Studies of the Initial Steps of Lysine Biosynthesis in *E.coli*

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Abbreviations

Amp	ampicillin
ASA	aspartate semialdehyde
BSA	bovine serum albumin
Da	dalton
DAP	diaminopimelate
DEAE	diethyl aminoethyl
DHDPA	dihydrodipicolinic acid

DHDPA synthase units One unit of enzyme activity is defined as the amount which catalyses an increase in absorbance at 270 nm of 0.001/min

DTT	dithiothreitol
EDTA	ethylene diamine tetra acetate
EtBr	ethidium bromide
FMN	flavin mononucleotide
f.p.l.c.	fast protein liquid chromatography
h.p.l.c.	high pressure liquid chromatography
MM	minimal medium
M_r	molecular weight
PAGE	polyacrlamide gel electrophoresis
RE	restriction endonuclease
RT	room temperature
SDS	sodium dodecyl sulphate
SpA	specific activity
TBE	Tris-borate/EDTA electrophoresis buffer
TE	Tris-EDTA
THDPA	tetrahydrodipicolinic acid
UV	ultra violet
Ve	elution volume

Summary

1. A pUC9 derived plasmid, pDA2, containing the gene encoding for DHDPA synthase was obtained from Dr. P.Stragier in Paris. The *E.coli* strain MV1190 (*RecA*$^-$) was transformed by pDA2 to produce an overexpressing strain for DHDPA synthase. The specific activity of this crude extract showed 200-fold more activity than that from the wild-type.

2. The DHDPA synthase was purified from both the wild-type and mutant *E.coli* strains. The purification from the transformed strain required fewer steps to acheive homogeneity. 35 mgs of homogeneous enzyme was obtained from 24 g of transformed cells compared to 39 μg obtained from 18 g of wild-type cells. This corresponds, in practical terms, to over 600-fold overexpression.

3. The assay used to monitor purification and inhibition tests involves measuring a hitherto uncharacterised product which absorbs at wavelength 270 nm. The product of the reaction, *in vivo*, is DHDPA which is unstable and could not be the 270 nm absorbing product. DHDPA is oxidised in air to dipicolinic acid (DPA) which absorbs at 270 nm. Therefore it has been proposed that the UV absorbing product of the assay is DPA. The assay product has been isolated by h.p.l.c. methods and it has been identified as DPA by UV and NMR spectroscopy.

4. DHDPA synthase has been used to screen various product analogues as potential inhibitors. The compounds were assayed at four final concentrations 10, 1, 0.5, and 0.1 mM. The results of a small selection of these compounds are shown in Chapter 3.

5. To investigate the next enzyme, DHDPA reductase, and subsequent enzymes on the pathway THDPA was made for use as a substrate for DHDPA reductase in the reverse reaction. Two enzymatic approaches were carried out to attempt to isolate THDPA, using either DHDPA reductase or meso-diaminopimelate (DAP) dehydrogenase. The

DHDPA reductase was isolated from an *E.coli* strain containing a plasmid from Dr. P.Stragier which has an insertion of the gene for DHDPA reductase. The meso-DAP dehydrogenase was partially purified from *Bacillus sphaericus*.

Chapter 1 A General Introduction To Lysine Biosynthesis

1.1 Introduction

Lysine is an essential amino acid for human nutrition and since many plant proteins have a relatively low lysine content it is frequently found to be the limiting amino acid in the nutritional value of plant proteins.

The lysine biosynthetic pathway occurs in bacteria, higher plants and fungi. Lysine is needed in all species for protein synthesis. In bacteria, lysine and its immediate precursor, diaminopimelate (DAP) are also essential building blocks for the peptidoglycan of the cell wall. In plants, lysine is also present in cell wall material but meso-DAP is not known to be a structural component.

Since lysine biosynthesis does not occur in animals, the individual steps of the pathway in bacteria and higher plants are of considerable importance as possible targets for antimicrobial agents and herbicides.

There are two distinct pathways used to synthesise lysine, the diaminopimelate (DAP) pathway and the homocitrate aminoadipate (AA) pathway. The former is the more common pathway and is found in bacteria and in higher plants (see later). It starts from aspartate, which is itself derived from the tricarboxylic acid (TCA) cycle (see Figure 1.1). The DAP pathway to lysine shares its intial two reaction steps with the biosynthesis of threonine, methionine, and isoluecine and these, together with lysine form the aspartate family of amino-acids (Figure 1.2). The three pathways separate after aspartate semialdehyde and the pathway towards lysine forms a unique seven step pathway via diaminopimelate (DAP), hence the pathway is named the DAP pathway (see Figure 1.3).

The AA pathway has only been observed in euglenoids and fungi such as *Schizosaccharomycetes pombe* and *Neuspora crassa* (Ye & Bhattacharjee, 1988 and Ramos *et al.*,1988). In this pathway lysine is synthesised via the intermediate aminoadipic acid (AA) (hence the name of the pathway) which is derived from 2-oxoglutarate and acetyl CoA (also from the TCA cycle). The pathway consists of eight

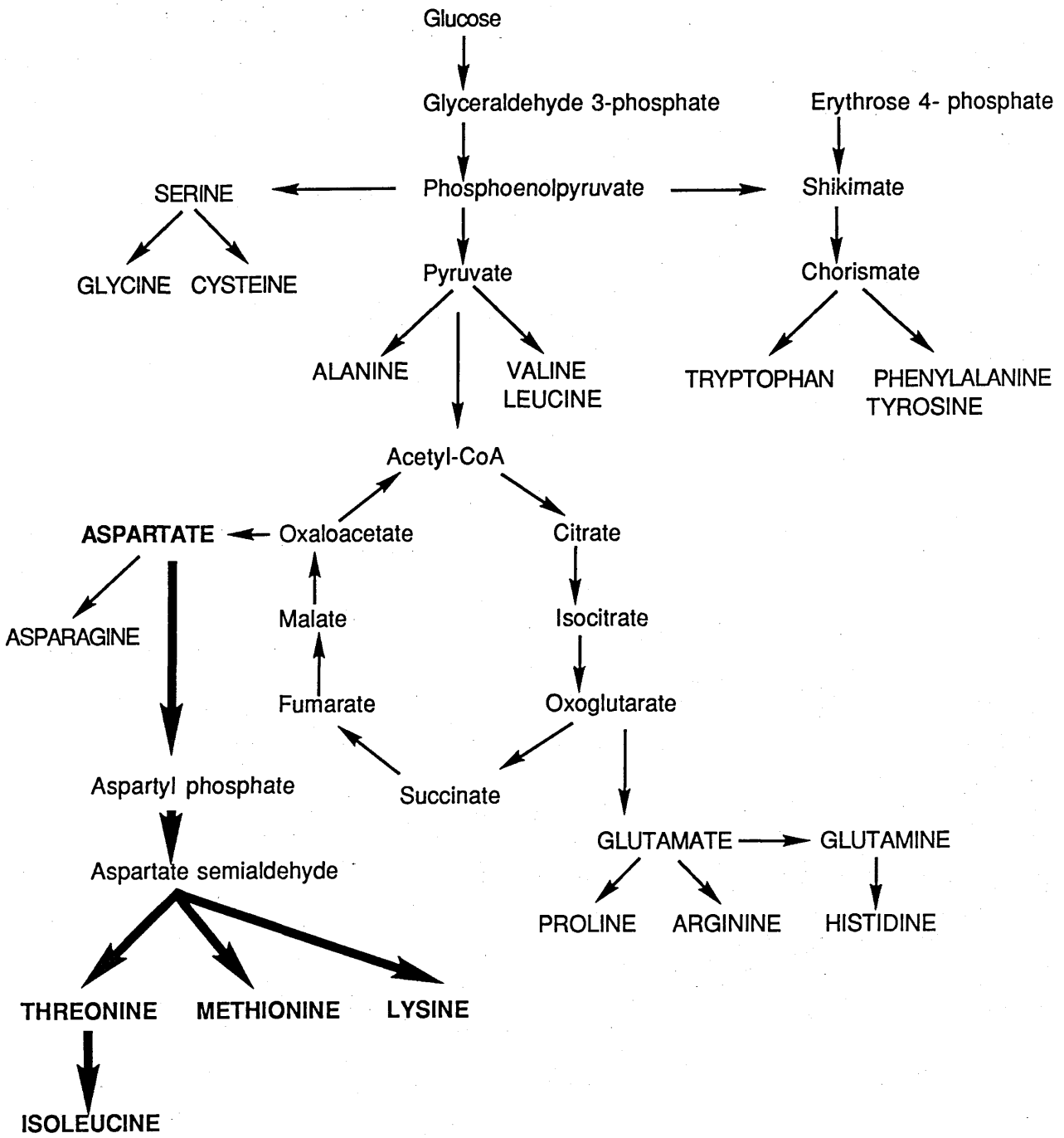


Figure 1.1 An Outline of amino acid Biosynthesis in Bacteria

This scheme summaries the biosynthesis of most of the amino acids in bacteria. This is possibily also found in plants. As is illustrated the lysine biosynthesis is directly from aspartate which is a side product of the central TCA cycle.

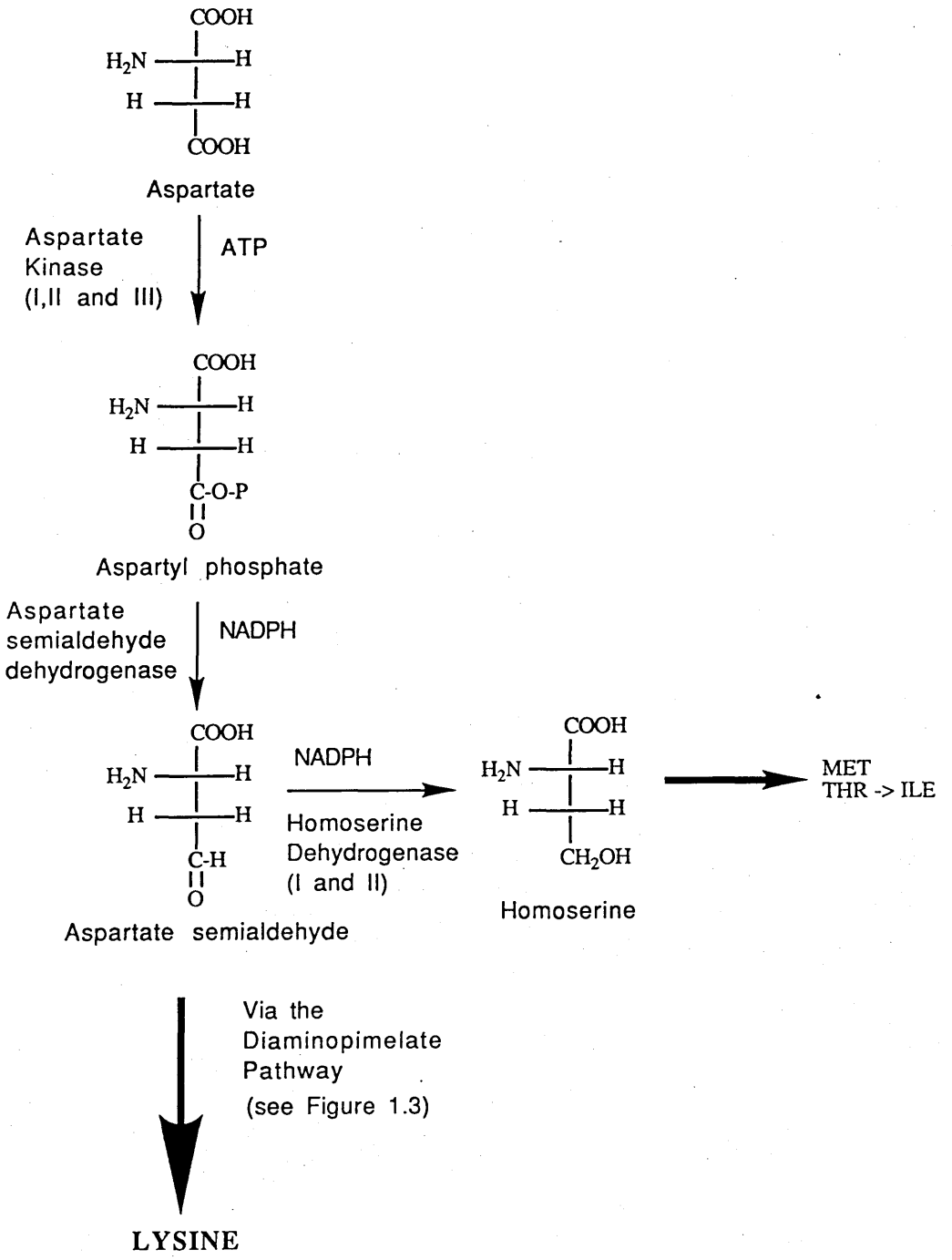


Figure 1.2 The Initial Common Steps on the pathway for the Biosynthesis of the Aspartate Family of amino acids

The first two enzymatic steps of the biosynthesis of lysine,threonine, (isoleucine), and methionine are the same. There are three isoenzymes of aspartate kinase, each representing an arm of the later pathway, but only one unique ASA dehydrogenase. After ASA the pathway branches either to homoserine on the pathways towards threonine, isoleucine and methionine biosynthesis or follows an unique pathway to lysine (see Figure 1.3).

enzymatic steps to produce lysine and bears no resemblence to the DAP pathway and will not be dicussed further.

In this chapter the DAP pathway is described and initially compared among bacteria and then briefly, in higher plants. The regulation of the pathway in both these types of organism is also described.

1.2 The Common Steps on the pathway for the Biosynthesis of the Aspartate Family of amino acids

The aspartate family of amino acids are all derived from aspartate and this is illustrated in Figure 1.2. Before the pathways split into three branches for lysine, methionine, and threonine (isoleucine is derived from threonine) there are two common enzymatic steps. These are the phosphorylation of aspartate followed by its reduction with NADPH (Cohen & Saint-Girons, 1987).

The phosphorylation of aspartate is catalysed by aspartate kinase which is encoded by three different genes which give rise to three distinct isoenzymes. Each enzyme relates to one of the three branches of the aspartate family pathway. The two enzymes encoded by *thrA* and *metL* genes, which relate to threonine and methionine biosynthesis repectively, are bifunctional proteins with both aspartate kinase and homoserine dehydrogenase activity. The latter activity catalyses a step later in the threonine and methionine biosynthetic pathways (see Figure 1.2). While the third isofunctional enzyme, encoded by *lysC* gene, has only aspartate kinase activity, and is involved in the lysine biosynthetic pathway.

The aspartate kinase-I- homoserine dehydrogenase I (encoded by the *thrA* gene) is a tetrameric protein of approximately 355 KDa and is inhibited by threonine. The aspartate kinase-II-homoserine dehydrogenase II (encoded by met L gene) is a dimer of approximately 88 KDa and is inhibited by methionine. The aspartate kinase III (encoded by lys C gene) is a dimer of approximately 50 KDa and its activity is inhibited and its synthesis repressed by lysine.

At first glance these iso-functional enzymes seem to be varied especially in size.

However when the DNA sequence of the genes was determined it became clear that the enzymes were related and had almost certain ly evolved from a common ancestral protein. The entire amino acid sequence of the aspartate kinase III is homologous to the N-terminal part of the two other enzymes (Cassan,1986). This implies that both the bifunctional enzymes have the aspartate kinase activity in the N-terminal region, which means that the homoserine dehydrogenase must reside in the C-terminal region.

Aspartate kinase III is inhibited by other amino acids such as leucine and phenylalanine as well as lysine, which implies that lysine biosynthesis is inhibited in the presence of these amino acids, without inhibiting threonine and methionine biosynthesis. Experiments with mutants of aspartate kinase III suggested that it was the main point for lysine feedback inhibition (Boy, 1979). Some of these mutants were lysine analog resistant and would excrete only lysine but not threonine or methionine. Therefore specific substrate channelling of aspartyl phosphate to DAP was investigated by attempting to demonstrate the presence of protein aggregates but none were found (Cohen & Saint-Girons, 1987). Lysine biosynthesis is also influenced by the inhibition of homoserine dehydrogenase by threonine. This directs aspartate semialdehyde towards lysine biosynthesis as opposed to threonine and methionine synthesis.

The second shared step of the aspartate family is the reduction of aspartyl phosphate to aspartate semialdehyde (ASA) which is catalysed by one enzyme for all three branches of the pathway, and is encoded by the *asd* gene. The protein is a dimer of identical subunit size of 77 KDa. It catalyses a reversible reaction which requires NADPH for the synthesis of ASA and NADP for the reverse reaction.

After ASA, the homoserine dehydrogenase activity of the bifunctional enzymes catalyse the reduction of ASA to homoserine in both threonine and methionine biosynthesis, and then these two pathways split. Meanwhile the lysine biosynthesis continues in its unique pathway after ASA.

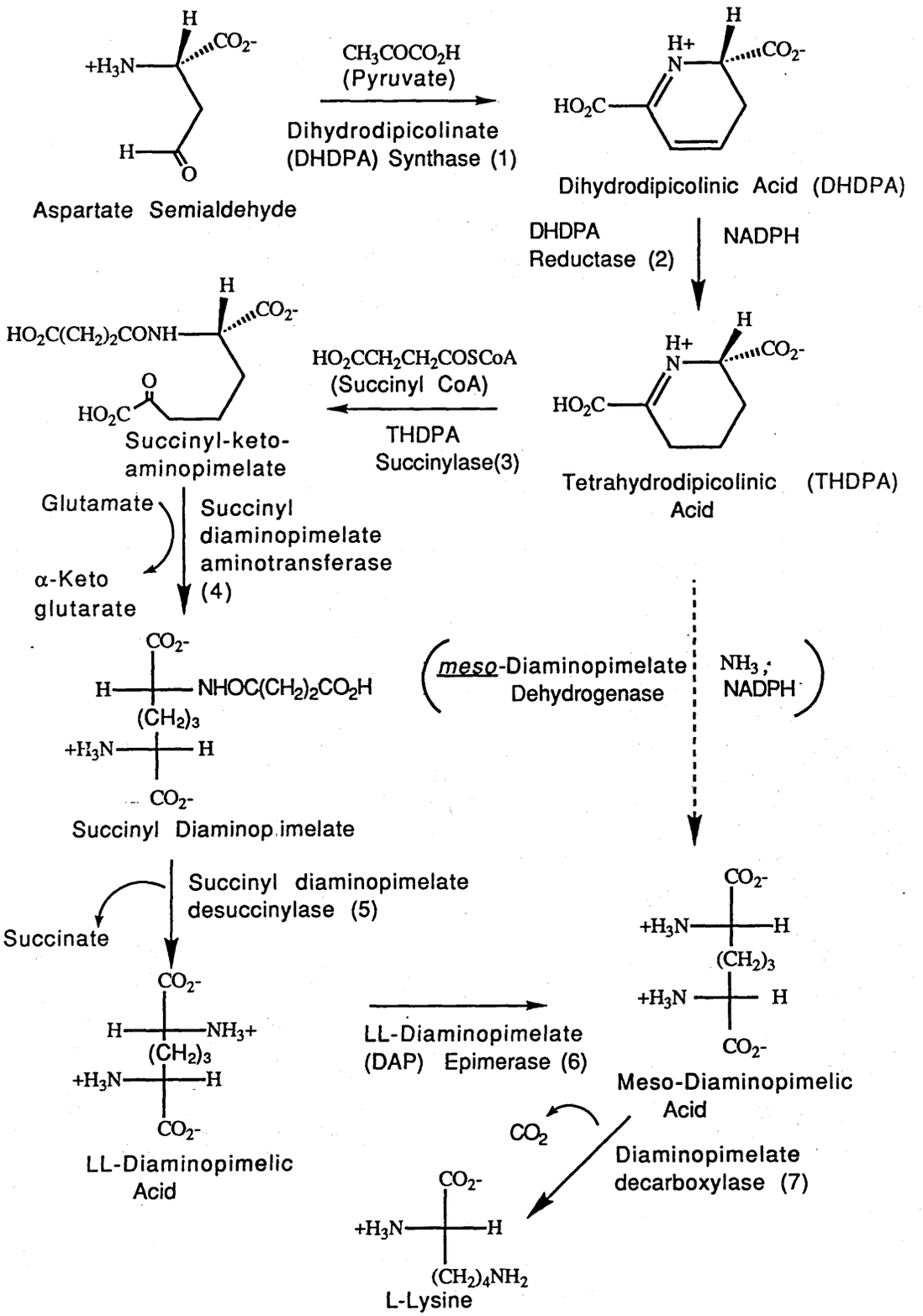
1.3 The Unique Steps of the pathway to Lysine Biosynthesis

The sequence of the seven enzyme catalysed steps leading from ASA to lysine and

Figure 1.3 The DAP (Diaminopimelate) Pathway to Lysine Biosynthesis

From ASA (synthesis is shown in Figure 1.2) seven enzymatic steps lead to the biosynthesis of lysine, these are numbered 1-7, above. ASA condenses with pyruvate to form a six-membered ring containing a nitrogen atom. The ring is broken by a succinylation reaction which is followed by an aminotransferase reaction. Then the intermediate is converted through DAP to lysine. These steps are thought to occur in chloroplasts in plants (see Section 1.5).

The dotted arrow represents the step catalysed by meso-DAP dehydrogenase which is found in Gram positive bacteria (see Section 1.4). Therefore neither the succinylated nor the acetylated intermediates, nor the enzymes required for these steps and also DAP epimerase are found in Gram positive organisms (see Section 1.5).


Aspartate Semialdehyde
CH3COCO2H (Pyruvate)
Dihydrodipicolinate (DHDPA) Synthase (1)
Dihydrodipicolinic Acid (DHDPA)
DHDPA Reductase (2)
NADPH
Tetrahydrodipicolinic (THDPA) Acid
HO2CCH2CH2COSCoA (Succinyl CoA)
THDPA Succinylase(3)
Succinyl-keto-aminopimelate
Glutamate
α-Keto glutarate
Succinyl diaminopimelate aminotransferase (4)
Succinyl Diaminopimelate
meso-Diaminopimelate Dehydrogenase
NH3 ; NADPH
Succinyl diaminopimelate desuccinylase (5)
Succinate
LL-Diaminopimelic Acid
LL-Diaminopimelate (DAP) Epimerase (6)
Meso-Diaminopimelic Acid
CO2
Diaminopimelate decarboxylase (7)
L-Lysine

the structures of the metabolic intermediates are illustrated in Figure 1.3. This shows the pathway eludicated for *E.coli* (Bukhari & Taylor, 1971) and the dotted arrow represents an enzymatic difference of the pathway found in other bacteria which is discussed later.

Mutants in *E.coli* have been isolated which block at almost every enzymatic step on the pathway, these mutants require either DAP or lysine for growth (Bukhari & Taylor, 1971). The mapping and subsequent isolation of the genes encoding the enzymes in the pathway has shown that these genes are scattered around the *E.coli* chromosome (Bachmann & Low,1980). This is in contrast to the genes for the enzymes of some other biosynthetic pathways; for example the genes encoding the enzymes of the pathway of the synthesis of trptophan from chorismate are contiguous in the *E.coli* chromosome and controlled by one promoter in a multigenic operon. Since the genes for the enzymes of lysine biosynthesis are scattered around the *E.coli* chromosome, there must be separate control sequences for each gene (Cohen & Saint-Girons,1987). Since they are controlled separately the mechanism probably involves DNA protein interactions initiated by a lysine-mediated transcriptional mechanism; however no evidence for this has been found, as yet.

The regulation of the pathway is controlled partly by the feedback inhibition of lysine on aspartate kinase III but also on the other steps of the pathway. The first unique step of the lysine biosynthetic pathway is catalysed by dihydrodipicolinic acid (DHDPA) synthase. This catalyses the condensation of ASA and pyruvate to form a six membered carbon ring compound, DHDPA (see Figure 1.3). DHDPA synthase is the rate determining step in the pathway after aspartate kinase III because as *dapA* gene (gene encoding DHDPA synthase) copy number is increased in a *lysC* (gene encoding asparatate kinase III) mutant, lysine excretion increases in parallel with DHDPA synthase activity.

The *dapA* gene has been cloned and sequenced from *E.coli* and its regulation (as with other genes encoding enzymes on the pathway) has been studied by either directly measuring the gene product or by using *lacZ* or *galK* fusion products (Richaud *et al.*,1986). Unlike the majority of genes encoding enzymes on the lysine pathway the

synthesis of DHDPA synthase is not regulated by the lysine pool, although lysine has a noncompetitive inhibitory effect on the DHDPA synthase activity (Yugari & Gilvarg, 1965). DHDPA synthase has been purified from wild-type *E.coli* to homogeneity (Shedlarski & Gilvarg,1970) and its native M_r is 134 KDa. It appears to be a tetramer with subunit size of 33 KDa (see Chapter 3).

The second unique step of the lysine biosynthesis in *E.coli* is the reduction of DHDPA to tetrahydrodipicolinic acid (THDPA) which is catalysed by DHDPA reductase. This has been isolated from *E.coli* and has a M_r of 115,000 and is tetrameric (Farkas &Gilvarg, 1965). Since dipicolinic acid and isophthalic acid were competitive inhibitors of the enzyme, it was suggested that the substrates form a ring when binding to the active site of the enzyme (Tamir & Gilvarg, 1974).

The *dapB* gene encoding DHDPA reductase is repressed by the lysine pool and a 153 bp region downstream of the gene has been shown to be involved with this regulation (Bouvier et al.,1984). As six of the seven genes encoding for lysine pathway enzymes (*dapA* is the only gene not regulated by lysine) are regulated by lysine there may be homology between their promotors (Richauds et al.,1984 (a)). However when this was investigated for *dapB*, *asd* (encodes for ASA dehydrogenase) and *dapD* (encodes for succinyl DAP aminotransferase) the downstream sequences did not show identity between sequences (Bouvier et al.,1984). However there maybe several lysine controlled trans-acting factors which activate or repress the promoter of these genes, which would recognise different DNA binding sequences.

The enzymes of the central portion of the pathway are not present in the bacterial cyctoplasm in high abundance. The next three steps of the lysine biosynthetic pathway, after DHDPA reductase, involve the succinylation and acetylation of intermediates and convert THDPA to LL-DAP. THDPA succinylase catalyses the formation of succinyl keto-L-pimelate and CoA from succinyl CoA and THDPA (see Figure 1.3). This enzyme is encoded by the *dapC* gene and is a dimer with a subunit size of 31,000 (Simms et al.,1984).

Succinyl DAP aminotransferase catalyses transfer of an amino group from glutamate to the succinyl keto aminopimelate. The enzyme is encoded by the *dapD*

gene and its synthesis is repressed by lysine (as mentioned above). It has a M_r of 30,000 and is a monomer (Richaud et al.,1984 (b)).

Succinyl DAP desuccinylase catalyses the hydrolysis of succinyl-DAP to LL-DAP and succinate, and it has a high substrate specificity. This enzyme, encoded by the *dapE* gene, has a subunit size of 40,000 and exists as a mixture of dimers and tetramers. It requires colbalt and zinc for activity (Lin et al.,1988).

LL-DAP is converted to meso-DAP by DAP epimerase. Meso DAP is a major component of the peptidoglycan in bacterial cell walls and therefore is essential for bacteria, for without it they cannot multiply. DAP epimerase is a monomer of M_r 34,000 (Wiseman et al.,1984).

Finally DAP decarboxylase catalyses the reaction of DAP to lysine. This enzyme is specific for meso-DAP and does not recognise LL-DAP as a substrate. Meso-DAP decarboxylase is a tetramer of native M_r 200,000, and the gene encoding the polypeptide is the *lysA* gene.

1.4 A Shorter Lysine Biosynthetic pathway in other Bacteria

There are several differences between the lysine biosynthetic pathway in *E.coli* (Gram negative) compared to Gram positive bacteria, such as the Bacillus species. Firstly, the Gram positive organisms have either one or two aspartate kinase enzymes as compared to the three independently regulated iso-functional proteins in *E.coli*. Depending on the species these aspartate kinases vary in their sensitivity towards lysine (Barlett & White ,1986). In *Bacillus licheniformis* the single asparate kinase is not inhibited nor repressed by lysine but it is inhibited and repressed by threonine and methionine (Halling & Stahly,1976), thereby allowing lysine synthesis when other amino acid pathways are shut down. Where there are two aspartate kinases present the lysine sensitivity of the enzymes varies from species to species, some have one lysine sensitive and one insensitive isoenzyme (for example *Bacillus cereus*). Others have enzymes which are sensitive to a combination of lysine and other amino-acids for example *Bacillus brevis* (Bartlett & White,1986).

The major difference is that all Gram positive bacteria studied have a shorter pathway than that in *E.coli*. These bacteria have a single enzyme which catalyses the reaction from THDPA to meso-DAP thereby by-passing four enzymatic steps of the pathway which occur in *E.coli* (illustrated by the dotted line in Figure 1.3). This enzyme, meso-DAP dehydrogenase, catalyses a reversible reaction which is controlled by the intracellular pH aswell as the availability of the substrates and cofactors (see Chapter 6).

