Biosynthetic and enzymic studies on natural products.

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During the course of my studies at Edinburgh I attended bioorganic group seminars and evening postgraduate seminars. I also attended lecture courses on nmr and mass spectrometry, on organic chemistry and on molecular biology organised within the department. I presented work in Edinburgh and at Norwich.

I attended a course on molecular biology at Hatfield Polytechnic and a NATO Advanced Study Institute on the Enzyme Catalysis Process in Italy.

I attended the First International Symposium on the separation of Chiral Molecules in Paris, the RSC Annual Chemical Congress at Canterbury, Kent in 1988, the annual meeting of the Society for General Microbiology in Southampton in 1988, a meeting on the Kinetic Analysis of Biological Systems in Bristol in 1987, the Second International Natural Products Symposium in Nottingham in 1986 and the Natural Product Chemistry meeting at Heriot Watt University, Edinburgh in 1987.

I presented posters on the work contained in this thesis in Italy, Bristol, Nottingham and Edinburgh.

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I confirm that this thesis has been composed by myself and that the work contained in it is my own.

In this thesis three aspects of the biosynthesis of natural products have been considered. In the first case, stable isotope labelling has been used in conjunction with nmr spectroscopy to study the biogenesis of 3-nitropropanoic acid from L-aspartic acid and the post biosynthetic cycling of the former compound with 3-nitroacrylic acid. The intermediacy of 2-nitrosuccinic acid has been demonstrated using ^{15}N labelling. Chiral deuterated and ^{13}C-labelled L-aspartic acids have been prepared and their stereochemical fate determined and discussed in terms of the likely mechanism of formation of 3-nitropropanoic acid. The use of chiral amide derivatives to distinguish pro R and pro S deuteration by deuterium nmr and the secondary isotope effect of deuterium on the ^{13}C nmr resonance of the metabolite were used. Finally, the role of 3-nitroacrylic acid was determined to be that of a post biosynthetically cycled product rather than an intermediate and these findings have been discussed in the context of the post biosynthetic cycling of 3-nitropropanoic acid and its unsaturated derivative.

In the second study the biosynthesis of the pyrimidine ring of thiamine (Vitamin B_1) in Saccharomyces cerevisiae has been studied using stable isotopes in conjunction with m.s. Since the metabolite is only produced in microgram amounts, a method has been developed for the cleavage of thiamine using the enzyme thiaminase I from Bacillus thiaminolyticus. This method was then applied to a stable isotope investigation which demonstrated that amination of the pyrimidine ring at the 4 position occurs prior to ring formation. A precursor which contains all the necessary nitrogen atoms is thereby implicated and the results have been discussed in terms of potential biosynthetic precursors.

Finally, the cleavage of DNA in a low water environment containing an ionic surfactant has been studied. While restriction has been shown to occur using a range of restriction enzymes, some apparent increase in specificity as evidenced by reproducible cleavage at certain nominally equivalent sites and not at others has been observed. Preliminary studies on the structure of the low water system suggest that the DNA may be constrained and the implications for the restriction enzyme cleavage of any modification in DNA structure have been considered. The potential of increased specificity for isolation of specific DNA fragments and its likely implication for the cloning of enzymes has been discussed.

CONTENTS

Introduction

The study of the biosynthesis of primary and secondary metabolites is crucial to an understanding both of how biologically important products are formed and, perhaps more importantly, of how such pathways and products can be manipulated in order to facilitate improved yields, better growth and even, in some cases, novel product formation[1].

Traditional studies of the biosynthetic pathways in both prokaryotes and eukaryotes concentrated upon feeding radiolabelled early precursors and isolating the required product[2]. A complex degradation pathway is then necessary in order to determine the precise site of incorporation of labelled atoms in the compound of interest. This, essentially chemical approach, yielded a great deal of information concerning the biosynthetic interrelationships between compounds and groups of compounds.

The next stage in this strategy was the synthesis of putative intermediates containing a radioactive label which could then be fed and their incorporation tested as above[3]. By such methods, details of the major biosynthetic pathways were elucidated and educated estimates made as to likely biosynthetic intermediates. While this approach is sensitive, general, powerful and provides substantial insight into the route of formation of naturally-occurring compounds, a number of limitations - both in a practical sense and inherent in the technique - are clearly present. First of all, while multiple-labelled compounds can be used, their analysis provides no information on the biosynthetic relationship of one atom to another throughout the pathway, i.e. there is no way of determining bond

formation, bond breaking or bond retention between any two atoms in a biosynthetic pathway. For this reason, while a mechanism of formation of a compound can often by inferred from the data obtained in radiochemical experiments, proof of the proposed mechanism is rarely possible except in cases where stereospecific tritium loss or retention can be demonstrated. Perhaps more crucially, there are no suitable radioisotopes of nitrogen available and it is, to all intents and purposes, invisible to radiochemical techniques. On a practical level, the synthesis of specifically single- or multiple-radiolabelled compounds can pose considerable laboratory problems and the complex degradation pathways which are necessary in order to determine the site of labelling are frequently inconvenient and technically demanding.

The advent of high resolution nuclear magnetic resonance (nmr) and mass spectrometry (ms) opened up a new potential avenue of research. Biosynthetic precursors and intermediates could be prepared which contained a non-radioactive isotope (^{13}C, ^{2}H, ^{15}N) and the position of the label in the product could then be determined directly[4]. Furthermore, both nmr and ms can be used to determine multiple incorporation directly. The former is particularly powerful for determining not only the site of incorporation, but also the incorporation (or otherwise) of adjacent atoms. Thus, for example, stereospecific deuterium incorporation can be determined directly by preparation of suitable chiral analogues[4] and the presence of ^{2}H, ^{15}N or ^{18}O adjacent to ^{13}C can be determined directly by consideration of the ^{13}C nmr resonance of the carbon of interest[5]. A considerable volume of very valuable and specialised work has been, and continues to be carried out using stable isotopes in conjunction, principally, with nmr and, to a lesser extent, with ms. In addition, the handling

and preparation of precursors and intermediates which contain a stable isotopic label is far easier practically than the corresponding radiolabelled compound.

There are, however, a number of limitations which are principally a consequence of the sensitivity of the detection systems used. Nmr is basically an insensitive technique which requires milligram amounts of material and low dilution of isotopic enrichment in order that the effects can be visualised in the spectrum. This precludes the study of many important compounds which are produced in only small amounts and intermediates which are poorly incorporated either per se or as a result of the form in which they are presented to the organism under investigation. Ms is substantially more sensitive than nmr with microgram amounts of material often sufficient and thus suitable for measurements of isotopic enrichments where only small amounts of material are available. This is still considerably more than is required for radiochemical studies. In addition, despite recent advances in soft ionisation techniques such as chemical ionisation (CI) and fast atom bombardment (FAB), a significant number of compounds are simply not amenable to analysis using ms. A further limitation is imposed because ms, unlike nmr, is not a selective technique - confining its application to situations where the dilution of isotopic species is low. The information provided by ms is, even in the best designed experiments, generally much less than that available from nmr studies.

In parallel with the chemical approach to the study of biosynthesis, a biological approach has also developed. Early studies involved the isolation of species which were deficient in sequential parts of biosynthetic pathways and which would therefore accumulate certain

intermediates. The creation and characterisation of mutants and the examination of their accumulated intermediates from incomplete biosynthetic pathways, remains a very powerful weapon in bioorganic chemistry[5]. Such studies are largely haphazard in nature, with the control being exercised at a later stage during the assay and selection of mutants. Frequently, however, suitable assay systems are difficult to design or the biosynthetic intermediate from an incomplete pathway is often not simply accumulated in a convenient form. In an ideal situation, the enzyme which carries out a specific transformation in a pathway will accumulate - together with its product - in the cell. More usually, the enzymes of interest are only produced in extremely small amounts. Under these conditions, recent advances in molecular biology offer an opportunity to investigate the reactions catalysed by these enzymes in detail. The gene which codes for the enzyme is isolated then cloned into a suitable piece of carrier DNA (vector). This conjugate is then introduced into the cell - where in some cases it may be produced in multiple copies - and the protein subsequently overproduced[6-7]. The overproduced enzyme can then be isolated using traditional protein purification techniques and its activity, substrate specificity and mechanism of action investigated in detail <u>in vitro</u>[5,8]. There are some difficulties in this latter approach. A great deal of preliminary work must be done in order to isolate the gene of interest. In many cases, simple strategies for cutting and joining the appropriate pieces of DNA to a suitable vector do not exist and other more complex techniques must be employed[9]. The overproduced protein may be difficult to isolate in a highly purified state and may be unstable to the purification conditions which must be employed. Nonetheless, allowing for these disadvantages, the possibilities for rapidly increasing knowledge of existing and future

interesting pathways makes this a method which should be considered to be essential to the future direction of bioorganic chemistry.

This thesis investigates three different methodological approaches to the study of biosynthetic pathways in prokaryotes, eukaryotes and *in vitro*. The biosynthesis of 3-nitropropanoic acid, a fungal metabolite, is investigated using stable isotopes and, principally, nmr. The pyrimidine ring of thiamine (vitamin B_1) is studied using ms. Finally, the *in vitro* manipulation of DNA in a novel environment is considered.

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Chapter 1

1. 3-Nitropropanoic acid - Biosynthesis

1.1 Introduction

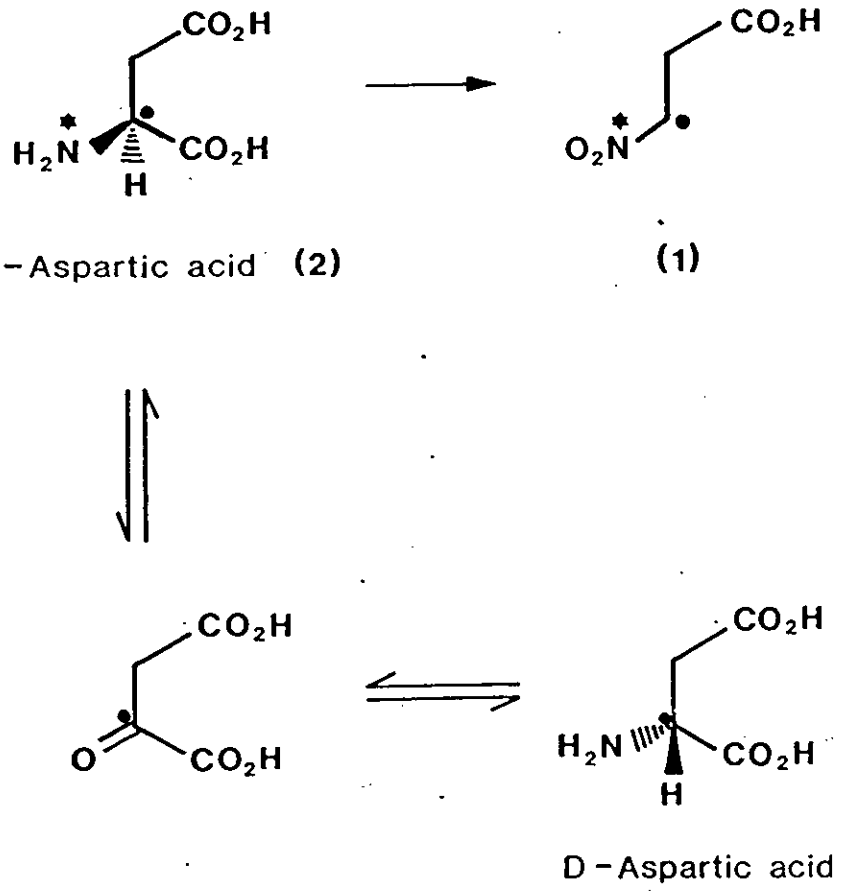
3-Nitropropanoic acid (1) is a toxic compound found in plants of the Fabaceae family[1] and in certain fungi[2]. It has been shown to inhibit mammalian succinate dehydrogenase irreversibly and fumarase reversibly[3,4,5]. The principal toxicity appears to occur as a result, therefore, of depression of energy metabolism through tricarboxylic acid cycle inhibition. Indeed, it has been suggested[6] that administration of 3-nitropropanoic acid (1) may serve as a useful model of brain damage following energy deficiency.

In plants, it occurs as a glycoside (hiptagin) from which 3-nitropropanoic acid (1) can be readily released by hydrolysis[7]. The glycoside was first isolated in 1920 from Hiptage mandablota Gaertn[8]. and later from Carynocarpus laevigata[9]. Acid hydrolysis of the glycoside gave hiptagenic acid (3-nitropropanoic acid), carbon dioxide, ammonia and a sugar residue[9]. In contrast to fungi, the biosynthesis of 3-nitropropanoic acid in plants has been relatively little studied. In indigo (Indigofera spicata, (Jacq.) Forsk.) malonic acid was found to be a precursor as was malonylmonohydroxamate[10]. The label from [2-^{14}C]-malonic acid was located in both C-2 and C-3 of the isolated 3-nitropropanoic acid and the cycling of malonic acid with oxaloacetic acid in the roots was suggested. There was no evidence of incorporation of [3-^{14}C]-L-aspartic acid or of constituents of the tricarboxylic acid cycle in 3-nitropropanoic acid (1) in indigo[10].

In fungi, the free acid is produced in large amounts (2.4g per litre of medium in the case of Penicillium atrovenetum)[12]. Raistrick and Stossl also found that the acid was formed in the earliest stages of growth then reached a maximum before subsequent decline[12].

3-Nitropropanoic acid (1) may be an intermediate in a fungal nitrification process where reduced organic nitrogen compounds are oxidised rather than nitration occurring directly[12]. This is analogous to the oxidation of ammonia to nitrite carried out by Nitromonas bacteria[13]. Ammonium salts were found to stimulate production while nitrate appeared to be inhibitory[12]. This early study was followed by an investigation by Birch et al.[14] on the biosynthetic precursors of 3-nitropropanoic acid (1) in P. atrovenetum in which [4-^{14}C]- aspartic acid was implicated as a direct precursor. The same workers noted that [1-^{14}C]-β-alanine was not incorporated while [^{14}C]-sodium bicarbonate was also a precursor of the carboxyl carbon atom. Gatenbeck and Forsgren showed[15] that uniform labelled [^{14}C]- aspartic acid was incorporated in a uniform fashion and this was suggested to implicate a dicarboxylic acid via the tricarboxylic acid cycle. Previously Hylin and Matsumoto[16], in a non-labelled investigation, studied the effectiveness of various carboxylic acids as precursors of 3-nitropropanoic acid (1). They found that maximal production required the presence of ammonium ion and a suitable, four-carbon, carboxylic acid. They implied, from these results, that, while aspartic acid (2) may be a precursor, it is effectively deaminated prior to incorporation and the dicarboxylic acid thereby formed enters the general acid pool and is subsequently incorporated. In addition, it was suggested that hydroxylamine addition to fumaric acid (3) (figure 1.7) may be a viable pathway.

Figure 1.1 **BIOSYNTHESIS OF 3-NITROPROPANOIC ACID (1)**



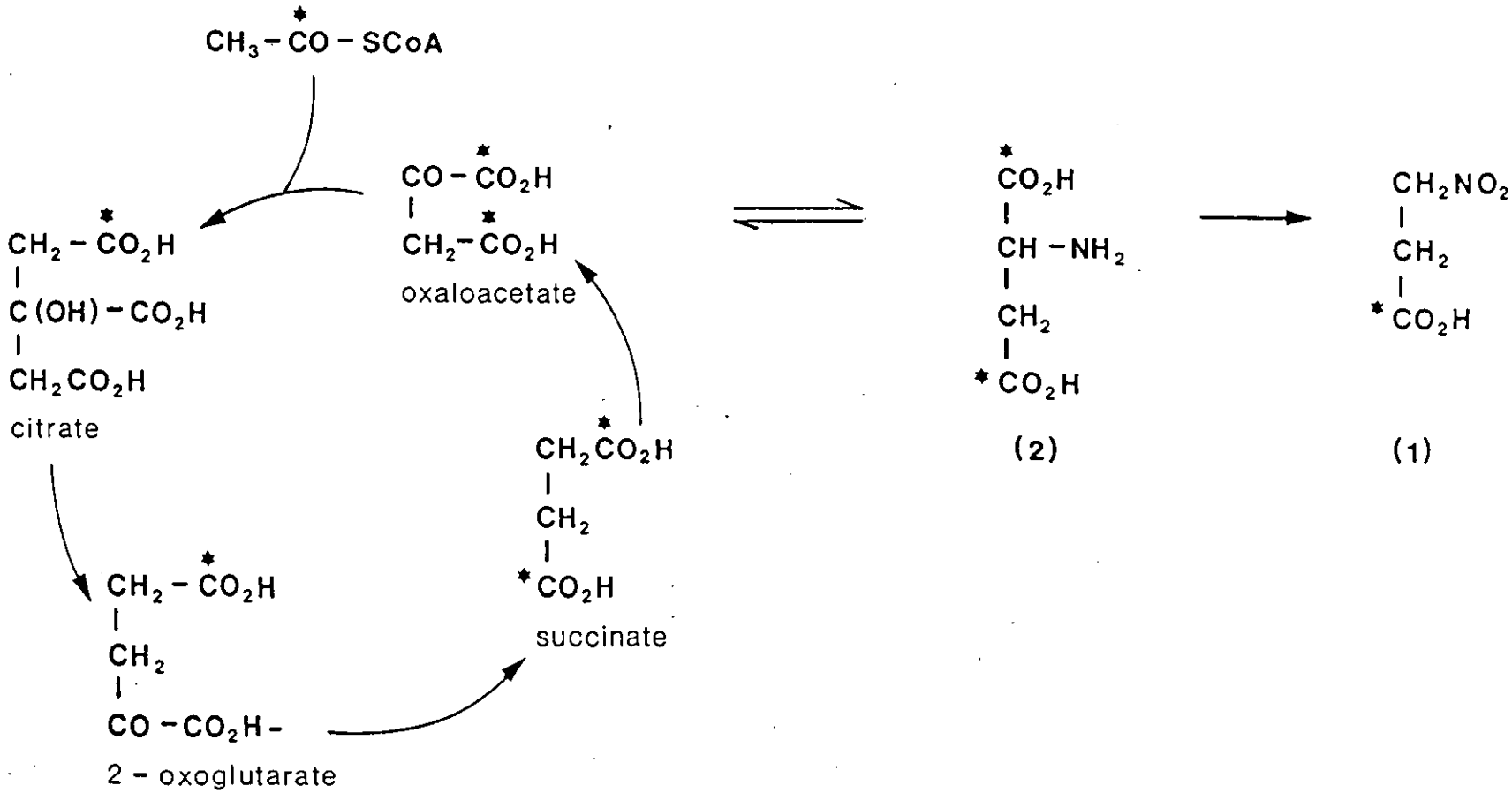
L-Aspartic acid (2) (1)

D-Aspartic acid

Such speculation was largely terminated by the double-labelled study of Gatenbeck and Forsgren[15] which showed that [^{15}N, U-^{14}C]-aspartic acid was incorporated suggesting that the amino group is oxidised *in situ*. Transamination exchange of the nitrogen of aspartic acid with ammonium ions in the medium served to complicate matters, however the results were sufficiently unequivocal for the intact incorporation of aspartic acid (2) to be generally accepted. This matter was not finally settled however until the nmr study of Baxter *et al*[17] who showed that [2-^{13}C, ^{15}N]-aspartic acid was incorporated with conservation of the carbon-nitrogen bond. The presence of doubly-labelled 3-nitropropanoic acid (1) was determined from the ^{15}N coupled ^{13}C nmr spectrum. These authors also found an upper limit for exchange of nitrogen from aspartic acid (2) to the medium of *ca* 20%; moreover the lack of enhancement of the C-1 and C-2 signals in the isolated 3-nitropropanoic acid (1) eliminated the possibility of randomisation of the label. Since DL-aspartic acid was fed and the D form is incorporated *via* oxaloacetic acid (with concommitant loss of the ^{15}N label), it is clear that equilibration of the ^{13}C label into C-2 and C-3 of oxaloacetic acid through malate dehydrogenase and aspartase activity cannot occur (Fig. 1.1). Similarly, randomisation through the tricarboxylic acid cycle is also eliminated as a possibility.

These results substantiate the earlier findings from ^{14}C labelled pyruvic and acetic acid feeding studies[18]. In this work, [2-^{14}C] pyruvic acid was shown to be incorporated equally into C-1 and C-3 with C-2 being about half of this. Such a result is predicted if pyruvate is converted to oxaloacetic acid and hence *via* aspartic acid (2) to 3-nitropropanoic acid (1). If a symmetrical, dicarboxylic acid was involved then equal labelling of C-2 and C-3 in the product

Figure 1.2 INCORPORATION OF [1-^{14}C]-ACETATE INTO 3-NITROPROPANOIC ACID (1)



would be expected. Examination of the pyruvate-derived aspartic acid in the medium showed that, after subtraction of the contribution from C-4, the specific radioactivity and distribution was almost identical with that of the isolated 3-nitropropanoic acid (1). Similar results with specifically labelled acetic acid (see Fig. 1.2) indicate that it too, is incorporated *via* oxaloacetic acid after metabolism through the tricarboxylic acid cycle.

It should, perhaps, be noted at this juncture, that, in the vast majority of cases, feeding of labelled precursors was carried out either at the time of innoculation, in the lag phase or early in the log phase of growth and isolation of 3-nitropropanoic acid (1) was carried out once the stationary phase had been reached. The significance of this point will become clear in the later discussion.

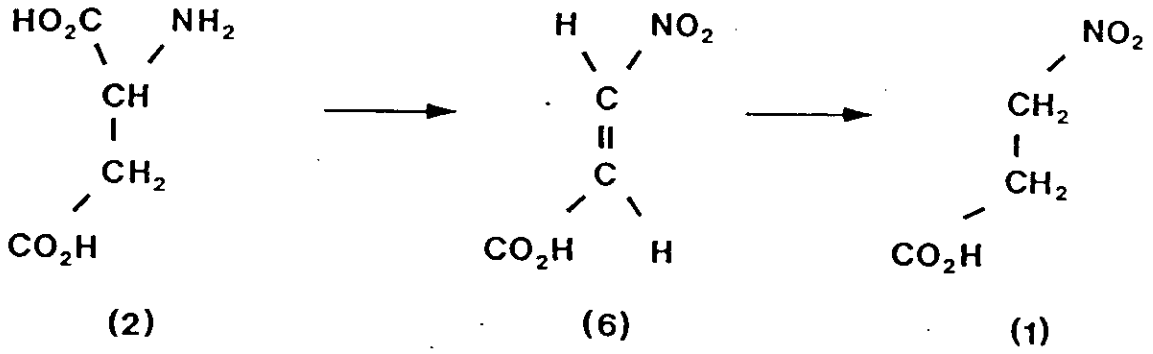
While *in situ* oxidation of the amino functionality in aspartic acid (2) is the biosynthetic route of choice, the nature of the intermediates remains unclear. Hydroxylamine has been detected in the culture medium[15], although addition of this compound did not promote production of 3-nitropropanoic acid (1) due, presumably, to the toxicity of hydroxylamine. Gatenbeck and Forsgren[15] also detected nitrite in the culture medium although neither 3-nitrososuccinic acid nor β-oximinopropionic acid were found to stimulate production. Both nitrite and nitrate are produced from 3-nitropropanoic acid (1) by an enzyme system isolated from *P. atrovenetum* and production is accompanied by a loss of the acid in the medium in the post-stationary phase of growth[19]. Inorganic, oxidised nitrogen compounds appear to be products from the subsequent breakdown of 3-nitropropanoic acid (1) rather than biosynthetic intermediates.

In contrast to the majority of the above results, Shaw and Wang[20] reported that neither β-alanine (4) nor aspartic acid (2) alone stimulated production of 3-nitropropanoic acid (1). These authors suggested an inorganic nitrogen precursor was implicated and found production to be stimulated both by hydroxylamine and nitrite. It should be noted, however, that, in contrast to other studies, these workers used shake flasks rather than surface cultures and the results were later contradicted by the same author[21] who suggested the composition of the medium to be of prime importance. Clearly ammonium ion can be incorporated *via* transamination with oxaloacetic acid, but this has been found to occur only to a limited extent.

A further and related point of interest in the biosynthesis of 3-nitropropanoic acid (1) is the derivation of the nitro oxygen atoms. Shaw and McCloskey have shown[21] that [^{18}O]- nitrate is not incorporated and that exogenous hydroxylamine does not depress the incorporation of [^{15}N]- aspartic acid. Lack of incorporation of [^{18}O]- nitrate may be due to oxygen exchange of some intermediate with water. This question has recently been answered in a further nmr study by Baxter and Greenwood[22]. In this investigation, cultures of *P. atroventum* were grown in an atmosphere of $^{18}O_2/^{16}O_2$ and with [^{15}N]- ammonium chloride in the medium. The proton decoupled ^{15}N nmr spectrum showed three resonances corresponding to $^{15}N^{16}O_2$, $^{15}N^{16}O^{18}O$ and $^{15}N^{18}O_2$ indicating that both oxygen atoms of the nitro group derive from dioxygen. This suggests involvement of one or two dioxygenase enzymes in the biosynthesis[23].

The isolation of a NADPH-dependent "reductase" enzyme from *P. atrovenetum* which catalyses the conversion of 3-nitroacrylic acid (6) to 3-nitropropanoic acid (1)[24] implicated the former as a

Figure 1.3 **PROPOSED BIOSYNTHESIS OF 3-NITROPROPANOIC ACID (1)**

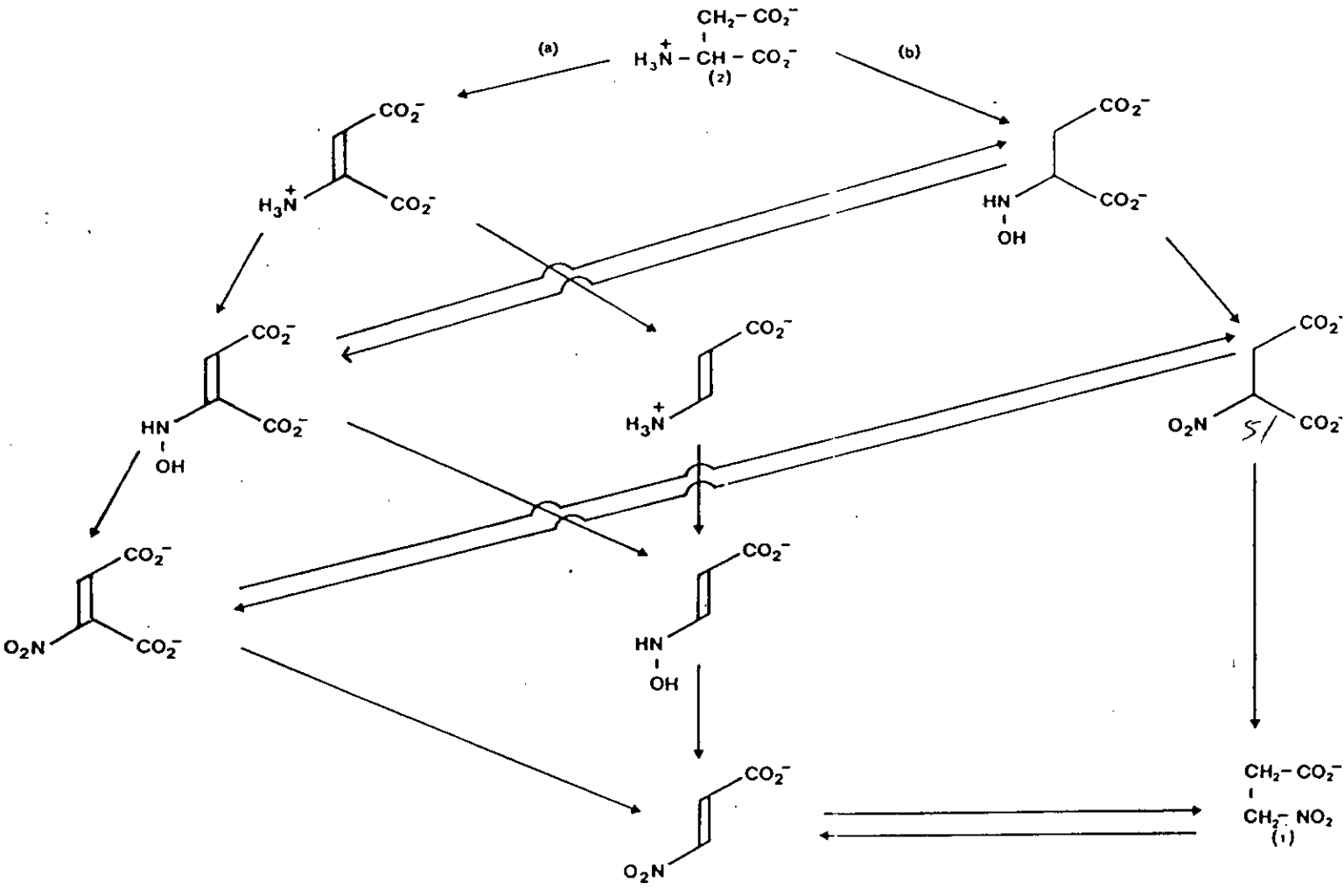


biosynthetic precursor (Fig 1.3). While 3-nitroacrylic acid (6) is toxic to the culture (being, presumably, an inhibitor of succinate dehydrogenase - an activity which 3-nitropropanoic acid (1) itself exhibits in vitro), at low levels it reduces the production of 3-nitropropanoic acid (1) and, furthermore, completely inhibits incorporation of [4-^{14}C]-aspartic acid into (1). In the presence of unlabelled aspartic acid, [1-^{14}C]-3-nitroacrylic acid (6) was incorporated by cells of P. atrovenetum (albeit only one third of the expected incorporation). The fact that the isolated enzyme, "3-nitroacrylate reductase", is unable to catalyse effectively the reverse reaction, oxidation of (1) to (6), has been interpreted as evidence of a precursoral relationship.

The primary precursor of 3-nitropropanoic acid (1) appears, therefore, to be L-aspartic acid (2), the nitro oxygen atoms are derived from molecular oxygen and the nitrogen is oxidised in situ with retention of the carbon-nitrogen bond. Additionally, Shaw et al[24] have shown that 3-nitroacrylic acid (6) may be an intermediate.

The information available from previous studies, summarised above, eliminates a number of possible routes between L-aspartic acid (2) and 3-nitropropanoic acid (1). Nonetheless, a number of potential pathways and stereochemical possibilities remain viable and some of these are outlined in scheme 1.1

Each of the transformations depicted has some analogy with known secondary metabolism pathways. Dehydroamino acids, for example, have been reported as fungal metabolites[2,25] and intermediates in the biosynthesis of certain cyclic and depsipeptides including the



Scheme 1.1

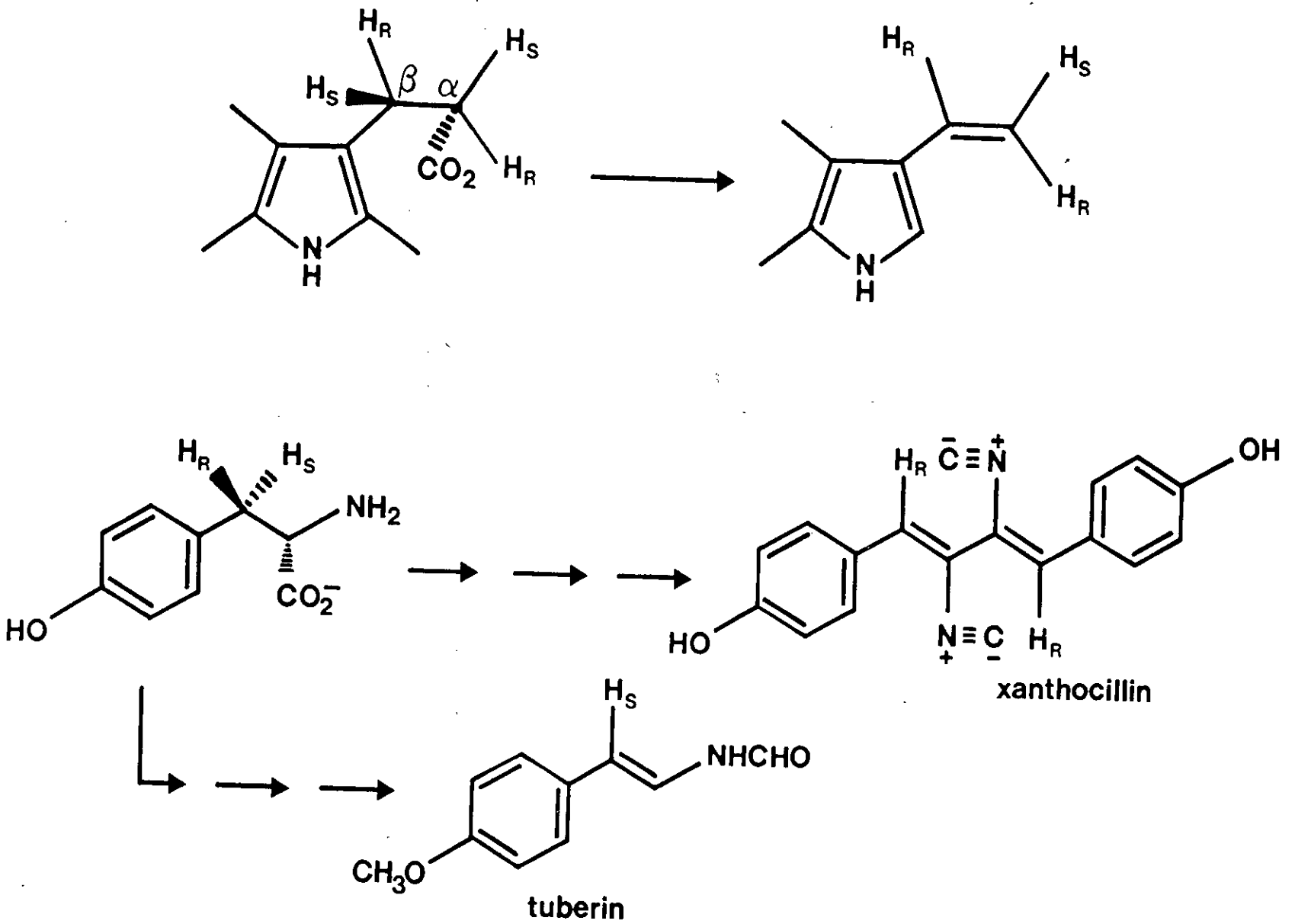
commercially important antibiotic nisin from Streptococcus lactis and the related subtilin from Bacillus subtilis[26].

The failure to incorporate β-alanine precludes decarboxylation as a first step in the sequence however it could occur at a number of oxidation levels as shown in scheme 1.1. In addition, several different mechanisms of decarboxylation are possible.

No definitive literature precedent for the decarboxylation of a dehydroamino acid is known, however a pyridoxal phosphate mechanism analogous to that involved in α-amino acid decarboxylation is possible which would give dehydroβ-alanine. Oxidation to the corresponding N-hydroxy dehydroamino acid and thence to 3-nitroacrylic acid (6) would afford a known in vitro precursor.

A similar oxidative pathway can occur with decarboxylation of N-hydroxy aspartic acid or of nitrosuccinic acid and several types of decarboxylative mechanism can be envisaged. Decarboxylation could occur directly, generating a carbanion intermediate which is then quenched by proton abstraction from water. This reaction could proceed with either retention or inversion of stereochemistry at the position derived from C-2 of L-aspartic acid. An analogy for direct decarboxylation can be found in the reversible action of propionyl CoA carboxylase which proceeds with overall retention of configuration[27]. In contrast, decarboxylation of UDP-glucuronate occurs with inversion of configuration at C-2[28].

An alternative mechanism which involves concerted abstraction of a β-proton to give an acrylate is also possible. This mechanism


HR
HS
β
α
HS
CO2
HR
N
H
HR
HS
HR
N
H
HR
HS
NH2
CO2−
HO
HR C≡N
OH
HO
N≡C
HR
xanthocillin
HS
NHCHO
CH3O
tuberin

Scheme 1.2

involving antiperiplanar elimination of hydrogen and the carboxyl functionality is analogous to that involved in the generation of the ethylidene side chain in porphyrin biosynthesis where the pro-S β proton is abstracted (Scheme 1.2)[29]. Herbert[30] has proposed a similar mechanism for the decarboxylation of L-tyrosine in the biosynthesis of xanthocillin and tuberin (Scheme 1.2). In the former case the 3-pro-S proton is lost while in the latter it is retained and the 3-pro-R is lost.

