STUDIES ON DIHYDROPTERIDINE REDUCTASES

A THESIS SUBMITTED TO
THE AUSTRALIAN NATIONAL UNIVERSITY
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

SUBHASH GUPTHA VASUDEVAN

Department of Biochemistry

John Curtin School of Medical Research

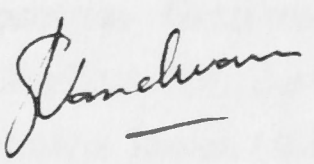
The Australian National University

Canberra

DECEMBER 1988

CERTIFICATE OF ORIGINALITY

The work described in this thesis is my own, except in specific cases where references are made in the text.



Subhash Guptha Vasudevan

ACKNOWLEDGEMENTS

It is a great pleasure to express my gratitude to Dr. Wilfred Armarego for his helpful supervision, support and encouragement throughout the course of this work. His kind and enthusiastic nature made the working environment pleasant and stimulating. Drs. Nick Dixon (Research School of Chemistry) and Chris Elvin (Bond University) are thanked for their helpful advice and guidance with the molecular biological studies in this work. I would like to express my gratitude to all the members of the Biochemistry Department for their help, and the pleasure and honour which it has been to be part of that department.

I would also like to specially thank; Dr. Denis Shaw for his advice and for carrying out the protein sequencing; Dr. John Morrison for useful discussions and gifts of enzymes and plasmids; Dr. R.G.H. Cotton (Murdoch Research Institute) for gifts of human DHPR antibodies; and Professor Robert Poole (Kings' College, London) for helpful discussions and obtaining the spectra of the haemoprotein. Gratitude is also expressed to Dr. Peter Jeffrey (analytical ultracentrifugation studies), Mr. Barry Webb (logistics), Mr Bella Paal (expert technical assistance), Mr. Lewis James (amino acid analysis), Mr. Gary Mayo (oligonucleotide synthesis) and the staff of the JCSMR Photography unit, for their contributions in this work.

The fellow students (past and present), Akiko, Hardy, Bobby, Munn, Joanne, Robert, Janet, Carolyn, Maria and Jeremy Garfield, are thanked for their unfailing support and camaraderie. The Melbourne and Cyprus connections are gratefully acknowledged for their love and friendship, which has made life 'downunder' a pleasant experience. Helen Vasudevan is specially thanked for providing the life support system during the most crucial stage of this work (the writing) and proof reading this thesis.

The love, devotion and immeasurable support of my family has enabled me to venture this far, and it is to them that I dedicate this thesis.

PREFACE

The work presented in this thesis was carried out between July 1985 and August 1988, in the Department of Biochemistry, The John Curtin School of Medical Research, and in the Research School of Chemistry, at the Australian National University. Financial support was by an Australian National University Ph.D. Scholarship. Parts of this thesis have been published and presented at conferences.

Publication

Vasudevan, S.G., Shaw, D.G. and Armarego, W.L.F. (1988) Dihydropteridine reductase from *Escherichia coli*. *Biochem.J.*, **225**, 581-588.

Vasudevan, S.G, Armarego, W.L.F., Shaw, D.C., Dixon, N.E. and Poole, R.K. (1989) A haemoglobin-like protein from *Escherichia coli*, in preparation.

Vasudevan, S.G. and Armarego, W.L.F. (1989) Dihydrofolate reductase activity of *E.coli* dihydropteridine reductase, in preparation.

Conference Papers

S.G.Vasudevan, S.G. and Armarego, W.L.F. (1986) Preliminary report of dihydropteridine reductase from *Escherichia coli*. *Proceedings of the Australian Biochemical Society,* **18**, p 17.

Vasudevan , S.G. and Armarego, W.L.F. (1986) Isolation and characterisation of dihydropteridine reductase with pterin-independent odixase activity from *Escherichia coli*. Contemporary themes in biochemistry, *Proceedings of the 4th Federation of Asian and Oceanian Biochemists Congress*, **volume 6**, Cambridge University Press, Cambridge, England, pp 80-81.

Vasudevan, S.G., Dixon, N.E. and Armarego, W.L.F. (1988) Cosmid cloning of the dihydropteridine reductase gene from *E.coli*. *Proceedings of the thirteenth annual Lorne conference on Protein structure and function,* Lorne, Australia.

ABBREVIATIONS

Amp^R, Ap^R	Ampicillin resistance
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
Bistris	Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
bp	Base pair
BSA	Bovine serum albumin
BH_4	5,6,7,8-Tetrahydrobiopterin
cDNA	Copy DNA
cpm	Counts per minute
2D	Two dimensional
dATP	Deoxyadenosine 5'-triphosphate
DEAE	Diethylaminoethyl
DHFR	Dihydrofolate reductase
DHPR	Dihydropteridine reductase
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
ε	Molar absorptivity
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
FAD, $FADH_2$	Flavin adenine dinucleotide and its reduced form
FITC	Fluorescin isothiocyanate
FNM	Flavin mononucleotide
f.p.l.c.	Fast protein liquid chromatography
GTP	Guanosine 5'-triphosphate
h	Hour
h.p.l.c.	High pressure liquid chromatography
IEF	Isoelectric focusing
IPTG	Isopropylthiogalactoside
Kb	Kilobase
kD	KiloDaltons
K_d	Binding constant
Ki	Inhibition constant
Km	Michaelis constant
LB	Luria-Bertani
2-Me	2-Methyl

MHPA	Malignant hyperphenylalaninaemia
M_r	Relative molecular weight
MTT	(3-[4,5-Dimethylthiazol-2-yl])-2,5-diphenyltetrazolium bromide (Thiazolyl blue)
NAD^+, NADH	Nicotinamide adenine dinucleotide and its reduced form
$NADP^+$, NADPH	Nicotinamide adenine dinucleotide phosphate and its reduced form
NEM	*N*-Ethylmaleimide
NMR	Nuclear magnetic resonance
OD	Optical density
PA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PAH	Phenylalanine hydroxylase
PBS	Phosphate buffered glycol
PCMB	*p*-Chloromercuribenzoic acid
PEG	Polyethylene glycol
PH_4	Tetrahydropteridine
pI	Isoelectric point
PKU	Phenylketonuria
PMSF	Phenyl methyl sulphonyl fluoride
P:O	Phosphate oxygen
PTH	Phenylthiohydantoin
q-BH_2	Quinonoid 7,8-dihydro(6*H*)biopterin
q-PH_2	Quinonoid dihydropteridine
RF	Relative mobility
RFLP	Restriction fragment linked polymorphism
RNA	Ribonucleic acid
s	Second
SDS-PAGE	Sodium dodecyl sulfate-PAGE
SHMT	Serine hydroxymethyl transferase
ss	Single-stranded
SSC	Buffer containing NaCl and sodium citrate
TBE	Buffer containing Tris, boric acid and NaCl
TBS	Buffer containing Tris, boric acid and EDTA
TE	Buffer containing Tris and EDTA
TEMED	*N,N,N',N'*-Tetramethylethylenediamine
Tet^R	Tetracycline resistance
t.l.c.	Thin Layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
TTBS	Buffer containing Tween-20, Tris, boric acid and EDTA

u.v.	Ultraviolet
V_{max}	Maximum velocity
X-gal	5-Bromo-4-chloro-3-indolyl-ß-galactoside

ABBREVIATIONS FOR AMINO ACIDS

AMINO ACID	THREE LETTER ABBREVIATION	ONE LETTER SYMBOL
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ABSTRACT

The enzyme dihydropteridine reductase (DHPR) plays the crucial role of recycling quinonoid dihydropterins to tetrahydropterins, which are cofactors for the aromatic amino acid hydroxylases. DHPR from *Escherichia coli* K12, first reported in this study, was investigated so that it could be used as a good model system for revealing alternative role(s) of this ubiquitous reductase in a cell that is devoid of aromatic amino acid hydroxylases.

The reducase from *E coli* was initially purified to apparent homogeneity in four steps by ammonium sulfate fractionation, followed by anion exchange chromatography through DE52 DEAE-cellulose and f.p.l.c Mono-Q columns, and finally by chromofocusing with a f.p.l.c Mono-P column. The protocol was developed based on experiences of other workers in purifications of DHPRs from several sources. The yield and stability of this bacterial reductase was conciderably improved by replacing Tris buffer with Hepes buffer at pH 7.4 and using a three-step purification method involving a flat bed isoelectric focusing step.

Unlike the reductases from other sources, the native enzyme from *E. coli* is a monomer with a molecular weight of about 27 000. The purified enzyme is a flavoprotein containing tightly bound (non-covalent) FAD, which endows the protein a yellow colour when concentrated. This is the first report of a DHPR with a prosthetic group. Consequently, artificial electron acceptors such as $K_3Fe(CN)_6$ tetrazolium-MTT are substrates, in a non-pterin dependent oxido-reductase reaction catalysed by the protein. The compound $K_3Fe(CN)_6$ exhibits saturation kinetics. In addition, the bacterial enzyme can also reduce 7, 8-dihydrofolate, a function that is normally unique to dihydrofolate reductases (DHFRs) and some bifunctional enzymes that possess both DHFR and thymidylate synthase activities. The enzymes DHPR and DHFR catalyse *superficially* similar reactions; the former reduces the pterin ring by transferring the hydride from the

pyridine nucleotide cofactor to position N-5, whereas in the latter case the hydride is transferred to position C-6. The ability to transfer the hydride to both positions is unique to the reductase from this study. This apparent lack of discrimination between N-5 and C-6 of the pterin ring suggests that the FAD prosthetic group may mediate the transfer of hydride. Preliminary kinetic data and the use of a site-specific inhibitor of FAD, support the veiw that FAD is involved in the catalysis. Hence it is proposed that the reaction catalysed by the enzyme may occur by the "ping-pong" mechanism.

The reductase from *E coli* has an isoelectric point of 5.3, an unprotected amino terminus and a pterin-binding site that is recognised by an anti-idiotype (pterin) antibody. It is also established that L-*threo* neopterin (monapterin) is the most abundant naturally-occurring pterin in the organism.

The cosmid cloning approach was explored in attempts to isolate the gene that encodes *E coli* DHPR. A clone (WA266) that exhibited a six-fold increase of DHPR specific activity compared to the background (wild-type) strain was investigated further. Two soluble proteins with molecular weights of about 45 000 and 47 000 were expressed in high levels under the direction of the plasmids pWA 266 (from the primary clone) and pWA 406 (subclone). The two proteins were partially purified and identified by *N*-terminal amino acid sequencing in order to investigate the suspicion that a fusion protein was inadvertently formed. The larger protein was serine hydroxymethyl transferase (SHMT) which was encoded by the *glyA* gene (Plamann *et al.*, 1983). The smaller protein was the previously described product of an unnamed gene which was adjacent to the *glyA* gene, but transcribed in the opposite direction (Plamann and Stauffer, 1983). The *N*-terminal sequence of the smaller protein showed extensive homology with that of haemoglobin from the filamentous myxobacterium *Vitreoscilla*, which has molecular weight of about 18 000 (Wakabayashi *et al.*, 1986).

The visible absorption spectra of the 45-kD protein are characteristic of a CO-binding *b*-type haem containing protein, that is high-spin in the reduced state. The gene that codes for the 45-kD protein is designated as *hmp* (for haemoprotein) and is located near the 55-min region of the *Escherichia coli* linkage map. The *in vivo* function of this soluble haemoprotein is not clear but based on the indirect evidence obtained from immunochemical investigations, this newly identified protein has a pterin binding site and is probably responsible for the six-fold increase in DHPR specific activity in the cosmid clone.

TABLE OF CONTENTS

		page
Title		i
Certificate of originality		ii
Acknowledgements		iii
Preface		iv
Abbreviations		v
Abstract		ix
CHAPTER 1 GENERAL INTRODUCTION		
1.1	DISCOVERY OF DIHYDROPTERIDINE REDUCTASE	1
1.2	ASPECTS OF PTERIDINE CHEMISTRY AND BIOLOGY	4
1.3	THE AROMATIC AMINO ACID HYDROXYLATION PATHWAY	5
1.4	CLINICAL SIGNIFICANCE	7
1.4.1	Classical PKU	8
1.4.2	Malignant hyperphenylalaninaemia (MHPA)	9
1.5	OCCURRENCE AND DISTRIBUTION OF DHPR	11
1.6	ASSAYS FOR DHPR	14
1.7	SUBSTRATES FOR DHPR	16
1.7.1	Structure-activity relationship of pterin cofactors	17
1.7.2	Pyridine nucleotide substrates for DHPR	20
1.8	PHYSICAL PROPERTIES OF DHPR	20
1.8.1	Molecular size and subunit structure	20
1.8.2	Isoelectric point	21
1.8.3	Amino acid composition	22
1.9	INHIBITORS OF DHPR	22
1.10	MECHANISM OF DHPR CATALYSIS	25
1.11	DIHYDROFOLATE REDUCTASE (DHFR)	25
1.12	STRUCTURE OF DHPR	27
	AIM OF THIS RESEARCH PROGRAMME	28

page

CHAPTER 2 PURIFICATION OF DIHYDROPTERIDINE REDUCTASE FROM *ESCHERICHIA COLI*

2.1 INTRODUCTION 30

2.2 RESULTS AND DISCUSSION 32

2.3 SUMMARY 40

2.4 MATERIALS 40

2.5 EXPERIMENTAL METHODS 41

2.5.1 Measurement of enzyme activity 41

2.5.1.1 The DHPR assay 41

2.5.1.2 The oxido-reductase assay 42

2.5.2 Protein determination 42

2.5.3 Electrophoretic methods 43

2.5.3.1 SDS-PAGE 43

2.5.3.2 Non-denaturing - PAGE and DHPR activity stain 43

2.5.3.3 Two-dimensional gel electrophoresis 44

2.5.3.4 Silver stain of protein gels 45

2.5.4 Growth of cells 46

2.5.5 Preparation of cell-free extract 47

2.5.6 Ammonium sulfate fractionation 47

2.6 DEVELOPMENT OF PURIFICATION PROCEDURE 47

2.6.1 Naphthoquinone affinity chromatography 48

2.6.2 Screening with dye Matrex columns 49

2.6.3 DE 52 anion-exchange chromatography 49

2.6.4 Ultragel AcA-34 gel filtration 49

2.6.5 F.p.l.c Superose 12 gel filtration 49

2.6.6 F.p.l.c Mono-Q anion exchange chromatography 50

2.6.7 F.p.l.c Mono-P chromatography 50

2.6.8 DEAE-Fractogel chromatography 50

2.6.9 Preparative granulated bed isoelectric focusing 51

page

CHAPTER 3 PROPERTIES OF *ESCHERICHIA COLI* DHPR

3.1 INTRODUCTION 52

3.2 RESULTS AND DISCUSSION 53

3.2.1 Enzyme activities of purified *E. coli* DHPR 53

3.2.2 Molecular weight of the reductase 56

3.2.3 *N*-terminal sequence of the reductase 58

3.2.4 Amino acid analysis 60

3.2.5 Identification of prosthetic group 61

3.2.6 Naturally occurring pterins in *E. coli* 62

3.2.7 Activity of substrates 64

3.2.8 Effect of methotrexate 66

3.2.9 Potential substrates and the effects of pterins on oxido-reductase activity 67

3.2.10 An active site-directed suicide inhibitor 69

3.2.11 Immunoreactivity of *E. coli* DHPR 71

3.3 SUMMARY 72

3.4 MATERIAL AND METHODS 74

3.4.1 Dihydrofolate reductase assay 74

3.4.2 Molecular weight determination 74

3.4.2.1 Gel filtration method 74

3.4.2.2 Analytical ultracentrifugation 75

3.4.3 *N*-terminal amino acid analyses 76

3.4.4 Amino acid analysis 76

3.4.5 Identification of the prosthetic group 77

3.4.6 Stoichiometry of NADH oxido-reductase activity 77

3.4.7 Analysis of naturally-occurring pterins 78

3.4.8 Activity of substrate and kinetic analysis 78

3.4.9 Synthesis of dimethylpropargylamine 79

page

3.4.10 Inhibition by dimethylpropargylamine 80

3.4.11 Effect of tetrahydropterin on pterin-independent oxido-reductase activity 80

3.4.12 Non-denaturing PAGE and activity staining of gels 80

3.4.13 Protein-blotting and *N*-terminal amino acid sequencing 81

3.4.14 Enzyme-linked immunosorbent assay (ELISA) 83

3.4.15 Raising polyclonal antibodies to *E. coli* DHPR 84

3.4.16 Western blot analysis 84

CHAPTER 4 ATTEMPTS TO ISOLATE THE GENE ENCODING *E. COLI* DHPR BY COSMID CLONING

4.1 INTRODUCTION 86

4.2 RESULTS AND DISCUSSION 87

4.2.1 Cosmid cloning 87

4.2.2 Screening directly for increased DHPR specific activity 91

4.2.3 Comparison of plasmids pWA 265 and 266 92

4.2.4 Sub-cloning the gene for *E. coli* DHPR 92

4.2.5 Analysis of the gene product 95

4.2.6 Protein synthesis directed by plasmid DNA 97

4.3 SUMMARY 98

4.4 MATERIALS 99

4.4.1 Enzymes antibiotics and compounds 99

4.4.2 Media used for growing bacteria 99

4.5 METHODS 100

4.5.1 Preparation of chromosomal DNA 100

4.5.2 *Sau* 3A partial digests of bacterial DNA 101

4.5.3 Restriction endonuclease digestion of DNA 102

4.5.4 Agarose gel electrophoresis 103

page

4.5.5 Dephosphorylation of DNA 103

4.5.6 Phenol extraction of DNA 104

4.5.7 Ethanol precipitation of DNA 104

4.5.8 Ligation of DNA 105

4.5.9 Packing *in vitro* of concatemers into bacteriophage λ particles 105

4.5.10 Transfection of packaged extract 106

4.5.11 Construction of the *rec* A derivative of *E. coli* strain D3-157 106

4.5.12 Direct screening for increased DHPR specific activity 107

4.5.13 Preparation of plasmid DNA 108

4.5.14 Rapid small scale isolation of plasmid DNA 109

4.5.15 Transformation of competent cells with plasmid DNA 110

4.5.16 Mini-cell isolation and *in vivo* protein synthesis 111

CHAPTER 5 IDENTIFICATION AND CHARACTERIZATION OF THE TWO MAJOR PROTEINS SYNTHESIZED UNDER THE DIRECTION OF PLASMIDS pWA 266 AND p WA 406

5.1 INTRODUCTION 113

5.2 RESULTS AND DISCUSSION 114

5.2.1 Partial purification and identification of 45-kD and 47-kD polypeptides 114

5.2.2 Restriction mapping of plasmid pWA 406 121

5.2.3 Oligonucleotide probing and DNA sequencing of 5' region of the gene responsible for coding the 44-kD protein 122

5.2.4 Characterization of the 44-kD protein by absorption spectroscopy 127

5.2.5. Is the increased DHPR specific activity in WA 266 due to the 44 kD haemoprotein? 131

page

5.3 SUMMARY 132

5.4 EXPERIMENTAL METHODS 134

5.4.1 Partial purification of protein from WA 266 extract 134

5.4.2 Isolation of DNA fragments from agarose gel 134

5.4.3 Preparation of synthetic oligonucleotides 135

5.4.4 End-labelling of oligonucleotide with [γ-^{32}P] ATP 135

5.4.5 Southern blot transfer of DNA 137

5.4.6 Hybridization of Southern and dot blot filters 138

5.4.7 Dideoxy sequencing of DNA 139

5.4.7.1 Composition of sequencing gels 139

5.4.7.2 Inserting DNA into M13 bacteriophage replicative form 140

5.4.7.3 Transformation of *E. coli* strain JM101 with M13 phage replicative form 140

5.4.7.4 Preparation of single stranded template DNA 141

5.4.7.5 Annealing, polymerisation and gel-electrophoresis of sequencing reactions 142

5.4.8 Phage dot-blot hybridization 144

5.4.9 Complementation of *gly*A mutation in GS 245 144

CHAPTER 6 IN SEARCH OF THE GENE THAT ENCODES THE 27-kD DHPR FROM *ESCHERISCHIA COLI*

6.1 INTRODUCTION 146

6.2 RESULTS AND DISCUSSION 147

6.2.1 Dot blot hybridization of cosmid pools 147

6.2.2 Direct screening for DHPR specific activity 150

6.3 SUMMARY 150

page

6.4 METHODS 151

6.4.1 Preparation of cosmid pools 151

6.4.2 Dot blot hybridization of cosmid pools 151

6.4.3 Complementation of *fol* mutation in strain D 3-157 152

CHAPTER 7 GENERAL DISCUSSION 153

REFERENCES 157

CHAPTER 1

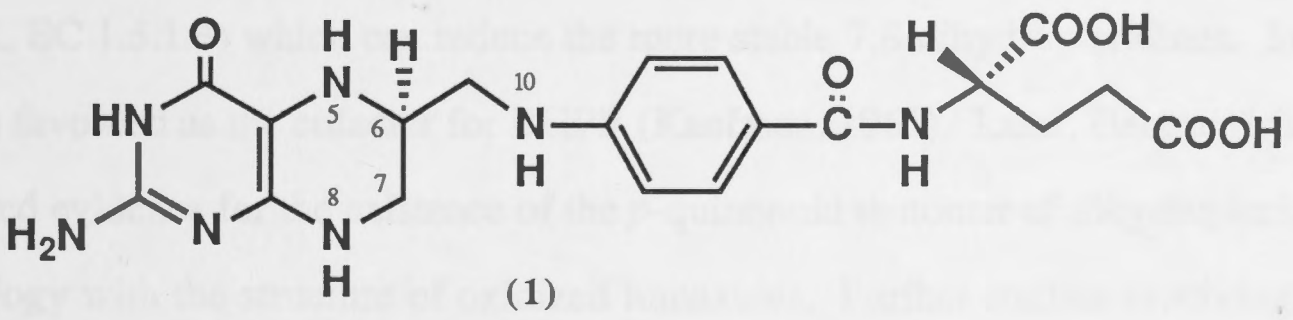
GENERAL INTRODUCTION

1.1 DISCOVERY OF DIHYDROPTERIDINE REDUCTASE

The conversion of phenylalanine into tyrosine was demonstrated *in vitro* as early as 1913 (Embden and Baldes, 1913) but the details of the reaction remained obscure for a further half a century. Except for the brief respite where it was wrongly shown that the soluble rat-liver extract contained an enzyme that carried out the conversion of phenylalanine into tyrosine in the presence of NAD^+ and an alcohol or aldehyde (Udendfriend and Cooper, 1952); the classic work describing the hydroxylation of phenylalanine into tyrosine which ultimately led to the discovery of *dihydropteridine reductase* (DHPR; Enzyme commission number, EC 1.6.99.7) was carried out in the laboratory of Seymour Kaufman. Kaufman (1957) showed that the conversion of phenylalanine into tyrosine required two separate ammonium sulfate aggregated fractions from the rat-liver extract, neither of which was active on its own and that the system required a reduced pyridine nucleotide to donate electrons. Fortunately the sheep-liver *extract* unlike the sheep-liver *homogenate* could not carry out the conversion of phenylalanine into tyrosine. This was because one of the components of the hydroxylating system which was present in the sheep-liver homogenate was not readily solubilized. The sheep-liver extract replaced the activity of the higher ammonium sulfate fraction from the rat-liver extract. In the system where the extracts from two animal sources were used to convert phenylalanine into tyrosine, NADPH was found to be the preferred pyridine nucleotide. With the information that phenylalanine stimulated the oxygen uptake of a dog-liver extract (Lang and Westphal, 1942) in hand, an overall equation for the conversion of phenyalanine into tyrosine was postulated;

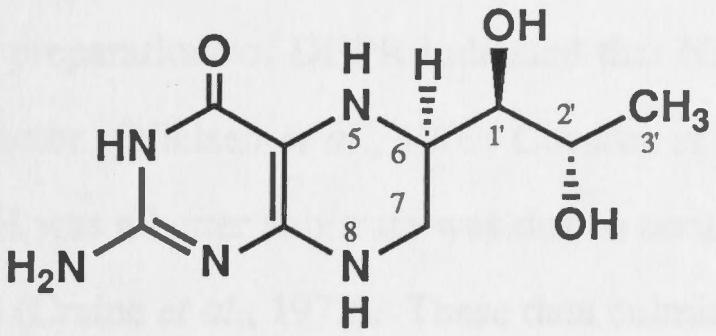
$$NADPH + H^+ + O_2 + \text{phenylalanine} \longrightarrow NADP^+ + H_2O + \text{tyrosine}$$

The demonstrated requirement of O_2 and NADPH in the hydroxylation reaction indicated that the hydroxylase is a mixed-function oxidase and that the phenolic oxygen in tyrosine was probably derived from molecular oxygen. Evidence for this came from carrying out the hydroxylation in the presence of O_2^{18} or H_2O^{18}, where tyrosine was only labelled in the presence of the former. During preliminary kinetic analysis of the hydroxylase it became evident that another factor that could be oxidized by air was also required for the conversion of phenylalanine into tyrosine. Direct evidence for the involvement of a cofator other than NADPH was gleaned during attempts to further purify the rat liver enzyme. The hydroxylase activity was completely lost and was only restored when boiled rat-liver extract was included in the assay. Early chemical evidence suggested that the non-protein component in the boiled rat-liver extract might be a pteridine. The well known pteridine cofactor in several biological reactions, 5,6,7,8-tetrahydrofolate (1) exhibited cofactor activity when included in the assay, whereas folic acid, 5-formyltetrahydrofolate and 7,8-dihydrofolate were inactive. Chemical studies of



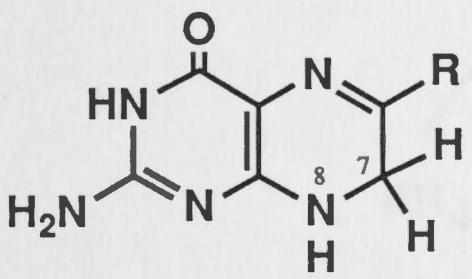
(1)

the natural cofactor indicated that it did not contain the *p*-aminobenzoylglutamate side chain. By a series of elegant experimental studies it was showed that the 'labile' rat-liver enzyme was phenylalnine hydroxylase (PAH), the 'stable' sheep-liver enzyme was dihydropteridine reductase (DHPR) and the cofactor in the boiled extract was the unconjugated pteridine 5,6,7,8-tetrahydrobiopterin (2). The last of these discoveries was significant because it was the first report of a biochemical role for an unconjugated pteridine (Kaufman, 1957,1958 a,b, 1959, 1961, 1963; Kaufman *et al.*, 1962).

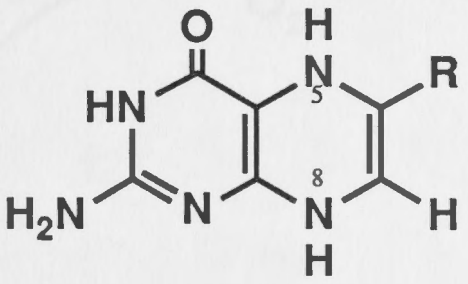


(2)

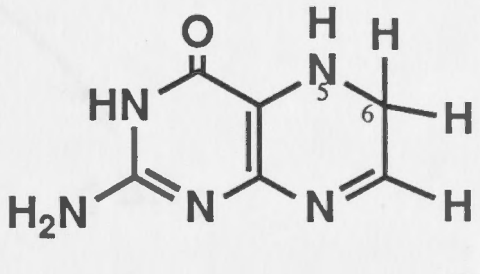
Several tautomers of dihydropteridine were postulated as possible oxidation products of the phenylalanine hydroxylase catalysed reaction, hence substrates for DHPR (3-5).



(3)

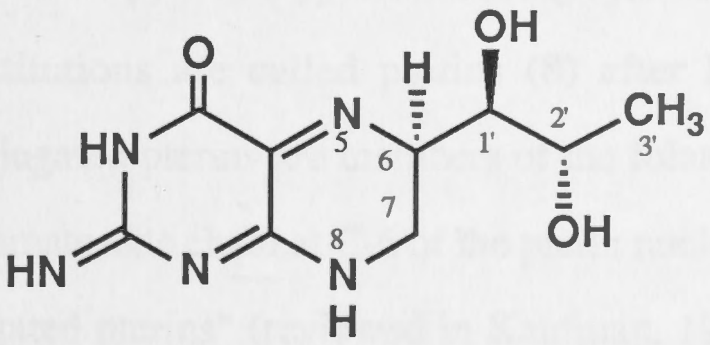


(4)



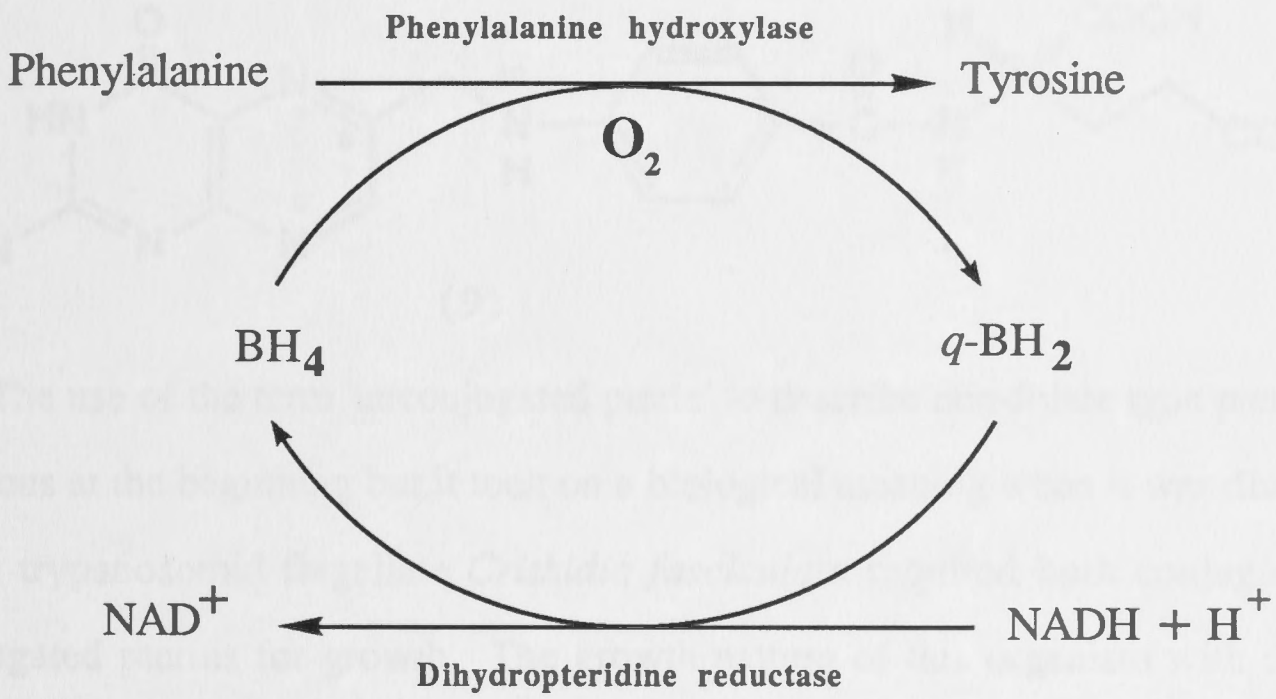
(5)

Structures (3) and (4) were eliminated on the basis of chemical studies, and it was realised that the sheep-liver enzyme was contaminated with dihydrofotate reductase (DHFR, EC 1.5.1.3) which can reduce the more stable 7,8-dihydropteridines. Structure (5) was favoured as the cofactor for DHPR (Kaufman, 1961). Later, Hemmerich (1963) presented evidence for the existence of the *p*-quinonoid tautomer of dihydropteridine (6) by analogy with the structure of oxidized lumazines. Further studies involving tritium labelling experiments enabled Kaufman (1964) to show that the dihydropteridine product of the hydroxylase reaction and hence the substrate for DHPR, was a *p*-quinonoid dihydropteridine (6).



(6)

Work with purer preparations of DHPR indicated that NADH was the preferred pyridine nucleotide cofactor (Nielsen *et al.*, 1969; Cheema *et al.*, 1973). The earlier observation that NADPH was a better substrate was due to contamination by DHFR in the enzyme preparations (Craine *et al.*, 1972). These data culminated in the proposition of Scheme 1.1 for the complex phenylalanine hydroxylation system and the discovery of dihydropteridine reductase..

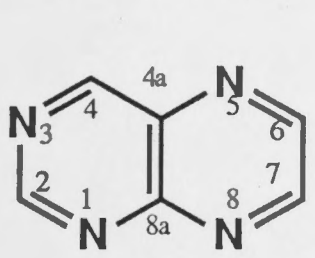


Scheme 1.1 The phenylalanine hydroxylation system (Craine *et al.*, 1972)

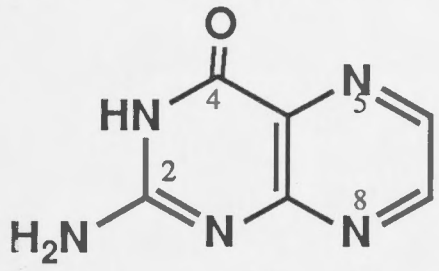
1.2 ASPECTS OF PTERIDINE CHEMISTRY AND BIOLOGY

Pteridines (7) are fused pyrazino-pyrimidine ring systems and pteridines with 2-amino and 4-oxo substitutions are called pterins (8) after Pfleiderer's suggestion (Pfleiderer, 1964). Conjugated pterins are members of the folate series (9) that contain the *p*-aminobenzoylglutamate side chain at C-6 of the pterin nucleus; all other pterins are referred to as "unconjugated pterins" (reviewed in Kaufman, 1967a). Since biological roles for conjugated pterins have been known and studied for a long time, this class of pterins is still referred to as the folates. The biologically active tetrahydrofolates exist in

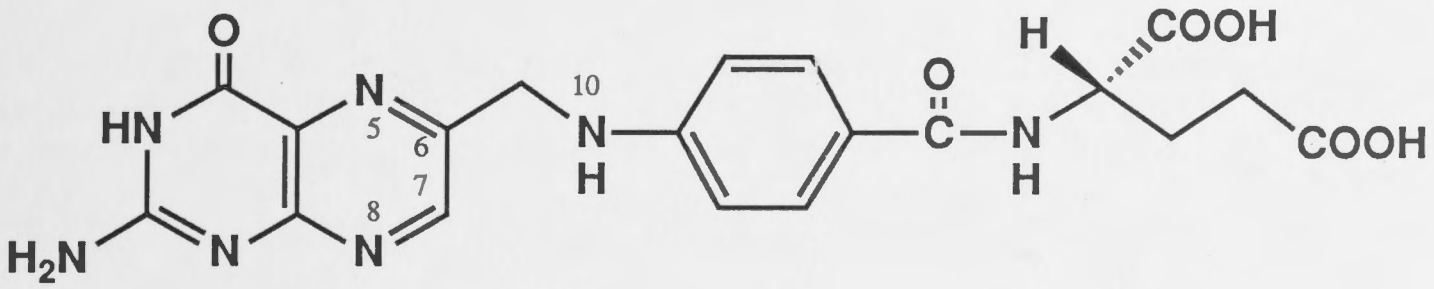
several oxidation levels by substitutions at N-5 and/or N-10 so that they can carry out their crucial role in several biosynthetic pathways by donating a carbon atom, including in the *de novo* biosynthesis of purines and thymidine (reviewed in Benkovic, 1980).



(7)



(8)

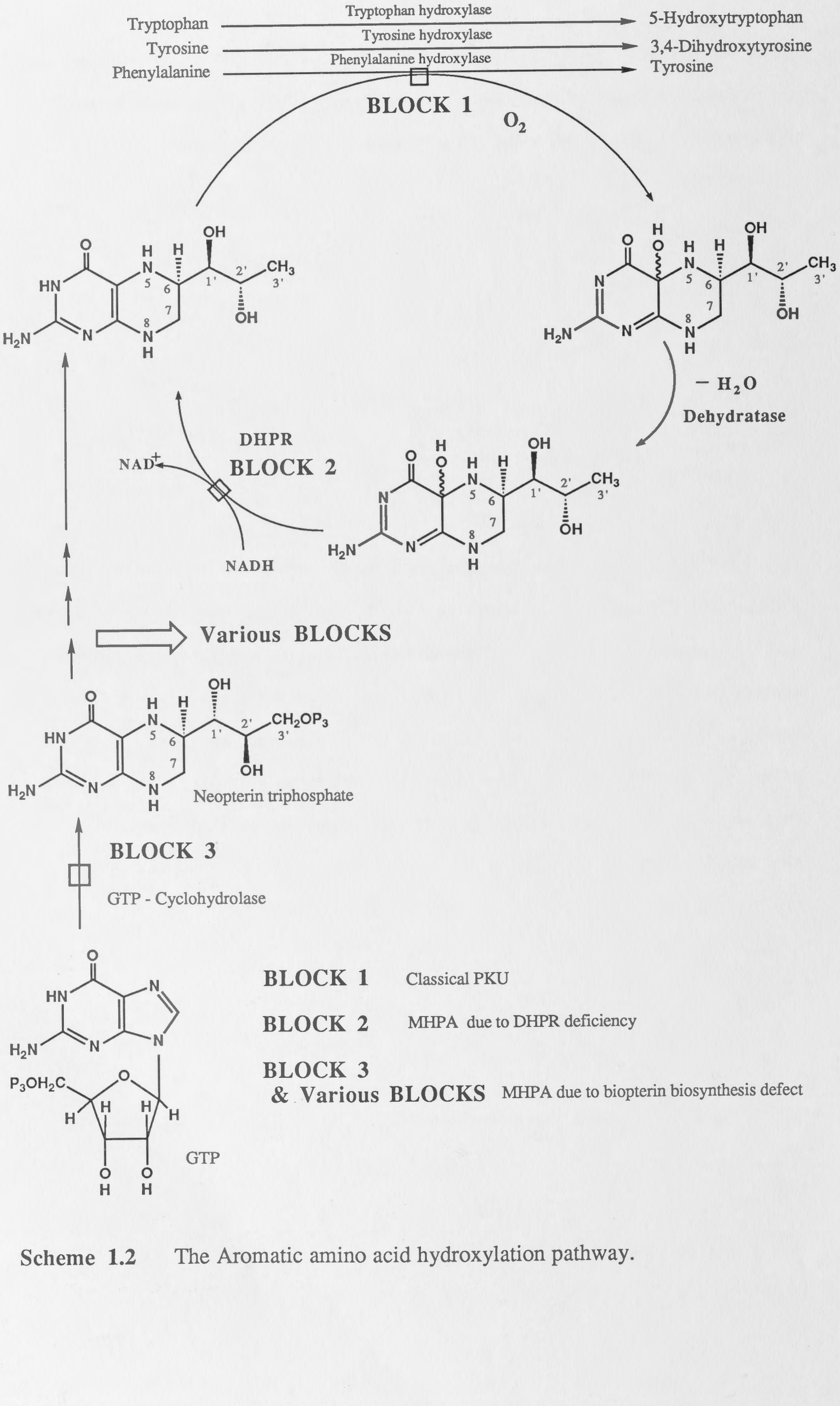


(9)

The use of the term 'unconjugated pterin' to describe non-folate type pterins was ambiguous at the beginning but it took on a biological meaning when it was discovered that the trypanosomid flagellate *Crithidia fasciculata* required both conjugated and unconjugated pterins for growth. The growth pattern of this organism with different types of pterins has become a sensitive assay for identification of naturally occurring pterins (e.g., L-erythrobiopterin, D-erythroneopterin, L-threobiopterin). The structure of the phenylalanine hydroxylase cofactor, L-*erythro*-tetrahydrobiopterin (2) was identified, after oxidation, by the *Crithidia* assay. This assay still remains as one of the most sensitive assays for biopterin, albeit the most time consuming (Dewey and Kidder, 1971).

1.3 THE AROMATIC AMINO ACID HYDROXYLATION PATHWAY

The discovery that phenylalanine hydroxylase required a pterin cofactor was thought to be a strange phenomenon especially since it was an oxygen-activated system. The purification and characterisation of tyrosine and tryptophan hydroxylases gave further evidence that oxygen-activated aromatic amino acid hydroxylases required a tetrahydropterin cofactor (Nagatsu *et al.*, 1964; Friedman *et al.*, 1972).



BLOCK 1 Classical PKU

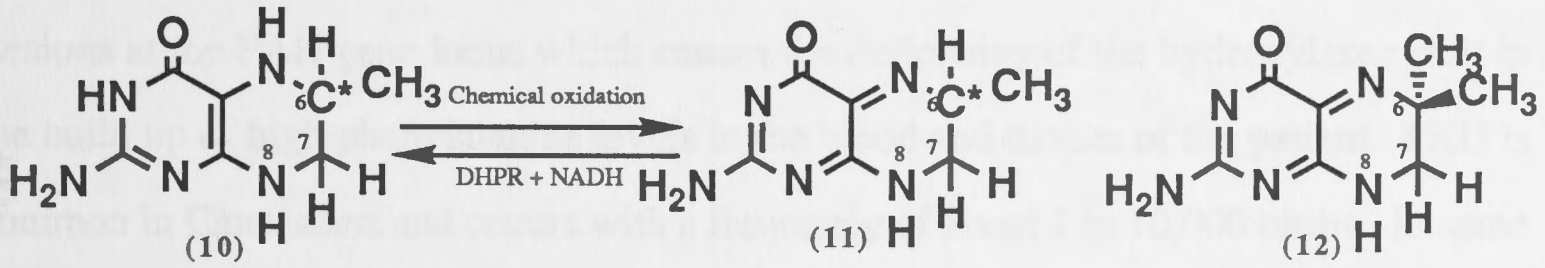
BLOCK 2 MHPA due to DHPR deficiency

BLOCK 3
& Various BLOCKS MHPA due to biopterin biosynthesis defect

Scheme 1.2 The Aromatic amino acid hydroxylation pathway.

Tetrahydrobiopterin was also the natural cofactor for tyrosine and tryptophan hydroxylases (see Scheme 1.2). The reactions catalysed by these two aromatic amino acid hydroxylases are important because they are the rate-limiting steps in the biosynthesis of neurotransmitters such as *dopamine*, *norepinephrine*, *serotonin* and *indolamine*.

^{13}C NMR studies of the pterin cofactor during the conversion of phenylalanine into tyrosine indicated that when 90% of tyrosine was formed, the predominant pterin was a 4a-carbinolamine which readily loses a molecule of water non-enzymatically (Lazarus *et al.*, 1982) to form the quinonoid dihydropterin which is the substrate for DHPR. Further evidence favouring the quinonoid dihydropterin structure as substrate for DHPR includes the preservation of the chiral centre* at C-6 of the pterin nucleus during the *in situ* oxidation of tetrahydropterin (10) and the subsequent reduction of the oxidized dihydropterin (11) by DHPR in the presence of NADH (Hasegawa *et al.*, 1979). The fact that quinonoid 6,6-dimethyldihydropterin (12) was a substrate for DHPR, provided added proof for the quinonoid species (Armarego and Waring, 1983).



(10) (11) (12)

The removal of H_2O from the primary product formed during the hydroxylase reaction to give the quinonoid dihydrobiopterin can also be catalysed by a dehydratase enzyme found in liver (Huang *et al.*, 1973 and Lazarus *et al.*, 1983; see Scheme 1.2). The natural tetrahydropterin cofactor for the aromatic amino acid hydroxylases can be biosynthesized in several organisms including man by apparently similar routes (Curtius *et al.*, 1985; Nichol *et al.*, 1985). The confirmation that the primary precursor for tetrahydrobiopterin biosynthesis is guanosine triphosphate (GTP) was evidenced by the ability of several organisms to convert [^{14}C] guanosine into [^{14}C] tetrahydrobiopterin, whereas [2-^{14}C] folic acid was not converted into [^{14}C] tetrahydrobiopterin. The first

step towards the synthesis of tetrahydrobiopterin involves a complex ring opening of the guanosine ring, elimination of C-8 and a ring closing reaction that can be catalysed by the enzyme GTP-cyclohydrolase (Fan and Brown, 1976; Blau and Niederwiesser, 1983). The product of this reaction is dihydroneopterin triphosphate and the mechanism of its subsequent conversion to tetrahydrobiopterin by a series of intramolecular rearrangements, elimination and reduction reactions, is still not completely clear. However, the involvement of DHFR in the biosynthesis was precluded because tetrahydrobiopterin synthesis was demonstrated *in vivo* when the reductase activity was inhibited by methotrexate (Duch *et al.*, 1983).

1.4 CLINICAL SIGNIFICANCE

The observation by Folling (1934) that urine samples of mentally retarded patients had high levels of phenylpyruvate, which is a breakdown product of phenylalanine, marked the discovery of the metabolic disorder Phenylketonuria (PKU). The cause of this genetic disorder which is transmitted in an autosomal recessive manner is the deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH; see Scheme 1.2). Lesions at the PAH gene locus which causes the deficiency of the hydroxylase result in the build up of high phenylalanine levels in the blood and tissues of the patient. PKU is common in Caucasians and occurs with a frequency of about 1 in 10,000 births. In some communities the ratio can be as high as 1 in 6,000, e.g. in Ireland (Tourian and Sidbury, 1983). The wide range of elevated levels of phenylalanine in the blood of PKU sufferers (from 2.5 mg to 60 mg/100 ml) indicated the heterogenous nature of the disease (Trefz *et al.*, 1985). The complexity of the phenylalanine hydroxylation system also indicated the possibility of several biochemical lesions that could result in PKU-like symptoms (see the Block sites in Scheme 1.2). It is now known that the lesion of PAH results in classical PKU and lesion of DHPR or the enzymes involved in biosynthesis of tetrahydrobiopterin results in PKU variants known as malignant hyperphenylalaninaemia (MHPA; Blocks 2,3 etc. in Scheme 1.2).

1.4.1 Classical PKU

Since 1965 most western countries have used a routine assay known as the Guthrie inhibition assay (Guthrie and Susie, 1963) to screen newborn infants for increased phenylalanine levels (>2.5 mg/100 ml blood). Guthrie-positive newborn infants are further investigated by direct determination of the level of serum phenylalanine and a phenylalanine load test (reviewed by Cotton, 1985). Very little was known about PKU at the molecular level because phenylalanine hydroxylase is quite labile and hence difficult to isolate and study. However, the cloning and sequencing of the full-length human PAH cDNA (Kwok *et al.*, 1985) has contributed immensely to the understanding of classical PKU. Restriction fragment linked polymorphism (RFLP) analysis of a PKU population identified twelve RFLP haplotypes, of which four mutations accounted for 90% of PKU cases in a sample population (Konecki *et al.*, 1988). This finding readily explains the reasons for the wide range of elevated levels of phenylalanine in blood and serum of positive PKU cases and also can serve as an excellent complement to confirm PKU positives that are identified by the Guthrie test. RFLP haplotype information has the potential of being used for neonatal screening of high risk groups and also for the purpose of identifying 'carriers' in the general population. A further development in this direction has been made by the demonstration that subnanogram quantities of DNA can be amplified by the Polymerase Chain Reaction and analysed for RFLP haplotypes (DiLella *et al.*, 1988).

The clinical symptoms as a result of high levels of phenylalanine in blood and tissues of classical PKU sufferers range from the impairment of their mental ability, by lowering the level of other aromatic amino acids available for the normal development of the brain, to inhibition of protein synthesis, especially of those proteins involved in myelination, hence causing disruption of the whole neuronal circuitry (Davison, 1973). Classical PKU has been controlled to a large extent by placing the affected infant on a strict phenylalanine-free diet (Bickel *et al.*, 1953). When the dietary treatment is

commenced before the affected infant is 3 weeks of age and continued for at least the first decade of its life, brain damage and other associated symptoms are effectively prevented (reviewed in Cotton, 1985).

1.4.2 Malignant hyperphenylalaninaemia (MHPA)

When all the components of the phenylalanine hydroxylating system were elucidated (Kaufman, 1967b), it became apparent that other forms of PKU that are not due to lesions of PAH can exist, . However, it was not until 1974-75 that several reports about PKU variants that were not responding to dietary treatment started to appear in the literature (Bartholome, 1974). The first confirmed report of MHPA was a patient lacking in DHPR (Kaufman *et al.*, 1975). This patient showed no DHPR activity when tissue samples were assayed under conditions that can detect 1% of normal DHPR acitivity. Antibodies raised against sheep-liver DHPR which gave a precipitin line with control human-liver extracts did not give any precipitin line with the extract from the patient's liver (Milstein and Kaufman, 1975). In addition it was found that the phenylalanine hydroxylase activity in the cases where there were lesions in DHPR or the enzymes involved in biosynthesis of tetrahydrobiopterins was depressed to about 20% of the normal activity. Scheme 1.2 clearly shows that DHPR and tetrahydrobiopterin are involved in the reactions catalysed by tyrosine and tryptophan hydroxylases, which are rate limiting steps for the biosynthesis of the neurotransmitters. Therefore it is not surprising that biochemical lesions that cause MHPA result in severe neurological deterioration and death by the time the patient is 6-7 years of age (Danks *et al.*, 1978). Several cases of MHPA due to tetrahydrobiopterin deficiency have been identified and in most cases the biochemical lesions have been related to deficiency of enzyme(s) involved in conversion of dihydroneopterin triphosphate to tetrahydrobiopterin (reviewed by Cotton, 1985). One case of tetrahydrobiopterin deficiency due to lack of GTP-cyclohydrolase has also been identified (Niederwieser *et al.*, 1984).

On the diagnosis front, Guthrie-positive infants that are suspected MHPA sufferers are further tested to establish the type of biochemical deficiency. The sensitivity of the DHPR assay has allowed direct measurement of the reductase activity in cultured skin fibroblasts, continuous lymphoid cells, erythrocytes and liver biopsies. This has been useful to determine if the patient suffers from MHPA due to DHPR deficiency. Levels of pterins in the serum of patients are also a good indicator of the type of biochemical defect. DHPR deficiency is normally characterised by high levels of dihydroxanthopterin, neopterin and biopterin with almost negligible levels of tetrahydrobiopterin. Defects in the biosynthesis of the pterin cofactor on the other hand result in a high neopterin to biopterin ratio in serum when the defect is due to enzyme(s) involved in converting dihydroneopterin triphosphate into tetrahydrobiopterin; and an overall decrease in levels of neopterin, biopterin and other pterins when the defect is due to deficiency in GTP-cyclohydrolase activity (reviewed by Cotton, 1985). The cloning and sequencing of the cDNA of human liver DHPR (Dahl *et al.*, 1987; Lockyer *et al.*, 1987) and the RFLP haplotype analysis of mutant genes (Dahl *et al.*, 1988) introduces the possibility of neonatal screening for DHPR deficiency in high-risk pregnancy cases. These new tools could also help in the early identification of the different MHPA types so that more progress could be made in the treatment of patients suffering from the syndrome.

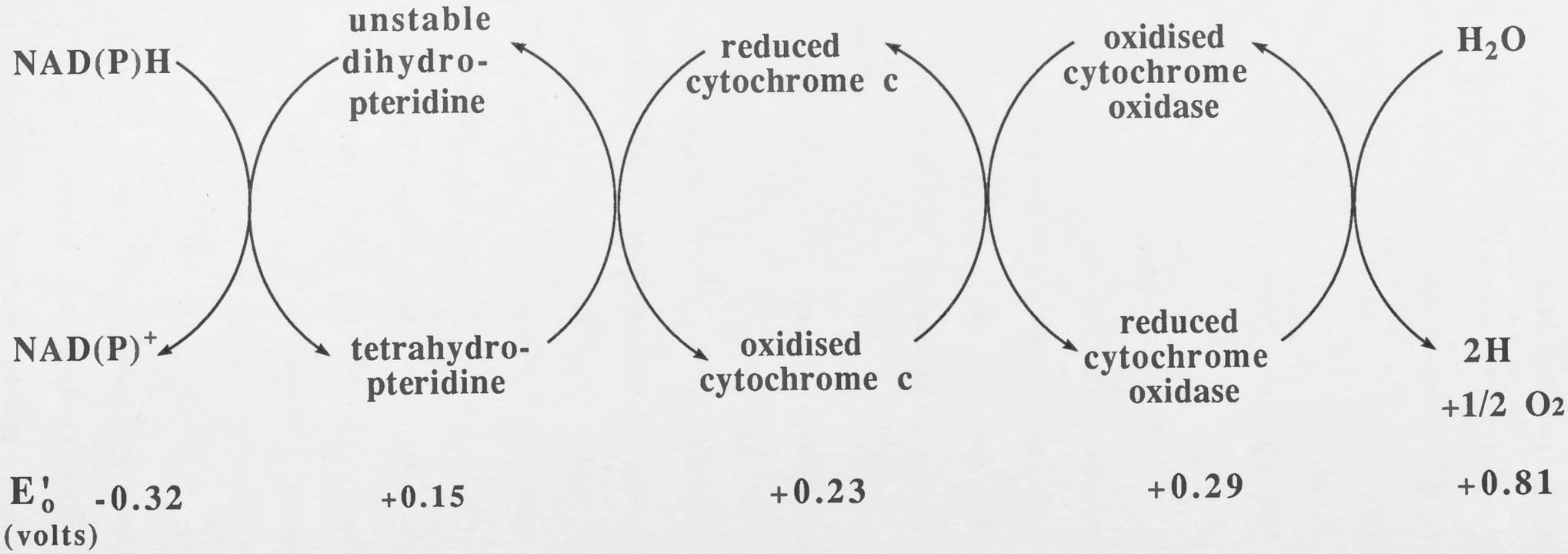
The treatment for MHPA involves the use of neurotransmitter replacement therapy, tetrahydrobiopterin therapy or the combination of both. The neurotransmitter replacement therapy using L-DOPA, L-5-hydroxytryptophan and carbidopa has been quite effective in conjunction with dietary restriction of phenylalanine to prevent symptoms caused by high phenylalanine levels in serum. Tetrahydrobiopterin treatment has been effective in several cases of MHPA but DHPR-deficient cases require higher doses of tetrahydrobiopterin because its effective concentration in cells and tissues is much lower than that predicted from the actual dose (reviewed by Armarego *et al.*, 1984; Cotton, 1985). The reason for this is the poor penetration of tetrahydrobiopterin across the blood-brain barrier. Synthetic tetrahydropterins such as 6-methyltetrahydropterin

cross the blood-brain barrier ten times better than tetrahydrobiopterin because of higher fat solubility arising from the lipophilic charateristic endowed by the methyl substituent at C-6 (Kapatos and Kaufman, 1981). Since the available protocols for MHPA therapy can be improved, a fuller understanding of the unknown roles of tetrahydropterins and DHPR *in vivo* will be a definite advantage.

1.5 OCCURRENCE AND DISTRIBUTION OF DHPR

DHPR is widely distributed in mammalian cells and has been purified from a wide variety of sources including a *Pseudomonas* species (reviewed by Armarego *et al.*, 1984). The only well-characterised physiological role for DHPR is to recycle the pterin cofactor involved in the hydroxylation of the aromatic amino acids. However, DHPR is known to occur in higher levels than the aromatic amino acid hydroxylases in some tissues. The reductase also occurs in human cultured fibroblasts, continuous lymphoid cells, peripheral blood lymphocytes, granulocytes and platelet cells, all of which are known to lack the aromatic amino acid hydroxylases. Molecular and immunological comparison of purified human liver DHPR with DHPR from human cultured fibroblasts and continuous lymphoid cells indicated that the same structural gene encoded DHPR irrespective of the tissue distribution and function (Firgaira *et al.*, 1981b). Also tetrahydrobiopterin, whose only established physiological role is as the natural cofactor for the aromatic amino acid hydroxylases, is also present in some tissues which have almost no aromatic amino acid hydroxylase activities (Fukushima and Nixon, 1980). These observations indicate that DHPR and tetrahydrobiopterin may be involved in other biochemical reactions. The literature points to several systems where putative roles for DHPR and pterins have been implied.

Alkylglycerols are glycerols that are attached to a fatty acid side chain by an ether bond and are known to be present as components of the myelin sheath and involved in haemopoeisis. The cleavage of the ether bond in alkylglycerols by the microsomal enzyme glyceryl etherase was shown to require molecular oxygen and tetrahydropterin.



Scheme 1.3 Proposed completely soluble electron transport chain (Rembold and Buff, 1972b).

The activity of this enzyme was shown to be maximal when the soluble fraction and NADH were added to the assay. The etherase activity was also stimulated when tetrahydropterin was added to washed subcellular preparations (Tietz *et al.*, 1964; Snyder *et al.*, 1973). In the case of phenylalanine hydroxylase, the addition of the fraction that contained DHPR activity enhanced the hydroxylase activity. By analogy, since the glyceryl etherase activity was also enhanced by the addition of the soluble fraction, it is most likely that DHPR may be involved in recycling the pterin cofactor required for the etherase activity .

The proline-rich collagen molecules are known to undergo processing by hydroxylation of proline residues while they are still attached to the ribosomes. This reaction is catalyzed by proline hydroxylase, which requires molecular oxygen, α-ketoglutarate and a reducing agent such as ascorbate. Tetrahydropterins, cysteine and dithiothreitol are also active as reducing agents. Although ascorbate is generally considered as the physiological reductant, Peterkofsky *et al.*. (1980) showed that proline hydroxylase was active in laboratory cultured L-929 cells which did not contain measurable ascorbate but contained pterins. The involvement of molecular oxygen and tetrahydropterin once again implies a possible role for DHPR.

The quinonoid dihydropterin-tetrahydropterin couple has a lower reduction potential than the oxidized cytochrome *c* - reduced cytochrome *c* couple (see Scheme 1.3) and this fact has been used for the measurement of dihydropteridine reductase where oxidized cytochrome *c* generates the quinonoid dihydropterin *in situ* (Hasegawa, 1977). Rembold and Buff (1972a,b) demonstrated that the addition of tetrahydropterin to rat liver mitochondria resulted in an increase in the net substrate-dependent oxygen consumption without the concomitant production of ATP. Their evidence that the electrons from the pterin couple were being fed into the mitochondrial respiratory chain at the level of cytochrome *c* came from preliminary low-temperature-difference spectral studies which indicated that the pterins caused the reduction of cytochrome *c* but not cytochrome *b*. Based on these preliminary results, a possible mechanism by which the quinonoid

dihydropterin-tetrahydropterin couple may be involved in the mitochondrial electron transfer chain was postulated (see Scheme 1.3). A later study of the above phenomenon demonstrated that ATP production was coupled to the increase in respiration produced by tetrahydropterin with a P:O ratio of 0.85, as expected for reduction at the level of cytochrome *c* (Taylor and Hochstein, 1975).

A previous study of the involvemant of pterins in electron transfer in the photosynthetic centre in postulation of an electron transfer mechanism which involved the quinonoid dihydropterin-tetrahydropterin couple (Fuller and Nugent, 1969). The recent identification of a pterin derivative as the second prosthetic group (flavin moeity was the first group that was identified) in *Escherichia coli* DNA photolyase, an enzyme that catalyses the monomerization of dimers formed between adjacent pyrimidine residues when DNA is exposed to ultraviolet light, has been hailed as the first positive implication pterin in photobiological processes (Wang *et al.*, 1988). In all these cases where the quinonoid dihydropterin-tetrahydropterin couple is involved, DHPR has the putative role of recycling the oxidized quinonoid dihydropterin with NAD(P)H providing the reducing equivalent.

Another function for DHPR became apparent when it was found that a patient suffering from MHPA due to DHPR deficiency had low levels of serum folate and the brain biopsy of this patient indicated deficiency in tetrahydrofolates (Pollock and Kaufman, 1978). Under physiological conditions, tetrahydrofolates can be oxidized to quinonoid dihydrofolate, and since the latter is a substrate for DHPR (Waring and Armarego, 1987), its role in maintenance of tetrahydrofolate levels becomes clear. For this reason, DHPR-deficient patients have been treated with folinic acid in conjunction with other treatment protocols (reviewed by Cotton, 1985).

Apart from the wide cellular distribution of DHPR and pterins in mammals, they are also known to be widely distributed throughout the living system. The need for good experimental models of PKU and MHPA has led to the study of DHPR in the ciliated

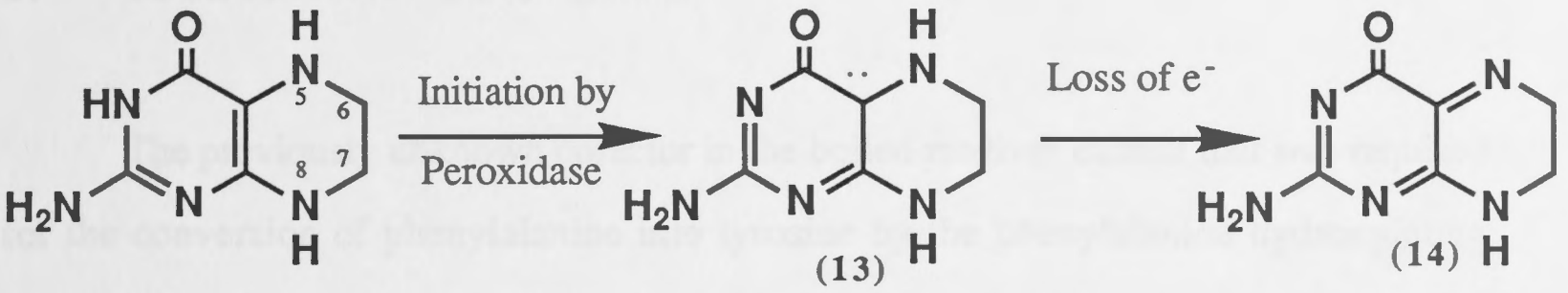
protozoan *Tetrahymena* (Sanford and Orias, 1981), the trypanosomid flagellate *Crithidia fasciculata* (Kidder and Nolan, 1973) and a *Pseudomonas* species (Williams *et al.*, 1976).

1.6 ASSAYS FOR DHPR

Since DHPR was first discovered in conjunction with the phenylalanine hydroxylating system, the early assay for DHPR used the coupled enzyme approach where the reductase reaction was coupled to the hydroxylase reaction and the rate of tyrosine formation was monitored (Craine *et al.*, 1972). The identification of the reduced pterin cofactor for phenylalanine hydroxylase activity, and the eventual elucidation that the oxidized pterin product of the hydroxylase reactions was the transiently-stable quinonoid dihydropterin species which was the substrate for DHPR led to the development of several direct enzymic assays. These direct DHPR assays ensured the accuracy of the kinetic parameters obtained for the DHPR-catalysed reaction because it became the rate-limiting step. The reduction of one mole of quinonoid dihydropterin requires one mole of NAD(P)H, therefore the initial rates of the enzymic reaction can be determined by following the rate of oxidation of NAD(P)H at 340 nm and using 6200 $M^{-1}cm^{-1}$ as its molar extinction coefficient.

The unstable nature of the quinonoid species (e.g. $t_{1/2}$ of ~20 min for quinonoid 6-methyl-dihydropterin; Armarego, 1984) required that the pterin substrate for DHPR be produced rapidly and quantitatively *in situ*. Several oxidants have been reported in the literature but the most commonly employed method uses peroxidase-hydrogen peroxide as the oxidant (Nielsen *et al.*, 1969). Peroxidase alone under aerobic conditions can rapidly oxidize tetrahydropterin to the quinonoid species (Armarego *et al.*, 1983). It was suggested that hydrogen peroxide keeps the peroxidase in the right oxidation state to carry out the initiation of oxidation to produce the C-4a radical (13) which could lose an electron to produce the quinonoid species (14) as in Scheme 1.4 (Armarego, 1984). The other oxidants that have been used include bromine (Benkovic *et al.*, 1985), 2,6-dichlorophenolindophenol (Craine *et al.*, 1972), ferric iron (Archer *et al.*, 1972),

potassium ferricyanide (Nielsen *et al.*, 1969; Archer *et al.*, 1972) and ferri-cytochrome c (Hasegawa, 1977).



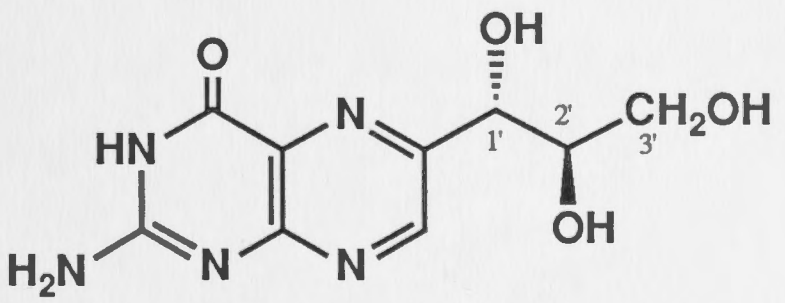
Scheme 1.4 Generation of quinonoid dihydropterin *in situ*

In all the cases, the oxidant is used in excess so that the tetrahydropterins are quantitatively oxidized to the quinonoid species and rapidly reoxidize the tetrahydropterins regenerated by DHPR during the enzymic assay. Thus, it can be assumed that the concentration of the quinonoid species during the first few minutes of the assay is equal to the concentration of tetrahydropterin that was added in the reaction cuvette for *in situ* oxidation. In most of the studies on DHPR reported in the literature the pterin substrate is often a synthetic analog of tetrahydrobiopterin. This is because synthetic pterin analogs with substitutions in the 6 and/or 7 positions of the pterin nucleus are often cheaper and more widely available. The corresponding quinonoid dihydropterins prepared from the synthetic tetrahydropterin substrates often vary in their stability, hence it is often a good practice for their stability to be determined spectroscopically prior to their use as substrates in enzyme assays. The stability of the quinonoid species is pH-dependent and the maximum half-life has been observed at pH 7.4 $\pm$ 1.0. Thus the optimal pH used for DHPR assay is within the range of 7.0 to 7.5, where there is negligible auto-oxidation of NAD(P)H and maximum stability of the quinonoid dihydropterin. Rearrangement of the quinonoid dihydropterin to the 7,8 dihydropterin (which is not a substrate for DHPR) is slowest in Tris-HCl buffer, hence it is generally used in the assay of the enzyme (Archer and Scrimgeour, 1970). Since the DHPR activity is quantified by observing the rate of

oxidation of NAD(P)H at 340nm, it is important that the oxidants used to generate the quinonoid dihydropterins *in situ* are transparent at this wavelength.

1.7 SUBSTRATES FOR DHPR

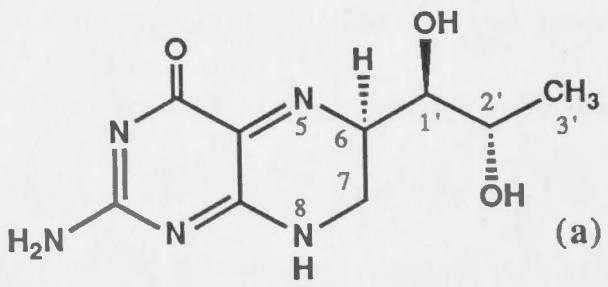
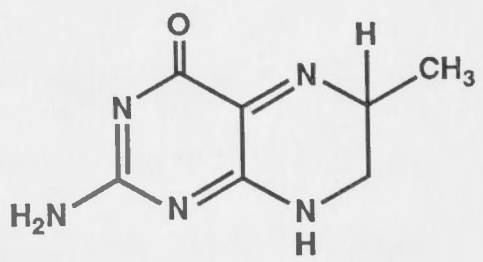
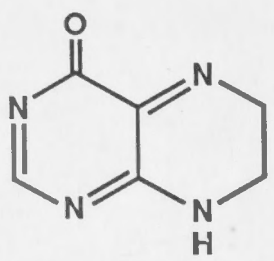
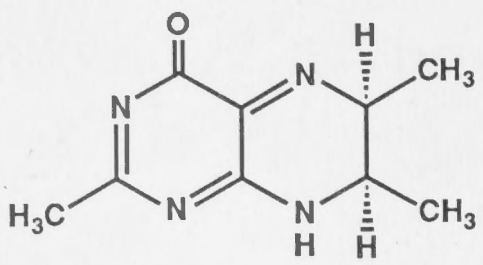
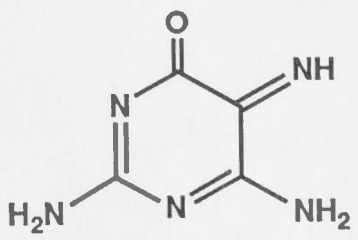
The previously unknown cofactor in the boiled rat-liver extract that was required for the conversion of phenylalanine into tyrosine by the phenylalanine hydroxylating system was shown to be 5,6,7,8-tetrahydrobiopterin and the unusual quinonoid species produced by this reaction is the substrate for DHPR (see Scheme 1.2). The *Crithidia* assay identified the configuration of the dihydroxypropyl side chain at C-6 to be L-*erythro* (Kaufman, 1963). Quinonoid 7,8-dihydrobiopterin is the natural substrate for all the dihydropteridine reductases reported in the literature except for the *Pseudomonas* species DHPR (Williams *et al.*, 1976). The analysis of naturally-occurring pterins in *Pseudomonas* species clearly showed that the major component was 6-trihydroxypropyl(L-*threo*)pterin or L-*threo*-neopterin (15). The evidence for this came from ultra-violet spectra, potassium permanganate ($KMnO_4$) oxidation and comparative paper chromatography. The *Crithidia* assay was consistent with the L-*threo* configuration and this substrate was about 150 times less effective when compared with L-*erythro* biopterin on a equimolar basis (Guroff and Rhoads, 1969).



(15)

Early evidence that NADPH was the preferred pyridine nucleotide was shown to be in error due to contamination by DHFR. Purified samples of dihydopteridine reductases from most sources use NADH about 15 to 45 times more effectively than

Table 1.1 Comparison of Km and Vmax values of the natural substrate (a) with synthetic quinonoid dihydropterin substrates (b - e) to show the minimum structure required for DHPR activity. (summarised by Armarego, 1987)

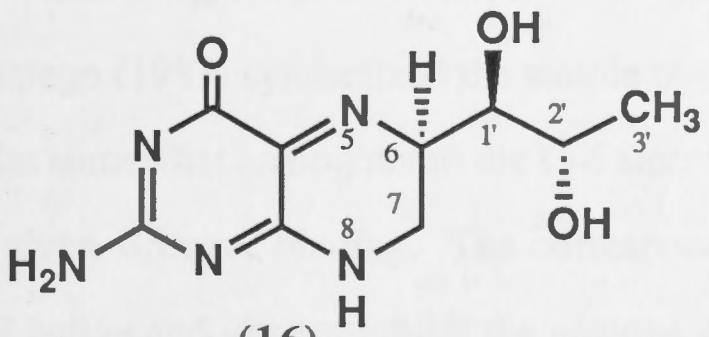
	Km (μM)	Vmax (U/mg)
 (a)	1.3	27
 (b)	11.0	491
 (c)	669	234
 (d)	38.8	10.1
 (e)	76.1	163.6

NADPH. However, there are reports in the literature of NADPH-specific dihydropteridine reductases with different molecular properties (Hasegawa, 1977).

1.7.1 Structure-activity relationship of pterin cofactors

The ambiguities with respect to the structure of the quinonoid dihydropterin substrate and the instability of tetrahydrobiopterin at physiological pH ($t_{1/2}$ ~3 min; Armarego, 1984) prompted several laboratories to synthesize more stable analogs and test their effectiveness as substrates with a view to using them for therapeutic purposes.

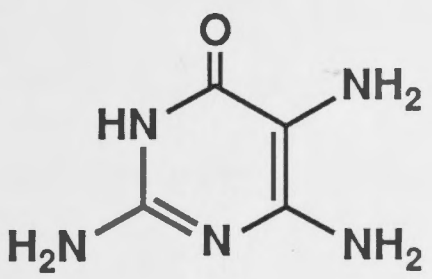
The *para* quinonoid structure (6) that was initially proposed by analogy with oxidized flavins has an exocyclic double bond at C-2 (Kaufman, 1964). This representation of the quinonoid species indicates that the protonation step during the DHPR-catalysed reduction may occur at the imino group on C-2. Evidence against the *para* quinonoid structure has been accumulating from several studies. Substitutions of pterins at C-2 with 2-MeNH, 2-MeS or 2-Me did not eliminate the substrate activities of the corresponding quinonoid species with DHPR. A series of simple quinonoid pteridines without the NH_2 group on C-2 were good substrates for DHPR and gave kinetic constants that were comparable with quinonoid 6-methyl dihydropterins (summarised in Table 1.1; Armarego and Waring, 1983; Armarego *et al.*, 1986). ^{15}N NMR studies of quinonoid 6,7-dimethyl-dihydropterin enriched at N-1, N-3, N-5 and NH_2 on C-2 indicated that the endocyclic quinonoid structure (16) was the predominant



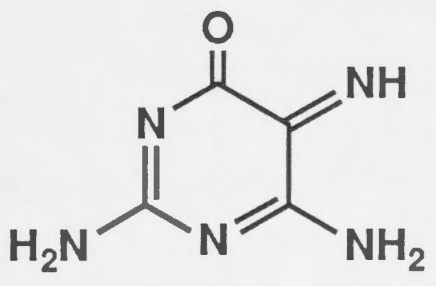
(16)

species in neutral aqueous buffer (Benkovic *et al.*, 1985). The results summarised in Table 1.1 show that the 2-NH_2 group is not essential for substrate activity with DHPR and the endocyclic quinonoid species is the correct tautomeric structure that becomes reduced by the enzyme in the presence of NADH.

A methyl group at N-8 makes the pterin an inefficient substrate for DHPR, and a methyl group at N-3 prevents the formation of significant quantities of the quinonoid species. The generation of a positive charge at N-3 subjects it to rearrangement by acid catalysis, resulting in rapid decomposition of the pterin molecule (Armarego and Waring, 1982; Mager, 1975). During the search for the minimum structural requirement for the pterin cofactor, the simple pyrimidine, 2,5,6-triamino-4-oxopyrimidine (17), was found to be a substrate for phenylalanine hydroxylase and its product 2,6-diamino-4-oxo-5-iminopyrimidine (18) was a substrate for DHPR (Kaufman, 1979). This indicated that the ethylene bridge between N-5 and N-8 is not crucial for the hydroxylase or DHPR activity.



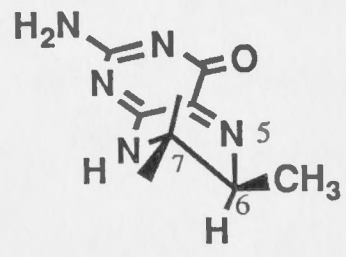
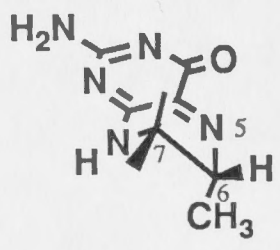
(17)



(18)

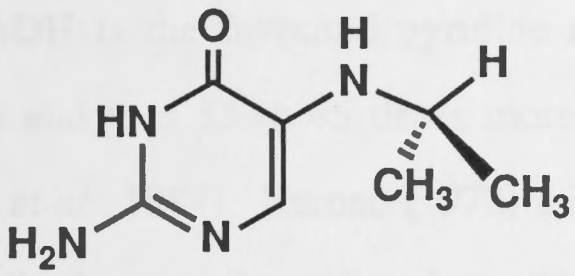
The endocyclic quinonoid structure of the natural substrate is retained in (18), which accounts for its favourable K_m (76 μM) with human-liver DHPR (Armarego *et al.*, 1983). Waring and Armarego (1987) synthesized the simple pyrimidine (19) so that the secondary propane will be somewhat analogous to the C-6 stereochemistry in the pterin moeity and might have given stronger binding. The corresponding quinonoid species however were not DHPR active and did not inhibit the enzyme activity even at 0.5 mM (Waring and Armarego, 1987). The inactivity was attributed to the formation of a structure such as (20) which would not bind tightly at the active site. The replacement of the 4-oxo group by 4-thioxo on the pterin nucleus was also tolerated by the enzyme, but

Table 1.2 Kinetic parameters of enantiomeric and racemic quinonoid dihydropterins with monkey-liver DHPR[a]

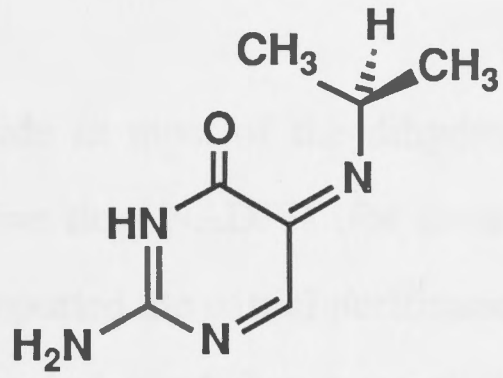
Quinonoid dihydropterin substrate	Km(μM)	Vmax
 S(-) - 6 -methyl	15.9	55.6
 R(+) - 6 -methyl	17.4	63.0
RS(±) - 6 -methyl	15.0	47.0
6,6-dimethyl	634	121

a. The DHPR assay was carried out 25°C at pH 7.5 (Armarego and Waring, 1982).

quinonoid 6-methyl-7,8-dihydrolumazine (21) was DHPR inactive (Armarego *et al.*, 1986). This must be because the electronic properties of the lumazine moiety are different from those of the pterin moiety.

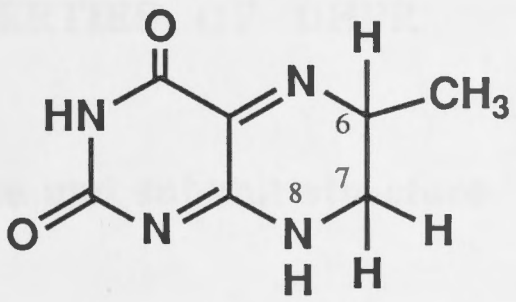


(19)



(20)

Armarego (1979) measured the kinetic parameters of the optically pure 6-R and 6-S enantiomers of quinonoid 6-methyl-dihydropterins with the view of obtaining some stereochemical information about the active site of DHPR. The enantiomers were equally effective as substrates. Interestingly, quinonoid 6,6-dimethyl-dihydropterin was a poorer substrate and bound some 30-fold less effectively than the quinonoid 6-methyl-dihydropterins, assuming that Km ≈ K_d (summarised in Table 1.2). The conclusion from these stereochemical studies was that the pterin-binding region at the active site of the enzyme does not discriminate between the 6-R and 6-S quinonoid 6-methyl-dihydropterins presumably because the bulky methyl group can occupy the equatorial



(21)

plane in both S(-) and R(+) enantiomers. On the other hand, with quinonoid 6,6-dimethyl-dihydropterin, one of the bulky methyl groups will always be in the axial plane, thus decreasing the binding efficiency due to steric hiderance. These results imply that the dihydroxypropyl side chain of quinonoid dihydrobiopterin at C-6 must occupy the equatorial plane when it is bound to the active site of the enzyme (see Table 1.2; Armarego, 1979; Armarego and Waring, 1982, 1983).

