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**The Enzymology of N-Deoxyribosyltransferase from  
*Lactobacillus leichmannii***

**By**

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**Submitted for the Degree of Doctor of Philosophy**

**University of Warwick**

**Department of Chemistry**

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*To Mum and Dad*

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## Declaration

The work described in this thesis is the original of the author, except where acknowledgement has been made to results and ideas previously published. The work was carried out at the Department of Chemistry, University of Warwick, between October 1st 1987 and September 30th 1990 and has not previously been submitted for a degree at any other institution.

## SUMMARY

Nucleoside deoxyribosyltransferase catalyses the transfer of a 2'-deoxyribose sugar between purine and pyrimidine bases. In nature, their occurrence appears to be confined to selected lactic acid bacteria, notably those from the genus *Lactobacillus*.

*L. leichmannii* possess two distinct nucleoside deoxyribosyltransferases: N-deoxyribosyltransferase-I, which mediates the transfer of the glycosyl group between purine bases exclusively, and N-deoxyribosyltransferase-II which catalyses the transfer of the 2'-deoxyribose sugar to and from purine and pyrimidine bases.

These enzymes are capable of accepting a wide number of heterocyclic base analogues as substrates and the necessary structural requirements for a competent acceptor have been defined. Similarly, it has been established that the N-deoxyribosyltransferase enzymes possess relatively little tolerance towards substrates with modified sugar moieties.

The transfer reaction proceeds via an enzyme-substitution or ping-pong bi-bi mechanism and kinetic and radiolabelling experiments provide reasonable support for the existence of a covalent glycosyl intermediate. Chemical modification of N-deoxyribosyltransferase-II with specific chemical reagents suggest that a histidine and, or carboxyl residues may participate in binding and catalysis at the active site of the enzyme.

## Abbreviations

|                |   |
|----------------|---|
| ATCC           | American Type Culture Collection          |
| °C             | degrees Centigrade                        |
| FPLC           | Fast Protein Liquid Chromatography        |
| g              | grams                                     |
| h              | hour                                      |
| HPLC           | High Pressure Liquid Chromatography       |
| K <sub>m</sub> | Michaelis constant                        |
| l              | litre                                     |
| mg             | milligrams                                |
| ml             | millilitre                                |
| M              | molar                                     |
| nm             | nanometres                                |
| M <sub>r</sub> | molecular mass                            |
| min            | minute                                    |
| rpm            | Revolutions per minute                    |
| Me             | methyl                                    |
| Et             | ethyl                                     |
| Pr             | propyl                                    |
| iPr            | iso-propyl                                |
| Ph             | phenyl                                    |
| Pipes          | [1,4-piperazinebis(ethanesulphonic acid)] |
| Tris           | Tris(hydroxymethyl)aminomethane           |

*' Yes, I have a pair of eyes,' replied Sam, 'and that's just it. If they wos a pair o' patent double million magnifyin' gas microscopes of hextra power, p'raps I might be able to see through a flight o' stairs and a deal door; but bein' only eyes, you see, my wisdom's limited'*

*The Pickwick Papers      Charles Dickens (1812-1870)*



# CHAPTER 1

## INTRODUCTION

### 1.1 GENERAL BACKGROUND

There are many different kinds of proteins in living systems most of which play crucial roles in maintaining the efficiency of biological processes. These macromolecules function in the mechanisms of motion, transport and storage, as major structural components, within the central nervous system, and as highly specific proteins which mediate immune response. But perhaps the most noteworthy class of proteins are enzymes, which catalyse the chains of chemical reactions comprising the pathways of metabolism.

The elucidation of the structures and mechanisms of action of these proteins leads us to a general understanding of how enzymes function in nature and as chemical catalysts *in vitro*. It enables us to predict roles for specific enzymes in metabolism and allows an explanation of some of the quite extraordinary properties peculiar to these molecules, such as their exceptionally high rates of catalysis and a remarkable specificity towards certain ligands.

Catalysis can be considered from several viewpoints. Indirect evidence of the mechanism by kinetic analyses provides information on the order in which substrates bind to the molecule how the products are released and the velocity at which these reactions occur. However, this fails to give any

detail of the structural elements involved and what specific groups participate in the chemistry of substrate binding and catalysis. Structural analysis, on the other hand, can convey a knowledge of both the overall three dimensional and subunit structure of the enzyme molecule in addition to the binding site, where specific amino-acid side chains and prosthetic groups may be involved in catalysis.

Inferences about the specific nature of these groups involved in the catalytic mechanism and whether these functionalities interact directly with substrates and products or play roles in the secondary bonding and stabilization of reactants and intermediates, can be made from an investigation of substrate specificity and from the action of inhibitors and reagents which attack particular chemical groups.

The key feature of the enzyme-catalysed reaction, is that it occurs at a well defined binding site. This *active site* is a three-dimensional region in the enzyme constructed from amino-acids which may be from different parts of the primary sequence.

The residues which comprise this active site can be catalytic or binding groups. The catalytic groups participate directly in the chemical changes at the site. Ligands with a particular affinity for the active site can bind reversibly to form an enzyme-substrate complex. These substrates are bound by relatively weak forces including covalent bonds, electrostatic attraction, hydrogen bonding, and hydrophobic affinity [1].



The specificity for binding is dependent on the arrangement of residues in the site. Emil Fischer first explained the marked stereospecificity of enzymes in terms of a lock-and-key hypothesis [2]. Using a series of stereoisomeric sugars, glycosidase enzymes were imagined to have binding sites complementary in nature to their natural substrates. It was later observed that not all enzymes molecules possessed such a rigid structure which led to the proposal of the induced-fit model by Koshland [3]. The binding of a substrate was now envisaged to induce a change in protein conformation, leading to a structure optimal for catalysis. Release of the product then allowed the enzyme to revert to its original shape. Thus, the active site can be viewed as a dynamic system.

### APPLICATIONS

An understanding of protein catalysts can also help to predict their potential usefulness as tools to assist in the development of synthetic procedures. Currently enzymes are playing major roles in certain industries and one of the most widely recognised uses of enzymes in industrial biotransformations is in the food industry.

Enzymes are also widely used in agriculture and medicine, enhancing traditional processes or replacing expensive and time-consuming operations. In the pharmaceutical industry, the major product of enzyme technology is the production of semi-synthetic penicillins. In addition to their contribution in

the field of antibacterial medicine, they have also found application in the area of antiviral therapy.

## 1.2 ANTIVIRAL THERAPY

With the incidence of acquired immune deficiency disease (AIDS), increasing rapidly in the last five years, the search for new antiviral drugs has assumed new and much greater proportions. Not only is there a need for drugs to inhibit or prevent the replication of viruses, but in the case of the Human Immunodeficiency Virus (HIV), to treat the life-threatening opportunistic infections associated with this immunosuppressive condition [4, 5].

Because the virus replicative cycle is intimately associated with the host cell biochemical mechanisms, successful treatment requires the interruption of viral multiplication by specific inhibition of viral enzymes. Increasing knowledge of the virus structure and its life cycle, has enabled the identification of biochemical events and pathways unique to these parasites and made feasible the rational design of chemotherapeutic agents to combat and control viral infections [6-12].

There are several stages in the viral life cycle where drugs can be targeted, but the majority of current successes have centered around interrupting nucleic acid synthesis and most of the promising drugs have been nucleoside analogues [13, 14, 15,

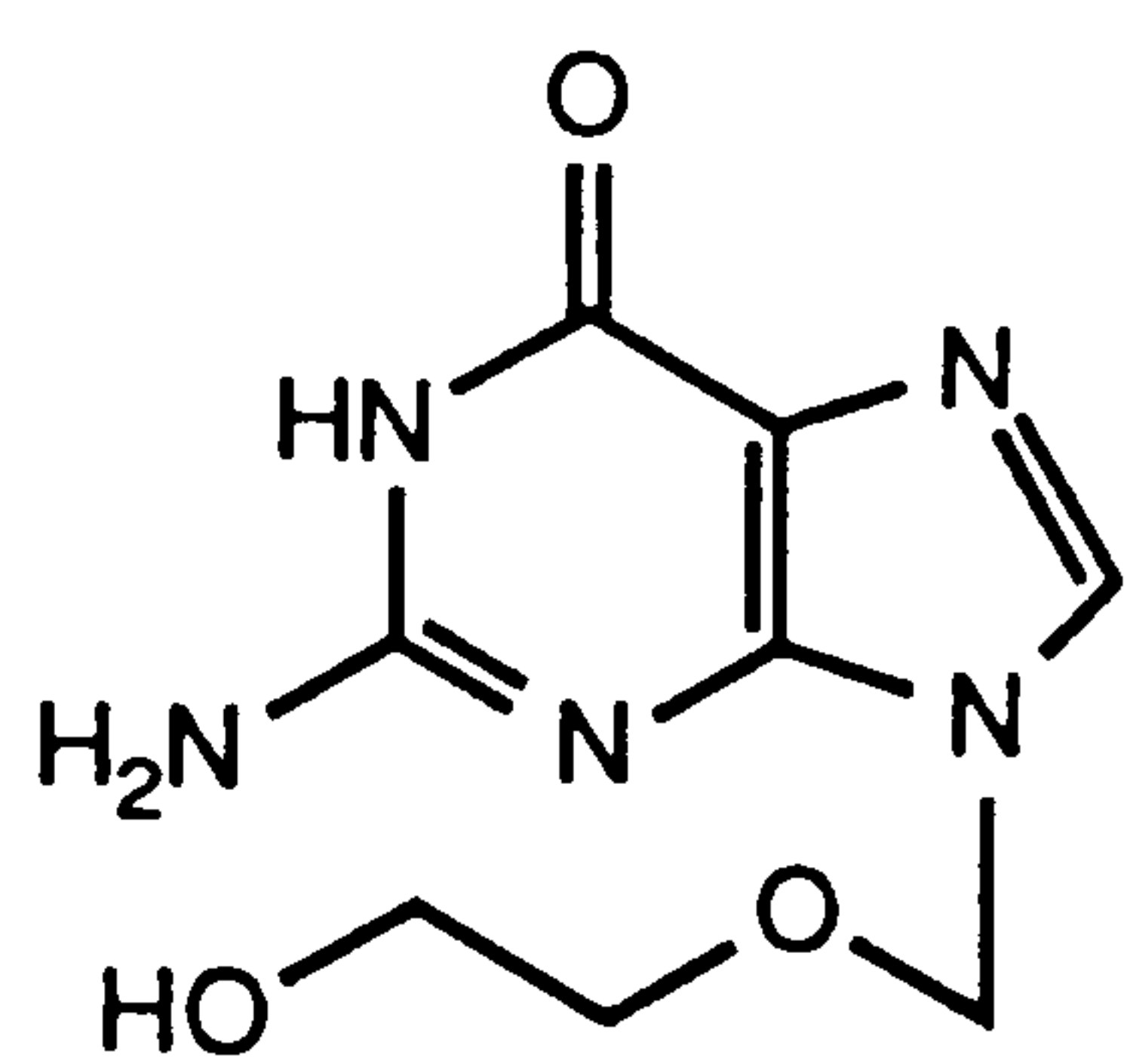


16]. Members of this class of compounds exhibit significant *in vitro* and *in vivo* antiviral activity.

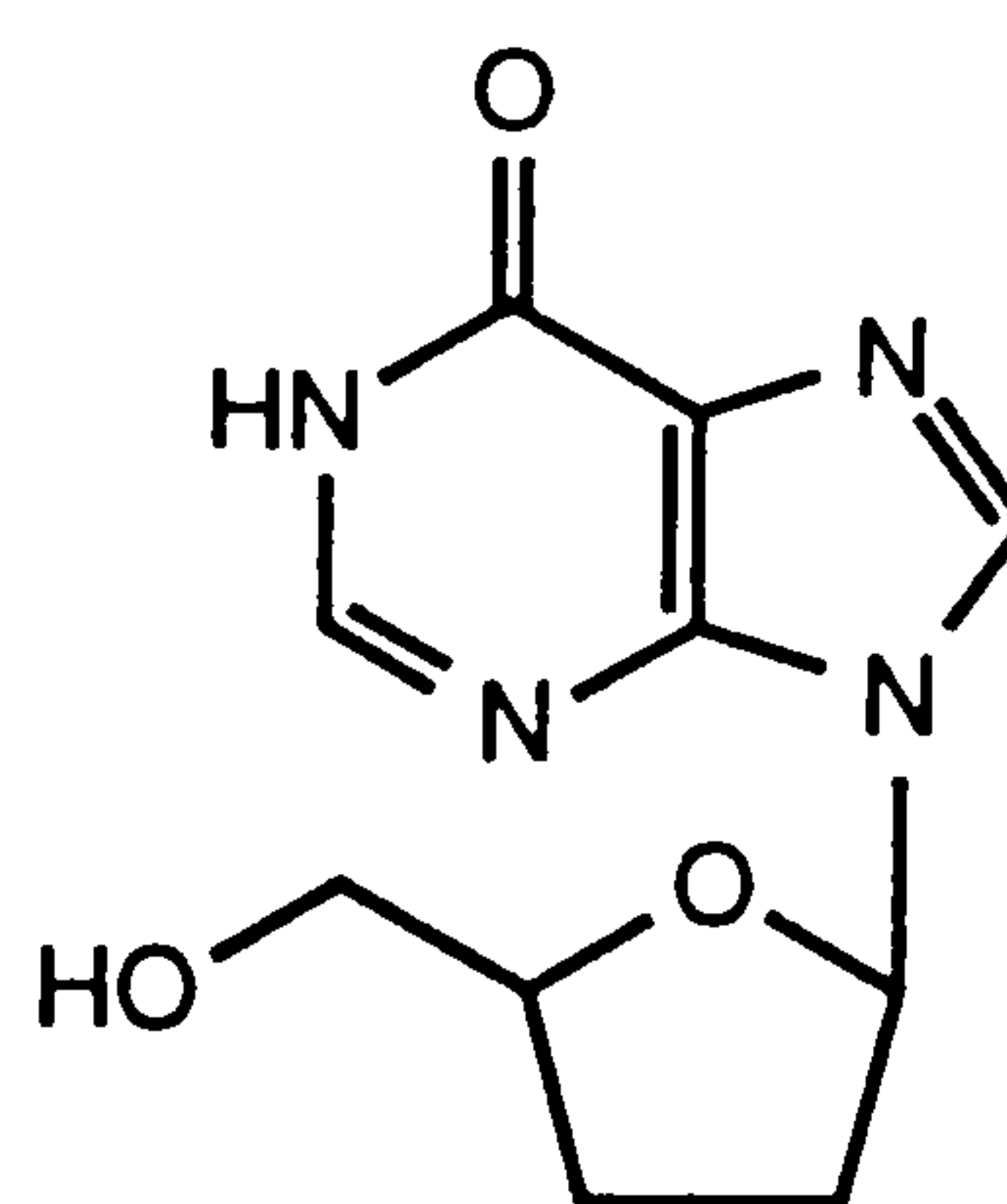
### 1.3 NUCLEOSIDE ANALOGUES

Purine and pyrimidine nucleoside analogues have been developed as inhibitors of the replication of both DNA and RNA viruses [17, 18, 19, 20, 21]. These compounds inhibit DNA replication by mimicking the structure of natural nucleosides - the precursors of DNA and RNA. They can block viral replication by acting as chain terminators [21, 22,] or by acting as inhibitors of methylation reactions essential to the transcription of mRNA [18, 23]. They can also become permanently associated with viral polymerases and bind to the terminated DNA template [24]. This last action renders the enzyme molecule incapable of further reaction. Thus, the role of these compounds is to prevent nucleic acid synthesis in the infected cell. The selective effect of these drugs often relies on the virally-encoded enzymes [25, 26] which activate the nucleoside analogues and on the high affinity of the viral polymerase to the compound compared to the host cell DNA polymerase.

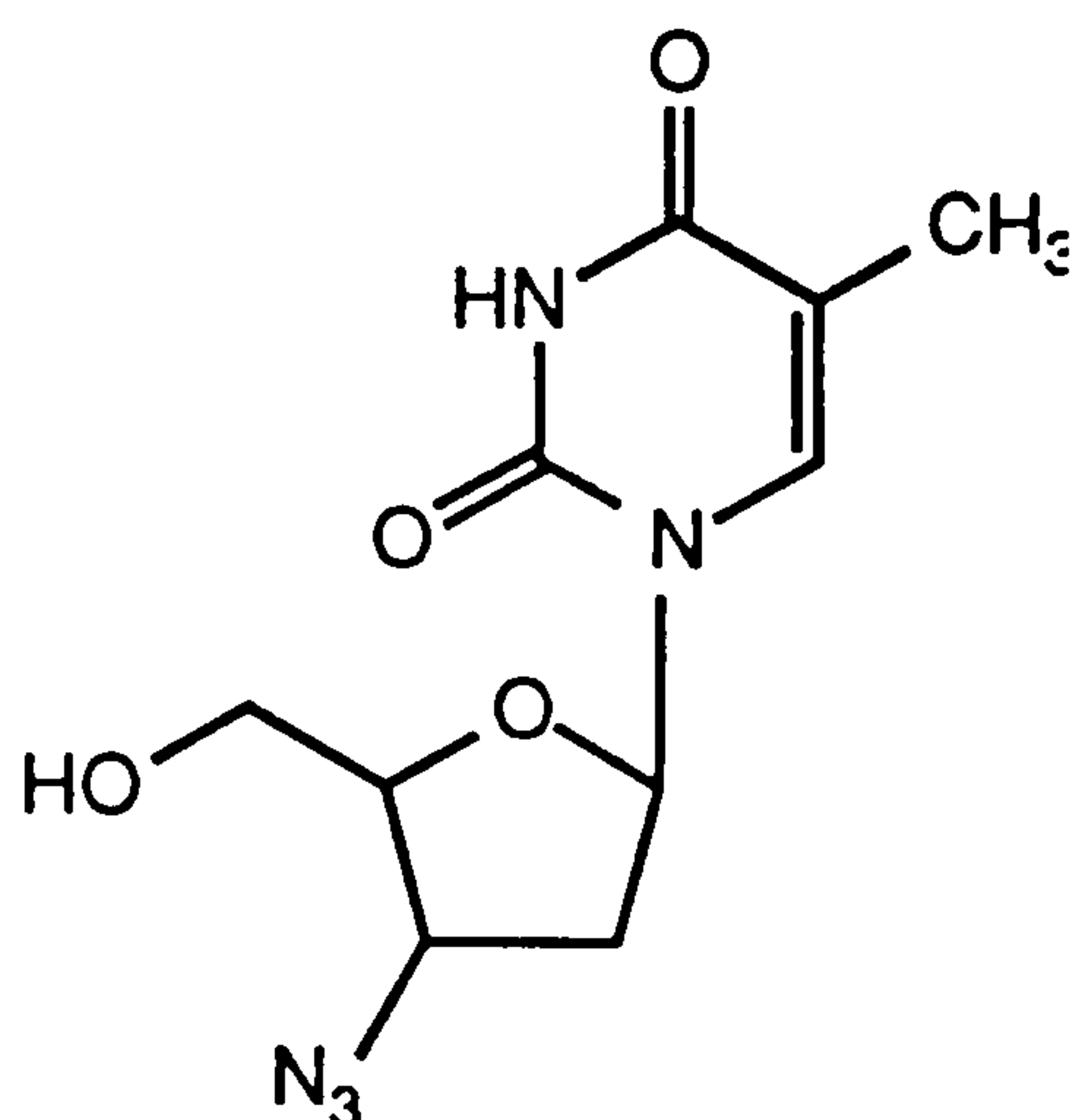
The guanosine nucleoside analogue 9-(2-hydroxyethoxymethyl) guanine or acyclovir (1), [14, 27] has proved to be very successful as a potent inhibitor of several viruses including those of the herpes simplex family; (HSV).



(1)



(2)



(3)

Dideoxynucleoside analogues such as 2',3'-dideoxyinosine (ddI), (2), and 3'-azido-3'-deoxythymidine (AZT), (3) [20, 21] can be metabolised by cellular enzymes to produce their triphosphates which, in turn, act as potent inhibitors of the retroviral enzyme reverse transcriptase [7]. However, despite these successes, few antiviral drugs are currently in approved clinical use. and there is a great deal of interest in expanding the range of these clinically useful therapeutic agents. This demand has been heightened with the awareness of antiviral drug resistance [28].

#### 1.4 CHEMICAL SYNTHESIS OF NUCLEOSIDE ANALOGUES

The production of nucleoside analogues by chemical means falls into two categories: the modification of a preformed nucleoside



or the *de novo* synthesis of a novel nucleoside by the fusion of a sugar group with a base.

#### (A) FUSION OF HETEROCYCLIC BASE AND SUGAR

The most widespread methods of chemical nucleoside syntheses are those employing condensation reactions using protected base and sugar derivatives [29, 30]. The Koenigs Knorr or heavy metal procedure employs salts of purines and pyrimidines together with a protected sugar halide. In the reaction of purines with the sugar group, the initial product is the N-3 glycosyl derivative. This then undergoes an intermolecular rearrangement to give the N-9- $\beta$ -nucleoside (Figure 1.1). With pyrimidines the first fusion product is the O-glycoside derivative which rearranges to the final N-glycoside [30]. In the synthesis of deoxynucleosides however, anomeric mixture are produced.

Like the Koenigs Knorr reaction, many of the traditional methods of synthesizing ribo- and deoxyribonucleosides also produce mixtures of the  $\alpha$ - and the  $\beta$ -anomers of the nucleoside analogue. Resolution of these isomers can result in a drop in the overall yield. Some stereochemical control can be gained in the synthesis of deoxynucleosides using suitable reaction conditions. In the synthesis of pyrimidine nucleosides using silylated heterocyclic bases, judicious choice of solvent was observed to promote the formation of the natural  $\beta$ -anomer. Non-polar solvents such as carbon tetrachloride were used to good effect to increase the  $\beta/\alpha$  anomer product ratio. The use of an  $\alpha$ -bromo-sugar instead of a peracylated sugar in the synthesis

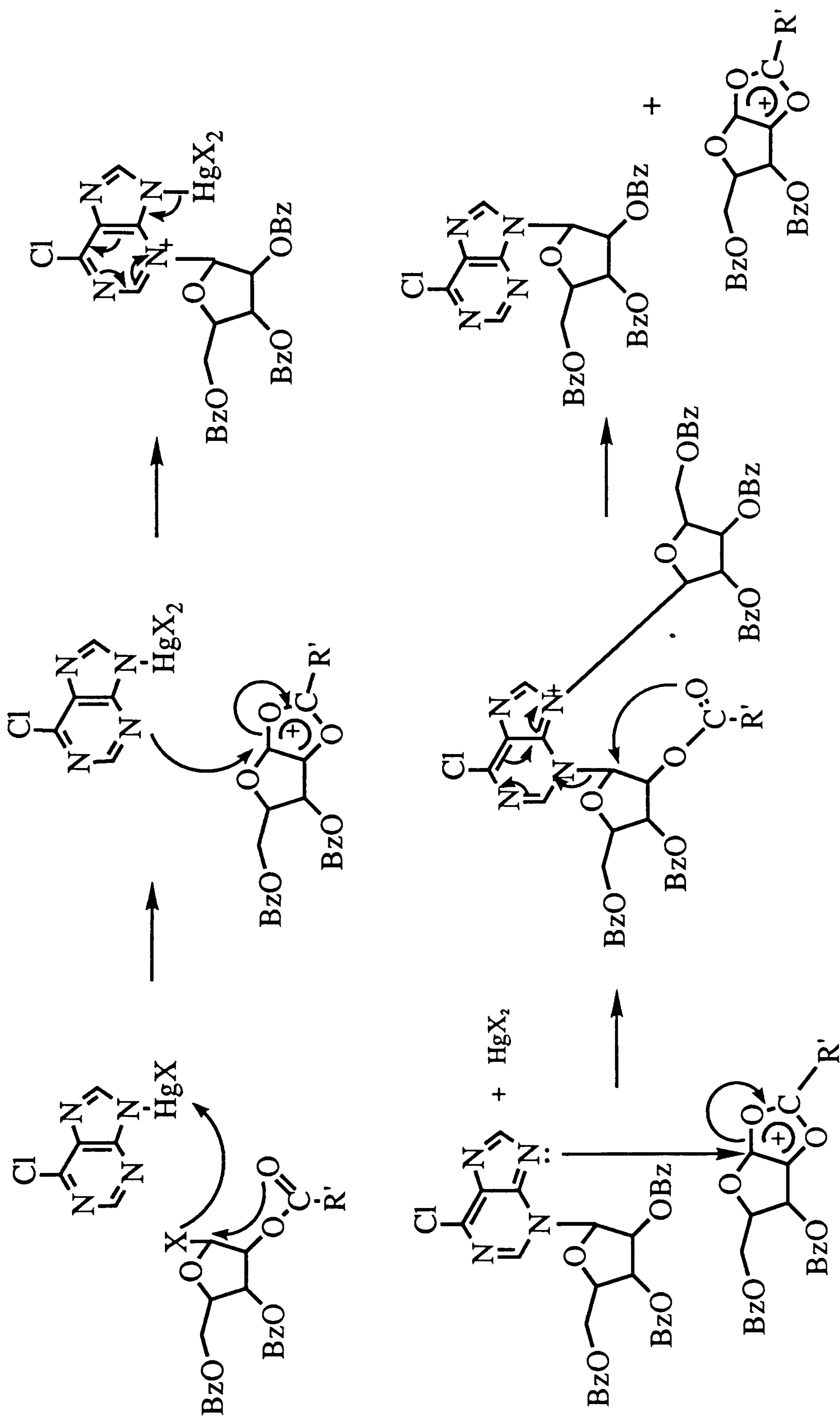


Figure 1.1 Purine Nucleoside Synthesis - Knoenigs Knorr Reaction



of deoxyribonucleosides has also led to the almost exclusive synthesis of the  $\beta$ -anomer of selected nucleoside analogues [31]. Protection is often necessary to provide regioselectivity in the chosen modification. However, protection and deprotection adds more steps to the operation and the yields can be quite substantially reduced.

### (B) CHEMICAL MODIFICATION OF AN EXISTING NUCLEOSIDE

There has been much interest recently in the synthesis of novel nucleosides containing unusual sugar moieties. Methods have been developed for the production of these compounds from preformed nucleosides by the chemical introduction of groups to generate the desired compound. The fluorinating agent diethylaminosulphur trifluoride (DAST) has been employed with some success in this fashion in the chemical synthesis of 3'-fluoro-2',3'-dideoxynucleosides [21]. Although the reaction of a range of different purine and pyrimidine 2'-deoxynucleosides give good to moderate yields of the 3'-fluoro-2',3'-dideoxynucleosides these methods still require strict operating conditions in addition to the use of protecting agents, all of which result in reduced final yields.

In the same way, the synthesis of nucleosides with unusual bases involves the introduction of substituents onto the heterocyclic base.

These chemical methods have often proved laborious and complex. In view of this situation, it is possible to make use of some of the unique properties of enzymes, either in

combination with chemical steps to improve existing procedures, or to develop methods where the use of a biocatalyst obviates the need for many of these non-specific, multistep processes. Exploitation of the stereo- and regioselectivity of enzymes together with their ability to catalyse reactions under mild conditions, seem to suggest a far more amenable way to extend the range of potentially therapeutically useful compounds in this class. The use of enzymes in glycosyl transfer reactions leads exclusively to the  $\beta$ -anomer of the nucleoside and usually only one position on the heterocyclic base is glycosylated [32].

Methods using biocatalysts in these syntheses are relatively new compared to the traditional chemical processes and there are still disadvantages, especially with respect to the large-scale production of nucleoside analogues which often relies on substantial amounts of enzymes. Limited enzyme production in some species of microorganisms due to low biomass could be addressed to some degree with the use of genetic manipulation to create mutants and over-producing strains. The use of immobilized whole cells or enzymes on a variety of supports could also prove to be preferable to manipulation of enzymes in batch culture [33]. There also has to be some attention directed towards solving the problem of low solubility of some substrates and products in these enzymatic systems.

The yields of these enzymatic reactions can still be relatively low, but a knowledge of enzyme stability and the optimal



operating conditions of specific enzymes can enable development of methodology to give respectable yields.

In order to find a compound with good antiviral activity, thousands of drugs have to be screened. Much time and effort has gone into designing and synthesizing nucleoside analogues that might possess antiviral activity. Since many enzymes also accept analogues of their natural substrates, the capability for simple, rapid, small-scale synthesis of a broad range of base- and sugar-modified nucleosides has become both feasible and attractive. Thus, by using enzymatic synthesis, perhaps part of the answer of how to make this screening operation more efficient can be found.

## 1.5 ENZYMATIC SYNTHESIS NUCLEOSIDE ANALOGUES

### (A) ENZYMATIC MODIFICATION OF EXISTING NUCLEOSIDE ANALOGUES

Enzymes have had limited use as catalysts in the modification of existing nucleosides [34, 35]. One of the most useful applications has been in the resolution of racemic mixtures of nucleoside analogues with antiviral activity [36, 37, 38].

The production of racemates is one of the problems frequently encountered in chemical syntheses. In therapeutically active racemates it is often observed that one enantiomer is significantly more biologically active than the other. It therefore becomes important to obtain an isomerically pure preparation, not only in terms of efficacy, but also with regard to its safety. An example of this omission is highlighted in the

marketing of the drug thalidomide. Originally synthesized as a racemate, it was only after its neurotoxic effects became recognised that the isomers were examined. It was found that the teratogenic effects at least were restricted to the (-)-S-thalidomide.

The potent anti-herpes nucleoside carbocyclic 2'-arabino-fluoro guanosine has been synthesized chemically to produce, in good yield, a mixture of active enantiomers. To determine which enantiomer had the maximum efficacy, the mixture was enzymatically resolved by enantioselective hydrolysis [38]. The preparation was initially monophosphorylated using phosphate and thymidine kinase from HSV-1 cells followed by treatment with 5'-nucleotidase from *Crotalus atrox* venom. This led to the selective removal of the 5'-phosphate group from the dextrorotatory enantiomer. The relative rapidity of the reaction of the (+) enantiomer as compared to that of the (-) isomer was thought to be because of its resemblance to the natural substrate.

A separate test of the two enantiomers against HSV-1 demonstrated that the dextrorotatory isomer had twice as much biological activity as the original racemate, whereas the laevorotatory isomer exhibited a two-fold decrease.

## (B) ENZYMATIC SYNTHESIS OF NUCLEOSIDE ANALOGUES USING GLYCOSYL TRANSFER REACTIONS

Two classes of enzymes are used in the synthesis of nucleoside analogues; nucleoside phosphorylases and N-deoxyribosyltransferases



### 1.5.1 NUCLEOSIDE PHOSPHORYLASES

The idea of using enzymatic processes to synthesize novel or unusual nucleosides is not a new one. The discovery of nucleoside phosphorylases and a description of their general mechanism made feasible the possibility that such a method could be used [39, 40]. Found in a wide variety of mammals and microorganisms, [41, 42, 43] they have already shown themselves to be capable as competent biocatalysts in the small scale production of these compounds [44, 45, 46].

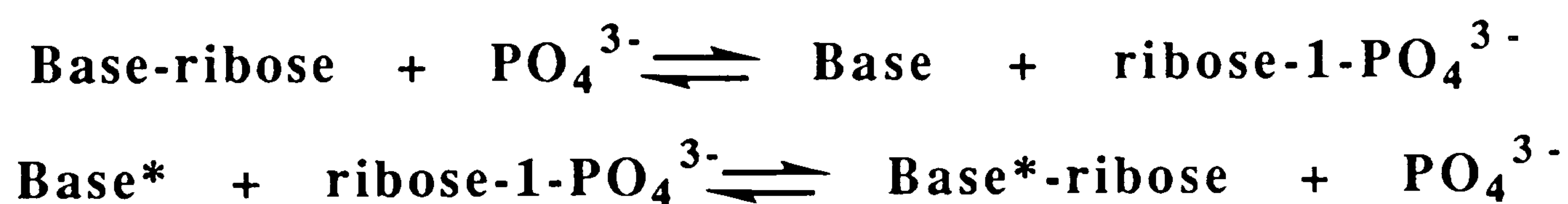
An extensive search has been made in diverse families of microorganisms [47] for an enzyme activity which has the ability to transfer ribose and 2'-deoxyribose groups from nucleosides to pyrimidine and purine bases. Cell-free extracts from almost all of the members of the family of *Enterobacteriaceae* and some strains from the genera *Micrococcaceae*, *Bacilliaceae* and *Cornybacteriaceae* were found to catalyse a phosphate-dependent transfer. Both pyrimidine- and purine-specific phosphorylases were reported.

Revelations from substrate specificity studies using uridine and thymidine donors for the transferase reaction with extracts from several bacteria led to the proposal that two discrete pyrimidine phosphorylase enzymes existed. The participation of distinct purine and pyrimidine phosphorylases was verified by the purification of purine nucleoside phosphorylase - PNPase - (EC 2.4.2.1), uridine phosphorylase - URPase - (EC 2.4.2.3), and thymidine phosphorylase - TdRPase - (EC 2.4.2.4.) from

*Aerobacter aerogenes* IFO 3321 [47]. Probably best characterized are those from the enteric bacterium *E. coli* [48, 49, 50].

A general mechanism for PNPase was first proposed in 1945 [39]. The nucleosidase was observed to act as a phosphorolytic enzyme, liberating purine bases and  $\alpha$ -D-ribose-1-phosphate from purine nucleosides. Since this was an equilibrium reaction, it meant that the addition of a new base to the system could generate a new nucleoside. This was demonstrated by incubating ribose-1-phosphate with hypoxanthine in the presence of nucleoside phosphorylase from rat liver. All of the hypoxanthine disappeared and was found as the hypoxanthine riboside.

The mechanism was proposed to have proceeded as follows:



Where: Base = heterocyclic base; (purine or pyrimidine)

Figure 1.2

Thus, the reaction did not proceed in the absence of phosphate ions [40].

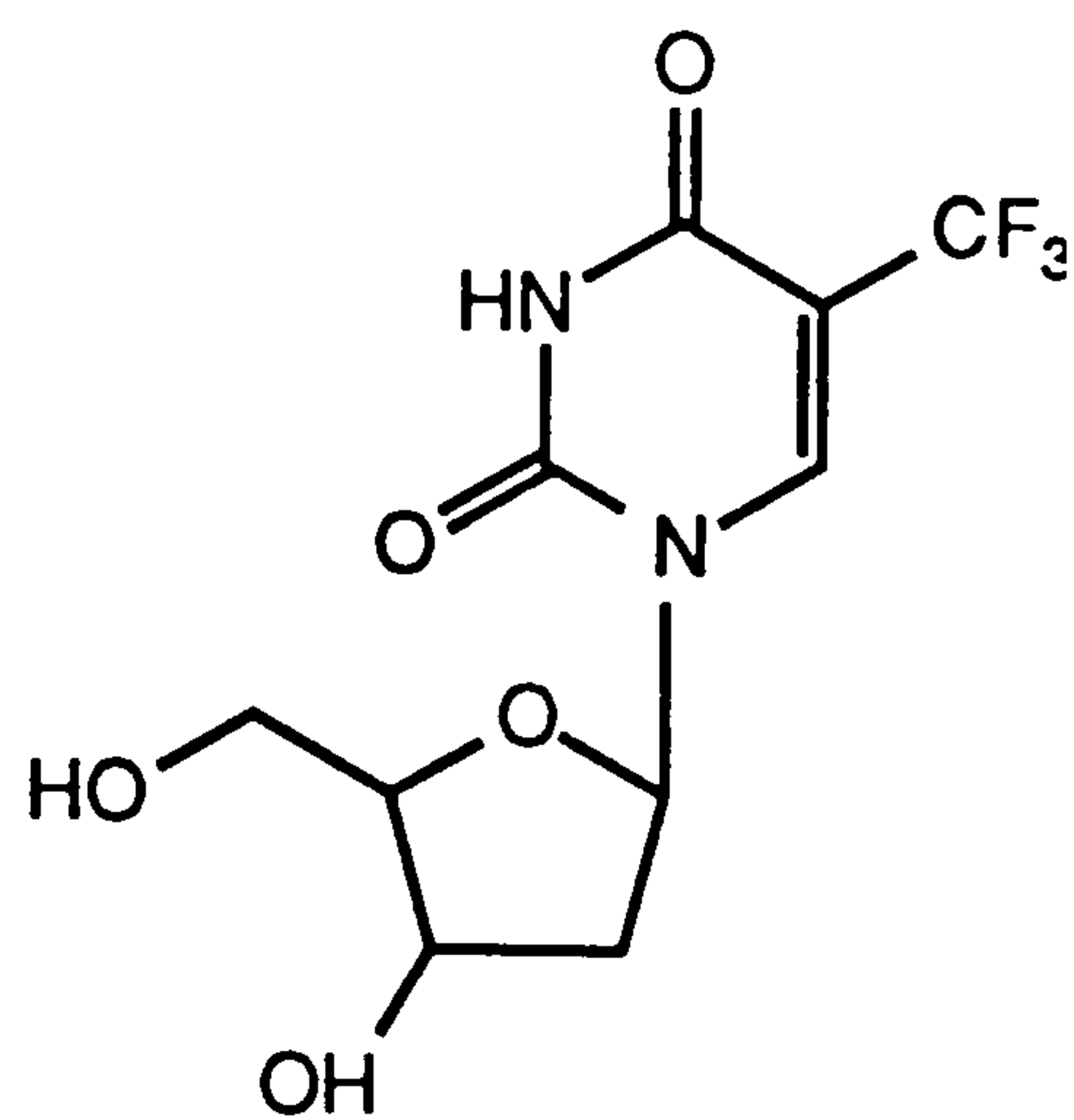


There was also speculation that deoxyribonucleosides might participate in the same type of reaction. The formation of a deoxyribose-1-phosphate intermediate was demonstrated when thymidine was incubated with an extract derived from rat liver [51]. This phosphorylation was attributed to the action of a pyrimidine nucleoside phosphorylase. The reaction was then coupled to purine nucleoside phosphorylase and, with the addition of hypoxanthine base, the 2'-deoxyinosine nucleoside was synthesized. The same intermediate has also been isolated from the phosphorylation of purine deoxyribonucleosides [52].

#### ENZYMATIC SYNTHESIS WITH NUCLEOSIDE PHOSPHORYLASES

One of the first enzymatic syntheses of a nucleoside analogue employed crude extract from *E. coli* B cells [53]. The antimetabolite, 5-trifluoromethyl-2'-deoxyuridine (4) was produced in low yields by the exchange reaction between the pyrimidine base analogue, 5'-trifluoromethyluracil, and thymidine. Since the enzymatic conversion occurred only in the presence of inorganic phosphate, the exchange reaction between deoxynucleoside and base was assumed to have been mediated by phosphorylase enzymes.

Since then nucleoside phosphorylases from a variety of sources have been engaged to make a wide range of analogues.



(4)

Whole cells from *E. coli* BM-11 have been used to synthesize enzymatically the antiviral compounds 5-ethyl-(2-bromovinyl)-2'-deoxyuridine and (E)-5-(2-bromovinyl)-2'-deoxyuridine, (EDU and BVDU) [54]. The catalysis was performed by a combination of (TdRPase) and PNPase where either thymidine or 2'-deoxyguanosine functioned as the donor and (E)-5-(2-bromovinyl) uracil as acceptor. One problem was that of very low productivity of the BVDU. This was ascribed to the poor solubility of the bromovinyluracil base. The addition of DMSO to the mixture improved these figures quite considerably.

The poor solubility of the nucleoside antibiotic and antiviral compound, guanine 7-N-oxide has led to efforts to improve its solubility, and hence activity, by preparation of its corresponding nucleoside guanosine 7-N-oxide. Wet cell paste from *Bacillus subtilis* catalyses the reaction between ribose-1-phosphate and guanine-7-N-oxide [55] and the activity is consistent with the action of PNPase.

A variety of 3-deazapurine ribo- and deoxynucleosides have been prepared using the coupled enzyme system of TdRPase, URPase and PNPase purified from *E. coli* [45]. The initial



phosphorylation to produce the 2'-deoxyribonucleoside-1-phosphate was mediated by TdRPase, while the ribose-1-phosphate intermediate resulted from the action of UPase on uridine [Figure 1.3].

The broad spectrum antiviral compound 1-( $\beta$ -D-ribofuranosyl)-1,2,4-triazole-3-carboxamide, (ribavirin, virazole), has also been prepared using phosphorylase enzymes [46].

A two-step reaction using purified purine nucleoside phosphorylase from *Enterobacter aerogenes* AJ 11125 has been used where the glycosyl donor was inosine and the initial step involved the production of ribose-1-phosphate from the enzymatic phosphorolysis of the purine nucleoside [46]. The ribose-1-phosphate was isolated by ion exchange chromatography onto a column before it was combined with 1,2,4-triazole-3-carboxamide (TCA). A transribosylation reaction then produced the nucleoside virazole.

The rationale behind the method takes into account the relatively low affinity of the TCA for the purine nucleoside phosphorylase as compared to that of the hypoxanthine base. ( $K_m$ -TCA, 167mM;  $K_m$ -HX, 5.6mM) If the hypoxanthine was retained in the reaction, it would compete with the TCA for the enzyme, and hamper its chances of recognition as a competent substrate.

A number of other bacteria have been tested for their ability to make virazole. Several were cited as producing good yields of either the ribose-1-phosphate intermediate, or the new nucleoside. In one study to increase the productivity of the

Coupled Enzymatic Synthesis of Ribonucleosides and 2'-Deoxyribonucleosides with Pyrimidine and Purine Nucleoside Phosphorylases from *Escherichia coli*.

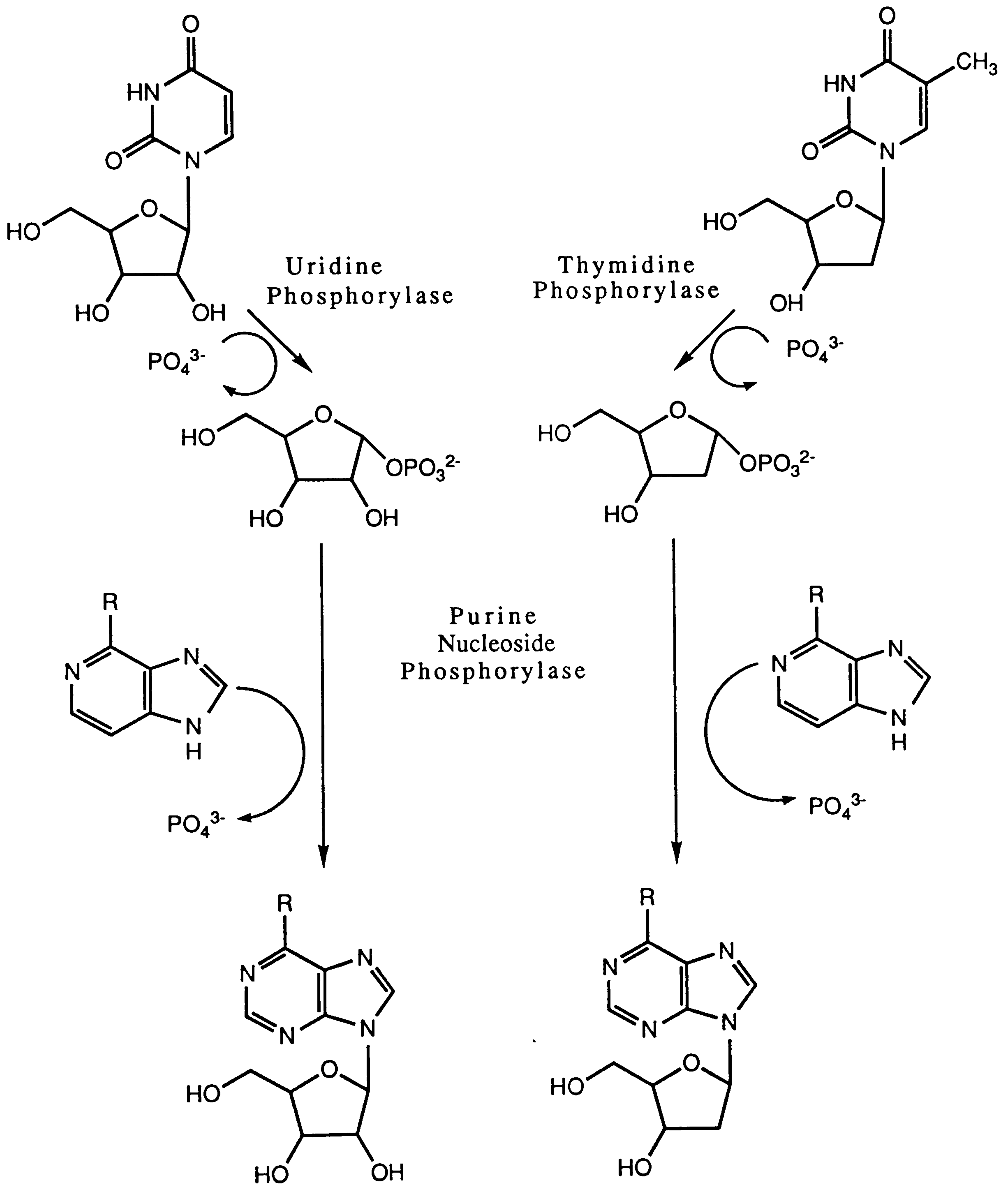


Figure 1.3



virazole reaction, a combination of microorganisms were used [56]. The enzymes from *Erwinia carotovora* catalysed the phosphorolysis of inosine, and those from *Bacillus brevis* the ribosylation of TCA. This proved to be a more efficient and productive system than the one using *Enterobacter aerogenes* AJ 11125 alone. Whole cells of *Erwinia carotovora* can also use orotidine as a ribosyl donor in this reaction [57].

The avoidance of a system which requires the presence of both purine and pyrimidine phosphorylase enzymes, and the multistep procedure involving the isolation of the ribose-1-phosphate for glycosyl donation would serve to improve the simplicity of this enzymatic route. A method has been developed so that only PNPase is necessary for the efficient synthesis of purine nucleoside analogues [58]. Based on a proposal of how the phosphorylation step might occur at the active site, a 7-methyl-ribonucleoside donor has been designed. Its affect is to make the initial phosphorylysis essentially irreversible since the 7-methylpurine does not acts as a nucleophile in the reverse reaction [59]. The yields for producing compound such as 3-deazaadenosine and virazole by this method were very high. Thus, by exploiting the coupled nature of the enzymatic route, synthetic procedure can be improved.

In addition to phosphorylases being used as catalysts in the biotransformation of unusual heterocyclic bases with ribosyl and deoxyribosyl donor nucleosides, they have also been functional in the synthesis of nucleosides with modified sugar moieties [60].

Whole cells from several bacterial sources, including *E.coli*, *Enterobacter aerogenes* AJ 11125 and *Erwina herbicola* have been used in the synthesis of 2'-amino-2'-deoxyribosides compounds, some of which have antibacterial, antitumour and antiviral activity [61]. When 2'-amino-2'-deoxyuridine was used as the glycosyl donor, in the presence of inorganic phosphate, the wet cell paste of the *Enterobacter aerogenes* AJ 11125 transformed the purine bases adenine, hypoxanthine, 2-chlorohypoxanthine and guanine into their respective 2'-amino-2'-deoxyribonucleotides [62]. Since no reaction occurred in the absence of phosphate ions it is justifiable to assume that the transglycosylation is catalysed by nucleoside phosphorylases.

Applying a combination of chemical and enzymatic techniques, 2',3'-dideoxyuridine has been used as a glycosyl donor with resting whole cells of *E. coli* AJ 2595 for the synthesis of the 2',3'-dideoxynucleosides of adenine and hypoxanthine [63]. 2',3'-Dideoxythymidine could also be produced by this reaction. It was observed that the yields of the 2',3'-dideoxyadenosine reaction were significantly improved on the addition of polyethylene glycol, (PEG). It is possible that this helps to improve productivity in several ways. The PEG could help to stabilise the proteins, as well as aid in the permeability of the cells to the substrates. It could also assist in the dissolution or dispersion of the substrates, making them more available to the enzymes.

$\beta$ -D-Arabinofuranosyl nucleosides have also been synthesised using nucleoside phosphorylase enzymes. 9-( $\beta$ -D-



Arabinofuranosyl) guanine is active against viruses of the herpes family and has been prepared with glutaraldehyde-treated cells from *E. coli* BM-11 [64]. This method uses 2'-deoxyguanine as a source of guanine base, and 1-( $\beta$ -D-arabinofuranosyl) cytosine (ara-C) as the sugar donor. Transarabinosylation, via 1-( $\beta$ -D-arabinofuranosyl) uracil (ara-U), is performed with reasonable efficiency to give modest yields. 2'-Deoxyguanosine was used as a substrate for PNPase to overcome problems of guanine base solubility.

The stereospecific synthesis of the antiviral 9-( $\beta$ -D-arabinofuranosyl) derivative of adenine (ara-A) [65], has been carried out with *Enterobacter aerogenes* AJ 11125 cells [46]. The reaction was highly dependent on the concentration of phosphate in the incubation medium and appeared to exhibit maximum productivity when performed at 60-65°C. The transformation was attributed to the combined action of URPase and PNPase in the absence of adenosine deaminase activity. This procedure takes advantage of the very low solubility of the product, allowing it to precipitate out, facilitating its easy isolation. The yield in this reaction was high; as much as six grams per litre.

### SUMMARY

Purine and pyrimidine nucleoside phosphorylase enzymes have proved to be very useful biocatalysts in the preparation of moderate yields of nucleoside analogues. They are stable and have a wide substrate range [66, 67], with the exception of adenine intolerance in mammalian PNPase [68], and have been

shown to be useful tools of synthesis as both purified and crude extracts.

The discovery of purine phosphorylase enzymes specific towards inosine and guanosine nucleosides, [50] and to adenine increases [69] the range of tools available to the chemist, and begs the question as to whether the range and potential of enzymes that could be accessible and well-suited to synthetic purposes have yet been realised.

The major disadvantages of these reactions lie in the two stage nature of the mechanism and it is not uncommon to find that the rate of a reaction is severely limited by the differential specificities of the enzymes for a substrate.

### 1.5.2 N-DEOXYRIBOSYLTRANSFERASES

The view that these transglycosylation reactions proceeded via a coupled phosphorylase mechanism persisted until the discovery that the 2'-deoxyribose group of a natural nucleoside could be transferred **directly** to a heterocyclic base in the absence of phosphate ions using the extracts of certain lactic acid bacteria.

This phosphate-independent transfer of the sugar moiety was first reported by McNutt in 1950 [70, 71]. It was known that selected species of bacteria required deoxyribonucleosides for growth, notably members of the genus *Lactobacillaceae*, [72, 73] and that any one of the four natural deoxyribonucleosides could serve equally well as growth promoting factors. It therefore seemed probable that the dependent organism converted the



administered deoxyribonucleoside to the other deoxyribose derivatives in order to make a full complement for nucleic acid synthesis.

McNutt demonstrated that dialysed, and therefore essentially phosphate-free, extracts from *Lactobacillus helveticus* performed the transglycosylation reaction when incubated with deoxyribonucleoside and a free purine or pyrimidine base [71]. Contrary to expectation, deoxyribose-1-phosphate did not participate in this reaction. When it was employed as a glycosyl donor, with a variety of acceptor bases, no appreciable deoxyribonucleoside was produced. Preparations of two other strains, *L. acidophilus* and *L. delbrueckii*, were also found to catalyse the exchange. The enzyme was proposed to be acting as a **trans-N-glycosidase**.

Since the reaction proceeded in the absence of inorganic phosphate, there was no evidence to suggest that nucleoside phosphorylases played any role in this transfer of the deoxyribose moiety.

The discovery that the mechanism did not involve a phosphorylated intermediate, led to the speculation that the reaction mechanism involved an initial hydrolysis of the glycosidic bond followed by resynthesis to give the new nucleoside rather than the product arising from trans-N-glycosylation. Comparison of the rates of hydrolysis of the donor nucleoside and transfer to produce a new nucleoside dismissed this hypothesis of an primary hydrolysis step before resynthesis [71].



Another possibility was that the transfer involved secondary enzymes. There was a chance that, in the reaction of 2'-deoxyinosine and adenine, 2'-deoxyadenosine was produced not by trans-N-glycosylation but by a transamination reaction involving the 6-position of the inosine donor. That this was untrue was verified by performing this reaction using  $^{14}\text{C}$ -radiolabelled adenine in the 8-position [74]. It was observed that the hypoxanthine formed was totally devoid of the  $^{14}\text{C}$  label and that the radioactivity lay exclusively in the adenine nucleoside formed. Thus, the transglycosidase mechanism was confirmed and the order of the reaction was depicted as follows:



Where:      base = heterocyclic base; (purine or pyrimidine)  
               dRibose = 2'-deoxy- $\beta$ -D-ribose

Figure 1.4

An extensive search was carried out to investigate the occurrence of the N-deoxyribosyltransferase enzymes. A wide range of microorganisms and animal tissues were examined, but the activity was reported to have a limited distribution. It appears in high levels only in bacteria from the genus *Lactobacillaceae* which lack significant levels of nucleoside phosphorylase enzymes [75].

N-deoxyribosyltransferases have been purified or partially purified from a number of *Lactobacillus* [76-81]. The physical

and kinetic properties [82, 83] as well as the substrate range of these enzymes have been investigated. Their suitability for synthetic biotransformations has also been tested and they have been employed as whole cells and crude or partially purified extracts in the enzymatic syntheses of unusual or novel nucleosides [32, 84, 85].

The N-deoxyribosyltransferase activity from *L. helveticus* was the first to be studied in detail. Investigations by Roush and Betz [80] provided preliminary information on the enzyme finding it to be stable to heat and to possess a tolerance towards a narrow range of acceptor bases. In confirmation with earlier studies, there was no evidence for nucleoside phosphorylase activity - the rates of reaction, the same in the presence or absence of inorganic phosphate, were consistent with its absence. They did, however, observe the very important differential inhibition of transfer activities in Tris buffer. The transfer of the deoxyribosyl sugar group to and from pyrimidine bases was strongly inhibited by the Tris, whereas at the same concentration the transfer to and from purine bases was only weakly affected. This observation was indicative of two distinct enzymes in the partially purified preparation.

This proposal was confirmed by Holguin and Cardinaud [81] who were the first to demonstrate the existence of two discrete N-deoxyribosyltransferase activities in *L. helveticus*. The two enzymes were separated by affinity chromatography using a derivatized purine nucleoside analogue as a ligand.

N-deoxyribosyltransferase-I catalysed the transfer of the deoxyribosyl moiety to and from purine base exclusively,



whereas exclusively N-deoxyribosyltransferase-II transferred the sugar group to and from purine and pyrimidine bases (Figure 1.5).

### N-deoxyribosyltransferase-I



### N-deoxyribosyltransferase-II



Where: Pur = Purine  
 Pyr = Pyrimidine  
 dRib = 2'-deoxy-D-ribose

Figure 1.5

The two enzymes showed different physical properties and disparate inhibition towards Tris buffer.

The transdeoxyribosylation has also been described in *Lactobacillus leichmannii* [78, 79]. The enzyme from *L. leichmannii* was observed to be capable of all types of transfer activities, independent of the concentration of the phosphate ions present. However, unlike *L. helveticus*, only one enzyme was identified [78, 79, 85]. Detailed kinetics of this transfer mechanism were investigated by Danzin and Cardinaud using



purified extracts of N-deoxyribosyltransferase from *L. helveticus* [82, 83]. It was established that the reaction proceeded via a double-displacement ping-pong bi bi mechanism, where the first product is released before the second substrate binds to the enzyme. These results satisfied the criteria set out by Cleland to characterize such a pathway [86].

The following scheme depicts the release of the base from the nucleoside donor, prior to the entry of the acceptor base into the active site (Figure 1.6.).

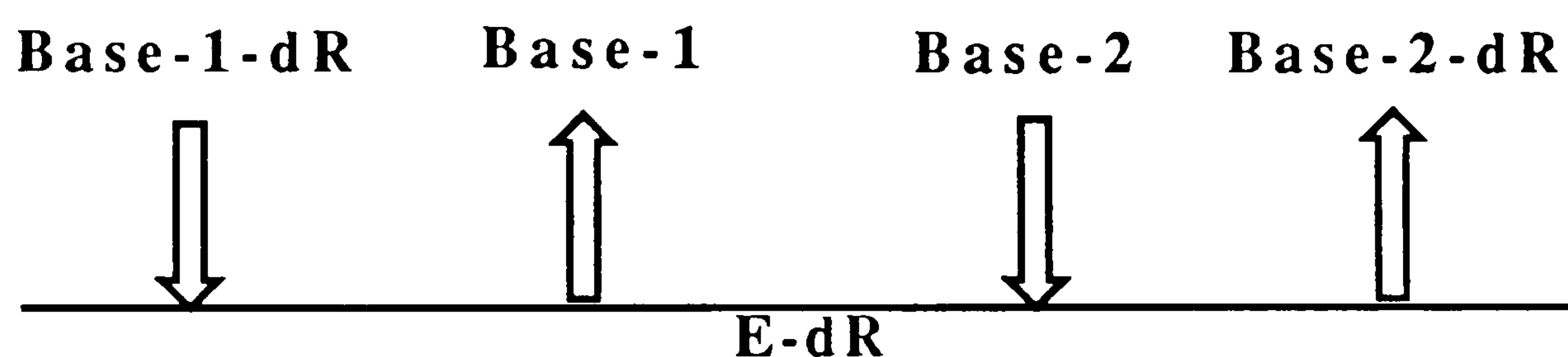


Figure 1.6

This route suggests the formation of a glycosyl intermediate, [87] but as yet this hypothesis has not been confirmed. The substrate specificity of these transferase enzymes have also been tested [88]. A wide range of heterocyclic bases have been found to act as competent acceptors, but extensive modifications in the sugar moiety are not tolerated. An attempt has also been

made to assign a function for the N-deoxyribosyltransferase enzyme in the general metabolism of *Lactobacilli*.

### FUNCTION OF N-DEOXYRIBOSYLTRANSFERASE ACTIVITY AND REPRESSION CONTROL

The metabolic function of N-deoxyribosyltransferases is thought to be intimately associated with nucleic acid synthesis [89, 90]. It was already known that some species of bacteria required exogenously supplied deoxyribonucleosides for growth, but it was found that in *L. leichmannii* this demand could be satisfied by the supply of vitamin B<sub>12</sub> [91].

When vitamin B<sub>12</sub> is absent in the growth medium of *L. leichmannii*, the synthesis of a full set of DNA precursors is dependent on the catalysis by N-deoxyribosyltransferase of deoxyribose groups from the administered deoxyriboside to free bases. Three other species of *Lactobacillus* where N-deoxyribosyltransferase activity is found display a growth requirement like this; *L. lactis*, *L. acidophilus* and *L. delbrueckii*. However, only *L. leichmannii* and *L. lactis* have the ability to be alternatively nourished with vitamin B<sub>12</sub> [73, 92, 93].

Starvation of vitamin B<sub>12</sub> or deoxyribonucleoside is found to result in impaired DNA synthesis [91, 94]. Under these limiting conditions the levels of N-deoxyribosyltransferase activity were observed to rise significantly, concurrent with a fall in the levels of the intracellular deoxyribosyl pool. Elevated levels of



enzyme with diminishing deoxyribosyl concentration implies that the enzyme synthesis is controlled by feedback inhibition. Whether this repression is dependent a specific combination of deoxyribosyl residues or one single type is unclear.

The enzyme and vitamin B<sub>12</sub> are thought to have a complementary role in nucleic acid synthesis (Figure 1.7).

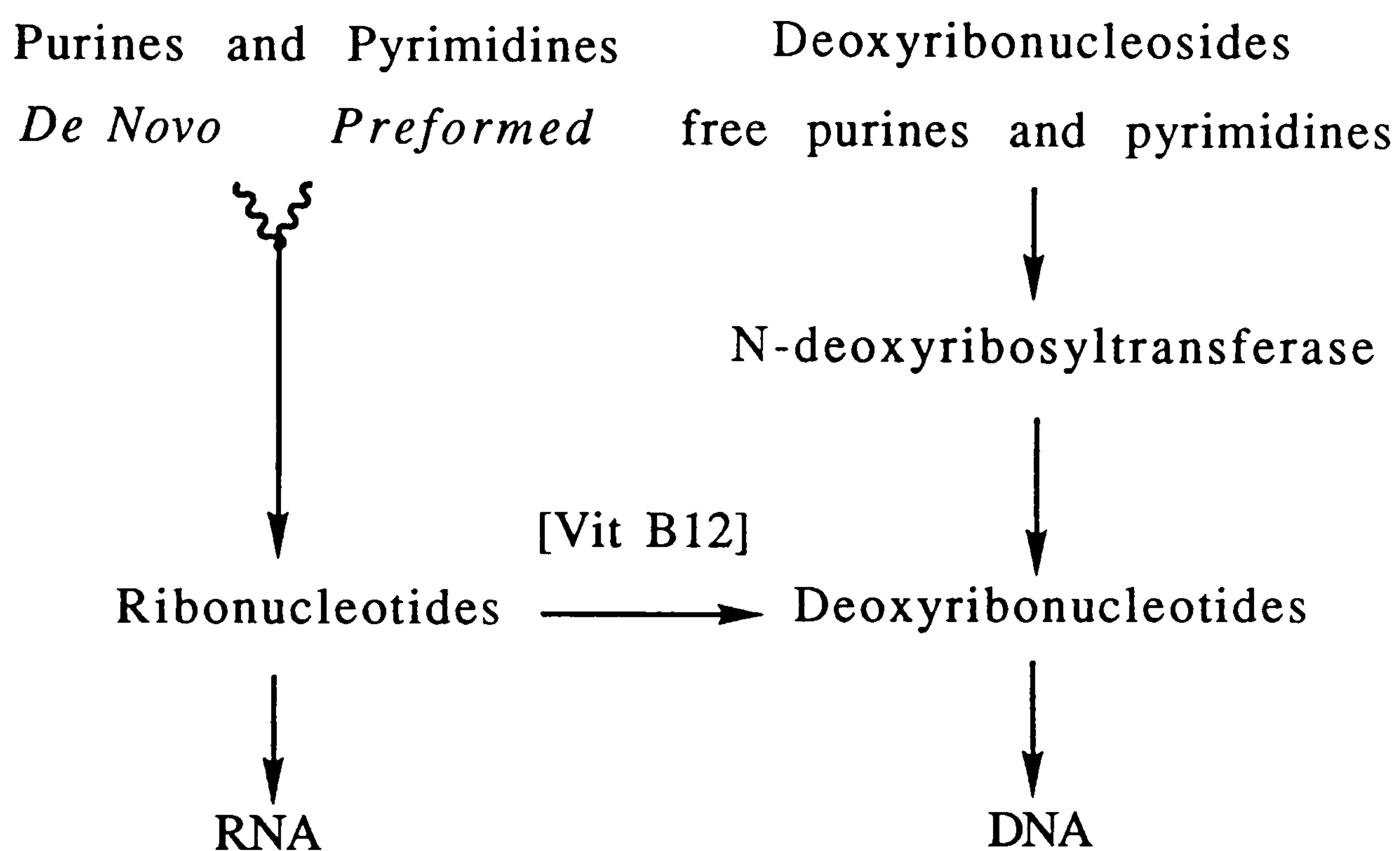


Figure 1.7

Vitamin B<sub>12</sub> participates in the reduction of ribonucleotides to deoxyribonucleotides [95]. Thus, the biological function of N-deoxyribosyltransferase is to provide a complement of deoxyribonucleotides to fulfil the demands of the cell for DNA synthesis in the absence of Vitamin B<sub>12</sub>.

*L. leichmannii* seem to favour using their deoxyribonucleosides for the synthesis of deoxyribonucleotide triphosphates in contrast with certain enteric bacteria such as *E. coli* which prefer to catabolize their deoxyribonucleosides. Perhaps this is



why *Lactobacillus* do not possess catabolizing enzymes such as deoxyriboaldolase, and the nucleoside phosphorylases TdRPase and PNPase.

### N-DEOXYRIBOSYLTRANSFERASES IN THE SYNTHESIS OF NUCLEOSIDE ANALOGUES

N-deoxyribosyltransferase has shown itself to be a useful alternative to using nucleoside phosphorylase enzymes as biocatalysts in the preparation of nucleoside analogues. Extracts from several *Lactobacilli* have been used in these preparations [84, 85]. For the purpose of synthesis, the two transferase activities are not usually separated, but used as a relatively crude mixture.