The work by Shaw[24] on the in vitro enzymic reduction of 3-nitroacrylic acid (6) to 3-nitropropanoic acid (1) suggests that a concerted decarboxylation mechanism is possible, the reduction of 3-nitroacrylic acid (6) could also be interpreted as evidence of a post-biosynthetic process akin to the metabolism of (1) in mammalian tissue in which 3-nitropropanoic acid (1) and its dehydro derivative are in equilibrium[3-5].

A strategy was developed for delineating which of the pathways in scheme 1.1 was involved and hence which of the mechanisms discussed above is implicated. It is essential, in the first instance, to determine at which stage decarboxylation of L-aspartic acid occurs. The incorporation of 2-nitrosuccinic acid (5) into 3-nitropropanoic acid (1) would preclude decarboxylation at a lower oxidation state and eliminate a number of possible pathways outlined in scheme 1.1 therefore this compound was evaluated first.

1.2 Nitrosuccinic acid (5)

1.2.1 Synthesis and feeding

Attempts to synthesise 2-nitrosuccinic acid (5) directly by nitration

of bromosuccinic acid were unsuccessful. Nitrite esters are an important side product in the reaction of sodium nitrite with primary alkyl bromides and phloroglucinol has been used to minimise ester production[31]. Bromosuccinic acid was treated with ethanolic HCl to give the diethylester which was then nitrated with [^{15}N]-sodium nitrite in dimethylsulphoxide in the presence of phloroglucinol giving [^{15}N]-DL-nitrosuccinic acid diethylester (20). Attempts to hydrolyse the ester gave only a poor yield of the required product. The diester (20) was therefore pulse fed to surface cultures of P. atrovenetum over 36 hours beginning 48 hours after innoculation. It was hoped that by adopting this protocol to obtain maximum incorporation with minimum dilution of the label resulting from metabolism of 3-nitropropanoic acid (1).

1.2.2 Results

The proton decoupled ^{15}N DEPT (distortionless enhanced polarisation transfer) nmr spectrum[32] of the isolated 3-nitropropanoic acid (1) showed an intense signal at 3.1 ppm with a fourteen fold increase in intensity over natural abundance. Incorporation was confirmed by the occurrence of ^{15}N satellites of both methylene signals in the ^{1}H nmr of the isolated 3-nitropropanoic acid (^{2}J NH 2.2 Hz, ^{3}J NH 3.7Hz) of an intensity corresponding to a twenty-fold dilution of the ^{15}N enrichment from that of the racemic diester.

1.2.3 Discussion and conclusions

The incorporation of [^{15}N]-DL-diethyl nitrosuccinic acid (20) suggests that the biosynthesis of 3-nitropropanoic acid (1) involves direct oxidation of L-aspartic acid (2). This study does not preclude

the possibility that N-hydroxy aspartic acid is also an intermediate but it does show that complete oxidation of the amino group of L-aspartic acid to a nitro group must occur prior to decarboxylation.

It has been observed that the C-N bond is conserved in the biosynthesis of 3-nitropropanoic acid (1) from L-aspartic acid (2) thus the biosynthetic pathway must proceed from L-aspartic acid (2) to nitrosuccinic acid (5) (perhaps via some other oxidised species) and thence, after decarboxylation, to 3-nitropropanoic acid (1). Furthermore, the non-intermediacy of β-alanine is confirmed.

1.3 Stereochemistry of nitrosuccinic acid incorporation

While nitrosuccinic acid (5) has been shown to be an intermediate, its stereochemistry remains unclear. The incorporation of deuterium atoms from L-[2,3,3-$^2H_3$]-aspartic acid (19) will serve to further delineate which of the pathways shown in scheme 1.1 is correct.

1.3.1 Feeding

L-[2,3,3-$^2H_3$]-Aspartic acid (19) was administered to cultures of P. atrovenentum in three equal amounts (8 mg) at 42, 48 and 54 hours after inoculation. 3-Nitropropanoic acid (1) was isolated after 72 hours as described in the experimental section.

1.3.2 Results and discussion

The ^2H nmr spectrum of 3-nitropropanoic acid (1) derived from the feeding of perdeuterated L-aspartic acid (19) showed two peaks at

2.97 ppm and 4.69 ppm due to deuterium incorporation at C-2 and C-3 respectively. Deuterium is therefore retained at both C-2 and C-3 positions of 3-nitropropanoic acid (1). If inversion of stereochemistry had occurred at the carbon derived from C-2 of L-aspartic acid (2) during the course of formation of nitrosuccinic acid then no retention of deuterium at the 3 position of the metabolite (1) would be expected. It follows, then, that the stereochemistry of L-aspartic acid is retained in the oxidation which gives nitrosuccinic acid. This, together with the previous result from ^{15}N-nitrosuccinic acid (5), implies that the biosynthetic pathway involves oxidation of L-aspartic acid (2) to give L-nitrosuccinic acid prior to decarboxylation. The retention of H-2 of L-aspartic acid (2) eliminates the possible intermediacy of dehydroaspartic acid derived intermediates (route (a) of Scheme 1.1) in the biosynthesis. The mechanism and stereochemistry of the decarboxylation however remains unclear.

1.4 Mechanism of decarboxylation

To evaluate which of the two plausible decarboxylation mechanisms (direct decarboxylation or decarboxylation with loss of a β-hydrogen yielding an acrylate intermediate) is involved, the retention of the hydrogen atoms from C-3 of L-aspartic acid (2) in the biosynthetic conversion was examined. Measurement of hydrogen retention or loss can be determined by making use of the β-isotope shift effect on the ^{13}C nmr signal caused by deuteration on an adjacent C atom[42]. Each deuterium atom shifts the position of resonance of a directly attached carbon nucleus by 0.3 to 0.6 ppm upfield. A deuterium which is two bonds away has a similar, though smaller (ca 0.01-0.1 ppm per

deuterium) effect[43], although variability has been noted especially for carbonyl carbon atoms[44]. The multiplicity of carboxyl ^{13}C resonances can thus be used to determine the extent of deuteration at C-2. The incorporation of deuterium from L-[4-^{13}C, 3-$^{2}H_2$]-aspartic acid would determine whether an acrylate intermediate is involved in the biosynthesis of 3-nitropropanoic acid (1) from L-nitrosuccinic acid (5).

1.4.1 Synthesis and feeding

DL-[4-^{13}C, 3-$^{2}H_2$]-Aspartic acid (19) was prepared by treatment of the methiodide of diethyl α-formamido-α-dimethylaminomethylmalonate with $Na^{13}CN$ followed by acid hydrolysis in ^{2}HCl. Acetylation followed by methylation of the acid groups afforded DL-[4-^{13}C, 3-$^{2}H_2$]-N-acetyl dimethyl aspartic acid. Examination by nmr suggested a deuterium content of 1.7 per molecule with 85% $^{2}H_2$ and 15% $^{2}H_1$ species.

DL-[4-^{13}C, 3-$^{2}H_2$]-aspartic acid (19) (24 mg) was pulse fed to growing cultures of P. atrovenetum and 3-nitropropanoic acid (1) isolated after 96 hours.

1.4.2 Results and discussion

The ^{13}C nmr resonance of the carboxyl carbon of the isolated 3-nitropropanoic acid (1) showed two distinct peaks at 175.934 ppm and 175.925 ppm - a difference of 0.009 ppm. These correspond to d_o and d_1 species. The difference between d_o and d_2 at the 2 position in [1-^{13}C]-3-nitropropanoic acid was determined elsewhere to be of

the order of 0.025 ppm (vide infra). The loss of a single deuterium implies a concerted loss of H and the carboxyl group thus suggesting that an acrylate intermediate is involved.

Earlier work on tuberin and xanthocillin biosynthesis discussed above[30] has determined that the loss of β-proton is a stereoselective process. A similar mechanism may be involved in this case, and if so, we would expect either the 3-pro-R or the 3-pro-S proton of L-aspartic acid (2) would be lost in the biosynthesis of 3-nitropropanoic acid (1). This was investigated by preparation and feeding of chirally deuterated L-aspartic acid and examination of the product using ^{2}H nmr.

1.5 Stereochemistry of incorporation of L-aspartic acid (2).

1.5.1 Introduction

The lack of chirality of 3-nitropropanoic acid (1) means that assignment of the C-2 or C-3 hydrogens requires that a chiral derivative is prepared to enable the prochiral methylene hydrogens to be distinguished. While any chiral derivative might be expected to lead to chiral distinctions, stereochemical assignment is more complex. Once approach which was considered was the construction of a cyclic derivative based on L-lactic acid (Figure 1.4). Model-building suggests that both the diastereotopic $3H_R$ and $3H_S$ and $2H_R$ and $2H_S$ would be sufficiently distinct at 200 MHz in the nmr spectrum. Furthermore, the stereochemistry is locked in the cyclic lactic acid derivative and thus the conformation might be determined by x-ray crystallography. Clearly a significant amount of chemical

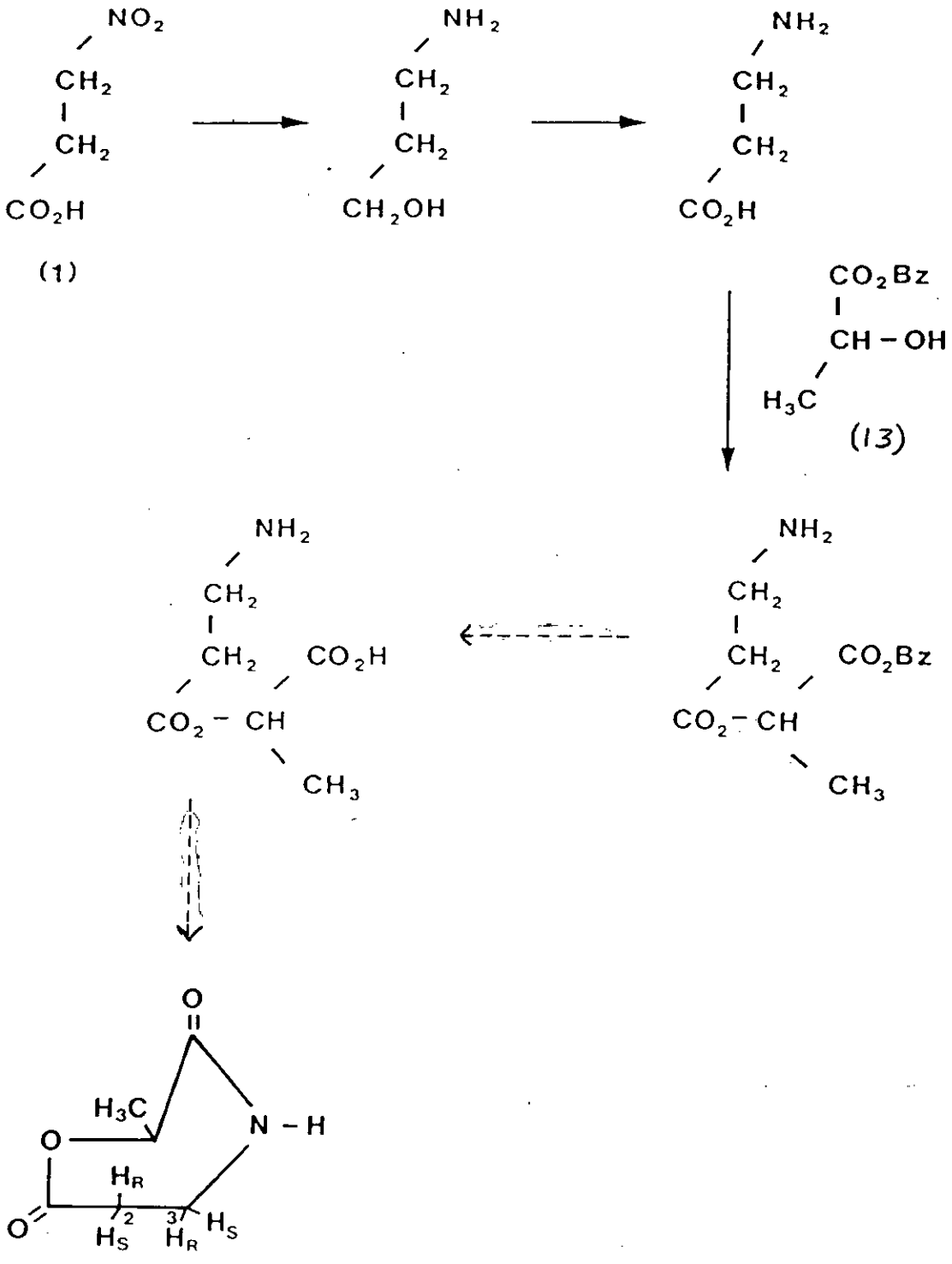

NO2
CH2
CH2
CO2H
(1)
NH2
CH2
CH2
CH2OH
NH2
CH2
CH2
CO2H
CO2Bz
CH – OH
H3C
(13)
NH2
CH2
CH2
CO2H
CO2 – CH
CH3
NH2
CH2
CH2
CO2Bz
CO2 – CH
CH3
O
H3C
N – H
O
HR
O
HS
2
3
HR
HS

Figure 1.4

manipulation of the isolated 3-nitropropanoic acid (1) is required. The acid (1) must be converted to a suitable form for derivatisation with a chiral agent and protection and deprotection steps with retention of methylene proton configuration were essential.

An alternative method makes use of chiral deuterated β-alanine (4) for conformational studies [33]. This approach has been applied by Young et al.[33] who prepared β-alanine (4) with stereospecific deuterium incorporation from the corresponding deuterated aspartic acids. The camphanyl amide of β-alanine (16), prepared by reaction with camphanyl chloride, showed distinction of the 3R and 3S hydrogen atoms in the ^{1}H nmr spectrum. In this case the pro-R hydrogen resonated at higher frequency than the pro-S and thus, in the case of 3-nitropropanoic acid (1), it ought to be possible to determine the stereochemistry of deuterium incorporation from feeding deuterated aspartic acid at C-3 by conversion of the product to β-alanine, preparing the camphanyl amides of the corresponding β-alanines and comparing with literature values. Although Gani and Young[33] were unable to find a suitable derivatising agent for chiral distinction at C-2 in β-alanine (4), it has been shown[34] that the methyl valine amides of carboxylic acids can give chiral distinction α to the carboxyl group. In addition, Brown and Parker[35] have shown that chiral distinction of the C-2 methylene protons can be observed in the S-methyl mandelate derivatives of simple carboxylic acids.

Comparison of the methyl valine amide or the methyl mandelate derivative of β-alanine derived from 3-nitropropanoic acid isolated after feeding perdeuterated L-aspartic acid to P. atrovenetum with the same β-alanine derivatives of known conformation prepared independently will confirm both the intermediacy or otherwise of

3-nitroacrylic acid (6) in the biosynthesis of 3-nitropropanoic acid (1) and elucidate the stereochemistry of the biosynthesis at C-2 of the product in an absolute fashion.

1.5.2 Synthesis

(a) Attempted synthesis of the cyclic derivative

Attempted reduction of 3-nitropropanoic acid (1) with lithium aluminium hydride did not give any of the expected amino alcohol. Reduction with tin in hydrochloric acid gave only a small yield of β-alanine (4) and this route was clearly of no use when dealing with labelled material since further reaction was necessary to prepare the cyclic derivative. Reaction of (1) with palladium/charcoal and dry ammonium formate[36] gave β-alanine (4) in good yield.

L-(+)-Lactic acid (13) was chosen as the chiral agent for construction of the cyclic derivative. Reaction of this compound directly with 3-nitropropanoic acid (1) however gave a mixture of products. The corresponding benzyl ester of lactic acid (13) was prepared by reaction with benzyl alcohol in tetrahydrofuran in the presence of 1,3-dicyclohexylcarbodimide (DCC) but this material could not be condensed with 3-nitropropanoic acid (1) under a range of conditions. There may be steric reasons for this. L-(+)-Lactic acid (13) was protected by reaction with 2,4'-dibromoacetophenone[37]. This protected derivative also failed to react with 3-nitropropanoic acid (1) under a range of conditions including the mixed anhydride procedure with triflouroacetic anhydride which activates the acid prior to esterification[38]. In the light of work described below, attempts to prepare cyclic derivatives were abandoned.

(b) Chiral L-aspartic acid

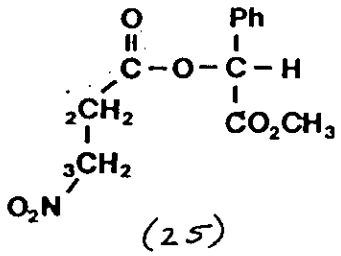
The difficulties in the cyclisation strategy led to a consideration of the two alternative methods of determining the route and stereochemistry of the incorporation of deuterated L-aspartic acid (2) into 3-nitropropanoic acid (1). These both require distinction of the pro-R and pro-S hydrogen atoms at C-2 in the product by preparation of a suitable chiral derivative which can be compared directly with an authentic sample of known stereochemistry.

β-Alanine was treated with freshly sublimed (-)- camphanic acid chloride in aqueous sodium hydroxide and the product isolated as an oil which was used without further purification. The N-camphanyl -β-alanine (16) was treated with methyl valine and DCC and the diderivatised β-alanine (17) isolated. This product could not be crystallised. Examination by ^{1}H nmr showed that the pro-R and pro-S hydrogen atoms at C-2 and C-3 of the corresponding 3-nitropropanoic acid (1) could be distinguished. Decoupling of each resonance in turn confirmed the assignments.

Deuterated 3-nitropropanoic acid (1) was prepared by deuterated acid hydrolysis of bromopropionitrile followed by nitration (vide infra).

Unfortunately, treatment with ammonium formate and palladium charcoal catalyst led to loss of the deuterium atoms and attempts to carry out the reduction by other methods in which the deuterium would be retained were unsuccessful. The use of chiral β-alanine derivatives was abandoned and a method sought whereby distinction of the pro-R and pro-S methylene protons could be made without reduction of the nitro group of (1).

Figure 1.5



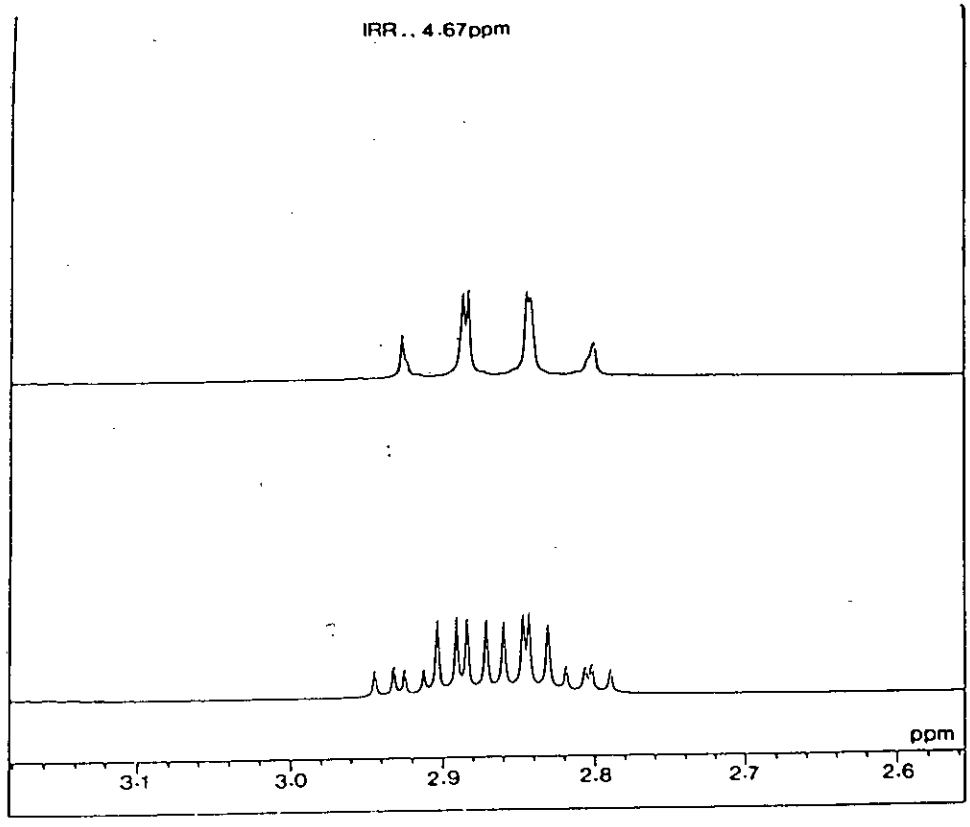
(25)

Note: For the sake of clarity, resonances due to C–2H and C–3H only are shown; other resonances have been deleted.

3-Nitropropanoic acid (1) was converted to the corresponding methyl mandelate ester by treatment with trifluoroacetic anhydride then with methyl mandelate at room temperature. Esterification using 2-ethoxy-1-ethoxy carbonyl-1,2-dihydroquinoline (EEDQ) or dimethylaminopyridine (DMAP) and dicyclehexylcarbodimide (DCC) gave only starting material. Examination by ^{1}H nmr showed that the pro-R and pro-S hydrogen atoms at C-3 were distinguishable from one another while those at C-2 remained unresolved (Fig. 1.5).

The methyl valine amide of 3-nitropropanoic acid was prepared by coupling the metabolite with L-methyl valine using DCC in THF. The ^{1}H nmr spectrum showed a complex pattern for the hydrogens at C-2 characteristic of an AA'BB' system[39]. Decoupling of the resonance due to the C-3 hydrogens at 4.72 ppm leads to collapse of the virtually symmetrical complex into an AB pattern. A similar pattern is obtained for the hydrogen at C-3 and decoupling of the resonance due to the hydrogens at C-2 (2.95 ppm) causes a similar collapse. (Fig. 1.6).

Failure to prepare β-alanine from 3-nitropropanoic acid (1) with retention of deuterium at C-2 of (1) made direct comparison of a suitable chiral derivative using ^{2}H nmr with the same derivative prepared with known stereochemistry impossible. Administration of chirally deuterated L-aspartic acids and preparation of the corresponding methyl valine amides from the isolated 3-nitropropanoic acid (1) offered an alternative method of distinguishing stereochemistry of the hydrogen atoms at C-2 of (1) derived from those at C-3 of L-aspartic acid.



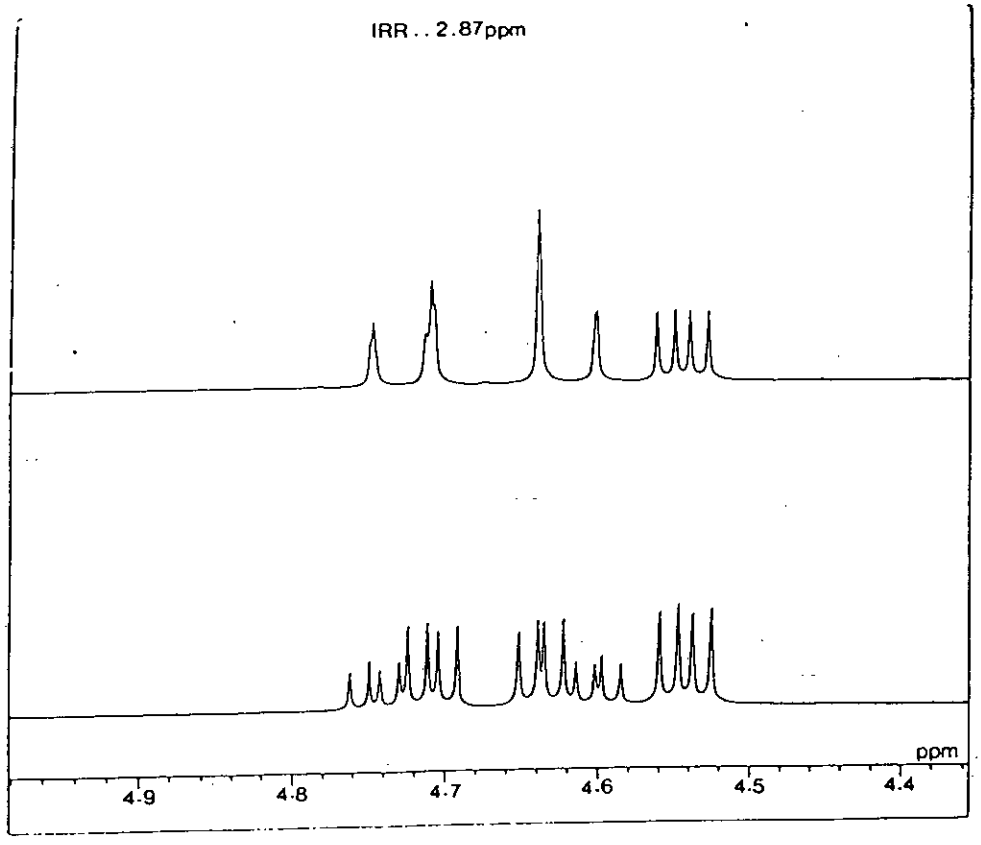


Figure 1.6

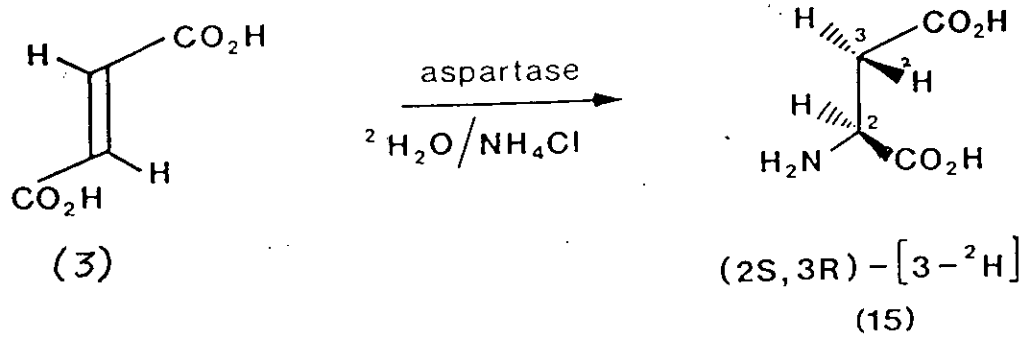
Aspartase catalyses the formation of L-aspartic acid from fumaric acid. The reaction is stereospecific, therefore using fumaric acid as substrate in deuterated water gives 2S, 3R-[3^2H]-aspartic acid while 2,3-dideutero fumaric acid (27) is converted to 2S, 3S-[2,3-$^2H_2$]-aspartic acid (Fig. 1.7). The dideuterated fumaric acid (27) was prepared from diacetylene dicarboxylate by reaction with triphenylphosphine in the presence of ^2H_2O followed by hydrolysis of the diester[40]. The chirally deuterated L-aspartic acids were then prepared by incubation with aspartase as described by Field and Young[41] (Fig. 1.7).

The ^{1}H nmr spectrum of the labelled material showed the expected resonances, 2S,3S-[2-3-$^2H_2$]-aspartic acid (14) giving a singlet at 2.65 ppm while the monodeuterated derivative, (2S, 3R) - [3-^{2}H] - aspartic acid (15), gave a 1H doublet at 3.50 ppm (H-2 J=8Hz) and a 1H doublet at 2.30 ppm (H-3 J=8Hz). Incorporation of ^{2}H was also confirmed by negative ion FAB mass spectral analysis which gave a small molecular ion in each case and significant peaks due to loss of CO_2H and showed that a little (<5%) non-deuterated material was also present.

Each of the labelled L-aspartic acids was fed to cultures of P. atrovenetum in 3 equal amounts (8mg/50cm^3 culture) at 42, 48 and 54 hours after innoculation. The 3-nitropropanoic acid (1) was isolated after 72 hours as described in the experimental section.

The 3-nitropropanoic acid (1) isolated from the stereospecifically deuterated-L-aspartic acid feeding experiments was examined by ^{2}H nmr then converted to the corresponding methyl valine amides and examined

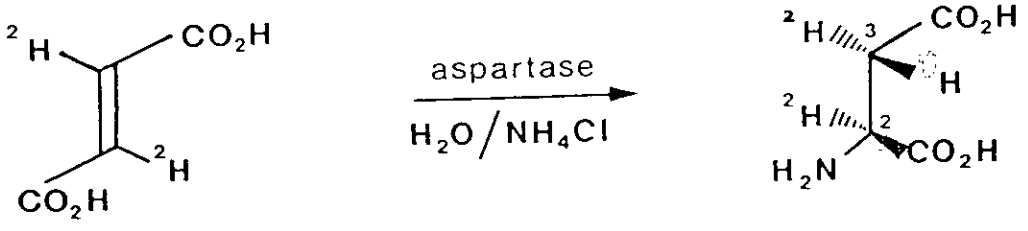
THE PREPARATION OF STEREOSPECIFICALLY DEUTERATED L-ASPARTIC ACIDS



(3)

(2S,3R)-[3-^{2}H]

(15)



(27)

(2S,3S)-[2,3-$^2H_2$]

(14)

Figure 1.7

once again. A sample of (2S-[2,3-$^2H_3$]-aspartic acid (15a) was also fed as described above and the derived 3-nitropropanoic acid examined by ^2H nmr. A sample of (2S,3R)- [3-^2H]-aspartic acid (15) was fed to two cultures and the 3-nitropropanoic acid (1) isolated after 72 and 96 hours then the methyl valine amides compared by ^2H nmr. Finally, [4-^{13}C, 3-$^2H_2$]-L-aspartic acid (18) was administered and the 3-nitropropanoic acid isolated after 72 hours and examined by ^{13}C nmr of the carboxyl resonance and FAB ms.

1.5.3 Results

Examination of 3-nitropropanoic acid (1) obtained from feeding 2S,3S-[2,3-$^2H_2$]-aspartic acid (19) by ^2H nmr showed two peaks at 2.97 and 4.69 ppm due to deuterium incorporation at C-2 and C-3 respectively. The methyl valine amides of this compound gave resonances at 2.96 ppm and 3.04 ppm due to the pro-R and pro-S deuterium atoms at C-2. The deuterium at C-3 was lost by exchange during the preparation of this compound. The proportions of the two resonances at C-2 were ca 2:1 (Fig 1.8); that at 3.04ppm being the smaller.

The isolated acid (1) from administered (2S,3S)-[2-^2H, 3-^2H]-L-aspartic acid (14) also showed two resonances in the ^2H nmr spectrum at 2.98ppm and 4.70 ppm. The corresponding methyl valine amide had a single peak at 3.04 ppm (Fig 1.9). The acid isolated after feeding (2S,3R)-[3-^2H]-L-aspartic acid (15) produced a single resonance in the ^2H nmr at 2.99 ppm while the corresponding methyl valine amide had a peak at 2.94 ppm and a very small resonance at 3.04 ppm (Fig 1.9). These results are summarised in Table 1.1.

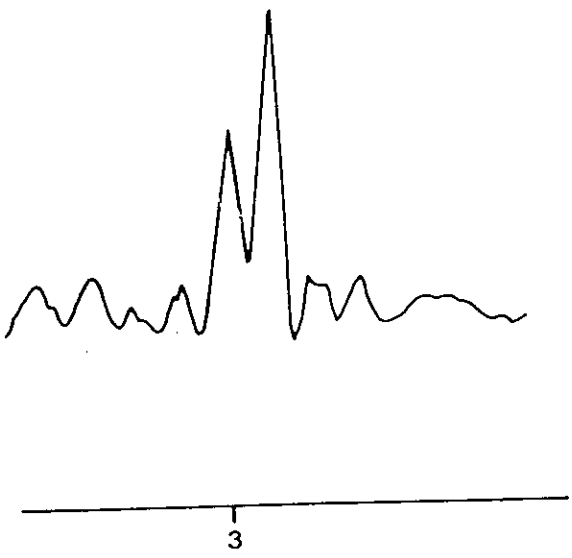


Figure 1.8

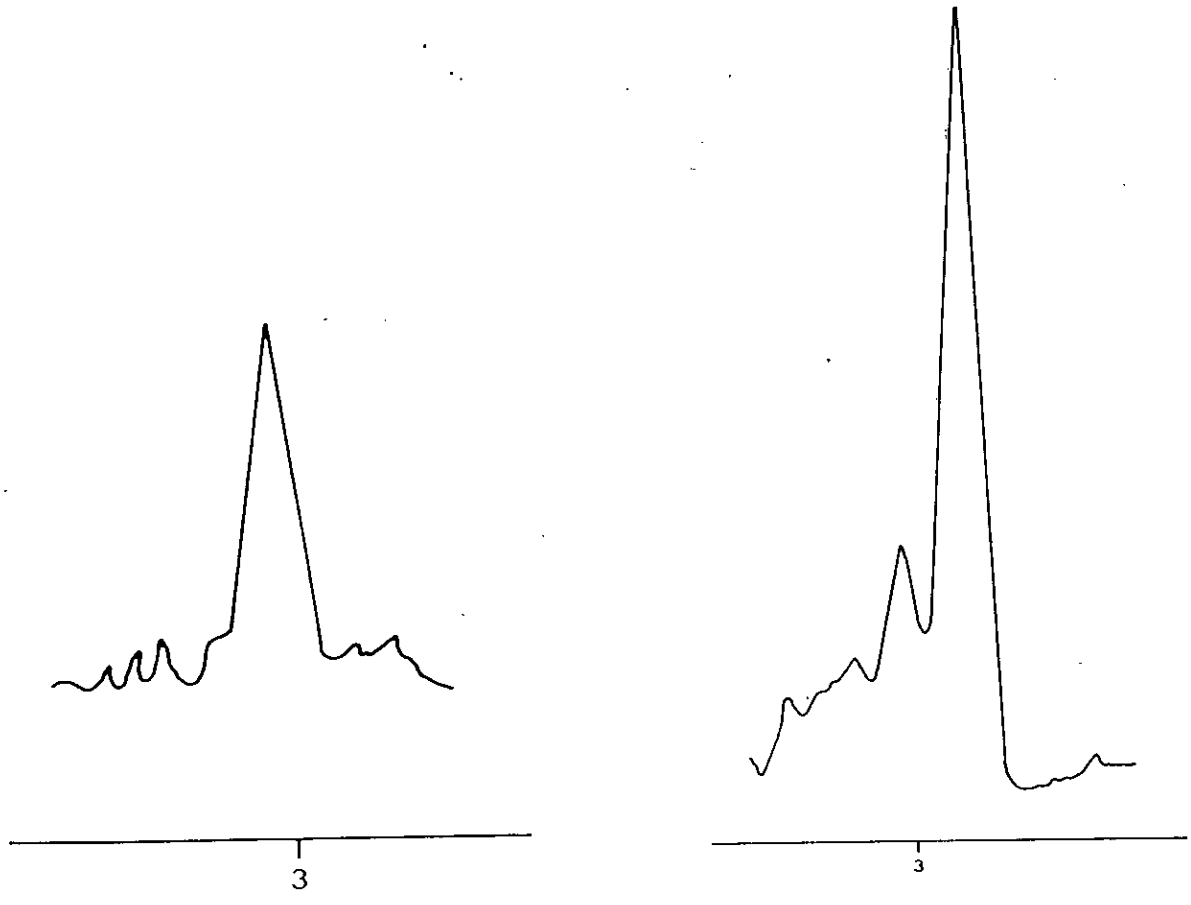
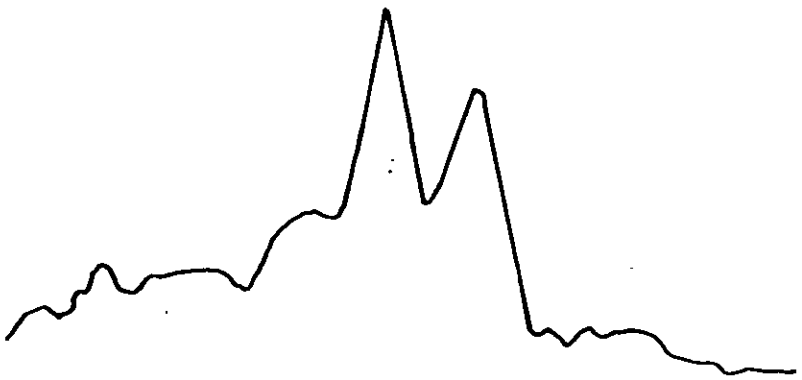


Figure 1.9

96h.



