The distribution and diversity of nucleoside *N*-deoxyribosyltransferase among lactic acid bacteria.

by

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Declaration

The work described in this thesis is the original of the author except where acknowledgement has been made to the results and ideas of others.

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Abstract

Nucleoside *N*-deoxyribosyltransferases are capable of synthesising stereo- and regioselectively a variety of 2'-deoxy- and 2',3'-dideoxyribonucleosides. These compounds could be effective as inhibitors of reverse transcriptase, by acting as DNA chain terminators.

The enzyme has been found previously only in a small number of microorganisms of the genus *Lactobacillus*. In the present study, phosphate-independent nucleoside *N*-deoxyribosyltransferase activity was detected in cell-free extracts from the genera *Aerococcus, Pediococcus, Leuconostoc, Streptococcus , Lactococcus* and also more widely among the subgroups of *Lactobacillus*. From this screening study, it was found that only purine-specific *N*-deoxyribosyltransferase activity was present in *Lactobacillus salivarius* subsp. *salivarius,* suggesting that this strain lacked an enzyme analogous to *N*-deoxyribosyltransferase II. *Leuconostoc mesenteroides* subsp. *cremoris* was found to express the highest *N*-deoxyribosyltransferase specific activity towards pyrimidines and purines.

As *N*-deoxyribosyltransferase activity had not been detected in strains of *Leuconostoc* before, the enzyme was purified and characterised from *Leu. mesenteroides* subsp. *cremoris*. A single multifunctional enzyme capable of carrying out the transfer of the deoxyribosyl moiety from either pyrimidine or purine nucleosides to either pyrimidine or purine bases was found to be present in this strain of *Leuconostoc*.. A four-step procedure employing affinity chromatography was used to purify the enzyme to homogeneity.

Kinetic studies carried out on the purified enzyme showed that the transfer reactions ocurred via a ping-pong mechanism. Evidence was also provided by radiolabelling studies of a glycosyl-enzyme intermediate forming when the first substrate, the nucleoside donor, bound to the enzyme.

Abbreviations

A	Adenine
ACV	Acyclovir
AIDS	Acquired immunodeficiency syndrome
AZT	Zidovudine (3'-azido-2',3'-dideoxythymidine)
AZU	3'-azido-2',3'-dideoxyuridine
BVDU	(*E*)-5-(2-bromovinyl)-2'-deoxyuridine
C	Cytosine
Ci	Curie
CM	Carboxymethyl
cm	Centimetres
Cyt	Cytidine
d	Doublet
d4T	2',3'-didehydro-2',3'-dideoxythymidine
dA	2'-deoxyadenosine
DAST	(Diethylamino)sulphur trifluoride
°C	Degrees centrigrade
dC	2'-deoxycytidine
ddA	2',3'-dideoxyadenosine
ddC	2',3'-dideoxycytidine
ddG	2',3'-dideoxyguanosine
ddI	2',3'-dideoxyinosine
DEAE	Diethylaminoethyl
dI	2'-deoxyinosine
(d)R1P	(Deoxy)ribose-1-phosphate
dT	2'-deoxythymidine
ε_{290}	Extinction coefficient at 290 nm
FPLC	Fast Protein Liquid Chromatography

g	Centrifugal force
g	Grammes
GCV	Ganciclovir
h	Hour
HIV	Human immunodeficiency virus
^{1}HNMR	Proton nuclear magnetic resonance
HPLC	High Pressure Liquid Chromatography
HSV	Herpes simplex virus
Hx	Hypoxanthine
Hz	Hertz
IDU	Idoxuridine
IEF	Isoelectric focussing
Ino	Inosine
J	Coupling constant
kDa	Kilodaltons
l	Litres
M	Molar
MES	4-Morpholine ethanesulphonic acid
µg	Microgrammes
mg	Milligrammes
min	Minutes
µl	Microlitres
ml	Millilitres
mM	Millimolar
mm	Millimetres
µmol	Micromole
mmol	Millimole
mU	Milliunits
ng	Nanogrammes

nm	Nanometres
NMR	Nuclear magnetic resonance
NuPase	Nucleoside phosphorylase
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
Pi	Inorganic phosphate
pI	Isoelectric point
Pipes	Piperzine-N,N'-bis[2-ethanesulphonic acid]
ppm	Parts per million
psi	Per square inch
pur	Purine
PurNPase	Purine nucleoside phosphorylase
pyr	Pyrimidine
PyrNPase	Pyrimidine nucleoside phosphorylase
s	Singlet
sec	Second
subsp.	Subspecies
TCA	1,2,4-triazole-3-carboxamide
TFT	Trifluridine
ThdNPase	Thymidine nucleoside phosphorylase
tlc	Thin layer chromatography
U	Units
Ur	Uracil
Urd	Uridine
UrdNPase	Uridine nucleoside phosphorylase

"The goddess of learning is fabled to have sprung full grown from the brain of Zeus, but it is seldom that a scientific conception is born in its final form, or owns a single parent. More often it is the product of a series of minds, each in turn modifying the ideas of those that came before, and providing material for those that came after."

George P. Thomson

Chapter 1

Introduction

1.1 Nucleoside *N*-deoxyribosyltransferase

The intracellular enzyme nucleoside *N*-deoxyribosyltransferase (trans-*N*-deoxyribosylase; nucleoside : purine (pyrimidine) *N*-deoxyribosyltransferase EC 2.4.2.6), catalyses the direct transfer of a deoxyribosyl group from a purine (or a pyrimidine) deoxyribonucleoside to a purine (or a pyrimidine) base, as shown by the following scheme:

B, B' = Heterocyclic bases

This enzyme was first shown to be present in certain species of the lactic acid bacteria of the genus *Lactobacillus* by MacNutt (1952) who showed the enzyme to be present in dialysed extracts of *L. helveticus, L. delbrueckii,* and *Thermobacter acidophilus* (now known as *L. acidophilus*). These bacteria all required deoxyribonucleosides as growth-promoting substances, which suggested that the enzyme played a role in the synthesis of other deoxyribonucleosides in the bacteria.

Production of the deoxyribonucleoside was also shown not to proceed through a mechanism of hydrolysis followed by resynthesis, because the only two compounds, which might have expected to act as intermediates, deoxyribose and deoxyribose-1-phosphate, were not substrates. Hence the enzyme was considered to be acting as a trans-*N*-glycosylase. In a transfer reaction where deoxyadenosine was produced from adenine and

deoxyinosine with the enzyme from L. helveticus, the C_8 atom of the adenine moiety was labelled with ^{14}C in order to determine whether the enzyme was performing a transamination or trans-N-glycosylation reaction (Kalcker et al., 1952). The isotopic activity was found to be present solely in the deoxyadenosine produced and with a molar concentration as high as that of the initial adenine. Hence, the enzyme was catalysing a trans-*N*-glycosylation reaction where only the purine bases were exchanged and not groups on the bases.

Cell free extracts from *L. lactis, L. delbrueckii, L. leichmânnii* and *L. acidophilus* (Beck & Levin, 1963) were also shown to contain *N*-deoxyribosyltransferase activity. Partial purifications of the enzyme have been carried out from *L. helveticus* (Roush & Betz, 1958), *L. leichmannii* (Minghetti, 1960), *L. acidophilus* (Marsh & King, 1959) and *L. delbrueckii* (Kanda & Takagi, 1959). However, transferase activity was found to be absent from *L. casei* and a thymine-requiring strain of *Escherichia coli* (Beck & Levin, 1963). The enzyme was also not detected from mammalian sources such as chicken kidney and rat liver (Marsh & King, 1959).

Roush and Betz (1958) were the first to suggest the existence of two distinct transferase enzymes from *L. helveticus*. They demonstrated that Tris buffer completely inhibited transfers involving pyrimidines and partly inhibited those where only purines were involved. This observation led to the suggestion that there were two kinds of transferase enzymes present in *L. helveticus*. This hypothesis was later confirmed by Holguin and Cardinaud (1975), who carried out the successful separation of the two transferase enzymes using affinity chromatography.

The two types of nucleoside *N*-deoxyribosyltransferases which have been isolated from *L. helveticus* are; nucleoside *N*-deoxyribosyltransferase I which catalyses the transfer between purine bases exclusively, and nucleoside *N*-deoxyribosyltransferase II which transfers the sugar moiety between purines and pyrimidines as well as from a purine to a pyrimidine, shown by the following scheme:

Transferase I:

$$\text{Deoxyribosyl-pur}_1 + \text{pur}_2 \rightleftharpoons \text{deoxyribosyl-pur}_2 + \text{pur}_1$$

Transferase II:

$$\text{Deoxyribosyl-pur} + \text{pyr} \rightleftharpoons \text{deoxyribosyl-pyr} + \text{pur}$$

$$\text{Deoxyribosyl-pur}_1 + \text{pur}_2 \rightleftharpoons \text{deoxyribosyl-pur}_2 + \text{pur}_1$$

$$\text{Deoxyribosyl-pyr}_1 + \text{pyr}_2 \rightleftharpoons \text{deoxyribosyl-pyr}_2 + \text{pyr}_1$$

Where pur = purine bases

pyr = pyrimidine bases

A nucleoside *N*-deoxyribosyltransferase enzyme from *L. helveticus*, displaying all three transfer activities, was the first to be purified to homogeneity from which microcrystals were produced (Uerkwitz, 1971). This enzyme was shown to have a molecular weight of 82 000 (± 5%). Later, two separate transferase enzymes were purified from *L. helveticus* using affinity chromatography with two types of ligands (Holguin & Cardinaud, 1975). The molecular weight of the purine-specific transferase enzyme was found to be 86 000 (± 4000). Holguin & Cardinaud (1975) also carried out an inhibition study with Tris buffer showing that the purine-specific enzyme was inhibited at much higher concentrations than transferase II.

Nucleoside *N*-deoxyribosyltransferase from *L. leichmannii* has also been purified using affinity chromatography, by a modified procedure of Holguin and Cardinaud (Huang *et al.*, 1981). More recently however, the gene coding for the enzyme in *L. leichmannii* has been cloned and expressed in *E. coli* (Cook *et al.*, 1990). Crystals of the recombinant bacterial transferase enzyme have also been grown for X-ray studies. The native molecular weight of the recombinant enzyme was found to be 110 000 and the subunit molecular weight was approximately 18 000. The enzyme was reported to be a hexamer of six identical subunits, but the authors did not specify whether the cloned transferase enzyme was transferase I or II. However, the isolation of two distinct *N*-deoxyribosyltransferases from *L. leichmannii* has been reported and their physical properties investigated (Heath, 1991).

1.1.2 Substrate Specificity

Nucleoside *N*-deoxyribosyltransferases are not specific for the naturally occurring deoxyribonucleosides and bases, but they are able to accept a wide variety of modified nucleosides and bases (Beck & Levin, 1963; Roush & Betz, 1958; Baranski *et al.*, 1969; Holguin *et al.*, 1975). From substrate specificity studies, general rules have been devised to predict whether a base would act as a competent acceptor for these transferase enzymes (Holguin *et al.*, 1975). For purine bases a tautomeric proton should be present on the imidazole ring, the usual shift being between position 9 and 7 (Figure 1.1). The position of this tautomeric proton directs the site of substitution of the deoxyribosyl moiety. Finally for steric reasons substituents are not usually allowed on position 8, since a mixture of products are formed (Huang *et al.*, 1983). Huang and co-workers showed that when purines with electron-withdrawing groups at the 8 position are

used as substrates, such as 8-bromo- or 8-chloroadenine, a mixture of 3- and 9-deoxyribonucleosides were produced.

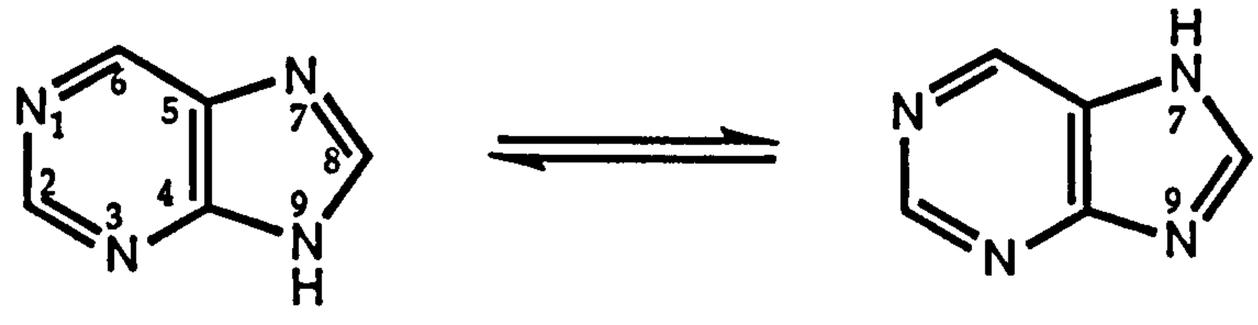


Figure 1.1 Tautomeric positions in purine bases

For pyrimidine analogues, a lactim-lactam type tautomerism must exist between the nitrogen atom N-1 and a suitable group on position 2 (or 6) (Figure 1.2). Therefore no other substituent can be allowed on position 6 (or 2) for steric reasons. The aromatic properties of the ring must also be preserved.

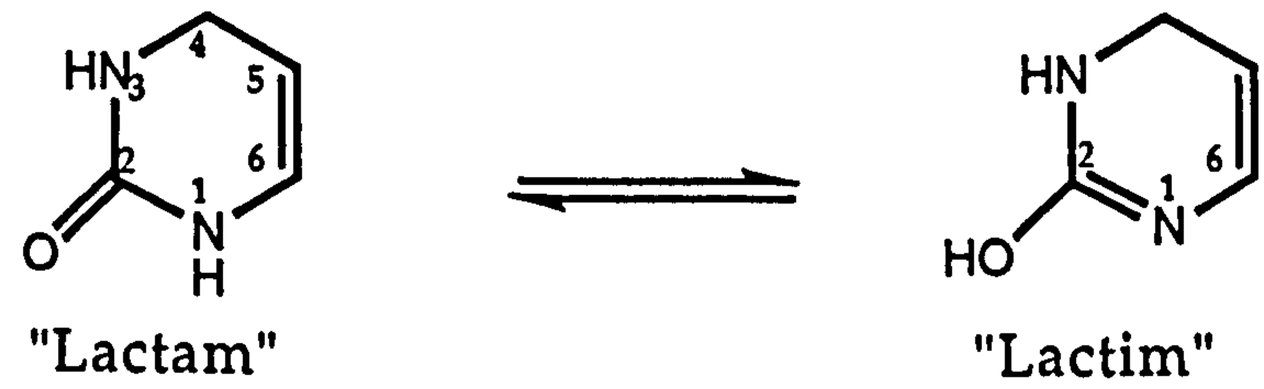
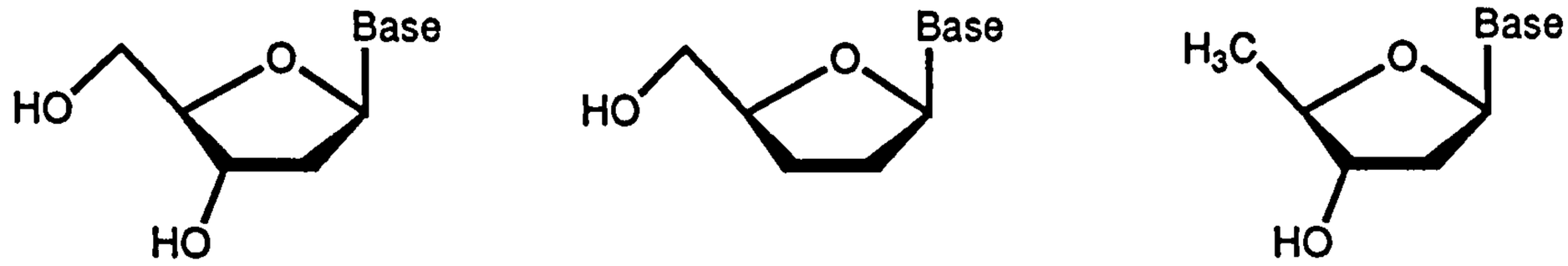


Figure 1.2 Tautomeric positions in pyrimidine bases

In contrast to the wide variety of bases that the transferase enzymes can receive as substrates, both enzymes can tolerate only a few modifications on the deoxyribosyl moiety of the donor nucleoside. The transferase enzymes from lactobacilli had been thought to be specific only for the 2'-deoxyribosyl group, however 2',3'-dideoxyribosyl (Figure 1.3) has also been shown to be a substrate for the enzymes. But the rate of transfer of the 2',3'-dideoxyribosyl moiety occurs at a much slower rate than the natural 2'-deoxyribosyl group (Carson & Wasson, 1988). Ribosides,

arabinosides and glucosides were not transferred by the enzymes from *L. helveticus* (Holguin, 1974).



2'-deoxyribonucleoside 2',3'-dideoxyribonucleoside 2',5'-dideoxyribonucleoside

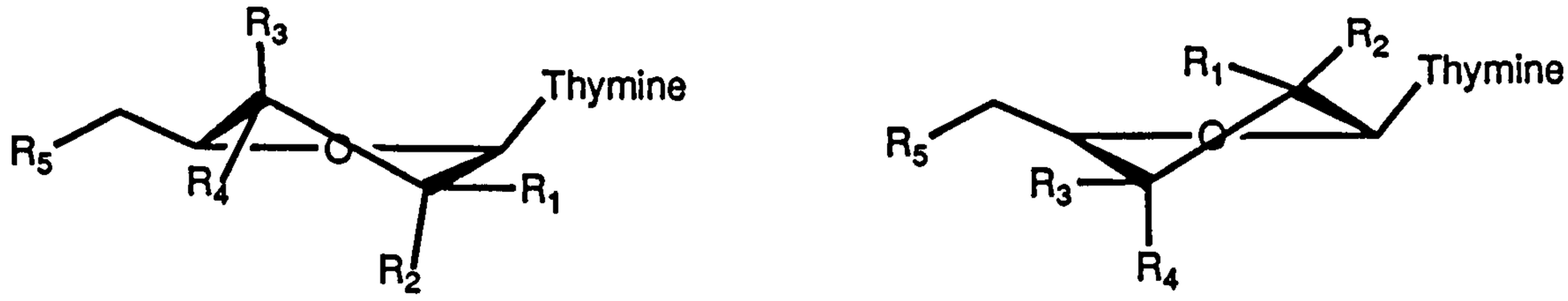
Figure 1.3 Deoxyribose residues accepted by *N*-deoxyribosyltransferases from *Lactobacillus*

Certain modifications on the 5'-position of the sugar group can also be accepted, such as fluorine (Cardinaud, 1978). The absence of the hydroxyl group at the 5'-position generates the 2',5'-dideoxynucleosides (Figure 1.3), which can also act as substrates (Carson & Wasson, 1988; Betbeder *et al.*, 1991).

In the absence of acceptors, recombinant nucleoside *N*-deoxyribosyltransferase from *L. leichmannii* has been shown to catalyse the hydrolysis of 2'-deoxyribonucleosides to produce initially D-ribal which then becomes hydrated (Smar *et al.*, 1991). D-ribal was also found to be an acceptor of the transferase enzyme because when incubated with adenine, in the presence of the transferase enzyme, 2'-deoxyadenosine was generated. Hence, a novel method of synthesising deoxyribonucleosides was suggested, where the purification of the new nucleoside was simplified by eliminating the separation step of the substrate nucleoside from the reaction mixture.

NMR studies at various temperatures have been carried out on some pyrimidine deoxyribonucleosides (Hicks *et al.*, 1991), where the

pseudorotation conformations of the deoxyribosyl ring of the nucleosides were investigated (Figure 1.4).



Type N
[2'exo, 3'endo]

Type S
[2'endo, 3'exo]

Figure 1.4 Pseudorotation conformations of the deoxyribosyl ring

These studies showed that substrates, such as thymidine, 5'-deoxythymidine and 2',3'-dideoxythymidine, were competent glycosyl donors for the transferase enzymes from lactobacilli because they possessed a relatively flexible glycosyl ring. This property may play an important role at the active site of the enzyme by allowing the correct conformation to be attained for binding and catalysis. Nucleoside analogues, such as 3'-deoxy-3'-azidothymidine (AZT) and 3'-deoxy-3'-fluorothymidine, were found to have a rigid, type S ring conformation (Figure 1.4). Hence, it appears that modifications on the glycosyl residue of nucleosides which enhance the rigidity of the deoxyribosyl ring may be inactive as substrates of the transferase enzymes because they possess an inflexible glycosyl ring. Other factors may also contribute to these nucleosides being non-substrates, such as the size of the group on the sugar ring may be too large for the active site to accept.

1.1.3 Mechanistic Studies

Extensive studies have been carried out on purified extracts of nucleoside *N*-deoxyribosyltransferase I (Danzin & Cardinaud, 1974) and on nucleoside *N*-deoxyribosyltransferase II (Danzin & Cardinaud, 1976) from *L. helveticus*. In both cases the initial velocity experiments have shown the transfer of the deoxyribosyl moiety to occur via a ping-pong bi-bi mechanism, according to the criteria laid down by Cleland (1963). The kinetic mechanism is ping-pong because the first product is released before the second substrate combines and bi-bi because there are two kinetically important substrates and products (Figure 1.5). This mechanism of transfer action has also been demonstrated in *N*-deoxyribosyltransferases from *L. leichmannii* (Heath, 1991).

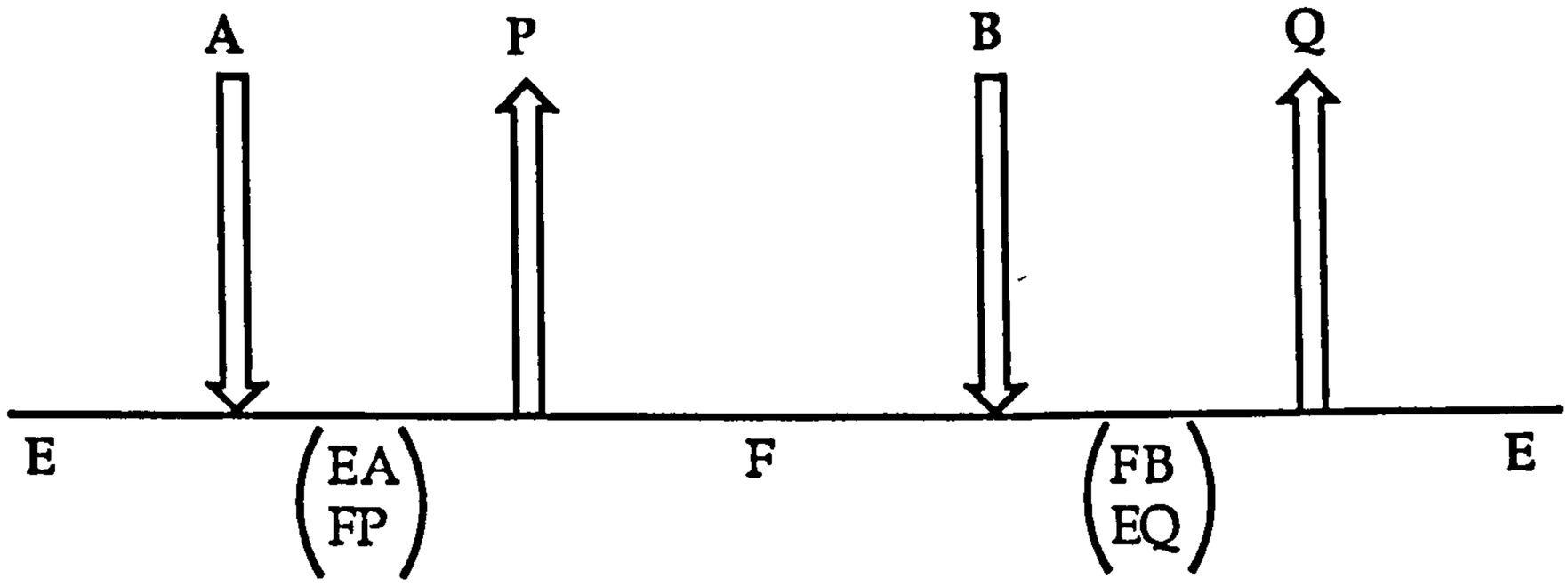


Figure 1.5 Ping-pong bi-bi reaction mechanism (Cleland, 1963a)

The mechanism depicted above suggests the formation of a glycosyl-enzyme intermediate. Preliminary experiments have been carried out on the isolation of such an intermediate in *L. leichmannii* (Heath, 1991), but conclusive evidence was not provided.

Chemical modification of specific amino acid residues have been carried out on *N*-deoxyribosyltransferase II from *L. leichmannii* (Heath, 1991). The

results of which suggested that an histidine and/or carboxyl groups may take part in the binding and catalysis at the active site of the enzyme. Figure 1.6 illustrates a proposed mechanism of action of the transferase enzymes from *L. leichmannii*, showing the involvement of the histidine and carboxyl groups, and the formation of a glycosyl-enzyme intermediate.

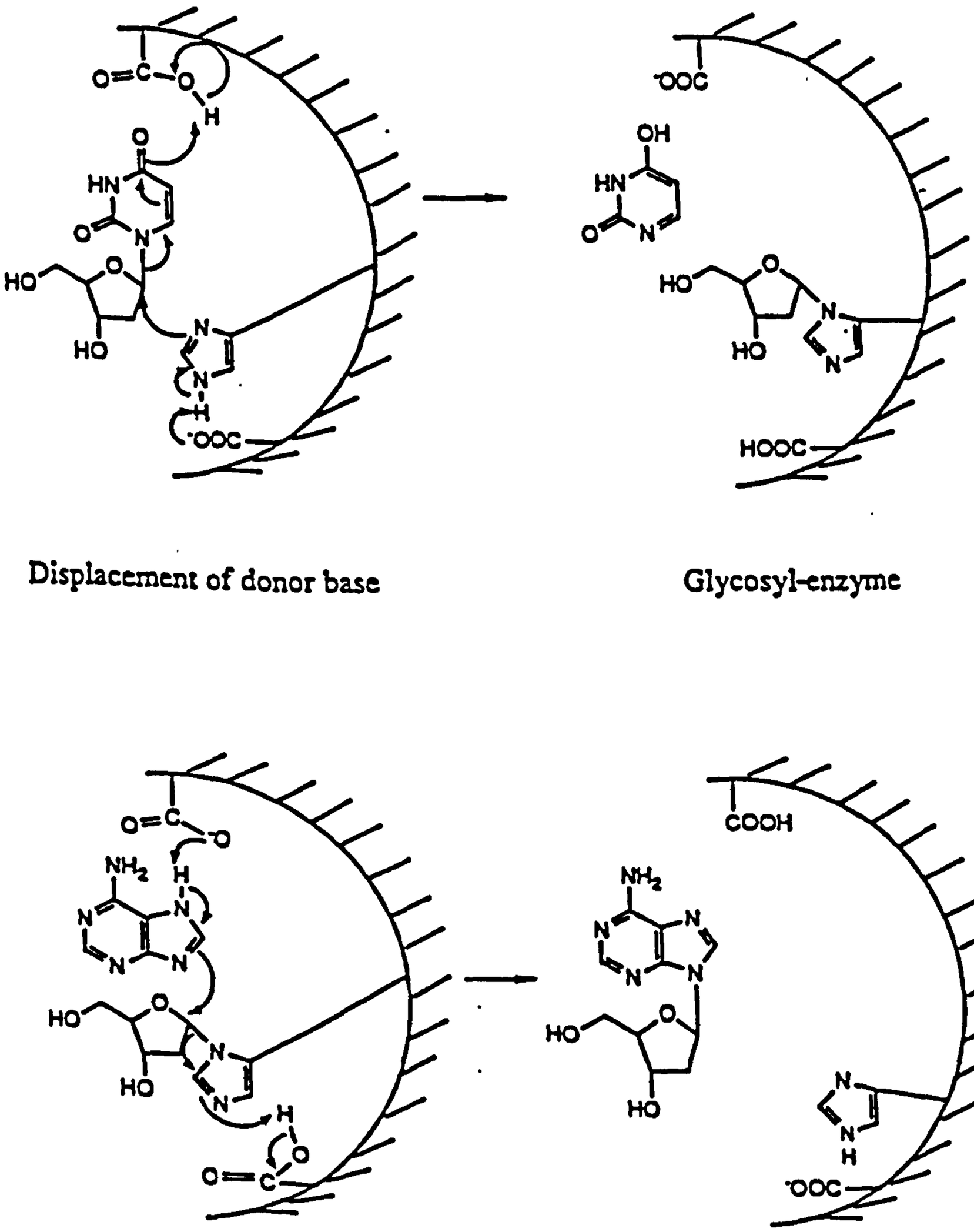


Figure 1.6 Proposed mechanism of action of *N*-deoxyribosyltransferases (Heath, 1991)

1.1.4 Biological Function of *N*-deoxyribosyltransferases

As described earlier, the biological function of nucleoside *N*-deoxyribosyltransferases in lactobacilli has been shown to be associated with the synthesis of deoxyribonucleic acids (Beck & Levin, 1962; 1963; Uerkvitz, 1971). The four species of lactobacilli studied, *L. leichmannii*, *L. lactis*, *L. acidophilus* and *L. delbrueckii*, all required at least one deoxyribonucleoside for growth (Beck & Levin, 1963). However, with *L. leichmannii* and *L. lactis* the deoxyribonucleoside requirement can be replaced by vitamin B_{12} (Beck & Levin, 1963; Wright *et al.*, 1948).

The biological role of vitamin B_{12} in *L. leichmannii* has been shown to be concerned with deoxyribonucleotide synthesis (Beck & Levin, 1962; Beck *et al.*, 1962). Vitamin B_{12} was originally thought to be the only compound that participated in the reductive conversion of ribonucleotides to deoxyribonucleotides (Downing & Schweigert, 1956; Spell & Dinning, 1959; Manson, 1960). It is now known that a dissociable cofactor, adenosylcobalamin from vitamin B_{12}, together with ribonucleotide reductase catalyse the reduction of ribonucleotides to deoxyribonucleotides (Thelander & Reichard, 1979).

Since the ribonucleotide reductase from *L. leichmannii* has an absolute requirement for coenzyme B_{12}, in the absence of vitamin B_{12} deoxyribonucleoside synthesis must depend on *N*-deoxyribosyltransferases. Beck & Levin (1962; 1963) investigated transferase levels in *L. leichmannii* during vitamin B_{12} starvation. Transferase activity was found to increase in the vitamin B_{12}-limited cultures compared to the optimally nourished controls in which the level of transferase activity remained essentially constant. Similar results were

demonstrated with deoxyribonucleoside-starved cultures (Beck & Levin, 1962). Transferase activity was also shown to be elevated in *L. leichmannii,* when limited in free bases (Beck & Levin, 1963).

Vitamin B_{12} and deoxyribonucleoside starvation resulted in impaired DNA synthesis and unbalanced growth producing long, non-viable, filamentous cells. The addition of excess vitamin B_{12} to cultures previously limited in vitamin B_{12} caused an abrupt increase in the levels of acid-soluble deoxyribosyl compounds and also in DNA (Beck *et al.*, 1962). Similary, these acid-soluble deoxyribosyl pools have been shown to decrease in vitamin B_{12}- and deoxyribonucleoside-starved cultures of *L. leichmannii,* but no effect can be seen on this pool size with pyrimidine and purine limitations.

The rise in transferase activity under nutrient limiting conditions and the fall in intracellular deoxyribosyl pool suggested that enzyme synthesis was controlled by a repression system. However, it was unclear whether repression was due to one or more of the acid-soluble deoxyribosyl compounds or upon the size or composition of the deoxyribosyl pool. Evidence for the direct feedback inhibition has not been determined.

Therefore, in the absence of vitamin B_{12}, nucleoside *N*-deoxyribosyltransferase provides an alternative salvage pathway for the production of deoxyribonucleotides in *L. leichmannii* and *L. lactis* (Figure 1.7). Deoxyribonucleotide metabolism in lactobacilli differs considerably from that in other bacteria and in its inability to catabolize deoxyribonucleosides. In contrast to *E. coli* which prefer to breakdown their deoxyribonucleosides, lactobacilli would rather use their

deoxyribonucleosides for the synthesis of other deoxyribonucleoside triphosphates.

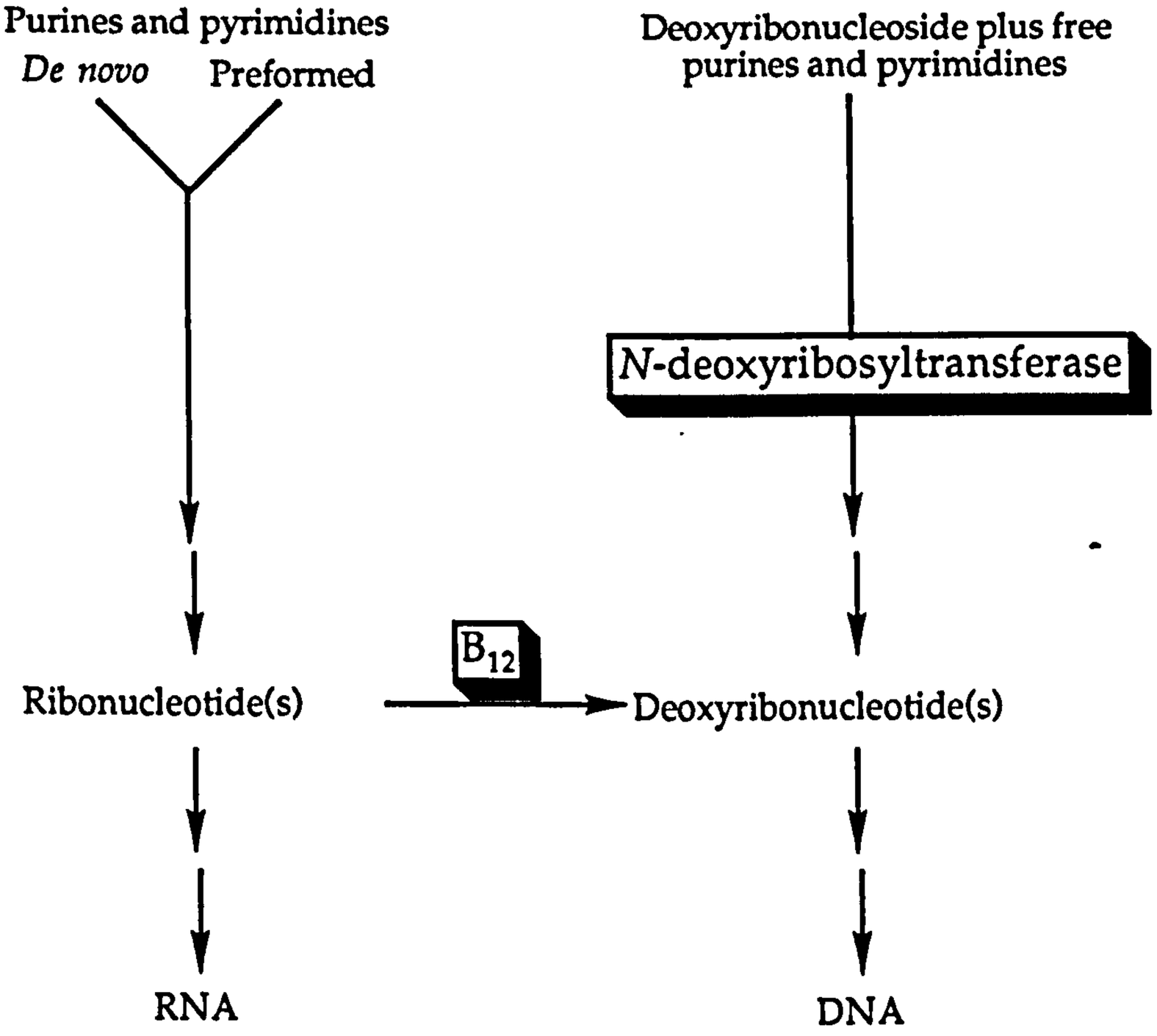


Figure 1.7 Alternative pathways proposed for deoxyribonucleotide synthesis in *L. leichmannii* (Beck & Levin, 1962)

These facts all confirm that the biological role of nucleoside *N*-deoxyribosyltransferase is to provide the cell with various deoxyribonucleosides through trans-deoxyribosylation reactions which ultimately leads to DNA synthesis.

1.1.5 Distribution

Nucleoside *N*-deoxyribosyltransferase activity has only been investigated in the genus *Lactobacillus* of the lactic acid bacteria. *Lactobacillus* comprises of about fifty species and many subspecies. They are identified as Gram-positive, non-spore forming rods, usually non-motile, which utilise

glucose fermentatively. They can either break down glucose homofermentatively, producing more than 85% lactic acid, or heterofermentatively, producing lactic acid, carbon dioxide, ethanol and/or acetic acid. Lactobacilli have been further divided into three subgroups based on their fermentation products; obligately homofermentative, facultatively heterofermentative and obligately heterofermentative (Table 1.1).

Glucose fermented almost entirely (85%) to lactic acid		Glucose fermented to lactic acid (50%)+ CO_2 + acetic acid + ethanol
Obligately Homofermentative	Facultatively Heterofermentative	Obligately Heterofermentative
L. acidophilus	*L. casei*	
L. delbrueckii		
L. bulgaricus		
L. lactis		
L. leichmannii		
L. helveticus		

Table 1.1 Subdivision of the genus *Lactobacillus* containing only the species investigated for nucleoside *N*-deoxyribosyltransferase activity.

The organisms which have been shown to possess nucleoside *N*-deoxyribosyltransferase activity fall into the obligately homofermentative group (Table 1.1). *L. casei*, which belongs to the facultatively heterofermentative group, has been reported to have no

transferase activity (Marsh & King, 1959; Beck & Levin, 1963). The present investigation confirms this report.

Recently, *N*-deoxyribosyltransferase activity has been reported in the protozoa, *Crithidia luculiae* (Steenkamp, 1991). Three nucleosidase activities were found to be present in this organism, two were ribonucleosidases and the third activity was a purine-2'-deoxyribonucleosidase. Trans-*N*-deoxyribosyltransferase activity was found to be an inherent property of the purine-2'-deoxyribonucleosidase from *C. luculiae*. Hence showing that a single enzyme contained both transferase and nucleosidase activities which were specific for purine deoxyribonucleosides.

1.2 Nucleoside Phosphorylases

In contrast to the direct transfer of deoxyribose groups by the *N*-deoxyribosyltransferases of lactobacilli, these transfer reactions can also be catalysed by another set of enzymes, the nucleoside phosphorylases. These phosphorylases carry out the transfer of glycosyl moieties by an indirect method, where the reversible phosphorolysis of either purine or pyrimidine nucleosides yields pentose-1-phosphate and a free base. This two-step mechanism can be depicted as follows:

$$\text{Pentose-1-Base}_1 + \text{Pi} \rightleftharpoons \text{Pentose-1-Pi} + \text{Base}_1$$

$$\text{Pentose-1-Pi} + \text{Base}_2 \rightleftharpoons \text{Pentose-1-Base}_2 + \text{Pi}$$

Where, Pi = inorganic phosphate

Base = purine or pyrimidine

Pentose = ribose or deoxyribose

Previously known as nucleosidases, the mechanism of these enzymes was not clarified until 1945 (Kalcker, 1945). Kalcker (1945; 1947a) demonstrated that a purine nucleosidase from rat liver was cleaving inosine phosphorolytically rather than hydrolytically, to give ribose-1-phosphate and hypoxanthine. This nucleosidase was later named purine nucleoside phosphorylase. Later, Manson and Lampen (1951) also noticed that enzyme preparations from calf kidney split thymidine, via a phosphorolytic mechanism, releasing a sugar phosphate ester, later identified as 2-deoxyribose-1-phosphate.

1.2.1 Distribution and Types of Nucleoside Phosphorylases

Nucleoside phosphorylases are widely distributed in nature, occurring in a number of animal tissues and in many species of bacteria. There are two main types of phosphorylases, which each have a broad range of substrates. These are the pyrimidine and purine nucleoside phosphorylases which are both able to cleave ribo- and deoxyribonucleosides. Other phosphorylases are known which are more specific in their choice of substrates and these include uridine, thymidine, inosine-guanosine and adenine phosphorylases.

1.2.1.1 Purine Phosphorylase [EC 2.4.2.1]

Purine phosphorylases have been isolated from a variety of animal and bacterial sources (Parks & Agarwal, 1972; Bzowska *et al.*, 1990) but the enzyme isolated from *E. coli* has been extensively studied (Jensen & Nygaard, 1975; Jensen, 1976). The base specificity of the purine phosphorylase is dependent on the source from which it is isolated. Hypoxanthine, guanine and xanthine and their nucleosides are efficient

substrates for most mammalian purine phosphorylases, but adenine and adenosine are not good substrates (Krenitsky, 1967; Zimmerman *et al.*, 1971).

Two purine nucleoside phosphorylases have been reported to occur in *E. coli*, one is specific for inosine and guanosine and their bases, known as inosine-guanosine phosphorylase (Koszalka *et al.*, 1988a), while the other is less restricted and cleaves adenosine, inosine and guanosine (Doskocil & Holy, 1977; Jensen, 1976). But xanthosine was found to be an inefficient substrate for these enzymes. *Bacillus subtilis* also contains two distinct purine phosphorylases, one specific for adenosine only termed adenosine phosphorylase (Senesi *et al.*, 1976), and the other for inosine and guanosine (Jensen, 1978), which is similar to the one found in *E. coli*.

Purine phosphorylases from both mammalian sources and *E. coli* are able to cleave ribo-, 2'-deoxyribo-, 5'-deoxyribo-, arabino- and 2',3'-dideoxyribonucleosides. However, the inosine-guanosine phosphorylase was unable to cleave hypoxanthine arabinoside, whereas purine nucleoside phosphorylase is already an efficient catalyst for the synthesis of purine arabinonucleosides (Utagawa *et al.*, 1980; Krenitsky *et al.*, 1981a).

1.2.1.2 Pyrimidine Phosphorylase [EC 2.4.2.2]

A pyrimidine phosphorylase has been isolated and purified from the thermophile *B. stearothermophilus* (Saunders *et al.*, 1969), which was found to be stable up to 60°C. This single enzyme has been found to be specific for both uridine and thymidine, hence able to catalyse the transfer of both ribose and deoxyribose moieties. *Haemophilus influenzae* is another source from which pyrimidine phosphorylase has been isolated

and studied extensively (Scocca, 1971; 1978). The enzyme from this bacteria also contained uridine and thymidine phosphorylase activities, but did not cleave cytidine.

1.2.1.3 Uridine Phosphorylase [EC 2.4.2.3]

The presence of uridine phosphorylase was first determined in *E. coli* by Paege and Schlenk (1952). Later the enzyme was separated from another pyrimidine phosphorylase, thymidine phosphorylase, by Razzell and Khorana (1958), from *E. coli* and also from mammalian tissues (Krenitsky *et al.*, 1964; Yamada, 1968). Uridine phosphorylase is able to cleave both ribo- and deoxyribonucleosides and is specific for uridine, deoxyuridine and thymidine from both mammalian and bacterial origins.

1.2.1.4 Thymidine Phosphorylase [EC 2.4.2.4]

The existence of thymidine phosphorylase was first demonstrated to be present in *E. coli* by Manson and Lampen (1951) and later found in a number of mammalian tissues (Friedkin & Roberts, 1954). Thymidine phosphorylases from *E. coli* (Razzell & Khorana, 1958; Schwartz, 1978) and *Salmonella typhimurium* (Blank & Hoffee, 1975; Hoffee & Blank, 1978) have been well documented and have been shown to be specific only for the pyrimidine nucleosides, thymidine and deoxyuridine. Deoxycytidine as well as purine ribonucleosides and deoxyribonucleosides were not substrates for the enzymes.

E. coli and *S. typhimurium* have been shown to possess purine, thymidine, and uridine phosphorylases while *B. stearothermophilus* and *H. influenzae* contain pyrimidine phosphorylase, which acts on both

phosphate-independent enzyme for carrying out glycosyl transfer reactions. Enzymatic phosphorolysis of nucleosides has not been demonstrated in many lactobacilli (O'Donovan & Neuhard, 1970). However, *L. casei* which was earlier shown not to possess *N*-deoxyribosyltransferase activity, has been reported to contain thymidine and uridine phosphorylases (Avraham *et al.*, 1988; 1990). Thymidine phosphorylase activity has also been reported in *L. acidophilus* (Sawula & Zamenhoff, 1974; Sawula *et al.*, 1975).

1.3 Biological Role

Nucleosides and nucleotides are too important to cells to be wasted in catabolic reactions and most organisms possess pathways to salvage their nucleosides (-tides) as well as having enzymes that can carry out the *de novo* biosynthesis of these compounds. These class of important compounds include ATP, the biological 'quantum' of energy, and the immediate source of energy for most cellular processes. AMP, ADP and ATP influence most metabolic pathways since these nucleotides are allosteric effectors of many key regulatory enzymes. Nucleoside triphosphates are also the activated precursors of DNA synthesis. Hence, it would be very wasteful for an organism not to be able to reuse these compounds once they were degraded.

In most bacteria, nucleoside phosphorylases are responsible for the degradation and the salvaging of nucleosides for nucleic acid synthesis. In *E. coli* and *S. typhimurium* thymidine phosphorylases play an important role in the metabolism of thymine auxotrophs and are necessary for the conversion of exogenous thymine to thymidine. Lactobacilli, on the other hand, seem to use the enzymes, nucleoside *N*-deoxyribosyltransferases, in

their salvage pathway for synthesising new nucleosides. In contrast to bacteria such as *E. coli* however, lactobacilli do not appear to have the ability to breakdown their deoxyribonucleosides, they would rather use them to synthesise other deoxyribonucleosides. This attractive property of *N*-deoxyribosyltransferases will be shown later to be useful in the enzymatic syntheses of nucleoside analogues.

1.3 Targets for Antiviral Therapy

1.3.1 Acquired Immunodeficiency Syndrome (AIDS)

Interest in antiviral therapy has escalated in the last ten years due to the sudden appearance and rapid spread of the debilitating disease, Acquired Immunodeficiency Syndrome (AIDS). The cause of this disease was discovered to be a new human virus, now known as human immunodeficiency virus (HIV) (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). Once inside the host, HIV infect the helper T-cells, which are a part of the immune system that help to destroy the invasion of any foreign particles. These T-cells are later progressively destroyed when the new virus particles are formed and released for further infection. As the immune system becomes impaired, the host becomes more susceptible to opportunistic infections such as pneumonia and certain types of neoplasms, eg. Kaposi's sarcoma (Wong-Staal & Gallo, 1985).

Like all viruses, HIV is an intracellular parasite that cannot replicate or do any damage until it enters a host cell. The genetic material of a retrovirus, such as HIV, is in the form of RNA, which has to be converted to DNA so that the viral components can be expressed for multiplication. Conversion of the viral genetic information from RNA to DNA is catalysed by the the

virally-encoded enzyme, reverse transcriptase. Once the virus has bound and fused to the membrane of the T-cell, the viral RNA and reverse transcriptase are released into the host cell's cytoplasm, where the reverse transcriptase uses the viral RNA as a primer to make a negative strand of DNA copy of the viral genome. Once the RNA of the RNA-DNA hybrid has been degraded by the inherent ribonuclease H activity of reverse transcriptase, the enzyme can then catalyse the formation of a positive strand of DNA. The double-stranded DNA circularizes and either remains in unintegrated form or is inserted into the cellular genome.

The different stages in the replicative cycle of viruses, including HIV, present a variety of potential targets for antiviral agents (Figure 1.8 and Table 1.2). Increased knowledge on the life cycle of viruses has also led to a more rational strategy in designing antiviral drugs (DeClercq, 1986; Mitsuya & Broder, 1987; Jeffries, 1989; Nasr *et al.*, 1990; Mitsuya *et al.*, 1990; 1991).

With HIV a vast amount of attention was given to compounds that inhibit the virally-encoded enzyme, reverse transcriptase. By targetting this enzyme the virus would be unable to make the DNA copy needed for producing more viruses for further replication. The only drug that is clinically approved for use against AIDS comes from a promising class of compounds known as nucleoside analogues. Hence, these compounds have emerged as potent antiviral agents.

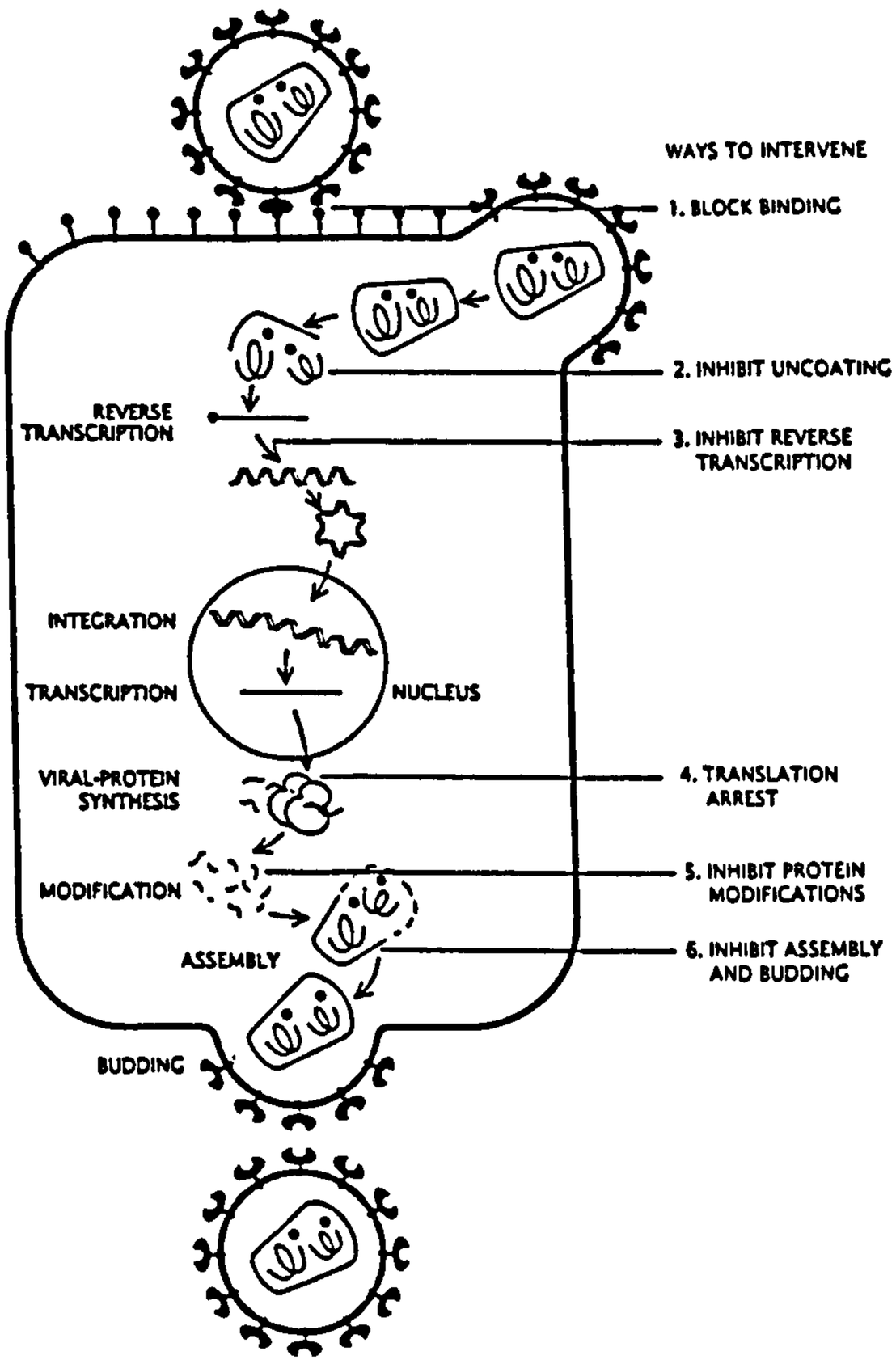


Figure 1.8 Stages in the HIV life cycle which can be targetted by drugs (Yarchoan *et. al.*, 1988)

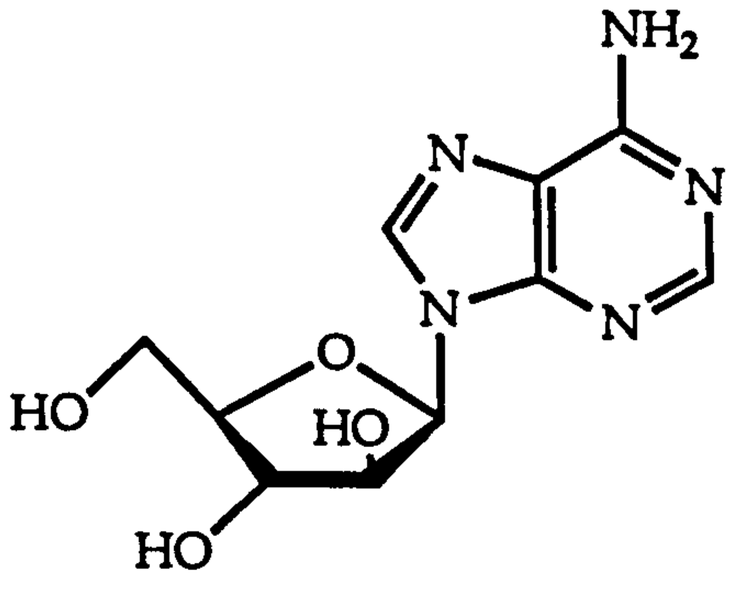
Stages	Potential intervention
Binding to target cell	Antibodies to the virus or cell receptor
Early entry into target cell	Drugs that block fusion or interfere with retroviral uncoating
Transcription of RNA to DNA by reverse transcriptase	Reverse transcriptase inhibitors
Degradation of viral RNA in an RNA-DNA hybrid	Inhibitors of RNase H activity
Integration of DNA into host genome	Drugs which inhibit *pol* gene-mediated 'integrase' function
Expression of viral genes	'Anti-sense' constructs; inhibitors of the *tat*-III protein or *art / trs* protein
Viral component production and assembly	Myristylation, glycosylation and protease inhibitors or modifiers
Budding of virus	Interferons

Table 1.2 Potential targets for therapeutic intervention (Mitsuya & Broder, 1987)

1.3.2 Nucleoside Analogues

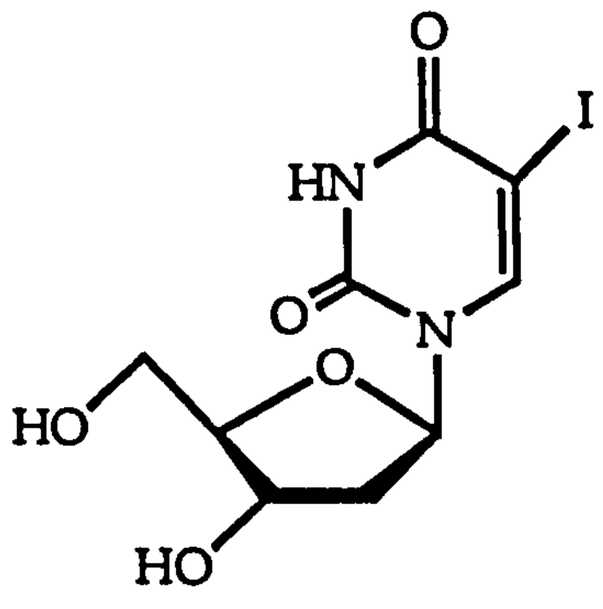
Following the discovery of reverse transcriptase in certain viruses (Baltimore, 1970; Temin & Mizutani, 1970) and their role in DNA synthesis from an RNA template, led to the production of numerous purine and pyrimidine nucleoside analogues possessing antiviral activity (Shugar, 1974; Smith & Gallo, 1974). The importance of this class of compounds in antiviral therapy can be appreciated since seven out of the eight drugs clinically approved for antiviral therapy are nucleoside analogues (Figure 1.9).

