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ENZYMATIC STUDIES OF ANHYDRONUCLEOSIDES AND ANHYDRONUCLEOTIDES

by

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To my husband, Alex.

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ABSTRACT

A study has been undertaken of anhydro analogs of adenosine and uridine with respect to their substrate and/or inhibitor activities toward certain important enzymes.

The anhydro analogs of adenosine were tested with adenosine aminohydrolase from calf intestinal mucosa. It was found that the enzyme seemed to show conformational specificity, hydrolysing compounds in the anti conformation, but remained inactive toward compounds in the syn conformation, such as 8-bromoadenosine[1]. Test compounds in the anti conformation found to be substrates or competitive inhibitors were 8,2'-anhydro-8-mercapto-0-β-D-arabinofuranosyladenine, 8,2'-anhydro-8-oxy-9-β-D-arabinofuranosyladenine, 8,2'-anhydro-8-amino-9-β-D-arabinofuranosyladenine, 8,5'-anhydro-8-oxy-9-β-D-ribofuranosyladenine, and 8,5'-anhydro-8-mercapto-9-β-D-ribofuranosyladenine. Furthermore, in the 8,2'-anhydro-series reactivity increased along the series $N < O < S$. Inhibition by these compounds seemed to follow a reversed order of their reactivity as substrates.

Dinucleotides containing the anhydro analogs of uridine or inosine were tested for their reactivity toward bovine spleen phosphodiesterase and snake venom

phosphodiesterase. Where the anhydro analog was the first base encountered by these enzymes, there was either very little activity or no activity at all. Of the dinucleotides tested, O^2,2'-anhydrouridylyl-(3'-5')-isopropylidineuridine was found to be completely resistant to spleen phosphodiesterase, while thymidylyl-(3'-5')-3'-O-acetyl-O^2,2'-anhydrouridine and thymidylyl-(3'-5')-3'-O-benzoyl-8,2'-thioanhydroinosine showed partial hydrolysis by snake venom phosphodiesterase after 24 hours at 37^o compared to complete hydrolysis of normal dinucleotides after only 5 hours at 37^o.[2]

Conformation does not appear to play a part in the phophodiesterase activity, since both 8-bromoadenosine and 8,2'-thioanhydroadenosine inhibited snake venom phosphodiesterase. Inhibition by the test dinucleotides followed the same order as substrate reactivity. The anhydro purines seemed to be less resistant than the anhydro pyrimidines.

Also reported is the synthesis of the trinucleotide, bis-β-cyanoethyl-deoxyadenylyl-(3'-5')-thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine.

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ABREVIATIONS

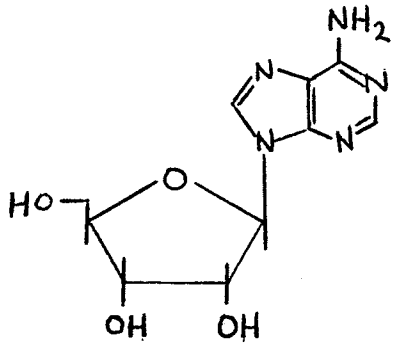
8,2'-SAnA.....	8,2'-Anhydro-8-mercapto-9-β-D-arabinofuranosyladenine
8,2'-NAnA.....	8,2'-Anhydro-8-amino-9-β-D-arabinofuranosyladenine
8,2'-OAnA.....	8,2'-Anhydro-8-oxy-9-β-D-arabinofuranosyladenine
8,5'-OAnA.....	8,5'-Anhydro-8-oxy-9-β-D-ribofuranosyladenine
8,5'-SAnA.....	8,5'-Anhydro-8-mercapto-9-β-D-ribofuranosyladenine
8,2'-SAnI.....	8,2'-Anhydro-8-mercapto-9-β-D-arabinofuranosylinosine
PNP-pT.....	p-Nitrophenyl thymidine 5'- phosphate
Tp-PNP.....	p-Nitrophenyl thymidine 3'- phosphate
$PNP\text{-}pT^{Ac}$.....	3'-O-Acetyl-p-nitrophenyl thymidine 5'-phosphate
AU.....	O^2,2'-Anhydrouridine
AU^{Ac}.....	3'-O-Acetyl-O^2,2'-anhydrouridine
$AUpU^{Iso}$.....	O^2,2'-Anhydrouridylyl-(3'-5')-3'-O-isopropylidineuridine
$TpAU^{Ac}$.....	Thymidylyl-(3'-5')-3'-O-acetyl-O^2, 2'-anhydrouridine
$TpSAnI^{Bz}$.....	Thymidylyl-(3'-5')-3'-O-benzoyl-8,2'-thioanhydroinosine
MTrT.....	5'-O-Monomethoxytritylthymidine
MTrTp(ce).....	β-Cyanoethyl-5'-O-monomethoxytritylthymidine 3'-phosphate
T(s).....	3'-O-(2,4-Dinitrobenzenesulfenyl) thymidine

MTrTp(ce)T(s).....	β-Cyanoethyl ester of 5'-O-monomethoxytritylthymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine
MTrdA^{Bz}p(ce).....	β-Cyanoethyl-N-benzoyl-5'-O-monomethoxytrityldeoxyadenosine 3'-phosphate
MTrdA^{Bz}p(ce)Tp(ce)T(s).	Bis β-cyanoethyl ester of N-benzoyl-5'-O-monomethoxytrityl-deoxyadenylyl-(3'-5')-thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine
Tp(ce)T(s).....	β-Cyanoethyl ester of thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine
dA^{Bz}p(ce)Tp(ce)T(s)....	Bis β-cyanoethyl ester of N-benzoyldeoxyadenylyl-(3'-5')-thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine
8-BrA.....	8-Bromoadenosine

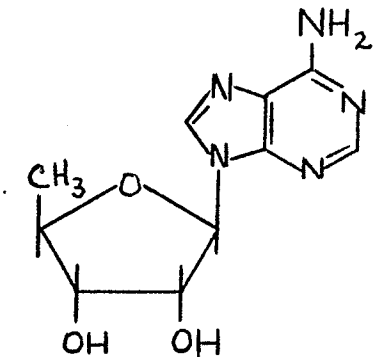
INTRODUCTION

A.) Nucleoside analogs, particularly of the adenosine variety, are of interest in chemotherapy. A great number of potentially useful chemotherapeutic agents have been synthesized which contain nucleoside units. Unfortunately, many of these compounds are rapidly inactivated by deamination in vivo. Cytosine arabinoside and adenine arabinoside have been found to have antitumor and antiviral activity [3,4], but both are deaminated to relatively inactive compounds by the respective cytidine or adenosine deaminase [5,6]. It is of importance, therefore, to determine which modifications on the nucleoside molecule decrease or abolish the susceptibility of adenosine or cytidine analogs to deamination. With such knowledge it would be possible to design effective chemotherapeutic agents that would either be resistant to or inhibit mammalian deaminases.

Previous studies with calf intestine adenosine deaminase have shown that various modifications can be made in the carbohydrate moiety of adenosine to produce analogs that still retain all or part of their ability to be bound to the enzyme. Only the 5'-OH group of adenosine (I) and its analogs plays a special role in the deamination reaction. The 5'-deoxynucleosides of adenine (II) do not undergo deamination unless a properly

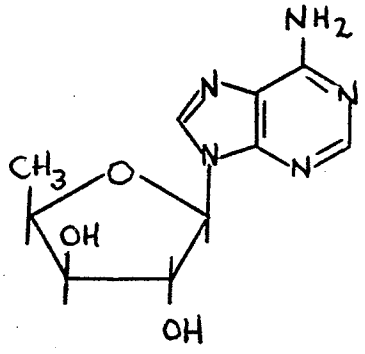


I.

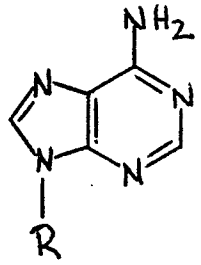


II.

positioned hydroxyl group is present at C-3', as in 9-(5'-deoxy-β - D - xylofuranosyl) adenine (III), such that the 3'-OH group can assume the function of the 5'-OH group of adenosine [7-9].



III.



IV.R, $-CH_2CH_2CH_3$

V.R, $-CH_2CHOHCH_3$

VI.R, $-CH_2CH_2CHOH$

VII.R, $-CH_2CHOHCHOH$

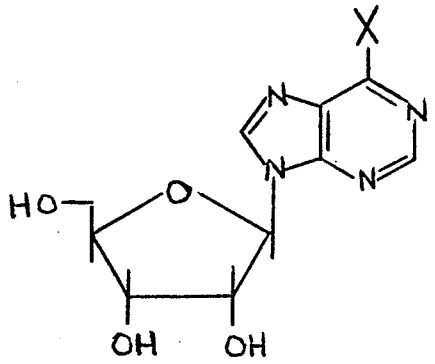
The 2'- and 3'-OH groups of adenosine do not play a critical role in substrate activity when a 5'-OH is present, for it has been found that 2'-deoxy-, 3'-deoxy-, and even 2',3'-dideoxyadenosine undergo deamination [10].

A comparison of the 6-amino purines which were substituted at the 9-position by n-propyl (IV), 2-hydroxypropyl (V), 3-hydroxypropyl (VI), and 2,3-dihydroxypropyl (VII) groups established that there is only one hydroxyl binding site on adenosine deaminase in the area two to three carbons removed from the 9-position of the purine nucleus [11]. Schaeffer [12] has shown that the mode of binding of the hydroxyl group on the alkyl chain at the 9-position of adenine is by means of a hydrogen bond. The H of the hydroxyl on the alkyl chain forms a H-bond with an electronegative atom on the enzyme.

Other acyclic analogs of adenosine, the 9-alkyladenines, were found to bind to adenosine deaminase only as inhibitors [13]. The amount of inhibition increases as the 9-alkyl group is lengthened from methyl to n-octyl. Furthermore, it appears that the 9-alkyl group of 9-alkyladenines binds to the enzyme outside of the active site. Because of the length and flexibility of the 9-alkyl group, for example, in 9-n-octy-

ladenine, it is possible for the 9-n-octyl group to bridge to a nonpolar region of the enzyme to which the normal 9-substituent (β -D-ribofuranosyl) of the substrate is neither attracted to nor able to reach. Schaeffer's work then, has revealed two facts about the enzyme which may prove useful in the synthesis of inhibitors. There is a hydrophobic region just outside the active site to which 9-alkyl groups up to n-octyl may bind and also, there exists a polar region within the active site two to three carbons removed from the 9-position of adenine which binds a hydroxyl group.

The availability of the calf intestine enzyme enabled other workers to examine the properties of this enzyme from the point of view of ring-modifications. A number of ring-modified compounds have been studied as substrates and/or inhibitors of the enzyme. Adenosine deaminase exhibits considerable nonspecificity with respect to the leaving group in the 6-position. It catalysed the hydrolytic cleavage of nitrogen-, halogen-, oxygen-, and sulfur- leaving groups from 6-substituted purine ribonucleosides [14,15] (VIII - XI). On the other hand, the enzyme has little bulk tolerance for groups on the 6-amino group of the purine nucleus [16]. For instance, N^6-n-propyladenosine, N^6,N^6-diethyladenosine and N^6-benzoyladenosine are neither substrates nor inhibitors.



VIII., X = NH_2

IX., X = Cl,Br,I

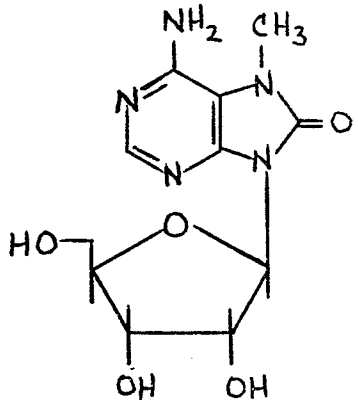
X., X = OCH_3

XI., X = SH

Drummond [17] proposed that the basic mechanism of action of the enzyme was not a specific amino hydrolysis, but appeared to constitute the catalysis of a nucleophilic displacement by hydroxyl at the 6-position of the purine system. Wolfenden [18] suggested a mechanism involving rate-limiting attack on the 6-position of purine derivatives to form a tetrahedral intermediate of the kind encountered in nucleophilic aromatic substitution. If nucleophilic attack constitutes a part of the rate-limiting step in the reaction, Vmax values might be expected to be raised by electron-withdrawing groups in the ring system. Wolfenden found that the Vmax for adenine was enhanced by the substitution of O and S atoms for the 8-carbon, and the Vmax for deoxyadenosine was enhanced by substitution of N for the 8-carbon [19].

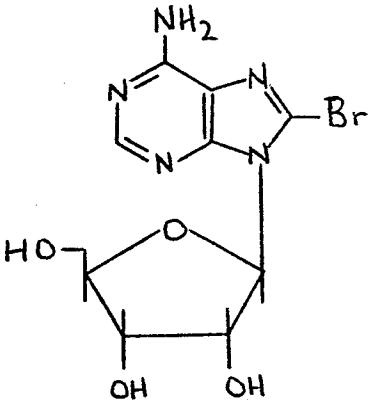
Robins[1] states that the non-activity of some 8-substituted adenosine molecules may be due to steric

hindrance; for example, 8-bromoadenosine and 8-thioadenosine, or due to a change in electronic configuration, such as in the case of 1-methyl-8-oxoadenosine (XII).

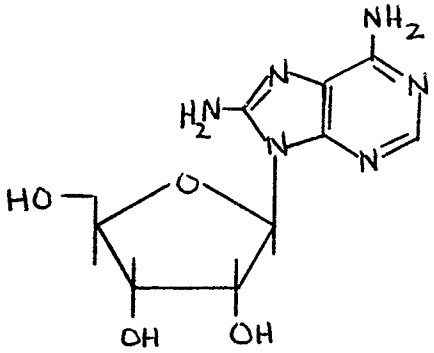


XII.

So far, in this short review of the literature, no mention has been made of the conformational specificity of adenosine deaminase, if any. It is the object of this work to establish whether such specificity does indeed exist for nucleoside subtrates of the enzyme. Robins' work indicates that molecules such as 8-bromoadenosine (XIII) are not substrates because they exist in the <u>syn</u> conformation. Other molecules such as 8-aminoadenosine (XIV) where the substituent is small enough to permit the purine ring to remain in an <u>anti</u> conformation act as substrates.

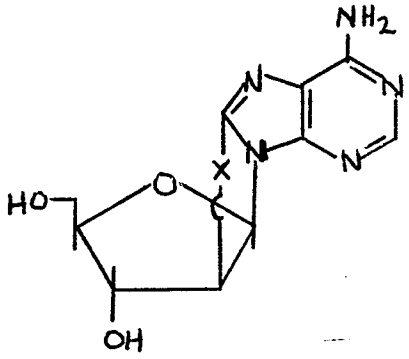


XIII.



XIV.

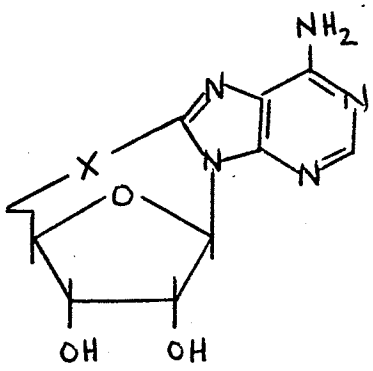
The anhydronucleoside derivatives (XV-XX) of adenosine are ideal in this type of study since the base is constrained in an anti conformation by the anhydro linkage.



XV., X = S

XVI., X = O

XVII., X = NH



XIX., X = S

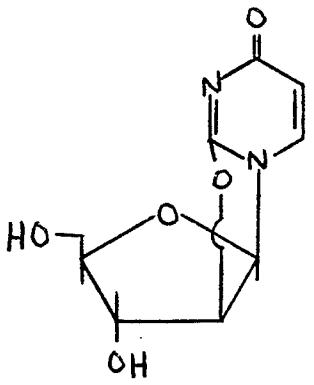
XX., X = O

A number of these molecules were available for our conformational studies. From the reactivity of these nucleoside derivatives towards adenosine deaminase we may be able to propose some type of conformational specificty for the enzyme. It may also be possible to make a correlation between reactivity and the nature of the hetero atom in the anhydro linkage.

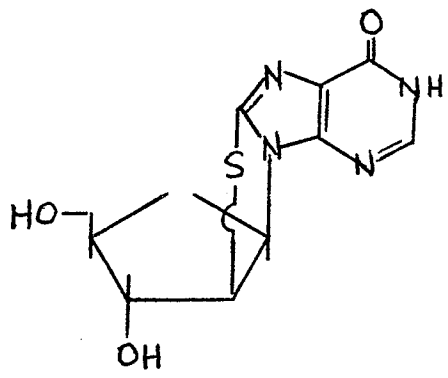
Thus, if the conformational specificity of adenosine deaminase could be established, the design of chemotherapeutic agents of the nucleoside type might be facilitated.

B.) Polynucleotides resistant to nucleases are of great importance to biochemists. Such polymers allow the storage of information in a rather stable form and might be useful as templates for polymerases and messenger - RNA. The only reported polyribonucleotides showing some resistance to nucleases have been ones containing either alternating phosphate and thiophosphate [20] groups or thiophosphate [21] groups exclusively. These polymers are also biologically active as messengers[22] in <u>in vitro</u> protein synthesis. Eckstein's [23] work on thiophosphate polymers has shown that they stimulate interferon production. This increase in antiviral activity is associated with increased resistance to

enzymatic degradation by phosphodiesterases and nucleases. Now, we have discovered another type of molecule that shows some resistance to the phosphodiesterases, the anhydro analogs of uridine and inosine. Several dinucleotides were synthesized which contained the anhydro analog of uridine (XXI) or inosine (XXII). These were available for enzymatic studies with spleen and snake venom diesterase.



XXI.



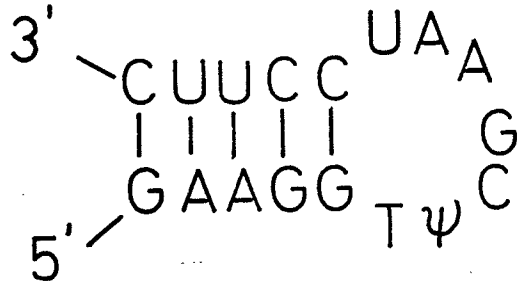
XXII.

On the basis of Eckstein's work, it is very possible that polymers of anhydrouridine might also stimulate interferon production. However, this polymer could not act as a messenger in <u>in vitro</u> synthesis of proteins, since the anhydrouridine base structure does not allow proper Watson-Crick base-pairing.

Thus, the polymers of anhydrouridine may prove to be another source of anti-viral agents.

C.) Another study of interest to us was the possible eluciation of the role played by the TΨCG loop common to all transfer RNAs. If the gene for the TΨCG loop could be synthesized, then binding studies could be conducted in an in vitro protein synthesis system. One would expect the gene to bind to its complement on the transfer-RNA, thereby eliminating the TΨCG loop from being able to participate in possible binding to the ribosomes. This decrease in binding to the ribosome by t-RNA could be detected by a decrease in the incorporation of labelled amino acid in a polypeptide chain.

The TΨCG loop of the transfer-RNA for tyrosine from E. coli is:



The gene coding for this is the complementary 17 base oligodeoxyribosenucleotide: 5' d-GAAGGATTCGAACCTTC 3' end. The proposed synthesis of this gene utilizes the method of phosphotriester intermediates [24]. An attempt will be made to synthesize this oligodeoxyribosenucleotide in two halves, starting from the 3' end. The

final condensation of these two molecules would involve the first half blocked at the 5' end and the second half blocked at the 3' end.

Such an oligodeoxyribosenucleotide would be of great biological importance not only in binding studies, but also may give an insight into the loop conformations characteristic of transfer-RNA.

EXPERIMENTAL

General Methods

Descending paper chromatography was carried out using Whatman 3MM paper. The solvent systems employed were: Solvent A, isopropyl alcohol-concentrated ammonium hydroxide-water (7:1:2); Solvent L, ethanol-water (7:3); Solvent I, butanol-water (86:14) and Solvent C, 1M ammonium acetate-ethanol (3:7). The solvents were prepared on a volume basis. Thin-layer chromatography was carried out employing the ascending technique in closed jars which were not coated with absorbent paper. All thin-layer chromatography was run on Eastman Chromagram Sheets 6060, silica gel with fluorescent indicator, on strips 10cm. x 2cm. Thick layer chromatography was carried out on glass plates (20cm. x 20cm.) coated with a 2mm. thick layer of silica gel DSF-5 (Mondray Chemicals Ltd.). Nucleosides and their derivatives were detected on paper chromatograms, thin and thick layer sheets using an ultraviolet light source (Mineralite, output $\sim$ 254nm.). Compounds containing p-monomethoxytrityl groups were detected by spraying the papers or thin layer sheets with 10% perchloric acid and drying them in a stream of warm air.

Infrared spectra were obtained on a Perkin-Elmer 337 recording instrument using KBr disks for sample preparation. Ultra-violet spectra were obtained on a Cary 14 instrument using a concentration of 2.5 - 3.0mg of compound dissolved in 100ml of solvent (water or 95% ethanol).

Melting points were determined on a Fisher-Johns melting point apparatus and are reported uncorrected.

Reagents and Chemicals

Reagent grade pyridine was distilled from p-toluenesulphonyl chloride, redistilled from calcium hydride, and stored over Linde Molecular Sieves. Reagent grade dimethyl formamide was distilled from calcium hydride and stored over Linde Molecular Sieves. Reagent grade acetic anhydride was distilled from phthalic anhydride and stored in the dark.

Adenosine deaminase from calf intestinal mucosa, bovine spleen phosphodiesterase and snake venom phosphodiesterase were obtained as lyophilized powders from Sigma Chemical Co. Thymidine, adenosine, 2'-deoxyadenosine, 2'-deoxycytidine and 2'-deoxyguanosine were purchased from Calbiochem. p-Nitrophenyl phosphorodichloridate, 2-mesitylenesulfonyl chloride, 2,4,6-triisopropylbenzenesulfonyl chloride and p-anisyl-

chlorodiphenylmethane were purchased from Aldrich Chemical Co., whereas 2,4-dinitrobenzenesulfenyl chloride came from the Eastman Organic Chemical Co. O^2,2'-Anhydrouridine, 3'-O-acetyl-O^2,2'-anhydrouridine and O^2, 2'-anhydrouridylyl-(3'-5')-3'-O-isopropylidine uridine were kindly donated by Don Iwacha of the University of Manitoba. 8,2'-O-anhydro adenosine was kindly supplied by Dr. Kelvin K. Ogilvie of the University of Manitoba. 8,2'-aminoanhydroadenosine, 8,5'-O-anhydroadenosine, and 8,5'-thioanhydroadenosine were generous gifts from Lewis Slotin of the University of Manitoba.

Synthetic Methods

p-Nitrophenyl thymidine 3'-phosphate and p-nitrophenyl thymidine 5'-phosphate

Both of these compounds were prepared by a modification of the literature procedure [25,26]. All reagents were dissolved in dry pyridine and dried by pumping off the pyridine. The reaction was carried out in the dark and the product isolated by paper chromatography in solvent A. The product had an R_f value of 0.65.

Thymidylyl-(3'-5')-3'-O-acetyl-O^2,2'-anhydrouridine [27], 8,2'-thioanhydroadenosine [28], and 8,2'-thioanhydroinosine [29] were prepared by standard procedures.

5'-O-Monomethoxytrityl-8,2'-thioanhydroinosine

8,2'-Thioanhydroinosine (85mg, 0.31mmole) was dissolved in a mixture of dry pyridine (1ml) and dimethylformamide (2ml) and treated with p-monomethoxytrityl chloride (185mg, 0.60mmole) for 4 days at room temperature. The reaction mixture was concentrated in vacuo and extracted with a mixture of chloroform (10ml) and water (10ml). The chloroform layer was separated, washed with water, and dried over sodium sulfate. This dried chloroform solution was concentrated in vacuo and applied to thick layer plates. The plates were developed in ethyl acetate-ethanol (9:1). The band at R_f 0.10 and the origin band were scrapped off and eluted with tetrahydrofuran (100ml each). Both bands contained a single monomethoxytritylated material with R_f 0.09 (tlc, ethyl acetate) and R_f 0.25 (tlc, ethyl acetate-ethanol, 9:1). The tetrahydrofuran eluants were concentrated in vacuo and precipitated with hexane. The yield was 25% and melting point 164-165°.

An ultra violet spectrum in 95% ethanol showed a maximum at 265nm (ϵ13,700) and a shoulder at 230nm (ϵ20,600).

The infrared spectrum showed a general broadening in the region 2.75 - 3.20$\mu$and principal bands at 5.95, 6.25, 6.65, 8.00, 8.50, 12.10 and 14.4μ.

5'-O-Monomethoxytrityl-3'-O-Benzoyl-8,2'-thioanhydroinosine

5'-O-Monomethoxytrityl-8,2'-thioanhydroinosine (40mg, 0.07mmole) was treated with benzoic anhydride (35mg, 0.15mmole) in 2ml of dry pyridine for 2 days at room temperature. The reacture mixture was concentrated in vacuo and applied to thick layer plates. The plates were developed in ethylacetate-ethanol (9:1). The band at R_f 0.?5 was scrapped off and the product eluted from the silica gel with tetrahydrofuran (50ml). The tetrahydrofuran eluant was concentrated in vacuo and precipitated with hexane. The white powder was collected in a 63% yield, melting point 142-145°. The thin layer chromatographic properties were R_f 0.16 (ethyl acetate) and R_f 0.40 (ethyl acetate-ethanol 9:1).

The ultraviolet spectrum in 95% ethanol showed maxima at 260nm (ϵ 15,400) and 232nm (ϵ 23,800). The infrated spectrum showed a general broadening in the region 2.8 - 3.4μ as well as principal bands at 5.75, 5.95, 6.65, 7.95 (broad), 8.50, 12.10, and 14.20μ(broad).