1.7.2 Pyridine nucleotide substrates for DHPR

NADH is the favoured pyridine nucleotide in most of the dihydropteridine reductases and it is 15 to 45 times more effective than NADPH (for summary, see Armarego *et al.*, 1984). Harono (1972) initially reported the partial purification of four different diaphorases from *Bombyx* silkworm and showed that three of them used NADPH more effectively than NADH. Quinonoid dihydrobiopterin was not a cofactor for these enzymes (up to a concentration of 0.7 mM), whereas the quinonoid dihydro forms of 6,7-dimethylpterin, 6-methylpterin and neopterin were all substrates (Harano, 1972). There has been no further report of this observation, so the reason why the quinonoid dihydrobiopterin was not a substrate is not clear. This was followed by a report by Hasegawa (1977) on the existence of a NADPH-specific DHPR in bovine liver with different physicochemical properties. With this protein, purified to homogeneity, the K_m values for NADH and NADPH with quinonoid 6-methyl dihydropterin as the second substrate were estimated to be 2900 μM and 1.4 μM respectively (Nakanishi *et al.*, 1986a).

1.8 PHYSICAL PROPERTIES OF DHPR

1.8.1 Molecular size and subunit structure

Generally the native dihydropteridine reductases from most sources are dimers of identical subunits with molecular weight of 50 000 to 52 000 as determined by gel filtration chromatography, non-denaturing PAGE or analytical ultracentrifugation studies. Cross-linking studies using dimethyl suberimidate have been used to confirm that the native protein is dimeric (Firgaira *et al.*,1981a). A subunit molecular weight of 25 000 to 27 000 as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is also a characteristic of the mammalian dihydropteridine reductases. These values are consistent

with the subunit molecular weights of human-liver DHPR (Dahl *et al.*, 1987; Lockyer *et al.*, 1987) and rat-liver DHPR (Shabaz *et al.*, 1987), determined from the amino acid sequence deduced from the nucleotide sequences of their genes. SDS-PAGE of human DHPR sometimes showed two bands around 25 kD and this was probably due to the presence of strongly-bound NADH in some subunits that retards its mobility (Firgaira *et al.*, 1981a). The identical subunits are not covalently bound because they dissociate in the presence of SDS even in the absence of sulphydryl reagents. The DHPR isolated from *Pseudomonas* species has an estimated molecular weight of 44 000 for the native protein but this enzyme was not purified to homogeneity and denaturing PAGE analysis has not been carried out (Williams *et al.*, 1976).

The NADPH-specific DHPR from bovine liver and human liver consist of two identical subunits that are 35 kD each. These reductases are also active as dimers (Nakanishi *et al.*, 1986a,b). There is apparently no NADPH-specific DHPR in human brain (S.G.Vasudevan and W.L.F.Armarego, unpublished results).

1.8.2 Isoelectric point

The isoelectric points of some of the dihydropteridine reductases have been measured and they range from 7.0 for the human-liver DHPR (Firgaira *et al.*, 1981a) to 5.4 for the enzyme from sheep liver (Webber *et al.*, 1978). Several reports of multiple bands during isoelectric focusing of purified dihydropteridine reductases have been attributed to tight nucleotide binding and other modifications that may arise during manipulations in the purification process. A detailed study of purified rat-liver DHPR indicated that three forms of the enzyme with approximate isoelectric points of 6.5, 5.9 and 5.7 can exist. The three forms were isolated by preparative chromatofocusing and shown to exhibit similar kinetic parameters with quinonoid dihydrobiopterin and NADH. However two distinct subunits, α and β, were identified when the different forms were subjected to denaturing isoelectric focusing in the presence of 6 M urea. The different forms of the enzyme represent the three possible combinations of the subunits, which are

αα, αβ and ββ. The αα form of the enzyme can be converted to the β subunit under acid conditions. Webber *et al.* (1986) suggest that differential hydrolysis of asparagine and glutamine residues may be responsible for the conversion of the α subunit (amide form) to the β subunit (carboxyl form). These amide to carboxyl changes probably occur during the purification manipulations.

1.8.3 Amino acid composition

The amino acid compositions of several dihydropteridine reductases have been reported and they show no unusual properties. The *N*-terminal amino acid residue of DHPR from mammalian sources is blocked by a covalent modification. *N*-Acetylation of isoleucine and cyclization of glutaminyl or glutamyl residues to give pyroglutamic acid were suggested as the possible nature of the *N*-blocked residue (Korri *et al.*, 1977; Webber *et al.*, 1987). The nucleotide sequence of the human-liver and rat-liver cDNAs coding for DHPR predict that the blocked residue may be *N*-acetylalanine (Dahl *et al.*, 1987; Shabaz *et al.*, 1987). Positive identification of the *N*-terminal amino-acid residue would only be obtained from NMR or mass spectrometric studies.

1.9 INHIBITORS OF DHPR

The similarities of the pterin nucleus of the natural cofactors for DHPR and DHFR led to the well-known inhibitors of the latter being investigated as inhibitors of the former. Most dihydropteridine reductases are inhibited by aminopterin, amethopterin (methotrexate) and other folate analogs, but the inhibition constants are several hundred-fold higher than those for DHFR. The mechanism of inhibition by aminopterin and amethopterin is competitive in most of the cases studies (for summary, see Armarego *et al.*, 1984). The powerful anticancer drug methotrexate which inhibits DHFR strongly, is also an inhibitor of DHPR. The *Pseudomonas* enzyme shows competitive inhibition (K_i 260 μM) by methotrexate with respect to pterin (Williams *et al.*, 1976). DHPR also exhibits substrate inhibition by pterins at high concentrations.

Sulphydryl-specific reagents such as *p*-chloromercuribenzoate (PCMB), mercuric chloride ($HgCl_2$) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were inhibitors of human-liver DHPR in the lower μM range, whereas N-ethylmaleimide (NEM) and iodoacetamide were inhibitors in the mM range. Pretreatment of the enzyme with NADH protected it totally from inactivation by these sulphydryl agents (Firgaira *et al.*, 1981a). The reductase from *Pseudomonas* species was also inhibited by some of the above sulfhydryl reagents in the mM range. Webber and Whiteley (1981) titrated four cysteine residues per subunit for the rat-liver DHPR by using sulfhydryl reagents. They also showed that only six (three per subunit) thiol residues were accessible to PCMB. When the reductase was complexed with NADH (1 : 1), only four thiols were titrated with PCMB. This suggested that two thiol groups (one per subunit) are inaccessible to PCMB and a further two are either close to or at the nucleotide binding site. In a more recent study it was shown that platinum (II) complexes inactivated human-brain DHPR in a time-dependent manner. Once again it was found that NADH could completely protect the enzyme from inactivation (Armarego and Ohnishi, 1987). These results indicate that thiol groups which are present at or close to the active site may be involved in catalysis.

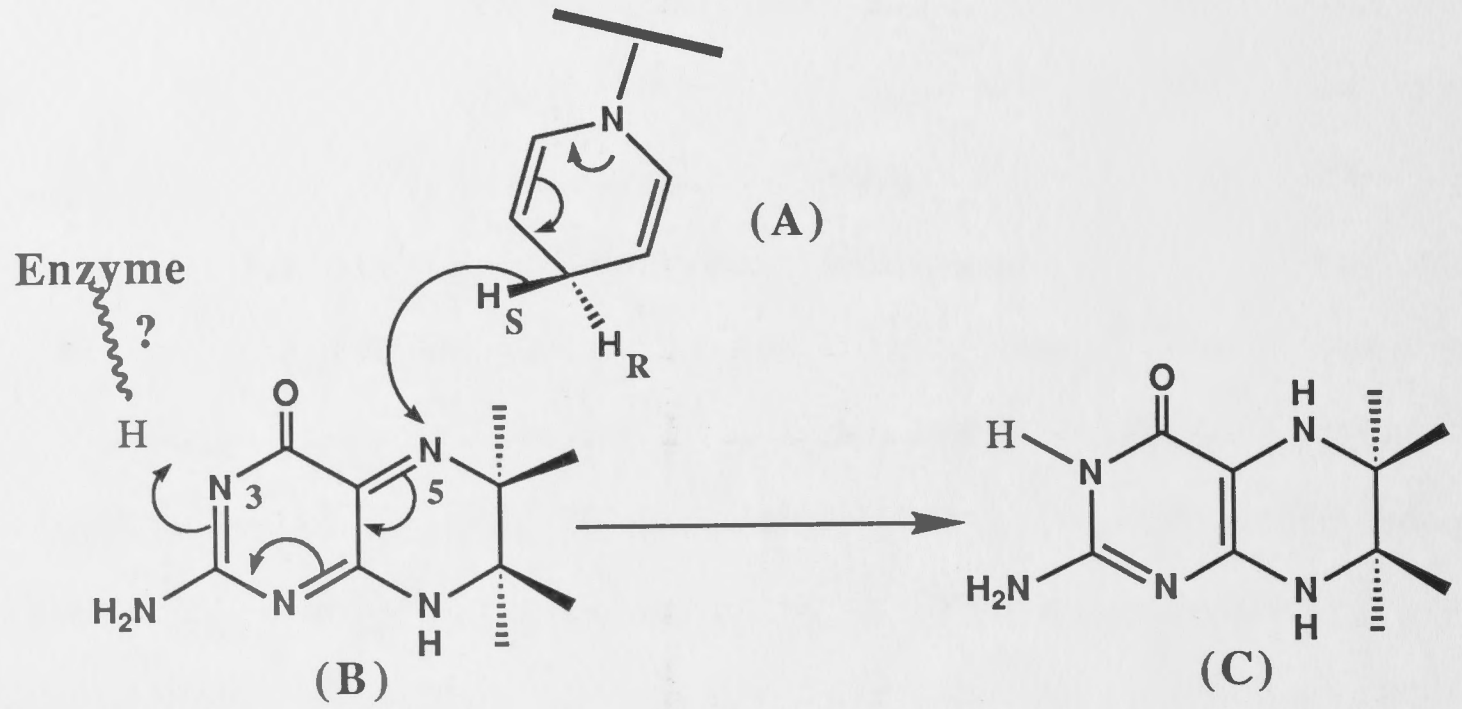
Metal ions such as of lead and aluminium have been shown to inhibit up to 50% of rat brain DHPR activity around 50 -100 μM levels (Purdy *et al.*, 1981; Leeming and Blair, 1979). As an extension of that observation, Altmann *et al.* (1987) claimed that encephalopathy associated with aluminium intoxication in patients undergoing haemodialysis was due to inhibition of DHPR in erythrocytes and proposed that a similar reduction in activity may occur in the brain. They indicated that treatment of patients with the aluminium chelating agent desfereoxamine showed a doubling of DHPR activity in the erythrocytes. However, it was pointed out later that the serum aluminium concentration may not reflect the erythrocyte aluminium concentration and that a single dose of desfereoxamine results in doubling of erythrocyte DHPR activity for 28 days even though the aluminium levels have been reduced to normal (Sprague and Umans, 1987). In a

more direct criticism, Milstein and Kaufman (1987) alleged that the results of Altmann *et al.* were due to artefacts in their DHPR assay.

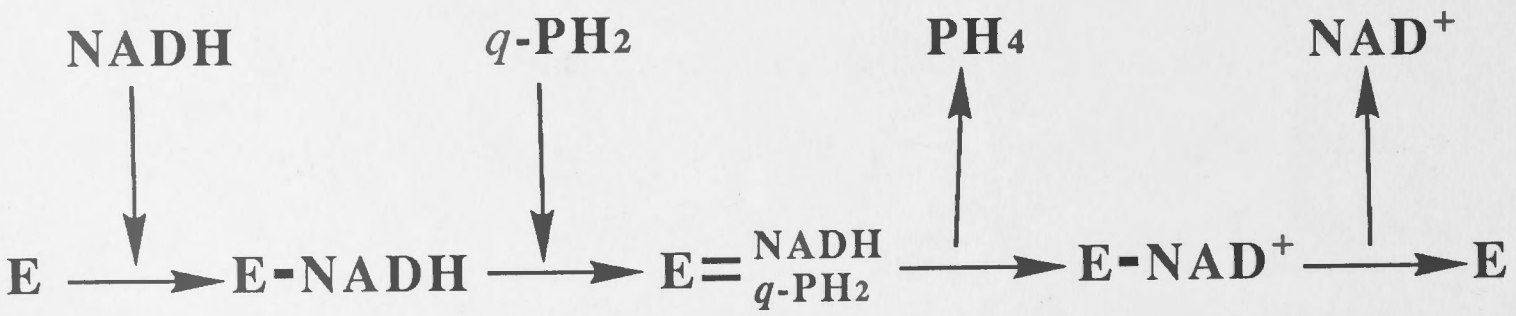
Several aromatic amino acid metabolites and their derivatives inhibit DHPR. The aminochrome oxidation products of the catecholamines (dopamine, adrenalin and noradrenalin) are potent reversible inhibitors of human-brain DHPR. It was proposed that the inactivation was due to structural changes brought about by disulfide bond formation between two surface sulphydryl groups (Armarego and Waring, 1983; Waring, 1986). The nigrostriatal neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the compound present in "synthetic heroin" that causes Parkinsonian symptoms, does not inhibit DHPR in the lower millimolar range but its hydroxylated derivative can inhibit the enzyme at the micromolar range. However, hydroxylated versions of MPTP have not been identified in humans or experimental animals (Abell *et al.*, 1984).

The inhibition of DHPR by quinones was the basis for the development of the naphthoquinone affinity column to purify human liver DHPR and human brain DHPR (Cotton and Jennings, 1978). Pyridine nucleotide analogs such as NAD^+, AMP, Cibacron Blue and Blue Dextran were all competitive inhibitors of bovine kidney DHPR with K_i values of 300 μM, 1 μM, 0.3 μM and 0.3 μM, respectively. These dyes have been used as affinity adsorbents for the purification of several mammalian dihydropteridine reductases (Korri *et al.*, 1977; Chauvin *et al.*, 1979).

Antibodies raised against DHPR from several sources inhibited their enzymic activities to varying degrees. Polyclonal antibodies raised against the bovine-brain enzyme crossreacted with the reductase isolated from the brain, kidney and liver tissues of rat. The antiserum against sheep liver DHPR crossreacted with DHPR isolated from various human, rat and bovine tissues (Snady and Musacchio, 1978; Milstein and Kaufman, 1975). On the other hand, the antibodies raised against bovine-liver NADPH-specific DHPR did not inhibit the NADH-specific DHPR and conversely, the antibodies



Scheme 1.5 The mechanism of transfer of the *pro*-**S** hydrogen from the **B** face of the nicotinamide ring (**A**) of the NADH to N-5 of the quinonoid dihydropterin (**B**) that results in the protonation of N-3 to give the tetrahydropterin (**C**).



Scheme 1.6 The reaction pathway for the DHPR catalysed reaction

against the latter enzyme did not inhibit the former. This indicates that the structures of the two proteins may be very different (Nakanishi *et al.*, 1986b).

1.10 MECHANISM OF DHPR CATALYSIS

The first committed step in the DHPR-catalysed reaction is the extremely tight binding to NADH which is only partially stable to exhaustive dialysis. The binding stoichiometry of NADH to the enzyme is 1:1, i.e. 1 subunit binds 1 mol. of NADH (Webber and Whiteley, 1978; Chauvin *et al.*, 1979; Armarego and Ohnishi, 1987). Armarego (1979) showed that the pro-S hydrogen from the B-face of the nicotinamide ring of NADH is transferred to N-5 on the pterin nucleus during the DHPR-catalysed reaction. The mechanism of the hydride transfer from NADH to the quinonoid dihydropterin (q-PH_2) is presented in Scheme 1.5. A comparison of deuterated NADH (NADD) with NADH as substrate for DHPR with quinonoid 6,7-dimethyl tetrahydropterin as the second substrate showed that there was no significant difference in K_m and V_{max}, implying that the rate-limiting step does not involve C - H bond breaking (C - D bond energy > C - H bond energy; Ferscht, 1985a). Therefore the rate determining step must occur before or after hydrogen transfer (Poddar and Henkin, 1984). Further studies using stopped-flow, rapid quench and temperature-jump techniques indicated that the kinetic mechanism involves non-equilibrium ordered binding of substrates, where the rate determining step may be the isomerization of the ternary complex which precedes hydride transfer (see Scheme 1.6; Poddar and Henkin, 1984; Randles, 1986).

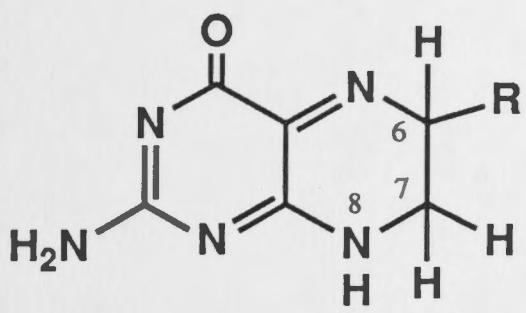
1.11 DIHYDROFOLATE REDUCTASE (DHFR)

A brief introduction of DHFR is necessary because several references have been made to it either to distinguish it from or compare it with some of the features of DHPR, which is the main topic of this thesis.

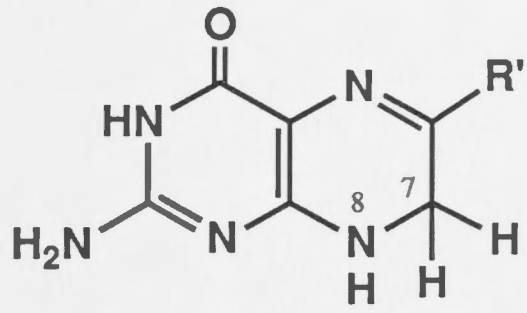
DHFR catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate,

and the enzyme from various sources can catalyse the reduction of folate to 7,8-dihydrofolate. However, since the rate of the former reaction is much faster than the latter, the enzyme is commonly referred to as dihydrofolate reductase (Blakley, 1984). DHFR plays a crucial role in the biosynthesis of the building blocks of DNA. Therefore, it was chosen as a target for the anti-cancer drugs which are designed to challenge the uncontrolled proliferation of cells (McCormack, 1981). Methotrexate is the most potent inhibitor of this enzyme with an inhibition constant (K_i) in the lower nanomolar range. A multitude of reviews and monographs cover the avalanche of articles describing various aspects of DHFR, ranging from X-ray crystallographic analysis, site-directed mutagenesis and detailed kinetic analysis (for a recent review see Morrison, 1988)

DHFR from most bacterial sources is monomeric with a molecular weight of about 18 000 and the mammalian enzymes are about 22 kD (Blakley, 1984). The Type I and II R-plasmid encoded DHFRs (trimethoprim resistant) are an exception because they have subunits with molecular weight of about 8 000-11 000 and are active either as dimers or tetramers (Matthews *et al.*, 1986). The superficial relationship between DHPR and DHFR that is often referred to is the fact that both the enzymes catalyse the reduction of dihydropterins to tetrahydropterins. In both cases the chemistry of the enzymatic reaction is confined to the pyrazine ring of the pterin nucleus. However, the dihydropterin substrate for DHPR is the transiently stable quinonoid dihydropterin (22) and that for DHFR is the more stable 7,8-dihydropterin (23) tautomer. NADPH is the preferred nucleotide cofactor for DHFR from all the sources and it is clear that the hydride is transferred onto C-6 of the pterin moeity as N-5 becomes protonated. In the case of *E.coli* DHFR, the protonation is carried out by an aspartate residue at position 27 (Howell *et al.*, 1986). In contrast, in the DHPR-catalysed reaction, the hydride is transferred from NADH to N-5 of the pterin moiety and protonation occurs at N-3. The substrate



(22)



(23)

specificity range for DHFRs is narrow. Even in the case of the R-plasmid encoded DHFR, which bears no homology with other DHFRs, the requirement for 7,8-dihydropterin is absolute and it is completely inactive towards quinonoid 6-methyl-dihydropterin (S.G. Vasudevan and J.F. Morrison, unpublished results).

1.12 STRUCTURE OF DHPR

Primary structural data for DHPR has been long overdue and the main reason for this may be that the *N*-terminal amino acid residue is blocked. Recently, there have been three reports of the complete amino acid sequence deduced from the nucleotide sequence of cDNA clones from human liver (Dahl *et al.*, 1987; Lockyer *et al.*, 1987) and rat liver (Shabaz *et al.*, 1987). The human-liver cDNA clones were identified by screening in one case with antibodies raised against human-liver DHPR (Dahl *et al.*, 1987) and in another, by using antibodies raised against sheep-liver DHPR (Lockyer *et al.*, 1987). The two human-liver sequences were identical except for a serine to threonine change at amino acid residue 51 (counting the methionine start codon as position 1) which may be due to polymorphism. However, an error in reading the nucleotide sequence cannot be ruled out because polymorphic sites usually arise from single nucleotide changes in the sequence, whereas in this case it is a GC inversion (AGC for serine to ACG for threonine). Moreover, one of the human-liver DHPR sequences (Dahl *et al.*, 1987) is identical with the rat-liver DHPR sequence at position 51 (see Figure 1.1). The rat-liver cDNA was obtained by hybridization screening using a low-degeneracy radiolabelled oligonucleotide probe predicted to encode a short internal peptide sequence of a cyanogen bromide

Figure 1.1 The amino acid sequence of human-liver DHPR predicted from the open reading frame in the nucleotide sequence of cDNA (Dahl *et al.*, 1987). (*) Position 51 is a threonine residue in the human-liver DHPR sequence obtained by Lockyer *et al.* (1987). The residues typed in bold are substitutions that occur in the rat-liver DHPR sequence (Shahbaz *et al.*, 1987). The boxed sequence indicate the human-brain DHPR peptides that were independently obtained in this laboratory (A. Ohnishi, S.G.Vasudevan and W.L.F. Armarego, unpublished results).

M A A Ā Ā Ā S Ā G E A R R V L V Y G G R G A L G S R C V Q A F R A R N W W V A S I V 40

D V V E N E E A S A S * V I I V K M T D S F T E Q A D Q V T A E V G K L L G D E Q E K V 80

D A I L C V A G G W A G G N A K S K S L F K N C N L M W K Q S I W T S T I S S H 120

L A T K H L K E G G L L T L A G A K A A L D G T P G M I G Y G M A K G A V H Q L 160

C Q S L A G K N S G M P S P G A A A I A V L P V T L D T P M N R K S M P E A D F S 200

S W T P L E F L V E T F H D W I T G N K N R P S N S G S L I Q V V T T E D G K R T E L T 240

P A Y F

cleavage fragment. The rat sequence differs from the human-liver DHPR sequence of Dahl *et al.* (1987) by 10 residues. The changes in most instances are conservative but the most significant difference is around the *N*-terminal region where the human liver enzyme has a string of three additional alanines and a serine to alanine replacement (see Figure 1.1). The high conservation of sequence between rat and human reductases is astounding and implies that the structure of the protein is very tightly linked with its function.

The DHPR sequences have been compared with the DHFR sequence to determine regions of homology since the two enzymes carry out superficially similar reactions. Only a small region of homology was observed between the human DHPR and the human DHFR around Cys^{104} for the former and Gly^{20} for the latter (Dahl *et al.*, 1987). This region in DHFR is known to be involved in the binding of the pyridine nucleotide. Several DHFRs have been crystallised and subjected to high resolution X-ray structural analysis. Thus crystal structure of DHPR will be desirable to indicate if the tertiary structure of these two related proteins have any resemblance that is pertinent to their function. Preliminary X-ray diffraction data of rat-liver DHPR that crystallised in the monoclinic system with a C_2 space group has been reported (Matthews *et al.*, 1986).

AIM OF THIS RESEARCH PROGRAMME

In the light of the rapidly accumulating data that uncongugated pterins and DHPR are distributed widely even in cells that do not contain aromatic amino acid hydroxylases, further investigations are necessary to identify alternative functions. Since it is well known that in *Escherichia coli* the aromatic amino acids phenylalanine, tyrosine and tryptophan are synthesized from a common precursor, chorismate *via* the shikimate pathway (Umbarger and Davis, 1962; Gibson and Pittard, 1968), the organism will be a good model system to investigate alternative role(s) for DHPR. As there are no aromatic amino acid hydroxylases in *E. coli,* and there is no information available on the presence of a dihydropteridine reductase in the organism, the primary tasks of this study are as follows:

a) Show the presence of DHPR in the organism.

b) Isolate and purify *E. coli* DHPR to electrophoretic homogeneity.

c) Characterise the protein and compare it with other known DHPRs.

d) Investigate other enzymic activities for the purified DHPR.

e) Obtain *N*-terminal sequence data for the new protein.

f) Determine kinetic constants for the various substrates and identify the natural substrates for the enzyme.

f) Isolate the gene that encodes DHPR in the organism so that it will provide a genetic handle for the use of established mutagenesis methods to investigate effects of deletion of the gene from *E. coli*. The experimental approach to isolate the gene coding for DHPR would involve:

i) Construction of a cosmid library containing large inserts of chromosomal DNA from *Escherichia coli* (~35-40 kb).

ii) Transfection of a suitable strain and selection of clones by screening for increased DHPR specific activity.

iii) Sub-cloning the DHPR gene and characterise the gene product to confirm that it is similar to the protein purified and characterised in the first instance.

iv) Attempt to sequence the complete gene that encodes DHPR so it can be compared with the mammalian DHPR sequence and with the *E. coli* DHFR sequence to determine if there is any significant relationship.

CHAPTER 2

PURIFICATION OF DIHYDROPTERIDINE REDUCTASE FROM *ESCHERICHIA COLI*

2.1 INTRODUCTION

Dihydropteridine reductase has been isolated in a pure form from several mammalian tissues. The reductase has also been partially purified from other sources, e.g. from a *Pseudomonas* species (reviewed by Armarego *et al.*, 1984 and Shiman, 1985). The only addition to the comprehensive list of sources from which DHPR has been purified, cited in the review by Armarego *et al.*, 1984, is the NADPH-specific DHPR purified from bovine and human livers (Nakanishi *et al.*, 1986a,b). The isolation of DHPR from these various sources was spurred on by the discovery of a variant form of phenylketonuria where the biochemical defect was in the reductase (reviewed by Danks *et al.*, 1978) and also because little was known about the enzyme. The enzyme from sheep that was described by Kaufman (1959) was purified to apparent homogeneity for the first time from the liver tissue (Craine *et al.*, 1972). Three fractionation steps (ammonium sulfate precipitation, zinc-ethanol fractionation and alkaline ammonium sulfate precipitation) followed by calcium phosphate gel elution, DEAE-cellulose chromatography and Sephadex G-100 gel filtration, were used to attain a 160-fold purified reductase with a final specific activity of 15.6 U/mg. Since then, a wide range of procedures have been used to purify DHPR from other sources. The use of affinity chromatography featured strongly in most of these new procedures. An affinity column based on the fact that the reductase is inhibited by methotrexate, which is a pterin analogue, has been used to purify the sheep and rat-liver enzyme (Webber *et al.*, 1978) with methotrexate-aminohexyl Sepharose affinity adsorbent. This method has not been adopted in other laboratories and even the authors' laboratory has reported an alternate method (Webber *et al.*, 1986). The main drawback with a methotrexate-based affinity

column, despite the fact that it somewhat speeded up the purification of DHPR, is the real problem of contamination by dihydrofolate reductase.

The reductase from bovine liver has been shown to be strongly inhibited (K_i of 0.3 μM for each) by Cibacron Blue (F3GA) and Blue Dextran (Chauvin *et al.*, 1979). Binding of proteins to immobilized Cibacron Blue columns has been ascribed to the interaction between the nucleotide binding domain of the protein and the dye ligand. It is well known that this specific interaction is due to the similarity in the three-dimensional structure of NAD and Cibacron Blue F3GA (Thompson *et al.*, 1975). Further evidence for this came from the X-ray crystallographic structure of Cibacron Blue F3GA bound to liver alcohol dehydrogenase, where the dye was clearly shown to be interacting with the nucleotide binding domain (Biellmann *et al.*, 1979). This dye ligand-nucleotide binding domain interaction was exploited in the use of immobilized Cibacron Blue F3GA (Chauvin *et al.*, 1979) and Matrex Gel Blue A (Shen and Abell, 1981; Webber *et al.*, 1986) to purify large quantities of the reductase from bovine kidney, human platelets and rat liver, respectively.

However, by far the most successful affinity adsorbent available for the purification of DHPR is 1,2-napththoquinone-4-sulfonic acid coupled to aminohexyl-Sepharose. The ligand, 1,2-napthoquinone-4-sulfonic acid is a potent inhibitor of phenylalanine hydroxylase and the affinity column was initially designed to purify that enzyme (R.G.H. Cotton, private communication). However the hydroxylase did not bind to the affinity column. It was suggested that the ligand may have undergone a physical change during the cross-linking reaction with the Sepharose, thus losing its capacity to interact with the hydroxylase. Fortunately the naphthoquinone column proved to be an excellent method for purifying DHPR (Cotton and Jennings, 1978). This method has been used to purify DHPR from human liver, fibroblasts and continuous lymphoid cells (Firgaira *et al.*, 1981a,b), from human brain (Armarego and Waring, 1983) and from rat liver (Purdy and Blair, 1980). Sheep-liver DHPR apparently did not bind to the naphthoquinone affinity column (W.L.F. Armarego and H. Taguchi,

unpublished results). In all cases, homogenous preparations were obtained after the passage of the affinity purified protein through at least one other chromatographic step.

Bearing in mind the various methods that have been used to purify the reductase from other sources, the experimental approach described in this chapter was to explore the possibility of purifying the *E. coli* reductase by an affinity chromatographic procedure. This turned out to be unsuccessful and the procedure of Williams *et al.* (1976) which described the partial purification of the only other bacterial DHPR (from *Pseudomonas* species ATCC 11299a) was first adopted to purify the reductase from *Eschericha coli.* This method was not ideal but a new method developed around it allowed the purification of *E. coli* DHPR to apparent homogeneity. This successful attempt was considerably improved by employing a preparative flat bed isoelectric focusing step that increased the yield and speeded the isolation procedure.

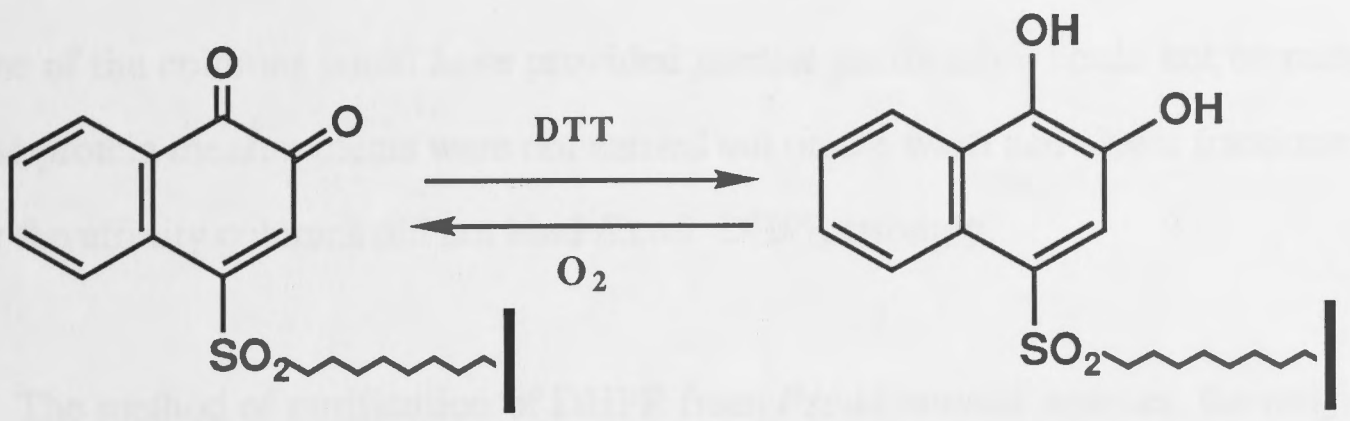
2.2 RESULTS AND DISCUSSION

In the attempted purification using the naphthoquinone affinity column the *E. coli* DHPR activity (90%) appeared in the void volume, unlike the human liver and brain enzyme that were bound to the column and were eluted by the DTT-containing buffer (see section 2.5.1; ca. Firgaira *et al.*, 1981a; Armarego and Waring, 1983). Normally, the DHPR-NADH complex binds to the mahagony-coloured oxidized quinone form of the ligand and upon washing with the DTT-containing buffer the bound ligand becomes reduced to the green-coloured diol form, which causes a weakening in its affinity for the reductase complex (see Scheme 2.1). To confirm that the affinity adsorbent was functioning properly, 80 units (enzyme activity) of human brain DHPR were applied to the column and eluted as described in Section 2.5.1. More that 90% of the human brain DHPR activity applied to the column was held and eluted by the DTT containing buffer, thus confirming that the naphthoquinone affinity adsorbent does not bind the *E. coli* DHPR-NADH complex.

Table 2.1 Trial purification using the Amicon Dye Matrex Kit

	Control	BlueA	RedA	OrangeA	GreenB	BlueB
% DHPR activity in void volume	98	84	70	80	90	100
% DHPR activity in high salt eluate	-	5	20	5	-	-

Assay: The DHPR assay was carried out as described in Section 2.5.1.1 at 25°C in Tris-HCl (pH7.4).



Sheme 2.1 The oxidised and reduced forms of the naphthoquinone ligand (Armarego *et al.*, 1984)

At this stage discovery of the inability of *E.coli* DHPR to bind the naphthoquinone Sepharose column was not surprising because not all dihydropteridine reductases are held by this column. Armarego and Taguchi (unpublished results) indicated that sheep-liver DHPR did not bind to it. However, the high homology between the predicted (from nucleotide sequence) amino acid sequences of rat-liver DHPR (Shabaz *et al.*, 1987) and human-liver DHPR (Dahl *et al.*, 1987), as well as the extensive homology between the available amino acid sequence of sheep-liver DHPR and the predicted amino acid sequence of human-liver DHPR (Lockyer *et al.*, 1987), indicates that mammalian DHPR structures are highly conserved. In this light, the lack of binding of sheep liver DHPR needs to be re-evaluated. In that particular case it is possible that deterioration of the affinity ligand may have been the reason. Although the reasons for the binding of DHPR to the naphthoquinone column are not very clear, the fact that the NADH-bound mammalian dihydropteridine reductases are held on the column suggests that the pterin binding domain may be the site of interaction. The lack of binding of *E. coli* DHPR may be due to lack of structural homology with the mammalian enzyme or the presence of some factor in the extract that inhibited the binding of this reductase, since it had been shown that the column was in working condition.

The commercially available Dye Matrex columns (Amicon Corporation) were surveyed to identify a suitable dye affinity column that would bind the reductase from *E.coli* and provide a good first step in purification. However the results in Table 2.1 indicated that none of the dye columns were suitable for binding DHPR. The possibility

that one of the columns could have provided *partial* purification could not be ruled out because protein measurements were not carried out on the wash and eluate fractions since clearly the affinity columns did not bind *E.coli* DHPR strongly.

The method of purification of DHPR from *Pseudomonas* species, the only other bacterial DHPR that had been reported (Williams *et al.*, 1976), was followed. The fractions that were eluted from the DE52 column were assayed for DHPR activity using $K_3Fe(CN)_6$ as the oxidant to generate the pterin substrate, quinonoid 6-methyl 7,8-dihydropterin. Two fractions that exhibited consumption of NADH in the DHPR assay were isolated. At first it was thought that the two species were isoenzymes because the band that was eluted at 100 mM NaCl (band I in Figure 2.1) had 50% of the reductase activity with NADPH as cofactor compared to that with NADH as the cofactor. The second band (band II in Figure 2.1) that was eluted at about 200 mM NaCl, on the other hand, was totally NADH-dependent and did not oxidise NADPH. However, upon further investigation of the two active bands using peroxidase/H_2O_2 as the oxidant to generate the quinonoid dihydropterin cofactor *in situ*, it was found that only band I exhibited pterin-dependent oxidation of NADH, whereas band II was completely inactive. This result indicated that both bands I and II had NADH oxido-reductase activity (with ferricyanide as the electron acceptor; Dixon and Webb, 1979), but only band I had DHPR activity. NADH oxido-reductases had been isolated and characterised from several sources and they are mostly involved in electron transport processes. More recently, a H_2O-forming NADH oxidase from *Streptococcus faecalis* was isolated and found to be a flavoprotein. This enzyme was also specific for NADH and completely inactive towards NADPH (Schmidt *et al.*, 1985). It is supected that the protein in band I may be similar to the *S. faecalis* enzyme because their natural molecular weights appeared to be the same (about 50 Kd). The general caution that ferricyanide should not be used as an oxidant to generate the quinonoid pterin substrate when studying DHPR in bacterial extracts should be raised. The cell-free extract from human brain *did not* exhibit pterin-independent NADH oxido-reductase activity when $K_3Fe(CN)_6$ was used as substrate (S.G. Vasudevan and W.L.F. Armarego, unpublished results).

Figure 2.1 **DE-52 anion exchange chromatography of *E. coli* dihydropteridine reductase as described in Section 2.6.3**

Key to symbols : ▲ conductivity; △ protein concentration; ◯ dihydropteridine reductase activity, assayed as described in Section 2.5.1.1; □ oxido-reductase activity, assayed as decribed in Section 2.5.1.2

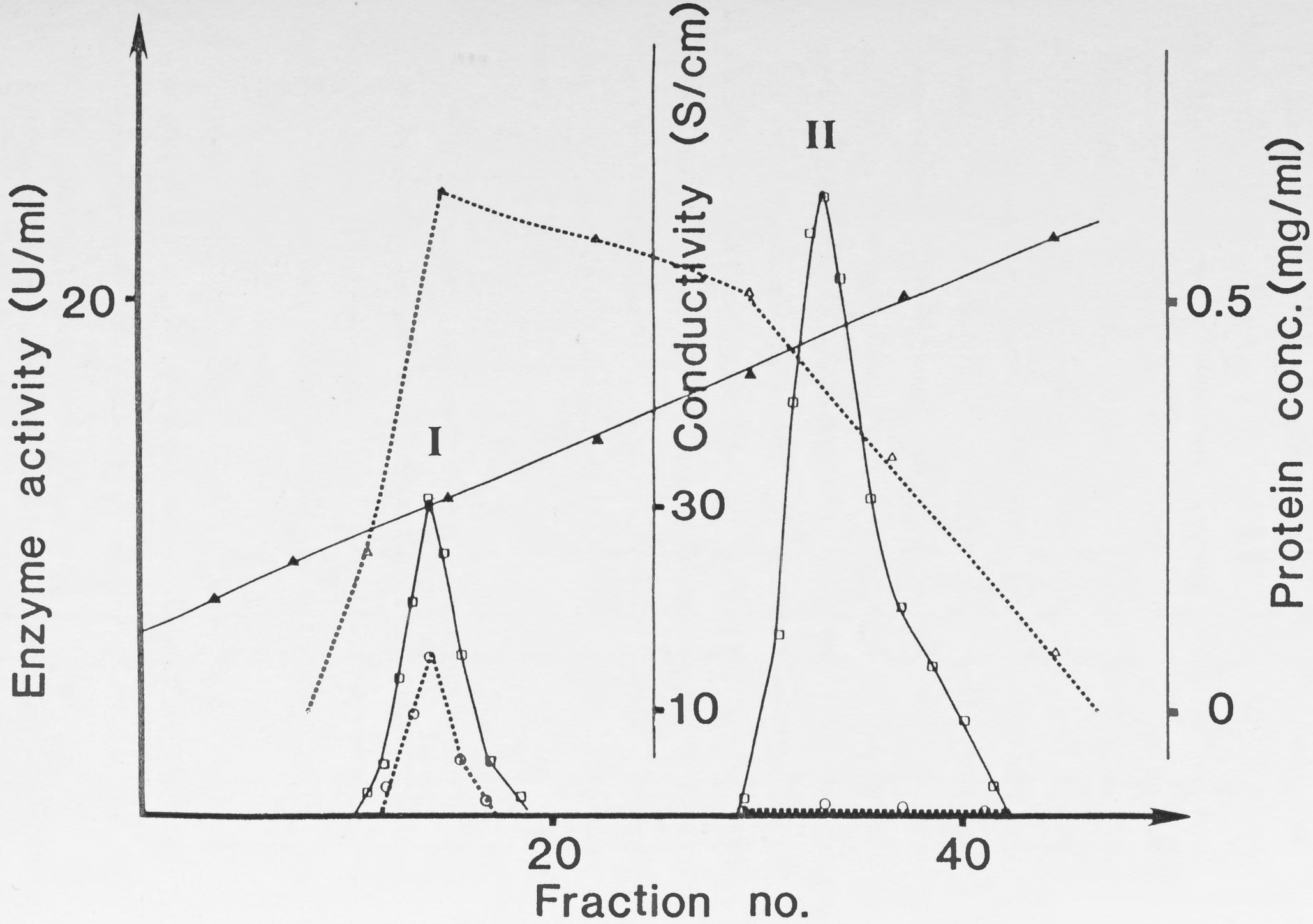

Enzyme activity (U/ml)
20
I
II
Conductivity (S/cm)
30
10
0.5
Protein conc.(mg/ml)
0
20
40
Fraction no.

Table 2.2 Requirements of pterin-dependent and pterin-independent NADH oxidation in Tris-HCl (pH7.4) at 25°C

Omission	Pterin-dependent[a] NADH oxidation[c]	Pterin-independent [b] NADH oxidation[c]
None	4.58	5.16
Enzyme	0.42	0.38
6-Methyltetrahydropterin	0.23	4.52
NADH	0.00	0.00
Peroxidase/peroxide	0.48	------
$K_3Fe(CN)_6$	------	0.45

a. DHPR assay carried out as described in Section 2.4.1.1

b. Oxidoreductase assay carried out as described in Section 2.4.2.2

c. µmoles NADH oxidized per min per ml of enzyme

The requirements for pterin-dependent and pterin-independent NADH oxidase activities were investigated and are summarised in Table 2.2. Clearly, quinonoid dihydropterin is the substrate for the pterin-dependent oxidation of NADH because when the constituents of the assay mixture such as the enzyme or the substrate (6-methyl-tetrahydropterin) or the oxidant (peroxidase/H_2O_2) were individually omitted, the level of NADH oxidation was <10% of that when all the requirements for the pterin-dependent reaction were present. The small level of NADH oxidation in the reactions without all the requirements for the DHPR assay can be ascribed to non-enzymic aerobic oxidation. In the pterin-independent oxido-reductase activity, the rate of oxidation of NADH in the absence of 6-methyl-tetrahydropterin was about 90% of that when the pterin subtrate was present. The requirement of $K_3Fe(CN)_6$ for NADH oxidation in the pterin-independent reaction was clearly demonstrated. In summary, although both peroxidase/H_2O_2 and $K_3Fe(CN)_6$ have been used as oxidants for assaying DHPR activity of mammalian enzymes, use in the purification of DHPR from *E. coli* is limited to the former. From this point on, the *pterin-dependent NADH oxidation* will be referred to as *DHPR activity* and the *pterin-independent NADH oxidation* will be known as *NADH oxido-reductase activity*.

The presence of both DHPR activity and NADH oxido-reductase activity in the partially purified band I, thought to be due to contamination by another protein with the latter activity. The DHPR-active band I lost >80% of the reductase activity that was applied to the Ultragel AcA 33 gel filtration column despite the presence of 2mM DTT and 20 μM NADH throughout the separation. The long separation time (>24 h) involved in the AcA.33 gel filtration was not suitable for the purification of *E. coli* DHPR. The *Pseudomonas* enzyme also lost >50% of the activity that was applied to each gel-filtration chromatography (Williams *et al.*, 1976).

The fast protein liquid chromatography (f.p.l.c.) system allowed rapid trials of various columns. The enzyme from the DE52 column was subjected to gel filtration (60

min) at 15°C on a Superose 6 column that had a separation range of 5 to 5 000 kD followed by a second gel filtration on Superose 12 column that had a separation range of 1000 to 3 000 kD. When the active fractions after the gel filtration purification were finally purified through a Mono-Q anion exchange column (see Figure 2.2) the resulting DHPR-active fraction also contained NADH oxido-reductase activity. Analysis of the active fractions by SDS/PAGE revealed two protein bands with M_r values of 20 000 and 27 kD (see Figure 2.3). The two protein bands were not separated after an additional step of f.p.l.c. Mono-S cation-exchange chromatography was introduced. Non-denaturing PAGE of this mixture gave one band when stained for protein. The same band also stained for DHPR activity. When the band corresponding to the protein and activity stain was cut out and extracted into 50 mM Tris/HCl pH 7.4 (500 µl), it was found to possess both DHPR activity and NADH oxido-reductase activities (see Figure 2.4).

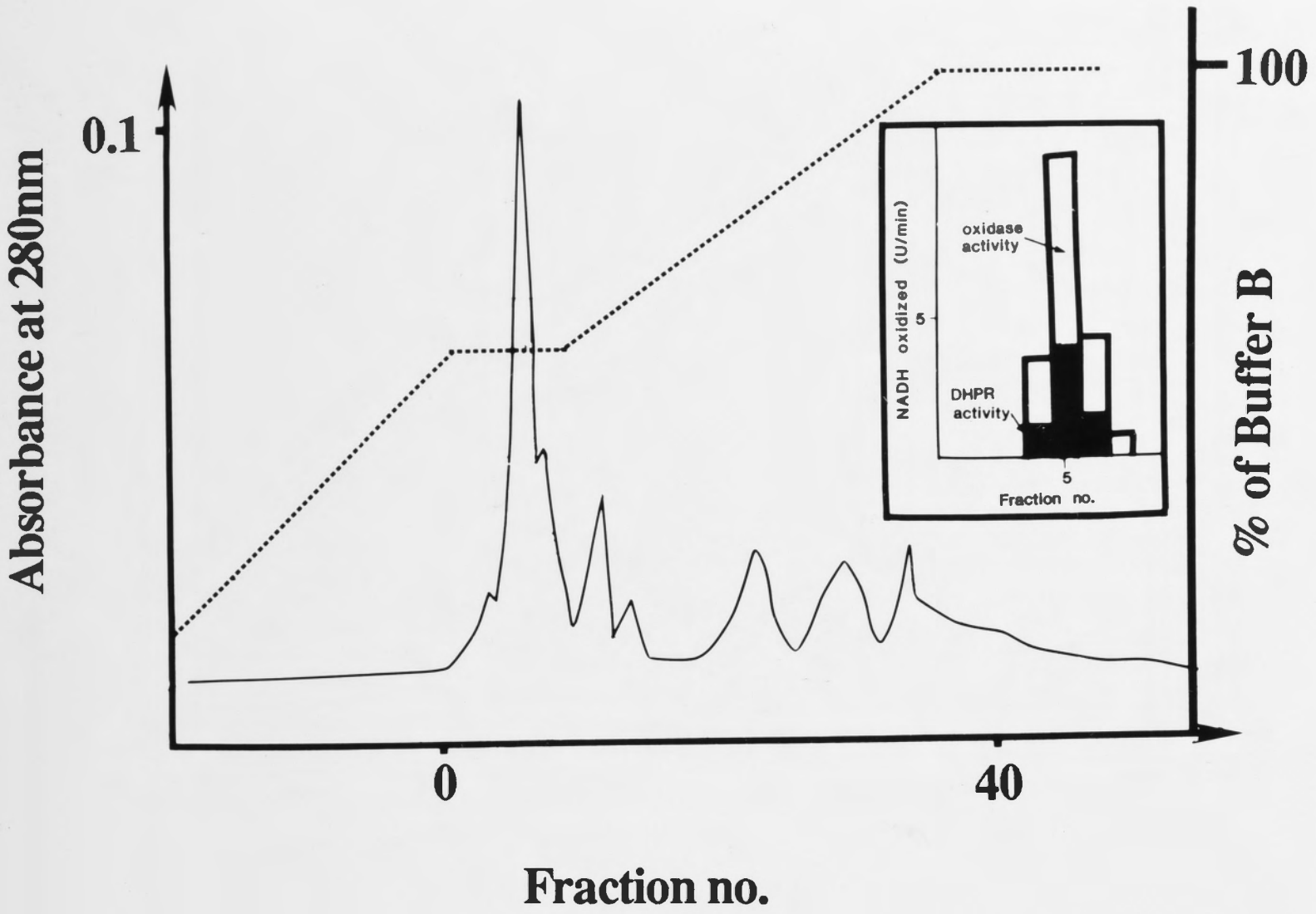
At this stage it was suspected that the protein could be a dimer with subunits of unequal M_r values, where one subunit was responsible for the DHPR activity and the second subunit was responsible for the NADH oxido-reductase activity. Further evidence that a single protein had both the activities came from looking at the ratios of the two activities during purification, which remained unchanged (see also the inset in Figure 2.2). Finally, when the two activities were measured in the presence of methotrexate, which was known to inhibit DHPR activity, both the DHPR and the NADH oxido-reductase activities were inhibited (see Figure 2.5). The concomitant loss of the two enzymic activities in the presence of an inhibitor which is a pterin analogue was taken as an indication that their active sites must be closely situated or identical.

However, when this apparently pure heterodimer was subjected to non-denaturing isoelectric focusing and stained for protein, two bands that were close to the anode were apparent. A second dimension (SDS-PAGE) of the isoelectric focused disc gel clearly showed that these were two different proteins (see Figure 2.6). If the protein was a true heterodimer, then non-denaturing isoelectric focusing should not have separated the subunits. The protein mixture was finally purified to apparent homogeneity by f.p.l.c.

Figure 2.2 Mono-Q anion exchange chromatography of *E. coli* DHPR

Partially purified DHPR (after chromatography through Superoses 6 and 12, gel filtration columns) was chromatographed on a Mono-Q column, essentially as described in Section 2.6.6. Buffer A was 50 mM Tris/HCl (pH7.4) and Buffer B contained 200 mM NaCl (in Buffer A). The column was eluted with a linear gradient of NaCl (0 to 100 mM) at a flow rate of 1 ml/min for 5 min, followed by isocratic elution at 100 mM NaCl at the same flow rate for 3 min and 0.5 ml fractions were collected. The linear gradient elution was then continued for a further 12 min. The DHPR and the oxido-reductase (oxidase) activities co-eluted between fractions 4 and 6 (Inset : Histogram shows the levels of the two enzymatic activities measured as described in Sections 2.5.1.1 and 2.5.1.2, respectively.

Figure 2.2 SDS-PAGE of samples from fractions 4, 5 and 6 after Mono-Q anion exchange chromatography.


Absorbance at 280nm
0.1
0
40
Fraction no.
100
% of Buffer B
NADH oxidized (U/min)
5
oxidase activity
DHPR activity
5
Fraction no.

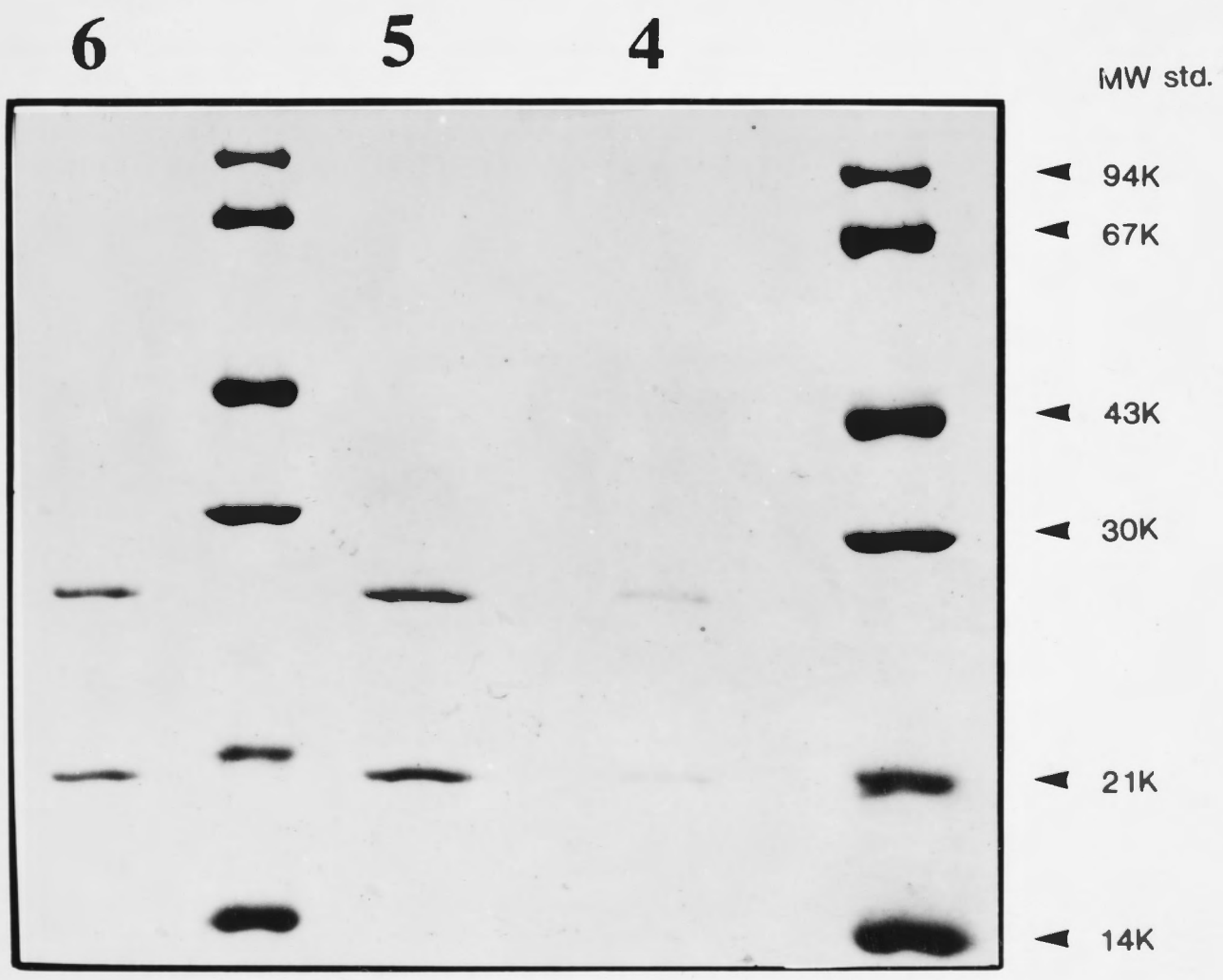
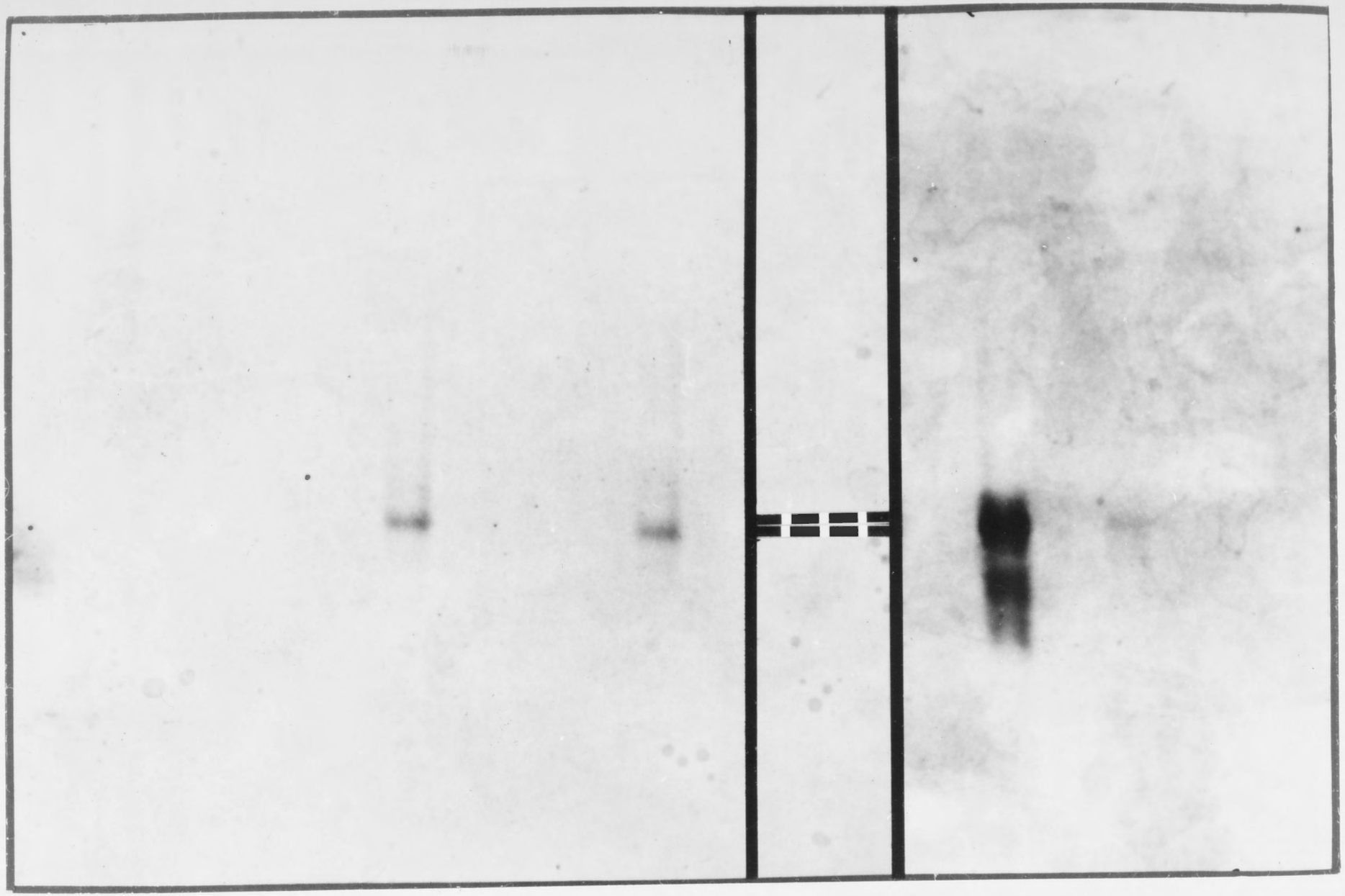

6
5
4
MW std.
94K
67K
43K
30K
21K
14K

Figure 2.4 Non-denaturing-PAGE of dihydropteridine reductase

The partially-purified protein (2 μg per track) was subjected to non-denaturing-PAGE as described in Section 2.5.3.2. The gel was cut into three sections (a, b and c). Section (a) was stained for protein using Coomassie Blue (see Section 2.5.3.1). The shaded portion in Section (b) was cut out with a scalpel and extracted into 50 mM Tris/HCl (pH7.4). The extracted protein contained both DHPR and oxido-reductase activities when measured spectrophotometrically, as described in Sections 2.5.1.1 and 2.5.1.2, respectively. Section (c) was stained for DHPR activity as described in Section 2.5.3.2. The broad band was attributed to diffusion of DHPR in the gel during the staining.



(a) (b) (c)

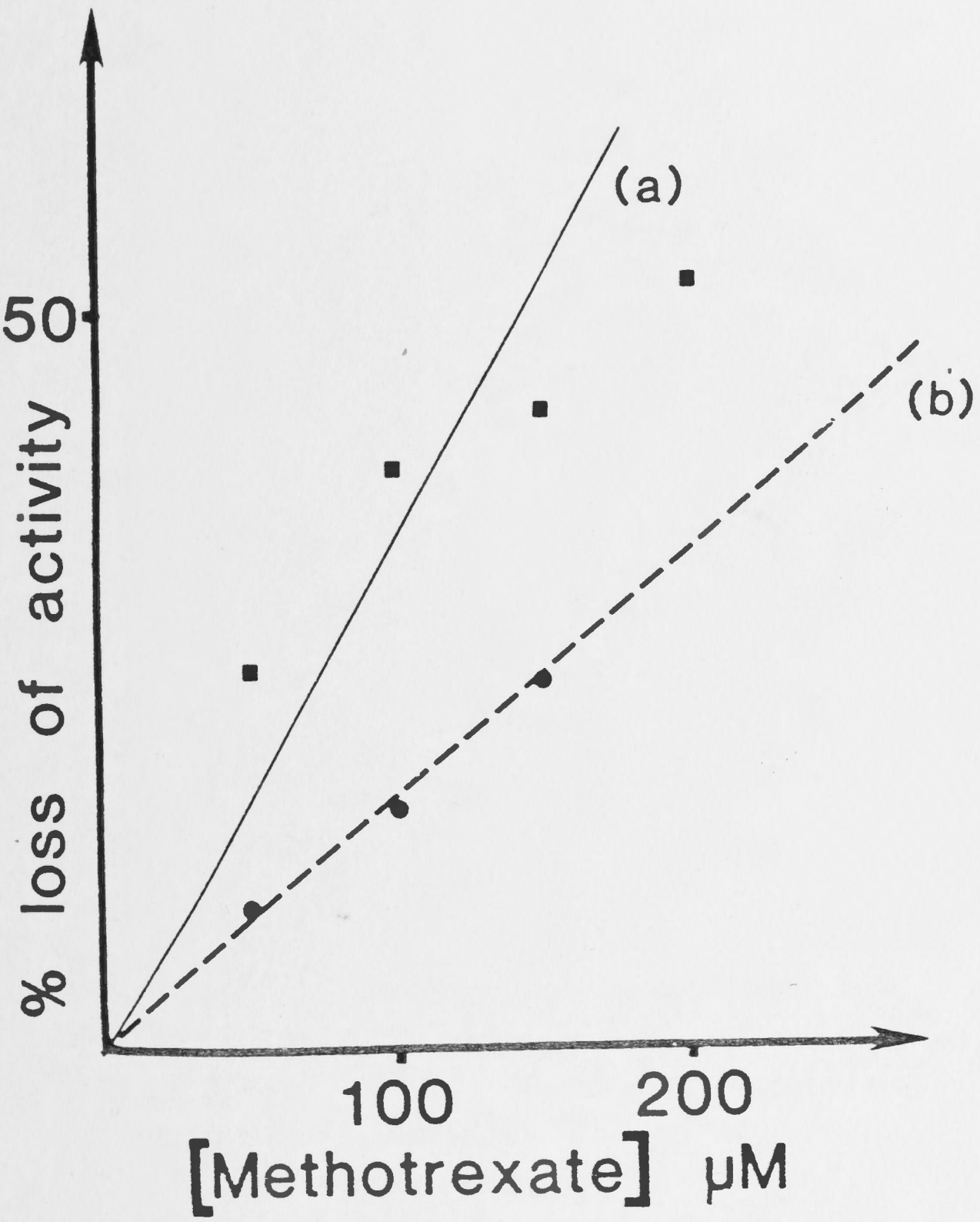


Figure 2.5 Effect of methotrexate on *E. coli* DHPR

(a) Percentage loss of pterin-independent NADH oxido-reductase activity [assay described in Section 2.5.1.2]. (b) Percentage loss of DHPR activity [assay described in Section 2.5.1.1].

Figure 2.6 Two-dimensional gel electrophoresis of apparently-pure heterodimer

~5 μg of apparently-pure *E. coli* DHPR (consisting of 27-kD and 20-kD subunits) was electrophoresed in the first dimension by non-denaturing IEF (Section 2.5.3.3) followed by a second dimension separation on a 12% (w/v) SDS-PA gel. The arrowhead 'a' indicates the 27-kD *E. coli* DHPR and the arrowhead 'b' indicates the contaminating protein (20 kD; discussed in text).

1st dimension (non-denaturing IEF)

+

2nd dimension (SDS/PAGE)

+

Table 2.3 Purification of dihydropteridine reductase from *Escherichia coli*

Purification step[a]	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	% yield
Cell-free extract	300	632	15800	0.04	1	-
Ammonium sulfate fraction[b]	80	355	4300	0.08	2	55
DE 52 anion exchange	8	80	160	0.50	12	14
Mono-Q anion exchange[c]	6	50	5.2	8.64	216	8
Mono-P chromatofocusing[c,d]	3	31	1.5	20.7	517	5

a. The ratio of DHPR and NADH oxido-reductase activity remained constant after the ammonium sulfate fractionation step.

b. The 35-50% ammonium sulfate saturation fraction was used.

c. Carried out using a complete Pharmacia f.p.l.c. system.

d. The pH range of 5.5 to 4.5 was used.

Mono-P chromatofocusing. A narrow pH range (between pH 4.5 and 5.5) was used after an unsuccessful attempt to separate them using a broad pH range (between pH 3 and 10 with the same column). The purified protein that migrated as a single band at M_r 25 000 on SDS-PAGE, contained both the DHPR and NADH oxido-reductase activies. The 20-kD band, on the other hand, did not contain either of these activities.

With this information at hand, the *E.coli* DHPR was purified to apparent homogeneity as judged by SDS-PAGE (Figure 2.7) and *N*-terminal amino acid sequencing (to be discussed in Chapter 3). A summary of the purification steps and the yields at each step is presented in Table 2.3. Gel filtration chromatography was omitted because a second anion exchange chromatography in Bistris buffer achieved a much better result as it gave a better separation at this stage and also obviated the need for additional purification steps. The two bands co-eluted through the Mono-P chromatofocusing column when a pH gradient of 3 to 10 was used. The pI values of the contaminating protein and the reductase were very close; therefore a narrow pH range chromatofocusing step was employed for the final purification. The enzyme was stable in the buffers used for chromatofocusing during the period of the separation. The pH was adjusted tp 7.0 immediately after purification and the fractions were rapidly dialysed against 50 mM Tris-HCl (pH 7.4) containing 2 mM DTT and 20 μM NADH. The purified enzyme lost 50% of its reductase activity at -70°C in ~3 months.

The reductases from mammalian sources are stable in the presence of NADH and DTT at -20°C for several years (for example human-brain DHPR that was purified in 1985 had not lost any activity in 1988). There has been no previous example to date of an unstable DHPR from any of the sources from which it has been purified (reviewed in Armarego *et al.*, 1984). In all cases, the presence of 2mM DTT and 20 μM NADH stabilized the protein. It was suspected that the reductase from this study might have been cold-sensitive because it lost enzyme activity even at -70°C. Addition of glycerol (20% w/v) to the enzyme did not protect it from inactivation. Pascal *et al.* (1983) have observed that the flavoenzyme orotate dehydratase from the trypanosomid flagellate *Crithidia*

Figure 2.7 SDS-PAGE of samples taken at different stages of purification of DHPR using the protocol summarised in Table 2.3

Lane (1) is the Pharmacia low molecular weight proteins (standard), ran concurrently with; (2) cell-free extract from strain H 712; (3) after DE-52 anion exchange chromatography; (4) after Mono-Q anion exchange (following the buffer exchange from Tris/HCl (pH 7.5) to Bistris/HCl (pH 6.5) and (5) after Mono-P chromatofocusing

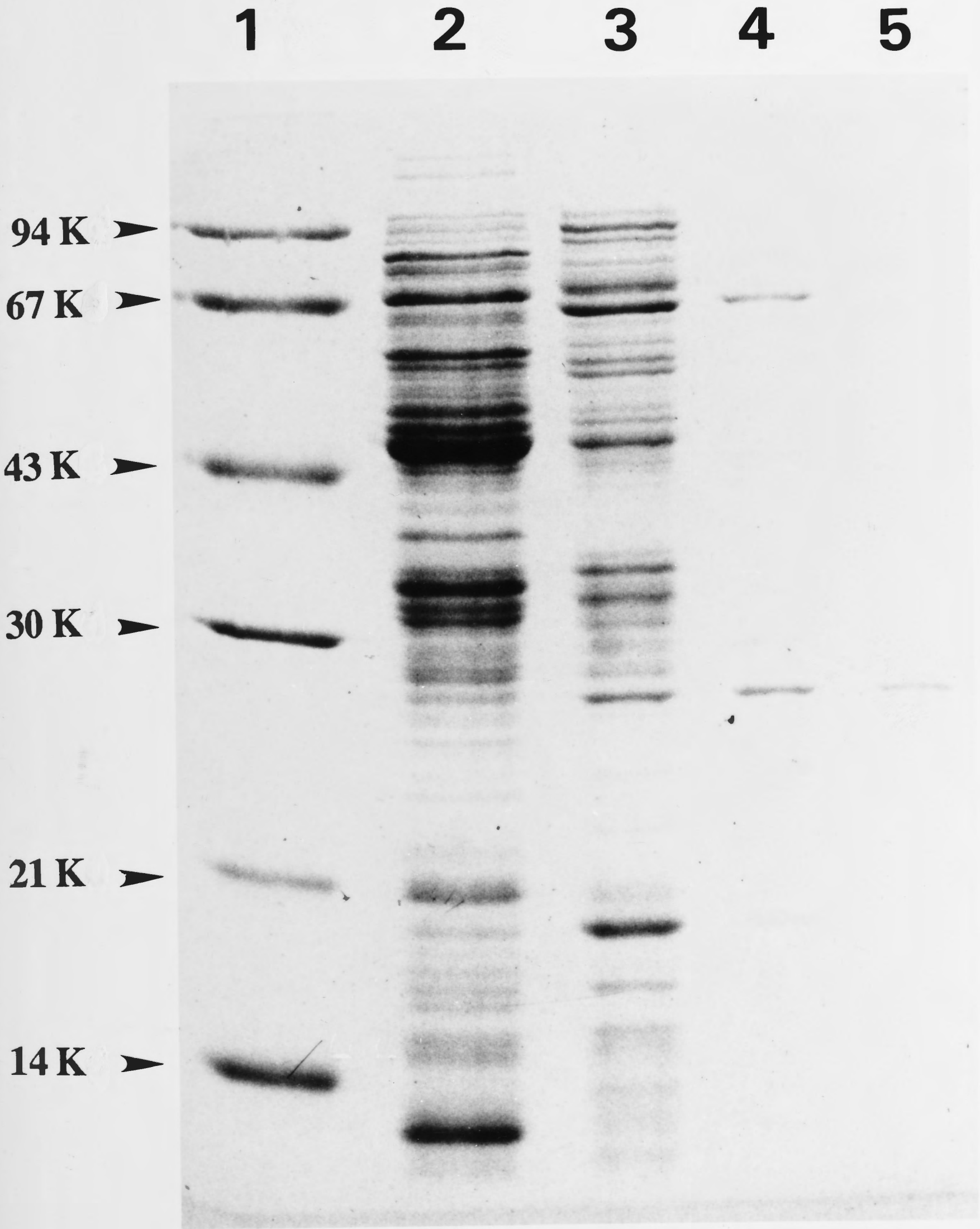

1
2
3
4
5
94 K
67 K
43 K
30 K
21 K
14 K

Table 2.4 Survey of the stability of dihydropteridine reductase from *E.coli* [a]

Condition	Temperature (°C)	DHPR activity[b] after 0 h[c]	24 h	48 h	72 h
DHPR without any addition	-20	7.8	6.9	7.3	6.8
DHPR + DTT (2mM)	-20	5.3	3.7	2.8	2.3
DHPR + DTT (2mM)	25	5.3	1.2	0.3	0.0
DHPR + NADH (20μM)	-20	6.9	7.1	7.1	7.1
DHPR + NADH (20μM)	25	6.5	7.2	6.8	6.5
DHPR + DTT (2mM) + NADH (20μM)	-20	5.8	5.7	5.4	4.7
DHPR + DTT (2mM) + NADH (20μM)	25	5.8	5.3	4.6	3.8
DHPR + Glycerol (20% w/v)	25	5.4	6.2	5.2	4.8
DHPR + BSA (1% w/v)	25	6.7	6.3	5.2	4.3

a. DHPR assay was carried out as described in Section 2.4.1.1

b. Activity units are expressed as μmoles NADH consumed per min per ml of enzyme.

c. The activity at time = 0 h was carried out to correct for reduction of activity due to volume changes.

fasciculata was more stable in Hepes-KOH pH 7.4 than in Tris-HCl pH 7.4. Since it was obvious by this stage that the reductase from *E.coli* may contain a flavin prosthetic group that gives rise to the NADH oxido-reductase activity (discussed in Chapter 3), the Tris-HCl buffer was replaced with Hepes buffer throughout all the purification steps .

The fractionation with ammonium sulfate was much better in Hepes buffer and more than 80% of the DHPR activity was in the 35-45% saturation fraction compared with 55% of the starting activity that was obtained in the 35-50% saturation fraction in 50 mM Tris-HCl pH 7.4 (compare Table 2.3 with Table 2.5). Furthermore, the high yield of DHPR activity in the 35-45% saturation fraction essentially excluded the protein responsible for the NADH specific oxido-reductase activity that was present in band II (discussed earlier) because it aggregates at a higher ammonium sulfate concentration (>45%, saturation). The DEAE-Fractogel chromatography gave a 30-fold purification with an overall activity yield of 70% which was better than the corresponding step using DEAE-cellulose DE-52 chromatography. When the fractions from the anion-exchange column that contained DHPR activity were pooled, it had a tinge of yellow colour. The colour disappeared upon addition of NADH and DTT to final concentrations of 20 μM and 2 mM, respectively. This observation was investigated further by adding NADH or DTT separately to partially-purified fractions that did not contain the two componds. The addition of NADH to a final concentration of 20 μM caused the immediate disappearence of the yellow colour but there was no change in DHPR activity. The loss of colour upon addition of NADH indicates that the flavin group must be in the oxidized form in the resting stage (this is shown spectroscopically in Chapter 3). There was no visible change in colour upon the addition of DTT to the solution, although about 20% of the total DHPR activity present at the start was lost when the reductase activity was measured 10 min after the addition of the thiol reagent.

The stability of the reductase from *E.coli* under various conditions was surveyed (see Table 2.4). It is clear that the DHPR activity was being affected by the presence of DTT. The loss of activity at 25°C in the presence of DTT is more rapid than that at 20°C.

Table 2.5 Purification of dihydropteridine reductase[a] from *Escherichia coli*

Purification step[a]	Volume (ml.)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	% yield
Cell free extract	100	303	5830	0.05	1	-
Ammonium sulfate fraction[b]	40	256	1350	0.19	4	84.5
DEAE anion exchange[c]	26	210	142	1.48	30	63.3
Preparative bed isoelectric focusing[d]	9	120	4.4	26.7	534	39.6

a. Hepes-KOH (pH 7.4) was used throughout the purification in replacement of Tris-HCl (pH 7.4)

b. A 35-45% ammonium sulfate saturation fraction was used.

c. The DEAE-Fractogel (Merck) resin was used in replacement of DE-52 (Whatman).

d. Using the Bio-Phoresis system (Bio-Rad) with a pH gradient of 4.5 to 7.5.

NADH did slow down the inactivation by DTT but it did not afford total protection. Perhaps the slow air oxidation of the pyridine nucleotide might have removed its protecting ability. There are two possible reasons for inactivation of the reductase by DTT. There may be a crucial disulfide bond in the protein that is cleaved by the thiol reagent thus causing it to unfold and become inactivated. Also it is known that thiol reagents can reduce oxidized flavins and act as a substrate for some flavoproteins and the caveat has been raised, "that the general practice of enzymologists to add some DTT to stablize soluble proteins needs to be reconsidered for flavoenzymes" (Desa, 1976). The reason for inactivation by DTT is not clear, but the reagent was omitted from all buffers used during the purification in later preparations and no loss of enzyme activity was apparent.

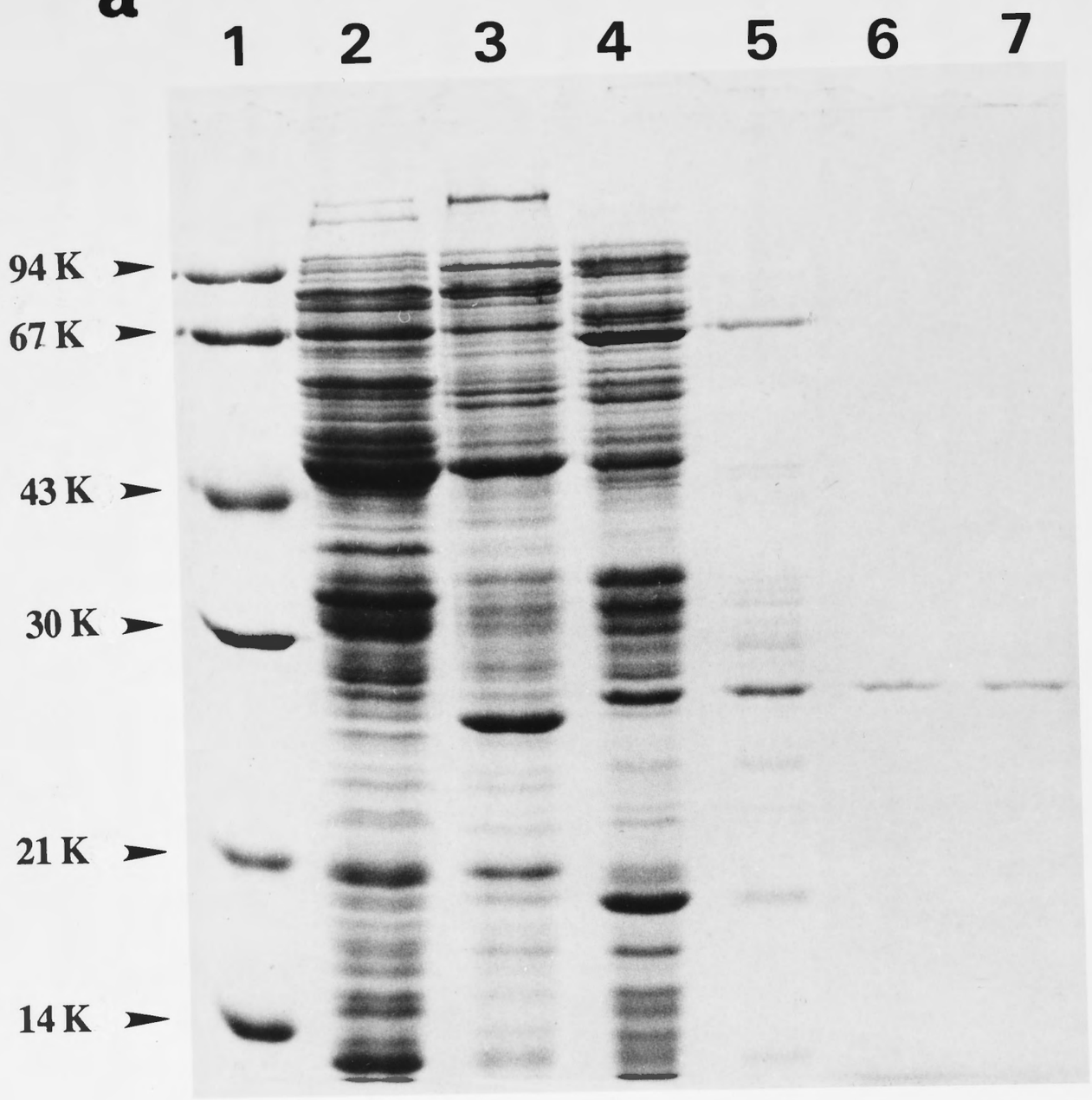
A further modification to the purification protocol summarised in Table 2.3 was the replacement of the f.p.l.c. Mono P chromatofocusing step with preparative granulated flat-bed isoelectric focusing, because of several disadvantages in using the former. The major disadvantage was that to obtain reproducible results, the Mono-P column required time-consuming stringent washing procedures: also the capacity and the volume of sample that could be applied to the column was low. Preparative flat-bed isoelectric focusing, on the other hand, allowed sample applications of up to 200 mg protein in a large sample volume (~10 ml) and required no operator attention once the separation was started. The yellow colour of the flavin prosthetic group allowed ready visualization of the required protein band. The three-step purification (see Figure 2.8) was rapid and reproducible. The yield of activity after final purification was almost 8-fold better than that summarised in Table 2.3 (compare with Table 2.5).