Compounds (1)-(6) are active against the various herpes simplex viruses (HSV), a DNA virus, whereas AZT (7) is an anti-AIDS drug. Ribavirin, also known as virazole, acts as a broad spectrum antiviral agent (Witkowski *et al.*, 1972), active against both DNA and RNA viruses *in vivo* (Sidwell *et al.*, 1972), but is ineffective against herpes encephalitis infections due to its inability to cross the blood-brain barrier. The discovery of the anti-herpes drug, acyclovir (ACV) (Schaeffer *et al.*, 1978), prompted the synthesis of another acyclonucleoside, ganciclovir (GCV) which was shown to be active against cytomegalovirus as well as some HSV, particularly herpes encephalitis.



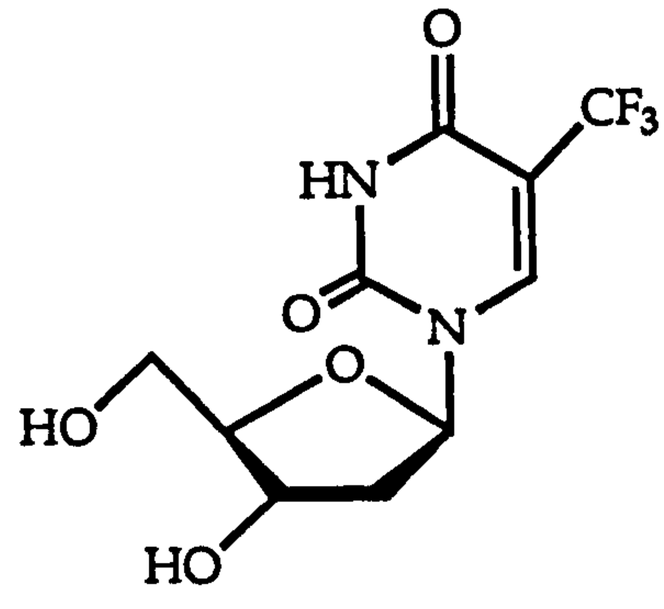
(1)

Vidarabine (ara-A)
[9-β-arabinofuranosyladenine]



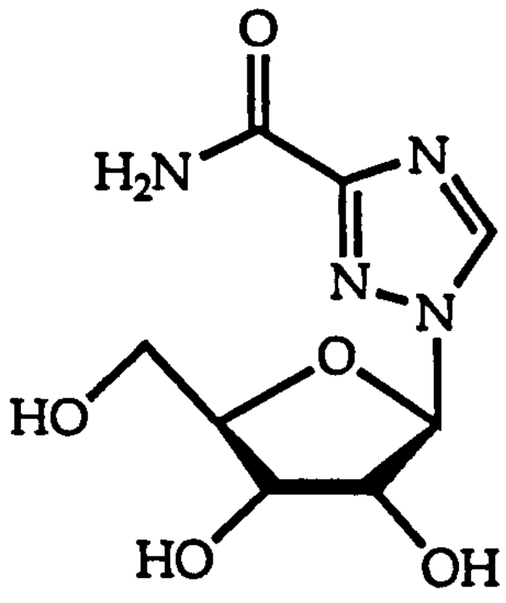
(2)

Idoxuridine (IDU)
[5-iodo-2'-deoxyuridine]



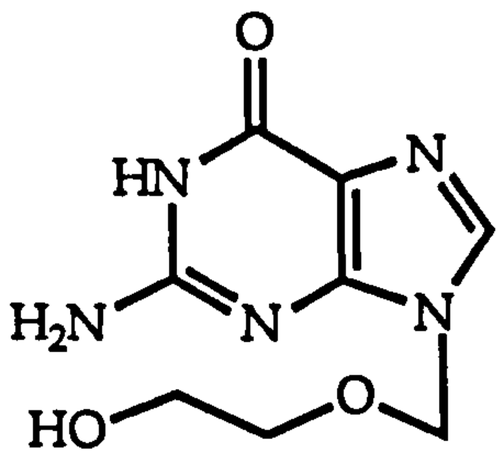
(3)

Trifluridine (TFT)
[5-trifluoromethyl-2'-deoxyuridine]



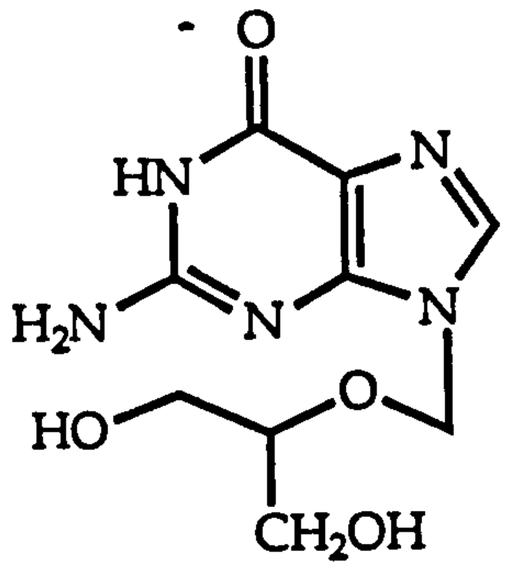
(4)

Ribavirin
[1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide]



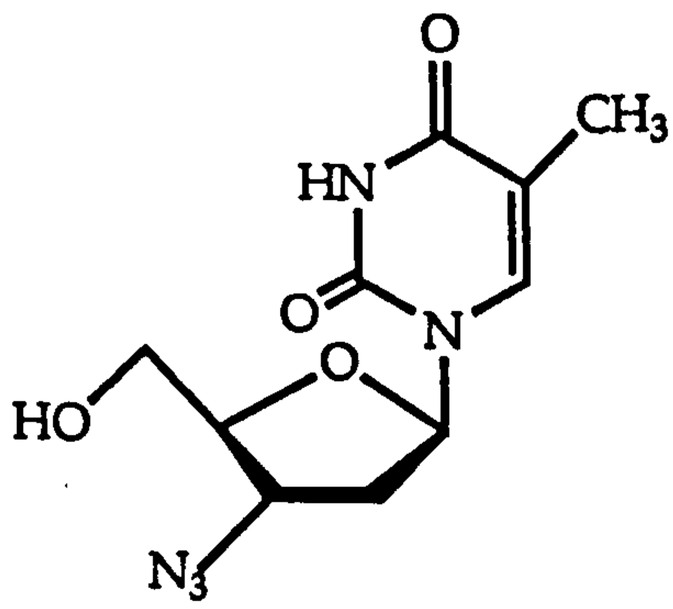
(5)

Acyclovir (ACV)
[9-(2-hydroxyethoxy-methyl)-guanine]



(6)

Ganciclovir (GCV)
[9-(1,3-dihydroxy-2-propoxy-methyl)-guanine]

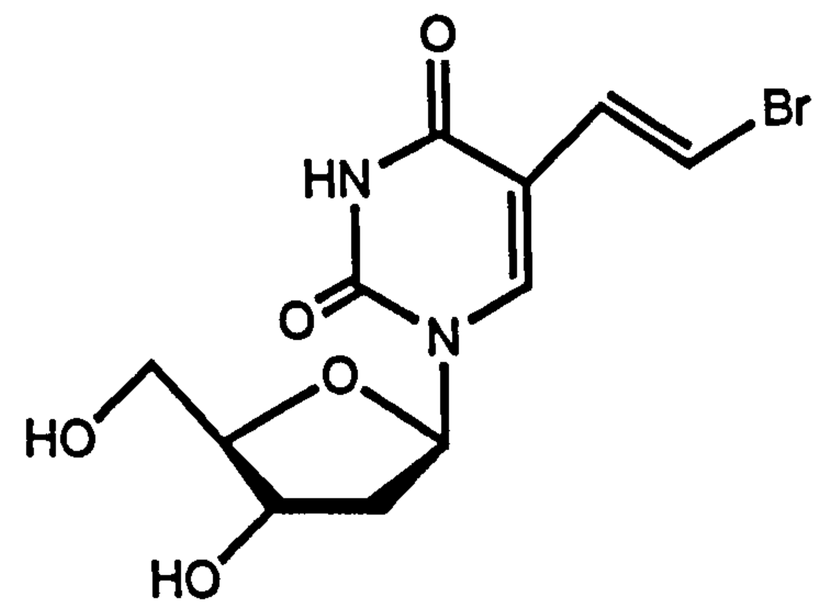


(7)

Zidovudine (AZT)
[3'-azido-2',3'-dideoxythymidine]

Figure 1.9 Clinically Approved Synthetic Antiviral Drugs (Nasr *et al.*, 1990)

IDU (2) and TFT (3) are also among the best known antiviral agents used in the topical treatment of local HSV infections, such as herpes keratitis and "cold sores". But a more effective and less toxic compound which has been developed is the bromovinyl analogue of 2'-deoxyuridine (8) (DeClercq *et al.*, 1979).



(8)

BVDU
(*E*)-5-(2-bromovinyl)-2'-deoxyuridine

AZT, on the other hand, was originally synthesised as a potential anti-cancer drug (Horwitz *et al.*, 1964), but was found to be unsuccessful in this case. However, its inhibitory effects were later demonstrated on the replication of the retrovirus, Friend lymphatic leukemia helper virus (Ostertag *et al.*, 1974). When AIDS was first identified as an entity, random screening of compounds active against this disease were carried out. One compound, AZT, was identified by Mitsuya and co-workers (1985) to stop HIV replication *in vitro*, and later shown to prolong and improve the quality of life for AIDS patients.

1.3.3 Mode of Action

The mode of action of these various nucleoside analogues are not all identical. For example ara-A (1) is first intracellularly converted to the

5'-triphosphate, which then inhibits ribonucleotide reductase and DNA polymerase (Furth & Cohen, 1968), but is not incorporated into RNA or DNA (LePage, 1970).

The deoxyuridine derivatives, IDU (2) and TFT (3), also undergo anabolic phosphorylation. The 5'-monophosphate of these compounds then act as anti-metabolites by inhibiting thymidylate synthetase (Dannenberg & Heidelberger, 1973), an enzyme responsible for the conversion of dUMP to dTMP. Inhibition of thymidylate synthetase slows down DNA biosynthesis or stops it altogether. BVDU (8) is thought to interfere with one or more stages of DNA biosynthesis other than the dTMP synthetase reaction.

The mode of action of ACV (5) and GCV (6) are similar in that both compounds are phosphorylated by virally- induced thymidine kinase, not by cellular thymidine kinases, making the compounds highly specific for the infected cell. The 5'-monophosphate of ACV and GCV are then phosphorylated to their triphosphates by cellular kinases, which can then act as potent inhibitors of the virus induced DNA polymerase rather than the α-DNA polymerase of the host. ACV was shown to act as a viral DNA chain terminator (Derse *et al.*, 1981), due to the lack of a 3'-hydroxyl group. Since ACV is similar in structure to guanine, ACV triphosphate can also act as a competitive inhibitor of dGTP. GCV, however, was found to be incorporated into both internal and terminal positions of a growing viral DNA chain (Cheng *et al.*, 1984). This suggests that GCV was not a strict DNA chain terminator. Incorporation of GCV at the terminal position of the viral DNA chain has been shown to slow down the rate of elongation of the DNA chain relative to the unmodified primer (Frank *et al.*, 1984).

The mode of action of ribavirin (4) is still unclear. It is readily phosphorylated to the 5'-triphosphate by host cellular kinases. No incorporation of ribavirin into RNA or DNA has been detected in mammalian or viral systems. However, the mechanism of antiviral action is thought to proceed by inhibiting guanylyl and N^7-methyl transferases, the enzymes involved with the formation of the 5'-cap of viral mRNA (Goswami *et al.*, 1979). This inhibition affects viral, but not cellular, protein synthesis. Ribavirin triphosphate also competitively inhibits inosine 5'-monophosphate dehydrogenase, decreasing the biosynthesis of GTP.

After AZT was discovered to be an anti-HIV agent, a rapid investigation was carried out on the mechanism of action of this drug. AZT was shown to be anabolically phophorylated to the 5'-triphosphate by the host cellular kinases, which then acts as a competitive inhibitor of thymidine triphosphate, the natural substrate of DNA synthesis. Once incorporation of AZT into the growing viral DNA chain has been achieved by reverse transcriptase, it then acts as a chain terminator with the formation of a dead-end complex (Heidenreich *et al.*, 1990). DNA chain termination by this method is due to the lack of an essential 3'-hydroxyl group on the deoxyribose moiety of a nucleoside to form the next 5',3'-phosphodiester linkage in DNA synthesis (Figure 1.10). The virus cannot repair this mistake so viral DNA synthesis comes to a halt.

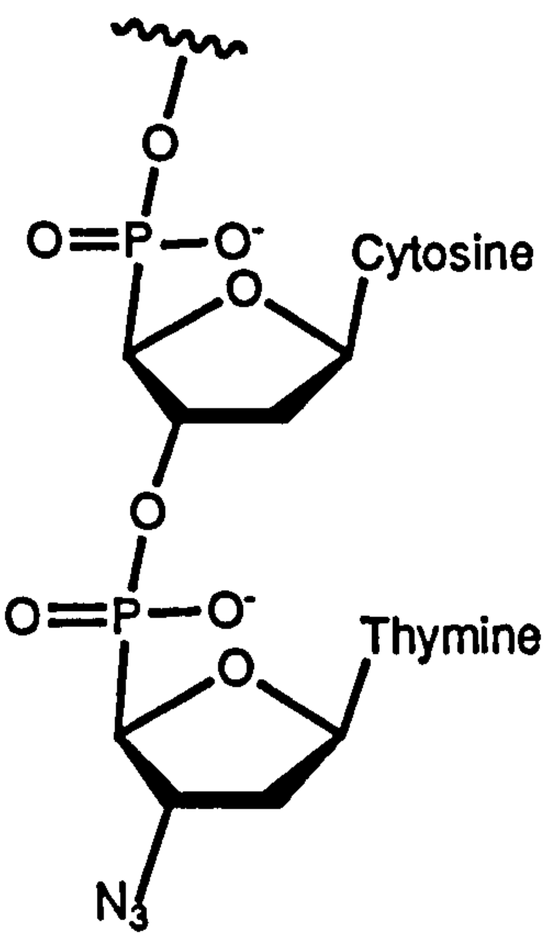
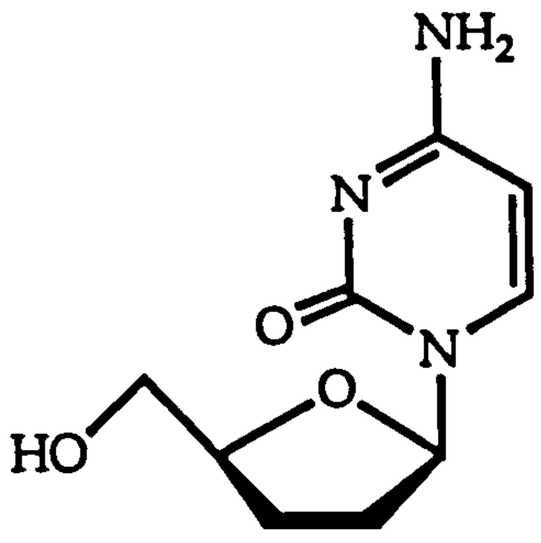
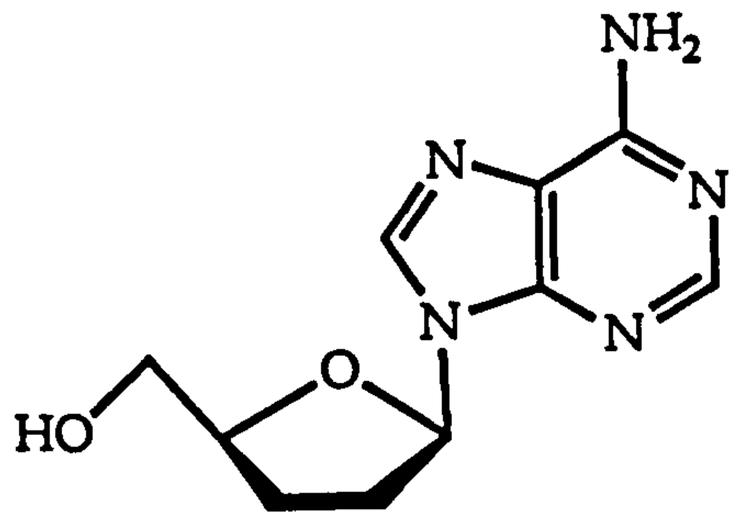


Figure 1.10 Mechanism of DNA chain termination by AZT

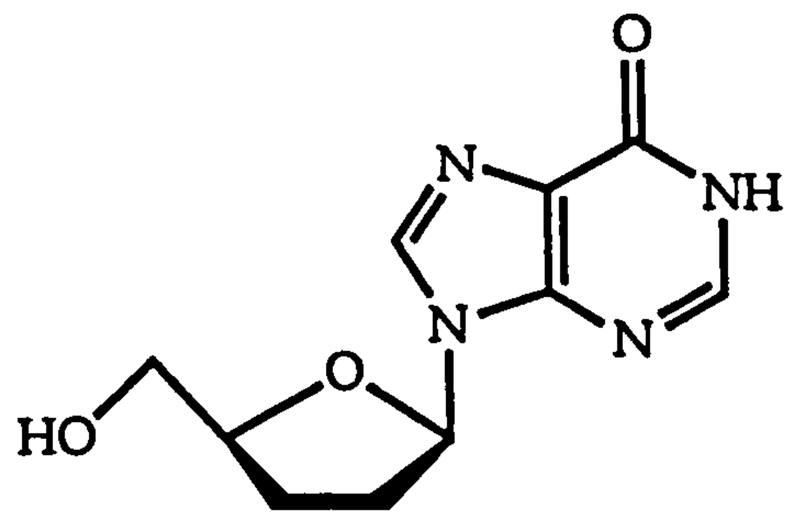
Once AZT was shown to inhibit HIV replication *in vivo*, other 2',3'-dideoxynucleosides were synthesised and tested for anti-HIV activity. These include the 2',3'-dideoxynucleosides of cytosine (ddC, 9), adenine (ddA, 10), hypoxanthine (ddI, 11) and guanine (ddG, 12).



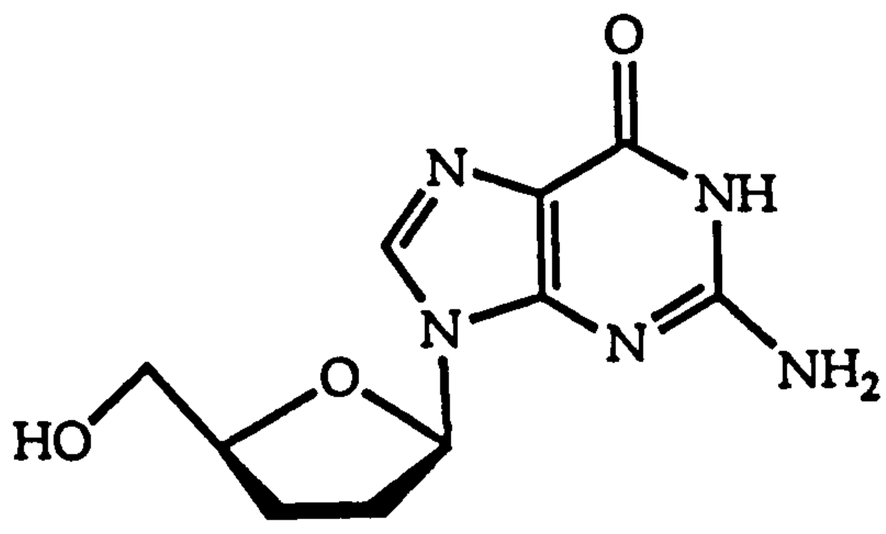
(9) ddC



(10) ddA



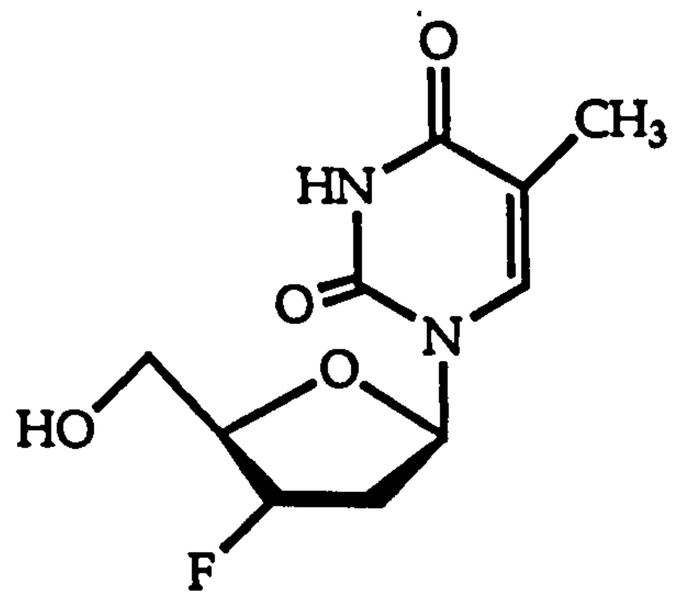
(11) ddI



(12) ddG

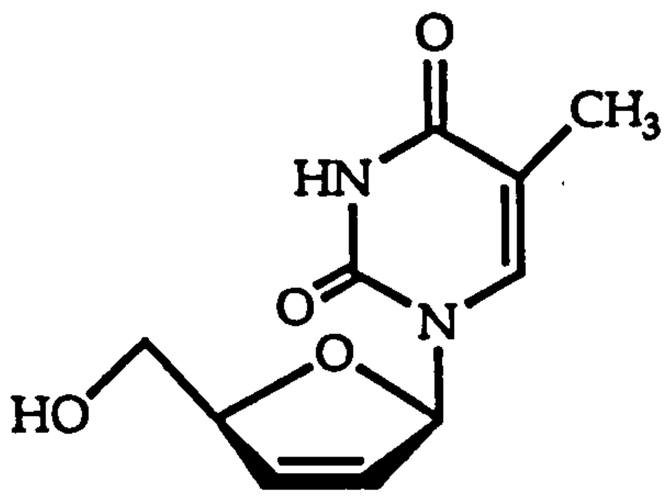
These compounds have been shown to be active against HIV replication *in vitro* in T-helper cells, monocytes and macrophages (Mitsuya *et al.*, 1990). This family of 2',3'-dideoxynucleosides are of great interest because they prove that a simple chemical modification in the sugar group can in some circumstances convert a normal substrate for nucleic acid synthesis into a potent inhibitor of HIV replication.

Another promising compound that was shown to posses potent anti-HIV activity was 3'-fluoro-2',3'-dideoxythymidine (13), unfortunately this compound was found to be highly toxic. AZT also has several toxic side effects, the most frequent toxicity being bone marrow suppression.

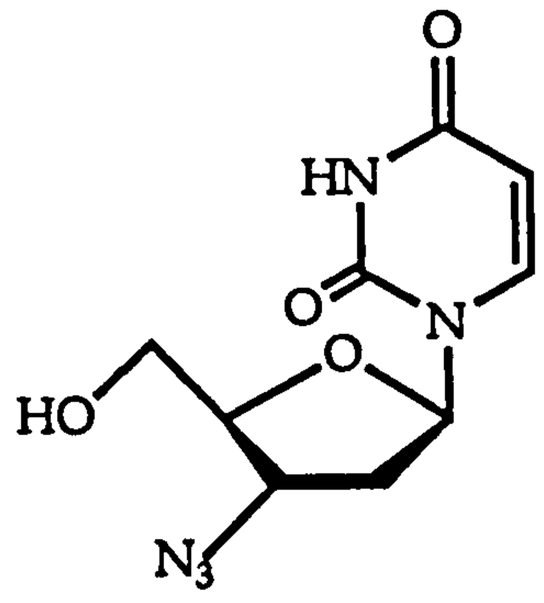


(13)
3'-fluoro-2',3'-dideoxythymidine

Two other members of the dideoxynucleosides that are currently under study with AIDS patients are 2',3'-didehydro-2',3'-dideoxythymidine (d4T, 14) and 3'-azido-2',3'-dideoxyuridine (AZU, 15) (Mitsuya *et al.*, 1991).

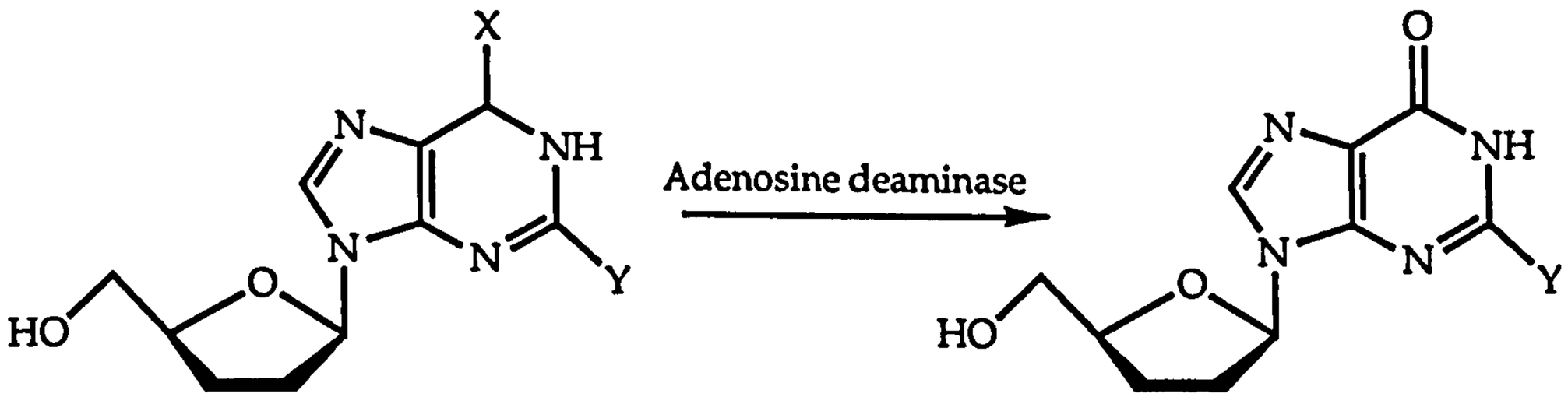


(14) d4T
2',3'-didehydro-2',3'-dideoxythymidine



(15) AZU
3'-azido-2',3'-dideoxyuridine

However, the need to develop nucleoside analogues as drugs with less toxic side effects, and more lipophilicity and stability *in vivo* without loss of anti-retroviral activity, is still of great importance. Recently, 2',3'-dideoxypurine nucleosides with a halogen atom at the 6-position of the base (16) was shown to have greater lipophilic character (Shirasaka *et al.*, 1990), and once inside cells they were converted to either ddI or ddG by the ubiquitous adenosine deaminase. Hence, these 6-halo compounds may be important as lipophilic prodrugs for ddI and ddG.



(16)
2',3'-dideoxypurine nucleoside
X = F or Cl
Y = H or NH_2

If Y = H, then ddI
NH_2, then ddG

Combination therapy is also being applied as a useful chemotherapeutic strategy. In this case a combination of antiviral drugs with different toxicity and efficacy profiles are given to patients, which can result in

enhancing the effectiveness of the combined drugs. For example, acyclovir (ACV, 5), an antiherpes drug but with weak anti-retroviral activity, has been shown to have an additive effect on the anti-retroviral activity of AZT *in vitro* (Mitsuya & Broder, 1987).

1.4 Synthesis of Nucleoside Analogues

The need to screen compounds with potential antiviral activity has prompted a great amount of interest in the procedures employed to synthesise this important class of compounds. Chemical or enzymatic methods, or a combination of both can be used to prepare these nucleoside analogues.

1.4.1 Chemical Synthesis

Nucleosides have traditionally been prepared by various chemical methods and these procedures have been extensively reviewed and compiled by various authors (Vorbrüggen, 1979; Walker, 1979; Townsend & Tipson, 1986). These procedures fall into three major categories, firstly the condensation reactions, secondly the transglycosylation reactions and thirdly modification of the base or sugar groups from a preformed nucleoside.

1.4.1.1 Condensation Reactions

A very popular method of preparing nucleosides was by the Hilbert-Johnson-silyl method (Hilbert & Johnson, 1930), which was later perfected by Niedballa & Vorbrüggen (1976) and Vorbrüggen & Bennua (1978). Both purine and pyrimidine nucleosides can be synthesised by this method. The procedure involves the reaction of a silylated heterocyclic base with a peracylated sugar in the presence of a Lewis acid (Figure 1.11).

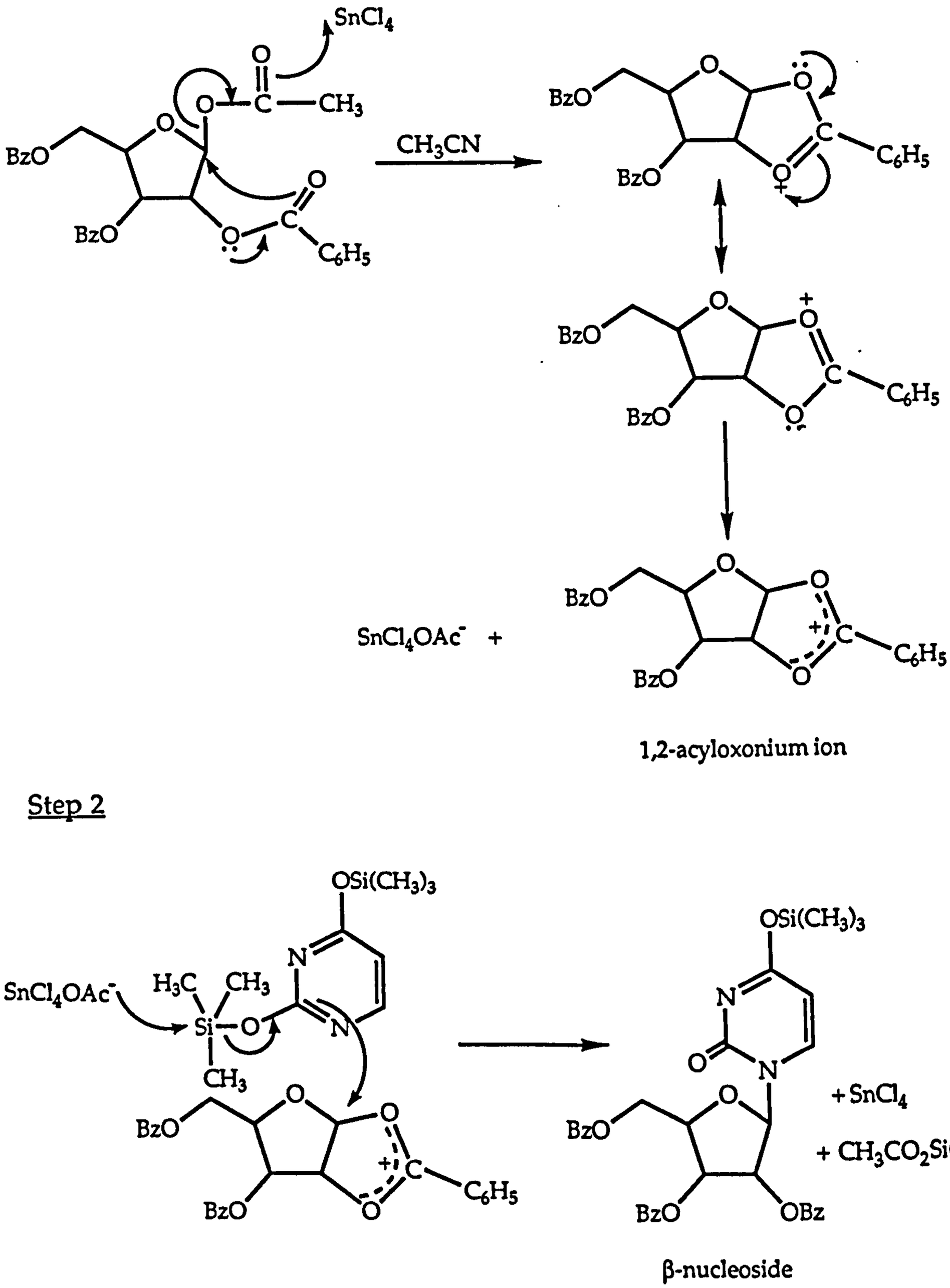
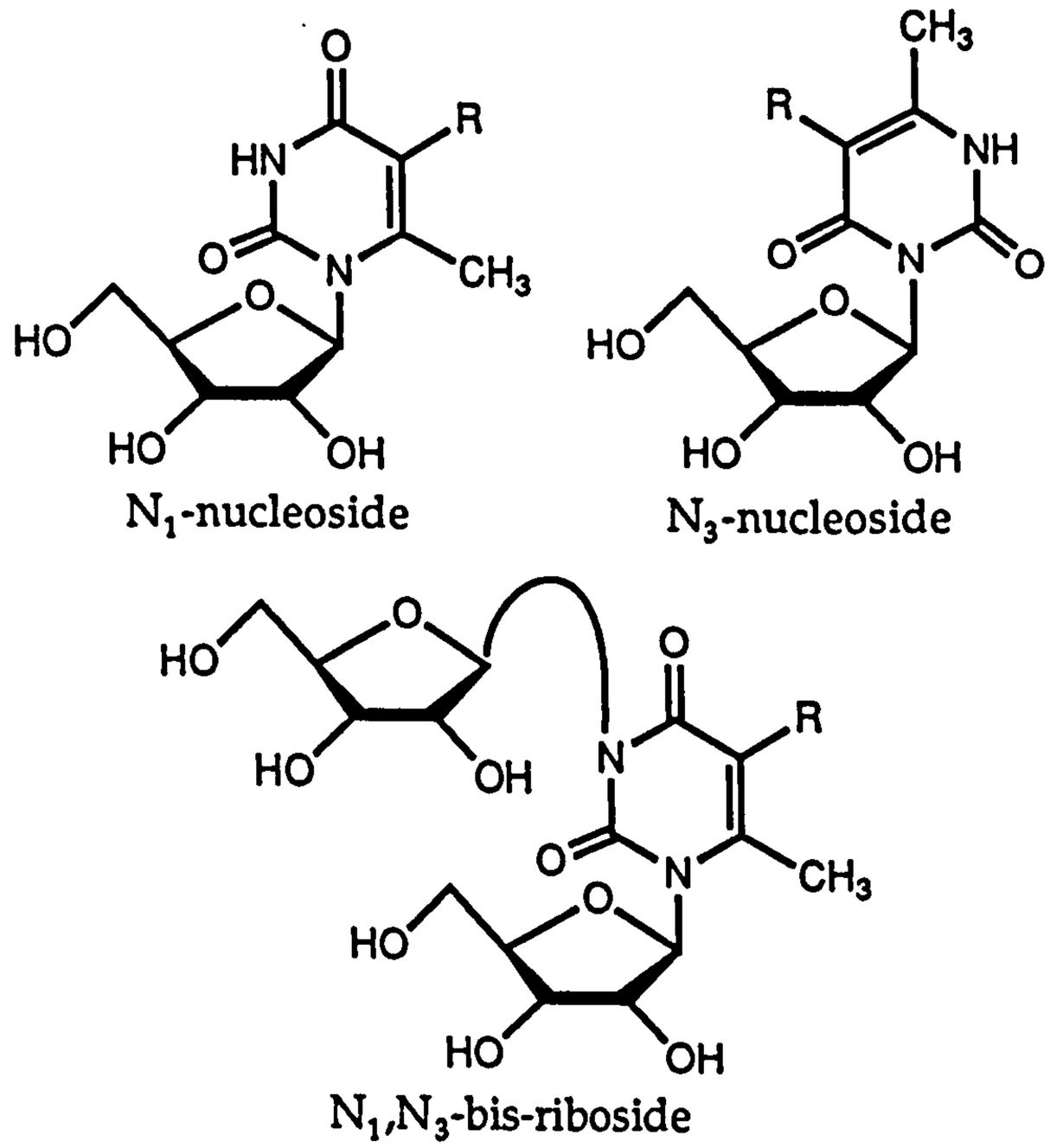


Figure 1.11 Hilbert-Johnson-silyl method of nucleoside synthesis

This method involves a two step procedure which requires the silylation of the base before reacting it with the peracylated sugar. However, Vorbrüggen and Bennua (1978) have demonstrated a simplified version of Figure 1.11, whereby the silylation of the heterocyclic base is carried out *in situ* with the peracylated sugar, giving fairly good yields of the β-nucleoside.

Substitution at the 5- and 6-position of uracil has also been shown to affect the condensation reaction by producing a mixture of N-substituted nucleosides. Niedballa and Vorbrüggen (1976) found ribosylation of a silylated uracil, with a methyl substituent at the 6-position as well as substitutions on the 5-position with various groups, yielded N_1-, N_3- and N_1,N_3-bis-riboside nucleosides (Figure 1.12).



N_1-nucleoside

N_3-nucleoside

N_1,N_3-bis-riboside

Figure 1.12 Nucleosides formed due to the substitution of uracil at the 5- and 6-positions

Hence the purification of the desired N_1-β-nucleoside becomes more complex and time-consuming, resulting in lower yields.

Synthesis of deoxynucleosides by this procedure can be carried out using a C_1-halogenated deoxyribose residue, known as an halogenose, and reacting this with a silylated purine or pyrimidine base (Figure 1.13).

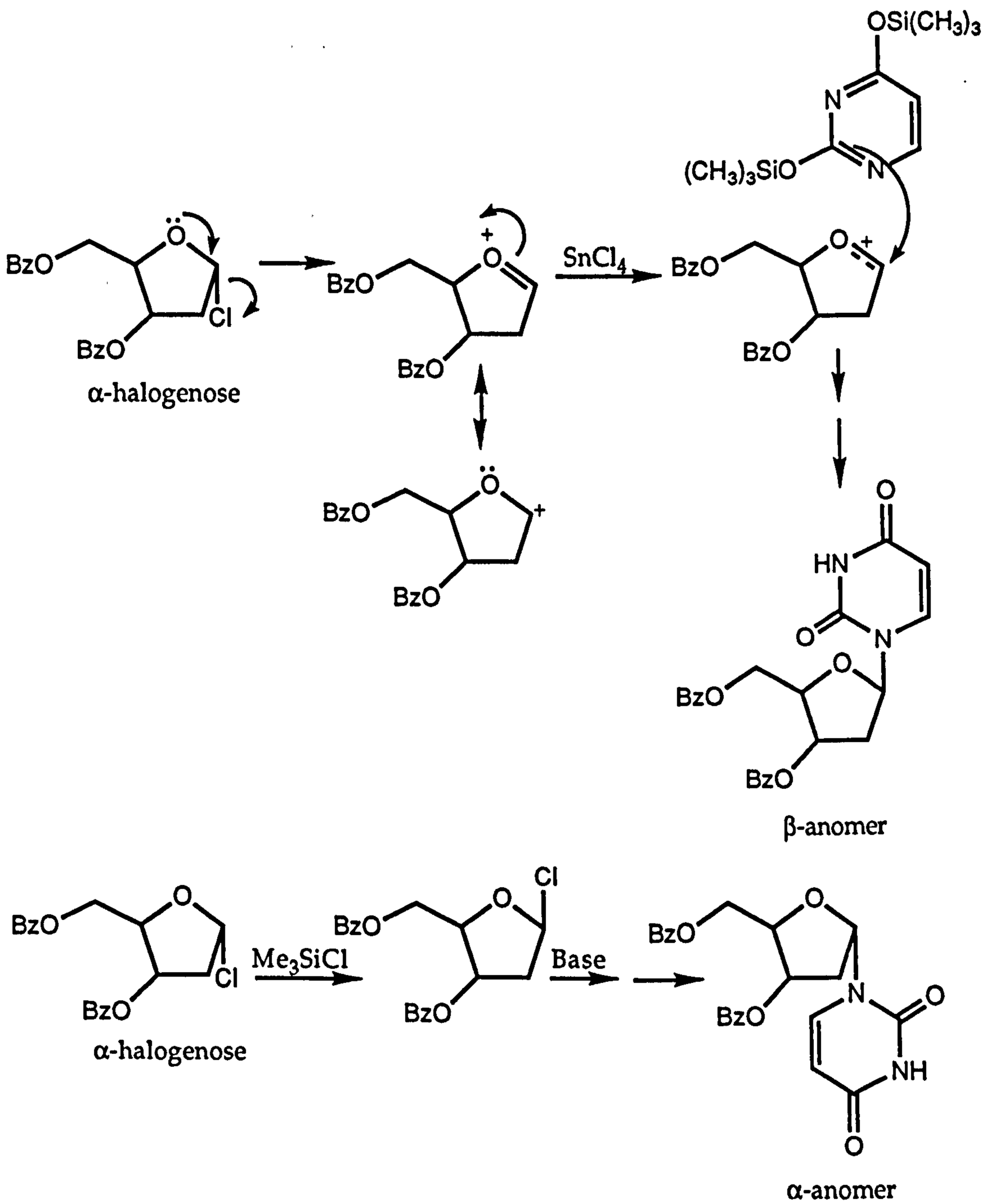


Figure 1.13 Synthesis of deoxyribonucleosides by the condensation method

The α-anomer nucleoside in Figure 1.13 is formed by a direct S_N2 displacement of the β-halogenose, which is in turn formed by the trimethylsilyl chloride-catalysed anomerisation of the α-halogenose.

Recently, an example of a condensation reaction using the silylated procedure was demonstrated by Howell and co-workers (1988), where the synthesis of 2'-fluoro-arabino-2'-deoxyribonucleoside produced almost exclusively the β-anomer in high yields. Since the stereospecific formation of the α-bromo sugar plays a key step in producing the β-nucleoside, the authors found that a solvent with a low dielectric constant, such as carbon tetrachloride, favoured the formation of this α-bromo sugar, via an S_N2 reaction.

Another type of condensation reaction used is the Helferich fusion method, which involves the fusion of a peracylated sugar with various purines under vacuum and in the presence of a catalytic amount of toluene-*p*-sulphonic acid. The yields vary widely and anomeric mixtures are produced. An important application of the fusion method is in the synthesis of purine 2'-deoxyribonucleosides (Robins & Robins, 1965). In general the fusion method has been much more useful for the synthesis of purine nucleosides than for pyrimidine nucleosides. The main advantage of the fusion procedure is that commercially available, stable, peracylated sugars and free purine bases can be used.

1.4.1.2 Transglycosylation Reactions

Nucleoside synthesis using transglycosylation reactions involves the transfer of a sugar residue from a nucleoside to another heterocyclic base. In order to undergo facile cleavage of the glycosidic bond, pyrimidine

nucleosides have usually been acylated at the basic and glycosyl residue and then heated with purine bases in the presence of acid catalysts. However, Imazawa and Eckstein (1978) have produced nucleosides without having to protect the starting pyrimidine nucleoside. The glycosyl residue of 3'-azido-2',3'-deoxythymidine was transferred to adenine and since no acylated group was present on the 2'-position of the glycosyl residue, a mixture of α- and β-anomers were formed.

1.4.1.3 Modification of the base or sugar moieties of a preformed nucleoside

Nucleoside synthesis by modification of a pre-existing nucleoside has been thoroughly covered by various authors (Goodman, 1974; Walker, 1979) and individual examples of this type of synthesis are included in a large section in a treatise on nucleosides by Townsend and Tipson (1986).

The synthesis of a whole range of 3'-substituted 2',3'-dideoxynucleoside analogues, potential anti-HIV agents, has been reported by Herdewijn and co-workers (1987). The nucleosides were synthesised using previously published procedures or modified versions in order to obtain better yields. The authors also reported on a general method of producing 3'-fluoro-2',3'-dideoxynucleosides, which have also been shown to be good anti-HIV agents. The fluorinating agent, (diethylamino)sulphur trifluoride (DAST), was used on four different 2'-deoxynucleosides giving good to moderate yields (59-65%).

Nucleosides with modified sugar residues are generating a great deal of interest, especially the 2',3'-dideoxynucleosides and the 3'-fluoro-2',3'-dideoxynucleosides. As discussed earlier, these compounds

have been shown to act as potent anti-HIV agents. However, other types of such compounds need to be prepared, in order to improve the selectivity of the final drug in targetting for the virally-infected cells and not the host cells, and thus reducing the toxic side effects.

There are many other chemical procedures of synthesising nucleosides which have been covered by the authors already mentioned. However, synthesis of nucleosides by these chemical methods requires many intermediary steps, such as glycosyl activation, protection and deprotection of the sugar and base moieties, which results in a several step synthesis producing low yields of the product. Other problems are the stereospecific and regiospecific control of the reaction to give the desired β-anomer and N_1- or N_9-glycosylated nucleoside.

1.4.2 Enzymatic Synthesis

Enzymes are fast becoming the focal point of biotechnological processes. These remarkably effective catalysts are responsible for the numerous co-ordinated chemical reactions involved in biological processes of living systems. One of the outstanding features of enzymes in comparison to chemical catalysts is the specificity towards their substrate(s). They are specific both for type of reaction(s) catalysed and for the substrate(s) accepted. The degree of substrate specificity can be variable, since many enzymes can accomodate synthetic substrate analogues as well as the naturally occurring substrates. Another benefit of enzymatically catalysed procedures is that the reactions can be carried out at moderate temperatures, near-neutral pH and be readily controlled.

The class of enzymes that are greatly used for the enzymatic preparation of nucleosides are the glycosyl transferase enzymes. There are two main types of these enzymes, the nucleoside phosphorylases and nucleoside *N*-deoxyribosyltransferases. These enzymes are able to catalyse the transfer of a glycosyl residue from a donor nucleoside to an acceptor base.

1.4.2.1 Nucleoside Phosphorylases

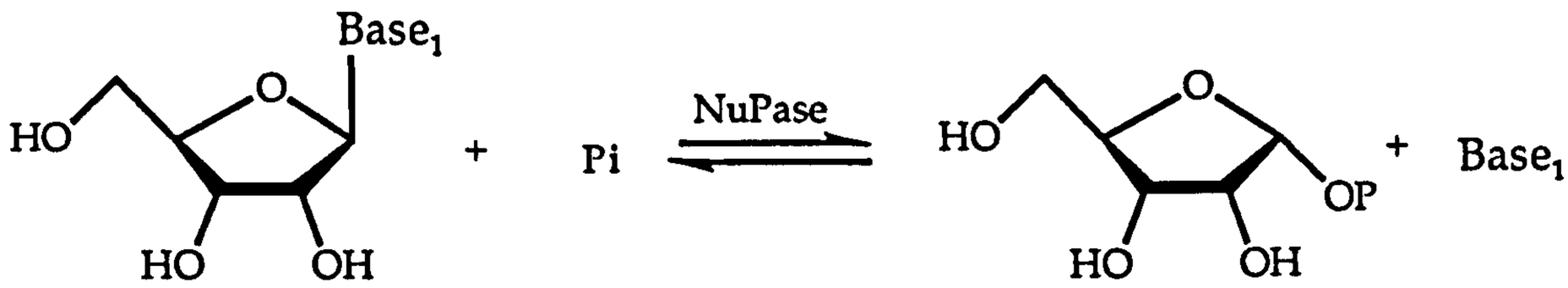
The use of nucleoside phosphorylases as biocatalysts in the synthesis of nucleosides has been widely employed (Hutchinson, 1990; Drueckhammer *et al.*, 1991; Hanrahan & Hutchinson, 1992). These enzymes are able to synthesise nucleosides by a reversible two-step reaction in the presence of inorganic phosphate. The glycosyl moiety of a nucleoside is transferred to a different purine or pyrimidine base via a glycosyl-1-phosphate intermediate with the equilibrium lying towards nucleoside formation.

Extensive screening programmes have shown the ubiquity of these nucleoside phosphorylases among bacteria (Imada & Igarasi, 1967). Enzymes catalysing ribosyl and deoxyribosyl transfer reactions from a pyrimidine nucleoside to a purine base were shown to be present in almost all strains belonging to the family Enterobacteriaceae and in some strains from Pseudomonadaceae, Corynebacteriaceae, Micrococcaceae, Bacteriaceae and Bacilliaceae. The transfer reactions studied were found to be nucleoside phosphorylases because of the increased activity in the presence of phosphate. The authors also showed the involvement of coupled nucleoside phosphorylases in carrying out the transfer reactions. No single enzyme was catalysing the transfer reactions.

Hence it is not surprising that three nucleoside phosphorylases from *E. coli*, uridine (UrdNPase), thymidine (ThdNPase) and purine nucleoside phosphorylase (PurNPase), have been cloned (Fischer & Short, 1982) and well characterized (Ott & Werkman, 1957; Schwartz, 1971; Jensen & Nygaard, 1975; Jensen, 1976; Leer *et al.*, 1977).

These phosphorylase enzymes from *E. coli* have been used in coupled phosphorylase systems to prepare a variety of nucleoside analogues. One of the first enzymatic synthesis of nucleosides was the preparation of the anti-metabolite 5-trifluoromethyl-2'-deoxyuridine (TFT, 3) (Heidelberger *et al.*, 1964). Crude cell free extracts of *E. coli* B carried out the phosphate-dependent transglycosylation reaction from thymidine to 5-trifluoromethyuracil.

There are two basic methods employed for the synthesis of nucleosides using nucleoside phosphorylases. The first method involves the production and isolation of the (deoxy)ribose-1-phosphate ((d)R1P) intermediate. Once a good yield of this intermediate has been isolated, it can then be used for the glycosylation of an heterocyclic base, which would also be a suitable substrate for the phosphorylases (Figure 1.14).



$Base_1$, $Base_2$ = purine or pyrimidine
NuPase = nucleoside phosphorylases

Figure 1.14 Two-step reaction for the production of ribonucleosides using nucleoside phosphorylases

The second method does not involve the isolation of (d)R1P but is generated *in situ* and base exchange of (d)R1P is carried out in the presence of catalytic amount of inorganic phosphate (Figure 1.15).

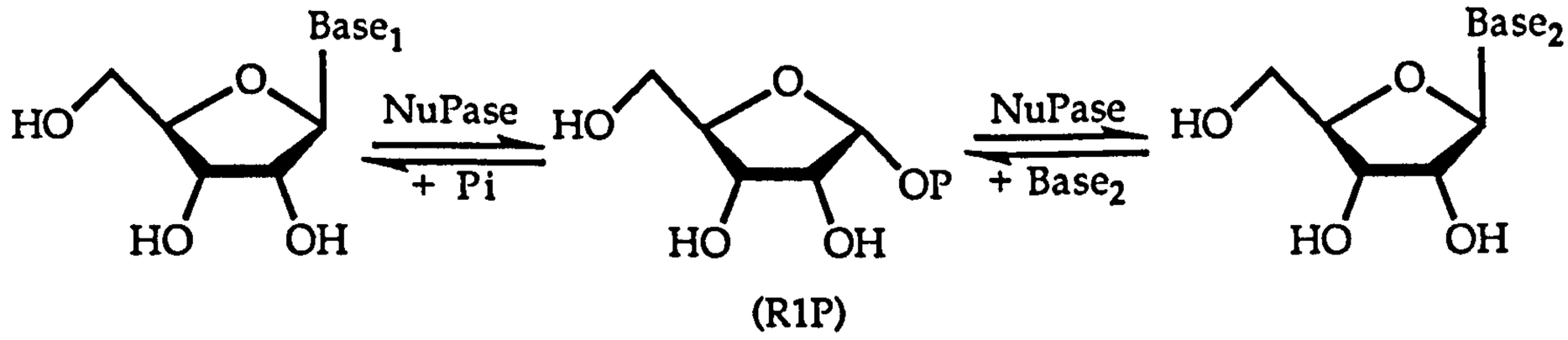


Figure 1.15 *In situ* production of ribonucleosides using nucleoside phosphorylases

The above methods have been applied to the production of ribavirin (4), the broad spectrum antiviral compound. This compound was first synthesised enzymatically by Utagawa and co-workers (1986) by using whole cells of *Enterobacter aerogenes* AJ 11125 following Figure 1.14.

