(I) CO(III) COMPLEX PROMOTED HYDROLYSIS OF PHOSPHATE DIESTERS.

(II) DESIGN AND SYNTHESIS OF COVALENTLY LINKED DNA BASES.

By

Xiang Zou

A thesis submitted to the Faculty of Graduate Studies
and Research in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Department of Chemistry
McGill University
Montreal, Canada

September 1987

(I) CO(III) COMPLEX PROMOTED HYDROLYSIS OF PHOSPHATE DIESTERS.

(II) DESIGN AND SYNTHESIS OF COVALENTLY LINKED DNA BASES.

To my parents

To my wife

ABSTRACT

Co(III) complexes, $[Co(cyclen)(OH_2)(OH)]^{2+}$, $[Co(trien)(OH_2)(OH)]^{2+}$ and $[Co(en)_2(OH_2)(OH)]^{2+}$, are shown to be highly efficient in promoting the hydrolyses of bis-(*p*-nitrophenyl)phosphate (BNPP) and adenosine-cyclic-(3'-5')-monophosphate (c-AMP) in neutral water. The cobalt bound phosphate diesters are hydrolyzed up to 10^9 times faster than the free diesters. The most efficient cobalt complex is thus 3500 times more reactive than free hydroxide in promoting the hydrolysis of BNPP. Kinetic studies indicate that the reactive species are weakly basic aquohydroxo cobalt complexes. The mechanism of the cobalt promoted hydrolysis involves binding of the phosphate diester to the cobalt complex followed by rate-determining intramolecular nucleophilic attack by the cobalt bound hydroxide to the cobalt bound phosphate diester. There is a close relationship between the structures of the cobalt complexes and their efficiencies in promoting the hydrolysis of the phosphate diesters.

The synthesis of covalently linked cytosine residues is described. Two cytosine residues have been covalently linked with 1,3-diaminopropane and four cytosine residues linked with tripropylenetetraamine. Synthesis toward covalently linked nucleic acid bases with a peptide backbone is discussed. A monomer unit is prepared with a cytosine residue attached to an ornithine.

RESUME

Des complexes de Co(III) tels que le $[Co(cyclen)(OH_2)(OH)]^{2+}$, $[Co(trien)(OH_2)(OH)]^{2+}$ et le $[Co(en)_2(OH_2)(OH)]^{2+}$ démontrent une grande capacité à hydrolyser le bis-(*p*-nitrophenyle)phosphate (BNPP) et l'adénosine-cyclique-(3'-5')-monophosphate (c-AMP) en milieu aqueux. Les esters liés au cobalt s'hydrolysent jusqu'à 10^9 fois plus rapidement que les esters libres. Le plus efficace des complexes de cobalt est 3500 fois plus réactif que l'ion libre d'hydroxide envers l'hydrolyse de BNPP. Des études de cinétique ont démontré que l'espèce active est formée du complexe de cobalt aquahydroxo, une base faible. Le méchanisme d'hydrolyse implique, dans un premier temps, la liaison du diester de phosphate au complexe de cobalt, suivi d'une attaque nucléophile intramoléculaire par l'ion hydroxide au diester de phosphate. Il existe une relation directe entre la structure du complexe de cobalt et le rendement de la réaction d'hydrolyse.

La synthèse de résidus de cytosine liés de façon covalente est décrite. Deux résidus de cytosine ont été liés de façon covalente à partir du 1,3-diaminopropane et quatre résidus de cytosine à partir du tripropylènetétraamine. La discussion comprend également une synthèse d'acide nucléique attaché de façon covalente à un support polymérique. Un monomère est préparé à partir d'un résidu de cytosine lié à l'ornithine.

ACKNOWLEDGMENT

I am indebted to Dr. Jik Chin for his advice, guidance and encouragement throughout the course of this study.

I would also like to thank:

Mariusz Banaszczyk, Vrej Jubian, Stephen Kawai and Andrew Moore for careful and critical reading of the manuscript and for friendship and helpful discussions.

Dr. Francoise Sauriol for the instruction in running NMR spectra.

Marlene Bouvier for the translation of the abstract.

Clifford C. F. Wong for the financial support in the form of a fellowship (1983-1985).

Finally, I wish to thank my wife, Yaping Ma, and my parents, Gongwei Zou and Gongrong Chan, for their constant love, support and understanding.

PREFACE

The objective of this research was to develop simple catalysts that would promote the hydrolysis of phosphate diesters efficiently and specifically, and to study the mechanism of the hydrolysis reaction.

Phosphate esters are found in many biologically important molecules, such as DNA and RNA backbones and phospholipids in membranes. Cleavage of phosphate ester bonds is involved in many important biological processes, and is the basis for DNA sequencing.

DNases and RNases cleave phosphate diesters very efficiently at neutral pH. However, the numbers and specificities of restriction enzymes remain limited. The mechanisms of the enzymatic catalysis are far from being understood. In the past, metal ion and metal complex promoted hydrolyses of phosphate esters and polynucleotides have been studied extensively. Nonetheless, none of the studied systems are comparable with enzymes in efficiency and specificity.

Simple synthetic molecules that can cleave phosphate diester bonds efficiently and specifically would be very useful in DNA sequencing. They would also be important in their own right as models for understanding the mechanisms of enzymatic hydrolysis and of catalytic reactions in general.

Site specific DNA binding molecules are of importance in drug design and in studying the properties of DNA molecules. Small DNA binding molecules, such as *cis*-dichlorodiammineplatinum, have long been used as anticancer drugs. The binding properties of small molecules to DNA also make them very useful as chemical probes in the study of DNA structure and conformation.

There are two parts to this study. The first consists of developing simple molecules that can promote the hydrolysis of phosphate diesters efficiently. The second consists of designing molecules that bind to single strand DNA or RNA sequence specifically. Both parts are important in the development of artificial restriction enzymes. Since the two areas are relatively independent of each other, they will be discussed separately in part I and part II. Part I is concerned with the development of the catalyst. Part II is concerned with the synthesis of the DNA binding molecules.

GLOSSARY OF ABBREVIATIONS

A	adenine
AACGUU	adenylyl(3'-5')adenylyl(3'-5')cytiylyl(3'-5') guanylyl(3'-5')uridylyl(3'-5')uridine
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ApA	adenylyl(3'-5')adenosine ammonium salt
b	broad
BNPP	bis-(*p*-nitrophenyl)phosphate mono anion
BDNPP	bis-(2,4-dinitrophenyl)phosphate mono anion
C	cytosine
c-AMP	adenosine-cyclic-(3'-5')-monophosphate
CBz	benzyl carboxyl
CD	circular dichrosome
CI	chemical ionization
cyclen	cyclic-1,4,7,10-tetraazadodecane
cyt	cytosine
DCC	dicyclohexylcarbodiimide
dec	decomposition
d(GGCC)	2'-deoxyguanylyl(3'-5')-2'-deoxyguanylyl(3'-5')-2'-deoxycytiylyl(3'-5')-2'-deoxycytidine
d(GGGG)	2'-deoxyguanylyl(3'-5')-2'-deoxyguanylyl(3'-5')-2'-deoxyguanylyl(3'-5')-2'-deoxyguanine
dien	diethylenetriamine
DMF	dimethylformamide
DMP	dimethylphosphate mono anion
DNA	2'-deoxylribonucleic acid
en	ethylenediamine

FAB	fast atom bombardment
h	hour(s)
G	guanine
k	rate constant
K	equilibrium constant
k_{obs}	observed rate constant
min	minute(s)
mL	milliliter(s)
mp	melting point
NMR	nuclear magnetic resonance
NPP	*p*-nitrophenylphosphate dianion
MS	mass spectrum
orn	ornithine
PNP	*p*-nitrophenol
RNA	ribonucleic acid
T	thymine
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran
T_m	melting temperature
trien	N,N'-bis-(2-aminoethyl)ethylenediamine
U	uridine
UV	ultraviolet-visible
uL	microliter(s)

TABLE OF CONTENTS

PART I. CO(III) COMPLEX PROMOTED HYDROLYSIS OF PHOSPHATE DIESTERS

1. INTRODUCTION

1.1 Literature Review

A. Phosphate Esters in Biological System

Phosphate esters are found in many biologically important molecules, such as DNA, RNA, ATP, phosphoenolpyruvate and phospholipids. DNA and RNA record genetic information. Phosphoenolpyruvate and ATP are the main sources of biological energy. Phospholipids are essential components in cell membranes. These are phosphate mono- or diesters of fundamental importance in biological systems.

Phosphorylation and dephosphorylation are important in the regulation of enzyme activities. For example, the level of c-AMP, which is a mediator of hormone action and modulator of enzyme activity[1], is a result of a balance between its rate of synthesis catalyzed by adenyl cyclase and its rate of hydrolysis catalyzed by cyclic 3',5'-nucleotides phosphodiesterase. Because of the great importance of phosphate esters, there have been numerous studies concerning the hydrolysis

1. (a) Robison, G. A.; Butcher, R. W.; Sutherland, E. W. *Ann. Rev. Biochem.* **1968**, *37*, 149-174.
(b) Sutherland, E. W.; Robison, G. A.; Butcher, R. W. *Circulation* **1968**, *37(2)*, 279-306.

reactions of these molecules[2].

B. Relative Reactivities of Phosphate Esters

One major difficulty in studying the mechanisms of hydrolysis of phosphate esters is that the rates of hydrolysis are extremely slow at neutral conditions in water, compared to those of carboxylic esters. Among the three types of phosphate esters (mono-, di- and tri-), the hydrolysis of phosphate diesters is the slowest. For example, the hydrolysis (P-O bond cleavage) of dimethylphosphate is estimated to be one billion times slower than the hydrolysis of methyl acetate in neutral water. The relative reactivities of carboxylic and phosphate methyl esters are summarized in Table 1.1.

The slow rate of hydrolysis of phosphate diesters has biological significance in that DNA and RNA, which carry genetic information, must survive at near neutral conditions in the cell for long periods of time without being hydrolyzed[2e]. The slow rate is also understandable chemically in that phosphate diesters are less labile towards nucleophilic attack than phosphate triesters because the diesters are negatively charged. On the other hand, phosphate diesters cannot be hydrolyzed through the metaphosphate mechanism which is common for phosphomonoesters (Figure 1.2).

---

2. Leading references may be found in following articles and references therein. (a) Kochetkov, N. K.; Budovskii, E. I.; Eds. "Organic Chemistry of Nucleic acids" A and B, Plenum Press, N. Y., **1971**. (b) Jones, D. R.; Lindoy, L. F.; Sargeson, A. M. *J. Am. Chem. Soc.* **1983**, *105(25)*, 7327-7336. (c) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1(3)*, 70-78. (d) Cox Jr., J. R.; Ramsay, O. B. *Chem. Rev.* **1964**, *64(4)*, 317-352. (e) Westheimer, F. H. *Science* **1987**, *235*, 1173-1178.

Table 1.1 Rates of hydrolysis of carboxylic and phosphate methyl esters in water[a]

Esters	First order rate constants(s^{-1})	Relative rate[b]	Reference
CH_3COOCH_3	9×10^{-10}	1	c
$(CH_3O)_3P=O$	2×10^{-8}	20	d
$(CH_3O)_2PO(O^-)$	1×10^{-21}	1×10^{-12}	e,f
$(CH_3O)PO(O^-)_2$	9×10^{-11}	1×10^{-1}	d

a) Rates for P-O bond cleavage, at pH7, 25°C in water.
b) Rate relative to that of CH_3COOCH_3.
c) Guthrie, J. P.; Cullimore, P. A. *Can. J. Chem.* **1980**, *58(13)*, 1281-1294.
d) Barnard, P. W. C.; Bunton, C. A.; Llewellyn, D. R.; Vernono, C. A.; Welch, V. A. *J. Chem. Soc.* **1961**, 2670-2676.
e) P-O bond cleavage, extrapolated (appendix 2) from data reported by Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)*, **1970**, 510-513. Rate of C-O bond cleavage is in the order of 10^{-14} s^{-1}.
f) Guthrie estimated this rate to be 2×10^{-14} s^{-1} (see appendix 2 for discussion). Guthrie, J. P. *J. Am. Chem. Soc.* **1977**, *99(12)*, 3991-4001.

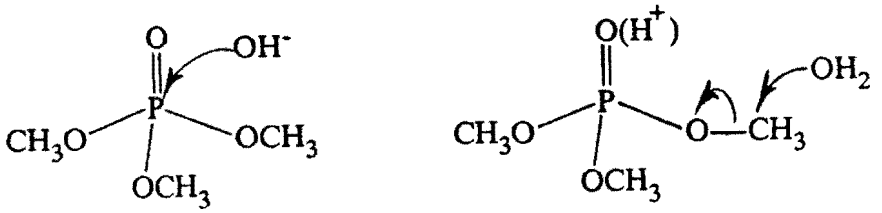
C. Mechanisms of Phosphate Ester Hydrolysis

In order to study the effect of simple catalysts on the hydrolysis of phosphate diesters, it is necessary to study the phosphate ester hydrolysis in the absence of any catalysts. The hydrolyses of phosphate esters are in many respects similar to those of carboxylic esters.

a. Triesters

In basic solution, trimethyl phosphate hydrolyzes by P--O bond cleavage through a $B_{AC}2$ like mechanism (figure 1.1) comparable to that for carboxylic esters[3]. In neutral and acidic conditions, alkyl-oxygen fission by an $A_{AL}2$ or a $B_{AL}2$ like mechanism[4] (figure 1.1) is important.

The hydrolysis of phosphate triesters is usually faster than the hydrolysis of the corresponding diesters. The lability of the triesters can be explained by the increased partial positive character at the phosphorus atom as well as the decreased charge-charge and/or charge-dipole repulsions between the phosphoryl group and the incoming nucleophile.



$B_{AC}2$ $A_{AL}2$ or $B_{AL}2$

Figure 1.1 $B_{AC}2$ mechanism and $B_{AL}2$ or $A_{AL}2$ mechanism.

Cyclic triesters with a five membered ring hydrolyze faster than their acyclic analogs. This has been attributed to the release of angle strains[5] in the cyclic ring

3. Bunton, C. A. *Acc. Chem. Res.* **1970**, *3(8)*, 257-265.
4. Thain, M. *J. Chem. Soc.* **1957**, 4694-4699.
5. Gerlt, J. A.; Westheimer, F. H.; Sturtevant, J. M. *J. Biol. Chem.* **1975**, *250(13)*, 5059-5067.

when it makes the transition from the ground state to a pentacoordinate intermediate. Stereoelectronic effects may also be important[6].

b. Diesters

The mechanisms of phosphodiester hydrolysis have not been studied as much as those of phosphate triesters and monoesters, probably because of the inconveniently slow rates of hydrolysis of the diesters. Among those diesters which have been studied, bis-(*p*-nitrophenyl)phosphate and bis-(2,4-dinitrophenyl)phosphate are known to hydrolyze by a mechanism which involves bimolecular nucleophilic attack at the phosphorus by the solvent water or by hydroxide to form a pentavalent intermediate[7]. The hydrolysis of these diesters with good leaving groups results in P-O bond fission over the whole pH range.

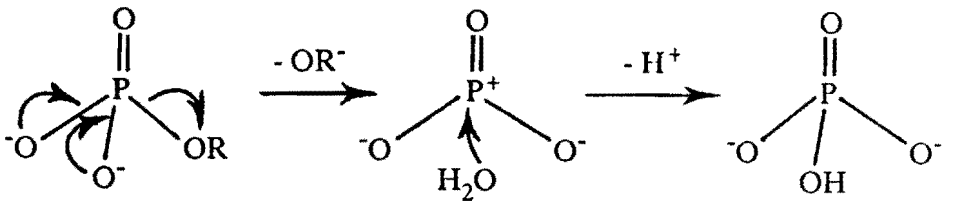
Dimethyl phosphate is typical of simple dialkyl phosphates. Its hydrolysis by P-O bond fission is so slow (table 1.1) that C-O bond cleavage is dominant at acidic and neutral pH[8]. P-O bond cleavage is only observed at strongly basic conditions, where the free hydroxide promoted P-O bond cleavage becomes important.

c. Monoesters

Phosphomonoesters with simple alkyl or aryl substituents hydrolyze by acid catalyzed P-O bond cleavage in strongly acidic conditions. In weakly acidic, neutral

6. Fanni, T.; Taria, K.; Gorenstein, D. G.; Vaidyanathaswary, R.; Verkada, J. G. *J. Am. Chem. Soc.* **1986**, *108(20)*, 6311-6314.
7. (a) Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)*, **1970**, 510-513. (b) Bunton, C. A.; Farber, S. *J. Org. Chem.* **1969**, *34(4)*, 767-772.
8. Bunton, C. A.; Mhala, M. M.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc.* **1960**, 3293-3301.

and basic pH, the metaphosphate mechanism is operative[9,2c] (figure 1.2). In this mechanism, the metaphosphate intermediate formed is stabilized by the two negative charges of the oxygens. Evidence supporting the mechanism includes the hydrolysis of monophenyl phosphate in methanol-water mixed solvent[10]. It was found that the proportion of methyl phosphate formed is almost equal to the mole fraction of the methanol in the solvent suggesting that a reactive and unselective intermediate (or transition state[11]) is formed.



A

Figure 1.2 Metaphosphate mechanism.

D. Metalloenzyme Models

a. Mechanisms of metal catalysis

Many hydrolytic enzymes contain tightly bound metal ions at the active

9. (a) Benkovic, S. J.; Schray, K, J. "*Enzymes*" 3rd Ed. **1973**, *8*, 201-238. (b) Westheimer, F. H. *Chem. Rev.* **1981**, *81(4)*, 313-326.
10. Chanley, J. D.; Feageson, E. *J. Am. Chem. Soc.* **1963**, *85(8)*, 1181-1190.
11. It is still controversial whether A exists in a dilute solution. Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1986**, *108(25)*, 7938-7946.

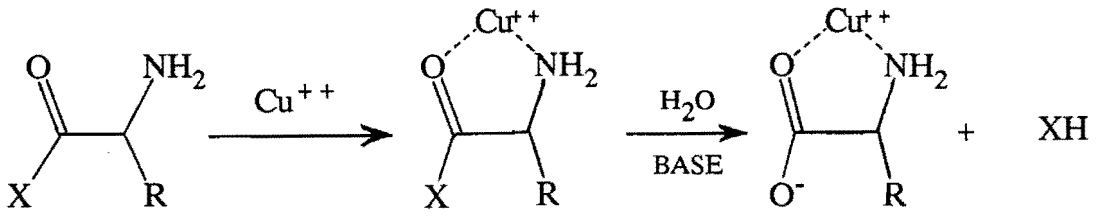
centers[12]. In order to understand the role of the metal ions in enzymes, numerous studies have been reported on simple metal ion promoted hydrolysis reactions[13]. It is generally accepted that in metal ion or metal complex promoted hydrolysis reactions, the metal acts as a Lewis acid[14] or the metal coordinated hydroxide acts as a nucleophile[15] (or both). The two mechanisms are shown in Figure 1.3.

Lewis acid **Metal hydroxide**

Figure 1.3 Lewis acid and metal hydroxide mechanisms.

The Lewis acid mechanism can be illustrated by copper(II)-promoted hydrolysis of esters of -amino acids[16] (Figure 1.4). The esters are chelated *via* the amino and carbonyl groups to the metal. Polarization of the carbonyl group by the metal cation results in an enhanced susceptibility of the carbonyl group to nucleophilic attack by a base. As a result, the rates of the hydrolysis in the presence of metal ions are about 10^6 times faster than those in the absence of the metal ions.

12. "The Inorganic Chemistry of Biological Processes", 2nd Ed., Hughes, M. N., p51 and p89, John Wiley & Son, N. Y., **1981**.
13. (a) Frey, C. M.; Stuehr, J. *Metal ions in Biological System.* **1974**, *1*, 52-116. (b) Klinman, J. P.; Samuel, D. *Biochem.* **1971**, *10(11)*, 2126-2131.
14. Example could be found in decarboxylation of acetone dicarboxylic acid; (a) Pure, J. E. *J. Chem. Soc.* **1952**, 2331-2338. (b) Steinberger, R.; Westheimer, F. H. *J. Am. Chem. Soc.* **1951**, *73(1)*, 429-435.
15. Wells, M. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1977**, *99(16)*, 5341-5356.
16. Bender, M. L.; Turnquest, B. W. *J. Am. Chem. Soc.* **1957**, *79(8)*, 1889-1893.



X = RNH or EtO

Figure 1.4 Hydrolysis of esters and amides; Lewis acid mechanism.

Different metal ions have different abilities to polarize the carbonyl groups depending largely on their size, overall charge, coordination number, and ease of displacement of a weakly coordinated ligand.

The metal hydroxide mechanism can be demonstrated by the hydrolysis[17] of *cis*-$[Co(en)_2(OH)(GlyNRR')]^{2+}$ (Figure 1.5). In this case, the hydroxide coordinated to the metal can directly attack the carbonyl group. The rate enhancement for this reaction is 10^7 fold over the uncatalyzed hydrolysis of the amide.

Positively charged metal ions or complexes are good electrophiles. When water is one of the ligands to the metal, the pK_a of the water is usually reduced by several orders of magnitude. The deprotonated water can become a good nucleophile in promoting a variety of reactions.

17. Buckingham, D. A.; Foster, D. M.; Sargeson, A. M. *J. Am. Chem. Soc.* **1970**, *92(21)*, 6151-6158.

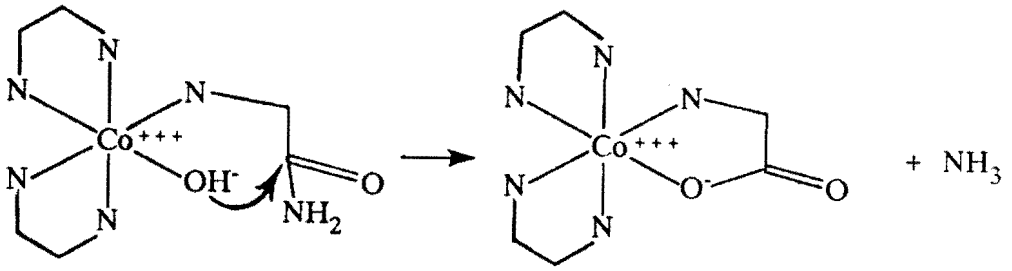
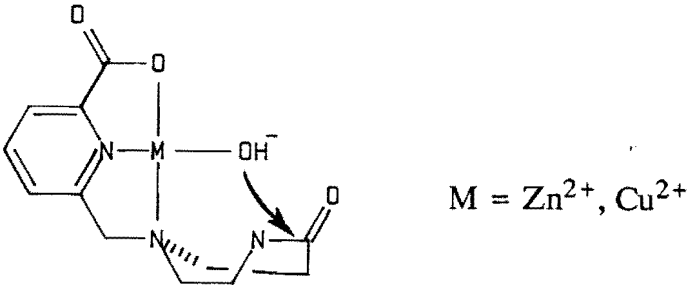


Figure 1.5 Hydrolysis of amide; metal hydroxide mechanism.

It is usually difficult however to distinguish, kinetically, between the Lewis acid mechanism and the metal hydroxide mechanism when labile divalent metal cations or metal complexes are used[18]. The few exceptions are when rigid structures of the ligands force the metal to act in a certain way. For example, in the hydrolysis of amide 1 in figure 1.6, the inflexible ligand holds the metal in such a position that only metal hydroxide attack at the amide bond is observed[19].



1

Figure 1.6 Hydrolysis through metal hydroxide mechanism involving complex with rigid ligand.

18. Breslow, R.; Fairweather, R.; Keana, J. *J. Am. Chem. Soc.* **1967**, *89(9)*, 2135-2138.
19. Groves, J. T.; Dias, R. M. *J. Am. Chem. Soc.* **1979**, *101(4)*, 1033-1035.

Metal ions in the active site of enzymes should not be substitutionally inert since the products of enzyme catalyzed reactions must dissociate efficiently. However, in simple chemical systems, it is sometimes useful to use substitutionally inert metal ions in order to elucidate the mechanism of the catalytic process, which is difficult when labile metal ions are used.

The ambiguity involved with labile metal ions can be seen in the hydrolysis of diisopropyl-phosphorofluoridate (DFP) promoted by a wide variety of metal chelates[20]. It is very difficult in this case to distinguish mechanisms **A** and **B** (figure 1.7) by kinetic, equilibrium or labelling methods. This kind of problem sometimes can be avoided if inert metal complexes are used instead of divalent metal complexes.

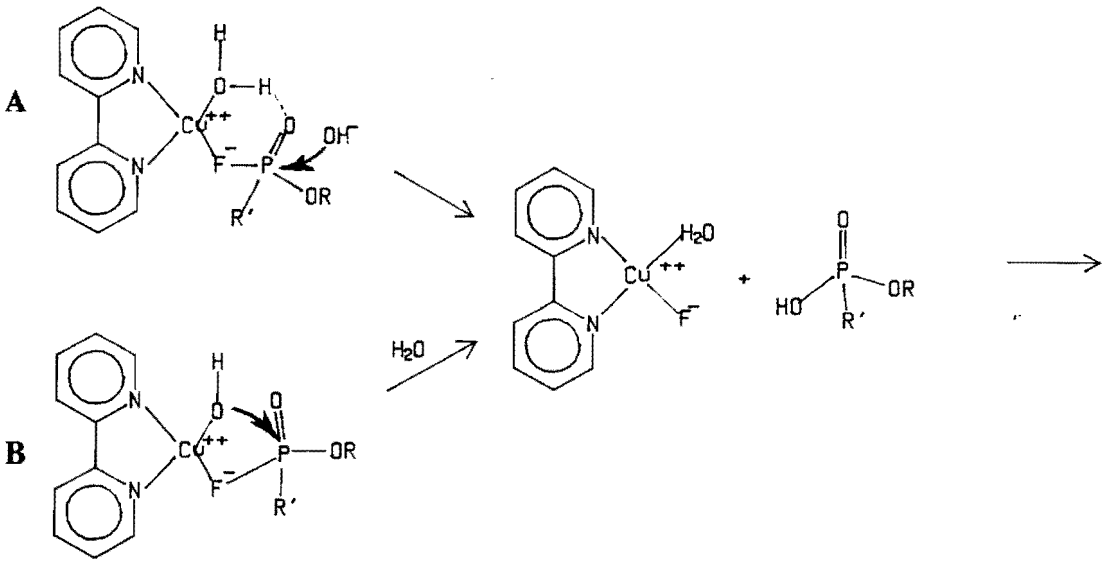


Figure 1.7 Hydrolysis of DFP promoted by Cu(II) complex.

20. (a) Gustafson, R. L.; Chaberek, S.; Martell, A. E. *J. Am. Chem. Soc.* **1963**, *85(5)*, 598-601.
(b) Gustafson, R. L.; Martell, A. E. *J. Am. Chem. Soc.* **1962**, *84(12)*, 2309-2316.

b. Zinc(II) complexes

Model studies using zinc complexes are worth some special attention because the Zn(II) cation is involved in the catalytic processes of many hydrolytic enzymes[12]. One well known example of a metalloenzyme model containing a zinc(II) ion is **ZnCR-OH** (2) as shown in Figure 1.8. In this complex, the pK_a of the coordinated

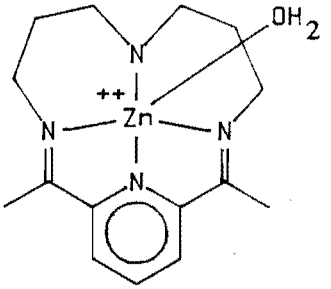


Figure 1.8 ZnCR-OH, 2.

water on the metal is dramatically reduced to 8.6. It was first shown by Woolley that the stable zinc complex efficiently promotes the hydration of acetaldehyde and carbon dioxide[21]. We demonstrated that the hydrolysis of carboxylic esters with poor leaving groups is promoted by 2[22]. More recently, Breslow reported that 2 is also a good catalyst for the hydrolysis of a phosphate triester[23]. The **ZnCR-OH** promoted hydration of carbon dioxide serves as a model for the hydration reaction

21. (a) Woolley, P. *Nature* (London) **1975**, *258*, 677-682. (b) Woolley, P. *J. Chem. Soc.,Perkin. Trans.* 2, **1977**, 318-324.
22. Chin, J.; Zou, X. *J. Am. Chem. Soc.* **1984**, *106(12)*, 3687-3688. See Appendix 3.
23. Gellman, S. H.; Petter, R.; Breslow, R. *J. Am. Chem. Soc.* **1986**, *108(9)*, 2388-2394.

catalyzed by carbonic anhydrase where the active site of the enzyme is believed to be a zinc hydrate (figure 1.9).

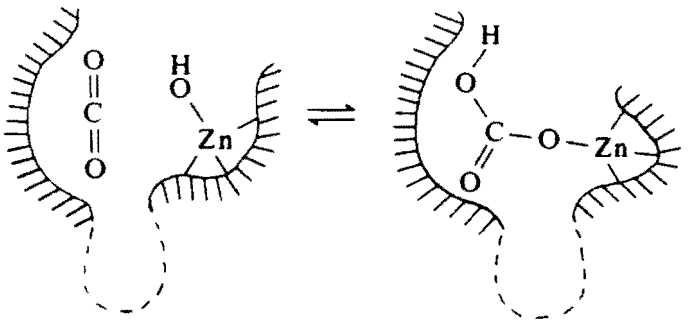


Figure 1.9 Hydration of CO_2 catalyzed by carbonic anhydrase.

The Zn(II) complex (2) was originally reported by Woolley to be a pentacoordinate molecule[21]. The metal bound hydroxide acts as a unifunctional nucleophile in promoting the hydration[21] of CO_2 and the hydrolysis[22] of carboxylate esters. However, it has been proposed recently[24,23] that the complex may be capable of forming an octahedral structure. It seems that more work needs to be done to establish the exact structure of the complex.

c. Co(III) complexes

In recent years considerable effort has been directed toward the studies of inert metal complexes, especially cobalt(III) complexes[25]. Co(III) complexes have

24. Norman, P. R. *Inorg. Chim. Acta* **1987**, *130*, 1-4.
25. (a) Hubner, P. W. A.; Milburn, R. M. *Inorg. Chem.* **1980**, *19(5)*, 1267-1272. (b) Anderson, B.; Milburn, R. M.; Sargeson, A. M. *J. Am. Chem. Soc.* **1977**, *99(8)*, 2652-2661. (c) Kenley,R. A.; Fleming, R. H.; Laine, R. M.; Tse, D. S.; Winterle, J. S. *Inorg. Chem.* **1984**, *23(13)*, 1870-1876.

been shown to promote the hydrolyses of ADP[26], ATP[26], phosphonates[25c], peptides[17] and phosphate monoesters[27].

The Co(III) ion is not found in natural hydrolytic metalloenzymes. However, in simple non-enzymic systems, cobalt(III) complexes are often more reactive[28] than other metal ions in promoting the hydrolysis of amides and esters. Co(III) complexes are substitutionally inert and, therefore, it is usually easier to evaluate the mechanism involved in the actual catalytic process.

Co(III) ion or complex promoted reactions are usually stoichiometric rather than catalytic since the products of hydrolysis are tightly bound to the metal. It has been found that when active Co(II) carbonic anhydrase[29] and Co(II) alkaline phosphatase[30] are oxidized to their corresponding Co(III) enzymes, their reactivities are lost completely. Nonetheless, Co(III) has been used extensively in simple chemical systems, since it can mimic divalent metal ions in polarizing different substrates and activating the coordinated water. It has been shown by Lincoln et al.[31] that expulsion of inorganic phosphate from a Co(III) complex, *cis*-$[Co(en)_2(OH)(PO_4)]^{2+}$ is relatively fast (k_{H2O} = $2x10^{-3}$ min^{-1}, 60°C).

---

26. (a) Milburn, R. M.; Gautam-Basak, M.; Tribolet, R.; Sigel, H. *J. Am. Chem. Soc.* **1985**, *107(11)*, 3315-3321. (b) Rawji, G.; Hediger, M.; Milburn, R. M. *Inorg. Chim. Acta* **1983**, *79*, 247-248. (c) Milburn, R. M.; Tafesse, F. *Inorg. Chim. Acta* **1987**, *135(2)*, 119-122.
27. See section 1.1.E.a, Monoester.
28. Buckingham, D. A.; Keene, F. R.; Sargeson, A. M. *J. Am. Chem. Soc.* **1974**, *96(15)*, 4981-4983.
29. Shinar, H.; Navon, G. *Biochem. Biophys. Acta* **1974**, *334(2)*, 471-475.
30. Anderson, R. A.; Vallee, B. L. *Proc. Nat. Acad. Sci. U.S.A.* **1975**, *72(1)*, 394-397.
31. (a) Lincoln, S. F.; Stranks, D. R. *Aust. J. Chem.* **1968**, *21(7)*, 1733-1743. (b) Lincoln, S. F.; Stranks, D. R. *Aust. J. Chem.* **1968**, *21(7)*, 1745-1756.

E. Co(III) Complex Promoted Hydrolysis of Phosphate Esters

a. Monoesters

A large part of the work on Co(III) complex promoted hydrolysis of phosphate esters has been focused on phosphate monoesters. Sargeson and his co-workers have investigated Co(III) complex promoted hydrolyses of peptides and phosphate monoesters for the last two decades[32,2b].

The strategy they have used is to synthesize phosphate monoesters coordinated to tetraamine cobalt(III) complexes and to study the mechanisms of the hydrolysis of the metal bound phosphate monoesters by ^{31}P and ^{1}H NMR as well as UV methods. In an elegant ^{18}O tracer experiment[2b], the hydrolysis of ^{18}O labelled *cis*-$[Co(en)_2(^{18}OH_2)O_3POC_6H_4NO_2]^+$ (**3**, Figure 1.10) was studied in water. The rate enhancement for the hydrolysis of the cobalt bound monoester is 10^5 fold over that of the unbound ester at **pH** 9. It was found that there was a complete retention of the label in the $[Co(en)_2PO_4]H_2O$ product after the hydrolysis. When the unlabelled compound **4** (Figure 1.10) was hydrolyzed in ^{18}O enriched water, 84% of the product was **unlabelled**. This work shows convincingly that the rate acceleration of the ester hydrolysis is mostly (at least 84%) due to the intramolecular nucleophilic attack at the phosphorus center by the cobalt coordinated hydroxide. A pentacoordinate intermediate is formed, followed by the release of a *p*-nitrophenolate.

---

32. Harrowfield, M. J.; Jones, D. R.; Lindoy, L. F.; Sargeson, A. M. *J. Am. Chem. Soc.* **1980**, *102(26)*, 7733-7741, and references therein.

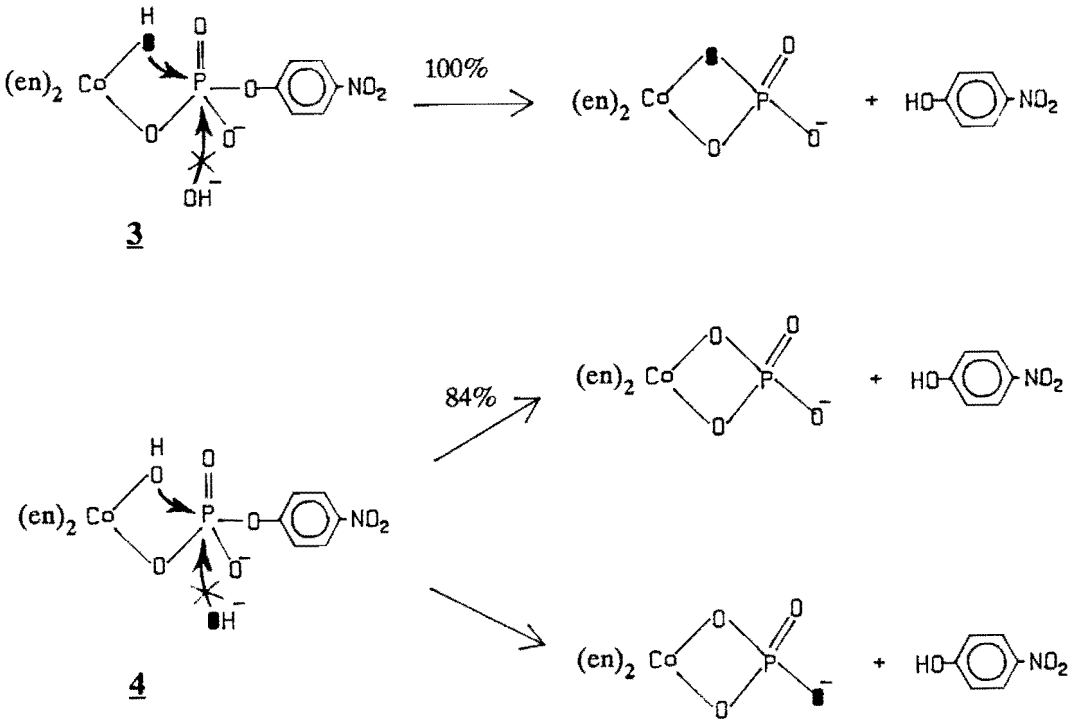


Figure 1.10 Co(III) promoted hydrolysis of phosphate mono ester; inter- *vs* intra-molecular mechanisms.

b. Diesters

Very little work has been done on the catalytic hydrolysis of phosphate diesters compared to that of phosphate monoesters. The earliest attempt to study Co(III) complex promoted hydrolysis of phosphate diesters was reported in 1963 by Schmidt and Taube[33] who prepared pentaamminecobalt(III) bound mono, di and trimethyl phosphates in acidic aqueous solutions. Ester hydrolysis was not detected for any of these species. The only reaction they observed was the breakdown of the

33. Schmidt, W.; Taube, H. *Inorg. Chem.* **1963**, *2(4)*, 698-705.

bound ester from the complex to form hydrated Co(III) complex and the free phosphate ester. The effects of bonding between the ester and the Co(III) complex, which can only act as a Lewis acid, are obviously too weak to assist the hydrolysis of phosphate esters with such poor leaving groups.