It is interesting to note that the final specific activities of the enzyme purified by the methods summarised in Tables 2.3 and 2.5 were of the same order despite the very low yield of activity in the former method. The low recovery by the first method may be due to the inactivation of the enzyme by DTT and Tris. The physico-chemical properties of the denatured DHPR must be different to those of the native protein during purification

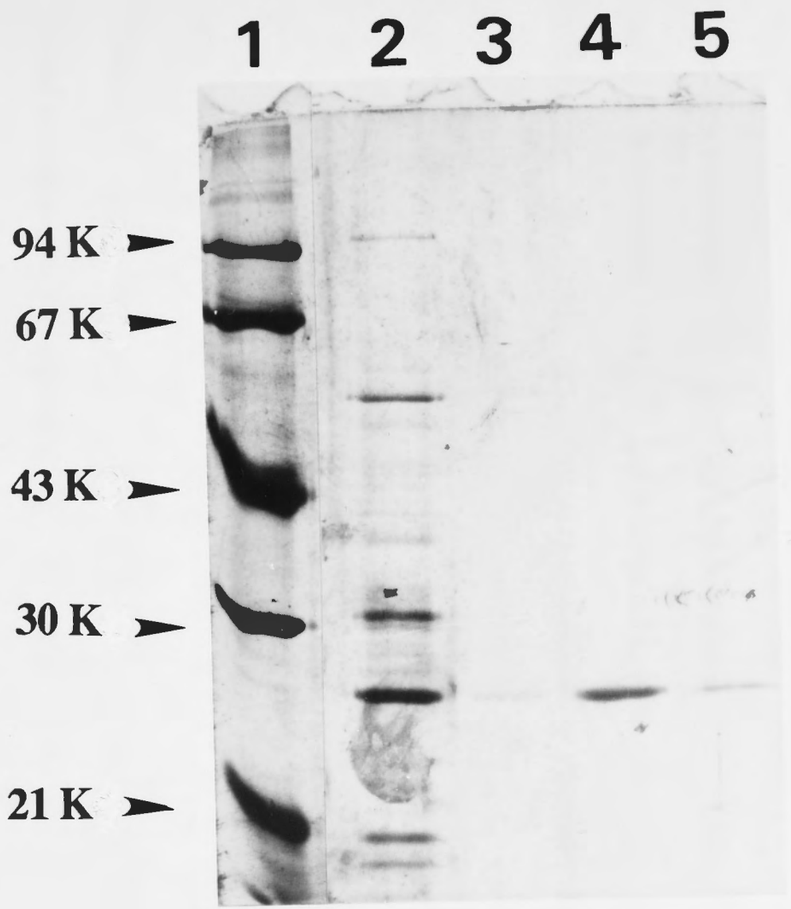
Figure 2.8 **SDS-PAGE of samples taken at different stages of purification of DHPR using the protocol summarised in Table 2.5**

In the Coomassie blue-stained gel (**a**): lane (1) is the Pharmacia low molecular weight standard; lane (2) the cell-free extract; lane (3) the greater than 35-45% ammonium sulfate fraction; lane (4) the 35-45% ammonium sulfate fraction that contains about 80% of DHPR activity; lane (5) a sample after DEAE-Fractogel chromatography and lanes (6 & 7) are fractions after preparative flat-bed isoelectric focusing.
In the silver-stained gel (**b**): lane (4) clearly shows a single band at about 27 kD for DHPR after preparative bed isoelectric focusing.

a



b



because if it were not, then the specific activity of the reductase purified by the former method (that summarised in Table 2.3) would have been considerably lower due to the presence of inactive enzyme. The reductase that was purified by preparative flat-bed isoelectric focusing was stable to storage in 50 mM Hepes-KOH (pH 7.4) containing 20 μM NADH at -20°C.

2.3 SUMMARY

After unsuccessful attempts using previously published methods, the *Escherichia coli* DHPR was purified to apparent homogeneity by two methods summarised in Tables 2.3 and 2.5. The latter method was rapid and gave a higher yield. The enzyme was more stable in Hepes-KOH buffer (pH 7.4) at every stage in the purification. Unlike other dihydropteridine reductases that have been described in the literature, the *E. coli* DHPR contains a tightly-bound prosthetic group (see Chaper 3). The presence of the prosthetic group enabled the *E. coli* reductase to carry out pterin-independent oxidation of NADH or NADPH when an artificial electron acceptor such as $K_3Fe(CN)_6$ was present. The susceptibility of the reductase from *E.coli* to inactivation by DTT is another feature that has not been encountered previously.

2.4 MATERIALS

The *E.coli* strains used for purification of DHPR: H712 (F^-, *guaB22 xyl-7 rpsL125*) and D3-157 (F^-, as H712 *fol200* xyl^+) were generous gifts from Dr Sara Singer of Burroughs Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A. (Singer *et al.*, 1985). All the 5,6,7,8-tetrahydropterins used in the enzyme assays throughout this work were prepared by standard procedures (Armarego and Schou, 1978) and were kind gifts from Dr Armarego. The human-brain DHPR used in this study was purified in this work using the naphthoquinone affinity column procedure

(Firgaira *et al.*, 1981a) and DEAE-Fractogel chromatography. The water used throughout this work was either glass distilled or Milli-Q reagent water (Millipore).

2.5 EXPERIMENTAL METHODS

2.5.1 Measurement of enzyme activity

Assays of DHPR activity (pterin-dependent oxidation of NADH or NADPH) and oxido-reductase activity (pterin-independent oxidation of NADH or NADPH) were carried out spectrophotometrically at 25°C on a Cary 219 or a Unicam SP 1800 double-beam spectrophotometer.

2.5.1.1 The DHPR assay

The stock solutions of pterin substrate (8-10 mM in 4 mM HCl) and NADH or NADPH (8-10 mM in 0.1 M Tris-HCl, pH 7.4) were stable for at least two months when stored frozen. The solutions for the assay were diluted appropriately to ~2 mM when required and these were stable at 4°C for several hours. The standard reaction mixture for the measurement of DHPR activity contained Tris-HCl, pH 7.4 (0.1 M), peroxidase (11 μg), H_2O_2 (5 μM), 6-methyl-5,6,7,8-tetrahydropterin (0.1 mM in 4 mM HCl) and NADH or NADPH (0.1 mM in 0.1 M Tris/HCl pH 7.4) in a total volume of 1 ml in each of two cuvettes. The substrates (pterin and NADH or NADPH) were kept on ice and added 2-3 minutes before the initiation of the reaction in small volumes (50 μl) so that the temperature can equilibrate rapidly and the quinonoid dihydropterin can be formed *in situ*. The DHPR reaction was initiated by injecting the enzyme solution (5-10 μl, 0.25-0.5 μg) into one cuvette only and the rate of change in absorbance at 340 nm due to the pterin-dependent oxidation of NADH or NADPH was recorded. The enzyme activity was calculated by using the molar absorption coefficient at 340nm of 6220 $M^{-1}cm^{-1}$ for NADH or NADPH.

2.5.1.2 The Oxido-reductase assay

The standard reaction mixture for the oxido-reductase activity (pterin-independent activity) contained Tris-HCl, pH 7.4 (0.1 M), $K_3Fe(CN)_6$ (0.3 mM) and NADH or NADPH (0.1 mM) in a total of 1 ml in each of two cuvettes. The assay was initiated by injecting the enzyme solution into one cuvette only and the rate of oxidation of the pyridine nucleotide was used to calculate the oxido-reductase activity, as described in Section 2.5.1.1.

One unit of enzyme activity for DHPR or oxido-reductase activities was defined as the amount of enzyme that caused the oxidation of 1 µmole of NAD(P)H per minute under the conditions described for the above assays.

2.5.2. Protein determination

Protein concentration was determined essentially by the method of Bradford (1976) using the Bio-Rad protein assay kit with bovine serum albumin as a standard. To 800 µl of bovine serum albumin in clean 1 ml plastic cuvettes (Sarstedt, W.Germany) at a series of concentrations between 2 and 20 µg/ml, was added 200 µl of the Bio-Rad cocktail. The solutions were mixed well by inverting the cuvette after sealing with parafilm. The absorbance at 595 nm was measured with a Perkin-Elmer Lambda 1 single beam spectrometer after the mixture had been standing at room temperature for about 10 minutes. The blank solution contained 800 µl of the buffer without bovine serum albumin and a standard curve was plotted. Sample protein solutions were diluted until the absorbance reading was in the middle range of the bovine serum albumin standard curve.

Routine protein measurement of fractions eluted from columns were estimated by absorbance at 280 nm where it was assumed that an absorbance of 1.0 at 280 nm was equivalent to a protein concentration of 1 mg/ml.

2.5.3 Electrophoretic methods

2.5.3.1 SDS-PAGE

Unless otherwise stated SDS-PAGE (Weber and Osborn, 1969) was carried out on slab gels 100 mm high by 150 mm wide by 1.5 mm thick. The separating gel contained 12% (w/v) acrylamide (from a stock solution containing 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide). 0.375 M Tris-HCl (pH 8.8) and 0.1% (w/v) SDS, in a final volume of 15 ml. (The mixture was degassed under a water pump for five minutes before the SDS was added). The polymerization was initiated by adding 50 µl of 10% (w/v) ammonium persulfate and 5 µl of TEMED. The stacking gel contained 5% (w/v) acrylamide (from the above 30:0.8 stock solution), 125 mM Tris-HCl (pH 6.8) and 0.1% SDS in a final volume of 5 mls. Ammonium persulfate (40 µl of 10% stock solution) and TEMED (4 µl)were added to polymerize the stacking gel. Sample wells 15 mm deep and 4 mm wide were made by inserting a comb into the stacking gel prior to polymerization. The separating gel was sometimes left standing overnight at 4°C with a 0.2% SDS overlay, but the stacking gel was always prepared 1-2 h before use. The sample was diluted (1:1) in the sample loading buffer (containing 0.08% (w/v) bromophenol blue, 25% (w/v) glycerol, 0.4 M Tris base, 67 mM DTT and 2% SDS) and heated at 100°C for 2 mins. After the samples were loaded into the sample wells, they were subjected to electrophoresis at a constant voltage of 60 V for 1 h, then at a constant voltage of 120 V for 3 h in a running buffer that contained 50 mM Tris, 385 mM glycine and 0.1% (w/v) SDS. The gel was fixed and stained simultaneously in solution that contained 0.25% (w/v) Coomassie Brilliant Blue G-250, 50% (v/v) methanol and 9% (v/v) acetic acid. Destaining of the gel was carried out in 10% (v/v) isopropanol and 10% (v/v) acetic acid. The fixing/staining and destaining steps were carried out at room temperature. The destained gel was stored in 7% acetic acid with 5% glycerol until it was photographed.

2.5.3.2 Non-denaturing-PAGE and DHPR activity stain

Non-denaturing-PAGE (Ornstein, 1964; Davis, 1964) was carried out in slab gels. The separating gel contained 6% (w/v) acrylamide (from the 30:0.8 stock solution)

and 0.375 M Tris-HCl (pH 8.8) in a total volume of 15 ml and polymerized with 40 µl of 10% (w/v) ammonium persulfate and 5 µl of TEMED. The stacking gel contained 2.5% acrylamide (from the above 30:0.8 stock solution) and 125 mM Tris-HCl (pH 6.8) in a total volume 5 mls. Polymerization was initiated with 40 µl of 0.04% (w/v) riboflavin and 4 µl TEMED. The gel was cooled to 4°C before loading the samples which were prepared by diluting the protein sample (1:1) in the loading buffer [which contained 0.08% (v/v) bromophenol blue, 25% (w/v) glycerol and 50 mM Tris-HCl (pH 8.8)]. Electrophoresis was carried out at a constant voltage of 30 V for 15 h at 4°C. The gel was cut into three sections and used for protein staining, enzyme activity measurement and DHPR activity stain, respectively. The DHPR activity stain was essentially as described by Cotton and Jennings (1978). The stain solution contained 1 mM NADH, 1 mM tetrazolium MTT (Sigma), and 50 mM Tris-HCl (pH 7.4) in a total of 9 ml at 37°C. The substrate, 1 mM quinonoid 6-methyl 7,8-dihydropterin was generated using dichlorophenolindophenol as the oxidant according to the method developed by Kaufman (1961). This was added to the above mixture and the gel was immersed in the solution with gentle rocking for five minutes. The stained gel was washed with two changes of distilled H_2O (200 ml) and photographed immediately. (Note that the free acid of dichlorophenolindophenol should be used to generate the quinonoid dihydropterin substrate and not the sodium salt).

2.5.3.3 Two-dimensional gel electrophoresis

Two dimensional gel electrophoresis was carried out using the method of O'Farrell (1975). In the first dimension, isoelectric focusing in tube gels separated proteins on the basis of their isoelectric points. The cylindrical gels (5 mm diameter x 130 mm long) consisted of 6% (w/v) acrylamide:bisacrylamide [from a 30% (w/v) stock solution containing 28.4% (w/v) acrylamide and 1.6% (w/v) bisacrylamide], 10% (v/v) glycerol and 2% (w/v) ampholines [a mixture of 1.6% (w/v) ampholines with a pH range of 5-7 and 0.4% (w/v) ampholines with a pH range of 3.5-10 from Pharmacia] in a total volume of 15 ml. The mixture was polymerized by adding 35 µl of 10% (w/v) ammonium persulfate and 7 µl of TEMED. The gel was overlaid with 200 µl of 10%

(v/v) glycerol containing 2% (w/v) ampholines. Samples (200-300 µl) were loaded and subjected to electrophoresis for 4 h starting at 180 V and maintaining constant current until the voltage reached 800 V for the remainder of the run. The cathode solution (20 mM NaOH) was in the top tank and the anode solution (10 mM phosphoric acid) was in the bottom tank. After electrophoresis the disc-gels were placed in SDS sample buffer (2% SDS, 62.5 mM Tris-HCl, 50 mM DTT, 10% glycerol, pH 6.9) and frozen at -20°C. The second dimension SDS-PAGE was set up and run essentially as described in section 2.5.3.1. The stacking gel did not contain sample wells. The first dimension disc-gel (from IEF) was placed carefully along a groove between the two glass plates and immobilized with 1% (w/v) agarose.

2.5.3.4 Silver stain of protein gels.

In cases where a sensitive staining procedure was required to visualise protein bands on a PA gel, silver staining was carried out essentially as described by Oakley *et al.* (1980). Gloves were worn throughout the procedure because fingerprints otherwise showed up. The gel was prefixed in a solution containing 50% methanol and 10% acetic acid (500 ml) for 30 min. This was followed by a wash in a solution (500 ml) containing 5% methanol and 7% acetic acid for 2 h or more. The gel was then soaked in 10% glutaraldehyde (80 ml of reagent stock + 120 ml water) for 30 min with gentle agitation on a gyratory shaker. After this the gel was rinsed in four changes of water (30 min each change), with agitation. While the gel was being rinsed, fresh ammoniacal silver solution was made as follows; to a stirred mixture of 2.8 ml of 23% NH_4OH solution and 42 ml of 0.36% NaOH was added 8 ml of 19.4% $AgNO_3$ dropwise. The solution was then made up to 200 ml with water. The gel was stained in the ammoniacal solution for 15 min with agitation. This was followed by a wash in water for 2 min with agitation and then the gel was developed in a dish containing freshly made reducer solution (0.2 ml 5% citric acid and 0.1 ml 37% formaldehyde made up to 200 ml) for about 5 min. The gel was then washed with three changes of water (30 min each time) and stored in 5% acetic acid until it was photographed.

2.5.4 Growth of Cells

LB (Luria-Bertani) medium containing the appropriate supplements was used to culture the bacterial strains used in this study. LB contained (per litre):

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
NaOH (1N)	2.5 ml

The pH of the medium was ~ 7.4 before sterilization by autoclaving. All media used for culturing bacteria were sterilised by autoclaving at 121°C for 30-45 min. *Escherichia coli* strains H712 and D3-157 were routinely cultured in LB medium supplemented with 0.2% (w/v) glucose (from a 20% w/v stock solution) and 50 μg/ml thymine (from a 2.5 mg/ml stock solution). The supplementations were more crucial for the *folA* strain D3-157.

Large quantities of strains H712 and D3-157 for isolation of DHPR were prepared using the following procedure: The strains were streaked onto a LB agar plate (1.5% (w/v) agar in LB) and incubated at 37°C for 15 h. Single colonies from the plate were used to inoculate 10 ml LB media containing appropriate supplements in a 150-ml capacity Erlenmeyer flask and cultured with aeration at 37°C for 10 h in a shaker waterbath. This culture (5 ml each) was used to inoculate two 2-L capacity Erlenmeyer flasks each containing 1 L of LB medium containing supplements and cultured with aeration at 37°C for 15 h in a gyratory environmental shaker. The cell suspension was in turn used to inoculate 38 L of sterile growth medium (1% (w/v) yeast extract, 0.9% (w/v) glucose*, 1% (w/v) K_2HPO_4, 0.18% (w/v) KH_2PO_4 and 5 x 10^{-5}% (w/v) thymine) in a 40 L fermenter (New Brunswick Scientific). The culture was incubated at 37°C with stirring and 30-50 mls were removed aseptically every 1 hr to determine the growth rate by measuring the turbidity at OD_{595}. The cells were normally harvested at the end of the log phase of growth (~5 h) and sedimented in a Sharpels continuous-flow centrifuge. The wet weight of cells obtained was between 100 and 130 g.

*Glucose (20% w/v) stock was sterilised separately and added aseptically.

2.5.5. Preparation of cell-free extract

The procedure for the lysis of cells and preparation of cell-free extract was essentially as described by Fuller and Kornberg (1983). Briefly, the cells from the large-scale growth were washed twice with 50 mM Tris/HCl (pH 7.4) containing 1 mM EDTA, 200 mM KCl and 2 mM DTT (buffer A) and suspended in the same buffer (2 ml/g of cells). This cell suspension was divided into 20-ml portions into 60Ti Beckman centrifuge tubes. Chicken egg-white lysozyme (Sigma, 50 mg/ml in buffer A) was added to the cell suspension to a final concentration of 5-10 mg/ml and the suspension wasshaken vigorously. The suspension was incubated at 0°C for 30 min, frozen in liquid nitrogen and thawed at 0°C to produce a gelatinous suspension. This was centrifuged at 150,000 g for 1 h at 4°C to give the cell-free extracts that were used for purification of the reductase. The protease inhibitor PMSF (Sigma) was added routinely to cell free extracts at this stage to a final concentration of 0.5 mM. This method of preparation of cell-free extract had the advantage that contamination by nucleic acids was minimal, thus obviating the need for an additional step in streptomycin sulfate precipitation. Note: The Tris/HCl was replaced with Hepes/KOH in later preparations.

2.5.6 Ammonium sulfate fractionation

Ground solid ammonium sulfate was added slowly over 30 min to bring the extracts to the required saturation (see Scopes, 1982), and stirred for a further 1 h to allow complete equilibration to take place between the dissolved and aggregated proteins. The aggregated protein was separated by centrifugation at 6000 x g for 20 min at 4°C. When the cell-free extract was in Tris buffer, a 35-50% ammonium sulfate saturation fraction contained about 60% of the total DHPR activity present in the starting extract and when it was in Hepes buffer, a 35-45% ammonium sulfate cut contained about 80% of the starting activity.

2.6 DEVELOPMENT OF PURIFICATION PROCEDURE

Unless otherwise stated all the purification steps were carried out at 4°C.

2.6.1 Naphthoquinone affinity chromatography

A sample (10 ml, 50 units of DHPR activity) of the 35-50% ammonium sulfate fraction in 50 mM Tris-HCl (pH 7.8) containing 0.8 MNaCl and 0.1 mM NADH, was applied to a naphthoquinone affinity column [5 cm x 1.5 cm; prepared by Mr Bella Paal using the method of Cotton and Jennings (1978)] that was previously equilibrated with the same buffer. After the passage of the extract, the column was washed successively with 40 ml of 50 mM Tris-HCl (pH 7.4), containing 0.8 M NaCl and 0.1 mM NADH (buffer B; the eluate was Fraction 1); 30 ml of 0.1 M Na_2CO_3 (pH 10) containing 0.8 M NaCl and 0.1 mM NADH (buffer C; the eluate was fraction 2); 30 ml of buffer C without NADH (the eluate was fraction 3). After a wash with 20 ml of buffer B without NADH (this eluate was fraction 4), a final wash with 30 ml of buffer B without NADH but with 5 mM DTT (this eluate was fraction 5) was carried out. The column was regenerated as described by Cotton and Jennings (1978) after it was washed with 8 M urea (20 ml) followed by 50 mM Tris-HCl pH 7.4 (200 ml).

2.6.2 Screening with dye Matrex columns.

The dye Matrex kit (Amicon) consisted of 6 polypropylene columns (9mm x 32 mm), each containing Matrex Gel Blue A, Matrex Gel Red A, Matrex Gel Orange A, Matrex Gel Green A, Matrex Gel Blue B and Matrex Gel control (without ligand). The columns were washed with 12 ml each of 8 M urea in 0.5 M NaOH (buffer D) to remove any free dye that may have resulted from very slow hydrolysis of the dye-agarose linkage. After draining buffer D the columns were washed with 30 ml each of 50 mM Tris-HCl (pH 7.4; buffer E). The dialysis residue of the 35-45% ammonium sulfate saturation fraction (0.5 ml, 10 units of DHPR activity) in buffer E was applied to each column, allowed to drain, and left standing for 0.5 h to allow binding equilibration which otherwise might not have occurred because of high flow rate. The columns were each washed with 10 ml of buffer E and the eluate was collected (void volume fraction). The bound proteins were eluted with 10 ml each of buffer E containing 1 to 5 M NaCl (high salt eluate fraction).

2.6.3 DE-52 anion exchange chromatography

The dialysis residue of the 35-50% ammonium sulfate saturation fraction in 50 mM Tris-HCl (pH 7.4; 100 ml; buffer E) was applied to a DEAE-cellulose DE-52 column (2.0 cm x 15 cm; Whatman) which was previously equilibrated with buffer E. The column was eluted with this buffer at a flow rate of 1 ml/min until the u.v. absorbance of the effluent at 280 nm was less than 0.1. A linear gradient between 250 ml of buffer E and 250 ml of buffer E containing 250 mM NaCl (formed with a Gradient mixer; Pharmacia) was used to elute the enzyme, which appeared at about 100 mM NaCl. Fractions of the eluate (8 ml) were collected in tubes containing DTT and NADH to give final concentrations of 2 mM and 20 μM respectively. The active fractions were pooled and concentrated to 8 ml in an ultrafiltration stirrer cell (Amicon) using a PM-10 membrane (10,000 MW cut-off) at a N_2 pressure of 45 psi. The concentrate was centrifuged (3000 x g) to remove some precipitated protein and the supernatant was stored in glass vials (1 ml aliquots) at -70°C.

2.6.4 Ultragel AcA-34 gel filtration

A sample of the enzyme (1 ml) from Section 2.6.3 was applied to a column of Ultragel AcA 34 (2.5 cm x 80 cm; LKB) that had been previously equiliberated with 50 mM Tris-HCl containing 2 mM DTT and 20 μM NADH (pH 7.4, buffer F). The fractionation range of Ultragel AcA 34 was 20,000-400,000 and when the column was run isocratically with buffer F at a flow rate of 20 ml/h, the DHPR activity appeared between 340-360 ml (void volume).

2.6.5 F.p.l.c Superose 12 gel filtration

The partially-purified sample (500 μl, after DE52-chromatography) was applied to a Superose 12 HR $^{10}/_{30}$ column (Pharmacia; a complete f.p.l.c. system was used) that was previously equilibrated with 50 mM Tris-HCl containing 2 mM DTT, 20 μM NADH and 200 mM NaCl (pH 7.4; buffer G, 30 ml). The enzyme was eluted with buffer G at a flow rate of 0.25-0.5 ml per min. The enzyme activity appeared between 18-20 ml.

2.6.6 F.p.l.c. Mono-Q anion-exchange chromatography

A sample (1 ml, after DE-52 chromatography) was dialysed against 50 mM Bistris/HCl pH 6.5, containing 2 mM DTT and 20 μM NADH (buffer H, 2 x 500 ml) using a Spectrapor (Spectrum Medical Industries, LA, U.S.A.) dialysis tube. The dialysis residue (1 ml) was applied to the Mono-Q HR $^{5}/_{5}$ column (Pharmacia). After a 5-min wash with buffer H at flow rate of 1 ml/min, a linear gradient of 0 to 200 mM NaCl in the same buffer (30 ml) was applied. The enzyme was eluted around 100 mM NaCl and the active fractions (3 ml) were stored at -70°C.

2.6.7 F.p.l.c. Mono-P chromatography

The active fraction (3 ml) after f.p.l.c. Mono-Q chromatography was dialysed against 25 mM piperazine-HCl (pH 6.3; 2 x 500 ml). The sample (1 ml) was then applied to a Mono-P HR $^{5}/_{20}$ column (Pharmacia), which had been treated and pre-equilibrated as specified by the manufacturers. The column was eluted with 10% (v/v) Polybuffer 74-HCl (Pharmacia, pH 4.5, 30 ml) at a flowrate of 1 ml/min to form a pH gradient between 5.5 and 4.5. The reductase activity was eluted at a pH of 5.3. The active fractions were adjusted to pH 7.4 with 50 mM Tris-HCl containing 2 mM DTT and 20 μM NADH and dialysed against the same buffer (2 x 500 ml). The purified active fractions were stored in 500 μl aliquots at -70°C.

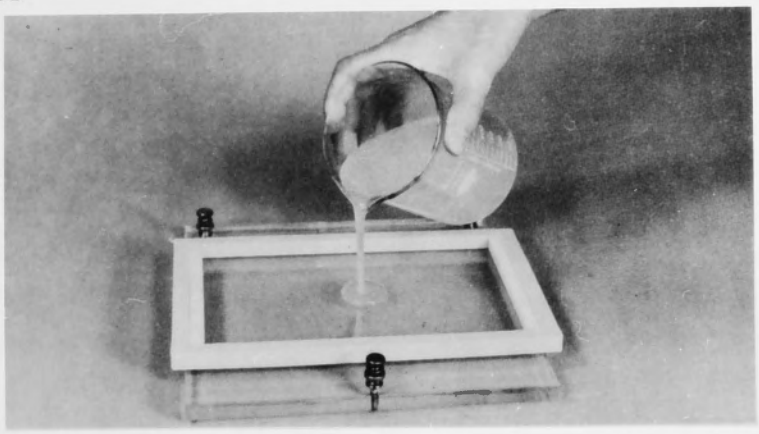
2.6.8 DEAE-Fractogel chromatography

The 35-45% ammonium sulfate precipitate which contained 80% of the DHPR activity was suspended in 50 mM Hepes/KOH pH 7.4 (buffer I) and dialysed against the same buffer (3 x 2 L). The sample (100 ml) was applied to a DEAE-Fractogel column (2 cm by 15 cm, Merck) which was previously equilibrated with buffer I at a flow rate of 2.5 ml/min. The column was washed with about 10 column volumes of the same buffer. A linear gradient formed with 250 ml of buffer I and 250 ml of buffer I containing 200 mM NaCl was used to elute the enzyme activity around 100 mM NaCl. The active

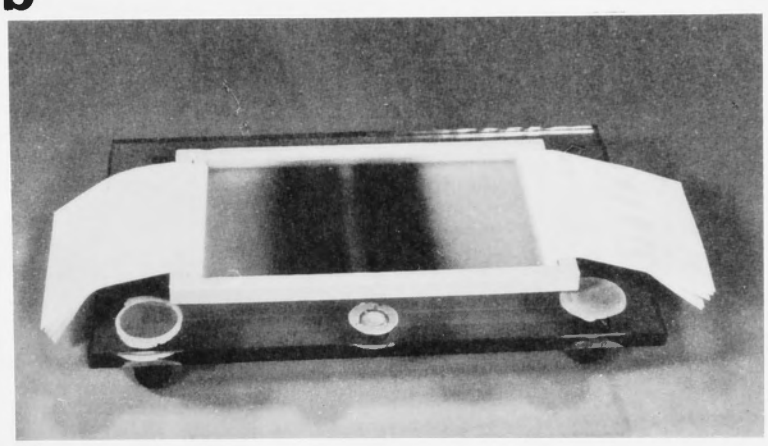
Figure 2.9 The steps involved in setting up and carrying out the flat bed isoelectric focusing procedure is presented pictorially (reproduced with kind permission from Bio-Rad Laboratories)

In (a) the gel + ampholyte slurry is being poured into the gel tray with wet electrofocusing wicks at either ends of it; (b) the excess moisture from the slurry is removed by the wicking method; (c) the sample is applied in the middle of the gel; (d) the uppermost wicks on either sides are replaced with wicks that are wet with the anodic and cathodic buffers; (e) the tray is placed on the cooled, wet stage and focused as required and in (f) it can be clearly seen that coloured proteins can be readily identified and isolated after placing a grid over the electrofocused gel and harvesting the band of interest.

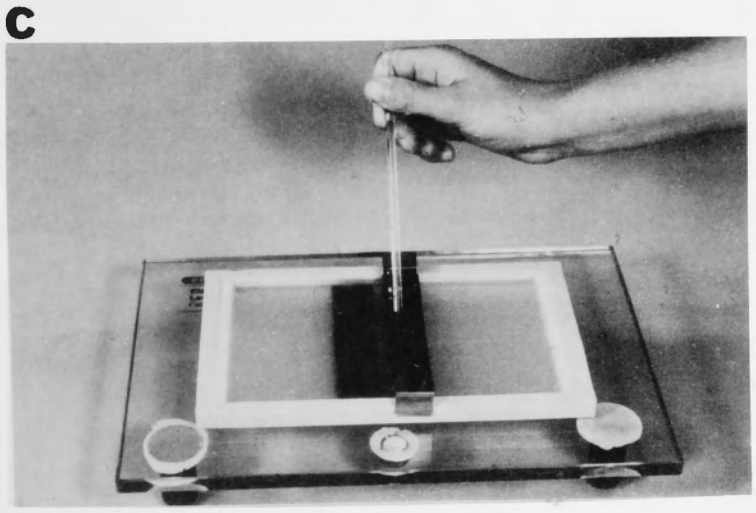
a



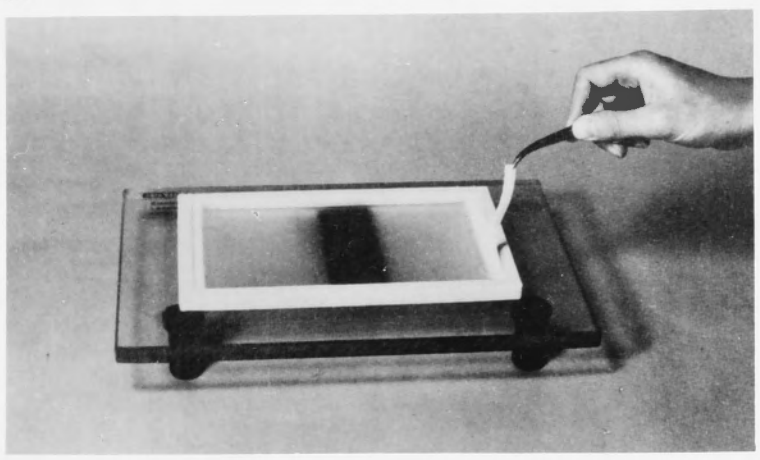
b



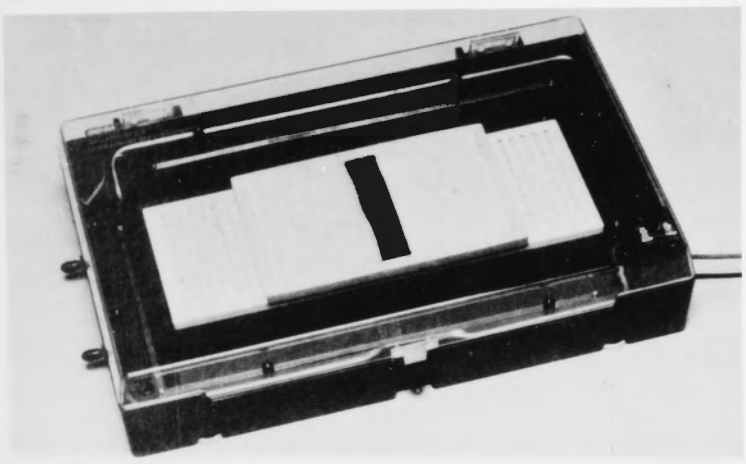
c



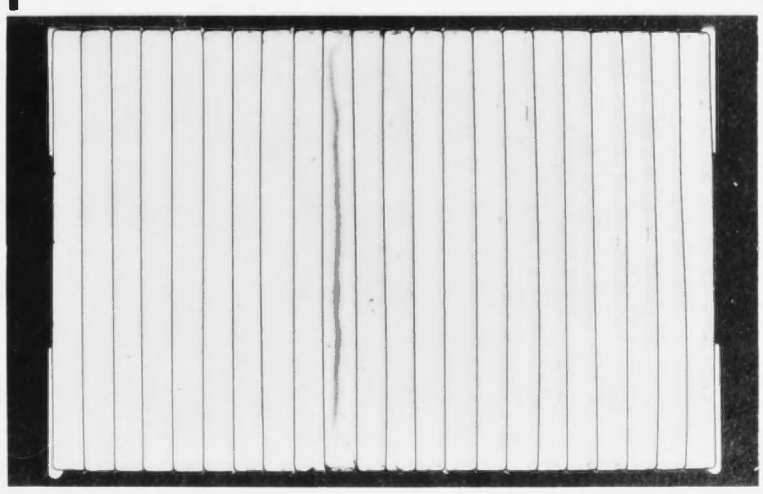
d



e



f



fractions were pooled and the total protein was precipitated with ammonium sulfate (80% w/v) and separated by centrifugation (6000 g). If not used directly for the next step, the pellet was suspended in buffer I and dialysed against it (3 x 500 ml) and stored in the presence of 20 mM NADH at -20°C.

2.6.9 Preparative granulated bed isoelectric focusing

The ammonium sulfate precipitate (80% w/v) of the pooled active fractions from the DEAE-Fractogel step was dissolved in 5 ml of Milli Q water . The enzyme (2.5 ml) was immediately loaded on two PD-10 columns (Sephadex G25, Pharmacia) that were pre-equilibrated with Milli Q water. The columns were eluted with Milli Q water (3.5 ml each) and the eluate (pooled total of 7 ml) was made to 4% w/v in ampholines (0.15 ml of 40 w/v Biolyte $^{3}/_{10}$ and 0.35 ml of 40% w/v Biolyte $^{5}/_{7}$; LKB). This sample was applied onto a preparative granulated flat-bed isoelectric focusing slab gel (20 by 11 by 0.8 cm) as described by the manufacturer (Bio-Rad: Bio-Phoresis Instruction Manual, 1985; see Figure 2.9 reproduced with permission from Bio-Rad Laboratories).

Pre-swollen Bio-Lyte electrofocusing gel (150 ml, Bio-Rad) made 4% in ampholine (6 mls of 40% w/v Biolyte $^{3}/_{10}$ and 9 ml of 40% w/v Biolyte $^{5}/_{7}$) was set in a gel tray (20 x 11 x 0.8 cm) by wicking off the excess liquid from both ends of the tray. The sample was applied in two additions (~4 ml each) in the middle of the gel bed and dried by wicking at the ends until the gel bed was firm upon tilting the tray to 45° inclination. The anode (1 N H_3PO_4) and cathode (1 N NaOH) were applied on thin filter strips and placed at each end of the tray. Electrofocusing was carried out in Biophoresis cell (Bio-Rad) with a stage cooled to 4°C at a constant voltage of 250 V for 2 h, followed by 18 h at 700 V. The stage was wet with 1% Triton X-100 solution so that it would be in even contact with the tray. The gel containing the protein (a sharp yellow band near the anodic end) was removed with a spatula, extracted with 50 mM Hepes-KOH pH 7.4 (Buffer I) containing 200 mM NaCl (by centrifugation) and dialysed against buffer I (2 x 500 ml). The enzyme was stored in 1-ml aliquots with 20 μM NADH at -20°C.

CHAPTER 3

PROPERTIES OF *ESCHERICHIA COLI* DHPR

3.1 INTRODUCTION

The properties of the dihydropteridine reductases from various sources that have been reported in the literature have been reviewed in Chapter 1. It is common practice to characterise a new protein once it has been isolated and purified to homogeneity. I will endeavour in this chapter to describe some of the properties of the reductase from *E. coli* so that it will serve as an useful preamble in the investigation of 'role(s)' of the enzyme in an organism that is devoid of aromatic amino acid hydroxylases. During the purification of this enzyme it became evident that it differed from dihydropteridine reductases from other sources in several respects. The most important difference is the NADH oxido-reductase activity in the absence of the pterin substrate when an artificial electron acceptor such as $K_3Fe(CN)_6$ is present. By analogy with other enzymes that exhibit NADH oxido-reductase activity, it was suspected that the reductase might contain a prosthetic group. The prosthetic group was isolated and identified as FAD. This is the first report of a DHPR with a prosthetic group, hence attempts have been made to relate its presence in the protein to the catalytic activity, especially the reduction of quinonoid dihydropterin.

Another novel feature of this reductase that is revealed by the investigations reported in this chapter is that *E.coli* DHPR can function as a dihydrofolate reductase (DHFR) *in vitro* ; i.e., it can reduce 7,8 dihydrofolate, albeit at a much slower rate than *E. coli* DHFR. The superficial similarity in the chemistry of the reactions catalysed by the two enzymes had led to the general belief that the primary structure of DHPR may have a high homology with that of DHFR. However this belief was quashed when the predicted primary structures of the human-liver DHPR (Dahl *et al.*, 1987; Lockyer *et al.*, 1987) and rat-liver DHPR (Shahbaz *et al.*, 1987) were compared with that of human DHFR (Masters and Attardi, 1983). There was no extensive similarity between the two

Table 3.1 The three different enzyme activities of purified *E. coli* DHPR in Tris/HCl buffer pH 7.4 at 25°C

Enzyme activity	Specific activity (U/mg)	Substrate
DHPR[a]	25.5	quinonoid 6-methyl-dihydropterin (100 μM)
Oxido-reductase[a]	110.	$K_3 Fe(CN)_6$ (300 μM)
DHFR[a]	1.25	7,8-dihydrofolate (100 μM)

a. NADH (100 μm) was used as the nucleotide cofactor.

sequences except for a small region around the proposed nicotinamide binding region of human DHFR (Dahl *et al.*, 1987). In this light, the discovery that the reductase from *E.coli* possesses both DHPR and DHFR activities reopens the question of the nature of evolution of these two proteins in higher organisms.

3.2 RESULTS AND DISCUSSION

3.2.1 Enzyme activities of purified *E. coli* DHPR

The results summarised in Table 2.2 established the *in vitro* assay requirements for *E. coli* DHPR. Unlike the reductases from the other sources, this enzyme is able to oxidize both NADH and NADPH independently of the quinonoid dihydropterin cofactor. More remarkably, the homogenous preparation of DHPR from *E. coli* can also reduce 7,8-dihydrofolate; a catalytic function that is normally exclusive to DHFR. The chemistry of DHFR- and DHPR-catalysed reactions bear one crucial similarity in that both enzymes are involved in the reduction of the pterin nucleus of two tautomers of 7,8-dihydropterin. None of the DHPRs reported in the literature can reduce 7,8-dihydro(3*H*)pterins (conjugated or unconjugated) and conversely none of the DHFRs can reduce quinonoid 7,8-dihydro(6*H*)pterins. In the DHFR-catalysed reaction the hydride is transferred from NADPH onto position C-6 of the pterin nucleus and protonation occurs at N-5. In contrast, in the DHPR reaction the hydride is transferred from NADH to N-5 of the pterin nucleus with protonation taking place at N-3 (see Scheme 1.5). The three different enzyme activities of *E. coli* DHPR are summarised in Table 3.1 and the ratio of the three specific activities is 1 : 4.4 : 0.05 (for DHPR, oxido-reductase and DHFR respectively).

These results with the protein purified from the wild type *E. coli* imply that the DHFR-deficient mutant of *E. coli* (strain D3-157) isolated by Singer *et al.* (1985) must be a double mutant, *i.e.* $DHFR^-$ and $DHPR^-$. This was hypothesized on the basis that if DHPR was present in the mutant strain, then its ability to recycle 7,8-dihydrofolate ought to complement the DHFR deficiency of the strain. This is however not true because in

Table 3.2 Comparison of DHFR[a] activity with NADPH[b] and NADH[b] in Tris/HCl buffer pH 7.4 at 25°C

		DHFR activity (mU/mg protein)	
Strain	DHFR Source	NADPH	NADH
H712	wild type	28.8	6.27
JFM228	overproducer	729	24.3
D3-157	**mutant**	**2.55**	**7.19**

a. DHFR activity was assayed as described in Section 3.4.1

b. [NADPH] or [NADH] was 100 μM

this work it is found that the strain D3-157 exhibits almost the normal level of DHPR activity that is present in the wild-type strain H712 from which the former was derived by mutagenesis with ethyl methane sulfonate. The reason for the inabilty of DHPR to complement the lack of DHFR activity in the strain D3-157 is not clear, but one possibility is that the catalytic efficiency of the enzyme is too low *in vivo*. Apart from the direct evidence that the purified DHPR from *E.coli* can reduce 7,8-dihydrofolate, further evidence that a protein other than the well-characterised DHFR can carry out a similar function comes from the comparison of DHFR activity in the cell-free extracts prepared from strains D3-157, H712 or JFM228 (an overproducer of chromosomal DHFR) using NADH or NADPH as the pyridine nucleotide cofactor. The DHFR specific activities in cell free extracts from H712 and JFM228 are higher when NADPH is the electron donor compared with NADH. This is consistent with the fact that the K_m values for NADPH are more favourable than those for NADH in all DHFRs, and in most cases the V_{max} values with NADH are only 10-30% of those for NADPH (reviewed by Blakley, 1984). In contrast, the DHFR specific activity in the cell-free extract from strain D3-157 is higher with NADH than with NADPH (see Table 3.2). Most DHPRs, on the other hand (with the exception of the physically distinct NADPH-specific DHPR), show more favourable Michaelis constants with NADH than with NADPH (reviewed by Shiman, 1985). Based on this, it is argued that the DHFR activity observed in the extract from strain D3-157 must be due to DHPR.

Singer *et al.* (1985) used antisera competition experiments to show that there was no significant amount of inactive DHFR in the extract from strain D3-157. They used an assay where active chromosomal DHFR incubated with DHFR antisera gave 57% inhibition of activity, and when 0.5 mg of extract from D3-157 was added the percentage inhibition was not changed. It can be inferred from this that the higher DHFR activity of the extract from D3-157 with NADH is not due to partial revertants which may have mutations that result in a more favourable binding site for NADH. Attempts were made in this work to investigate the presence of catalytically-inactive protein by comparing the 2D-electrophoretic (first dimension was IEF and the second dimension was 12% SDS PAGE

as described in Section 2.5.3) separation of the cell-free extract from JFM 228 with that from the strain D3-157. The rationale was that JFM 228 overproduces DHFR, hence the protein may be identified on the 2D gel by using its molecular weight (18 000) and isoelectric point (pI 4.5). A band that fitted the criteria for wild-type DHFR was identified on the 2D gel of the extract from JFM 228 visualised by silver staining. The corresponding region on the 2D gel of the extract from D3-157 showed *no* protein band (data not shown). However, a mutation that may cause changes in the total number of charged amino acid residues may have considerably altered the mobility of the protein in a 2D gel (for example, when Asp-27 of wild type *E. coli* DHFR was altered by site-directed mutagenesis to Asn, the pI value of the mutant was 4.8; Villafranca *et al.*, 1983). The most persuasive evidence that *E. coli* DHPR can reduce 7,8-dihydrofolate comes from the presence of DHFR activity in the apparently homogenous preparation, and also that in the extract from strain D3-157 the DHFR specific activity is higher with NADH than NADPH as the pyridine nucleotide cofactor.

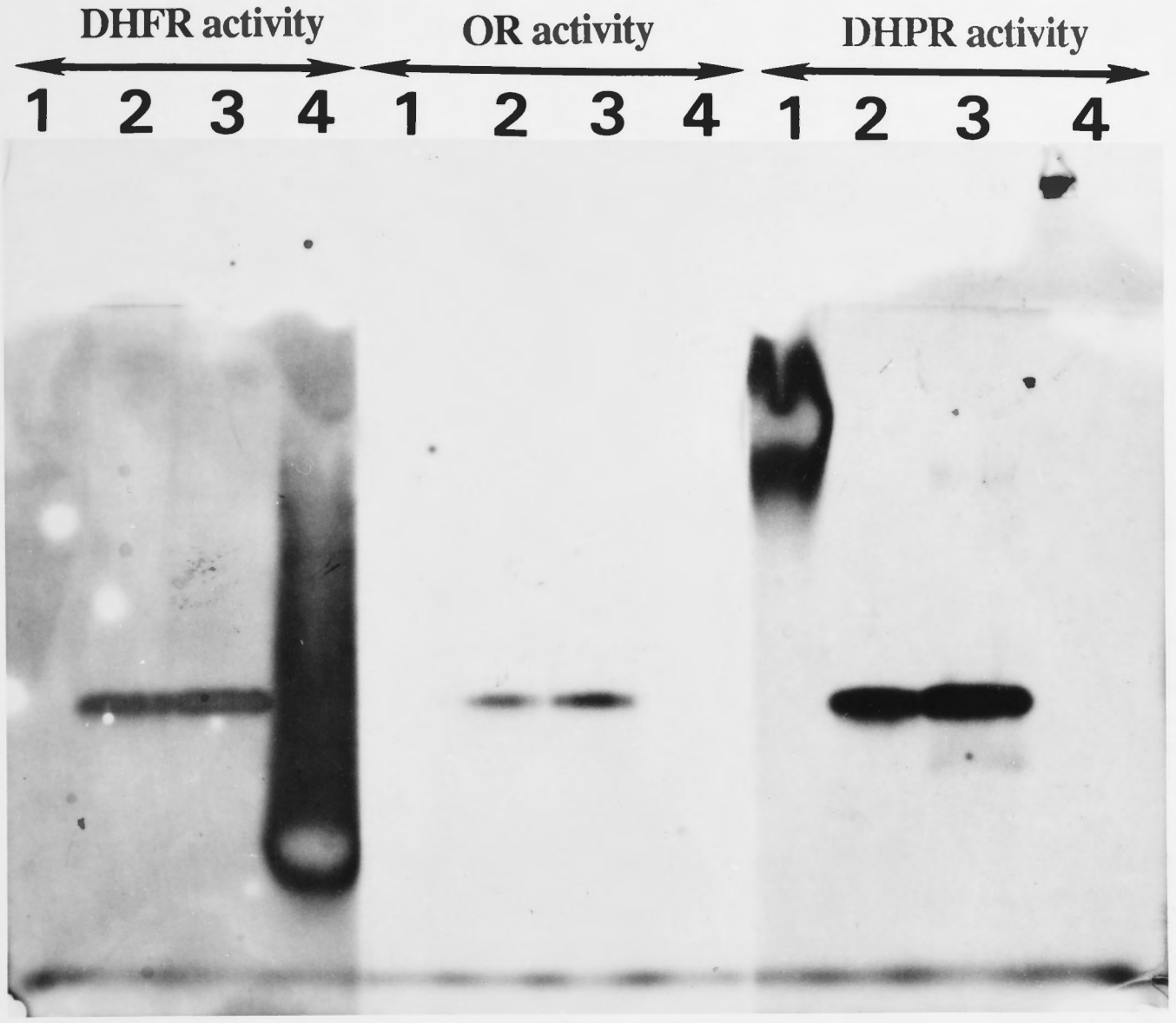
Enzyme activity staining of a sample of partially purified DHPR from *E. coli* was presented in Chapter 2 to demonstrate the presence of DHPR activity in a native PA gel. The basis of the activity stain method is that tetrahydropterins, which are products of both the DHPR- and DHFR-catalysed reactions, can reduce the soluble tetrazolium MTT to yield the insoluble blue coloured formazan (Gunlak *et al.*, 1966). In an endeavour to further demonstrate the presence of both DHPR and DHFR activity of *E. coli* DHPR, the tetrazolium MTT activity stain approach was examined in this study. During preliminary investigations it became evident the tetrazolium-MTT could also be used to indicate the NADH oxido-reductase activity of *E. coli* DHPR. Using apparently homogeneous *E. coli* DHFR (kind gift from Dr. J. Morrison) and human-brain DHPR as standards, the three different activities of *E. coli* DHPR were demonstrated by enzyme activity staining (see Figure 3.1A) on a single slab PA gel. This was achieved by using a simple modification of the published assay methods (Nixon and Blakley, 1968; Hasegawa, 1977; Cotton and Jennings, 1978) whereby the reactants for each of the three enzymic activites are overlayered on the PA gel in 0.3% agarose so that they become immobilised.

Figure 3.1 Activity staining of wild type *E. coli* DHFR(18-kD), *E. coli* DHPR (27-kD) and human brain DHPR (50-kD), for DHFR, oxido-reductase and DHPR activities, respectively.

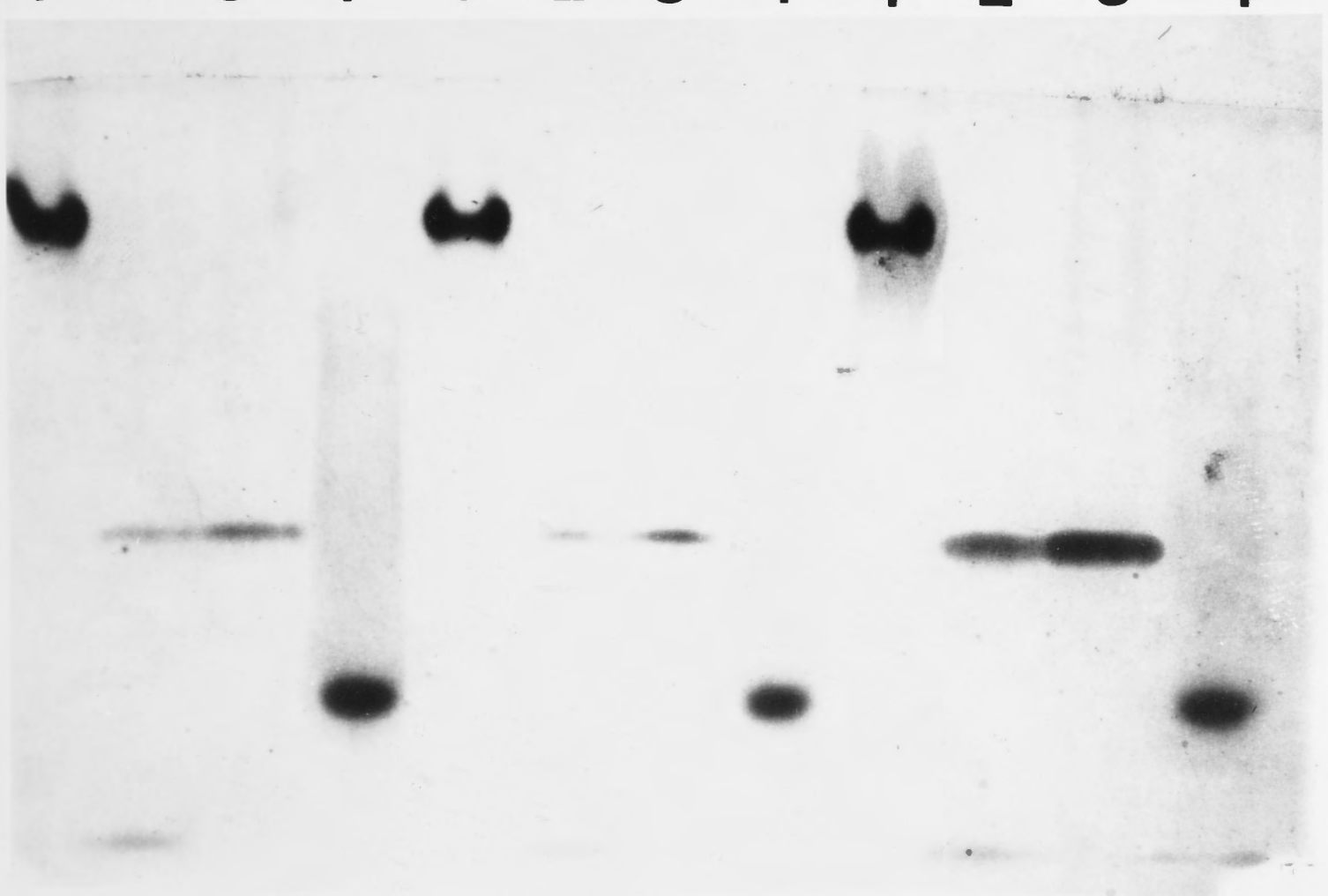
a. Detection of the three enzyme activities by staining was carried out as described in Sections 2.5.3.2 and 3.4.12. The lanes marked '1' contain human brain DHPR (5 μg) each; the lanes marked '2' and '3' contain purified *E. coli* DHPR (0.75 μg and 2 μg respectively); and the lanes marked '4' contain *E. coli* DHFR (3μg)

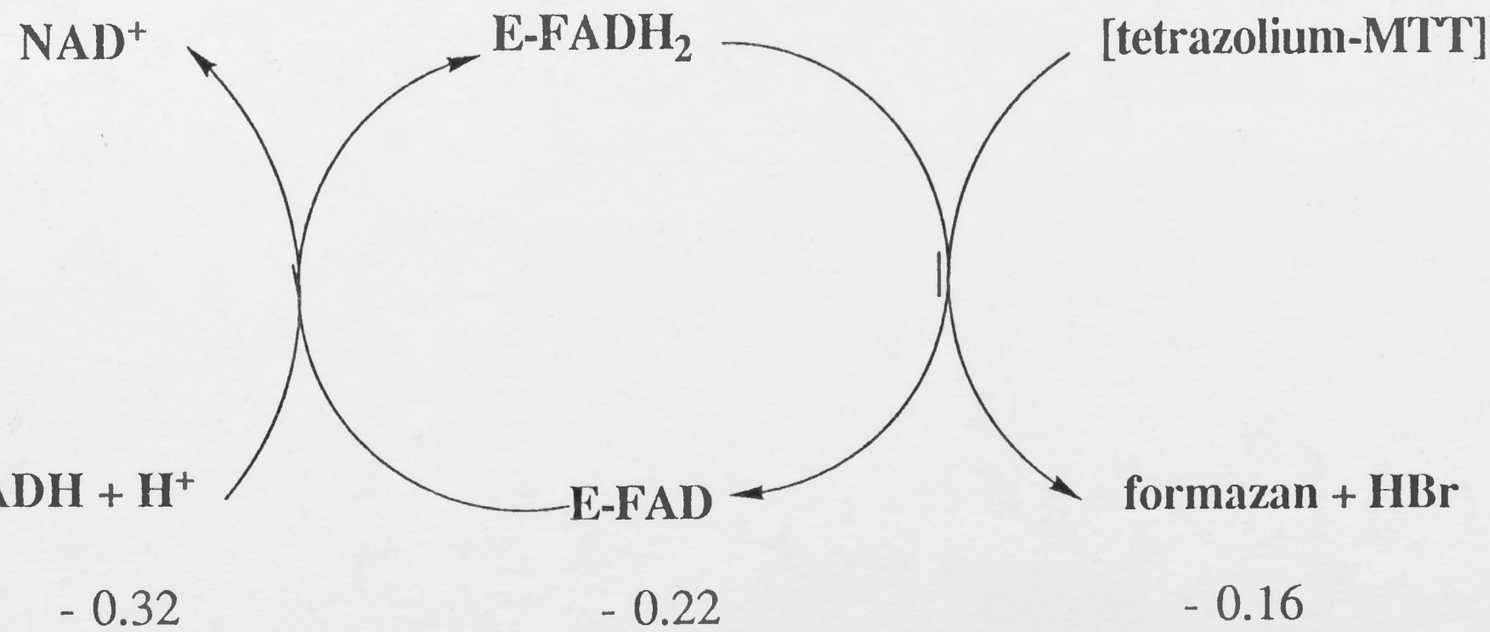
b. The activity-stained gel (without complete destaining to remove the formazan) was stained with Coomassie Blue and destained as described in Section 2.5.3.1.

a


DHFR activity
OR activity
DHPR activity
1 2 3 4 1 2 3 4 1 2 3 4

b


1 2 3 4 1 2 3 4 1 2 3 4



Scheme 3.1 Mechanism of oxido-reductase activity with tetrazolium - MTT as substrate.

The DHFR-stained section showed that *E. coli* DHPR and *E. coli* DHFR reduced 7,8-dihydrofolate to tetrahydrofolate which in turn was oxidized by tetrazolium MTT, whereas DHPR from human brain was completely inactive. Conversely quinonoid 6-methyl-7,8-dihydropterin which was used in the DHPR-stained section was reduced by human-brain DHPR and *E. coli* DHPR, and gave a blue colour which is due to the insoluble formazan. Authentic *E. coli* DHFR was completely inactive towards the DHPR stain reaction. In the oxido-reductase stain the pterin substrate was excluded, but the formazan formation around the *E. coli* DHPR spot suggested that the reduced flavin prosthetic group can reduce the tetrazolium to the formazan.

The reason for formazan formation around the *E.coli* DHPR spot was not obvious in the beginning, but upon further deliberation it became clear that a tightly-bound flavin prosthetic group upon reduction by NADH could serve as an electron donor to tetrazolium-MTT. The flow of electrons from NADH through to tetrazolium-MTT is favoured by the reduction potentials of the three oxidized-reduced couples that are involved (see Scheme 3.1). This result indicates that the formazan formation by *E.coli* DHPR due to the oxido-reductase activity must occur in the sections stained for DHFR and DHPR. However it is argued that the intensity of the activity stain is greater for the DHPR and DHFR activities compared with the oxido-reductase activity in this apparently multifunctional enzyme. (Note that the three staining reactions were carried out for the same length of time). This indirectly shows that 7,8-dihydrofolate and quinonoid 6-methyl-7,8-dihydropterin are reduced by *E. coli* DHPR. The relatively faster rates of enzymatic reactions near the centre of the spot compared with the tetrahydropterin-dependent reduction of tetrazolium-MTT at the periphery of the spots may possibly account for the colourless region within the respective protein spots (compare with Figure 3.1B).

3.2.2 Molecular weight of the reductase

The molecular weight of native *E. coli* DHPR as determined by gel-filtration chromatography was 54 000. This value was comparable with values obtained for other reductases from mammalian sources (reviewed by Armarego *et al.*, 1984). The molecular weight reported in the literature for the partially-purified *Pseudomonas* reductase was about 44 000 with no reference to the nature of its subunits (Williams *et al.*, 1976). The NADPH-dependent reductase from bovine liver however had a molecular weight of about 72 000 and consisted of two identical subunits (Nakanishi *et al.*, 1986a). The subunit molecular weight value of about 27 000 for the *E. coli* reductase as determined by SDS-PAGE (see Figure 2.8) was in good agreement with values reported for other dihydropteridine reductases. The mammalian reductases are presumed all to be active in the dimeric form by analogy with human-liver DHPR which was shown to be active after covalent linking of the subunits by cross-linking with suberimidate (Firgaira *et al.*, 1981a).

The subunits in most of the dihydropteridine reductases are held together by non-covalent bonds because treatment in 2% SDS without heating is sufficient to denature them to the monomeric form in the absence of DTT (reviewed by Armarego *et al.*, 1984). Since the native molecular weight and subunit molecular weight of *E. coli* DHPR were in good agreement with the reported reductases, it was at first assumed that the new protein could be similar to known reductases in that it would be active as a dimer and perhaps inactive as a monomer. The loss of 80% of DHPR activity when the enzyme assay was carried out in the presence of 0.2% SDS was erroneously taken as further evidence that the *E. coli* enzyme was similar to the other dihydropteridine reductases. In contrast to the previous results, the enzyme activity stain of *E. coli* DHPR on a non-denaturing PA slab gel (see Figure 3.1A) when compared with native human-brain DHPR (M_r 54 000) and native *E. coli* DHFR (M_r 18 000) indicated that *E. coli* DHPR was *active* as a monomer (M_r 27 000); the band at 27 000 was stained.

Table 3.3 ***N*-terminal amino acid sequence of *E.coli* DHPR[a,b,c] compared with those of related proteins**

Protein	Sequence of the first 20 residues																			
E.coli DHPR[a]	M	D	I	I	C	V	A	L	K	C	I	V	X	X	A	F	**D**	A	M	K
E.coli DHPR[b]	M	A	I	I	C	V	A	L	K	C	I	V	T	K	A	F	**D**	A	M	K
E.coli DHPR[c]	M	D	I	I	C	V	A	L	K	C	T	V	T	K	A	F	**D**	A	M	K
E.coli DHFR[d]	M	I	S	L	I	A	A	L	A	V	D	R	V	I	G	M	**E**	N	A	M
S.faecium DHFR[d]	M	F	I	S	M	W	A	Q	D	K	N	G	L	I	G	L	**D**	G	L	L
Human liver DHPR[e]	M	A	A	A	A	A	A	G	E	A	R	R	V	L	V	Y	**G**	G	R	G
Rat liver DHPR[f]	M	-	-	-	A	A	S	G	E	A	R	R	V	L	V	Y	**G**	G	R	G

a . Sequence obtained from a Beckman model 890M2 spinning cup sequencer.

b.& c. Sequence obtained from an Applied Biosystems 477A Pulse-Liquid Phase Sequencer

c. The protein sample was transblotted from SDS-PA gel onto Polybrene coated glass fibre sheet as described in Section 3.4.13

e. Obtained from review by Blakley, (1985)

f. Predicted sequence from Dahl *et al.*, (1987)

g. Predicted sequence from Shahbaz *et al.*, (1987)

To resolve the conflicting results, a sample of *E.coli* DHPR (purified preparative isoelectric focussing in the presence of ampholines) was examined in a meniscus-depletion sedimentation equilibrium experiment (Yphantis, 1964). The molecular weight of the protein, after allowing for the presence of ampholines, was found to be 25.7 00. There was no indication of any other species in the specimen that was examined. (The sedimentation equilibrium experiment and the analysis was kindly carried out by Dr. Peter Jeffrey, JCSMR). The protein sample had not lost its DHPR activity at the completion of the sedimentaion equilibrium experiment. This confirmed that *E. coli* DHPR to be a monomer in its native state. The gel-filtration chromatography of *E. coli* DHPR along with the molecular weight standards, described at the beginning of this section (which indicated a M_r of 54 000) were carried out in the presence of 0.2 M NaCl to minimise hydrophobic interactions. In hindsight it seems possible that the presence of DTT in the elution buffer may have caused the protein to dimerise because less than 40% of the total activity that was applied to the column appeared in the peak fraction that indicated a molecular weight of about 54 000.

3.2.3 *N*-Terminal sequence of the reductase

The *N*-terminal amino acid of the *E. coli* reductase is not protected. This is in contrast with the *N*-terminal amino acid residues of several mammalian dihydropteridine reductases (reviewed by Armarego *et al.*, 1984) and human-brain DHPR (S.G. Vasudevan and W.L.F. Armarego, unpublished work), which are irreversibly blocked by acylation. The *E. coli* reductase was subjected to amino-acid sequence analysis on a Beckman model 890 M2 spinning cup sequencer and the resulting PTH derivatives, which were analysed as described in Section 3, readily gave a short sequence (Sequence A in Table 3.3). At the time this sequence was obtained, there was no sequence information in the literature about other dihydropteridine reductases. This sequence showed no significant homology with other proteins in the National Biomedical Research Foundation Protein Data Bank. Although *N*-terminal amino acid-protected proteins are

rare in *E. coli,* the possibility that there may have been an *N*-protected protein in the sample that co-purified with the unprotected protein to yield sequence A was ruled out because sequencing of the cyanogen bromidecleaved reaction mixture (without separation of polypeptides) gave recoveries of additional amino acids that were comparable with those obtained for the native protein. Also, the number of extra sequences obtained was reasonably consistent with the methionine content of the protein (see Table 3.4). In addition to giving the *N*-terminal sequence of *E. coli* DHPR, data from the above experiments clearly indicated that the protein was essentially pure.

The *N*-terminal amino acid sequence of *E. coli* DHPR was also obtained using the Applied Biosystems 477A Pulse-Liquid Phase Sequencer with samples purified from preparative flat-bed isoelectric focusing. Whereas sequence B was obtained after direct application of the appropriately-treated sample, sequence C was obtained by electroblotting *E. coli* DHPR separated on a (12%) SDS-PA gel (method of Vandekerckhove *et al.*, 1985) onto a Polybrene -coated glass fibre sheet and sequencing of the appropriate band (see Section 3). This last method was very convenient and its full potential was realised when *N*-terminal amino acid sequences of partially-purified proteins were obtained (see Chapter 5). There were small differences in assignment of residues between the three sequences but residues 12-20 were consistent in sequences B and C (see Table 3.3).

Although the first 20 residues of *E.coli* DHPR did not show any significant homology with those of *E. coli* DHFR, *S. faecium* DHFR (reviewed by Blakley, 1984), human-liver DHPR (Dahl *et al.*, 1987) and rat-liver DHPR (Shahbaz *et al.*, 1987), they share a common feature in that their *N*-terminal regions are mainly composed of hydrophobic residues. The secondary structure prediction for human liver DHPR using the Chou-Fasman rules concurs with this observation (Cotton *et al.*, 1987). The amino acid at position 17 in all the bacterial dihydrofolate reductases is an aspartate or glutamate. Although there are presently no known functions for this residue, it is interesting that *E.*

Table 3.4 Amino acid composition (calculated for a 27-kD protein)

Amino acid	*E. coli* DHPR	Rat DHPR[a]	Human DHPR[b]
Lysine	17	15	14
Histidine	5	4	4
Arginine	7	8	8
Cysteine	3[c]	4	4
Aspartic acid	24	19	9
Asparagine			7
Threonine	11	17	17
Serine	11	20	19
Glutamic acid	27	19	14
Glutamine			7
Proline	8	8	9
Glycine	24	25	25
Alanine	29	29	33
Valine	23	18	18
Methionine	5(6)	8	7
Isoleucine	12	9	9
Leucine	19	21	21
Tyrosine	3	3	3
Phenylalanine	7	7	7
Tryptophan	N.D[d].	7	7

a From Shabaz *et al.*, 1987.

b From Dahl *et al.*, 1987.

c Determined as cysteic acid in this work.

d Not determined

coli DHPR also had an aspartate at this position, whereas the human and rat proteins have glycine residues in this position.

3.2.4 Amino acid analysis

The amino acid composition of the reductase was determined by the method of Spackman *et al.* (1958). Cysteine was determined as cysteic acid following oxidation of the protein with performic acid prior to hydrolysis. The amino acid ratios were obtained based on the determined concentration of phenylalanine, as it is one of the more stable amino acids in the analysis procedure, and the composition was calculated for a 27-kD protein (see Table 3.4). The sequence determination of cyanogen bromide-cleaved fragments of the reductase indicated at least six fragments whereas the analysis indicates five residues. However, considering that methionine residues can be oxidized to methionine sulfone under conditions used for amino acid analysis (Glazer *et al.*, 1976), the number of methionine residues in the protein is probably six. The amino acid compositions of human-liver DHPR (Dahl *et al.*, 1987) and rat-liver DHPR (Shahbaz *et al.*, 1987) deduced from the nucleotide sequences of their respective genesare also presented for comparison. The lower threonine and serine content and the higher asparagine/aspartate and glutamine/glutamate content are the main differences between *E. coli* DHPR and the two mammalian enzymes. The higher content of the latter residues indicates that there may be more acidic residues in the bacterial reductase. This may account for its isoelectric point of 5.3 which is lower than that for human-liver DHPR (pI = 7.0; Firgaira *et al.*, 1981a) and rat-liver DHPR (pI = 6.4; Webber *et al.*, 1978).

3.2.5 Identification of prosthetic group

The possibility that *E. coli* DHPR had a tightly-bound prosthetic group became clear early in this work when it was noticed that the reductase consumed NADH in the absence of the pterin cofactor and the nature of its u.v.-visible absorption spectrum (see below). This pterin-independent consumption of NADH was elicited only when the assay mixture contained $K_3Fe(CN)_6$ which is normally present to oxidize tetrahydropterin to the quinonoid dihydropterin form *in situ* (see Table 2.2). This ferricyanide-mediated NADH oxidation by the enzyme was quite well known among flavoproteins (Dixon and Webb, 1979), which led to the early belief that the reductase contained a flavin prosthetic group. Since quinonoid dihydropterin is unstable and needs to be generated *in situ* for measurement of enzyme activity, the peroxide-peroxidase method was used for its generation. Peroxide-peroxidase did not oxidize NADH or NADPH in the absence of the pterin cofactor or enzyme at any appreciable rate (<5%).

The purified reductase was yellow in colour when concentrated. The yellow colour was used in the prepartive flat-bed isoelectric focusing step to visualise and obtain high yields of apparently pure *E. coli* DHPR (see Chapter 2). The u.v.-visible absorption spectrum of the pure protein exhibited a maximum at 449 nm, with a shoulder at about 475 nm (see Figure 3.2). This is characteristic of an oxidized flavin tightly bound to protein. When NADH was added to the enzyme, the peak at 449 nm and the shoulder at 475 nm disappeared. After about 5-10 min in air, the original spectrum of the protein was restored except that the intensity of the peak at 449 nm was reduced slightly, probably due to the presence of NAD^+ in the enzyme solution (see Figure 3.2). This result clearly suggested the flavin prosthetic group in the enzyme to be in the oxidized form. When NADH was added, the oxidized flavin became reduced to $FADH_2$, which does not absorb in the visible region. The prosthetic group was isolated by trichloroacetic acid denaturation of the protein. This method of freeing the prosthetic group ruled out any strong covalent association between the flavin moiety and the protein. The isolated prosthetic group gave a u.v.-visible absorption spectrum that was similar to authentic free

Figure 3.2 U.v.-visible absorption spectra of purified *E. coli* DHPR

(A) spectrum of protein (15 μM) in 50 mM Hepes buffer at pH 7.4 and 25°C; (B) as in (A) ~ 1 min after adding 100 μM NADH; (C), (D) and (E) spectra after standing in air for about 10 min after addition of NADH (100 μM); (F) base linewith 50 mM Hepes buffer (pH 7.4) at 25°C.

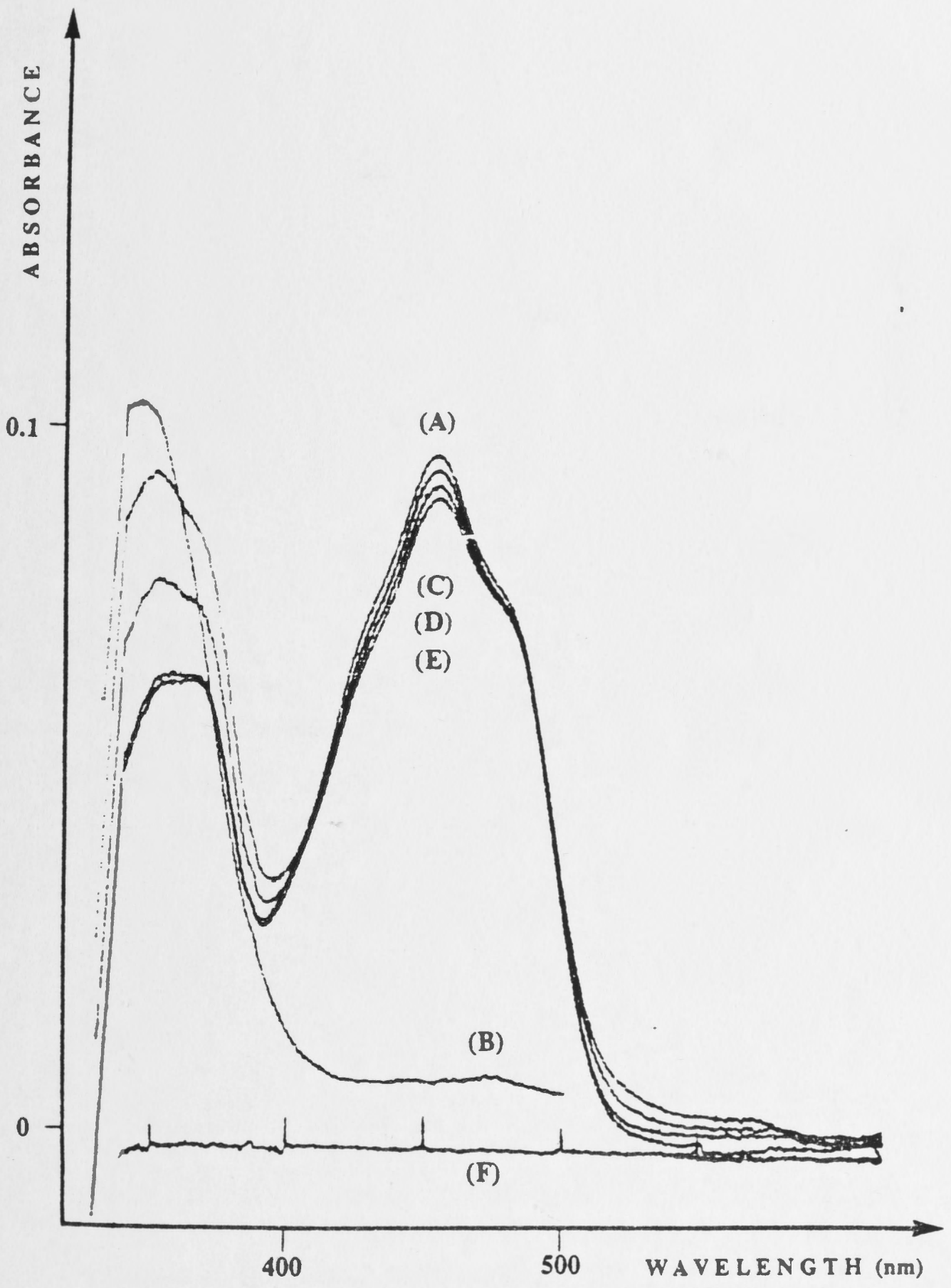
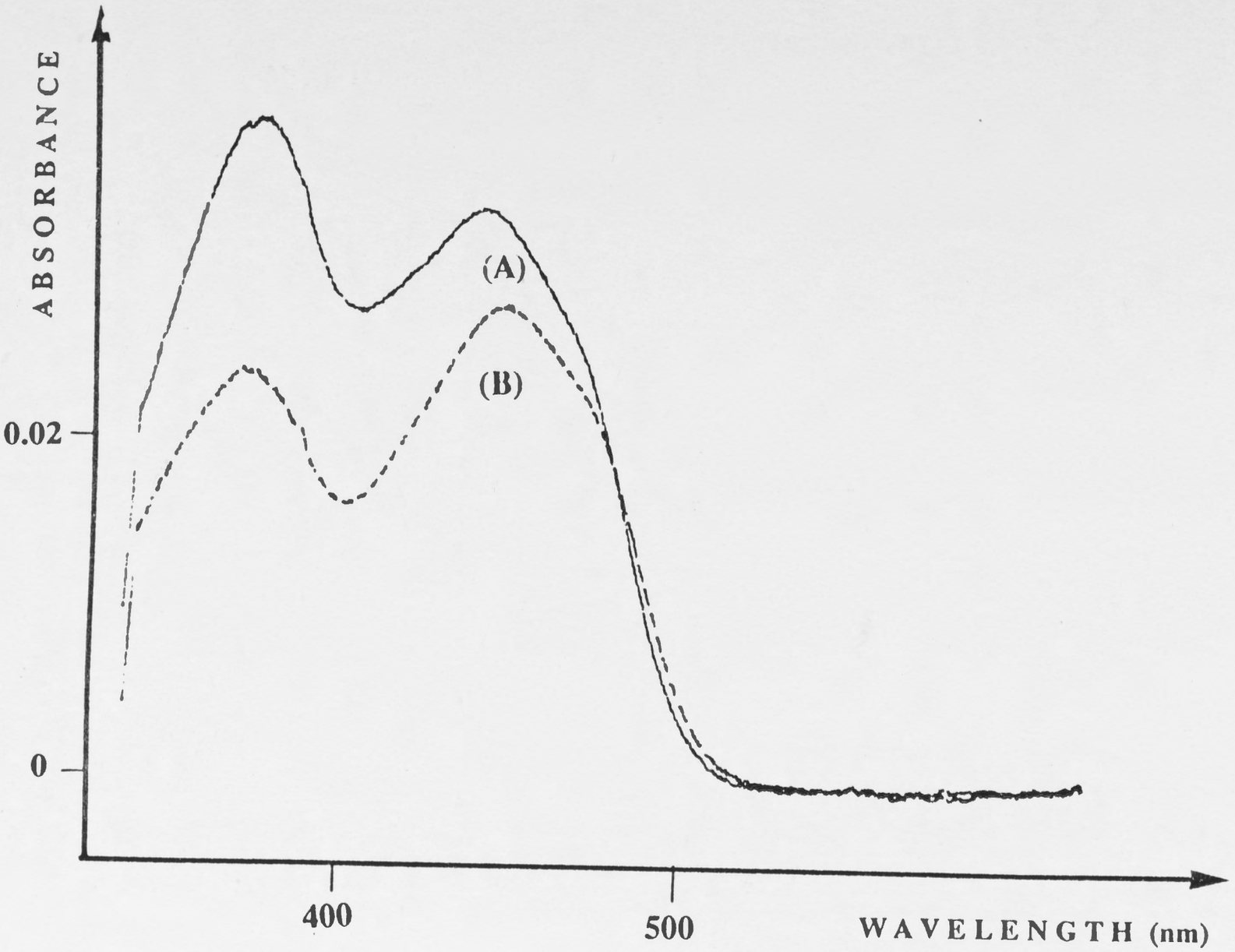
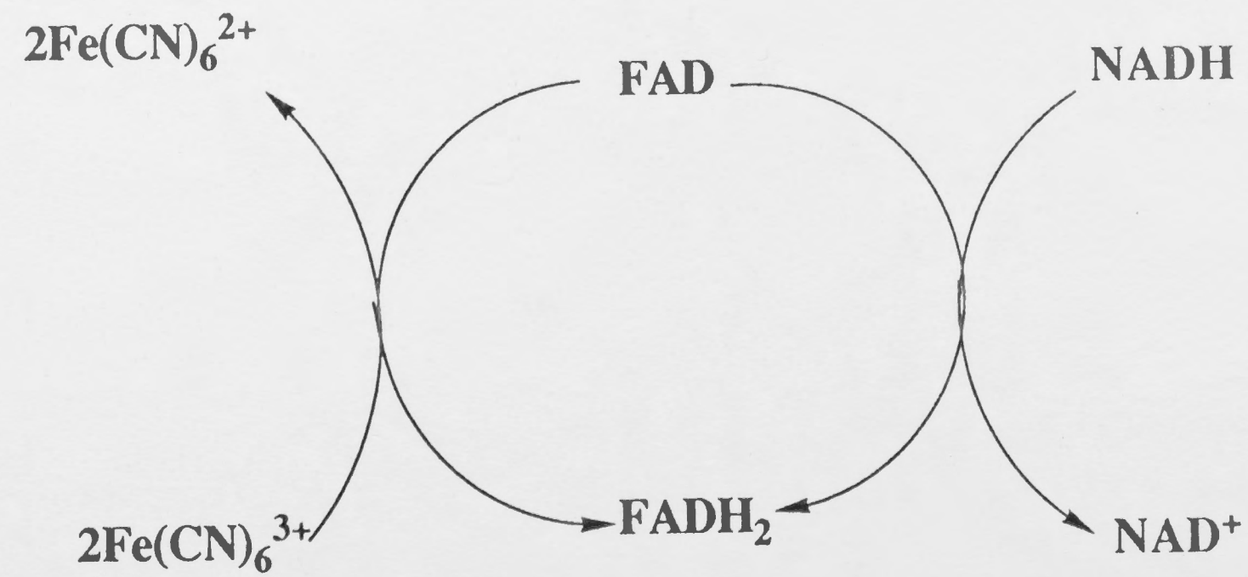

ABSORBANCE
0.1
0
(A)
(B)
(C)
(D)
(E)
(F)
400
500
WAVELENGTH (nm)

Figure 3.3 U.v.-visible absorption spectrum of the prosthetic group isolated from purified *E. coli* DHPR

(A) is the spectrum of the prosthetic group isolated from 400 μg of purified protein, in 50 mM Hepes buffer at pH 7.4 and 25°C; (B) is the spectrum of authentic FAD (20 μM) in 50 mM Hepes buffer at pH 7.4 and 25°C.