First partially purified PurNPase from *Ent. aerogenes* was used to phosphorolytically cleave inosine in the presence of inorganic phosphate, producing good yields of R1P. This was then used as the ribosyl donor for ribavirin production with 1,2,4-triazole-3-carboxamide (TCA, the aglycon component of ribavirin). However, the authors were unable to carry out ribavirin synthesis directly from inosine and TCA, due to competitive inhibition by hypoxanthine (Km=5.6 mM) with TCA (Km=167 mM).

Other workers (Shirae *et al.*, 1988a) then tried to improve on the above method by screening micro-organisms which could produce R1P and ribavirin more efficiently. Through this screening study, *Erwinia caratovora* AJ 2992 and *Bacillus brevis* AJ 1282 were selected as active producers of R1P from inosine and ribavirin from R1P and TCA, respectively.

To avoid the problem of competitive inhibition by hypoxanthine, Shirae and co-workers (1988b) developed a novel enzymatic procedure of producing ribavirin directly from a pyrimidine base, rather than a purine base, and TCA catalysed by intact cells of *Ent. aerogenes* AJ 11125. Since the reaction is catalysed by two different enzymes, firstly pyrimidine nucleoside phosphorylase (PyrNPase) and then PurNPase, the released pyrimidine base does not inhibit the PurNPase enzyme. The same authors (1988c) also reported on the increased production of ribavirin from orotidine, as a producer of R1P, which was catalysed by intact cells of *Erw. caratovora* AJ 2992.

High ribavirin production has been synthesised directly from purine nucleosides and TCA by intact cells of *Brevibacterium acetylicum* ATCC 954 (Shirae *et al.*, 1988d). Guanosine and xanthosine were shown to

be the best substrates as glycosyl donors. The released bases did not inhibit the PurNPase activity, needed for ribavirin production because of the poor solubilities of these compounds in the reaction mixture compared to TCA. Nonetheless *B. acetylicum* demonstrated good ribavirin production when inosine was used as the glycosyl donor, suggesting that the PurNPase from *B. acetylicum* has a different affinity for hypoxanthine than the PurNPase from *Ent. aerogenes*.

Other workers (Krenitsky *et al.*, 1981b; 1982; 1986; Rideout *et al.*, 1982) have also developed efficient one-pot syntheses of purine nucleosides by employing pyrimidine nucleoside donors and purine base analogues as acceptors with the appropriate nucleoside phosphorylases from *E. coli*. 3-Deazapurine nucleosides have been prepared by coupled nucleoside phosphorylases (Krenitsky *et al.*, 1986), producing one (17) that contained potent anti-inflammatory activity (Figure 1.16).

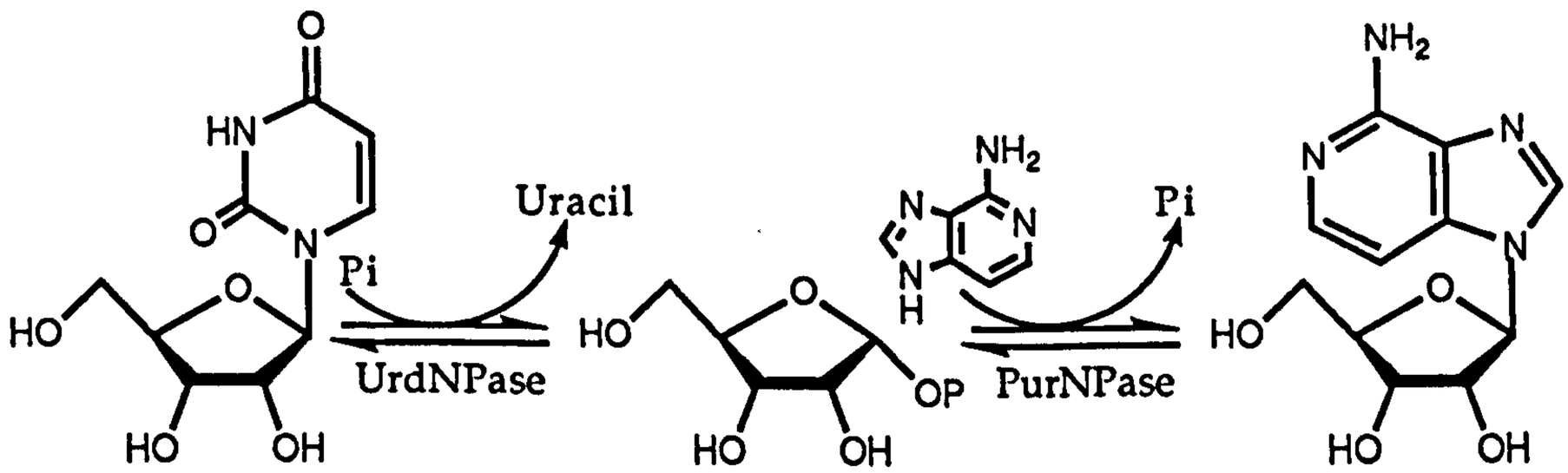


Figure 1.16 Utilisation of coupled nucleoside phosphory lase in the production of 3-deazapurine nucleosides

Whole cells of *E. coli* BM-11 have been used as biocatalysts in the synthesis of the antiviral agent BVDU (8) (Zinchenko *et al.*, 1990a). The intact cells of *E. coli* were modified with glutaraldehyde, partially immobilising the cells so that they could be used repeatedly. Thymidine and 2'-deoxyguanosine

were used as the glycosyl donors with the corresponding bases and catalysed by the appropriate phosphorylase enzymes from the glutaraldehyde-treated cells.

Thymidine has been successfully synthesised by the thermophile, *Bacillus stearothermophilus*, at a reaction temperature of 65°C (Hori *et al.*, 1989). At this high temperature, enzymatic side reactions are inhibited as well as growth of other bacteria.

Nucleoside phosphorylases have also been used extensively in the preparation of arabinonucleosides. Ara-A (1), an anti-herpes drug, has been synthesised by intact cells of *Ent. aerogenes* AJ 11125 (Utagawa *et al.*, 1980; 1985a) The reaction can be summarised by Figure 1.17.

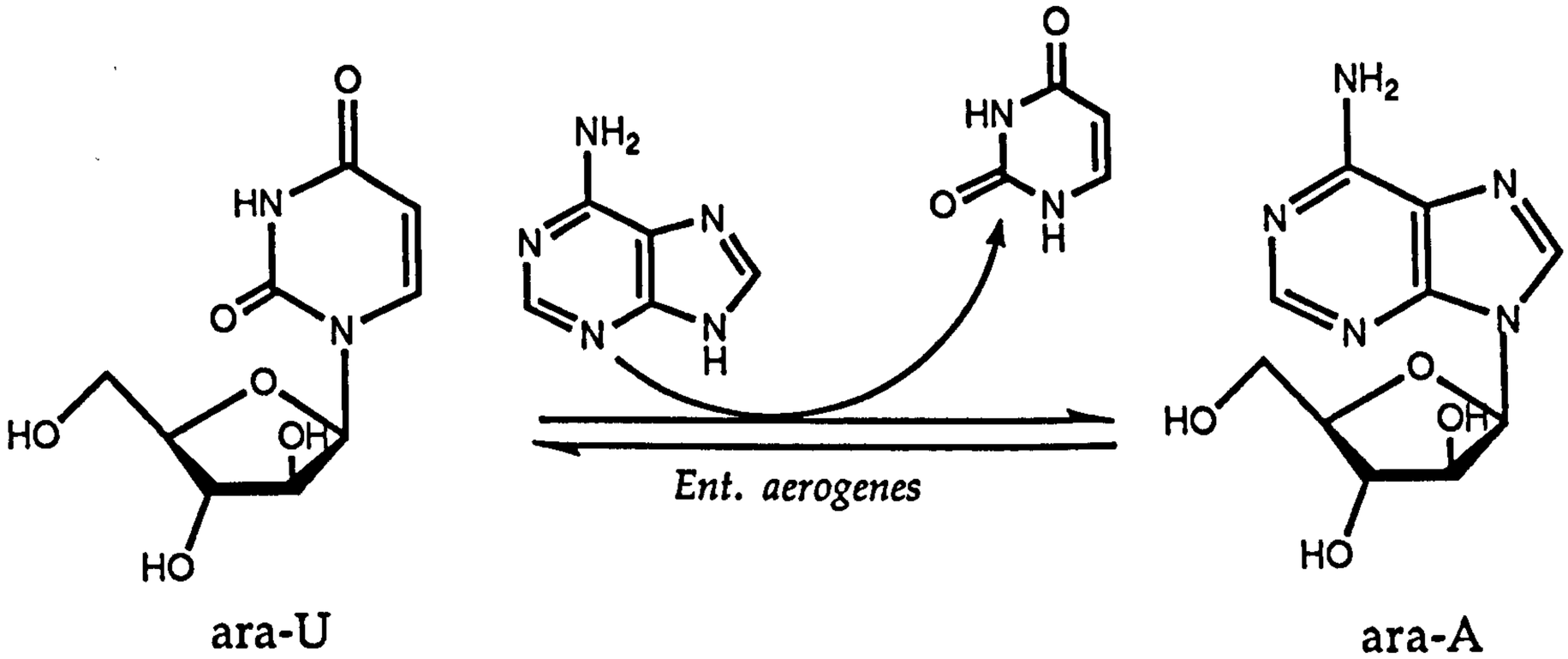


Figure 1.17 Preparation of arabinonucleosides using whole cells of *Enterobacter aerogenes* AJ 11125

The synthesis was carried out at high temperatures in order to avoid side reactions such as deamination of adenine by adenine deaminase which would be present in the organism. A combination of chemical and enzymatic reactions have been employed to synthesise purine

arabinonucleosides (Morisawa *et al.*, 1980; Krenitsky *et al.*, 1981a; Zinchenko *et al.*, 1990b).

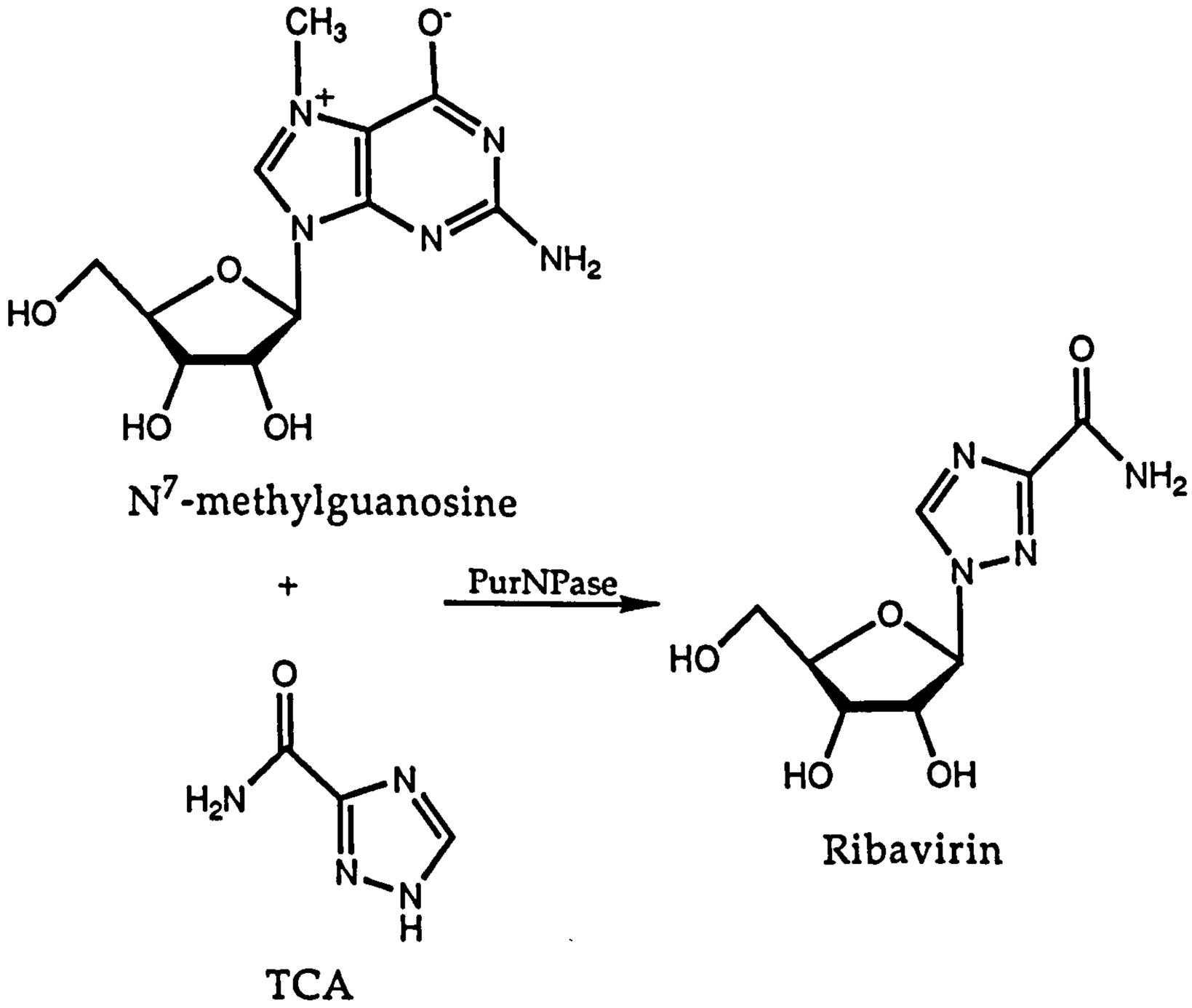
Other base modified substrates of the nucleoside phosphorylases are the 3'-amino- and 2'-amino-2',3'-dideoxyribonucleosides. The synthesis of some 3'-amino-2',3'-dideoxyribonucleosides of pyrimidines, catalysed by the ThdNPase of *E. coli*, has been reported by Krenitsky and co-workers (1983). *Erw. herbicola*, *Salmonella schottmuelleri*, *Ent. aerogenes* and *E. coli* have been employed to carry out the phosphate-dependent transaminoribosylation reaction of 2'-amino-2',3'-dideoxyuridine to various purine bases (Utagawa *et al.*, 1985b).

The potent anti-HIV agents, 2',3'-dideoxynucleosides, are good substrates for the nucleoside phosphorylase enzymes from many micro-organisms. By screening micro-organisms for production of ddA (10) from the relevant substrates, *E. coli* AJ 2595 was selected as the best producer of ddA from 2',3'-dideoxyuridine (ddU) (Shirae *et al.*, 1989). The donor nucleoside, ddU, was chemically synthesised from uridine. Polyethylene glycol (PEG) was used in the enzymatic reaction mixture, since it improved the production of ddA. The effect of PEG is thought to be related to an increase in the solubility of adenine in the reaction mixture and perhaps also to affect the membrane of the *E. coli* cells so that the uptake of substrates into the cells was enhanced.

The preparation of 2',3'-dideoxynucleosides with 6-substituted purine bases, potential anti-HIV agents, has been reported (Koszalka *et al.*, 1988b). The synthetic procedure utilised the purified enzymes, ThdNPase and PurNPase, adsorbed onto DEAE cellulose by a method described previously by Krenitsky and co-workers (1981b).

Whole cells of *E. coli* have also been used for preparing 2'-deoxythymidine from thymine and various natural purine and pyrimidine deoxynucleosides, obtained from DNA hydrolysis (Zinchenko *et al.*, 1990b).

An important factor in these coupled phosphorylase systems is for the equilibrium of the reaction to lie well towards nucleoside formation. So in order to achieve maximum synthesis of the new nucleoside, Hennen and Wong (1989) have developed a novel method of producing ribavirin which has N^7-methylguanosine as the glycosyl donor. Gram quantities of 3-deazaadenosine as well as ribavirin were produced from the irreversible synthesis of N^7-methylguanosine and 3-deazaadenine or TCA with PurNPase (Figure 1.18).



N^7-methylguanosine

TCA

Ribavirin

Figure 1.18 Improved synthesis of ribavirin by using an irreversible nucleoside donor

Kulikowska and co-workers (1986) had reported that 7-methylguanosine and 7-methylinosine were substrates for the calf-spleen PurNPase, but that the corresponding bases were not substrates in the reverse reaction, or in the enzymatic synthesis of other nucleosides. Therefore, 7-methylguanosine and 7-methylinosine can be used as excellent glycosyl donors for the *in situ* production of R1P and in the subsequent production of the new nucleoside.

In summary nucleoside phosphorylases are widely distributed in nature and have a broad substrate specificity from both bacterial and mammalian sources, with the exception of mammalian PurNPases which do not accept adenine or adenosine as substrates. Some bacterial phosphorylases have been found to be thermostable, allowing their use at high temperatures and hence inhibiting side reactions from occurring. The presence of other specific nucleoside phosphorylases such as inosine-guanosine (Koszalka *et al.*, 1988a) and adenosine (Senesi *et al.*, 1976) phosphorylases increases the range of enzymes available for synthetic use.

However, coupled phosphorylase systems have their disadvantages in that the two-step reactions can often require the use of two different enzymes from either the same source or from different sources. This could involve extra work in the synthetic protocol, such as purifying intermediates for use in the next step in the reaction, especially when one enzyme is inhibited by the product of the second enzyme.

1.4.2.2 Nucleoside *N*-deoxyribosyltransferases

Nucleoside *N*-deoxyribosyltransferases have also been employed in the syntheses of nucleosides (Hanrahan & Hutchinson, 1992). These

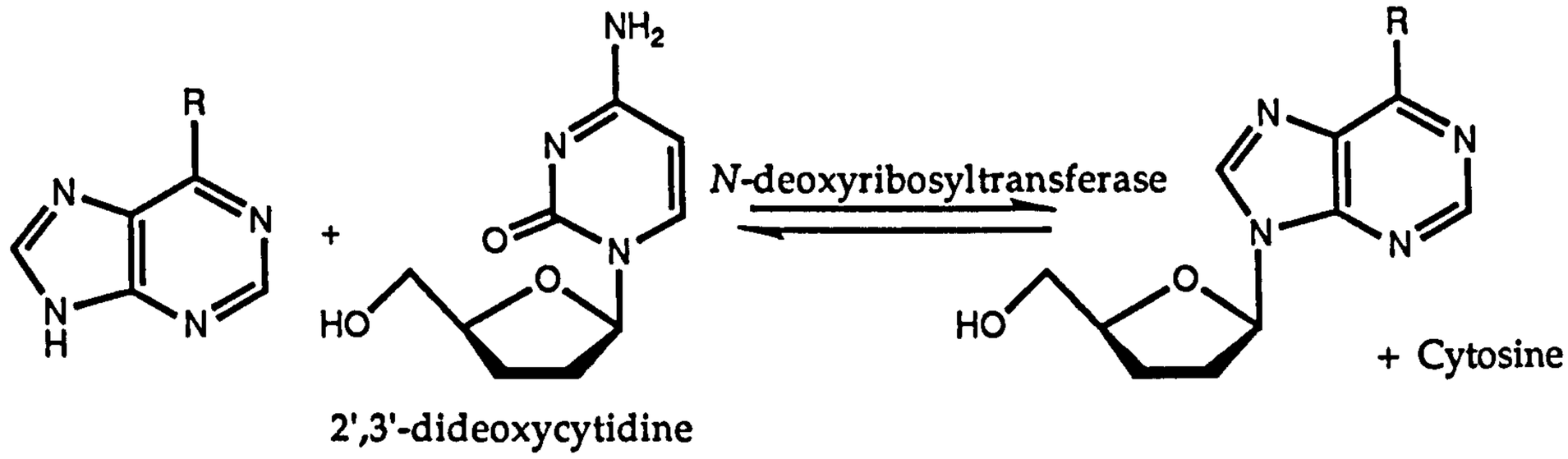
transferase enzymes carry out the syntheses through a single-step, phosphate-independent reaction. As with the phosphorylases, the reaction is highly stereo- and regiospecific, forming only the β-nucleoside with glycosylation occurring at the N_1- and N_9-positions of pyrimidines and purines, respectively.

As discussed earlier, there are two types of transferase enzymes which have been isolated from *L. helveticus* (Holguin & Cardinaud, 1975) and *L. leichmannii* (Heath, 1990). Crude enzyme preparations from *L. helveticus* and *L. leichmannii* have generally been used for synthesis of nucleoside analogues. Heidelberger and co-workers (1964) used an enzyme from *L. leichmannii* to prepare the radioactively labelled nucleoside, 2-[^{14}C]-2'-deoxyribofuranosyl-5-trifluoromethyluracil.

Although the transferase enzymes from *L. helveticus* and *L. leichmannii* are unable to tolerate a great deal of modification on the sugar moiety, they can accept considerable structural variation in the acceptor bases. Partially purified transferase enzyme from *L. helveticus* has been shown to accept 5-mercaptouracil (Baranski *et al.*, 1969) and 2-halogenated adenines (Carson *et al.*, 1980; 1984). The compounds, 2-chloro- and 2-fluoro-2'-deoxyadenosine, were shown to possess anti-leukemic and immunosuppressive activity. The 2',3'-dideoxynucleoside derivatives of the 2-halo-2'-deoxynucleosides have also been prepared by the catalytic activity of the partially purified transferase enzymes from *L. helveticus* (Haertle *et al.*, 1988). Carson and Wasson (1988) have demonstrated that transferase enzymes from *L. helveticus* can be used to prepare radioactively labelled 2',3'-dideoxynucleoside, the label being on the sugar residue. These compounds can then be used for studying drug metabolism.

Huang and co-workers (1981) have extensively purified a transferase enzyme from *L. leichmannii*, where the final stages of purification were by affinity chromatography. The purified enzyme was then used to synthesise twelve analogues of 2'-deoxyadenosine on a 100-400 mg scale, giving an average yield of 64% of the new nucleoside. Since only purines were used as substrates, it was not clear whether the enzyme was transferase I or II. Stout and co-workers (1976) have been able to demonstrate the broad substrate range of the transferase enzymes from *L. leichmannii*. They were able to synthesise nucleosides of 2,6-diaminopurine and 6-azathymine with crude preparations of *L. leichmannii*, whereas Marsh and King (1959) were unable to synthesise these compounds with a purified enzyme from *L. acidophilus*.

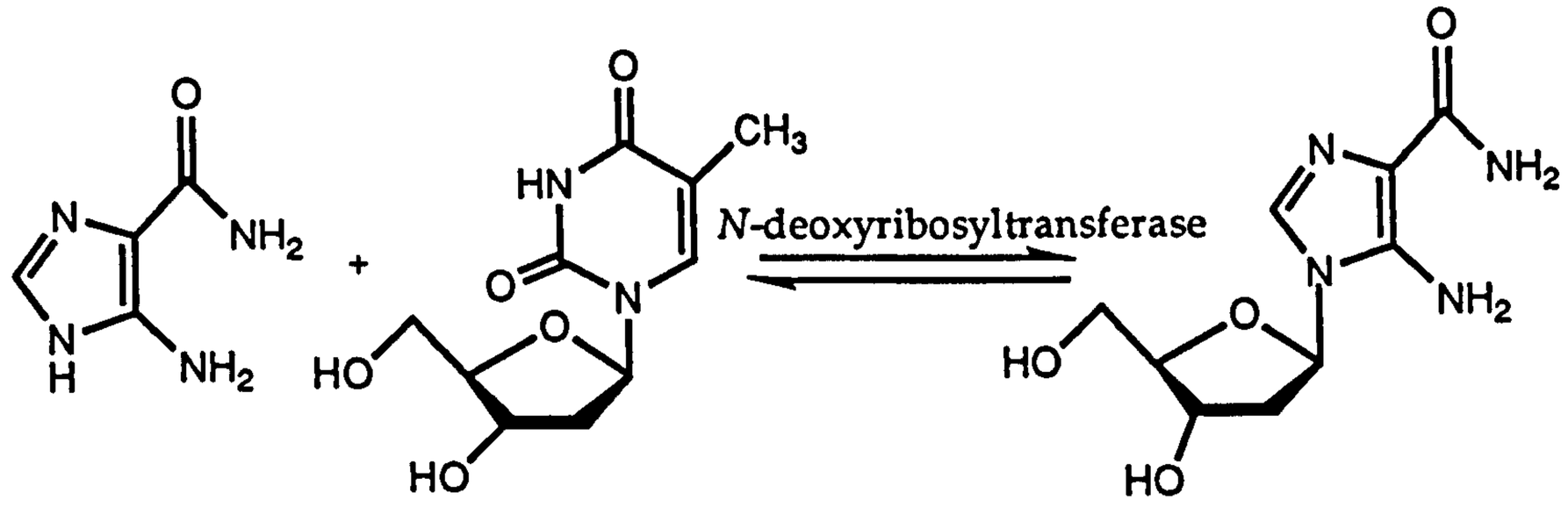
In this department, nucleoside analogues have been synthesised exclusively by crude extracts of *L. leichmannii*, where only one product was synthesised from thymidine and 1-deazapurine (Betbeder *et al.*, 1989). The 1-deazapurine nucleoside did not possess any anti-HIV activity, but the N^6-substituted purines of 2',3'-dideoxynucleosides, prepared by the same method but using 2',3'-dideoxycytidine as the donor (Figure 1.19), were found to be inhibitors of HIV type 1 (Betbeder *et al.*, 1990a), and also possessed low *in vitro* toxicity (compounds **18-21**).



Where R is:- $-N(CH_3)_2$ (18)
$-N(C_2H_5)_2$ (19)
$-NHCH_3$ (20)
$-NH\text{-}n\text{-}C_6H_{13}$ (21)

Figure 1.19 Preparation of N^6-substituted purines of 2',3'-dideoxynucleosides using crude *N*-deoxyribosyltransferases from *L. leichmannii*

Other compounds prepared by Betbeder and co-workers (1990b), by the same type of enzymatic procedure include 2'-deoxyribofuranosyl-5-aminoimidazole-4-carboxamide (22) and 2'-deoxyribofuranosyl-benzimidazole (23), as illustrated in Figure 1.20.



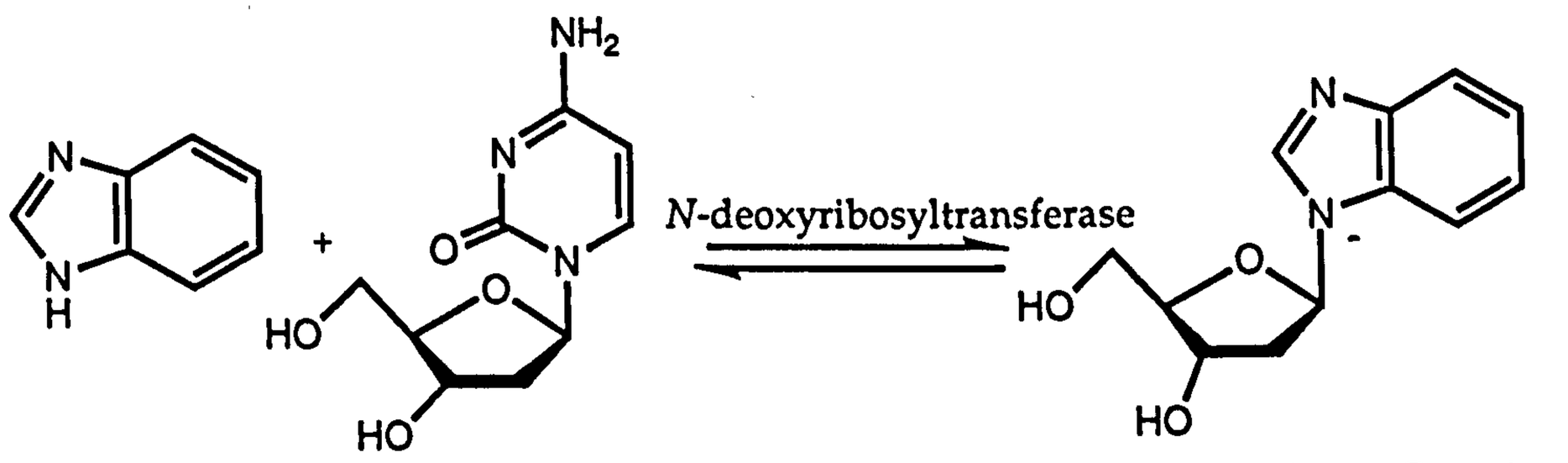


Figure 1.20 Preparation of imidazole deoxynucleosides using crude *N*-deoxyribosyltransferases from *L. leichmannii*

Crude *N*-deoxyribosyltransferase enzymes from *L. leichmannii* have also been employed to synthesise 2',5'-dideoxynucleoside containing N^6-methylaminopurine as a base **(24)** in high yield (98%) from 2',5'-dideoxythymidine (Betbeder *et al.*, 1991) (Figure 1.21).

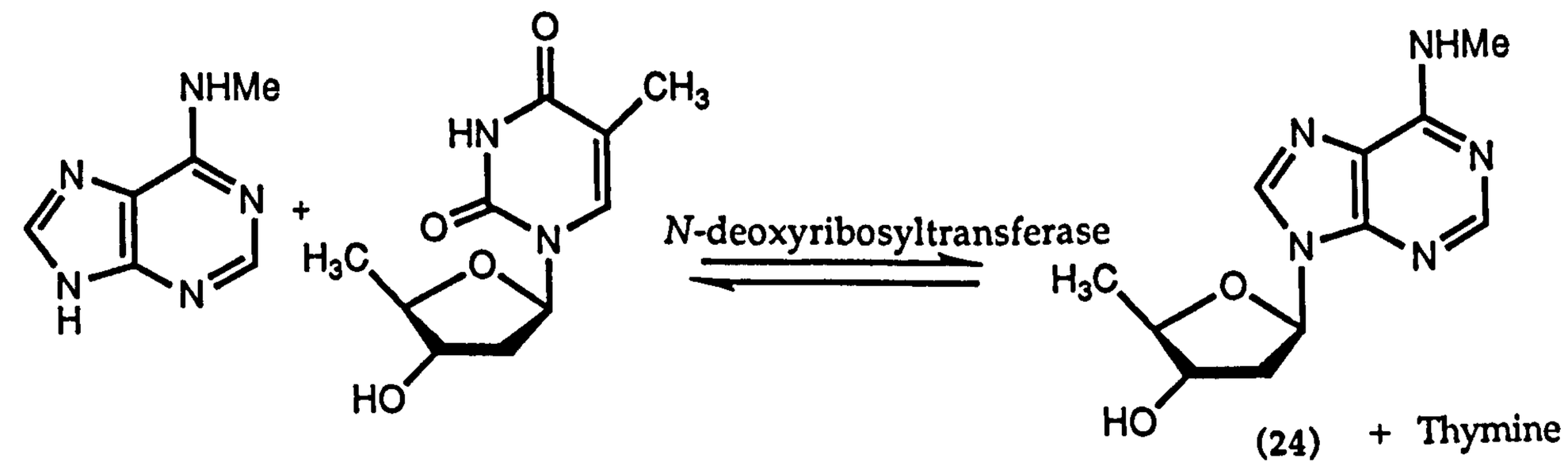


Figure 1.21 Preparation of 2',5'-dideoxynucleoside using crude *N*-deoxyribosyltransferases from *L. leichmannii*

Thymidine or 2'-deoxycytidine have generally been used as glycosyl donors in the transfer reactions with crude extracts of *L. leichmannii*. However, crude extracts contain many other enzymes which might interfere with the reaction, such as deaminases which would degrade the deoxycytidine to deoxyinosine or hydrolases which would cleave the nucleosides leaving an unreactive glycosyl residue. Recently, Huard and Hutchinson (1992) have demonstrated the suppressive activities of ethylene glycol. When 10% of ethylene glycol was added to the reaction mixture the degradation of the nucleoside products and starting materials was almost completely abolished. The glycosyl transfer was not affected if the concentration of the ethylene glycol was kept low.

The synthesis of the weakly anti-HIV active agent, 9-β-D-2',3'-dideoxyribofuranosyl 2-aminopurine has been synthesised with crude extracts of *L. leichmannii* in the presence of ethylene glycol (Hicks *et al.*, 1992).

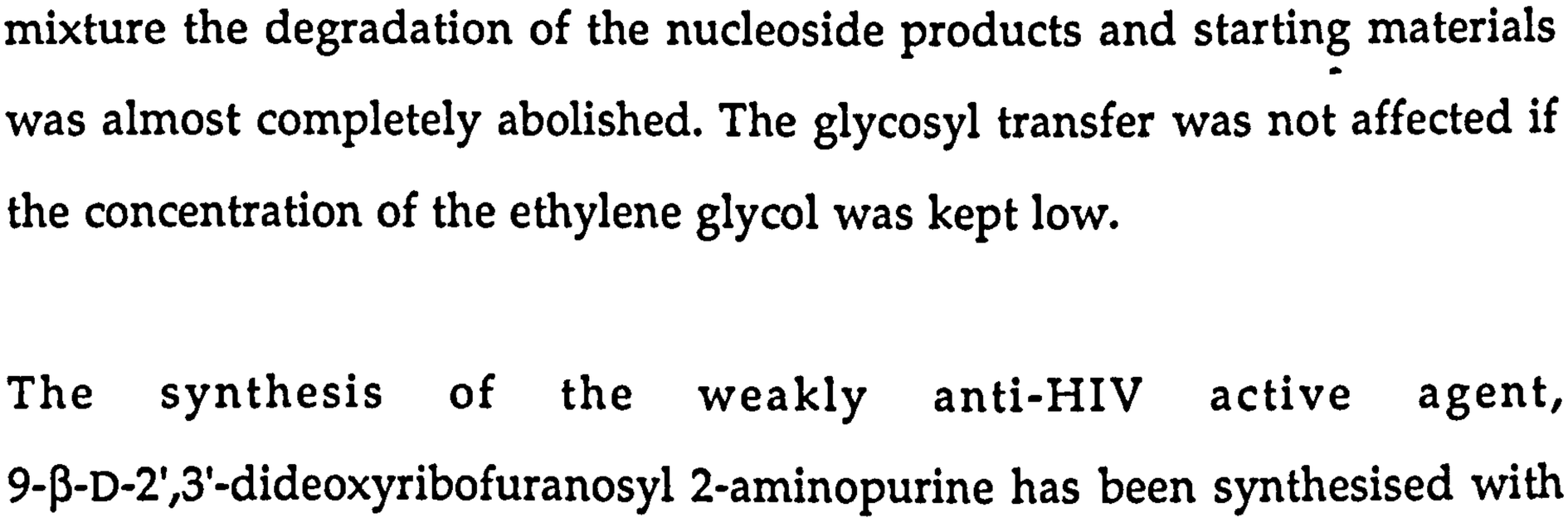


Figure 1.22 Preparation of 2',3'-dideoxynucleoside using crude *N*-deoxyribosyltransferases from *L. leichmannii*

Recently, Smar and co-workers (1991) have shown that in the absence of acceptors, genetically engineered nucleoside *N*-deoxyribosyltransferase from *L. leichmannii* hydrolyses 2'-deoxyribonucleosides producing D-ribal first, which then becomes hydrated (Figure 1.23). They were then able to

demonstrate the usefulness of D-ribal as a substrate in the synthesis of nucleosides by synthesising 2'-deoxyadenosine from D-ribal and adenine, and simplifying the purification of the new nucleoside by eliminating the substrate nucleoside from the reaction.

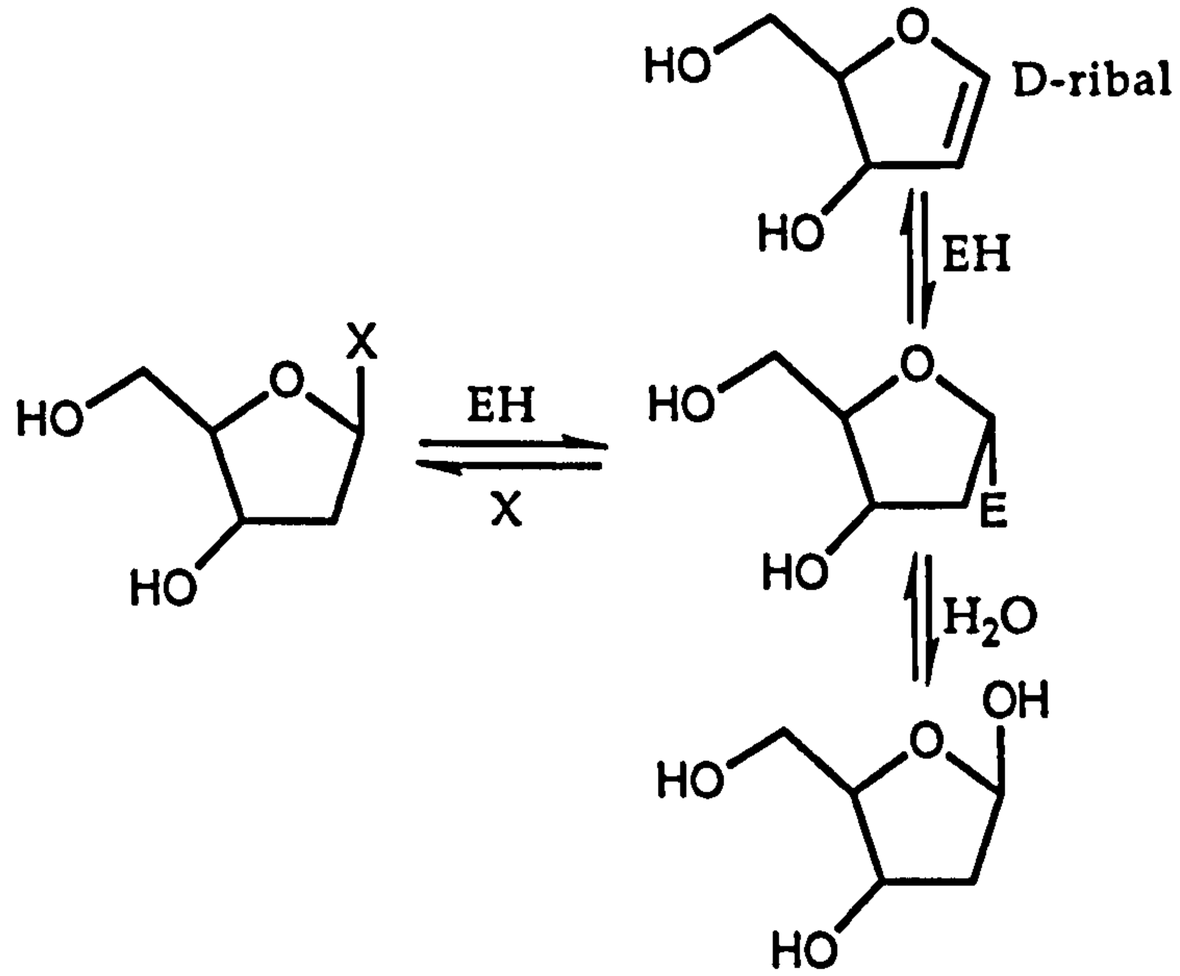


Figure 1.23 Production of D-ribal by nucleoside *N*-deoxyribosyltransferase from *L. leichmannii* (Smar *et al.*, 1991)
(X represents a purine or pyrimidine base and EH represents a nucleophilic group at the active site of the enzyme.)

The synthesis of 2',3'-dideoxynucleosides of purines, pyrazolo[3,4-*d*]pyrimidine and triazolo [4,5-*d*]pyrimidine and their derivatives with purified *N*-deoxyribosyltransferase from *L. leichmannii* has been described (Fischer *et al.*, 1990a; 1990b). These compounds are thought to possess potential chemotherapeutic activity, especially as antiviral agents.

In summary it can be seen that nucleoside *N*-deoxyribosyltransferases are becoming very useful as synthetic tools for preparing 2'-deoxyribonucleosides and 2',3'-dideoxyribonucleosides. Advantages of using *N*-deoxyribosyltransferases are that the syntheses involve

single-step reactions which are highly stereo- and regiospecific forming only the β-anomer and glycosylation at the N_1 and N_9 positions of pyrimidine and purine bases, respectively. Since a single enzyme contains all three transfer activities the need to purify the enzyme is not generally essential for synthetic purposes.

1.5 Objectives

Since *N*-deoxyribosyltransferases have not been so extensively studied as the phosphorylases, it would be of interest to determine whether they are more widely distributed among microorganisms than just in the lactobacilli, and whether different types of *N*-deoxyribosyltransferases exist. *N*-deoxyribosyltransferases possessing broad substrate specificity would greatly enhance the enzymatic syntheses of these nucleoside analogues.

Nucleoside *N*-deoxyribosyltransferase has been found only in certain lactic acid bacteria belonging to the obligately homofermentative subgroup of the genus *Lactobacillus*. However, many species of *Lactobacillus* have not been examined. Other closely related genera of lactic acid bacteria, such as *Streptococcus* and *Leuconostoc* have similarly been overlooked. Therefore, the primary objective of this project was to identify organisms that produce a high concentration of active nucleoside *N*-deoxyribosyltransferase per unit biomass, and secondly to discover nucleoside *N*-deoxyribosyltransferases that accept an altered (expanded) range of substrates, especially those that accept 3'-modified nucleosides.

Chapter 2

Screening for *N*-deoxyribosyltransferases among lactic acid bacteria

2.1 General Introduction

Minimal media for many species of *Lactobacillus* require a deoxyribonucleoside, along with some purine and pyrimidine bases (Rogosa *et al*, 1961; Beck & Levin, 1962). In *L. leichmannii*, other deoxyribonucleosides required for the synthesis of DNA are produced intracellularly by transfer of a deoxyribosyl group from the nucleoside to the free bases (Beck & Levin, 1962; 1963), which is catalysed by nucleoside *N*-deoxyribosyltransferase [EC 2.4.2.6]. Similar enzyme activity has been observed in preparations from *L. acidophilus*, *L. delbrueckii* (MacNutt, 1952; Beck & Levin, 1963) and *L. lactis* (Beck & Levin, 1963). As mentioned in Chapter 1, two forms of the enzyme have been purified to homogeneity from *L. helveticus* (Uerkwitz, 1971; Holguin & Cardinaud, 1975) and *L. leichmanii* (Heath, 1991). Apart from the current work, attempts to demonstrate nucleoside *N*-deoxyribosyltransferase activity in other organisms have proved fruitless (Holguin, 1974; O'Donovan & Neuhard, 1970; Marsh & King, 1959).

In most other bacteria, transfer of the deoxyribosyl group is catalysed by the coupled reactions of thymidine phosphorylase and purine nucleoside phosphorylase (Imada & Igarasi, 1967). Gram negative bacteria also catalyse ribosyl transfer reactions, employing purine nucleoside phosphorylase and uridine phosphorylase (Kamimura *et al.*, 1973; Imada & Igarasi, 1967). In some mammals and in bacteria such as *B. stearothermophilus* and

H. influenzae, a single pyrimidine nucleoside phosphorylase can catalyse the phosphorolysis of either uridine or thymidine (Krenitsky *et al.*, 1964; Scocca, 1978). By contrast the four species of *Lactobacillus* which possess nucleoside *N*-deoxyribosyltransferases appear to lack the phosphorylases (Cardinaud, 1978).

The majority of the lactic acid producing bacteria belong to the two families of Lactobacillaceae and Streptococcaceae. The genera of the lactic acid bacteria are *Lactobacillus* from Lactobacillaceae and *Streptococcus, Lactococcus, Leuconostoc, Pediococcus, Aerococcus* from Streptococcaceae. Recently the genus *Streptococcus* has been subdivided into three genera *Streptococcus, Enterococcus* and *Lactococcus*, where the genus *Lactococcus* comprises of all the lactic streptococci (Schleifer & Kilpper-Balz, 1987).

These bacteria grow in similar environments and produce lactic acid as a major fermentation product. Organisms from the family Streptococcaceae have been found in some of the same selective habitats and often growing on the same selective media as the lactobacilli, such as the oral cavity of humans and animals. The genus *Lactobacillus* has been shown to be part of a phylogenetic cluster with close relations to *Streptococcus, Leuconostoc* and *Pediococcus*, but more distant relations with *Aerococcus* (London, 1976; Stackebrandt *et al.*, 1983).

Both the *N*-deoxyribosyltransferases and the phosphorylases have been employed as biocatalysts for the preparation of nucleoside analogues with antiviral and anti-tumor activity (Utagawa *et al.*, 1986; Huang *et al.*, 1981; Carson & Wasson, 1988; Betbeder *et al.*, 1989; Hicks *et al.*, 1992). Screening programmes have identified strains of enteric bacteria (Utagawa *et al.*, 1986; Shirae *et al.*, 1989) and thermophiles containing exceptional

phosphate-dependent activities (Hori *et al.*, 1989). Lactic acid bacteria appear to have been overlooked in these screening programmes.

In the present study the mechanism of pentosyl transfer employed by 22 strains of lactic acid bacteria was investigated, many of which were chosen on the basis of nucleoside and base requirements for growth (Sharpe, 1981; Teuber & Geis, 1981; Kandler & Weiss, 1986).

In the past, the majority of the assays employed to measure the activity of *N*-deoxyribosyltransferases have centred on spectrophotometric methods. Direct spectrophotometric determinations have been used which involve selecting a suitable wavelength for a substrate couple (eg. deoxycytidine and adenine) and determining the difference in extinction coefficients of the substrate couples with the product couples (eg. deoxyadenosine and cytosine) at a given concentration. Hence the absorbance change detected over time would be proportional to the difference in extinction coefficients of the substrate and product couples (Danzin & Cardinaud, 1976).

Another common method used for assaying *N*-deoxyribosyltransferase activities was by using coupled enzyme systems (Kalcker, 1947), which made use of measuring differences in absorbance resulting from the action of an auxiliary enzyme. For example, to measure the transfer reaction with deoxyinosine and adenine (dI:A), xanthine oxidase can be used as the auxiliary enzyme to oxidise the hypoxanthine, produced from the deoxyribosyl transfer reaction, to uric acid. The change in absorbance due to the appearance of the uric acid can then be followed at 290 nm when most common bases and nucleosides have low extinction coefficients (Fig 2.1).

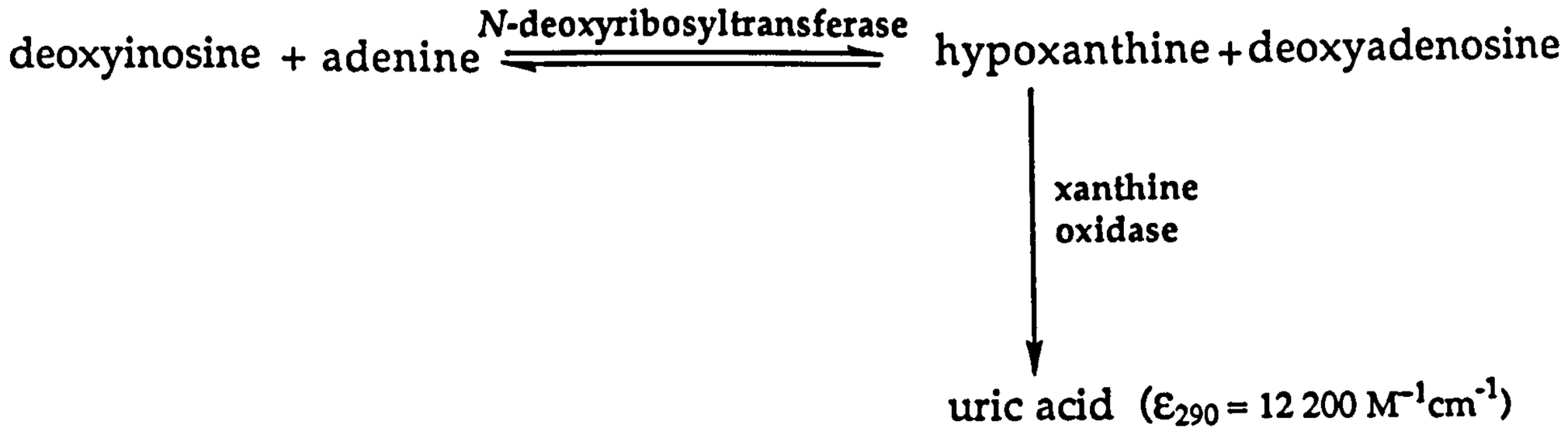


Figure 2.1 Use of coupled enzyme system with xanthine oxidase to assay for *N*-deoxyribosyltransferase activity

A colorimetric assay has also been used to determine *N*-deoxyribosyltransferase activities by measuring the concentration of free 2-deoxyribose in solution (Blakley, 1966). The reaction relies upon the acid stability of the pyrimidine nucleosides compared to the acid lability of purine nucleosides, which when treated with diphenylamine reagent, liberates the 2'-deoxyribose group. The blue colour which develops is then conveniently measured at 595 nm. The main disadvantage of this assay would be that pur:pur and pyr:pyr activities of *N*-deoxyribosyltransferase could not be measured.

The most convenient method employed to measure *N*-deoxyribosyltransferase activities has made use of detecting and separating the substrates and products of the transfer reaction by high pressure liquid chromatography (HPLC), using C_{18} or C_8 reverse phase columns with a mobile phase of water and methanol. Detection can be carried out at 254 nm, where most nucleosides and bases have high extinction coefficients, and the new nucleoside formed from the transfer reaction can be quantified by measuring the peak area produced with the peak areas of known amounts of the same compound.

2.2 Materials and Methods

2.2.1 Microorganisms and media

All lactic acid microorganisms (Appendix 1) studied were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland, UK), except *L. leichmannii* which was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and *L. helveticus* which came from the National Collection of Dairy Organisms (NCDO, Reading, Berkshire, UK).

The organisms were cultivated, without agitation or pH control, in capped 300 ml bottles, containing 200 ml of MRS (DeMan *et al.*, 1960) broth (Oxoid, Basingstoke, Hampshire, UK; Appendix 2). *Aerococcus viridans* was grown in the same manner but in glucose-buffered broth (Appendix 2). Growth of the bacteria was monitored by measuring the optical density of the cultures at 600 nm. Measurements were made until the OD_{600} was fairly constant, indicating that optimum growth had been achieved. After incubation for 24 h near their optimal growth temperatures (Appendix 1), the organisms were harvested by centrifugation (6000 *g*, 15 min) and washed twice in 0.02 M Pipes-NaOH buffer (pH 6.5). Cultures of *Leuconostoc mesenteroides* subsp. *cremoris* (NCIMB 12008) were harvested after 48 h of incubation at 30°C. All subsequent operations were carried out at 4°C except where otherwise indicated.

2.2.2 Preparation of extracts

Cell pellets were suspended to a final volume of 5 ml in 0.02 M Pipes-NaOH buffer (pH 6.5). Sodium dithiothreitol (1 mM) was added to

protect phosphorylase activity (Koszalka *et al.*, 1988a). The bacteria were disrupted at 20 000 psi in a French pressure cell (x 3), and the extracts clarified by centrifugation (20 000 *g*, 30 min). Cell-free extracts were dialysed overnight against 100 volumes of 0.02 M Pipes-NaOH buffer (pH 6.5), containing sodium dithiothreitol (1 mM) and sodium azide (0.05% w/v), which was added to prevent microbial growth. Dialysates were maintained at 4°C for 2-4 weeks without significant loss of activity.

2.2.3 Enzyme assays

The concentration of nucleosides and bases formed during pentosyl transfer reactions were determined using reverse phase HPLC on a Lichrosorb C18 column (0.46 x 25 cm; Jones Chromatography, Hengoed, UK). Samples (20 µl) were removed from the reaction mixtures after incubating for a certain length of time and applied to the HPLC column which was eluted using a mobile phase of methanol and water and products detected at 254 nm.

2.2.3.1 Nucleoside *N*-deoxyribosyltransferase assays

Nucleoside *N*-deoxyribosyltransferase activity was measured by three different assays to take account of strain specific differences in activity towards purines compared with pyrimidines. The assays were carried out at pH 6.5 in a final volume of 0.5 ml and at 40°C. Reactions were stopped by placing them in dry ice.

The rate of deoxyribosyl transfer from a purine nucleoside to a purine base (pur:pur) (dI:A) was measured by following the formation of deoxyadenosine from adenine (0.2 µmol) and deoxyinosine (0.2 µmol) in a

Pipes-NaOH buffer (50 µmol). The reaction was initiated by the addition of the dialysed extract.

Similarly, the rate of deoxyribosyl transfer between a pyrimidine nucleoside and purine base (dC:A) was monitored by following the formation of deoxyadenosine from deoxycytidine and adenine (pyr:pur). The production of deoxyadenosine in pur:pur and pyr:pur transfer reactions were measured by HPLC with a mobile phase of methanol:water (17:83) at a flow rate of 1 ml/min.

N-deoxyribosyltransferase activity between pyrimidines (pyr:pyr) was measured by following the production of deoxyuridine from uracil and thymidine (dT:Ur). Production of deoxyuridine was measured by an elution gradient from 10% to 40% methanol:water at a flow rate of 1 ml/min.

2.2.3.2 Nucleoside phosphorylase assays

Four types of nucleoside phosphorylase activities were measured; one type of purine phosphorylase and three types of pyrimidine phosphorylases.

Purine Phosphorylase: The rate of production of inosine from hypoxanthine and ribose-1-phosphate (Hx:Ino) determined the activity of purine nucleoside phosphorylase. The reaction mixture contained hypoxanthine (1 µmol), ribose-1-phosphate (2.5 µmol) and Pipes-NaOH buffer (50 µmol, pH 6.5). The reaction was initiated by the addition of dialysed extract to give a final volume of 0.5 ml and incubated at 40°C. Production of inosine was determined by elution with methanol:water (10:90) at a flow rate of 2 ml/min.

Uridine Phosphorylase: Uridine phosphorylase activity (Ur:Urd) was measured by estimating the concentration of uridine formed from uracil and ribose-1-phosphate at 40°C. The assay was carried out as described for purine phosphorylase, substituting uracil (1 µmol) for hypoxanthine. Analysis was carried out by HPLC with elution by methanol:water (7:93) at a flow rate of 1.5 ml/min.

