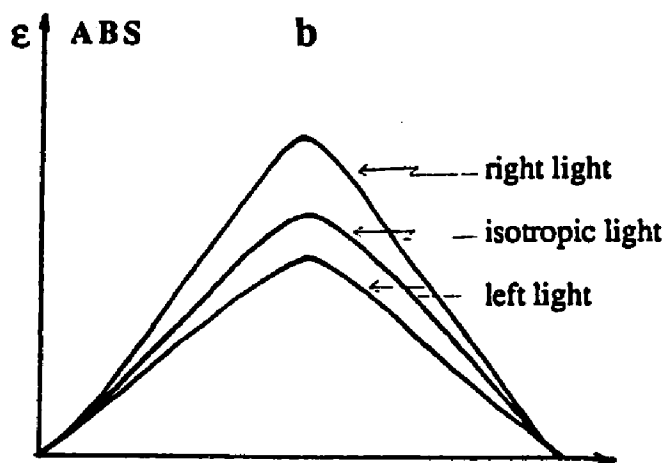
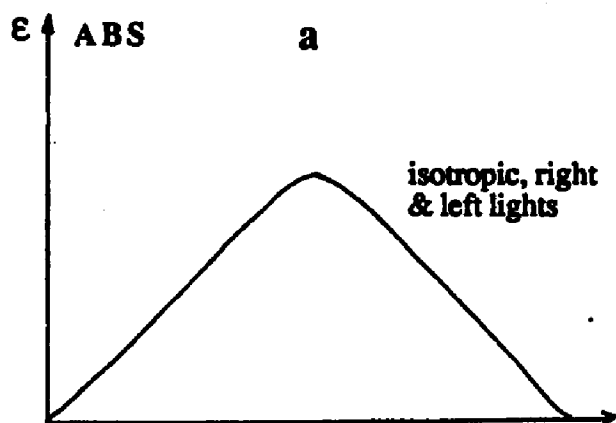
Normal absorption spectroscopy uses unpolarized light. For optically inactive substances randomly oriented (as in the gas or solution), linearly polarized light will give the same absorption spectrum as unpolarized light (figure 4-1a). However, an asymmetric molecule will absorb right circularly polarized light differently from the left circular polarized light, even though the molecules are randomly oriented (figure 4-1b). This specific feature is the basis of the circular dichroism technique; it depends upon the fact that the molar absorptivity of an optically active compound is different with two types of circularly polarized light (figure 4-1c). It is a popular spectroscopic technique because the measurement is extremely sensitive to the conformation of the asymmetric molecule and because the measurement can be conveniently applied to randomly oriented molecules in solution.

This technique utilizes the wavelength dependence of $(\epsilon_L - \epsilon_R)$, where ϵ_L and ϵ_R are the respective molar absorptivities of an optically active substance for left and right circularly polarized light. Beer's law will be obeyed for circularly polarized light of either rotation. Thus the difference in absorbance is given by:

$$\Delta A = A_L - A_R = \epsilon_L bc - \epsilon_R bc = \Delta \epsilon bc \quad (4-1)$$

where the subscripts "L" and "R" denote the left and right rotation of the light and A, ϵ , b and c have the same meaning as in the original Beer's law, that is, they are respect to absorbance, extinction coefficient, the length of light pass and the concentration of the sample measured in molarity, respectively. The convention of measuring $A_L - A_R$ is arbitrary, since either rotation might be absorbed more strongly. Circular dichroism bands can be either positive or negative.

Figure.4-1. The comparison between absorption (ABS) and circular dichroism (CD) spectroscopy: (a). absorption spectra for a symmetric substance irradiated by isotropic, right and left lights; (b). absorption spectrum for a asymmetric substance irradiated by right, isotropic and left lights , respectively (the larger ellipticity caused by right light than by left light is arbitrary); (c). circular dichroism spectrum for a asymmetric substance irradiated by polarized light.



Wavelength

If new circular dichroism peaks appear when an optically active substance is added to an optically inactive substance in solution, the phenomenon is called induced circular dichroism.

Nucleic acids are optically active substances, they show circular dichroism peaks in the ultraviolet region, but not in the region of the principal absorption peaks of the porphyrin (400 to 700 nm). The porphyrins discussed in this dissertation are not optically active substances and do not absorb right and left circularly polarized light differently in any region. If the induced circular dichroism spectrum shows up in the visible region when a nucleic acid is added to the porphyrin solution, it means that a porphyrin-nucleic acid complex has been formed. Thus, circular dichroism is very useful technique to judge the interactions between optically active (nucleic acids in this work) and optically inactive (our porphyrins in this work) substances.

After investigating a series of synthetic meso-substituted porphyrins, Pasternack concluded that the formation of a truly intercalated species requires G-C base pairs ⁹. Intercalation of the porphyrins he studied into G-C base pairs produces a negative induced circular dichroism band in the region of the principal absorption peaks of the porphyrin (400 to 500 nm). He found that either a partially intercalated complex or an outside-bound complex is formed within A-T regions of DNA. This type of binding produces a positive induced circular dichroism band (in the region of 400 to 500 nm) at small values of the ratio of the total porphyrin concentration to the total nucleic acid concentration, $[\text{Porphyrin}]_t / [\text{Nucleic acid}]_t \leq 0.03$ suggested by Pasternack ⁹.

Visible absorption spectroscopy is also an informative technique which may provide some information about the binding properties of porphyrins to nucleic acids. For the intercalated complex, it is observed ⁹ that (i) the red shifts of the Soret maximum (> 15 nm) are larger than the shifts for the non-intercalated complex (about 4-7 nm) and (ii) there is substantial hypochromicity ($> 35\%$) in the most prominent band

in the visible spectrum of a porphyrin, occurring at about 400-420 nm for most porphyrins (called the Soret band).

The changes in the location and intensity of the Soret band and therefore, the differential absorbance in the induced circular dichroism spectrum of a porphyrin is very informative and is often invoked as evidence of the nature of an interaction. In this section, the circular dichroism and visible absorption spectra as well as fluorescence spectra are discussed. These techniques were used to investigate the binding characteristics of our newly synthesized tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) with nucleic acids. In addition, the tetra-(4-methylpyridyl)porphyrin (TMPyP-4) used by Pasternack is also examined in absorption and fluorescence experiments. The latter porphyrin was used to compare results with new porphyrins and to better understand the binding pattern of newly synthesized porphyrins with nucleic acids.

4.2 Materials and Methods

Calf thymus DNA (CT.DNA), Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) were purchased from Sigma Chemical Co. Calf thymus DNA was further purified as previously described by Pasternack⁹ with slight modifications. Samples of 1 mg of CT.DNA / ml were dissolved by stirring for 40 hrs at about 4 °C in acetate buffer (the total concentration of acetic acid and sodium acetate was 0.05 M and the concentration of sodium chloride was 0.1 M; pH = 5.5). Protein was removed from the CT.DNA by repeated extraction with a chloroform - isoamyl alcohol solution (24:1) using bradford reagent (purchased from Biorad) as the indicator to check the amount of protein in the extract layer. After the final centrifugation, the top two-thirds of the solution was carefully pipetted off and exhaustively dialyzed against the pH 5.5 buffer described above. Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) were

used without further purification. Stock solutions of these two synthetic polymers were prepared by dissolving the materials in 0.05 M acetate buffer at room temperature and ionic strength (contributed by sodium chloride) of 0.1 M. Solutions of approximately 6×10^{-4} M were frozen until use. The integrity of the double stranded nucleic acid was determined by comparing their ultraviolet visible spectra with published results ¹⁵⁻¹⁷. Concentrations, calculated in base pairs, were determined spectrophotometrically with $\epsilon_{260} = 1.31 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for calf thymus DNA ¹⁵, $\epsilon_{262} = 1.32 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for Poly(dA-dT) • Poly(dA-dT) ¹⁶ and $\epsilon_{254} = 1.68 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for Poly(dG-dC) • Poly(dG-dC) ¹⁷.

All other chemicals were reagent grade and were used without further purification. Circular dichroism experiments were carried out at room temperature and absorption and fluorescence experiments were carried out at 25 °C. The same acetate buffer containing 0.05 M of acetic acid and sodium acetate, 0.1 M sodium chloride, pH=5.5, was used in all three experimental techniques.

Circular dichroism spectra were recorded on the Jobin Yvon spectropolarimeter at a spectral band-pass of 20 Å. Absorption spectra were carried out on the Beckman Du-8 spectrophotometer with a thermostated cell compartment. Fluorescence spectra were recorded on a Amino-Bowman 125 SPF corrected spectrophotofluorometer with a thermostated compartment. The reference, exit, entrance and signal PM tube rotary slits at 0.5, 0.5, 1.0 and 0.5 mm, respectively were used. The wavelength of excitation used was 410 nm and the wavelength of the emission was scanned at about 220 nm / min.

4.3 Results

The interaction of tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP) with nucleic acids: calf

thymus DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC), were examined with circular dichroism, visible absorption and fluorescence spectra.

4.3.1 Circular Dichroism Spectra of Porphyrin — Nucleic Acid System.

Figures 4-2 and 4-3 show the circular dichroism spectra of TAMPP and N-MTAMPP with DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) at a ratio of the total concentration of porphyrin to that of nucleic acid in base pairs of 0.03. The ratio used in this work is same as Pasternack suggested ⁹. All six porphyrin — nucleic acid systems show induced circular dichroism spectra within the visible region near the Soret band position of the porphyrins (the porphyrins considered in this dissertation do not display circular dichroism spectra by itself). Therefore, both TAMPP and its N-methyl derivative, N-MTAMPP, can interact or bind to these three different nucleic acids.

The very obvious phenomenon shown from figure 4-2 is that TAMPP gives a very similar induced circular dichroism spectrum when it interacts with Poly(dA-dT) • Poly(dA-dT) or CT.DNA, but it displays a completely different induced circular dichroism spectrum when it interacts with Poly(dG-dC) • Poly(dG-dC). Both the TAMPP — CT.DNA and TAMPP — Poly(dA-dT) • Poly(dA-dT) systems display only positive circular dichroism spectra, that is, a larger positive band located at 442 nm with a small shoulder near 420 nm for TAMPP — CT.DNA complex and a larger positive band located at 445 nm with a small shoulder also at 421 nm for TAMPP — Poly(dA-dT) • Poly(dA-dT) complex. While TAMPP gives rise to relatively small but evident negative (located at 435 nm) and positive peaks (located at 449 nm) of about the same magnitude in the circular dichroism spectrum when it is mixed with Poly(dG-dC) • Poly(dG-dC) (figure 4-2c). The interesting and noticeable phenomenon is that the induced circular dichroism spectrum of TAMPP — Poly(dG-dC) • Poly(dG-dC)

Figure.4-2. The circular dichroism (CD) spectra of systems (a). TAMPP — CT.DNA, (b) TAMPP — Poly(dA-dT) • Poly(dA-dT) and (c). TAMPP — Poly(dG-dC) • Poly(dG-dC). The concentrations of species in solution are: [TAMPP] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.

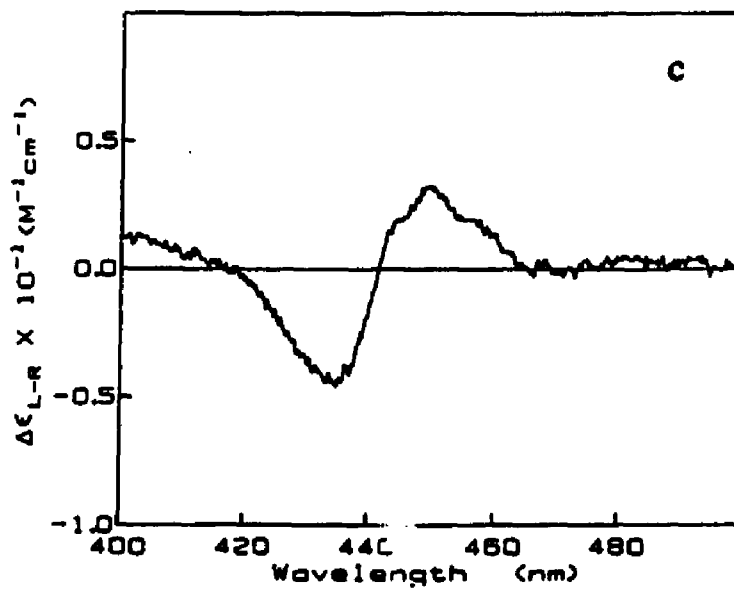
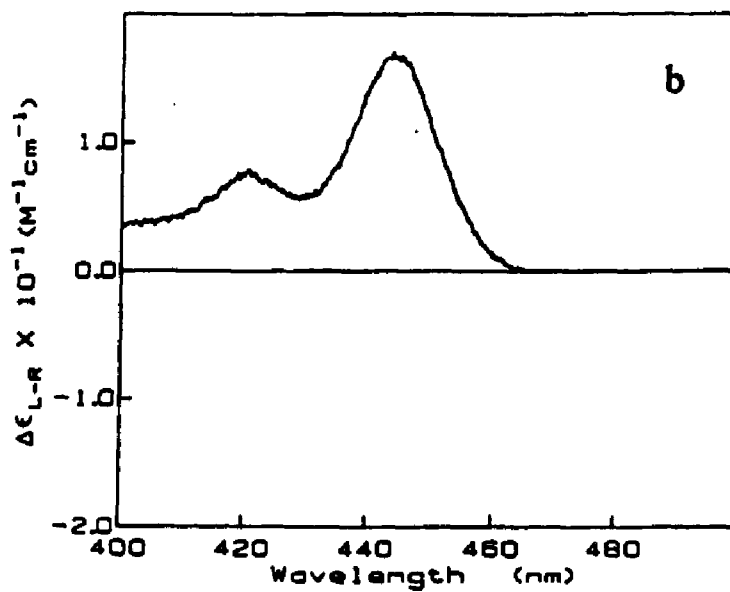
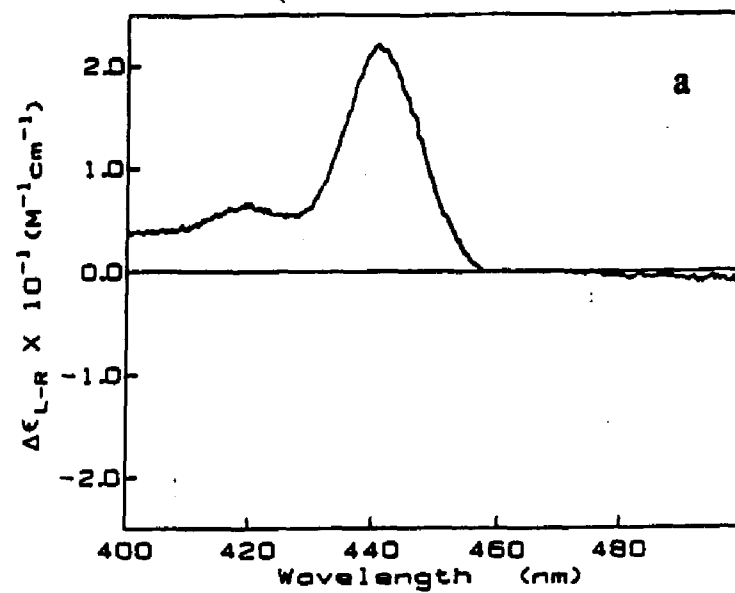
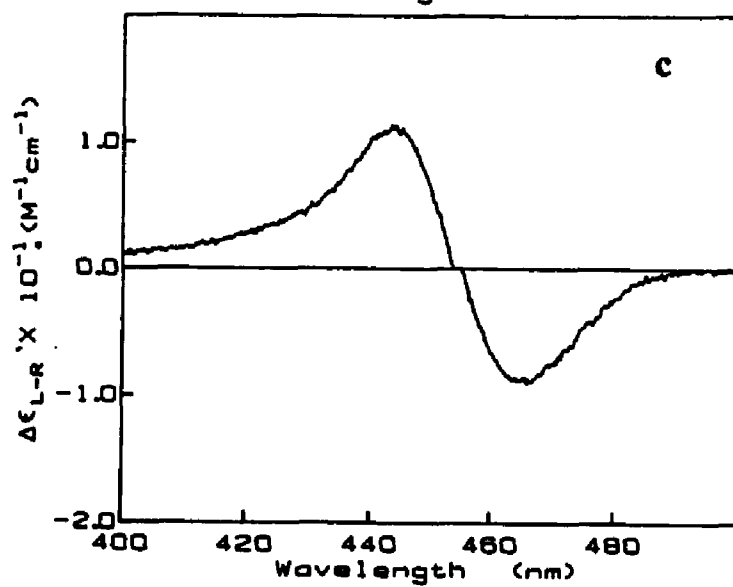
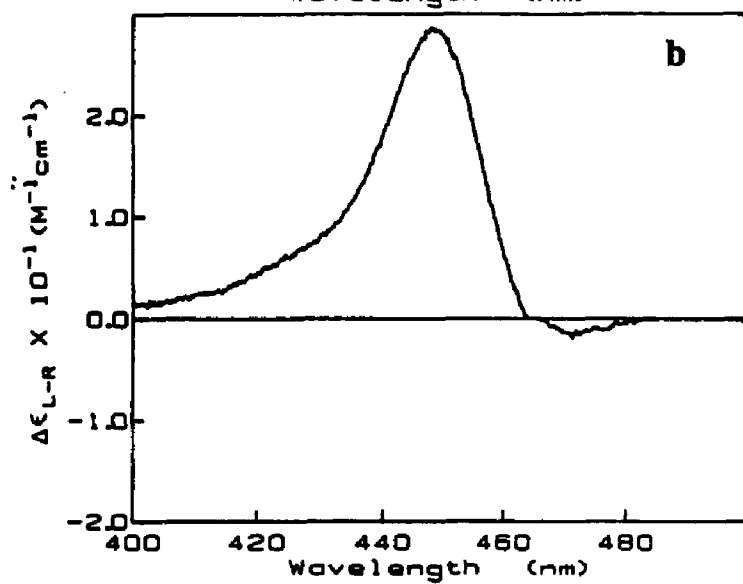
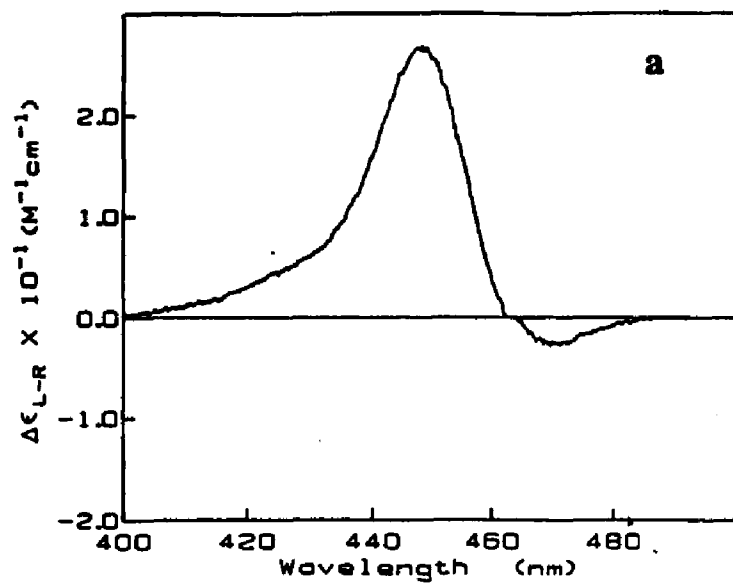


Figure.4-3. The circular dichroism (CD) spectra of systems (a). N-MTAMPP — CT.DNA, (b) N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) and (c). N-MTAMPP — Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are: [N-MTAMPP] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.



complex shows a negative phase, that is, its negative band appears at a lower wavelength than its positive band. The results of similar intensity and shape of circular dichroism spectra in figures 4-2a and 2b suggest that TAMPP binds much easier with AT regions than with GC regions within calf thymus DNA.

Similar phenomena as found for TAMPP — nucleic acid complexes happen with N-MTAMPP — nucleic acid systems. When mixed with CT.DNA or Poly(dA-dT) • Poly(dA-dT), N-MTAMPP shows very similar induced circular dichroism spectra. That is, a larger positive circular dichroism peak appears at the same wavelength, 448 nm, and much smaller negative circular dichroism peak is located at about 470 nm when N-MTAMPP binds with CT. DNA or Poly(dA-dT) • Poly(dA-dT), respectively (figures 4-3a and 3b). However, when N-MTAMPP binds to Poly(dG-dC) • Poly(dG-dC), its induced circular dichroism spectrum gives rise to peaks as about the same magnitude, a positive one at 443 nm, and a negative one at 465 nm (figure 4-3c). The phenomena of similar shape and intensities of circular dichroism in figures 4-3a and 3b suggest that N-MTAMPP preferably binds to AT-rich regions of DNA too. The induced circular dichroism spectra for all of three N-MTAMPP—nucleic acid systems show a positive phase, that is, their positive bands appear at lower wavelength than the negative bands.

4.3.2 Absorption Spectra of Porphyrin — Nucleic Acid complexes.

The Soret band 1 in visible absorption spectra of figure 4-4a or 4b or 4c is displayed by the porphyrin TAMPP in pH 5.5 buffer solution. The Soret bands 2 in the visible absorption spectra for complexes of TAMPP with CT.DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) are shown in of figures 4a, 4b and 4c, respectively. The same concentrations were used for the Soret bands 1 and 2 as in the circular dichroism experiments discussed in section (4.3.1). Both spectra 1 and 2 show

Figure.4-4. The visible absorption spectra of systems (a). 1- TAMPP, 2- TAMPP — CT.DNA; (b). 1- TAMPP, 2- TAMPP — Poly(dA-dT) • Poly(dA-dT); (c). 1- TAMPP, 2- TAMPP — Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are: [TAMPP] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.

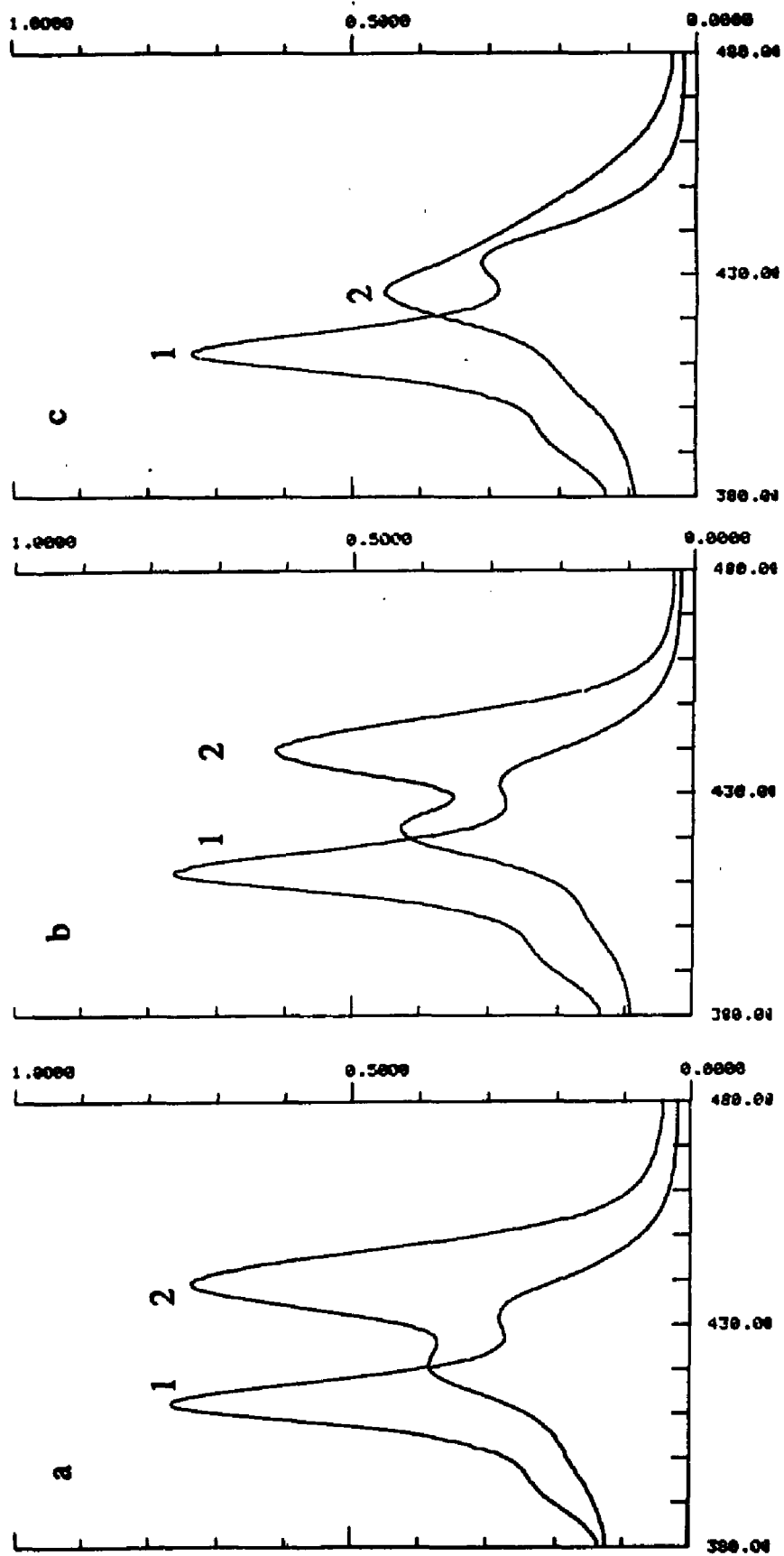
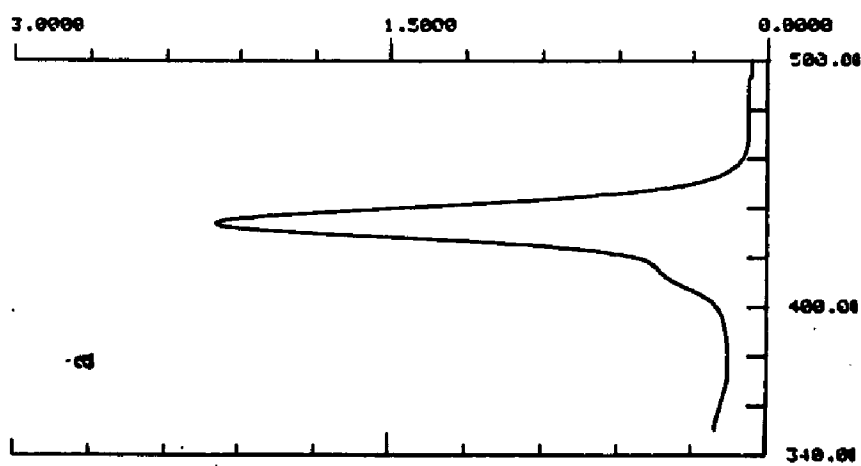
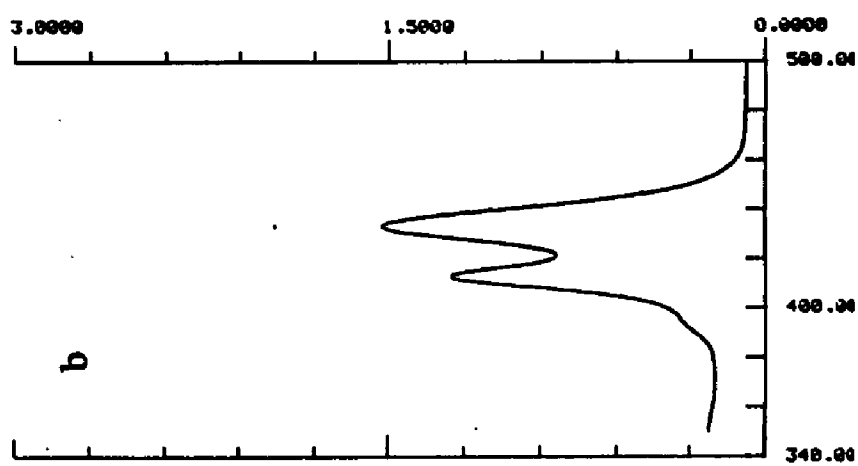
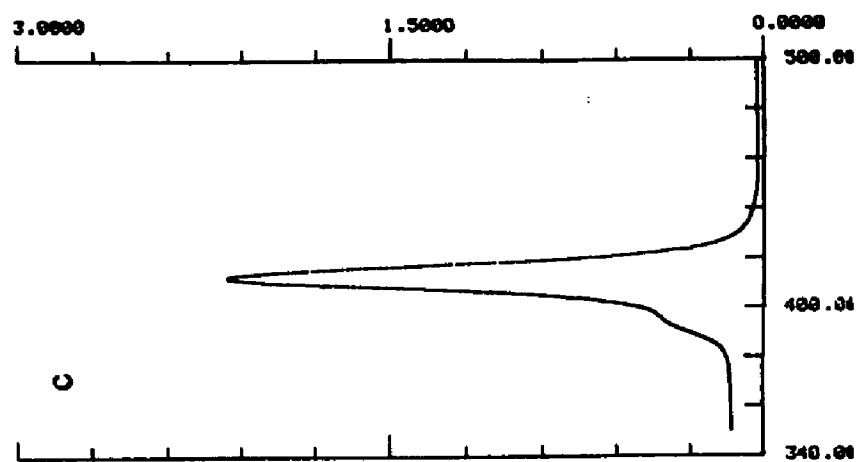


Figure.4-5. The visible absorption spectra of TAMPP in (a) diprotonated form, pH = 1.5; (b). mixture of diprotonated and neutral forms, pH = 4.1; (c). neutral form, pH = 6.0.



small shoulders implying that there are two species in each solution. In order to better understand of the spectra, spectra of TAMPP at pH 1.5, 4.1, and 6.0 are displayed as (a), (b) and (c) of figure 4-5. It is quite clear that the Soret bands at wavelength 435 (figure 4-5a) and 413 nm (figure 4-5c) are indicated by diprotonated and neutral TAMPP, respectively. Therefore, TAMPP in the absence of any nucleic acid is mostly in its neutral form in pH 5.5 aqueous buffer solution and nucleic acid bound TAMPP in the same buffer seems to be mostly in its protonated form.

Quite similar Soret bands are evident for TAMPP — CT.DNA and TAMPP — Poly(dA-dT) • Poly(dA-dT) systems, but a much different Soret band pattern is displayed by TAMPP — Poly(dG-dC) • Poly(dG-dC) complex. This phenomenon is coincident with the results of circular dichroism experiment. The TAMPP — Poly(dG-dC) • Poly(dG-dC) complex gives substantial hypochromicity (taken as the relative change of the extinction coefficient at the wavelength of maximum absorbance) of the Soret maximum band (58%) compared with the TAMPP — Poly(dA-dT) • Poly(dA-dT) complex (19%) and the TAMPP—DNA complex (4%). TAMPP gives the larger red shifts of the Soret maximum with CT.DNA and Poly(dA-dT) • Poly(dA-dT) (27.5 nm) than the one with Poly(dG-dC) • Poly(dG-dC) (20.8 nm), all these shifts being substantial when compared with Pasternack's results for tetra-(4-methylpyridyl)porphyrin (TMPyP-4) and its metal complexes ⁹.

Figures 4-6a, 6b and 6c show the visible absorption spectra of complex N-MTAMPP with CT.DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC). Like TAMPP, N-MTAMPP shows larger hypochromicity of the Soret maximum band with Poly(dG-dC) • Poly(dG-dC) (34%) than with Poly(dA-dT) • Poly(dA-dT) (2%) and CT.DNA (10%). Much smaller and more similar red shifts of Soret maximum were obtained for N-MTAMPP with Poly(dG-dC) • Poly(dG-dC) (7.5 nm),

Figure.4-6. The visible absorption spectra of systems (a). 1- N-MTAMPP, 2- N-MTAMPP — CT.DNA; (b). 1- N-MTAMPP, 2- N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) ; (c). 1- N-MTAMPP, 2- N-MTAMPP — Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are : [N-MTAMPP] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.

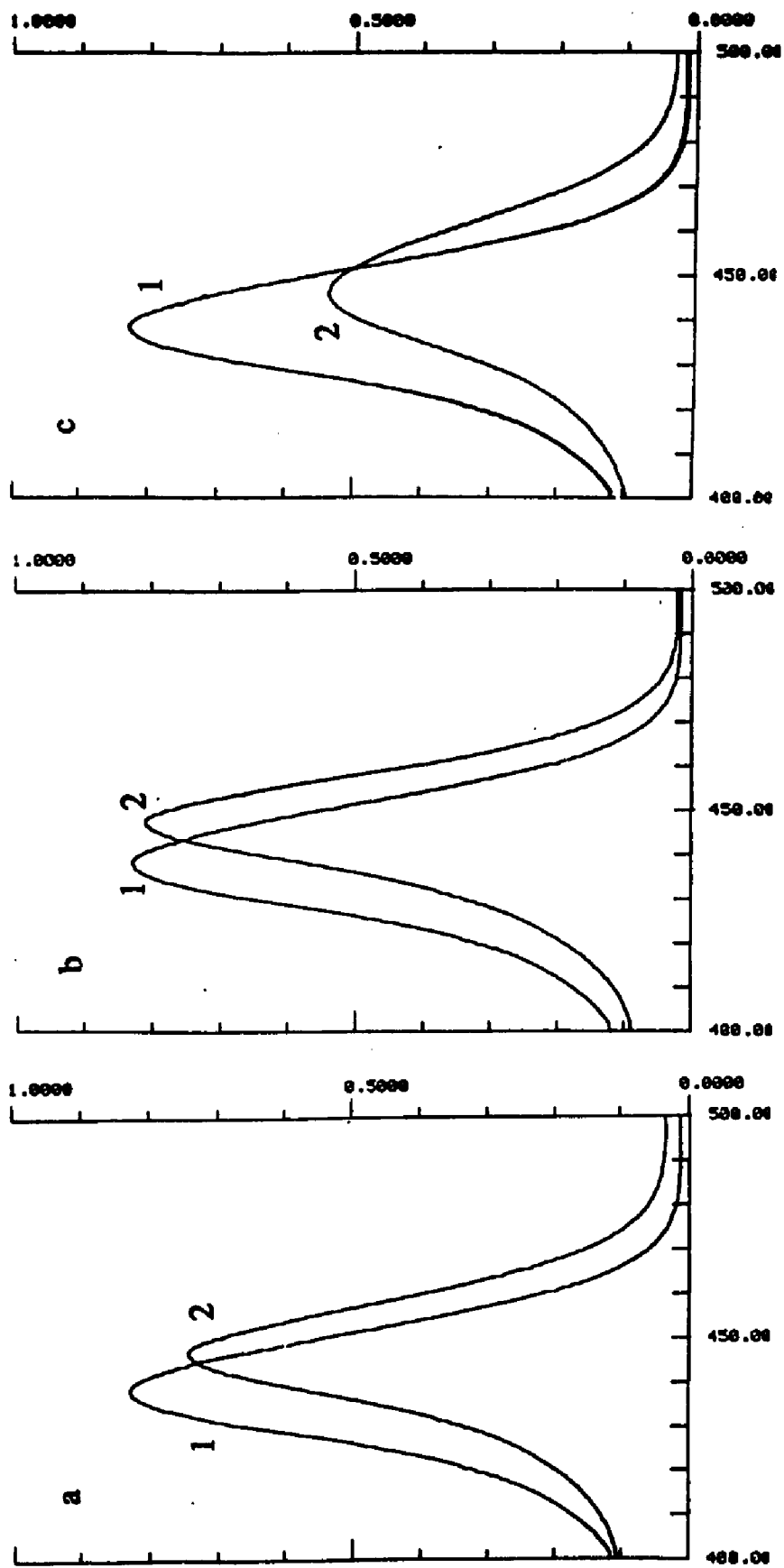
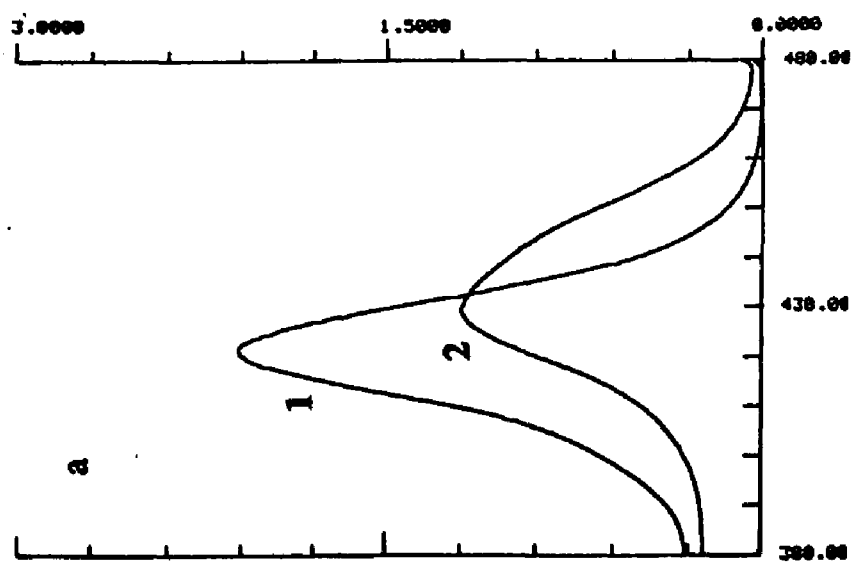
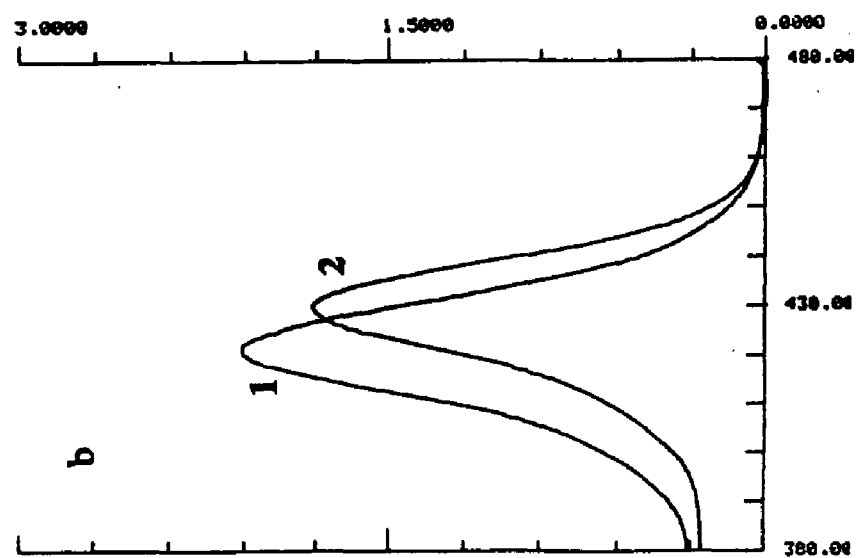
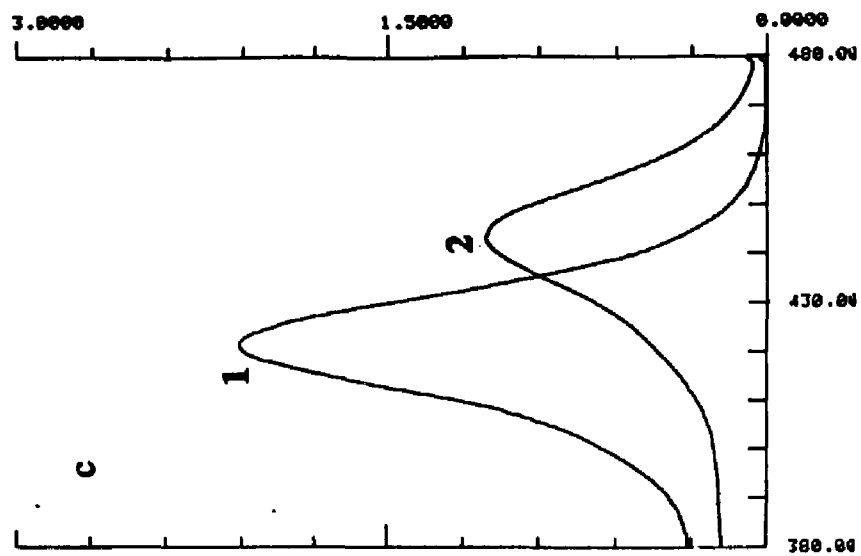


Figure.4-7. The visible absorption spectra of systems (a). 1- TMPyP-4, 2- TMPyP-4 — CT.DNA; (b). 1- TMPyP-4, 2- TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) ; (c). 1- TMPyP-4, 2- TMPyP-4 — Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are : [TMPyP-4] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.



Poly(dA-dT) • Poly(dA-dT) (9.2 nm) and CT.DNA (8.3 nm) systems. As with TAMPP, the character of the Soret bands is similar for N-MTAMPP with Poly(dA-dT) • Poly(dA-dT) and CT.DNA systems and a quite different type of Soret band is shown by the N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) system. However, the difference between the two kinds of Soret spectra here is smaller than the case of TAMPP. This phenomenon is consistent with circular dichroism results.

Figures 4-7a, 7b and 7c show the Soret bands of visible spectra for TMPyP-4 with CT.DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC). The red shifts (8.3 nm for TMPyP-4 — DNA and TMPyP-4 — Poly(dA-dT) • Poly(dA-dT), 21.7 nm for TMPyP-4 — Poly(dG-dC) • Poly(dG-dC) system) and hypochromicity results (43% for TMPyP-4 — DNA, 14% for TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) and 47% for TMPyP-4 — Poly(dG-dC) • Poly(dG-dC) systems) are in good agreement with Pasternack's results⁹. Therefore, the pH 5.5 buffer offers a dependable medium for comparison studies of porphyrin — nucleic acid interactions. In these TMPyP-4 — nucleic acid systems, the complex of the porphyrin with Poly(dG-dC) • Poly(dG-dC) also shows the largest hypochromicity and red shift, while the TMPyP-4 — DNA system displays a closer hypochromicity value to the TMPyP-4 — Poly(dG-dC) • Poly(dG-dC) complex and about the same extent of red shift as TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) complex.

4.3.3 Fluorescence Spectra of Porphyrin — Nucleic Acid Complexes.

Figures 4-8, 9 and 10 show the fluorescence emission spectra of TAMPP — nucleic acid, N-MTAMPP — nucleic acid and TMPyP-4 — nucleic acid systems, respectively. The very obvious characteristics are that (1) the fluorescence intensities are less for all three TAMPP — nucleic acid systems than the fluorescence spectral intensity of TAMPP alone, i.e. a hypochromic character (the relative fluorescence

Figure.4-8. The fluorescence spectra of systems (1). TAMPP only, (2). TAMPP + CT.DNA, (3) TAMPP + Poly(dA-dT) • Poly(dA-dT) and (4). TAMPP + Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are : [TAMPP] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.