ABSORBANCE
0.02
0
(A)
(B)
400
500
WAVELENGTH (nm)



Scheme 3.2 Stoichiometry of the NADH oxido-reductase activity.

FAD (see Figure 3.3). The fluorescence emission spectrum of the isolated prosthetic group had a maximum at 530 nm (data not shown) when the excitation wavelength was fixed at 450, nm which is the same as that observed for free flavins (Morton, 1975). However, the spectroscopic data did not distinguish between FAD and FMN. Unequivocal evidence that the flavin was FAD was obtained by t.l.c. The R_F value of the isolated fluorescent cofactor relative to riboflavin was 0.16 and that for authentic free FAD was also 0.16. In contrast, the R_F value for FMN with respect to riboflavin was 0.31 (cf. Spitzer and Weiss, 1985). The possibility that oxygen or superoxide may be involved in the oxido-reductase reaction was also investigated. The NADH oxido-reductase activity was not affected by bubbling nitrogen through the assay mixture for 10 min prior to the assay. It was similarly shown that superoxide dismutase had no influence on the rate of the reaction.

Theoretically the oxidation of one molecule of NADH requires two molecules of ferricyanide. The experimentally obtained stoichiometry in this study, as evidenced by the consumption of one mol. of NADH per mol. of ferricyanide, is in agreement with the theoretical value. The rate of oxidation of NADH (from the rate of change of absorbance at 340 nm) and the rate of reduction of ferricyanide (from the rate of change of absorbance at 420 nm) were the same and followed first order kinetics for more than 80% of the reaction. Rate constants of 8.8×10^{-3} s^{-1} for the former reaction and 8.6×10^{-3} s^{-1} for the latter reaction (at 25°C and Tris/HCl pH 7.4) were calculated. These data (almost identical rate constants) taken together with the fact that oxygen and/or superoxide are not involved in the NADH oxido-reductase reaction suggest that the enzymatic reactions presented in Scheme 3.2 are tightly coupled.

3.2.6 Naturally occurring pterins in *E. coli*

The absence of well-characterised reduced pterin-requiring enzymes such as phenylalanine hydroxylase in *E. coli* indicated the need to investigate the nature of the reduced pterins in this organism. The cell-free extract, deproteinized, oxidized and

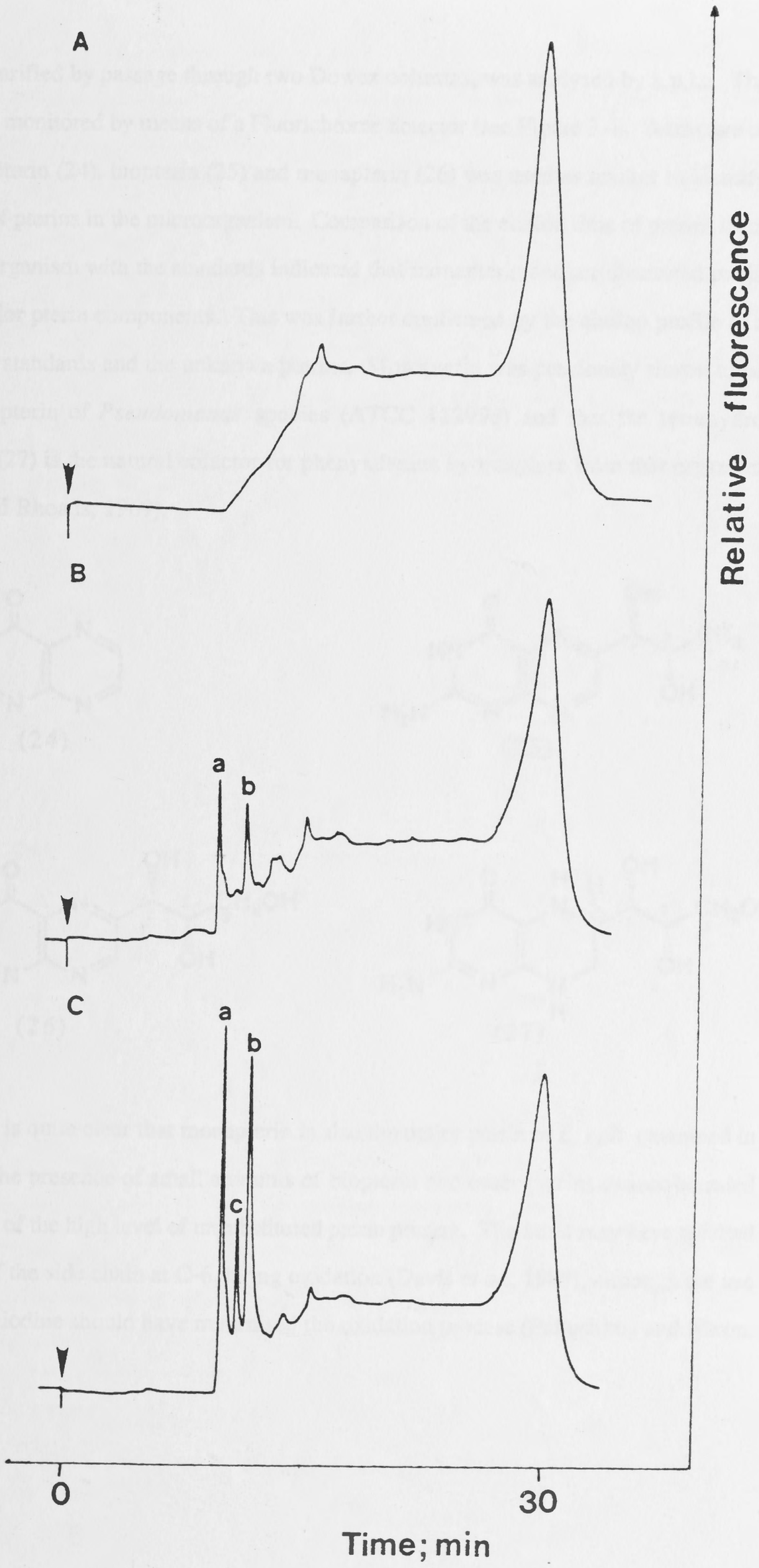
Figure 3.4 The h.p.l.c. analysis of oxidized pterins from *E. coli*

A is the blank h.p.l.c. elution profile of the Whatman ODS (0.46 cm x 25 cm) column, eluted with 5% methanol in water at 0.5 ml/min and detected by fluorescence (arbitrary units).

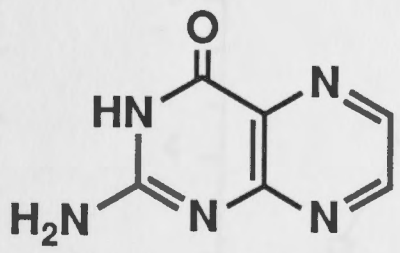
B is the elution profile of oxidized pterins from *E. coli*, obtained as described in Section 3.5.7, through the column as described above.

C is the elution profile of a 3:1 mixture respectively, of oxidized pterins from *E. coli* and authentic monapterin (a), biopterin (c) and pterin (b).

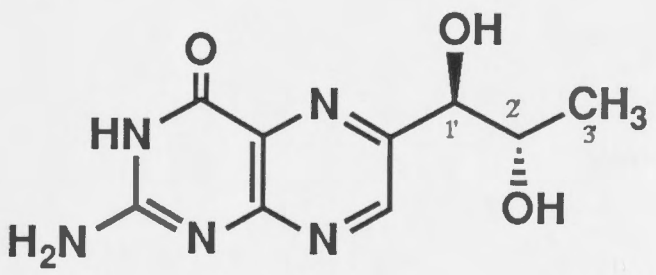
The peaks marked (a) and (b) in the elution profile of naturally-occurring pterins from *E. coli* were identified as monapterin (a) and pterin (b).


A
B
a
b
C
a
b
c
Relative fluorescence
0
30
Time; min

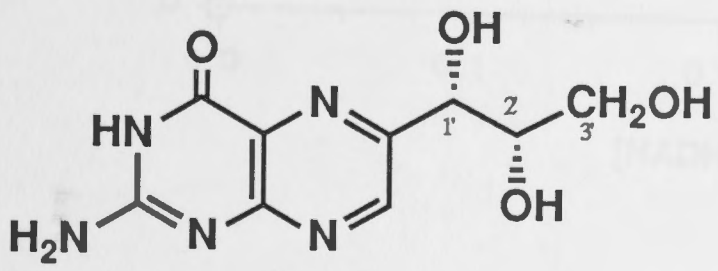
partially purified by passage through two Dowex columns, was analysed by h.p.l.c. The eluate was monitored by means of a Fluorichrome detector (see Figure 3.4). A mixture of synthetic pterin (24), biopterin (25) and monapterin (26) was used as marker to identify the types of pterins in the microorganism. Comparison of the elution time of pterins from the microorganism with the standards indicated that monapterin and unsubstituted pterin are the major pterin components. This was further confirmed by the elution profile of a mixture of standards and the unknown pterins. Monapterin was previously shown to be the major pterin of *Pseudomonas* species (ATCC 11299a) and that the tetrahydro derivative (27) is the natural cofactor for phenylalanine hydroxylase from that organism (Guroff and Rhoads, 1969).



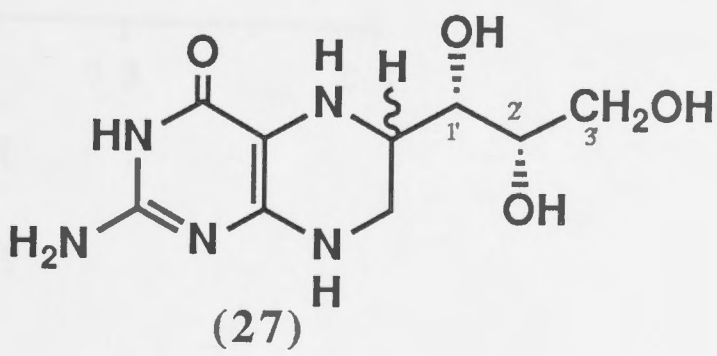
(24)



(25)



(26)



(27)

Although it is quite clear that monapterin is also the major pterin in *E. coli* examined in this work, the presence of small amounts of biopterin and other pterins cannot be ruled out because of the high level of unsubstituted pterin present. The latter may have resulted from loss of the side chain at C-6 during oxidation (Davis *et al.*, 1988), although the use of acidified iodine should have minimised the oxidation process (Fukushima and Nixon, 1980).

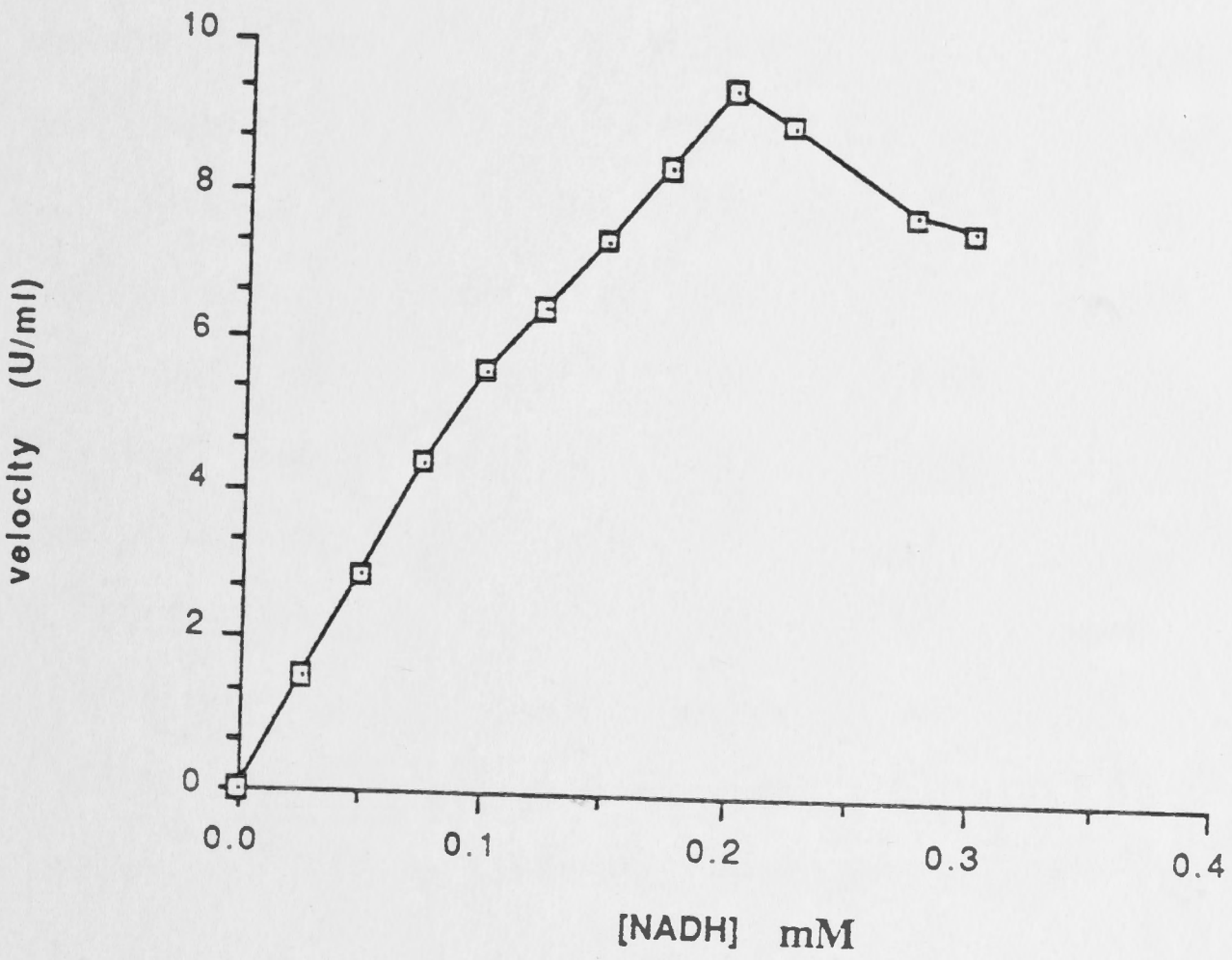


Figure 3.5 Dihydropteridine reductase activity of *E.coli* DHPR as a function of [NADH].

DHPR activity was determined as described in Section 2.5.1.1 except that the NADH concentration was varied from 0.025mM to 0.3 mM.

Table 3.5 Kinetic data for *E. coli* DHPR in Tris-HCl pH 7.4, at 25 °C

Substrate	Apparent K_m (μM)[a]	Apparent V_{max}[a,b]	Second substrate (100 μM)
NADH	91 (90)[c]	5.1	quinonoid 6-methyl-DHP
NADPH	290	5.2	quinonoid 6-methyl-DHP
quinonoid dihydrobiopterin	226 (160)[c]	2.6	NADH
quinonoid dihydroneopterin	1050	10.1	NADH
quinonoid dihydromonapterin	1320 (1450)[c]	10.1	NADH
quinonoid 6-methyl DHP	128	4.1	NADH
quinonoid 6,7-dimethyl DHP	523 (430)[c]	8.6	NADH

a. Standard errors are within ± 5%.

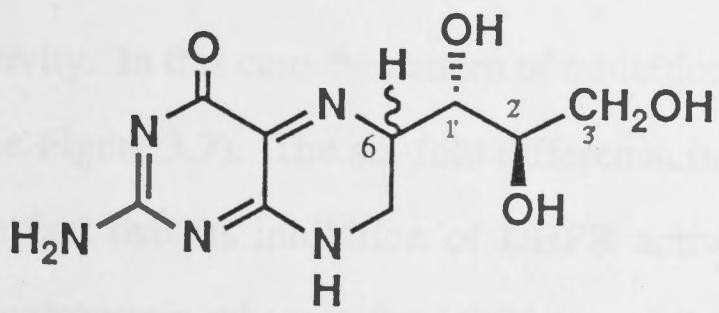
b. μmole of NADH (or NADPH) oxidized per min per 0.5 μg protein.

c. Data for *Pseudomonas* sp. DHPR (Williams *et al.*, 1976)

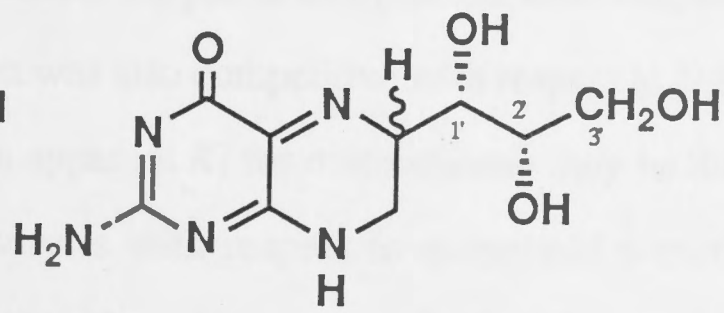
3.2.7 Activity of substrates

The rate of DHPR activity in the enzyme from *E. coli* is dependent on the concentrations of both NADH and quinonoid dihydropterin, i.e. both substrates show Michaelis-Menten type saturation kinetics. When the concentration of quinonoid 6-methyl-dihydropterin was 100 μM, increasing the concentration of NADH from 10 μM to 100 μM resulted in increased DHPR activity (see Figure 3.5). Increasing the concentration above 100 μM NADH resulted in reduced DHPR activity. A similar phenomenon was observed with the *Pseudomonas* DHPR (Williams *et al.*, 1976), and may be due to substrate inhibition. The K_m value of 91 μM for NADH with respect to quinonoid 6-methyldihydropterin was calculated from a double reciprocal plot and using the computer programme of Cornish-Bowden (Cornish-Bowden and Edrenyi, 1981). This value compares favourably with the K_m value of 90 μM for the above *Pseudomonas* enzyme. Mammalian dihydropteridine reductases generally have much lower K_m values for NADH, and they range from 1-30 μM (reviewed by Armarego *et al.*, 1984). The reductase from *E. coli* is active also with NADPH and the rates are about 50% of those with NADH under the same reaction conditions. The K_m value for NADPH is 290 μM. Despite a three fold lower affinity (by comparing K_m values, assuming that $Km \approx K_d$) in binding by NADPH compared with NADH, their maximum velocities are the same (see Table 3.5). On the other hand, the reductase activity of the *Pseudomonas* enzyme with NADPH was less than 5% of that with NADH. It is interesting that NADPH and NADH were equally effective in protecting the *Pseudomonas* reductase from heat inactivation (Williams *et al.*, 1976). The mammalian dihydropteridine reductases generally use NADPH about 15-fold less efficiently than NADH. The only exceptions to this are the NADPH-dependent dihydropteridine reductases isolated from bovine and human livers (Nakanishi *et al.*, 1986a,b). In these examples, the K_m values for NADPH and NADH were 1.4 μM and 2900 μM respectively, with quinonoid 6-methyldihydropterin as the second substrate.

The ability of a range of quinonoid pterins to act as subtrates was also examined in the present work and the kinetic parameters are presented in Table 3.5. Despite the fact that monapterin has been implicated as the predominant naturally occurring pterin in *E. coli* , its K_m for quinonoid dihydromonapterin (1320 μM) is about six times higher than that for quinonoid dihydrobiopterin (226 μM). The *Pseudomonas* enzyme also had a lower K_m value with quinonoid dihydrobiopterin (160 μM) compared with quinonoid dihydromonapterin (1450 μM) (Williams *et al.*, 1976). The characteristic feature about the natural substrate for dihydropteridine reductases from both bacterial sources is that its maximum velocity is higher than those of other substrates tested (see Table 3.5). However, the concentration of monapterin in *Pseudomonas* was estimated to be 0.1mM (Williams *et al.*, 1976) and at this level there is no apparent advantage to be gained by a high K_m and favourable maximum velocity. The K_m value for quinonoid dihydroneopterin (28) {stereoisomer of quinonoid dihydromonapterin (29)} is also in the mM range for the reductase from *E. coli*. Judging from the K_m values for pterin substrates with various substitutions at C-6 (see Table 3.5), the active site of the enzyme seems to indicate tighter binding with pterins that contain a more hydrophobic sidechain (quinonoid dihydroneopterin and dihydromonapterin are the least hydrophobic because they contain a trihydroxypropyl group). This qualitative observation is supported by the lower K_m values for quinonoid dihydrobiopterin and quinonoid 6-methyl and 6,7-dimethyl dihydropterins. The three-fold higher value for the K_m of quinonoid 6,7-dimethyl-dihydropterin compared with that of quinonoid 6-methyl-dihydropterin suggests that the methyl group at C-7 in the former may present some steric hindrance.



(28)



(29)

3.2.8 Effect of methotrexate

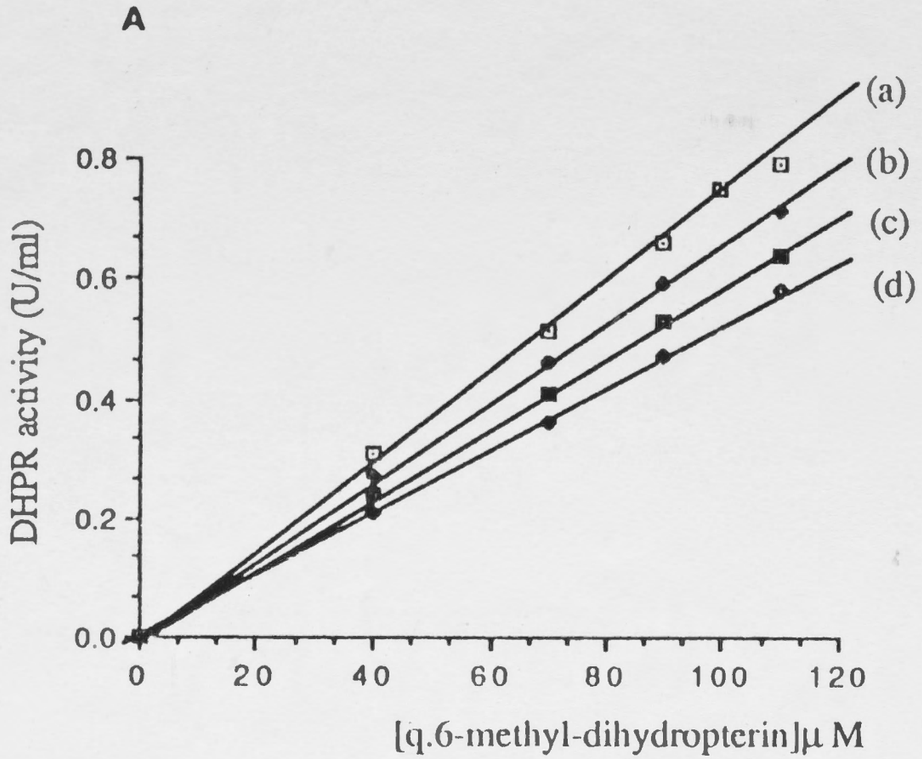
Methotrexate is successfully used in cancer chemotherapy because it inhibits DHFR (the essential enzyme in the biosynthesis of purines and thymine) at the pM level (Sather *et al.*, 1979; Carter *et al.*, 1981). It also inhibits dihydropteridine reductases from mammalian sources but at a much higher concentration (in the μM range; reviewed by Armarego *et al.*, 1984). The inhibition of pterin-dependent DHPR and pterin-independent oxido-reductase activities by methotrexate has been discussed in Chapter 2 (see Figure 2.5). The inhibition of both the activities was taken as evidence that the reductase from *E. coli* has more than one activity associated with it and that their active sites are either the same or contiguous. An apparent K_i of 0.26 mM at 25°C was calculated from the rate data for the pterin-dependent DHPR activity using the Cornish-Bowden programme (Cornish-Bowden and Endrenyi, 1981). Double-reciprocal plots of the data (see Figure 3.6) gave a pattern of lines consistent with competitive inhibition with respect to quinonoid 6-methyl-dihydropterin. This type of inhibition was also observed for the *Pseudomonas* enzyme where an apparent K_i of 0.21mM was reported (Williams *et al.*, 1976)

The hypothesis at that point in time was that the apparent K_i for methotrexate in the pterin-independent NADH oxido-reductase must be identical with that for the pterin-dependent reductase reaction if the active site for the two activities are identical. The reason for the difference in slopes (Fig. 2.5) is probably because the binding abilities of the pterin substrate and potassium ferricyanide are different. An apparent K_i of 35 μM for methotrexate was calculated from the data for the pterin-independent oxido-reductase activity. In this case the pattern of inhibition was also competitive with respect to NADH (see Figure 3.7). The six-fold difference in apparent K_i for methotrexate may be due to the fact that its inhibition of DHPR activity is with respect to quinonoid 6-methyl-dihydropterin whereas the inhibition of the oxido-reductase activity is with respect to NADH. The pattern of inhibition by methotrexate in the oxido-reductase reaction was expected to be a series of parallel lines with respect to NADH (when analysed graphically

Figure 3.6 Inhibition of *E. coli* DHPR activity by methotrexate

A DHPR activity was determined as described in Section 2.5.1.1, in the absence of methotrexate (a); or in the presence of 50 μM (b), 100 μM (c) and 150 μM (d) methotrexate, respectively. The concentration of quinonoid 6-methyl-dihydropterin was varied from 40 to 110 μM for each concentration of the inhibitor.

B The double reciprocal plot of **A.**


A
DHPR activity (U/ml)
0.0
0.2
0.4
0.6
0.8
0
20
40
60
80
100
120
[q.6-methyl-dihydropterin]μ M
(a)
(b)
(c)
(d)

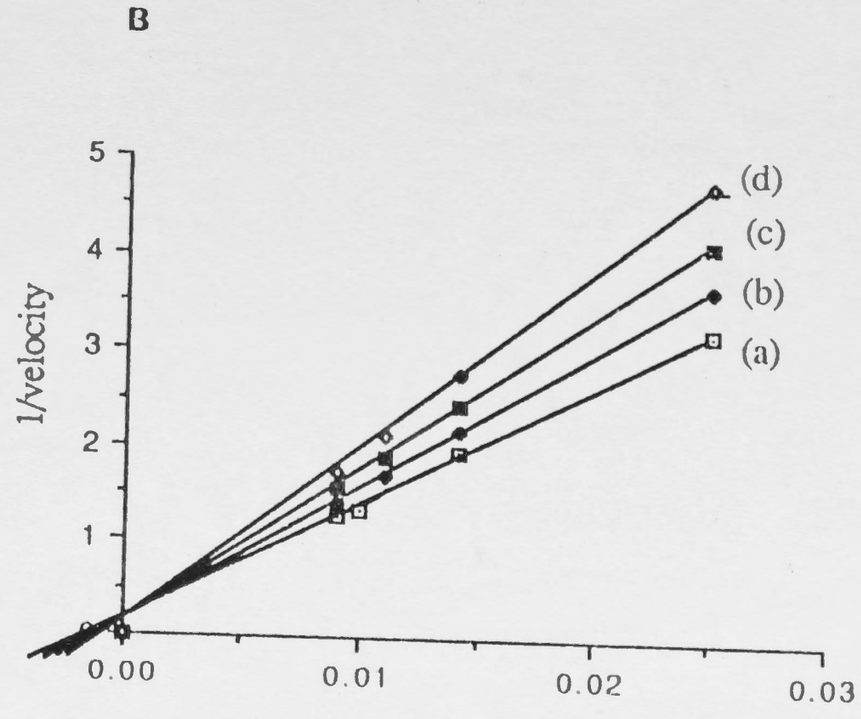
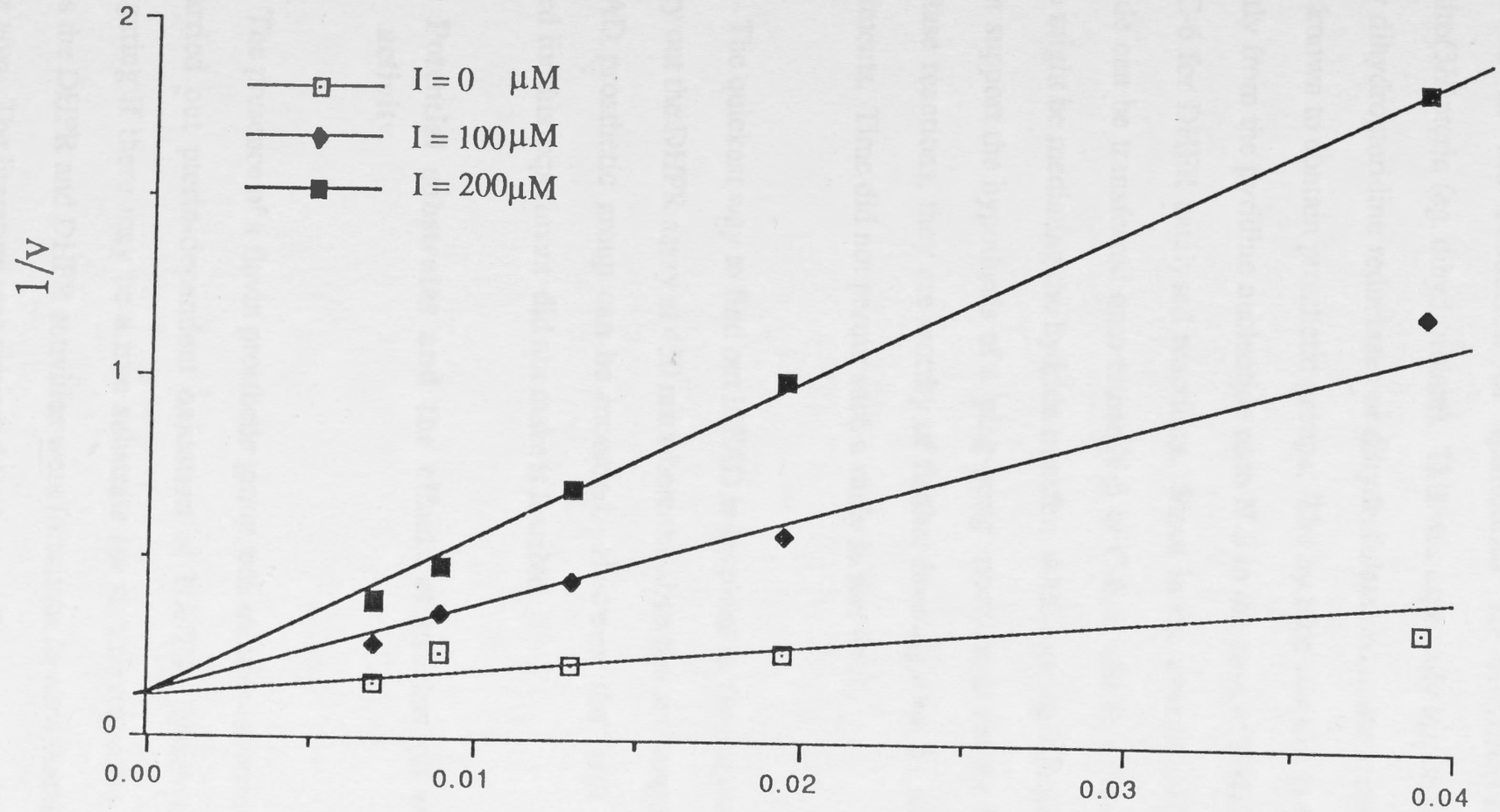

B
1/velocity
1
2
3
4
5
0.00
0.01
0.02
0.03
1/[q.6-methyl-dihydropterin]
(d)
(c)
(b)
(a)

Figure 3.7 The double reciprocal plot data on the inhibition of oxido-reductase activity by methotrexate

The oxido-reductase activity was measured essentially as described in Section 2.5.1.2 in the absence (I = 0 μM) or presence (I = 100 μM an I = 200 μM, respectively) of methotrexate, where 'I' is the concentration of methotrexate. The concetration of NADH was varied from 0 to 150 μM, for each of the inhibitor concentration.


I = 0 μM
I = 100μM
I = 200μM
1/v
2
1
0
0.00
0.01
0.02
0.03
0.04
1/[s]

in a double reciprocal plot), by analogy with other flavoenzymes (W. W. Cleland, Wisconsin, USA, personal communication). The question that was being addressed when carrying out the inhibition studies was whether the flavin prosthetic group is involved in the reduction of quinonoid 7,8-dihydro(6*H*)pterins and 7,8-dihydro(3*H*)pterin (eg. dihydrofolate). This was especially important because none of the other dihydropteridine reductases or dihydrofolate reductases reported in the literature were known to contain prosthetic groups. The hydride transfer in these enzymes occurs directly from the pyridine nucleotide onto N-5 in the case of DHPR catalysed reactions and C-6 for DHFR catalysed reactions. Since in the case of DHPR from *E. coli* the hydride can be transferred onto either N-5 or C-6, it was felt that the flavin prosthetic group might be mediating the hydride transfer. Although the kinetic data from this study do not support the hypothesis of a 'ping-pong' mechanism for the reductase and oxido-reductase reactions, they are worthy of further investigation by more involved kinetic experiments. Time did not permit such a study in this work.

The quickest way to find out if FAD is involved in the enzymic reaction would be to carry out the DHPR assay at 450 nm where the absorbance changes due to reduction of the FAD prosthetic group can be recorded. However the large amount of enzyme required for this experiment did not make it feasible.

3.2.9 Potential substrates and the effects of pterins on oxido-reductase activity

The presence of a flavin prosthetic group was an unusual occurrence in an enzyme that carried out pterin-dependent oxidation of NADH. Much effort was put into investigating if there may be a true substrate for this flavoprotein in *E. coli* and that perhaps the DHPR and DHFR activities were fortuitous *in vitro* reactions that do not take place *in vivo*. The literature was surveyed for known flavoproteins from any sources that had molecular weights of about 25 kD. Flavoproteins that used phenol, salicylate, 4-hydroxybenzoate, 4-hydroxyphenylacetate, orcinol and 2,6-dihydroxypyridine were the

Figure 3.8 $K_3Fe(CN)_6$ is a substrate for the oxido-reductase reaction catalysed by *E. coli* DHPR, and shows saturation kinetics

The oxido-reductase activity was carried out essentially as described in Section 2.5.1.2, except that the concentration of $K_3Fe(CN)_6$ was varied from 0 to 300 μM.

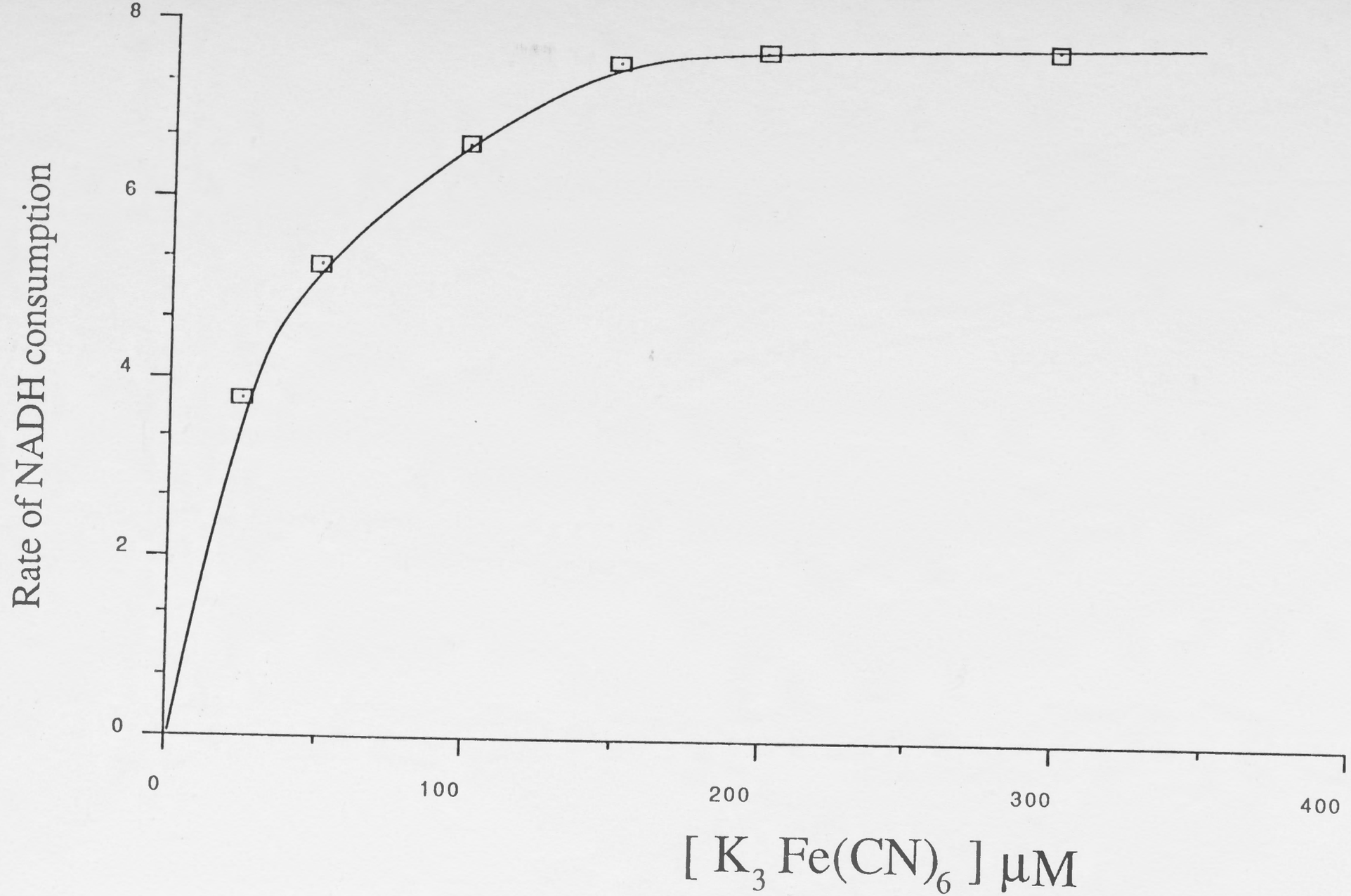
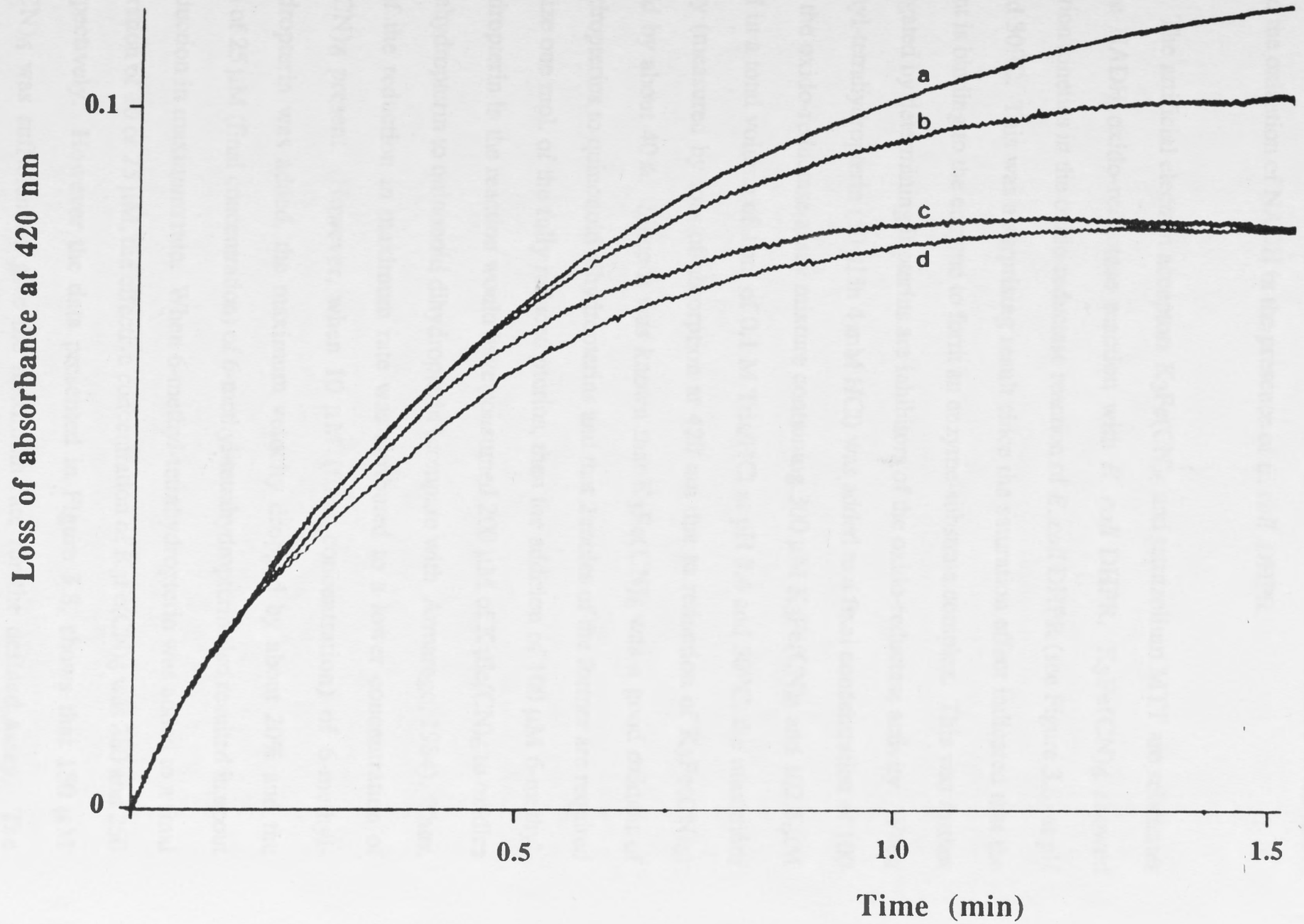

Rate of NADH consumption
8
6
4
2
0
0
100
200
300
400
[K3 Fe(CN)6] μM

Figure 3.9 Effect of 6-methyltetrahydropterin on the oxido-reductase reaction catalysed by *E. coli* DHPR

The oxido-reductase assay described in Section 2.5.1.2 was slightly modified, in that the visible wavelength of 420 nm was used to follow the loss of absorption, due to reduction of $K_3Fe(CN)_6$. The effect of the pterin substrate on the oxido-reductase activity was measured in the absence (a), or presence of 10 μM (b), 25 μM (c) and 100 μM (d) of 6-methyltetrahydropterin.


Loss of absorbance at 420 nm
0.1
0
a
b
c
d
0.5
1.0
1.5
Time (min)

best candidates from the literature survey, so their substrate activity was examined individually with the enzyme purified in this study. None of the reactants listed above caused the oxidation of NADH in the presence of *E. coli* DHPR.

The artificial electron acceptors $K_3Fe(CN)_6$ and tetrazolium MTT are substrates for the NADH oxido-reductase reaction with *E. coli* DHPR. $K_3Fe(CN)_6$ showed saturation kinetics in the oxido-reductase reaction of *E. coli* DHPR (see Figure 3.8) at pH 7.4 and 30°C. This was a surprising result since the saturation effect indicated that the reactant is binding to the enzyme to form an enzyme-substrate complex. This was further investigated by determining if pterins are inhibitors of the oxido-reductase activity. When 6-methyl-tetrahydropterin (50 µl in 4 mM HCl) was added to a final concentration of 100 µM to the oxido-reductase assay mixture containing 300 µM $K_3Fe(CN)_6$ and 102.5 µM NADH in a total volume of 1 ml of 0.1 M Tris/HCl at pH 7.4 and 30°C, the maximum velocity (measured by loss of absorption at 420 nm due to reduction of $K_3Fe(CN)_6$) dropped by about 40%. Since it was known that $K_3Fe(CN)_6$ was a good oxidant of tetrahydropterins to quinonoid dihydropterins and that 2 moles of the former are required to oxidize one mol. of the fully reduced pterins, then the addition of 100 µM 6-methyl-tetrahydropterin in the reaction would have consumed 200 µM of $K_3Fe(CN)_6$ to oxidize the tetrahydropterin to quinonoid dihydropterin (compare with Armarego, 1984). Thus, some of the reduction in maximum rate was attributed to a lower concentration of $K_3Fe(CN)_6$ present. However, when 10 µM (final concentration) of 6-methyl-tetrahydropterin was added, the maximum velocity dropped by about 20% and the addition of 25 µM (final concentration) of 6-methyl-tetrahydropterin also resulted in about 40% reduction in maximum rate. When 6-methyl-tetrahydropterin was added to a final concentration of 10 or 25 µM, the effective concentration of $K_3Fe(CN)_6$ was 280 and 250 µM respectively. However the data presented in Figure 3.8, shows that 150 µM $K_3Fe(CN)_6$ was sufficient to give the maximum rate for the defined assay. The suggestion from the qualitative results (see Figure 3.9) obtained by adding increasing amounts of 6-methyl-tetrahydropterin to the pterin-independent oxido-reductase assay is that the effective enzyme concentration is reduced, thereby causing the decrease of the

Table 3.6 Effect of *N*,*N*-dimethylpropargylamine (2.2mM) on the pterin-dependent oxidation of NADH[a] (DHPR activity) in 0.1 M Tris/HCl pH 7.4 at 25°C

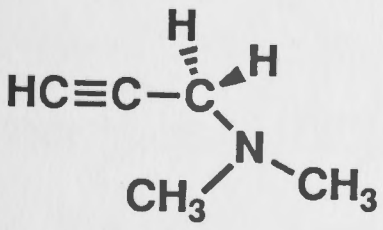
	% loss of DHPR activity	
Time (min)	*E. coli* DHPR + inhibitor	*E. coli* DHPR (control)
0	--	--
5	58	4
10	74	7
15	79	11
20	89	13
25	95	14

a. The inhibition experiment was carried out as described in Section 3.4.10.

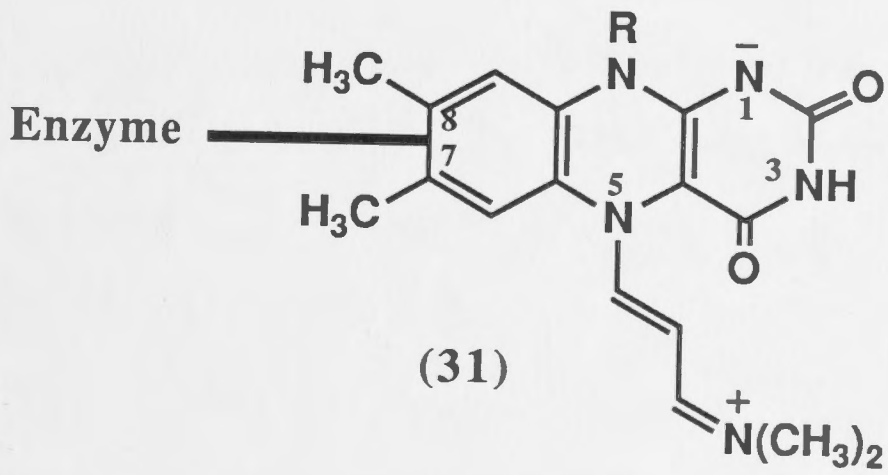
oxido-reductase activity. Since both 25 and 100 μM 6-methyl-tetrahydropterin caused the same level of inhibition, the hypothesis was that there may be two binding sites for $K_3Fe(CN)_6$ because when the enzyme is saturated with the pterin for which there is presumably only one binding site, the maximum velocity of the NADH oxido-reductase activity is more than 50%. (Note: $K_3Fe(CN)_6$ does not oxidize NADH non-enzymically). Another conclusion that may be drawn from this result is that the prosthetic group is not buried deeply in the protein, and could be reduced non-enzymically.

3.2.10 An active site-directed suicide inhibitor

Since the kinetic studies did showed no clear evidence of the involvement of the flavin prosthetic group in the DHPR reaction, attention was turned to the use of a suitable inhibitor. It is well known that several psychiatric and neurological diseases are depicted by imbalances in monamine oxidases (flavoproteins) which are involved in the deamination of substances such as dopamine and serotonin. Most of the drugs that are used to treat psychiatric and neurological diseases (e.g. Parkinson's disease) are based on acetylinic suicide inhibitors that inhibit the monamine oxidases by covalently binding to

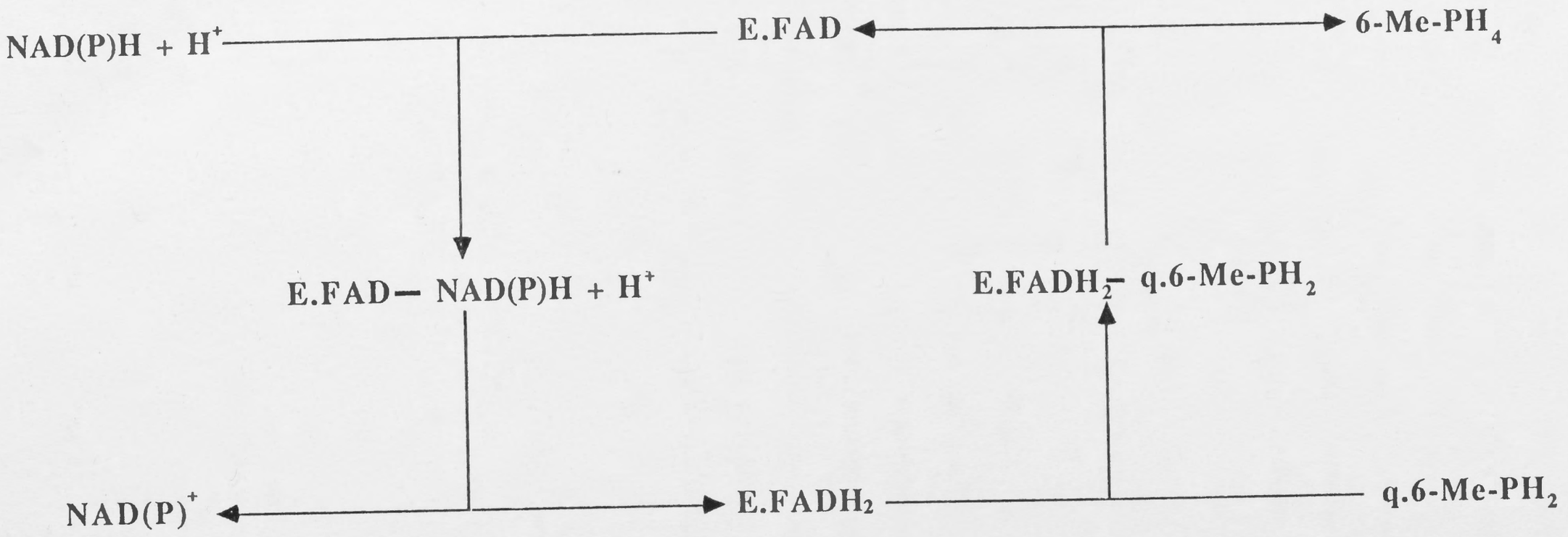


(30)



(31)

the flavin prosthetic group (Ferscht, 1885a; Youdim and Salach, 1978). *N*,*N*-dimethylpropargylamine (30) is a well-known inhibitor of several flavoproteins (Maycock *et al.*, 1976). The compound was prepared in this study and was >95% pure on the basis of ^1H NMR analysis. The data presented in Table 3.6 show that the pterin-dependent



Scheme 3.3 The proposed 'ping-pong' mechanism for DHPR from ***E. coli***

oxidation of NADH is inhibited by the acetylenic suicide inhibitor. This is direct evidence that the reduction of quinonoid 6-methyldihydropterin is mediated by the flavin prosthetic group. The loss of 50% of the enzyme activity after a five-minute treatment with the compound is evidence of its strong inhibitory action and almost all the DHPR activity was lost within 25 min at 25°C. The mechanism of inhibition, by analogy with other flavoproteins, involves alkylation of the flavin moiety (31) at N-5 (Maycock *et al.*, 1976). The alkylation reaction probably occurs by a Michael addition reaction (Ferscht, 1985b).

This is the first evidence that the flavin prosthetic group mediates the transfer of electrons from the pyridine nucleotide to the dihydropterin substrate. This then explains the reason why *E. coli* DHPR does not distinguish between positions N-5 and C-6 of the pyrazine ring of the pterin nucleus and can transfer a hydride to either of the positions, thus exhibiting both DHPR and DHFR activities. More evidence for this argument comes from the kinetic parameters for NADH and NADPH in the DHPR reaction for the *E. coli* enzyme presented in Table 3.5. Although the K_m value for NADH was about 3-fold lower than for NADPH, the maximum velocities with both the pyridine nucleotide cofactors were identical. The DHPRs from most of the sources that have been studied use NADH in preference to NADPH, and the V_{max} value with the former substrate is much higher than that with the latter. This is also true in the case of DHFRs from most sources which use NADPH in preference to NADH where the V_{max} value with the former is higher. Therefore it may be reasoned that both NADH and NADPH exhibit identical apparent maximum velocities despite the 3-fold lower efficiency in the binding of the latter (which is probably due to lack of a site for the binding of the 2' phosphate group of NADPH) because their role in catalysis is mediated *via* the reduction of the flavin prosthetic group which in turn reduces the dihydropterins. These results are good support for the ping-pong mechanism of catalysis which is presented in Scheme 3.3.

3.2.11 Immunoreactivity of *E. coli* DHPR

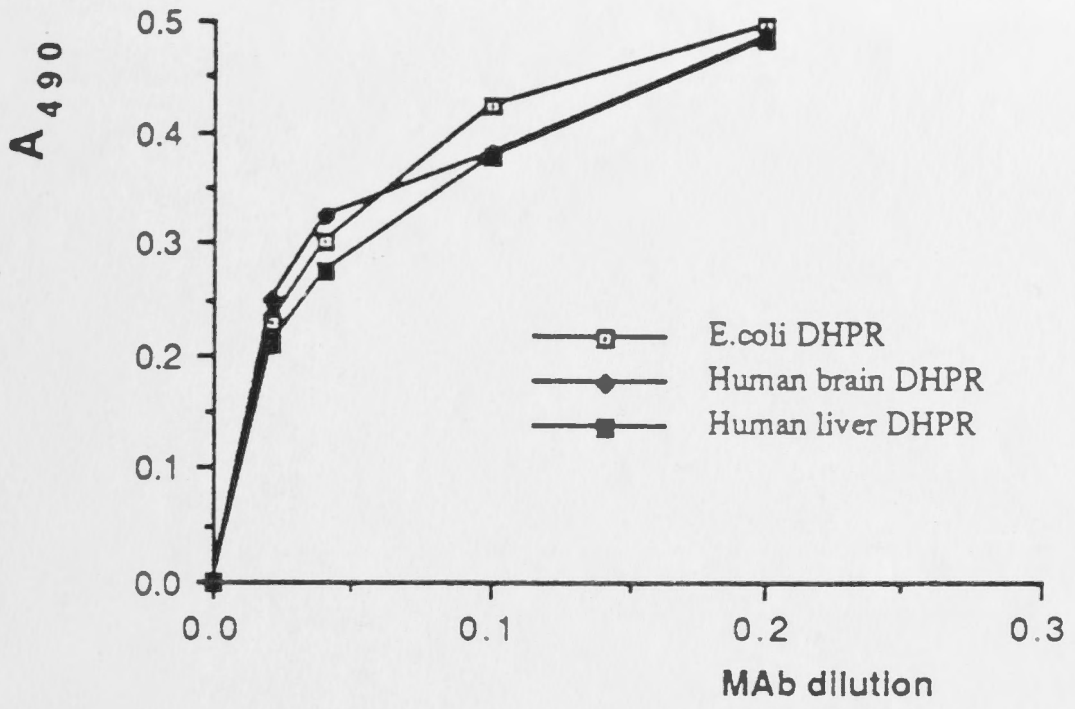
An ideal method of investigating structural relationships of enzymes that carry out a similar function and are separated by evolution (or by a creationist time-scale?) is to compare their cross-reactivity with antibodies raised against either of the antigens. Since the results discussed in the preceding sections indicated that the physical and kinetic properties of *E. coli* DHPR are considerably different from those of mammalian DHPRs reported in the literature, it was felt that cross reactivity studies with antibodies raised against human DHPR should be investigated to determine if there are any surface structural similarities between them. The sensitive enzyme-linked immunosorbent assays (ELISA; Engvall, 1980) were carried out for *E. coli* DHPR, human-brain DHPR and human-liver DHPR with monoclonal antibodies raised against human liver DHPR (monoclonal and polyclonal antibodies against human liver DHPR were kindly supplied by Dr. R.G.H. Cotton, The Murdoch Institute, Melbourne). Of the four monoclonal antibodies examined (#DHPR-1 to #DHPR-4), three of them (#DHPR-1,2 and 3) recognised a conserved epitope present in the three reductases that were examined (see Figure 3.10). Judging from the versatility of the reductase from *E. coli* (i.e. its multifunctionality) and the single known function of recycling quinonoid dihydropterin for human DHPR, perhaps the epitope being recognised may be a pterin binding site. However, when the *E. coli* reductase was compared with the human reductases by a dot blot experiment probed with monoclonal antibody # DHPR-1, there was no indication of antigen-antibody reaction. This was not surprising because other workers have reported problems with reacting monoclonal antibodies with antigens blotted onto nitrocellulose (Szewczyk and Summers, 1987). It was felt tha perhaps the monospecificity of these antibodies may be lost because the antigen may not be in its native conformation.

E. coli DHPR and human DHPR were also examined by Western blots with polyclonal antibodies raised against human DHPR (see Figure 3.11). Human-brain DHPR showed a much stronger signal compared with that of the apparently homogenous preparation of *E. coli* DHPR. The absence of a signal from the *E. coli* DHPR band in the

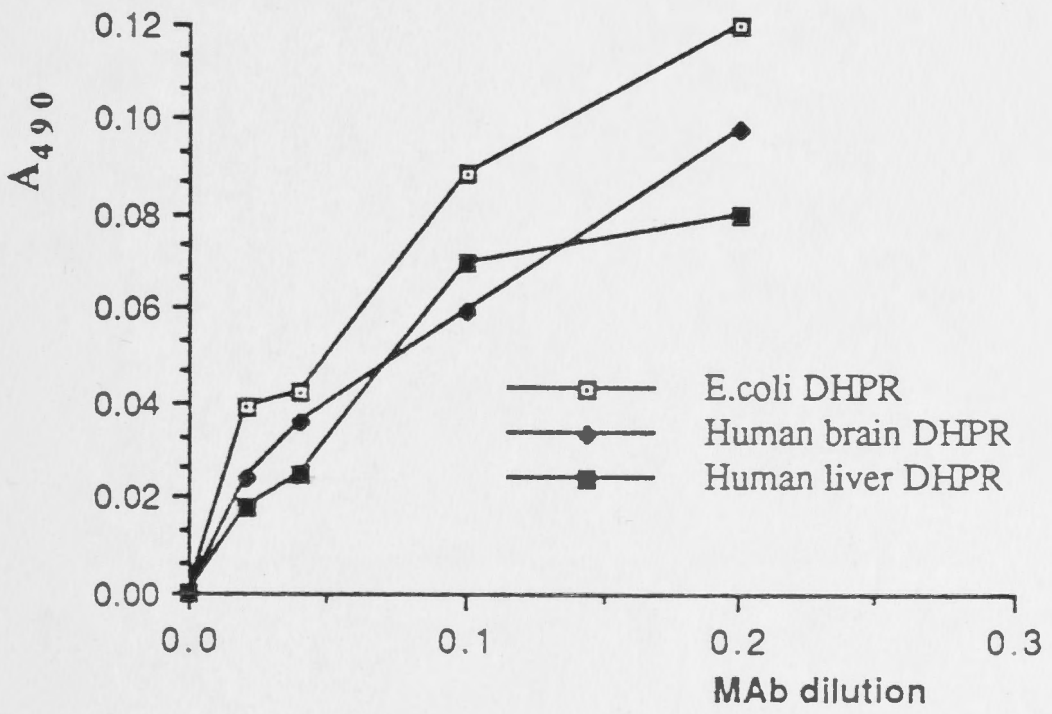
Figure 3.10 **Enzyme-linked immunosorbent assay of *E.coli* DHPR, human-brain DHPR and human-liver DHPR, using monoclonal antibodies (# DHPR-1, # DHPR-2 or # DHPR-3)**

The ELISA experiment was carried out as described in Section 3.4.14.

DHPR-1



DHPR-2



DHPR-3

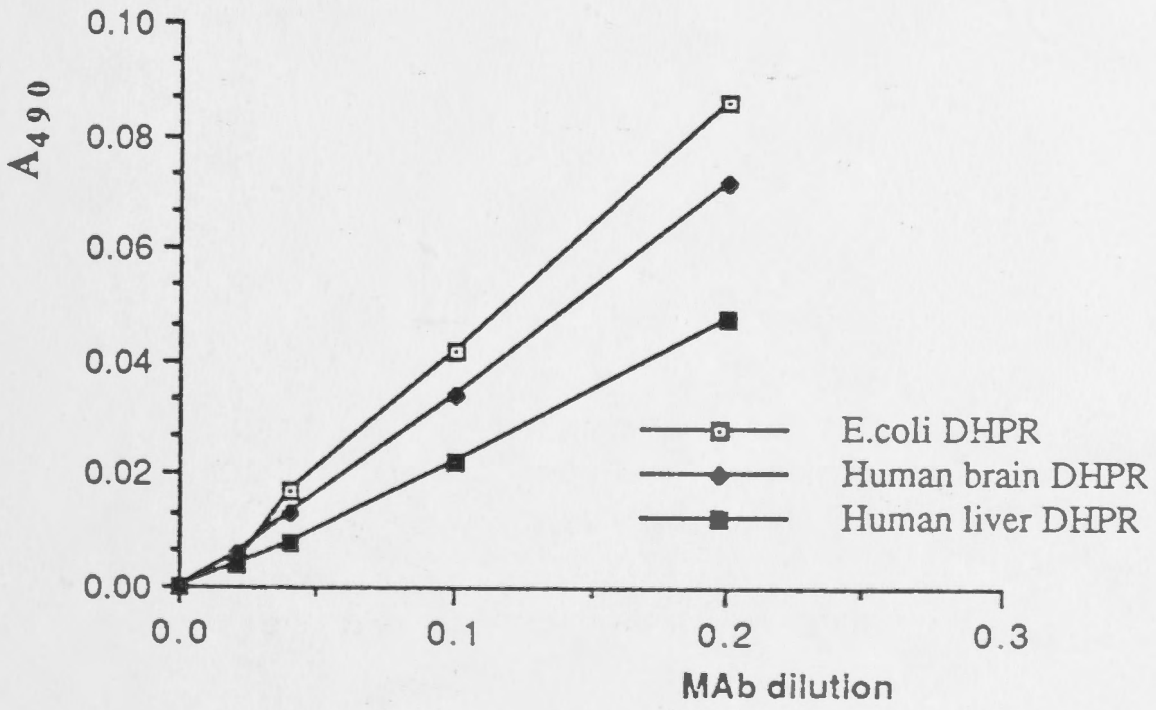


Figure 3.11 Western blot analysis of purified human-brain DHPR (b), purified *E. coli* DHPR (c) and cell-free extract from strain H712 (d), using polyclonal antibodies to human-liver DHPR

Human-brain DHPR (2 μg), *E. coli* DHPR (2 μg) and cell-free extract from strain H712 (100 μg) were run concurrently with Bio-Rad pre-stained molecular weight standards (a) on a 12% w/v SDS-PA gel, blotted onto a nitrocellulose filter and probed with polyclonal antibodies to human liver DHPR (1 in 2000 dilution of antiserum), as described in Section 3.4.16.

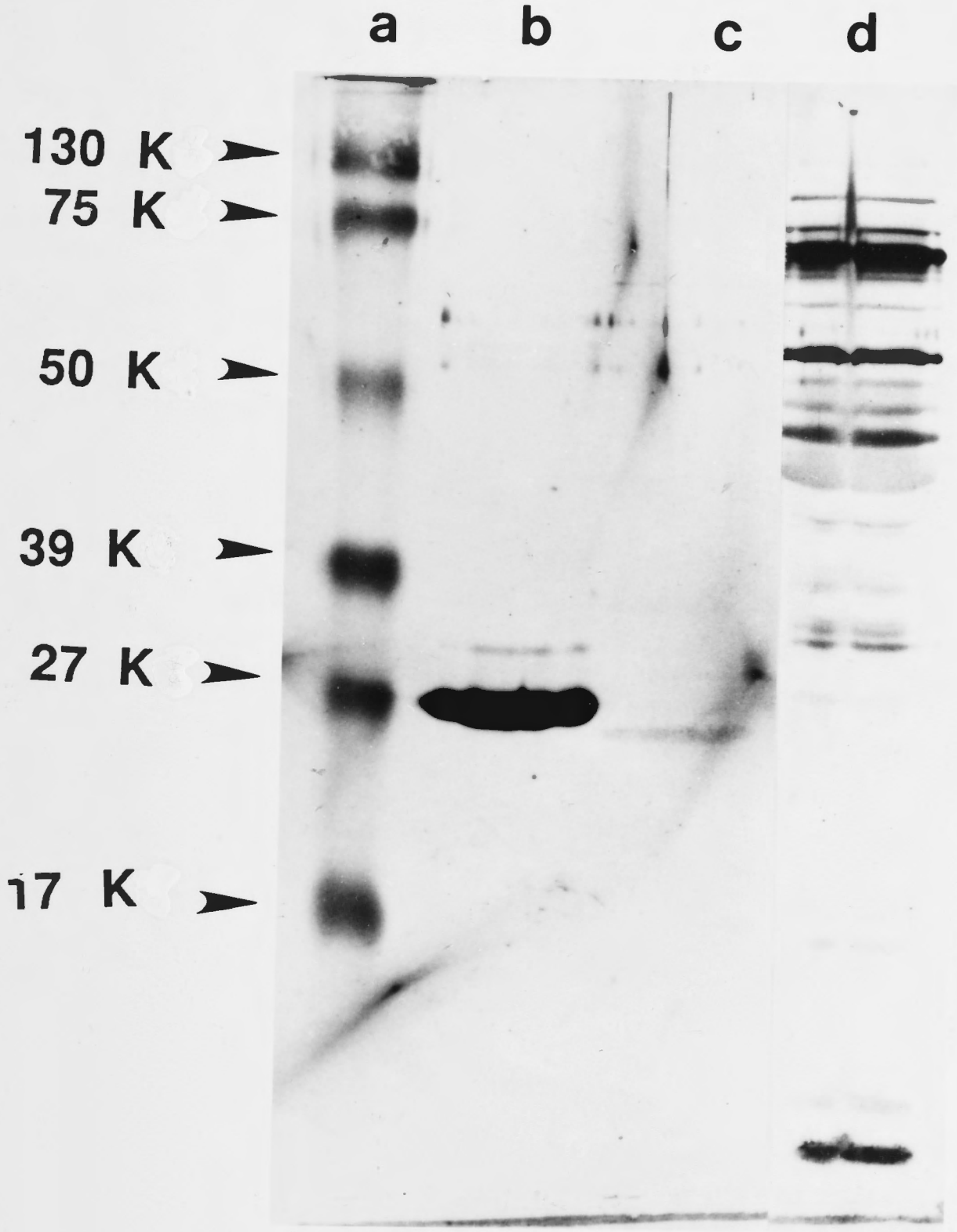

a
b
c
d
130 K
75 K
50 K
39 K
27 K
17 K

Table 3.7 Neutralisation of DHPR activity by polyclonal antibodies raised against *E. coli* DHPR

Vol. of antiserum (μl)	% loss of DHPR activity	
	E. coli DHPR[a]	Human-brain DHPR[a]
1	23	-
2	48	4
3	73	7
4	92	8
5	-	10

a 0.5 μg of antigen was added in the assay together with the appropriate volume of antiserum in the enzyme assay carried out as described in Section 2.5.1.1,

in the cell-free extract suggests that the antigenic properties of the two reductases are probably different.

At a later stage in this work, polyclonal antibodies against *E.coli* DHPR were raised specifically to investigate if a fusion protein had been inadvertently formed during attempts to clone the gene that encoded the reductase (discussed in the following Chapters). The crude antiserum obtained after removal of the coagulated blood cells was used in a Western blot experiment, to indicate that antibodies raised were specific for *E. coli* DHPR. These polyclonal antibodies gave a strong signal with the apparently pure *E. coli* DHPR as well as with the reductase band in the cell-free extract. The human-brain DHPR however showed a weak signal (data not shown). Further evidence that the antibodies were specific for *E. coli* DHPR came from its abilty to neutralise the reductase activity. Under conditions where 2µl of antiserum gave about 50% inhibition of bacterial DHPR activity, only 4% of enzyme activity of human brain DHPR was lost (see Table 3.7). It may be concluded from the above results that the mammalian and the bacterial reductases are antigenically diverse. However there are suggestions of a small degree of surface similarity which most probably is in the region of the pterin binding site. This notion also receives support from the preliminary studies with an anti-idiotype monoclonal antibody which behaves like a pterin substrate, which showed cross-reactivity with the *E. coli* reductase (R.G.H. Cotton and I. Jennings, private communication).

3.3 SUMMARY

Dihydropteridine reductase from *E. coli* is different from all the previously characterised reductases from mammalian sources (reviewed by Armarego *et al.*, 1984) and NADPH-specific DHPR from bovine and human liver (Nakanishi *et al.*, 1986a,b) in several respects. The reductase from this study is a 27 kD protein which is active as a monomer. In addition to its ability to reduce quinonoid dihydropterins to the corresponding tetrahydropterins, the *E. coli* reductase can also reduce 7,8 dihydrofolate to

tetrahydrofolate. *E. coli* DHPR has FAD as prosthetic group which is non-covalently bound to the enzyme. The presence of the FAD prosthetic group gives rise to the non-pterin dependent oxidase activity of NADH in the presence of artificial electron acceptors such as $K_3Fe(CN)_6$ and tetrazolium-MTT. The possibility that the protein from this work may have a major *in vivo* function which is unrelated to the *in vitro* activities was examined using phenol, salicylate, 4 hydroxybenzoate, 4-hydroxyphenylacetate, orcinol and 2,6-dihydroxypyridine as possible substrates, however none of them was active. Stoichiometric studies indicated that the pterin-independent oxidation of NADH in the presence of $K_3Fe(CN)_6$ is a tightly coupled reaction because 1 mole of NADH is oxidized when two moles of $Fe(CN)_6^{3+}$ are reduced. The oxidation of NADH and the reduction of ferricyamide follow first-order kinetics. The reduction of quinonoid 6-methyl-dihydropterin to the corresponding tetrahydropterin by *E. coli* DHPR is probably mediated by the FAD prosthetic group because the acetylenic inhibitor propargylamine inhibits the reaction. The enzymatic reaction is probably by the 'ping-pong' mechanism.

The major naturally occurring pterin cofactor in *E. coli* is monapterin (L-*threo*-neopterin). This pterin was previously identified as the cofactor for phenylalanine hydroxylase in *Pseudomonas* species (ATCC 11299a) (Guroff and Rhoades, 1969) and was the major pterin in that organism. The kinetic parameters for several quinonoid dihydropterins with respect to NADH as the second cofactor for *E. coli* DHPR compare well with those from the reductase from *Pseudomonas* (Williams *et al.*, 1976). With both the bacterial reductases, quinonoid dihydrobiopterin has ~6-fold lower K_m value than the natural substrate quinonoid dihydromonapterin. The *E. coli* reductase prefers NADH as the pyridine nucleotide substrate but can also use NADPH to an equal maximum velocity, although the Km value is lower. The *N* -terminus of DHPR from *E. coli* is not blocked by acetylation and methionine is the first residue. The sequence of the first 20 amino-acids of *E. coli* DHPR do not residues in common with the corresponding regions of human DHPR (Dahl *et al.*, 1987) and rat DHPR (Shahbaz *et al.*, 1987). The immunochemical studies may indicate that the bacterial reductase contain a pterin binding site.

3.4 MATERIAL AND METHODS

3.4.1 Dihydrofolate reductase assay

The DHFR activity assay, which measures the folate dependent oxidation of NADPH or NADH, was carried out spectrophotometrically on a Cary 219 double beam spectrophotometer (Wilmanns, 1974). The stock solution of 7,8-dihydrofolate (DHF) in 50 mM Tris-HCl pH 7.4 was made freshly on the day of the experiment. The standard reaction mixture contained Tris-HCl pH 7.4 (0.1 M), DHF (0.1 mM) and NADH or NADPH (0.1 mM) in a total volume of 1 ml each in two cuvettes. The enzyme activity was initiated by injecting the enzyme solution into one cuvette only. The enzyme activity was calculated by using the reaction molar absorption coefficient of 12,440 $M^{-1}cm^{-1}$ (because molar absorption coefficient for NADH or NADPH is 6220 $M^{-1}cm^{-1}$ and that for DHF is also 6220M^{-1} cm^{-1}) and the absorbances of both the substrates are decreased simultaneously in the enzyme reaction.

3.4.2 Molecular weight determination

3.4.2.1 Gel filtration method

A Superose-12 gel filtration column with the f.p.l.c system (Pharmacia) and 50 mM Tris-HCl pH 7.5 containing 2 mM DTT and 20 μm NADH as solvent was used to determine the native molecular weight of the enzyme. The column was calibrated with a mixture (0.2 ml in the above buffer containing 100 μg of each protein) of phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400), at a flow rate of 0.5 ml/min. The molecular weight of native DHPR (0.5 mg) was estimated from the calibration plot of logarithm of molecular weight *versus* elution volume, to be 54000± 2000. SDS/PAGE

run concurrently with low molecular weight standards (Pharmacia) was carried out to estimate the molecular weight of the denatured enzyme.

3.4.2.2 Analytical ultracentrifugation

Analytical centrifugation of the purified protein was examined in a meniscus-depletion sedimentation equilibrium experiment (Yphantis, 1964) by Dr Peter Jeffrey of the Protein Chemistry Group, JCSMR. DHPR purified by preparative bed isoelectric focusing (0.5 mg/ml) was dialysed against 50 mM Hepes pH 7.5 (500 ml, 2 days). The speed of 40 000 rpm was calculated to be appropriate for a solute of molecular weight 25 000. The experiment was performed in a double-sector aluminium-filled cpm cell with sapphire windows at a controlled temperature of 20 $\pm$ 0.1°C with the dialysed protein solution (0.10 ml) in one sector and its equilibrium dialysate (0.11 ml) in the other. A photograph was taken after sedimentation equilibrium had been attained (8 h) using refractometric optics of the Rayleigh interference fringe pattern. The fringe patterns were measured using a Nikon microcomparator and the results expressed as *ln* (fringe displacement) vs. r^2 where r is the distance from the axis of rotation. The weight average molecular weight was evaluated from the expression:

$$\overline{M}_w = \frac{2\,RT}{(1 - \bar{\upsilon}\,\rho)\,\omega^2} \quad \frac{d\ln y}{d\,(r^2)}$$

where

y	is the fringe displacement
R	is the gas constant
T	is the absolute temperature
υ	is the partial specific volume of the protein
ρ	is the density of the solution
ω	is the angular velocity of rotation

The partial specific volume was assumed to be 0.73 ml/g (for a normal soluble protein). The density was 1.001 g/ml.

3.4.3 *N*-terminal amino acid analyses

The purified reductase (0.4 ml, 50 µg) was dialysed against 0.5% NH_4HCO_3 (w/v, 3 x 500 ml) and finally against MQ water (Millipore, 500 ml). The sequence analyses were carried out by Dr Denis Shaw, Protein Chemistry Group, JCSMR. One half of the dialysis residue was applied directly on to a Beckman model 890M2 spinning-cup sequencer, with automatic conversion to release the phenylthiohydantoin (PTH) derivative of the *N*-terminal amino acid after each cycle. The amino acid PTH derivatives were analysed on a du Pont PTH column (with elution by sodium acetate-acetonitrile) using a Hewlett-Packard 1084B h.p.l.c system with an Altex 165 variable-wavelength detector. The second half of the dialysis residue was made 70% (v/v) in formic acid, treated with CNBr (200 µg) and analysed without separation of peptides. The reaction mixture was applied to the spinning cup and washed with ethyl acetate before the sequencing was commenced.

3.4.4 Amino acid analysis

E. coli DHPR(75 µg) in 1 ml of 50 mM Hepes-KOH pH 7.4 was dialysed exhaustively against the same buffer (2 x 500 ml) followed by dialysis against water (2 x 500 ml). The protein (dialysis residue) was aliquoted (~0.4 ml) into three hydrolysis tubes and freeze dried. HCl (6 M, 1 ml) was added to each tube containing the freeze-dried protein and degassed (using a freeze-thaw cycle) with an oil vacuum pump before sealing the tube. The protein was hydrolysed at 110°C for 24, 48 and 72 h. After the hydrolysis the seals were broken and the acid was removed by rotary evaporation. The residue was taken up in 0.15 ml System 6300 Na-S™ buffer (Beckman) and applied to the Beckman System 6300 High Perfomance Analyzer by Mr L.B. James, Biochemistry Department, JCSMR.

The performic acid oxidation of protein to obtain the analysis of cysteine as cysteic acid was carried out by adding 1 ml of performic acid (prepared from 9.5 ml of 90% formic acid and 0.5 ml of 30% hydrogen peroxide) to a hydrolysis tube containing 25 µg of protein treated as above. The oxidation was allowed to proceed for 6 hrs at 0°C and the solvent was removed by freeze drying. The oxidized protein was then hydrolysed and analysed as above.

3.4.5 Identification of the prosthetic group

The purified reductase (400 µg, 3 ml) in 50 mM Hepes-KOH pH 7.4 was denatured by the addition of trifluoroacetic acid (Aldrich; 90 µl of 99+%), centrifuged (2000 x g, 30 min) and the supernatant and was filtered through a Centricon YM10 (Amicon Mr cut-off 10000) by centrifugation (4000 x g, 30 min). The visible absorption spectrum of the filtrate (protein-free) was determined on a Cary 219 spectrophotometer, and the fluorescence spectrum was measured on a Perkin-Elmer 3000 fluorescence spectrometer with the excitation wavelength set at 450 nm. Authentic FAD (Sigma; 2 µM) solution was used for comparison. The solution from the spectroscopic measurements was freeze-dried, dissolved in 20 µl of water and an aliquot was run on a silica-gel 60 (Merck) plate together with authentic FAD, FMN and riboflavin. The Rf values of the isolated fluorescent cofactor, and authentic FAD and authentic FMN, relative to riboflavin were 0.16, 0.16 and 0.31, respectively (cf. Spitzer and Weiss, 1985).