In 1969, Spiro and his co-workers studied the effects of $[Co(trien)(OH_2)(OH)]^{2+}$ on the rate of hydrolysis of mono- and dimethylphosphate[34] at neutral pH. They found that the rate of hydrolysis of monomethyl phosphate in the presence of the cobalt complex was accelerated by a modest 130 fold over the uncatalyzed reaction. No catalysis was observed for the hydrolysis of the dimethyl ester nor was it observed that $[Co(NH_3)_5(OH)]^{2+}$ had any significant effect on the hydrolysis of methyl phosphate and dimethyl phosphate. This is not surprising since the rate of hydrolysis of dimethyl phosphate is at least 10^8 times slower (Table 1.1) than that of methyl phosphate. Even if there is rate enhancement in the same order as in the case of methyl phosphate, it would still be difficult to detect it under their experimental conditions.

The two mechanisms they suggested for $[Co(trien)(OH_2)(OH)]^{2+}$ promoted hydrolysis of monomethyl phosphate are shown in Figure 1.11. Although it was

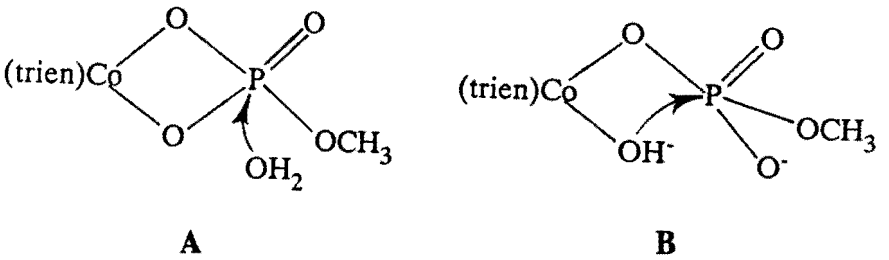


Figure 1.11 Mechanism of $[Co(trien)(OH_2)(OH)]^{2+}$ complex promoted hydrolysis of methyl phosphate.

34. Farrell, F. J.; Kjellstrom, W. A.; Spiro, T. G. *Science* **1969**, *164*, 320-321.

proposed that the bidentate intermolecular nucleophilic attack (mechanism A) might be important, Sargeson's work[2b] suggests that mechanism B is more likely to contribute to the catalysis.

Rate acceleration in the hydrolysis of bis-(2,4-dinitrophenyl)phosphate has been observed in the presence of aquohydroxo-(3,3',3"-triaminotripropylamine) cobalt(III) complex[26b]. However, a detailed interpretation of the mechanism involved was not reported. In some model systems, divalent metal ions such as Zn^{2+} and Mg^{2+} have been shown to promote the hydrolysis of cyclic phosphate diesters[35]. Nevertheless, the rate enhancements are low in those cases (in the order of 10^2 fold), and the mechanistic aspects of the catalysis remain to be clarified.

F. Nonmetallic Systems

Nonmetallic molecules such as surfactants[36] and bilayer membranes[37] have been reported to promote the hydrolysis of phosphate esters. Rate enhancements for the hydrolyses of phosphate triester and diester promoted by these molecules are in the order of 10^1-10^2 fold. Cyclodextrin[38] and -chymotrypsin[39], which mimic the active sites of enzymes, have been shown to promote the hydrolysis of catechol cyclic phosphate.

35. Steffens, J. J.; Sampson, E. J.; Siewers, I. J.; Benkovic, S. J. *J. Am. Chem. Soc.* **1973**, *95(3)*, 936-938.
36. (a) Bunton, C. A.; Ionescu, L. G. *J. Am. Chem. Soc.* **1973**, *95(9)*, 2912-2917. (b) Moss, R. A.; Swarup, S. *J. Am. Chem. Soc.* **1986**, *108(17)*, 5341-5342. (c) Moss, R. A.; Alwis, K. W.; Shin, J. S. *J. Am. Chem. Soc.* **1984**, *106(9)*, 2651-2655.
37. Okahata, Y.; Ihara, H.; Kunitake, T. *Bull. Chem. Soc. Japan* **1981**, *54(7)*, 2072-2078.
38. (a) Mochida, K.; Matsui, Y.; Ota, Y.; Arakawa, K.; Date, Y. *Bull. Chem. Soc. Japan* **1976**, *49(11)*, 3119-3123. (b) Breslow, R.; Doherty, J. B.; Guillot, G.; Lipsey, C. *J. Am. Chem. Soc.* **1978**, *100(10)*, 3227-3229.
39. Kaiser, E. T.; Lee, T. W. S.; Boer, F. P. *J. Am. Chem. Soc.* **1971**, *93(9)*, 2351-2353.

The aims of these studies were set to mimic the local environments of enzyme active sites. However, at the present stage of investigation, high efficiency of catalysis has not yet been achieved.

G. Site Specific Cleavage of DNA

The systems discussed so far are focused on the effects of simple metal complexes on the hydrolysis of simple phosphate esters. In general, the metal complexes cannot be used in the cleavage of natural DNA molecules. The few attempts to develop molecules that cleave natural DNA or RNA sequence specifically have been relatively recent.

Dervan and his co-workers have developed some very interesting metal containing molecules[40] which consist of a binding part and a cleaving group. The cleaving part is an EDTA-Fe(II) complex. One of the binding molecules they have used is the crescent-shaped distamycin (Figure 1.12), which is known to bind to the minor groove of B-DNA with a strong preference for AT-rich sequences[41]. By connecting distamycin with the DNA cleaving part, the compound is capable of binding and **oxidatively** cleaving DNA in the presence of oxygen. Although the mechanism of the cleavage is not yet clear, it has been suggested that a Fenton-type reaction with diffusible superoxide ion (O_2^-) or hydroxyl radical ($OH\cdot$)[42] is involved.

---

40. (a) Youngqurst, R. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1985**, *107(19)*, 5528-5529. (b) Dervan, P. B. *Science* **1986**, *232*, 464-471. (c) Hertzberg, R. P.; Dervan, P. B. *Biochem.* **1984**, *23(17)*, 3934-3945.
41. (a) Marky, L. A.; Blumenfeld, K. S.; Breslauer, K. J. *Nucleic Acids Res.* **1983**, *11(9)*, 2857-2870. (b) Zakrzewska, K.; Lavery, R.; Pullman, B. *Nucleic Acids Res.* **1983**, *11(24)*, 8825-8839. (c) Patel, D. J.; Canuel, L. L. *Proc. Natl Acad. Sci. U.S.A.* **1977**, *74(12)*, 5207-5211.
42. (a) Hertzberg, R. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, *104(1)*, 313-315. (b) Graham, D. R.; Marshall, L. E.; Reich, K. A.; Sigman, D. S. *J. Am. Chem. Soc.* **1980**, *102(16)*, 4919-4921.

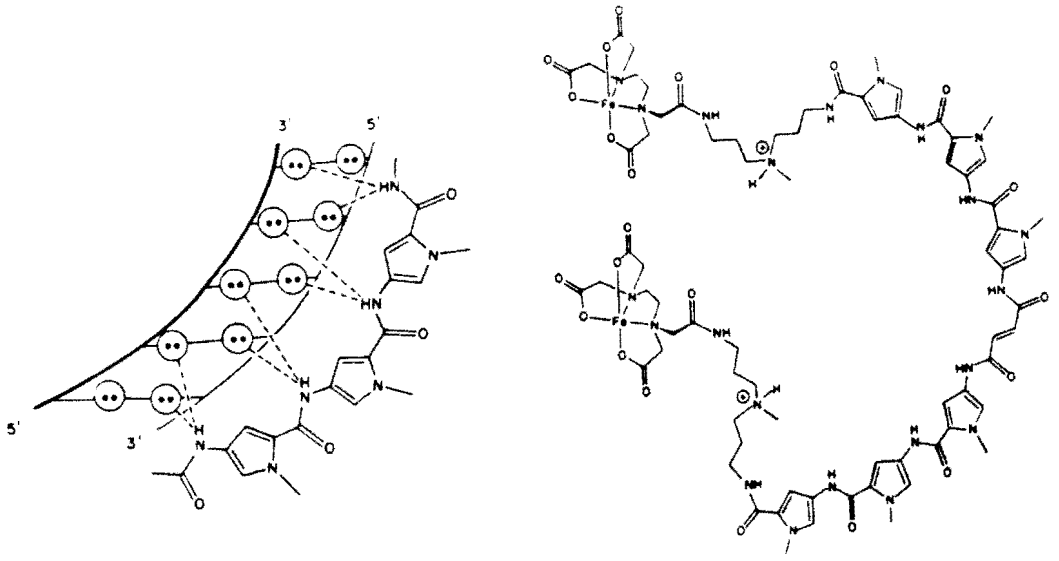


Figure 1.12 Dervan's DNA binding and cleaving molecule.

The importance of this work is that it initiated a new idea of designing potential DNA cleaving molecules. However, in the oxidative cleavage which involves the easily diffusible superoxide or hydroxyl radical, it is clearly very difficult[43] to control the cleavage sites on the DNA sequence (figure 1.13). During the process, the cleaved nucleotides are oxidized. In enzymatic reactions, the cleavage of DNA is **hydrolytic**, not oxidative, so that the products of the hydrolytic cleavage can be easily reconnected by ligases[44] and reused in the biological systems[2e]. Hydrolytic cleavage is also a basic requirement for DNA and RNA repair[45].

43. Chu, B. C. F.; Orgel, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82(2)*, 963-967.
44. "Enzyme-catalysed Reactions", Gray, C. J., VNR, London, **1971**.
45. Hamawalt, P. C.; Cooper, P. K.; Ganesan, A. K.; Smith, C. A. *Ann. Rev. Biochem.* **1979**, *48*, 783-836.

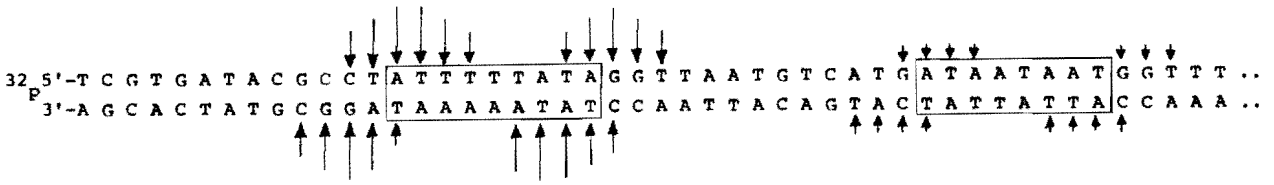


Figure 1.13 DNA binding (box) and cleaving (arrows) sites by distamycin-EDTA-Fe(II).

Other binding molecules used in the sequence-specific cleavage of DNA include oligodeoxynucleotides[46,43] which are also connected to EDTA-Fe(II). Although the specificity of the binding is improved by this approach, the drawback, in addition to the oxidative cleavage, is the possibility of self cleavage of the molecules when they are used to cleave natural DNA and RNA.

A similar strategy has been used by Barton on chiral triphenanthroline Co(III) complexes (Figure 1.14). In this case, the binding to DNA is through

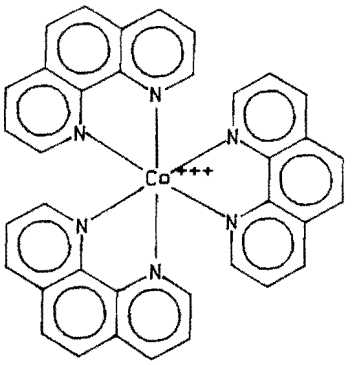


Figure 1.14 Triphenanthroline Co(III) complex.

46. Dreyer, G. B.; Dervan, P. B *Proc. Natl. Acad. Sci. U. S. A.* **1985**, *82(4)*, 968-972.

the ligands of the chiral inorganic complex, and the cleavage is again oxidative[47] involving oxygen. A system of porphyrin-Fe(II)-spermine has also been reported to cleave DNA molecules oxidatively[48].

It seems essential that in designing DNA cleaving molecules, two moieties are necessary, one for binding with DNA and the other for cleaving. Compounds that lack a DNA recognition moiety have almost no DNA cleaving ability[40c,48]. It is somewhat surprising that oxidative cleavage is invariably used in all these systems. The strategic differences in designing the DNA cleaving molecules have only been in the use of different binding groups. This probably reflects the fact that simple molecules that can efficiently hydrolyze phosphate diesters and DNA are not yet available, and there are demands for such molecules.

1.2 Plan of My Work

My plan was to study the kinetics and mechanisms of Co(III) complex promoted hydrolysis of phosphate diesters. It had been shown that Co(III) complexes are highly efficient in promoting the hydrolysis of amides and phosphate monoesters[17,2b]. However, there had been no systematic studies on Co(III) complex promoted hydrolysis of phosphate diesters.

The aim of the project was to determine the rate limiting steps of the hydrolysis reaction. This could be done on a selected series of phosphate diesters

47. Barton, J. K.; Raphael, A. L. *Proc. Natl. Acad. Sci. U.S.A.*, **1985**, *82(19)*, 6460-6464.
48. Hashimoto, Y.; Lee, C. S.; Shudo, K.; Okamoto, T. *Tet. Lett.* **1983**, *24(14)*, 1523-1526.

with different reactivities. The rates of hydrolysis in neutral water for some phosphate diesters are summarized in Table 1.2.

By using this series of phosphate diesters, the mechanisms of hydrolysis promoted by cobalt complexes could be examined systematically. Once the mechanism of the cobalt complex promoted hydrolysis is understood, the factors that influence the efficiencies of different cobalt complexes could be analyzed using a series of cobalt complexes with related structures. The relationship between the structures of the cobalt complexes and their efficiencies in promoting hydrolysis reactions had not been addressed before.

The cobalt complexes used in this study are mainly $[Co(N_4)(OH_2)(OH]^{2+}$ type complexes, where N_4 represents one tetradentate or two bidentate nitrogen ligands connected by ethylene bridges.

Table 1.2 First order rate constants for the hydrolyses of some phosphate diesters at pH7, 50°C (P-O bond cleavage).

Esters	k (s^{-1})	$t_{1/2}$ (h)	ref
BDNPP	$2.1x10^{-6}$	$1.3x10^{2}$	a
BNPP	$3.0x10^{-10}$	$9.3x10^{5}$	b
c-AMP	$1.0x10^{-14}$	$2.8x10^{10}$	c
ApA	10^{-17}-10^{-19}	10^{13}-10^{15}	d
$(MeO)_2P(O)O$	10^{-19}	$2.8x10^{15}$	e

a) Bunton, C. A.; Farber, S. J. *J. Org. Chem.* **1969**, *34(4)*, 767-772.
b) Kirby, A. J.; Younas, M. *J. Chem. Soc. (B), 1970,* 510-513.
c) See text in section 3.3 for discussion.
d) Rate of hydrolysis is not known, but it should not be more stable than dimethyl phosphate.
e) See Table 1.1, p4.

2. RESULTS

2.1 General

A. Co(III) Complexes

Table 2.1 shows the acid dissociation constants of the aquo cobalt complexes used in this study. Literature values are also given for comparison. Determination of the pK_a values is complicated by the *cis-trans* isomerizations of these complexes. Different isomers of $[Co(en)_2(OH_2)(OH)]^{2+}$ have been shown to have quite different pK_a values[49]. The data reported here were determined under conditions where isomerizations had already proceeded to equilibrium levels. The ionic strength of the cobalt complex solution was maintained at 0.1 with NaCl. The constants for $[Co(cyclen)(OH_2)_2]^{3+}$, which conform to the general trend of the dissociation constants of this series of complexes, have not been previously reported. Only one pK_a value was obtained for $[Co(dien)(OH_2)_3]$. At high pH the complex decomposes to give a precipitate of the metal hydroxide.

Table 2.2 gives the visible absorption spectral data of the Co(III) complexes, along with literature values.

49. Bjerrum, J.; Rasmussen, S. E. *Acta Chem. Scand.* **1952**, *6*, 1265-1284.

Table 2.1 Dissociation constants for aquo Co(III) complexes used in this study (25°C).

Complexes	Found		Literature value	
	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
$[Co(en)_2(OH_2)_2]^{3+}$	5.7	7.9	5.8[a]	8.1[a]
$[Co(trien)(OH_2)_2]^{3+}$	5.9	8.0	5.9[b]	8.1[b]
$[Co(cyclen)(OH_2)_2]^{3+}$	6.1	7.8	-----	-----
$[Co(en)_2(NH_3)(OH_2)]^{3+}$	6.1		6.00[c]	
$[Co(dien)(OH_2)_3]^{3+}$	6.8		----	

a) A mixture of *cis-trans* equilibrium in 1N $NaNO_3$. Bjerrum, J.; Rasmussen, S. E. *Acta Chem. Scand.* **1952**, *6*, 1265-1284.
b) Ionic strength not specified. Kenley, R. A.; Fleming, R. H.; Laine, R. M.; Tse, D. S.; Winterle, J. S. *Inorg. Chem.* **1984**, *23(13)*, 1870-1876.
c) Ionic strength not specified. Pearson, R. G.; Meeker, R. E.; Basolo, F. *J. Am. Chem. Soc.* **1956**, *78(4)*, 709-713.

B. Kinetics

Pseudo first order rate constants were obtained by fitting the first three half lives of the kinetic data to a first order equation (correlation coefficient > 0.995). Each rate constant reported in Table 2.3 represents the average value of at least three runs with deviation within $\pm$5%.

In all the hydrolysis reactions of phosphate diester promoted by Co(III) complexes, kinetic studies by UV and ^{1}H NMR methods showed that there was no build-up of phosphate monoester intermediate. Two equivalents of free *p*-

Table 2.2 Visible absorption spectral data for Co(III) complexes.

Complexes	Found λ max (ε) (nm, $l \cdot M^{-1}cm^{-1}$)		Literature value λ max (ε) (nm, $l \cdot M^{-1}cm^{-1}$)	
cis-$[Co(en)_2Cl_2]$	388(85)	538(97)	390(78)[a]	535(100)[a]
cis-$[Co(en)_2(OH_2)_2]$	354(71)	492(79)	355(60)[a]	492(81)[a]
cis-α-$[Co(trien)Cl_2]$	384(127)	544(137)	385(145)[a]	545(135)[a]
cis-β-$[Co(trien)(OH_2)_2]$	356(93)	488(113)	357(85)[b]	487(122)[b]
$[Co(cyclen)Cl_2]$	390(159)	558(182)	390(165)[a]	560(185)[a]
$[Co(cyclen)(OH_2)_2]$	362(165)	513(188)	-----	-----
cis-$[Co(en)_2(NH_3)Br]$	-----	548(80)	-----	550(82)[c]
cis-$[Co(en)_2(NH_3)(OH_2)]$	348(62)	482(65)	345(60)[c]	480(65.2)[c]
$[Co(dien)(OH_2)_3]$	486(104)		-----	

a) Poon, C. K.; Tobe, M. L. *J. Chem. Soc. (A)* **1968**, 1549-1555.
b) Sargeson, A. M.; Searle, G. H. *Inorg. Chem.* **1967**, *6(4)*, 787-796.
c) Nyholm, R. S.; Tobe, M. L. *J. Chem. Soc.* **1956**, 1707-1718.

nitrophenolate were released in the hydrolysis of bis-(*p*-nitrophenyl)phosphate (BNPP). Two equivalents of free 2,4-dinitrophenolate were released for bis-(2,4-dinitrophenyl)phosphate (BDNPP) hydrolysis. These were indicated by the final optical absorbances of the free *p*-nitrophenolate and 2,4-dinitrophenolate products and by the lack of ^1H NMR signals due to the monoesters.

NaF or NaCl (0.1M) solution has no observable effect on the rate of the BNPP hydrolysis promoted by cobalt complex. Therefore, the ionic strength of the reaction solution is not important, and was not controlled in the kinetic studies.

2.2 Co(III) Complex Promoted Hydrolysis of Phosphate Diesters with Good Leaving Groups[50]

A. Rates of Co(III) Complex Promoted Hydrolysis of BNPP and BDNPP

Figure 2.1 shows the UV optical absorbance change during the hydrolysis of bis-(2,4-dinitrophenyl)phosphate (BDNPP) promoted by $[Co(trien)(OH_2)(OH)]^{2+}$. The Co(III) complex absorbs strongly below 370nm (>3) when the hydrolysis reaction was monitored by UV. The formation of free 2,4-dinitrophenolate was confirmed by a parallel ^1H NMR experiment.

Figure 2.2 shows $[Co(trien)(OH_2)(OH)]^{2+}$ promoted hydrolysis of bis-(*p*-nitrophenyl)phosphate (BNPP), monitored by the ^1H NMR signals due to the free *p*-nitrophenolate and BNPP. The reaction was followed at 25°C. In 0.1 M cobalt complex solution at 50°C, the rate of hydrolysis of BNPP is too fast ($t_{1/2}$ ~ 2 min) to be monitored by the NMR method.

The observed pseudo first order rate constants for the hydrolyses of BNPP and BDNPP as well as for the hydrolysis of (*p*-nitrophenyl)phosphate (NPP) promoted by different cobalt complexes are summarized in table 2.3. The rate constants marked $< 10^{-6}$ indicate the upper limit of those slow reactions.

50. Chin, J.; Zou, X. *J. Am. Chem. Soc.* In press.

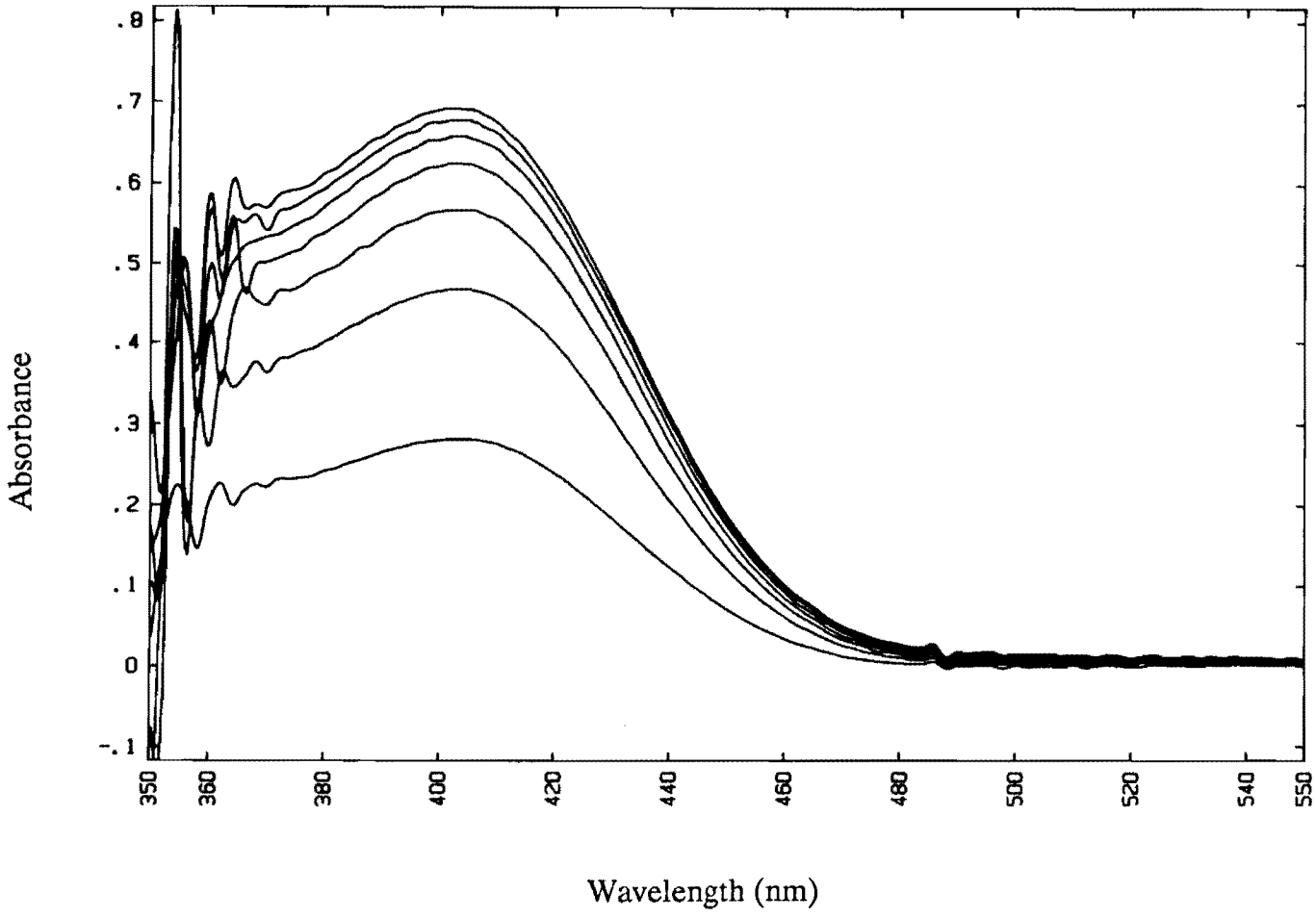


Figure 2.1 UV spectrum of the hydrolysis of BDNPP ($3x10^{-5}M$) promoted by $[Co(trien)(OH_2)(OH)]^{2+}$ (0.01M) at pH7, 50°C.

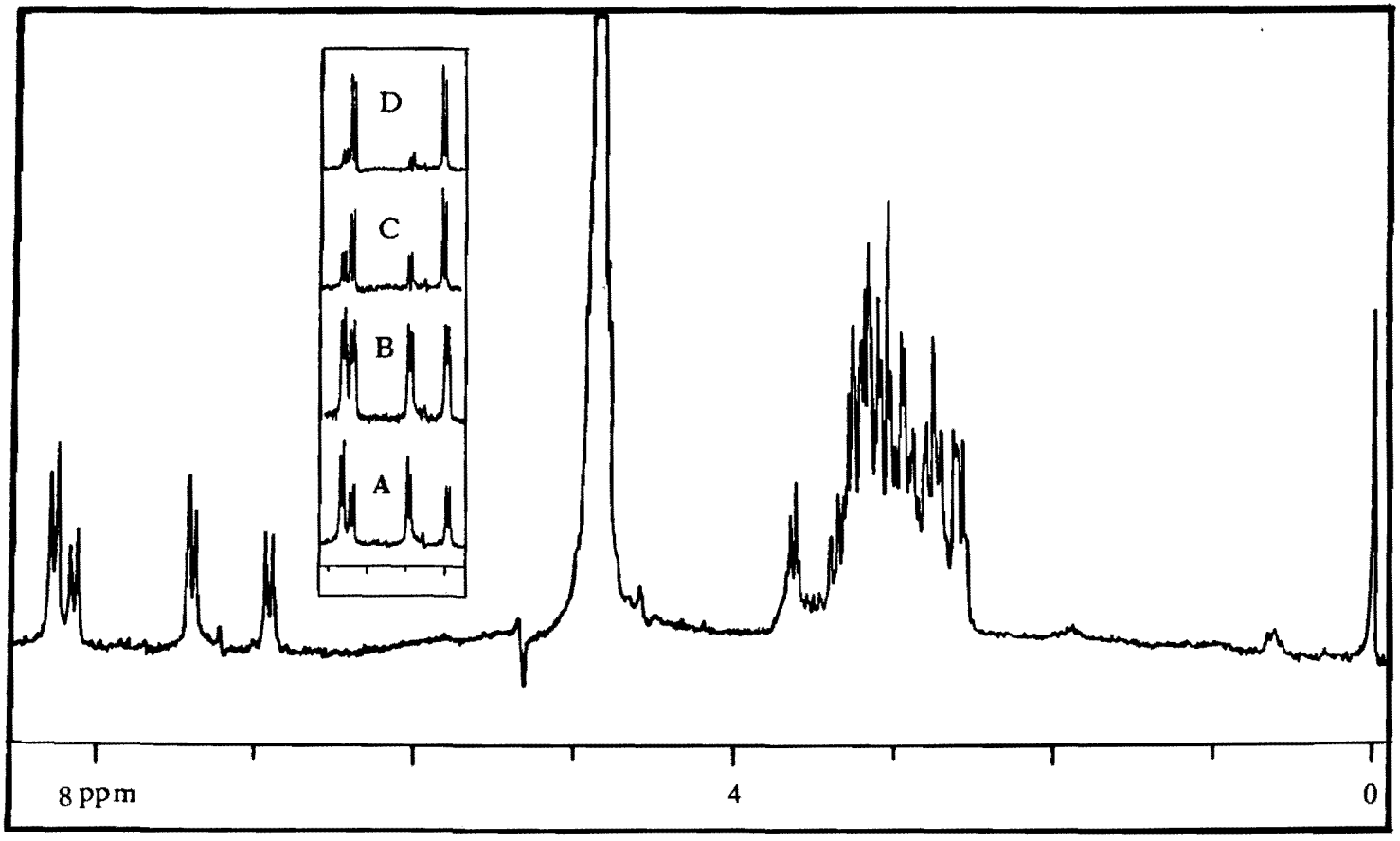


Figure 2.2 NMR spectrum of hydrolysis of BNPP (0.01M) promoted by $[Co(trien)(OH_2)(OH)]^{2+}$ (0.1M) in D_2O at pD7, 25°C, taken one hour after the reaction started. Inset: time dependence of aromatic C-H signals; A) 1 h, B) 3 h, C) 7 h, D) 10 h.

Table 2.3 Observed pseudo first order rate constants (s^{-1}) for the hydrolysis of phosphate esters promoted by cobalt complexes (0.01M) at pH7, 50°C.

Substrates / Catalysts	BNPP	BDNPP	NPP
$[Co(cyclen)(OH_2)(OH)]^{2+}$	$4.4x10^{-3}$	$1.1x10^{-2}$	$2.5x10^{-2}$
$[Co(trien)(OH_2)(OH)]^{2+}$	$4.8x10^{-4}$	$5.2x10^{-3}$	$5.6x10^{-3}$
$[Co(en)_2(OH_2)(OH)]^{2+}$	$2.7x10^{-5}$	$4.2x10^{-4}$	$3.0x10^{-4}$
$[Co(dien)(OH_2)(OH)]^{2+}$	$< 10^{-6}$	$4.3x10^{-2}$	$< 10^{-6}$
$[Co(en)_2(NH_3)(OH)]^{2+}$	$< 10^{-6}$	$1.2x10^{-4}$	$< 10^{-6}$
none[a]	$3.0x10^{-10}$ [b]	$2.1x10^{-6}$ [b]	$6.0x10^{-8}$ [c]
NaOH (1M)[d]	$1.6x10^{-4}$	$2.8x10^{-2}$ [e]	

a) Water rates. Extrapolated to 50°C from literature value.
b) For references, please see Table 1.2.
c) Kirby, A. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87(14)*, 3209-3216.
d) In 1M NaOH, BNPP hydrolyzes to NPP, the monoester. Subsequent hydrolysis of NPP is slow at this pH. Reference e) i.
e) i) Bunton, C. A.; Farber, S. *J. Org. Chem.* **1969**, *34(4)*, 767-772. ii) Reference c).

Figures 2.3 and 2.4 show the pH-rate profile for $[Co(trien)(OH_2)(OH)]^{2+}$ promoted hydrolyses of BDNPP and BNPP. The data points for the hydrolysis of BDNPP fit a theoretical curve (see discussion on p49) with pK_{a1} = 5.4, pK_{a2} = 7.9 and k_2 = 5.8 x $10^{-1}M^{-1}s^{-1}$. For BNPP, the data fit a theoretical curve with pK_{a1} = 5.6, pK_{a2} = 7.5 and k_2 = 6.6 x 10^{-2} $M^{-1}s^{-1}$.

During the hydrolysis reactions, the reactivities of some of the cobalt complexes decreased slowly because of the decomposition reactions of the

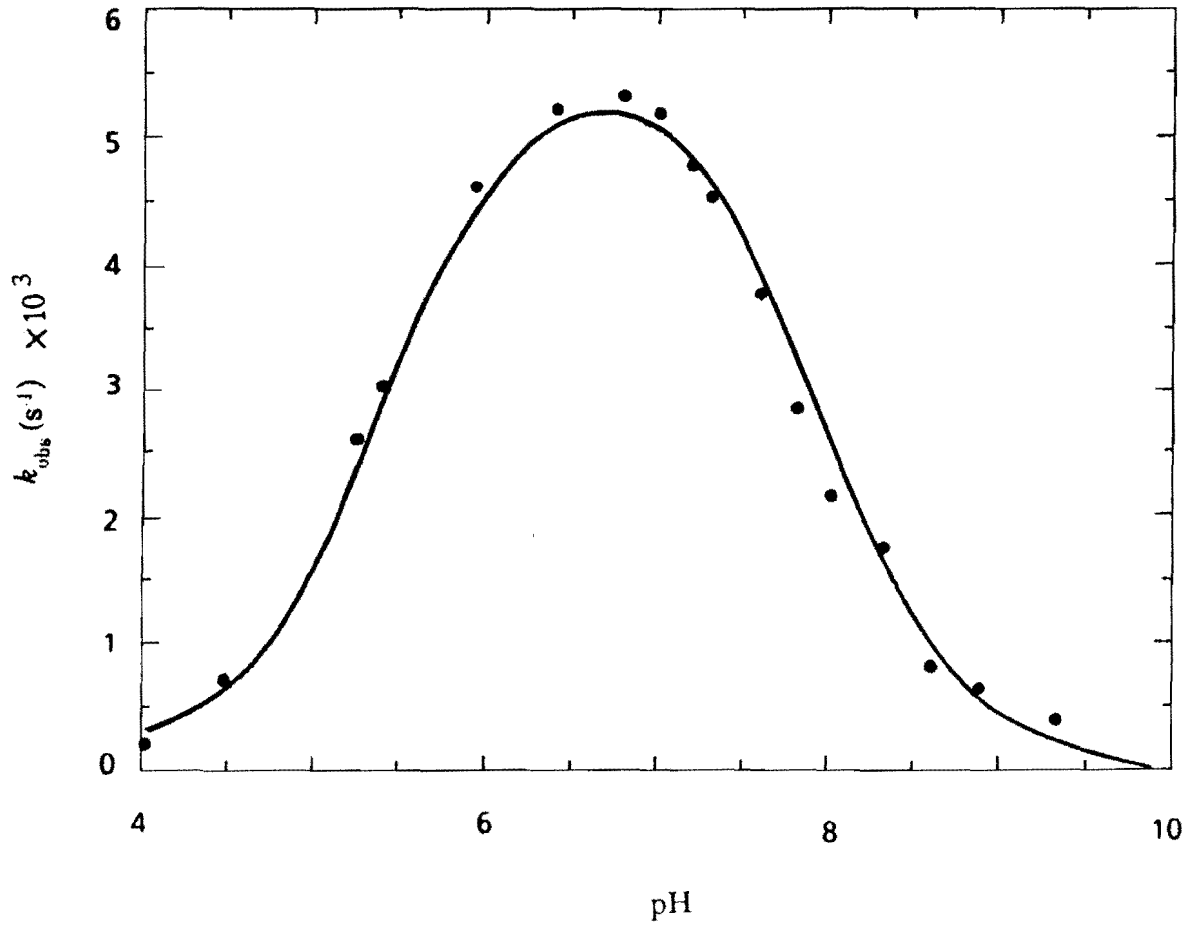


Figure 2.3 pH-rate profile for the hydrolysis of BDNPP promoted by $[Co(trien)(OH_2)(OH)]^{2+}$ (0.01M).

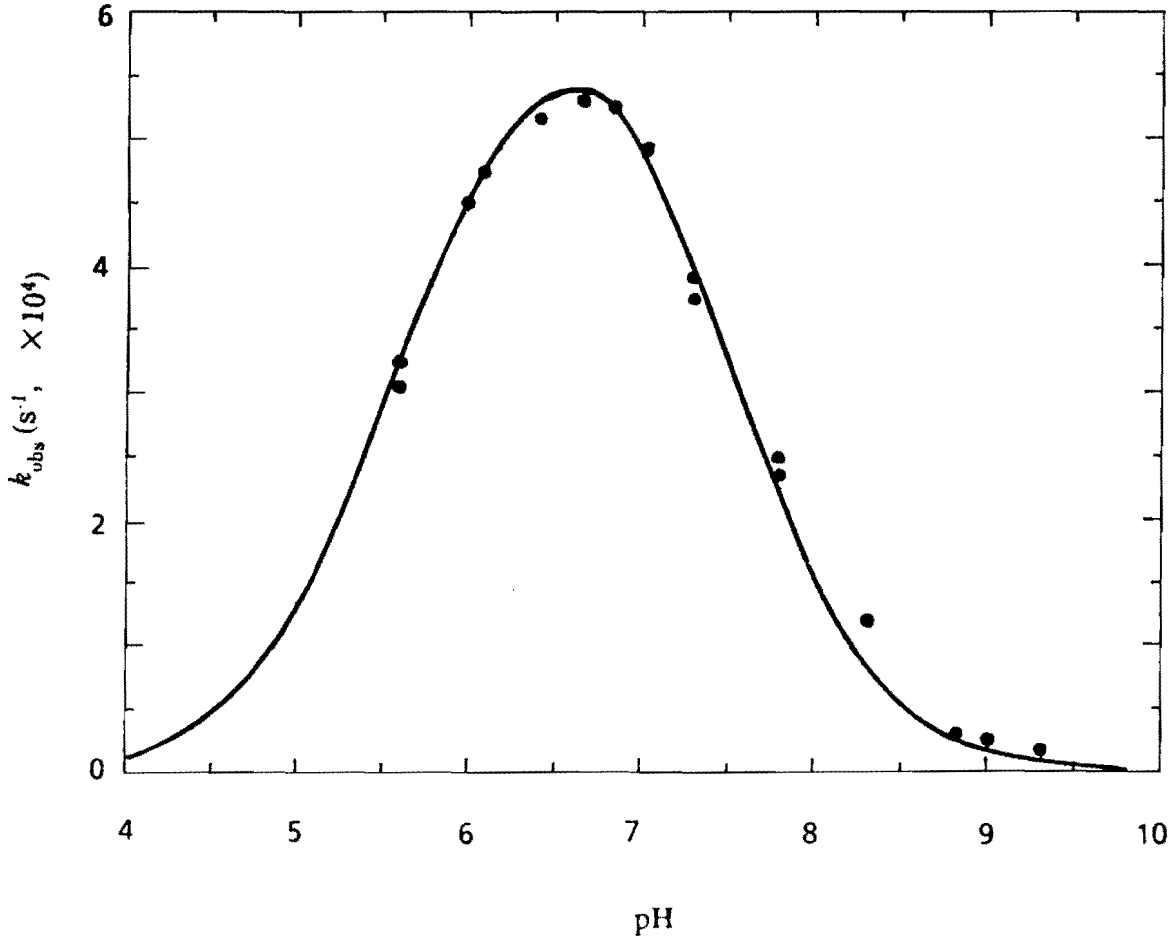


Figure 2.4 pH-rate profile for the hydrolysis of BNPP promoted by $[Co(trien)(OH_2)(OH)]^{2+}$ (0.01M).

complexes[51]. $[Co(dien)(OH_2)(OH)]^{2+}$ is the least stable. Its reactivity reduced to 50% after the stock solution had been kept at 50°C for 1 h. Solutions of $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ are stable for at least one day.