N-deoxyribosyltransferase from *L. leichmannii* has been instrumental in the synthesis of a number of both base-modified and sugar-modified nucleoside analogues. A diverse range of bases were observed to be competent acceptors for the enzyme and were used with thymidine as a glycosyl donor in a biotransformation reaction to synthesize a sequence of 2'-deoxyadenosine nucleoside analogues [85]. The analogues were able to be produced on a scale of 100-400mg with an average yield of 64%. The good thermal stability of the enzyme preparation was exploited to the advantage of the preparation.

9- $\beta$ -D-2'-Deoxyribofuranosyl 1-deazapurine has been prepared from crude extract of *L. leichmannii* with thymidine as a glycosyl donor [32]. This synthesis confirms the high degree of

regio- and stereoselectivity that the enzyme conveys on the reaction. In both circumstances the glycosyl group is transferred exclusively to the N-9 position on the purine acceptor, and the glycosidic link of the new deoxynucleoside shown to have the  $\beta$ -configuration.

The broad substrate specificity of *L. helveticus* has been exploited in the synthesis of a series of 2'-deoxy-2-halopurine compounds [96]. Partially purified extract was incubated with thymidine and 2-halopurine acceptor bases to achieve the facile, large scale preparation of these anti-leukemic analogues [97]. An interesting property of these 2-halopurine nucleosides is their ability to resist the action of adenosine deaminase [85]. This observation was used later in designing the antiviral 2-halo-2',3'-dideoxynucleoside analogues [98].

The efficacy of the potent antiviral drugs 2',3'-dideoxyadenosine and 2',3'-dideoxyinosine [99] are diminished inside cells by attack from adenine deaminase and mammalian PNPase respectively. 2-Halo-2',3'-dideoxyadenosine derivatives are found to be resistant to this catabolism, and a combination of chemical and biocatalytic steps have been used to make these compounds. N-Deoxyribosyltransferase extract from *L. helveticus* was used in the direct and simple synthesis of 2-halo-2'-deoxyadenosine nucleosides. Chemical methods were then used to produce the corresponding 2-halo-2',3'-dideoxynucleosides.

N-deoxyribosyltransferase is also able to accept some modified sugar groups in donor nucleosides. 2',3'-Dideoxynucleosides,



several of which have shown to be very potent inhibitors of the HIV-1 virus, have been prepared by enzymatic transglycosylation using partially purified extract from *L. helveticus* [100].

2',3'-Dideoxycytidine acted as the pentosyl group donor to a variety of 2- and 6-substituted purine acceptor bases. The substrate specificity of acceptor bases in this reaction with the 2',3'-dideoxyribose moiety seems to be analagous to the 2'-deoxyribose transfer system.

The ability of the N-deoxyribosyltransferase to accept the 2',3'-dideoxyribosyl group as a substrate has led to its use in the very specific synthesis of sugar- and base-radiolabelled nucleosides for routine use in the analysis of drug metabolism of therapeutic nucleosides [53, 85].

### SUMMARY

N-deoxyribosyltransferases have been shown to be practical in the synthesis of a number of nucleoside analogues. The enzymes are able to accept a large number of different 'unnatural' substrates with modifications both in the sugar and the heterocyclic base.

Some of the problems which arise from using phosphorylases in a coupled, phosphate-dependent system for these biotransformations can be overcome with N-deoxyribosyltransferases.

With one reported exception [75], all members of the *Lactobacilli* family have a N-deoxyribosyltransferase activity



all of which catalyse the glycosyl transfer between purines and pyrimidines. In this respect they seem to possess some advantage over the phosphorylase system which generally relies on the presence of both purine- and pyrimidine-specific enzymes.

Further study of the substrate specificity of these and other strains of the *Lactobacilli* genus and members of other genera of lactic acid bacteria could help to reveal enzymes which can use highly modified sugars as substrates in the transfer reaction. Characterization of the specific activities which perform these transformations will enable them to be used in circumstances where very selective catalysis is required, such as the removal or addition of particular groups to an existing molecule or the resolution of enantiomers.

Information about the physical properties of enzymes and the way they behave outside of the living cell could enable the improvement of the stability of these biocatalysts and hence boost the yield of enzymatic syntheses [101].

Knowledge of the high thermal stability of the N-deoxyribosyltransferase extract from *L. leichmannii* has enabled this property to be exploited in the synthesis of certain compounds [85].

## 1.6 OBJECTIVES

The objectives of this project were to obtain a greater understanding of the N-deoxyribosyltransferase reaction in *L. leichmannii*. The active site was mapped by examining its substrate specificity in order to determine the nature and stereochemistry of binding.

Chemical modification using group-specific reagents was used to reveal which amino-acid residues might participate in binding and catalysis.

To date, studies on *L. leichmannii* have not revealed the existence of two discrete enzymes analogous to those of *L. helveticus*. We hoped that by the development of a different purification scheme we could demonstrate the presence of further enzymes.

## 1.7 PROPOSALS FOR THE ACTIVE SITE OF GLYCOSYL TRANSFER ENZYMES

Models for the active site of both phosphorylase and N-deoxyribosyltransferase enzymes have been proposed [102, 103, 104].

Detailed studies of substrate and inhibitor of PNPase from mammalian and bacterial sources have led to the proposal that particular amino acid residues are in or close to the active site and play a big role in binding and catalysis.



Mammalian PNPase is imagined to contain essential histidyl [105] and carboxyl residues (Figure 1.8), which interact with positions O(6) and N(1) on the six-membered ring of a purine base or nucleoside respectively.

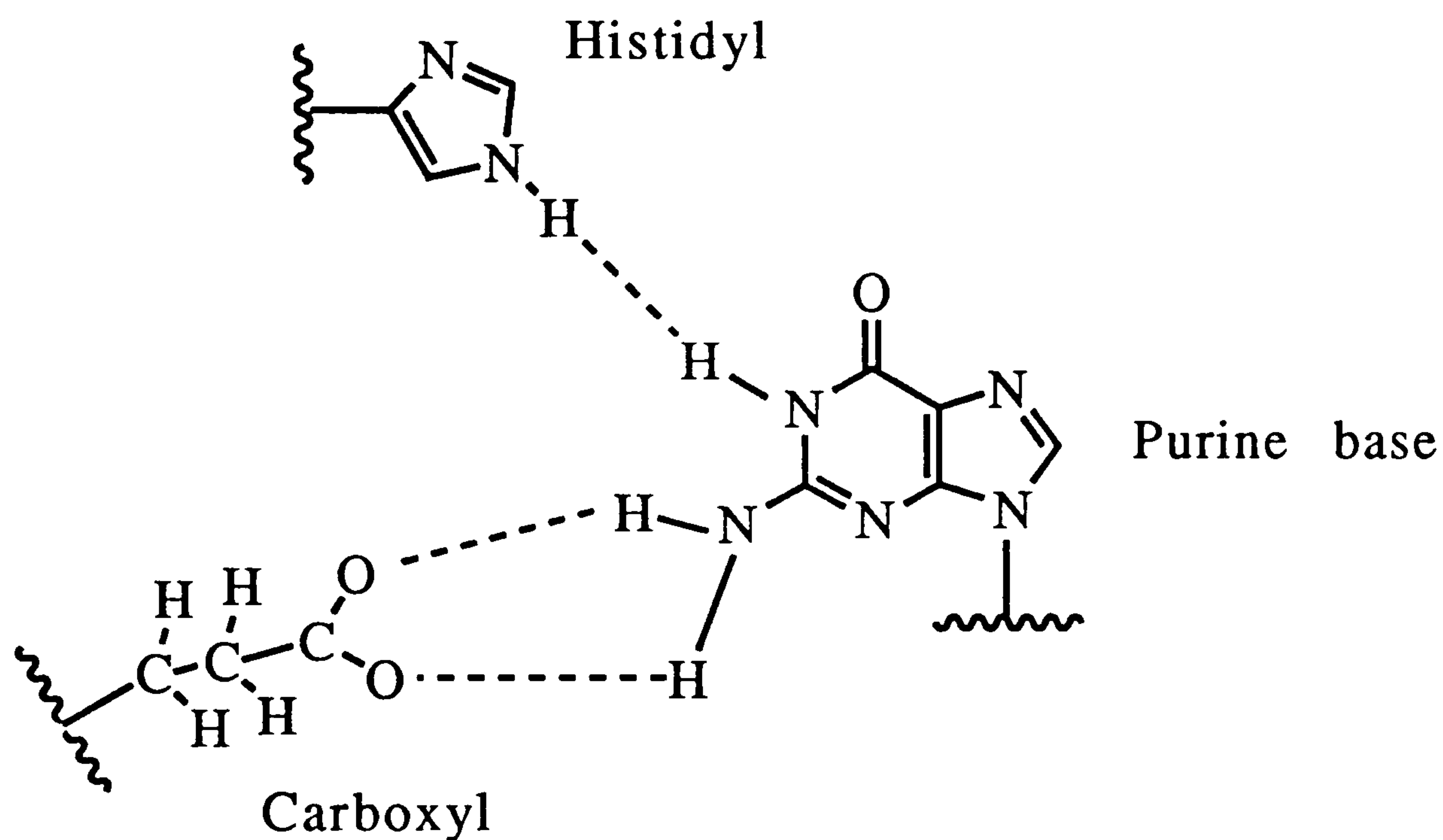


Figure 1.8

On the basis of substrate specificity studies in *L. helveticus* [88], Holguin proposed that the imidazole ring of the purine base was essential for binding (Figure 1.9).

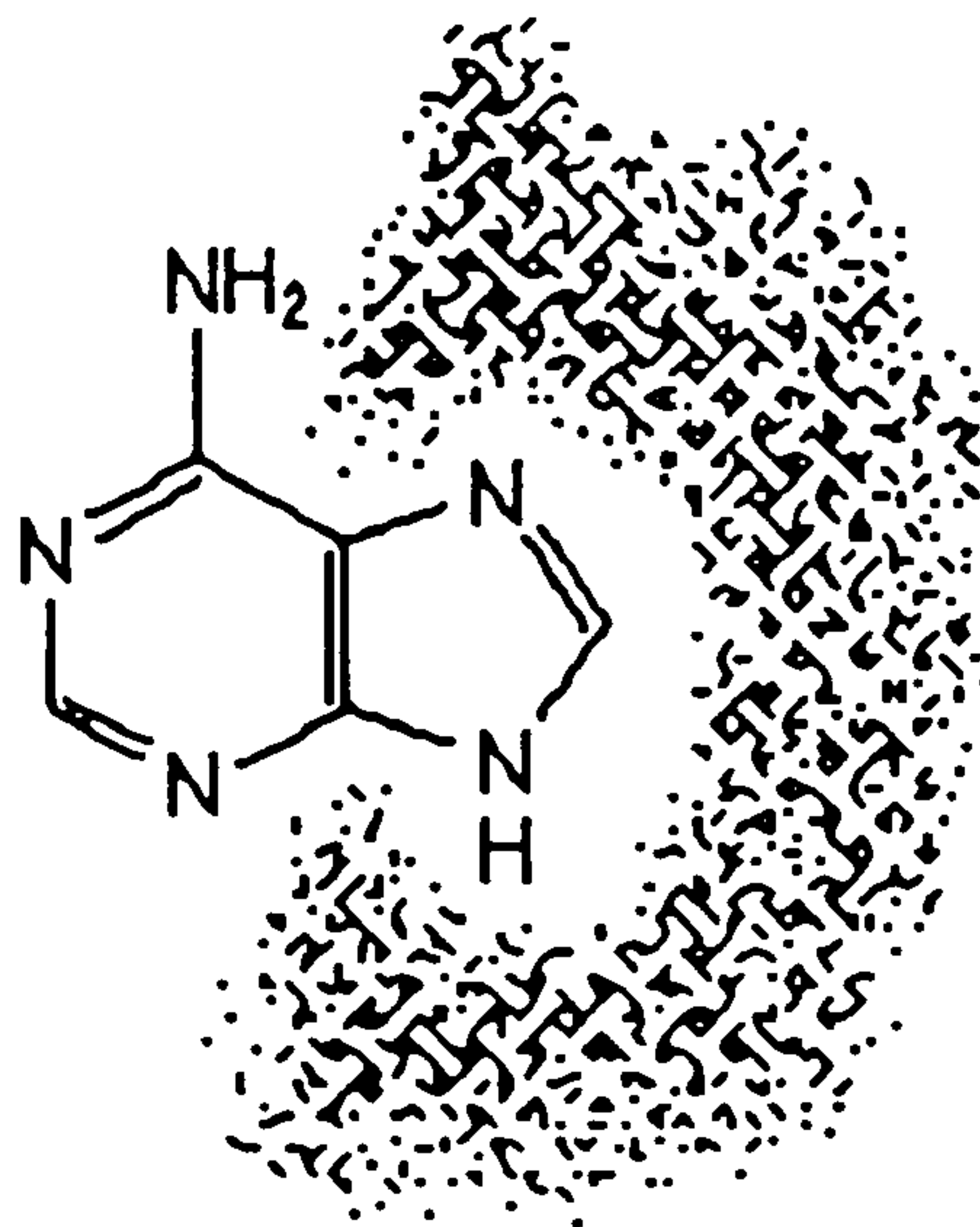


Figure 1.9

## CHAPTER 2

### PURIFICATION OF N-DEOXYRIBOSYLTRANSFERASES FROM *L. LEICHMANNII*

#### 2.1 GENERAL BACKGROUND

In order to study individual enzymes in detail they must be pure. Isolation of an enzyme from all other proteins and from polysaccharides enables conclusive results to be obtained in the investigation of the biocatalysis of one particular chemical reaction. Only when a reaction is catalysed by a pure preparation can it be said to be mediated by a single enzyme. The products of a reaction can also be substrates for other enzymes and it is essential to make sure that none of these are degraded or derivatised by contaminating proteins.

The first successful enzyme purification was in 1926 when Sumner crystallized the protein urease [105]. There followed a period of investigation which concentrated on characterizing crystalline proteolytic enzymes, work which served to dispel any doubt that enzymes were indeed proteins. A large number of both intracellular and extracellular enzymes have now been purified, and although some have been found to require non-protein co-factors, each one has proved to be a protein. The purification of some enzymes has enabled their detailed characterization, both as chemical catalysts in isolation, and as components of the metabolic mechanism of the living cell.



N-Deoxyribosyltransferase enzymes from a number of different species of *Lactobacillus* have been purified (Table 2.1).

The early approaches to the purification of N-deoxyribosyltransferases were relatively crude, consisting solely of salt fractionation and heat denaturation steps [76, 77, 80]. The extent of purification of these enzymes was not outstanding and final preparations were always mixtures of the three transfer activities:



*Lactobacillus* are generally grown in adapted, complex media, for example, De Mann, Rogosa, Sharpe (MRS) medium [106] which is designed for the profuse growth of lactic acid bacteria. The cells are grown without aeration and agitation and harvested in the stationary phase. After harvesting the biomass, the first stage of purification involves the homogenization of the cells. This entails disrupting the cells to release the cellular contents, including enzymes, into solution. There are many approaches to this problem from gentle enzymatic proteolysis of the cell walls with enzymes such as lysozyme [107] and mutanolysin [108] which produces protoplasts, to more vigorous techniques like sonication, or passage through a french pressure cell which breakdowns cells by shear forces. The most popular methods to disrupt the *Lactobacillus* cells have employed sonication [79, 81, 89] and bead-milling [76, 80]. After disintegration, the cell debris of the

Table 1

Stages in the Purification of N-Deoxyribosyltransferases from *Lactobacillus* species.

|                                      |                       | Successive stages of purification  |
|--------------------------------------|-----------------------|--|
| Roush and Betz (1958)<br>[80]        | <i>L. helveticus</i>  | Ammonium sulphate fractionation (x2), Heat denaturation.<br><i>Purification: x 11.25</i>   |
| Marsh and King (1959)<br>[76]        | <i>L. acidophilus</i> | MgCl <sub>2</sub> , Ammonium sulphate fractionation, Ethanol Precipitation,<br>Ammonium sulphate fractionation. <i>Purification: x 52.8</i>  |
| Kanda and Takagi (1959)<br>[77]      | <i>L. delbrueckii</i> | MgCl <sub>2</sub> , Ammonium sulphate fractionation Acetone precipitation.<br><i>Purification: x 19</i>  |
| Beck and Levin (1963)<br>[79]        | <i>L. leichmannii</i> | Heat denaturation, Ammonium sulphate fractionation, DEAE-<br>cellulose ion exchange chromatography (x2). <i>Purification: x 151.0</i>  |
| Uerkvitz (1971)<br>[89]              | <i>L. helveticus</i>  | Heat denaturation, Ammonium sulphate fractionation, DEAE-<br>Sephadex ion exchange chromatography (x2).  |
| Holguin and Cardinaud (1975)<br>[81] | <i>L. helveticus</i>  | Ammonium sulphate fractionation, Protamine sulphate<br>fractionation, Ammonium sulphate fractionation, Sephadex G-100<br>gel permeation chromatography, SE-Sephadex-, CM-Cellulose-,<br>DEAE-Sephadex-ion exchange chromatography.<br><i>Purification: pur/pur x 1421; pur/pyr x 240</i> |
| Danzin and Cardinaud (1975)<br>[82]  | <i>L. helveticus</i>  | Ammonium sulphate fractionation, Protamine sulphate<br>fractionation, Heat denaturation, Affinity column.  |
| Huang (1981)<br>[85]                 | <i>L. leichmannii</i> | Protamine sulphate fractionation, Ammonium sulphate<br>fractionation, Sephadex G-100 gel permeation chromatography,<br>Ammonium sulphate fractionation, dGTP-Sepharose, Affinity<br>Column.  |



homogenate is removed by centrifugation.

Since large volumes are generally involved, the concentration of the crude extract by salt fractionation is commonly the next step. This procedure allows large quantities of material to be dealt with and proteins are generally precipitated with ammonium sulphate. Bacterial extracts derived from vigorous disruption procedures such as sonication or passage through a pressure cell can appear viscous due to the presence of DNA. This accounts for the use of a second precipitation step with protamine sulphate in order to eliminate nucleic acid material [81, 82, 85]. The inclusion of heat denaturation steps in some purification methods exploits the property of the high thermal stability of some N-deoxyribosyltransferase activities. This was used routinely in earlier procedures before any detailed physical characterization of the activities [79, 80]. These classical methods have remained important in the preliminary stages of the purification of these enzymes because of their ability to cope with reasonable amounts of extract and their relative insensitivity to any non-protein contaminants.

Ion exchange chromatography was introduced in later N-deoxyribosyltransferase purification schemes [79, 81]. This adsorption column chromatography step separates protein molecules on the basis of their net charge at a given pH. Proteins bind reversibly to an insoluble ion-exchange matrix by electrostatic forces which exist between surface charges of the proteins and charged groups on the support. Salt gradients are the most common way of eluting proteins adsorbed to ion exchange matrices, although raising and lowering the pH of the

eluting buffer can be employed to the same effect.

Initial attempts to purify discrete activities from *L. helveticus* using a combination of precipitation and ion exchange steps were somewhat successful allowing a partial separation of the purine:purine activity on a DEAE-Sephadex support [81]. Affinity chromatography using a modified purine analogue as a ligand was required for the complete isolation of this activity [82]. This affinity adsorption technique aims to exploit the unique properties of individual enzymes on the basis of their biological specificity. Ligands, usually substrate analogues and competitive inhibitors of the desired enzyme, are covalently attached to a support so as to be available for selective combination with the protein of choice. This method requires that the affinity of a ligand for the enzyme is very high and that the binding is non-reactive and reversible. Affinity adsorption methods have also been employed in the purification of the N-deoxyribosyltransferase activities from *L. leichmannii* [85].

Gel permeation or molecular exclusion chromatography, which has the least capacity of all the column techniques, separates proteins on the basis of molecular size and shape. This is normally employed as the final step in a purification.

Hydroxyapatite, a form of calcium phosphate, has also proved itself to be useful in the fractionation of proteins. Its mode of action is not clear, but the surface of the matrix is made up of charged ions, and electrostatic attraction is thought to be important in the selective adsorption of proteins. Since positive and negative charges are closely associated in the support, the



binding is likely to arise from dipole-dipole interactions rather than any ion exchange effects. Although it has not previously been used in the purification of N-deoxyribosyltransferases, we decided that it might be a valuable complement to other separation methods.

Our purification procedure has centred on trying to develop a suitable route to isolate the purine-specific N-deoxyribosyltransferase activity in *L. leichmannii* analogous to that from *L. helveticus*.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Analytical Methods

#### Unit of enzyme Activity.

A unit of activity is defined as the amount of protein which catalyses the formation of 1.0mmol of 2'-deoxyadenosine in one minute at 40°C.

#### Assay of N-Deoxyribosyltransferase activity by HPLC.

Deoxyribosyl transfer between purines was assayed routinely by measuring the formation of 2'-deoxyadenosine from 2'-deoxyinosine and adenine at 40°C using HPLC. The reaction mixture contained 0.1µmol 2'-deoxyinosine, 0.1µmol adenine, 25µmol of Pipes buffer, pH 6.5, in a volume of 0.25 ml. The reaction was initiated by the addition of approximately 2 milliunits (mU) of enzyme. At intervals of 10 minutes, samples (20µl) of the reaction mixture were injected onto a Lichrosorb

C<sub>18</sub> reverse phase column (Jones Chromatography, Hengoed, U.K.) The nucleosides and bases were readily resolved by elution with a 15% aqueous solution of methanol and detected by absorbance at 254nm.

Transfer reactions between other pairs of nucleosides and bases were assayed under similar conditions. Pyrimidine:purine transfer was assayed by monitoring the formation of 2'-deoxyadenosine from 2'-deoxycytidine and adenine. Pyrimidine:pyrimidine deoxyribosyltransfer activity was assayed by following the formation of thymidine from 2'-deoxycytidine and thymine.

Assay of N-Deoxyribosyltransferase activity by Diphenylamine Colour Reaction.

Deoxyribosyltransfer between purines and pyrimidines was assayed by measuring the concentration of free 2'-deoxyribose in solution using diphenylamine reagent [109]. Commercial diphenylamine was recrystallized from petroleum ether. The colour reagent was prepared by dissolving 1g diphenylamine in 100ml glacial acetic acid and adding 3ml of sulphuric acid (18.4M). This solution was kept at 4°C. Two volumes of reagent were added to one volume of the standard assay mixture and incubated at 37°C for 2 hours. The blue colour which appeared was measured spectrophotometrically at 595nm.

Assay of Deoxyribosyltransferase activity coupled to Xanthine Oxidase.

A coupled spectrophotometric assay [110] was employed for



sensitive measurements, i.e. the determination of apparent  $K_m$ . In the measurement of the  $K_m$  values for adenine, xanthine oxidase was used as an auxiliary enzyme. The hypoxanthine formed was rapidly oxidized to uric acid by the xanthine oxidase and the reaction followed by recording the absorbance change at 290nm ( $\epsilon_{290}$  for uric acid was taken as  $12,200 \text{ M}^{-1} \text{ cm}^{-1}$ ). All reactions were carried out at  $40^\circ\text{C}$  in a quartz cuvette of 10mm path length (final volume 1.5ml). 2'-Deoxyinosine solution was mixed together with  $50\mu\text{mol}$  of Pipes buffer, pH 6.5 and 3 mU units of xanthine oxidase. The mixture was equilibrated at  $40^\circ\text{C}$  for 10 minutes to ensure that any trace amounts of hypoxanthine in the 2'-deoxyinosine were completely oxidised. The adenine was added and the increase in absorbance due to the slow oxidation of the base recorded. The enzyme solution (10mU) was added to start the reaction and the absorbance change followed with time.

#### Protein Determination

Protein determinations were carried out using the Bio-Rad protein assay according to the method of Bradford [111, 112]. Bovine serum albumin was used as a standard.

#### pH Optimum

The enzyme was incubated under the standard conditions for 20 minutes in 0.1M buffer at various pH values. Citrate-phosphate buffer was used in the range from pH 3.0 - 7.0, and phosphate buffer in the range from pH 6.0 - 8.5.

### 2.2.2 Cultivation of Organism.

*L. leichmannii* ATCC 4797 were grown in a modified MRS medium [106]. Dehydrated MRS medium was dissolved in 20 l distilled water ( $26\text{g l}^{-1}$ ) to give half the recommended concentration. The medium was adjusted to pH 6.5 with 5M NaOH solution and sterilized by autoclaving at  $121^{\circ}\text{C}$  for 50 minutes. A solution, of 200g of glucose and 100g of bacteriological peptone was sterilized separately by autoclaving at  $110^{\circ}\text{C}$  for 10 minutes and added aseptically to the broth. The medium was inoculated with 200ml of a 16h starter culture grown in MRS broth, and incubated at  $37^{\circ}\text{C}$ , without aeration or agitation. Growth was monitored by following the decrease in pH and the increase in optical density at 600nm.

The bacteria were harvested in the stationary phase after 23 hours of growth. The bacterial suspension was concentrated to 1 l in a continuous centrifuge (American Instrument Company) then harvested by centrifugation at 17,700g for 15 minutes. The pellet was resuspended and washed twice in equal volumes of 0.02M Pipes buffer, pH 6.5, and stored at  $-20^{\circ}\text{C}$  as a cell paste.



## 2.3 RESULTS

### 2.3.1 PURIFICATION PROCEDURE

#### Preparation of Cell-free Extract

The paste was thawed and resuspended at a concentration of 1g ml<sup>-1</sup> in 0.02M Pipes buffer, pH 6.5 containing 5mg ml<sup>-1</sup> lysozyme and 50 Uml<sup>-1</sup> mutanolysin to digest the cell walls [108]. The preparation was incubated at 37°C for 3 hours and protoplast formation was followed by light microscopy. Disruption was completed by two passages through a French pressure cell (American International Company, Maryland, USA.) at 20,000 psi, and the preparation cooled to 4°C in an ice bath. All subsequent operations were carried out at this temperature unless otherwise indicated. Cell debris were removed by centrifugation at 18,000g for 20 minutes. The pellet was resuspended in 0.02M Pipes buffer, pH 6.5, and subjected to two further passages through the pressure cell, then centrifuged under the same conditions. The supernatant fluids were pooled and assayed for nucleoside deoxyribosyltransferase activity.

#### Ammonium Sulphate Precipitation

The crude extract was fractionated by the addition of solid ammonium sulphate. The material precipitating between 40-80% saturation was collected by centrifugation at 38,000 g for 25 minutes. This was dissolved in 0.02M Pipes buffer, pH 6.5, and dialysed against 10mM Bis-Tris/iminodiacetic acid buffer, pH 7.0.

## Fast Protein Liquid Chromatography (FPLC)

### Anion Exchange on MONO-Q

The dialysed material was applied to a Mono-Q high resolution, anion-exchange column (100 x 10mm) pre-equilibrated with 10mM Bis-Tris/iminodiacetic acid buffer, pH 7.0, and eluted with a linear 0 - 0.35M NaCl gradient in the same buffer. The gradient was run at 4ml min<sup>-1</sup> for 54 min. Eluant fractions, (5.0ml), were assayed under standard conditions. Enzyme activity appeared in two, partially separated, peaks eluting between 0.21 and 0.33M NaCl. The active fractions were pooled and dialysed against 10mM phosphate buffer, pH 6.0.

### DEAE-2SW

This dialysate was applied to a TSK DEAE-cellulose 2SW column (7.8 x 300mm) which had been equilibrated with 10mM phosphate buffer, pH 6.0. The column was eluted at 1ml min<sup>-1</sup> with a linear 0 - 0.5M NaCl gradient over 100 minutes. Eluant fractions (2.0ml) were assayed under standard conditions. Enzyme activity was resolved into two distinct peaks: IV(A), eluting between 0.3 - 0.4M NaCl and IV(B) between 0.4 - 0.5M NaCl (Figure 2.6). Most of the active fractions of each peak were pooled as fractions IV(A), N-deoxyribosyltransferase-I, and IV(B), N-deoxyribosyltransferase-II, and dialysed separately against 10mM phosphate buffer, 0.3mM CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 6.8.

### Hydroxyapatite-HPHT

The dialysed fractions of N-deoxyribosyltransferase-I, IV(A), and N-deoxyribosyltransferase-II, IV(B), activity were applied,



in turn, to a Bio-Gel Hydroxyapatite HPHT column (Bio-Rad.) (7.8 x100 mm) and eluted at 0.5 ml min<sup>-1</sup> with a linear gradient of 0.01-0.4M phosphate buffer, 0.1mM CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 6.8 (Figures 2.7[i] and 2.7[ii]). Eluant fractions were assayed under standard conditions and the active fractions pooled and dialysed against 10mM phosphate buffer, pH 6.0 to give fractions V(A) and V(B).

#### Gel Filtration Chromatography

Pooled fractions V(A) and V(B) of each enzyme were loaded individually onto a TSK G4000SW gel filtration column (ToyaSoda, Tokyo, Japan) (7.8 x 300mm) and eluted with 10mM phosphate buffer, pH 7.0. The elution of the activities was followed by the assay of eluant samples under the standard conditions. There was insufficient protein in the recovered samples of VI(A) and VI(B) to carry out accurate determinations of specific activity. However, the samples were used to determine the molecular masses of the enzymes.

#### Native-PAGE

The purity of N-deoxyribosyltransferase-II was confirmed by native polyacrylamide gel electrophoresis (native-PAGE) using the Phastsystem method developed by Pharmacia.

5ng (approximately) of sample VI(B) was loaded onto a Phast Gel gradient 10-15 with Pharmacia molecular weight markers comprising a mixture of proteins in the range 67,000-669,000. The gel was run against native agarose buffer strips and silver stained according to the method set out by Pharmacia [113].

Table 2.2

Purification of N-deoxyribosyltransferase activities  
from *Lactobacillus leichmannii*

*Transfer between Purines*

|     |                    | <i>Protein Activity</i> |          |               |               |               |
|-----|--------------------|-------------------------|----------|---------------|---------------|---------------|
|     |                    | <u>mg</u>               | <u>U</u> | <u>Sp.Act</u> | <u>Purif.</u> | <u>Yield%</u> |
|     |                    | [dI/Ade]                |          |               |               |               |
| I   | Crude Extract      | 1404                    | 120.9    | 0.086         | 1.00          | 100.0         |
| II  | Ammonium Sulphate  | 767.1                   | 78.0     | 0.102         | 1.19          | 64.5          |
| III | FPLC Mono-Q        | 97.2                    | 29.3     | 0.30          | 3.50          | 24.2          |
| IV  | HPLC DEAE 2SW (A)  | 9.10                    | 23.0     | 2.52          | 29.3          | 19.0          |
|     | (B)                | 0.16                    | 1.37     | 8.50          | 99.5          | 1.40          |
| V   | Hydroxyapatite (A) | 0.70                    | 12.5     | 17.85         | 207.6         | 10.3          |
|     | (B)                | 0.03                    | 0.268    | 8.90          | 103.8         | 0.22          |

*Transfer between Purines and Pyrimidines*

|     |                    | <i>Protein Activity</i> |          |               |               |              |
|-----|--------------------|-------------------------|----------|---------------|---------------|--------------|
|     |                    | <u>mg</u>               | <u>U</u> | <u>Sp.Act</u> | <u>Purif.</u> | <u>Yield</u> |
|     |                    | [dC/Ade]                |          |               |               |              |
| I   | Crude Extract      | 1404                    | 301.0    | 0.21          | 1.00          | 100          |
| II  | Ammonium Sulphate  | 767.1                   | 178.5    | 0.23          | 1.10          | 59.3         |
| III | FPLC Mono-Q        | 97.2                    | 39.6     | 0.41          | 2.05          | 13.1         |
| IV  | HPLC DEAE 2SW (A)  | 9.1                     | 0 0      | -             | 0             |              |
|     | (B)                | 0.16                    | 18.9     | 118.2.        | 562.8         | 6.20         |
| V   | Hydroxyapatite (A) | 0.70                    | 0 0      | -             | 0             |              |
|     | (B)                | 0.03                    | 8.54     | 284.5         | 1355.5        | 2.8          |

dI = 2'-deoxyinosine, dC = 2'-deoxycytidine, Ade = adenine.  
(A) - N-deoxyribosyltransferase-I; (B) - N-deoxyribosyltransferase-II.



### SDS-PAGE

The subunit composition of N-deoxyribosyltransferase-II was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the Phastsystem method developed by Pharmacia.

5ng (approximately) of sample VI(B) was heated for 5 minutes at 100°C with 2.5% sodium dodecyl sulphate and 5.0%  $\beta$ -mercaptoethanol. Bromophenol blue was added to approximately 0.01% and any insoluble material removed by centrifugation. The sample was then loaded onto a Phast Gel, gradient 10-15 with Pharmacia SDS molecular weight marker proteins in the range 53,000- 212,000. The gel was run using SDS agarose buffer strips and stained according to a modified high sensitivity silver staining method for SDS-PAGE Phast Gel media [114].

### 2.3.2 Physical Characteristics

The relative molecular masses of N-deoxyribosyltransferase-I and N-deoxyribosyltransferase-II were determined on a TSK-Gel G4000SW column (7.8 x 300mm) which was equilibrated with 10mM phosphate buffer, pH 7.0, with a flow rate of 0.4 ml min<sup>-1</sup>. The following calibration markers were applied to the column separately: blue dextran, ( $M_r$  2,000,000) thyroglobulin (bovine), ( $M_r$  669,000) apoferritin (horse spleen,  $M_r$  443,000),  $\beta$ -amylase (sweet potato,  $M_r$  200,000), alcohol dehydrogenase (yeast,  $M_r$  150,000), albumin (bovine serum,  $M_r$  66,000). Elution volume was determined by peak absorbance at 280nm.

The molecular masses of N-deoxyribosyltransferase-I and N-deoxyribosyltransferase-II are 100,000 +/- 4,000 and 92,000 +/- 4,000 respectively (Figure 2.1). The values are an average of 3 runs. The subunit molecular weight of N-deoxyribosyltransferase-II was confirmed by SDS-PAGE (Figure 2.2).

The purine:purine activity of N-deoxyribosyltransferase-I was purified 207-fold and that of N-deoxyribosyltransferase-II, 103-fold. The pyrimidine:purine activity was increased 1355-fold. N-deoxyribosyltransferase-II was purified to homogeneity (Figure 2.3).

#### Isoelectric Focusing

The isoelectric point of the N-deoxyribosyltransferase-II was determined by isoelectric focusing (IEF) using the Phastsystem method developed by Pharmacia. 5ng (approximately), was loaded onto a PhastGel IEF 3-9 with Pharmacia pI calibration markers comprising a mixture of proteins with pIs in the range 3.50 - 9.30.

The gel was silver stained according to the method set out by Pharmacia [113].

The pI of the N-deoxyribosyltransferase-II was calculated to be 5.2 (Figure 2.4).



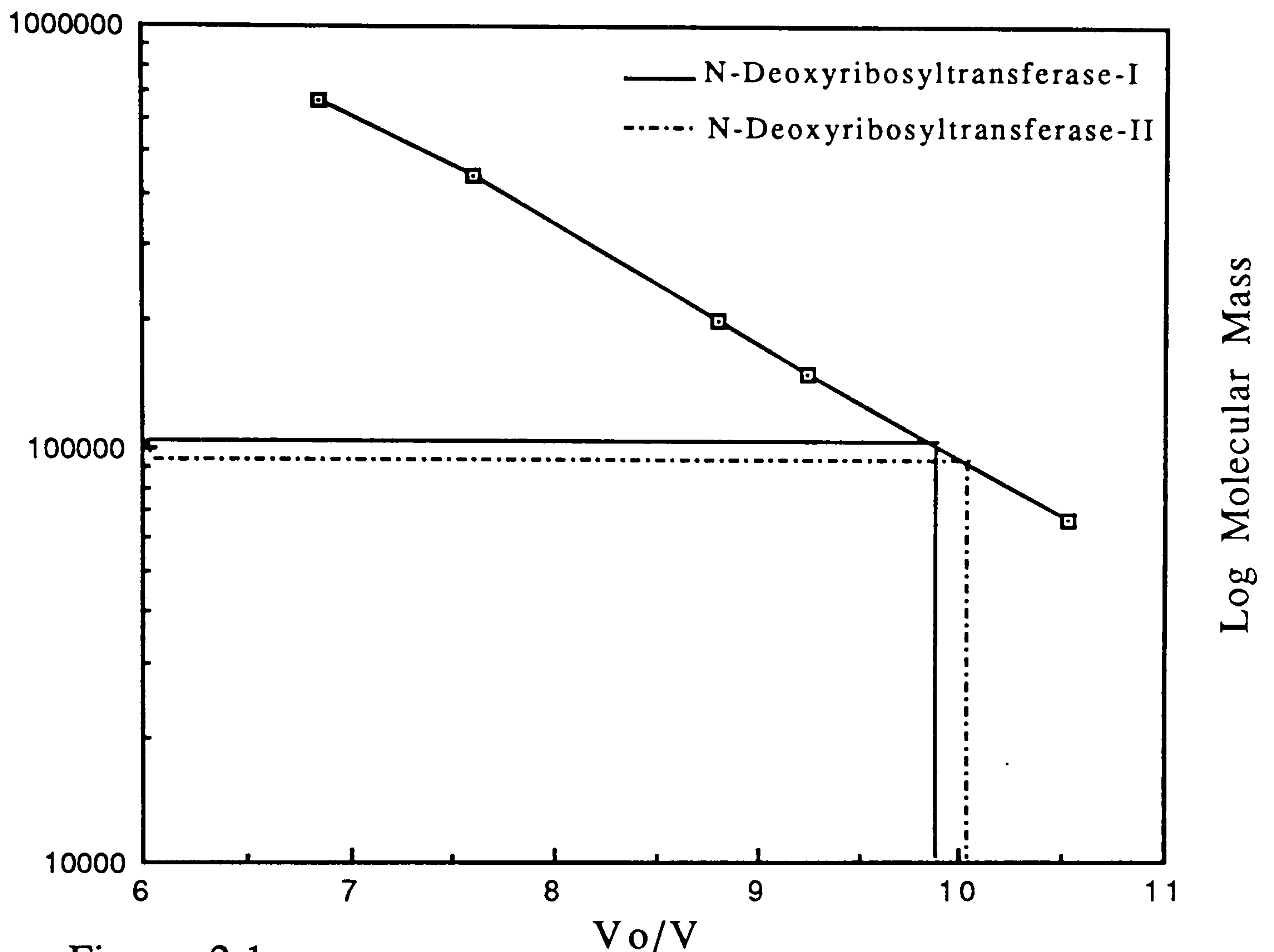


Figure 2.1

Determination of the Molecular Masses of  
N-Deoxyribosyltransferase-I and -II  
Conditions as described in the text

The thermal stability of the enzymes was compared by incubation at various temperatures between 35°C and 85°C prior to enzyme assay (Figure 2.4). These experiments demonstrate that distinct differences exist in the stability of the enzymes. At 60°C, for example, N-deoxyribosyltransferase-I was inactivated after 20 minutes incubation, whereas N-deoxyribosyltransferase-II retained half its activity.

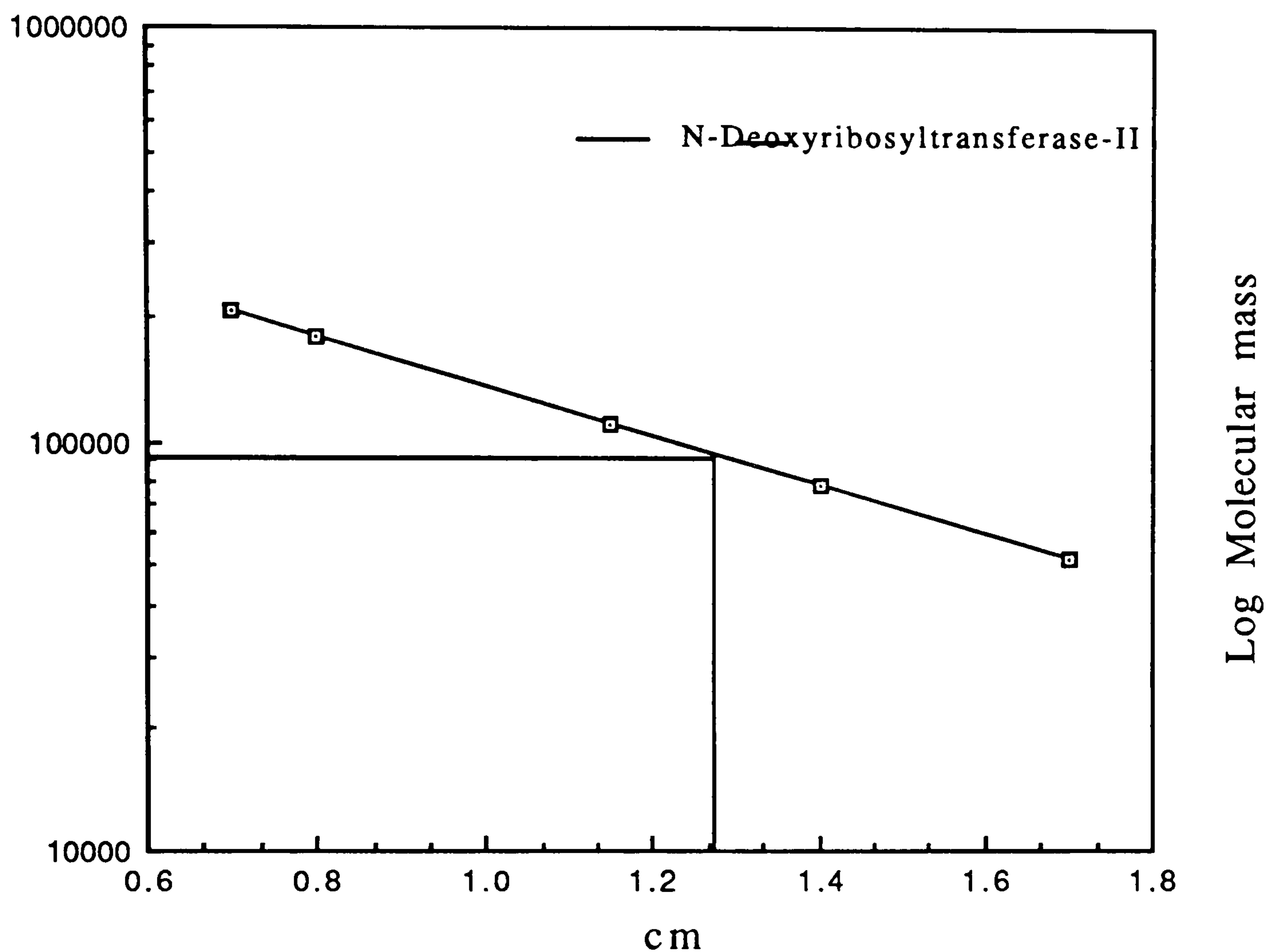


Figure 2.2 Determination of Molecular Mass of N-Deoxyribosyltransferase-II by SDS-PAGE. Conditions as described in the text

The influence of pH on the activity of the enzyme was seen by performing the standard reaction conditions under a variety of pH conditions from pH 3.0 - 8.5 (Figure 2.5). The pH optimum of N-deoxyribosyltransferase-I is pH 6.0 and that of N-deoxyribosyltransferase-II is pH 5.6 (Table 2.3). Neither enzyme demonstrated activity below pH 3.0 or above pH 8.1.



Polyacrylamide Gel Electrophoresis of Native  
N-deoxyribosyltransferase-II

Lanes 1, 3, 5, 7: High Mol. Wt. Std; 2, 4, 6: Transferase-II

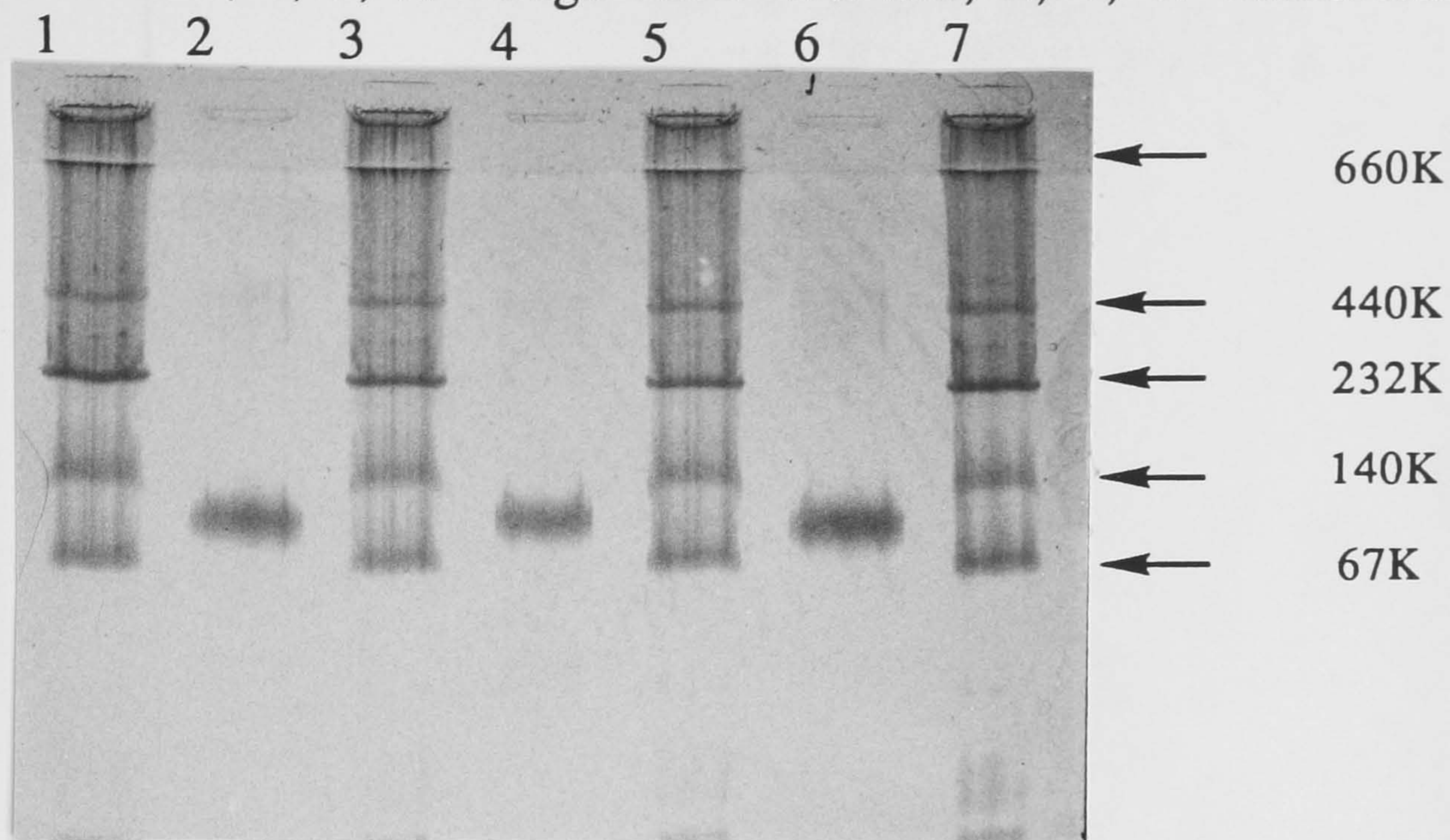


Figure 2.3

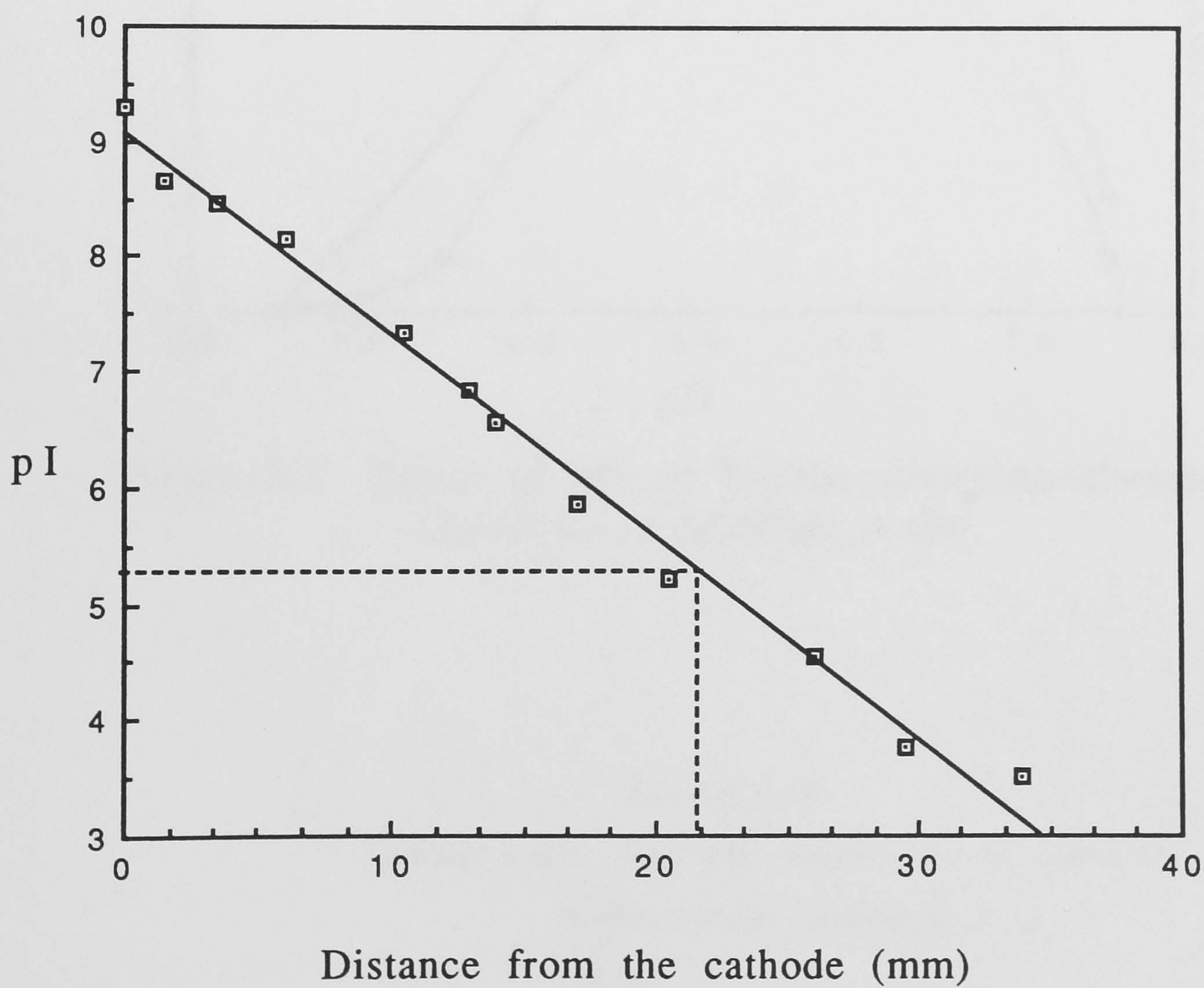


Figure 2.4 Determination of pI N-Deoxyribosyltransferase-II



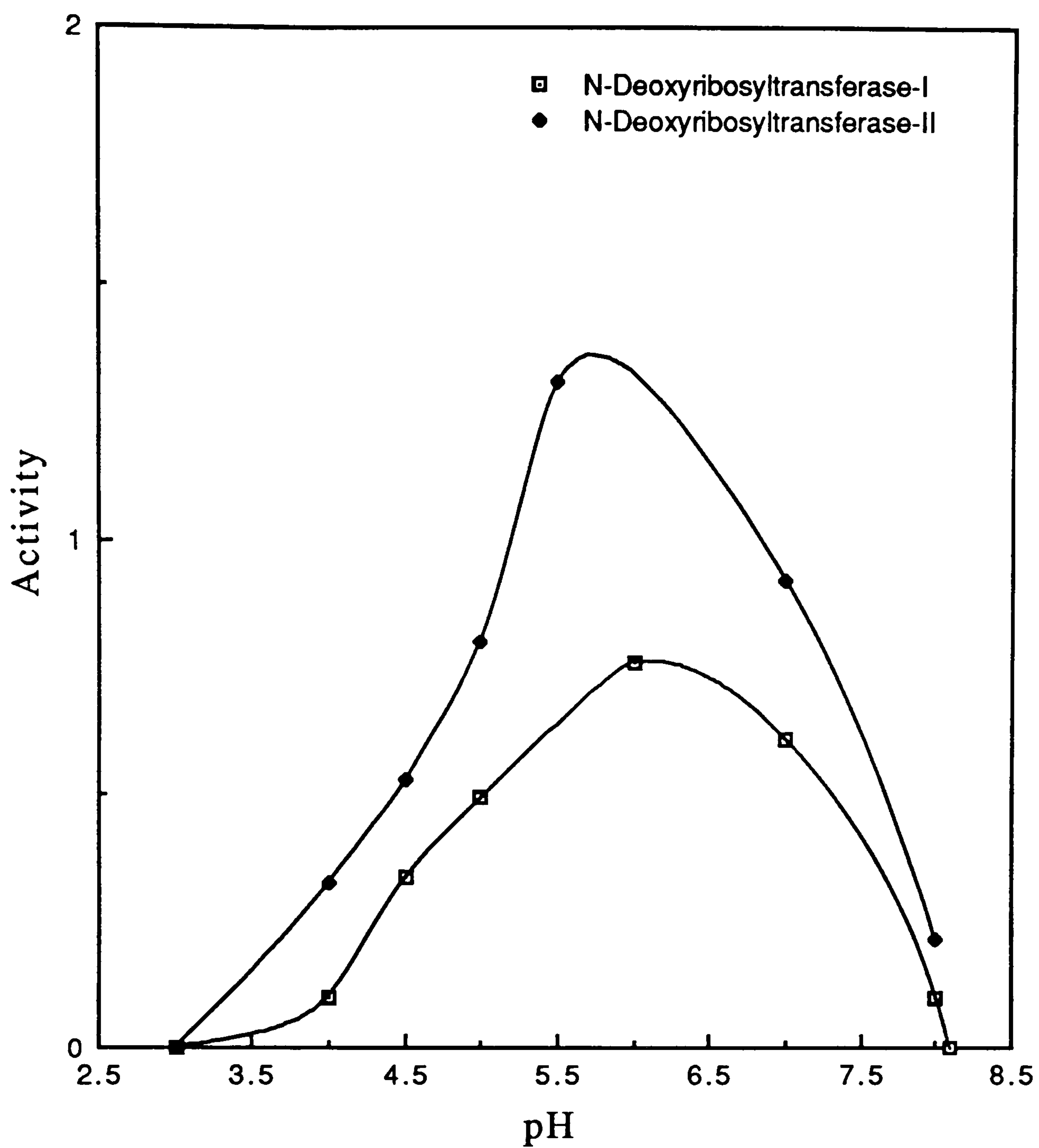


Figure 2.5 Effect of pH on N-deoxyribosyltransferase-I and -II. Conditions as described in text.

Table 2.3

*Temperature and pH Maxima and Optima*

|                             | N-deoxyribosyltransferase |     |
|-----------------------------|---------------------------|-----|
|                             | I                         | II  |
| Temp. optimum/ $^{\circ}$ C | 45                        | 65  |
| Temp. Maximum/ $^{\circ}$ C | 65                        | 85  |
| pH minimum                  | 3.0                       | 3.0 |
| pH optimum                  | 6.0                       | 5.6 |
| pH maximum                  | 8.0                       | 8.1 |



## 2.4 DISCUSSION

*L. leichmannii* contains two forms of nucleoside deoxyribosyltransferase which are analogous in their physical and catalytic properties to those resolved from *L. helveticus*. Attempts have been made to purify nucleoside deoxyribosyltransferase from *L. acidophilus* [76], *L. delbrueckii* [77], and *L. leichmannii* [79, 87], but few detailed studies have been published.

Several reports describe the preparation of N-deoxyribosyltransferase from *L. leichmannii* and inhibition studies have often been used to demonstrate that more than one deoxyribosyl transferase was present. Minghetti [78] noted that the reactions involving pyrimidines were inhibited when using Tris buffer. This was probably due to the inhibition of N-deoxyribosyltransferase-II by the Tris. The rates of purine:purine transfer were less inhibited, suggesting the presence of two or more enzymes. These phenomena have been observed in other species [76, 80, 81].

Beck and Levin [79] purified an N-deoxyribosyltransferase activity from *L. leichmannii* 151-fold by anion exchange, whilst Huang *et al* [87] attained a 260-fold purification using affinity chromatography. Both of these authors used thymidine as the donor nucleoside in the transfer reaction to assay all fractions during purification, and so failed to detect the purine specific enzyme. All the fractions obtained during this purification were assayed routinely for both purine:purine and purine:pyrimidine activity using 2'-deoxyinosine and 2'-deoxycytidine respectively as donors by the HPLC method described above.



The present results indicate that two distinct enzyme species are readily separable by ion-exchange chromatography on high resolution HPLC columns (Figure 2.6).

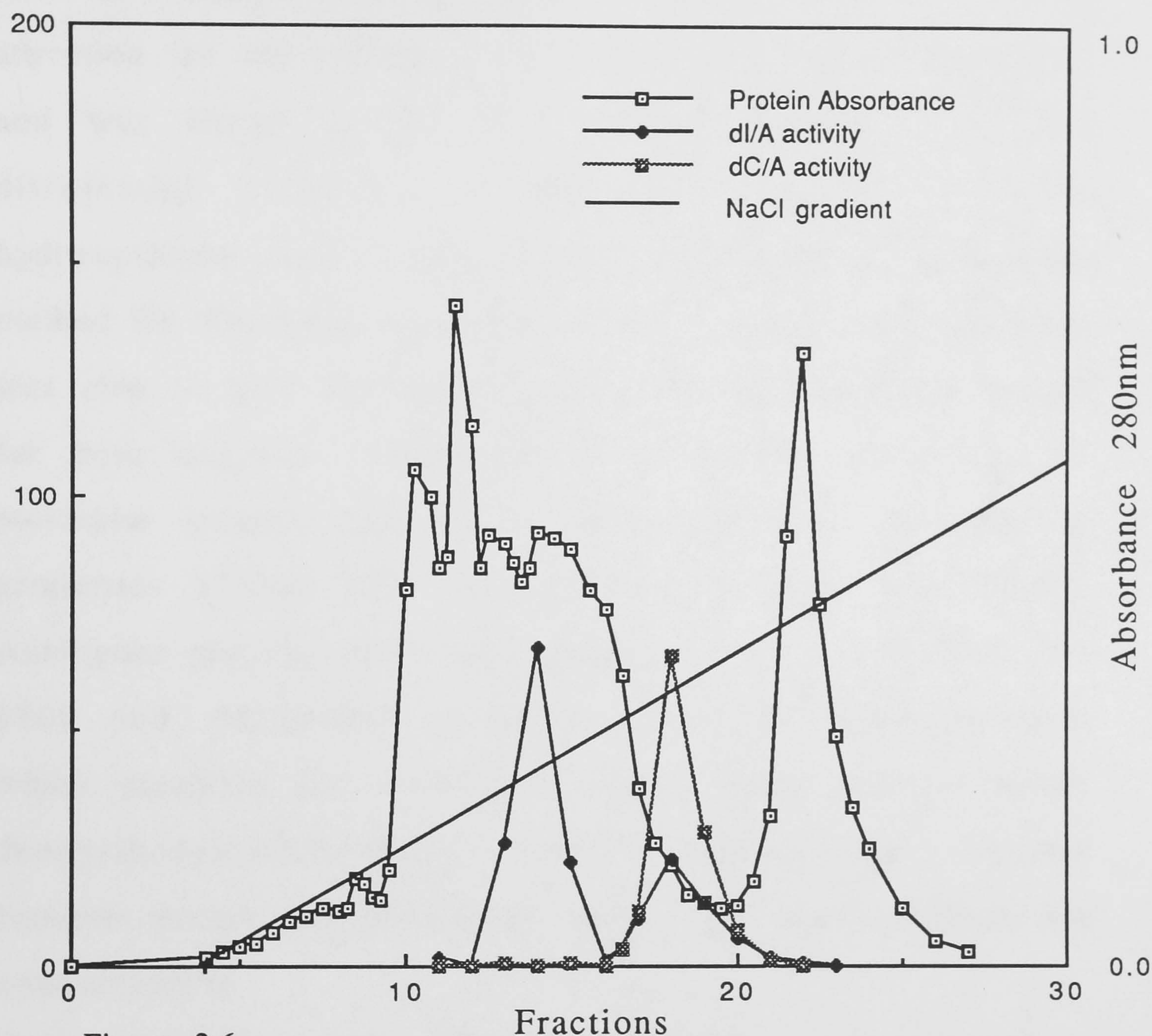


Figure 2.6

HPLC on DEAE 2SW ion exchange of a mixture of N-Deoxyribosyltransferase-I and -II. Transferase activities were eluted with a linear 0-0.5M NaCl gradient in 10mM phosphate buffer, pH6.0.

The Fast Protein Liquid Chromatography system facilitated the proficient handling of large amounts of material in a relatively short time span. Exploration of different types of column compatible with this system could help to accelerate the whole scheme, improving the efficiency and the yield of the



purifications. The hydroxyapatite-HPHT column step was introduced after the separation of the two activities in order to clean up the preparations. This was especially effective in the case of N-deoxyribosyltransferase-I which had little or no attraction for the column, even at very low salt concentration, and was eluted in the void volume (Figure 2.7[i]) The differential affinities of the two enzymes for the hydroxyapatite matrix suggests that this could be a possible method for the initial separation of the activities. Gel filtration was able to give reproducible values of the molecular masses for both enzymes. Although these proteins are of similar molecular masses, they can be distinguished by their thermal properties (Table 2.3) and reactivity towards pyrimidines. Analogous enzymes have been purified from *L. helveticus* [81, 110] and designated nucleoside deoxyribosyltransferase-I, which catalyses the transfer of purine bases and nucleoside deoxyribosyltransferase-II, which catalyses the transfer between purine and pyrimidine bases. We have adopted this nomenclature.

N-Deoxyribosyltransferase-II was purified to homogeneity as demonstrated by a single band on a native polyacrylamide gel. Its molecular mass was determined by gel permeation chromatography and confirmed by SDS-polyacrylamide gel electrophoresis. The SDS gel also defined the subunit composition of the enzyme and the appearance of a single band on this gel implies that the enzyme is a single polypeptide. Holguin's preliminary results indicated that purine specific enzyme from *L. helevticus* was a polymeric enzyme.