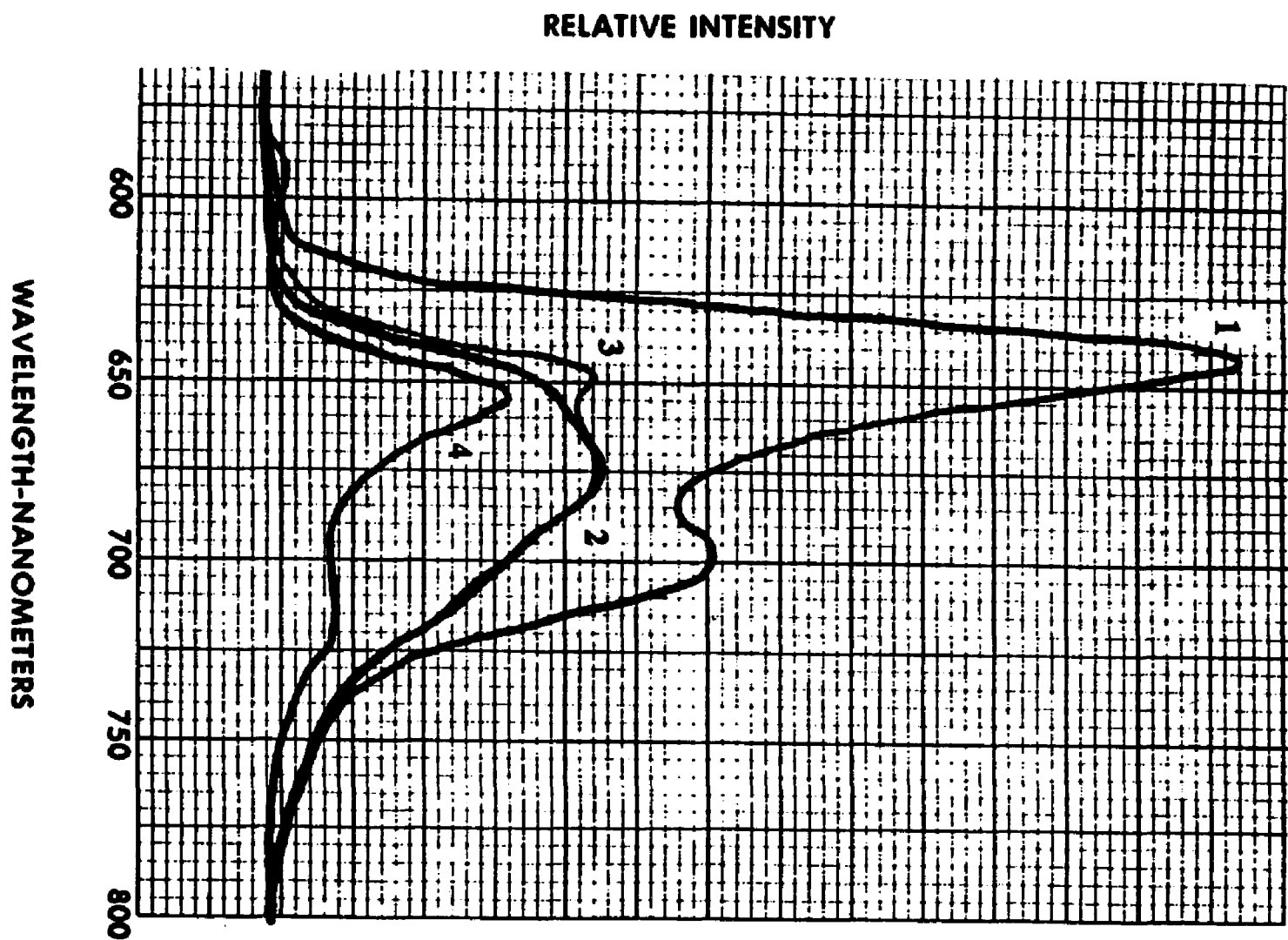


Figure.4-9. The fluorescence spectra of systems (1). N-MTAMPP only, (2). N-MTAMPP + CT.DNA, (3) N-MTAMPP + Poly(dA-dT) • Poly(dA-dT) and (4). N-MTAMPP + Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are: [N-MTAMPP] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.

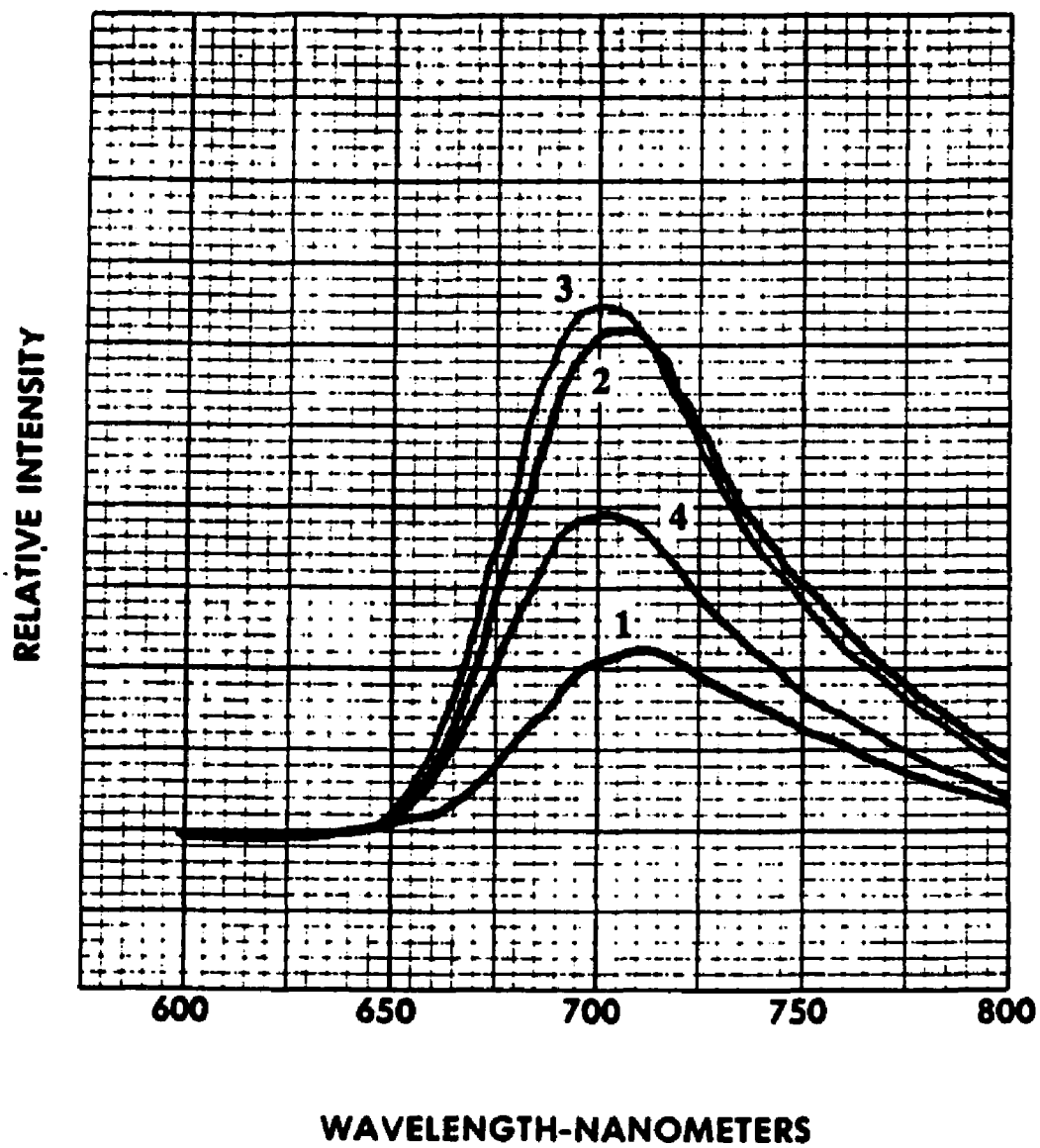
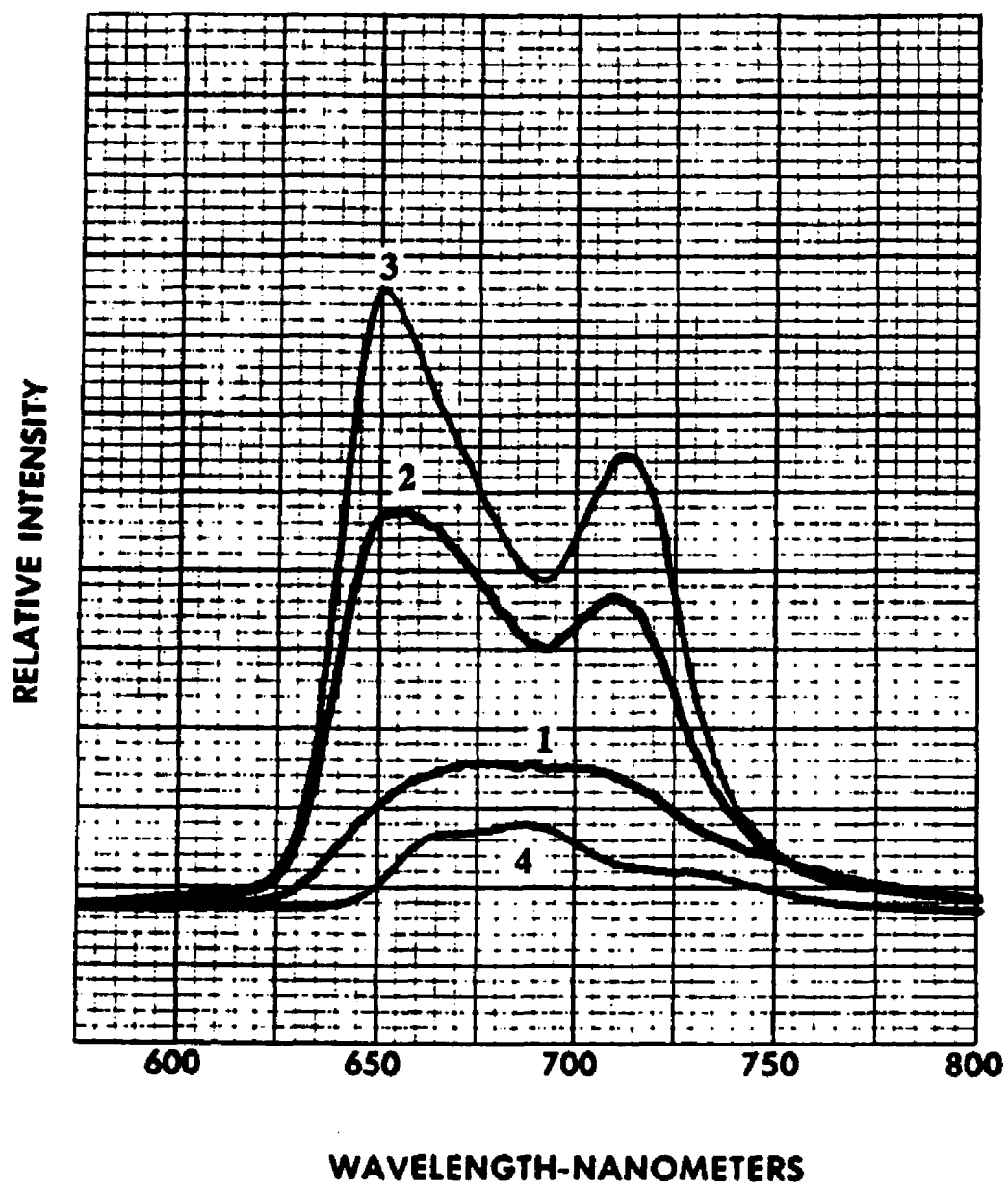


Figure.4-10 The fluorescence spectra of systems (1). TMPyP-4 only, (2). TMPyP-4 + CT.DNA, (3) TMPyP-4 + Poly(dA-dT) • Poly(dA-dT) and (4). TMPyP-4 + Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are: [TMPyP-4] = 8.79×10^{-6} M, [Nucleic Acid] = 2.64×10^{-4} M. pH 5.5 buffer solution.



intensity goes down 65%, 65% and 74% when TAMPP binds with CT.DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC), respectively); (2) the fluorescence intensities are greater for all of three N-MTAMPP nucleic acid systems compared to the fluorescence spectral intensity of N-MTAMPP alone, i.e. a hyperchromic character (the relative fluorescence intensity goes up relatively 157%, 162% and 48% when N-MTAMPP binds with CT.DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC), respectively); (3) the relative fluorescence intensity is greater for TMPyP-4 — DNA and TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) (hyperchromicity) but less for TMPyP-4 — Poly(dG-dC) • Poly(dG-dC) (hypochromicity) systems than the intensity of the fluorescence spectrum of TMPyP-4 alone at the same concentration of porphyrin (the relative fluorescence intensity goes up relatively 180% and 340% when TMPyP-4 binds with CT.DNA, Poly(dA-dT) • Poly(dA-dT) and goes down 42% when TMPyP-4 binds with Poly(dG-dC) • Poly(dG-dC), respectively); (4) For all of these porphyrins, especially for TAMPP and N-MTAMPP, the fluorescence spectra are more similar to one another when the porphyrins interact with CT-DNA and Poly(dA-dT) • Poly(dA-dT) than when they interact with Poly(dG-dC) • Poly(dG-dC); (5) All of the three porphyrin — Poly(dG-dC) • Poly(dG-dC) complexes give the lowest fluorescence intensity compared to the corresponding porphyrin — DNA or porphyrin — Poly(dA-dT) • Poly(dA-dT) systems.

4.4 Discussion

Based on the results of chapter (3), little monoprotonated TAMPP accumulates as TAMPP is protonated, so the expression $K_{app}(TAMPP) = K_3 K_4 = 10^{-9}$ at $[I] = 0.12M$ is used. Therefore the concentration ratio of neutral and diprotonated TAMPP is calculated in pH 5.5 buffer as:

$$\frac{[\text{PH}_2]}{[\text{PH}_4^{2+}]} = \frac{K_{\text{app}}}{[\text{H}^+]^2} = \frac{10^{-9}}{10^{-5.5 \times 2}} = 100 \quad (4-2)$$

That is, TAMPP is mostly in its free base form.

However, absorption spectra demonstrate that in the presence of the nucleic acid, TAMPP is changed to its diprotonated form even in pH 5.5 buffer.

On the other hand, the pK_3 and pK_4 values of N-MTAMPP are more widely separate (8.1 and 3.1 respectively at $[\text{I}] = 0.12 \text{ M}$), giving:

$$\frac{[\text{PH}_3^+]}{[\text{PH}_4^{2+}]} = \frac{K_4}{[\text{H}^+]} = \frac{10^{-3.1}}{10^{-5.5}} = 251 \quad (4-3)$$

$$\frac{[\text{PH}_3^+]}{[\text{PH}_2]} = \frac{[\text{H}^+]}{K_3} = \frac{10^{-5.5}}{10^{-8.1}} = 398 \quad (4-4)$$

and

$$\frac{[\text{PH}_4^{2+}]}{[\text{PH}_2]} = \frac{[\text{H}^+]^2}{K_3 K_4} = \frac{10^{-5.5 \times 2}}{10^{-11.2}} = 1.6 \quad (4-5)$$

That is, $[\text{PH}_3^+] \gg [\text{PH}_4^{2+}] > [\text{PH}_2]$ and N-MTAMPP is mostly in its monoprotonated state when pH 5.5 buffer is used.

The results of porphyrins bound with nucleic acids in pH 5.5 buffer derived from circular dichroism, visible absorption and fluorescence spectra are summarized in Table 4-1. Comparing the results of three different experimental techniques, it seems that

Table 4-1

Summary of Circular Dichroism, Absorbance and Fluorescence Results in the Visible Region

TMPyP-4					TAMPP					N-MTAMPP				
Visible			Fluorescence		CD	Visible			Fluorescence		CD	Visible		
$\lambda_{\max}$	$\pm\Delta A\%$		$\lambda_{\max}$	$\pm\Delta I\%$	$\pm\lambda_{\max}$	$\lambda_{\max}$	$\pm\Delta A\%$		$\lambda_{\max}$	$\pm\Delta I\%$	$\pm\lambda_{\max}$	$\lambda_{\max}$	$\pm\Delta A\%$	$\lambda_{\max}$
None	422	0	680	0	+0	413	0		642	0	+0	440	0	709
			-0						-0					0
DNA	430	-43	655	+180	+441	441	-4		675	-65	+448	448	-10	705
					-0						-470(s)			+157
Poly	431	-14	650	+340	+445	441	-19		648	-65	+448	449	-2	700
(dA-dT) ₂					-0						-471(s)			+162
Poly	444	-47	685	-42	+449	427	-58		655	-74	+443	447	-34	700
(dG-dC) ₂			-435						-465					+48

(1) TAMPP binds much more readily into A-T rich region in calf thymus DNA than into G-C rich region of calf thymus DNA, which is demonstrated from very similar induced circular dichroism, visible and fluorescence spectra for TAMPP — DNA and TAMPP — Poly(dA-dT) • Poly(dA-dT) systems. The newly synthesized porphyrin TAMPP is not like the planar porphyrin TMPyP-4 studied by Pasternack ⁹ in this respect. The TMPyP-4 — DNA system shows features of both TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) complexes in its induced circular dichroism, absorption and fluorescence spectra (figures 4-7a, 7b, 7c and 10 and Pasternack's circular dichroism results).

(2) The nature of the interactions of TAMPP with Poly(dG-dC) • Poly(dG-dC) and Poly(dA-dT) • Poly(dA-dT) may be quite different from each other, because their induced circular dichroism spectra show completely different shapes and their visible absorption and fluorescence spectra are also markedly different. A similar phenomenon was reported by Pasternack ⁹. When binding with Poly(dA-dT) • Poly(dA-dT) or Poly(dG-dC) • Poly(dG-dC), the newly synthesized TAMPP gives rise to induced circular dichroism and absorption properties similar to those of the planar porphyrins TMPyP-4, Cu(II)TMPyP-4 and Ni(II)TMPyP-4 bound to corresponding same polynucleic acid studied by Pasternack ⁹. For induced circular dichroism spectra: with Poly(dA-dT) • Poly(dA-dT), TAMPP displays only positive CD bands with relative large ellipticity; while with Poly(dG-dC) • Poly(dG-dC), TAMPP displays both positive and negative CD bands with relatively small ellipticities. Similar observations were obtained for TMPyP-4 and other planar porphyrins ⁹ except that they give rise to induced circular dichroism spectra with positive phase when they bind with Poly(dG-dC) • Poly(dG-dC). In contrast, TAMPP — Poly(dG-dC) • Poly(dG-dC) shows a negative phase in the circular dichroism spectrum. For absorption spectra: TAMPP — Poly(dA-dT) • Poly(dA-dT) shows less hypochromicity (19%) and a larger red shift

(27.5 nm), while TAMPP — Poly(dG-dC) • Poly(dG-dC) shows a larger hypochromicity (58%) and a small red shift (14.2 nm). Similar phenomena were observed for TMPyP-4 and the other planar porphyrins (this work and Pasternack's results). For fluorescence experiments, the spectrum of TAMPP with Poly(dA-dT) • Poly(dA-dT) shows less hyperchromicity and an abnormally broad peak (without typical peak values, it spans 650-700 nm), while the spectrum of TAMPP with Poly(dG-dC) • Poly(dG-dC) shows a larger hypochromicity and a normal narrow peak (the peak position is 654 nm). The fluorescence results for TMPyP-4 derived from this work show a larger hyperchromicity (+340%) for TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) complex and a small hypochromicity (-42%) for TMPyP-4 — Poly(dG-dC) • Poly(dG-dC) complex, very different experimental phenomena for these two complexes.

(3) N-MTAMPP interacts strongly with AT-rich regions of DNA as well since the characteristics of induced circular dichroism, absorption and fluorescence spectra are alike for N-MTAMPP — CT.DNA and N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) systems. But it seems that some of N-MTAMPP may bind to GC-rich regions of DNA because the hypochromicity of the N-MTAMPP — DNA system is between those of N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) and N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) complexes in visible absorption spectra (figures 4-6a, 6b and 6c) and also because there appears to be some contribution of the spectrum of the N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) complex in induced circular dichroism spectrum (figure 4-3). The fluorescence spectra (figure 4-9) for the mixture of N-MTAMPP and calf thymus DNA systems also show some similarity. Compared with TAMPP, N-MTAMPP seems more like TMPyP-4 when it binds with DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC), respectively.

(4) There may not be much difference between the nature of interaction of N-MTAMPP with Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) because

there are no essential differences apparent in the shapes of the induced circular dichroism spectra and the visible absorption spectra. The fluorescence spectra of the two complexes are also similar in shape.

(5) It appears that the types of interactions of TAMPP — Poly(dG-dC) • Poly(dG-dC) and N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) are somewhat different because the induced circular dichroism spectra and the fluorescence spectra of these two complexes are quite different. The phase of circular dichroism spectrum for TAMPP — Poly(dG-dC) • Poly(dG-dC) system is negative, while it is positive for N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) complex. The TAMPP — Poly(dG-dC) • Poly(dG-dC) complex shows a large hypochromicity (-74%) of the relative fluorescence intensity while the N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) complex shows a larger hyperchromicity (+48%) in its the fluorescence spectrum. In contrast TMPyP-4, its Poly(dG-dC) • Poly(dG-dC) complex shows a positive phase circular dichroism spectrum⁹ and a hypochromicity (-42%) in its relative fluorescence intensity. It seems that these three porphyrin — Poly(dG-dC) • Poly(dG-dC) systems have some common points between them and also some differences.

(6) TAMPP — Poly(dA-dT) • Poly(dA-dT) and N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) give quite different fluorescence spectra. The former is obviously hypochromic (-65%) and the latter is markedly hyperchromic (+162). On the other hand, their induced circular dichroism spectra are similar except for an extra small positive shoulder peak for TAMPP — Poly(dA-dT) • Poly(dA-dT) system and a small negative band for N-MTAMPP — Poly(dA-dT) • Poly(dA-dT). The above phenomena seem to imply that there are some common points of interaction between TAMPP and N-MTAMPP with Poly(dA-dT) • Poly(dA-dT) and there are also some differences between them.

After studying circular dichroism and absorption spectra of planar porphyrins TMPyP-4, Cu(II)TMPyP-4 (without axial ligands) and non-planar porphyrins Zn(II)TMPyP-4, Co(III)TMPyP-4, Fe(III)TMPyP-4 and Mn(III)TMPyP-4 (with axial ligands), Pasternack⁹ suggested that the type of complex formed with GC-rich regions is fundamentally different from that formed in AT-rich regions and for this types of porphyrin, at small ratio of total concentration of porphyrin to that of nucleic acid (≤ 0.03 in his work), a positive CD is indicative of interactions with AT sites which may be either a partially intercalated complex or an outside-bond complex and a negative CD band with GC sites which forms a truly intercalated species.

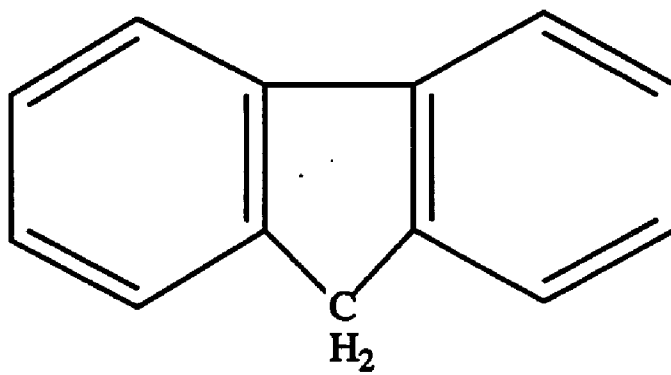
According to Pasternack's empirical correlations: (1) TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) is a outside-bound complex and TMPyP-4 — Poly(dG-dC) • Poly(dG-dC) is a intercalated complex; (2) with Poly(dA-dT) • Poly(dA-dT), TAMPP shows only positive CD bands which implies that it may only bind outside of Poly(dA-dT) • Poly(dA-dT) and with Poly(dG-dC) • Poly(dG-dC), TAMPP shows both positive and negative CD bands (but note its induced circular dichroism spectrum has a negative phase) which implies that it may be either intercalated into and also outside-bound to Poly(dG-dC) • Poly(dG-dC); (3) For N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) and N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) systems, their induced circular dichroism spectra suggest that N-MTAMPP may be mostly outside-bound to Poly(dA-dT) • Poly(dA-dT), while it may be either intercalated and outside-bound to Poly(dG-dC) • Poly(dG-dC) in equivalent tendency.

However, the fluorescence emission spectra (figures 4-8, 9 and 10) demonstrate that the TAMPP—Poly(dA-dT) • Poly(dA-dT) system is hypochromic (-65%), while TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) and N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) systems are hyperchromic (+340% and +162% respectively). It seems that the outside binding interactions between TAMPP, N-MTAMPP and TMPyP-4 with

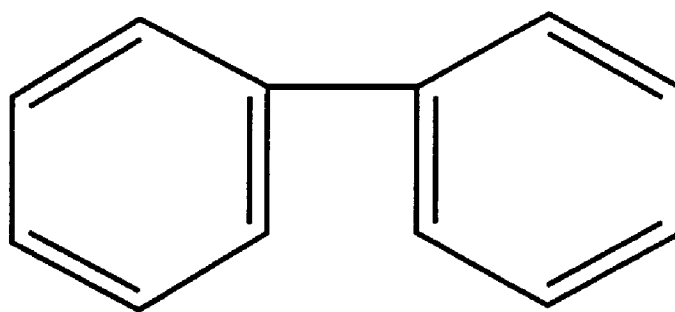
Poly(dA-dT) • Poly(dA-dT) are different. The outside interaction may be a geometrically non-specific electrostatic attraction or may be a more specific interaction that leads to insertion of the porphyrin into the groove of the nucleic acid. The TAMPP — Poly(dA-dT) • Poly(dA-dT) outside-bound complex may be a non specific electrostatic one, which would be consistent with the hypochromicity of its fluorescence spectrum. The electrostatic interaction between TAMPP and Poly(dA-dT) • Poly(dA-dT) may allow more paths for excited molecules to deactivate radiationlessly to its ground state, decreasing the fluorescence quantum yield and leading to hypochromicity. N-MTAMPP may insert into the groove of Poly(dA-dT) • Poly(dA-dT) which would increase structural rigidity and lower the internal conversion rate, i.e. limit the vibration relaxation of porphyrin. It is found experimentally that fluorescence is particularly favored in molecules that possess rigid structures ¹⁸. For instance, the quantum yield for fluorene and biphenyl (figure 4-11) are nearly 1.0 and 0.2, respectively, under similar conditions ¹⁸. Insertion into the groove also decreases the chance of radiationless deactivation of excited porphyrins to solvent and solute. Thus, insertion into the groove of the nucleic acid may increase the fluorescence quantum yield of the system. Based on this explanation, TMPyP-4 may also bind in the groove of Poly(dA-dT) • Poly(dA-dT) since the TMPyP-4 — Poly(dA-dT) • Poly(dA-dT) system shows a positive CD spectrum and hyperchromic fluorescence (Pasternack's result and figure 4-10 in this work).

With Poly(dG-dC) • Poly(dG-dC), TMPyP-4 and TAMPP display negative induced circular dichroism bands and hypochromic fluorescence spectra. This leads to the conclusion that the intercalation of a porphyrin in a polynucleic acid can cause hypochromic character in its fluorescence spectra. Pasternack's empirical correlation would suggest that intercalation occurs in the TMPyP-4 — Poly(dG-dC) • Poly(dG-dC) and TAMPP — Poly(dG-dC) • Poly(dG-dC) systems. If this is true, there may be

Figure.4-11. The molecular structures of (a). Fluorene, its quantum yield is about 1.0 and (b). Biphenyl, its quantum yield is about 0.2.



(a)



(b)

similar decrease in internal conversion where intercalation occurs, as the case for outside binding by electrostatic attraction. In that case the quantum yield of the system is decreased and hypochromic features are evident. Based on the above considerations, N-MTAMPP may be either intercalated (since it shows a negative circular dichroism band and hypochromic character in the fluorescence spectrum) and also bound on the outside of the groove (since it shows positive circular dichroism band and hyperchromic character in the fluorescence spectrum) when it interacts with Poly(dG-dC) • Poly(dG-dC). Then there will be two contrary factors reflected in the fluorescence spectrum of the N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) system: that is, the intercalation lowers the fluorescence intensity (hypochromic character) while binding into groove increases the fluorescence intensity (hyperchromic character). Therefore, the small hyperchromicity of the N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) system is dominant which means that N-MTAMPP binding into the groove of Poly(dG-dC) • Poly(dG-dC) is more prevalent than intercalation into Poly(dG-dC) • Poly(dG-dC). The intermediate fluorescence intensity value of TMPyP — 4-DNA (figure 4-14) implies that TMPyP-4 can both intercalate (probably in GC-rich regions) and also bind in the groove (probably on AT-rich regions) of DNA. The circular dichroism results for this system are also consistent with this explanation.

The information disclosed by figure 4-4a, 4b, 4c and 4-5 implies also that TAMPP is outside-bound to DNA and Poly(dA-dT) • Poly(dA-dT). TAMPP in the absence of nucleic acids is mostly in its neutral form in pH 5.5 buffer. On the contrary, when it interacts with CT.DNA and Poly(dA-dT)•Poly(dA-dT), TAMPP is mostly in its protonated form. Only when binding outside or interacting by electrostatic attraction to the phosphate groups of nucleic acid, can TAMPP be induced to its protonated form, which allows TAMPP to bind efficiently and strongly on the outside of CT.DNA and Poly(dA-dT)•Poly(dA-dT). In contrast, when binding to Poly(dG-dC) • Poly(dG-dC),

TAMPP is still in its neutral form, which implies that TAMPP is intercalated into Poly(dG-dC) • Poly(dG-dC) because neutral porphyrins stay in a planar conformation and it is easy for TAMPP to intercalate between nucleic acid base pairs.

4.5 Conclusion

Comparing the results demonstrated by induced circular dichroism, fluorescence spectra and the observations of Pasternack ⁹, it seems that (1) positive CD bands indicate outside-binding to nucleic acid either by electrostatic attraction or binding in the groove. The former will cause a hypochromic signature of the fluorescence spectra and the latter will show a hyperchromic signature for its fluorescence spectra; (2) negative CD bands indicate intercalation into nucleic acid base pairs. There may also be hypochromicity of the fluorescence spectrum.

Based upon above empirical correlations, for porphyrins TAMPP, N-MTAMPP and TMPyP-4: (1) TAMPP is able to intercalate into Poly(dG-dC) • Poly(dG-dC) and to bind by non-specific electrostatic attraction on the outside of Poly(dA-dT) • Poly(dA-dT). On the other hand, TAMPP binds preferentially at AT-rich regions of calf thymus DNA; (2) N-MTAMPP may bind in a groove of Poly(dA-dT) • Poly(dA-dT) and it can also intercalate into Poly(dG-dC) • Poly(dG-dC). N-MTAMPP preferentially populates AT-rich regions of calf thymus DNA; (3) TMPyP-4 may bind in the groove of Poly(dA-dT) • Poly(dA-dT) and may intercalate into Poly(dG-dC) • Poly(dG-dC). From Pasternack's work, TMPyP-4 interacts significantly with both GC and AT region of calf thymus DNA.

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Chapter 5

The Affinity of Tetrakis(4-aminomethylphenyl)Porphyrin and its N-methyl Derivative for Poly-Nucleic Acids

5.1 Introduction

Pasternack ¹, Fiel ² and Le Pecq ³ have examined the interaction of tetrakis(4-N-methylpyridyl)porphine (TMPyP-4) and some of its derivatives with nucleic acids. They found that those porphyrins with positive charge on their peripheral groups have a high affinity for nucleic acids.

Because porphyrins can interact with nucleic acids at different positions and in different ways ^{1, 2, 4}, the competition binding method using fluorescence spectroscopy is a better way to determine the affinity than a direct method using absorption spectroscopy. The competition method uses a probe whose binding constant with the corresponding nucleic acid can be well established by Scatchard equation and which has a fluorescence intensity that changes significantly and reproducibly when it interacts with a nucleic acid. The well-known probe candidate is the drug ethidium bromide ^{3, 5}. Ethidium bromide — nucleic acid complexes involve small molecular ligands bound to nucleic acids with no cooperativity among ligands. The experimental titration data for ethidium bromide obey Scatchard treatment very well, with linearity over a broad concentration range ^{3, 5} (figure 5-8 in this section). The fluorescence change when a porphyrin of unknown affinity constant is added can be used to determine the overall binding constant of the porphyrin with a nucleic acid ³. The direct method using visible absorption spectroscopy is typically restricted to the affinity of the strongest binding complex ². In addition, constants for the same system reported by different groups have been very different from each other ^{1, 2}.

The competition method has been used by Le Pecq and his coworkers ^{3, 5}. Their data processing method can be summarized as follows. For the competitive binding of two different drugs, D_1 and D_2 (both of them non-cooperative) at the same site of the nucleic acid base pairs, with binding constants K_1 and K_2 , the following equation applies:

$$\frac{r_1}{[D_1]} = \frac{K_1}{1 + K_2 [D_2]} (n - r_1) \quad (5-1)$$

in which r_1 is equal to $[D_1]_b / [NA]_t$, i.e., the concentration of bound drug D_1 over the total concentration of nucleic acid and n is the number of binding sites offered by each nucleic acid base pair. Plotting $r_1 / [D_1]$ versus r_1 at different concentrations of D_2 , a series of straight lines would be obtained. Replotting the reciprocal of the slope versus its corresponding $[D_2]$, the K_2 will be resolved if K_1 is a known constant.

However, the equation (5-1) given by Le Pecq ⁵ should be actually rewritten as:

$$\frac{r_1}{[D_1]_f} = \frac{K_1}{1 + K_2 [D_2]_f} (n - r_1) \quad (5-2)$$

based on the appropriate simplest equilibrium expressions in absence of cooperativity among ligands. The detailed derivation (5-2) is given in appendix (5). According to equation (5-2), $[D_1]$ and $[D_2]$ in (5-1) are actually the free (unbound) concentration of drugs D_1 and D_2 . Therefore, several problems are involved if equation (5-1) is used directly. First, $[D_1]$ is not a constant in one titration trial because when titration of D_1 is going on, there will be more and more D_1 to replace bound D_2 and $[D_2]_f$ would be larger and larger; Second, $[D_2]_f$ should not be replaced by total concentration of drug D_2 ($[D_2]_t$). $[D_2]_f$ is the difference between the total ($[D_2]_t$) and bound ($[D_2]_b$)

concentration of drug D_2 and $[D_2]_b$ should not be too small compared with $[D_2]_t$ if competition binding experiment is selected. Otherwise, a large relative error will be introduced because it is not possible to determine a small $[D_2]_b$ with high accuracy. Thirdly, equation (5-1) or (5-2) implies that drug D_2 will occupy the same number of nucleic acid base pairs as the drug D_1 . However, in most cases, the structure, the molecular size, and the nature of the electron distribution of D_1 and D_2 will differ from each other. It is not necessary to use the restricted condition that D_1 and D_2 take the same number of nucleic acid base pairs: these two values may be different when different drugs (for example, ethidium bromide and porphyrin) interact with nucleic acids. Thus, equation (5-2) is not suitable for competition systems in general.

McGhee and von Hippel derived a approach to analyze competition - type experiments, in which the binding of an unknown (or difficult to measure) ligand is monitored as a function of the binding of other ligand (which has distinct optical properties) for systems involving noncooperative ligand binding system ⁶. The need to use a McGhee - von Hippel approach to data analysis instead of the Scatchard analysis, used by Le pecq and used also in a modified form in this work, can be determined by the fit of data to the Scatchard plot, which we will show is adequate, eliminating the need for a McGhee - von Hippel analysis.

In this work, a model was developed to determine affinity constant for competitive binding system in which two different binding competitors may eliminate a different number of nucleic acid base pairs. For simplicity, the model worked here only deals with those non - competitive binding. We will show that this is adequate because both the interaction of ethidium ^{3, 5} and of the positive charged porphyrin ^{1, 2, 3} with DNA appears to be non-cooperative under our conditions.

5.2 Materials and Instruments

Calf thymus DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) were purchased from Sigma Chemical Co. Calf thymus DNA was purified as previously described by Pasternack ¹ with slight modifications described in chapter (4). Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) were used without further purification. Ethidium bromide was purchased from Aldrich Chemical Co. Inc. All other chemicals were reagent grade and used without further purification. All experiments were carried out at 25 °C and pH 5.5 using acetic acid buffer containing 0.05M acetic acid and sodium acetate in total and 0.1 M sodium chloride. The integrity of the nucleic acid duplexes was determined by comparing their ultraviolet spectra with published results ^{13, 14, 15}. Concentrations, always calculated in base pairs, were determined spectrophotometrically with $\epsilon_{260} = 1.31 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for calf thymus DNA ¹³, $\epsilon_{262} = 1.32 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for Poly(dA-dT) • Poly(dA-dT) ¹⁴ and $\epsilon_{254} = 1.68 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for Poly(dG-dC) • Poly(dG-dC) ¹⁵.

Fluorescence spectra were obtained using a Amino-Bowman SPF 125 Spectrophotofluorometer with a thermostated cell compartment. The reference, exit, entrance and signal PM tube slits used were 0.5, 0.5, 1.0 and 0.5 mm respectively. Fluorescence titration experiments were conducted by adding aliquots of a concentrated stock solution of the ethidium bromide (EB) directly into a 4 ml cuvette whose starting volume was 2 ml. The wavelength of excitation was 520 nm and the wavelength of the emission was 600 nm. The signal high voltage was 925 volts. For the fluorescence scanning experiments, the wavelength of excitation was set at 520 nm and the wavelength of the emission was changed with time by about 220 nm / min.

Ethidium bromide is highly poisonous and should be handled carefully with gloved hands. The used ethidium bromide solution should not be desposed of into the plumbing system but saved for commercial desposal.

5.3 Analytical methods

5.3.1 Derivation of a general equation for competitive binding of a molecule to nucleic acid

Suppose a drug D_1 binds to a nucleic acid (NA) in absence of cooperativity manner and obeys the well-known Scatchard equation:

$$\frac{[D_1]_b}{[D_1]_f [NA]_t} = K_{D_1} \left(n - \frac{[D_1]_b}{[NA]_t} \right) \quad (5-3)$$

where $[D_1]_b$ and $[D_1]_f$ are the concentrations of bound and free drug D_1 , $[NA]_t$ is the total concentration of nucleic acid, K_{D_1} is the affinity constant of D_1 with the nucleic acid and n is the number of binding sites for D_1 per nucleic acid base pair. The value of n is also the number of moles of bound D_1 per mole of nucleic acid base pair because one D_1 molecule requires one D_1 binding site on the nucleic acid. Therefore, the number of nucleic acid base pairs occupied by one bound D_1 molecule equal to $1/n$.

When drug D_2 coexists in solution with drug D_1 and can bind non-cooperatively and competitively to the nucleic acid base pairs against D_1 , the following equilibria apply:



and the corresponding relationships are obtained:

$$K_{D_1} = \frac{[D_1]_b}{[D_1]_f \cdot n[NA]_f} \quad (5-6)$$

$$K_{D_2} = \frac{[D_2]_b}{[D_2]_f \cdot n[NA]_f} \quad (5-7)$$

where $[D_1]_b$, $[D_1]_f$, K_{D_1} and n have the same meaning as in equation (5-3); $[D_2]_b$ and $[D_2]_f$ are the concentration of bound and free drug D_2 respectively and $[NA]_f$ is the concentration of free nucleic acid in base pairs. Therefore, the term " $n[NA]_f$ " means the concentration of binding sites on nucleic acid capable of binding drug D_1 that remain free. K_{D_2} is the affinity constant of drug D_2 with nucleic acid. Combining equations (5-6) and (5-7), we have:

$$K_{D_1} \frac{[D_1]_f}{[D_1]_b} = K_{D_2} \frac{[D_2]_f}{[D_2]_b} \quad (5-8)$$

and then

$$\frac{[D_1]_f}{[D_1]_b} = \frac{K_{D_2}}{K_{D_1}} \left(\frac{[D_2]_t}{[D_2]_b} - 1 \right) \quad (5-9)$$

where $[D_2]_f$ is replaced by

$$[D_2]_f = [D_2]_t - [D_2]_b \quad (5-10)$$

Now a new parameter M_{drug} is introduced and defined as the number of D_1 binding sites required by each specific drug molecule. If we use D_1 as our reference material (because the parameter n is dependent on particular D_1 used), then M_{D_1} will equal to one, that is one D_1 molecule needs one binding site on nucleic acid

and will still occupy the number of $1/n$ nucleic acid base pairs exactly the same as the case of D_1 alone in solution with the nucleic acid. A molecule D_2 does not necessary take the same number of binding sites (or same number of nucleic acid base pairs) as its competitor, D_1 because D_1 and D_2 can have different molecular and/or electronic structures. D_2 may also bind to nucleic acids in of different manner than does D_1 . But it can still efficiently prevent D_1 from binding to nucleic acid. For example, it could cover the passage by which D_1 approaches its binding site, or it could deform the helical structure of the nucleic acid. Each D_2 molecule may take more, equal, and even fewer nucleic acid base pairs than its competitor D_1 . Therefore, M_{D_2} may be larger, equal and smaller than one. The number of nucleic acid base pairs occupied by one D_2 molecule is M_{D_2} / n . Based on discussions above, the concentration of binding sites on the nucleic acid occupied by bound D_2 , or inaccessible binding sites on nucleic acid because of bound D_2 molecules, $[BS]_{D_2}$, will equal:

$$[BS]_{D_2} = M_{D_2} [D_2]_b \quad (5-11)$$

Introducing (5-11) into equation (5-9), the last equation can be rewritten as:

$$\frac{[D_1]_f}{[D_1]_b} = \frac{K_{D_2}}{K_{D_1}} \left(\frac{M_{D_2} [D_2]_t}{[BS]_{D_2}} - 1 \right) \quad (5-12)$$

in which $[BS]_{D_2}$ can be described in more detail in terms of material balance:

$$[BS]_{D_2} = n[NA]_t - n[NA]_f - [BS]_{D_1} \quad (5-13)$$

where $[BS]_{D_1}$ is the concentration of binding sites on the nucleic acid occupied by bound D_1 , or inaccessible binding sites on nucleic acid because of bound D_1 molecules and it equals:

$$[BS]_{D_1} = M_{D_1}[D_1]_b = [D_1]_b \quad (5-14)$$

because M_{D_1} is equal to 1. Substitution of equations (5-14) and (5-6) into (5-13), gives:

$$[BS]_{D_1} = n[NA]_t - [D_1]_b - \frac{[D_1]_b}{K_{D_1}[D_1]_f} \quad (5-15)$$

Therefore, if we can determine $[D_1]_b$, the concentration of bound drug D_1 , K_{D_2} and M_{D_2} can be derived by applying equations (5-12) and (5-15).

The general equations describing two competitively binding drugs are expressed by equations (5-12) and (5-15), in which drug D_1 is taken as a reference binding material. Its affinity constant with the nucleic acid (K_{D_1}) as well as the number of binding sites for D_1 per nucleic acid base pair (n) are obtained using Scatchard's equation (5-3) to treat data from separate experiments using only D_1 in nucleic acid buffer solution. D_2 is a competitor of D_1 in solutions of nucleic acids. The affinity constant of D_2 with the specific nucleic acid (K_{D_2}) and the number of binding sites needed for each D_2 molecule on the nucleic acid (M_{D_2}) can be determined provided that the constants K_{D_1} and n of the corresponding competitor are known. D_1 takes *one* binding site and the number of nucleic acid base pairs it occupies is $1/n$. D_2 occupies M_{D_2} binding sites and the number of nucleic acid base pairs it occupies is M_{D_2}/n .

Using equations (5-12) and (5-15) to determine K_{D_2} and M_{D_2} , specific experiments must be designed to obtain a series of related $[D_1]_b$, $[D_1]_f$, $[D_2]_f$ and

$[NA]_t$ data. Since the values of K_{D_1} and n can be obtained independently from equation (5-3), plots of $[D_1]_f / [D_1]_b$ versus $[D_2]_f / [BS]_{D_2}$ will give K_{D_2} and M_{D_2} from the corresponding slope and y-intercept based on equation (5-12):

$$K_{D_2} = K_{D_1} \cdot \text{y-intercept} \quad (5-16)$$

$$M_{D_2} = \frac{\text{slope}}{\text{y-intercept}} \quad (5-17)$$

The precondition of determining K_{D_2} and M_{D_2} by the competition method is that the affinity constants K_{D_1} and K_{D_2} of drugs D_1 and D_2 must not be too different from each other. Otherwise, one of them can not compete with the other even after concentration adjustment, and a large error will be introduced.