72h.

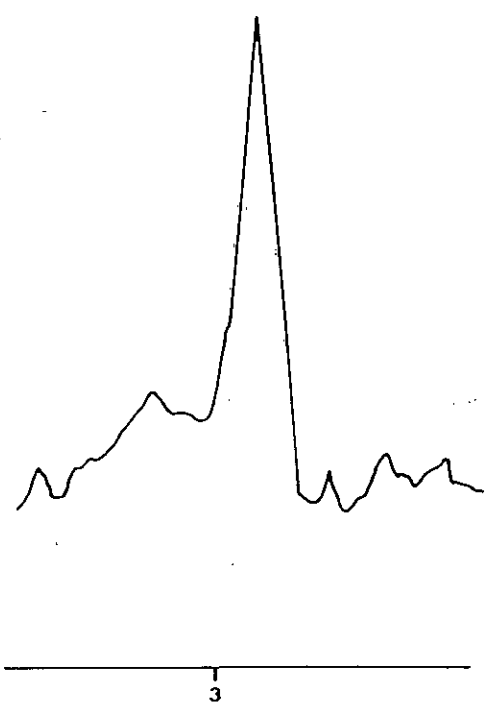


Figure 1.10

In the time course experiments, deuterium was found to be incorporated in 3-nitropropanoic acid isolated after 72 and 96 hours growth. In the case of the 72 hours isolate, the methyl valine amides exhibited a single resonance at 2.94 ppm, and a very small peak at 3.08 ppm (Fig. 1.10). The 96 hours incubation gave 3-nitropropanoic acid in which the methyl valine amide showed two peaks at 2.94 ppm and 3.02 ppm (Fig. 1.10), corresponding to the pro R and pro S deuterium atoms at C-2, of approximately equal size. These results are summarised in Table 1.1.

Table 1.1 Results from feeding deuterated aspartic acids to P. atrovenetum.

L-Aspartic acid	^{2}H Chemical shift of (1) (ppm)	^{2}H Chemical shift of amide derivative
[2-^{2}H, 3-^{2}H$_2$]	2.97, 4.69	2.96, 3.04
(2S, 3S)[2-^{2}H, 3-^{2}H]	2.98, 4.70	3.04
(2S, 3R)[3-^{2}H]	2.99	2.94
(2S, 3R)[3-^{2}H] (72h)	2.96	2.94
(2S, 3R)[3-^{2}H] (96h)	2.99	2.94, 3.02

1.5.4. Discussion

The ^{2}H nmr spectrum of the methyl valine amide of 3-nitropropanoic acid (Fig. 1.8) isolated from feeding [2-^{2}H, 3-^{2}H$_2$]-L-aspartic acid (19) suggests that, if an unsaturated intermediate is involved, either desaturation or reduction is a non-stereospecific process. Both the 3 pro-R and the pro-S deuterium atoms of deuterated

aspartates are retained at C-2 of the isolated 3-nitropropanoic acid (1) giving resonances at 2.96 ppm and 3.04 ppm in the spectrum of the methyl valine amide. Thus stereospecific hydrogen loss and subsequent gain is precluded. If 3-nitroacrylic acid (6) is involved as a biosynthetic precursor it may be produced by loss of hydrogen either in a specific or a random fashion but the subsequent hydrogen addition to give (1) must be non-stereospecific process to give a mixture of 2 pro-R and 2 pro-S deuterated 3-nitropropanoic acid (1). If a saturated intermediate is involved then the retention of deuterium from C-2 deuterated L-aspartic acid at C-3 of 3-nitropropanoic acid (1) implies overall retention of stereochemistry at this position. It should be noted that while pyridoxal phosphate-mediated interconversion of amino acids and α-keto acids can occur in vivo, this would also cause loss of deuterium from C-2 of L-aspartic acid which is clearly not evident in this case.

One of the C-2 deuterium atoms in 3-nitropropanoic acid (1) from the perdeuterated aspartate feeding occurs in ca 50% lower abundance than the other (Figure 1.8). This observation is important to later arguments but, for the moment, it implies that the pro-R and pro-S hydrogen atoms at C-3 of L-aspartic acid, even when isotopically identical, are not completely equivalent in the biosynthesis of 3-nitropropanoic acid (1).

Feeding L-aspartic acids in which either the pro-R or pro-S hydrogen at C-3 are replaced by deuterium gives incorporation in both cases. This result suggests that any hydrogen loss through the intermediacy of an acrylate analogue must be non-stereospecific since either the

pro-R or the pro-S can be retained. Preparation of the methyl valine amides of the isolated 3-nitropropanoic acid (1) from each L-aspartic acid feeding shows that a different hydrogen is retained in each case. For the pro-R ^{2}H case, a single resonance at 2.94 ppm (Figure 1.9) was observed in the chiral amide while in the 3-nitropropanoic acid (1) derived from feeding pro-S ^{2}H aspartic acid the corresponding peak is at 3.04 ppm (Figure 1.9). This is consistent with the results from the perdeuterated aspartic acid feeding in which resonances were observed at 2.96 and 3.04 ppm. There is a small peak in the pro R-^{2}H feeding at 3.04 ppm corresponding to the other isomer which may occur as a result of post-biosynthetic cycling of the 3-nitropropanoic acid (1), in a non-stereospecific fashion, with 3-nitroacrylic acid (6). It is not possible to determine, by inspection, if the deuterium is being retained at C-2 of 3-nitropropanoic acid (1) with retention of configuration or inversion.

Gani and Young have shown[33] that, in the camphanoyl amide of β-alanine, the pro-R hydrogen adjacent to the amide occurred at higher chemical shift than the pro-S hydrogen. Brown and Parker, in a study of stereospecific deuteration[35], found that the S-methyl mandelate esters of L-deuterated carboxylic acids showed chiral distinction with the pro-R hydrogen at higher chemical shift than the pro-S. In the case of the methyl valine amide of 3-nitropropanoic acid the deuterium derived from pro-R deuterated L-aspartic acid occurs at a higher chemical shift than that from pro-S thus while retention is implied, it remains unproven. The retention of stereospecificity at C-3 of 3-nitropropanoic acid (1) mentioned above however, and the non-intermediacy of the acrylate implies retention of configuration at C-2.

In the time course experiment, deuterium incorporation from (2S, 3R)-[3-^{2}H]-L-aspartic acid (15) was observed after 72 and 96 hours (Figure 1.10). The corresponding methyl valine amide for 96 hours gave two peaks in the ^{2}H nmr which corresponded to the resonances of the products from pro-R deuterated and pro-S deuterated L-aspartic acid feedings. Since the "reductase" enzyme is known to be active at this time and not at 72 hours (vide infra) this suggests that post-biosynthetic randomisation of the stereochemistry at C-2 of 3-nitropropanoic acid (1) is occurring. There are two possible explanations for this observation. In the conversion to 3-nitroacrylic acid (6), one hydrogen is lost. If the loss is stereospecific then this hydrogen must be the pro-R hydrogen from L-aspartic acid (such a stereospecific loss may be due to an isotope effect). The addition of hydride from NADPH, to form 3-nitropropanoic acid (1) occurs with inversion of configuration so the pro-R derived site is now deuterated. This will lead to a population of pro-R and pro-S deuterated 3-nitropropanoic acids (1) and hence two peaks in the methyl valine amides. A second possibility is that the hydrogen loss and subsequent gain is a completely random process which leads to a population of pro-R and pro-S deuterated 3-nitropropanoic acids (1) of approximately equal amounts. However, the nmr suggests that more of the pro-R derived deuterium is present than the pro-S and this implies that randomisation does not occur and consequently that inversion is the preferred mechanism.

The carboxyl resonance in the ^{13}C nmr spectrum of 3-nitropropanoic acid (1) derived from feeding DL-[4-^{13}C, 3-$^{2}H_{2}$]-aspartic acid (18) has already been shown to be shifted by 0.009 ppm - indicative of a

single deuterium loss. This implies that if any [1-^{13}C, 2-$^{2}H_2$]-3-nitropropanoic acid is present, then it is in insufficient amounts to be detected by the β-isotopic shift effect on C-1. The FAB ms of the isolated 3-nitropropanoic acid (1) shows significant ions at m/z 118, 119, 120, 121 and 122. Non-labelled 3-nitropropanoic acid (1) gives ions at m/z 118, 119 and 120 due to M-1, M and M+1 respectively. The relative ratios are as shown in Table 1.2. From the intensities of the ions in the reference spectrum, it is possible to determine the contribution of each of the labelled 3-nitropropanoic acids to each of the ions observed. These results are detailed in Table 1.2. A small contribution due to [1-^{13}C, 2-$^{2}H_2$]-3-nitropropanoic acid is apparent.

Table 1.2 FAB ms of 3-nitropropanoic acid (1) and 3-nitropropanoic acid from [4-^{13}C, 3-$^{2}H_2$]-L-aspartic acid feeding.

Label in (1)	ion (m/z)				
	118	119	120	121	122
[M-1] $^{13}C_0$, $^{2}H_0$	33254				
[M] $^{13}C_0$, $^{2}H_0$		8812			
[M+1] $^{13}C_0$, $^{2}H_0$			546		
[M-1] $^{13}C_0$, $^{2}H_0$		7038			
[M] $^{13}C_0$, $^{2}H_0$			145		
[M+1] $^{13}C_0$, $^{2}H_0$				9	
[M-1] ^{13}C, $^{2}H_1$			359		
[M] ^{13}C, $^{2}H_1$				95	
[M+1] ^{13}C, $^{2}H_1$					9
[M-1] ^{13}C, $^{2}H_2$				457	
[M] ^{13}C, $^{2}H_2$					121

1.5.5 Conclusions

It has been shown that feeding L-aspartic acid (2) containing deuterium in either the pro-R or pro-S positions at C-3 leads to stereospecific incorporation at C-2 of 3-nitropropanoic acid (1). The experiment with perdeuterated L-aspartic acid, where both 2 pro-R and 2 pro-S deuterons appear in the product effectively precludes the possibility that an acrylate intermediate is involved since, in this case, the overall reaction would be required to be non-stereospecific and this is at variance with the results from the stereospecifically labelled aspartic acid experiments. This implies that a saturated intermediate is involved. The most plausible mechanism would involve complete oxidation of the amino functionality of L-aspartic acid (2) followed by decarboxylation with retention of configuration at C-2 of the resulting 3-nitropropanoic acid (1).

The occurrence of an ion in the FAB ms of 3-nitropropanoic acid (1) derived from feeding [4-^{13}C, 3-$^{2}H_2$]-DL-aspartic acid which contains a ^{13}C label and two deuterium atoms is further confirmation of the pathway. This result implies that L-aspartic acid (2) is incorporated into 3-nitropropanoic acid (1) with retention of the C-H bonds at C-3 of L-aspartic acid (C-2 of 3-nitropropanoic acid). Subsequent loss of deuterium may occur as a result of post-biosynthetic cycling (vide infra) which produces [1-^{13}C, 2-$^{2}H_1$] and [1-^{13}C, 2-$^{2}H_0$]-3-nitropropanoic acid. This implies that the post-biosynthetic cycling does not occur with retention of configuration and that either inversion or scrambling occurs with ultimately, complete loss of deuterium from 3-nitropropanoic acid (1).

Post-biosynthetic cycling of 3-nitropropanoic acid

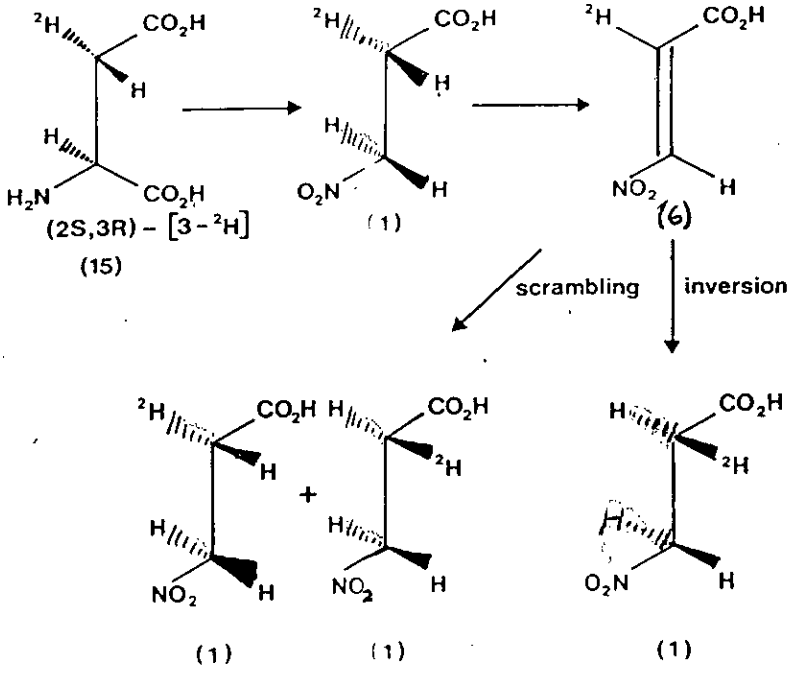


Figure 1.11

While an isotope effect cannot be excluded, the inequality of the peaks in the perdeuterated L-aspartic acid (2) feeding would imply that, in an isotopically identical situation, there is a degree of specificity exhibited in the post biosynthetic cycling with the pro-R deuterium lost preferentially.

Finally, the isolation of 3-nitropropanoic acid (1) from cultures of P. atrovenetum at different times after innoculation and feeding with (2S, 3R)-[3-^{2}H]-L-aspartic acid (15) and the examination of the corresponding methyl valine amides sheds light upon the stereochemistry of the proposed post-biosynthetic cycling of 3-nitropropanoic acid (1) with the 3-nitroacrylic acid (6). When isolated soon after feeding the 3-nitropropanoic acid (1) contains deuterium stereospecifically at C-2. When 3-nitropropanoic acid (1) is isolated 96 hours after incubation then two peaks are observed in the ^{2}H nmr spectrum of the methyl valine amides corresponding to deuteration at the pro-R and the pro-S positions. This suggests that "reductase" activity is not present early in the growth cycle of P. atrovenetum and the enzyme is only formed late in the growth cycle. Furthermore, either the post-biosynthetic cycling randomises the label or that inversion occurs during the cycling. If the latter is the case then the pro-R hydrogen from L-aspartic acid is retained during hydrogen loss. These results are summarised in figure 1.11. The greater peak size of the pro-R derived resonance implies that scrambling (which could only produce, at best, a 50:50 mixture) cannot be occurring and that inversion is the likely mechanism.

1.6 Post-biosynthetic cycling

Post-biosynthetic cycling of 3-nitropropanoic acid (1) via the acrylate (6) will necessarily result in loss of hydrogen at C-2 and C-3. The earlier evidence for the incorporation of 3-nitroacrylic acid (6) suggests that a concerted pathway with loss of H from C-3 may be involved in the decarboxylation of L-asparatic acid (2) to give 3-nitropropanoic acid (1). An alternative, suggested by the incorporation experiments from stereospecifically deuterated L-aspartic acid, is that (1) and (6) are in post-biosynthetic equilibrium in the cell.

The occurrence of [1-^{13}C, 2-^{2}H]-3-nitropropanoic acid in the presence of normally biosynthesised 3-nitropropanoic acid (1) can be determined by the secondary isotope effect induced in the C-1 nmr signal of the carboxyl by deuterium labelling 3 bonds away[42].

1.6.1 Synthesis and feeding

The synthesis of [1-^{13}C, 2-$^{2}H_2$]-3-nitropropanoic acid (7) was carried out by insertion of the ^{13}C label fairly early in the synthetic sequence. While such methods are generally, not preferred, attempts to insert a label at a later stage were unsuccessful.

Tosylation of 2-nitroethanol was carried out successfully in tetrahydrofuran/triethylamine to give the tosyl derivative (21) after an initial failure using pyridine as solvent. It was found, however, that substitution of the tosyl group by cyanide in dimethyl formamide and 18-crown-6 was only accomplished in low yield (<10%) and this was

unacceptable. The substitution reaction failed completely in dimethyl sulphoxide[45] both at room temperature and at 110°C. Direct preparation of 3-nitropropionitrile from 2-nitroethanol using triphenylphosphine, potassium cyanide and a phase transfer catalyst[46] was also unsuccessful.

A second approach to the synthesis was made via 1-(N,N-dimethyl)-amino-2-nitroethene (22). The enamine (22) was readily formed by reaction of nitromethane with dimethylformamide dimethylacetal in dimethylformamide. Attempts to add iodoacetonitrile[47] or bromoacetic acid to nitromethane in a base-mediated condensation gave only starting material.

Hydrolysis of 3-bromopropionitrile (8) with hydrochloric acid gave 3-bromopropanoic acid (9) which was isolated in good yield. This compound could, in turn, be nitrated with sodium nitrite in dimethylsulphoxide to give (1) in about 50% yield. The conditions and time for the nitration had to be carefully controlled, stirring at room temperature for 2 hours being found to produce the optimum yields. Longer periods of time or higher temperatures gave mixtures of products. These reactions were repeated with the incorporation of a deuterium label at C-3. 3-Bromopropionitrile (8) was hydrolysed with deuterium bromide to afford the corresponding acid which was then treated with sodium nitrite in dimethylsulphoxide as described above to afford the deuterated 3-nitropropanoic acid (10). This material was taken up in aqueous sodium carbonate buffer (pH 10) and stirred at room temperature overnight in order to exchange any deuterium incorporated at C-3 of the final product. Examination by

nmr and mass spectrometry showed that only C-2 was deuterated. The ^{1}H nmr spectrum showed a small triplet at 4.45 ppm and a large singlet corresponding to hydrogens at C-3 whilst the resonance for those at C-2 was a small triplet at 2.65 ppm whose integral suggested 90% deuteration. It should be noted, however, that although fast atom bombardment (FAB) mass spectrometry with a glycerol matrix gives a peak due to [2-$^{2}H_{2}$]-3-nitropropanoic acid (10) initially, (121, M^{-}) the deuterium atoms gradually exchange with hydrogen atoms in the glycerol while the sample is on the probe (m/z 119, M^{-}). After a period of 30 minutes, virtually no deuterium is left in the sample.

Since 3-bromopropionitrile (8) could be effectively converted to [2-$^{2}H_{2}$]-3-nitropropanoic acid (10) by this route, the remaining problem was the preparation of [1-^{13}C]-3-bromopropionitrile. Reaction of dibromoethane with sodium cyanide in dimethylsulphoxide gave the dinitrile rather than the mononitrile under a range of conditions.

An alternative method which retained the strategy of nitration as the final step in the synthetic procedure was the hydrolysis of 3-hydroxypropionitrile (11) with deuterium bromide giving deuterated 3-bromopropanoic acid (9) which could be nitrated as outlined previously. 3-Hydroxypropionitrile (11) was prepared from sodium cyanide and 2-chloroethanol. This reaction was most successful (>60%) in the presence of phase transfer catalyst (18-crown-6) and at 80-90°[37,48]. Heating of the product with hydrobromic acid afforded 3-bromopropanoic acid (9) in reasonable yield. This reaction sequence was carried out using [^{13}C] sodium cyanide to give [1-^{13}C]-3-hydroxypropionitrile (24) which was refluxed with deuterium

PREPARATION OF $[1-^{13}C, 2-^{2}H_2]$-3-NITROPROPANOIC ACID (7)

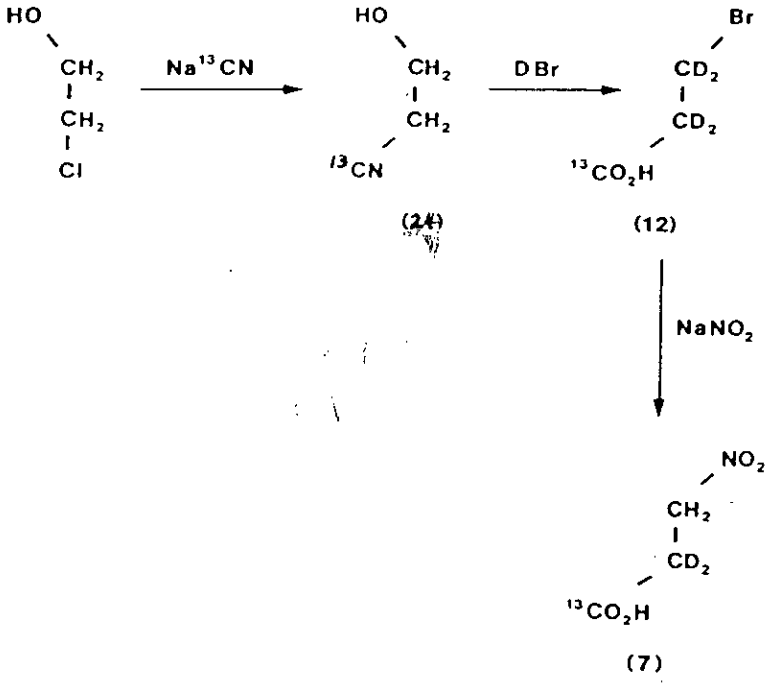


Figure 1.12

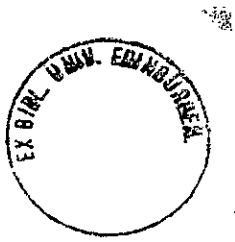
bromide to give [1-^{13}C, 2-$^{2}H_2$]-3-bromopropanoic acid (12). Nitration with sodium nitrite afforded [1-^{13}C, 2-$^{2}H_2$]-3-nitropropanoic acid (7) (Figure 1.12). The ^{13}C and deuterium incorporation was verified by mass spectrometry (m/z 122,M^-, 121,$M-1^-$).

P. atrovenetum (ATCC 13351) was assayed for "reductase" activity at various times after inoculation as described by Shaw[24]. Activity was found to be present in mycelium harvested between 96 and 120 hours. Dark grown mycelium and cultures grown in shake flasks did not contain any active enzyme. It has subsequently been noted[49] that cultures of P. atroventum secrete an enzyme capable of oxidising 3-nitropropanoic acid (1) to 3-carboxyacetaldehyde. The mechanism proposed for this oxidation involves isomerisation to the aci-nitro compound and such a transformation is clearly not possible with 3-nitroacrylic acid (6).

[1-^{13}C, 2-$^{2}H_2$]-3-Nitropropanoic acid (7) was fed to growing cultures of P. atrovenetum 24 hours after innoculation and incubation continued for a further 76 hours. 3-Nitropropanoic acid (1) was isolated and the product analysed by ^{13}C nmr.

1.6.2. Results

The ^{13}C nmr of the isolated 3-nitropropanoic acid (1) gave two resonances due to the carboxyl group with a difference of 0.0123 ppm (Fig 1.13(a)). The sample was spiked with synthetic [1-^{13}C, 2-$^{2}H_2$] - 3-nitropropanoic acid (7). In this case, the carboxyl resonance in the ^{13}C nmr spectrum consisted of three components with chemical shift differences of 0.012 and 0.016ppm (Fig 1.13(b)).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The FAB mass spectrum of the isolated 3-nitropropanoic acid (1) shows peaks at m/z 119 [M^-] and 118[M-1]$^-$ and 121 and 120. The latter two are due to [M^-] and [M-1]$^-$ ions of singly deuterated, ^{13}C-labelled 3-nitropropanoic acid (1). There is also a very small peak at m/z 122 which corresponds to the uncycled dideuterated compound (Fig. 1.14) at ca. 5% over background.

1.6.3 Conclusions

The ^{13}C nmr spectrum of the carboxyl resonance of the isolated 3-nitropropanoic acid indicates a mixture of C-2 monodeuterated and non-deuterated material. This implies that at least one deuterium is lost from the C-2 position of the fed metabolite. The nmr and ms data from this experiment implies that (1) and (6) are rapidly interconverted.

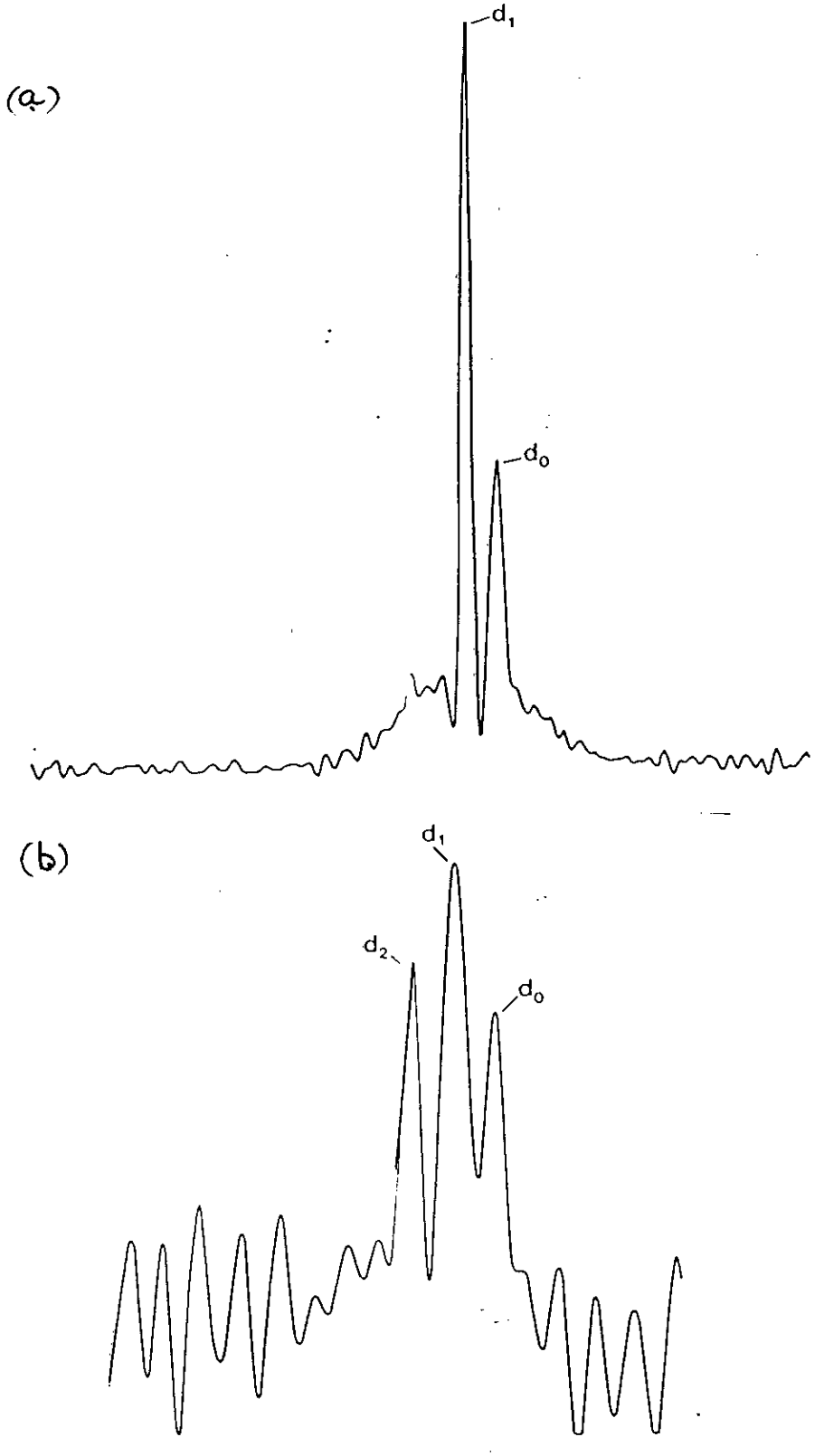
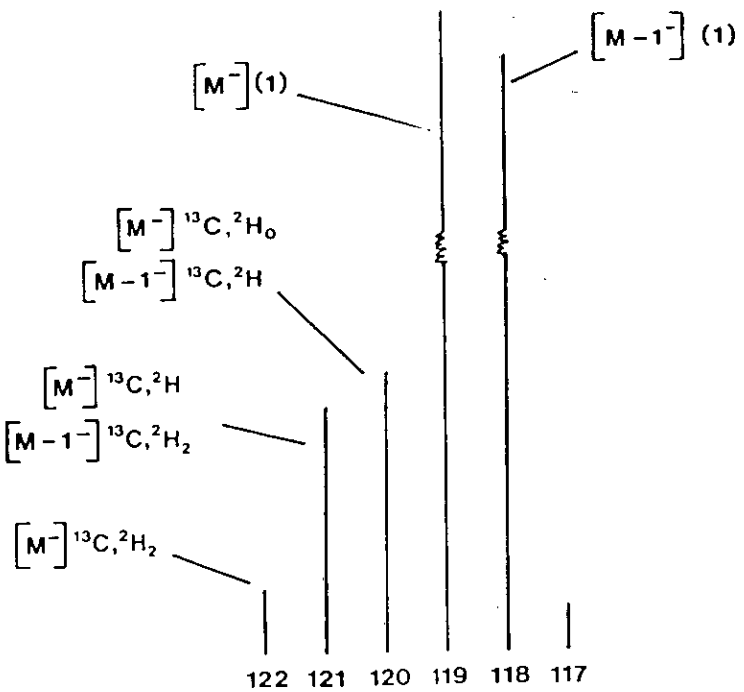


Figure 1.13



FAB mass spectrum of 3-nitropropanoic acid
from administration of $\left[1-{}^{13}C, 2-{}^{2}H_2\right]$-3-nitropropanoic acid

Figure 1.14

1.7 3-Nitroacrylic acid reductase

While in the above experiments post-biosynthetic cycling of 3-nitropropanoic acid (1) and 3-nitroacrylic acid (6) appears to occur the reaction has been claimed by Shaw to be non-reversible[24] when isolated enzyme is used. It was therefore important to prepare 3-nitroacrylic acid (6) and to incubate this material with the isolated enzyme in deuterated water in order to determine if any specificity is inherent in the reaction. Also in view of the *in vivo* result the claimed irreversibility of the reaction *in vitro* was also worth reinvestigation.

1.7.1 Synthesis, enzyme isolation and results

3-Nitroacrylic acid (6) was synthesised as described by Shaw[21] and the purified product stored under nitrogen at 0°C until required. The reductase enzyme was isolated and assayed according to the procedure outlined by Shaw[24]. A time course study of the fungi showed that the reductase was present between 84 and 110 hours after inoculation. The crude ammonium sulphate-precipated enzyme (40%-65% saturation) was used since attempts to purify the enzyme further using DEAE Sephadex resulted in loss of activity.

The enzyme was incubated with the acrylate (6) and NADPH at pH 7.3 over a period of 2 hours and monitored spectrophotometrically at 231 nm[24]. Examination of the product isolated by ether extraction after this time showed it to be identical with an authentic sample of 3-nitropropanoic acid (1). When the incubation was carried out in deuterated water, deuterium incorporation was detected only at C-3 as assessed by ^{1}H and ^{2}H nmr. (4.76 ppm, $CHDNO_2$).

In agreement with Shaw's results incubation of 3-nitropropanoic acid with the crude enzyme preparation in deuterated water and NAD or NADP failed to show any incorporation of deuterium. This result is summarised in Figure 1.15.

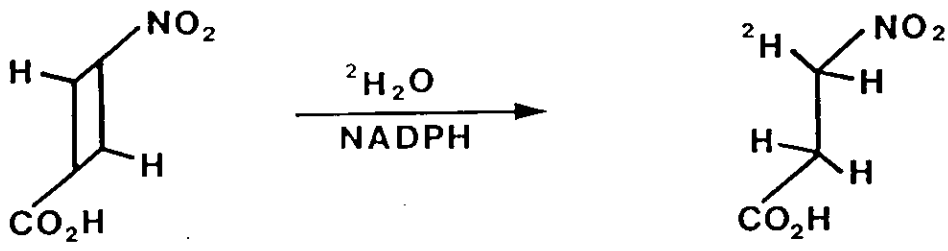
1.7.2 Conclusions

The results described above confirm that, under a range of conditions, the reaction catalysed by "3-nitroacrylic acid reductase" is irreversible *in vitro*. Furthermore, it appears that hydrogen incorporation at C-2 and C-3 occurs regiospecifically with the former presumably derived from NADPH and the latter from the water (Fig 1.16). There is, however, no information about the stereospecificity of the addition in this experiment. The regiospecificity of the reaction implies that the enzyme is not succinate dehydrogenase and this confirms the study by Shaw[24] in which succinic acid was found not to be a substrate. A likely mechanism would involve abstraction of a proton from the medium at C-3 followed by hydride attack from NADPH at C-2 or, possibly, H^- attack in a Michael fashion at C-2 followed by H^+ quenching of the intermediate stabilised carbanion.

1.8 3-Nitropropanoic acid biosynthesis - conclusions

This study has demonstrated that 3-nitrosuccinic acid (5) is an intermediate in the biosynthesis of 3-nitropropanoic acid (1) from L-aspartic acid (2). A range of biosynthetic pathways which require a dehydroamino acid intermediate are thereby eliminated. The first step in the biosynthesis has been shown to be oxidation of the amine functionality (possibly *via* N-hydroxy-L-aspartic acid) to give

PREPARATION OF 3-NITROPROPANOIC ACID (1) FROM 3-NITROACRYLIC ACID (6) WITH REDUCTASE ENZYME



(6) (1)

Figure 1.15

3-nitrosuccinic acid (5) with retention of stereochemistry followed by decarboxylation. Retention of a single deuterium in 3-nitropropanoic acid (1) from administration of [4-^{13}C, 3-$^{2}H_2$]-L-aspartic acid (18) initially suggests that loss of the carboxyl group is a concerted process with loss of H from C-3. Further investigation by ms however suggests that a non-acrylate pathway is operating.

Feeding of deuterated L-aspartic acids (14,15,19) has shown that an unsaturated acrylate cannot be a biosynthetic intermediate. Deuteration of both the pro-R and the pro-S hydrogens at C-3 independently in L-aspartic acid gives 3-nitropropanoic acid (1) stereospecifically labelled at C-2 thus implying that, if desaturation/resaturation is part of the pathway then the process must be stereospecific. This result is precisely opposed to the observation that, on feeding perdeuterated L-aspartic acid (19), the label at C-2 of 3-nitropropanoic acid (1) is present in both the pro-R and the pro-S positions thus implying scrambling of the label in a desaturation/resaturation process. It is clear from these results, therefore, that 3-nitroacrylic acid (6) cannot be a biosynthetic intermediate and is thus involved only in post biosynthetic cycling.

An indirect confirmation comes from a time course feeding of (2S,3R) - [3-^{2}H] - aspartic acid (15). When 3-nitropropanoic acid (1) is isolated from the culture before it has begun to produce the "reductase" (72 hours), the deuterium is located stereospecifically at C-2. Investigation of the metabolite produced after feeding (2S, 3R)-[3-^{2}H]-L-aspartic acid (15) to a culture which is producing

reductase (96 hours) shows deuterium incorporation at both the pro-R and pro-S positions at C-2 implying post-biosynthetic cycling and scrambling of the label at C-2. This may result if the post-biosynthetic cycling is a non-stereospecific process or if it occurs with inversion.

While conclusive proof of retention of stereochemistry in the conversion of L-aspartic acid (2) into 3-nitropropanoic acid (1) has not been presented, clearly the process is stereospecific and occurs via 3-nitrosuccinic acid (7) in which stereochemistry is retained. Studies on other systems by Gani and Young[33] and Brown and Parker[35] suggest by comparison of ^{2}H nmr data that the overall process may involve retention of configuration at C-2.