3'-O-Benzoyl-8,2'-thioanhydroinosine

5'-O-Monomethoxytrityl-3'-O-benzoyl-8,2'-thioanhydroinosine (40mg) was treated with 80% acetic acid

(2ml) at room temperature. After 1/2 hour, 3'-O-benzoyl-8,2'-thioanhydroinosine began to precipitate out. The reaction was continued for 4 more hours and the product filtered and washed with ether. The white powder obtained decomposed gradually upon heating above 205°. The yield was 62%. The thin layer chromatographic properties of 3'-O-benzoyl-8,2'-thioanhydroinosine were: R_f 0.09 (ethyl acetate) and R_f 0.25 (ethyl acetate-ethanol, 9:1).

An ultra violet spectrum in 95% ethanol showed a maximum at 263nm (ϵ 12,400). The infrared spectrum showed a general broadening in the region 2.75-3.5μ and principal bands at 5.80, 6.15, 7.40, 7.95 (broad), 12.70 and 14.00μ.

Thymidylyl-(3'-5')-3'-O-Benzoyl-8,2'-thioanhydroinosine

5'-O-Monomethoxytritylthymidine 3'-phosphate (90mg,0.16mmole), prepared by standard methods [30], and 3'-O-benzoyl-8,2'-thioanhydroinosine (40mg,0.11mmole) were dried by evaporation of dry pyridine (six 2-ml portions). A mixture of dry pyridine (0.5ml) and dry dimethylformamide (0.5ml) was added, but the 3'-O-benzoyl-8,2'-thioanhydroinosine was only sparingly soluble. Mesitylenesulfonyl chloride (44mg, 0.2mmole)

and pyridine (0.2ml) were added and the resulting mixture was stirred. After 15 min. of stirring the 3'-O-benzoyl-8,2'-thioanhydroinosine dissolved and the resulting solution was stirred for 10 hr. After addition of cold water (0.5ml), stirring was continued for 24 hr. The reaction mixture was then diluted with cold water (0.5ml) and extracted with chloroform (three 5-ml portions). The chloroform extracts were combined, washed with water, dried over sodium sulfate and concentrated. 80% Acetic acid (2ml) was added to remove the monomethoxytrityl group. After 6 hr. at room temperature the solvent was evaporated and the reaction mixture was extracted with ether to remove monomethoxytritanol. The residue was dissolved in ethanol and applied to papers developed in solvent L. The band at R_f 0.70 was eluted with water. The eluant was concentrated and reapplied to papers developed in solvent A. Two bands resulted: one at R_f 0.12 corresponding to unreacted thymidine 3'-phosphate and the other at R_f 0.55 corresponding to the dinucleotide thymidylyl-(3'-5')-3'-O-benzoyl-8,2'-thioanhydroinosine. Both bands were eluted with water and lyophilized. The thymidine 3'-phosphate represented a 21% recovery of starting material. The product gave a yield of 20%. Electrophoresis carried out in 0.05M triethyl ammonium bicarbonate (pH 7.5) for

TABLE I. Chromatographic Properties of 8,2'-Thioanhydroinosine Derivatives

Compound	Ethyl Acetate[a]	Ethyl Acetate-Ethanol (9:1)[a]	Solvent A[b]	Solvent L[b]
8,2'-Thioanhydroinosine	0.00	0.07	0.34	0.69
5'-O-MTr-8,2'-SAnI	0.09	0.25	-	-
5'-O-MTr-3'-OBz-8,2'-SAnI	0.16	0.40	-	-
3'-O-Bz-8,2'-SAnI	0.09	0.25	0.67	0.78
Thymidine 3'-phosphate	0.00	0.00	0.12	0.56
Tp-3'-O-Bz-8,2'-SAnI	0.00	0.00	0.55	0.70

[a] Thin-layer chromatography. [b] Paper chromatography. MTr = monomethoxytrityl, Bz = benzoyl, SAnI = thioanhydroinosine, Tp = thymidine 3'-phosphate.

1 hr. gave a Rm 0.26 for the dinucleotide relative to thymidine 3'-phosphate (Rm 1.00). The ultraviolet spectrum in water showed a maximum at 265nm (ϵ22,000).

The product was further characterized by spleen enzyme. It was completely degraded by spleen phosphodiesterase to thymidine 3'-phosphate R_f 0.12 (solvent A) and 3'-O-benzoyl-8,2'-thioanhydroinosine R_f 0.67 (solvent A).

Attempted Synthesis of the 17-Base Oligodeoxyribosenucleotide Coding for the TΨCG Loop in t-RNA (tyrosine)

β-Cyanoethyl-5'-O-monomethoxytritylthymidine-3'-phosphate (MT Tp(ce)) was synthesized by standard procedures [24] on a 2mmole scale. 3'-O-(2,4-Dinitrobenzenesulfenyl) thymidine (Tp(s)) was synthesized by the method of Letsinger [32] with the exception that the p-monomethoxytrityl group was used instead of trityl as the blocking group of the 5'-OH. N-Benzoyl-5'-O-monomethoxytrityldeoxyadenosine ($MTrdA^{Bz}$) was prepared by Khorana's procedure [30].

a) β-Cyanoethyl Ester of 5'-O-Monomethoxytrityl-thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine (MTrTp(ce)T(s))

β-Cyanoethyl-5'-O-monomethoxytritylthymidine 3'-phosphate MTrTp(ce), prepared on a 2mmole scale, was not isolated as a solid, but was stored in a chloroform solution (4ml). This chloroform solution was evaporated to a gum which was dried by evaporation of pyridine (four 4-ml portions). 3'-O-(2,4-dinitrobenzenesulfenyl) thymidine, Tp(s), (0.90g, 2.05mmoles), triisopropylbenzene-sulfonyl chloride (1.21g, 4mmoles) and anhydrous pyridine (4ml) were added, and the mixture stirred at room temperature for 24 hr. Upon addition of cold water (4ml), a yellow solid precipatated. The mixture was stirred for another 10 hr. and then diluted with water (4ml). The yellow solid was filtered and yielded 102mg. It decomposed upon heating above 196°. Paper chromatography in solvent I gave an R_f 0.00 (R_f^I 0.83, 3'-O-(2,4-dinitrobenzene sulfenyl) thymidine) and in solvent A, this yellow precipitate had an R_f 0.00 (R_f^A 0.84, 5'-O-monomethoxytritylthymidine). Thin layer chromatography revealed the presence of at least 3 compounds R_f 0.86, R_f 0.22 and R_f 0.00 (ethyl acetate). Electrophoresis showed that a mono-

charged compoundplus some uncharged material were present. After treatment with 80% acetic acid for 20 min. over a steam bath and then with ammonium hydroxide for 1 hr. to remove blocking groups, the reaction mixture was put on a paper developed in solvent A. Four bands were eluted off the paper with water. The material at R_f 0.12 was dicharged (Rm 1.00 relative to Tp) and its ultraviolet spectrum showed a maximum at 267nm (ε9600). This compound appears to be Tp, so that the blocked compound present in the yellow precipitate must have been unreacted MTrTp(ce). The R_f 0.64 band was uncharged and was identified as thymidine from its ultraviolet and chromatographic properties. The blocked compound then was unreacted MTrT. The other 2 minor bands at R_f 0.74 and R_f 0.87 also showed a maximum at 267nm and were uncharged, but positive identification could not be assigned to these bands.

The filtrate from the condensation reaction was extracted with chloroform (3 x 20ml). The chloroform extracts were concentrated in vacuo, washed with water and dried over sodium sulfate. The chloroform solution was applied to a column (60 x 3cm) of silica gel in ethyl acetate. The column was eluted with ethyl acetate (3.5 l) and then with tetrahydrofuran (1 l.). The first 1000ml of eluent contained some of the desired product

along with impurities. Pure MTrTp(ce)T(s) was obtained in the tetrahydrofuran fractions. Mixed fractions were concentrated and applied to thick layer plates developed in ethyl acetate. The band at R_f 0.16 was scrapped off the plates and eluted with tetrahydrofuran (200ml). The eluent was concentrated and the product MTrTp(ce)T(s) precipitated as a white powder (100mg) upon addition of hexane. The tetrahydrofuran fractions were concentrated and precipitated with hexane to yield 525mg of MTrTp(ce)T(s). The total yield was 625mg (30%), melting point 136-138° (softening from 126°). The thin layer chromatographic properties were R_f 0.75 (tetrahydrofuran), R_f 0.16 (ethyl acetate) and R_f 0.82 (ethanol).

The ultraviolet spectrum in 95% ethanol showed a maximum at 265nm (ϵ18,300) and shoulders at 305nm (ϵ4140) and 230nm (ϵ22,200). The principal bands in the infrared spectrum occurred at 2.91, 4.45(w), 6.00(broad), 6.67, 7.42, 7.80, 9.45(broad) and 14.5μ.

b) <u>β-Cyanoethyl Ester of Thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine (Tp(ce)T(s)</u>

MTrTp(ce)T(s) (500mg, 0.47mmole) was heated in 20ml of 80% acetic acid on a steam bath for 20 min. The solvent was removed at reduced pressure and the last

traces of acetic acid was removed by evaporation of ethanol. The residue was dissolved in chloroform and applied to a column (30 x 2cm) of silica gel in hexane. The column was eluted with ethyl acetate (300ml) and then with tetrahydrofuran (1 l.). Tp(ce)T(s) was obtained by concentration of the tetrahydrofuran fractions and precipitation with hexane: yield; 310mg (84%), melting point 125° (softening at 119°). The thin layer chromatographic properties of the product were: R_f 0.12 (ethyl acetate), R_f 0.55 (tetrahydrofuran) and R_f 0.67 (ethanol).

The ultraviolet spectrum in 95% ethanol showed a maximum at 264nm (ϵ 18,700) and a shoulder at 305nm (ϵ1550). The principal bands in the infrared spectrum occurred at 2.9, 5.9, 6.8, 7.8 and 9.7μ(broad).

c) <u>β-Cyanoethyl Ester of N-Benzoyl-5'-O-Monomethoxytrityldeoxyadenosine 3'-phosphate (MTrdAp^{Bz}(ce))</u>

MTrdA^{Bz} (0.50g, 0.8mmole) and pyridinium mono-β-cyanoethyl phosphate (4ml, 2mmoles) were mixed and dried by dissolving in pyridine and removing the pyridine under vacuum. Mesitylenesulfonyl chloride (0.70g, 3.2mmoles) and pyridine (2ml) were added and the solution stirred

for 8 hr., whereupon 2ml of water was added. After 24 hr. of additional stirring, the solution was diluted with 2ml of water and extracted with chloroform (3 x 10ml). The chloroform extracts were combined, washed with water, concentrated to a gum, dried by evaporation of pyridine (4 x 5ml), and finally taken up in 2ml of dry pyridine. The product was checked by electrophoresis. An aliquot (0.2ml) of the pyridine solution was treated with 80% acetic acid (0.5ml) at room temperature for 3 hr. The reaction mixture was then extracted with ether to remove monomethoxytritanol. The residue was spotted on paper, and its electrophoretic mobility at pH 7.5 was determined to be Rm 0.35 relative to Tp (Rm 1.00). The thin layer chromatographic properties of $MTrdAp^{Bz}(ce)$ were: R_f 0.04 (ethyl acetate) and R_f 0.18 (tetrahydrofuran).

d) Bis-β-Cyanoethyl Ester of N-Benzoyl-5'-O-Monomethoxytrityldeoxyadenylyl-(3'-5')-thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzene sulfenyl) thymidine ($MTrdAp^{Bz}(ce)Tp(ce)T(s)$).

Tp(ce)T(s) (204mg, 0.26mmole) was dried by dissolving in dry pyridine and evaporating the pyridine under vacuum. Triisopropylbenzenesulfonyl chloride (151mg, 0.5mmole) was added and the drying procedure repeated. $MTrdAp^{Bz}(ce)$ (1ml, 0.4mmole) and pyridine (1ml) were

added and the solution stirred for 32 hr. at room temperature. Water (1ml) and pyridine (0.5ml) were added and stirring continued for 18 hr. The solution was then extracted twice with chloroform (2 x 5ml), the extracts were washed with water (5ml), dried over sodium sulfate and evaporated. The residual gum was dissolved in 2ml of tetrahydrofuran and applied to a column (27 x 2cm) of silica gel in ethyl acetate. The column was eluted first with ethyl acetate (500ml), then with tetrahydrofuran (500ml). However, thin-layer chromatography showed that all fractions were mixed and no separation of the trinucleotide from contaminants had occurred. A new column (65 x 2cm) of silica gel in ethyl acetate was prepared. The solid collected upon evaporation of the fractions from the first column was redissolved in tetrahydrofuran (2ml) and applied to the column. The column was eluted with ethyl acetate (800ml), ethyl acetate-tetrahydrofuran mixture 8:2 (600ml), ethyl acetate-tetrahydrofuran mixture 6:4 (600ml), and tetrahydrofuran (600ml). Fractions of 200ml were collected. All fractions contained a charged nucleotide R_f 0.05 (tetrahydrofuran) and R_f 0.00 (ethyl acetate). Fractions 5-13 contained two compounds, R_f 0.05 and R_f 0.46 (tlc, tetrahydrofuran). These fractions were combined and evaporated. The residue was dissolved in

tetrahydrofuran and applied on thick layer plates developed in tetrahydrofuran. The origin and the band at R_f 0.40 were scrapped off the plates and eluted with ethanol (100ml) and tetrahydrofuran (100ml) respectively. The eluent from the band at R_f 0.40 was concentrated and precipitated with hexane, yielding 40mg of the product, $MTrdAp^{Bz}(ce)Tp(ce)T(s)$. This represented a yield of 11%, melting point 150-152° (softening from 141°). The thin layer chromatographic properties were: R_f 0.46 (tetrahydrofuran) and R_f 0.14 (ethyl acetate).

The ultraviolet spectrum in 95% ethanol showed 2 maxima, at 274nm (ε 37,000) and 232nm (ε51,000). The principal bands in the infrared spectrum occurred at 2.88, 5.89, 6.20, 6.57, 6.83, 7.15, 7.90, 14.20, and 14.7μ.

The ethanol eluent from the origin contained a single compound with R_f 0.08 (tlc, tetrahydrofuran). The ultraviolet spectrum in 95% ethanol showed a maximum at 280nm (ε20,000) and a shoulder at 232nm (ε22,000). The compound appears to be unreacted $MTrdAp^{Bz}(ce)$. $MTrdAp^{Bz}(ce)$ was further characterized by removal of the monomethoxytrityl and β-cyanoethyl groups and examining the electrophoretic and chromotographic properties of the deblocked material. An aliquot (0.5ml) was treated with 80% acetic acid (1ml) for 3 hr., solvents evaporated

and 1ml of ammonium hydroxide added and stirred overnight. Electrophoresis at pH 7.5 showed a dicharged compound with Rm 0.90 relative to Tp. A spot of the ammonium hydroxide solution was applied to a paper run in solvent A. A single spot appeared at R_f 0.16.

e) Bis-β-Cyanoethyl Ester of N-Benzoyl-deoxyadenylyl-(3'-5')- thymidylyl-(3'-5')-3'-O-(2,4-dinitrobenzenesulfenyl) thymidine (dAp^{Bz}(ce)Tp(ce)T(s)).

MT-dAp^{Bz}(ce)Tp(ce)T(s) (40mg, 0.028mmole) was treated with 80% acetic acid (4ml) at room temperature for 18 hr. The mixture was poured into ether (5ml) and the precipitate collected by centrifugation. The white precipitate was extracted twice with tetrahydrofuran (2 x 5ml). The extractions were concentrated and dAp^{Bz}(ce)Tp(ce)T(s) precipitated upon addition of hexane. The yield was 43%. The product decomposed slowly upon heating above 189°. The thin layer chromatographic properties were: R_f 0.39 (tetrahydrofuran), R_f 0.07 (ethyl acetate) and R_f 0.06 (ether).

β-cyanoethyl groups were removed by treatment with ammonium hydroxide (3mg of dAp^{Bz}(ce)Tp(ce)T(s) dissolved in 1ml) at room temperature for 6 hr. A spot of the ammonium hydroxide solution was applied to a

TABLE II. Paper Chromatographic Properties of Nucleotide Derivatives of thymidine and Adenosine

COMPOUND	Solvent A	Solvent C
Tp*	0.09	0.23
Tp(s)	0.06	0.49
TpT*	0.37	0.54
TpT(s)	0.31	0.69
TpTpT*	0.13	0.33
$dAp^{Bz}TpT(s)$	0.13	0.63

* R_f values obtained from K.K. Ogilvie [51].

paper developed in solvent A and to another paper developed in solvent C. Spots of Tp(s) and TpT(s) were also applied. The results are listed in Table II.

Electrophoresis of $dAp^{Bz}TpT(s)$ at pH 7.5 gave an Rm 0.69 relative to Tp. This may be compared to the electrophoretic mobility of TpTpT (Rm 0.52)[51].

Enzyme Assays

Adenosine Deaminase

The assay procedure is a modification of the general procedure described by Kaplan[33]. The reaction mixture contained 2.0ml of substrate and 0.50ml of adenosine deaminase in 0.055M KH_2PO_4-NaOH buffer (pH 7.5). Deaminase activity was measured by the decrease in absorbance at 259nm due to the conversion of adenosine to inosine, at 275.5nm due to the conversion of 8,2'-thioanhydroadenosine to 8,2'-thioanhydroinosine, at 273nm for 8,2'-aminoanhydroadenosine and at 260nm for 8,2'-O-anhydroadenosine. The reaction was carried out at room temperature, approximately 25°C, in a spectrophotometric quartz cell of 1.0cm path length. Readings were taken in a Cary 14 spectrophotometer every 15 sec. after addition of enzyme. The enzyme concentration was adjusted to give a linear decrease in absorbancy over a one minute interval for substrate concentrations varying

from 25μM to 100μM. For adenosine, an enzyme concentration of 0.10mg/ml was required, whereas 8,2'-thioanhydroadenosine required a 20-fold increase of deaminase concentration to 2mg/ml. For both 8,2'-aminoanhydroadenosine and 8,2'-O-anhydroadenosine, the Vmax were so low that the enzyme concentration had to be increased 100-fold and readings were made every min. for 5 minutes. The molar extinction coefficient for the decrease in extinction between adenosine and inosine was calculated as 7400 at 259nm; for 8,2'-thioanhydroadenosine, 8,2'-aminoanhydroadenosine, and 8,2'-O-anhydroadenosine, the change in extinction coefficients for the deamination reaction were 10,200 at 275.5nm, 8100 at 273nm and 3400 at 260nm respectively. Michaelis and maximum velocity constants were calculated by the method of Lineweaver and Burk[34]. Initial velocities were calculated over the first minute of the reaction, since this was shown to be linear.

Compounds were tested for their ability to inhibit the deamination of adenosine in the following manner. The reaction mixture contained 1.0ml of adenosine (8.64×10^{-5}M) as substrate, 1.0ml of test compound at five different concentrations and 0.50ml of adenosine deaminase (0.10mg/ml). The reaction was followed at 265nm, where $\Delta\epsilon = 8600$ [35]. An equal amount

of the test compound was added to the solvent blank. The rate of reaction was determined after addition of enzyme and compared with the uninhibited reaction. The ratio of the concentration of inhibitor to the concentration of substrate for 50% inhibition $(I/S)_{0.5}$ ie., the index of inhibition, was used to compare the inhibitory properties of the various compounds. In order to determine the concentration of inhibitor required for 50% inhibition, a plot of Vo/V vs. I was made, where Vo = initial velocity of the uninhibited reaction, V = initial velocity of the inhibited reaction at various inhibitor concentrations, and I = the various concentrations of inhibitor [36]. In the case where 8,2'-NAnA was tested on 8,2'-SAnA as substrate, measurements were made at 275.5nm. The enzyme concentration required in this case was 2.0mg/ml.

All test compounds were tested as competitive inhibitors in the following manner. Reaction velocities were measured over a range of substrate concentrations from 8.64×10^{-5}M to 20.5×10^{-5}M, in the presence of a constant amount of inhibitor. A comparison of the Lineweaver-Burk plots under these conditions with those obtained with substrate alone, showed whether or not the inhibition was competitive.

SPLEEN PHOSPHODIESTERASE

Spectrophotometric assay of spleen phosphodiesterase was done using p-nitrophenyl-Tp as a substrate and measuring the rate of reaction by the release of p-nitrophenol [37]. The basic incubation mixture contained 0.20ml of p-nitrophenyl-Tp (0.2 - 1.8 mole) in ammonium acetate buffer pH 5.7 and 0.05ml (0.75 unit) of spleen enzyme in 0.01M sodium pyrrophosphate buffer, pH 6.5. One unit of enzyme is equivalent to the amount of enzyme which will hydrolyse one μmole of substrate in 1 min. at 37°. The mixture, without enzyme, was preincubated for 2 minutes in a water bath at 37°, enzyme added, and 0.05ml aliquots transferred to 2.0ml of 0.1M NaOH at intervals and mixed. The absorbance of the resulting solution was measured in a spectrophotometer (Cary 14, 1.0cm path length) at 400nm. The Emax for p-nitrophenol was assumed to be 12,000 [37]. The best straight lines were obtained in a double-reciprocal plot (1/V vs. 1/S) using regression analysis. Km, Ki and Vmax were calculated from the straight-line equations.

The inhibition studies of spleen phosphodiesterase were carried out with a reaction mixture containing 0.20ml of p-nitrophenyl-Tp as substrate and 0.05ml of the inhibitor. A blank was also prepared containing

substrate and inhibitor but no enzyme. In determining the inhibition index the reaction mixture contained 0.10ml of substrate, 0.10ml of inhibitor and 0.05ml of spleen phosphodiesterase.

SNAKE VENOM PHOSPHODIESTERASE

Spectrophotometric assay [38] of snake venom diesterase was done using p-nitrophenyl-pT and 3'-O-acetyl-p-nitrophenyl-pT as substrates and measuring the course of the reaction by the release of p-nitrophenol at 400nm in a 1mm cell. The hydrolysis was carried out in small test tubes containing 0.20ml of substrate and 0.10ml of venom diesterase (0.5mg/ml). At this enzyme concentration the reaction was linear for the first 2 minutes. All solutions were made up in Tris buffer pH 8.9 for p-nitrophenyl-pT and pH 8.0 for 3'-O-acetyl-p-nitrophenyl-pT as substrate. The lower pH was to reduce hydrolysis of the acetyl group. After incubation at 37^{o}, the reaction was stopped by addition of 1.0ml of 0.1N NaOH. Again Km and Vmax were determined from a linear regression analysis of the Lineweaver-Burk plots.

Three different assays were used to study inhibitory effects of various test compounds.

Assay 1: Incubation mixture contained 0.20ml of substrate, 0.08ml of test compound; initial velocites were

measured at varying enzyme concentrations.

Assay 2: To determine competitive inhibition the incubation mixture contained 0.20ml of substrate, 0.08ml of inhibitor and 0.10ml of enzyme. The initial velocities were then measured over a range of substrate concentrations (3.0mM - 6.0mM).

Assay 3: To determine the index of inhibition, initial velocities were measured with varying inhibitor concentrations. The incubation mixture contained 0.10ml of substrate, 0.10ml of inhibitor and 0.10ml of enzyme.

Enzymic Degradation of Thymidylyl-(3'-5')-3'-O-benzoyl-8,2' - thioanhydroinosine

The procedure described by Ralph [39], et al., was followed for spleen and venom diesterase hydrolysis. After incubation at 37° for 5 hr., a spot of the solutions were applied to Whatman 3MM paper and developed in solvent A. Since the venom diesterase reaction showed no degradation products after 5 hr., the reaction was repeated for 24 hr. and the solution worked up as described by Letsinger [40]. After 24 hr. the dinucleotide was only partially degraded by snake venom phosphodiesterase as shown by the 3 bands which sepa-

rated after development in solvent A: R_f 0.09, R_f 0.55 and R_f 0.67. These bands were eluted from the paper with water and identified by their ultraviolet spectra and electrophoretic properties. The work-up yielded 0.70 O.D. unit of thymidylyl-(3'-5')-3'-O-benzoyl-8,2'-thioanhydroinosine (R_f^A 0.55), 0.30 O.D. unit of 8,2'-thioanhydroinosine-5'-phosphate (R_f^A 0.09) and 0.37 O.D. unit of thymidine (R_f^A 0.67); nucleoside/nucleotide = 1.23. Electrophoresis of 8,2' - thioanhydroinosine 5'-phosphate gave an Rm 0.89 relative to Tp (Rm 1.00). The recovered thymidylyl-(3'-5')-3'-O-benzoyl-8,2'-thioanhydroinosine represented 54% of the starting material.

Hydrolysis by spleen phosphodiesterase gave 2 products. The work-up yielded 0.59 O.D. unit of 3'-O-benzoyl-8,2'-thioanhydroinosine (R_f^A 0.67) and 0.70 O.D. unit of Tp (R_f^A 0.12); nucleoside/nucleotide = 0.84.

The ultraviolet spectra of thymidine and Tp both have a maximum at 267nm (9600), but of course, only Tp showed any electrophoretic mobility (Rm 1.00 as compared to a standard of Tp).

RESULTS AND DISCUSSIONS

A.) ADENOSINE DEAMINASE STUDIES

The aim of the adenosine deaminase studies was to establish whether or not conformational specificity exists for this enzyme. Compounds in the anti conformation were chosen for this investigation because compounds in the syn conformation have been found to be neither substrates nor inhibitors[1]. Five anhydro analogs of adenosine were studied: 8,2'-O-,-S-, and -NH- anhydroadenosine, 8,5'-O- and -S- anhydroadenosine. (XV-XX)

SUBSTRATE ACTIVITIES

8,2'-SAnA, 8,2'-OAnA and 8,2'-NAnA were tested as substrates. The results are shown in Tables 1.1 - 1.3 and plotted in Fig. 1.1 - 1.3.