Cytidine Phosphorylase: Production of cytidine from cytosine and ribose-1-phosphate determined the presence of cytidine phosphorylase. The assay was carried out as above, but using cytosine (1 µmol) as the base substrate. HPLC conditions for determining the cytidine were methanol:water (7:93) at a flow rate of 0.75 ml/min.

Thymidine Phosphorylase: Thymidine phosphorylase activity (dT:T) in the extracts was measured by determining the concentration of thymine produced by phosphorolysis of thymidine at 40°C. The reaction mixture contained thymidine (1 µmol) and sodium phosphate buffer (40 µmol, pH 6.5), with the reaction being initiated by the addition of dialysed extract to give a final volume of 0.5 ml. The phosphorolysis reaction was resolved by eluting with methanol:water (15:85) at a flow rate of 1 ml/min.

Retention times of the above compounds separated and identified by HPLC are all listed in Appendix 3.

2.2.3.3 Definition of Unit and Specific Activity

One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1.0 µmol of product formed in 1 min in the

above-mentioned assay conditions. Specific activity was defined as units per milligram of protein.

2.2.4 Protein determination

The concentration of protein was estimated using the dye-binding BioRad protein assay (BioRad, Watford, Hertfordshire) according to the method of Bradford (Bradford, 1976). The colorimetric property of Coomassie blue G-250 is employed, where the absorbance maximum of the dye changes from 465 nm to 595 nm when binding to protein occurs. Bovine serum albumin was used as a standard.

2.2.5 Whole cell screening of 3′-modified nucleoside-producing microorganisms

Erwinia

Nine strains of *Erwinia*† (Appendix 1) were grown in Luria-broth (Appendix 2) with shaking at 30°C, initiated with an 18 h inoculum (1%) grown in the same broth. After 24 h the cells were harvested by centrifugation (10 000 *g*, 10 min) and washed twice with 50 mM potassium phosphate buffer (pH 7), keeping the temperature at 4°C. The wet cell paste were then used as the enzyme source.

Escherichia coli

E. coli IAM 1204§ was grown in nutrient broth (Appendix 2) and in medium defined by Shirae and coworkers (Appendix 2) with shaking.

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† Kind gift from Dr G P C Salmond, University of Warwick, England.
§ Kind donotion from the Institute of Applied Microbiology (IAM), Tokyo University, Japan.

Growth was initiated with an 18 h inoculum (1%) grown in the same media at 30°C. After 24 h the cells were harvested by centrifugation (10 000 *g*, 10 min) and washed twice with 50 mM potassium phosphate buffer (pH 7). The wet cell paste was resuspended in 50 mM potassium phosphate buffer (pH 7) to give a final concentration of 50 mg/ml.

2.2.5.1 Reaction assays

The standard reaction mixture contained 3-fluoro-2′,3′-dideoxythymidine (1 µmol) as the donor nucleoside and one of four acceptor bases (1 µmol) were used, these included adenine, cytosine, 5-methylcytosine or 6-methyladenine with potassium phosphate buffer (12.5 µmol, pH 7). The reactions were initiated with wet cell paste (25 mg) as enzyme source The final volume of the reactions were 0.5 ml and these were incubated at 50°C with no shaking.

At intervals of 0, 0.5, 1, 3, 6, 12, 24, 36 and 48 h the cells were centrifuged down using a micro-centrifuge (11 600 *g*, 5 min), and supernatant (50 µl) was removed. The reaction products were analysed by tlc on silica gel plates which were developed with ethanol:dichloromethane (1:3) solution and the spots were detected under UV light.

2.2.5.2 Thymine-requiring mutants

E. coli # 5022 and KL262 were grown in nutrient broth supplemented with thymine (Appendix 2) at 30°C with shaking, *E. coli* (NCIMB 8583) was grown in AC broth (Appendix 2) at 37°C with shaking and *Enterobacter*

Kind gift from Dr D A Hodgson, University of Warwick, England.

aerogenes (NCIMB 10102) was grown in nutrient broth only (Appendix 2) at 30°C with shaking. All microorganisms were initiated with an 18 h inoculum (1%) and incubated at the appropriate temperatures for 24 h. The cells were harvested by centrifugation (10 000 *g*, 10 min) and washed twice with 50 mM potassium phosphate buffer (pH 7), and the resulting wet cell paste was used as the enzyme source.

2.2.5.3 Reaction assays for the thymine-requiring mutants

The standard reaction mixture contained 1 µmol each of the nucleoside donor and acceptor base. Four nucleosides were tested which include thymidine, 2′,3′-didehydro-2′,3′-dideoxythymidine[¥], 3′-fluoro-2′,3′-dideoxythymidine[*] and 3′,3′-difluoro-2′,3′-dideoxythymidine[¥]. The acceptor base used in each case was 6-dimethyladenine. Potassium phosphate buffer (12.5 µmol, pH 7) was added and the reaction was initiated with wet cell paste (500 µl, 11.6 mg of protein) in a final volume of 1 ml. The reaction mixtures were incubated at 35°C.

Samples of supernatant were taken as described in section 2.2.5.1 at intervals of 1, 3 and 6 days and analysed by HPLC. The concentration of the nucleoside donor (substrate nucleoside) was measured in each case by an elution gradient of 3% methanol:water for 10 min followed by a rise to 70% methanol:water over 20 min, remaining at 70% for 5 min and then immediately dropped to 3% methanol:water within 0.5 min. Flow rate was at 1 ml/min and the nucleosides were detected at 254 nm.

Retention times are listed in Appendix 3.

¥ Kind gift from Nicky Hicks, University of Warwick, England.
* Kind donation from Dr N G Johansson, Medivir, Stockholm, Sweden.

2.2.5.4 Effect of phosphate concentration on the cleavage of 3′-fluoro-2′,3′-dideoxythymidine by *E. coli* 5022

The reaction mixture contained 3′-fluoro-2′,3′-dideoxythymidine (1 µmol), 0, 20, 40, 60, 80, 100 µl of potassium phosphate buffer (pH 7, 0.5 M) in a final volume of 0.5 ml. The reaction was initiated with wet cell paste of *E. coli* 5022 (8.5 mg of protein). At zero concentration of phosphate buffer, Pipes-NaOH buffer (50 µmol, pH 7) was added. The reactions were incubated at 35°C for 3 days. After which time the cells were separated from the supernatants as described above and the supernatant analysed by HPLC as described in section 2.2.5.3.

2.2.6 Protein determination of whole cells

The protein concentration of whole cells was determined by boiling cells (100 µl) in H_2O (400 µl) and NaOH (500 µl, 1 M) for 5 min, allowing them to cool and then the BioRad protein assay was carried out, according to the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

2.3 Results

The specific rates of deoxyribosyl transfer varied 1.5 to 5-fold in extracts from a given strain grown on separate occasions. These variations were small, compared to the 10 to 500-fold differences between species, and may have resulted from variations in the composition of the complex media. The highest specific rates obtained for each reaction are presented below (Tables 2.1-2.4).

In order to account for the the variation between species, the phylogenetic relationship between the strains was considered. Tables 2.1-2.3 present the data obtained from the three main subgroups of *Lactobacillus*: the obligately homofermentative thermobacteria, the facultatively heterofermentative streptobacteria, and the obligately heterofermentative betabacteria (Orla-Jensen, 1919; Sharpe, 1981; London, 1976). The strains in Table 2.4 are representative of the family Streptococcaceae (Deibel & Seeley, 1974), which form a loose phylogenetic association.

2.3.1 Obligately homofermentative lactobacilli

Eight representatives of obligately heterofermentative strains of *Lactobacillus* were tested (Table 2.1). All exhibited *N*-deoxyribosyltransferase activities which were 100 to 500 times higher than the phosphorylase activities in the same extracts. None of these strains produced a detectable inosine phosphorylase. However, *L. helveticus* and *L. salivarius* subsp. *salivarius* catalysed exceptionally high rates of uridine synthesis and thymidine phosphorolysis, suggesting the presence of phosphorylase enzymes. *L. salivarius* subsp. *salivarius* was

also exceptional in that the strain lacked *N*-deoxyribosyltransferase activities towards pyrimidines (pyr:pur and pyr:pyr).

Strain	Specific rate of pentosyl transfer (mU/mg of protein)					
	dC:A	dI:A	dT:Ur	Ur:Urd	dT:T	Hx:Ino
L. delbrueckii [a]	164.4	9.3	12.9	0.3	0.3	0
L. lactis [a]	57.7	4.6	4.8	0.8	0.4	0
L. bulgaricus [a]	144.2	24.5	10.0	2.0	1.5	0
L. leichmannii [a]	336.8	18.5	20.5	0.9	0.6	0
L. helveticus	450.7	49.6	19.3	5.2	3.6	0
L. acidophilus	156.8	22.9	7.3	2.8	1.9	0
L. agilis	128.1	178.0	14.9	0	0.4	0
L. salivarius subsp. *salivarius*	0	51.5	0	8.2	3.6	0

Table 2.1 Specific rates of pentosyl transfer measured in extracts from representative strains of the obligately homofermentative lactobacilli.

[a]These strains have recently been accorded the status of subspecies of *L. delbrueckii*.

Symbols : Transfer of deoxyribose from deoxycytidine to adenine (dC:A), from deoxyinosine to adenine (dI:A), from thymidine to uracil (dT:Ur). The synthesis of inosine from hypoxanthine and ribose-1-phosphate (R1P) (Hx:Ino), of uridine from uracil and R1P (Ur:Urd), and the phosphorolysis of thymidine to thymine and R1P (dT:T).

2.3.2 Facultatively heterofermentative lactobacilli

By contrast, the facultatively heterofermentative lactobacilli showed elevated phosphorylase activities and little phosphate-independent activity (Table 2.2). It was also shown that three of the representatives in

this division catalysed the synthesis of inosine from ribose-1-phosphate and hypoxanthine, an activity which was absent from extracts of all other lactobacilli tested in this study.

The thymidine and uridine phosphorylase specific activities of 27 mU/mg and 16 mU/mg, respectively, in *L. casei* subsp. *casei* were significantly high in this organism, but reduced in the other representatives (Table 2.2). In the absence of phosphate however, the extracts catalysed little transfer from thymidine to uracil (pyr:pyr), except in the case of *L. alimentarius*.

Strain	Specific rate of pentosyl transfer (mU/mg of protein)					
	dC:A	dI:A	dT:Ur	Ur:Urd	dT:T	Hx:Ino
L. casei subsp. *casei*	0	2.3	23.3	16.2	26.8	9.1
L. casei subsp. *rhamnosus*	0	0	4.4	6.3	9.6	34.0
L. plantarum	62.7	7.4	6.8	6	1	3
L. alimentarius	2	17.5	171.0	9.4	1.3	0

Table 2.2 Specific rates of pentosyl transfer catalysed by extracts from representative strains of facultatively heterofermentative lactobacilli.
(Symbols as for Table 2.1)

2.3.3 Obligately heterofermentative lactobacilli

Table 2.3 shows the highest rates of pentosyl transfer measured in extracts from representatives of the obligately heterofermentative lactobacilli. In reactions between purines, these organisms resembled the obligately homofermentative lactobacilli (Table 2.1). All three strains lacked inosine phosphorylase, but catalysed phosphate-independent deoxyribosyl transfer from deoxyinosine to adenine (Table 2.3). However, unlike the

thermobacteria (Table 2.1), these betabacteria (Table 2.3) catalysed reactions involving pyrimidines both in the presence and absence of phosphates. Uridine and thymidine phosphorylase specific activities were nearly as high as the specific rates of deoxyribosyl transfer from thymidine to uracil.

Strain	Specific rate of pentosyl transfer (mU/mg of protein)					
	dC:A	dI:A	dT:Ur	Ur:Urd	dT:T	Hx:Ino
L. fermentum	380.1	260.0	36.3	10.3	3.0	0
L. buchneri	57.9	27.8	3.8	0.6	6.0	0
L. hilgardii	17.9	12.3	3.3	0.5	2.0	0

Table 2.3 Specific rates of pentosyl transfer catalysed by extracts from representative strains of obligately heterofermentative lactobacilli.
(Symbols as for Table 2.1)

2.3.4 Other lactic acid bacteria

2.3.4.1 Streptococci

To test the hypothesis that *N*-deoxyribosyltransferases are restricted to lactobacilli, representatives of four allied genera of lactic acid bacteria were screened (Table 2.4). *Streptococcus salivarius* subsp. *thermophilus* and *Streptococcus lactis* subsp. *lactis* (now known as *Lactococcus lactis* subsp. *lactis*) both exhibited high purine nucleoside phophorylase activity, and low thymidine and uridine phosphorylase activities. No traces of deoxyribosyl transfer were detected in the absence of phosphate. The streptococci were unique in this respect.

2.3.4.2 Leuconostoc

All representatives of the three remaining genera (Table 2.4) exhibited phosphate-independent *N*-deoxyribosyltransferase activities. *Leu. mesenteroides* subsp. *dextranicum* expressed a pyr:pur activity which was as high as that of the homofermentative *L. bulgaricus* and *L. delbrueckii*, while *Leu. mesenteroides* subsp. *cremoris* expressed the highest *N*-deoxyribosyltransferase specific activity measured in this study. These *Leuconostoc* species also catalysed pur:pur and pyr:pyr deoxyribosyltransferase. Uridine phosphorylase activities were comparable to the rates of pyr:pyr *N*-deoxyribosyltransferase. Thymidine and inosine phosphorylase activities were absent.

2.3.4.3 Pediococci and Aerococci

Extracts from two representatives of the genus *Pediococcus* and one representative of the genus *Aerococcus* were also analysed (Table 2.4). All three species catalysed deoxyribosyl transfer from deoxyinosine to adenine, but showed no inosine phosphorylase activities. In reactions involving pyrimidines, these organisms showed unusually high selectivity. *P. pentosaceus* catalysed transfer from deoxycytidine to adenine, but not between thymidine and uracil. The reverse was true of extracts from *A. viridans*.

Strain	Specific rate of pentosyl transfer (mU/mg of protein)					
	dC:A	dI:A	dT:Ur	Ur:Urd	dT:T	Hx:Ino
S. salivarius subsp. *thermophilus*	0	0	0	0.9	0.8	165.0
S. lactis subsp. *lactis*	0	0	0	3.0	2.3	87.5
Leu. mesenteroides subsp. *dextranicum*	142.1	45.2	3.9	3.1	0.8	0
Leu. mesenteroides subsp. *cremoris*	523.3	86.8	4.6	5.5	0	0
P. pentosaceus	30.7	36.7	0.3	0.4	0.1	0
P. acidilactici	39.0	46.2	2.3	0	0	0
A. viridans	0	18.1	16.3	4.0	1.7	0

Table 2.4 Specific rates of pentosyl transfer in extracts from representative strains of four genera of the Streptococcaceae : *Streptococcus* (*S*), *Leuconostoc* (*Leu*), *Pedicoccus* (*P*), *Aerococcus* (*A*). (Other symbols as for Table 2.1)

2.3.5 Cytosine phosphorylase

All extracts were also challenged with cytosine and ribose-1-phosphate. No extracts catalysed the synthesis of cytidine.

2.3.6 Whole cell screening for 3′-modified nucleoside producers

Strains of *Erwinia* and *E. coli* IAM 1204 were tested for their ability to transfer 3′-fluoro-2′,3′-dideoxythymidine with various acceptors, but a new nucleoside containing the 3′-fluoro-deoxyribose moiety was not produced by the microorganisms investigated. However, when thymine-requiring

mutant strains of *E. coli* and *Ent. aerogenes* were challenged with a variety of 3′-modified nucleosides a decrease in the substrate nucleoside was observed (Fig 2.2).

It can be seen from Figure 2.2 that thymidine and 2′,3′-didehydrodideoxythymidine are easily cleaved by *E. coli* 5022, whereas the presence of a fluorine group at the 3′-position greatly hinders the enzyme from cleaving the thymine base from the nucleoside. The presence of the acceptor base, 6-dimethyladenine did not affect the rate of cleavage to any significant amounts. This type of decrease in the substrate nucleoside was observed in the other thymine-requiring mutant species, where thymidine was cleaved by 100% and the 3′-fluoro-modified nucleosides were cleaved at a lesser extent. However, decrease in the concentration of substrate nucleoside was not followed by an increase in a new nucleoside being formed.

The effect of phosphate concentration on the rate of cleavage of 3′-fluorothymidine by intact cells of *E. coli* 5022 can be illustrated by Figure 2.3. The rate of cleavage of the nucleoside appears to be independent of phosphate concentration, since cleavage of the nucleoside is occurring in the absence as well as in the presence of phosphate.

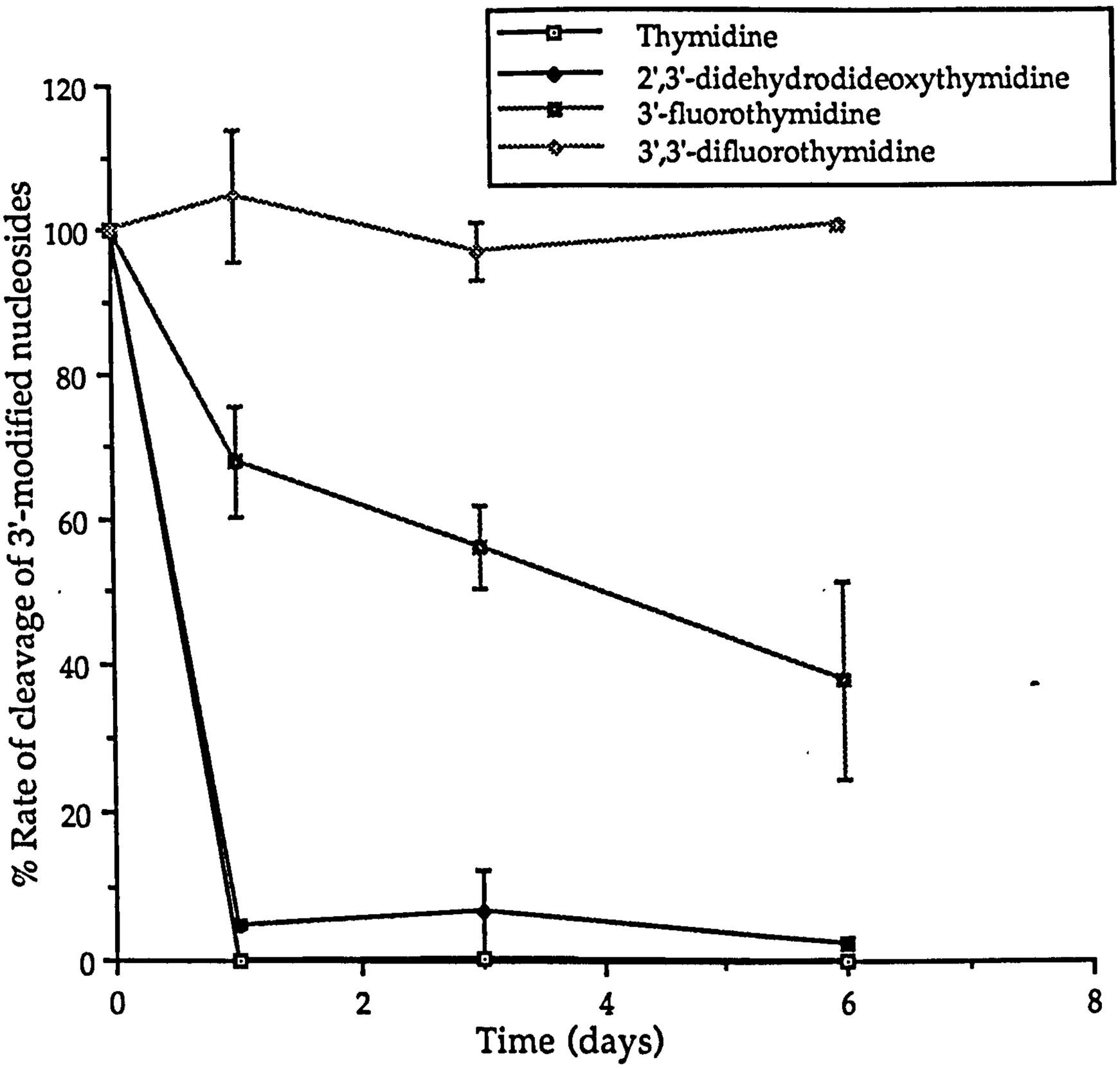


Figure 2.2 Rate of cleavage of 3'-modified nucleosides by intact cells of *E. coli* 5022

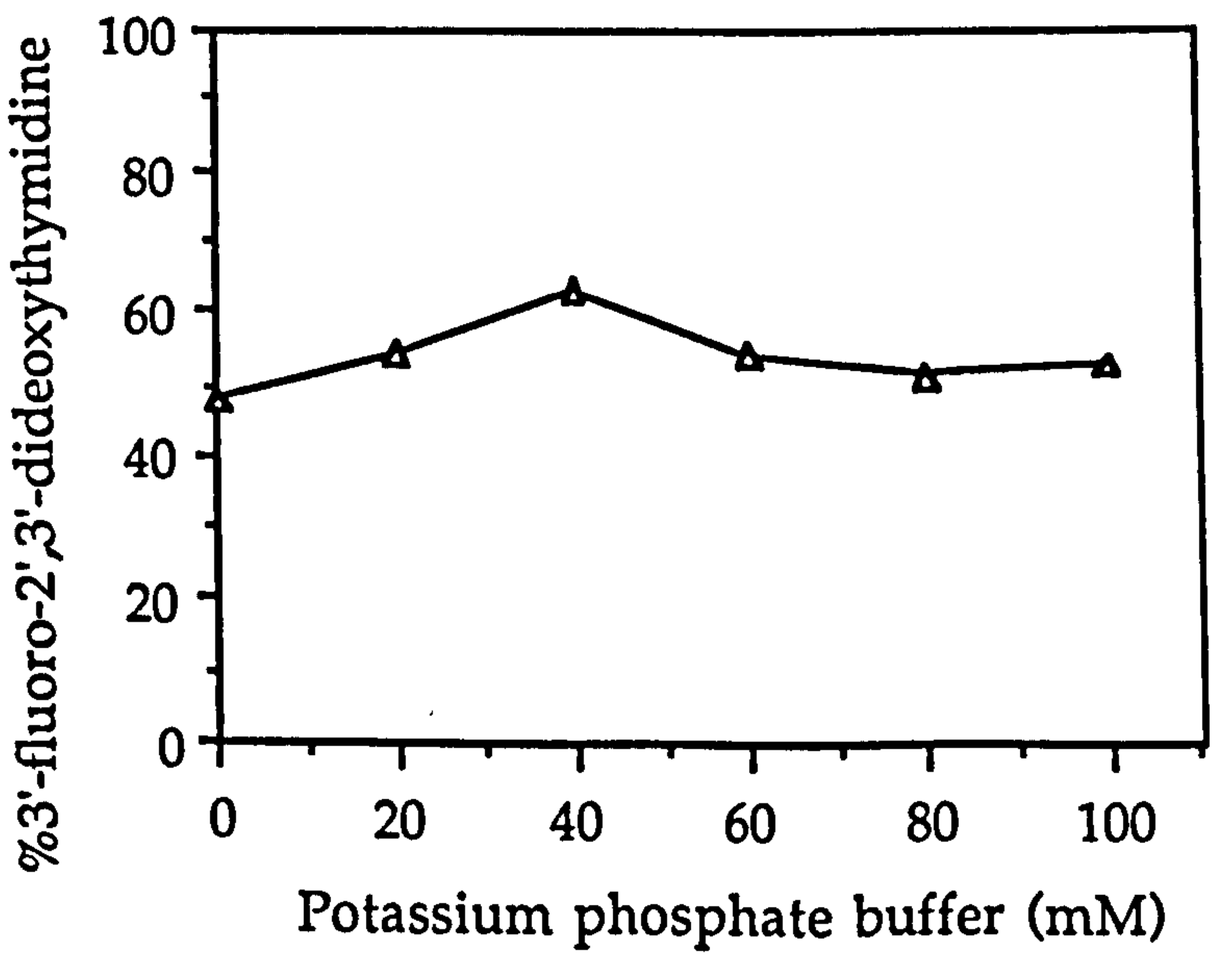


Figure 2.3 Effect of phosphate concentration on the rate of cleavage of 3'-fluoro-2',3'-dideoxythymidine by intact cells of *E. coli* 5022

2.4 Discussions

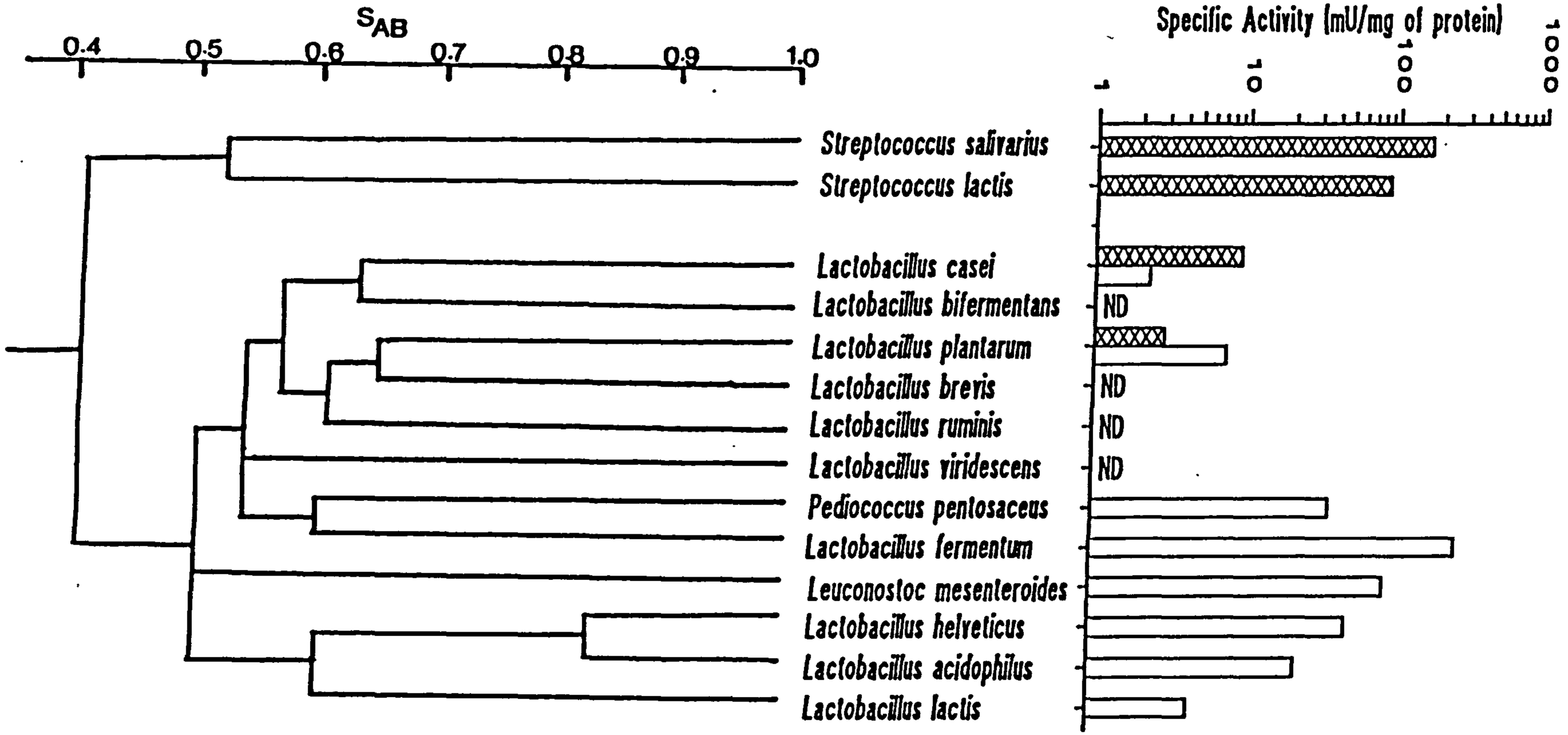
Phylogenetic correlations (The distribution of enzymes between species)

The data in Table 2.1 to 2.4 may be unsuitable for incorporation into a quantitative numerical taxonomy of lactic acid bacteria because of the initial bias toward strains known to require bases for growth. However, a surprising degree of consistency within groups is already evident, suggesting correlations with conventional taxonomic schemes.

Recently, the taxonomic relationships between the families of lactic acid bacteria have been reviewed on the basis of nucleic acid hybridization studies (Kandler, 1984; Kandler & Weiss, 1986). Analysis of oligonucleotide catalogues of ribosomal 16S RNAs were unable to separate *Lactobacillus* from *Pediococcus* and *Leuconostoc mesenteroides* (Stackebrandt *et al.*, 1983; Martinez-Murcia & Collins, 1990). However, *S. salivarius* and *S. lactis* were placed in a distinct cluster with other streptococci (Ludwig *et al.*, 1985). This correlates with the data in Table 2.4 which show the streptococci to be unique in lacking *N*-deoxyribosyltransferases.

Figure 2.4 shows that the measurements of inosine phosphorylase and purine-specific *N*-deoxyribosyltransferase activities support this taxonomy. A gradation of activities from the streptococci to *L. lactis* can be discerned in Figure 2.4. The inosine phosphorylase activity decreased from the streptococci to *L. casei* and *L. plantarum,* and are absent from other lactobacilli.

From a study of cross reactions of aldoses from lactic acid bacteria with anti-*Pediococcus cerevisiae* aldolase serum and anti-*Streptococcus faecalis*



⊠ Inosine phosphorylase activity ☐ Purine specific *N*-deoxyribosyltransferase activity ND = not determined

The phylogenetic scheme is according to Stackebrandt *et al.* (1983; modified).

Figure 2.4 Phylogenetic relationship in lactic acid bacteria and pentosyl transfer

serum, London (1976) came to a similar conclusion. The streptococci and *L. helveticus* were placed at extreme ends of a linear phylogenetic map, agreeing with the present study (Figure 2.4). Similarly, *L. acidophilus* and the *L. delbrueckii/L. leichmannii* group were mapped near *L. helveticus*, while *L. casei* mapped close to the streptococci. The detection of inosine phosphorylase activity in both *L. casei* subsp. *casei* and *L. casei* subsp. *rhamnosus* supports their close relationship with the streptococci.

At the genospecies level, DNA-DNA homology and the G+C mol% of DNA have been used as criteria to indicate that *L. leichmannii*, *L. lactis* and *L. bulgaricus* are subspecies of *L. delbrueckii* (Weiss *et al.*, 1983). Table 2.1 shows that these organisms are distinctive in possessing high *N*-deoxyribosyltransferase activities, and insignificant phosphorylase activities (usually < 1 mU/mg of protein). The related species *L. helveticus*, *L. acidophilus* and *L. salivarius* subsp. *salivarius* showed phosphorylase activities of 2-8 mU/mg of protein.

Distribution of *N*-deoxyribosyltransferases

Previously, nucleoside *N*-deoxyribosyltransferase activity was considered to be restricted to five species of obligately homofermentative lactobacilli. However, in the present study comparable activities have been found in obligately heterofermentative but not facultative heterofermentative lactobacilli. In addition, high nucleoside *N*-deoxyribosyltransferase activities were detected in two representatives of the genus *Pediococcus*, two strains of *Leuconostoc mesenteroides* and in *Aerococcus viridans*.

Purified *N*-deoxyribosyltransferases show no dependence on phosphates, and are specific for deoxyribosides. Transfer of deoxyribose was considered to be catalysed by *N*-deoxyribosyltransferases when the reaction occurred in the absence of phosphate; and particularly when phosphorylase activities in the same extracts were comparably low. In *L. salivarius* subsp. *salivarius* (Table 2.3) the absence of *N*-deoxyribosyltransferase activity towards pyrimidine nucleosides suggests that the strain lacks an enzyme analagous to *N*-deoxyribosyltransferase II (Cardinaud, 1978).

Coordination of nucleoside anabolism and catabolism

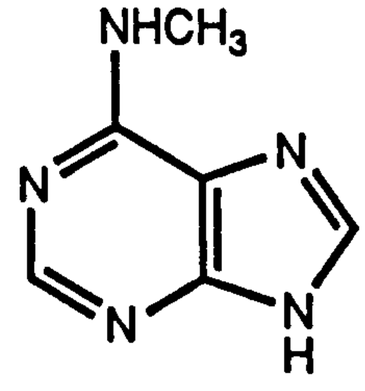
It is possible that nucleoside phosphorylases and nucleoside *N*-deoxyribosyltransferases do not co-exist *in vivo*. Nucleoside *N*-deoxyribosyltransferases are repressed when the intracellular nucleoside concentrations of *L. leichmannii* are high (Beck & Levin, 1962; Beck *et al.*, 1962). By contrast, the thymidine phosphorylase of *E. coli* is induced under these conditions (Rachmeler *et al.*, 1960; O'Donovan & Neuhard, 1970). It has also been suggested that nucleoside *N*-deoxyribosyltransferases function in nucleoside anabolism, whereas the phosphorylases are catabolic enzymes (O'Donovan & Neuhard, 1970). These findings may explain why phosphorylase activities were universally low in cells containing high *N*-deoxyribosyltransferase activities, and why phosphorylases have been difficult to detect in stationary phase cultures. However, strains which lacked pyr:pur and pyr:pyr *N*-deoxyribosyltransferase activities showed significant pyrimidine phosphorylase activities. These include the streptococci (Table 2.4) as well as *L. salivarius* subsp. *salivarius* (Table 2.1) and the *L. casei* species (Table 2.2). Similarly, strains lacking pur:pur *N*-deoxyribosyltransferase activity showed significant purine nucleoside phosphorylase activity, and

these included the streptococci (Table 2.4) and the *L. casei* species (Table 2.2). This distribution of pentosyl transferring enzymes may indicate a diversity of strategies employed by lactic acid bacteria in the regulation of purine and pyrimidine metabolism.

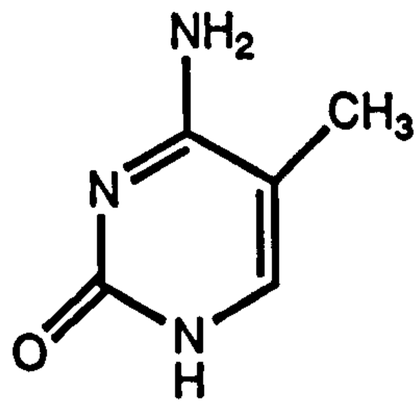
Screening for 3′-modified nucleoside-producing microorganisms

As discussed in Chapter 1, the most effective antiviral compounds came from the class of nucleosides which were modified at the 3′-position of the deoxyribosyl moiety, such as 3′-fluoro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine (AZT). So the need to find organisms which were capable of transferring these 3′-modified nucleosides was of great importance in order to synthesise a variety of these compounds enzymatically.

Crude extracts of the 22 lactic acid bacteria were subjected to 3′-fluoro-2′,3′-dideoxythymidine as the donor nucleoside and various acceptors, such as adenine, cytosine 6-methyladenine and 5-methylcytosine. Methylated bases of adenine and cytosine (Figure 2.5) were used in order to inhibit the actions of deaminases present in the crude extracts, especially as longer incubation periods were being employed.



6-methyladenine



5-methylcytosine

Figure 2.5 Some methylated derivatives of adenine and cytosine

The crude extracts and substrates were incubated at 40°C for 7 days and analysed for the production of a new nucleoside by HPLC. However, the production of a new 3′-fluoro-modified nucleoside was not observed.

As the *N*-deoxyribosyltransferases did not accept the 3′-fluoro-modified nucleosides as substrates, attention was diverted to microorganisms containing phophorylases. Shirae and co-workers (1989) had shown that a vast number of microorganisms capable of producing 2′,3′-dideoxyadenosine (ddA) from 2′,3′-dideoxyuridine (ddU). These workers used whole cells to screen for the production of ddA. From the investigation carried out by these workers it was clear that strains of *E. coli* and *Erwinia* were active in phosphorylases capable of accepting 2′,3′-dideoxy-modified nucleosides as substrates. Hence, these species of microorganisms were selected to screen for the 3′-fluoro-modified nucleoside producers. The results of the screening in strains of *Erwinia* and *E. coli* were disappointing since none of them were able to produce 3′-fluoro-modified nucleosides.

Studies were also carried out with thymine-requiring auxotrophs. These organisms are unable to synthesise their own thymine and so have to depend on extracting the base from their surroundings. The thymine requirement has been shown to be fulfilled by thymidine and 5-methyldeoxycytidine for a thymine-requiring mutant strain of *E. coli* (Barner & Cohen, 1954; Cohen & Barner, 1957).

Three strains of *E. coli* and one of *Enterobacter aerogenes* were challenged with three types of 3′-modified nucleosides as well as thymidine. A decrease in the substrate nucleosides was observed but no new nucleoside was produced. The thymine base was cleaved by almost 100% from

thymidine and 2′,3′-didehydro-2′,3′-dideoxythymidine, by about 60% from the 3′-fluoro-2′,3′-dideoxythymidine, but hardly at all from 3′,3′-difluoro-dideoxythymidine (Figure 2.6).

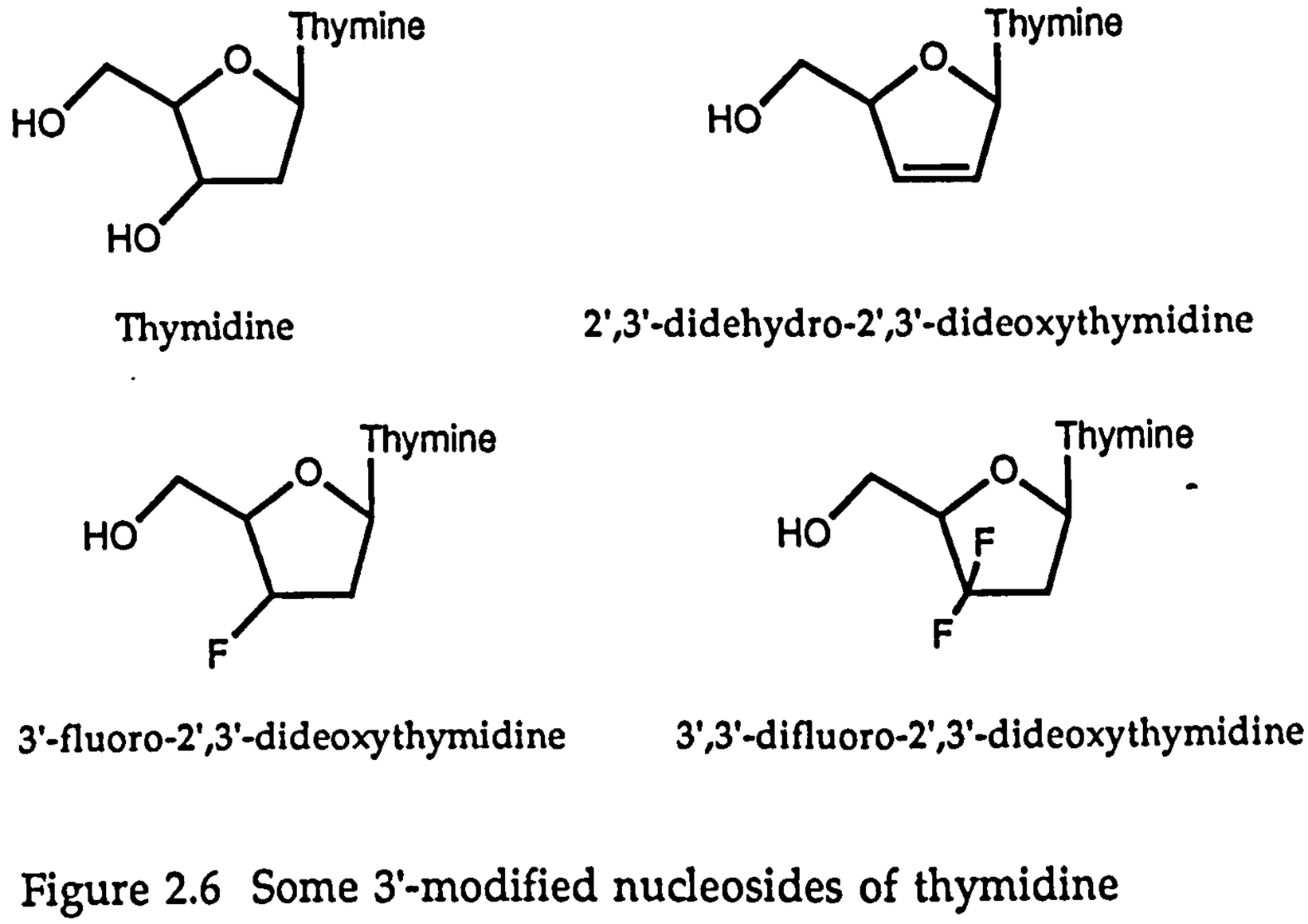


Figure 2.6 Some 3'-modified nucleosides of thymidine

The cleavage of the thymine base appeared to be occurring hydrolytically rather than phosphorolytically, since cleavage of thymine, from 3′-fluorothymidine by *E. coli* 5022, occurred independent of phosphate addition.

Therefore, it appears that both *N*-deoxyribosyltransferases and phosphorylases are unable to accept modifications, such as fluorine atoms, at the 3′-position of the deoxyribosyl moiety for carrying out the transfer reaction with another base. It can be proposed that the conformation of the deoxyribosyl moieties is affected by the 3′-fluoro- and 2′,3′-didehydro-groups because the deoxyribosyl moieties may not be in the correct conformation for binding and catalysis to occur by *N*-deoxyribosyltransferases and perhaps also the phosphorylases.

Although work has not been carried out on the acceptability of the conformational positions of the sugar group for the phosphorylases. Hence, groups present on the deoxyribosyl moiety may play an important role in defining whether a nucleoside is to be accepted by these enzymes.

Chapter 3

Purification of nucleoside *N*-deoxyribosyltransferase from *Leuconostoc mesenteroides* subspecies *cremoris*

3.1 General Introduction

Enzymes can potentially be extracted from any biological source, but have been most commonly isolated from microorganisms. Microorganisms have become far more appealing as producers of enzymes than plant or animal sources because of their biochemical diversity and the ease with which enzyme concentrations can be increased by genetic manipulations. Microbial cells generally need only short fermentation times and inexpensive media to be able to grow. The existence of distinct proteins from different strains which can catalyse the same reaction, allows for flexibility in the choice of fermentation conditions. Especially since these different enzymes may possess different pH and temperature stabilities as well as having different substrate specificities.

The manufacture of commercially viable products can require the need of a single enzyme. Hence extensive purification is sometimes necessary to remove other contaminating enzymes from the crude mixture, if such a single enzyme activity has to be achieved.

The study on the distribution of nucleoside *N*-deoxyribosyltransferase activities demonstrated the existence of this enzyme in lactic acid bacteria other than the lactobacilli. The highest pyr:pur specific activity was expressed in *Leu. mesenteroides* (*cremoris*), therefore it was of interest to determine whether the transferase enzyme from this species differs in any

way than from the transferase enzymes purified from the lactobacilli species.

As discussed earlier, *N*-deoxyribosyltransferase has been shown to exist in two distinct types when purified from *L. helveticus* (Holguin & Cardinaud, 1975) and *L. leichmannii* (Heath, 1991): (a) a purine specific enzyme (transferase I) and (b) a multifunctional enzyme catalysing all three transferase activities (transferase II). Hence, it was of interest to determine whether the transferase activity exhibited in *Leu. mesenteroides* (*cremoris*) consists of both types or just a single multifunctional transferase enzyme.

Early procedures employed to purify *N*-deoxyribosyltransferase from species of *Lactobacillus* involved the use of salt or solvent precipitation followed by heat denaturation of contaminating proteins (Roush & Betz, 1958; Marsh & King, 1959; Kanda & Takagi, 1959; Minghetti, 1960). Precipitation of proteins at high salt concentrations is one of the most widely used techniques in enzyme purification, and is very useful for concentrating the desired enzyme from large volumes of the crude mixture. Ammonium sulphate is the most commonly used salt and also has the advantage of stabilising the proteins. However, salt precipitation can be a very long and tedious procedure resulting in significant losses of product.

The heat denaturation step employed in these earlier purification strategies took into account the thermal stablilty of transferase II, but at the same time destroyed the relatively heat labile transferase I. This heat denaturation step was probably the cause of transferase I being undetected in these early attempts of purification.

Later, ion exchange chromatography was introduced into the purification strategy (Uerkvitz, 1971; Danzin & Cardinaud, 1974; Holguin & Cardinaud, 1975; Huang *et al.*, 1981). This separation technique is based on the different charges exhibited by proteins. The process involves the reversible binding of proteins to an ion exchange column due to electrostatic attractions which exist at a given pH between surface charges on the protein and the charged groups on the exchange support. Anion and cation exchange chromatography are often used in purification procedures, where the exchange materials are diethylaminoethyl (DEAE) and carboxymethyl (CM), respectively.

For example a DEAE anion exchange column, containing positively charged groups, will reversibly bind proteins with an overall net negative charge. Elution can be achieved by displacing the protein with a counter-ion such as chloride ion by adding a linear gradient of NaCl solution. Hence, ion exchange chromatography can be used efficiently in the initial purification steps for removing the bulk of the contaminating proteins present in the crude mixture.

The technique of affinity chromatography has played an important role in the detection of the two transferase enzymes from *Lactobacillus*. This type of column chromatography demonstrates the specific affinity of enzymes for their substrates. Holguin and Cardinaud (1975) synthesised a variety of purine and pyrimidine ligand adsorbents which were then coupled to a Sepharose support containing a suitable spacer arm. The spacer arm is useful in setting the ligand away from the support so that it is more accessible to the enzyme. Elution of the enzyme was achieved by adding solutions of free ligand or mild denaturing agents such as urea and guanidine hydrochloride.

Holguin and Cardinaud (1975) were able to resolve the two distinct deoxyribosyl transfer activities from *L. helveticus* by using affinity chromatography on a CN-Br activated Sepharose support coupled to a rigid spacer arm link, *m*-phenylenediamine, which in turn was attached to the ligand 6-(*p*-aminobenzyl)aminopurine (Figure 3.1). Elution of transferase I was achieved by 1 mM deoxyinosine solution and transferase II was eluted with 1 M guanidine hydrochloride. Danzin and Cardinaud (1974) also used the same affinity adsorbent to purify transferase I from *L. helveticus*, where elution of transferase I was achieved with 10 mM solution of deoxyinosine.

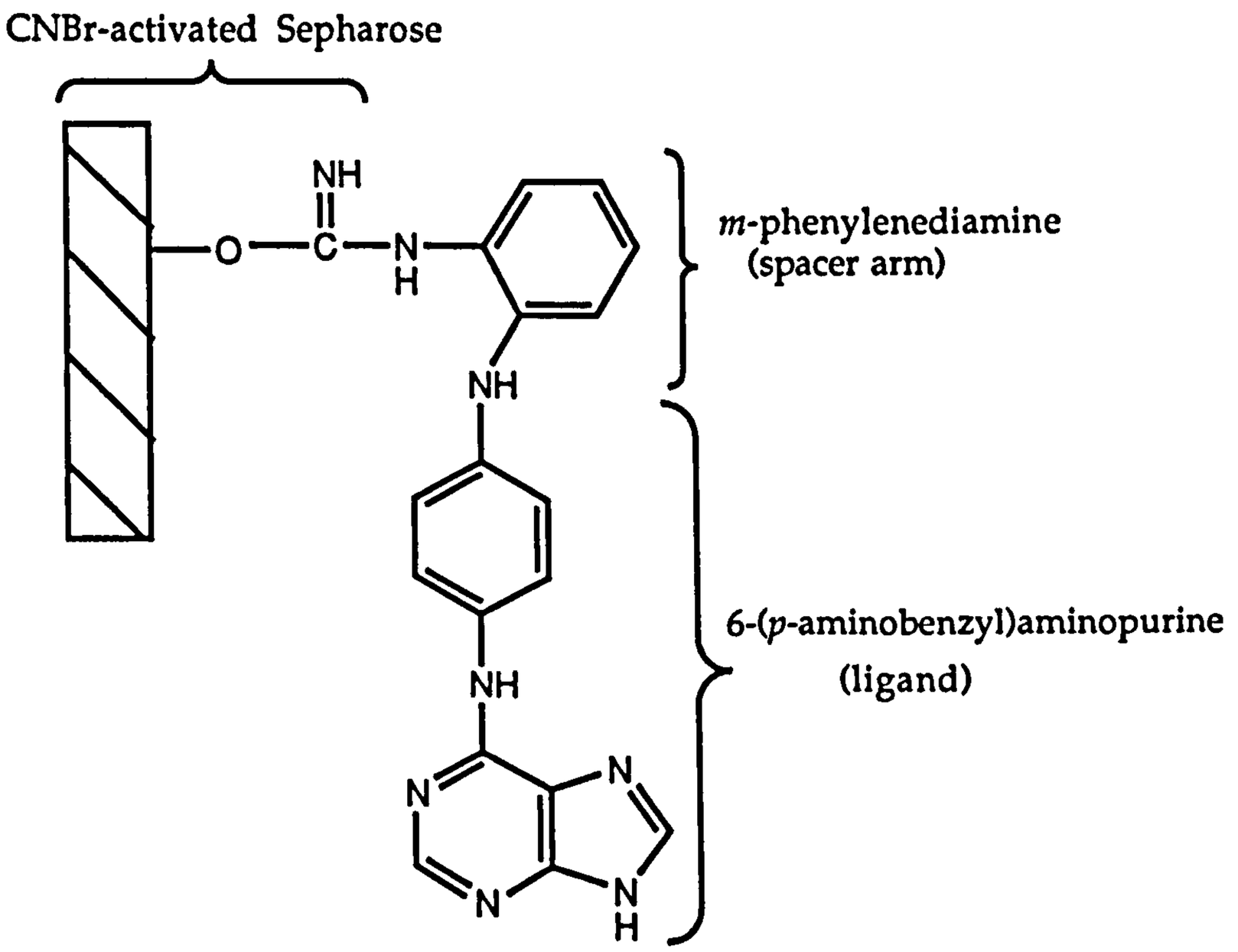


Figure 3.1 Affinity material employed to resolve *N*-deoxyribosyltransferase I and II from *L. helveticus* (Holguin & Cardinaud, 1975)

Huang and co-workers (1981), however, used the same purine ligand as Holguin and Cardinaud, but were unable to synthesise the ligand moiety by the procedure described by them. Therefore, a different procedure was used to obtain the same ligand, which was used to purify only

transferase II from *L. leichmannii*. Elution of the enzyme was achieved with different concentrations of guanidine hydrochloride. Therefore, it can be seen that affinity chromatography is a very powerful tool because it relies on specific interactions between the enzyme and the affinity ligand. Hence, this technique is useful in the purification of proteins and is commonly used in the latter stages of the purification strategy.

Later, the presence of two distinct transferase enzymes from *L. leichmannii* was demonstrated by Heath (1991). The purification strategy employed was salt fractionation followed by two anion exchange chromatography steps, one using the Mono Q column on the FPLC and the other using an HPLC DEAE column, and finally an HPLC hydroxyapatite column chromatography step. The hydroxyapatite column completely resolved the two transferase enzymes when eluted with a linear gradient of phosphate buffer. The ion exchange chromatography steps only partially separated the two enzymes. Although the mode of action of the hydroxyapatite is not clearly understood, the surface of the matrix, which is composed of a gelatinous form of calcium phosphate, is made up of charged ions and so electrostatic attraction must play an important factor in the reversible binding of proteins.

The purification scheme for *Leu. mesenteroides* (*cremoris*) was based on a short and efficient route for isolating both types of transferase enzymes, if present, and to obtain reasonably high yields of the purified enzyme. Therefore, affinity chromatography was utilised efficiently to purify the transferase enzyme from *Leu. mesenteroides* (*cremoris*).

3.2 Materials and Methods

3.2.1 Reagents

All compounds were obtained from Sigma, BDH or Aldrich. CNBr-activated Sepharose 4B was purchased from Pharmacia and DE-52 cellulose from Whatman.

3.2.2 Determination of optimal *N*-deoxyribosyltransferase activity during the growth cycle of *Leuconostoc mesenteroides* (*cremoris*)

Stages in the growth cycle of *Leu. mesenteroides* (*cremoris*) NCIMB 12008 were first established. 100 ml of MRS broth was sterilised by autoclaving at 121°C for 15 min. The medium was inoculated with a 1% 16 h culture grown in the same medium, and incubated at 30°C without any aeration. Samples were taken at intervals for optical density measurements at 600 nm. A plot of log OD_{600} readings against time showed the different stages in the growth cycle of the bacteria (Figure 3.2).