In the post-biosynthetic cycling with 2 pro-R deuterated material the 2-pro-R hydrogen from 3-nitropropanoic acid (1) appears to be lost preferentially. The 3-nitropropanoic acid (1) isolated after cycling has occurred has deuterium in both the 2 pro-R and 2 pro-S positions suggesting that inversion or scrambling has occurred. Random scrambling of the label would give, at best, a 50:50 mixture of 2 pro-R and 2 pro-S deuterated material and the 2 pro-S deuterated material could never predominate. Since the ^{2}H nmr spectrum of the methyl valine amides shows that the 2 pro-S deuterium species predominates then the cycling must occur with inversion. The same effect could occur, however, as a result of an isotope effect with the C-^{2}H bond preferentially retained. This would still only result in, at best, a 50:50 mixture of the R and S deuterated acid.

1.9 Future work

While these results have served to delineate a substantial amount of detail about the biosynthesis of 3-nitropropanoic acid (1) from L-aspartic acid (2), a number of factors remain to be investigated. While conservation of stereochemistry rather than inversion seems likely, this remains to be proven conclusively. Oxidation of stereochemically deuterated β-alanines of known conformation may provide a means of confirming the stereochemistry of the product.

Administering [1-^{13}C, 2-$^{2}H_2$]-3-nitropropanoic acid to the culture and isolation of [1-^{13}C, 2-$^{2}H_1$]-3-nitropropanoic acid suggests that 3-nitropropanoic acid (1) and 3-nitroacrylic acid (7) are cycled *in vivo*. The isolated reductase enzyme shows specificity in its catalytic activity but the stereochemistry of the process is unclear.

The stereochemistry of the cycling process can be investigated by feeding 3 pro-S deuterated L-aspartic acid in a time course experiment and determining the stereochemistry at C-2 of 3-nitropropanoic acid (1) after cycling. If scrambling is occurring then both pro-R and pro-S hydrogens will be labelled while if inversion occurs then only the original uncycled material will contain deuterium. This will also serve to confirm any putative isotope effect in the recycling process. The second half of the cycling process can be investigated *in vitro* by incubating the reductase enzyme with 3-nitroacrylic acid (6) and NADP^{2}H and finding if the isolated 3-nitropropanoic acid (1) is stereochemically deuterated at C-2 by preparation of the corresponding methyl valine amides as before.

The nature of any other intermediates remains unclear. While acrylate-type compounds have been eliminated N-hydroxy-L-aspartic acid remains a potential biosynthetic precursor. The instability of this compound militates against synthesis and feeding as a means of determining if it is an intermediate in the biosynthetic pathway between L-aspartic acid (2) and 3-nitropropanoic acid (1).

Chapter 2

2. Thiamine

2.1 Introduction

The biosynthesis and physiological role of thiamine (30) (Fig. 2.1) has been extensively studied and reviewed[50-52]. Thiamine (30) was isolated in 1926[53] from 100 kilograms of rice bran and subsequently synthesised[54,55]. It exists in vivo largely as its diphosphate (31) (Fig. 2.2) (thiamine pyrophosphate, cocarboxylase) in which form it is the cofactor for decarboxylation of pyruvic acid[56]. In addition, in animal tissue, small amounts of free thiamine, thiamine monophosphate and thiamine triphosphate are also found[51].

2.1.1. Chemistry and Physiology

The chemistry and physiology of thiamine (30) have been extensively studied[50-52,57], indeed early work on this vitamin was based upon isolation of an anti-beriberi factor from rice. Ugai demonstrated that benzoin formation from benzaldehyde can be catalysed by thiamine[58,59] (30) and subsequent studies showed that it could catalyse the decarboxylation of pyruvate non-enzymically.

Thiamine pyrophosphate (31) can carry out three types of reaction, non-oxidative decarboxylation of α-keto acids, oxidative decarboxylation of α-keto acids and formation of α-ketols (acyloins). The mechanism of the reactions of thiamine (30) is a consequence of the characteristics of the two ring systems and, in particular, the

Biological activity of thiamine

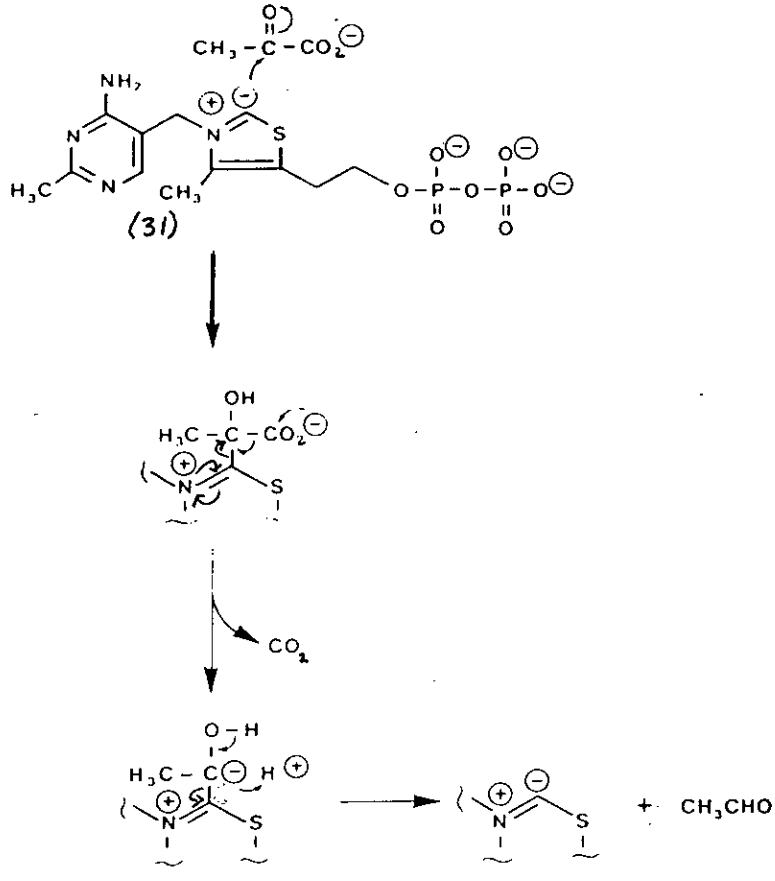


Figure 2.1

The mechanism of thiaminase I activity

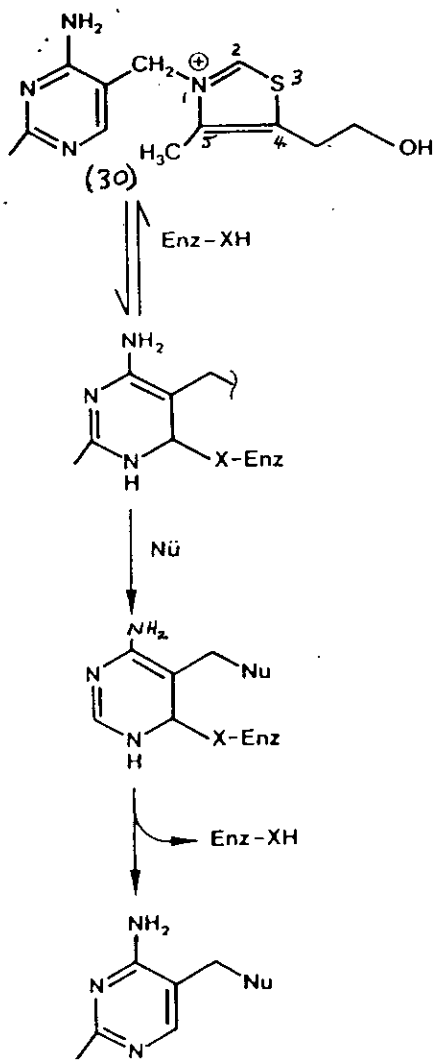


Figure 2.2

thiazole ring. Initial progress in this area resulted from Breslow's discovery[60] that H-2 (Fig. 2.1) of the thiazolium ring would readily exchange into deuterium in deuterated water. This suggests that the reactivity of thiamine (30) occurs as a result of reaction at a C-2 carbanion. The mechanism shown in Fig 2.1 was postulated[61]. This was subsequently substantiated by the observation that several 2α-hydroxyalkylthiamine pyrophosphates function, as expected, as intermediates when added to suitable enzyme preparations[62,63].

The mechanism of nucleophilic substitution on thiamine (30) has been extensively studied - in particular the reaction with aqueous sulphite ion[64]. Thiamine (30) also reacts readily with nucleophiles such as aniline in the presence of an enzyme, thiaminase I to give the pyrimidyl aniline analogue[65,66] (32). An analogous mechanism to that proposed for bisulphite cleavage of thiamine has been suggested, viz. nucleophilic addition across the 1,6 bond of the pyrimidine ring creating a dihydropyrimidine intermediate which can then be substituted at the benzylic methylene with elimination of the thiazole followed by addition of bisulphite (or some other nucleophile) and reversal of the 1,6 addition giving a substituted pyrimidine (Figure 2.2). Hutter and Slama[67] have recently tested this mechanism by preparing a β-chloropyrimidine which, they postulate, forms an enzyme-substrate complex then eliminates chloride thus remaining irreversibly bound to the enzyme as an inhibitor of activity. It appears, therefore, that thiaminase I reacts with thiamine after first forming a 1,6-pyrimidyl enzyme intermediate rather than any other mechanism which would have a strict requirement for the 4-amino functionality in the ring[68] or may lead to hydrogen exchange at the benzylic methylene. The importance of this observation will be discussed in greater detail below.

Biosynthesis of the thiazole ring in eukaryotes

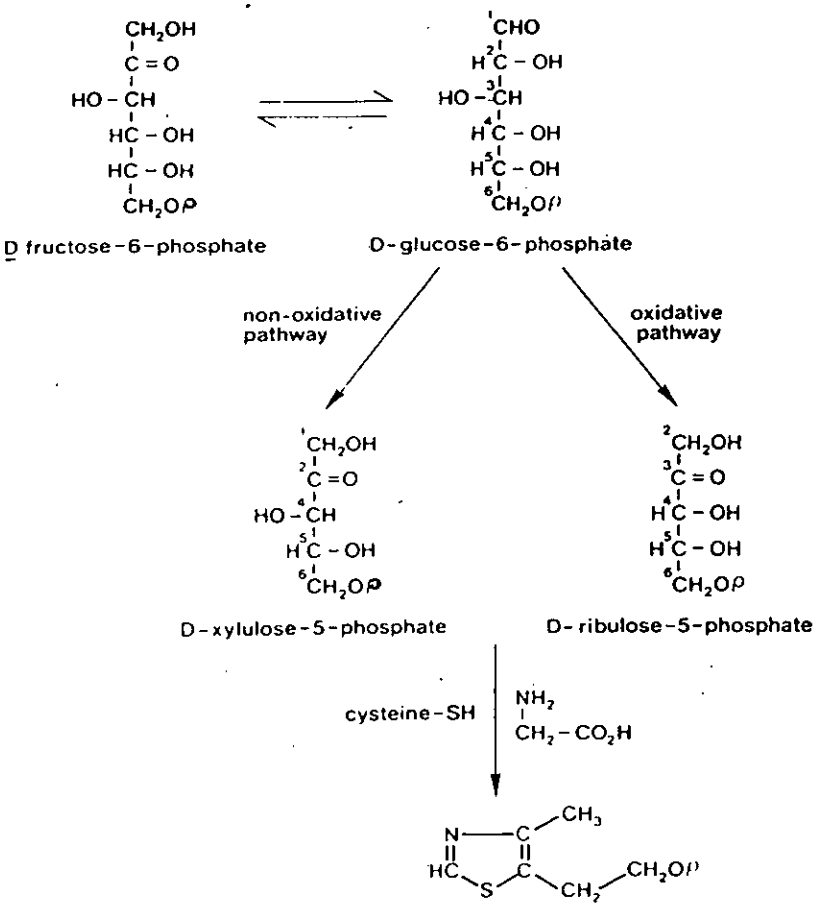


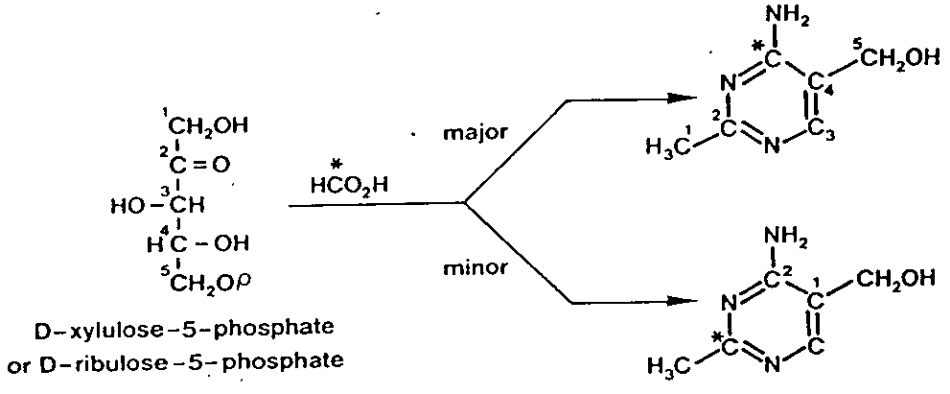
Figure 2.3

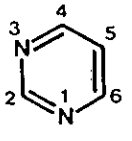
2.1.2 Biosynthesis

The biosynthesis of thiamine (30) has been the subject of significant scientific endeavour since its discovery and structural elucidation. Work has been reviewed frequently with reviews by Brown and Williamson[50], Leder[51] and Young[52] being the most recent. This present work is concerned with the biosynthesis of the pyrimidine ring of thiamine (30) in yeast (Saccharomyces cerevisiae) therefore studies carried out in prokaryotes (in particular, Escherichia coli) will be referred to only briefly and where they shed light or provide contrast to the biosynthesis in yeast. The biosynthesis of the thiazole moiety in yeast will be dealt with since it has some bearing on studies of the pyrimidine pathway.

In the thiazole ring in prokaryotes, the nitrogen atom and the adjacent carbon are derived from tyrosine[69,70] with the remaining five carbon unit coming from pyruvate[71] and a triose[72]. The sulphur atom comes from cysteine[73,74]. In eukaryotes, the thiazole ring is derived from glycine and a 2-pentulose[75] (Figure 2.3). As a result of labelled glycerol and glucose feeding experiments, White and Spenser[75] proposed that incorporation could occur by the oxidative pentose phosphate pathway via D-ribulose-5-phosphate or by the non-oxidative pentose phosphate pathway by way of xylulose-5-phosphate. The proportions of the label from specifically labelled glucose found in the product thiazole are dependent upon which of the two pathways predominate. This result is relevant to their later studies on the biosynthesis of the pyrimidine ring of thiamine.

The biosynthesis of the pyrimidine moiety of thiamine in Saccharomyces cerevisiae





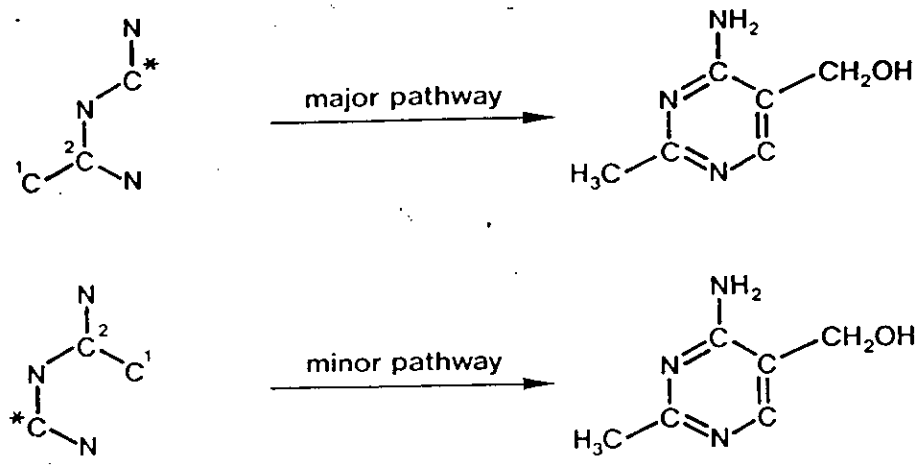
Numbering system for pyrimidine

Figure 2.4

In the biosynthesis of the pyrimidine moiety, a different set of precursors is involved in prokaryotes as compared with eukaryotes. In procarytes, the pyrimidine moiety and purines share a common biosynthetic precursor i.e. 5'-aminoimidazoleribonucleotide (5'-AIR). Formate is therefore incorporated into C-2 of the ring.

In yeast, however, incorporation of formate is at C-4 although a minor pathway has been suggested which involves C-2 incorporation[76]. In a detailed study of the yeast pathway by Grue Sorensen et al. which made use of specifically [^{14}C]-labelled glucose, glycerol and formate the biosynthetic pathway shown in fig 2.4 was elucidated[76]. Two different routes were postulated to operate - one in which C-4 is formate derived, C-2 is from C-2 or C-3 of glucose, C-2' is from C-1 or C-2 of glucose and C-5' is derived from C-6 of glucose. The proportions of C-1 and C-2 incorporated and the site of incorporation are dependent upon the relative proportions of the oxidative and non-oxidative pathways which operate (as seen in the thiazole experiments earlier) and convert glucose to either xylulose-6-phosphate or to ribulose-6-phosphate. These results were confirmed using labelled glycerol. In the minor pathway C-2 was derived from formate, C-2' was from some unknown source and C-4 and C-5 derived from C-1 or C-2 of glucose with the proportions dependent upon the oxidative and non-oxidative pathways as described above. All the other carbon atoms and the nitrogens in this pathway are from some unknown source. On the basis of these experiments, Grue Sorensen et al.[76] suggested that the biosynthetic breakdown of each pathway implicated similar intermediates. In each case a C_2 unit derived from C-1, C-2 of a pentose unit is linked, via the C-2 of the pentose, to two nitrogen atoms one of which, in turn, is attached to a formate-derived carbon atom (figure 2.5).

Putative precursor for the pyrimidine moiety of thiamine



* – formate derived
1 – C-1 glucose derived
2 – C-2 glucose derived

Figure 2.5

Both pathways may, therefore, have the same biosynthetic precursor unit. A 5-aminoimidazole unit was proposed as a possible precursor which could be cleaved in either of two ways in order to give the appropriate partial structure which could then be elaborated to give thiamine (30). Further work by the same group[77] on the biosynthesis of purines in yeast showed that purines and thiamine (30) do not share a common precursor. The carbon skeleton of the purines of Saccharomyces cerevisiae were shown to arise from bicarbonate, formate and glycine and therefore a common precursor does not exist for purines and the pyrimidine ring of thiamine in yeast.

Other studies[78] have shown that N-3 and N-4 in both yeast and E. coli are derived from glutamine. Tazuya et al[79] have recently shown however that administered histidine dilutes the incorporation of [^{15}N]-ammonium chloride in yeast. These workers suggested, analogously to the situation in prokaryotes, that an imidazole ring (that of histidine rather than 5'-AIR) may be a precursor of both carbon and nitrogen atoms of the pyrimidine moiety.

It is clear, therefore, that many of the details of the biosynthesis of the pyrimidine moiety of thiamine (30) remain unknown and therefore warrant detailed investigation. It is clearly important to determine the nature of any intermediates which lie between the pentose unit and the pyrimidine ring. Since, in the major proposed pathway, C-4 is derived from formate, the amino functionality (C-4') could be introduced after or prior to pyrimidine ring formation. A putative precursor in the former case, analogous to the purine amination pathway, would be the desamino pyrimidine.

While such an investigation might be carried out using a

radiolabelled precursor, ease of handling and simplicity of assay would suggest that the corresponding deuterated or [^{13}C]-labelled compound may offer advantages. In order to make use of such a precursor it is important to determine if the product thiamine (30), which is produced in very small amounts, can be assayed accurately using mass spectrometric techniques.

This study is, therefore, designed firstly to develop a reliable method for determining stable isotope incorporation from labelled precursors into the pyrimidine moiety of thiamine in Saccharomyces cerevisiae. The method developed necessarily requires that incorporation will be monitored by mass spectral examination of either the intact thiamine (using Fast Atom Bombardment - FAB) or of a suitable volatile derivative (using Electron Impact (EI) or Chemical Ionisation (CI).

The technique developed can then be applied to a study of the intermediacy of a 4-desamino pyrimidine in the biosynthesis. Initial results from Tazuya et al[78] suggest that glutamine is a precursor and, under these circumstances, amination could be a post-cyclisation event. If, on the other hand, the imidazole ring of histidine is a direct precursor[79], then the 4-amino functionality is likely to be present in this precursor and post-cyclisation amination will not occur.

2.1.3. Analysis and mass spectrometry

While administering stable isotope precursors combined with mass spectrometry of the biosynthetic product have a number of advantages

over radiolabelled investigations - direct analysis of the product with little modification necessary, simplicity of precursor synthesis and product isolation - a number of disadvantages are also inherent in the technique. The sensitivity of mass spectrometry is considerably less than that of radionucleotide detection. The molecule must be presented in a form suitable for analysis. While soft ionisation techniques such as Fast Atom Bombardment (FAB) have greatly increased the range of molecules which can be detected and successfully analysed by ms, a problem nonetheless remains in certain cases. It should be noted that, while pyridinium cations have been shown to be amenable to analysis by FAB ms[80], thiamine itself has never been investigated using this technique.

The detailed analysis of thiamine (30) derived from a labelled precursor by mass spectrometry is, in principle, simpler than the complex degradation and assay procedures required by radioisotope incorporation experiments. It is necessary, however, to separate the desired compound from other components and, while protocols for dealing with very small amounts of thiamine (30) in, for example, foodstuffs exist, the final separation generally makes use of high performance liquid chromatography (hplc)[81]. The use of ms linked to hplc is still at a fairly primitive stage and, although it has tremendous potential for the detailed analysis of vitamins and other micronutrients[82], the method of choice is gas chromatography mass spectrometry (gc/ms). This technique requires that the thiamine (30) be presented in a volatile form.

2.2. Assay of thiamine

2.2.1 Introduction

Since most studies of the biosynthesis of thiamine (30) have made use of radioisotopes, the standard methods of cleavage adopted have concentrated upon separation of the individual carbon atoms. In a very few cases ^{15}N and ^{13}C labelling has been carried out and these have made use of suitable volatile thiol derivatives. In particular, de Moll and Shive[83] investigated the origin of the sulphur in the thiazole part of thiamine by gc/ms using [^{34}S]-sulphate and L-[sulphane-^{34}S]-thiocystine as labelled, non-radioactive precursors. White used ^{13}C and ^{2}H labelled sugars[71] and ^{15}N labelled L-tyrosine to investigate the biosynthesis of the thiazole ring which was cleaved from thiamine using bisulphite then taken up in triflouroacetic anhydride/methylene chloride prior to g.c/ms analysis. While this cleavage gives a volatile thiazole derivative, the pyrimidine part of the molecule forms an involatile sulphonate[84] which is clearly unsuitable for gc/ms analysis.

Direct analysis of the pyrimidine part of thiamine has been carried out by reaction of thiamine in a sealed tube with a suitable alkylthiol[85,86]. Thiamine is particularly susceptible to nucleophilic attack with concomitant liberation of substituted pyrimidine, however the reaction with alkylthiols requires high temperature and pressure. An alternative hydrolysis reaction is catalysed by the enzyme, thiaminase I, (thiamine: base 2-methyl-4-aminopyrimidine-5-methenyl transferase E.C. 2.5.1.2)[65] which has been isolated and characterised[66,67].

Our strategy was, therefore, to determine, firstly, if thiamine can be assayed directly by FAB ms. If this technique is unsuitable then ms linked to gc becomes the method of choice, for which a suitable volatile pyrimidine derivative would be required.

2.2.2. Direct ms analysis

The analysis of thiamine (30) and thiamine pyrophosphate (31) was carried out using FAB-ms The samples were taken up in water and examined in a glycerol matrix under negative and positive ionisation conditions. In neither case could a molecular ion or the pyrimidine part of the molecule be detected under negative conditions. Under positive FAB, however, the molecular ion was seen for both compounds together with a fragment at 123 due to the pyrimidine part of the thiamine molecule. The fragmentation could be followed with peaks at 110 and 93 corresponding to loss of CH_2 and NH_2, however detailed fragmentation was lost in background. It would appear, therefore, that while direct analysis of thiamine (30) and thiamine pyrophosphate (31) is possible there are, nonetheless, difficulties in obtaining detailed spectra of the pyrimidine moiety in FAB mode. In addition, small levels of incorporation of isotopes would be very difficult to detect given the high level of background noise in the spectrum.

Both thiamine (30) and thiamine pyrophosphate (31) can be detected by electron impact (EI)ms This technique was used to elucidate the fragmentation pathway of thiamine using daughter ion analysis. This gave the breakdown pathway shown in fig 2.6. Of particular interest is the loss of CH_3CN from the substituted pyrimidine moiety. This

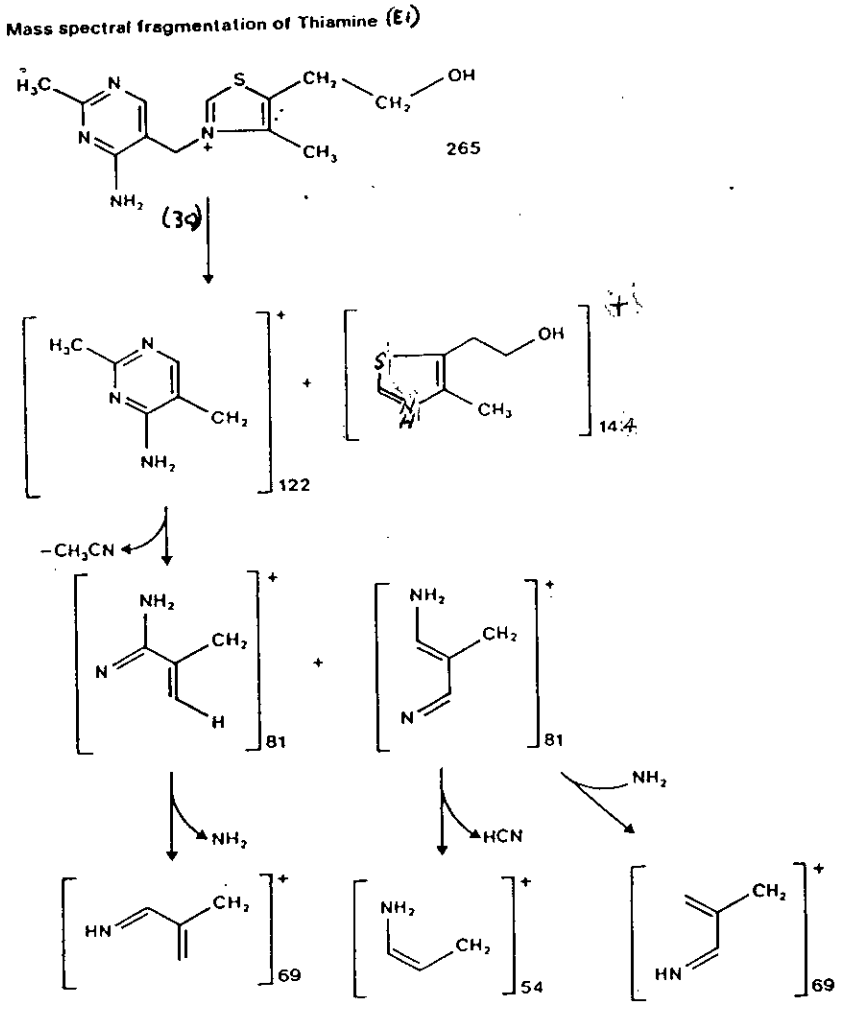

Mass spectral fragmentation of Thiamine (EI)
265
122
144
81
81
69
54
69

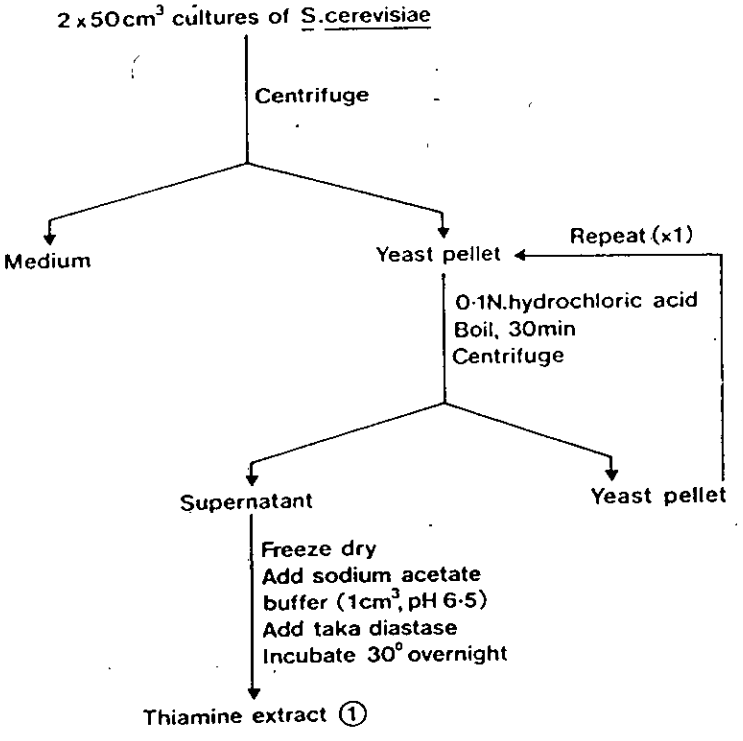
Figure 2.6

can occur by one of two pathways as shown thus unambiguous assignment of all the fragments is not possible. The detection limit for thiamine and thiamine pyrophosphate by EI-ms was rather poor with ca 10 μg of material required in order to obtain a suitable spectrum. This is more than the total amount of thiamine produced in a 50 cm^3. culture of Saccharomyces cerevisiae. In addition to minimise background interference, thiamine (30) has to be in a purified form for ms analysis. The best protocol involved thiamine (30) (or the pyrophosphate (31)) being absorbed onto an ion exchange column then eluted with aqueous buffers. A more efficient and effective strategy is based upon preparation of a suitably volatile thiamine (30) derivative which can then be examined directly by gc/ms

2.2.3 Isolation and Cleavage of thiamine from yeast.

Saccharomyces cerevisiae was grown on a supplemented vitamin-free medium as described in the experimental section and found, by hplc, to produce ca 2-3μg of thiamine (30) per 50 cm^3 of culture after 24 hours growth. Considerable losses were noted if purification by ion exchange was attempted. A protocol was developed for isolation of thiamine (30) based upon methods of White and Spencer[87] and is shown in scheme 2.1. Attempts to reproduce the work of White and others[86] in which thiamine (30) is cleaved with an alkylthiol then analysed directly by gc/ms were unsuccessful. Reaction of thiamine (30) with ethanethiol in a sealed tube at elevated temperature produced a range of volatile products in which the peaks due to the pyrimidine thiol (33), while discernible, were almost swamped by those of other compounds and fragments. Various conditions and thiols were attempted with little success.

Thiamine isolation from Saccharomyces cerevisiae



Scheme 2.1

The breakdown of thiamine (30) can be catalysed by the addition of sodium metabisulphite[84]. In a model reaction 0.1 mole equivalent of sodium metabisulphite was reacted with thiamine (30) and aniline in 60% aqueous ethanol and the mixture heated under reflux. The isolated product was found, from ms, to be the required aniline pyrimidine compound[88] (fig. 2.7). The gc/ms characteristics of the compound were investigated and conditions developed under which it could be purified and analysed (see experimental section). The thiamine extract from yeast in 60% aqueous ethanol was treated with 0.2 μg of sodium metabisulphite and excess aniline then heated as described previously. A control experiment was also carried out in which a similar sample of pure thiamine was treated in an identical manner to the yeast-derived sample. In neither case was any material corresponding to the aniline derivative (32) detected by ms Repetition of the experiment under a range of conditions (different incubation times, temperatures, sealed tube reactions) also failed to give any of the required compound. The difficulties apparent in this approach led to consideration of an alternative procedure based on the enzymatic hydrolysis of thiamine.

Thiaminase I was isolated from Bacillus thiaminolyticus as described by Wittliff and Airth[66]. The bacterium was grown on nutrient agar slants and then transferred to nutrient broth and grown in shake flasks for 20 hours. An aliquot was then transferred to the defined medium of Douthit and Airth[89] and shaken for a further 20 hours. The enzyme activity was assayed by monitoring production of the aniline derivative (32) spectrophotometrically[89] as described below. Initially the enzyme was active against both thiamine (30) and thiamine pyrophosphate (31), however, the activity against the latter

Mass spectral analysis of 2 – methyl – 4 – amino – 5 – anilinomethyl pyrimidine (EI)

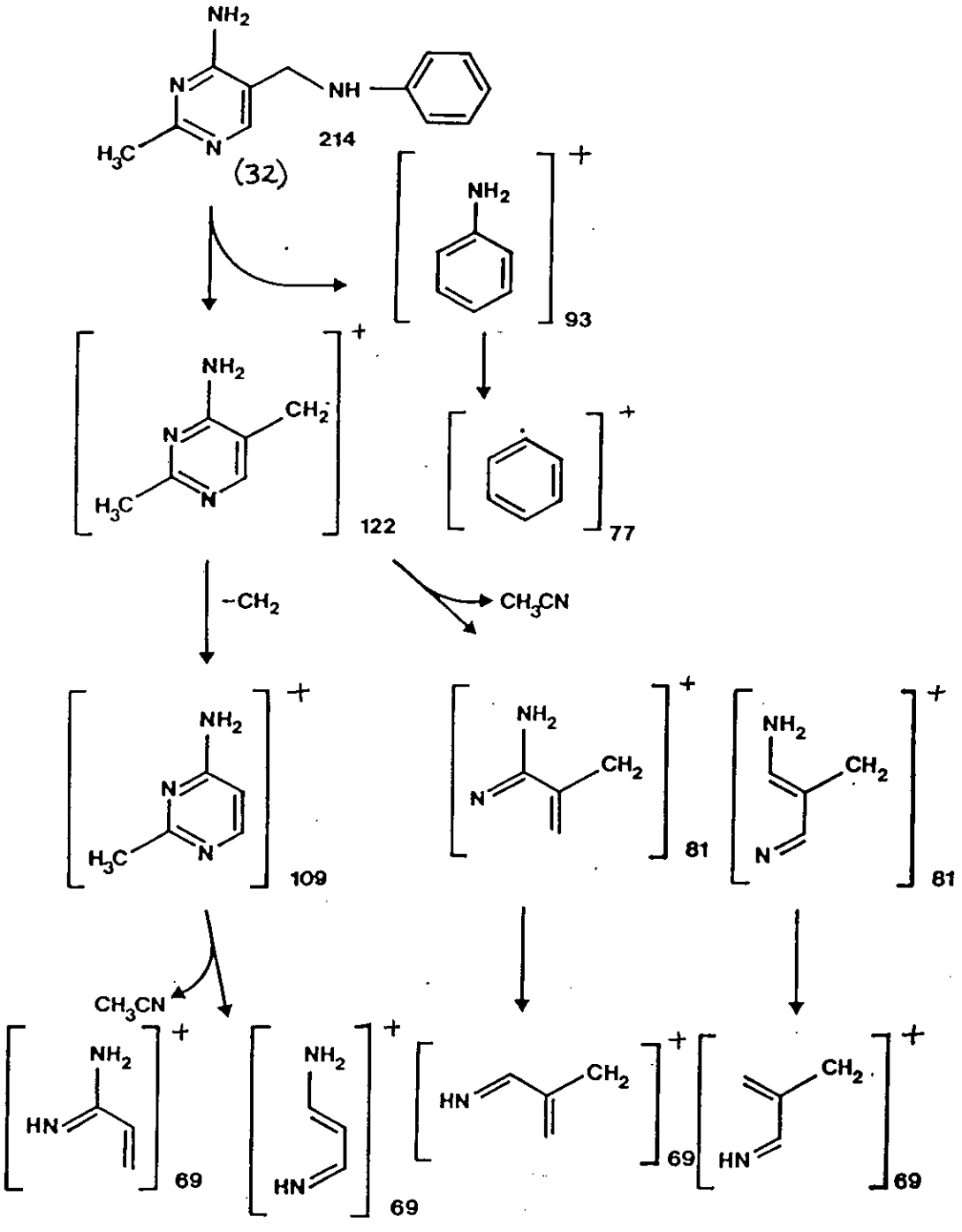


Figure 2.7

substrate was found to diminish fairly rapidly with storage at -25° in buffer (Table 2.1). Activity diminished even more rapidly when the enzyme was stored at 0° thus the loss in activity was not solely a result of the freezing of the protein. The crude enzyme mixture may contain a labile pyrophosphatase as well as thiaminase I. Alternately thiaminase I may exist in two forms; the more stable of which uses thiamine (30) as substrate while the less stable can also (or only) cleave the pyrophosphate (31).

