A KINETIC AND PROTON NMR STUDY OF O2- AND O4-METHYLATED NUCLEOSIDES AND THEIR HYDROLYSIS PRODUCTS

A Thesis

Submitted to

The Faculty of Graduate Studies and Research

at the University of Manitoba

in Partial Fulfillment

of the Requirements for the Degree of

MASTER OF SCIENCE

by

BRIAN D. ALLORE

Winnipeg, Manitoba

October, 1982

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I am forever grateful to my parents and family for their continued support in all that I do.

I am grateful to the Department of Chemistry and the University of Manitoba for financial support.

All things in the glory of our Lord and God.

ABSTRACT

A series of O-methylated pyrimidine 2'-deoxyribonucleosides, m^2dU, m^4dU, m^2dT and m^4dT, were synthesized. These modified nucleosides, as well as the ribosides m^2U and m^4U, were used in kinetic and proton nuclear magnetic resonance studies. The ^1H NMR studies have shown the trends in the chemical shifts (δ) for the base which result from O-alkylation. These data are useful for the identification of alkylation products from nucleic acids as the general trends in chemical shifts observed in the methylated nucleosides would be expected to be similar for other alkylated nucleic acid products. An examination of proton chemical shifts as a function of pH was carried out to evaluate the ionization behaviour of the O-methylated molecule.

Kinetic studies of the acid and alkaline hydrolysis of the modified nucleosides were carried out and pH profiles obtained. The hydrolysis of the O-methylated nucleosides in alkaline solutions suggest a direct attack of hydroxide ion on the nucleoside resulting in demethylation of the O-alkyl group. From these data k_{OH^-} values have been calculated. In acid solutions, m^4U, m^4dU, m^4dT and m^2U were shown to undergo demethylation and, from the results, approximate pK_a values were obtained. The 2'-deoxyribosides m^2dU and m^2dT were shown to undergo depyrimidination in acid solutions to form the O^2-methylated

base and free sugar. The kinetic NMR investigation of the depyrimidination reactions suggests the hydrolysis to proceed via a preequilibrium base protonation followed by cleavage of the N-glycosyl bond. Again the data was useful in determining approximate pK_a values. The O-methylated nucleosides were shown to be stable in the pH range 3-12 with no hydrolysis observed after a period of several days.

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Listed below are the abbreviations and the symbols used for the molecules of major importance in this thesis.

		Abb.	Symbol
1.	Uridine	U	
2.	2'-deoxythymidine	dT	
3.	2'-deoxyuridine	dU	
4.	O^4-methyluridine	m^4U	●
5.	O^4-methyl-2'-deoxyuridine	m^4dU	○
6.	O^4-methyl-2'-deoxythymidine	m^4dT	◉
7.	O^2-methyluridine	m^2U	■
8.	O^2-methyl-2'-deoxyuridine	m^2dU	□
9.	O^2-methyl-2'-deoxythymidine	m^2dT	▣
10.	uracil base	Ura	
11.	thymine base	Thy	

For Lynn

I. INTRODUCTION

A. ALKYLATION OF NUCLEOSIDES

The subject of modified nucleosides has been an area of research interest for many years[1,2,3]. The many different types of alterations to the base and sugar moieties which may occur have both chemical and biological significance. Of primary concern in this thesis is the alkylation of pyrimidine bases. Structures of the eight common nucleosides are shown in Figure 1.

Nucleosides are amongst the most versatile and essential of biomolecules. Joined together through a phosphate link between the sugar moieties, these molecules are the building blocks for nucleic acids which are involved in the storage and transmission of genetic information.

The alkylation of nucleic acids may affect the hydrogen bonding ability of the bases and it has been suggested that such modifications may lead to mutagenesis and carcinogenesis. Initial interest was focussed on N(7)-methylguanine (m^7G),[4-8] Figure 2, which had been isolated by Loveless from acid hydrolysates of treated DNA[9]. Some doubt was cast on the significance of m^7G in mutagenesis by Shooter *et al.*[6] where m^7G in bacteriophage RNA did not alter its hydrogen bonding properties. Minor alkylation products such as N(1)-methyladenosine (m^1A) and N(3)-methylcytidine (m^3C), Figure 2, in which the modification involves a

FIGURE 1

Common nucleosides:

I. Uridine, U.

II. Thymidine, dT.

III. Cytidine, C.

IV. 2'-Deoxycytidine, dC.

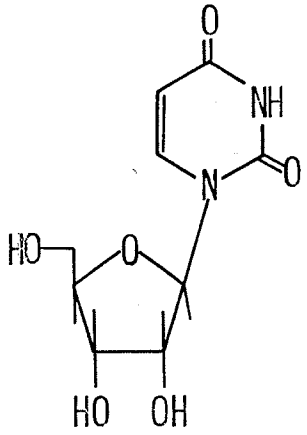
V. Guanosine, G.

VI. 2'-Deoxyguanosine, dG.

VII. Adenosine, A.

VIII. 2'-Deoxyadenosine, dA.

I



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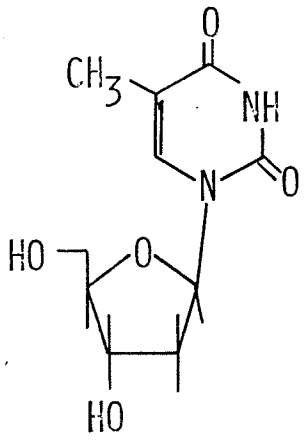
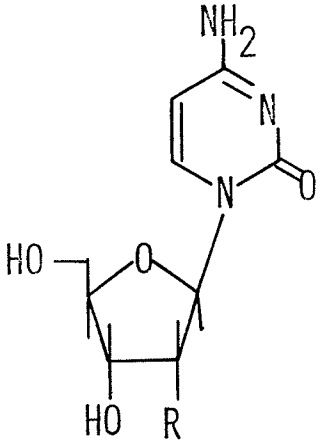
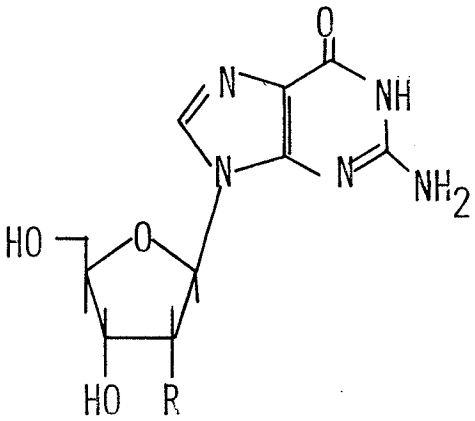




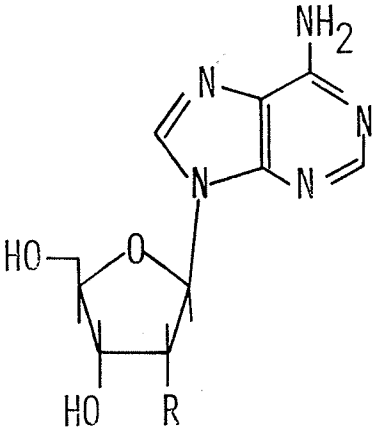
III, R = OH

IV , R = H



V, R = OH

VI, R = H



VII, R = OH

VIII, R = H

FIGURE 2

Alkylated nucleosides:

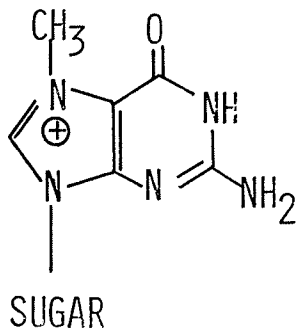
I. N(7)-methylguanosine, m^7G.

II. N(1)-methyladenosine, m^1A.

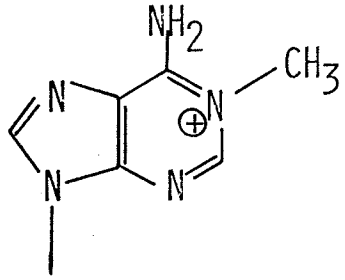
III. N(3)-methylcytidine, m^3C.

IV. O^6-methylguanosine, m^6G.

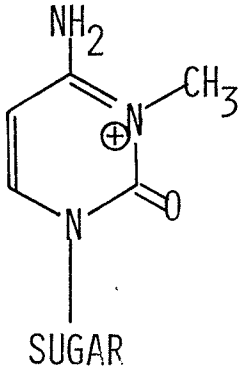
I


CH3
O
N
NH
N
N
NH2
SUGAR

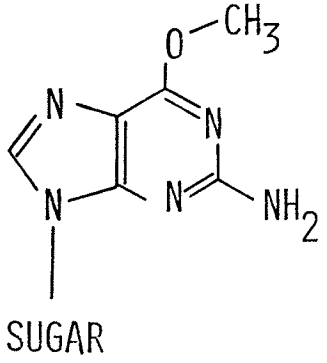
II


NH2
CH3
N
N
N
N
SUGAR

III


NH2
CH3
N
O
N
SUGAR

IV


O
CH3
N
N
N
N
NH2
SUGAR

Watson-Crick hydrogen bonding site, were shown to have ambiguous base pairing properties[10,11].

Initially it was believed that alkylation occurred only on the base nitrogens but this was eventually shown to be incorrect. Failure to isolate O-methylated nucleosides was due to hydrolysis of these alkylation products under the harsh isolation procedures used in early work. Friedman et al.[12,13], using milder experimental conditions, were able to isolate O^6-methylguanosine (m^6G) as a product of the reaction of guanosine with diazomethane. Upon alkylation, the m^6G base is no longer expected to show base pairing specificity with the cytosine base, but the formation of an m^6G-T complex involving a pair of hydrogen bonds can be visualized[9,14]. In fact, when used in a template for RNA polymerase, m^6G was shown to cause mispairing and it has been suggested that G:C→A:T transitions may occur, Figure 3[15-17].

Interest in the O-alkylation of pyrimidine nucleosides is also developing[2]. Using 1-methyluracil, Wong et al.[18] observed the formation of 1,O^4-dimethyluracil upon treatment with diazomethane. Subsequently the isolation of both O^2- and O^4-methylthymidine (m^2dT, m^4dT), Figure 4, led to the suggestion that O-alkylation of the thymine (or uracil) base would destroy the base pairing for adenine since a pair of hydrogen bonds can no longer be formed[3,19-21]. A new specificity for the G base seems possible because a pair of hydrogen bonds could form between

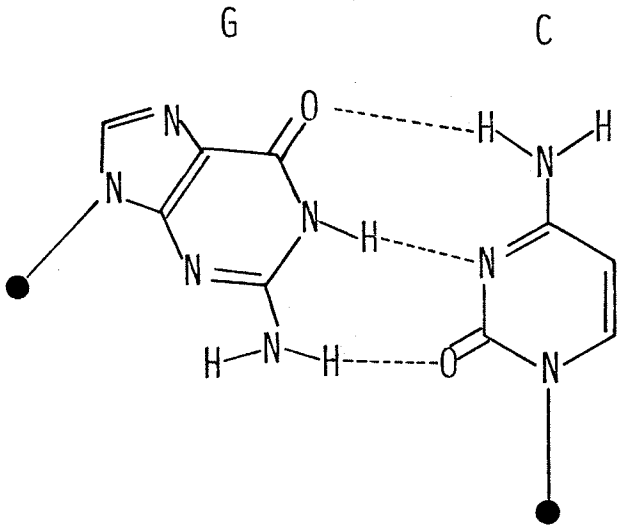
FIGURE 3

Proposed base pair mutation as a result of O-alkylation of G.

I. Normal Watson-Crick G≡C base pair.

II. Proposed base pair between m^6G and dT.

I



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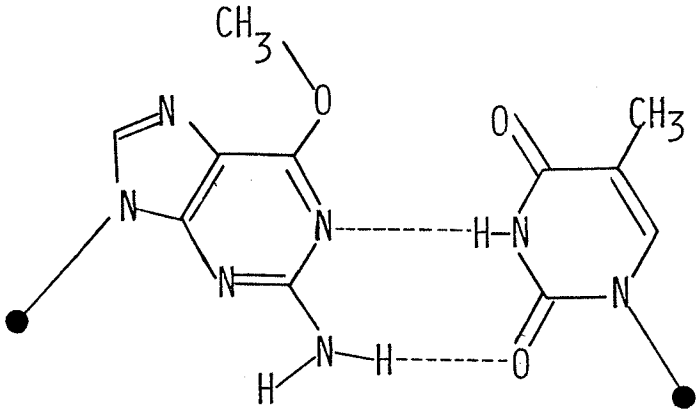


$_{m}{}^{6}G$ dT

FIGURE 4

O-Methylated pyrimidine nucleosides.

I. O^4-methyluridine, m^4U.

II. O^2-methyluridine, m^2U.

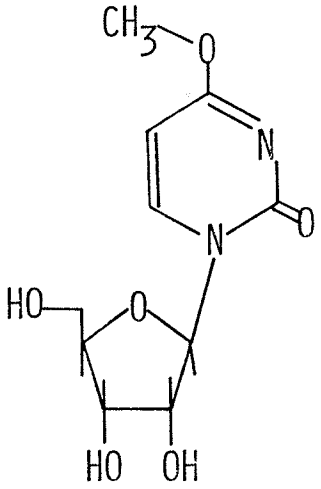
III. O^4-methyl-2'-deoxyuridine, m^4dU.

IV. O^2-methyl-2'-deoxyuridine, m^2dU.

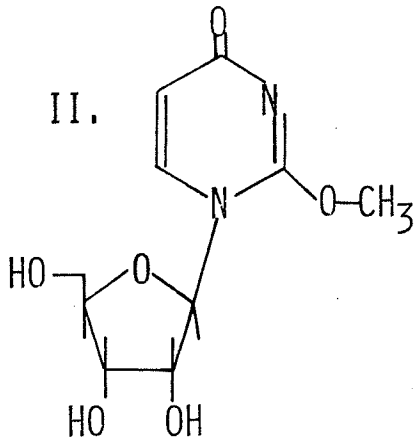
V. O^4-methylthymidine, m^4dT

VI. O^2-methylthymidine, m^2dT.

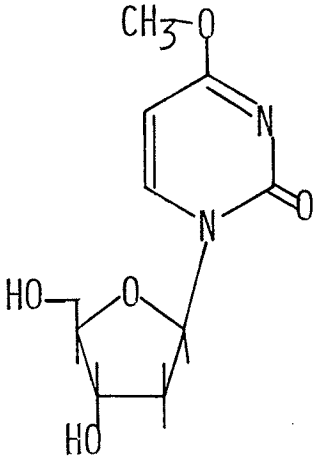
I.



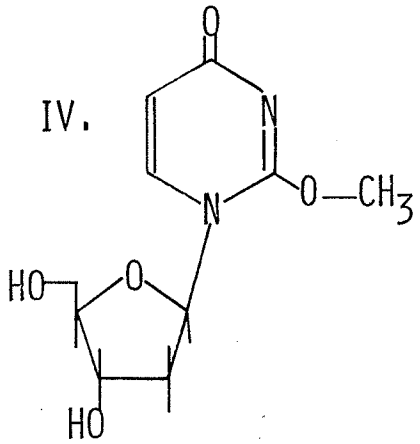
II.



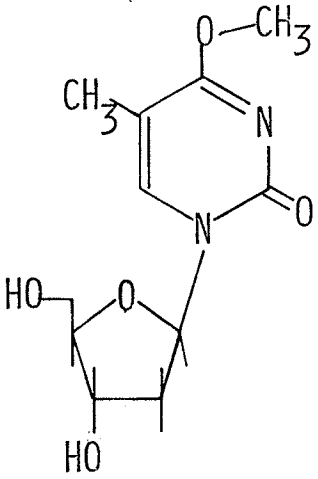
III.



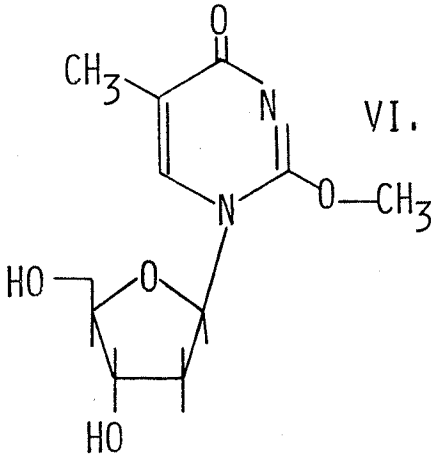
IV.



V.



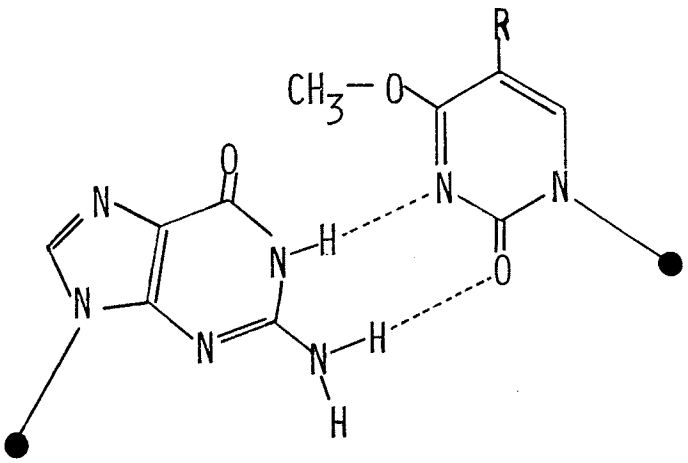
VI.



an O^2- or O^4-methylthymine and guanine, Figure 5[22]. However, it may be important to consider the steric effects of the bulky methyl group since it may influence the base stacking and conformational properties of the nucleosides resulting in the observed ambiguous base pairing[23-27].

FIGURE 5

Proposed base pairing as a result of O^4-methylation of U(R=H) or dT($R=CH_3$). Two hydrogen bonds are possible between m^4U or m^4dT and G.


R
CH3
O
O
N
N
N
H
N
N
N
H
O
N
H

B. STABILITY AND HYDROLYSIS OF NUCLEOSIDES

Investigation of the stability of purine and pyrimidine nucleosides is a continuing study [28-30]. Despite the research effort in this work the mechanism for the acid catalyzed hydrolysis of the N-glycosyl bond has not been totally resolved. A detailed knowledge of this mechanism is important since hydrolysis in acid is a standard degradation technique for determining the base composition and sequence in polynucleotides[31,32]. Two principal pathways have been suggested, these will be discussed below.

1. Hydrolysis of the N-Glycosyl Bond via a Schiff-Base Intermediate

In this suggested mechanism, rapid protonation of the ring oxygen of the sugar (II) preceeds formation of a Schiff-base, Figure 6 [33-35]. Ring opening of the sugar is rate determining and leads to the Schiff-base intermediate (III). This intermediate may react by two different pathways;

(1) hydrolysis of the N-glycosyl bond to produce the free base and sugar (V) (Pathway A) or,

(2) ring closure of the C1' carbonium ion (VI) to generate α and β furanose and pyranose nucleosides (Pathway B).

This mechanism was initially proposed because of the structural similarity to glycosylamines which are believed to hydrolyze via a Schiff-base[36], Figure 7(III), but there was little

FIGURE 6

Acid catalyzed hydrolysis of the N-glycosyl bond via a Shiff base intermediate.