5.3.2 Specific equation for this work

In this work, ethidium bromide (figure 5-1), whose interaction with DNA obeys Scatchard's equation very well (figure 5-8), was used as the reference material (D_1 in equations (5-3), (5-12), and (5-15)) and a porphyrin was used as the competitor drug (D_2 in equations (5-12) and (5-15)). The specific equations for this work are:

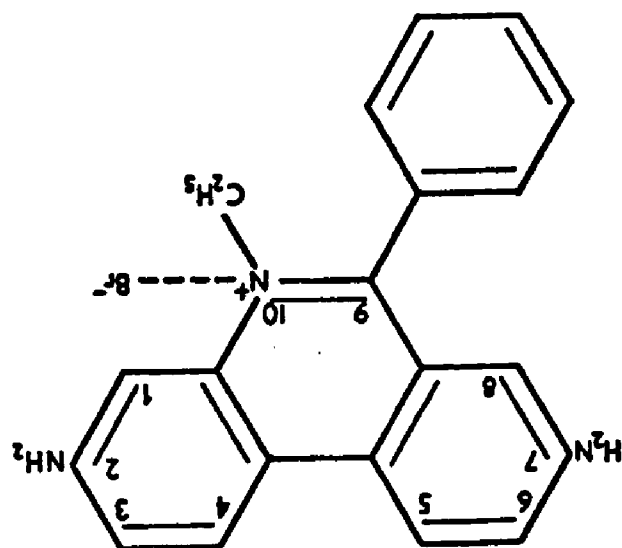
$$\frac{[EB]_b}{[EB]_f [NA]_t} = K_{EB} \left(n - \frac{[EB]_b}{[NA]_t} \right) \quad (5-18)$$

$$\frac{[EB]_f}{[EB]_b} = \frac{K_{Por}}{K_{EB}} \left(\frac{M_{Por} [Por]_t}{[BS]_{Por}} - 1 \right) \quad (5-19)$$

and

$$[BS]_{Por} = n[NA]_t - [EB]_b - \frac{[EB]_b}{K_{EB} [EB]_f} \quad (5-20)$$

**Figure. 5-1. The molecular structure of ethidium bromide: 2,7
diamino - 9 - phenyl - 10 - ethyl phenanthridinium bromide**



Therefore, plotting $[EB]_f / [EB]_b$ vs. $[Por]_t / [BS]_{Por}$ will give K_{Por} and M_{Por} from the corresponding slope and y-intercept:

$$K_{Por} = K_{EB} \cdot \text{y-intercept} \quad (5-21)$$

$$M_{Por} = \frac{\text{slope}}{\text{y-intercept}} \quad (5-22)$$

The porphyrins used for studying affinity constants are tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and its N-methyl derivative N-MTAMPP. The nucleic acids used are calf thymus DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC).

5.3.3 Examination of the extent of energy transfer from ethidium bromide to porphyrin

Compared to free ethidium bromide, ethidium bromide bound to a nucleic acid shows a larger fluorescence intensity (figures 5-2a and 2c; 5-3a and 3c; 5-4a and 4c). When the porphyrin TAMPP or N-MTAMPP is bound to a nucleic acid, it shows little fluorescence intensity change from the corresponding free state (figures 5-2d and 2e, 5-3d and 3e, 5-4d and 4e for TAMPP; figures 5-5d and 5e, 5-6d and 6e, 5-7d and 7e for N-MTAMPP). The change seen in ethidium bromide fluorescence is much larger than the change seen in the porphyrin fluorescence. In addition, almost no fluorescence intensity is contributed by free or bound TAMPP or N-MTAMPP at 600 nm when the excitation wavelength is 520 nm. The fact that the only changes in fluorescence when the excitation wavelength of 520 nm is used is due to ethidium bromide allows one to determine the affinity constant of porphyrin—nucleic acid complexes by competition binding of ethidium bromide and porphyrin to nucleic acid using fluorimetric methods. Important considerations are that the amount of energy transfer from ethidium bromide to porphyrin is small enough to be neglected and their affinities for nucleic acids are not

Figure. 5-2. The fluorescence spectra of (a). ethidium bromide (EB) alone, (b). ethidium bromide + calf thymus DNA + TAMPP, (c). ethidium bromide + calf thymus DNA, (d). TAMPP alone and (e). TAMPP + calf thymus DNA. The composition of solution concentrations for all of spectra: $[\text{EB}] = 3.46 \times 10^{-6} \text{ M}$, $[\text{CT.DNA}] = 4.28 \times 10^{-6} \text{ M}$, $[\text{TAMPP}] = 1.84 \times 10^{-6} \text{ M}$.

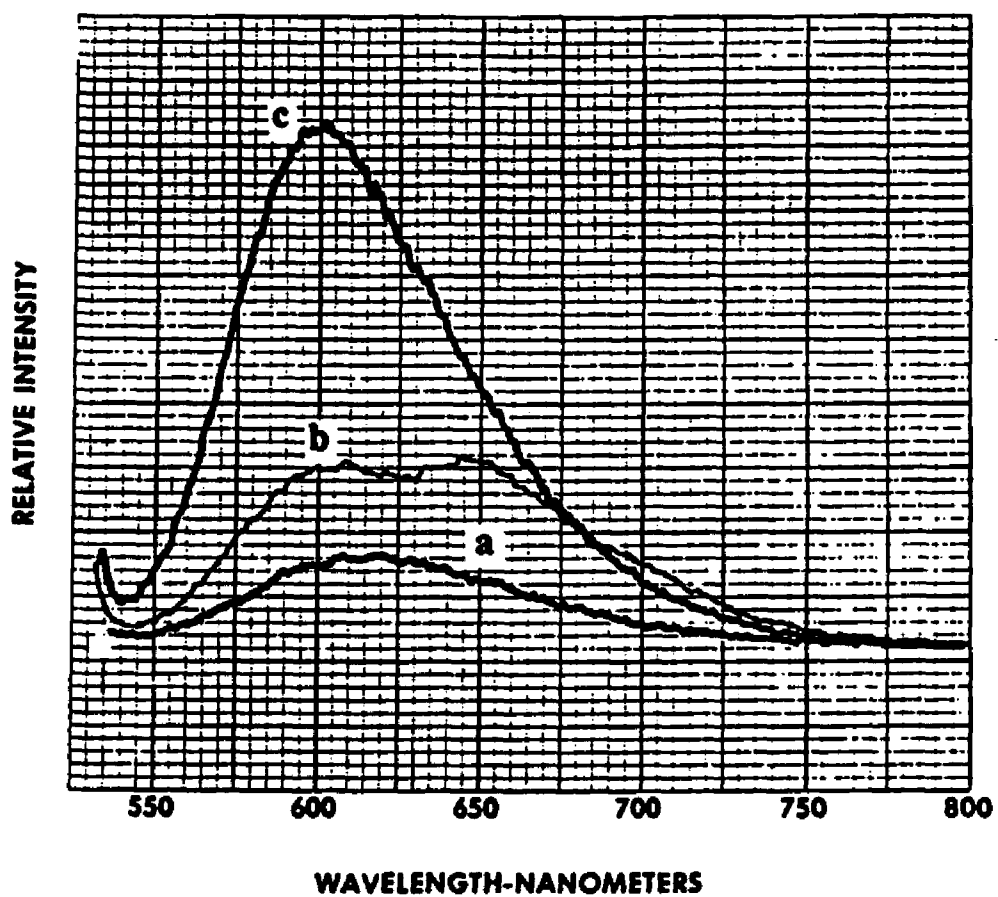
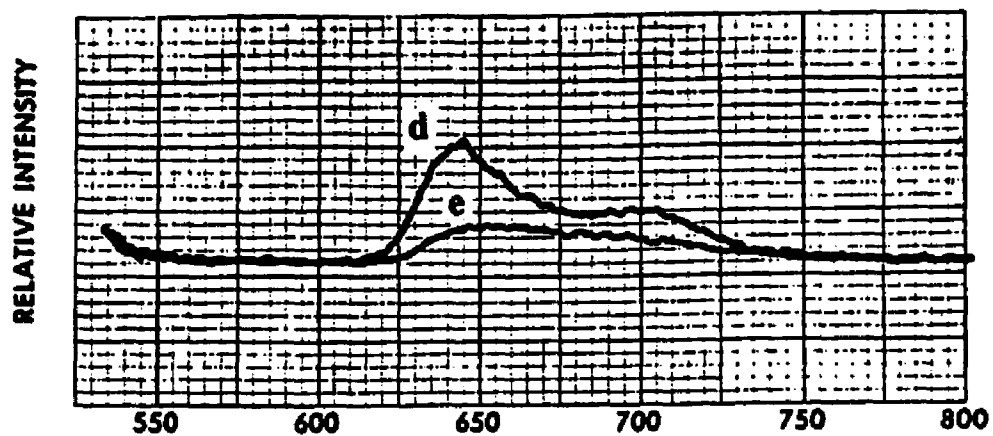


Figure. 5-3. The fluorescence spectra of (a). ethidium bromide (EB) alone, (b). ethidium bromide + Poly(dA-dT) • Poly(dA-dT) + TAMPP, (c). ethidium bromide + Poly(dA-dT) • Poly(dA-dT) , (d). TAMPP alone and (e). TAMPP + Poly(dA-dT) • Poly(dA-dT) . The composition of solution concentrations for all of spectra: [EB] = 3.46×10^{-6} M, [Poly(dA-dT) • Poly(dA-dT)] = 4.28×10^{-6} M, [TAMPP] = 1.84×10^{-6} M.

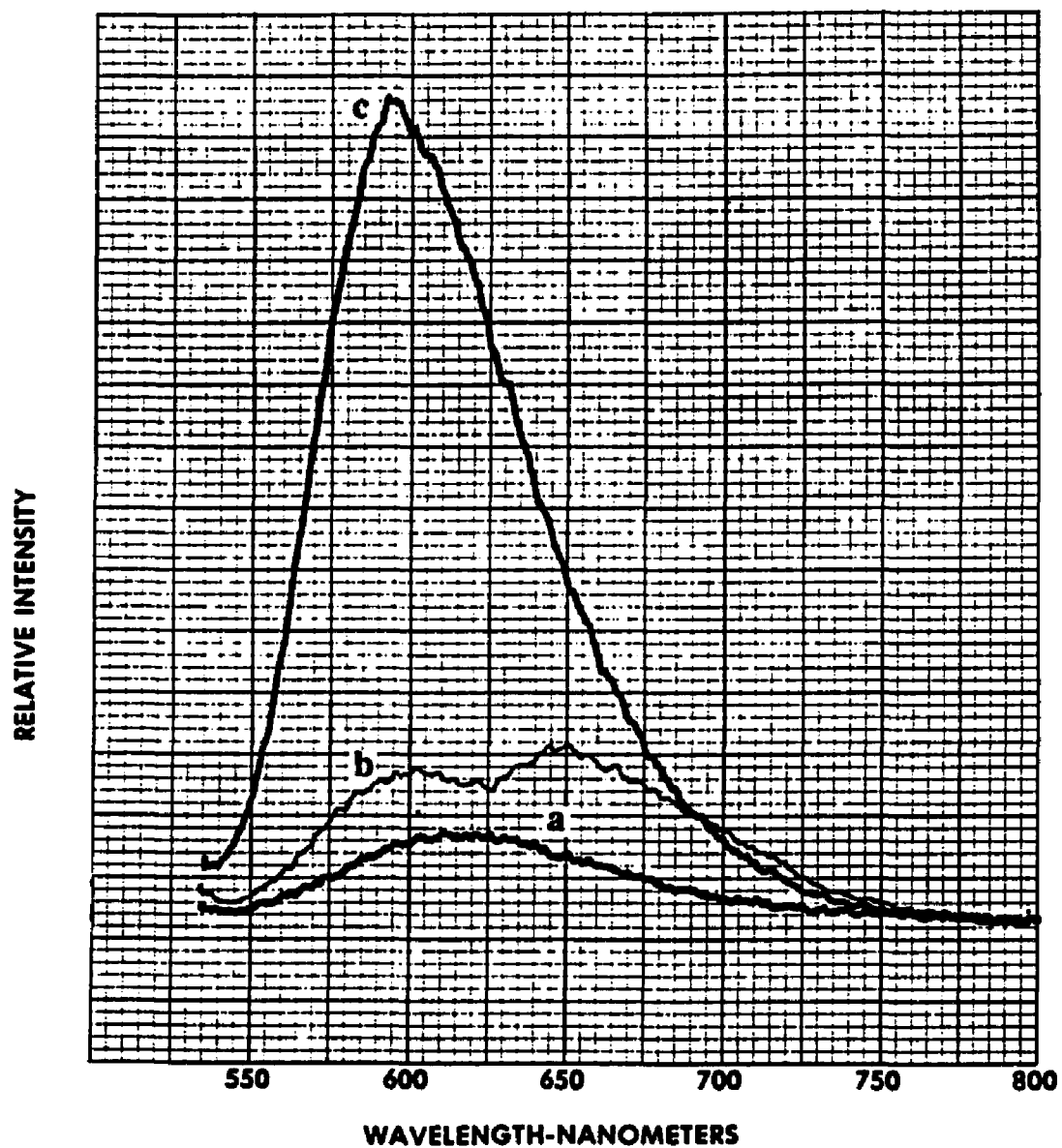
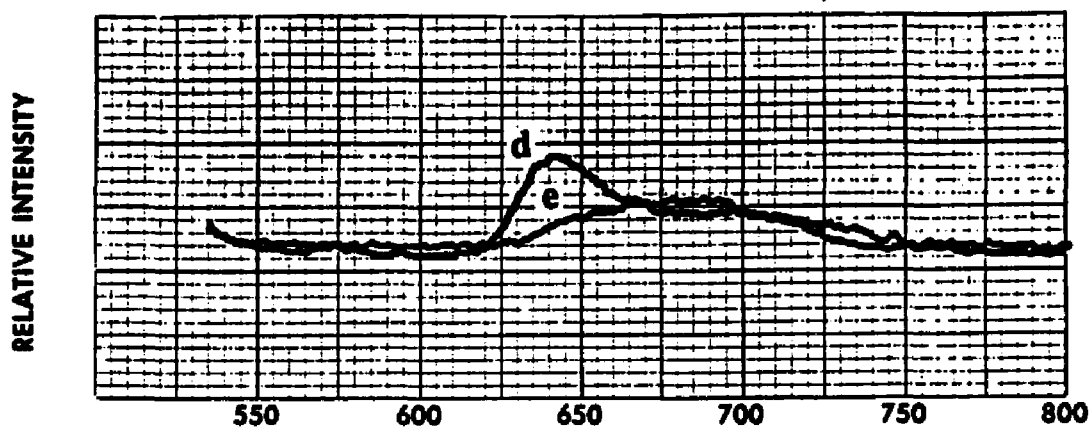


Figure. 5-4. The fluorescence spectra of (a). ethidium bromide (EB) alone, (b). ethidium bromide + Poly(dG-dC) • Poly(dG-dC) + TAMPP, (c). ethidium bromide + Poly(dG-dC) • Poly(dG-dC) , (d). TAMPP alone and (e). TAMPP + Poly(dG-dC) • Poly(dG-dC) . The composition of solution concentrations for all of spectra: [EB] = 3.46×10^{-6} M, [Poly(dG-dC) • Poly(dG-dC)] = 4.28×10^{-6} M, [TAMPP] = 1.84×10^{-6} M.

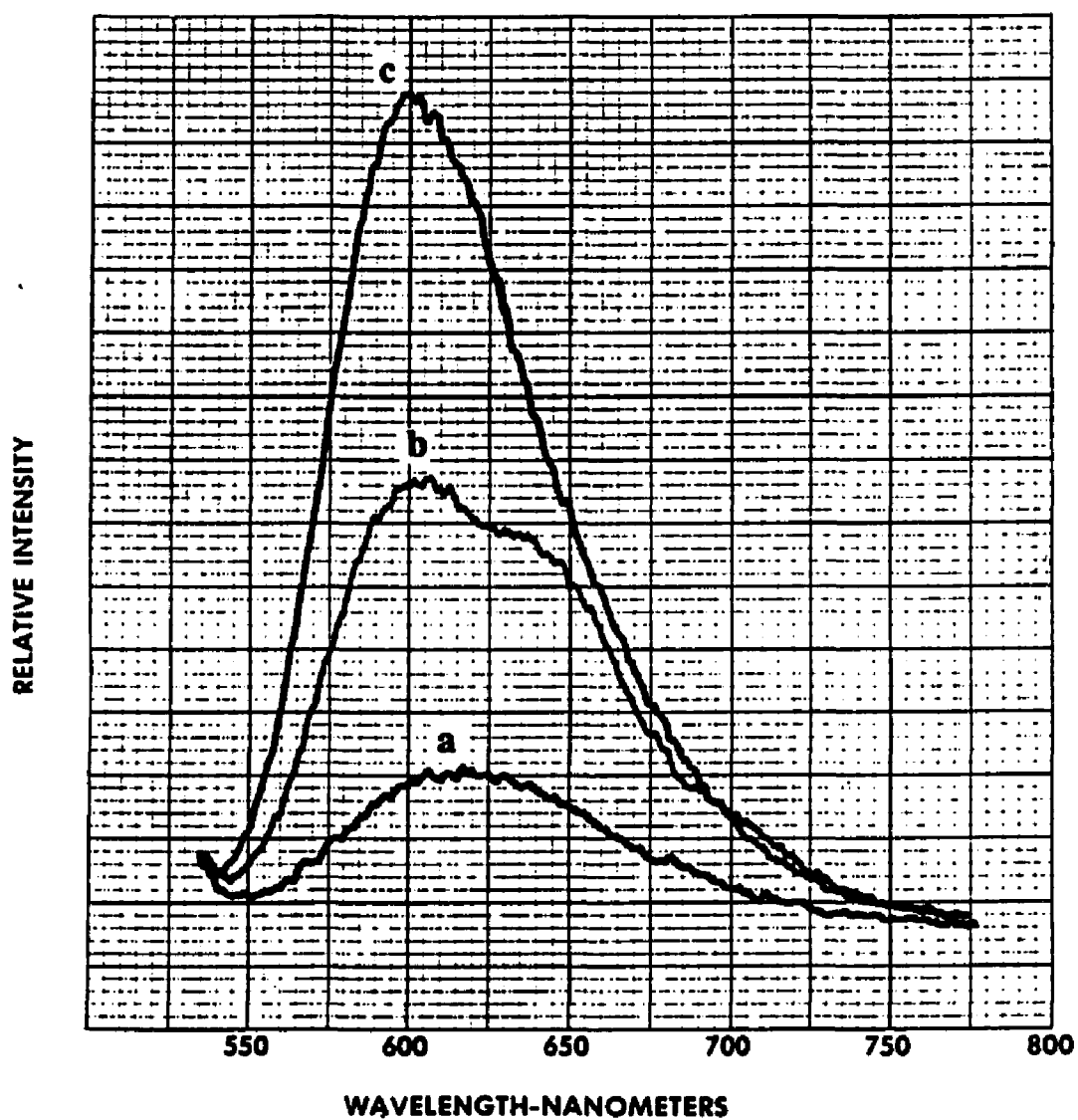
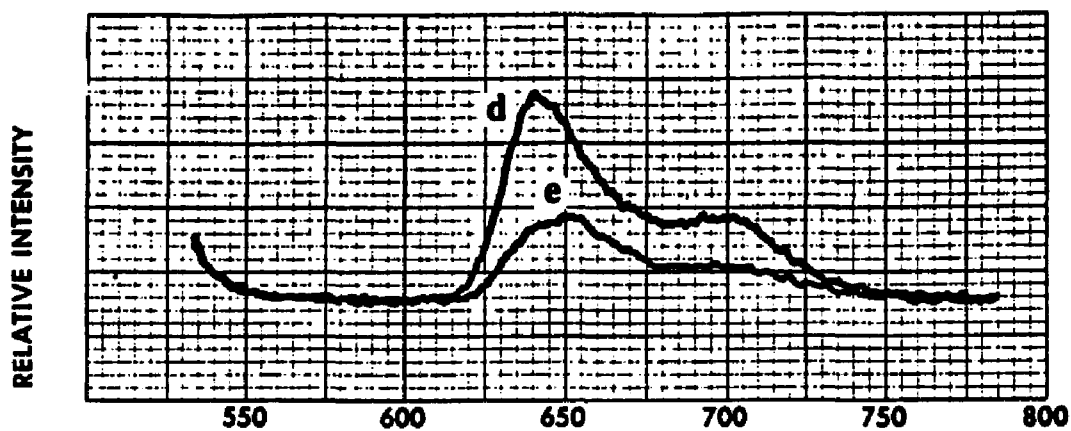


Figure. 5-5. The fluorescence spectra of (a). ethidium bromide (EB) alone, (b). ethidium bromide + calf thymus DNA + N-MTAMPP, (c). ethidium bromide + calf thymus DNA, (d). N-MTAMPP alone and (e). N-MTAMPP + calf thymus DNA. The composition of solution concentrations for all of spectra: [EB] = 3.46×10^{-6} M, [CT.DNA] = 4.28×10^{-6} M, [N-MTAMPP] = 4.76×10^{-7} M.

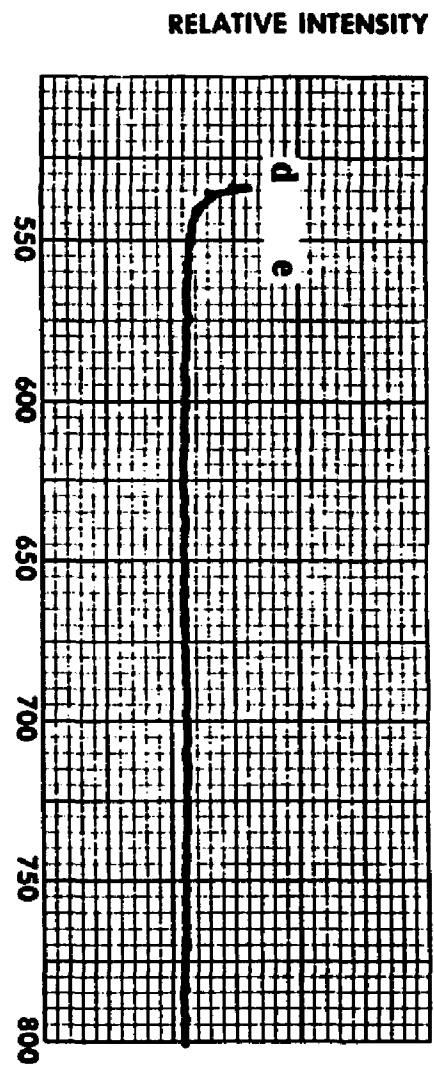
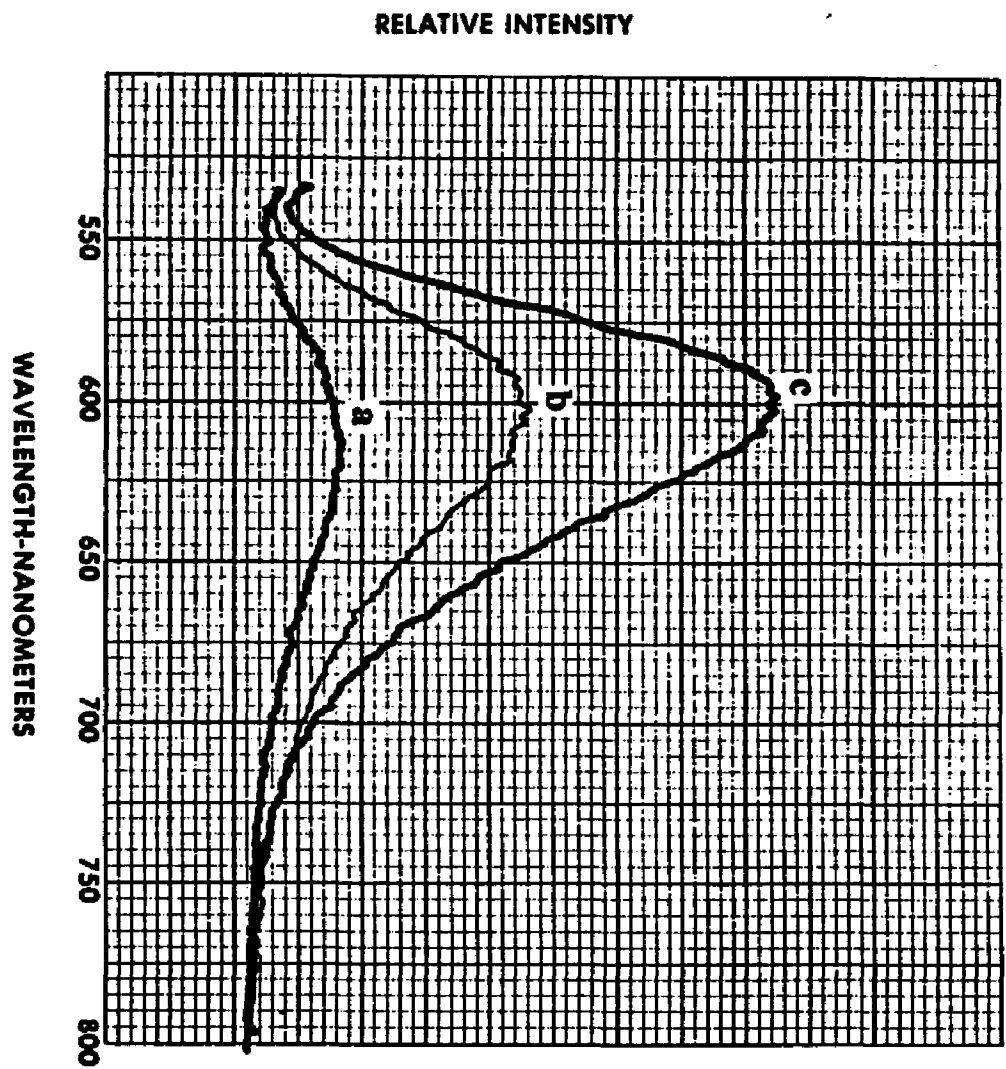


Figure. 5-6. The fluorescence spectra of (a). ethidium bromide (EB) alone, (b). ethidium bromide + Poly(dA-dT) • Poly(dA-dT) + N-MTAMPP, (c). ethidium bromide + Poly(dA-dT) • Poly(dA-dT) , (d). N-MTAMPP alone and (e). N-MTAMPP + Poly(dA-dT) • Poly(dA-dT). The composition of solution concentrations for all of spectra: $[EB] = 3.46 \times 10^{-6} \text{ M}$, $[Poly(dA-dT) \bullet Poly(dA-dT)] = 4.28 \times 10^{-6} \text{ M}$, $[N-MTAMPP] = 4.76 \times 10^{-7} \text{ M}$.

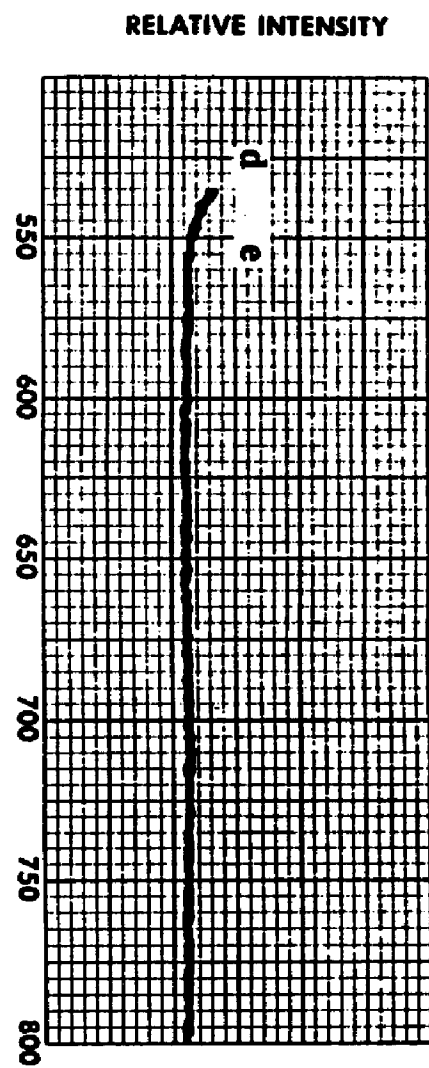
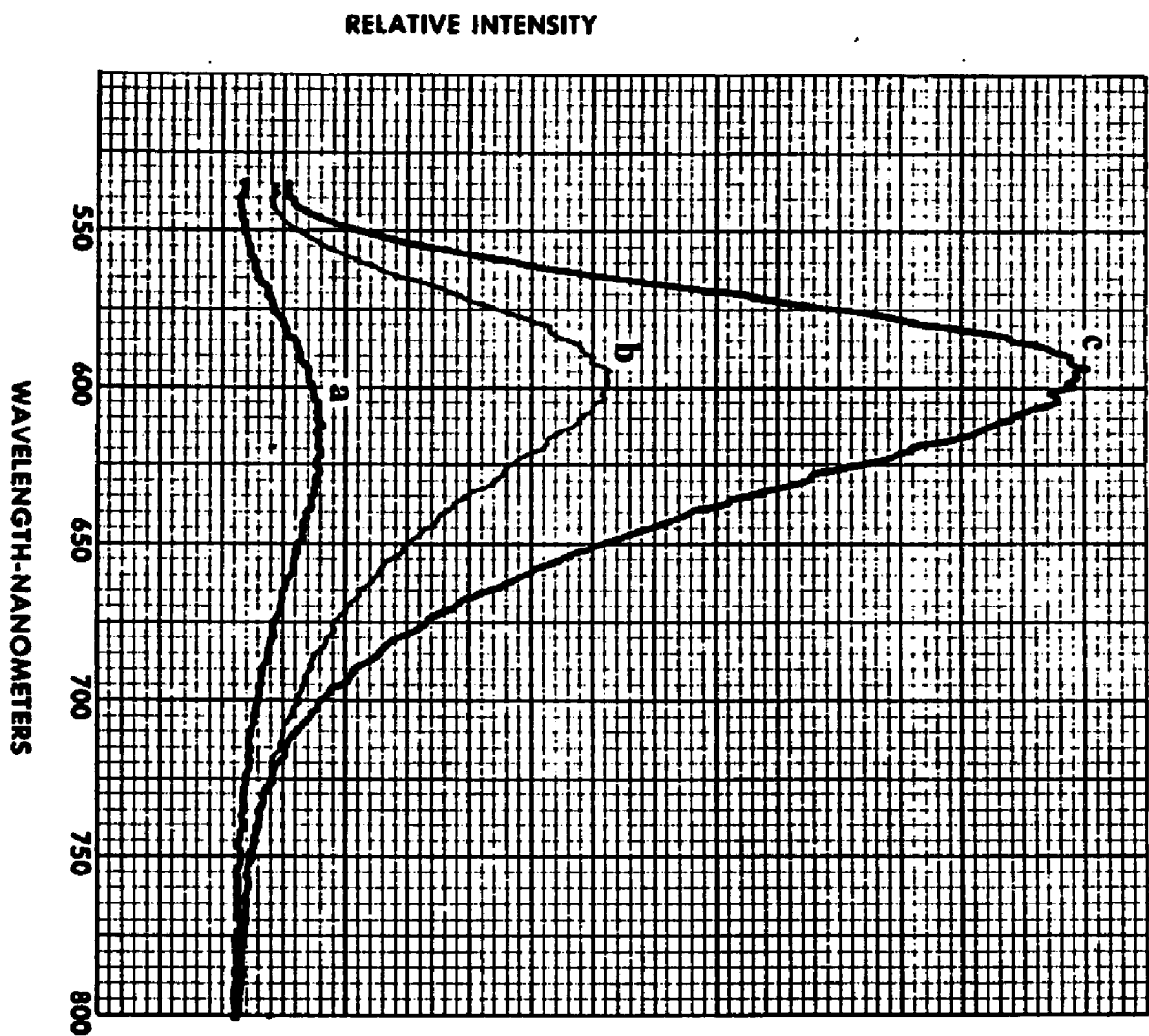
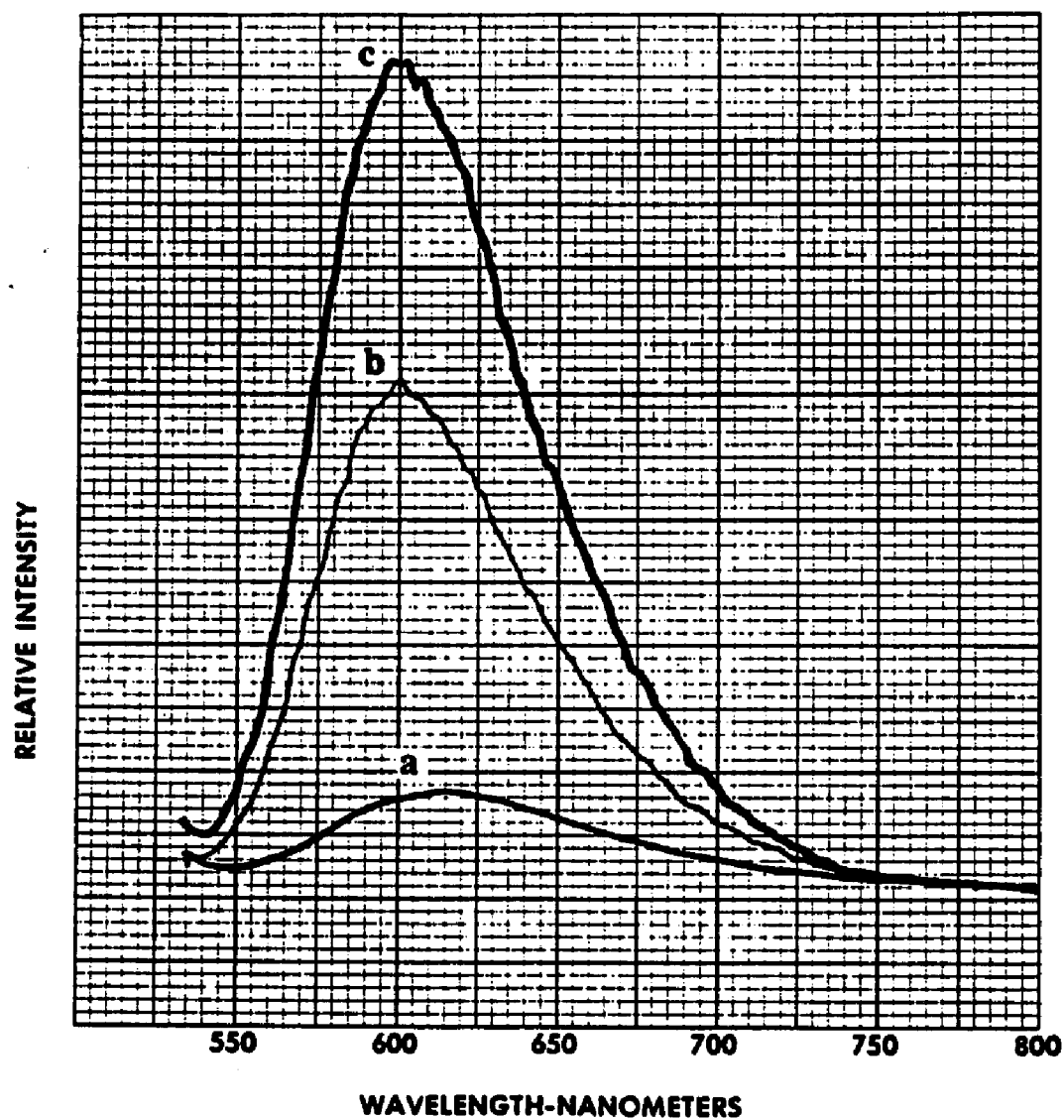
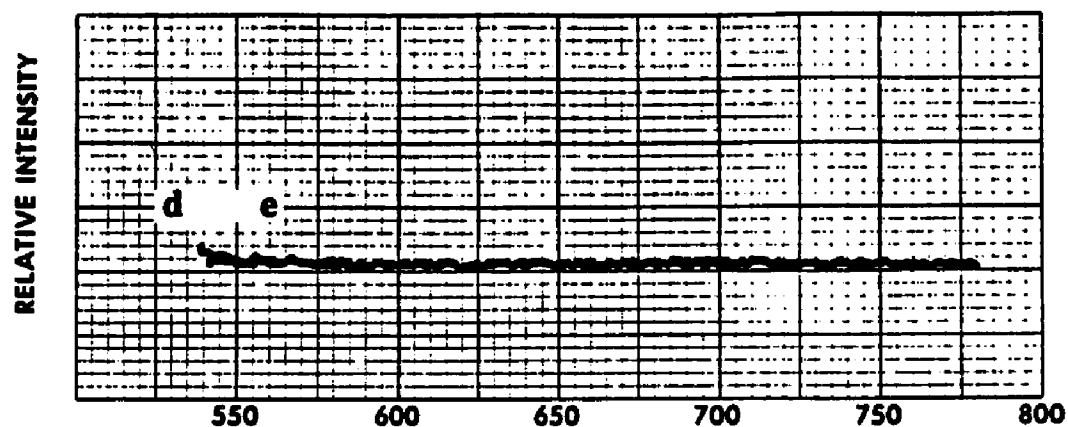


Figure. 5-7. The fluorescence spectra of (a). ethidium bromide (EB) alone, (b). ethidium bromide + Poly(dG-dC) • Poly(dG-dC) + N-MTAMPP, (c). ethidium bromide + Poly(dG-dC) • Poly(dG-dC) , (d). N-MTAMPP alone and (e). N-MTAMPP + Poly(dG-dC) • Poly(dG-dC) . The composition of solution concentrations for all of spectra: $[EB] = 3.46 \times 10^{-6} \text{ M}$, $[Poly(dG-dC) \bullet Poly(dG-dC)] = 4.28 \times 10^{-6} \text{ M}$, $[N-MTAMPP] = 4.76 \times 10^{-7} \text{ M}$.



very different.

If a porphyrin can bind competitively to nucleic acids against ethidium bromide, then the relative fluorescence intensity of ethidium bromide—nucleic acid solution will decrease if the porphyrin is added. The fluorescence intensity decrease can be used to evaluate K_{por} and M_{por} using equations (5-19) and (5-20). If the fluorescence intensity decreases to some extent due to energy transfer from ethidium bromide to porphyrin, the data treatment of equations (5-19) and (5-20) will provide misleading results. In this section an attempt will be made to determine if there is an energy transfer between bound ethidium bromide and the porphyrins.

Figures 5-2, 3 and 4 show the relative fluorescence emission spectra for ethidium bromide alone (a), ethidium bromide in DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) (b), ethidium bromide plus TAMPP with the corresponding nucleic acid (c), porphyrin TAMPP only (d), and TAMPP in the presence of DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) (e), respectively. Comparing the spectrum (b) (solution of EB + TAMPP + nucleic acid) with the spectra of (a) (solution of EB only), (c) (Solution of EB + nucleic acid), (d) (solution of TAMPP only) and (e) (solution of TAMPP + nucleic acid) in figures 5-2, 3 and 4, it is evident that there is no abnormal fluorescence increase at wavelength 640 nm, the position where TAMPP gives rise to its own fluorescence (figures 5-2d, 2e, 3d, 3e, 4d, and 4e): the spectra 5-2b, 3b and 4b are simply the addition of corresponding spectra a, c, d and e in different proportions. Similar but simpler phenomena occur with N-MTAMPP (figures 5-5b, 6b and 7b) since the bound and free N-MTAMPP show little fluorescence intensity (figures 5-5e, 5d, 6e, 6d, 7e and 7d). The solution composition used for all of spectra (b) were designed to be exactly the same as the one in the middle of the titration of ethidium bromide in the competition experiment,

conditions at which binding would be high. Therefore, no energy transfer from ethidium bromide to porphyrin is evident.

We can take advantage of the sensitive fluorescence intensity difference of bound and free ethidium bromide to determine the affinity constants for porphyrins with nucleic acid using an excitation wavelength at 520 nm and emission wavelength at 600 nm, because at these wavelengths no fluorescence is evident for either TAMPP or N-MTAMPP in its either bound or free forms.

5.3.4 Spectrofluorometric Determination of the Bound Concentration of Ethidium Bromide

The affinity constant K_{por} and the related parameter M_{por} can be obtained by linear regression analysis of the decrease in fluorescence intensity of ethidium bromide based on equations (5-19) and (5-20), when ethidium bromide and porphyrin coexist in solution with the nucleic acid.

The key task in using (5-19) and (5-20) is to derive the concentration of bound ethidium bromide ($[EB]_b$) since $[Por]_t$, $[NA]_t$ and $[EB]_t$ are directly determined experimentally.

If ethidium bromide is titrated into plain buffer, the fluorescence intensity I_0 of solution is linearly related to the accumulated titrated ethidium bromide concentration $[EB]_0$ by:

$$I_0 = k_f [EB]_0 \quad (5-23)$$

provided that the total concentration of titrated ethidium bromide is low and there are no self - quenching and self - absorption happened to the system. In equation (5-23), k_f is the fluorescence coefficient for free form of ethidium bromide and I_0 is the

corresponding fluorescence intensity. After corresponding amount of ethidium bromide (in concentration $[EB]_t$) is titrated in buffer with nucleic acid (for determining K_{EB} and n) or buffer with nucleic acid plus porphyrin (competition experiment for determining K_{Por} and M_{Por}), if I_t is defined as the total fluorescence intensity at the same concentration of ethidium bromide as the titration ethidium bromide into plain buffer (5-23), that is $[EB]_t = [EB]_o$ in each titration step (the total amount of ethidium bromide is equal in both with and without nucleic acid titration trials) using the same excitation and emission wavelengths, temperature and solvent as used in obtain I_o , and if, I_b and I_f are defined to be the fluorescence intensity contributed by bound and free ethidium bromide, and $[EB]_b$ and $[EB]_f$ are the corresponding concentrations, then the expression for I_t is:

$$I_t = I_b + I_f \quad (5-24)$$

$$[EB]_t = [EB]_o = [EB]_b + [EB]_f \quad (5-25)$$

and

$$I_f = k_f [EB]_f \quad (5-26)$$

$$I_b = k_b [EB]_b = \nu k_f [EB]_b \quad (5-27)$$

It should be noted that ν has been defined as:

$$\nu = k_b / k_f \quad (5-28)$$

a constant ratio. $[EB]_o$ in (5-25) is the total concentration of ethidium bromide in the titration trial without nucleic acid. $[EB]_t$, $[EB]_f$ and $[EB]_b$ in (5-25) are the total, free

and bound concentrations of ethidium bromide in the titration trial with nucleic acid, respectively. Combine equations (5-23), (5-24) and (5-25), we have

$$I_t = I_b + k_f [EB]_f \quad (5-29)$$

Replacing $[EB]_f$ by (5-25):

$$I_t = I_b + k_f ([EB]_t - [EB]_b) \quad (5-30)$$

and considering $[EB]_t = [EB]_o$ of the experimental condition used which is shown in (5-23) and (5-25):

$$I_t = I_b + k_f [EB]_o - k_f [EB]_b \quad (5-31)$$

Substitution of (5-23) and $[EB]_b$ derived from (5-27) into (5-31), give:

$$I_t = I_b + I_o - I_b / v \quad (5-32)$$

Reorganization of (5-32) leads to:

$$I_b = \frac{I_t - I_o}{1 - 1/v} \quad (5-33)$$

After substitution of (5-33) into (5-27), the concentration of bound ethidium bromide $[EB]_b$ can be expressed by detectable variables:

$$[\text{EB}]_b = \frac{I_b}{v k_f} = \frac{I_t - I_o}{(v-1) k_f} \quad (5-34)$$

in which v is derived from $v = k_b / k_f$, k_f and k_b are fluorescence coefficients for free and bound ethidium bromide as defined in equations (5-26) and (5-27), I_o is fluorescence intensity when only ethidium bromide is present in buffer, and I_t is the fluorescence intensity when ethidium bromide is added to buffer containing a nucleic acid or into a buffer containing porphyrin and nucleic acid.