This shorter pathway is found in both sporulating and non-sporulating Gram positive bacteria. The regulation of the pathway in Gram positive bacteria differs among and between the sporulating and non-sporulating bacteria. The feedback inhibition of lysine on DHDPA synthase is variable: in most Bacillus species, for example *Bacillus licheniformis* the DHDPA synthase is insentive to lysine (Halling & Stahly,1976) while the *Bacillus sphaericus* enzyme is noncompetively inhibited by lysine (Barlett & White,1986). Both of these bacteria are examples of sporulating bacteria. However the specific activity of DHDPA synthase increases in sporulating bacteria cells during sporulation thereby combating any feedback inhibition caused by lysine on the enzyme.

Some sporulating bacteria do not require meso-DAP in the cell wall during the vegetative stage but it is present in the cell wall of the spores (Bartlett & White, 1985). Therefore the regulation of the lysine pathway towards meso-DAP production may be activated by the onset of sporulation. This could be indicated by the change in intracellur pH (see Chapter 6 &7). The genes encoding the enzymes of the lysine pathway in most Gram positive bacteria are constitutive and therefore not regulated by the lysine pool (Yeh et al.,1988). In *Bacillus licheniformis* the binding of the substrates to DHDPA synthase are co-operative, the pyruvate concentration has to be above a certain level to activate the enzyme (Stahly,1969). This is not apparent for the binding of the substrates of the *E.coli* DHDPA synthase.

A by-product of the shorter pathway in sporulating bacteria is dipicolinic acid (DPA) produced by the oxidation of DHDPA (Kimura & Sasakawa,1975) and this is also an important component of spores (see Chapter 7 for detail). Since the reaction

uses DHDPA this causes a branch point in the pathway after this substrate. Therefore there has to be a regulatory enzyme to allow lysine production to continue, as DPA is produced from DHDPA. This enzyme is DHDPA reductase which in sporulating bacteria is very different to the enzyme found in *E.coli*. For example DHDPA reductase from *Bacillus subtilis* has FMN as a prothetic group and is a much smaller protein (74,000) compared to the *E.coli* DHDPA reductase (110,000) which uses NADPH (see Chapter 5).

However as with the other enzymes on the pathway the reductase varies from species to species and in some Gram positive bacteria, for example in *Bacillus sphaericus*, the DHDPA reductase is similar to the *E.coli* enzyme (Bartlett & White, 1986).

1.5 The Lysine Biosynthetic pathway in plants compared to *E.coli*

The lysine biosynthetic pathway has not been elucidated completely in plants. However various pieces of evidence suggest that lysine biosynthesis in plants occurs in chloroplasts and follows the DAP pathway as described for *E.coli*. By following ^{14}C-labelled aspartate intact isolated chloroplasts have been shown to synthesise all the aspartate-derived amino acids, including lysine, in the presence of light (Mills et al.,1980). This implies that the pathway is localised to the chloroplast.

Some of the enzymes catalsying the steps of the lysine pathway in higher plants have been isolated. Meso-DAP decarboxylase, the enzyme catalysing the last step of the pathway, was the first to be found in maize (Mazelis et al.,1976). After this asparate kinase, ASA dehydrogenase and DHDPA synthase were isolated and partially purified from chloroplasts of many plants (for example Mazelis et al.,1977). However aminoadipic acid and saccharopine, which are key intermediates of the AA pathway found in fungi, have also been isolated from some plants suggesting that this pathway may also occur.

Since the only enzymes found in higher plants have been ones catalysing steps at the begining of the pathway and DAP decarboxylase at the end of the pathway, it was

impossible to conclude whether the pathway was like that in *E.coli* or like that found in the Gram positive bacteria. If the latter were true none of the four intermediate enzymes would be found in plants which would instead have only the by-pass enzyme, meso-DAP dehydrogenase.

However DAP epimerase (Tyagi *et al.*,1982) and DHDPA reductase were isolated from maize (Tyagi *et al.*,1983) and this provided good evidence that the pathway was similar to that found in *E.coli* (Figure 1.3).

Aspartate kinase, which catalyses the first reaction for all the aspartate family amino acids has been studied in both dicotyledous and monocotyledous plants. In the former such as *Nicotiana sylvestris* (tobacco) there are two iso-enzymes for aspartate kinase, one sensitive and the other insensitive to lysine (Kumpsai, 1987). In monocots, for example wheat, there is only one aspartate kinase. This enzyme is only partially inhibited by lysine but in the presence of lysine and S-adenosyl-methionine (SAM) it is strongly inhibited and therefore blocks the synthesis of all the aspartate amino-acids. SAM is a derivative of methionine and so only when lysine and methionine both accumulate in the plant cell does the above mechanism shut down their biosynthesis (Wallsgrove & Mazilis 1981 and Rognes *et al.*,1980). If either lysine or methionine accumulate individually it does not shut down the other amino-acid's biosynthesis. There has to be another step in the lysine biosynthesis which can regulate only the lysine synthesis and as with *E.coli* this enzyme is DHDPA synthase which catalyses the first unique step in the lysine biosynthetic pathway.

The first studies on a plant DHDPA synthase were in maize seedlings (Mazelis et al.,1977). The pH optimum of the enzyme is similar to that of the *E.coli* DHDPA synthase. It was also very sensitive to lysine and therefore could shut down only the lysine branch of aspartate family biosynthesis.

The substrates of the plant DHDPA synthase bound co-operatively and required the pyruvate concentration to be a certain level for the enzyme to function (Mazelis et al.,1977). This is similar to the *Bacillus licheniformis* enzyme (Halling & Stahly, 1976). Co-operative binding was also shown for the spinach DHDPA synthase (Wallsgrove et al.,1981). Later studies with wheat suspension cells showed that the mechanism of DHDPA synthase to be similar to the *E.coli* enzyme with the pyruvate

binding to the active site via a Schiff's base mechanism (Kumpaisal, 1987), and this is discussed in more detail in Chapter 3.

The plant DHDPA synthases are tetramers of M_r ≈120-160,000 (Kumpaisal 1987) and thus similar in size and subunit make up to the tetrameric *E.coli* enzyme of M_r 143,000. Therefore it seems likely that the higher plant DHDPA synthasese are functionally and structurally homologous to the *E.coli* enzymes (Gishlain, 1990). Recent sequence information supports this (Kaneko *et al.*, 1990).

The general regulation of the lysine pathway in higher plants is more complex than found in bacteria, due to the compartmentalisation of eukaryotic cells. This almost certainly plays a major role in the regulation of amino acid biosynthesis in plant cells, especially with the key enzymes of the biosynthetic pathways. An example of a key enzyme of another pathway is DAHP (D-arabino-heptulosonic acid 7- phosphate) synthetase. It catalyses the rate determining step of the shikimate pathway which leads to the biosynthesis of aromatic amino-acids (Mousdale & Coggins,1991). In lysine biosynthesis the DHDPA synthase plays a similar role, being the rate determining step of the pathway.

The genes for both these key enzymes are encoded in the nuclear chromosome, as are the genes encoding the other enzymes of their pathways. Therefore a polypeptide precursor must be synthesised in the cytoplasm and then translocated into the chloroplast to allow the pathway to initiate in chloroplasts. No direct evidence for translocation has yet been found for DHDPA synthase (Gishlain,1990).

DHDPA reductase was isolated and partially purified from maize (Tyagi *et al.*,1983). This enzyme has a M_r of 80,000 which is slightly smaller than the *E.coli* enzyme (M_r of 110,000). The plant DHDPA reductase is inhibited by DPA, therefore suggesting the substrate is in a ring form which is agreement with the *E.coli* enzyme (Tyagi *et al.*,1983).

The isolation of DAP epimerase in maize, although a major break-through as evidence that the chloroplastic pathway to lysine was following the *E.coli* lysine pathway, was unusual (Tyagi *et al.*,1982). Plants do not use meso-DAP in their cell wall material, unlike bacteria, therefore it is strange that higher plants have not developed a mechanism in which L-DAP could be converted to lysine without the

meso-DAP intermediate.

To date only three out of the nine enzymes required for the lysine biosynthetic pathway have not been isolated in plants. These enzymes catalyse the central steps of the pathway and are not abundant in *E.coli*, and if this was the case in plants this would account for the difficulty of finding them in plants.

1.6 Aims of This Project

DHDPA synthase is the first unique step of the lysine biosynthetic pathway, and therefore compounds which inhibit this enzyme would block lysine biosynthesis and kill the bacteria. These compounds could be used as antimicrobial agents and possibly herbicides if the inhibitory effect was also found for the plant enzyme.

The aims of this project are:-

1. To produce a large amount (miligram quantities) of purified DHDPA synthase from *E.coli*, by constructing an overexpression strain.
2. To set up an assay for DHDPA synthase (Chapter 4).
3. To use the assay to screen potential inhibitors of the enzyme (Chapter 3).
4. To obtain partially purified DHDPA reductase by overexpressing the enzyme in *E.coli* (Chapter 5).
5. To obtain THDPA to be used as a substrate for the reverse reaction of DHDPA reductase since the substrate, DHDPA is unstable. This will require an enzymatic approach using either DHDPA reductase or meso-DAP dehydrogenase (Chapters 5 &6).

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and Biochemicals

Tris, NADPH (tetrasodium salt), NADP, and N,N,N',N'- tetramethylethylene diamine (TEMED) were obtained from Boehringer Corp., Lewes, East Sussex.

DTT, imidazole, disodium EDTA, 2-mercaptoethanol, NH_4Cl, Na_2HPO_4, $MgSO_47H_20$, $CaCl_2$ and all reagents for gel electrophoresis were obtained from BDH Chemicals,Poole, Dorset.

Pyruvic acid (sodium salt), Amp, Thiamine (Vitamin B_1), and Coomassie Brilliant Blue G250 were obtained from Sigma Chemical Co., Poole, Dorset.

KCl, KH_2PO_4, K_2HPO_4, SDS, acrylamide, and bis-acrylamide were obtained from FSA Laboratory Supplies, Loughborough.

The chemicals and equipment for Phastgels were obtained from Pharmacia, Milton Keynes.

All other chemicals were of analytical reagent grade or of the highest available purity.

2.1.2 Chromatographic Media

DEAE-Sephacel, phenyl-Sepharose and G-15 Sephadex were obtained from Pharmacia, Milton Keynes. The f.p.l.c. was preformed using Mono Q (HR5/5 and HR10/10) and Superose 6 (10/30) prepacked columns on a standard Pharmacia f.p.l.c. system.

2.1.3 Enzymes and Proteins

BSA was obtained from Sigma Chemical Co., Poole, Dorset.

Proteins used for Superose 6 calibration were pig lactate dehydrogenase and malate dehydrogenase and rabbit muscle pyruvate kinase and aldolase; these were obtained from Boehringer Corp., Lewes East Sussex.

All restriction endonucleases were obtained from BRL, Gibco Ltd., Paisley, Scotland.

2.1.4 Bacterial Strains and Plasmids

The *E.coli* K12 is wild-type ATCC 14048, F^-, lysogenic; it originated from Amercian Type Culture Collection (Rockville, Maryland, USA). *E.coli* MV1190 is a M13 susceptible *RecA*$^-$ strain obtained from BioRad, Watford and is as described in BioRad Molecular Biology Reports (1987),1(1),5-6. *Bacillus sphaericus* IFO 3525 strain was obtained from NCIMB Ltd., Aberdeen.

The plasmids pDA2 and pDB17 containing *dapA* and *dapB* genes respectively were a gift from Dr. P.Stragier, Instut de Biologie Physico-Chimique, Paris.

2.1.5 Enzyme Substrates

Aspartate semialdehyde (ASA) was prepared by the ozonolysis of allylglycine (Black & Wright, 1954) by L.Couper and D.Tudor, Department of Chemistry, University of Glasgow.

Tetrahydrodipicolinic acid (THDPA) was prepared chemically (Couper,1991) by L.Couper.

Meso-diaminopimelate (DAP) was prepared by L. Couper. It was obtained by fractional crystallisation of a mixture of meso-DAP, DD and LL DAP isomers which had been purchased from Sigma.

All the DHDPA and pyruvate analogues were synthesised chemically by L.Couper and D.Tudor.

2.2 General Methods

2.2.1 pH and Conductivity measurements

pH measurements were made with a Corning pH probe, calibrated at room temperature.

Conductivity measuremets were made at 4°C with a Radiometer Conductivity Meter type 2CDM2e (Radiometer Copenhagen, Denmark).

2.2.2 Protein Estimation

Protein concentration was measured by the method of Bradford (1976) with BSA as the standard.

2.2.3 Dialysis Membranes

Dialysis membranes were boiled for 30 min in 3 mM-EDTA and 18 mM-$NaHCO_3$ then rinsed thoroughly with distilled water and stored in 50% ethanol at 4°C.

2.2.4 Sterilisation Techniques

Glass and plastic ware were autoclaved at 15 psi for 20 min. Some solutions and media were autoclaved at 5 psi for 50 min and others were filter sterilised through Minisart syringe filters (pore size 0.2 μm).

2.3 Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) with 5% stacking gel and 15% running gel. The ratio of acrylamide:bis-acrylamide was 30:0.8 and polymerisation was induced by 0.06% (v/v) TEMED and 0.1% ammonium persulphate.

The gels were stained for protein with 0.1% Coomasie brillant blue G250 in 50% (v/v) methanol and 10% (v/v) glacial acetic acid for 1-2 hrs at room temperature, then destained in 10% methanol and 10% glacial acetic acid.

2.4 Pharmacia Phastsystem

12.5% gels were used with SDS buffer strips and run on a Phastsystem under the conditions described in the Pharmacia Phastgel manual.

The gels were stained by silver stain containing 0.25% (w/v) silver nitrate as described in the Pharmacia Phastgel manual. The molecular weight markers used for M_r estimation were obtained from Sigma; seven proteins with M_r in the range of 14.2-66 KDa were used:- bovine milk lactalbumin (14,200), soybean trypsin inhibitor (20,100), bovine pancreas trypsinogen (24,000), bovine erythrocytes carbonic anhydrase (29,000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36,000), egg albumin (45,000), and bovine albumin (66,000).

2.5 Enzyme Assays

Assays were performed at 25°C in a total volume of 1.0 ml. The instrument used was a Gilford model 2600 Spectrophotometer equiped with Gilford photoelectric detector and recorder.

2.5.1 DHDPA Synthase Assay (270 nm Assay)

The assay used to measure DHDPA synthase during its purification was modified from Yugari & Gilvarg (1965) and is described in Chapter 4. The assay cocktail contained (final concentrations):- 100 mM-imidazole buffer pH 7.4, 5 mM-pyruvate, 2 mM-ASA (neutralised with KOH immediately before use), and enzyme. The assay used to screen for inhibitors was a modified version of the above with 1 mM-ASA, 1 mM-pyruvate and 20 units of DHDPA synthase. The units were calculated as described in Chapter 4.

2.5.2 DHDPA Reductase Assay

DHDPA reductase was measured using a coupled assay with DHDPA synthase and has been modified from Farkas &Gilvarg (1965) and is described in Chapter 5. The assay cocktail contained (final concentrations):- 100 mM-imidazole buffer pH 7.4, 2 mM-ASA (neutralised), 40 units DHDPA synthase, 0.25 mM-NADPH, 10 mM-pyruvate and enzyme. Activity was measured by monitoring the oxidation of NADPH at 340 nm (ε=6,200 $M^{-1}cm^{-1}$).

2.5.3 Meso-Diaminopimelate Dehydrogenase Assays

Meso-DAP dehydrogenase was measured using the assay modified from Misono *et al.*, 1979 and this is described in Chapter 6. The assay cocktail contained (final concentrations):- 200 mM-glycine/KCl buffer pH10.5, 10 mM-meso-DAP, 1 mM-NADP and enzyme. The reverse reaction (see Chapter 6) was also assayed using THDPA as the substrate. The assay cocktail contained (final concentrations):- 100 mM-Tris pH7.4, 2 mM-NADPH, 200 mM-NH_4Cl, 10 mM-THDPA and the enzyme. Both the activities were measured by either NADP or NADPH conversion at 340 nm (ε=6,200 $M^{-1}cm^{-1}$).

2.6 Media

2.6.1 Minimal Medium

Minimal medium was used for the growth of *E.coli* K12 and *E.coli* MV1190/ *RecA*$^-$ cells. This medium contains M9 salts (per litre of distilled water):- 1 g NH_4Cl, 0.13 g $MgSO_4.7H_2O$, 3 g KH_2PO_4 and 6 g Na_2HPO_4. This was autoclaved at 5 psi for 50 min. 0.4% glucose and 0.1 mM-$CaCl_2$ (final concentrations) which had been autoclaved seperately were added after the medium had cooled. For the *Rec A*$^-$ strain the filter sterilised supplements added were 2 µg/ml thiamine (Vitamin B_1) and 50 µg/ml Amp, for plasmid selection.

For plates, 12.5 g agar in 488 ml distilled water was autoclaved separately, and added to 500 ml of sterile 2 x conc. M9 salts, with supplements, to a final voume of 1 litre.

2.6.2 MM63 Medium

MM63 medium was used to grow the transformed *E.coli* cells. This medium consists of, per litre:- 13.6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, and 0.5 mg $FeSO_4.7H_2O$ adjusted to pH 7.0 with KOH and autoclaved at 5 psi for 50 min. Then the following supplements were added :- 1 mM-$MgSO_4.7H_2O$, 0.4% glucose, 7.5 µg/ml thiamine (Vitamin B_1) and 50 µg/ml Amp.

2.6.3 L-Amp

L-Amp was used for the growth of transformed *E.coli* MV1190 cells. The L-broth consists of (per litre):- 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl. This was autoclaved at 5 psi for 50 min, cooled, and 50 µg/ml Amp added. For plates 15 g Bacto-agar was added to L-broth before autoclaving.

2.6.4 Medium for *Bacillus sphaericus* IFO 3525 (Medium 4)

Bacillus sphaericus IFO 3525 was grown in a medium containing, per litre,:- 15 g peptone, 2 g KH_2PO_4, 2 g K_2HPO_4, 2 g NaCl, and 1 g yeast extract. The pH was then adjusted to pH 7.2 with NaOH and it was autoclaved at 5 psi for 50 min. After cooling, sterile solutions of 0.1% glycerol and 0.01% $MgSO_4.7H_2O$ (final concentrations) were added to produce Medium 4.

2.7 The Transformation of *E.coli* with Plasmids

All DNA work was carried out with pre-autoclaved plastic ware and sterile solutions.

The *E.coli* MV/1190 *RecA*$^-$ cells were made competent by the $CaCl_2$ method, as described by Sambrook *et al.*, (1989). The cells were transformed with plasmid with an expression time on ice of 30 min (Sambrook *et al.*,1989), and the cells spread onto L-Amp plates (to express Amp^R plasmid) and incubated at 37^{o}C overnight.

2.8 Small Scale Plasmid Preparation

A single colony from a plate of the above grown cells was picked and used to inoculate a 2 ml L-Amp culture which was then grown at 37^{o}C overnight. Afterwards the culture was centrifuged and stored at 4^{o}C. The cells were lysed by the alkali method and the plasmid DNA isolated by phenol extraction and ethanol precipitation (Sambrook *et al.*,1989). The plasmid was resuspended in TE (Tris/EDTA) and stored at -20^{o}C.

2.9 Identification of Plasmid

2.9.1 Restriction Digests

Restriction digests were set up in a total voume of 20 µl which contained:- 12 µl H_2O, 2 µl 10x React Buffer (Gibco salt buffers depending on the RE's preferred salt concentration), 5 µl DNA solution (<1 µg) and 1 µl RE (to give 5 u/µg DNA). This was incubated at 37^{o}C for 1 hour. For double digests the RE with the lower salt buffer was incubated first, then the higher salt salt buffer was added with the second RE and a futher 1 hour incubation was allowed. If the same salt concentration was required, then the REs were incubated simultaneously.

2.9.2 Agarose Gels

A 1% agarose gel was cast with 1x TBE (45 mM Tris borate: 1 mM EDTA) with the addition of 0.5 µg/ml EtBr (final concentration). The running buffer used was 1x TBE also containing this concentration of EtBr. The restriction digests were prepared

with 5 µl ficoll loading buffer (0.25% (w/v) bromophenol blue, 0.25% xylene cyanal, and 15% Ficoll type 400: Pharmacia) as were the 1 kbase ladder markers which were obtained from Gibco. The gel was run at a current of 100 mA for 45 min.

The DNA fragments were observed on a UV transilluminator and photographed through a red filter using a Polaroid CU-5 camera with 665 +ve/-ve film.

2.10 Growth of Bacterial Cells

2.10.1 Growth of *E.coli* Cells

E.coli K12 cells were grown in minimal medium (M9 salts) while transformed *E.coli* MV1190/*RecA*$^-$ cells were grown in MM63 medium (with Amp), however both were grown in a similar large scale manner.

From either a plate or a glycerol stock a 10 ml L-broth (or Amp) shaking culture was grown overnight at 37^{o}C. A 1 ml aliquot of this culture was added to 50 ml L-broth (or Amp) in a 250 ml conical flask and shaken at 37^{o}C for 16 hours. 200 µl aliquots from this culture were used to innoculate 50 ml of MM (or MM63) in 250 ml conical flasks which were incubated by shaking at 37^{o}C overnight.

2 x 50 ml cultures (of the above) were used to innoculate each 4 x 4 litres of MM (or MM63) in 4 x 10 litre flasks. These were incubated at 37^{o}C, stirring at 200 rpm and aerated at 400 ml/min until $A_{420} \approx 3.5/4.0$ which was 6-8 hours for *E.coli* K12 and 10-12 hours for the mutant strain.

The cells were harvested by centrifugation in a MSE 6 x 750 ml rotor at 4^{o}C at 8,000 g for 15 min and stored at -20^{o}C. All strains of *E.coli* gave a yield of 12-15 g (wet weight) of cells per 4 litres of medium.

2.10.2 Growth of *Bacillus sphaericus* IFO 3525

Bacillus sphaericus IFO 3525 was grown in 2 x 10 ml Medium 4, and shaken at 37^{o}C overnight. Each of these was used to innoculate 2 x 500 ml of Medium 4 in 2 litre conical flasks. These were shaken at 37^{o}C for 6-8 hours until A_{420} was 6-8.

The cells were harvested by centrifugation in a MSE 6 x 750 ml rotor at 4°C at 8,000 g for 15 min followed by a wash with 0.85% NaCl before storage at -20°C. The yield achieved was ≈ 2.5 g per 500 ml culture.

2.11 Bacterial Cell Breakage

Both *E.coli* and *B.sphaericus* cells for enzyme purification were resuspended in ice cold extraction buffer (see Chapters 3, 5, 4, and 6) and broken by three passages through an automatic French pressure cell at 95 MPa (14,000 psi internal pressure).

2.12 Calibration of Superose 6 with Molecular Weights Markers

The Superose 6 column on a f.p.l.c. system was used for the final step of the purification of DHDPA synthase from wild-type *E.coli* and to confirm its subunit M_r (see chapter 3). Therefore, the following proteins were used to produce a standard curve of $V_{elution}$ vs log M_r:- pyruvate kinase; 240,000, aldolase; 160,000, lactate dehydrogenase; 140,000 and malate dehydrogenase;70,000. These were applied (100µl) to the Superose 6 column (which had been pre-equilibrated with Millipore filtered 10 mM Tris pH 7.4) separately and eluted at a rate of 1 ml/min with 1 ml fractions collected.
Superose 6 (HR 10/30) had a bedsize of 1 x 30cm

2.13 G-15 Sephadex Set-up and Calibration

G-15 Sephadex separates, by gel filtration, small compounds of M_r ≈0-1,500. 100 g solid G-15 sephadex was swelled to 300 ml with degassed, distilled water during 1 hour gentle shaking at 90°C, then put at 4°C to cool with intermittent shaking. A 80 x 2.5 cm column was poured and equilibrated in degassed 100 mM Tris pH 7.4 buffer; the flow rate was 7 ml/hr and 5 ml fractions were collected.

The column void volume (120 ml) was measured using Blue Dextran (M_r of 2,000,000) and the total volume (1160 ml) by nitrophenol (M_r of 139). The total volume may be distorted by the adsorption of nitrophenol to the Sephadex.

2.14 UV Spectra

UV spectra were determined at 25^{o}C using a Philips (PU 8720 UV/VIS) scanning spectrophotometer with attached Philips printer. Samples were measured in 1 ml cuvettes.

2.15 H.P.L.C. for Separation of 270 nm Assay Components

The two columns used on the h.p.l.c. were the organic acids column; Aminex Ion Exclusion HPX-87H (300 x 7.8 mm) and fast acids analysis column (HPAH 100 x 7.8 mm) both obtained from BioRad. These were used with a standard cartridge holder containing aminex resin as a guard column to protect the column. The columns were run on a Gilson model 303 h.p.l.c.

The 25 mM-H_2SO_4 used to elute the columns was pre-filtered through a Millipore System using filters with 0.22 μm pore size which was carried out on an aquavac. The eluent was passed through a UV detector and the output recorded on a BBC recorder; fractions were collected on a Gilson model 201-202 controller fraction collector.

Chapter 3 : Studies of Dihydrodipicolinic Acid Synthase from wild-type *E.coli* and a Plasmid Bearing Strain of *E.coli* (MV1190/pDA2)

3.1 Introduction

The first reported partial purification of dihydrodipicolinic acid (DHDPA) synthase (E.C. 4.2.1.52) from *E.coli* was by Yugari & Gilvarg in 1965. Five years later they purified the enzyme to homogeneity from *E.coli* (strain W, ATCC 637) using a seven step purification proceedure which resulted in a 5000 fold purification from crude extract. More recently Laber (1987) who worked on several enzymes of the lysine pathway, purified DHDPA synthase using a six step scheme which gave a 200 fold purification. Although both investigators claimed to have homogeneous preparations there is major disrepancy between the purification factors reported.

In this chapter a purification scheme for DHDPA synthase from wild-type (wt.) *E.coli* K12 following a modified version of Laber's work is described. The yield from this proceedure was not enough to permit detailed characterisation of the enzyme and so a method was set up to over produce DHDPA synthase in *E.coli* cells.

The gene encoding DHDPA synthase is *dapA*; this has been cloned and sequenced by Richaud *et al.*,1986. Dr. Stragier, from this group, kindly provided a pUC 9 derived plasmid which contains a 1.2 kbp *Bst*NI fragment carrying the entire *dapA* gene (Figure 3.1).

The transformation of a suitable *E.coli* host strain with this plasmid and the growth and subsequent purification of the enzyme from the resulting overexpressing strain is described in the second part of this chapter.

3.2 Purification Proceedures From wild-type *E.coli* K12

The growth and harvesting conditions for *E.coli* strain K12 are described in Chapter 2. After breaking the cells all steps were carried out at 4^{o}C except those involving the f.p.l.c. which were carried out at room temperature. The 270 nm assay

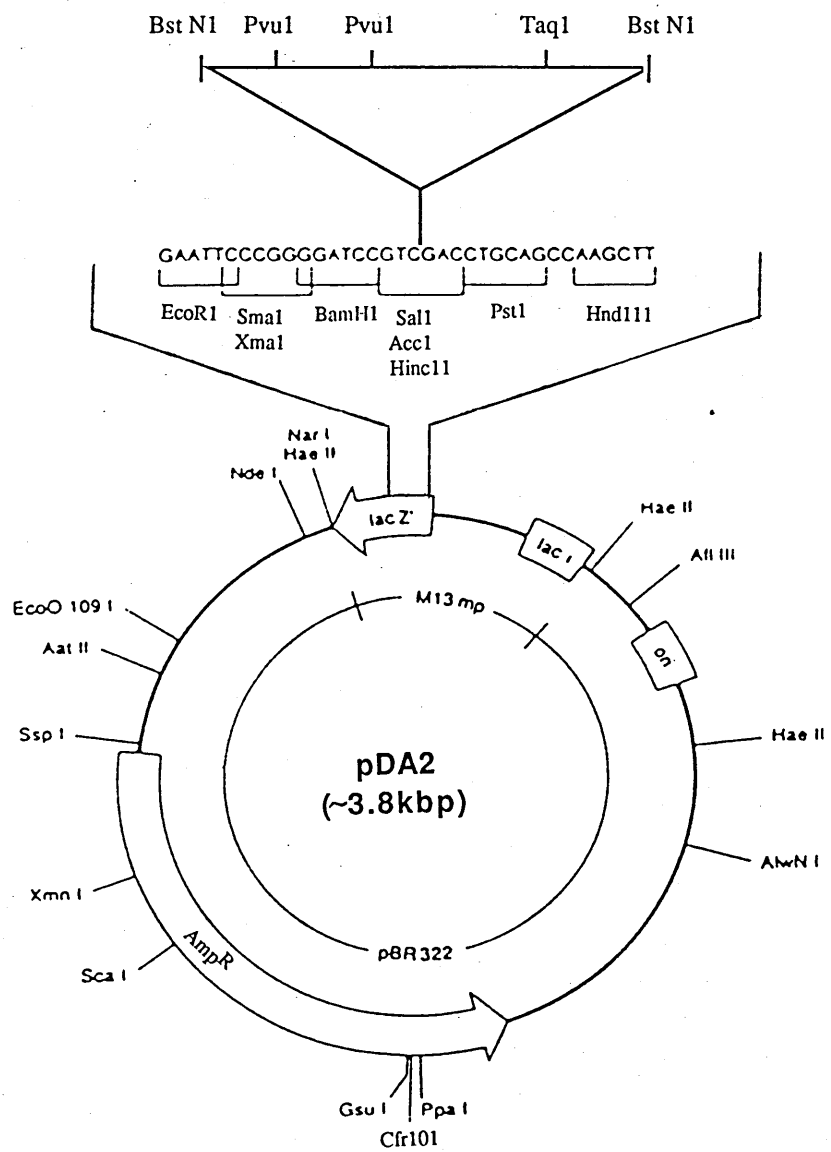


Figure 3.1 The Plasmid Map of pDA2

The plasmid, pDA2, was constructed in the following way:- a 1.2 kb *Bst NI* fragment containing the *dapA* gene was subcloned into the *HincII* site of a pUC9 plasmid. This fragment was present in a previously constructed plasmid (pDA1), which was digested by endonuclease *Bst NI* and the cohesive ends were filled in by DNA polymerase and the restriction fragment ligated into *HincII* digested pUC9. On digestion the *BstN1* sites are lost (Richaud *et al.*,1986).

was used to monitor activity during the purification (see Chapter 4) The assay mixture contained:-100 mM-imidazole buffer pH 7.4, 5 mM-pyruvate, 2 mM-ASA (neutralised with 10 mM-KOH), and enzyme in a final volume of 1 ml; activity was measured at 25°C.