B. Dependence of BNPP Hydrolysis Rate on the Concentration of Co(III) Complex

The rate of hydrolysis of BNPP increases linearly with the increase of the concentration of the cobalt complex. Figure 2.5 shows the relationship between the rate of BNPP hydrolysis and the concentration of $[Co(trien)(OH_2)(OH)]^{2+}$. The rate of hydrolysis of BDNPP revealed the same dependence on the concentration of $[Co(trien)(OH_2)(OH)]^{2+}$ as shown in Figure 2.6.

C. Anation of Inorganic Phosphate to Cobalt Complexes

The results of studies of anation reaction of inorganic phosphate ($5x10^{-4}$ M) to cobalt complexes (0.01 M) at 50°C are given in Table 2.4. The reactions were followed at 530 nm for $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ as the optical absorption increased due to the formation of $[Co(N_4)PO_4]^o$ products[31].

51. Schwarzenbach, G.; Boesch, J.; Egli, H. *J. Inorg. Nucl. Chem.* **1971**, *33(7)*, 2141-2156.

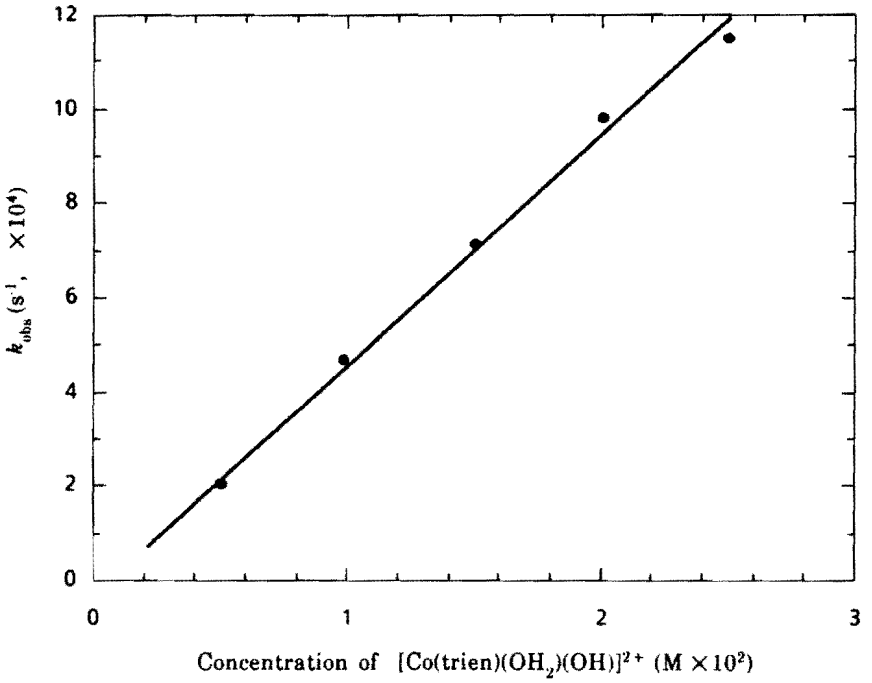


Figure 2.5 Observed first order rate constants of BNPP hydrolysis under different concentrations of $[Co(trien)(OH_2)(OH)]^{2+}$.

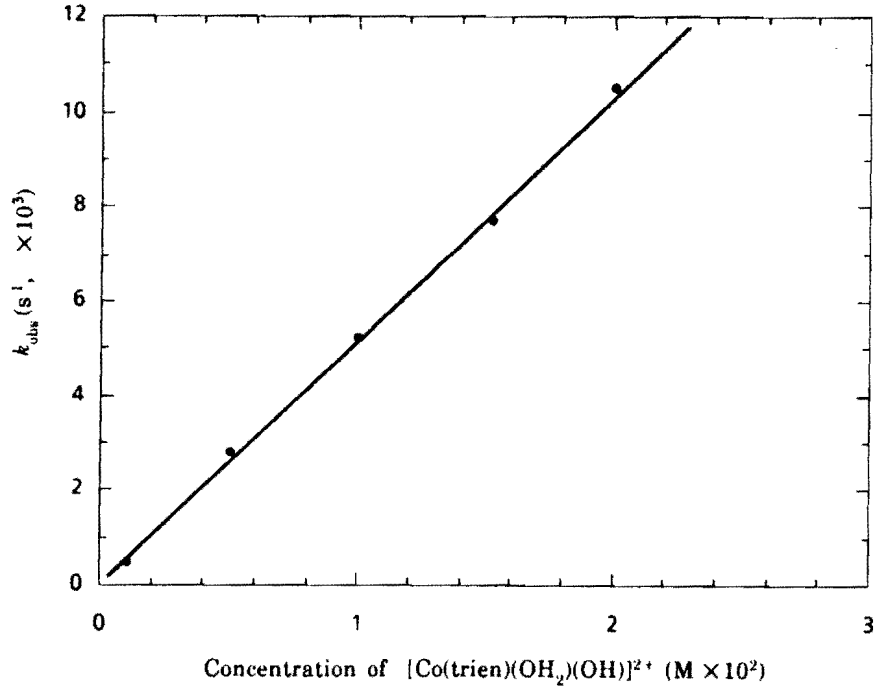


Figure 2.6 Observed first order rate constants of BDNPP hydrolysis under different concentrations of $[Co(trien)(OH_2)(OH)]^{2+}$.

Table 2.4 Rates of anation of inorganic phosphate to Co(III) complexes at pH 7, 50°C.

Complex	k_{obs} (s^{-1})
$[Co(en)_2(OH_2)(OH)]^{2+}$	$9x10^{-4}$
$[Co(trien)(OH_2)(OH)]^{2+}$	$3x10^{-2}$
$[Co(cyclen)(OH_2)(OH)]^{2+}$	2.87[a]
$[Co(dien)(OH_2)_2(OH)]^{2+}$	b

a. Extrapolated to 50°C from data obtained at 10°C, 15°C and 20°C ($2.32x10^{-2}s^{-1}$, $4.55x10^{-2}s^{-1}$ and $8.74x10^{-2}s^{-1}$).
b. Change of absorbance too small to be measured by the same method.

2.3 Co(III) Complex Promoted Hydrolysis of Phosphate Diesters with Poor Leaving Groups[52]

Hydrolysis of adenosine-cyclic-(3'-5')-monophosphate (c-AMP, 0.01M) promoted by $[Co(trien)(OH_2)(OH)]^{2+}$ (0.2M) was monitored by the ^1H NMR signals due to c-AMP and the adenosine product. As the 1'-H signals of c-AMP was decreasing, signals due to free adenosine increased (Figure 2.7). The observed pseudo first order rate constant for the hydrolysis is $1.2x10^{-6}s^{-1}$ at 50°C.

As a control, hydrolysis of c-AMP in 0.2M phosphate buffer was carried out at 100°C (Figure 2.8). In the absence of the cobalt complex, c-AMP is very resistant to hydrolysis in phosphate buffer solution at pH 7. There was virtually no hydrolysis even after the reaction solution had been heated for one month at 100°C.

52. Chin, J.; Zou, X. *Can. J. Chem.* **1987**, *65(8)*, 1882-1884.

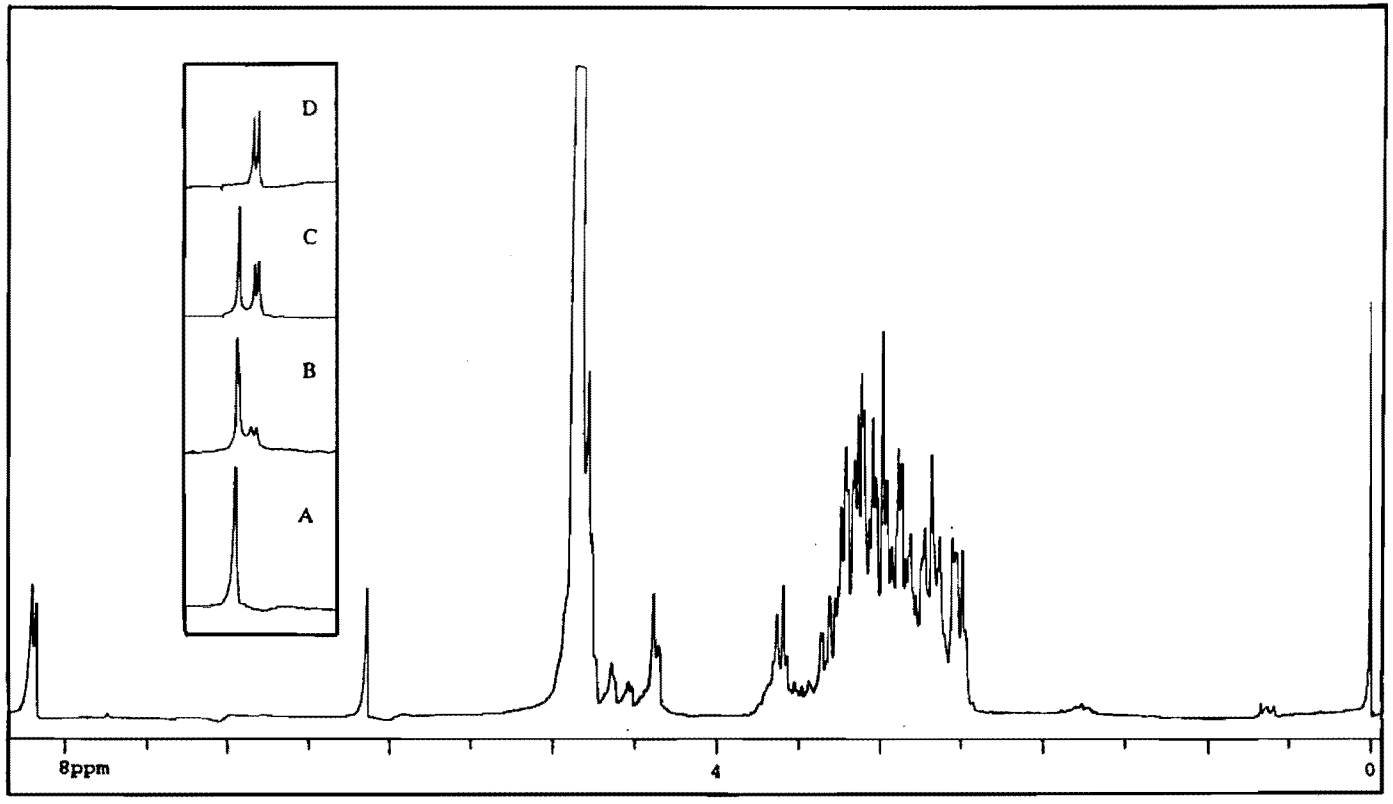


Figure 2.7 ^{1}H NMR spectrum of c-AMP in D_2O solution of $[Co(trien)(OH_2)(OH)]^{2+}$ (0.2M) at pH 7. Inset: time dependence of the 1'-H proton signals of c-AMP: A) 0 h, B) 30 h, C) 110 h, D) pure adenosine. Reaction carried out at 50°C.

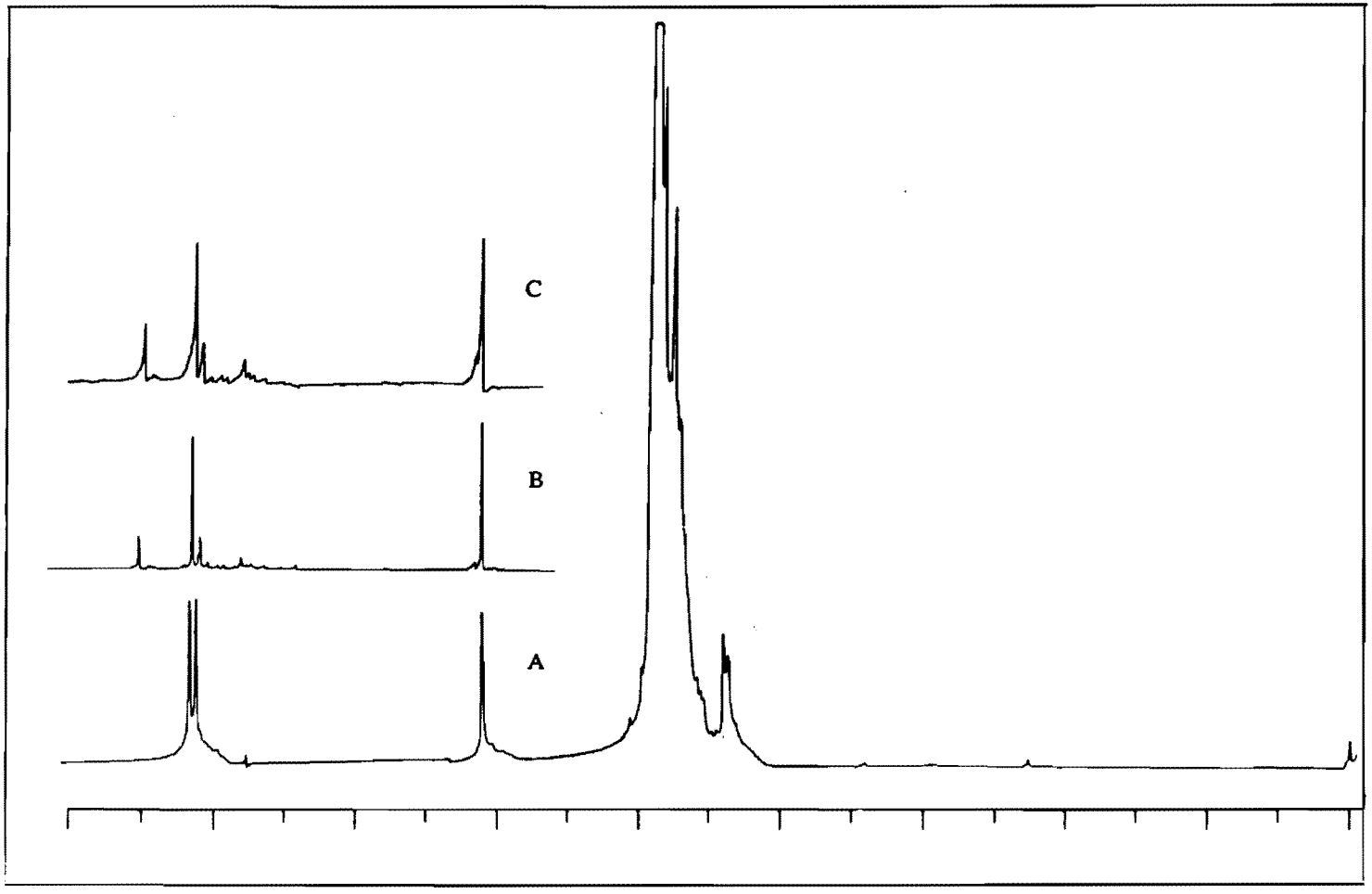


Figure 2.8 ^{1}H NMR spectra of c-AMP in 0.2M phosphate buffer solution at pH 7. A) 0 h, B) 360 h, C) 660 h. Reaction carried out at 100°C.

After prolonged heating at 100°C (>300 h), c-AMP underwent some unidentified decomposition reactions resulting in the formation of precipitates in the NMR tube.

$[Co(trien)(OH_2)(OH)]^{2+}$ (0.1M) promoted hydrolysis of adenylyl(3'-5')adenosine (ApA, 0.01M) was attempted at 50°C by the ^1H NMR method. It failed to give clear cut results because the rate of hydrolysis was still slow in the presence of the cobalt complex. After prolonged heating, the ^1H NMR signals due to ApA were broadened.

2.4 ZnCR-OH Promoted Hydrolysis of BDNPP

The pH-rate profile of **ZnCR-OH** (5 mM) promoted hydrolysis of BDNPP is shown in Figure 2.9. The maximum solubility of **ZnCR-OH** in water is close to 10 mM. At 5 mM concentration, there is no precipitation problem of the complex. The hydrolysis of BNPP in the presence of **ZnCR-OH** was too slow to be measured under the experimental conditions. A rate constant of $10^{-6}s^{-1}$ is the upper limit for the hydrolysis of BNPP promoted by **ZnCR-OH**.

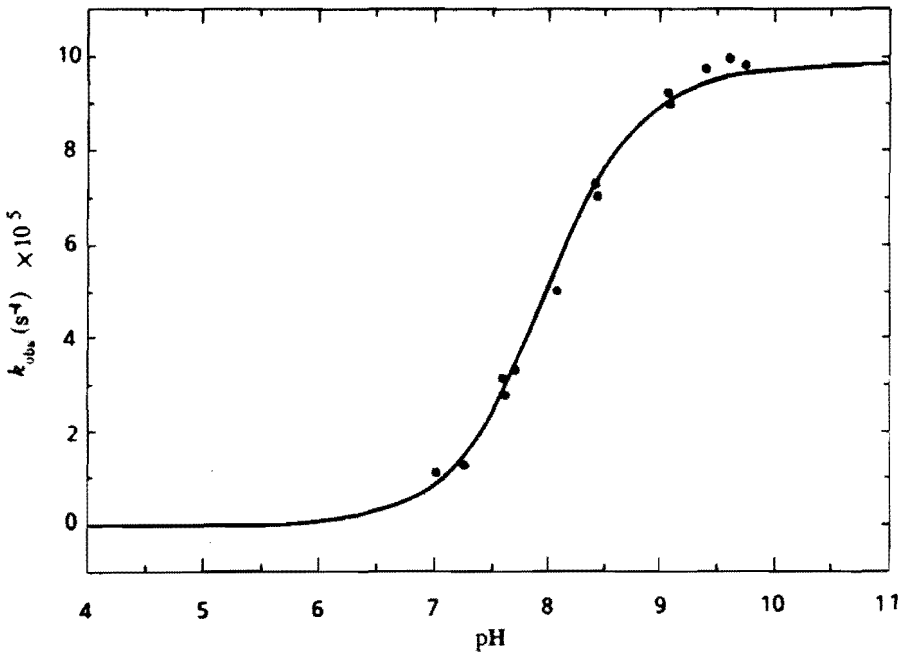


Figure 2.9 pH-rate profile for **ZnCR-OH** promoted hydrolysis of BDNPP at 50°C.

3. DISCUSSION

3.1 Co(III) Complex Promoted Hydrolysis of Phosphate diesters with Good Leaving Groups

A. *cis-trans* Isomerization of Co(III) Complexes

Numerous Co(III) complexes have been synthesized over the years. Their structures and properties have been well characterized. In simple model systems, some have been shown to promote the hydrolysis of phosphate monoesters[53,25b], peptides[17] and triphosphates[54,25a]. In order to study cobalt complex promoted hydrolysis of phosphate diesters, it is essential to understand the general features of these cobalt complexes in solution.

The structure of $[Co(en)_2(Cl)_2]^{1+}$ has been characterized[55]. The ligand substitution reactions of the chlorides by water are well understood processes[56,55c]. The substitutions for both the first and the second chlorides by water or hydroxide

53. (a) Harrowfield, J. M.; Jones, D. R.; Lindoy, L. F.; Sargeson, A. M. *J. Am. Chem. Soc.* **1980**, *102(26)*, 7733-7741.
54. (a) Norman, P. R.; Cornelius, R. D. *J. Am. Chem. Soc.* **1982**, *104(9)*, 2356-2365. (b) Suruki, S.; Higashiyama, T.; Nakahara, A. *Bioinorg. Chem.* **1978**, *8*, 277. (c) Hediger, M.; Milburn, R. M. *ACS Symp. Ser. No. 171*, 211, **1981**.
55. (a) Bailar, Jr., J. C.; Auten, R. W. *J. Am. Chem. Soc.* **1934**, *56(4)*, 774-776. (b) Bailar, Jr., J. C.; Peppard, D. F. *J. Am. Chem. Soc.* **1940**, *62(4)*, 820-823. (c) Baldwin, M. E.; Chan, S. C.; Tobe, M. L. *J. Chem. Soc.* **1961**, 4637-4645.
56. (a) Sargeson, A. M.; Searle, G. H. *Nature* **1963**, *200*, 356-357. (b) Hay, R. W.; Norman, P. R. *J. Chem. Soc., Chem. Comm.* **1980**, 734-735. (c) Chan, S. C.; Tobe, M. L. *J. Chem. Soc.* **1962**, 4531-4540. (d) Martin, D. F.; Tobe, M. L. *J. Chem. Soc.* **1962**, 1388-1396. (f) Pearson, R. G.; Basolo, F. *J. Am. Chem. Soc.* **1956**, *78(19)*, 4878-4883.

are fast (completed within minutes) for either the *cis* or *trans*-$[Co(en)_2(Cl)_2]^{1+}$ in neutral and basic solutions[57]. During the reactions, the *cis* form will have complete retention of the configuration, and the *trans* form will mostly rearrange to the *cis* form[55c].

Like other inorganic complexes, $[Co(N_4)(OH_2)_2]^{3+}$ type complexes have several equilibria in water solution. For example, $[Co(en)_2(OH_2)_2]^{3+}$ has the following equilibria[31b] as shown in figure 3.1. At pH 7, the second equilibrium is the dominant one. The *cis*-aquohydroxo form of the complex exists as 59% of all

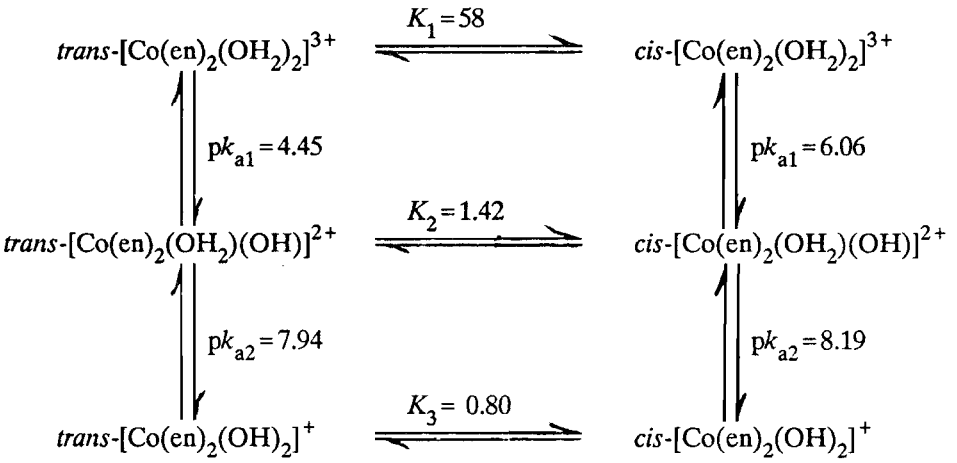
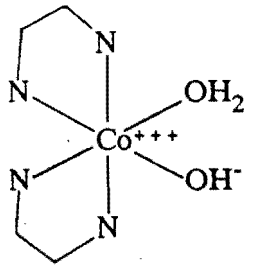


Figure 3.1 Equilibria of $[Co(en)_2(OH_2)_2]^{3+}$ in water.

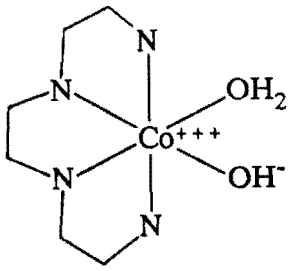
57. (a) Pearson, R. G.; Meeker, R. E.; Basolo, F. *J. Am. Chem. Soc.* **1956**, *78(4)*, 709-713. (b) Basolo, F.; Pearson, R. G. "Mechanisms of Inorganic Reactions" John Wiley & Sons, N. Y., 1987.

the species in equilibria. The rate of isomerization from *trans* to *cis* in the second equilibrium has a half life of 6 minutes at 25°C[58].

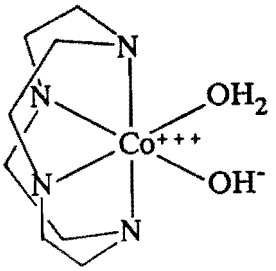
The aquohydroxo cobalt complexes used are shown in Figure 3.2. $[Co(cyclen)(OH_2)(OH)]^{2+}$ can only exist in the *cis* configuration. $[Co(en)_2(OH_2)(OH)]^{2+}$ has three different forms; trans, *l-cis* and *d-cis*. The two *cis* forms are mirror images of each other. The isomeric possibilities in $[Co(trien)(OH_2)(OH)]^{2+}$ are quite complex. There are nine configurations in



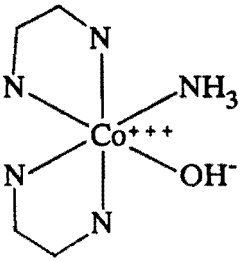
$[Co(en)_2(OH_2)(OH)]^{2+}$



$[Co(trien)(OH_2)(OH)]^{2+}$



$[Co(cyclen)(OH_2)(OH)]^{2+}$



$[Co(en)_2(NH_3)(OH)]^{2+}$

Figure 3.2 Some cobalt complexes used in this study.

solution; *trans*-SS, *trans*-RR, *trans*-RS, *cis*-α -SS, *cis*-α -RR, *cis*-β -RR, *cis*-β -SS, *cis*-β -RS and *cis*-β -SR. In diaquo complexes, the stabilities of the

58. Kruse, W.; Taube, H. *J. Am. Chem. Soc.* **1961**, *83*(6), 1280-1284.

different configurations have the following order: *cis*- β > *cis*- α > trans[59]. Within the β configuration, *cis*-β -RR and *cis*-β -SS are known to be more stable than *cis*-β -RS and *cis*-β -SR[60]. Isomerization and aquation reactions are fast for both the *cis* and *trans* $[Co(trien)(OH_2)(OH)]^{2+}$ in neutral and basic conditions, and the ß -forms of the complex exist as 85% in the equilibria[59]. In promoting the hydrolysis of phosphate esters, the *cis* forms are expected to be more reactive than the *trans* forms because the two coordinate sites (aquo and hydroxide) in the *cis*-complex are close together and the coordinated hydroxide is so placed that it can reach a bound phosphate diester easily in a bifunctional mechanism. The reactivities of different configurations in the *cis*-complex may be different. However, it is very difficult to determine which configuration is more reactive because all these forms are in equilibrium in solution.

B. Mechanism of $[Co(trien)(OH_2)(OH)]^{2+}$ and $[Co(en)_2(OH_2)(OH)]^{2+}$ promoted hydrolysis of BNPP

In the hydrolysis of BNPP promoted by $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$, two equivalents of *p*-nitrophenolate were released from the diester without accumulation of the phosphate monoester. This was indicated by the final UV absorbance and the lack of the ^1H NMR signals due to the monoester. These results showed that the hydrolysis of phosphate monoester is faster than the hydrolysis of the diester under the experimental conditions. BNPP is hydrolyzed directly to form a inorganic phosphate and two equivalents of *p*-nitrophenolate. This was also confirmed clearly by the hydrolysis of the

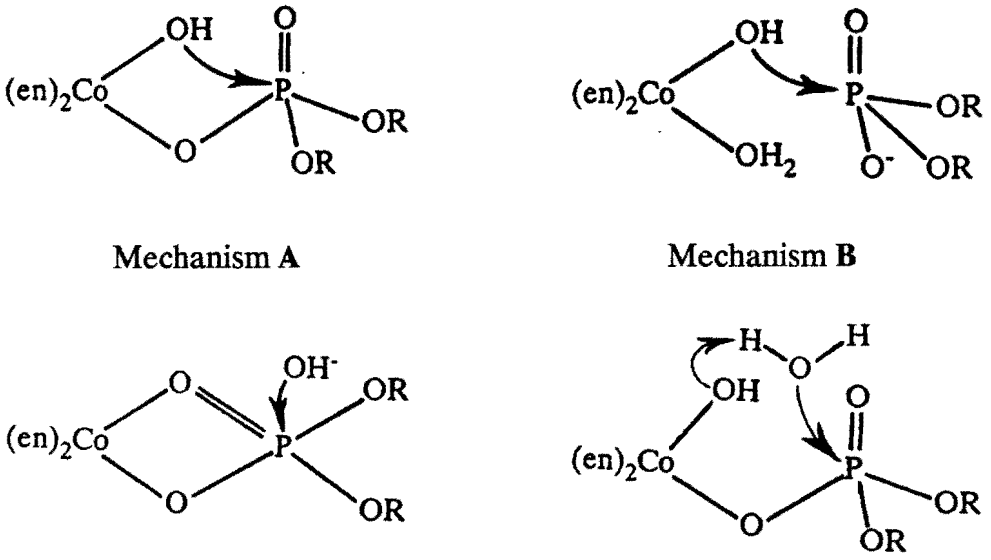
59. Sargeson, A. M.; Searle, G. H. *Inorg. Chem.* **1967**, *6(4)*, 787-796.
60. (a) Buckingham, D. A.; Mazilli, P. A.; Sargeson, A. M. *Inorg. Chem.* **1967**, *6(5)*, 1032-1041. (b) Sargeson, A. M.; Searle, G. H. *Inorg. Chem.* **1967**, *6(12)*, 2172-2180.

corresponding phosphate monoester, NPP, in the presence of the cobalt complexes (Table 2.3). The rates of hydrolysis of NPP promoted by the two complexes are about 10 times faster than the corresponding rates for the hydrolysis of BNPP promoted by the two cobalt complexes.

The pH-rate profile of the hydrolysis of BNPP (Figure 2.4) shows a 'bell' shaped curve from pH 5.5 to pH 9 when $[Co(trien)(OH_2)(OH)]^{2+}$ was used. The maximum rates were found between pH 6.5 to 7 where the aquohydroxo form of the cobalt complex is at its maximum, suggesting that the reactive species is the aquohydroxo form of the cobalt complex. The diaquo and dihydroxo forms of the complex are much less reactive.

Several possible mechanisms are shown in Figure 3.3 for the hydrolysis reaction. In mechanism **A**, the cobalt complex first acts as a Lewis acid to bind the phosphate diester. The hydroxide which is coordinated to the metal then attacks the phosphate diester intramolecularly to form a pentacoordinate intermediate followed by the release of a *p*-nitrophenolate. In mechanism **B**, the bound hydroxide acts only as a simple nucleophile. It attacks intermolecularly at the phosphate diester. Mechanisms **C** goes through a pure Lewis acid mechanism with two oxygens of the phosphate diester chelated to the cobalt. The hydrolysis is accomplished by intermolecular attack by a solvent water or hydroxide. Mechanism **D** is a general base mechanism. The cobalt bound hydroxide acts as a general base to assist the attack by a solvent water on the bound phosphate diester. These mechanisms are kinetically indistinguishable.

Mechanism **B** can be eliminated because the pH-rate profile of $[Co(trien)(OH_2)(OH)]^{2+}$ promoted hydrolysis (Figure 2.4) shows a substantial



$R = C_6H_4NO_2$

Figure 3.3 Possible mechanisms of BNPP hydrolysis promoted by $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$.

decrease at the pH range above 7.5. If the hydrolysis of BNPP is accomplished by mechanism **B**, no rate decrease of the hydrolysis should be observed under those basic conditions. We also studied the reactivity of $[Co(en)_2(NH_3)(OH)]^{2+}$ in promoting the hydrolysis of BNPP (Table 2.3). No rate acceleration was observed for the hydrolysis of BNPP in the presence of $[Co(en)_2(NH_3)(OH)]^{2+}$, also indicating that mechanism **B** is not important because $[Co(en)_2(NH_3)(OH)]^{2+}$ is a unifunctional molecule and rate acceleration for BNPP hydrolysis by this complex could only be accomplished by mechanism **B** (Figure 3.3).

$[Co(trien)(OH_2)(OH)]^{2+}$ and $[Co(en)_2(OH_2)(OH)]^{2+}$ have similar structures (Figure 3.2) and pK_a values (Table 2.1). Yet, the reactivities of the two cobalt complexes on the hydrolysis of BNPP are quite different (4.8×10^{-4} s^{-1} and 2.7×10^{-5} s^{-1}, Table 2.3). The general base mechanism (mechanism **D**, Figure 3.3) could not explain the different reactivities of the two cobalt complexes. If mechanism **D** is important is important, we should expect to see similar reactivities for the two complexes. A nucleophilic catalysis is usually more sensitive to the structural change of the catalysts and more efficient than a general base catalysis[61]. The effective molarities of intramolecular nucleophilic catalysis range from 10^4 to 10^8 M or even higher for ring closure reactions. On the other hand, the highest effective molarity for general base catalysis[61] is only 80 M.

Sargeson et al. showed by isotope labelling experiments (Figure 1.10) that the hydrolysis of the cobalt bound phosphate monoester, $[Co(en)_2(OH)(OP(O_2)OC_6H_4NO_2)]^{2+}$ occurs by an intramolecular nucleophilic attack similar to mechanism **A** (Figure 3.3). A mechanism by the formation of a cobalt-phosphate four membered ring followed by intermolecular attack by the solvent molecule analogous to mechanism **C** was not observed (Figure 1.10).

In general, intramolecular nucleophilic catalysis is much more efficient than its intermolecular counterparts[62,61]. For example,the hydrolysis of succinanilic acid[63] at pH 5 is 10^5 times more rapid than that of acetanilide (Figure 3.4). The faster rate of hydrolysis of succinanilic acid is a result of participation of the carboxyl group through intramolecular nucleophilic attack. In the hydrolysis of BNPP, the bound hydroxide should be much more efficient than external hydroxide or water due to its favorable position (and thus entropy and high effective

61. Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183-278.
62. Menger, F. M. *Acc. Chem. Res.* **1985**, *18(5)*, 128-134, and reference therein. .
63. Higuchi, T.; Eberson, L. Herd, A. D. *J. Am. Chem. Soc.* **1966**, *88(16)*, 3805-3808.

molarity) to attack. Based on these analysis, we propose that it is likely mechanism A is also important in the cobalt complex promoted hydrolysis of phosphate diester.

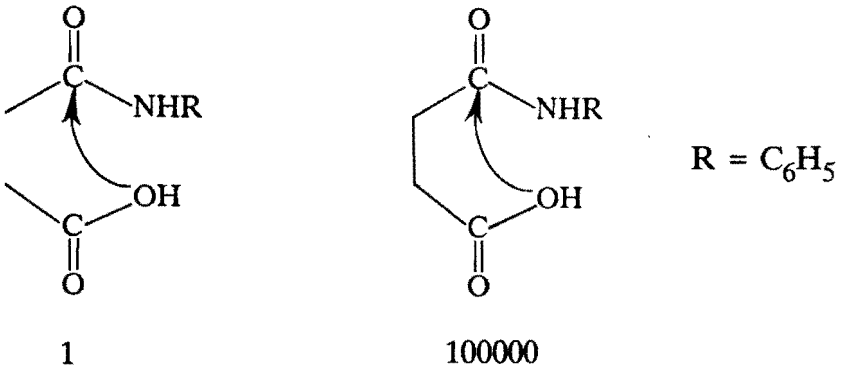


Figure 3.4 Inter *vs* intra molecular hydrolysis.

There are two steps involved in the hydrolysis of BNPP promoted by the bifunctional cobalt complex. Firstly, the phosphate diester replaces the coordinated water in an anation step that makes the phosphate diester more susceptible toward nucleophilic attack. Secondly, the cobalt bound phosphate diester is attacked intramolecularly by the cobalt bound hydroxide. The overall reaction cycle of the $[Co(en)_2(OH_2)(OH)]^{2+}$ promoted hydrolysis of BNPP is illustrated in Figure 3.5.

We measured the anation rates of inorganic phosphate to different aquohydroxo cobalt complexes under the conditions used for the phosphate diester hydrolysis by a UV method[31]. The pseudo first order rate constants of anation by the phosphat ($5x10^{-4}$ M) to $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ (0.01 M) were found to be $9x10^{-4}$ s^{-1} and 3×10^{-2} s^{-1} at 50°C (Table 2.4). The mechanism of anation reactions to Co(III) complex is known to be dissociative (I_d) and the rate of anation

is not sensitive to the entering groups[64]. The rates of anation of BNPP to the cobalt complexes (k_1, Figure 3.5) should be comparable to the rate of anation of inorganic phosphate to cobalt at neutral pH. It follows that the anation rates of phosphate diester to $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ are faster than the

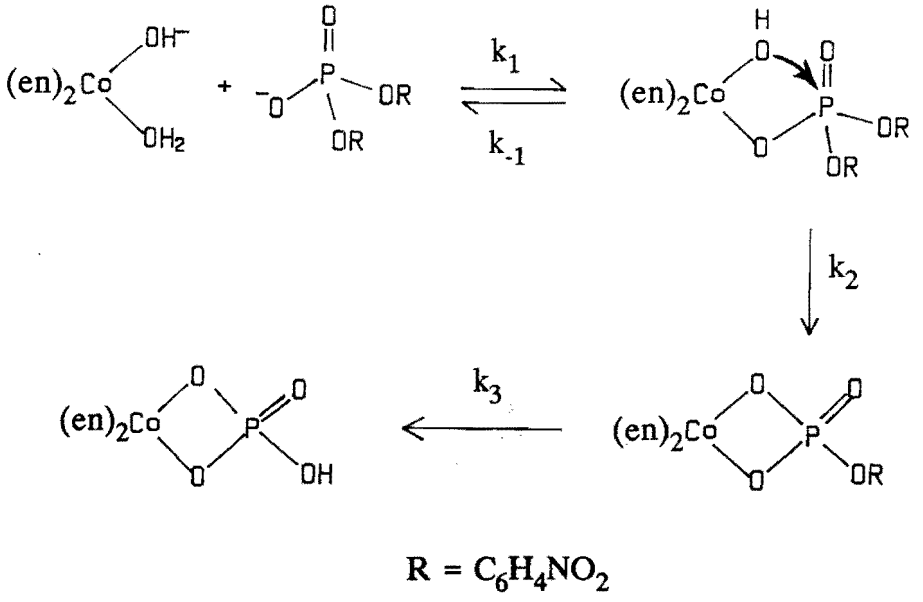


Figure 3.5 Reaction cycle of cobalt complex promoted hydrolysis of BNPP.

rates of the cobalt complex promoted BNPP hydrolysis ($2.7 \times 10^{-5} s^{-1}$ and $4.8 \times 10^{-4} s^{-1}$, Table 2.3). As has been discussed earlier, the hydrolysis of the cobalt bound monoester is faster than the hydrolysis of the bound diester (Table 2.3, k_3, Figure 3.5). Therefore, the second step, the intramolecular nucleophilic attack of the cobalt bound hydroxide on bound diester, is the rate determining step in the overall hydrolysis reaction of the phosphate diester.