TABLE 1.1: Effect of increasing substrate, S, (8,2'-SAnA) concentrations on initial velocities (v). Enzyme conc., 2mg/ml. Reaction time, 1 min. (See Figure 1.1)

Concentration of 8,2'-SAnA ($Mx10^6$)	1/S ($M^{-1}x10^{-4}$)	Change in Absorbance, at 275.5nm	Change in Concentration ($Mx10^6$)	V/mg $x10^2$	1/V/mg $x10^{-1}$
30.6	3.27	0.076	7.45	1.86	5.38
42.0	2.38	0.100	10.20	2.54	3.95
51.1	1.96	0.120	11.70	2.94	3.40
60.4	1.66	0.130	12.75	3.19	3.14
75.8	1.32	0.150	14.70	3.68	2.72
97.4	1.03	0.194	19.00	4.76	2.10

TABLE 1.2: Effect of substrate (8,2'-NanA) concentrations on initial velocities. Enzyme concentration, 10mg/ml. Reaction time, 5 min. (See Figure 1.2).

Concentration of 8,2'-SAnA ($Mx10^6$)	1/S ($M^{-1}x10^{-4}$)	Change in Absorbance, at 273nm	Change in Concentration ($Mx10^6$)	V/mg $x10^3$	1/V/mg $x10^{-2}$
53.0	1.89	0.083	10.25	1.23	8.14
58.0	1.73	0.094	11.60	1.39	7.20
64.3	1.56	0.106	13.10	1.57	6.37
78.6	1.27	0.122	15.10	1.81	5.53
92.0	1.09	0.141	17.40	2.09	4.78

TABLE 1.3: Effect of substrate (8,2'-OAnA) concentrations on initial velocities. Enzyme concentration, 10mg/ml. Reaction time, 4 min. (See Figure 1.3).

Concentration of 8,2'-OAnA ($Mx10^6$)	1/S ($M^{-1}x10^{-4}$)	Change in Absorbance, at 260nm	Change in Concentration ($Mx10^6$)	V/mg $x10^3$	1/V/mg $x10^{-2}$
43.2	2.22	0.051	15.1	1.88	5.32
43.5	2.20	0.054	15.9	1.95	5.13
64.8	1.54	0.065	19.1	2.39	4.19
86.4	1.16	0.090	26.4	3.30	3.06
94.0	1.06	0.076	22.4	3.69	2.71
108.0	0.93	0.110	32.4	4.05	2.47

Figure 1.1 Lineweaver-Burk plot for 8,2'-SAnA as substrate for adenosine deaminase (2mg/ml). Vmax = 0.143μmole per min per mg

1.2 Lineweaver-Burk plot for 8,2'-NAnA as substrate for adenosine deaminase (10mg/ml). V =μmole per min

Vmax = 0.018μmole per min per mg

FIG. 1.1

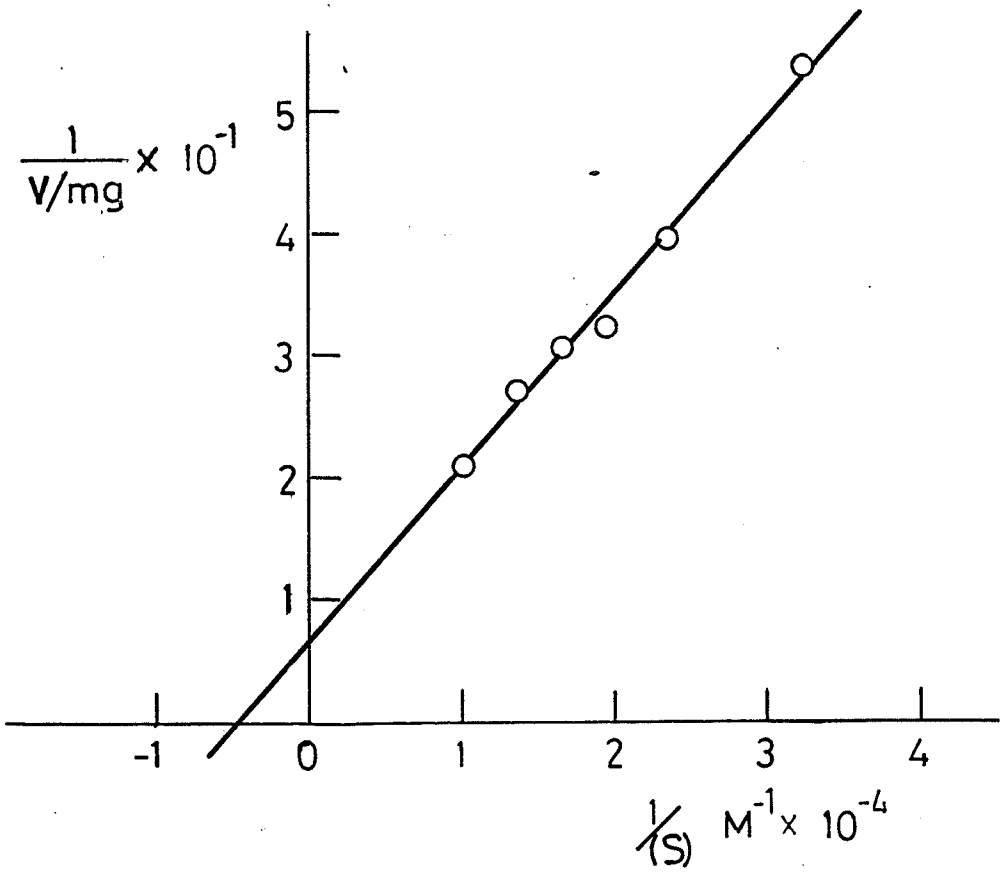

1/V/mg x 10^-1
5
4
3
2
1
-1
0
1
2
3
4
1/(S) M^-1 x 10^-4

FIG. 1.2

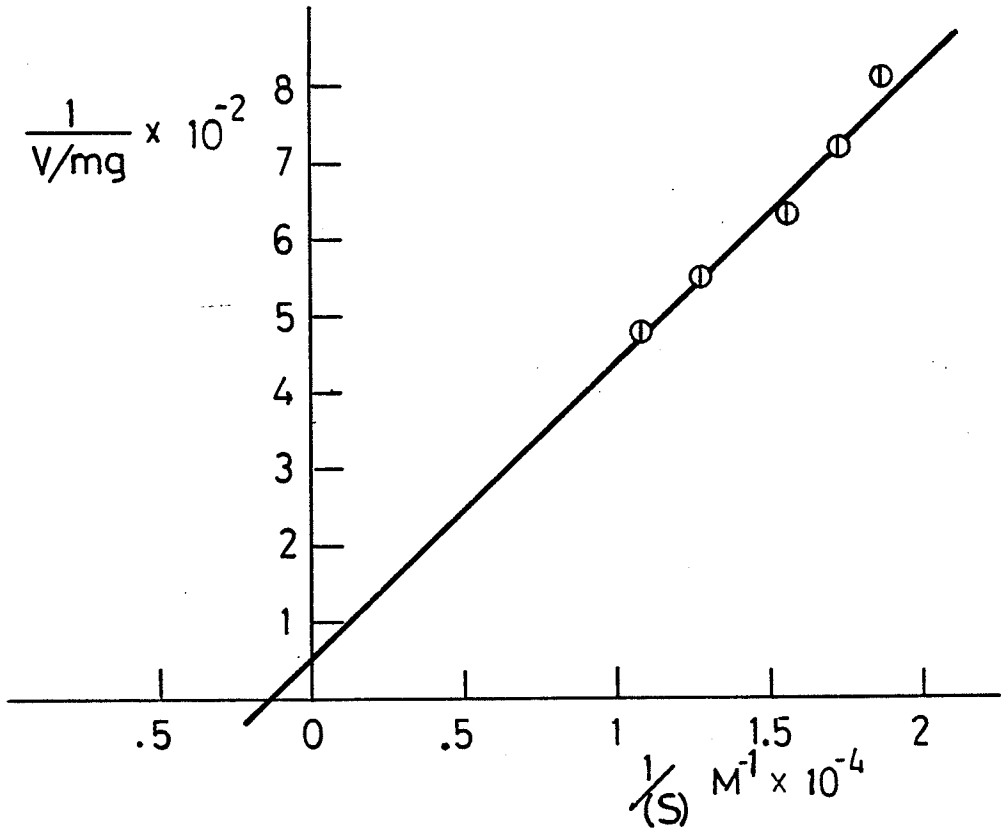

1/V/mg x 10^-2
8
7
6
5
4
3
2
1
.5
0
.5
1
1.5
2
1/(S) M^-1 x 10^-4

TABLE 2.1: Effect of substrate (adenosine) concentrations on initial velocities. Enzyme concentration, 0.1mg/ml. Reaction time, 1 min. (See Figure 2).

Concentration of Adenosine ($Mx10^6$)	1/S ($M^{-1}x10^{-4}$)	Change in Absorbance, at 259nm	Change in Concentration ($Mx10^6$)	V/mg x10	1/V/mg
24.6	4.06	0.135	18.2	9.10	1.100
33.2	3.01	0.158	21.4	10.70	0.937
39.6	2.52	0.174	23.5	11.76	0.850
40.0	2.50	0.173	23.4	11.72	0.852
44.7	2.24	0.181	24.5	12.40	0.805
60.0	1.67	0.214	28.8	14.40	0.694
62.8	1.59	0.229	30.9	15.44	0.647
74.0	1.35	0.238	32.0	16.00	0.625

TABLE III. Kinetic Constants for Alternate Substrates of Adenosine Deaminase

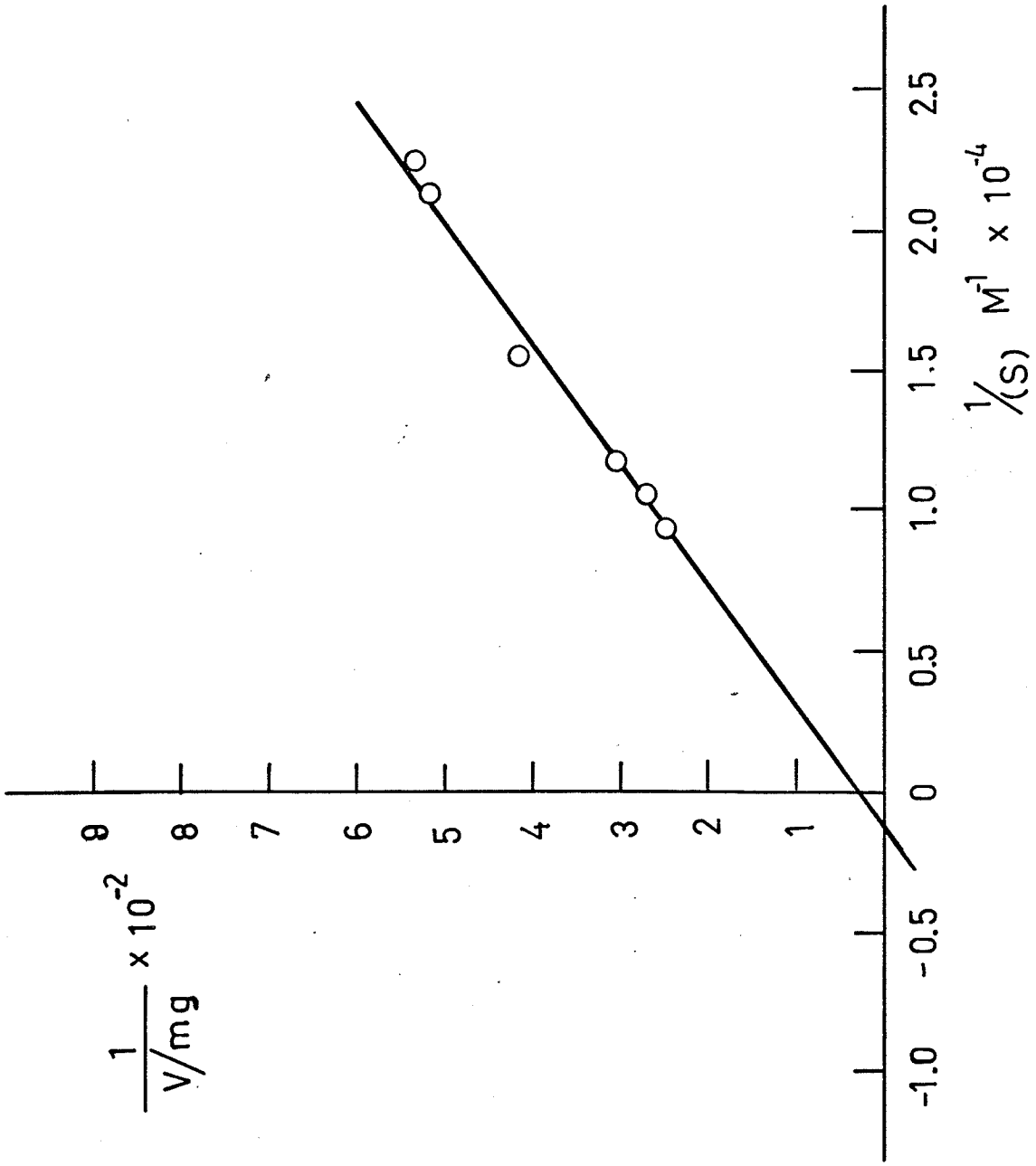
Substrate	Km x 10^5	Rel Vmax
Adenosine	4.76	1.00
8,2'-Thioanhydroadenosine	0.20	0.06
8,2'-Oxyanhydroadenosine	0.80	0.0126
8,2'-Aminoanhydroadenosine	0.67	0.0070

Figure 1.3 Lineweaver-Burk plot for 8,2'-OAnA as substrate for adenosine deaminase (10mg/ml)

V = μmole per min

Vmax = 0.033μmole per min per mg

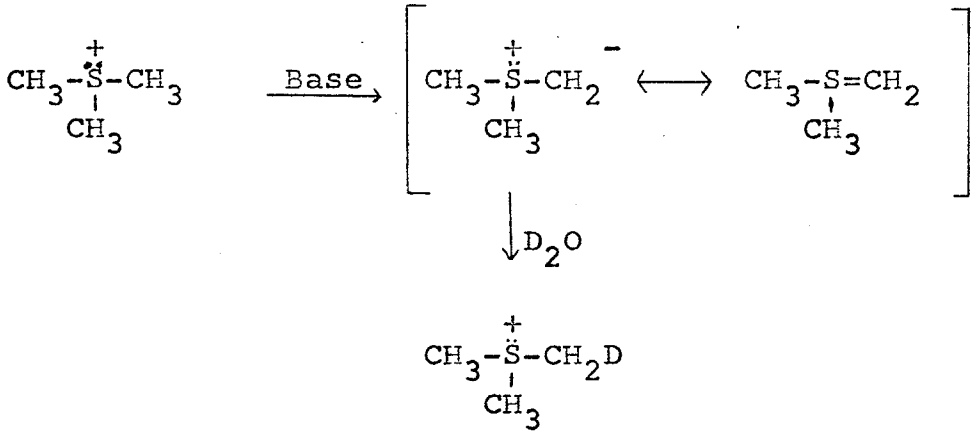
FIG. 1.3

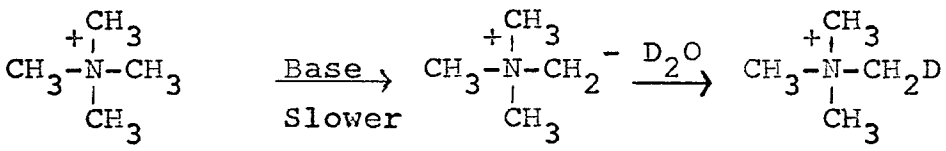


All the 8,2'-anhydro analogs of adenosine were found to be substrates of adenosine deaminase. As summarized in Table III, the Km and Vmax of the anhydro compounds indicate that there is no correlation between the apparent affinity of the substrate for the catalytic site (Km) and the rate of decomposition of the enzyme-substrate complex (Vmax). However, the rate of reaction increases along the series N < O < S. If the enzymic hydrolysis is considered to be a nucleophilic displacement reaction, then the rate of hydrolysis will be enhanced by electron - withdrawing groups, as shown by Wolfenden [15]. However, taking only inductive effects into account gives a different order of reactivity, ie, O < S, N. From the point of view of electronegativities, oxygen would again be the best participant in hydrogen bonding to some part of the enzyme in the active site. Therefore, to explain the greater reactivity of S in relation to O, we must look for other possible interactions.

Atoms beyond the second period of the periodic table can accomodate more than eight outer-valence electrons by utilizing their d orbitals. In particular, sulfur is able to expand its octet and delocalize electrons from adjacent centres of electron density. If observed stabilization of carbanions by mercapto

groups is in fact due to such resonance forms as $-\ddot{\underset{\cdot\cdot}{S}}=C-$, then atoms of the first period will be less effective than S eventhough they have comparable electronegativities. Consistent with this, Doering and Hoffmann[41] found that trimethylsulfonium ion exchanges hydrogen for deuterium much faster than the tetramethylammonium ion. Thus hydrogens α to a S are more acidic than those α to a N, presumably because of resonance.





Another observation which indicates that sulfur stabilizes a carbanion is that mercapto groups activate a methylene group for condensation reactions[42].

$$CH_2(SC_2H_5)_2 \xrightarrow[NaNH_2, NH_3]{RCl} R\text{-}CH(SC_2H_5)_2$$

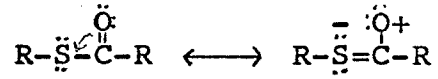
The mechanism involves displacement of Cl^- by the stabilized carbanion $-CH(SC_2H_5)_2$. If this stabilization is partly because of resonance, then S should be more effective than is O. Baliah[43] finds this to be the case in a related system: phenylmercaptoacetic acid condenses with benzaldehyde in acetic acid with piperidine catalysis.

$$C_6H_5S\text{-}CH_2\text{-}CO_2H + C_6H_5\text{-}CHO \rightarrow C_6H_5S\text{-}\overset{\overset{CHC_6H_5}{\|}}{C}\text{-}COOH$$

Under these conditions, the O analog does not react.

$$C_6H_5\text{-}O\text{-}CH_2\text{-}CO_2H + C_6H_5CHO \longrightarrow \text{No reaction}$$

Infrared spectra of divalent sulfur compounds also have been rationalized by use of d-orbitals[44]. A study of the infrared spectra of thiolesters, R-S-CO-R has been reported by Baker and Harris[45]. The carbonyl frequency in thioesters is 40 to 60cm^{-1} lower than in the corresponding ester. The thioester carbonyl group is judged to be less basic than an ordinary ester carbonyl since it hydrogen bonds to phenylacetylene to a lesser extent. Thus, the sulfur interacts with the carbonyl to remove electrons.

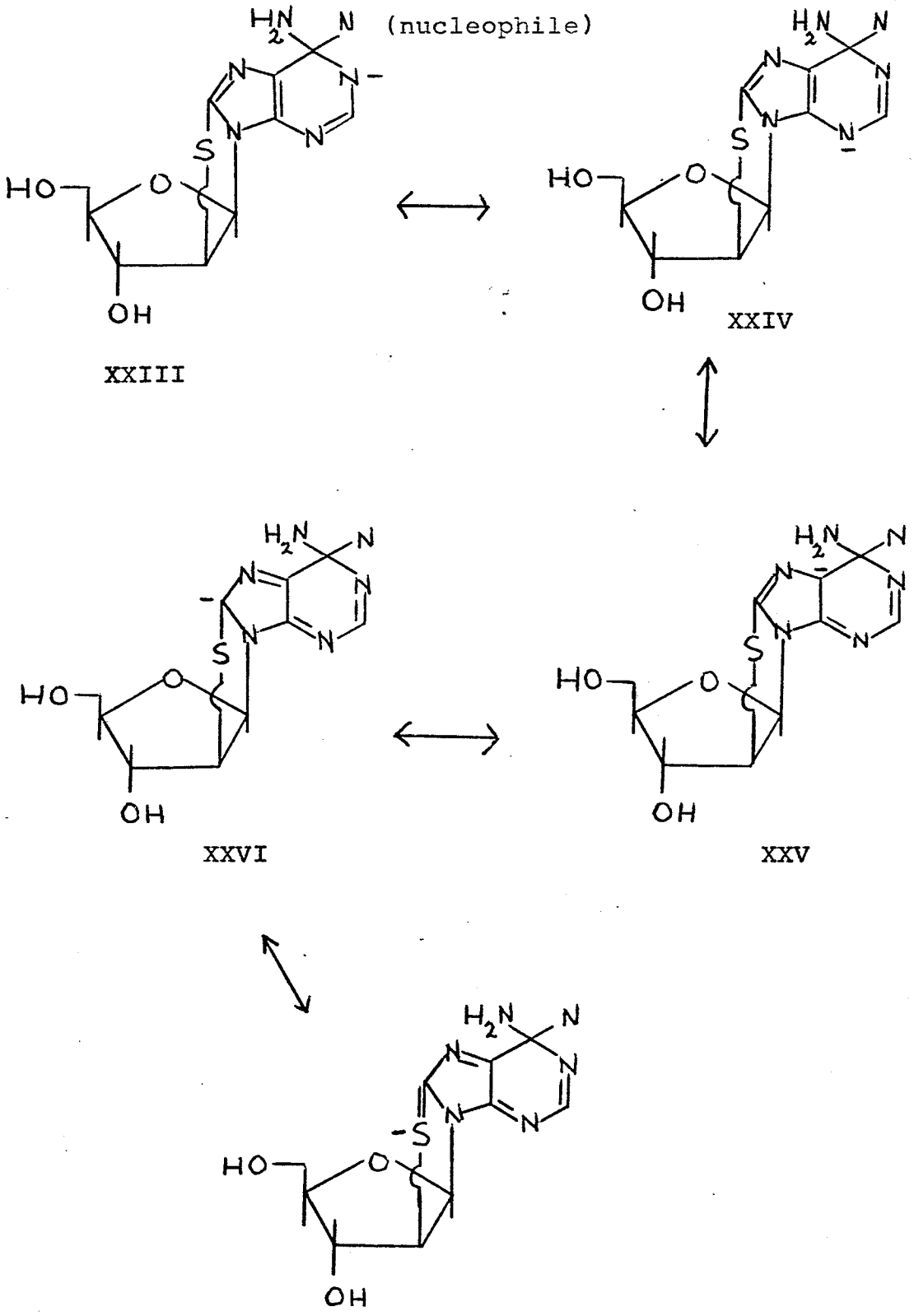


46-48

Ultraviolet spectral data also support these conclusions.

In the deaminase reaction we propose a similar explanation for the reactivity S>O. The intermediate proposed by Wolfenden[15] is shown in Scheme I, along with its resonance forms (XXIII-XXVII). If we assume that the sulfur atom in XXVII lies in the same plane as the adenine ring so that maximum overlap is possible between the S d-orbitals and the p-orbital of the 8-carbon, then resonance structure XXVII is possible. In a similar case, NMR studies have shown that the oxygen in the anhydro linkage of anhydrouridine is coplanar with the base ring[49]. Structure XXVII is only possible with the sulfur atom. It is this additional resonance form that makes the 8,2'-thioanhydroadenosine more reactive due to stabilization of the tetrahedral intermediate. For both oxygen and nitrogen, only 4 resonance structures can be written. Therefore, the order of reactivity, S>O, may be explained by the ability of sulfur to stabilize carbanions (at the 8-carbon of adenine, XXVI) by utilization of d-orbitals. The order O>N may be explained by inductive effects, since oxygen and nitrogen have approximately equal valence orbital stabilization. The fact that all the 8,2'-anhydro analogs were substrates is in agreement with the idea that adenosine deaminase may require nucleoside substrates to be in the anti conformation.

Scheme I



Inhibitor Studies

The three 8,2'-anhydro analogs, (XV-XVII) 8,5'-thioanhydroadenosine (XIX) and 8,5'-O-anhydrodenosine (XX) were tested as inhibitors of adenosine deaminase. Measurements were made at the isobestic point, the wavelength at which the conversion of the inhibitor into product does not result in a change in absorbance. Therefore, any decrease in absorbance may be attributed to the conversion of the substrate, adenosine, to inosine. Since the Vmax values were so low for 8,2'-aminoanhydroadenosine and 8,2'-O-anhydroadenosine, it was possible to make inhibition measurements without determining the isobestic points. The reactions were followed at 265nm, since there would be no detectable hydrolysis of 8,2'NAnA or 8,2'-OAnA at the enzyme concentration used for adenosine as substrate. In the case of 8,2'-thioanhydroadenosine , which is a reasonable substrate of adenosine deaminase, the isobestic point, where $\Delta\epsilon=0$ for the conversion of 8,2'-thioanhydroadenosine to 8,2'-thioanhydroinosine, was calculated to be at 267.5mμ. However, under the assay conditions described in the experimental section, no inhibition by XV of adenosine deaminase with adenosine as substrate could be detected.

The effect on the Lineweaver-Burk plot of the presence of a constant amount of inhibitor can be seen in Fig. 2. The experimental regression lines for 8,2'-aminoanhydroadenosine, 8,2'-O-anhydroadenosine, 8,5'-thioanhydroadenosine, and 8,5'-O-anhydroadenosine when compared with adenosine alone, show the classical case of competitive inhibition[54]. Inhibitor constants (Ki) were calculated from the equation:

$$Ki = \frac{i}{\frac{Kp}{Km} - 1}$$

where i is the inhibitor concentration in moles/litre and Kp is the apparent Michaelis constant in the presence of inhibitor. The data for the Lineweaver-Burk plots in Fig. 2 are recorded in Tables 2.2 - 2.5 below.