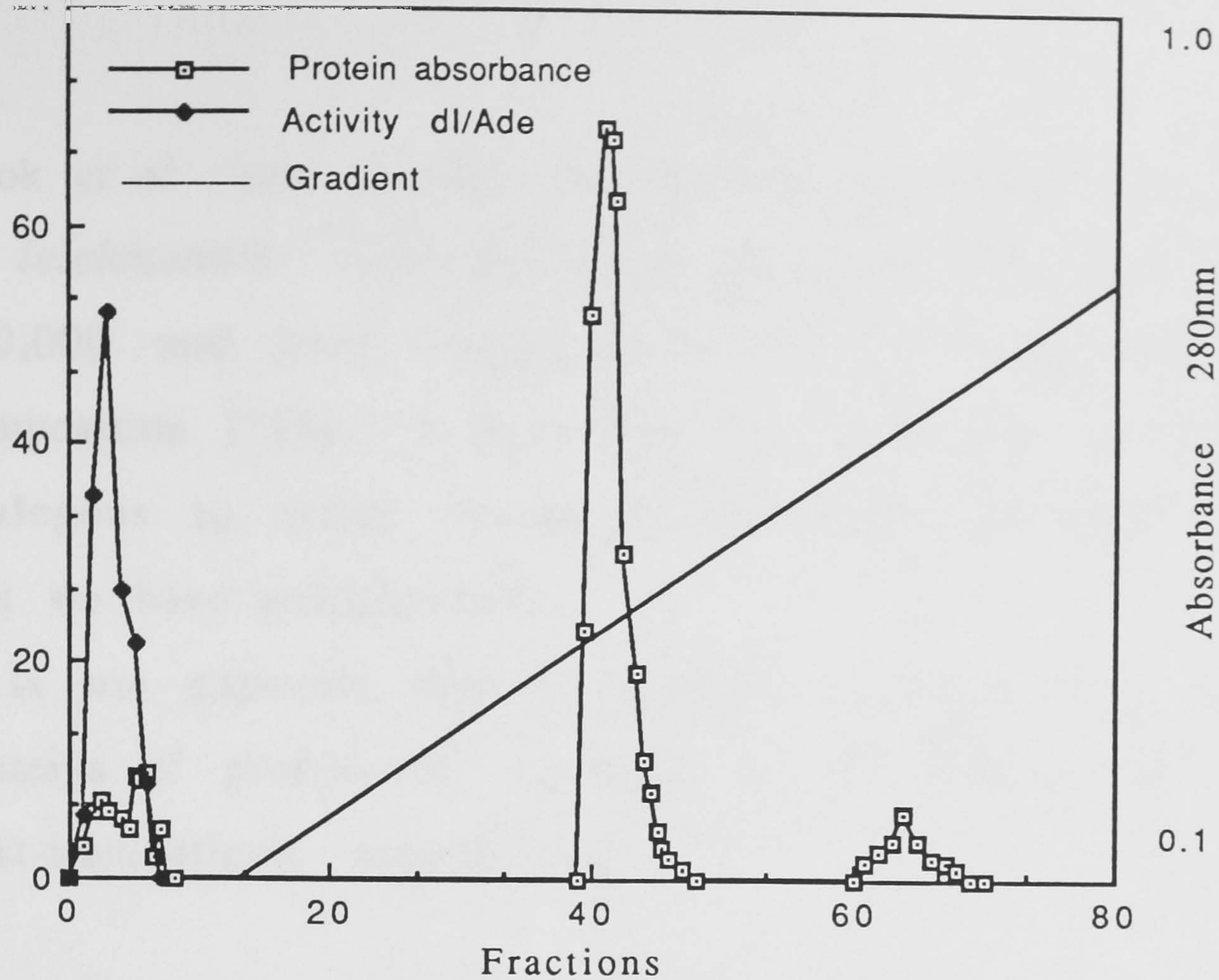


Figure 2.7[i]

HPLC on Hydroxyapatite-HPHT of  
N-deoxyribosyltransferase-I

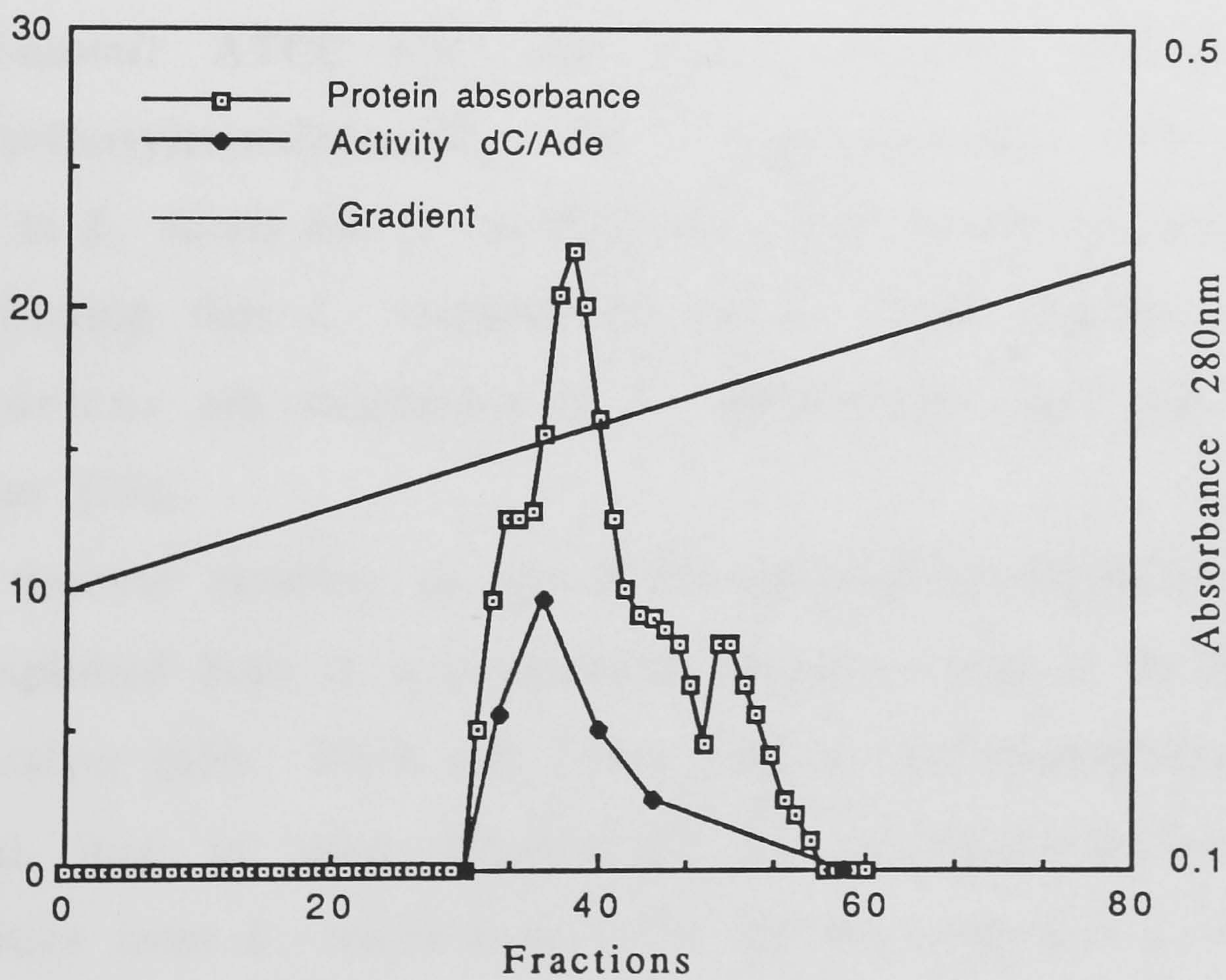


Figure 2.7[ii]

HPLC on Hydroxyapatite-HPHT of  
N-deoxyribosyltransferase-II

The N-deoxyribosyltransferase activities were eluted from their respective columns with a linear 0.01-0.4M gradient phosphate buffer, 0.1mM calcium chloride, 0.05% sodium azide, pH 6.8



Cook *et al* have reported the molecular weight of a recombinant *L. leichmannii* nucleoside deoxyribosyltransferase enzyme to be 110,000 and have shown it to have a hexameric subunit composition [115]. It is unclear as to whether this enzyme is analogous to either of the N-deoxyribosyltransferase enzymes that we have purified here.

It is not expected that this protein is glycosylated since the proteins of prokaryotic organisms do not undergo this type of post-translational modification.

The N-deoxyribosyltransferase-II enzymes in both *L. leichmannii* (Figure 2.8) and *L. helveticus* [81, 105], are more stable to heating than the corresponding N-deoxyribosyltransferase-I enzymes. An extremely stable enzyme is reported to be present in *L. leichmannii* ATCC 4797 [85] and is probably identical to N-deoxyribosyltransferase-II. It is suggested that two enzymes exist in *L. lactis* and *L. delbrueckii*. This prediction is based on the finding that *L. leichmannii* and *L. lactis* together with *L. bulgaricus* are subspecies of *L. delbrueckii*, and not separate species [75].

The thermal stability of the N-deoxyribosyltransferase-II could be exploited both in a purification procedure and in its role as a biocatalyst [85]. Beck and Levin used a heat denaturation as the initial step in their purification of N-deoxyribosyltransferase activities from *L. leichmannii* [79]. By this method it is likely that they destroyed quite a large fraction of the heat-labile purine-specific enzyme, decreasing the chances of its detection under suitable assay conditions. Its value as a purification step in this instance would arise after the separation of the two enzymes.

The pH curves for N-deoxyribosyltransferase-I and N-deoxyribosyltransferase-II are very similar, suggesting that the same type of amino acid groups might participate in binding and catalysis at the active site.

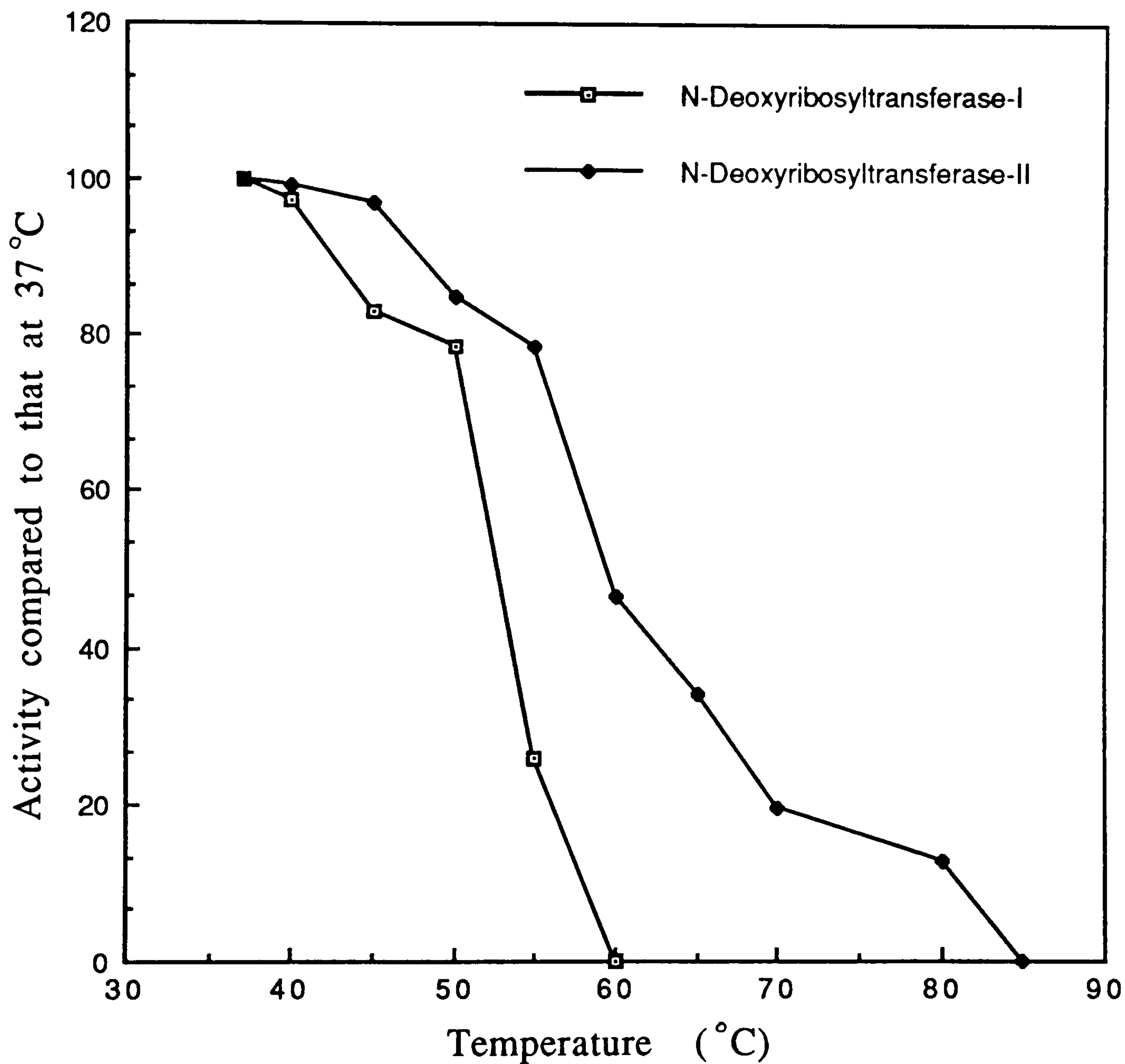


Figure 2.8 Thermal inactivation profiles of N-Deoxyribosyltransferase-I and II. Conditions as described in the text



The Michaelis constants we determined of  $4\mu\text{M}$  and  $10\mu\text{M}$  in the transfer reaction from 2'-deoxyinosine to adenine for N-deoxyribosyltransferase-I and -II respectively, were surprisingly low. By contrast, a value of  $41\mu\text{M}$  was obtained for N-deoxyribosyltransferase-I from *L. helveticus* [83]. However, direct comparison is difficult since the  $K_m$  values for 2'-deoxyinosine vary with the concentration of adenine [79, 110] and some deoxyribonucleosides [79]. The apparent  $K_m$ s of these reactions were determined using a xanthine oxidase coupled enzyme assay system. This procedure, however, has some practical disadvantages compared with the standard HPLC method. Care must be taken to ensure that the xanthine oxidase does not limit the rate of conversion of hypoxanthine to uric acid and, since adenine is a substrate of the xanthine oxidase, the conditions must be controlled so as to minimize this side reaction and corrections made for the conversion of adenine to 2',8-dihydroxyadenine. Detailed studies are required to elucidate these effects.

The majority of the assay systems for measuring N-deoxyribosyltransferase activities have concentrated on spectrophotometric methods [82] using coupled enzyme systems or colour reactions [77, 79] which measure the concentration of free 2'-deoxyribose in solution. Although convenient for reconnaissance experiments, the diphenylamine colour reaction [109] was not able to be usefully employed throughout the purification procedure. The reaction relies upon the acid stability of the pyrimidine nucleosides compared to the acid lability of the purine nucleoside which, when treated with diphenylamine

reagent, liberates the 2'-deoxyribose group. Thus, it is unable to monitor the purine:purine transfer reaction. The blue colour which is generated by the reaction of the reagent with the 2'-deoxyribose, is conveniently measured spectrophotometrically. The high performance liquid chromatography method was developed with C<sub>8</sub> and C<sub>18</sub> reverse phase columns. The bases and nucleosides were generally eluted isocratically using a mobile phase of water and methanol but for some compounds it was necessary to develop a gradient program using a combination of different solvents.

Although this purification scheme was successful in separating two distinct nucleoside deoxyribosyltransferase activities from *L. leichmannii*, there are several ways in which the initial purification steps could be modified to secure an improved yield. The microaerophilic growth of *L. leichmannii*, proved to be easy, and relatively large amounts of biomass were obtained with small to medium scale growth. However, the deoxyribosyltransferase enzymes constitute less than 1% of the total intracellular protein. One way of boosting the levels of enzyme activity would be to take advantage of the vitamin B<sub>12</sub> salvage pathway and grow the *L. leichmannii*, under vitamin B<sub>12</sub>- or deoxyribonucleoside-starvation conditions [92, 95]. The ratios of activities towards reactions (a), (b) and (c) varies from strain to strain however, and it is unclear which of these activities would be increased [75].

Methods used to break the cells including sonication and the french pressure cell lack the ability to cope with large volumes of



material, and samples have to be treated batchwise, and quite often the process has to be repeated. Disruption of the cell walls using the combination of lysozyme and mutanolysin proved to be very effective and reduced the required number of passages of the cells through the french pressure apparatus. A combination of sonication and french press methods have been tried in subsequent preparations of crude extracts from *L. leichmannii*. This has resulted in extracts with specific activities of 0.155units  $\text{mg}^{-1}$  and 0.866units  $\text{mg}^{-1}$  for the purine:purine and pyrimidine:purine activities respectively for the new method, compared with 0.086units  $\text{mg}^{-1}$  and 0.21units  $\text{mg}^{-1}$  for the purine:purine and pyrimidine:purine activities respectively for the initial method of disruption.

Speculations on the existence of a pyrimidine-specific enzyme have been made and Cardinaud and Holguin have managed to separate a discrete pyrimidine:pyrimidine activity from an extract of *L. helveticus* using an affinity column [116]. A distinctive third band of activity was observed to be present in one purification of the N-deoxyribosyltransferase enzymes from *L. leichmannii*, eluting from the FPLC MONO-Q high resolution anion exchange column immediately after the N-deoxyribosyltransferase-II enzyme. This activity was not seen in subsequent preparations.

## CHAPTER 3

### SUBSTRATE SPECIFICITY

#### GENERAL BACKGROUND

The marked specificity towards particular types of ligands is one of the characteristic properties of some proteins which enables them to distinguish between closely related molecules. This ability allows antibodies to differentiate between self and non-self, while an enzyme is able to select only those ligands which fulfil the structural criteria defined by the active site.

The nature of the forces involved in the binding and specificity of these ligands are well understood. Binding is thought to be governed by hydrophobic interactions. In the same way that hydrogen bonding plays a vital part in maintaining the conformation and structure of proteins and nucleic acids, the specificity of an individual enzyme for its substrate is provided by *inter alia* hydrogen bonding interactions and salt bridge formation [117]. Attempts have been made to quantify the relative contribution that each of these forces makes [118].

One way to chart the active site of an enzyme is to establish the three dimensional layout of the binding pocket. This can be achieved by examination of the specificity of the individual enzyme. A series of analogues can be constructed by making systematic modifications to a natural substrate. These are tested to see if they are suitable substrates, giving information on the ligand positions essential to binding and specificity. With these



data a model can be constructed to illustrate how these ligands might interact with specific amino acids in the active site. Even when an analogue fails to act as a substrate, it can still yield valuable information. In these cases it has to be established whether the non-substrate actually binds to the protein at the active site and inhibits the approach or binding of the natural substrate; whether it fails to contact the binding pocket due to steric constraints on the part of the protein or the ligand, or, having reached the active site, is unable to secure a stable binding position.

In the N-deoxyribosyltransferase reaction, substrate specificity studies must explore the enzymes' selectivity for both sugar and heterocyclic base. Substrate-activity studies with nucleoside phosphorylases from mammalian and bacterial sources have aided the construction of models of active site geometries in several of these enzymes [102]. In certain cases, it has enabled predictions to be made regarding the involvement of selected amino acids in the binding and specificity of substrates. Substrate specificity for N-deoxyribosyltransferase enzymes has been examined in a number of species, but as yet only detailed studies have been carried out on the N-deoxyribosyltransferase-II enzyme from *L. helveticus* [103].

## EXPERIMENTAL METHODS

Compounds were commercially available except where indicated in the tables.

### Deoxyribosyl transfer to Base Analogues:

Deoxyribosyltransfer from 2'-deoxyinosine (N-deoxyribosyltransferase-I), and 2'-deoxycytidine (N-deoxyribosyltransferase-II) to various base analogues was measured by the monitoring the production of new 2'-deoxynucleoside with HPLC. The reaction mixture contained the same concentrations as the standard mixture with the exception of the donor concentration which was raised to 0.3 $\mu$ mol, to give a final donor: acceptor ratio of 3:1. The reaction was initiated by the addition of approximately 10mU enzyme. After 3h the mixture was eluted with a 15 minute linear gradient of methanol 7-35% on a Technopak HPLC column (7.8 x 100mm) and the production of the new 2'-deoxynucleoside recorded. Products were identified by their elution times using authentic nucleosides as standards. Certain reactions were followed by base release from the substrate nucleoside or by the disappearance of the acceptor substrate. This allowed reactions to be followed where either the acceptor base demonstrated a weak absorbance at 254nm or where the nucleoside product product had a strong affinity for the C<sub>18</sub> material and elution from the HPLC column was as a broad, diffuse band of product. Novel nucleoside products were identified by ultraviolet and nmr spectroscopy.



### Deoxyribosyl transfer from Sugar Analogues:

In the case of deoxyribosyl transfer from nucleosides with modified pentosyl groups to natural base acceptors, the reaction mixture contained 25 $\mu$ mol Pipes, pH 6.5, 0.1 $\mu$ mol donor and 0.05 $\mu$ mol acceptor in a total volume of 0.5ml.

### Hydrolysis of ribose group from 7-Deazaadenosine.

7-Deazaadenosine (50mg) was refluxed in 100ml of 10M HCl for 48h. The hydrolysis reaction was followed by monitoring the release of the base by HPLC. The base and nucleoside were readily separated on the gradient system described above.

### Identification of Nucleoside Products of 8-Bromoadenine and Xanthine. (N-deoxyribosyltransferase-II)

Two products were isolated from each of the separate reactions between the donor nucleoside 2'-deoxycytidine and the purine bases xanthine and 8-bromoadenine. The initial transfer reactions were carried out as described above. Fractions were collected of two discrete product peaks from each reaction as they eluted from the HPLC column. Those fractions from the 2'-deoxycytidine/xanthine transfer were designated X<sub>1</sub> and X<sub>2</sub> and those from the 2'-deoxycytidine/8-bromoadenine reaction A<sub>1</sub> and A<sub>2</sub>. These fractions were examined spectrophotometrically (Tables 3.9 & 3.10) and tested as competent donors of the glycosyl group. For the reverse transfer each reaction mixture contained 0.2 $\mu$ mol acceptor base, 50 $\mu$ mol of Pipes buffer, pH 6.5, in a volume of 0.5ml of the eluant sample. The base for X<sub>1</sub> and X<sub>2</sub> was adenine and that for A<sub>1</sub> and A<sub>2</sub> cytosine. The reaction was initiated by the addition of approximately 4mU of enzyme.

The N-deoxyribosyltransferase reaction was assayed by measuring the formation of the new nucleoside at 40°C using HPLC.

## RESULTS

All purine compounds were examined for their ability to act as substrates in the transfer reaction with both N-deoxyribosyltransferase-I and -II enzymes. Pyrimidine compounds were tested as potential acceptors with N-deoxyribosyltransferase-II, and nucleosides with modified sugar groups were tested with both enzymes.

### Purine Analogues

#### *Effect of Modification on Positions 1 and 3*

This experiment demonstrated that with one exception, the removal or replacement of the N-1 or N-3 with a carbon, did not affect the ability of the purine base to act as an acceptor (Table 3.1). Thus, compounds such as 1-deazapurine, benzimidazole, and benzotriazole were substrates. In contrast, the 3-deazaadenine was found not to be an acceptor. It was also observed that when the pyrimidine ring structure is incomplete as in the case of ethyl 4-aminoimidazole-5-carboxylate and its 4-hydroxy counterpart, the base was still able to participate in the transfer reaction (Table 3.2). Complete removal of the pyrimidine ring resulted in a much reduced activity as seen with 1,2,4-triazole. Imidazole is not a substrate.



### Effect of Modification on Position 6

A number of varied 6-substituted purine analogues were examined as substrates (Table 3.3, 3.4) Without exception these were found to act as competent acceptors for the glycosyl group in both the purine:purine and pyrimidine:purine reactions. Even compounds with bulky substituents such as 6-furfurylaminopurine and N<sub>6</sub>-ethenoadenine, and those with hydrophobic side chains were observed to be good substrates. It was observed that on increasing the size of the substituent on the dialkylsubstituted purines, that the reaction velocity decreased.

The 2,6-disubstituted purines, xanthine and 2,6-diaminopurine were also good acceptors but their respective velocities compared to the natural mono-substituted purines hypoxanthine and adenine, were considerably lower. The transfer reaction of xanthine with both purine and pyrimidine donors generated two products X<sub>1</sub> and X<sub>2</sub>.

### Effect of Modification on Position 7

Purine bases substituted in the 7-position were not acceptors in the reactions catalysed by either N-deoxyribosyltransferase-I or -II (Table 3.5). The compounds 7-deazaadenine and indole, where the N-7 was replaced by a carbon, were also non-substrates. The 7-deaza-8-aza compounds 4-aminopyrazolo[3,4-d]pyrimidine and its 4-hydroxy counterpart however, were substrates for both enzymes.

### Effect of Modification on Position 8

All but one of the 8-modified analogues tested were substrates (Table 3.6). While 8-aza compounds were very competent

acceptors, the 8-substituted analogue 8-bromoadenine had a much a decreased reaction velocity compared to the parent adenine. 8-bromoadenine, like xanthine, also gave rise to two discrete products, A<sub>1</sub> and A<sub>2</sub>. A decreased velocity for 8-bromoadenine, and the observation that uric acid was not a substrate, indicates that 8-substituted analogues are unable to reach the active site readily because of steric hinderance.

### Pyrimidines Analogues

#### Effect of Modification on Position 1

Substitution at the N-1, the primary site of glycosylation, was not tolerated as observed by the non-reaction of 1-methylthymine.

#### Effect of Modification on Position 5

A number of modifications were tolerated at the 5-position of uracil, from a number of small halogen groups to large, hydrophobic alkyl substituents (Table 3.7, 3.8). Alkyl substitution was however, tolerated only to a small degree indicating that there might be a binding pocket of defined size at, or near this position.

Replacing the carbon at the 5-position was found to abolish the acceptor activity. 5-azacytosine was found to be a weak inhibitor of the pyrimidine:purine transfer reaction.



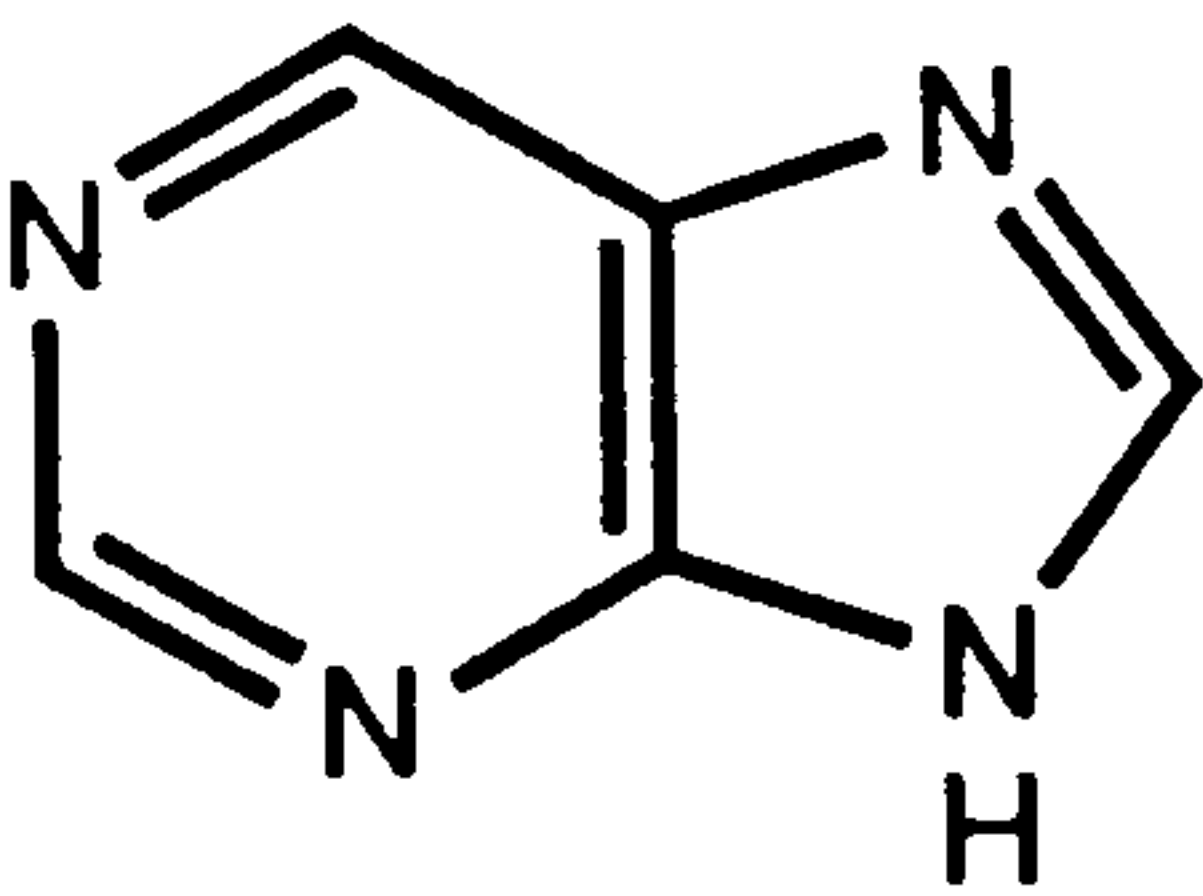
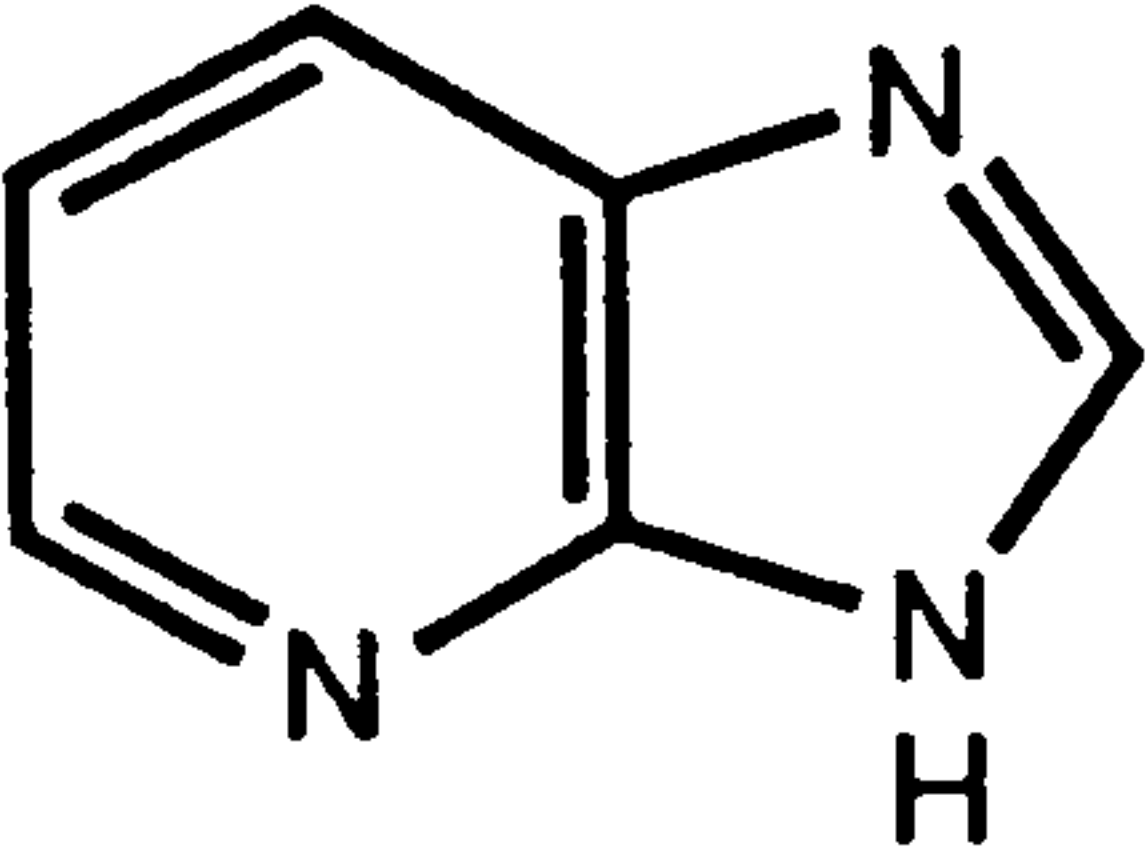
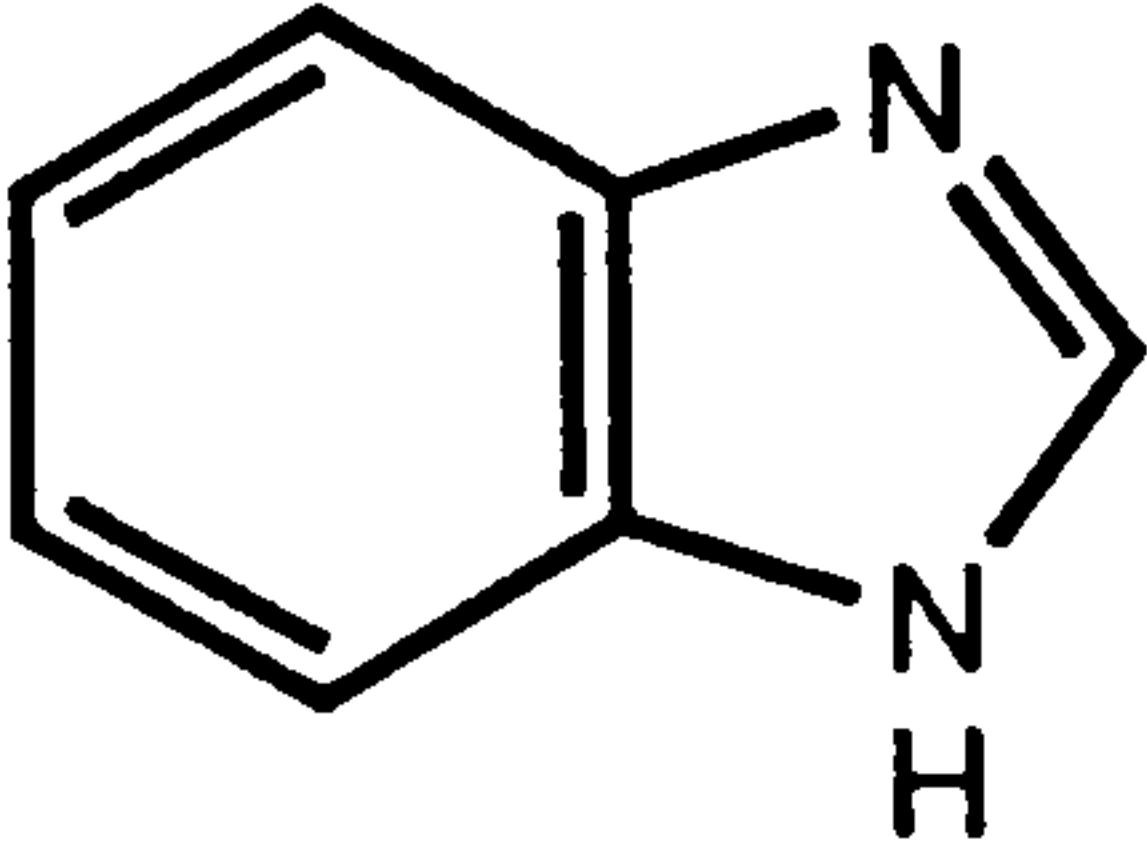
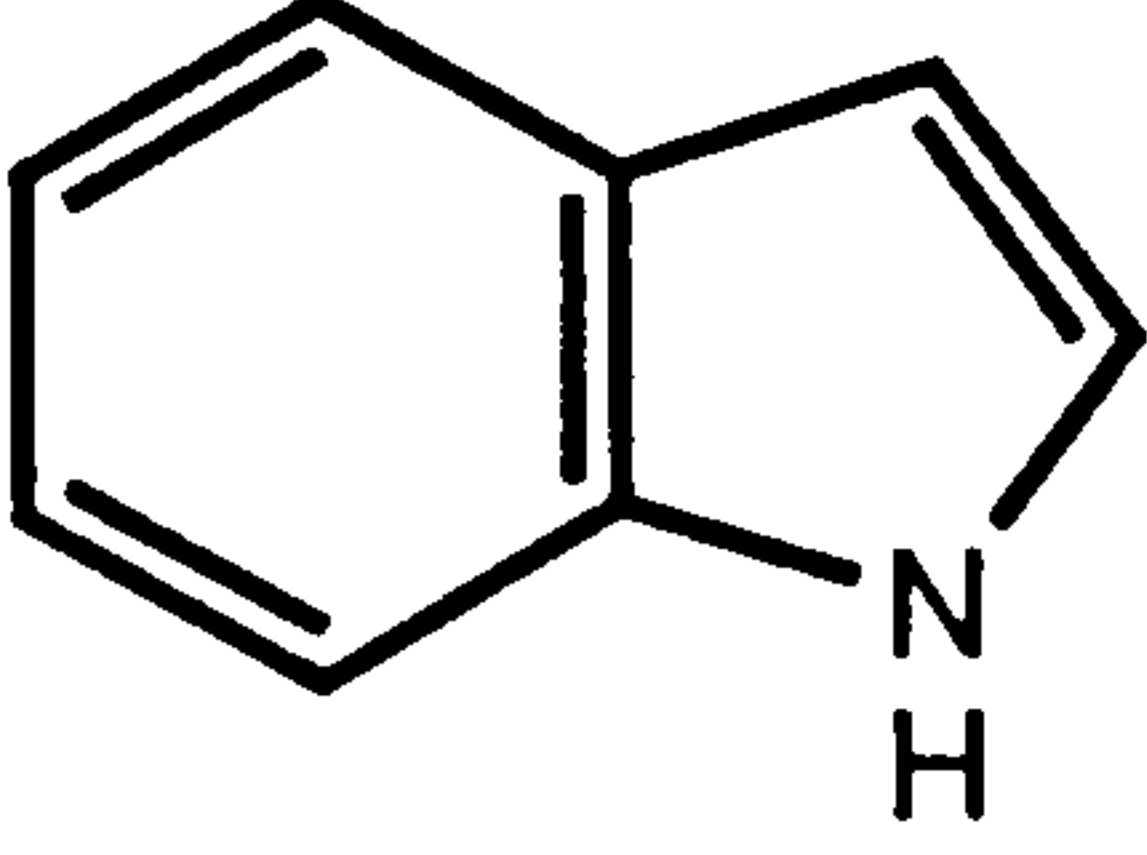
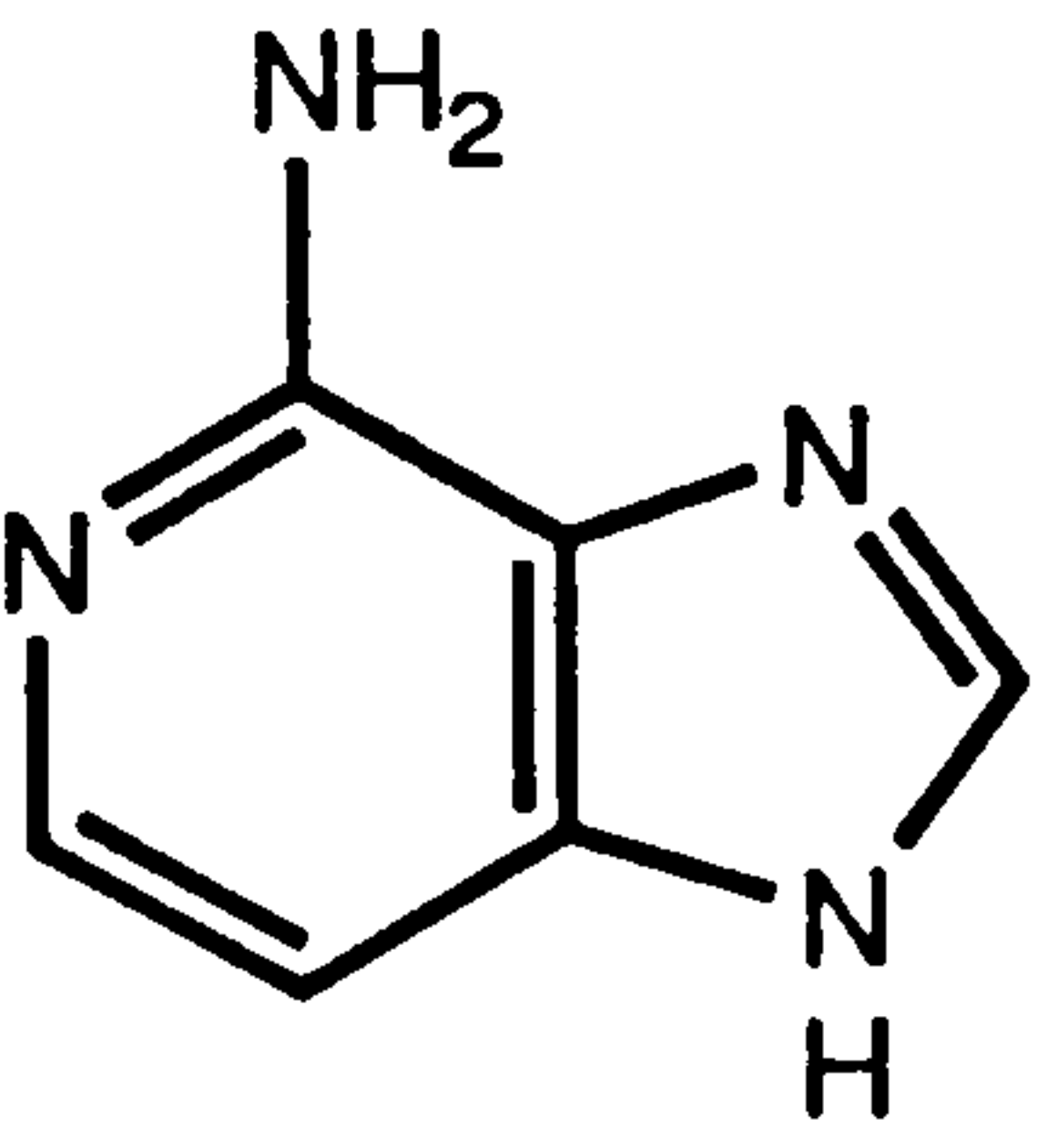
Effect of Modification on Positions 2 and 6

Three disubstituted cytosine analogues examined were found to be non-substrates, indicating that these positions are conserved for binding and catalysis. A series of 5-alkyl-2-thioluracils were also tested and found not to act as acceptors (Table 3.7).

In tables 3.1-3.7, % 3h-I and %3h-II denote the percentage of new N-deoxyribosyl nucleoside product formed in three hours in the transfer reactions employing purified N-deoxyribosyl-transferase-I and -II respectively.

Table 3.1

## Effect of Modification on Positions 1 and 3

| Compound       | Structure  | %3h-I | %3h-II |
|----------------|--|-------|--------|
| Purine         |    | 86.6  | 87.3   |
| 1-Deazapurine  |   | 90.7  | 86.8   |
| Benzimidazole  |  | 25.1  | 27.2   |
| Indole         |  | 0     | 0      |
| 3-Deazaadenine |  | 0     | 0      |

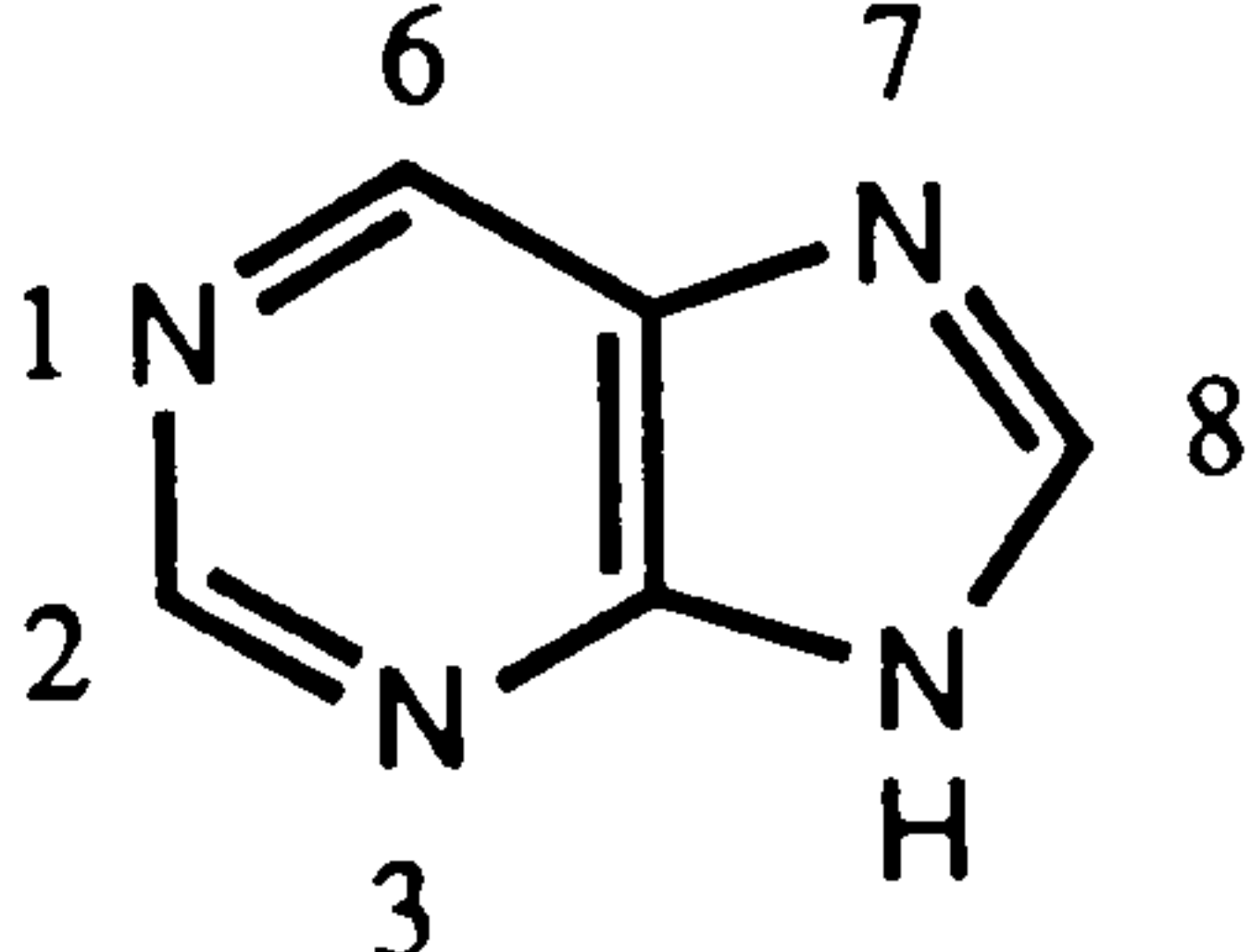
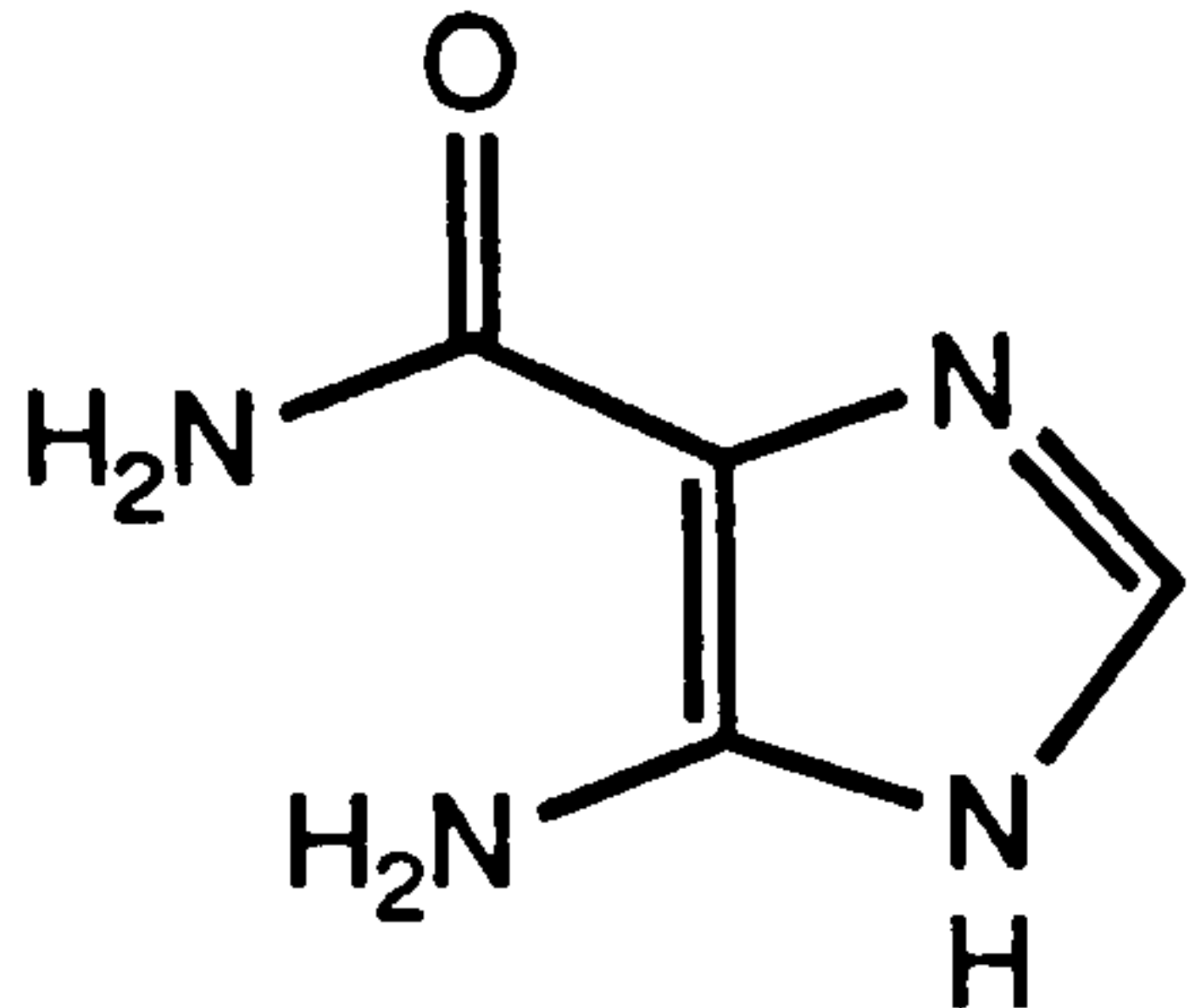
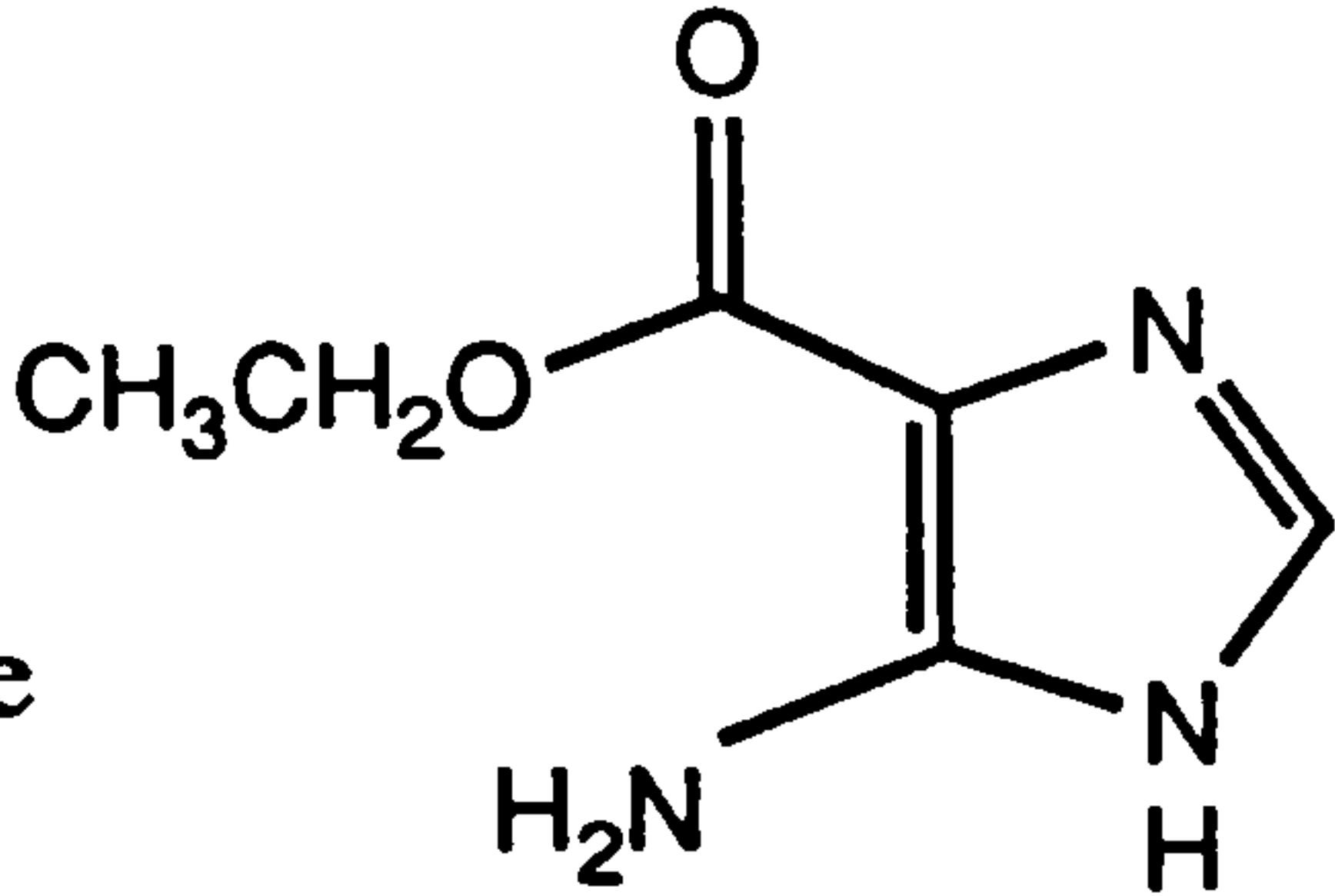
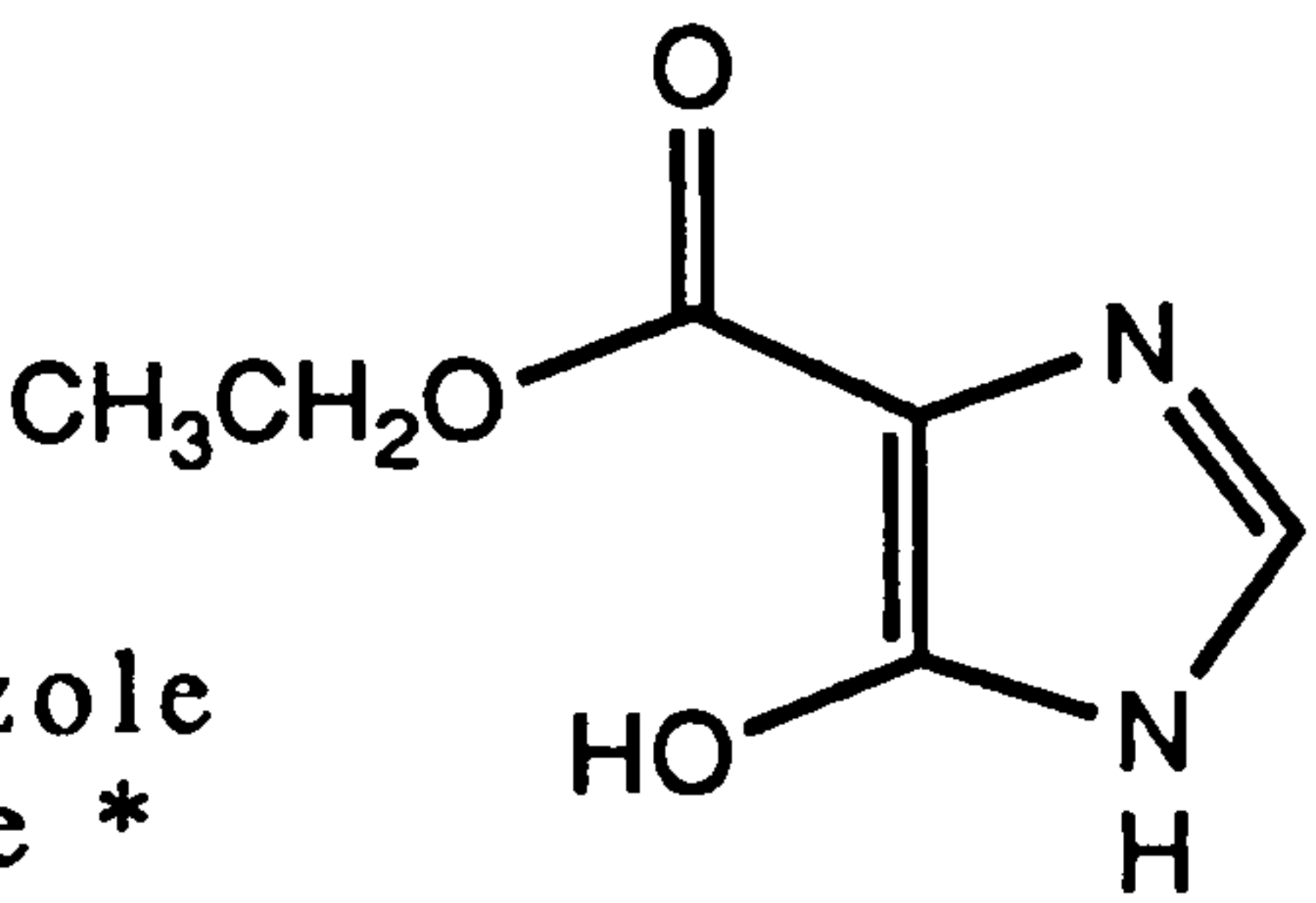
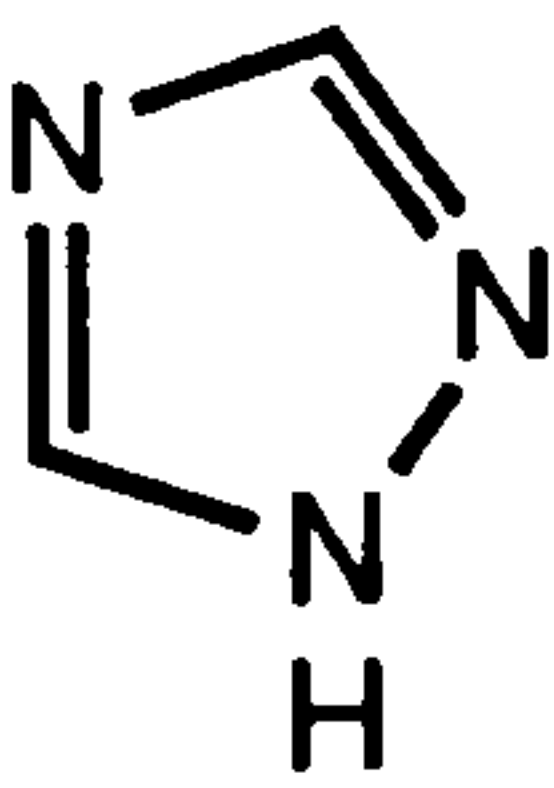
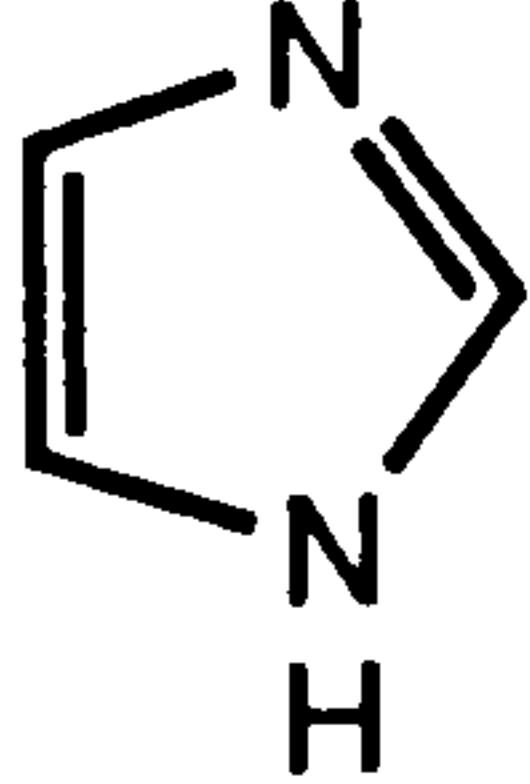