64. Different anionic ligands such as Cl^-, $H_2PO_4^-$, $SO_4^=$, NCS^-, H_2O and so on have been studied. "Concepts and Models of Inorganic Chemistry", 2nd. Ed., Douglas, B. E.; McDaniel, D. H. Alexander, J. J., John Wiley & Sons, N. Y., 1983

The rate and the observed first order rate constant of the hydrolysis reaction can be expressed by equation 3.1 and 3.2, where k_1 and k_{-1} are the rate constants for the forward and reverse anation reactions and k_2 is the rate constant for the hydrolysis step. $[Co(OH_2)(OH)]$ and [S] are the concentrations of the aquohydroxo cobalt complex and BNPP respectively.

$$R = \frac{k_1 \cdot k_2}{k_{-1}} [Co(OH_2)(OH)] \cdot [S] \qquad (3.1)$$

$$k_{obs} = \frac{k_1 \cdot k_2}{k_{-1}} [Co(OH_2)(OH)] \qquad (3.2)$$

Equation 3.3 and 3.4 give the equilibrium constants for the dissociations of the diaquo cobalt complex, where $[Co(OH_2)_2]$, $[Co(OH_2)(OH)]$ and $[Co(OH)_2]$ are the concentrations of the cobalt complex in different protonation forms. The

$$K_{a1} = \frac{[Co(OH_2)(OH)] \cdot [H^+]}{[Co(OH_2)_2]} \qquad (3.3)$$

$$K_{a2} = \frac{[Co(OH)_2] \cdot [H^+]}{[Co(OH_2)(OH)]} \qquad (3.4)$$

total concentration of the cobalt complex (C_o) used can be expressed by equation 3.5. From these equations, the concentration of the reactive aquohydroxo species

$$C_o = [Co(OH_2)_2] + [Co(OH_2)(OH)] + [Co(OH)_2] \qquad (3.5)$$

can be expressed as a function of the acidity of the solution (equation 3.6). Substituting equation 3.6 into equation 3.2, the observed rate constant (k_{obs}) for the hydrolysis reaction becomes equation 3.7.

$$[Co(OH_2)(OH)] = \frac{C_o \cdot K_{a1} \cdot [H^+]}{K_{a1} \cdot K_{a2} + K_{a1} \cdot [H^+] + [H^+]^2} \qquad (3.6)$$

$$k_{obs} = \frac{k_1 k_2}{k_{-1}} \times \frac{C_o \cdot K_{a1} \cdot [H^+]}{K_{a1} \cdot K_{a2} + K_{a1} \cdot [H^+] + [H^+]^2} \qquad (3.7)$$

Equations 3.6 and 3.7 can be related to the 'bell' shaped pH-rate profile for the cobalt complex promoted hydrolysis of BNPP. At high acidity, the term $[H^+]$ is large. Equation 3.8 holds. The first term in the denominator of equation 3.7 is therefore not important. k_{obs} can be written as equation 3.9.

$$K_{a1} \cdot K_{a2} << K_{a1} \cdot [H^+] + [H^+]^2 \qquad (3.8)$$

$$k_{obs} = \frac{k_1 k_2}{k_{-1}} \times \frac{C_o \cdot K_{a1}}{K_{a1} + [H^+]} \qquad (3.9)$$

Under these conditions, the concentration of the aquohydroxo complex as well as the rate of the hydrolysis will decrease as the acidity of the solution increases. On

$$K_{a1} \cdot K_{a2} >> K_{a1} \cdot [H^+] + [H^+]^2 \qquad (3.10)$$

$$k_{obs} = \frac{k_1 k_2}{k_{-1}} \times \frac{C_o \cdot [H^+]}{K_{a2}} \qquad (3.11)$$

the other hand, equations 3.10 and 3.11 become important in basic conditions. It follows that the concentration of the aquohydroxo complex and the rate of the hydrolysis will also decrease as the concentration of the dihydroxo complex increases. The highest reactivity of the cobalt complex is thus found at the pH range from 6.5 to 7, where the concentration of the aquohydroxo complex is the highest.

The diaquo complex is less reactive than the aquohydroxo complex because the coordinated water is not very nucleophilic. The dihydroxo complex is not very reactive since it is difficult to replace a hydroxide from the cobalt complex in the anation step.

The observed pseudo first order rate constants for the hydrolysis of BNPP promoted by $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ (0.01M) are 2.7×10^{-5} s^{-1} and 4.8×10^{-4} s^{-1} respectively (Table 2.3). The equilibria for the anation of anionic species to Co(III) complex have been studied. There is a linear free energy relationship between the equilibrium constant of anation on Co(III) and the basicity of the anionic ligand[65]. From this relationship, the equilibrium constant for the anation reaction (k_1/k_{-1}) of phosphate diesters to $[Co(en)_2(OH_2)(OH)]^{2+}$ can be estimated[52] to be $1M^{-1}$. Thus, the rate constant (k_2, Figure 3.5) in the nucleophilic attack step is estimated to be $3.2 \times 10^{-3} s^{-1}$ for $[Co(en)_2(OH_2)(OH)]^{2+}$ promoted hydrolysis of BNPP. This rate is ten million

65. (a) Langford, C. H.; Gray, H. B. "Ligand Substitution Processes", Benjamin Inc., N. Y., 1965. (b) Tobe, M. L. "Inorganic Reaction Mechanisms", Thomas Nelson and Son Ltd., Great Britain, 1972.

(10^7) times faster than that of uncoordinated BNPP hydrolysis under neutral conditions (Table 2.3). For $[Co(trien)(OH_2)(OH)]^{2+}$ promoted hydrolysis of BNPP,
the rate enhancement is even greater (10^8 fold).

In the study of cobalt complex promoted hydrolysis of phosphate monoesters, Sargeson and his co-workers prepared phosphate monoesters ligated to Co(III) complex and studied the hydrolysis reaction of the bound species[2b]. This is possible since the rate determining step is the first step in cobalt promoted hydrolysis of phosphate monoesters. On the other hand, this approach is not applicable in the case of phosphate diesters because the rate determining step is the second step of the nucleophilic attack in the cobalt promoted hydrolysis of BNPP. Cobalt bound phosphate diester should break down to free phosphate diester and the complex more rapidly than hydrolysis.

More than one metal may participate in the catalysis in cobalt complex promoted hydrolysis of ATP, DNA and RNA since these phosphate esters have multiple binding sites[66]. In the hydrolysis of simple phosphate diesters, however, it is likely that only one metal complex is participating in the catalysis because only one binding site is available in phosphate diesters. This is supported by the concentration dependence of the rates of BNPP hydrolysis promoted by $[Co(trien)(OH_2)(OH)]^{2+}$. When different concentrations of the complex (0.0025 to 0.025 M) were used, there is a good linear relationship between the concentration of the cobalt complex and the rate of BNPP hydrolysis (Figure 2.4).

Both the ligation of phosphate diester to the cobalt and the intramolecular attack by the coordinated hydroxide are important in promoting the hydrolysis of BNPP. This type of double activations may also be important in enzymatic

66. Suh, J.; Han, O.; Chang, B. *J. Am. Chem. Soc.* **1975**, *108*(*8*), 1839-1842.

hydrolysis of RNA and phosphate diesters. A common strategy of enzymatic catalysis is to bind two substrates in close proximity and in a specific orientation, therefore facilitating their reactions. This is what is observed here in Co(III) complex promoted hydrolysis of phosphate diester.

C. Comparison of the Reactivities of the Co(III) Complexes, ZnCR-OH, Free Hydroxide and Hydrolytic Enzymes

a. Comparison of different Co(III) complexes

We have studied the reactivities of several structurally related cobalt complexes toward the hydrolysis of BNPP (Figure 3.2, Table 2.3). Interestingly, different cobalt complexes promote the hydrolysis of BNPP with different efficiencies. $[Co(cyclen)(OH_2)(OH)]^{2+}$ is 10 times more efficient than $[Co(trien)(OH_2)(OH)]^{2+}$, and $[Co(trien)(OH_2)(OH)]^{2+}$ about 20 times more efficient than $[Co(en)_2(OH_2)(OH)]^{2+}$. These results showed that the structure of the amine ligands not only influences the rate of the anation reaction of the cobalt complex (Table 2.4), but also enormously affects the rate of the hydrolysis step(k_2, Figure 3.5).

No quantitative data has been reported in the literature for the rates of anation and ligand exchange for the different cobalt complexes used in this study. We thus measured the rate of anation of inorganic phosphate to each of the cobalt complexes used (Table 2.4). The rate of anation by the inorganic phosphate to $[Co(cyclen)(OH_2)(OH)]^{2+}$ is 100 times faster than to $[Co(trien)(OH_2)(OH)]^{2+}$, and the rate to $[Co(trien)(OH_2)(OH)]^{2+}$ is about 30 times faster than to $[Co(en)_2(OH_2)(OH)]^{2+}$. Nevertheless, as discussed earlier the

anation step is **not** the rate determining step during the hydrolysis of BNPP and any increase in the rate of anation should not change the overall rate of BNPP hydrolysis. The driving force for the efficiency of the cobalt complexes therefore should come from factors other than anation. This will be discussed in detail in a separate section (p59).

b. Comparison of Co(III) complexes and ZnCR-OH

Many hydrolytic enzymes use Zn(II) ions in their catalytic processes[12]. Naturally we wanted to compare the reactivities of cobalt complexes with zinc complexes. The reactivity of **ZnCR-OH**, on the hydrolysis of BNPP was examined because **ZnCR-OH** had been shown to be efficient in promoting the hydration of aldehydes and carbon dioxide[21] as well as the hydrolysis of carboxylic esters[22]. To our knowledge, **ZnCR-OH** has the lowest pK_a value (8.6) of the bound water among known Zn(II) complexes. However, **ZnCR-OH** showed only a modest reactivity in promoting the hydrolysis of BDNPP (Figure 2.9), an extremely reactive phosphodiester. Rate enhancement in the hydrolysis of BNPP was not observed. These results showed that **ZnCR-OH** is much less reactive than the $[Co(N_4)(OH_2)(OH)]^{2+}$ type complexes in promoting the hydrolysis of phosphate diesters.

When this work was in progress, Breslow reported that **ZnCR-OH** is 10 times more reactive than free hydroxide[23] in promoting the hydrolysis of a phosphate triester, diphenyl *p*-nitrophenyl phosphate. The reactivity of **ZnCR-OH** was attributed to a bifunctional mechanism, where **ZnCR-OH** acts both as a Lewis acid and as a nucleophile (Figure 3.6).

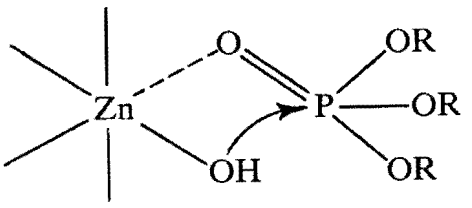


Figure 3.6 Mechanism of **ZnCR-OH** promoted hydrolysis of phosphate triester.

Although this bifunctional mechanism may be operative in **ZnCR-OH** promoted hydrolysis of phosphate triesters (there is no direct evidence to support this hypothesis), the lack of reactivity of **ZnCR-OH** in promoting the hydrolysis of BNPP seems to suggest that **ZnCR-OH** may only act as a simple nucleophile by metal bound hydroxide in promoting the hydrolysis of phosphodiesters. This can be seen by the comparable reactivity of a cobalt complex, $[Co(en)_2(NH_3)(OH)]^{2+}$ (table 2.3), which can only act as a simple nucleophile by the cobalt bound hydroxide. $[Co(en)_2(NH_3)(OH)]^{2+}$ is also unreactive in promoting the hydrolysis of BNPP and gives only a modest rate enhancement in promoting the hydrolysis of BDNPP.

c. Comparison of Co(III) complexes, OH^- and hydrolytic enzymes

It would be interesting to compare the rates of Co(III) complex promoted hydrolysis of BNPP with those promoted by hydroxide and by hydrolytic enzymes. The second order rate constants for BNPP hydrolysis promoted by hydroxide, cobalt complex, and by some hydrolytic enzymes are summarized in table 3.1.

Table 3.1 Second order rate constants for BNPP hydrolysis promoted by different catalysts.

Catalysts	k ($M^{-1}s^{-1}$)	Reference
None	$5.5x10^{-12}$	a
NaOH (1.0 M)	$1.6x10^{-4}$	a
$[Co(trien)(OH_2)(OH)]^{2+}$	$5.7x10^{-2}$	b
$[Co(cyclen)(OH_2)(OH)]^{2+}$	$5.7x10^{-1}$	b
Acid Phosphatase II	$2.8x10^{-1}$	c,d
Bovine Intestine	$1.3x10^{1}$	c,e

a) Rates at 50°C. See Table 2.3. Rate of hydrolysis without any catalyst has been divided by the concentration of water (55M).
b) Rates at 50°C, 0.01M of Co(III) complex.
c) Calculated by $k_{cat} = V_{max}/[enzyme]$. "Biochemistry" p140, Zubay, G., Addison-Wesley, 1983.
d) PH 5.3, 37°C. Yoshida, H. *J. Biochem.* **1973**, *73(1)* 23-29.
e) PH 8.0, 30°C. Kelly, S. J.; Dardinger, D. E.; Butler, L. G. *Biochem.* **1975**, *14(22)*, 4983-4988.

For NaOH promoted hydrolysis of BNPP, the second order rate constant is 1.6×10^{-4} $M^{-1}s^{-1}$. The corresponding rate constant for $[Co(trien)(OH_2)(OH)]^{2+}$ is $5.7x10^{-2}$ $M^{-1}s^{-1}$ (Table 3.1). Thus, $[Co(trien)(OH_2)(OH)]^{2+}$ is some 350 times more efficient than free hydroxide in promoting the hydrolysis of BNPP at neutral conditions. The difference in reactivities between $[Co(cyclen)(OH_2)(OH]^{2+}$ and free hydroxide is 3500 fold. The remarkable efficiency of the cobalt complexes can be explained in that free hydroxide can only act as a simple nucleophile while the cobalt(III) complexes can activate both the bound ester and the bound water.

BNPP is not a natural substrate for enzymes but it has been used over the years as a standard artificial substrate for determining the reactivities of hydrolytic enzymes. It is very interesting to see that in promoting the hydrolysis of a phosphate diester with good leaving groups, the reactivity of the most efficient

cobalt complex, $[Co(cyclen)(OH_2)(OH)]^{2+}$, is only 10 to 100 times less reactive than some of the phosphoesterases (Table 3.1).

D. Hydrolysis of BDNPP Promoted by Co(III) Complexes

In the two-step BNPP hydrolysis, the second step (k_2, Figure 3.5) is the rate limiting step, but the rates of the two steps are not substantially different. It is possible that the rate limiting step will shift to the anation step (k_1, Figure 3.5) if a phosphate diester with a better leaving group, such as 2,4-dinitrophenol, is used to 'speed up' the second step. This was studied using bis-(2,4-dinitrophenyl)phosphate as the substrate. 2,4-Dinitrophenol is an extremely good leaving group. The pK_a of 2,4-dinitrophenol is 4.12[67] compared to 7.14 of *p*-nitrophenol.

The rate constants for several cobalt complex promoted hydrolysis of BDNPP are also given in Table 2.3. In the case of $[Co(en)_2(OH_2)(OH)]^{2+}$ promoted hydrolysis of BDNPP, the rate constant for anation ($\sim 9x10^{-4}s^{-1}$, Table 2.4)[64] and the rate constant for hydrolysis ($4.2x10^{-4}s^{-1}$, Table 2.3) are comparable, suggesting that the anation step is at least partially rate determining in the hydrolysis.

It should be noted in Table 2.3 that $[Co(en)_2(NH_3)(OH)]^{2+}$ does promote the hydrolysis of BDNPP at a rate comparable to that of $[Co(en)_2(OH_2)(OH)]^{2+}$. It seems that when the leaving groups become extremely good as is the case for 2,4-dinitrophenol, it is possible that the hydrolysis can take place by the direct nucleophilic attack mechanism (**B**, Figure 3.3) However, this mechanism should be less efficient than the bifunctional mechanism **A** (Figure 3.3). For the aquohydroxo

67. Jencks, W. P.; Regenstein, J. "Handbook of Biochemistry and Molecular Biology" 3rd. Ed. Phy. Chem. Data *1*, 305, Fasman, G. D. Ed. CRC Press, Ohio, 1976.

complexes used, mechanism **A** should still be important in the hydrolysis of BDNPP as evidenced by the p**H**-rate profile of $[Co(trien)(OH_2)(OH)]^{2+}$ promoted hydrolysis of BDNPP (figure 2.3). The rate of hydrolysis of BDNPP decreased substantially at both high and low p**H** conditions, suggesting that the most reactive species is still the aquohydroxo complex.

$[Co(dien)(OH_2)(OH)]^{2+}$ is the most efficient complex in promoting the hydrolysis of BDNPP (table 2.3). However, the complex is very unstable. The exact mechanism of the hydrolysis remains unclear.

E. Hydrolysis of NPP Promoted by Co(III) Complexes

In order to clarify the mechanisms involved in the hydrolysis of BNPP, we examined the hydrolysis reaction of mono *p*-nitrophenyl phosphate (NPP) promoted by the cobalt complexes. It was found that the rate of hydrolysis of NPP promoted by each of the aquohydroxo cobalt complex is in general ten times faster than that of the corresponding diester, BNPP (table 2.3). In the absence of any catalysts, NPP is cleaved much faster than BNPP in neutral conditions[68,7].

For cobalt complex promoted reactions, Co(III) bound phosphate diester, BNPP, is cleaved 10^7-10^9 times more rapidly than the unbound phosphate diester. In contrast, cobalt complex bound phosphate monoester, NPP, is cleaved only 10^4 to 10^6 times more rapidly than the free ester. The larger rate acceleration for cobalt complex promoted hydrolysis of BNPP over that of NPP (table 2.3) is mainly due to the availability of the metaphosphate mechanism for the hydrolysis of the free

68. Kirby, A. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87(14)*, 3209-3216.

monoester, NPP[9,50]. As discussed earlier, the metaphosphate mechanism is not possible for the hydrolysis of BNPP.

3.2 Reactivities and Structures of Co(III) Complexes

In the cobalt complex promoted hydrolysis of BNPP, the rate determining step is the second step (k_2, Figure 3.5). $[Co(cyclen)(OH_2)(OH)]^{2+}$ is ten times more efficient than $[Co(trien)(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ is about 20 times more reactive than $[Co(en)_2(OH_2)(OH)]^{2+}$. It is interesting that such small structural changes can lead to such large differences in reactivities.

To account for the different reactivities of the structurally related cobalt complexes, we should look more closely at the hydrolysis process. There are two factors contributing to the rate enhancement in the cobalt complex promoted hydrolysis. One is the coordination of the phosphate ester to the cobalt and the other is the nucleophilic attack by the cobalt bound hydroxide on the bound phosphate ester. The effects of coordination of BNPP to different Co(III) complexes on the rate of hydrolysis should be about the same for all the aquohydroxo cobalt complexes used since the Co(III) complexes have same charge and similar structures. The difference in the reactivities of the different cobalt complexes in promoting BNPP hydrolysis therefore should be due to the step of the nucleophilic attack which is the rate determining step. When the coordinated hydroxide attacks the bound phosphate diester, it will form a four membered ring intermediate. It is well known that the formation of a four membered ring system will impose a considerable amount of ring strain. X-ray crystallographic studies showed that in a cobalt and phosphate four membered ring system, the O-Co-O angle is 76° and the O-P-O angle is 98.7° (Figure 3.7)[25b]. The angle between the

two adjacent ligands should be 90° in a normal hexa-coordinated cobalt complex that has an octahedral arrangement around the metal's hybrid orbitals. During the hydrolysis, when the cobalt bound hydroxide attacks the phosphate center, it has to

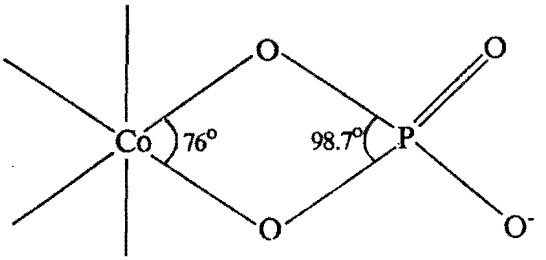
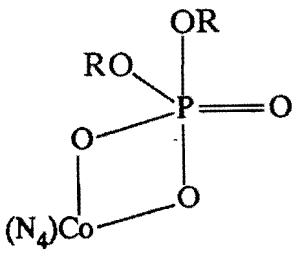


Figure 3.7 X-ray structure of $[Co(en)_2PO_4]$.

bend over to reduce the O-Co-O angle to about 76°C to reach the cobalt bound phosphate diester in the transition state. Form the transition state, the molecule will go on to form a trigonal-bipyramid intermediate **10**, as shown in Figure 3.8.

In the trigonal-bipyramid pentaphosphorus intermediate, the leaving group is expelled from the apical position and the electron donating groups occupy the equatorial positions[2c,69]. The sum of the four angles in the four membered ring intermediate (**10**) should be less than the sum of the four strain free angles (90°+90°+109.5°+109.5°). Therefore, the O-Co-O angle should be less than 90° in intermediate **10**. It is apparent that if the O-Co-O angle is small in a Co(III) complex, there will be less strain imposed on the four membered ring system both in the transition state and in the pentacoordinate intermediate **10**.

69. Trippett, S. *Pure Appl. Chem.* **1974**, *40(4)*, 595-605.

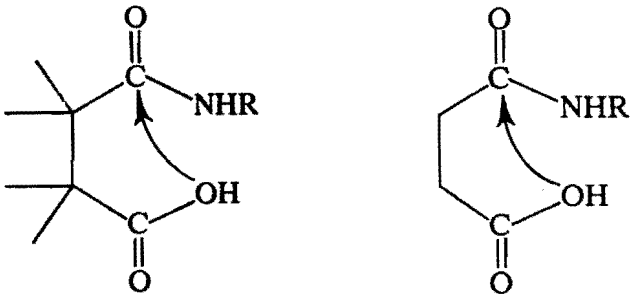


R = $C_6H_4NO_2$

10

Figure 3.8 Phosphate-cobalt intermediate.

Consequently, a smaller O-Co-O angle in a $[Co(N_4)(OH_2)(OH)]^{2+}$ type complex will result in a more stable intermediate than one with a larger O-Co-O angle, because the transition energy to generate the intermediate of the former will be



R = C_6H_5

1000 1

Figure 3.9 Hydrolyses of tetramethyl maleamic and maleamic monoamides.

smaller. It follows that the former cobalt complex should be more efficient in promoting the hydrolysis of a phosphate ester than the latter when the 'ring formation' step is the rate determining step.

Small structural change can lead to large change in reactivities. For example, the hydrolysis of tetramethylsuccinanilic acid occurs some 1000 times more rapidly than the hydrolysis of the unsubstituted succinanilic acid[63] (Figure 3.9). This is due to the proximity effects of the substitutions by the methyls that bring the carboxyl group closer to the amide.

X-ray structural data for the A-Co-B angles of $[Co(N_4)(A)(B)]$ type complexes are given in Table 3.2 along with the observed pseudo first order rate constants for the hydrolysis of BNPP, promoted by the corresponding aquohydroxo cobalt complexes. N_4 represents $(en)_2$, trien and cyclen. A and B are monodentate ligands. Changing the monodentate ligands, A and B, to monodentate ligands, OH_2 and OH^-, should not lead to large deviation of the OH_2-Co-OH^- angle from the A-Co-B angle.

In the series of $[Co(N_4)(OH_2)(OH)]^{2+}$ type complexes, the reactivity increases successively with a decrease in the angle between the two reactive sites. During the hydrolysis, a small O-Co-O angle in the complex between the OH^- and OH_2 will place the coordinated hydroxide in a close proximity to the coordinated phosphate diester and, therefore, the complex will be more efficient in promoting the hydrolysis of BNPP. The angle of O-Co-O in $[Co(cyclen)(OH_2)(OH)]^{2+}$ is about 84.5° which is 8 degrees and 4 degrees smaller than those in $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ respectively. As a result, the reactivity of $[Co(cyclen)(OH_2)(OH)]^{2+}$ is about 160 and 10 times greater than those of the other two complexes in promoting BNPP hydrolysis. The overall rate enhancement for the hydrolysis of $[Co(cyclen)(OH_2)(OH)]^{2+}$ bound BNPP is one billion (10^9) fold over that of the unbound diester.

Table 3.2 X-ray data for A-Co-B angles of $[Co(N_4)(A)(B)]$ type complexes and the reactivities of their corresponding aquohydroxo complexes toward BNPP hydrolysis.

Complexes	k_{obs} (s^{-1})[a]	Angles[b]	Reference
[Co(cyclen)(A)(B)]	$4.4x10^{-3}$	84.5°	c
[Co(trien)(A)(B)]	$4.8x10^{-4}$	88.6°	d
$[Co(en)_2(A)(B)]$	$2.7x10^{-5}$	92-93°	e

a) See table 2.3 for reaction conditions.
b) Angles between the two non-cyclic ligands, A and B.
c) A=B=NO_2, Iitaka, Y.; Shina, M.; Kimura, E. *Inorg. Chem.* **1974**, *13(12)*, 2886-2891.
d) A=Cl, B=H_2O, Freeman, H. C.; Maxwell, I. E. *Inorg. Chem.* **1969**, *8(6)*, 1293-1298.
e) A=B=Cl, Matsumoto, K.; Ooi, S.; Kuroya, H. *Bull. Chem. Soc. Japan* **1970**, *43(12)*, 3801-3804.

We actually started out with $[Co(en)_2(OH_2)(OH)]^{2+}$ and $[Co(trien)(OH_2)(OH)]^{2+}$ complexes, and arrived at the reactivity pattern. This finding directed us to make the more reactive $[Co(cyclen)(OH_2)(OH)]^{2+}$ complex which, to our knowledge, has the smallest A-Co-B angle (table 3.2) among all Co(III) complexes that have been analyzed by X-ray as of yet. Further rate enhancement can be expected if the angle between the two monodentate ligands in the Co((III) complex is further reduced. Making a five membered ring system could also release the strain that is involved in the four membered ring system.

3.3 Co(III) Complex Promoted Hydrolysis of Phosphate Diesters with Poor Leaving Groups

Simple hydrolytic catalysts which are efficient in promoting the hydrolysis of esters with good leaving groups can be millions of times less efficient in promoting the hydrolysis involving poor leaving groups[22,70]. Having succeeded in finding cobalt complexes that give up to 10^9 fold rate enhancement in the hydrolysis of phosphate diesters with good leaving groups, we tested them on phosphate diesters with poor leaving groups.

C-AMP was chosen as the substrate because of its biological importance and its suitable uncatalyzed rate of hydrolysis. It serves as an intermediate stage in the study before the complexes will be tested in the hydrolysis of natural DNA molecules.

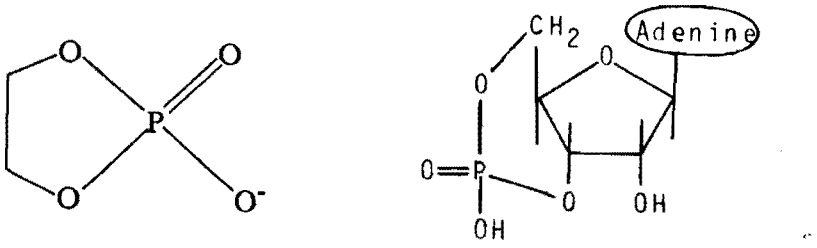


Figure 3.10 Ethylene phosphate and c-AMP.

Phosphate diesters with five membered ring structures are usually more reactive than their corresponding acyclic counterparts. For example, ethylene phosphate, which is an analog of 2',3'-cyclic AMP, undergoes P-O bond cleavage

70. Kirsch, J. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1964**, *86(5)*, 837-846.

some 10^7 times faster than dimethyl phosphate. For an acyclic diester, when it makes the transition from the ground state to a trigonal bipyramidal intermediate, the O-P-O angle will change from an angle of 109° in the tetrahedral ground state to 90° in the five coordinated transition state[2c]. In ethylene phosphate, the corresponding endocyclic O-P-O angle is already compressed to 98° in the ground state because of the strain imposed by the ring. The activation energy to attain the trigonal bipyramidal is thus reduced. Although c-AMP is a six membered ring system, the *trans*-conformation within the six membered ring system, however, does make the cyclic phosphate diester bonds quite unstable compared to the phosphate diester bonds in RNA and dimethyl phosphate[71]. The uncatalyzed rate of hydrolysis of c-AMP is much lower than that of BNPP.

In the presence of 0.2M $[Co(trien)(OH_2)(OH)]^{2+}$ at pH 7 and 50°C, the observed pseudo first order rate constant for the hydrolysis of c-AMP was found to $1.2 \times 10^{-6} s^{-1}$. In a control experiment in 0.2M phosphate buffer solution, there was no sign of hydrolysis of c-AMP even after the reaction solution had been heated at 100°C for a month. Thus, we could not directly obtain the uncatalyzed rate constant for c-AMP hydrolysis at neutral conditions.

Prior to this study, c-AMP has only been hydrolyzed in strongly acidic, basic and enzymic conditions. In order to estimate the uncatalyzed rate, we assayed[52] the hydrolysis of ethylene phosphate at pD7 and 100°C. The pseudo first order rate constant for the hydrolysis of ethylene phosphate was found to be $1.0 \times 10^{-7} s^{-1}$. For every 10°C temperature drop, the rate was reduced by forty percent. Hence, the rate constant should be about $1.0 \times 10^{-9} s^{-1}$ at 50°C. Ethylene phosphate is 10^5 times more reactive than c-AMP under basic conditions[5]. If we assume that this ratio is

71. Greengard, P.; Rudolph, S. A.; Sturtevant, J. M. *J. Biol. Chem.* **1969**, *244(17)*, 4798-4800.

maintained in neutral water as well, the rate constant for the hydrolysis of c-AMP at 50°C should be $1.0 \times 10^{-14} s^{-1}$.

The mechanism for the hydrolysis of c-AMP should be the same as that for the hydrolysis of BNPP (i.e. the second step (k_2, Figure 3.5) is the rate determining step) since c-AMP has poorer leaving groups than BNPP. During the course of the hydrolysis, no intermediate was detected. C-AMP might cleave at the 3'-O-P bond or at the 5'-O-P bond first. Either way, the subsequent hydrolysis of monoester should be rapid[72]. As has been discussed earlier (p46), the equilibrium constant for anation of a phosphate diester to Co(III) complex can be estimated to be $1M^{-1}$. Taking into account the concentration of the reactive aquohydroxo form of the cobalt complex, k_2 is calculated to be $7.1 \times 10^{-6} M^{-1} s^{-1}$. The rate enhancement for the hydrolysis of the cobalt(III) bound c-AMP is thus at least 10^8 fold over the hydrolysis of c-AMP in the absence of the cobalt complex.

Compared to NaOH, $[Co(trien)(OH_2)(OH)]^{2+}$ is 1000 times (Table 3.3) more efficient in promoting the hydrolysis of c-AMP. For enzyme catalyzed hydrolysis of c-AMP, the second order rate constants (k_{cat}) are about $3 \times 10^{-2} M^{-1} s^{-1}$ for 3',5'-cyclic nucleotide phosphodiesterase at 37°C (Table 3.3) and $10^1 M^{-1} s^{-1}$ for bovine intestine at 30°C (Table 3.3). The enzymes are thus about 10^4-10^6 time more efficient than $[Co(trien)(OH_2)(OH)]^{2+}$.

We have also tested $[Co(trien)(OH_2)(OH)]^{2+}$ on the hydrolysis of a natural dinucleotide, ApA. However, the rate of hydrolysis of ApA in the presence of $[Co(trien)(OH_2)(OH)]^{2+}$ was still very slow. No hydrolysis was observed in the presence of 0.1M complex at 50°C. The ^1H NMR signals of ApA broadened after prolonged heating. The uncatalyzed rate constant of ApA hydrolysis should be in

72. Milburn, R. M.; Gautam-Basak, M.; Tribolet, R.; Sigel, H. *J. Am. Chem. Soc.* **1985**, *107*(*11*), 3315-3321.

the order of 10^{-17} to 10^{-19} s^{-1} at room temperature (Table 1.2). Under our experimental conditions, even if there was a rate enhancement of 10^8 fold, it would be still very difficult to follow the hydrolysis.

Table 3.3 Second order rate constants for hydrolysis of c-AMP promoted by different catalysts.

Catalyst	$k(M^{-1}s^{-1})$	Reference
None	$1.8x10^{-16}$	a
NaOH	$4.7x10^{-9}$	b
$[Co(trien)(OH_2)(OH)]^{2+}$	$7.1x10^{-6}$	c
Bovine intestine	$1.2x10^{1}$	d
3',5'-Cyclic nucleotide phosphodiesterase	$3.3x10^{-2}$	e

a) Water rate at 50°C. See text. The rate has been divided by the concentration of water (55M).
b) Estimated from the rate of ethylene phosphate hydrolysis at 25°C. See text. Kumamoto, J.; Cox, J. R. Jr.; Westheimer, F. H. *J. Am. Chem. Soc.* **1956**, *78(18)*, 4858-4860.
c) This work.
d) PH 8.0, 30°C. See Table 3.1 reference e.
e) PH 7.5, 37°C. Assuming the molecular weight of the enzyme to be 60,000. Nair, K. G. *Biochem.* **1966**, *5(1)*, 150-157.

3.4 Further Work

We have showed that $[Co(N_4)(OH_2)(OH)]^{2+}$ type complexes promote the hydrolysis of c-AMP and BNPP very efficiently. Rate enhancement of up to 10^9

fold has been achieved in the hydrolysis of BNPP. Considering the rate acceleration of enzymatic catalysis, we believe that further improvements to the most efficient cobalt complex of an additional factor of 10-100 would enable it to be used in the hydrolysis of natural DNA and RNA. The O-Co-O angle found in a cobalt phosphate four membered ring system is about 76° (figure 3.7). The smallest angle in the cobalt complexes used in this study is about 84.5°C (table 3.2). Further improvement on the efficiency of the complexes lies in the fine tuning of the tetraamine ligands to reduce the angle between the two monodentate ligands.

There are many prospects in using such artificial catalysts. The one that is particularly appealing is in the modification of DNA structures. An efficient phosphate cleaving catalyst can be connected with a sequence specific DNA binding molecule. This combination will result in a sequence specific DNA cleaving molecule. This molecule could target at single or double stranded DNA, depending on the binding moiety used. The use of such a catalyst aimed at a double stranded DNA is illustrated in figure 3.11. A segment of DNA or RNA molecule can be cleaved site specifically by the catalyst. Another different sequence of oligonucleotides can then be joined by ligases. This operation will produce a modified DNA or RNA molecule whose properties will be different from its parent molecule. Clearly, this kind of sequence specific cleaving molecule will also be very useful in DNA or RNA sequencing.

In order to generate such an efficient catalyst, sequence specific DNA binding molecules have to be developed. This is one of the reasons we had directed our attention to the design and synthesis of DNA binding molecules which will be discussed in the second part of this thesis.

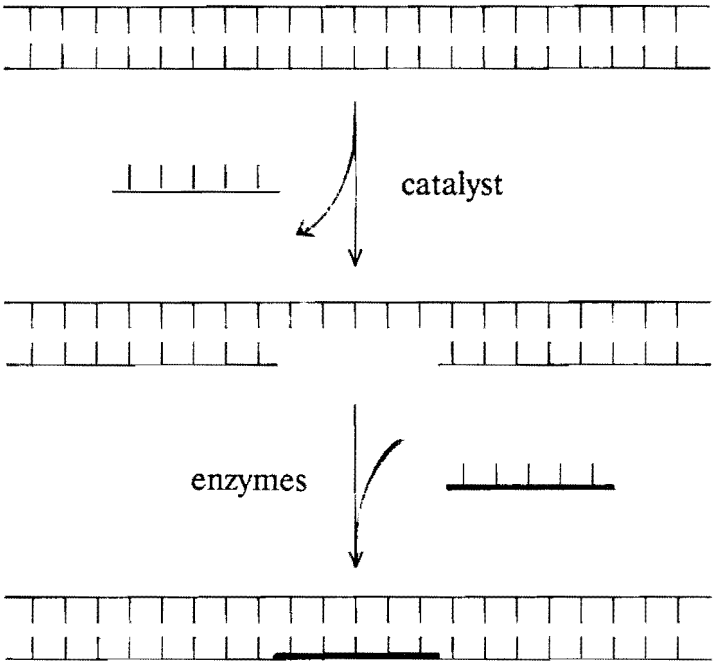


Figure 3.11 Modification of DNA structures.

4. EXPERIMENTAL SECTION

4.1 Instruments

Nuclear magnetic resonance (NMR) spectra were taken either on a Varian XL-200 or a Varian XL-300 spectrometer. Chemical shifts were measured relative to tetramethylsilane (TMS) in deuterated organic solvents and to 3-(trimethylsilyl)-1-propane sulfonic acid sodium salt (DSS) in deuterium oxide. Ultraviolet-visible spectra of kinetic measurements were recorded on a Hewlett Packard 8451A Diode Array Spectrophotometer equipped with a Brinkman MGW Lauda RM6 thermostat circulator for temperature control. pH values were measured using a Copenhagen Radiometer PHM63 digital pH meter. The acid dissociation constants of the cobalt complexes were obtained on a Copenhagen Radiometer RTS822 titration system.