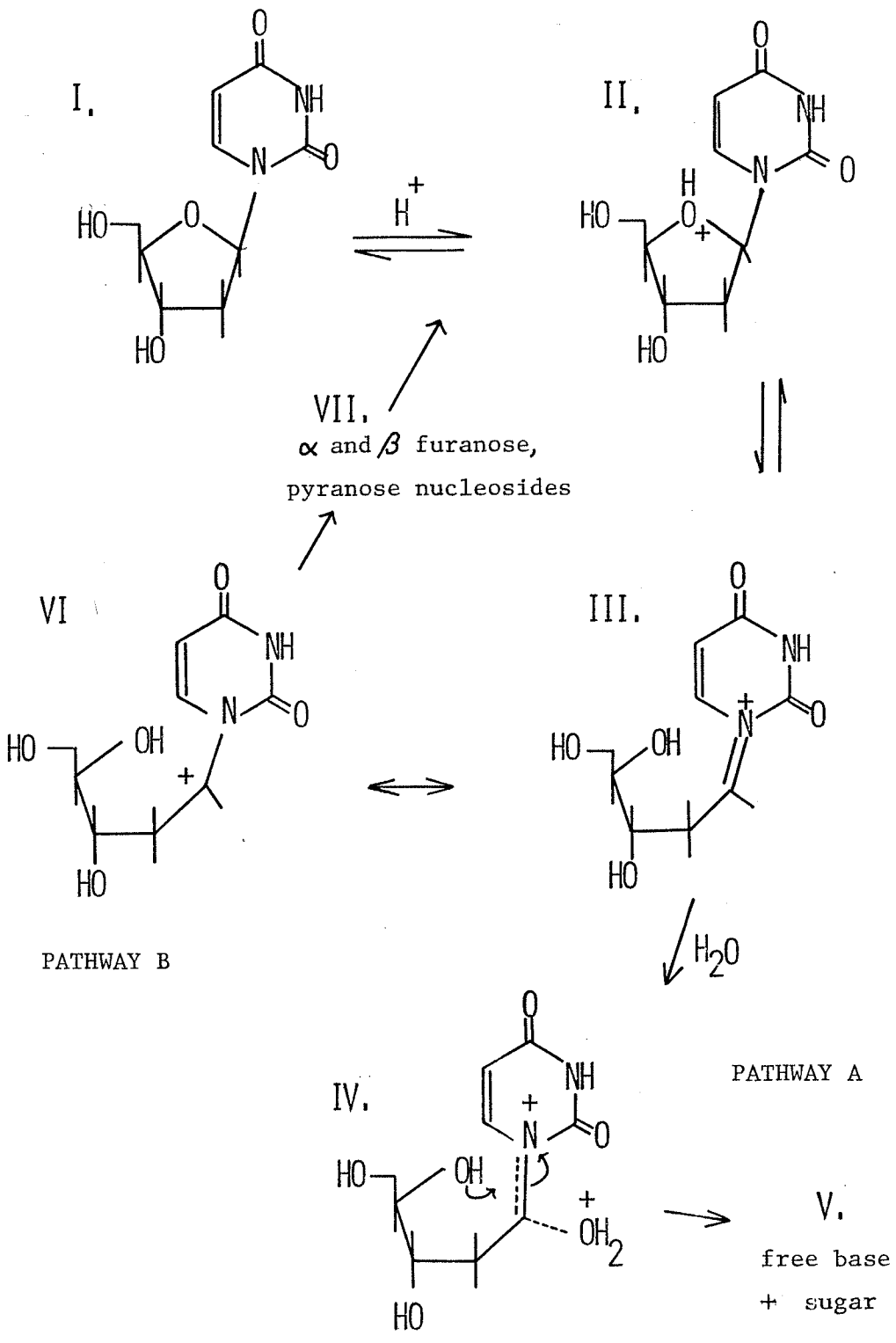
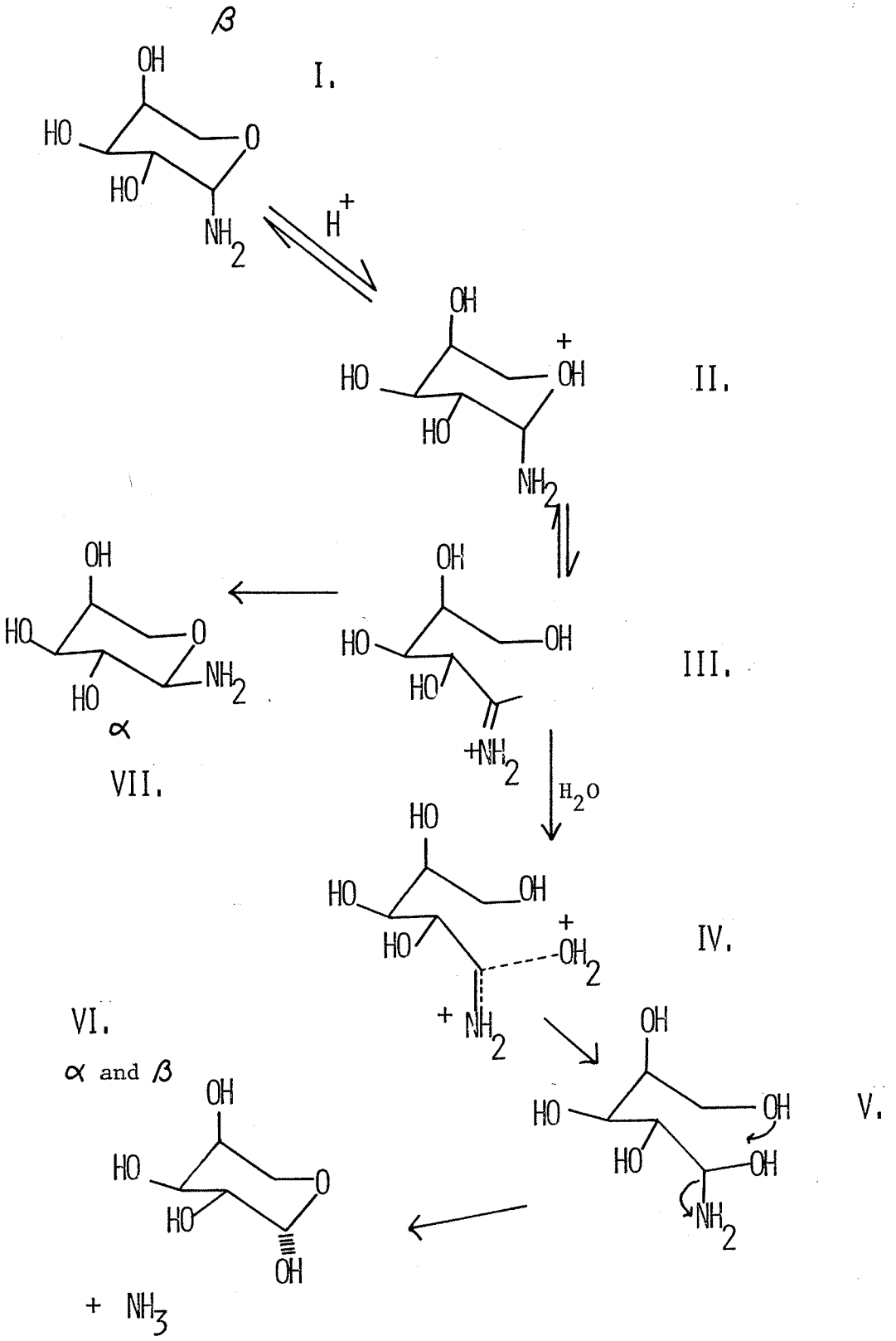

I.
II.
VII.
α and β furanose,
pyranose nucleosides
VI
III.
PATHWAY B
PATHWAY A
IV.
V.
free base
+ sugar

FIGURE 7

Acid catalyzed hydrolysis of glycosylamines.


β
I.
OH
HO
O
HO
NH2
H+
OH
+
OH
HO
HO
NH2
II.
OH
HO
O
NH2
HO
α
VII.
OH
HO
OH
HO
+NH2
III.
H2O
HO
HO
OH
+
OH2
+ NH2
IV.
VI.
α and β
OH
HO
O
HO
OH
+ NH3
OH
HO
OH
HO
OH
NH2
V.

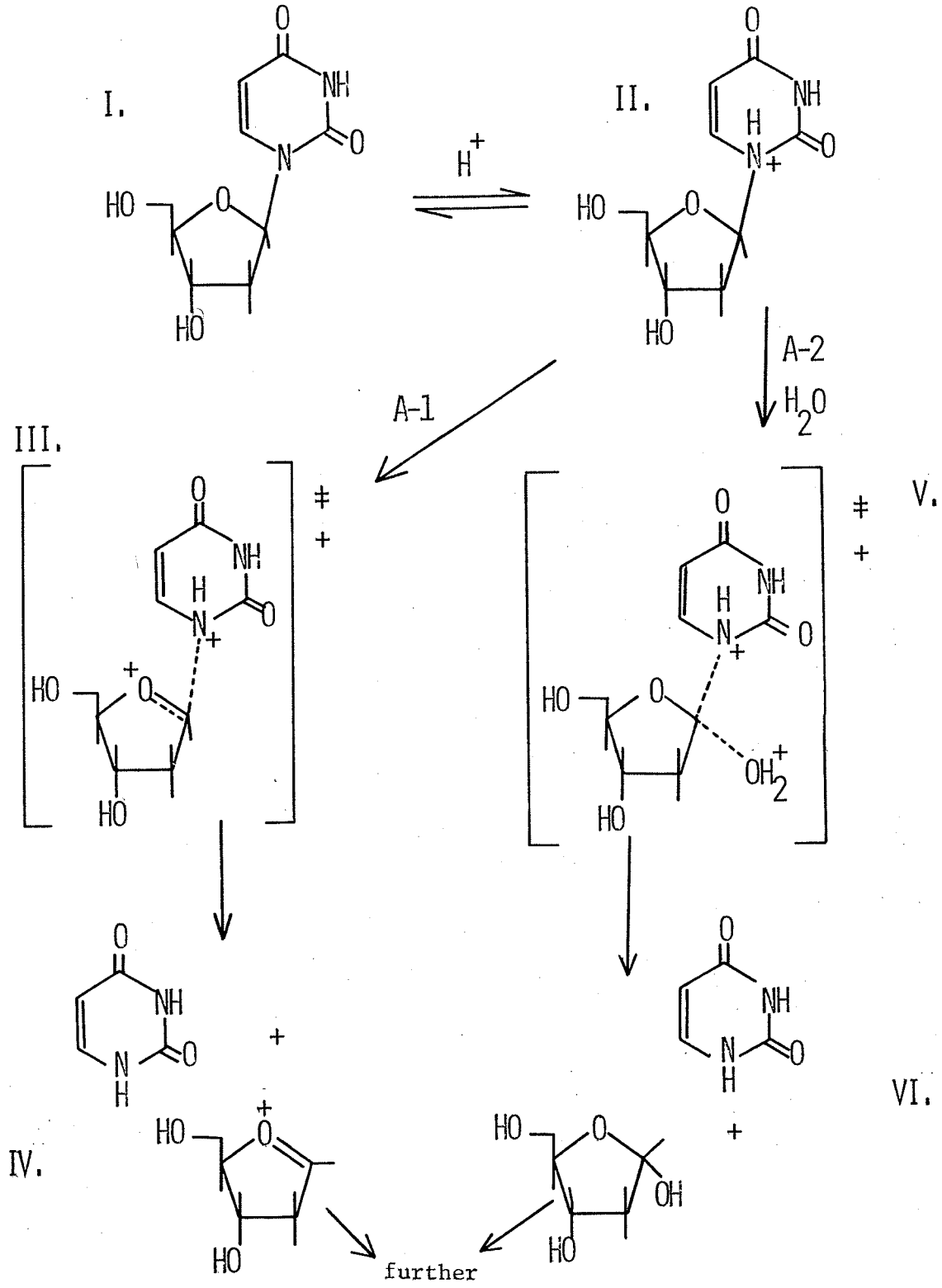
experimental evidence to support this proposition.

2. Hydrolysis of the N-Glycosyl Bond via Base Protonation

This mechanism, Figure 8, postulates the initial protonation of a nitrogen of the base moiety (II) which labilizes the N-glycosyl bond[37,38]. Cleavage of the N-glycosyl bond follows via either an A-1 or A-2 pathway. The A-1 mechanism is a unimolecular reaction in which the rupture of the N-glycosyl bond results in formation of the free base and a positively charged cyclic sugar (IV). In the bimolecular A-2 mechanism, water aids in the cleavage of the N-glycosyl bond (V). Both of the reaction products are neutral (VI).

FIGURE 8

Acid catalyzed cleavage of the N-glycosyl bond via base protonation.


I.
II.
H+
A-2
H2O
A-1
III.
V.
IV.
VI.
further
degradation

C. INVESTIGATION INTO THE MECHANISM OF NUCLEOSIDE HYDROLYSIS

1. Proof of the Schiff-base pathway required the isolation of α and β, furanose and pyranose nucleosides from the acid hydrolysis of a natural nucleoside. Results from the majority of studies do not contain evidence for the formation of α and β, furanose and pyranose nucleosides and, therefore, the Schiff-base process does not seem to be a viable pathway. However, one isolated case has been reported in which the treatment of dU and dT with perchloric acid led to the isolation of the different anomers and isomers of the pyrimidine nucleosides[39]. All remaining evidence presented here supports hydrolysis by base protonation.

2. The pH-rate profiles for acid hydrolysis of nucleosides are sigmoidal and accommodate well the base protonation pathway [40]. They do not at all resemble the profiles of glycosylamines which give bell-shaped curves with well-defined maxima [35,41].

3. Stability of the N-glycosyl bond has shown a dependance on the sugar structure[28,34,42]. The 2'-hydroxyl group, being an equal number of bonds away from the sugar ring oxygen and the base ring nitrogen, would be expected to decrease the basicity of both sites to a similar extent [43]. As a result, a comparison of hydrolysis rates for the ribo- and 2'-deoxyribonucleosides would not preferentially favour either of the proposed mechanisms.

Information obtained from the hydrolysis of a 3'-deoxyribonucleoside is useful when compared with similar results for the 2'-deoxy analog. In studies by Garrett and Mehta[43], they suggest that a 2'-deoxy- and a 3'-deoxyribose sugar would affect the rate of hydrolysis of the nucleoside to a similar extent if protonation occurred on the sugar ring oxygen. Their results, however, showed only a slight rate increase for the 3'-deoxyribonucleoside when compared with results for the ribonucleoside, the rate increase for the 2'-deoxy compound was a factor of several hundred times. These data are consistant with the hydrolysis of the N-glycosyl bond by base protonation rather than a Schiff-base process.

Other studies[37,38] which have examined the question of acid hydrolysis of nucleosides suggest the hydrolysis mechanism to proceed by base protonation. The isolated case by Cadet and Teoule[39] suggests that the Schiff-base mechanism might occur, however, special experimental conditions are required.

A secondary consideration, in the hydrolysis of nucleosides, is the role of water in the transition state leading to cleavage of the N-glycosyl bond, Figure 8. Studies of the hydrolysis of alkyl and aryl glycosides showed that unimolecular processes (A-1) were associated with positive entropies ($\Delta S^{\ddagger}$) whereas bimolecular systems (A-2) had negative $\Delta S^{\ddagger}$ [29]. Shapiro et al.[29] reported entropy of activation values for dU derivatives which were positive and compatible with an A-1 mechanism.

D. STABILITY OF O-ALKYLATED NUCLEOSIDES

Examination of the stability of O-alkylated nucleosides was the primary aim of this thesis. Singer et al.[44,45] have carried out similar work on O-ethylated pyrimidine nucleosides.

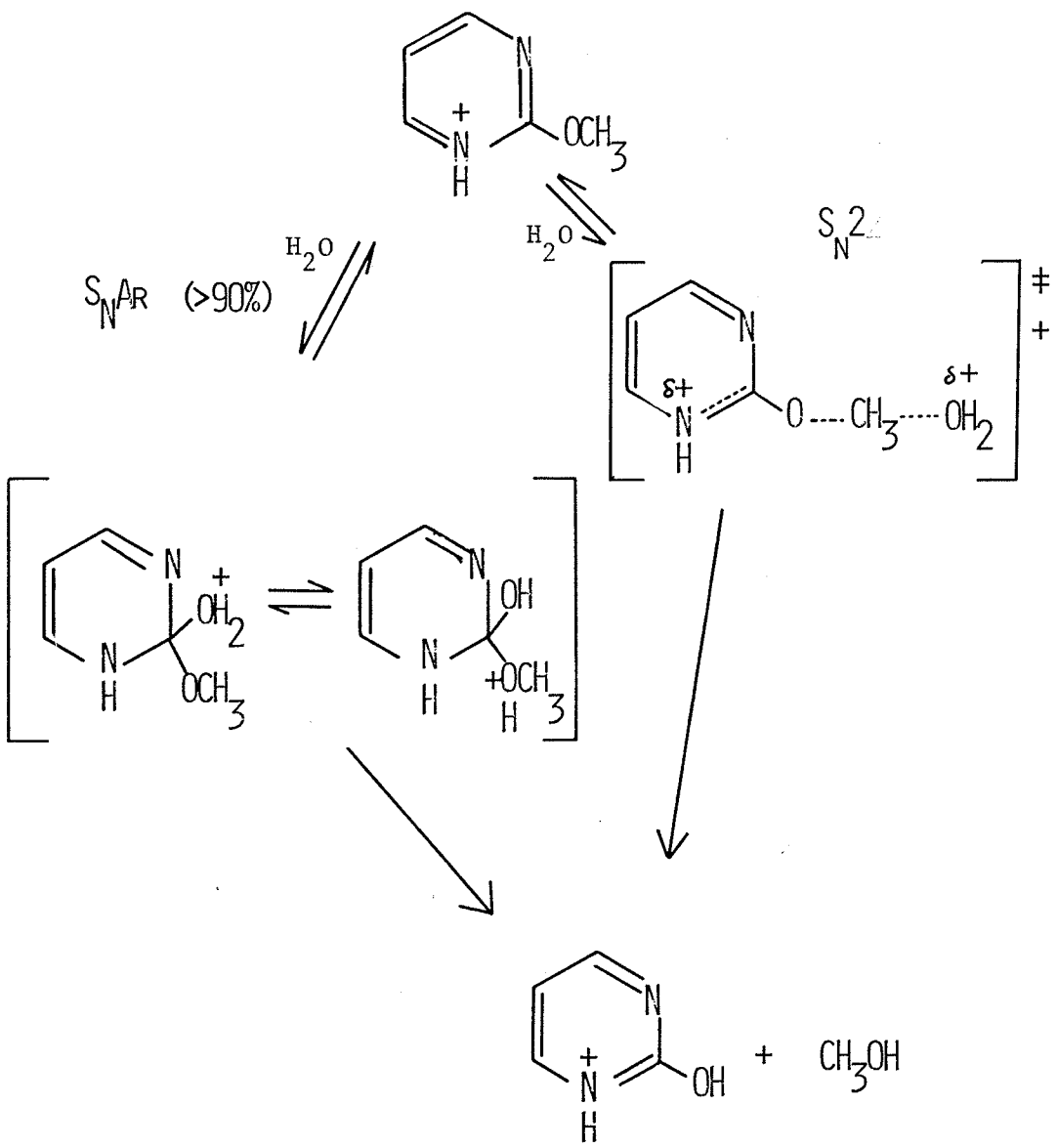
Their results suggest that the site of alkylation and the type of sugar have a marked effect on the stability of the nucleoside[44]. For both the ribose and the 2'-deoxyribonucleoside alkylation of the O^4-position did not affect the stability of the N-glycosyl bond. In acid and alkaline medium, the hydrolysis of the O^4-ethyl group was observed as the major reaction.

O^2-Alkylation of the 2'-deoxyribonucleosides was shown to labilize the N-glycosyl bond to acid and alkaline hydrolysis[44]. For the analogous O^2-alkylated ribonucleoside, Et^2U, similar experimental conditions resulted in both depyrimidination and dealkylation, the extent to which each process occurred varied with pH. By comparison of reaction half-lives, the preferred routes in acid and alkaline medium were; for O^2-alkylated 2'-deoxyribonucleosides, depyrimidination and, for O^2-alkylated ribonucleosides, dealkylation.

The study by Daniels et al.[30] of the dealkylation of O^2-methoxypyrimidine in acid media is a useful model system for comparison with nucleosides. Their results, Figure 9, indicated that the removal of the methoxy group proceeded primarily (>90%) by an S_NAr pathway with a minor contribution from an S_N2 route.

FIGURE 9

Acid catalyzed dealkylation of 2-methoxy-pyrimidine.

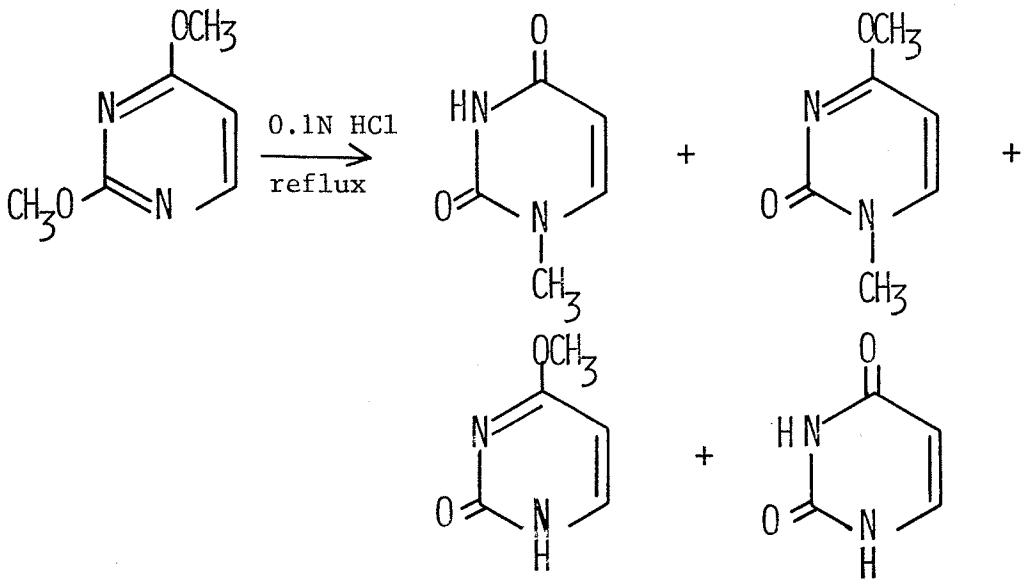

S_NAR (>90%)
H2O
H2O
S_N2
δ+
δ+
+OCH3
CH3OH

Wong et al.[46], carrying out similar work with 2,4-dialkoxypyrimidine, showed that both dealkylation and isomerization occurred in aqueous acid, Figure 10(I). Isomerization was attributed to the combined effect of the high concentration of base used and reflux temperatures. The hydrolysis mechanism was in agreement with that proposed by Daniels et al.[30] for 2-methoxypyrimidine. Refluxing of 2,4-dialkoxypyrimidine in alkaline solution resulted only in hydrolysis, with hydroxide ion showing a selectivity towards the 4-position, Figure 10(II)[46]. Similar product ratios were observed for 2,4-dialkoxy-5-methylpyrimidine suggesting that the 5-methyl substituent does not sterically hinder the attacking nucleophile. The total alkaline hydrolysis to either uracil or thymine was found to be slow as a result of the stability of the 2-alkoxy group. Displacement of the alkoxy group was shown to proceed exclusively via S_NAr. The attack by hydroxide ion is believed to be influenced by electronic repulsion from the two nitrogen atoms adjacent to the 2-position, this effect resulting in the stability of the 2-alkoxy group.