Once the growth cycle was established a 4 l culture of the bacteria was initiated as described above. 650 ml samples were taken out aseptically after 3, 6, 10, 24, 36 and 48 h. The cells were harvested by centrifugation (10 000 *g*) and washed (x2) with 0.02 M Pipes-NaOH buffer (pH 6.5). The cells were resuspended in the minimum amount of the same buffer in order to obtain a suspension of cells. The cells were broken by passing the suspension through a French pressure cell at 20 000 psi (x3), keeping the temperature below 10°C. The supernatant was separated from the cell debris by centrifugation (10 000 *g*) and dialysed overnight against 100

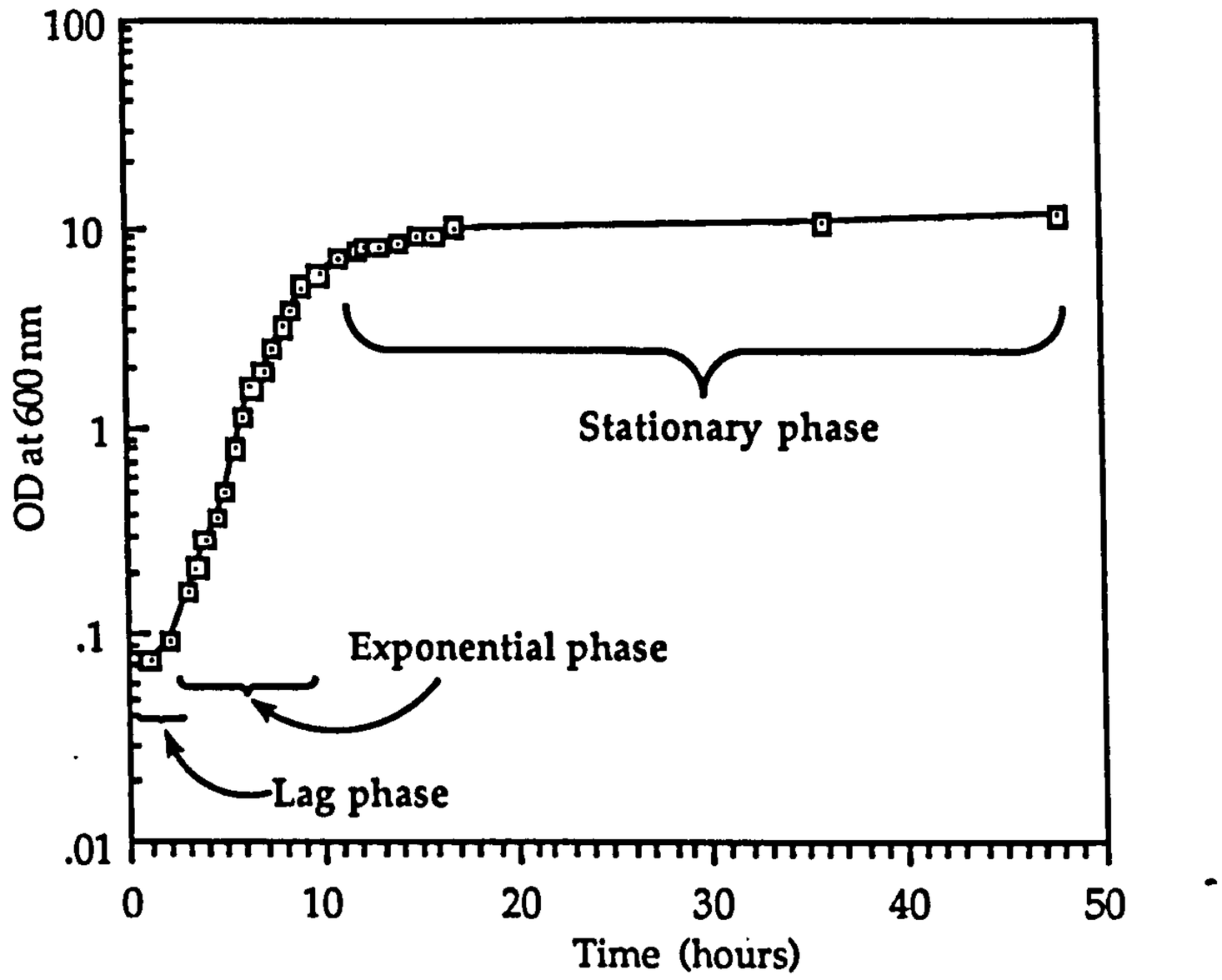


Figure 3.2 Stages in the growth cycle of *Leu. mesenteroides* (*cremoris*)

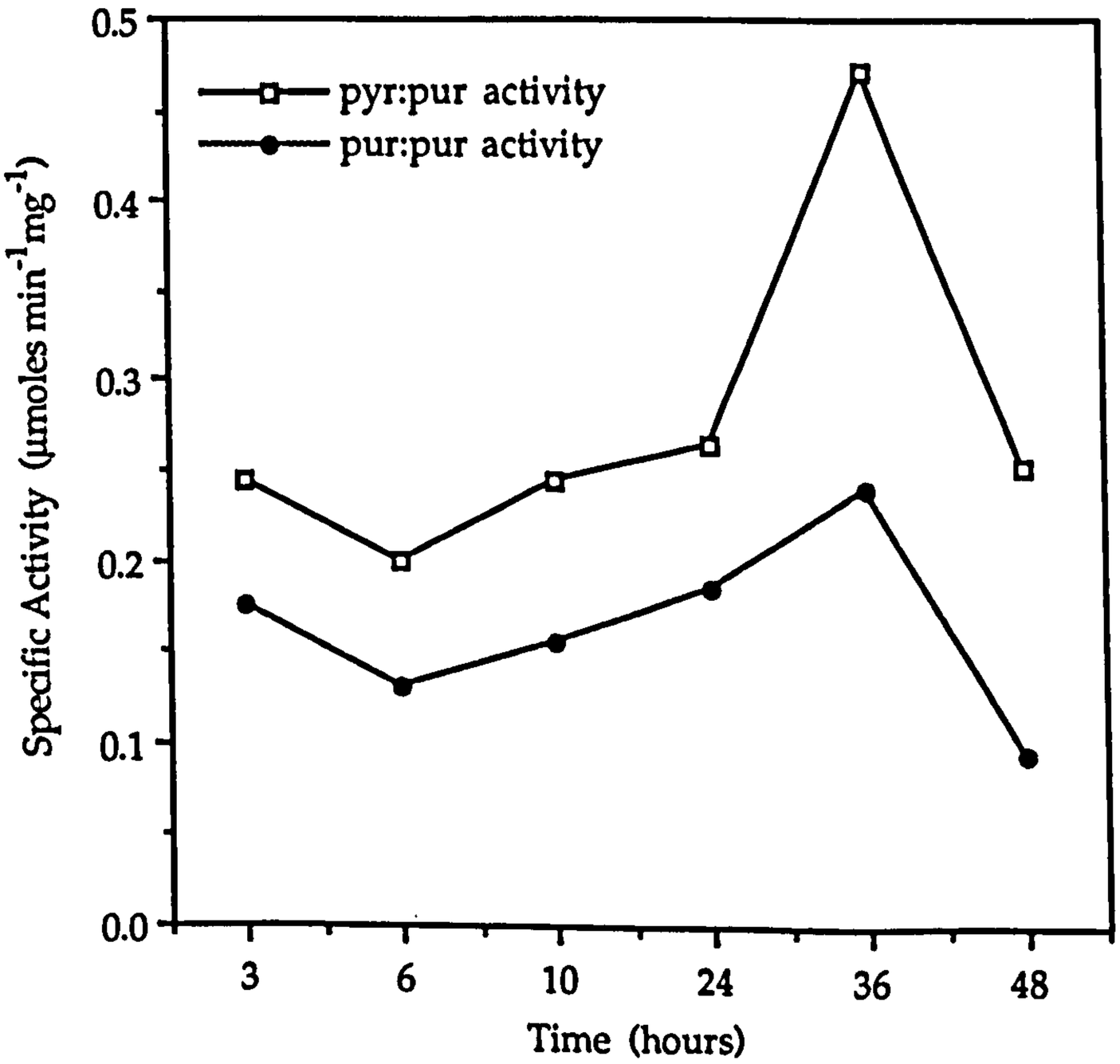


Figure 3.3 Levels of *N*-deoxyribosyltransferase activity during the growth cycle of *Leu. mesenteroides* (*cremoris*)

volumes of 0.02M Pipes-NaOH buffer (pH 6.5) containing NaN_3 (0.05% w/v) at 4°C, with a change of buffer after about 5 h.

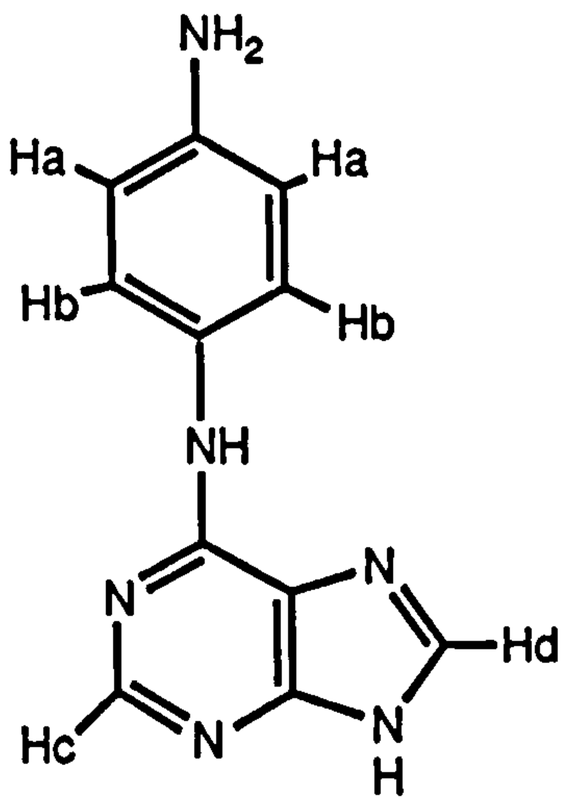
Protein determinations were carried out as described in Chapter 2. The cell-free extracts were measured for transferase activities, pyr:pur and pur:pur, using the standard enzyme assays described in section 2.2.3.1 in Chapter 2. A plot of specific activity against time determined the stage in the growth cycle of the bacteria which contained maximal transferase activity (Figure 3.3.).

3.2.3 Preparation of a ligand for an affinity column

Holguin and Cardinaud (1975) and Huang and co-workers (1981) have reported on the preparation of several affinity ligands for the purification of *L. helveticus*. A simpler and effective affinity ligand can be prepared by the following procedure.

6-Chloropurine (1.3 g, 8 mmoles), phenylenediamine (1.75 g, 16 mmoles) and sodium carbonate (0.85 g, 8 mmoles) were suspended in *N,N*-dimethylformamide (13 ml) and stirred at 60°C. Heating was continued and samples were removed at intervals to examine the progress of the reaction by tlc on silica plates which were developed with dichloromethane-methanol (9:1). The reaction was stopped after 24 h and the solvent removed under reduced pressure on a rotary evaporator. A portion of the residue (0.7 g) was redissolved in the minimum amount of dichloromethane-methanol (9:1) solvent. The desired product, 6-(4'-aminophenylamino)purine **(26)**, was purified from this crude mixture by flash chromatography using silica gel (70 g) and eluted with dichloromethane-methanol (9:1). 25 ml fractions were collected and those

containing a compound with an R_f of 0.1, in the tlc system described above, were pooled and the solvent removed under vacuum on a rotary evaporator. A dark purple crystalline product with a yield of 0.141 mg (0.6 mmoles, 8%) was obtained. The purified material was identified by ^1H NMR to be 6-(4'-aminophenylamino)purine. ^1H NMR at 400 MHz in CD_3OD with the chemical shifts in ppm: 6.80, 6.83 (2H, d, J_{ortho} 8.8 Hz, Ha); 7.44, 7.46 (2H, d, J_{ortho} 8.8 Hz, Hb); 8.17 (1H, s, H_d); 8.29 (1H, s, H_c).



(26)

The affinity ligand (26) was coupled to CNBr-activated Sepharose 4B. Freeze-dried CNBr-activated Sepharose beads (2 g) were swollen for 15 min with 1 mM HCl on a sintered glass filter (porosity 3) and then washed with 1 mM HCl (400 ml), added in several aliquots with the supernatant being removed between successive additions. HCl preserves the activity of the reactive groups which hydrolyse at alkaline conditions. The gel was then washed with 0.1 M $NaHCO_3$ buffer (10 ml, pH 8.3) and immediately transferred to a solution of the ligand (140 mg) in 0.1 M $NaHCO_3$ buffer (14 ml, pH 8.3). A gel to buffer ratio of 1:2 gave a suitable suspension for coupling. The mixture containing the ligand and swollen gel were left to rotate end-over-end overnight at 4°C.

Unreacted active groups remaining on the gel after coupling were blocked by adding an excess of 1 M ethanolamine in 0.1 M $NaHCO_3$ buffer (pH 8.3) and leaving the mixture for 2 h at room temperature. The blocking agent and any uncoupled ligand were washed away with the coupling buffer (0.1 M $NaHCO_3$ buffer, pH 8.3). The ligand coupled to CNBr-activated Sepharose 4B was stored at 4°C in 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.05% (w/v) NaN_3.

3.2.4 Definition of Unit and Specific Activity

A unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of 2'-deoxyadenosine in the case of pyr:pur and pur:pur activities, or 2'-deoxyuridine in the case of pyr:pyr activity in 1 min at 40°C. Specific activity was expressed as units per mg protein.

3.2.5 *N*-deoxyribosyltransferase assays

N-deoxyribosyltransferase activities were measured routinely by the method described in section 2.2.3.1 in Chapter 2. Protein concentrations were determined by the method of Bradford (1976) using the BioRad protein dye and bovine serum albumin as a standard.

3.2.6 Cultivation of bacteria

Leu. mesenteroides (*cremoris*) NCIMB 12008 was grown in MRS medium (DeMan-Rogosa-Sharpe, 1960) with a slight modification. 20 l of MRS broth was made up to half the concentration (26 g/l) in a 25 l container and the pH was adjusted to 6.7 with 10 M NaOH solution. A large magnetic stirring bead was added and the opening of the container was loosely sealed with a cotton wool plug and foil, this was then autoclaved at 121°C

for 50 min. 50% solutions of glucose (200 g/400 ml) and of peptone (100 g/200 ml) were prepared in separate containers and sterilised at 121°C for 15 min. The glucose and peptone solutions were added aseptically to the 20 l container of MRS broth and incubated at 30°C with gentle stirring.

The medium was inoculated with 1% of a 16 h culture of the bacteria, grown in the same medium and incubated at 30°C with gentle stirring without aeration. The cells were harvested after 36 h, which was the optimal time for maximal transferase activity determined from experiment 3.2.3. The cells were concentrated in a continuous Westfalia centrifuge (Westfalia Separator Ltd., Milton Keynes) and then centrifuged (17 700 *g*) for 15 min. All subsequent operations were carried out at 4°C except where otherwise indicated. The cells were washed twice with 0.02 M Pipes-NaOH buffer (pH 6.5) and the pellet stored at -20°C if not used immediately.

3.3 Results of the Purification procedure

3.3.1 Preparation of cell-free extract

The cell paste (125 g wet weight) was thawed and resuspended in 0.02 M Pipes-NaOH buffer (pH 6.5) to give a final concentration of 1 g/ml. Cell degrading enzymes, lysozyme (5 mg/ml) and mutanolysin (50 U/ml) were added stepwise to the cell suspension. The mixture was incubated at 30°C for 2 h and protoplast formation was followed by light microscopy.

The "softened" cells were then disrupted by passing the suspension through a continuous cell disrupter (Constant Systems, Warwick, UK) at 20 000 psi at 4°C. This process was repeated and cell debris removed by

centrifugation (17 700 *g*) for 30 min. The cell-free extract was dialysed overnight against 100 volumes of 0.02 M Pipes-NaOH buffer (pH 6.5) containing NaN_3 (0.05% w/v), with a change of buffer after 5 h. Protein concentration of the supernatant was determined and then assayed for *N*-deoxyribosyltransferase activities.

3.3.2 DEAE-cellulose anion exchange chromatography

A column of DEAE-cellulose DE 52 (80 ml, 2.7 x 17 cm) was washed and equilibrated with 0.02 M Pipes-NaOH buffer (pH 7) at 4°C. The cell-free extract (1812 mg, 155 ml) was dialysed overnight against 100 volumes of 0.02 M Pipes-NaOH buffer (pH 7) containing NaN_3 (0.05% w/v) with a change of buffer after 5 h, after which the protein concentration was determined.

Dialysed, cell-free extracts were loaded onto the equilibrated anion exchange column at a flow rate of 1 ml/min, in batches not exceeding more than 1 g of total protein. The column was washed with 1 bed volume of 0.02 M Pipes-NaOH buffer (pH 7) and elution was performed with a linear gradient of 0-1.0 M NaCl in the same buffer at a flow rate of 1 ml/min for 1000 min. 10 ml eluant fractions were collected and assayed for pyr:pur and pur:pur activities.

Both enzymatic activities appeared between 0.2-0.7 M NaCl (Figure 3.4). The active fractions were pooled and concentrated using a stirred ultrafiltration cell (Amicon Ltd., Stonehouse, Gloucestershire) with a 10K molecular weight cut-off membrane. Protein content and specific rate of *N*-deoxyribosyltransferase activities were determined after dialysis was carried out as described above.

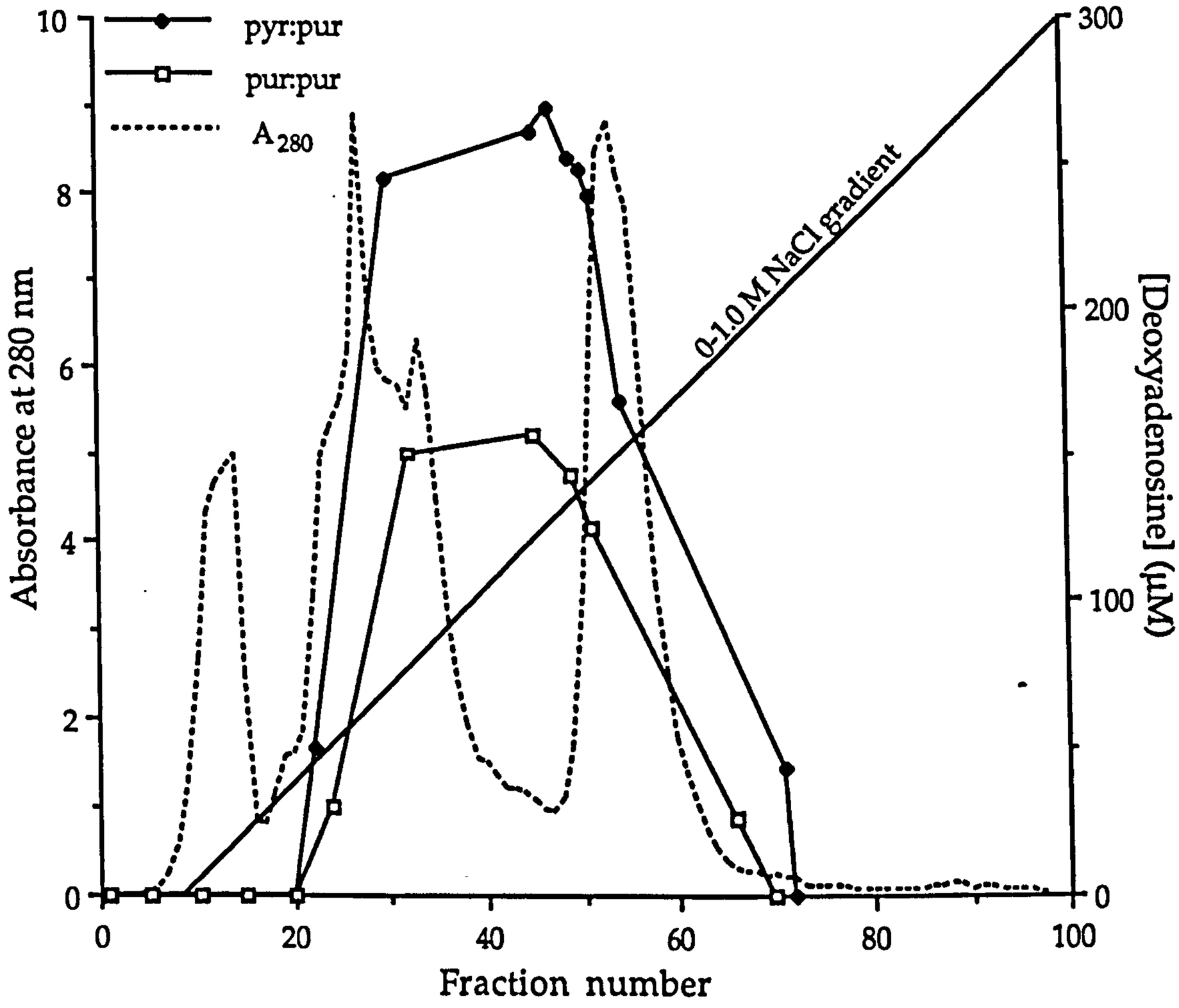


Figure 3.4 Chromatography profile of crude extract of *Leu. mesenteoides* (*cremoris*) with DEAE-cellulose (DE-52)

Reaction mixtures contained deoxycytidine (2 μmol) and adenine (2 μmol) for pyr:pur and deoxyinosine (2 μmol) and adenine (2 μmol) for pur:pur with Pipes-NaOH buffer (pH 6.5, 50 μmol) and enzyme extract (100 ml) in a final volume of 0.5 ml. The reaction mixtures were incubated for 60 min at 40°C and then assayed by HPLC as described in Chapter 1.

3.3.3 Fast Protein Liquid Chromatography (FPLC)

Mono Q anion exchange chromatography

The dialysed and concentrated material from the DEAE column (819 mg, 233 ml) was next applied in batches, not exceeding more than 150 mg, to a high resolution Mono Q (Pharmacia, Uppsala, Sweden) anion exchange column (1 x 10 cm), which had been previously equilibriated with 0.02 M Pipes-NaOH buffer (pH 7). A linear gradient of elution from 0.4-0.8 M NaCl in the same buffer was applied to the column at a flow rate

of 2 ml/min for 55 min. Eluant fractions (4 ml) were collected and immediately stored on ice and later assayed for pyr:pur and pur:pur activities. Both transferase activities eluted between 0.42-0.75 M NaCl (Figure 3.5).

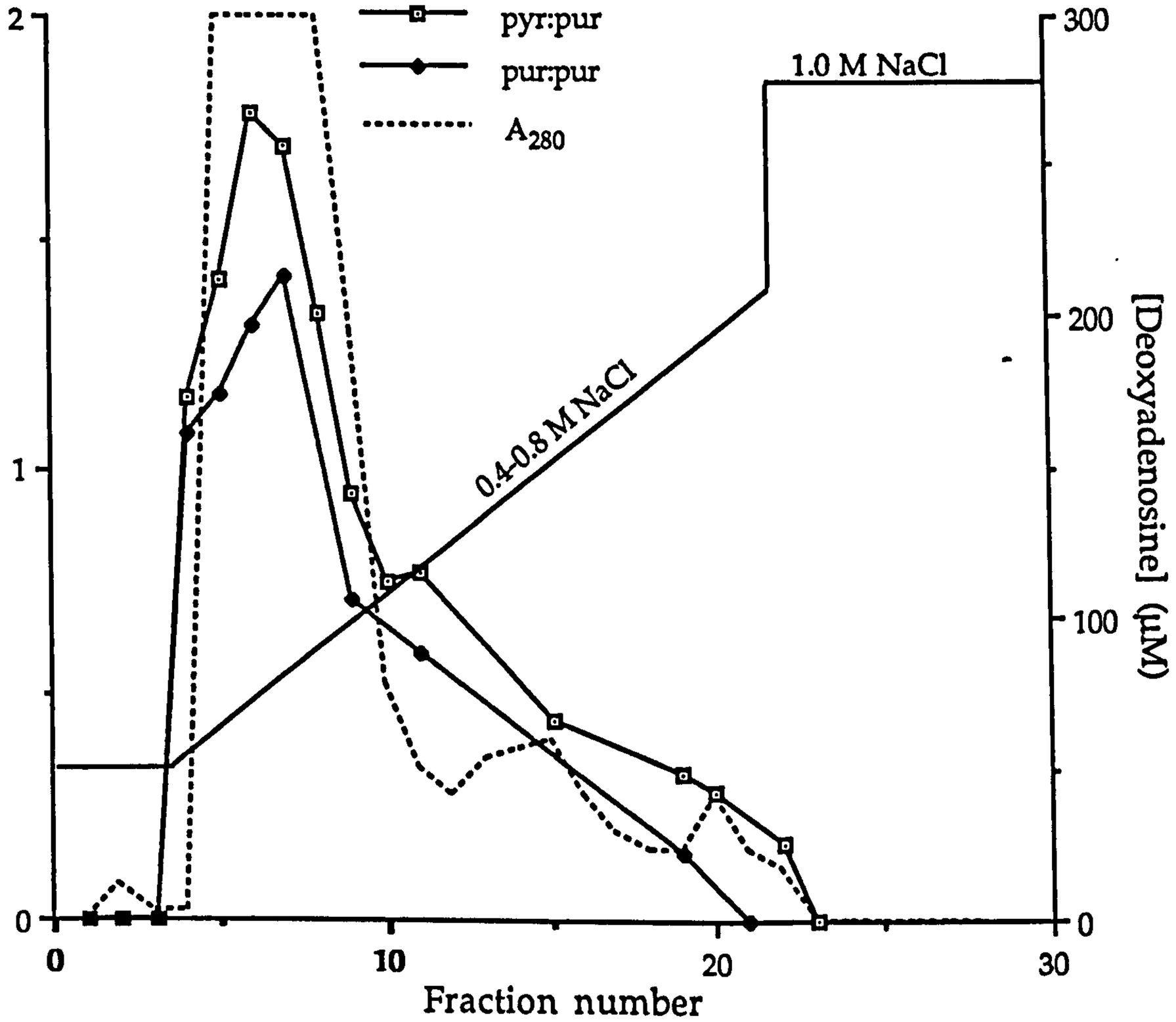


Figure 3.5 Chromatography profile of partially purified extract of *Leu. mesenteroides* (*cremoris*) on Mono Q anion exchange column using the FPLC

Reaction conditions are as described for Figure 3.4.

The active fractions were pooled and concentrated, and then dialysed overnight against 0.02 M Pipes-NaOH buffer (pH 6.5) as described earlier. Protein content and specific rates were also determined as before.

3.3.4 Affinity chromatography

This separation was carried out at 4°C in a cold room. An affinity column (7 ml, 1.7 x 15 cm) of the specific adsorbent of Sepharose 4B beads coupled

to 6-(4'-aminophenylamino)purine was washed with 10 bed volumes of 1 M NaCl in 0.02 M Pipes-NaOH buffer (pH 6.5), and then equilibrated with 0.02 M Pipes-NaOH buffer (pH 6.5) until the OD_{280} of the eluate was constant and/or zero. The dialysed eluant (532 mg, 159 ml) from the Mono Q column was applied to the affinity adsorbent in batches, not loading more than 150 mg of protein at one time. Once the protein was loaded, the column was washed with 3 bed volumes of 0.02 M Pipes-NaOH buffer (pH 6.5) to elute any non-specifically bound protein, and then with 10 bed volumes of 1 M NaCl in 0.02 M Pipes-NaOH buffer (pH 6.5), to elute any ionically bound protein, and then again with 7 bed volumes of 0.02 M Pipes-NaOH buffer (pH 6.5) until the reading at OD_{280} was constant.

The column was then brought up to room temperature (19-20°C) and eluted with 14 bed volumes of 20 mM adenine in 0.02 M Pipes-NaOH buffer (pH 6.5). 10 ml fractions were collected and immediately placed on ice. The column was further washed with 0.02 M Pipes-NaOH buffer (pH 6.5) until the OD_{280} reading was constant. Fractions of 10 ml were assayed for pyr:pur and pur:pur activities. The active fractions were then pooled, concentrated and dialysed against 0.02 M Pipes-NaOH buffer (pH 6.5) as described earlier.

After dialysis the eluant was run down a G25 (Pharmacia, Uppsala, Sweden) FPLC gel filtration column (1 x 11 cm) to remove the remaining adenine ligand from the protein. Protein concentration and specific activities were determined after the eluant from the G25 column had been concentrated using the stirred ultrafiltration cell with a 10K membrane. The purification steps are summarized in Table 3.1.

Step	Volume of fraction (ml)	Protein concentration (mg/ml)	Total protein (mg)	Total activity* (Units)	Specific activity (Units/mg of protein)	Yield* (%)	Purification factor* (fold)
Crude cell-free extract	160	11.3	1812	1536	0.848 (pyr:pur) 0.331 (pur:pur) 0.0015 (pyr:pyr)	"100"	"1.0"
DEAE Anion exchange chromatography	233	3.5	819	827	1.01 (pyr:pur) 0.707 (pur:pur) 0.0025 (pyr:pyr)	53.8	1.2
Mono Q Anion exchange chromatography	159	3.3	532	638	1.2 (pyr:pur) 0.923 (pur:pur) 0.0032 (pyr:pyr)	41.5	1.4
Affinity chromatography	5.5	0.19	1.06	631	596 (pyr:pur) 121.0 (pur:pur) 0.441 (pyr:pyr)	41	702

* Values refer to the pyr:pur (dC:A) transfer.

Note : pur:pur represents dI:A transfer and pyr:pyr represents dT:Ur transfer.

Table 3.1 Purification of nucleoside *N*-deoxyribosyltransferase from *Leuconostoc mesenteroides* (*cremoris*)

3.3.5 Enzyme purity

Native-PAGE

The purity of the multi-functional *N*-deoxyribosyltransferase enzyme from *Leu. mesenteroides* (*cremoris*) was checked by carrying out native polyacrylamide electrophoresis (native-PAGE) using the Phastsystem (Pharmacia, Uppsala, Sweden).

370 ng of concentrated sample from the affinity chromatography step was loaded onto a polyacrylamide PhastGel with gradient 8-25, together with 167 ng of high molecular weight markers (Pharmacia HMW calibration kit proteins) containing a mixture of proteins in the molecular weight range of 67 000-669 000. The gel was run using native agarose buffer strips and stained with Coomassie blue (Neuhoff *et al.*, 1985) according to the method set out by Pharmacia. The purity of the enzyme was confirmed by the appearance of a single band on the gel (Figure 3.6).

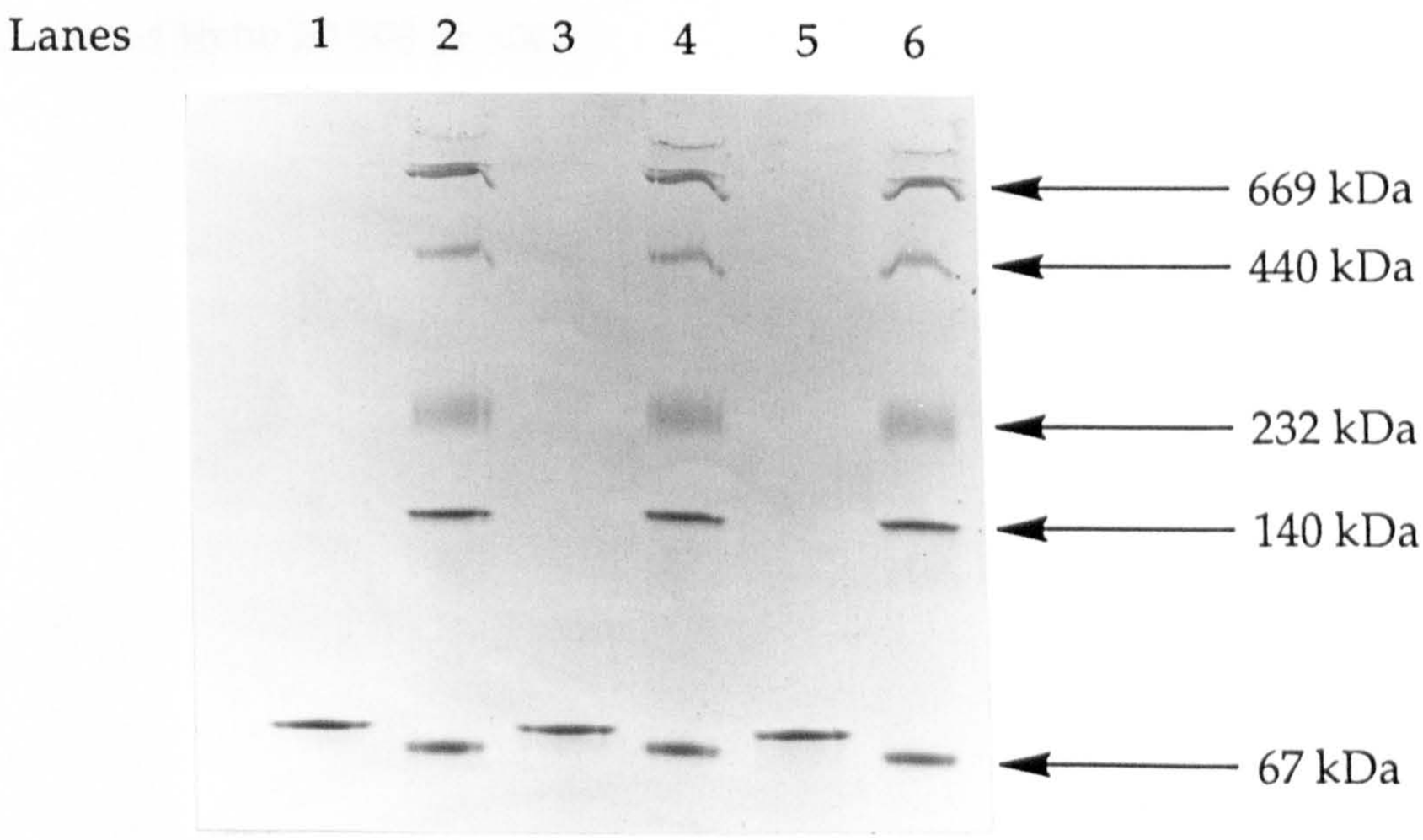


Figure 3.6 Native gradient gel (8-25) with high molecular weight protein markers (lanes 2, 4 & 6) and *N*-deoxyribosyltransferase (lanes 1, 3 & 5). Gel stained with Coomassie blue.

SDS-PAGE

The molecular weight of the subunit composition of the purified enzyme was also determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Phastsystem.

A portion of the enzyme was heated for 5 min at 100°C with 2.5% sodium dodecyl sulphate and 5% b-mercaptoethanol. Bromophenol blue was added to approximately 0.01% to the cooled mixture. 259 ng of the denatured enzyme was loaded onto a PhastGel gradient 10-15 with SDS low molecular weight marker proteins, covering the molecular weight range of 14 000-94 000. The gel was run using SDS agarose buffer strips and stained with Coomassie blue (Neuhoff *et al.*, 1985) according to the method set out by Pharmacia. The SDS gel (Figure 3.7) showed the appearance of a single band. The molecular weight of the subunit composition of the purified enzyme was calculated from the plot of molecular weight of the protein markers against the relative mobilities (R_f) of the protein markers (Figure 3.8). The subunit molecular mass was calculated to be 20 500 (± 500).

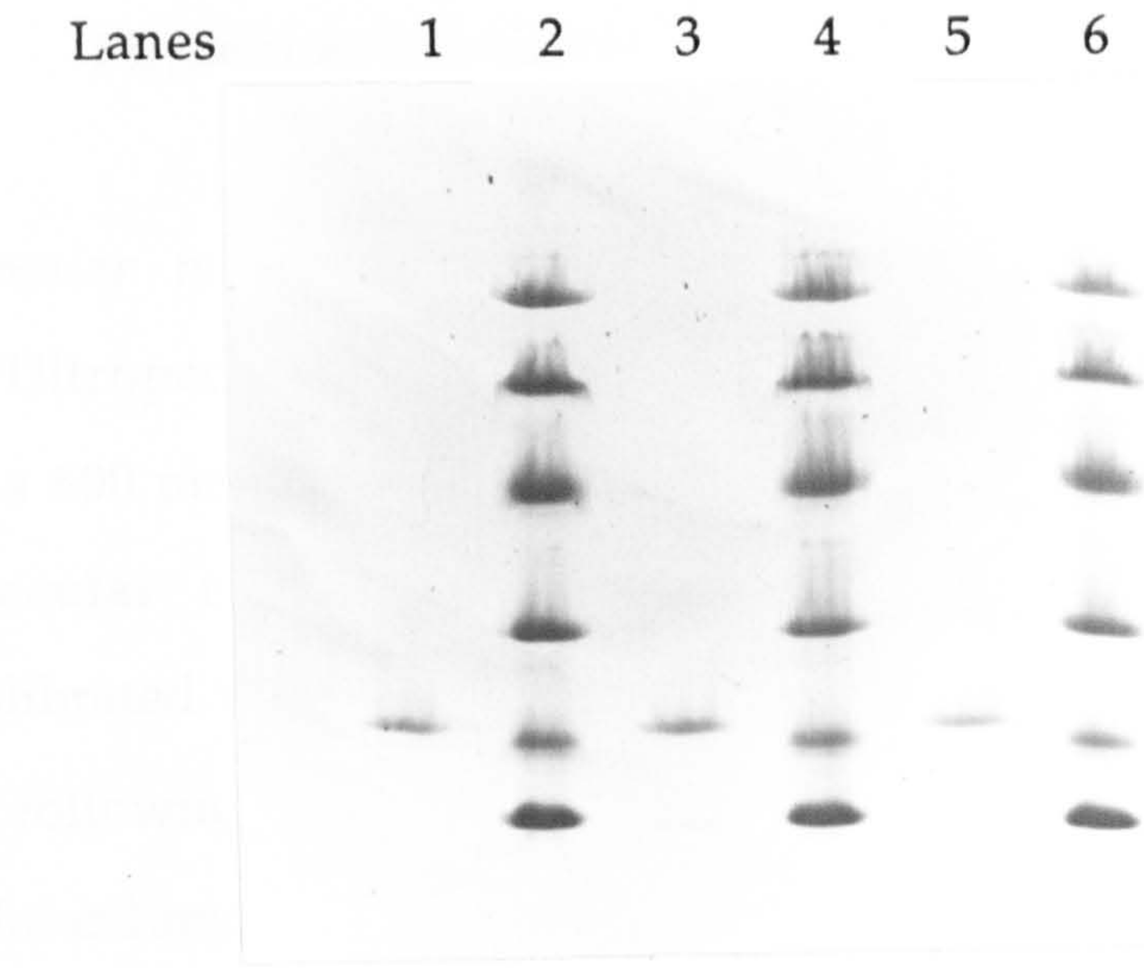


Figure 3.7 SDS gradient gel (10-15) with low molecular weight protein markers (lanes 2, 4 & 6) and *N*-deoxyribosyltransferase (lanes 1, 3 & 5). Gel stained with Coomassie blue.

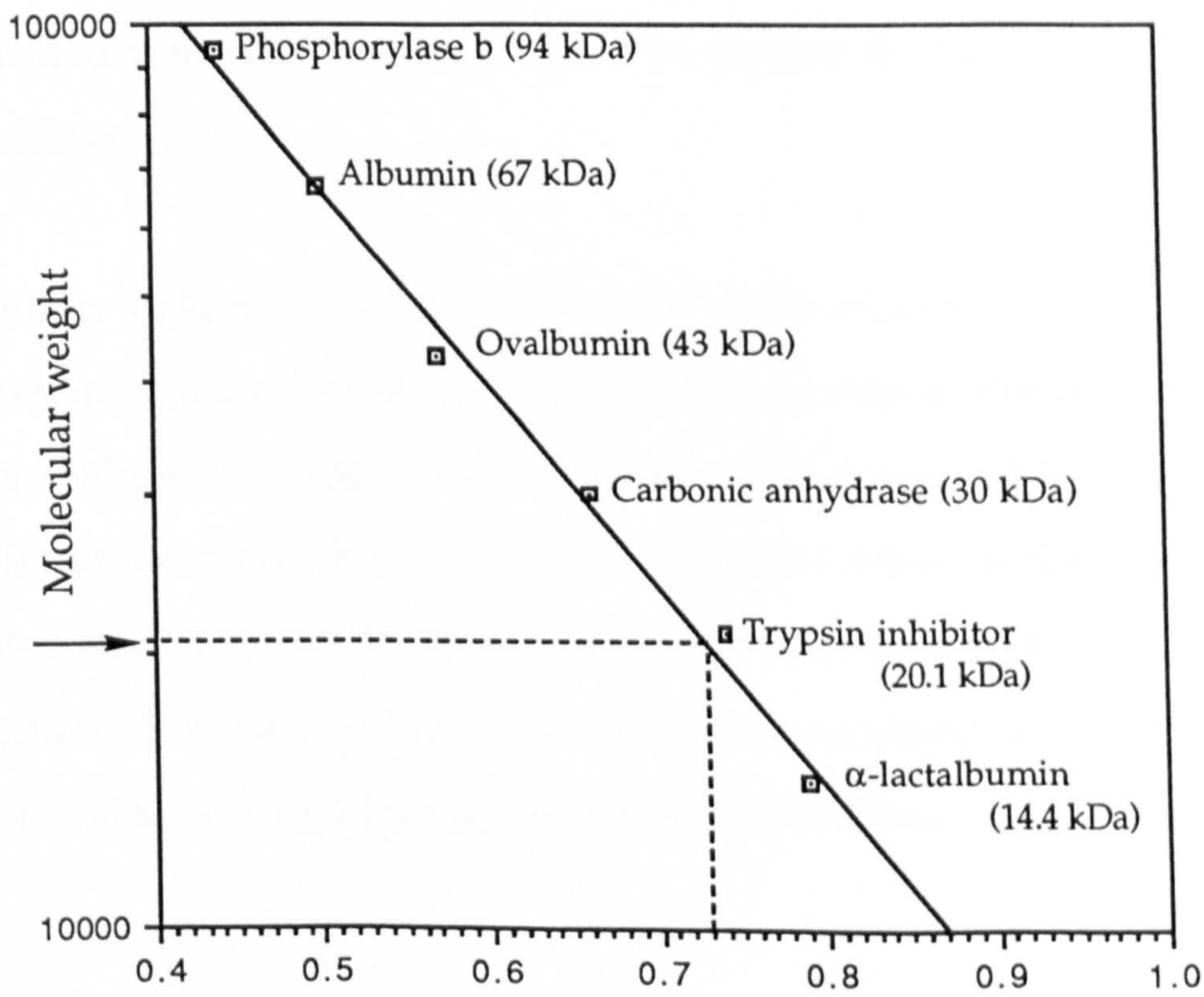


Figure 3.8 Subunit molecular weight determination of *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*), calculated from the above SDS-PAGE gel. Arrow marks the position of *N*-deoxyribosyltransferase

3.3.6 Physical properties

Molecular mass

An Ultropac TSK G4000SWG analytical HPLC gel filtration column (7.5 x 600 mm) from Pharmacia was used to determine the relative molecular mass of the native purified enzyme. The column was equilibrated with 20 mM MES buffer (pH 5.5) at a flow rate of 0.5 ml/min. The following protein calibration markers (66.6 mg, 200 ml) were applied to the column separately at the same flow rate and made up in the same buffer system: blue dextran (Mr 2 000 000), thyroglobulin (Mr 669 000), horse spleen apoferritin (Mr 443 000), sweet potato β-amylase (Mr 200 000), yeast alcohol dehydrogenase (Mr 150 000), bovine serum albumin (Mr 67 000), carbonic anhydrase (Mr 29 000). The same amount of the purified transferase enzyme was also applied to the column at the same conditions.

Elution volume was determined by peak absorbance at 280 nm. Blue dextran was used to determine the void volume of the column. A plot of the molecular masses of the protein markers against V_e/V_o provides a calibration curve from which the molecular mass of the enzyme can be calculated (Figure 3.9), where V_e was the elution volume and V_o the void volume. The native molecular mass was calculated to be 95 000 (± 475). This value was an average of three determinations.

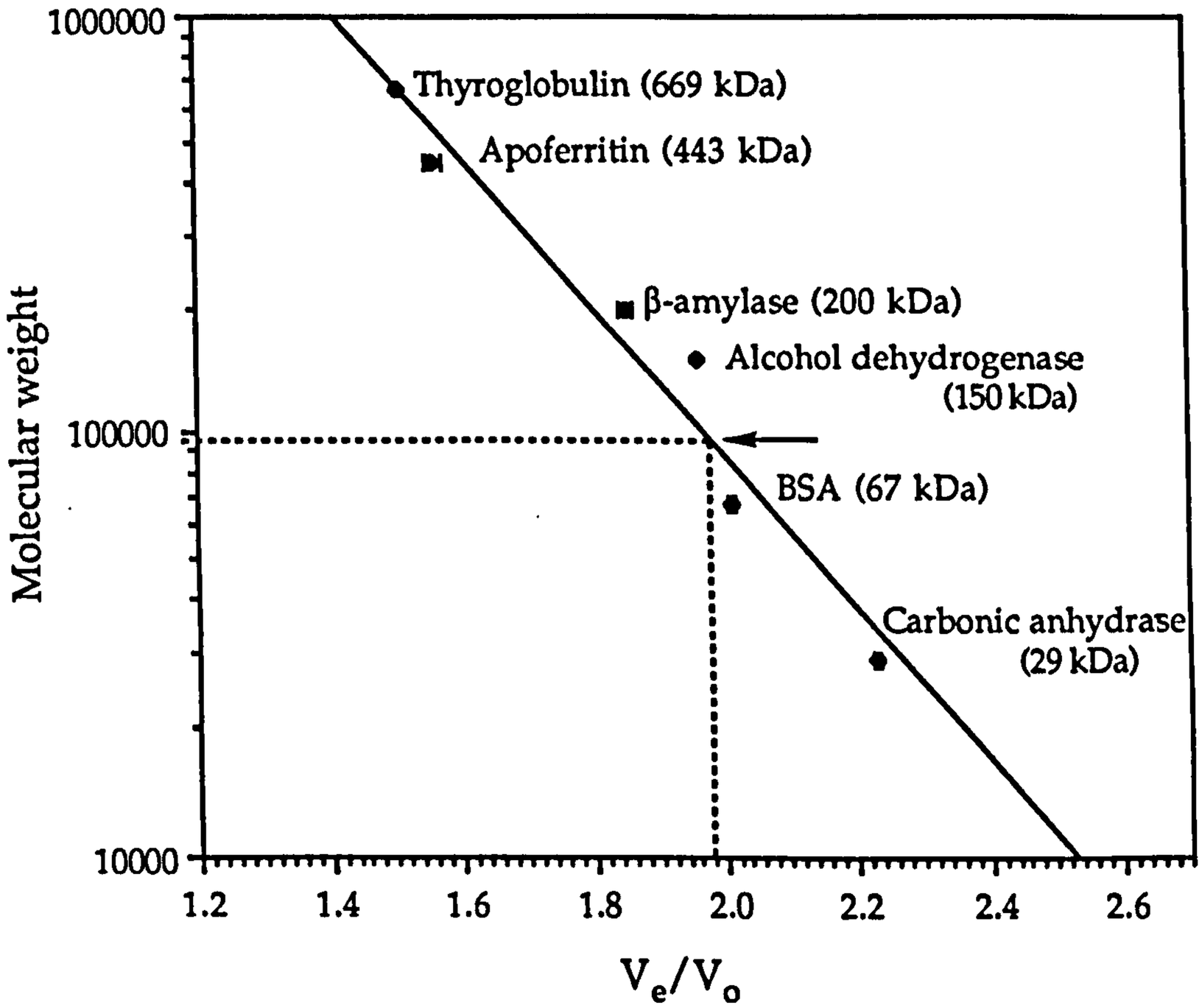


Figure 3.9 Native molecular weight determination of *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*).
Ultropac TSK G4000SWG (7.5 x 600 nm) was equilibrated with 20 mM MES buffer pH 5.5. Elution was at 0.5 ml/min, and elution volume (V_e) was measured by peak absorbance at 280 nm.
Arrow marks the position of *N*-deoxyribosyltransferase.

pH profile

The effect of pH on the activity of the enzyme could was studied by carrying out a pyr:pur reaction under the standard enzyme assay conditions over the pH range 3.5-8.5. 4-Morpholine ethanesulphonic acid (MES) buffer was used for the pH range of 3.5-6.5 and potassium phosphate buffer for the pH range of 6.0-8.5. The pH optimum was found to be in the range of 5.5-6.5 (Figure 3.10).

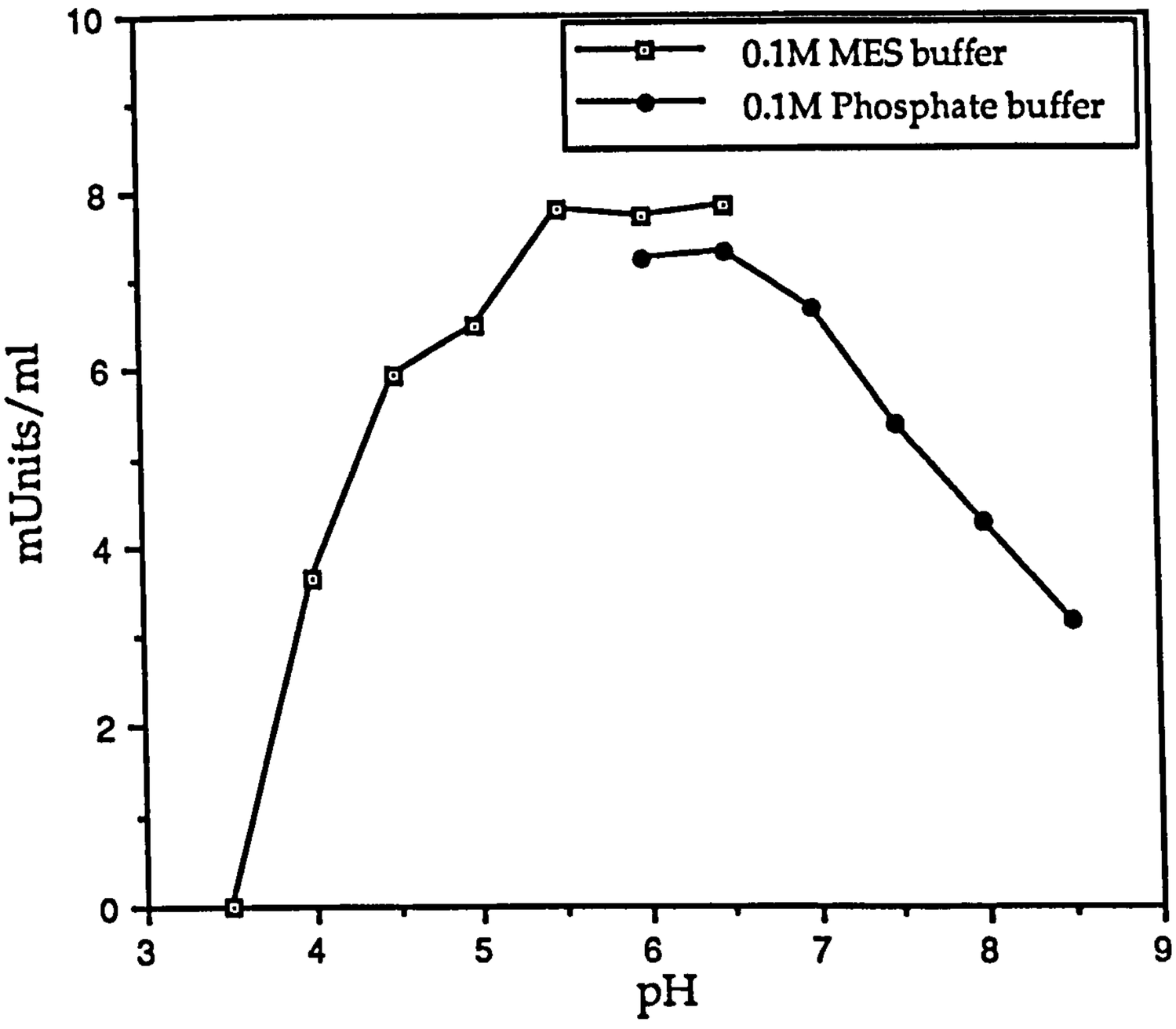


Figure 3.10 Effect of pH on the pyr:pur activity of *N*-deoxyribosyltransferase from *Leu. mesenteroides (cremoris)*

The pyr:pur reaction contained 0.4 µmoles of deoxycytidine and adenine, 100 µmoles of MES or phosphate buffer, initiated with 0.11 units of enzyme in a final volume of 1 ml and incubated at 40°C for 30 min. The products were assayed by HPLC as described in Chapter 2.

Thermal stability profile

The thermal stability of the enzyme was studied by heating the buffered enzyme at various temperatures for 10 min, and then cooling on ice. The mixture was kept on ice until the residual pyr:pur activity could be measured at 30°C for an incubation time of 30 min (Figure 3.11). The transferase enzyme was found to retain more than half its activity between 55°-65°C when incubated at these temperatures for 10 min.