TABLE 2.2: Effect of 8,2'-aminoanhydroadenosine on varying concentrations of adenosine. Enzyme = 0.12mg/ml V = μmole per min.

Concentration of Adenosine ($Mx10^6$)	1/S ($M^{-1}x10^{-4}$)	Change in Absorbance	Change in Concentration ($Mx10^6$)	V/mg	1/V/mg
34.6	2.89	0.172	20.0	0.834	1.200
44.8	2.23	0.216	25.1	1.045	0.956
63.3	1.58	0.267	31.0	1.290	0.776
74.0	1.35	0.292	34.0	1.420	0.705
82.0	1.22	0.305	35.5	1.480	0.676

Figure 2 Lineweaver-Burk plots showing the effect of four competitive inhibitors on adenosine as substrate. Calf enzyme was used (0.1mg/ml), O-O, adenosine; ⊖-⊖, adenosine plus 8,2'-NAnA (1.05×10^{-4}M); Θ-Θ, adenosine plus 8,5'-OAnA (0.99×10^{-4}M); ●-●, adenosine plus 8,2'-OAnA (2.88×10^{-4}M); ◐-◐, adenosine plus 8,5'-SAnA (1.57×10^{-4}M) (Vmax calculated for adenosine was 2.62 μmoles per min per mg).

FIG. 2

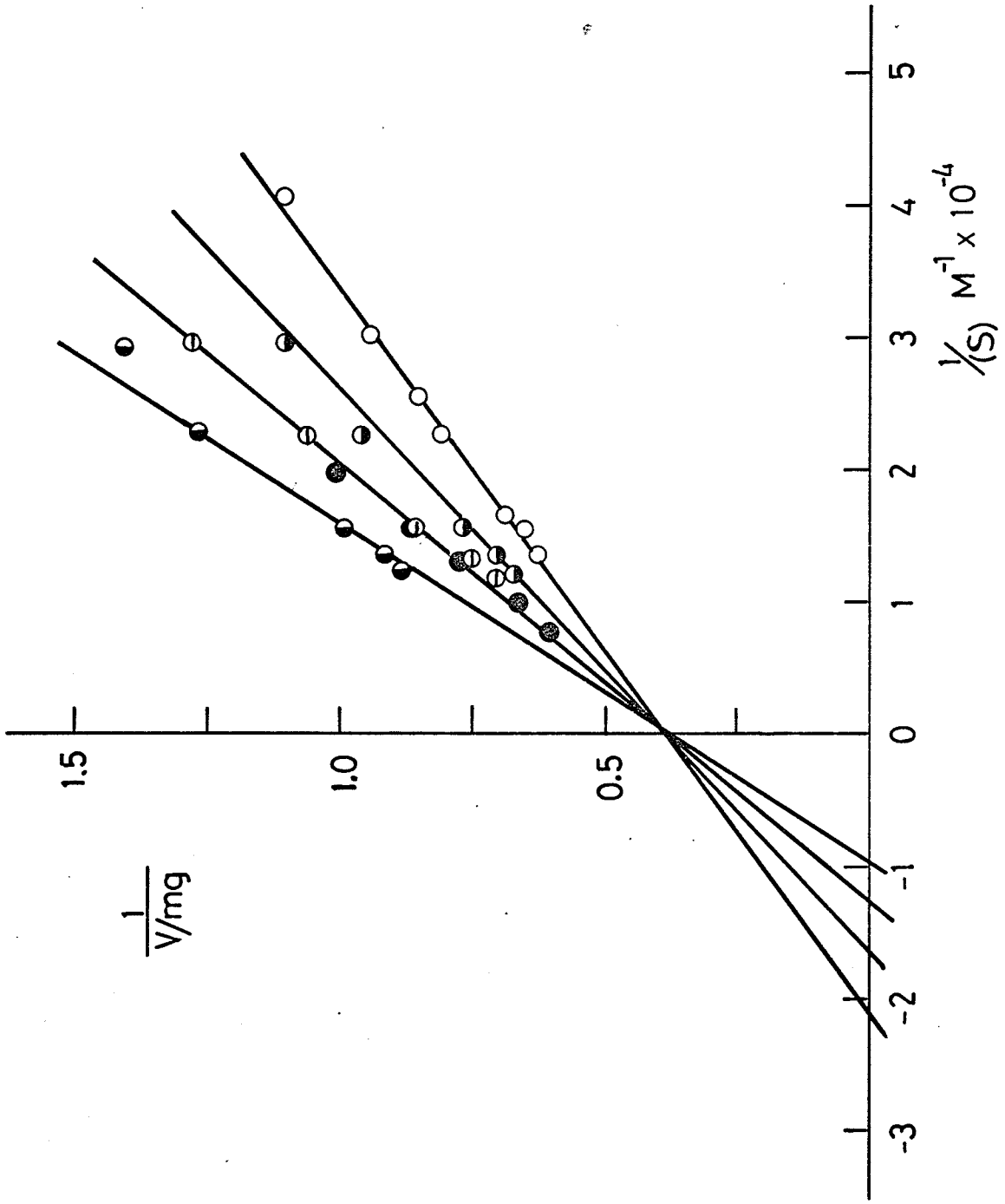


TABLE 2.3: Effect of 8,2'-O-anhydroadenosine on varying concentrations of adenosine. Enzyme = 0.12mg/ml; V = μmole per min.

Concentration of Adenosine ($Mx10^6$)	1/S ($M^{-1}x10^{-4}$)	Change in Absorbance	Change in Concentration ($Mx10^6$)	V/mg	1/V/mg
51	1.96	0.202	23.5	0.98	1.020
64	1.56	0.248	28.8	1.20	0.834
77	1.30	0.281	32.7	1.36	0.735
102	0.98	0.318	37.0	1.54	0.650
128	0.78	0.345	40.1	1.67	0.600

TABLE 2.4: Effect of 8,5'-O-anhydroadenosine on varying concentrations of adenosine. Enzyme = 0.12mg/ml; V = μmole per min.

Concentration of Adenosine ($Mx10^6$)	1/S ($M^{-1}x10^{-4}$)	Change in Absorbance	Change in Concentration ($Mx10^6$)	V/mg	1/V/mg
34.6	2.89	0.162	18.8	7.84	1.275
44.8	2.23	0.192	22.4	9.35	1.070
63.3	1.58	0.244	28.4	11.80	0.847
74.0	1.35	0.278	32.3	13.45	0.744
82.0	1.22	0.300	34.9	14.50	0.690

TABLE 2.5: Effect of 8,5'-S-anhydroadenosine on varying concentrations of adenosine. Enzyme = 0.11mg/ml; V = μmole per min.

Concentration of Adenosine ($Mx10^6$)	1/S ($M^{-1}x\ 10^{-4}$)	Change in Absorbance	Change in Concentration ($Mx10^6$)	V/mg	1/V/mg
34.6	2.89	0.132	15.4	0.703	1.420
44.8	2.23	0.149	17.4	0.794	1.260
63.3	1.58	0.193	22.4	1.020	0.980
74.0	1.35	0.208	24.2	1.100	0.910
82.0	1.22	0.214	24.9	1.140	0.878

The 8,5'-anhydroanalogs were found not to be substrates at an enzyme concentration of 10mg/ml, as expected, since the 5'-hydroxyl essential for activity[7-9] is no longer present. But since these compounds are constrained in an almost perfect anti conformation, they were expected to be, and were found to be, competitive inhibitors in accordance with our anti conformation theory for substrate specificity. Therefore, we may conclude that compounds with an anti conformation will bind at the active site as substrates and/or inhibitors.

The inhibitory effects of these inhibitors were compared by determining the inhibition index. The data for the Vo/V vs. I plots are contained in Tables 3.1(a)-(c) and 3.2(a),(b).

TABLE 3.1(a): Effect of 8,2'-aminoanhydroadenosine as an inhibitor on adenosine. Vo = 4.75×10^{-2} μmole per min. (See Fig. 3.1).

Concentration of 8,2'-NAnA ($M \times 10^6$)	Change in Absorbance	Change in Concentration ($M \times 10^6$)	$V \times 10^2$ μmole/min	Vo/V
65.2	0.163	19.00	3.96	1.20
103.0	0.130	15.10	3.77	1.26
206.0	0.099	11.50	2.88	1.65
260.8	0.092	10.65	2.67	1.78
326.0	0.084	9.78	2.44	1.95

TABLE 3.1(b): Effect of 8,5'-O-anhydroadenosine as an inhibitor on adenosine. Vo = 4.75×10^{-2} μmole per min. (See Fig. 3.1).

Concentration of 8,5'-OAnA ($M \times 10^6$)	Change in Absorbance	Change in Concentration ($M \times 10^6$)	$V \times 10^2$ μmole/min	Vo/V
71	0.155	18.0	4.50	1.06
142	0.142	16.5	4.13	1.15
213	0.136	15.8	3.95	1.20
284	0.122	14.2	3.55	1.34
355	0.125	14.5	3.62	1.31

TABLE 3.1(c): Effect of 8,5'-S-anhydroadenosine as an inhibitor on adenosine. Vo = 4.07×10^{-2} µmole per min. (See Fig. 3.1).

Concentration of 8,5'-SAnA ($M \times 10^6$)	Change in Absorbance	Change in Concentration ($M \times 10^6$)	$V \times 10^2$ µmole/min	Vo/V
39.2	0.138	16.1	4.02	1.01
157	0.130	15.1	3.78	1.08
236	0.128	14.9	3.72	1.09
314	0.125	14.6	3.64	1.12
392	0.125	14.6	3.64	1.12

TABLE 3.2(a): Effect of 8,2'-O-anhydroadenosine as an inhibitor on adenosine. Vo = 5.52×10^{-2} µmole per min. (See Fig. 3.2).

Concentration of 8,2'-OAnA ($M \times 10^6$)	Change in Absorbance	Change in Concentration ($M \times 10^6$)	$V \times 10^2$ µmole/min	Vo/V
115	0.176	20.4	5.10	1.08
144	0.172	20.0	5.00	1.00
173	0.170	19.8	4.95	1.12
230	0.165	19.2	4.80	1.15
288	0.160	18.6	4.65	1.19

TABLE 3.2(b): Effect of 8,2'-N-anhydroadenosine as an inhibitor on 8,2'-S-anhydroadenosine. Vo = 2.7×10^{-2} μmole per min. (See Fig. 3.2).

Concentration of 8,2'-NAnA ($M \times 10^6$)	Change in Absorbance	Change in Concentration ($M \times 10^6$)	$V \times 10^2$ μmole/min	Vo/V
73.5	0.110	10.80	2.70	1.00
97.5	0.098	9.60	2.40	1.12
170.5	0.084	8.24	2.06	1.30
260.8	0.078	7.65	1.91	1.41
322.0	0.070	6.86	1.72	1.57

TABLE IV. Index of Inhibition and Ki of Certain Anhydroadenosine Derivatives

Compound	Conc. of Adenosine ($M \times 10^6$)	Conc. of Inhibitor (I)0.5	(I/S)0.5	$Ki \times 10^4$
8,2'-N-anhydroadenosine	86.4	335	3.89	3.89
8,2'-O-anhydroadenosine	102	1200	11.80	4.44
8,5'-O-anhydroadenosine	86.4	930	10.70	1.52
8,5'-S-anhydroadenosine	89.3	3000	33.60	1.31
8,2'-N-anhydroadenosine*	119	594	4.98	

* Substrate was 8,2'-S-anhydroadenosine

Figure 3.1. Vo/V vs. I plots to determine the inhibition index for ●-●, 8,2'-NAnA; O-O, 8,5'-OAnA; ⊖-⊖, 8,5'-SAnA with adenosine as substrate.

3.2. ⊖-⊖, 8,2'-OAnA as inhibitor on adenosine as substrate; ●-●, 8,2'-NAnA as inhibitor on 8,2'-SAnA as substrate.

FIG. 3.1

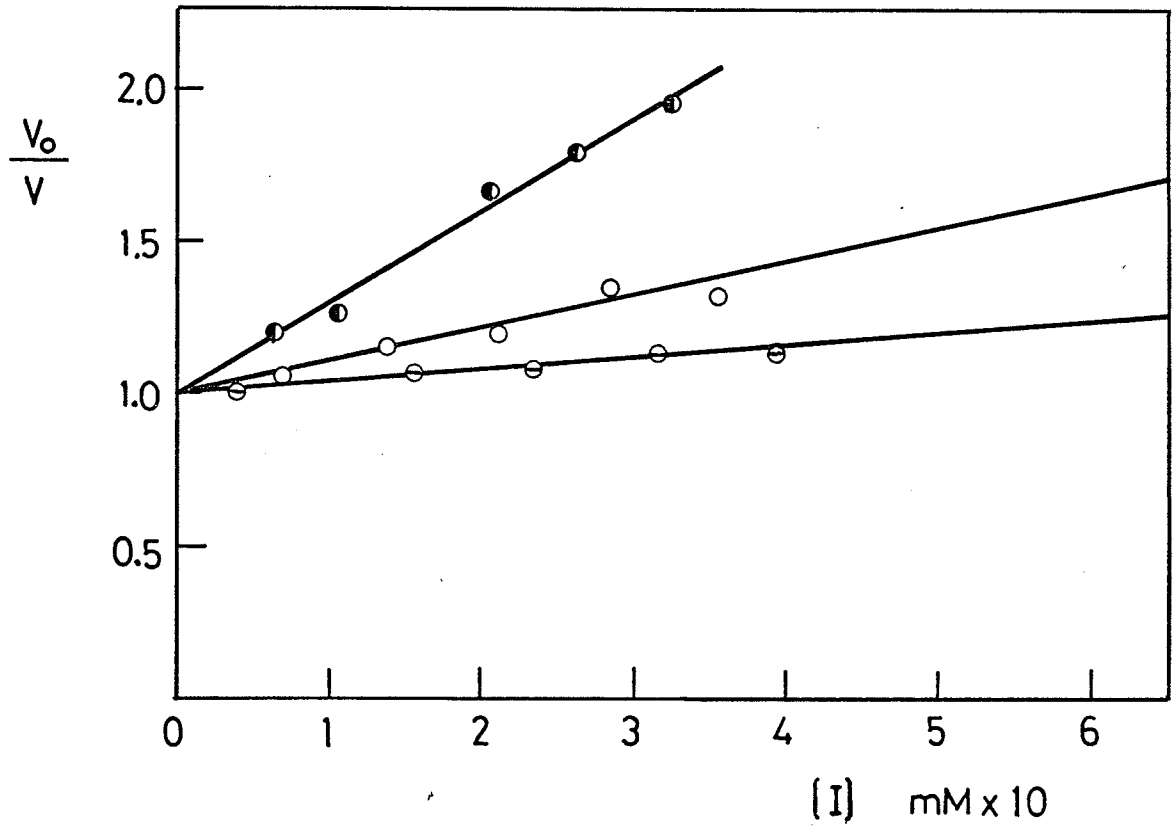
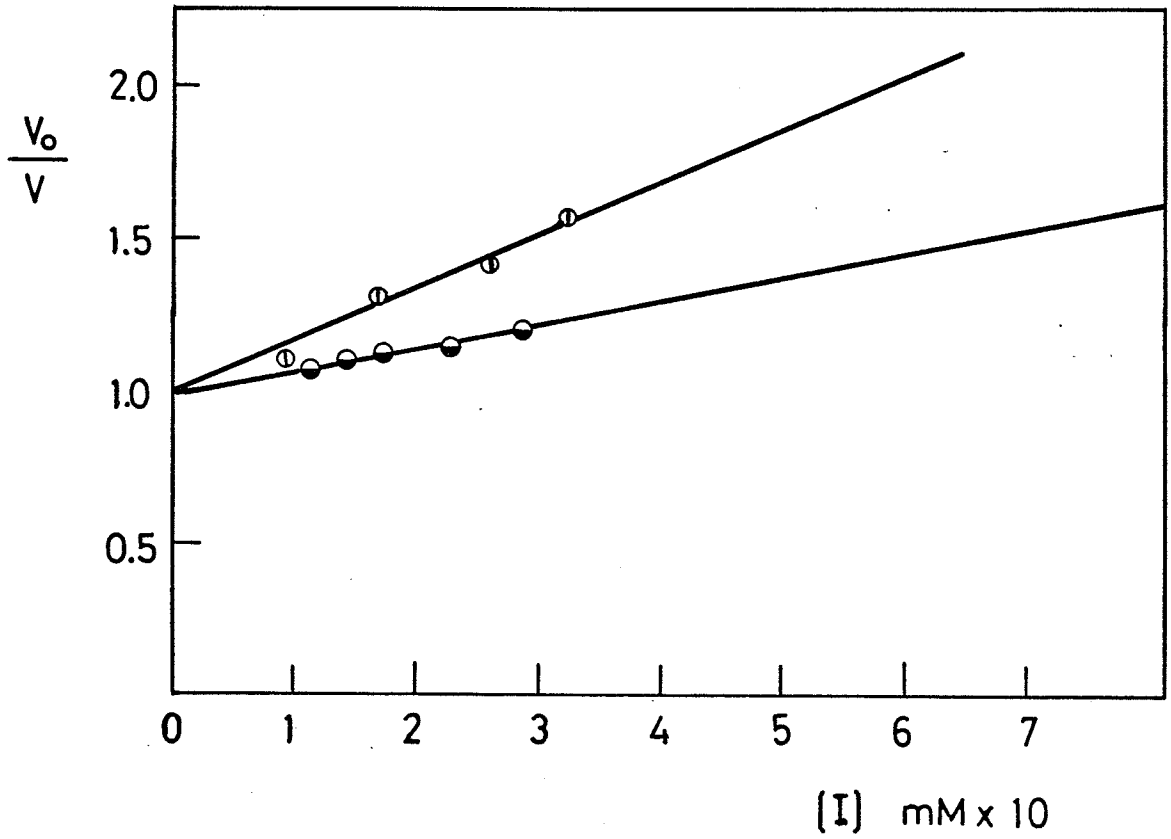

$\frac{V_o}{V}$
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
[I] mM x 10

FIG. 3.2

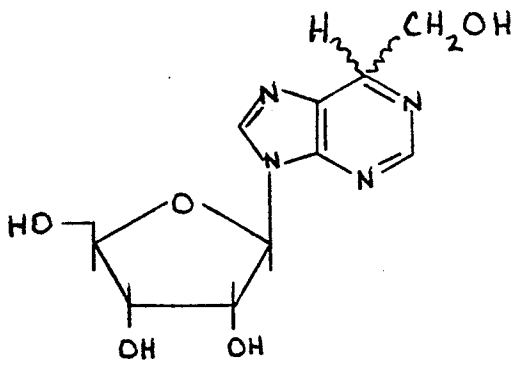

$\frac{V_o}{V}$
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
[I] mM x 10

The inhibition indices listed in Table IV were calculated from an extrapolation of the plots in Fig. 3.1 and 3.2. The order of inhibitor activity seems to follow a reverse order of substrate reactivity, ie., N > O > S. Unfortunately, we cannot compare all these three kinds of analogs within one series; 8,5'-N-anhydroadenosine was not available while 8,2'-S-anhydroadenosine could not be measured. If we assume that 8,2'-S-anhydroadenosine is such a poor inhibitor that its effect could not be detected, then inhibition of adenosine deaminase may be dependent on the Vmax, the rate of dissociation of the enzyme-substrate complex, and not dependent on the binding to the catalytic site. In other words, the faster the Vmax of a compound, then the poorer the inhibitory effects exhibited by that compound. For the best inhibitor, 8,2'-N-anhydroadenosine, the Vmax is very low so that the active site is being occupied by the inhibitor longer than in the case of 8,2'-S-anhydroadenosine. If the initial binding of the inhibitor to the enzyme is the rate-determining step, then the best substrate should be the best inhibitor and the order of inhibitor reactivity would follow along the series N < O < S, which is the reverse of our findings. The non-detectable inhibitory effects of 8,2'-S-anhydroadenosine may

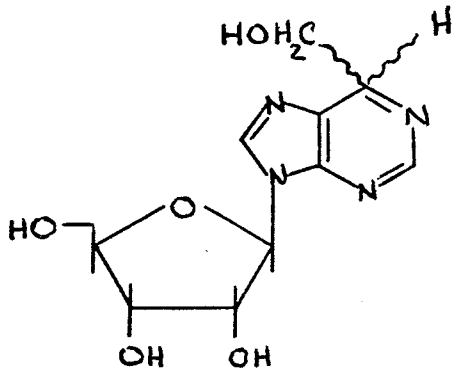
be explained by the fact that since it is a fairly good substrate, it will be displaced from the catalytic site much faster than the 8,2'-O- or 8,2'-N- anhydroadenosine; therefore, making it the poorest inhibitor of the three. With the 8,5'-anhydro analogs, the above situation does not exist, since these compounds are not substrates. We can only surmise that the sulfur analog is a poorer inhibitor than the corresponding oxygen analog due to some sort of interaction between these compounds and the enzyme.

The work described above gives sufficient evidence to support the anti conformation theory of adenosine deaminase activity. We have shown that all the compounds studied, which are considered to be in the anti conformation, bind to the active site either as substrates, as in the case of 8,2'-anhydro analogs, or as competitive inhibitors, as with the 8,5'-anhydro analogs.

Further evidence to support our theory of conformational specificity for adenosine deaminase comes from the work of Wolfenden[55]. Two diastereomers of 1,6-dihydro-6-hydroxymethylpurine ribonucleoside (XXVIII, XXVIII') were tested as inhibitors of adenosine deaminase. Compound XXVIII was found to be an exceptionally powerful competitive inhibitor of adenosine



XXVIII



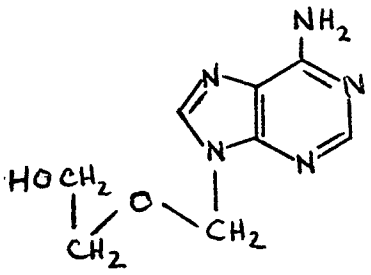
XXVIII'

deaminase, while XXVIII' was relatively ineffective as an inhibitor. These results may be rationalized if the enzymatic reaction involves stereospecific attack from only one side of the purine ring. Relating this to the conformational aspect of adenosine substrates, we see that different sides of the purine ring are exposed to enzymic hydrolysis when adenosine is in the anti or syn conformations. From our findings that only compounds in the anti conformation are substrates, we can conclude that this is the required conformation for stereospecific attack in the deaminase reaction.

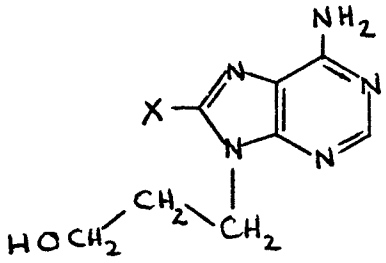
PROPOSED FUTURE STUDIES

An acyclic analog of adenosine, 9-(2-hydroxy-ethoxymethyl) adenine XXIX , was shown to be a substrate of calf intestinal mucosal adenosine deaminase

by Schaeffer[50]. These acyclic analogs are important



XXIX



XXX, X=Br
XXXI, X=SH

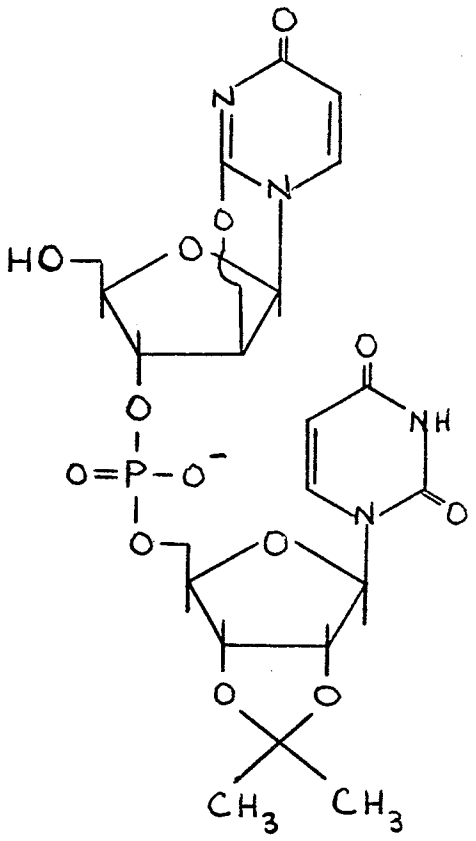
because they do not exist in the <u>syn</u> or <u>anti</u> conformation since there is no sugar ring present. These types of molecules may be utilized in studies on steric effects at the 8-position of the adenine ring. For example, 9-(3-hydroxypropyl)-8-bromoadenine (XXX) and 9-(3-hydroxypropyl)-8-mercaptoadenine (XXXI), may be tested as substrates for adenosine deaminase. The corresponding adenosine derivatives were found to be neither substrates nor inhibitors[1]. If compounds XXX and XXXI were found to be substrates, then we have conclusive evidence that steric effects are not important but that conformational effects are.