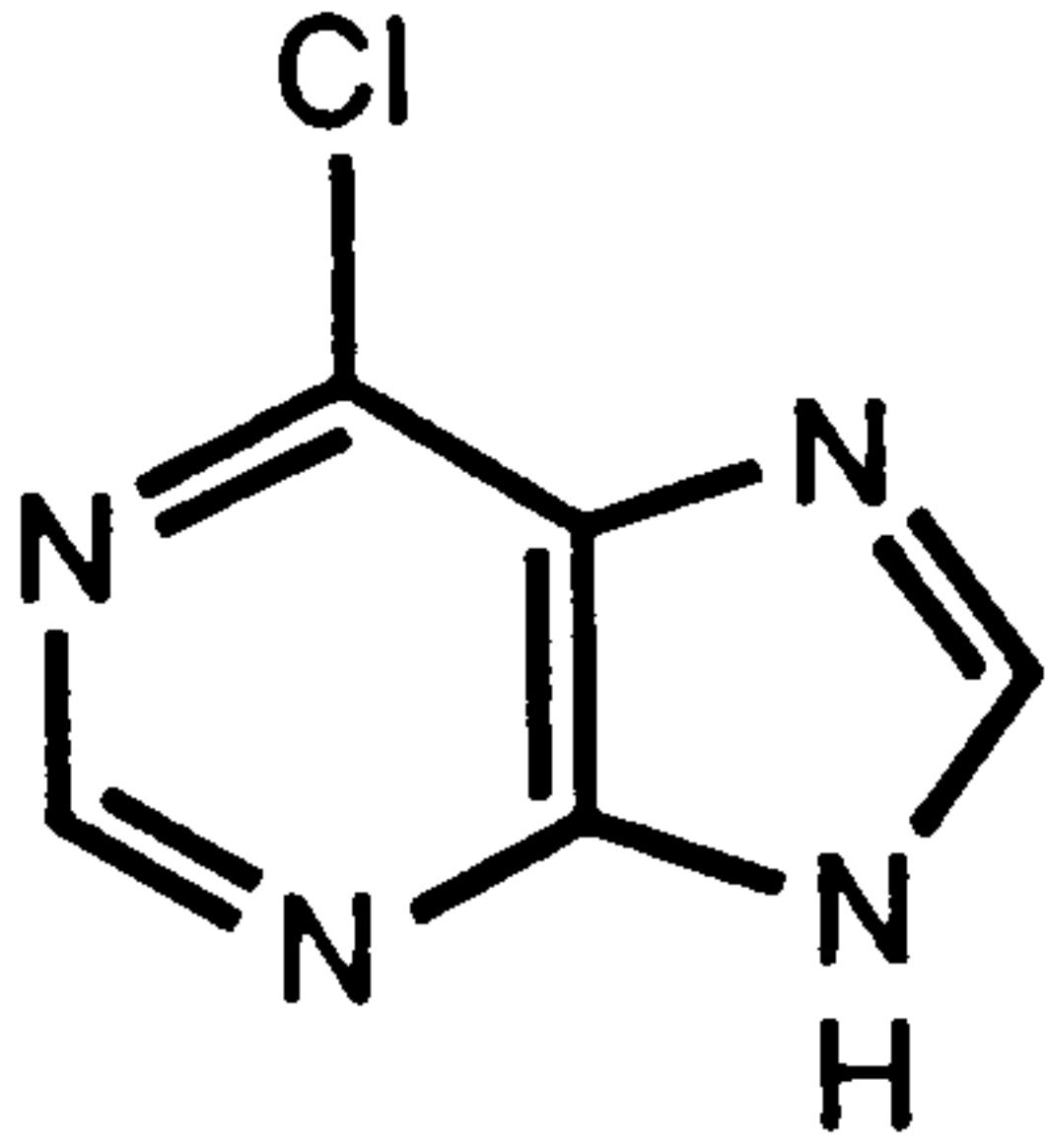
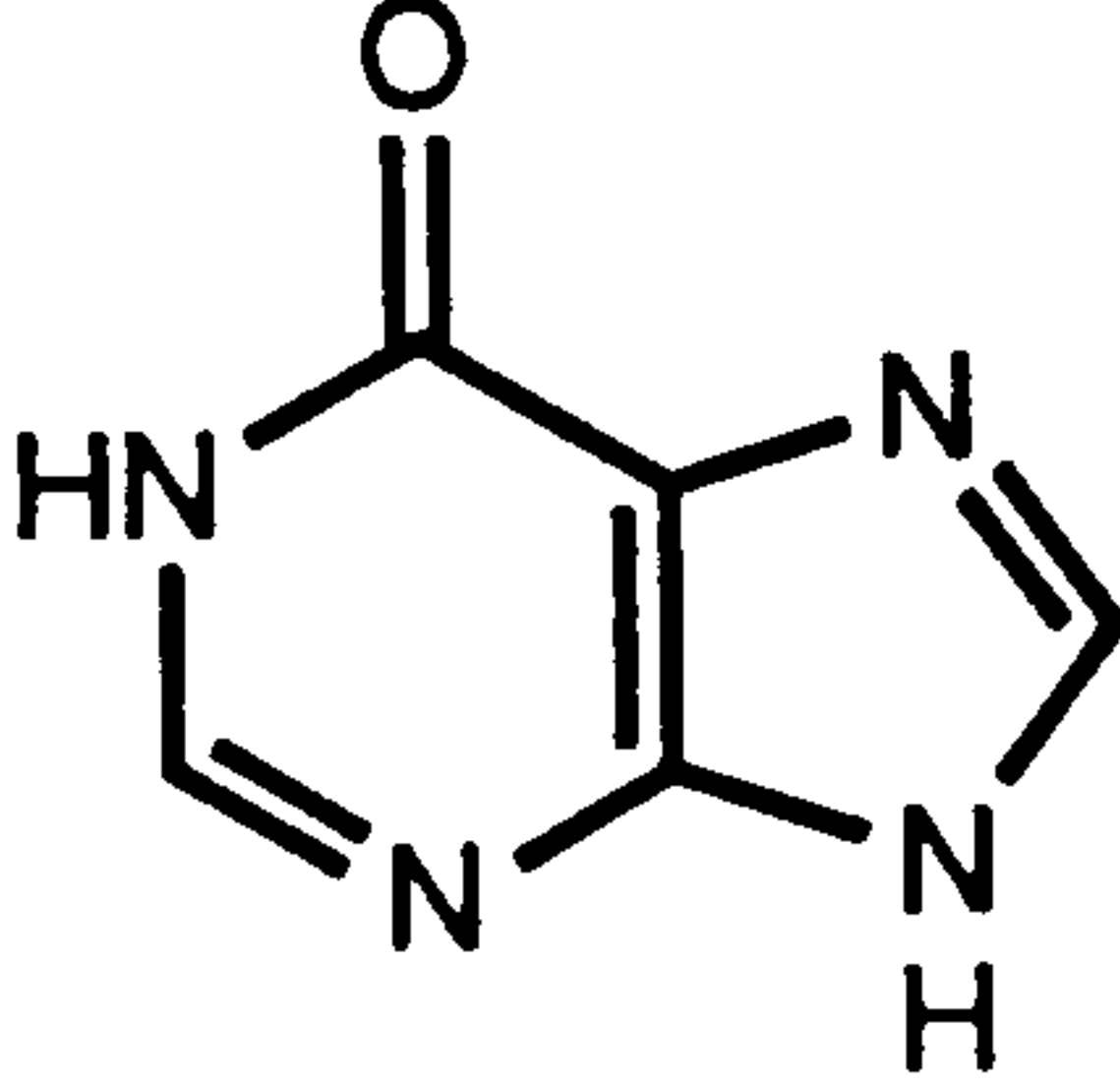
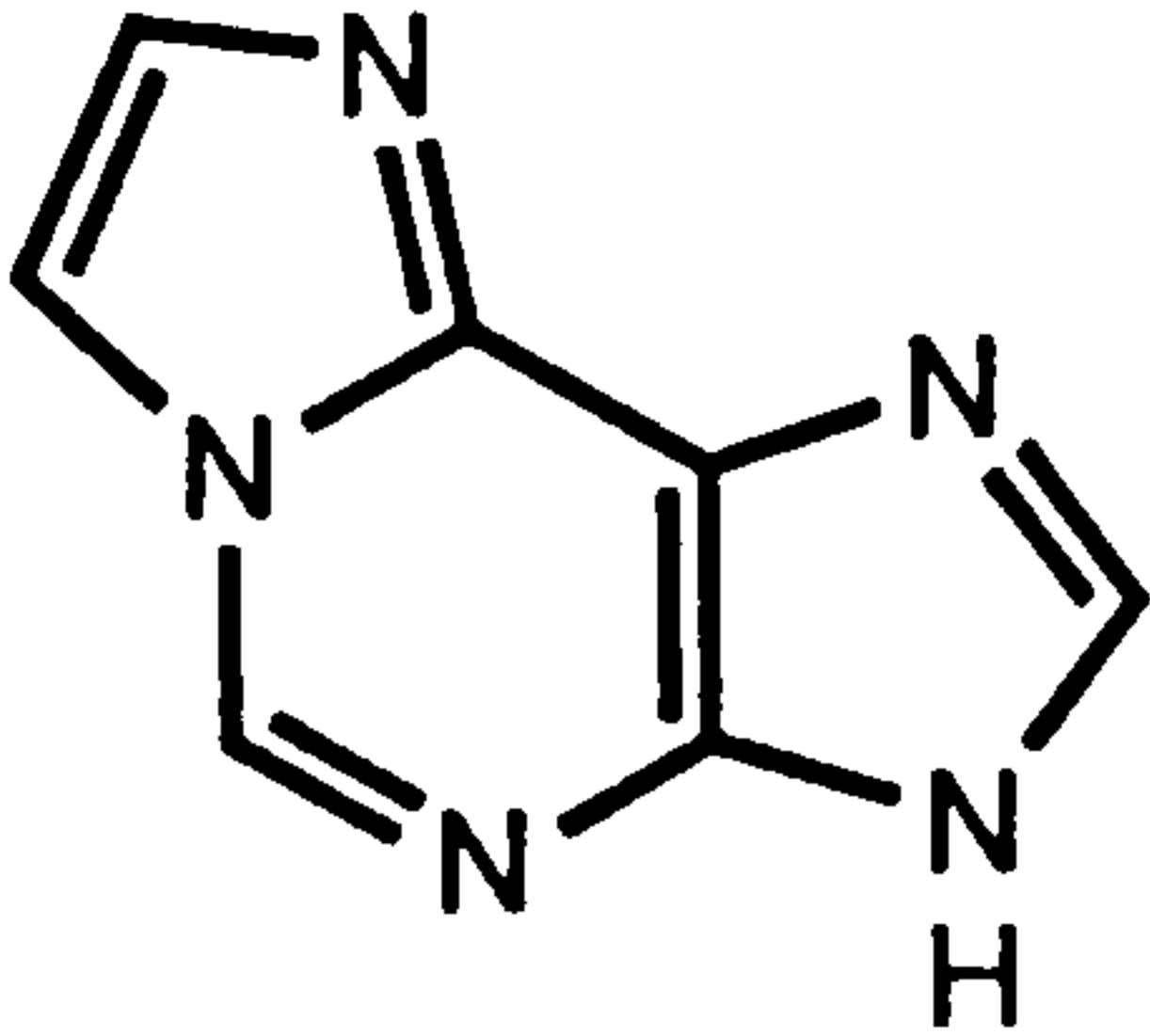
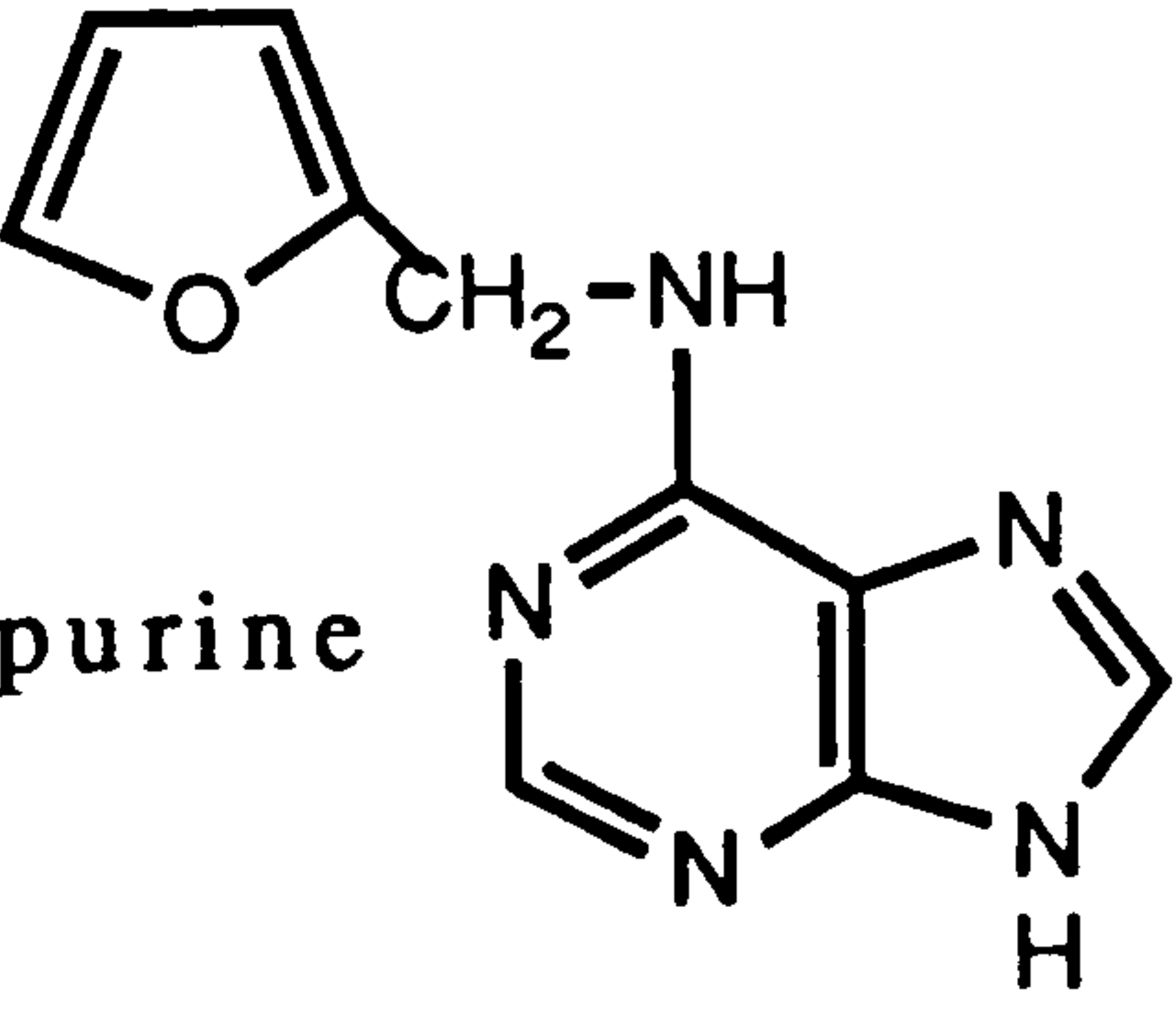
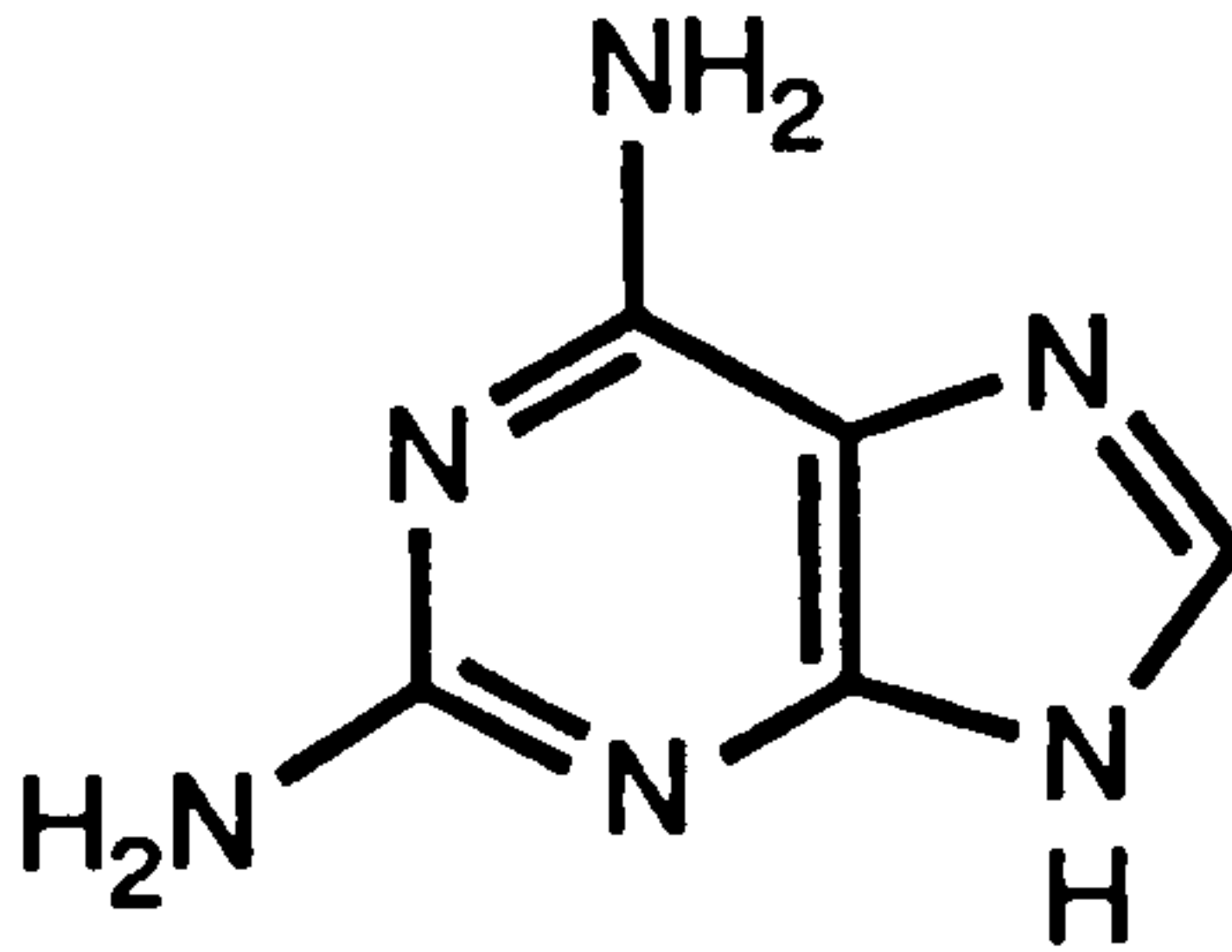
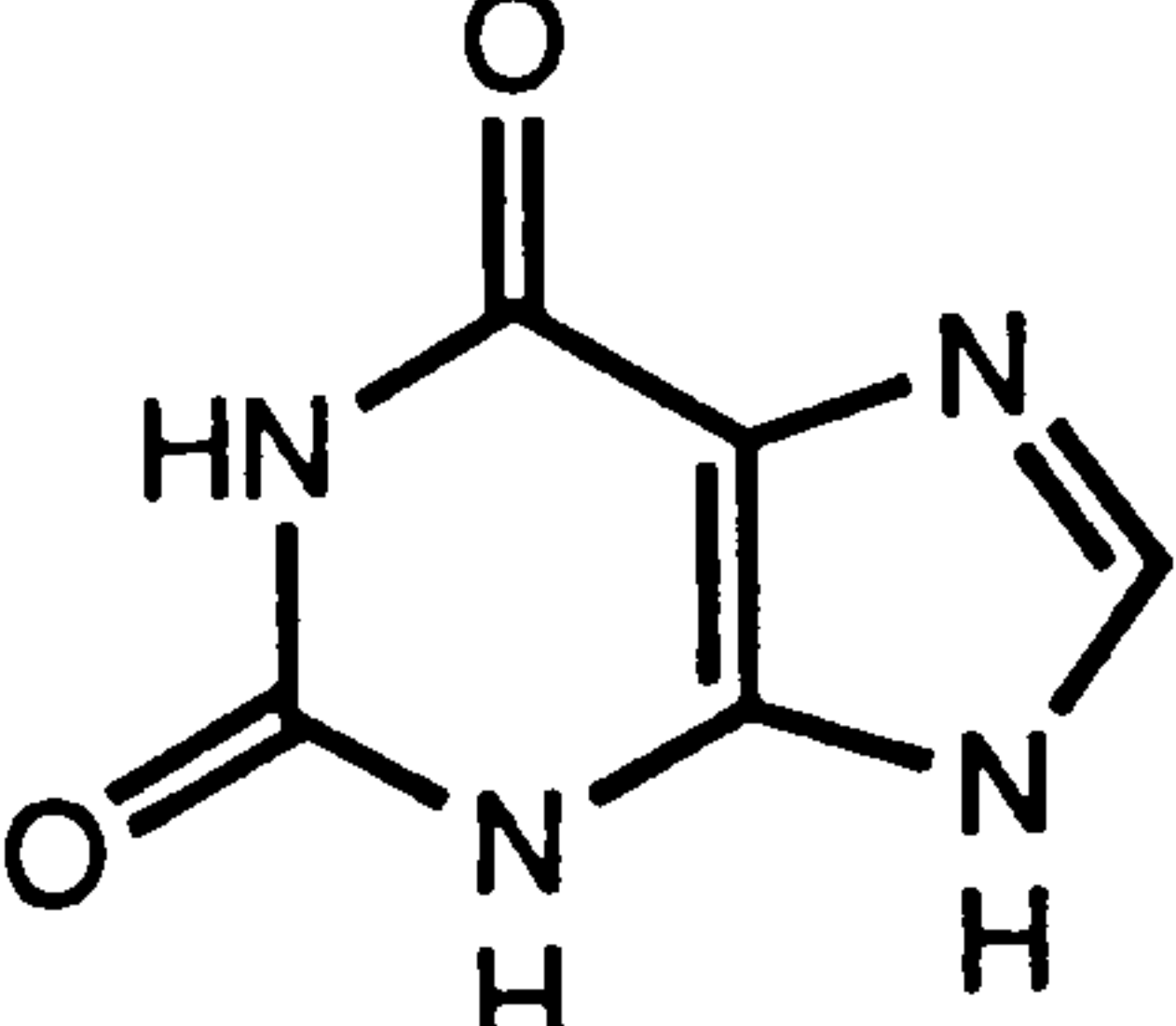
Table 3.2

## Effect of Modification on the Purine ring

| Compound   | Structure  | %3h-I | %3h-II |
|--|--|-------|--------|
| 4-aminoimidazole<br>-5-carboxamide               |    | 86.8  | 68.3   |
| Ethyl-4-<br>aminoimidazole<br>-5-carboxylate     |   | 24.7  | 64.5   |
| Ethyl-4-<br>hydroxyimidazole<br>-5-carboxylate * |  | 20.2  | 38.6   |
| 1,2,4-Triazole                                   |  | 17.9  | 22.4   |
| Imidazole  |  | 0     | 0      |

\* Gift of Dr. G. MacKenzie,  
Humberside College Further Education, Hull.

Table 3.3  
Effect of Modification on Position 6

| Compound                      | Structure  | %3h-I | %3h-II |
|-------------------------------|--|-------|--------|
| 6-Chloropurine                |    | 89.9  | 90.3   |
| Hypoxanthine                  |   | 85.8  | -      |
| N <sub>6</sub> -ethenoadenine |  | 32.1  | 38.9   |
| 6-furfurylamino-purine        |  | 37.5  | 24.7   |
| 2,6-Diaminopurine             |  | 48.2  | 41.8   |
| Xanthine                      |  | 55.5  | 41.3   |

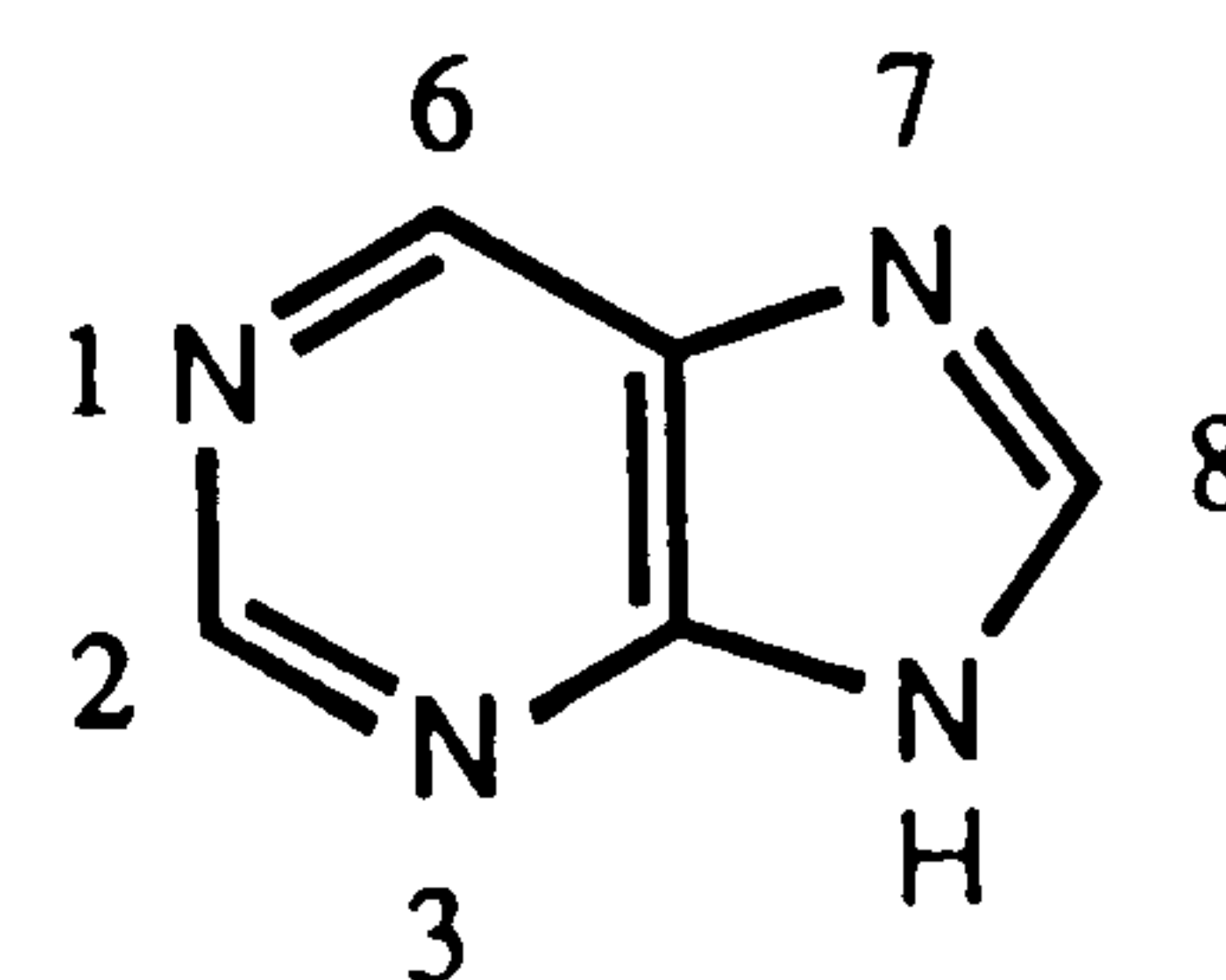
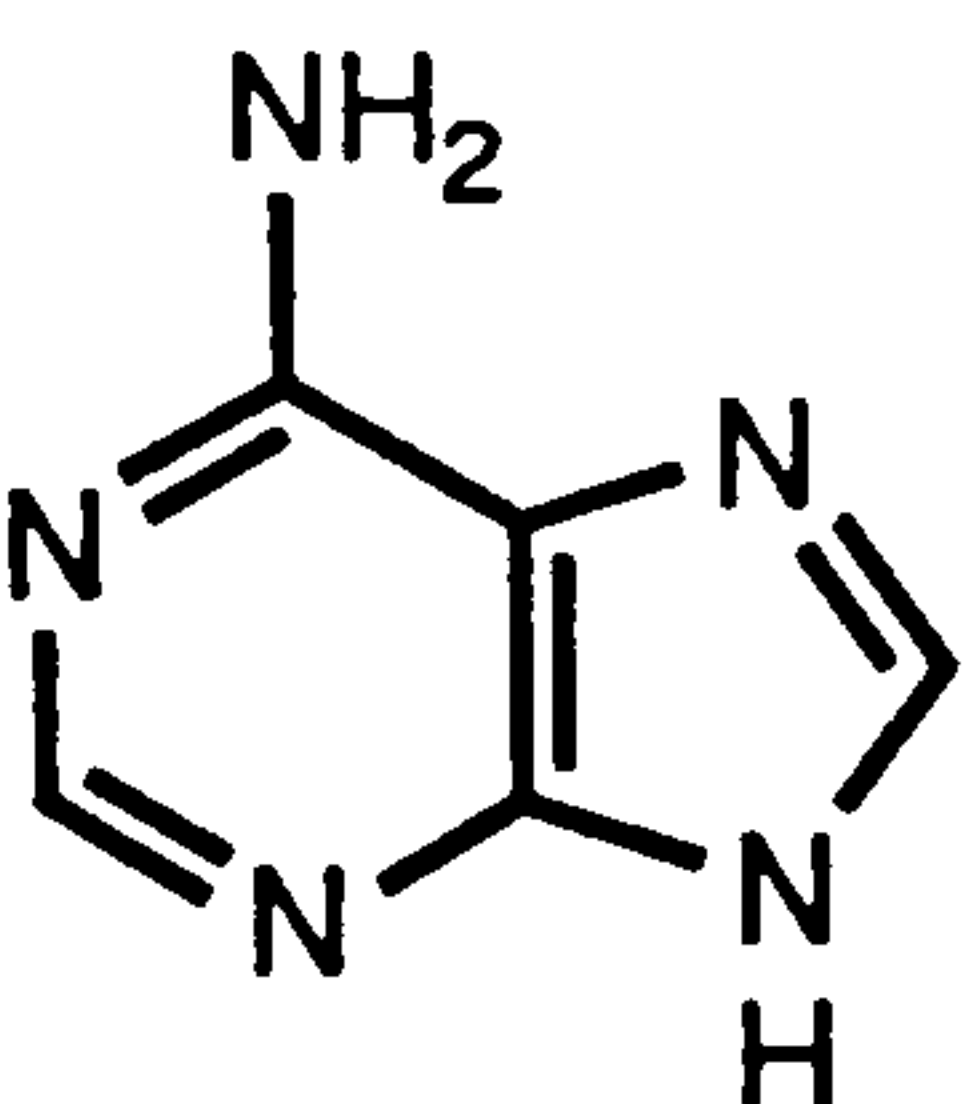
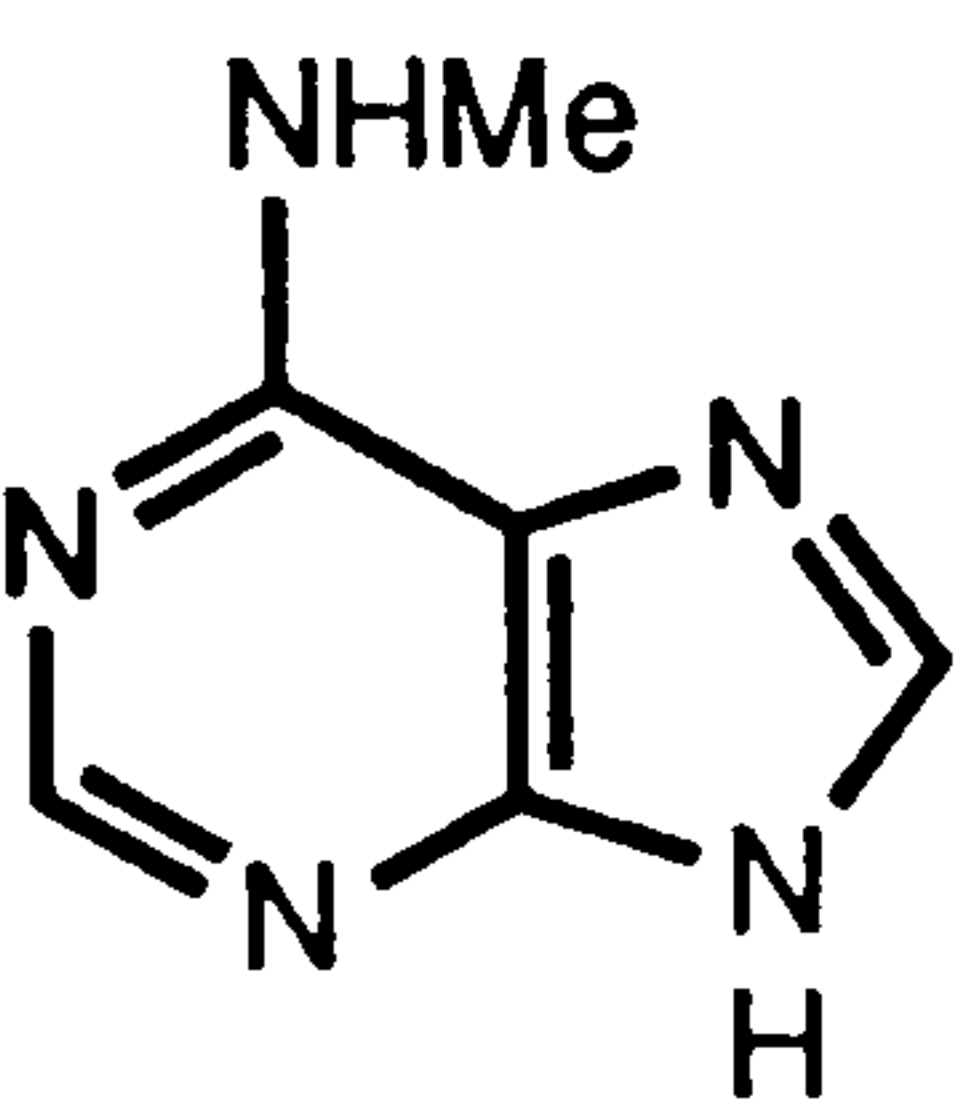
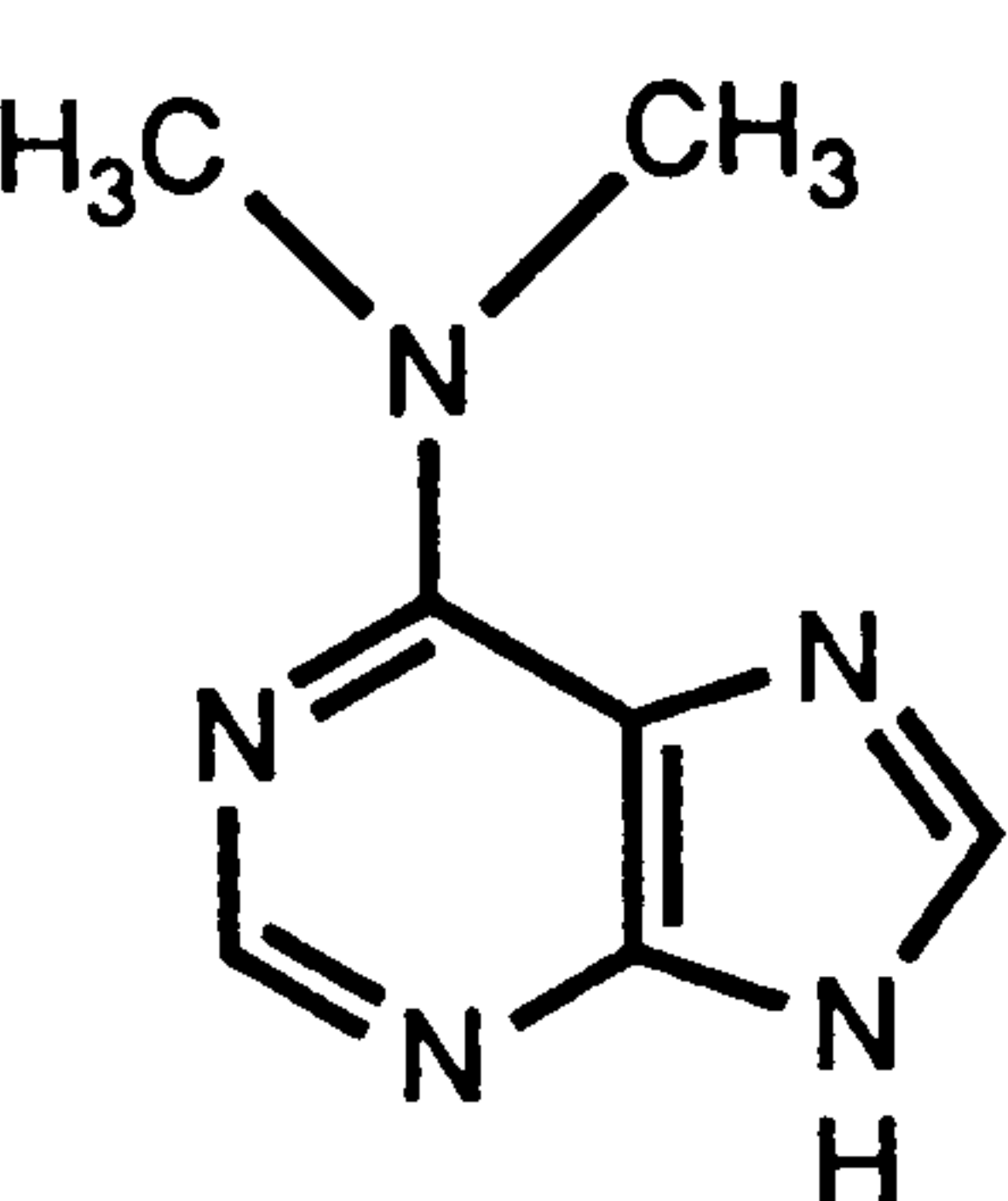
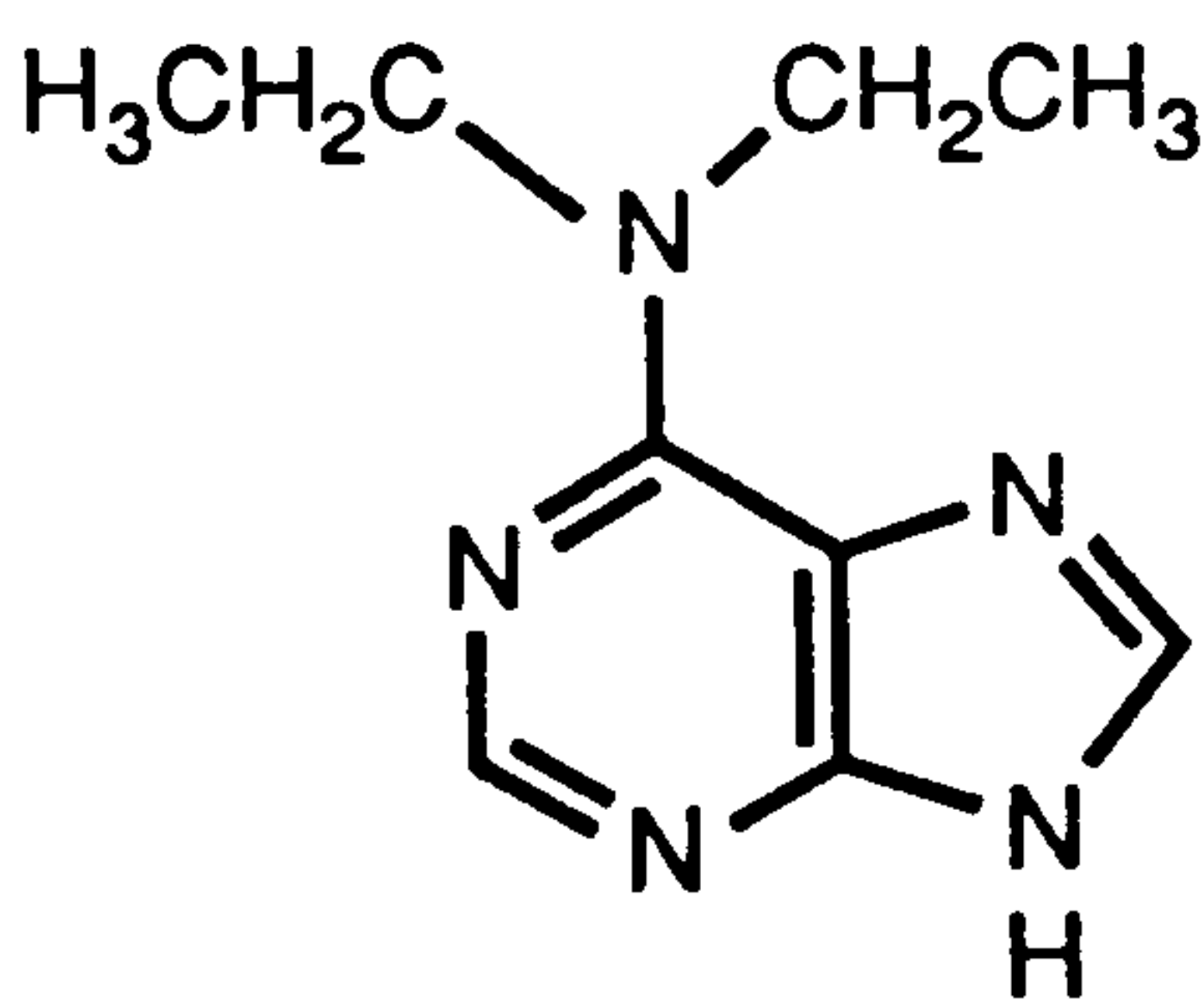
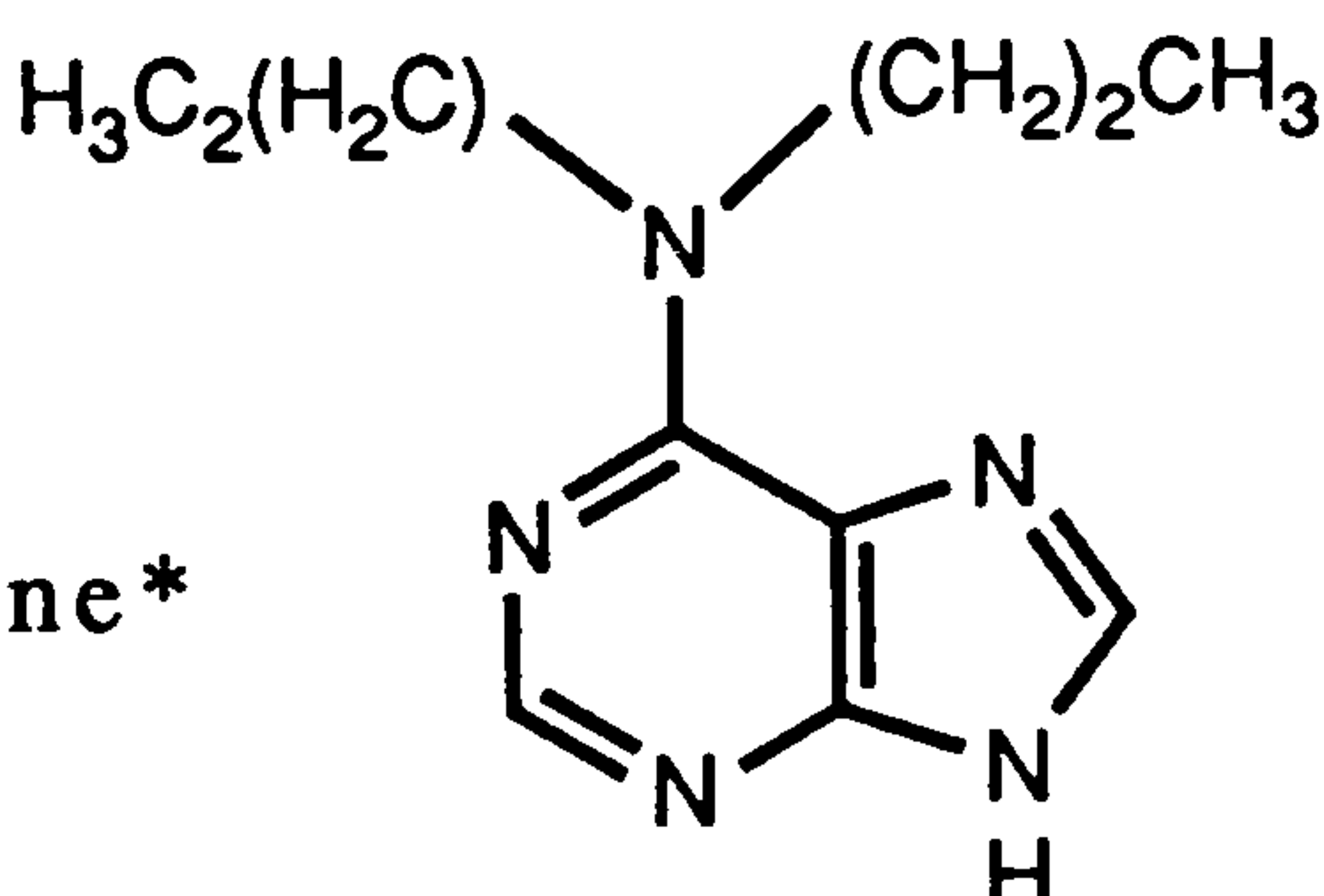
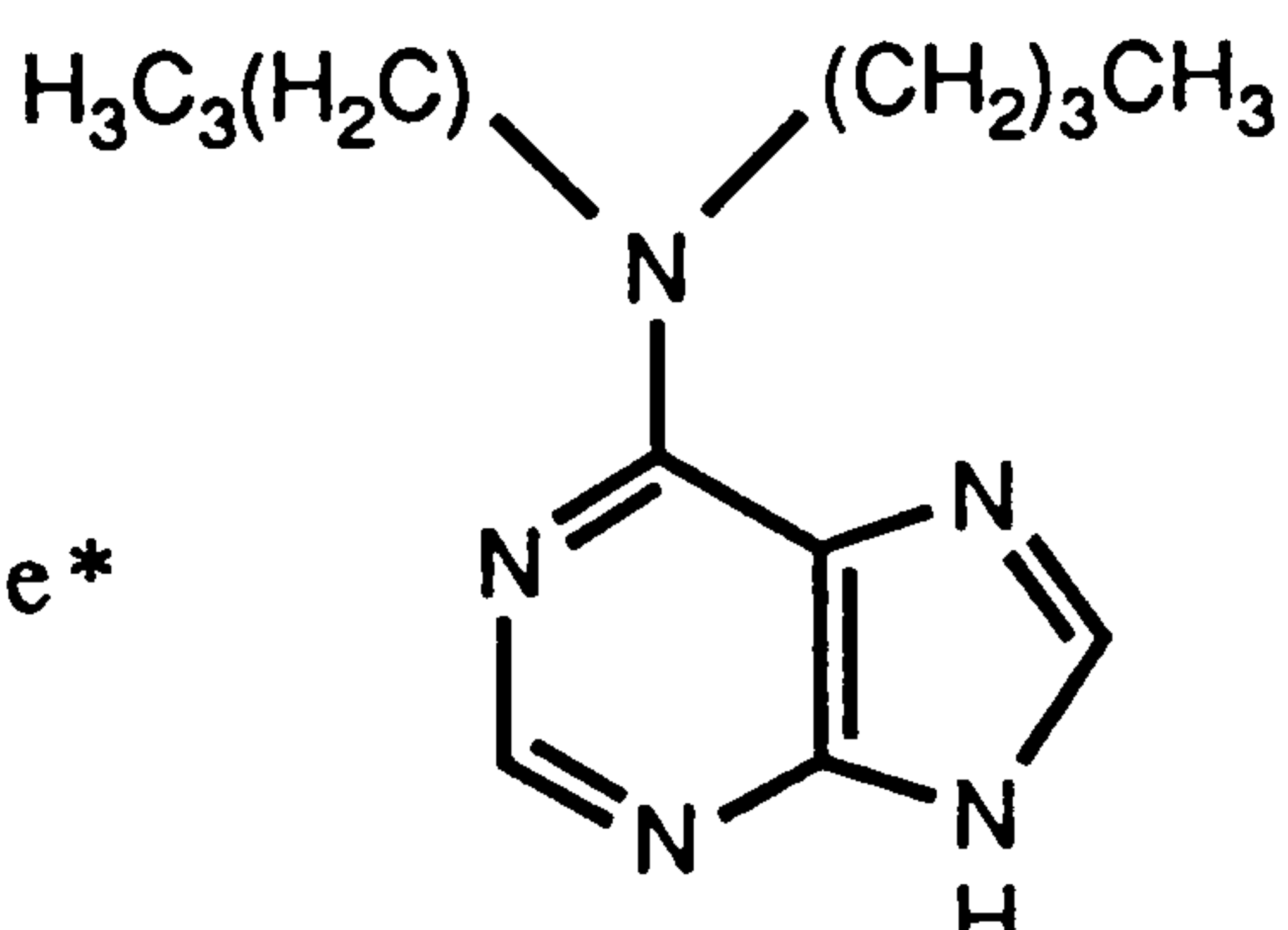




Table 3.4

## Effect of Modification on Position 6

| Compound         | Structure  | %3h-I | %3h-II |
|------------------|--|-------|--------|
| Adenine          |    | 97.5  | 98.1   |
| Methyladenine    |   | 99.0  | 85.7   |
| Dimethyladenine  |  | 98.1  | 77.9   |
| Diethyladenine*  |  | 62.1  | 48.8   |
| Dipropyladenine* |  | 52.3  | 38.9   |
| Dibutyladenine*  |  | 44.9  | 36.1   |

\*Gift of Ms. S. Brennan, University of Warwick.

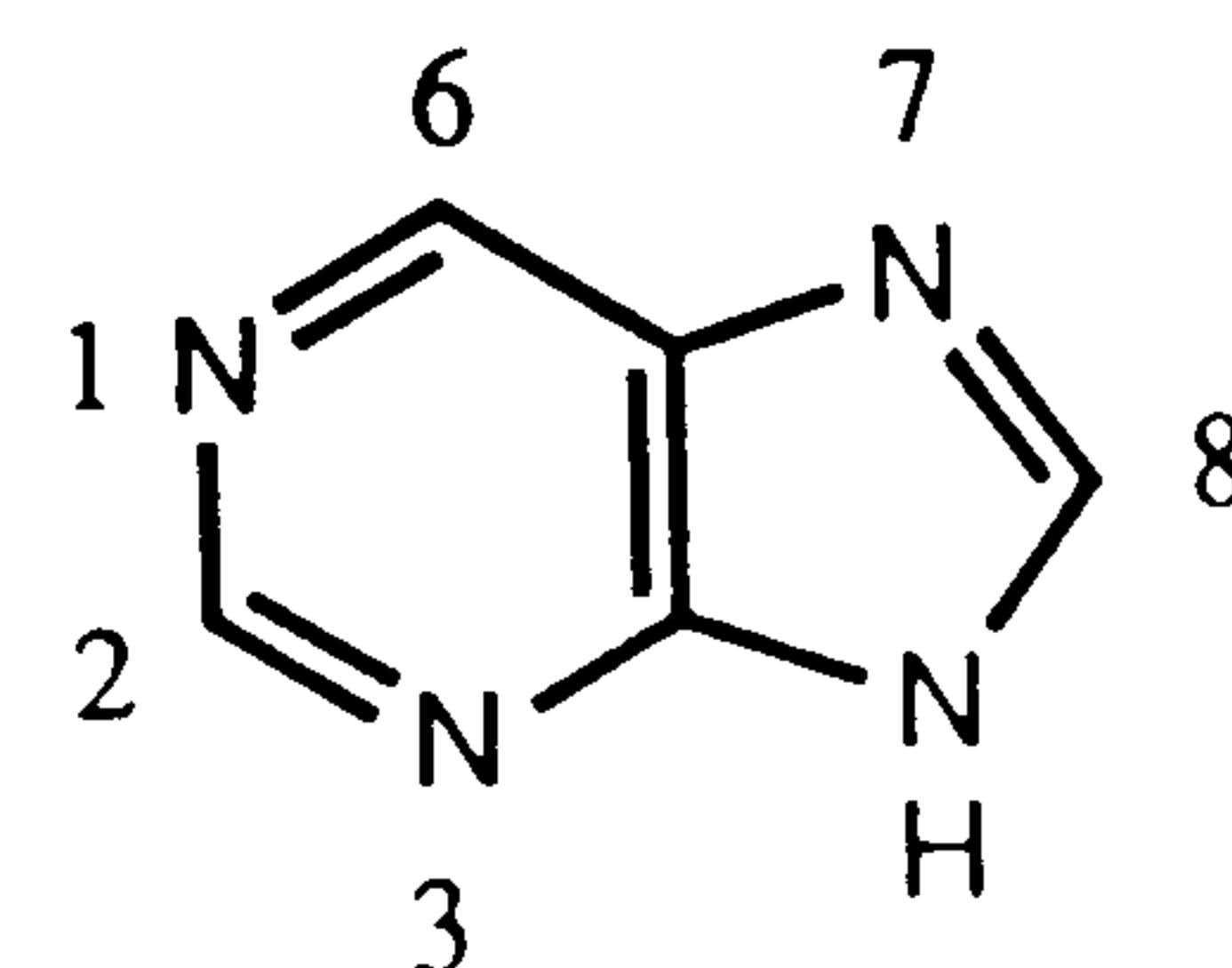
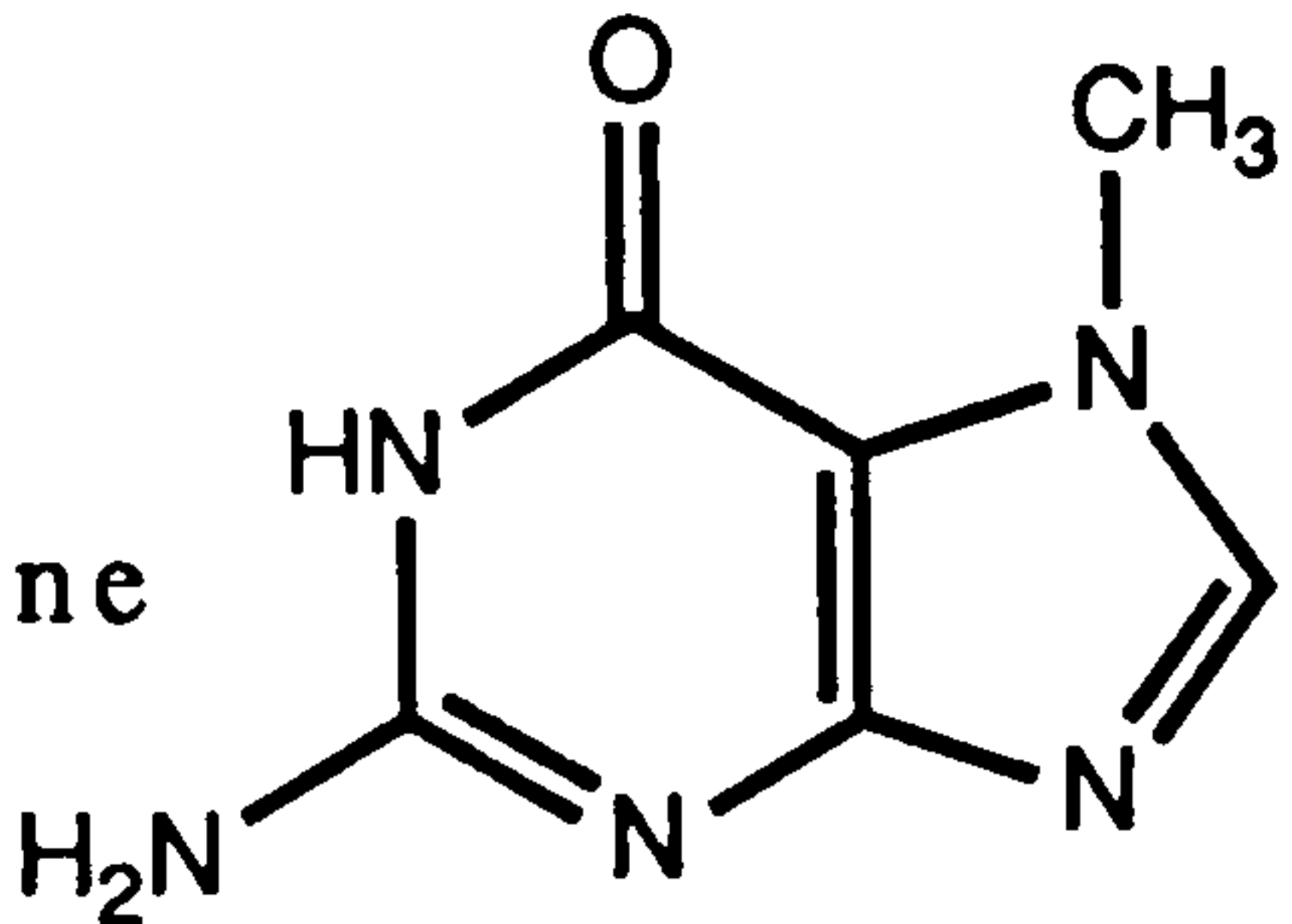
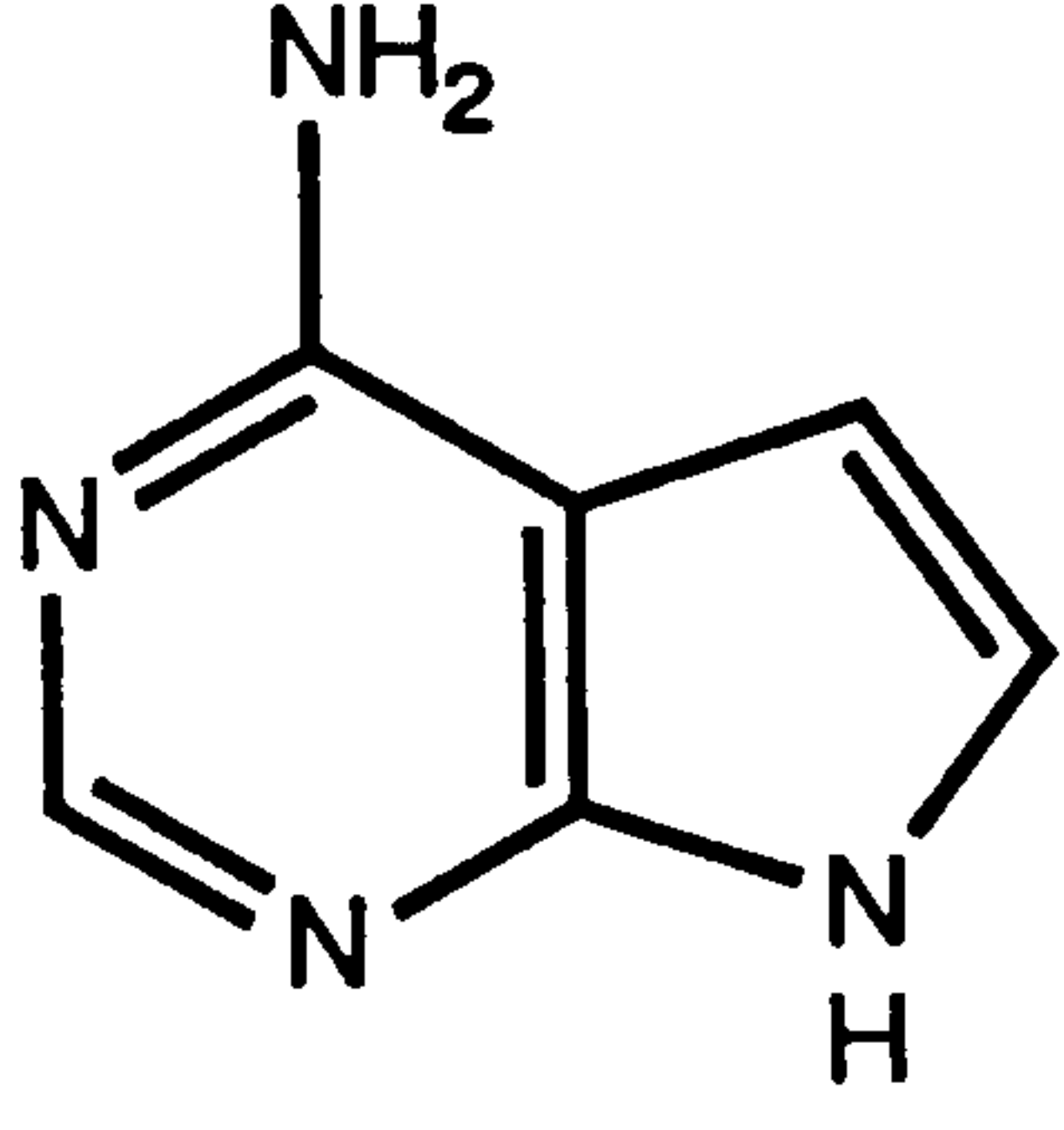
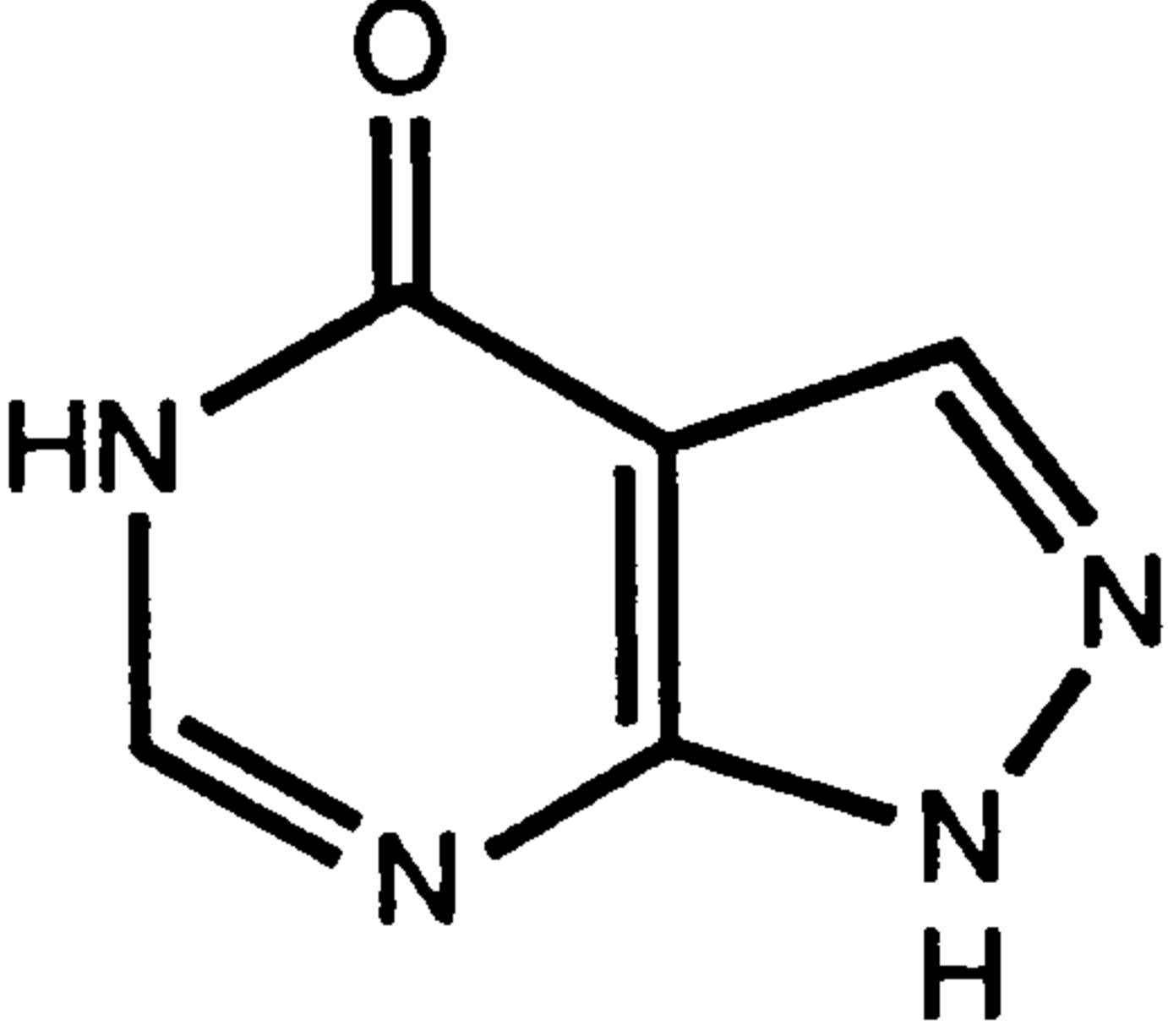
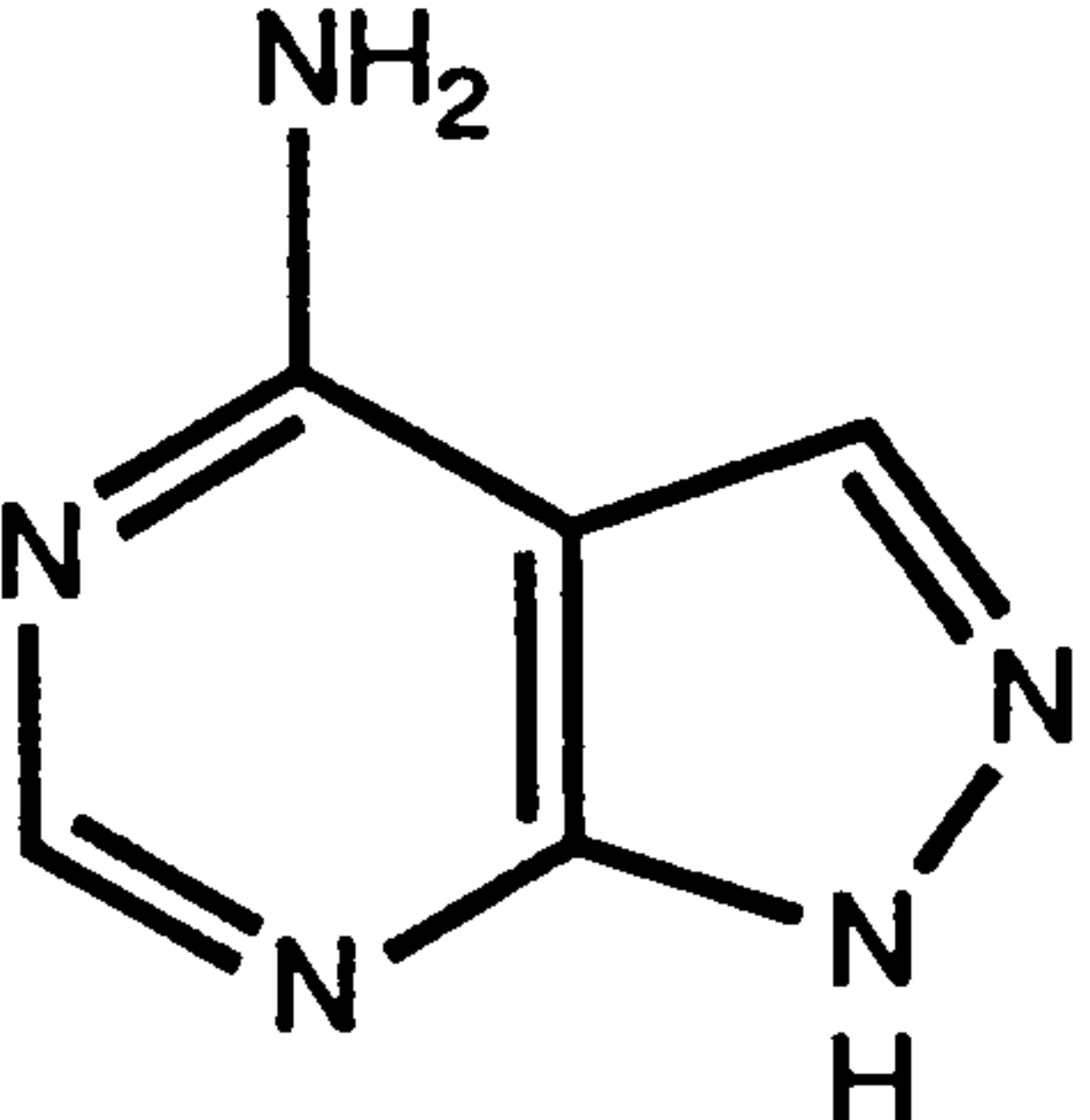


Table 3.5

## Effect of Modification on Position 7

| Compound                             | Structure  | %3h-I | %3h-II |
|--------------------------------------|--|-------|--------|
| 7-Methylguanine                      |    | 0     | 0      |
| 7-deazaadenine                       |   | 0     | 0      |
| 4-hydroxypyrazolo-[3,4-d]-pyrimidine |  | 42.6  | 11.2   |
| 4-aminopyrazolo-[3,4-d]-pyrimidine   |  | 80.2  | 86.6   |