Step 1. Breakage of *E.coli* cells

18 g (wet weight) of cells were resuspended in 35 mls (total volume) of 20 mM-Tris HCl pH 7.4, 10 mM-EDTA, 0.4 mM-DTT and 100 mM-KCl (Buffer A) with an addition of 10 mM-sodium pyruvate.

Cells were broken by passage through the French press three times as described in Chapter 2. The resulting suspension was then centrifuged at 28,000 **g** for 30 min and the pellet was discarded.

Step 2. Heat step

The supernatant was treated to a heat step at 70°C for 2 min and precipitated protein removed by centrifugation, as above. The supernatant was dialysed against Buffer A, overnight.

Step 3. DEAE-Sephacel chomatography

The dialysed protein was applied to a DEAE-Sephacel column (30 x 3 cm) which was pre-equilibrated with Buffer A. The column was washed with this buffer at a flow rate of 1ml/min until the A_{280} of the eluent was below 0.1.

The protein was eluted with a linear gradient (900 mls) of 0.1 -0.5 M-KCl in Buffer A at 1 ml/min and 12 ml fractions were collected.

The fractions containing the highest enzyme activity (elution profile shown in Figure 3.2) were pooled and dialysed against Buffer A overnight.

Step 4. Phenyl-Sepharose chromatography

The dialysed sample was treated with $(NH_4)_2SO_4$, by slow stirring to achieve a final concentration of 0.5 M-$(NH_4)_2SO_4$. The sample was applied to a phenyl-

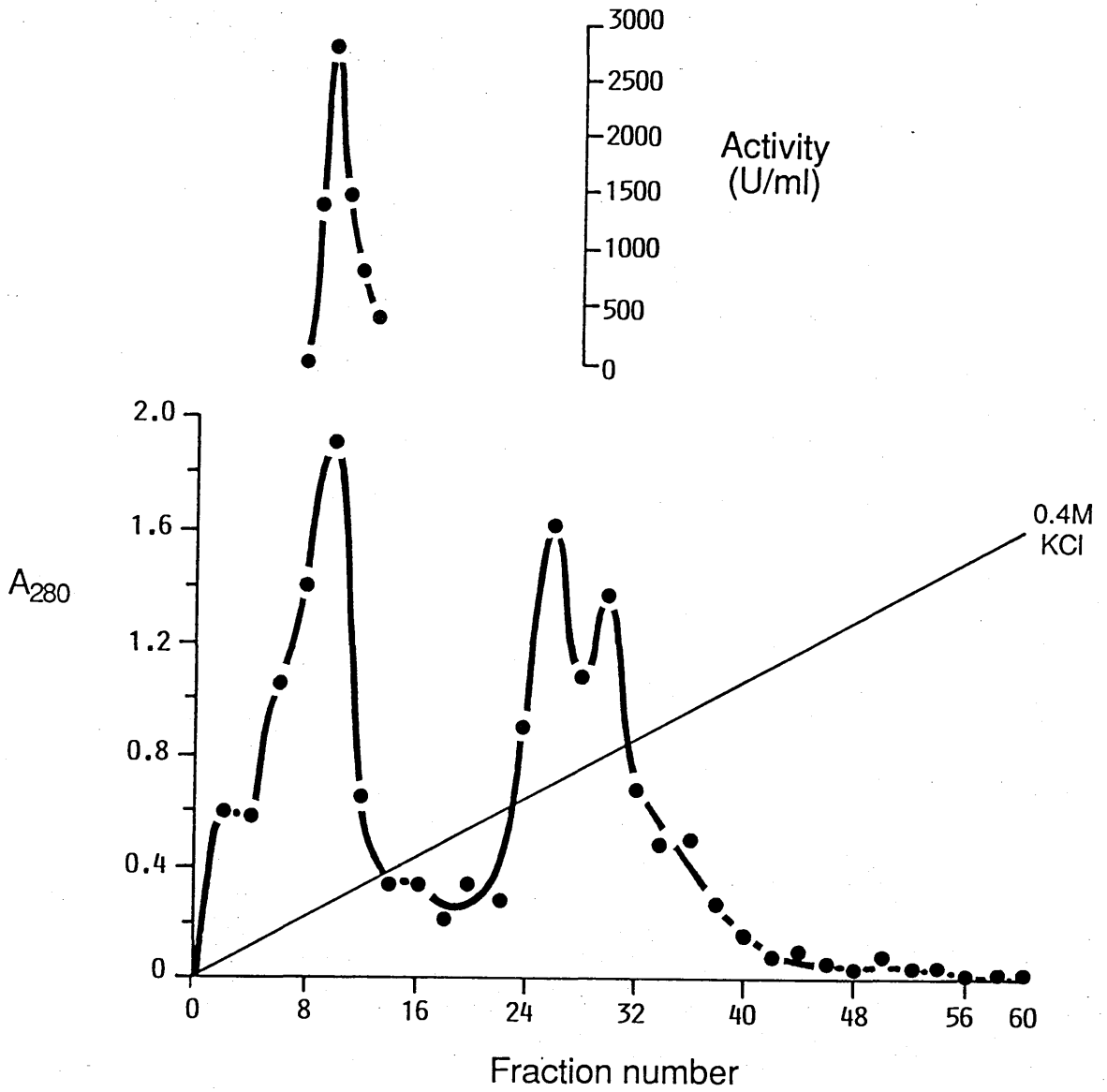


Figure 3.2 The DEAE-Sephacel Elution Profile of DHDPA Synthase from wild-type *E.coli* K12.

The sample from the heat step (38,400 units in 24 mls) was loaded onto a DEAE-Sephacel column (30 x 3 cm) which had been equilibrated with Buffer A. The column was washed with this buffer until the A_{280} of the eluate was below 0.1. The column was eluted in a gradient of 0.1-0.5 M-KCl (900ml), with a flow rate of 1 ml/min and 12 ml fractions collected. Activity was found in fractions 8-11 which corresponded to protein eluted at ≈0.16 M-KCl.. The total activity recovered was 96,000 units which had increased from the loaded sample due to purifying away enzyme inhibitors.

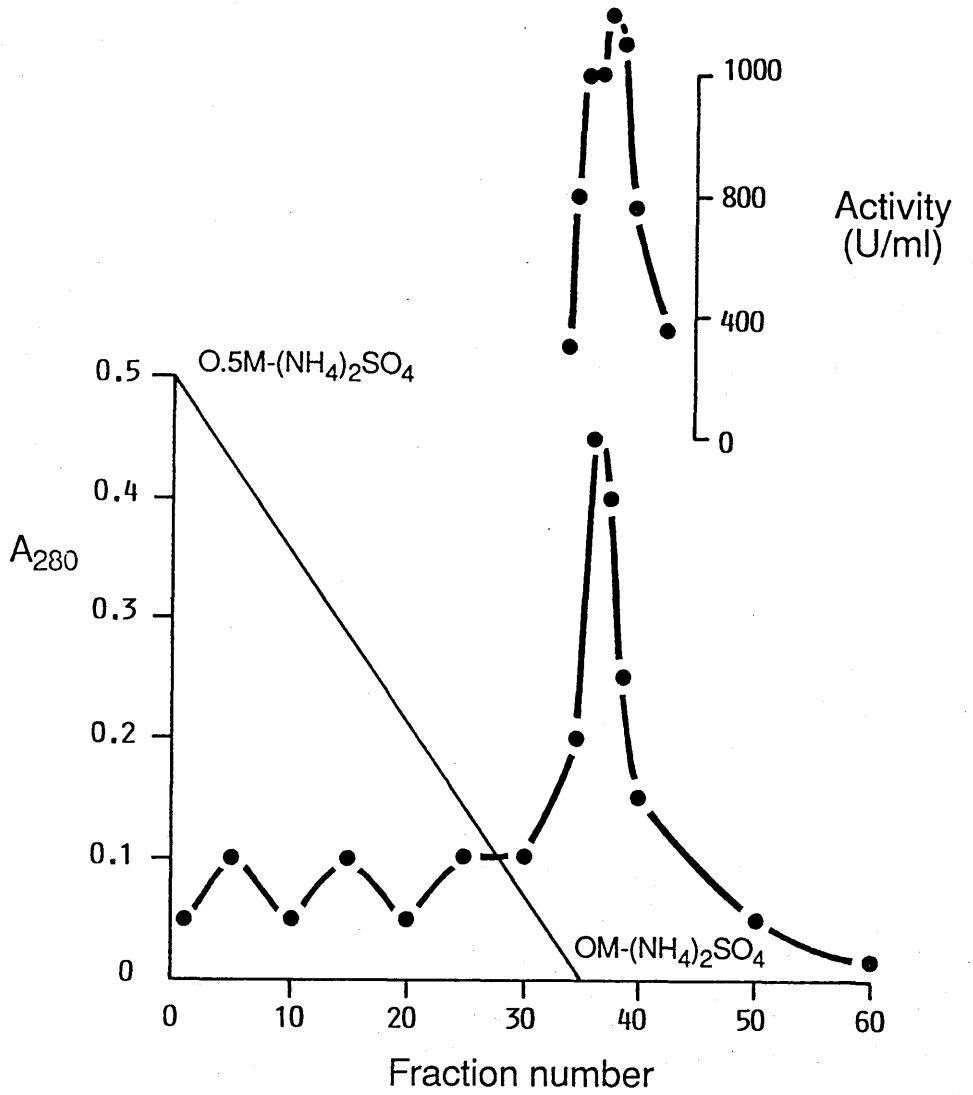


Figure 3.3 The Phenyl-Sepharose Elution Profile of DHDPA Synthase from wild-type *E.coli* K12.

The sample from the DEAE-Sephacel step (96,000 units in 60 mls) was loaded onto a phenyl-Sepharose column (15 x 4 cm) which had been equilibrated with Buffer A plus 0.5 M-$(NH_4)_2SO_4$. The column was washed with this buffer until the A_{280} of the eluate was below 0.1. The column was eluted in linear negative gradient of 0.5-0 M-$(NH_4)_2SO_4$ (500ml), with a flow rate of 1.5 ml/min and 10 ml fractions collected. Activity was found in fractions 34-45 which corresponded to protein eluted at the end of the gradient. The total activity recovered was 45,000 units which is 47 % of the sample loaded.

Sepharose column (15cm x 4cm) which had been pre-equilibrated with Buffer A plus 0.5M-$(NH_4)_2SO_4$. The column was washed at the flow rate of 1.5 ml/min with Buffer A plus 0.5 M-$(NH_4)_2SO_4$ until the A_{280} was below 0.1.

The protein was eluted with a decreasing linear gradient (500 mls) of 0.5-0 M-$(NH_4)_2SO_4$ in Buffer A at 1.5 ml/min and 10 ml fractions were collected.

The fractions with the highest activity (the elution profile is shown in Figure 3.3) were pooled and dialysed against Buffer A.

Step 5. Chromatography on Mono Q

This step was carried out at room temperature using a Pharmacia fast protein liquid chromatography (f.p.l.c.) system. The dialysed enzyme was applied to a MonoQ (1ml, HR 5/5) anion exchange column; the column was eluted with a linear gradient of 0.1-0.5 M-KCl in Buffer A. The flow rate was 1 ml/min and 1 ml fractions were collected: however no activity was eluted. The concentration was raised to 1 M-KCl and activity was eluted together with other proteins but no purification was achieved. The fractions with activity were pooled and dialysed again against Buffer A.

This sample was applied again to the Mono Q column which was eluted with a linear gradient of 0.1-1 M-KCl in Buffer A with the above flow rate and fraction size. This did not achieve complete purification and so the fractions were collected and dialysed as before.

Finally the Mono Q step was repeated using a third gradient system consisting of a linear gradient of 0.1-0.5 M-KCl for 10 min followed by 0.5-1 M-KCl for 80 min.

The fractions with activity from this final Mono Q step were pooled (profile illustrated in Figure 3.4) and dialysed as before. This sample was analysed on a Phastgel (see Chapter 2) and since other protein bands were observed it was clearly not homogeneous (Figure 3.5). Further purification was required to acheive homogeneity.

Step 6. Chromatography on Superose 6.

This step was carried out at room temperature using a Pharmicia f.p.l.c. system. The gel filtration Superose 6 column was pre-calibrated using known molecular weight

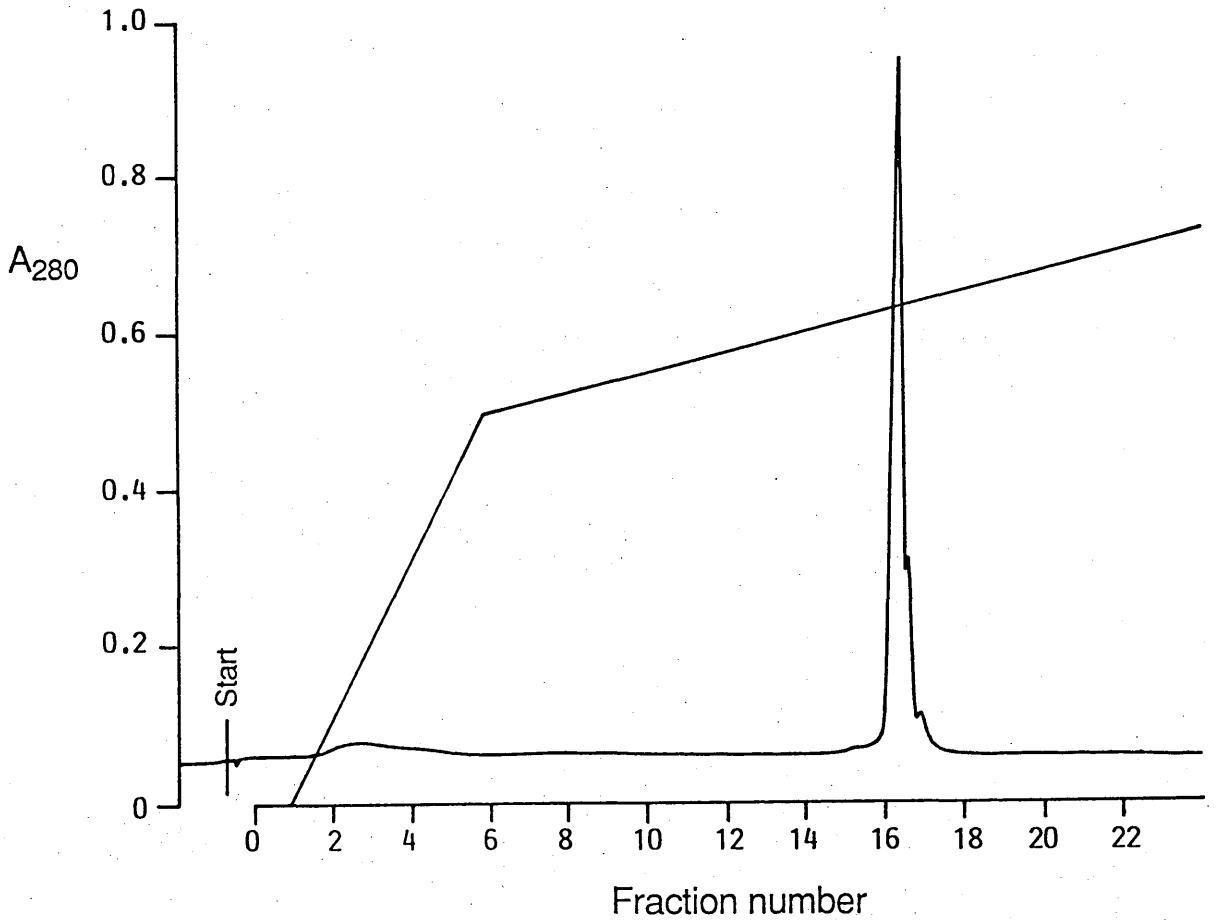


Figure 3.4 The Mono Q Elution Profile of DHDPA Synthase from Wild-Type *E.coli* K12.

The sample from previous Mono Q runs (19,000 units in 2 ml) was loaded onto a 1 ml Mono Q column which had been equilibrated with Buffer A. The column was eluted in a linear two phase gradient of KCl (0.1-0.5 M) in 10 min, followed by 0.5-1 M-KCl for 80 min, with a flow rate of 1 ml/min and 1 ml fractions collected. The protein was eluted in a single peak with a couple of shoulders at ≈0.65 M-KCl. The fractions 16-17 showed most activity and these were pooled and dialysed against Buffer A overnight. The total activity recovered was 15000 units which is 78% of the loaded sample but some was lost during the previous repeated Mono Q columns.

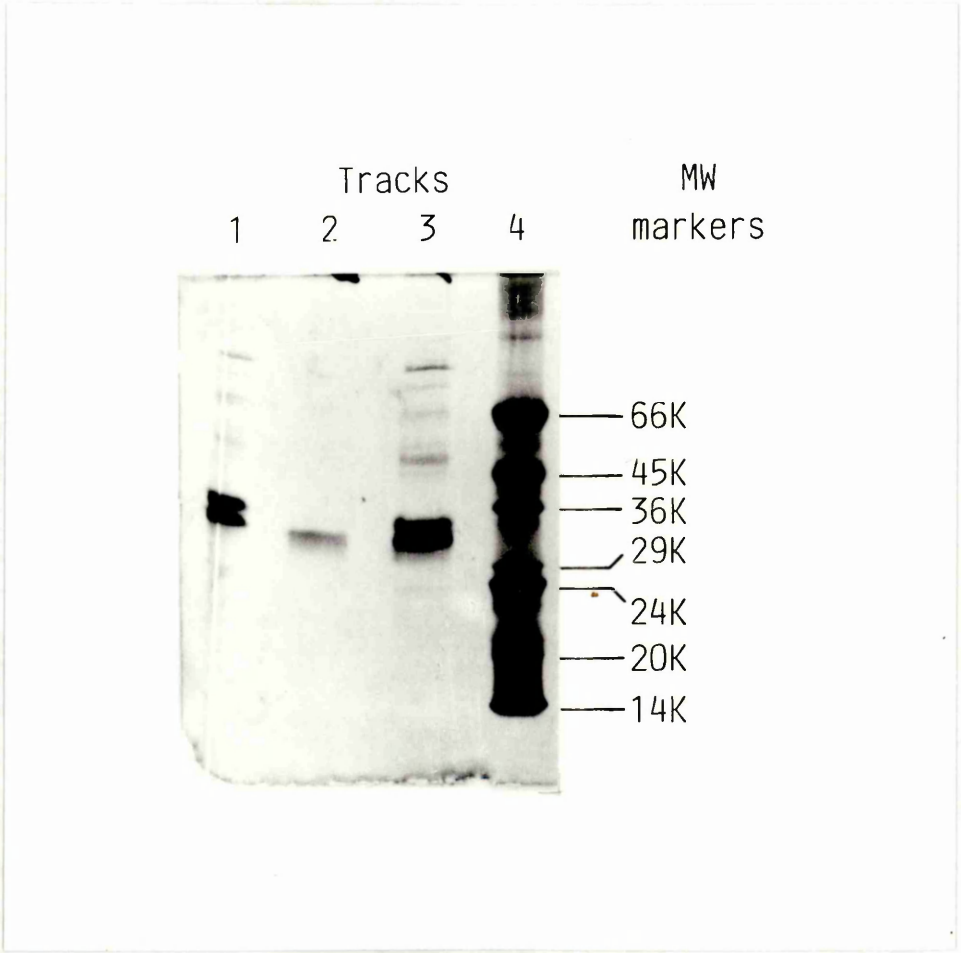


Figure 3.5 A 12.5% SDS Phastgel of the Last Steps of DHDPA Synthase Purification from wild-type *E.coli* K12.

The purification is summarised in Table 3.1 and a sample from the Mono Q and Superose 6 steps were loaded onto a 12.5% SDS Phastgel and stained with silver stain (see Chapter 2). The tracks contain the following amounts of protein estimated by Bradford method (Bradford, 1970):- 1. & 3. 0.3 ng Mono Q step and 2. 0.6 ng Superose 6. Track 4 has molecular weight markers as shown (see Chapter 2).

markers in Buffer A and eluted at 1 ml/min (see Chapter 2). After calibration the dialysed enzyme from the Mono Q step was applied to the Superose 6 column and was eluted in 16.3 mls (elution profile shown in Figure 3.6) which coincided with the elution volume of lactate dehydrogenase, M_r 140,000. This established the M_r of DHDPA at about 140,000 in agreement with previous results of Laber (1987).

The enzyme was dialysed against Buffer A, overnight, and then dialysed against the same buffer containing 50% (v/v) glycerol. Once in this buffer it could be stored at -20°C for several months.

3.3 Summary of wild-type Purification

A SDS phastgel showed a single band (silver staining for protein) after the gel fitration step which can be compared to the Mono Q step in which several bands were visible (Figure 3.5). The position of the band present on the SDS Phastgel corresponded to a subunit M_r of 33,000. This together with the native M_r of 140,000 established by gel filtration indicates that the enzyme is a tetramer.

Table 3.1 summaries a typical purification of DHDP synthase from wild-type *E.coli* K12 cells; 39 µg of protein were obtained from 18 g of cells with a purification factor of 9500-fold.

3.4 Transformation of *E.coli* cells by pDA2

A plasmid (Figure 3.1) containing the *dapA* gene which encodes DHDPA synthase was given to us by Dr Stragier. It had been constructed in the following way:- a 1.2 kb Bst N1 fragment containing the *dapA* gene was subcloned into the *Hinc*II site of a pUC9 plasmid. This fragment was present in a previously constructed plasmid (pDA1), which was digested by endonuclease *Bst* NI and the cohesive ends were filled in by DNA polymerase and the restriction fragment ligated into *Hinc*II digested pUC9 (Richaud *et al.*,1986).

The plasmid,pDA2, was transformed into *E.coli* MV1190 Rec A^- cells by the

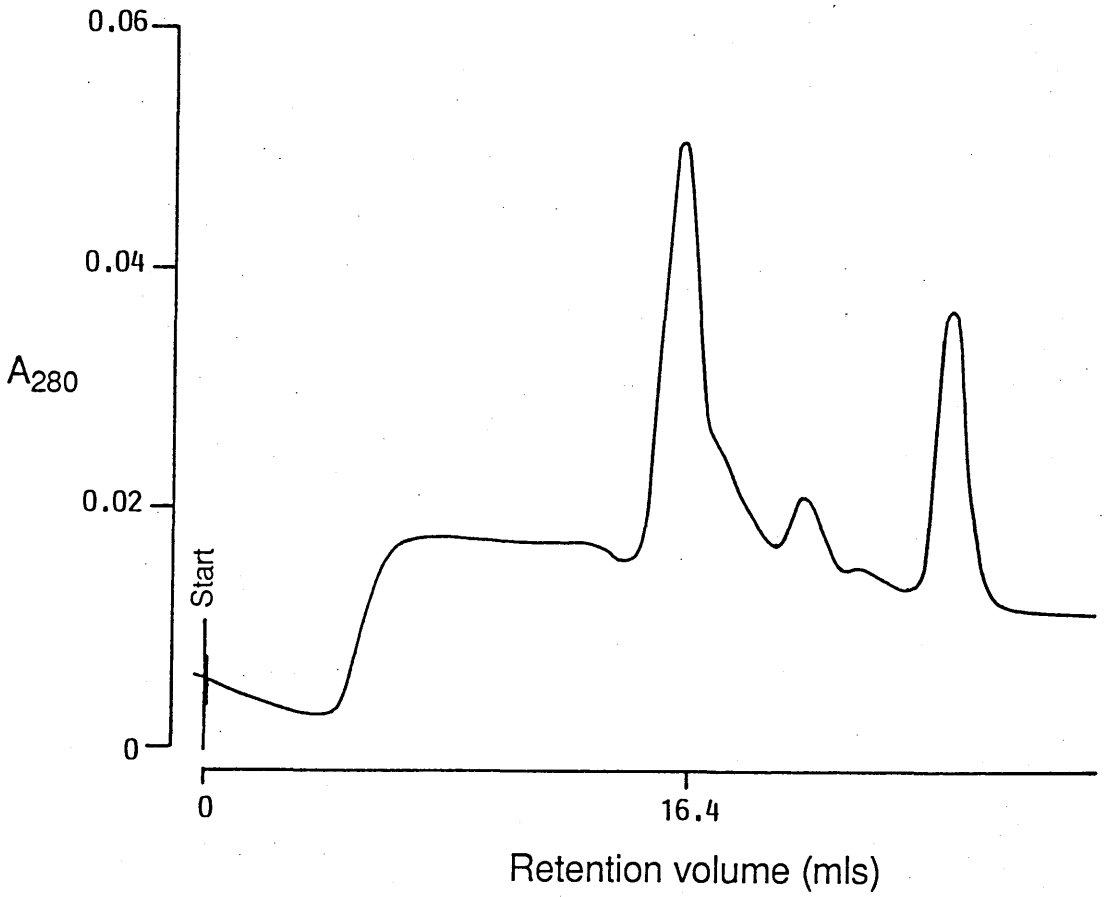


Figure 3.6 The Superose 6 Elution Profile of DHDPA Synthase from Wild-Type *E.coli* K12.

A 200 µl aliquot from Mono Q column (3,000 units) was loaded onto Superose 6 column which had been equilibrated with Buffer A and pre-calibrated as described in Chapter 2. The protein was eluted with washing with Buffer A The retention time of 16.4 min for a protein peak corresponded to the DHDPA synthase activity. This was repeated with the rest of the Mono Q sample and the active fractions pooled and shown to be pure on the phastgel shown in Figure 3.5.

Step	Vol. (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Activity (U/ml)	Activity Total	Sp.A (U/mg)	Yield (%)	P.F.
Crude extract	35	50	1750	800	28000	16	100	1
Heat step	24	19.25	474	1600	38400	81	137	5.1
DEAE-Sephacel	60	1.08	65	1600	96000	1481	342	92.5
Phenyl-Sepharose	50	0.05	2.5	900	45000	18000	160	1125
Mono Q	1	0.45	0.45	15000	15000	33333	53	2083
Superose 6	0.8	0.047	0.038	7166	5733	151000	20	9500

Table 3.1 The Purification of DHDPA synthase from 18 g (wet wt.) wild-type *E.coli* K12

Note: The units (Us) were calculated using the 270 nm assay. One unit of activity is defined as the amount of enzyme catalyzing an absorbance increase of 0.001/min (Yugari & Gilvarg, 1965). Protein was measured by the Bradford method (Bradford, 1970). SpA. is specific activity and P.F. is purification factor.

$CaCl_2$ method as described in Chapter 2. After the transformation, mini plasmid preparations were made and restriction enzyme digests (see Chapter 2 for methods) carried out to confirm the identity of the plasmid and insert.

Digestion with the enzyme *Pst*I linearised the plasmid to give a 3.8 kbp band (Track 2, Figure 3.7). Double digestion with *Pst*I and *Bam*HI resulted in fragments of 2.6 kbp and 1.3 kbp (Track 3). These fragments confirm the presence of the 1.2 kbp insert containing the *dapA* gene in the pUC 9 plasmid.

3.5 Purification of DHDPA Synthase From *E.coli* MV1190/pDA2

The *E.coli* MV1190/pDA2 cells were grown in MM63 medium with Amp, for plasmid selection. Growth occured in a similar manner to wild-type *E.coli*, see Chapter 2 for growth conditions.

As with the purification from the wild-type cells all steps were carried out at $4^{o}C$ except those involving f.p.l.c. chromatography, which were carried out at room temperature.

Step 1: Breakage of *E.coli* cells

24 g (wet weight) of cells were resuspended in 50 ml of 20 mM-Tris HCl pH 7.4, 10 mM-EDTA, 0.4 mM-DTT and 100 mM-KCl (Buffer A) with an addition 10 mM-sodium pyruvate.

Cells were broken by passage through the French press three times as described in Chapter 2. The resulting suspension was then centrifuged at 28,000 g for 30 min and the pellet was discarded.