5.4 Results and Experimental Approach

In order to obtain the K_{Por} (the affinity constant for the porphyrin—nucleic acid complex) and M_{Por} (the number of ethidium bromide binding sites needed for each bound porphyrin molecule on the nucleic acid) by the competition binding method using equations (5-19), (5-20) and (5-34), K_{EB} (the affinity constant of the ethidium bromide — nucleic acid complex) and n (number of ethidium bromide binding sites per nucleic acid base pair) must be determined by separate experiments first, treating the data using equations (5-18) and (5-34). A typical set of experimental data for the combination of ethidium bromide and calf thymus DNA and the results based on equations (5-18) and (5-34) are listed in Table (5-1) to show the details of experimental approach. Results for other ethidium bromide—nucleic acid combinations are listed in appendixes (6-1), (7-1) and (8-1).

Columns (1), (2) and (3) of Table (5-1) are measured experimental data. The corresponding experimental conditions (solution compositions before titration) are given above each column. $I_o(\text{EB})$ values in column (2) are the relative fluorescence intensities recorded after each addition of ethidium bromide in plain buffer (the accumulated volume of ethidium bromide titrated is shown in column (1)). Values of $I_t(\text{EB-DNA})$ in

Table (5-1)

Data for Affinity Constant Study of EB — CT.DNA Complex

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.9 ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(DNA)[DNA]}{V(tol)}$	$\frac{I_t - I_o}{(I_o - 1)k}$	$\frac{[EB]_t - [EB]_b}{[DNA]_t}$	$\frac{[EB]_t}{[DNA]_t}$	$\frac{[EB]_b}{[EB]_t [DNA]_t}$
EB in ml	V(DNA)=0 ml	V(DNA)=0.1 ml						
V(EB)	$I_o(EB)$	$I_t(EB-DNA)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_t(10^{-6})$	$X(10^{-1})$	$Y(10^4)$
0.01	1.8	18.4	0.71	4.49	3.26	0.38	0.73	10.3
0.02	3.5	30.7	1.41	4.47	5.33	0.88	1.19	8.48
0.03	5.3	39.6	2.10	4.45	6.73	1.43	1.51	7.21
0.04	7.1	46.5	2.78	4.42	7.72	2.01	1.74	6.27
0.05	8.8	52.0	3.46	4.40	8.46	2.61	1.92	5.55
0.06	10.6	56.8	4.14	4.38	9.05	3.23	2.07	4.99
0.07	12.2	60.9	4.80	4.36	9.54	3.85	2.19	4.56
0.08	14.0	64.3	5.46	4.34	9.85	4.47	2.27	4.16
0.09	15.6	67.5	6.11	4.32	1.02	5.09	2.35	3.85
0.10	17.1	70.1	6.76	4.30	1.04	5.72	2.42	3.57

The concentration of stock solution: $[EB]_s = 1.42 \times 10^{-4}$ M, $[DNA]_s = 9.03 \times 10^{-5}$ M

Buffer composition: 0.05 M (acetic acid + sodium acetate), 0.1 M NaCl, pH=5.5

$V(EB-DNA) = 21.1$; $k = 2.55 \times 10^6$

Linear regression results: $K = 3.97 \times 10^5$, $\Pi = 0.33$. ($r = 0.999$)

column (3) are recorded relative fluorescence intensities after the corresponding amount of ethidium bromide as in column (2) was titrated in buffer with DNA present.

Columns (4) to (9) are results derived from experimental data shown in columns (1) to (3) and the corresponding relevant equations are shown above of each column. $[EB]_s$ and $[DNA]_s$, the stock concentrations of ethidium bromide and DNA used in this work, are given at the bottom of the table. $V(EB)$, $V(DNA)$ and $V(BUF)$ given in column (1) and above of columns (2) and (3), are the accumulated volumes of ethidium bromide titrated and the volumes of DNA and buffer used, respectively. $V(tol)$ is the total reaction volume, the summation of buffer volume, volume of DNA and the accumulated titration volume of ethidium bromide. The parameter v is defined as k_b / k_f by equation (5-28) and is a constant for a specific ethidium bromide — nucleic acid system for a specific excitation and emission wavelength. In order to obtain k_b , the fluorescence constant of bound ethidium bromide, a separate experiment was conducted. Ethidium bromide was titrated into a large excess of nucleic acid solution ($[DNA]_t / [EB]_t \geq 100$). If I_t versus $[EB]_t$ is plotted and the plot is linear, the slope of the straight line will be k_b (otherwise, the amount of nucleic acid must be increased). The term k_f is the fluorescence coefficient of unbound (free) ethidium bromide and is obtained by titration of ethidium bromide in plain buffer.

The values of $[EB]_b / [DNA]_t$ and $[EB]_b / [DNA]_t [EB]_f$ in equation (5-18) are obtained from the appropriate calculation and shown in the last two columns in Table (5-1) as X and Y, respectively. The linearity of the plot of X vs. Y is evident in figure (5-8). Based on Scatchard's equation (5-18), the negative slope gives the affinity constant K_{EB} which is 3.97×10^5 for the ethidium bromide — DNA complex and its abscissa intercept gives the parameter n, the number of binding sites per nucleic acid base pair, which is 0.33 for EB — DNA complex. Therefore, one bound ethidium bromide molecule needs 3 DNA base pairs ($1 / n = 3$).

Figure 5-8. The Scatchard plot of table 5-1: $[EB]_b / [EB]_f \cdot [DNA]_t$ versus $[EB]_b / [DNA]_t$ for titration of ethidium bromide into buffer with DNA present.

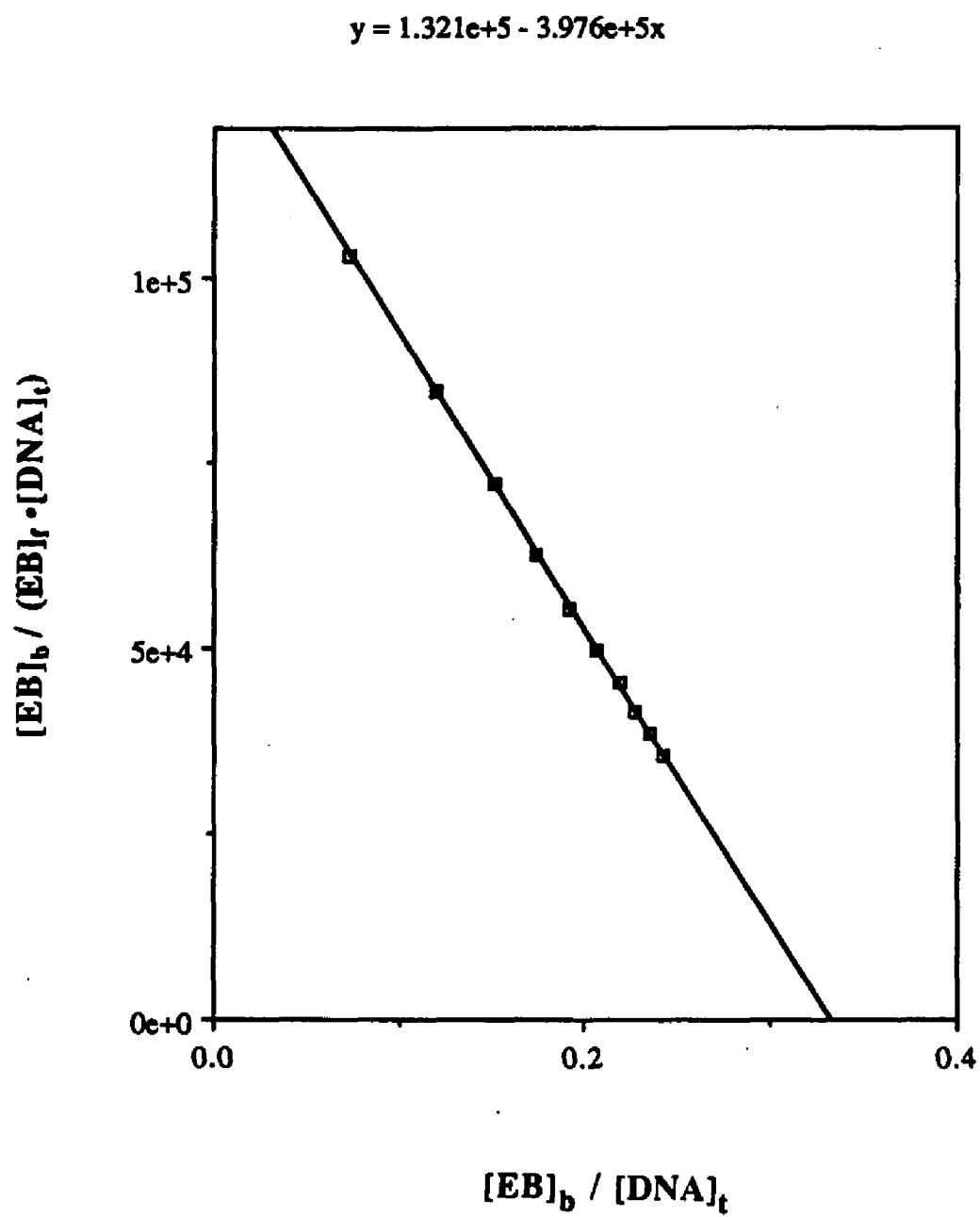


Table (5-2) lists one typical group of experimental data set for deriving the affinity constant K_{Por} and the parameter M_{Por} for the TAMPP — DNA complex. It is composed of results from two trials also. First, ethidium bromide (the cumulative titration volume is shown in column (1)) is added into a buffer solution, giving a series of $I_o(EB)$ values shown in column (2); Second, ethidium bromide was titrated into a buffer containing DNA and TAMPP, giving a series of $I_t(EB-DNA-TAMPP)$ values shown in column (3). The total volumes ($V(tol)$) must be kept the same for each corresponding titration step for both trials. Table (5-2) is organized according to equations (5-19), (5-20) and (5-34) similar to the arrangement of table (5-1). The solution composition of the trials and relevant equations used are given on top of the appropriate column. Two new variables shown in table (5-2) not used in table (5-1), are $[Por]_t$, the total concentration of porphyrin and $[BS]_{Por}$, the number of binding sites occupied by porphyrin molecules, which are defined by equation (5-20). The ratio $[Por]_t / [BS]_{Por}$ is given as X and the ratio $[EB]_t / [EB]_b$ as Y in Table (5-2). These ratios are defined by equation (5-19) and have a different meaning from the X and Y shown in table (5-1) (defined by equation (5-18)). All other variables shown in Table (5-2) have the same meaning as in Table (5-1). Plotting Y ($[EB]_t / [EB]_b$) versus X ($[Por]_t / [BS]_{Por}$), gives the straight line shown in figure (5-9). For the TAMPP — DNA complex, the average affinity constant K_{Por} from four trials is 4.4×10^6 and the average parameter M_{Por} , the number of binding sites needed by one TAMPP molecule, is 3 based on equations (5-21) and (5-22). Therefore one bound TAMPP molecule takes 9 DNA base pairs (M_{Por} / n), or more accurately, every nine base pairs of DNA will be inaccessible after one TAMPP molecule bind to DNA.

Additional data and results for the TAMPP-DNA system are shown in appendix (6-2). The experimental data and calculated values for TAMPP — Poly(dA-dT) • Poly(dA-dT) and TAMPP — Poly(dG-dC) • Poly(dG-dC) complexes are shown in

Table (5-2)

Data for Affinity Constant Study of TAMPP — CT.DNA Complex

1	2	3	4	5	6	7	8	9	10	11
Titrated	V(Buf)=2.0 ml V(DNA)=0 ml	V(Buf)=1.83 ml V(DNA)=0.1 ml	$\frac{V(EB) \times [EB]_s}{V(tot)}$	$\frac{V(DNA) \times [DNA]}{V(tot)}$	$\frac{I_0 - I}{(I_0 - I)k}$	$[EB]_i - [EB]_b$	$\frac{V(Por) \times [Por]}{V(tot)}$	$\frac{n[DNA]_i - [EB]_b}{[EB]_b / K_{EB} [EB]_i}$	$\frac{[Por]_i}{[BS]_{Por}}$	$\frac{[EB]_i}{[EB]_b}$
EB in ml	V(TAP)=0 ml	V(TAP)=0.07 ml	V(tot)	V(tot)	(I-I)k					
V(EB)	I ₀ (EB)	I _i (EB-DNA-TAP)	[EB] _i (10 ⁻⁶)	[DNA] _i (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁷)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	1.6	6.6	0.71	4.49	1.15	.59	4.40	8.77	0.502	5.14
0.02	3.1	12.2	1.41	4.47	2.09	1.20	4.38	8.25	0.531	5.72
0.03	4.6	16.9	2.10	4.45	2.83	1.82	4.36	7.92	0.550	6.42
0.04	6.10	21.0	2.78	4.42	3.43	2.44	4.34	7.64	0.568	7.12
0.05	7.5	24.7	3.46	4.40	3.96	3.07	4.32	7.32	0.589	7.75
0.06	8.9	28.5	4.14	4.38	4.51	3.69	4.30	6.87	0.625	8.17
0.07	10.4	31.7	4.8	4.36	4.90	4.31	4.27	6.63	0.645	8.80
0.08	11.8	34.6	5.46	4.34	5.24	4.94	4.25	6.40	0.665	9.41
0.09	13.2	37.3	6.11	4.32	5.54	5.56	4.23	6.20	0.683	10.03
0.10	14.6	40.0	6.76	4.30	5.84	6.18	4.21	5.96	0.707	10.57

The concentration of stock solution: [EB]_s=1.42x10⁻⁴ M, [DNA]_s=9.03x10⁻⁵ M, [TAMPP]_s=1.264x10⁻⁵ M.

Buffer composition: 0.05 M (acetic acid + sodium acetate), 0.1 M NaCl, pH=5.5

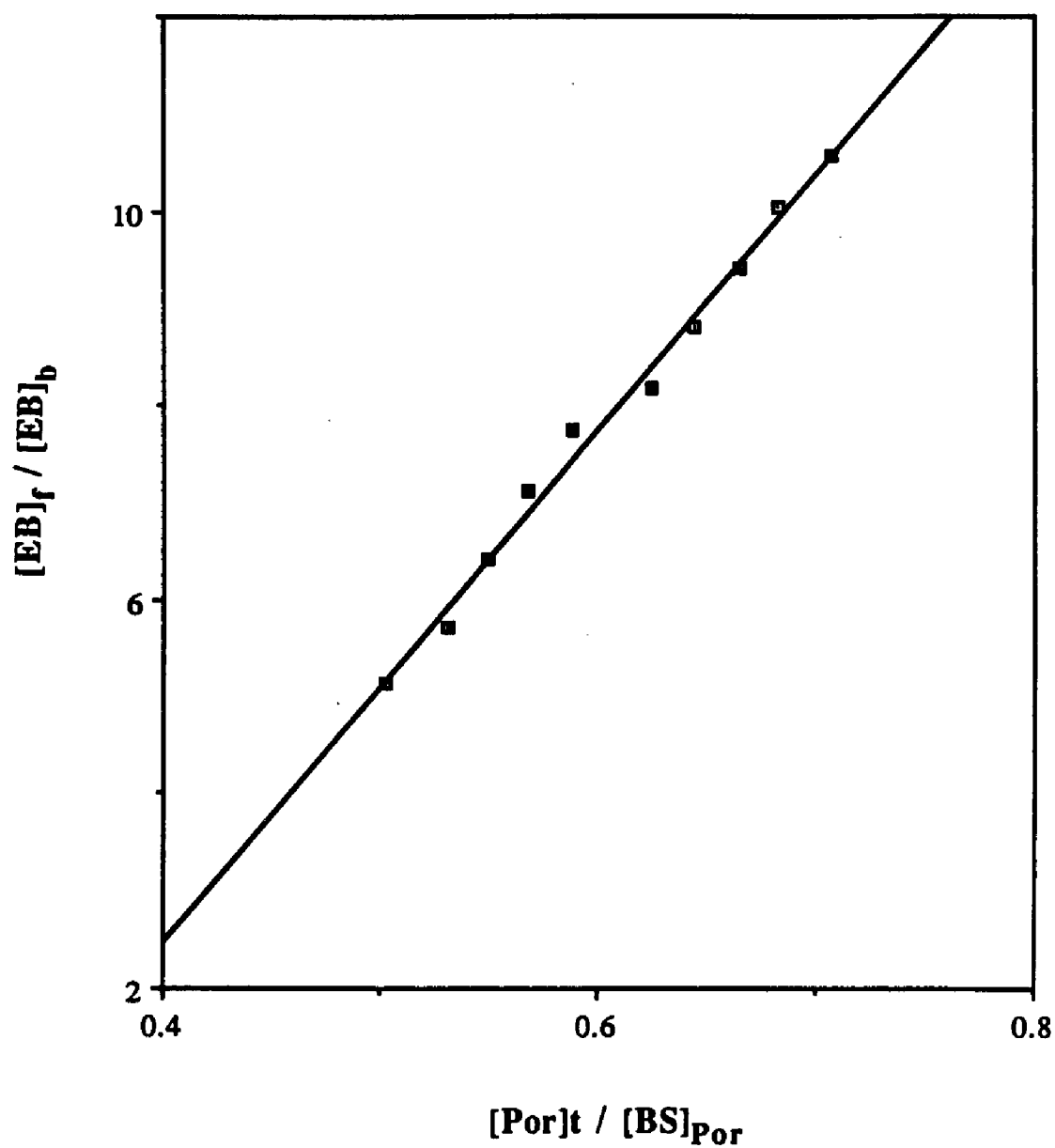
V(EB-DNA)=21.1; k=2.16x10⁶

Note: pK₃ and pK₄ values of TAMPP have been changed and TAMPP is mostly in its diprotonated state after TAMPP bind outside to DNA (see the discussion on chapter 4).

Linear regression results: K=3.2x10⁶, M=3.3. (r=0.996)

Figure 5-9. The plot of table 5-2: $[\text{EB}]_f / [\text{EB}]_b$ versus $[\text{Por}]_t / [\text{BS}]_{\text{por}}$ for titration of ethidium bromide into buffer with DNA and TAMPP present.

$$y = -8.0789 + 26.3676x$$



appendixes (7-2) and (8-2). The data and results for N-MTAMPP — DNA, N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) and N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) complexes are given in appendixes (6-3), (7-3) and (8-3), respectively.

Linear regression results of the all six porphyrin — nucleic acid complexes, each derived from four trials, are summarized in table (5-3) for TAMPP and table (5-4) for N-MTAMPP. The term r is the relative coefficient of linear regression. It can be seen that a good linear relationship for each trial and reproducibility among trials was obtained for these porphyrin-nucleic acid systems.

In order to get reliable results, several points must be considered in performing the experiments. First, the ratio of $[EB]_t / [NA]_t$ in the experiment to determine K_{EB} and n must be the same as that in the experiment to determine K_{Por} and M_{Por} . Otherwise, the linear regression analysis will not give a satisfactory result. If there is more than one binding site on the nucleic acid (which has been noted previously¹⁶), K_{EB} and n will be different for different ratios of $[EB]_t / [NA]_t$. The $[EB]_t / [DNA]_t$ ratio spans a range from 0.16 to 1.6 (between first and last titration points) for all the titration experiments in this work. Second, the total concentration of porphyrin cannot be too big or too small compared with ethidium bromide. The binding ability of a porphyrin and ethidium bromide with a nucleic acid should match each other when the competitive method is used to determine K_{Por} and M_{Por} . A large error will be introduced if one molecule does not have the ability to compete with the other to bind to the nucleic acid. Their competitive ability not only depends on their affinities for the nucleic acid (K_{EB} and K_{Por}) but also on their concentration ratio ($[EB]_t / [Por]_t$). Therefore, the concentration of porphyrin should be adjusted until its competitive strength is close to that of ethidium bromide, that is, until a linear relationship and reproducible results are obtained. Thirdly, the pair of titration trials, that is, titration of ethidium bromide into plain buffer and into buffer containing a nucleic acid (for determining K_{EB} value, columns 2 and 3 in

Table (5-3)**Affinity Constants of TAMPP — Nucleic Acid Complexes**

Nucleic acid	$K_{EB}(10^5)$	n	$K_{Por}(10^6)$	M_{Por}	r
CT.DNA ($\nu=21.1$)	3.97	0.33	3.21	3.26	0.996
			3.78	3.24	0.974
			6.55	2.57	0.984
			4.07	3.04	0.970
Poly(dA-dT) ($\nu=24.2$)	2.77	0.72	2.16	5.93	0.996
			1.57	5.88	0.992
			3.09	5.86	0.996
			2.68	6.21	0.998
Poly(dG-dC) ($\nu=16.8$)	3.88	0.42	4.12	0.95	0.963
			4.07	0.97	0.985
			3.18	1.24	0.941
			6.01	1.12	0.962

Table (5-4)**Affinity Constants of N-MTAMPP — Nucleic Acid Complexes**

Nucleic acid	$K_{EB}(10^5)$	n	$K_{Por}(10^6)$	M_{Por}	r
CT.DNA ($\nu=21.1$)	3.97	0.33	1.56	5.67	0.975
			2.28	5.75	0.974
			1.97	6.23	0.991
			3.11	5.57	0.997
Poly(dA-dT) ($\nu=24.2$)	2.77	0.72	2.33	9.92	0.978
			2.79	12.49	0.991
			3.14	13.57	0.978
			2.64	14.33	0.957
Poly(dG-dC) ($\nu=16.8$)	3.88	0.42	1.25	1.48	0.923
			2.24	1.49	0.980
			0.85	1.65	0.972
			2.68	1.04	0.903
			1.71	1.42	0.905

Table (5-1) or into plain buffer and into buffer containing a nucleic acid and a porphyrin titration pair (for determining K_{por} value, columns 2 and 3 in Table 5-2), must be worked in same reference photomultiplier tubes (PMT) votage value (except for those conditions mentioned in section (5-2)). The fluorescence intensity is dependent on the value of Reference PMT voltage. Thus, if both I_0 and I_t (the fluorescence intensity of ethidium bromide in plain buffer and buffer with a nucleic acid present or buffer containing nucleic acid as well as a porphyrin (in Tables (5-1) and (5-2))), are recorded in same reference PMT voltage, the concentration of bound ethidium bromide, $[EB]_b$, and the resulting K_{EB} or K_{por} values in equation (5-19) will be independent of the reference PMT voltage. Therefore, a pair of titrations should be finished while the reference PMT voltage value remains stable. Finally, the thermostated temperature of the cell compartment should be very close to room temperature. In that way, the amount of porphyrin bound on nucleic acids before ethidium bromide is added is stable (K_{por} depends on temperature). In addition, the titration can be finished in a short time for several related trials. Otherwise, it takes some time to reach thermal equilibrium after each addition of ethidium bromide.

5.5 Discussion

The average K_{por} and M_{por} values for the six porphyrin—nucleic acid complexes are summarized in Table (5-5). The corresponding uncertainties are specified as the average deviation from the mean.

It can be seen that all six porphyrin — nucleic acid systems give large and similar affinity constants. The differences between them are small, within the range of reproducibility of measurement. In contrast, there is quite a large difference in M_{por} values for different porphyrins and nucleic acid complexes.

Table (5-5)**Affinity Constants of Porphyrin — Nucleic Acid Complexes**

Nucleic acid	$K_{EB}(10^5)$	n	Porphyrin	$K_{Por}(10^6)$	M_{Por}
CT.DNA	3.97	0.33	TAMPP	4.4($\pm$ 1.1)	3.0($\pm$ 0.2)
			N-MTAMPP	2.2($\pm$ 0.5)	5.8($\pm$ 0.2)
Poly(dA-dT)	2.77	0.72	TAMPP	2.4($\pm$ 0.5)	6.0($\pm$ 0.1)
			N-MTAMPP	2.7($\pm$ 0.3)	12.6($\pm$ 1.4)
Poly(dG-dC)	3.88	0.42	TAMPP	4.3($\pm$ 0.8)	1.1($\pm$ 0.1)
			N-MTAMPP	1.7($\pm$ 0.6)	1.4($\pm$ 0.2)

Table (5-6)**Number of Nucleic Acid Base Pairs per Bound Drug (M/n)**

Drug	CT.DNA	Poly(dA-dT)	Poly(dG-dC)
EB	3.0	1.4	2.4
TAMPP	9.1	8.3	2.6
N-MTAMPP	17.6	17.5	3.3

It is interesting to see that the parameter M_{por} changes a great deal for different porphyrin — nucleic acid systems. First, the values of M_{por} are much larger for porphyrin — CT.DNA and porphyrin — Poly(dA-dT) • Poly(dA-dT) systems than that for porphyrin — Poly(dG-dC) • Poly(dG-dC) systems. Ethidium bromide is a well-known intercalator into nucleic acids⁷⁻¹². The larger M_{por} means that a porphyrin will require more ethidium bromide binding sites when it interacts with nucleic acids. Therefore, the larger M_{por} value implies that the interaction between the porphyrin and CT.DNA or Poly(dA-dT) • Poly(dA-dT) is of a different manner than that of ethidium bromide with CT.DNA or Poly(dA-dT) • Poly(dA-dT). The most reasonable possibility is TAMPP and N-MTAMPP binds at the outside of CT.DNA and Poly(dA-dT) • Poly(dA-dT) (they do not intercalate). In that way, a porphyrin can cover more nucleic acid base pairs and/or distort the helical structure of nucleic acid (see below) to prevent ethidium bromide from intercalating. The results shown in chapter (4) confirm that TAMPP and N-MTAMPP bind on the outside of CT.DNA and Poly(dA-dT) • Poly(dA-dT) instead of intercalating.

Second, based on the M_{por} values, when interacting with CT.DNA and Poly(dA-dT) • Poly(dA-dT), the N-methylated porphyrin seems to cover twice the amount of nucleic acid base pairs as the non-N-methylated form TAMPP. The two possibilities of outside binding are insertion into a groove of the nucleic acids and electrostatic attraction between the positively charged porphyrin and the negatively charged surface of nucleic acids. Analyzing DNA's structure (figure 5-10), it seems that by inserting into the major groove of DNA, the porphyrin can cover more nucleic acid base pairs and more efficiently prevent ethidium bromide from binding to DNA compared with binding by electrostatic attraction to the outside of DNA. Therefore, the larger M_{por} values for N-MTAMPP — DNA and Poly(dA-dT) • Poly(dA-dT) complexes imply that N-MTAMPP may insert into major groove of DNA. The

relatively smaller M_{por} values for TAMPP with CT.DNA and Poly(dA-dT) • Poly(dA-dT) complexes imply that TAMPP may bind on the outside of the negatively charged phosphate groups of DNA. This is consistent with the results shown in chapter (4).

Third, both TAMPP — Poly(dG-dC) • Poly(dG-dC) and N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) complexes give M_{por} values close to one. This implies that these two porphyrins take almost the same number of nucleic acid base pairs and bind in a manner similar to ethidium bromide when they interact with Poly(dG-dC) • Poly(dG-dC). That is, TAMPP and N-MTAMPP can intercalate into Poly(dG-dC) • Poly(dG-dC). This is also the conclusion derived from the induced circular dichroism experiments.

Fourth, from the results of circular dichroism, visible absorption and fluorescence spectra, it is known that TAMPP and N-MTAMPP interact mostly on the AT regions of DNA. Table (5-6) is worked out from the table (5-5) and shows the number of nucleic acid base pairs required for each bound drug. It can be seen that TAMPP occupies a similar number of nucleic acid base pairs when it interacts with CT.DNA and Poly(dA-dT) • Poly(dA-dT). Similar experimental results are shown also for N-MTAMPP — DNA and Poly(dA-dT) • Poly(dA-dT) systems. This phenomenon means that when TAMPP and N-MTAMPP interact with DNA, they seem mostly to interact to AT regions of DNA. These results correlate well with the results derived from the circular dichroism, visible absorption and fluorescence spectra.

Finally, the large M_{por} values mean that each porphyrin molecule interacts with more nucleic acid base pairs than ethidium bromide does. A porphyrin with a larger M_{por} value may show a larger induced circular dichroism ellipticity when it is mixed with a nucleic acid (the ellipticity also depends to some extent on geometry and to a large extent on the distance to the chiral center). The smaller M_{por} value means that fewer nucleic acid pairs interact with each porphyrin molecule and the corresponding induced

circular dichroism spectrum of the porphyrin — nucleic acid mixture should exhibit a lower ellipticity. Comparing the circular dichroism spectra in chapter (4), it is true that porphyrin — DNA and porphyrin — Poly(dA-dT) • Poly(dA-dT) systems with larger M_{por} values have higher induced circular dichroism band ellipticities than that of the same porphyrin with Poly(dG-dC) • Poly(dG-dC) (which gives a smaller M_{por}).

Based on the discussion above , a great deal of information can be derived from the parameter M_{por} . The facts disclosed by M_{por} are confirmed well by the results from circular dichroism, absorption and fluorescence spectra. Therefore, the parameter M_{por} introduced in this chapter is important.

What should be noticed is that the number of nucleic acid base pairs needed by each bound drug (M_{Drug} / n) in table (5-6) is not the number of nucleic acid base pairs in physical contact with each bound drug. Both parameters M_{por} and n_{EB} are a kind of averaged, or apparent value. For example, it takes three nucleic acid base pairs for each bound ethidium bromide, based on its n_{EB} . But this result does not mean that each ethidium bromide molecule will exactly cover three nucleic acid base pairs physically, but means that every three nucleic acid base pairs are inaccessible on DNA by binding one ethidium bromide molecule. The parameter n_{EB} has already averaged the nucleic acid base pairs covered directly by a ethidium bromide molecule and the gap segment between each bound ethidium bromide. The structure of DNA does not allow a very high binding density because each molecule that binds distorts the helix of the nucleic acid to some extent. Similarly, the " M_{por} / n " values for TAMPP and N-MTAMPP in Table (5-6) are not the number of nucleic acid base pairs physically covered by each porphyrin molecule. Instead, every " M_{por} / n " nucleic acid base pairs can hold one porphyrin molecule or the number of " M_{por} / n " nucleic acid base pairs will not accessible because of binding one porphyrin molecule. Therefore, the larger " M_{por} / n_{EB} " for porphyrin-CT.DNA and Poly(dA-dT) • Poly(dA-dT) complexes imply that the

binding density is lower for them than for porphyrin — Poly(dG-dC) • Poly(dG-dC) systems.

A tentative conclusion from $M_{\text{Por}} / n_{\text{EB}}$ values in Table (5-6) is that if each major groove is composed of about 6 nucleic acid base pairs and each narrow groove is composed of about 3 nucleic acid base pairs (every periodic repeat in DNA is composed of one major and one narrow groove containing 10 nucleic acid base pairs in total), then (1) one N-MTAMPP molecule may be insert into one major groove of DNA and cause the two adjacent grooves to be deformed. Thus, N-MTAMPP can insert into alternate major grooves of DNA (figure 5-10) and about every 18 nucleic acid base pairs on DNA bind one N-MTAMPP molecule. This may be the reason for the $M_{\text{Por}} / n_{\text{EB}}$ experimental value of N-MTAMPP (17.6) shown in table (5-6) when N-MTAMPP interacts with DNA. (2) one TAMPP molecule may bridge two strands of DNA at the narrow groove interacting at the same time with two adjacent negatively charged phosphate groups (figure 5-11). In that manner of electrostatic attraction, TAMPP binds stably on the DNA surface. Therefore, about every 9 nucleic acid base pairs bind one TAMPP molecule. This interpretation would explain the experimental value of $M_{\text{Por}} / n_{\text{EB}}$ (9.1) for TAMPP binding to DNA. It can be seen that both of the binding interactions discussed for N-MTAMPP and TAMPP with DNA can efficiently prevent ethidium bromide from intercalating into nucleic acid base pairs.

It will be interesting to determine the influence of the ionic strength on N-MTAMPP — DNA and TAMPP — DNA systems in futher studies. Such studies might disclose some useful information about the degree of electrostatic interaction of the binding systems ¹⁷.

Figure. 5-10. The possible binding manner of N-MTAMPP on DNA: N-MTAMPP inserted into alternate major groove of DNA.

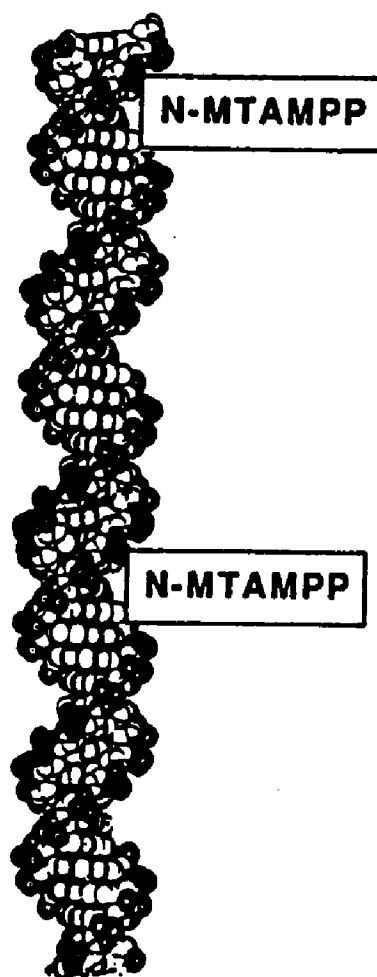
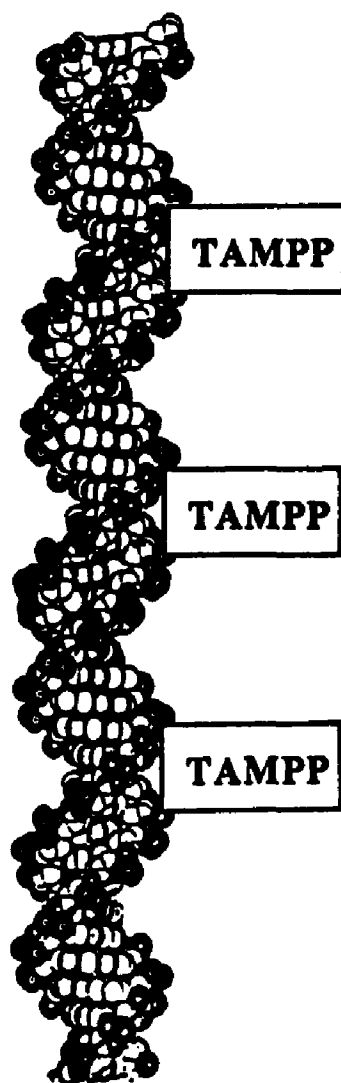


**Figure. 5-11. The possible binding manner of TAMPP on DNA:
TAMPP bridge two strands of DNA at the position of narrow
groove.**



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Chapter 6

The Synthesis and Interaction Studies of Mn(III)•TAMPP and Co(II)•N-MTAMPP with Nucleic Acids

6.1 Introduction

Some metalloporphyrins have been investigated as DNA cleavage reagents ^{1,2}. The most extensively studied porphyrin derivatives are certain water - soluble, cationic metalloporphyrins. It has been found that they can interact with some natural nucleic acids, such as calf thymus DNA (CT.DNA) and some synthetic polynucleotides, such as Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) ^{1,3}.

For the non - methylated water - soluble metalloporphyrins, most studies have involved metal complexes of tetrakis(4-N-methylpyridyl)porphine ^{1, 3-6}. It was observed that the mode of binding and the base preference of the metalloporphyrins are strongly influenced by the porphyrin structure, the coordinated metal ion and the nature of the nucleic acid. Pasternack, Ward and coworkers reported that those metalloporphyrins which are planar and have no axial bound ligands, such as Ni(II) and Cu(II)-TMPyP-4, can intercalate in GC rich regions of DNA and those metalloporphyrins which are nonplanar and have axially bound ligands, such as Mn(III), Fe(III), Co(III) and Zn(II) - TMPyP-4, can not intercalate, but instead bind on the outside of AT rich regions of DNA ^{1,3}. It was also observed that in the presence of some kind of activators, such as potassium superoxide, Mn(III), Fe(III) and Co(III) - TMPyP-4 can cleave DNA ¹. Bromley ² examined the DNA cleavage specificity of a series of outside binding cationic porphyrin complexes of manganese in the presence of potassium superoxide and found that changes in total charge and the location of charge on the framework of meso-substituent of porphyrin do not significantly influence

binding specificity. However, the studies showed the importance of ligand charge and shape on DNA sequence recognition.

The studies of the interaction of N-methylated water-soluble metalloporphyrins with polynucleic acids, a very interesting project, has not yet been reported because of the difficulty of synthesis as stated previously.

DNA strand scission by the non-N-methylated water-soluble metalloporphyrins which can bind on the outside of DNA may be very useful in identifying the interaction sites of DNA with other binding ligands (drugs or proteins, for example) and in uncovering certain elements of DNA structure and conformation ⁷⁻⁹. The N-methylated metalloporphyrin can lose its N-substituent when strong and efficient nucleophiles coexist in solution. This specific property may permit the site specific selective alkylation of DNA bases if the water-soluble N-methylated metalloporphyrins can bind to DNA with the assistance of some "DNA anchor" reagent, such as acridine. The nitrogenous atom of a base on DNA will be the nucleophile in this case. The attraction of this nucleophile for the N-methylated nitrogenous atom of pyrrolic group of the porphyrin is expected to be efficient because they would be very close each other when the porphyrin binds to DNA.

In this chapter, the synthesis of manganese (III) tetrakis(p-aminomethylphenyl)porphyrin (Mn(III)-TAMPP) and cobalt (II) N-methyl tetrakis(p-aminomethylphenyl)porphyrin (Co(II)-N-MTAMPP) and studies of interaction of these water-soluble metalloporphyrins with DNA, Poly(dA-dT) • Poly(dA-dT) and Poly(dG-dC) • Poly(dG-dC) are reported. These results provide a useful basis for further studies of DNA scission and DNA specific alkylation.

6.2 Materials and methods

6.2.1 Materials

Manganese (II) acetate tetrahydrate ($(\text{CH}_3\text{CO}_2)_2\text{Mn}\cdot 4\text{H}_2\text{O}$) and anhydrous cobalt chloride (CoCl_2) were purchased from Aldrich Chemical Company, Inc. Absolute ethyl alcohol was purchased from AAPER Alcohol and Chemical Co. All these chemicals are reagent grade and were used without further purification. Water-soluble non-N-methylated tetrakis(p-aminomethylphenyl)porphyrin (TAMPP) and N-methyl tetrakis(p-aminomethylphenyl)porphyrin (N-MTAMPP), which were synthesized as reported in Chapter (2), were used as the starting materials for synthesizing two metalloporphyrins, $\text{Mn(III)}\cdot\text{TAMPP}$ and $\text{Co(II)}\cdot\text{N-MTAMPP}$.

Calf thymus DNA, $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ and $\text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ were purchased from Sigma Chemical Co. Calf thymus DNA was purified as previously described by Pasternack ² with slight modifications given in chapter (4). $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ and $\text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ were used directly without further purification. Ethidium bromide was purchased from Aldrich Chemical Co. Inc. All other chemicals were reagent grade and used without further purification. All experiments were carried out at 25 °C using a pH 5.5 acetic acid buffer containing 0.05M of acetic acid and sodium acetate in total and 0.1 M sodium chloride for the studies of $\text{Mn(III)}\cdot\text{TAMPP}$ — nucleic acid systems or a pH 7.4 tris buffer containing 0.05M of tris and 0.1 M sodium chloride for the studies of $\text{Co(II)}\cdot\text{N-MTAMPP}$ — nucleic acid systems. The integrity of the nucleic acid duplexes was determined by comparing their ultraviolet spectra with published spectra ^{10, 11, 12}. Concentrations, always calculated in base pairs, were determined spectrophotometrically with $\epsilon_{260} = 1.31 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for calf thymus DNA ¹⁰, $\epsilon_{262} = 1.32 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ ¹¹ and $\epsilon_{254} = 1.68 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for $\text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ ¹².

6.2.2 Synthesis of metalloporphyrins

6.2.2a Synthesis of Mn(III)•TAMPP

Mn(III)•TAMPP was synthesized *in situ*. The non-N-methylated porphyrin TAMPP was dissolved in pH 5.5 acetic acid buffer (0.05M of acetic acid and sodium acetate in total and 0.1 M sodium chloride) at a concentration of less than 5×10^{-5} M and then the solution was warmed to reflux. Equimolar $(\text{CH}_3\text{CO}_2)_2\text{Mn} \cdot 4\text{H}_2\text{O}$ which was dissolved in the same pH 5.5 buffer first, was added dropwise. The reaction system was refluxed for 24 hours. The Mn(III)•TAMPP derived in this manner was directly used in the studies of Mn(III)•TAMPP — nucleic acid systems.

Figure 6-1b is the Soret band of newly synthesized Mn(III)•TAMPP in pH 5.5 acetic acid buffer solution. Compared with figure 6-1a, the Soret band of TAMPP in the same pH 5.5 buffer solution, a new prominent peak appears at a wavelength of 467 nm, which is the position typical of manganese (III) derivatives of tetraarylporphyrins ¹³. Figure 6-2 shows the fluorescence spectrum of starting reactant TAMPP (a), and its manganese product (b) in pH 5.5 buffer solution. It is quite clear that TAMPP reacted almost completely with manganese because it is a typical characteristic of the paramagnetic metalloporphyrins that there is no fluorescence ¹⁴. This test is much more sensitive than the change in the visible spectrum as the metalloporphyrin forms (as little as 0.1% unreacted porphyrin is typically detectable from its fluorescence). It is used to determine if a metal complexation reaction is complete. The more important finding is that this is the first time that a manganese (III) porphyrin complex has been simply synthesized in an aqueous medium.

The extinction coefficient of Mn(III)•TAMPP at a wavelength of 467 nm is $\epsilon_{467} = 2.48 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ in pH 5.5 aqueous buffer solution.

Figure. 6-1. The visible absorption spectra of (a). TAMPP in pH = 5.5 buffer solution; (b). product Mn(III)•TAMPP in pH = 5.5 buffer solution.