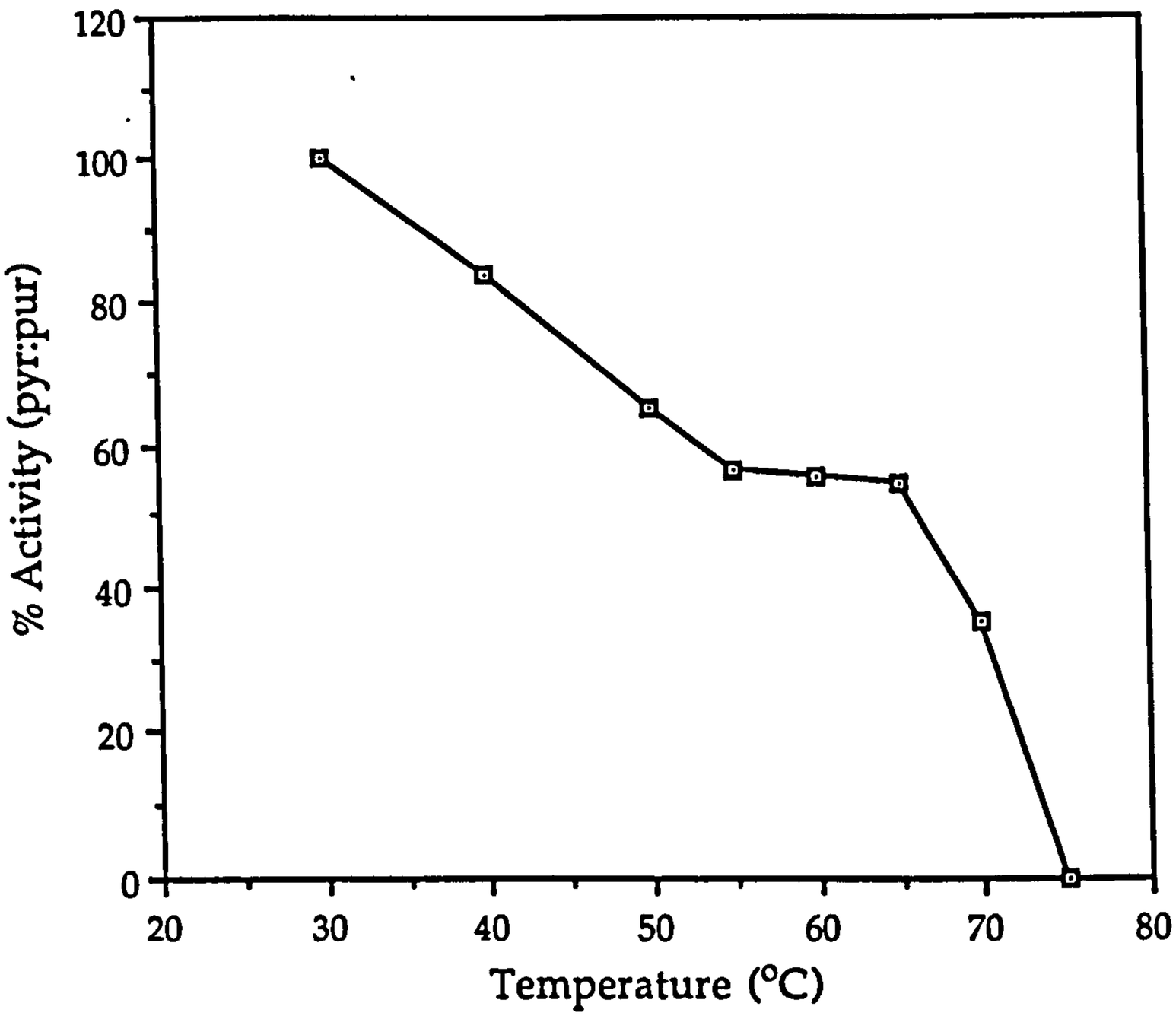


Figure 3.11 Thermal inactivation of *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*).

Purified enzyme (0.1 ml, 0.078 units) was added to 50 µmoles of MES buffer pH 5.5 and held in a water bath at the indicated temperatures for 10 min, cooled and kept on ice until the residual activity was measured. 0.2 µmoles of deoxycytidine and adenine were added to the buffered enzyme mixtures giving a final volume of 0.5 ml and incubated at 30°C for 30 min and the products analysed by HPLC as described in Chapter 2.

Isoelectric focusing

The isoelectric focusing (IEF) method developed by Pharmacia was used on the Phastsystem to determine the isoelectric point (pI) of the transferase enzyme. 1.48 mg of protein was loaded onto an IEF PhastGel (pH 4-6.5) with low pI calibration markers ranging from (2.80-6.55) and stained with Coomassie blue (Figure 3.12). A plot of pI against the relative mobilty (R_f) of the protein markers can be used to determine the pI of the purified transferase (Figure 3.13). The pI was calculated to be 4.4.

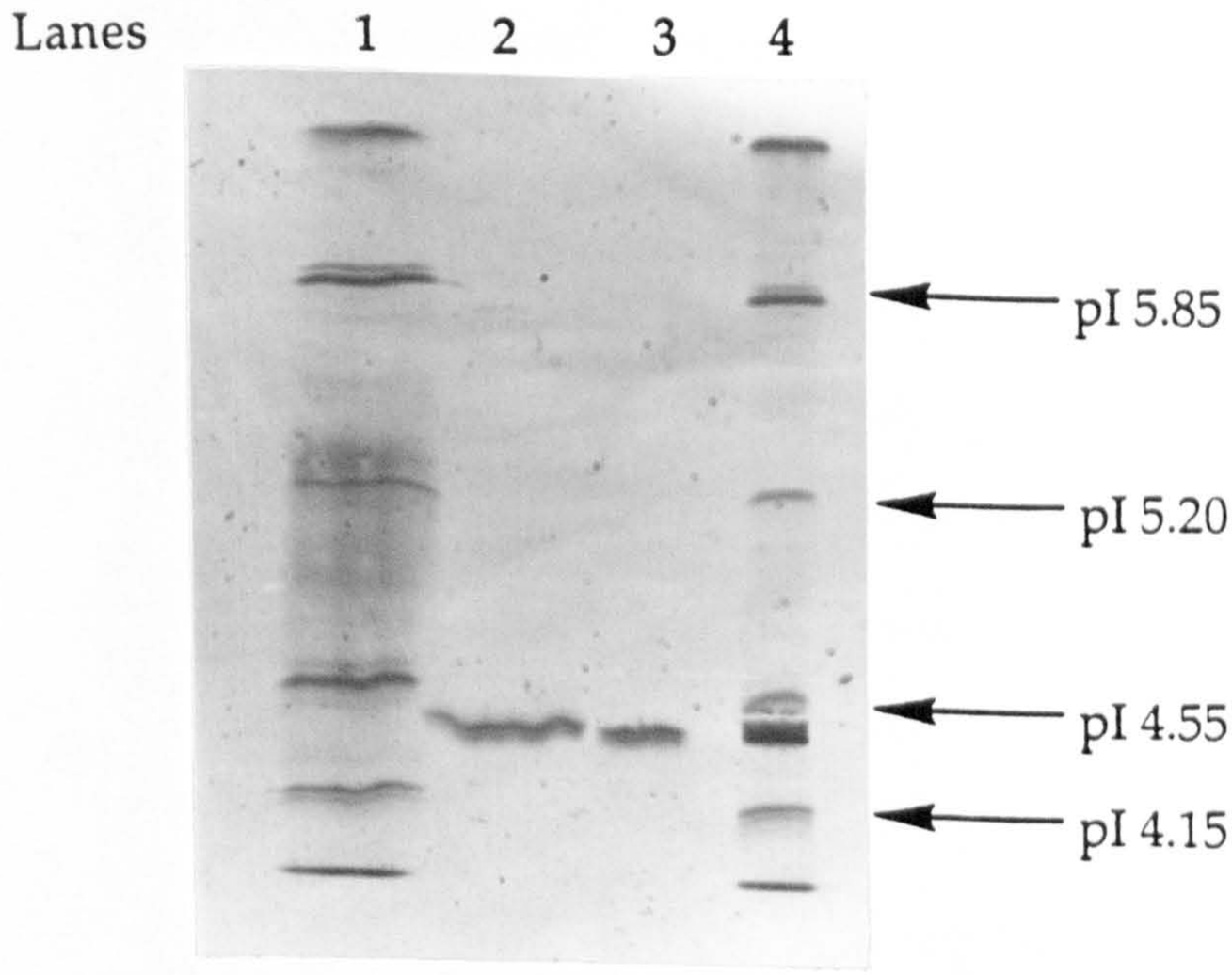


Figure 3.12 IEF gel (pH 4-6.5) with low pI calibration markers (lanes 1 & 4) and *N*-deoxyribosyltransferase (lanes 2 & 3). Gel stained with Coomassie blue.

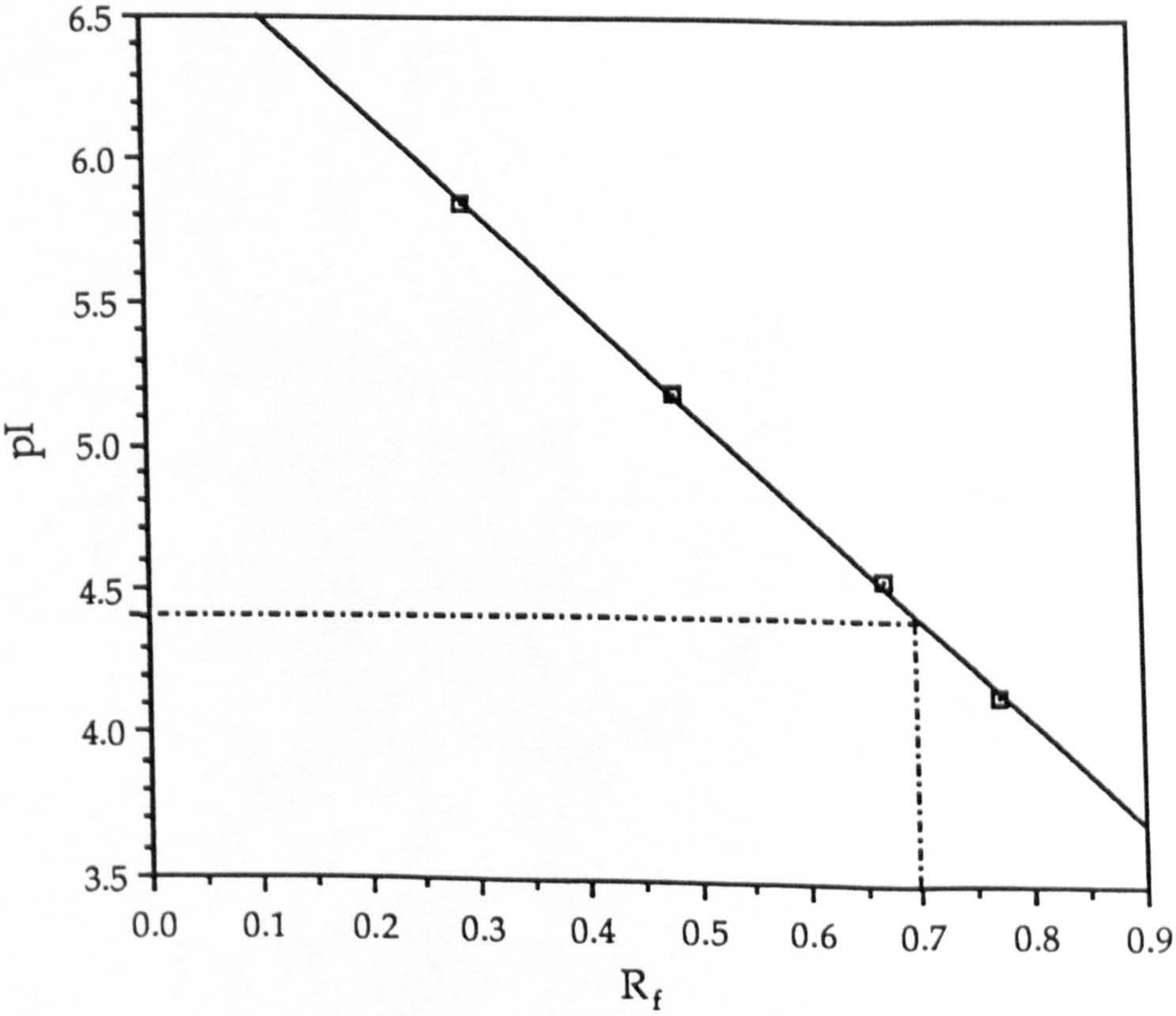


Figure 3.13 Determination of the isoelectric point (pI) of *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*), calculated from the above IEF gel.

3.4 Discussion

A single, multifunctional nucleoside *N*-deoxyribosyltransferase was purified to homogeneity from *Leuconostoc mesenteroides* subspecies *cremoris*. A four-step purification procedure was employed which led to an overall recovery yield of 41% with a 702-fold purification. The strategy used to purify the enzyme was rapid and effective, resulting in good yields of the purified enzyme.

If the specific activities of the three transferase activities of the enzyme are compared during the purification steps (Figure 3.14), it can be seen that the most productive step was the affinity chromatography.

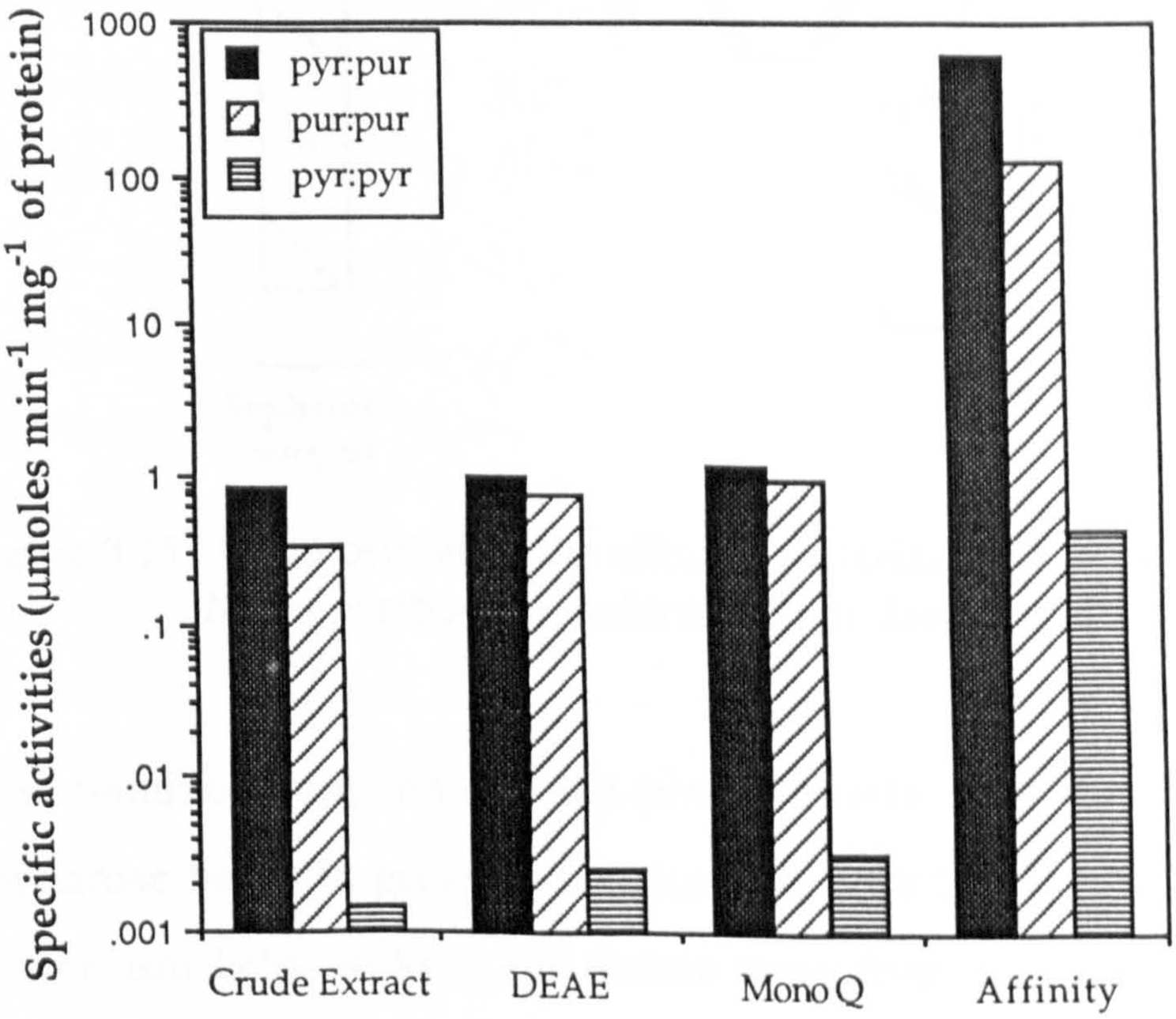


Figure 3.14 Variation in the levels of transferase activities during the purification of *N*-deoxyribosyltransferase from *Leuconostoc mesenteroides* (*cremoris*)

The second anion exchange chromatography step did not yield a significant amount of purification of the enzyme and could probably be left out. A great deal of protein and activity were also lost during the DEAE anion exchange chromatography step (see Table 3.1), hence this step could have been replaced by another procedure such as gel filtration.

Affinity chromatography using Sepharose as the support material and 6-(4'-aminophenylamino)purine as the ligand and spacer arm (Figure 3.15) was a very specific step in the purification strategy.

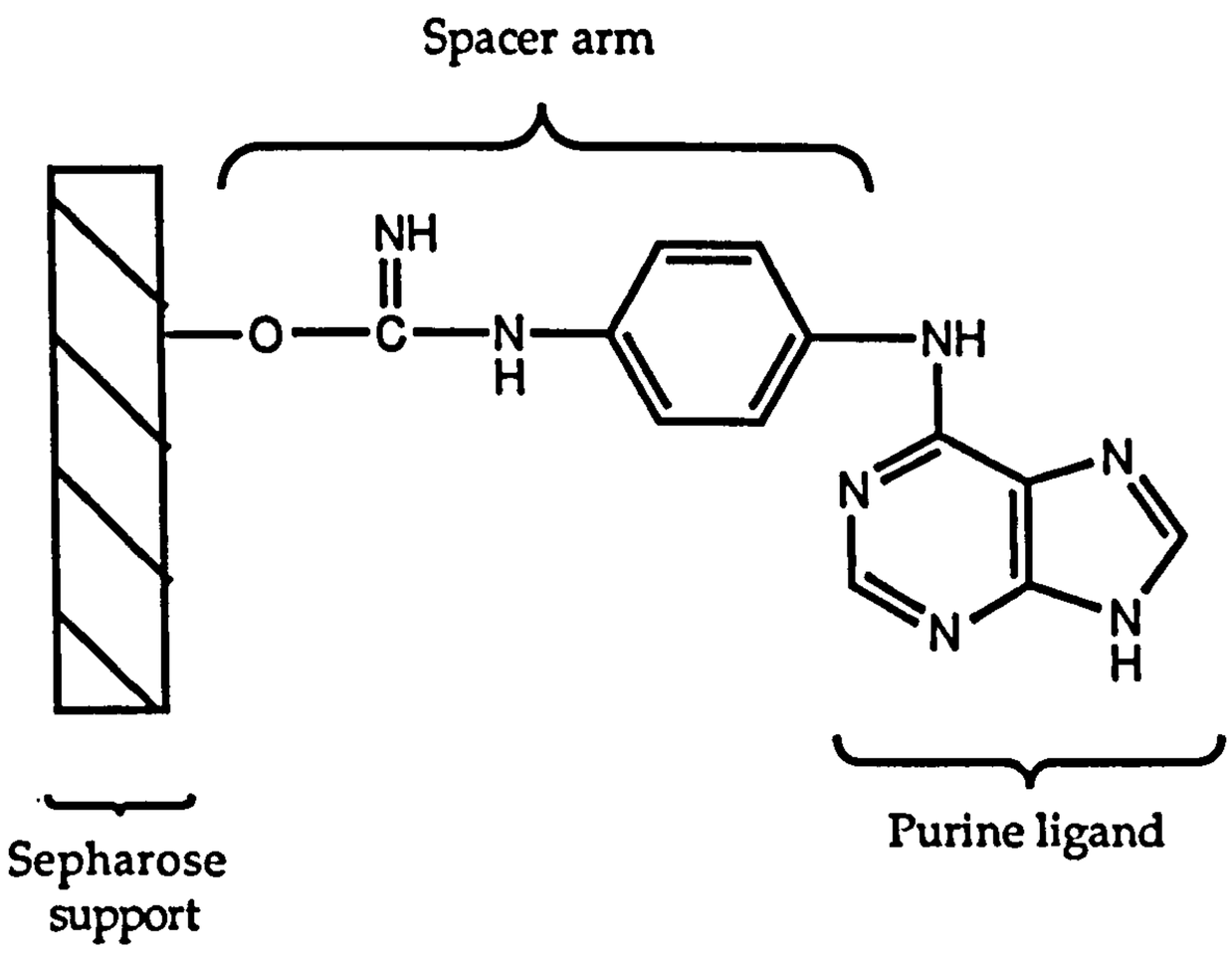


Figure 3.15 Composition of the affinity material used to purify *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*)

The 4-amino group on the the phenyl reacts with the CNBr-activated Sepharose beads to give the structure illustrated in Figure 3.15. The rigid spacer arm helps to keep the matrix away from the ligand and allows a greater accessibility of the ligand to the active site of the enzyme.

Affinity chromatography was used successively by Holguin and Cardinaud (1975) to purify two nucleoside *N*-deoxyribosyltransferases of different

specificities from *L. helveticus*. They used base analogues rather than deoxynucleosides to construct the affinity ligand. The reason for this could be due to the fact that the acceptor bases, used in transfer reactions for kinetic studies, generally have lower K_m values than the donor deoxyribonucleosides (Holguin & Cardinaud, 1975; Cardinaud, 1978), resulting in a fairly strong binding of the enzyme to the ligand. Adenine (a purine analogue) has been shown to be a competitive inhibitor in transfer reactions where it is the acceptor base (Beck & Levin, 1963; Holguin & Cardinaud, 1975). This makes adenine or a derivative of it a suitable compound to use as an affinity ligand.

Hence, a purine base analogue was chosen for the synthesis of the affinity ligand and being a purine derivative it would be suitable for the adsorption of both transferase I and II type activities. Elution of transferase II type activity was achieved by adding 20 mM adenine solution to the column at room temperature, after contaminating proteins had been washed off with high salt concentration. Care had been taken to assay for the presence of a purine-specific enzyme, transferase I, throughout the purification protocol, but no separation of the enzymes was observed. After transferase II type activity had been eluted from the affinity column, 20 mM deoxyinosine was added to the column to see whether a transferase I type activity would elute with deoxyinosine rather than adenine. Deoxyinosine was chosen as the eluting compound because this compound was used by Holguin and Cardinaud (1975) to elute transferase I from *L. leichmannii*. However, the dialysed eluate from the column did not contain any transferase activity. 2 M Urea solution was also added to the column to strip any tightly bound proteins, the dialysed eluate was again assayed for transferase I activity but no activity was detected.

The enzyme ran as a single band on native polyacrylamide gel confirming its purity. The native molecular mass of the enzyme was determined by gel filtration/permeation chromatography and was found to be approximately 95 000 (± 475). Gel filtration chromatography is routinely used to determine protein masses and is based on separating proteins according to their size only, where the proteins elute in the order of the largest to the smallest. There are drawbacks to using gel filtration, especially if the protein is non-globular, as these type of proteins will appear to be larger than globular proteins of the same molecular mass and hence will elute earlier than expected. Essentially a variety of different gel filtration columns should be used as well as other methods, such as ultracentrifugation studies, for determining the molecular mass more accurately.

The oligomeric protein was subjected to SDS polyacrylamide gel electrophoresis to determine the nature and molecular mass of the subunits. The molecular mass of the monomer was calculated to be approximately 20 500 (± 500), whereas Cook and co-workers (1990) determined the subunit molecular mass of the genetically engineered *N*-deoxyribosyltransferase from *L. leichmannii* to be approximately 18 000. This method of determining the molecular weight of the subunits in the presence of SDS is far more accurate than gel filtration because the polypeptide chains generally bind similar amounts of SDS, *eg.* ~1.4 g SDS/1 g polypeptide (Reynolds & Tanford, 1970). At the same time the polypeptide chains become highly negatively charged by the addition of strongly acidic sulphonic groups, resulting in the protein having a relatively constant charge-to-mass ratio. Polypeptide chains saturated with SDS have been shown to migrate upon electrophoresis in polyacrylamide gel according to their molecular weight (Weber & Osborn, 1969).

The presence of a single band on the SDS polyacrylamide gel also suggests that the oligomeric protein is composed of one type of polypeptide chain. It is difficult at present to conclude whether *N*-deoxyribosyltransferase II from *L. mesenteroides (cremoris)* is a tetramer or pentamer because the ratio of the molecular masses of the intact protein to that of the subunit is about 4.6. However, the subunit composition of the native protein could have been calculated by a method, which is becoming increasingly common, involving cross-linking with bifunctional reagents, such as dimethyl suberimidate followed by SDS-PAGE and staining (Davies & Stark, 1970). If the oligomer is composed of identical monomers then cross-linking of the monomers will result in a number of bands on SDS polyacrylamide gel with molecular weights representing integral multiples of the monomer molecular weight up to the molecular weight of the oligomer.

Initial cross-linking experiments were carried out to determine the exact number of monomers in the native transferase enzyme following the method described by Davies and Stark (1970), but the results were disappointing as no cross-linking of the monomers occurred. This could be due to a variety of reasons such as the scarcity of amino groups in the protein to react with the bifunctional reagent (Figure 3.16), or perhaps the bifunctional reagent was not long enough to cross-link the amino groups.

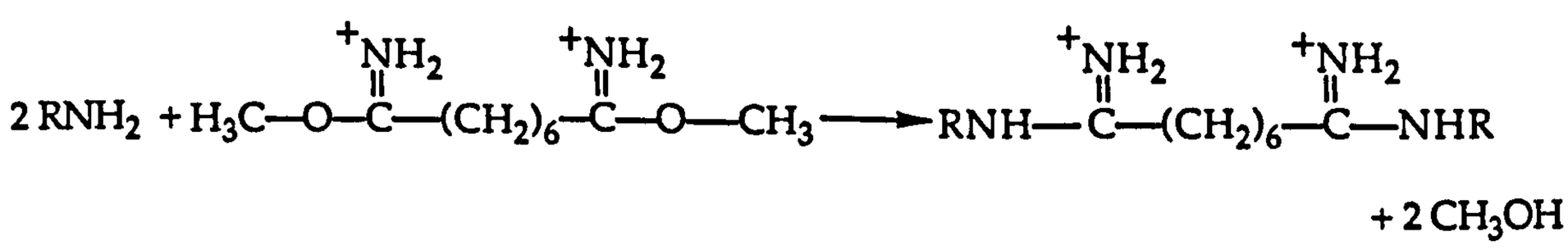


Figure 3.16 Amidination reaction with primary amine groups of proteins

Hence, more experiments would need to be carried out to determine the number of monomers present in the oligomeric protein, by using a variety of bifunctional reagents. The pH of the reaction should also be maintained above 8, as this is an important factor for the amidination of the protein to occur.

The physical properties of the transferase II enzyme from *Leu. mesenteroides (cremoris)* are similar to the ones isolated from the lactobacilli species. The pH optimum was found to be in the range of 5.5-6.5 when using MES buffer. When phosphate buffer was used to measure the pyr:pur activity from pH 6-8.5 and MES buffer was used to measure the same activity between pH 4-6.5, where the two buffers overlapped (pH 6 and 6.5), the activity of the enzyme was reduced by the phosphate buffer (section 3.3.6 and Figure 3.10). The pH optima values of transferase II enzymes from the lactobacilli species were found to be 6.5 from *L. acidophilus* (Marsh & King, 1959); 5.8 (Roush & Betz, 1959) and 6.5 (Holguin & Cardinaud, 1975) from *L. helveticus*; 5.8-5.9 (Beck & Levin, 1963) and 5.6 (Heath, 1991) from *L. leichmannii*, which suggests that the enzymes are very similar and could be catalysed by the same type of amino acid groups in the active site. When the enzyme was subjected to isoelectric focusing a pI value of 4.4 was obtained.

When the thermal stability of the enzyme was studied, there was a rapid decrease in the residual activity from 30°-55°C after which the enzyme appeared stable until 70°C by retaining more than half its activity when compared to 30°C. However Heath (1991) showed that transferase II from *L. leichmannii* retained 50% of the pyr:pur activity at 60°C. The highest temperature before total inactivation of the protein was reached was 75°C, where only 35% of the pyr:pur activity remained.

Since nucleoside *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*) represents about 0.1% of the total cell protein, it would be worthwhile investigating methods of elevating the concentration of enzyme level. This could be achieved by cloning the gene into an over-expressing strain of *E. coli* or by investigating the effect of nutrient limiting conditions on levels of the transferase enzyme. Since lactobacilli and leuconostoc species are found living in the same habitats and utilising the same media, an alternate pathway for DNA synthesis may exist in the leuconostoc species as it does in *L. leichmannii* (Beck & Levin, 1963).

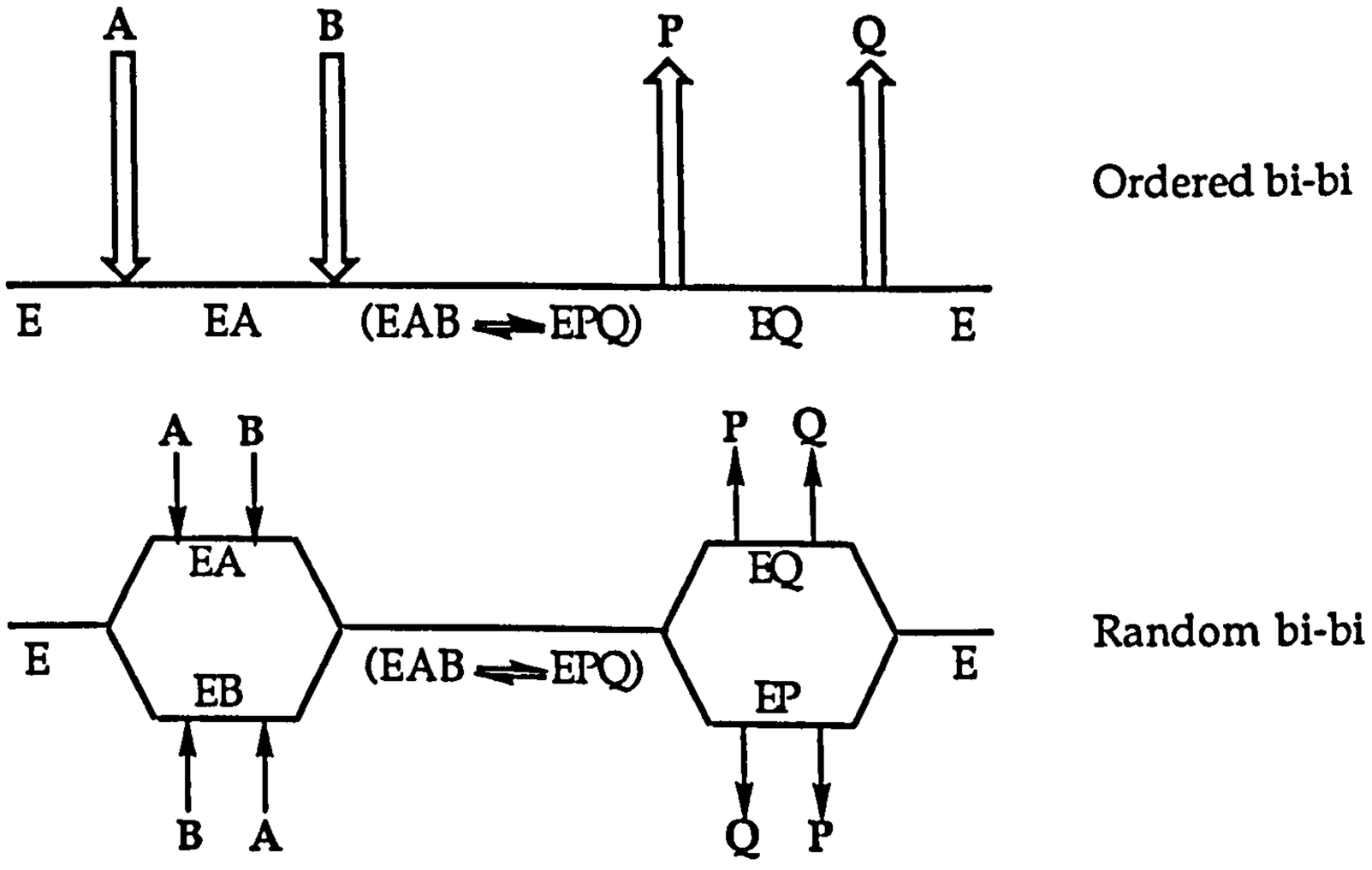
In conclusion the purification scheme reported here allows for an efficient and rapid method for purifying nucleoside *N*-deoxyribosyltransferase II and perhaps *N*-deoxyribosyltransferase I. The use of this purification strategy for purifying *N*-deoxyribosyltransferase I could be confirmed by purifying the transferases from *L. leichmannii*. Although the transferase II enzyme from *Leu. mesenteroides* (*cremoris*) was found to be similar in its physical properties to those isolated from the lactobacilli, only one transferase enzyme was isolated containing all three transfer activities. The molecular mass of these types of transferase enzymes seem to vary depending on the source from which it was isolated. Although the substrate specificity of the enzyme was not studied, the purified enzyme was not able to accept 3'-fluoro-deoxyribonucleosides as was observed with the enzyme from *L. leichmannii*. It is proposed that the enzyme from *Leu. mesenteroides* (*cremoris*) must require that the deoxyribonucleoside ring should be in the same type of planar confirmation during the transfer reaction as required by lactobacilli transferase enzymes (see Chapter 1).

Chapter 4

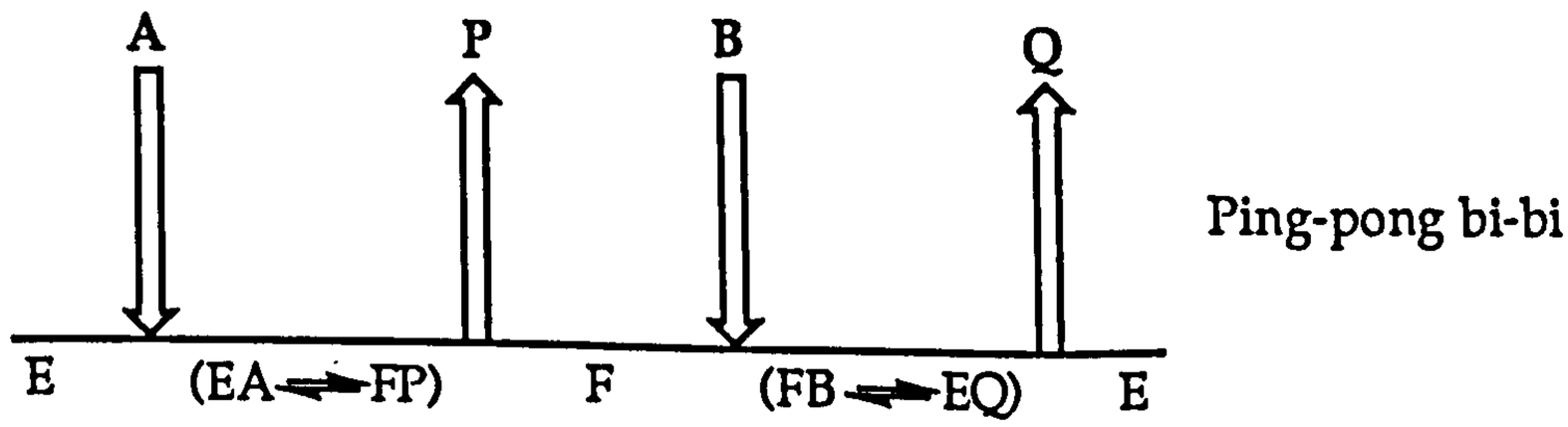
Mechanistic studies on *N*-deoxyribosyltransferase from *Leuconostoc mesenteroides* (*cremoris*)

4.1 General Introduction

Kinetic mechanisms in multisubstrate systems fall into two major groups; **sequential** (Figure 4.1(a)), where all substrates must combine with the enzyme before any products can be released, and **ping-pong** (Figure 4.1(b)), where one or more products are released before all substrates have added (Cleland, 1970).



(a) Two types of sequential mechanisms



(b) Ping-pong mechanism

Figure 4.1 Types of mechanisms present in multisubstrate systems

It can be seen from Figure 4.1 that seqential mechanisms contain only one stable enzyme form, E, whereas in a ping-pong mechanism the enzyme oscillates between two stable forms, E and F. Bi-bi refers to the number of substrates added and products released, in this case two of each.

Cleland (Cleland, 1963a; 1963b;1963c) has laid down certain rules for the characterisation of a ping-pong bi-bi mechanism, such as (a) the generation of parallel lines with double-reciprocal plots for different concentrations of the fixed substrates, (b) when both substrates are present at the same concentration, the reciprocal of the rate against the reciprocal of the substrate concentrations, gives a straight line, and (c) each product is a competitive inhibitor of the corresponding substrate and a non-competitive inhibitor of the other.

Mechanistic studies carried out on *N*-deoxyribosyltransferases from *L. helveticus* (Danzin & Cardinaud, 1974; 1976) and *L. leichmannii* (Heath, 1991) have been confined to kinetic studies employing spectrophotometric and isotopic exchange methods. Double-reciprocal plots of the initial velocity kinetic experiments have always generated parallel lines, implying that a ping-pong bi- bi mechanism was being used by the enzymes. From these kinetic analysis, the same workers postulated that a covalent glycosyl-enzyme intermediate must exist, if the mechanism of action of the enzyme is via ping-pong.

Equation 1 is the rate law for ping-pong kinetics when no competitive inhibition of substrates is observed.

Equation 1

$$v_o = \frac{V[A][B]}{K_b[A] + K_a[B] + [A][B]}$$

where, v_o = initial velocity

[A], [B] = concentration of substrates

K_a, K_b = Michaelis constants

V = maximum velocity in the forward direction

The double-reciprocal or Lineweaver-Burk form of equation 1 would give the following equation.

Equation 2

$$\frac{1}{v_o} = \frac{K_a}{V}\left(\frac{1}{[A]}\right) + \frac{1}{V}\left(1 + \frac{K_b}{[B]}\right)$$

Therefore, plots of $1/v_o$ versus $1/[A]$ at varying fixed concentrations of B would generate sets of parallel lines (Figure 4.2).

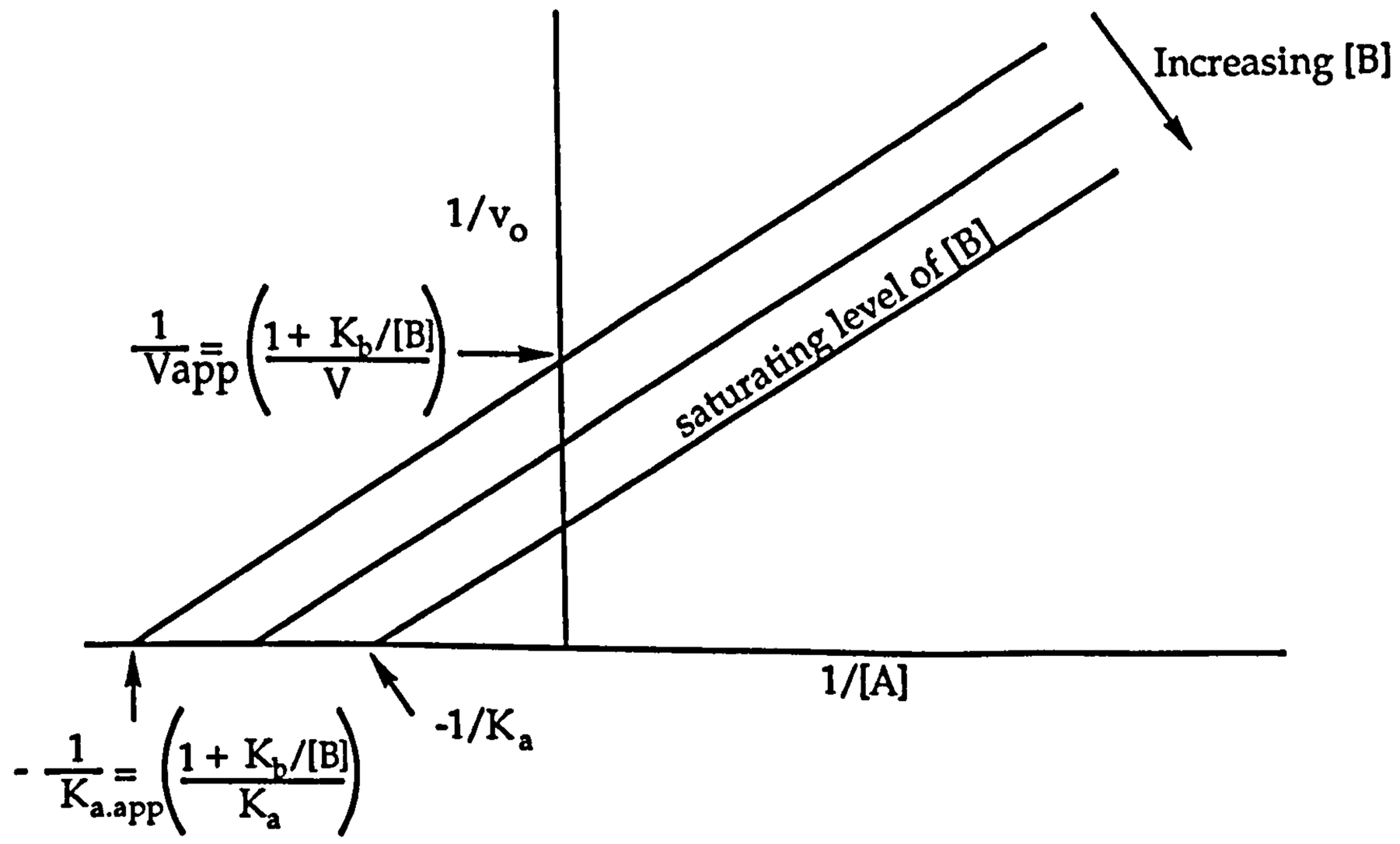


Figure 4.2 Double-reciprocal (Lineweaver-Burk) plot for a ping-pong mechanism

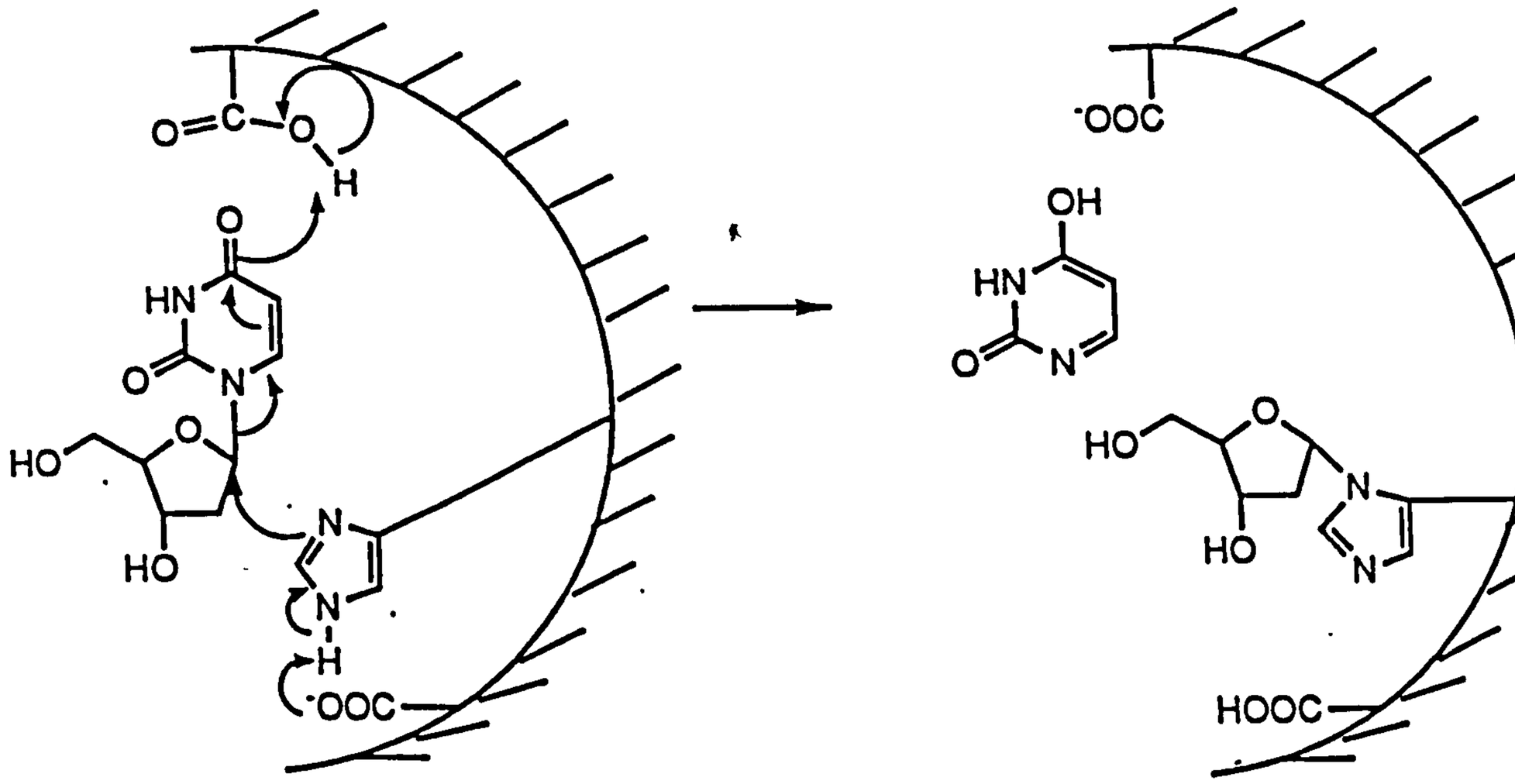
These type of results have also been observed for yeast nucleoside diphosphate kinase (Garces & Cleland, 1969), where the kinetic mechanism of the enzyme is ping-pong. The authors were also able to isolate a stable phosphoenzyme intermediate which forms during the reaction between adenine and uridine nucleotides (Figure 4.3).

$$MgATP + MgUDP \underset{\text{Diphosphate Kinase}}{\overset{\text{Yeast Nucleoside}}{\rightleftharpoons}} MgADP + MgUTP$$

Figure 4.3 Phosphorylation of nucleoside diphosphates by yeast nucleoside diphosphate kinase

Mechanisms on how the substrates react with certain groups in the active site of *N*-deoxyribosyltransferase from *L. leichmannii* have also been proposed (Heath, 1991). When active site modification experiments were conducted, it was found that mainly carboxylic and histidine residues affected the catalytic activity of the enzyme (Figure 4.4). If the mechanism of action is occurring as in Figure 4.4, then a covalent glycosyl-enzyme intermediate should form, which might be stable enough to be isolated.

Heath (1991) also carried out some preliminary experiments to determine the existence of a glycosyl-enzyme intermediate, using (5'-^{3}H)-thymidine. *N*-deoxyribosyltransferase II from *L. leichmannii* and bovine serum albumin, for stability, were incubated with (5'-^{3}H)-thymidine and then co-precipitated in ice-cold trichloroacetic acid (TCA). The resulting precipitate was collected on fibre filters and the excess radiolabelled thymidine was washed off with some more TCA. The precipitate was dried, solubilized and then measured for radioactivity. From her results it could not be proved conclusively that the incorporation of the radioactivity, detected in the protein fraction, was due to the presence of



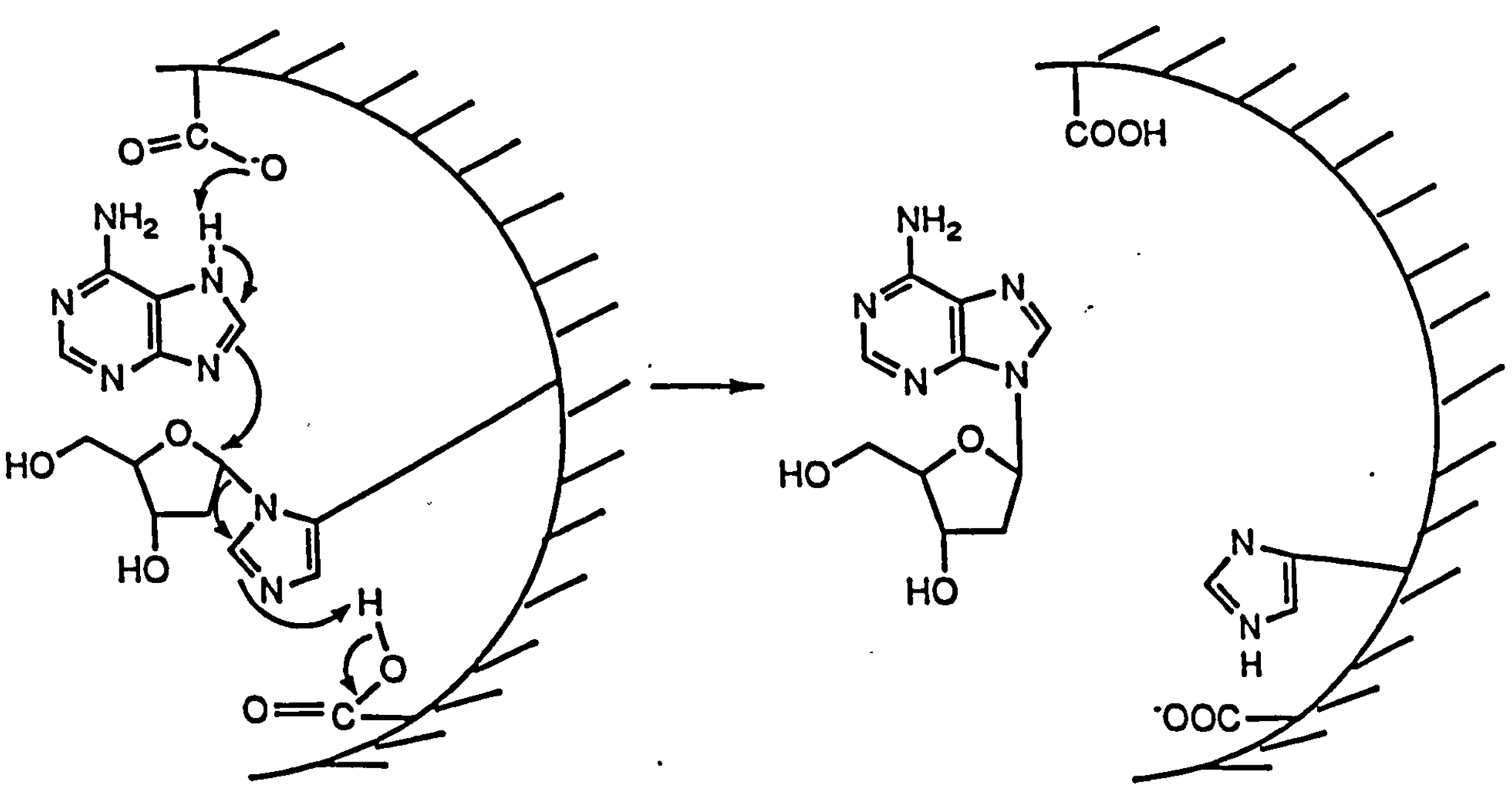


Figure 4.4 Proposed mechanism of action of *N*-deoxyribosyltransferases (Heath, 1991).

only the glycosyl residue binding to the active site of the enzyme. Further experiments need to be carried out where a different radioactive label is present on the base as well as on the glycosyl residue. Experimental conditions also need to be altered so as to achieve a better incorporation of the label.

If the glycosyl-enzyme intermediate could be trapped, it would be interesting to see by isolating this intermediate, whether the second part of the transfer reaction could be achieved by adding a new base and monitoring the production of the new nucleoside. This would invariably prove the existence of the glycosyl-enzyme intermediate.

Fluorescent probes can also be very useful in determining certain aspects of an enzyme's mechanism. Fluoresence spectroscopy relies on the properties of absorption and subsequent emission of radiation of a particular molecule. When a fluorophore (the molecular group giving rise to fluorescence) absorbs energy (radiation), the potential energy levels of the molecule move from a lower to a higher energy level. Upon returning to the ground state radiation is emitted, which results in the process of fluorescence (Figure 4.5). Fluorescence spectra usually appear at longer wavelength to the absorption wavelength because energy is lost in dropping to the lowest level of the first excited state (S_1), hence the emitted light will have less energy than the absorbed light.

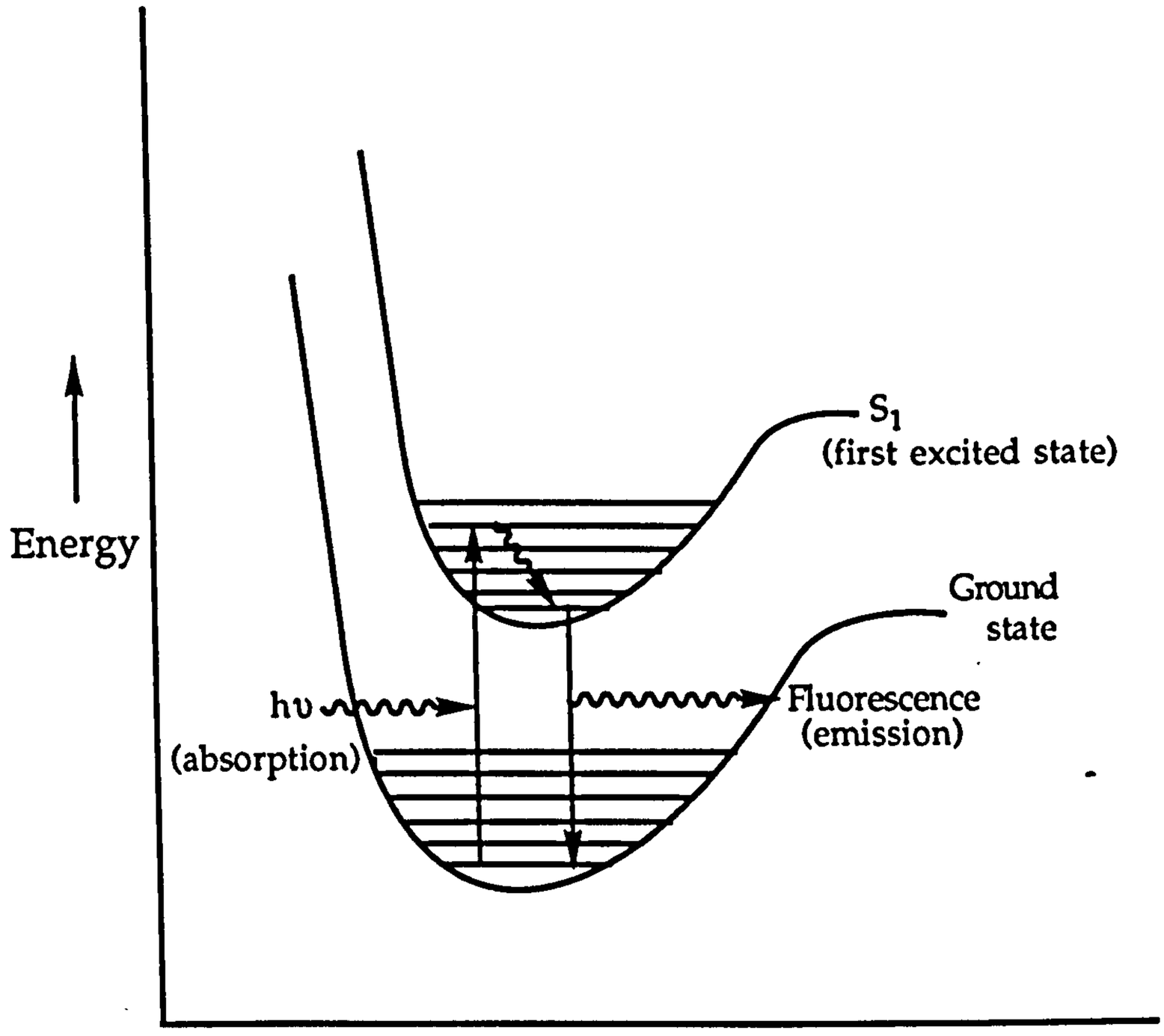


Figure 4.5 Process leading to fluorescence

Generally, natural nucleic acids do not have fluorescence activity, however, ribosyl and 2′-deoxyribosyl (Figure 4.6) derivatives of 1,N^6-ethenoadenine have been shown to be highly fluorescent (Secrist,III *et al.*, 1972; Spencer *et al.*, 1974).