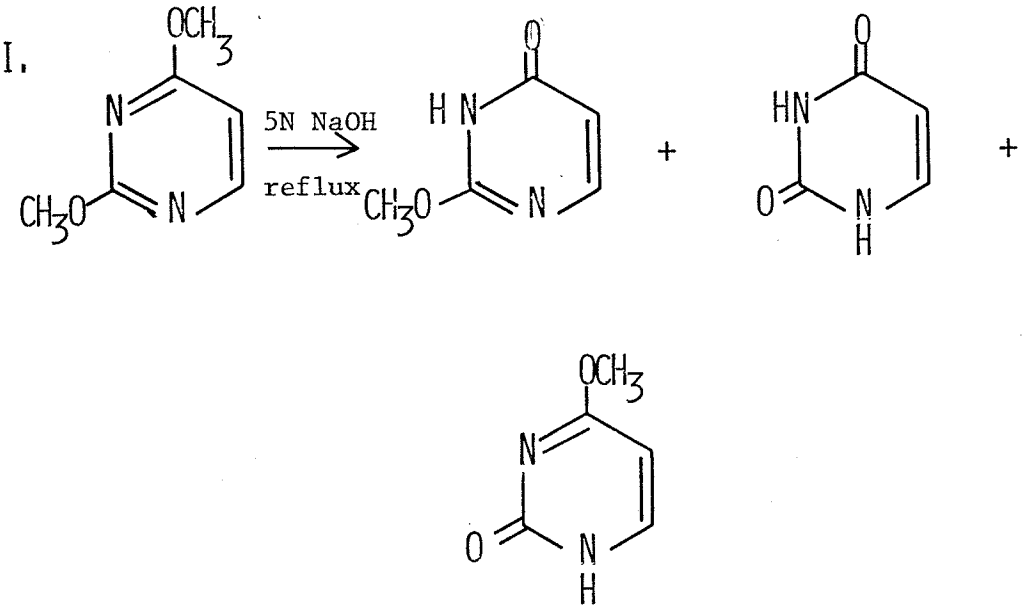
FIGURE 10

The hydrolysis and isomerization of 2,4-dimethoxypyrimidine in aqueous acid and alkaline solutions.

I.


OCH3
N
CH3O
N
0.1N HCl
reflux
O
HN
O
N
CH3
+
OCH3
N
O
N
CH3
+
OCH3
N
O
N
H
+
O
HN
O
N
H

II.


OCH3
N
CH3O
N
5N NaOH
reflux
O
HN
CH3O
N
+
O
HN
O
N
H
+
OCH3
N
O
N
H

E. NATURE OF THE PROBLEM

The study of the effects of alkylation of nucleic acids is an expanding field and its possible biological significance has been well documented[1,2]. It is of interest to study alkylated nucleosides incorporated into oligomer strands in an effort to better understand the effects of the modification on the stacking and H-bonding properties of nucleic acids.

In order to study the O-alkylated pyrimidine nucleosides at the oligomer level, a more complete understanding of the monomer nucleosides is necessary. Work by Singer et al.[44] has examined the stability of O-ethylated pyrimidine nucleosides. However, a more extensive study was undertaken in this work to determine the pH profile of the O-methylated nucleosides m^4U, m^4dU, m^4dT, m^2U, m^2dU and m^2dT. The results from this work will be useful in determining pK_a values for the methylated nucleosides and, by comparison with Singers work[44], will indicate the effect of changing the O-alkyl group from a methyl group to an ethyl group.

Identification of the effects of O-alkylation of pyrimidine nucleosides on the proton chemical shifts in NMR work would be a useful tool for the characterization of modified nucleic acids. We have, therefore, also undertaken the ^1H NMR study of the O-methylated pyrimidine nucleosides to determine the

effects of O-alkylation on the base proton chemical shifts.

CHAPTER II

CHAPTER II. EXPERIMENTAL METHODS

A. MATERIALS

1. REAGENTS AND CHEMICALS

The nucleosides used in this study were purchased from Sigma Chemical Co. All blocked nucleosides and the O^2 and O^4-methylated dU and dT were synthesized by procedures outlined below. The m^2- and m^4-U were synthesized in our lab by Wayne J. P. Blonski and Gilbert G. Prive. The commerical sources for other synthetic reagents have been given in the synthetic procedures.

The D_2O (99.8%) used in the NMR studies was purchased from Stohler Isotope Chemicals. The DCl and NaOD were purchased from Merck, Sharp and Dohme Canada Ltd.

Solutions used in UV kinetic studies were prepared from DILUT-IT analytical concentrate (HCl, NaOH) by J. T. Baker Chemical Co. The ionic strength for all solutions was maintained at 2N with NaCl.

Dry DMF was prepared by reflux of reagent grade DMF over purified calcium hydride (Fisher) and then distilled at atmospheric pressure directly into a reagent bottle containing molecular sieves.

Dry pyridine was prepared by refluxing technical grade

pyridine over chlorosulphonic acid (12 ml/l) for 6 hours. Using a Vigreux column and excluding moisture the pyridine was distilled directly into a flask containing calcium hydride, and refluxed again. Final distillation was done using a Vigreux column, the distillate being collected directly in a reagent bottle containing molecular sieves.

Dry chloroform was prepared by extraction of reagent grade chloroform (x6) with about half its volume of water. The chloroform was dried over anhydrous calcium chloride, decanted and distilled directly into a dark reagent bottle containing molecular sieves. Dry chloroform was prepared freshly for each use due to phosgene formation.

Dry methanol was prepared by heating reagent grade methanol over iodine and magnesium turnings and refluxing for 1 hour. Using a Vigreux column, the solvent was distilled directly into reagent bottles containing molecular sieves.

Reagent grade benzene was dried over sodium wire.

The molecular sieves used were "Linde" type 3A, diameter 1/16", as supplied by Metheson Coleman and Bell.

Sodium methoxide was prepared by dissolving sodium wire in dry methanol. The methoxide was prepared fresh and used directly after preparation.

2. GENERAL METHODS

Nucleosides used in synthetic procedures were dried by first dissolving in dry pyridine followed by the evaporation of the solvent at reduced pressure, air was admitted at the end through a drying tube containing anhydrous calcium sulphate.

Paper chromatography was performed by the descending technique using Whatman 3MM papers, the paper size was 57 cm x 23 cm. The solvent system used was n-butanol-ethanol-water, volume ratio = 9:1:2. Whenever required, a particular band was cut out and eluted in water and lyophilised to dryness.

Thin layer chromatography was run on Eastman 13254 Cellulose Chromatogram sheets and Silica gel 60 F_{254} (EM reagents) both with fluorescent indicator. Dimensions of the strips were 6.5 cm x 2 cm. Thick layer chromatography was done on glass plates (20 cm x 20 cm) coated with a 1 mm thick layer of MN-Kieselgel P/UV_{254} (Macherey Nagel and Co.).

For detecting nucleosides on tlcs and papers, an ultraviolet light source was used (Mineralight, 254 nm).

B. KINETICS AND NMR METHOD

The kinetics of the dealkylations were monitored at 25.0 ± 0.02°C in a Beckman DU spectrophotometer modified so that its output could be digitised by a voltmeter (Dana, model 5330) and stored in a Wang 600 calculator. Rate constants were calculated automatically by a least squares method.

The kinetic data for the depyrimidination of m^2dU and m^2dT were obtained from the time dependance of the H6 proton nmr intensities obtained at 90.02 MHz on a Bruker WH90DS spectrometer equipped with a Nicolet 1180 computer. The probe was maintained at a higher temperature (32 ± 0.5°C) than in the spectrophotometric measurements to lessen precipitation of the relatively insoluble bases formed in the reactions.

C. SYNTHESIS

1. Synthesis of O^4-Methyl Pyrimidine 2'-Deoxyribonucleosides

(a) Discussion (FIGURE 11).

The initial step in the synthesis was protection of the 3'- and 5'-hydroxyl groups of the sugar moiety using benzoyl chloride (BDH)[47]. Benzoylation was carried out in dry pyridine, yields were generally 90-95% (II).

The previous compound, II, was chlorinated at the 4-position of the base ring using thionyl chloride (Fisher) and catalytic amounts of DMF in pyridine[48,49]. The reaction time and the amount of DMF used seemed to be a governing factor in the ease of isolation of and the yield of product (III). The reaction yields were 45-55%.

Treatment of III in a solution of methanol and sodium methoxide under reflux conditions[48] afforded the O^4-methylated pyrimidine 2'-deoxyribonucleoside (IV) in yields of 25-30%.

(b) Experimental Procedures

i. 3',5'-Dibenzoyl-2'-deoxyribonucleoside (II).

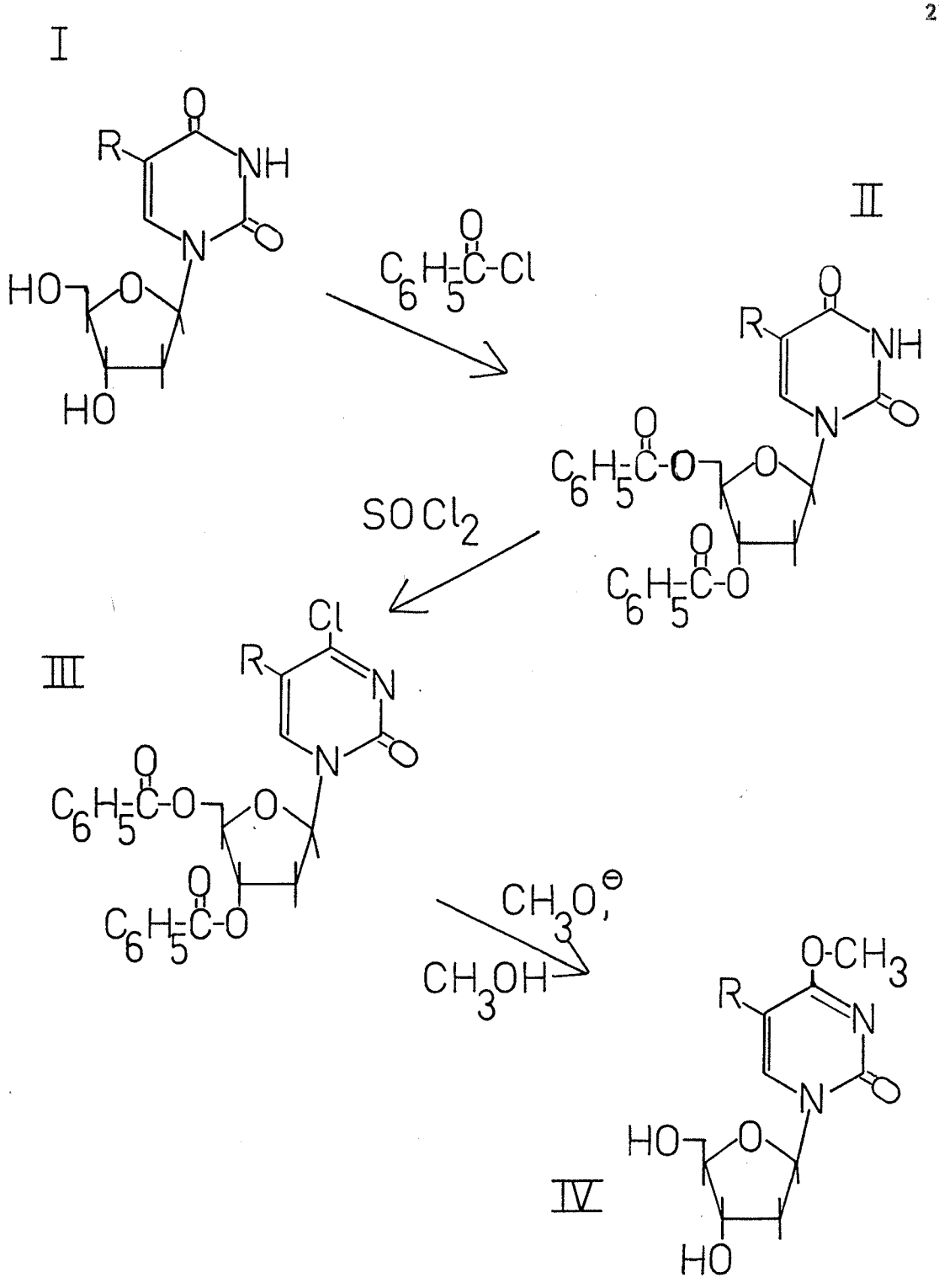
The nucleoside was dissolved in pyridine (1 ml/mmole nucleoside) and the mixture cooled in an ice bath. Benzoyl chloride (2.5 eq/eq.nucleoside) was added dropwise and the solution brought to room temperature when addition was complete.

FIGURE 11

Reaction sequence for the synthesis of O^4-methylated pyrimidine 2'-deoxyribonucleosides.

R = H, dU.

R = CH_3, dT.


I
II
III
IV
R
NH
N
O
HO
HO
$C_6H_5C(=O)Cl$
$C_6H_5C(=O)O$
$SOCl_2$
Cl
$CH_3O^{\ominus}$
CH_3OH
$O\text{-}CH_3$

After five hours, the reaction was quenched with ice and then water added to bring the total volume to 75 mls. The solution was extracted with $CHCl_3$ (4 x 25 mls) and the combined organic fractions coevaporated with toluene. Recrystallization was from hot CH_2Cl_2 with the addition of heptane until the mixture became turbid. Identification was by tlc ($CHCl_3$:EtOH, 24:1), Table 1, and yields were 90-95%.

ii. 4-Chloropyrimidine-3',5'-dibenzoyl-2'-deoxyribonucleoside(III).

The protected nucleoside (II) was combined with $SOCl_2$ (10 eq./eq.nucleoside) and about 0.5 mls of dry DMF in chloroform (20 ml/mmole nucleoside) and refluxed for four hours. The solvent was removed and the yellow oil obtained was placed on the vacuum pump for twelve hours. Purification of the resulting residue could be carried out either by trituration with dry benzene or by thick layer chromatography ($CHCl_3$:EtOH, 24:1). The product, recovered in yields of 35-45%, was identified by tlc (Table 1) and its characteristic light blue colour under U.V. light.

iii. O^4-methylpyrimidine-2'-deoxyribonucleoside (IV).

The chloro compound (III) was refluxed in dry methanol (5 ml/mmole nucleoside) with sodium methoxide (3.5 eq./eq. nucleoside) for twenty minutes and then stirred at room temperature for twelve hours. The pH of the solution was adjusted to near neutral with IRC-50 resin and the mixture filtered and

TABLE 1

R_f Values for Compounds Involved in the Synthesis of m^4dU and m^4dT.

	R_f	
	1	2
dU	0.0	0.25
dT	0.0	0.36
3',5'-di-O-benzoyldU	0.22	
3',5'-di-O-benzoyldT	0.46	
3',5'-di-O-benzoyl-4-CldU	0.33	
3',5'-di-O-benzoyl-4-CldT	0.64	
m^4dU		0.51
m^4dT		0.63

1) $CHCl_3$:EtOH, 24:1
2) $CHCl_3$:EtOH, 2:1

TABLE 2

U.V. Spectral Identification for the Synthesis of m^4dU and m^4dT.

	λ_{max}	λ_{min}
dU	262	231
m^4dU	276	288
dT	267	235
m^4dT	281	240

the resin washed well with methanol. The combined methanol fractions were taken to dryness at reduced pressure and the resulting residue dissolved in 100 ml of water. The solution was extracted with ether (4 x 25 ml) and $CHCl_3$ (1 x 25 ml). The aqueous layer taken to dryness and placed on the vacuum pump for twelve hours. The resulting solid was purified by extraction with boiling ethyl acetate (6 x 50 ml). The combined liquid fractions were filtered and the solvent removed under reduced pressure. The product was washed with petroleum ether (40° - 60°) (2 x 10 ml) and ether (1 x 5 ml) and then lyophilized. Product identification was by tlc ($CHCl_3$:EtOH, 2:1), Table 1, and by U.V. analysis [19,48], Table 2. Further purification was necessary for m^4dU and this was done by paper chromatography.

2. Synthesis of O^2-Methylated Pyrimidine 2'-Deoxyribonucleosides

(a) Discussion

Three reaction sequences were proposed for the synthesis of the O^2-methylated nucleosides, each scheme having the same general route, Figure 12. Each sequence has been discussed but experimental procedures are given only for the successful route.

i. Reaction Sequence A, Figure 13.

Selective blocking of the 5'-hydroxyl group was done

FIGURE 12

General scheme for the synthesis of O^2-methylated pyrimidine 2'-deoxyribonucleosides.

R = H, U.

R = CH_3, dT.

BL = blocking group.

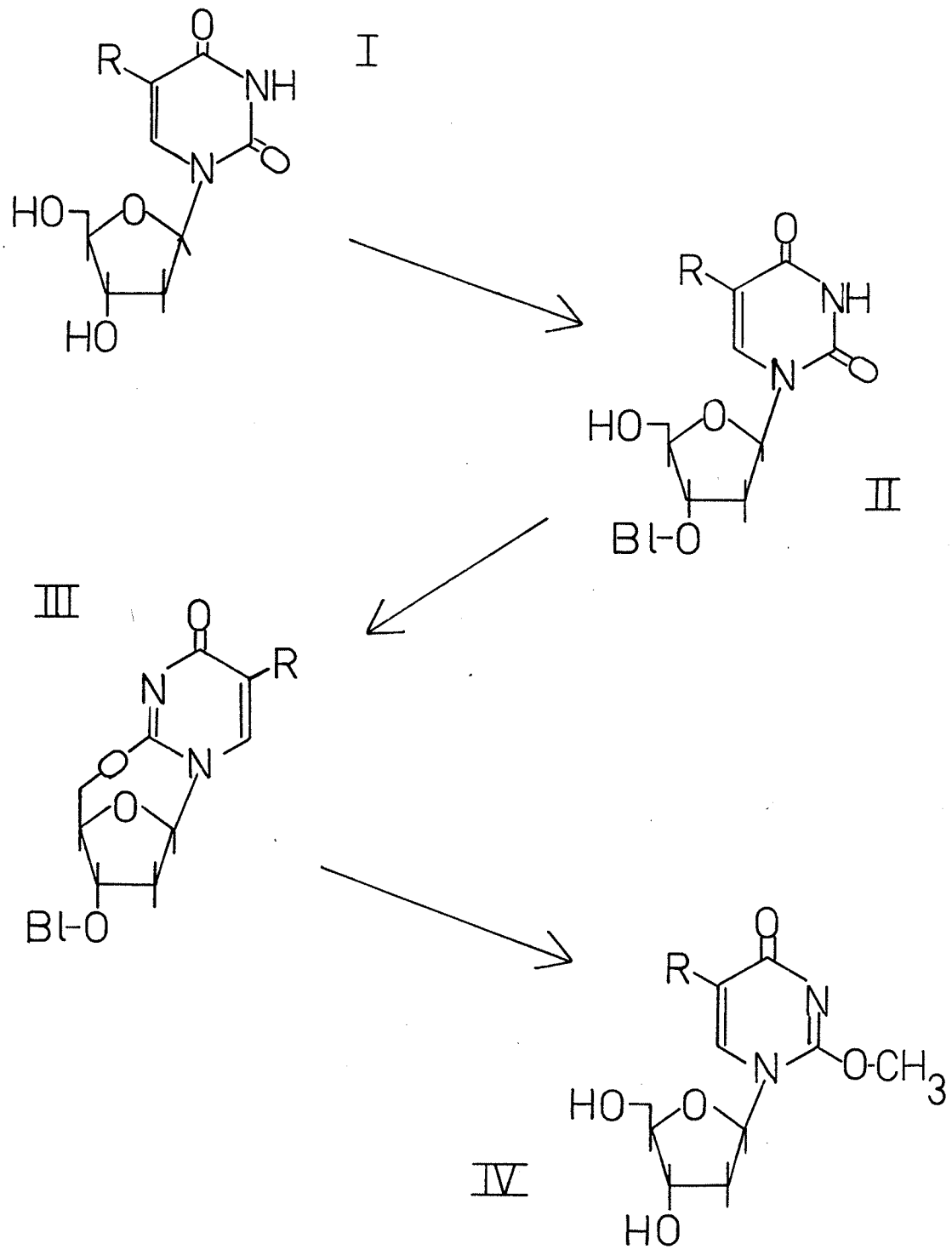
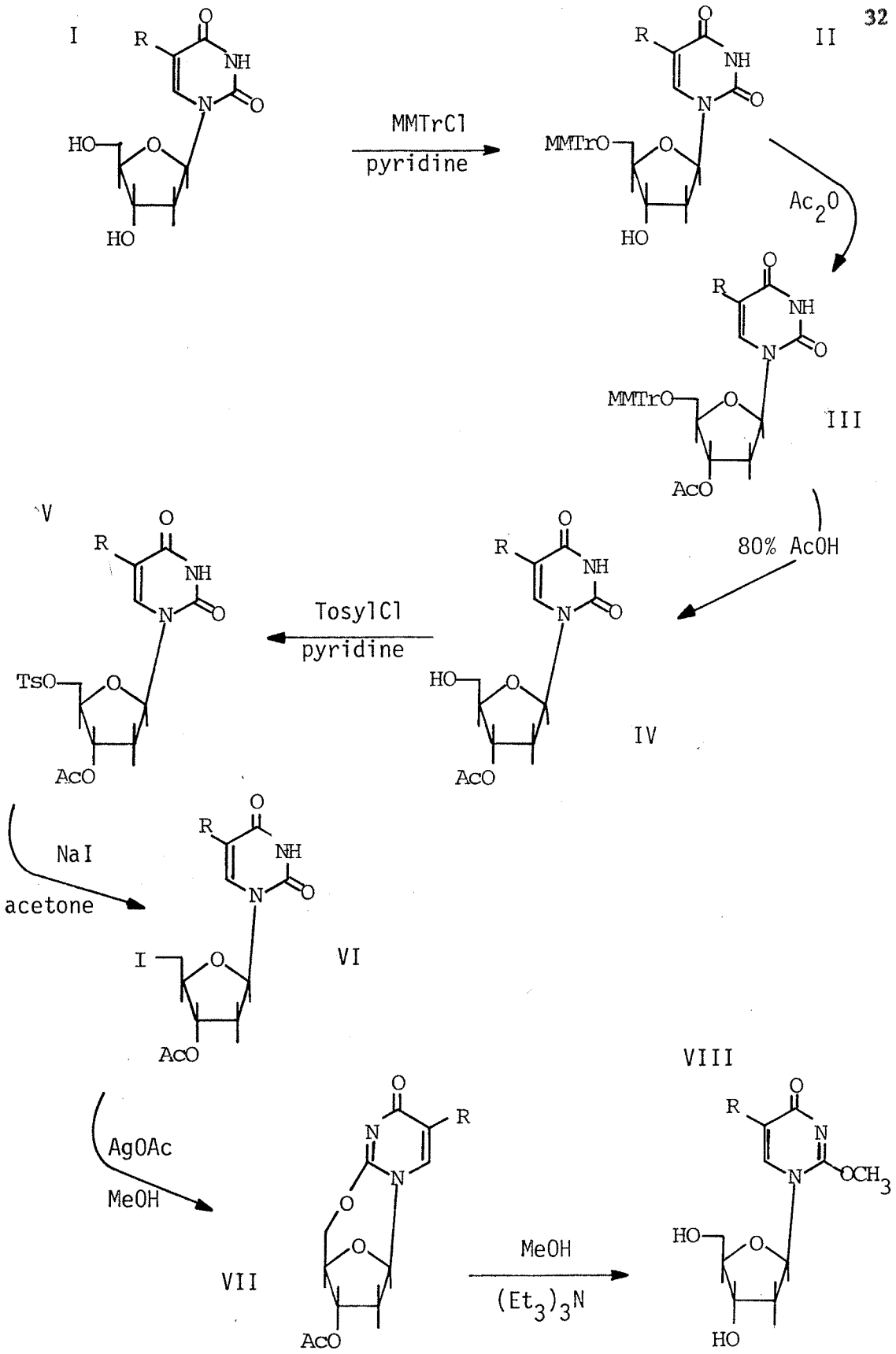