3.4.6 Stoichiometry of NADH oxido-reductase activity

The oxido-reductase activity assay (see Section 2.5.1.2) was used to investigate the stoichiometry. The enzyme (0.5 µg) was added to one of the cuvettes to start the reaction. The rate of decrease of absorbance at 340 nm (due to NADH oxidation) and the rate of decrease of absorbance at 420 nm (due to reduction of ferricyanide, ε 1050 cm^{-1} M^{-1}) were recorded on a Cary 219 double-beam spectrometer driven by an Apple II-

PLUS computer using a multiscan kinetic program. The absorbance readings were taken at 22 s intervals, and the readings at 340 nm were taken 11 s after the readings at 420 nm. Data were collected for 15 min. The infinity absorbance values for the two wavelengths were calculated by using the Curfit program provided by Dr David Randles (Department of Community Services and Health, Phillip, Canberra, A.C.T.).

3.4.7 Analysis of naturally occurring pterins

Escherichia coli strain H712 (20 g) cultured as described in Section 2.5.4 was suspended in 0.1 M H_3PO_4 (40 ml) and lysed in a Sorvall Ribi cell smasher [290 kPa (2000 1b.in^{-2})]. The cell-free extract was obtained by centrifugation (2000 x g, 20 min) and concentrated (to 4 ml) by freeze drying. The sample was oxidized in a mixture of trichloroacetic acid (1 ml, 2 M) and acidified iodine (2 ml of 9 mg I_2, 1.8 mg KI/ml solution in 0.1 M HCl) for 1 h at 25°C in subdued light. The suspension was centrifuged (2000 x g, 10 min) and the supernatant was concentrated to 2 ml by freeze-drying. The sample was applied to Dowex 50W x 4 (0.2 ml, 100-200 mesh, H^+ form) resin in a Pasteur pipette. After washing with water (5 ml) the pteridines were eluted with 0.25 M NH_4OH (6 ml) directly onto a Dowex 1 column (0.2 ml, 50-100 mesh, OH^- form) also in a Pasteur pipette. This was washed with water (3 ml with suction) and the pterins were eluted with 1 M acetic acid (0.6 ml). The effluent was analysed for pterins on an ODS column (0.46 cm x 25 cm) fitted to a Varian 5000 h.p.l.c system with a Fluorichrome detector. Standard solutions of monapterin, biopterin, neopterin and pterin were used to identify the unknown pterins.

3.4.8 Activity of substrate and kinetic analyses

All the substrates examined in this work exhibited Michaelis-Menten type saturation kinetics. The kinetic parameters for the pyridine nucleotide substrate in the DHPR reaction were obtained by varying its concentration from 10 μM to 100 μM (at least five different concentrations, e.g. 10, 20, 40, 60, 80 μM) and keeping the

concentration of the pterin substrate constant at 100 μM. Conversely, the concentration of the pyridine nucleotide was kept constant when kinetic parameters with pterin substrates were measured. The assays were carried out in duplicate for each concentration value. The velocities obtained were plotted graphically in the double reciprocal form (Lineweaver and Burk plot) to check for linearity before analysis with the computer programme of Cornish-Bowden (Cornish-Bowden and Endrenyi, 1981).

The inhibition constants with methotrexate were measured by keeping the concentration of one of the substrates constant (e.g. NADH = 100 μM) and varying the second substrate from 10 μM to 100 μM (at least five points), in the absence of methotrexate (I = 0) and repeated in the presence of 100 μM, 200 μM or 300 μM methotrexate respectively. (Note: The inhibitor was added in both cuvettes). The sets of data were graphically analysed by the double reciprocal plot (e.g. see Figure 3.8) and analysed by the computer programme of Cornish-Bowden by fitting into appropriate rate equations to determine if the pattern of inhibition were linear competitive, non-competitive or uncompetitive. The pattern of inhibition obtained by computer analysis agreed with that determined graphically. All kinetic constants (Km, V_{max} and Ki) had standard errors less than $\pm$ 5%.

3.4.9 Synthesis of dimethylpropargylamine

Dimethylamine (0.45 g, 1.36 ml in ethanol) was slowly added to a 50 ml round-bottomed flask containing propargylbromide (1.11 g, 1.31 mls). The flask was stoppered after the white cloudiness disappeared and the solution was left stirring overnight at room temperature. Ethanol (2 ml) was added to the reaction mixture and the product was crystallized by adding ether dropwise and scratching the glass at 0°C. The crystals that were filtered were dissolved in the minimum volume of ethanol, recrystallized by addition of ether and washed with ether (3 x 10 mls) before drying over P_2O_5. The product dimethyl propargylamine was > 95% pure as determined by ^1H NMR (see spectrum in the Aldrich "NMR spectra" catalogue).

3.4.10 Inhibition by dimethylpropargylamine

Dimethylpropargylamine (2 mg) was dissolved in a mixture containing Tris/HCl (1 M, 0.6 ml), distilled water (5.9 ml) and *E. coli* DHPR (30 µg; 60 µl) at 25°C. The control experiment was carried out without the inhibitor. An aliquot (0.75 ml) was removed immediately after addition of enzyme and at five minute intervals thereafter. DHPR activity was determined from the change in absorbance at 340 nm followingaddition of peroxidase (20 µg; 100 µl), H_2O_2 (11 µmol; 50µl), 6-methyltetrahydropterin (100 µM; 50 µl) and NADH (100 µM; 50 µl) to start the reaction.

3.4.11 Effect of tetrahydropterin on pterin-independent oxido-reductase activity

The pterin-independent oxido-reductase activity assay (Section 2.5.1.2) was carried out. The substrate 6-methyl-tetrahydropterin was added to give final concentrations of 0, 10, 25 or 75 µM to the sample and reference cuvettes. The enzyme was added to the sample cuvette to start the reaction. The rate of decrease of absorbance at 420 nm (due to the reduction of ferricyanide, ε 1050 cm^{-1} M^{-1}) was recorded on a Cary 219 double-beam spectrophotometer.

3.4.12 Non-denaturing PAGE and activity staining of gels

The non-denaturing PAGE was carried out as described in Section 2.5.3.2. Since the reductase from *E. coli* possessed three different enzyme activities, a modified method of staining for all the activities on a single gel was used. Basically, it involved immobiliation of the constituents for a particular reaction in a gel medium so that it could be layered over the PA gel. When staining for 3 different activities on a single gel, spacers were used to guide the layering of the reaction mixture over a particular section.

For the DHPR stain, stock solutions of 10 mM NADH (1 ml), 10 mM tetrazolium MTT (1 ml), and 10 mM quinonoid 6-methyl-7,8-dihydropterin were warmed briefly to ~37°C and added to molten agarose (0.3% w/v) in 7 ml of 140 mM Tris HCl (pH 7.4). The mixture was lukewarm when layered over the PA gel section that was analysed for DHPR activity. For the DHFR stain the reaction constituents were 10 mM NADPH (1 ml), 10 mM tetrazolium MTT (1 ml) and 10 mM 7,8-dihydrofolate (1 ml), mixed with molten agarose prepared as above. For the pterin-independent NADH oxido-reductase activity the reaction mixture contained 10 mM NADH (1 ml), 10 mM tetrazolium MTT (1 ml), distilled water (1 ml), added with molten agarose as for the DHPR stain.

The agarose gel overlay containing the reactants for the three enzymic reactions were allowed to be in contact with the PA gel for 5-10 min at room temperature. Once the bands were visualised, the overlay was removed and the gel was photographed immediately for record. The activity stained gels were stained for protein using the Coomassie G-250 method described in Section 2.5.3.1.

3.4.13 Protein-blotting and *N*-terminal amino acid sequencing

The Applied Biosystems 477A gas-liquid phase sequencer was used for microsequencing kindly carried out by Dr Denis Shaw, JCSMR.

The partially purified protein (~4 µg per track) was separated by SDS-PAGE on a 12% gel as described in Section 2.5.3.1. Meanwhile, the sheets of glass microfiber (GF/C; Whatman, U.K.) were cut to the size of the PA gel and evenly wetted with 3 ml/ml solution of Polybrene in water. The impregnated sheets were dried in air for 4 to 5 hours. When the electrophoresis was completed, the glass plates were carefully separated. The stacking gel was removed with a scalpel and the separating gel was placed in a "sandwich" assembly (perspex sheet, Scotchbrite pad, 2 sheets of 3 MM paper cut to the size of the Scotchbrite pad, the PA gel, two sheets of Polybrene-coated GF/C placed

back to back, two sheets of 3 MM paper, one sheet of Scotchbrite pad and finally the perspex sheet). The Polybrene coated GF/C was washed in 100 ml water for 5 min to remove excess unimpregnated Polybrene before it was mounted in the "sandwich"). All the constituents of the "sandwich" were wetted with the transfer buffer (50 mM sodium borate, pH 8.0, 20% methanol and 0.02% 2-mercaptoethanol) and the bubbles from each layer of the "sandwich" were removed by rolling over it gently with a cylindrical tube. The "sandwich" was fastened by clips between the perspex sheets and immersed in an electro-blotting apparatus (Bio-Rad Trans-Blot Cell) containing the transfer buffer, and the electrophoretic transfer was carried out at room temperature for 15 h at 25 V (constant). Care was taken to ensure that the Polybrene coated GF/C within the sandwich was at the anodic end before the transfer was begun. After the transfer was completed, the constituents of the "sandwich" were carefully removed to expose the Polybrene coated GF/C. The sheets were marked so that the front and back sheets could be distinguished and superimposed after staining and drying. The sheets were subsequently washed for 10 min with 150 ml of 25 mM NaCl, 10 mM sodium borate (repeated four times) and finally washed in water. They were then dried by being suspended in a clean cupboard for 3-4 h. The dried sheets were sprayed with a freshly-prepared acetone solution of fluorescamine (1 mg/300 ml acetone; Fluka A.G) and dried again (~30 min). Proteins were visualized under to u.v. illumination. The relevant bands were lightly outlined with a pencil and cut out with a clean scalpel. This was then mounted in the reaction chamber of the Applied Biosystems 477A gas-liquid phase sequencer essentially as described by Hewick *et al.* (1981). The liberated PTH derivatives of amino acids were analysed on-line in an Applied biosystems 120A h.p.l.c. fitted with a PTH-C18 reverse phase column using a gradient elution system essentially as described by Hunkapiller and Hood (1983). It should be stressed that gloves should be worn throughout and tall precautionary steps should be taken to prevent introduction of contaminating proteins. The dry GF/C membrane was has handled carefully because it was very brittle.

3.4.14 Enzyme-linked immunosorbent assay (ELISA)

The purified *E. coli* DHPR, human-brain DHPR and human-liver DHPR (10 µg/ml each in 0.05 M Na_2CO_3, pH 9.6) were examined by ELISA using monoclonal antibodies raised against human-liver DHPR (kind gift of Dr R.G.H. Cotton). The monoclonal antibodies #DHPR 1, 2, 3 and 4 and the pre-immune control supernatant were examined. The protein antigens (50 µl) were placed in the appropriate wells of a microtitre plate (96 U shaped wells with cover; Linbro, U.S.A), allowed to stand overnight at 4°C and covered to prevent evaporation. The excess unbound antigen was removed and the wells were washed (twice) with phosphate buffered saline (PBS, pH 7.2). The wells were blocked with 100 µl of 1% BSA (Fraction V, 99% pure; Boehringer, Mannheim) for 1 h at 4°C. The excess was removed by tapping the plate. The monoclonal antibodies and the control were diluted (in PBS containing 1% BSA) to form a range from neat to 1/50 and added to the appropriate wells. The plate was incubated for 1 h at 4°C. The excess antibody was then removed and the wells were washed (3 times) with PBS-Tween 20 (0.02%). The second antibody was peroxidase-coupled goat antimouse IgG (diluted 1 in 2000 in PBS containing 1% BSA; Sigma), 50 µl of which was added to each well and left standing at 4°C for 1 h. This was removed and the wells were washed (twice) with PBS-Tween 20 and twice with water.

The substrate for the perioxidase assay was prepared by dissolving 5 mg of *o*-phenylenediamine in 0.25 ml of methanol followed, and just prior to addition to the wells, by addition of 12.25 ml of citrate-phosphate buffer, pH 5.4 (see later) and 1.25 µl of 30% H_20_2. This solution was added to each well and allowed to stand for 15 min at room temperature in the dark. The reaction was stopped with 50 µl of 8 N H_2SO_4 and the absorbance at 490 nm was read using the Titertek Multi-Scan spectrophotometer.

Buffers: 0.05 M Na_2CO_3, pH 9.6 contained 1.59 g Na_2CO_3, 2.9 g $NaHCO_3$ made up to 1 l in water. Citrate-phosphate, pH 5.4 was made by adding 49.7 ml of phosphate

solution (17.8 g $Na_2HPO_4.2H_20$ in 500 ml of water) to 48.3 ml of citrate solution (10.5 g of citric acid in 500 ml of water).

3.4.15 Raising polyclonal antibodies to *E. coli* DHPR

Homogeneous *E. coli* DHPR (350 µg) in 0.5 ml of 50 mM Hepes-KOH (pH 7.4) was mixed with an equal volume of Freund's complete adjuvant (Commonwealth Serum Laboratories, Australia) and injected subcutaneously on the left thigh of a male rabbit. The rabbit had previously been bled from an ear vein to obtain pre-immune serum. Ten days later the rabbit was boosted with 200 µg of *E. coli* DHPR in 1 ml of Freund's incomplete adjuvant by subcutaneous injection on the right thigh. After a further 10 days, the rabbit was bled from an ear vein to give 30 ml of blood. The blood samples were allowed to clot overnight at 4°C and were then centrifuged at 25000 x g for 20 min at 4°C. The antiserum was removed carefully with a Pasteur pipette into a fresh container and stored at -20°C until used. The pre-immune serum was obtained similarly. The presence of antibodies against *E. coli* DHPR in the antiserum was established by Western blot and neutralization of enzyme activity by antiserum in the DHPR assay.

3.4.16 Western blot analysis

Proteins were analysed by Western blot after separation on SDS-PA gel followed by electrophoretic transfer using the Bio-Rad Trans-Blot apparatus. The trans-blot buffer was 20 mM Tris, 150 mM glycine containing 20% methanol. The proteins were transferred onto a nitrocellulose membrane cut to the size of the gel and placed in a sandwich arrangement as described for protein blotting onto Polybrene coated GF/C. The electrophoretic transfer was carried out at 25 volts (constant) for 15 h. Pre-stained molecular weight markers (Bio-Rad) were transferred concurrently to indicate that the

transfer process was complete). The nitrocelluiose filters containing the transferred proteins were sometimes stained with fluorescene isothiocyanate (FITC) as described by Szewazyk and Summers (1987) as this method did not affect the antibody reaction. [Nitrocellulose membranes to which antigens were applied by spotting (manually) or electroblotting were treated similarly from this step onwards]. The nitrocellulose membrane was immersed in the blocking solution (100 ml of 3% gelatin in 20 mM Tris-HCl pH 7.5, 500 mM NaCl,; TBS) and agitated gently for 30 min to 1 h. The membrane was then transferred into a tray containing TTBS [Tween-20 (0.05%) in TBS] and washed (twice) for five minutes each. Following this the membrane was exposed to the primary antibody (1 in 2000 dilution) (either polyclonal antibodies against human DHPR or *E. coli* DHPR) in 100 ml of TTBS containing 1% gelatin with gentle agitation from 5-15 h. The membrane was washed with TTBS (twice) to remove unbound first antibody. The membrane was then exposed to the second antibody, goat anti rabbit-alkaline phosphate conjugate (1 in 3000 dilution; Bio Rad) in 100 ml of TTBS containing 1% gelatin and agitated gently for 2 h. Then the membrane was washed with TTBS for 5 min (2 times) and finally with TBS to remove residual Tween-20 detergent from the membrane surface.

The alkaline phosphatase colour reaction was set up by separately dissolving 30 mg of nitroblue-tetrazolium in 1 ml of 70% DMF, and 15 mg of bromochloroindolyl phosphate into 1 ml of DMF (separately). Just prior to use these two 1 ml solutions were mixed into 100 ml of carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8), and the membrane was immersed into it. The antigens were normally visible after 5-10 min of colour development. The membrane was immersed in 2 changes of water (10 min each) to stop the development. It was then photographed wet so as to enhance the purple coloured bands.

CHAPTER 4

ATTEMPTS TO ISOLATE THE GENE ENCODING *E. COLI* DHPR BY COSMID CLONING.

4.1 INTRODUCTION

The limiting factor in the study of the catalytic properties and mechanism of action of an enzyme is often the amount of the active protein available. Although the 27-kD *E.coli* DHPR is a fairly abundant protein in the wild type organism, the final yield of pure protein starting from a 40 litres of culture was about 2-4 mg (see Chapter 2). Isolation of the gene that encodes *E.coli* DHPR is the first step towards producing richer sources of the protein. The cloning of the gene into a multi-copy plasmid expression vector such that it is placed downstream from an efficient promoter can result in a single protein constituting more than 50% of the total cell protein in the engineered organism (Dixon *et al.*, 1988). Large amounts of protein produced by genetic engineering could ultimately assist in the solution of the X-ray crystallographic structure of this flavin containing dihydropteridine reductase. Since an *in vivo* function for the reductase is not known, isolation of the gene would also enable construction of mutants for genetic studies. The complete nucleotide sequence would allow the deduction of the primary amino acid sequence of *E. coli* DHPR which could be compared with the known sequences of human-liver DHPR (Dahl *et al.*, 1987; Lockyer *et al.*, 1987) and rat liver DHPR (Shahbaz *et al.*, 1987). Such a primary structure comparison could comment on the nature of evolution of DHPR and identify the functionally important amino acid residues within the sequence that are conserved.

Some preliminary attempts were made in this work to obtain a strain that was deficient in dihydropteridine reductase using the tetrazolium-MTT dye, by assuming that the reductase may not be essential for growth of the organism. The method was similar to that used for the isolation and mapping of glutathione reductase-negative mutants of *E. coli* KI2 using the compound 5,5'-dithiobis-(2-nitrobenzoic acid) (Davis *et al.*, 1982).

The tetrazolium-MTT staining method was not successful however. It stained all colonies blue despite attempts to reduce the background by spraying the lysed colonies with Methotrexate. This was done to inhibit dihydrofolate reductase which also forms the insoluble formazan with the MTT indicator.

Given the current status of molecular biological techniques, the cloning of the gene from *E. coli* should in theory be trivial. Elegant methods such as the use of synthetic oligonucleotide probe to isolate the gene (Ullrich *et al.*, 1984) were overlooked in favour of the cosmid cloning strategy (Hohn and Collins, 1980). The attractive feature of this method was that there was a high probability of obtaining the complete DHPR gene (~ 0.8.kb based on the molecular weight of the protein) because of the large insert fragments (35-45 kb) that can be cloned into the cosmid vectors. In this chapter, I describe the construction of a cosmid library of the genome of *E. coli* strain D3-157 (Singer *et al.*, 1985). The genomic DNA from this strain was chosen because it is *folA*. It was hoped that overproduction of *E.coli* DHPR could complement the DHFR mutation in the strain D3-157, thus enabling selection of recombinants on minimal nutrient plates containing no folate-dependent end-products such as methionine, glycine, thymidine, adenine and pantothenate. However this screening method could not be used because the strain D3-157 was found to be resistant. After using a suitable strain for transfection, 300 recombinants, each bearing 35-45 kb DNA fragments in the cosmid vector pHC79 (Hohn and Collins, 1980), were purified. Their cell-free extracts were individually prepared and screened directly for increased DHPR specific activity compared with that from the background strain. Two cosmid clones thus obtained were studied in further detail.

4.2 RESULTS AND DISCUSSION

4.2.1 Cosmid cloning

Cosmids are plasmids that contain the *cos* sequence (*cos* stands for cohesive site) from bacteriophage λ DNA required for packaging DNA into the λ capsid (Hohn and

Murray, 1977). The cosmid vector pHC79 (Hohn and Collins, 1980) used in this study is a derivative of the plasmid vector pBR322 (Bolivar *et al.*,1977). The pHC79 vector contains an ~ 2-kb DNA fragment containing the bacteriophage *cos* sequence inserted in a non-essential region of pBR322 (4.4 kb). The cosmid vector has features of pBR322 such as the tetracycline and ampicillin resistance genes, several unique cloning sites and an origin of replication (*ori*) which is similar to that of Col E1 (see Figure 4.1).

The chromosomal DNA used for the construction of the cosmid library was isolated from the *folA* strain D3-157 (Singer *et al.*, 1985) as described in Section 4.5.1. Large DNA fragments for cloning into cosmid vectors are normally generated by partial digests of the genomic DNA with an appropriate restriction endonuclease. In this study the restriction endonuclease *Sau* 3A was used for the partial digests of chromosomal DNA from strain D3-157. This enzyme recognises a four base sequence -N-G-A-T-C-N-, which occurs frequently (about once every 256 bps assuming restriction endonuclease sites are distributed randomly) in any given DNA sequence. Another feature that makes *Sau* 3A a suitable candidate for generating large fragments for cloning into the cosmid vector is that the 5' overhang sequence that it produces is complementary to that produced by the restriction endonuclease *Bam* H1, which recognises the six base sequence -G-G-A-T-C-C-. The cosmid vector pHC 79 has a unique *Bam* H1 site located within the *tet* resistance gene.

Trial partial digests of the chromosomal DNA with *Sau* 3A were carried out to optimize the conditions required for obtaining fragments ranging from 35 to 45 kb (see Section 4.5.2). The result in Figure 4.2 indicates that 0.016 units of *Sau* 3A digested 0.5 µg of DNA under the conditions used to generate fragments of the required size range. This partial digest was scaled-up appropriately to digest 60 µg of chromosomal DNA. After the reaction was terminated by adding EDTA (pH 8.0) to a final concentration of 20 mM, the digest was fractionated according to size by sodium chloride gradient centrifugation, and the contents of the tubes were distributed into 10 Eppendorf tubes (see Section 4.5.2). Electrophoresis of samples (10 µl) from each fraction on a

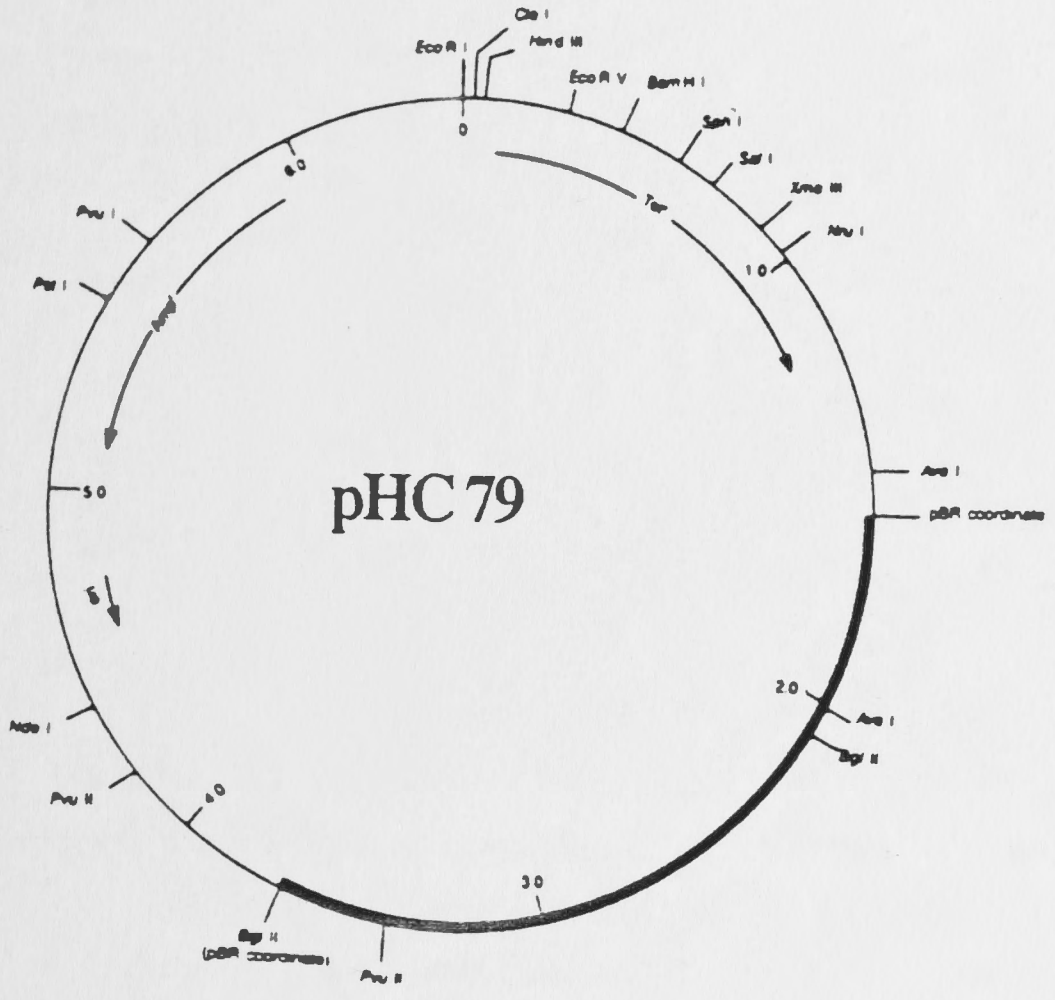


Figure 4.1 The stucture of cosmid vector pHC79(~6.4 kb) showing its restriction enzyme cleavage sites (Hohn and Collins, 1980).

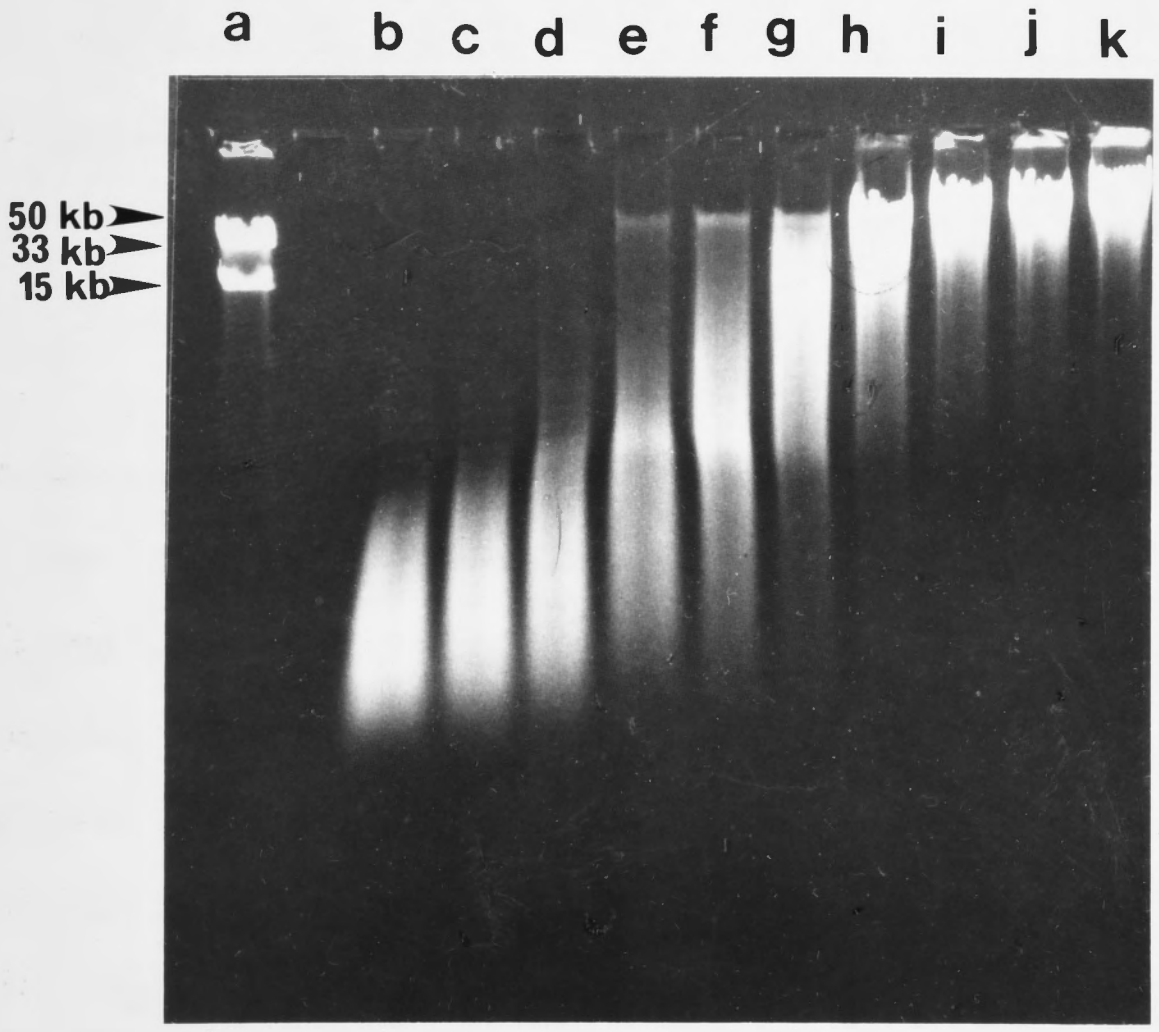
The direction of transcription of the ampicillin (Amp^r) and tetracycline (Tet^r) resistance genes, and the origin of replication (ori) are indicated by thin arrows within the circle. The thick line on the circle (flanked by the pBR 322 coordinates) contains the cos sequence from λ DNA.

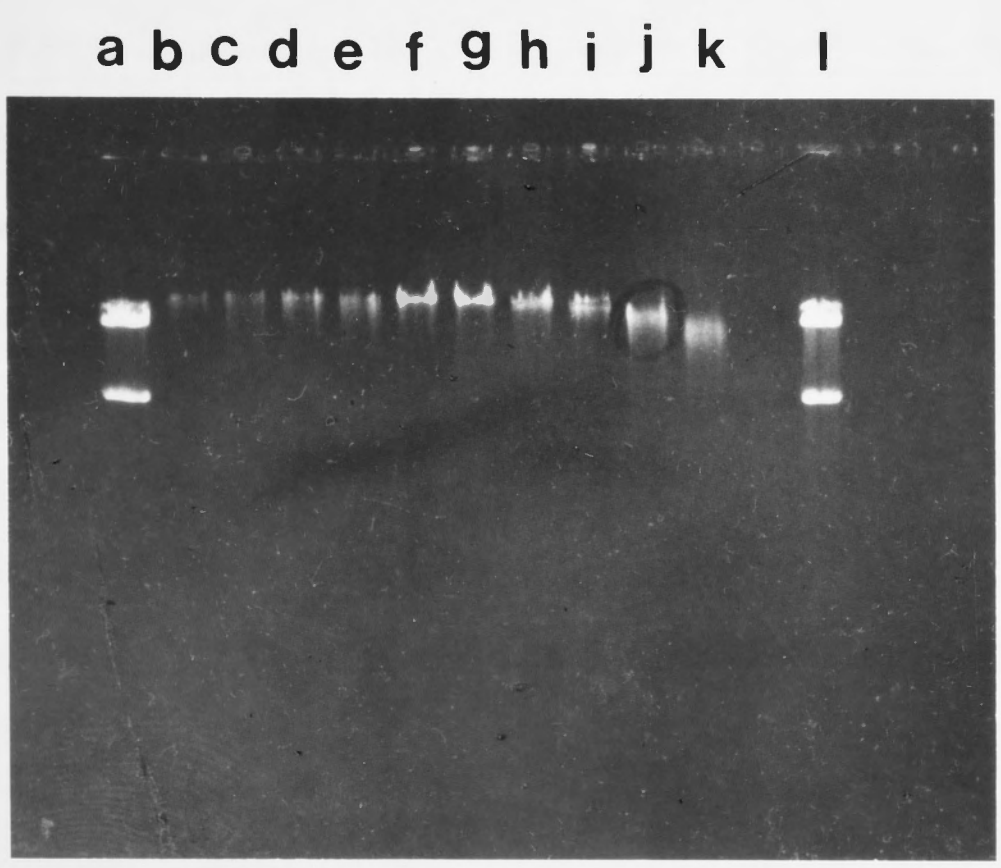
Figure 4.2 Establishing the conditions for partial digestion of chromosomal DNA from strain D3-157, with *Sau* 3A.

The experimental details for the partial digestion are described in Section 4.5.2. The samples were analysed by agarose gel electrophoresis (0.3% w/v at 4°C for 15h), 'a' λ DNA cut with *Xho* I is the known size marker. Lanes 'b' to 'j' contain 1 μg of DNA digested with 2 to 0.08 units of *Sau* 3A at 37°C for 20 min. Lane 'k' is undigested chromosomal DNA. The conditions for lane 'i' were scaled up for the large scale digestions.

Figure 4.3 Agarose gel electrophoresis of samples after NaCl gradient centrifugation of large-scale partial digestion, as described in Section 4.5.2.

Lane 'a' and 'l' are λ DNA (cut with *Xho* I) size markers. Lanes 'b' to 'k' are samples from the fractions collected after the gradient centrifugation. Fraction 'i' was used for making the cosmid library.


a b c d e f g h i j k
50 kb
33 kb
15 kb


a b c d e f g h i j k l

0.3% (w/v) agarose gel (see Figure 4.3) showed that fraction "i" contained the ideal-size fragments required for the construction of the cosmid library. Fractions "j" and "k" contained fragments that were < 35 kb, which might form non-contiguous inserts with fragments from two different regions of the *E. coli* chromosome (Old and Primrose, 1985).

After dialysis against TE, half the volume of fraction "i" (~1 μg DNA) was co-precipitated with 2 μg of pHC79 (that had been previously digested with *Bam* HI and dephosphorylated to remove its 5' terminal phosphates; a gift from Dr N. E. Dixon) by addition of ethanol. The vector DNA was dephophorylated to prevent self-ligation and a 2:1 ratio of vector DNA to insert DNA was used so that the formation of hybrid concatenated molecules would be favoured. This mixture was ligated with the joining enzyme T4 DNA ligase.

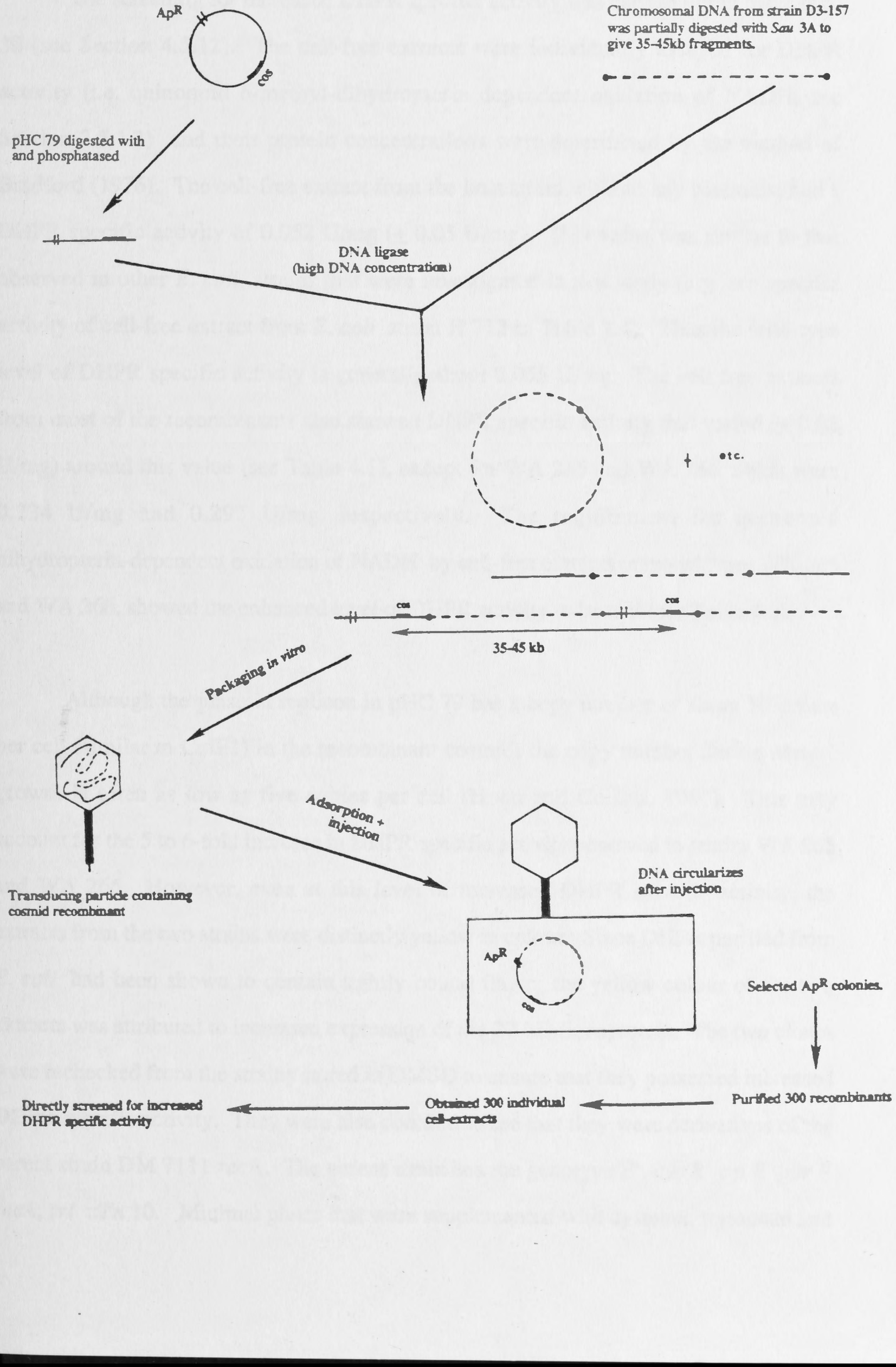
The ligation mixture was packaged *in vitro* (see Section 4.5.9). During *in vitro* packaging the concatemeric DNA (ie. a DNA fragment flanked by lefthand and righthand vector) is nicked at the *cos* sites by an endonuclease which is a product of gene *A* of bacteriophage λ. These nicks are on the opposite strands of the righthand and lefthand vectors. They form the cohesive termini (or sticky ends) which allow the concatenated molecule to circularise upon adsorption and injection into a suitable host by the transducing phage particle. The *in vitro* packaged extract was used to transfect the host strain D3-157 *rec A* (see Section 4.5.11 for details of construction of the *rec A* strain) as described in Section 4.5.10. It was important that the host strain was *rec A* to prevent the large insert fragments from undergoing homologous recombination into the chromosome of the host. The strain D3-157 *rec A* was selected as the host because it was shown in this work to grow poorly on LB plates containing 100 μM Methotrexate. It was argued that recombinants that carry the gene coding for DHPR would show better growth as a result of complementation of the DHFR mutation of this strain. The basis for this argument is that homogenous preparations of *E. coli* DHPR show dihydrofolate reductase activity (see Section 3.2.1). If the transfection had been successful, it was

intended to plate the transfected cells on LB plates containing ampicillin and Methotrexate (100 μM) to give an arbitrary size selection of the colonies. However, this was not successful because there were no recombinants when the transfected cells were plated on LB-plates containing ampicillin. This was later shown to be due to the λ resistance of the host strain (the strain grew across a λ *Ch* 28 streak). The host strain in cosmid cloning has to be λ sensitive so that the transducing phage particles can adsorb and its DNA be injected into the cell.

An alternative host strain DM7111 *rec*A (a gift from Dr N. E. Dixon) which was known to be λ sensitive (confirmed by streaking across λ *Ch* 28 and observing no growth along or after the λ *Ch* 28 streak) was transfected with the *in vitro* packaged mixture (only 1/5 of the original packaged extract was used for each transfection). In the former case (i.e. with D3-157 *rec*A as host) only the large colonies were going to be selected and screened directly for increased DHPR specific activity. With DM7111 *rec*A as the host, it was theoretically determined (based on the size of the insert in the cosmid vectors and the size of the *E. coli* chromosome ~4600 kb) that screening at least 300 recombinants should yield at least two that had the complete gene encoding DHPR.

DM7111 *rec*A transfected with the packaged extract was suspended in 500 μl of LB broth and plated on LB plates containing ampicillin (250, 150, 100, 50 and 25 μl per plate). There were > 1000 recombinants on the plate spread with 150 μl of the transfected cell suspension. The steps involved in cloning using the cosmid vector are presented schematically in Figure 4.4. One thousand colonies were toothpicked (sterile) on to LB plates containing ampicillin (100 per plate) and incubated at 37°C overnight. About 95% of these colonies were ampicillin resistant. From these, 300 colonies were purified for singles on four-sector LB plates containing ampicillin. The single colonies were streaked on 8-sector plates and a loopful from each sector was suspended in 1.5 ml-capacity glass screw-cap vials containing 1 ml LB medium and 77 μl DMSO (sterile). Two hundred and ninety-seven recombinants (WA101-WA397) were stored at -70°C. WA101-WA397 are DM7111 *rec*A bearing pWA101-pWA397, respectively.

Figure 4.4 The Scheme of events for cloning large DNA fragments from strain D3-157 into the cosmid vector pHC79 and screening for DHPR clones.


Ap^R
cos
pHC 79 digested with
and phosphatased
Chromosomal DNA from strain D3-157
was partially digested with Sau 3A to
give 35-45kb fragments.
DNA ligase
(high DNA concentration)
etc.
cos
cos
35-45 kb
Packaging in vitro
Adsorption +
injection
Transducing particle containing
cosmid recombinant
DNA circularizes
after injection
Ap^R
cos
Selected Ap^R colonies.
Purified 300 recombinants
Obtained 300 individual
cell-extracts
Directly screened for increased
DHPR specific activity

4.2.2 Screening directly for increased DHPR specific activity

The screening for increased DHPR specific activity was carried out in batches of 30 (see Section 4.5.12). The cell-free extracts were individually assayed for DHPR activity (i.e. quinonoid 6-methyl-dihydropterin dependent oxidation of NADH; see Section 2.5.1.1) and their protein concentrations were determined by the method of Bradford (1976). The cell-free extract from the host strain without any plasmids, had a DHPR specific activity of 0.052 U/mg (± 0.05 U/mg). This value was similar to that observed in other *E. coli* strains that were investigated in this study (e.g. see specific activity of cell-free extract from *E. coli* strain H 712 in Table 2.4). Thus the wild-type level of DHPR specific activity is generally about 0.055 U/mg. The cell free extracts from most of the recombinants also showed DHPR specific activity that varied (± 0.02 U/mg) around this value (see Table 4.1), except for WA 265 and WA 266 which were 0.234 U/mg and 0.292 U/mg, respectively. The requirement for quinonoid dihydropterin-dependent oxidation of NADH by cell-free extracts prepared from WA 265 and WA 266, showed the enhanced level of DHPR activity to be real (see Table 4.2).

Although the plasmid replicon in pHC 79 has a copy number of about 50 copies per cell (similar to ColE1) in the recombinant cosmids the copy number during normal growth is often as low as five copies per cell (Hohn and Collins, 1980). This may account for the 5 to 6-fold increase in DHPR specific activity observed in strains WA 265 and WA 266. However, even at this level of increased DHPR specific activity, the extracts from the two strains were distinctly yellow in colour. Since DHPR purified from *E. coli* had been shown to contain tightly bound flavin, the yellow colour of the two extracts was attributed to increased expression of the 27-kD flavoprotein. The two clones were rechecked from the strains stored in DMSO to ensure that they possessed increased DHPR specific activity. They were also checked to see that they were derivatives of the parent strain DM 7111 *rec*A. The parent strain has the genotype F^-, *cys B trp E pur F recA, srl ::Tn* 10. Minimal plates that were supplemented with cysteine, trytophan and

Table 4.1: Direct screening of cosmid clones for increased DHPR activity at 30°C in Tris/HCl at pH 7.4.

S/No. (WA)	S/A U/mg	S/No. (WA)	S/A U/mg	S/No. (WA)	S/A U/mg	S/No. (WA)	S/A u/mg	S/No. (WA)	S/A U/mg
101	0.052	134	0.057	167	0.058	200	0.055	233	0.055
102	0.056	135	0.052	168	0.057	201	0.052	234	0.055
103	0.056	136	0.057	169	0.055	202	0.055	235	0.055
104	0.052	137	0.055	170	0.052	203	0.055	236	0.052
105	0.058	138	0.052	171	0.052	204	0.058	237	0.055
106	0.052	139	0.052	172	0.055	205	0.055	238	0.055
107	0.055	140	0.052	173	0.055	208	0.055	239	0,052
108	0.055	141	0.057	174	0.048	207	0.052	240	0.057
109	0.053	142	0.055	175	0.052	208	0.048	241	0.057
110	0.055	143	0.064	176	0.057	209	0.043	242	0.052
111	0.057	144	0.051	177	0.057	210	0.052	243	0.052
112	0.057	145	0.055	178	0.052	211	0.061	244	0.055
113	0.057	146	0.057	179	0.045	212	0.053	245	0.048
114	0.057	147	0.057	180	0.058	273	0.058	246	0.052
115	0.057	148	0.052	181	0.052	214	0.055	247	0.055
116	0.052	149	0.052	182	0.052	215	0.057	248	0.058
117	0.050	150	0.052	183	0.055	216	0.057	249	0.058
118	0.057	151	0.050	184	0.036	217	0.052	250	0.057
119	0.057	152	0.057	185	0.052	219	0.056	251	0.055
120	0.035	153	0.057	186	0.057	219	0.052	252	0.055
121	0.057	154	0.057	187	0.052	220	0.052	253	0.055
122	0.057	155	0.057	188	0.052	221	0.048	254	0.055
123	0.053	156	0.055	189	0.057	222	0.057	255	0.046
124	0.055	157	0.057	190	0.055	223	0.057	256	0.052
125	0.055	158	0.052	191	0.055	224	0.057	257	0.052
126	0.052	159	0.073	192	0.055	225	0.052	258	0.052
127	0.046	160	0.058	193	0.057	226	0.057	259	0.036
128	0.055	161	0.096	194	0.055	227	0.057	260	0.052
129	0.055	162	0.057	195	0.052	228	0.052	261	0.055
130	0.057	163	0.062	196	0.057	229	0.052	262	0.053
131	0.083	164	0.057	197	0.052	230	0.057	263	0.048
132	0.053	165	0.058	198	0.055	231	0.052	264	0.055
133	0.055	166	0.055	199	0.055	232	0.052	**265**	**0.234**

continued-/

Table 4.1: Direct screening of cosmid clones for increased DHPR activity

S/No. (WA)	S/A U/mg	S/No. (WA)	S/A U/mg	S/No. (WA)	S/A U/mg	S/No. (WA)	S/A u/mg
266	**0.292**	299	0.057	332	0.058	365	0.058
267	0.058	300	0.052	333	0.063	366	0.057
268	0.057	301	0.052	334	0.062	367	0.062
269	0.057	302	0.057	335	0.057	368	0.065
270	0.052	303	0.052	336	0.055	369	0.062
271	0.055	304	0.057	337	0.058	370	0.058
272	0.055	305	0.052	338	0.057	371	0.060
273	0.055	306	0.052	339	0.057	372	0.062
274	0.055	307	0.057	340	0.057	373	0.058
275	0.057	308	0.055	341	0.057	374	0.062
276	0.057	309	0.055	342	0.056	375	0.065
277	0.052	310	0.072	343	0.055	376	0.062
278	0.057	311	0.055	344	0.058	377	0.060
279	0.057	312	0.057	345	0.045	378	0.065
280	0.057	313	0.057	346	0.055	379	0.058
281	0.093	314	0.057	347	0.055	380	0.047
282	0.055	315	0.057	348	0.055	381	0.063
283	0.055	316	0.048	349	0.052	382	0.062
284	0.049	317	0.057	350	0.057	383	0.062
285	0.052	318	0.068	351	0.057	384	0.058
286	0.055	319	0.052	352	0.057	385	0.058
287	0.055	320	0.052	353	0.058	386	0.065
288	0.038	321	0.055	354	0.057	387	0.062
289	0.055	322	0.057	355	0.052	388	0.062
290	0.058	323	0.057	356	0.052	389	0.058
291	0.062	324	0.052	357	0.052	390	0.058
292	0.076	325	0.055	358	0.057	391	0.058
293	0.078	326	0.055	359	0.062	392	0.058
294	0.058	327	0.055	360	0.058	393	0.060
295	0.052	328	0.052	361	0.057	394	0.062
296	0.052	324	0.057	362	0.065	395	0.060
297	0.048	330	0.052	363	0.058	396	0.058
298	0.052	331	0.550	364	0.058	397	0.058

Table 4.2 Requirements of DHPR activity in cell-free extracts from WA 265 and 266 in Tris/HCl (pH 7.4) at 25°C

Omission	WA 265[a]	WA 266[a]
None	5.26	6.98
Cell free extract	0.28	0.30
6-methyl-tetrahydropterin	0.32	0.43
NADH	0.00	0.00
Perioxidase/H_2O_2	0.38	0.45

a . µmoles NADH oxidized per min per ml.

uracil allowed the growth of WA 265, WA 266 and DM 7111 *rec*A. When cysteine or uracil were omitted from the minimal plates, none of the three strains grew. The clones WA 265 and 266 were selected for further study.

4.2.3 Comparison of plasmids pWA 265 and 266

The cellular content of plasmids pWA 265 and pWA 266 from the recombinants WA 265 and WA 266 were amplified (by adding spectinomycin at mid-log phase during growth to inhibit protein synthesis) and the plasmids were isolated by caesium chloride gradient centrifugation. The two plasmids were digested with the restriction endonucleases *Eco* RI, *Bam* HI and *Hind* III, individually, and in combination. The digested DNAs were analysed on a 0.7% (w/v) agarose gel (see Figure 4.5). The suspicion that WA 265 and WA 266 were siblings that may have arisen before the initial recombinants were selected for ampicillin resistance, was confirmed by the restriction digest pattern of their respective plasmids. Although some of the digests had not gone to completion, the sizes of the smaller (< 6.5 kb) fragments in the double and triple digests were identical for the two plasmids. Two unrelated plasmids with the gene of interest in an overlapping region would have been useful for sub-cloning the gene encoding the reductase. In this case, since the two clones were siblings, the strain WA 266 and its plasmid pWA266 were used for all further work..

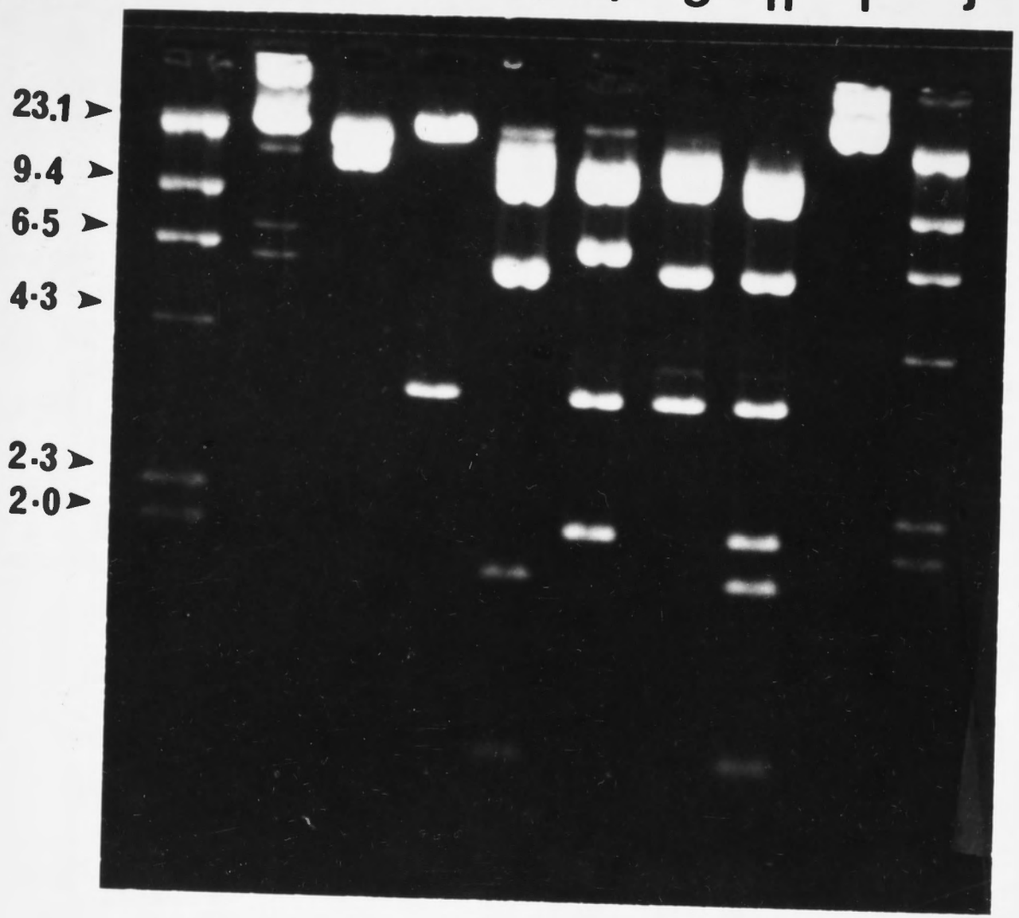
4.2.4 Sub-cloning the gene for *E coli* DHPR

It was argued that the 5 to 6-fold increase in specific activity observed in cell-free extracts prepared from WA 266 might be increased if the size of the insert in plasmid pWA266 (~35-45 kb) were to be reduced so that the pHC79 replicon may function at a higher efficiency. Six-fold increase in the specific activity of the reductase was already sufficient to distinguish the recombinant clone WA 266 from other cosmid containing strains due to the intense yellow colour of the colonies. It was surmised that the bright yellow colour could be used as the selection criterion during sub-cloning. Since the insert

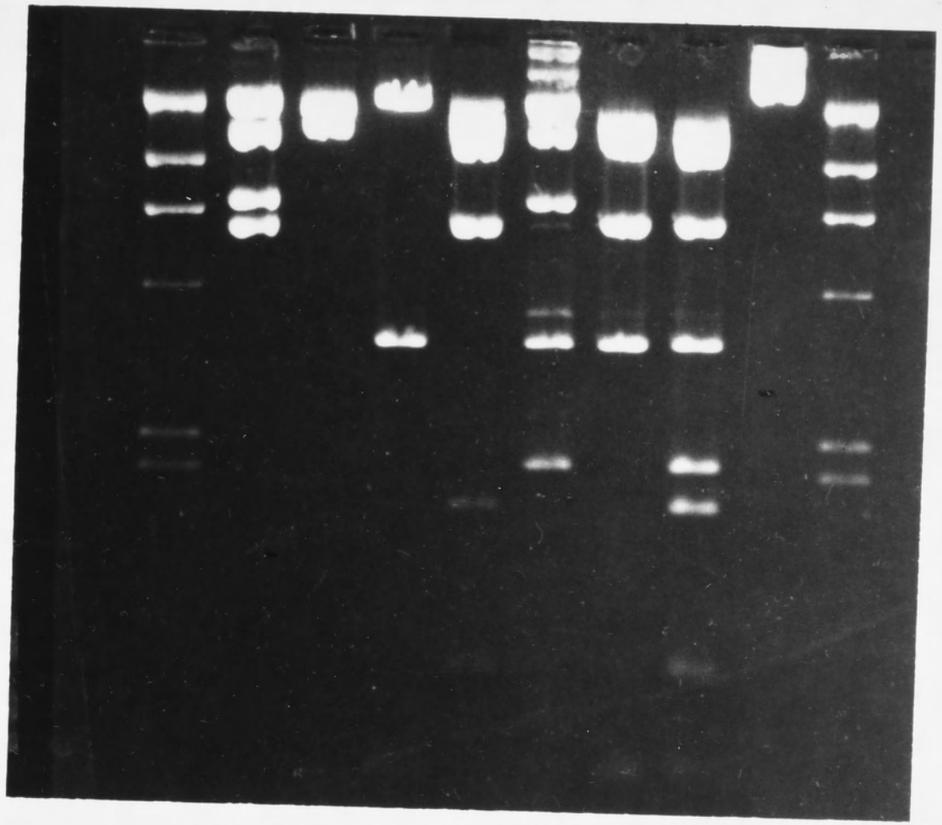
Figure 4.5 Agarose gel electrophoresis of restriction endonuclease cleavage fragments of plasmids pWA 265 (A) and pWA 266 (B).

Samples were run on a 0.7% (w/v) agarose gel as described in Section 4.5.4. Lanes 'a', 'j', 'k' and 't', are the known size markers (λ DNA digested with *Hind* III); 'b' and 'l' were digested *Eco* RI; 'c' and 'm' were digested with *Bam* HI; 'd' and 'n' were digested with *Hind* III; 'e' and 'o' were double digested with *Eco* RI and *Bam* HI; 'f' and 'p' were double digested with *Eco* RI and *Hind* III; 'g' and 'q' were double digested with *Bam* HI and *Hind* III; 'h' and 'r' were triple digested with *Eco* RI, *Bam* HI and *Hind* III; 'i' and 's' were digested with *Xho* I.

A



B



in pWA266 had not been mapped, the sub-cloning strategy was to digest the large plasmid separately with several commonly used restriction enzymes that recognise a six-base pair sequence, and ligate to the resulting fragments with a plasmid vector that had been digested with the same or compatible restriction enzyme. The plasmid vector pUC8 (Vieira and Messing, 1982) was selected because it has several unique cloning sites in its polylinker region. This vector has also the strong *lac* promoter which can be manipulated to induce high level expression of genes products whose coding sequences (DNA) are downstream from it.

The plasmid pWA 266 (2 μg per digest) was separately digested with the restriction endonucleases *Eco* RI, *Bam* HI, *Hind* III and *Sal* I and ligated with pUC8 (0.2 μg per digest) which had been linearized with the respective enzymes and dephosphorylated. The ligation mixtures were used separately to transform DM 7111 *rec*A that had been made competent by $CaCl_2$ treatment (see Section 4.5.16). The transformation mixtures were plated out on LB plates containing ampicillin and incubated at 37°C. The yield of transformants was low (about 20 colonies per transformation). The *Sal* I or *Hind* III digests of pWA 266 ligated with similarly-digested pUC8 did not yield yellow colonies. The transformants resulting from *Bam* HI and *Eco* RI digests of pWA 266 ligated into pUC8 gave some yellow colonies which were of the same intensity as WA 266. The plasmids from the transformants were isolated by a rapid lysis method (see Section 4.5.14) and analysed on a 0.7% (w/v) agarose gel (result not shown). The white colonies from the above transformations contained reclosed pUC8 vectors and the yellow colonies contained very large plasmids. The plasmids from the yellow colonies of the above transformations were not studied any further.

An alternative strategy, which if successful can result in the gene of interest being localised in a small fragment, was carried out. The method was essentially similar to the first step in the construction of the cosmid library, where a frequently-cutting restriction enzyme was used to generate fragments of the required size range. In this instance *Sau* 3A was used in a trial partial digest of plasmid pWA266 to generate fragments ranging

Table 4.3 Direct screening of sub-clones for increased DHPR specific activity in Tris-HCl (pH 7.4) at 25°C.

Strain	Specific activity (U/mg)	Strain	Specific activity (U/mg)
AN 1459 (control)	0.052	**HM 018**	**0.131**
DM 7111 (control)	0.057	HM 023	0.034
HM 002	**0.11**	HM 025	0.057
HM 004	0.057	HM 027	0.057
WA 401	**0.19**	HM 028	0.052
HM 402	**0.225**	HM 029	0.049
HM 012	0.049	WA 405	0.086
WA 403	**0.189**	HM 032	0.042
WA 404	**0.196**	**WA 406**	**0.252**

from 5 to 10 kb. The partial digest was scaled up appropriately to digest 4 μg of plasmid pWA266. One half of the *Sau* 3A partial digest was ligated with pUC8 that had been linearized with *Bam*HI and dephosphorylated (with calf intestinal alkaline phosphatase to prevent reclosure). The second half was reclosed by intramolecular ligation. The two separate ligation mixtures were used to transform *E. coli* strain AN1459 that had been made competent by calcium chloride treatment. (The strain AN 1459 had been noted to have a high transformation efficiency; Dr N. E. Dixon, personal communication). The plasmid pWA266, partially digested with *Sau* 3A and ligated with *Bam* HI digested pUC8, gave several hundred transformants, but none was clearly yellow. The reclosed *Sau* 3A partial digests on the other hand yielded more transformants and about two dozen of them were bright yellow. A total of 36 transformants from the partial digest ligated with pUC8, were streaked on 8 sector plates. Plasmid DNA from these transformants was isolated by rapid lysis and analysed on a 0.7% (w/v) agarose gel (result not shown). More than 50% of the transformants were reclosed vectors, thus indicating that the dephosphorylation of the vector was not complete. The transformants with inserts from this experiment showed no increase in DHPR specific activity when their cell-free extracts were assayed.

The yellow colonies obtained from transformation with the reclosed *Sau* 3A partial digest, together with some that were not intensely coloured, were also analysed following rapid lysis (see Figure 4.6). The yellow colonies in the plate containing the transformants from the reclosed partial *Sau* 3A digest fluoresced (see Figure 4.7) when viewed under short wavelength u.v. irradiation (after the colonies had been picked and streaked onto 8-sector plates). The fluorescence was ascribed to the increased level of flavin prosthetic group due to increased level of the reductase. The direct screening for increased DHPR specific activity of some of the transformants from the reclosed *Sau* 3A partial digest (see Table 4.3) showed clones that had only 2 to 5-fold increase over the levels in thebackground strain (AN 1459). In the face of the earlier argument that the reduction in size of the insert in pWA266 might result in an increase in DHPR specific activity due to

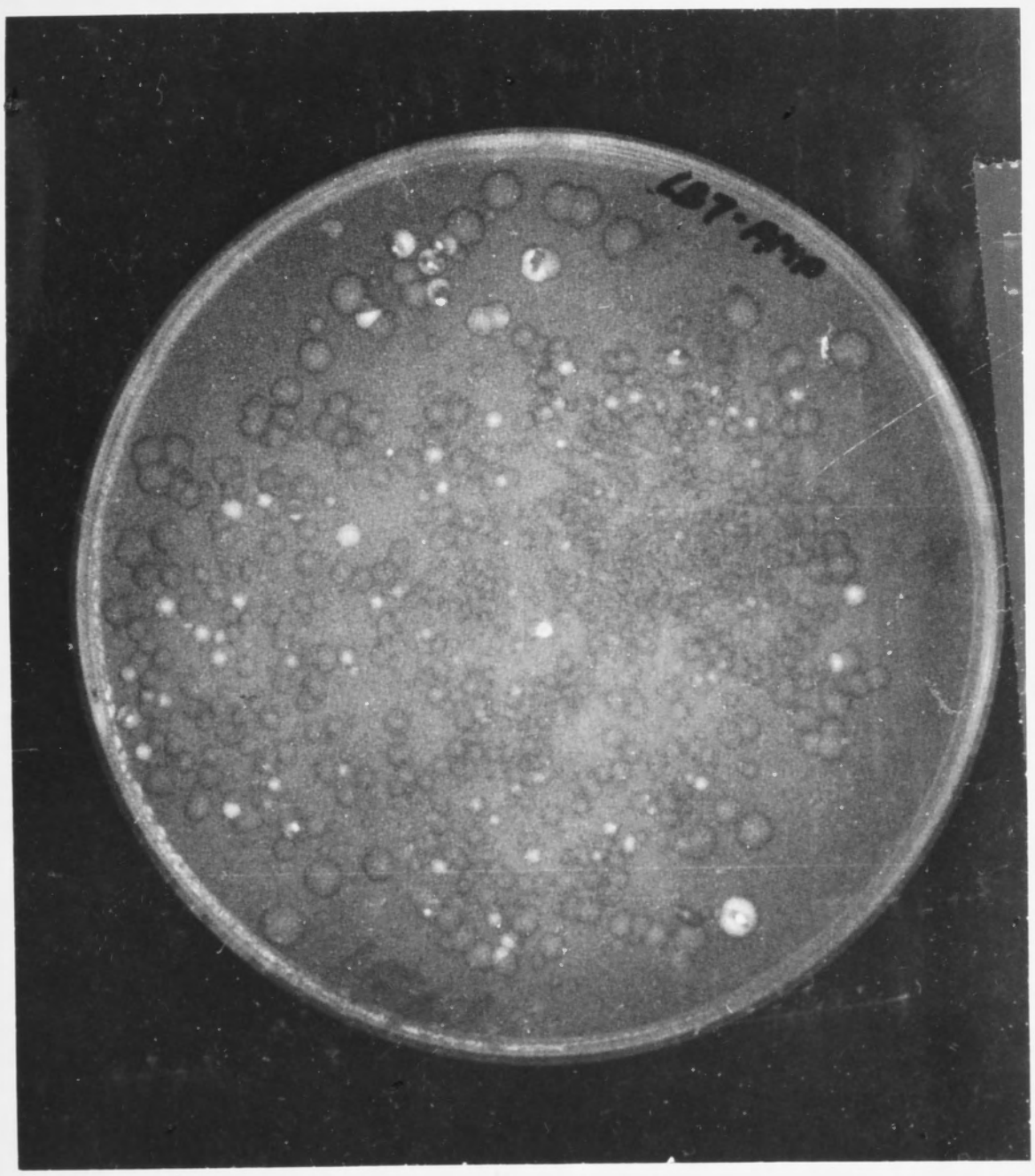
Figure 4.6 Agaraose gel electrophoresis of rapid lysates of 36 transformants from sub-cloning experiment.

The undigested plasmid DNA obtained from 36 transformants (that were purified and subjected to rapid lysis as described in Section 4.5.14), were analysed by agarose gel electrophoresis (0.7% w/v) together with undigested plasmids pUC 8 (2.6 kb), ori C (12.2 kb) and pBR 322 (4.36 kb), as size markers . Lane '33' is plasmid pWA 406 which was selected for further studies.

25
36
pUC 8
ori C
pBR322
13
24
pUC 8
ori C
pBR322
1
12
pUC 8
ori C
pBR322

Figure 4.7 Yellow colonies fluoresce under u.v. light

The yellow colonies which showed 2 to 6-fold increase in DHPR specific activity (in their cell-free extracts) appear as bright spots when viewed under short wave u.v. irradiation.



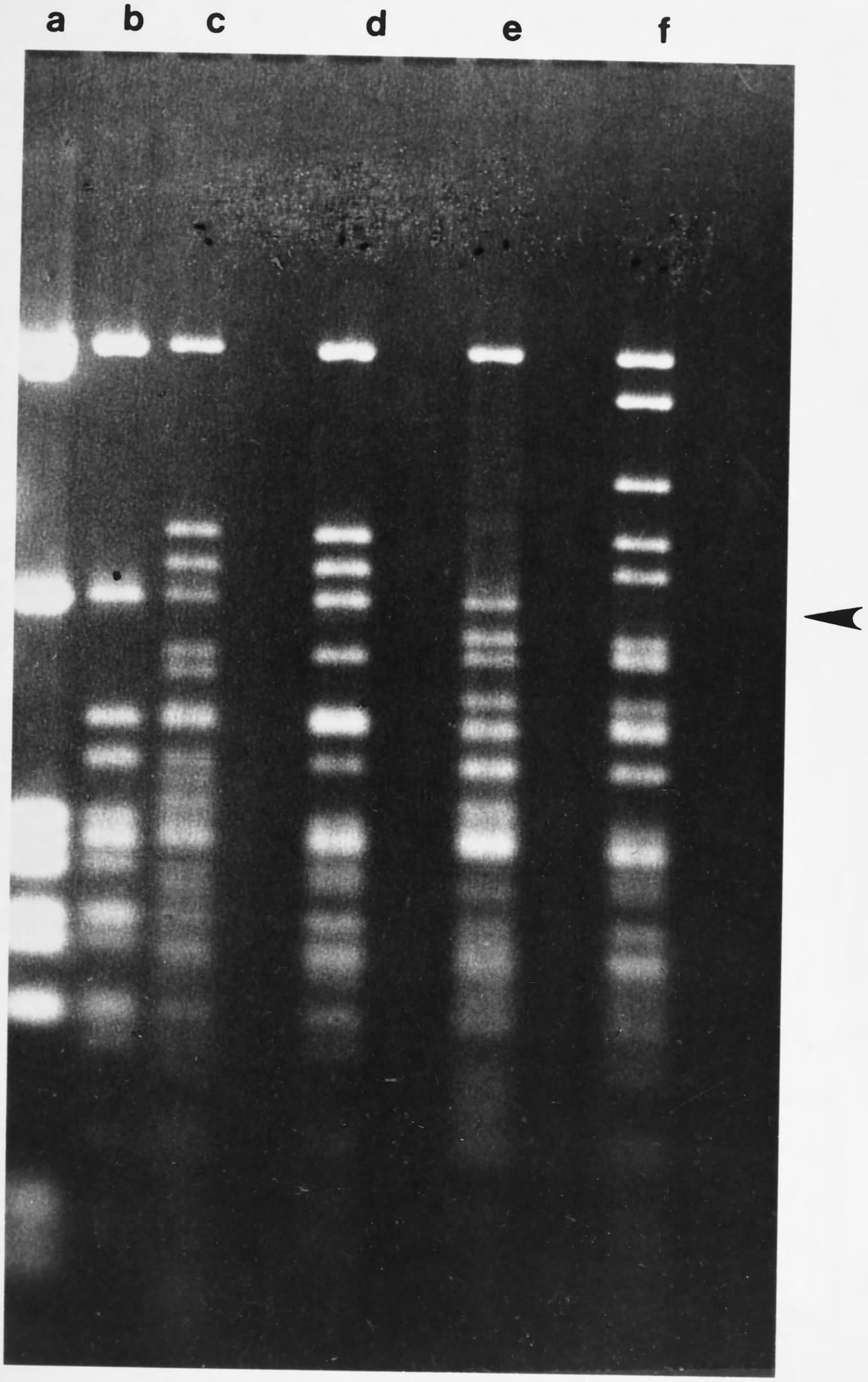
more copies of the plasmid occurring in the recombinant, the above result cannot be easily explained.

Plasmid DNA from three of the sub-clones WA401, WA403 and WA406 were isolated from large-scale preparations. The sizes of the plasmids pWA401, pWA403 and pWA406 from the sub-clones were estimated to be 12.5, 9 and 15 kb respectively, by summation of the sizes of fragments produced by several restriction endonucleases. Complete *Sau* 3A digests of the three plasmids were compared with those of pUC 8, pHC 79 and pBR 322 by electrophoresis on an 1.5% (w/v) agarose gel (see Figure 4.8), to check for deletions within the vector sequence. The result indicated that in plasmid pWA 406, the cosmid vector pHC 79 has lost a 665 bp *Sau* 3A fragment that contained an *Eco* RI site .

4.2.5 Analysis of the gene product

The only indication so far that the gene coding for *E. coli* DHPR had been isolated were the increased specific activity which is quinonoid dihydropterin-dependent (see Table 4.2) and the bright yellow colour of the colonies (ascribed to the flavin content of DHPR). It was possible that the reason for no significant increase in DHPR specific activity despite reduction of the size of the insert from 35 to 45 kb (in pWA266) and 8 to 10 kb (in pWA406), may have been formation of the apoprotein. Perhaps the flavin biosynthesis in the organism may not have kept up with the high levels of DHPR produced in the clones. This hypothesis was investigated firstly by growing WA 266 and WA 406 in LB broth containing ampicillin and supplemented with riboflavin (100 μM). There was no significant increase in the specific activity of the reductase in the extracts prepared from WA 266 and WA 406 grown with riboflavin supplementation. Secondly, when the extracts of the clones were incubated with FAD (10 μM) at 37°C for 0.5 h, neither showed any change in specific activity. These results suggested that apoprotein formation may not be the reason for the lack of increase in DHPR specific activity.

Figure 4.8 Agarose gel electrophoresis of complete *Sau* 3A digests of plasmids pUC 8 (a), pBR 322 (b), pHC 79 (c), pWA 401 (d), pWA 403 (e) and pWA 406 (f) The plasmids were digested completely with *Sau* 3A (37°C, 2h) and electrophoresed on an agarose gel (1.2% w/v). The arrowhead indicates that the 665-bp *Sau* 3A fragment present in the plasmid vectors (a, b and c), was missing from the plasmid pWA 406.


a
b
c
d
e
f

The cell-free extracts of several strains bearing plasmids that direct synthesis of proteins to give increase DHPR specific activity were compared with those of the *E. coli* strain AN1459 (background level of DHPR specific activity) by 12% (w/v) SDS-PAGE analysis. Approximately equal amounts of total protein were applied to each track so that they could be compared after staining and destaining. It was expected that the clones showing 4 to 6-fold increase in specific activity would show a more intensely-staining protein band around 27 kD (molecular weight of wild type DHPR). The result in Figure 4.9 indicated there to be no obvious overproduction of the 27-kD DHPR in the clones, compared with the level in the background strain. There may be three possible explanations for the absence of any observable increase in the DHPR at the expected region on the SDS-PA gel:

a) The levels of over-production of DHPR were not sufficiently high to differentiate visually between extracts from clones and the background strain by SDS-PAGE analysis.

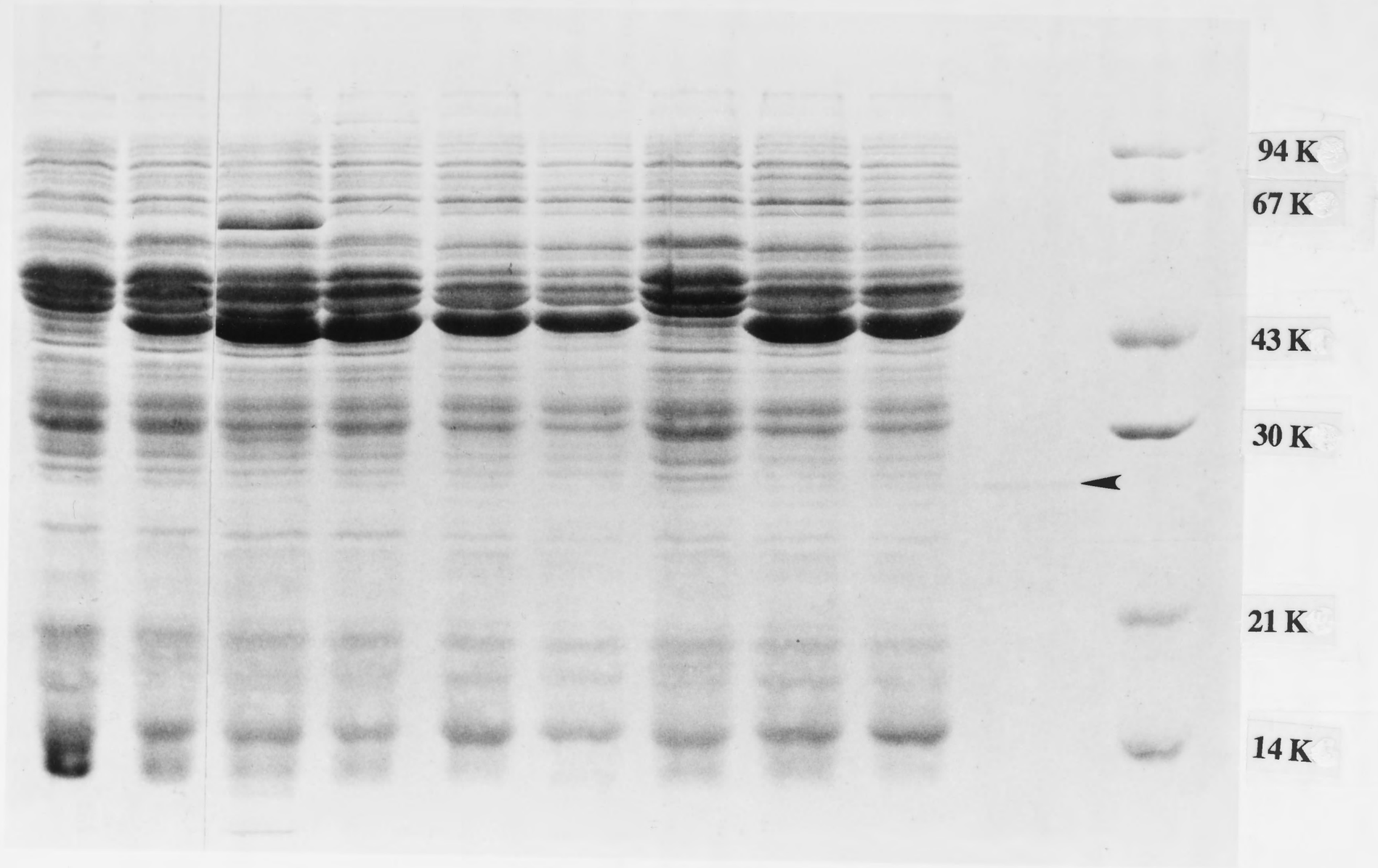
b) There may be a second previously-unidentified protein that can reduce quinonoid 6-methyl-dihydropterin, which is responsible for the increased activity in WA 266 and the others that were derived from it during sub-cloning.

c) The gene for DHPR may have fused with a second gene to produce a partially-active fusion protein of higher molecular weight.

It was at first believed that the first explanation could not be true because the clones and their extracts were bright yellow in colour. If the colour were due to increased levels of the 27 kD flavoprotein identified in this study as *E. coli* DHPR, then the protein should be visible as a more intense band in the SDS-PA gel. The second explanation might have been true except that when the reductase from *E. coli* was being purified, every attempt was made to look for possible isoenzymes in the membrane and soluble fraction to compare with the NADPH specific DHPR isolated from bovine and human livers (Nakanishi *et al.* , 1986a,b). Judging from the SDS-PA gel (see Figure 4.9), all the clones that gave yellow colonies and produced yellow cell-free extracts showed an

Figure 4.9 Polyacrylamide gel electrophoresis of cell-free extracts prepared from several *E. coli* strains.

The cell-free extracts (100 μg each) from AN 1459, the wild type strain (a), WA 266, the primary cosmid clone; and WA 405 (c), WA 406 (d), WA 401 (e), WA 403 (f), HM 023 (g), HM 002(h) and HM 018 (i), which are strains that contain plasmids derived from pWA 266 with insert DNA of various sizes, . The arrowhead indicates the position of purified *E. coli* DHPR (27 kD). Pharmacia low molecular weight markers were also run concurrently. The extracts (b, c, d, e, f, h and i) which had enhanced DHPR specific activities, did not show any sign of overproduction of the 27-kD protein, but they have an intense band aroun 45 kD which is absent in AN 1459.


a
b
c
d
e
f
g
h
i
MW std.
94 K
67 K
43 K
30 K
21 K
14 K

intense band at about 45 kD (compared with the Pharmacia low molecular weight standards). It was more intense than the corresponding band from the extract of the background strain (AN 1459). This observation provoked the hypothesis that a gene fusion may have been formed inadvertently during the construction of the cosmid library with the consequent production of a higher molecular-weight fusion protein. Further support for this possibility came from the fact that the increased reductase activity present in the extracts prepared from WA266 or WA406 was rapidly lost during attempts to purify the protein responsible for the activity (unlike that from the wild type strain discussed in Chapter 2).