B.) SPLEEN AND SNAKE VENOM PHOSPHODIESTERASE STUDIES

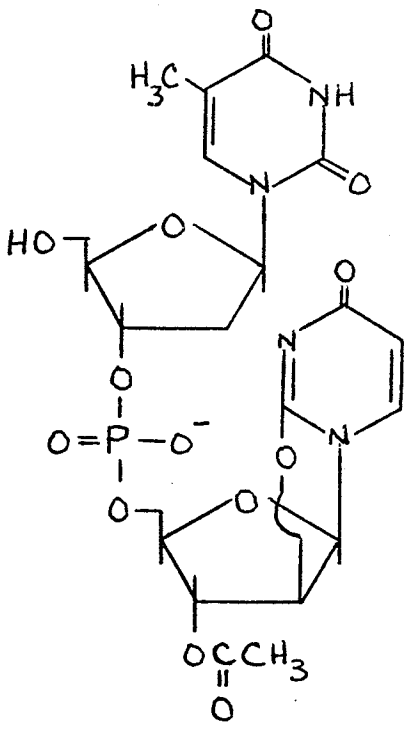
A number of dinucleotides containing anhydronucleosides have been found to be fairly resistant to the phosphodiesterases. The dinucleotide O^2,2'-anhydrouridylyl-(3'-5')-isopropylidine uridine ($AUpU^{Iso}$,XXXII) was resistant to spleen phosphodiesterase,while thymidylyl-(3'-5')-3'-O-acetyl-O^2,2'-anhydrouridine ($TpAU^{Ac}$,XXXIII) and thymidylyl-(3'-5')-3'-O-benzoyl-8,2'-thioanhydroinosine ($TpSAnI^{Bz}$,XXXIV) were partially resistant to snake venom phosphodiesterase. These compounds are important because of their potential anti-viral activities. Eckstein[23] has shown that increased interferon production could be induced by polymers which had increased resistance to phosphodiesterases. Compounds XXXV (PNP-pT), XXXVI ($PNP\text{-}pT^{Ac}$) and XXXVII (Tp-PNP) were chosen as convenient substrates of snake venom or spleen enzymes respectively. Inhibition studies were conducted with $AUpU^{Iso}$ and $TpAU^{Ac}$ on both spleen and snake venom enzymes.

SPLEEN PHOSPHODIESTERASE STUDIES

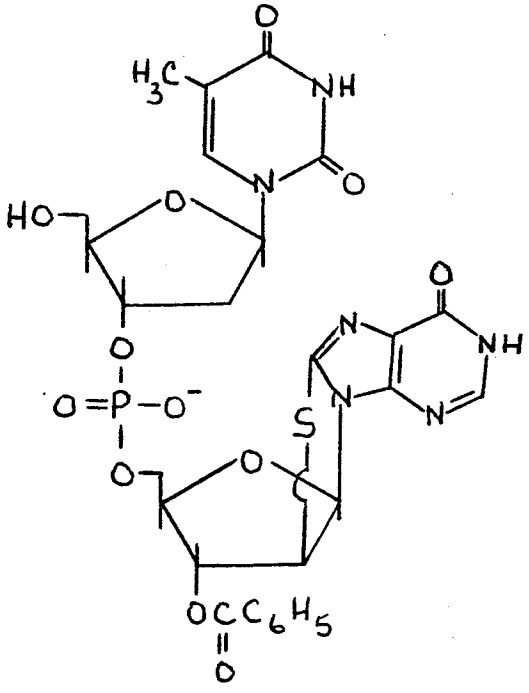
A plot of the amount of substrate reacted versus time was made for Tp-PNP (1mM-6mM) to determine the maximum time during which the enzyme reaction was linear. For the substrate concentrations studied the reaction



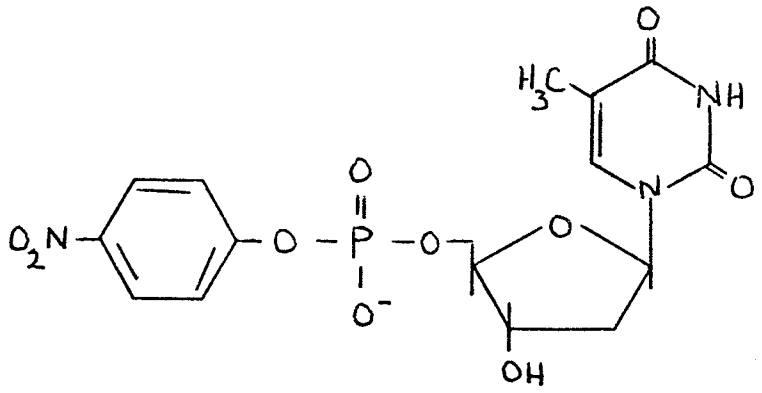
XXXII, $AUpU^{Iso}$



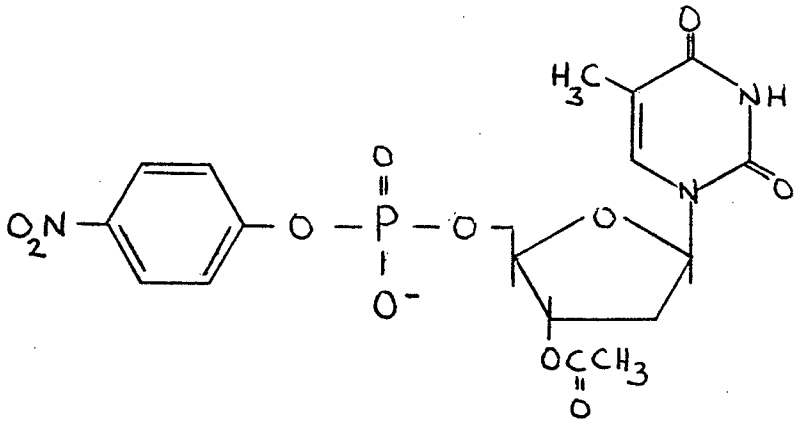
XXXIII, $TpAU^{Ap}$



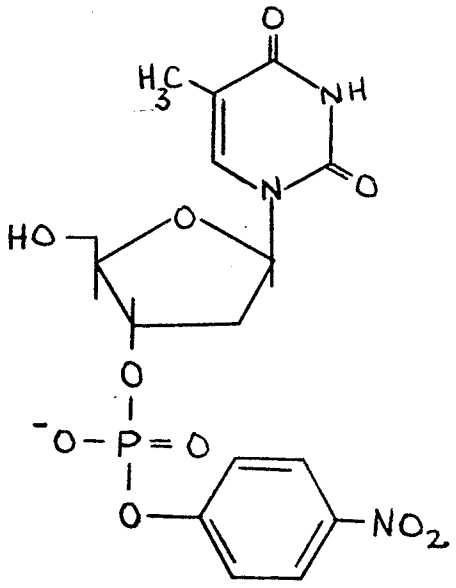
XXXIV, $TpSAnI^{Bz}$



XXXV, PNP-pT



XXXVI, PNP-pT^{Ac}



XXXVII, Tp-PNP

was linear up to 2 min. The data for the time course of hydrolysis of Tp-PNP are recorded in Tables 4(a)-(e) and the curves are plotted in Fig. 4.

TABLE 4(a): Variation with time of the hydrolysis of Tp-PNP, 1mM.

Time (min.)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No. μmoles Reacted x 10
2	0.150	12.5	1.26
4	0.200	16.7	1.69
6	0.170	14.2	1.44
11	0.200	16.7	1.69
19	0.245	20.4	2.06
30	0.215	17.9	1.81
45	0.220	18.4	1.86
60	0.240	19.2	1.94

TABLE 4(b): Variation with time of the hydrolysis of Tp-PNP, 2mM.

Time (min.)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No. μmoles Reacted x 10
2	0.248	20.7	2.09
4	0.312	26.0	2.63
6	0.315	26.2	2.65
12	0.467	38.9	3.92
20	0.456	38.0	3.84
30	0.415	34.6	3.50
45	0.455	38.0	3.84
60	0.561	46.8	4.72

TABLE 4(c): Variation with time of the hydrolysis of Tp-PNP, 3mM.

Time (min.)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No. μmoles Reacted x 10
2	0.254	21.2	2.14
4	0.366	30.5	3.08
6	0.357	29.8	3.02
10	0.526	43.9	4.44
21	0.650	54.3	5.49
31	0.640	53.4	5.40
40	0.690	57.5	5.81

TABLE 4(d): Variation with time of the hydrolysis of Tp-PNP, 4mM.

Time (min.)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No. μmoles Reacted x 10
2	0.375	31.2	3.15
4	0.435	36.2	3.66
6	0.598	49.8	5.03
10	0.775	64.6	6.53
20	0.809	67.4	6.80
30.5	0.809	67.4	6.80
60	0.925	77.0	7.77
75	1.030	86.0	8.69

TABLE 4(e): Variation with time of the hydrolysis of Tp-PNP, 6mM.

Time (min.)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No. μmoles Reacted x 10
2	0.475	39.6	4.06
4	0.495	41.3	4.17
6	0.770	64.2	6.49
10	1.110	92.6	9.36
20	0.910	75.8	7.65
30.5	1.170	97.5	9.85
75	1.470	122.5	12.40

Initial velocities as determined from Fig. 4 were used in the Lineweaver-Burk plot (Fig.5.1) for the calculation of Km and Vmax of Tp-PNP.

TABLE 5.1(a): Variation of initial velocities with substrate concentration. (See Fig. 5.1).

Concentration of Tp-PNP (mM)	Vx10 μmole/min.	1/Tp-PNP ($M^{-1}x10^{-2}$)	$1/Vx10^{-1}$
1.0	0.63	10.00	1.59
2.0	1.05	5.00	0.95
3.0	1.07	3.33	0.93
4.0	1.58	2.50	0.63
6.0	2.03	1.67	0.49

Figure 4. Time course of hydrolysis of Tp-PNP (1mM-6mM) by spleen phosphodiesterase (0.75 unit). ●-●, 1mM; O-O, 2mM; ⊖-⊖, 3mM; ◐-◐, 4mM; ⊖-⊖, 6mM.

FIG. 4

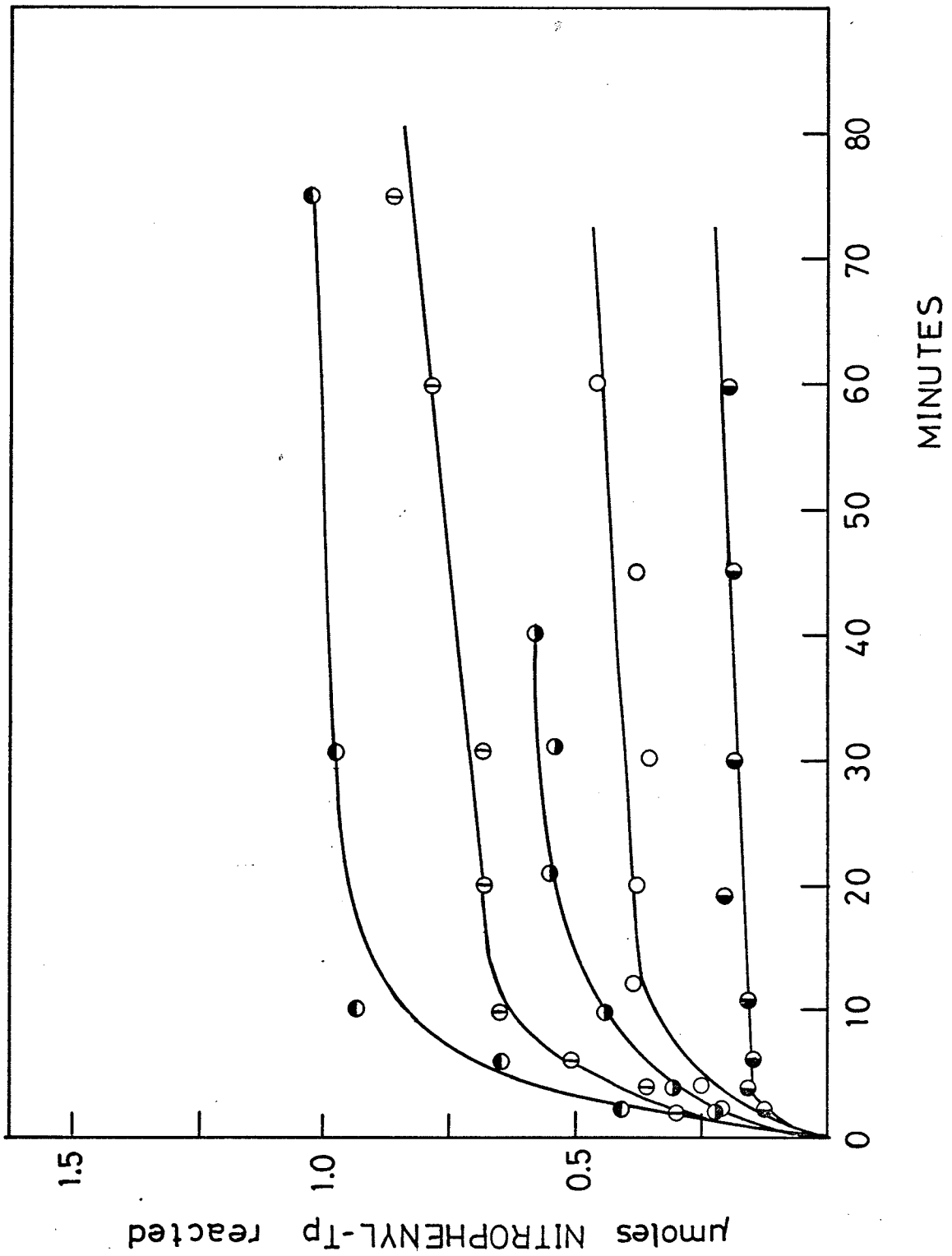
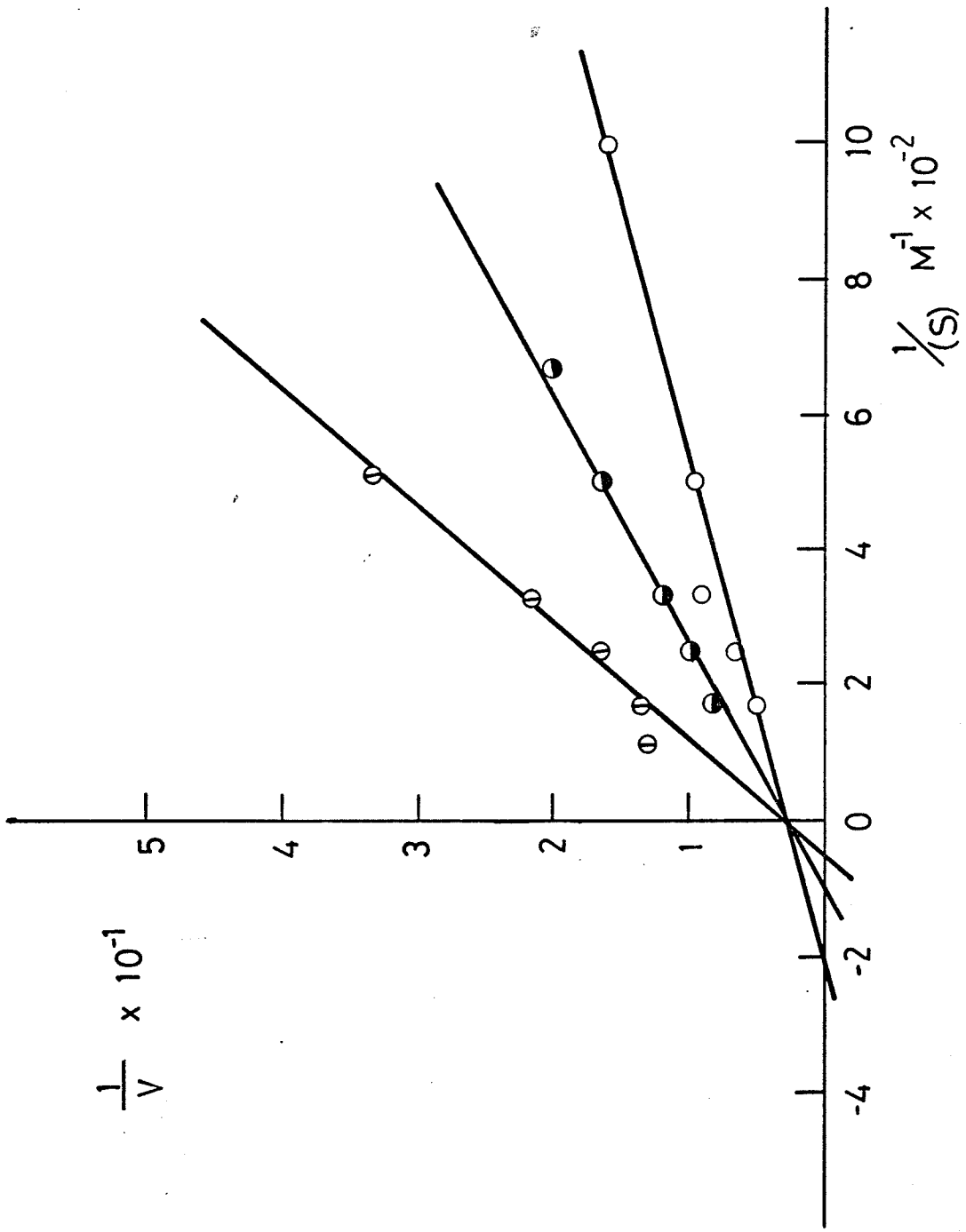

μmoles NITROPHENYL-Tp reacted
1.5
1.0
0.5
0
10
20
30
40
50
60
70
80
MINUTES

Figure 5.1. Lineweaver-Burk plots showing the effect of $TpAU^{Ac}$ and $AUpU^{Iso}$ as competitive inhibitors with Tp-PNP as substrate. Spleen phosphodiesterase was used. O-O, nitrophenyl-Tp; ⊕-⊕, nitrophenyl-Tp plus $TpAU^{Ac}$ (6.3×10^{-4}M); ⊖-⊖, nitrophenyl-Tp plus $AUpU^{Iso}$ (6.5mM).

V = μmole/min.

FIG. 5.1



The Km for Tp-PNP was calculated to be 4.55×10^{-3}M and the Vmax was 0.33 μmole per min.

Inhibition studies were carried out on AU, $AUpU^{Iso}$ and $TpAU^{Ac}$. The effect of each of these compounds on the initial velocity of Tp-PNP was measured at varying concentrations of enzyme. $TpAU^{Ac}$ was found to be the best inhibitor. There was 10% inhibition at S/I = 4, as shown in Fig. 5.2. Anhydrouridine showed only 5% inhibition at S/I = 1, while $AUpU^{Iso}$ did not appear to slow down the hydrolysis of Tp-nitrophenyl at all (see Fig. 6). $TpAU^{Ac}$ is 8x as effective an inhibitor as AU.

The data for the above studies are recorded in Tables 5.2(a),(b); 6(a)-(c).

TABLE 5.2(a): Effect of enzyme concentration on Tp-PNP (4.0mM). Reaction carried out for 4 min. at 37°. Absorbance taken with 1mm cells.

Concentration of Spleen(ml)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No.μmoles Reacted x 10	$Vx10^2$ μmole/min
0.050	1.50	125	2.82	7.05
0.075	1.80	150	3.41	8.52
0.100	2.00	167	3.84	9.60
0.150	2.30	192	4.50	11.25
0.200	2.52	210	5.04	12.60

Figure 5.2. Effect of $TpAU^{Ac}$(I) on Tp-PNP(S) at varying spleen enzyme concentrations. S/I = 4; O-O, Tp-nitrophenyl (4mM), Θ-Θ, Tp-PNP plus $TpAU^{Ac}$

5.3. A Vo/V vs. I plot for $TpAU^{Ac}$ to determine the inhibition index. Tp-PNP concentration = 2mM.

FIG. 5.2

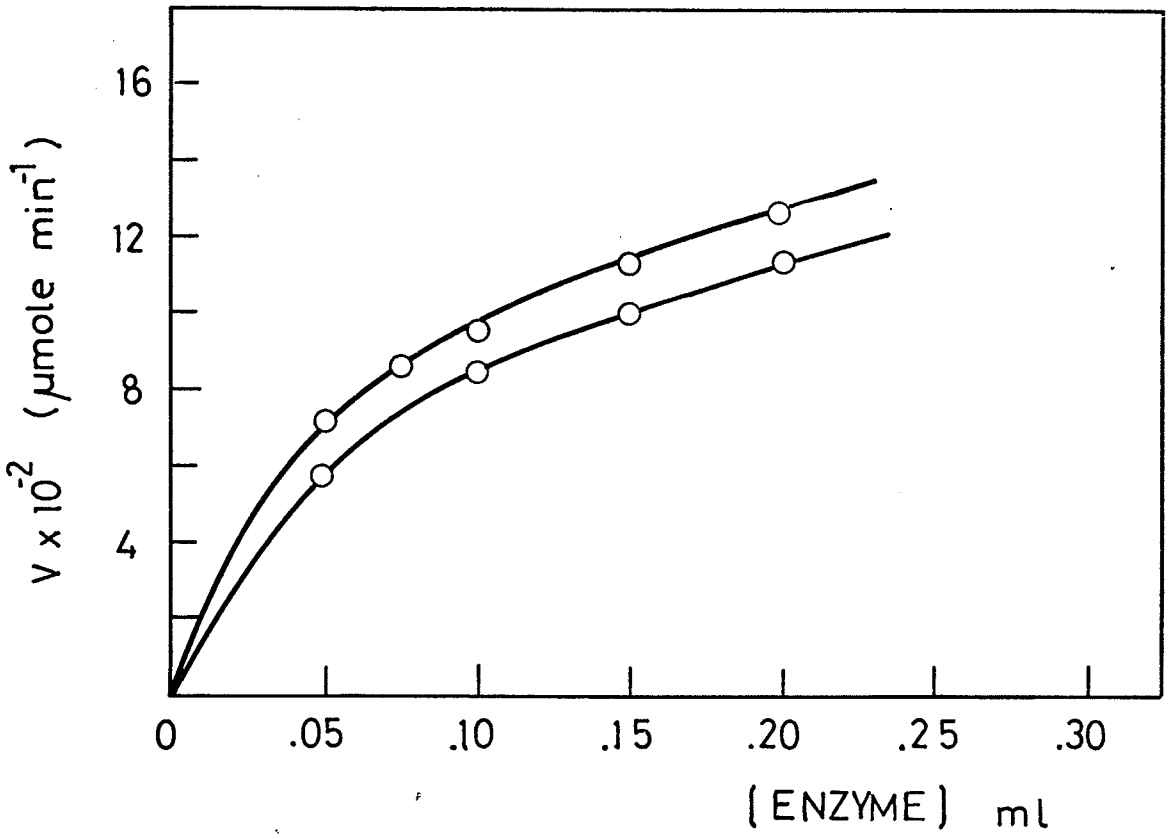

16
12
8
4
V x 10^-2 (μmole min^-1)
0
.05
.10
.15
.20
.25
.30
[ENZYME] ml

FIG. 5.3

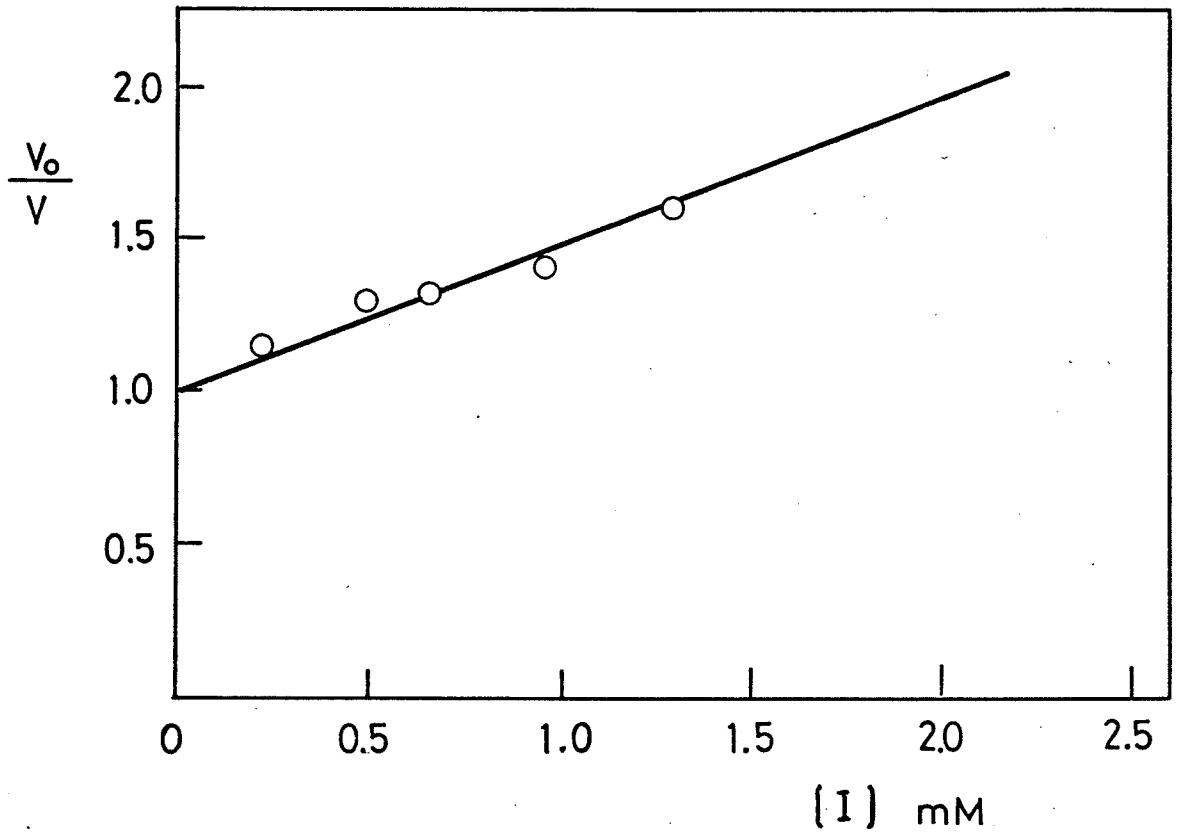

2.0
1.5
1.0
0.5
Vo/V
0
0.5
1.0
1.5
2.0
2.5
[I] mM

TABLE 5.2(b): Effect of enzyme concentration on Tp-PNP (4.0mM) in the presence of $TpAU^{Ac}$. S/I = 4.