4.2 Materials

A. General

All chemicals used were reagent grade or better. Disodium *p*-nitrophenylphosphate (NPP), sodium bis-(*p*-nitrophenyl)phosphate (BNPP),

adenosine cyclic-3',5'-phosphate mono (c-AMP) and adenylyl(3'-5')adenosine phosphate ammonium salt (ApA) were purchased from Sigma. Ethylenediamine (en), triethylenetetraamine (trien), diethylenetriamine (dien), ethylene glycol and tosyl chloride were purchased from Aldrich. They were used without further purification. Bis-(2,4-dinitrophenyl)phosphate (BDNPP) was synthesized as a pyridinium salt by the method reported by Rawji et al.[73]. The preparation of ZnCR-OH, (figure 1.8) has been reported elsewhere[74,22].

B. Co(III) complexes

Cis-dichlorobis(ethylenediamine)cobalt(III) chlorides[75], *cis*-α-dichloro(triethylenetetraamine)cobalt(III) chloride[59], *cis*-bromoamminebis(ethylenediamine)cobalt(III) bromide[76], trichloro(diethylenetriamine)cobalt(III)[77] were prepared according to well known procedures. Some of the physical properties of these cobalt complexes are given in tables 2.1 and 2.2 in the result section. Cyclic-1,4,7,10-tetraazadodecane was prepared using a similar strategy reported by Hay et al.[78] with some modifications as outlined in figure 4.1.

73. Rawji, G.; Milburn, R. M. *J. Org. Chem.* **1981**, *46(6)*, 1205-1206.
74. Prince, R. H.; Stotter, D. A.; Woolley, P. R. *Inorg. Chim. Acta* **1974**, *9(1)*, 51-54.
75. Bailar Jr., J. C. *Inorg. Syn.* **1960**, *2*, 222-225.
76. Tobe, M. L.; Martin, D. F. *Inorg. Syn.* **1966**, *8*, 198-202.
77. Crayton, P. H.; Mattern, J. A. *J. Inorg. Nucl. Chem.* **1960**, *13(3)*, 248-253.
78. Hay, R. W.; Norman, P. R. *J. Chem. Soc. Dalton Trans.* **1979**, 1441-1445.

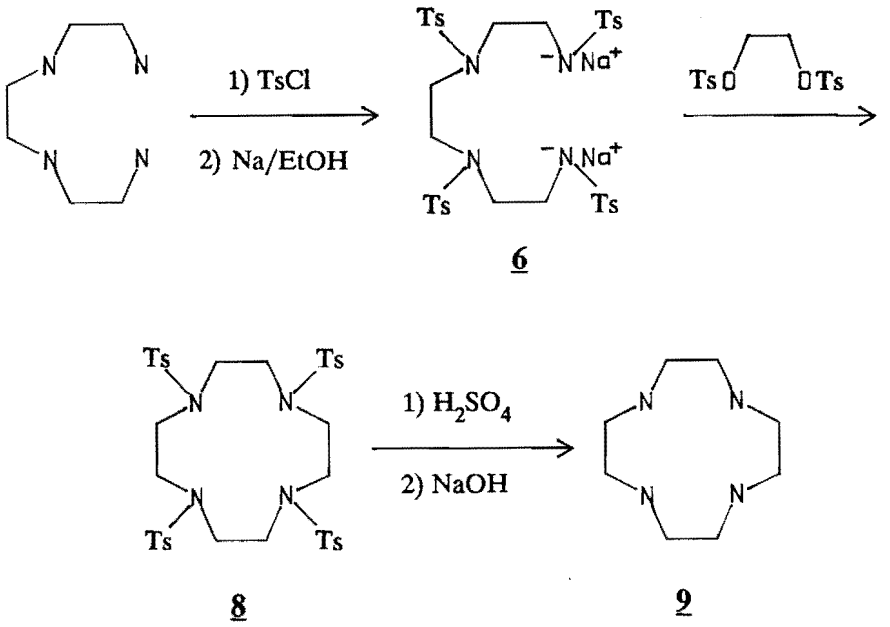
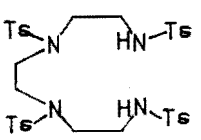


Figure 4.1 Synthesis of cyclic-1,4,7,10-tetraazadodecane.

Tetratosyl triethylenetetraamine (5)[79].

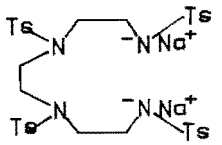
To a solution of tosyl chloride (7.63g, 40 mmol) in 10 mL of pyridine at room temperature with stirring was added triethylenetetraamine (1.46g, 10 mmol) in 3 mL of pyridine over a one hour period. The solution was stirred for 10 h at room temperature. At the end 4 mL of water was added dropwise to the reaction solution to effect a white precipitate. The suspension was stirred for another 10 h and kept in a refrigerator for 1 h. After filtration the solid was washed two times with water and was dried to give 3.85g of product, in 50% yield. mp: 216-218°C. ^{1}H NMR (DMSO): 7.69 (d, 4H, **CH**-tosyl), 7.63 (d, 4H, **CH**-tosyl), 7.38 (d, 4H, **CH**-tosyl), 7.36 (d, 4H, **CH**-tosyl), 3.10 (m, 8H, **CH_2**), 2.89 (m, 4H ,**CH_2**), 2.44 (s, 6H, **CH_3**-tosyl), 2.41 (s, 6H, **CH_3**-tosyl).



79. Atkins, T. J.; Richman, J. E.; Oettle, W. F. *Org. Syn.* **1978**, *58*, 86-98.

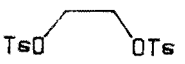
Disodium tetratosyl trien (6).

A sample of 3.85g of 5 was suspended in 10 mL of ethanol and heated to 70°C. A solution of 7 mL of sodium ethoxide in ethanol (1.5N) was added dropwise through a separatory funnel. The solution became clear at the beginning and turned into a slurry in a few minutes. It was cooled to room temperature slowly and was left in the refrigerator for 1 h. After filtration, the solid collected was washed with ethanol to give 3.65g of 6, in 90% yield. mp: 276-279°C. 6 was used without further purification.



Ditosyl ethylene glycol (7).

Prepared by tosylation[80] of ethylene glycol, in 95% yield. mp: 124-125°C. ^{1}H NMR ($CDCl_3$): 7.74 (d, 4H, **CH**-tosyl), 7.34 (d, 4H, **CH**-tosyl), 4.11 (s, 6H, **CH_3**-tosyl), 2.46 (s, 4H, **CH_2**).

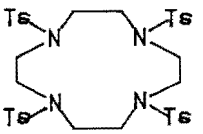


Tetratosyl cyclic-1,4,7,10-tetraazadodecane (8).

To a 2000 mL round bottom flask with 600 mL of pre-dried DMF maintained at 105°C, a sample of 16.14g **of** 6 (20 mmol) in 300 mL of DMF and a sample of 7.41g of 7 (20 mmol) in 300 mL of DMF were added dropwise at the same rate, over a 2 h period, from two separatory funnels. The solution was stirred for another 2 h

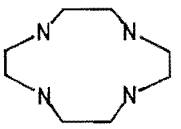
80. Busch, D. H.; Olszanski, D. J.; Stevens, J. C.; Schammel, W. P.; Kojima, M.; Herron, N.; Zimmer, L. L.; Holter, K. A. *J. Am. Chem. Soc.* **1981**, *103(6)*, 1472-1478.

at 105°C and cooled to room temperature. The solvent was reduced to about 300 mL. Water (300 mL) was added dropwise to the residue to effect precipitation. After stirring for two more hours at room temperature, the precipitate was filtered and washed with water to give 13g of **8**, in 78% yield. mp: 172-175°C. ^{1}H NMR ($CDCl_3$): 7.69 (d, 8H, **CH**-tosyl), 7.33 (d, 8H, **CH**-tosyl), 3.43 (s, 16H, **CH_2**), 2.45 (s, 12H, **CH_3**-tosyl). ^{13}C NMR: 143.96, 133.91, 129.89, 127.72, 52.33, 21.56.



Cyclic-1,4,7,10-tetraazadodecane (9)[81].

A sample of 4.5g of **8** was dissolved in 80 mL of concentrated H_2SO_4 with 2 mL of water. The solution was heated to 100°C for 40 h. After cooled to room temperature, it was washed two times with chloroform. Ether (100 mL) was added to the solution to induce a precipitate which was filtered and washed with ether (two times). The solid was air-dried to give 2.8g of sulfuric salt of **9**. The crude product was dissolved in 12 mL of water. Eight equivalents of NaOH were added. The precipitate formed was filtered and washed with water to give 0.81g of **9** after drying (81%). mp: 79.5-81.5°C. ^{1}H NMR (D_2O): 3.36 (s, **CH_2**),(lit.[78]: 3.34). ^{13}C NMR ($CDCl_3$): 46.01. (lit.[24]: 43.3)



Cis-dichloro cyclic-1,4,7,10-tetraazadodecane Cobalt(III) was prepared by the usual procedure for the preparation of cobalt(III) complex[82]. Some of the physical properties of this complex are given in Tables 2.1 and 2.2.

81. Koyama, H.; Yoshino, T. *J. Chem. Soc. Japan*, **1972**, *45(2)*, 481-484.
82. Hung, Y.; Busch; D. H. *J. Am. Chem. Soc.* **1977**, *99(15)*, 4977-4984.

C. Preparation of $[Co(N_4)(OH_2)(OH)]^{2+}$ Complexes

The aquohydroxotetraamine cobalt complexes used were prepared by hydrolyzing the corresponding dihalide complexes prior to use. The preparation of $[Co(en)_2(OH_2)(OH)]^{2+}$ is given below as an example.

Aquohydroxo(ethylenediamine)cobalt(III).

A sample of 28.5 mg (0.1 mmol) of *cis*-dichloro (ethylenediamine)cobalt(III) chloride was dissolved in 9 mL of deionized water, and the pH of the solution was brought to 9-10 by adding 1.5 equivalents of 1M NaOH solution. The solution was kept at 50°C for 5-10 min and the pH was adjusted to 7 with HCl solution. The volume of the solution was then brought to 10 mL. The rates of ligand substitution by hydroxide and water are very fast at pH 9 for all of the dichloro cobalt complexes used. The reactions will complete within minutes[56,55c].

The acid dissociation constants of the diaquo cobalt complexes were determined at 25°C, using a sample of 10 mmol of the cobalt complex. The ionic strength of the cobalt complex solution was maintained at 0.1 with NaCl.

4.3 Kinetics

A. Conditions

The hydrolysis of the phosphate esters in the presence of the cobalt complex

was carried out in water under pseudo first order conditions with excess cobalt complex at pH 7 unless other wise indicated.

The diaquo-cobalt complexes used in this study have pK_{a1} below and pK_{a2} above 7 [25c,56] (Table 2.1). Under the conditions used for kinetic studies, these complexes are self buffered at pH 7. The cobalt complex buffer system was sufficient to maintain the pH within a range of 7± 0.05 during the entire hydrolysis reaction. The buffer system of *cis*-aquohydroxobis(ethylenediamine) cobalt(III) is shown in Figure 4.2. Commonly used inorganic buffer systems were avoided to eliminate competitive binding to and inhibition of the cobalt catalyst.

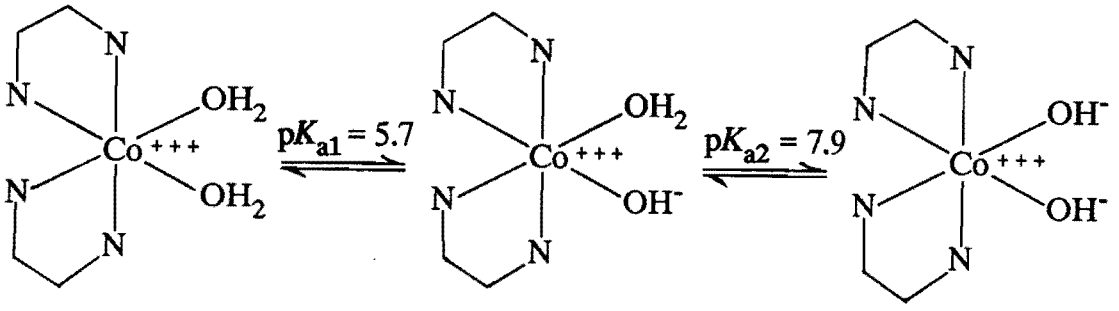


Figure 4.2 Buffer system of $[Co(en)_2(OH_2)(OH)]^{2+}$.

Concentrations of phosphate esters used were 10^{-5}M to 10^{-4}M in UV and 10^{-2}M in NMR experiments. The cobalt complex used in kinetic studies is at least in 10 times excess to the phosphate ester substrate.

B. UV Method

The hydrolyses of NPP, BNPP and BDNPP were monitored by following the increase of the UV absorbance at 400nm due to the formation of *p*-nitrophenolate or 2,4-dinitrophenolate. In a typical UV experiment, 3 mL of freshly prepared $[Co(trien)(OH_2)(OH)]^{2+}$ solution (0.01M) was placed in a three-milliliter quartz cuvette and allowed to stand in the sample holder of the spectrometer for 10 min at 50°C. The hydrolysis reaction was initiated by addition of 10-30 uL of a stock solution (0.01M) of phosphate ester in an appropriate solvent[83]. The change of absorbance with time was recorded at 400 nm. At 0.025 M concentration, the cobalt complexes absorb strongly at 400nm (>2). The hydrolysis reactions at that concentration of cobalt complex were followed at 410nm.

Reactions were followed at least five half lives (97% completion) for fast reactions. For slow reactions the initial rate method was used. Final absorbance was taken at least after ten half lives or after heating the reaction solution at a higher temperature until the change of absorbance had stopped. All reactions were carried out at 50°C unless otherwise stated. The pH of the solution was measured before and after the hydrolysis reaction. All the cobalt complex promoted hydrolysis reactions of the phosphate esters showed good pseudo first order behavior. The data reported for the kinetic studies is the average values of at least three runs with deviations within $\pm$5%.

83. BNPP and NPP in water. BDNPP in acetonitrile (final concentration of acetonitrile: 0.3-0.6%).

C. NMR Method

The hydrolysis of the *p*-nitrophenyl esters can also be readily followed by observing the ^1H NMR signals due to the phosphate esters and the products. In a typical NMR experiment, the hydrolysis of 0.01M BNPP in a freshly prepared $[Co(trien)(OH_2)(OH)]^{2+}$ D_2O solution (0.1M) was followed by taking spectra at specific time intervals. The kinetics were monitored by observing the appearance of the ^1H NMR signals due to the free *p*-nitrophenolate (6.97,d; 8.17,d) and the signals due to the disappearance of BNPP (7.41,d; 8.27,d). The hydrolysis of BDNPP was too fast to be monitored by the NMR method, but the NMR spectrum was taken to confirm the nature of the products.

Hydrolysis of c-AMP was monitored by NMR. The formation of free adenosine and the disappearance of c-AMP were followed by the 1'-H signals of adenosine (5.99, d) and the 1'-H signals of c-AMP (6.087, s) in the presence of $[Co(trien)(OH_2)(OH)]^{2+}$ (0.2 M) at 50°C. The product was confirmed by addition of a genuine sample of adenosine to the reaction mixture at the end of the hydrolysis reaction. As a control, hydrolysis of c-AMP was also followed in 0.2M phosphate buffer solution at pH 7, 100°C. Attempted hydrolysis of ApA was followed by ^1H NMR method under the same conditions used for the hydrolysis of c-AMP.

D. Anation Reactions

The measurements of the anation reactions of inorganic phosphate to different cobalt complexes followed the work of Lincoln et al.[31b] who reported the

anation of phosphate to $[Co(en)_2(OH_2)(OH)]^{2+}$. The reactions could be carried out with the phosphate in excess or the cobalt complex in excess. In both cases, the anation reactions showed good pseudo first order behavior. Figure 4.3 shows the UV spectra of the anation reaction of inorganic phosphate to $[Co(cyclen)(OH_2)(OH)]^{2+}$ with phosphate in excess. The UV absorbance changes with $[Co(cyclen)(OH_2)(OH)]^{2+}$ in excess is shown in Figure 4.4. Anation reactions for $[Co(trien)(OH_2)(OH)]^{2+}$ and $[Co(en)_2(OH_2)(OH)]^{2+}$ were monitored by following the increase of the optical absorbance at 530nm due to the formation of $[Co(N_4)PO_4]^o$ products. The reaction was carried out by adding a sample of inorganic phosphate (30 uL, 0.05 M, pH 7) to a thermostated solution of aquohydroxo cobalt complex (3 mL, 0.01 M, pH 7) at 50°C. The conditions used here are identical to those used in the kinetic study of phosphate diester hydrolysis in the UV experiment. For the anation to $[Co(cyclen)(OH_2)(OH)]^{2+}$, the reactions were followed at 370nm and 550nm. The rate of anation to $[Co(cyclen)(OH_2)(OH)]^{2+}$ by inorganic phosphate is very fast at 50°C ($t_{1/2} < 1s$). Therefore, anation reactions to this cobalt complex were followed at 10°C, 15°C and 20°C. The data was extrapolated to 50°C.

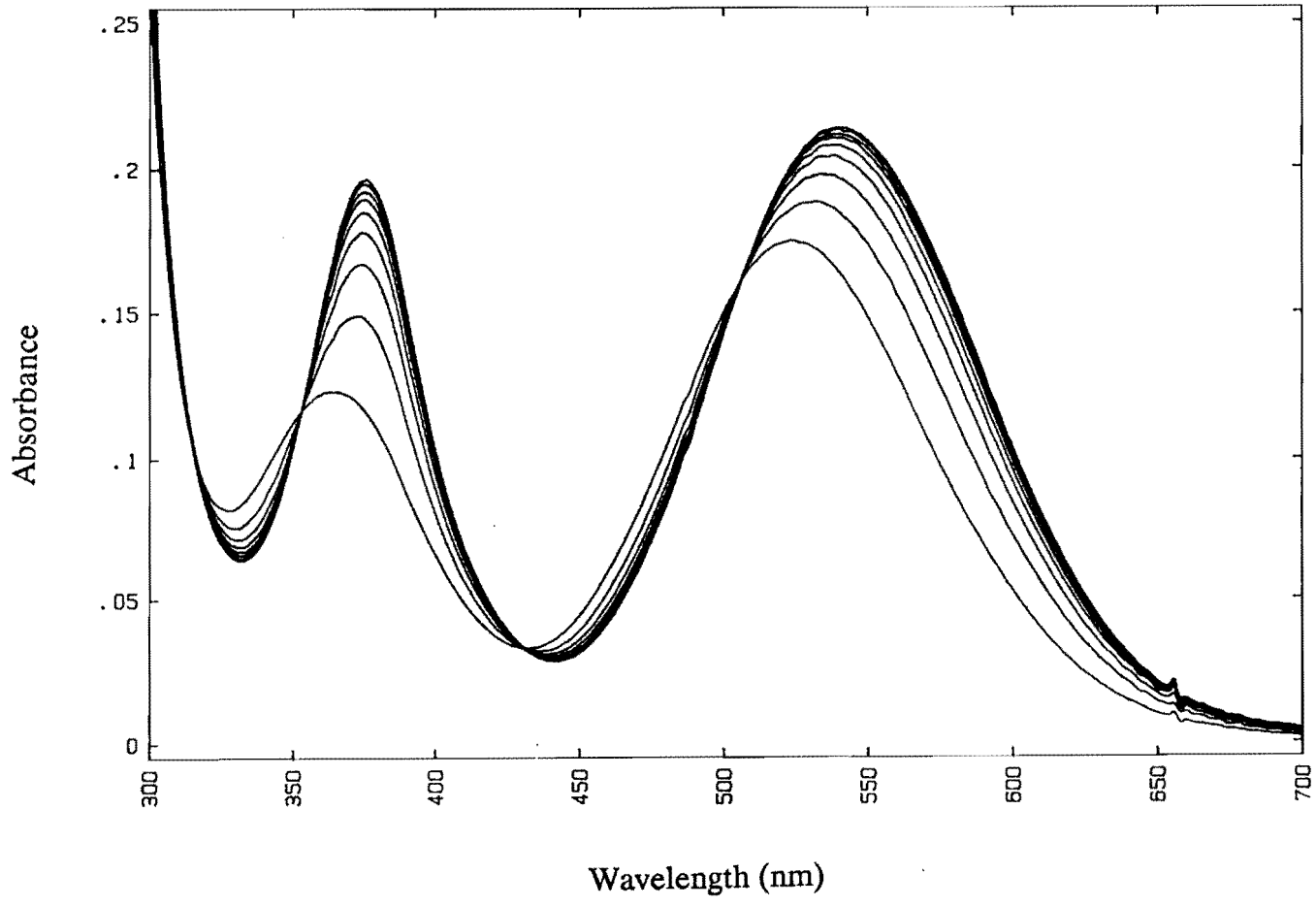


Figure 4.3 Anation reaction to $[Co(cyclen)(OH_2)(OH)]^{2+}$ (10^{-3}M) by inorganic phosphate ($5x10^{-3}$M) at 15°C, pH7.

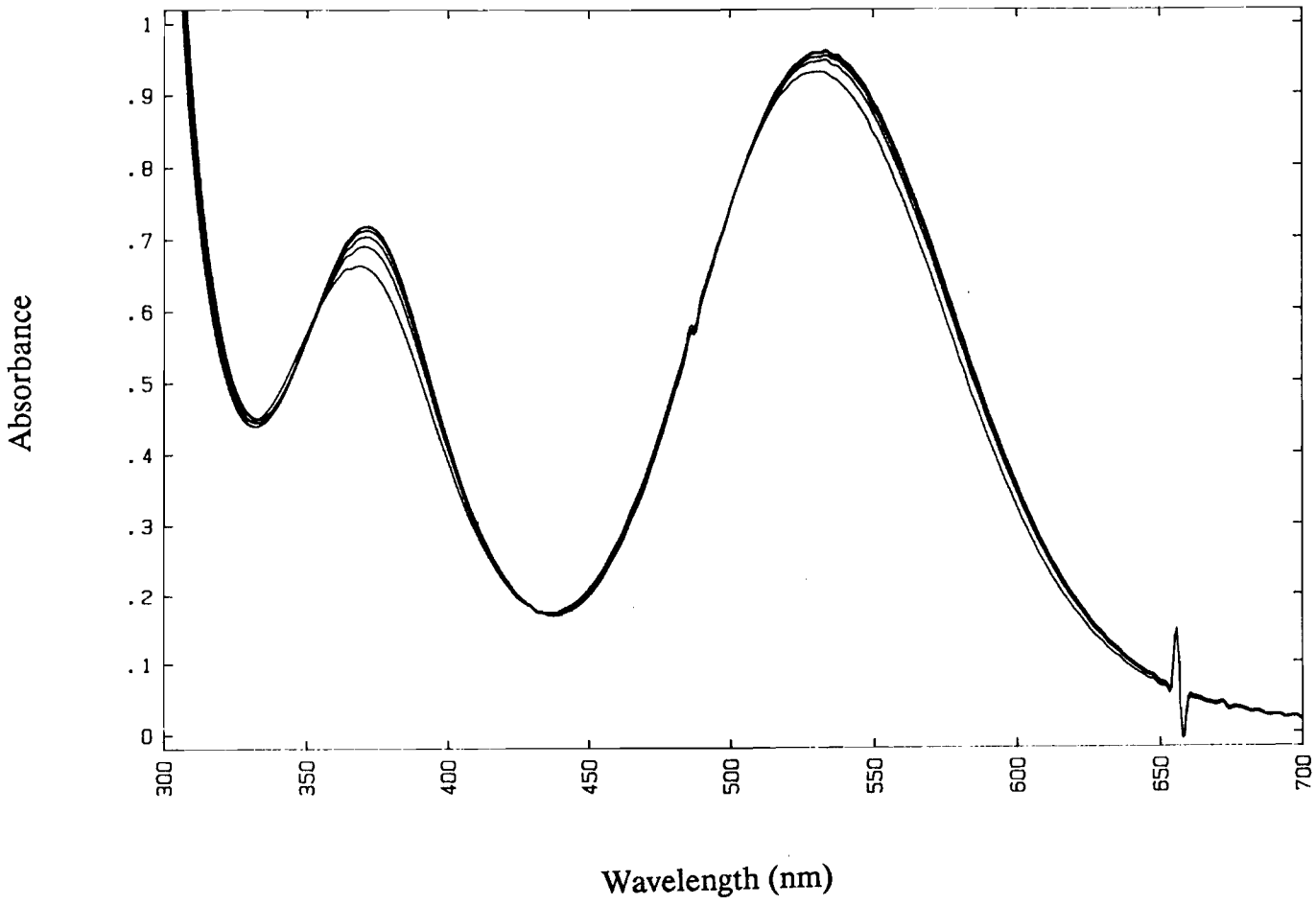


Figure 4.4 Anation reaction to $[Co(cyclen)(OH_2)(OH)]^{2+}$ ($5x10^{-3}M$) by inorganic phosphate ($5x10^{-4}M$) at 20°C, pH7.

5. CONCLUSION AND CONTRIBUTIONS TO KNOWLEDGE.

1) It is shown that the hydrolyses of stable phosphate diesters both with good and poor leaving groups are efficiently promoted by several simple Co(III) complexes. The hydrolysis of $[Co(cyclen)(OH_2)(OH)]^{2+}$ bound bis-(*p*-nitrophenyl)phosphate gives up to one billion fold rate enhancement over the hydrolysis of the unbound ester. The best cobalt complex, $[Co(cyclen)(OH_2)(OH)]^{2+}$, is about 3500 times more efficient than hydroxide and about 100 times less reactive than hydrolytic enzymes in promoting the hydrolysis of BNPP. For the hydrolysis of $[Co(trien)(OH_2)(OH)]^{2+}$ bound c-AMP, the rate enhancement is 10^8 fold. The cobalt complexes used in this study are by far the most efficient synthetic molecules in promoting the hydrolysis of simple phosphate diesters.

2) The mechanism of the cobalt complex promoted hydrolysis of phosphate diesters is explained in terms of a bifunctional mechanism. The rate limiting step of the hydrolysis is found to be the nucleophilic attack by the cobalt bound hydroxide on the bound diester.

3) The relationship between the structures of the Co(III) complexes and their efficiencies in promoting the hydrolysis reactions is evaluated. This will be very useful in directing further improvement of the catalysts.

4) The prospects of using these simple complexes are discussed. It is shown that once the simple complexes are connected with RNA or DNA binding molecules, they will be valuable tools in the determination and modification of DNA and RNA structures.

PART II. DESIGN AND SYNTHESIS OF COVALENTLY LINKED DNA BASES

6. INTRODUCTION

6.1 Base Pairing and Stacking in DNA and RNA Molecules

Both DNA and RNA may exist in single stranded or double stranded forms. The three dimensional structure of double stranded DNA was first revealed by Watson and Crick in 1953[84]. This and subsequent work[85] have firmly established that the two strands in a double stranded DNA have complementary base sequences and are anti-parallel to each other in a double helical structure. The forces holding the two single strands together are hydrogen bonding[86] through base pairing and hydrophobic interaction[87] through base stacking. Hydrogen bonding between purine and pyrimidine bases in nucleic acids occurs in a very specific fashion known as Watson-Crick base pairing. Cytosine pairs with guanine forming three hydrogen bonds and adenine pairs with thymine forming two hydrogen bonds. In RNA, thymine is replaced by uracil.

The preference to form Watson-Crick base pairs is due to the suitable distance between the base pairs to form stable hydrogen bonds. The distances of

84. Watson, J. D.; Crick. F. H. C. *Nature* **1953**, 737-738.
85. For a historical review on the subject, see "Molecular Associations in Biology" Pallman, B., Ed., Academic, N.Y. 1968, Hoogstern, K. pp21-38, Ts'o, P. O. P. pp39-74.
86. "Biophysical Chemistry" Part 1, p155, Cantor, C. R.; Schimmel, P. R., Freeman, San Francisco, 1980.
87. (a) Reference 3, p238. (b) "Biomolecular Stereodynamics", Vol. 1, p429, Turner, D. H.; Petersheim, M.; Albergo, D. D.; Dewey, T. G.; Freier, S. M., Adenine Press, Guilderland, N.Y., 1981. (c) "Physical Chemistry of Nucleic Acids", Bloomfield, V. A.; Crothers, D. M.; Tinoco Jr., I., Harper and Row, NY, 1974.

A-T and G-C pairs measured from the points of attachments of the bases to the sugars are almost identical. The double-helix geometry can accommodate both of the base pairs without any distortion or loss in symmetry. Hydrogen bonds between other combinations of base pairs are possible but they are usually not as stable as those found in Watson-Crick base pairs. Oligonucleotides and even dinucleotides[88] have shown the same preference. A G-C base pair binds much more tightly than either an A-T or A-U base pair[88] because it forms one more hydrogen bond than the latter two pairs.

Hydrophobic interaction between adjacent bases plays an important role in the stability of the polynucleotide double helix. The interaction arises from the planar structure of nucleic acid bases which are approximately perpendicular to the plane of the sugar rings and are parallel to each other in the double helix. These arrangements make it possible for the base pairs to stack one upon the other, further stabilizing the helical structure of DNA or RNA. Purine bases have stronger interactions with each other than pyrimidine bases since the sizes of the aromatic rings in purines are bigger.

The stability of DNA has been the subject of many studies over the years because of its fundamental importance. It has been widely accepted that hydrogen bonding and hydrophobic interaction are the primary sources contributing to the stability of the nucleotide double helix. However, it is still not clear how important the structure of the backbone is in the formation of the nucleotide double helix.

88. Young, M. A.; Krugh, T. R. *Biochem.* **1975**, *14(22)*, 4841-4847.

6.2 Stability of Base Pairing and Stacking in DNA Double Helix

The stability of a DNA double helix reflects the strength of the bonding forces generated from base pairing and base stacking between the two strands. This stability is determined by the compositions and the length of the two sequences in a double helix. In solution, the double helix can be separated into two single strands if the temperature is elevated. The dissociation constant for the separation is customarily expressed in the form of melting temperature (T_m). T_m is characteristic of the stability of the base pairing and/or base stacking in a double or single stranded nucleotide sequence. The measurement of T_m is based on the fact that a solution of stacked polynucleotide has a lower optical density than will an equimolar solution of the monomeric units, a phenomenon generally referred to as hypochromism. T_m is usually determined by monitoring the change of optical absorbance with temperature (by UV, CD or ORD) of a single or double stranded nucleotide solution. In this way, a 'melting curve' can be obtained and T_m is the midpoint at which the transition occurs. For single stranded DNA or RNA, T_m only reflects the stability of base stacking while for double stranded DNA or RNA, it refers to both the base stacking and base pairing.

Longer sequences of nucleotides will bind more tightly than shorter ones and hence will have a higher T_m. The order of the base sequence in a given oligonucleotide also influences the T_m of an oligonucleotide. For example, the self complementary sequences, 5'-CCGG-3' and 5'-GGCC-3', have exactly the same composition of base units, but the T_m values of the two are 27.4°C and 35°C

respectively at 10^{-4} M concentration[89]. This 'nearest neighbor effect' is obviously a complex phenomenon, and no conclusive explanation has been reached[90,88].

Semi-empirical methods have been used for calculating T_m of oligo- and poly- nucleotide double helixes of known sequences[91]. These methods are based on the melting behavior of a series of short oligonucleotides to obtain the sequence dependent contribution of base pairs to the thermodynamic parameters of the helix formation. These parameters are then used to estimate the T_m of other oligonucleotide sequences. The estimations are usually in good agreement with experimental results. For example, the T_m of AACGUU (10^{-4} M nucleotide concentration) is 22°C by experimental measurement[89]. The calculated value is 19°C. Clearly, these methods are very useful in predicting the stability of a given oligo- and polynucleotide.

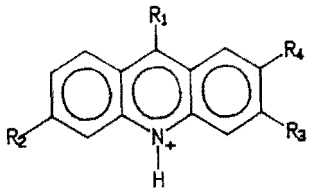
6.3 DNA Binding Molecules

In order to study the structure of DNA and RNA, there has been a lot of effort directed toward the development of small molecules which can bind to DNA at specific sites[92]. Many of these molecules are drugs showing antibactierial and

89. (a) Freier, S. M.; Burger, B. J.; Alkema, D. Neilson, T.; Turner, D. H. *Biochem.* **1983**, *22(26)*, 6198-6206. (b) Freier, S. M.; Alkema, D.; Sinclair, A.; Neilson, T.; Turner, D. H. *Biochem.* **1985**, *24(17)*, 4533-4539.
90. (a) Petersheim, M.; Turner, D. H. *Biochem.* **1983**, *22(2)*, 256-263. (b) Bubienko, E.; Cruz, P.; Thomason, J. F.; Borer, P. N. *Prog. Nucl. Acid Res. Mol. Biol.* **1983**, *30*, 41-90.
91. Borer, P. N.; Dengler, B.; Tinoco, I., Jr.; Uhlenbeck, O. C. *J. Mol. Biol.* **1974**, *86(4)*, 843-853. A detailed discussion and the evaluation of the methods are given in **Appendix 1**.
92. Lochmann, E. R.; Micheler, A. "Physico-chemical Properties of Nucleic Acids" Vol. *1*, 223-267, Duchesne, J. Ed. Academic Press, N.Y. **1973**.

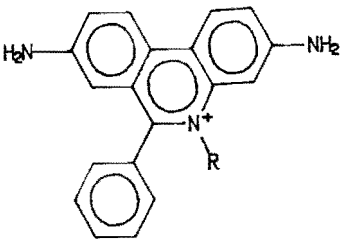
antiviral activities[93,94]. They bind to DNA by intercalation, hydrogen bonding or direct chemical reactions.

A large majority of the molecules developed to date bind with DNA by intercalation. These molecules have planar chromophores which can insert between the base pairs in the DNA double helix. They are referred to as intercalators[93] and bind tightly to the DNA by electrostatic[95] and hydrophobic[96]



1) $R_1=R_4=H$, $R_2=R_3=NH_2$
Proflavine

2) $R_2=R_3=R_4=H$, $R_1=NH_2$
9-aminoacridine



1) $R=C_2H_5$, Ethidium

2) $R=(CH_2)N^+(CH_3)(C_2H_5)_2$
Propidium

Figure 6.1 DNA binding molecules.

93. For a review see Berman, H. M.; Young, P. R. *Ann. Rev. Biophys. Bioeng.* **1981**, *10*, 87-114.
94. (a) Dickinson, L.; Chantrill, D. H.; Inkley, G. W.; Thompson, M. J. *Brit. J. Pharmacol.* **1953**, *8(2)*, 139-142. (b) Newton, B. A. *Adv. Chemother.* **1964**, *1, 35-83.* (c) Wilson, W. D.; Jones, R. L. *J. Am. Chem. Soc.* **1986**, *108(22)*, 7113-7114.
95. (a) Manning, G. S. *Q. Rev. Biophys.* **1978**, *11*, 179-246. (b) Record, M. T., Jr.,; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* **1978**, 11, 103-178.
96. (a) Douthart, R. J.; Burnett, J. P.; Beasley, F. W.; Frank, B. H. *Biochem.* **1973**, *12(2)*. 214-220. (b) Waring, M. J. *J. Mol. Biol.* **1965**, *13(1)*, 269-282. (c) Le Pecq, J. B. "Methods of Biochem. Anal." **1971**, *20*, 41-86.

interactions. Intercalators have also been used extensively as chemical probes in the study of DNA and RNA structures and conformations[97]. Some of the best known examples of intercalators[98] are shown in Figure 6.1. The binding of ethidium bromide with DNA double helix is depicted in Figure 6.2. Inorganic analogs of intercalators have been developed recently. Enantiomers of chiral metal complexes such as tris-phenanthroline Co(III)[99] (Figure 1.14), diphenanthroline Cu(II)[100] and

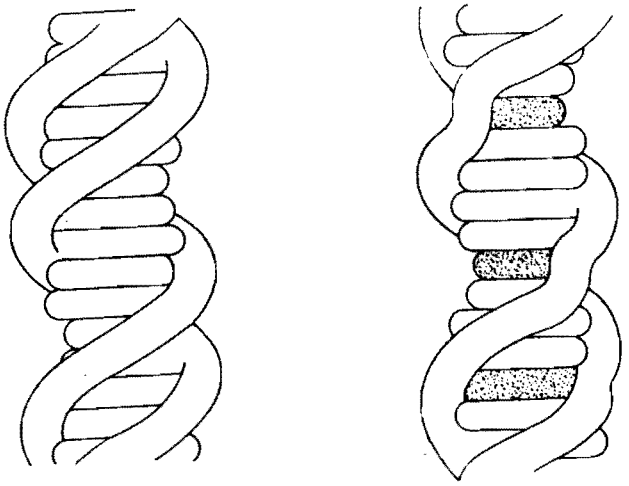


Figure 6.2 Binding of ethidium bromide with double stranded DNA. Normal DNA (left) and DNA with ethidium bromide (right).

97. Pope, L. E.; Sigman, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81(1)*, 3-7. (b) Cartwright, I. L.; Hertzberg, R. P.; Dervan, P. B.; Elgin. S. C. R. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80(11)*, 3213-3217. (c) Ephrussi, A.; Church, G. M.; Tonegawa, S.; Gilbert, W. *Science* **1985**, *227*, 134-140.
98. (a) Patel, D. J.; Cannel, L. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74(7)*, 2624-2628. (b) Coddington, J. M.; Alkema, D.; Bell, R. A.; Hughes, D. W.; Neilson, T. *Chem. Biol. Interactions* **1984**, *50*, 97-100. (c) Lerman, L. S. *J. Mol. Biol.* **1961**, *3(1)*, 18-30.
99. Barton, J. K.; Raphael, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82(19)*, 6460-6464.
100. Spassky, A.;Sigman, D. S. *Biochem.* 1985, 24(27), 8050-8056

tris-(tetramethylphenanthroline)Ru(II)[101] complexes have been shown to bind stereoselectively to different forms of DNA molecule.