4.2.6 Protein synthesis directed by plasmid DNA

Proteins that are synthesized under the direction of plasmids containing the cloned DNA fragments can be detected by *in vitro* transcription and translation (Pratt *et al.*, 1981) or by *in vivo* methods using mini-cells (Dougan and Kehoe, 1984) or maxi-cells (Sancar *et al.*, 1979). In this study the mini-cell expression system was used to investigate the proteins that were synthesized under the direction of the plasmids pWA 266 and pWA 406. Mini-cells are small, spherical and anucleate vesicles that continually bud-off from certain mutant *E.coli* strains. When these strains are transformed with plasmids that can occur as multiple copies, the mini-cells that bud-off from them often contain a copy of the plasmid. Hence the mini-cell producing strain DS 410 (Dougan and Sherrat, 1977) was made competent with calcium chloride and transformed separately with plasmids pWA 266, pWA 406 and the cosmid vector pHC 79. The transformants were cultured separately and mini-cells produced were isolated by sucrose gradient centrifugation. The purified mini-cells containing the plasmids pWA 266, pWA 406 or pHC 79 were allowed to synthesize proteins in the presence of ^{35}S-methionine (to radiolabel the newly-synthesized proteins) and their products were subsequently analysed by SDS-PAGE and fluorography. Both plasmids pWA 266 and pWA 406 contained a gene that resulted in five to six-fold increase in DHPR specific activity in the cell-free extracts of strains trasformed with them [eg. strain WA 266 (DHPR specific activity is

Figure 4.10 Identification of proteins synthesized by plasmid DNA *in vivo* in the presence of ^{35}S-methionine.

(A) SDS-PA gel (12%w/v) of 'a' Pharmacia low molecular weight standards; 'b' human brain DHPR (25 kD); 'c' and 'd' *E. coli* DHPR (27 kD); 'e', 'f' and 'g' are minicells containing plasmids pHC 79, pWA 266 and pWA 406, respectively (the experiment is described in Section 4.5.16).

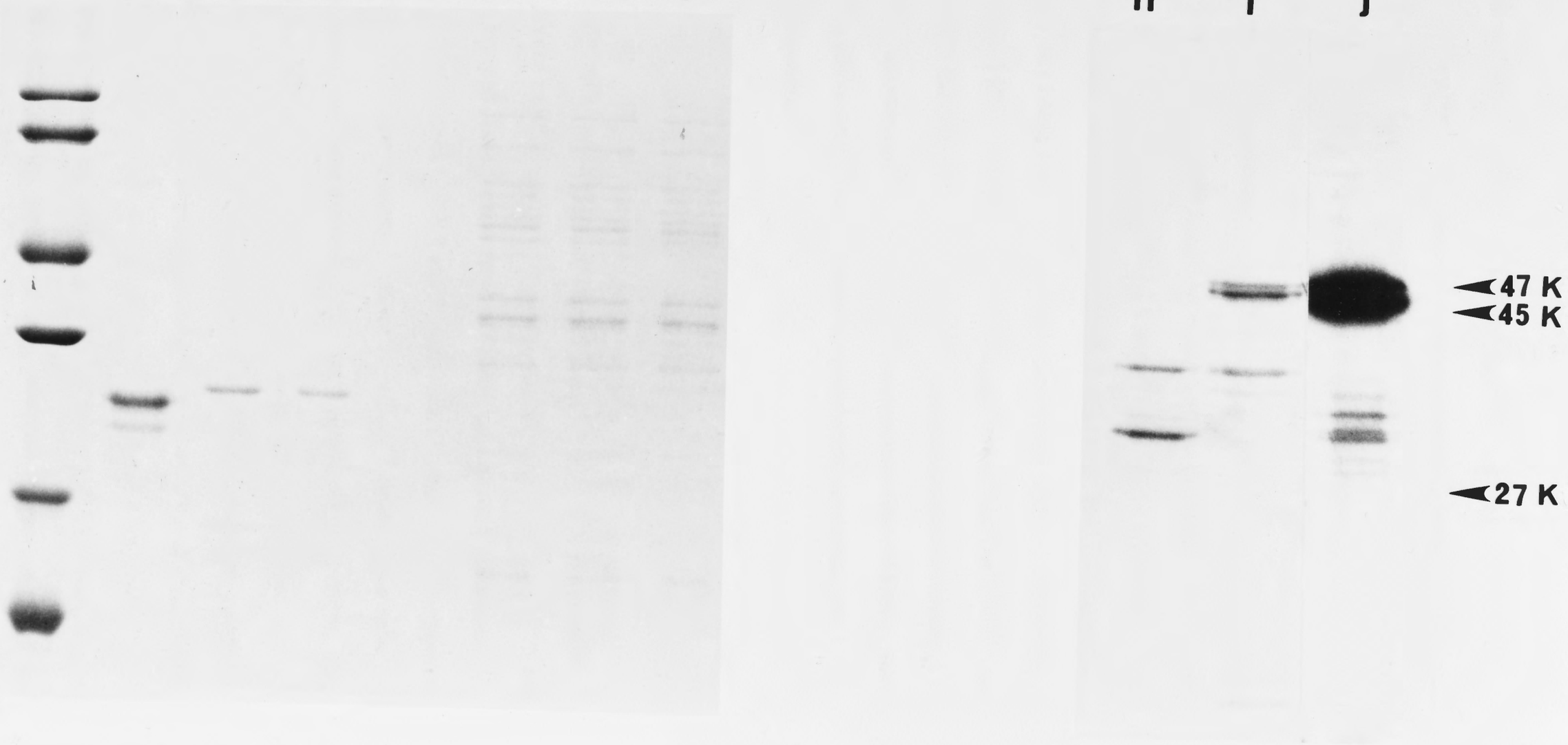
(B) The gel (A) was dried down and the proteins that were synthesized under the direction of the plasmids in the mini cells were visualised by fluorography, as described in Section 4.5.16. 'h' is the same as lane 'e' and shows the proteins that were encoded by pHC 79; 'i' is the same as lane 'f' and shows the proteins encoded by plasmid pWA 266; 'j' is the same as lane 'g' and shows the proteins encoded by plasmid pWA 406. The 27-kD *E. coli* DHPR was not synthesized under the direction of plasmids pWA 266 or pWA406, although both plasmid directed the synthesis of two proteins with molecular weights of about 45 Kd and 47 kD.

A

a b c d e f g

B

h i j



0.292 U/mg) which is DM 7111 *rec* A (DHPR specific activity is 0.052 U/mg) transformed with plasmid pWA 266]. However, the result (in Figure 4.10) indicated clearly that the 27-kD *E. coli* DHPR was not being synthesized from the plasmids pWA 266 and pWA 406 in the minicells. When the vector-encoded proteins (mini-cell + pHC 79) were taken into account, the proteins at about 45 kD and 47 kD were the major products expressed from the inserts in plasmids pWA 266 and pWA 406. It was felt that either the 45 kD or the 47 kD protein might have been responsible for the increased DHPR specific activity because the SDS-PAGE analysis of cell-free extracts prepared from several strains, including WA 266 and WA 406 (Figure 4.9), also indicated that a protein of about 45 kD was being over-produced (in comparison with extracts prepared from wild type strain AN 1459). It was argued that the protein band on the SDS-PA gel that was approximately 45 Kd (Figure 4.9) might contain both the proteins (45 Kd and 47 Kd, see Figure 4.10) that are encoded by the cloned DNA fragments (35 to 45 kb in pWA 266 and about 10 kb in pWA 406). The over-expressed proteins were considered to be worthy of further investigation.

4.3 SUMMARY

A cosmid library was constructed by cloning 35 to 45-kb DNA fragments generated by partial digestion with *Sau* 3A into the *Bam* H1 site of the cosmid vector pHC79. Three hundred transfectants were selected and screened directly for increase in DHPR specific activity. Two clones WA265 and WA266 had DHPR specific activity of 0.232 U/mg and 0.292 U/mg, respectively. These were 5 to 6-fold higher than the specific activity of the reductase in the extracts of the wild-type background strain. The plasmids pWA 265 and pWA 266 were identical when analysed by restriction endonuclease digestion. The plasmid pWA 266 was sub-cloned by *Sau* 3A partial digest followed by intramolecular religation to give plasmid pWA 406 which had a 9 to 10-kb insert in the vector pHC 79 which was in turn missing a 632-bp *Sau* 3A fragment. SDS-PAGE analysis of the extracts from strains bearing pWA 266 and pWA 406 indicated that a 45-kD protein was expressed in high levels. *In vivo* synthesis of proteins under the

direction of pWA 266 and pWA 406 produced two proteins that were 47 kD and 45 kD. The 27-kD *E. coli* DHPR was not synthesized by the plasmids. It was thought possible that one of the proteins (47 kD or 45 kD) might have been a fusion product of *E. coli* DHPR and a product of another gene (see however Chapter 5).

4.4 MATERIALS

4.4.1 Enzymes, antibiotics and compounds

The restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from Boehringer-Mannheim, GmbH, W. Germany. Tetracycline was from Sigma Chemical Co., St. Louis, Mo. U.S.A. Spectinomycin hydrochloride (Trobicin) was obtained from Upjohn Co., Kalamazoo, U.S.A. and ampicillin was from Beecham Research Laboratories, Victoria, Australia. L-[^{35}S]-methionine, α–[^{32}P]-deoxyadenosine triphosphate and γ–[^{32}P]-adenosine triphosphate were obtained from from Amersham Australia Pty. Ltd.

4.4.2 Media used for growth of bacteria

LB media and plates were prepared as described in Section 2.5.4. Antibiotics were filter sterilised and used at the following final concentrations: 15 µg/ml tetracycline, and 50 µg/ml ampicillin. The minimal medium 56 was essentially as described by Monod *et al.* (1951) and contained (per liter):

10.6g Potassium hydrogen orthophosphate
6.1g Sodium dihydrogen orthophosphate
0.2g Magnesium sulphate
2.0g Ammonium sulphate

The medium was prepared as a five-times concentrated stock with chloroform added to 0.05% (v/v).

4.5 METHODS

4.5.1 Preparation of chromosomal DNA

E. coli strain D3-157 cells (Singer *et al.*, 1985) from a 500-ml culture (LB + 50 µg/ml thymine, 0.2% (w/v) glucose, 100 µg/ml streptomycin sulfate) were harvested (at OD_{600} ~1.0) by centrifugation at 6000 rpm for 10 min at 4°C (in a Sorvall RC-2B Centrifuge with a GS3 rotor). The pellet was rinsed with 20 ml of 50 mM Tris-Cl pH 7.4 (containing 20% (w/v) sucrose, 1 mM EDTA and 2 mM DTT), and suspended in 10 ml of the same buffer. Note that the cell suspension could be stored at -70°C without serious damage to them. Lysozyme (2.5 ml of a 10 mg/ml solution in the above buffer) was added with swirling on ice for 5 min followed by 2.5 ml of 0.5 M EDTA pH 8.5. After a further 5 min, 15 ml of Triton X-100 solution (0.1% (v/v) Triton X-100, 62.5 mM EDTA, 50 mM Tris/Cl pH 8.0) was added. The mixture was kept for 15 min on ice. The lysate (36 ml) was added to 34.2 g of CsCl and 3.6 ml of ethidium bromide (10 mg/ml). The mixture was gently shaken under subdued light to dissolve the CsCl and transferred equally to two polyallomer ultracentrifuge tubes (Beckman). The tubes were filled to the top with TE (10 mM Tris/Cl, pH 7.8 and 1 mM EDTA) containing 0.95 g/ml CsCl sealed, and centrifuged at 39 k rpm for 40 h at 15°C in a Beckman L8-70 Ultracentrifuge in a vertical Ti-50 rotor. The chromosome band was briefly visualized with a long-range u.v. light source and drawn from the tube with a pasteur pipette after the top of the tube was carefully cut with a sterile scalpel. The DNA was transferred into a second polyallomer ultracentrifuge tube with 0.5 ml of ethidium bromide solution and

TE containing 0.95 g/ml CsCl. The tubes were sealed and centrifuged at 35 k rpm for 36 h at 15°C in a fixed-angle 50 Ti rotor in a Beckman L8-70 Ultracentrifuge. The chromosome band was visualized and isolated as above. The ethidium bromide was removed after six extractions with NaCl-saturated isopropanol (50:50 mixture of isopropanol and 5 M NaCl in TE). The colourless aqueous phase was dialysed overnight against TE (2 x I L) at 4°C. The concentration of DNA (stored at 4°C) was 100 µg/ml (on the assumption that a 50 µg/ml DNA solution will give an absorbance of 1.0 at 260 nm).

4.5.2 *Sau*3A partial digests of bacterial DNA

A trial partial digest was carried out as follows: 135 µl of stock DNA (10 µg) and 15 µl of 10 x *Sau*3A buffer (final concentration 7.5 mM Tris/Cl pH 7.4, 15 mM $MgCl_2$, 60 mM NaCl and 6 mM 2-mercaptoethanol) were mixed in a 1.5 ml Eppendorf tube. This was portioned out into 9 tubes (that were labelled Tubes 1-9). The mixture (30 µl) was transferred into tube 1 and 15 µl into each of the remaining 8 tubes. Four units of *Sau*3A were added to tube 1 and mixed well. Thus, in tube 1, 2 units of enzyme was used to digest 1 µg of DNA. By working rapidly, 15 µl were transferred from tube 1 to tube 2 and mixed well. This two-fold serial dilution was continued to tube 9. The tubes were transferred from the ice-bath to a 37°C water bath and incubated for 20 min. The reaction was stopped by adding 0.5 M EDTA (pH 8.0) to a final concentration of 20 mM. To each tube was added 5 µl of gel loading buffer (see Section 4.5.4) and the samples (20 µl) were loaded in order into the wells of a 0.4% (w/v) agarose gel. Bacteriophage λ DNA digested with *Xho*I and undigested chromosomal DNA were included as markers.

Scale up of the partial digest was carried out by using the optimum conditions determined in the trial. The scale up (ten-fold) was performed under exactly the same conditions as the above trial experiment keeping volume, temperature and time of digest constant. The large fragments (35-45 kb) required for the cosmid cloning experiment were further purified by NaCl gradient centrifugation. After the partial digest was

stopped, 0.5 ml of the mixture was carefully layered onto a SW41 polypropylene centrifuge tube (Beckman), containing 5-25% NaCl in TE (10 ml; the gradient was formed using a gradient mixer). The tubes were mounted into detachable buckets of the SW41 swing-out rotor and centrifuged at 35K rpm for 3 h at 12°C in a Beckman model L8-70 ultracentrifuge. After centrifugation the polypropylene tubes were punctured at the bottom with a sterile 18-gauge needle (with care being taken not to introduce any bubbles), 0.5-ml aliquots were withdrawn and placed in 1.5 ml Eppendorf tubes. Small aliquots of these fractions were analysed by agarose gel electrophoresis (0.4% w/v). The fractions of interest were dialysed *vs*. TE, DNA was recovered by ethanol precipitation.

4.5.3 Restriction endonuclease digestion of DNA

DNA (0.5-10 µg) was digested, with the appropriate restriction endonuclease, in the buffers prescribed by the manufacturers, or in the general buffers: "low salt buffer" (ie. 10 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT); "medium salt buffer" (as in "low salt buffer" + 50 mM NaCl) and "high salt buffer" (50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 100 mM NaCl). The buffers were made up as 10-fold concentrates, sterilised by filtration and stored at -20°C. The digestions were normally set up in a volume of 20 µl where the DNA solution, in a sterile Eppendorf tube was made up to 18 µl with sterile H_20 and 2 µl of the appropriate 10 x buffer was added. The restriction enzyme (1 µl of a stock ranging in activity from 5-10 U/µl) was added last; reactions were mixed well and incubated at 37°C for 2-4 h. If the digested DNA was to be analysed by agarose gel electrophoresis, 6 µl of gel-loading dye (see Section 4) was added and the mixture was loaded into a gel slot. The digestion reaction was stopped by addition of 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM or by heating the mixture at 70°C for 5 min.

4.5.4 Agarose gel electrophoresis

Agarose gels were cast in horizontal gel tanks (180 cm long and 140 cm wide). Wells (3-6 mm deep and 5 mm wide) formed with combs immersed in the melted agarose were used for loading samples. Agarose gels, which were generally 0.7% (w/v), were made by melting 1.4 g agarose in 200 ml (+ 10 ml to allow for reduction in volume during melting) TBE (Tris-borate buffer contains 0.089 M Tris-borate, 0.089 M boric acid and 2 mM EDTA) in a microwave oven. Upon cooling to ~50^{o}C, ethidium bromide solution (final concentration of 0.5 µg/µl) was added to the clear agarose. The agarose was then cast in the horizontal gel tank with comb in place to form wells. The tank was filled with TBE containing 0.5 µg/ml ethidium bromide until the buffer covered the gel by 1-2 mm (to prevent dehydration). The loading buffer for agarose gels was 0.25% bromophenol blue in 40% (w/v) sucrose dissolved in water (6 x concentrate). The gels were normally run at 50 volts until the dye reached the anode region. The gels were photographed (Polaroid camera; Polaroid Type 57 film) using a transilluminator to transmit u.v. light through the gel.

4.5.5 Dephosphorylation of DNA

The 5' terminal phosphate of DNA was removed using calf intestinal alkaline phosphatase (CIP). The DNA in a minimum volume of TE (3-6 µl) was mixed with 5 µl of 10 x CIP buffer (0.5 M Tris-Cl, pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$ and 10 mM spermidine) and made up to 48 µl with sterile water. CIP (2 µl, 5-10 U/µl) was added and the mixture incubated at 37^{o}C for 30 min. A second aliquot of CIP was added and incubation was continued for a further 30 min before the reaction was stopped by addition of 5 µl of 10% (w/v) SDS and heating at 70^{o}C for 15 min. The DNA was purified by phenol extraction (see Section 4.5.6) and precipitated with ethanol (see Section 4.5.7).

4.5.6 Phenol extraction of DNA

Plasmid DNA isolated by rapid lysis (see Section 4.5.14) was further purified by phenol/chloroform extraction as described in Maniatis *et al.* (1982). The aqueous DNA sample was mixed with an equal volume of phenol (equilibrated with 0.1M Tris-Cl pH 8.0 containing 0.1% (w/v) hydroxyquinoline and 0.2% (w/v) β-mercaptoethanol) and centrifuged in an Eppendorf Microfuge for 3 min. The aqueous layer was removed with a Pipetman tip (discarding the interface and lower organic phase) and placed in a fresh Eppendorf tube. An equal volume of a 1:1 mixture of phenol (as above) and chloroform (24.1 (v/v) mixture of chloroform and isoamyl alcohol) was added and mixed by vortexing before centrifugation in a Microfuge for 3 min. The aqueous layer was removed as above and mixed with an equal volume of chloroform (as above) to remove traces of phenol from the previous steps. The aqueous layer was separated by centrifugation in a Microfuge for 3 min and the DNA was precipitated with ethanol as described in the following Section).

4.5.7 Ethanol precipitation of DNA

DNA was precipitated from aqueous solutions (100-300 µl) by adding 3 M sodium acetate (1/10th of the volume of aqueous solution) and absolute ethanol (2 times the volume of aqueous solution) in 1.5 ml Eppendorf tubes. The contents were mixed well and stored in ice or at -70°C for 30 min (if nanogram quantities were being precipitated the tubes were left overnight) and then centrifuged in a Microfuge for 5 min. The supernatant was discarded and 1 ml of 70% (v/v) ethanol (stored at -20°C) was added to the tube without resuspending the pellet. After two or three careful inversions, the tubes were centrifuged for 5 min in a Microfuge. The supernatant was aspirated off and the pellets were dried in vacuum desiccator for ~10 min. The pellet was resuspended in the appropriate solution or stored at -20°C until required.

4.5.8 Ligation of DNA

Ligations were generally done in a total volume of 20 µl. The compositions of blunt-end ligation buffer (10 x concentrate) and the sticky-end ligation buffer (10 x concentrate) are given below. The fragments of DNA to be ligated were placed in an Eppendorf tube and made up to 18 µl with sterile H_2O. The appropriate "ligation buffer" (2 µl of 10 x concentrate) was mixed with the DNA solution, 1 µl of T4 DNA ligase (1 U/µl) was added and the reaction was allowed to proceed for 12-20 h at about 14°C.

	10 x Blunt end buffer	10 x Sticky end buffer
Tris-Cl pH 7.4	250 mM	500 mM
$MgCl_2$	50 mM	100 mM
DTT	50 mM	100 mM
Spermidine	2.5 mM	10 mM
ATP	10 mM	10 mM
Hexamine cobalt chloride	10 mM	-
BSA	0.1 mg/ml	1 mg/ml

Buffer concentrates were sterilised by filtration and stored in 50 µl aliquots at -70°C.

4.5.9 Packaging *in vitro* of concatemers into bacteriophage λ particles

The ligation of large *Sau*3A fragments (generated by partial digestion) with *Bam*HI-digested cosmid vector pHC79 (dephosphorylated) results in high molecular weight concatameric DNA. The phage particles for the packaging were obtained as freeze-thaw lysates and sonicated extracts (gifts from Dr Barbara van Leeuwen, JCSMR). The freeze-thaw lysate was prepared from an induced lysogen with an amber mutation in gene E that prevents formation of the phage head and the sonicated extract was prepared from an induced lysogen with an amber mutation in gene D that results in accumulation of phage in the pre-head stage. When the two extracts are mixed with the concatemers,

genetic complementation occurs, and the DNA is packaged into λ particles (Hohn and Murray, 1977).

The freeze-thaw lysate (10 µl) was carefully placed by the side of a 1.5-ml Eppendorf tube that contained 5 µl of ligated mixture at the bottom. The sonicated extract (15 µl) was dropped directly onto the ligated mixture and, using the same Pipetman tip, the freeze-thaw lysate was mixed in without introducing bubbles. Note that bubbles tend to reduce drastically the packaging efficiency. The mixture was set aside at room temperature for 1 h, and 500 µl of SM buffer (which contained per liter: 50 ml of 1M Tris-Cl (pH 7.5), 5.8g NaCl, 2g $MgSO_4$ and 5ml of 2% gelatin) was added to it prior to storage at 4°C.

4.5.10 Transfection of packaged extract

The strain to be transfected was grown in LB medium (30 ml) supplemented with maltose (10 mM) at 37°C, overnight. The cells were harvested by centrifugation (6000 rpm, 10 min) and suspended in 5 ml of LB medium containing 10 mM $MgSO_4$. The packaged extract (100 µl) was diluted with 100 µl of SM buffer in a 1.5-ml Eppendorf tube. The cell suspension (200 µl) was mixed with the diluted packaged extract and incubated at 37°C. After 20 min, 1 ml of LB medium was added and the incubation was continued for a further hour to allow for expression of drug resistance. The transfected cells were pelleted by centrifugation in a Microfuge and suspended in 100 µl of LB. Various amounts of this were spread on LB plates supplemented with ampicillin and incubated at 37°C.

4.5.11 Construction of the *rec*A derivative of *E. coli* strain D3-157

The Pl lysate used in the above experiment was a kind gift from Dr C. M. Elvin and was made by growing bacteriophage P1 on *E. coli* strain NK 5304 (*srl-1300* :: Tn*10* *rec*A56).

Strain D3-157 (25 ml cultures in LB broth) was grown to an OD_{595} ~0.6 and transferred (5 ml each) to 4 screw-capped tubes. They were centrifuged at 3200 rpm for 10 min in a bench-top centrifuge and the supernatants were discarded. The following additions were made to the 4 tubes:

	Tube 1	Tube 2	Tube 3	Tube 4
LB broth	0.5 ml	0.5 ml	0.5 ml	0.5 ml
$CaCl_2$(50 mM)/$MgCl_2$(30 mM)	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Pl lysate	0.5 ml	0.1 ml	0.02 ml	-
LB broth	-	0.4 ml	0.48 ml	0.5 ml

The tubes were incubated at 37°C for 20 min, then the cells were pelleted by centrifugation at 3200 rpm for 10 min in a bench-top centrifuge. The cell pellets were suspended in minimal salts medium (diluted two-fold) and pelleted by centrifugation (the wash step was repeated). The pellets were suspended in 50 µl of LB broth, spread on LB plates containing 15 µg/ml tetracycline, and incubated at 37°C overnight. There were no colonies from Tube 4. Transductants from Tube 3 were screened for the presence of the *rec*A allele by checking for sensitivity to u.v. irradiation. This was carried out by streaking the colonies on LB plates containing tetracycline and exposing the plate for 30 sec to short wavelength u.v. source followed by overnight incubation at 37°C. The *rec*A strains were sensitive to the u.v. irradiation, whereas the *rec*+ strain (D3-157) used as a control survived the 30 s exposure.

4.5.12 Direct screening for increased DHPR specific activity

The recombinants that were being screened for increased DHPR specific activity were grown overnight in 30 ml of LB medium supplemented with ampicillin. The cells were pelleted by centrifugation in SS-34 tubes using the SS-34 rotor in a Sorvall RC-5C centrifuge at 6000 rpm for 10 min at 4°C. The cells were suspended in 50 mM Tris-Cl, pH 7.4, containing 1 mM EDTA and 200 mM KCl (0.8 ml) and transferred into 1.5-ml capacity TLA-100 (Beckman) tubes. Chicken egg-white lysozyme (200 µl, 20 mg/ml in

the above buffer) was added and mixed in well with a 1-ml Pipetman tip. The suspension was kept at 0°C for 1 hr and then frozen in liquid N_2. This was allowed to thaw at 4°C and centrifuged at 75 000 rpm with a TLA-100 rotor in a Beckman model T-100 benchtop ultracentrifuge at 4°C for 30 min. The cell-free extract (0.75 ml) was removed and stored in labelled glass vials. Aliquots were taken to assay for DHPR activity (Section 2.5.1.1) and protein concentration was determined as described in Section 2.5.2. By this method, thirty recombinants were screened at a time.

4.5.13 Preparation of plasmid DNA

The preparation of amplifiable plasmid was essentially as described by Silhavy *et al.* (1984). An overnight culture (5 ml) of the plasmid-bearing strain in LB broth containing 50 µg/ml ampicillin was used to inoculate 1 L of minimal medium supplemented with 0.5% (w/v) casamino acids, 40 mM glucose; other growth requirements and ampicillin (or appropriate antibiotic). Growth at 37°C with aeration in a gyratory environmental shaker was monitored by measuring OD_{590}. When the OD_{590} was ~0.7, spectinomycin hydrochloride (0.3 g/L) was added to inhibit protein synthesis. Cultures were grown for a further 12-16 h for plasmid amplification. The cells were pelleted by centrifugation (as in Section 4.5.1) and resuspended in 20 ml of 25 mM Tris-Cl pH 8.0 containing 50 mM glucose and 10 mM EDTA (at 4°C). Lysozyme (50 mg) was added whilst swirling and the mixture left on ice for 5 min. NaOH-SDS solution (40 ml, 0.2 M NaOH and 1% SDS) was added, mixed in well by inverting the capped tube until the cell suspension was clear and left on ice for 5 min. Potassium acetate solution pH 4.8 (30 ml; 3 M potassium acetate and 3 M glacial acetic acid) was added to the tube with mixing. A white precipitate was formed while standing on ice for 15 min. The supernatant (75 ml) was collected after centrifugation at 10 000 rpm for 30 min in a Sorvall RC-5C centrifuge with a GSA rotor and the DNA was precipitated by adding isopropanol (45 ml). The mixture was frozen in a dry ice-ethanol bath, thawed and the DNA pellet was separated by centrifugation at 10000 rpm for 30 min. The pellet was rinsed with cold 70% (v/v) ethanol and dried in a desiccator attached to a water pump for

10 min. The pellet was suspended in TE containing CsCl and ethidium bromide (8 ml TE + 8 g CsCl + 1 ml of 10 mg/ml ethidium bromide) and transferred into a polyallomer tube (Beckman) to fit the fixed angle Ti-50 rotor and centrifuged at 39 000 rpm for 36 h at 15°C in a Beckman L8-70 Ultracentrifuge. The supercoiled plasmid band (well separated from the band of relaxed DNA) was visualised under long-range u.v. light and withdrawn from the tube with a sterile disposable 5-ml syringe attached to an 18-gauge needle. The ethidium bromide was completely removed after three extractions with aqueous NaCl-saturated isopropanol and the aqueous layer was dialysed overnight (4°C) against TE (2 x 1 L). The DNA yield by this method was usually about 500 µg/L of culture

4.5.14 Rapid small scale isolation of plasmid DNA

Strains containing plasmids were streaked on 8-sectored LB plates containing ampicillin (50 µg/ml) and incubated overnight (at 37°C). A loopful of cells was scraped from a sector and transferred to a 1.5-ml Eppendorf tube containing 1 ml of LB broth. The cells were pelleted by centrifugation in an Eppendorf Microfuge for 5 min. The supernatant was discarded and the tubes were stored on ice. The cell pellet was suspended in 200 µl of lysozyme solution (25 mM Tris-Cl pH 8.0, 50 mM glucose, 10 mM EDTA and 5 mg/ml lysozyme) using a Pipetman tip and incubated on ice for 5 min. NaOH-SDS stock solution (0.3 ml, 0.2 M NaOH and 1% SDS) was added to the tube and mixed in well by inverting the capped tube several times. The clear cell suspension was chilled in ice for 5 min. Potassium acetate pH 4.8 (0.3 ml; 3 M potassium acetate and 3 M glacial acetic acid) was added to the tube. The contents were mixed gently by inversion and frozen in dry-ice for ~5 min. This was then thawed to room temperature and centrifuged for 10 min in a Microfuge. The supernatant (0.75 ml) was carefully removed (making sure not to transfer any of the white precipitate) and transferred into a fresh 1.5-ml Eppendorf tube. Isopropanol (0.45 ml) was added to the supernatant and mixed well. After 20 min at room temperature, the precipitated DNA was pelleted by centrifugation in a Microfuge for 10 min. The supernatant was discarded and 1 ml of 70% (v/v) ethanol was added carefully without resuspending the pellet. The tubes were

centrifuged for a further 10 min in a Microfuge and the supernatant was aspirated off using a drawn-out Pasteur pipette. The pellet was dried in a vacuum desiccator (~10 min) until it became translucent, then suspended in 50 µl of TE containing 20 µg/ml RNaseA using a Pipetman tip. After incubated at 37°C for 15 min and then at 70°C for 15 min, the plasmid DNA was stored frozen at -20°C.

A large number of transformants can be analysed for insertions by the above method. The size of the plasmid DNA from the above rapid lysates was analysed by mixing 5 µl of the RNaseA-treated solution with 10 µl of TE and 5 µl of loading buffer and subjecting them to agarose gel (0.7-1.0% (w/v)) electrophoresis (see Section 4.5.4). The plasmid DNA prepared as described above could also be used for transformation of competent cells. For restriction enzyme analysis, the plasmid DNA samples were further purified by phenol extraction (see Section 4.5.6).

4.5.15 Transformation of competent cells with plasmid DNA

The transformation procedure was carried out essentially as described by Lederberg and Cohen (1974). Cells were grown overnight in 10 ml of LB medium. A sub-culture was grown by adding 200 µl of overnight culture to 20 ml of LB medium, followed by growth at 37°C to an OD_{595} ~0.7. The medium containing the cells was chilled on ice then centrifuged at 6000 rpm for 10 min at 4°C. The cell pellet was suspended in an equal volume of cold 0.1 M $MgSO_4$. The cells were pelleted by centrifugation, suspended in 10 ml of cold 0.1 M $CaCl_2$ and kept on ice for 30 min. This was followed by centrifugation to pellet the cells, which were suspended in 1 ml of cold 0.1 M $CaCl_2$ and left standing on ice for 40 min. These cells were competent for transformation. The plasmid DNA or ligation mixture (made up to 200 µl with TE) to be used for the transformation was chilled on ice and 200 µl of competent cells were added. The mixture was allowed to stand on ice for 30 min and then heat shocked for 2 min at 42°C. LB medium (1 ml) was added to the mixture, which was then incubated for 1-2 h to allow time for expression of drug resistance. The cells were pelleted by centrifugation

in a Microfuge for 3 min and suspended in 100 µl of LB medium. Aliquots were spread on selective medium and incubated for 1-2 days at 37°C.

4.5.16 Mini-cell isolation and *in vivo* protein synthesis

The strain DS410 (Genotype : *minA*, *minB*, *thi*, *lac*; Dougan and Sherrat, 1977)was transformed with plasmids pWA 266, pWA 406 or vector pHC 79 and were grown separately overnight at 37°C in a 1:1 mixture of LB:56 media (500 ml total) containing 0.4% glucose and ampicillin (50 µg/ml). The cells were chilled on ice and harvested by centrifugation at 8000 rpm for 15 min at 4°C with a GS-3 rotor in a Sorvall RC-5C centrifuge. The pellets containing the whole cells and the mini-cells were suspended in 10 ml of RM medium (described later) and shaken vigorously for 2 h at 4°C. The cell suspension (5 ml per gradient) was layered on a sucrose gradient (Dougan and Kehoe, 1984) which was formed by freezing 30% w/v sucrose (in RM medium) in clear SS 34 Centrifuge tubes (40 ml per tube) and allowing it to thaw slowly at 4°C. Tubes were centrifuged at 6000 rpm at 4°C for 20 min using a HB-4 rotor (swing-out) in a Sorvall RC-5C centrifuge. The mini-cells which formed an opaque band around the middle of the tube were well separated from the whole cells which pelleted at the bottom of the tube. The mini-cell band (~5 mls) was collected with a Pasteur pipette and pelleted by centrifugation at 13 000 rpm at 4°C for 10 min. The mini-cell pellet was resuspended in RM buffer and the sucrose gradient centrifugation procedure was repeated. The pellet was suspended in 3 ml of RM buffer and kept on ice. This preparation was checked for contamination with parent cells by viewing through a phase-contrast microscope (magnified 400x). There was about 1 parent cell per 10 fields viewed at that magnification (Note: 1 cell per field viewed at 400 x magnification indicates a concentration of ~1 x 10^6 cells/ml which is about 10 times more than is acceptable for *in vivo* expression studies). The mini-cells were pelleted by centrifugation, suspended in RM buffer containing 30% glycerol to an OD_{595} of 2 and stored as 200 µl aliquots at -70°C (after instant freezing in liquid N_2) until required.

For *in vivo* protein synthesis, 20 µl of the stored mini-cells were collected by centrifugation in 1.5-ml Eppendorf tubes and then resuspended in 20 µl of RM medium containing vitamins, nucleotides and 19 amino acids (omitting methionine). After preincubation for 1 h at 37°C, 25 µCi of ^{35}S-methionine (1,390 Ci/mmol) was added and the mixture was incubated for a further hour, followed by a 15 min chase with unlabelled methionine (100 µM). The mini-cells were pelleted by centrifugation and suspended in 30 µl of "SDS cracking buffer" (300µl of 50% aqueous glycerol, 100µl of 0.4% aqueous bromophenol blue, 200µl of 0.5 M DTT, 400µl 0f 2 M Tris-base, 30µl of aqueous 10% SDS and 200µl of water), heated at 100°C for 3 min and electrophoresed on a 12% SDS-PA gel. The gel was stained with Coomassie Blue and destained (see Section 2.5.3) to visualise the Pharmacia low molecular weight standard markers and the purified *E. coli* DHPR.

In order to detect the ^{35}S-methionine labelled proteins, the gel was soaked in water to remove residual acid from the destaining step and then impregnated with 0.8 M sodium salicylate (Chamberlain, 1979) by soaking for 40 min. The gel was washed briefly with distilled water before it was dried onto Whatman 3 MM paper at 60°C using a BioRad #483 slab gel drier, and exposed to Kodak XAR-5 film for 2-4 days.

RM salts (10-fold concentration): 10g NH_4Cl + 15 g KCl dissolved in 100 ml water
57.1 g Na_2HPO_4 + 24.9 g KH_2PO_4 were added
Brought to 1 liter with water and autoclaved.

RM-medium: RM salts (10x) 5 ml

containing: Glucose solution (20%; 1 ml), water (43 ml), 1M $MgSO_4$ (0.5 ml), (0.5ml), 0.1M $CaCl_2$ (0.05 ml), 0.01M $FeCl_3$ (0.01 ml), D-Cycloserine (10mg/ml; 0.1 ml), and all other amino acids (ommitting methonine at 50µg/ml each. .Vitamins: thiamine (B1) and pantothenic acid each at 0.001% w/v. Nucleotides (ATP, GTP, CTP and TTP) each at 5mM.

CHAPTER 5

IDENTIFICATION AND CHARACTERIZATION OF THE TWO MAJOR PROTEINS SYNTHESIZED UNDER THE DIRECTION OF PLASMIDS pWA266 AND pWA406

5.1 INTRODUCTION

The *in vivo* expression of proteins under the direction of the plasmids pWA266 and pWA406 clearly showed that two proteins with relative molecular weights of 47 000 and 45 000 were synthesized in fairly large amounts. The 27 kD *E.coli* DHPR was not apparently being synthesized under the direction of the plasmids. Since much effort had been invested in obtaining the primary cosmid clone (Chapter 4), it was decided that the six-fold increase in DHPR specific activity in the cell-free extracts prepared from strains WA266 and WA406 required further investigation. Several scenarios were envisaged for the increased DHPR activity (discussed in Chapter 4). It was reasoned that the most probable of these was that a gene fusion may have occurred inadvertently during the construction of the cosmid library to give rise to a higher molecular weight protein. It was suspected that fusion between the 5' region of the gene that encodes DHPR and the 3' part of the *tet* gene of the cosmid vector pHC79 may have taken place. The reason for this was that the large DNA fragments generated by *Sau* 3A partial digest for the construction of the cosmid library were inserted into the *Bam* HI site which occurs within the *tet* gene of the vector. The loss of the termination codon of the gene that codes for DHPR (during the partial digestion with *Sau* 3A to obtain large DNA fragments for cosmid library construction) could then have resulted in the formation of an extended reading frame. If this were the case, then part of the DHPR gene would be on one end of the plasmid pWA266. It was reasoned that if the occurrence of the fusion could be established, then the complete gene encoding DHPR could be readily isolated from a new

cloning experiment by using a radiolabelled fragment from the cosmid clone as a hybridization probe.

This chapter describes attempts to purify the protein responsible for the increased DHPR activity in the extracts prepared from the recombinant strains WA266 or WA406. A purification method for *E.coli* DHPR, which is a 27-kD flavoprotein, has been established in this study (see Chapter 2). However the rapid loss of reductase activity during the early steps in the attempted isolation of the protein responsible for the increased enzyme activity did not permit purification of the protein to homogeneity. Partial purification was achieved by using steps similar to those summarised in Table 2.3. Two proteins with relative molecular weights of about 45 000 and 47 000 that co-purified through the three purification steps were studied further because one them was suspected to be responsible for the increased DHRP specific activity in the strain WA266. Since the *N*-terminal amino acid sequence of DHPR from *E.coli* has been determined, it was felt that sequencing the *N*-termini of the two proteins would be one way to confirm the fusion protein hypothesis. The amino termini of the two proteins were determined by microsequencing using the method of Vanderkerckhove *et al.*, (1985). The 45-kD protein, which in all probability is the source for the increased DHPR activity proved to be a natural haemoprotein in *E. coli* whose *N*-terminal amino acid sequence was significantly homologous with haemoglobin from the filamentous myxobacterium *Vitreoscilla* (Wakabayashi *et al.*, 1986). The 47-kD protein on the other hand was serine hydroxymethyltransferase.

5.2 RESULTS AND DISCUSSION

5.2.1 Partial purification and identification of 45-kD and 47-kD polypeptides

Procedures similar to those used for the purification of *E. coli* DHPR described in Chapter 2 were followed to purify the suspected fusion protein from the cell-free extract prepared from WA 406. After some initial work it became clear that the protein

responsible for the increased DHPR activity was behaving very differently from the 27-kD *E. coli* DHPR. The reductase activity in the extracts prepared from WA 266 was similar to that from WA 406. Since the decrease in the size of the insert from 35 to 45 kb in pWA 266 to about 10 kb in pWA 406 did not increase the level of DHPR in the strain WA 406, there was no apparent advantage in purifying the fusion protein from it. Also it was argued that purification of the suspected fusion protein from WA 266 would directly establish that fusion occurred during the cosmid cloning; however further evidence would be required to confirm this if the the protein was isolated from WA 406. In the latter case it could be argued that a fusion may have resulted during sub-cloning and not during the original cosmid library construction. Therefore it was decided that the protein responsible for the increased DHPR activity had to be purified from extracts made from WA 266.

The increased DHPR specific activity present in the cell-free extract prepared from the strain WA 266 was stable in 50 mM Hepes-KOH (pH 7.4) containing 200 mM KCl and 1mM EDTA at 4°C for 1-2 days. The activity was also stable to storage for several months at -20°C. Repeated thawing of the extracts to 4°C and freezing to -20°C did not affect the reductase activity. However, when the extract was fractionated with ammonium sulfate, the 35-45% saturation fraction which normally separated more than 80% of the total DHPR activity in strains H-712 and D3-157 (compare with Table 2.4), contained less than 15% of the total activity that was present in the initial extract. The supernatant, after the extract was brought to 45% saturation with ammonium sulfate had about 15% of the activity contained in the original extract. There was almost no DHPR activity in the 0-35% ammonium sulfate saturation fraction. This shows that the increased DHPR specific activity was lost during the ammonium sulfate fractionation. In the light of the fact that ammonium sulfate fractionation is normally employed as the first step in isolation of proteins because of its stabilization effects and ability to give some degree of purification when starting from a cell free extract, the loss of DHPR activity could not be readily explained. Moreover the reductase isolated and characterised as DHPR in this study has been shown to be stable to ammonium sulfate precipitation during the purification. Thus this phenomenon was investigated further.

The extracts from strains WA 266 and D3-157 were saturated to 0.4 M ammonium sulfate and incubated at 25^0C. At this level of saturation there was no protein precipitation. The extract from WA 266 lost DHPR activity rapidly with time. More than 80% of the activity present in the original extract was lost in three hours. During the same period, the extract from WA 266 incubated under identical conditions without ammonium sulfate, lost about 40% of reductase activity. The (lower level of) DHPR activity in D3-157 was stable when in ammonium sulfate (10% of saturation) and compared under the same conditions as for WA 266 (see Figure 5.1). Dialysis of the extract fromWA 266 containing ammonium sulfate against 50mM Hepes-KOH did not restore the reductase activity. Also, dialysis of the starting extract against 50mM Hepes-KOH (pH 7.4) resulted in the loss of more than 80% of DHPR activity . Unsuccessful attempts were also made to reactivate the reductase by adding freshly-prepared cell-free extract from strains D3-157 or WA 266 to investigate if unknown factor(s) were being removed during the fractionation. The reason for the loss of DHPR activity is not yet clear, but the anomalous behaviour was taken as further support that a fusion protein *may* have been formed.

At this stage of the work it was decided that it is more important to isolate the two major proteins (45-kD and 47kD) that were synthesized under the direction of the plasmid DNA, even if it is at the expense of the reductase activity, and to characterise them by *N*-terminal amino acid sequencing. The ammonium sulphate fractionation of extract from WA 266 was carried out rapidly (about 1 h) so that some idea of the distribution of the DHPR activity into different fraction could be ascertained. Working quickly was an important criterion because the reductase activity seemed to be lost in a time dependent manner after the addition of ammonium sulfate (see Figure 5.1). About 65% of the total reductase activity that was present in the starting extract appeared in the 45-60% ammonium sulfate saturation fraction and only about 20% of the activity remained in the 35-45% saturation fraction. DHPR activity in the 45-60% saturation fraction was very unstable and all the enzyme activity was lost within 3-5 h while standing in ice. On the

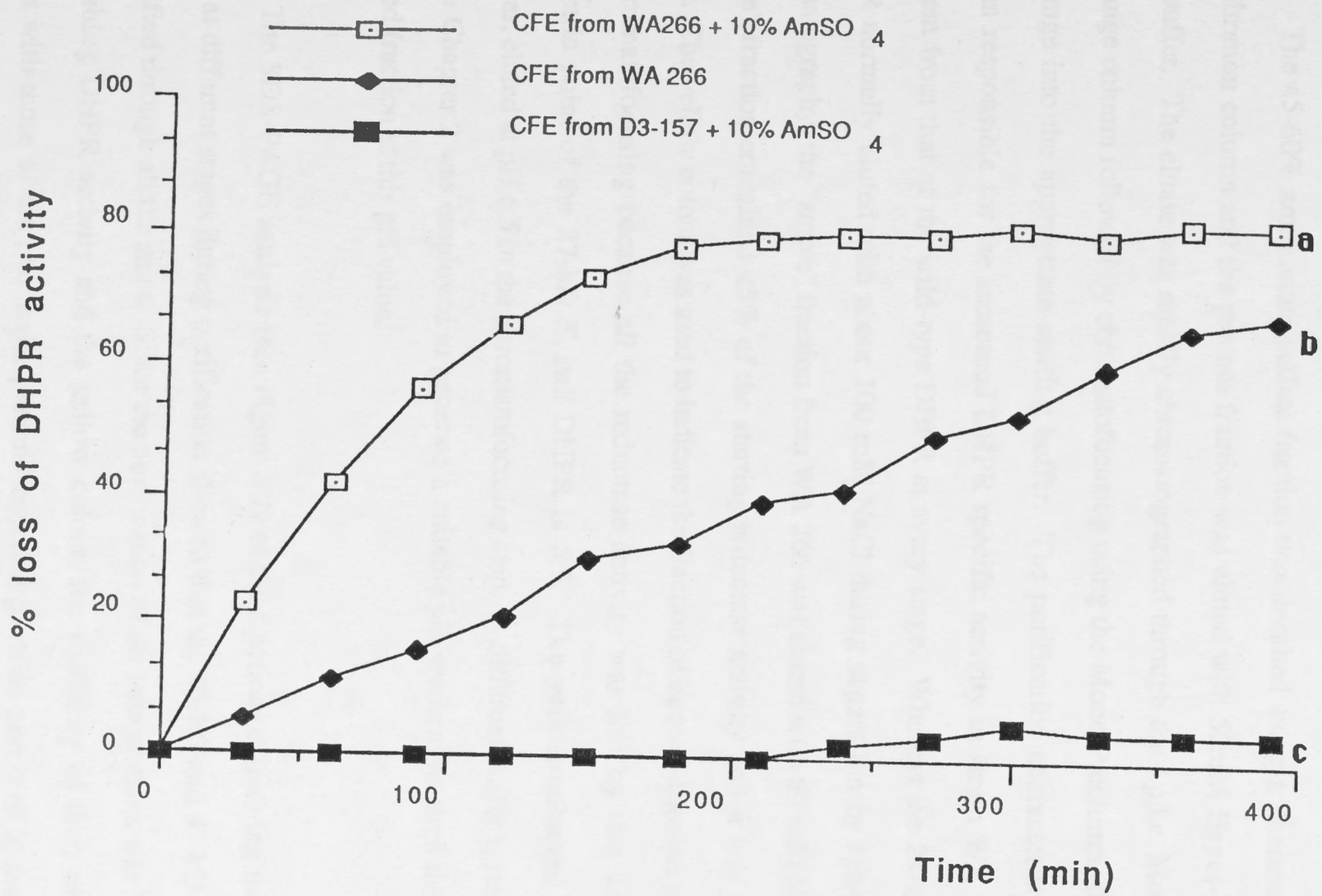
Figure 5.1 Effect of ammonium sulfate on DHPR activity in cell-free extract prepared from strain WA 266 at 25°C

The DHPR activity of samples were measured as described in Section 2.5.1.1.

(a) cell-free extract from WA 266 + 0.4 M saturated ammonium sulfate was incubated at 25°C, and aliquots were removed for enzyme activity assay at 30 min intervals.

(b) as in (a), except that no ammonium sulfate was added.

(c) cell-free extract from strain D3-157 + 0.4 M saturated ammonium sulfate was carried out as described for (a).


CFE from WA266 + 10% AmSO 4
CFE from WA 266
CFE from D3-157 + 10% AmSO 4
% loss of DHPR activity
100
80
60
40
20
0
0
100
200
300
400
Time (min)
a
b
c

other hand the reductase activity in the 35-45% saturation fraction was stable. This indicates that the product of the cloned gene which has DHPR activity appears in the 45-60% ammonium sulfate saturation fraction and the wild type DHPR (27-kD) is contained in the 35-45% saturation fraction as established in Chapter 2.

The 45-60% ammonium sulfate fraction was desalted using a Pharmacia PD-10 gel filtration column and the protein fraction was eluted with 50mM Hepes-KOH (pH 7.4) buffer. The eluate was rapidly chromatographed through the f.p.l.c. Mono-Q ion-exchange column followed by chromatofocusing using the Mono-P column, after buffer exchange into the appropriate starting buffer. The purification characteristics of the protein responsible for the increased DHPR specific activity in strain WA 266 were different from that of the wild-type DHPR at every stage. Whereas the 27-kD *E. coli* DHPR normally eluted with about 100 mM NaCl during separation by ion-exchange chromatography, the "active" fraction from WA 266 was eluted with 60 mM NaCl. The "active" fraction contained <5% of the starting reductase activity and it was yellow in colour. The yellow colour was used to indicate the fraction of interest after the separation by chromatofocusing because all the reductase activity was lost by this time. The isoelectric point of the 27-kD *E. coli* DHPR is 5.3. The yellow-coloured fraction, however, eluted at pH 6.5 in the chromatofocusing step. A different buffer system to that used in Chapter 2 was employed to generate a suitable pH gradient to elute the yellow-coloured fraction at this pH value.

The SDS-PAGE analysis (see Figure 5.2) of the fractions containing the yellow colour at different stages during purification showed that the 45-kD and 47-kD proteins co-purified through all the steps. Since the purification of the two proteins was based on diminishing DHPR activity and the yellow colour, the similarity of their molecular weights with those of the two major plasmid-encoded proteins observed in the *in vivo* transcription and translation system (see Chapter 4, Figure 4.10), was taken as support for the notion that one of them may be the suspected fusion protein. The partially-purified proteins were separated on a 12% (w/v) PA gel and electroblotted onto a polybrene

Figure 5.2 Polyacrylamide gel electrophoresis of proteins.

(a) Pharmacia low molecular weight standards; (b) purified *E. coli* DHPR (27 kD); (c) cell free extract from WA 266; (d) 0 - 45% ammonium sulfate saturation fraction of extract from WA 266; (e) 45 - 60% ammonium sulfate fraction of the extract from WA 266; (f) and (g) show fractions after Mono-Q anion exchange chromatography and (h)is the sample after Mono-P chromatofocusing.

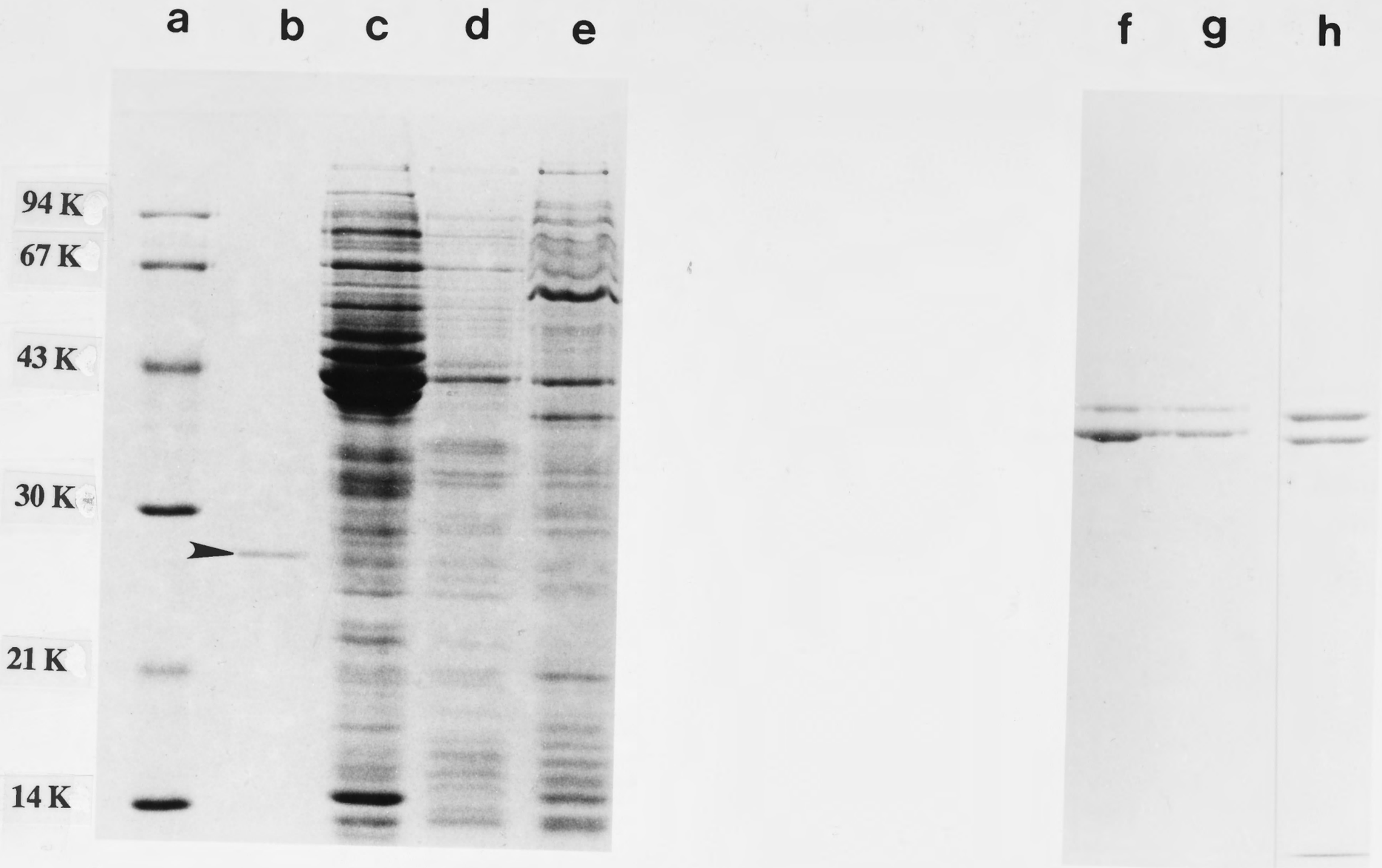
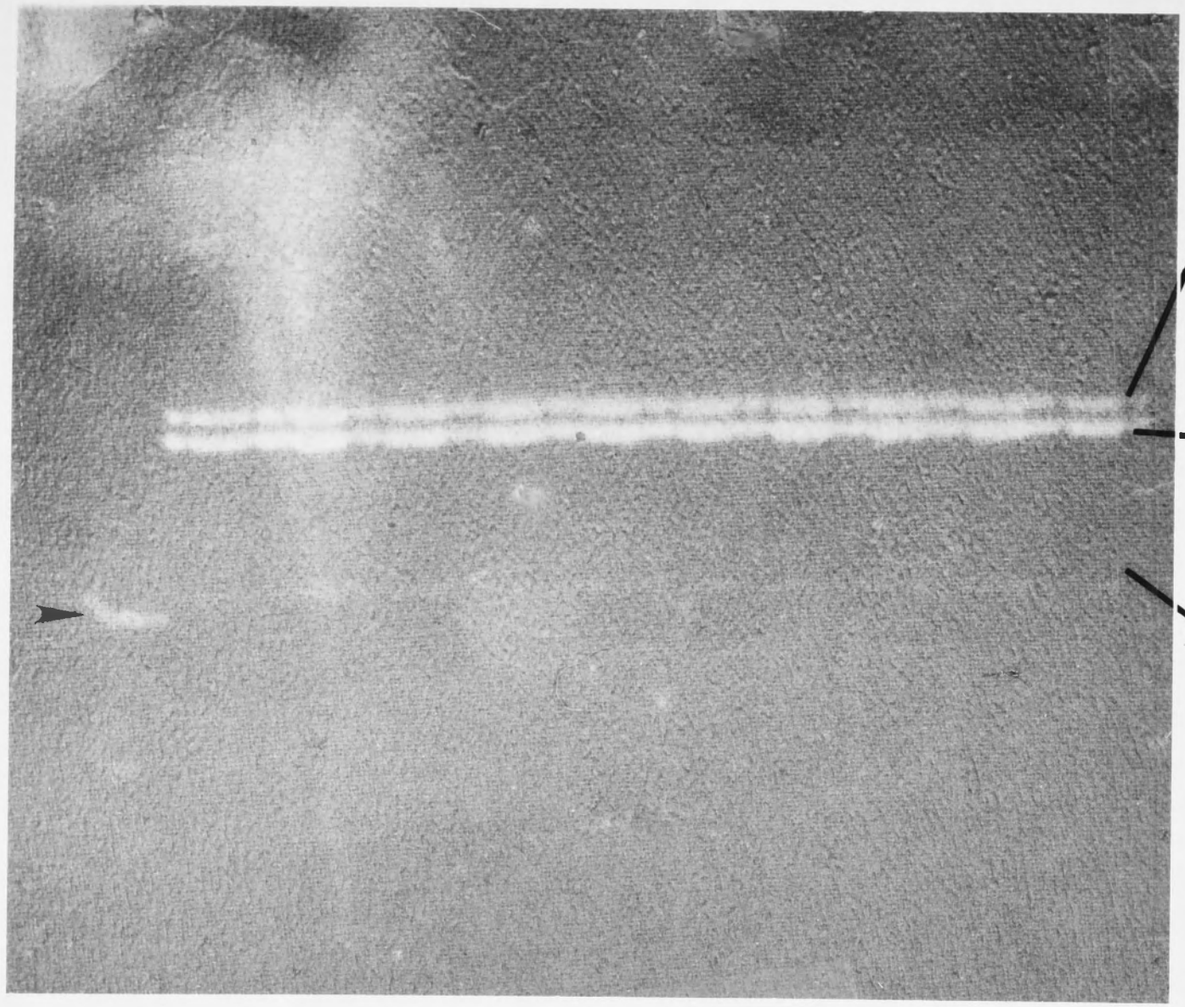

a
b
c
d
e
f
g
h
94 K
67 K
43 K
30 K
21 K
14 K

Figure 5.3 Polybrene glass blot of partially purified proteins from strain WA 266, stained with fluorescamine.

The sequence of the 47 Kd and 45 Kd proteins were obtained by microsequencing in an Applied Biosystem 477A Protein Sequencer. The arrowhead indicates the position of *E. coli* DHPR (27Kd). The *N*-terminal amino acid residues of the 47 Kd and 45 Kd proteins are presented and are different from that obtained for the 27 Kd *E.coli* DHPR (compare with Table 3.3)



47 K protein
M L K C E M N I A D Y D A E L E Q

45 K protein
M L D A Q T I A T V K A T I P L L K E T

30 K protein
No sequence obtained

(quarternized ammonium polybase)-coated glass fibre sheet . The protein bands were visualised under u.v. light after staining with fluorescamine (see Figure 5.3), where the intensity of the fluorescamine stain is dependent on the number of reactive primary amino groups available on the immobilized protein. Traces of an ~30-kD protein that was not observed in the Coomassie-stained protein bands in Figure 5.2 were seen with the fluorescamine stain.

The 45-kD protein (bands from four tracks were carefully excised with a scalpel and mounted into the reaction chamber of the Applied Biosystems 477A sequenator) was subjected to microsequencing. The *N*-terminal sequence of the first 20 amino acid was readily obtained with an average amino acid repetitive yield of 90%. The initial yield of about 45 pmol (the lower limit for sequencing with this machine is about 10 pmol) of protein enabled confident identification of the amino acid residues. The *N*-terminal sequence was not similar to that of the 27-kD reductase. The *N*-terminal 17 residues of the 47-kD protein, obtained by microsequencing, also showed no similarity with the sequence of the 27-kD DHPR. The 30-kD band did not give any sequence data. The earlier suspicion that a fusion protein may have been formed and that one of the two major proteins products of the plasmid DNA may contain the *N*-terminal region of *E.coli* DHPR is *not* true. If a fusion protein had been formed (by the fusion of the gene that codes for the 27-kD DHPR and the *tet* gene) then its *N*-terminal amino acid would have been the same as that of the 27-kD *E.coli* DHPR (see Figure 5.3).

Two other approaches that were concurrently undertaken with the protein sequencing approach to hasten the isolation of the complete DHPR gene were also unsuccessful. One of them involved obtaining about 1 kb of DNA sequence data in the region where the gene fusion was suspected to have occurred (see the restriction map of plasmid pWA 406 in Figure 5.6). There was no open reading frame which corresponded to that of the gene encoding the 27-kD DHPR as predicted from its *N*-terminal amino acid sequence (data not shown). The second approach was involved *insitu* hybridization of more than 1000 recombinants (from the comid library prepared in this study) replicated

onto nitrocellulose membranes, with the radiolabelled SalI-Bam HI fragment (*Sal* I partial digest to isolate the 2.6-kb fragment followed by a *Bam* HI digest) indicated on the restriction map of pWA 406 (see Figure 5.6). This method was performed essentially as decribed in Maniatis *et al.* (1982). It was felt that if part of the gene that codes for DHPR were within the region of DNA used as a probe, then some of the colonies with which it would hybridize should contain the complete gene for DHPR. Sixty colonies that hybridized with the probe were grown from the master plates and directly screened for increased DHPR activity but without any success (data not shown).

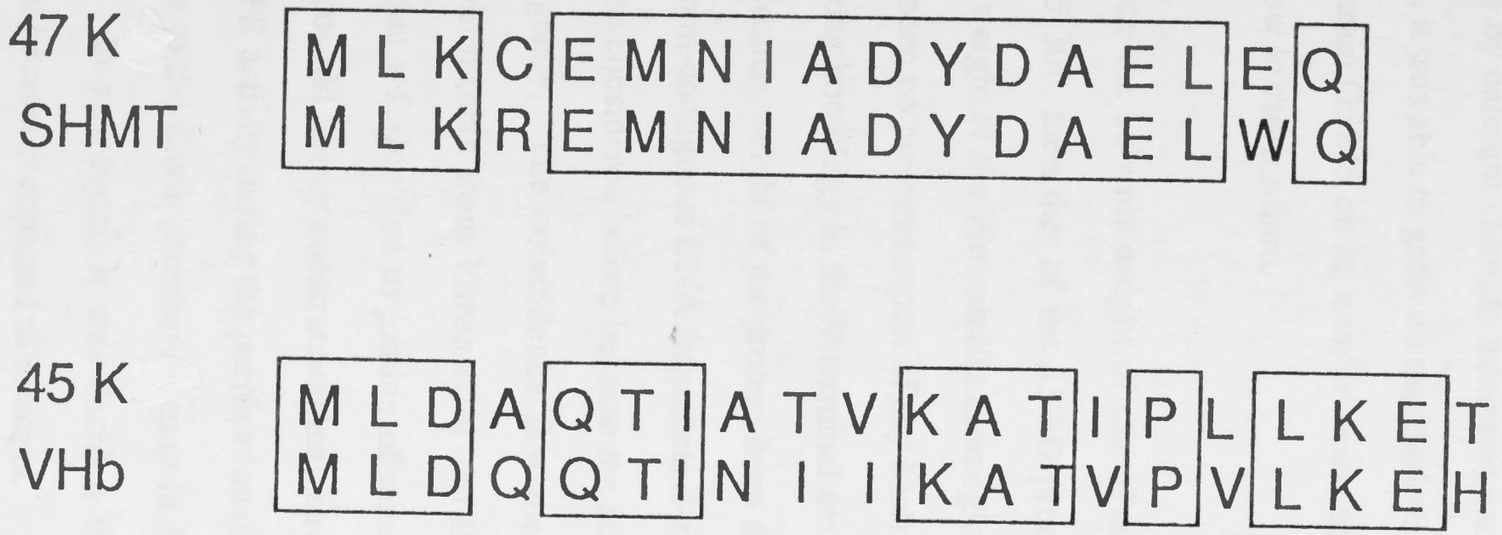
The *N*-teminal sequences of the 45-kD and 47-kD proteins were compared with those of other protein sequences stored in the National Biomedical Research Foundation Protein Data Bank to determine if they corresponded to any known *E.coli* proteins. The 45-kD protein was found to be highly homologous with haemoglobin from the filamentous myxobacterium *Vitreoscilla* (Wakabayashi *et al.*, 1986). Of the 20 amino acid residues that were compared, 14 residues occurred in identical positions as the haemoglobin from *Vitreoscilla* and 3 of the remaining residues were conservative substitutions involving valine-isoleucine changes in two of the postions and valine-leucine change in the remaining position (see Figure 5.4). The 47-kD protein was identical at 15 positions (of the 17 that were compared) with serine hydroxymethyl transferase, the *glyA* gene product from *E. coli* (Stauffer *et al.*, 1981, Plamann *et al.*, 1983). The amino acid residue at position 4 of the 47-kD protein was preliminarily assigned as cysteine. However, arginine has been predicted to occur at this position from the DNA sequence of the *glyA* gene (Plamann *et al.*,1983). The PTH-derivatives of both cysteine and arginine are known to escape detection by h.p.l.c., therefore it is likely that cysteine may have been wrongly assigned at this position in the first instance. The amino acid residue at position 16 was analysed as glutamate whereas the predicted residue for serine hydroxymethly transferase is tryptophan.

The molecular weight of the 47-kD protein obtained by SDS-PAGE comparison with Pharmacia low molecular weight standard is in close agreement with the published

Figure 5.4 Comparison of *N*-terminal sequences of the 47-kD and 45-kD proteins with known proteins

The N-terminal amino acid sequences of the 47-kD and 45-kD proteins were compared with sequences of other proteins in the National Biomedical

Research Foundation Protein Data Bank (see text)


47 K
M L K C E M N I A D Y D A E L E Q
SHMT
M L K R E M N I A D Y D A E L W Q
45 K
M L D A Q T I A T V K A T I P L L K E T
VHb
M L D Q Q T I N I I K A T V P V L K E H

molecular weight of *E. coli* serinehydroxymethyl transferase (45 300) which was calculated from the predicted amino acid sequence of the *glyA* gene (Plamann *et al.*, 1983). The indirect evidence for the presence of the *glyA* gene in plasmids pWA 266 and pWA 406 was confirmed by carrying out genetic complementation experiments using a *glyA* mutant strain GS245 (Stauffer *et al.*, 1981). When this mutant strain made competent by calcium chloride treatment was transformed with plasmids pWA 266 or pWA 406, it was able to grow on minimal plates that did not contain serine and glycine, whereas strain GS245 on its own or when transformed with the cosmid vector pHC79 did not grow in this medium.

Since the subunit weight of the 47-kD protein (measured by SDS-PAGE) is actually 45 300, then that of the 45-kD protein must be about 44 000. The subunit molecular weight of the *Vitreoscilla* haemoglobin is 15 800 (Wakabayashi *et al.*, 1986). There has been no previous report of any haemoglobin like protein in *E. coli*. Although there is strong homology in the *N*-terminal amino acid sequence of the two proteins, the higher molecular weight of the protein from this study needs to be accounted for. The joining of non-contiguous DNA fragments during the construction of the cosmid library has not been considered before because the selection of the insert was carried out with great stringency. The coincidence that the sum of the molecular weights of the haemoglobin protein from *Vitreoscilla* and *E. coli* DHPR is about 44 000, led to the suspicion that a fusion due to joining of non-contiguous regions of the chromosome during the cosmid library construction may have occurred. Other evidences such as the loss of DHPR activity during the purification of the protein and the yellow colour which may be due to the flavin prosthetic group in *E. coli* DHPR, favour the fusion protein arguement. In any event, it was decided that the 44-kD protein meritted further investigation because it appeared to be novel.

5.2.2 Restriction mapping of plasmid pWA 406

The restriction mapping of the plasmid pWA 406 was carried out so that the gene(s) responsible for the coding of the 44-kD protein could be isolated. The plasmid DNA or fragments isolated from it, were analysed by single, double or triple digests with commonly used restriction endonucleases (for example see Figure 5.5a). The restriction digests of plasmids pWA 401 and pWA 403 were also carried out (see Figure 5.5b) to support the location of some of the sites on pWA 406.

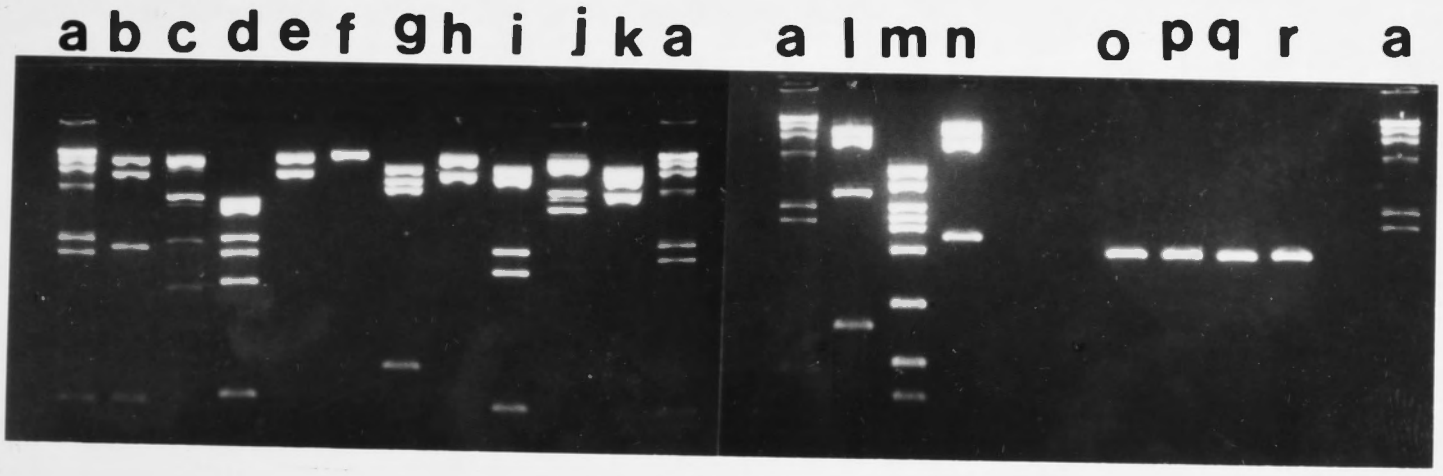
The *Sal* I digest of pWA 406 gave 4 fragments (8 kb, 6 kb, 2.1 kb and 0.6 kb) which indicated that there must be 4 such restriction sites. One of the *Sal* I sites was located within the vector pHC79 (compare with Figure 4.1). The 8-kb and 6-kb *Sal* I fragments were isolated, reclosed by intramolecular ligation and separately transformed into AN 1459. The rationale here was that only the reclosed vector containing the complete vector DNA would yield recombinants. The reclosed 6-kb fragment gave recombinants on a LB plate containing ampicillin. This information was used to locate the second *Sal* I site (*) on the map of pWA 406. Since the size of the vector in pWA 406 is about 5.7 kb, the second *Sal* I site was placed such that a 6-kb fragment would contain most of the vector DNA (see Figure 5.6). The remaining two *Sal* I sites were located by comparison with the *Sal* I restriction pattern of plasmids pWA 401 and pWA 403. The *Eco* RI digest of pWA 406 generated 2 fragments of about 10 kb and 8 kb. Both sites must have been in the insert because the *Eco* RI site of the vector was lost in the 665 bp fragment that was deleted during the sub-cloning (see Figure 4.7). The first *Bam* HI site that occurred within the 0.6-kb *Sal* I fragment was formed fortuitously during the cosmid construction because the ligation of a *Bam* HI cut 5' overhang with a *sau* 3A cut 5' overhang had a 25% probability of forming a new *Bam* HI site. The second *Bam* HI site that occurs at the junction where the 665-bp fragment has been deleted was formed by intramolecular ligation of two *Sau* 3A generated 5' overhangs. However the T nucleotide residue at position +4045 of the vector (from the DNA sequence of pBR322; Sutcliffe, 1979) will not form a *Bam* HI recognition sequence. Deletion of two further 36-bp and

Figure 5.5 Agarose gel electrophoresis of plasmid DNA

(A) Plasmid pWA 406 was digested with *Sal* I (b), *Eco* RI (c), *Pvu* II (d), *Bam* HI (e) *Xho* I (f), *Hpa* II (g), *Sma* I (h), *Sal* I - *Xho* I (i), *Eco* RI + *Xho* I (j), *Bam*HI + *Xho* I (k) *Bam*HI + *Eco* RI (l), *Sal* I + *Pvu* II (m), and *Eco* RI + *Sma* I (n). A 1.6-kb *Pvu* II fragment isoltaed from pWA 266 was digested with *Sma* I, *Xho* I, *Eco* RI and *Sal* I respectively ('o' - 'r'), to check for the presence of these restriction sites. The lanes marked (a) contain λ DNA digested with *Hind* III, and were run as known size markers.

(B) Plasmids pWA 401 (b - h), pWA 403 (i - o) or pWA 406 (p - v) were digested with *Pvu* II + *Eco* RI, *Pvu* II + *Sal* I, *Eco* RI +*Sal* I, *Pvu* II + *Eco* RI + *Sal* I, *Eco* RI and *Sal* I, respectively. Lanes marked 'a' contain λ DNA digested with *Hind* III, size markers.

A



B

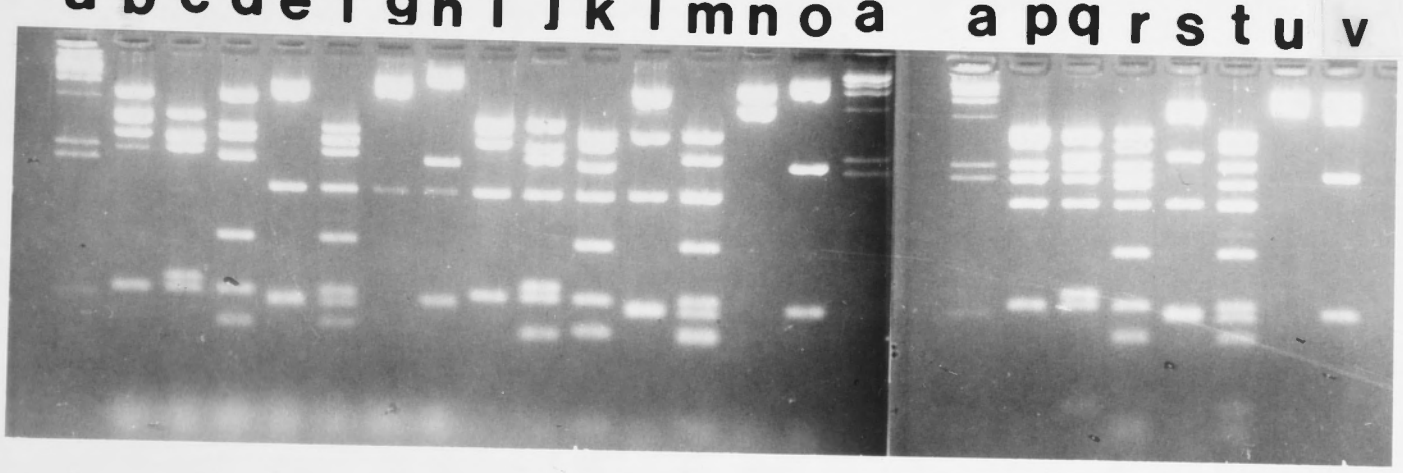
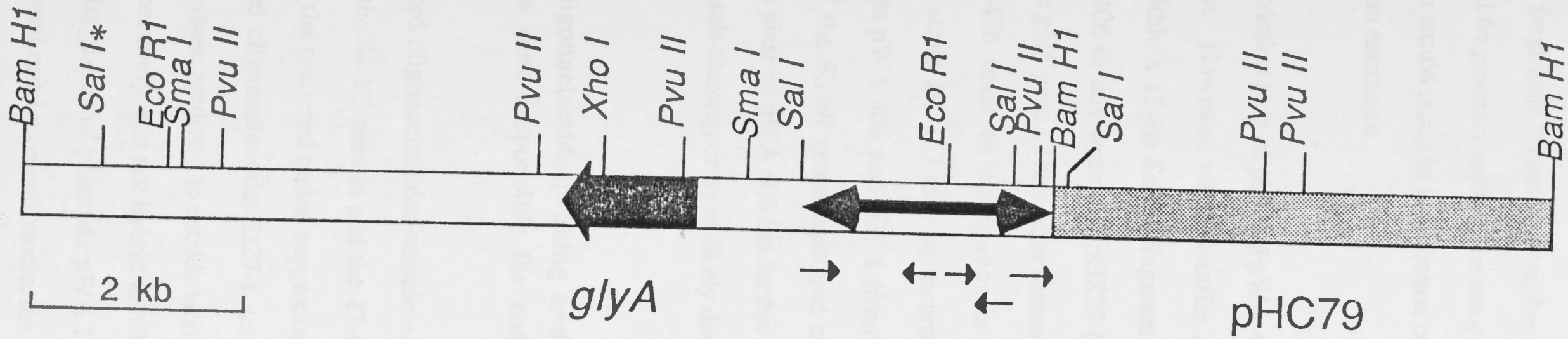


Figure 5.6 Restriction map of plasmid pWA 406.

The double arrowhead (within the ~2.3 kb Sal I - Bam HI fragment) indicates the region that was used as probe DNA in the *in situ* hybridization and the small arrows in dicate the regions that were sequenced in the attempt to identify an open reading frame for *E. coli* DHPR gene (explained in the text). The position of *glyA* gene and pHC 79 vector DNA are also indicated.


Bam H1
Sal I*
Eco R1
Sma I
Pvu II
Pvu II
Xho I
Pvu II
Sma I
Sal I
Eco R1
Sal I
Pvu II
Bam H1
Sal I
Pvu II
Pvu II
Bam H1
2 kb
glyA
pHC79

17-bp *Sau* 3A fragments however, would result in a new *Bam* HI site. The deletion of these two small fragments could not be resolved in Figure 4.7. It was reasoned that any further deletion would result in inactivation of the *bla* gene which in turn would result in loss of ampicillin resistance.

The restriction map of plasmid pWA 406 shows that the *glyA* gene is on the 8-kb *EcoRI* fragment. However, when Stauffer *et al.* (1981) cloned the *glyA* gene it was shown to be within a 13-kb *EcoRI* fragment. Comparison of the restriction maps of plasmid pWA 406 in this work and pGS29 (Stauffer *et al.*, 1981) enabled the correct placement of the *glyA* gene. The earlier assumtion that the 10-kb insert DNA fragment in plasmid pWA 406 must be contiguous in comparison with its occurrence in the chromosome of strain D3-157 may be incorrect because comparison of the restriction maps of plasmids pWA 406 and pGS 1 (from Stauffer *et al.*, 1981) with the complete physical map of the *E.coli* genome around minute 55 (see Figure 5.7; Kohara *et al.*, 1987) shows that plasmid pWA 406 has further deletions in the insert that may have taken place during the sub-cloning, or more likely due to restriction site polymorphism D3-157 *vs* W3110.