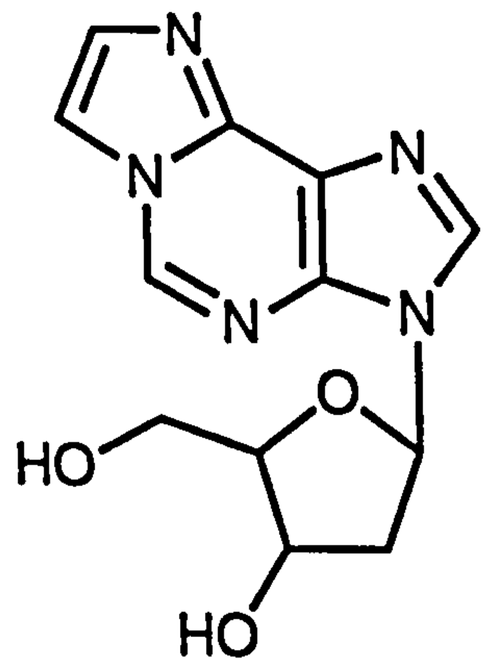


Figure 4.6 1,N^6-etheno-2'-deoxyadenosine

An advantage of using fluorescent nucleosides with *N*-deoxribosyltransferase would be to measure the increase or decrease in fluorescence when a substrate is catalysed to form another product, i.e. resulting in either the substrate or product losing its fluorescent activity. In the case of 1,N^6-etheno-2′-deoxyadenosine, the nucleoside is much more fluorescent than the corresponding base, 1,N^6-ethenoadenine. Hence, studies can be carried out with this fluorescent nucleoside to further substantiate the ping-pong mechanism of the *N*-deoxribosyltransferase enzyme.

4.2 Materials and methods

4.2.1 Reagents

Xanthine oxidase, Grade I from buttermilk, and 1,N^6-etheno-2′-deoxyadenosine and 1,N^6-ethenoadenine were purchased from Sigma. (5'-^{3}H)-thymidine and (2-^{14}C)-thymidine were purchased from Amersham and NEN-Biotechnology Systems Dupont, respectively.

4.2.2 Kinetic analysis

Velocity measurements were carried out using a coupled enzyme system, where the auxiliary enzyme was xanthine oxidase and deoxyinosine was the donor nucleoside in the reaction. Xanthine oxidase rapidly oxidises the hypoxanthine formed from deoxyinosine to uric acid (Kalcker, 1947; Danzin & Cardinaud, 1974). Uric acid was measured by recording the absorbance change at 290 nm, using the Pye SP1800 spectrophotometer. All reactions were carried out at 40°C in 0.1 M MES buffer (pH 6) in a quartz cuvette of 1 cm path length in a final volume of 1.5 ml.

The reaction mixtures contained deoxyinosine (0.15-1.5 µmol) and cytosine (0.15-0.9 µmol) with 0.1 M MES buffer (pH 6) and xanthine oxidase (0.33 U). These mixtures were incubated for 10 min at 40°C, to check whether any hypoxanthine, occassionally present in deoxyinosine, was completely oxidised before initiating the reaction with *N*-deoxyribosyltransferase (0.063 µg/ml; 0.095 µg). The absorbance change at 290 nm per minute was determined in order to calculate the initial rates of the reactions, when ε_{290} for uric acid was 12 200 $M^{-1}cm^{-1}$. Assays were carried out in duplicate.

4.2.3 Substrate binding experiments

4.2.3.1 Fluorescence labelling

All reactions were carried out on a Perkin Elmer Luminescence Spectrometer LS 50 and at room temperature (23°-24°C). Fluorescent compounds used were the nucleoside, 1,N^6-etheno-2'-deoxyadenosine, and base, 1,N^6-ethenoadenine. The maximum excitation and emission wavelengths of these two compounds were determined in 0.1 M MES buffer (pH 6). The progress of the reaction was therefore monitored by a time-dependent decrease in fluorescence intensity at 410 nm, where the difference in absorbances between nucleoside and the corresponding base were maximal (Figure 4.7), with an excitation wavelength of 300 nm.

The reaction mixture contained 1,N^6-etheno-2'-deoxyadenosine (2.5 nmol) in 0.1 M MES buffer (pH 6) and initiated with *N*-deoxyribosyltransferase (0.66 µg) in a final volume of 1 ml. Control experiments were also carried out containing (i) only 1,N^6-etheno-2'-deoxyadenosine, (ii) only transferase enzyme, (iii) denatured transferase enzyme with 1,N^6-etheno-2'-deoxyadenosine, and (iv) transferase with the base, 1,N^6-ethenoadenine. 1,N^6-etheno-2'-deoxyadenosine was also incubated with catalase and bovine serum albumin.

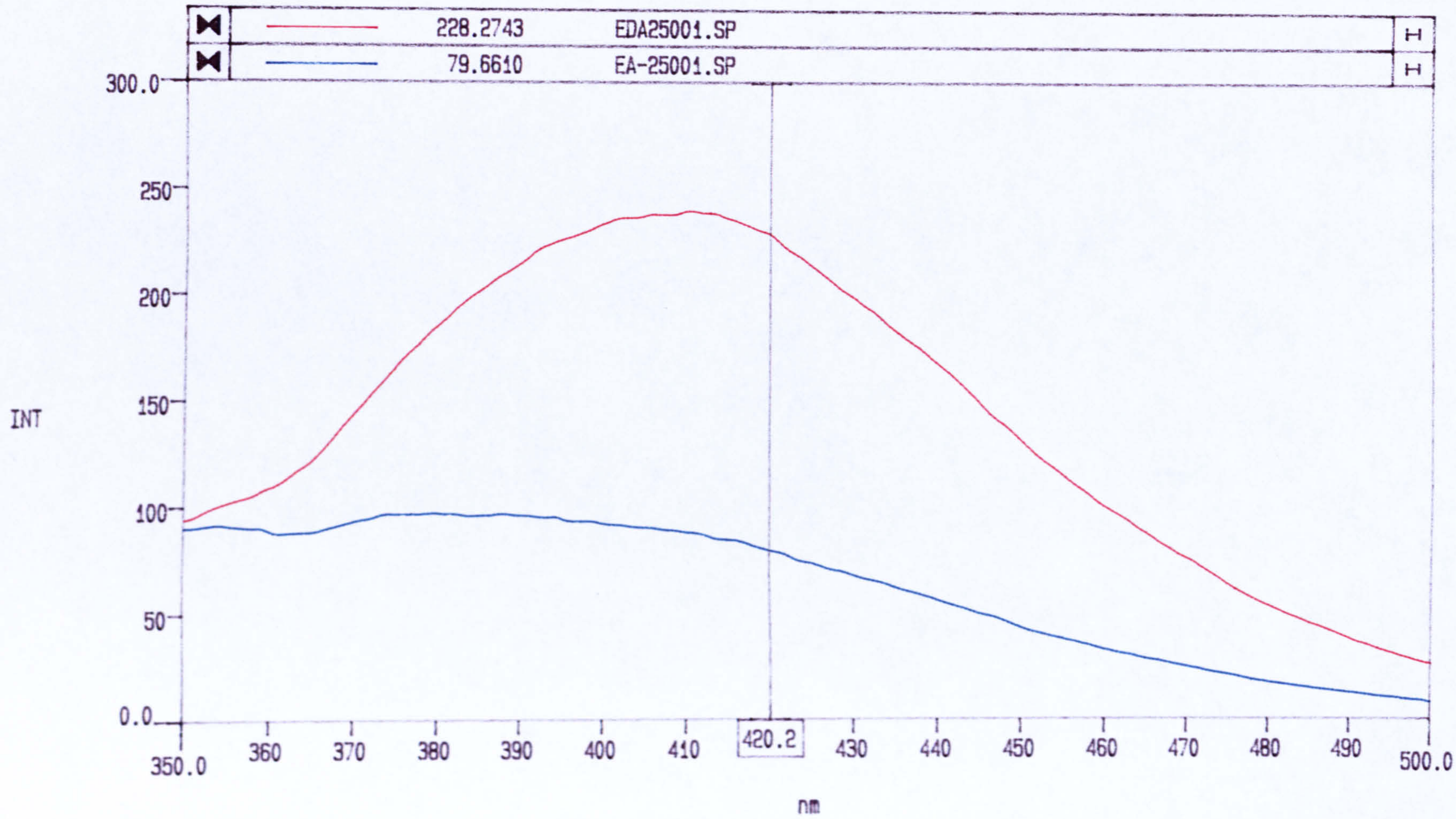


Figure 4.7 Fluorescence spectrum of 1,N^6-etheno-2'-deoxyadenosine (red) and ethenoadenine (blue). Excitation wavelength at 300 nm.

4.2.3.2 Radiolabelling

The reactions were incubated at 30°C for 15 min containing (5'-^{3}H)-thymidine (4.5 µmoles, 31.39 Ci/mole) or (2-^{14}C)-thymidine (4.5 µmoles, 59 Ci/mole) with 0.1 M MES buffer (pH 5.5) in a final volume of 0.5 ml, after which time the reactions were initiated with *N*-deoxyribosyltransferase (25.9 µg). After 5 and 10 seconds the reactions were stopped by adding 10% acetic acid (0.5 ml), bringing the pH down to 2.3. The mixture was then run down a G25 FPLC gel filtration column (1 x 11 cm) at 1 ml/min with 10% acetic acid, to separate the protein from the excess radioactively-labelled compounds. 0.5 ml fractions were collected, Optiphase scintillation fluid (3 ml) was added to 100 µl of each fraction and the radioactivity counted on a scintillation counter (LKB, Wallac Oy, Finland). A control experiment was carried out where bovine serum albumin (25.9 µg) was used instead of *N*-deoxyribosyltransferase, using the above procedure.

Radioactively-labelled protein fractions were pooled from which a 100 µl aliquot was counted for radioactivity as described above. A further 2 x 100 µl aliquots were lyophilised and the protein concentration determined by the method of Lowry (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard.

4.3 Results

4.3.1 Kinetic data

Kinetic analysis was carried out by using the Enzfitter programme (Elsevier-BIOSOFT, Cambridge, UK) for a non-linear regression curve to

fit equation 3, the Michaelis-Menten equation.

Equation 3 $v_o = V_{max}[S]/(K_m + [S])$

where, v_o = initial velocity
V_{max} = maximum velocity
K_m = Michaelis constant
[S] = variable substrate concentration

Figure 4.8 shows the initial velocity pattern for *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*) under conditions where the cytosine concentration was varied, while the deoxyinosine was held at different constant concentrations. The Lineweaver-Burk, double-reciprocal plots were found to be linear for a given deoxyinosine concentration and at different deoxyinosine concentrations.

The lines can be seen to be parallel to each other, which suggests that a ping-pong bi bi mechanism is being employed by the enzyme. The kinetic constants, K_m and V_{max}, for cytosine can be determined from Figure 4.8 when the concentration of deoxyinosine was saturating, eg. at 1 mM. If a replot of the intercepts ($v_{o\ app}$) of the Lineweaver-Burk plots in Figure 4.8 are plotted against the of the deoxyinosine concentrations used (Figure 4.9), then the K_m and V_{max} values for deoxyinosine can be calculated. The kinetic analysis for the replots were carried out using the Enzfitter programme and displayed as a Haynes plot, eg. [substrate]/v_o versus [substrate].

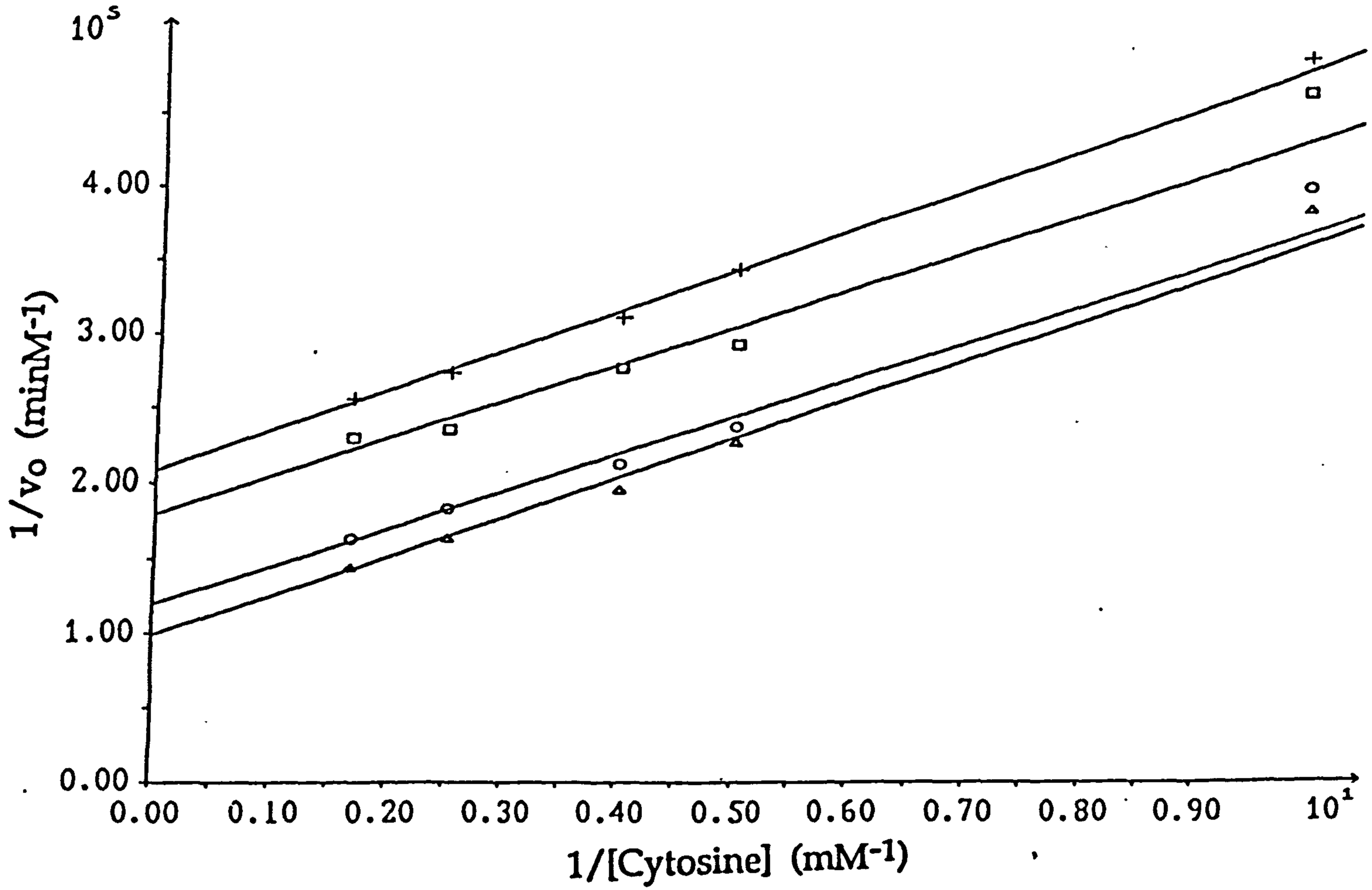


Figure 4.8 Initial velocity pattern for the dI:C reaction with cytosine as the variable substrate. Protein concentration was at 0.063 µg/ml. Deoxyinosine was the fixed substrate: (+) 0.1 mM; (□) 0.2 mM; (○) 0.6 mM; (△) 1.0 mM.

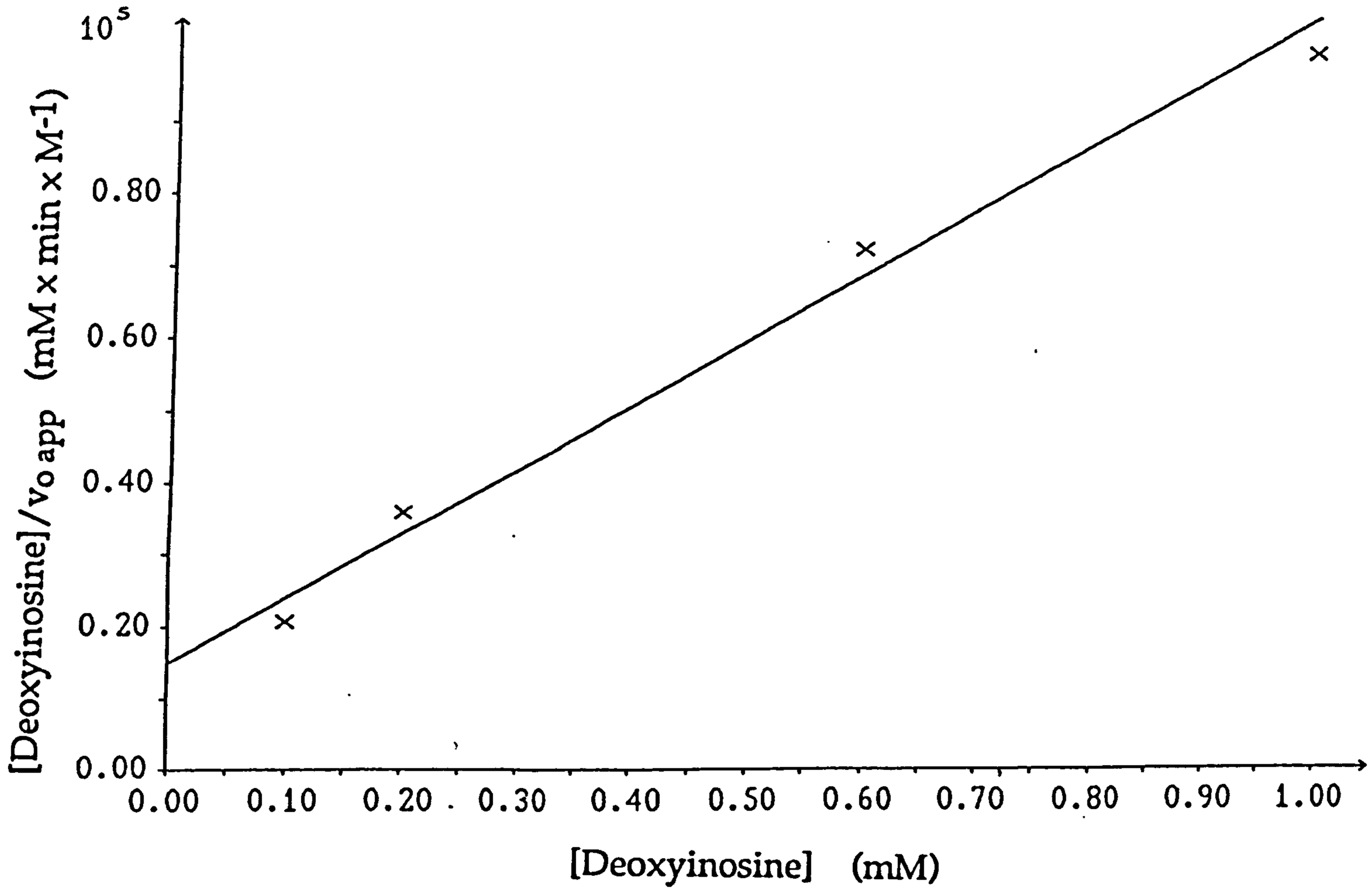


Figure 4.9 Replot of the initial velocity data (intercepts) from Figure 4.8 with deoxyinosine as the variable substrate.

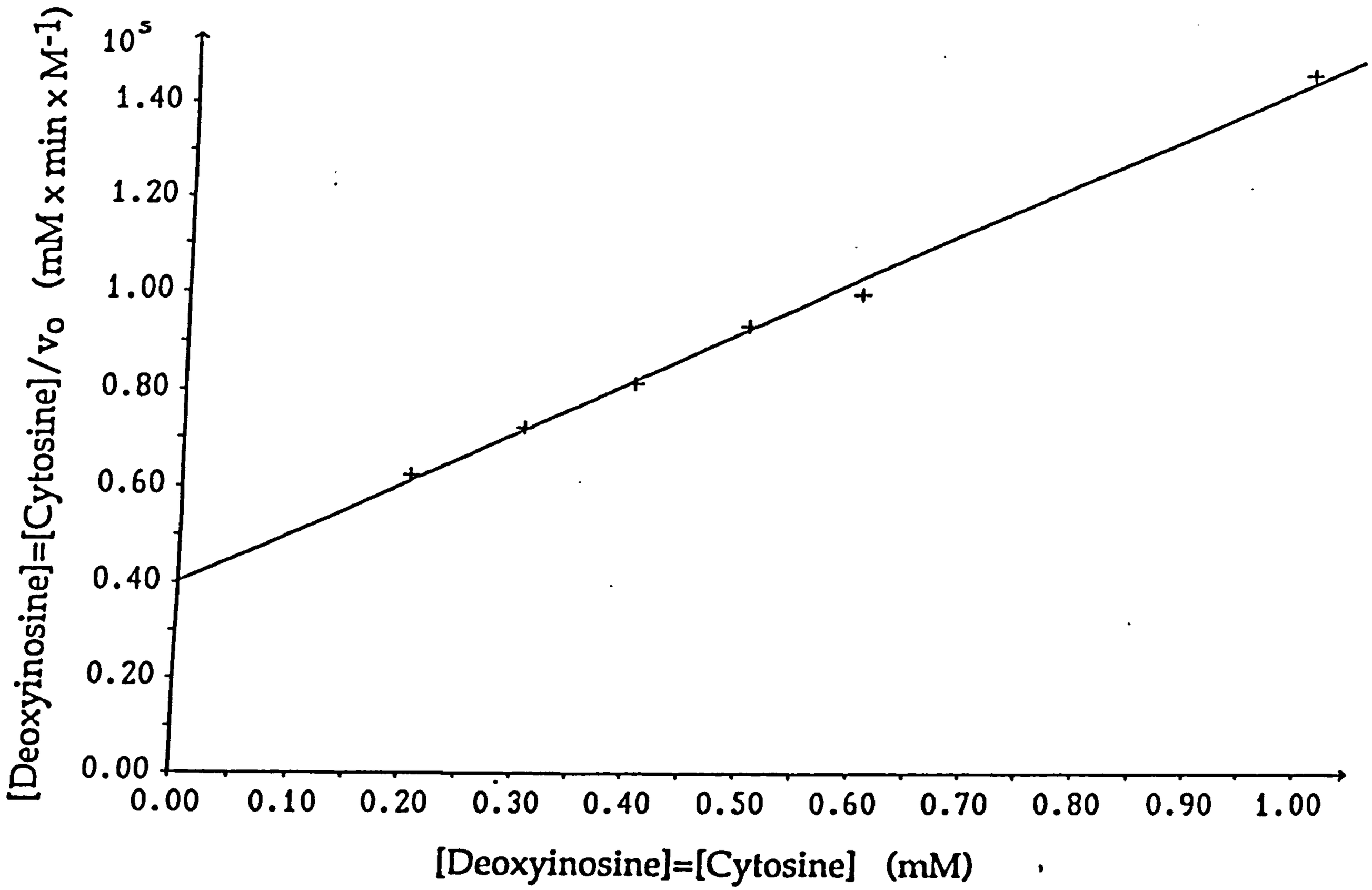


Figure 4.10 Initial velocity pattern for identical concentrations of both deoxyinosine and cytosine substrates. Protein concentration was 0.063 µg/ml.

The true V_{max} and k_{cat} values for the reaction, dI:C, can be determined when the concentrations of the two substrates were held constant (Figure 4.10). The concentration of the transferase enzyme was calculated by taking the molecular weight of the subunit, eg. 20 500 daltons. The kinetic constants for the forward reaction of dI:C can be summarised in Table 4.1.

Type of constant	Deoxyinosine	Cytosine	For the reaction dI:C
K_m (mM)*	0.17 (±3%)	0.26 (±9%)	-
V_{max} ($nmolesmin^{-1}$)*	16.8 (±9%)	15.0 (±4%)	14.3 (±3%)
k_{cat} (min^{-1})*	3628.5 (±9%)	3239.7 (±4%)	3088.6 (±3%)

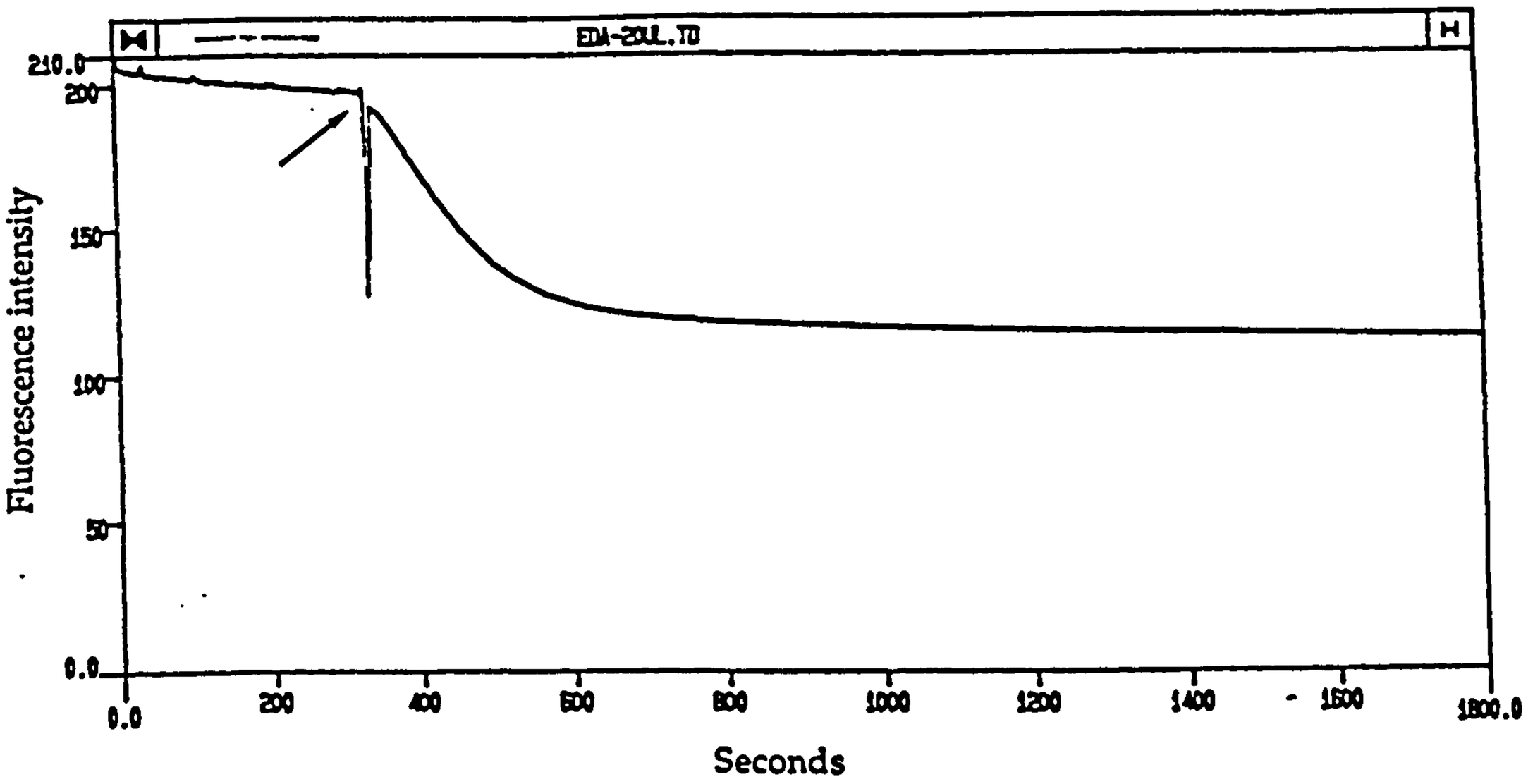
* Values are an average of 3 determinations

Table 4.1 Kinetic constants for nucleoside *N*-deoxyribosyltransferases from *Leu. mesenteroides* (*cremoris*)

4.3.2 Fluorescence labelling data

Figure 4.11 A shows the decrease in fluorescence intensity when *N*-deoxyribosyltransferase was incubated with the nucleoside, $1,N^6$-ethenodeoxyadenosine, at room temperature. This decrease in activity was not observed when denatured (boiled) transferase enzyme was used (Figure 4.11 B), nor when the transferase enzyme was replaced with catalase (Figure 4.12 A) or bovine serum albumin (Figure 4.12 B). Incubation of $1,N^6$-etheno-2'-deoxyadenosine without any transferase enzyme also did not affect the fluorescence intensity (Figure 4.13 A), during the time span of the experiment. Incubating the transferase

A



B

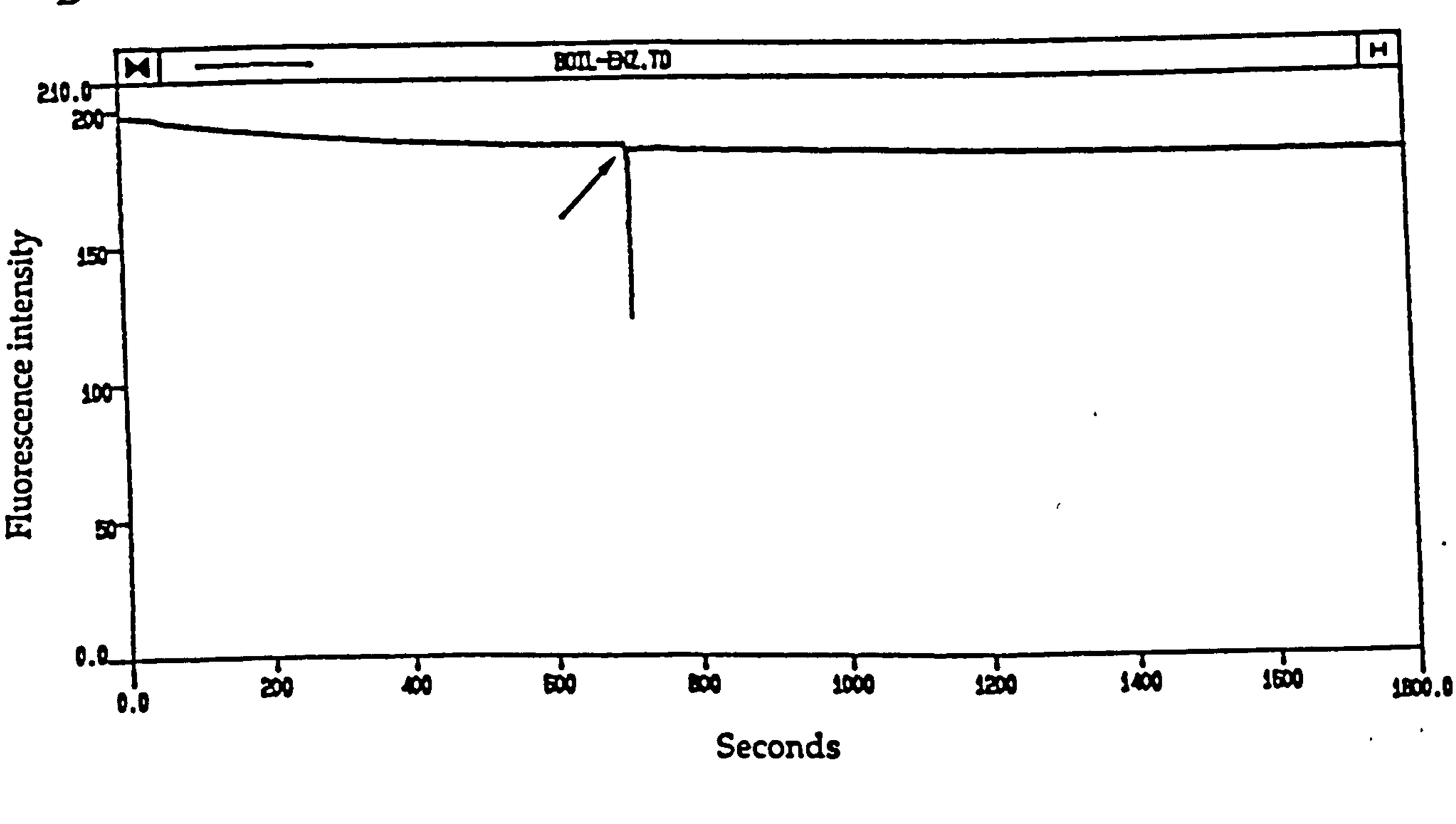
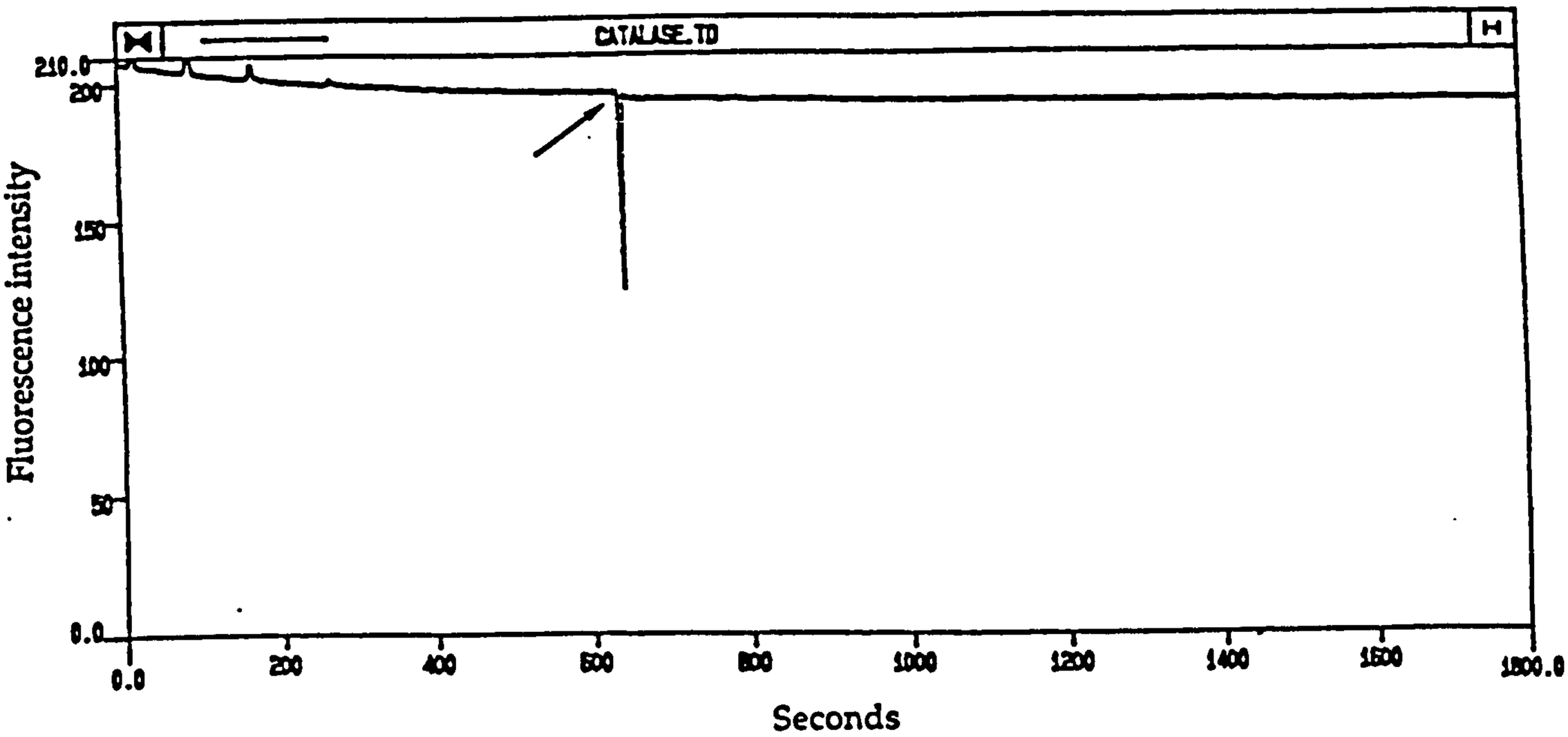


Figure 4.11 Fluorescence spectra of 1,N^6-etheno-2'-deoxyadenosine (2.5 nmol) in 0.1 M MES buffer (pH 6) with (A) *N*-deoxyribosyltransferase (0.66 µg) and (B) with denatured enzyme (0.66 µg). Excitation wavelength was at 300 nm and emission wavelength at 410 nm. Arrow marks the point of addition of substrate.

A



B

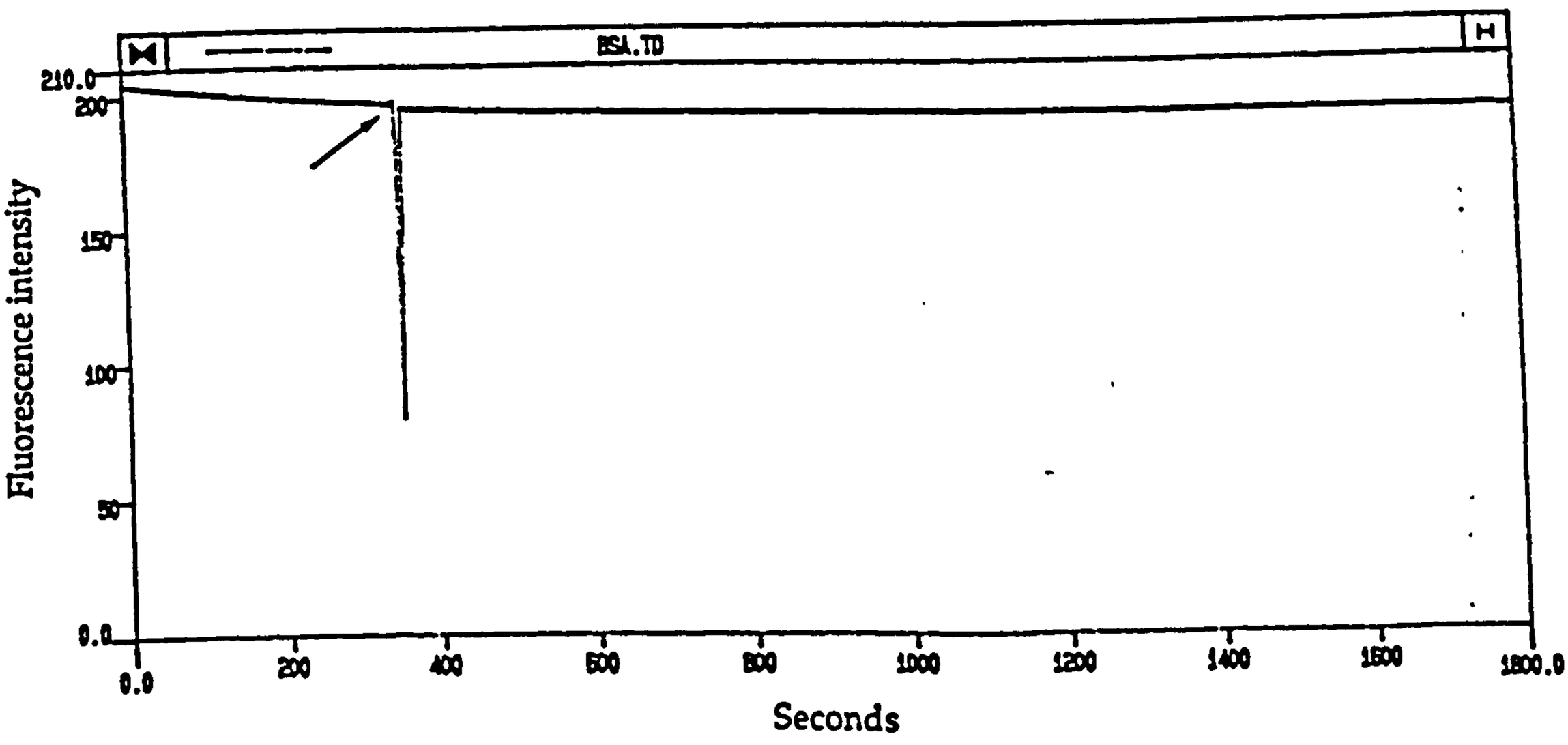
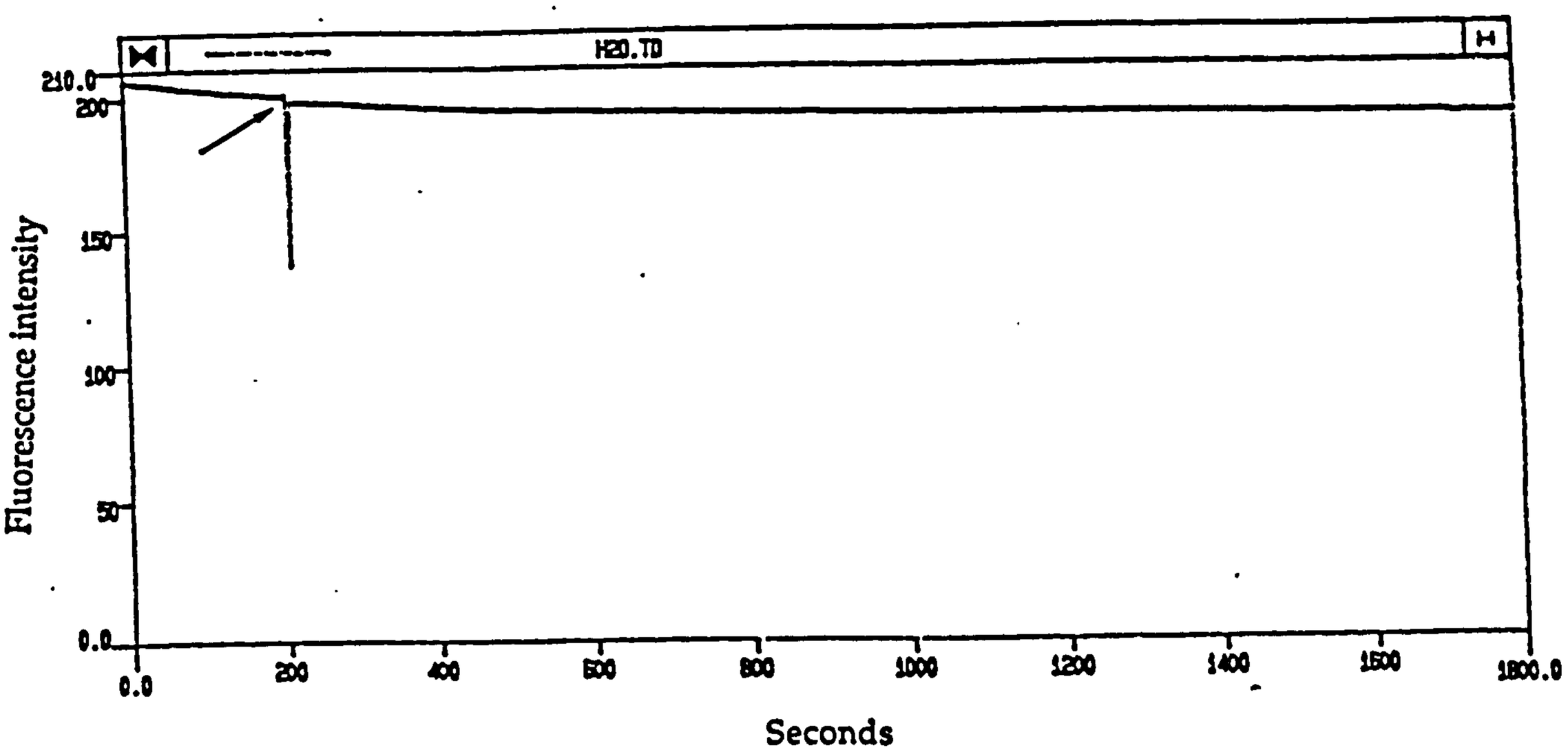


Figure 4.12 Fluorescence spectra of 1,N^6-etheno-2'-deoxyadenosine (2.5 nmol) in 0.1 M MES buffer (pH 6) with (A) catalase (0.66 µg) and (B) with bovine serum albumin (0.66 µg). Excitation wavelength was at 300 nm and emission wavelength at 410 nm. Arrow marks the point of addition of substrate.

A



B

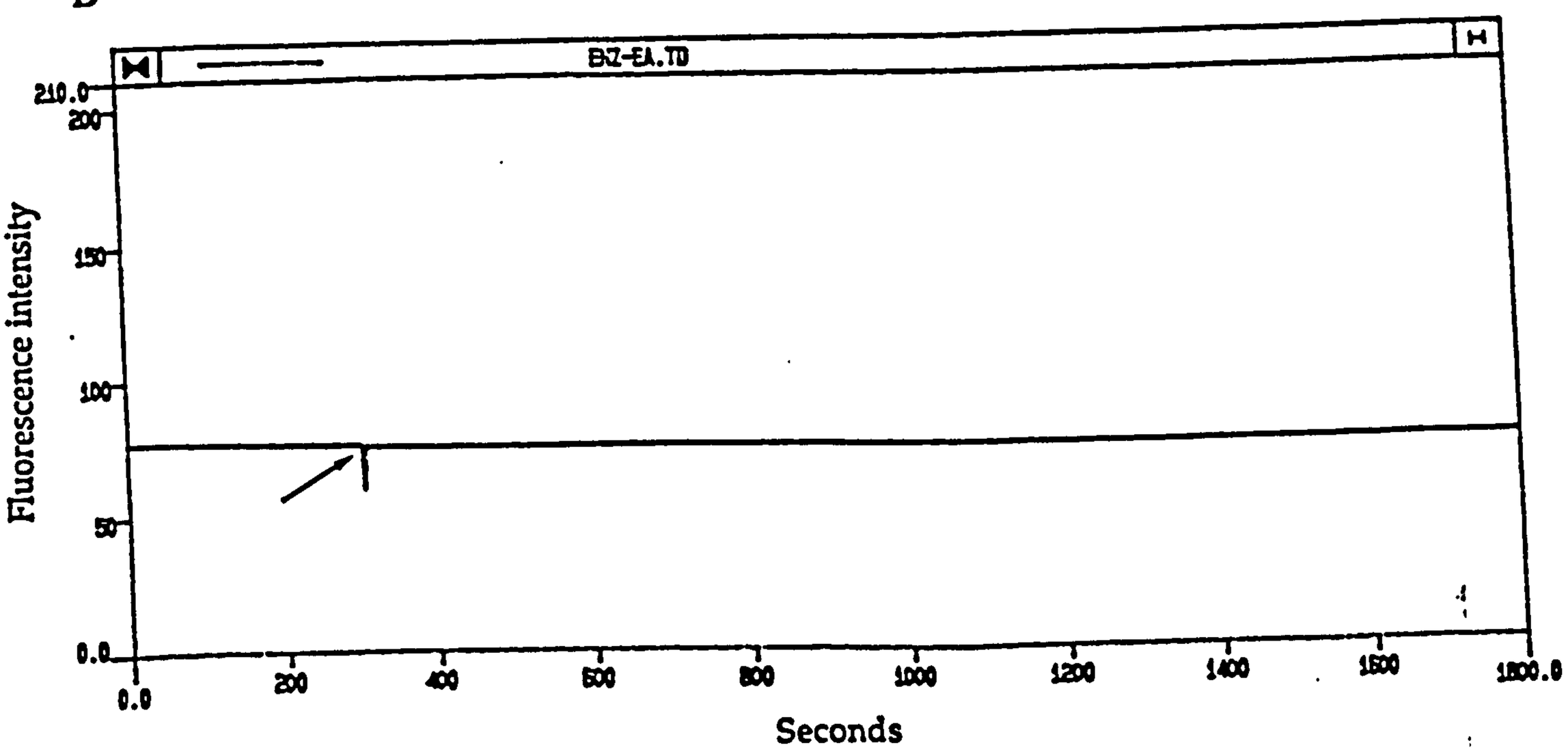


Figure 4.11 Fluorescence spectra of (A) 1,N^6-etheno-2'-deoxyadenosine (2.5 nmol) in 0.1 M MES buffer (pH 6) with no enzyme (water) and (B) with 1,N^6-ethenoadenine (2.5 nmol) with *N*-deoxyribosyltransferase (0.66 µg) in 0.1 M MES buffer (pH 6). Excitation wavelength was at 300 nm and emission wavelength at 410 nm. Arrow marks the point of addition of substrate.

enzyme with the base, 1,N^6-ethenoadenine, again did not affect the fluorescence intensity (Figure 4.13 B).

4.3.3 Radiolabelling data

Table 4.2 shows the amount of incorporation of radioactivity when the enzyme was incubated with (5'-^{3}H)-thymidine, (2-^{14}C)-thymidine, and when the enzyme was replaced with bovine serum albumin and incubated with the same radioactive compounds

	% of protein labelled with tritium			
	N-deoxyribosyltransferase*		Bovine serum albumin*	
	5 sec	10 sec	5 sec	10 sec
(5'-^{3}H)-thymidine	18.7	26.1	0	0
(2-^{14}C)-thymidine	0	0	0	0

*Values are from single determinations.

Table 4.2 Incorporation of the (5'-^{3}H)-deoxyribosyl moiety of thymidine by *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*)

Figure 4.14 shows a typical chromatogram, after the reaction mixture had been stopped and run down a G25 column on the FPLC and the radioactivity had been counted. The first peak to appear on the chromatogram is the protein followed by the excess labelled nucleoside and finally the third peak is the liberated thymine and perhaps some hydrolysed glycosyl groups.

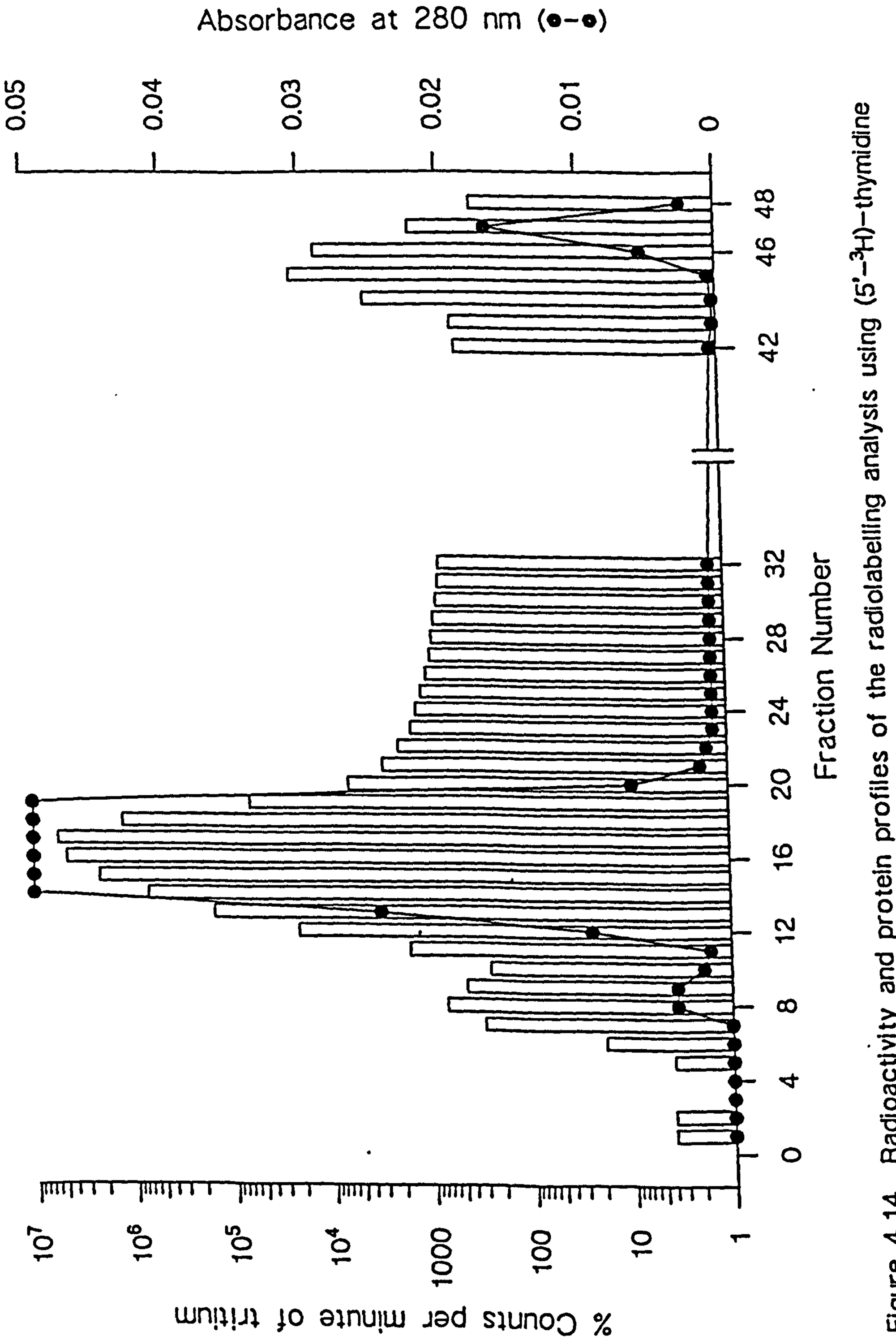


Figure 4.1 Radioactivity and protein profiles of the radiolabelling analysis using (5'–^{3}H)–thymidine

4.4 Discussion

The parallel initial velocity patterns observed from the kinetic studies, illustrate that the nucleoside *N*-deoxyribosyltransferase isolated from *Leu. mesenteroides* (*cremoris*) carries out the transfer reaction via a ping-pong bi-bi mechanism. This mechanism is analogous to the ones determined for the deoxyribosyltransferases from *L. helveticus* (Danzin & Cardinaud, 1974; 1976) and *L. leichmannii* (Heath, 1991). The reaction scheme used in the present kinetic experiments can be summarised as in Figure 4.15.