Step 2. Heat Step and Step 3. DEAE-Sephacel Chomatography

These steps were carried out as described for the wild type *E.coli*; the elution profile for the DEAE-Sephacel (same size of column as before) step is illustrated in Figure 3.8.

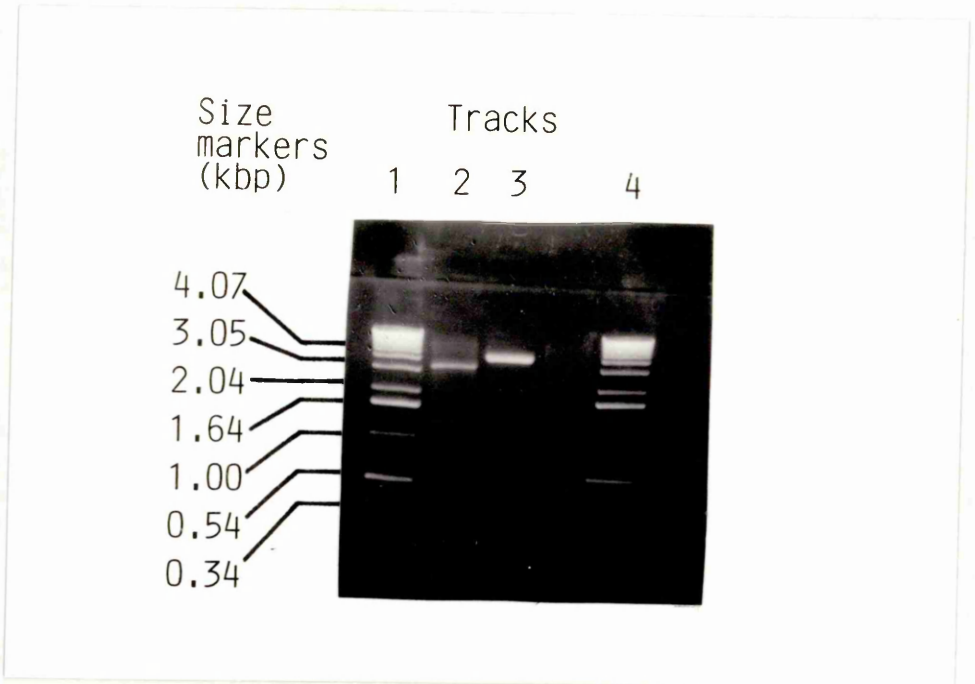


Figure 3.7 The Restriction Digestion of pDA2

The plasmid, pDA2, is derived from a pUC9 plasmid as described in Section 3.4 and is illustrated in Figure 3.1. The plasmid was transformed into *E.coli* MV1190 *RecA*$^-$ cells and mini plasmid preparations were made. To confirm the identity of the plasmid, restriction digests were carried out and the result run on a 1% agarose gel with EtBr (see Chapter 2 for details on method). A digest with *Pst1* yielded a 3.8 kbp fragment size (Track 3) and a double digest with *Pst1* and *BamH1* gave fragments of sizes 2.6 and 1.3 kbp (Track 2). The size markers (Tracks 1 & 4) are a 1 kbp ladder obtained from Gibco.

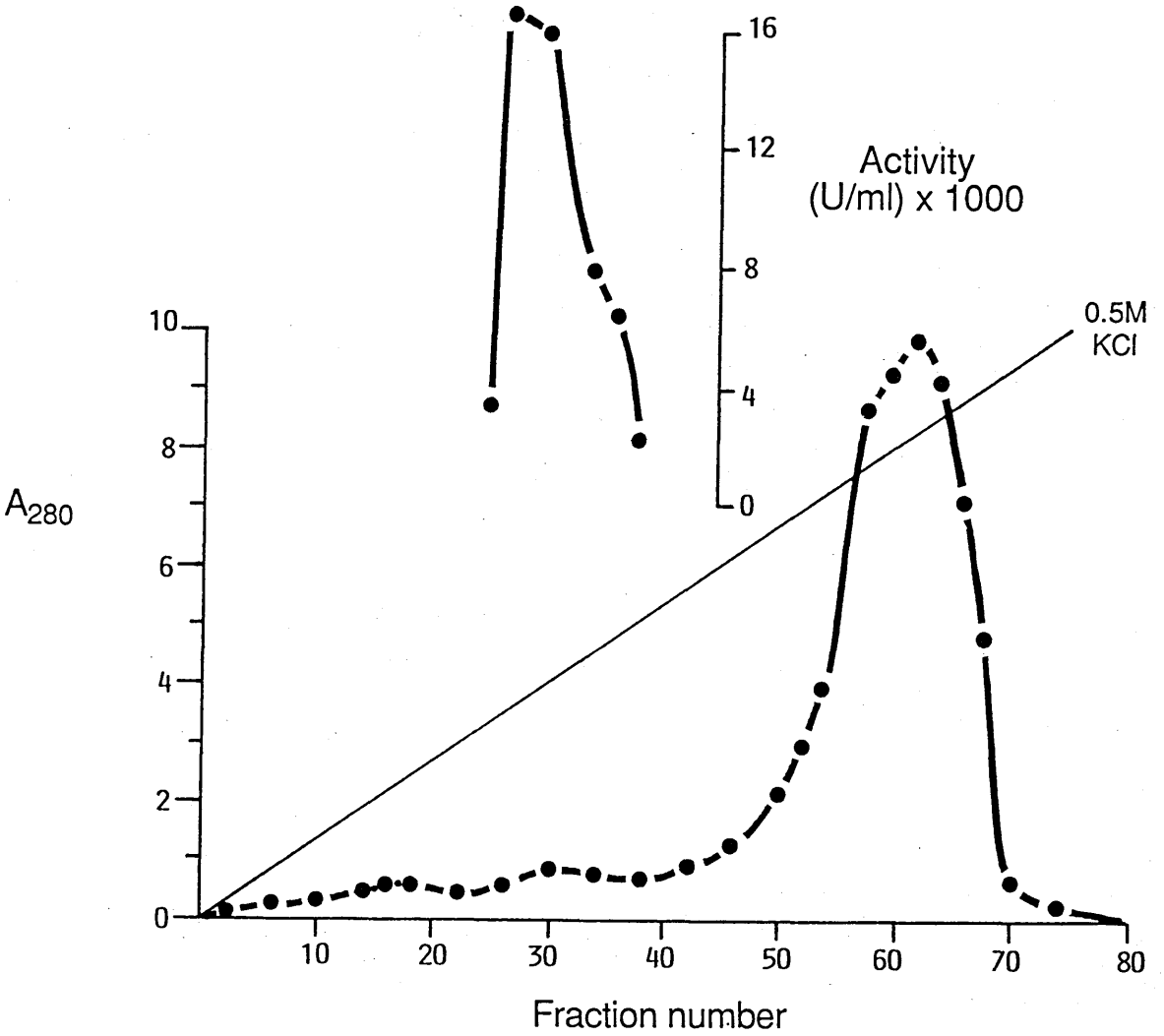


Figure 3.8 The DEAE-Sephacel Elution Profile of DHDPA Synthase from *E.coli* MV1190/pDA2.

The sample from the heat step (3,750,000 units in 30 mls) was loaded onto a DEAE-Sephacel column (30 x 3 cm) which had been equilibrated with Buffer A. The column was washed with this buffer until the A_{280} of the eluate was below 0.1. The column was eluted in a gradient of 0.1-0.5 M-KCl (900ml), with a flow rate of 1 ml/min and 12 ml fractions collected. Activity was found in fractions 25-37 which corresponded to protein eluted at ≈0.3 M-KCl. The total activity recovered was 10,837,000 units which had increased from the loaded sample due to purifying away inhibitors of the enzyme.

Step 4. Phenyl-Sepharose Chromatography

The phenyl-Sepharose step was carried out in a similar manner to the wild-type enzyme purification (same size of column was used) except that 14 ml fractions were collected and the fractions containing the highest activity had to be diluted up to 1/100 fold to obtain readable assay rates. The elution profile is illustrated in Figure 3.9 and fractions containing activity were pooled and dialysed against Buffer A.

Step 5: Chromatography on Mono Q

The dialysed enzyme was applied to a large Mono Q anion exchange column (10 ml,size HR 10/10), because more than 50 mgs of protein were present in the sample. The activity was eluted by a two phase gradient of 0.1-0.5-M-KCl in 20 min followed by 0.5-1 M-KCl in 40 min; the flow rate was 3 ml/min and 1 ml fractions were collected. The elution profile of the Mono Q column is shown in Figure 3.10.

The fractions with the highest activity were pooled and intially dialysed against Buffer A overnight, and then again with this buffer containing 50% (v/v) glycerol for a second night. Upon storage at -20^{o}C the activity of the enzyme is maintained at 100% for six months in this buffer; during subsequent storage the activity decreased slowly.

3.6 Summary of the Purification

The purification of DHDPA synthase from *E.coli* MV1190/pDA2 is summarised in Table 3.2. The purification were monitored on a 15% SDS gel (Figure 3.11) and the protein appeared to be homogeneous after the phenyl-Sepharose step (Track 4). The Mono Q step was carried out, as a Phastgel (not shown) showed a minor impurity (with a M_r smaller than DHDP synthase) in the material from the phenyl Sepharose column. Figure 3.11 suggests that the Mono Q step is redundant since the enzyme is essentially pure after phenyl-Sepharose chromatography. The Mono Q step does serve to concentrate the protein.

The purification of DHDPA synthase from *E.coli* MV1190/pDA2 requires only three steps after cells breakage, compared to the five steps required for wild-type

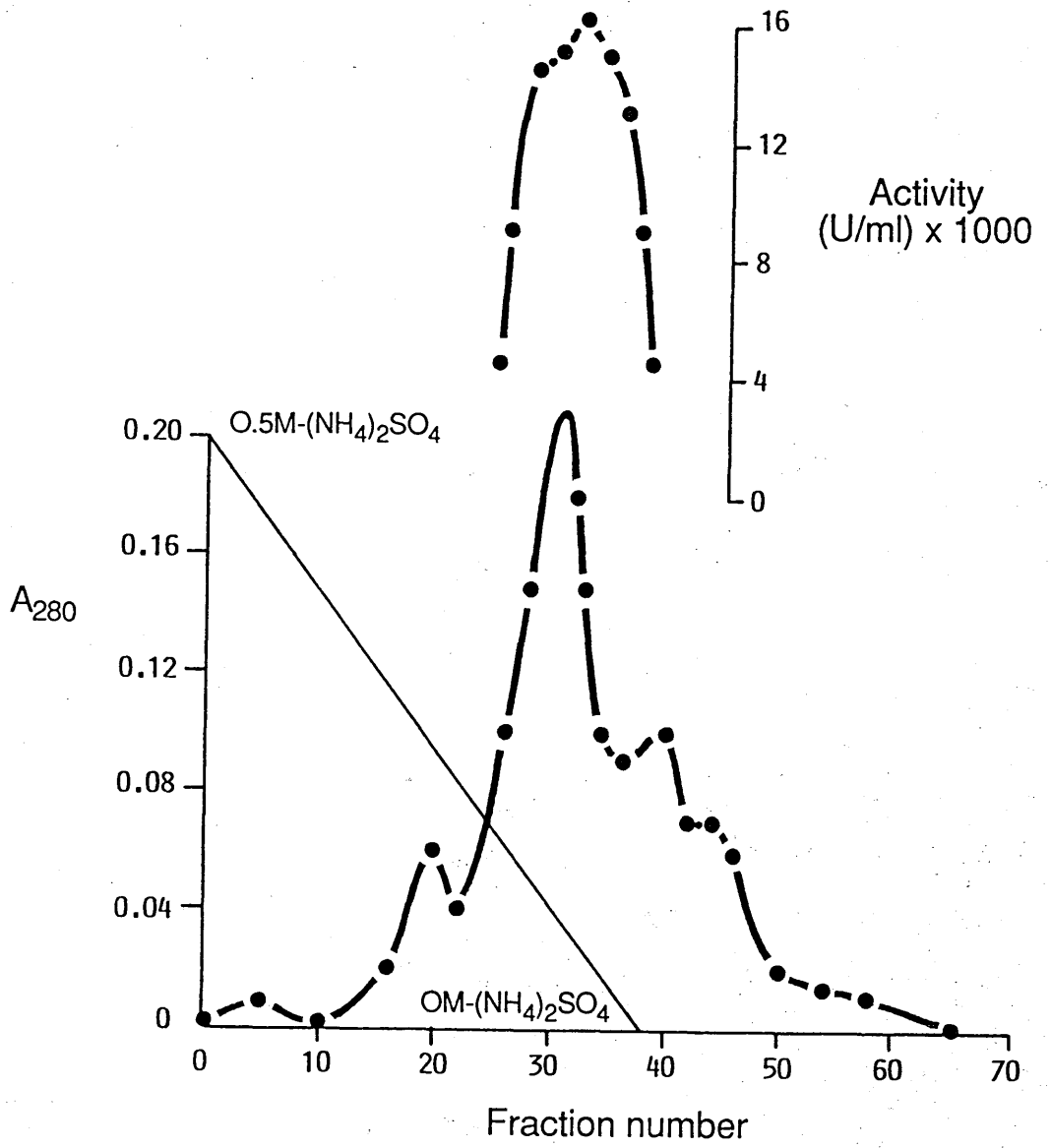


Figure 3.9 The Phenyl Sepharose Elution Profile of DHDPA Synthase from *E.coli* MV1190/pDA2.

The sample from the DEAE-Sephacel step (10,837,000 units in 170 mls) was loaded onto a phenyl-Sepharose column (15 x 4 cm) which had been equilibrated with Buffer A plus 0.5 M-$(NH_4)_2SO_4$. The column was washed with this buffer until the A_{280} of the eluate was below 0.1. The column was eluted in linear negative gradient of 0.5-0 M-$(NH_4)_2SO_4$ (500ml), with a flow rate of 1.5 ml/min and 10 ml fractions were collected. Activity was found in fractions 25-37 which corresponded to protein eluted at ≈0.16 M-$(NH_4)_2SO_4$. The total activity recovered was 8,448,000 units which is 77 % of the sample loaded.

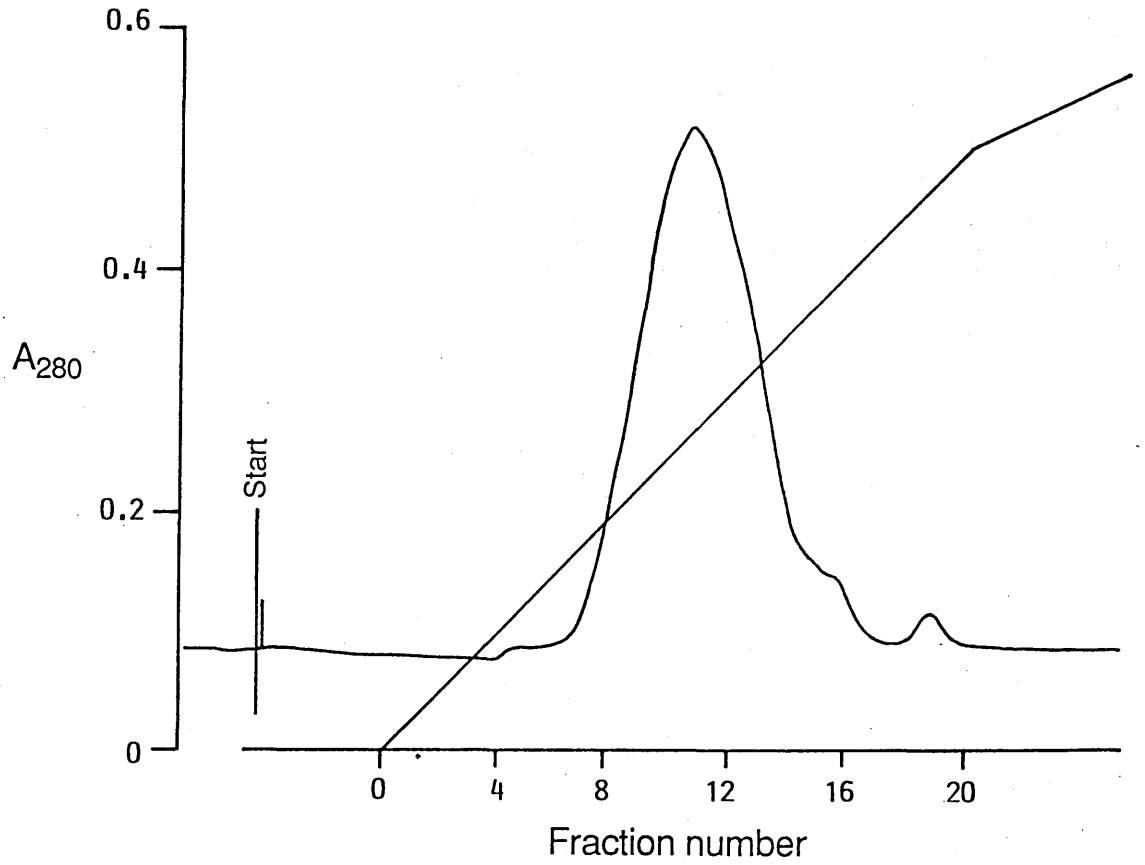


Figure 3.10 The Mono Q Elution Profile of DHDPA Synthase from *E.coli* MV1190/pDA2

The sample from the phenyl-Sepharose column (8,448,000 units in 192 mls) was loaded onto a 10 ml Mono Q column which had been equilibrated with Buffer A. The column was eluted in a two phase linear gradient of KCl (0.1-0.5 M) in 20 min followed by 0.5-1 M-KCl in 40 min, with a flow rate of 3 ml/min and 3 ml fractions were collected. A mass of protein was eluted at ≈0.25 M-KCl, and most of the DHDPA synthase activity was found in fractions 6-17. However this protein was shown to be pure on a SDS polyacrlamide gel, see Figure 3.11. The total activity recovered was 7,262,0000 units which is 86% of the loaded sample

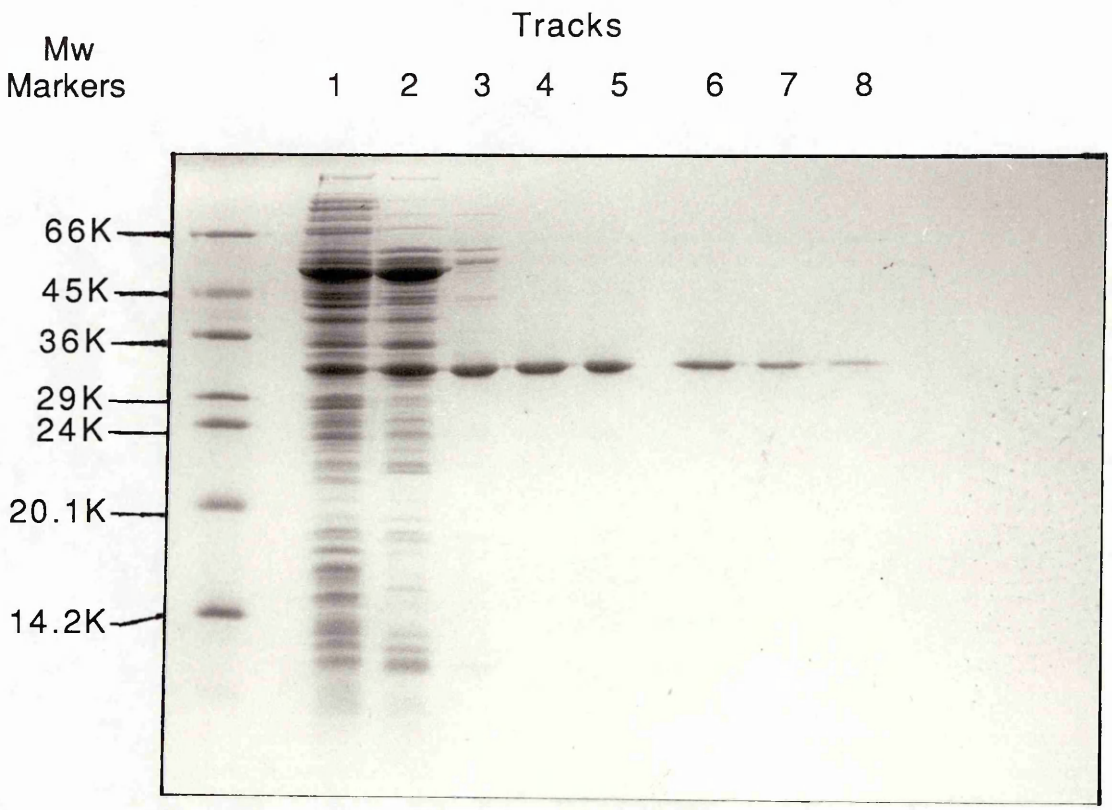


Figure 3.11 15% SDS PAGE Gel of DHDPA Synthase Purification from *E.coli* MV1190/pDA2

The purification is summarised in Table 3.2 and a sample from each step was loaded onto a 15 % SDS polyacryamide gel and stained with Coomassie Blue (see Chapter 2). The tracks contain the following amounts of protein, estimated by the Bradford method (Bradford,1970):- 1: 50 µg crude extract, 2: 50 µg Heat treated sample, 3: 16 µg DEAE Sephacel sample, 4: 10 µg phenyl-Sepharose sample, 5: 10 µg Mono Q sample, 6: 5 µg Mono Q sample, 7: 2.5 µg Mono Q sample, and 8: 1.25 µg Mono Q sample.

Step	Vol. (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Activity (U/ml)	Activity Total (X1000)	Sp.A (U/mg)	Yield	P.F.
Crude extract	37.5	31.25	1172	132	4969	4240	100	1
Heat step	30.0	10.0	300	125	3750	12500	75	3
DEAE-Sephacel	170	0.43	72.3	63.8	10837	149896	218	35
Ph.-Sepharose	192	0.25	48.0	44.0	8448	176000	170	42
Mono Q	35.0	1.00	35.0	207.5	7262	207486	146	49

Table 3.2 The Purification of DHDPA synthase from 24 g (wet wt.) overproducing Strain *E.coli* MV1190/pDA2

Note: The units (Us) were calculated using the 270 nm assay. One unit of activity is defined as the amount of enzyme catalyzing an absorbance increase of 0.001/min (Yugari & Gilvarg, 1965). Protein was measured by the Bradford method (Bradford, 1970).

SpA. is specific activity, P.F. is purification factor and Ph. is short for phenyl.

E.coli cells. The subunit M_r of 33,000 agrees exactly with that found for the enzyme from wild-type cells.

3.7 Test Screening of Compounds For Inhibition of DHDPA Synthase

DHDPA synthase (from the overexpressed strain) was used to screen various potential inhibitors synthesised by L.Couper and D.Tudor. The 270 nm assay was modified to use 1 mM-ASA, 1 mM-pyruvate and 20 units DHDPA synthase and over 70 compounds were screened at four concentrations: 10, 1, 0.5, and 0.1 mM.

About 20 of these compounds showed inhibition at 0.5 mM or lower and examples of these are shown in Figure 3.12 (compounds 1-4); these are all product analogues.

Some pyruvate analogues were also tested for inhibition in a similar manner. A couple of examples are shown in Figure 3.12 (5,6) and these could mimic an intermediate of the enzyme reaction.

3.8 Discussion

The comparison of the purification of DHDPA synthase from the two strains is best illustrated by Tables 3.1 and 3.2. The specific activity of the crude extract from the overproducing strain is approximately 200 fold higher than the wild-type *E.coli* extract.

The overexpression in the strain containing the plasmid pDA2 is caused by the high copy number (200), of the plasmid. In the plasmid the expression of the *dapA* gene is controlled by its own promoter.

35 mgs of homogeneous enzyme were obtained from 24 g of cells compared to 39 µg obtained from 18 g of wild-type cells. This corresponds, in practical terms to over 600 fold overexpression. This figure may be exaggerated due to the easier assay of larger amounts of activity and the quicker purification method.

It had been shown in previous purifications of *E.coli* DHDPA synthase that the enzyme remains most stable when 100 mM-KCl is included in the buffer (Shedlarski &

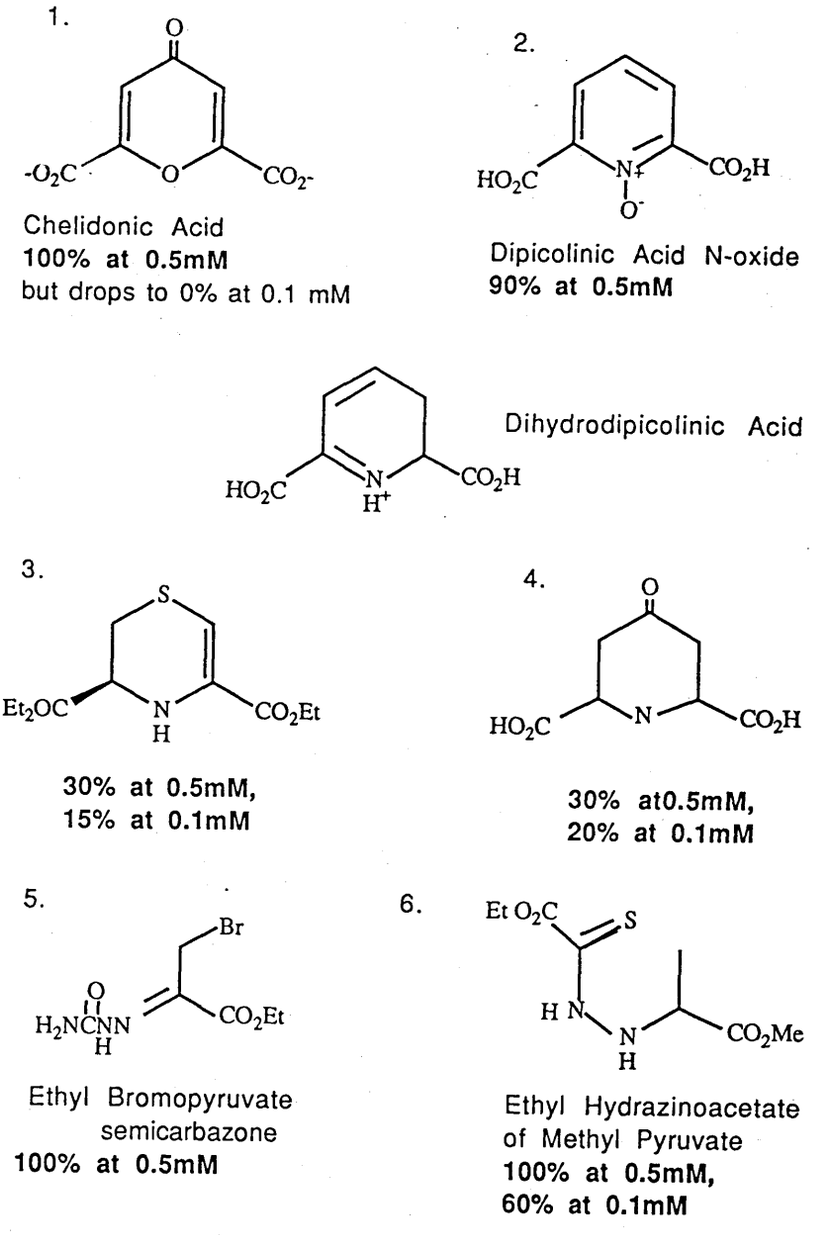


Figure 3.12 Examples of Compounds Tested for DHDPA Synthase Inhibition.

The compounds 1-4 are DHDPA analogues and 5 & 6 are pyruvate analogues. Chelidonic acid (1) is a commercially available compound and inhibits as does the N-oxide of the aromatic dipicolinic acid (2). The sulphur analogue with diester (3) shows slightly less inhibition at 0.5mM but this maintained at 0.1 mM as does the fourth example.

The pyruvate analogues mimic possible intermediates in the reaction between ASA and pyruvate.

Gilvarg, 1970). The reason for this is not known.

The enzyme can withstand high temperatures as demonstrated by the heat step in the purification. During this step, other non-stable proteins such as dehydrogenases and oxidases are denatured and precipitated out. There is a 75% recovery of the DHDPA synthase during this step from the over-expressing strain but a higher recovery from the wild-type strain. This may be due to purifying away inhibitors of the enzyme or components which interfered with the assay for activity.

Ion-exchange chromatography on DEAE-Sephacel was used as the first purification step. The enzyme was eluted with a KCl gradient, wild-type emerging at 0.14 M-KCl and the overexpressed enzyme at 0.25 M.

The second chromatographic step was a hydrophobic column (phenyl-Sepharose). The majority of activity eluted with the main protein peak during the negative gradient elution proceedure employed. Evidence from the SDS gel (Figure 3.10) for the enzyme from the overexpressed strain showed that it was homogeneous after this step (track 4). However the SDS phastgel from the phenyl-Sepharose stage of the wild-type enzyme purification still showed several bands to be present.

The Mono Q step was essential for the purification of wild-type enzyme and required optimisation but still did not give homogeneous enzyme (Figure 3.5). This required a final gel fitration step. The Mono Q step for the enzyme from the overexpressed strain was not required for purification since the enzyme from the phenyl-Sepharose step was homogeneous as judged by SDS PAGE. It was useful for concentrating the product. The enzyme was eluted from the Mono Q column at 0.25 M-KCl, which is much earlier than the wild-type enzyme (eluted at 0.65 M-KCl) perhaps due to the quantity of protein present.