Hydrogen bonding is another strategy used in the design of DNA binding molecules. This is demonstrated by the interactions between DNA and distamycin which binds to the minor groove of AT rich sequences[41]. The use of distamycin as a 'carrier' in the oxidative cleavage of DNA has been discussed in part I of this thesis (figure 1.12, page 20).

cis-Diamminedichloroplatinum(II) (figure 6.3) shows yet another mode of binding. It binds to DNA by an intrastrand cross-link where the N(7) atoms of

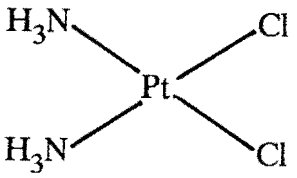


Figure 6.3 *cis*-Diamminedichloro platinum (II).

adjacent guanine bases in DNA replace the chloride ions in the metal complex[102]. Its activity as an anticancer drug is believed to be due to its inhibition of DNA replication[103].

101. Mei, H. Y.; Barton, J. K. *J. Am. Chem. Soc.* **1986**, *108(23)*, 7414-7416.
102. Pinto, A. L.; Lippard, S. J. *Biochem. Biophys. Acta* **1985**, *780(3)*, 167-180.
103. "Platinum Coordination Compounds in Cancer Chemotherapy" Hacker, M. P.; Douple, E. B.; Krakoff, I. H., Eds., Nijhoff, Boston, **1984**.

The DNA binding molecules discussed so far are targeted at double stranded DNA molecules. The specificities of some of these molecules are quite impressive. For example, the right handed tris-phenanthroline cobalt(III) complex only binds to B-DNA, while its left handed enantiomer favors left handed Z-DNA[104]. On the other hand, molecules that bind to double stranded DNA can hardly achieve site specific binding with DNA. The best these molecules can do is to bind to certain ranges of DNA sequences.

6.4 Design of DNA Binding Molecules

A. General Considerations

Our involvement in the synthesis of DNA binding molecules stemmed from our interest to study the importance of DNA backbone in the formation of the DNA double helix. We were also interested in the possibility of using small binding molecules to carry the DNA cleaving molecules developed in part I of this thesis to cleave single stranded DNA at specific sites[105]. Since the existing DNA and RNA binding molecules in the literature could not meet these criteria, we decided to develop a new class of compounds to study their binding properties with normal nucleotides and to accomplish the high and flexible binding specificity we needed.

The target molecule would have an artificial backbone with the same or different purine and pyrimidine bases attached to it. The specificity would be accomplished by binding of the covalently linked nucleic acid bases to a specific

104. Barton, J. K. *Science* **1986**, *233*, 727-734.
105. (a) Knorre, D. G.; Vlassov, V. V. *Prog. Nucl. Acid Res. Mol. Biol.* **1985**, *32*, 291-321. (b) Reference 86, p163.

sequence in a single stranded DNA by Watson-Crick base pairing, a concept nature has selected. By changing the base composition and the base sequence, and varying the length of the sequence in the synthetic molecules, it should be possible to select the binding sites on a given single stranded DNA. The effects of the backbone on binding can also be evaluated by studying the stability of the binding between the synthetic molecule and its complementary single stranded DNA sequence.

B. Choice of the Backbones

There has been a lot of interest in modifying the nucleotide backbones of DNA or RNA. The interest is generated from the possibility that studies of the molecules with modified backbones may shed light on the understanding of nucleic acid conformations and the interaction between DNA and proteins, which is one of the central processes in living cells[107]. It is also because nonionic oligonucleotide analogs can penetrate cells more easily than natural polynucleotides[112b]. Phosphorothioate[106], carbonate[107], carbamate[108], oxyacetamide[109] phosphoramidate[110], silyl[111] and methylphosphonate[112] internucleoside links have

106. Koziolkiewicz, M.; Uznanski, B.; Stec, W. J. *Chemica Scripta* **1985, 26**, 251-260.
107. (a) Mertes, M. P.; Coats, E. A. *J. Med. Chem.* **1969**, *12(1)*, 154-157. (b) Tittensor, J. R. *J. Chem. Soc. (C)*, **1971**, 2656-2662.
108. (a) Coull, M. J.; Carlson, D. V.; Weith, H. L. *Tet. Lett.* **1987**, *28(7)*, 745-748. (b) Mungall, W. S.; Kaiser, J. K. *J. Org. Chem.* **1977**, *42(4)*, 703-706.
109. (a) Gait, M. J.; Jones, A. S.; Walker, R. T. *J. Chem. Soc. Perkin I*, **1974**, 1684-1686. (b) Gait, M. J.; Jones, A. S.; Jones, M. D.; Shepherd, M. J.; Walker, R. T. *J. Chem. Soc. Perkin I*, **1979**, 1289-1294.
110. (a) Letsinger, R. L.; Mungall, W. S. *J. Org. Chem.* **1970**, *35(11)*, 3800-3803. (b) Nemer, M. J.; Ogilvie, K. K. *Tet. Lett.* **1980**, *21*, 4149-4152.
111. (a) Ogilvie, K. K.; Cormier, J. F. *Tet. Lett.* **1985**, *26(35)*, 4159-4162. (b) James F. Cormier's PhD thesis, McGill University.
112. (a) Miller, P. S.; Yano, J. Yano, E.; Carroll, C.; Jayaraman, K.; Ts'o, P. O. P. *Biochem.* **1979**, *18(23)*, 5134-5143. (b) Jayaraman, K.; McParland, K.; Miller, P.; Ts'o, P. O. P. *Proc. Nalt. Acad. Sci. U. S. A.* **1981**, *78(3)*, 1537-1541.

been reported. Some of these molecules are shown in Figure 6.4. Generally speaking, these synthetic molecules have only small deviations from the natural DNA backbone. The changes are mostly focused on the phosphate ester linkage. They were not synthesized specifically for binding studies.

The most dramatic change of backbone was reported by Inaki and his co-workers[113] who synthesized series of polyethyleneimine with grafted adenines or

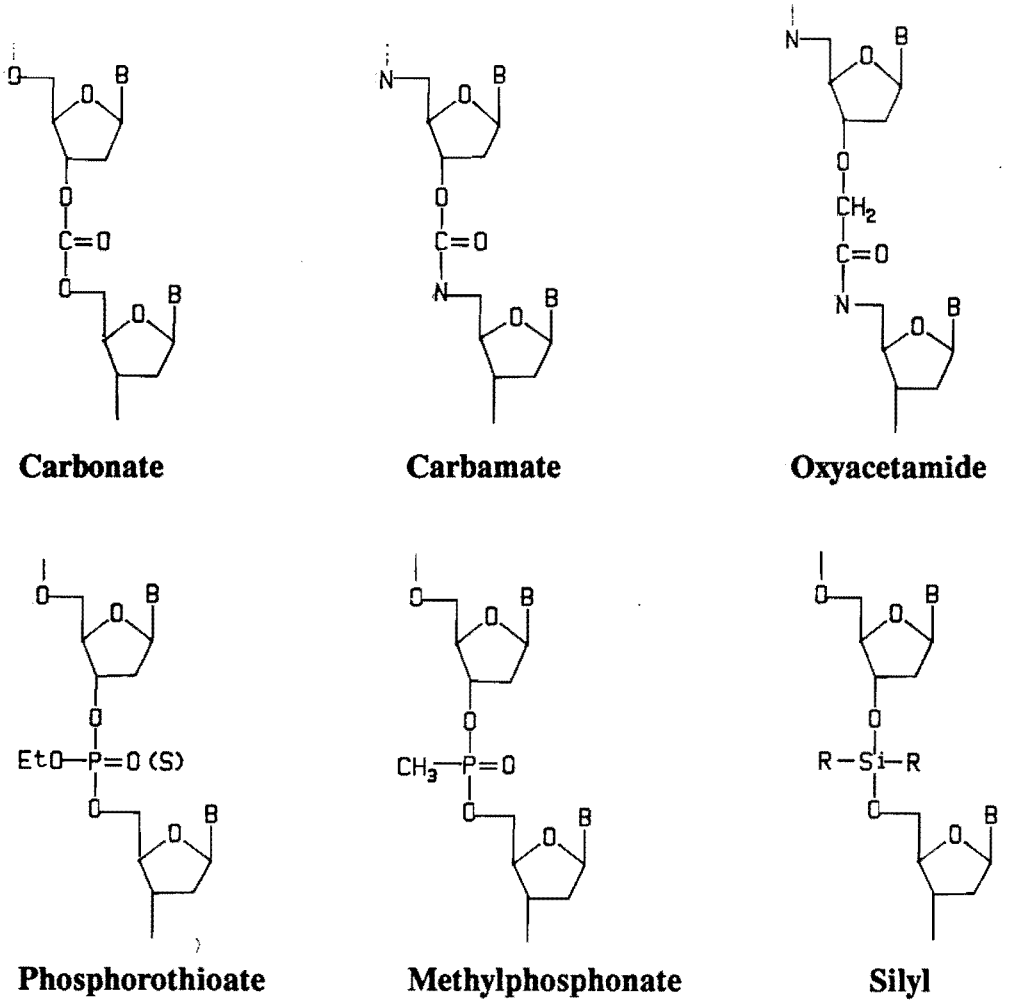


Figure 6.4 Different internucleoside linkages.

113. (a) Overberger, C. G.; Inaki, Y. *J. Polym. Sci. Polym. Chem. Ed.* **1979**, *17*, 1739-1758. (b) Inaki, Y.; Sakuma, Y.; Suda, Y; Takemoto, K. *J. Polym. Sci. Polym. Chem. Ed.* **1982**, *20*, 1917-1933.

thymines. These polynucleotides with the artificial backbone showed the same hypochromicity as those with the natural phosphate-sugar backbone, indicating that base stacking is indeed occurring between the attached bases on the backbone. However, binding of the synthetic polynucleotides with complementary natural polynucleotides was not reported.

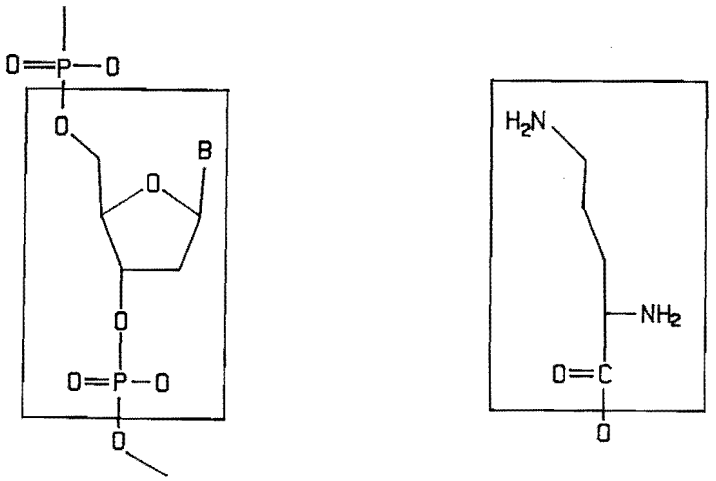
Other acyclic nucleotide analogs have been prepared by free radical polymerization such as 1-vinyluracil[114], 4-vinylpyridine[115] and vinylalcohol[116]. This methodology however can only be used for the preparation of homopolymers and can not be easily adopted to the synthesis of oligonucleotide analogs with specific sequence.

In designing our DNA binding molecules, one approach was to use the readily available oligo-propyleneamine as the backbone. The advantage of using this type of backbone is the simplicity in the synthesis for short nucleotide analogs. The base can be directly grafted to the backbone. Synthesis of simple molecules with the amine containing backbone could also serve as models to develop the methodologies for attaching nucleic acid bases to other backbones. The drawback is that the length of the nucleotide is pre-set by the length of the backbone used and only one type of nucleic base can be attached to the backbone.

---

114. Maggiora, L.; Boguslawski, S.; Mertes, M. P. *J. Med. Chem.* **1977**, *20(10)*, 1283-1287
115. Shimidzu, T.; Murakami, A.; Konoshita, Y.; Minami, M. *Bull. Chem. Soc. Japan* **1987**, *51(3)*, 821-825.
116. Seita, T.; Yamauchi, K.; Kinoshita, M.; Imoto, M. *Die Makromolekulare Chemie* **1973**, *164(1)*, 15-23.

Another more systematic and completely different approach is to use a peptide backbone. This involves attaching the nucleic acid base to an amino acid to produce monomers. The monomers then are used as building blocks to construct oligomers by the usual methods of peptide synthesis. It would be possible to synthesize oligomers of desired length and base sequence with a peptide backbone once the synthetic methods have been developed for attaching the four different nucleic acid bases to an amino acid and for coupling these monomers together. Of all the natural amino acids available, ornithine is unique in that it is the only amino acid having a six-atom skeleton with two amino groups. Two amino groups are necessary in this approach because one would be used to form the peptide backbone and the other for the connection with the nucleic acid base. In natural DNA and RNA, the mononucleotide has six atoms in its backbone skeleton. This relationship is shown in Figure 6.5. One advantage of this approach is that many amino acids are



DNA **Ornithine**

Figure 6.5 Comparison of ornithine and DNA skeletons.

commercially available. Modification of the backbone is just a matter of changing from one amino acid to another.

C. Choice of the Base and the Length of the Sequence

All four DNA bases would be used eventually in order to achieve specific binding with a complementary DNA sequence. However, our general approach to the choice of the base unit at this point was to make the base sequence as short and as simple as possible for the reason of simplicity in synthetic work. Since the G-C pair is known to bind much more tightly than the A-T pair, naturally the G-C pair was selected. Within the two bases, cytosine was the first choice since it is usually simpler to carry out synthesis using cytosine than using guanine because of the solubility problem with the latter.

From the above considerations, we planned to make an all-cytosine base sequence with cytosine residues attached to an oligopropyleneimine backbone in the first approach. In the peptide backbone approach, a self complementary oligomer containing covalently linked guanine residues and cytosine residues was projected.

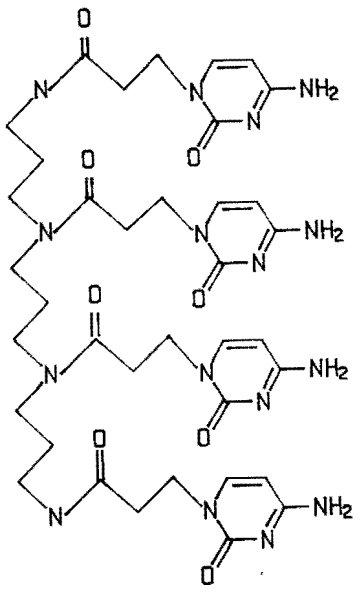
The last thing that had to be decided was the length of the base sequence. As discussed earlier, the stability (T_m) of an oligonucleotide double helix is chain length dependent. The T_m for the binding of the synthetic molecule with its complementary oligonucleotide should be within the convenient limit of 0°-100°C, at about 10^{-4} M concentration. Thus, the T_m of the binding for G-C oligomers was estimated by the semi-empirical calculation (appendix 1). The calculation showed that the T_m of binding for a tetraguanine nucleotide with a tetracytosine nucleotide

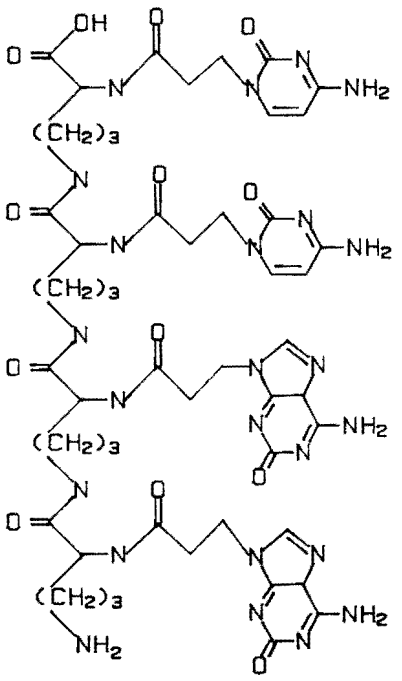
is 50°C at 10^{-4} M concentration. The T_m is 8°C for a guanine trimer with a cytosine trimer. This indicates that at least a tetramer is required in order to have reasonably stable binding for the synthetic molecule with its complementary single stranded DNA sequence.

Different approaches could be used to link a cytosine with an amine containing backbone. One possibility is to use an alkyl group to connect the two parts. Formation of two amide bonds or one amide and one alkyl is also possible. Amide bone formation at the N(1) position of cytosine is undesirable since it will alter the aromatic system of the base too much and thus reduce the binding ability of the cytosine with a guanine. An alkyl substitution at the N(1) position of cytosine is suitable for our purpose. At the other end of the linkage to the amine backbone, formation of an amide bond should be the easiest.

The first target molecule thus was a tetra-cytosine sequence with a 1,4,8,12-tetraaza dodecane backbone. The second target molecule was a self complementary GGCC tetramer with ornithine as the backbone units. These two molecules are shown in figure 6.6.

It was hoped that once these molecules were prepared, they would enable us to devise a proper test for the role of the DNA backbone in the stability of the double helix structures. The synthetic strategies used for the preparation of these molecules will be discussed in the next few sections.





20 **34**

Figure 6.6 Tetranucleotides with amine backbone and peptide Backbone.

7. SYNTHESIS OF A CYTOSINE TETRAMER WITH AMINE BACKBONE

7.1 Through Activated Ester

The activated ester method has been used extensively in peptide synthesis. It has also been used successfully to attach adenine and thymine to polyimine backbone[114]. Figure 7.1 shows the initial plan for the synthesis of the target molecule **20** (figure 6.6) using the activated ester method.

A. Synthesis of 3-(1-(N-4-acetylcytosine))-Methyl Propionate (16)

Introduction of alkyl groups to nucleic bases by Michael addition has been common in the preparation of adenine[117], thymine[114a] and cytosine[118] derivatives. Both ethanol/sodium and pyridine-water can be used as solvent. In the preparation of **16**, the addition reaction was started with N(4) protected cytosine. A large excess of methyl acrylate was used as the adduct to ensure complete reaction in

117. Lira, E. P.; Huffman, C. W. *J. Org. Chem.* **1966**, *31(7)*, 2188-2191.
118. Ueda, T.; Fox, J. J. *J. Org. Chem.* **1964**, *29(7)*, 1762-1769.

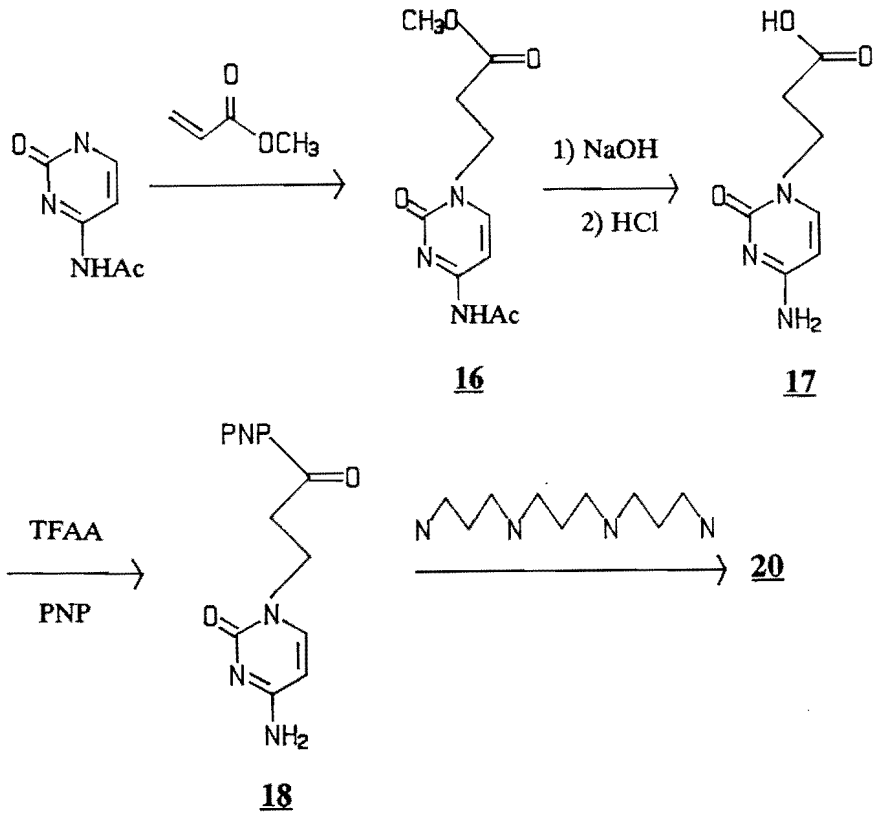
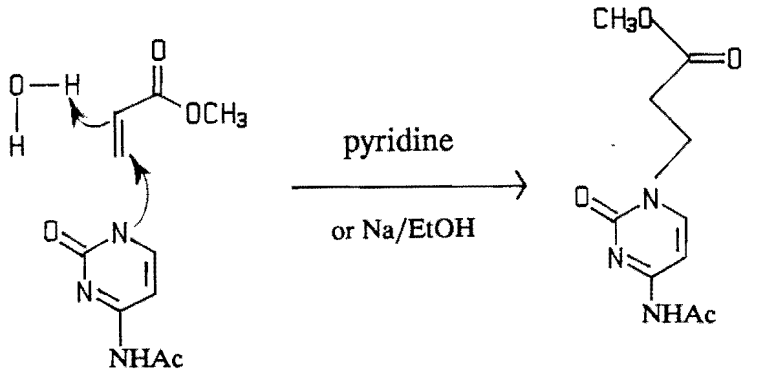


Figure 7.1 Route through activated ester.

pyridine-water. **16** was obtained in 87% yield after the reaction solution had been refluxed for an hour. Ethanol/sodium conditions had also been used to carry out the addition reaction. The yield under these conditions was 80%.

With **16** in hand, directly replacement of the ester group in **16** by an alkylamine was attempted. It was hoped that the formation of tetramer **20** could be achieved by ester-amide exchange. However, a model study using diethylamine to replace the methyl ester was unsuccessful. The exchange reaction was too slow both in water and in chloroform.



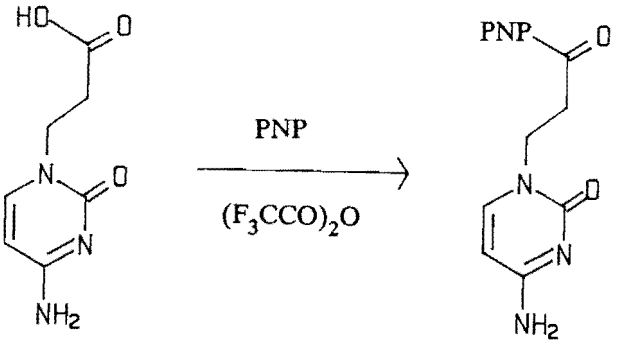
16

Figure 7.2 Synthesis of **16** by Michael addition.

B. Synthesis of 3-(1-cytosyl)-p-nitrophenylpropionate (18)

Hydrolysis of the methyl group was accomplished by treating ester **16** with two equivalents of sodium hydroxide in water to afford the free acid **17**. It is worth noting that the amino protecting group of the cytosine residue was also hydrolyzed during the process. Addition of one equivalent of sodium hydroxide gave a mixture of hydrolysis products. Therefore, selective cleavage of the methyl ester of the ornithine was not pursued since protection of the N(4)-amino of cytosine is not crucial in subsequent reactions. It had been shown by Overberger and co-workers[114a] that grafting of unprotected adenine derivatives to polyethyleneimine did not present any problem.

Originally, we planned to make *p*-nitrophenyl trifluoroacetate (**35**), and to use this reagent to prepare **18**. Compound **35** was prepared according to known



17 18

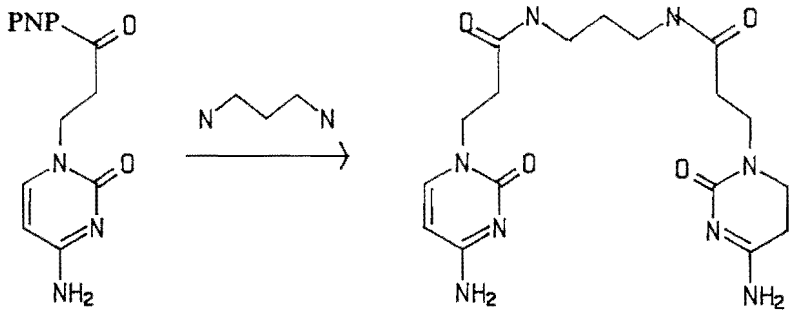
Figure 7.3 Preparation of the activated ester, 18

procedures[119]. However, 35 proved to be very unstable. It decomposed very quickly during the process of purification or upon storage at room temperature for one day. Because of this problem, a modified method was used to make 18 by preparing 35 in *situ* and adding 17 immediately after 35 was formed in pyridine solution. This approach proved to be very efficient. The one-pot reaction gave close to quantitative yield of the desired ester (18) (figure 7.3).

C. Attempted Synthesis of Cytosine Tetramer 20 from 18

In the literature, the grafting reaction of polyethyleneamine by adenosine or thymine derivatives[114a] did not go to completion. The base content in the polymer after the reaction was reported to be about 63%. In a model study, 18 did react with 1,3-diaminopropane to give 22, as shown in figure 7.4. However, the yield of

119. Sakakibara, S.; Inukai, N. *Bull. Chem. Soc. Japan* **1964**, *37(8)*, 1231-1232.



18 22

Figure 7.4 Reaction of 1,3-diaminopropane with 18.

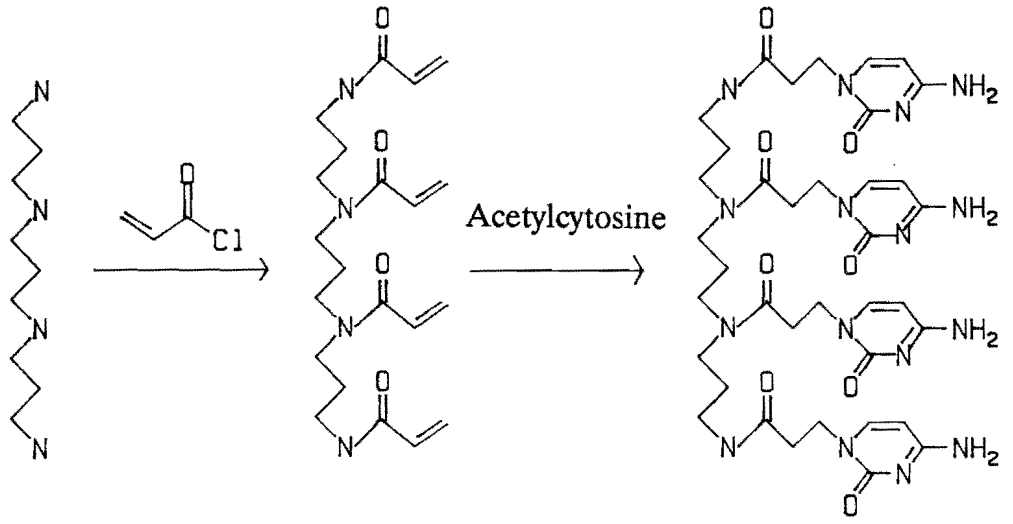
this reaction was only 40% even though imidazole had been used as a catalyst. When the same reaction was applied to attach four cytosine derivatives to the tetraamine in either DMSO or pyridine as solvent, less than 2% of the product was separated. Addition of excess of 18 gave no improvement in the yield. The reason for the low yield is probably due to the low reactivity of the two secondary amines. After the reaction, most of the compounds separated were the mono- and di-cytosine-substituted side products.

Due to the low yield in the grafting step, an alternative route by direct Michael addition was used to prepare the target molecule 20.

7.2 Direct Michael Addition by Acetylcytosine

A. Strategy

A reversed reaction sequence was used for the preparation of **20** as outlined in Figure 7.5. In this approach the acrylolyl group was added to the tetraamine backbone first. Cytosine was directly added to the acrylolyl group through Michael addition in a subsequent step. One advantage of this route is its simplicity in that only two steps are involved in the entire synthesis. Model studies to prepare a dimer



19 **20**

Figure 7.5 Synthesis of **20** by direct Michael addition.

with two cytosine residues attached to 1,3-diaminopropane were undertaken to determine the best conditions suited for the synthesis of tetramer **20**. It was found that the reaction of 1,3-diaminopropane and acrylolyl chloride gave a reasonably good yield (60%) in a benzene and water mixed solvent with NaOH as the base. The yield was poor when the reaction was carried out under benzene and triethylamine conditions. For the Michael addition step, the reaction of N,N'-diacrylolyl 1,3-diaminopropane (**21**) with acetylcytosine under Na/EtOH conditions gave the dicytosine adduct **22**, in 57% yield.

B. Synthesis of Tetraacrylolyl Tetraamine (19)

The reaction of acrylolyl chloride and tripropylenetetraamine went smoothly in benzene and water mixed solvent with NaOH. The yield of addition was 37%. One thing worth mentioning is that the proton NMR signals of the acrylolyl groups in **19** were broad at room temperature. The signals started to sharpen out when the temperature was elevated to 55°C. This is clearly an indication that the acrylolyl groups are not freely rotating at room temperature.

C. Direct Michael Addition by Acetylcytosine

The Michael addition step for **19** by acetylcytosine (figure 7.5) required longer reaction time than that for its dimer analog **21**. The substrates did not totally dissolve in ethanol solution even upon refluxing. The solvent therefore was

changed to methanol with addition of a small amount of sodium to maintain the basic conditions. Under these conditions, the amino protecting group of cytosine was hydrolyzed during the reaction. It seems that the hydrolysis occurred after the addition had taken place since attempts to carry out the addition by unprotected cytosine to **19** failed to give any product **20**. The yield for the Michael addition step was 13%. Mono, di and tri adducts were separated. Steric hindrance is probably responsible for the low yield of **20**. Restricted rotation was also observed in **20**. This was indicated by the broadening and complication of ^{1}H NMR signals of **20** at room temperature. Clear and sharp NMR signals could not be attained before the temperature was raised to 90°C. At high temperature, **20** clearly shows two sets of cytosine signals in the ^{1}H NMR spectrum (figure 7.6) since the cytosines at the end and the ones in the middle of the backbone have slightly different chemical shifts. The identity of compound **20** was also confirmed by FAB mass spectroscopy.

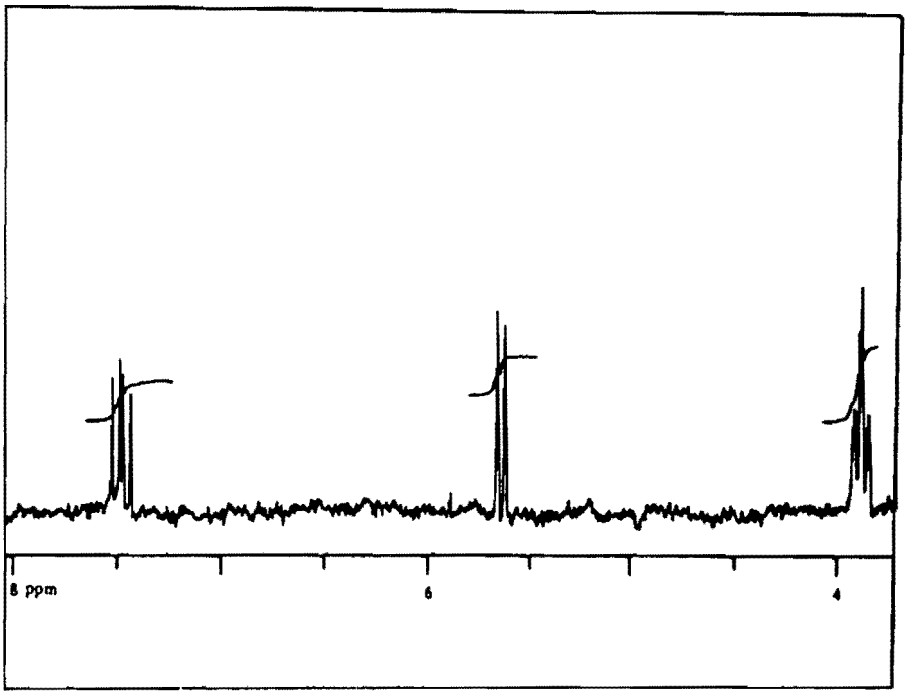


Figure 7.6 ^{1}H NMR spectrum of the aromatic signals of cytosine-tetramer (**20**) in DMSO at 90°C.

8. TOWARDS THE SYNTHESIS OF DNA BINDING MOLECULES WITH A PEPTIDE BACKBONE

8.1 General

In the approach to synthesize oligonucleotides with peptide backbone, it is necessary to develop methodologies for the preparation of monomers first and then to couple the monomers together. One special feature of the synthesis is that in constructing the peptide backbone, the ornithine derivative will be coupled through the -amino group. In proteins, peptide bonds are formed with the α-amino groups of amino acids. Ornithine protected at the δ-position was used so as to attach the base at the α-position. The commercially available δ-carbobenzoxyl ornithine (**32**) was chosen as the substrate.

The strategy used in the preparation of the monomer units closely followed the methodology developed in the preparation of **20**. This route also offers the possibility of introducing other nucleic acid bases. The synthetic scheme for the preparation of a cytosine monomer unit is shown in Figure 8.1.

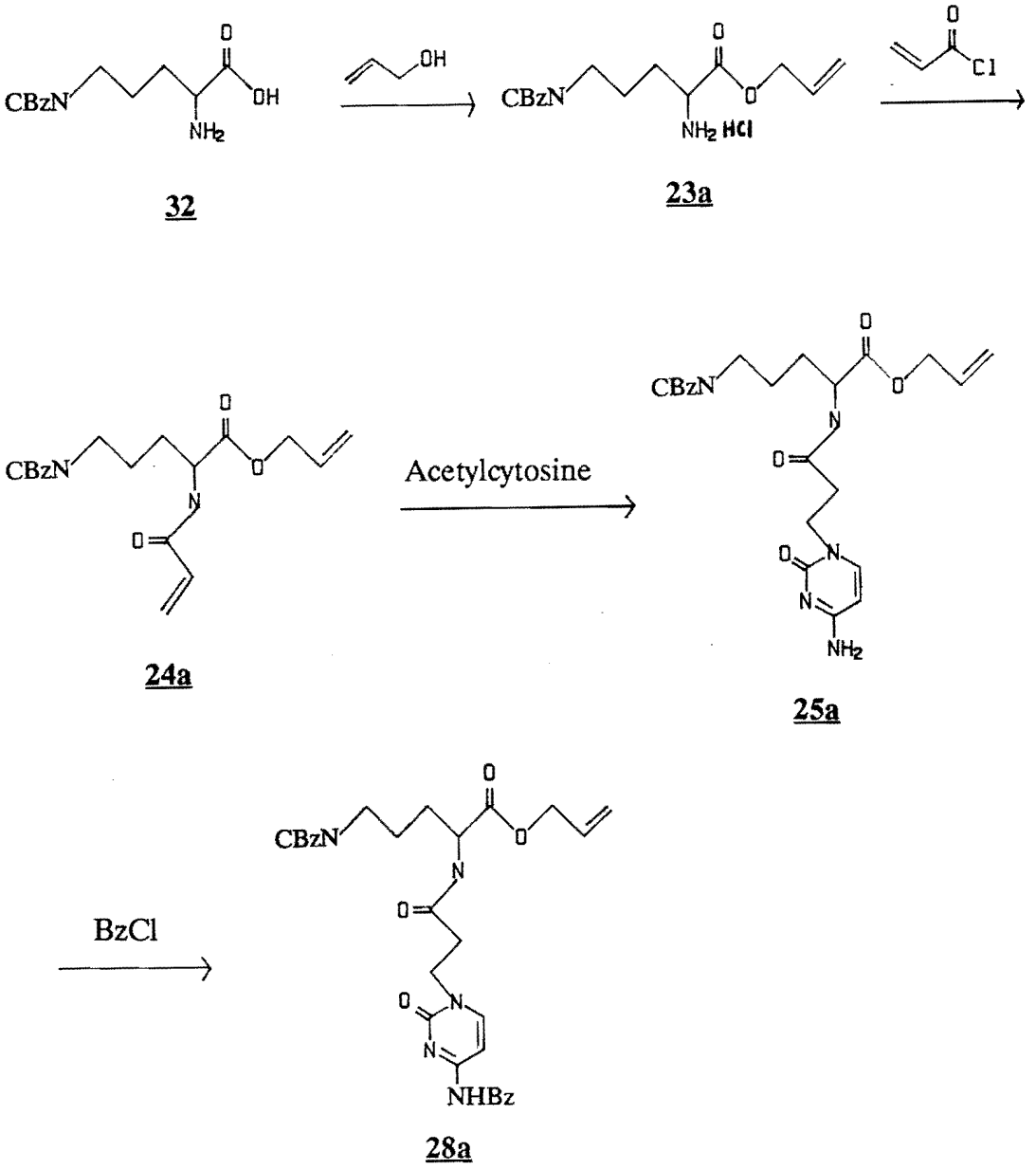


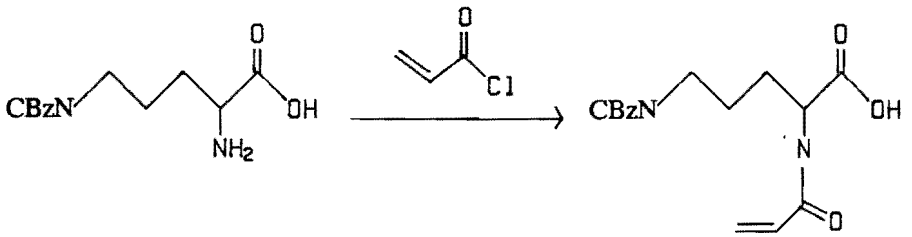
Figure 8.1 Route used in the synthesis of cytosine monomer unit.

8.2 Synthesis of α-Acrylolyl- δ-CBz-Ornithine Allyl Ester

Attempts to prepare the acrylolyl derivative **36** directly from **32** failed (Figure 8.2). The presence of the free acid in **32** complicated the reaction. The low solubility of **32** in organic solvent was another problem. Therefore, the acid in **32** was protected before the introduction of the acrylolyl group.

Allyl alcohol was chosen as the protecting group of the acid since it can be easily removed when the free acid group is required[120,121]. The reaction to prepare the allyl ester (**23a**, Figure 8.1) was straightforward. The free α-amino group of ornithine did not interfere with the reaction.