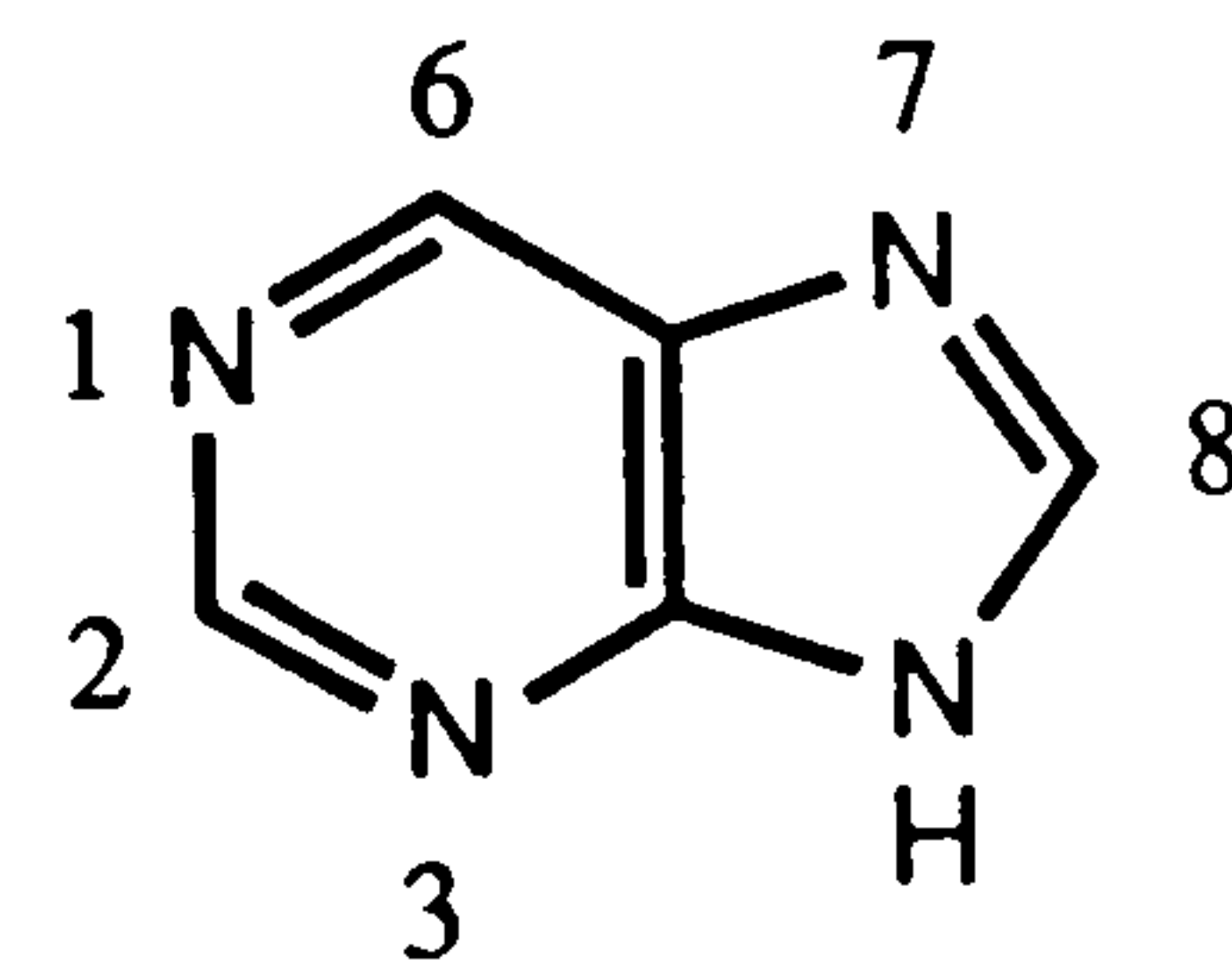
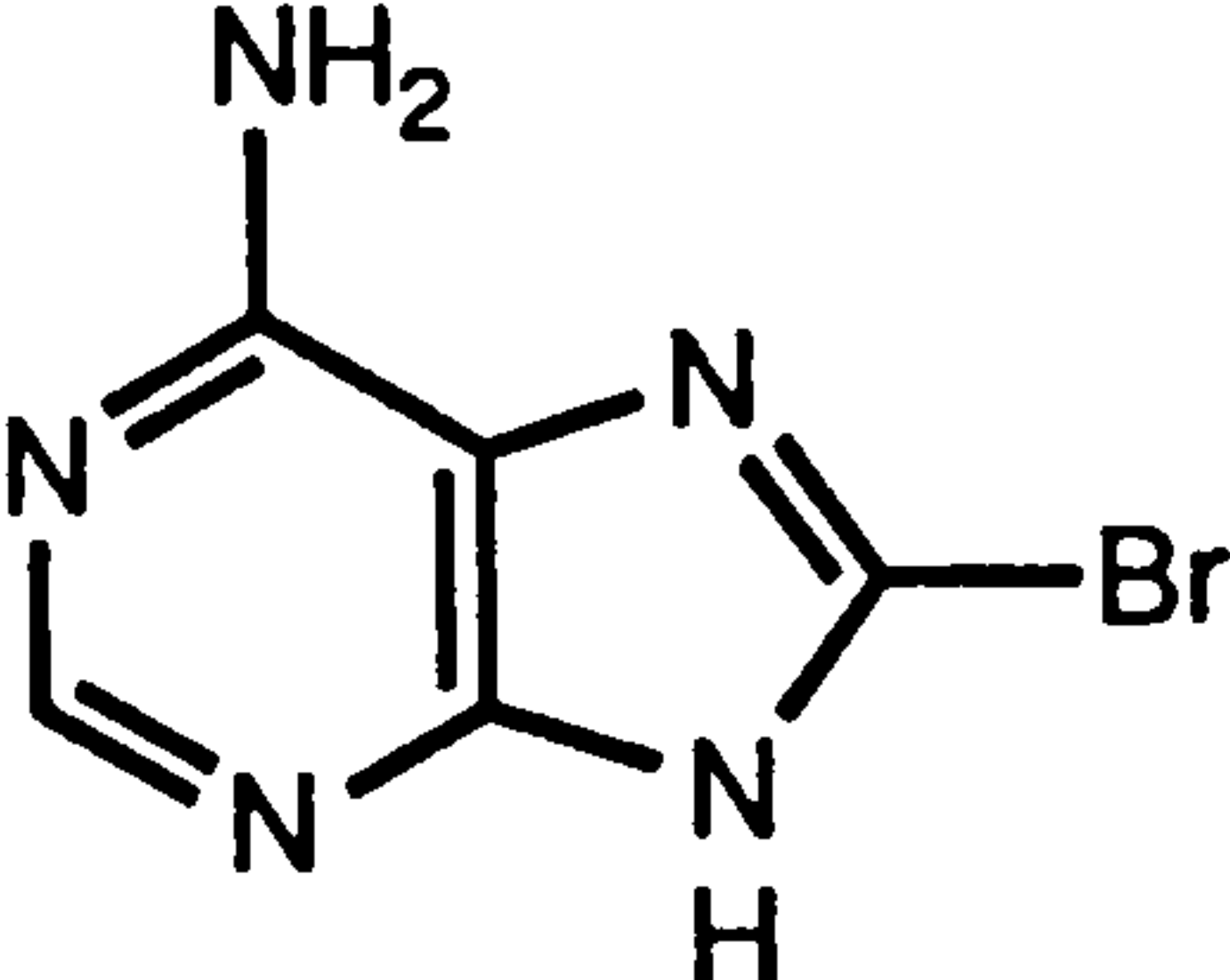
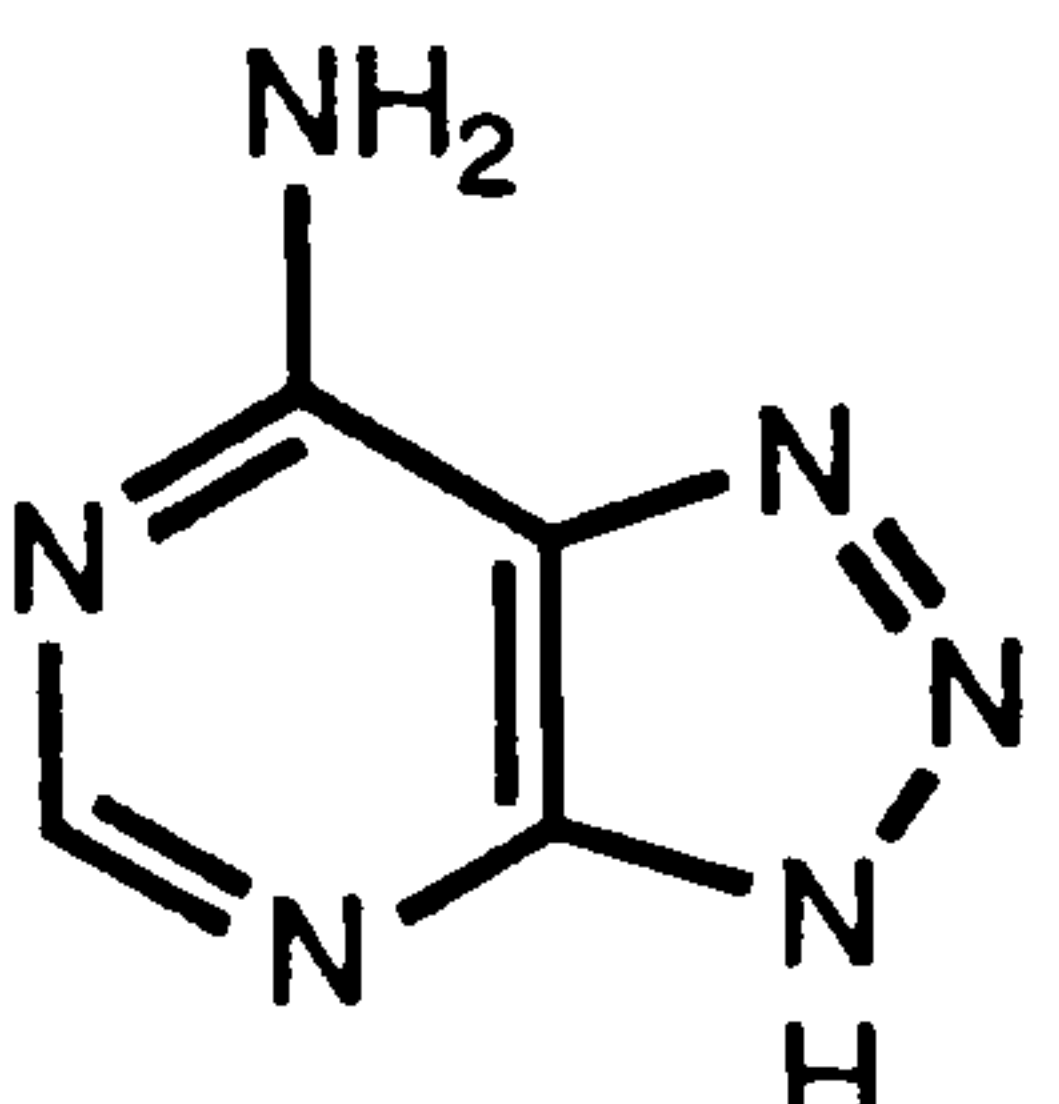
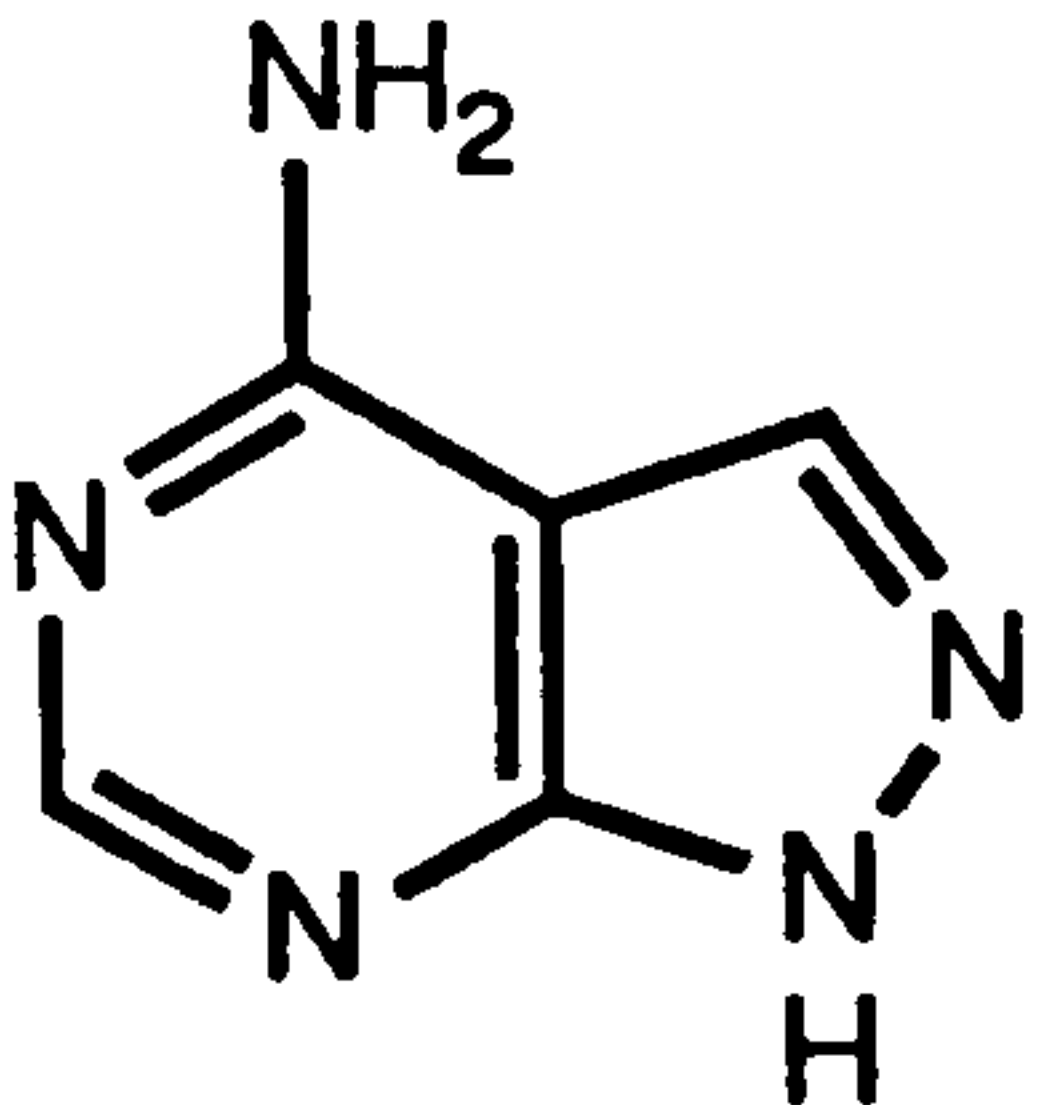
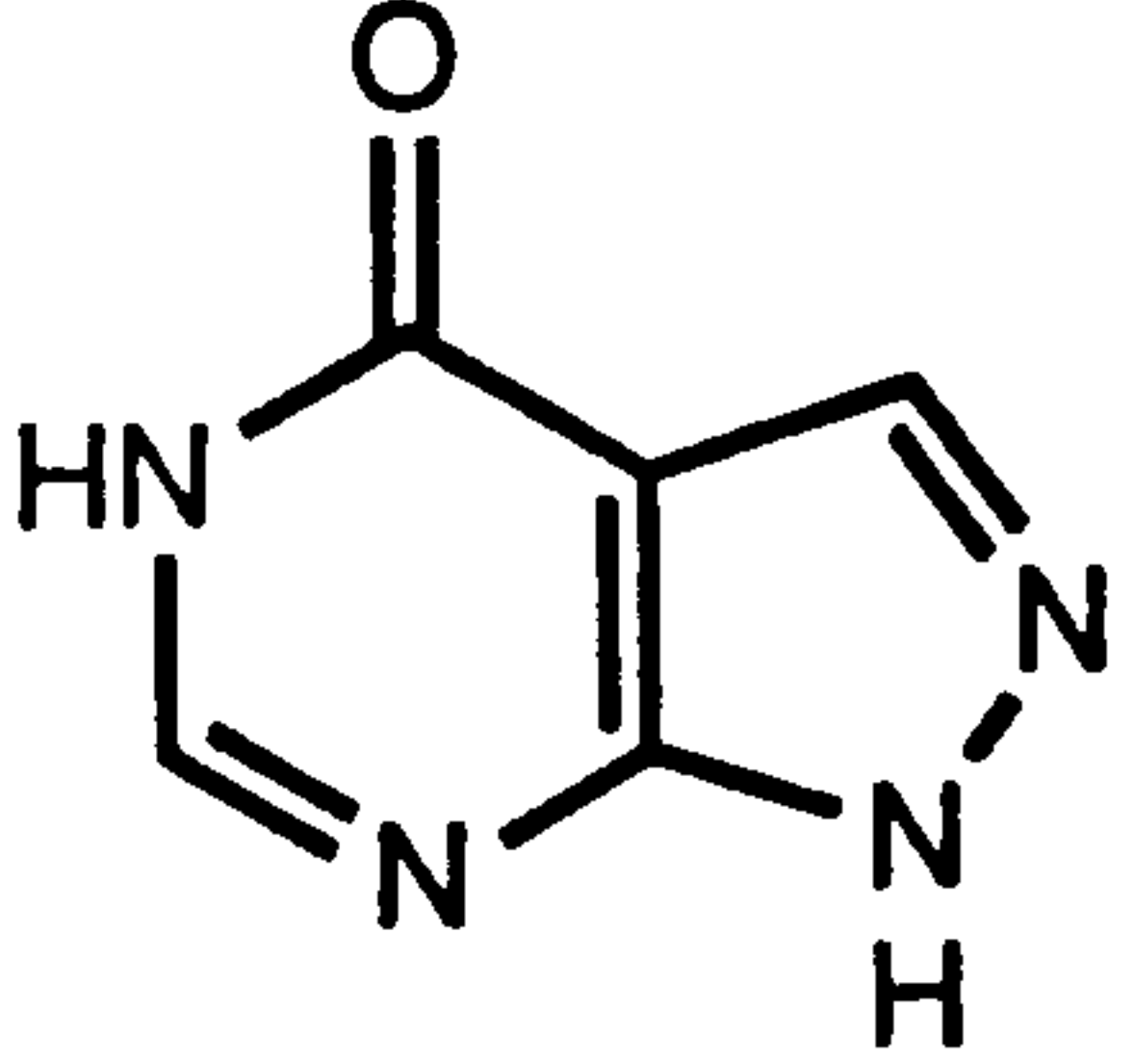
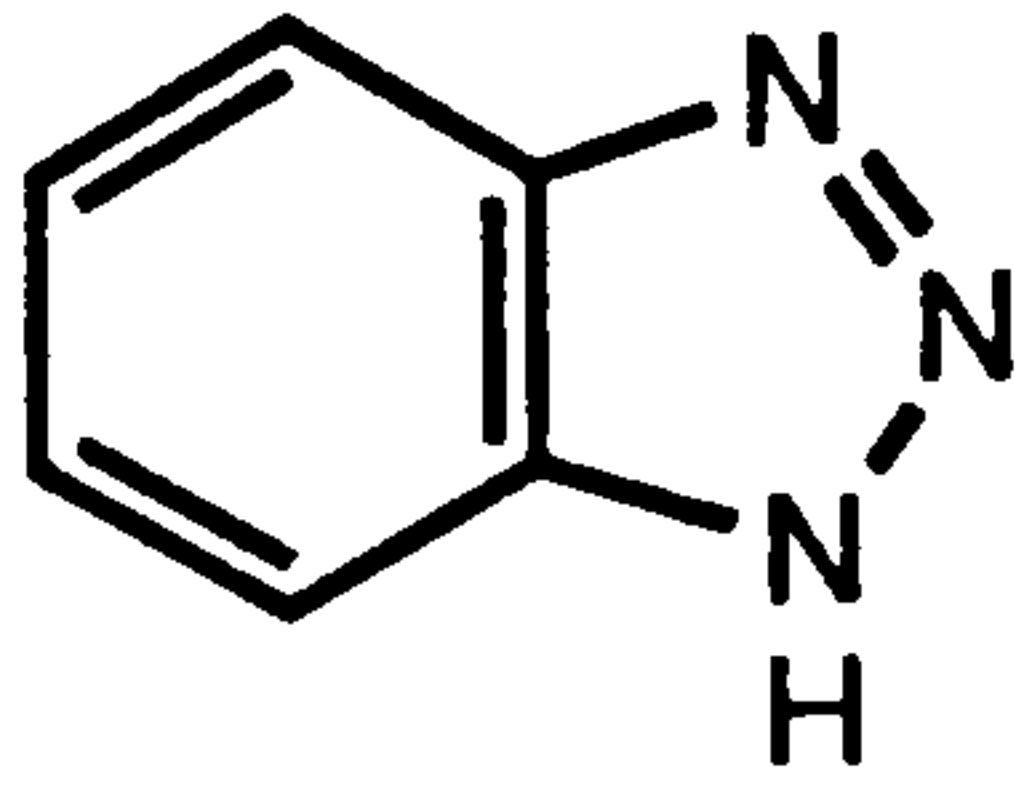
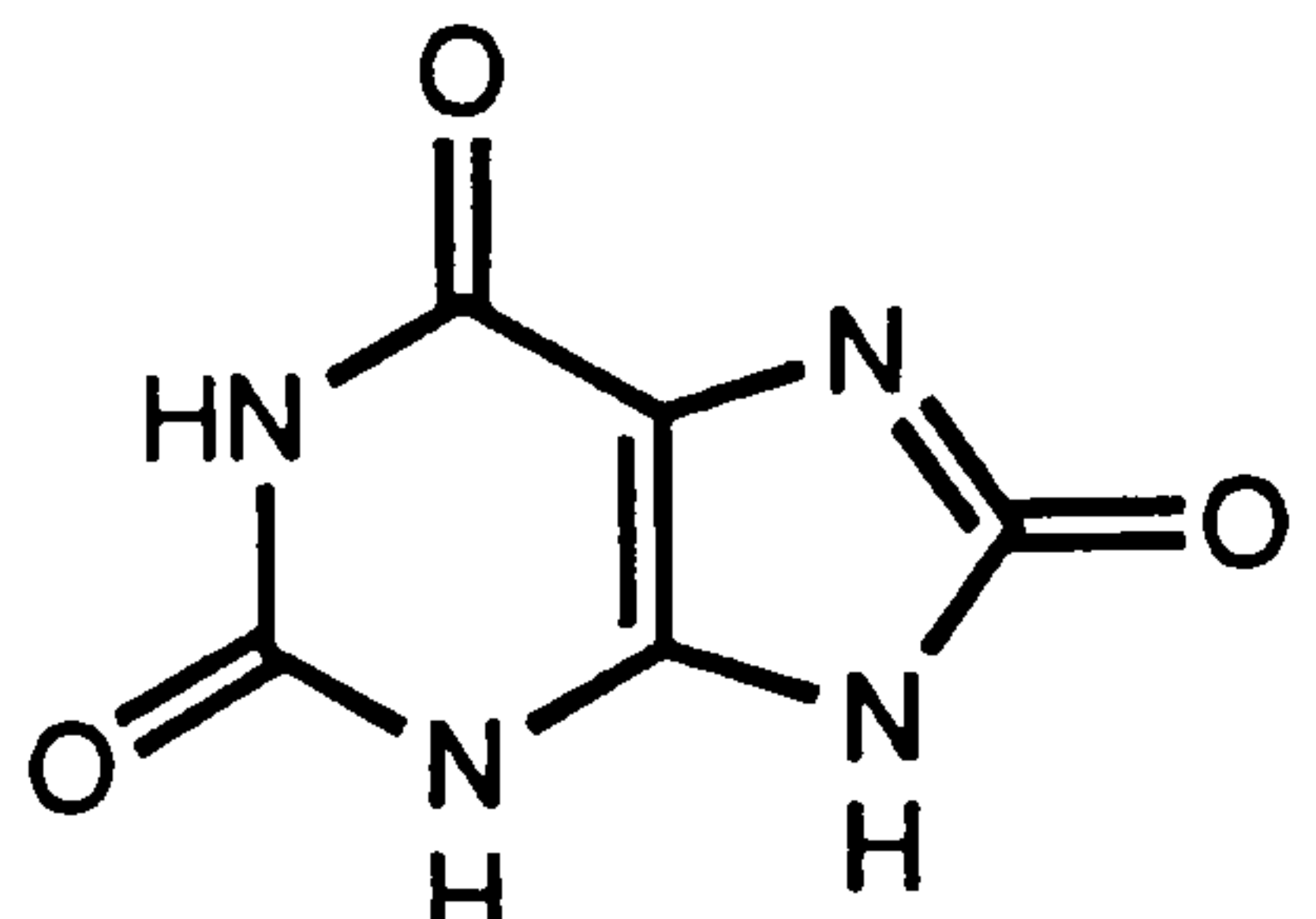




Table 3.6

## Effect of Modification on Position 8

| Compound                              | Structure  | %3h-I | %3h-II |
|---------------------------------------|--|-------|--------|
| 8-Bromoadenine                        |    | 28.2  | 35.6   |
| 8-Azaadenine                          |   | 69.2  | 21.1   |
| * 4-aminopyrazolo-[3,4-d]-pyrimidine  |  | 80.2  | 86.6   |
| *4-hydroxypyrazolo-[3,4-d]-pyrimidine |  | 42.6  | 11.2   |
| Benzotriazole                         |  | 20.2  | 17.5   |
| Uric Acid                             |  | 0     | 0      |

\*included in Tables 3.5 and 3.6 as they are modified at both the 7- and the 8-positions

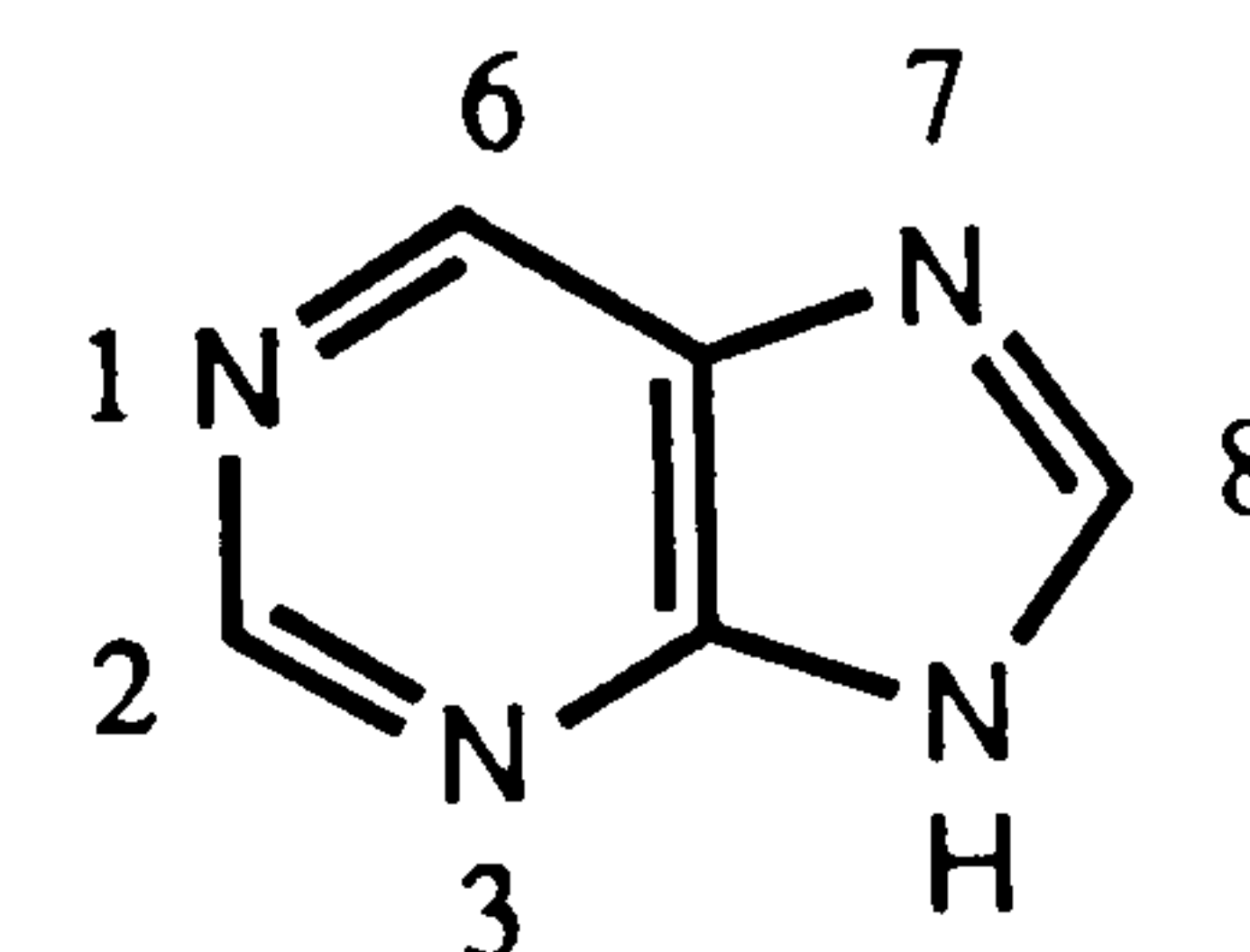


Table 3.7

Effect of Modification on Positions 2, 4, 5 and 6  
on the Pyrimidine ring

| 2                              | 4                | 5   | 6                 | +/- |
|--------------------------------|------------------|---|-------------------|-----|
| *S                             | -OH              | H   | H                 | -   |
| *S                             | -OH              | CH <sub>3</sub>                                 | H                 | -   |
| *S                             | -OH              | CH <sub>2</sub> CH <sub>3</sub>                 | H                 | -   |
| *S                             | -OH              | (CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub> | H                 | -   |
| *S                             | -OH              | CH <sub>2</sub> (CH <sub>3</sub> ) <sub>2</sub> | H                 | -   |
| *S                             | -OH              | (CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub> | H                 | -   |
| <sup>+</sup> -NH <sub>2</sub>  | -NH <sub>2</sub> | H   | -OH               | -   |
| <sup>+</sup> -OH               | -NH <sub>2</sub> | H   | -OH               | -   |
| <sup>+</sup> -Cl               | -NH <sub>2</sub> | H   | -Cl               | -   |
| <sup>+</sup> -OCH <sub>3</sub> | -NH <sub>2</sub> | H   | -OCH <sub>3</sub> | -   |

\*Gift of Ms. N. Hicks, University of Warwick.

<sup>+</sup>Gift of Dr. J.P. Madley, Courtaulds Research plc., Coventry.



Table 3.8

Effect of Modification on Position 5 of Uracil

| Compound                   | 2 / 4 | 5   | +/- |
|----------------------------|-------|---|-----|
| Uracil                     | -OH   | H   | +   |
| 5-Methyluracil             | -OH   | CH <sub>3</sub>                                 | +   |
| *5-Ethyluracil             | -OH   | CH <sub>2</sub> CH <sub>3</sub>                 | +   |
| *5-Propyluracil            | -OH   | (CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub> | -   |
| *5-i-Propyluracil          | -OH   | CH <sub>2</sub> (CH <sub>3</sub> ) <sub>2</sub> | -   |
| *5-Butyluracil             | -OH   | (CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub> | -   |
| 5-Fluorouracil             | -OH   | F   | +   |
| 5-Chlorouracil             | -OH   | Cl  | +   |
| 5-Bromouracil              | -OH   | Br  | +   |
| 5-Iodouracil               | -OH   | I   | +   |
| 5-Trifluoromethyluracil    | -OH   | CF <sub>3</sub>                                 | +   |
| 5-Aminouracil              | -OH   | NH <sub>2</sub>                                 | +   |
| 5-Nitouracil               | -OH   | NO <sub>2</sub>                                 | +   |
| 5-Carboxyuracil            | -OH   | COOH  | -   |
| (E)-5-(2-bromovinyl)uracil | -OH   | CHBr  | -   |

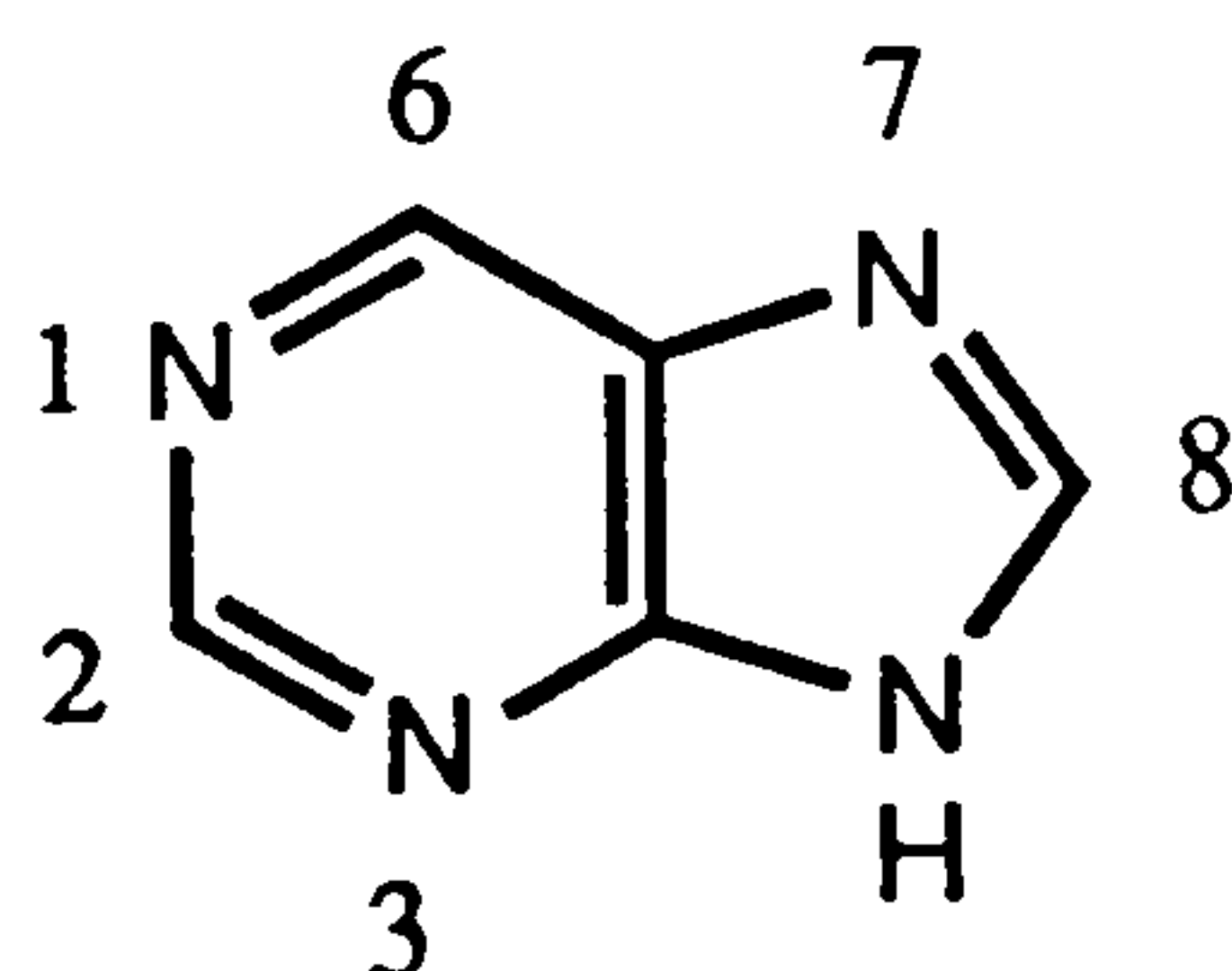
\*Gift of Ms. N. Hicks, University of Warwick.

## DISCUSSION

Several conclusions with regard to the structure-activity relationship between the heterocyclic rings and the sugar group of the donor nucleoside with the N-deoxyribosyltransferase enzymes can be made from these results. Diverse purine analogues have been shown to be substrates of N-deoxyribosyltransferase-II from *L. leichmannii* and *L. helveticus*, but the substrate range of N-deoxyribosyltransferase-I has not been described.

## PURINE ANALOGUES

Tables 3.1-3.6 show that N-deoxyribosyltransferase-I utilised the same range of purine analogues as N-deoxyribosyltransferase-II.



It can be concluded that the positions 1, 2, 3 and 6 are not essential for binding. Indeed when the pyrimidine structure is removed, the analogue, although at a much reduced reaction rate, still acts as a substrate. The ability of the enzyme to accept such a variety of 6-substituted purine analogues suggests that these probably reside in a large binding pocket. The fact that the reactions employing analogues with larger side chains proceed at



a relatively slow velocity could be due to some steric hinderance on the part of the substituent or to tighter binding of the substrate if the region is hydrophobic in character. The reasons why the 3-deazaadenine is not a substrate are unclear as it has been shown quite catagorically that this position is unnecessary to allow the molecule to participate in the transfer. Holguin and Cardinaud [88] have shown that substitution at this position with a methyl group gives a very slow acceptor. Replacement of the N-3 with a carbon could put the molecule under some conformational strain which prevents it from either entering the active site easily or being able to bind. The discovery that two imidazole-5-carboxylates and one imidazole carboxamide [119], which lack the six membered ring, were also competent acceptors confirms that the action of this part of the base in binding is of secondary significance. Therefore, the important part of the structure, and one which is common to all proficient acceptors, is the imidazole ring.

From the non-reactivity of the 7-modified purine analogues it is possible to conclude that either the nitrogen at N-7 is critical for binding, or that a tautomeric proton is required in the imidazole ring. Although there are four possible tautomers of purine, in solution the purine will exist as the 7-H and the 9-H tautomers. Since the site of glycosyl substitution must possess a proton, this suggests that the 9-H tautomer is the dominant one.

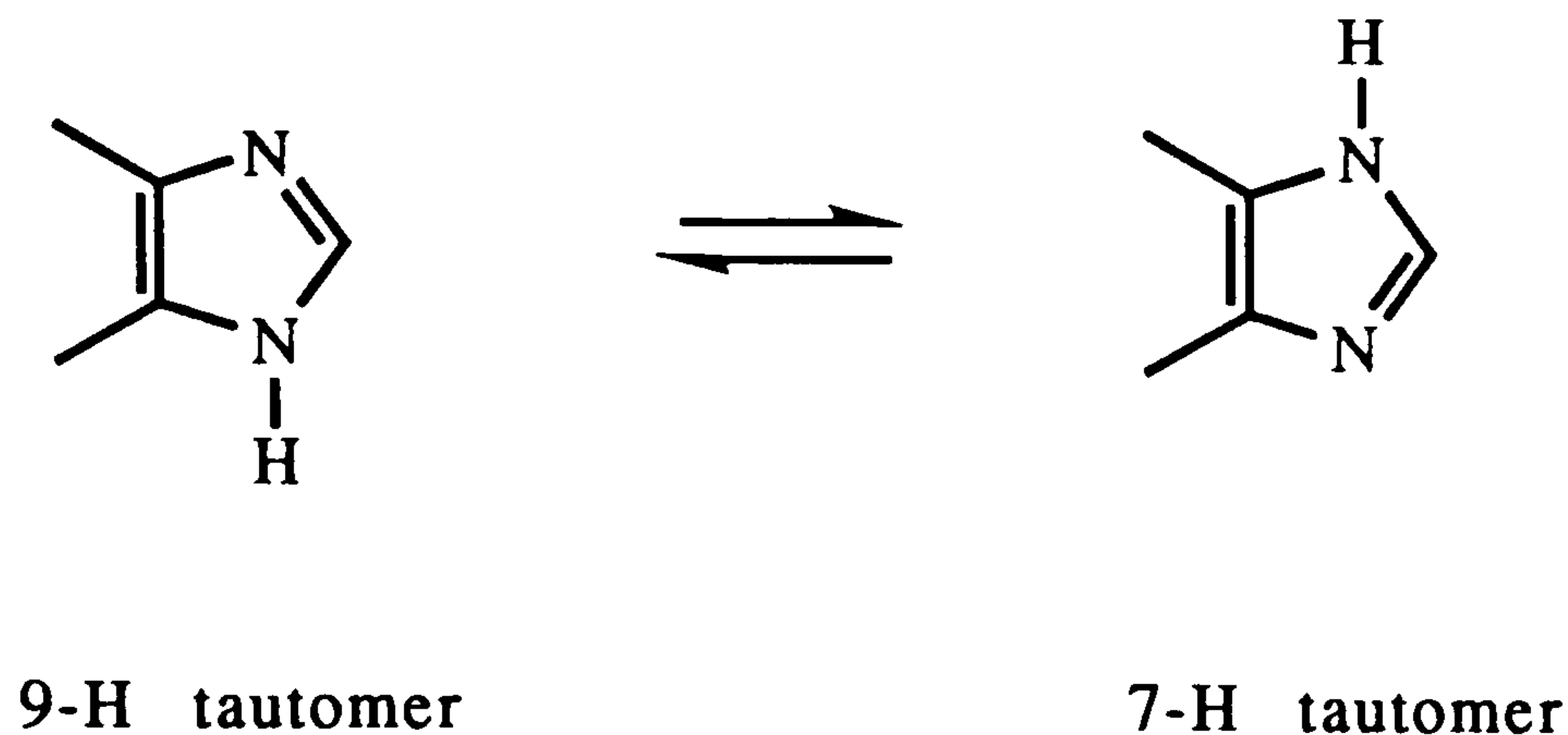


Figure 3.1

When the nitrogen is replaced by a carbon in the case of 7-deazaadenine, or modified, 7-methylguanine, this tautomeric shift is unable to take place. The observation that 4-aminopyrazolo[3,4-d]pyrimidine and its corresponding 4-hydroxy analogue were competent acceptors suggests that this tautomeric shift can occur between the 8 and the 9 positions in the absence of the 7-position nitrogen. This last observation supports the theory for N-7 as necessary for tautomeric shift rather than essential binding. Although the 4-hydroxypyrazolo[3,4-d]pyrimidine was a very poor substrate for N-deoxyribosyltransferase-II, its reaction with the purine:purine enzyme was much better. These results are contrary to those obtained by Holguin who found this compound was not accepted by the N-deoxyribosyltransferase-II from *L.helveticus* [88]. The 8-azaadenine proved to be a reasonable acceptor which is in agreement with examination of these compounds with the N-deoxyribosyltransferase of other species [76, 77, 80, 120].

8-Substituted compounds, were observed to be very slow acceptors or non-substrates. In the glycosyl transfer to 8-bromoadenine, the slow reaction generated two products. The reduction in the reaction velocity could be due to the bromine



substituent sterically hindering the approach of the purine molecule into the active site. In this instance it is thought that the molecule seeks an alternative access to normal binding and docks into the protein catalytic site via the pyrimidine rather than the imidazole ring. This solution means that a tautomeric shift can now exist between the 9- and the 3-positions, allowing the production of the 3- $\beta$ -D-deoxyribonucleoside. Isolation of the two products eluting from the HPLC column enabled a brief characterization. A UV spectrum of one fraction demonstrated a large shift of  $\lambda_{\max}$  in accordance with the presence of the 3- $\beta$ -D-deoxyribonucleoside (Table 3.9). This is in agreement with the results of Huang *et al.* [87] who examined a number of 8-halo-substituted adenine analogues and found two products: the 3- and the 9- $\beta$ -D-deoxyribonucleoside.

Table 3.9

pH 11.0

| Compound       | $\lambda_{\max}$ | $\lambda_{280/260}$ |
|----------------|------------------|---------------------|
| 8-bromoadenine | 271.8            | 0.98                |
| A <sub>1</sub> | 294.8            | 1.38                |
| A <sub>2</sub> | 266.9            | 0.56                |

Huang also observed that a trend existed between the ratios of the two products and the electronegativity of the group at the 8-position: the greater the electronegativity of the substituent, the greater the proportion of the 3- $\beta$ -D-deoxyribonucleoside formed. In all cases these reactions were found to proceed at much slower

rates compared to the parent adenine.

The fractions separated from this reaction mixture were also tested independently for their ability to act as glycosyl donors in the transfer reaction. Both were able to function competently donating the deoxyribose group to a cytosine acceptor. When uric acid was used as an acceptor there were no products. This is most likely due to obstruction by the 8-hydroxy group making the active site inaccessible.

In view of the initial results that the pyrimidine ring did not seem to play a large part in governing either the ability for an analogue to act as an acceptor or influencing the site of glycosyl substitution, the observation that xanthine gave two products was unexpected. Holguin and Cardinaud [121] reported that in their reaction of thymidine with xanthine using the N-deoxyribosyltransferase-II enzyme from *L. helveticus*, the major product was the 7- $\beta$ -D-deoxyribonucleoside, as identified by UV spectroscopy. The two products collected from the reaction between 2'-deoxycytidine and xanthine were also found to have distinct spectral properties. Examination of the X<sub>1</sub> product demonstrated a marked spectral shift from the xanthine free base and the 9- $\beta$ -D-deoxyribonucleoside (Table 3.10 ).

Table 3.10

pH 11.0

| Compound       | $\lambda_{\max}$ | $\lambda_{250/260}$ | $\lambda_{280/260}$ | $\lambda_{290/260}$ |
|----------------|------------------|---------------------|---------------------|---------------------|
| Xanthine       | 277.4            | 1.44                | 1.87                | -                   |
| X <sub>1</sub> | 291.7            | 0.86                | 3.03                | 3.5                 |
| X <sub>2</sub> | 274.2            | -                   | 0.946               | 0.57                |



This is similar in magnitude to the UV shift shown by the A<sub>1</sub> product isolated from the 8-bromoadenine reaction and it is suspected that this X<sub>1</sub> product might also be the 3-β-D-deoxyribonucleoside. Mammalian pyrimidine phosphorylases, despite their strict structural ligand requirements, are able to accept xanthine as a substrate, and give the 3-β-D-deoxyribonucleoside product [122, 123]. This would suggest that the enzyme is viewing the purine as a pyrimidine, recognising the six-membered ring as a uracil analogue and placing the glycosyl group onto the N-1 pyrimidine position of the ring. It could be considered that the N-deoxyribosyltransferase-II enzyme from *L. leichmannii* is identifying the xanthine in the same way, allowing the substrate to interact and bind via the 2- and 6-hydroxyl groups to the active site with the concurrent formation of the 3-β-D-xanthine deoxyribonucleoside. Since the enzyme is recognised to have the ability to express some flexibility toward tautomerism, as witnessed by its acceptance of 7-deaza-8-aza compounds, it is thought that the synthesis of this compound is more likely to occur than the production of the 7-β-D-deoxyribonucleoside proposed by Holguin and Cardinaud. This conclusion could be extended to include a compound such as guanine which, like xanthine, possess a 2-hydroxy substituent. It is possible that this base could also bind in a similar fashion, reacting to give two distinct products. Very little detailed work however, has been performed on guanine and its base analogues because of problems of solubility in aqueous media. Further studies are required to clarify these phenomena. The 2,6-diaminopurine base has been shown to be a non-substrate for the N-deoxyribosyltransferase enzymes from *L. helveticus* [71], and

*L. acidophilus* [76]. However, in agreement with the conclusions drawn so far, the base is an acceptor for both enzymes from *L. leichmannii*. These data have enabled a model to be constructed for the binding of a purine base or nucleoside with the N-deoxyribosyltransferase enzymes from *L. leichmannii* (Figure 3.2).

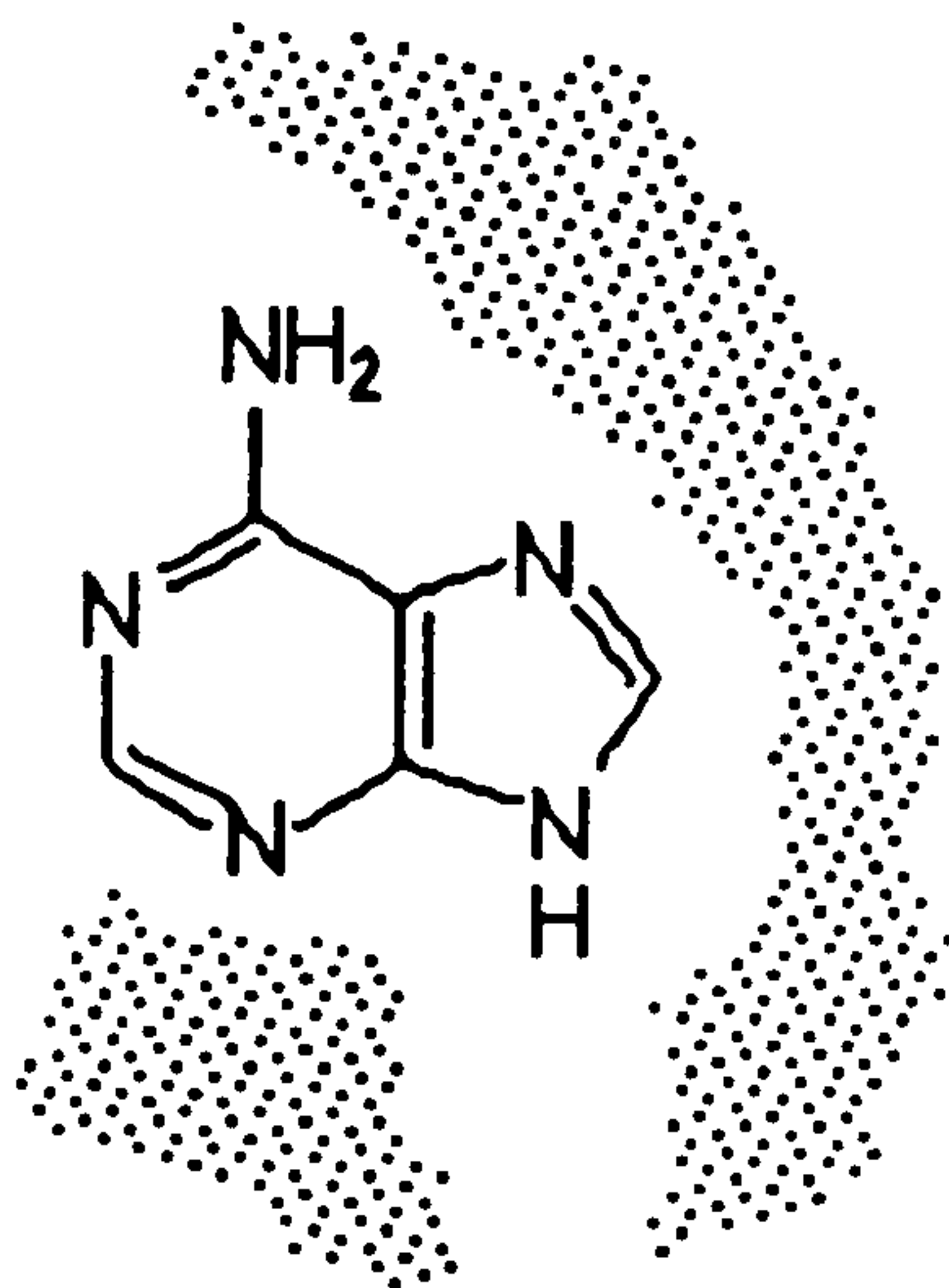
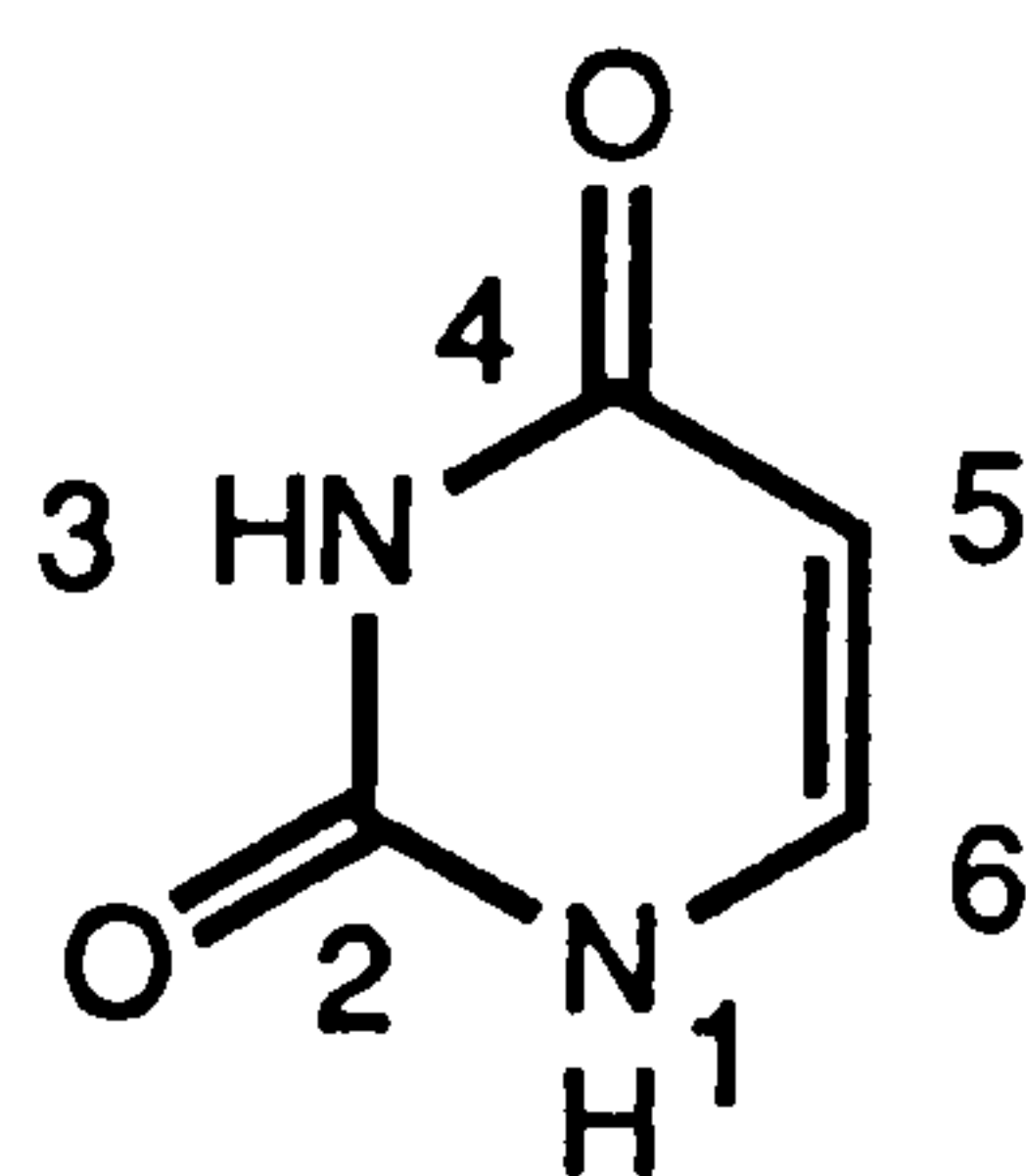


Figure 3.2

### PYRIMIDINE ANALOGUES



Substitutions on the pyrimidine structure of such a wide variety, are not so easily accepted by the N-deoxyribosyltransferase-II enzyme as those made on the six membered ring of the purine. Modification to the N-1 position meant loss of acceptor capability; similarly substitutions at the C-2 position did not permit transfer. From the observations it can be concluded that tautomerism



between the 1- and the 2- positions is necessary for activity (Figure 3.3).

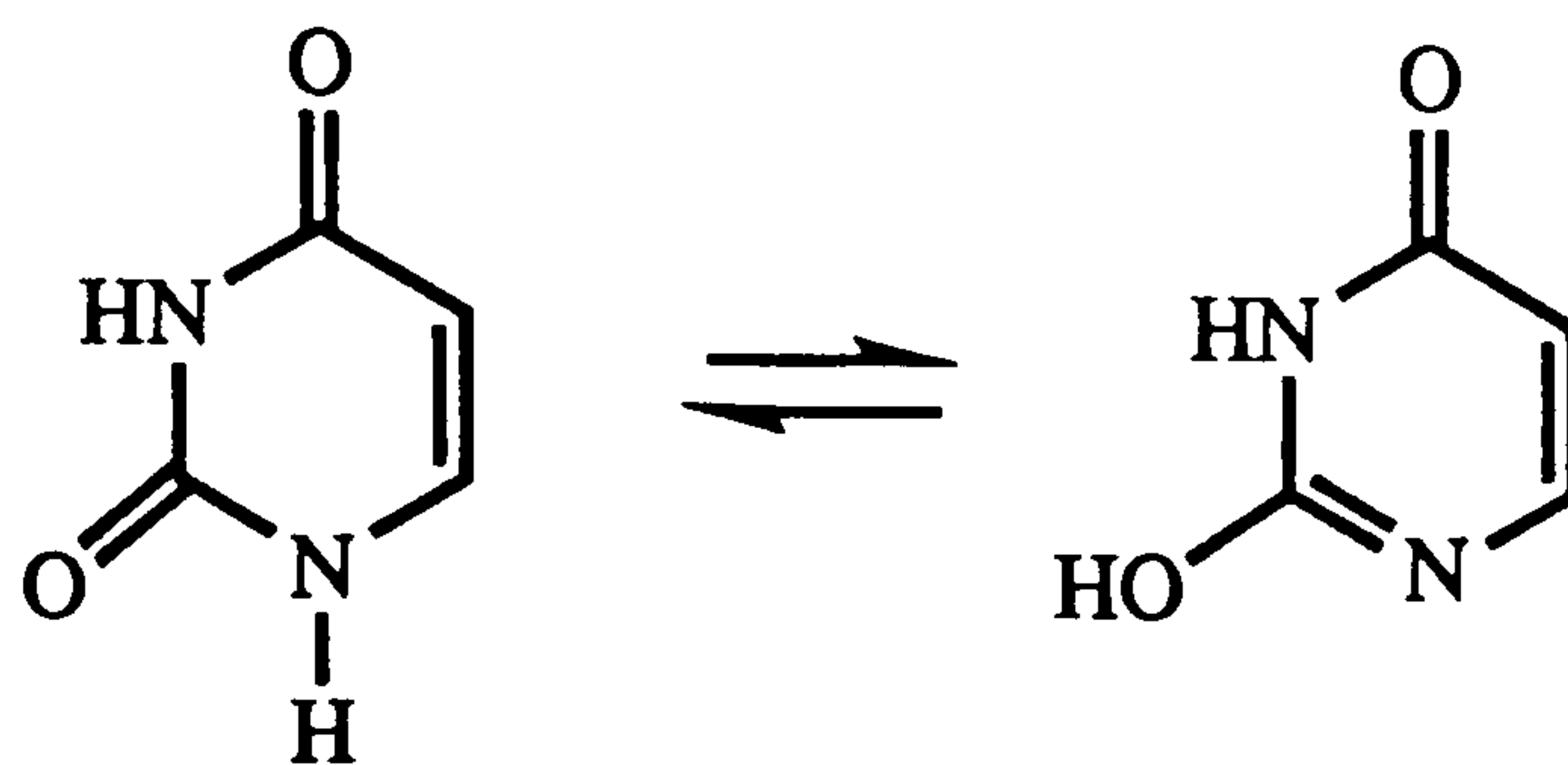


Figure 3.3

Removal of the hydroxyl at the 2-position altogether, as in pyrimidine or pyrazines\* gave analogues which were non-substrates. Disubstituted compounds at the 2- and the 6-positions were also inactive, indicating that all of these sites seem to be crucial in competent acceptors (Table 3.7). Modifications at the 5-position seemed to suggest that this might be analogous to the 6-position on purines with respect to its versatility. Various changes were made at this position and, with few exceptions, all the analogues were substrates (Table 3.8). Smaller substituents like the halogens were observed to react readily, but some larger alkyl groups, were unable to do so. It appeared that once the size of the alkyl substituent had reached a certain size, any further increase resulted in an inactive compound. This seems to suggest the presence of a binding pocket of defined size and shape close to this position which is reluctant to accommodate very bulky groups.

\*Gift of Mr. A.O'L. Richards, University of Warwick.

The introduction of the carboxyl group probably changes the pyrimidine ring structure debilitating efficient binding and renders the base unacceptable as a substrate. The (E)-5-(2-bromovinyl) group is probably sterically constrained and unable to fit into the binding pocket. More information on the exact dimensions and nature of this pocket could be gained from comparison of binding constants with the electronegative and hydrophobic characteristics of 5-substituted analogues. From these results it might be envisaged that the pyrimidine ring binds in the active site as depicted as follows (Figure 3.4), anchoring itself by hydrogen bond interactions between the substituents at positions 2-, 4- and 5.