Thiamine pyrophosphate (31) present was hydrolysed prior to incubation, incubations were monitored spectrophotometrically, and enzyme was discarded after one month and fresh stocks reisolated. The purification procedure developed included dialysis of the ammonium sulphate precipitated protein at 0° overnight. This led to a large variation in the activity of the freshly isolated protein. Attempts to purify the enzyme by Sephadex column chromatography as described by Wittliff and Airth[66] gave only inactive fractions. The crude precipitated protein was therefore desalted by concentration in an Amicon filter then made up to the original volume with distilled water. This procedure minimised the amount of time the enzyme was held at 0° and gave enzyme of consistently higher activity than that obtained after dialysis. These findings confirm those of Hutter and Slama[67] who also found prolonged exposure to dialysis conditions led to enzyme inactivation.

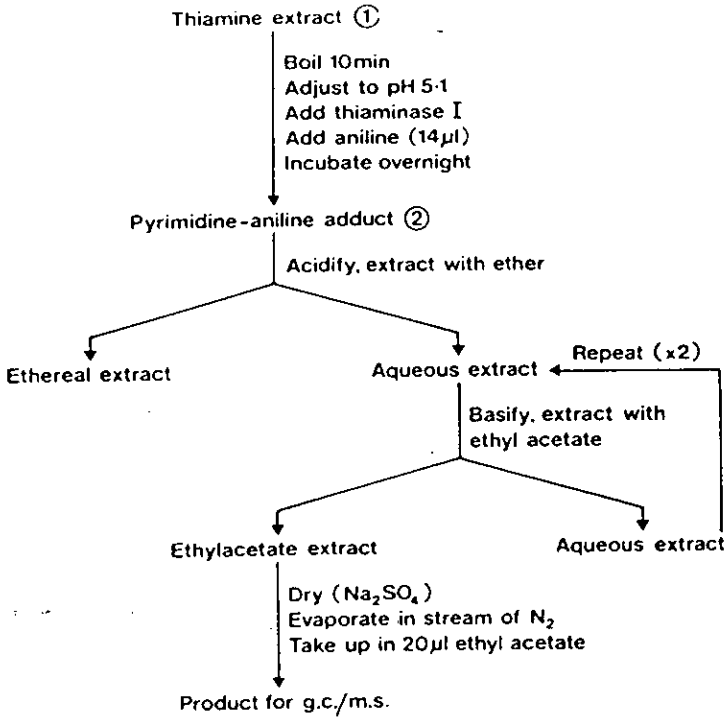
Table 2.1 Activity of thiaminase I with thiamine and thiamine pyrophosphate as substrates.

	Activity*	
Time	Thiamine	Thiamine pyrophosphate
1d	1.00	1.00
5d	0.98	0.92
32d	0.70	0.58

* Activity at 1d taken as 1.00.

Crude thiamine isolate was incubated with thiaminase I and aniline and the formation of the pyrimidine aniline product monitored spectrophotometrically[66]. The aniline-pyrimidine adduct (32) was extracted into ethyl acetate. Drying the ethyl acetate extract with magnesium sulphate led to complete loss of the pyrimidine/aniline compound (32) therefore sodium sulphate was used, the solution filtered and the filtrate concentrated by removing the ethyl acetate in a stream of nitrogen. The residue was taken up in 20μl of ethyl acetate and half of the volume applied to the gc/ms. Direct injection into the ms spectrometer gave EI spectra corresponding to the pyrimidine moiety and the thiazole ring, however when a BP-5 packed column was used, the pyrimidine/aniline compound appeared to be preferentially adsorbed. Capillary column gc/ms enabled the required product to be separated. When the incubation was repeated using similar amounts of pure thiamine (30) under identical conditions the same gc/ms profile was observed. The isolation and analysis protocol outlined in scheme 2.2 was therefore adopted.

Isolation of the pyrimidine-aniline adduct of thiamine



Scheme 2.2

2.3 Biosynthetic studies

2.3.1 Introduction

Having developed a method for the analysis of microgram amounts of thiamine from S. cerevisiae, the biosynthesis of the pyridimine moiety could then be studied. The stage in the biosynthesis at which amination at C-4 occurs was investigated by preparation and administration of normal and deuterated 2-methyl-4-hydroxy-5-hydroxymethyl pyrimidine (34). This compound was chosen by analogy with the purine biosynthetic pathway where inosine monophosphate is aminated to give adenosine monophosphate or via xanthosine monophosphate to give guanosine monophosphate[90]. In both cases, a hydroxyl functionality is present at the point of amination. The question being addressed, therefore, is whether a cyclised pyrimidine (analogous to inosine monophosphate) is aminated or if the amine functionality is present prior to cyclisation as is the case for the pyrimidine moiety in prokaryotes.

The synthesis and incorporation of normal (35) and deuterated 2-methyl-4-amino- 5-hydroxymethyl pyrimidine (36) must be carried out in order that it may be used as a standard. If the deuterated amino compound (36) - which is a known precursor - is not incorporated to an extent sufficient to permit examination by ms then there would be little point in attempting to study the incorporation of the [5'-^{2}H]-2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34). If this were the case, the corresponding ^{14}C or tritiated compound would have to be used.

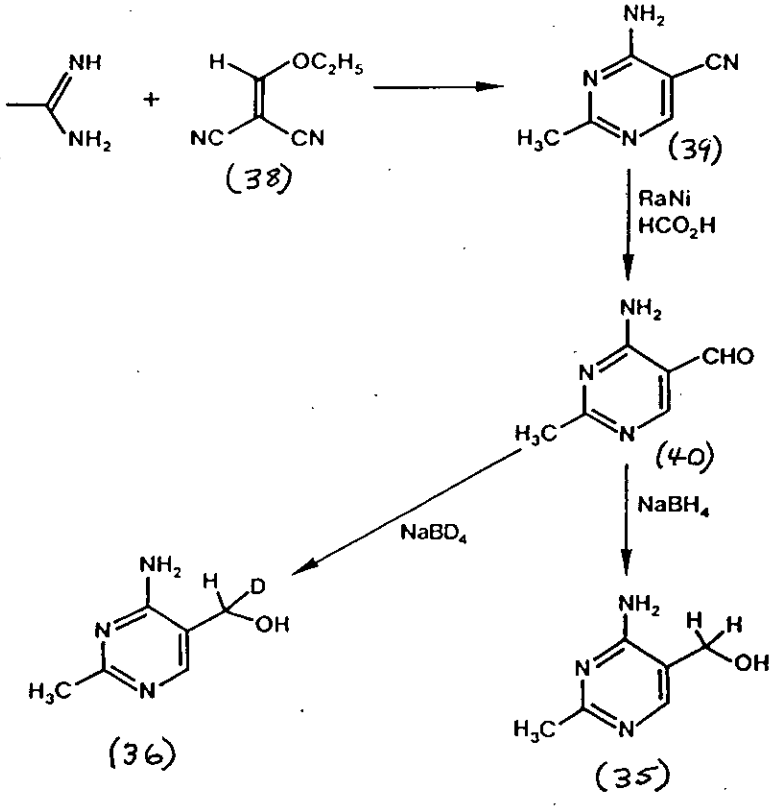
The most convenient position to incorporate a label was considered to be at C-5′ by preparation of a suitable derivative which could be reduced using a deuterated reducing agent such as sodium borodeuteride or lithium aluminium deuteride.

The chemistry of 4-hydroxypyrimidines has been extensively reviewed[91]. They can exist in a keto or an enol form (lactim/lactam isomerisation) and this clearly has a significant effect on the chemistry both of the ring itself and on any substituents attached to it[92]. In normal pyrimidines, the 2, 4 and 6 positions of the ring are electron deficient. Substitution with any group which is electron releasing (eg. hydroxyl) will give the pyrimidine a more aromatic character. The chemistry of 2, 4 and 6 hydroxylated pyrimidines is similar giving, for example, principally N-substituted products in alkylation reactions. This is a direct consequence of keto-enol tautomerism and the favouring of the keto form. In addition, it has been reported that 4-hydroxypyrimidines can associate in solution because of hydrogen bonding[93] and this may have an effect upon reactions at the 5-position.

2.3.2 Synthesis of 2-methyl-4-amino-5-hydroxymethyl pyrimidine

Acetamidine hydrochloride was treated with sodium ethoxide to generate the free base then reacted with ethoxymethylene malonitrile[94] (38) to afford immediately 2-methyl-4-aminopyrimidine-5-carbonitrile (39) as a dense precipitate which was recrystallised from ethanol. This product was converted to the corresponding aldehyde (40) by treatment with Raney nickel in formic acid[95]. The aldehyde had identical tlc characteristics to the nitrile in chloroform/methanol (4:1) but was identified on tlc by spraying with

Synthesis of 2-methyl-4-amino-5-hydroxymethyl pyrimidine



Scheme 2.3

2,4-dinitrophenylhydrazine. The crude aldehyde was reduced using sodium borohydride to afford the corresponding 5-hydroxymethyl compound (35) which was characterised by nmr and ms. The aldehyde could also be reduced with sodium borodeuteride to afford the monodeuterated product (36) (Scheme 2.3). The extent of deuteration was determined by ^{1}H nmr spectroscopy and found to be ca 90% single deuteration at the C-5′ position.

2.3.3 Synthesis of 2-methyl-4-hydroxy-5-hydroxymethyl pyrimidine (34)

The first attempt to prepare deuterated 2-methyl-4-hydroxy-5′-hydroxymethylpyrimidine (37) made use of the corresponding 5-ethyl ester (41) which can be prepared easily from acetamidine and diethylethoxymethylenemalonate[96]. This gave ethyl-2-methyl-4-hydroxypyrimidine-5-carboxylate (41) in reasonable yield. Attempts to reduce the ester using sodium borohydride under a variety of conditions (eg. with polyethylene glycol[97], with methanol and tert-butanol[98], with excess borohydride[99], in the presence of calcium chloride[100], diglyme with lithium bromide[101]) gave only starting material. Direct reduction with lithium aluminium hydride in tetrahydrofuran[102] afforded 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) however the yield was low (< 20%) therefore alternative methods were investigated.

Attempted preparation of ethyl 2-methyl-4-chloro pyrimidine-5-carboxylate (42) by reaction with thionyl chloride gave no isolable product. The hydroxy group could be readily substituted with a chloro group by reaction with phosphorus oxychloride[103]. It was found to be important to keep the reaction at ice temperature during quenching of excess reagent and work up otherwise hydrolysis to give the non-chlorinated product

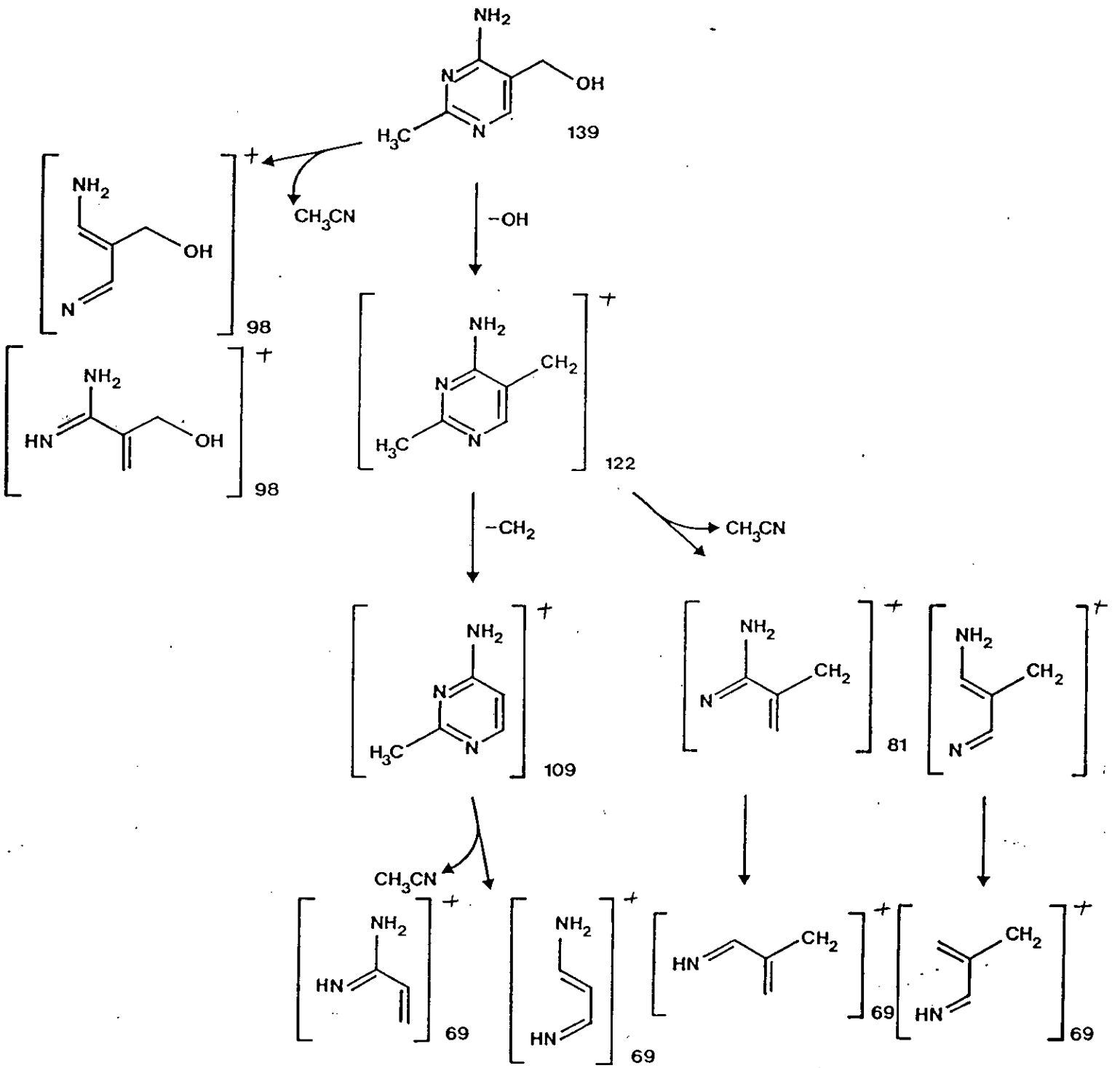
occurred. Ethyl 2-methyl-4-chloropyrimidine-5- carboxylate (42) was unstable and thus was used rapidly after minimal clean up.

Reaction of (42) with sodium methoxide in methanol[104] gave ethyl 2-methyl-4- methoxypyrimidine-5-carboxylate (43) which could be readily reduced with sodium borohydride in the presence of methanol and t-butanol[98] to afford the corresponding 5-hydroxymethyl compound (44). The mono deuterated compound (45) could be prepared by reaction with sodium borodeuteride under identical conditions. Unfortunately attempts to regenerate the 4-hydroxy analogue (37) by heating with trifluoroacetic acid or with hydrochloric acid (6N) gave only starting material and this route was abandoned.

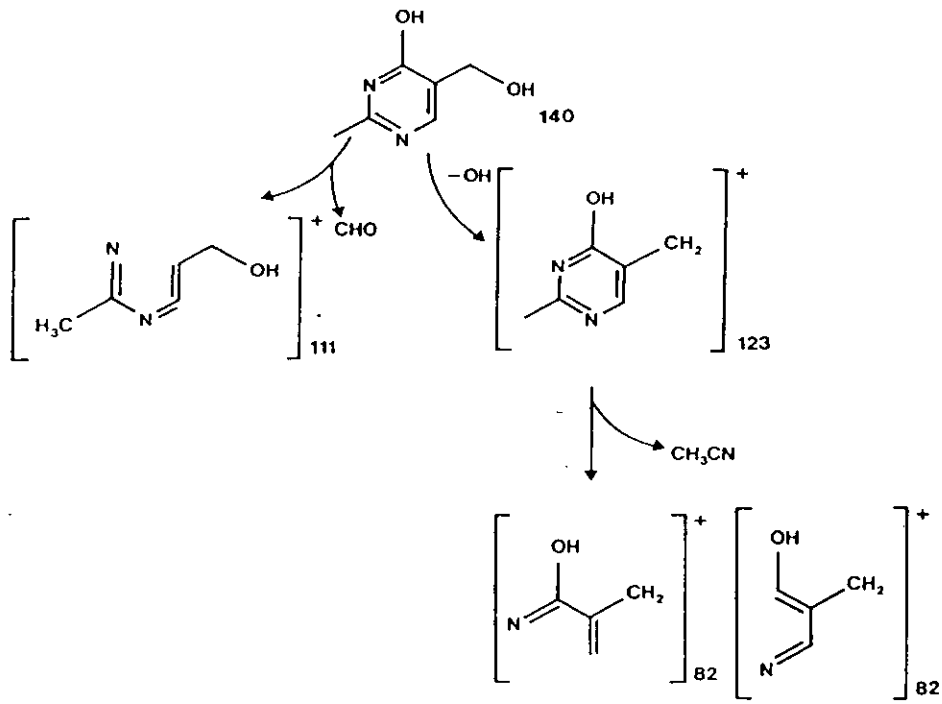
Cyanoethoxymethylene acetate[105] (46) was reacted with acetamidine hydrochloride to afford 2-methyl-4-hydroxypyrimidine-5-carbonitrile[94] (47) however attempts to reduce this compound to the corresponding aldehyde (48) using Raney nickel gave only starting material.

Treatment of 2-methyl-4-amino-5-hydroxymethylpyrimidine (35) with hot hydrochloric acid gave the corresponding 4-hydroxy compound (34) in good yield. The same reaction carried out with the [5′-^{2}H] analogue (36) showed retention of the deuterium in reaction times up to 30 minutes (80% retention by ms) however longer reaction times led to exchange. This method was, therefore, used to prepare 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) and [5′-^{2}H]-2-methyl-4- hydroxy-5-hydroxymethylpyrimidine (37). Hydrolysis of the 4-amino aldehyde (40) to the corresponding 4-hydroxy aldehyde (48) using 6N hydrochloric acid was carried out but the yield was poor (<30%).

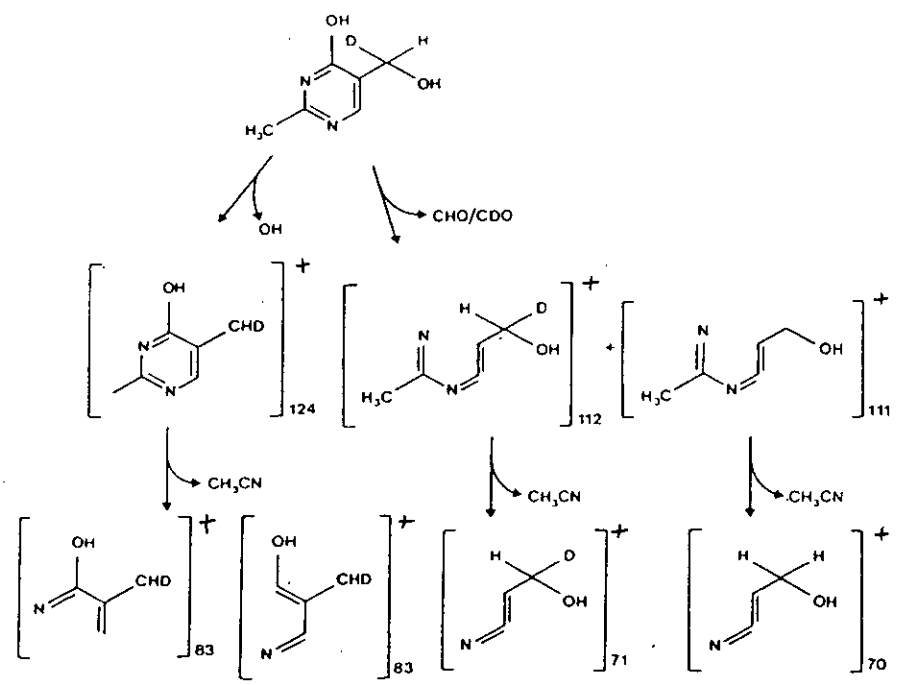
Scheme 2-4
Mass spectral analysis of 2-methyl-4-amino-5-hydroxy methyl pyrimidine (EI)



Mass spectral analysis of 2 – methyl – 4 – hydroxy – 5 – hydroxymethyl pyrimidine



Mass spectral analysis of [5' - ^{2}H] – 2 – methyl – 4 – hydroxy – 5 – hydroxymethyl pyrimidine



Scheme 2.5

The mass spectral analysis of 2-methyl-4-amino-5-hydroxymethyl pyrimidine (35) gives the expected fragmentation pattern (scheme 2.4) with loss of OH followed by ring cleavage or loss of the benzylic CH_2.

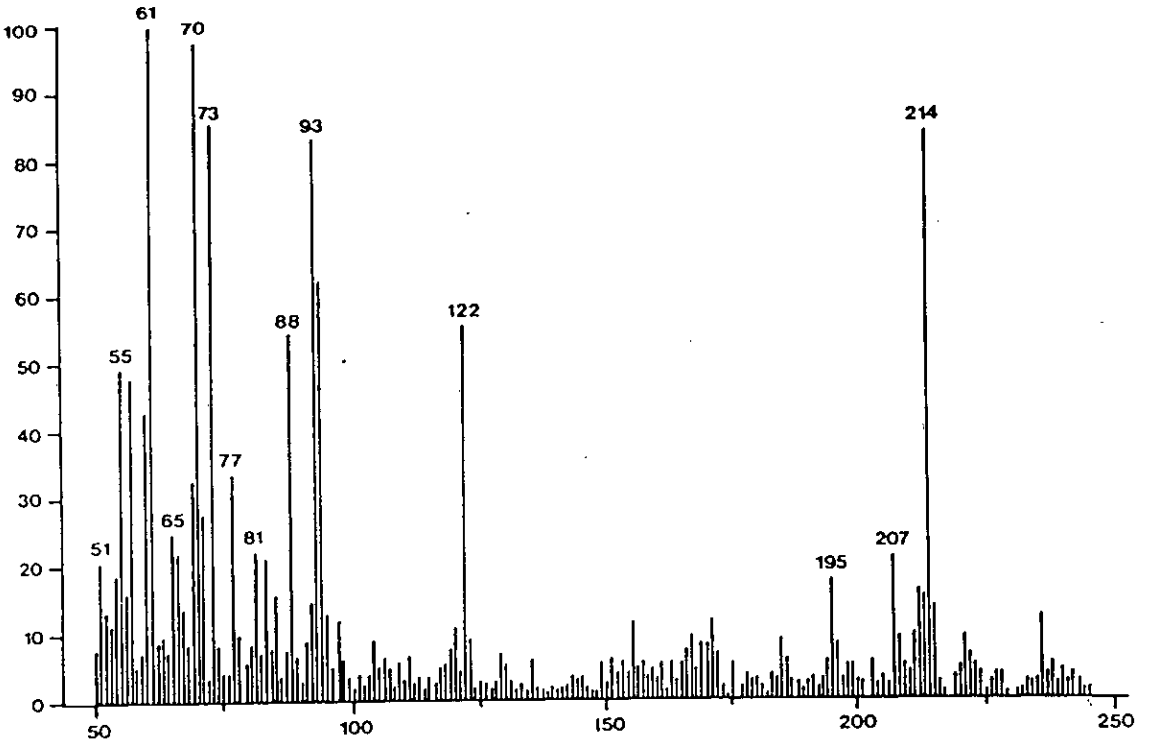
In the case of the corresponding 4-hydroxy compounds (34) and (37), fragmentation involves loss of H_2O (scheme 2.5) giving an $[M-OH]^+$ at 123. In the deuterated analogue (37) fragments 123 $[M-OD]^+$ and 124 $[M-OH]^+$ are observed clearly. A most characteristic fragment occurs as a result of loss of CHO. The accepted mechanism for such a loss in phenolic compounds[106] involves elimination of oxygen together with its adjacent ring carbon atom. Substantial hydrogen scrambling occurs with CHO loss and this is confirmed in the case of the hydroxy pyrimidine by loss of deuterium from C-5′ (scheme 2.5) which may occur by a similar mechanism to that shown. Further fragmentation then occurs as expected. Such fragmentation and the loss of deuterium suggests that the keto form is preferred.

2.3.4. Results

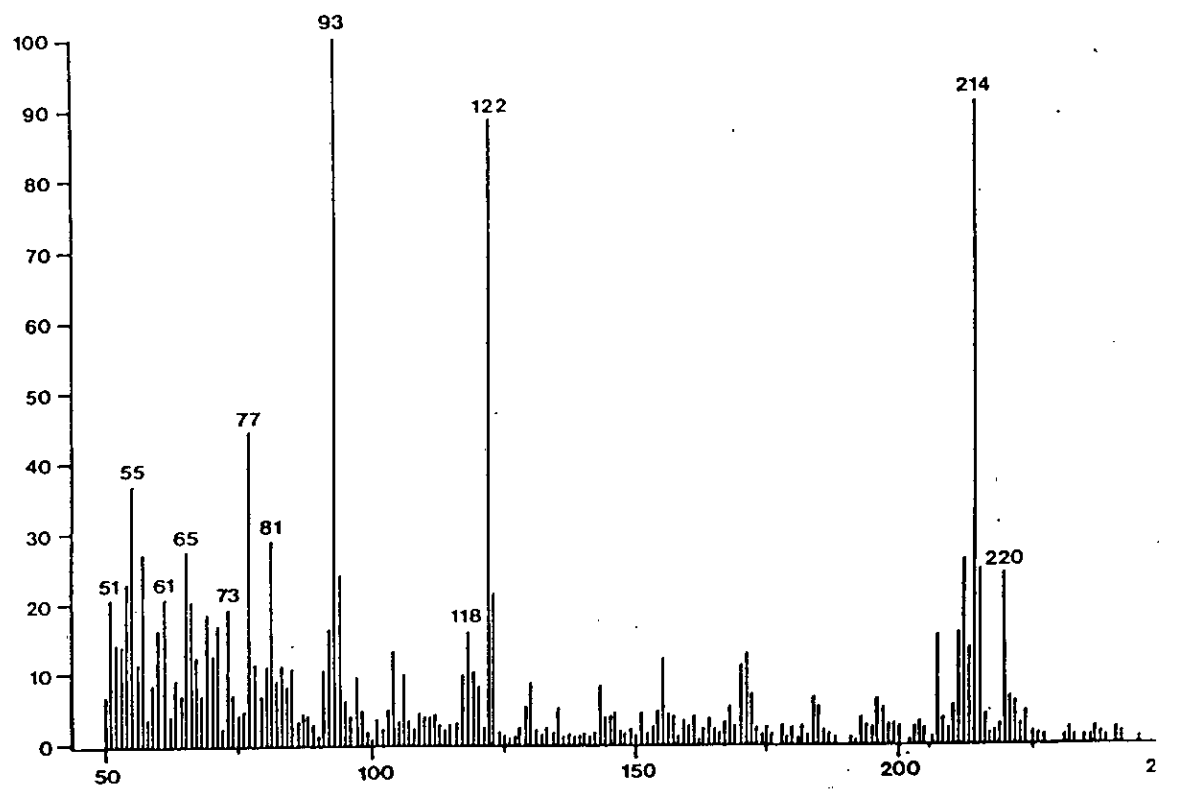
Protiated and deuterated 2-methyl-4-amino-5-hydroxymethyl pyrimidine (35) (36)and 2-methyl-4-hydroxy-5-hydroxymethyl pyrimidine (34),(37) were fed to growing cultures of Saccharomyces cerevisiae. The isolated thiamine (30) was incubated as before, in the presence of aniline, with freshly prepared thiaminase I and the crude ethyl acetate extract examined by capillary gc/ms.

Peaks due to the molecular ion at 214 and a major fragment at 122 were examined for deuterium incorporation. The background figure for the M + 1 peak in the unlabelled feeding trial is 4.5% for 215 and 1.8% for 123 (Fig.2.8(a)). The product from feeding the labelled 4-amino pyrimidine (36) shows increases to 12.5% and 9.1% for each of

(a)


100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
61
70
73
93
214
122
88
55
77
65
81
51
195
207

(b)


100
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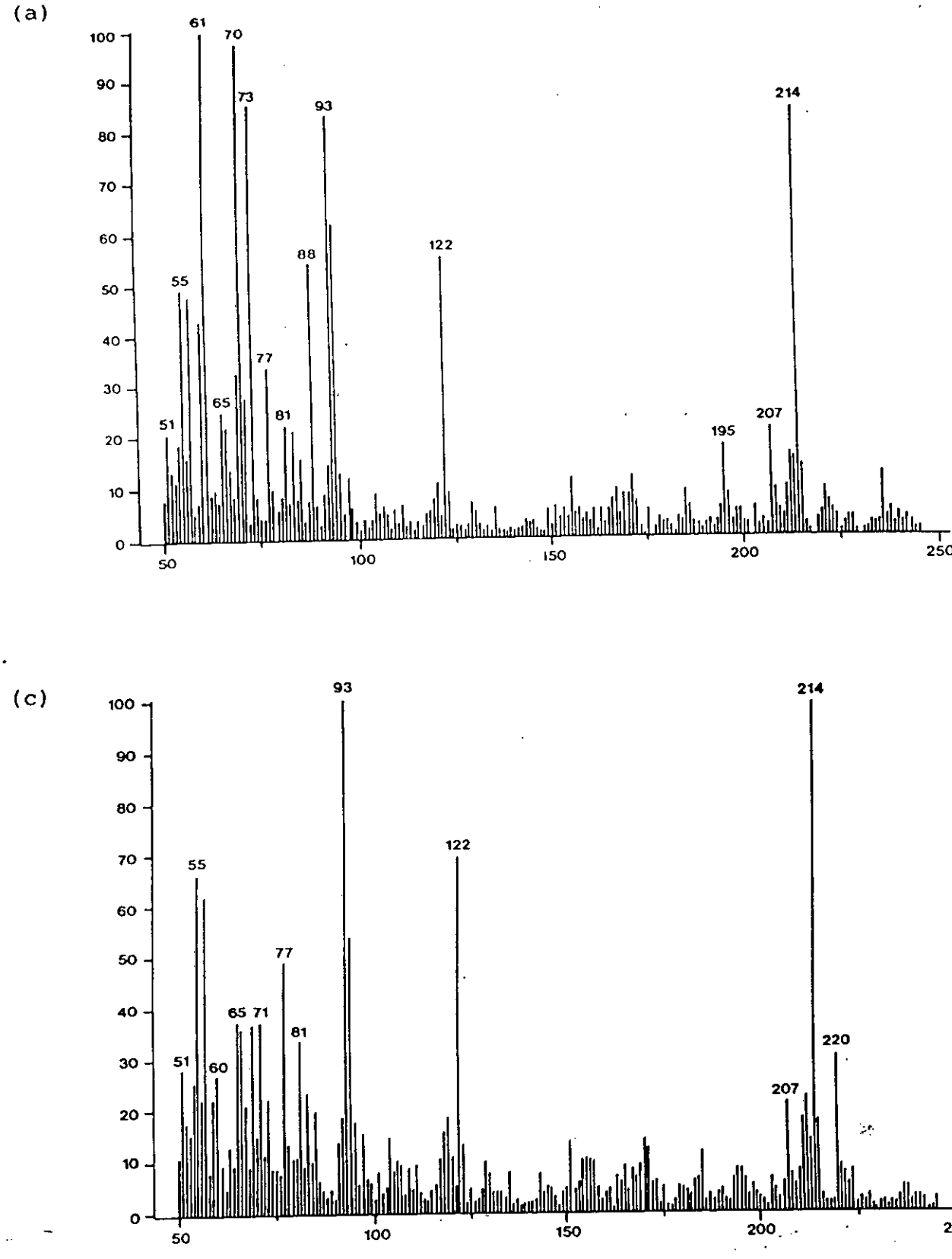
118

Figure 2.8


(a)
100
90
80
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40
30
20
10
0
50
100
150
200
250
61
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73
93
214
122
88
55
77
65
81
51
207
195
(c)
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
93
214
122
55
77
65
71
81
220
51
60
207

Figure 2.8

these peaks respectively (Fig. 2.8(b)) corresponding to net incorporation of 8% and 7.3%. Feeding the corresponding 5-deutero 4-hydroxy compound (37), at the same level gave no net incorporation (Figure 2.8(c)).

When the level of administered [5'-^{2}H]-2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (37) is increased, an inhibitory effect on the growth of the yeast is observed. At 8 mg/50cm^3 of culture, normal growth is observed, however at 25 mg/50cm^3 of culture, growth, as assessed by optical density of cells, is reduced by 60% while at 50mg/50cm^3 of culture, virtually no growth occurs.

2.3.5. Discussion

These results suggest that 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34), while a yeast growth inhibitor, is not a precursor of the pyrimidine moiety of thiamine. This implies that the 4-amino group is not incorporated subsequent to pyrimidine ring formation. The lack of incorporation would also suggest that the enzymes which are involved in the biosynthesis of thiamine have a relatively high substrate specificity - the 4-hydroxy compound is not incorporated to form an oxythiamine analogue of thiamine (30) as might be expected if the substrate specificity were fairly low. However, it should be noted that oxythiamine has been shown to be toxic[107] and it may be formed in amounts too small to detect while being sufficient to prevent yeast growth. The oxythiamine analogue has not been tested as a substrate for thiaminase I and may not be cleaved although the postulated mechanism does not require a 4-amino functionality.

Spenser et al. have already noted[77] that, in contrast to prokaryotes, the ring skeleton of the pyrimidine in thiamine (30) and the purines in yeast do not have a common precursor. Since the purines have been shown to have a common biogenesis in eukaryotes and prokaryotes, (from bicarbonate, formate and glycine), it necessarily follows that the carbon skeleton of the pyrimidine moiety of thiamine and that of purines in yeast are not linked. A unit which contains all three nitrogens already would appear to be favoured by this result. The attachment of a nitrogen to the formate-derived carbon at C-4 is clearly an early biosynthetic step occurring prior to pyrimidine ring formation.

2.3.6. Conclusions

A method has been developed which can be used to study the biosynthesis of thiamine (30) at the microgram level using gc/ms and stable isotopically labelled precusors. Such a method has certain advantages over radiochemical methods including the ease of precursor preparation and the direct analysis of the product thiamine without the necessity of complex degradation and analysis of breakdown products. While this method is considerably less sensitive than using radiolabelled biosynthetic intermediates, when late precursors are being studied and where good incorporation would be expected, the levels of labelled material are adequate. From the background observed in this present series, an incorporation of the order of 6% is required in order to be easily detected over background using gc/ms.

The method developed has been used to investigate the possible intermediacy of 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) in

the biosynthesis of the pyrimidine moiety of thiamine. The corresponding 4-amino compound (35) is clearly incorporated, as expected. The apparent non-incorporation of the 4-hydroxy compound (34) eliminates it as a precursor of the 4-aminopyrimidine and, hence, of thiamine (30). The result implies that post-ring amination does not take place and that the 6 atom skeleton proposed by Spenser et al.[76] involving an amino functionality on the formate-derived carbon atom or a histidine precursor[79] may be correct. 2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) is, however, a potent inhibitor of growth of yeast[108]. The precise nature of this inhibition is unclear however, the 4'-amino group of the pyrimidine ring is essential to thiamine pyrophosphate coenzyme activity and the 4-hydroxy analogue of thiamine has been shown to be totally inactive as a coenzyme[109]. If the biosynthetic pathway is capable of incorporating 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) to make the corresponding 4-hydroxy thiamine analogue, then this may account for the observed inhibition of growth although this analogue was not detected in the incubate. The 4-hydroxy analogue of thiamine causes a 50% decrease in the activity of pyruvate decarboxylase after simultaneous incubation of the enzyme with thiamine pyrophosphate and the analogue[110]. 2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) may thus be incorporated into 4-hydroxythiamine and then act as an inhibitor of cell growth as a consequence of its effect on thiamine-dependent enzymes or on the biosynthesis of thiamine itself. Below the level necessary for inhibition, it appears not to be substantially incorporated although a failure to detect it may be due to the failure of thiaminase I to cleave the 4-hydroxythiamine analogue or to the low levels formed.