Concentration of Spleen(ml)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No.μmoles Reacted x 10	$Vx10^2$ μmole/min
0.050	1.20	100	2.33	5.83
0.075	1.80	150	3.53	8.83
0.100	1.72	143	3.41	8.53
0.150	2.06	172	4.00	10.00
0.200	2.25	188	4.66	11.65

TABLE 6(a): Effect of enzyme concentration on Tp-PNP (6mM). Reaction carried out for 2 min. at 37^o.

Concentration of Spleen(ml)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No.μmoles Reacted x 10	$Vx10^2$ μmole/min
0.025	0.097	8.03	0.74	3.70
0.050	0.150	12.5	1.26	6.30
0.075	0.167	13.9	1.56	8.98
0.100	0.201	16.7	2.06	10.30
0.150	0.211	17.6	2.53	12.65
0.175	0.208	16.9	2.60	13.00

TABLE 6(b): Effect of enzyme concentration on Tp-PNP (6mM) in the presence of AU. S/I = 1.

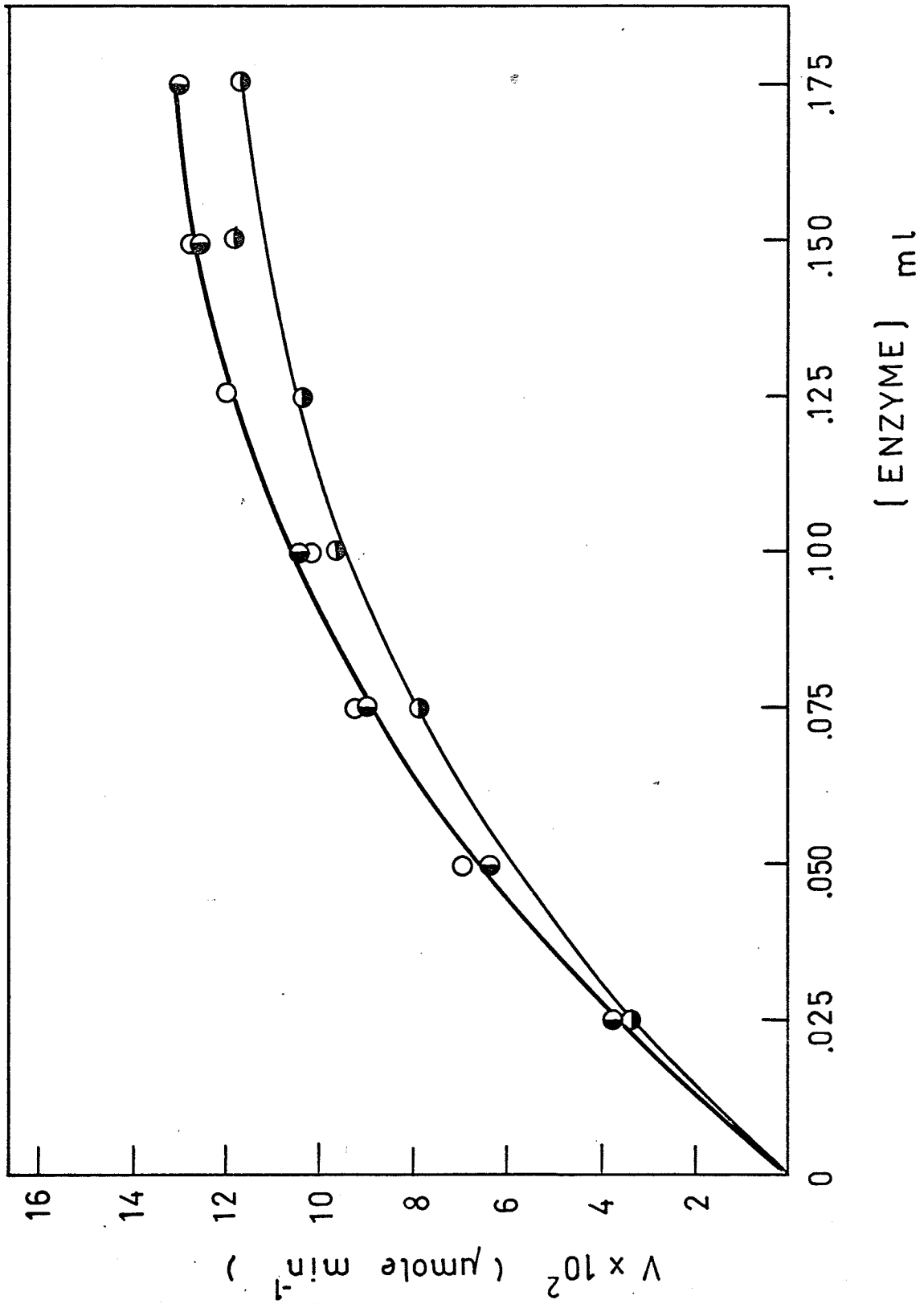
Concentration of Spleen(ml)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No.μmoles Reacted x 10	$Vx10^2$ μmole/min
0.025	0.072	6.07	0.68	3.40
0.050	0.134	11.20	1.37	6.85
0.075	0.135	11.30	1.50	7.50
0.100	0.162	13.50	1.93	9.65
0.125	0.162	13.50	2.06	10.30
0.150	0.173	14.40	2.36	11.80
0.175	0.163	13.60	2.36	11.80

TABLE 6(c): Effect of enzyme concentration on Tp-PNP (6mM) in the presence of $AUpU^{Iso}$. S/I = 4.

Concentration of Spleen(ml)	Absorbance (at 400nm)	Concentration ($Mx10^6$)	No.μmoles Reacted x 10	$Vx10^2$ μmole/min
0.025	0.154	12.8	1.44	4.8
0.050	0.205	17.1	2.10	7.0
0.075	0.272	22.6	2.82	9.4
0.100	0.270	22.4	3.24	10.8
0.125	0.285	23.7	3.66	12.2
0.150	0.281	23.4	3.84	12.8

Figure 6. Effect of inhibitors AU and $AUpU^{Iso}$ on Tp-PNP with varying spleen enzyme concentrations. O-O, Tp-PNP (6mM); ◐-◐, Tp-PNP plus $AUpU^{Iso}$ (S/I = 4); ⊖-⊖, Tp-PNP plus AU (S/I = 1).

FIG. 6


V x 10² (μmole min⁻¹)
16
14
12
10
8
6
4
2
0
.025
.050
.075
.100
.125
.150
.175
[ENZYME] ml

The effect on the Lineweaver-Burk plot of the presence of a constant amount of inhibitor can be seen in Fig. 5.1. The experimental regression lines for $TpAU^{Ac}$ and $AUpU^{Iso}$, when compared with Tp-PNP alone, show the classical case of competitive inhibition. The data for the Lineweaver-Burk plots are shown in Tables 5.1(b),(c).

TABLE 5.1(b): Effect of $TpAU^{Ac}$ (6.3×10^{-3}M) as an inhibitor of Tp-PNP at varying substrate concentrations.

Concentration of Tp-PNP Mx10^3	Absorbance (at 400nm)	Concentration (Mx10^6)	Vx10^2 μmole/min	1/V
2.0	0.62	51.6	3.00	33.3
3.0	0.96	79.5	4.64	21.5
4.0	1.22	102	5.95	16.8
6.0	1.53	128	7.45	13.4
9.0	1.60	133	7.78	12.9

TABLE 5.1(c): Effect of $AUpU^{Iso}$ (6.5mM) as an inhibitor of Tp-PNP.

Concentration of Tp-PNP mM	1/S ($M^{-1}x10^{-2}$)	A400	Concentration ($Mx10^{6}$)	$Vx10^{2}$ μmole/min	1/V $x10^{-1}$
1.5	6.67	0.510	42.5	4.89	2.04
2.0	5.00	0.622	51.8	5.95	1.68
3.0	3.33	0.860	71.8	8.25	1.21
4.0	2.50	1.040	86.6	9.95	1.01
6.0	1.67	1.210	101	11.60	0.86

The Ki was calculated from Fig. 5.1 for $AUpU^{Iso}$. For $TpAU^{Ac}$, Ki was determined by the method of Dixon[52]. The data for the Dixon plots are recorded in Tables 5.4(a)-(d) and are plotted in Fig. 5.4.

TABLE 5.4(a): Effect of inhibitor ($TpAU^{Ac}$) concentration on the rate of hydrolysis of Tp-PNP (2mM).

Concentration $TpAU^{Ac}$(mM)	A400	Concentration ($Mx10^{6}$)	$Vx10^{2}$ μmole/min	1/V
0.60	0.799	66.6	3.83	26.1
1.20	0.700	58.4	3.40	29.4
1.65	0.686	57.1	3.33	30.0
2.41	0.644	53.6	3.13	32.0
3.30	0.574	47.8	2.78	36.0

TABLE 5.4(b): Effect of inhibitor ($TpAU^{Ac}$) concentration on the rate of hydrolysis of Tp-PNP (3mM).

Concentration of $TpAU^{Ac}$(mM)	A400	Concentration ($Mx10^6$)	$Vx10^2$ μmole/min	1/V
0.60	1.134	94.6	5.43	18.4
1.20	0.942	78.5	4.58	21.8
1.65	0.960	80.0	4.65	21.5
2.41	0.943	78.4	4.55	22.0
3.30	0.864	72.0	4.20	23.8

TABLE 5.4(c): Effect of inhibitor ($TpAU^{Ac}$) concentration on Tp-PNP (4mM).

Concentration of $TpAU^{Ac}$(mM)	A400	Concentration ($Mx10^6$)	$Vx10^2$ μmole/min	1/V
1.20	1.332	111	6.48	15.4
1.65	1.380	115	6.67	15.0
2.41	1.300	108	6.30	15.9
3.30	1.260	105	6.13	16.8

TABLE 5.4(d): Effect of inhibitor ($TpAU^{Ac}$) concentration on Tp-PNP (6mM).

Concentration of $TpAU^{Ac}$(mM)	A400	Concentration (Mx10^6)	Vx10^2 μmole/min	1/V
0.60	1.59	132	7.70	13.0
1.20	1.64	137	8.00	12.5
1.65	1.59	132	7.00	13.0
2.41	1.47	123	7.15	14.0
3.30	1.64	137	8.00	12.5

By fitting regression lines to the Dixon plots of Fig.5.4, Ki was calculated to be 3.85×10^{-3}M for $TpAU^{Ac}$ (XXXIII).

To compare the inhibitory effects of $TpAU^{Ac}$ and $AUpU^{Iso}$, the inhibition index was determined for each. The data for the Vo/V vs. I plots are in Tables 5.3 and 10.2(a). The corresponding graphs are represented in Fig. 5.3 and 10.2.

Figure 5.4: A Dixon plot to determine Ki for $TpAU^{Ac}$ with Tp-PNP as substrate.

	Tp-PNP
⊖-⊖,	2mM
O-O,	3mM
⊖-⊖,	4mM
◐-◐,	6mM

V = μmole/min

FIG. 5.4

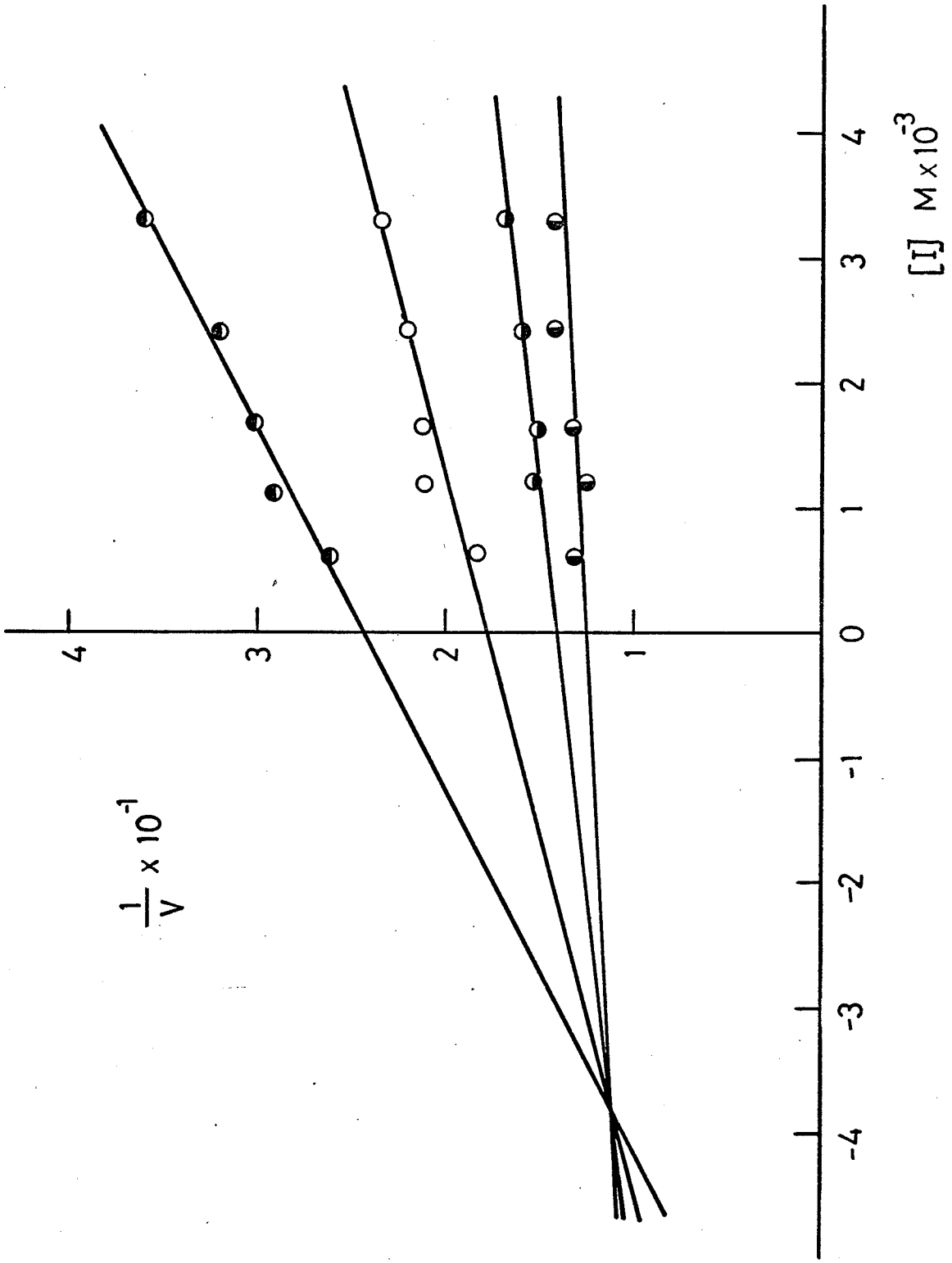


TABLE 5.3: Effect of $TpAU^{Ac}$ concentrations on Tp-PNP (2mM, Vo = 0.044μmole/min.) Reaction time 4 min.

Concentration of $TpAU^{Ac}$(mM)	A400	Concentration (Mx10^6)	Vx10^2 μmole/min	Vo/V
0.24	0.799	66.6	3.83	1.15
0.48	0.700	58.4	3.40	1.29
0.66	0.695	57.9	3.33	1.32
0.96	0.644	53.6	3.13	1.40
1.32	0.574	47.8	2.78	1.58

TABLE 10.2(a): Effect of $AUpU^{Iso}$ concentration on Tp-PNP (1.55mM, Vo = 0.035μmole/min.). Reaction time 4 min.

Concentration of $AUpU^{Iso}$(mM)	A400	Concentration (Mx10^6)	Vx10^2 μmole/min	Vo/V
1.8	0.695	57.9	3.33	1.05
2.7	0.664	55.3	3.18	1.10
3.5	0.695	57.9	3.33	1.05
4.0	0.609	50.8	2.92	1.20
5.8	0.618	51.4	2.96	1.18

TABLE V. Index of Inhibition and Ki of Dinucleotide Derivatives of Anhydrouridine

Compound	Concentration of Tp-PNP (mM)	Concentration of Inhibitor $(I)_{0.5}$	$(I/S)_{0.5}$	$Kix10^3$
$TpAU^{Ac}$	2.0	2.6	1.3	3.85
$AUpU^{Iso}$	1.55	22.4	14.7	8.50

The dinucleotide $TpAU^{Ac}$, with the anhydrouridine at the 3'-end, was completely degraded by spleen enzyme, while $AUpU^{Iso}$, with anhydrouridine at the 5'-end, was resistant to the enzyme[53]. A comparison of the $(I/S)_{0.5}$ in Table V shows that the good substrate $TpAU^{Ac}$ is ten times more effective as an inhibitor than the non-substrate $AUpU^{Iso}$. It can be concluded that although $AUpU^{Iso}$ is not degraded by spleen enzyme, it is capable of binding to the enzyme as a competitive inhibitor (Fig. 5.1) although a very poor one. These results led us to believe that the change in electronic configuration of the uridine ring

may affect the binding of the molecule to the active site to such an extent that substrate activity is eliminated but inhibitor activity was still possible. The fact that anhydrouridine itself is an inhibitor, although only one-eighth as effective as $TpAU^{Ac}$, proves that the anhydronucleoside is capable of binding at the active site. The important feature here is the fact that $AUpU^{Iso}$ is resistant to spleen, which hydrolyses oligonucleotides from the 5'-hydroxyl end to give nucleoside 3'-phosphates.

SNAKE VENOM PHOSPHODIESTERASE STUDIES

Snake venom phosphodiesterase acts in a manner complementary to spleen diesterase. This enzyme begins hydrolysis of oligonucleotides from the 3'-hydroxyl resulting in the successive release of nucleoside 5'-phosphates. Unlike spleen phosphodiesterase which requires a free terminal 5'-hydroxyl group for substrate activity, snake venom diesterase also hydrolyses, but at a slower rate, oligonucleotides which are substituted at the terminal 3'-hydroxyl[56].

AU and $AUpU^{Iso}$ were tested as inhibitors of $PNP\text{-}pT^{Ac}$ while $TpAU^{Ac}$, 8,2'-SAnA and 8-bromoadenosine were tested as inhibitors of PNP-pT. The Km and Vmax

for PNP-pT and PNP-pT^{Ac} were determined from a linear regression analysis of the Lineweaver-Burk plots. The data for these plots are shown in Tables 7.1(a) and 8 and the plots are shown in Fig.7.1 and 8.

TABLE 7.1(a): Effect of varying PNP-pT concentrations on initial velocities. (See Fig.7.1). Enzyme concentration = 0.5mg/ml.

Concentration of Nitrophenyl-pT ($Mx10^{4}$)	V x 10 μmole/min	V/mg	1/S ($M^{-1}x10^{-3}$)	1/V/mg x 10
3.38	1.52	3.04	2.960	3.29
6.45	2.06	4.12	1.550	2.43
9.00	2.27	4.54	1.110	2.20
12.90	2.67	5.24	0.776	1.91
27.20	2.87	5.74	0.368	1.74
30.75	3.21	6.42	0.262	1.56

TABLE 8(a): Effect of varying PNP-pT^{Ac} concentrations on initial velocities. (See Fig.8). Enzyme concentration = 0.5mg/ml.

Concentration of Nitrophenyl-pT^{Ac} ($Mx10^3$)	Vx10 μmole/min	V/mg	1/S ($M^{-1}x10^{-2}$)	1/V/mg x 10
3.0	1.62	3.24	3.33	3.09
3.5	1.65	3.30	2.86	3.03
4.0	1.76	3.51	2.50	2.85
4.8	1.92	3.84	2.08	2.61
6.0	2.04	4.08	1.67	2.46

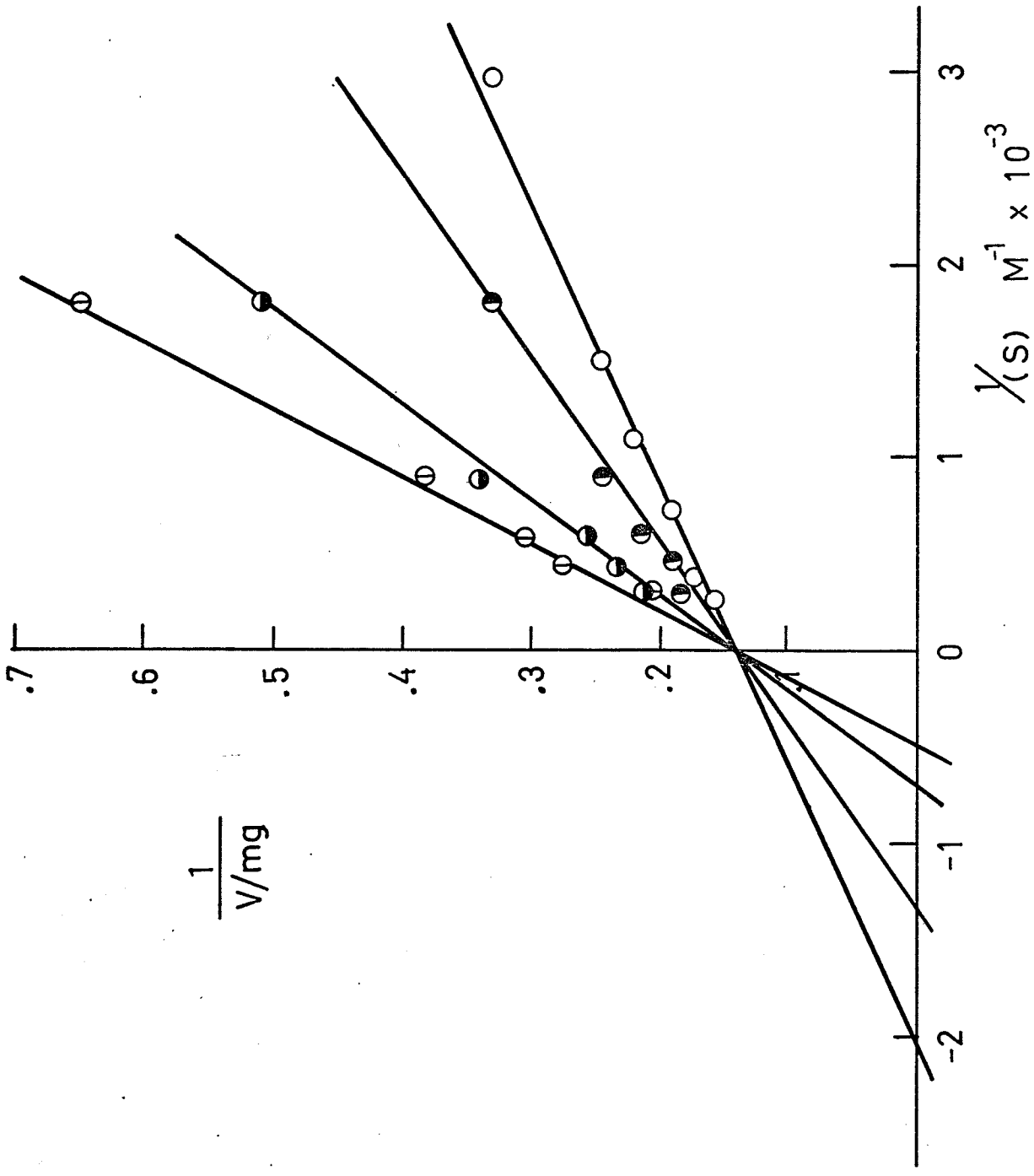
TABLE VI. Kinetic Constants of p-Nitrophenyl Thymidine 5'-Phosphate Derivatives

Compound	Km(M)	Vmax(μmole/min/mg)
Nitrophenyl-pT	4.78×10^{-4}	7.25
Nitrophenyl-pT^{Ac}	2.13×10^{-3}	5.39

Figure 7.1. Lineweaver-Burk plots showing the effect of three competitive inhibitors on PNP-pT as substrate. Venom diesterase = 0.5mg/ml
O-O, PNP-pT; ◐-◐, PNP-pT plus $TpAU^{Ac}$ (10mM); ⊖-⊖, PNP-pT plus 8,2-SAnA (3mM); ◐-◐, PNP-pT plus 8-bromoadenosine (3mM).

V = μmole/min.

FIG. 7.1



The effect of 8-bromoadenosine, 8,2'-SAnA and $TpAU^{Ac}$ on PNP-pT as substrate was measured at varying enzyme concentrations. From Fig.7.2 we see that 8-bromoadenosine is a better inhibitor than 8,2'-SAnA at low enzyme concentrations, but at high enzyme concentrations 8,2'-SAnA is the better inhibitor. $TpAU^{Ac}$, on the other hand, does not appear to slow down the hydrolysis of PNP-pT, as shown in Fig.7.4. The data for Fig.7.2 and 7.4 are recorded in Tables 7.2(a)-(c) and 7.4(a),(b) below.

TABLE 7.2(a): Effect of enzyme concentration on PNP-pT (1.51mM).

Concentration of Venom Diesterase (ml)	Final Total Volume (ml)	A400	Concentration ($M \times 10^6$)	V x 10 μmole/min
0.025	2.225	0.650	54.1	1.20
0.050	2.250	0.890	74.2	1.67
0.075	2.275	1.090	91.0	2.07
0.100	2.300	1.125	93.8	2.16
0.150	2.350	1.165	97.2	2.28

TABLE 7.2(b): Effect of enzyme concentration on PNP-pT in the presence of 8-BrA; S/I = 3.

Concentration of Venom Diesterase (ml)	Final Total Volume(ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
0.025	2.275	0.470	39.2	0.89
0.050	2.300	0.546	45.5	1.05
0.075	2.325	0.901	75.0	1.74
0.100	2.350	1.051	87.5	2.06
0.150	2.400	1.145	95.3	2.28

TABLE 7.2(c): Effect of enzyme concentration on PNP-pT in the presence of 8,2'-SAnA; S/I = 3.