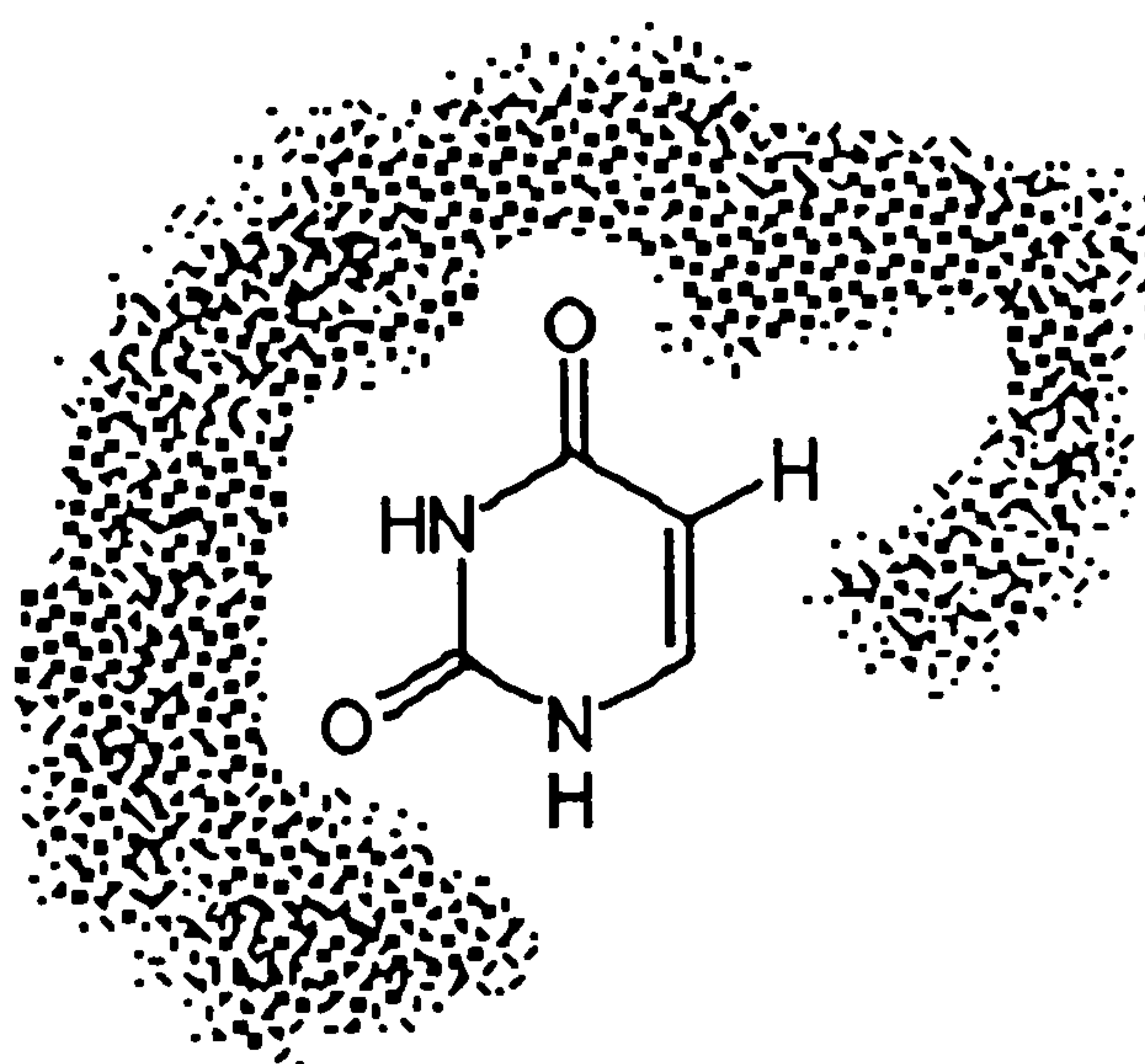
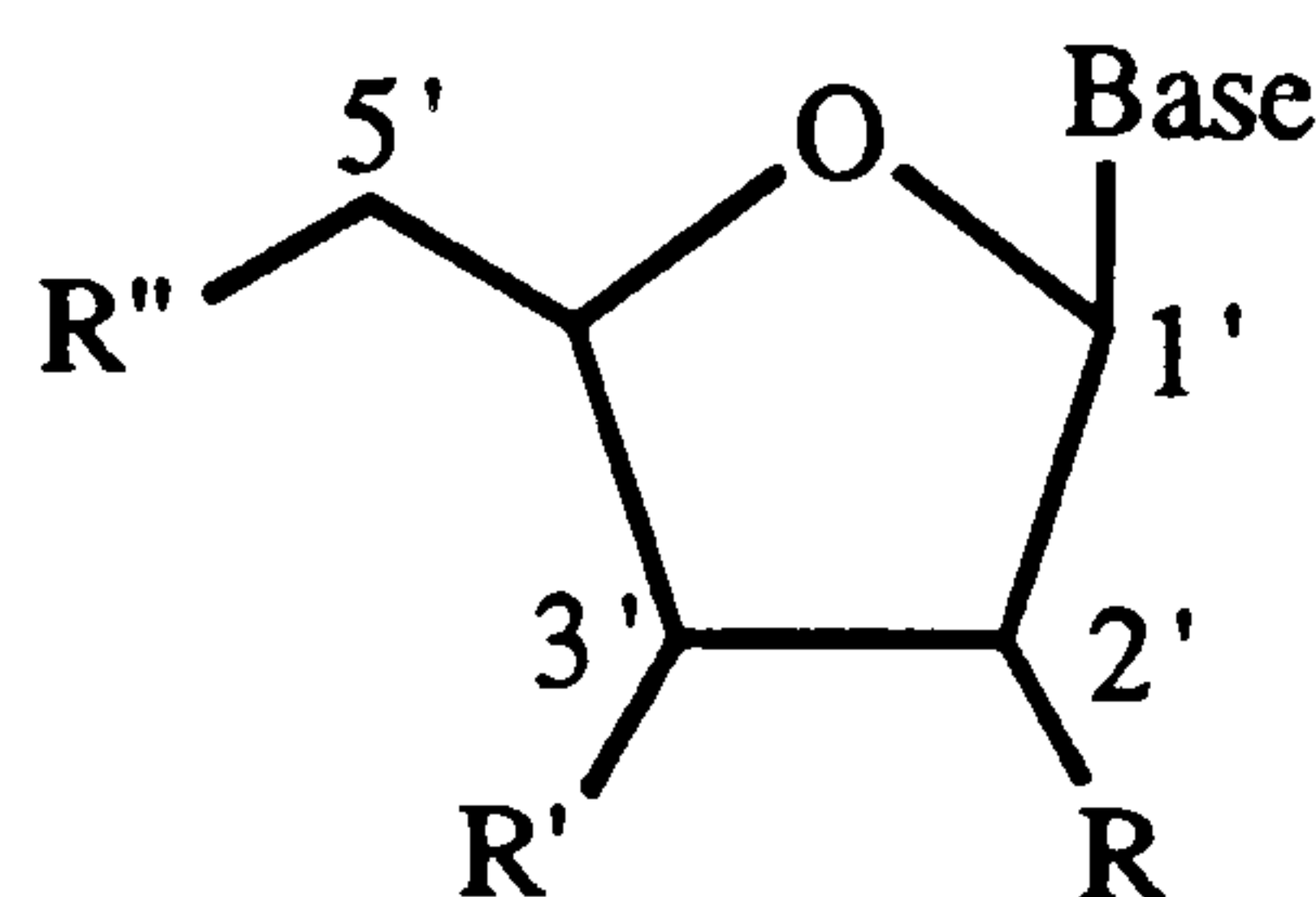


Figure 3.4



## MODIFIED SUGAR GROUPS

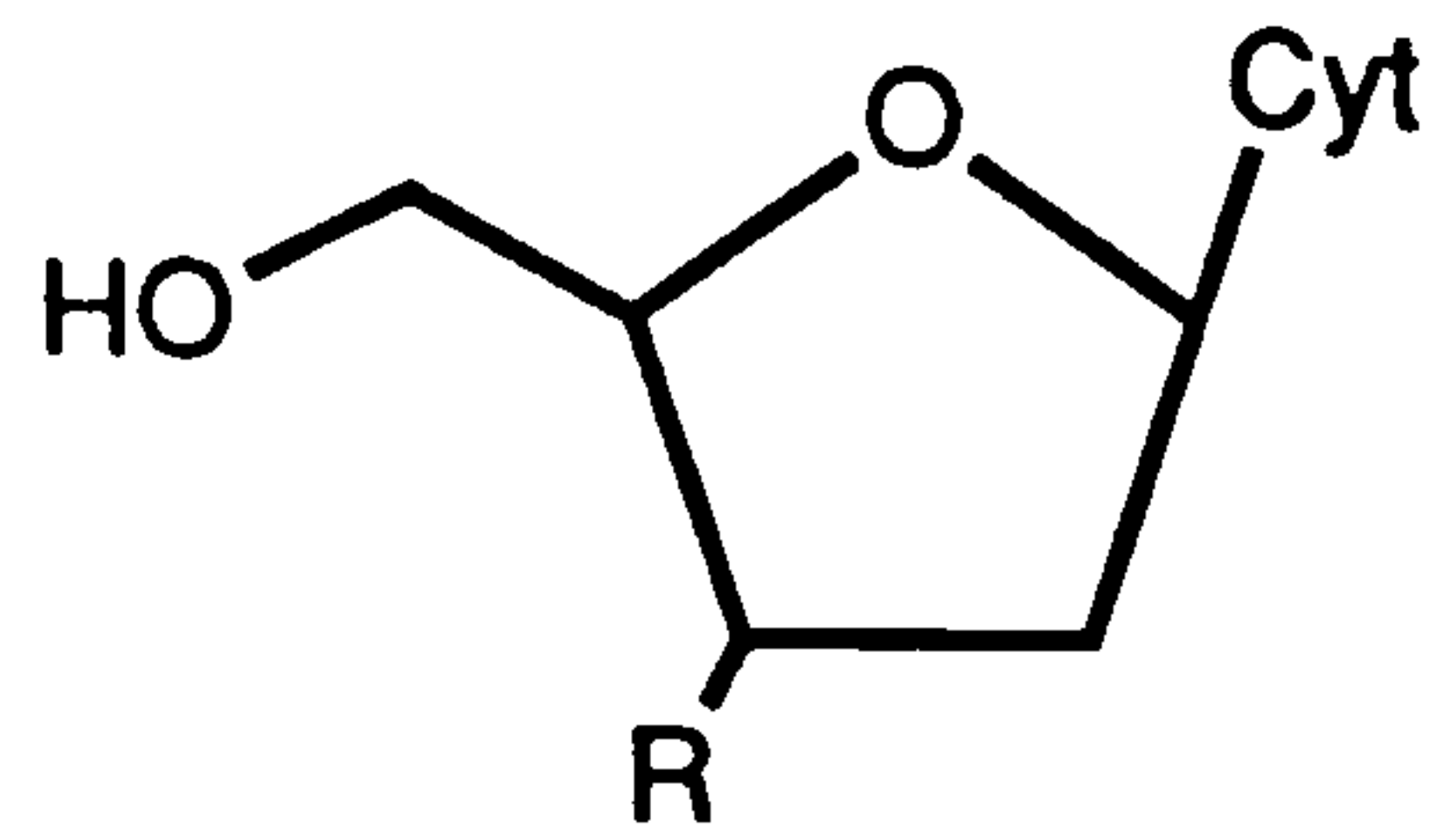


In contrast to the wide range of bases that act as competent acceptors with N-deoxyribosyltransferase-I and -II, few modifications on the sugar moiety are tolerated. The results demonstrate that analogues that lack a hydroxyl group in the 3'- or 5'- positions are able to be transferred to purines and pyrimidines. With the 3'-position, only a hydrogen can replace the hydroxyl group to retain transfer capability, but at the 5'- site a number of substitutions are tolerated by the enzymes (Figure 3.5). Similar reactions have been demonstrated with 2',5'-dideoxy-5'-fluorothymidine [110], and 2',3'-dideoxyribonucleosides [100], with nucleoside N-deoxyribosyltransferases from *L. helveticus*. Substitutions of hydrogen atoms on the pentose ring with hydroxyl groups abolishes activity. Two carbocyclic deoxyribonucleoside analogues of unknown structure were provided by Glaxo Group Research, but neither was active as a substrate. Likewise, no arabinose sugar tested was accepted by the enzymes. Observations that a number of 3'-substituted-2'-deoxyribose sugars were not capable of participating in the transfer, led to the examination of 3'-fluoro substituted sugars (1).

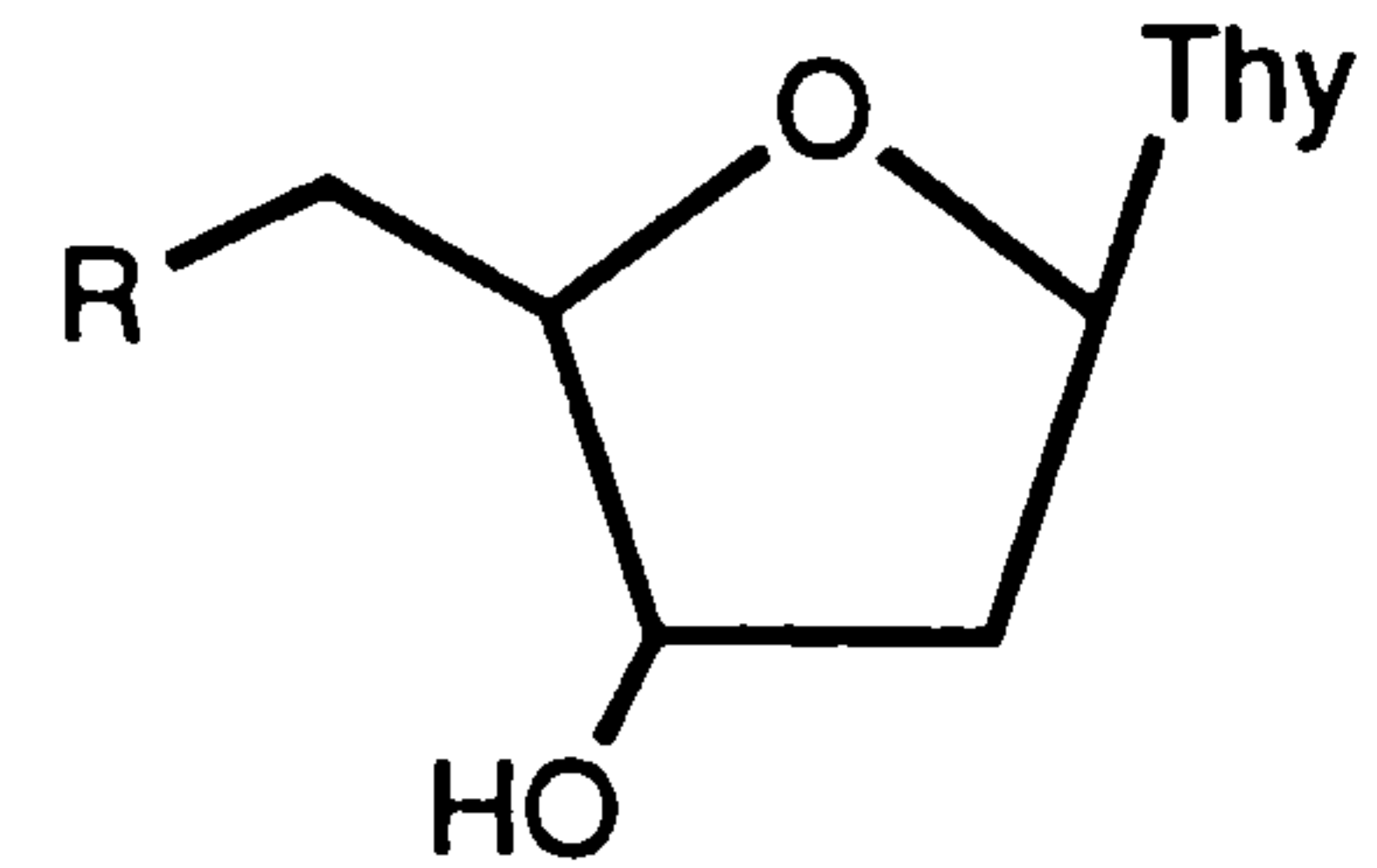
Figure 3.5

STERIC REQUIREMENTS OF SUGARS

**Active**

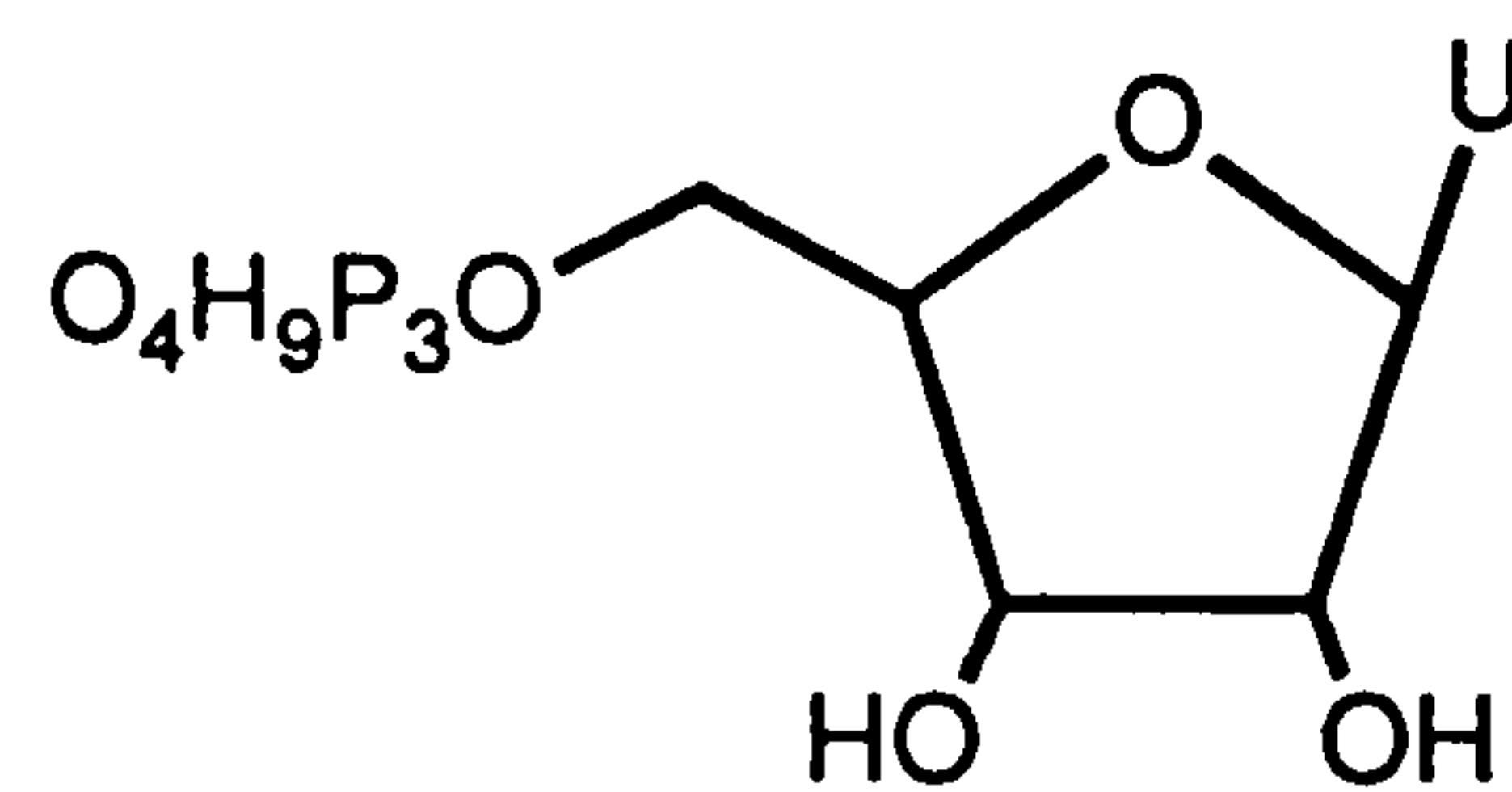
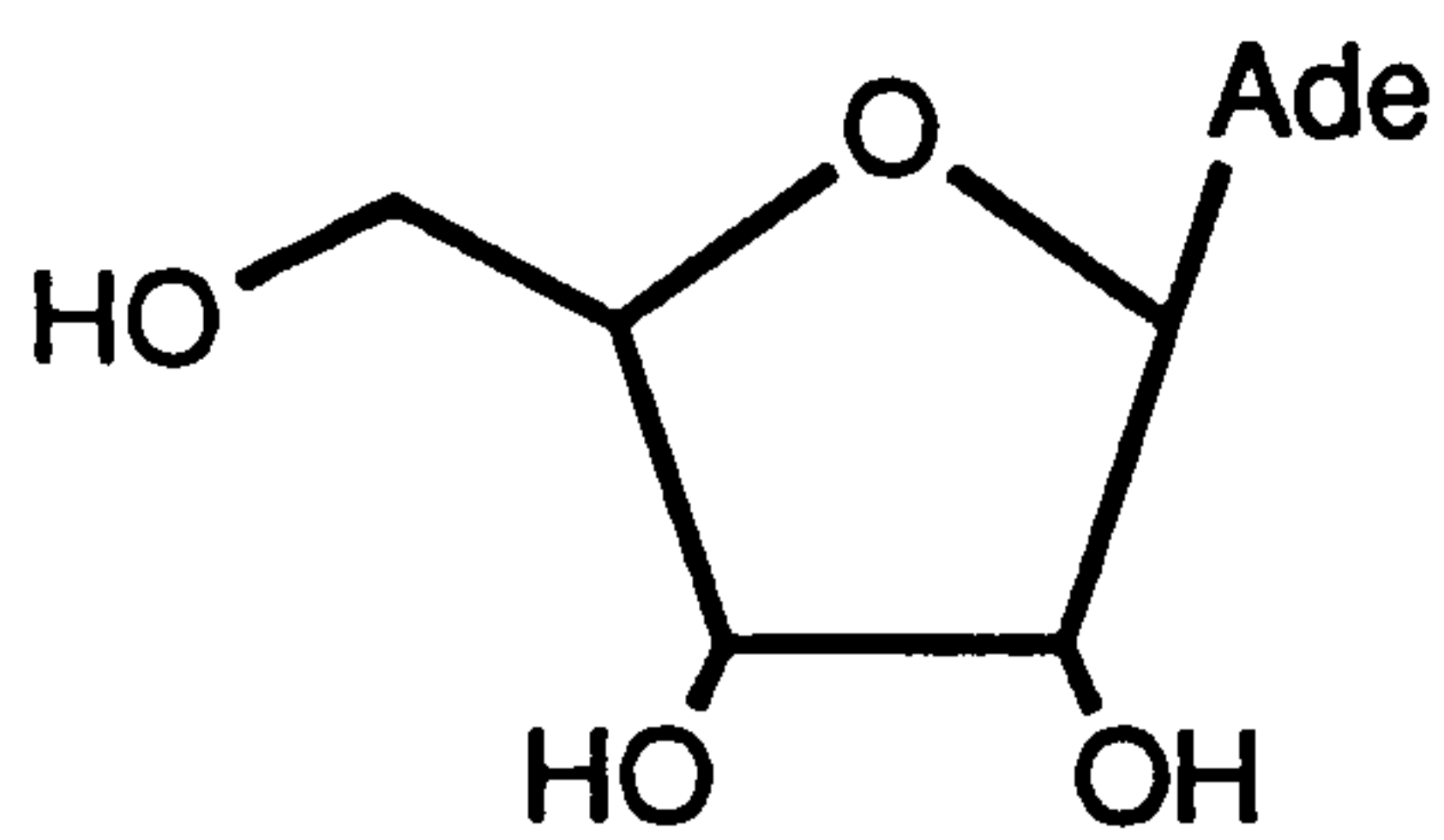


where R = H, OH

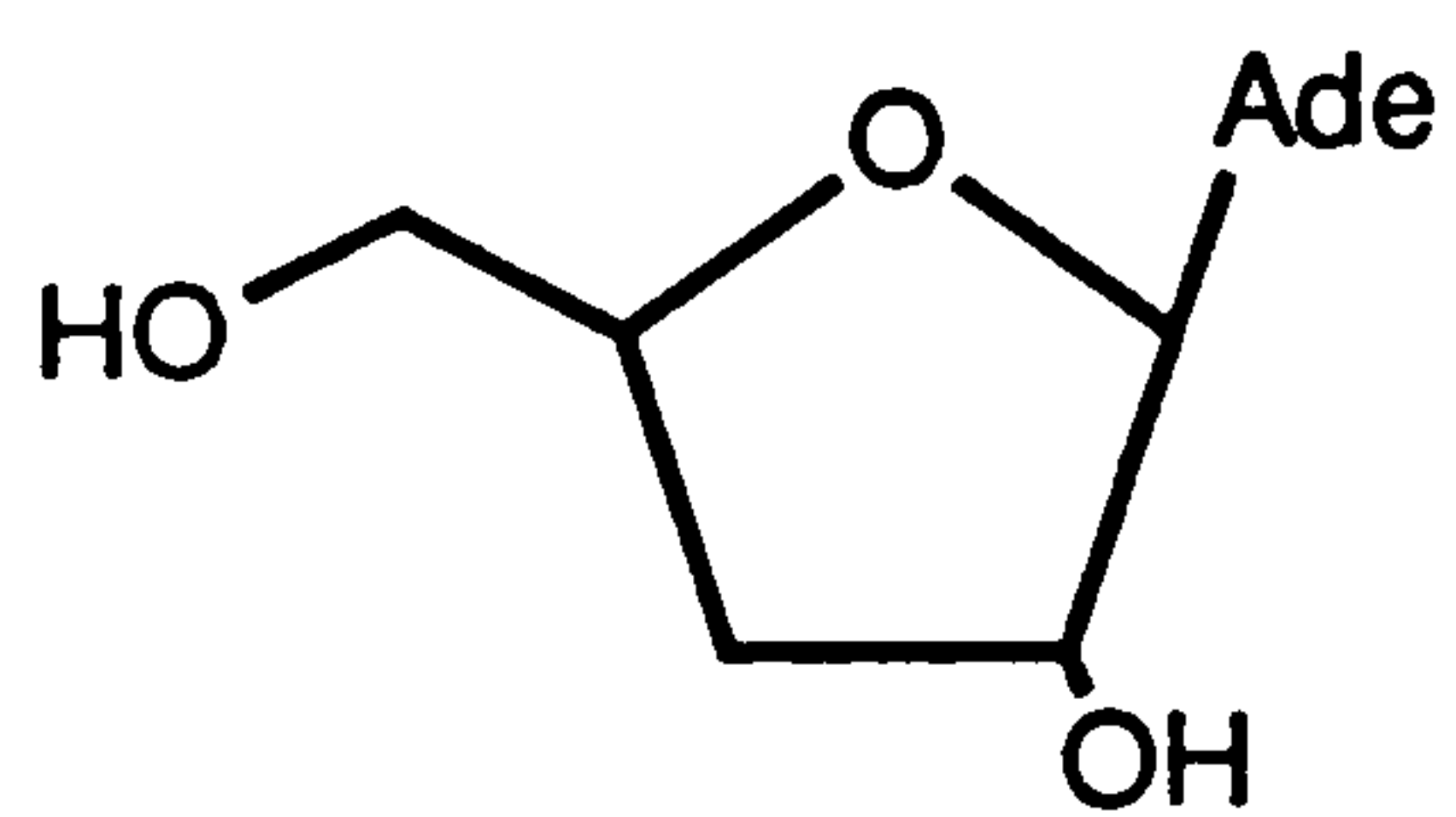


where R = H, OH, Cl, I

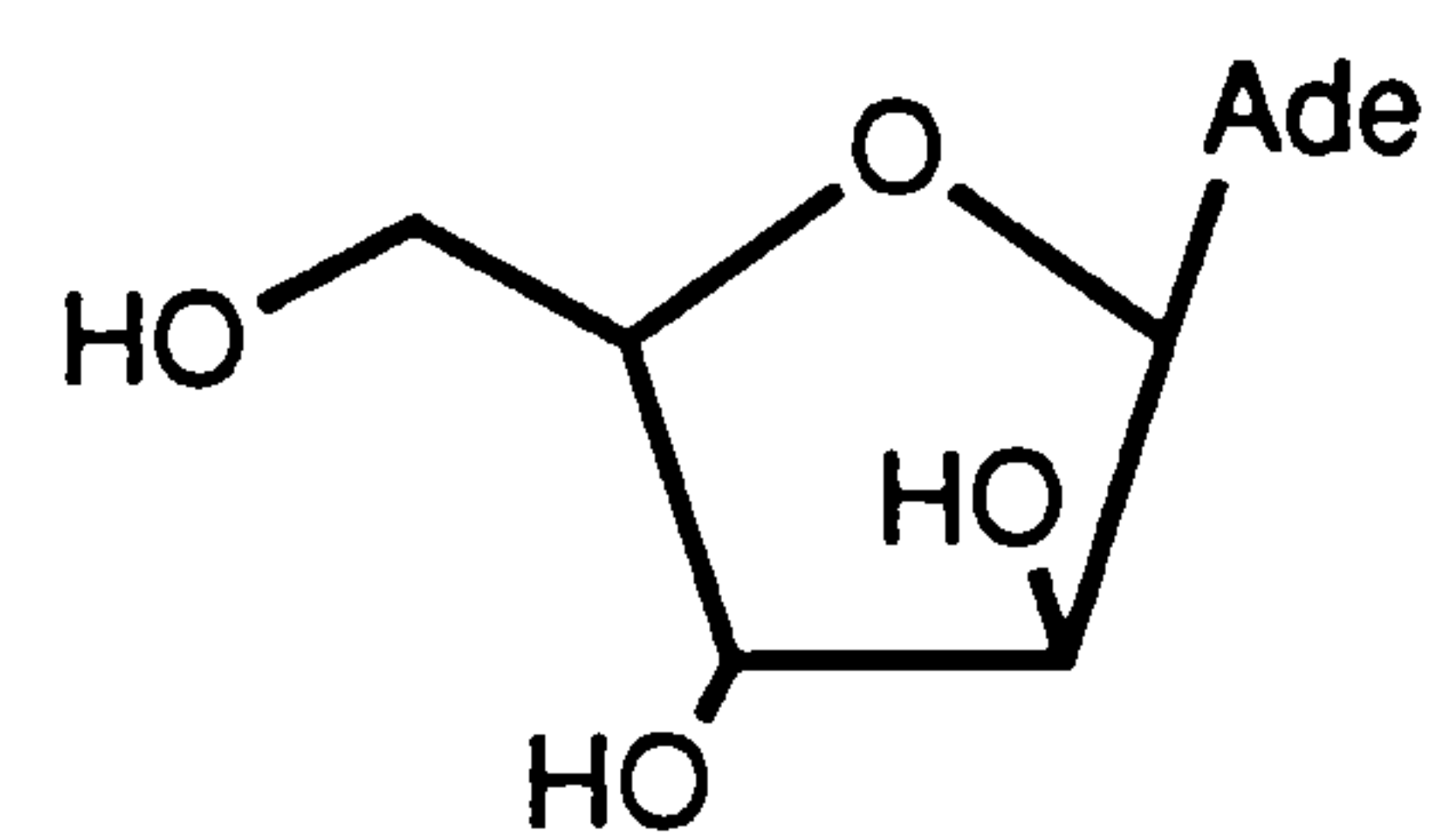
**Inactive**



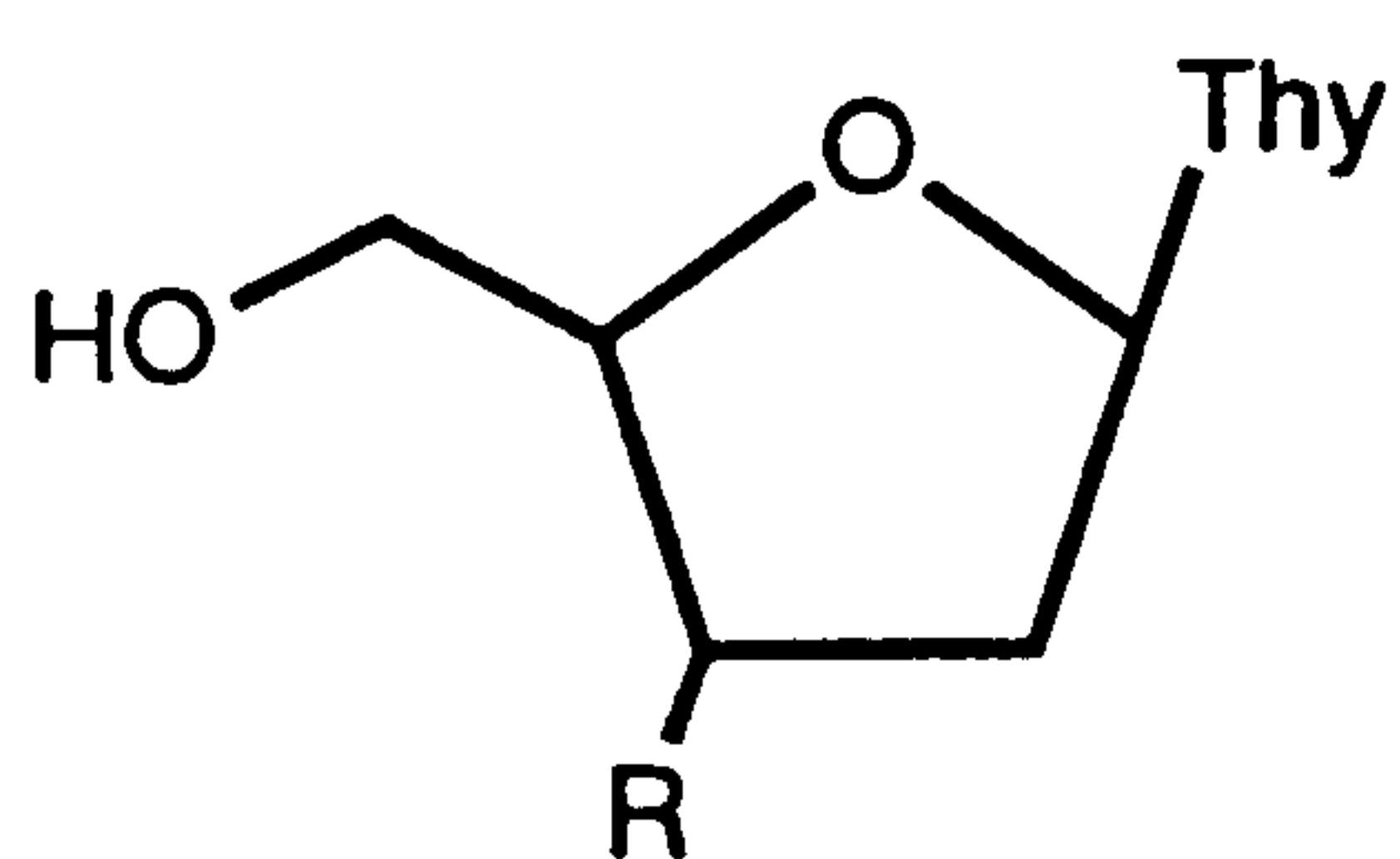
Adenine, uracil etc. ribosides or their 5'-triphosphates



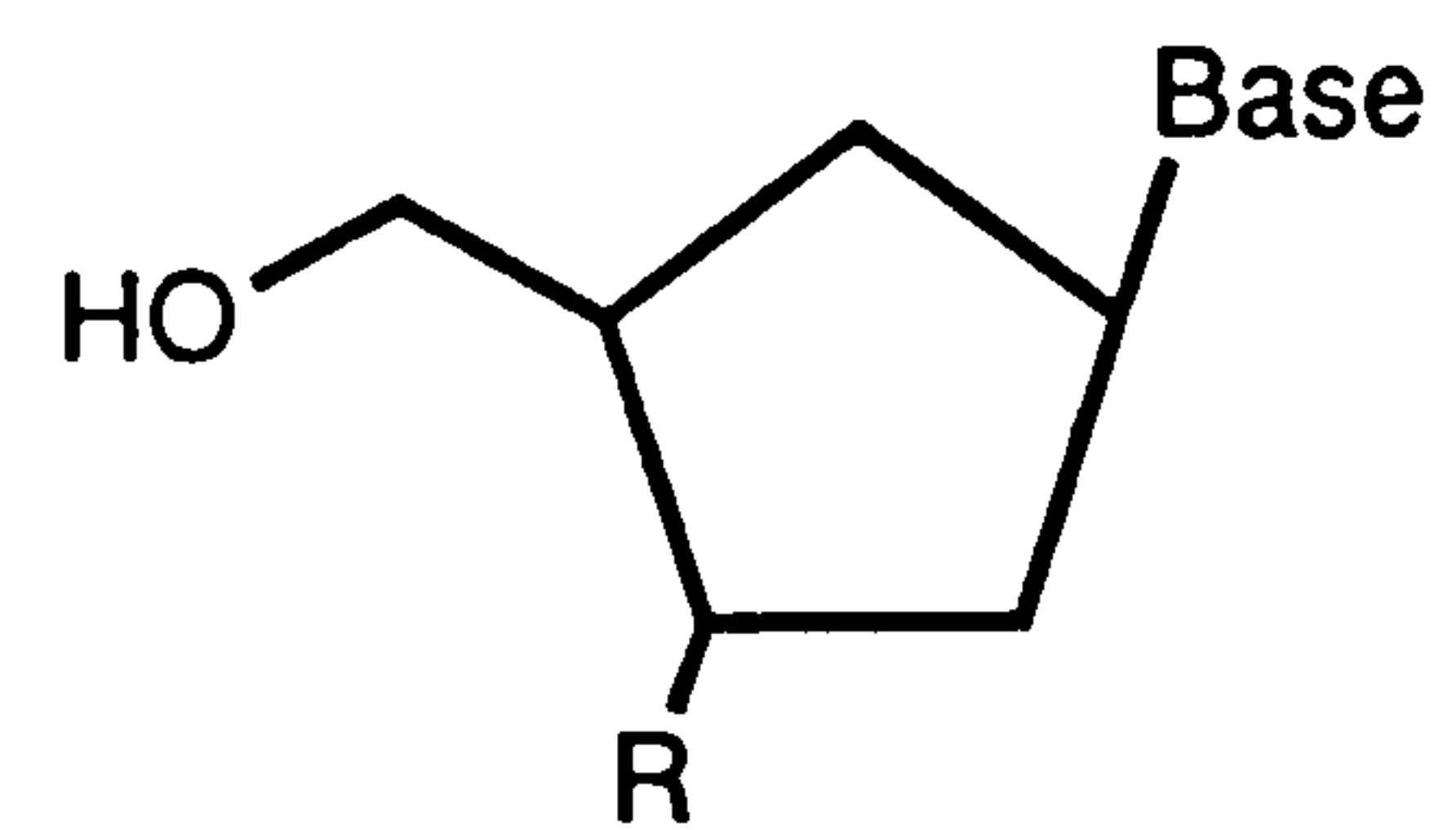
Adenine-9-(3'-deoxyribosides)



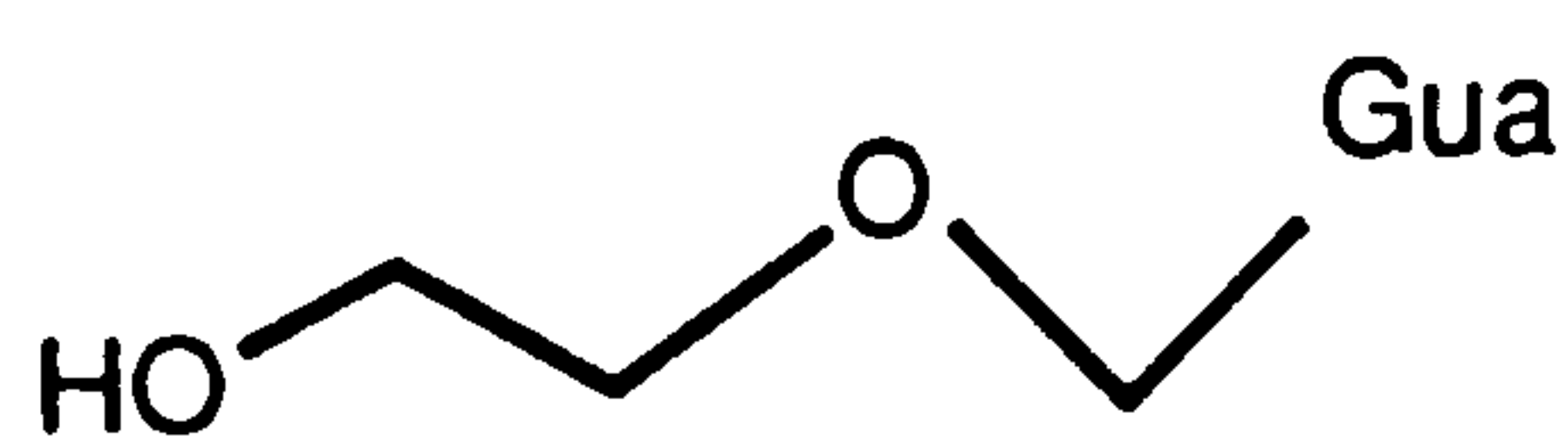
Adenine arabinoside



where R = NH<sub>3</sub>, N<sub>3</sub>, F, Cl, I

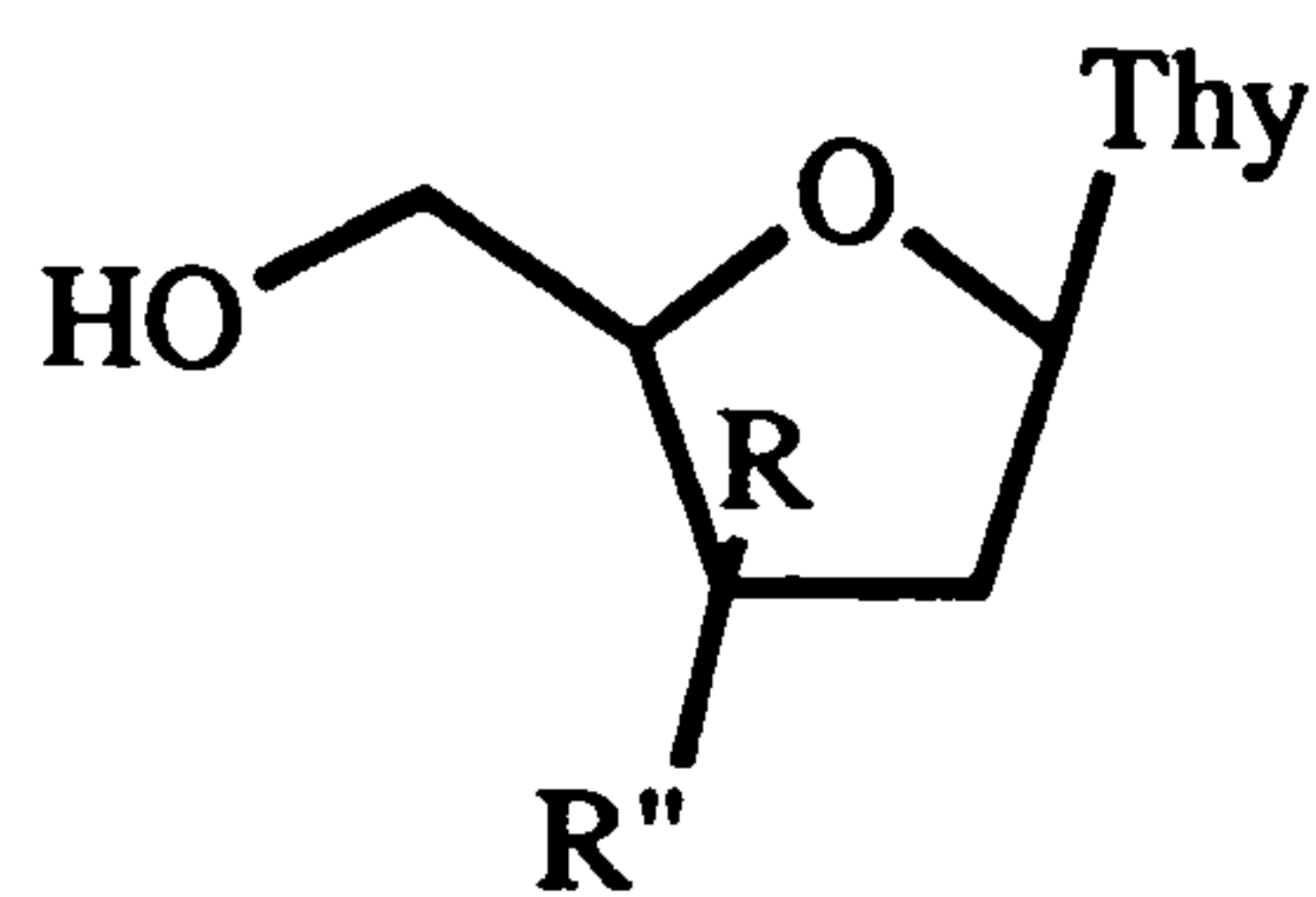


carbocyclic analogues



acyclic nucleosides





(1)

Because the fluorine group is of very similar size to the hydroxyl group it replaces, the aim was that such a substitution would provide information on the role of the 3'-hydroxyl in hydrogen bond interactions. The fluorine cannot act as a hydrogen bond donor, although it can be weak acceptor. Although none of the fluoro-substituted sugars were able to be transferred, some degree of hydrolysis was observed indicating that the compound actually binds to the enzyme. This could be because the sugar is binding weakly or in the wrong conformational state for catalysis. It is recognised that, depending on the substituents, the pentose ring can adopt different conformations. This preference may account for the unstable binding of the fluoro-substituted sugars. Thus, the 3'-hydroxyl plays a central role in deoxynucleoside recognition and binding of the sugar moiety for efficient transfer activity.

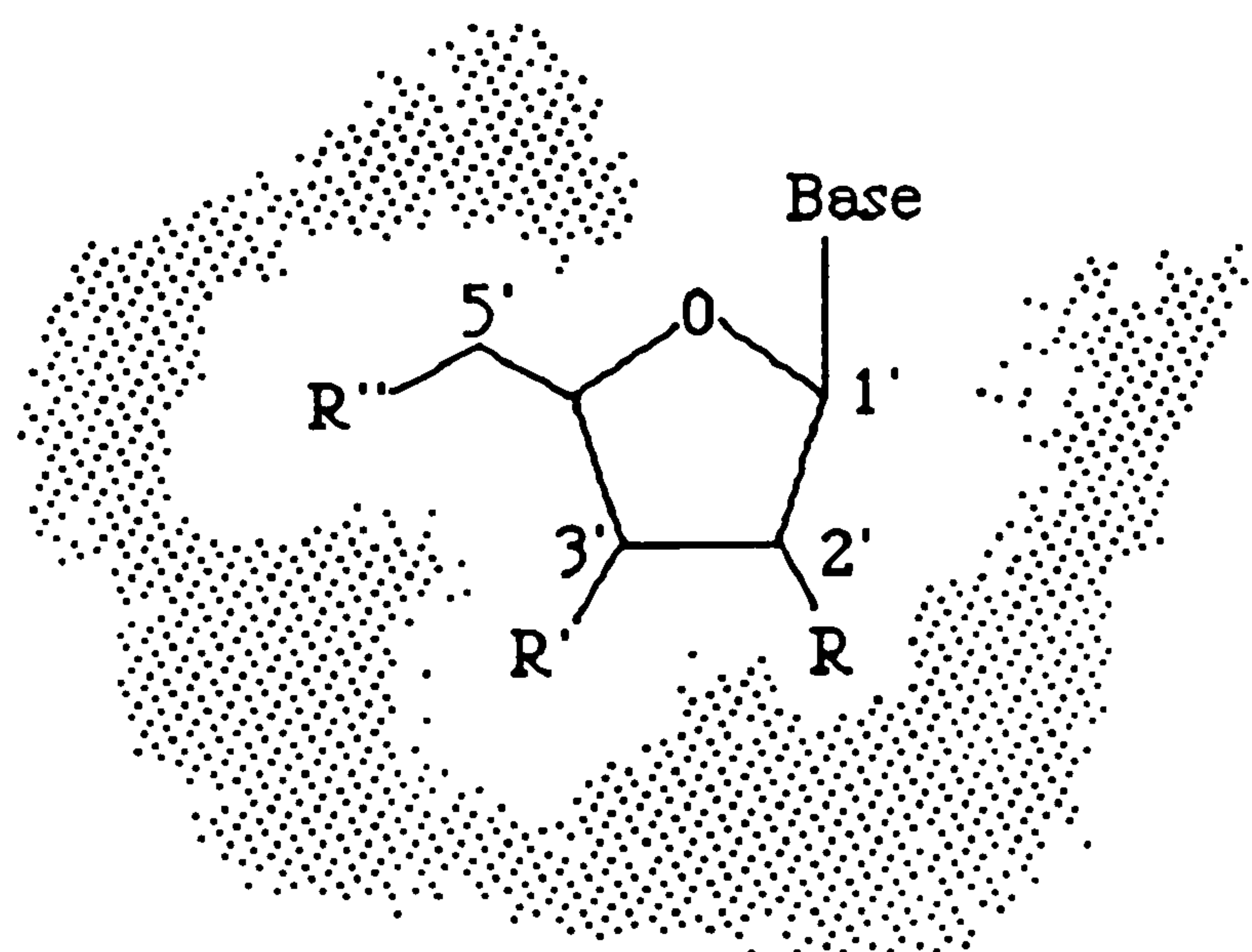


Figure 3.6

The structural features necessary for activity with these enzymes have been defined by this study. Not only is this information important in assessment of the enzyme recognition and binding, but also when N-deoxyribosyltransferases are used as biocatalysts in the preparation of nucleoside analogues. Although both forms of N-deoxyribosyltransferase from *L. leichmannii* are suitable in these biotransformations, N-deoxyribosyltransferase-II is preferred as a result of its greater thermal stability (Figure 2.7).

Crude extracts have been used to synthesize milligram quantities of the deoxyribosides 1-deazapurine [32] and 4-amino-imidazole-5-carboxamide, [119] and the 2',3'-dideoxynucleosides of N-(6-hexyl-)substituted purines [124]. Since there appears to be some correlation between the conformational states of the sugar rings and the antiviral activity of the resultant nucleoside [125], knowledge of the preference exhibited by the N-deoxyribosyltransferase enzymes for these different conformers would also be useful in the design of therapeutically active nucleoside analogues.

Consideration of this aspect of the active site also allows the structural features required by an inhibitor to be identified. This enables the design of inhibitors for studies such as chemical modification, and the construction of affinity adsorption columns for enzyme purification.



## CHAPTER 4

### ACTIVE SITE MODIFICATION

#### 4.1 GENERAL BACKGROUND

The principal step in an enzymatic reaction is the recognition and binding of a specific ligand or substrate. As detailed in the Introduction, the resulting interaction occurs at a defined binding site at or near the surface of the enzyme which is shaped by a number of amino acid side residues whose side chains contact the substrate. The residues in or around this active site can either participate in the bond-breaking and bond-making events that proceed during chemical catalysis or contribute to the flexibility and stability of the binding pocket. Auxilliary amino acids are involved in securing the precise orientation of the substrate for catalysis. Other residues outside the active site provide a foundation for the binding groups maintaining the necessary three-dimensional protein structure in solution to facilitate efficient catalysis [1]. These amino acids play no dynamic role in the enzyme's action.

The detailed characterization of the active site has remained of central importance in the investigation of the structure-function relationship of enzymes providing a major contribution to our understanding of the chemical events of catalysis. Information about the nature of reaction intermediates and transition states can be extrapolated to define the geometry of this site. In the same way the unique properties of high catalytic efficiency and

the decisive specificity of individual enzymes can be explained by the participation of several distinct functional amino acid groups within the active site.

There are many different techniques which are used to locate and define functional groups at catalytic sites of enzymes. One of the most powerful is X-ray crystallography. Application of this method to native enzymes and enzyme-substrate complexes can provide detailed three-dimensional analyses of protein structure and topography. However, there are practical limitations to its routine use and in order to perform this expensive and time-consuming analysis the protein must first be available in a crystalline state. Questions are still raised however, as to the validity of models for enzymes in aqueous solutions constructed from these data. There are more simple procedures however, that can provide good preliminary information about the nature of the enzyme structure and action.

- (1) Substrate specificity studies examine the interaction of the enzyme with chemically modified substrates or inhibitors.
- (2) Chemical modification of specific amino acid residues using group selective reagents.
- (3) Affinity labelling using "designer" substrate analogues to effect specific binding.

The aim of this chapter is to show how the technique of chemical modification can be usefully employed to predict what amino acid groups may reside at the active site of the N-deoxyribosyltransferase-II enzyme. In doing so an attempt will be made to assign specific roles for these residues in the processes of binding and glycosyl transfer.



## 4.2 CHEMICAL MODIFICATION

Chemical modification of an enzyme involves the covalent binding of a chemical reagent to an amino acid side chain in the enzyme. Modification is usually accompanied by a change in the enzyme, often observed as a fall in the enzyme activity. This loss is seen if an 'essential' residue is blocked, if substrate binding is sterically impeded or if the conformation of the native protein has been altered leading to a distortion of the active site pocket. Ideally the loss of activity should be seen as a result of selective binding of a reagent to a single amino acid or amino acid type in conditions where conformational perturbations arising from the effects of the reagent are kept to a minimum.

Only those amino acids with polar side-chains are usually receptive to these modifying agents and their reactivity is primarily a function of their nucleophilicity. Residues with reactive side-chains can be classified into those with acidic moieties (aspartate & glutamate), basic groups (arginine, histidine & lysine), polar, uncharged side-chains (cysteine, serine & tyrosine), and those amino acids with reactive moieties on their side-chains (methionine & tryptophan) [126, 127].

In practice the selectivity of the reagents is not absolute and reaction can occur with more than one type of residue. Modification affecting non-essential amino acid residues not directly involved in catalysis or in the binding and recognition of ligands can result in loss of activity due to conformation changes to the enzyme. Therefore, careful assessment of results must be made before the assignment of roles to any residues suspected of

catalytic participation. However, diligent application of this technique to probe the active site can provide a great deal of information in the initial stages of mechanistic interpretation. Further information can be gained by performing these modifications in the presence or absence of substrates or inhibitors of the enzyme and monitoring their affect on the velocity and degree of modification. These compounds can act as protecting agents, masking vulnerable amino acid side chains from the effect of modifying reagents. The exploitation of certain requirements and properties of these reactions such as their pH dependence and the ability to reverse selected modifications to recover altered biological activity also enables more detailed analysis. An example of this type is seen with the base-mediated reversal of the diethyl pyrocarbonate modification of histidyl groups, where hydroxylamine can be used to displace the carbethoxy group from the covalent carbethoxy-imidazole complex (Figure 4.1)

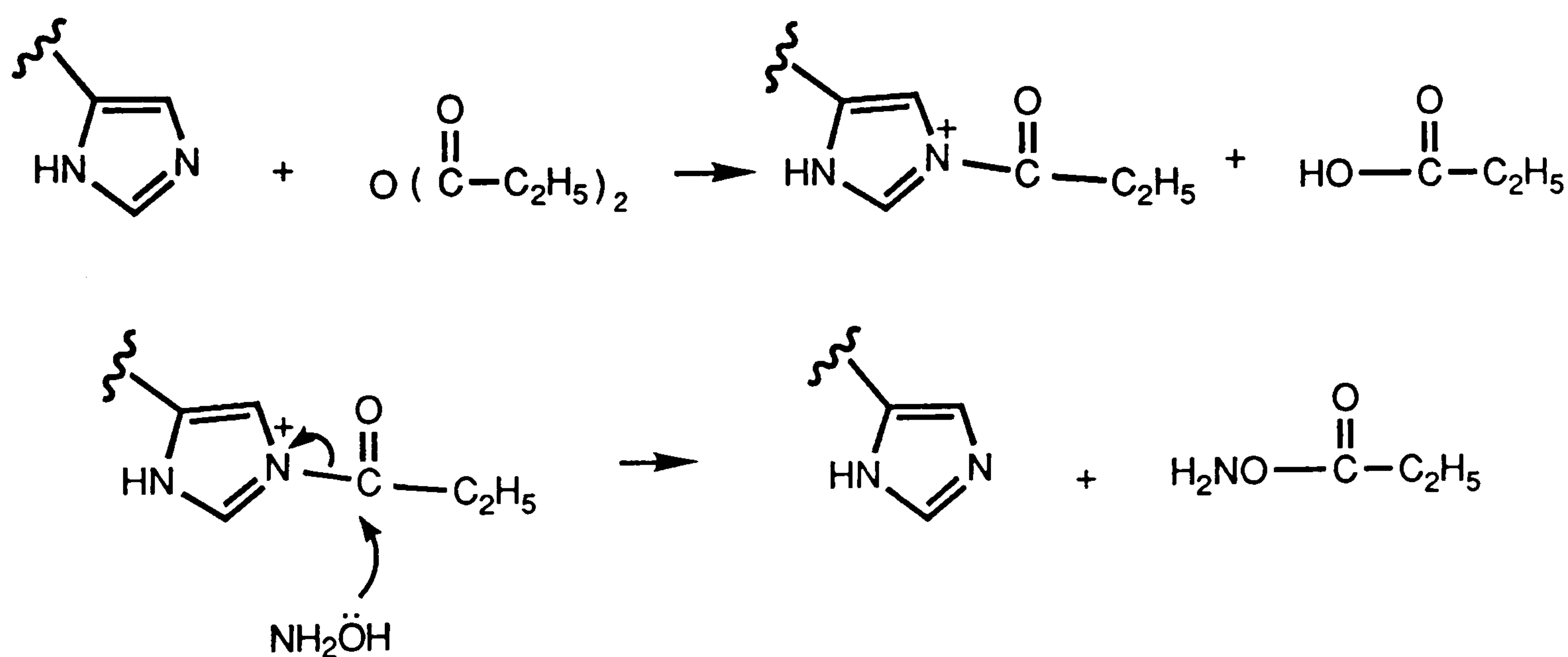


Figure 4.1



There are a number of variables arising from the influence of the protein environment which can affect the reactivity of certain functional groups. Enzymes possess microenvironments where functional groups may exhibit reactivities quite different from those expected for side chains of isolated amino acids. The active site is such a niche and commonly this results in groups at the site with widely different pKa values to those predicted and this situation provides the means to take advantage of the pH dependence of most modification reactions. Most of the reactions are carried out at or around the pKa of the target group where the best selectivity can be achieved. Hydrogen bonding can act to stabilise species to either enhance or depress group reactivity and the protein matrix can lower the rate of certain reactions by providing protection from the reagent.

In practice the native enzyme is incubated with a large excess of a group-specific reagent over that of the protein concentration in conditions suitable to the reactivity of the target residue and the enzyme stability. At intervals, aliquots of the reaction mixture are removed and assayed under standard assay conditions for enzyme activity.

Despite extensive investigations of the kinetic mechanism of the transfer reactions of N-deoxyribosyltransferases, very little work has been carried out to examine the nature of the precise groups involved in catalysis. Uerkvitz [89] demonstrated that when deoxyribosyltransferase from *L. helveticus* was treated with the sulphhydryl modifying reagents, p-chloromercuribenzoate and iodoacetamide, no reduction in enzyme activity was observed and

thus deduced that residues with thiol groups in the enzyme were not essential for the transfer activity.

Outlined below are the methods chosen for the specific chemical modification of selected amino acids.

#### 4.2.1 CHEMICAL MODIFICATION REACTIONS EMPLOYED

##### 4.2.1.1 *Modification of Arginine Groups*

The highly basic guanidium group of arginine is usually modified by condensation with dicarbonyl compounds [128]. The majority of procedures employ reagents like 1,2-cyclohexanedione (CHD), [129, 130] 2,3,-butanedione or phenylglyoxal [131]. The phenylglyoxal reaction is irreversible and can be performed under mild conditions in alkaline media. The derivative contains two phenylglyoxal moieties per guanidium group. The  $\epsilon$ -amino groups of lysine are also susceptible to modification under these conditions, but react slowly compared to arginine groups. The products of this reaction can be reversed by incubation with hydroxylamine.

The guanidium group of arginine residues can also condense with 1,2-cyclohexanedione at pH 8-9 in sodium borate buffer. Reaction in this buffer favours the production of a single, stable product (Figure 4.2).



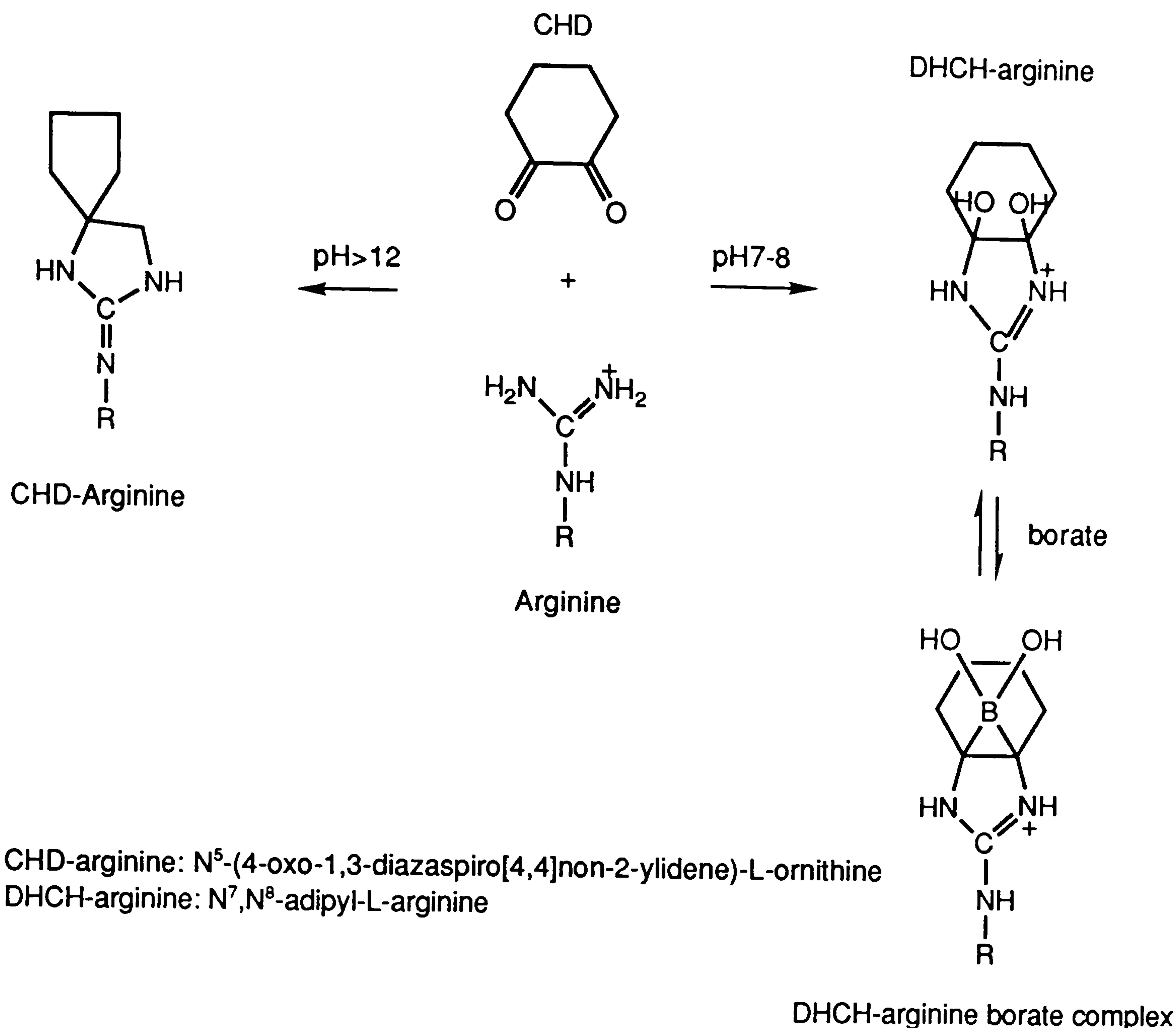


Figure 4.2

In strong alkali, a single derivative, CHD-arginine is formed. At pH 7-8 the reaction also produces a single product DHCH-arginine [130]. Arginyl residues can be regenerated from DHCH-arginine by incubation in hydroxylamine at neutral pH.

#### 4.2.1.2 Modification of Carboxyl Groups

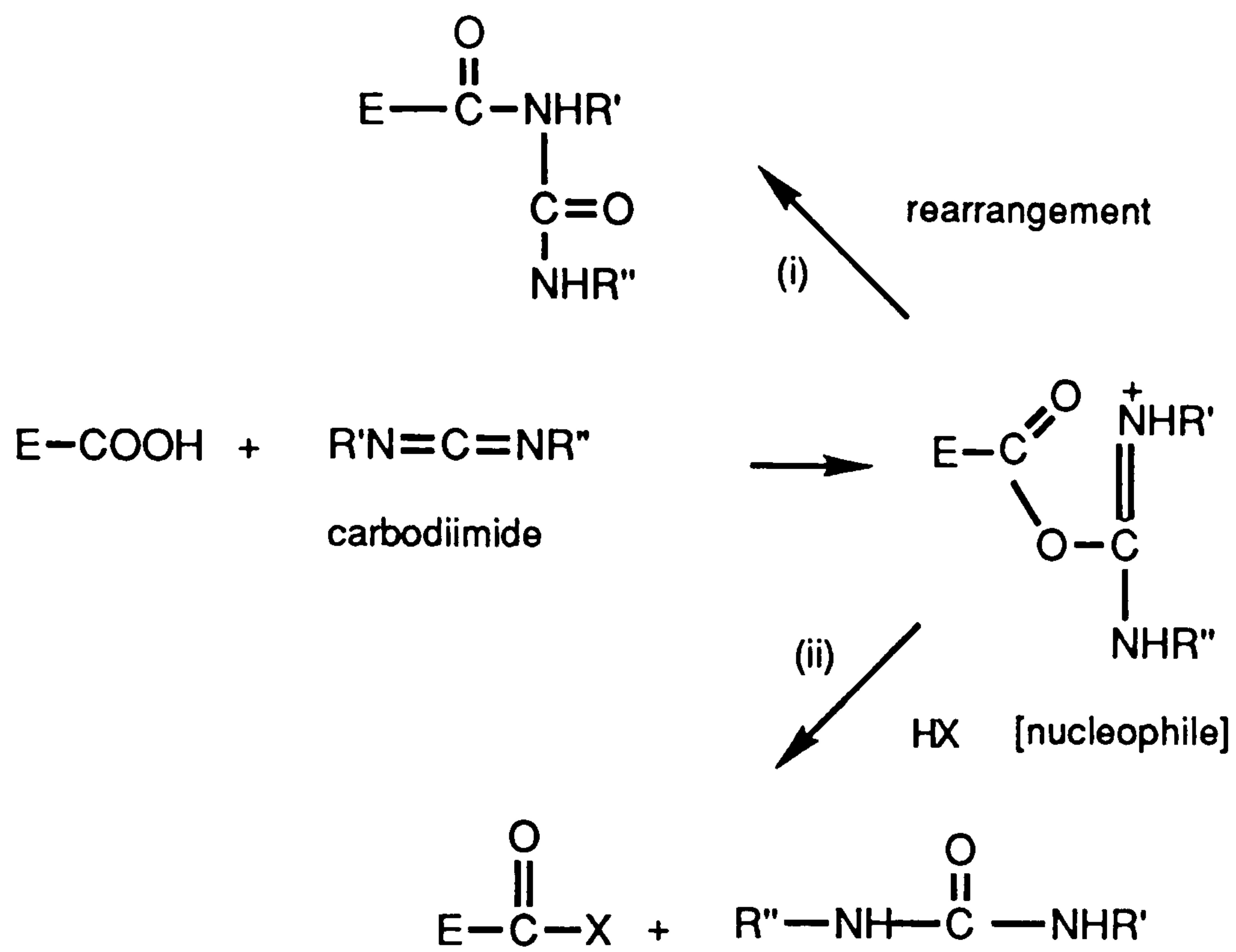
Most methods for the modification of carboxyl groups in proteins involve the use of water-soluble carbodiimides [132, 133]. These react with carboxyl moieties at acidic pH to generate an activated O-acylisourea intermediate. There are two possible routes of reaction for this intermediate: rearrangement to generate an N-

acylurea via intramolecular acyl transfer, or reaction with a nucleophile to yield the acyl-nucleophile product plus urea (Figure 4.3). It has been demonstrated that if the reaction mixture contains a high concentration of the nucleophile, the path to rearrangement is virtually abolished [134, 135]. Side reactions can occur with tyrosyl [136] and sulphhydryl [137] groups.