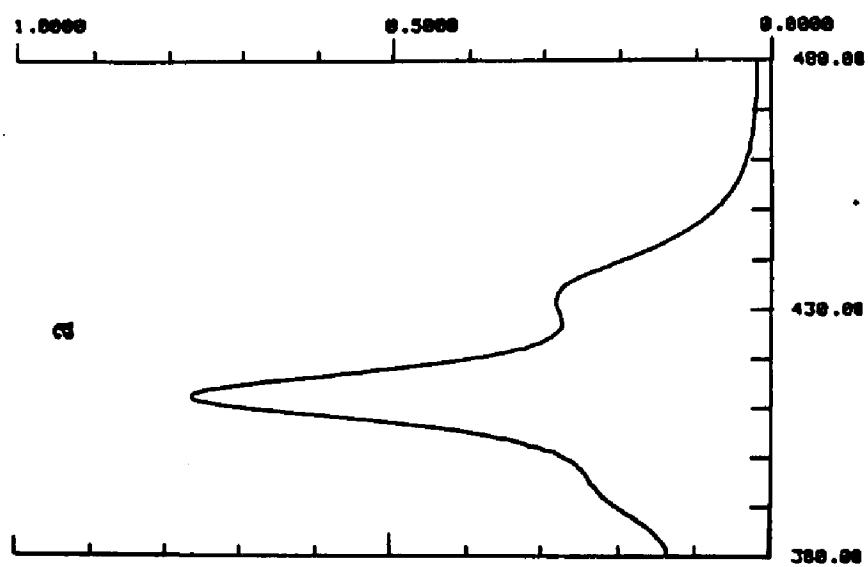
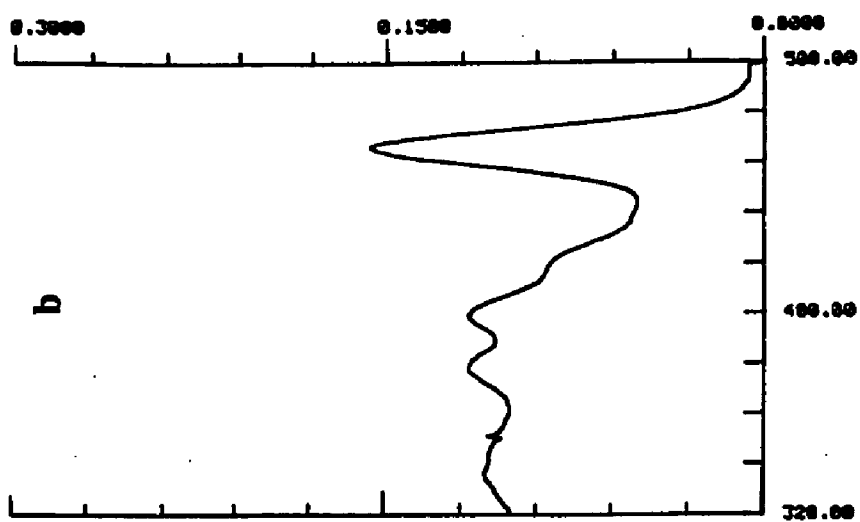
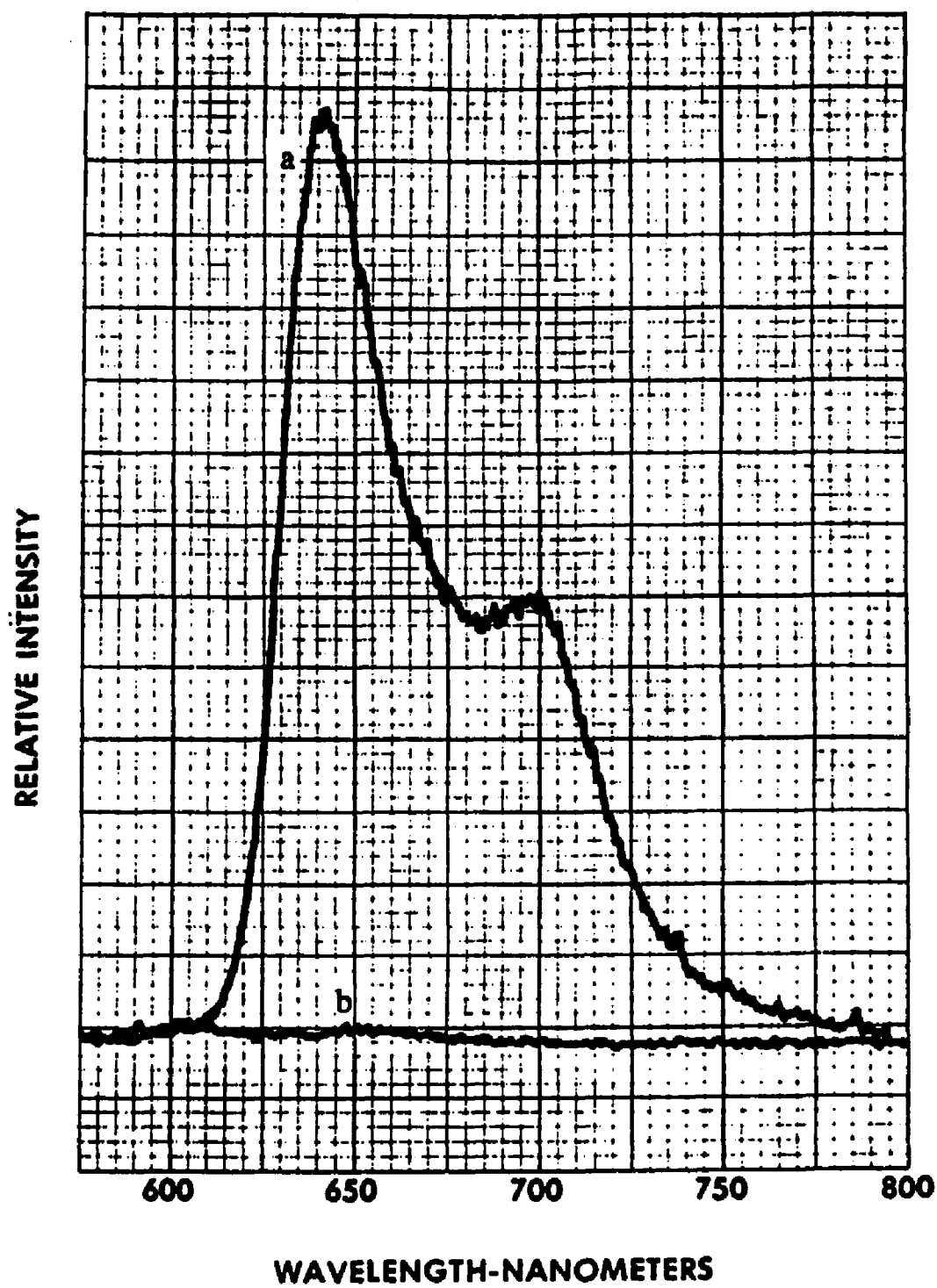


Figure. 6-2. The fluorescence absorption spectra of (a). TAMPP in 1.76×10^{-5} M, pH = 5.5 buffer solution; (b). product Mn(III)•TAMPP in 1.47×10^{-5} M, pH = 5.5 buffer solution.



6.2.2b Synthesis of Co(II)•N-MTAMPP

Co(II)•N-MTAMPP was synthesized in an anhydrous solvent. N-MTAMPP was dissolved in absolute ethanol (about 5×10^{-5} M) and warmed to reflux. Anhydrous cobalt chloride in absolute ethanol (about 5×10^{-3} M) was added quickly in equimolar proportion to the porphyrin. The reaction mixture was refluxed until there was no further change in the visible or fluorescence spectra (about four hours). The solvent was removed under the reduced pressure and the compound produced was directly used without further purification.

Figure 6-3 shows the visible spectra of the starting reactant N-MTAMPP (a) and the cobalt complexed product (b) in absolute ethanol. The shape of spectrum (6-3b) is typical of that of a cobalt complex of a tetraarylporphyrin^{15,16}. Figure 6-4 gives the fluorescence spectra of N-MTAMPP at 1.20×10^{-5} M (a) and its cobalt complexed product at 8.00×10^{-6} M (b) in pH 7.4 tris buffer. It can be seen from the area ratios of spectra 6-3a and 6-3b that the reaction is not complete and about 5% of N-MTAMPP does not form the cobalt metalloporphyrin derivative. It is very difficult to separate the non metallic N-MTAMPP and Co(II)•N-MTAMPP because it was found that Co(II)•N-MTAMPP dissociates very easily to form the free ligand in weak acidic solvents. The complex is not stable even in distilled water. In equimolar ratios of cobalt chloride and free N-MTAMPP, we were fortunate to obtain 95% conversion to Co(II)•N-MTAMPP. Higher ratios of cobalt chloride to porphyrin lead to lower yields of the complex, perhaps due to hydrolysis of traces of water by the metal ion and subsequent dissociation of the metal from the porphyrin due to the acid produced by the hydrolysis.

The similar shapes of visible spectra for Co(II)•N-MTAMPP in pH 7.4 buffer (figure 6-5b) and in ethanol (figure 6-3b) were observed. The extinction coefficients of Co(II)•N-MTAMPP in pH 7.4 tris buffer are: $\epsilon_{436} = 5.08 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{560} = 7.26 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{609} = 7.81 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, and $\epsilon_{653} = 6.25 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Figure. 6-3. The visible absorption spectra of (a). N-MTAMPP in absolute ethanol; (b). product Co(II)•N-MTAMPP in absolute ethanol.

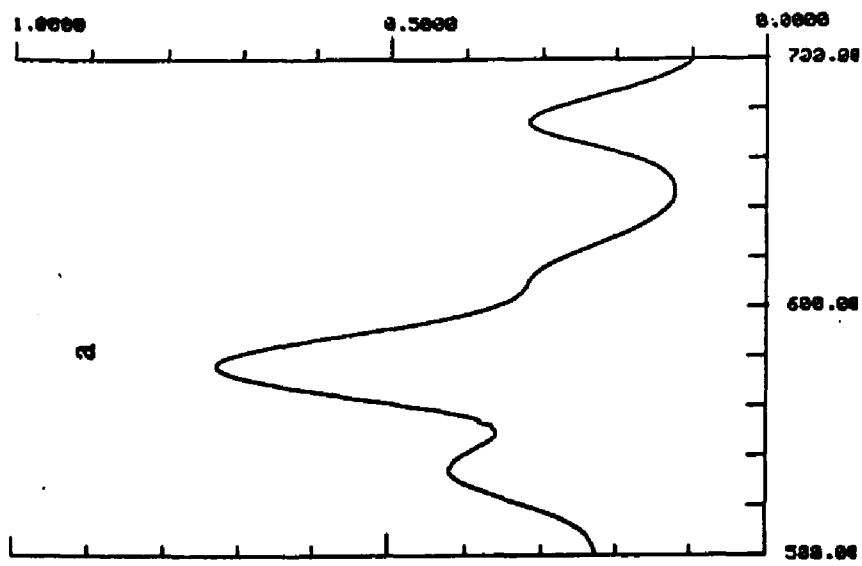
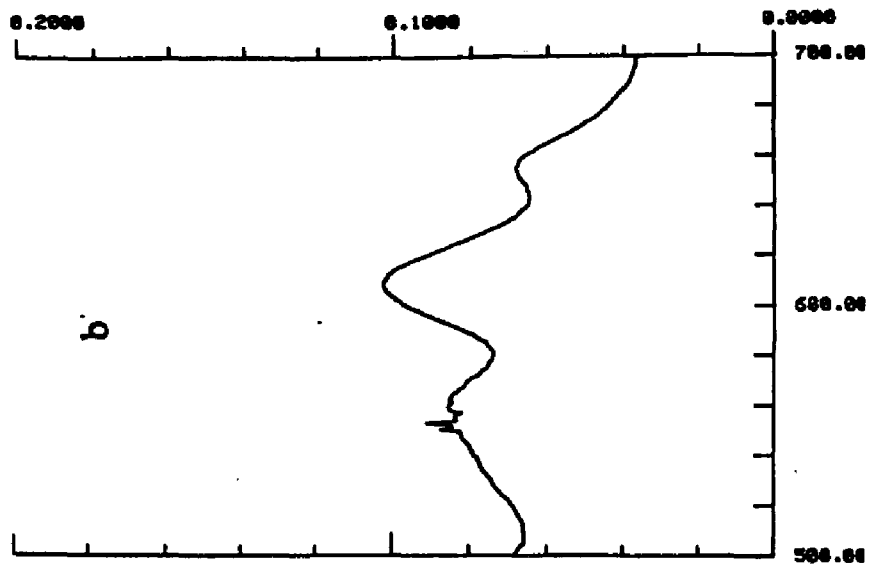


Figure. 6-4. The fluorescence absorption spectra of (a). N-MTAMPP in 1.20×10^{-5} M, pH = 7.4 buffer solution; (b). product Co(II)•N-MTAMPP in 8.00×10^{-6} M, pH = 7.4 buffer solution.

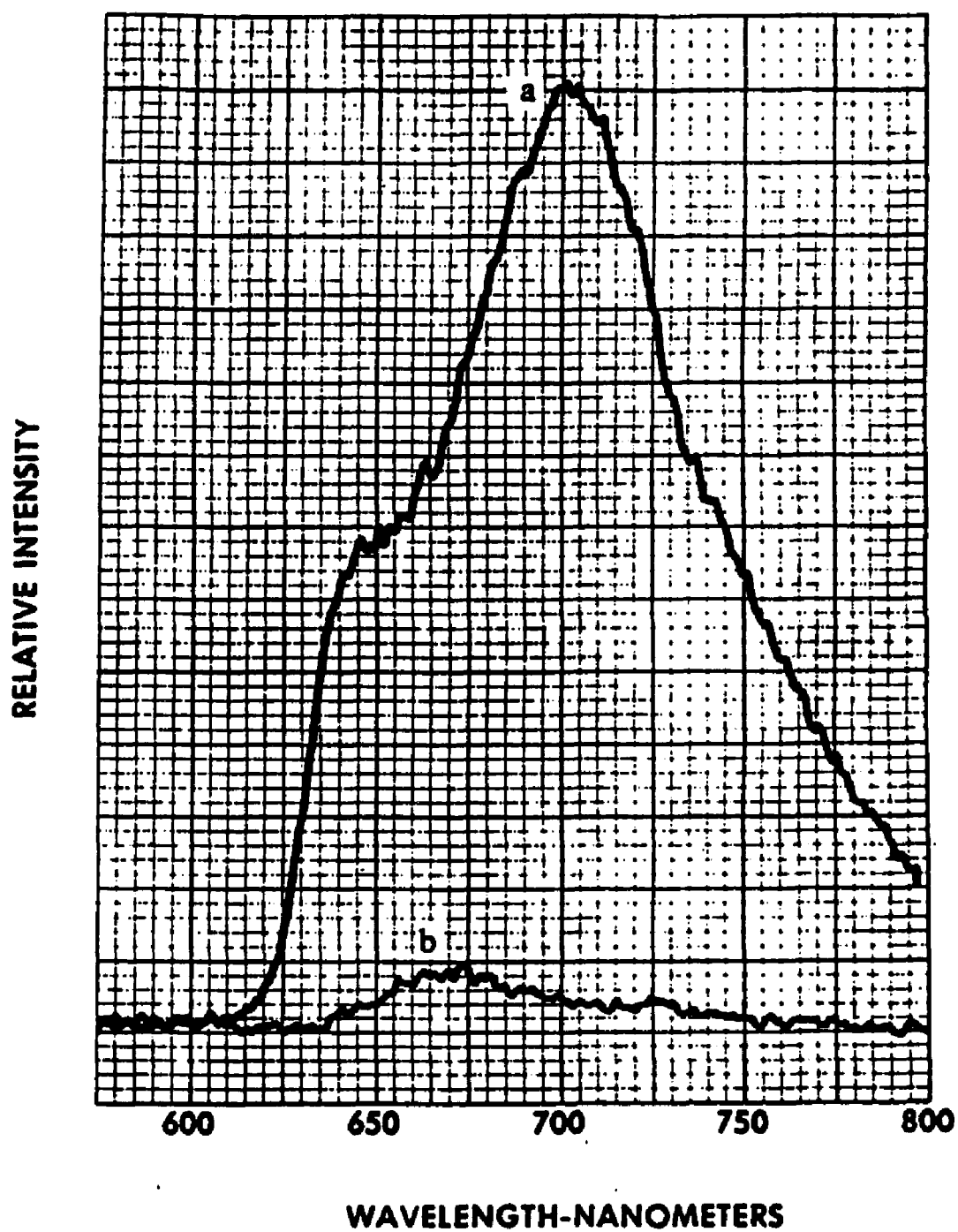
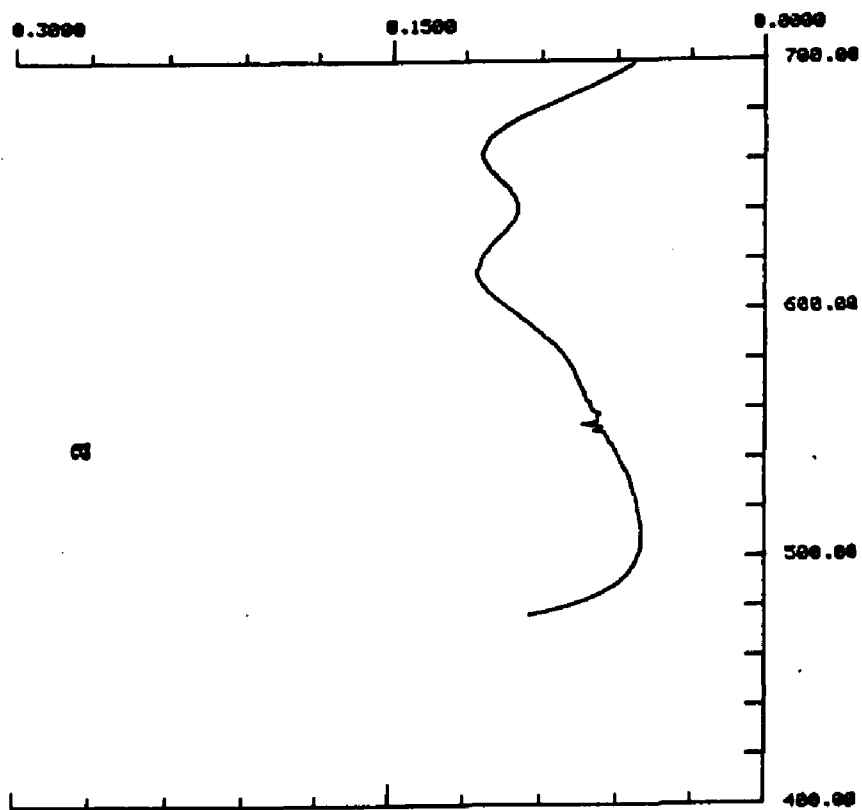
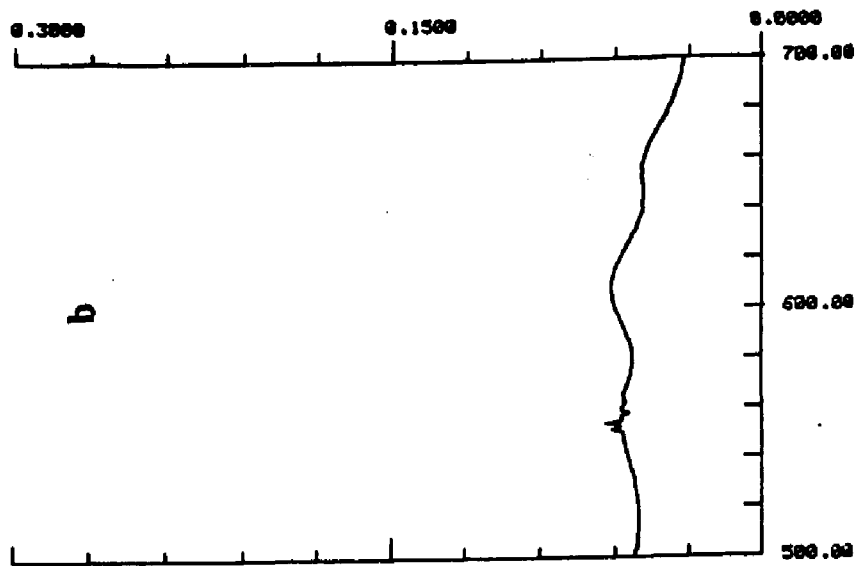


Figure. 6-5. The visible absorption spectra of (a). N-MTAMPP in pH = 7.4 buffer solution, 1.20×10^{-5} M; (b). product Co(II)•N-MTAMPP in pH = 7.4 buffer solution, 8.0×10^{-6} M.



6-2.3 Instruments

Fluorescence spectra were obtained using a Amino-Bowman SPF 125 Spectrophotofluorometer with a thermostated cell compartment. The reference, exit, entrance and signal PM tube slits used were 0.5, 0.5, 1.0 and 0.5 mm respectively. Fluorescence titration experiments were conducted by adding a concentrated stock solution of the ethidium bromide (EB) directly into a 4 ml cuvette whose starting volume was 2 ml. The wavelength of excitation was 520 nm and the wavelength of the emission was 600 nm. The signal high voltage was 925 volts. The temperature was thermostated at 25 °C. For the fluorescence scanning experiments, the wavelength of excitation was set at 520 nm and the wavelength of the emission was changed with time by about 220 nm / min. Circular dichroism spectra were recorded on the Jobin Yvon spectropolarimeter at a spectral band-pass of 20 Å. Absorption spectra were carried out using a Beckman Du-8 spectrophotometer with a thermostated cell compartment.

6-3 Results

6-3.1 Circular Dichroism Spectra of Metalloporphyrin — Nucleic Acid Systems

Figures 6-6 and 6-7 are the induced circular dichroism spectra of systems Mn(III)•TAMPP and Co(II)•N-MTAMPP with calf thymus DNA (a), Poly(dA-dT) • Poly(dA-dT) (b) and Poly(dG-dC) • Poly(dG-dC) (c) at a ratio of the total concentration of metalloporphyrin to that of nucleic acid in base pairs of less than 0.03.

Like its corresponding free ligand, Mn(III)•TAMPP gives very similar induced circular dichroism spectra when interacting with CT.DNA and Poly(dA-dT) • Poly(dA-dT). Both Mn(III)•TAMPP — DNA and Mn(III)•TAMPP — Poly(dA-dT) • Poly(dA-dT) systems give only positive circular dichroism bands with the peak position at 467 nm. In contrast, the system of Mn(III)•TAMPP — Poly(dG-dC) • Poly(dG-dC)

Figure. 6-6. The circular dichroism spectra of (a). Mn(III)•TAMPP — CT.DNA , (b). Mn(III)•TAMPP — Poly(dA-dT) • Poly(dA-dT) and (c). Mn(III)•TAMPP — Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are: [Mn•TAMPP] = 6.3×10^{-6} M, [nucleic acid] = 2.6×10^{-4} M. (sensitivity: 1×10^{-5} ; step size(nm): 0.2; responsible time: 0.5; number of cycles: 20)

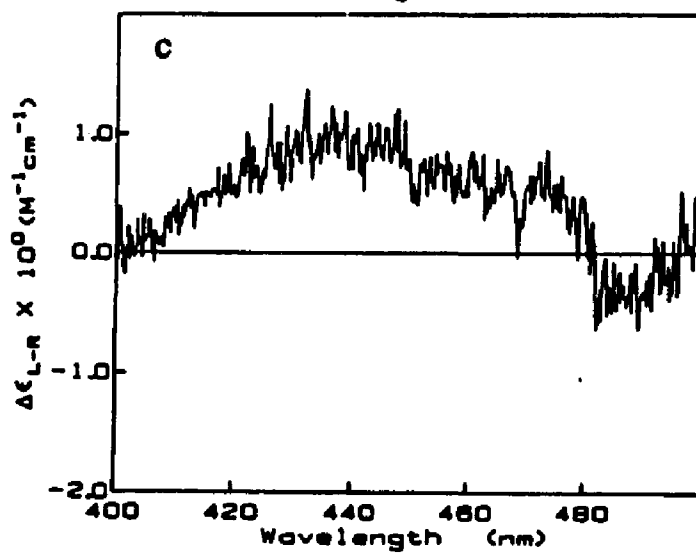
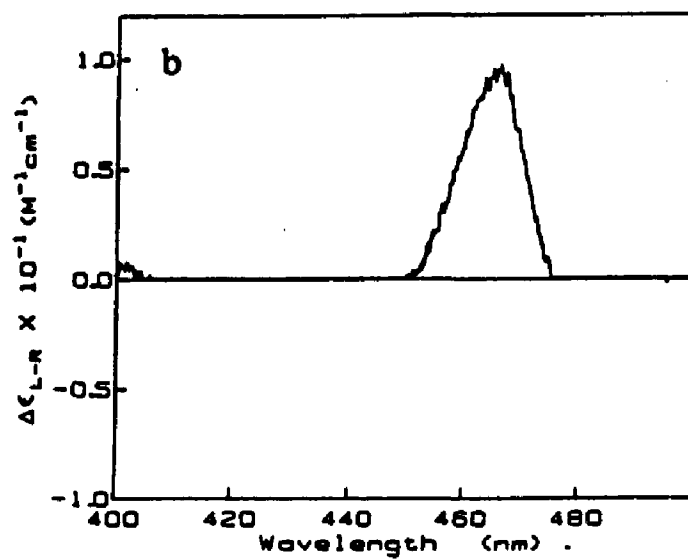
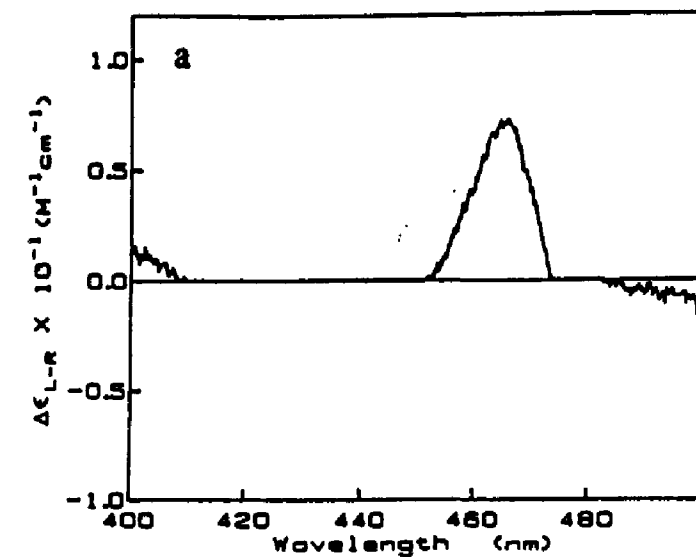
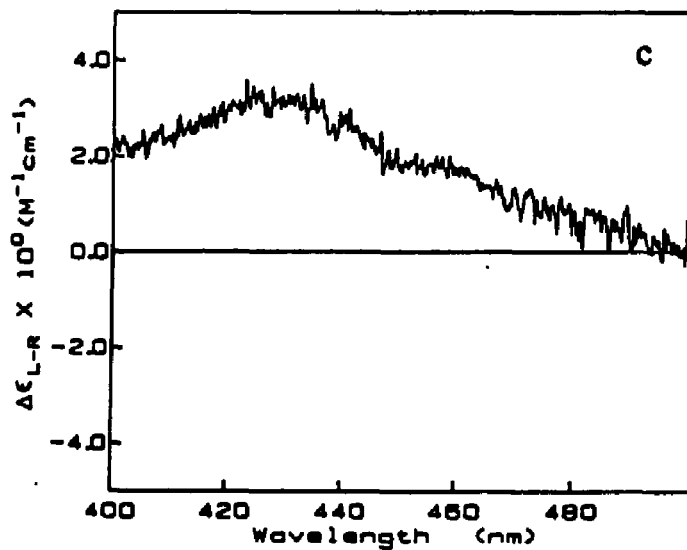
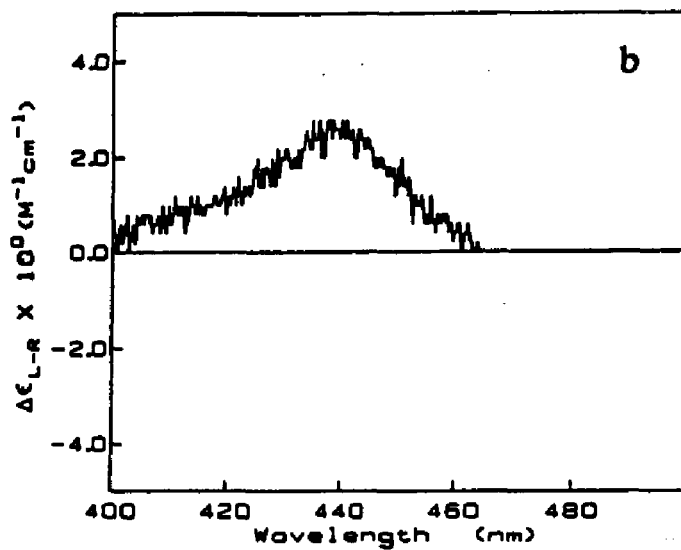
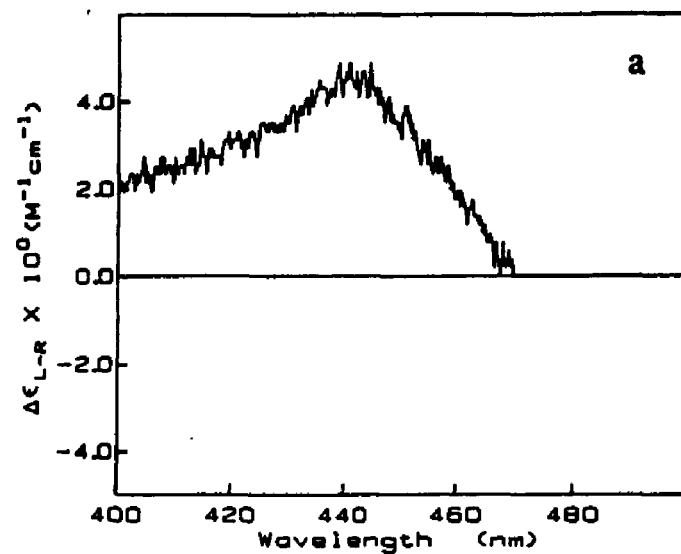


Figure. 6-7. The circular dichroism spectra of (a). Co(II)•N-MTAMPP — CT.DNA , (b). Co(II)•N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) and (c). Co(II)•N-MTAMPP — Poly(dG-dC) • Poly(dG-dC). The concentration of species in solution are: [Co•N-MTAMPP] = 6.3×10^{-6} M, [nucleic acid] = 2.6×10^{-4} M. (sensitivity: 1×10^{-5} ; step size(nm): 0.2; responsible time: 0.5; number of cycles: 20)



displays a very small molar ellipticity in the circular dichroism spectrum, implying that the electronic interaction between $\text{Mn(III)}\cdot\text{TAMPP}$ and $\text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ is very weak.

A similar phenomenon was found also for the N-methylated metal coordinated compound: $\text{Co(II)}\cdot\text{N-MTAMPP}$. When interacting with CT.DNA and $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$, $\text{Co(II)}\cdot\text{N-MTAMPP}$ gives rise to only positive circular dichroism bands with the peak position at 440 nm. $\text{Co(II)}\cdot\text{N-MTAMPP}$ shows a very small positive CD band when it interacts with $\text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$.

6-3.2 Visible Absorption Spectra of Metalloporphyrin — Nucleic Acid Systems

Figures 6-8 and 6-9 are the visible absorption spectra of systems $\text{Mn(III)}\cdot\text{TAMPP}$ and $\text{Co(II)}\cdot\text{N-MTAMPP}$ with calf thymus DNA (a), $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ (b) and $\text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ (c) at a ratio of the total concentration of metalloporphyrin to that of nucleic acid in base pairs of less than 0.03. The changes in visible absorption spectra that occur when the metalloporphyrins $\text{Mn(III)}\cdot\text{TAMPP}$ and $\text{Co(II)}\cdot\text{N-MTAMPP}$ interact with nucleic acids compared to the changes that occur when the free ligand forms of the porphyrins interact with the same nucleic acid are small.

There are no detectable red shift of the solet band when $\text{Mn(III)}\cdot\text{TAMPP}$ interacts with CT.DNA and $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$, and a small red shift (about 2 nm) for $\text{Mn(III)}\cdot\text{TAMPP} - \text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ system. $\text{Mn(III)}\cdot\text{TAMPP} - \text{DNA}$ and $\text{Mn(III)}\cdot\text{TAMPP} - \text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ systems display a small hypochromic behavior (-7% and -9%). In contrast, the $\text{Mn(III)}\cdot\text{TAMPP} - \text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ system gives rise to a hyperchromic changes (+26%).

All three $\text{Co(II)}\cdot\text{N-MTAMPP} - \text{nucleic acid}$ systems show a similar small red shift of the Soret band (about 4 nm). $\text{Co(II)} \cdot \text{N-MTAMPP} - \text{DNA}$ and $\text{Co(II)} \cdot$

Figure. 6-8. The visible absorption spectra of (a). 1- Mn(III)•TAMPP , 2- $\text{Mn(III)•TAMPP} - \text{CT.DNA}$, (b). 1- Mn(III)•TAMPP , 2- $\text{Mn(III)•TAMPP} - \text{Poly(dA-dT) • Poly(dA-dT)}$ and (c). 1- Mn(III)•TAMPP , 2- $\text{Mn(III)•TAMPP} - \text{Poly(dG-dC) • Poly(dG-dC)}$. The concentration of species in solution are: $[\text{Mn•TAMPP}] = 6.3 \times 10^{-6} \text{ M}$, $[\text{nucleic acid}] = 2.6 \times 10^{-4} \text{ M}$, pH 5.5 buffer solution.

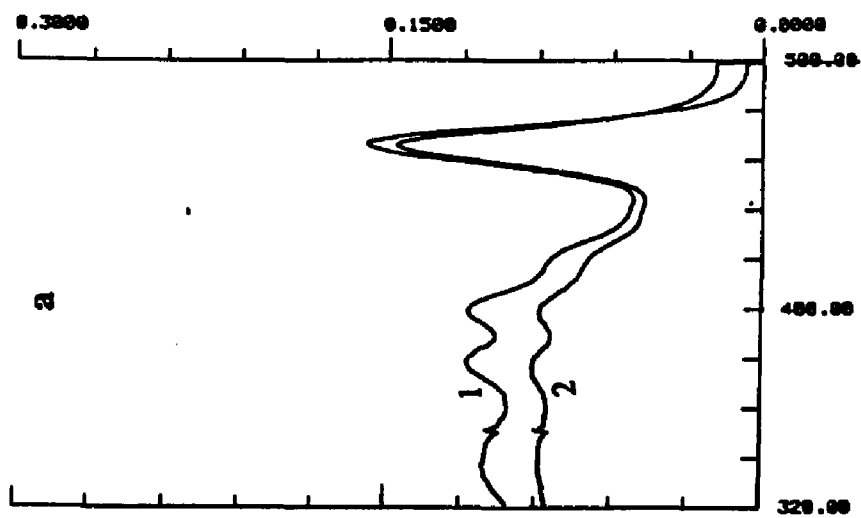
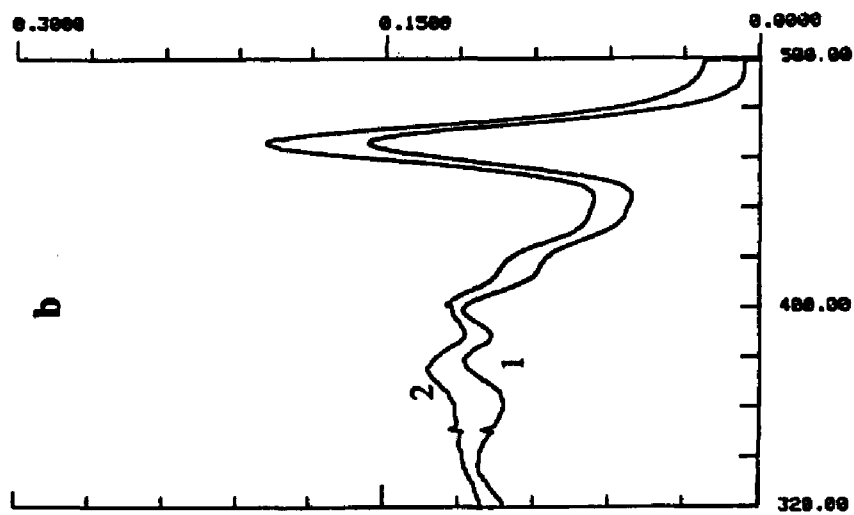
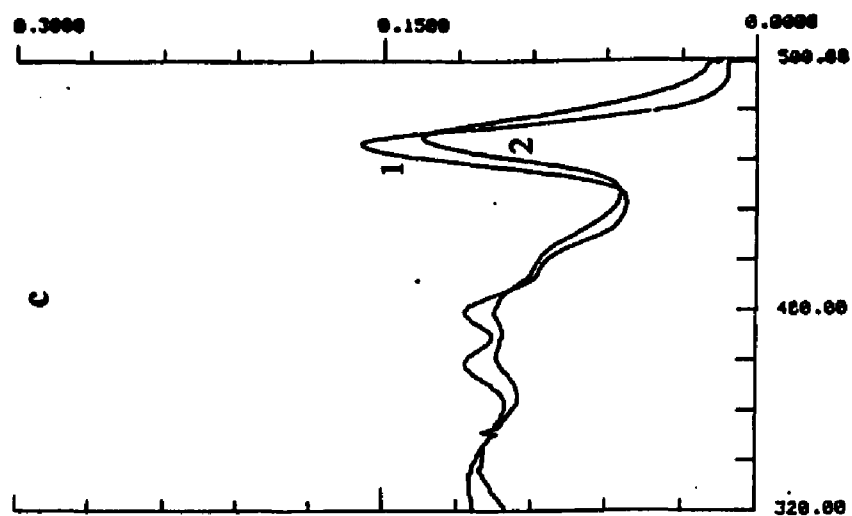
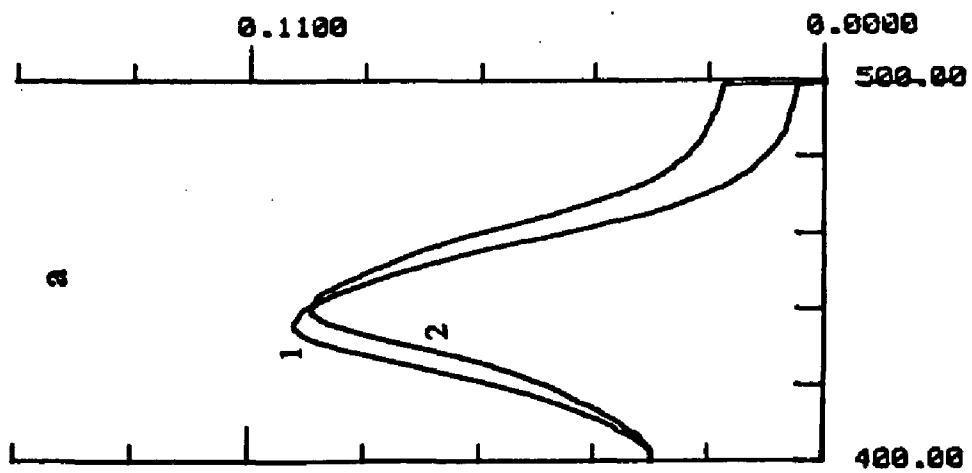
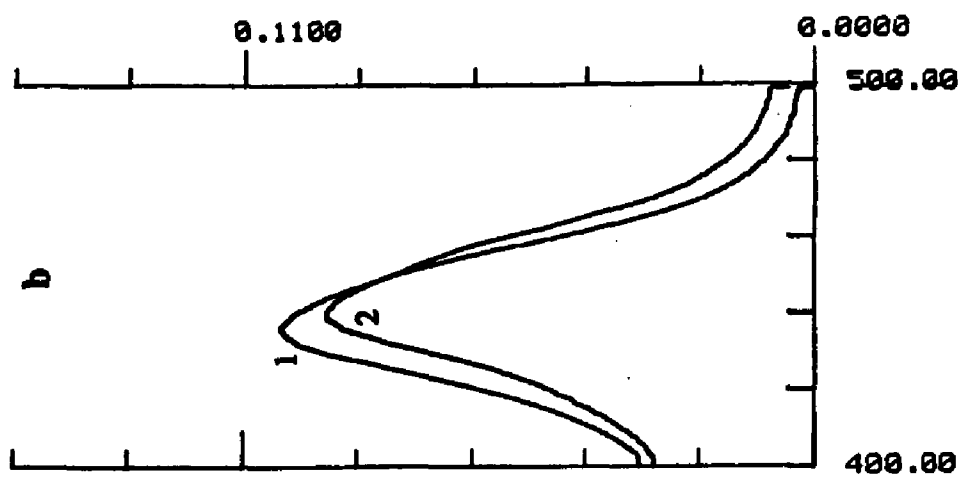
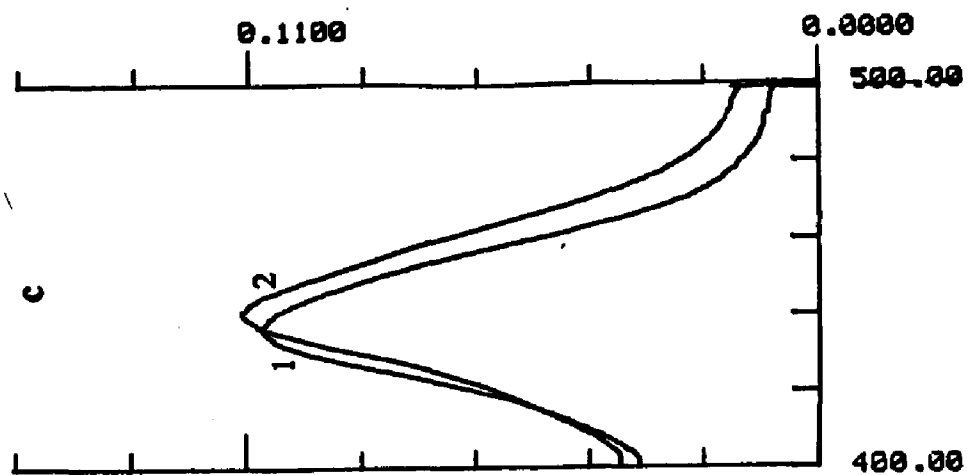


Figure. 6-9. The visible absorption spectra of (a). 1- $\text{Co(II)} \cdot \text{N-MTAMPP}$, 2- $\text{Co(II)} \cdot \text{N-MTAMPP} - \text{CT.DNA}$, (b). 1- $\text{Co(II)} \cdot \text{N-MTAMPP}$, 2- $\text{Co(II)} \cdot \text{N-MTAMPP} - \text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ and (c). 1- $\text{Co(II)} \cdot \text{N-MTAMPP}$, 2- $\text{Co(II)} \cdot \text{N-MTAMPP} - \text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$. The concentration of species in solution are: $[\text{Co} \cdot \text{N-MTAMPP}] = 6.3 \times 10^{-6} \text{ M}$, $[\text{nucleic acid}] = 2.6 \times 10^{-4} \text{ M}$, pH 7.4 buffer solution.



N-MTAMPP — Poly(dA-dT) • Poly(dA-dT) show small hypochromicity (-4 and -8%) and the Co(II)•N-MTAMPP — Poly(dG-dC) • Poly(dG-dC) system shows a small hyperchromicity (+4%).

6-3.3 Affinity Constant Studies of Metalloporphyrin — CT.DNA Systems

The same competition method as discussed as in Chapter (5) was used to determine the affinity constant of complexes of Mn(III)•TAMPP — DNA and Co(II)•N-MTAMPP — DNA. Equations (5-18), (5-19), (5-20), (5-21), (5-22) and (5-34) were used to process the data derived from competition experiments. The detailed experimental approach and data treatment were discussed in Chapter (5).

Table 6-1 summarizes the affinity constants of the system Mn(III)•TAMPP — DNA and Co(II)•N-MTAMPP — DNA derived from processing data of competition experiments. The average affinity constants for the systems Mn(III)•TAMPP — DNA and Co(II)•N-MTAMPP — DNA are $2.60 (\pm 0.34) \times 10^7 \text{ M}^{-1}$ and $1.28 (\pm 0.18) \times 10^7 \text{ M}^{-1}$. The average parameter M_{por} , the number of ethidium bromide binding sites required by each metalloporphyrin molecule, are 0.54 ± 0.01 and 0.84 ± 0.03 for the system Mn(III)•TAMPP — DNA and Co(II)•N-MTAMPP — DNA, respectively. The uncertainties shown in brackets are specified as the average deviation from the mean. The more detailed experimental data are reported in appendix (9).