A more detailed investigation of the biological effect of 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) is clearly warranted, as is a study of the biosynthetic pathway between the pentose unit and the complete pyrimidine ring of thiamine (30).

Chapter 3

3. Studies in the Genetic Manipulation of Biosynthetic Pathways

Modifying the activity of restriction enzymes - a preliminary study.

3.1 Introduction

Biosynthetic pathways are composed of individual steps mediated by one or more enzymes. The detailed study of the individual steps (and hence the intermediates) involved in the biosynthesis of a particular product is crucial to understanding the mechanism and the factors affecting the production of the biosynthetic target molecule. Many studies have been carried out using mutants which contain blocks in the biosynthetic pathway and hence accumulate particular intermediates[110]. Such an approach is relatively tedious and, essentially, random.

In many cases the enzymes for any particular biosynthetic step in a pathway are strictly controlled. The sequence of amino acids in the enzyme are coded for by groups of three bases (codons) in ribonucleic acid (RNA) which in turn are read directly from the corresponding deoxyribonucleic acid (DNA). Control of the expression of the gene for a particular enzyme generally takes place at the DNA level. Most of the DNA in a cell is accumulated in the nucleus however, small, autonomously replicating circular pieces of DNA (plasmids) exist in cells. Plasmids can be replicated many times over in the cell, they are also passed on to daughter cells and the protein they code for is produced by the cell using the normal cellular apparatus.

Furthermore, the production of this protein is not generally subject to the control factors which govern normal chromosomal cellular protein production thus it can be overproduced and subsequently isolated[111-113].

It is clearly implicit in the above that the simple introduction of a piece of DNA into a cell will not result in transcription and translation to give the protein which is coded for by the DNA. The DNA which codes for a particular protein (enzyme) in a biosynthetic pathway must be located and isolated. The isolation process is carried out using enzymes which recognise and cleave DNA at specific sites (Type II restriction enzymes). A great number of these restriction enzymes with different site specificities have been isolated[113]. A plasmid* is then opened up using the same restriction enzyme(s) and the piece of isolated DNA inserted and ligated thus reclosing the plasmid. The ends of the insert and the plasmid must be compatible to ensure maximum incorporation (ligation) and this means that they ought to be cut with the same restriction enzymes. From the point of view of the plasmid, this is easily accomplished by preparing a suitable strong promoter with a series of restriction sites following it. There is however no guarantee that a unique restriction site occurs at the beginning of the gene to be inserted. In such cases, more complex manipulation using linkers must be carried out. The extra piece of DNA which is introduced into the plasmid must be placed under the control of a suitable mechanism (a promoter) which directs the cellular machinery to produce large

* Note: A plasmid is an autonomous, self-replicating piece of closed circular DNA which carries the piece of DNA of interest into the cell and then infiltrates the cellular machinery so that the protein coded for is produced. Multiple copies of plasmids can occur in any one cell thus leading to overexpression.

amounts of the corresponding RNA otherwise no protein will be synthesised. The modified plasmid is introduced into the host cell and, if successful, is replicated and produces large amounts of the required enzyme which can be isolated and studied.

This is a greatly simplified picture however. In the preliminary stage of the process the DNA sequence which codes for the protein of interest must be uniquely identified and then excised from the genomic DNA molecule. This latter procedure can involve a large number of manipulations since suitable restriction sites are rarely conveniently placed in natural sequences.

It would thus, clearly be of value to be able to modify the activity of restriction enzymes so that particular sites can be favoured or disfavoured. There are a number of observations which suggest that this may be possible. In the case of a large piece of DNA derived from phage lambda (ca 43 k base pairs), the cleavage of the DNA at its known restriction sites by the enzymes EcoRI and Hind III is not a simple process. Some preference is shown for particular sites[114,115]. Additionally, a non-specific endonuclease which generally cleaves DNA at random, nonetheless shows preferences for particular base sequences[116]. A further group of site-specific restriction enzymes (notably Nar I,Nae I, Sac II and Xma III) show dramatic site preferences between recognition sites in the plasmid pBR322. No correlation with surrounding nucleotides has been observed and the reason for these differences remains unclear. If it is possible to further modify restriction enzyme specificity in a predictive and reproducible fashion in order to get cleavage at certain recognition sites only or, indeed, to alter slightly the recognition sites themselves, then alternative cloning strategies may

be possible with concomitant advantages in the study of natural product biosynthesis. The activity of certain restriction enzymes can be modified by the presence of organic solvents or in high salt concentrations[117,118]. Such "star" activity generally leads however to a loss in specificity and greatly increased frequency of cleavage. One possibility is to constrain either the cleavage sites in the DNA or the activity of the enzyme such that certain sites are disfavoured compared with others thereby increasing the specificity of the enzyme.

Enzymes have frequently been used in an immobilised form and, more recently, in low water systems[119]. A favoured method of creating low water systems is to dissolve the enzyme in a very small amount of water and then to distribute this aqueous solution into an apolar organic solvent (generally an alkane) in which a surfactant (eg sodium dioctyl sulphosuccinate (AOT)) has been dissolved. The surfactant serves to protect the enzyme from the organic solvent and causes the formation of very small water droplets in which the enzyme is encapsulated (fig 3.1). These droplets comprise a thermodynamically stable system which is optically transparent is of low viscosity and is termed a reversed micelle[120,121]. The water droplets are generally of the order of 2-20nm. in diameter and the interfacial area is very large (tens of m^2 per cm^3). These systems have been used to "solubilise" enzymes in organic solvents, to enable organic substrates to come into contact with enzymes and, on occasion, to improve enzyme stability and efficiency. In addition, novel enzyme activity has been noted in certain cases[122].

Despite the small size of reverse micelles (typically of the order of 10-15 nm), large enzymes[123], nucleic acids[124] and even bacterial

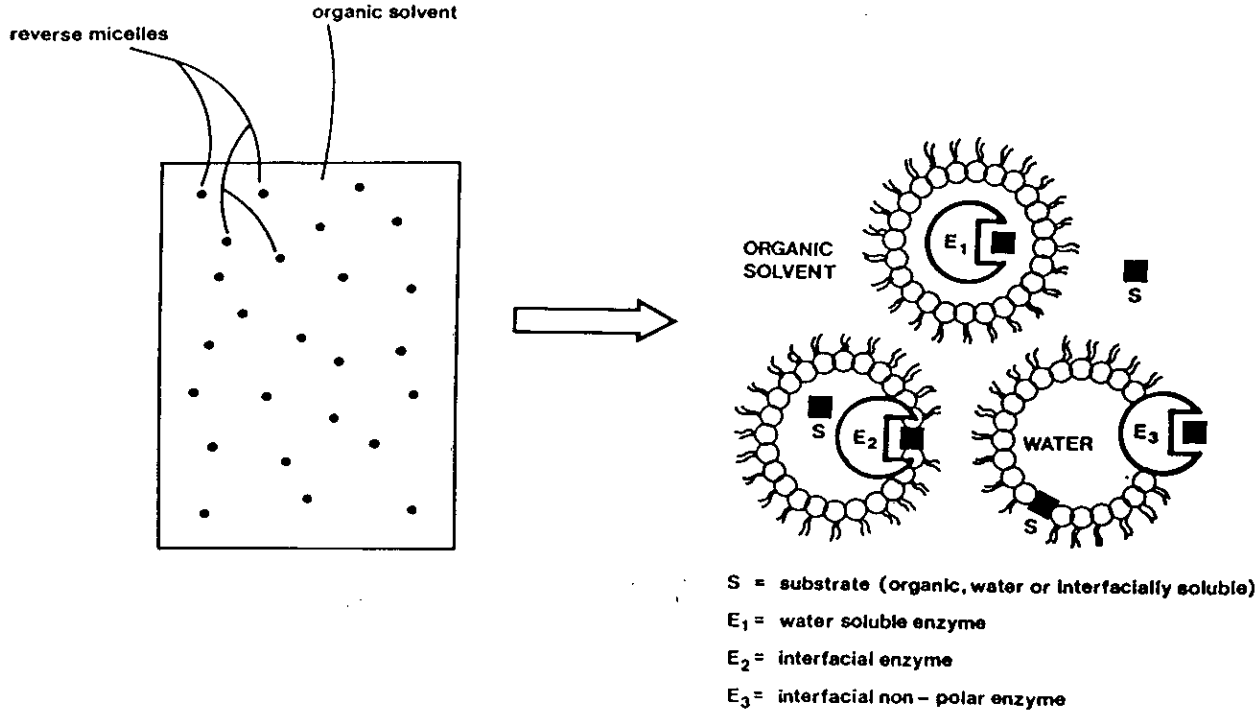

reverse micelles
organic solvent
ORGANIC SOLVENT
E1
S
S
E2
WATER
E3
S
S = substrate (organic, water or interfacially soluble)
E1 = water soluble enzyme
E2 = interfacial enzyme
E3 = interfacial non – polar enzyme

Figure 3.1

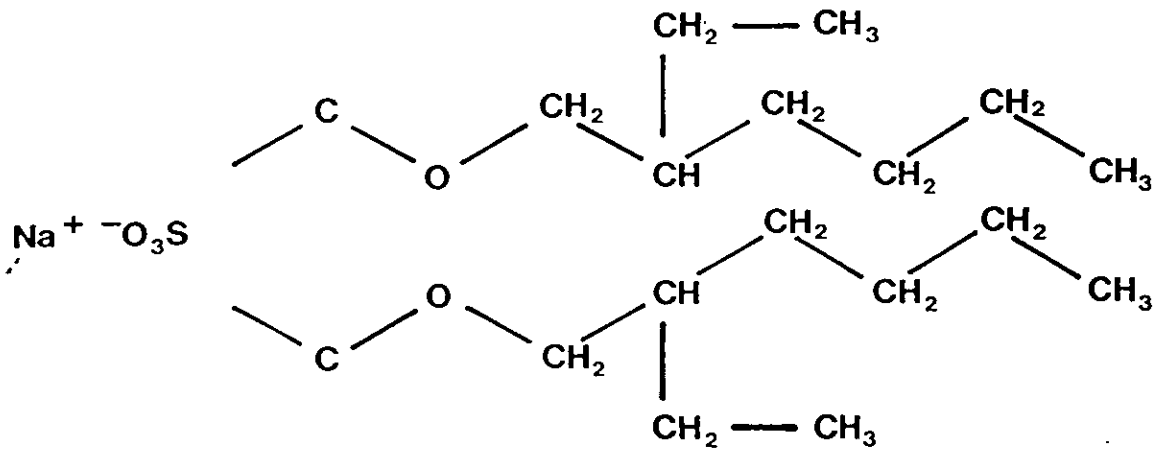
cells[125] have been taken up in reverse micelle systems and found to function normally. The precise nature of the system in these cases remains unclear, however it seems likely that conformational restraints on both the enzyme and the nucleic acid exist.

These conformational restraints on large molecules may lead to a modification in the substrate specificity of the entrapped enzyme or changes in the availability of the substrate. In the case of restriction enzymes and nucleic acids, this may lead to unusual fragments due to changes in the recognition site or in partial disfavouring of certain sites due to the folding of the DNA molecule in the reverse micelle system. In this study we examined the action of a series of type II restriction endonucleases on DNA in reverse micelles to see if enzyme activity is retained in these systems and, if so, then whether modification of site specificity is exercised.

3.2 Results

3.2.1 Cleavage of plasmid pUC 8

The plasmid pUC 8 is a member of the family of pUC plasmids developed by Messing et al[126]. It is derived from pBR322 - the first unnatural plasmid vector constructed with specific defined characteristics. The pUC series contain the replication origin and ampicillin resistance gene of pBR322 with a portion of the β-galactosidase (lac Z') gene of E. coli. The value of pUC plasmids resides in the number of unique restriction sites within the lac gene. When the plasmid is transformed into a suitable strain of E. coli, blue colonies are produced on plates which contain a specific indicator. The cells



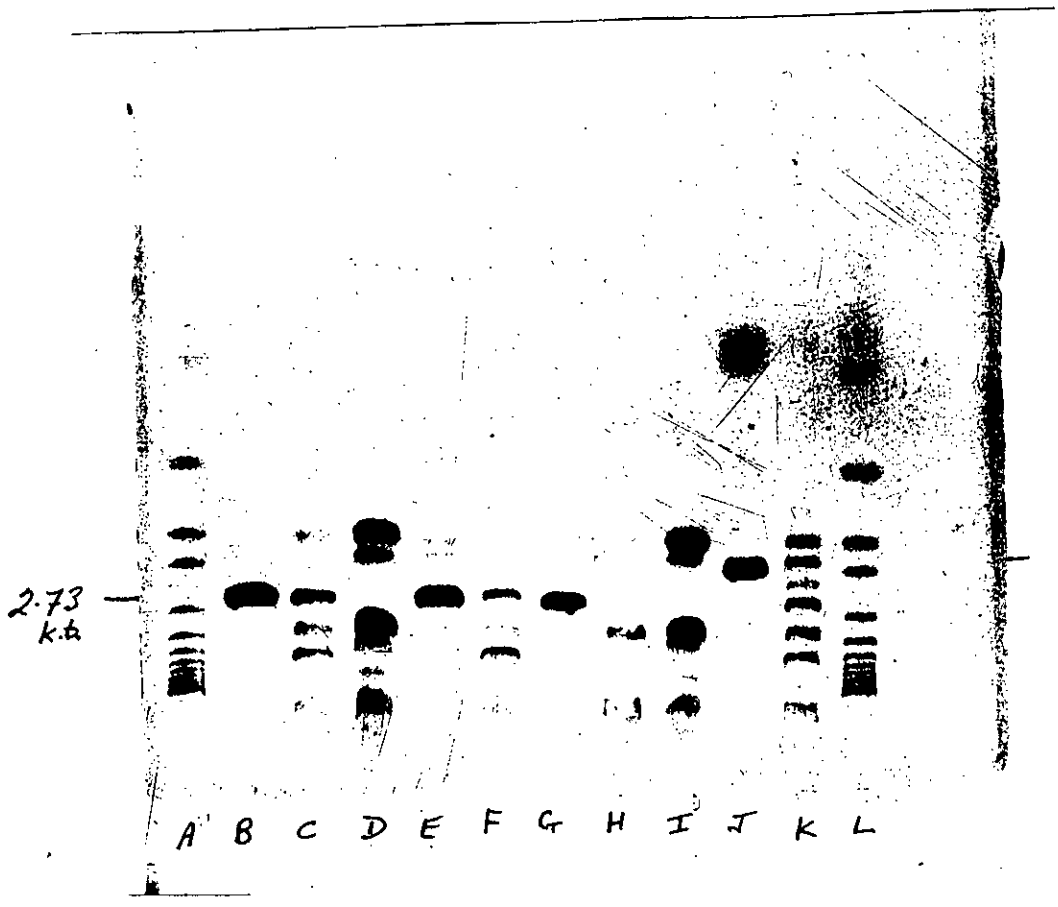
Sodium dioctylsulphosuccinate(AOT)

Figure 3.2

with the plasmid are also resistant to ampicillin. The insertion of foreign DNA by ligation into one (or more) of the unique restriction sites in the lac gene usually inactivates the gene and hence results in colourless colonies which retain ampicillin resistance. This insertional inactivation is a very powerful technique for rapidly monitoring the integration of a plasmid containing a desired insert into cells[113,127].

The agarose gel electrophoresis of the fragments from the cleavage of the plasmid pUC8 with a range of restriction enzymes in both aqueous buffer and in sodium dioctylsulphosuccinate (AOT) (Fig. 3.2) reverse micelle systems is shown in Fig. 3.3. The pUC 8 plasmid has single restriction sites from Bam HI, Hind III and Eco RI and three sites for the enzyme Hae II (Fig. 3.4). Both Bam HI and Eco RI give principally a single linear fragment from the range of partially supercoiled and fully supercoiled DNA. Supercoiled, nicked, (partially supercoiled) and circular plasmid DNA migrate at different rates through the agarose gel hence the series of bands obtained in the non-restricted sample. Cleavage with EcoRI under normal conditions, gives a major linear fragment of ca 2.7k. base pairs and some bands due to partially relaxed (nicked) DNA. In the reverse micelle system a single fragment of linear DNA is observed for both enzymes together with other nicked fragments observed in the natural, unrestricted nucleic acid.

Restriction with Hind III should, as above, give a single linear fragment of 2.73k base pairs and this is observed in the aqueous buffer system. In the reverse micelle, however, no cleavage is observed and a pattern identical to that of unrestricted plasmid is seen (Fig. 3.3).



A – Ladder

B – BamHI, aqueous

C – BamHI, reverse micelle

D – pUC8, uncut

E – EcoRI, aqueous

F – EcoRI, reverse micelle

G – Hind III, aqueous

H – Hind III, reverse micelle

I – pUC8, uncut

J – Hae II, aqueous

K – Hae II, reverse micelle

L – Ladder

Figure 3.3

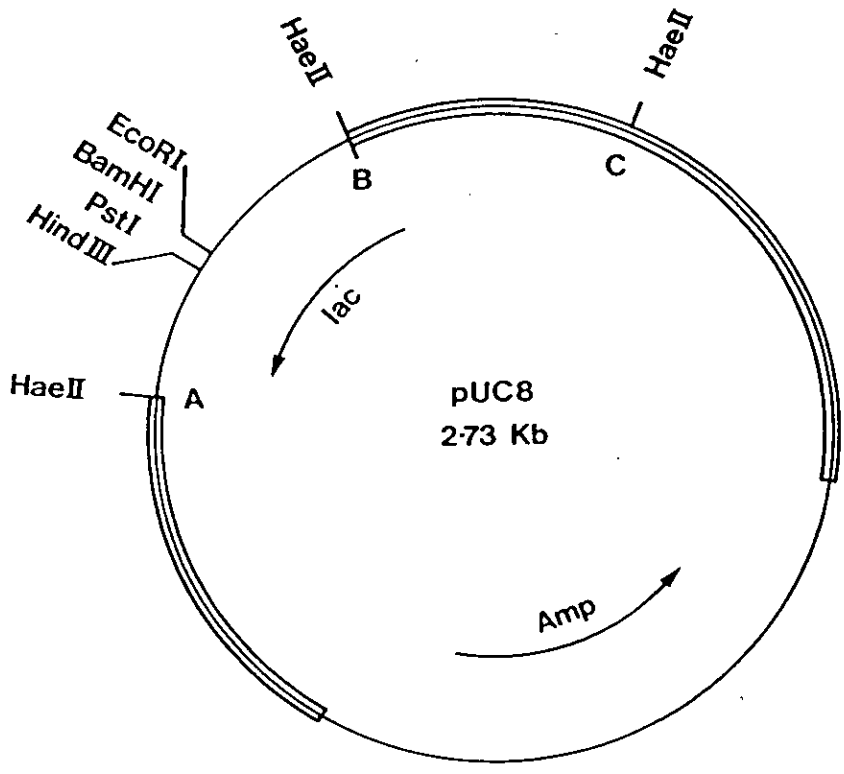

HaeII
HaeII
EcoRI
BamHI
PstI
HindIII
B
C
lac
HaeII
A
pUC8
2·73 Kb
Amp

Figure 3.4

The cleavage of pUC 8 with Hae II gives 3 fragments as shown in fig 3.3. In both the aqueous buffer and the reverse micelle systems the expected fragment sizes are observed although, in the latter, fragments symptomatic of incomplete cleavage are also found.

3.2.2. Cleavage of lambda and lambda bio 1

Lambda bio 1 is a double stranded linear DNA with a molecular weight of the order of 31.5×10^6 Daltons and 12 base pair single stranded cohesive ends[128]. It contains two genes of the biotin operon (bio A and bio B) (Fig. 3.5). Lambda bio 1 was prepared in Edinburgh in the course of other work and the availability of this material and accurate mapping of the restriction sites in the DNA sequence made it a convenient model for our studies. The restriction map of lambda bio 1 is shown in figure 3.5.

Treatment with Hind III in aqueous buffer gave the expected fragments (Table 3.1). When this was repeated in 50 mM surfactant, once again, fragments of the expected sizes were seen after electrophoreseis. At 100mM, surfactant concentration, however, cleavage at a single site only appears to occur.

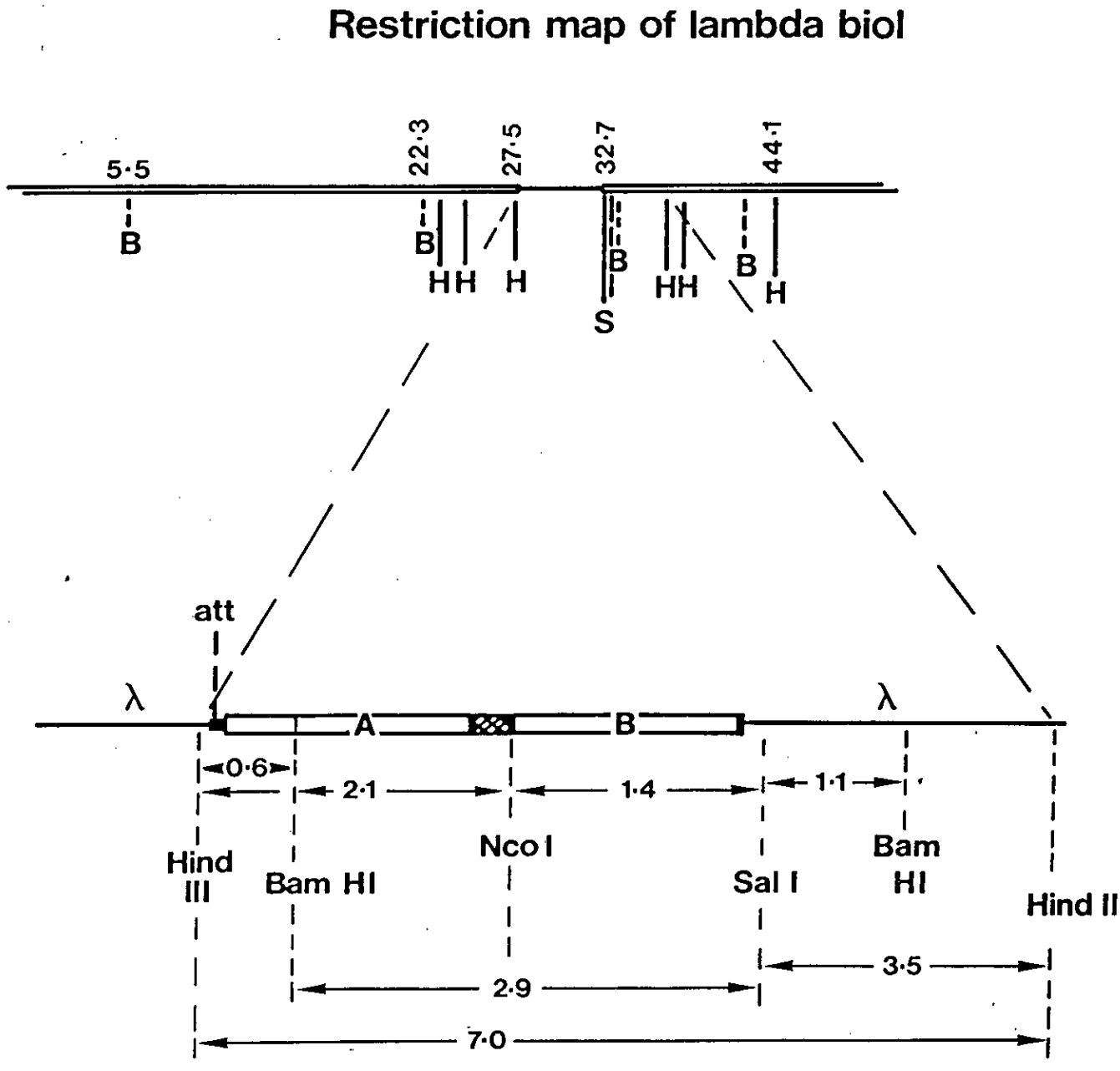

Restriction map of lambda biol
5·5
22·3
27·5
32·7
44·1
B
B
H H
H
B
S
H H
B
H
att
λ
A
B
λ
0·6
2·1
1·4
1·1
Hind III
Bam HI
Nco I
Sal I
Bam HI
Hind III
3·5
2·9
7·0

Figure 3.5

Table 3.1 Cleavage of lambda bio 1 DNA with Hind III

Aqueous buffer (k.base pairs)	Reverse micelle (50mM) (k.base pairs)	Reverse micelle (100 mM) (k.base pairs)
23.1	23.1	23
2.0	2.0	25
2.3	2.3	
7.8	7.8	
0.5	0.5	
0.1	0.1	
6.6	6.6	
4.3	4.3	

Cleavage with Eco RI, Hae II and Bam HI at 100mM surfactant concentration and in aqueous buffer gave the expected fragment sizes only.

Cleavage of normal lambda DNA with Hind III in 100 mM surfactant gave fragments at about 24k base pairs, 4.3 k base pairs and 2.0 k base pairs rather than the expected fragments. The effect of pH upon the efficiency of cleavage was monitored using Hind III and lambda bio 1 at pH 7 and pH 9. No cleavage was observed at pH 9, however the expected fragments were found at pH 7 in the reverse micelle. The incubations were carried out at 50mM surfactant concentration.

3.2.3 Cleavage and annealing of lambda Hind III

The specificity of the restriction enzymes was further tested in the

reverse micelle environment by incubating lambda DNA, which had already been exhaustively digested with Hind III, with Eco R1 and Bam H1. Incubation in the reverse micelle appeared to promote reversible annealing of the cohesive ends and to circumvent this samples were heated to 65°C prior to gel electrophoresis. In both 50mM and 100mM AOT, incubation with both enzymes gave only fragments of an identical size to those observed in a double digest in aqueous buffer. It would appear, therefore, that with smaller pieces of DNA, all the restriction sites for Eco R1 and Bam H1 are available for cleavage.

3.3 Measurement of reverse micelle size

The size of samples of nucleic acid in reverse micelles was measured by photon correlation spectroscopy (PCS). This technique relies upon the scattering of a beam of light by a dilute micellar solution. The scattering intensity increases with increasing size and concentration of particles and with refractive index differences between the solvent and the dispersed phase. The diffusion of particles in and out of a small volume (ca 0.1 mm^3) can be measured and give information that can be used to relate fluctuations in scattered light intensity to the diffusion coefficient and hence to the hydrodynamic radius of the particles. The output is transformed into an exponential decay curve which is analysed to obtain the radius. An optimal sampling time for particles of a specific size exists and thus, in a bimodal system, two (or more) runs with different sampling times are necessary in order to obtain realistic data. In the case of nucleic acids in reverse micelles, the nucleic acid-containing micelles were considerably larger than those which contain water only, therefore multiple scans were necessary.

The reverse micelles which contained only water and surfactant were found to be of the expected sizes with diameters of the order of 10 nm. When reverse micelles which contain lambda were examined, diameters of the order of 250-300 nm were routinely observed. When samples containing pUC 8 or small fragments (<9k base pairs) were examined, results were variable but rapid aggregation appeared to occur and the size of the particles in solution outstripped the range of the instrument.

3.4 Discussion

3.4.1 Restriction of pUC 8

The plasmic pUC 8 is a small (2.7k base pairs) circular piece of DNA which exists in both supercoiled and relaxed forms. Repeated freezing and thawing and the action of certain enzymes results in nicks in one of the chains giving rise to various relaxed and semi-coiled forms of DNA. Plasmid DNA *in vivo* is negatively supercoiled and, because of its small, compact nature, migrates more quickly in agarose gel electrophoresis than more relaxed forms. The rate of migration of DNA is thus a measure of the degree of supercoiling as well as the size. Under normal conditions, plasmid DNA will exist in a number of forms varying between completely supercoiled and totally relaxed. This, in turn, gives rise to a series of bands when the sample is examined by agarose gel electrophoresis. In addition, ethidium bromide intercalates into DNA and generates positive superhelical turns. Clearly, once the DNA strands are cut through completely by the action of a restriction endonuclease a linear piece of DNA results and supercoiling cannot occur.

The identical size of the cleavage product from incubation of pUC 8 with Eco Rl and Bam Hl in reverse micelles shows that cleavage at extra sites - as sometimes occurs in the presence of high salt concentrations and organic solvents - is not a feature of the action of restriction enzymes under these conditions. The efficiency of cleavage is, however, reduced in the reverse micelle system and complete digestion does not occur. However, while cleavage at more than one site appears not to occur, the precise site of restriction is unknown and these results in themselves do not indicate that site specificity is retained in this system. The failure of Hind III to cleave pUC 8 in the reverse micelle suggests either that the enzyme is particularly susceptible to the reverse micelle environment or that the plasmid is folded in such a way that the recognition site for Hind III is not readily available. Given the proximity of the Hind III recognition site to the Eco RI and the Bam Hl recognition sites (fig. 3.4), the latter explanation would appear to be less likely.

All three restriction enzymes require Mg^{2+} and the exchange with Na^{+} in the reverse micelle system may reduce drastically the amount of Mg^{2+} available and thus substantially diminish the activity. This may account for the prolonged (ca 12 hours) incubation times required to achieve comparable cleavage to the aqueous system.

The restriction of pUC 8 with an enzyme which cuts at more than one site (Hae II), to produce the expected range of fragments together with partial cleavage products does however indicate that restriction is occurring at the same recognition sites in the reverse micelle system as in aqueous buffer. The major fragment (1.934k base pairs) and a linear whole piece are clearly present however, there is also some indication of a fragment of between 2.73 k base pairs (linear)

and 1.934 k base pairs. This piece of DNA may arise as a result of incomplete cleavage at either of the three sites in conjunction with complete cleavage at one other (Fig. 3.5). No fragment of 0.787 k base pairs was detected suggesting that complete restriction at site C is not occurring. These results, therefore imply that restriction occurs readily at site B giving the linear whole fragment. Further restriction at sites A and C does occur but at different rates thus giving rise to fragments of 2.304k base pairs and 2.351 k base pairs (which would be indistinguishable on the gel) and, where both are cut, a fragment of 1.934 k base pairs corresponding to the major fragment in the normal aqueous buffer incubations. Both of the small fragments (0.417 and 0.370 k base pairs) are also observed.

The results from the single restriction site enzymes and using Hae II suggest that the specificity of restriction enzymes remains high in a reverse micelle environment. Clearly Hind III is either more sensitive to the reverse micelle system or its restriction site is not readily available. This latter option is supported by the results from Hae II digestions. Clearly all three sites in the plasmid are not equal and, while this may be due to some greater degree of specificity invoked by the environment, the net effect is that of preferential cleavage at certain of the sites. It is noteworthy that the base sequence surrounding the Hae II sites A and B are similar while that of site C has a higher level of thymidine residues.

3.4.2 Restriction of lambda bio 1

The cleavage of lamda bio 1 in aqueous buffer gave fragments of the expected sizes. The same experiment carried out with Eco R1 and Bam H1 again gave the expected fragements at 100Mm surfactant

concentrations. When this incubation was carried out with Hind III, only two fragments of approximately equal sizes were observed. This would suggest that Hind III restriction is occurring at one of three possible sites in a reproducible fashion. When the cleavage was repeated using normal lambda without the biotin insert incomplete cleavage was also evident although different fragments were observed on this occasion. When the Hind III experiment with lambda bio 1 was repeated at 50 mM surfactant concentration, the expected restriction pattern was seen. These results suggest that the amount of surfactant present has a direct effect on the specificity of cleavage. The micelle size and the amount of free water is dependant upon the ratio of water to surfactant with less free water being present at higher surfactant concentrations. From the point of view of the Hind III experiments, this lack of free water may cause conformational restraints upon the DNA thus only allowing a relatively small number of sites might near the centre of the lambda bio 1 to be exposed. Initial cleavage at one of these sites will not lead to dissociation and subsequent cleavage at other sites because the DNA is held so tightly in the reverse micelle environment. In the case of normal phage lambda, the absence of the biotin insert would appear to expose other sites, in particular, that at 4300 base pairs.

The cleavage of lambda bio 1 using the other restriction enzymes (Eco R1 and Bam H1) are difficult to justify in terms of the above analysis. All three restriction enzymes have restriction sites in the similar domains and thus should all be subject to the same difficulties of enzymes-DNA interactions as Hind III in carrying out the cleavage. It is possible that the environment has having an effect upon the enzyme itself (as suggested earlier by the experiments with pUC 8). The noticable difference observed between lambda and

lambda bio 1 hydrolysis would suggest that a relatively small alteration in the composition of the DNA substrate has a considerable effect on the cleavage. Hind III is a relatively large enzyme (m.w. 80,000 Da) compared with Eco R1 (2 x 37,000 Da) this may have an effect upon the amount of free water available and hence on the cleavage at different sites. The lowered availability of Mg^{2+} in the reverse micelle system may also be more crucial to Hind III than to the other enzymes.

Certain restriction enzymes are particularly susceptible to the presence of high salt concentrations (which can occur in impure AOT reverse micelle systems) or organic solvents. In enzymes such as Eco R1 and Hind III this can induce "star" activity in which some specificity is lost and restriction occurs at sites other than the generally recognised restriction sites. Clearly this is not happening in this case.

The rate of cleavage at different sites in lambda DNA can vary as much as 14 fold with Hind III and Eco R1[115], however even such differences in rate do not explain why only a single site is apparently cleaved at 100 mM surfactant concentration with Hind III and no abnormal effect is noted with Eco R1. Other enzymes (in particular Nar I, Nae I, Sac II and Xma III) exhibit even greater site specificity with fifty-fold and greater differences observed between certain sites in lamda. Conformational restraints upon the DNA or the enzyme (or both) would appear to be a likely explanation for the results observed. It remains possible, however, that local base sequences may cause certain sites to be considerably less accessible than others and that the cleavage sites for Hind III are particularly susceptible to this conformational constraint.

3.5 Conclusions

This study was designed to find out if DNA could be cleaved in a specific and reproducible fashion using restriction enzymes in reverse micelles and to examine whether the site-specificity of restriction was altered under these conditions. In most cases, cleavage produces identical fragments both in small, plasmid DNA and in large modified lambda DNA. The exception occurs with the enzyme Hind III which has very low activity in the reverse micelle enviroment with plasmid DNA and cleaves at a single site only in lambda bio 1 DNA. This may simply be due to the low activity of the enzyme per se, however this would not explain the reproducible nature of the cleavage in the multiple site modified lambda. Furthermore, in normal phage lambda without the biotin genes insert, the cleavage is, once again, abnormal but in a different manner to that observed for the modified lambda. It is more likely therefore, that the principal cause of the differences observed is inherent in the DNA rather than the enzyme. Since many of the overall cleavage domains are similar for the different restriction enzyme sites, a more localised effect would appear to be indicated. The nature of the restriction sites themselves is not sufficiently different to explain the results thus it may be some reflection of the neighbouring base sequences. The exchange of Mg^{2+} with Na^{+} in the reverse micelle system with concomitant lowered availability of Mg^{2+} may be more important to some enzymes than to others. The size of the restriction enzyme, the amount of water it requires to order around itself and the nature of the active site may be of particular importance.

While the specificity of the enzymes appears to be maintained in reverse micelles it remains to be proven that the site of recognition

and cleavage is precisely identical in aqueous and reverse micelle systems and further work involving, perhaps, sequencing of the termini of fragments is necessary to confirm this observation.

Further studies are also necessary to determine if there is an optimal surfactant/water ratio for a range of restriction enzymes and if the sites of cleavage can be predicted. Also of interest may be the cleavage of DNA using "non-specific" endonucleases such as deoxyribonuclease I (E.C. 3.1.21.1). While this enzyme is used for general DNA assays, it does exhibit some substrate specificity[116] and it may be possible to tailor this specificity to certain types of sequences, eg CG-rich regions which are associated with particular parts of the gene. Certain sequences of nucleotides are conserved in, for example, the Shine-Dalgarno (S-D) sequence which occurs prior to the bacterial start codon for a gene. Careful manipulation of the enzyme and the conditions of incubation may lead to greater, reproducible specificity of cleavage.