Tyrosyl modification can be slowly reversed with hydroxylamine but sulphhydryl groups require protection prior to carbodiimide treatment. An advantage of this method is the two stage nature of the reaction, allowing greater versatility towards varying reactants and conditions. Carbodiimides do have some practical limitations in these reactions including their instability in aqueous solution [138] and a tendency to cause some proteins to polymerize [139]. The use of a very strong attacking nucleophile has been used to good effect in lessening the degree of polymerization [139].

Isoxazolium salts are also used to determine the presence of carboxyl groups [140, 141, 142]. The reagent N-ethyl-5-isoxazolium-3'-sulphonate or Woodward's Reagent-K (WRK), has been used in a number of different modifications reactions with proteins both alone, and in conjunction with carbodiimides to demonstrate the existence of two different functional carboxyl groups [143].





Where E = enzyme; R' and R'' are alkyl groups

Reaction of protein carboxyl groups with carbodiimide followed by (i) rearrangement or (ii) nucleophilic attack

Figure 4.3

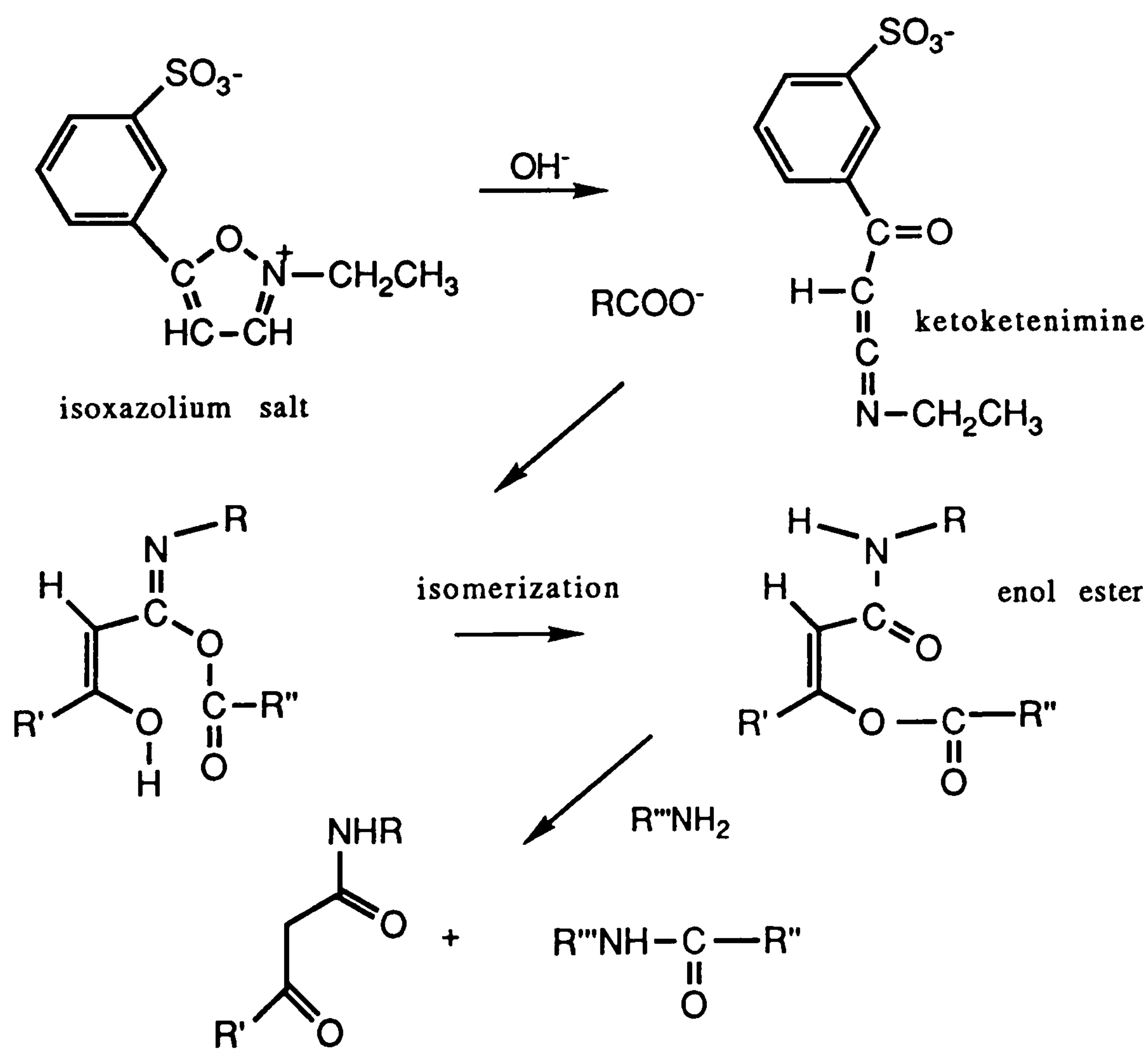


Figure 4.4

The active species in this reaction is thought to be a highly reactive intermediate ketoketenimine [140, 142] (Figure 4.4). A method has been developed so that the progress of the reaction can be followed spectrophotometrically [144]. However, Woodward's Reagent-K is not without its problems as a modifying agent, one of its major limitations being that it is very unstable above pH 3 [140, 142].

#### 4.2.1.3 Modification of Tryptophan Groups

The modification of tryptophan residues usually employ oxidative methods whereby the indole moiety is transformed to the oxindole group (Figure 4.5). The generation of a significantly weaker chromophore enables the oxidation to be followed spectrophotometrically by monitoring the decrease in absorbance at 280nm and allows quantitative analysis of the modification. N-bromosuccinimide (NBS) [145] is often the reagent of choice but is so reactive that it remains somewhat unselective and side reactions include peptide bond cleavage in addition to the oxidation of tyrosine, methionine, cysteine, histidine and arginine side-chains. Thus, modification by this method must be interpreted with some caution.

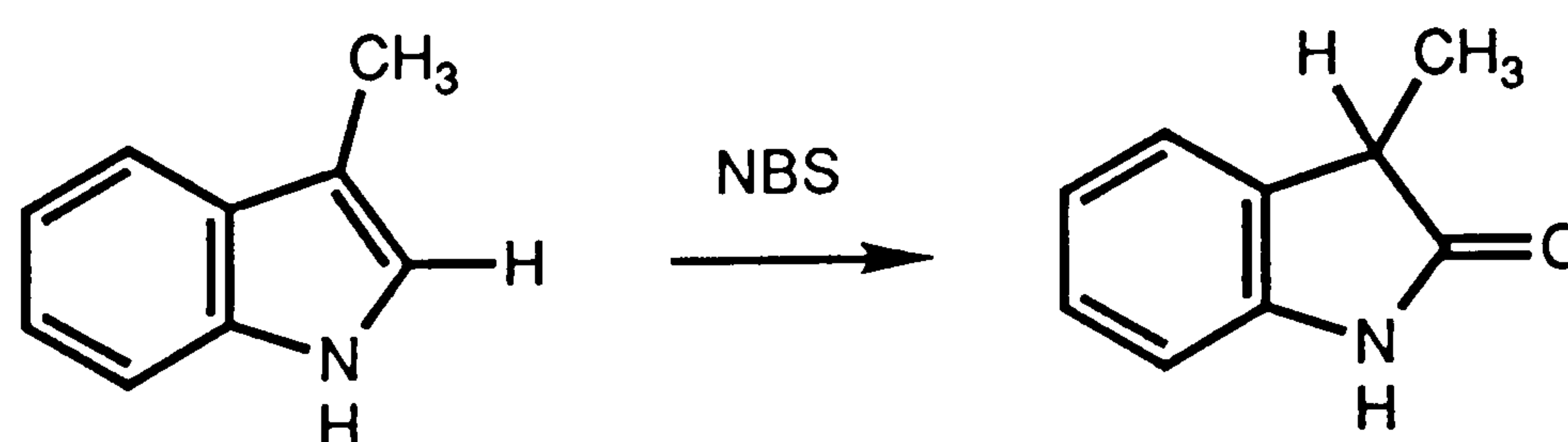


Figure 4.5



Other reagents for modifying tryptophan side chains include alkyl and benzyl halides. 2-Hydroxy-5-nitrobenzyl bromide (HNB-Br) or Koshland's reagent-I, is known to react rapidly and selectively over a wide range of pH with tryptophan residues [146, 147, 148]. The mechanism by which this reagent works is complex and a number of products can be generated from its reaction with tryptophan groups in a protein. The primary reaction is thought to involve the formation of a 1:1 adduct across the 3-position of the indole ring, but the final products seem to depend on the nature of the groups in the neighbourhood of the tryptophan which is being modified [149].

#### 4.2.1.4 Modification of Lysine Groups

2,4,6-Trinitrobenzenesulphonate (TNBS) is used under alkaline conditions to facilitate the modification of  $\epsilon$ -amino groups [150, 151] (Figure 4.6).

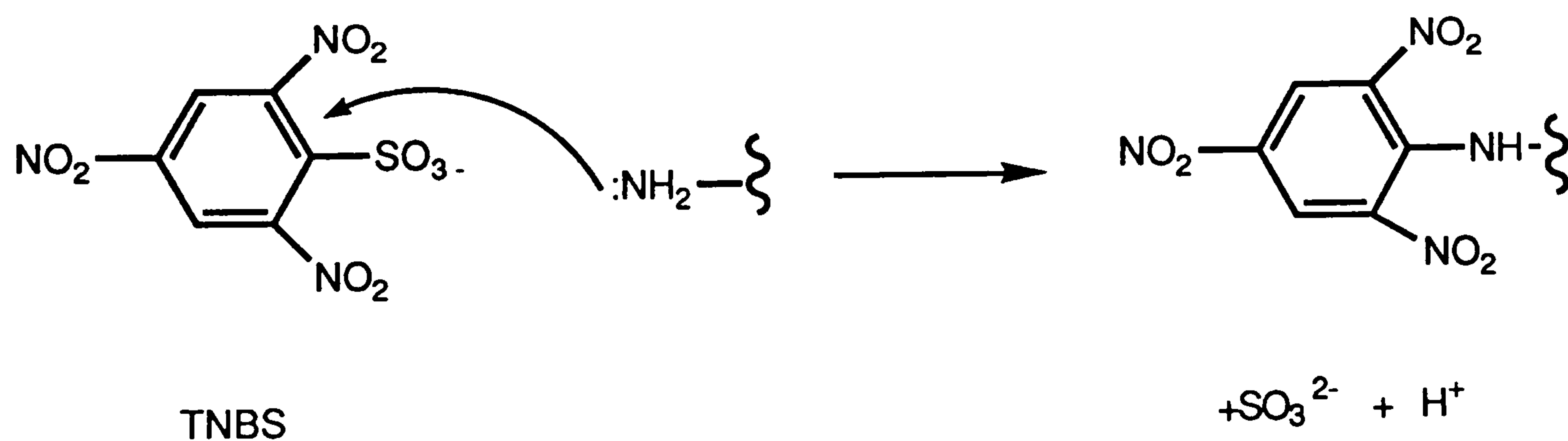
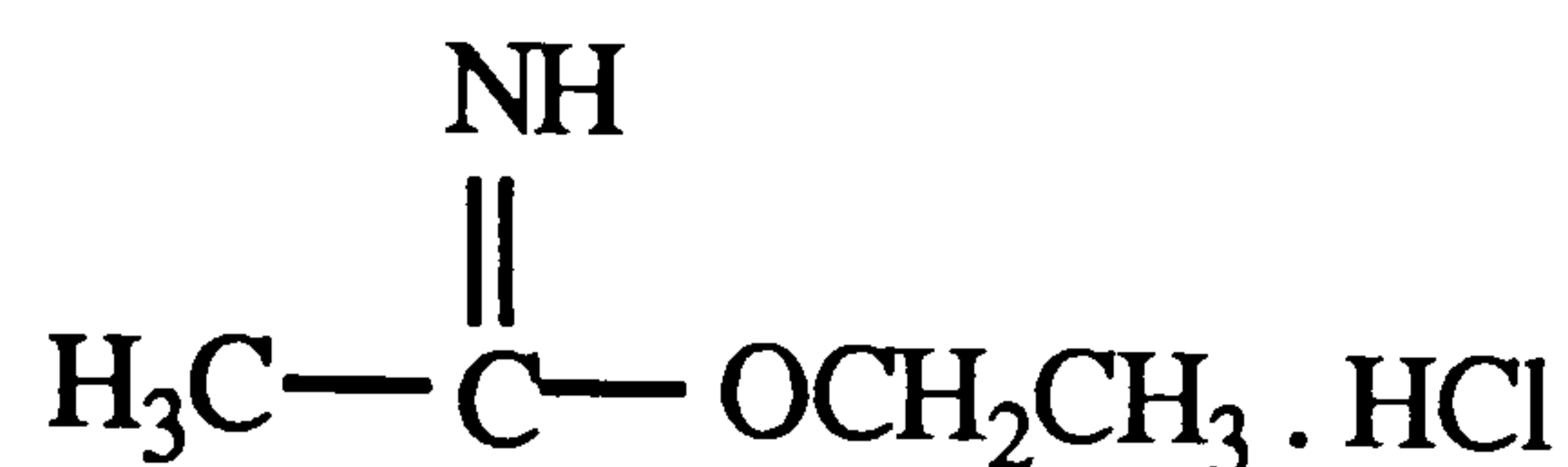


Figure 4.6

Imidoesters such as ethylacetimidate (1) are also used around at high pH to modify amino groups [152].



(1)

#### 4.2.1.5 Modification of Cysteine Groups

The high degree of reactivity of the thiol groups on cysteine residues has permitted the design of a number of modification agents. Among these are a few which are selective and which react rapidly under mild conditions [153]. *p*-Chloromercuribenzoate (*p*-CMB) [154, 155] and *N*-ethylmaleimide (NEM) [153, 156], are often the reagents of choice. Reaction of thiol groups in proteins with these reagents gives rise to spectral changes which enables the modification reaction to be easily followed and quantified. Thiol groups can be readily regenerated from mercaptide derivatives produced by their reaction with organomercurials.

The reagent *N*-ethylmaleimide exhibits good specificity towards cysteine residues at neutral pH and generates stable thiol esters. The reaction is readily quantified due to the change in spectral characteristics on the formation of the adduct (Figure 4.7).

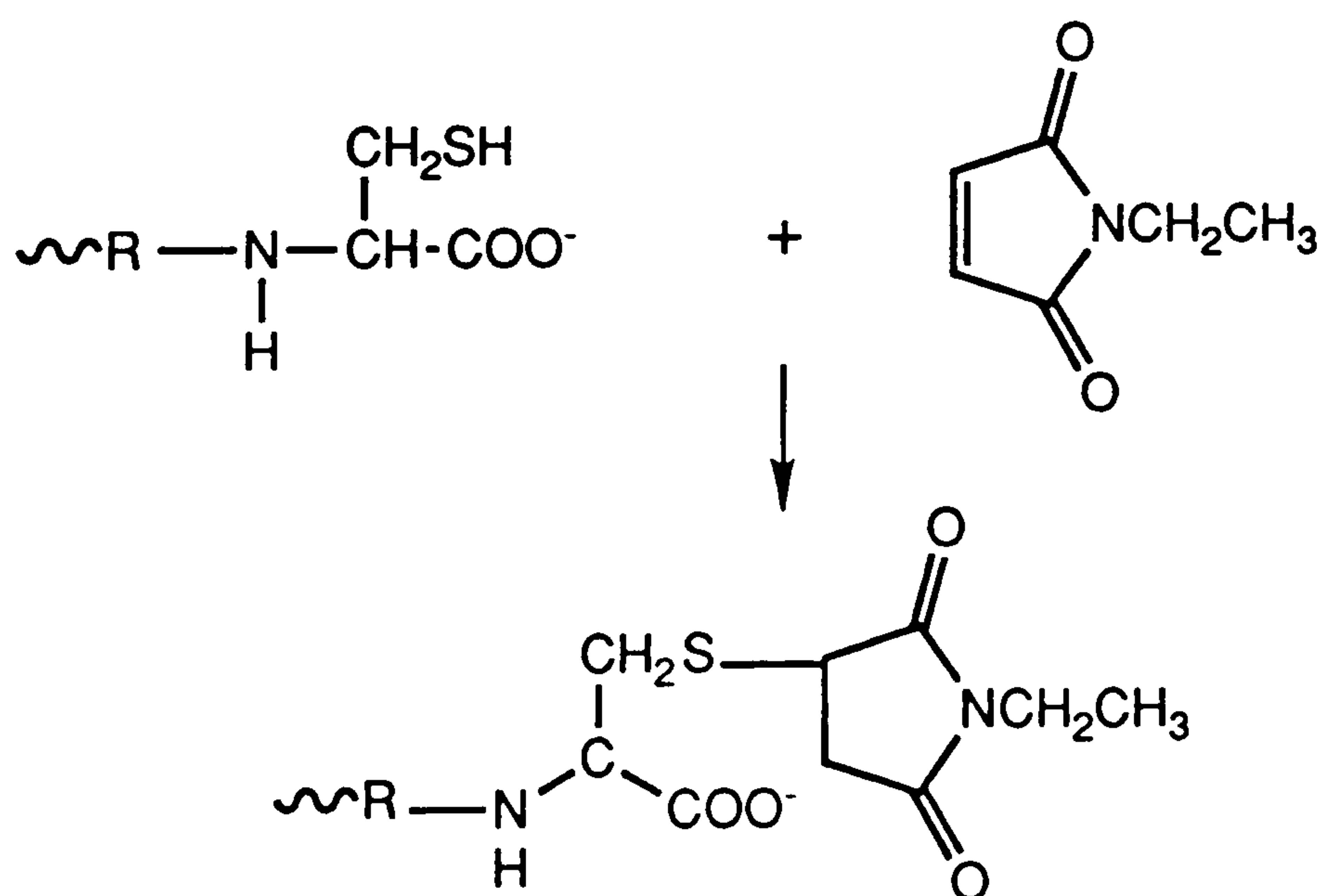


Figure 4.7



#### 4.2.1.6     Modification of Tyrosine Residues

Nitration using tetranitromethane (TNM) is the usual approach to modifying tyrosyl groups on proteins [157, 158, 159]. This highly selective reagent attacks the phenol group of the side chain in alkaline media to produce the O-nitrotyrosine. This proceeds under very mild conditions and the extent of modification can be monitored spectrophotometrically since the O-nitrophenolate anion absorbs maximally at 428nm. Polymer formation due to crosslinking of peptides has been found to be a problem with some proteins despite mild conditions and strict control of the concentration of reagent used [160, 161].

#### 4.2.1.7     Modification of Histidine Groups

The imidazole group of histidine has traditionally been modified by photo-oxidation in the presence of oxygen and a photosensitive dye such as methylene blue or Rose Bengal [162, 163]. The mechanism of the reaction is complex and evidence points to a number of possible reactive species. However, this is not a selective method and a variety of amino acids are susceptible to photooxidation including methionine, cysteine, tryptophan and tyrosine in addition to histidine. Some degree of specificity can be gained by control of the pH of the medium [164] and choice of photosensitizing dye [162, 165]. At neutral pH histidine is the most likely to undergo oxidation. In acidic media methionine, cysteine and tryptophan are all subject to oxidation. That the choice of dye is an important determinant for specific reaction has been demonstrated with the high selectivity of Rose Bengal for histidine residues as compared to methylene blue and other dyes.

Diethyl pyrocarbonate has also been used widely as a histidine-acylating agent for proteins [166-169]. When used near neutral pH, it has a high specificity for the imidazole group of histidine residues. The progress of the reaction can be followed by the increase in absorbance at 240nm due to the formation of the carbethoxyhistidyl group (Figure 4.1). Biological activity can be restored by incubating the modified protein with a nucleophile such as hydroxylamine [168]. DEPC is however, unstable in aqueous media, especially at high pH [170].

### 4.3 KINETICS OF THE N-DEOXYRIBOSYLTRANSFERASE REACTION

To elucidate the mechanism of an enzyme reaction it is essential to delineate the reaction pathway, determining the sequence and nature of these processes and the rates at which they proceed; this includes the detection and characterization of reaction intermediates.

Of those two-substrate reactions which do not involve the formation of a ternary complex, the most important class consist of reactions which proceed via ping pong or enzyme substitution mechanisms. Kinetic studies of the purine:purine and the pyrimidine:purine N-deoxyribosyltransferase activities from *L. helveticus* [82, 83], have shown that the enzymes transfer the deoxyribosyl moiety from a purine or a pyrimidine base by a ping-pong bi-bi mechanism (Figure 4.8).



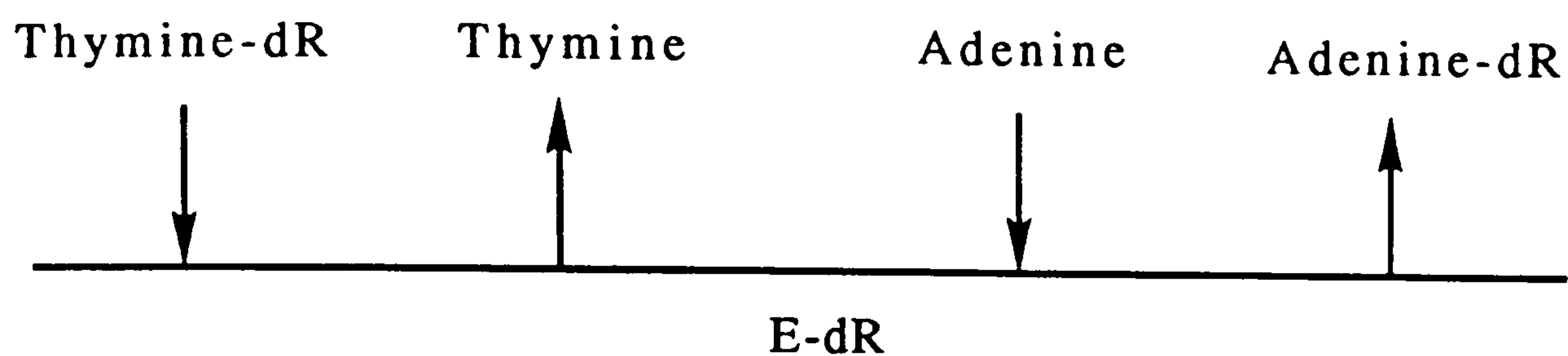
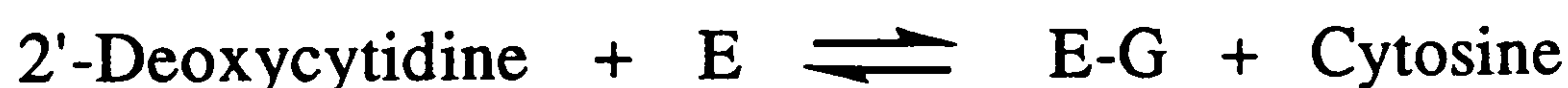


Figure 4.8

There are a number of criteria laid down for the kinetic characterization of this type of mechanism [86], and one of the most important indications that enzyme substitution is operative can be seen when parallel lines are generated in double reciprocal plots for different concentrations of fixed substrates. Other evidence can be obtained by establishing the patterns of substrate and product inhibitions. Holguin showed with the enzymes from *L. helveticus*, that each product is a competitive inhibitor of the corresponding substrate and a non-competitive inhibitor of the other. Having shown that such a reaction route might be functional, confirmatory evidence can be obtained by (a) the demonstration of partial reactions (Figure 4.9):



Where E = enzyme and E-G = Glycosyl-enzyme intermediate

Figure 4.9

and (b) the isolation of the modified intermediate form of the enzyme.

By definition, the ping-pong bi-bi mechanism implies the formation of a covalent-intermediate. Kinetics can be a useful tool in providing evidence for intermediates, but proof is only

satisfied when a suspected intermediate is isolated and characterized. The aim of this section was to determine the pathway of the N-deoxyribosyltransferase reaction and to demonstrate the existence of a glycosyl-enzyme intermediate using radiolabelled 2'-deoxyribonucleoside substrate.

#### 4.4 EXPERIMENTAL PROCEDURE

This section details the experimental procedure used to modify amino acid residues of N-deoxyribosyltransferase-II from *L. leichmannii*. In all cases control experiments were carried out. The deoxyribosyltransferase activity was assayed under standard conditions.

##### Modification of Arginine Residues.

###### *(A) 1,2-Cyclohexanedione*

1,2-Cyclohexanedione was recrystallized from petroleum ether. The reaction mixture containing 1,2-cyclohexanedione (Table 4.1) and enzyme in sodium borate buffer, pH 8.0, was incubated at 37°C for 2 hours. Excess reagent was removed by dialysis against pipes buffer, pH 6.5 at 4°C, and the dialysate assayed for transferase activity.

###### *(B) Phenylglyoxal*

This modifying reaction was carried out in the same way as described for 1,2-cyclohexanedione.



## Modification of Carboxyl Residues.

### *(A) Carbodiimide*

The modification was carried out according to the method of Carraway and Koshland [132]. The reaction mixture contained 10mM glycine methyl ester, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide, (Table 4.1) and the enzyme in phosphate buffer, pH 4.75. Samples were removed, diafiltered through a Microcentricon 10,000 filtration apparatus and the retentate assayed for N-deoxyribosyltransferase activity.

### *(B) Woodward's Reagent-K*

Because of the instability of the reagent above pH 3, a fresh solution was made for each individual reaction. The modification was carried out in phosphate buffer, pH 7.9 at 25°C. Aliquots were taken at intervals and excess reagent removed by centrifugation through Sephadex G-25. The eluant fraction was assayed for enzyme activity.

## Modification of Tryptophan Residues.

### *(A) 2-Hydroxy-5-nitrobenzyl bromide (Koshland's Reagent-I)*

The reagent, dissolved in 1,4-dioxane, was added to the reaction mixture containing the enzyme in citrate phosphate buffer, pH 4.0. This was incubated at 25°C, for 40 minutes and the excess reagent removed by a passage through Sephadex G-25, before the eluant was assayed for activity.

\*Due to the high molecular weight of the protein it was taken that all activity would be eluted from the column. Control experiments supported this assumption.

## Modification of Lysine Residues.

### *(A) Ethylacetimidate.HCl*

The enzyme was incubated with ethylacetimidate.HCl in buffer, pH 8.5 at 25°C for 2 hours. The reaction was stopped by the addition of 5-fold ammonium acetate, and excess reagent removed by centrifugation through a 1cm<sup>3</sup> Sephadex G-25 column. The filtrate was assayed for N-deoxyribosyltransferase activity.

### *(B) 2,4,6,-Trinitrobenzenesulphonate*

The TNBS reagent was recrystallized according to the method of Fields [151]. The reaction mixture containing TNBS and enzyme in phosphate buffer, pH 8.0, was held at 25°C for 3 hours. Aliquots were taken every 30 minutes and passed through Sephadex G-25 before assaying for activity.

## Modification of Cysteine Residues.

### *(A) p-Chloromercuribenzoate*

Stock solutions of p-chloromercuribenzoate were prepared in 0.1M pipes buffer, pH 7.0, and 0.1M sodium acetate buffer, pH 4.6, according to the method of Riordan and Vallee [153] and the concentrations determined by the change in absorbance 233nm on the addition of cysteine. The enzyme was incubated at pH 7.0 and 4.6 at 25°C for 2 hours. These solutions were then passed through Sephadex G-25 and the activity of the eluant determined.



*(B) N-Ethylmaleimide*

The reaction was carried out in pipes buffer, pH 7.0 at 37°C for 2 hours and passed through Sephadex G-25 before the extent of modification was determined.

Modification of Tyrosine Residues.

Stock solutions of tetranitromethane were made up freshly in 95% ethanol. The modification reaction was initiated by the addition of TNM to a solution of the enzyme in phosphate buffer, pH 7.8, and the mixture kept at 25°C for 40 minutes. Aliquots were removed at intervals and immediately assayed for activity. The concentration of ethanol did not exceed 5% (v/v) in the reaction mixture.

*(B) N-Bromosuccinimide*

The was performed under the same conditions as described for 2-hydroxy-5-nitrobenzyl bromide. Aliquots from the reaction mixture were removed at intervals, quenched with 5-fold L-tryptophan and the extent of modification determined by the standard enzyme assay.

Modification of Histidine Residues.

*(A) Photoxidation.*

Rose Bengal (Table 4.1) was incubated with the enzyme in 0.05M phosphate buffer, pH 7.0. The reaction mixture was held at 25°C for 16 minutes at a distance of 20cm in front of a 1000 watt tungsten lamp whilst oxygen was gently bubbled over the surface of the solution. Aliquots (100 $\mu$ l) were withdrawn at intervals and passed through a column (1cm<sup>3</sup>) of Sephadex G-25\*. The eluant

fraction was assayed for activity according to the standard procedure. The control reaction was performed in the dark with the same concentration of Rose Bengal.

*(B) Diethylpyrocarbonate.*

The DEPC was freshly diluted with ice-cold ethanol (95%) before each experiment. The concentration of the reagent was determined from the increase in absorbance at 240nm after reaction with 10mM imidazole, pH 7.5, where  $\epsilon 3,200\text{M}^{-1}\text{cm}^{-1}$ . The reaction was initiated by the addition of the DEPC to the enzyme in 0.05M pipes buffer, pH 6.5. This was incubated for 8 minutes at 22°C. The concentration of ethanol in the reaction mixture did not exceed 5% (v/v). Control experiments containing enzyme, buffer and ethanol, equivalent to that used to add DEPC, demonstrated it had no effect on enzyme activity during the time course of the reaction. Aliquots were removed from the reaction mixture at intervals and quenched with 0.01M L-histidine. The extent of modification was determined by measuring the enzyme activity.

*Kinetic measurements.*

All kinetic measurements were carried out according to the standard HPLC assay.

*Detection of a Glycosyl-Enzyme Intermediate using Radiolabelled Thymidine.*

Specific activity of 5'-<sup>3</sup>H thymidine: 15.2 Ci/mmol.

16 $\mu$ l 5'-<sup>3</sup>H thymidine added per ml protein. (41 $\mu$ g/ml.)



The incorporation of radioactive reagent was determined by coprecipitating 20.5 $\mu$ g of labelled N-deoxyribosyltransferase-II with 150 $\mu$ g bovine serum albumin (BSA) in ice-cold 10% (w/v) trichloroacetic acid (TCA). After the addition of the TCA the protein was allowed to precipitate at 0°C. The resulting precipitate was collected on GF/C microglass fibre filters and washed with a further 20-40ml of ice-cold 10% TCA. The filter was then air dried and the collected precipitate solubilized from the GF/C filter by incubating in 1.0ml of NCS tissue solubilizer overnight at room temperature or at 55°C for 2 hours. The radioactivity was measured by scintillation counting in 9ml econofluor scintillation fluid. Glacial acetic acid (35 $\mu$ l) was added to quench chemiluminescence. Control experiments were performed to eliminate the effects of non-specific binding of the labelled substrate to the BSA. The counting efficiency was determined by the addition of a  $^3\text{H}_2\text{O}$  internal standard.

In the first experiment only native enzyme was coprecipitated with BSA and the solution left for 30 minutes in TCA. In the second experiment, in addition to the native enzyme (a), 20.5 $\mu$ g of the N-deoxyribosyltransferase-II was heat-treated prior to labelling (b). It was kept at 85°C for 30 minutes then cooled to room temperature before use.

## 4.5 RESULTS AND DISCUSSION

### 4.5.1 GLYCOSYL-ENZYME INTERMEDIATE

In the results from the kinetic experiments it was observed that a double reciprocal plot in the transfer reaction between adenine and 2'-deoxycytidine generated parallel lines (Figure 4.10).

When both of these substrates were present at the same concentrations a double reciprocal plot of initial velocity against substrate concentration gave a straight line (Figure 4.11). These results agree with those described by Holguin for the N-deoxyribosyltransferase enzymes from *L. helveticus* [82, 83], and confirm that the ping-pong bi-bi mechanism is operative.

### Radiolabelling Experiment.

#### Experiment 1.

Incorporation = 1.12% or 0.01moles  $5'$ - $^3\text{H}$  Thd\*/mole protein

#### Experiment 2.

Incorporation (a) = 5.6% or .0056moles  $5'$ - $^3\text{H}$  Thd\*/mole protein

Incorporation (b) = 3.1% or .0031moles  $5'$ - $^3\text{H}$  Thd\*/mole protein

The rationale behind this method was to establish by the incorporation of radioactivity, using  $5'$ - $^3\text{H}$  thymidine, the existence of a covalent glycosyl enzyme intermediate in the transfer of 2'-deoxyribosyl moiety by N-deoxyribosyltransferase-II. The mechanism of this transfer reaction has been described in *L. helveticus*, [83] but this has been a purely kinetic approach and experiments to show that a glycosyl enzyme participates in the transfer have not been attempted. Reasonable support for the presence of such an intermediate is provided by these results.

\*Thymidine.



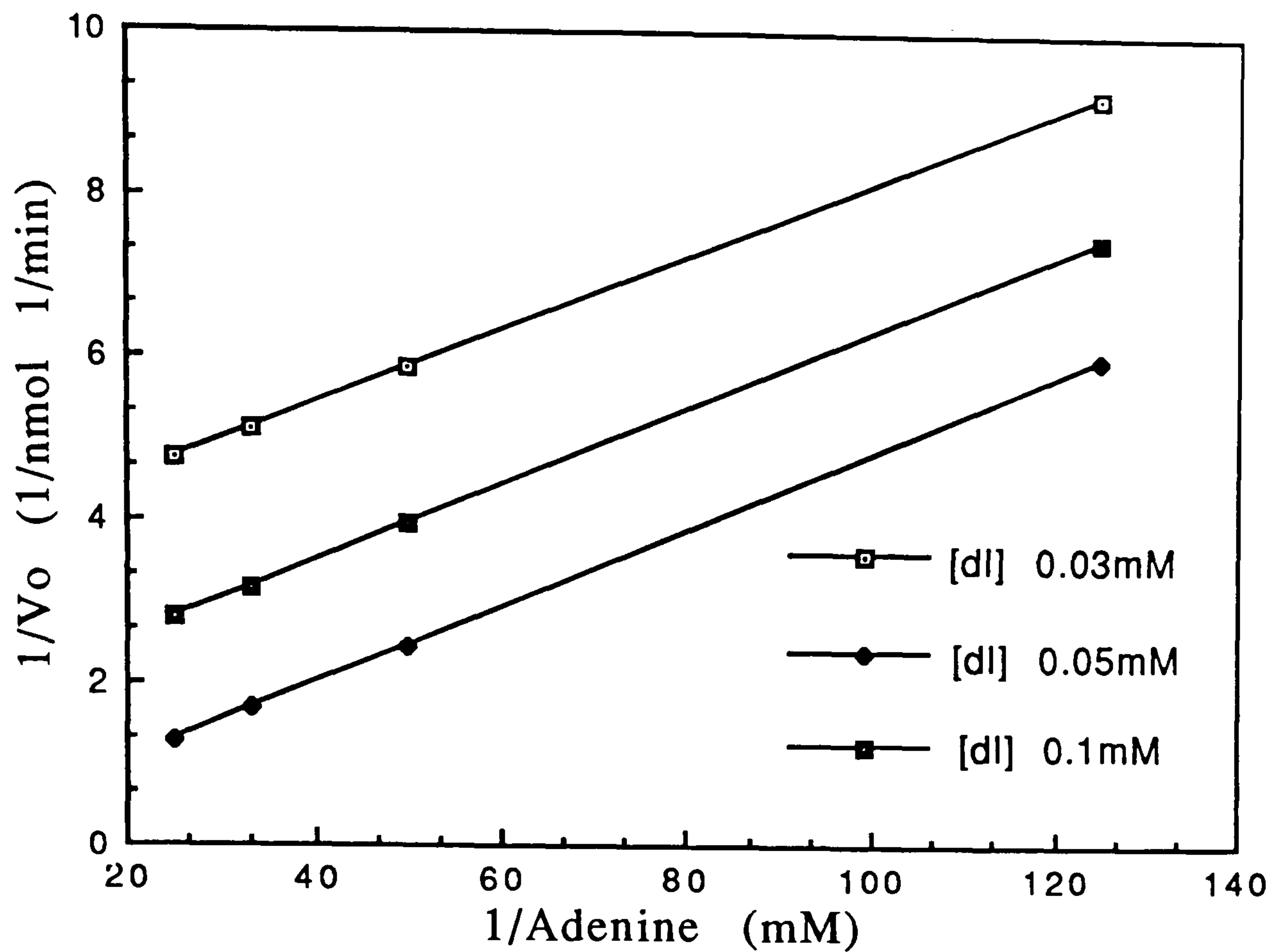


Figure 4.10  
Initial velocity pattern for the (dC->A) reaction with N-deoxyribosyltransferase-II, with adenine as the varying substrate.

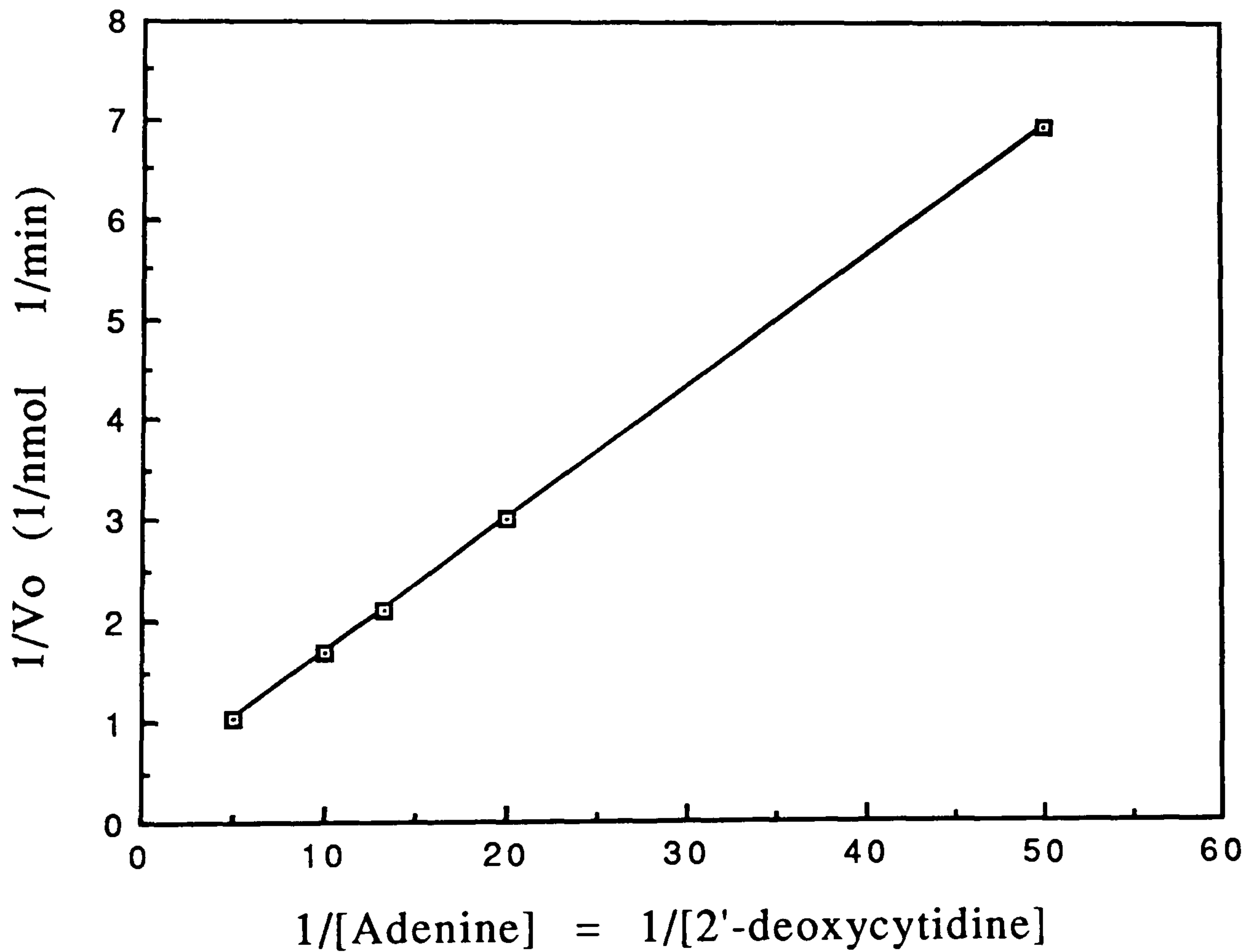


Figure 4.11  
Initial velocity pattern for identical concentrations of substrates 2'-deoxycytidine and adenine.

When the thymidine is radiolabelled exclusively in the 5'-position of the deoxyribose group and incubated without an acceptor base, it would be expected that any incorporation of radioactivity into the protein would be due to the formation of a covalently-bound, glycosyl-enzyme intermediate. In the first experiment, an incorporation of 1.12% or 0.01moles 5'-<sup>3</sup>H thymidine/mole protein was recorded. Repeat of the procedure with native and heat-treated enzyme, under slightly altered reaction conditions, showed a 5-fold increase in incorporation of the labelled thymidine into the native enzyme. The heat-treated enzyme demonstrated an incorporation of 3.1%, 55% of that of the native enzyme under the same conditions. This diminution of incorporation was attributed to the partial denaturation of the enzyme by heat treatment, and lend further support to the hypothesis of the covalent glycosyl-enzyme intermediate. The covalent nature of the enzyme is assumed because of the TCA step in the procedure. In the second experiment the reactions conditions were also adjusted to reduce the duration of the TCA precipitation. The incorporation of radioactivity rose by 80% suggesting that the glycosyl-enzyme is acid labile.

These preliminary experiments provide reasonable support for the existence of the glycosyl intermediate postulated by kinetics. That this is clearly a glycosyl- rather than a nucleoside-enzyme intermediate, needs to be corroborated by dual labelling experiments. Attempts to radiolabel this enzyme with [<sup>14</sup>C]-base- and [<sup>3</sup>H]-sugar-labelled nucleosides should show the exclusive incorporation of tritium. Verification of its existence could be obtained by its isolation. However, there are often problems



associated with this procedure, the most common being the stability of the intermediate form.

In his study of the formation of 2'-deoxynucleosides from 8-substituted purines by nucleoside deoxyribosyltransferase from *L. leichmannii*, Huang found that in certain instances both the 3- and the 9-(2'-deoxyribofuranosyl) nucleosides were formed [87]. In the case of the transfer reaction between thymidine and 8-bromoadenine, however, it was observed that prolonged incubation of the reaction mixture with the enzyme resulted in the decrease of the 3-(2'-deoxyribofuranosyl) nucleoside concentration and an increase in the 9-(2'-deoxyribofuranosyl) product. In the absence of this enzyme no interconversion was observed. Significant amounts of hydrolysis were also found to occur in the presence of the N-deoxyribosyltransferase, because of the long time of reaction (Figure 4.12). This hydrolysis was proportional to the concentration of enzyme and suggests the hydrolysis of a deoxyribosyl intermediate form. The presence of the 2'-deoxyribose was detected by the diphenylamine assay procedure [109].

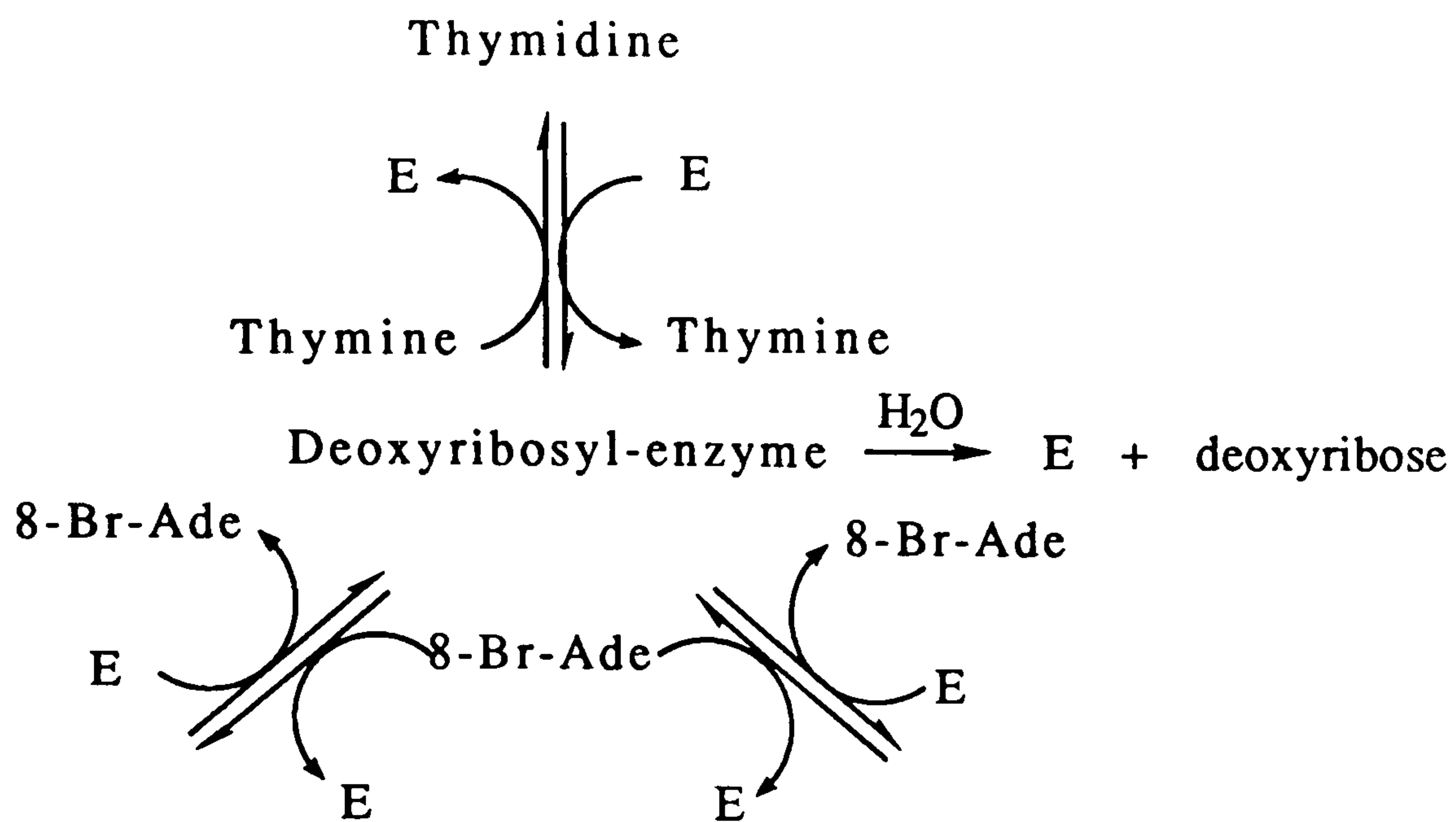


Figure 4.12

Huang also demonstrated that these rates of hydrolysis were independent of the nature of the purine base. These results point toward the existence of a glycosyl intermediate in the transfer reaction mediated by this enzyme, (Figure 4.12).

Evidence of a sugar-enzyme intermediate has also been confirmed by kinetic and selective labelling methods in the glycosyl transfer enzyme, sucrose phosphorylase [171]. In this instance, the intermediate was found to be extremely unstable at neutral pH and proved hard to isolate. If the complex can be isolated, it must be demonstrated that this modified form of the enzyme is able to act as a kinetically competent intermediate in the transfer reaction (Figure 4.13).

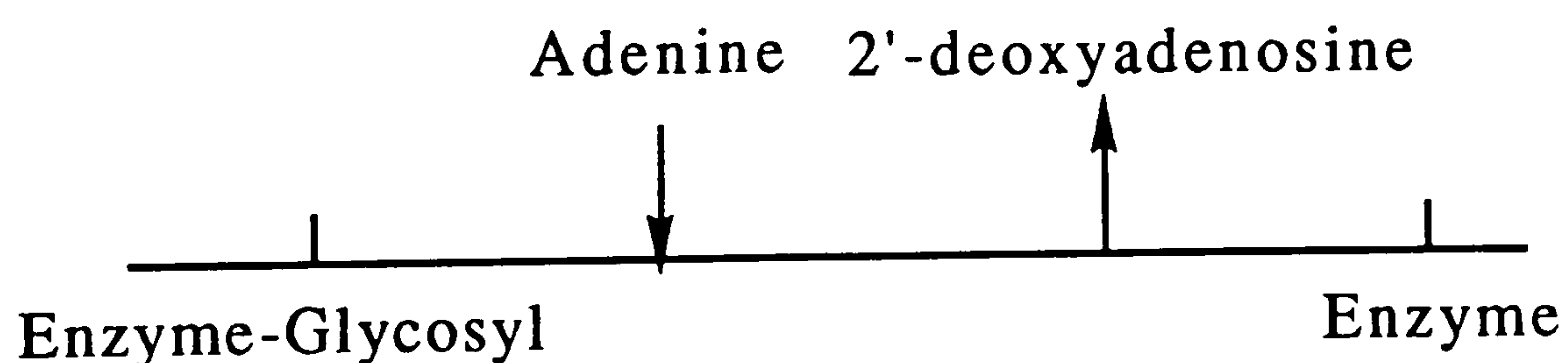


Figure 4.13



An investigation of the exact configurational state of the intermediate by nmr should also aid in the definition of the transfer mechanism. However, large amounts of material are required to perform this task.

Isolation in a stable form also extends the possibility of segregating the active site peptide. Information about the sequence of this fragment could provide information on the amino acids at or near the active site which participate in binding and catalysis.

#### 4.5.2 CHEMICAL MODIFICATION

It can be observed (Table 4.1) that a number of the modification reactions had little or no effect on the original activity of the enzyme. Treatment with the sulphhydryl reagents N-ethylmaleimide and p-chloromercuribenzoate, caused no significant decrease in activity. This is in agreement with Uerkevitz [89] who reported that sulphhydryl reagents had no effect on deoxyribosyltransferase activity of *L. helveticus*. Similarly, specific reagents directed against lysine and arginine residues had little effect upon the original activity. Thus, these residues obviously play no direct role in either binding substrates, catalysis or stabilization of the active site pocket.

N-Bromosuccinimide reduced the activity of the enzyme to a substantial degree but when a second tryptophan-specific reagent, 2-hydroxy-5-nitrobenzyl bromide was employed in the

Table 4.1  
Effect of Group Specific Regents on N-deoxyribosyltransferase-II from *L. leichmannii*.

| Target Residue          | Reagent             | Molar Ratio<br>Reagent/Enzyme | Time (min) | % Residual Activity | % Residual with<br>Protection<br>(50mM 2'-dC) |
|-------------------------|---------------------|-------------------------------|------------|---------------------|---|
| Arginine                | 1,3-CHD             | $5.0 \times 10^3$             | 120        | 92.0                | ---   |
|                         | Phenyglyoxal        | $5.0 \times 10^3$             | 120        | 98.0                | ---   |
| Glutamate/<br>Aspartate | EDC + Glycine ester | $1.5 \times 10^4$             | 180        | 30.0                | 98.0  |
|                         | Woodwards-K         | $1.2 \times 10^4$             | 2          | 24.5                | 52.0  |
| Cysteine                | p-CMB               | $1.0 \times 10^5$             | 120        | 95.0                | ---   |
|                         | NEM                 | $3.2 \times 10^4$             | 120        | 98.0                | ---   |
| Histidine               | Rose Bengal         | 16.5                          | 16         | 50.0                | 67.5  |
|                         | DEPC                | $8.6 \times 10^3$             | 8          | 48.0                | 73.0  |
| Lysine                  | TNBS                | $1.25 \times 10^4$            | 180        | 94.0                | ---   |
|                         | Ethylacetimidate    | $1.0 \times 10^4$             | 120        | 99.0                | ---   |
| Tryptophan              | NBS                 | $1.2 \times 10^2$             | 20         | 55.4                | 54.0  |
|                         | HNB-Br              | $1.5 \times 10^2$             | 40         | 95.8                | ---   |
| Tyrosine                | TNM                 | $6 \times 10^2$               | 40         | 51.5                | 51.0  |



modification reaction, there was virtually no loss of activity, suggesting tryptophan is not a catalytically important residue. As N-bromosuccinimide is known to be a highly reactive and somewhat unselective reagent, the partial inactivation of the enzyme observed in this case may be due to conformational changes from peptide cleavage, rather than modification of an essential amino acid function.

The most pronounced inactivation is observed when histidyl, carboxyl and tyrosyl-specific agents are used. The reaction were observed to follow pseudo-first-order kinetics over a range of concentrations. To support the observations that essential imidazole and carboxyl groups might participate in catalysis, two different modifying reagents were used to examine each case. When treatment of the N-deoxyribosyltransferase with Rose Bengal caused a diminution in activity (Figure 4.14), the theory that there might be an essential histidine residue was substantiated by the use of the selective agent, DEPC which was observed to have the same effect of reducing the activity (Figure 4.15). In each case, when the concentration of the modifying agent was increased, the rate of inactivation increased.

Indications that an important carboxyl residue may reside in or near the active site were given by the observation that there is a loss of activity from the reaction of carbodiimide with the N-deoxyribosyltransferase (Figure 4.16). This too was supported by using a second carboxyl-specific reagent, the isoxazolium salt, Woodward's Reagent-K, which reduced transferase activity significantly (Figure 4.17).

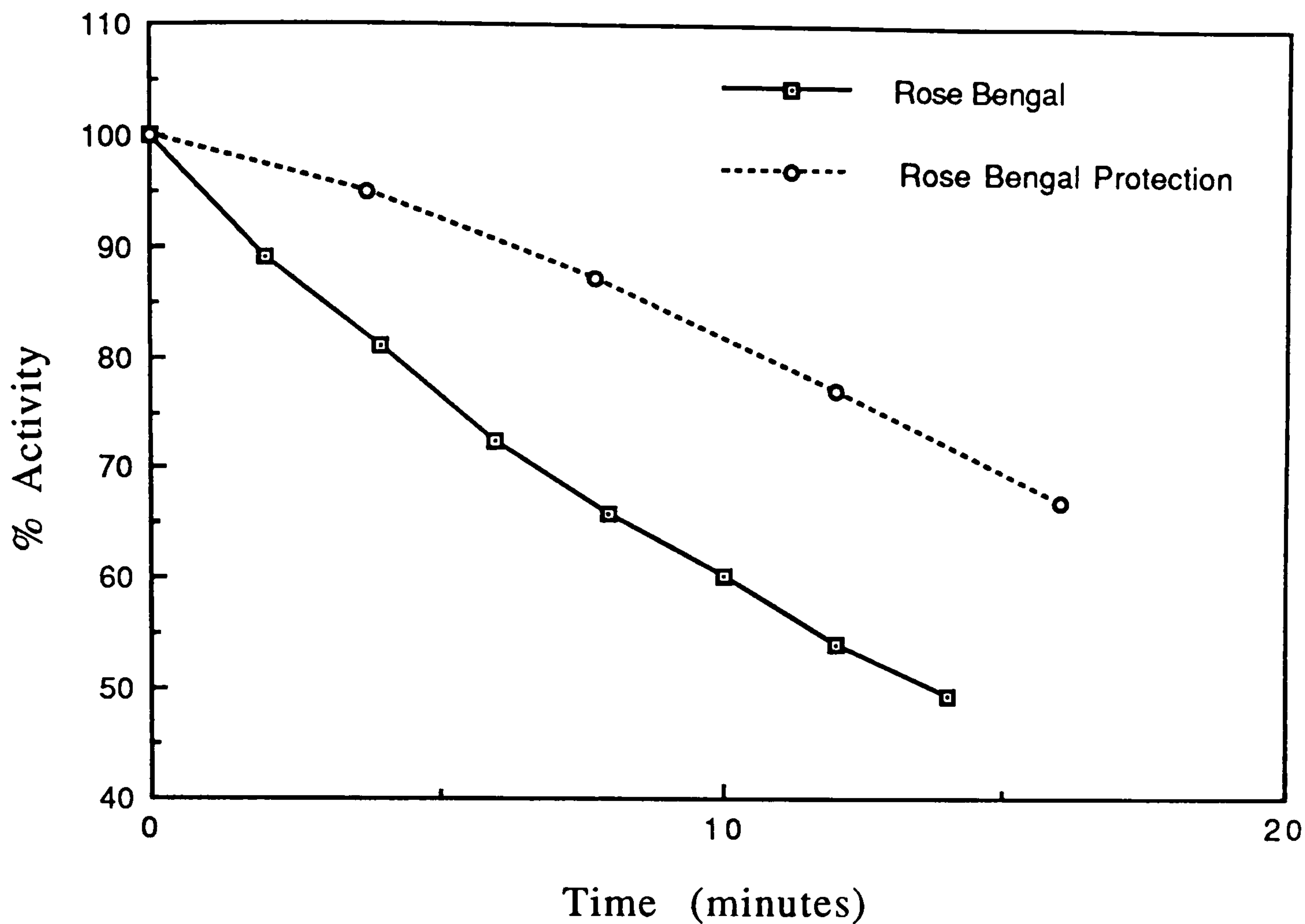


Figure 4.14

Inactivation of N-deoxyribosyltransferase-II by Rose Bengal in the presence and absence of 2'-deoxycytidine (50mM). Reaction performed in phosphate buffer, pH 7.0, 25C.

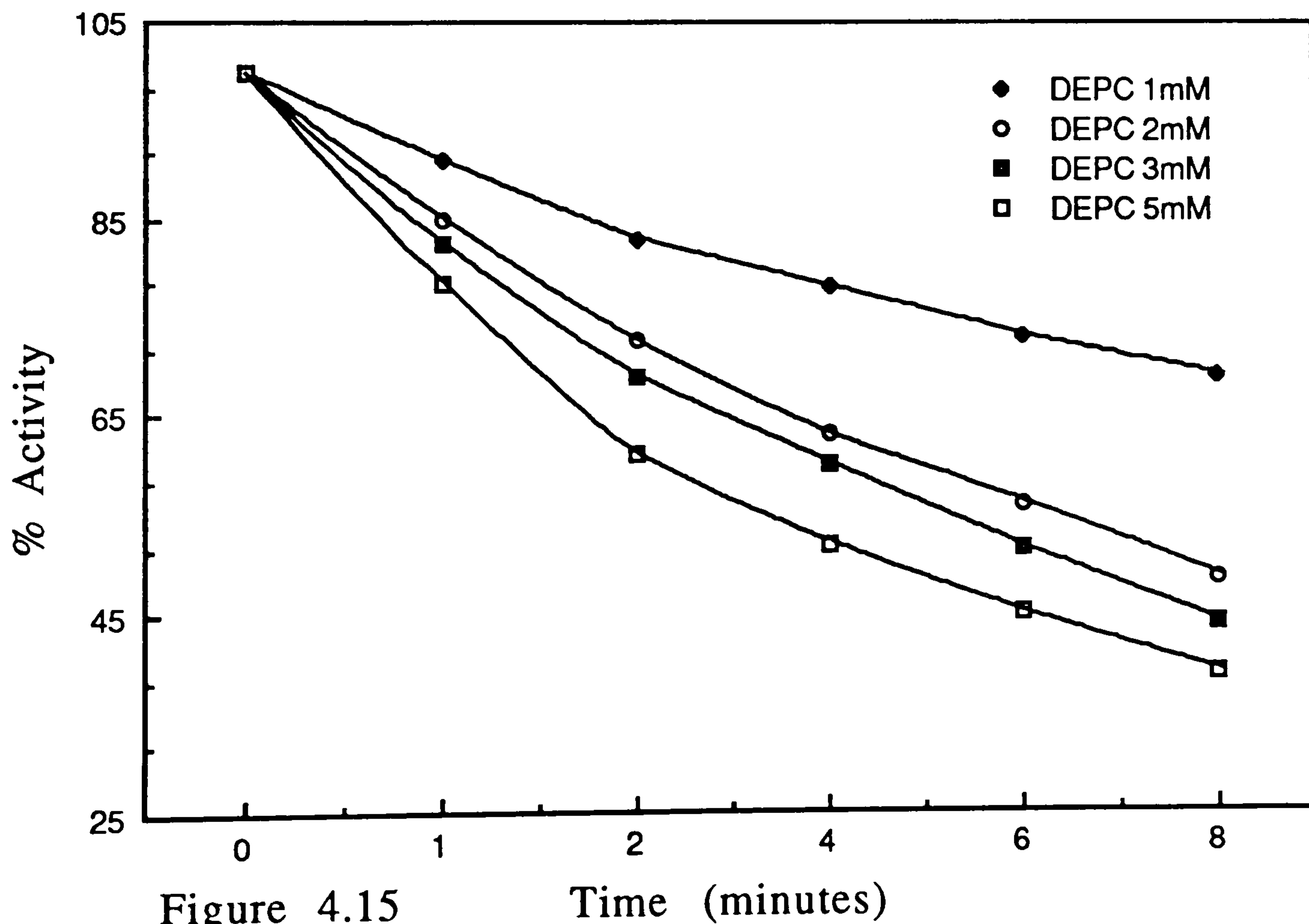


Figure 4.15

Inactivation of N-deoxyribosyltransferase-II by DEPC. Reaction performed in 50mM pipes buffer, pH 6.0, 22C. Reaction quenched with 10mM L-histidine.



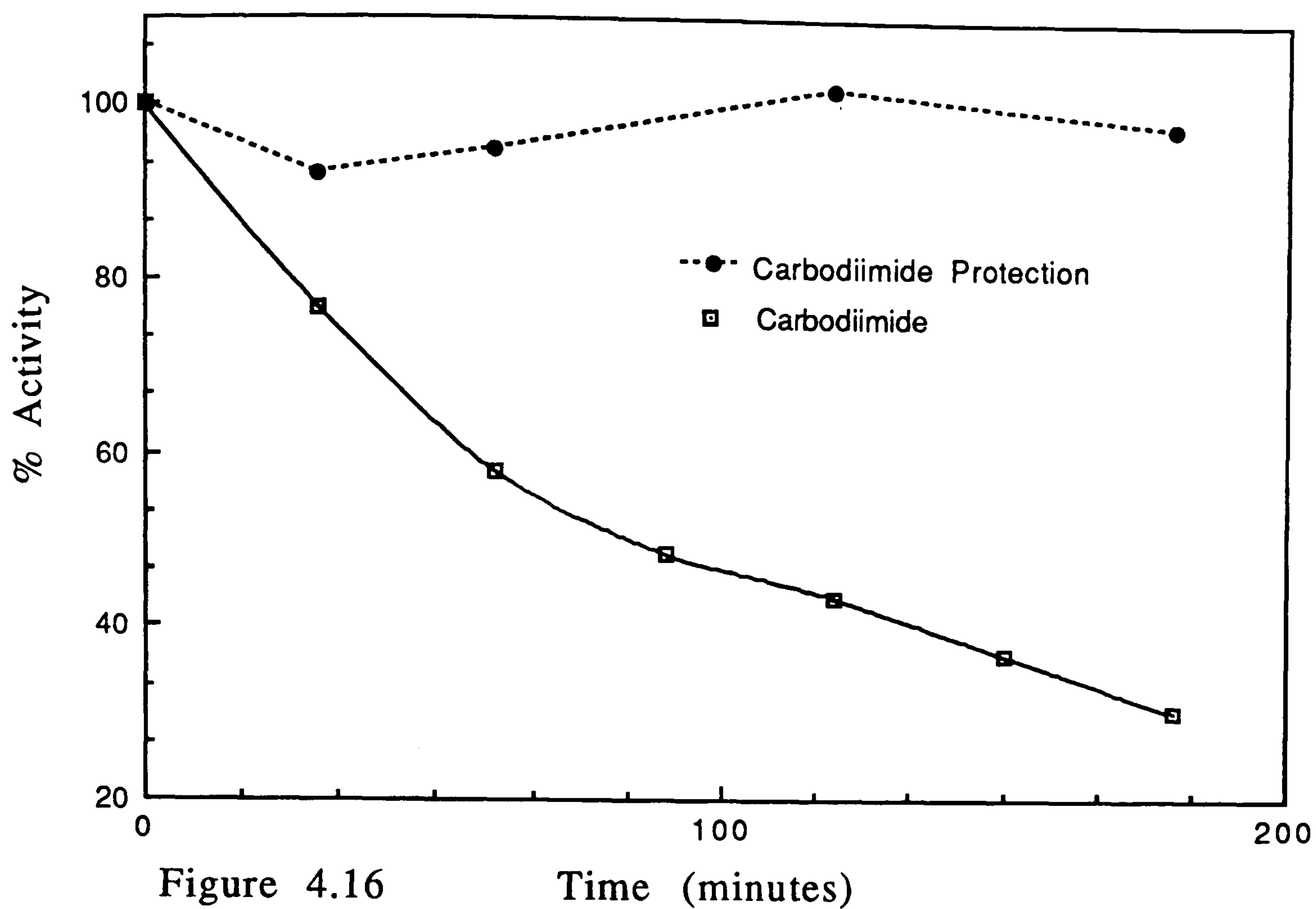


Figure 4.16 Inactivation of N-Deoxyribosyltransferase-II by 1-(3-Dimethylaminopropyl)3-ethyl-carbodiimide, in the presence and absence of 2'-deoxycytidine (50mM). Reaction mixture performed in phosphate buffer, pH 4.75, 25C.