Previous gel filtration results had estimated the size of DHDPA synthase to be 132-134,000 (Shedlarski and Gilvarg,1970) and therefore the final step to purify the enzyme from wild-type was a Superose 6 column on the f.p.l.c. The protein eluted with a similar elution volume to lactate dehydrogenase (140 kDa) and was shown to have a single band on a SDS phastgel of M_r 33,000. This corresponds to the band for the homogeneous protein from the overexpressing strain. The enzyme is clearly

tetrameric.

The mechanism of DHDPA synthase in *E.coli* has not been explored in great detail The binding of pyruvate has been shown to be via a Schiff's base reaction at the enzymes active site (Shedlarski & Gilvarg, 1970). Kinetic data suggests that the enzyme works by a ping-pong mechanism (Kumpaisal et al.,1987) in which a form of the enzyme containing activated pyruvate is formed first and then reacts with ASA. Two possible mechanisms of this reaction are shown in Figure 3.13 (Shedlarski & Gilvarg 1970). The precise nature of the later intermediates of the reaction are unknown. The use of DHDPA and pyruvate analogues (see Figure 3.12) will hopefully help to elucidate the second part of the enzyme's mechanism when these are more closely examined, for the types of inhibition that occur.

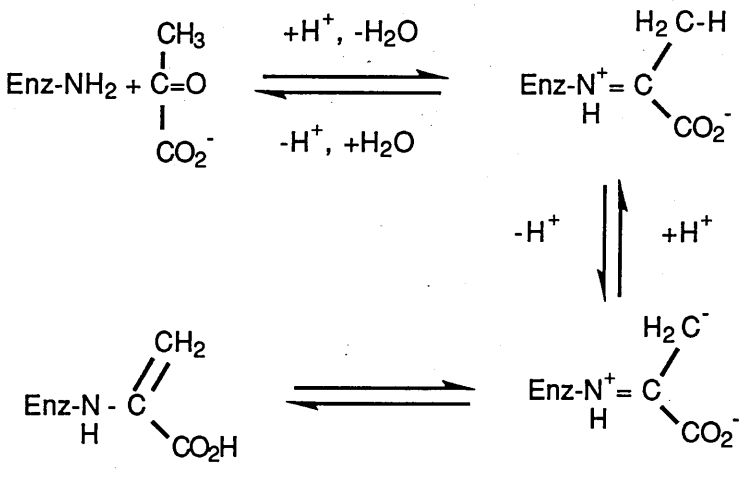
Figure 3.13 Proposed Mechanisms the Active Site for DHDPA Synthase

Part 1 denotes the pyruvate activation. This is by the formation of a Schiff base bond between the carbonyl group of pyruvate and an amino group of a lysine residue at the active site of DHDPA synthase. During this reaction water is released.

Part 2 illustrates two alternative mechanisms for the condensation of the activated pyruvate and ASA. The first mechanism (A) involves the nucleophilic attack by the carbanion (of the pyruvate) onto the carbonyl carbon of ASA. After this another nucleophilic attack by the amino group onto the highly reactive carbon atom of pyruvate causes a transimination thereby releasing the product (3) from the enzyme.

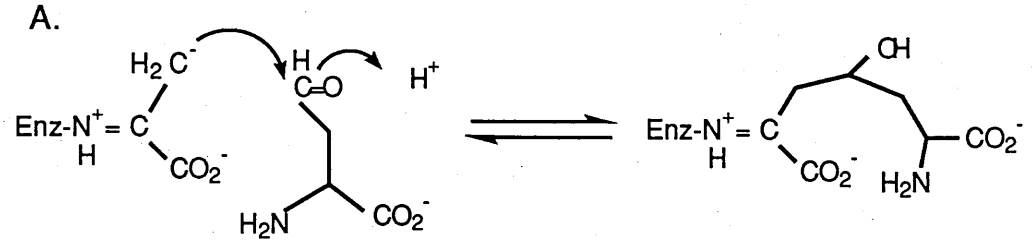
An alternative route (B) is the displacement of the amino nitrogen of the lysine (on the enzyme) by the amino nitrogen of the ASA which would produce an enzyme bound conjugate of the two substrates. The generation of a carbanion followed by condensation would cause ring closure to product (3).

1.

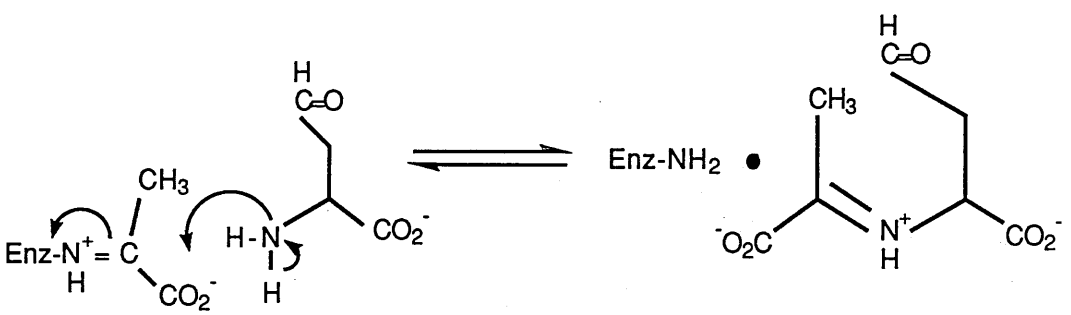

Enz-NH2 + C=O
CH3
CO2-
+H+, -H2O
-H+, +H2O
H2 C-H
Enz-N+= C
H
CO2-
-H+
+H+
CH2
Enz-N - C
H
CO2H
H2 C-
Enz-N+= C
H
CO2-

2.

A.


H2 C-
H
C=O
H+
Enz-N+= C
H
CO2-
H2N
CO2-
OH
Enz-N+= C
H
CO2-
CO2-
H2N

B.


H
C=O
CH3
Enz-N+= C
H
CO2-
H-N
H
CO2-
Enz-NH2
H
C=O
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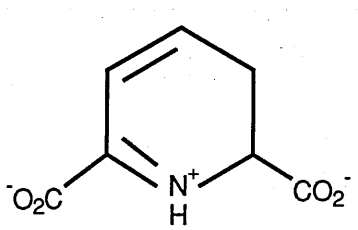
-O2C
N+
H
CO2-

3.


-O2C
N+
H
CO2-

Chapter 4 : The Investigation of the 270 nm Absorbing Product of the DHDPA Synthase Reaction

4.1 Introduction

The first reported partial purification of DHDPA synthase (E.C. 4.2.1.52) from *E.coli* was by Yugari & Gilvarg in 1965 and later Shedlarski & Gilvarg (1970) described the preparation of homogeneous enzyme. A modified and improved purification of DHDPA synthase was described in the previous chapter.

The reaction catalysed by the enzyme is the condensation of aspartate semialdehyde (ASA) and pyruvate to form the unstable cyclic compound dihydrodipicolinic acid (DHDPA). The enzyme was initially called the condensing enzyme by Yugari & Gilvarg, but since the product of the reaction was determined, it has been known as DHDPA synthase. An assay to monitor the enzyme's activity was required and the first assays were described by Yugari & Gilvarg (1965).

Initially an assay was set up to monitor the pyruvate usage: this consisted of measuring the change in pyruvate concentration, after incubation with ASA and DHDPA synthase, by using the enyzme lactic dehydrogenase. This was a stopped assay and therefore tedious and of limited accuracy.

When Yugari and Gilvarg became more aware of the reactions following DHDPA synthase they produced a coupled assay with the next enzyme on the lysine biosynthetic pathway, DHDPA reductase (this enzyme is described in Chapter 5). DHDPA reductase reduces the product of DHDPA synthase via NADPH to form tetrahydrodipicolinic acid (THDPA). When excess reductase is present in the assay reaction the rate determining step is the formation of DHDPA, and so the oxidation of NADPH ($\varepsilon = 6{,}200\ M^{-1}cm^{-1}$) can be measured at 340 nm. However this assay is not suitable for determinations in crude extracts which contain NADPH oxidases. Another problem is that DHDPA reductase has to be obtained relatively pure and free of other dehydrogenases if reliable measurements are to be made.

Yugari & Gilvarg (1965) speculated that the condensation of ASA and pyruvate

would form a nitrogen containing ring compound. A chemical known to form coloured, usually yellow, adducts with pyrroline and piperidine compounds is o-aminobenzaldhyde and this was tested in an assay with DHDPA synthase and its substrates. A purple adduct was detected at 540 nm and after a lag time of 20-30 min the colour change was found to be proportional to the enzyme concentration. One unit of enzyme activity was defined as the amount giving an increase in absorbance at 540 nm of 0.001/min after maximum velocity is reached (Shedkarski, 1971). A disadvantage of this assay is that the rate of reaction is limited by the solubility of o-aminobenzaldehyde (Shedlarski, 1970). Also the adduct formed in the reaction is unknown (the expected colour for the reaction product with o-aminobenzaldehyde was yellow, but the colour observed was purple).

Finally when investigating the reaction with DHDPA synthase and its substrates it was shown that a product was obtained which absorbed at 270 nm when the assay was carried out in imidazole buffer (Yugari & Gilvarg 1965). It was also claimed that this compound was not formed when using Tris or phosphate buffer although this is contradicted by results described later in this chapter. Once a linear rate (there was a slight lag depending on the amount of enzyme present) was obtained the rate was directly proportional to the amount of enzyme added. One unit of enzyme activity was defined as the amount which catalyses an increase in absorbance at 270 nm of 0.001/min after maximum rate was reached (Shedlarski, 1970). This is a simple and quick assay and was therefore used to monitor the activity of the enzyme during the purification (see Chapter 3). Since this assay was used routinely for the work in this thesis it was important to determine the nature of the 270 nm absorbing compound.

There have been several investigations of this compond. Yugari & Gilvarg, 1965 suggested that the product was one of the five isomers of dihydrodipicolinic acid. They reached this conclusion by comparing products of hydrogenation. The 270 nm assay product was hydrogenated and compared to the hydrogenation product of THDPA (the product of the next step on the pathway) and found to be identical. To isolate which isomer of DHDPA was used in the pathway, lysine was broken down with amino-acid oxidase to give 3,6-dihydrodipicolinic acid. This compound when treated with o-aminobenzaldehyde gives a similar absorbance to that of the adduct

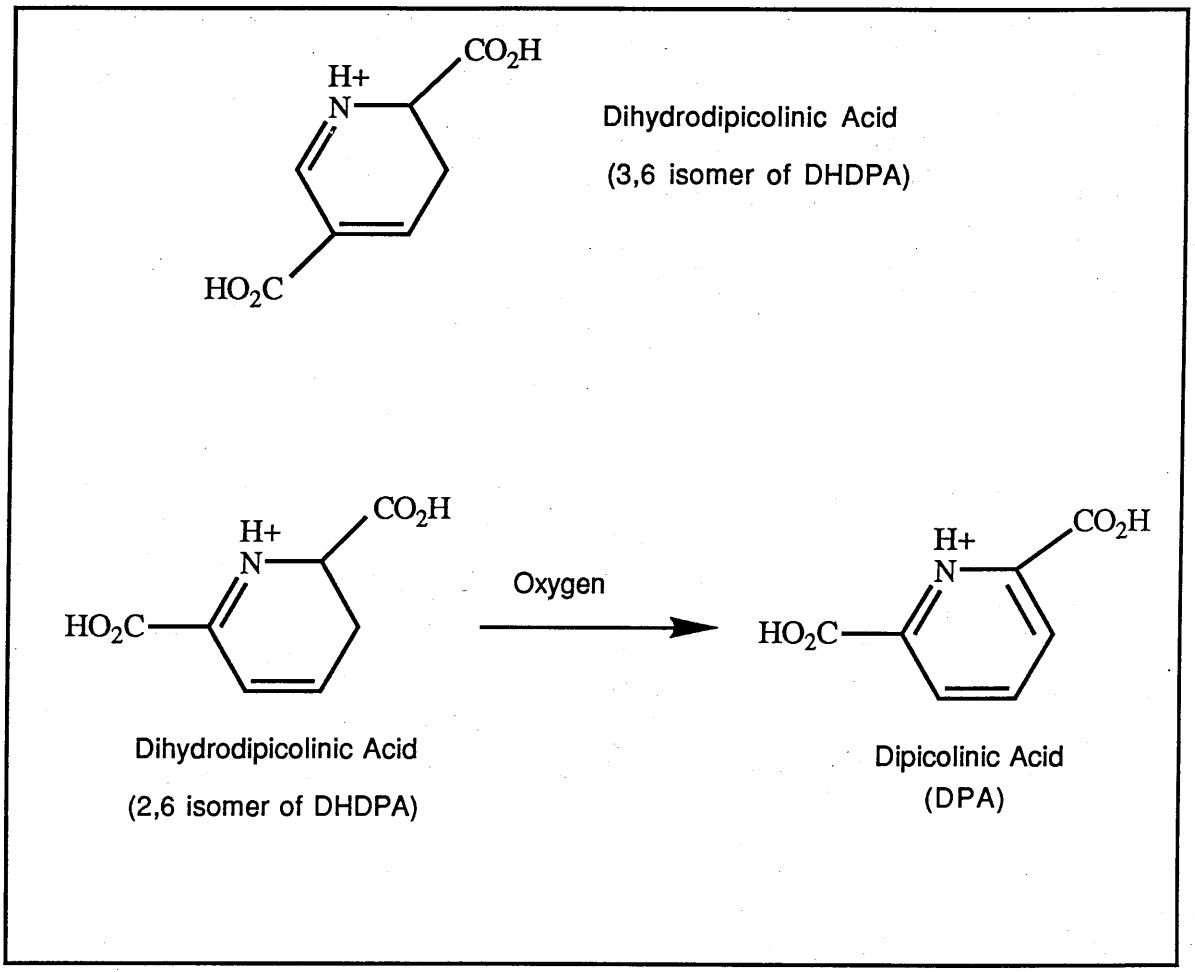


Figure 4.1 The 3,6 isomer of DHDPA and the Spontaneous Oxidation of the 2,6 isomer of DHDPA to DPA

Initially the product of DHDPA synthase reaction was thought to be the 3,6 isomer of DHDPA (Yugari & Gilvarg,1965) (see Section 4.1). However further studies showed it was the 2,6 isomer (Kimura,1974).

The spontaneous oxidation of DHDPA in air has been reported in the Bacillus species of bacteria and this reaction is shown. The reaction forms the basis of the 270 nm assay used to measure the activity of the DHDPA synthase. DPA absorbs with a maximum peak at 270 nm (see Figure 4.2), which suggests that the increasing absorption at 270 nm during the assay is due to the production of DPA.

produced in the o-aminobenzaldehyde assay. Therefore the structure of the product of the reaction was proposed to be 3,6-dihydrodipicolinic acid, see Figure 4.1.

Since then more work on the lysine pathway as been carried out and now the complete pathway in bacteria has been established (Bukhari, 1971). The substrates for each enzyme are known and it follows that the product of DHDPA synthase in nature is the 2,6 isomer of DHDPA (see Figure 4.1). But this compound is very unstable and could not be the 270 nm product which has been shown to remain stable for several days, in solution, at room temperature.

In this chapter it is proposed that the 270 nm absorbing product is the result of the spontaneous oxidation of DHDPA (2,6 isomer) in air to the more stable fuuly aromatic compound, dipicolinic acid (DPA). This oxidation reaction is illustrated in Figure 4.1.

4.2 The substrates of the reaction : Aspartate Semialdehyde and Pyruvate

DHDPA synthase requires two substrates to form the ring of DHDPA, these are pyruvate and aspartate semialdehyde (ASA). Pyruvate is commercially available as a sodium salt but ASA had to be synthesised and therefore calibrated before used in the assay. ASA was prepared by L.Couper and D.Tudor by the ozonolysis of allylglycine in HCl (Black & Wright 1954). The product of the ozonolysis was analysed for ASA using homoserine dehydrogenase. This enzyme converts ASA to homoserine via the oxidation of NADPH. A partial preparation of purified homoserine dehydrogenase was required for this purpose and was obtained using the method of Bachi & Cohen (1969).

4.2.1 The Partial Purification of Homoserine Dehydrogenase From *E.coli* K12

The growth and harvesting conditions for *E.coli* K12 are described in Chapter 2. After breaking the cells all steps were carried out at 4^{o}C. The assay to monitor the

activity of homoserine dehydrogenase during the purification was measured at 25°C and the assay cocktail consisted of:- 500 µl of buffer mix (200 mM-potassium phosphate pH 7.2, 2 M-KCl, 50 mM-EDTA and 10 mM-lysine), 100 µl 2.5 mM-NADPH, 100 µl 1/100 ASA (this was later to be calculated as 0.4 mM and so in later assays 2 mM-ASA was used) and homoserine dehydrogenase extracts made up to 1 ml with H_2O, Assay A (Bachi & Cohen, 1969). The rate was measured at 340 nm, monitoring the decrease in absorbance of NADPH (ε = 6,200 $M^{-1}cm^{-1}$) as it was oxidised.

Step 1. Breakage of *E.coli* Cells

10 g (wet weight) of cells were resuspended in 20 mls (total volume) of 20 mM-potassium phosphate buffer pH 7.4, 150 mM-KCl, 0.4 mM-DTT,10 mM-EDTA, and 1 mM-L-threonine (Buffer A).

Cells were broken by passage through the French press three times as described in Chapter 2. The resulting suspension was then centrifuged at 28,000 **g** for 30 min and the pellet was discarded.

Step 2. $(NH_4)_2SO_4$ Fractionation

The sample was treated with $(NH_4)_2SO_4$ by slow stirring to acheive a final saturation of 25% $(NH_4)_2SO_4$ (113 g/litre). The mixture was stirred for 30 min to allow full precipitation and then the precipitated proteins were removed by centrifugation for 30 min at 28,000 **g**. The supernatant was then adjusted to 40% (60 g/litre) saturation with $(NH_4)_2SO_4$ and centrifuged in the same manner as above. The precipitated protein was resuspended in Buffer A and was dialysed against Buffer A, overnight.

Step 3. DEAE-Sephacel chromatography

The dialysed protein was applied to a DEAE Sephacel column (5 x 2.5 cm) which had been pre-equilibrated with Buffer A. The column was washed with this buffer and the enzyme eluted at 1 ml/min collecting 1 ml fractions. The fractions containing the activity were pooled and dialysed against Buffer A containing 50% (v/v) glycerol

Step	Vol. (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Activity (U/ml)	Activity Tot.	Sp.A (U/mg)	Yield (%)	P.F.
Crude extract	20	19.25	385	1.66	33.2	0.0862	100	1
0-25% $(NH_4)_2SO_4$ (supernatant)	19	13.5	256	1.46	27.8	0.108	83.8	1.26
25-40% $(NH_4)_2SO_4$ (pellet)	5	9.44	47.2	3.61	18.03	0.382	54.3	4.4
DEAE-Sephacel	17.5	0.22	3.85	0.68	11.9	3.10	36	36

Table 4.1 The Partial Purification of Homomserine Dehydrogenase from 10 g (wet wt.) wild-type *E.coli* K12

The units (Us) are calculated from the conversion of NADPH (ε= 6,200 $M^{-1}cm^{-1}$) to NADP (Bachi & Cohen, 1969) and the protein was measured by Bradford method (Bradford, 1970). SpA. is specific activity and P.F. is purification factor.

overnight, and stored at -20°C for several months. The partial purification of homoserine dehydrogenase is summarised in Table 4.1.

4.2.2 The Determination of ASA Concentration

The ASA concentration was measured by determining the amount of NADPH oxidised when it was completely reduced by homoserine dehydrogenase using Assay A, above, with ASA as the substrate. The ASA sample was diluted in the range 1/100-1/800 fold and aliquots tested with 40 munits of the dehydrogenase. The reaction was initiated with the ASA and the absorbance decrease at 340 nm was observed. Once the ASA in the sample was exhausted by the enzyme (the time taken for this varied between 10 and 30 min depending on the ASA concentration) the absorbance remained stable.

The change in concentration of NADPH during the reaction is given by : $\Delta C = \Delta A/6.2$ (6.2 represents the absorbance coefficient for NADPH). The equilibrium strongly favours aspartate semialdehyde reduction at pH 7.2 (Mitoma & Weissbach, 1962) and since 1mole of NADPH reacts with 1mole of ASA the concentration of ASA is equivalent to the change of concentration of NADPH (the final concentration of ASA in the cuvette is very low). The concentration of the stock solution of the ozonolysed ASA can thus be determined. The concentration of ASA in the ozonolysed samples varied between 0.25 and 0.4 M which suggested the samples contained 25-40% ASA. Attempts to purify ASA on ion-exchange and h.p.l.c. columns have failed and so the crude ASA was used in the assay.

Before using the ASA in the DHDPA synthase reaction assay, the HCl in the sample has to be neutralised by adding approximately 10 mM-KOH (final concentration) immediately before use. Neutralised ASA is unstable and degrades in a few hours at 4°C, whereas acidic ASA can be stored in at -20°C for several months and also remains resonably stable at 4°C (for several days).

4.3 The Assay Product

The assay reaction for DHDPA synthase (purified to homogeneity, see Chapter 3) used during the purification of the enzyme consisted (final concentrations) of :-100mM imidazole buffer pH 7.4, 5 mM-pyruvate, 2mM-ASA (neutralised with 10 mM-KOH) and 20-40 units of enzyme (Assay B) incubated and measured at 25°C.

The absorbing product at 270 nm is stable for several hours which strongly suggested that it could not be the unstable DHDPA. It was proposed that another reaction occured which was the spontaneous oxidation of DHDPA to the more stable aromatic compound, dipicolinic acid (DPA) and the rest of this chapter describes the attempted proof of this hypothesis.

The UV spectrum of 0.1 mM-DPA, in a slightly alkali solution, is a shouldered peak with a maximum at ≈270 nm as illustrated in Figure 4.2.

A time course was set up using the 270nm assay in a 1 ml cuvette at 25°C. The maximum rate observed between 5-15 min incubation was 125,000 u/ml. The UVspectra of the assay were taken at time intervals during a 20 min incubation and these are shown in Figure 4.3 and the observed maximum during the time course was at 265nm. The comparison of UV spectra of DPA and the assay was not conclusive. Therefore the separation of the product from the other assay components was necessary.

4.4 The Separation of Assay Product by H.P.L.C.

The method used for the separation of the assay components of Assay B was ion exhange chromatography on an organic acids column. The column used was an Aminex Ion Exclusion HPX-87H column (300 x 7.8 mm), see Chapter 2 for further details. This was operated at 25°C on a Gilson High Pressure Liquid Chromatography (h.p.l.c.) system.

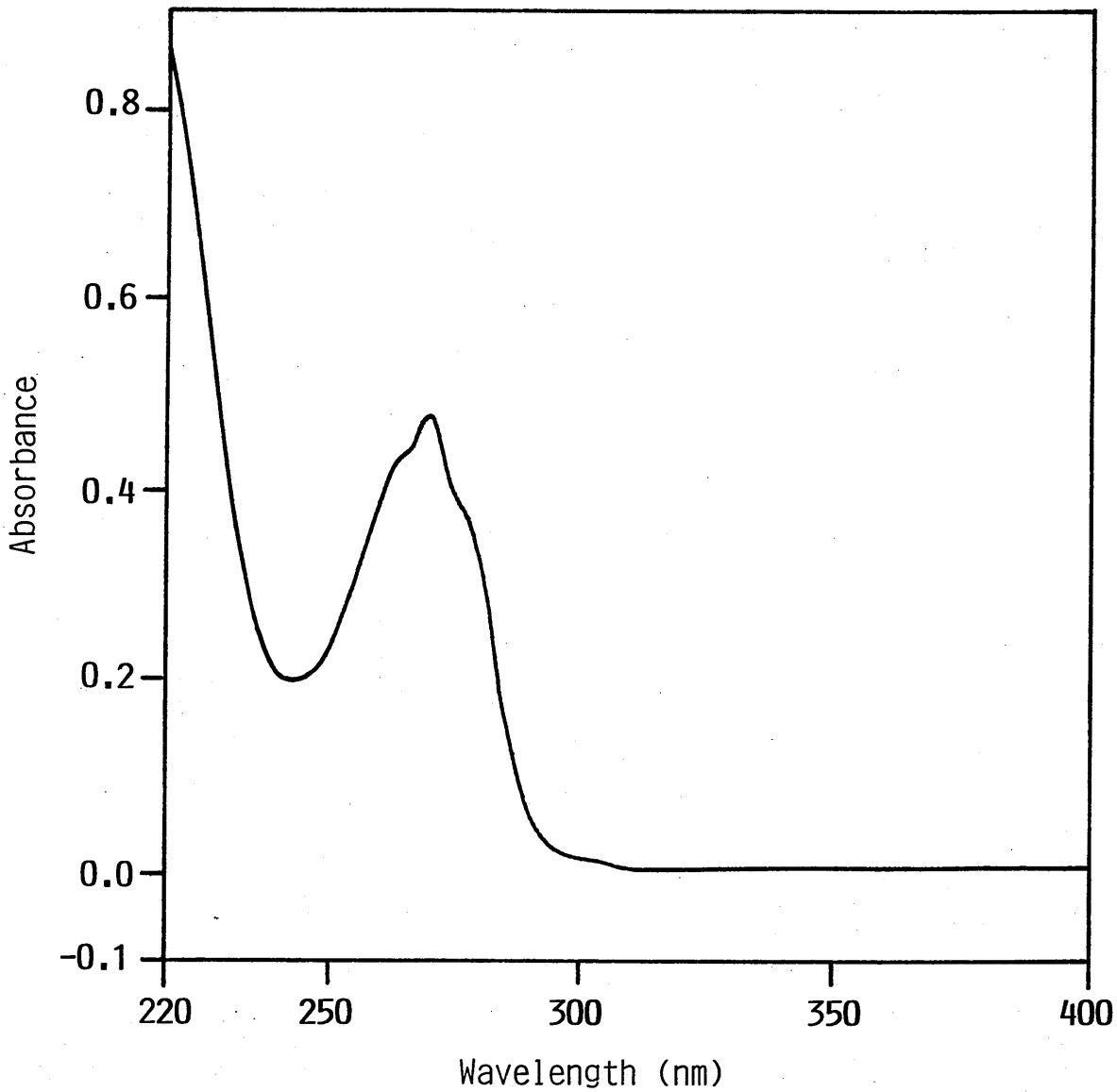


Figure 4.2 The UV Absorbance Spectrum of Dipicolinic Acid

As first shown by Powell,1953, DPA has a characteristic UV spectra. It has a maximum peak at ≈ 270 nm with a shoulder at either side (see Section 4.3).

A slightly alkali solution of 0.1 mM-DPA was set up in a 1 ml cuvette and the UV spectra scanned on a Philips scanning spectrophotometer. The maximum observed was at 271.2 nm.