I
II
III
IV
R
NH
N
O
HO
Bl-O
O-CH3

FIGURE 13

Proposed reaction sequence for the synthesis of O^2-methylated pyrimidine 2'-deoxyribonucleosides.


I
II
MMTrCl
pyridine
MMTrO
HO
HO
Ac2O
III
MMTrO
AcO
80% AcOH
IV
HO
AcO
TosylCl
pyridine
V
TsO
AcO
NaI
acetone
VI
I
AcO
AgOAc
MeOH
VII
AcO
VIII
MeOH
(Et3)3N
OCH3
HO
HO
R
NH
N
O

using p-anisychlorodiphenylmethane (MMTrCl)(Sigma) in pyridine[50]. The reaction mixture was stirred for twelve hours at room temperature and then quenched with ice water. The product (II) was isolated in yields of 70-75%.

The 3'-hydroxyl group of II was blocked by reaction with acetic anhydride (AC_2O) (Aldrich) in pyridine[51,52].Reaction conditions and work up were the same as for II. The product III was isolated in yields of 90-95%.

Removal of the 5'-hydroxyl protecting group was done by stirring III in 80% acetic acid at 75-80°C for twenty minutes[50]. The mixture was cooled and the solvent removed by coevaporation with toluene. Product yields, IV, were generally 90-95%.

Attempts to synthesize V by reaction of IV with p-toluene-sulfonylchloride (TosylCl) (Aldrich) were not successful. The procedure was carried out several times, however, acceptable results were not obtained and the scheme was abandoned.

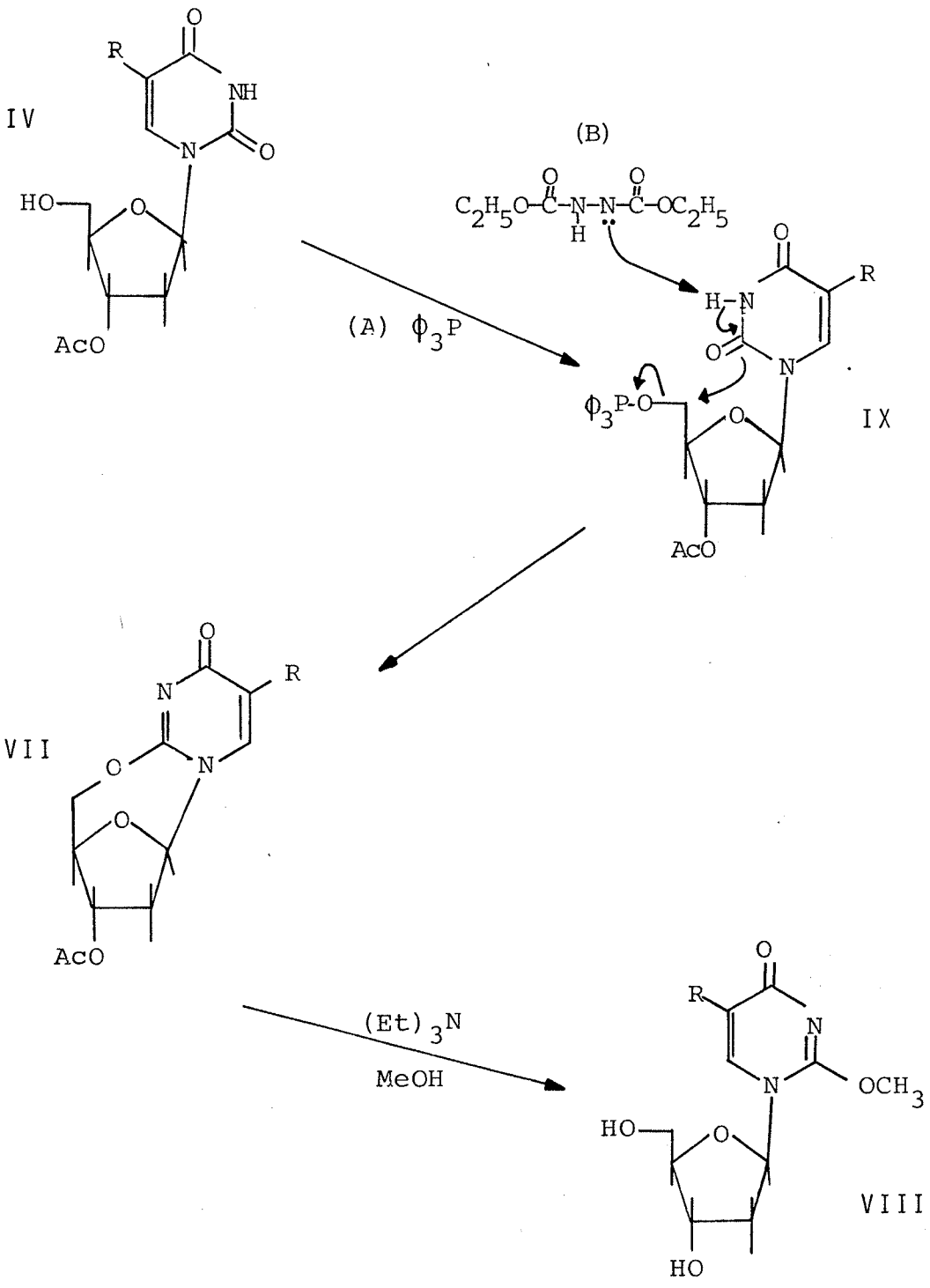
ii. Reaction Sequence B, Figure 14.

This proposed route has been reported for the synthesis of O^2,5'-anhydropyrimidineribonucleosides [53-56]. The starting material, IV, was prepared as described in Sequence A.

The proposed one step cyclization reaction was carried out by treatment of IV with triphenylphosphine (A)(J. T. Baker Chemicals) and diethylazodicarboxylate (B)(Aldrich). The

FIGURE 14

Proposed one step O^2-C5' cyclization for the synthesis of O^2-methylated pyrimidine 2'-deoxyribonucleosides.


IV
(B)
(A) ϕ_3P
IX
VII
$(Et)_3N$
MeOH
VIII

reaction was monitored by tlc, and although some reaction occurred, an intractable mixture resulted. Similar findings have been reported by Watanabe et al.[57].

iii. Reaction Sequence C, Figure 15.

Two pathways are discussed here, their difference being the blocking groups used to protect the hydroxyl groups.

PATHWAY A

The 5'-hydroxyl group of IV was blocked using methanesulfonylchloride (MesylCl)(Aldrich) in pyridine at room temperature[50]. After stirring the mixture for twelve hours, the reaction was quenched with ice water and the product X, recovered in yields of 80-85%.

Cyclization of X was carried out in acetonitrile using 1,5-diazabicyclo[5,4,0]undec-5-ene (DBU)(Aldrich)[57,58]. The mixture was refluxed for about twenty hours, then cooled and the solvent evaporated at reduced pressure. Yields of VII were 60-65%.

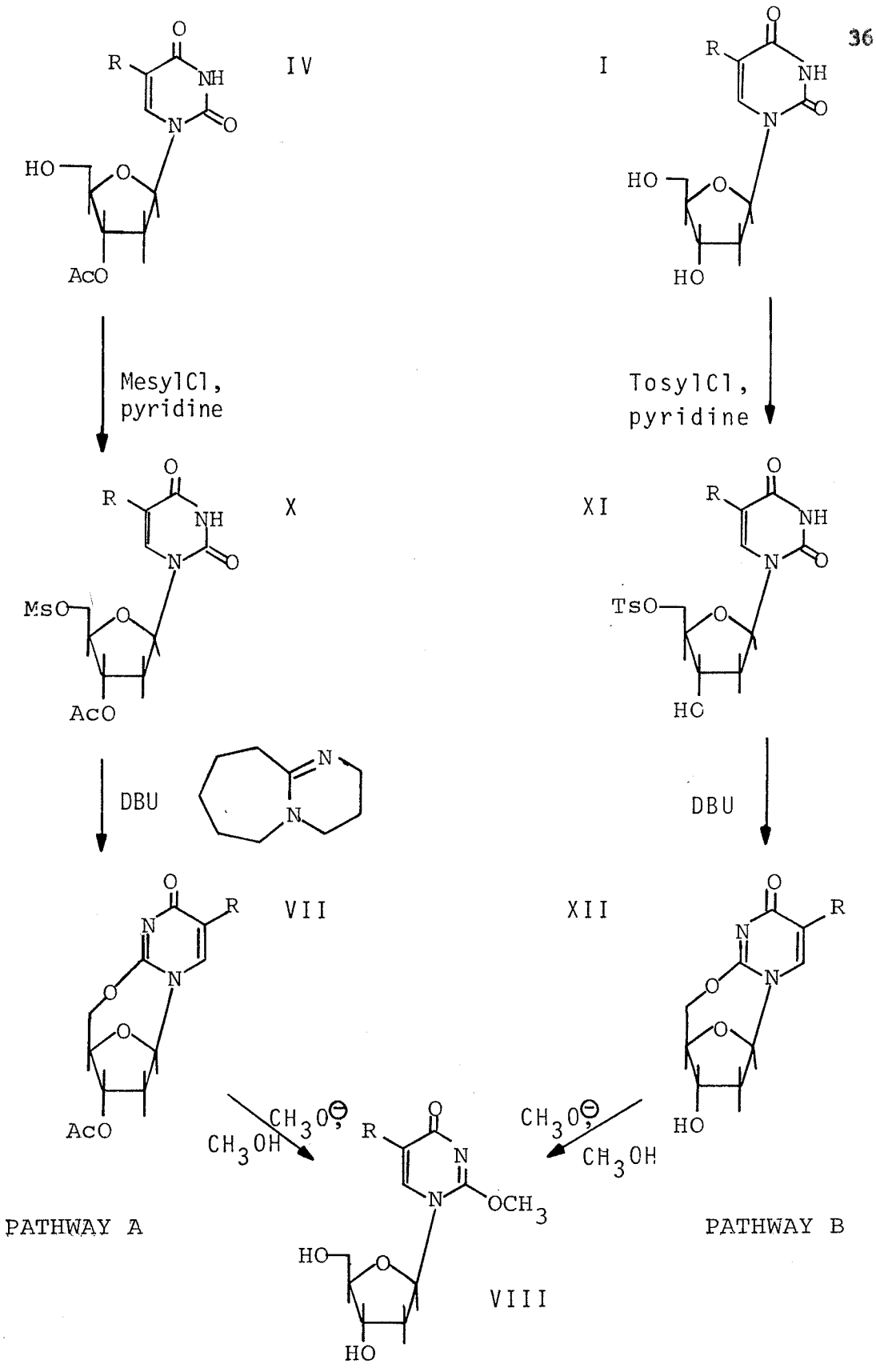
Treatment of VII at room temperature with sodium methoxide in methanol removed the 3'-acetyl blocking group, leaving the O^2,5'-anhydro link intact. When the solution was heated the anhydro bridge was cleaved and VIII was isolated in yields of (75-80%).

PATHWAY B

The 5'-hydroxyl group of I was protected using p-toluene-

FIGURE 15

Reaction sequence for the synthesis of O^2-methylated pyrimidine 2'-deoxyribonucleosides.


IV
I
MesylCl, pyridine
TosylCl, pyridine
X
XI
DBU
DBU
VII
XII
$CH_3O^{\ominus}$, CH_3OH
$CH_3O^{\ominus}$, CH_3OH
PATHWAY A
PATHWAY B
VIII

sulfonylchloride (TosylCl)(Aldrich) in pyridine at 0°C. The mixture was stirred for twelve hours at room temperature, quenched with ice water and the solvent removed. Yields of XI were 45-50%.

Cyclization of XI followed by alkylation of XII were the same procedures as used in PATHWAY A. Yields of XII and VIII were 45-50% and 75-80% respectively.

Comparison of PATHWAY A and B for the synthesis of O^2-methylated pyrimidine 2'-deoxyribonucleosides shows B to involve fewer synthetic steps and less time.

b. Experimental Procedures

i. 5'-Monomethoxytrityl-2'-deoxyribonucleoside (II).

A pyridine solution (10 ml/mmole nucleoside) of MMTrCl (1.3 eq./eq.nucleoside) and the nucleoside (I) was stirred for twelve hours at room temperature. The mixture was quenched with ice water and the insoluble product filtered and washed with cold water. The product, II, identified by tlc ($CHCl_3$:EtOH, 8:1) was visible as a yellow spot after the developed slide was sprayed with perchloric acid (Table 3). Yields of II were 70-75%.

ii. 3'-Acetyl-5'-monomethoxytrityl-2'-deoxyribonucleoside (III).

This reaction may be carried out in the same solution as II without previous isolation of the starting material. Acetic-anhydride (10 eq./eq.nucleoside) was added to the above

solution and the mixture stirred for twelve hours at room temperature. Product isolation was as for II, the product being identified by tlc ($CHCl_3$:EtOH, 2:1) followed by perchloric acid spray (Table 3). Yields of III were 85-90%.

iii. 3'-Acetyl-2'-deoxyribonucleoside (IV).

Treatment of III with 80% acetic acid (30 ml/mmole nucleoside) at 80°C for twenty minutes removed the 5'-hydroxyl protecting group. The reaction was quenched with ice water and the insoluble, displaced blocking group was filtered off. The solvent was coevaporated with toluene and the product recovered in yields of 90-95%. The product, IV, was identified by tlc ($CHCl_3$:EtOH, 8:1), Table 3.

iv. 3'-Acetyl-5'-mesyl-2'-deoxyribonucleoside (X).

Compound IV was dissolved in pyridine (20 ml/mmole nucleoside) and methanesulfonylchloride (1.2 eq./eq.nucleoside) was added dropwise. The mixture was stirred for twelve hours at room temperature and then quenched with ice water. The aqueous mixture was extracted with $CHCl_3$ (3 x 25 ml) and then the organic layers were combined and extracted with a saturated bicarbonate solution (1 x 50 ml) followed by water (1 x 50 ml). The $CHCl_3$ layer was dried over sodium sulfate and the solvent then removed by coevaporation with toluene. The product was identified by tlc ($CHCl_3$:EtOH, 8:1)(Table 3) and yields were 80-85%.

v. 5'-Tosyl-2'-deoxyribonucleoside (XI).

Tosyl chloride (1.1 eq./eq.nucleoside) was added to a pyridine solution (20 ml/mmole nucleoside) of the nucleoside at 0°C. The reaction mixture was then stirred for twelve hours at room temperature. Isolation of the product was similar to that described in (iv), yields were 45-50%.

vi. O^2,5'-Anhydropyrimidine-3'-acetyl-2'-deoxyribonucleoside (VII), or O^2,5'-Anhydropyrimidine-2'-deoxyribonucleoside (XII).

The starting material for VII was X and for XII, XI was used. The protected nucleoside (X or XI) was dissolved in acetonitrile (30 ml/mmole nucleoside) along with DBU (1.2 eq./eq.nucleoside) and the mixture refluxed for twenty hours. The solvent was removed under reduced pressure and the solid triturated with acetone (1 x 5 ml). Product identification was by U.V. analysis, Table 4, and yields were, 60-65% for VII, and 45-50% for XII.

vii. O^2-methylpyrimidine 2'-deoxyribonucleoside (VIII).

The procedure is the same for both VII and XII as starting materials. In a solution of dry methanol (5 ml/mmole nucleoside) and sodium methoxide (2.2 eq./eq.nucleoside), the bridged nucleoside (VII or XII) was refluxed for one hour. The work up procedure is the same as that used in 1, iii for O^4-methylpyrimidine-2'-deoxyribonucleosides. Identification of the product was by U.V. analysis and NMR, these data are given in the Results and Discussion, A1.

TABLE 3

R_f values for compounds involved in the synthesis of m^2dU and m^2dT.

Compound	R_f	
	1	2
dU	0.10	
dT	0.15	
5'-mmtr dU*	0.50	0.60
5'-mmtr dT*	0.61	0.65
3'-Ac-5'-mmtr dU*	0.75	0.73
3'-Ac-5'-mmtr dT*	0.95	0.80
3'-AcdU	0.40	
3'-AcdT	0.61	
3'-Ac-5'-mesyldU	0.48	
3'-Ac-5'-mesyldT	0.70	

1. $CHCL_3$:EtOH, 8:1 2. $CHCL_3$:EtOH, 2:1

* visible as yellow spot after perchloric acid spray.

TABLE 4

U.V. Spectral Analysis of O^2,5'-Anhydropyrimidine 2'-deoxyribonucleosides

	λ_{max}	λ_{min}
3'-Ac-O^2,5'-anhydrodT	250	218
O^2,5'-anhydrodT	250	218
3'-Ac-O^2,5'-anhydrodU	250	218

CHAPTER III

CHAPTER III. RESULTS AND DISCUSSION

A. O-METHYLATED NUCLEOSIDES

The O-methylated nucleosides used in the kinetics and NMR studies were synthesized in our lab (see Synthesis). Structure identification of the methylated nucleosides was by ^{1}H NMR and UV spectral analysis.

The UV spectral characteristics, Table 5, for the methylated nucleosides are in good agreement with published data for the same derivatives[19,45,48,59].

The ^{1}H spectral assignments were made by comparison with U, dU[60-1] and dT[62-3]. Also useful were data for C-, N-, and O-methylated pyrimidine bases[46] and $m^{4}U$ [48]. Resonances for H6, H5 (or C(5)-CH_3), H1', and -O-CH_3 are particularily useful for the identification of the methylated nucleosides, Table 6.

TABLE 5

UV Absorptions for Nucleosides and O-Methylated Derivatives at neutral pH.