6-4 Discussion

The circular dichroism technique is a very useful and informative for studying the interaction between a porphyrin (an optically inactive substance) and nucleic acids (optically active substance). Based on the Pasternack's empirical correlation³ and the discussion in Chapter (4) and (5), the small positive circular dichroism bands for metalloporphyrins interacting with DNA and Poly(dA-dT) • Poly(dA-dT) shown in

Table (6-1)
Affinity Constants of
Mn•TAMPP — CT.DNA and Co•N-MTAMPP — DNA Complexes

Metalloporphyrin	$K_{Por}(10^7 \text{ M}^{-1})$	M_{Por}	r
Mn•TAMPP	2.27	0.56	0.981
	3.23	0.53	0.972
	2.36	0.54	0.962
	2.82	0.51	0.975
	2.32	0.54	0.943
Average value	2.60 ± 0.34	0.54 ± 0.01	
Co•N-MTAMPP	1.22	0.87	0.970
	1.18	0.87	0.984
	1.07	0.83	0.982
	1.14	0.80	0.995
	1.49	0.87	0.973
	1.62	0.78	0.983
Average value	1.28 ± 0.18	0.84 ± 0.03	

figure 6-5a, 5b and 6-6a, 6b imply that both $\text{Mn(III)}\cdot\text{TAMPP}$ and $\text{Co(II)}\cdot\text{N-MTAMPP}$ (1) bind on the outside of DNA and $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ (positive circular dichroism band); (2) prefer to bind on AT rich regions of DNA (the circular dichroism spectral intensities and shape are similar for metalloporphyrins with DNA and $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$) and (3) require few nucleic acid base pairs when they bind on DNA or $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ (small ellipticity for this two metalloporphyrins DNA and $\text{Poly(dA-dT)} \cdot \text{Poly(dA-dT)}$ systems). The positive circular dichroism band of the $\text{Mn(III)}\cdot\text{TAMPP} - \text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ and $\text{Co(II)}\cdot\text{N-MTAMPP} - \text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$ systems implies that the two metalloporphyrins can not intercalate into $\text{Poly(dG-dC)} \cdot \text{Poly(dG-dC)}$.

The most evident results from acidity constant studies of $\text{Mn(III)}\cdot\text{TAMPP} - \text{DNA}$ and $\text{Co(II)}\cdot\text{N-MTAMPP} - \text{DNA}$ systems are (1) their affinity constants are about one unit higher than the corresponding free form of porphyrin bound to DNA (2.7×10^7 for $\text{Mn(III)}\cdot\text{TAMPP} - \text{DNA}$ and 4.4×10^6 for $\text{TAMPP} - \text{DNA}$; 1.3×10^7 for $\text{Co(II)}\cdot\text{N-MTAMPP} - \text{DNA}$ and 2.2×10^6 for $\text{N-MTAMPP} - \text{DNA}$) and (2) the parameters M_{por} of these two metalloporphyrin — DNA systems are much smaller than their corresponding free form of porphyrin binding on DNA (0.5 for $\text{Mn(III)}\cdot\text{TAMPP} - \text{DNA}$ and 3.0 for $\text{TAMPP} - \text{DNA}$; 0.8 for $\text{Co(II)}\cdot\text{N-MTAMPP} - \text{DNA}$ and 5.8 for $\text{N-MTAMPP} - \text{DNA}$ system).

The small M_{por} values of $\text{Mn(III)}\cdot\text{TAMPP} - \text{DNA}$ and $\text{Co(II)}\cdot\text{N-MTAMPP} - \text{DNA}$ are consistent with their corresponding circular dichroism results which indicate that the two metalloporphyrin require fewer nucleic acid base pairs to bind to DNA. However, the positive circular dichroism bands imply that the metalloporphyrins are bound outside the DNA and the results of Chapter (5) suggest that outside binding requires more nucleic acid base pairs than intercalation. Considering the differences in circular dichroism spectra, the parameter M_{por} , and the affinity constants K_{por} between

these metalloporphyrins and their corresponding free ligands when they interact with DNA, the metalloporphyrins are bound outside the DNA but the manner in which they interact may be different from that of free form of the porphyrin.

After coordinating with metal, there are more positive charge introduced on the porphyrin ring, which may cause a larger affinity for the metalloporphyrin to bind electrostatically to the outside of DNA compared with the free form of porphyrin. Because of coordinated metal, this outside binding may cause less deformation of the structure of DNA and/or the complex may occupy fewer nucleic acid base pairs. It is quite possible that a phosphate group of the nucleic acid could be as the axial ligand for either the Mn(III) or the Co(II) porphyrin complexes. In this case, the metalloporphyrin would require few base pairs (consistent with the small M_{por} value). Also, this could place the porphyrin ring relatively far from the nucleic acid bases, leading to relatively weaker electrostatic interaction, consistent with the small observed molar ellipticity in the circular dichroism spectra. In this binding manner, the affinity between porphyrin and nucleic acid could be increased a lot (consistent with the higher K_{por} value). The results described in this section are preliminary and not sufficient to derive specific conclusion about the way in which these metalloporphyrin bind to nucleic acids.

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Appendix 1

The Experimental Data for Spectrophotometric Titration to TAMPP

1-1. [I] = 0.12 M

1-1.1 Titration with HNO₃

V _{HNO₃} (ml)	A ₅₁₆	A ₆₄₈	pH	A ₆₄₈ /A ₅₁₆
0.003	0.0473	1.237	2.96	26.2
0.008	0.0469	1.244	2.58	26.5
0.058	0.0436	1.238	1.87	28.4
0.158	0.0420	1.209	1.49	28.8
0.358	0.0397	1.146	1.15	28.9

The starting concentration of TAMPP was 3.613×10^{-5} M;

The starting volume was 3 ml;

The concentration of HNO₃ titrated is 1.00 M.

V_{HNO₃}: The accumulated volume of HNO₃ added;

A₅₁₆: Absorbance at wavelength 516 nm;

A₆₄₈: Absorbance at wavelength 648 nm.

1-1.2 Titration with NaOH

V_{NaOH} (ml)	A_{516}	A_{648}	pH	A_{648}/A_{516}
0	0.0576	1.168	3.48	20.3
0.007	0.0732	1.070	3.83	14.6
0.012	0.1849	0.6227	4.46	3.37
0.014	0.3388	0.1753	5.02	0.52
0.016	0.3884	0.0705	6.17	0.18

The starting concentration of TAMPP was 3.613×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated is 0.0922 M.

The experimental data shown in 1-1.1 and 1-1.2 give: $\text{pK}_3 \sim \text{pK}_4 = 4.5$
for TAMPP in $[\text{I}] = 0.12$ M.

1-2 [I] < 0.01 M**1-2.1 Titration with HNO₃**

V_{HNO_3} (ml)	A ₅₁₆	A ₆₄₈	pH	A ₆₄₈ /A ₅₁₆
0	0.0648	0.948	2.81	14.6
0.010	0.0567	1.026	2.44	18.1
0.020	0.0542	1.031	2.28	19.0
0.060	0.0507	1.033	1.92	20.4
0.160	0.0477	1.005	1.55	21.1
0.260	0.0462	0.9771	1.34	21.2
0.460	0.0417	0.9276	1.18	22.2
0.760	0.0400	0.8588	0.99	21.5

The starting concentration of TAMPP was 3.118×10^{-5} M;

The starting volume was 3 ml;

The concentration of HNO₃ titrated was 1.00 M.

1-2.2 Titration with NaOH

V_{NaOH} (ml)	A_{516}	A_{648}	pH	A_{648}/A_{516}
0.010	1.228	0.9842	2.86	8.01
0.020	0.1295	0.9411	2.95	7.27
0.030	0.1367	0.8927	3.06	6.53
0.036	0.1483	0.8463	3.15	5.71
0.042	0.1618	0.7711	3.27	4.77
0.048	0.1970	0.6476	3.43	3.29
0.050	.1942	0.5782	3.50	2.98
0.052	0.2361	0.5139	3.62	2.18
0.054	0.2451	0.4037	3.70	1.65
0.056	0.2932	0.3004	3.86	1.02
0.058	0.3554	0.2047	4.10	0.58
0.060	0.4051	0.1012	4.70	0.25
0.062	0.4171	0.0992	5.96	0.24

The starting concentration of TAMPP was 3.118×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

The experimental data shown in 1-2.1 and 1-2.2 give: $pK_3 \approx pK_4 = 3.6$ for TAMPP in $[I] \leq 0.01$ M.

Appendix 2

The Experimental Data of Potentiometric Titration to TAMPP

2-1 [I] = 0.12 M

2-1.1 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V_{NaOH}
0	3.48		
0.007	3.83	50	0.0035
0.012	4.46	126	0.0095
0.014	5.02	280	0.013
0.016	6.17	575	0.015
0.018	6.83	330	0.017
0.020	7.30	235	0.019
0.022	8.34	520	0.021
0.024	9.03	345	0.023
0.026	9.40	185	0.025
0.028	9.66	130	0.027
0.030	9.84	90	0.029

The starting concentration of TAMPP was 3.613×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

$\text{p}K_3 \sim \text{p}K_4 = 4.2$, $\text{p}K_a = 6.0$

2-1.2 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	3.50		
0.005	3.68	35.4	0.0025
0.009	3.95	66.3	.0070
0.012	4.34	130	0.0105
0.013	4.53	198	0.0125
0.014	4.77	237	0.0135
0.015	5.10	325	0.0145
0.016	5.51	413	0.0155
0.017	5.98	469	0.0165
0.018	6.91	933	0.0175
0.019	7.58	665	0.0185
0.020	7.83	250	0.0195
0.021	8.16	330	0.0205
0.022	8.54	385	0.0215
0.023	8.82	276	0.0225
0.024	9.03	216	0.0235
0.026	9.36	164	0.0250
0.028	9.62	128	0.0270
0.030	9.81	99	0.0290
0.032	9.98	81	0.0310

The starting concentration of TAMPP was 3.613×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

$\text{p}K_3 \approx \text{p}K_4 = 4.7$, $\text{p}K_a = 6.5$

2-1.3 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	3.47		
		39.4	0.0025
0.005	3.67	63.8	.0070
0.009	3.92	133.5	0.0100
0.011	4.19	146.5	0.0120
0.013	4.48	241.0	0.0135
0.014	4.72	341.0	0.0145
0.015	5.06	323.0	0.0155
0.016	5.39	584.0	0.0165
0.017	5.97	757.0	0.0175
0.018	6.73	748.0	0.0185
0.019	7.48	239.0	0.0195
0.020	7.72	173.0	0.0205
0.021	7.89	314.0	0.0215
0.022	8.20	211.0	0.0225
0.023	8.41	238.0	0.0235
0.024	8.65	131.0	0.0245
0.025	8.78	149.0	0.0255
0.026	8.93	123.0	0.0265
0.027	9.05	115.5	0.0280
0.029	9.29	79.0	0.0300
0.031	9.44	58.0	0.0345
0.036	9.77	48.3	0.0380
0.040	9.96		

The starting concentration of TAMPP was 3.613×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

$\text{p}K_3 = \text{p}K_4 = 4.6$, $\text{p}K_a = 6.2$

2-2. [I] < 0.01 M

2-2.1 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	2.80		
0.010	2.86	5.3	0.0050
0.020	2.95	9.0	0.0150
0.030	3.06	11.1	0.0250
0.036	3.15	14.8	0.0330
0.042	3.27	20.5	0.0390
0.048	4.43	27.3	0.0450
0.050	3.50	35.5	0.0490
0.052	3.616	56.5	0.0510
0.054	3.70	43.5	0.0530
0.056	3.86	80.5	0.0550
0.058	4.10	115.5	0.0570
0.060	4.70	300.0	0.0590
0.062	5.96	632.5	0.0610
0.064	6.89	464.0	0.0630
0.066	7.92	514.0	0.0650
0.068	8.90	492.0	0.0670
0.070	9.29	195.0	0.0690
0.072	9.56	135.0	0.0710
0.077	9.96	79.0	0.0745
0.087	10.4	40.2	0.0820

The starting concentration of TAMPP was 3.118×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

$\text{p}K_3 \sim \text{p}K_4 = 3.1$, $\text{p}K_a = 5.7$

2-2.2 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	2.82		
0.020	2.97	7.8	0.0010
0.040	3.25	14.4	0.0300
0.045	3.38	25.4	0.0425
0.049	3.51	30.8	0.047
0.051	3.59	41.5	0.050
0.053	3.68	46.5	0.052
0.055	3.80	59.0	0.054
0.057	3.94	69.0	0.056
0.058	4.04	100.0	0.0575
0.059	4.16	119.0	0.0585
0.060	4.30	144.0	0.0595
0.061	4.50	201.0	0.0605
0.062	4.91	408.0	0.0615
0.063	5.52	607.0	0.0625
0.064	6.31	794.0	0.0635
0.065	6.90	583.0	0.0645
0.066	7.38	485.0	0.0655
0.067	7.73	345.0	0.0665
0.068	8.01	286.0	0.0675
0.069	8.24	232.0	0.0685
0.070	8.55	304.0	0.0695
0.071	8.87	323.0	0.0705
0.073	9.25	188.0	0.0720
0.075	9.45	104.0	0.0740
0.080	9.86	80.2	0.0775
0.085	10.1	47.0	0.0825

The starting concentration of TAMPP was 3.118×10^{-5} M;
 The starting volume was 3 ml;
 The concentration of NaOH titrated was 0.0922 M
 $\text{p}K_3 \sim \text{p}K_4 = 3.8$, $\text{p}K_a = 6.9$

2-2.3 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	2.80		
		8.6	0.0200
0.040	3.14	23.8	0.0450
0.050	3.38	44.4	0.0525
0.055	3.60	85.3	0.0570
0.059	3.94	116.0	0.0595
0.060	4.06	215.0	0.0605
0.061	4.28	340.0	0.0615
0.062	4.62	620.0	0.0625
0.063	5.24	954.0	0.0635
0.064	6.19	643.0	0.0645
0.065	6.83	500.0	0.0655
0.066	7.33	268.0	0.0665
0.067	7.60	294.0	0.0675
0.068	7.89	271.0	0.0685
0.069	8.17	338.0	0.0695
0.070	8.50	249.0	0.0705
0.071	8.75	226.0	0.0715
0.072	8.98	130.0	0.0725
0.073	9.11	122.5	0.0740
0.075	9.35	94.5	0.0760
0.077	9.54	74.0	0.0785
0.080	9.76	56.0	0.0820
0.084	9.99		

The starting concentration of TAMPP was 3.118×10^{-5} M;
 The starting volume was 3 ml;
 The concentration of NaOH titrated was 0.0922 M.

$\text{p}K_3 \approx \text{p}K_4 = 3.7$, $\text{p}K_a = 6.6$

Appendix 3

The Experimental Data of Spectrophotometric Titration to N-MTAMPP

3-1 [I] = 0.12 M

3-1.1 Titration with HNO_3

V_{HNO_3} (ml)	A_{588}	A_{618}	A_{666}	pH	A_{666}/A_{588}	A_{666}/A_{618}
0	0.3129	0.5804	1.131	3.50	3.61	1.95
(+ 0.010 ml NaOH)	0.3675	0.6440	0.7690	4.39	2.09	1.19
0.002	0.2850	0.5407	1.296	3.20	4.54	2.40
0.004	0.2534	0.5003	1.487	2.90	5.86	2.97
0.006	0.2369	0.4782	1.598	2.72	6.74	3.34
0.010	0.2122	0.4489	1.696	2.50	7.99	3.78
0.020	0.1931	0.4238	1.791	2.22	9.27	4.23
0.060	0.1736	0.3967	1.827	1.81	10.52	4.61
0.360	0.1542	0.3533	1.719	1.13	11.15	4.87
0.660	0.1407	0.3229	1.599	0.87	11.36	4.95

The starting concentration of N-MTAMPP was 4.330×10^{-5} M;

The starting volume was 3 ml;

The concentration of HNO_3 titrated was 1.00 M;

V_{HNO_3} : The accumulated HNO_3 volume added;

A_{588} : Absorbance at wavelength 588 nm;

A_{618} : Absorbance at wavelength 618 nm;

A_{666} : Absorbance at wavelength 666nm.

3-1.2 Titration with NaOH

$V_{\text{NaOH}}(\text{ml})$	A_{588}	A_{618}	A_{666}	pH	A_{666}/A_{588}	A_{666}/A_{618}
0	0.3126	0.5845	1.134	3.50	3.63	1.94
0.004	0.3343	0.6047	1.010	3.67	3.02	1.67
0.006	0.3451	0.6173	0.9444	3.86	2.74	1.53
0.008	0.3536	0.6297	0.8686	4.02	2.45	1.38
0.010	0.3623	0.6397	0.8004	4.43	1.95	1.25
0.012	0.3718	0.6498	0.7251	5.63	1.95	1.12
0.014	0.3907	0.5751	0.6035	7.31	1.54	1.05
0.016	0.4135	0.5138	0.5013	8.10	1.21	0.98
0.018	0.4288	0.4545	0.3875	8.28	0.90	0.85
0.020	0.4715	0.4423	0.3300	8.42	0.70	0.75
0.022	0.5292	0.4437	0.2721	8.68	0.51	0.61
0.024	0.5494	0.4498	0.2618	9.12	0.48	0.58
0.034	0.5960	0.4860	0.2830	10.00	0.47	0.58

The starting concentration of N-MTAMPP was 4.330×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M;

Combine the experimental data shown in 3-1.1 and 3-1.2:

A_{666}/A_{588} vs. pH: $\text{pK}_3 = 8.1$, $\text{pK}_4 = 3.1$,

A_{666}/A_{618} vs. pH: $\text{pK}_3 = 8.1$, $\text{pK}_4 = 3.1$.

3-2 [I] < 0.01 M

3-2.1 Titration with HNO_3

V_{HNO_3} (ml)	A_{588}	A_{618}	A_{666}	pH	A_{666}/A_{588}	A_{666}/A_{618}
0	0.2223	0.3992	0.4783	3.40	2.15	1.20
0.002	0.2254	0.3864	0.5337	3.00	2.37	1.38
0.005	0.2168	0.3731	0.6206	2.81	2.86	1.66
0.008	0.1999	0.3547	0.6940	2.64	3.47	1.96
0.012	0.1812	0.3344	0.7720	2.50	4.26	2.31
0.017	0.1706	0.3199	0.8583	2.38	5.03	2.68
0.023	0.1594	0.3044	0.9280	2.28	5.82	3.05
0.035	0.151	0.2904	1.002	2.13	6.64	3.45
0.055	0.1372	0.2725	1.074	1.99	7.83	3.94
0.115	0.1281	0.2573	1.118	1.71	8.73	4.35
0.215	0.1163	0.2406	1.106	1.430	9.51	4.60
0.415	0.1042	0.2210	1.051	1.19	10.09	4.76
0.815	0.0975	0.2000	0.9501	0.940	9.74	4.75

The starting concentration of N-MTAMPP was 2.869×10^{-5} M;

The starting volume was 3 ml;

The concentration of HNO_3 titrated was 1.00 M;

V_{HNO_3} : The accumulated HNO_3 volume added;

A_{588} : Absorbance at wavelength 588 nm;

A_{618} : Absorbance at wavelength 618 nm;

A_{666} : Absorbance at wavelength 666 nm.

3-2.2 Titration with NaOH

$V_{\text{NaOH}}(\text{ml})$	A_{588}	A_{618}	A_{666}	pH	A_{666}/A_{588}	A_{666}/A_{618}
0.002	0.2269	0.3919	0.4694	3.45	2.07	1.20
0.004	0.2269	0.3916	0.4638	3.55	2.04	1.18
0.010	0.2301	0.3922	0.4409	4.11	1.92	1.12
0.013	0.2264	0.3822	0.4202	5.28	1.86	1.10
0.014	0.2343	0.3752	0.4050	6.30	1.73	1.08
0.015	0.2379	0.3536	0.3735	6.90	1.57	1.06
0.016	0.2616	0.3201	0.3206	7.21	1.23	1.00
0.017	0.2708	0.3044	0.2937	7.60	1.08	0.96
0.018	0.2748	0.2796	0.2511	7.83	0.91	0.90
0.019	0.2762	0.2540	0.2067	8.07	0.75	0.81
0.021	0.2741	0.2160	0.1346	8.26	0.49	0.62
0.025	0.2943	0.2307	0.1324	8.87	0.45	0.57
0.027	0.3103	0.2482	0.1456	9.12	0.47	0.59
0.029	0.3222	0.2616	0.1572	9.36	0.49	0.60
0.031	0.3245	0.2644	0.1597	9.55	0.49	0.60

The starting concentration of N-MTAMPP was 2.869×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M;

Combine the experimental data shown in 3-2.1 and 3-2.2:

A_{666}/A_{588} vs. pH: $\text{pK}_3 = 7.4$, $\text{pK}_4 = 2.4$,

A_{666}/A_{618} vs. pH: $\text{pK}_3 = 7.4$, $\text{pK}_4 = 2.4$.

Appendix 4

The Experimental Data of Potentiometric Titration to N-MTAMPP

4-1 [I] = 0.12 M

4-1.1 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	3.50		
		42.5	0.0020
0.004	3.67	95.0	0.0050
0.006	3.86	80.0	0.0070
0.008	4.02	205.0	0.0090
0.010	4.43	600.0	0.0110
0.012	5.63	840.0	0.0130
0.014	7.31	395.0	0.0150
0.016	8.10	90.0	0.0170
0.018	8.28	70.0	0.0190
0.020	8.42	130.0	0.0210
0.022	8.68	220.0	0.0230
0.024	9.12	156.0	0.0265
0.029	9.90	20.0	0.0315
0.034	10.00		

The starting concentration of TAMPP was 4.330×10^{-5} M;
 The starting volume was 3 ml;
 The concentration of NaOH titrated was 0.0922 M.
 $\text{pK}_a = 7.8$

4-1.2 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	3.55		
0.002	3.64	41.5	0.0010
0.004	3.74	52.0	0.0030
0.006	3.87	66.5	0.0050
0.008	4.07	98.5	0.0070
0.010	4.40	162.5	0.0090
0.011	4.72	325.0	0.0105
0.012	5.56	836.0	0.0115
0.013	6.67	1114.0	0.0125
0.014	7.69	1021.0	0.0135
0.015	8.12	431.0	0.0145
0.016	8.29	171.0	0.0155
0.017	8.44	147.0	0.0165
0.018	8.55	105.0	0.0175
0.019	8.64	93.0	0.0185
0.020	8.76	118.0	0.0195
0.021	8.96	206.0	0.0205
0.022	9.23	263.0	0.0215
0.023	9.42	191.0	0.0225
0.024	9.56	142.0	0.0235
0.025	9.70	137.0	0.0245
0.026	9.80	103.0	0.0255
0.028	9.96	82.0	0.0270
0.030	10.1	65	0.0290

The starting concentration of TAMPP was 4.330×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

pKa = 7.9

4-1.3 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	3.50		
0.004	3.66	39.8	0.0020
0.006	3.77	55.0	0.0050
0.008	3.94	84.5	0.0070
0.010	4.20	130.0	0.0090
0.012	4.90	350.5	0.0110
0.0133	5.80	692.3	0.01265
0.0140	6.43	900.0	0.01365
0.0150	6.98	549.0	0.0145
0.0160	7.33	347.0	0.0155
0.0170	7.71	378.0	0.0165
0.0180	7.90	196.0	0.0175
0.0190	8.04	138.0	0.0185
0.020	8.17	124.0	0.0195
0.021	8.22	56.0	0.0205
0.022	8.26	40.0	0.0215
0.023	8.35	84.0	0.0225
0.024	8.37	28.0	0.0235
0.025	8.39	18.0	0.0245
0.026	8.42	24.0	0.0255
0.027	8.46	41.0	0.0265
0.028	8.53	68.0	0.0275
0.030	8.67	72.5	0.0290

Continued from (4-1.3)

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0.031	8.70	80.0	0.0305
0.033	8.91	104.5	0.0320
0.035	9.15	124.0	0.034
0.037	9.28	63.5	0.036
0.039	9.34	30.5	0.038
0.043	9.64	73.5	0.041
0.047	9.83	47.5	0.045
0.051	9.98	40.0	0.049

The starting concentration of TAMPP was 4.330×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

pKa = 7.8

4-2 [I] < 0.01 M

4-2.1 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	3.39		
		29.5	0.0010
0.002	3.45	53.0	0.0030
0.004	3.55	92.3	0.0070
0.010	4.11	391.7	0.0115
0.013	5.28	1023.0	0.0135
0.014	6.30	597.0	0.0145
0.015	6.90	310.0	0.0155
0.016	7.21	393.0	0.0165
0.017	7.60	231.0	0.0175
0.018	7.83	236.0	0.0185
0.019	8.07	95.0	0.020
0.021	8.26	152.5	0.023
0.025	8.87	125.0	0.026
0.027	9.12	120.0	0.028
0.029	9.36	93.0	0.030
0.031	9.55	105.0	0.032
0.033	9.90		

The starting concentration of TAMPP was 2.869×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

$\text{pK}_a = 7.3$

4-2.2 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	2.90		
0.005	2.92	3.4	0.0025
0.015	3.03	10.8	0.0100
0.025	3.13	10.5	0.0200
0.035	3.29	15.9	0.0300
0.045	3.56	26.5	0.0400
0.049	3.73	43.8	0.0470
0.051	3.86	64.0	0.0500
0.053	4.05	95.0	0.0520
0.055	4.36	158.0	0.0540
0.056	4.63	265.0	0.0555
0.057	5.15	518.0	0.0565
0.058	5.75	600.0	0.0575
0.059	6.51	760.0	0.0585
0.060	7.23	723.0	0.0595
0.061	7.79	560.0	0.0605
0.062	8.12	330.0	0.0615
0.063	8.34	224.0	0.0625
0.064	8.53	190.0	0.0635
0.065	8.67	134.0	0.0645
0.066	8.88	212.0	0.0655
0.067	9.03	145.0	0.0665
0.068	9.15	125.0	0.0675

Continued from (4-2.2)

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
		130.0	0.0685
0.069	9.28	65.0	0.0695
0.070	9.35	109.0	0.0705
0.071	9.45	86.0	0.0715
0.072	9.54	100.0	0.0725
0.073	9.64	60.0	0.0735
0.074	9.70	62.5	0.0750
0.076	9.83	52.5	0.0770
0.078	9.93	50.0	0.0790
0.080	10.0	41.0	0.0825
0.085	10.2	23.2	0.0875
0.090	10.4	18.9	0.0950
0.100	10.5	11.5	0.0105
0.110	10.7		

The starting concentration of TAMPP was 2.869×10^{-5} M;
 The starting volume was 3 ml;
 The concentration of NaOH titrated was 0.0922 M.
 $\text{pK}_a = 7.5$

4-2.3 Titration with NaOH

V_{NaOH} (ml)	pH	$\Delta\text{pH}/\Delta V$	V'_{NaOH}
0	2.81		
		6.4	0.0100
0.020	2.94	13.3	0.0300
0.040	3.21	32.9	0.0450
0.050	3.53	43.0	0.0525
0.055	3.75	357.0	0.0565
0.058	4.82	807.5	0.0590
0.060	6.44	588.0	0.0605
0.061	7.02	254.0	0.0615
0.062	7.28	351.0	0.0625
0.063	7.63	257.0	0.0635
0.064	7.89	106.0	0.0645
0.065	7.991	157.0	0.0655
0.066	8.15	90.0	0.0665
0.067	8.24	33.0	0.0675
0.068	8.27	164.5	0.0690
0.070	8.60	141.0	0.0710
0.072	8.88	114.0	0.0730
0.074	9.11	77.5	0.0750
0.076	9.27	86.0	0.0775
0.079	9.52	58.0	0.0810
0.083	9.76	47.8	0.0855
0.088	9.99		

The starting concentration of TAMPP was 2.869×10^{-5} M;

The starting volume was 3 ml;

The concentration of NaOH titrated was 0.0922 M.

$\text{pK}_a = 7.2$

Appendix 5

The Equation for the Competitive Binding of Two Different Drugs

When two different drugs, D_1 and D_2 compete binding at the same sites on a nucleic acid, with binding constant K_1 and K_2 , the following equilibrium exist in solution:



where D_1 , D_2 and NA indicate two different drugs and nucleic acid, $D_1 - NA$ and $D_2 - NA$ mean the formed binding complex between D_1 , D_2 with nucleic acid. The following equilibrium equation which describe the corresponding process (A5-1) and (A5-2) can be given:

$$K_1 = \frac{[D_1 - NA]}{[D_1][NA]} = \frac{[D_1]_b}{[D_1]_f n[NA]_f} \quad (A5-3)$$

$$K_2 = \frac{[D_2 - NA]}{[D_2][NA]} = \frac{[D_2]_b}{[D_2]_f n[NA]_f} \quad (A5-4)$$

where $[D_1]_f$, $[D_1]_b$ and $[D_2]_f$, $[D_2]_b$ are the concentrations of free (unbound) and bound drugs D_1 , D_2 respectively; $[NA]_f$ is the concentration of free (unbound) nucleic acid in base pairs; n is the number of binding sites for D_1 and D_2 per nucleic acid base pair. Therefore, the term " $n[NA]_f$ " means the concentration (in M) of free binding site on

nucleic acid which is the same in (A5-3) and (A5-4). then, the combined equation can be derived:

$$\frac{[D_1]_b}{[D_1]_f K_1} = \frac{[D_2]_b}{[D_2]_f K_2} \quad (A5-5)$$

In terms of material balance, we have:

$$n[NA]_t = n[NA]_f + [D_1]_b + [D_2]_b \quad (A5-6)$$

where $[NA]_t$ is the total concentration of nucleic acid and then " $n[NA]_t$ " means the total concentration of binding site on nucleic acid. Rearrange (A5-6) and substitute $n[NA]_f$ by (A5-3), we have:

$$[D_2]_b = n[NA]_t - [D_1]_b - \frac{[D_1]_b}{[D_1]_f n K_1} \quad (A5-7)$$

Replacing $[D_2]_b$ in (A5-5) by equation (A5-7), gives:

$$\frac{[D_1]_b}{[D_1]_f K_1} = \frac{n[NA]_t - [D_1]_b - \frac{[D_1]_b}{[D_1]_f n K_1}}{[D_2]_f K_2} \quad (A5-8)$$

Multiply both sides by $K_1 / [NA]_t$:

$$\frac{[D_1]_b}{[D_1]_f [NA]_t} = \frac{n K_1 - K_1 \frac{[D_1]_b}{[NA]_t} - \frac{[D_1]_b}{[D_1]_f [NA]_t}}{[D_2]_f K_2} \quad (A5-9)$$

Let $r_1 = [D_1]_f/[NA]_t$ (A5-9) is reduced to:

$$\frac{r_1}{[D_1]_f} = \frac{nK_1 - r_1K_1}{[D_2]_fK_2} - \frac{\frac{r_1}{[D_1]_f}}{[D_2]_fK_2} \quad (A5-10)$$

Reorganization equation (A5-10) gives:

$$\frac{r_1}{[D_1]_f} = \frac{K_1 (n - r_1)}{[D_2]_fK_2} \frac{1}{1 + 1/[D_2]_fK_2} \quad (A5-11)$$

and

$$\frac{r_1}{[D_1]_f} = \frac{K_1 (n - r_1)}{[D_2]_fK_2} \frac{[D_2]_fK_2}{1 + [D_2]_fK_2} \quad (A5-12)$$

Cancel the term " $[D_2]_fK_2$ " appered in nomenator and denominator, the last equation derived:

$$\frac{r_1}{[D_1]_f} = \frac{K_1}{1 + [D_2]_fK_2} (n - r_1) \quad (A5-13)$$

Appendix 6

Experimental Data for Affinity Constant Studies of TAMPP — CT.DNA and N-MTAMPP — CT.DNA Complexes Using Competition Binding against Ethidium Bromide Method

The concentration of stock solution: $[EB]_s = 1.42 \times 10^{-4}$ M, $[DNA]_s = 9.03 \times 10^{-5}$ M,
 $[TAMPP]_s = 1.264 \times 10^{-5}$ M, $[N-MTAMPP]_s = 3.187 \times 10^{-6}$;

Buffer composition: 0.05 M (acetic acid + sodium acetate), 0.1 M NaCl, pH=5.5.

Signal high voltage: 925 volts;

Excitation wavelength: 520 nm;

Emission wavelength: 600 nm;

Cell Compartment Temperature: 25 °C.

Data shown in (6-1) are the experimental and calculated results for determining parameter ν , n_{EB} and K_{EB} of ethidium bromide — calf thymus DNA system;

Data shown in (6-2) are the experimental and calculated results from the four trails, specified as 6-2.1, 6-2.2, 6-2.3 and 6-2.4 respectively, for determining $K_{TAP-DNA}$ and $M_{TAP-DNA}$ of TAMPP — calf thymus DNA system;

Data shown in (6-3) are the experimental and calculated results from the four trails, specified as 6-3.1, 6-3.2, 6-3.3 and 6-3.4 respectively, for determining $K_{NMP-DNA}$ and $M_{NMP-DNA}$ of N-MTAMPP — calf thymus DNA system.

6-1 EB-CT.DNA

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.9 ml	$V(EB)[EB]_s$	$V(DNA)[DNA]$	$I_t - I_o$	$[EB]_t - [EB]_b$	$[EB]_t$	$[EB]_b$
EB in ml	V(DNA)=0 ml	V(DNA)=0.1 ml	V(tol)	V(tol)	$(U-1)k$		$[DNA]_t$	$[EB]_f [DNA]_t$
V(EB)	$I_o(EB)$	$I_t(EB-DNA)$	$[EB]_t (10^{-6})$	$[DNA]_t (10^{-6})$	$[EB]_b (10^{-7})$	$[EB]_t (10^{-6})$	X (10^{-1})	Y (10^4)
0.01	1.8	18.4	0.71	4.49	3.26	0.38	0.73	10.3
0.02	3.5	30.7	1.41	4.47	5.33	0.88	1.19	8.48
0.03	5.3	39.6	2.10	4.45	6.73	1.43	1.51	7.21
0.04	7.1	46.5	2.78	4.42	7.72	2.01	1.74	6.27
0.05	8.8	52.0	3.46	4.40	8.46	2.61	1.92	5.55
0.06	10.6	56.8	4.14	4.38	9.05	3.23	2.07	4.99
0.07	12.2	60.9	4.80	4.36	9.54	3.85	2.19	4.56
0.08	14.0	64.3	5.46	4.34	9.85	4.47	2.27	4.16
0.09	15.6	67.5	6.11	4.32	1.02	5.09	2.35	3.85
0.10	17.1	70.1	6.76	4.30	1.04	5.72	2.42	3.57

Experamental data for determing $k_b(EB)$ (Start conditions: $[DNA]_t = 9.0 \times 10^{-5} M$, $V(tol)=2 ml$):

V(EB)	0.005	0.010	0.015	0.020
$I_b(EB)$	19.2	37.6	56.8	75.3
$[EB]_b \times 10^{-6}$	0.354	0.706	1.057	1.406

Linear regression result: $k_f(EB) = 2.54 \times 10^6$; $k_b(EB) = 5.36 \times 10^7$; $U(EB-AT)=21.1$

$k_f(EB) = 2.54 \times 10^6$; $K_{EB}=3.97 \times 10^5$, $n_{EB}=0.33$. ($r=0.999$)

6-2.1 TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(TAP)=0	V(Buf)=1.83 ml V(DNA)=0.1 ml V(TAP)=0.07 ml	$\frac{V(EB)(EB)_i}{V(tol)}$	$\frac{V(DNA)(DNA)}{V(tol)}$	$\frac{I_t - I_0}{(1-k)}$	$\frac{[EB]_i - [EB]_b}{[EB]_i}$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n(DNA)_i - [EB]_b}{[EB]_i / K_{EB} [EB]_f}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-TAP)$	$[EB]_i(10^{-6})$	$[DNA]_i(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_f(10^{-6})$	$[Por]_i(10^{-7})$	$[BS](Por)(10^{-7})$	X	Y
0.01	1.6	6.6	0.71	4.49	1.15	0.59	4.40	8.77	0.502	5.14
0.02	3.1	12.2	1.41	4.47	2.09	1.20	4.38	8.25	0.531	5.72
0.03	4.6	16.9	2.10	4.45	2.83	1.82	4.36	7.92	0.550	6.42
0.04	6.1	21.0	2.78	4.42	3.43	2.44	4.34	7.64	0.568	7.12
0.05	7.5	24.7	3.46	4.40	3.96	3.07	4.32	7.32	0.589	7.75
0.06	8.9	28.5	4.14	4.38	4.51	3.69	4.30	6.87	0.625	8.17
0.07	10.4	31.7	4.8	4.36	4.90	4.31	4.27	6.63	0.645	8.80
0.08	11.8	34.6	5.46	4.34	5.24	4.94	4.25	6.40	0.665	9.41
0.09	13.2	37.3	6.11	4.32	5.54	5.56	4.23	6.20	0.683	10.03
0.10	14.6	40.0	6.76	4.30	5.84	6.18	4.21	5.96	0.707	10.57

Linear regression results: $k=2.16 \times 10^6$

$$K_{TAMPP}=3.2 \times 10^6, M_{TAMPP}=3.3. \quad (r=0.996)$$

6-2.2 TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(TAP)=0	V(Buf)=1.77 ml V(DNA)=0.1 ml V(TAP)=0.13 ml	$\frac{V(EB)EB_i}{V(tol)}$	$\frac{V(DNA)DNA}{V(tol)}$	$\frac{I_t - I_0}{(1-I)}$	$\frac{[EB]_t - [EB]_b}{[EB]_t}$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[DNA]_t - [EB]_b}{[EB]_t / K_{EB}[EB]_f}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-TAP)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_t(10^{-6})$	$[Por]_t(10^{-7})$	$[BS](Por)(10^{-7})$	X	Y
0.01	1.6	4.0	0.71	4.49	0.55	0.65	8.18	12.1	0.674	11.80
0.02	3.1	7.7	1.41	4.47	1.06	1.30	8.13	11.6	0.699	12.29
0.03	4.6	11.4	2.10	4.45	1.56	1.94	8.09	11.1	0.731	12.42
0.04	6.1	14.5	2.78	4.42	1.93	2.59	8.05	10.8	0.747	13.41
0.05	7.5	17.6	3.46	4.40	2.32	3.23	8.02	10.4	0.771	13.91
0.06	8.9	20.4	4.14	4.38	2.65	3.87	7.98	10.1	0.790	14.63
0.07	10.4	23.3	4.8	4.36	2.97	4.51	7.94	9.76	0.813	15.18
0.08	11.8	25.8	5.46	4.34	3.22	5.14	7.90	9.52	0.829	15.96
0.09	13.2	28.3	6.11	4.32	3.47	5.77	7.86	9.26	0.849	16.60
0.10	14.6	30.3	6.76	4.30	3.61	6.40	7.82	9.15	0.855	17.72

Linear regression results: $k=2.16 \times 10^6$

$$K_{TAMPP}=3.8 \times 10^6, M_{TAMPP}=3.2. \quad (r=0.974)$$

6-2.3 TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(TAP)=0	V(Buf)=1.80 ml V(DNA)=0.1 ml V(TAP)=0.10 ml	$\frac{V(EB)[EB]_t}{V(tol)}$	$\frac{V(DNA)[DNA]}{V(tol)}$	$\frac{I_t I_0}{(0.1)k}$	$[EB]_t - [EB]_0$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[DNA]_t - [EB]_0}{[EB]_t / K_{EB}[EB]_t}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_t}{[EB]_0}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-TAP)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_0(10^{-7})$	$[EB]_t(10^{-6})$	$[Por]_t(10^{-7})$	$[BS](Por)(10^{-7})$	X	Y
0.01	1.6	4.9	0.71	4.49	0.76	0.63	6.29	11.0	0.570	8.31
0.02	3.1	9.3	1.41	4.47	1.43	1.26	6.26	10.5	0.597	8.86
0.03	4.6	13.3	2.10	4.45	2.00	1.90	6.23	10.0	0.622	9.49
0.04	6.1	16.6	2.78	4.42	2.42	2.54	6.20	9.79	0.632	10.53
0.05	7.5	20.0	3.46	4.40	2.88	3.18	6.17	9.37	0.658	11.05
0.06	8.9	23.0	4.14	4.38	3.24	3.81	6.14	9.07	0.676	11.75
0.07	10.4	25.8	4.8	4.36	3.54	4.45	6.11	8.84	0.690	12.56
0.08	11.8	28.3	5.46	4.34	3.80	5.08	6.08	8.64	0.703	13.39
0.09	13.2	30.7	6.11	4.32	4.03	5.71	6.05	8.45	0.715	14.19
0.10	14.6	33.0	6.76	4.30	4.23	6.34	6.02	8.27	0.728	14.98

Linear regression results: $k=2.16 \times 10^6$

$$K_{TAMPP}=6.6 \times 10^6, M_{TAMPP}=2.6. \quad (r=0.985)$$

6-2.4 TAMPP—CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated	$V(Bu)=2.0$ ml	$V(Bu)=1.80$ ml	$\frac{V(EB)EB}{V(DNA)=0$ ml	$\frac{V(DNA)DNA}{V(EB)}$	$\frac{I_0}{(n-1)k}$	$\frac{[EB]_t[EB]_b}{[EB]_t[EB]_b}$	$\frac{V(Pox)Pox}{V(EB)}$	$\frac{\eta(DNA)_t[EB]_b}{[EB]_t K_{EB}[EB]_t}$	$\frac{[Pox]_t}{[BS](Pox)}$	$\frac{[EB]_t}{[EB]_b}$
EB in ml	$V(TAP)=0$	$V(TAP)=0.10$ ml	$V(EB)$	$V(DNA)$	$(n-1)k$	$[EB]_t[EB]_b$	$V(EB)$	$[EB]_t[EB]_b$	$[BS](Pox)$	$[EB]_b$
$V(EB)$	$I_0(EB)$	$I_0(EB-DNA-TAP)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_t(10^{-6})$	$[Pox]_t(10^{-7})$	$[BS](Pox)(10^{-7})$	x	y
0.01	1.6	4.9	0.71	4.49	0.76	0.63	6.29	11.0	0.570	8.31
0.02	3.1	9.4	1.41	4.47	1.45	1.26	6.26	10.4	0.602	8.70
0.03	4.6	13.7	2.10	4.45	2.09	1.89	6.23	9.79	0.636	9.03
0.04	6.1	17.4	2.78	4.42	2.60	2.52	6.20	9.41	0.659	9.71
0.05	7.5	20.5	3.46	4.40	2.99	3.16	6.17	9.16	0.673	10.58
0.06	8.9	23.8	4.14	4.38	3.43	3.79	6.14	8.76	0.701	11.07
0.07	10.4	26.6	4.8	4.36	3.73	4.43	6.11	8.54	0.714	11.89
0.08	11.8	29.2	5.46	4.34	4.00	5.06	6.08	8.33	0.730	12.65
0.09	13.2	31.7	6.11	4.32	4.26	5.69	6.05	8.11	0.746	13.37
0.10	14.6	34.1	6.76	4.30	4.49	6.34	6.02	7.91	0.761	14.07