Experimental

General methods

Starting materials from commercial sources were used without further purification unless otherwise indicated. Solvents were either Analar grade or redistilled prior to use with the exception of ethyl acetate, chloroform, petroleum ether and diethyl ether which were laboratory grade. Organic extracts were dried with anhydrous magnesium sulphate or anhydrous sodium sulphate prior to evaporation. Comparison of synthesised material with commercially available samples or with literature values (m.pt., ms, nmr etc.) was carried out whenever possible. Melting points are uncorrected. ^{1}H nmr spectra were obtained on a Bruker spectrometer operating at 80.13 M.Hz. or on a Bruker WH200 operating at 200.13 MHz. ^{13}C Nmr spectra were obtained on the latter instrument at 50.32 M.Hz. ^{2}H and ^{15}N nmr spectra were obtained at 40 and 36.5 M.Hz respectively. Mass spectra were obtained routinely on a Kratos MS30 or MS60 mass spectrometer operating in CI, EI or FAB mode. All ms and gc/ms were run in EI mode unless indicated otherwise. Capillary gc/ms was carried out using an BPI capillary column (12.5m x 0.33mm i.d.) connected to a Kratos MS60 mass spectrometer. Photon correlation spectroscopy was carried out on a Malvern 7026 spectrometer at the Institute of Food Research, Norwich, by Alan Mackie.

Plasmid and lambda DNA was obtained either from a commercial source or from Dr. N. Murray, Department of Molecular Biology, University of Edinburgh. Restriction enzymes were obtained from commercial sources. Cultures of *Penicillium atrovenetum* and *Saccharomyces cerevisiae* (ATCC 29403) were obtained from the Commonwealth Mycological Institute, Kew

Gardens. The former was maintained on Czapek Dox slants and subcultured as little as possible since this was found to result in the loss of 3-nitropropanoic acid synthesising capacity. The yeast was maintained on agar slants and subcultured every 4-6 weeks. Fresh slants were used for each experiment.

Penicillium atrovenetum was grown on modified Raulin Thom medium as described by Shaw[20]. Saccharomyces cerevisiae was grown on Difco vitamin free medium supplemented with pantothenic acid, biotin and myo-inositol[75]. The isolation of thiaminase I was carried out from Bacillus thiaminolyticus which was grown in the defined medium of Douthit and Airth[89].

"A sample of the amino acid (19, 200mg) in water (1 cm^3) was neutralised to pH 7.6 (NaOH, 4N) then cooled in ice and treated with acetic anhydride (140μl). The pH was adjusted to >5 (NaOH, 4N) and the mixture was treated again with acetic anhydride (140μl) and the pH adjusted to >5 (NaOH, 4N). The mixture was stirred for 30 min. then applied to an ion exchange column (IR 120(H), 20 Mesh, 2 x 30 cm). The eluate was freeze dried to afford the acetate as an oil. A sample of the acetate was treated with diazomethane in methanol then evaporated to dryness <u>in vacuo</u> to afford the acetylated dimethylester. δ_H (80MHz, $CDCl_3$), 6.74 p.p.m. (1H, br.d., J=7.8Hz, NH), 4.76 p.p.m. (1H, d, J=8.4Hz, CH (anomeric)), 3.67 p.p.m. (3H, S, CO_2CH_3), 3.60 p.p.m. (3H, D, J=3.9Hz, $^{13}CD_2CH_3$), 2.68 p.p.m. (0.3H, $CHD+CH_2$), 1.96 p.p.m. (3H, s, $NHCOC\underline{H}_3$). Apparent deuteration <u>ca</u>. 85%. ms (E1) m/z, 206 (M^+, ^{13}C, d_2), 205 (M^+, ^{13}C, d_1), 204 (M^+, ^{13}C, d_1). Apparent deuteration <u>ca</u>. 60% d_2, 20% d_1, 20% d_0".

[4-^{13}C,3-$^{2}H_2$, 2-^{2}H]-DL-Aspartic acid (19): Diethylacetamide malonate (21.7g, 0.1 moles) and formaldehyde (4.0%, 8.2 cm^3) were added to a solution of dimethylamine (15 cm^3, 0.1 moles) in glacial acetic acid (15 cm^3) at 0°C. The reaction mixture was left at room temperature for 30 minutes, made alkaline by addition of sodium hydroxide solution (20%) and the Mannich base crystallised out (27.4g). This produce was dissolved in ethanol (60 cm^3) and treated with methyl iodide to give the corresponding methiodide. The methiodide (4.2g, 10 m.moles) was dissolved in water and added to a solution of [^{13}C]-sodium cyanide (0.6g, 12 m.moles) in water (20 cm^3) and the mixture heated under reflux for 16 h. The reaction mixture was evaporated to dryness in vacuo then treated with ^{2}HCl (20% in $^{2}H_2O$) for 18 h. The reaction was evaporated to dryness in vacuo, the residue dissolved in methanol and the sodium iodide removed by filtration. Addition of pyridine in two stages gave [4-^{13}C, 3-$^{2}H_2$, 2-^{2}H]-DL-aspartic acid (19) (0.97g, 73%).

insert A →

1-(4-methyltoluenesulphonyl)-3-nitro-ethane (21): A solution of 2-nitroethanol (4.75g, 0.052 moles) in dry, redistilled tetrahydrofuran (25cm^3) was cooled in an ice bath then treated with triethylamine (2cm^3) and 4-toluenesulphonyl chloride (10.5g, 0.55 moles) and the mixture allowed to reach room temperature and stirred overnight. The reaction mixture was filtered, applied to a column of silica (Kieselgel 60-230 Mesh) and eluted successively with hexane, hexane/ethyl acetate (60:40), ethyl acetate, methanol and ethanol. The alcoholic fractions were combined and evaporated under reduced pressure to afford an oil which slowly solidified. The crude material was recrystallised from chloroform/ethanol to afford a purified product (5.24g, 47%) δ_H (80 MHz, $CDCl_3$), 7.20ppm (2H, d, J = 8Hz, m.pt. 122-124°C)

aromatic), 6.72ppm (2H, d, J = 8Hz, aromatic), 3.52ppm (3H, s, CH_3), 3.05ppm (2H, t, J = 6Hz CH_2 NO_2), 2.60ppm (2H, t, J = 6Hz, CH_2 OTs) ms (EI) m/z, 213 [M^+].

1-N,N-dimethylamino-2-nitroethene (22) Nitromethane (5g, 0.1 moles) in dry dimethyl formamide (100 cm^3) was treated with N,N-dimethyl formamide dimethylacetal (11.1g, 0.12 moles) and pyrrolidine (0.04g, 0.6m.moles). The mixture was heated to 70-80°C for 3h by which time the initially pale yellow solution had turned deep red. Dimethyl formamide was removed by distillation in vacuo with the temperature kept below 40°C. The residual red oil was triturated with isopropanol to afford an orange solid which was recrystallised from ethanol to give pure 1-N,N-dimethylamino-2-nitroethene (22, 6.1g, 54%) m.pt. 92-4°C δ_H (80MHz, $CDCl_3$), 7.80 ppm (1H, d, J = 8Hz, $CHNO_2$), 6.15ppm (1H, d, J = 8Hz, CH-N $(CH_3)_2$), 2.85 ppm (3H, s, N-CH_3), 2.55ppm (3H, s, N-CH_3) ms (EI) m/z, 116 [M^+], 71 [M-NO_2], 58 [M-$CHNO_2$]. $C_4H_{10}N_2O_2$ requires C, 41.4%; H, 8.6%, N, 24.1%. Found C, 41.9%; H, 8.3%; N, 23.8%.

[2-$^2H_2$, 3-$^2H_2$]-3-Bromopropanoic acid (23): 3-Bromopropionitrile (0.2g 1.5m.moles) was treated with deuterium bromide (37% in ^2H_2O, 4 cm^3) and the mixture refluxed for 3h then allowed to cool, diluted with ^2H_2O (5cm^3) and extracted with diethyl ether (10cm^3 x 3). The ethereal extracts were combined, dried and evaporated under reduced pressure to afford an oil with identical tlc characteristics to an authentic sample of 3-bromopropanoic acid[129] (23, 0.22g) ms m/z 156 [M^+], 110 [M-CO_2H].

[2-$^2H_2$]-3-Nitropropanoic acid (10): Crude [2-^2H, 3-$^2H_2$]-3-bromopropanoic acid (22) (0.2g, 1.3m.moles) from the above

reaction was added to a suspension of sodium nitrite (0.16g, 2.3m.moles) in dry dimethylformamide ($3cm^3$) at 0°C. The mixture was allowed to reach room temperature and stand for 3h. then diluted with water ($5\ cm^3$), acidified (6 N hydrochloric acid, $1cm^3$), dried and evaporated under reduced pressure to afford a brown oil which was purified by sublimation (60–65°, 0.1mm) to give m.pt. 63–64°C, lit[14], 63–65°C [2-$^2H_2$]-3-nitropropanoic acid (10, 0.08g, 50%) which was identified by tlc comparison with an authentic sample. δ (80 MHz, $(CD_3)_2CO$) 4.45 ppm (2H, t, J=7.5Hz, d, J=6Hz, CH_2NO_2), 2.65ppm (0.2H, t, J=7.5Hz, CH_2CO_2H).

3-Hydroxypropionitrile (11): (a) A solution of sodium cyanide (0.31g, 6.3m.moles) in water ($5cm^3$) was treated dropwise with 2-chloroethanol (0.6g, 7.4m.moles), and the mixture stirred at 45°C for 1h. The reaction mixture was heated to 55°C and stirred for a further 3h., allowed to cool to room temperature, treated with citric acid (10%, $5cm^3$) and extracted with ethyl acetate ($10cm^3$ x 2). The organic layers were combined, dried and evaporated under reduced pressure to afford an oil (0.26g) which appeared, from ^1H nmr, to contain only about 20% of the desired product. Prolonged heating gave a mixture of products.

(b) A mixture of sodium cyanide (1.55g, 31.5 m.moles) and 18-crown-6 (0.15g, 0.55m.moles) was treated with 2-chloroethanol (3.0g, 37.0m.moles) and water ($10cm^3$) and the reaction stirred at 80–90°C for 3h. The mixture was allowed to cool to room temperature, treated with citric acid solution (10%, $25cm^3$) and extracted with ethyl acetate ($50cm^3$ x 3). The organic fractions were combined, dried and the solvent removed by evaporation under reduced pressure giving a crude

product (2.8g) which was distilled under reduced pressure to afford pure 3-hydroxypropionitrile (11, 1.64g, 63%). B.pt. 74-76°C/1mm, lit [130] 107-109°C/12mm. δ_H (80MHz, $CDCl_3$), 3.85ppm (2H, t, J=6Hz, CH_2-OH) 2.80ppm (2H, t, J=6Hz, CH_2CN). ir ν 3350cm^{-1} (OH stretch), 2250cm^{-1} (C ≡ N stretch).

3-Bromopropanoic acid (9): (a) 3-Hydroxypropionitrile (11) (0.3g, 4.2m.moles) in hydrobromic acid (40%, 5cm^3) was refluxed for 2 hours. The reaction mixture was then allowed to cool to room temperature and extracted with ethyl acetate (5cm^3 x 3). The organic fractions were combined, dried and evaporated to dryness to afford a crude product which was crystallised from aqueous ethanol to give (9) (0.32g, 62%) m.pt. 62-63°C, lit [129] 62.5 - 63.5°C. δ_H (60MHz, CD_3CN) 2.5ppm (2H, t, J = 6Hz, CH_2Br), 1.7ppm (2H, t, J=6Hz, CH_2CO_2H) which was identical to an authentic sample.

(b) 3-Bromopropionitrile (8) (0.1g, 0.75m.moles) in hydrochloric acid (20%, 3cm^3) was refluxed for 3h. then allowed to cool to room temperature and extracted with ether (5cm^3x3). The ethereal extracts were combined, dried and evaporated to dryness in vacuo to afford the product as an oil which solidified overnight (0.06g). Recrystallisation from aqueous ethanol gave pure 3-bromopropanoic acid (9, 0.045g, 40%) which had identical tlc, m.pt. and nmr characteristics to an authentic sample.

3-Nitropropanoic acid (1): 3-Bromopropanoic acid (9) (0.77g, 5m.moles) in dry dimethylsulphoxide (10cm^3) was treated with sodium nitrite (0.55g, 8m.moles) and the mixture stirred at room temperature for 1.5h. The reaction mixture was then poured into ice water (10cm^3)

acidified to pH 1-2 with 6N hydrochloric acid and extracted with ether ($10cm^3$ x 3). The ethereal extracts were combined, dried and the solvent removed by evaporation in vacuo to afford a mixture of starting material and the desired product. The crude mixture was sublimed at 60-65°/0.1mm and the crystalline sublimate recrystallised from chloroform to afford 3-nitropropanoic acid (1, 0.15g, 25%) which was identified by comparison (m.pt, ms, tlc, nmr) with an authentic sample. M.pt. 62-64°C lit[131] 63-65°C. δ_H (80MHz, CD_3CN), 4.45ppm (2H, t, J=7Hz, CH_2NO_2), 2.65ppm (2H, t, J = 7Hz, CH_2CO_2H) ms (FAB -ve) m/z 119 $(M)^-$, 118 $(M-H)^-$.

[1-^{13}C]-3-Hydroxypropionitrile (24): A mixture of [^{13}C]-sodium cyanide (0.1g, 2.0 m.moles), 2-chloroethanol (0.25 cc^3) and 18-crown-6 (0.03g, 0.11μmoles) was stirred at 90°C for 3h. The cooled reaction mix was absorbed on dry silica (2g) and eluted with ethyl acetate (2 cm^3 x 5). The organic eluate was evaporated under reduced pressure to afford the crude product as an oil (0.08 g). The product had identical tlc characteristics to an authentic sample of 3-hydroxypropionitrile (11) and was used without further purification.

[1-^{13}C]-[2-^{2}H$_2$, 3-^{2}H$_2$]-3-Bromo-propanoic acid (12): [1-^{13}C]-3-Hydroxypropionitrile (0.156g, 2.0 m.moles) in deuterobromic acid (40%, 3.0 cm^3) was refluxed for 2 hours. The reaction was allowed to cool to room temperature, diluted with ^2H_2O (3.0 cm^3) and extracted with ether (10 cm^3 x 3). The ethereal extracts were combined, dried and the solvent removed by evaporation under reduced pressure to afford a brown oil which solidified upon agitation and cooling (0.12 g). This crude product had identical tlc characteristics to an authentic sample of 3-bromopropanoic acid and was used without further purification.

[1-^{13}C, 2-$^{2}H_2$]-3-Nitropropanoic acid (7): Crude bromopropanoic acid (12) from the above reaction (0.1g) was added to a suspension of sodium nitrite (0.075g) in dry dimethylsulphoxide (5 cm^3) and the mixture stirred at room temperature for 1.5h. The reaction mixture was then diluted with water (10cm^3), acidified to pH 1-2 with hydrochloric acid (6N) and extracted with ether (15 cm^3 x 5). The ethereal extracts were combined, dried and evaporated to dryness *in vacuo* to afford the crude product (35 mg) which was recrystallised from chloroform to give [1-^{13}C, 2-$^{2}H_2$]-3-nitropropanoic acid (7, 0.025g, 28%) m.pt. 62-3°C (lit[14], 63-65°C) δ_H (80MHz, $(CD_3)_2CO$) 4.45 ppm (2H, br, CH_2NO_2). ms (FAB +ve) m/z 122 [M^-], 121 [M-1]$^-$.

3-Aminopropanoic acid (4): (a) 3-Nitropropanoic acid (1) (15 mg, 0.125 m.moles) was added to tin powder (80mg) in hydrochloric acid (35%, 3 cm^3) and the mixture refluxed for 2 hours. The cooled reaction mixture was evaporated to dryness *in vacuo* to afford a solid residue which was washed repeatedly with ether to leave a colourless solid (4 mg) with identical tlc and nmr characteristics to authentic 3-aminopropanoic acid (4).

(b) A stirred solution of 3-nitropropanoic acid (1) (0.06g, 0.5 m.moles) in dry methanol (3 cm^3) was treated with palladium on charcoal (10%, 0.1g) and the suspension added to anhydrous ammonium formate (0.14g, 2.3 m.moles). The reaction was stirred at room temperature for 20 min then filtered through celite and evaporated to dryness under reduced pressure to afford a colourless crystalline solid (0.058g) which was identified by comparison (m.pt., tlc, ^{1}H nmr) with an authentic sample of 3-aminopropanoic acid (4).

3-Nitropropanoic acid methylmandelate ester (25): (a) A stirred solution of 3-nitropropanoic acid (1) (0.12g, 1 m.mole) in dichloromethane (5 cm^3) was treated with 2-dimethyl aminopyridine (5 mg 0.04 m.moles), S-(+)-methyl mandelate (0.25g, 1.5 m.moles) and 1,3-dicyclohexylcarbodiimide (0.15g, 0.73 m.moles) at 0°C. The reaction mixture was stirred at 0°C for 5 min. then allowed to warm to room temperature and stirred for a further 3h. The reaction mixture was then filtered and the solvent removed by evaporation in vacuo to afford an oil (0.21g) which appeared, from tlc and nmr to consist of starting material.

(b) 3-Nitropropanoic acid (1) (0.055g, 0.46 m.moles) in dry tetrahydrofuran (3 cm^3) was treated with 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.14g, 0.57 m.moles) and S-(+)-methylmandelate (0.08g, 0.48 m.moles). The reaction mixture was stirred at room temperature for 5 d after which time examination by tlc indicated that only starting material was present.

(c) 3-Nitropropanoic acid (1) (0.1g, 0.8 m.moles) was treated with triflouroacetic anhydride (0.18g, 0.8 m.moles) and the mixture shaken for 2 min. then intermittently over 1 h. R-(-)-Methyl mandelate (0.2g, 0.85 m.moles) was added and the reaction mixture left standing at room temperature overnight. The reaction mixture was then cooled to 0°C, neutralised (pH 7-9) with potassium hydroxide (3N) and extracted with ethyl acetate (5 cm^3 x 3). The organic fractions were combined, dried and evaporated to dryness in vacuo to afford an oil. The crude material was purified by preparative tlc using chloroform/ethanol (95:5) as the mobile phase to afford 3-nitropropanoic acid methyl mandelate ester (25, 0.08g, 37%) m.pt.

120–122°C δ(80MHz, C_6D_6) 7.00 ppm (5H, aromatic), 5.50 ppm (1H, s, $CH-CO_2CH_3$), 3.90 (1H, m ($AA'B_2$), CH_2NO_2), 3.70 ppm (1H, m ($AA'B_2$) $CHNO_2$), 3.40 ppm (3H, s, CO_2 CH_3) 2.45 ppm (2H, t, J=10Hz, CH_2CO_2R). ms (EI) m/z, 267 [M^+].

3-Nitropropanoic acid methyl valine amide (26): 3-Nitropropanoic acid (1) (0.2 g, 1.7 m.moles) in dry tetrahydrofuran (5 cm^3) was treated with L-valine methyl ester (0.33g, 2.0 m.moles) generated from the corresponding hydrochloride by shaking an ethyl acetate suspension with saturated sodium bicarbonate. 1,3-Dicyclohexylcarbodiimide (0.2g, 1.0 m.moles) was added and the reaction mixture stirred at room temperature for 2 h. The filtered reaction mixture was evaporated in vacuo to afford an oil which was purified by flash chromatography using chloroform/methanol as the mobile phase, giving an oil which slowly crystallised. Crystallisation from ethyl acetate/hexane gave 3-nitropropanoic acid methyl valine amide (26, 0.208g, 53%) m.pt. 42–44°C. δ(200MHz, $CDCl_3$) 6.25 ppm (1H, d, J=9Hz, NH), 4.70 ppm, (3H, m (AA'B) CH_2NO_2).4.60 ppm (1H, dd, J=10Hz, 5Hz, CH-NH), 3.75 ppm (3H, s, CO_2CH_3), 2.86 ppm (2H, m, (AA'B), CH_2CO_2H), 2.14 ppm (1H, m, $CH(CH_3)_2$), 0.92 ppm (3H, d, J=5.5 Hz, CH_3), 0.89 ppm (3H, d, J=5.5 Hz, CH_3) ms (EI) m/z, 232 [M^+], 172 [$M-CO_2CH_3$], 125 [$172-NO_2$]. Exact mass measurement gives 232.1069 corresponding to $C_9H_{16}N_2O_5$.

[2-^{2}H, 3-^{2}H]-Fumaric acid (27): Dimethylacetylene dicarboxylate (2.1 g, 14.8 m.moles) in tetrahydrofuran (10 cm^3, preshaken with 0.5 cm^3 deuterated water) was cooled to 0°C then treated dropwise with triphenylphosphine (3.9 g, 15 m.mles) in dry tetrahydrofuran (4 cm^3). The initially colourless solution turned deep red and was allowed to warm to room temperature over 30 min. then refluxed for 3h. The reaction mixture was then allowed to cool to room temperature, dried

and evaporated to dryness in vacuo to afford an orange solid which was purified by sublimation to give [2-^{2}H, 3-^{2}H]-dimethylfumarate (28, 0.78g) m.pt. 90-92°C ms (EI) 134 [M^+], 115 [$M-OCH_3$]$^+$. A sample of this product (0.5 g, 0.34 m.moles) was treated with aqueous sodium hydroxide (10 cm^3, 12%) and the reaction mixture stirred at room temperature for 72 h. The reaction was cooled in ice, acidified (pH 1-2) with hydrochloric acid (6N) and the product isolated by filtration and crystallised from aqueous ethanol [2-^{2}H, 3-^{2}H]-fumaric acid (26, 0.4g, 36%) m.pt. 298-300°C lit[40] 299-300°C ms (EI) m/z 118 [M^+], 100 [$M-H_2O$].

[2S-^{2}H, 3S-^{2}H]-L-Aspartic Acid (14): [2-^{2}H, 3-^{2}H]-Fumaric acid (27) (0.4g, 3.5 m.moles) in water (5 cm^3) was treated with magnesium sulphate heptahydrate (0.08g, 0.3 m.moles) and TRIZMA base (0.11g) then a solution of ammonium chloride (0.38g, 7.0 m.moles) in water (5 cm^3) was added. The mixture was made up to 20 cm^3 with water, adjusted to pH 8.0-8.5 by addition of aqueous sodium hydroxide (2N) then treated with aspartase (4 units) and the mixture incubated at 30°C for 10 d. The incubate was then immersed in boiling water for 30 min., cooled and treated with cupric sulphate heptahydrate (0.85g, 3.5 m.moles) and the blue precipitate isolated by filtration. The solid was suspended in water (10 cm^3) and treated with hydrogen sulphide for 3-4 min. The black precipitate was removed by filtration through celite and the filtrate treated with ethanol (20 cm^3) and stored at 0°C for 4d over which time [2S-^{2}H, 3R-^{2}H]-L-aspartic acid (14) crystallised out and was isolated by centrifugation (0.11g, 24%) δ(80 MHz, Na OD/D_2O) 2.65 ppm (s, 3R-H) ms (FAB +ve) 136 m/z [M^+], 90[$M-CO_2H$]$^+$, 72[$-NH_2$]$^+$.

[3R-^{2}H]-L-aspartic acid (15): Fumaric acid (0.8g, 6.9 m.moles) was added to a mixture of magnesium sulphate heptahydrate (0.15g, 0.6 m.moles) and TRIZMA base (01.11g) in deuterated water (10 cm^3). The mixture was heated at 80°C for 10 min. then allowed to cool to room temperature. The same procedure was carried out for a solution of ammonium chloride (0.74g, 15 m.moles) in deuterated water (5 cm^3). Both solutions were evaporated to dryness in vacuo and the former dissolved in deuterated water (15 cm^3) and added to a solution of the latter in deuterated water (10 cm^3). The combined mixture was adjusted to pH 8.1 with sodium deuteroxide (2N) then incubated with aspartase (2 units) at 30°C for 10d. After this time the reaction mixture was treated in an identical fashion to that described above with cupric sulphate heptahydrate (1.9g, 7.0 m.moles) and [3S-^{2}H]-L-aspartic acid isolated as colourless crystals (15, 0.26g, 28%). ms m/z (FAB +ve), 135 [M^+], 89 [$M-CO_2H]^+$, 71[$-NH_2]^+$. δ_H (80 MHz, $NaOD/D_2O$), 2.35 p.p.m. (1H, br. d., J=8 Hz, CHD), 3.55 p.p.m. (1H, d, J=8 Hz, CH-NH_2).

3-Nitroacrylic acid (6): Fuming sulphuric acid (12.3g, 90%, 0.15 moles) was added dropwise to a stirred solution of fuming nitric acid (10.0g, 90%, 0.15 moles) at 0°C. The reaction mixture was stirred vigorously at 0°C while chlorosulphonic acid (17.0g, 0.14 moles) was added slowly over 2-3h and the evolved nitryl chloride was collected in an acetone/CO_2 trap. Nitryl chloride was allowed to warm and bubbled into stirred glacial acrylic acid (6.4g, 0.09 moles) at 0°C. The reaction mixture was stirred at 0°C for 1h and then stored at 0°C until required.

A sample of 2-chloro-3-nitropropanoic acid prepared above (0.7g, 4.6 m.moles) in dry ether (50cm^3) was treated slowly with anhydrous sodium acetate (0.43g, 5.2 m.moles) and the reaction mixture heated under

reflux for 1h. The solution was then allowed to cool to room temperature and reduced by evaporation in vacuo to afford an oil which was purified by flash chromatography on silica using redistilled chloroform as eluant. The purified product was crystallised from redistilled chloroform to afford 3-nitroacrylic acid (6) as off-white crystals (0.08g, 15%) m.pt. 135-136°C lit[132] 134-136°C. δ(80MHz, CD_3CN) 7.25 ppm (1H, d, J = 12Hz $CHNO_2$), 6.55 ppm (1H, d, J = 12Hz, $CHCO_2H$) ms m/z (FAB -ve) 117 $[M]^-$, 116 $[M-H]^-$.

3-Bromopropionitrile (8): Dibromoethane (0.71g, 5 m.moles) in dry dimethyl sulphoxide (20 cm^3) was treated with sodium cyanide (0.30 g, 4.8 m.moles) and the mixture stirred at 110°C for 1h. After this time the reaction was allowed to cool to room temperature, quenched with citric acid solution (15%, 20 cm^3) and extracted with ethyl acetate (20 cm^3 x 3). The organic layers were combined, dried and evaporated in vacuo to afford an oil which was identified by comparison (tlc, ir, ms) with an authentic sample of succinonitrile.

[2-$^2H_2$]-3-Nitropropanoic acid (10): 3-Hydroxy propionitrile (11) (0.2g, 2.8 m.moles) in deuterium bromide (40%, 5 cm^3) was refluxed for 2h. The reaction mixture was allowed to cool to room temperature, diluted with deuterated water and extracted with ether (10 cm^3 x 3). The ethereal extracts were combined, dried and evaporated to dryness in vacuo to afford an oil which solidified overnight and was identical (tlc) to an authentic sample of 3-bromopropanoic acid (9).

Crude 3-bromopropanoic acid (9) prepared above was dissolved in dry dimethylsulphoxide (10 cm^3) and treated with sodium nitrite (0.3g). The reaction mixture was stirred at room temperature for 1.5h then diluted with water, acidified with hydrochloric acid (2N) and extracted with ether (10cm^3 x 5). The ethereal extracts were

combined, dried and evaporated to dryness under reduced pressure to afford an oil which was taken up in buffer (15 cm^3, pH10) and stirred at room temperature overnight. The reaction mixture was acidified with hydrochloric acid (2N) and extracted with ether (10 cm^3 x 5). The ethereal extracts were combined, dried and evaporated to dryness in vacuo to afford an off-white crystalline solid which was recrystallised from chloroform to afford [2-$^2H_2$]-3-nitropropanoic acid (10, 0.05g, 15%) which was identified by comparison (tlc, m.pt.) with an authentic sample. δ(80M.Hz, CD_3CN), 4.65 ppm (2H, br.m., CH_2NO_2) ms m/z (FAB, -ve) 121 [M^-], 120 [M-H]$^-$.

[3-$^2H_2$]-3-Nitropropanoic acid (28): 3-Nitropropanoic acid (1, 0.05g, 0.42 m.moles) in deuterated water (2 cm^3) was treated with anhydrous sodium carbonate (0.03g, 0.28 m.moles) and the mixture allowed to stand at room temperature. After 24 h the reaction mixture was acidified (deuterium chloride, 35%) to pH 1-2 and extracted with ether (5 cm^3 x 3). The ethereal extracts were combined, dried and evaporated to dryness under reduced pressure to afford [3-$^2H_2$]-3-nitropropanoic acid (28, 0.04g). The identity of the product was confirmed by comparison (tlc, m.pt) with an authentic sample. The ^1H nmr indicated that ca 70% exchange had occurred at C-3 with no exchange at C-2 δ(80MHz, $CDCl_3$) 4.4 ppm (0.6H, t, J = 8 Hz, CH_2NO_2).

[2-$^2H_2$]-3-aminopropanoic acid (29): [2-$^2H_2$]-3-Nitropropanoic acid (0.02g, 0.17 m.moles) in methanol (3 cm^3) was treated with anhydrous ammonium formate (0.035g, 0.55 m.moles) and 10% palladium on charcoal (0.02g) and the mixture stirred at room temperature for 30 min. The reaction mixture was then filtered through celite and evaporated to

dryness in vacuo to afford a colourless solid (0.02g). Examination by ^{1}H nmr and ms showed this sample to be identical with an authentic sample of 3-aminopropanoic acid (4) with no deuterium incorporation.

N-Camphanoyl-3-aminopropanoic acid (16): (a) 3-Aminopropanoic acid (0.09g, 1 m.mole) in dichloromethane (5 cm^3) was treated with triethylamine (0.14g, 1.5 m.moles) and (-)-camphanyl chloride (0.23g, 1.1 m.moles) at 0°C and the reaction mixture stirred for 3h. Further camphanyl chloride (0.12g, 0.5 m.moles) was added and the reaction mixture stirred at room temperature overnight however tlc examination after this time showed that no reaction had occurred. ms (FAB, +ve) m/z 198 $[M^+]$-camphanic acid, 153 $[M-CO_2H]^+$.

(b) (-)-Camphanyl chloride (0.27g, 1.2 m.moles) in toluene (1 cm^3) was treated with a solution of 3-aminopropanoic acid (0.08g, 0.9 m.moles) in aqueous sodium hydroxide (0.5 cm^3, 2N plus 0.75 cm^3, 3N) and the mixture stirred at room temperature overnight. The reaction mixture was washed with chloroform, the aqueous layer acidified (pH 1-2) with 6N hydrochloric acid and then extracted with chloroform (2 cm^3 x 3). The chloroform extracts were combined, dried and evaporated to dryness under reduced pressure to afford an off white solid which consisted of starting material and camphanic acid only.

(c) Freshly sublimed (-)-camphanyl chloride (0.09g, 0.45 m.moles) in toluene (0.2 cm^3) was treated with a solution of 3-aminopropanoic acid (0.0254g, 0.3 m.moles) in aqueous sodium hydroxide (0.17cm^3, 2N plus 0.17cm^3, 3N) and the mixture stirred vigorously for 3 h. The reaction mixture was extracted with chloroform 2 cm^3 x 3) and the aqueous portion acidified (pH 1-2) with 6N hydrochloric acid then extracted with chloroform (5

cm^3 x 3). The organic phase was dried and evaporated to dryness under reduced pressure to afford an oil which slowly solidified (0.085g). The crude material was recrystallised from chloroform/40-60° petroleum ether to afford N-camphanoyl-3-aminopropanoic acid (16) (0.055g, 65%) m.pt 129-131°C lit[33] 128-130°. δ(80MHz, $CDCl_3$) 7.0 ppm (1H, br.s, COOH) 3.5 ppm (2H, m, CH_2NHCO), 2.7 ppm (2H, t, J = 6 Hz, CH_2CO_2H), 1.8 ppm (2H, m, camphamyl CH_2) 1.7 ppm (2H, m, camphanyl CH_2) 1.1 (6H, s, camphanyl 2 x CH_3), 0.8 ppm (3H, s, camphanyl CH_3) ms (EI) m/z 269 [M^+].

N-Camphonyl-3-aminopropanoic acid methyl valine amide (17):
N-Camphonyl-3- aminopropanoic acid (0.050g, 0.18 m.moles) from the above reaction in dry tetrahydrofuran (2 cm^3) was treated with 1, 3-dicyclohexylcarbodiimide (0.05g, 0.24 m.moles) and added to methyl valine (0.05g, 0.3 m.moles) in dry ethyl acetate (4 cm^3). The mixture was stirred at room temperature overnight then filtered and evaporated to dryness in vacuo to afford a crude product which was purified by flash column chromatography on silica gel (Kieselgel H type 60) using chloroform as the mobile phase. The purified product was recrystallised from chloroform/40°-60° petroleum ether to give N-camphonyl-3-aminopropanoic acid methyl valine amide (17, 0.04g, 56%) m.pt. 142-144°C δ(80MHz, $CDCl_3$), 7.0 ppm (H, d, J = 10 Hz, NHCO Camphanyl), 6.25 ppm (1H, brs, NH-methyl valine), 4.5 ppm (1H, dd, J = 6Hz, CH_2NHCO), 2.4 ppm (2H, m, CH_2CONH), 1.8 ppm (2H, m, camphonyl CH_2), 1.7 ppm (2H, m, camphonyl CH_2), 1.1 ppm (6H, s, 2 x CH_3), 0.8 ppm (3H, s, camphonyl CH_3). ms (EI) m/z382 [M^+].

Feeding experiments

[1-^{13}C, 2-$^{2}H_2$]-3-Nitropropanoic acid (7). Penicillium atrovenetum was cultured on Czapek Dox slopes and then grown in modified Raulin Thom medium (50 cm^3 x 2) in static culture. Growth was maintained in the light at 30°C for 24 h. and the culture then treated with [1-^{13}C, 2-$^{2}H_2$]-3-nitropropanoic acid (7) (10 mg per flask) and the incubation continued for a further 72 h. the mycelium was removed by filtration, the medium acidified with conc. hydrochloric acid (ca pH 1-2) and extracted with ether (100 cm^3 x 5). The ethereal fractions were combined, dried and evaporated in vacuo to give a yellow crystalline solid which was recrystallised from chloroform to afford 3-nitropropanoic acid (1, 80 mg). δ 400 MHz, ($CDCl_3$) carboxyl region only 173.334 ppm, 172.716 ppm. ms (FAB -ve) m/z 122 (small), 121, 120.

Deuterated aspartic acids

Modified Raulin Thom medium (50 cm^3 x 2) was inoculated with Penicillium atrovenetum from Czapek Dox slopes and the culture incubated under static conditions at 30°C for 42 h. then pulse fed with the deuterated L-aspartic acid in distilled water at 42, 48 and 54 h. after innoculation (total 20 mg). The flasks were worked up as described above 96h. after the initial inoculation and 3-nitropropanoic acid isolated and recrystallised from chloroform.

In the time course experiments, flasks were worked up 72 and 96h. after inoculation and the 3-nitropropanoic acid isolated as described above. The product was examined by ^{2}H nmr spectroscopy (see table in results and discussion).

3-Nitropropanoic acid methyl valine amides: 3-Nitropropanoic acid (1) isolated from the Penicillium atrovenetum was treated with 1,3-dicyclohexylcarbodiimide (1.1 mol. equiv) and with methyl valine (1.1 mol. equiv.) in dry THF/ethyl acetate (1:1) and the reaction mixture stirred at room temperature overnight. After this time the reaction mixture was filtered and the solvent removed in vacuo to afford the crude product which was purified by flash column chromatography using chloroform/methanol as the mobile phase. The product was identified by comparison (tlc) with an authentic sample prepared previously.