	λ_{max}(nm)	λ_{min}(nm)
U	262	230.5
dU	262	231
dT	267	235
m^2U[a]	251,222	237,212
m^4U	274	238
m^2dU[a]	252,222	236,<220
m^4dU[a]	276	238
m^2dT[a]	255,227	235,215
m^4dT[b]	281	240

a) H_2O

b) MeOH

TABLE 6

Proton Chemical Shifts (δ) and Coupling Constants (J) for Natural and O-Methylated Nucleosides

	pH	δ6	δ5, δ5-CH_3	δ1'	δO-CH_3	J_{56}, J_{M6}	$J_{1'2'}$; Σ
m^2U	7	8.08	6.17	5.96	4.06	7.6	3.4
m^4U	7	8.17	6.25	5.93	3.95	7.5	3.1
m^2dU	7	8.01	6.16	6.30	4.04	7.6	12.8
m^4dU	7	8.14	6.25	6.27	3.95	7.5	12.8
m^2dT	7	7.85	1.95	6.31	4.03	1.1	13.0
m^4dT	7	7.90	1.99	6.28	3.97	1.1	13.0
U	0.5	7.85	5.92	5.92	-	8.1	4.5
	7	7.85	5.93	5.94	-	8.1	4.5
	13.5	7.67	5.84	5.83	-	7.7	~5
dU	0.5	7.84	5.90	6.27	-	8.2	13.4
	7	7.84	5.92	6.29	-	8.1	13.4
	13.5	7.70	5.83	6.29	-	7.6	12.6
dT	0.5	7.63	1.90	6.28	-	1.1	13.6
	7	7.61	1.90	6.31	-	1.1	13.6
	13.5	7.49	1.87	6.30	-	1.0	13.2

Data at 270 MHz (m^2U, , m^4U, m^2dU, m^4dU, m^2dT, m^4dT) (25°C) and 90 MHz (U, dU, dT) (32°C) relative to TSP

Σ= J(1'-2') + J(1'-2")

B. TRENDS IN THE BASE ^{1}H CHEMICAL SHIFTS

Some general trends which are observed in the ^{1}H spectral results and may be useful for identification of O-alkylated nucleosides are discussed. While similar data have been reported by Hruska and Blonski[64] we have, because of minor differences in data and for completeness in this study, included this work.

From the data in Table 6, we have constructed Figure 16 which clearly indicates the effect of O-alkylation of the pyrimidine base on the (H6) and (H5) proton shifts. Included in Figure 16 are data for U, dU, dT and their O^2- and O^4-methylated derivatives as well as, for comparison, cytidine (C) and its 2'-deoxy analog. The data used are from solutions of neutral pH.

1. From Figure16 the chemical shifts for H6 and H5 are observed to exhibit parallel behaviour for both the ribose (R) and 2'-deoxyribose (D) series of molecules. This result suggests that the nature of the sugar moiety has little influence on the H5 and H6 chemical shifts.

2. For a particular base the dependance on the nature of the sugar is greater for δ(H6) than for δ(H5), in line with the relative separation of these protons from the sugar. (Compare, for example, U and dU). The largest variation for δ(H6) (0.07 ppm) is seen in m^2U; a smaller variation is seen

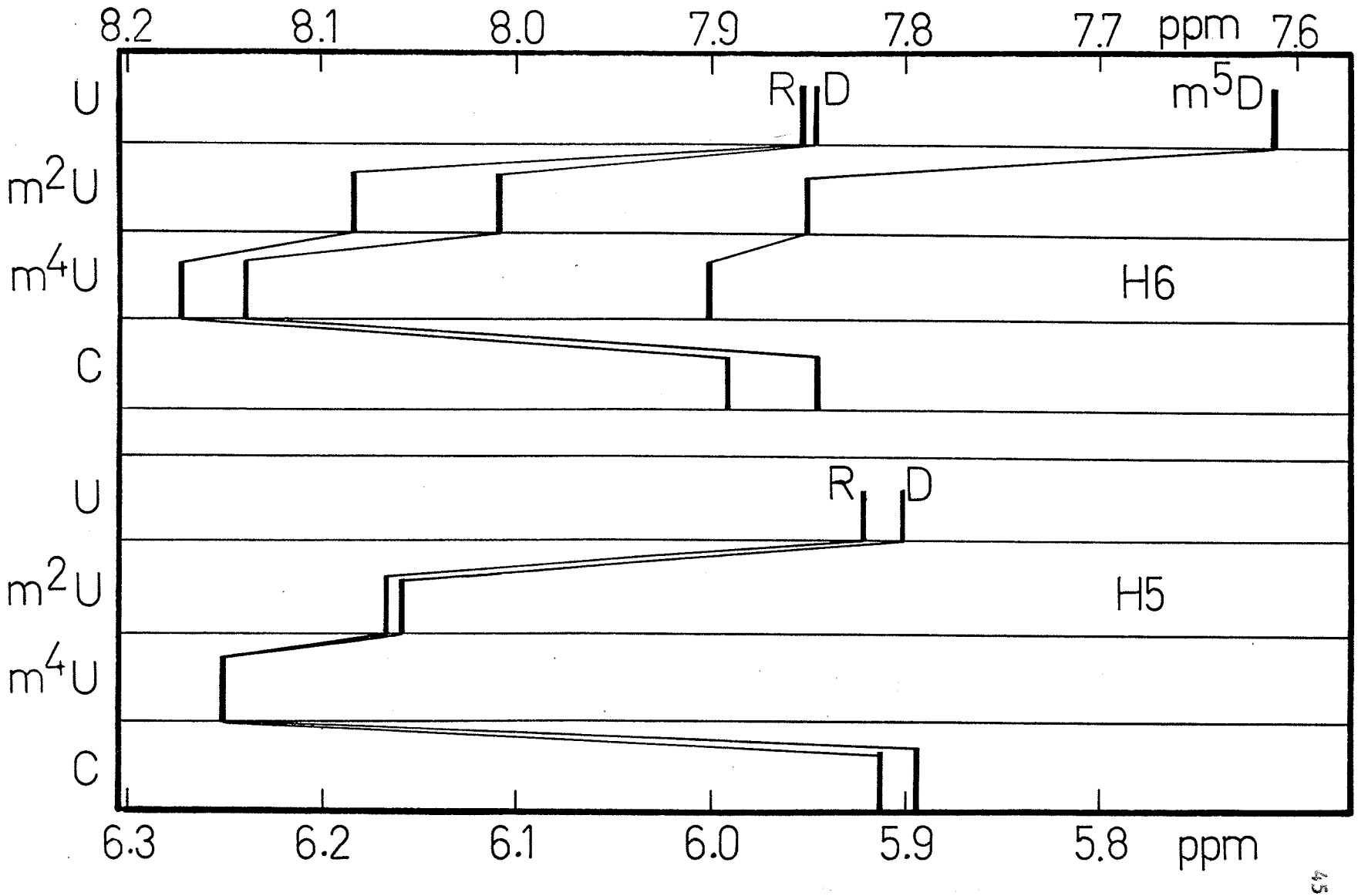
FIGURE 16

Trends in the base ^{1}H chemical shifts of pyrimidine nucleosides as a result of O-alkylation.

Shifts relative to TSP.

R - ribose

D - 2'-deoxyribose


8.2
8.1
8.0
7.9
7.8
7.7
ppm
7.6
U
R
D
m5D
m2U
m4U
H6
C
U
R
D
m2U
H5
m4U
C
6.3
6.2
6.1
6.0
5.9
5.8
ppm

in m^4U (0.03 ppm).

3. Comparison of dU and m^5dU(dT) indicates that C(5)-methylation has a shielding effect on H6 (0.16 - 0.24 ppm), in line with the observed shielding increments for olefinic protons effected by cis alkylation[65].

4. Upon O^4-methylation, H6 and H5 are deshielded by ~0.30 ppm whereas upon O^2-methylation they are deshielded by ~0.2 ppm and 0.25 ppm respectively. This suggests that O-methylation is reflected in the electronic properties of the C5-C6 bond. In the cytosine (C) base H6 is deshielded, relative to U, to a lesser extent than the isoelectric m^4U base and in contrast the H5 of C is shielded slightly.

5. The methyl proton shifts lie in the range 1.9 - 4.1 ppm (Table 5), with the order of increasing shielding O^2-Me < O^4-Me < C5-Me.

C. NMR TITRATION OF O-METHYLATED PYRIMIDINE NUCLEOSIDES

In conjunction with the kinetic results, obtained for the acid catalyzed hydrolysis of the O-methylated nucleosides, an NMR titration study was carried out to determine pKa values and to aid in the interpretation of the hydrolysis mechanism of the O-methylated nucleosides.

To investigate the ionization behaviour of the O-methylated nucleosides the ^{1}H chemical shifts for H6, H5, C5-CH_3 and 2(4)-OCH_3 have been obtained as a function of pH, Figure 17, (a)-(c), Table 7. The study was restricted to acid solutions since the bases have no acidic sites which would undergo ionization in basic solution. (However, ionization of the sugar hydrolys occurs at pH > 12.) These studies could not be extended to pH values below 1 because, under these conditions, rapid hydrolysis of the methylated nucleosides occurs.

For all O-methylated molecules, except m^4dT, the H6, H5, C5-CH_3 and 2(4)-OCH_3 chemical shifts remain constant over the range pH 2-7 and then, in more acidic solutions, move downfield. (Acutally a small variation (< 0.03 ppm) occurs over the range pH 2-7 which can be attributed in part to the protonation of the carboxylate group of the TSP reference (pKa ≈ 5).) Unfortunately, a complete sigmoidal titration curve is not obtained in the pH range examined. As a result, the

FIGURE 17

Effect of pH on the ^{1}H chemical shifts of O- methylated pyrimidine nucleosides.

I. $-OCH_3$.

II. H6.

III. H5 or $C(5)-CH_3$.

m^4U ●

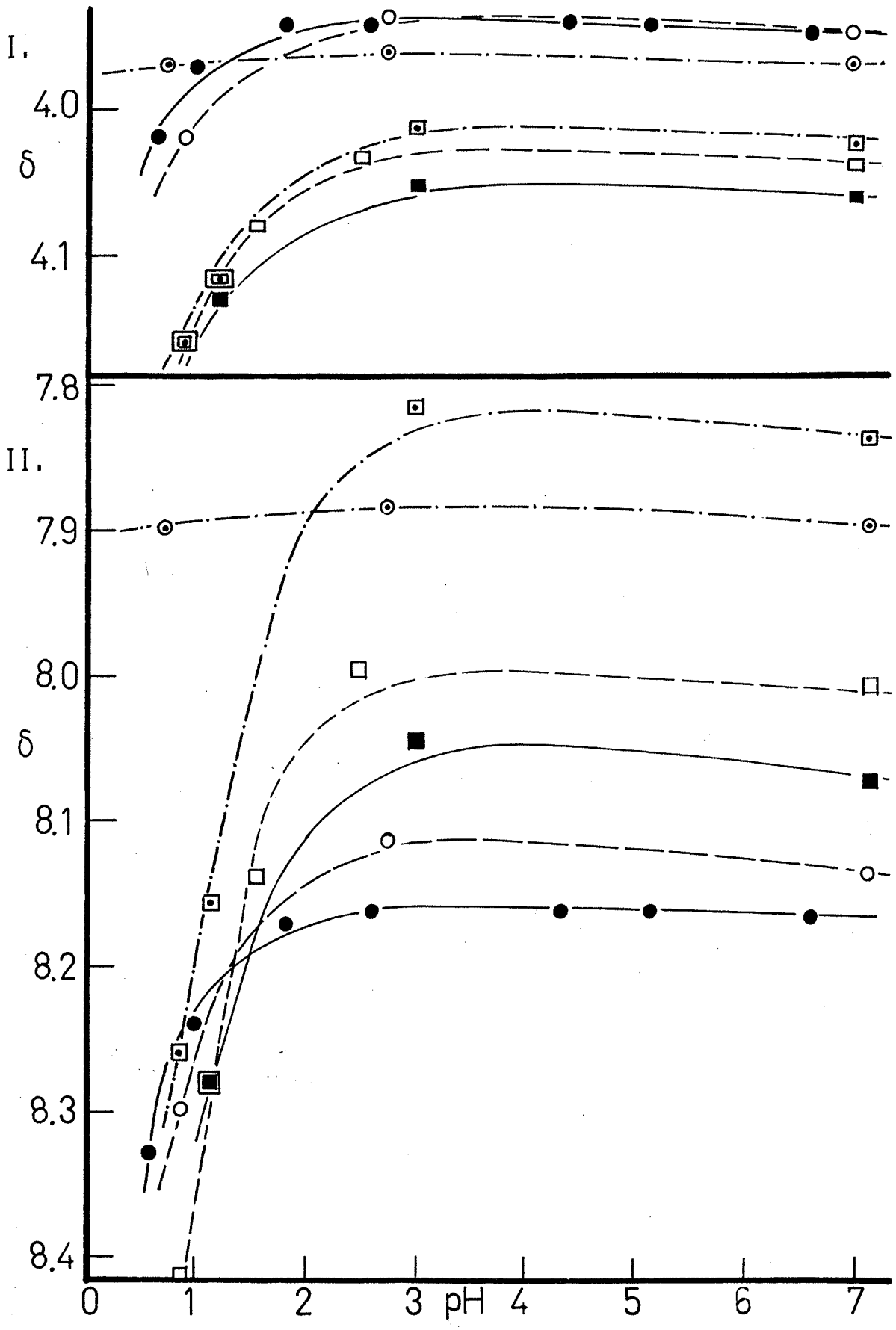
m^4dU ○

m^4dT ⊙

m^2U ■

m^2dU □

m^2dT ⊡


I.
4.0
δ
4.1
7.8
II.
7.9
8.0
δ
8.1
8.2
8.3
8.4
0
1
2
3
pH
4
5
6
7

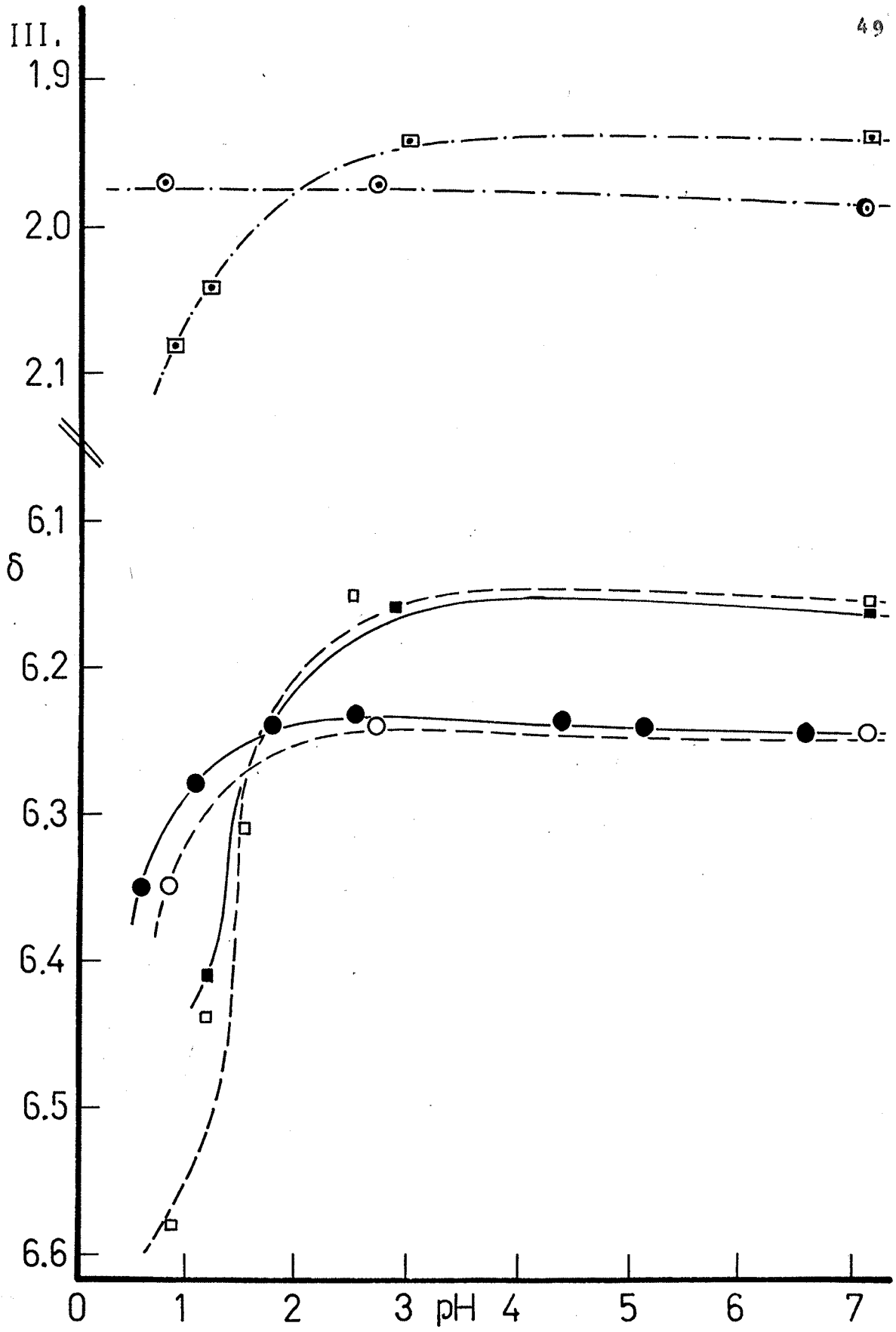

III.
1.9
2.0
2.1
6.1
δ
6.2
6.3
6.4
6.5
6.6
0
1
2
3
pH
4
5
6
7

TABLE 7

Titration of O-Methylated Pyrimidine Nucleosides

	pH	H6 (ppm)	H5 (ppm) C(5)-CH_3	O-CH_3 (ppm)
m^2U	1.2	8.28	6.41	4.13
	2.9	8.04	6.16	4.05
	7*	8.08	6.17	4.06
m^2dU	0.9	8.42	6.58	4.16
	1.2	8.28	6.44	4.12
	1.5	8.14	6.31	4.08
	2.5	7.99	6.15	4.03
	7*	8.01	6.16	4.04
m^2dT	0.9	8.26	2.08	4.16
	1.2	8.16	2.04	4.12
	3.0	7.81	1.94	4.01
	7*	7.85	1.95	4.03
m^4dU	0.9	8.30	6.35	4.02
	2.7	8.11	6.24	3.93
	7*	8.14	6.25	3.95
m^4dT	0.8	7.90	1.97	3.97
	2.7	7.88	1.97	3.96
	7*	7.90	1.99	3.97
m^4U	0.7	8.33	6.35	4.02
	1.1	8.24	6.28	3.97
	1.8	8.17	6.24	3.94
	2.6	8.16	6.23	3.94
	4.4	8.16	6.24	3.94
	5.1	8.16	6.24	3.94
	6.6	8.17	6.25	3.95
	7.3	8.17	6.24	3.95

Data at 90 MHz (m^2U, m^2dU, m^2dU, m^4dT) (32°C), m^4U(27°C) and * at 270MHz (25°C), relative to TSP.

chemical shifts of the fully protonated cation and the pKa value for its ionization cannot be determined. However, the general appearance of the curves suggests pKa values less than about 1.5 in all cases. The chemical shifts for m^4dT remain constant over the entire pH range examined (0.8 - 7) and do not exhibit the downfield shift near pH 2. This result suggests that the pKa of m^4dT is noticeably less than m^4dU and may in fact be negative. These values are in line with those obtained for the O-ethylated deoxyribosides by Singer et al.[44]: $e^2dU(0.92)$, $e^2dT(0.5)$, $e^4dU(0.66)$ and $e^4dT(-0.32)$.

In studying N(1), O^4-dialkyluracils, UV spectral analysis under neutral and acidic conditions led Kusmierek et al.[45] to conclude that the protonation site is N3 as in N1-alkylated cytosines[66]. Comparison of the NMR titration behaviour of m^4U and m^4dU (Figure 17) with that of cytidine (C) support their proposal. Thus, upon formation of the N3 protonated cation, H6 and H5 of cytidine move downfield by 0.33 ppm and 0.24 ppm respectively (relative effect H6:H5 ≃ 1.4). With reference to their neutral resonance positions, H6 and H5 of m^4U in the most acidic solution have downfield shifts of 0.18 ppm and 0.12 ppm respectively (relative effect H6:H5 ≃ 1.5). A similar comparison for m^4dU shows downfield shifts of 0.19 ppm H6, and 0.11 ppm H5 (relative effect H6:H5 ≃ 1.7). The similarity in the ratios for C, m^4U and m^4dU suggests an

identical site for primary protonation, this being at N3. It seems reasonable to assume that the protonation of m^4dT also occurs at N3. As for the case of the O^2-alkylated derivatives, the H6 and H5 are identically shielded by base protonation. Titration curves on model compounds is required for comparison to determine the site of protonation.