Introduction of an acrylolyl group to **23a** by acrylolyl chloride in the presence of triethylamine gave close to quantitative yield. One precaution that has to be taken is not to use too much acrylolyl chloride. In some runs, the use of too much acrylolyl chloride gave very poor yields.



32 **36**

Figure 8.2 Attempted synthesis of ornithine acrylolyl derivative.

120. Brook, M. A.; Chan, T. H. *Synthesis* **1983**, 201-203.
121. Kamber, M.; Just, G. *Can. J. Chem.* **1985**, *63(4)*, 823-827.

8.3 Synthesis of N(4)-Unprotected Cytosine Monomer

Michael addition of acetylcytosine to **24a** in allyl alcohol only gave 25% yield. Due to the limited solubility of acetylcytosine in allyl alcohol and for economical reasons, large excess of any of the two substrates was not feasible. Attempts to use other solvents (pyridine and pyridine/water) were not successful. Because of the low yield in the addition step and the low solubility of acetylcytosine in allyl alcohol, an alternative approach involving methanol as the acid protecting group was used. The methyl ester could be replaced by an allyl alcohol in a subsequent ester exchange step. This change allowed the use of methanol as the solvent for the addition reaction. Indeed, the yield of Michael addition was improved to 53% when the methyl ester **24b** was used. All the acetylcytosine was dissolved when the solution was refluxed. The subsequent reaction to exchange the methyl ester to allyl ester also gave good yield of **25a** in the presence of triethylamine.

There is a possibility that in the slightly basic conditions employed for the Michael addition reaction, the chiral amino acid derivative might lose its chirality. However, the chirality of the monomer seems to have been preserved. The specific rotation of the monomer **25b** is -18·4° compared to -16.5° of the starting material **24b**. The bulky substituents on the ornithine may have prevented the monomer from racemizing.

8.4 Deprotection of Cytosine Monomer

In order to form the peptide bonds, the CBz group and the allyl group have to be removed selectively from monomer **25a**. The removal of allyl ester can be achieved either by catalytic hydrogenation or by $Pd[P(Ph_3)]_4/P(Ph)_3/$ potassium 2-ethylhexanoate in ethyl acetate[121]. The latter method was used because the CBz group can also be removed by catalytic hydrogenation[122].

Under mild conditions at room temperature, Pd(0) complex is very effective and selective in removing allyl esters without complication. The product **(27)** was easily separated by filtration and the yield of this reaction was over 90%. The mechanism of this reaction is believed to be by activation of the leaving allyl group which coordinates to the metal[123].

The CBz protecting group was removed from **25a** by an old, but effective procedure using hydrobromic acid in glacial acetic acid[124] to give **26a**. Catalytic hydrogenation[122] was not used for the same reason as discussed before. At room temperature, CBz was removed smoothly by 15%HBr in acetic acid within two hours. The allyl ester was not affected by HBr. Attempt to use HCl-AcOH showed that it was less efficient than HBr-AcOH and required refluxing the reaction solution for 10 hours before the reaction went to completion.

---

122. (a) Bergmann, M.; Zervas, L. *Ber.* **1932**, *65*, 1192-1202. (b) Boissonnas, R. A.; Preitner, G. *Helv. Chim. Acta* **1953**, *36(4)*, 875-886.
123. Jeffrey, P. D.; McCombie, S. W. *J. Org. Chem.* **1982**, *47(3)*, 587-590.
124. (a) Ben-ishai, D.; Berger, A. *J. Org. Chem.* **1952**, *17(12)*, 1564-1570. (b) Ben-ishai, D. *J. Org. Chem.* **1954**, *19(1)*, 62-66.

8.5 Protection of the N(4)-amino Group of Cytosine Monomer

The N(4) amino group of the cytosine residue in the monomer may interfere during coupling reactions. In order to protect the free amino group, a benzoyl protecting group was used. The protected monomers **28a** and **28b** were prepared from **25a** and **25b** respectively by reaction with benzoyl chloride in the presence of triethylamine in THF. The products (**28a** and **28b**) were oils even after purification by column chromatography. Upon standing in refrigerator for several hours, both hardened to form semi-solids. Attempts to crystallize the products in different solvents failed. The solid forms of the compounds could be obtained as powders by lyophilization of benzene solutions of the protected monomer.

The deprotections of the δ-amino group and the acid to form **29b** and **30** followed the same procedures used for the preparation of **27** and **26a**. The benzoyl group was stable in both deprotection reactions.

9. PROPERTIES OF CYTOSINE TETRAMER 20

The cytosine tetramer **20** has four cytosine residues linked to the tripropylenetetraamine backbone. In solution, the bases may stack one upon the other to form ordered structures. This possibility was studied by UV spectroscopy to look for hypochromicity of the compound. However, preliminary studies showed that there is no observable hypochromism of **20** in phosphate buffer (10 mM), EDTA (0.1 mM) and sodium chloride (1 M) solution at pH 7. When the solution was heated from 5°C to 80°C, the absorbance at 260nm decreased slightly. The lack of an observed hypochromicity indicates that base stacking is not occurring in **20**. This probably can be explained by the restricted rotation of the attached bases. If the bases can not freely rotate, there would be no chance for them to show stacking interactions.

It has been shown by Michelson and his co-workers that oligocytidylates at neutral pH possess a single-stranded base-stacked helical conformation at low temperature[125]. The transition from ordered to disordered structures can be observed by CD spectra when the temperature is increased. However, CD spectrum of **20** should not give us any information because there are no chiral centers in **20**.

A more proper compound for the binding studies should be the self complementary GGCC tetramer with the peptide backbone since it has been shown

125. Brahms, J.; Maurizot, J. C.; Michelson, A. M. *J. Mol. Biol.* **1967**, *25(3)*, 465-480.

that in binding studies, poly-G and guanine-rich oligomers will form self associated complexes[126] and, therefore, it is usually very difficult for them to form double helixes with complementary base sequence.

126. Podder, S. K. *Indian J. Biochem. Biophys.* **1971**, *8*, 239-246.

10. EXPERIMENTAL SECTION

10.1 General

Acrylolyl chloride, allyl alcohol, trifluoroacetic anhydride, N,N'-bis-(3-aminopropyl)-1,3-propanediamine, tetrakis(triphenylphosphine)palladium(0), triphenyl phosphine, chlorotrimethylsilane, trifluoroacetic anhydride and benzoyl chloride were purchased from Aldrich. Cytosine, N- δ -CBz-L-ornithine, d(GGCC), and d(GGGG) were obtained from Sigma. The chemicals were used without further purification.

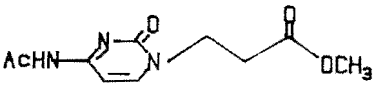
Measurements of the specific rotations were performed on a JASCO DIP-40 digital polarimeter in absolute ethanol at 25°C. Other instruments used for analysis have been described in part I of this thesis.

10.2 Synthesis

3-(1-(4-acetylcytosine))methylpropionate (16)[118].

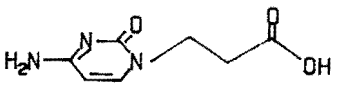
To a suspension of acetyl cytosine (4.4g, 28.8 mmol) in 100 mL of pyridine and 50 mL of water was added dropwise methyl acrylate (35 mL). After the

mixture had been refluxed for 1 h, the solvent was removed under reduced pressure to give an oily product. The product was washed with benzene twice to removed unreacted acrylate to give 6 g of 16, in 87% yield. The product was used without further purification. A pure sample was obtained by column chromatography on silica gel (CH_2Cl_2: ethanol/ 5:1). mp: 132-134°C. ^{1}H NMR ($CDCl_3$): 7.86 (d, 1H, **CH** cyt), 7.42 (d, 1H, **CH** cyt), 4.14 (t, 2H, **CH_2**-N ethylene),3.68 (s, 3H, **CH_3**-O ester), 2.89 (t, 2H, **CH_2**-C=O ethylene), 2.29 (s, 3H, **CH_3**-C=O Ac).



3-(1-cytosyl)propanic acid (17).

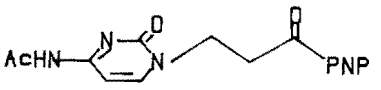
A sample of 1g (4.2 mmol) of 16 was added to two equivalents of sodium hydroxide in 70 mL of water. The solution was refluxed for 6 h. The solvent was evaporated to dryness to give the sodium salt of 17, which was redissolved in water and neutralized with 1M HCl to pH 3.5. The solid that precipitated out was filtered and washed with water. The white solid obtained was dried to give 0.65g of 17, in 85% yield. mp: >250°C. ^{1}H NMR (D_20, NaOH): 7.57 (d, 1H, **CH** cyt), 5.94 (d, 1H, **CH** cyt), 3.96 (t, 2H, **CH_2**-N ethylene), 2.55 (t, 2H, **CH_2**-C=O ethylene).



3-(1-cytosyl)p-nitrophenylpropionate (18)[119].

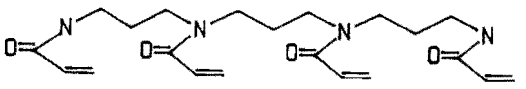
To a solution of p-nitrophenol (PNP) (0.3g, 2.2 mmol) in 8 mL of dry pyridine was added trifluoroacetic anhydride (0.46 g, 2.2 mmol) under nitrogen. The solution was allowed to stir at room temperature for 10 min. A sample of 0.37g (2 mmol) of 17 was added to the solution. It was stirred at room temperature for half an hour. At the end, all 17 was dissolved. Another portion of trifluoroacetic

anhydride (0.46 g) was added to the solution, and it was stirred for another 10 min. The solvent was, then, removed under reduced pressure to give an oily product, which was digested with ethanol. The undissolved solid was filtered and washed with ethanol to give 0.48g of product, in 97% yield. mp: 175-176°C. ^{1}H NMR (pyridine): 8.21 (d, 2H, **CH** PNP), 7.88 (d, 1H, **CH** cyt), 7.40 (d, 2H, **CH** PNP), 6.09 (d, 1H, **CH** cyt), 4.32 (t, 2H, **CH$_2$**-N ethylene), 3.32 (t, 2H, **CH$_2$**-C=O ethylene).



N,N'N,"N,"'-tetraacrylolyl N,N'-bis-(3-aminopropyl)-1,3-propanediamine (19).

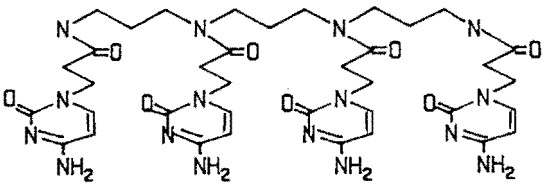
A sample of 0.376g of N,N'-bis-(3-aminopropyl)-1,3-propanediamine (2 mmol) was dissolved in a mixture of 5 mL of benzene and 5 mL of water with 0.8g (20 mmol) of sodium hydroxide in an ice-water bath at 0°C. Acrylolyl chloride (0.9g, 10 mmol) in 2 mL of benzene was added dropwise to the mixture. After stirring for 10 min, the mixture was extracted with chloroform (3x15mL). The combined chloroform solutions were washed with water, dilute HCl and water, and then dried. The solvent was removed under reduced pressure to give the crude product of **19**. The sample was purified by column chromatography on silca gel with 30% methanol in ethyl acetate to give an oil (0.3g, 37%) which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 55°C): 6.06-6.60 (m, 8H, **CH$_2$**=), 5.57-5.73 (m, 4H, CH=), 3.26-3.50 (m, 12H, **CH$_2$**-N propylene), 1.72-1.91 (m, 6H, **CH$_2$**-CH$_2$-N propylene).



N,N',N",N"'-tetra-(3-(1-cytosyl)ethylcarboxyl)-N,N'-bis-(3-aminopropyl)-1,3-propanediamine (20).

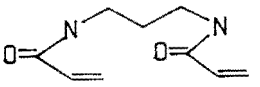
A sample of 0.337g of acetyl cytosine (2.2 mmol) in 40 mL of methanol was

added 4mg of sodium. After the sodium had all dissolved, 19 (0.22g, 0.54 mmol) in 2 mL of methanol was added to the suspension at room temperature. It was then refluxed for 35 h. At the end, the solvent was removed under reduced pressure. The solid obtained was digested in 10mL of methanol, and the undissolved solid (cytosine) was filtered. The filtrate was evaporated to dryness. The product was separated by column chromatography on silca gel (eluents: 1:1 methanol/ethyl acetate; 60% methanol, 35% ethyl acetate and 5% triethylamine) to give 60mg of product in 13% yield. mp: >200°C (dec). ^{1}H NMR (DMSO, 90°C): 7.52 (d, 2H, **CH** cyt inner), 7.45 (d, 2H, **CH** cyt), 5.62 (d, 4H, **CH** cyt), 3.85 (t, 4H, **CH_2**-N inner ethylene), 3.84 (t, 4H, **CH_2**-N ethylene), 3.12-3.25 (m, 12H, **CH_2**-N propylene), 3.03 (m, 4H, **CH_2**-C=O inner ethylene), 2.65 (t, 4H, **CH_2**-C=O ethylene), 2.58 (m, 6H, **CH_2**-CH_2-N propylene). ^{13}C NMR: 174.38, 174.12, 169.71, 160.58, 160.45, 150.03, 149.66, 96.95, 96.87, 48.61, 48.54, 46.48, 46.25, 39.58, 39.52, 37.52, 31.37, 30.20. FAB-MS (glycerol/Na): e/m 871 (M+23), 849 (M+1).



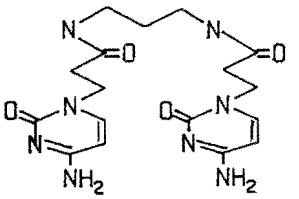
N,N'-Diacrylolyl 1,3-diaminopropane (21).

To an ice cooled mixture of diaminopropane (0.74g, 10 mmol) in 5 mL of benzene and 9 mL of 5 N NaOH aqueous solution was added dropwise a solution of acrylolyl chloride (1.81g, 20 mmol) in 4 mL of dry benzene. The addition took about 10 min. The white precipitate formed was then filtered and washed with water two times. The solid was dried to give 0.45g of 21, in 60% yield. It was used without further purification. mp: 114°C (dec). ^{1}H NMR ($CDCl_3$): 6.67 (b, 2H, **NH**), 6.08-6.35 (m, 4H, **CH_2**=), 5.66 (dd, 2H, **CH**=), 3.38 (dt, 4H, **CH_2**-N propylene), 1.70 (m, 2H, **CH_2**-CH_2-N propylene).



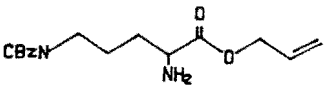
N,N'-Bis-(3-(1-cytosyl)ethylcarboxyl)-1,3-diaminopropane (22).

To a solution of 5 mL of ethanol with acetyl cytosine (0.31g, 2mmol) was added sodium (2mg). After the sodium had all dissolved, a solution of 21 (0.18g, 1 mmol) in 1 mL of ethanol was added. The suspension was refluxed for 20 h and all the solid was dissolved. The reaction solution was cooled to room temperature, and the precipitate formed was filtered and washed with ethanol. After it was dried, the white solid gave 0.45g of 22, in 57% yield. mp: 216-219°C. ^{1}H NMR (DMSO): 7.89 (t, 2H, **NH**), 7.46 (d, 2H, **CH** cyt), 6.97 (b, 4H, **NH_2**), 5.59 (d, 2H, **CH** cyt), 3.80 (t, 4H, **CH_2**-N ethylene), 2.99 (m, 4H, **CH_2**-N propylene), 2.42 (t, 4H, **CH_2**-C=O ethylene), 1.45 (m, 2H, **CH_2**-CH_2-N propylene). ^{13}C NMR: 169.69, 165.95, 155.63, 146.41, 92.84, 45.77, 36.22, 34.38, 29.02.



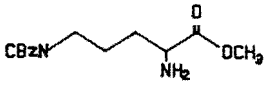
N-δ-CBz-L-ornithine allyl ester hydrochloride (23a)[120].

To a suspension of N-δ-CBz-ornithine (0.13g, 0.5 mmol) in dry allyl alcohol (2 mL) was added chlorotrimethylsilane (4 mL) with stirring. The suspension became clear during the first half addition and turned into a white slurry at the end. The mixture was heated to 50°C for 2 h to effect a clear solution. After removal of the solvent, it gave 0.15g of the product as a white solid, in 87% yield. mp: 104-105°C. ^{1}H NMR ($CDCl_3$): 8.76 (b, 2H. **NH_2**), 7.30 (m, 5H, **CH** CBz), 5.82 (m, 1H, **CH**=), 5.05-5.33 (q, 2H, **CH_2**=), 5.05(s, 2H, **CH_2** CBz), 4.61 (d, 2H, **CH_2**-O allyl), 4.19 (m, 1H, **CH**-NH_2 orn), 3.20(t, 2H, **CH_2**-N orn), 2.09 (m, 2H, **CH_2**-CH orn), 1.74 (m, 2H, **CH_2**-CH_2-N orn). ^{13}C NMR: 169.34, 156.72, 136.73, 130.88, 128.48, 127.98, 119.66, 67.08, 66.51, 52.88, 40.11, 27.46, 25.26.



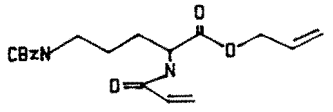
N-δ-CBz-L-ornithine methyl ester (23b).

Obtained from N- δ-CBz-L-ornithine *via* the procedure for the preparation of **23a**, in close to 100% yield. mp: 138-139°C. $[\alpha]_D$: 8.0° (c=0.5), ^{1}H NMR (DMSO): 8.52 (b, 3H, NH_2,NH), 7.75 (m, 5H, CH CBz), 5.01 (s, 2H, CH_2 CBz), 4.04 (m, 1H, CH-NH_2 orn), 3.74 (s, 3H, CH_3), 3.01 (dt, 2H, CH_2-N orn), 1.78 (m, 2H, CH_2-CH orn), 1.49 (m, 2H, CH_2-CH_2-N). ^{13}C NMR: 169.82, 156.08, 137.13, 128.30, 127.71, 127.65, 65.12, 52.69, 51.62, 38.63, 27.35, 24.90.



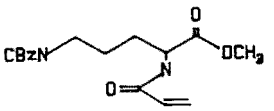
N-δ-CBz-N'-α-acrylolyl-L-ornithine allyl ester (24a).

To a solution of **23a** (0.171g, 0.5 mmol) in 10 mL of dry THF with triethylamine (0.074g, 0.75 mmol) was added acrylolyl chloride in 2 mL of THF over 5 min. The suspension was stirred for 20 h at room temperature. The precipitate formed was filtered (triethylamine hydrochloride), and the filtrate was evaperated to dryness to give an oil which solidified upon standing at room temperature. The crystal collected was washed with hexane to give 0.16g of **24a**, in 89% yield. mp: 81-83°C. ^{1}H NMR ($CDCl_3$): 7.35 (m, 5H, CH CBz), 6.11-6.37 (m, 2H, CH_2= acrylolyl), 5.84-5.98 (m, 1H, CH= allyl), 5.69 (m, 1H, CH= acrylolyl), 5.30-5.37 (m, 2H, CH_2= allyl), 5.09 (s, 2H, CH_2 CBz), 4.72 (m, 1H, CH-N orn), 4.65 (d, 2H, CH_2-O allyl), 3.23 (dt, 2H, CH_2-N orn), 1.59-1.94 (m, 4H, CH_2 orn). ^{13}C NMR: 171.93, 165.21, 156.53, 136.52, 131.35, 130.26, 128.52, 128.13, 127.37, 119.21, 66.72, 66.19, 51.94, 40.44, 29.73, 26.06.



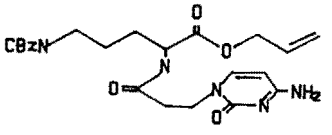
N-δ-CBz-N'-αacrylolyl-L-ornithine methyl ester (24b).

Obtained from 23b *via* the procedure for the preparation of 24a, in 93% yield. mp: 116-118°C. $[\alpha]_D$: - 16.5° (c=0.72). ^{1}H NMR ($CDCl_3$): 7.35 (m, 5H, **CH** CBz), 6.37 (b, 1H, **NH**), 6.08-6.38 (m, 2H, $\mathbf{CH_2}$=), 5.69 (m, 1H, **CH**=), 5.09 (s, 2H, $\mathbf{CH_2}$ CBz), 4.93 (b, 1H, **NH**), 4.70 (m, 1H, **CH**-N orn), 3.75 (s, 3H, $\mathbf{CH_3}$), 3.23 (dt, 2H, $\mathbf{CH_2}$-N orn), 1.52-1.95 (m, 4H, $\mathbf{CH_2}$ orn). ^{13}C NMR: 172.85, 165.44, 156.64, 136.50, 129.11, 128.51, 128.09, 125.28, 66.69, 52.58, 51.93, 40.42, 29.47, 26.03.



N-δ-CBz-N'-α(β-(1-cytosyl)ethylcarboxyl)ornithine allyl ester (25a).

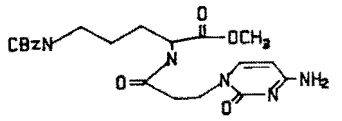
Acetyl cytosine (0.06g, 0.325 mmol) was suspended in 6 mL of allyl alcohol. 1 mg of sodium was added to the suspension. After the sodium had all reacted, 0.072g (0.2 mmol) of 24a in 1 mL of allyl alcohol was added. The mixture was refluxed for 12 h. The solvent was, then, removed to give an oil, which was digested in 5 mL of chloroform with 20% methanol. The precipitate was filtered (cytosine), and the filtrate was evaporated to dryness. The residue was purified by column chromatography on silica gel (10% methanol in chloroform). Yield: 0.024 g (25%). mp: 167-168°C. ^{1}H NMR (CD_3OD): 7.48 (d, 1H, **CH** cyt), 7.33 (m, 5H, **CH** CBz), 5.89 (m, 1H, **CH**= allyl), 5.77 (d, 1H, **CH** cyt), 5.18-5.35 (m, 2H, $\mathbf{CH_2}$= allyl), 5.06 (s, 2H, $\mathbf{CH_2}$ CBz), 4.58 (d, 2H, $\mathbf{CH_2}$-O allyl), 4.37 (m, 1H, **CH**-N orn), 4.00 (dt, 2H, $\mathbf{CH_2}$-N ethylene), 3.10 (t, 2H, $\mathbf{CH_2}$-N orn), 2.67 (dt, 2H, $\mathbf{CH_2}$-C=O ethylene), 1.45-1.88 (m, 4H, $\mathbf{CH_2}$ orn). ^{13}C NMR: 174.61, 169.64, 164.67, 160.46, 149.87, 149.74, 140.02, 134.87, 131.03, 130.52, 130.34, 120.23, 97.03, 68.93, 68.30, 55.23, 49.44, 42.71, 36.96, 31.17, 28.89. CI-MS(NH_3): e/m 472 (M+1) 6.85%, 361 (M-110) 100%, 253 (M-220) 25%, 112 (M-359) 49%.



25a was also prepared by refluxing **25b** in allyl alcohol for 2 h in the presence of triethylamine. The ester exchange reaction gave 90% yield.

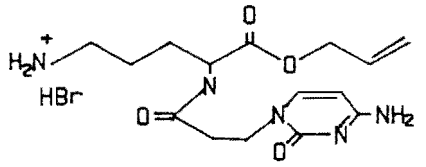
N-δ-CBz-N'-α-(β-(1-cytosyl)ethylcarboxyl)ornithine methyl ester (25b).

Obtained from **24b** *via* the procedure for the preparation of **25a**, in 53% yield. Methanol was used in place of allyl alcohol. mp: 150-151.5°C. $[\alpha]_D$: - 18.4° (c=0.125). ^{1}H NMR (CD_3OD): 7.48 (d, 1H, **CH** cyt.), 7.33 (m, 5H, **CH** CBz), 5.78 (d, 1H, **CH** cyt.), 5.06 (s, 2H, CH_2 CBz), 4.35 (m, 1H, **CH**-N orn), 4.00 (t, 2H, CH_2-N ethylene), 3.67 (s, 3H, CH_3), 3.10 (t, 2H, CH_2-N orn), 2.67 (dt, 2H, CH_2-C=O ethylene), 1.78-1.43 (m, 4H, CH_2 orn). ^{13}C NMR: 175.43, 174.59, 169.64, 160.45, 149.79, 149.75, 140.02, 131.03, 130.53, 130.34, 97.01, 68.93, 55.14, 54.28, 49.47, 42.73, 36.96, 31.17, 28.87.



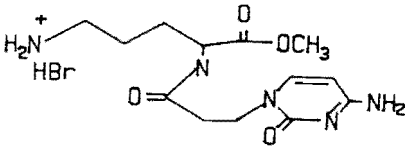
N'- α-(β-(1-cytosyl)ethylcarboxyl)ornithine allyl ester hydrobromide salt (26a)[124].

A sample of 47mg (0.1 mmol) of **25a** was dissolved in a 2 mL solution of 15% HBr in acetic acid. The solution was stirred overnight at room temperature. Ether (10 mL) was added to the reaction mixture to produce a white precipitate which was filtered and washed with ether. The hygroscopic solid was dried to give 40mg of **26a**, in 95% yield. ^{1}H NMR (CD_3OD): 7.92 (d, 1H, **CH** cyt), 6.01 (d, 1H, **CH** cyt), 5.85-6.04 (m, 1H, **CH**= allyl), 5.20-5.39 (m, 2H, CH_2= allyl), 4.60-4.64 (m, 2H, CH_2-O allyl), 4.39-4.45 (m, 1H, **CH**-N orn), 4.13 (t, 2H, CH_2-N ethylene), 2.96 (t, 2H, CH_2-N orn), 2.68-2.86 (m, 2H, CH_2-C=O ethylene), 1.71-1.99 (m, 4H, CH_2 orn).



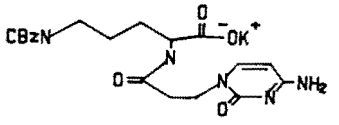
N'-α-(β-(1-cytosyl)ethylcarboxyl)ornithine methyl ester hydrobromide salt (26b).

Obtained from **25b** *via* the procedure for the preparation of **26a**, in 98% yield, as a hygroscopic solid. ^{1}H NMR (DMSO): 9.50 (b, 1H, **NH**), 8.41 (b, 1H, **NH**), 8.27 (b, 1H, **NH**), 7.96 (d, 1H, **CH** cyt), 7.78 (b, 3H, **NH_3**), 6.09 (d, 1H, **CH** cyt), 4.24 (m, 1H, **CH**-N orn), 3.99 (t, 2H, **CH_2**-N ethylene), 3.63 (s, 3H, **CH_3**), 2.79 (m, 2H, **CH_2**-N orn), 2.62 (t, 2H, **CH_2**-C=O ethylene), 1.57-1.79 (m, 4H, **CH_2** orn). ^{13}C NMR: 171.89, 169.64, 159.58, 150.47, 147.25, 92.75, 51.79, 51.54, 45.95, 38.38, 33.23, 27.73, 23.49.



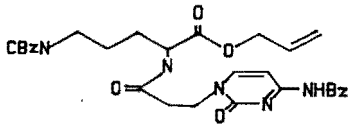
N- δ-CBz-N'- α-(β -(1-cytosyl)ethylcarboxyl)ornithine potassium salt (27)[123].

To 47mg **of 25a** (0.1 mmol) and 44 mg of potassium 2-ethylhexanoate (0.24 mmol) in 1 mL of ethyl acetate was added tetrakis(triphenylphosphine) palladium(0) (7.3mg, 6mol%) and triphenyl phosphine (2.1mg, 8mol%). The suspension was stirred overnight at room temperature. The solid separated was filtered and washed with ether and ethyl acetate several times to give 30mg product after drying, in 70% yield. mp: 165°C(dec). **27** was used as it was without further purification. ^{1}H NMR (CD_3OD): 7.52 (d, 1H, **CH** cyt), 7.33 (m, 5H, **CH** CBz), 5.78 (d, 1H, **CH** cyt), 5.05 (s, 2H, **CH_2** CBz), 4.22 (dt, 1H, **CH**-N orn), 4.02 (m, 2H, CH_2-N ethylene), 3.10 (t, 2H, **CH_2**-N orn), 2.65 (m, 2H, **CH_2**-C=O ethylene), 1.41-1.90 (m, 4H, **CH_2** orn). ^{13}C NMR: 178.88, 172.11, 168.83, 168.14, 158.94, 148.22, 138.60, 129.53, 128.99, 128.92, 95.54, 67.38, 55.92, 47.98, 41.59, 36.15, 31.22, 27.22.



N-δ-CBz-N'-α-(β-(1-(4-benzoyl)cytosyl)ethylcarboxyl) ornithine allyl ester (28a).

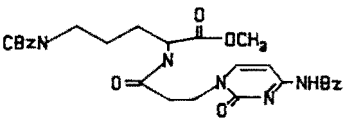
A sample of 47mg (0.1mmol) of **25a** and 20mg (0.2 mmol) of triethylamine was dissolved in 2 mL of dry THF. The solution was cooled to 0°C in an ice-water bath as benzoyl chloride (20mg, 0.14 mmol) in 1 mL of dry THF was added to it. The mixture was stirred at room temperature for 6 h. The precipitate formed (triethylamine hydrochloride) was filtered and washed with THF. The filtrate and the washings were combined and evaporated to dryness. The residue was dissolved in chloroform and washed with water, 5% sodium bicarbonate, water, dilute HCl and water successively. The solution was then dried (Na_2SO_4) and the solvent was removed to give the crude product of **28a**. The product was purified by column chromatography on silica gel to give an oily product. The solid form of the product was only obtained by lyophilization of a benzene solution of the oil. Yield: 40mg (70%). mp: 55-58°C (soften at 48°C). ^{1}H NMR ($CDCl_3$): 7.17-7.88 (m, 12H, cyt, CBz, Bz), 5.67-5.83 (m, 1H, **CH**= allyl), 5.09-5.23 (m, 2H, **CH_2**= allyl), 4.92 (s, 2H, **CH_2** CBz), 4.47 (d, 2H, **CH_2**-O allyl), 4.41-4.51 (m, 1H, **CH**-N orn), 4.04 (t, 2H, **CH_2**-N ethylene), 3.10 (dt, 2H, **CH_2**-N orn), 2.72 (dt, 2H, **CH_2**-C=O ethylene), 1.74 (m, 2H, **CH_2**-CH-N orn), 1.50 (m, 2H, **CH_2**-CH_2-N orn).



N-δ-CBz-N'-α-(β-(1-(4-benzoyl)cytosyl)ethylcarboxyl) ornithine methyl ester (28b).

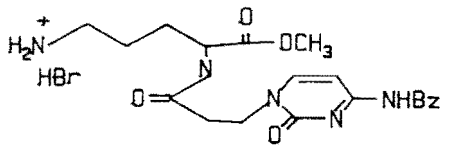
Obtained from **25b** *via* the procedure for the preparation of **28a**, in 77% yield, as a white powder from lyophylization of benzene solution. mp: 92-94°C (soften at 85°C). $[\alpha]_D$:-5.45° (c=0.55). ^{1}H NMR ($CDCl_3$): 7.25-8.10 (m, 12H, cyt. CBz. Bz), 5.00 (s, 2H, **CH_2** CBz), 4.42 (dt, 1H, **CH**-N orn), 4.16 (t, 2H, **CH_2**-N ethylene),

3.65 (s, 3H, CH_3), 3.08-3.21 (m, 2H, CH_2-N orn), 2.83 (t, 2H, CH_2-C=O ethylene), 1.79 (m, 2H, CH_2-CH orn), 1.54 (m, 2H, CH_2-CH_2-N orn). ^{13}C NMR: 172.88, 170.24, 167.55, 161.51, 156.65, 153.27, 151.50, 136.64, 133.85, 131.85, 129.18, 128.87, 128.48, 128.04, 96.35, 66.54, 52.62, 52.49, 48.07, 40.46, 34.15, 28.58, 26.16.



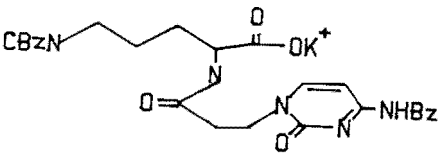
N-α-(β-(1-(4-benzoyl)cytosyl)ethylcarboxyl) ornithine methyl ester hydrobromide salt (29b).

Obtained from **28b** *via* the procedure for the preparation of **26a**, in 85% yield, as a hygroscopic solid. ^{1}H NMR (DMSO): 7.20-8.51 (m, 7H, cyt, Bz), 4.23 (m, 1H, CH-N orn), 4.00 (t, 2H, CH_2-N ethylene), 3.59 (s, 3H, CH_3), 2.75 (m, 2H, CH_2-N orn), 2.63 (t, 2H, CH_2-C=O ethylene), 1.51-1.73 (m, 4H, CH_2 orn). ^{13}C NMR: 171.98, 169.82, 167.33, 162.64, 154.41, 151.00, 133.07, 132.66, 128.51, 128.36, 95.48, 51.80, 51.30, 46.59, 38.29, 33.26, 27.61, 23.48.



N-δ-CBz-N'-α-(β-(1-(4-benzoyl)cytosyl)ethylcarboxyl)ornithine potassium salt (30).

Obtained from **28a** *via* the procedure for the preparation of **27**, in 78% yield. mp: 145-148°C. ^{1}H NMR (DMSO): 7.16-8.09 (m, 12H, Bz, CBz, cyt), 4.96 (s, 2H, CH_2 CBz), 3.97 (t, 2H, CH_2-N ethylene), 3.69 (m, 1H, CH-N orn), 2.90 (m, 2H, CH_2-N orn), 2.54 (t, 2H, CH_2-C=O ethylene), 1.18-1.59 (m, 4H, CH_2 orn). ^{13}C NMR: 172.62, 168.01, 167.46, 163.06, 155.94, 154.98, 150.61, 137.27, 133.43, 132.46, 128.86, 128.32, 128.26, 127.60, 95.60, 64.93, 53.98, 46.82, 40.49, 34.11, 30.13, 25.72.



11. CONCLUSION AND CONTRIBUTIONS TO KNOWLEDGE

1. Oligonucleotide analogs with two cytosine residues attached to 1,3-diaminopropane and four cytosine residues attached to tripropylenetetraamine have been prepared. Preliminary studies showed that there is no base stacking within the tetramer in water solution. Complicating factors that may be responsible for the lack of base stacking were discussed.

2. A monomer unit was synthesized with a cytosine residue covalently linked to ornithine. Synthesis of this monomer unit is the first step towards making sequence specifically linked DNA bases with peptide backbones. It is shown that the methodology can be adopted for the preparation of other monomer units with different nucleic acid bases.

APPENDIX 1. Method of T_m Estimation

Semi-empirical method for the calculation of T_m is quite useful in predicting the stability of a given base sequence. Borer et al.[91] developed the following method based on the melting behavior of a set of oligoribonucleotide helixes to obtain the sequence dependent contribution of the base pairs to the free energy, enthalpy and entropy of helix formation. The evaluated thermodynamic parameters are tabulated [91].

It is assumed that the formation of a double helix is a concentration-dependent binding process of the first base pair, followed by its nearest neighbor base pair, and so on. The formation of the first base pair only involves hydrogen bonding between the two bases. The subsequent ones involve both hydrogen bonding and base stacking interactions.

The first base pair formation is assumed to have negligible enthalpy change ($\Delta H^o = 0$). From thermodynamic equations, we have

$$\Delta G^o = \Delta H - T(\Delta S + \Delta S^o_i) = - RT\ln K \qquad (1)$$

where ΔG^o and ΔS^o_i are the standard free energy and entropy for the first step ($\Delta H^o_i = 0$), and ΔH and ΔS are the sums of enthalpies and entropies of the subsequent steps of the base pair formation (tabulated in reference 91). For self complementary strands, the equilibrium constant is given by:

$$K = \frac{f}{2(1-f)^2 c} \qquad (2)$$

where f is the fraction of paired bases, and c is the concentration of the total oligonucleotides. At T_m, f equals 1/2. Equation (1) becomes:

$$\Delta H - T_m(\Delta S + \Delta S^o_i) = RT_m \ln c \qquad (3)$$

and

$$T_m(^oC) = \frac{\Delta H}{R\ln c + \Delta S + \Delta S^o_i} - 273 \qquad (4)$$

ΔS^o_i was assigned a value of 5 kcal/deg/mol for G-C initiated and 6 kcal/deg/mol for A-U initiated base pair from early studies[127]. For non-identical strands c is replaced by $c/4$. The calculations have showed good agreement with experimental results. It should be pointed out that all these calculations are more accurate for longer oligonucleotides than for shorter ones.

127. (a) Applequist, J.; DamLe, V. *J. Am. Chem. Soc.* **1965**, *87(7)*, 1450-1458. (b) Crothers, D. M.; Kallenbach, N. R.; Zimm, B. H. *J. Mol. Biol.* **1965**, *11(4)*, 802-820.

APPENDIX 2 Estimation of the Hydrolysis Rate of Dimethylphosphate

The rate of hydrolysis of dimethylphosphate (P-O bond cleavage) has not been directly studied at neutral pH because under those conditions, the C-O bond cleavage is dominant. There have been several attempts to estimate this rate constant. Kirby and Younas[7a] examined the hydrolysis of a series of diaryl phosphate diesters with pK_a ranging from 4 to 8.35 at 100°C, pH 7. They found that there is a good linear free energy relationship between the first order rate constant for the hydrolysis of diaryl phosphate (P-O bond cleavage) and the pK_a of the conjugate acid of the leaving group (eq 1).

$$\log k_{hyd} = 1.57 - 0.97\, pK_a \qquad (1)$$

From the relationship, it can be calculated that the first order rate constant for the hydrolysis of dimethyl phosphate (pK_a = 15.54) is 5.2×10^{-16} s^{-1} at 100°C. Assuming that the activation entropy for the hydrolysis of dimethyl phosphate is -25.5 e.u. which is the same as for the hydrolysis of bis(*p*-nitrophenyl)phosphate[7a], the activation enthalpy is thus calculated to be 38.6 kcal/mol. The first order rate constant for the hydrolysis of dimethylphosphate should be about 10^{-21} s^{-1} at 25°C at pH 7.