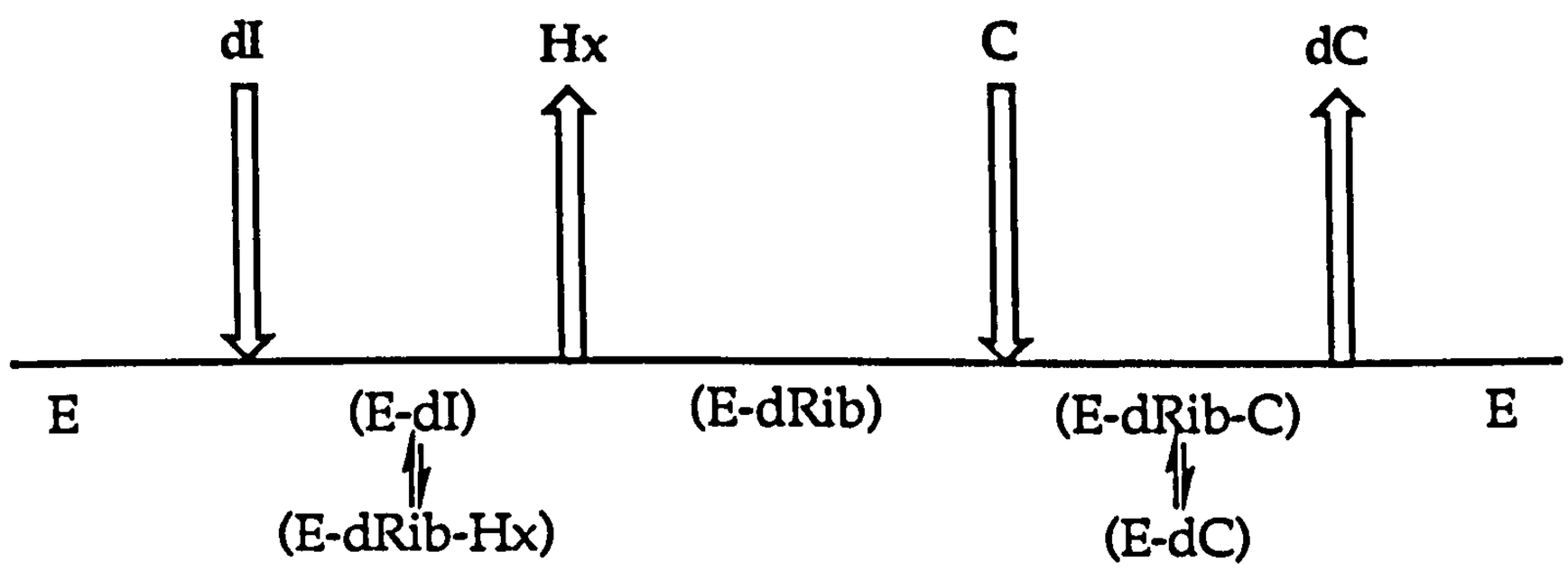


Figure 4.15 Reaction scheme for the transfer of deoxyinosine to cytosine

In the above scheme, E is the free enzyme, E-dRib is the glycosyl-enzyme intermediate, dI and Hx are deoxyinosine and hypoxanthine, and C and dC are cytosine and deoxycytidine, respectively. The above scheme also depicts the enzyme as being in two stable forms, E and and E-dRib, where the first product, Hx, is released from the enzyme before the second substrate, C, is able to bind to E-dRib.

When the concentration of both substrates, deoxyinosine and cytosine, were held in constant ratio, and initial velocities of the reactions were determined, a plot of [substrate]/v_o versus [substrate], gave a straight line.

This type of plot is indicative of a ping-pong mechanism being operated, rather than a sequential one. A similar plot for an enzyme employing a sequential mechanism would generate a plot that concaves upwards at low concentrations of substrate. So care would need to be taken when carrying out such experiments that the correct area of substrate concentration has been covered. The advantage of such a plot is that the true maximum velocity and catalytic constant of the reaction, in the forward direction in this case, can be derived. Care was also taken to eliminate substrate inhibition, as this would distort the plots derived from the data. When the initial kinetic experiments were being conducted, it was noticed that at concentrations exceeding 0.8 mM an inhibitory effect of cytosine was observed. This was probably due to the formation of a dead-end complex when cytosine combines with free enzyme. Hence, all experiments were conducted below this value of cytosine concentration. However, no competitive inhibition by deoxyinosine was observed even at concentrations as high as 2.0 mM.

Replots of the data from the Lineweaver-Burk plots can determine conveniently the kinetic constants of the second substrate, deoxyinosine. The K_m values for deoxyinosine and cytosine are 0.17 mM (±3%) and 0.26 mM (±9%), respectively. These values can be compared with those obtained for the same reaction by Danzin and Cardinaud (1976), using deoxyribosyltransferase from *L. helveticus*, which were 3.5 mM and 0.073 mM for deoxyinosine and cytosine, respectively. Hence, deoxyinosine appears to be a good donor substrate for the transferase enzyme from *Leu. mesenteroides* (*cremoris*), but cytosine is not as good an acceptor base. The true maximum velocity and catalytic constant, k_{cat}, for the reaction (dI:C), were calculated to be 14.3 $nmolesmin^{-1}$ and 3088.6 min^{-1}, determined from the plot where the two substrate

concentrations were held constant.

1,N^6-etheno-2'-deoxyadenosine has been shown to be highly fluorescent (Secrist III *et al.*, 1972; Spencer *et al.*, 1974), but the corresponding base is not as fluorescent, as can be seen from the scan of the two compounds (section 4.2, Figure 4.7). These compounds were first tested with the pure transferase enzyme from *Leu. mesenteroides* (*cremoris*) to see how good a substrate they were compared to the normal substrates, 2'-deoxyadenosine and adenine. 1,N^6-etheno-2'-deoxyadenosine was as efficient a donor as 2'-deoxyadenosine but 1,N^6-ethenoadenine was only 60% as efficient as an acceptor compared to adenine. Hence 1,N^6-etheno-2'-deoxyadenosine was a good substrate to use for the fluorescence labelling experiments.

The results from the fluorescence experiments showed that when the fluorescent nucleoside, 1,N^6-etheno-2'-deoxyadenosine, was incubated with the transferase enzyme, a decrease in fluorescence intensity was observed. This suggests that the nucleoside reacts with the enzyme first, since no change in fluorescence was observed when only the base, 1,N^6-ethenoadenine, and the enzyme were incubated. Although the kinetic analysis suggests the mechanism of the enzyme is via ping-pong bi-bi, it does not specify which substrate binds with the enzyme first. However, from the type of reaction being considered here, it can be correctly assumed that the first substrate to bind would be the nucleoside as this would release the first product. If more than two substrates were present then it would be more difficult to deduce which order the substrates would add to the enzyme.

The decrease in fluorescence intensity also suggests that the nucleoside was splitting up by releasing the base, 1,N^6-ethenoadenine, which would

probably leave the glycosyl residue attached to the active site of the enzyme. Since the fluorescent nucleoside showed no decrease in intensity with denatured enzyme or with catalase or bovine serum albumin, it can be assumed that the loss in fluorescence is not due to non-specific binding of the compound to the enzyme. 1,N^6-etheno-2'-deoxyadenosine was also found to be stable over the time period of the experiments. Therefore, in the absence of the acceptor the transferase enzyme was able to release the first product from the donor nucleoside.

The radiolabelling experiments provided significant evidence for the glycosyl-enzyme intermediate being formed during the course of the reaction. Although incorporation of the label to the level of 100% was not seen, there was a significant amount of incorporation of the (5'-^{3}H)-glycosyl residue of thymidine and no incorporation of the (2-^{14}C)-base residue of thymidine present in the protein fractions under the same conditions. The low amount of incorporation could be due to the slow hydrolysis of the glycosyl (or 2'-deoxyribose) moiety from the active site of the transferase enzyme when no acceptor was present. This phenomenon has been noticed by Huang and co-workers (1983) and more recently by Smar and co-workers (1991). Huang and co-workers (1983) found that when transferase from *L. leichmannii* was incubated with either thymidine, deoxyadenosine or 8-bromodeoxyadenosine but no acceptors, the rate of formation of the glycosyl moiety was constant with each of the three different nucleosides, and suggested that the same type of species was undergoing this hydrolysis, namely the glycosyl-enzyme intermediate (Figure 4.16).

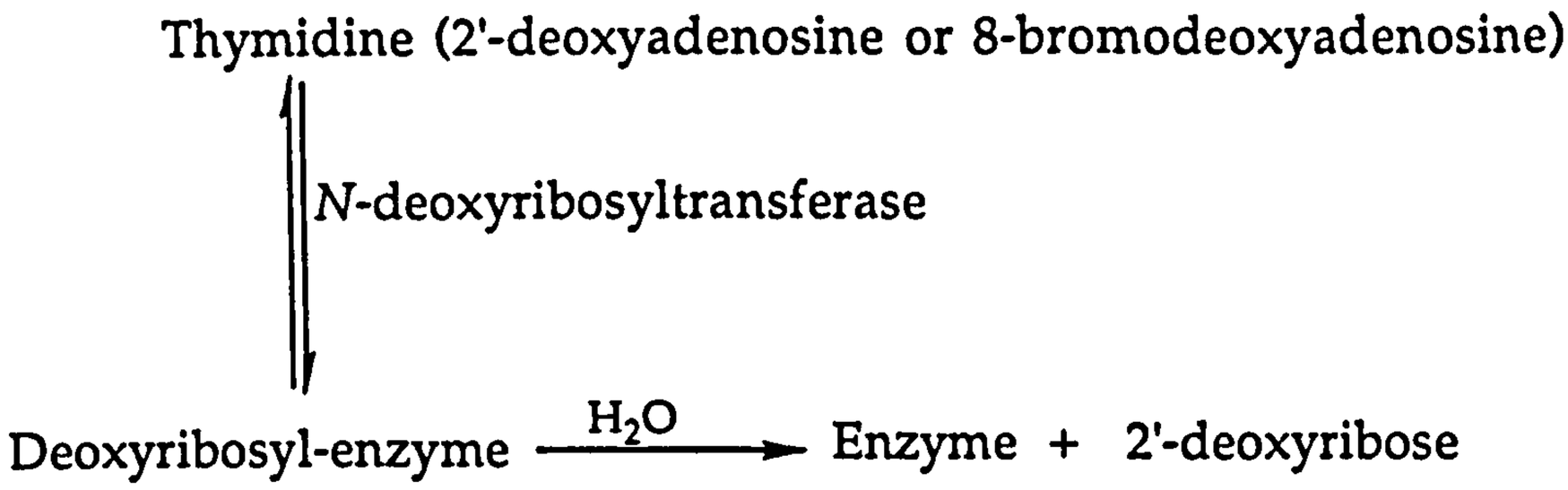


Figure 4.16 Formation of deoxyribose by the hydrolytic activity of nucleoside *N*-deoxyribosyltransferase from *L. leichmannii*

This hydrolytic activity could be a characteristic feature of the *N*-deoxyribosyltransferase enzymes, and also of the transferase enzyme from the *Leuconostoc* species. So 100% incorporation of the radioactive label would not be expected to occur. The important point is that incorporation of the tritiated thymidine was observed but no incorporation of the ^{14}C-thymidine was noticed. Non-specific binding of the label to the protein was also ruled out since incorporation of tritium was not observed when bovine serum albumin was used instead of the transferase enzyme.

Once incorporation of radioactivity had been established further experiments were conducted to react the glycosyl-enzyme intermediate with an acceptor such as adenine and determine whether a radioactively-labelled nucleoside would form. The glycosyl-enzyme intermediate was brought back to pH 5.5 by the slow addition of 2 M potassium hydroxide and then incubated at 40°C with adenine, and the reaction was monitored by HPLC. Unfortunately the production of deoxadenosine was not detected by UV nor by radioactive counting of collected fraction.

The important factor that was considered when the intermediate was

trapped was to stop the apparent intrinsic hydrolytic activity of the transferase enzyme, by decreasing the pH it appears that the enzyme becomes irreversibly denatured. Milder conditions need to be found where only partial denaturation occurs, so that the formation of the new nucleoside from the intermediate can be detected. Unfortunately, due to the lack of time further experiments could not be conducted.

The presence of an hydrolytic activity in the transferase enzyme can be further verified by observing Figure 4.14, where a second increase in tritiated radioactivity was detected near the third peak. This increase of radioactivity could not be tritiated thymidine as the peak for this compound appears much earlier than for the thymine peak. Since the glycosyl residue is smaller in size than thymidine, and as there is such an excess of thymidine in the reaction, it would be expected that the glycosyl residues would appear after the thymidine had passed through the gel filtration matrix. The appearance of the glycosyl residue could not be detected by UV spectroscopy, but running labelled 2′-deoxyribose down the gel filtration column would have determined when the glycosyl residue would have eluted.

Although conditions for trapping the intermediate need to be improved, it can still be concluded that a glycosyl-enzyme intermediate was isolated and the glycosyl residue was not just binding to the active site pocket by non-specific binding. Detection of radioactivity when the radioactive label was on the sugar moiety and not on the base moiety further supports the argument for a glycosyl-enzyme intermediate being formed during the reaction with tritiated thymidine and *N*-deoxyribosyltransferase from *Leu. mesenteroides* (*cremoris*).

Appendices

Appendix 1

Lactic acid Microorganisms	Strain Number	Growth Temperature
L. delbrueckii	NCIMB 8130	37°C
L. lactis	NCIMB 8170	37°C
L. bulgaricus	NCIMB 11778	37°C
L. leichmannii	ATCC 4797	37°C
L. helveticus	NCDO 30	40°C
L. acidophilus	NCIMB 11506	37°C
L. agilis	NCIMB 11716	37°C
L. salivarius subsp. *salivarius*	NCIMB 11975	37°C
L. casei subsp. *casei*	NCIMB 4113	37°C
L. casei subsp. *rhamnosus*	NCIMB 7473	37°C
iL. plantarum	NCIMB11974	30°C
L. alimentarius	NCIMB 11994	30°C
L. fermentum	NCIMB 11840	37°C
L. buchneri	NCIMB 8007	37°C
L. hilgardii	NCIMB 8040	30°C
S. salivarius subsp. *thermophilus*	NCIMB 8510	37°C
S. lactis subsp. *lactis*	NCIMB 8662	30°C
Leu. mesenteroides subsp. *dextranicum*	NCIMB 12007	30°C
Leu. mesenteroides subsp. *cremoris*	NCIMB 12008	30°C
P. pentosaceus AL	NCIMB 12012	37°C
P. acidilactici	NCIMB 12174	30°C
A. viridans	NCIMB 11775	30°C

Erwinia	Strain number	Growth Temperature
E. amylovora	SCRI* 454	30°C
E. quercina	SCRI 477	30°C
E. caratovora subsp. *atroseptica*	SCRI 1043	30°C
E. cypripedii	SCRI 440	30°C
E. herbicola	SCRI 463	30°C
E. nigrifluens	SCRI 496	30°C
E. rhapontici	SCRI 421	30°C
E. rhubrifaciens	SCRI 445	30°C
E. uredovora	SCRI 433	30°C

* Scottish Crop Research Institute

Appendix 2

MRS broth

Peptone	10 g
Beef exract	10 g
Yeast extract	5 g
Glucose	20 g
Tween 80	1 ml
K_2HPO_4	2 g
Sodium acetate	5 g
Triammonium citrate	2 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.2 g
Distilled water	1000 ml

Adjust pH to 6.7 and autoclave at 121°C for 15 min.

Luria-broth

Bacto-yeast extract	5 g
Tryptone	10 g
NaCl	10 g
Distilled water	1000 ml

Adjust pH to 7.5 and autoclave at 121°C for 15 min.

Nutrient broth

Lab-lemco beef extract	1 g
yeast extract	2 g
Peptone	5 g
NaCl	5 g
Distilled water	1000 ml

Autoclave at 121°C for 15 min.

Medium for *E. coli* IAM 1204 (Shirae *et al* ., 1989)

Peptone	10 g
Yeast extract	5 g
KNO_3	3 g
KH_2PO_4	1 g
K_2HPO_4	3 g
$FeSO_4 . 7H_2O$	0.01 g
$MnSO_4 . 4H_2O$	0.01 g
Meat extract	10 g

Adjust to pH 7 and autoclave at 121°C for 15 min.

Nutrient broth supplemented with thymine

Lab-lemco beef extract	1 g
yeast extract	2 g
Peptone	5 g
NaCl	5 g
Thymine	0.32 mM
Distilled water	1000 ml

Autoclave at 121°C for 15 min.

AC broth

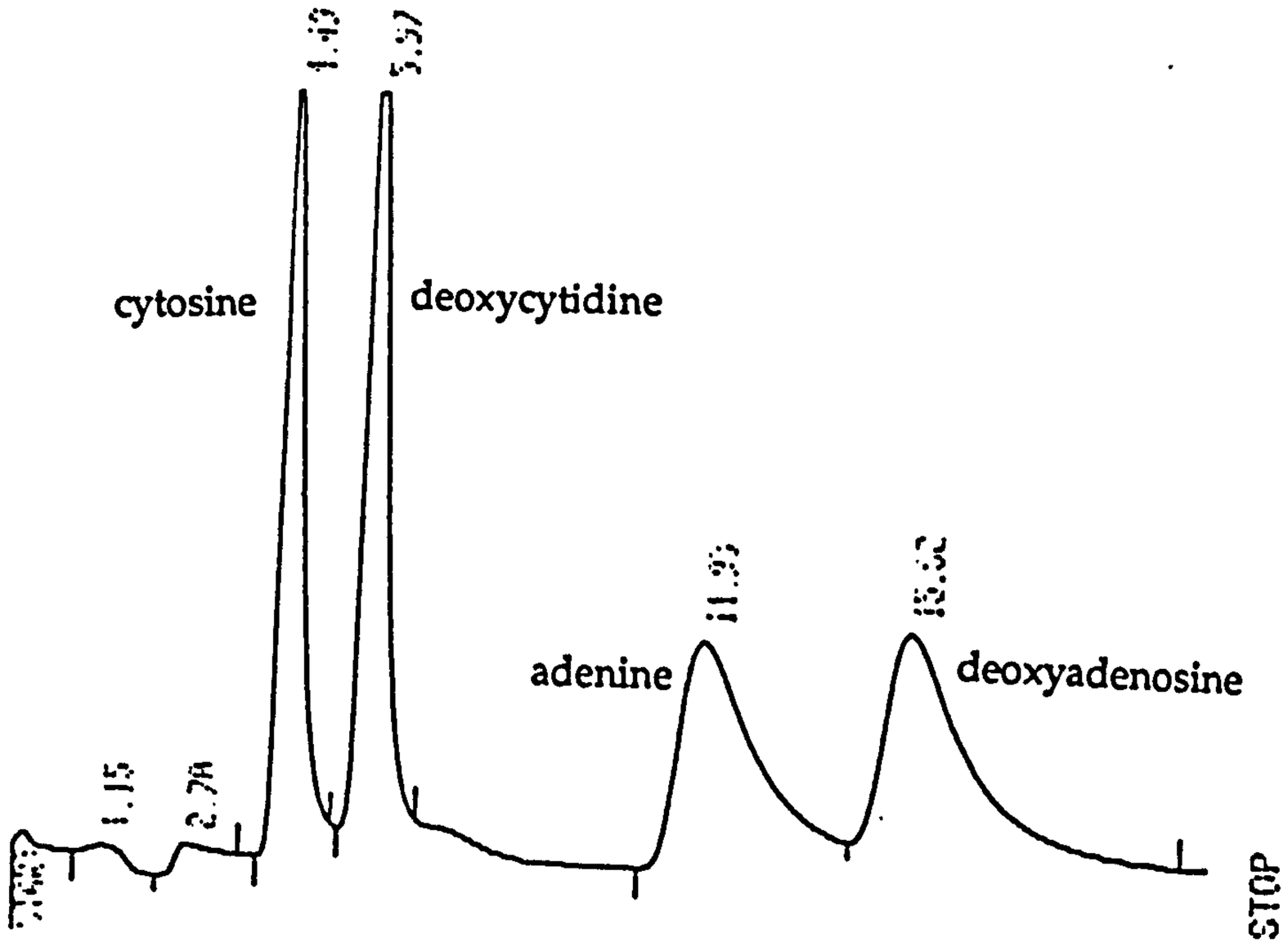
Difco protease peptone no. 3	2 g
bacto beef extract	3 g
Bacto yeast extract	3 g
Difco malt extract	3 g
Glucose	5 g
Ascorbic acid	0.2 g

Adjust to pH 7.2 and autoclave at 121°C for 15 min.

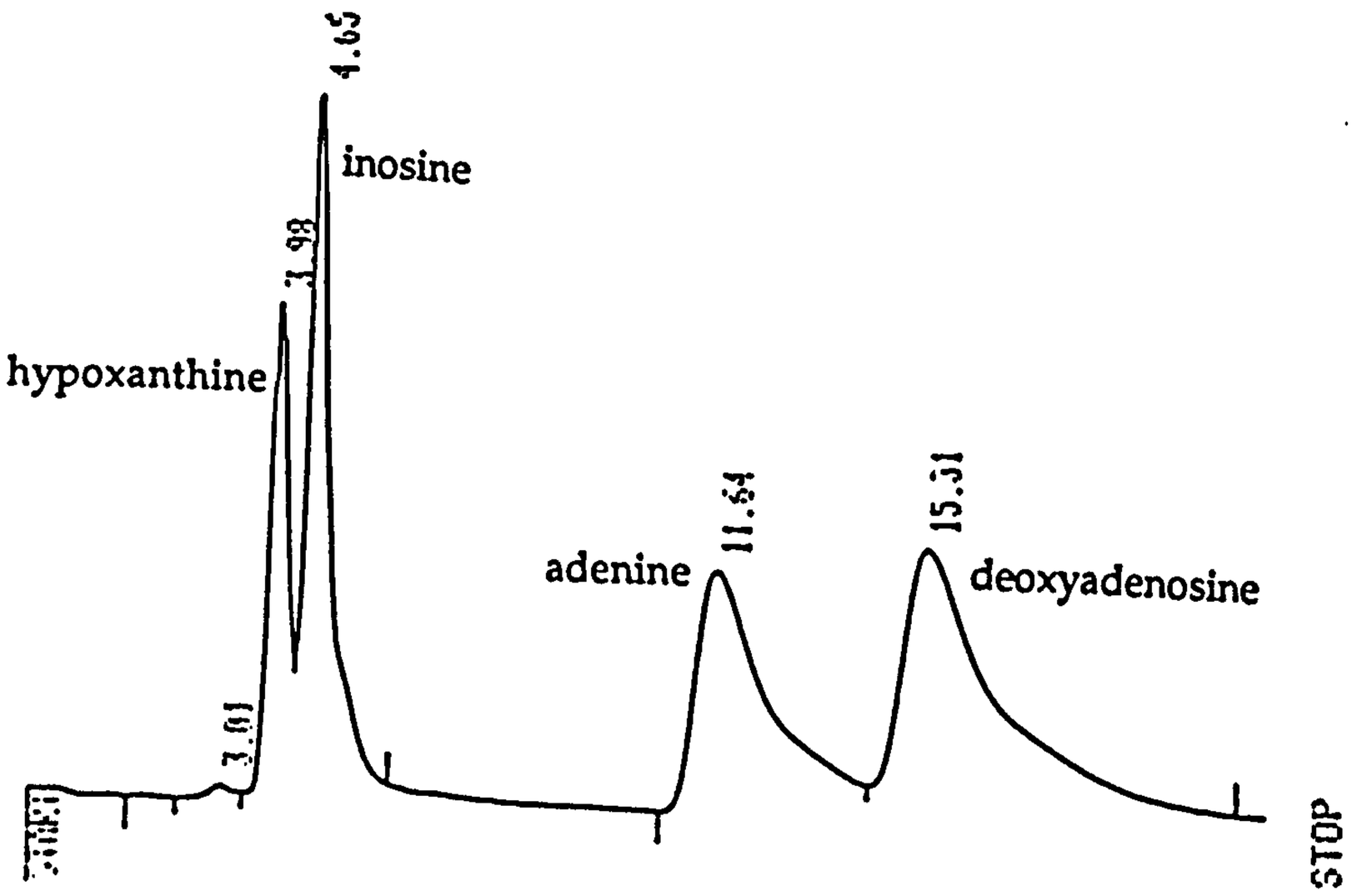
Appendix 3

The following HPLC traces show the separation of substrates and products from the reaction assays involving nucleoside *N*-deoxyribosyltransferase with the conditions mentioned in section 2.2.3.1.

dC:A dC + A ⇌ dA + C



dI:A dI + A ⇌ dA + Hx



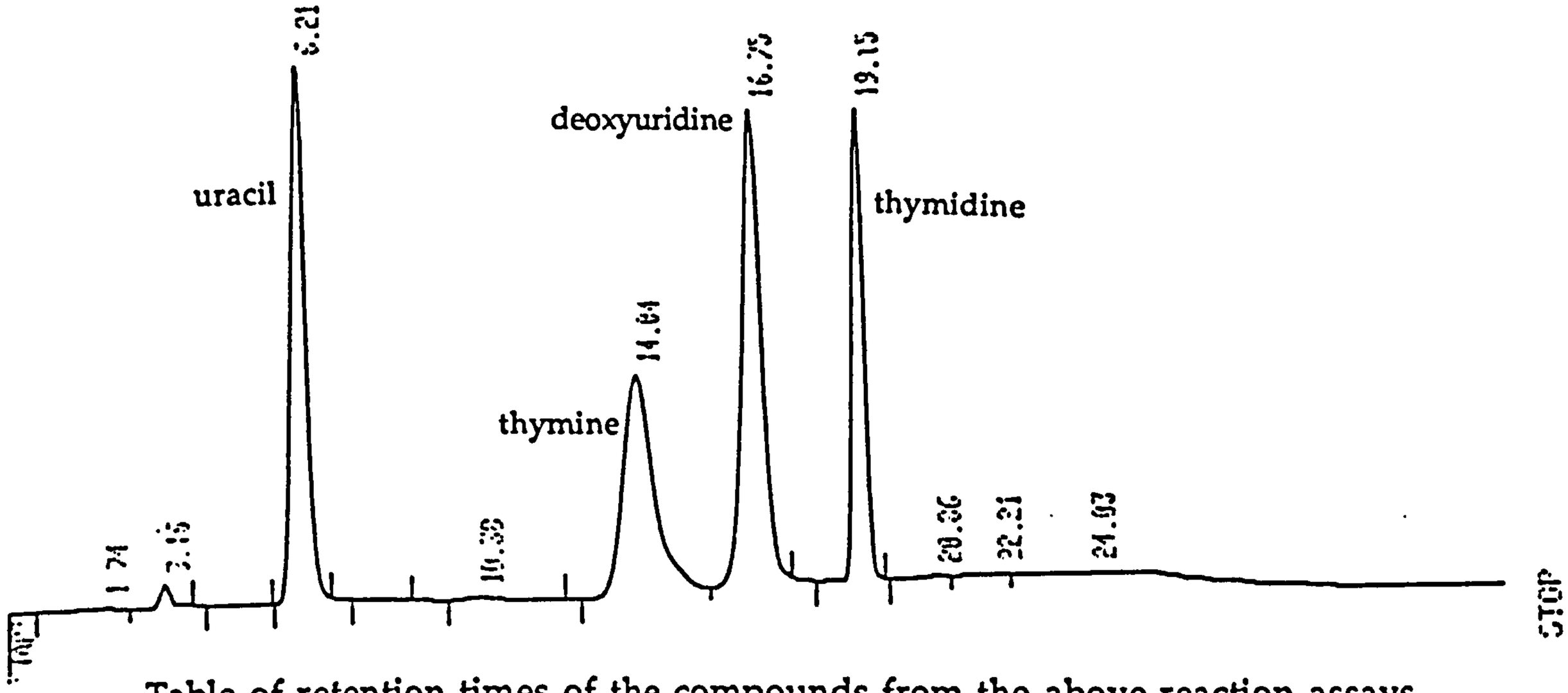


Table of retention times of the compounds from the above reaction assays as mentioned in section 2.2.3.2.

Compound	Retention Time (min)	Reaction
Cytosine	3.98	dC:A
2'-deoxycytidine	4.65	
Adenine	11.64	
2'-deoxyadenosine	15.31	
Hypoxanthine	4.49	dI:A
2'-deoxyinosine	5.97	
Adenine	11.93	
2'-deoxyadenosine	15.62	
Uracil	6.21	dT:Ur
2'-deoxyuridine	16.75	
Thymine	14.04	
2'-deoxythymidine	19.15	

Table of retention times of the compounds separated by HPLC involving nucleoside phosphorylases with the conditions mentioned in section 2.2.3.2.

Compound	Retention Time (min)	Reaction
Hypoxanthine	3.10	Hx:Ino
Inosine	4.38	Hx + R1P ⇌ Ino
Uracil	3.43	Ur:Urd
Uridine	4.87	Ur + R1P ⇌ Urd
Cytosine	7.34	C:Cyt
Cytidine	9.83	C + R1P ⇌ Cyt
Thymine	5.86	dT:T
Thymidine	8.55	dT ⇌ T + R1P

Table of retention times of compounds detected by HPLC as mentioned in section 2.2.5.3.

Compound	Retention Time (min)
2',3'-didehydrodideoxythymidine	25.48
3'-fluorothymidine	27.73
3',3'-difluorothymidine	28.43
6-dimethyladenine	31.80
Thymidine	23.69

References

Avraham, Y., Grossowicz, N., Yashphe, J. (1990) *Biochim. Biophys. Acta.*, **1040**, 287-293.

Avraham, Y., Yashphe, J., Grossowicz, N. (1988) *FEMS Microbiol. Lett.*, **56**, 29-34.

Baltimore, D. (1970) *Nature*, **226**, 1209-1211.

Baranski, K., Bardos, T. J., Bloch, A., Kalman, T. I. (1969) *Biochem. Pharmacol.*, **18**, 347-358.

Barner, H. D., Cohen, S. S. (1954) *J. Bacteriol.*, **68**, 80-88.

Barré-Sinoussi, F., Chermann, J. C., Rey, R., Nugeyre, M. T., Charmanet, S., Gruest, J., Dauget, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C. (1983) *Science*, **220**, 868-871.

Beck, W. S., Goulian, M., Hook, S. (1962) *Biochim. Biophys. Acta.*, **55**, 470-478.

Beck, W. S., Levin, M. (1962) *Biochim. Biophys. Acta.*, **55**, 245-247.

Beck, W. S., Levin, M. (1963) *J. Biol Chem.*, **238**, 702-709.

Betbeder, D., Heath, C. M., Hutchinson, D. W. (1991) *Nucleosides Nucleotides*, **10**, 465-468.

Betbeder, D., Hutchinson, D. W. (1990b) *Nucleosides Nucleotides*, **9**, 569-577.

Betbeder, D., Hutchinson, D. W., Richards, A. O'L. (1989) *Nucleic Acid Res.*, **17**, 4217-4222.

Betbeder, D., Hutchinson, D. W., Richards, A. O'L., Mahood, N., Kinchington, D. (1990a) *Antiviral Chem. Chemother.*, **1**, 249-253.

Blakley, R. L. (1966) *J. Biol. Chem.*, **241**, 176-179.

Blank, J. G., Hoffee, P. A. (1975) *Arch. Biochem. Biophys.*, **168**, 259-265.

Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.

Bzowska, A., Kulikowska, E., Shugar, D. (1990) Z. *Naturforsch.*, **45c**, 59-70.

Cardinaud, R. (1978) *Methods Enzymol.*, **51**, 446-455.

Carson, D. A., Wasson, D. B. (1988) *Biochem. Biophys. Res. Commun.*, **155**, 829-834.

Carson, D. A., Wasson, D. B., Beutler, E. (1984) *Proc. Natl. Acad. Sci.*, **81**, 2232-2236.

Carson, D. A., Wasson, D. B., Kaye, J., Ullman, B., Martin Jr., D. W., Robins, R. K., Montgomery, J. A. (1980) *Proc. Natl. Acad. Sci.*, **77**, 6865-6869.

Cheng, Y.-C., Grill, S. P., Dutschman, G. E., Frank, K. B., Chiou, J.-F., Bastow, K. F., Nakayama, K. (1984) *Antimicrob. Agents Chemother.*, **26**, 283-288.

Cleland, W. W. (1963a) *Biochim. Biophys. Acta.*, **67**, 104-137.

Cleland, W. W. (1963b) *Biochim. Biophys. Acta.*, **67**, 173-187.

Cleland, W. W. (1963c) *Biochim. Biophys. Acta.*, **67**, 188-196.

Cleland, W. W. (1970). Steady state kinetics. The Enzymes. Kinetics and mechanism. New York, Academic Press. Third ed., 1-65.

Cohen, S. S., Barner, H. D. (1957) *J. Biol. Chem.*, **226**, 631-642.

Cook, W. J., Short, S. A., Ealick, S. E. (1990) *J. Biol. Chem.*, **265**, 2682-2683.

Danenberg, P. V., Heidelberger, C. (1973) *J. Med. Chem*, **16**, 712-74.

Danzin, C., Cardinaud, R. (1974) *Eur. J. Biochem.*, **48**, 255-262.

Danzin, C., Cardinaud, R. (1976) *Eur. J. biochem.*, **62**, 365-372.

Davies, G. E., Stark, G. R. (1970) *Proc. Natl. Acad. Sci.*, **66**, 651-656.

DeClercq, E. (1986) *J. Med. Chem.*, **29**, 1561-1569.

DeClercq, E., Deschamps, J., Desomer, P., Barr, P. J., Jones, A. S., Walker, R. T. (1979) *Proc. Natl. Acad. Sci.*, **76**, 2947-2951.

Deibel, R. H., Seeley Jr., H. W. (1974). Gram-positive cocci. Family II. Streptococcaceae. Bergey's Manual of Determinative Bacteriology. Baltimore, Williams & Wilkins Co. Eighth ed., 490-517.

DeMan, J. C., Rogosa, M., Sharpe, M. E. (1960) *J. Appl. Bact.*, **23**, 130-135.

Derse, D., Cheng, Y.-C., Furman, P. A., St. Clair, M. H., Elion, G. B. (1981) *J. Biol. Chem.*, **256**, 11447-11451.

Doskocil, J., Holy, A. (1977) *Collect. Czech. Chem. Commun.*, **42**, 370-383.

Downing, M., Schweigert, B. S. (1956) *J. Biol. Chem.*, **220**, 521-526.

Drueckhammer, D. G., Hennen, W. J., Pederson, R. L., Barbas III, C. F., Gautheron, C. M., Krach, T., Wong, C.-H. (1991) *Synthesis*, **7**, 499-582.

Fischer, M., Short, S. A. (1982) *Gene*, **17**, 291-298.

Fischer, W., Kaun, E., Uwe, G. (1990a) *Ger. Offen.*, DE 3840160 A1.

Fischer, W., Kaun, E., Uwe, G. (1990b) *Internationale Veröffentlichungsnummer*, WO 90/06312.

Frank, K. B., Chiou, J. F., Cheng, Y.-C. (1984) *J. Biol. Chem.*, **259**, 1566-1569.

Friedkin, M., Roberts, D. (1954) *J. Biol. Chem.*, **207**, 245-256.

Furth, J. J., Cohen, S. S. (1968) *Cancer Res.* , **28**, 2061-2067.

Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Olesky, J., Safai, B. (1984) *Science* , **224**, 500-503.

Garces, E., Cleland, W. W. (1969) *Biochemistry* , **8**, 633-640.

Goodman, L. (1974). Chemical syntheses and transformations of nucleosides. Basic principles in nucleic acid chemistry. New York, Academic Press. First ed., 94-208.

Goswami, B. B., Borek, E., Sharma, O. K., Fujitaki, J., Smith, R. A. (1979) *Biochem. Biophys. Res. Commun.* , **89**, 830-836.

Haertle, T., Carrera, C. J., Wasson, D. B., Sowers, L. C., Richman, D. D., Carson, D. A. (1988) *J. Biol. Chem.* , **263**, 5870-5875.

Hanrahan, J. R., Hutchinson, D. W. (1992) *J. Biotech* , **23**, 193-210.

Heath, C. M., "The enzymology of *N*-deoxyribosyltransferase from *Lactobacillus leichmannii*", PhD Thesis, University of Warwick, 1991.

Heidelberger, C., Parsons, D. G., Remy, D. C. (1964) *J. Med. Chem.* , **7**, 1-5.

Heidenreich, O., Kruhøffer, M., Grosse, F., Eckstein, F. (1990) *Eur. J. Biochem.* , **192**, 621-625.

Hennen, W. J., Wong, C.-H. (1989) *J. Org. Chem.* , **54**, 4692-4695.

Herdewijn, P., Balzarini, J., DeClercq, E., Pauwels, R., Baba, M., Broder, S., Vanderhaeghe, H. (1987) *J. Med. Chem.* , **30**, 1270-1278.

Hicks, N., Howarth, O. W., Hutchinson, D. W. (1991) *Carbohydr. Res.* , **216**, 1-9.

Hilbert, G. E., Johnson, T. B. (1930) *J. Am. Chem. Soc.* , **52**, 2001-2007.

Hoffee, P. A., Blank, J. (1978) *Methods Enzymol.*, **51**, 437-442.

Holguin, J., "Etude Generale de la Trans-N-deoxyribosylase", PhD Thesis, Paris-Orsay, 1974.

Holguin, J., Cardinaud, R. (1975) *Eur. J. Biochem.*, **54**, 505-514.

Holguin, J., Cardinaud, R., Salemink, C. A. (1975) *Eur. J. Biochem.*, **54**, 515-520.

Hori, N., Watanabe, M., Yamazaki, Y., Mikami, Y. (1989) *Agric. Biol. Chem.*, **53**, 197-202.

Horwitz, J. P., Chua, J., Noel, M. (1964) *J. Org. Chem.*, **29**, 2076-2078.

Howell, H. G., Brodfuehrer, P. R., Brundidge, S. P., Benigni, D. A., Sapino Jr., C. (1988) *J. Org. Chem.*, **53**, 85-88.

Huang, M.-C., Hatfield, K., Roetker, A. W., Montgomery, J. A., Blakley, R. L. (1981) *Biochem. Pharmacol.*, **30**, 2663-2671.

Huang, M.-C., Montgomery, J. A., Thorpe, M. C., Stewart, E. L., Secrist III, J. A., Blakley, R. L. (1983) *Arch. Biochem. Biophys.*, **222**, 133-144.

Huard, C. N., Hutchinson, D. W. (1992) *Biocatalysis*, **6**, 201-210.

Hutchinson, D. W. (1990) *TIBTECH*, **8**, 348-353.

Imada, A., Igarasi, S. (1967) *J. Bacteriol.*, **94**, 1551-1559.

Imazawa, M., Eckstein, F. (1978) *J. Org. Chem.*, **43**, 3044-3048.

Jeffries, D. J. (1989) *J. Infect.*, **18**, 5-13.

Jensen, K. F. (1976) *Eur. J. Biochem.*, **61**, 377-386.

Jensen, K. F. (1978) *Biochim. Biophys. Acta.*, **525**, 346-356.

Jensen, K. F., Nygaard, P. (1975) *Eur. J. Biochem.*, **51**, 253-265.

Kalckar, H. M., MacNutt, W. S., Hoff-Jørgensen, E. (1952) *Biochem. J.*, **50**, 397-400.

Kalcker, H. M. (1945) *Fed. Proc.*, **4**, 248-252.

Kalcker, H. M. (1947a) *J. Biol. Chem.*, **167**, 477-486.

Kalcker, H. M. (1947b) *J. Biol. Chem.*, **167**, 429-443.

Kamimura, A., Mitsugi, K., Okumura, S. (1973) *Agric. Biol. Chem.*, **37**, 2063-2072.

Kanda, M., Takagi, Y. (1959) *J. Biochem.*, **46**, 725-732.

Kandler, O. (1984) *Ind. Microbiol.*, **25**, 109-123.

Kandler, O., Weiss, N. (1986). Regular nonsporing Gram-positive rods. Bergey's Manual of Systematic Bacteriology. Baltimore, Williams & Wilkins Co. First ed., 1208-1234.

Koszalka, G. W., Vanhooke, J., Short, S. A., Hall, W. W. (1988a) *J. Bacteriol.*, **170**, 3493-3498.

Koszalka, G. W., Krenitsky, T. A., Rideout, J. L., Burns, C. L. (1988b) *Eur. Pat. Appl.*, 0286425 A2.

Krenitsky, T. A. (1967) *Mol. Pharmacol.*, **3**, 526-536.

Krenitsky, T. A., Barclay, M., Jacquez, J. A. (1964) *J. Biol. Chem.*, **239**, 805-812.

Krenitsky, T. A., Freeman, G. A., Shaver, S. R., Beacham III, L. P., Selway, J. W. T. (1983) *J. Med. Chem.*, **26**, 891-895.

Krenitsky, T. A., Koszalka, G. W., Tuttle, J. V., Rideout, J. L., Elion, G. B. (1981a) *Carbohydr. Res.*, **97**, 139-146.

Krenitsky, T. A., Koszalka, G. W., Tuttle, J. V. (1981b) *Biochemistry* , **20**, 3615-3621.

Krenitsky, T. A., Rideout, J. L., Chao, E. Y., Koszalka, G. W., Gurney, F., Crouch, R. C., Cohn, N. K., Wolberg, G., Vinegar, R. (1986) *J. Med. Chem.* , **29**, 138-143.

Krenitsky, T. A., Rideout, J. L., Koszalka, G. W., Inmon, R. B., Chao, E. Y., Elion, G. B., Latter, V. S., Williams, R. B. (1982) *J. Med. Chem.* , **25**, 32-35.

Kulikowska, E., Bzowska, A., Wierzchowski, J., Shugar, D. (1986) *Biochim. Biophys. Acta.* , **874**, 355-363.

Leer, J. C., Hammer-Jespersen, K., Schwartz, M. (1977) *Eur. J. Biochem* , **75**, 217-224.

LePage, G. A. (1970) *Can. J. Biochem.* , **48**, 75-83.

London, J. (1976) *Ann. Rev. Microbiol.* , **30**, 279-301.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) *J. Biol. Chem.* , **193**, 265-275.

Ludwig, W., Seewaldt, E., Kilpper-Bälz, R., Schleifer, K. H., Magrum, L., Woese, C. R., Fox, G. E., Stackebrandt, E. (1985) *J. Gen. Microbiol.* , **131**, 543-551.

MacNutt, W. S. (1952) *Biochem. J.* , **50**, 384-397.

Manson, L. A. (1960) *J. Biol. Chem.* , **235**, 2955-2958.

Manson, L. A., Lampen, J. O. (1951) *J. Biol. Chem.* , **193**, 539-547.

Marsh, J. C., King, M. E. (1959) *Biochem. Pharmacol.* , **2**, 146-153.

Martinez-Murcia, A. J., Collins, M. D. (1990) *FEMS Microbiol. Lett.* , **70**, 73-84.

Minghetti, A. (1960) *Ital. J. Biochem.*, **8**, 224-230.

Mitsuya, H., Broder, S. (1987) *Nature*, **325**, 773-778.

Mitsuya, H., Weinhold, K. J., Furman, P. A., Clair, M. H. S., Lehrman, S. N., Gallo, R. C., Bolognesi, D., Barry, D. W., Broder, S. (1985) *Proc. Natl. Acad. Sci.*, **82**, 7096-7100.

Mitsuya, H., Yarchoan, R., Broder, S. (1990) *Science*, **249**, 1533-1544.

Mitsuya, H., Yarchoan, R., Kageyama, S., broder, S. (1991) *FASEB*, **5**, 2369-2381.

Morisawa, H., Utagawa, T., Miyoshi, T., Yoshinaga, F., Yamazaki, A., Mitsugi, K. (1980) *Tetrahedron Lett.*, **21**, 479-482.

Nasr, M., Litterst, C., McGowan, J. (1990) *Antiviral Res.*, **14**, 125-148.

Neuhoff, V., Stamm, R., Eibl, H. (1985) *Electrophoresis*, **6**, 427-448.

Niedballa, U., Vorbrüggen, H. (1976) *J. Org. Chem.*, **41**, 2084-2086.

O'Donovan, G. A., Neuhard, J. (1970) *Bacteriol. Rev.*, **34**, 278-343.

Orla-Jensen, S. (1919). The lactic acid bacteria. Copenhagen, Anhr. Fred. Høst and Son.

Ostertag, W., Roesler, G., Krieg, C. J., Kind, J., Cole, T., Crozier, T., Gaedicke, G., Steinheider, G., Kluge, N., Dube, S. (1974) *Proc. Natl. Acad. Sci.*, **71**, 4980-4985.

Ott, J. L., Werkman, C. H. (1957) *Arch. biochem. Biophys.*, **69**, 264-276.

Paege, L. M., Schlenk, F. (1952) *Arch. Biochem. Biophys.*, **40**, 42-49.

Parks Jr., R. E., Agarwal, R. P. (1972). Purine nucleoside phosphorylase. The Enzymes. New York, Academic Press. 3rd ed., 483-514.

Rachmeler, M., Gerhart, J., Rosner, J. (1961) *Biochim. Biophys. Acta.*, **49**, 222-225.

Razzell, W. E., Khorana, H. G. (1958) *Biochim. Biophys. Acta.*, **28**, 562-566.

Reynolds, J. A., Tanford, C. (1970) *J. Biol. Chem.*, **245**, 5161-5165.

Rideout, J. L., Krenitsky, T. A., Koszalka, G. W., Cohn, N. K., Chao, E. Y., Elion, G. B., Latter, V. S., Williams, R. B. (1982) *J. Med. Chem.*, **25**, 1040-1044.

Robins, M. J., Robins, R. K. (1965) *J. Am. Chem. Soc.*, **87**, 4934-4940.

Rogosa, M., Franklin, J. G., Perry, K. D. (1961) *J Gen. Microbiol.*, **25**, 473-482.

Roush, A. H., Betz, R. F. (1958) *J. Biol. Chem.*, **233**, 261-266.

Saunders, P. P., Wilson, B. A., Saunders, G. F. (1969) *J. Biol. Chem.*, **244**, 3691-3697.

Sawula, R. V., Zamenhoff, P. J. (1974) *J. Bacteriol.*, **117**, 1358-1360.

Sawula, R. V., Zamenhoff, S., Zamenhoff, P. J. (1975) *Can J. Microbiol.*, **21**, 501-509.

Schaeffer, H. J., Beauchamp, L., DeMiranda, P., Elion, G. B., Bauer, D. J., Collins, P. (1978) *Nature*, **272**, 583-585.

Schleifer, K. H., Kilpper-Bälz, R. (1987) *System. Appl. Microbiol.*, **10**, 1-19.

Schwartz, M. (1971) *Eur. J. Biochem.*, **21**, 191-198.

Schwartz, M. (1978) *Methods Enzymol.*, **51**, 442-445.

Scocca, J. J. (1971) *J. Biol. Chem.*, **246**, 6606-6610.

Scocca, J. J. (1978) *Methods Enzymol.*, **51**, 432-437.

Secrist III, J. A., Barrio, J. R., Leonard, N. J., Weber, G. (1972) *Biochemistry* , **11**, 3499-3506.

Senesi, S., Falcone, G., Mura, U., Sgarrella, F., Ipata, P. L. (1976) *FEBS Lett* , **64**, 353-357.

Sharpe, M. E. (1981). The genus *Lactobacillus*. The Prokaryotes. A handbook on habitats, isolation and identification of bacteria. New York, Springer-Verlag. First ed., 1653-1679.

Shirae, H., Kobayashi, K., Shiragami, H., Irie, Y., Yasuda, N., Yokozeki, K. (1989) *Appl. Environ. Microbiol.* , **55**, 419-424.

Shirae, H., Yokozeki, K., Kubota, K. (1988a) *Agric. Biol. Chem.* , **52**, 295-296.

Shirae, H., Yokozeki, K., Kuboto, K. (1988b) *Agric. Biol. Chem.* , **52**, 1233-1237.

Shirae, H., Yokozeki, K., Kubota, K. (1988c) *Agric. Biol. Chem.* , **52**, 1499-1504.

Shirae, H., Yokozeki, K., Uchiyama, M., Kubota, K. (1988d) *Agric Biol. Chem.* , **52**, 1777-1783.

Shirasaka, T., Murakami, K., Ford Jr., H., Kelley, J. A., Yoshioka, H., Kojima, E., Aoki, S., Broder, S., Mitsuya, H. (1990) *Proc. Natl. Acad. Sci.* , **87**, 9426-9430.

Shugar, D. (1974) *FEBS Lett.* , **40**, S48-S62.

Sidwell, R. W., Huffman, J. H., Khare, G. P., Allen, L. B., Witkowski, J. T., Robins, R. K. (1972) *Science* , **177**, 705-706.

Smar, M., Short, S. A., Wolfenden, R. (1991) *Biochemistry* , **30**, 7908-7912.

Smith, R. G., Gallo, R. C. (1974) *Life Sci.* , **15**, 1711-1730.

Spell Jr., W. H., Dinning, J. S. (1959) *J. Am. Chem. Soc.* , **81**, 3804-3805.

Spencer, R. D., Weber, G., Tolman, G. L., Barrio, J. R., Leonard, N. J. (1974) *Eur. J. Biochem.*, **45**, 425-429.

Stackebrandt, E., Fowler, V. J., Woese, C. R. (1983) *System. Appl. Microbiol..*, **4**, 326-337.

Steenkamp, D. J. (1991) *Eur. J. Biochem.*, **191**, 431-439.

Temin, H. M., Mizutani, S. (1970) *Nature*, **226**, 1211-1213.

Teuber, M., Geiss, A. (1981). The family Streptococcaceae (non-medical aspect). The Prokaryotes. A handbook on habitats, isolation and identification of bacteria. New York, Springer-Verlag. First ed., 1614-1630.

Thelander, L., Reichard, P. (1979) *Ann. Rev. Biochem.*, **48**, 133-158.

Townsend, L. B., Tipson, R. S., Ed. (1986). Nucleic Acid Chemistry. New York, J. Wiley & Sons.

Uerkvitz, W. (1971) *Eur. J. Biochem.*, **23**, 387-395.

Utagawa, T., Morisawa, H., Miyoshi, T., Yoshinaga, F., Mitsugi, K. (1980) *FEBS Lett.*, **109**, 261-263.

Utagawa, T., Morisawa, H., Yoshinaga, F., Yamazaki, A., Mitsugi, K., Hirose, Y. (1985a) *Agric. Biol. Chem.*, **49**, 1053-1058.

Utagawa, T., Morisawa, H., Yamanaka, S., Yamazaki, A., Hirose, Y. (1985b) *Agric. Biol. Chem.*, **49**, 2711-2717.

Utagawa, T., Morisawa, H., Yamanaka, S., Yamazaki, A., Hirose, Y. (1986) *Agric. Biol. Chem.*, **50**, 121-126.

Vorbrügen, H. (1979). Methods of nucleoside synthesis. Nucleoside Analogues: Chemistry, Biology and Medicinal Applications. New York, Plenum Press. First ed., 35-69.

Vorbrüggen, H., Bennua, B. (1978) *Tetrahedron Lett.*, **15**, 1339-1342.

Walker, R. T. (1979). Nucleosides. The synthesis and reactions of organic compounds. Oxford, Pergamon Press. First ed., 53-104.
Weber, K., Osborn, M. (1969) *J. Biol. Chem.*, **244**, 4406-4412.

Weiss, N., Schillinger, U., Kandler, O. (1983) *System. Appl. Microbiol.*, **4**, 552-557.

Witkowski, J. T., Robins, R. K., Sidwell, R. W., Simon, L. N. (1972) *J. Med. chem.*, **15**, 1150-1154.

Wong-Staal, F., Gallo, R. C. (1985) *Nature*, **317**, 395-403.

Wright, L. D., Skeggs, H. R., Huff, J. W. (1948) *J. Biol. Chem.*, **175**, 475-476.

Yamada, E. W. (1968) *J. Biol. Chem.*, **243**, 1649-1655.

Yarchoan, R., Mitsuya, H., Broder, S. (1988) *Sci. Am.*, **259**, 88-97.

Zimmerman, T. P., Gersten, N. B., Ross, A. F., Miech, R. P. (1971) *Can. J. Biochem.*, **49**, 1050-1054.

Zinchenko, A. I., Barai, V. N., Bokut, S. B., Kvasyuk, E. I., Mikhailopulo, I. A. (1990a) *Appl. Microbiol. Biotechnol.*, **32**, 658-661.

Zinchenko, A. I., Barai, V. N., Bokut, S. B., Dudchik, N. V., Belyaeva, Y. V., Mikhailopulo, I. A. (1990b) *Biotechnol. Lett.*, **12**, 341-346.

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