Examination of the acidity of the cationic O^4-ethylated pyrimidine base by Singer et al.[44] showed that methylation of the 5-position lead to a large increase in acidity. (Compare pKa of e^4dU (0.66) and of e^4dT (-0.32).) In our work, methylation of the 5-position lead to a similar observation as is evident from the titration curves for m^4dU and m^4dT, Figure 17. In surprising contrast to these results, Borodavkin et al.[67] have shown that, 5-ethylation of the cytosine base leads to a small increase in its pKa. Continuing in this trend, 5-alkylation of the uracil base leads to a decrease in the acidity of the N(3)-H suggesting an electron donating role for the alkyl group. A simple explanation for the increased acidity of the cationic m^4dT and e^4dT is not evident to us. The conformational properties of the C(4)-O4 bond, which defines the position of the hydrophobic methoxy methyl group relative to the N(3) protonation site and which should be influenced by the size of the 5-substituent, may have some bearing here.

D. HYDROLYSIS REACTION PRODUCTS

1. Demethylation Reactions

Hydrolysis of the O-methylated nucleosides was carried out in both acid and alkaline media. Products of the hydrolysis reactions were identified using ^{1}H NMR and UV spectral analysis.

Identification by ^{1}H NMR was based primarily on the chemical shifts and coupling constants for the H6, H5 or C5-CH_3, and H1' resonances. Spectra from the demethylation reactions gave δ and J values in good agreement with literature data for the parent pyrimidine nucleosides [60-63], Table 6. Demethylation also results in the loss of the -O-CH_3 absorption paralleled by the formation of a singlet peak (δ3.35 ppm) for methanol.

The hydrolysis reactions were also followed by scanning UV spectroscopy. The data acquired was in agreement with the parent nucleosides at similar pH values.

2. Depyrimidination Reactions

The acid hydrolysis of m^2dU and m^2dT was shown to proceed via rupture of the N-glycosyl bond to form the O^2-methylated base and free sugar. The methylated base also proved to be unstable in the reaction medium, decomposing to the free base of the parent nucleoside and methanol. The hydrolysis products

for both reactions were identified using ^{1}H NMR.

The hydrolysis of the N-glycosyl link was characterized using the chemical shifts and coupling constants for H6, H5 or C(5)-CH_3, H1' and O-CH_3, Table 8. The ^{1}H spectral assignments were made by comparison with data for C, N and O-methylated pyrimidine bases[46]. As shown in Figure 18(a)-(c), rupture of the N-glycosyl bond of m^2dU is observed with the formation of a second set of H6 and H5 peaks representing m^2Ura as well as the loss of the resonance for the anomeric proton. The upfield shift ($\Delta\delta$) experienced by both H6 and H5 is similar to that observed between U and Ura in work done in this lab.

The loss of the -O-CH_3 group from the methylated base was identified using the chemical shifts and coupling constants for H6 and H5. The data obtained, Table 8, is in good agreement with ^{1}H spectral analysis done in this lab on Ura and Thy in aqueous acid media. The upfield shift experienced by δH6 and δH5 is seen in Figure 18(c)-(d). Also observed in the spectra for the demethylation reaction was the loss of the -OCH_3 peak accompanied by the formation of a peak for methanol (δ3.35 ppm).

TABLE 8

Proton Chemical Shifts (δ) Coupling Constants (J) for m^2dU, m^2Ura, m^2dT, m^2Thy, Ura and Thy.

	pM	δ6	δ5, $\delta5\text{-}CH_3$	δ1'	$\delta\text{-}OCH_3$	$J^{5,6}_{M6}$	$J_{1',2'} + J_{1',2''}$
m^2dU	0.9	8.42	6.58	6.33	4.16	7.5	11.8
m^2Ura	0.9	8.04	6.50	-	4.14	7.0	-
Ura	0.9	7.56	5.81	-	-	7.7	-
m^2dT	1.2	8.16	2.04	6.31	4.12	1.0	12.4
m^2Thy	1.2	7.80	2.02	-	4.14	1.1	-
Thy	1.2	7.38	1.8	-	-	1.2	-

FIGURE 18

^{1}H spectra from the acid catalyzed depyrimidination of $m^{2}dU$, pH = 0.9.

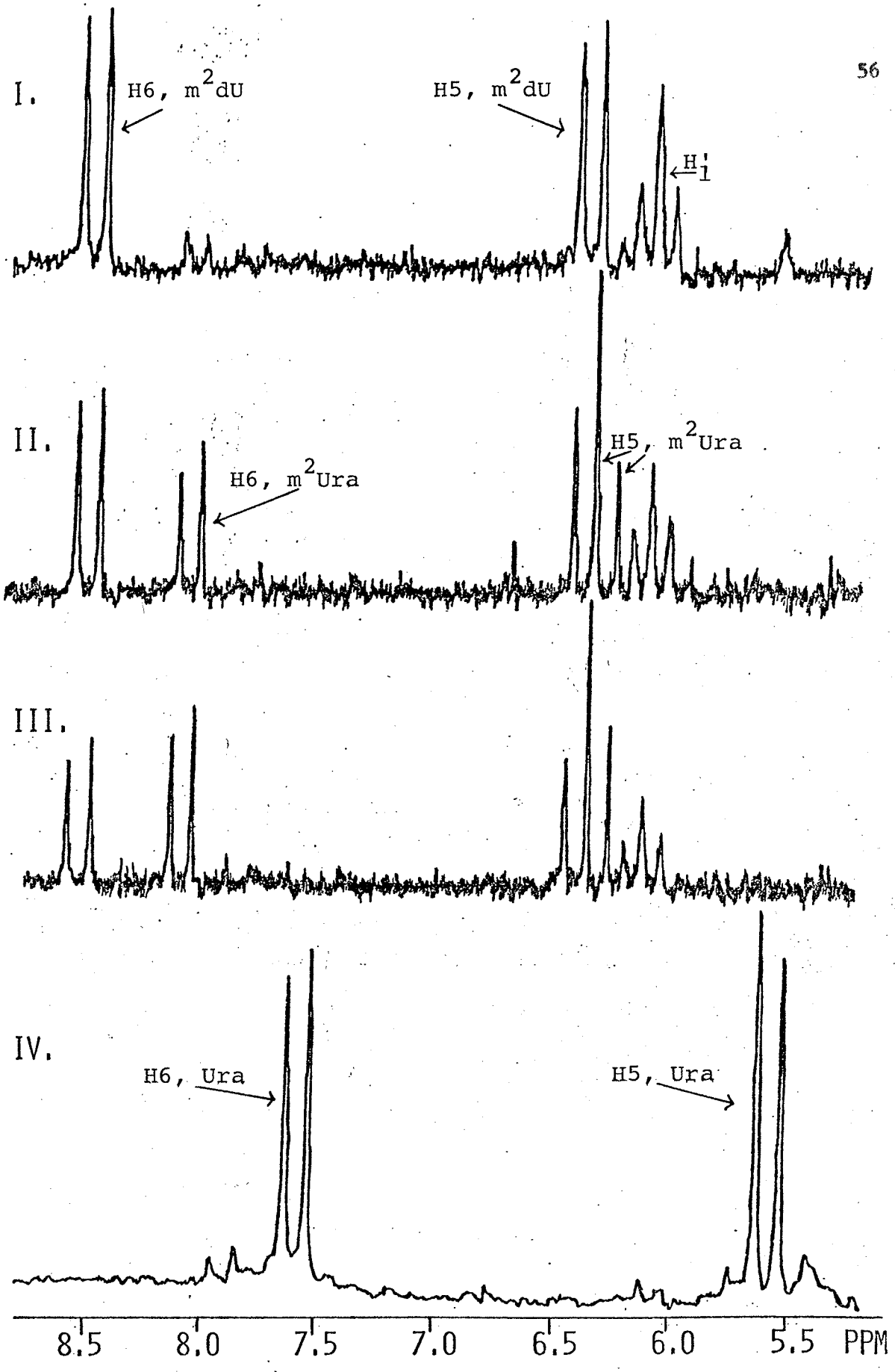
Reaction Times:

I. 2.8 min

II. 6.4 min

III. 8.2 min

IV. after 15 hr


I.
H6, m2dU
H5, m2dU
H1'
II.
H6, m2Ura
H5, m2Ura
III.
IV.
H6, Ura
H5, Ura
8.5
8.0
7.5
7.0
6.5
6.0
5.5
PPM

E. KINETICS

1. Hydrolysis of O^2 and O^4-methylated Nucleosides in Alkaline Solutions

The O^2 and O^4-methylated nucleosides stood for several days in the presence of sodium hydroxide at pH 14. The NMR data showed that the only products formed were methanol and the corresponding nucleoside with no evidence for cleavage of the N-glycosyl link.

The kinetic experiments were carried out at 25°C and 2M ionic strength. Plots of k_{obs} vs pH are shown in Figure 19. The corresponding plots of k_{obs} vs $[OH^-]$(Figure 20) are linear. As a result, the rate dependance on base concentration can be represented by:

$$k_{obs} = k_{H_2O} + k_{OH}[OH^-] \qquad (1)$$

where k_{obs} is the experimental pseudo first order rate constant for the overall reaction. Values of k_{OH} and k_{H_2O} were obtained from Figure 2 and are listed in Table 9. The value of k_{H_2O} for the alkaline hydrolysis study was approximately zero for all the O-methylated nucleosides (Table 9) and should be the same for the acidic catalyzed hydrolysis.

Figure 21 shows two possible mechanisms by which basic hydrolysis of the O^4-methylated nucleosides might occur. One of these, pathway A, is described by the term S_NAr process and the other, pathway B, by the term S_N2 process.

FIGURE 19

Plots of $k_{obs}(s^{-1})$ vs pH for the hydrolysis of O-methylated pyrimidine nucleosides.

m^4U ●

m^4dU ○

m^4dT ⊙

m^2U ■

m^2dU □

m^2dT ⊡

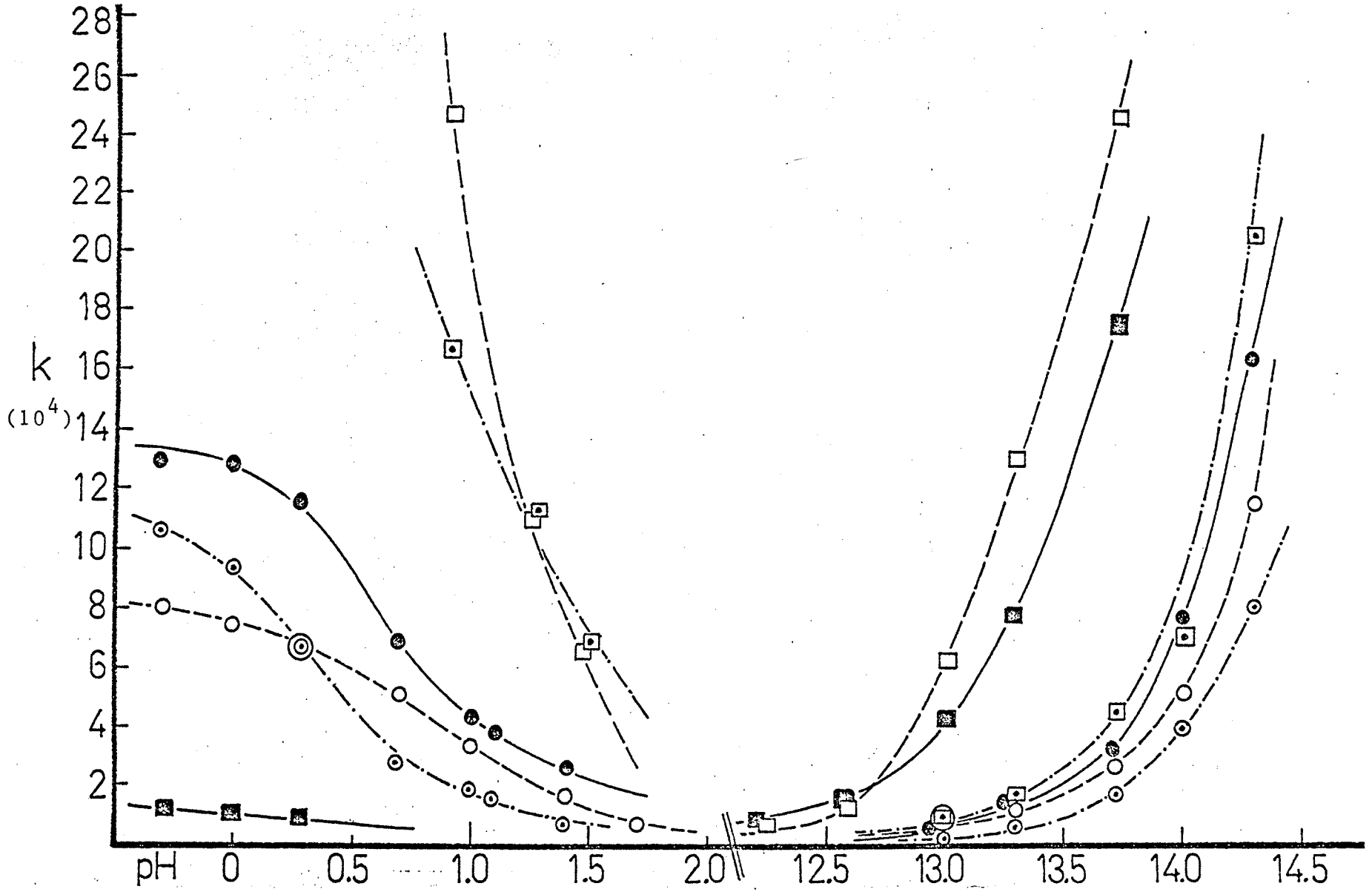

28
26
24
22
20
18
k 16
(10^4) 14
12
10
8
6
4
2
pH 0 0.5 1.0 1.5 2.0 12.5 13.0 13.5 14.0 14.5

FIGURE 20

Plots of k_{obs} (s^{-1}) vs $[OH^-]$ for the alkaline hydrolysis of O-methylated pyrimidine nucleosides.

m^4U ●

m^4dU ○

m^4dT ⊙

m^2U ■

m^2dU □

m^2dT ⊡

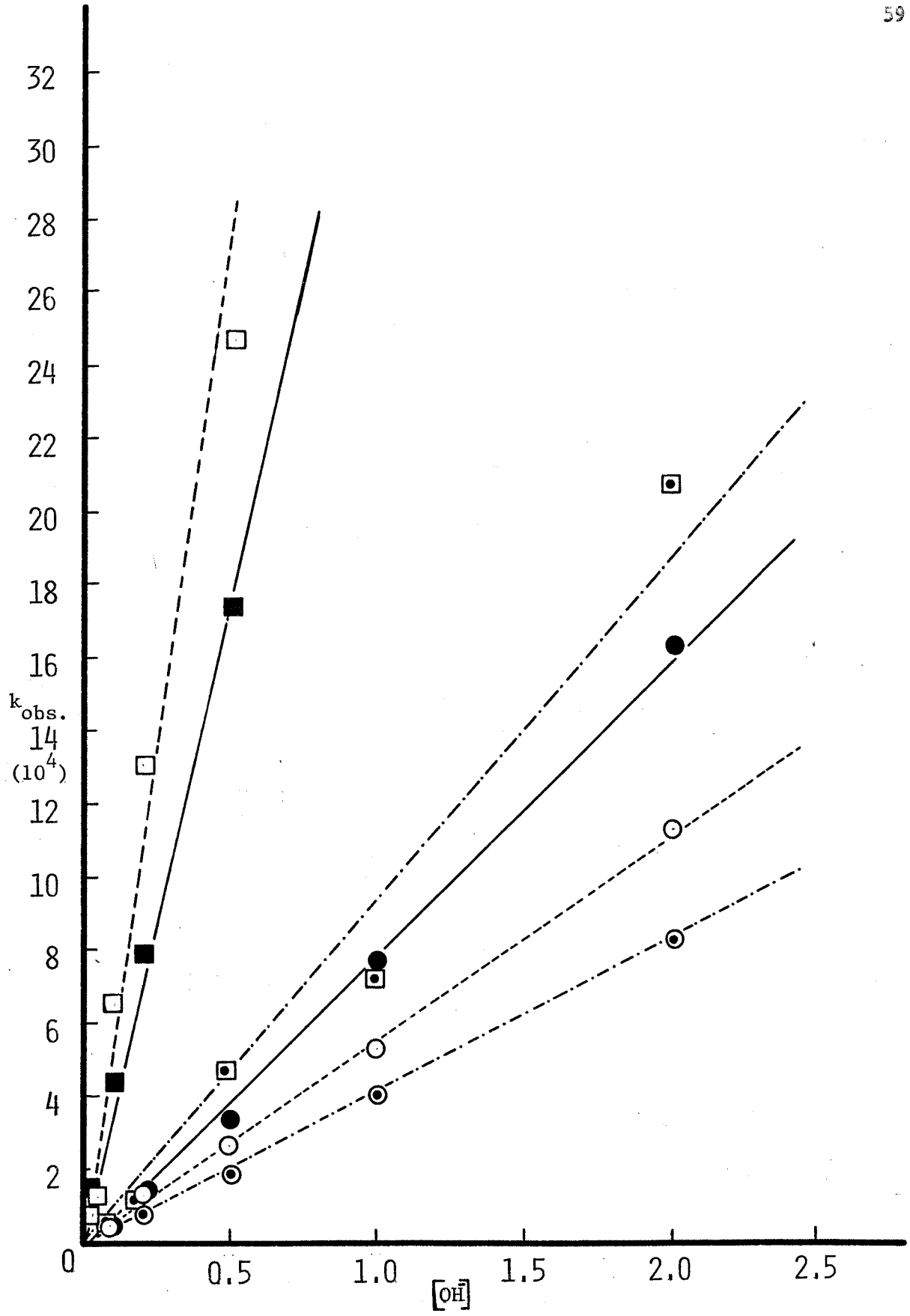

32
30
28
26
24
22
20
18
16
$k_{obs.}$
14
(10^4)
12
10
8
6
4
2
0
0.5
1.0
1.5
2.0
2.5
[OH⁻]

TABLE 9

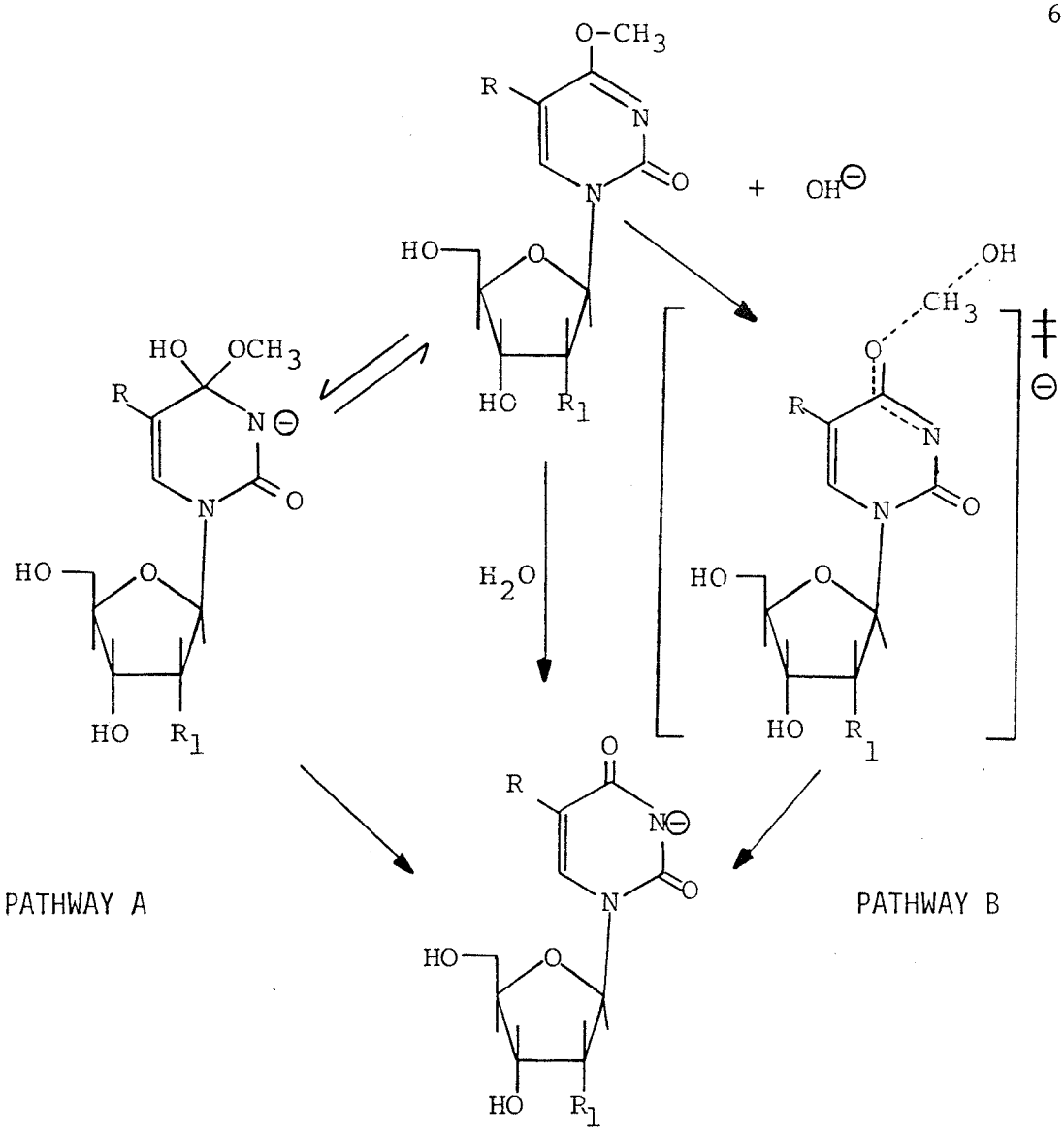
Rate Constants for the Alkaline Hydrolysis of O-Methylated Nucleosides at 25°C.

	k_{OH} ($\times 10^4$ $l.m^{-1}.s^{-1}$)	k_{H_2O} ($\times 10^4$ s^{-1})
m^4U	8.24 ± 0.28	-0.20 ± 0.29
m^4dU	5.63 ± 0.18	0.12 ± 0.15
m^4dT	4.13 ± 0.06	-0.05 ± 0.06
m^2U	34.27 ± 1.07	0.59 ± 0.26
m^2dU	49.75 ± 4.50	0.69 ± 1.10
m^2dT	10.37 ± 0.99	-0.85 ± 1.03

FIGURE 21

Proposed reaction pathways for the alkaline hydrolysis of O^4- methylated pyrimidine nucleosides.