5.2.3 Oligonucleotide probing and DNA sequencing of 5' region of the gene responsible for coding the 44-kD protein.

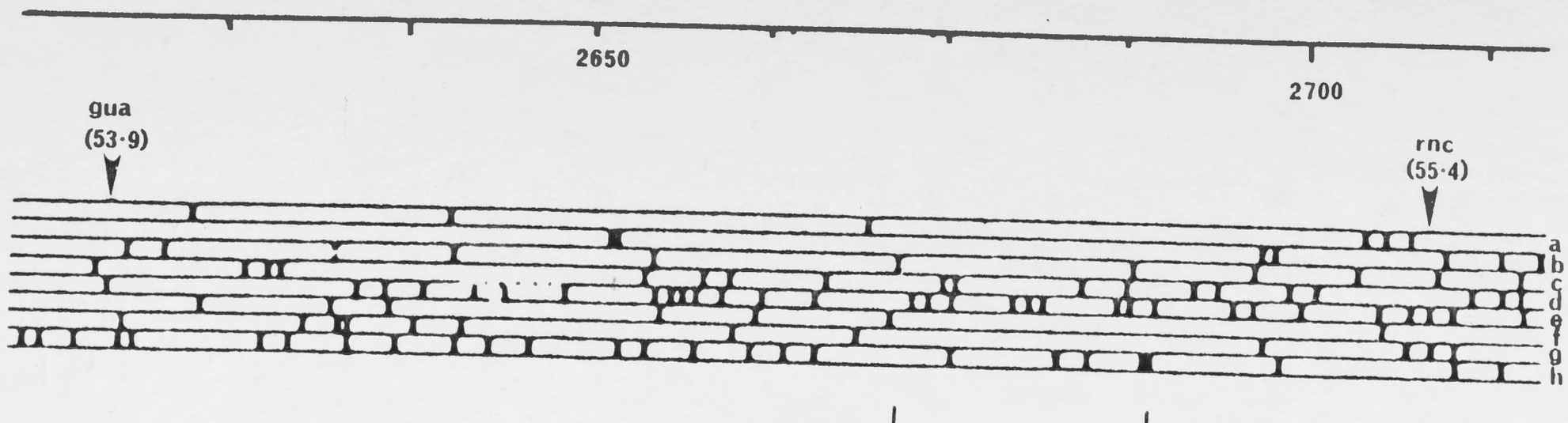
Three mixed oligonucleotides containing sequences that were complementary to the *N*-termini of the 44-kD protein and the 27-kD *E. coli* DHPR were synthesized (see Figure 5.8) using the preferred codon usage catalog for *E. coli* (Grantham *et al.*, 1981). The γ-^{32}P-labelled oligonucleotides D27-1 and D27-2 (containing sequences that are complementary to those predicted to encode amino acid residues 1 to 6 and 14 to 20 of the 27 Kd DHPR respectively) did not hybridize with any bands on the nitrocellulose filter which contained fragments of plasmids pWA 266 and pWA 406 obtained by separate digestion with several restriction endonucleases. This result indicates that the increased DHPR specific activity in the cell free extracts of strains bearing plasmids pWA 266 and

Figure 5.7 Comparison of restriction map of pWA 406 (3) with the physical map of *E. coli* chromosome around the 55 min region.

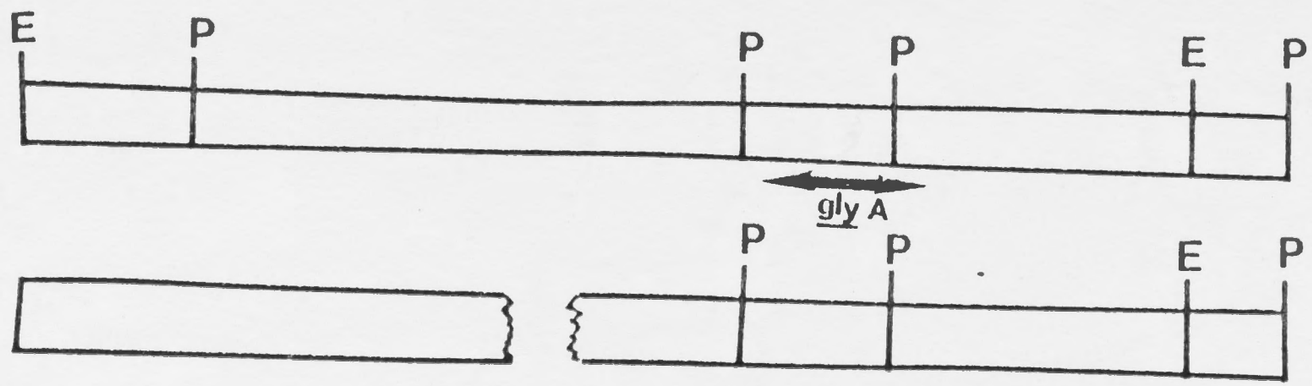
The physical map of the entire *E. coli* genome was obtained using *Bam* HI (a), *Hind* III (b), *Eco* RI (c), *Eco* RV (d), *Bgl* I(e), *Kpn* I (f), *Pst I* (g) and *Pvu* II (h) by Kohara *et al.* (1987). The map around the 55-min region which contains the ~13-kb *Eco* RI fragment in plasmid pGS 1 (Stauffer *et al.*, 1981) is shown in the figure. The position of *glyA* is also indicated. By comparison, in the restriction map of pWA 406, the ~ 10-kb insert DNA of the plasmid may not be contiguous in the chromosome [as indicated by the break in (3)].

'E' is *Eco* RI; P is *Pvu* II; gua is the mnemonic for guanine and rnc is the mnemonic for RNAase.

1



2



3

Figure 5.8 **The sequences of the synthetic mixed oligonucleotides predicted from the *N*-terminal amino acid sequence of *E. coli* DHPR (D27-1) and D27-2; compare with Table 3.3) and 45-kD protein (D45-1; compare with Figure 5.3) (N = G, A, T and C).**

D27-1

```
5'-  ATG  GAC   ATC   ATC   TGC  GT  -3'
       T    T     T     T     T
                  A     A
```

D27-2

```
5'-  TTT  CAT   NGC   ATC   AAA   NGC   TTT  -3'
                        G     G
```

D45-1

```
5'-  GC  AAT  AGT  CTG  NGC  ATC  CAG CAT  - 3'
         G    G    T         G
```

pWA 406 may be due to another protein that is capable of oxidizing NADH in a quinonoid dihydropterin-dependent reaction or that the 5' region of the gene that codes for *E.coli* DHPR may be missing in the clones.

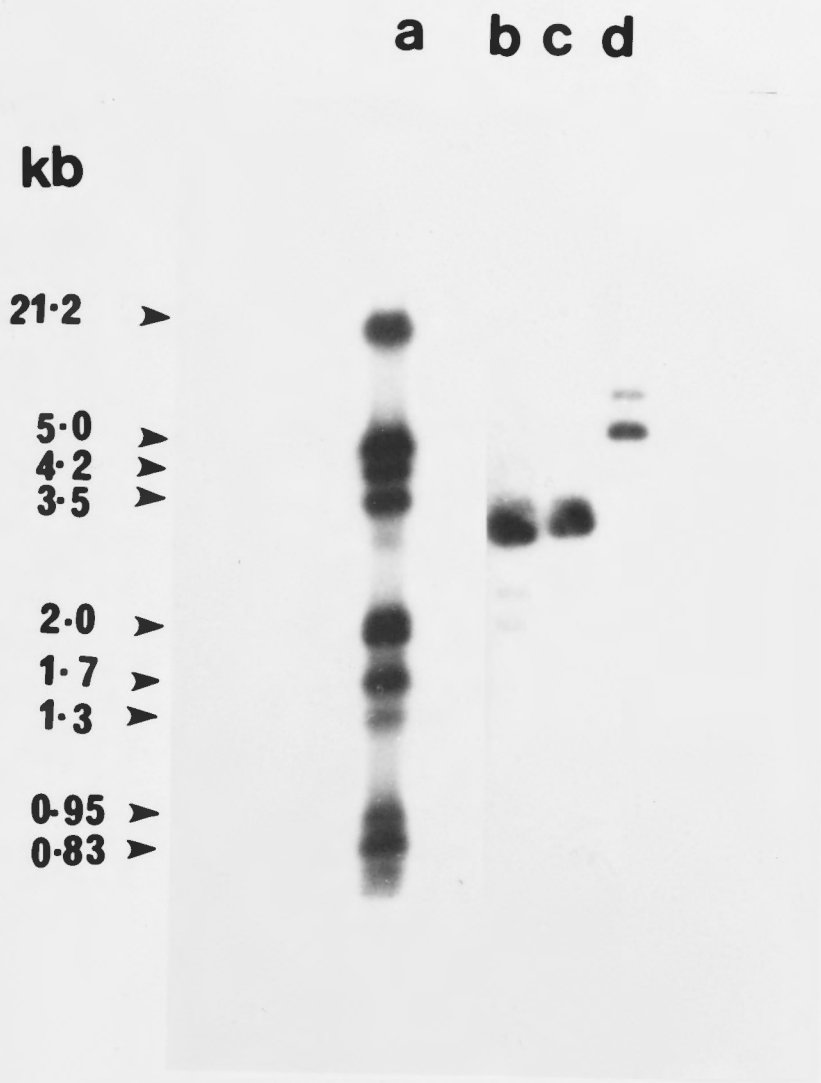
The gene that codes for the 44-kD polypeptide was localised in a 3.2-kb *Pvu* II fragment by hybridization with γ^{32}-P labelled mixed oligonucleotides that were complementary to the 5' region of the gene as predicted from the protein data (see Figure 5.9). The probe also hybridized with a 3.2-kb *Pvu* II fragment of chromosomal DNA from *E. coli* strain ND82 that was completely digested with the restriction endonuclease (see Figure 5.10). This implies that the *Pvu* II fragment containing the gene for the 44-kD polypeptide is most probably a contiguous DNA fragment found in the chromosome. The 3.2-kb *Pvu* II fragment (~2 μg) was isolated after pWA 406 (~10 μg) had been digested completely with the restriction endonuclease *Pvu* II. This fragment was in turn digested completely with the restriction endonuclease *Sau* 3A and ligated with the sequencing vector M13 mp18 (100 ng; Viera and Messing, 1982) digested with *Bam* HI. The *E. coli* strain JM101 was transformed with the ligation mixture and 48 clear plaques were selected to identify a M13 clone bearing the 5' region of the gene encoding the 44-kD protein by dot blot hybridization with γ^{32}-P labelled mixed oligonucleotide D44-1. One of the M13 clones containing the *Sau* 3A-generated fragment hybridized with the probe. Also the 3.2-kb *Pvu* II fragment and on M13 clone containing the fragment also hybridized with the radiolabelled oligonucleotide (see Figure 5.11). The size of the insert in the ss DNA prepared from the M13 clone containg the *Sau* 3A fragment could not be estimated by comparison with the ss DNA from the M13 vector on 0.6% agarose gel (data not shown). It was felt that the small insert in the M13 clone should be sequenced to confirm that the gene encoding the 44-kD protein is responsible for the hybridization signal with the mixed oligonucleotide probe. The complete sequence of the insert which is 163 base pairs long was obtained by the M13 dideoxy sequencing (Sanger et al.,1977) using ^{35}S detection (see Figure 5.12).

Figure 5.9 Southern blot hybridization of plasmid DNA.

(a) λ DNA digested with *Eco* RI and *Hind* III; (b) Plasmid pWA 406 digested with *Pvu* II; (c) ~ 10-kb *Bam* HI fragment from plasmid pWA 406, digested with *Pvu* II; and (d) ~ 8-kb *Eco* RI fragment from plasmid pWA 406 digested with *Sal* I. The samples were separated on a 0.7% (w/v) agarose gel and transferred onto a nitrocellulose filter by Southern blotting, as described in Section 5.4.5. The DNA was baked on the filter and hybridized to 5'-labelled mixed oligonucleotide probe D45-1. The autoradiograph shows the ~ 3.2-kb *Pvu* II fragment hybridized strongly with the labelled probe (in lanes 'b' and 'c'). In 'd' an ~ 5.5-kb *Sal* I fragment also hybridized with the probe because it contained most of the 3.2-kb *Pvu* II fragment (compare with the restriction map of pWA 406 in Figure 5.6). The ~ 8-kb *Eco* RI fragment was not digested completely with *Sal* I, hence the large fragment also hybridized with the probe.

Figure 5.10 Southern blot hybridization of chromosomal DNA from strain ND 82 digested with a variety of restriction endonucleases.

The chromosomal DNA from strain ND 82 was digested completely with *Bam* HI (b), *Eco* RI (c), *Hind* III (d), *Pvu* II (e) or *Sal* I (f), and electrophoresed on a 0.7% agarose gel followed by Southern blot transfer onto a nitrocellulose filter. The DNA was baked on the filter and hybridized with the labelled mixed oligonucleotide D45-1. The autoradiograph indicated that the ~ 3.2-kb *Pvu* II fragment hybridized with the probe DNA.


a b c d
kb
21·2
5·0
4·2
3·5
2·0
1·7
1·3
0·95
0·83

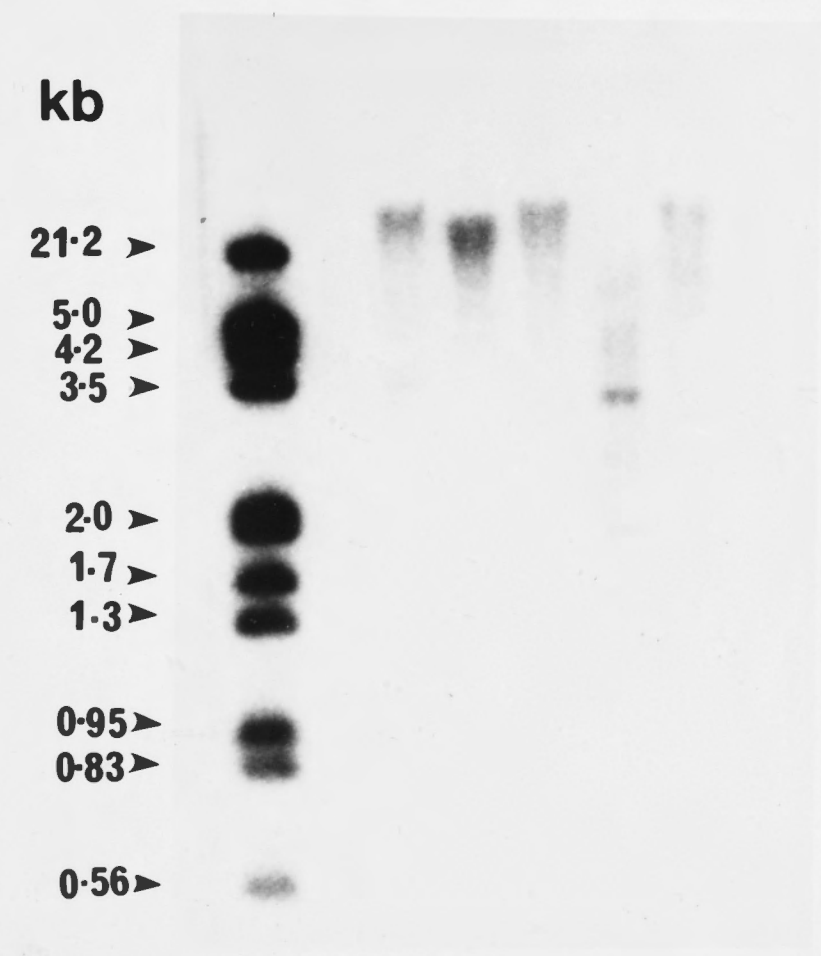
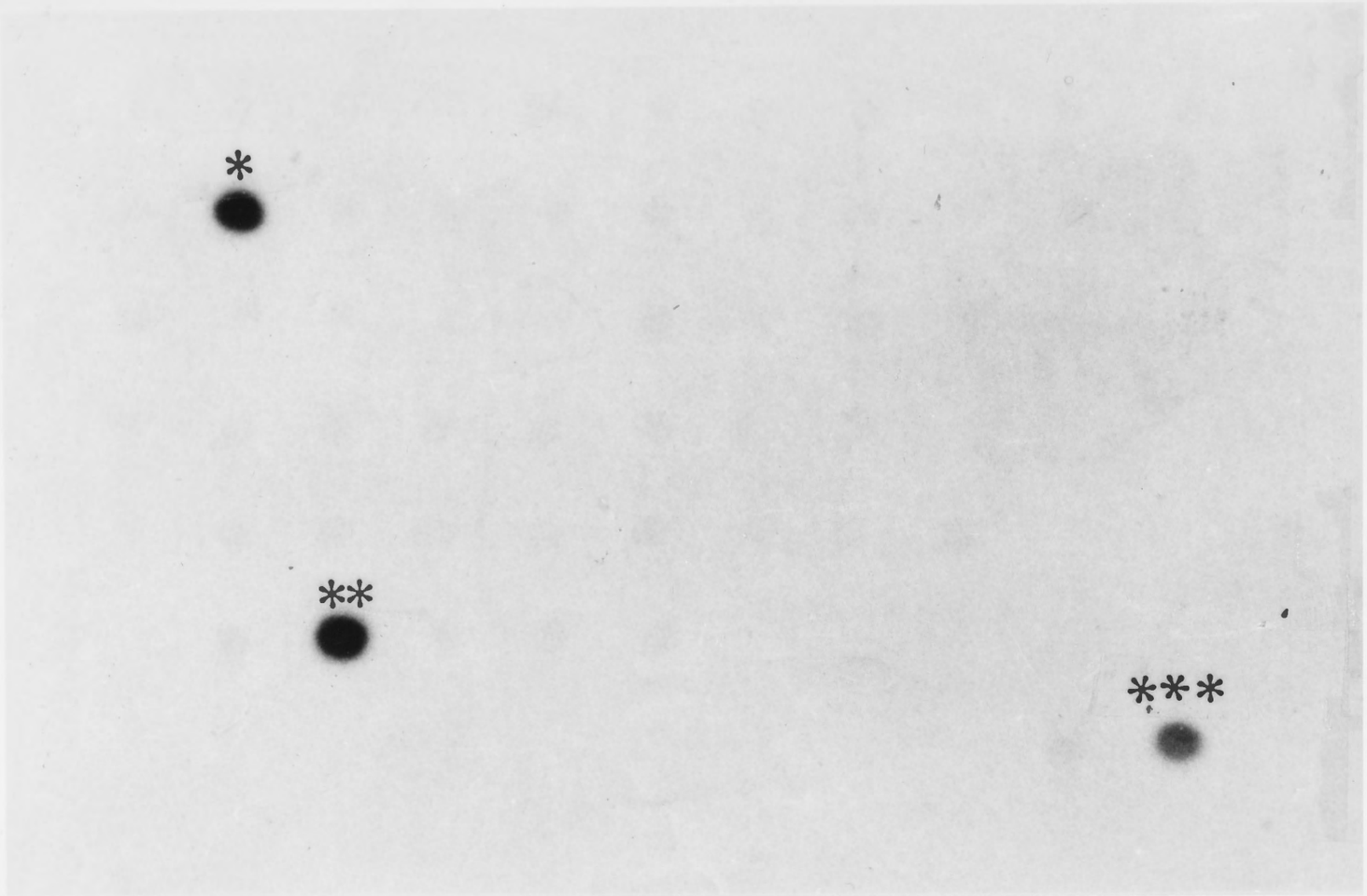

a b c d e f
kb
21·2
5·0
4·2
3·5
2·0
1·7
1·3
0·95
0·83
0·56

Figure 5.11 Screening for M13 clones containing the 5'-region of the gene that encodes the 45-KD protein.

The 3.2-kb *Pvu* II fragment (~2 μg) from plasmid pWA 406 was isolated as described in Section 5.4.2 and digested completely with *Sau* 3A. The fragments were cloned into M13mp18 cut with *Bam* HI and transformed into a suitable strain (JM101). 48 clear plaques (M13 clone #01-48) were selected and analysed by dot blot hybridization with the labelled mixed oligonucleotides D45-1. The autoradiograph indicated that one M13 clone [#13(*)] hybridized strongly with the probe. The probe also hybridized with a M13 clone containing a 3.2-kb *Pvu* II fragment (A2,**) and the denatured 3.2-kb *Pvu* II (B2, ***) fragment that was included as a positive control.



◄ M13 clones 01 to 11

◄ M13 clones 12 to 21

◄ M13 clones 22 to 30

◄ M13 clones 31 to 38

◄ M13 clones 39 to 48

◄ M13 clones A1 to A6

◄ B1 and B2

Figure 5.12 Dideoxy nucleotide sequencing of M13 clone # 13.

The preparation of template DNA and the sequencing using ^{35}S detection was carried as described in Section 5.4.7. The figure shows the complete sequence of the non-coding strand of the 163-bp fragment derived from plasmid pWA406.


G A T C
G A T C
1
20
40
60
80
100
120
140
CCTAGTATACGAACTGCGAGTTTGGTAGCGATGTGATTTCGGTG
GTAGGGCCCTGACCACCTTTGCCCGGTTTCAATTGGCGGGTAAA
GATGGCTGGCATACAAATGAGTATTGGGTCTTGAGTTTCTTTAAAA
ATTGTACTGCATTGGTCGCATTACCGCCTAGG

The nucleotide sequence 5'-GCGATTGGTT.....on the non-coding strand starting from position ~ 154 is identical at 22 out of the 23 bases with one of the species of the mixed oligonucleotide D44-1, (5'-GCRATRGTRTGNGCRTCCAGCAT). The sequence of the 163 bases are likely, therefore, to be from the non-coding strand of the gene that encodes the 44-kD protein. The information was used to predict the amino acid sequence of the *N*-terminal region. Except for the lysine residue at position 18 of the preliminary *N*-terminal amino acid sequence (of the first 20 amino acid resisues) obtained for the 44-kD protein (in this study), it was identical with the corresponding region of the amino acid sequence predicted from the DNA sequence (*N*-terminal 52 residues). This confirmed that the DNA sequence obtained is the 5' region of the gene that codes for the 44-kD protein that was being investigated.

The predicted *N*-terminal 52 amino acid residues of the 44-kD protein showed extensive homology with the sequence of the *Vitreosscilla* haemoglobin (Wakabayashi *et al.*, 1986). The sequences are identical in 23 positions (43%) and conservative substitutions occur at 8 of the remaining positions (15%; see Figure 5.13). The complete primary structure of haemoglobins from several species is known and the comparison of these sequences indicate that only two residues are common to all. One is the histidine (helix position F8) that forms a covalent link with the haem iron and the other is a phenylalanine that occurs in position 1 of the loop made by helices C and D which is responsible for wedging the haem molecule into its pocket. Most haemoglobins also contain a histidine that is distal to the haem (helix position E7), which can form a hydrogen bond with the bound oxygen. In *Vitreoscilla* haemoglobin, a glutamine residue occurs in the place of histidine at this position. However it is suggested that the amino group of glutamine could form the hydrogen bond with the bound oxygen in the same way as the imidazole from histidine (Perutz, 1986; Perutz *et al.*, 1965; Phillips and Shoenborn, 1981).

The phenylalanine residue at position 33 of the available sequence for the 44 kD protein occurs in the same position in *Vitreoscilla* haemoglobin. The sequence alignment

Figure 5.13 The predicted sequence of the *N*-terminal region of the 45-KD protein designated as HMP (haemoprotein) is compared with the corresponding region of *Vitreoscilla* haemoglobin. The shaded box indicates the matched residues and the clear box shows the residues that are conservative substitutions. Phenylalanine at position 31 (*) is conserved in all haemoglobins (compare with Figure 5.14).

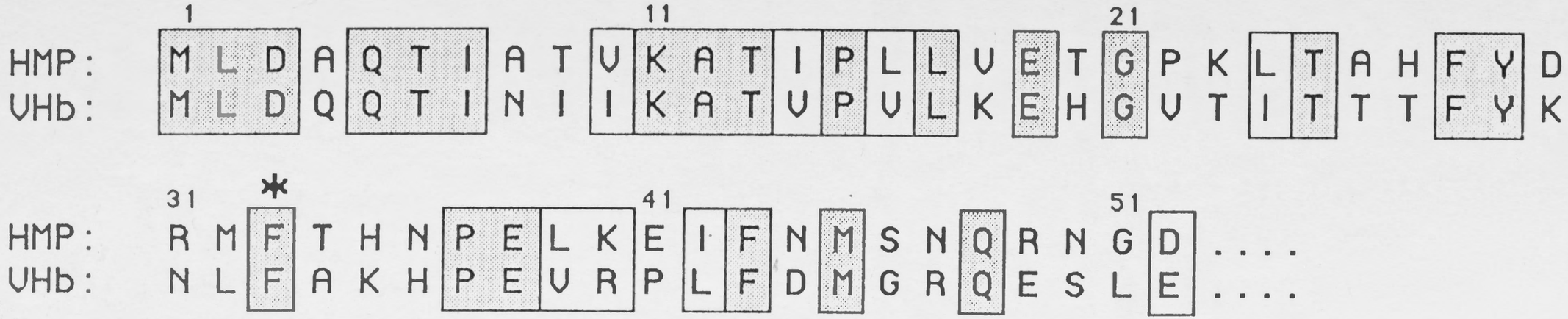

1
11
21
HMP: M L D A Q T I A T V K A T I P L L V E T G P K L T A H F Y D
VHb: M L D Q Q T I N I I K A T V P V L K E H G V T I T T T F Y K
31
*
41
51
HMP: R M F T H N P E L K E I F N M S N Q R N G D ....
VHb: N L F A K H P E V R P L F D M G R Q E S L E ....

Figure 5.14 Comparison of the *N*-terminal 52 amino acid residues of the 45-KD haemoprotein with other haemoglobins.

Human haemoglobin chains (HHbα and HHbβ), Sperm whale myoglobin (SWMb), Glycera haemoglobin (GHb), Lupin leghaemoglobin (LLb), *Vitreoscilla* haemoglobin (VHb) and the 45-KD haemoprotein (Hmp) from this study. All the sequences, except for that of *Hmp*, were alligned by Wakabayashi *et al.* (1986).

Residue no. (*R*)	Helix posn (*H*)	HAEMOGLOBIN						
		HHbα	HHbβ	SWMb	GHb	LLb	VHb	Hmp
1			V			G		
2	NA1	V	H	V	G	A		
3	NA2	L	L	L	L	L		
4	A1	S	T	S	S	T		
5	A2	P	P	E	A	E		
6	A3	A	E	G	A	S		
7	A4	D	E	E	Q	Q		
8	A5	K	K	W	R	A		
9	A6	T	S	Q	Q	A	M	M
10	A7	N	A	L	V	L	L	L
11	A8	V	V	V	I	V	D	D
12	A9	K	T	L	A	K	Q	A
13	A10	A	A	H	A	S	Q	Q
14	A11	A	L	V	T	S	T	T
15	A12	W	W	W	W	W	I	I
16	A13	G	G	A	K	E	N	A
17	A14	K	K	K	D	E	I	T
18	A15	V	V	V	I	F	I	V
19	A16	G		E	A	N	K	K
20	AB1	A		A	G	A	A	A
21					N			
22					D			
23	B1	H	N	D	N	N	T	T
24	B2	A	V	V	G	I	V	I
25	B3	G	D	A	A	P	P	P
26	B4	E	E	G	G	K		
27	B5	Y	V	H	V	H		
28	B6	G	G	G	G	T		
29	B7	A	G	Q	K	H	V	L
30	B8	E	E	D	D	R	L	L
31	B9	A	A	I	C	F	K	V
32	B10	L	L	L	L	F	E	E
33	B11	E	G	I	I	I	H	T
34	B12	R	R	R	K	L	G	G
35	B13	M	L	L	H	V	V	P
36	B14	F	L	F	L	L	T	K
37	B15	L	V	K	S	E	I	L
38	B16	S	V	S	A	I	T	T
39	C1	F	Y	H	H	A	T	A
40	C2	P	P	P	P	P	T	N
41	C3	T	W	E	E	A	F	F
42	C4	T	T	T	M	A	Y	Y
43	C5	K	Q	L	A	K	K	D
44	C6	T	R	E	A	D	N	R
45	C7	Y	F	K	V	L	L	M
46	CD1	F	F	F	F	F	F	F
47	CD2	P	E	D	G	S	A	T
48	CD3	H	S	R			K	H
49	CD4	F	F	F	F	F	H	N
50	CD5		G	K	S	L	P	P
51	CD6	D	D	H	G	K	E	E
52	CD7	L	L	L		G	V	L
53	CD8	S	S	K			R	K
54	D1		T	T		T		
55	D2		P	E		S	P	E
56	D3		D	A	A	E	L	I
57	D4		A	E	S	V	F	F
58	D5		V	M		P	D	N
59	D6	H	M	K		Q	M	M
60	D7	G	G	A		N	G	S
61	E1	S	N	S		N	R	N
62	E2	A	P	E	D	P	Q	Q
63	E3	Q	K	D	P	E	E	R
64	E4	V	V	L	A	L	S	Q
65	E5	K	K	K	D	Q	L	G
66	E6	G	A	K	L	A	E	D
67	E7	H	H	H	G	H	Q	
68	E8	G	G	G	A	A	P	
69	E9	K	K	V	K	G	K	
70	E10	K	K	T	V	K	A	
71	E11	V	V	V	L	V	L	
72	E12	A	L	L	A	F	A	
73	E13	D	G	T	E	K	M	
74	E14	A	A	A	I	L	T	
75	E15	L	F	L	G	V	V	
76	E16	T	S	G	V	Y	L	
77	E17	N	D	A	A	E	A	
78	E18	A	G	I	V	A	A	
79	E19	V	L	L	S	A	A	
80	E20	A	A	K	H	I	Q	
81	EF1	H	H	K	L	Q	N	
82		V	L			L	I	
83						E	E	

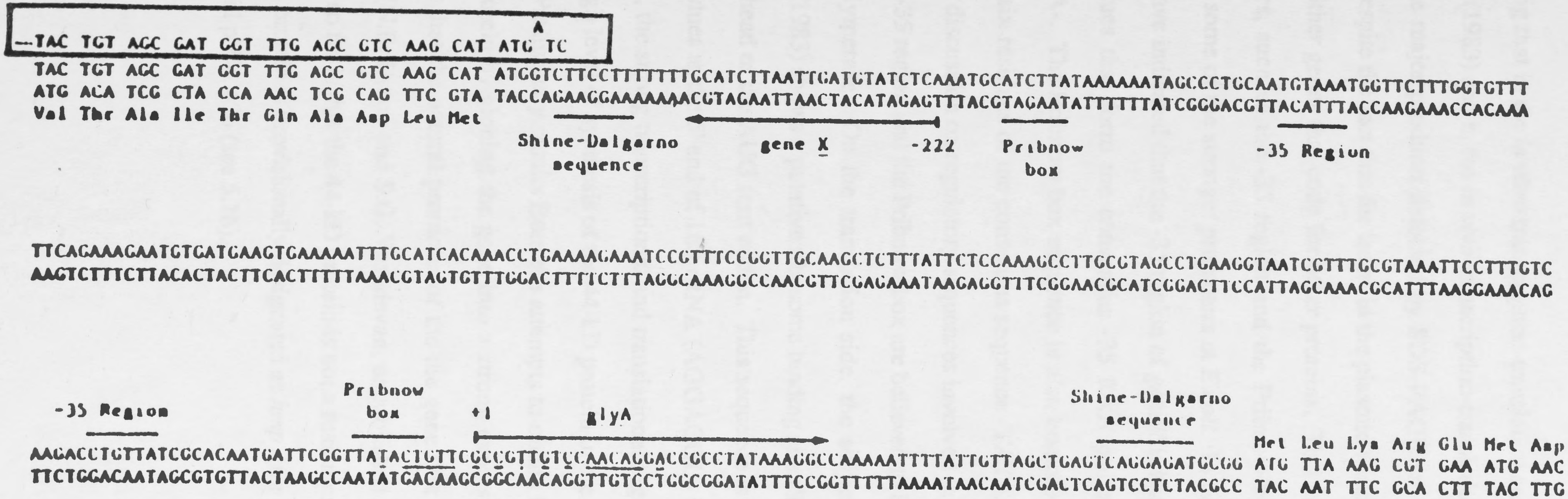
of *Vitreoscilla* haemoglobin placed this phenylalanine residue at the position CD1 (see Figure 5.14). Only further sequencing will tell if the remaining two crucial amino acid residues are also present in the 44-kD protein. Most known haemoglobin subunits are about 13-15 kD, thus the size of the 44-kD protein requires further explanation.

At this stage further information was necessary to establish if the protein was naturally 44 kD or the suspected fusion product. However this doubt was dispelled when the 162-bases-long nucleotide sequence was compared with the European Molecular Biology gene data bank. The first 36 bases (see Figures 5.12 and 5.15), except for the T residue at position 3 which generates the *Sau* 3A recognition sequence were identical with the corresponding 5' sequence of an unknown *geneX* whose product was also reported to have a molecular weight of 44 000 (Plamann and Stauffer, 1983). This gene is situated adjacent to the *glyA* gene but is transcribed in the opposite direction (see Figure 5.15). The *in vitro* transcription-translation of the plasmid pGS1, which carries the *E. coli glyA* gene and other genes on a 13-kb *Eco* RI DNA fragment, as well as that of several other derivatives bearing smaller DNA fragments, directed the synthesis of two proteins with relative molecular weights of about 46 500 and 45 500 (Plamann and Stauffer, 1983). This is in agreement with the independent observation (made in this study) that the inserts in plasmids pWA266 and pWA 406 direct the synthesis of two polypeptides of similar molecular weight in the *in vivo* transcription-translation experiment using minicells (see Figure 4.10). Plamann and Stauffer (1983) identified the protein that is coded by the *glyA* gene by inactivating the gene using the transposable element Tn5, and showed that the 46.5-kD protein was not being made in an *in vitro* transcription-translation system. The concomitant loss of serine hydroxymethyl transferase activity confirmed that the larger of the two proteins detected in SDS-PA gels of the *in vitro* transcription-translation products was the *glyA* gene product.

The nucleotide sequence of the transcription and translation control regions for *glyA* and *geneX* that codes for the 44-kD protein (Plamann and Stauffer, 1983; Figure 5.15) clearly shows that the two genes are being transcribed in opposite directions. It is

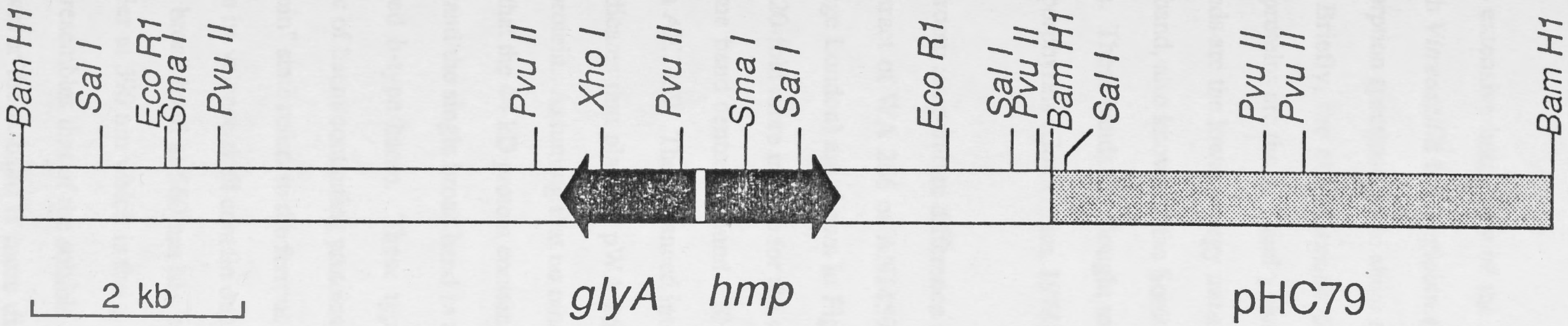
Figure 5.15 The first 36 bp at the 5' end of the sequence obtained for the gene that encodes the 45-KD protein compared with the available sequence of gene 'X'

The sequence of the control region of *glyA* and gene 'X' (identified as haemoprotein gene in this study) was obtained by Plamann and Stauffer (1983).


A
---TAC TGT AGC GAT GGT TTG AGC GTC AAG CAT ATG TC
TAC TGT AGC GAT GGT TTG AGC GTC AAG CAT ATGGTCTTCCTTTTTTTGCATCTTAATTGATGTATCTCAAATGCATCTTATAAAAAATAGCCCTGCAATGTAAATGGTTCTTTGGTGTTT
ATG ACA TCG CTA CCA AAC TCG CAG TTC GTA TACCAGAAGGAAAAAAACGTAGAATTAACTACATAGAGTTTACGTAGAATATTTTTTATCGGGACGTTACATTTACCAAGAAACCACAAA
Val Thr Ala Ile Thr Gln Ala Asp Leu Met
Shine-Dalgarno sequence
gene X
-222
Pribnow box
-35 Region
TTCAGAAAGAATGTGATGAAGTGAAAAATTTGCATCACAAACCTGAAAAGAAATCCGTTTCCGGTTGCAAGCTCTTTATTCTCCAAAGCCTTGCGTAGCCTGAAGGTAATCGTTTGCGTAAATTCCTTTGTC
AAGTCTTTCTTACACTACTTCACTTTTTAAACGTAGTGTTTGGACTTTTCTTTAGGCAAAGGCCAACGTTCGAGAAATAAGAGGTTTCGGAACGCATCGGACTTCCATTAGCAAACGCATTTAAGGAAACAG
-35 Region
Pribnow box
+1
glyA
Shine-Dalgarno sequence
Met Leu Lys Arg Glu Met Asp
AAGACCTGTTATCGCACAATGATTCGGTTATACTGTTCGCCGTTGTCCAACAGGACCGCCTATAAAGGCCAAAAATTTTATTGTTAGCTGAGTCAGGAGATGCGG ATG TTA AAG CGT GAA ATG AAC
TTCTGGACAATAGCGTGTTACTAAGCCAATATGACAAGCGGCAACAGGTTGTCCTGGCGGATATTTCCGGTTTTTAAAATAACAATCGACTCAGTCCTCTACGCC TAC AAT TTC GCA CTT TAC TTG

interesting that in the *in vitro* transcription-translation studies carried out by Plamann and Stauffer (1983) and in the *in vivo* transcription-translation experiments carried out in this work, the major products detected by SDS-PAGE are the 44-kD and 45-kD proteins. This is despite the fact that the insert in the plasmid templates contains (based on its size) several other genes that code for other proteins. This suggests that their transcriptional regulators, such as the -35 region and the Pribnow box in the promoter region, are amongst some of the stronger promoters in *E.coli*. In this respect Plamann and Stauffer (1983) have indicated that the -35 region of *geneX* is homologous at five residues to the six residues that form the consensus -35 RNA polymerase recognition sequence -TTGACA-. The Pribnow box sequence is also homologous at four residues compared with the six residues of the consensus sequence -TATAAT- (see Rosenberg and Court, 1979, for discussion on regulatory sequences involved in the promotion of transcription). Both the -35 region and the Pribnow box are believed to be involved in the binding of the RNA polymerase. On the translation side, the sequence obtained by Plamann and Stauffer (1983) shows a putative ribosome binding site (Shine-Dalgarno sequence) that is 4 bases ahead of the AUG start codon. This sequence is complementary at 6 residues to the 7 residues at the 5' end of 16S rRNA (AGGAGGU) involved in mRNA binding. In summary, the strong transcriptional and translational regulation sequences must account for the high level of synthesis of the 44-kD protein in the *in vivo* transcription-translation system. Preliminary results from the attempts to obtain high levels of expression of the 44-kD protein by cloning the gene into a strong expression vector supports the above argument that the natural promoter of the the gene that codes for the 44-kD protein is efficient (N.E. Dixon and S.G. Vasudevan, unpublished results). The implication from the result so far is that the 44-kD protein is not a fusion product. The gene that codes for the 44 Kd protein is provisionally designated as *hmp* (for haemoprotein) and is located in the plasmid pWA 406 (see 5.16).

Figure 5.16 Restriction map of plasmid pWA 406 showing the positions of *glyA* and *hmp* genes.


Bam H1
Sal I
Eco R1
Sma I
Pvu II
Pvu II
Xho I
Pvu II
Sma I
Sal I
Eco R1
Sal I
Pvu II
Bam H1
Sal I
Pvu II
Pvu II
Bam H1
2 kb
glyA
hmp
pHC79

5.2.4 Characterization of the 44-kD protein by absorption spectroscopy.

The extensive homology of the *N*-terminal amino acid sequence of the 44-kD protein with *Vitreoscilla* haemoglobin (Wakabayashi *et al.*, 1986) was investigated by visible absorption spectroscopy to show the presence of the haem group and determine its structure. Briefly, the characteristic features of visible absorption spectra of haem containing proteins are the α, β and γ bands which are due to the metalloporphyrins. The α and β bands are the lower energy transitions and generally occur between 500 and 600 nm. The γ band, also known as the Soret peak, is an intense band that occurs around 380 and 420 nm. These bands are thought to originate from π to π* electronic transitions of the metalloporphyrins (Gouterman, 1978).

The room temperature difference spectra of the dithionite reduced *minus* oxidized cell free extract of WA 266 or AN1459 (kindly carried out by Professor R.K. Poole, Kings College London) are shown in Figure 5.17. The intensity of γ band at 435 nm is about 16 to 20-fold more intense for the spectrum of the extract from WA 266 compared with the same band centred around 420 nm in the spectrum of the similarly prepared extract from AN1459. The enhanced intensity of the γ band in the soluble extract from WA 266 indicates that plasmid pWA 266 is directing the synthesis of a soluble haem containing protein. Assuming that no other haemoprotein is encoded by the plasmid, this is evidence that the 44-kD protein contains a haem group. The broad symmetrical γ-band (at 435 nm) and the single broad band in the α-β region at 560 nm is indicative of "high-spin" reduced *b*-type haem. These types of "high-spin" reduced *b*-type haems are characteristic of haem-containing proteins such as myoglobin (reviewed by Wood, 1984). The "high-spin" state refers to the ferrous ion in the haemoprotein, where four of the five *3d* orbitals in its valence shell contain one unpaired electron each (Cotton and Wilkinson, 1980). The broad band at 560 nm has been assigned as the β band because there is a weak shoulder at 590 nm which is the α band. In this respect the the spectrum of WA 266 closely resembles that of the soluble haemoprotein *b* -590 from *E.coli* (Poole *et al.*, 1986). However the α-band is more distinct in haemoprotein *b*-590 and its subunit

Figure 5.17 Reduced (dithionite) *minus* oxidized (ammonium persulfate) difference absorption spectra of soluble extracts of WA 266 (a) and AN 1459 (b) recorded at room temperature.

The spectrum of an oxidized sample was stored in a digital memory of a Johnson Foundation dual wavelength spectrophtometer, with 500 nm as reference, and was subtracted from subsequent spectral scans. The oxidized *minus* oxidized baseline was featureless (not shown). The protein concentration of (a) was 4 mg/ml and (b) was 3 mg/ml. The pathlength was 1 cm, the scan rate 2.9 nm s^{-1} and spectral bandwidth was 4 nm.

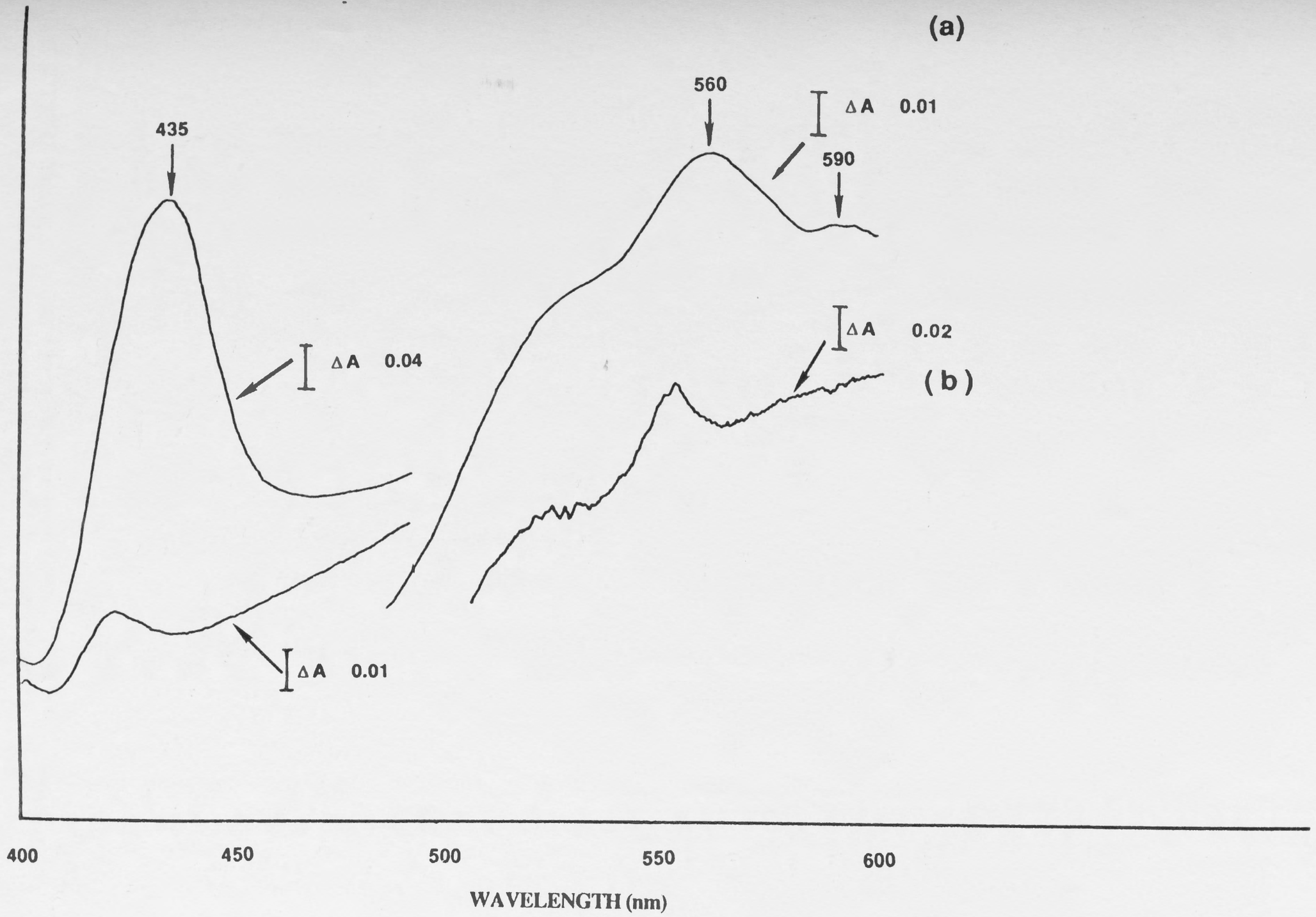
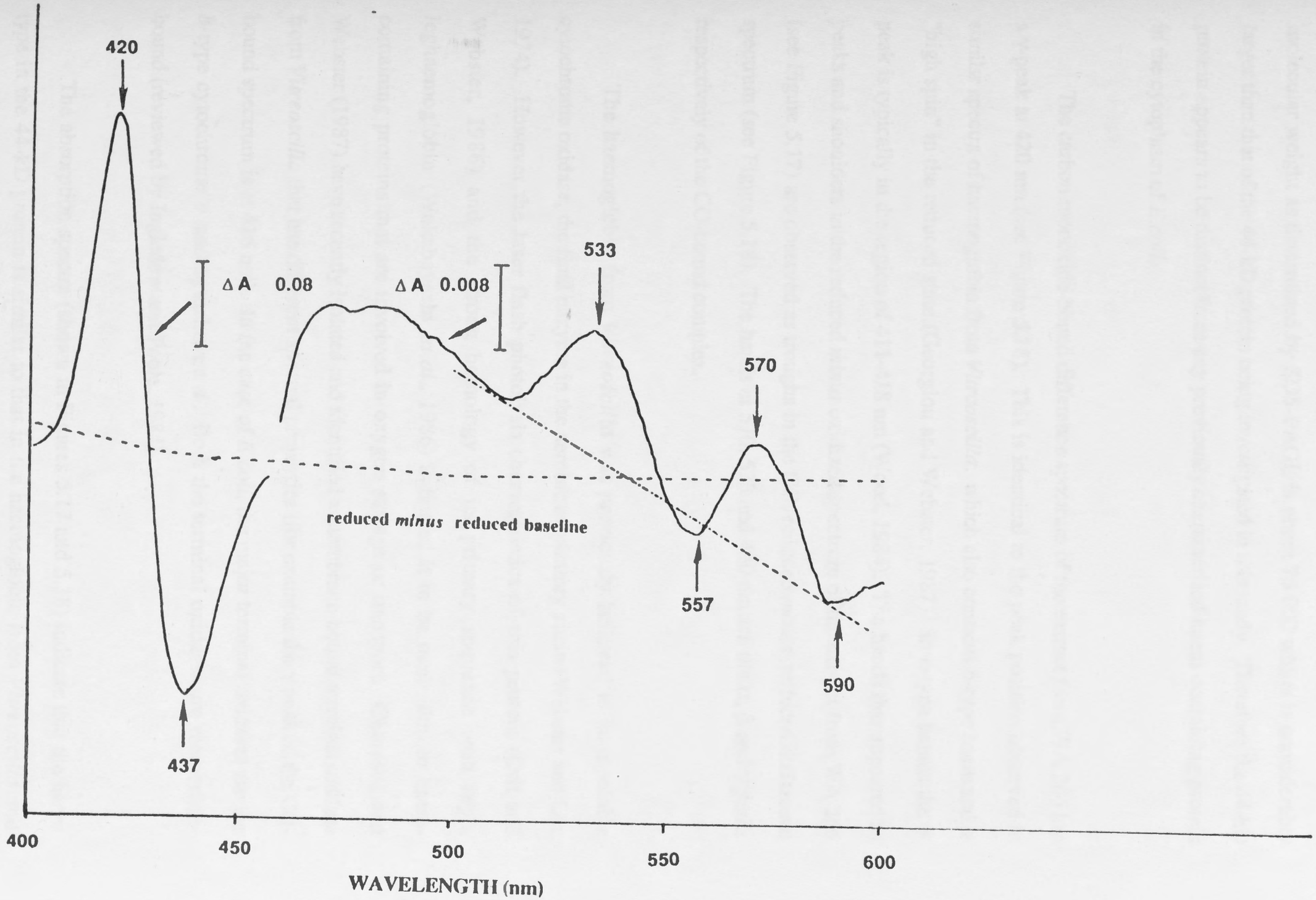

(a)
435
560
590
ΔA 0.01
ΔA 0.04
ΔA 0.02
(b)
ΔA 0.01
400
450
500
550
600
WAVELENGTH (nm)

Figure 5.18 CO-reduced *minus* reduced difference absorption spectra of soluble extract of WA 266 recorded at room temperature.

The spectrum of the reduced sample was stored in a digital memory of a Johnson Foundation dual wavelength spectrophtometer and used to generate a featureless baseline. The sample was bubbled with a thin stream of CO for 2 min and the difference spectrum was recorded. The protein concentration was 4 mg/ml. The pathlength was 1 cm, the scan rate 2.9 nm s^{-1} and spectral bandwidth was 4 nm.


420
ΔA 0.08
ΔA 0.008
533
570
reduced *minus* reduced baseline
557
590
437
400
450
500
550
600
WAVELENGTH (nm)

molecular weight as determined by SDS-PAGE is about 75 000 which is considerably larger than that of the 44-kD protein being investigated in this study. Therefore the 44-kD protein appears to be distinct from any previously characterised haem-containing protein in the cytoplasm of *E.coli.*

The carbon monoxide-bound difference spectrum of the extract from WA 266 has a γ-peak at 420 nm (see Figure 5.18). This is identical to the peak position observed in similsr spectra of haemoglobin from *Vitreoscilla,* which also contains *b*-type haem and is "high spin" in the reduced state (Georgiou and Webster, 1987). In *c*-type haems the γ-peak is typically in the region of 411-418 nm (Wood, 1984). The bands that appeared as peaks and shoulders in the reduced *minus* oxidized spectrum of the extract from WA 266 (see Figure 5.17) are observed as troughs in the CO+reduced *minus* reduced difference spectrum (see Figure 5.18). The bands at 570, 533 and 420 nm are the α, β and γ peaks respectively of the CO-bound complex.

The haemoglobin from *Vitreoscilla* was previously believed to be a soluble cytochrome oxidase, the final enzyme in the aerobic respiratory chain (Webster and Liu, 1974). However the laser flash photolysis characteristics of this protein (Orii and Webster, 1986) and the strong homology of its primary sequence with lupin leghaemoglobin (Wakabayashi *et al.*, 1986) indicated it to be more akin to haem-containing proteins that are involved in oxygen storage or transport. Georgiou and Webster (1987) have recently isolated and identified a membrane-bound terminal oxidase from *Vitreoscilla* that has different spectral properties (for example the γ peak of the CO-bound spectrum is at 416 nm). In the case of *E.coli*, the major terminal oxidases are the *b*-type cytochrome *o* and cytochrome *d* . Both the terminal oxidases are membrane-bound (reviewed by Ingledew and Poole, 1984).

The absorption spectra (shown in Figures 5.17 and 5.18) indicate that the haem type in the 44-kD protein is similar to that in the haemoglobin from *Vitreoscilla* and distinct from the terminal oxidases that occur in *E.coli.* However, this does not imply that

they carry out the same function. An *in vivo* function for the *Vitreoscilla* haemoglobin is still in the area of speculation but a recent report suggested that the heterologous expression of *Vitreoscilla* haemoglobin gene improves the growth properties of recombinant *E. coli* (Khosla and Bailey, 1988)! The authors found that *E. coli* strain JM101 containing the plasmid vector pUC9 showed lower cell yield than one that contained a plasmid pRED2 which expressed *Vitreoscilla* haemoglobin.

With the view of investigating whether the expression of the gene encoding the 44-kD haemoprotein from this study has any effect on cell yield, the strains DM7111, WA 266 and WA 101 were grown under aerobic and anaerobic conditions (by bubbling a constant stream of nitrogen) in rich medium. Strain DM7111 is the background for WA 266 and WA 101 which contain plasmids pWA 101 and pWA 266. The rationale for using WA 101 was that the gene encoding the 44-kD haemoprotein is not present in plasmid pWA 101. Both plasmids pWA 101 and pWA 266 contain about 35-45 kb of insert DNA (cloned into *BamHI* site of cosmid vector pHC79). The growth yields (as judged by wet weight) of DM7111, WA 101 and WA 266 were similar for aerobically grown cells. In the oxygen depleted cultures, the wet weight of cells were also similar for the three strains (data not shown). Khosla and Bailey (1988) showed that the cell yield when *Vitreoscilla* haemoglobin was expressed in *E. coli* was twice that of *E. coli* strain containing plasmid vector pUC9. However, the preliminary results in this study indicate that overproduction of the 44-kD haemoprotein does not result in an increase in cell yield, in spite of the facts that it contains a similar haem type, and shows extensive *N*-terminal homology with the *Vitreoscilla* haemoglobin (Wakabayashi *et al.*, 1986).

Lamba and Webster (1980) showed that under optimal growth conditions the haem content in *Vitreoscilla* can be increased. When the oxygen concentration in the growth medium was below 10% of atmospheric, an increase in *Vitreoscilla* haemoglobin level was observed (Wakabayashi *et al.*, 1986). Whole cells of he strains DM7111 and WA266 that were grown under aerobic and anaerobic conditions were compared by absorption spectroscopy to see if the levels of the 44-kD haemoprotein were enhanced in

the anaerobically grown cells. The reduced *minus* oxidized whole cell spectra of DM7111 and WA 266 grown aerobically and anaerobically (spectra were kindly obtained by Professor Robert Poole whilst on study leave in Canberra) are compared in Figures 5.19 and 5.20. Since the whole cell spectrum is complex and includes all the cytochromes and haemoproteins in *E. coli,* this discussion will be limited to the two main spectral features due to the 44-kD haemoprotein, which are the γ-peak around 434 nm and the broad β peak centred around 560 nm.

In the spectra of DM7111, the anaerobically grown cells show a prominent shoulder at about 434 nm in γ band compared with the aerobically grown cells (see Figure 5.19). This shoulder, however, has contributions from cytochrome b595 and haemoprotein b590 (Poole *et al.*, 1986). There is no clear evidence of broadening of the base of the peak around 560 nm that can be assigned to the β band due to the 44-kD haemoprotein. Hence anaerobic growth of DM7111 does not show any spectroscopically observable increase in the levels of this new haemoprotein. Spectra of WA 266 cells have a shoulder around 434 nm and a broad β band which is centred around 560 nm for both the aerobically and anaerobically grown cells (see Figure 5.20). There is a trough around 578 nm which may be accentuated due to the increased levels of the 44-kD haemoprotein which overlaps the b-595 component of the cytochrome *bd* complex (R. K. Poole, personal communication). This trough is not present in the spectra of DM7111.

If the function of the 44-kD haemoprotein discovered in this study is similar to that of the *Vitreoscilla* haemoglobin, then one would expect to see some of its spectral features in wild type *E. coli* grown under anaerobic conditions. DM7111 showed no indications of its presence. In WA 266 the 44-kD protein was synthesized under aerobic and anaerobic conditions but there is no indication of higher levels of the protein in the anaerobically grown cells. It is clear that further spectral characterizations must be carried out before a specific function can be assigned to the 44-kD haemoprotein. The complete DNA sequence of the gene will permit comparison with the *Vitreoscilla* haemoglobin and determine if the position of the distal histidine and proximal histidine or glutamine are

Figure 5.19 Reduced *minus* oxidized difference absorption spectra of whole cells of strain DM 7111 grown under aerobic (A) and anaerobic conditions, recorded at 77K.

The spectra was measured using a Aminco dualwavelength spectrophotometer. The protein concentration was 13 mg/ml in (A) and 7.5 mg/ml in (B) [determined by the method of Markwell *et al*. (1978)].

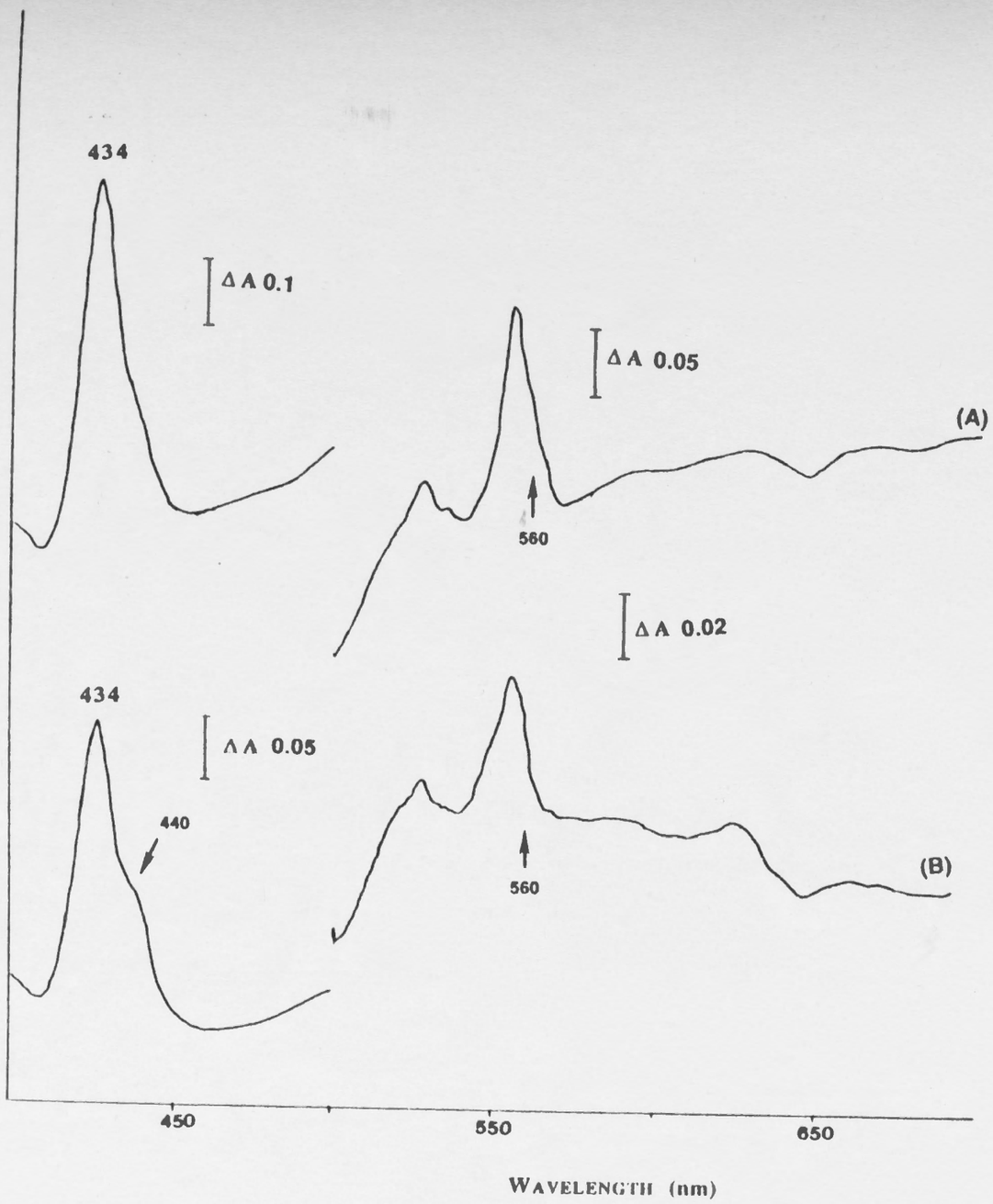
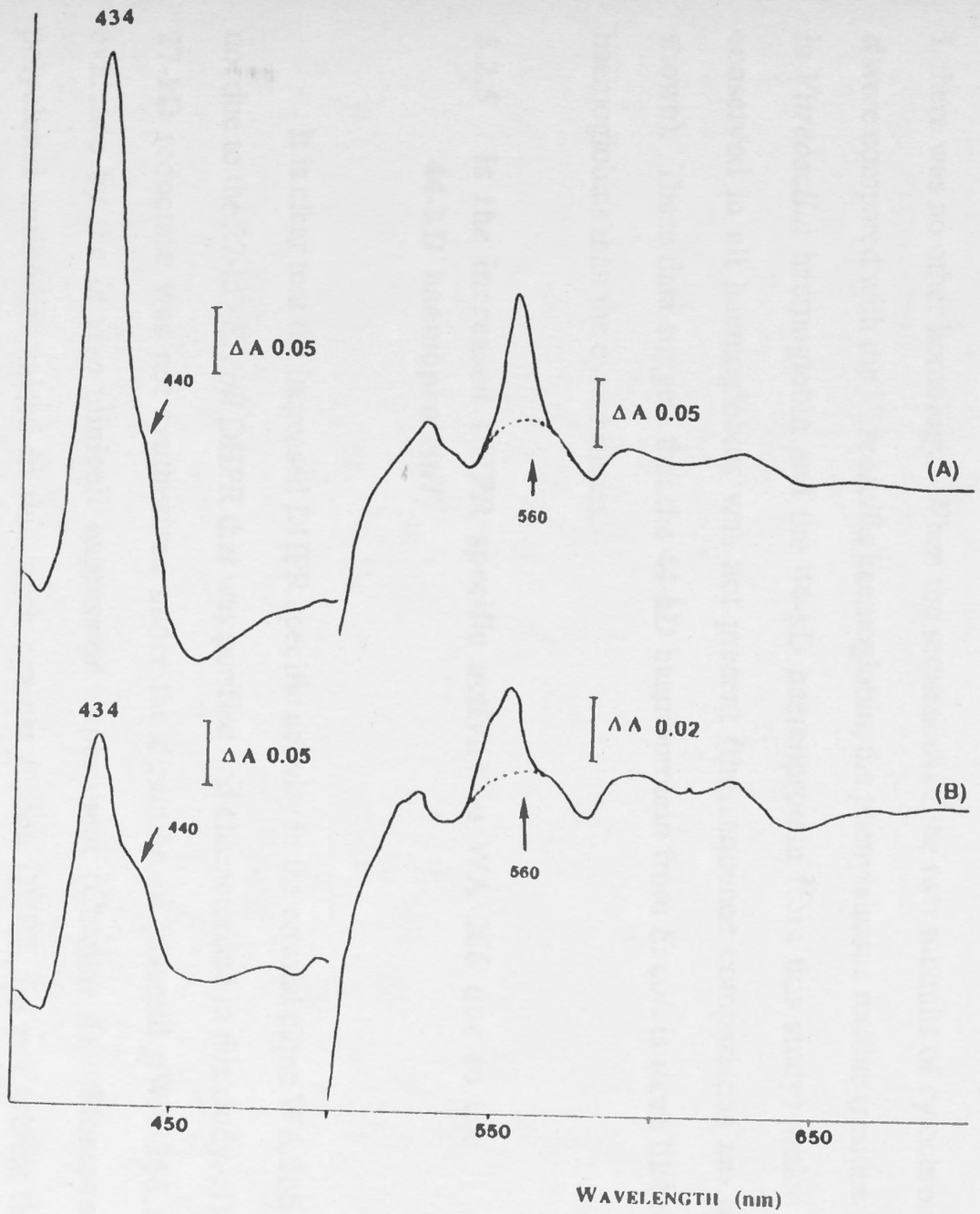

434
ΔA 0.1
ΔA 0.05
(A)
560
ΔA 0.02
434
ΔA 0.05
440
560
(B)
450
550
650
WAVELENGTH (nm)

Figure 5.20 **Reduced *minus* oxidized difference absorption spectra of whole cells of strain WA 266 grown under aerobic (A) and anaerobic conditions, recorded at 77K.**

The spectra was measured using a Aminco dualwavelength spectrophotometer. The protein concentration was 9 mg/ml in (A) and 7.5 mg/ml in (B) [determined by the method of Markwell *et al.* (1978)]. The characteristic broad peak centred around 560 nm observed in the extract prepared from WA 266, is present.


434
ΔA 0.05
440
ΔA 0.05
560
(A)
434
ΔA 0.05
440
ΔA 0.02
560
(B)
450
550
650
WAVELENGTH (nm)

conserved at the same positions in the haemoprotein. It will also establish the point at which the two sequences diverge and may assist in accounting for the function of the *C*-terminal region of the new haemoprotein.

The sequence of the *N*-terminal 52 amino acid residues of the 44-kD haemoprotein was compared with the corresponding regions of the recently published sequence of subunits I and II of the membrane-bound cytochrome *d* terminal oxidase from *E. coli* (Green *et al.*, 1988). Except for the first three residues which are Met-Leu-Asp in subunit I, there was no other homology. When the sequences of the two subunits of cytochrome *d* were compared with the *Vitreoscilla* haemoglobin, the phenylalanine residue (residue 33 in *Vitreoscilla* haemoglobin and the 44-kD haemoprotein from this study) which is conserved in all haemoglobins was not present (the sequence comparisons are not shown). These data suggest that the 44-kD haemoprotein from *E. coli* is more like the haemoglobins than the cytochromes.

5.2.5 Is the increased DHPR specific activity in WA 266 due to the 44-kD haemoprotein?

It is clear that the increased DHPR specific activity in the cosmid clone WA 266 is not due to the 27-kD *E. coli* DHPR that was purified and characterised in this study. The 27-kD reductase was not synthesized under the direction of plasmid pWA 266 as evidence by the *in vivo* minicell expression experiment (Chapter 4). Moreover, polyclonal antibodies raised in this work against *E. coli* DHPR did not inhibit the increased DHPR specific activity in the extract prepared from WA 266 as strongly as the enzyme activity observed in the extract prepared from DM 7111. Comparison of the two extracts by Western blot detection did not show any strong antigenic signal around the 44-kD region above the background levels (data not shown).

However, it is known that the *glyA* gene product serine hydroxymethyl transferase (which catalyses the interconversion of serine and glycine) requires 5,10-

Figure 5.21 Western blot analysis of protein sample from WA 266

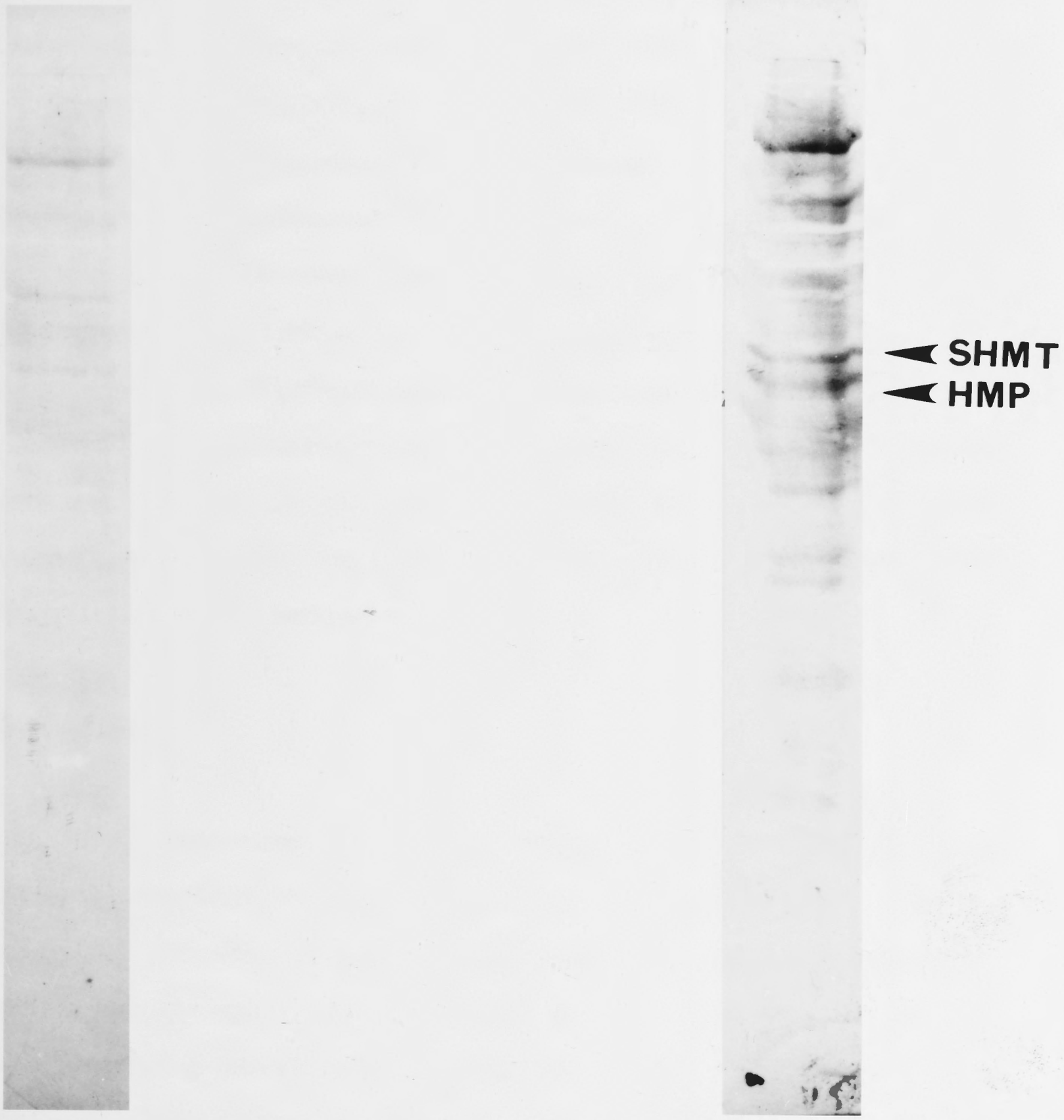
The 45 - 60% ammonium sulfate saturation fraction (in track a and b) was electrophoresed in a SDS-PA gel (12%w/v) and transferred onto a nitrocellulose filter as described in Section 3.4.16. The nitrocellulose filter was sent to Mr Ian Jennings(The Murdoch Institute), who carried out the probing with the antibodies.

(a) was probed with pre-immune serum.

(b) was probed with the anti-idiotype antibody NS-6. The protein band identified as SHMT and HMP were of molecular weights of about 47 000 and 45 000 (respectively) as judged by molecular weight markers that were run concurrently (not shown).

a

b



methylentetrahydrofolate for its function (Scrimgeour and Huennekens, 1962; Mudd and Cantoni, 1964). This indicates that serine hydroxymethyl transferase must also have a pterin binding site. It was felt that the presence of serine hydroxymethyl transferase would serve as good internal control to investigate if the 44-kD haemoprotein possessed a pterin-binding site by using the anti-idiotype antibody (pterin analogue). Some preliminary evidence from Western blot detection using anti-idiotype antibody NS-6 suggests that both serine hydroxymethyl transferase and the 44-kD haemoprotein which are present in high levels in extracts prepared from WA 266 contain pterin binding sites. The Western blot (kindly carried out by Mr. Ian Jennings, The Murdoch Institute, Royal Melbourne Children's Hospital) was carried out on the 45-60% ammonium sulfate saturation fraction of the extract from WA 266 (see Figure 5.21). Other proteins were recognised as well and this may have been due to non-specific interactions of the anti-idiotype antibody with proteins that bind substrates that bear some similarity with the pterin nucleus, e.g. flavin and other purine-derived molecules. Attempts to visualise the increased DHPR specific activity in the extracts of WA 266 by activity staining of non-denaturing polyacrylamide gels were unsuccessful because of high background caused by the presence of NADH oxidases.

5.3 SUMMARY

Partial purification of the proteins from the cell-free extract of WA 266 was achieved by monitoring the residual DHPR enzyme activity and the yellow colour. Two proteins of molecular weights around 47 000 and 45 000 were present after the final purification through a chromatofocusing column. *N*-terminal amino acid microsequencing identified the slower moving band on a SDS-PA gel as serine hydroxymethyl transferase, the *gly*A gene product (Plamann *et al.*, 1982). The faster moving band on the other hand is a new protein discovered in this work and its *N*-terminal amino acid sequence (52 residues) shows significant homology with the haemoglobin from the filamentous myxobacterium *Vitreoscilla*.

The presence of the *gly*A gene in plasmid pWA 406 was confirmed by restriction endonuclease mapping. Also, the plasmids pWA 266 and pWA 406 were able to complement the *gly*A mutation in *E. coli* strain GS 245. The 16 to 20-fold enhancement of the characteristic Soret band in the cell-free extract prepared from WA 266 and the nucleotide sequence of a 163-bp fragment derived from plasmid pWA 406 is evidence that the gene that encodes the 44-kD haemoprotein is also present in the plasmids pWA 266 and pWA 406. Except for the substitution by a T residue for the C residue 4 bp upstream from the ATG start codon, the nucleotide sequence of the first 36 bp of the gene that encodes the 44-kD haemoprotein matched all the reported sequence of an unknown *geneX* which is transcribed in the opposite direction to *gly*A and present immediately adjacent to it (Plamann and Stauffer, 1983). The *N*-terminal 52 amino acid residues predicted from the nucleotide sequence show strong homology with the *Vitreoscilla* haemoglobin (Wakabayashi *et al.*, 1986). The phenylalanine residue at position 33 in the 44-kD haemoprotein is conserved in all haemoglobins. The absorption spectra of the protein indicated that it contains a *b*-type haem which is "high spin" in the reduced state similar to the *Vitreoscilla* Hb and myoglobins (Wood, 1983). This protein is probably not the haemoprotein b-590 isolated from the cell free extract of *E. coli* (Poole *et al.*, 1986) due to the fact that their molecular weights are different and also because the α-peak in the reduced spectrum of the 44-kD protein is less prominent than that of the former protein. The 44-kD protein is provisionally designated *E. coli* haemoprotein.

An anti-idiotype antibody which mimics the pterin nucleus appears to recognises both serine hydroxymethyl transferase and the haemoprotein identified in this study. Further work is required to ascertain if either of these two proteins is responsible for the increased DHPR specific activity in the extracts prepared from WA 266.

5.4 EXPERIMENTAL METHODS

5.4.1 Partial purification of protein from WA 266 extract.

The ammonium sulfate fractionation, DEAE-Fractogel or Mono-Q anion-exchange chromatography, and the Mono-P chromatofocusing, were carried out essentially as described in Section 2.6, except that for the chromatofocusing step, the start buffer was 25 mM Bis-tris/Iminodiacetic acid (pH 7.1) and the eluent buffer was 10% Polybuffer TM 74/Iminodiacetic acid (pH 4.1).

5.4.2 Isolation of DNA fragments from agarose gel.

When fragments of DNA were required, either for sub-cloning DNA sequencing or for confirming the presence of restriction sites contained within, they were isolated from agarose gels. The DNA that has been digested with the appropriate restriction endonuclease was mixed in the sample loading buffer (see Section 4.5.4) and applied to slots (20 mm wide, 6 mm deep) in a 0.7% agarose gel. The gel and the running buffer contained ethidium bromide (0.5 μg/ml) to allow visualization of bands under long-range u.v. light during electrophoresis. When the fragment of interest was sufficiently separated from others, a scalpel was used to make a cut ahead of the band or the area around the band was cut out completely and inserted in another area of the gel. A strip of NA45 paper (DEAE membrane; Schleicher and Schuell D-3354, Dassel, W. Germany) was placed in the slit ahead of the band and electrophoresis was continued. Long range u.v. light was used to monitor the migration of the DNA fragment onto the NA45 paper. The paper which retained all the DNA was removed from gel, rinsed with TE buffer and placed in a 1.5 ml microfuge tube containing 0.45 ml of NaCl (1 M) and arginine (50 mM) . The paper was completely immersed in the buffer and the tube was placed in a heating block at 70^{o}C for 1 h. The NaCl dissociates the DNA bound to the NA45 paper and the arginine competes for the negative charge on the paper thereby displacing the DNA into the solution. The paper was removed and 0.9 ml of ethanol was added to the

tube. The DNA was precipitated by leaving the tube at -70°C (3-15 hrs) and later centrifuging it in an microfuge for 10 min to pellet the precipitated DNA. The pellet was rinsed with cold 70% (v/v) ethanol by centrifugation and dried at a water vacuum pump for 10 min. The DNA was resuspended in TE buffer and stored at -20°C until required.

5.4.3 Preparation of synthetic oligonucleotides.

The synthetic oligodeoxyribonucleotides used in this study were prepared using an Applied Biosystems 380B DNA synthesizer (by Mr Garry Mayo, Medical Molecular Biology Group, JCSMR). The synthetic oligonucleotides were deblocked by treating with 2 ml of ammonium hydroxide for 6 h at room temperature followed by heating at 65°C overnight. When required for probing DNA fragments, one drop of the oligonucleotide mixture (~30-50 µl) was placed on a clean watchglass and allowed to evaporate to dryness. The residue was dissolved in a total of 60 µl of 80% acetic acid and allowed to stand at room temperature for 30 min. Diethyl ether (1 ml) was added to the tube, which was allowed to stand for a further 30 min before the white precipitate was collected by centrifugation in a microfuge for 10 min. The precipitate was dried in air and dissolved in water (1 ml). The absorbance at 260 nm was used to quantify the oligonucleotide (1 OD ~20 µg), and the solution was diluted appropriately to give a stock concentration of about 50 ng/µl.

5.4.4 End-labelling of oligonucleotide with [γ-^{32}P]ATP.

The oligonucleotide mixture (500 ng) was made up to 15 µl with the phosphorylation buffer (66 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$ and 10 mM β-mercaptoethanol). To this was added 4 µl of [γ-^{32}P]ATP (1.4 Mbq; 370 Mbq/ml; 2000 Ci/mmol) and 1 µl of phage T4 polynucleotide kinase (~20 units). The reaction mixture was incubated at 37°C for 1 h and the reaction was terminated by adding 7 µl of formamide loading buffer (80% (v/v) formamide, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue).