Guthrie (see table 1.1 ref f) estimated this rate to be 2×10^{-14} s^{-1}. The estimation was based on studies[8] examined in solutions from strongly acidic up to pH 4. Under those conditions, the hydrolysis of the anion of the diester is very slow, and involves C-O bond cleavage.

APPENDIX 3. Hydrolysis of Carboxylic Esters with Poor Leaving Groups

The following article is a result during the course of our study on metal hydroxide promoted hydrolysis of esters. It was published in the Journal of American Chemical Society, 1984, 106, 3687-3688.

Relationship between Effective Nucleophilic Catalysis in the Hydrolysis of Esters with Poor Leaving Groups and the Lifetime of the Tetrahedral Intermediate

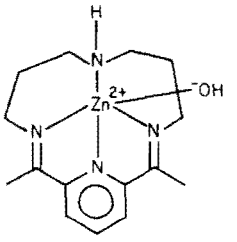
Jik Chin and Xiang Zou

Department of Chemistry, McGill University
Montreal, Canada H3A 2K6

Received December 12, 1983
Revised Manuscript Received April 17, 1984

Many enzymes (chymotrypsin,[1] carboxypeptidase,[2] carbonic anhydrase[3]) catalyze the hydrolysis of esters with good leaving groups as well as esters with poor leaving groups. However, simple hydrolytic catalysts are often tested only on esters with good leaving groups. Simple catalysts that are almost as active as hydroxide in cleaving esters with good leaving groups can be millions of times less active than hydroxide in cleaving esters with poor leaving groups.[4]

In this paper we report the catalytic hydrolysis of methyl trifluoroacetate. In a typical run, hydrolysis of 1 μL of the substrate in 10 mL of water at pH 8.0 and 25.0 °C was monitored to completion by the pH stat method. The reaction was monitored in the presence and absence of varying amounts of the zinc hydroxide (ZOH) prepared according to known procedures.[5] As



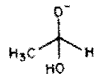
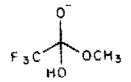
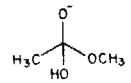
ZOH

a control, initial hydrolysis of 0.2 mL of methyl acetate was monitored by the method described above in the presence and absence of ZOH. In the presence of 50 mg of ZOH, the rate of hydrolysis of methyl trifluoracetate is increased sixfold whereas for the hydrolysis of methyl acetate there is no observable increase in the rate. No rate enhancement is observed with 10 mM borate buffer or imidazole buffer. Therefore, any rate increase by general base catalysis must be small.[4] For all of the above reactions, the kinetic data were corrected for a small background rate (rate without substrate).

In general, nucleophiles do not catalyze the hydrolysis of esters efficiently if the basicity of the nucleophile is significantly less than that of the leaving group.[4] If the base strength of the nucleophile is significantly greater than the base strength of the leaving group, transacylation to the nucleophile is fast but the

(1) Bender, M. L.; Clement, G. E.; Gunter, C. R.; Kezdy, F. J. *J. Am. Chem. Soc.*, **1964**, *86*, 3697.
(2) Breslow, R.; Chin, J.; Hilvert, D.; Trainor, G. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4585–4589.
(3) Pocker, Y.; Sarkanen, S. *Adv. Enzymol.*, **1978**, *47*, 149.
(4) Jencks, W. P.; Kirsch, J. F. *J. Am. Chem. Soc.*, **1964**, *86*, 837.
(5) Prince, R. H.; Stotter, D. A.; Woolley, P. R. *Inorg. Chim. Acta* **1974**, *9*, 51–54.

Table I. Catalytic Efficiency of ZOH and the Lifetime ($1/k$) of the Intermediate

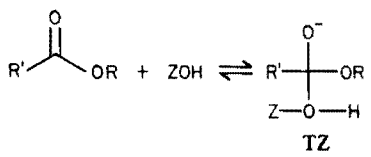
intermediate structure	k_{cat}/k_{OH}	lifetime of C–O bond, s
	6×10^{-2} [a]	10^{4} [b]
	3×10^{-3} [a]	10^{-3} [c]
	6×10^{-3}	10^{-1} [d]
	$<10^{-5}$	10^{-6} [d]

[a] Reference 9. [b] Sirs, J. A. *Trans. Faraday Soc.* **1958**, *54*, 201. [c] Reference 13. [d] Guthrie, J. P.; Cullimore, P. A. *Can. J. Chem.* **1980**, *58*, 1281–1294.

subsequent hydrolysis is slow.[6] Nucleophilic catalysis for hydrolysis of amides or esters involving strongly basic leaving groups and weakly basic nucleophiles had not been observed except when the nucleophile is bound to the substrate.[7,8]

The conjugate acid of ZOH (ZOH_2) has a pK_a of 8.6 and the metal hydroxide has been shown to catalyze the hydration of acetaldehyde and carbon dioxide, and the hydrolysis of propionic anhydride by a nucleophilic mechanism.[9,10]

Equation 1 shows the addition of ZOH to an ester. The adduct

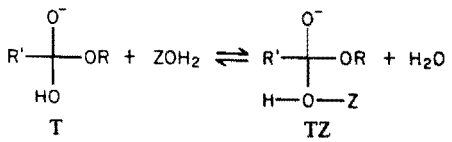


(1)

TZ

(TZ) has three bonds that can be cleaved rapidly around a single oxygen. In general, adducts between esters with poor leaving group and weakly basic nucleophiles revert back to starting material rapidly.[4] Reversion of TZ to starting material can be significantly reduced if the metal–oxygen bond or the hydrogen–oxygen bond is cleaved more rapidly than the carbon–oxygen (metal hydrate oxygen) bond. The lifetimes ($1/k$) of the three bonds can be estimated since metal hydrate exchange rates,[11] proton transfer rates,[12] and lifetimes of tetrahedral intermediates[13] can be measured or estimated.

The lifetime of the C–O bond in TZ is more difficult to estimate than the lifetime of the C–O bond in T (eq 2). The lifetime of



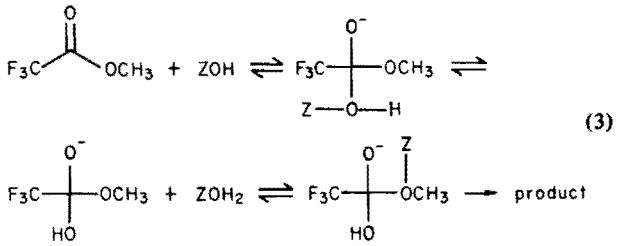
(2)

T TZ

the C–O bond in T can also be used to predict the efficiency of the catalyst. In accordance with the principle of microscopic reversibility, if the metal hydroxide can catalyze the formation of T from the substrate, it should also catalyze the formation of the substrate from T. There will be no catalysis if T in eq 2 is so short lived that the metal–oxygen exchange cannot take place. At 10 mM catalyst concentration, the formation of TZ from T should have a pseudo-first-order rate constant of about 10^4 s^{-1}. In order to have any chance at catalysis, T must be long lived (lifetime > 10^{-4} s).

Table I shows the relationship between the lifetime of the anionic intermediate in various reactions and the catalytic efficiency of ZOH with respect to hydroxide. All of the anions live long enough to bind with the catalyst except the anion formed from methyl acetate. Knowing the tetrahedral intermediate stability is important in understanding the mechanism of these reactions, and there has been much progress in measuring and estimating the lifetimes of these species.[13–15]

At equilibrium, the ratio TZ/T (eq 2) is equal to ZOH_2/H_2O if it is assumed that the Z–O bond strengths are the same.[16–18] At 10 mM ZOH, TZ/T is about 10^{-4}. In order to account for the observed ZOH-catalyzed hydrolysis of methyl trifluoroacetate (sixfold rate enhancement under these conditions) the lifetime of T must be longer than the lifetime of TZ by a factor of 10^4–10^5. Since the lifetime of T is 10^{-1} s (Table I), the lifetime of the C–O bond in TZ is about 10^{-5} to 10^{-6} s. The basicity of the leaving group in T is higher than that in TZ by a factor of 10^7. The metal hydroxide not only catalyzes the formation T but also the breakdown of T to form the products (eq 3) since the expulsion



(3)

of methoxide should be at least partially rate determining for the uncatalyzed process.[19,20]

In conclusion, a weakly basic nucleophile has been shown, for the first time, to be highly effective in catalyzing the hydrolysis of an ester with a poor leaving group. This catalysis is effected by rapid metal–oxygen ligand exchange processes. The effectiveness of the catalyst is related to the lifetime of the tetrahedral intermediate involved and can be estimated. We are currently investigating the reactions of other esters and metal hydrates.

Acknowledgment. This work was supported by the Natural Sciences and Engineering Research Council of Canada and a Clifford Wong fellowship to X.Z. We thank Professor B. Belleau for permitting us to use his equipment.

Registry No. ZOH, 90065-64-8; methyl trifluoroacetate, 431-47-0; methyl acetate, 79-20-9.

(6) Cram. D. J.; Katz, H. E. *J. Am. Chem. Soc.*, **1983**, *105*, 135–136.
(7) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183.
(8) Kluger, R.; Chin, J. *J. Am. Chem. Soc.*, **1982**, *104*, 2891–2897.
(9) Woolley, P. *Nature (London)* **1975**, *258*, 677–682.
(10) Prince, D. H.; Woolley, P. R. *J. Chem. Soc., Perkin, Trans. 2* **1977**, 318–324.
(11) McWinnie, W. R.; Miller, J. D. *Adv. Inorg. Chem. Radiochem.* **1969**, *12*, 135–215.
(12) Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1964**, *3*, 1–19.
(13) Guthrie, J. P. *Acc. Chem. Res.* **1983**, *16*, 122–129.
(14) Jencks, W. P. *Acc. Chem. Res.* **1980**, *13*, 161–169.
(15) McClelland, R. A.; Santry, L. J. *Acc. Chem. Res.* **1983**, *16*, 394–399.
(16) The interaction between the metal ion and the oxy anion in the tetrahedral intermediate is ignored since this interaction is not important in the reaction pathway. Experimental evidence for this is that different metal hydroxides and unbound hydroxide fall on the same Brønsted plot for various reactions.[10]
(17) Langford, C. H.; Gray, H. B. "Ligand Substitution Processes"; W. A. Benjamin: New York, 1965.
(18) This assumption is a first approximation. The substituent effect for binding of substituted amines to metal ions is small. Hine, J. "Structural Effects on Equilibria in Organic Chemistry"; Wiley: New York, 1975.
(19) Barnett, R. E. *Acc. Chem. Res.* **1973**, *6*, 41–46.
(20) Moffat, A.; Hunt, H. *J. Am. Chem. Soc.* **1959**, *81*, 2082.

APPENDIX 4 Supplemental Material

Table A PH-rate profile of $[Co(trien)(OH)(OH_2)]^{2+}$ (0.01M) promoted hydrolysis of BNPP and BDNPP (0.05mM) at 50°C, pH 7 (Figures 2.3 and 2.4)

pH	$k_{hyd}(s^{-1})$ (BDNPP)	pH	$k_{hyd}(s^{-1})$ (BNPP)
4.0	0.20×10^{-3}	5.6	3.1×10^{-4}
4.5	0.72×10^{-3}		3.25×10^{-4}
5.25	2.6×10^{-3}	6.0	4.5×10^{-4}
5.35	3.03×10^{-3}	6.1	4.7×10^{-4}
5.9	4.62×10^{-3}	6.4	5.15×10^{-4}
6.4	5.7×10^{-3}	6.7	5.28×10^{-4}
6.8	5.8×10^{-3}	6.85	5.23×10^{-4}
7.0	5.65×10^{-3}	7.05	4.9×10^{-4}
7.2	5.38×10^{-3}	7.35	3.73×10^{-4}
7.3	4.52×10^{-3}		3.86×10^{-4}
7.6	3.75×10^{-3}	7.8	2.32×10^{-4}
7.8	2.8×10^{-3}		2.45×10^{-4}
8.0	2.15×10^{-3}	8.3	1.15×10^{-4}
8.35	1.7×10^{-3}	8.8	0.3×10^{-4}
8.6	0.78×10^{-3}	9.0	0.25×10^{-4}
8.85	0.63×10^{-3}	9.3	0.15×10^{-4}
9.35	0.35×10^{-3}		

Table B. First order rate constants (s^{-1}) for the hydrolysis of BNPP and BDNPP (0.05mM) in different concentrations of $[Co(trien)(OH)(OH_2)]^{2+}$ at pH 7, 50°C (Figures 2.5 and 2.6)

Concentration of $[Co(trien)(OH(OH_2)]^{2+}$ (M)	k_{BNPP}	k_{BDNPP}
.001		0.39×10^{-3}
.005	2.1×10^{-4}	2.65×10^{-3}
.010	4.5×10^{-4}	5.20×10^{-3}
.015	7.2×10^{-4}	7.5×10^{-3}
.020	9.8×10^{-4}	10.4×10^{-3}
.025	11.5×10^{-4}	

Table C PH-rate profile for **ZnCR-OH** (5mM) promoted hydrolysis of BDNPP (0.05mM) at 50°C (Figures 2.9)

pH	7.0	7.2	7.6	7.65	8.05	8.4	9.1	9.4	9.6	9.75
$k(s^{-1})$	1.1	1.30	2.92 3.05	3.35	4.9	7.02 7.2	9.03 9.17	9.73	10.0	9.75

Bibliography

1. (a) Robison, G. A.; Butcher, R. W.; Sutherland, E. W. *Ann. Rev. Biochem.* **1968**, *37*, 149-174. (b) Sutherland, E. W.; Robison, G. A.; Butcher, R. W. *Circulation* **1968**, *37(2)*, 279-306.
2. Leading references may be found in following articles and references therein. (a) Kochetkov, N. K.; Budovskii, E. I.; Eds. "Organic Chemistry of Nucleic acids" A and B, Plenum Press, N. Y., **1971**. (b) Jones, D. R.; Lindoy, L. F.; Sargeson, A. M. *J. Am. Chem. Soc.* **1983**, *105(25)*, 7327-7336. (c) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1(3)*, 70-78. (d) Cox Jr., J. R.; Ramsay, O. B. *Chem. Rev.* **1964**, *64(4)*, 317-352. (e) Westheimer, F. H. *Science* **1987**, *235*, 1173-1178.
3. Bunton, C. A. *Acc. Chem. Res.* **1970**, *3(8)*, 257-265.
4. Thain, M. *J. Chem. Soc.* **1957**, 4694-4699.
5. Gerlt, J. A.; Westheimer, F. H.; Sturtevant, J. M. *J. Biol. Chem.* **1975**, *250(13)*, 5059-5067.
6. Fanni, T.; Taria, K.; Gorenstein, D. G.; Vaidyanathaswary, R.; Verkada, J. G. *J. Am. Chem. Soc.* **1986**, *108(20)*, 6311-6314.
7. (a) Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)*, **1970**, 510-513. (b) Bunton, C. A.; Farber, S. *J. Org. Chem.* **1969**, *34(4)*, 767-772.
8. Bunton, C. A.; Mhala, M. M.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc.* **1960**, 3293-3301.
9. (a) Benkovic, S. J.; Schray, K. J. "*Enzymes*" 3rd Ed. **1973**, *8*, 201-238. (b) Westheimer, F. H. *Chem. Rev.* **1981**, *81(4)*, 313-326.
10. Chanley, J. D.; Feageson, E. *J. Am. Chem. Soc.* **1963**, *85(8)*, 1181-1190.
11. It is still controversial whether A exists in a dilute solution. Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1986**, *108(25)*, 7938-7946.
12. "The Inorganic Chemistry of Biological Processes", 2nd Ed., Hughes, M. N., p51 and p89, John Wiley & Son, N. Y., **1981**.
13. (a) Frey, C. M.; Stuehr, J. *Metal ions in Biological System.* **1974**, *1*, 52-116. (b) Klinman, J. P.; Samuel, D. *Biochem.* **1971**, *10(11)*, 2126-2131.
14. Example could be found in decarboxylation of acetone dicarboxylic acid; (a) Pure, J. E. *J. Chem. Soc.* **1952**, 2331-2338. (b) Steinberger, R.; Westheimer, F. H. *J. Am. Chem. Soc.* **1951**, *73(1)*, 429-435.
15. Wells, M. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1977**, *99(16)*, 5341-5356.
16. Bender, M. L.; Turnquest, B. W. *J. Am. Chem. Soc.* **1957**, *79(8)*, 1889-1893.
17. Buckingham, D. A.; Foster, D. M.; Sargeson, A. M. *J. Am. Chem. Soc.* **1970**, *92(21)*, 6151-6158.
18. Breslow, R.; Fairweather, R.; Keana, J. *J. Am. Chem. Soc.* **1967**, *89(9)*, 2135-2138.
19. Groves, J. T.; Dias, R. M. *J. Am. Chem. Soc.* **1979**, *101(4)*, 1033-1035.
20. (a) Gustafson, R. L.; Chaberek, S.; Martell, A. E. *J. Am. Chem. Soc.* **1963**, *85(5)*, 598-601. (b) Gustafson, R. L.; Martell, A. E. *J. Am. Chem. Soc.* **1962**, *84(12)*, 2309-2316.
21. (a) Woolley, P. *Nature* (London) **1975**, *258*, 677-682. (b) Woolley, P. *J. Chem. Soc.,Perkin. Trans.* 2, **1977**, 318-324.
22. Chin, J.; Zou, X. *J. Am. Chem. Soc.* **1984**, *106(12)*, 3687-3688. See Appendix 2.
23. Gellman, S. H.; Petter, R.; Breslow, R. *J. Am. Chem. Soc.* **1986**, *108(9)*, 2388-2394.
24. Norman, P. R. *Inorg. Chim. Acta* **1987**, *130*, 1-4.
25. (a) Hubner, P. W. A.; Milburn, R. M. *Inorg. Chem.* **1980**, *19(5)*, 1267-1272. (b) Anderson, B.; Milburn, R. M.; Sargeson, A. M. *J. Am. Chem. Soc.* **1977**, *99(8)*, 2652-2661. (c) Kenley,R. A.; Fleming, R. H.; Laine, R. M.; Tse, D. S.; Winterle, J. S. *Inorg. Chem.* **1984**, *23(13)*, 1870-1876.
26. (a) Milburn, R. M.; Gautam-Basak, M.; Tribolet, R.; Sigel, H. *J. Am. Chem. Soc.* **1985**, *107(11)*, 3315-3321. (b) Rawji, G.; Hediger, M.; Milburn, R. M. *Inorg. Chim. Acta* **1983**, *79*, 247-248. (c) Milburn, R. M.; Tafesse, F. *Inorg. Chim. Acta* **1987**, *135(2)*, 119-122.
27. See section 1.1.E.a, Monoester.
28. Buckingham, D. A.; Keene, F. R.; Sargeson, A. M. *J. Am. Chem. Soc.* **1974**, *96(15)*, 4981-4983.
29. Shinar, H.; Navon, G. *Biochem. Biophys. Acta* **1974**, *334(2)*, 471-475.
30. Anderson, R. A.; Vallee, B. L. *Proc. Nat. Acad. Sci. U.S.A.* **1975**, *72(1)*, 394-397.
31. (a) Lincoln, S. F.; Stranks, D. R. *Aust. J. Chem.* **1968**, *21(7)*, 1733-1743. (b) Lincoln, S. F.; Stranks, D. R. *Aust. J. Chem.* **1968**, *21(7)*, 1745-1756.
32. Harrowfield, M. J.; Jones, D. R.; Lindoy, L. F.; Sargeson, A. M. *J. Am. Chem. Soc.* **1980**,

102(26), 7733-7741, and references therein.
33. Schmidt, W.; Taube, H. *Inorg. Chem.* **1963**, *2(4)*, 698-705.
34. Farrell, F. J.; Kjellstrom, W. A.; Spiro, T. G. *Science* **1969**, *164*, 320-321.
35. Steffens, J. J.; Sampson, E. J.; Siewers, I. J.; Benkovic, S. J. *J. Am. Chem. Soc.* **1973**, *95(3)*, 936-938.
36. (a) Bunton, C. A.; Ionescu, L. G. *J. Am. Chem. Soc.* **1973**, *95(9)*, 2912-2917. (b) Moss, R. A.; Swarup, S. *J. Am. Chem. Soc.* **1986**, *108(17)*, 5341-5342. (c) Moss, R. A.; Alwis, K. W.; Shin, J. S. *J. Am. Chem. Soc.* **1984**, *106(9)*, 2651-2655.
37. Okahata, Y.; Ihara, H.; Kunitake, T. *Bull. Chem. Soc. Japan* **1981**, *54(7)*, 2072-2078.
38. (a) Mochida, K.; Matsui, Y.; Ota, Y.; Arakawa, K.; Date, Y. *Bull. Chem. Soc. Japan* **1976**, *49(11)*, 3119-3123. (b) Breslow, R.; Doherty, J. B.; Guillot, G.; Lipsey, C. *J. Am. Chem. Soc.* **1978**, *100(10)*, 3227-3229.
39. Kaiser, E. T.; Lee, T. W. S.; Boer, F. P. *J. Am. Chem. Soc.* **1971**, *93(9)*, 2351-2353.
40. (a) Youngqurst, R. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1985**, *107(19)*, 5528-5529. (b) Dervan, P. B. *Science* **1986**, *232*, 464-471. (c) Hertzberg, R. P.; Dervan, P. B. *Biochem.* **1984**, *23(17)*, 3934-3945.
41. (a) Marky, L. A.; Blumenfeld, K. S.; Breslauer, K. J. *Nucleic Acids Res.* **1983**, *11(9)*, 2857-2870. (b) Zakrzewska, K.; Lavery, R.; Pullman, B. *Nucleic Acids Res.* **1983**, *11(24)*, 8825-8839. (c) Patel, D. J.; Canuel, L. L. *Proc. Natl Acad. Sci. U.S.A.* **1977**, *74(12)*, 5207-5211.
42. (a) Hertzberg, R. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, *104(1)*, 313-315. (b) Graham, D. R.; Marshall, L. E.; Reich, K. A.; Sigman, D. S. *J. Am. Chem. Soc.* **1980**, *102(16)*, 4919-4921.
43. Chu, B. C. F.; Orgel, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82(2)*, 963-967.
44. "Enzyme-catalysed Reactions", Gray, C. J., VNR, London, **1971**.
45. Hamawalt, P. C.; Cooper, P. K.; Ganesan, A. K.; Smith, C. A. *Ann. Rev. Biochem.* **1979**, *48*, 783-836.
46. Dreyer, G. B.; Dervan, P. B *Proc. Natl. Acad. Sci. U. S. A.* **1985**, *82(4)*, 968-972.
47. Barton, J. K.; Raphael, A. L. *Proc. Natl. Acad. Sci. U.S.A.*, **1985**, *82(19)*, 6460-6464.
48. Hashimoto, Y.; Lee, C. S.; Shudo, K.; Okamoto, T. *Tet. Lett.* **1983**, *24(14)*, 1523-1526.
49. Bjerrum, J.; Rasmussen, S. E. *Acta Chem. Scand.* **1952**, *6*, 1265-1284.
50. Chin, J.; Zou, X. *J. Am. Chem. Soc.* In press.
51. Schwarzenbach, G.; Boesch, J.; Egli, H. *J. Inorg. Nucl. Chem.* **1971**, *33(7)*, 2141-2156.
52. Chin, J.; Zou, X. *Can. J. Chem.* **1987**, *65(8)*, 1882-1884.
53. (a) Harrowfield, J. M.; Jones, D. R.; Lindoy, L. F.; Sargeson, A. M. *J. Am. Chem. Soc.* **1980**, *102(26)*, 7733-7741.
54. (a) Norman, P. R.; Cornelius, R. D. *J. Am. Chem. Soc.* **1982**, *104(9)*, 2356-2365. (b) Suruki, S.; Higashiyama, T.; Nakahara, A. *Bioinorg. Chem.* **1978**, *8*, 277. (c) Hediger, M.; Milburn, R. M. *ACS Symp. Ser. No. 171*, 211, **1981**.
55. (a) Bailar, Jr., J. C.; Auten, R. W. *J. Am. Chem. Soc.* **1934**, *56(4)*, 774-776. (b) Bailar, Jr., J. C.; Peppard, D. F. *J. Am. Chem. Soc.* **1940**, *62(4)*, 820-823. (c) Baldwin, M. E.; Chan, S. C.; Tobe, M. L. *J. Chem. Soc.* **1961**, 4637-4645.
56. (a) Sargeson, A. M.; Searle, G. H. *Nature* **1963**, *200*, 356-357. (b) Hay, R. W.; Norman, P. R. *J. Chem. Soc., Chem. Comm.* **1980**, 734-735. (c) Chan, S. C.; Tobe, M. L. *J. Chem. Soc.* **1962**, 4531-4540. (d) Martin, D. F.; Tobe, M. L. *J. Chem. Soc.* **1962**, 1388-1396. (f) Pearson, R. G.; Basolo, F. *J. Am. Chem. Soc.* **1956**, *78(19)*, 4878-4883.
57. (a) Pearson, R. G.; Meeker, R. E.; Basolo, F. *J. Am. Chem. Soc.* **1956**, *78(4)*, 709-713. (b) Basolo, F.; Pearson, R. G. "Mechanisms of Inorganic Reactions" John Wiley & Sons, N. Y., 1987.
58. Kruse, W.; Taube, H. *J. Am. Chem. Soc.* **1961**, *83(6)*, 1280-1284.
59. Sargeson, A. M.; Searle, G. H. *Inorg. Chem.* **1967**, *6(4)*, 787-796.
60. (a) Buckingham, D. A.; Mazilli, P. A.; Sargeson, A. M. *Inorg. Chem.* **1967**, *6(5)*, 1032-1041. (b) Sargeson, A. M.; Searle, G. H. *Inorg. Chem.* **1967**, *6(12)*, 2172-2180.
61. Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183-278.
62. Menger, F. M. *Acc. Chem. Res.* **1985**, *18(5)*, 128-134, and reference therein. .
63. Higuchi, T.; Eberson, L. Herd, A. D. *J. Am. Chem. Soc.* **1966**, *88(16)*, 3805-3808.
64. Different anionic ligands such as Cl^-, $H_2PO_4^-$, $SO_4^=$, NCS^-, H_2O and so on have been studied.

"Concepts and Models of Inorganic Chemistry", 2nd. Ed., Douglas, B. E.; McDaniel, D. H. Alexander, J. J., John Wiley & Sons, N. Y., **1983**

65. (a) Langford, C. H.; Gray, H. B. "Ligand Substitution Processes", Benjamin Inc., N. Y., **1965**. (b) Tobe, M. L. "Inorganic Reaction Mechanisms", Thomas Nelson and Son Ltd., Great Britain, **1972**.
66. Suh, J.; Han, O.; Chang, B. *J. Am. Chem. Soc.* **1975**, *108(8)*, 1839-1842.
67. Jencks, W. P.; Regenstein, J. "Handbook of Biochemistry and Molecular Biology" 3rd. Ed. Phy. Chem. Data *1*, 305, Fasman, G. D. Ed. CRC Press, Ohio, 1976.
68. Kirby, A. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87(14)*, 3209-3216.
69. Trippett, S. *Pure Appl. Chem.* **1974**, *40(4)*, 595-605.
70. Kirsch, J. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1964**, *86(5)*, 837-846.
71. Greengard, P.; Rudolph, S. A.; Sturtevant, J. M. *J. Biol. Chem.* **1969**, *244(17)*, 4798-4800.
72. Milburn, R. M.; Gautam-Basak, M.; Tribolet, R.; Sigel, H. *J. Am. Chem. Soc.* **1985**, *107(11)*, 3315-3321.
73. Rawji, G.; Milburn, R. M. *J. Org. Chem.* **1981**, *46(6)*, 1205-1206.
74. Prince, R. H.; Stotter, D. A.; Woolley, P. R. *Inorg. Chim. Acta* **1974**, *9(1)*, 51-54.
75. Bailar Jr., J. C. *Inorg. Syn.* **1960**, *2*, 222-225.
76. Tobe, M. L.; Martin, D. F. *Inorg. Syn.* **1966**, *8*, 198-202.
77. Crayton, P. H.; Mattern, J. A. *J. Inorg. Nucl. Chem.* **1960**, *13(3)*, 248-253.
78. Hay, R. W.; Norman, P. R. *J. Chem. Soc. Dalton Trans.* **1979**, 1441-1445.
79. Atkins, T. J.; Richman, J. E.; Oettle, W. F. *Org. Syn.* **1978**, *58*, 86-98.
80. Busch, D. H.; Olszanski, D. J.; Stevens, J. C.; Schammel, W. P.; Kojima, M.; Herron, N.; Zimmer, L. L.; Holter, K. A. *J. Am. Chem. Soc.* **1981**, *103(6)*, 1472-1478.
81. Koyama, H.; Yoshino, T. *J. Chem. Soc. Japan*, **1972**, *45(2)*, 481-484.
82. Hung, Y.; Busch, D. H. *J. Am. Chem. Soc.* **1977**, *99(15)*, 4977-4984.
83. BNPP and NPP in water. BDNPP in acetonitrile (final concentration of acetonitrile: 0.3-0.6%).
84. Watson, J. D.; Crick. F. H. C. *Nature* **1953**, 737-738.
85. For a historical review on the subject, see "Molecular Associations in Biology" Pallman, B., Ed., Academic, N.Y. 1968, Hoogstern, K. pp21-38, Ts'o, P. O. P. pp39-74.
86. "Biophysical Chemistry" Part 1, p155, Cantor, C. R.; Schimmel, P. R., Freeman, San Francisco, **1980**.
87. (a) Reference 3, p238. (b) "Biomolecular Stereodynamics", Vol. 1, p429, Turner, D. H.; Petersheim, M.; Albergo, D. D.; Dewey, T. G.; Freier, S. M., Adenine Press, Guilderland, N.Y., **1981**. (c) "Physical Chemistry of Nucleic Acids", Bloomfield, V. A.; Crothers, D. M.; Tinoco Jr., I., Harper and Row, NY, **1974**.
88. Young, M. A.; Krugh, T. R. *Biochem.* **1975**, *14(22)*, 4841-4847.
89. (a) Freier, S. M.; Burger, B. J.; Alkema, D. Neilson, T.; Turner, D. H. *Biochem.* **1983**, *22(26)*, 6198-6206. (b) Freier, S. M.; Alkema, D.; Sinclair, A.; Neilson, T.; Turner, D. H. *Biochem.* **1985**, *24(17)*, 4533-4539.
90. (a) Petersheim, M.; Turner, D. H. *Biochem.* **1983**, *22(2)*, 256-263. (b) Bubienko, E.; Cruz, P.; Thomason, J. F.; Borer, P. N. *Prog. Nucl. Acid Res. Mol. Biol.* **1983**, *30*, 41-90.
91. Borer, P. N.; Dengler, B.; Tinoco, I., Jr.; Uhlenbeck, O. C. *J. Mol. Biol.* **1974**, *86(4)*, 843-853. A detailed discussion and the evaluation of the methods are given in **Appendix 1**.
92. Lochmann, E. R.; Micheler, A. "Physico-chemical Properties of Nucleic Acids" Vol. *1*, 223-267, Duchesne, J. Ed. Academic Press, N.Y. **1973**.
93. For a review see Berman, H. M.; Young, P. R. *Ann. Rev. Biophys. Bioeng.* **1981**, *10*, 87-114.
94. (a) Dickinson, L.; Chantrill, D. H.; Inkley, G. W.; Thompson, M. J. *Brit. J. Pharmacol.* **1953**, *8(2)*, 139-142. (b) Newton, B. A. *Adv. Chemother.* **1964**, *1, 35-83.* (c) Wilson, W. D.; Jones, R. L. *J. Am. Chem. Soc.* **1986**, *108(22)*, 7113-7114.
95. (a) Manning, G. S. *Q. Rev. Biophys.* **1978**, *11*, 179-246. (b) Record, M. T., Jr.,; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* **1978**, 11, 103-178.
96. (a) Douthart, R. J.; Burnett, J. P.; Beasley, F. W.; Frank, B. H. *Biochem.* **1973**, *12(2)*. 214-220. (b) Waring, M. J. *J. Mol. Biol.* **1965**, *13(1)*, 269-282. (c) Le Pecq, J. B. "Methods of Biochem. Anal." **1971**, *20*, 41-86.

97. Pope, L. E.; Sigman, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81(1)*, 3-7. (b) Cartwright, I. L.; Hertzberg, R. P.; Dervan, P. B.; Elgin. S. C. R. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80(11)*, 3213-3217. (c) Ephrussi, A.; Church, G. M.; Tonegawa, S.; Gilbert, W. *Science* **1985**, *227*, 134-140.
98. (a) Patel, D. J.; Cannel, L. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74(7)*, 2624-2628. (b) Coddington, J. M.; Alkema, D.; Bell, R. A.; Hughes, D. W.; Neilson, T. *Chem. Biol. Interactions* **1984**, *50*, 97-100. (c) Lerman, L. S. *J. Mol. Biol.* **1961**, *3(1)*, 18-30.
99. Barton, J. K.; Raphael, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82(19)*, 6460-6464.
100. Spassky, A.;Sigman, D. S. *Biochem.* 1985, 24(27), 8050-8056
101. Mei, H. Y.; Barton, J. K. *J. Am. Chem. Soc.* **1986**, *108(23)*, 7414-7416.
102. Pinto, A. L.; Lippard, S. J. *Biochem. Biophys. Acta* **1985**, *780(3)*, 167-180.
103. "Platinum Coordination Compounds in Cancer Chemotherapy" Hacker, M. P.; Douple, E. B.; Krakoff, I. H., Eds., Nijhoff, Boston, **1984**.
104. Barton, J. K. *Science* **1986**, *233*, 727-734.
105. (a) Knorre, D. G.; Vlassov, V. V. *Prog. Nucl. Acid Res. Mol. Biol.* **1985**, *32*, 291-321. (b) Reference 86, p163.
106. Koziolkiewicz, M.; Uznanski, B.; Stec, W. J. *Chemica Scripta* **1985**, **26**, 251-260.
107. (a) Mertes, M. P.; Coats, E. A. *J. Med. Chem.* **1969**, *12(1)*, 154-157. (b) Tittensor, J. R. *J. Chem. Soc. (C)*, **1971**, 2656-2662.
108. (a) Coull, M. J.; Carlson, D. V.; Weith, H. L. *Tet. Lett.* **1987**, *28(7)*, 745-748. (b) Mungall, W. S.; Kaiser, J. K. *J. Org. Chem.* **1977**, *42(4)*, 703-706.
109. (a) Gait, M. J.; Jones, A. S.; Walker, R. T. *J. Chem. Soc. Perkin I*, **1974**, 1684-1686. (b) Gait, M. J.; Jones, A. S.; Jones, M. D.; Shepherd, M. J.; Walker, R. T. *J. Chem. Soc. Perkin I*, **1979**, 1289-1294.
110. (a) Letsinger, R. L.; Mungall, W. S. *J. Org. Chem.* **1970**, *35(11)*, 3800-3803. (b) Nemer, M. J.; Ogilvie, K. K. *Tet. Lett.* **1980**, *21*, 4149-4152.
111. (a) Ogilvie, K. K.; Cormier, J. F. *Tet. Lett.* **1985**, *26(35)*, 4159-4162. (b) James F. Cormier's PhD thesis, McGill University.
112. (a) Miller, P. S.; Yano, J. Yano, E.; Carroll, C.; Jayaraman, K.; Ts'o, P. O. P. *Biochem.* **1979**, *18(23)*, 5134-5143. (b) Jayaraman, K.; McParland, K.; Miller, P.; Ts'o, P. O. P. *Proc. Nalt. Acad. Sci. U. S. A.* **1981**, *78(3)*, 1537-1541.
113. (a) Overberger, C. G.; Inaki, Y. *J. Polym. Sci. Polym. Chem. Ed.* **1979**, *17*, 1739-1758. (b) Inaki, Y.; Sakuma, Y.; Suda, Y; Takemoto, K. *J. Polym. Sci. Polym. Chem. Ed.* **1982**, *20*, 1917-1933.
114. Maggiora, L.; Boguslawski, S.; Mertes, M. P. *J. Med. Chem.* **1977**, *20(10)*, 1283-1287
115. Shimidzu, T.; Murakami, A.; Konoshita, Y.; Minami, M. *Bull. Chem. Soc. Japan* **1987**, *51(3)*, 821-825.
116. Seita, T.; Yamauchi, K.; Kinoshita, M.; Imoto, M. *Die Makromolekulare Chemie* **1973**, *164(1)*, 15-23.
117. Lira, E. P.; Huffman, C. W. *J. Org. Chem.* **1966**, *31(7)*, 2188-2191.
118. Ueda, T.; Fox, J. J. *J. Org. Chem.* **1964**, *29(7)*, 1762-1769.
119. Sakakibara, S.; Inukai, N. *Bull. Chem. Soc. Japan* **1964**, *37(8)*, 1231-1232.
120. Brook, M. A.; Chan, T. H. *Synthesis* **1983**, 201-203.
121. Kamber, M.; Just, G. *Can. J. Chem.* **1985**, *63(4)*, 823-827.
122. (a) Bergmann, M.; Zervas, L. *Ber.* **1932**, *65*, 1192-1202. (b) Boissonnas, R. A.; Preitner, G. *Helv. Chim. Acta* **1953**, *36(4)*, 875-886.
123. Jeffrey, P. D.; McCombie, S. W. *J. Org. Chem.* **1982**, *47(3)*, 587-590.
124. (a) Ben-ishai, D.; Berger, A. *J. Org. Chem.* **1952**, *17(12)*, 1564-1570. (b) Ben-ishai, D. *J. Org. Chem.* **1954**, *19(1)*, 62-66.
125. Brahms, J.; Maurizot, J. C.; Michelson, A. M. *J. Mol. Biol.* **1967**, *25(3)*, 465-480.
126. Podder, S. K. *Indian J. Biochem. Biophys.* **1971**, *8*, 239-246.
127. (a) Applequist, J.; DamLe, V. *J. Am. Chem. Soc.* **1965**, *87(7)*, 1450-1458. (b) Crothers, D. M.; Kallenbach, N. R.; Zimm, B. H. *J. Mol. Biol.* **1965**, *11(4)*, 802-820.

To my parents

To my wife