[^{15}N]-Nitrosuccinic acid (5): Modified Raulin Thom medium (50 cm^3 x 2) was inoculated with Penicillium atrovenetum a described above. The cultures were treated with [^{15}N]-nitrosuccinic acid (20 mg) after 42, 48 and 54h. and the 3-nitropropanoic acid (1) isolated after 96h. as described above. The recrystallised sample was identified by comparison (tlc) with authentic 3-nitropropanoic acid.

Isolation of 3-Nitropropanoic acid reductase

The method of Shaw[24] was used, with the exception that cultures were grown in a static state in the light since dark-grown cultures failed to produce reductase activity in crude extracts.

The crude mycelium from cultures grown in modified Raulin Thom medium (2 x 50 cm^3 per extraction) were harvested at various time intervals. Crude extracts were prepared from the frozen mycelium by grinding and extracting the mycelium at 0°C in phosphate buffer (pH 7.2). The cell debris was removed by centrifugation at 0-4°C (3,000 g, 20 min.) and

the crude extract assayed for enzyme activity (see below). Activity was found in extracts from mycelium harvested between 84 and 110 hours.

Extracts which possessed activity were combined and treated with solid ammonium sulphate (24.2g/100 cm^3) to 40% saturation then centrifuged (20,000g, 20 min.) and the supernatant retained. Treatment with additional ammonium sulphate (18.8g/100 cm^3) to 65% saturation and centrifugation (20,000g, 20 min.) gave a pellet of protein which was resuspended in phosphate buffer (0.05M) then dialysed at 2-4°C overnight. By this method, from 2g of mycelium, sufficient enzyme was isolated to hydrolyse 1 μmole of NADPH per min. The partially purified enzyme extract was stored frozen at -15°C until used.

Assay of 3-nitropropanoic acid reductase.

The assay procedure followed was essentially that of Shaw[24]. The assay mixture contained potassium phosphate (pH 5, 100 μmoles) NADPH (0.3 μmoles), 3-nitroacrylic acid (3.0 μ moles), enzyme extract (0.3 cm^3 equivalent to 0.075 g of wet mycelium) made up to a final volume of 1.5 cm^3 with water. The oxidation of NADPH was monitored spectrophotometrically at 340 nm.

Incubations with 3-nitropropanoic acid reductase.

The enzyme was incubated with 3-nitroacrylic acid (6) in both water and deuterated water in the presence of stoicheometric amounts of NADPH. The reaction was monitored spectrophotometrically and the 3-nitropropanoic acid isolated by acidification (pH 1-2, 6N

hydrochloric acid) and extraction into ether. The ethereal extracts were dried and evaporated to dryness in vacuo and the product identified by comparison (tlc) with an authentic sample of 3-nitropropanoic acid.

Ethyl 2-methyl-4-hydroxypyrimidine-5-carboxylate (41): Acetamidine hydrochloride (1.24g, 0.013 moles) was added to an ice cold solution of sodium (01.30g, 0.013 moles) in ethanol (10 cm^3) and the mixture stirred at 0°C until the acetamidine had dissolved. The reaction mixture was filtered through celite, treated with diethylethoxymethylene malonate (2.84g, 0.13 moles) and stirred at 0° for 3h. The reaction was then treated with a further aliquot of sodium (0.30g, 0.013 moles) in ethanol (10 cm^3) and stirred at room temperature overnight. The ethanol was removed by evaporation in vacuo and the yellow residue suspended in water (20 cm^3) washed with ether (10 cm^3 x 2)and the ether washings discarded. The aqueous portion was acidified with glacial acetic acid to pH 5-6 then extracted with ethyl acetate (20 cm^3 x 3). The organic fractions were combined, dried and evaporated to dryness in vacuo to afford the crude crystalline product which was recrystallised from acetone to afford ethyl 2-methyl-4-hydroxypyrimidine-5-carboxylate (41) 1.18g, (50%) m.pt. 190-192°C, lit[96] 191°C δ(80MHz, $CDCl_3$) 8.7 ppm (0.67 H, s, CH aromatic), 7.25 ppm (0.33H, s, CH aromatic), 4.50 ppm (2H, q, J = 7Hz, CH_2) 2.60 ppm (3H, s, aromatic CH_3), 1.35 ppm (3H, t, J = 7 Hz, CH_3). ms (EI) m/z 182 [M^+].

Ethyl 2-methyl-4-chloropyrimidine-5-carboxylate (42) :

(a) Ethyl 2-methyl-4-hydroxypyrimidine-5-carboxylate (41) prepared above (0.1g, 0.55 m.moles) was treated with phosphorous trichloride (0.25 cm^3) then sealed in a tube and heated to 100°C for 30 min.

Excess phosphorous trichloride was removed by evaporation under reduced pressure and the residue treated with ice water, neutralised with solid sodium carbonate and extracted with ethyl acetate (5 cm^3 x 3). The dried, evaporated organic fraction gave a white crystalline solid which was identical with starting material (tlc).

(b) Ethyl 2-methyl-4-hydroxypyrimidine-5-carboxylate (41) (0.11g, 0.55 m.moles) in chloroform (0.5 cm^3) was treated with thionyl chloride (0.5 cm^3, 0.8g, 6 m.moles) and the reaction mixture heated under reflux for 2h. then cooled in ice, treated dropwise with ice water, neutralised with solid sodium carbonate and extracted with ethyl acetate (5 cm^3 x 3). The dried and evaporated organic fraction gave a brown oil which appeared, from tlc, to contain a number of products.

(c) Ethyl 2-methyl-4-hydroxypyrimidine-5-carboxylate (41) (1.0g, 5.5 m.moles) was treated with phosphorous oxychloride (8 cm^3, 13 g, 0.85 moles) and heated to 78-80°C for 3h. Excess phosphorous oxychloride was removed by evaporation under reduced pressure and the residue cooled in ice and treated with ice water. The reaction mixture was then neutralised with sodium bicarbonate and extracted with chloroform (20 cm^3 x 3). The organic fractions were combined, dried and evaporated to dryness under reduced pressure to afford the product as an oil which was purified by flash chromatography to give ethyl 2-methyl-4-chloro pyrimidine-5-carboxylate (42) 0.42g (38%). This product was unstable in the presence of light and air and analytical data could not be obtained. Vacuum distillation led to decomposition. δ (80 MHz, $CDCl_3$) 9.0 ppm (1H, s, CH aromatic), 4.4 ppm (2H, q, J = 7Hz, CH_2) ms (EI), m/z 200 $[M^+]$, 165 $[M-Cl]^+$.

Ethyl 2-methyl-4-methoxypyrimidine-5-carboxylate (43):
Ethyl 2-methyl-4-chloropyrimidine-5-carboxylate prepared above (42, 0.1g, 0.5 m.moles) in methanol (5 cm^3) was treated with sodium methoxide (0.03g, 0.06 m.moles) and the mixture stirred at room temperature for 4 h. The reaction mixture was then filtered and evaporated to dryness in vacuo and the residue treated with water (5 cm^3) then extracted with ethyl acetate (5 cm^3 x 3). The organic fractions were combined, dried and evaporated to dryness under reduced pressure to afford a mixture of the ethyl and methyl esters of 2-methyl-4-methoxypyrimidine-5-carboxylate (43) (0.085g) m.pt. 100-101 ref[73] 101-102 δ(80MHz, $CDCl_3$) 9.4 ppm (1H, s, aromatic CH), 4.7 ppm (1H, q, J = 7Hz, CO_2CH_2), 4.4 ppm (3H, s, OCH_3), 4.2 ppm (1.9H, s, CO_2CH_3), 3.0 ppm, (3H, s, aromatic CH_3), 3.0 ppm, (3H, s, aromatic CH_3), 1.65 ppm (1.5H, t, J = 7Hz, $CO_2CH_2CH_3$). Separation of the two esters by tlc was not possible therefore the crude product was used without further purification.

2-Methyl-4-methoxy-5-hydroxymethylpyrimidine (44): The mixture of esters prepared above (0.1g) in t-butanol (3 cm^3) was treated with sodium borohydride (0.03g, 0.8 m.moles) and heated to 80°C. Methanol (1.5 cm^3) was added dropwise and the reaction mixture then refluxed for 3h. After this time the reaction mixture as allowed to cool to room temperature, acidified (pH 1-2, 2N hydrochloric acid), neutralised with saturated sodium bicarbonate and extracted with ethyl acetate (10 cm^3 x 3). The organic fractions were combined, dried and evaporated to dryness in vacuo to afford the crude product which was purified by flash column chromatography using chloroform/ethanol as eluant to give 2-methyl-4-methoxy-5-hydroxymethyl pyrimidine as a colourless crystalline solid (44, 0.04g), δ(80MHz, $CDCl_3$), 8.5 ppm (1H, s, CH aromatic), 5.3 ppm (1H, brs, OH), 4.65 ppm (2H, s, CH_2OH),

4.10 ppm (3H, s, OCH_3), 2.9 ppm (3H, s, CH_3) ms (EI), m/z 155 $[M+1]^+$, 140 $[M-CH_3]$.

[5'-^{2}H]-2-Methyl-4-methoxy-5-hydroxymethylpyrimidine (45) : The deuterated material was prepared in an identical fashion to the non-deuterated compound by reduction of the mixture of methyl and ethyl esters of 2-methyl-4-methoxypyrimidine-5-carboxylate (43) with sodium borodeuteride. The product was isolated as a colourless crystalline solid with identical tlc characteristics to the non-deuterated compound. δ (80MHz, $CDCl_3$) 8.50 ppm (1H, s, CH aromatic) 4.65 ppm (1H, s $CH^2{}_4HOH$), 4.10 pm (3H, s, OCH_3), 2.90 ppm (3H, s, CH_3) ms (EI) m/z 156 $[M+1]^+$, 141 $[M-CH_3]^+$.

2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine (34):

(a) Ethyl-2-methyl-4- hydroxypyrimidine-5-carboxylate (41, 0.46g, 2.5 m.moles) in dry tetrahydrofuran (50 cm^3) was added to a suspension of lithium aluminium hydride (0.45 g, 12 m.moles) in dry tetrahydrofuran (20 cm^3) and the mixture stirred at room temperature for 1h. then refluxed for 45 min. until a faint yellow colour was observed. The reaction mixture was cooled in ice, treated dropwise with water (0.5 cm^3) and the precipitate collected by filtration then added to phosphoric acid (d 1.71, 1.05 cm^3) in water (50 cm^3). The reaction mixture was heated at 100°C for 30 min. and the hot solution filtered, neutralised with aqueous sodium hydroxide (0.1M) and evaporated to dryness under reduced pressure to afford a colourless residue. This solid material was extracted with boiling ethanol (60 cm^3 x 3, 20 min.). The ethanolic extracts were combined and evaporated to dryness to afford a colourless solid which was recrystallised from dioxane to

give 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34) 0.02g, (6%) m.pt. 213-215°C, lit[102] 212-213°C δ(80MHz, d_6-DMSO), 7.80 ppm (1H, s, CH aromatic) 4.30 ppm (2H, s, CH_2OH), 2.54 ppm (3H, s, CH_3) ms (EI) m/z 140 [M^+], 123 [M-OH]$^+$, 82 [M-OH-CH_3CN]$^+$.

(b) 2-Methyl-4-amino-5-hydroxymethylpyrimidine (35, 0.2g, 1.4 m.moles) was taken up in hydrochloric acid (6N,m 5 cm^3) and the reaction mixture heated under reflux for 5h. then evaporated to dryness in vacuo. The colourless product was crystallised from ethanol/ethyl acetate to afford 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (34), 0.16g (82%) which had identical characteristics (tlc, m.pt, nmr) to an authentic sample prepared above.

[5'-$^2H_2$]-2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine (37): The above reaction was repeated using lithium aluminium deuteride and the product isolated and recrystallised as before (37) 0.025g, (7%) m.pt. 213-214°C δ(80MHz, d_6DMSO) 7.80 ppm (1H, s, CH aromatic), 4.30 ppm (0.5H, s, CH^2HOH), 2.54 ppm (3H, s, CH_3) ms (EI) m/z 142 [M^+], 124 [M-CDO], 83 [M-H_2O-CH_3CN]$^+$

Cyanoethoxymethylene acetate (46): Ethylcyanoacetate (28.25g, 0.25 moles) triethylorthoformate (36g, 0.25 moles) and acetic anhydride (51g, 0.5 moles) were refluxed together for 1.5 h. then unreacted starting material distilled off at 160°C. The residual oil slowly solidified giving yellow crystals which were recrystallised from ethanol to afford cyanoethoxymethylene acetate (46) 13g (30%) m.pt. 121-123°C lit[105] 122-123°C δ(80MHz, $CDCl_3$) 7.9 ppm (1H, s, CH), 4.25 ppm (4H, 2xq, J = 7Jz, CH_2 x 2), 1.3 ppm (6H, 2xt, J = 7Hz, CH_3 x 2).

2-Methyl-4-hydroxypyrimidine-5-carbonitrile (47): Acetamidine hydrochloride (6.7g, 0.07 m.moles) was added to a solution of sodium (1.7g, 0.07 m.moles) in ethanol (35 cm^3) at 0°C. After shaking to dissolve the acetamidine, the mixture was filtered through celite and the clear filtrate treated with cyanoethoxymethylene acetate (5.6g, 0.03 m.moles). The reaction mixture was stirred at room temperature for 2h. then stored at 0°C overnight and the orange/yellow crystals which formed were isolated by filtration, and recrystallised from ethanol to afford the intermediate dimer (49) 3.75g (28%) δ(80 MHz, $CDCl_3$) 7.8 ppm (1H, s, CH), 2.0 ppm (3H, s, CH_3) 1.95 ppm (3H, s, CH_3). A sample of this product (1.5g, 7.7 m.moles) in ethanol (10 cm^3) was treated with hydrochloric acid (0.5N, 15 cm^3) and the solution heated to 100°C for 10 min. then allowed to cool to room temperature overnight. The colourless crystals were isolated by filtration, washed with ice water and dried to afford 2-methyl-4-hydroxypyrimidine -5-carbonitrile (47) 0.4g, (38%) m.pt. 230-232°C lit[104] 233-235°C δ(80MHz, $CDCl_3$) 8.7 ppm (1H, s, CH aromatic), 2.75 ppm (3H, s, CH_3) ms m/z (EI) 135 $[M^+]$, 107 $[M-CO]^+$.

2-methyl-4-aminopyrimidine-5-carbonitrile (39): Acetamidine hydrochloride (0.8g, 8.5 m.moles) was added to a solution of sodium (0.21g, 9.1 m.moles) in ethanol (4 cm^3) at 0°C. The mixture was shaken to dissolve the acetamidine, filtered through celite and the clear filtrate treated with ethoxymethylene malonitrile (0.5g, 4.1 m.moles). A dense precipitate formed immediately which was separated by filtration and crystallised from ethanol to afford 2-methyl-4-aminopyrimidine-5-carbonitrile (39), 0.23g (42%) δ(80MHz, ^2H_2O+^2HCl) 8.75 ppm (1H, s, CH aromatic), 2.85 ppm (3H, s, CH_3) ms

(EI) m/z 134 [M^+], 94 [$M-CH_3CN]^+$. $C_6H_6N_4$ requires C, 53.7%; H, 4.5%; N, 41.8%. Found C, 53.4%, H, 4.6%; N, 42.0%.

2-Methyl-4-aminopyrimidine-5-carbaldehyde (40): Raney nickel (0.25g) was activated by stirring with aqueous sodium hydroxide (2N, 6 cm^3) for 30 min. then the aqueous portion decanted off and the residue washed with water (10 cm^3 x 2). The active catalyst was added to the nitrile prepared above (39, 0.5g, 1.1 m.moles) in formic acid (98-100%, 2.5 cm^3) and the mixture stirred at 80-100°C for 45 min. The reaction mixture was then filtered through celite and the celite washed with ethanol/water (3:2, 5 cm^3 x 2). The filtered reaction mixture and the washings were combined and the ethanol removed by evaporation in vacuo to leave an aqueous portion which was neutralised with sodium bicarbonate and extracted with ethyl acetate (10 cm^3 x 4). The organic fractions were combined, dried and evaporated to dryness to afford the aldehyde as an off white solid which was crystallised from ethanol to afford 2-methyl-4-aminopyrimidine-5-carbaldehyde (40), 0.075g m.pt. 192-194°C, lit[133], 195-196°C. δ (80MHz, d_6-DMSO) 9.8 ppm (1H, s, CHO), 8.6 ppm (1H, s, CH aromatic), 2.4 ppm (3H, s, CH_3) ms (EI) m/z, 137 [M^+] 109 [M-CO].

2-Methyl-4-amino-5-hydroxymethyl pyrimidine (35) :
2-Methyl-4-aminopyrimidine-5-carbaldehyde (40, 0.5g, 3.7m. moles) in methanol (25cm^3) was treated with sodium borohydride (0.2g, 5.3 m.moles) and the reaction mixture stirred at room temperature for 30 min. The reaction was then treated with further sodium borohydride (0.1g, 2.6 m.moles), stirred at 100m temperature for 30 minutes, then quenched with hydrochloric acid (2N, 3.2 cm^3). The solution was evaporated to dryness under reduced pressure and the residue extracted

with hot ethanol ($10cm^3$ x 3). The ethanol extracts were combined and evaporated to dryness in vacuo to afford a colourless solid which was recrystallised from ethanol to afford 2-methyl-4-amino-5-hydroxymethylpyrimidine (35) 0.48g (93%) m.pt. 198-200°C lit[133] 193-194°C δ(80 M.Hz., d_6 DMSO) 8.50 ppm (2H, br.s, NH_2), 8.05ppm (1H,s, CH aromatic), 4.35ppm (2H,S,CH_2), 2.50ppm (3H,s,CH_3) ms (EI), m/z 139 [M^+], 122 [M-OH], 110 [M-CHO].

[5^1-^{2}H]-2-Methyl-4-amino-5-hydroxymethylpyrimidine (36) : 2-Methyl-4-aminopyrimidine-5-carbaldehyde (40, 0.1g, 0.7 m.moles) in methanol ($8cm^3$) was treated with sodium borodeuteride (0.04g, 0.95 m.moles) and the reaction mixture stirred at room temperature for 30 minutes. The reaction was treated with further sodium borodeuteride (0.02g, 0.48 m.moles) and stirred at room temperature for 30 minutes then quenched with hydrochloric acid (2N, 0.6 cm^3) and evaporated to dryness in vacuo. The residue was extracted with hot ethanol ($5cm^3$ x 3) and the ethanol extracts combined then evaporated to dryness under reduced pressure to give the crude product which was recrystallised from ethanol to afford [5^1-^{2}H]-2-methyl-4-amino-5-hydroxymethylpyrimidine (36), 0.075g (76%). m.pt. 196-198°C. lit[133] 193-194°C δ (80MHz, d_6- DMSO 8.5ppm (2H, br.s, NH_2), 8.05ppm (1H,s,CH aromatic), 4.35ppm (1H, s, CHD), 2.50ppm (3H, s,CH_3) ms (EI) m/z, 140 [M^+], 110 [M -CHDOH), 82 [123 - CH_3 CN].

[5'-^{2}H]-2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine (37): [5'-^{2}H]-2-Methyl-4-amino-5-hydroxymethylpyrimidine (36) (0.3g, 2.16m.moles) was added to hydrochloric acid (6N, $5cm^3$) and the reaction mixture treated at 100°C for 30 min. After this time the reaction was evaporated to dryness in vacuo and the residue recrystallised from ethanol/ethyl

acetate to afford [5'-^{2}H]-2-methyl-4-hydroxy-5-hydroxymethylpyrimidine (37) 0.26g (77%) m.pt. 198-200°C δ(400 MHz, d_6 DMSO) 7.8 ppm (1H, s, CH aromatic) 4.20 ppm (1.1H, s, CH^2HOH), 2.50 ppm, (3H, s, CH_3) ms (EI) m/z, 41 [M^+], 124 [M-OH]$^+$. Heating for >45 min. lead to substantial loss of deuterium as measured by ms

2-Methyl-4-amino-5-methylanilinopyrimidine (32) : (a) Thiamine (1, 0.5g, 1.48 m.moles) in ethanol/water (3:2, 12cm^3) was treated with aniline (0.14g, 1.50 m.moles) and sodium metabisulphite (0.0g, 0.15 m.moles) and the mixture refluxed for 1 h. The cooled reaction mixture was acidified (6N hydrochloric acid), washed with ether, neutralised (4N sodium hydroxide) and extracted with ethyl acetate (20cm^{3}x3). The ethyl acetate fractions were combined, dried and evaporated to dryness under reduced pressure to afford an oil (0.15g) which was purified by preparative tlc using chloroform/methanol (9:1) as the mobile phase and recrystallised from ethanol to afford 2-methyl-4-amino-5-methylanilinopyrimidine (32), 0.08g. m.pt. 168-170°C, lit[88] 167-169°C ms, (EI), m/z 214 [M^+], 122 [M - C_6H_5NH] (b) Thiamine (1, 0.01g, 0.03 m.moles) was incubated with thiaminase I in phosphate buffer (pH5, 2cm^3) containing aniline (0.005g, 0.06 m.moles) and the appearance of (32) followed spectrophotometrically at 248 nm. After incubation overnight at 37°C, the mixture was acidified, (6 N hydrochloric acid) washed with ether, neutralised (2 N sodium hydroxide) and extracted with ethyl acetate (2cm^3 x 3). The ethyl acetate fractions were combined, dried and the solvent removed by a stream of nitrogen. The residue was examined by capillary gc/ms and found to be identical to the authentic product prepared above.

2-Methyl-4-amino-5-methylsulphonatepyrimidine (50) :

Thiamine hydrochloride (1, 0.5g, 1.48 m.moles) in water ($2cm^3$) was treated with sodium metabisulphite (0.3g, 1.6 m.moles) and the solution heated in a boiling water bath for 10 minutes. The cooled reaction mixture was left standing at room temperature overnight and the white crystals which formed were isolated by filtration, washed with ice water and dried in vacuo to afford 2-methyl-4-amino-5-methylsulphonatepyrimidine (50), 0.30g (98%).

Isolation of thiaminase I.

Bacillus thiaminolyticus from nutrient agar slopes was grown initially in nutrient broth in a shake culture for 20 h at 35°C. An aliquot ($5cm^3$) was then transferred to the $100cm^3$ of the defined medium of Douthit and Airth[87] and the culture grown in shake culture at 37°C for a further 20h. The culture was then centrifuged (30,000 rpm, 15 min.) at 0-4°C. The precipitate was discarded and the supernatant treated with ammonium sulphate (to 75% saturation, 516g l^{-1}) at 0-4°C. The precipitate which formed after 5-6h. was collected by centrifugation (30,000 rpm 20 min), taken up in distilled water and desalted using an Amicon filter unit. Desalting by dialysis led to an extract of low activity. The enzyme was stored frozen in aliquots at -20°C.

Assay of thiaminase I

The assay procedure was essentially that of Douthit and Airth[87]. Sodium phosphate buffer (0.1m, pH 5.8) and aniline (11.5×10^{-4}M, 10 $\mu l/100\ cm^3$) was treated with thiamine (5×10^{-7}M, $17mg/100cm^3$) or thiamine pyrophosphate ($5 \times 10^{-}$M, $23mg/\ 100cm^3$) and an aliquot of the crude enzyme ($0.2cm^3/2cm^3$) and the formation of the anilinopyrimidine product followed spectrophotometrically at 248nm. Activity against thiamine pyrophosphate was found to diminish more rapidly than activity against thiamine itself over a period of months (vide infra).

Production of thiamine by Saccaromyces cervisiae

The growth of the yeast in the thiamine-free medium was monitored using a haemocytometer. A short lag phase (2h) was followed by a

rapid growth phase lasting a further 7h. and this was followed by a static phase. After 20h. the incubate contained ca 3 x 10^7 cells/cm^3. Thiamine was isolated from the yeast cells after 20h. by boiling the centrifuged cells in 0.1 N hydrochloric acid (5cm^3). They were centrifuged and extracted once again and the pH of the supernatant adjusted to ca 6.5. The supernantant was then incubated with takadiastase which also possesses phosphatase activity. A sample of the supernatant (5cm^3, 25%) was analysed for thiamine using hplc.

Thiamine was converted to its thiochrome derivative by the addition of potassium ferricyanide (0.3M, 3cm^3) in sodium hydroxide (3.75M). The mixture was then shaken vigorously for 2 min. and left to stand in the dark for 10 min. after which time it was filtered through a millipore filter (0.45 μm) into a vial for hplc analysis. Standard solutions of known thiamine concentration were treated in an identical fashion. Ten microlitres from each sample was injected on a 25cm x 4.6mm μ - Bondapak C^{18} column using water : methanol (7:3) as mobile phase and running at 2cm^3/min. Peaks were detected using a Perkin-Elmer LS-5 luminescence spectrometer fitted with a 8μl flow cell with excitation at 365nm and emission at 435nm. Calibration curves were constructed by plotting peak heights against concentration. Using this method, the amount of thiamine per 50cm^3 incubation was found to be ca 2.4μg.

Capillary gc/ms analysis of thiamine as its aniline derivative

The thiamine isolated from yeast cells as described above were treated with thiaminase I as described previously and the pyrimidine/aniline derivative (32) extracted into ethyl acetate then blown to dryness in a stream of nitrogen. The residue was taken up in ethyl acetate

(20μl) and a sample (5μl) injected onto a BP1 fused silica capillary column (12.5m x 0.3mm i.d.) The temperature programme used started at 70°C and increased at 30°/min. to 220°C then at 5°/min to 280°C. The total ion current was monitored however it revealed little about the required product therefore ions of 214-215 (the M^- peak for the aniline derivative) and 122-123 (M-aniline) were also monitored. The column output was measured directly using a Kratos ms 80 RFA mass spectrometer operating in the EI mode. Under these conditions, the aniline pyridine compound (32) had a retention time of 8min.

Cleavage of nucleic acids in reverse micelles

Samples were prepared in duplicate containing 1 μl buffer (50 mM NaCl, 100 mM Tris Cl pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol), enzyme (1 μL ca 10u), DNA (1-2 μl, 1μg) and then made up to 10 μl with sterile distilled water. One sample was treated with a solution of sodium sulphosuccinate dioctyl ester (AOT) in hplc grade hexane (100 mM or 50 mM) and vortexed vigorously until visually clear while the other was vortexed. Both samples were then incubated at 37°C overnight. After incubation the samples were placed at -25°C for 2h or at -78°C for 30 min. then treated, while still cold, with water (10 μl) and loading buffer (2 μl). Following centrifugation in an eppendorf centrifuge (15000 rpm, 5 min.) the aqueous phase was removed and applied to a 1% agarose gel containing 0.5 $\mu g/cm^3$ ethidium bromide. Bands were visualised by ultraviolet illumination.

In the annealing experiments, samples were incubated as described above with and without enzyme and the reversibility of the annealing process demonstrated by heating the sample to 65°C prior to gel electrophoresis.

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Identification of L-Nitrosuccinate as an Intermediate in the Fungal Biosynthesis of 3-Nitropropanoic Acid

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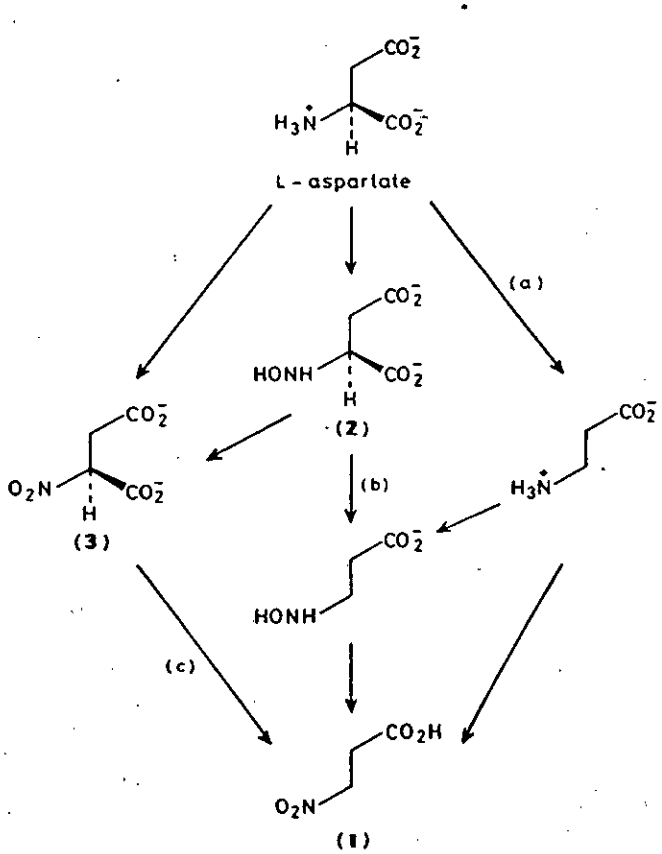
D,L-Diethyl [^{15}N]nitrosuccinate is efficiently incorporated into 3-nitropropanoic acid (1) by cells of *Penicillium atrovenetum*; incorporation of L-[2,3,3-$^{2}H_3$]aspartate into (1) with retention of the C-2 deuterium of the amino acid allows assignment of the chirality of the intermediate.

3-Nitropropanoic acid (1) is a toxin produced by a number of fungi[1] and several plants of the family *Fabaceae*.[2] The biosynthetic routes to this metabolite, although not characterised in detail, appear to be markedly different in the two types of organism.[3] The fungal pathway is of especial interest since 3-nitropropanoic acid is implicated as a key intermediate in the nitrification pathway of *Aspergillus* and *Penicillium* strains.[4] Previous investigations with *P. atrovenetum* have shown that the amino nitrogen and carbons-2, -3 and -4 of the L-aspartate skeleton are incorporated as an intact unit[5—7] and that both oxygens of the nitro group are derived from dioxygen.[8] On this basis, three distinct routes from L-aspartate to (1) are possible in theory (Scheme 1). While route (a), *via* β-alanine, can be discounted by the failure to incorporate label from β-alanine into (1),[5] the alternative routes, involving decarboxylation either of *N*-hydroxyaspartate (2), or of nitrosuccinate (3), have proven difficult to test because of the instability of these compounds.[9] We have examined the incorporation of ^{15}N from D,L-diethyl [^{15}N]nitrosuccinate into (1), reasoning that *in vivo* hydrolysis of the diester would be slow enough to liberate small quantities of the free acid within the cells and that subsequent incorporation of (3), while it would not preclude *N*-hydroxyaspartate as an earlier intermediate, would show that complete oxidation of the amino group of aspartate to a nitro group precedes decarboxylation.

Accordingly, D,L-diethyl [^{15}N]nitrosuccinate was prepared by treatment of diethyl bromosuccinate with $Na^{15}NO_2$ (94 atom % ^{15}N) in the presence of phloroglucinol[10] and the diester was pulse fed to surface cultures of *P. atrovenetum* over a period of 36 h beginning 48 h after inoculation. The ^{1}H-decoupled ^{15}N DEPT n.m.r. spectrum of the isolated 3-nitropropanoic acid in deuteriomethanol showed an intense signal at 3.4 p.p.m. (relative to $MeNO_2$) corresponding to a 14-fold increase of the ^{15}N signal over natural abundance. The enrichment of the nitro nitrogen was also evident from the appearance of ^{15}N satellites of both methylene signals in the ^{1}H n.m.r. spectrum of the metabolite ($^{2}J_{NH}$ 2.2 Hz, $^{3}J_{NH}$ 3.7 Hz) which corresponded in intensity to a 20 fold dilution of ^{15}N enrichment from the racemic diester.

The stereochemistry of the intermediate nitrosuccinic acid was determined indirectly by examining the incorporation of deuterium from L-[2,3,3-$^{2}H_3$]aspartic acid (98 atom % ^{2}H) into (1). The ^{2}H n.m.r. spectrum of the enriched 3-nitropropanoic acid shows that deuterium is retained at both the 2- and



Scheme 1

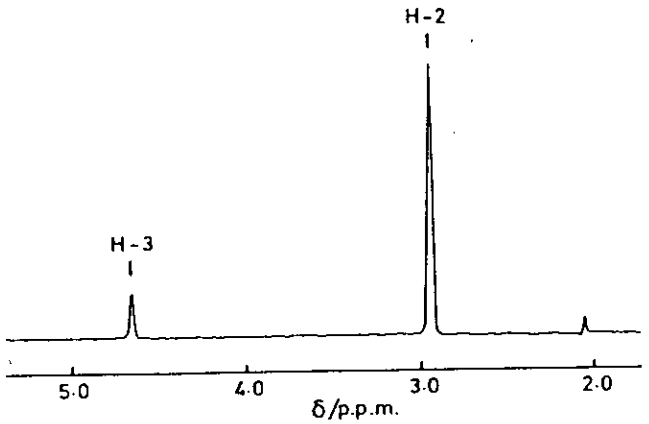


Figure 1. 55.3 MHz ^2H N.m.r. spectrum of 3-nitropropanoic acid obtained from material biosynthetically enriched with ^2H from L-[2,3,3-$^2H_3$]aspartic acid. The spectrum was measured on a 1 M solution in acetone, spectral width 600 Hz, acquisition time 0.85 s, 2000 transients.

3-positions of the metabolite (Figure 1).† If inversion of the stereochemistry at the carbon derived from the C-2 of L-asparatate had occurred in the formation of nitrosuccinic acid then no retention of deuterium of the 3-position of (1) would be expected. It follows that the stereochemistry at the C-2 of L-aspartate is probably retained in the metabolic oxidation of the amino acid to nitrosuccinic acid.

On the basis of these results it appears evident that the biosynthetic pathway involves oxidation of L-aspartate to L-nitrosuccinate prior to decarboxylation [route (c) in Scheme 1] and that the subsequent steps do not involve loss of the H-2 of this intermediate. The mechanism and stereochemistry of the decarboxylation step are however unknown.

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† Partial loss of ^2H derived from the 2-position of L-[2,3,3-^2H]asapartate may arise through aspartate aminotransferase activity[7] or by chemical exchange at a later stage in the pathway. In a control experiment (data not shown) the intensity ratio of the ^2H signals for 3-nitropropanoic acid isolated from a culture grown in medium containing 20% ^2H_2O was *ca.* 1:1, indicating that no significant exchange of ^2H at C-3 occurred during isolation of the metabolite.

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Correlation of the Energies of π-Bonds between Carbon and Other Elements with E.S.R. Hyperfine Coupling Constants

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A linear correlation is found between C=A π-bond energies in compounds of the type $H_2C{=}AR_n$ (or $HC{\equiv}AR_{n-1}$) and β-proton hyperfine coupling constants for the corresponding radicals $H_3C{-}\dot{A}R_n$ (or $H_2C{=}\dot{A}R_{n-1}$): an empirical method is therefore available for either estimating π-bond energies from e.s.r. data or predicting hyperfine coupling constants from bond energy data.

We have found that a linear correlation (r = 0.96) exists between C=A π-bond energies (estimated as the difference E(C=A) − E(C–A); A is an element) and the β-proton hyperfine coupling constants in the corresponding radicals $H_3C{-}\dot{A}R_n$ (or $H_2C{=}\dot{A}R_{n-1}$). [We have taken the β-proton couplings of methyl groups as the maximum, on the basis of a $B\cos^2\theta$ dependence (*i.e.* as the value of B, since θ = 0 for maximum coupling), in order to relate them directly to couplings in radicals of the type $H_2C{=}\dot{A}R_{n-1}$, where the geometry forces θ = 0.] The data used in the correlation are collated in Table 1.

Our qualitative interpretation of this is that as the formation of a π-bond requires overlap between a singly occupied C(2p) orbital and a singly occupied A(np) orbital, so part of the hyperfine coupling mechanism for β-protons involves hyperconjugation, which, in the cases considered here, requires overlap between a doubly occupied carbon-based group orbital of π-symmetry and a single occupied A(np) orbital: a relationship between these processes therefore appears reasonable. We attribute the intercept on the plot to the fact that our simple difference method does not separate the strengthening of the C–A σ-bond due to the change in hybridisation between C(sp^3)–C(sp^3) and C(sp^2)=C(sp^2), which will therefore be included in the derived π-bond energies.

However, our main point is that this correlation may be used in a purely empirical way for predictive or interpretive purposes, by employing either equation (1) or (2), as illustrated by the following examples which relate to some areas of current interest.