Similar pathways are available for the alkaline hydrolysis of O^2-methylated pyrimidine nucleosides.


O-CH3
R
N
O
+ OH⊖
HO
O
HO R1
HO OCH3
R
N⊖
O
N
O
HO
O
HO R1
H2O
OH
CH3
O
R
N
N
O
HO
O
HO R1
‡
⊖
R
O
N⊖
N
O
HO
O
HO R1
PATHWAY A
PATHWAY B

The mechanism of cleavage of the C(4)-O-CH_3 bonds differs in the two pathways, therefore, in principle it should be possible to distinguish between them using O^{18} labelled compounds. However, this work has not yet been carried out.

a) The effect of the sugar moiety

The values of k_{OH^-} decrease in the order

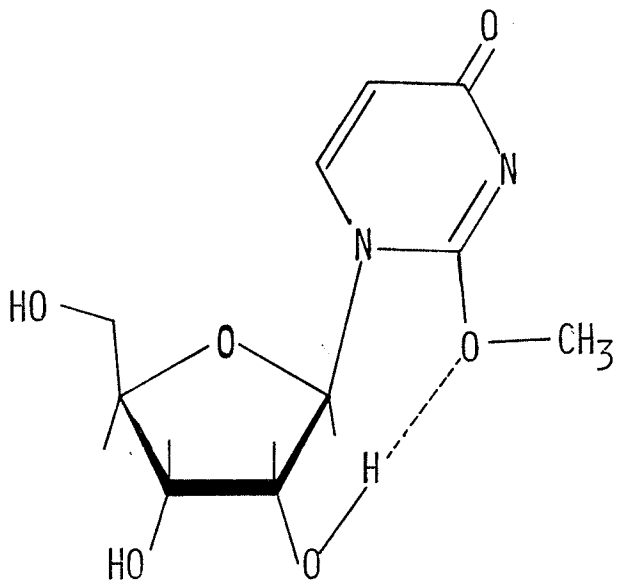
$$m^2dU > m^2U \gg m^2dT > m^4U > m^4dU > m^4dT$$

The observed rate difference between m^4U and m^4dU may be understood in terms of the electron withdrawing effect of the 2'-hydroxyl group present in the ribose nucleoside. The attack of the nucleophile, on either the base ring or on the methyl group, would be enhanced with respect to that for the deoxy analogue.

Consequently, the difference between m^2dU and m^2U requires some other explanation. As has been illustrated in Figure 22, an intramolecular hydrogen bonding interaction may occur for m^2U. As a result, there would be considerable steric hinderance to attack by hydroxide ions on C(2) of the base ring (Figure 21, pathway A). Such an effect would be absent in the deoxy analog and also unimportant in the O^4-methylated nucleosides. Direct evidence for an S_NAr mechanism for alkaline hydrolysis of methoxypyrimidines is not available, but the acid catalyzed

FIGURE 22

Possible intramolecular H-bonding scheme for m^2U.


O
N
N
HO
O
O
CH3
H
HO
O

hydrolysis of 2-methoxypyrimidines has been shown by O^{18} tracer studies to proceed via a similar mechanism[30].

b) The effect of the C(5)-Methyl Group

In both the O^2 and O^4-methyl series, k_{OH} (mdU) > k_{OH} (mdT). The observed decrease in rate as a result of C(5)-methyl group is, as would be expected, a consequence of the increased electron density in the pyrimidine rings.

2. Hydrolysis of O^4-Methylated Nucleosides in Acidic Solutions

The acid catalyzed hydrolysis of the O^4 and O^2-methylated nucleosides have been considered separately because, in the latter cases only, cleavage of the N-glycosidic link is an important reaction, in some cases preceeding demethylation.

NMR studies of the acid catalyzed hydrolysis of m^4U, m^4dU and m^4dT showed that, over a period of several days, the only products were methanol and the corresponding nucleoside. There was no evidence for the cleavage of the N-glycosidic bond.

Studies of the rates of hydrolysis were carried out at 25°C and 2M ionic strength. The plots of the pseudo first order rate constants, k_{obs}, against pH (Figure 19)were sigmoidal and showed inflection points close to the corresponding pKa values of the methylated nucleosides. The results suggest that

hydrolysis of the O^4-methylated nucleosides involved attack by water on the N(3)-protonated compounds as shown in Figure 23. Since the rates of reaction at pH values between 5 and 8 were very low, water catalyzed hydrolysis must be a very slow process.

On the basis of the mechanism in Figure 23, it can be shown (Appendix II) that:

$$k_{obs} = k_{H_2O} + \frac{k_1 [H_3O^+]}{K_a + [H_3O^+]} \qquad (2)$$

Since there was no evidence of demethylation with water at pH 7 over several days, k_{H_2O} must be small and may be neglected. This conclusion is supported by the plots in Figure 20, which indicate $k_{H_2O} \sim 0$. Hence we can write:

$$k_{obs} = \frac{k_1 [H_3O^+]}{K_a + [H_3O^+]} \qquad (3)$$

This, in turn, may be rewritten as:

$$\frac{1}{k_{obs}} = \frac{K_a}{k_1} \cdot \frac{1}{[H_3O^+]} + \frac{1}{k_1} \qquad (4)$$

In agreement with this equation, plots of k_{obs}^{-1} against $[H_3O^+]^{-1}$ were linear, Figure 24. Values of the pKa and k_1 for the hydrolysis of the various nucleosides are listed in Table 10.

The general trend observed for the NMR acid titration curves (see Section III, c), from which pKa values of less than 1 were suggested for m^4U and m^4dU, is in agreement with those values

FIGURE 23

Proposed acid-catalyzed hydrolysis of O^4-methylpyrimidine nucleosides.

R = H, R_i = OH, m^4U.

R = H, R_i = H, m^4dU.

R = CH_3, R_i = H, m^4dT.

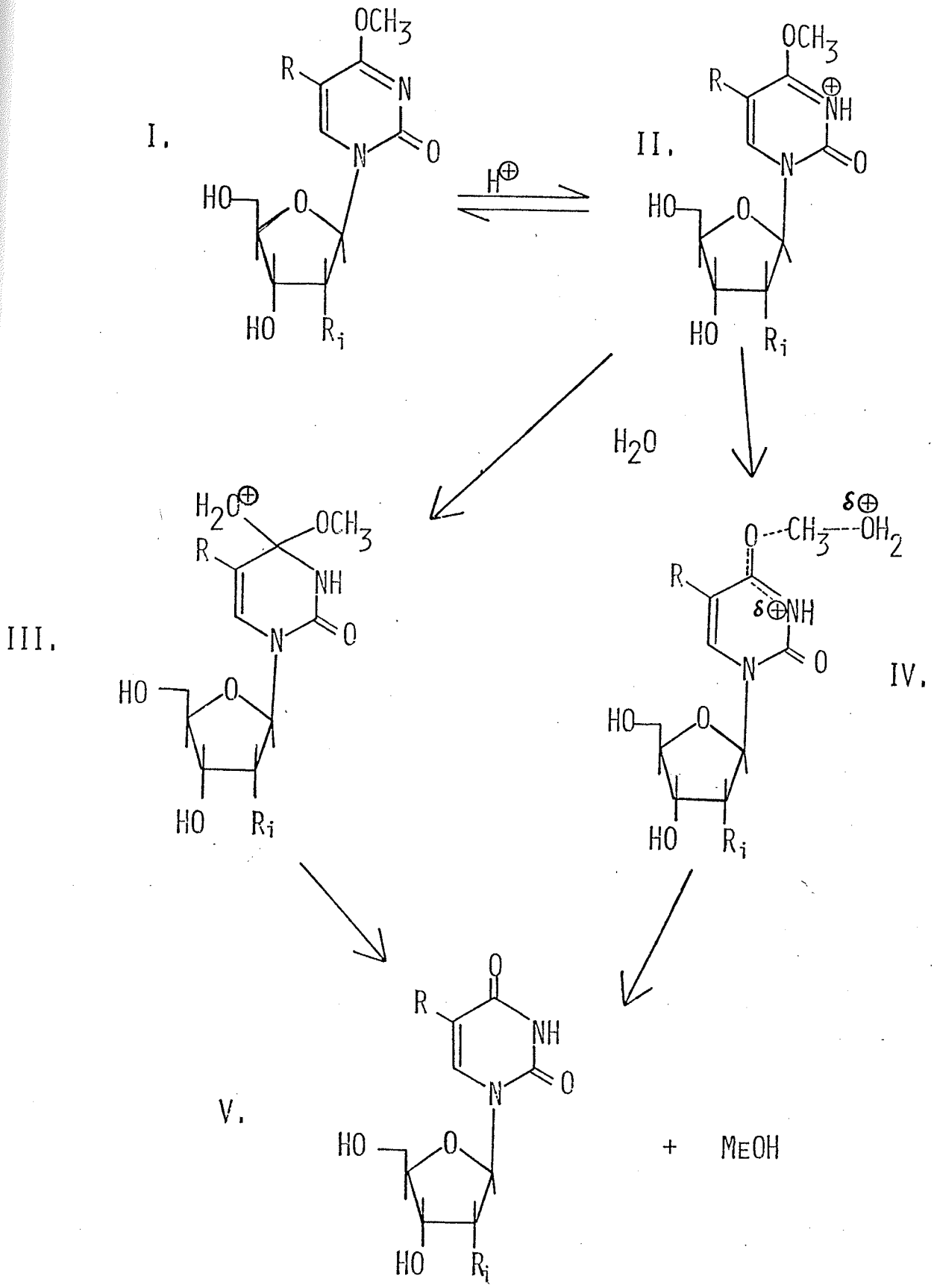

I.
II.
III.
IV.
V.
$H^{\oplus}$
H_2O
$H_2O^{\oplus}$
OCH_3
R
HO
R_i
NH
N
O
$\delta\oplus$
CH_3
OH_2
+ MeOH

TABLE 10

Rate Constants and Equilibrium constants for the Acid Catalyzed Hydrolysis of O-methylated Nucleosides at 25°C.

	k_1 ($\times 10^3$ s^{-1})	K_a	pK_a	K_b ($\times 10^{14}$)
m^4U	1.36 ± 0.13	0.184 ± 0.024	.735	5.43
m^4dU	1.05 ± 0.15	0.200 ± 0.038	.699	5.00
m^4dT	1.29 ± 0.30	0.556 ± 0.151	.255	1.80
m^2U	0.14 ± 0.01	0.223 ± 0.011	.652	4.48
m^2dU*	6.65 ± 5.90	0.270 ± 0.254	.569	3.70
m^2dT*	3.29 ± 0.28	0.111 ± 0.013	.955	9.01

*at 32°C

FIGURE 24

Plots of $\frac{1}{k_{obs}}$ (s) vs $\frac{1}{[H_3O^+]}$ ($l \cdot m^{-1}$) for the acid catalyzed hydrolysis of O-methylated pyrimidine nucleosides.

Two different Y-axes scales are shown to accomodate all data on one figure.

Left axis: m^4dT, m^4dU, m^2U.

Right axis: m^2dT, m^2dU, m^4U.

m^4U ●

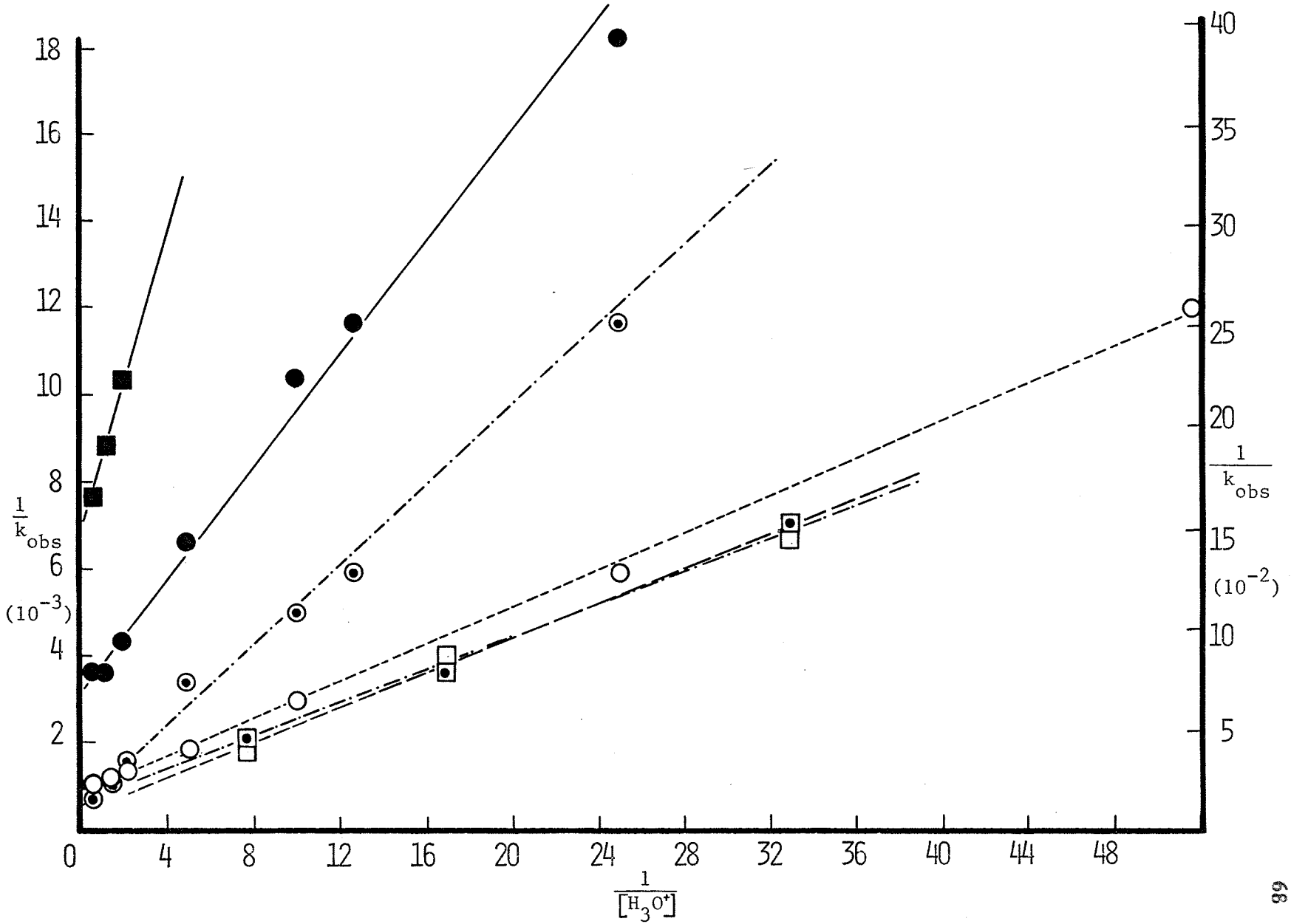
m^4dU ○

m^4dT ⊙

m^2U ■

m^2dU □

m^2dT ⊡


18
16
14
12
10
8
6
4
2
$\frac{1}{k_{obs}}$
(10^{-3})
40
35
30
25
20
15
10
5
$\frac{1}{k_{obs}}$
(10^{-2})
0
4
8
12
16
20
24
28
32
36
40
44
48
$\frac{1}{[H_3O^+]}$

given in Table 10. An inconsistancy is seen from m^4dT, the NMR data suggesting a pKa value < 0 while kinetic results give this value as 0.25. Spectrophotometric data by Singer et al.[44] for O^4-ethylated nucleosides gave pKa values of 0.66 (et^4dU) and -0.32 (et^4eT). The differences in the NMR and kinetic data for m^4dT may be the result of a salt or a temperature effect.

a) The effect of the sugar moiety

From the data in Table 10, the K_B values for m^4U and m^4dU are the same within experimental error. An almost similar result occurs for the k_1 values of m^4U and m^4dU, with m^4U having the slightly larger value. These results indicate that the effect of the different sugars is very small or negligible.

b) The effect of the C(5)-Methyl Group

The data in Table 10 show that m^4dT is a weaker base than m^4dU. This is unexpected since the C(5)-methyl group in m^4dT will release electrons and cause an increase in electron density at the ring nitrogens. However, molecular models show that the C(5)-methyl group inhibits the free rotation of the C(4)-OCH_3 group and forces it into close proximity to the nitrogen at position 3. Two effects can be considered to decrease the basicity of the N group at position 3:

i) Steric hinderance to protonation.

ii) The hydrophyllic proton might be repelled by the hydrophobic

environment around the nitrogen atom at position 3.

Such effects would be absent in m^4dU due to free rotation of the methoxy group.

The data in Table 10 also show that the k_1 values for m^4dT and m^4dU are the same within experimental error. The plot of k_{obs} vs pH (Figure 19), however, does indicate a difference in rates between m^4dT and m^4dU. This observed difference is reflected in the size of the K_a and the deciding rate factor, therefore, is the protonation reaction.

3. Hydrolysis of O^2-Methylated Nucleosides in Acidic Solutions

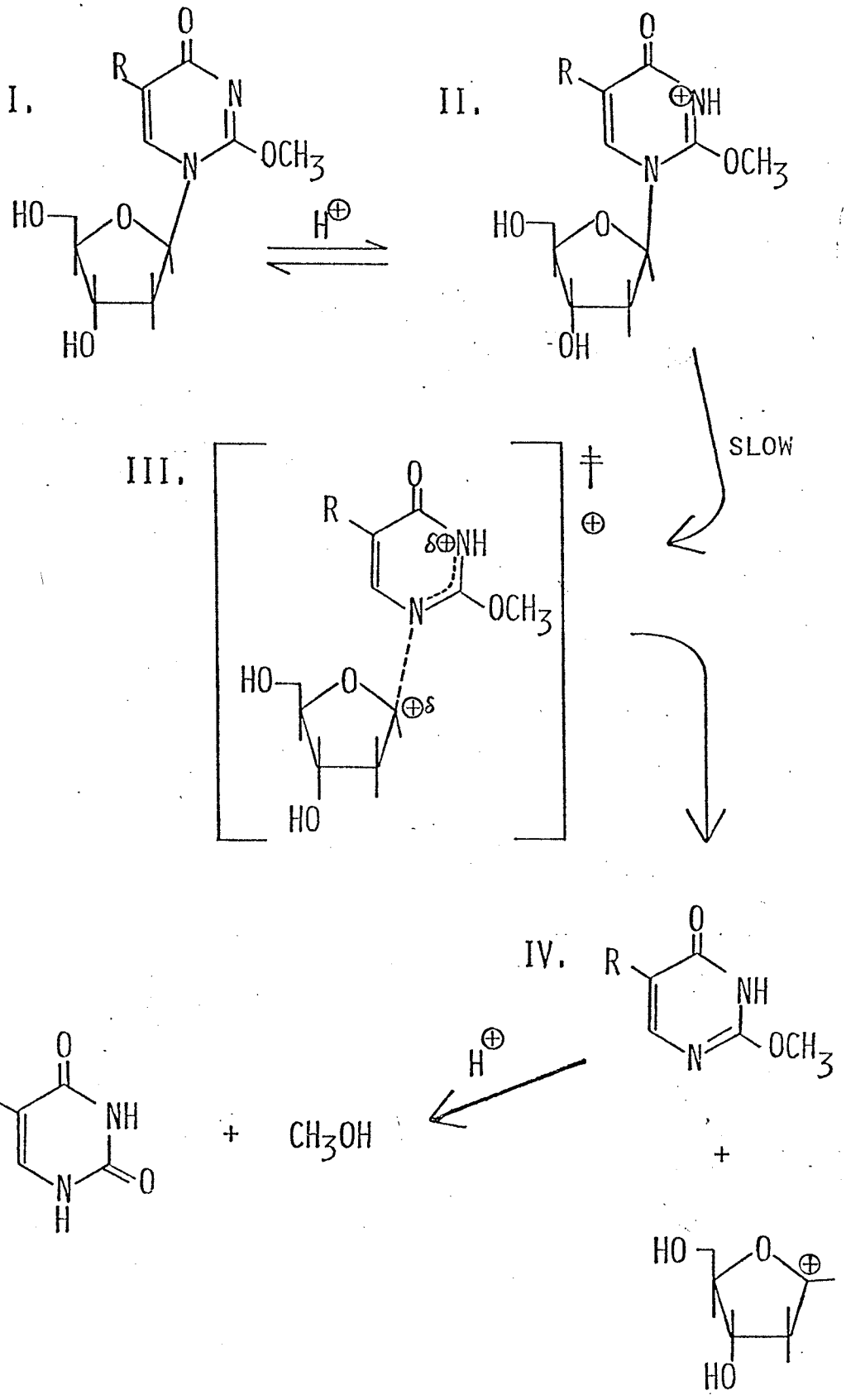
These nucleosides are considered separately because the 2'-deoxy compounds initially react at the N-glycosyl bond to yield 2'-deoxyribose and the corresponding O^2-methyl pyrimidines. On the other hand, m^2U reacted similarly to the O^4-methylated nucleosides to form methanol and the parent nucleoside. The difference between the ribose and 2'-deoxy compounds is readily understood on the basis of the proposed hydrolysis process of the N-glycosyl bond (Figure 25), for which the low reactivity of ribonucleosides is well documented[34,42]. This mechanism, discussed in Chapter I, B(2) involves the formation of a stabilized carbonium ion at the C1' position of the sugar. Such carbonium ions would be "de-stabilized" by the inductive, electron withdrawing

FIGURE 25

Proposed acid-catalyzed hydrolysis of O^2-methylpyrimidine 2'-deoxyribonucleosides.