The labelled oligonucleotide was separated from unincorporated [γ-^{32}P]ATP and smaller oligonucleotide (In the synthesis of oligonucleotide mixture, up to 5 percent of the oligonucleotides were smaller than the required length) by electrophoresis through a 16% (w/v) acrylamide gel using the sequencing gel plates (40 cm long by 18 cm wide) with 0.8 mm thick spacers and a comb that formed wells that were 1.5 cm deep and 1.5 cm wide. The 16% (w/v) acrylamide gel was made by mixing 40 ml of the 20% acrylamide stock for DNA sequencing with 10 ml of stock urea mix (see later). To this mixture was added 0.8 ml of 10% (w/v) ammonium persulfate and 75 µl TEMED. After mixing well, the mixture was poured between two sequencing gel plates (that were sealed by taping three sides) held at about 30^{o} inclination. The comb was inserted and the gel was allowed to polymerize (~1 h). The bottom tape was then removed and the gel was subjected to pre-electrophoresis for 2 h at 1500 volts, using TBE as running buffer. The comb was removed from the gel and the wells were thoroughly flushed with TBE. The oligonucleotide mixture containing the formamide dye loading buffer was heated at 90^{o}C for 3 min and loaded in the well using a drawn-out pipette tip. The mixture was electrophoresed through the gel at a constant voltage of 1500 volts for 3-4 h.

After the electrophoresis, one of the glass plates was removed carefully. The plate retaining the gel was covered with Saran wrap, and the position of the oligonucleotide within the gel was visualised by exposing the gel to a Kodak XAR-5 film for 2 min and marking the orientation of the gel on film. The film was developed using a Kodak M20 X-Omat processor. The developed film was used as a guide to position the oligonucleotide within the gel. (The oligonucleotide of interest was usually the slowest moving DNA band, which gave very high counts, indicating maximum incorporation of label). The gel was cut out carefully and sliced into smaller pieces in a 1.5 ml Eppendorf tube, after which 1 ml TE was added. The tube was briefly vortexed and incubated at 65^{o}C for 3-6 h to allow the oligonucleotides to leach out into the TE buffer, which was removed by using a 1 ml Pipetman tip. The labelled mixed oligonucleotides were used for hybridizations.

5.4.5 Southern blot transfer of DNA

The Southern blot transfer of DNA was carried out essentially as described by Southern (1975). The electrophoresis of the appropriate restriction endonuclease-digested DNA was carried out on agarose minigels (8 cm long and 4 cm wide) using TBE containing 0.5 µl/ml ethidium bromide as running buffer, until the blue dye marker reached the anodic end. The gel was photographed before the sides of the gel and the region around the wells were trimmed using a scalpel so that the gel face was flat. The gel was then placed in a tray containing 200 ml of 0.25 M HCl to allow depurination (this step is only necessary when transferring large fragments of DNA,>10 kb). As soon as the blue dye in the gel turned greenish yellow (~5 min), the gel was rinsed with water and immersed in a dish containing the denaturation buffer (0.5 M NaOH,.1 M NaCl) for 30-40 min. After a brief rinse in water the gel was immersed in 200 ml of neutralization buffer (1 M Tris-HCl pH 8.0, 0.6 M NaCl) for 30 min (this step was repeated).

A 5 mm thick glass plate 10 cm by 5 cm was wrapped with Whatman 3 MM paper and placed in a tray. The transfer buffer (20 x SSC; 3 M NaCl, 0.3 M sodium citrate) was poured into the tray so that it was about 4 mm deep, thus wetting the glass mount wrapped with Whatman 3 MM paper by capillary action (it is important not to submerge the paper in the buffer). The gel that had been treated as above was placed on the wet Whatman 3 MM paper and smoothed out by rolling over the gel with a clean test tube to remove bubbles trapped between the gel and the 3 MM paper. The nitrocellulose membrane cut to the size of the gel (floated in 2 X SSC until the upper surface became uniformly wet; ~30 min) was placed on the gel surface and bubbles were smoothed out as before. Three sheets of Whatman 3 MM paper wet with 2 x SSC were placed individually over the nitrocellulose membrane. Adsorbent towels that were cut to the same size as the gel were stacked (5-8 cm thick) on top of 3 MM paper and a 1 kg weight was placed on top of these. The whole set was covered with Saran wrap and the transfer

of DNA fragments onto the nitrocellulose membrane was allowed to take place at room temperature overnight .

The weight, towels and 3 MM paper were removed and the nitrocellulose membrane was placed between two sheets of dry 3 MM paper and baked at 80°C for 2 h in a vacuum oven. The compressed gel was routinely reswollen by soaking in 1 x TBE containing 0.5 µg/ml ethidium bromide and photographed in order to assess the efficiency of DNA transfer by comparison with a photograph of the preblotted gel. If not used directly, the nitrocellulose membrane was stored in a vacuum desiccator in a cool place.

5.4.6 Hybridization of Southern and dot blot filters

The hybridization procedure was carried out essentially as described by Maniatis *et al.* (1982). After baking, the nitrocellulose filter was floated on the surface of 6 x SSC until it was wet from beneath. It was then completely immersed in the solution for 2 min and transferred to a plastic dish (with lid) containing 30 ml of prehybridization fluid (6 x SSC, 0.5% SDS, 5 x Denhardt's solution and 100 µg/ml of denatured salmon sperm DNA; see below for contents of these solutions). The size of the dish was such that its base was completely covered by the 30 ml of prehybridization solution. The dish was covered and incubated in a water bath at 65°C (a weight was placed on the dish to keep in place) for 3-4 h with gentle agitation. The nitrocellulose filter was then transferred into 30 ml of hybridization solution (6 x SSC, 0.01 M EDTA, [γ-^{32}P]-labelled mixed oligonucleotide probe, 5 x Denhardt's solution, 0.5% SDS and 100 µg/ml of denatured salmon sperm DNA) and incubated at 65°C for 2-4 h in a water bath with gentle agitation. The temperature control of the bath was then adjusted so that the filter cooled to 37°C slowly to promote annealing. Hybridization was continued at 37°C for 2 h (or overnight).

The filter was transferred into a solution (100 ml) of 2 x SSC and 0.5% SDS at room temperature in a fresh tray. After 5 min the filter was transferred to another tray

containing a solution of 2 x SSC and 0.1% SDS and incubated for 15 min at room temperature with gentle agitation. The filter was finally placed in a tray containing a solution of 0.2 x SSC and 0.5% SDS and incubated from 30 min to 2 h (as required) at 37°C with gentle agitation. A second change of the final wash step was carried out if the background radioactivity level was high. The filter was dried on a sheet of Whatman 3 MM paper, wrapped with Saran Wrap and exposed to Kodak XAR-5 film in a Kodak X-Omatic C-2 cassette with intensifying screen for 3-24 h, at -70°C. The film was processed using the Kodak M20 X-Omat processor.

Constitution of Denhardt's solution (50x)	
Ficoll	5 g
polyvinylpyrrolidone	5 g
BSA (Pentax Fraction V)	5 g
water	to 500 ml

5.4.7 Dideoxy sequencing of DNA

Nucleotide sequencing was carried out by the dideoxy chain-terminating method of Sanger *et al.* (1980), using [α-^{32}P]-dATP or [^{35}S]-dATP with a Dideoxy Sequencing Kit purchased from BRESA (Biotechnology Research Enterprises S.A. Pty. Ltd., G.P.O. Box 498, Adelaide 5001, South Australia).

5.4.7.1 Composition of sequencing gels

20% Acrylamide sock	
acrylamide	96.5 g
methylene bisacrylamide	3.35 g
urea	.233.5 g
5 x TBE	100 ml

water	to 500 ml
Urea mix	
urea	233.5 g
5 x TBE	100 ml
water	to 500 ml

The appropriate percentage sequencing gels were prepared by mixing the acrylamide stock and the urea mix. Routinely, 10% ammonium persulfate (0.4 ml) and TEMED (50 µl) were added to polymerize the gel.

5.4.7.2 Inserting DNA into M13 bacteriophage replicative form

The DNA fragments for sequencing were generated by the appropriate restriction endonuclease digestion and inserted into the complementary site in the polylinker region of the replicative form of bacteriophage M13 mp 18 or mp 19. The replicative forms of M13 mp 18 and M13 mp 19 are identical except that the polylinker region is reversed from one with respect to the other. When DNA fragments were generated with two different restriction endonucleases, insertion into M13 mp 18 and mp 19 gave recombinant phage containing the fragment to be sequenced, in both orientations. This was useful so that both coding and non-coding strands of the DNA fragment could be readily sequenced.

5.4.7.3 Transformation of *E. coli* strain JM101 with M13 phage replicative form

E. coli strain JM101 was transformed with M13 phage replicative form ligated with fragments to be sequenced because the genetics of the strain allowed easy selection of recombinant phage. The host strain is normally maintained on minimal media so that the F episome it carries (which bears the *lac pro* region that has been deleted from its chromosome) will not be lost. The *lac* operon on the F episome contains two mutations which make it *lac*-. This strain was grown in minimal media (56 minimal medium containing 20 mM glucose, 1 mM $MgSO_4$ and 0.0001% (w/v) thiamine (B_1)) and made

competent for transformation. The ligated M13 phage replicative form was used to transform the competent cells (see Section 4.5).

The transformation mixture (10-100 µl) was mixed with 3 ml of 0.7% (w/v) soft agar (~45°C) containing 8 µl of 0.5 M IPTG (isopropylthiogalactoside), 40 µl of a 20 mg/ml stock solution of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 250 µl of a log phase culture of *E. coli* strain JM101. The soft agar was molten and by working rapidly, the mixture was poured onto LB plates and allowed to set. These were incubated at 37°C overnight.

The *lac*$^-$ host strain is complemented by *lacz'* expressed by non-recombinant M13 phage. In the presence of IPTG, infected cells make the β-galactosidase enzyme which hydrolyses X-gal to free the chromogenic substance that renders the plaques blue. Host strains with recombinant M13 phage however show colourless plaques because the insertion of DNA fragment into the polylinker region inactivates a factor that complements the *lac* mutation (carried on the F episome) of the strain, i.e. these strains do not produce β-galactosidase.

5.4.7.4 Preparation of single stranded template DNA

JM101 was grown overnight at 37°C with shaking in the minimal medium described above. This overnight culture was diluted 1 in 50 into 2 x YT medium (1% yeast extract, 1.6% tryptone, 0.5% NaCl) and 5 ml aliquots were dispensed into sterile screw-capped polypropylene tubes of 10 ml capacity. The clear plaques from Section 5.4.7.3 were picked individually with a sterile Pasteur pipette and inoculated into these tubes. The tubes were placed in racks and incubated at 37°C in an air shaker for 4-6 h.

The tubes were centrifuged at 6000 r.p.m. for 10 min at 4°C, using a SS34 rotor-head with jackets (to hold tubes in position) in a Sorvall RC-5 centrifuge. The

supernatant (4 ml; the 1 ml close to the cell pellet was discarded) was mixed well with 0.8 ml of 25% PEG 6000 (polyethyleneglycol) containing 2.5 M NaCl, in a Sorvall SE12 tube and incubated at 0°C for 1 h. The precipitated phage (viable at this stage) was pelleted by centrifugation (SE12 rotor, 15 000 r.p.m., 20 min, 4°C). The phage pellet was suspended well in 550 µl of TE containing 0.5% sarkosyl and transferred into a 1.5 ml microfuge tube. The tubes were centrifuged in a microfuge for 10 min and 0.5 ml of the supernatants were transferred into fresh microfuge tubes containing 0.1 ml of 25% PEG + 2 M NaCl. The tubes were mixed well and incubated in ice for 1 h before centrifugation for 10 min in a microfuge to pellet the phage. The supernatant was removed by aspiration with a drawn out pipette connected to a water pump. The tubes were centrifuged for a further 2 min to pack the pellet and any remaining supernatant was removed with a Pipetman tip (PEG can affect the quality of DNA sequence, so it must be removed as completely as possible). The pellets were suspended in 200 µl of TE containing 1% SDS and extracted twice each with phenol, phenol-chloroform and chloroform. The single-stranded template DNA in the aqueous phase was precipitated with ethanol, suspended in 50 µl of TE and stored at -20°C until used as template for DNA sequencing.

5.4.7.5 Annealing, polymerisation and gel-electrophoresis of sequencing reactions

To 5 µl of single-stranded DNA (prepared as above) was added 1 µl of M13 universal sequencing primer (2.5 ng/µl), 1 µl of 10 x annealing buffer (0.1 M Tris-Hcl, pH 8.0, 0.1 M $MgCl_2$) and 2 µl of sterile water. The contents were mixed well in a 0.5-ml capacity Eppendorf tube, placed in a beaker containing water at 75°C and allowed to cool to room temperature so that the annealing reaction could occur (~30 min). The tubes were briefly centrifuged to bring down the condensation and 1 µl of labelled dATP [α-^{32}P or ^{35}S] was added. A set of 'mastermix' tubes for each nucleotide reaction was made up depending on the number of templates being sequenced (using the protocol supplied with the BRESA DIDEOXY SEQUENCING KIT, BRESA, S. Australia). 2 µl of each

nucleotide mastermix were dispensed into the bottom of separate tubes marked G, A, T and C. 0.5 units of Klenow enzyme (large fragment of DNA polymerase I) was mixed with the annealed DNA and radionucleotide label mixture, and 2 µl aliquots were dispensed along the sides of the tubes G, A, T and C. The tubes were centrifuged in a microfuge for 2 min to allow the solutions to mix and were warmed at 37°C. After 15 min the polymerisation reactions were chased with 1 µl of chase solution (0.25 mM each of dGTP, dATP, dTTP and dCTP) and the reactions were continued for a further 10 min. The reactions were stopped by adding 4 µl of formamide dye loading mix to each tube and heated at 90°C for 5 min and placed in ice.

Gel electrophoresis of sequencing reactions were generally carried out on 6% (w/v) acrylamide gels (15 mls of 20% stock acrylamide solution containing 35 ml of urea mix, 0.4 ml of 10% w/v ammonium persulfate and 50 µl of TEMED) using Bio-Rad Sequigen apparatus (the gels were 36 cm long, 17 cm wide and 0.25 mm thick). Sample wells were 4 mm wide and were made by using a "shark tooth" comb. The gels were pre-electrophoresed for 1 hr at a constant voltage of 1500 volts using TBE as running buffer. The wells were flushed with buffer using a 10 ml syringe attached to a 24 gauge needle and 2 µl of each sequencing reaction was loaded carefully into separate wells using drawn-out capilliary tubes. The gel was run at 1750 volts (the gel temperature was about 45-50°C) for 1.5 h for obtaining sequences close to the universal primer. Sequences of up to 500 bases could be obtained by double and triple loading of the sequencing reaction after 2 and 4 h following the first sample load. After sufficient electrophoresis, the gel was removed from one of the glass plates by placing a sheet of Whatman 3 MM paper on the gel and using a test tube to rub the surface of the papers so that the gel would adhere to the paper more strongly than to the glass plate. The paper with the gel adhering to it was carefully peeled off, covered with Saran Wrap and dried by heating at 80°C under vacuum using a Bio-Rad model 483 slab-dryer. The gels were exposed at room temperature in a Kodak X-Omatic C-2 cassette without an intensifying screen when [α-^{32}P]-dATP was used or at -70°C in a cassette with intensifying screen when ^{35}S-dATP

was used. They were usually developed after 12-24 h using the Kodak M20 X-Omat processor. The sequences were read by using a manual digitizer.

5.4.8 Phage dot-blot hybridization

The clear plaques obtained as described in Section 5.4.7.4 were individually picked using sterile Pasteur pipettes and inoculated into separate 1.5-ml Eppendorf tubes each containing 1 ml of *E. coli* strain JM101 in 2 x YT medium (an overnight culture of JM101 grown in minimal medium was diluted 1 in 50 into 2 x YT as described in Section 5.4.7.4). The tubes were placed in a rack and shaken at 37°C in an air incubator for 6-8 h and centrifuged in a microfuge for 10 min. The supernatant (0.8 ml) was transferred into fresh Eppendorf tubes (taking care not to bring any of the pellet across) containing 200 µl of 25% PEG 6000/2 M NaCl. The tubes were mixed well and incubated on ice for 30 min. The phage was pelleted by centrifugation in a Microfuge for 10 min and the supernatant was removed by aspiration. The phage pellets were suspended in 100 µl TE buffer and stored at 4°C in labelled tubes.

About 2 µl of each of the phage stocks were applied directly onto a dry sheet of nitrocellulose membrane placed on a 96 well tissue culture tray over a light box (to guide the application of phage spot). The nitrocellulose membrane was dried in air and then baked at 80°C for 2 h in a vacuum oven. Hybridization with radio-labelled oligonucleotide was carried out as described in Section 5.4.6. The phage stock that hybridized with the oligonucleotide was used for the preparation of M13 single-stranded DNA for sequencing as described in Section 5.4.7.3.

5.4.10 Complementation of *gly*A mutation in GS 245

E. coli strain GS 245 (*phe*A 905, *ara*D 139, *Δlac*U 169, *Δgly*A, *str*A, *thi*) was a generous gift from Professor George Stauffer, University of Iowa, U.S.A. The glycine requirement of GS 245 was checked by streaking the strain grown on rich medium onto a

minimal plate supplemented with 0.4% (w/v) glucose, 200 µg/ ml phenylalanine, 1 µg/ml thiamine, 10 µg/ml each of thymine and uracil. The strain GS 245 did not grow in this minimal medium. However the strain GS 245 grew well when the minimal agar plates were supplemented with 200 µg/ml of glycine and serine.

The *gly*A complementation experiments were carried out by making GS 245 competent and transforming it with plasmids pWA 266 or pWA 406. These cells were plated out on the minimal plate with all the supplements (except for glycine and serine) and ampicillin. GS 245 containing plasmids pWA 266 or pWA 406 grew on ampicillin-containing minimal plates without glycine and serine supplementation. The negative control, GS 245 transformed with cosmid vector pHC 79, did not grow whereas GS 245 competent cells plated on minimal plates with all the supplements, including glycine and serine, grew well.

CHAPTER 6

IN SEARCH OF THE GENE THAT ENCODES THE 27-Kd DHPR FROM *ESCHERISCHIA COLI*

6.1 INTRODUCTION

In this study it has been shown that the subunit molecular weight of human DHPR and *E. coli* DHPR are about the same when their relative mobilities are compared on SDS-PA gel. The open reading frame for the human DHPR gene is 732 bp long (Dahl *et al.*, 1987; Lockyer *et al.*, 1987). Therefore, in the *simplest case* the gene encoding DHPR, including the transcription control regions, must be about 0.8 - 0.9 kb in length. The direct screening for increased DHPR specific activity (see Chapter 4) did not identify any clones that overexpressed the 27-kD protein, although one clone (WA 266) showed a 6-fold increase in DHPR specific activity that is probably due to overproduction of the 44-kD haemoprotein (see Chapter 5). The implication from the work in Chapters 4 and 5 is that the expression of the gene that encodes the 27-kD DHPR from *E. Coli* may be quite complex and perhaps it may be part of an operon.

The work outlined in this chapter decribes how the presence of the *hmp* and *gly*A genes in cosmid pWA 266 (and the sibling pWA 265) was exploited to set up methodology for isolating the gene that encodes the 27-kD DHPR from cosmid pools (see later in this Chapter). Six cosmid strains whose plasmids hybridized strongly with radiolabelled oligonucleotide D27-2 (synthetic mixed oligonucleotides that should be complementry to the 5' portion of the gene that encodes DHPR, as predicted from the protein sequence information) were selected for further study. The cell free extracts prepared from the six strains did not show significant increase in DHPR specific activity compared with the wild-type level. However, the strong hybridization reactions that were observed suggest that the gene may be present in these cosmids, but for some as yet unknown reason was not overexpressed. Nucleotide sequencing however would confirm this preliminary observation that the DHPR gene was present in the cosmid.

Table 6.1 The composition of the cosmid pools

Cosmid pool	Source strains	[DNA]µg/ml	Vol. used for DBH[a]
A	WA 100 - 112	116	1
B	WA 113 - 124	102	1
C	WA 125 - 136	43	2.5
D	WA 137 - 148	15	6
E	WA 149 - 160	32	4
F	WA 161 - 172	22	5
G	WA 173 - 184	61	2
H	WA 185 - 196	45	2.5
I	WA 197 - 208	38	2.5
J	WA 209 - 220	61	2
K	WA 221 - 232	33	3
L	WA 233 - 244	22	5
M	WA 245- 256	39	2.5
N	**WA 257 - 268**	**30**	**3.5**
O	WA 269 - 280	18	5
P	WA 281 - 292	11	10
Q	WA 293 - 304	38	2.5
R	WA 305 - 316	92	1
S	WA 317 - 328	97	1
T	WA 329 - 340	94	1
U	WA 341 - 352	78	1.5
V	WA 353 - 364	28	3.5
W	WA 365 - 376	115	1
X	WA 377 - 388	72	1.5
Y	WA 389 - 393	26	4
A to Y[b]		37	2.5

a. Volume used for dot blot hybridization.

b. 1.2µg of DNA from each pool was mixed to give the combined pool.

6.2 RESULTS AND DISCUSSION

6.2.1 Dot blot hybridization of cosmid pools

The cosmid pools (see Table 6.1) used in this study were made by Ms A. Hassan, Research School of Chemistry, ANU, using the cosmid library WA101 - WA 397 that was constructed in this study (see Section 6.4.1).

Preliminary dot blot hybridization was carried out to establish the methodology to identify new gene(s) from the cosmid pool using a radiolabelled mixed oligonucleotide probe that contained a sequence complementary to the gene predicted from the *N*-terminal protein sequence data. Since it was known that the cosmid pool N (pWA 257 - pWA 267) should hybridize with the oligonucleotide probe D45-1 because the *hmp* gene was present on pWA 266 (and the sibling pWA 265), it was used as a positive control. Cosmid pools N, O, P, Q, S and A - Y (mixture containing all the pools) were dot blot hybridized with D45-1 (method described in Section 6.4.2). Cosmid pool N showed a strong hybridization reaction whereas the cosmid pools O, P, Q, S and A - Y did not hybridize with D45-1. Taking this a step further, strain GS 245 that was made competent by $CaCl_2$ treatment, was transformed separately with cosmid pools N, O, P, Q, S or A - Y. The transformation mixture was plated out on minimal plates containing the necessary supplements, but without glycine and serine, to select for *gly*A complementation. There were about 50 transformants in the plate where the strain GS 245 was transformed with cosmid pool N and none for the other plates from transformations with cosmid pools O, P, Q, S or A - Y. This exploratory experiment clearly showed that dot blot hybridization with an appropriate oligonucleotide mixture was an ideal method for cloning new genes from the cosmid pool. Further, the complementation of the *gly*A mutation in the strain GS 245 by cosmid pool N confirmed that complementation of genetic traits could be used as a selection criterion for isolating a gene of interest. The cosmid pool designated A - Y did not hybridize with the oligonucleotide D45-1 and had no transformants in the complementation experiment despite the fact that pWA 266 (and the sibling pWA 265)

Figure 6.1 Screening cosmid pools by dot blot hybridization with ^{32}P labelled oligonucleotide D27-2

The dot blot hybridization was carried out as described in Section 6.4.2. The autoradiograph shows that cosmid pools P, Q, W and E hybridized with the probe DNA.

A B C D F G H I J K L M

N O P Q R S T U V W X Y

E A→Y

was present in it. This suggests that the size of the cosmid pool (i.e. the total number of different plasmids within a pool) may be a crucial factor.

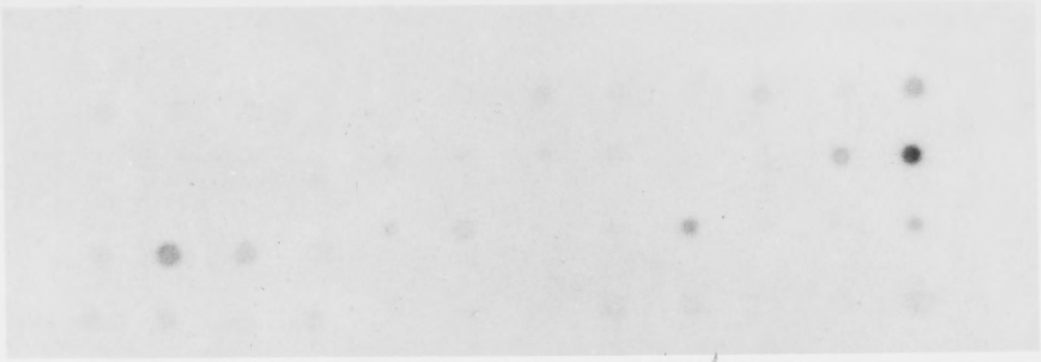
Dot blot hybridizations of the 25 cosmid pools (from A to Y) were carried out as described in Section 6.4.2, using γ-^{32}P-labelled mixed oligonucleotide D27-2 which contained the complementary sequence to the gene that encodes DHPR as predicted from the amino acid residues starting from position 14 to position 20 (Lys - Ala - Phe - Asp - Ala - Met - Lys; see Table 3.3). The cosmid pools E, P, Q and W (see Figure 6.1) hybridized with the probe DNA.

The first approach that was adopted to identify the plasmids within the pools that hybridized strongly with the probe DNA was to use the genetic complementation method. The rationale for using this approach came from the discovery in this study that *E. coli* DHPR can reduce 7,8-dihydrofolate. Since the cosmid library was made from the DHFR *minus (folA)* strain D 3-157, none of the plasmids within the cosmid pools would code for active wild-type DHFR. If the complete gene encoding *E. coli* DHPR was present in a plasmid and the product of the gene were sufficiently over-produced, then that plasmid may be able to complement the DHFR mutation in the *rec*A derivative of strain D3-157. The *rec*A derivative of strain D3-157 was made competent and transformed separately with cosmid pools E, P, Q or W and spread on minimal plates without any supplementation with folate dependent end-products such as methionine, glycine, thymidine, adenine and pantothenate. There were no transformants in any of the minimal plates, but there were >250 colonies when each transformation mixture was spread on LB plates containing ampicillin. Two positive controls were also carried out simultaneously by transforming the strain D3-157 separately with plasmids pJFM 8 or pR 67 [pJFM 8 contains the gene for chromosome-encoded DHFR described by Rood *et al.* (1980) and pR 67 contains the gene for type II R-plasmid encoded DHFR described by Smith *et al.* (1979); both were kind gifts from Dr J. F. Morrison]. Both these plasmids were able to complement the DHFR mutation, as judged by growth on minimal plates without folate dependent end products. However, the interesting implication from the positive controls is that the R-plasmid encoded DHFR which has a turnover number (catalytic efficiency)

Figure 6.2 Screening rapid lysates of strains that contributed to the cosmid pools E, P, Q and W, by dot blot hybridization.

Rapid lysates of strains that contributed to the cosmid pools were denatured and spotted on nitrocellulose filters (A and B). The filters were baked and then hybridized separately with D27-1 (A) and D27-2 (B). The six plasmids selected for further study based on the results in (A) and (B) were prepared in large scale as described in Section 4.5.13 and subjected to dot blot hybridization with the labelled probe D27-2 (C).

A



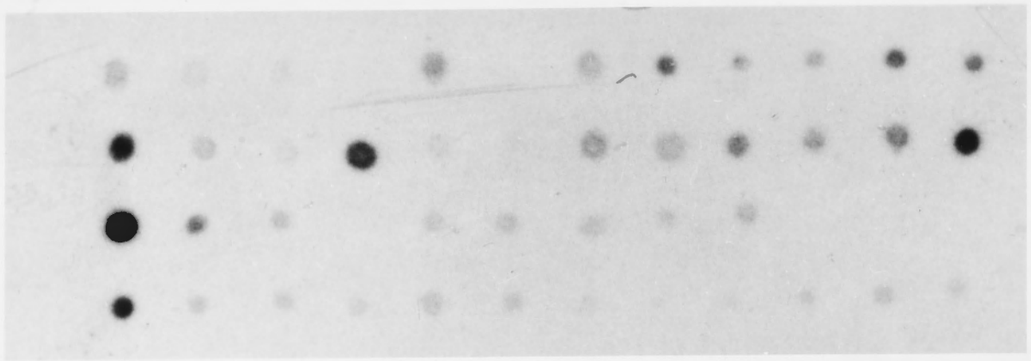
Plasmids pWA149 to pWA160

Plasmids pWA281 to pWA292

Plasmids pWA293 to pWA304

Plasmids pWA365 to pWA375

B



Plasmids pWA149 to pWA160

Plasmids pWA281 to pWA292

Plasmids pWA293 to pWA304

Plasmids pWA365 to pWA375

C

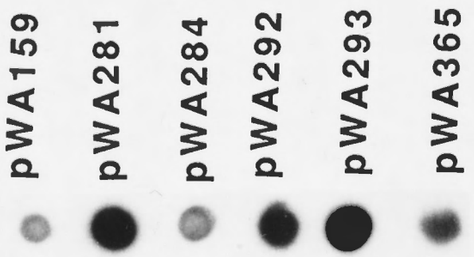


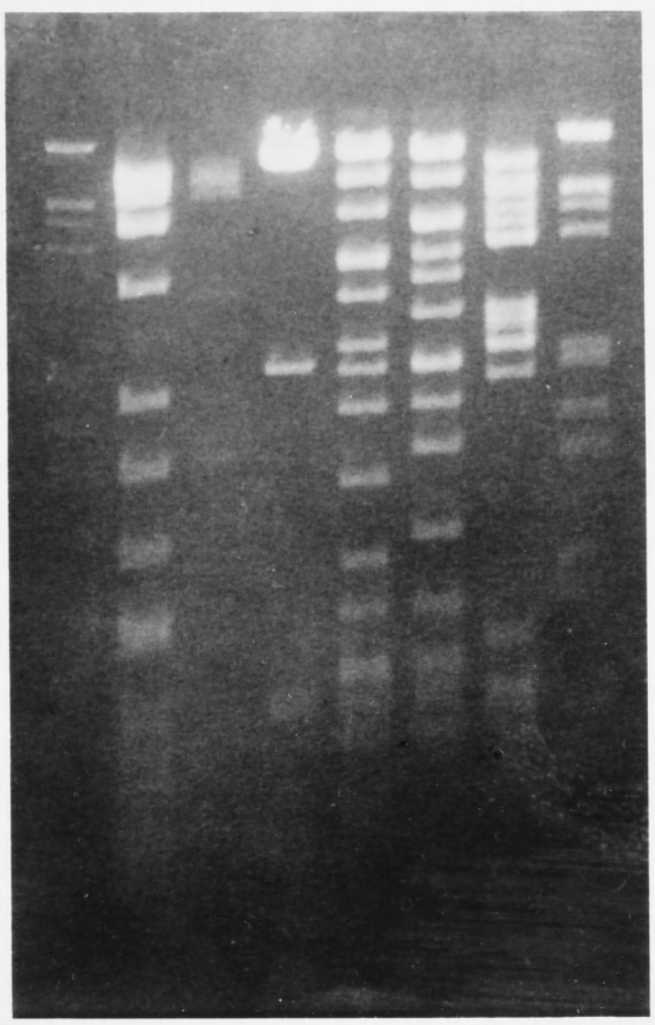
Figure 6.3 Agarose gel electrophoresis of plasmid DNA.

The plasmids pWA 159 (b), pWA 281 (c) pWA 284 (d), pWA 292 (e), pWA 293 (f) and pWA 365 (g) were digested completely with *Pvu* II and electrophoresed on a 0.7% (w/v) agarose gel (**A**), with λ DNA digested with *Eco* RI and *Hind* III, as size markers (a and h). (The size markers were end-filled in the presence of [α-^{32}P]-dATP, as described by Maniatis *et al.* 1982.)

The agarose gel (**A**) was blotted onto a nitrocellulose filter as described in Section 5.4.5.

(**B**) The nitrocellulose filter was baked and hybridized with labelled mixed oligonucleotides D27-2. The bands in (a) and (h) are the end-filled molecular weight markers. An ~ 1.4-kb fragment from plasmid pWA 159 hybridized strongly with the probe DNA, and an ~2.1-kb fragment from plasmid pWA 284 also hybridized significantly.

A


a b c d e f g h

B

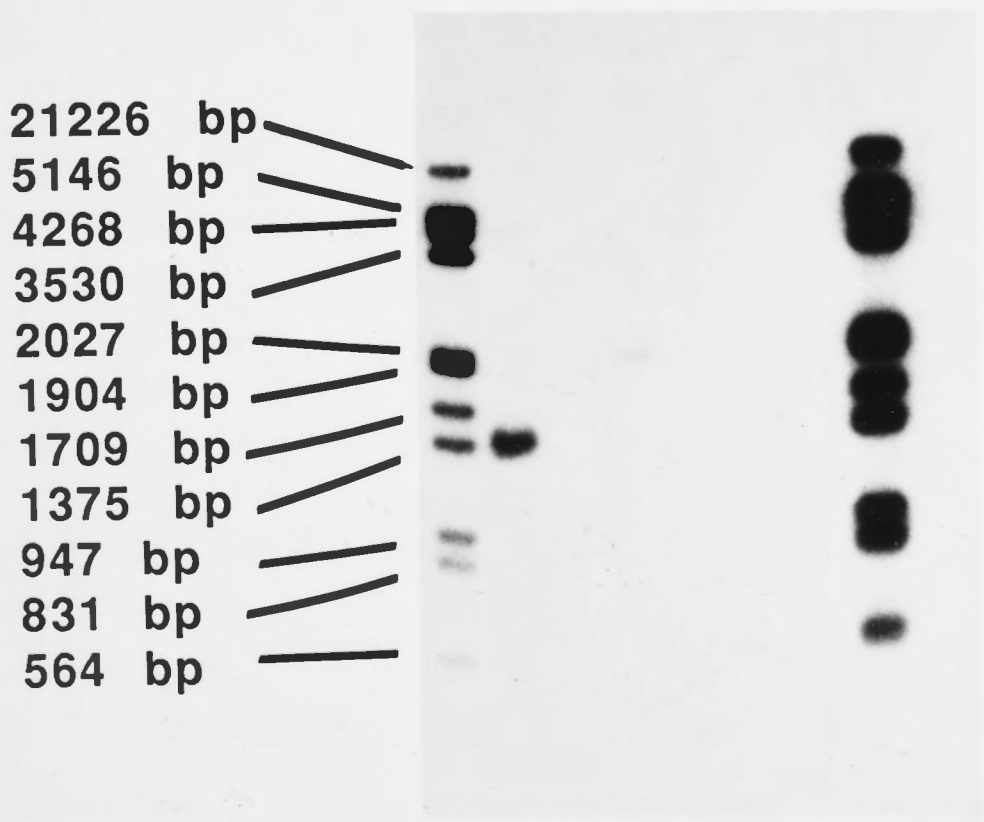

a b c d e f g h
21226 bp
5146 bp
4268 bp
3530 bp
2027 bp
1904 bp
1709 bp
1375 bp
947 bp
831 bp
564 bp

Table 6.2 Direct screening of DHPR specific activity[a,b]

Strain	Specific activity
DM 7111[c]	0.056 U/mg
WA 159	0.085 U/mg
WA 281	0.126 U/mg
WA 284	0.061 U/mg
WA 292	0.082 U/mg
WA 293	0.080 U/mg
WA 365	0.060 U/mg

a. The DHPR assay was carried out as described in Section 2.5.1.1 at 25°C in Tris/HCl pH 7.4

b. Protein estimations were performed using the Bio-Rad method (Bradford, 1976).

c. The background strain containing no cosmids.

of 0.5 s^{-1} (J. F. Morrison, personal communication) was able to complement the DHFR mutation in D3-157. From the data presented in Table 3.1, it was calculated that the turnover number for the reduction of 7,8-dihydrofolate by *E. coli* DHPR is also about 0.5 s^{-1}. Although the catalytic efficiencies of these two enzymes appear to be similar in the *in vitro* situation, their efficiency *in vivo* may be dependent on several factors.

The source strains for the cosmid pools E, P, Q and W (WA 149 - 160, WA 281 - 304 and WA 365 - 376) were grown on LB plates containing ampicillin. Small scale plasmid DNA preparations (as described in Section 4.5.14) of these strains were dot blot hybridized separately with the synthetic oligonucleotides D27-1 (predicted from the first seven *N*-terminal amino acid residues of *E. coli* DHPR; see Tabel 3.3) or D27-2. Plasmids pWA 159, pWA 281, pWA 284, pWA 292, pWA 293 and pWA 365 showed strong hybridization response with the probe D27-2 and only plasmid pWA 293 hybridized significantly with the probe D27-1 (see Figure 6.2). It was felt from the onset that the probe D27-1 may not be ideal because it was predicted from a short sequence where the assignment of two residues (at position 2 and 5) were uncertain (see Table 3.3). However, large scale plasmid preparations of the plasmids pWA 159, pWA 281, pWA 284, pWA 292, pWA 293 and pWA 365 were made and dot blot hybridizations with D27-2 showed that all of them hybridized strongly even after more stringent washing procedures. The six plasmids were digested completely with the restriction endonuclease *Pvu* II and analysed on a 0.7% (w/v) agarose gel (see Figure 6.3A). The restriction pattern of plasmids pWA 281 and pWA 284 were not clear because in the former, insufficient DNA was present (the DNA yield from strain WA 281 was very low, hence an ethanol-precipitated fraction was used for the restriction analysis without quantifying the DNA) and the latter may have been only partially digested. Plasmids pWA 292 and pWA 293 shared several bands in common but were not siblings. The fragments from the agarose gel were transferred onto a nitrocellulose filter and hybridized with the γ-^{32}P-labelled probe D27-2 (see Figure 6.3B). A 1.4-kb *Pvu*II fragment from pWA 159 hybridized strongly with the probe and also another approximately 2-kb fragment from plasmid pWA 284 showed moderate hybridization. The result from the Southern blot

hybridization is quite ambiguous because none of the fragments from plasmids pWA 292, pWA 293 or pWA 365 hybridized with the probe, whereas the plasmid dot blots showed strong hybridization signals (see Figure 6.2C).

6.2.2 Direct screening for increased DHPR specific activity

Cell free extracts were prepared from the strains that hybridized with the probe D27-2 and their DHPR levels were compared with the background level of DHPR in strain DM 7111, that was similarly prepared. The results (see Table 6.2) indicated that the DHPR specific activity in strain WA 281 is more than two fold higher than the background level and that of strains WA 292 and WA 293 were only marginally higher. Samples of the cell free extracts were analysed by SDS-PAGE to check if any apoprotein was being made (see Figure 6.4). There were no indications of high levels of protein around molecular weight of 27 000 being synthesized in any of the strains.

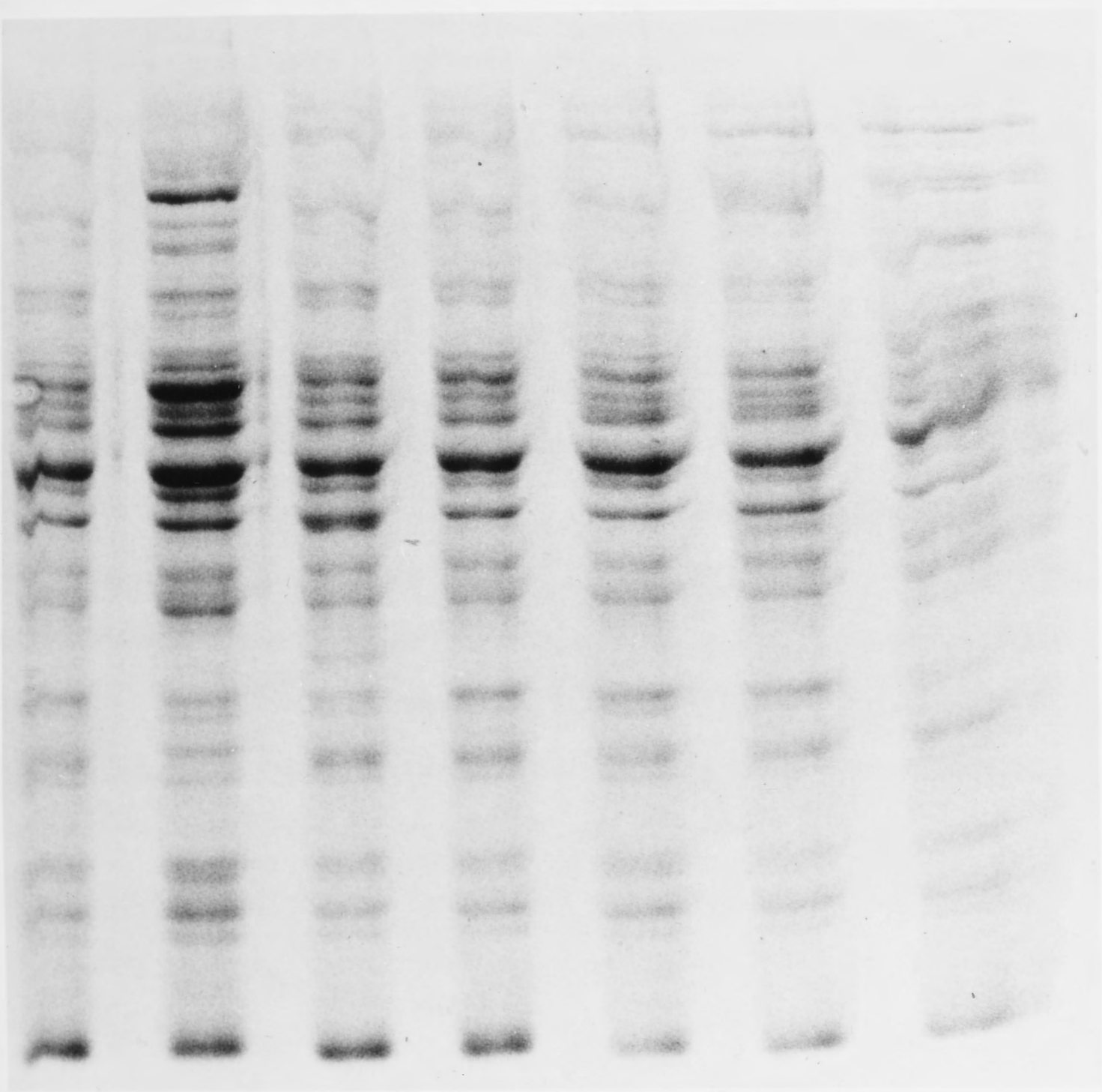
6.3 Summary

The strains that were identified as possible *E. coli* DHPR clones on the basis of strong hybridization signal with the γ-^{32}P labelled D27-2 probe, did not show any increase in DHPR specific activity. Neither the possible clone colonies nor the extracts prepared from them were yellow, as would have been expected in the event of increased expression of a protein containing a flavin prosthetic group. The *N*-terminal amino acid sequence of *E. coli* DHPR (position 14 to 20), which was used to predict the sequence for the oligonucleotide probe D27-2, were unambiguous assignments. Also the sequence of the amino acids (Lys - Ala - Phe - Asp - Ala - Met - Lys) was ideal for probe synthesis because of the limited codons that code for several of amino acid residues such as lysine, phenylalanine, aspartate and methionine (Grantham *et al.*, 1981). Based on the experience with the probing and ultimate sequencing of the 5' region of the *hmp* gene (described in Chapter 5), it is felt that the gene that codes for *E. coli* DHPR may be present in some of the plasmids that hybridized strongly with the DNA probe. However

Figure 6.4 Polyacrylamide gel electrophoresis of cell-free extracts from strains AN 1459 (1), WA 159 (2), WA 281 (3), WA 284 (4), WA 292 (5), WA 293 (6) and WA 365 (7).

Cell free extracts from the strains (100 μg each) were electrophoresed on a SDS-PA gel as described in Section 2.5.3.1. The arrowhead indicates the position of the 27-kD *E. coli* DHPR (not shown). There is no sign of any significant increase in the levels of the 27-kD protein in the cell-free extracts of the strains whose plasmids hybridized strongly with the oligonucleotide probes, compared with the wild type strain AN 1459.

1 2 3 4 5 6 7



this can only be proved by nucleotide sequencing after subcloning the plasmids identified in this study.

6.4 METHODS

6.4.1 Preparation of cosmid pools

The preparation of cosmid pools was carried out by Ms A. Hassan and were a kind gift from Dr N. E. Dixon.

The strains WA 101 - WA 393 that were stored at -70°C in 7% DMSO, were streaked on 8-sectored LB plates containing ampicillin. A loopful of colony from each sector was used to inoculate 20 ml of LB medium containing ampicillin and grown overnight. Cultures were pooled in groups of 12 and used to prepare plasmid DNA as described in Section 4.5.13.

6.4.2 Dot blot hybridization of cosmid pools

The general method described in Section 5.4.8 was applied for the dot blot hybridization of the cosmid pools. About 1 µg of DNA from each pool (see the volumes used in Table 6.1) was made up to 10 µl in TE and placed in a 96-well tissue culture plate (Linbro, Virginia, USA). The denaturation buffer (10 µl each; 1 M NaOH, 2 M NaCl) was added to each of the wells containing the individual cosmid pools and incubated at room temperature for 30 min. The denatured cosmid pools were spotted (4 µl/spot) onto nitrocellulose sheet placed on a clean 96-well tissue culture plate over a light box to guide the spotting process. The nitrocellulose filter was dried and treated as described in Section 5.4.6.

6.4.3 Complementation of *fol* mutation in strain D3-157

Competent cells of the *rec*A derivative of D 3-157 were made by the $CaCl_2$ method described in Section 4 and transformed with the appropriate test plasmids and control plasmids. The transformation mixtures were spread on minimal plates that contained the essential salts, 0.2% glucose, tryptophan, tyrosine, histidine, guanine (50 µg/ml each) guanine and thiamine (10 µg/ml). The strain D 3-157 did not grow in this minimum plate but when plasmids pJFM 8 [Ap^r, $DHFR^+$ (chromosomal)] or pR 67 (type II R-plasmid $DHFR^+$) were used to transform the strain, they were able to grow. The strain D 3-157 was able to grow when the above minimal agar was supplemented with methionine, glycine, thymidine (50 µg/ml each), adenine (20 µg/ml) and pantothenate (1 µg/ml).

CHAPTER 7
GENERAL DISCUSSION

The work described in this thesis has shown that DHPR from *E.coli* is a 27 kD flavoprotein. Apart from the 27-kD enzyme, another protein present in the cell-free extract prepared from strain WA 266 showed quinonoid dihydropteridine-dependent oxidation of NADH. The results from the experiments carried out in this work imply that the 44-kD haemoprotein is probably the second protein with DHPR activity. Although further work is required to confirm this, the reasons for the present belief will be discussed later.

This is the first report of a DHPR with a flavin (FAD) prosthetic group. All the DHPRs that were characterised previously are oxido-reductases that carry out the reduction of quinonoid dihydropterin by transferring hydride from the pyridine nucleotide to position N-5 of the pterin ring. The physical and kinetic properties of the reductase from *E.coli* are somewhat different from those of the mammalian reductases and may account for the reasons why it did not bind to the naphthoquinone affinity column. The reductases that bind to the affinity column are believed to do so by interaction of the pterin binding site of the conformationally altered NADH-enzyme complex with the affinity adsorbent. However, in the case of the 27-kD flavoprotein, the catalytic reaction probably involves a mechanism where the conformational change may not take place after the binding of NADH, because its function is to reduce the FAD prosthetic group. Also, the Km for the pterin substrates are much higher (~ 0.1 - 1.5 mM) for the *E.coli* DHPR, compared with the mammalian enzymes (which have values in the lower μM range). Hence the bacterial reductase does not associate strongly with the affinity ligand. The interaction between NAD(P)H and the flavoprotein is not very strong either and may account for the inability of the reductase to bind to the dye affinity columns.

The active-site region of *E.coli* DHPR has not been studied in detail, but the fact that the reductase is inactivated by DTT suggests that there may be crucial redox-active disulfide bond(s) near the catalytic site or in such proximity as to affect this site. The

presence and the function of the disulfide bonds need to be investigated further in future work.

It was pointed out in earlier chapters that the difference between the DHPR and DHFR reactions lies in the positions of the pterin ring (N-5 and C-6 respectively) to which the hydride from the reduced pyridine nucleotide is transferred during the catalysis. The 27-kD flavoprotein, which is clearly the protein responsible for the quinonoid dihydropterin-dependent oxidation of NADH, apparently does not distinguish between the positions N-5 and C-6, hence it can carry out both DHPR and DHFR reactions with the appropriate substrates. Perhaps this flavoprotein may be the evolutionary precursor of DHPRs and DHFRs that do not possess any prosthetic. It is also tempting to suggest that if DHPR or DHFR (without any prosthetic group) is altered by introduction of a flavin moiety near its active site by protein engineering, then the resultant semi-synthetic protein will be able to carry out the reduction catalysed by both enzymes. In this respect, Kokubo *et al.* (1987) have shown that it is possible to convert a haemoglobin to a flavohaemoglobin, where the latter can perform a new catalytic function.

The 27-kD DHPR from *E.coli* is similar to the reductase from *Pseudomonas* species in several respects. Both showed similar kinetic parameters with various pterin substrates (with respect to NADH), except that the *Pseudomonas* enzyme was almost inactive with NADPH. In both the organisms, monapterin was apparently the naturally occurring pterin substrate. The DHPR from *Pseudomonas* carries out the known function of recycling the pterin cofactor for phenylalanine hydroxylase which occurs in that organism. In the case of DHPR from *E.coli*, an *in vivo* function is still obscure because this microorganism does not have the aromatic amino acid hydroxylases. However, the similarities between the two bacterial reductases suggest that DHPR from *E.coli* must be the enzyme that reduces quinonoid dihydropterins from as yet uncharacterised systems which use a tetrahydropterin cofactor.

Attempts to clone the gene that encodes the 27-kD DHPR resulted in the cloning of the *glyA* gene and the newly identified *hmp* gene. Serine hydroxymethyl transferase is the product of the first gene, and is a crucial enzyme in the one-carbon metabolic pathway. The physiological reaction catalysed by SHMT is the interconversion of serine and glycine with tetrahydrofolate serving as the one-carbon carrier. In addition, SHMT can also catalyse other reactions such as decarboxylation of aminomalonate, aldol cleavage of allothreonine and transamination of D-alanine, because it is a pyridoxal phosphate-containing enzyme (Shostak and Schirch, 1988). If SHMT were responsible for the 6-fold increase in DHPR activity in the extract from strain WA 266, then it is not clear why the activity was lost during the purification. The notion that the cofactor that was required for the reductase was lost during the purification was discounted because the addition of freshly prepared extracts did not restore the DHPR activity in the partially-purified protein samples.

The more favoured candidate that may be responsible for the enhanced DHPR activity in the extract from strain WA 266 is the 44-kD haemoprotein which is encoded by the *hmp*gene. The *N*-terminal amino acid sequence of this protein showed extensive homology with the corresponding region of the haemoglobin from *Vitreoscilla.* In addition to the sequence data presented in Figure 5.14, the 44-kD haemoprotein also has a glutamine residue at the corresponding helix position E7 (N.E.Dixon, personal communication). This provides further evidence that it is related to haemoglobin-like proteins. The glutamine residue at that position is though to hydrogen bond with the oxygen molecule as in the case of myoglobin (Phillips and Schoenborn, 1981). There is no direct evidence for a distinct function for the 44-kD haemoprotein as yet. However, it appears to have a pterin binding site and seems very likely to be responsible for the enhanced DHPR activity. SHMT on the other hand is a well characterised protein, and from the extensive knowledge of its mechanism of action it is difficult to see how it could act as a reductase.

The molecular weight and spectral properties of the 44-kD haemoprotein are similar to those of the oxygen-binding flavohaemoprotein from the hydrogen bacterium *Alcaligenes eutrophus* (Probst *et al.*, 1979). The yellow colour of the extract from strain WA 266 and the partially purified protein is an indication that the haemoprotein from *E.coli* may also contain FAD. This haemoprotein also resembles the flavohaemoglobin from the yeast *Candida mycoderma* in molecular weight and spectral properties.

An interesting speculation at this point is that the 44-kD haemoprotein is a bifunctional protein. One half of the protein may be involved in the storage of oxygen, which is transported to the haeme site by a tetrahydropterin as a hydroperoxy group attached to the '4a' bridgehead position of the pterin ring (compare with Armarego, 1984). After the removal of the hydroperoxy group, the pterin substrate becomes oxidised to the quinonoid dihydropterin which is recycled by the reductase portion of the haemoprotein. This hypothetical model requires rigorous experimental evaluation. It may shed light on the *in vivo* physiological role for the protein. Wood (1984) classified the haemoglobin from *Vitreoscilla* and the flavohaemoprotein from *A.eutrophus*, together with a few other haem-containing proteins with similar spectral properties, as *putative oxygen buffers*. The 44-kD haemoprotein from *E.coli* may well have a similar function. The haemoprotein from this study is expressed at low levels in the wild-type bacteria and has escaped discovery despite exhaustive investigation. If this is meaningful, then it may well act as a biological switch for some unknown reactions.

REFERENCES

Abell, C. W., Shen, R.-S., Gessner, W. and Brossi, A. (1984) *Science* **224**, 405-407.

Altman, P., Salihi, F. Al-., Butter, K., Cutler, P., Blair, J., Leeming, R., Cunningham, J. and Marsh, F. (1987) *N. Engl. J. Med.* **317** 80-84.

Archer, M. C. and Scrimgeour, K. G. (1970) *Can. J. Biochem.* **48** 278-287.

Archer, M. C., Vonderschmitt, D. J. and Scrimgeour, K. G. (1972) *Can. J. Biochem.* **50**, 1174-1182.

Armarego, W. L. F. (1979) *Biochem. Biophys. Res. Commun.* **89**, 246-249.

Armarego, W. L. F. (1984) *Lects. Heterocycl. Chem.* **7**, 121-130.

Armarego, W.L.F. (1987) In: Papers dedicated to Professor W.Pfleiderer's 60th Birthday.

Armarego, W. L. F. and Ohnishi, A. (1987) *Eur. J. Biochem.* **164**, 403-409.

Armarego, W. L. F., Ohnishi, A. and Taguchi, H. (1986) *Biochem. J.* **234**, 335-342.

Armarego, W. L. F., Randles, D., and Taguchi, H. (1983) *Biochem. J.* **211**, 357-361.

Armarego, W. L. F., Randles, D. and Waring, P. (1984) *Med. Res. Rev.* **4**, 267-321.

Armarego, W. L. F. and Schou, H. (1978) *Aust. J. Chem.* **31**, 1081-1094.

Armarego, W. L. F. and Waring, P. (1982) *J.C.S. Perkin Trans.* **2**, 1227-1233.

Armarego, W. L. F. and Waring, P. (1983) In: *Chemistry and Biology of Pteridines* (Blair, J. A., ed.), W. de Gruyter, Berlin, pp. 429-433.

Bartholome, K. (1974) *Lancet* **2**, p. 1580.

Benkovic, S. J. (1980) *Ann. Rev. Biochem.* **49**, 227-251.

Benkovic, S. J., Sammons, D., Armarego, W. L. F., Waring, P. and Inners, R. (1985) *J. Am. Chem. Soc.* **107**, 3706.

Bickel, H., Gerrard, J. and Hickman, E. M. (1953) *Lancet* **2**, p. 812.

Biellmann, J. F., Samama, J. P., Branden, C. I. and Eklund, H. (1979) *Eur. J. Biochem.* **102**, 107-110.

Blakley, R. L. (1984) In: *Folates and Pterins,* vol. 1 (Blakley, R. L. and Benkovic, S. J., eds.), Wiley-Interscience, pp. 191-254.

Blau, N. and Niederwieser, A. (1983) *Anal. Biochem.* **128**, 446-452.

Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, J. H. and Falkow, S. (1977) *Gene* **2**, 95-113.

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

Carter, S. K., Bakowski, M. T. and Hellman, K. (1981) *Chemotherapy of Cancer, 2nd edn.*, John Wiley and Sons, New York, p. 379.

Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132-135.

Chauvin, M. M., Korri, K. K., Tirpak, A., Simpson, R. C. and Scrimgeour, K. G. (1979) *Can. J. Biochem.* **57**, 178-187.

Cheema, S., Soldin, S. J., Knapp, A., Hofmann, T. and Scrimgeour, K. G. (1973) *Can. J. Biochem.* **51**, 1229-1239.

Cornish-Bowden, A. and Endrenyi, L. (1981) *Biochem. J.* **193**, 1005-1008.

Cotton, R. G. H. (1985) In: *Folates and Pterins,* vol. 3, (Blakley, R. L. and Whitehead, V. M., eds.), Wiley-Interscience, New York, pp. 359-412.

Cotton, R. G. H., Hutchinson, W. M., Wake, S., Jennings, I. G., McAdam, W. J., Danks, D. M. and Dahl, H. -H. M. (1987) In: *Unconjugated Peterins and Related Biogenic Amines* (Curtis, H. -Ch., Blau, N. and Levine, R. A. , eds.), Walter de Gruyter, Berlin, pp. 275-281.

Cotton, R. G. H. and Jennings, I. (1978) *Eur. J. Biochem.* **83**, 319-324.

Cotton, F. A. and Wilkinson, G. (1980) *Advanced Inorganic Chemistry,* 4th edn., Wiley, New York.

Craine, J. E., Hall, E. S. and Kaufman, S. (1972) *J. Biol. Chem.* **247**, 6082-6091.

Curtius, H. -CH., Heintel, D., Ghisla, S., Kuste, TH., Leimbacher, W. and Niederwieser, A. (1985) *J. Inher. Metab. Dis.* **8 Suppl. 1** pp. 28-33.

Dahl, H. -H. M., Hutchinson, W., McAdam, W., Wake, S., Morgan, F. J. and Cotton, R. G. H. (1987) *Nucl. Acids Res.* **15**, 1921-1936

Dahl, H. -H. M., Wake, S., Cotton, R. G. H. and Danks, D. M. (1988) *J. Med. Genet.* **25**, 25-28.

Danks, D. M., Bartholome, K., Clayton, B. E., Curtius, H., Grobe, H., Kaufman, S., Leeming, R., Pfleiderer, W., Rembold, H. and Rey, F. (1978) *J. Inher. Metab. Dis.* **1** 49-53.

Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404-427.

Davis, N. K., Greer, S., Jones-Mortimer, M. C. and Perham, R. N. (1982) *J. Gen. Microbiol.* **128**, 1631-1634.

Davis, M. D., Kaufman, S. and Milstein, S. (1983) *Eur. J. Biochem.* **173**, 345-351.

Davison, A. N. (1973) In: *Inborn Errors of Metabolism* (Hommes, F. A. and Van den Berg, C. J., eds.), Academic, New York, p. 55, p. 67.

Desa, R. J. (1976) In: *Flavins and Flavoproteins,*, Proceedings of the 5th International Symposium (Singer, T. P., ed.), Elsevier, Amsterdam, pp. 720-725.

Dewey, C. V. and Kidder, G. W. (1971) *Methods Enzymol.*. **XVIIIB**, 618-624.

DiLella, A. G., Huang, W. M. and Woo, S. L. C. (1988) *The Lancet*, March 5, pp. 497-499.

Dixon, M. and Webb, E. C. (1979) In: *Enzymes* (3rd edn.), Academic Press, New York, pp. 479-485.

Dixon, N. E., Argall, M. E., Elvin, C. E., Hendry, P. and Lilley, P. E. M. (1988) *Proceedings of the Thirteenth Annual Conference on Protein Structure and Function,* Lorne, Australia.

Dougan, G. and Kehoe, M. (1984) *Methods Microbiol.* **17**, 233-258.

Dougan, G. and Sherrat, D. (1977) *Mol. Gen. Genet.* **151**, 151-160.

Duch, D. S., Lee, C. L., Edelstein, M. P. and Nichol, C. A. (1983) *Molec. Pharmacol.* **24**, 103-108.

Embden, G. and Baldes, K. (1913) *Biochem. Z.*, **55**, 301-322.

Engvall, E. (1980) *Methods Enzymol.* **70**, 419-439.

Fan, C. L. and Brown, G. M. (1976) *Biochem. Genet.* **14**, 259-270.

Ferscht, A. (1985a) *Enzyme Structure and Mechanism,* 2nd edn., W. H. Freeman & Co., New York, pp. 92-94.

Ferscht, A. (1985b) *Enzyme Structure and Mechanism,* 2nd edn., W. H. Freeman & Co., New York, pp. 260-261.

Firgaira, F. A., Cotton, R. G. H. and Danks, D. M. (1981a) *Biochem. J.* **197**, 31-43.

Firgaira, F. A., Choo, K. H., Cotton, R. G. H. and Danks, D. M. (1981b) *Biochem. J.* **197**, 45-53.

Folling, A. (1934) *Z. Physiol. Chem.* **227**, 169-176.

Friedman, P. A., Kappelman, A. H. and Kaufman, S. (1972) *J. Biol. Chem.* **247**, 4165-4173.

Fukushima, T. and Nixon, J. C. (1980) *Anal. Biochem.* **102**, 176-188.

Fuller, R. S. and Kornberg, A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5817-5821.

Fuller, R. C. and Nugent, N. A. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 1311-1318.

Georgiou, C. D. and Webster, D. A. (1987) *Biochemistry* **26**, 6521-6526.

Gibson, F. and Pittard, J. (1968) *Bacteriol. Rev.* **32**, 465-492.

Glazer, A. N., De Lange, R. J. and Sigman, D. S. (1976) In: *Lab. Tech. Biochem. Mol. Biol.*. vol. 4, (Work, T. S. and Work, E., eds.), Elsevier, New York, pp. 110-120.

Gouterman, M. (1978) In: *The Porphyrins*, vol. 3, (Dolphin, D., ed.), Academic Press, New York, pp. 12.

Grantham, R., Gautier, C., Gouy, M., Jacobzone, M. and Mercier, R. (1981) *Nucl. Acids Res.* **9**, 43-74.

Green, G. N., Fang, H., Lin, R. -J., Newton, G., Mather, M., Georgiou, C. D. and Gennis, R. B. (1988) *J. Biol. Chem.* **263**, 13138-13143.

Gunlack, B. G., Neal, G. E. and Williams, D. C. (1966) *Biochem. J.* **101**, 20.

Guroff, G. and Rhoads, C. A. (1969) *J. Biol. Chem.* **244**, 142-146.

Guthrie, R. and Susi, A. (1963) *Pediatrics*, **32**, 338-343.

Harono, T. (1972) *Insect. Biochem.* **2**, 385-398.

Hasegawa, H. (1977) *J. Biochem.* **81**, 169-177.

Hasegawa, H., Imaizumi, S., Ichiyama, A., Sugimoto, T, Matsuura, S., Oka, K., Kato, T., Nagatsu, T. and Akino, M. (1979) In: *Chemistry and Biology of Pteridines,* (Kislink, R. L. and Brown, G. M., eds.), Elsevier, Amsterdam, pp. 183-188.

Hemmerich, P. (1963) In: *Proceedings of the Third International Pteridine Symposium,* Pergamon Press, London, pp. 143-161.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E. and Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990-7997.

Hohn, B. and Collins, J. (1980) *Gene* **11**, 291-298.

Hohn, B. and Murray, K. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3259-3263.

Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J. and Kraut, J. (1986) *Science* **231**, 1123-1128.

Huang, C. Y., Max, E. E. and Kaufman, S. (1973) *J. Biol. Chem.* **248**, 4235.

Hunkapiller, M. W. and Hood, L. E. (1983) *Meth. Enzymol.* **91**, 486-494.

Ingledew, W. J. and Poole, R. K. (1984) *Microbiol. Rev.* **48**, 222-271.

Ishida, A. (1977) *J. Biochem (Tokyo),* **81**, 1869-1878.

Kapatos, G. and Kaufman, S. (1981) *Science* **212**, 955-956.

Kaufman, S. (1957) *J. Biol. Chem.* **226**, 511-524.

Kaufman, S. (1958a) *J. Biol. Chem.* **230**, 931-939.

Kaufman, S. (1958b) *Biochim. Biophy. Acta* **27**, 428-429.

Kaufman, S. (1959) *J. Biol. Chem.* **234**, 2677-2682.

Kaufman, S. (1961) *J. Biol. Chem.* **236**, 804-810.

Kaufman, S. (1963) *Proc. Natl. Acad. Sci. U.S.A.* **50**, 1085-1093.

Kaufman, S. (1964) *J. Biol. Chem.* **239**, 332-338.

Kaufman, S. (1967a) *Ann. Rev. Biochem.* **36**, 171-184.

Kaufman, S. (1967b) In: *Phenylketonuria and Allied Metabolic Diseases: Conference Proceedings,* (Anderson, J. A. and Swaiman, K. F., eds.), Washington D.C., pp. 205-213.

Kaufman, S. (1979) *J. Biol. Chem.* **254**, 5150-5154.

Kaufman, S., Bridges, W. F., Eisenberg, F. and Friedman, S. (1962) *Biochem. Biophys. Res. Commun.* **9**, 497-502.

Kaufman, S., Holtzman, N. A., Milstein, S., Butler, I. J. and Krumholz, A. (1975) *New Eng. J. Med.* **293**, 785-790.

Khosla, C. and Bailey, J. E. (1988) *Nature* **331**, 633-635.

Kidder, G. W. and Nolan, L. L. (1973) *Biochem Biophys. Res. Comm.* **53**, 929-936.

Kohara, Y., Akiyama, K. and Ison, K. (1987) *Cell* **50**, 495-508.

Kokubo, T., Sassa, S. and Kaiser, E.T. (1987) *J. Am. Chem. Soc.* **109**, 606-607.

Konecki, U. -L., Konecki, D. S., DiLella, A. G., Brayton, K., Marvit, J., Hahn, T. M., Trefz, F. K. and Woo, S. L. C. (1988) *Biochemistry* **27**, 2881-2885.

Korri, K. K., Chippel, D., Chauvin, M. M., Tirpak, A. and Scrimgeour, K. G. (1977) *Can. J. Biochem.* **55**, 1145-1152.

Kwok, S. C., Ledley, F. D., DiLella, A. G., Robson, K. J. and Woo, S. L. C. (1985) *Biochemistry* **24**, 556-561.

Lamba, P. and Webster, D. A. (1980) *J. Bacteriol.* **142**, 169-173.

Lang, K. and Westphal, U. (1942) *Z. Physiol. Chem.* **276**, 179.

Lazarus, R. A., Benkovic, S. J. and Kaufman, S. (1983) *J. Biol. Chem.* **258**, 10960.

Lazarus, R. A., DeBrosse, C. W. and Benkovic. S. J. (1982) *J. Am. Chem. Soc.* **104**, 6869-6871.

Lederberg, E. M. and Cohen, S. N. (1974) *J. Bacteriol.* **119**, 1072-1074.

Leeming, R. J. and Blair, J. A. (1979) *Lancet* **1**, 556.

Lockyer, J., Cook, R. G., Milstein, S., Kaufman, S., Woo, S. L. C. and Ledley, F. D. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3329-3333.

Mager, H. I. X. (1975) In: *Chemistry and Biology of Pteridines* , (Pfleiderer, W., ed.), W. de Gruyter, Berlin, pp. 753-773.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular cloning: a laboratory manual*, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) *Anal. Biochem.* **87**, 206-210.

Masters, J. N. and Attardi, G. (1983) *Gene* **21**, 59-63.

Matthews, D. A., Smith, S. L., Baccanari, D. P., Burchall, J. J., Oatley, S. J. and Krant, J. (1986) *Biochemistry* **25**, 4194.

Maycock, A. L., Abels, R. H., Salach, J. L. and Singer, T. P. (1976) *Biochemistry* **15**, 114-118.

McCormack, J. J. (1981) *Med. Res. Rev.* **1**, 303-331.

Milstein, S. and Kaufman, S. (1975) *Biochem. Biophys. Res. Commun.* **66**, 475-481.

Milstein, S. and Kaufman, S. (1987) *N. Engl. J. Med.* **317**, 1605.

Monod, J., Cohen-Bazire, G. and Cohn, M. (1951) *Biochim. Biophys. Acta* **7**, 585-599.

Morrison, J. F. (1988) In: *Study of Enzymes; Mechanism of Enzyme Action: A study from selected examples,* CRC Press, Florida, Chapter 4.B.

Morton, R. A. (1975) *Biochemical Spectroscopy,* Adam Hilger, London, pp. 427-435.

Mudd, S.H. and Cantoni, G.L. (1964) In: *Comprehensive Biochemistry*, **Vol. 15**, (Florkin, M. and Stotz, E. H., eds) Elsevier, Amsterdam, pp. 1-47.

Nagatsu, T., Levitt, M. and Udenfriend, S. (1964) *J. Biol. Chem.* **239**, 2910-2917.

Nakanishi, N., Hasegawa, H., Yamada, S. and Akino, M. (1986a) *J. Biochem. (Tokyo)* **99**, 635-644.

Nakanishi, N., Hasegawa, H., Akino, M. and Yamada, S. (1986b) *J. Biochem. (Tokyo),* **99**, 645-652.

Nichol, C. A., Smith, G. K. and Duch, D. S. (1985) *Ann. Rev. Biochem.* **54**, 729-764.

Niederwieser, A., Blau, N., Wang, M., Joller, P., Atares, M. and Cardes-Gareit, J. (1984) *Eur. J. Pediatr.* **141**, 208-214.

Nielsen, K. H., Simonsen, V. and Lind, K. E. (1969) Eur. J. Biochem. **9**, 497-502.

Nixon, P. F. and Blakley, R. L. (1968) *J. Biol. Chem.* **243**, 4722-4731.

Oakley, B. R., Kirsch, D. R. and Morris, N. R. (1980) *Anal. Biochem.* **105**, 361-363.

O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007-4021.

Old, R. W. and Primrose, S. B. (1985) *An Introduction to Genetic Engineering*, 3 edn., Blackwell Scientific Publications, London, pp 85-90.

Orii, Y. and Webster, D. A. (1986) *J. Biol. Chem.* **261**, 3544-3547.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321-349.

Oshino, R., Asakura,T., Tajio, K., Oshino, N. and Chance, B. (1973) *Eur. J. Biochem.* **39**, 581-590.

Pascal, Jr., R. A., LeTrang, N., Cerami, A. and Walsh, C. (1983) *Biochemistry* **22**, 171-178.

Pattishall, K. H., Acar, J., Burchall, J. J., Goldstein, F. W. and Harvey, R. H. (1977) *J. Biol. Chem.* **252**, 2319-2323.

Perutz, M. F. (1986) *Nature* **322**, 405.

Perutz, M. F., Kendrew, J. C. and Watson, H. C. (1965) *J. Molec. Biol.* **13**, 669-678.

Peterkofsky, B., Kalwinsky, D., Assad, R. (1980) *Arch. Biochem. Biophys.* **199**, 362-373.

Pfleiderer, W. (1964) *Angew . Chem., Int. Ed. Engl.* **3**, 114.

Phillips, S. E. V. and Schoenborn, B. (1981) *Nature* **292**, 81-82.

Plamann, M. D. and Stauffer, G. V. (1983) *Gene* **22**, 9-18.

Plamann, M. D., Stauffer, L. T., Urbanowski, M. L. and Stauffer, G. V. (1983) *Nucl. Acids Res.* **11**, 2065-2075.

Poddar, S. and Henkin, J. (1984) *Biochemistry* **23**, 3143-3148.

Pollock, R. J. and Kaufman, S. (1978) *J. Neurochem.* **30**, 253-256.

Poole, R. K., Baines, B. S. and Appleby, C. A. (1986) *J. Gen. Microbiol.* **132**, 1525-1539.

Pratt, J. M., Boulnois, G. J., Darby, V., Orr, E., Wahle, E. and Holland, I. B. (1981) *Nucl. Acids Res.* **9**, 4459-4474.

Probst, I., Wolf, G. and Schlegel, H. G. (1979) *Biochim. Biophys. Acta* **576**, 471-478.

Purdy, S. E. and Blair, J. A. (1980) *Biochem. Soc. Trans.* **8**, 565-566.

Purdy, S. E., Blair, J. A. and Barford, P. A. (1981) *Biochem J.* **195**, 769-771.

Randles, D. (1986) *Eur. J. Biochem.* **155**, 301-304.

Rembold, H. and Buff, K. (1972a) *Eur. J. Biochem.* **28**, 579-585.

Rembold, H. and Buff, K. (1972b) *Eur. J. Biochem.* **28**, 586-591.

Rood, J. I., Laird, A. J. and Williams, J. W. (1980) *Gene* **8**, 255-265.

Rosenberg, M. and Court, D. (1979) *Ann. Rev. Genet.* **13**, 319.

Sancar, A., Hack, A. M. and Rupp, W. D. (1979) *J. Bacteriol.* **137**, 692-693.

Sanford, Y.M. and Orias, E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7614-7618.

Sanger, F., Nicklen, S. and Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467.

Sather, M. R., Weber, Jr., C. E., Preston, J. D., Lynam, G. H. and Sleight, S. M. (1979) In: *Cancer Chemotherapeutic Agents: Handbook of Clinical Data*, Martinus Nijhoff, The Hague, pp. 85-88.

Schmidt, H. L., Stocklein, W., Danzer, J., Kirch, P. and Limbach, B. (1986) *Eur. J. Biochem.* **156**, 149-155.

Scopes, R. K. (1982) In: *Protein Purification: Principles and Practice*, Springer-Verlag, Berlin, pp. 43-52.

Scrimgeour, K. G. and Cheema, S. (1971) *Ann. N.Y. Acad. Sci.* **186**, 115- .

Scrimgeour, K. G. and Huennekens, F. M. (1962) *Methods Enzymol.* **5**, 838-843.

Shahbaz, M., Hoch, J. A., Trach, K. A., Hural, J. A., Webber, S. and Whiteley,.J. M. (1987) *J. Biol. Chem.* **262**, 16412-16416.

Shen,.R. S. and Abell, C. W. (1981) *J. Neurosci. Res.* **6**, 193-201.

Shiman, R. (1985) In: *Pterins and Folates*, vol. 2 (Blakley, R. L. and Benkovic, S. J., eds.), Wiley-Interscience, New York, pp. 179-250.

Shostak, K and Schirch, V (1988) *Biochemistry* **27**, 8007-8014.

Silhavy, T. J., Berman, M. L. and Enquist, L. W. (1984) In: *Experiments with Gene Fusions*, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, U.S.A.

Singer, S., Ferone, R., Walton, L. and Elwell, L. (1985) *J. Bacteriol.* **164**, 470-472.

Smith, S. L., Stone, D., Novak, P., Baccanari, D. P. and Burchall, J. J. (1979) *J. Biol. Chem.* **254**, 6222-6225.

Snady, H. and Musacchio, J. M. (1978) *Biochem. Pharmacol.* **27**, 1939-1945.

Synder, F., Malone, B. and Piantadosi, C. (1973) *Biochem. Biophys. Acta.* **316**, 259-265.

Southern, E. M. (1975) *J. Molec. Biol.* **98**, 503-517.

Spackman, D. H., Stein, W. H. and Moore, S. (1958) *Anal. Chem.* **30**, 1190-1206.

Spitzer, E. D. and Weiss, B. (1985) *J. Bacteriol.* **164**, 994-1003.

Sprague, S. M. and Umans, J. G. (1987) *N. Engl. J. Med.* **317**, 1604-1605.

Stauffer, G. V., Plamann, M. D. and Stauffer, L. T. (1981) *Gene* **14**, 63-72.

Sutcliffe, J.G. (1978) *Nucleic Acids Res.* **5**, 2721-2728.

Szewarzyk, B. and Summers, D. F. (1987) *Anal. Biochem.* **164**, 303-306.

Taylor, D. and Hochstein, P. (1975) *Biochem. Biophys. Res. Comm.* **67**, 156-162.

Thomson, S. T., Cass, K. H. and Stellwage, E. (1975) *Proc. Natl.. Acad. Sci.* **72**, 669-672.

Tietz, A., Lindberg, M. and Kennedy, E. P. (1964) *J. Biol. Chem.* **293**, 4081-4090.

Tourian, A. and Sidbury, J. B. (1983) In: *The Metabolic Basis of Inherited Diseases*, (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. and Brown, M. S., eds.), McGraw-Hill, New York, pp. 270-286.

Trefz, F. K., Schmidt, H., Bartholome, K., Mahle, M., Matthes, P. and Pecht, G. (1985) In: *Inherited Diseases of Amino Acid Metabolism* (Bickel, H. and Wachtel, U., eds.) Thieme, Stuttgart, pp. 86-100.

Udenfriend, S. and Cooper, J. R. (1952) *J. Biol. Chem.* **194**, 503-511.

Ullrich, A., Berman, C. H., Dull, T. J., Gray, A. and Lee, J. M. (1984) *EMBO J.* **3**, 361-364.

Umbarger, H. E. and Davis, B. D. (1962) In: *The Bacteria*, vol. 3, (Gunsalus, I. C. and Stanier, R. Y., eds.), Academic Press, New York, p. 167.

Vandekerckhove, J., Bauw, G., Puype, M., van Damme, J. and van Montagu, M. (1985) *Eur. J. Biochem.* **152**, 9-19.

Vieira, J. and Messing, J. (1982) *Gene* **19**, 259-268.

Villafranca, J. E., Howell, E. E., Voet, D. H., Strobel, M. S., Ogden, R. C., Abelson, J. N. and Kraut, J. (1983) *Science* **222**, 782-788.

Wakabayashi, S., Matsubara, H., and Webster, D A. (1986) *Nature* **322**, 481-483.

Wang, B., Jordan, S. P., Jorns, M. S. (1988) *Biochemistry* **27**, 4222-4226.

Waring, P. (1986) *Eur. J. Biochem.* **155**, 305-310.

Waring, P. and Armarego, W. L. F. (1987) *Eur. J. Med. Chem.* **22**, 83-90.

Webber, S., Deits, T. L., Snyder, W. R. and Whiteley, J. M. (1978) *Anal. Biochem.* **84**, 491-503.

Webber, S., Hural, J. A. and Whiteley, J. M. (1986) *Arch. Biochem. Biophys.* **248**, 358-367.

Webber, S., Hural, J. and Whiteley, J. M. (1987) *Biochem. Biophys. Res. Commun.* **143**, 582-586.

Webber, S. and Whiteley, J. M. (1978) *J. Biol. Chem.* **253**, 6724-6729.

Webber, S. and Whiteley, J. M. (1981) *Arch. Biochim. Biophys.* **206**, 145-152.

Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412.

Webster, D. A. and Liu, C. Y. (1974) *J. Biol. Chem.* **249**, 4257-4260.

Wilmanns, W. (1974) In: Methods in Enzymatic Analysis, volume 2 (Bergmeyer, H.U. ed.) Verlag Chemie Weinheim, New York, pp. 666-672.

Williams, C. D., Dicken, G., Letendre, C. H., Guroff, G., Haines, C. and Shiota, T. (1976) *J. Bacteriol.* **127**, 1197-1207.

Wood, P. M. (1984) *Biochim. Biophys. Acta* **768**, 293-317.

Youdim, M. B. H. and Salach, J. I. (1978) In: *Enzyme-activated irreversible inhibitors*, (Seiler, N., Jung M. J. and Koch-Weser, J , eds.), Elsevier, Amsterdam, pp. 235-251.

Yphantis, D. A. (1964) *Biochemistry* **3**, 297-316.