Concentration of Venom Diesterase (ml)	Final Total Volume(ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
0.025	2.275	0.552	46.0	1.05
0.050	2.300	0.724	59.6	1.37
0.075	2.325	0.820	68.4	1.59
0.100	2.350	0.934	77.8	1.83
0.150	2.400	1.026	85.5	2.05

Figure 7.2. Effect of inhibitors on PNP-pT at varying enzyme concentrations.
O-O, PNP-pT (1.51mM); ⊖-⊖, PNP-pT plus 8,2'-SAnA; Φ-Φ, PNP-pT plus 8-bromo-adenosine; S/I = 3.

FIG. 7.2

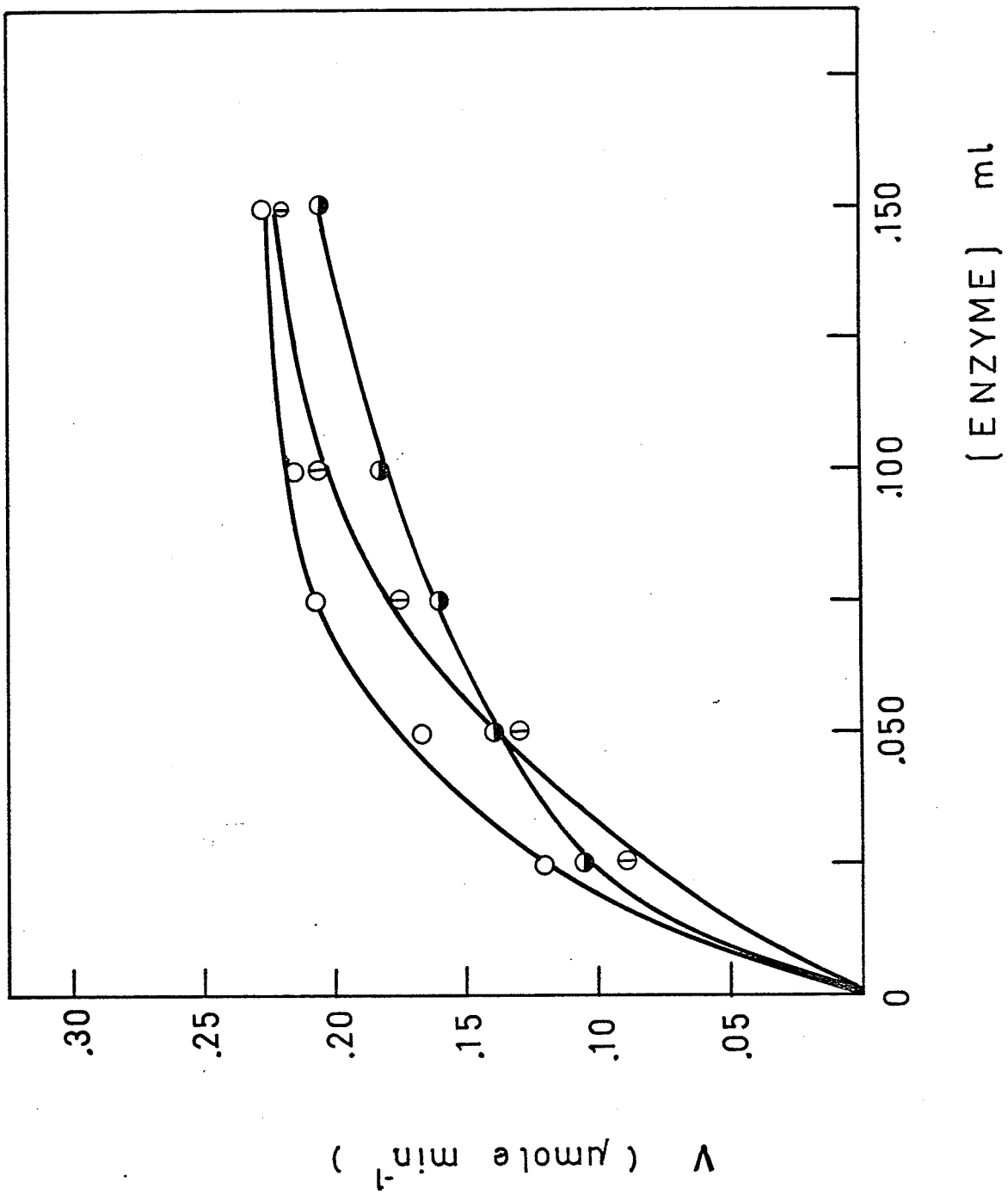


TABLE 7.4(a): Effect of enzyme concentration on PNP-pT (3.075mM) as substrate.

Concentration of Venom Diesterase (ml)	Final Total Volume (ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
0.025	2.225	0.628	52.3	1.16
0.050	2.250	1.240	103	2.32
0.075	2.275	1.668	139	3.16
0.100	3.300	1.383	115	3.81
0.150	3.350	1.707	142	4.76

TABLE 7.4(b): Effect of enzyme concentration on PNP-pT in the presence of $TpAU^{AC}$; S/I = 3.

Concentration of Venom Diesterase (ml)	Final Total Volume (ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
0.025	2.305	0.630	52.6	1.25
0.050	2.330	1.210	101	2.40
0.075	2.355	1.633	136	3.21
0.100	3.380	1.375	114	3.87
0.150	3.430	1.643	137	4.70

The effect on the Lineweaver-Burk plot of the presence of a constant amount of inhibitor can be seen in Fig.7.1. The experimental regression lines for $TpAU^{Ac}$, 8,2'-SAnA and 8-bromoadenosine, when compared with PNP-pT alone, show the classical case of competitive inhibition. The data for the Lineweaver-Burk plots are shown in Tables 7.1(b)-(d).

TABLE 7.1(b): Effect of 8-bromoadenosine (3mM) as an inhibitor of PNP-pT.

Concentration of PNP-pT(mM)	1/S ($M^{-1}x10^{-3}$)	A400	Concentration (Mx10^6)	Vx10 (μmole/min)	V/mg	1/V/mg x10
0.558	1.800	0.685	57	0.77	1.54	6.50
1.116	0.895	1.17	97.5	1.31	2.63	3.80
1.674	0.598	1.47	123	1.66	3.32	3.02
2.240	0.447	1.63	136	1.83	3.66	2.74
2.790	0.360	2.21	184	2.48	4.96	2.02

TABLE 7.1(c): Effect of 8,2'-SAnA (3mM) as an inhibitor of PNP-pT.

Concentration of PNP-pT(mM)	1/S ($M^{-1}x10^{-3}$)	A400	Concentration ($Mx10^6$)	Vx10 (μmole/min)	V/mg	1/V/mg x10
0.558	1.800	0.87	72.5	0.98	1.96	5.10
1.116	0.895	1.31	109	1.47	2.94	3.40
1.674	0.598	1.74	145	1.96	3.92	2.56
2.240	0.447	1.93	161	2.17	4.34	2.31
2.790	0.360	2.12	177	2.39	4.78	2.09

TABLE 7.1(d): Effect of $TpAU^{Ac}$(10mM) as an inhibitor on PNP-pT.

Concentration of PNP-pT(mM)	1/S ($M^{-1}x10^{-3}$)	A400	Concentration ($Mx10^6$)	Vx10 (μmole/min)	V/mg	1/V/mg x10
0.558	1.800	1.35	112.5	1.52	3.04	3.29
1.116	0.895	1.81	151	2.04	4.08	2.45
1.674	0.598	2.08	173	2.33	4.66	2.15
2.240	0.447	2.34	195	2.63	5.26	1.90
2.790	0.360	2.48	206	2.78	5.56	1.80

The effect of anhydrouridine on PNP-pT^{Ac} was measured at varying enzyme concentrations. A 10% inhibition was observed for I/S = 1.25, as shown in Fig.7.3 and Table 7.3 (a),(b).

Figure 7.3. Effect of AU on PNP-pT^{Ac}(4.8mM) at varying snake diesterase concentrations O-O, PNP-pT^{Ac}; ⊖-⊖, PNP-pT^{Ac} plus AU (I/S = 1.25).

7.4. Effect of TpAU^{Ac} on PNP-pT (3.075mM) at varying snake diesterase concentrations. O-O, PNP-pT; ●-●, PNP-pT plus TpAU^{Ac} (I/S = 0.33).

FIG. 7.3

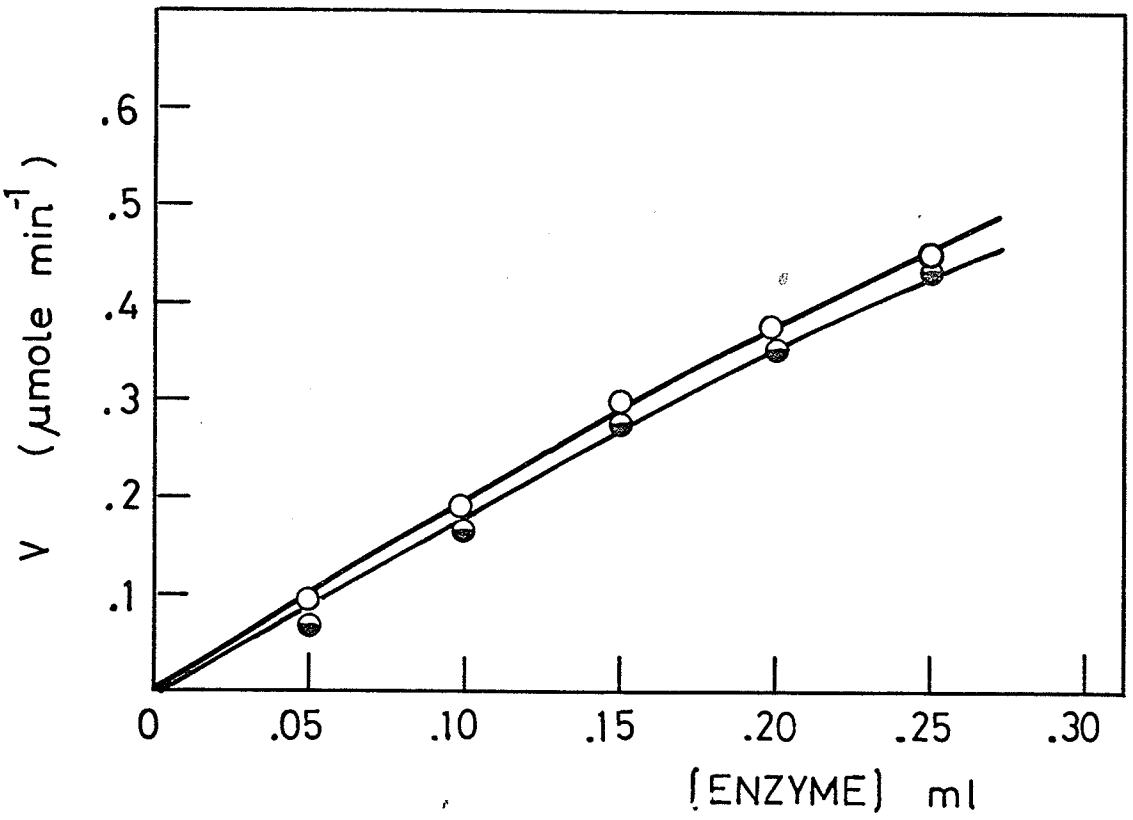

V (μmole min-1)
.6
.5
.4
.3
.2
.1
0
.05
.10
.15
.20
.25
.30
[ENZYME] ml

FIG. 7.4

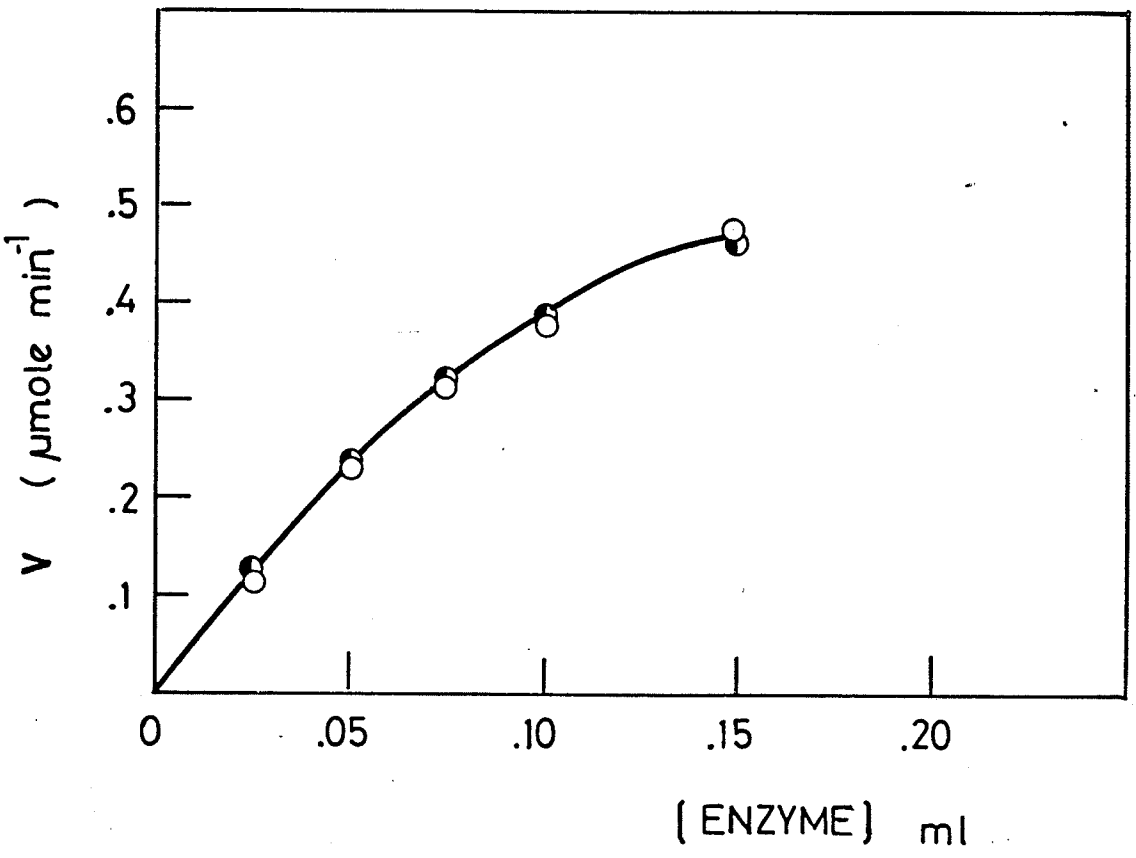

V (μmole min-1)
.6
.5
.4
.3
.2
.1
0
.05
.10
.15
.20
[ENZYME] ml

Figure 8. Lineweaver-Burk plots showing the effect of a competitive inhibitor, $AUpU^{Iso}$, on $PNP\text{-}pT^{Ac}$ as substrate. Venom diesterase = 0.5mg/ml. O-O, $PNP\text{-}pT^{Ac}$; Θ-Θ, $PNP\text{-}pT^{Ac}$; plus $AUpU^{Iso}$ (9.6×10^{-4}M).

FIG. 8

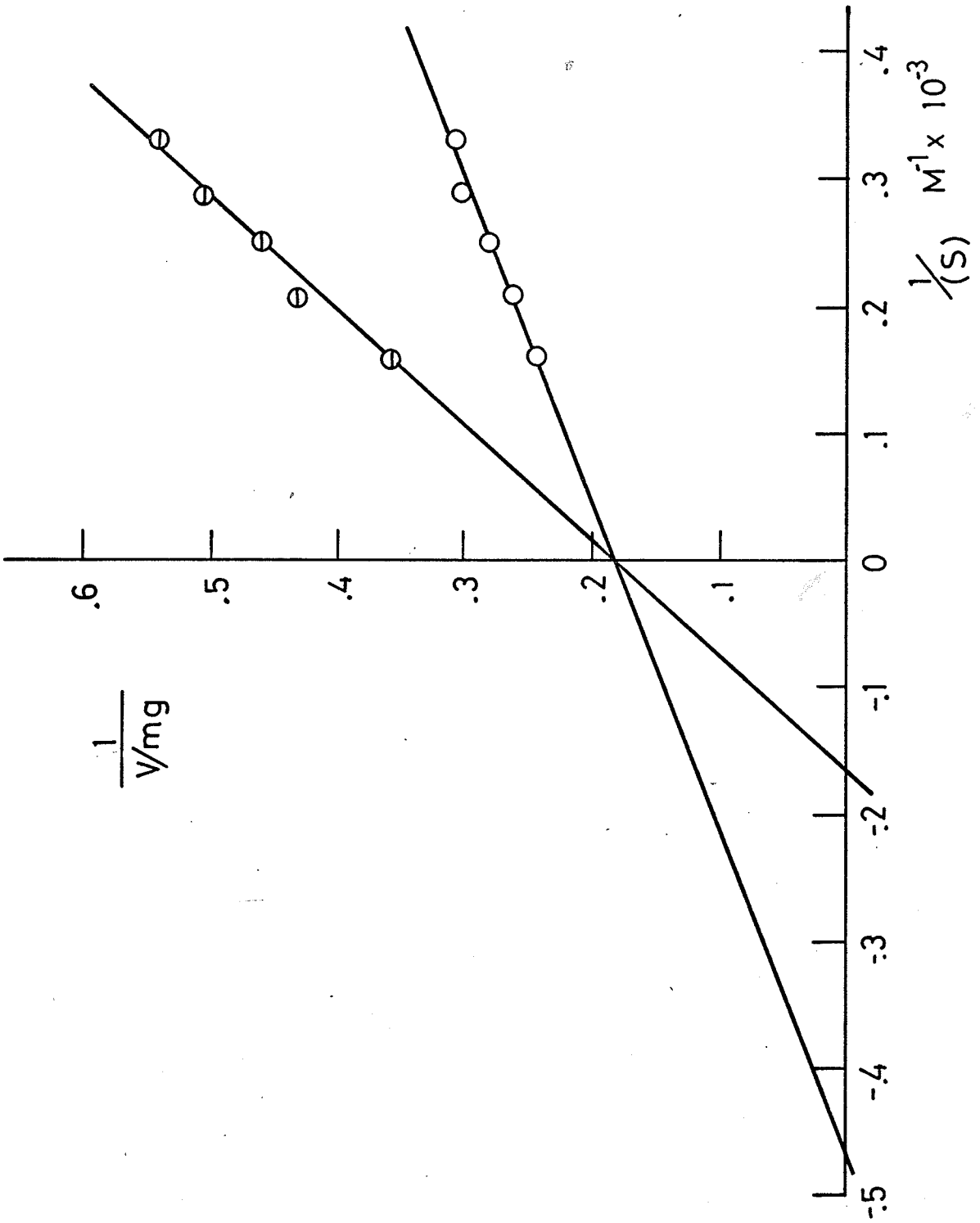


TABLE 7.3(a): Effect of enzyme concentration on PNP-pT^{Ac} (4.8mM) as substrate.

Concentration of Venom Diesterase(ml)	Final Total Volume (ml)	A400	Concentration ($Mx10^6$)	$V \times 10^2$ μmole/min
0.05	1.25	0.88	73.4	9.18
0.10	1.30	1.78	148	19.2
0.15	1.35	2.65	221	29.9
0.20	1.40	3.23	269	37.7
0.25	1.45	3.69	308	44.7

TABLE 7.3(b): Effect of enzyme concentration on PNP-pT^{Ac} in the presence of AU; I/S = 1.25.

Concentration of Venom Diesterase(ml)	Final Total Volume(ml)	A400	Concentration ($Mx10^6$)	$V \times 10^2$ μmole/min
0.05	1.30	0.71	59.2	7.7
0.10	1.35	1.55	129	17.4
0.15	1.40	2.39	199	27.9
0.20	1.45	2.95	246	35.7
0.25	1.50	3.65	306	45.6

The effect of $AUpU^{Iso}$ on PNP-pT and $PNP\text{-}pT^{Ac}$ as substrates was measured at varying enzyme concentrations. As seen in Fig.9.1 and 9.2, there is 21% inhibition of PNP-pT at S/I = 3 and 26% inhibition of $PNP\text{-}pT^{Ac}$ by $AUpU^{Iso}$ at S/I = 6. $AUpU^{Iso}$ is twice as good as inhibitor of $PNP\text{-}pT^{Ac}$ than it is of PNP-pT. The data for Figures 9.1 and 9.2 are recorded below in Tables 9.1(a),(b) and 9.2(a),(b).

TABLE 9.1(a): Effect of enzyme concentration on the activity of PNP-pT (2.77mM).

Concentration of Venom Diesterase(ml)	Final Total Volume (ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
Blank				1.92
0.05	1.75	2.73	228	3.98
0.10	1.80	3.19	266	4.79
0.15	1.85	3.38	282	5.21
0.20	1.90	3.80	317	6.03
0.25	1.95	3.67	306	5.97

TABLE 9.1(b): Effect of enzyme concentration on the activity of PNP-pT (2.77mM) in the presence of $AUpU^{Iso}$; S/I = 3.

Concentration of Venom Diesterase(ml)	Final Total Volume (ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
0.05	1.83	2.18	182	3.32
0.10	1.89	2.38	199	3.76
0.15	1.93	2.60	217	4.18
0.20	1.98	2.99	249	4.94
0.25	2.03	2.95	246	4.94

TABLE 9.2(a): Effect of enzyme concentration on the activity of PNP-pT^{Ac}(6mM).

Concentration of Venom Diesterase(ml)	Final Total Volume(ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
0.05	1.25	0.83	69.2	0.87
0.10	1.30	1.99	166	2.16
0.15	1.35	3.24	270	3.64
0.20	1.40	4.04	337	4.72
0.25	1.45	4.84	404	5.86

Figure 9.1. Effect of $AUpU^{Iso}$ on PNP-pT as substrate at varying snake venom enzyme concentration O-O, PNP-pT (2.77mM); ⊖-⊖, PNP-pT plus $AUpU^{Iso}$ (S/I = 3).

9.2. Effect of $AUpU^{Iso}$ on $PNP\text{-}pT^{Ac}$ as substrate at varying snake venom enzyme concentrations. ⊙-⊙, $PNP\text{-}pT^{Ac}$ (6mM); ●-●, $PNP\text{-}pT^{Ac}$ plus $AUpU^{Iso}$ (S/I = 6).

FIG. 9.1

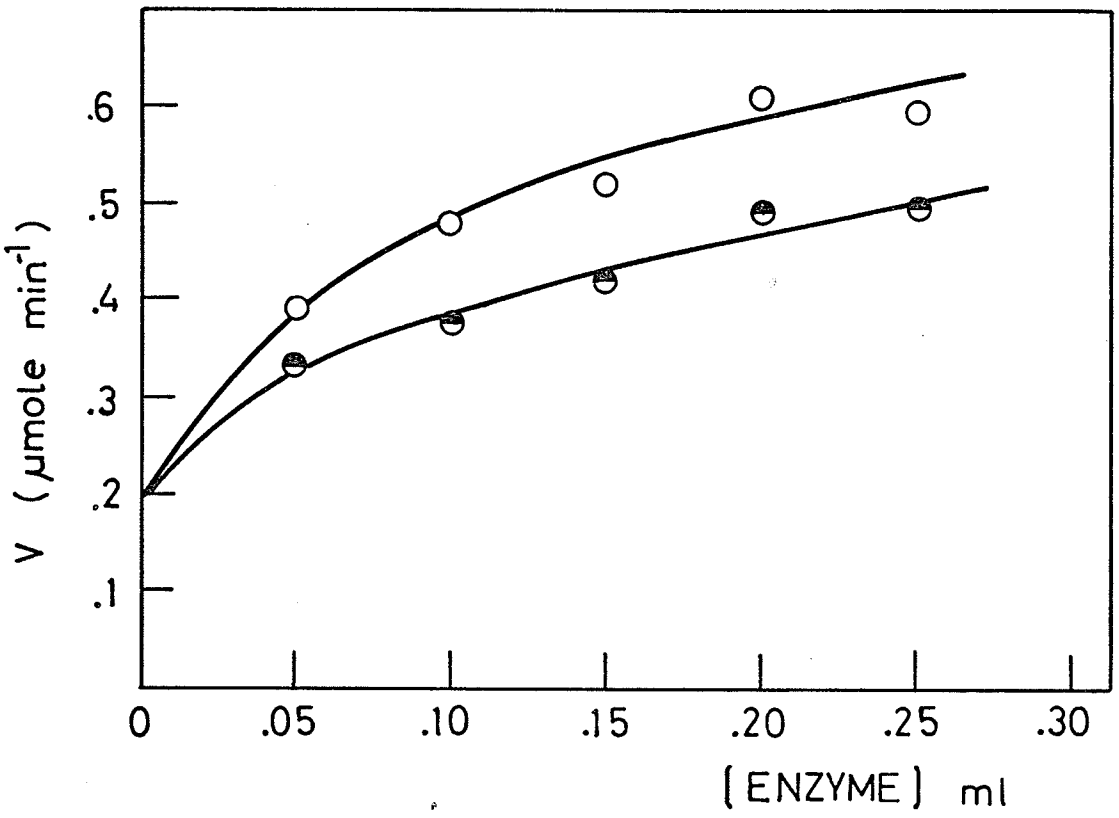

V (μmole min⁻¹)
.6
.5
.4
.3
.2
.1
0
.05
.10
.15
.20
.25
.30
[ENZYME] ml

FIG. 9.2

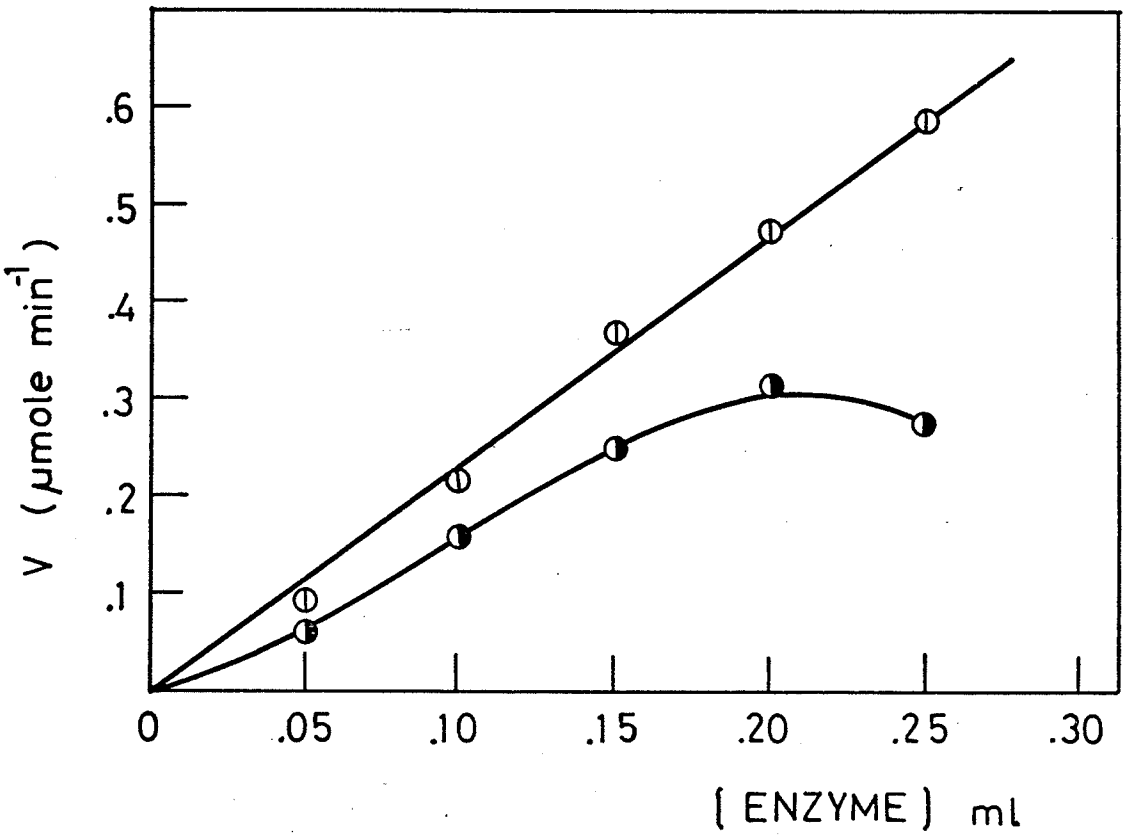

V (μmole min⁻¹)
.6
.5
.4
.3
.2
.1
0
.05
.10
.15
.20
.25
.30
[ENZYME] ml

TABLE 9.2(b): Effect of enzyme concentration on the activity of PNP-pT^{Ac} (6mM) in the presence of $AUpU^{Iso}$; S/I = 6.