Linear regression results: $k=2.16 \times 10^6$

$$K_{TAMPP}=4.1 \times 10^6, M_{TAMPP}=3.0. \quad (r=0.971)$$

6-3.1 N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(NMP)=0	V(Buf)=1.80 ml V(DNA)=0.10 ml V(NMP)=0.10 ml	$\frac{V(EB)I_0}{V(tot)}$	$\frac{V(DNA)I_0}{V(tot)}$	$\frac{I_0 - I}{(1-k)}$	$\frac{[EB]_i - [EB]_b}{[EB]_i}$	$\frac{V(Por)I_0}{V(tot)}$	$\frac{a(DNA)_i - [EB]_b - [EB]_i / K_{EB}[EB]_i}{[BS](Por)}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_i}{[EB]_b}$
V(EB)	I ₀ (EB)	I ₀ (E-DNA-NMP)	[EB] _i (10 ⁻⁶)	[DNA] _i (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁷)	[BS](Por)(10 ⁻⁷)	X	Y
0.03	6.5	31.7	2.10	4.45	4.07	1.69	1.57	4.53	0.347	4.15
0.04	8.4	39.4	2.78	4.42	5.01	2.28	1.56	4.06	0.385	4.55
0.05	10.5	46.1	3.46	4.40	5.76	2.89	1.55	3.75	0.414	5.02
0.06	12.6	51.7	4.14	4.38	6.32	3.50	1.55	3.59	0.431	5.54
0.07	14.7	57.1	4.8	4.36	6.86	4.12	1.54	3.34	0.461	6.00
0.08	16.8	61.5	5.46	4.34	7.23	4.74	1.53	3.25	0.471	6.56
0.09	18.9	65.5	6.11	4.32	7.53	5.36	1.52	3.18	0.480	7.12
0.10	21.0	69.7	6.76	4.30	7.87	5.97	1.52	2.99	0.508	7.59

Linear regression results: $k=3.08 \times 10^6$

$$K_{N-MTAMPP} = 1.56 \times 10^6, M_{N-MTAMPP} = 5.67. \quad (r=0.975)$$

6-3.2 N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(NMP)=0	V(Buf)=1.70 ml V(DNA)=0.10 ml V(NMP)=0.20 ml	$\frac{V(EB)(EB)_i}{V(tol)}$	$\frac{V(DNA)(DNA)}{V(tol)}$	$\frac{I-I_0}{(I_0-1)k}$	$(EB)_i(EB)_b$	$\frac{V(Por)(Por)}{V(tol)}$	$\frac{n(DNA)_i(EB)_b}{(EB)_i/K_{EB}(EB)_f}$	$\frac{(Por)_i}{(BS)(Por)}$	$\frac{(EB)_f}{(EB)_b}$
V(EB)	$I_0(EB)$	$I_i(E-DNA-NMP)$	$(EB)_i(10^{-6})$	$(DNA)_i(10^{-6})$	$(EB)_b(10^{-7})$	$(EB)_f(10^{-6})$	$(Por)_i(10^{-7})$	$(BS)(Por)(10^{-7})$	X	Y
0.01	1.7	7.2	0.71	4.49	1.12	0.59	3.17	8.92	0.355	5.28
0.02	3.4	13.0	1.41	4.47	1.96	1.21	3.16	8.69	0.363	6.16
0.03	5.2	18.1	2.10	4.45	2.64	1.83	3.14	8.41	0.373	6.96
0.04	6.8	23.0	2.78	4.42	3.31	2.45	3.12	7.89	0.396	7.40
0.05	8.5	27.3	3.46	4.40	3.84	3.08	3.11	7.54	0.412	8.01
0.06	10.2	31.3	4.14	4.38	4.31	3.70	3.09	7.21	0.429	8.59
0.07	11.8	34.9	4.80	4.36	4.72	4.33	3.08	6.92	0.445	9.17
0.08	13.4	38.1	5.46	4.34	5.05	4.96	3.06	6.70	0.457	9.81
0.09	14.9	41.5	6.11	4.32	5.44	5.57	3.05	6.35	0.480	10.2
0.10	16.1	44.8	6.76	4.30	5.87	6.18	3.04	5.92	0.513	10.5

Linear regression results: $k=2.43 \times 10^6$

$$K_{N-MTAMPP} = 2.28 \times 10^6, M_{N-MTAMPP} = 5.75. \quad (r=0.975)$$

6-3.3 N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(NMP)=0	V(Buf)=1.70 ml V(DNA)=0.10 ml V(NMP)=0.20 ml	$\frac{V(EB)[EB]_t}{V(tol)}$	$\frac{V(DNA)[DNA]}{V(tol)}$	$\frac{I_t - I_0}{(0.1)k}$	$[EB]_t - [EB]_b$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n(DNA)_t - [EB]_b}{[EB]_t / K_{EB}[EB]_t}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_t}{[EB]_b}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-NMP)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_t(10^{-6})$	$[Por]_t(10^{-7})$	$[BS](Por)(10^{-7})$	X	Y
0.01	1.5	6.2	0.71	4.49	1.16	0.60	3.17	9.31	0.341	5.66
0.02	3.2	11.8	1.41	4.47	1.94	1.21	3.16	8.77	0.360	6.25
0.03	4.7	16.8	2.10	4.45	2.73	1.83	3.14	8.18	0.384	6.69
0.04	6.2	21.0	2.78	4.42	3.34	2.45	3.12	7.83	0.399	7.34
0.05	7.7	25.3	3.46	4.40	3.97	3.07	3.11	7.30	0.426	7.72
0.06	9.2	28.3	4.14	4.38	4.31	3.71	3.09	7.22	0.428	8.60
0.07	10.6	31.8	4.80	4.36	4.78	4.32	3.08	6.82	0.451	9.04
0.08	12.0	35.1	5.46	4.34	5.21	4.94	3.06	6.45	0.475	9.48
0.09	13.5	37.8	6.11	4.32	5.48	5.57	3.05	6.29	0.485	10.2
0.10	14.8	40.5	6.76	4.30	5.80	6.18	3.04	6.03	0.504	10.7

Linear regression results: $k=2.21 \times 10^6$

$$K_{N-MTAMPP} = 1.97 \times 10^6, M_{N-MTAMPP} = 6.23. \quad (r=0.991)$$

6-3.4 N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(NMP)=0	V(Buf)=1.70 ml V(DNA)=0.10 ml V(NMP)=0.20 ml	$\frac{V(EB) \times [EB]_i}{V(tol)}$	$\frac{V(DNA) \times [DNA]}{V(tol)}$	$\frac{I_t - I_0}{(I_0 - I_t)}$	$\frac{[EB]_i - [EB]_b}{[EB]_i - [EB]_b}$	$\frac{V(Por) \times [Por]}{V(tol)}$	$\frac{n[DNA]_i - [EB]_b}{[EB]_i / K_{EB} [EB]_f}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-NMP)$	$[EB]_i(10^{-6})$	$[DNA]_i(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_f(10^{-6})$	$[Por]_i(10^{-7})$	$[BS](Por)(10^{-7})$	X	Y
0.01	1.7	6.4	0.71	4.49	0.96	0.61	3.17	9.91	0.320	6.38
0.02	3.5	12.3	1.41	4.47	1.79	1.23	3.16	9.27	0.340	6.84
0.03	5.1	17.2	2.10	4.45	2.47	1.85	3.14	8.85	0.355	7.51
0.04	6.8	21.7	2.78	4.42	3.04	2.48	3.12	8.48	0.368	8.17
0.05	8.5	25.8	3.46	4.40	3.53	3.11	3.11	8.15	0.382	8.82
0.06	10.1	29.6	4.14	4.38	3.97	3.74	3.09	7.81	0.396	9.41
0.07	11.8	33.0	4.80	4.36	4.32	4.37	3.08	7.58	0.406	10.1
0.08	13.4	36.6	5.46	4.34	4.73	4.99	3.06	7.21	0.425	10.6
0.09	14.9	39.5	6.11	4.32	5.01	5.61	3.05	6.99	0.436	11.2
0.10	16.4	42.3	6.76	4.30	5.28	6.23	3.04	6.77	0.448	11.8

Linear regression results: $k=2.44 \times 10^6$

$$- K_{N-MTAMPP} = 3.11 \times 10^6, M_{N-MTAMPP} = 5.57. \quad (r=0.997)$$

Appendix 7

Experimental Data for Affinity Constant Studies of TAMPP — Poly(dA-dT)₂ and N-MTAMPP — Poly(dA-dT)₂ Complexes Using Competition Binding against Ethidium Bromide Methold

The concentration of stock solution: $[EB]_s = 1.42 \times 10^{-4}$ M, $[TAMPP]_s = 1.264 \times 10^{-5}$ M, $[N-MTAMPP] = 3.187 \times 10^{-6}$; $[Poly(dA-dT)_2]_s = 8.76 \times 10^{-5}$ M in all (7-1) and (7-2) trials, $[Poly(dA-dT)_2]_s = 7.49 \times 10^{-5}$ M in (7-3.1) and (7-3.2) trials and $[Poly(dA-dT)_2]_s = 9.59 \times 10^{-5}$ M in (7-3.2) and (7-3.3) trials;

Buffer composition: 0.05 M (acetic acid + sodium acetate), 0.1 M NaCl, pH=5.5.

Signal high votage: 925 volts;

Excitation wavelength: 520 nm;

Emission wavelength: 600 nm;

Cell Compartment Temperature: 25 °C.

Data shown in (7-1.1) and (7-1.2) are the experemental and calculated results for determing parameter ν , n_{EB} and affinity constant K_{EB} of ethidium bromide — Poly(dA-dT)₂ system;

Average Result from (7-1.1) & (7-1.2) are: $\nu(EB-AT) = 24.2$, $K_{EB-AT} = 2.77 \times 10^5$, $n_{EB-AT} = 0.72$.

Data shown in (7-2) are the experemental and calculated results from the four trails, specified as 7-2.1, 7-2.2, 7-2.3 and 7-2.4 respectively, for determing K_{TAP-AT} and M_{TAP-AT} of TAMPP — Poly(dA-dT)₂ system.

Data shown in (7-3) are the experimental and calculated results from the four trials, specified as 7-3.1, 7-3.2, 7-3.3 and 7-3.4 respectively, for determining $K_{\text{NMP-AT}}$ and $M_{\text{NMP-AT}}$ of N-MTAMPP — Poly(dA-dT)₂ system.

7-1. 1 EB — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.9 ml	$V(EB) \backslash [EB]_s$	$V(AT) \backslash [AT]_t$	$I_t - I_0$	$[EB]_t - [EB]_b$	$[EB]_t$	$[EB]_b$
EB in ml	V(AT)=0 ml	V(AT)=0.1 ml	V(tol)	V(tol)	(U-1)k		$[AT]_t$	$[EB]_t \backslash [AT]_t$
V(EB)	$I_0(EB)$	$I_t(EB-AT)$	$[EB]_t(10^{-6})$	$[AT]_t(10^{-6})$	$[EB]_b(10^{-6})$	$[EB]_t(10^{-6})$	X	Y(10^4)
0.01	1.8	29.7	0.71	4.36	0.52	0.71	0.118	16.7
0.02	3.4	51.0	1.41	4.34	0.88	1.41	0.202	14.4
0.03	5.2	67.4	2.10	4.32	1.15	2.10	0.266	12.7
0.04	6.7	81.1	2.78	4.30	1.37	2.78	0.320	11.5
0.05	8.5	91.6	3.46	4.28	1.53	3.46	0.359	10.4
0.06	10.0	99.6	4.14	4.25	1.65	4.14	0.389	9.39
0.07	11.6	105.2	4.80	4.23	1.73	4.80	0.408	8.49
0.08	13.1	110.9	5.46	4.21	1.80	5.46	0.428	7.84
0.09	14.5	114.5	6.11	4.19	1.84	6.11	0.440	7.20
0.10	15.9	119.0	6.76	4.17	1.90	6.76	0.456	6.74

Experimental data for determining $k_b(EB)$ (Start conditions: $[Poly(dA-dT)_2]_t = 1.30 \times 10^{-5}$, $V(tol)=2$ ml):

V(EB)	0.030	0.032	0.034	0.036	0.038	0.040
$I_b(EB)$	120.5	127.5	134.5	141.5	148.9	156.8
$[EB]_b(10^{-6})$	2.098	2.236	2.374	2.511	2.648	2.784

Linear regression result: $k_f(EB) = 2.39 \times 10^6$; $k_b(EB) = 5.66 \times 10^7$; $U(EB-AT)=23.7$

$k_f(EB) = 2.39 \times 10^6$; $K_{EB} = 2.93 \times 10^5$, $n_{EB} = 0.70$. ($r=0.999$)

7-1 2. EB — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.9 ml	$\frac{V(EB)X[EB]_s}{V(tol)}$	$\frac{V(AT)X[AT]_s}{V(tol)}$	$\frac{I_t - I_o}{(U-1)k}$	$\frac{[EB]_t - [EB]_b}{[AT]_t}$	$\frac{[EB]_t}{[AT]_t}$	$\frac{[EB]_b}{[EB]_t[AT]_t}$
EB in ml	V(AT)=0 ml	V(AT)=0.1 ml						
V(EB)	I ₀ (EB)	I _t (EB-AT)	[EB] _t (10 ⁻⁶)	[AT] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁶)	[EB] _t (10 ⁻⁶)	X	Y(10 ⁶)
0.01	1.4	24.6	0.71	4.36	0.48	0.71	0.110	15.6
0.02	2.9	43.2	1.41	4.34	0.84	1.41	0.193	13.7
0.03	4.3	59.3	2.10	4.32	1.14	2.10	0.265	12.6
0.04	5.6	70.5	2.78	4.30	1.35	2.78	0.313	11.2
0.05	7.0	80.2	3.46	4.28	1.52	3.46	0.355	10.3
0.06	8.4	86.9	4.14	4.25	1.63	4.14	0.383	9.26
0.07	9.7	91.9	4.80	4.23	1.70	4.80	0.403	8.38
0.08	11.1	97.0	5.46	4.21	1.78	5.46	0.423	7.75
0.09	12.4	101.1	6.11	4.19	1.84	6.11	0.439	7.18
0.10	13.7	104.1	6.76	4.17	1.88	6.76	0.449	6.65

Experamental data for determing $k_b(EB)$ (Start conditions:[Poly(dA-dT)₂]_t = 1.8x10⁻⁵, V(tol)=2 ml):

V(EB)	0.022	0.024	0.026	0.028	0.030
I ₀ (EB)	78.8	85.2	91.6	98.0	104.4
[EB] _b (10 ⁻⁶)	1.545	1.684	1.822	1.961	2.099

Linear regression result: $k_f(EB) = 2.03 \times 10^6$; $k_b(EB) = 5.03 \times 10^7$; $\nu(EB-AT)=24.7$

$k_f(EB) = 2.03 \times 10^6$; $K_{EB} = 2.60 \times 10^5$, $n_{EB} = 0.73$. (r=0.999)

7-2.1 TAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(TAP)=0 ml	V(Buf)=1.83 ml V(dAT)=0.10ml V(TAP)=0.07ml	$\frac{V(EB)I_0}{V(tot)}$	$\frac{V(AT)I_0}{V(tot)}$	$\frac{I_t - I_0}{(1-I)}$	$\frac{[EB]_t - [EB]_0}{[EB]_t}$	$\frac{V(Por)I_0}{V(tot)}$	$\frac{n(AT)_t - [EB]_0}{[EB]_0 / K_{EB} [EB]_t}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_t}{[EB]_0}$
V(EB)	I ₀ (EB)	I _t (EB-AT-TAP)	[EB] _t (10 ⁻⁶)	[dAT] _t (10 ⁻⁶)	[EB] ₀ (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	[Por] _t (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.6	9.8	0.71	4.36	1.68	0.54	4.40	1.84	0.239	3.20
0.02	3.1	18.3	1.41	4.34	3.12	1.09	4.38	1.78	0.246	3.51
0.03	4.5	25.7	2.10	4.32	4.35	1.66	4.36	1.73	0.252	3.82
0.04	5.9	32.3	2.78	4.30	5.42	2.24	4.34	1.68	0.258	4.14
0.05	7.2	38.1	3.46	4.28	6.34	2.83	4.32	1.64	0.264	4.46
0.06	8.7	43.8	4.14	4.25	7.20	3.42	4.30	1.58	0.272	4.74
0.07	10.2	48.7	4.80	4.23	7.90	4.01	4.27	1.55	0.276	5.08
0.08	11.6	53.3	5.46	4.21	8.56	4.61	4.25	1.51	0.282	5.38
0.09	12.7	57.7	6.11	4.19	9.23	5.19	4.23	1.45	0.291	5.62
0.10	14.1	62.0	6.76	4.17	9.83	5.78	4.21	1.41	0.299	5.88

Linear regression results: $k=2.10 \times 10^6$

$$K_{TAP-AT} = 2.16 \times 10^6, M_{TAP-AT} = 5.93. \quad (r=0.996)$$

7-2.2 TAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(TAP)=0 ml	V(Buf)=1.83 ml V(dAT)=0.10ml V(TAP)=0.07ml	$\frac{V(EB)(EB)_i}{V(tol)}$	$\frac{V(AT)(AT)}{V(tol)}$	$\frac{I_i - I_0}{(0.1)k}$	$[EB]_i - [EB]_0$	$\frac{V(Por)(Por)}{V(tol)}$	$\frac{n(AT)_i - [EB]_0}{[EB]_0 / K_{EB} [EB]_i}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_i}{[EB]_0}$
V(EB)	I ₀ (EB)	I _i (EB-AT-TAP)	[EB] _i (10 ⁻⁶)	[dAT] _i (10 ⁻⁶)	[EB] ₀ (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.8	11.8	0.71	4.36	1.81	0.53	4.40	1.72	0.257	2.90
0.02	3.4	22.0	1.41	4.34	3.35	1.07	4.38	1.66	0.264	3.19
0.03	5.2	31.6	2.10	4.32	4.76	1.62	4.36	1.57	0.277	3.41
0.04	6.7	39.5	2.78	4.30	5.91	2.19	4.34	1.53	0.284	4.71
0.05	8.5	46.8	3.46	4.28	6.90	2.77	4.32	1.49	0.290	4.02
0.06	10.0	53.2	4.14	4.25	7.79	3.36	4.30	1.45	0.297	4.31
0.07	11.6	59.4	4.80	4.23	8.62	3.94	4.27	1.40	0.306	5.57
0.08	13.1	64.9	5.46	4.21	9.34	4.53	4.25	1.36	0.314	5.85
0.09	14.5	70.6	6.11	4.19	10.1	5.10	4.23	1.29	0.327	5.05
0.10	15.9	74.5	6.76	4.17	10.6	5.71	4.21	1.28	0.329	5.40

Linear regression results: $k=2.39 \times 10^6$

$$K_{TAP-AT} = 1.57 \times 10^6, M_{TAP-AT} = 5.88. \quad (r=0.992)$$

7-2.3 TAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(TAP)=0 ml	V(Buf)=1.83 ml V(dAT)=0.10ml V(TAP)=0.07ml	$\frac{V(EB)EB_i}{V(tol)}$	$\frac{V(AT)AT}{V(tol)}$	$\frac{I_t - I_0}{(0.1)k}$	$EB_i - EB_b$	$\frac{V(Por)Por}{V(tol)}$	$\frac{n(AT)_t - [EB]_b}{- [EB]_b / K_{EB} [EB]_f}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	I ₀ (EB)	I _t (EB-AT-TAP)	[EB] _i (10 ⁻⁶)	[dAT] _i (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _f (10 ⁻⁶)	[Por] _t (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.7	10.3	0.71	4.36	1.55	0.55	4.40	1.97	0.223	3.56
0.02	3.4	19.5	1.41	4.34	2.90	1.12	4.38	1.90	0.231	3.85
0.03	5.1	27.3	2.10	4.32	4.00	1.70	4.36	1.86	0.235	4.25
0.04	6.7	34.5	2.78	4.30	5.01	2.28	4.34	1.80	0.241	4.56
0.05	8.3	41.0	3.46	4.28	5.89	2.87	4.32	1.75	0.247	4.88
0.06	9.8	46.8	4.14	4.25	6.67	3.47	4.30	1.70	0.252	5.21
0.07	11.5	52.1	4.80	4.23	7.31	4.07	4.27	1.67	0.256	5.57
0.08	13.2	57.1	5.46	4.21	7.91	4.67	4.25	1.63	0.261	5.91
0.09	14.6	61.3	6.11	4.19	8.41	5.27	4.23	1.60	0.264	6.27
0.10	16.1	65.9	6.76	4.17	8.97	5.86	4.21	1.56	0.271	6.54

Linear regression results: $k=2.39 \times 10^6$

$$K_{TAP-AT} = 3.09 \times 10^6, M_{TAP-AT} = 5.86. \quad (r=0.996)$$

7-2.4TAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(TAP)=0 ml	V(Buf)=1.83 ml V(dAT)=0.10ml V(TAP)=0.07ml	$\frac{V(EB)X[EB]_t}{V(tol)}$	$\frac{V(AT)X(AT)}{V(tol)}$	$\frac{I_t - I_0}{(I - I_0)k}$	$[EB]_t - [EB]_b$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n(AT)_t - [EB]_b}{[EB]_b / K_{EB} [EB]_t}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_t}{[EB]_b}$
V(EB)	I ₀ (EB)	I _t (EB-AT-TAP)	[EB] _t (10 ⁻⁶)	[dAT] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	[Por] _t (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.7	10.3	0.71	4.36	1.52	0.56	4.40	2.00	0.220	3.65
0.02	3.5	19.6	1.41	4.34	2.84	1.12	4.38	1.92	0.228	3.95
0.03	5.1	27.6	2.10	4.32	3.97	1.70	4.36	1.87	0.233	4.28
0.04	6.8	34.9	2.78	4.30	4.96	2.29	4.34	1.81	0.239	4.61
0.05	8.5	41.5	3.46	4.28	5.83	2.88	4.32	1.77	0.244	4.94
0.06	10.1	47.2	4.14	4.25	6.55	3.48	4.30	1.73	0.248	5.31
0.07	11.8	53.0	4.80	4.23	7.27	4.07	4.27	1.68	0.255	5.60
0.08	13.4	58.0	5.46	4.21	7.87	4.67	4.25	1.64	0.260	5.94
0.09	14.9	62.5	6.11	4.19	8.40	5.27	4.23	1.60	0.264	6.28
0.10	16.4	67.2	6.76	4.17	8.97	5.87	4.21	1.56	0.271	6.54

Linear regression results: $k=2.44 \times 10^6$

$$K_{TAP-AT} = 2.68 \times 10^6, M_{TAP-AT} = 6.21. \quad (r=0.998)$$

7-3.1 N-MTAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(NMP)=0 ml	V(Buf)=1.78 ml V(AT)=0.12ml V(NMP)=0.10ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(AT)[AT]}{V(tol)}$	$\frac{I_t - I_0}{(0.1)k}$	$[EB]_t - [EB]_b$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[AT]_t - [EB]_b}{[EB]_b / K_{EB}[EB]_t}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_t}{[EB]_b}$
V(EB)	I ₀ (EB)	I _t (EB-AT-NMP)	[EB] _t (10 ⁻⁶)	[AT] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	[Por] _t (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.4	11.4	0.71	4.47	2.30	0.48	1.59	1.25	0.127	2.08
0.02	2.7	21.2	1.41	4.45	4.25	0.98	1.58	1.22	0.130	2.31
0.03	3.9	29.6	2.10	4.43	5.90	1.51	1.57	1.19	0.132	2.56
0.04	5.2	36.7	2.78	4.41	7.23	2.06	1.56	1.18	0.132	2.85
0.05	6.5	43.4	3.46	4.39	8.47	2.62	1.55	1.14	0.136	3.09
0.06	7.7	49.7	4.14	4.36	9.64	3.17	1.55	1.08	0.143	3.29
0.07	9.0	54.8	4.80	4.34	10.5	3.75	1.54	1.06	0.145	3.57
0.08	10.3	59.7	5.46	4.32	11.3	4.33	1.53	1.03	0.148	3.82
0.09	11.5	63.5	6.11	4.30	11.9	4.92	1.52	1.03	0.148	4.12
0.10	12.7	67.5	6.76	4.28	12.6	5.50	1.52	1.00	0.152	4.38

Linear regression results: $k=1.88 \times 10^6$

$$K_{NMP-AT} = 2.33 \times 10^6, M_{NMP-AT} = 9.91. \quad (r=0.978)$$

7-3.2 N-MTAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(NMP)=0 ml	V(Buf)=1.78 ml V(AT)=0.12ml V(NMP)=0.10ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(AT)[AT]}{V(tol)}$	$\frac{I_t - I_0}{(I_t - I_0)k}$	$\frac{[EB]_t - [EB]_b}{[EB]_t}$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[AT]_t - [EB]_b}{-[EB]_b / K_{EB} [EB]_f}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	I ₀ (EB)	I _t (EB-AT-NMP)	[EB] _t (10 ⁻⁶)	[AT] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	[Por] _t (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.5	10.4	0.71	4.47	1.99	0.51	1.59	1.60	0.099	2.54
0.02	2.8	19.5	1.41	4.45	3.74	1.03	1.58	1.52	0.104	2.76
0.03	4.2	27.2	2.10	4.43	5.15	1.58	1.57	1.50	0.105	3.03
0.04	5.6	33.9	2.78	4.41	6.34	2.15	1.56	1.47	0.106	3.39
0.05	6.7	40.0	3.46	4.39	7.46	2.72	1.55	1.42	0.109	3.64
0.06	7.8	45.2	4.14	4.36	8.38	3.30	1.55	1.39	0.112	3.94
0.07	9.2	50.3	4.80	4.34	9.21	3.88	1.54	1.35	0.114	4.22
0.08	10.5	54.6	5.46	4.32	9.88	4.47	1.53	1.33	0.115	4.53
0.09	11.7	58.8	6.11	4.30	10.6	5.06	1.52	1.29	0.118	4.80
0.10	13.0	62.5	6.76	4.28	11.1	5.65	1.52	1.27	0.120	5.10

Linear regression results: $k=1.92 \times 10^6$

$$K_{NMP-AT} = 2.79 \times 10^6, M_{NMP-AT} = 12.49. \quad (r=0.992)$$

7-3.3 N-MTAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(NMP)=0 ml	V(Buf)=1.809 ml V(AT)=0.091 ml V(NMP)=0.10 ml	$\frac{V(EB) \times [EB]_i}{V(tot)}$	$\frac{V(AT) \times [AT]}{V(tot)}$	$\frac{I_0 - I}{(0.1) \times}$	$[EB]_i - [EB]_b$	$\frac{V(Por) \times [Por]}{V(tot)}$	$\frac{n(AT)_i - [EB]_b}{[EB]_b / K_{EB} [EB]_i}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_i}{[EB]_b}$
V(EB)	I ₀ (EB)	I _i (EB-AT-NMP)	[EB] _i (10 ⁻⁶)	[AT] _i (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.7	11.8	0.71	4.34	1.82	0.52	1.59	1.69	0.094	2.87
0.02	3.4	21.7	1.41	4.32	3.30	1.08	1.58	1.67	0.094	3.26
0.03	5.0	30.2	2.10	4.30	4.55	1.64	1.57	1.64	0.096	3.61
0.04	6.6	38.0	2.78	4.28	5.67	2.22	1.56	1.59	0.098	3.91
0.05	8.2	45.1	3.46	4.25	6.66	2.80	1.55	1.54	0.101	4.20
0.06	9.7	51.5	4.14	4.23	7.55	3.38	1.55	1.49	0.104	4.48
0.07	11.5	57.2	4.80	4.21	8.25	3.98	1.54	1.46	0.105	4.82
0.08	13.2	62.1	5.46	4.19	8.83	4.58	1.53	1.44	0.106	5.19

Linear regression results: $k=2.39 \times 10^6$

$$K_{NMP-AT} = 3.14 \times 10^6, M_{NMP-AT} = 13.57 \quad (r=0.978)$$

7-3.4 N-MTAMPP — Poly(dA-dT)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(AT)=0 ml V(NMP)=0 ml	V(Buf)=1.809 ml V(AT)=0.091 ml V(NMP)=0.10 ml	$\frac{V(EB)(EB)_i}{V(tol)}$	$\frac{V(AT)(AT)}{V(tol)}$	$\frac{I_t - I_0}{(0.1)k}$	$\frac{[EB]_i - [EB]_0}{[EB]_i}$	$\frac{V(Por)(Por)}{V(tol)}$	$\frac{n(AT)_i - [EB]_0}{[EB]_0 / K_{EB} [EB]_i}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_i}{[EB]_0}$
V(EB)	I ₀ (EB)	I _t (EB-AT-NMP)	[EB] _i (10 ⁻⁶)	[AT] _i (10 ⁻⁶)	[EB] ₀ (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁷)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	1.7	11.9	0.71	4.34	1.85	0.52	1.59	1.66	0.095	2.83
0.02	3.4	21.3	1.41	4.32	3.24	1.08	1.58	1.70	0.093	3.34
0.03	5.0	29.9	2.10	4.30	4.51	1.65	1.57	1.66	0.095	3.66
0.04	6.6	37.7	2.78	4.28	5.63	2.22	1.56	1.60	0.098	3.95
0.05	8.2	44.5	3.46	4.25	6.57	2.81	1.55	1.56	0.100	4.27
0.06	9.7	51.4	4.14	4.23	7.55	3.38	1.55	1.49	0.104	4.48
0.07	11.5	57.2	4.80	4.21	8.27	3.98	1.54	1.46	0.106	4.81
0.08	13.2	62.2	5.46	4.19	8.87	4.57	1.53	1.43	0.107	5.16
0.09	14.6	67.0	6.11	4.17	9.48	5.17	1.52	1.39	0.109	5.45
0.10	16.0	71.3	6.76	4.15	10.0	5.76	1.52	1.36	0.111	5.76

Linear regression results: $k=2.38 \times 10^6$

$$K_{NMP-AT} = 2.64 \times 10^6, M_{NMP-AT} = 14.33 \quad (r=0.957)$$

Appendix 8

Experimental Data for Affinity Constant Studies of TAMPP — Poly(dG-dC)₂ and N-MTAMPP — Poly(dG-dC)₂ Complexes Using Competition Binding against Ethidium Bromide Methold

The concentration of stock solution: $[EB]_s = 1.42 \times 10^{-4}$ M, $[TAMPP]_s = 1.264 \times 10^{-5}$ M, $[N-MTAMPP] = 3.187 \times 10^{-6}$; $[Poly(dG-dC)_2]_s = 4.74 \times 10^{-5}$ M in (8-1), (8-2.1), (8-2.2), (8-2.3) and all (8-3) trials and $[Poly(dG-dC)_2]_s = 5.15 \times 10^{-5}$ M in (8-2.4) trial;

Buffer composition: 0.05 M (acetic acid + sodium acetate), 0.1 M NaCl, pH=5.5.

Signal high votage: 925 volts;

Excitation wavelength: 520 nm;

Emission wavelength: 600 nm;

Cell Compartment Temperature: 25 °C.

Data shown in (8-1) are the experemental and calculated results for determing parameter ν , n_{EB} and affinity constant K_{EB} of ethidium bromide — Poly(dG-dC)₂ system;

Data shown in (8-2) are the experemental and calculated results from the four trails, specified as 8-2.1, 8-2.2, 8-2.3 and 8-2.4 respectively, for determing K_{TAP-GC} and M_{TAP-GC} of TAMPP — Poly(dG-dC)₂ system;

Data shown in (8-3) are the experemental and calculated results from the four trails, specified as 8-3.1, 8-3.2, 8-3.3 and 8-3.4 respectively, for determing K_{NMP-GC} and M_{NMP-GC} of N-MTAMPP — Poly(dG-dC)₂ system;

8-1 EB — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.815 ml	V(EB)[EB] _s	V(GC)[GC] _s	I _t -I _o	[EB] _t -[EB] _b	[EB] _t	[EB] _b
EB in ml	V(GC)=0 ml	V(GC)=0.185 ml	V(tol)	V(tol)	(U-1)k		[GC] _t	[EB] _t [GC] _t
V(EB)	I _o (EB)	I _t (EB-GC)	[EB] _t (10 ⁻⁶)	[GC] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁶)	[EB] _t (10 ⁻⁶)	X	Y(10 ⁴)
0.01	2.8	26.7	0.71	4.37	0.40	0.71	0.091	12.9
0.02	5.5	44.9	1.41	4.34	0.66	1.41	0.151	10.7
0.03	8.2	57.9	2.10	4.32	0.83	2.10	0.191	9.11
0.04	10.8	68.0	2.78	4.30	0.95	2.78	0.221	7.94
0.05	13.4	76.4	3.46	4.28	1.05	3.46	0.245	7.06
0.06	16.0	82.7	4.14	4.26	1.11	4.14	0.260	6.29
0.07	18.6	88.7	4.80	4.24	1.17	4.80	0.275	5.72
0.08	20.7	93.7	5.46	4.22	1.21	5.46	0.288	5.27
0.09	23.0	98.8	6.11	4.20	1.26	6.11	0.300	4.91
0.10	25.4	102.8	6.76	4.18	1.29	6.76	0.308	4.55

Experamental data for determing k_b(EB) (Start conditions:[Poly(dG-dC)₂]_t = 1.8x10⁻⁵, V(tol)=2 ml):

V(EB)	0.01	0.012	0.014	0.016	0.018	0.020	0.030	0.040	0.050	0.060	0.070	0.080	0.090	0.100
I _b (EB)	19.5	23.6	27.2	30.8	34.5	38.3								
I _o (EB)	1.3					2.5	3.5	4.7	5.7	6.9	7.9	8.9	9.9	10.9
[EB] _b (10 ⁻⁶)	0.71	0.85	0.99	1.13	1.27	1.41	2.10	2.78	3.46	4.14	4.80	5.46	6.11	6.76

Linear regression result: k_f(EB) = 1.59x10⁶; k_b(EB) = 2.67x10⁷; U(EB-GC)=16.8

$$k_f(EB) = 3.81 \times 10^6; K_{EB} = 2.93 \times 10^5, n_{EB} = 0.70. \quad (r=0.999)$$

8-2.1 TAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
TarGCcd	V(Buf)=2.0 ml V(dGC)=0 ml	V(Buf)=1.60 ml V(dGC)=0.1851 ml	$\frac{V(EB)(EB)_b}{V(tol)}$	$\frac{V(GC)(GC)}{V(tol)}$	$\frac{I_t - I_0}{(1-1)k}$	$\frac{[EB]_t - [EB]_b}{[EB]_t}$	$\frac{V(Por)(Por)}{V(tol)}$	$\frac{n[GC]_t - [EB]_b}{[EB]_b / K_{EB} [EB]_f}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
EB in ml	V(TAP)=0 ml	V(TAP)=0.215 ml	V(tol)	V(tol)	(1-1)k		V(tol)		[BS](Por)	[EB] _b
V(EB)	I ₀ (EB)	I _t (EB-GC-TAP)	[EB] _t (10 ⁻⁶)	[dGC] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	[Por] _t (10 ⁻⁶)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	0.9	3.9	0.71	4.37	1.53	0.55	1.35	9.71	1.39	3.63
0.02	1.8	7.2	1.41	4.34	2.75	1.13	1.35	9.24	1.46	4.12
0.03	2.7	10.0	2.10	4.32	3.71	1.73	1.34	8.91	1.50	4.65
0.04	3.6	12.7	2.78	4.30	4.63	2.32	1.33	8.30	1.60	5.02
0.05	4.4	14.9	3.46	4.28	5.34	2.93	1.33	7.94	1.67	5.49
0.06	5.3	16.8	4.14	4.26	5.85	3.55	1.32	7.80	1.69	6.07
0.07	6.1	18.6	4.80	4.24	6.35	4.17	1.31	7.52	1.75	6.56
0.08	6.8	19.9	5.46	4.22	6.66	4.80	1.31	7.48	1.75	7.20
0.09	7.5	21.2	6.11	4.20	6.97	5.42	1.30	7.35	1.77	7.78
0.10	8.2	22.7	6.76	4.18	7.37	6.02	1.29	7.02	1.84	8.17

Linear regression results: $k=1.24 \times 10^6$

$$K_{TAP-GC} = 4.12 \times 10^6, M_{TAP-GC} = 0.95. \quad (r=0.963)$$

8-2.2 TAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
TarGCcd	V(Buf)=2.0 ml V(dGC)=0 ml	V(Buf)=1.60 ml V(dGC)=0.1851 ml	$\frac{V(EB)EB_i}{V(tol)}$	$\frac{V(GC)GC_i}{V(tol)}$	$\frac{I_i - I_0}{(0.1)k}$	$\frac{[EB]_i - [EB]_0}{[EB]_i}$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[GC]_i - [EB]_0}{[EB]_0 / K_{EB}[EB]_i}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_i}{[EB]_0}$
EB in ml	V(TAP)=0 ml	V(TAP)=0.215 ml								
V(EB)	I ₀ (EB)	I _i (EB-GC-TAP)	[EB] _i (10 ⁻⁶)	[dGC] _i (10 ⁻⁶)	[EB] ₀ (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁶)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	1.8	7.8	0.71	4.37	1.50	0.56	1.35	9.89	1.37	3.71
0.02	3.7	14.6	1.41	4.34	2.73	1.13	1.35	9.32	1.44	4.16
0.03	5.4	20.1	2.10	4.32	3.68	1.73	1.34	9.01	1.49	4.71
0.04	7.1	25.3	2.78	4.30	4.55	2.33	1.33	8.48	1.57	5.12
0.05	8.8	29.7	3.46	4.28	5.23	2.94	1.33	8.17	1.62	5.63
0.06	10.6	33.4	4.14	4.26	5.70	3.57	1.32	8.07	1.63	6.26
0.07	12.2	37.2	4.80	4.24	6.25	4.18	1.31	7.70	1.71	6.68
0.08	13.8	40.5	5.46	4.22	6.68	4.79	1.31	7.45	1.75	7.18
0.09	15.4	43.4	6.11	4.20	7.00	5.41	1.30	7.30	1.78	7.73
0.10	17.0	46.1	6.76	4.18	7.28	6.03	1.29	7.17	1.81	8.29

Linear regression results: $k=2.53 \times 10^6$

$$K_{TAP-GC} = 4.07 \times 10^6, M_{TAP-GC} = 0.97. \quad (r=0.985)$$

8-2.3 TAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
TitrGCed	V(Buf)=2.0 ml V(dGC)=0 ml	V(Buf)=1.58 ml V(dGC)=0.1851 ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(GC)[GC]}{V(tol)}$	$\frac{I_t - I_o}{(I - I_o)}$	$[EB]_t - [EB]_b$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[GC]_t - [EB]_b}{[EB]_b / K_{EB}[EB]_f}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
EB in ml	V(TAP)=0 ml	V(TAP)=0.23 ml	V(tol)	V(tol)	(U-1)*		V(tol)		[BS](Por)	[EB] _b
V(EB)	I _o (EB)	I _t (EB-GC-TAP)	[EB] _t (10 ⁻⁶)	[dGC] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	[Por] _t (10 ⁻⁶)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	1.70	5.8	0.71	4.37	1.16	0.59	1.48	12.1	1.22	5.08
0.02	3.25	11.2	1.41	4.34	2.25	1.18	1.47	11.1	1.33	5.24
0.03	4.75	16.0	2.10	4.32	3.19	1.78	1.46	10.3	1.41	5.58
0.04	6.25	20.2	2.78	4.30	3.95	2.39	1.46	9.85	1.48	6.04
0.05	7.75	23.7	3.46	4.28	4.52	3.01	1.45	9.59	1.51	6.66
0.06	9.25	27.0	4.14	4.26	5.03	3.63	1.44	9.29	1.55	7.22
0.07	10.75	29.8	4.80	4.24	5.40	4.26	1.43	9.14	1.57	7.89
0.08	12.25	32.8	5.46	4.22	5.82	4.88	1.43	8.82	1.62	8.38
0.09	13.60	35.2	6.11	4.20	6.12	5.50	1.420	8.64	1.64	8.99
0.10	15.00	37.8	6.76	4.18	6.46	6.12	1.41	8.36	1.69	9.46

Linear regression results: $k=2.23 \times 10^6$

$$K_{TAP-GC} = 3.18 \times 10^6, M_{TAP-GC} = 1.24. \quad (r=0.941)$$

8-2.4 TAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
TitrGCed	V(Buf)=2.0 ml V(dGC)=0 ml	V(Buf)=1.65 ml V(dGC)=0.170 ml	$\frac{V(EB)EB_i}{V(tol)}$	$\frac{V(GC)GC}{V(tol)}$	$\frac{I_t - I_0}{(I_t - I_0)}$	$\frac{[EB]_i - [EB]_b}{[EB]_i}$	$\frac{V(Por)Por}{V(tol)}$	$\frac{\eta[GC]_i - [EB]_b}{[EB]_b / K_{EB}[EB]_i}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_i}{[EB]_b}$
EB in ml	V(TAP)=0 ml	V(TAP)=0.180 ml	V(tol)	V(tol)	(U-1)k		V(tol)		[BS](Por)	[EB] _b
V(EB)	I ₀ (EB)	I _t (EB-GC-TAP)	[EB] _i (10 ⁻⁶)	[dGC] _i (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁶)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	1.1	4.4	0.71	4.36	1.41	0.57	1.13	10.4	1.084	4.000
0.02	2.2	8.2	1.41	4.34	2.57	1.15	1.13	9.88	1.140	4.473
0.03	3.2	11.5	2.10	4.31	3.55	1.74	1.12	9.31	1.204	4.905
0.04	4.2	14.2	2.78	4.29	4.28	2.36	1.12	9.06	1.231	5.503
0.05	5.2	16.7	3.46	4.27	4.92	2.97	1.11	8.75	1.269	6.034
0.06	6.2	18.8	4.14	4.25	5.39	3.60	1.10	8.59	1.285	6.667
0.07	7.1	20.8	4.80	4.23	5.87	4.22	1.10	8.32	1.322	7.187
0.08	8.0	22.5	5.46	4.21	6.21	4.84	1.09	8.17	1.339	7.797
0.09	9.0	24.1	6.11	4.19	6.47	5.47	1.09	8.09	1.346	8.458
0.10	9.9	25.7	6.76	4.17	6.76	6.09	1.08	7.88	1.374	8.996

Linear regression results: $k=1.48 \times 10^6$

$$K_{TAP-GC} = 6.01 \times 10^6, M_{TAP-GC} = 1.12. \quad (r=0.962)$$

8-3.1 N-MTAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(GC)=0 ml V(NMP)=0 ml	V(Buf)=1.58 ml V(dGC)=0.1851 ml V(NMP)=0.235 ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(GC)[GC]}{V(tol)}$	$\frac{I_t I_o}{(0.1)k}$	$[EB]_t - [EB]_b$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n(GC)_t - [EB]_b}{[EB]_b / K_{EB}[EB]_f}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	I ₀ (EB)	I _t (EB-GC-NMP)	[EB] _t (10 ⁻⁶)	[dGC] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _f (10 ⁻⁶)	[Por] _t (10 ⁻⁷)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	1.70	9.8	0.71	4.37	2.30	0.48	3.73	3.63	1.03	2.08
0.02	3.25	18.0	1.41	4.34	4.18	0.99	3.71	3.16	1.17	2.36
0.03	4.75	24.8	2.10	4.32	5.68	1.53	3.69	2.90	1.27	2.69
0.04	6.25	30.8	2.78	4.30	6.96	2.09	3.67	2.52	1.46	3.00
0.05	7.75	35.6	3.46	4.28	7.89	2.67	3.65	2.48	1.48	3.39
0.06	9.25	39.8	4.14	4.26	8.66	3.27	3.64	2.41	1.51	3.78
0.07	10.75	43.7	4.80	4.24	9.34	3.87	3.62	2.24	1.61	4.14
0.08	12.25	46.7	5.46	4.22	9.76	4.49	3.60	2.34	1.54	4.59
0.09	13.60	49.7	6.11	4.20	10.2	5.09	3.58	2.22	1.61	4.98
0.10	15.00	52.6	6.76	4.18	10.7	5.70	3.57	2.07	1.72	5.35