R = H, m^2dU.

R = CH_3, m^2dT.


I.
II.
III.
IV.
V.
SLOW
+ CH3OH
+

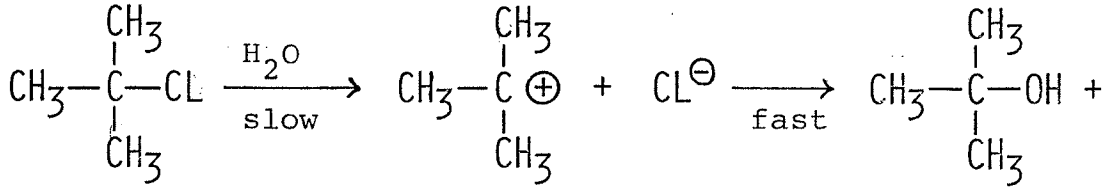
effect of a 2'-hydroxyl group so that the carbonium ion would be less readily formed for ribonucleosides than 2'-deoxy-ribonucleosides.

This adjacent group effect may be compared to work done by Robertson[69] on t-butylchloride and 1-methoxy-2-chloro-2-methylpropane (Figure 26). The inductive effect of the methoxy group (B), decreases the rate of formation of the carbonium ion by a factor 300 with respect to (A).

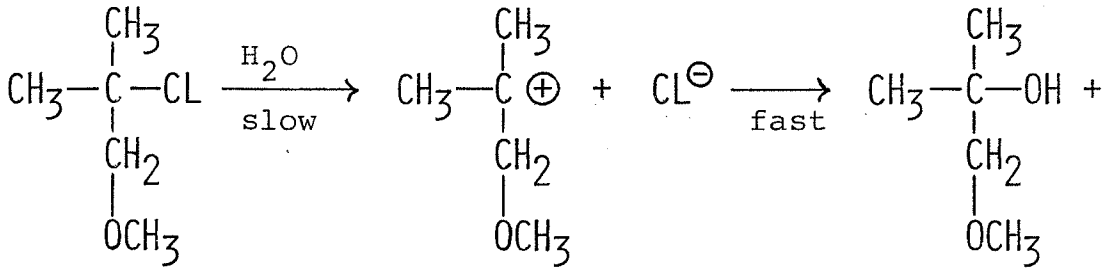
The m^2U reacted very slowly, even in 2M hydrochloric acid solution and it has not been possible to detect an inflection in the plot of k_{obs} vs pH (Figuer 19). Nevertheless, it seems likely that the reaction is similar to those of the 0^4 analogs and values of K_a and k_1 have been calculated from plots of k_{obs}^{-1} vs $[H_3O]^+$ (Figure 24). These values have been given in Table 10, where they can be compared with those for the 0^4 nucleosides. An independant value of K_a (0.13) was obtained for m^2U from plots of λ_{max} vs pH, (Figure 27) and is in fair agreement with that listed in Table 10. Unfortunately, this value is subject to errors because of the appreciable decomposition of m^2U which occurred at low pH values during the time required to carry out the spectral measurements. The K_A values obtained for m^2U are similar to those values obtained for the 0^4 nucleosides, implying that protonation of N3 is the important feature of demethylation in all nucleosides.

FIGURE 26

The effect of an adjacent, inductive, electron withdrawing group on the rate of formation of a carbonium ion.

A. 

$k_i = 300$

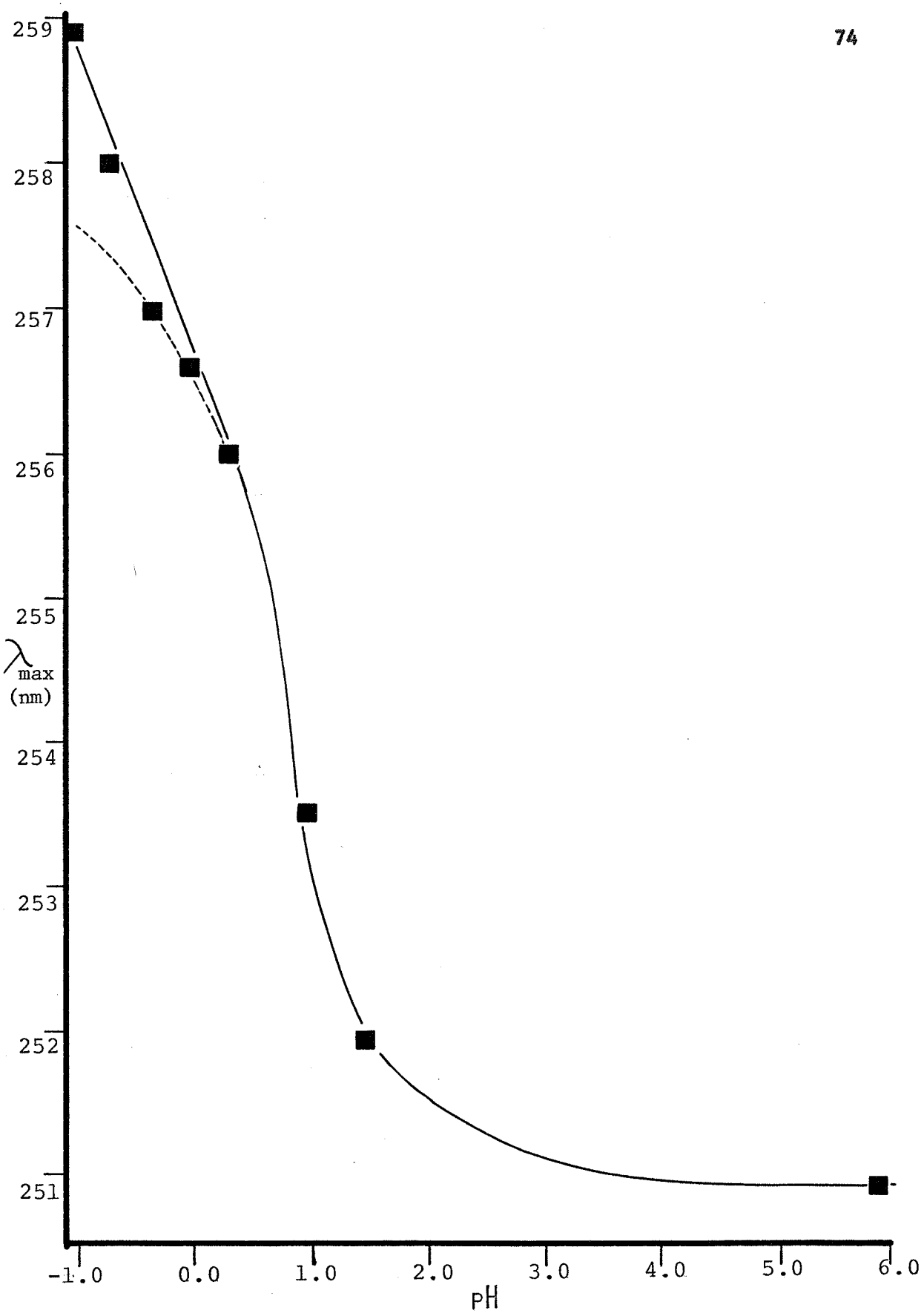
B. 

$k_{ii} = 1$

FIGURE 27

A plot of λ_{max} vs pH for m^2U.

A complete sigmoidal curve could not be obtained due to the rapid decomposition of m^2U for pH $\leq$ 0.5.


259
258
257
256
255
λmax (nm)
254
253
252
251
-1.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
pH

The low value of k_1 probably reflects considerable steric hinderance to attack by water on the methoxy group.

The hydrolysis of the m^2dU and m^2dT could not be followed by U.V. absorption spectroscopy because the chromophores in both reactants and products were methoxypyrimidines, which have similar spectra. The reactions were followed using FT-NMR which limited the study to reactions with $k_{obs} < 3 \times 10^{-3}$ s^{-1}. At pH = 0.5 the reaction was too fast to measure so that no inflection point could be observed in the k_{obs} vs pH plots (Figure 19). Nevertheless, approximate values of K_a and k_1 were obtained from the plots of k_{obs}^{-1} vs $[H_3O^+]^{-1}$ (Figure 24), (Table 10). Figure 25 gives a proposed mechanism for the hydrolysis of m^2dU and m^2dT.

4. Kinetics Summary

In this thesis we have determined the approximate pKa values for m^4dU, m^4dT, m^4U, m^2dU, m^2dT and m^2U at 25°C. Similar data has been obtained for the ethylated 2'-deoxy analogs by Singer et al.[44] and, in general, the data are comparable suggesting little effect on the pKa value in changing from an O-methyl to an O-ethyl substituent.

Rate profiles for all O-methylated nucleosides listed above were recorded in acidic and alkaline solutions. This data may be useful synthetically when these modified nucleosides are

incorporated into oligomer strands.

From the kinetic and NMR data for the depyrimidination reactions of m^2dU and m^2dT, results suggest the base protonation mechanism as there was no evidence for anomerization or isomerization. The remaining compounds, m^4U, m^4dU, m^4dT and m^2U underwent dealkylation in acid media.

In alkaline solutions, all O-methylated nucleoside studies underwent dealkylation. Contrary to this result Singer et al.[44] observed depyrimidination as the major process for et^2dT and et^2dU at pH - 12.5. The change of mechanism between the two systems may be attributed to the temperature difference, Singer's values being obtained at 100°C.

The observed reactivity in alkaline media was (m^2-nucleosides) > (m^4-nucleosides). Considering the work by Wong and Fuchs[46] on 2,4-dialkoxypyrimidines in alkaline solution, the observed rate order was surprising, as their results showed hydroxide ion to have a specificity for the 4-position. The inversion of reaction order may be a temperature effect as reflux conditions were employed by Wong and Fuchs[46].

CHAPTER IV

SUGGESTIONS FOR FUTURE RESEARCH

Kinetic and NMR studies of the hydrolysis of the O-methylated pyrimidine nucleosides have shown the importance of the structure of the sugar moiety in the mechanism of acid catalyzed hydrolysis. The O^2-methyl ribose compounds were shown to demethylate while the O^2-methyl-2'-deoxyribose compounds undergo depyrimidination. A similar study involving an arabinose nucleoside would be of interest especially considering the extra steric and electronic effects resulting from the endo 2'-hydroxyl group in the arabinose sugar.

The alkylation of nucleic acids has been implicated as a cause of mutagenesis and carcinogenesis. It would be of interest to study the effects of metal ions on the stability of the O-methylated pyrimidine nucleosides, as they may be involved in the removal of the modification. In this regard, studies have been undertaken in this lab to obtain information on the effects of O-alkylation of pyrimidine nucleosides on metal binding.

Also included in the future plans of this research group are the incorporation of these O-methylated nucleosides into oligomer strands and to determine the effects of the modified nucleoside on the physical chemical properties of the oligomer.

The data which have been provided in this study, combined with future results will aid in a better understanding of the effects of O-alkylation of nucleic acids.

APPENDIX

APPENDIX I

Rate Constants for the Hydrolysis of O-methylated pyrimidine nucleosides from U.V. spectrophotometric studies, 25°C.

A m^4U

B m^4dU

C m^4dT

D m^2U

E m^2dU

F m^2dT

A. m^4U

λ261 $[H_3O^+]$	pH	k_{obs} ($x10^4S^{-1}$)	r	k_{av} ($x10^4S-1$)
2.000	-0.3	12.72	0.9999	
		12.71	0.9999	12.72
		12.71	0.9999	
1.000	0.0	12.92	0.9999	
		12.94	0.9999	12.93
		12.92	0.9999	
0.500	0.3	11.17	0.9999	
		11.15	0.9999	11.16
		11.14	0.9999	
0.200	0.7	6.94	0.9999	
		6.94	0.9999	6.94
		6.94	0.9999	
0.100	1.0	4.41	0.9999	
		4.33	0.9998	4.38
		4.12	0.9999	
0.080	1.1	3.95	0.9999	
		3.96	0.9999	3.95
		3.93	0.9999	
0.040	1.4	2.49	0.9999	
		2.51	0.9999	2.51
		2.53	0.9999	
$[OH^-]$				
0.100	13.0	0.95	0.9999	
		0.93	0.9999	0.94
		0.94	0.9999	
0.200	13.3	1.67	0.9999	
		1.69	0.9998	1.69
		1.70	0.9999	
0.500	13.7	3.40	0.9997	
		3.33	0.9995	3.36
		3.36	0.9996	
1.000	14.0	7.83	0.9997	
		7.64	0.9997	7.78
		7.88	0.9998	
2.000	14.3	16.82	0.9977	
		15.31	0.9980	16.50
		17.36	0.9992	

B. m^4dU, λ261

$[H_3O^+]$	pH	$k_{obs}(10^4).S^{-1}$	r	ave.$k(10^4)S^{-1}$
2.000	-0.3	8.01	0.9960	8.01
		8.02	0.9998	
		8.01	0.9971	
1.000	0.0	7.18	0.9992	
		6.85	0.8959	7.27
		7.79	0.9972	7.49(1,3)
0.500	0.3	6.93	0.9997	
		6.69	0.9997	6.97
		7.29	0.9998	
0.200	0.7	5.11	0.9991	
		5.03	0.9996	5.04
		4.98	0.9998	
0.100	1.0	3.41	0.9996	
		3.39	0.9995	3.40
		3.41	0.9997	
0.040	1.4	1.65	0.9970	
		1.71	0.9993	1.70
		1.72	0.9995	
0.020	1.7	0.84	0.9984	
		0.83	0.9981	0.83
$[OH^-]$		0.82	0.9983	
0.100	13.0	0.86	0.9884	
		0.81	0.9928	0.82
		0.80	0.9928	
0.200	13.3	1.42	0.9977	
		1.35	0.9982	1.37
		1.34	0.9980	
0.500	13.7	2.92	0.9998	
		2.92	0.9999	2.92
		2.92	0.9999	
1.000	14.0	5.41	0.9999	
		5.23	0.9999	5.33
		5.36	0.9998	
2.000	14.3	12.31	0.9999	
		10.34	0.9979	11.57
		12.05	0.9998	

C m^4dT, λ261

$[H_3O^+]$	pH	$k_{obs}(10^4)S^{-1}$	r	av.k(10^4)S-1
2.000	-0.3	9.95	0.9979	10.59
		10.83	0.9998	
		11.00	0.9999	
1.000	0.0	9.39	0.9999	9.40
		9.35	0.9999	
		9,45	0.9999	
0.500	0.3	6.76	0.9997	6.76
		6.73	0.9997	
		6.79	0.9998	
0.200	0.7	2.82	0.9999	2.80
		2.79	0.9999	
		2.78	0.9998	
0.100	1.0	1.96	0.9998	1.97
		1.98	0.9999	
		1.98	0.9999	
0.080	1.1	1.75	0.9998	1.72
		1.70	0.9999	
		1.71	0.9999	
0.040	1.4	0.85	0.9999	0.85
		0.84	0.9999	
$[OH^-]$		0.87	0.9999	
0.100	13.0	0.44	0.9999	0.45
		0.45	0.9995	
		0.48	0.9992	
0.200	13.3	0.82	0.9999	0.82
		0.81	0.9999	
		0.83	0.9984	
0.500	13.7	1.90	0.9998	1.89
		1.88	0.9999	
		1.90	0.9999	
1.000	14.0	4.05	0.9995	4.05
		3.95	0.9993	
		4.15	0.9995	
2.000	14.3	8.33	0.9998	8.26
		8.29	0.9998	
		8.17	0.9998	

D m^2U, H_3O^+ at λ261, OH^- at λ257

$[H_3O^+]$	pH	k_{obs} ($x10^4 S^{-1}$)	r	k_{av} ($x10^4 S^{-1}$)
2.000	-0.3	1.28	0.9990	1.28
		1.27	0.9990	
		1.28	0.9990	
1.000	0	1.18	0.9993	
		1.09	0.9997	1.15
		1.17	0.9992	
0.500	0.3	0.99	0.9997	
		0.96	0.9998	0.97
$[OH^-]$		0.99	0.9995	
0.020	12.3	0.96	0.9997	
		0.96	0.9997	0.96
		0.97	0.9997	
0.040	12.6	1.71	0.9999	
		1.70	0.9999	1.71
		1.71	0.9999	
0.100	13.0	4.32	0.9999	
		4.36	0.9999	4.33
		4.32	0.9999	
0.200	13.3	7.90	0.9998	
		7.91	0.9998	7.91
		7.93	0.9997	
0.500	13.7	17.50	0.9996	
		17.60	0.9996	17.51
		17.44	0.9997	

E m^2dU, λ252

$[OH^-]$	pH	k_{obs} ($x10^4.S^{-1}$)	r	k_{av} ($x10^4.S^{-1}$)
0.020	12.3	0.90	0.9999	0.91
		0.91	0.9999	
		0.91	0.9999	
0.040	12.6	1.37	0.9999	1.36
		1.35	0.9999	
		1.37	0.9999	
0.100	13.0	6.39	0.9990	6.34
		6.35	0.9990	
		6.30	0.9991	
0.200	13.3	11.70	0.9990	13.02
		12.79	0.9980	
		14.55	0.9980	
0.500	13.7	25.61	0.9996	24.62
		23.65	0.9994	
		24.61	0.9990	

m^2dU, depyrimidination rates determined by NMR, 32°C

pM	k_{obs} ($x10^4$, S^{-1})	r
0.9	24.7	0.968
1.2	10.9	0.983
1.5	6.9	0.998

F m^2dT, λ252

$[OH^-]$	pH	k_{obs} ($x10^4.S^{-1}$)	r	k_{av} ($x10^4.S^{-1}$)
0.100	13.0	0.85	0.9998	0.85
		0.85	0.9998	
		0.86	0.9998	
0.200	13.3	1.53	0.9980	1.53
		1.52	0.9980	
		1.54	0.9980	
0.500	13.7	4.61	0.9996	4.67
		4.67	0.9994	
		4.71	0.9994	
1.000	14.0	7.05	0.9994	7.17
		7.19	0.9995	
		7.26	0.9996	
2.000	14.3	20.72	0.9999	20.90
		21.03	0.9998	
		20.96	0.9999	

m^2dT, depyrimidination rates determined by NMR, 32°C

pM	k_{obs} ($x10^4$, S^{-1})	r
0.9	16.7	0.992
1.2	10.9	0.994
1.5	6.7	0.988

APPENDIX II

Rate Equation for the Acid-Catalyzed Hydrolysis of O^4-methyl pyrimidine nucleosides.

For a case:

$$\underset{(a-x-y)}{R\text{-}OCH_3} + H_3O^+ \underset{}{\overset{\frac{1}{K_a}}{\rightleftharpoons}} \underset{y}{HR\text{-}OCH_3} + H_2O \xrightarrow{k_1} \underset{x}{ROH} + CH_3OH$$

$$y = (a-x)\frac{[H_3O^+]}{K_a + [H_3O^+]}$$ if step 1 >> faster than step 2.

$$\frac{d[ROH]}{dt} = k_1(y)$$

$$= k_1(a-x)\frac{[H_3O^+]}{K_a + [H_3O^+]} = k_{obs}(a-x)$$

$$k_{obs} = \frac{k_1[H_3O^+]}{K_a + [H_3O^+]}$$

A. when $y = \frac{a-x}{2}$, i.e. base 1/2 protonated

$K_a = [H_3O^+]$, $pK_a = pH$

$\therefore k_{obs} = \frac{k_1}{2}$.

B. when $[H_3O^+] >> K_a$

$$k_{obs} = k_1$$

C. when $[H_3O^+] << K_a$

$$k_{obs} \longrightarrow 0$$

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