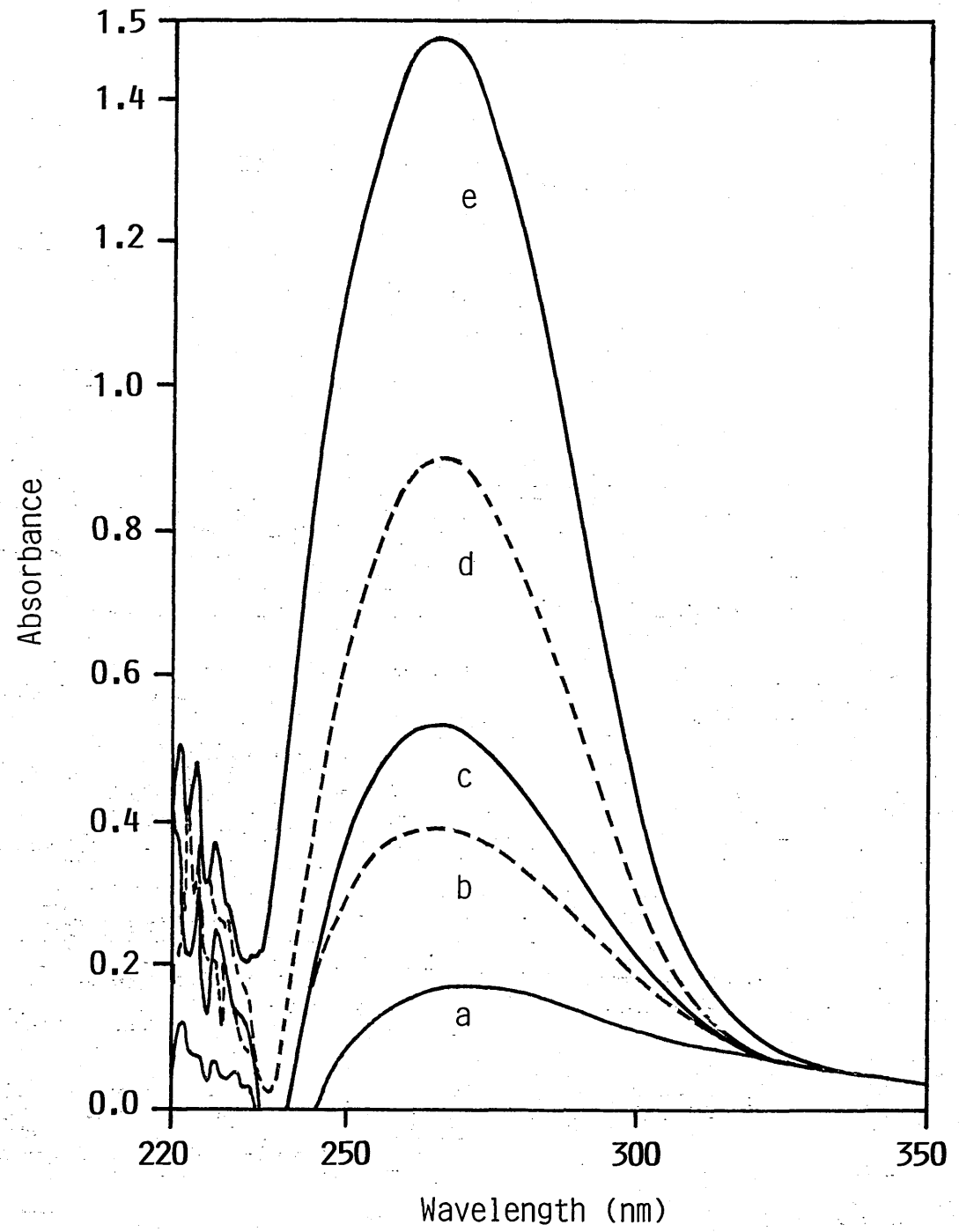


Figure 4.3 The UV Absorbance Spectra of The Increasing 270 nm Assay Product With Time

A time course (see Section 4.3) was set up using the assay consisting of :- 100 mM-imidazole buffer pH 7.4, 5 mM-pyruvate, 2 mM-ASA (nuetralised) and 20 units DHDPA synthase in a 1 ml cuvette. The assay was incubated at 25^{o}C for 1, 3, 5, 10, and 20 min represented by a-e, respectively. The UV spectra were scanned and the assay product was shown to increase at an absorbance of ≈265 nm. This is slightly less than 270 nm due to the other assay components interfering.

4.4.1 The Comparison of the Assay Product with DPA

The retention time for DPA on the h.p.l.c. column was determined by applying 50µl 1 mM-DPA to the organic acids column which had been pre-equilibrated with 25 mM -H_2SO_4. The sample was eluted by continuous wash with 25 mM -H_2SO_4 at a flow rate of 1 ml/min and the eluent was monitored at either 210 nm or 270 nm. At either wavelength a 40 min peak was observed and no other peaks detected.

The reaction mixture for assay B (1 ml) was for 24 hours. Aliquots (100 µl) were taken at intervals over this period and loaded onto the h.p.l.c. column and eluted in the same manner as above. When the column eluent was monitored at 210 nm several peaks were observed during the first 10 min but no later peak was observed either at 210 nm or 270 nm. Only some of the initial peaks were also detected at 270 nm.

To try and assign these intial peaks, the separate assay components and the assay mixture minus the enzyme were also run separately on the column. This would allow the products of chemical side reactions to be detected. The retention times for the assay components are summarised in Table 4.2, also the heights of the peaks during the time course to indicate which decrease or increase during the reaction. There are four significant observations from the time course of the assay:- the reduction of the substrate peaks, the increase of a peak at 9.2 min, the appearance of a peak at 10.8 min and its increase and the absence of the 40 min peak.

The ASA was impure and contained two peaks at 4.8 and 8.6 min both of which decreased with time during the assay reaction. The 9.2 min peak increased slightly with time, and so was investigated further. The retention time of acetate is ≈9 min and therefore a spiking experiment was set up. 100 µl of an 6 hour Assay B was mixed with 100 µl 10 mM-acetic acid and 50 µl of the mixture was applied to the h.p.l.c. column. The elution pattern was compared to the assay alone and it was shown that the 9.2 min peak increased with the spike present and so the 9.2 min peak was provisionally identified as actetate. The 10.8 min also increased during the reaction but no identification of the peak could be made.

There was no later peak (at ≈40 min) which could represent DPA and no peaks

Retention Time (min)	Intervals of Incubation of the Assay (min) Height of peaks(cm) 0	5	10	20	40	1440	Assay Component
4	4	3.9	3.4	3.1	3.1	4.3	solvent front
4.8	4.2	3.7	1.3	0.4	0	0	ASA
6.2	≈22	≈22	21	14.5	8.9	0	pyruvate
7.4	1.7	1.7	1.7	1.3	1.3	1.7	impurity
8.6	1.5	1.2	0.7	0.6	0.7	1.0	ASA
9.2	3.5	3.7	3.8	3.7	4.0	4.7	actetate
10.8	0.6	3.7	3.8	4.5	5.1	9.1	don't know

Table 4.2 The Change in Height of Peaks from the Organic Acid Analysis, during an Incubation of the 270 nm assay in Imidazole Buffer

A time course using the assay mixture :- 100 mM-imidazole buffer pH 7.4, 5 mM-pyruvate, 2 mM-ASA (neutralised) and 30 units of DHDPA synthase in a final volume of 1 ml. These were incubated at 25^{o}C and at the intervals shown, 50µl aliquots were taken and loaded onto an Organic Acids column (see Section 4.4). The column was eluted in 25 mM-H_2SO_4 with a flow rate of 1 ml/min and the eluent monitored at 210 nm. The heights of the absorbance peaks monitored were measured. Using the individual substrates of the assay, the peaks can be labelled. The growth of the 10.8 min peak is still a mystery.

were observed when monitoring at 270nm.

4.4.2 Changing the Assay Buffer

The rate of DHDPA synthase observed in Assay B is maximal with imidazole buffer. The rates observed with Tris HCl, pH 7.4 buffer or potassium phosphate, pH 7.4 buffer were respectively 40% and 60% of the maximum rate observed with imidazole buffer, pH 7.4. Although the rate was decreased when these other buffers were used there was still a substantial rate. This observation disagreed with Yugari & Gilvarg (1965) who had reported that the assay only gave an increase in absorbance at 270 nm in the presence of imidazole buffer.

A time course was set up using the following assay cocktail with final concentrations of:- 100 mM-Tris HCl pH 7.4, 5 mM-pyruvate, 2 mM-ASA (neutralised as before), and ≈30 units of DHDPA synthase (maximum calculated) in a 1ml cuvette (Assay C). The UV spectra were measured over a time course (similar to that described in Section 4.3) of 0-20 min but no specific 270 nm peak was seen although the absorbance at 270 nm did increase.

A 4 hour incubation was set up of Assay C and an aliquot was applied to the h.p.l.c. column and eluted as before. The intial peaks were similar to those observed with the imidazole buffer but also present was a peak at ≈40 min and this is illustated in Figure 4.4. An h.p.l.c. run of an assay mixture at zero time is shown in Figure 4.5. The 40 min peak did not appear but the substrate peaks were bigger; this shows that the 40 min peak is a genuine product peak. Note also that the 9.2 and 10.8 min peaks increased with time of incubation in a similar way to those observed for the imidazole buffer assay (see Table 4.2).

The h.p.l.c. run was repeated (with the 4 hour incubation) and the the eluent monitored at 270 nm and as expected the intial peaks were reduced but the 40 min peak increased. This peak was collected in 1 ml fractions and the UV spectra of the individual fractions proved dirty but a small maximum at 270 nm was seen. During a time course of 0 to 4 hours the 40 min peak observed on the h.p.l.c. trace increased in

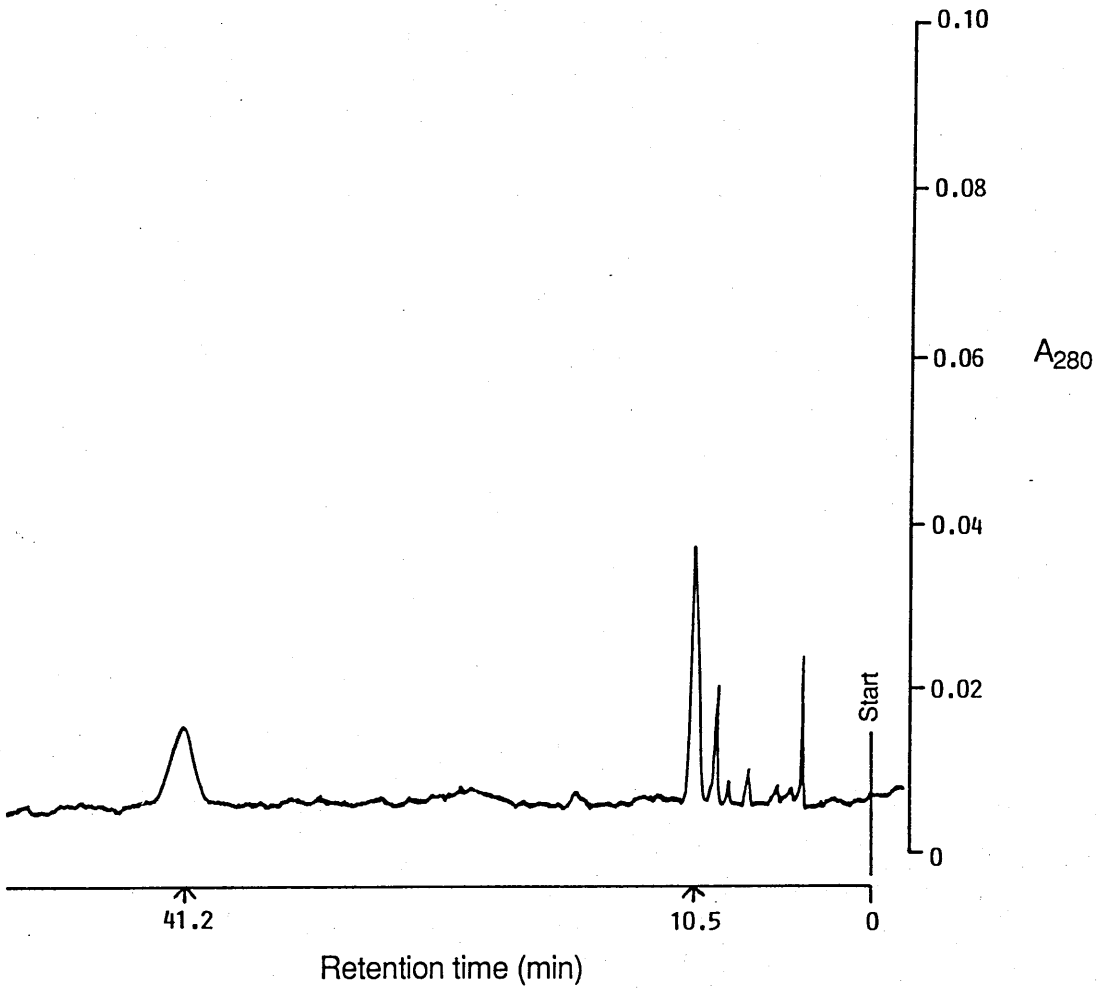


Figure 4.4 The H.P.L.C. Trace of The Tris Assay after a Four Hour Incubation

Assay C consists of 100mM-Tris buffer pH7.4, 5 mM-pyruvate, 2 mM-ASA (neutralised) and 30 units DHDPA synthase in a final volume of 1 ml and incubated at 25°C for 4 hours. A 50 µl aliquot was loaded on to an organic acids analysis (the big column, see Section 4.4.2). The column was eluted with 25 mM-H_2SO_4 and the eluent monitored at 210 nm. The retention times for two major peaks are indicated by the arrows. The 10.5 min peak was observed for the imidazole buffer assay, see Table 4.2 but has not been assigned. The 41.2 min peak has been identified as DPA by spiking experiments (see Section 4.4.2). Note also the decreased initial substrate peaks compared to Figure 4.5.

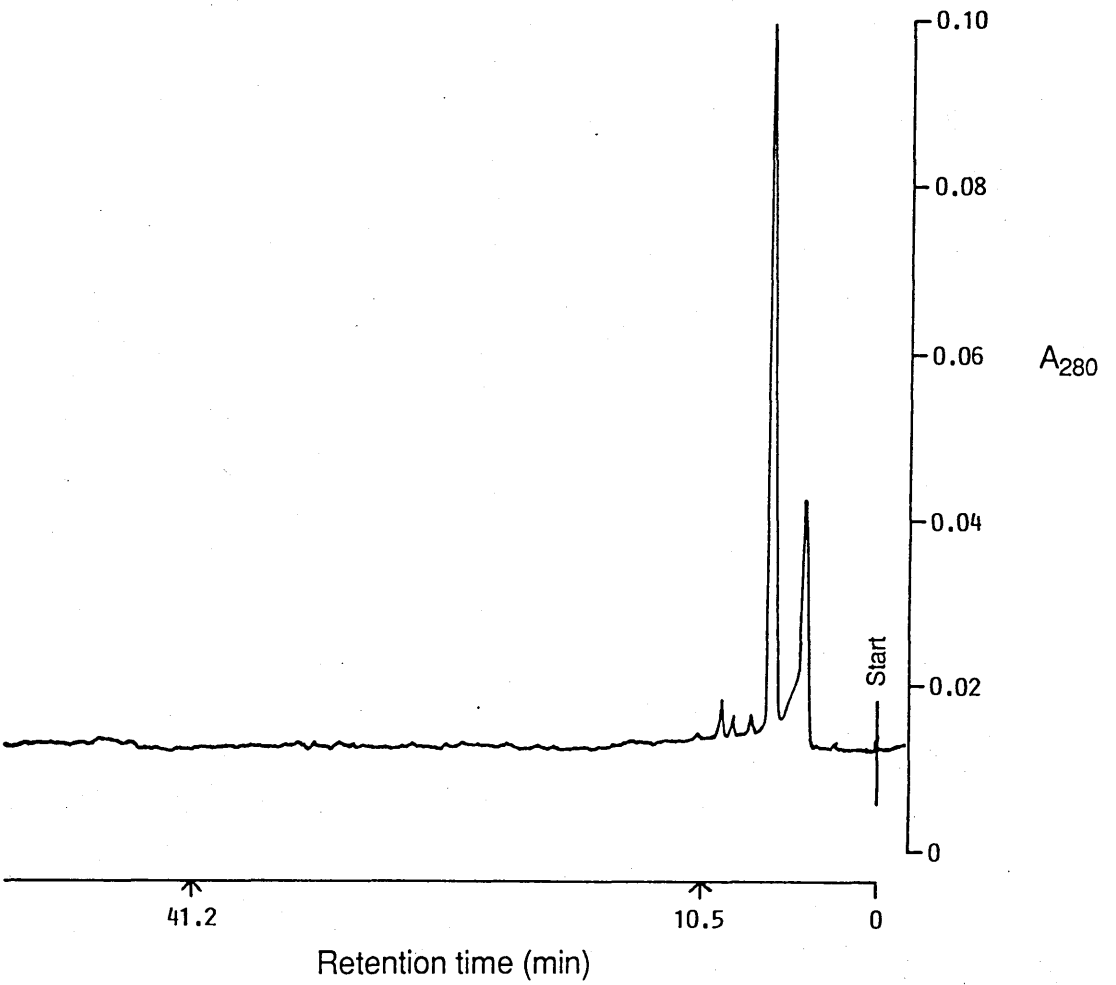


Figure 4.5 The H.P.L.C. Trace of The Tris Assay with No Incubation

To show that the 41.2 min peak in Figure 4.4 represented a genuine product the same assay (Assay C) was set up, but with no incubation. A 50 µl aliquot was loaded on to the organic acids analysis (see Section 4.4.2). The column was eluted with 25 mM-H_2SO_4 and the eluent monitored at 210 nm. The resulting trace shows neither the 10.8 nor 41.2 min peak, proving that these peaks must represent products of the reaction. Note also the tall peak at 6.2 min which represents pyruvate, this is used up in the reaction but also degrades at room temperature. One of the ASA peaks (4.8 min) has merged with the solvent front but decreases during the assay reaction (see Figure 4.4).

width rather than height, giving an increased area, overall. A spiking experiment was set up, similar to that for the 9.2 min peak. in which 50 µl 0.05 mM-DPA were added to 50 µl of the assay mixture and an aliquot (50 µl) was applied to the column. The result showed an increased 40 min peak and confirmed that DPA and the 40 min peak product elute at the same retention time.

Assay C was scaled up to a 20 ml total volume and incubated for 4 hours at 25°C. 1 ml aliquots were loaded onto a smaller organic acids column (fast acids analysis column, 100 x 7.8 mm, see Chapter 2); the assay product eluted with a retention time of 9 min (as did DPA) and the separation process was much quicker. The column was run 20-30 times and with each run the 9 min peak was collected in 1ml fractions. The UV spectra of the peak sample were similar to those shown before. The sample had to concentrated into a smaller volume and so fractions were treated with $BaCl_2$ (final concentration of 25mM) and stood at room temperature for 30 min before centrifugation for 5 min (in a microfuge). This precipitated the H_2SO_4, which would have interfered with freeze drying as the $BaSO_4$. The supernatants were collected, pooled, and freeze dried overnight. The dried sample was resuspended in 1 ml distilled water and the UV spectrum was taken. The scan was still dirty with a less of a peak at 270 nm than before the concentration process. Perhaps the product had been lost during the freeze drying process and it was decided to try an alternative method of isolation.

4.4.3 Changing H.P.L.C. Eluent

For a compound to survive freeze drying the solvent has to be more volatile than the compound. Formic acid is highly volatile and so can be used instead of H_2SO_4 as the h.p.l.c. solvent. The h.p.l.c. runs were repeated, as above, but with 25 mM-formic acid as the solvent. The 9 min peak was collected but the UV scan was still dirty for the indivual fractions. Pooling the fractions and freeze drying overnight gave sufficient material for repurification. The sample was resuspended in 5 ml water (total volume) and reloaded on to the small h.p.l.c. column in 1ml aliqouts and 1 ml fractions collected were collected, pooled and freeze dried overnight. The dried sample was resuspended

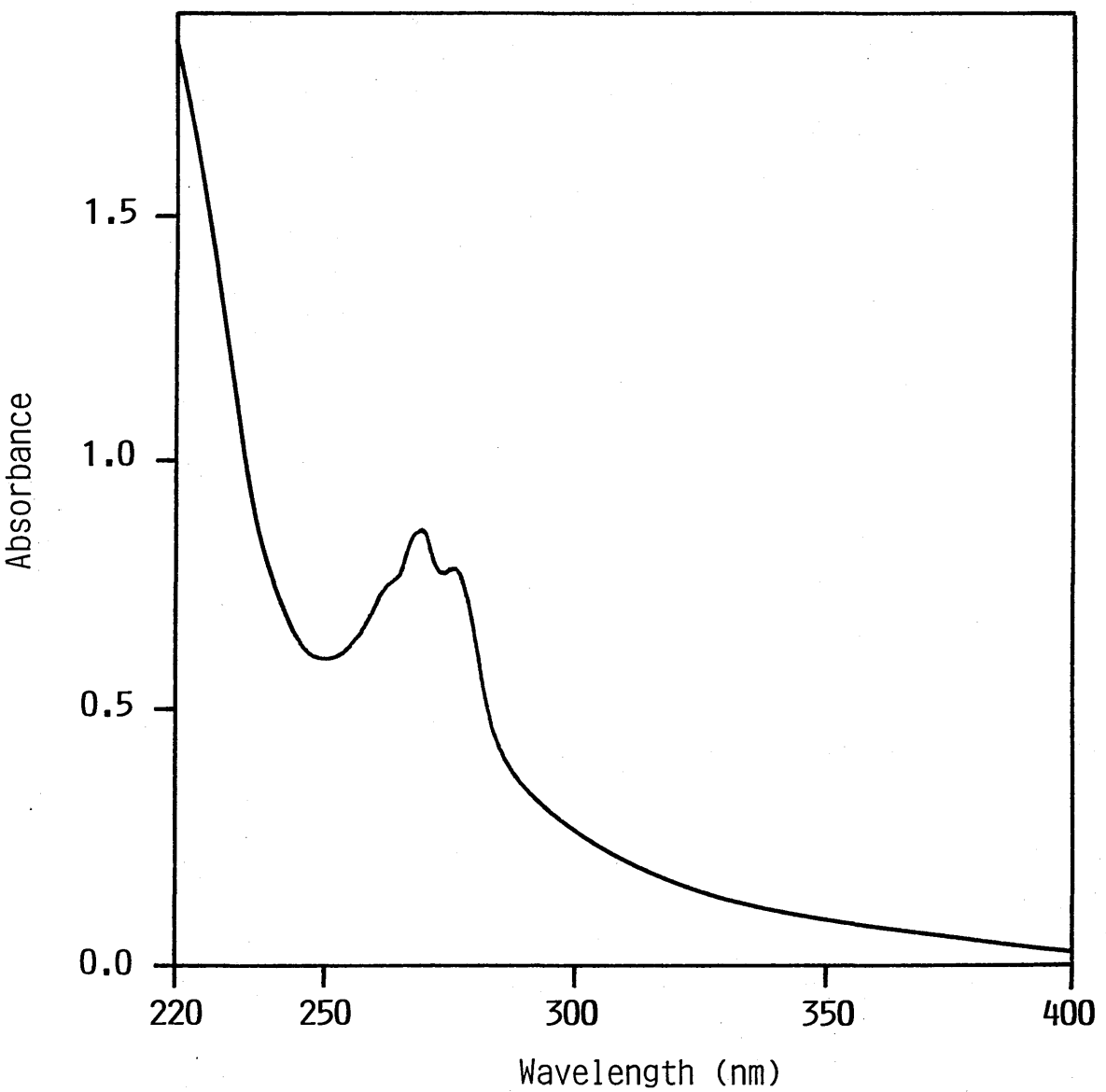


Figure 4.6 The UV Absorbance Spectrum of 'Pure' 270 nm Assay Product

The assay product peak of the Tris assay (Assay C) was collected during several (30) runs on the fast acids analysis (the small h.p.l.c. column, see Section 4.4.2) by elution with 25 mM-formic acid (see Section 4.4.3). The 9 min peak was collected in 1 ml fractions. The fractions were pooled, freeze dried and aliquots loaded back on to the h.p.l.c. column. Again the 9 min peak was collected, pooled, and re-freeze dried. Then the dried sample was resuspended in 2 ml and from that sample a 250 µl aliquot added to 750 µl water in a 1 ml cuvette. The UV spectrum was scanned and a peak at 270.1 nm is shown. This has the characteristic shoulders similar to the UV absorbance spectrum, for the standard DPA (Figure 4.2).

in 2 ml water and from that sample a 250 µl aliquot was taken and diluted to 1 ml with water and UV spectra measured. The resulting UV spectrum (shown in Figure 4.6) had a cleaner peak at 270 nm with the characteristic shoulders of DPA. All the sample was recovered and re-freeze dried overnight.

4.5 Other methods to prove the H.P.L.C. Product is DPA

4.5.1 NMR

DPA is an aromatic compound (see Figure 4.1) and its NMR spectrum consists of a low-field peak, which can only be resolved under very high resolution conditions. DPA was dissolved in D_2O and applied to a Brucker WP200SY 200 MHz NMR spectrometer and run at room temperature by J. Gall (Department of Chemistry, University of Glasgow). A low field peak at 8.3 PPM was observed together with a water peak at 4.6 PPM. The assay product sample (0.2 mgs calculated from UV comparisons with the standard) from Section 4.4.2 was treated with D_2O and the NMR spectrum measured; it was unfortuneately very noisy with several impurities present (see Figure 4.7). The peak marked at 7.95 PPM is the product, the larger peaks at 8.2 PPM and 3.5 PPM perhaps represent an impurity which could be methyl formate. The size of the 7.95 PPM peak (representing 0.2 mgs) indicated that to acheive a better NMR spectrum a much larger amount of product is required. This demonstrates the relatively low sensitivity of NMR in comparison with UV spectroscopy. It is possible to detect 0.01 mgs DPA by UV spectroscopy.

4.5.2 $CaCl_2$ Treatment

Another method to compare DPA with the assay product is by treatment with $CaCl_2$. The UV spectra of DPA is altered when $CaCl_2$ is added to give absorption maxima at 270 and 277.5 nm and a minimum at 274 nm. This compares to the single peak at ≈270 nm, shown without $CaCl_2$. The optical properities of Ca-DPA solution

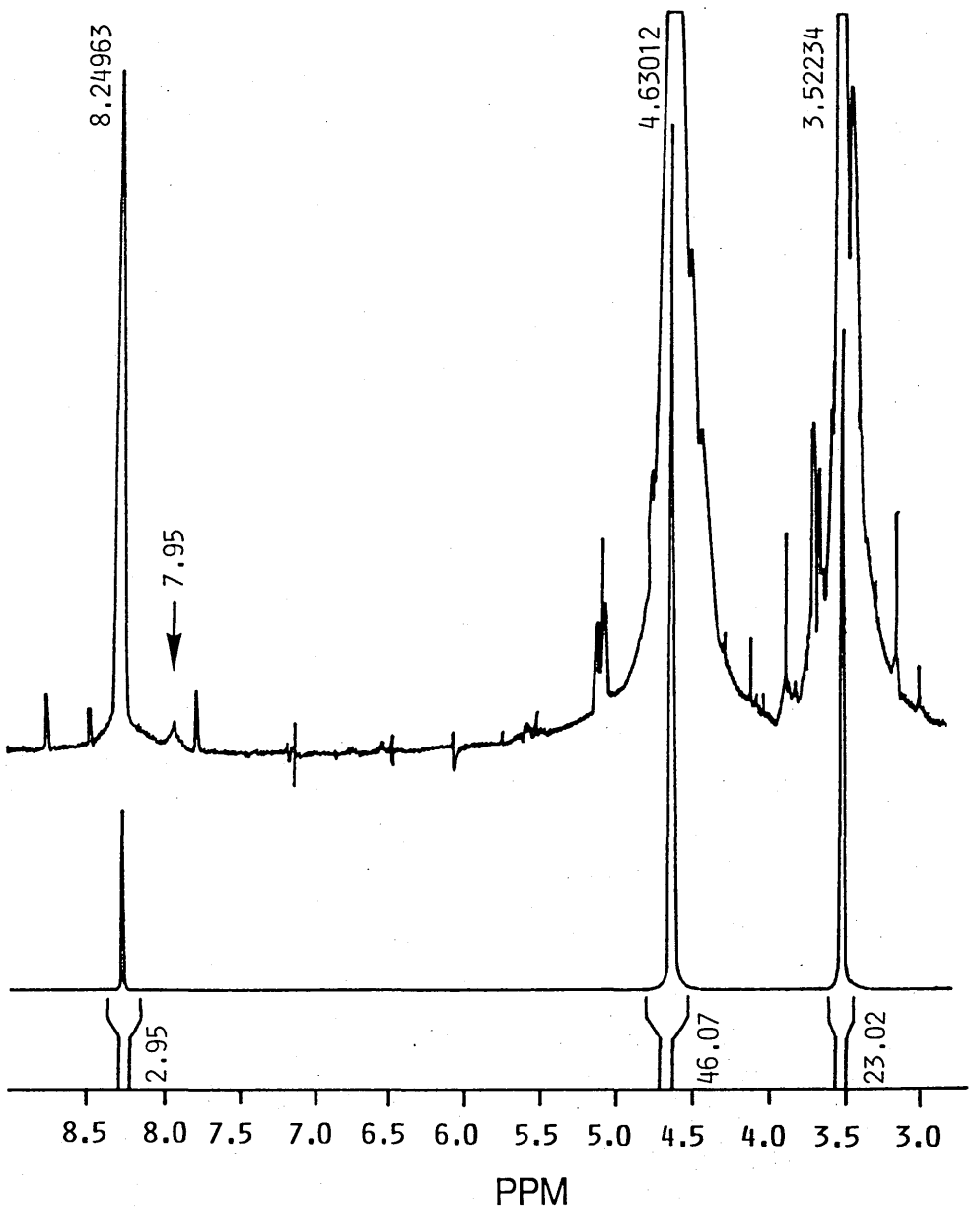


Figure 4.7 The NMR Spectrum of 'Pure' Assay Product

The freeze dried sample from the pooled peaks from the fast organic acid analysis (the small h.p.l.c. column, see Section 4.5.1), was resuspended in D_2O and loaded onto a Brucker WP200SY 200 MHz NMR spectrophotometer. The resulting spectrum shows several impurities, but a small low field peak at 7.95 PPM is shown and this indicates the presence of DPA in the sample. A previous NMR spectrum (not shown) with standard DPA showed a low field singlet at 8.3 PPM. The lower trace is a low sensitivity run and the upper trace a high sensitive run.

showed that ratios of OD_{270}/OD_{274} is 1.5 and $OD_{270}/OD_{277.5}$ is 1.1 in solutions which have the molar ratio of $CaCl_2$/DPA higher than 20. Also since there is a linear relationship between the amount of DPA and the absorbance, the amount of DPA can be calculated by the following equation:- $(OD_{277.5} - OD_{274})/1.3$ = μmoles of DPA/ml (Kimura, 1974).