Linear regression results: $k=2.23 \times 10^6$

$$K_{NMP-GC} = 1.25 \times 10^6, M_{NMP-GC} = 1.48. \quad (r=0.923)$$

8-3.2 N-MTAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(GC)=0 ml V(NMP)=0 ml	V(Buf)=1.60 ml V(dGC)=0.1851 ml V(NMP)=0.215 ml	$\frac{V(EB)(EB)_i}{V(tol)}$	$\frac{V(GC)(GC)}{V(tol)}$	$\frac{I_i - I_0}{(0.1)k}$	$[EB]_i - [EB]_b$	$\frac{V(Por)(Por)}{V(tol)}$	$\frac{n[GC]_i - [EB]_b}{[EB]_b / K_{EB} [EB]_f}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	I ₀ (EB)	I _i (EB-GC-NMP)	[EB] _i (10 ⁻⁶)	[dGC] _i (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁷)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	1.70	9.8	0.71	4.37	2.30	0.48	3.41	3.63	0.939	2.08
0.02	3.25	17.7	1.41	4.34	4.10	1.00	3.39	3.55	0.954	2.43
0.03	4.75	24.2	2.10	4.32	5.51	1.55	3.38	3.46	0.976	2.81
0.04	6.25	29.8	2.78	4.30	6.67	2.12	3.36	3.26	1.029	3.17
0.05	7.75	34.6	3.46	4.28	7.61	2.70	3.34	3.11	1.075	3.55
0.06	9.25	38.9	4.14	4.26	8.40	3.30	3.33	2.91	1.142	3.92
0.07	10.75	42.8	4.80	4.24	9.08	3.89	3.31	2.71	1.223	4.29
0.08	12.25	46.0	5.46	4.22	9.57	4.50	3.29	2.68	1.229	4.71
0.09	13.60	48.7	6.11	4.20	9.95	5.12	3.28	2.68	1.225	5.15
0.10	15.00	51.6	6.76	4.18	10.4	5.72	3.26	2.51	1.302	5.52

Linear regression results: $k=2.23 \times 10^6$

$$K_{NMP-GC} = 2.23 \times 10^6, M_{NMP-GC} = 1.49. \quad (r=0.980)$$

8-3.3 N-MTAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(GC)=0 ml V(NMP)=0 ml	V(Buf)=1.60 ml V(dGC)=0.1851 ml V(NMP)=0.215 ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(GC)[GC]}{V(tol)}$	$\frac{I_0 - I_b}{(0.1)^k}$	$\frac{[EB]_t - [EB]_b}{[EB]_t}$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[GC]_t - [EB]_b}{[EB]_b / K_{EB} [EB]_t}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_t}{[EB]_b}$
V(EB)	I ₀ (EB)	I _t (EB-GC-NMP)	[EB] _t (10 ⁻⁶)	[dGC] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	[Por] _t (10 ⁻⁷)	[BS](Por)(10 ⁻⁷)	x	y
0.01	1.5	9.6	0.71	4.37	2.41	0.47	3.41	2.61	1.305	1.936
0.02	3.1	17.4	1.41	4.34	4.25	0.98	3.39	2.83	1.197	2.309
2.641	0.03	4.6	24.0	2.10	4.32	5.76	1.52	3.38	2.63	1.283
0.04	6.0	29.5	2.78	4.30	6.98	2.09	3.36	2.46	1.367	2.988
0.05	7.4	34.3	3.46	4.28	7.99	2.66	3.34	2.25	1.484	3.333
0.06	8.8	38.5	4.14	4.26	8.82	3.25	3.33	2.08	1.603	3.687
0.07	10.2	42.3	4.80	4.24	9.54	3.85	3.31	1.88	1.762	4.035
0.08	11.7	45.7	5.46	4.22	10.1	4.45	3.29	1.77	1.864	4.406
0.09	13.0	48.5	6.11	4.20	10.5	5.06	3.28	1.71	1.914	4.797
0.10	14.3	51.1	6.76	4.18	10.9	5.67	3.26	1.64	1.985	5.184

Linear regression results: $k=2.13 \times 10^6$

$$K_{NMP-GC} = 0.85 \times 10^6, M_{NMP-GC} = 1.65. \quad (r=0.972)$$

8-3.4 N-MTAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(GC)=0 ml V(NMP)=0 ml	V(Buf)=1.60 ml V(dGC)=0.1851 ml V(NMP)=0.215 ml	$\frac{V(EB)[EB]_i}{V(tol)}$	$\frac{V(GC)[GC]}{V(tol)}$	$\frac{I_t - I_0}{(I_t - I_0)}$	$[EB]_i - [EB]_0$	$\frac{V(Por)[Por]}{V(tol)}$	$\frac{n[GC]_i - [EB]_0}{[EB]_0 / K_{EB}[EB]_f}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_0}$
V(EB)	I ₀ (EB)	I _t (EB-GC-NMP)	[EB] _i (10 ⁻⁶)	[dGC] _i (10 ⁻⁶)	[EB] ₀ (10 ⁻⁷)	[EB] _f (10 ⁻⁶)	[Por] _i (10 ⁻⁷)	[BS](Por)(10 ⁻⁷)	X	Y
0.01	1.45	9.3	0.71	4.37	2.42	0.47	3.41	2.51	1.357	1.923
0.02	3.00	16.9	1.41	4.34	4.28	0.98	3.39	2.68	1.264	2.285
	0.03	4.44	23.2	2.10	4.32	5.78	1.52	3.38	2.59	1.304
2.633										
0.04	5.79	28.3	2.78	4.30	6.93	2.09	3.36	2.59	1.296	3.017
0.05	7.14	33.1	3.46	4.28	7.99	2.66	3.34	2.25	1.486	3.333
0.06	8.49	37.0	4.14	4.26	8.78	3.26	3.33	2.17	1.535	3.711
0.07	9.84	40.3	4.80	4.24	9.38	3.86	3.31	2.17	1.527	4.120
0.08	11.29	43.3	5.46	4.22	9.86	4.48	3.29	2.19	1.507	4.541
0.09	12.54	46.1	6.11	4.20	10.30	5.08	3.28	2.06	1.593	4.917
0.10	13.80	48.7	6.76	4.18	10.70	5.69	3.26	1.93	1.688	5.292

Linear regression results: $k=2.06 \times 10^6$

$$K_{NMP-GC} = 2.68 \times 10^6, M_{NMP-GC} = 1.04. \quad (r=0.903)$$

8-3.5 N-MTAMPP — Poly(dG-dC)₂

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	$V(\text{Buf})=2.0 \text{ ml}$ $V(\text{GC})=0 \text{ ml}$ $V(\text{NMP})=0 \text{ ml}$	$V(\text{Buf})=1.60 \text{ ml}$ $V(\text{dGC})=0.1851 \text{ ml}$ $V(\text{NMP})=0.215 \text{ ml}$	$V(\text{EB})[\text{EB}]_i$ $V(\text{tol})$	$V(\text{GC})[\text{GC}]$ $V(\text{tol})$	$I_i I_o$ $(0-1)\%$	$[\text{EB}]_i - [\text{EB}]_b$	$V(\text{Por})[\text{Por}]$ $V(\text{tol})$	$\frac{n[\text{GC}]_i - [\text{EB}]_b}{[\text{EB}]_b / K_{\text{EB}} [\text{EB}]_f}$	$\frac{[\text{Por}]_i}{[\text{BS}](\text{Por})}$	$\frac{[\text{EB}]_f}{[\text{EB}]_b}$
V(EB)	$I_o(\text{EB})$	$I_i(\text{EB-GC-NMP})$	$[\text{EB}]_i(10^{-6})$	$[\text{dGC}]_i(10^{-6})$	$[\text{EB}]_b(10^{-7})$	$[\text{EB}]_f(10^{-6})$	$[\text{Por}]_i(10^{-7})$	$[\text{BS}](\text{Por})(10^{-7})$	X	Y
0.01	1.45	9.1	0.71	4.37	2.36	0.47	3.41	3.09	1.104	1.999
0.02	3.00	16.5	1.41	4.34	4.16	0.99	3.39	3.27	1.038	2.382
0.03	4.44	22.7	2.10	4.32	5.62	1.54	3.38	3.10	1.089	2.732
0.04	5.79	28.2	2.78	4.30	6.90	2.09	3.36	2.67	1.257	3.035
0.05	7.14	32.8	3.46	4.28	7.90	2.67	3.34	2.46	1.360	3.383
0.06	8.49	36.5	4.14	4.26	8.62	3.27	3.33	2.47	1.344	3.795
0.07	9.84	39.8	4.80	4.24	9.23	3.88	3.31	2.45	1.352	4.205
0.08	11.29	42.8	5.46	4.22	9.70	4.49	3.29	2.45	1.346	4.629
0.09	12.54	45.5	6.11	4.20	10.10	5.10	3.28	2.35	1.392	5.025
0.10	13.80	48.4	6.76	4.18	10.70	5.70	3.26	2.07	1.573	5.347

Linear regression results: $k=2.06 \times 10^6$

$$K_{\text{NMP-GC}} = 1.71 \times 10^6, M_{\text{NMP-GC}} = 1.42. \quad (r=0.905)$$

Appendix 9

Experimental Data for Affinity Constant Studies of Mn(III)•TAMPP — CT.DNA and Co(II)•N-MTAMPP — CT.DNA Using Competition Binding against Ethidium Bromide Methold

In pH = 5.5 buffer:

The concentration of stock solution: $[EB]_s = 1.42 \times 10^{-4}$ M, $[DNA]_s = 9.026 \times 10^{-5}$ M,
 $[Mn \cdot TAMPP]_s = 1.054 \times 10^{-5}$ M,

Buffer composition: 0.05 M (acetic acid + sodium acetate), 0.1 M NaCl, pH=5.5.

In pH = 7.4 buffer:

The concentration of stock solution: $[EB]_s = 1.42 \times 10^{-4}$ M, $[DNA]_s = 1.061 \times 10^{-4}$ M,
 $[Co \cdot N-MTAMPP]_s = 8.00 \times 10^{-6}$ M,

Buffer composition: 0.05 M tris, 0.1 M NaCl, pH=7.4.

Signal high votage: 925 volts;

Excitation wavelength: 520 nm;

Emission wavelength: 600 nm;

Cell Compartment Temperature: 25 °C.

The parameters ν , n_{EB} and K_{EB} of ethidium bromide — calf thymus DNA system in
pH=5.5 buffer were determined in appendix (6-1), which are:

$$\nu = 24.1, n_{EB} = 0.33, K_{EB} = 3.97 \times 10^6;$$

Data shown in (9-1) are the experimental and calculated results for determining parameter ν , n_{EB} and K_{EB} of ethidium bromide — calf thymus DNA system in pH=7.4 buffer; Average Result from (9-1.1) (9-1.2) & (9-1.3) are: $\nu(EB-DNA)=24.1$, $K_{EB-DNA}=3.04 \times 10^5$, $n_{EB-DNA}=0.37$.

Data shown in (9-2) are the experimental and calculated results from the five trails in pH 5.5 buffer, specified as 9-2.1, 9-2.2, 9-2.3, 9-2.4 and 9-2.5 respectively, for determining $K_{Mn \cdot TAP-DNA}$ and $M_{Mn \cdot TAP-DNA}$ of Mn(III)•TAMPP — calf thymus DNA system;

Data shown in (9-3) are the experimental and calculated results from the six trails in pH=7.4 buffer, specified as 9-3.1, 9-3.2, 9-3.3, 9-3.4, 9-3.5 and 9-3.6 respectively, for determining $K_{CoNMP-DNA}$ and $M_{CoNMP-DNA}$ of Co(II)•N-MTAMPP — calf thymus DNA system.

9-1.1 EB-CT.DNA(pH = 7.4)

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.915 ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(DNA)[DNA]}{V(tol)}$	$\frac{I_t - I_0}{(0.1)k}$	$\frac{[EB]_t - [EB]_b}{[DNA]_t}$	$\frac{[EB]_t}{[DNA]_t}$	$\frac{[EB]_b}{[EB]_t + [DNA]_t}$
EB in ml	V(DNA)=0 ml	V(DNA)=0.085 ml	V(tol)	V(tol)	(0.1)k			
V(EB)	$I_0(EB)$	$I_t(EB-DNA)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_t(10^{-6})$	$X(10^{-1})$	$Y(10^4)$
0.01	2.0	21.0	0.71	4.49	2.90	0.42	0.64	9.14
0.02	4.0	35.4	1.41	4.46	4.79	0.93	1.07	7.63
0.03	5.5	46.7	2.10	4.44	6.28	1.47	1.41	6.74
0.04	7.3	55.4	2.78	4.42	7.34	2.05	1.66	5.96
0.05	8.9	62.2	3.46	4.40	8.13	2.65	1.85	5.33
0.06	10.5	68.3	4.14	4.38	8.81	3.26	2.01	4.87
0.07	12.2	73.3	4.80	4.36	9.32	3.87	2.14	4.45
0.08	14.3	78.8	5.46	4.34	9.84	4.48	2.27	4.15
0.09	15.8	82.6	6.11	4.32	10.2	5.09	2.36	3.86
0.10	17.5	86.7	6.76	4.29	10.6	5.70	2.46	3.63

Experimental data for determining $k_b(EB)$ (Start conditions: $[DNA]_t = 4.51 \times 10^{-5} M$, $V(tol)=2$ ml):

V(EB)	0.01	0.012	0.014	0.016	0.018
$I_b(EB)$	48.4	57.6	67.1	76.6	86.6
$[EB]_b(10^{-6})$	0.706	0.847	0.987	1.127	1.267

Linear regression result: $k_f(EB) = 2.59 \times 10^6$; $k_b(EB) = 6.82 \times 10^7$; $\nu(EB-AT)=26.4$

$k_f(EB) = 2.59 \times 10^6$; $K_{EB}=3.03 \times 10^5$, $n_{EB}=0.36$. ($r=0.999$)

9-1 2 EB-CT.DNA(pH = 7.4)

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.915 ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(DNA)[DNA]}{V(tol)}$	$\frac{I_t - I_0}{(U-1)k}$	$\frac{[EB]_t - [EB]_b}{[DNA]_t}$	$\frac{[EB]_t}{[DNA]_t}$	$\frac{[EB]_b}{[EB]_t[DNA]_t}$
EB in ml	V(DNA)=0 ml	V(DNA)=0.085 ml						
V(EB)	$I_0(EB)$	$I_t(EB-DNA)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_t(10^{-6})$	$X(10^{-1})$	$Y(10^4)$
0.01	2.3	21.0	0.71	4.49	2.87	0.42	0.64	9.04
0.02	4.3	35.4	1.41	4.46	4.77	0.93	1.07	7.60
0.03	6.3	46.7	2.10	4.44	6.19	1.48	1.39	6.64
0.04	8.2	55.4	2.78	4.42	7.24	2.06	1.64	5.88
0.05	9.9	62.2	3.46	4.40	8.02	2.66	1.82	5.26
0.06	12.0	68.3	4.14	4.38	8.63	3.28	1.97	4.77
0.07	14.0	73.3	4.80	4.36	9.09	3.89	2.09	4.35
0.08	16.1	78.8	5.46	4.34	9.61	4.50	2.22	4.06
0.09	18.0	82.6	6.11	4.32	9.90	5.12	2.30	3.75
0.10	19.8	86.7	6.76	4.29	10.3	5.73	2.39	3.53

Experamental data for determing $k_b(EB)$ (Start conditions: $[DNA]_t = 4.51 \times 10^{-5} M$, $V(tol)=2 ml$):

V(EB)	0.01	0.012	0.014	0.016	0.018
$I_b(EB)$	48.4	57.6	67.1	76.6	86.6
$[EB]_b(10^{-6})$	0.706	0.847	0.987	1.127	1.267

Linear regression result: $k_f(EB) = 2.93 \times 10^6$; $k_b(EB) = 6.82 \times 10^7$; $U(EB-AT)=23.3$

$k_f(EB) = 2.93 \times 10^6$; $K_{EB}=3.16 \times 10^5$, $n_{EB}=0.35$. ($r=0.999$)

9-1 3 EB-CT.DNA(pH = 7.4)

1	2	3	4	5	6	7	8	9
Titrated	V(Buf)=2.0 ml	V(Buf)=1.915 ml	$\frac{V(EB)[EB]_s}{V(tol)}$	$\frac{V(DNA)[DNA]}{V(tol)}$	$\frac{I_t - I_o}{(I_t - I_o)_k}$	$\frac{[EB]_t - [EB]_b}{[DNA]_t}$	$\frac{[EB]_t}{[DNA]_t}$	$\frac{[EB]_b}{[EB]_t[DNA]_t}$
EB in ml	V(DNA)=0 ml	V(DNA)=0.085 ml	V(tol)	V(tol)	(I _t -I _o) _k	[EB] _t -[EB] _b	[DNA] _t	[EB] _t [DNA] _t
V(EB)	I _o (EB)	I _t (EB-DNA)	[EB] _t (10 ⁻⁶)	[DNA] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _t (10 ⁻⁶)	X(10 ⁻¹)	Y(10 ⁴)
0.02	4.6	39.2	1.41	4.46	5.17	0.89	1.16	8.23
0.03	6.7	51.2	2.10	4.44	6.64	1.44	1.50	7.13
0.04	8.8	60.7	2.78	4.42	7.75	2.00	1.75	6.29
0.05	10.8	69.2	3.46	4.40	8.72	2.59	1.98	5.72
0.06	12.8	76.0	4.14	4.38	9.43	3.20	2.16	5.21
0.07	14.9	82.2	4.80	4.36	10.0	3.80	2.31	4.80
0.08	17.0	87.9	5.46	4.34	10.6	4.40	2.44	4.47
0.09	18.8	91.6	6.11	4.32	10.9	5.02	2.52	4.12

Experimental data for determining $k_b(EB)$ (Start conditions: $[DNA]_t = 4.51 \times 10^{-5} M$, $V(tol)=2 ml$):

V(EB)	0.002	0.004	0.006	0.008	0.01
I _b (EB)	9.8	19.8	29.6	39.5	49.8
[EB] _b (10 ⁻⁷)	1.419	2.834	4.247	5.657	7.065

Linear regression result: $k_f(EB) = 3.11 \times 10^6$, $k_b(EB) = 7.01 \times 10^7$, $\nu(EB-AT)=22.6$;

$$k_f(EB) = 3.11 \times 10^6; K_{EB} = 2.93 \times 10^5, n_{EB} = 0.39. \quad (r=0.999)$$

9-2.1 Mn(III)•TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	$V(\text{Buf})=2.0 \text{ ml}$ $V(\text{DNA})=0 \text{ ml}$ $V(\text{MnPP})=0$	$V(\text{Buf})=1.40 \text{ ml}$ $V(\text{DNA})=0.1 \text{ ml}$ $V(\text{MnPP})=0.50 \text{ ml}$	$V(\text{EB})[\text{EB}]_0$ $V(\text{tol})$	$V(\text{DNA})[\text{DNA}]$ $V(\text{tol})$	$I_t - I_0$ $(I - I_0)k$	$[\text{EB}]_t - [\text{EB}]_b$	$V(\text{Por})[\text{Por}]$ $V(\text{tol})$	$n[\text{DNA}]_t - [\text{EB}]_b$ $[\text{EB}]_b / K_{\text{EB}}[\text{EB}]_t$	$[\text{Por}]_t$ $[\text{BS}](\text{Por})_t$	$[\text{EB}]_t$ $[\text{EB}]_{\text{tot}}$
V(EB)	$I_0(\text{EB})$	$I_t(\text{E-DNA-MnPP})$	$[\text{EB}]_t(10^{-6})$	$[\text{DNA}]_t(10^{-6})$	$[\text{EB}]_b(10^{-7})$	$[\text{EB}]_t(10^{-6})$	$[\text{Por}]_t(10^{-6})$	$[\text{BS}](\text{Por})(10^{-6})$	X	Y
0.01	3.1	7.6	0.71	4.49	0.55	0.65	2.61	1.21	2.150	11.88
0.02	5.9	14.5	1.41	4.47	1.05	1.30	2.60	1.17	2.228	12.41
0.03	8.7	20.4	2.10	4.45	1.43	1.96	2.59	1.14	2.267	13.71
0.04	11.6	25.6	2.78	4.42	1.71	2.61	2.57	1.12	2.288	15.31
0.05	14.3	30.8	3.46	4.40	2.01	3.26	2.56	1.10	2.336	16.22
0.06	17.0	35.5	4.14	4.38	2.26	3.91	2.55	1.08	2.370	17.34
0.07	19.7	39.8	4.80	4.39	2.45	4.56	2.54	1.06	2.396	18.60
0.08	22.3	44.2	5.46	4.34	2.67	5.19	2.52	1.04	2.437	19.46
0.09	24.8	48.1	6.11	4.32	2.84	5.83	2.51	1.02	2.467	20.53
0.10	27.3	51.4	6.76	4.30	2.94	6.47	2.50	1.01	2.474	22.02

Linear regression results: $k=4.08 \times 10^6$

$$K_{\text{Mn} \cdot \text{TAMPP}} = 2.25 \times 10^7, M_{\text{Mn} \cdot \text{TAMPP}} = 0.55. \quad (r=0.981)$$

9-2.2 Mn(III)•TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(MnP)=0	V(Buf)=1.40 ml V(DNA)=0.1 ml V(MnP)=0.50 ml	$\frac{V(EB)X[EB]_t}{V(tol)}$	$\frac{V(DNA)X[DNA]}{V(tol)}$	$\frac{I_t - I_0}{(0.1)k}$	$[EB]_t - [EB]_b$	$\frac{V(Por)X[Por]}{V(tol)}$	$\frac{n[DNA]_t - [EB]_b}{[EB]_t / K_{EB}[EB]_t}$	$\frac{[Por]_t}{[BS]X[Por]_t}$	$\frac{[EB]_t}{[EB]_{bt}}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-MnP)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_t(10^{-6})$	$[Por]_t(10^{-6})$	$[BS]X[Por]X(10^{-6})$	X	Y
0.01	3.1	7.6	0.71	4.49	0.55	0.65	2.61	1.22	2.146	11.97
0.02	5.9	14.2	1.41	4.47	1.00	1.31	2.60	1.18	2.202	13.00
0.03	8.7	19.8	2.10	4.45	1.34	1.96	2.59	1.16	2.228	14.62
0.04	11.6	25.1	2.78	4.42	1.63	2.62	2.57	1.14	2.260	16.05
0.05	14.3	29.9	3.46	4.40	1.89	3.27	2.56	1.12	2.289	17.35
0.06	17.0	34.3	4.14	4.38	2.09	3.93	2.55	1.10	2.312	18.76
0.07	19.7	39.1	4.80	4.36	2.35	4.57	2.54	1.07	2.360	19.46
0.08	22.3	42.3	5.46	4.34	2.42	5.22	2.52	1.07	2.352	21.57

Linear regression results: $k=4.11 \times 10^6$

$$K_{Mn \cdot TAMPP} = 3.23 \times 10^7, M_{Mn \cdot TAMPP} = 0.53. \quad (r=0.972)$$

9-2.3 Mn(III)•TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(MnP)=0	V(Buf)=1.35 ml V(DNA)=0.1 ml V(MnP)=0.55 ml	$\frac{V(EB)I_0}{V(tot)}$	$\frac{V(DNA)I_0}{V(tot)}$	$\frac{I_t - I_0}{(1-I)}$	$\frac{[EB]_t - [EB]_b}{[EB]_t}$	$\frac{V(Por)[Por]}{V(tot)}$	$\frac{n[DNA]_t - [EB]_b}{[EB]_t / K_{EB}[EB]_f}$	$\frac{[Por]_t}{[BS](Por)_t}$	$\frac{[EB]_f}{[EB]_{bt}}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-MnP)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_f(10^{-6})$	$[Por]_t(10^{-6})$	$[BS](Por)(10^{-6})$	X	Y
0.01	3.1	6.7	0.71	4.49	0.44	0.66	2.87	1.27	2.260	15.10
0.02	5.9	12.9	1.41	4.47	0.85	1.32	2.86	1.23	2.331	15.48
0.03	8.7	18.8	2.10	4.45	1.23	1.98	2.84	1.19	2.396	16.04
0.04	11.6	23.8	2.78	4.42	1.49	2.64	2.83	1.17	2.421	17.72
0.05	14.3	28.5	3.46	4.40	1.73	3.29	2.82	1.15	2.455	19.01
0.06	17.0	32.8	4.14	4.38	1.93	3.94	2.80	1.13	2.480	20.47
0.07	19.7	36.9	4.80	4.36	2.10	4.59	2.79	1.11	2.504	21.90
0.08	22.3	40.9	5.46	4.34	2.27	5.23	2.78	1.10	2.533	23.09
0.09	24.8	45.1	6.11	4.32	2.47	5.87	2.76	1.07	2.579	23.71
0.10	27.3	48.7	6.76	4.30	2.61	6.50	2.75	1.06	2.603	24.92

Linear regression results: $k=4.08 \times 10^6$

$$K_{Mn \cdot TAMPP} = 2.36 \times 10^7, M_{Mn \cdot TAMPP} = 0.54. \quad (r=0.962)$$

9-2.4 Mn(III)•TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(MnP)=0	V(Buf)=1.35 ml V(DNA)=0.1 ml V(MnP)=0.55 ml	$\frac{V(EB) \times [EB]_0}{V(tot)}$	$\frac{V(DNA) \times [DNA]}{V(tot)}$	$\frac{I_t - I_0}{(1-I)k}$	$[EB]_t - [EB]_b$	$\frac{V(Por)[Por]}{V(tot)}$	$\frac{n[DNA]_t \cdot [EB]_b - [EB]_t / K_{EB}[EB]_f}{[BS](Por)_t}$	$\frac{[Por]_t}{[BS](Por)_t}$	$\frac{[EB]_f}{[EB]_{bt}}$
V(EB)	I ₀ (EB)	I _t (E-DNA-MnP)	[EB] _t (10 ⁻⁶)	[DNA] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _f (10 ⁻⁶)	[Por] _t (10 ⁻⁶)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	3.1	7.1	0.71	4.49	0.49	0.66	2.87	1.25	2.305	13.49
0.02	5.9	13.5	1.41	4.47	0.93	1.31	2.86	1.20	2.374	14.17
0.03	8.7	19.3	2.10	4.45	1.29	1.97	2.84	1.17	2.426	15.24
0.04	11.6	24.4	2.78	4.42	1.56	2.63	2.83	1.15	2.452	16.84
0.05	14.3	29.2	3.46	4.40	1.82	3.28	2.82	1.13	2.489	18.07
0.06	17.0	33.9	4.14	4.38	2.06	3.93	2.80	1.11	2.531	19.08
0.07	19.7	37.8	4.80	4.36	2.21	4.58	2.79	1.10	2.543	20.76
0.08	22.3	41.9	5.46	4.34	2.39	5.22	2.78	1.08	2.576	21.86
0.09	24.8	45.7	6.11	4.32	2.55	5.86	2.76	1.06	2.605	23.00
0.10	27.3	49.1	6.76	4.30	2.66	6.50	2.75	1.05	2.620	24.44

Linear regression results: $k=4.08 \times 10^6$

$$K_{Mn \cdot TAMPP} = 2.82 \times 10^7, M_{Mn \cdot TAMPP} = 0.51. \quad (r=0.975)$$

9-2.5 Mn(III)•TAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(MnP)=0	V(Buf)=1.40 ml V(DNA)=0.1 ml V(MnP)=0.50 ml	$\frac{V(EB)X[EB]_t}{V(tot)}$	$\frac{V(DNA)X[DNA]}{V(tot)}$	$\frac{I_t - I_0}{(1-I)}$	$[EB]_t - [EB]_b$	$\frac{V(Por)[Por]}{V(tot)}$	$\frac{n[DNA]_t - [EB]_b}{[EB]_b / K_{EB}[EB]_f}$	$\frac{[Por]_t}{[BS](Por)_t}$	$\frac{[EB]_f}{[EB]_{bt}}$
V(EB)	I ₀ (EB)	I _t (E-DNA-MnP)	[EB] _t (10 ⁻⁶)	[DNA] _t (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _f (10 ⁻⁶)	[Por] _t (10 ⁻⁶)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	3.1	7.8	0.71	4.49	0.57	0.65	2.61	1.20	2.173	11.33
0.02	5.9	14.8	1.41	4.47	1.08	1.30	2.60	1.16	2.249	11.96
0.03	8.7	21.1	2.10	4.45	1.51	1.95	2.59	1.12	2.308	12.88
0.04	11.6	26.7	2.78	4.42	1.84	2.60	2.57	1.10	2.345	14.13
0.05	14.3	31.9	3.46	4.40	2.15	3.25	2.56	1.07	2.389	15.14
0.06	17.0	36.6	4.14	4.38	2.39	3.90	2.55	1.05	2.421	16.31
0.07	19.7	40.9	4.80	4.36	2.58	4.54	2.54	1.04	2.445	17.58
0.08	22.3	44.8	5.46	4.34	2.74	5.19	2.52	1.02	2.464	18.91
0.09	24.8	48.3	6.11	4.32	2.86	5.83	2.51	1.01	2.475	20.34
0.10	27.3	51.9	6.76	4.30	3.00	6.46	2.50	1.00	2.496	21.55

Linear regression results: $k=4.08 \times 10^6$

$$K_{Mn \cdot TAMPP} = 2.32 \times 10^7, M_{Mn \cdot TAMPP} = 0.54, (r=0.943)$$

9-3.1 Co(II)•N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(CoP)=0	V(Buf)=1.515 ml V(DNA)=0.085 ml V(CoP)=0.40 ml	$\frac{V(EB) \times [EB]_b}{V(tot)}$	$\frac{V(DNA) \times [DNA]_b}{V(tot)}$	$\frac{I_t - I_0}{(I_t - I_0)k}$	$[EB]_t - [EB]_b$	$\frac{V(Por) \times [Por]_b}{V(tot)}$	$\frac{n[DNA]_t - [EB]_b}{[EB]_t / K_{EB} [EB]_f}$	$\frac{[Por]_t}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	$I_0(EB)$	$I_t(E-DNA-CoP)$	$[EB]_t(10^{-6})$	$[DNA]_t(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_f(10^{-6})$	$[Por]_t(10^{-6})$	$[BS](Por)(10^{-6})$	X	Y
0.01	2.6	9.3	0.71	4.48	0.82	0.62	1.59	1.13	1.410	7.58
0.02	5.2	16.8	1.41	4.46	1.43	1.26	1.58	1.12	1.410	8.87
0.03	7.4	22.8	2.10	4.44	1.89	1.91	1.58	1.11	1.415	10.09
0.04	9.8	28.8	2.78	4.42	2.33	2.55	1.57	1.09	1.444	10.93
0.05	12.2	34.6	3.46	4.40	2.75	3.19	1.56	1.05	1.481	11.58
0.06	14.7	39.7	4.14	4.37	3.07	3.83	1.55	1.03	1.502	12.47
0.07	16.9	44.4	4.80	4.35	3.38	4.46	1.55	1.01	1.530	13.21
0.08	19.3	49.0	5.46	4.33	3.65	5.10	1.54	0.99	1.555	13.97
0.09	21.5	53.2	6.11	4.31	3.89	5.73	1.53	0.97	1.580	14.70
0.10	23.7	57.0	6.76	4.29	4.09	6.35	1.52	0.95	1.598	15.53

Linear regression results: $k=3.52 \times 10^6$

$$K_{Co \cdot N-MTAMPP} = 1.22 \times 10^7, M_{Co \cdot N-MTAMPP} = 0.87. \quad (r=0.970)$$

9-3.2 Co(II)•N-MTAMP — CT-DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated	$V(\text{Buf})=2.0 \text{ ml}$	$V(\text{Buf})=1.515 \text{ ml}$	$V(\text{EB})/[\text{EB}]_t$	$V(\text{DNA})/[\text{DNA}]_t$	$I_t^{-1} \cdot I_0$	$[\text{EB}]_t \cdot [\text{EB}]_b$	$V(\text{Por})/[\text{Por}]$	$a[\text{DNA}]_t \cdot [\text{EB}]_b$	$[\text{Por}]_t$	$[\text{EB}]_t$
$V(\text{DNA})=0 \text{ ml}$	$V(\text{DNA})=0.085 \text{ ml}$		$V(\text{tot})$	$V(\text{tot})$	$(n-1) \cdot k$		$V(\text{tot})$	$[\text{EB}]_b / K_{\text{EB}} [\text{EB}]_t$	$[\text{BS}] (\text{Por})$	$[\text{EB}]_b$
EB in ml	$V(\text{CoP})=0$	$V(\text{CoP})=0.40 \text{ ml}$								
$V(\text{EB})$	$I_0(\text{EB})$	$I_t(\text{E-DNA-CoP})$	$[\text{EB}]_t (10^{-6})$	$[\text{DNA}]_t (10^{-6})$	$[\text{EB}]_b (10^{-7})$	$[\text{EB}]_t (10^{-6})$	$[\text{Por}]_t (10^{-6})$	$[\text{BS}] (\text{Por}) (10^{-6})$	x	y
0.01	2.6	9.2	0.71	4.48	0.81	0.63	1.59	1.14	1.398	7.71
0.02	5.2	16.8	1.41	4.46	1.43	1.26	1.58	1.12	1.408	8.87
0.03	7.4	23.5	2.10	4.44	1.98	1.90	1.58	1.09	1.446	9.61
0.04	9.8	29.2	2.78	4.42	2.38	2.55	1.57	1.08	1.458	10.68
0.05	12.2	34.6	3.46	4.40	2.75	3.19	1.56	1.06	1.479	11.58
0.06	14.7	39.7	4.14	4.37	3.07	3.83	1.55	1.04	1.500	12.47
0.07	16.9	44.4	4.80	4.35	3.38	4.46	1.55	1.01	1.527	13.21
0.08	19.3	49.2	5.46	4.33	3.67	5.09	1.54	0.99	1.559	13.87
0.09	21.5	53.5	6.11	4.31	3.93	5.72	1.53	0.96	1.588	14.55
0.10	23.7	57.6	6.76	4.29	4.16	6.35	1.52	0.94	1.615	15.24

Linear regression results: $k=3.52 \times 10^6$

$$K_{\text{Co} \cdot \text{N-MTAMP}} = 1.18 \times 10^7, M_{\text{TAMP}} = 0.87. \quad (r=0.984)$$

9-3.3 Co(II)•N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(CoP)=0	V(Buf)=1.515 ml V(DNA)=0.085 ml V(CoP)=0.40 ml	$\frac{V(EB)I_0}{V(tot)}$	$\frac{V(DNA)I_0}{V(tot)}$	$\frac{I_0 - I}{(I_0 - I)k}$	$\frac{[EB]_f - [EB]_b}{[EB]_f}$	$\frac{V(Por)(Por)}{V(tot)}$	$\frac{n[DNA]_f - [EB]_b}{[EB]_f / K_{EB}[EB]_f}$	$\frac{[Por]_f}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	I ₀ (EB)	I ₀ (E-DNA-CoP)	[EB] _f (10 ⁻⁶)	[DNA] _f (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _f (10 ⁻⁶)	[Por] _f (10 ⁻⁶)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	2.6	9.9	0.71	4.48	0.90	0.62	1.59	1.08	1.476	6.88
0.02	5.2	17.8	1.41	4.46	1.55	1.25	1.58	1.08	1.471	8.08
0.03	7.4	24.8	2.10	4.44	2.14	1.88	1.58	1.04	1.511	8.82
0.04	9.8	30.8	2.78	4.42	2.58	2.53	1.57	1.03	1.525	9.79
0.05	12.2	36.5	3.46	4.40	2.99	3.16	1.56	1.01	1.552	10.60
0.06	14.7	42.0	4.14	4.37	3.35	3.80	1.55	0.98	1.584	11.33
0.07	16.9	46.8	4.80	4.35	3.67	4.43	1.55	0.96	1.612	12.07
0.08	19.3	51.7	5.46	4.33	3.98	5.06	1.54	0.94	1.646	12.72
0.09	21.5	55.8	6.11	4.31	4.21	5.69	1.53	0.92	1.667	13.51
0.10	23.7	60.1	6.76	4.29	4.47	6.31	1.52	0.90	1.701	14.12

Linear regression results: $k=3.52 \times 10^6$

$$K_{Co \cdot N-MTAMPP} = 1.07 \times 10^7, M_{Co \cdot N-MTAMPP} = 0.83. \quad (r=0.982)$$

9-3.4 Co(II)•N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(CoP)=0	V(Buf)=1.515 ml V(DNA)=0.085 ml V(CoP)=0.40 ml	$\frac{V(EB)EB_i}{V(tol)}$	$\frac{V(DNA)DNA_i}{V(tol)}$	$\frac{I_i - I_0}{(I - I_0)k}$	$[EB]_i - [EB]_b$	$\frac{V(Por)(Por)}{V(tol)}$	$\frac{n(DNA)_i - [EB]_b}{[EB]_b / K_{EB} [EB]_i}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_i}{[EB]_b}$
V(EB)	I ₀ (EB)	I _i (E-DNA-CoP)	[EB] _i (10 ⁻⁶)	[DNA] _i (10 ⁻⁶)	[EB] _b (10 ⁻⁷)	[EB] _i (10 ⁻⁶)	[Por] _i (10 ⁻⁶)	[BS](Por)(10 ⁻⁶)	X	Y
0.01	2.6	10.0	0.71	4.48	0.91	0.62	1.59	1.07	1.488	6.77
0.02	5.2	18.3	1.41	4.46	1.61	1.24	1.58	1.05	1.505	7.74
0.03	7.4	25.3	2.10	4.44	2.20	1.88	1.58	1.03	1.537	8.54
0.04	9.8	31.8	2.78	4.42	2.70	2.51	1.57	1.00	1.571	9.30
0.05	12.2	37.3	3.46	4.40	3.08	3.16	1.56	0.99	1.585	10.23
0.06	14.7	42.6	4.14	4.37	3.43	3.79	1.55	0.97	1.607	11.07
0.07	16.9	47.6	4.80	4.35	3.77	4.42	1.55	0.94	1.642	11.73
0.08	19.3	52.4	5.46	4.33	4.07	5.05	1.54	0.92	1.672	12.43
0.09	21.5	56.4	6.11	4.31	4.29	5.69	1.53	0.91	1.688	13.26
0.10	23.7	60.7	6.76	4.29	4.55	6.31	1.52	0.88	1.723	13.88

Linear regression results: $k=3.52 \times 10^6$

$$K_{Co \cdot N-MTAMPP} = 1.14 \times 10^7, M_{Co \cdot N-MTAMPP} = 0.80. \quad (r=0.995)$$

9-3.5 Co(II)•N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	$V(\text{Buf})=2.0 \text{ ml}$ $V(\text{DNA})=0 \text{ ml}$ $V(\text{CoP})=0$	$V(\text{Buf})=1.535 \text{ ml}$ $V(\text{DNA})=0.085 \text{ ml}$ $V(\text{CoP})=0.38 \text{ ml}$	$\frac{V(\text{EB})[EB]_i}{V(\text{tol})}$	$\frac{V(\text{DNA})[DNA]}{V(\text{tol})}$	$\frac{I_t - I_0}{(1-1)k}$	$\frac{[EB]_i - [EB]_b}{[EB]_i - [EB]_b}$	$\frac{V(\text{Por})[Por]}{V(\text{tol})}$	$\frac{n[\text{DNA}]_i - [EB]_b}{[EB]_i / K_{EB} [EB]_f}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	$I_0(\text{EB})$	$I_t(\text{E-DNA-CoP})$	$[EB]_i (10^{-6})$	$[DNA]_i (10^{-6})$	$[EB]_b (10^{-7})$	$[EB]_i (10^{-6})$	$[Por]_i (10^{-6})$	$[BS](Por) (10^{-6})$	X	Y
0.01	2.8	10.2	0.71	4.48	0.85	0.62	1.51	1.11	1.360	7.31
0.02	5.6	18.3	1.41	4.46	1.46	1.26	1.50	1.11	1.353	8.64
0.03	7.9	25.3	2.10	4.44	2.00	1.90	1.50	1.08	1.381	9.50
0.04	10.5	31.5	2.78	4.42	2.41	2.54	1.49	1.07	1.393	10.55
0.05	13.1	37.3	3.46	4.40	2.78	3.19	1.48	1.05	1.413	11.46
0.06	15.7	42.7	4.14	4.37	3.10	3.83	1.48	1.03	1.433	12.34
0.07	18.1	47.7	4.80	4.35	3.40	4.46	1.47	1.01	1.456	13.13
0.08	20.7	52.6	5.46	4.33	3.66	5.10	1.46	0.99	1.479	13.91
0.09	23.0	57.0	6.11	4.31	3.90	5.72	1.45	0.97	1.501	14.66
0.10	25.3	61.5	6.76	4.29	4.16	6.35	1.45	0.95	1.532	15.27

Linear regression results: $k=3.77 \times 10^6$

$$K_{\text{Co} \cdot \text{N-MTAMPP}} = 1.49 \times 10^7, M_{\text{TAMPP}} = 0.87. \quad (r=0.973)$$

9-3.6 Co(II)-N-MTAMPP — CT.DNA

1	2	3	4	5	6	7	8	9	10	11
Titrated EB in ml	V(Buf)=2.0 ml V(DNA)=0 ml V(CoP)=0	V(Buf)=1.495 ml V(DNA)=0.085 ml V(CoP)=0.42 ml	$\frac{V(EB) \times [EB]_i}{V(tot)}$	$\frac{V(DNA) \times [DNA]}{V(tot)}$	$\frac{I_i - I_0}{(I_i - I_0)}$	$[EB]_i - [EB]_b$	$\frac{V(Por) \times [Por]}{V(tot)}$	$\frac{n[DNA]_i - [EB]_b}{[EB]_i / K_{EB} [EB]_f}$	$\frac{[Por]_i}{[BS](Por)}$	$\frac{[EB]_f}{[EB]_b}$
V(EB)	$I_0(EB)$	$I_i(E-DNA-CoP)$	$[EB]_i(10^{-6})$	$[DNA]_i(10^{-6})$	$[EB]_b(10^{-7})$	$[EB]_i(10^{-6})$	$[Por]_i(10^{-6})$	$[BS](Por)(10^{-6})$	X	Y
0.01	2.6	9.4	0.71	4.48	0.84	0.62	1.67	1.12	1.490	7.46
0.02	5.2	17.2	1.41	4.46	1.47	1.26	1.66	1.11	1.504	8.54
0.03	7.4	23.5	2.10	4.44	1.98	1.90	1.66	1.09	1.518	9.61
0.04	9.8	29.4	2.78	4.42	2.41	2.54	1.65	1.07	1.539	10.56
0.05	12.2	34.7	3.46	4.40	2.76	3.19	1.64	1.05	1.557	11.53
0.06	14.7	39.7	4.14	4.37	3.07	3.83	1.63	1.04	1.575	12.47
0.07	16.9	44.5	4.80	4.35	3.39	4.46	1.62	1.01	1.607	13.16
0.08	19.3	48.8	5.46	4.33	3.62	5.10	1.62	1.00	1.624	14.07
0.09	21.5	52.9	6.11	4.31	3.86	5.73	1.61	0.98	1.647	14.85
0.10	23.7	57.2	6.76	4.29	4.12	6.35	1.60	0.95	1.682	15.43

Linear regression results: $k=3.52 \times 10^6$

$$K_{Co-N-MTAMPP}=1.62 \times 10^7, M_{Co-N-MTAMPP}=0.78. (r=0.983)$$