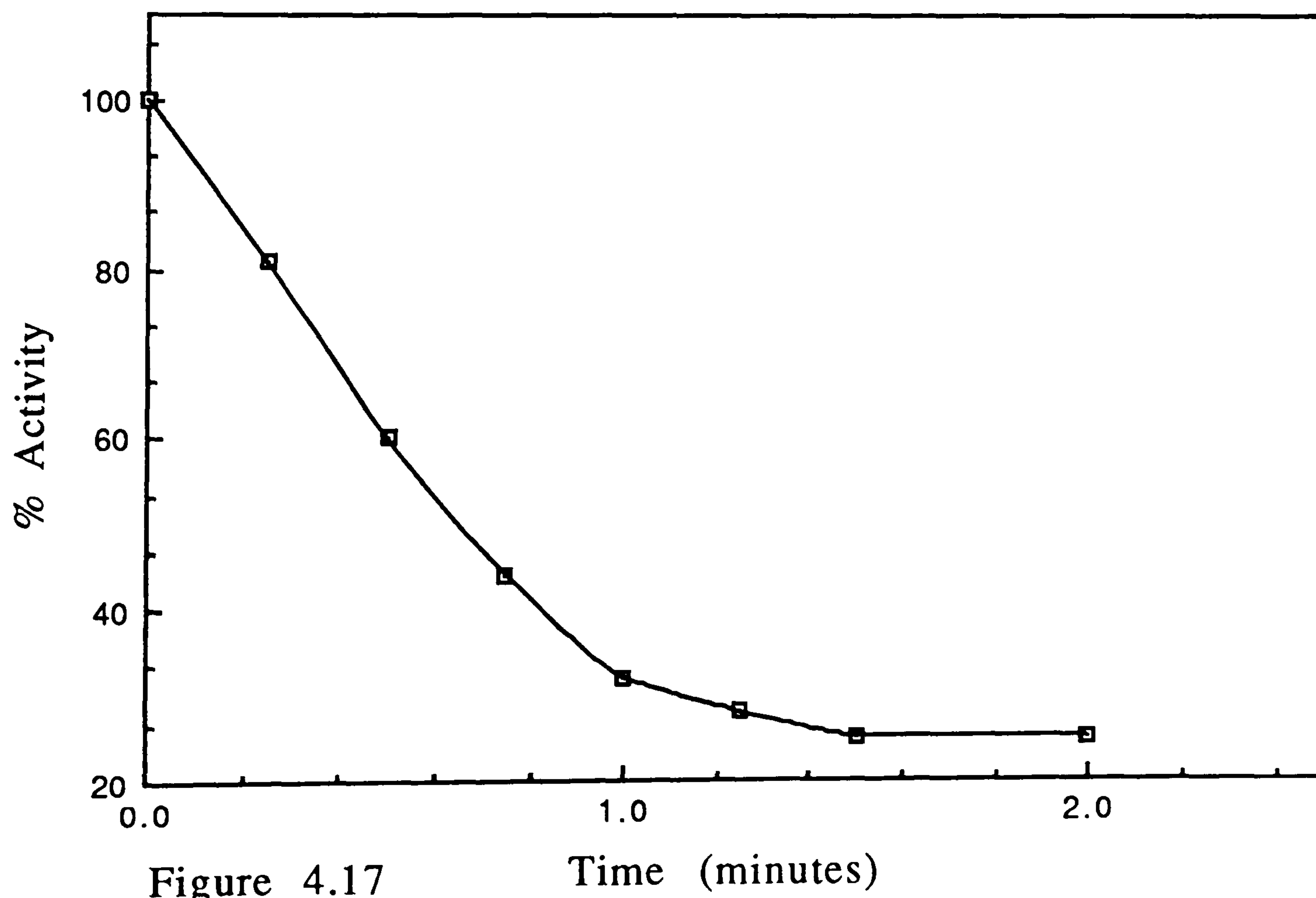


Figure 4.17 Inactivation of N-Deoxyribosyltransferase-II with Woodward's Reagent-K. Reaction performed in phosphate buffer, pH 7.9, 25C.

Further reactions carried out in the presence of the natural nucleoside substrate 2'-deoxycytidine demonstrated that certain modifications could be significantly retarded. This held for the inactivations by histidyl (Figure 4.18) and carboxyl reagents (Figure 4.16) but not for the modification with tetranitromethane (Figure 4.19).

Lack of protection of the enzyme with high concentrations of substrate from the tetranitromethane reagent suggest that a tyrosine residue is not involved in any direct action at the active site. It is possible however, is that a non-essential tyrosine may play an auxiliary role in stabilization or binding close to the active site. Alternatively, TNM may be inducing a change in tertiary structure, causing the peptides to crosslink and the protein to precipitate, bringing about a reduction in overall activity.

The observations that both the histidyl and carboxyl modifications reactions are able to be arrested by the inclusion of high concentrations of substrate in the reaction mixture, is consistent with their importance in catalysis. The modification by DEPC is normally able to be reversed by incubating the treated enzyme with high concentrations of hydroxylamine, at neutral pH for long periods of time. This displacement of the carbethoxy group from the modified histidine lends further support to the idea that it is a histidine that is being selectively modified, and that diminution of activity is not due to either conformational perturbations or the modification of another residue. Many proteins, however, remain sensitive to this nucleophile and in the case of the DEPC-induced modification of N-deoxyribosyltransferase-II, it was not possible to show any reaction reversal.



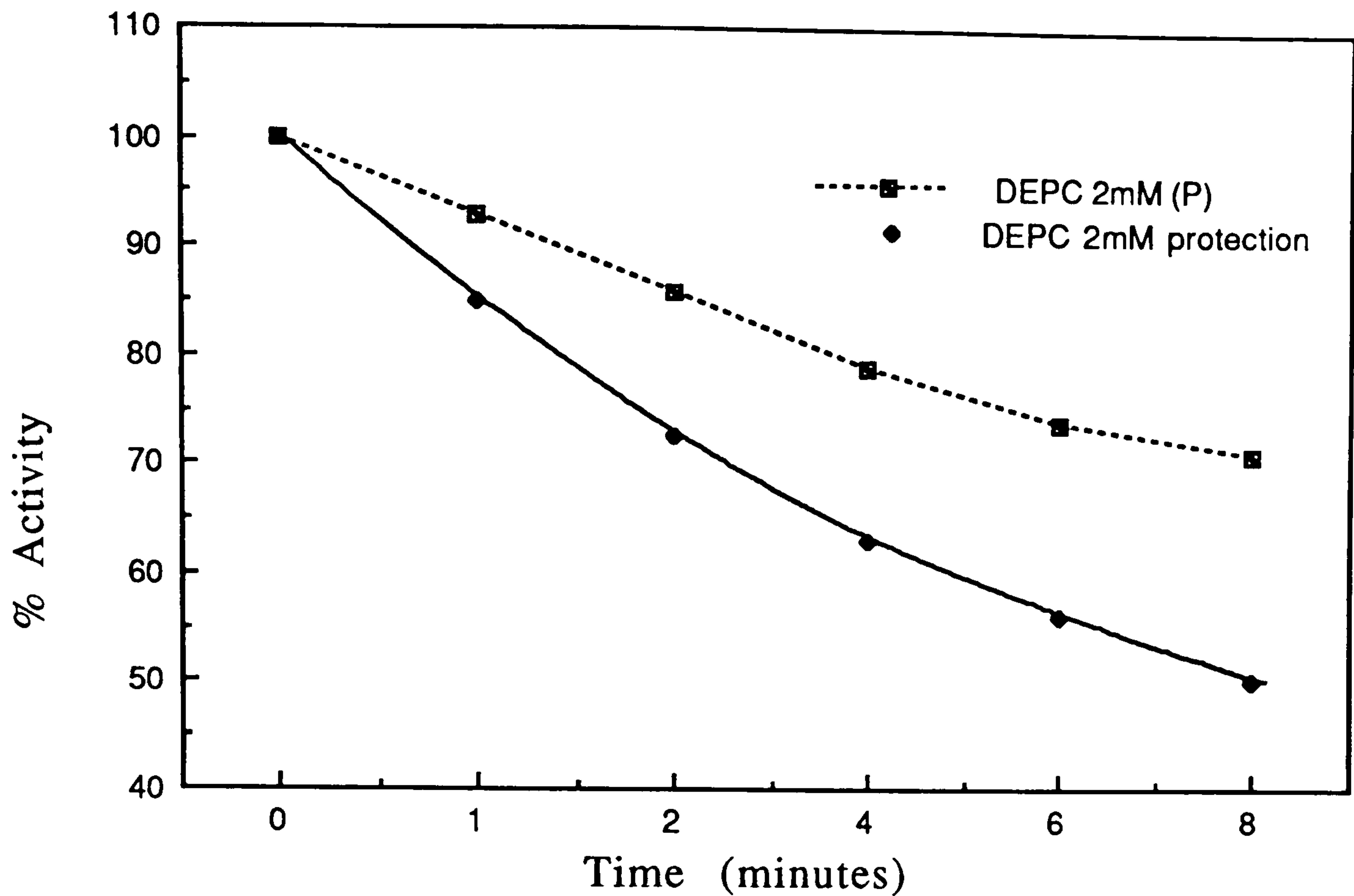


Figure 4.18 Inactivation of N-Deoxyribosyltransferase-II by DEPC in the presence and absence of 2'-deoxycytidine (50mM). Reaction conditions as described in text.

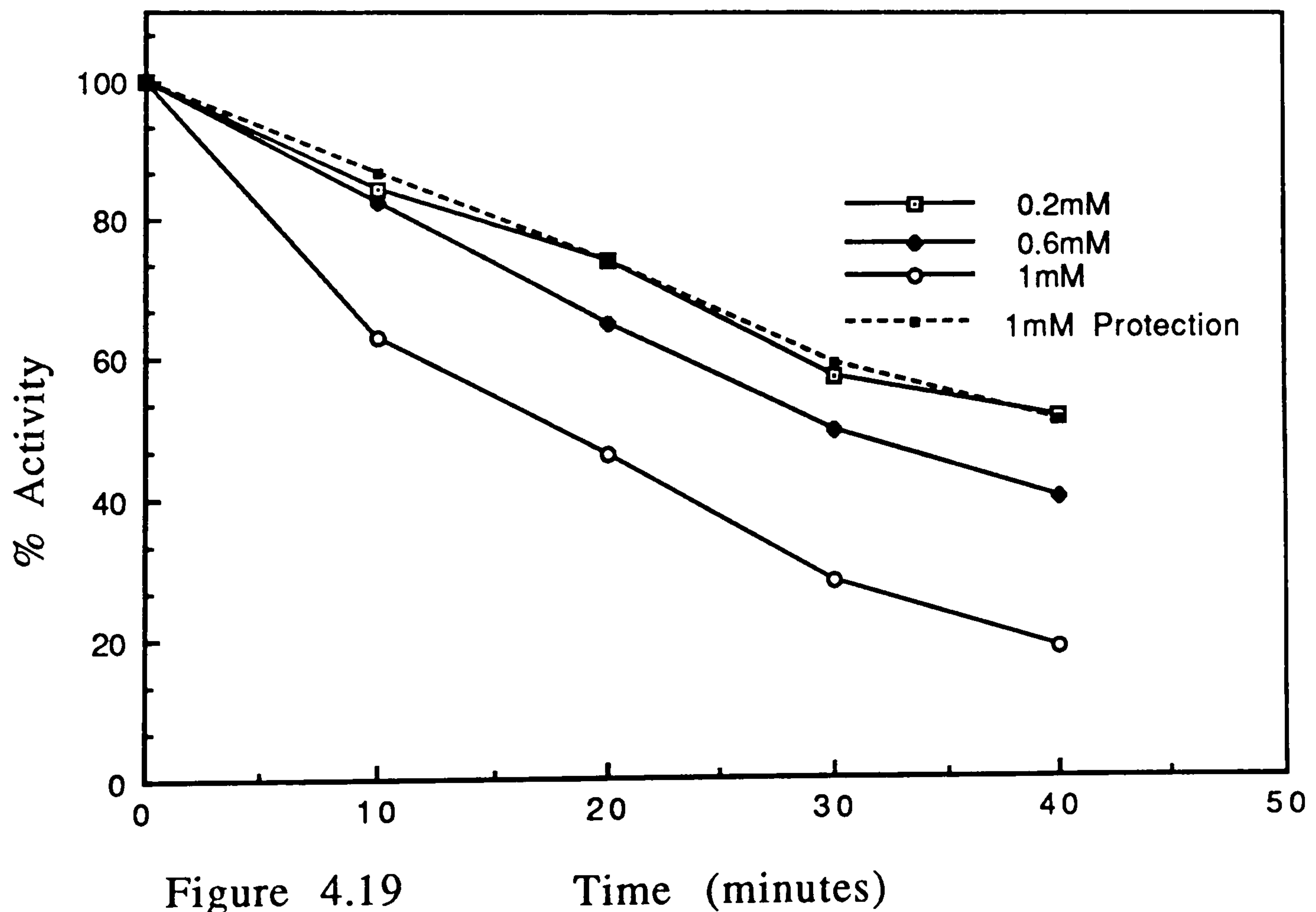


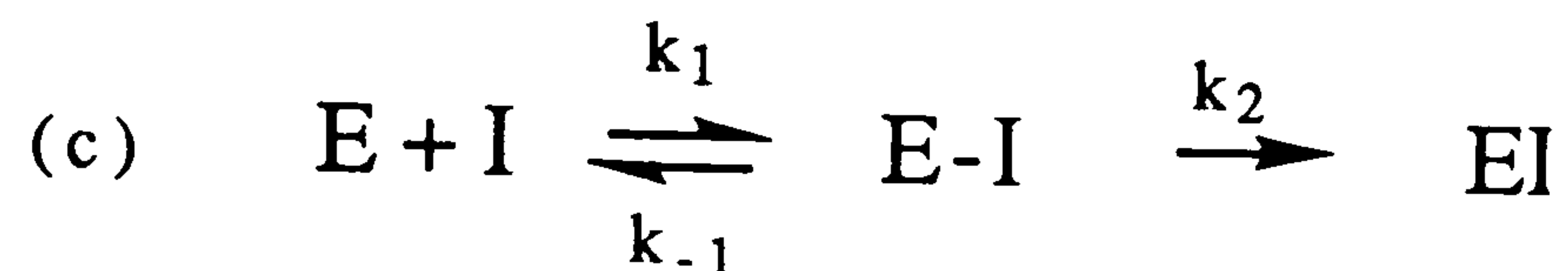
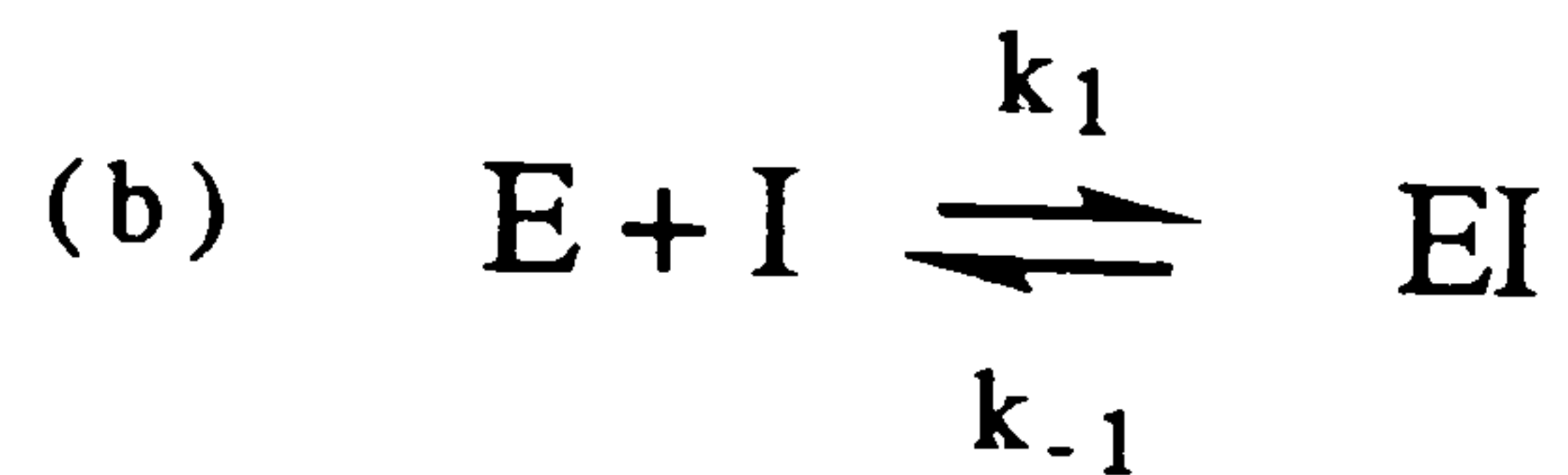
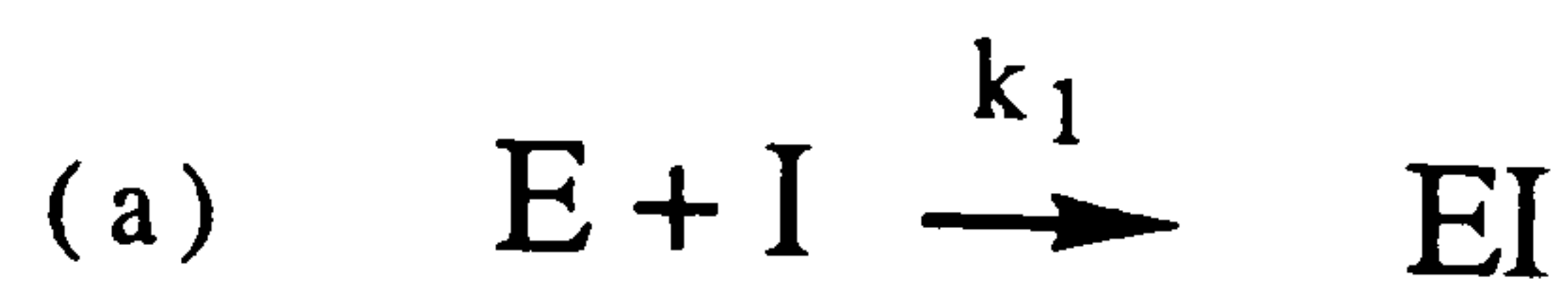
Figure 4.19 Inactivation of N-Deoxyribosyltransferase-II with TNM in the presence and absence of 2-deoxycytidine (50mM). Reaction performed in phosphate buffer, pH 7.8, 25C.

The protection that 2'-deoxycytidine conveyed was not complete in most of the reactions. In the Rose Bengal and the DEPC substrate protection experiments, the modification was reduced, on average between 20-30%. In the reaction of the enzyme with Woodward's Reagent-K, protection was only 25%. This incomplete protection could be due to the weak binding of the substrate in the vicinity of these groups at the active site, or modifications may be occurring at positions where the protein interaction with the substrate is not maximal. Modification in the presence of a pyrimidine or purine base alone or a combination of base and nucleoside was found not to increase protection. Ideally a strong binding, reversible inhibitor is required for full protection of vulnerable active site residues. The 2'-deoxycytidine did, however, give complete protection to the enzyme from carbodiimide, which indicates that this reagent might be modifying a different carboxyl residue to the Woodward's Reagent-K.

### Kinetics of Modification

When the enzyme (E) reacts with an inhibitor (I) it may do so in a number of ways. It can bind irreversibly, (a), to the protein, reversibly, (b), or via the formation of an intermediate complex, E-I, (c).





For the reversible reaction the forward reaction rate ( $v$ ) can be expressed as:

$$v = k_1[E][I]. \quad (d)$$

and where the concentration of  $I$  is much greater than the concentration of  $E$ ,

$$v = k_1[I] \quad \text{or} \quad k_{\text{obs}} = k_1[I] \quad (e)$$

Discriminations between the three mechanisms is possible by plotting  $k_{\text{obs}}$  as a function of the inhibitor concentration.

The rate constants measured over the first 8 minutes for the inactivation of the deoxyribosyltransferase with DEPC shows that there is a non-linear dependence of the observed rate constant on the DEPC concentration (Figure 4.20). The pseudo-first-order rate constants were calculated from slopes of linear inactivation curves over a range of concentrations of DEPC.

Analysis of the data suggests that it fits the model of (c) and that an intermediatery reversible complex is formed between the acylating DEPC and an imidazole group on the enzyme prior to covalent modification.

Assuming that the equilibrium condition  $k_2 \ll k_{-1}$  holds [172], it can be shown that:

$$k_{\text{obs}} = \frac{k_1 k_2 [\text{I}]}{k_1 [\text{I}] + k_{-1}} \quad (\text{f})$$

A plot of  $k_{\text{obs}}$  versus  $[\text{I}]$  (Figure 4.20) gives a rectangular hyperbola.

The reciprocal of equation (f) gives :

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k_2} + \frac{K_{\text{diss}}}{k_2} \frac{1}{\text{I}} \quad (\text{h})$$

When  $\frac{1}{k_{\text{obs}}}$  versus  $\frac{1}{\text{I}}$ , the slope is equal to  $\frac{K_{\text{diss}}}{k_2}$  and the intercept on the y-axis to  $\frac{1}{k_2}$ . (Figure 4.21).

Thus  $K_{\text{diss}} = 8.7 \times 10^{-5}$ .



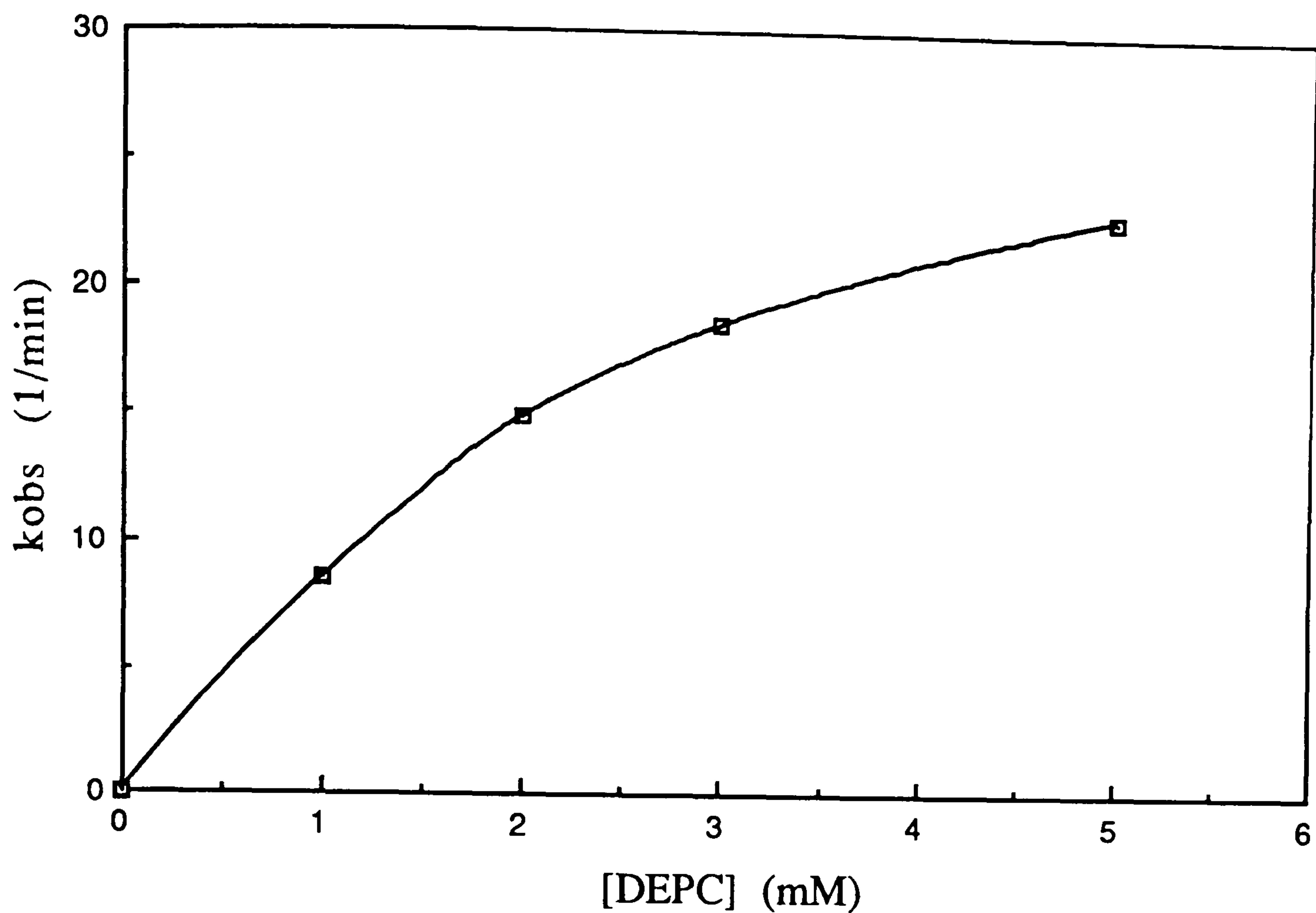


Figure 4.20 Plot of equation (f) for the inactivation of N-Deoxyribosyltransferase-II with DEPC. Reaction conditions as described in text.

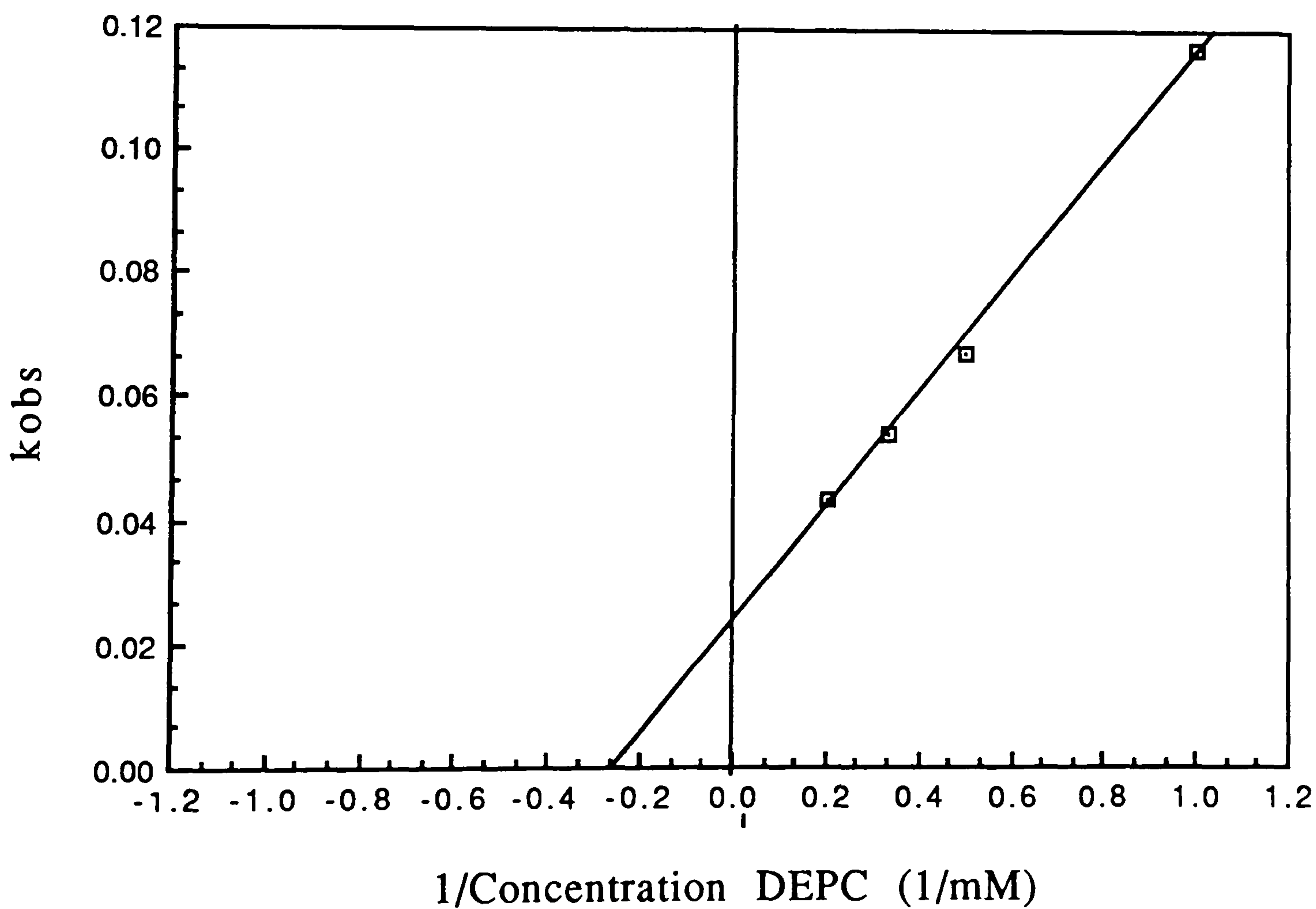


Figure 4.21 Dependence of the pseudo-first-order rate constant, k<sub>obs</sub>, for the inactivation of N-Deoxyribosyltransferase-II on DEPC concentration. Conditions as described in text.

Knowledge of the substrate requirements for correct binding and orientation, the enzyme substitution mechanism, the existence of a glycosyl-enzyme intermediate, and the conclusion that two specific residues are central to catalysis, allow the construction of several models. These models illustrate the sequence and nature of the transfer reaction of N-deoxyribosyltransferase-II based on the aforementioned results and conclusions. Since there is an overall retention of configuration, there are two possible mechanisms by which the transfer of the glycosyl group could occur. Firstly via an  $S_N1$  reaction, where the intermediate form is a carbonium ion (Figure 4.22). Here, the expulsion of the base from the donor nucleoside could be mediated by an unionized acid residue, while the enzyme-carbonium ion intermediate is stabilized by electrostatic interactions with an ionized acid group. However, since there is reasonable evidence for a covalent glycosyl-enzyme intermediate in the mechanism, it is unlikely that the reaction would proceed via the carbonium ion form. In the second case, the transfer might proceed by two successive  $S_N2$  steps such that the glycosyl intermediate has an  $\alpha$ -configuration about the C-1 glycosidic bond. The nature of the ping-pong bi-bi mechanism indicates a covalently bound intermediate participates in the reaction scheme we have shown evidence to suggest that this is likely to be the case in N-deoxyribosyltransferase-II. The glycosyl moiety could become attached to groups on one of the two residues postulated to exist at the active site; the imidazole group of a histidine residue or the carboxyl group of an acidic residue. Figures 4.23 and 4.24 depict what might happen in the glycosyl transfer between a pyrimidine nucleoside and a purine base. In the first case (Figure



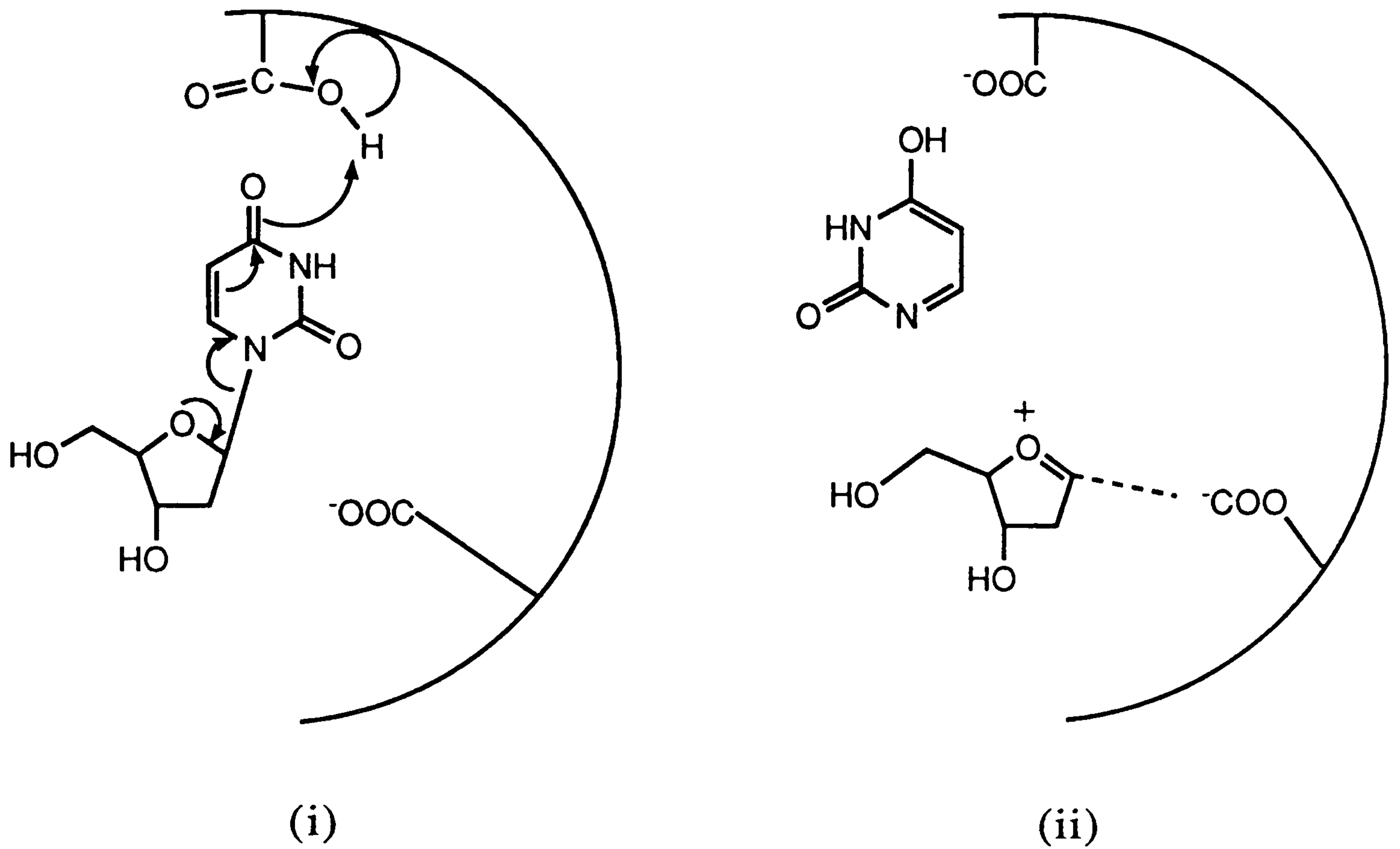
4.24), it is envisaged that a histidine acts as a nucleophile, generating the glycosyl-enzyme intermediate on the release of the pyrimidine base. A second base then displaces this sugar moiety by the reverse reaction. Two additional residues with acid groups act as proton donors and acceptors in this push-pull reaction. It is possible that there are two distinct carboxyl groups mediating in this transfer which might react differently to distinct modifying agents. This could explain this different degrees of protection observed for the carbodiimide and isoxazolium salt modifications respectively. In the second case (Figure 4.24), the glycosyl group is attached to the carboxyl of an acid residue. Examples of this are seen in the enzymes  $\alpha$ -galactosidase and  $\beta$ -galactosidase.

What is unclear at the moment is exactly which of the residues postulated as being crucial to catalysis, participates at each stage of the reaction mechanism. Confirmation of these hypotheses will require further detailed studies to clarify the role of each group at any point in the transfer.

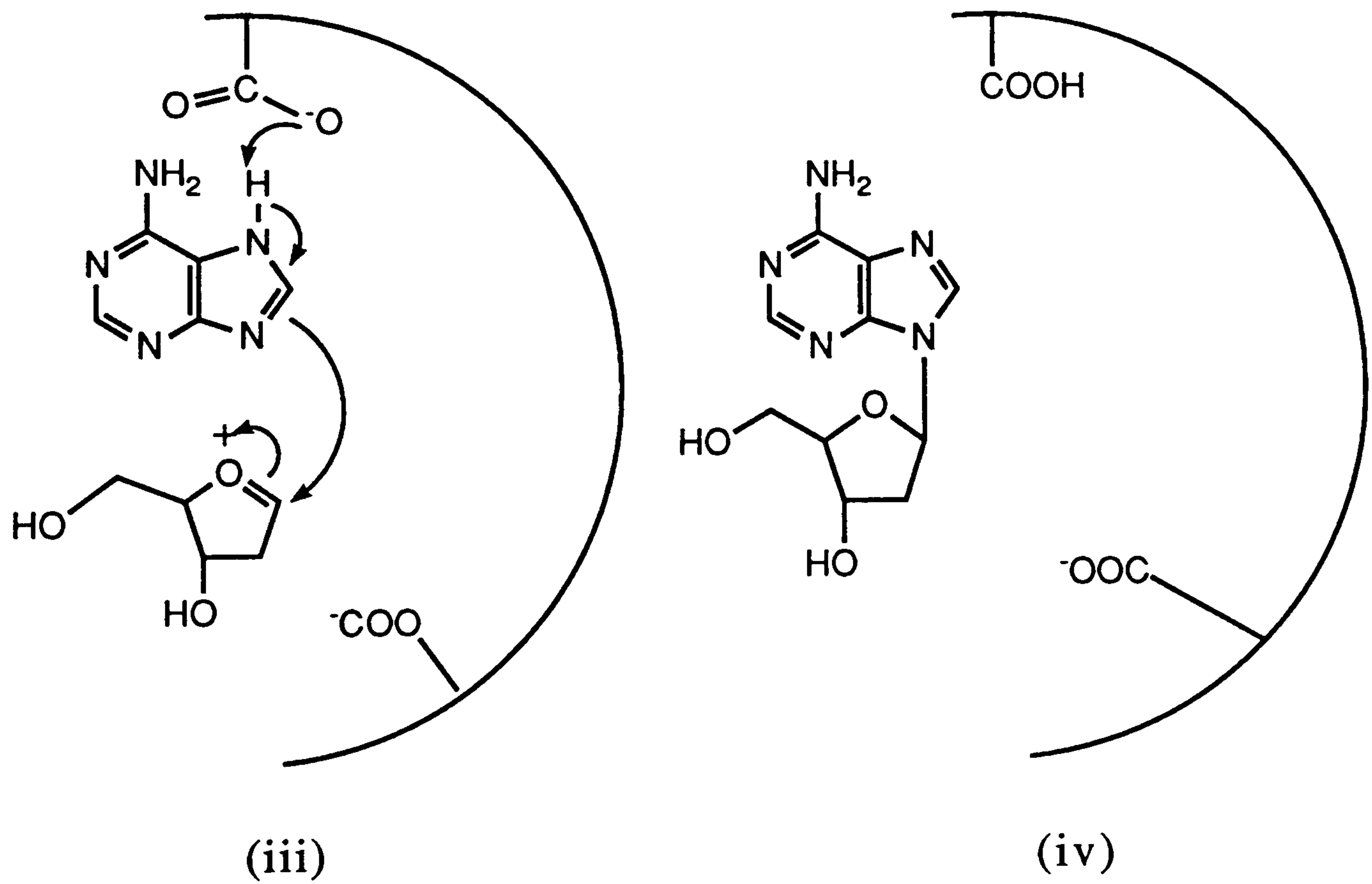
The acquisition of a strong inhibitor would greatly aid in this process, enabling quantitative modification to be carried out with radiolabelled reagents. Knowledge of the substrate specificity should also enable the synthesis of an affinity label. The design of a purine inhibitor or affinity label would allow the active sites of both N-deoxyribosyltransferase-I and -II to be scrutinized in detail. Both of these enzymes have very similar pH profiles which is an initial indication that they may possess similar catalytic groups at the binding site. What is likely to differ is the residues which secure the shape of the binding pocket and

Figure 4.22

Possible Model for Enzymatic Reaction of N-Deoxyribosyltransferase-II  
involving two carboxyl groups



Displacement of donor base

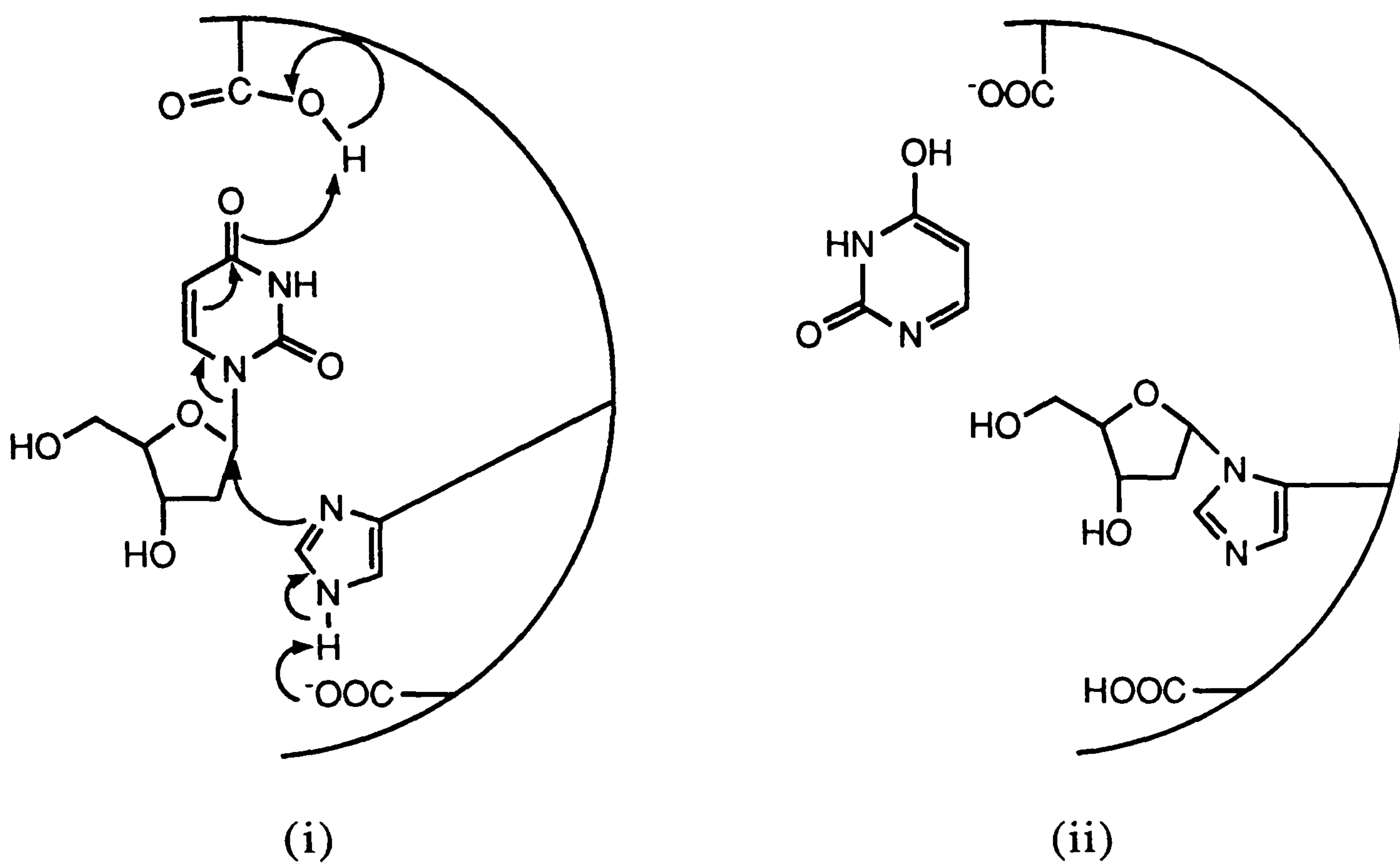


Formation of product



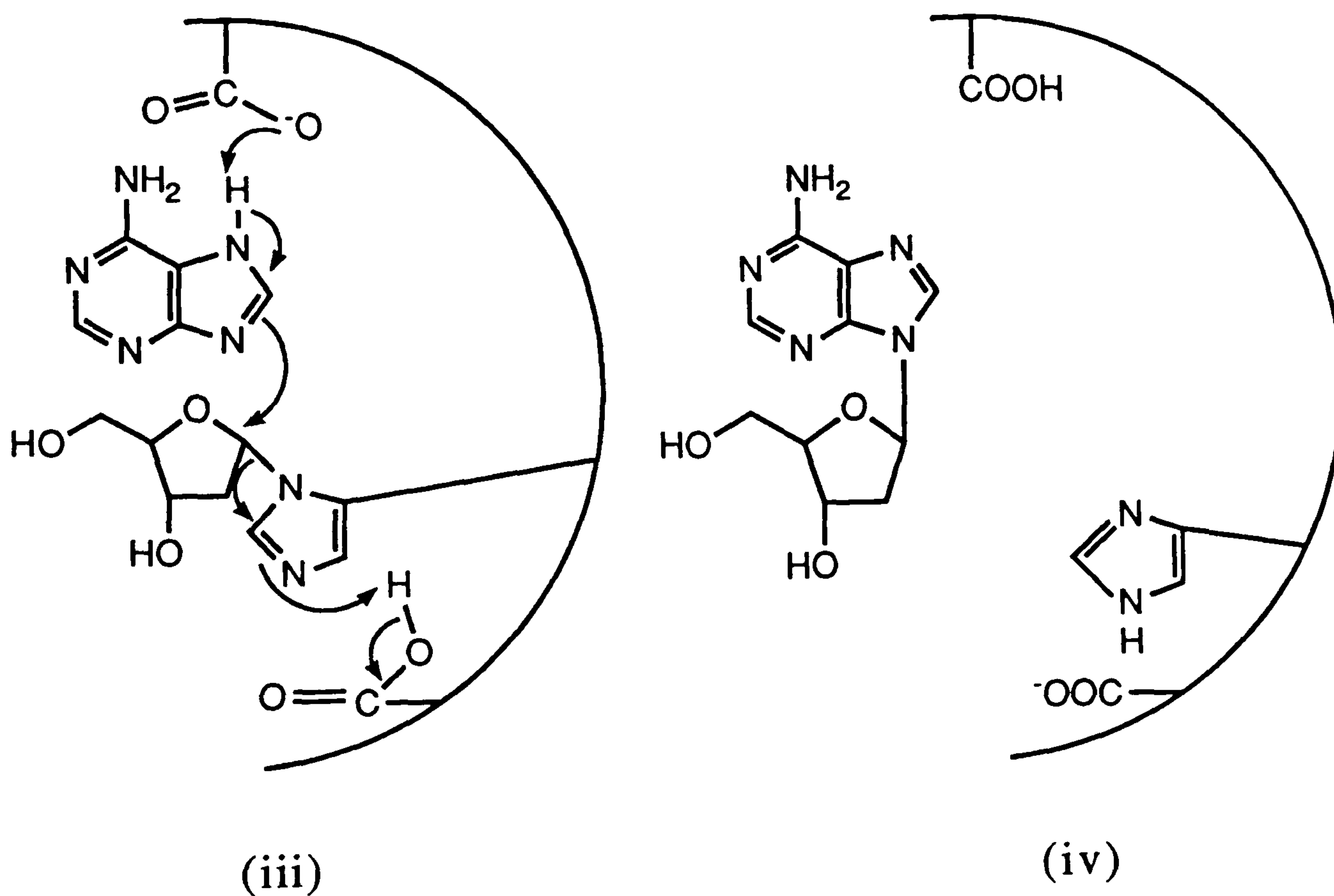
Figure 4.23

Possible Model for Enzymatic Reaction of N-Deoxyribosyltransferase-II involving a histidine, and carboxyl groups



Displacement of donor base

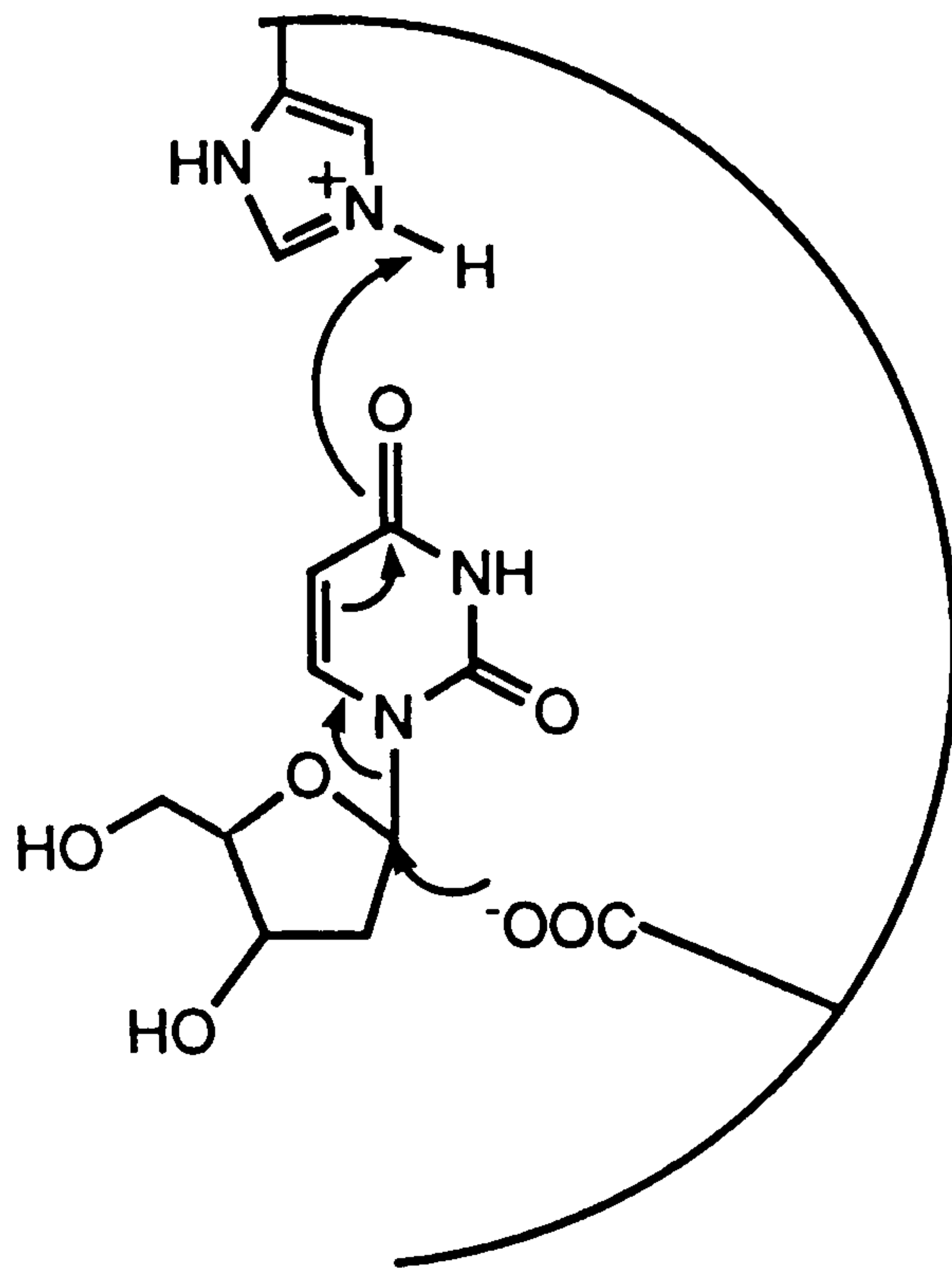
Glycosyl enzyme



Formation of product

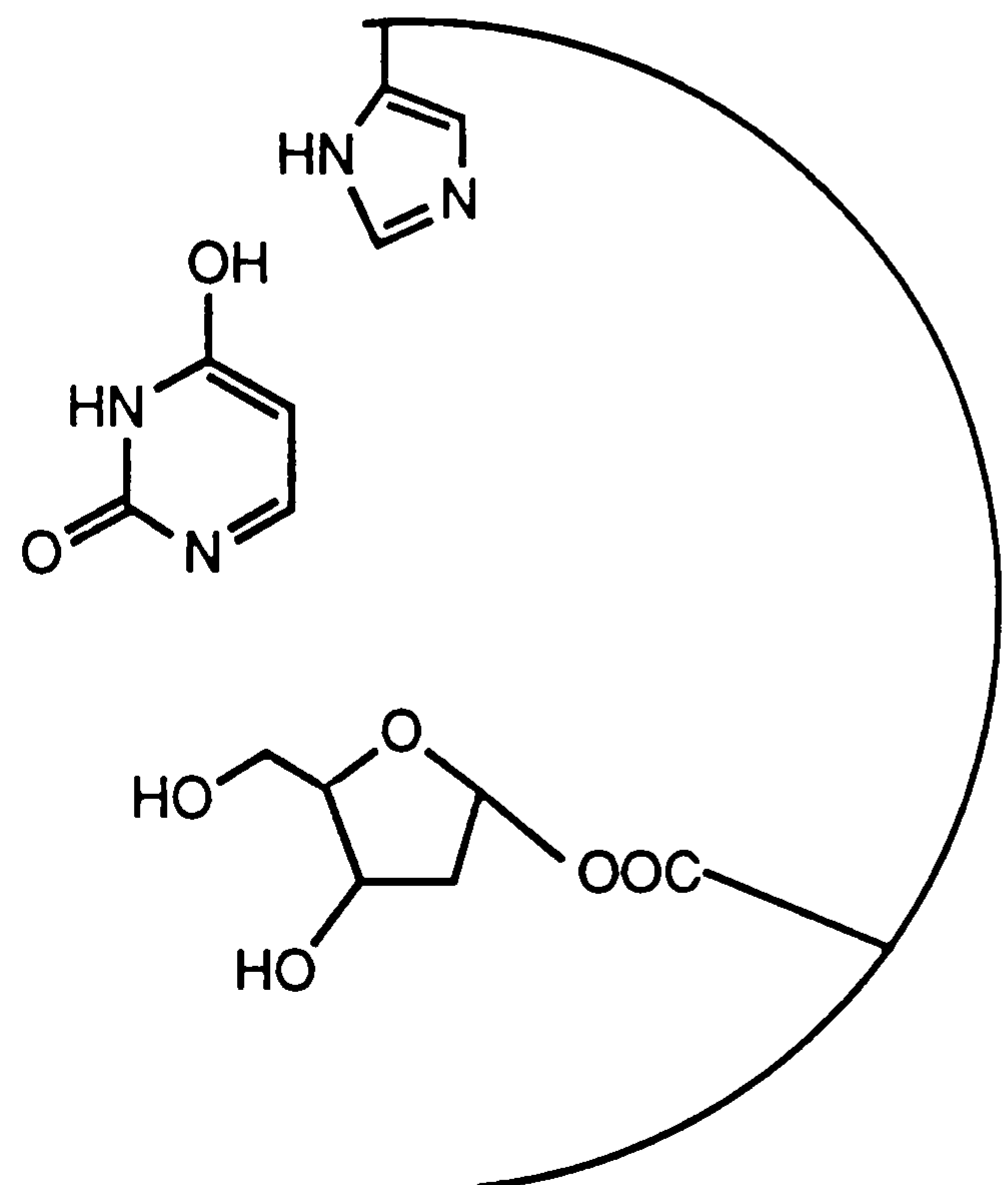
Figure 4.24

Possible Model for Enzymatic Reaction of N-Deoxyribosyltransferase-II  
involving a histidine and a carboxyl group



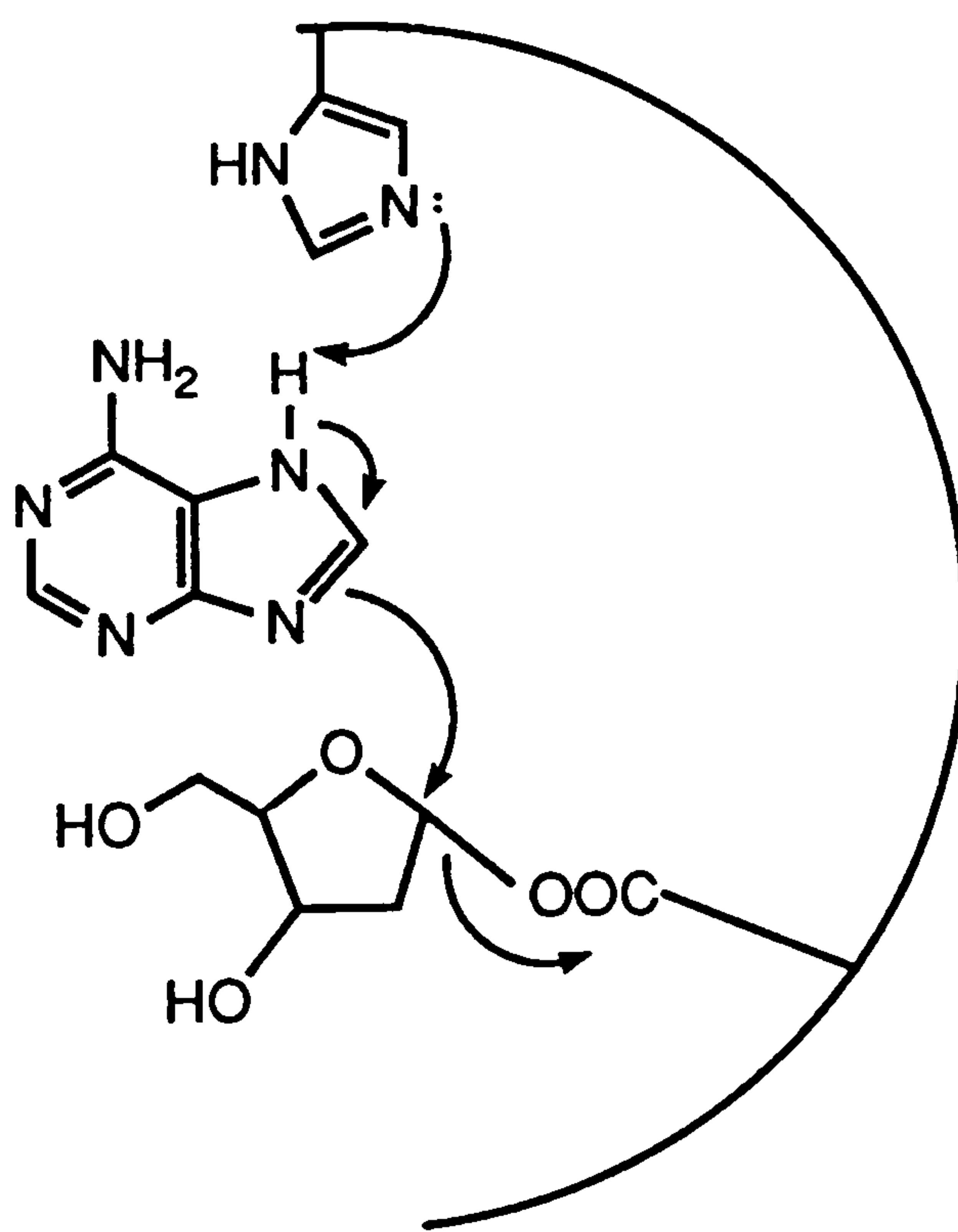
(i)

Displacement of donor base



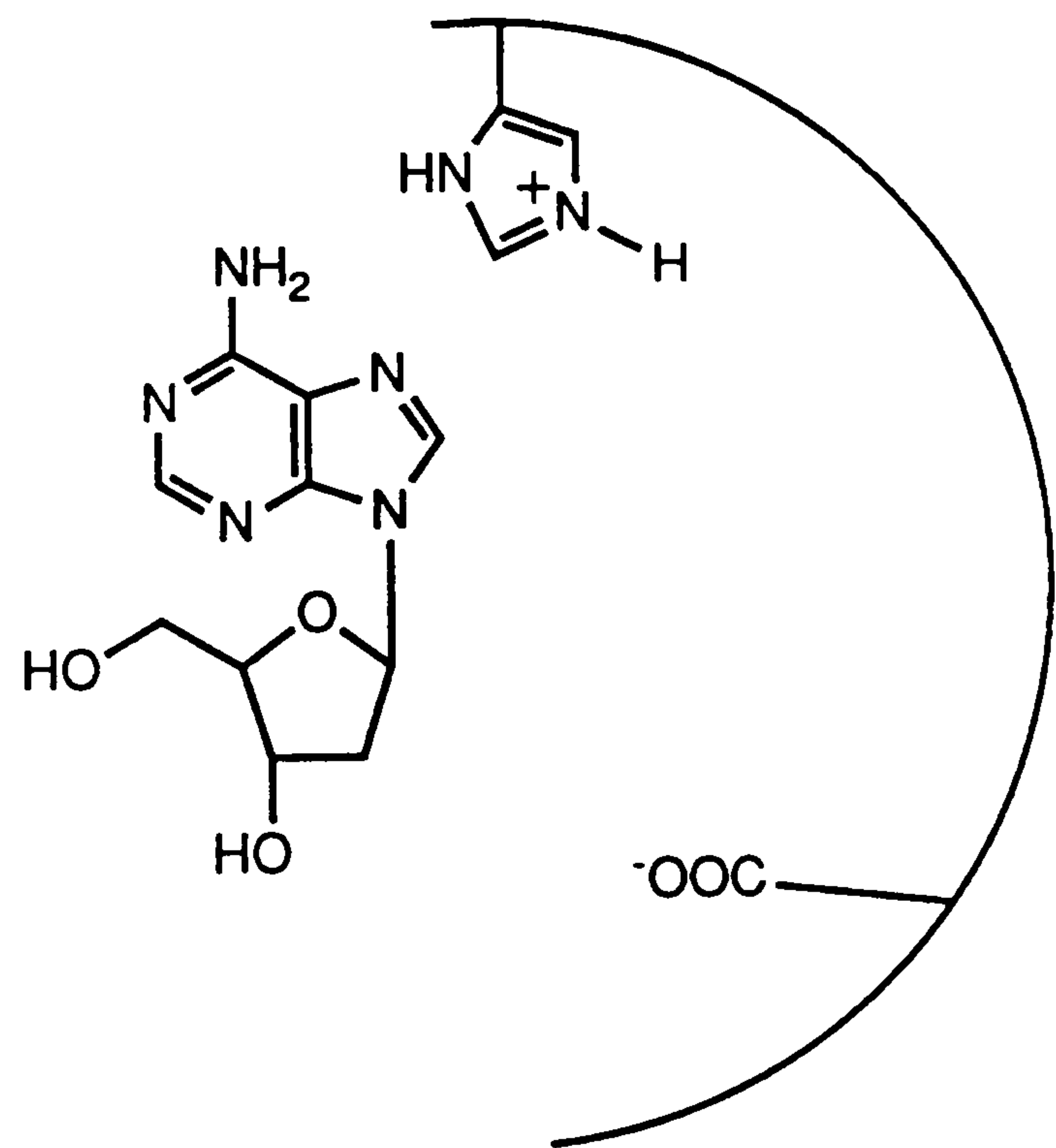
(ii)

Glycosyl enzyme



(iii)

Formation of product



(iv)



determine the specificity between these two N-deoxyribosyltransferases. Many of these modification agents are unstable in aqueous solution [170] and away from extremes of pH the degree of inactivation achieved was not always as high as anticipated, and strict conditions were often required to preserve the stability of the reagents and enzyme. An affinity label may obviate the need for many of these problems to be solved.

Uridine phosphorylase from *E. coli* has been shown to be sensitive to the reagent DEPC and inactivation of the enzyme indicates that there is an essential histidine residue at the active site [173]. The phosphorylase mechanism is known to proceed via the formation of an phosphate- $\alpha$ -1-ribose intermediate. It is possible that there is a covalent enzyme-ligand complex bound through a histidine residue similar to the one postulated for N-deoxyribosyltransferase-II from *L. leichmannii*.

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