To test these statements and the equation, 0.1 mM-DPA was made up in a 1 ml cuvette, a UV spectrum was taken, then 50μl of 0.1M-$CaCl_2$ (final concentration of 5 mM) was added to the cuvette and the UV spectrum was run, again. The two spectra are shown in Figure 4.8. The wavelengths at which the maxima and minimum appeared were slightly different from those reported by Kimura (1974) but the two peaks was observed. The OD_{270}/OD_{274} and $OD_{270}/OD_{277.5}$ ratio values were 1.4 and 1.1, respectively, which matched the previous observed ratios (Kimura 1974). The calculation of the amount of DPA present was 0.08 mM, which compares with the value of 0.1 mM, calculated from the weight of DPA used to make up the solution.

The product retrieved from the NMR was freeze dried and resuspended in 2 ml water. 250 μl was added to 1 ml cuvette containing 750μl water. 50μl of 0.1M $CaCl_2$ was added and the UV spectrum was observed before and after $CaCl_2$ treatment and the results are shown in Figure 4.9. The OD_{270}/OD_{274} and $OD_{270}/OD_{277.5}$ ratios were 1.2 and 1.1 respectively, which suggested DPA was present in the sample. The calculation suggested that there was 0.03 μmoles of DPA present in the 1 ml cuvette, which corresponds to a total of only 0.04 mgs DPA (some has been lost during the NMR and repeated freeze drying process).

4.6 Summary of The Separation and Identification of the Assay Product

The 270 nm assay has a product which absorbed at 270 nm when imidazole, Tris or phosphate buffer was used. The above product was investigated and proved to be dipicolinic acid (DPA). However on the separation of the product from the other assay components by h.p.l.c. on the organic acids column, it was only isolated in measurable amounts from assays containing Tris or phosphate buffers and not from the imidazole

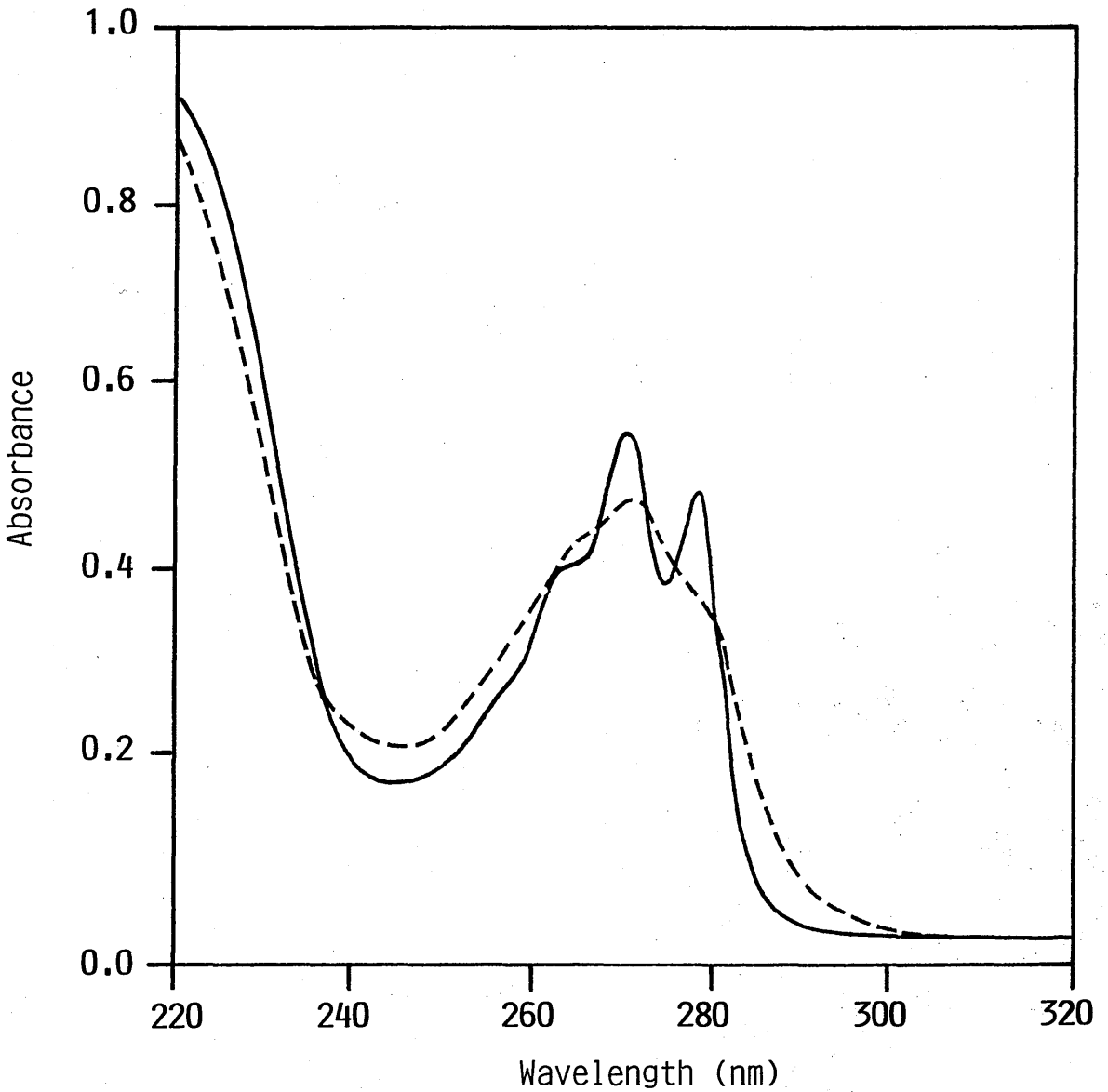


Figure 4.8 The $CaCl_2$ Effect on the UV Absorbance Spectrum of DPA

As first shown by Kimura, 1974, the addition of $CaCl_2$ to DPA splits the absorbance peak into two maxima and a minimum. In a simlar manner to that described in Figure 4.2 a slightly alkali solution of 0.1 mM-DPA was prepared and the UV spectrum represented by (---). Then 50 µl of 0.1 M-$CaCl_2$ was added (5 mM final concentration) to the DPA solution and the resulting UV spectrum is indicated by (——). The OD_{270}/OD_{274} and $OD_{270}/OD_{277.5}$ ratios are 1.4 and 1.1 repectively which are characteristic for DPA (see Section 4.5.2).

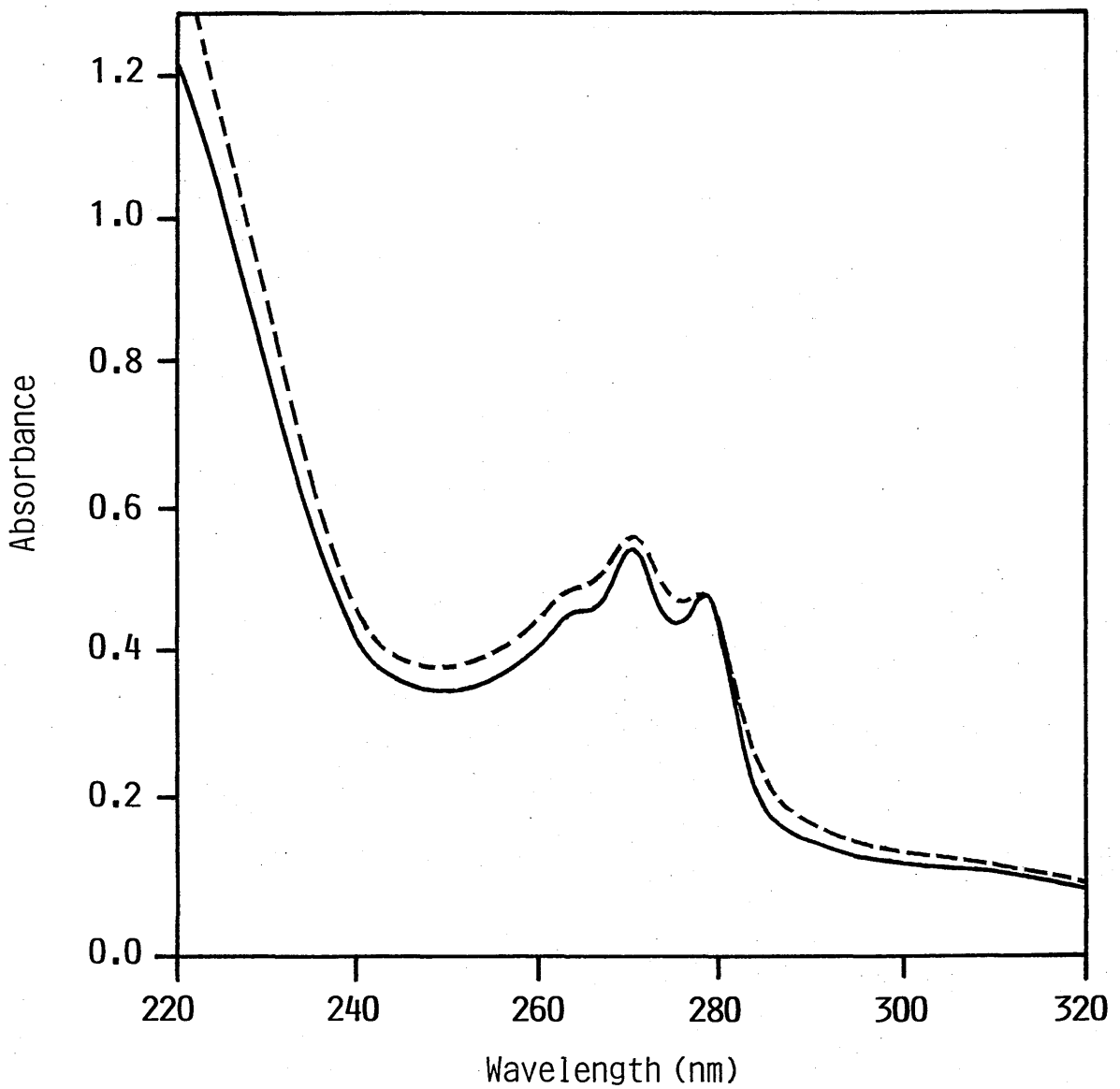


Figure 4.9 The $CaCl_2$ Effect on the UV Absorbance Spectrum of the 'Pure' Assay Product

The product retreived from the NMR was freeze dried and resuspended in 2 ml of water. A 250 µl aliquot was added to 750 µl water in a 1 ml cuvette. The UV spectrum was scanned and is represented by (---). Then 50 µl of 0.1 M-$CaCl_2$ was added (5 mM final concentration) to the product solution and the resulting UV spectrum is indicated by (——). The OD_{270}/OD_{274} and $OD_{270}/OD_{277.5}$ ratios are 1.2 and 1.1 repectively which are similar to the characteristics shown for DPA (see Section 4.5.2 and Figure 4.8).

buffer experiments.

The evidence showing that the assay product peak was DPA was that provided by UV and NMR spectra of the purified product, and the $CaCl_2$ effect on the UV spectrum of the product. The product of the 270 nm assay used in this Thesis to monitor DHDPA synthase activity was thus clearly identified as DPA.

DPAc has a molar coefficient of 4,000 $M^{-1}cm^{-1}$ and so the units of activity could be calculated in international units (μmoles product produced per minute) and not simply in arbitary units based in the absorbance change. Thus the arbitary enzyme units in Table 3.1 and 3.2 in Chapter 3 would be 4000 fold less if expressed as international units.

4.7 Discussion

DHDPA synthase is the first unique step on the lysine biosynthetic pathway, and therefore is a good target for inhibitors (see Chapter 3). To test various inhibitory compounds it was important to obtain a reasonable assay which is simple and understandable.

There are three assays available to monitor DHDPA synthase (Yugari &Gilvarg, 1965) and these are :- a coupled assay with DHDPA reductase (measures NADPH oxidation), the o-aminobenzaldehyde assay (measurement of an unknown adduct formed with this chemical), and the 270 nm assay (measured by the appearance of the previously uncharacterised 270 nm absorbing product).

Each assay required the presence of the two substrates of the reaction, pyruvate and aspartate semialdehyde (ASA). ASA has to be synthesised by the ozonolysis of allylglycine and its concentration determined using an enzymatic method. This consisted of monitoring the oxidation of NADPH during the complete conversion of ASA to homoserine by homoserine dehydrogenase. The enzyme used for this analysis, homoserine dehydrogenase, was partially purified from a wild-type *E.coli* K12 strain as summarised in Table 4.1.

The average yield of ASA from the ozonolysis varied between 25 and 40%

depending on the efficiency of the ozonolyser. It was important that ASA was neutralised before used in the DHDPA synthase assays as the buffering system can not control the low pH caused by the HCl present in the ASA sample.

The quickest and simplest assay was the 270 nm assay. However it was necessary to characterise the compound absorbing at 270 nm produced during the assay. It had been suggested (Yugari & Gilvarg, 1965) that the product of the assay was the 3,6-isomer of DHDPA. Since then the lysine pathway has been fully eludicated in *E.coli* and it has been shown that DHDPA (2,6 isomer) is the product of the enzymatic reaction in nature (for mutant eludication see, Bukhari 1971, for pathway see Figure 1.2, Chapter 1). However DHDPA is a very unstable compound and therefore could not be the product of the assay since the 270 nm absorbing product remained stable at 25^{o}C for several days. The reason that DHDPA is unstable is because it spontaneously oxidises in air to dipicolinic acid and this suggests that DPA is the 270 nm product (Kimura & Sasakawa, 1975), see Figure 4.1.

There have been several reports of the presence of DPA in sporulating bacteria, such as *B.cereus* var. *mycoides* and *B. subtilis* (Perry &Foster 1955) and *B.megatherium* (Powell 1953). There are two routes of biosynthesis of DPA studied in these sporulating bacteria. In 1955, Perry & Foster suggested DPA was formed from 2,6 diaminopimelic acid which was further investigated twenty years later by Kimura & Sasakawa (1975). They demonstrated that diketopimelic acid (possibly from diaminopimelic acid) was a precursor of dihydrodipicolinic acid (DHDPA) which could be either enzymatically or spontaneously oxidised into DPA in these bacteria. Another route via ASA and pyruvate condensation to form DHDPA was also shown to exist in the sporulating bacteria and therefore the DPA could be synthesised as a branch of the lysine biosynthetic pathway (Kimura, 1974). Therefore it was proposed that the DHDPA synthesised in *E.coli* could also be spontaneously oxidised to DPA after the condensation reaction of ASA and pyruvate in the DHDPA synthase assay.

The UV spectrum of DPA showed a shouldered peak at 270 nm as illustrated in Figure 4.2. The assay product monitored during the reaction by UV spectrum showed an increased peak at 265 nm (see Figure 4.3) however, since other assay components which might perturb the spectrum were present, no direct comparison to DPA could be

made. Therefore the assay product had to be separated from the other assay components such as unused ASA (pyruvate degrades rapidily at room temperature to non absorbing compounds and therefore was not present), buffer and any chemical reaction products,such as acetate which may have been formed, through the degradation of ASA.

The organic acids analysis on the h.p.l.c. separates organic acids by IMP (ion moderated partition) mechanisms, using primarily ligand exchange and adsorption and therefore could separate the product. The DPA gave a retention time of 40 min. To show DPA was the assay product an increasing peak at 40 min had to be detected when the assay was applied to the h.p.l.c. Unfortunately no such peak was observed with the assay in imidazole buffer.

The 270 nm assay can also be run with different buffers instead of imidazole at the same pH, for example Tris and phosphate. This contradicts the observations of Yugari & Gilvarg (1965) who had claimed that the assay only worked in imadazole buffer. The rates observed were smaller in Tris and phosphate but were still readily observed and were linear.

Once the imidazole buffer was replaced by Tris or phosphate buffer a 40 min peak was detected on the organic acids analysis. The reason why the peak did not appear with imidazole buffer may have been that this buffer was affected by the acidic eluent of the column. The 40 min peak was collected (for the Tris buffer) and as described in Sections 4.4.2 and 4.4.3 the h.p.l.c. eluent was changed to the more volatile formic acid which would allow the fractions to be freeze dried.

The UV spectrum (Figure 4.5) of the purer product after two repeated runs of the h.p.l.c., matched closely to the pattern of the UV spectrum of DPA (Figure 4.2). Further tests were done to prove that this compound was DPA. The NMR result (Figure 4.5) was not conclusive on its own, and would probably need to be repeated with a larger amount of compound. Nevertheless it did show that some DPA was present in the sample.

The $CaCl_2$ treatment (first shown by Powell, 1953 and re-examined by Kimura (1974) is a good test to show that the compound produced by the assay was DPA. The theory of the reaction of $CaCl_2$ and DPA is unknown but the formation of two

absorbance peaks can be easily detected by UV spectra. The ratios of $OD_{270/274}$ (see section 4.5.2) was slightly low perhaps due to impurities such as the methyl formate detected in the NMR spectra (Figure 4.5). Although the product peak has not been observed with imidazole as the buffer it has been proved undoubtedly by UV, NMR and $CaCl_2$ spectra that the product of the 270 nm assay is DPA.

The units of DHDPA synthase could be calculated in a more reasonable manner, by using the molar coefficient of DPA. This would reduce the units calculated for DHDPA synthase, in Chapter 3, by 4000 fold and so into more 'workable' figures. This gives a specific activity of 38 units/mg protein which corresponds to a turnover number of 1243 sec^{-1} for DHDPA synthase from the wild-type strain. For the enzyme from the overexpressed strain the specific activity was 52 units/mg and the turnover number was 1713 sec^{-1}.

Chapter 5 : Studies on DHDPA Reductase

5.1 Introduction

DHDPA reductase (E.C. 1.3.1.26) catalyses the second unique step of the lysine biosynthetic pathway in bacteria. The reaction is the reduction of the unstable DHDPA to the more stable, THDPA; the reducing agent is the coenzyme NADPH.

DHDPA reductase was first described by Farkas & Gilvarg (1965) who obtained evidence for its existence by studying blocked mutants in the pathway. By supplementing medium with specific substrates from the pathway it was determined that the *E.coli* strian M203 lacked enzymes between aspartate semialdehyde (ASA) and succinyl keto-aminopimelate (the fourth unique intermediate on the pathway, see Figure 1.2, Chapter 1).

In 1974, Tamir & Gilvarg purified DHDPA reductase from wild-type *E.coli* to 95% purity as judged on an SDS gel. The purification consisted of a 5 step scheme which yielded a 1000 fold purification from crude extract. The M_r of the protein is 115,000 and the subunit size is about 32,000 which suggests the protein is a tetramer.

DHDPA is an unstable compound, but its chemical synthesis has been reported (Kimura, 1974). The product of the condensation of ASA and pyruvate was separated by thin layer chromatography and shown to be DHDPA. DHDPA is readily oxidised in air to DPA, this caused difficulties when trying to reproduce these experiments (Couper, 1991). The oxidation of DHDPA to DPA in the presence of air is the basis of the DHDPA synthase assay (see Chapter 4). The assay used to test for DHDPA reductase activity is a coupled assay with DHDPA being formed enzymatically by DHDPA synthase (Farkas & Gilvarg, 1965).

The reverse reaction of the reductase would use the more stable THDPA as a substrate. Previous studies (Tamir & Gilvarg, 1974) using THDPA gave no detectable rate for the reduction of NADP with DHDPA reductase. They concluded that the equilibrium constant for the reaction must lie far in the direction of the THDPA formation.

In this chapter, I describe a partial purification of DHDPA reductase from an *E.coli* over-producing strain, containing a plasmid with the gene encoding the enzyme. This is followed by the description of the attempted isolation of THDPA from the reductase reaction and finally the reverse reaction is investigated over a range of pHs.

5.2 Activity Determination of DHDPA Reductase

The coupled assay using the enzymatic formation of DHDPA is similar to that proposed by Farkas & Gilvarg (1965). The assay mixture used to test the crude extracts from wild-type *E.coli* K12 is:- 100 mM-imadazole buffer pH 7.4, 2 mM-ASA (neutralised with 10 mM-KOH immediately before use), 40 units of DHDPA synthase (see Abbreviations for definition of these units), 0.25 mM-NADPH,10 mM-pyruvate and DHDPA reductase in a 1 ml cuvette. The activity in international units was calculated from the change in absorbance at 340 nm, by using the extinction coefficient of NADPH (ε= 6,200 $M^{-1}cm^{-1}$).

The activity observed for the crude extract of wild-type *E.coli* was 0.2 u/ml. However, there were large background rates due to NADPH oxidases and other enzymes in the crude extract. The *E.coli* MV1190/pDB17 strain (construction described in Section 5.3) crude extract was also tested using the same assay mixture. The activity obtained was 4 u/ml, which corresponds to about 20 fold over production of DHDPA reductase.

The background rate remained a problem in the overexpressing strain and so the assay was refined. The improved assay protocol with the reagents written in order of addition is :-100 mM-imidazole buffer pH 7.4, 5 mM-ASA (neutralised with 10 mM-KOH immediately before use), 104 units of DHDPA synthase (see Abreviations for definition of these units), 0.25-mM NADPH, DHDPA reductase and 10 mM-pyruvate in a 1 ml cuvette. Using this assay the enzyme activity for the crude extract of *E.coli* MV1190/pDB17 was 43 u/ml. This assay was used to monitor the activity of DHDPA reductase during the partial purification described in Section 5.4.

5.3 The Over Expressing Strain *E.coli* MV1190/pDB17

A plasmid, pDB17, containing the *dapB* gene, which encodes for DHDPA reductase, was kindly given to us by Dr.Stragier (see Chapter 3). The *dapB* gene had been previously isolated from an *Eco*R1 library of the *E.coli* genome cloned in the phage λgλtλ B. The bacterial *EcoR1* DNA fragment with the *dapB* gene was ligated into the *EcoR1* site of pBR322. The fragment containing *dapB* gene was localised to a 2.3 kbp *Hpa 1/Cla 1* fragment, which was subcloned into a *Cla 1* site in pBR322 (Bouvier *et al.*, 1984). This plasmid, pDB17, is illustrated in Figure 5.1.

The plasmid was transformed into *E.coli* MV1190 Rec A- cells by the $CaCl_2$ method as described in Chapter 2. After the transformation, mini-plasmid preparations were made and restriction digests (see Chapter 2) were carried out to confirm the identity of the plasmid and insert. The plasmid map in Figure 5.1 shows three *Bgl*1 sites, therefore upon restriction with *Bgl*1 three fragments are expected of 2.4, 1.2, and 1.1 kbp. These were all observed as illustrated in Figure 5.2.

5.4 The Partial Purification of DHDPA Reductase from *E.coli* MV1190/pDB17

The *E.coli* MV1190/pDA2 cells were grown in MM63 medium with Amp for plasmid selection. The growth occured in a similar manner to wild-type *E.coli*, (see Chapter 2 for growth conditions). The partial purification (from Laber 1987) was carried out at 4°C, and the assay used to monitor the activity is described above . Protein was measured by the method of Bradford (1970).

Step 1 Breakage of E.coli Cells

2.75 g (wet weight) of cells were resuspended in 5 mls (total volume) of 20 mM-Tris HCl pH7.4, 10 mM -EDTA and 0.4mM-DTT (Buffer A).

The cells were broken by passage through the French press three times as described in Chapter 2. The resulting suspension was then centrifuged at 28,000 g for 30 min and the pellet was discarded.

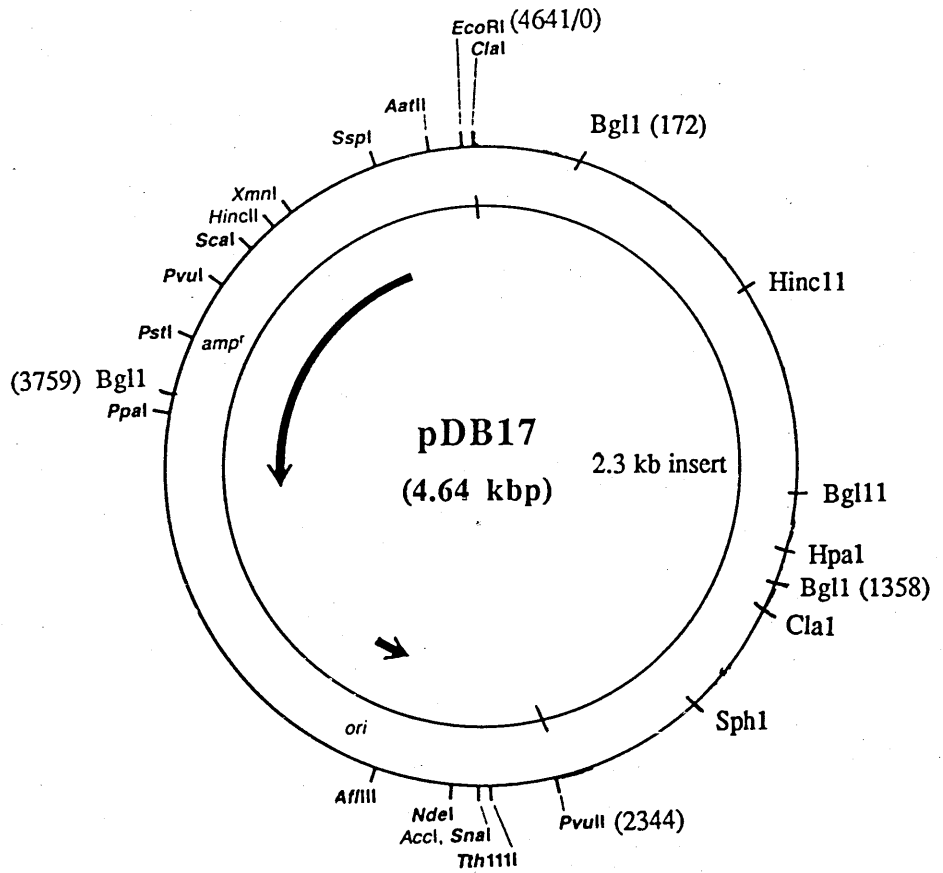


Figure 5.1 The Plasmid Map of pDB17

The plasmid, pDB17, was constructed in the following way;- the *dapB* gene, encoding for the *E.coli* DHDPA reductase, had been previously isolated in an *EcoR1* library of the *E.coli* genome cloned in the phage λgtλB. The bacterial *EcoR1* DNA fragment with the *dapB* gene was ligated into the *EcoR1* site of pBR322. The fragment containing *dapB* gene was localised to a 2.3 kbp *Hpa1/Cla1* fragment, which was subcloned into between *Cla1/Pvu11* sites in pBR322 (Bouvier *et al.*,1984). This process destroyed the *Hpa1* and *Pvu11* sites to leave a *Hpa1/Pvu11* hybrid, which is not cutable by either enzyme. The three *Bgl1* sites which were cut to confirm the identity of plasmid (see Figure 5.2) are also illustrated above.

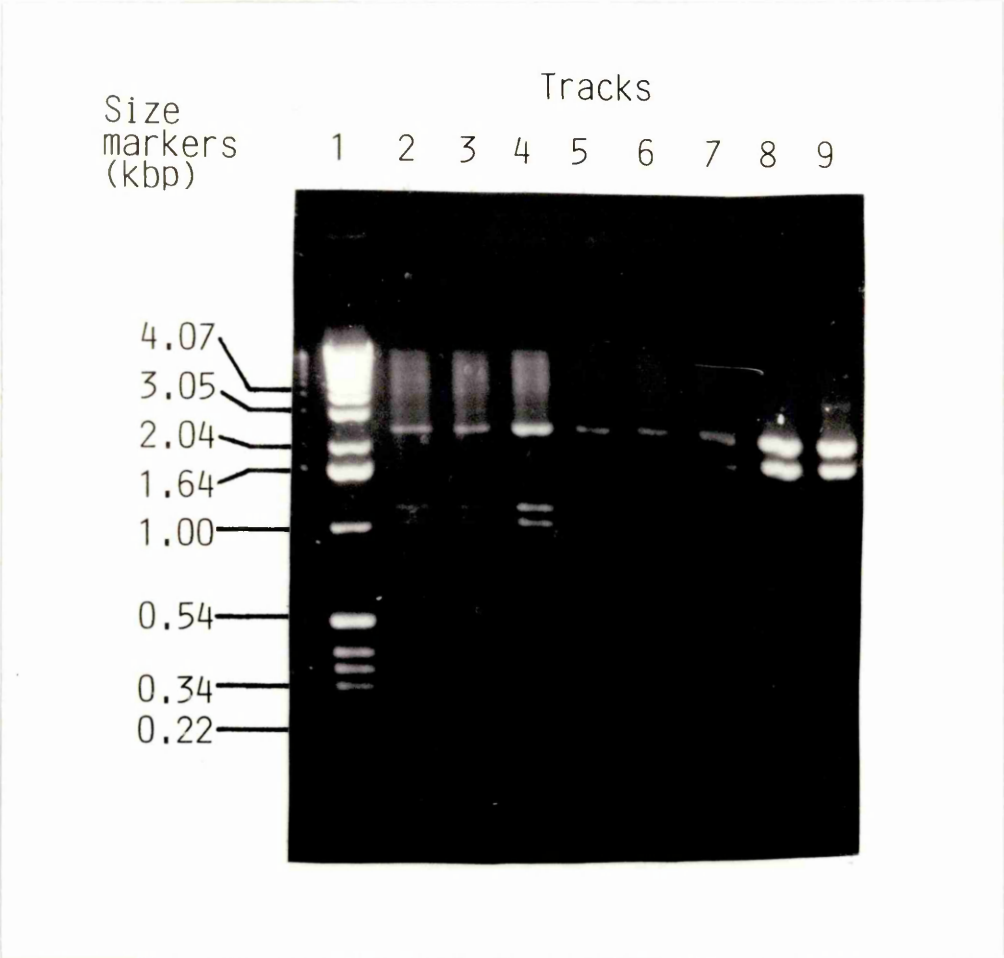


Figure 5.2 The Restriction Digestion of pDB17

The plasmid pDA2 is derived from a pBR322 plasmid as described in Section 5.3, and is illustrated in Figure 5.1. The plasmid was transformed into *E.coli* MV1190 *RecA*$^-$ cells and mini plasmid preparations were made. To confirm the identity of the plasmid, restriction digests were carried out and the result run on a 1% agarose gel (see Chapter 2 for details on method). A digest with *Bgl1* was carried out and yielded fragment sizes of 2.4, 1.2, and 1.1 kbp (Tracks 2-6). To show the difference from pBR322 which also has three *Bgl1* sites, it has been treated in the same manner. The fragment sizes yielded were 2.3, 1.8, and 0.2 kbp (Tracks 8&9) and therefore different from pDB17. Note the sample from Track 7 has leaked from either side thereby invalidating its result. The size markers (Track 1) are a 1 kbp ladder obtained from Gibco.