Concentration of Venom Diesterase(ml)	Final Total Volume (ml)	A400	Concentration ($Mx10^6$)	V x 10 μmole/min
0.05	1.33	0.52	43.4	0.58
0.10	1.38	1.36	113	1.58
0.15	1.43	2.08	173	2.48
0.20	1.48	2.53	211	3.12
0.25	1.53	2.14	178	2.72

The effect of a constant amount of $AUpU^{Iso}$ on the Lineweaver-Burk plot is shown in Fig.8. A comparison of the regression line for $AUpU^{Iso}$ with that for PNP-pT^{Ac} alone indicates that the inhibition is competitive. The data for the regression line in the presence of $AUpU^{Iso}$ are recorded in Table 8(b).

TABLE 8(b): Effect of $AUpU^{Iso}$ (9.6×10^{-4}M) as an inhibitor with PNP-pT^{Ac} as substrate.

Concentration of PNP-pT^{Ac} (mM)	1/S ($M^{-1} \times 10^{-2}$)	A400	Concentration ($M \times 10^{6}$)	$V \times 10^{2}$ (μmole/min)	V/mg	1/V/mg x10
3.0	3.33	0.805	67.0	9.25	1.85	5.42
3.5	2.86	0.870	71.6	9.90	1.98	5.05
4.0	2.50	0.950	79.2	10.90	2.18	4.58
4.8	2.08	1.010	84.3	11.60	2.32	4.31
6.0	1.67	1.210	100.6	13.90	2.78	3.60

A comparison of the inhibitory effects of the inhibitors $TpAU^{Ac}$ and $AUpU^{Iso}$ was made by determining the inhibition index for each. This was done by making two Vo/V vs. I plots, as shown in Figures 10.1 and 10.2. The data for these two figures are recorded below in Tables 10.1 and 10.2(b).

Figure 10.1 A plot of Vo/V vs. I for determination of inhibition index for $AUpU^{Iso}$ on $PNP\text{-}pT^{Ac}$ as substrate (6mM).

10.2 Determination of inhibition index for $TpAU^{Ac}$ with PNP-pT as substrate and for $AUpU^{Iso}$ on Tp-PNP as substrate. Θ-Θ, $TpAU^{Ac}$ (PNP-pT = 0.555mM); Θ-Θ, $AUpU^{Iso}$ (Tp-PNP = 1.55mM).

FIG. 10.1

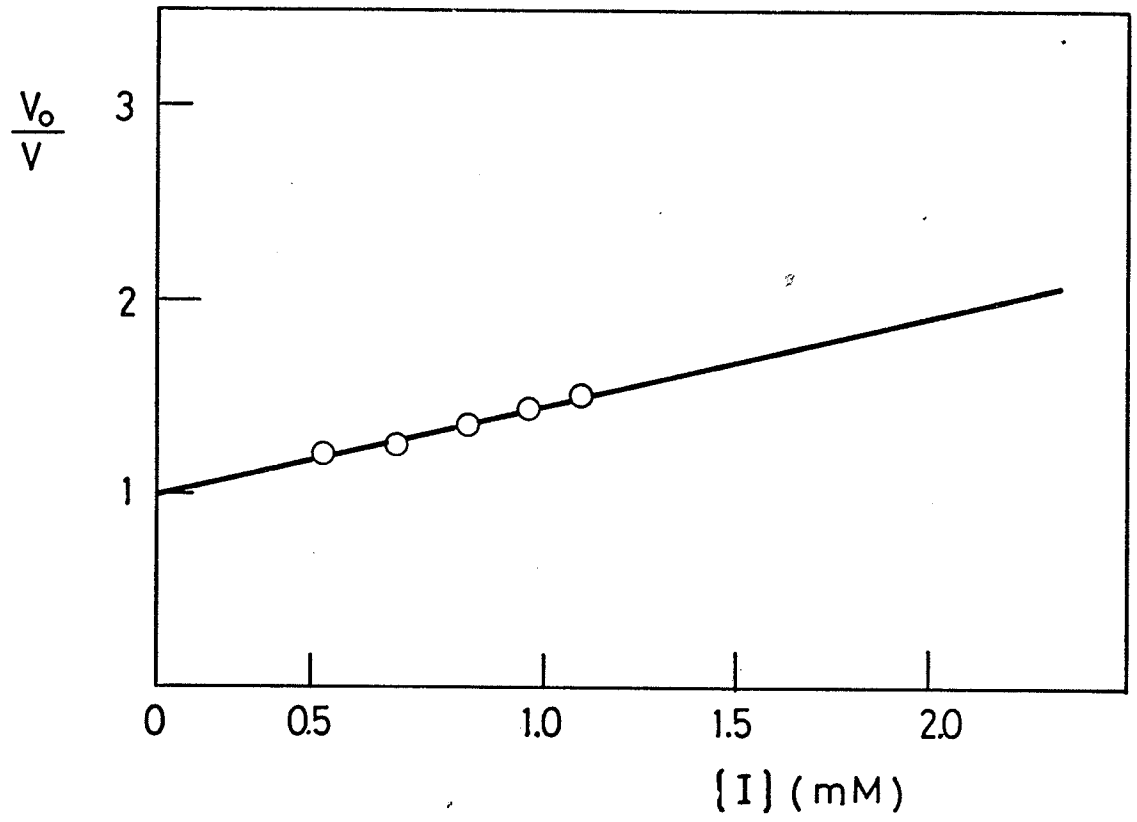


FIG. 10.2

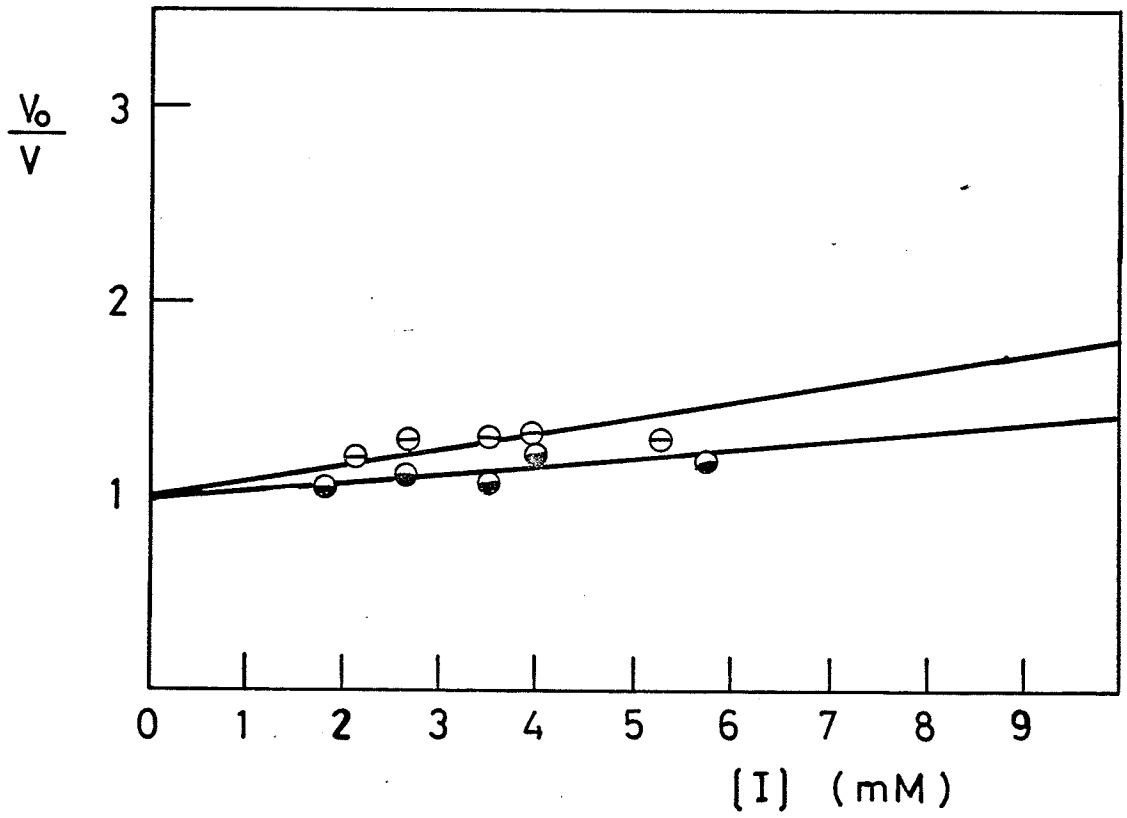


TABLE 10.1: Effect of $AUpU^{Iso}$ concentration on the activity of PNP-pT^{Ac} (6mM), Vo = 6.58 μmoles per min. per mg.

Concentration of $AUpU^{Iso}$ (mM)	A400	Concentration (Mx10^6)	Vx10 (μmole/min	V/mg	Vo/V
0.42	2.37	197	2.73	5.46	1.20
0.61	2.29	191	2.63	5.26	1.25
0.80	2.10	176	2.42	4.84	1.36
0.96	1.95	162	2.24	4.48	1.46
1.09	1.91	159	2.20	4.40	1.50

TABLE 10.2(b): Effect of $TpAU^{Ac}$ concentration on the activity of PNP-pT (0.555mM), Vo = 0.175 μmole per min.

Concentration of $TpAU^{Ac}$(mM)	A400	Concentration (Mx10^6)	Vx10 (μmole/min)	Vo/V
2.12	1.35	112	1.46	1.20
2.65	1.25	104	1.35	1.29
3.53	1.24	103	1.34	1.30
3.98	1.23	102	1.32	1.32
5.30	1.25	104	1.35	1.29

The Ki, as calculated from the Lineweaver-Burk plots, and $(I/S)_{0.5}$, as determined from the Vo/V vs. I plots, are listed in Table VII.

TABLE VII: Index of Inhibition and Ki for Dinucleotide Derivatives of Anhydrouridine and some Adenosine Derivatives.

Compound	Concentration of PNP-pT(mM)	Concentration of Inhibitor $(I)_{0.5}$	$(I/S)_{0.5}$	Ki
$TpAU^{Ac}$	0.555	12.5	22.6	17.6×10^{-3}
$AUpU^{Iso}$	6.0*	2.25	0.375	4.59×10^{-4}
8,2'-SAnA	-	-	-	15.10×10^{-4}
8-BrA	-	-	-	9.44×10^{-4}

* Substrate was PNP-pT^{Ac}.
8BrA = 8-bromoadenosine

The dinucleotide $AUpU^{Iso}$ was completely degraded by snake venom phosphodiesterase while $TpAU^{Ac}$, with anhydrouridine at the 3'-end, showed less than 5% hydrolysis after 7 hr. [53] Both dinucleotides were found to be competitive inhibitors, with $AUpU^{Iso}$ being the better inhibitor by a factor of 50. Taking into

account that PNP-pT^{Ac} is inhibited 2x more effectively than is PNP-pT, AUpU^{Iso} is then the better inhibitor by a factor of 25. Anhydrouridine itself is an inhibitor of snake venom phosphodiesterase. By comparing the amount of inhibition by AU and AUpU^{Iso} on PNP-pT^{Ac} (Fig.7.3 and 9.2) we see that AUpU^{Iso} is about 12x as effective as AU.

It was also possible in the venom diesterase studies to test 8,2'-SAnA and 8-BrA as inhibitors. Both were found to be competitive inhibitors, about 10x as effective as TpAU^{Ac}. Two factors must be considered. Firstly, 8,2'-SAnA is in an anti conformation while 8-bromoadenosine is in the syn conformation. Secondly, in these two molecules the electronic configuration of the base ring is not altered. From the comparison of the Ki values in Table VII, the following conclusions may be made. With the snake venom phosphodiesterase the conformation of the base ring does not play a critical part in the binding to the active site, since both 8,2'-SAnA and 8-BrA were competitive inhibitors. Furthermore, a change in the electronic configuration of the base ring does indeed affect the binding of molecules to the active site, since TpAU^{Iso} is only 1/25 as strong an inhibitor as AUpU^{Iso}.

An enzymatic degradation of a dinucleotide containing an anhydropurine derivative, Tp-8,2'-SAnI^{Bz}, showed similar results as with the anhydropyrimidine derivative, TpAU^{Ac}. Tp-8,2'-SAnI^{Bz} was completely degraded by spleen diesterase but showed only 46% hydrolysis by snake enzyme after 24 hr. TpAU also showed about 45% hydrolysis after 24 hr., but the degradation products were thymidine and arabinouridine 5'-phosphate [53]. The anhydro linkage had been hydrolysed during the long incubation period at pH 9.2. Therefore, the apparent 45% hydrolysis of TpAU was probably a result of the breaking of the anhydro linkage which would speed up the enzymic reaction. It is safe to conclude then, that TpSAnI^{Bz} is a much better substrate than TpAU, eventhough TpAU has a free 3'-OH which should have made it more reactive. Since no preferential hydrolysis of purine ribonucleoside 5'-phosphoryl esters over the pyrimidine counterparts has been shown for venom diesterase, as it has been for spleen diesterase[57], the increased hydrolysis of TpSAnI^{Bz} may be attributed to change in the electronic configuration of the uridine ring in AU. This change may be just sufficient to affect the binding ability of TpAU^{Ac} to the active site.

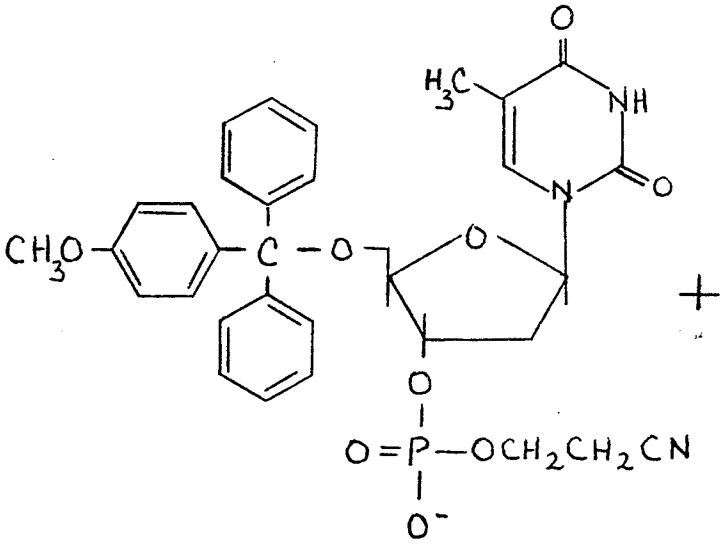
To sum up the results of the spleen and snake venom phosphodiesterase studies, we can say that dinucleotides containing anhydrouridine or thioanhydroinosine derivatives are fairly resistant to the phosphodiesterases but are very poor competitive inhibitors. Anhydro purine nucleotides appear to be less resistant than anhydro pyrimidine nucleotides. The fact that these anhydronucleotides show considerable resistance to the phosphodiesterases indicates that a polymer of anhydrouridine or thioanhydroinosine units will be of great interest and importance as anti-viral agents.

C.) Attempted Synthesis of the 17-Base Oligodeoxyribosenucleotide Coding for the TΨCG Loop in t-RNA (tyrosine)

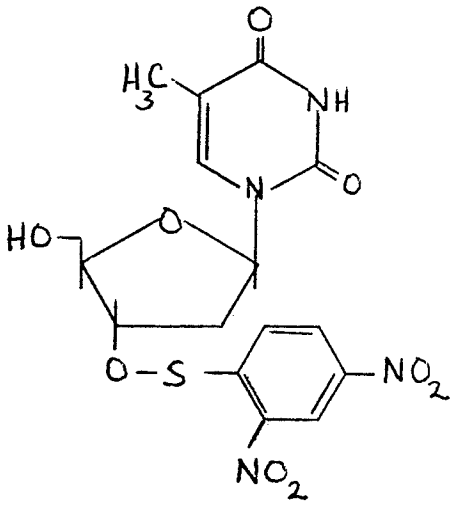
The phosphotriester method of Letsinger and Ogilvie[24] has been shown to be successful in the synthesis of polymers or copolymers of deoxyribosenucleotides. This was the method chosen for our synthesis of an oligodeoxyribosenucleotide of a specific sequence, d-GAAGGATTCGAACCTTC.

The following scheme was followed yielding a trinucleotide:

SCHEME II

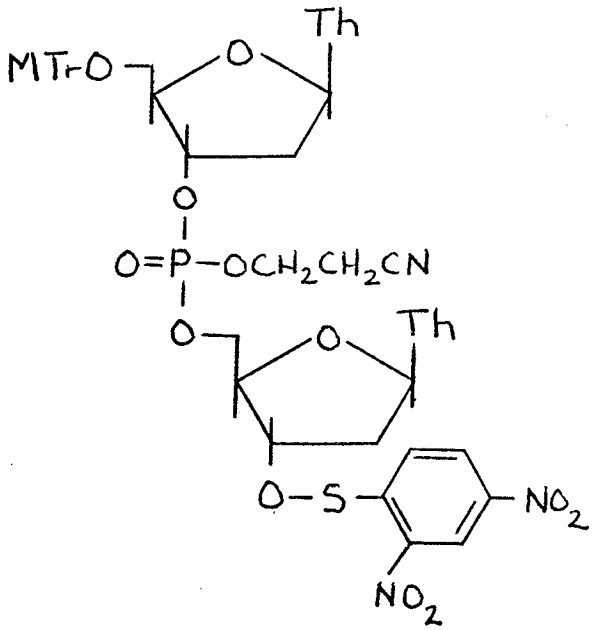


XXXVIII, MTrTp(ce)



XXXIX, Tp(s)

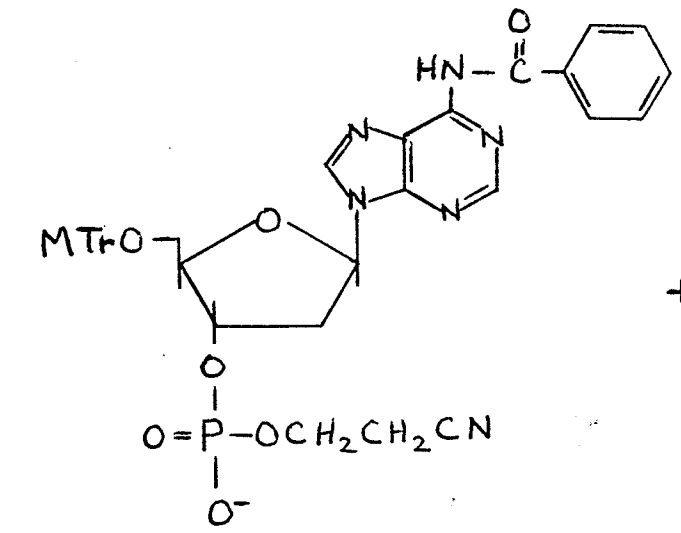
2,4,6 Triisopropylbenzenesulfonyl chloride



LX, MTrTp(ce)T(s)

80% Acetic Acid

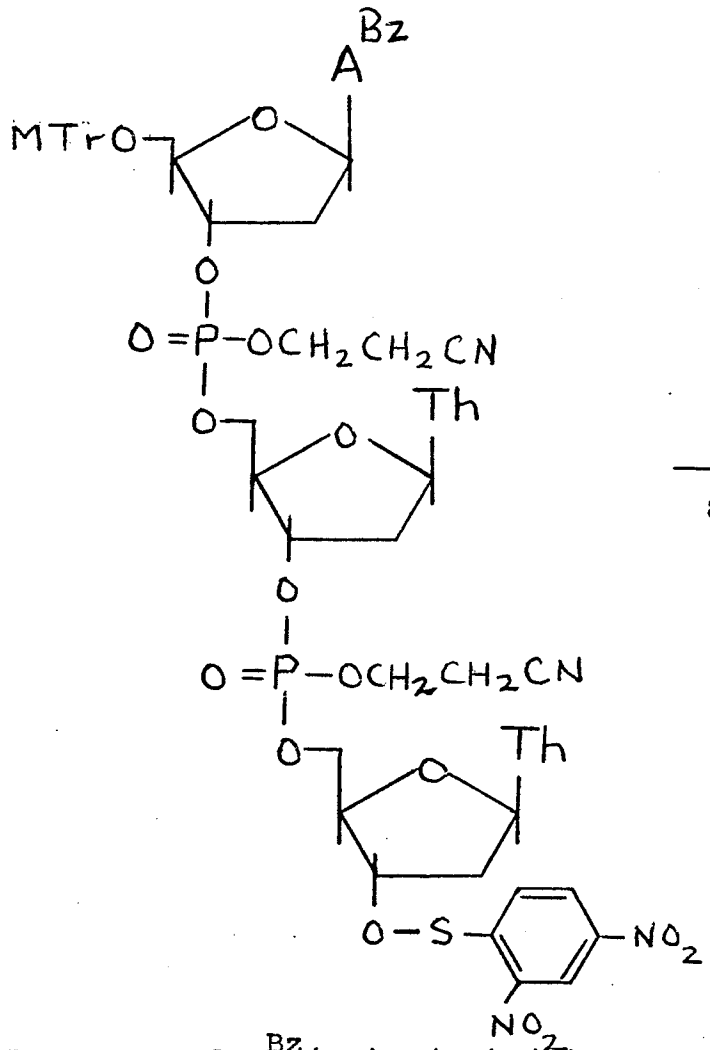
Tp(ce)T(s)
LXI



+ Tp(ce)T(s)
LXI

LXII, MTrdAp(ce)

TPS



LXIII, MTrdAp^{Bz}(ce)Tp(ce)T(s)

80% Acetic Acid

LXIV,
dAp^{Bz}(ce)Tp(ce)T(s)

Synthesis of the 17-base oligodeoxyribosenucleotide was stopped at the trinucleotide stage. The yields from the two coupling reactions had been very low, less than 30%. In the first condensation to give MTrTp(ce)T(s) and in the second condensation to give MTrdAp^{Bz}(ce)Tp(ce)T(s), there was difficulty in separating the unreacted mononucleotide (MTrTp(ce) or MTrdAp^{Bz}(ce)) from the desired product. In both cases there was a fair amount of starting material recovered, up to 20%. In the trinucleotide condensation reaction, isolation of the product was not possible on a silica gel column. The fractions eluted had to be concentrated and applied to thick layer plates. Such an extensive work-up would of course tend to cut down on the yield. Another factor contributing to the low yields may be that the mononucleotides, MTrTp(ce)(XXXVIII) and MTrdAp^{Bz}(ce) (LXII), were never isolated and the phosphorylations assumed to be complete. However, since both MTrT and MTrTp(ce) were recovered from the dinucleotide reaction, it would appear that the phosphorylation did not go to completion. Isolation of compounds XXXVIII and LXII might increase the yield of the respective condensation reactions.

After the synthesis of $dAp^{Bz}(ce)Tp(ce)T(s)$ in only a 10% yield, it did not seem feasible to continue. Had we been successful in the synthesis of the oligodeoxyribosenucleotide coding for the TΨCG loop in t-RNA (tyrosine), it would indeed have provided a molecule of extreme importance to chemists as well as molecular biologists.

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