Step	Vol. (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Activity (U/ml)	Activity Tot.	Sp.A (U/mg)	Yield (%)	P.F.
Crude extract	3.1	36.3	113	43	134	1.18	100	1
Heat Step	2.25	14.4	32.4	54	122	3.75	91	3
0-35% $(NH_4)_2SO_4$	2	13.8	27.6	48	96	3.48	72	3
35-60% $(NH_4)_2SO_4$ (pellet)	1.5	6.8	10.2	36.5	55	5.39	41	4.6
(supernatant)	2.1	3.5	7.4	41	86.1	11.6	65	9.8

Table 5.1 The Partial Purification of DHDPA reductase from 2.8 g (wet weight) overproducing Strain *E.coli* MV1190/pDB17

The units (Us) are calculated from the conversion of NADPH (ε= 6,200 $M^{-1}cm^{-1}$) to NADP (Farkas & Gilvarg, 1965) and the protein was measured by Bradford method (Bradford, 1970). SpA. is specific activity and P.F. is purification factor.

Step 2 Heat Step

The supernatant was treated to a heat step at 70°C for 2 min and precipitated protein removed by centrifugation, as above. The supernatant was dialysed against Buffer A, overnight.

Step 3 $(NH_4)_2SO_4$ Fractionation

The dialysed sample was treated with $(NH_4)_2SO_4$ by slow stirring to acheive a final saturation of 35% $(NH_4)_2SO_4$ (208 g/litre). The mixture was stirred for 30 min to allow complete precipitation and then precipitated proteins were removed by centrifugation for 30 min at 28,000 g. The supernatant was adjusted to 60% (163 g/litre) saturation with $(NH_4)_2SO_4$ and after 30 min stirring centrifuged as above. The sample was centrifuged as before. The precipitated protein was resuspended in Buffer A and both this sample and the supernatant were found to contain activity. The supernatant contained more activity and its specific activity was higher (11.6 u/mg compared to 5.4 u/mg for the pellet). The partial purification of DHDPA reductase is summarised in Table 5.1. The partially purified sample of DHDPA reductase in the supernatant was used in the following work.

5.5 Attempted Separation of THDPA from DHDPA Reductase Reaction

The product of DHDPA reductase is THDPA. The isolation of this compound would be useful for two main reasons; as a substrate for the reverse reaction of DHDPA reductase, and as a substrate for the next enzyme on the lysine pathway.

Simms *et al.* (1984) isolated THDPA by using a G15 Sephadex gel filtration column to separate the substrates and products of the reaction catalysed by the enzyme, meso-DAP dehydrogenase. This enzyme (described in Chapter 6) catalyses the reversible conversion of THDPA to meso-DAP.

An experiment was set up to try to separate THDPA from the substrates of the DHDPA reductase reaction on a G15 gel filtration column. The partially purified enzyme ($(NH_4)_2SO_4$ 35-60% supernatant) was used in a reaction mixture (5mls

volume) made up in the same way as the assay mixture used during the purification. This was loaded onto a G-15 Sephadex column (80 x 2.5 cm) pre-equlibrated with Buffer A and pre-calibrated as desribed in Chapter 2. The flow rate was 7 ml/hr and 5 ml fractions were collected. The fractions were monitored at 280 nm and 340 nm to measure protein/THDPA and NADPH elution respectively. The elution profile (not shown) didnot contain an extra peak at 280 nm for the THDPA as observed by Simms (1984). Despite this fractions were tested for THDPA using two different asaays; the first assay was the reverse reaction of DHDPA reductase (this assay did not work, see Section 5.6) and the other was the meso-DAP assay as described in Chapter 6.

5.6 The Reverse Reaction of DHDPA Reductase

The reverse reaction of DHDPA reductase would involve the oxidation of THDPA via NADP reduction. The potassium salt of THDPA was obtained by a multistep chemical scheme (Couper, 1991) and this was tested in the following assay : 200-mM-glycine/KCl pH10.5, 1-mM-NADP,10 mM-THDPA (potassium salt), and DHDPA reductase. Since the reduction of NADP generally occurs at high pH, the initial pH of the buffer was pH 10.5. When this failed to give a rate the pH was varied using other buffers (such as Tris and phosphate). The range covered was pH 6.5-10.5, but no rate was detected.

5.7 Discussion

The coupled assay for DHDPA reductase does not give a completely reliable measurement of its activity. The formation of the substrate DHDPA is dependent on DHDPA synthase and this may limit the observed rate. This made the study of the kinetics of the reductase reaction very difficult. To obtain a reliable assay for kinectic work DHDPA must be made but unfortunately the synthesis of this compound has proved to be difficult. It is a very unstable compound as it is easily oxidised in air to dipicolinic acid (DPA). DPA itself is not a substrate for DHDPA reductase but is a

competitive inhibitor (Tamir & Gilvarg, 1974). The coupled assay proved to be satisfactory for approximate determination of the amount of DHDPA reductase present, and it was used to monitor the purification of the enzyme.

A plasmid containing the *dapB* gene, encoding DHDPA reductase, pDB17, (see Figure 5.2) was obtained. After the transformation into *E.coli* cells the specific activity of the reductase using a refined assay was found to be 43 u/ml in the crude extract. This result is not directly comparable to the activity measured in the wild-type *E.coli* crude extract, as the assay conditions had been changed. However using the original assay a 20 fold over expression had been observed. If the wild-type *E.coli* activity had been measured using the optimised assay the extent of overexpression could be measured more accurately, due to decrease of background noise but this was not done.

DHDPA reductase was partially purified from the overproducing strain by a two step scheme which acheived approximately 10 fold purification. The second step, $(NH_4)_2SO_4$ fractionation did not work because activity was split between a precipitate (where it was expected) and a supernatant. The supernatant had the higher specific activity and it was used in the further studies.

The separation of the product of the reductase reaction from the substrates by Sephadex G-15 gel filtration was unsuccessful. Among the possible explanations for this are that the products of the reaction, THDPA (M_r, 171), DHDPA, (M_r, 169), and its oxidation product DPA, (M_r, 167), are all of similar molecular weight. This makes their separation difficult by gel filtration unless there are differential adsorbtion effects. Also if DPA was present in the sample it would strongly inhibit DHDPA reductase (Tamir & Givarg, 1974), and so little or no THDPA would be produced.

The other approach for making THDPA is by a complicated chemical synthesis involving a number of steps which produced its potassium salt (Couper,1991). This was tested as a substrate for the reverse reaction under a range of pH values but no rate was detectable. This suggests that the equilibrium constant for the reaction lies toward THDPA and supports the previous observations and conclusions of Tamir & Gilvarg (1974).

Chapter 6: Studies of meso-Diaminopimelate dehydrogenase

6.1 Introduction

Meso-diaminopimelate (meso-DAP) is a key intermediate in the lysine biosynthetic pathway in bacteria. It is also a major component in the cell wall of certain bacteria. In *E.coli* the lysine biosynthetic pathway consists of seven reaction steps from aspartate semialdehyde to lysine and involves a six membered ring formation and subsequent breakage. In this pathway meso-DAP is the immediate precursor to lysine and so is synthesised through a six step reaction pathway from ASA (see Figure 1.2, Chapter 1).

In Gram positive bacteria such as *Bacillus sphaericus* and *Corynebacterium glutamicum* the lysine pathway is shortened by a 'by-pass' enzyme (Misono, 1979). This enzyme is meso-DAP dehydrogenase (E.C. 1.4.1.16) which catalyses the reversible reduction and amination of tetrahydrodipicolinic acid (THDPA) to meso-DAP. The reaction is illustrated in Figure 6.1; the forward direction favoured at pH 7.5 is the reductive amination of THDPA with NADPH and ammonia. The oxidative deamination of meso-DAP can be observed at pH 10.5 and is accompanied by the reduction of NADP. This suggests the reaction of the enzyme will be affected by pH.

Meso-DAP does not occur in the cell wall of *B. sphaericus* IFO 3525 strain where the specific activity of meso-DAP dehydrogenase is high at 0.334 u/mg (Misono *et al.*, 1979). The physiological function of the enzyme is unknown but it probably plays a role in lysine biosynthesis as the enzyme is abundant in bacterial strains which are good lysine producers.

Meso-DAP has been shown by gel filtration to be a dimer with a native M_r of 80,000 (Misono & Soda, 1980).

In this chapter meso-DAP dehydrogenase is isolated and partially purified from *Bacillus sphaericus* strain IFO 3525 by the method described by Misono & Soda (1980). The partially purified enzyme was used to try to isolate THDPA from the assay reaction, by chromatography on Sephadex G15 as described by Simms *et al.*(1984). The enzyme was shown to work in the reverse reaction using chemically synthesised

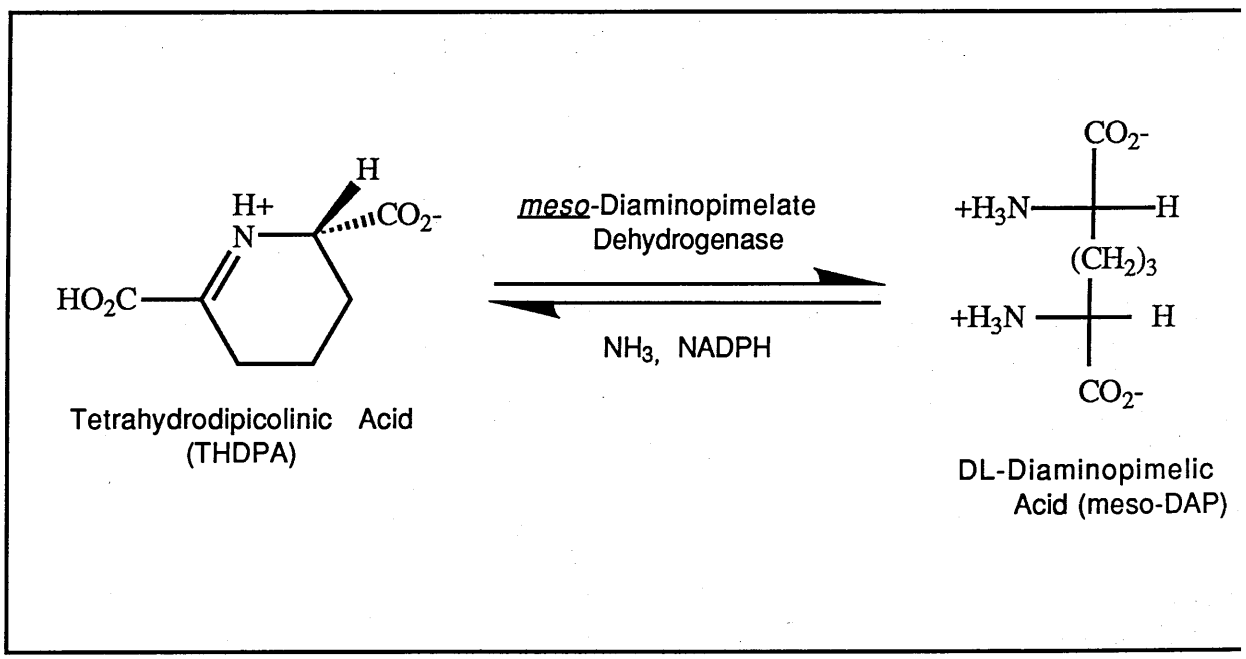


Figure 6.1 The Reaction catalysed by meso-DAP Dehydrogenase

This reaction is the by pass reaction which occurs in some Gram positive and sporulating bacteria such as *Bacillus subtilis* and *sphaericus*. The reaction is reversible; in neutral pH the direction goes towards meso-DAP, but towards THDPA in alkali conditions.

THDPA.

6.2 Enzyme Activity Determination

There are two assays available to measure the activity of meso-DAP dehydrogenase as either of the two substrates can be used, these are meso-DAP and THDPA. Meso-DAP can be crystallised and separated from the DL-DAP (obtained from Sigma), see Couper 1991. There have been problems with the chemical synthesis of THDPA (see later) and so the activity was initially determined using meso-DAP. The meso-DAP dehydrogenase activity was measured at 25°C and the rate of increase in the absorbance at 340 nm as NADP was reduced (ε =6,200 $M^{-1}cm^{-1}$). The standard reaction mixture consisted of 10 mM-DAP,1 mM-NADP, 200 mM-glycine/KCl Buffer pH 10.5 and enzyme in a final volume of 1 ml. (Misono *et al.*,1979)..

6.3 Partial Purification of meso-DAP dehydrogenase from *Bacillus Sphaericus* IFO 3525

The growth conditions of *B. sphaericus* are described in Chapter 2. Purification was carried out, on a small scale, according to the scheme described by Misono & Soda (1980), with the following changes:-

1. Only the first three steps were followed, up to the DEAE ion-exchange chromatography.
2. 2.5 g (wet weight) of cells were used.
3. A smaller DEAE-Sephacel (instead of cellulose) column of dimensions 3x30 cm was used.
4. Dialysis after the DEAE-Sephacel step was in 10 mM buffer (10 mM-poassium phosphate pH 7.4 and 0.01% 2-mercaptoethanol) overnight.

The partially pure enzyme was then dialysised again in this buffer with 50% (v/v) glycerol. This sample can be stored at -20°C for several months.

The partial purification of meso-DAP dehydrogenase is summarised in Table 6.1.

Step	Vol. (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	Activity (U/ml)	Activity Tot.	Sp.A (U/mg)	Yield (%)	P.F.
Crude extract	5	26.9	135	30	150	1.11	100	1
Dialysed cr. extract	5	21.9	110	27.5	138	1.26	92	1.1
0-50% $(NH_4)_2SO4$ (supernatant)	4.5	10	45	28.6	129	2.86	86	2.6
50-80% $(NH_4)_2SO4$ (pellet)	3.5	5.3	18.6	11.7	41	2.2	27	2
DEAE-sephacel	24	0.19	4.6	1.27	30.6	6.6	20	5.8

Table 6.1 The Partial Purification of meso-DAP dehydrogenase from 2.5 g (wet weight) *Bacillus Sphaericus* IFO 3525.

The units (Us) were calculated using the conversion of NADP (ε =6,200 $M^{-1}cm^{-1}$) to NADPH (Misono *et al.*,1979), and the protein was measured by Bradford method (Bradford,1970). SpA. is specific activity and P.F. is purification factor.

The specific activity of the crude extract at 1.11 u/mg is three times higher than previous reports (Misono *et al.*, 1979)

6.4 The Reverse reaction of meso-DAP dehydrogenase

The reaction catalysed by meso-DAP dehydrogenase is a reversible reaction (see Figure 6.1). Depending on the pH the enzyme can use either THDPA or meso-DAP as substrates. The meso-DAP can be synthesised chemically and is used during the purification of meso-DAP dehydrogenase (as described above). However, THDPA has proved a more difficult compound to make. Chapter 5 reports the unsuccessful isolation of THDPA using an enzymatic approach. A similar method using meso-DAP dehydrogenase is described below. Also a chemically synthesised of THDPA was tested.

6.4.1. Enzymatic method

The method described by Simms *et al.* (1984) for the isolation of THDPA from a DHDPA synthase reaction mixture (see Section 5.5) was adapted for the attempted isolation of THDPA from a meso-DAP dehydrogenase reaction mixture. The assay mixture for meso-DAP dehydrogenase (see Section 6.2) was scaled up to 5 mls (total volume) and the reaction was monitored by removing 1 ml aliquots to observe the increase in absorbance at 340 nm in a similar manner to the 1ml scale assay and then returning them to the reaction mixture.

After several minutes (maximum 10 min) the rate had decreased to zero and so the reaction was complete. The sample (5 ml) was applied to the G-15 Sephadex column used in Chapter 5, which had been pre-equilibrated in 200 mM-glycine/KCl pH 10.5. The column was eluted at 7 ml/hr and 5 ml fractions were collected. The fractions were monitored at 340 nm and 280 nm and the elution profile (not shown) was similar to that shown in Simms *et al.*(1984). The fractions were tested for the presence of THDPA using meso-DAP dehydrogenase in the reverse direction. The assay was carried out at

25°C and the reaction mixture consisted of:- 100 mM-Tris pH 7.4, 2 mM-NADPH, 200 mM-NH_4Cl, 28.6 munits of meso-DAP dehydrogenase and 100 µl of each fraction (THDPA). No decrease at 340 nm was observed for any of the samples and so this proceedure did not appear to give any THDPA.

6.4.2 Chemically

THDPA was chemically synthesised as the potassium salt (Couper, 1991). This compound had previously been tested as a substrate of DHDPA reductase in the reverse direction (Chapter 5) but this was not successful. The chemically synthesised THDPA was supplied and tested using a similar assay to that described in Section 6.4.1. Assuming the chemical reaction was 100%, 10 mM-K-THDP was used in the 1 ml assay with 28.6 munits of meso-DAP dehydrogenase (determined by the meso-DAP direction of assay). The rate of reaction was 7.5 mu/ml which corresponds to four times slower than the speed of the reverse reaction.

6.5 Discussion

Meso-DAP dehydrogenase is an abundant enzyme in the gram-positive bacterial strain, *Bacillus Sphaericus* IFO 3525. The crude extract was assayed in the meso-DAP direction and gave a specific activity of 1.11 which is two to three times higher than previous results (Misono *et al.*, 1979 and Misono *et al.*, 1980).

The enzyme was only partially purified using two steps after cell breakage. The specific activity of the DEAE- Sephacel step was 6.6 u/mg which was less than in the Misono *et al.*, (1980) work, perhaps due to the scale down of the preparation. However, the enzyme was pure enough to follow an adapted version of the method of Simms *et al.*.(1984) for the isolation of THDPA. This involved gel filtration of the products of the meso-DAP dehydrogenase reaction. The attempted isolation of THDPA was unsuccessful, as was a similar attempt with a DHDPA synthase reaction mixture in Chapter 5. The reason for the failure of the meso-DAP dehydrogenase reaction procedure may have been that it was carried out on too small a scale.

The chemically synthesised THDPA was tested and it was found that using the same amount of meso-DAP dehydrogenase the reaction worked four times quicker in the oxidation of meso-DAP than in the reduction of THDPA. In *Bacillus sphaericus*, meso-DAP is not used in the cell wall in the vegetative stage (Hungerer, *et al.*, 1969) therefore meso-DAP dehydrogenase uses meso-DAP to accumulate THDPA. However at the onset of sporulation in these bacteria NADPH and ammonia are made available and so more meso-DAP is synthesised (see Chapter 7, for more on sporulation).

The direction of the meso-DAP dehydrogenase reaction is favoured at different pHs; the reduction of THDPA is favoured at pH 7.5, while the meso-DAP oxidation is favoured at pH 10.5.

Chapter 7 : Final Discussion

7.1 Introduction

Lysine biosynthesis in *E.coli* follows a seven step pathway via diaminopimelate which is the immediate precursor to lysine. In some other bacteria this route is shortened by the enzyme, meso-DAP dehydrogenase, which catalyses the reaction of THDPA to meso-DAP thereby by-passing four of the steps found in the *E.coli* pathway (see Figure 1.2, Chapter 1).

In this work the first unique enzyme on the pathway, DHDPA synthase has been overexpressed in *E.coli* and purified to homogeneity. This was used to test various product analogues, examples of these are shown in Figure 3.12, Chapter 3. The 270 nm absorbing product of the DHDPA synthase assay was unknown until this investigation. The compound was identified as dipicolinic acid, DPA, which is produced by oxidation of DHDPA, the unstable product of the enzyme *in vivo*. Since DHDPA is unstable, studies on the following enzyme, DHDPA reductase were difficult because no stable substrate preparation was available (Chapter 5).

Meso-DAP dehydrogenase was also partially purified from *B.sphaericus* and the enzyme assayed in both the forward and backward directions. The forward reaction produces meso-DAP which is a major component of bacterial spores as is DPA and this is dicussed in the first part of this Chapter. The mechanism of DHDPA synthase is also summarised and future work is described.

7.2 DPA and meso-DAP- Important Products of Lysine Biosynthesis

DHDPA is unstable and has been shown to spontaneously oxidise in air to DPA (Kimura 1974). In order to investigate the substrates of the initial steps of the lysine biosynthesis pathway in *E.coli* it was necessary to isolate DHDPA synthase and DHDPA reductase. Since the product of DHDPA synthase, DHDPA, is unstable it must either be used rapidly in *E.coli* perhaps through some channelling mechanism, or

must be more stable inside the cell than it is in an assay cuvette. A channelling mechanism would imply that DHDPA synthase and reductase were organised very closely together in the bacteria so as not to allow the DHDPA to oxidise before the reductase had used it as a substrate. At the present time no evidence has been obtained for such enzyme organisation in *E.coli* (Cohen & Saint-Girons, 1987). Alternatively DHDPA may survive *in vivo* because the reducing conditions which exist within the cell prevent its spontaneous oxidation.

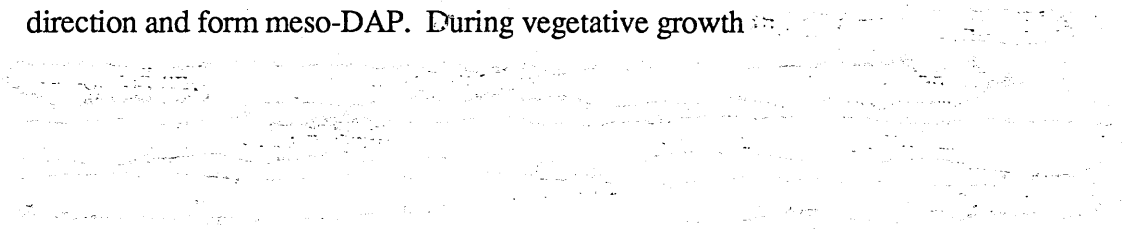
Lysine biosynthesis has also been studied in sporulating bacteria, such as Bacillus species where a major component of the spores is DPA. In these species (for example *Bacillus subtilis*) the enzyme DHDPA reductase appears to be very different from the *E.coli* enzyme. The biosynthesis of DPA seems to occur via two routes: the cyclisation and oxidation of diketopimelic acid or the oxidation of DHDPA from the lysine pathway. The route to DPA from diketopimelate involves a spontaneous condensation reaction with ammonia to produce an isomer of DHDPA which is either oxidised in air to DPA or converted enzymically to DPA (Kimura & Sasakawa, 1975). The other route, from another isomer of DHDPA which is the intermediate of the lysine pathway can be either spontaneous or enzymatic.

The enzyme catalysing the oxidation of DHDPA to DPA has been identified in sporulating bacteria as DHDPA reductase. This enzyme has been isolated and purified by Kimura and Sasakawa (1975) from *B.subtilis* and found to have FMN as a prosthetic group unlike the *E.coli* DHDPA reductase which is free of flavin (Tamir & Gilvarg, 1974). In *B.subtilis* DHDPA reductase is strongly inhibited by DPA. However the inhibited enzyme, which is a flavoprotein, can oxidise an isomer of DHDPA formed from diketopimelate and ammonia to DPA. In this reaction the DHDPA isomer appears to act as a reducing agent for FMN and is itself oxidised to DPA. The reduced FMN is reoxidised by air (Kimura & Sasakawa, 1975).

Unlike the sporulating bacteria, *E.coli* have no need to produce DPA. For the efficient production of lysine in *E.coli*, all the DHDPA should be converted to THDPA and the formation of DPA (by spontaneous oxidation) should be minimal. The conversion of DHDPA to THDPA is catalysed by a DHDPA reductase which is not a flavoprotein and uses NADPH as the reducing agent (see Chapter 6).

Another difference between *E.coli* and the sporulating bacteria and other Gram positive bacteria involves meso-DAP dehydrogenase. This enzyme (described in Chapter 6) by-passes four steps of the lysine pathway (which produce succinylated and acetylated intermediates of THDPA) and converts THDPA directly into meso-DAP. It is not found in *E.coli* but it occurs in Gram positive bacteria. Some Gram positive bacteria (for example, *B.sphaericus*) are 'good ' lysine producers (Misono & Soda, 1980), and meso-DAP is not used as a cell-wall compononent in the vegetative stage of these bacteria. However the peptidoglycan (a polysaccharide) of the cell wall of the bacterial spores does contain meso-DAP. Therefore meso-DAP is required for both lysine biosynthesis and for sporulation in these bacteria.

Meso-DAP dehydrogenase requires NADPH and ammonia to go in the reductive direction and form meso-DAP. During vegetative growth



DHDPA reductase activity may fall allowing the spontaneous conversion of DHDPA to DPA and so the two components required for sporulation are produced at the expense of lysine production.

7.3 DHDPA Synthase

Overexpression of *E.coli* DHDPA synthase was achieved by using a high copy number plasmid containing the gene encoding the enzyme. A copy number of about 200 is typical for a pUC type plasmid and this is consistent with the level of overexpression observed in strain MV1190/pDA2. The overexpression enabled large quantities of enzyme to be isolated and purified to homogeneity in less steps (Figure 3.11) than from a wild-type strain of *E.coli* (see Chapter 3).

The pure enzyme was used to screen many potential inhibitors of DHDPA synthase since it is the first unique step of the lysine pathway as described in Chapter 1. Several product analogues inhibited the enzyme relatively well (see Chapter 3) but further tests will have to be carried out to determine K_i values and the nature of the inhibition. The

compounds which are inhibitory could be useful as antimicrobial agents and may also be tested on DHDPA synthase in plants for herbicidal activity.

The M_r of the DHDPA synthase of *E.coli* is 134K (in agreement with Shedkarski & Gilvarg, 1970) and it is a tetramer of identical 33 K subunits. The wheat DHDPA synthase has a M_r of 123 K and the spinach a M_r 115 K (Kumpaisal *et al.*, 1987 and Wallsgrove & Mazelis, 1981). The genes encoding the *E.coli* (Richaud, 1986) and wheat DHDPA synthases have been cloned and sequenced and the amino acid sequences deduced. The wheat enzyme has 30% amino acid identity to the *E.coli* DHDPA synthase (Kaneko, 1990).

The mechanism of DHDPA synthase in *E.coli* has not been explored in great detail. The binding of the first substrate, pyruvate, to the active site was shown to be via Schiff's base formation (Shedlarski & Gilvarg, 1970) which was confirmed by treatment of the enzyme with pyruvate and sodium borohydride. This means that there must be a lysine residue at the enzyme's active site (Shedkarski & Gilvarg, 1970) and this is shown in Figure 3.13 in Chapter 3.

Amino acid sequence comparison of the wheat and *E.coli* enzymes showed that a lysine residue, Lys 182 in wheat and Lys 162 in *E.coli*, is conserved in both species. This lysine residue may be the active site lysine which forms the Schiff's base with pyruvate (Kaneko, 1990).

DHDPA synthase appears to work by a ping pong mechanism (see Figure 3.13, Chapter 3). An activated form of the enzyme containing bound pyruvate is formed first and this then reacts with aspartate semialdehyde (Kumpaisal *et al.*,1987).

7.4 Future Work

There are several ways in which this work could be continued. Initially the nature of the 'inhibitory' compounds of DHDPA synthase has to be investigated. This would lead to more information on the later part of the mechanism of this enzyme.

DHDPA reductase has also to be purified to homogeneity from the overproducing *E.coli* strain. A better assay is required to monitor the activity of DHDPA reductase and for use in the screenning of DHDPA analogues as inhibitors. The results of these

tests and further kinetic studies would provide information on the mechanism of DHDPA reductase.

Both DHDPA synthase and reductase have been isolated from plants (Tyagi, *et at.*,1983, and Wallsgrove & Mazelis, 1980). These two plant enzymes could therefore now be compared with the bacterial enzymes, for example the effect of the compounds that inhibit the *E.coli* enzymes could be investigated with the plant enzymes.

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