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STUDIES ON THE 5-ENOLPYRUVYL SHIKIMATE 3-PHOSPHATE SYNTHASE
OF ESCHERICHIA COLI.

by

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Thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy.

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November, 1984.

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ABBREVIATIONS

The abbreviations used are those recommended in the Biochemical Journal "Instructions to authors, 1983" with the following additions:

AT	aminotransferase.
bisTris	bis(2-hydroxyethyl)imino-tris-(hydroxymethyl) methane.
BrPyr	3-bromopyruvate.
DAHP	3-deoxy-D-arabinoheptulosonate 7-phosphate.
DHQ	3-dehydroquate.
DTT	dithiothreitol.
EPSP	5-enolpyruvylshikimate 3-phosphate.
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid.
HPLC	high pressure liquid chromatography.
MM + glc	minimal medium + glucose.
OHYPyr	3-hydroxypyruvate.
PAGE	polyacrylamide gel electrophoresis.
PEP	phosphoenolpyruvate.
PLP	pyridoxal phosphate.
PMSF	phenylmethylsulphonyl fluoride.
P-OHPyr	3-phosphohydroxypyruvate.
PSAT	phosphoserine aminotransferase.
PTH-	phenylthiohydantoin-
SDS PAGE	PAGE in the presence of sodium dodecyl sulphate.
shik 3-P	shikimate 3-phosphate.
TEA·HCl	triethanolamine hydrochloride.
UDPGlcNAc	UDP N-acetylglucosamine.
V _e	elution volume.
V _o	void volume.
DAHP synthase (Trp)	Tryptophan sensitive DAHP synthase
DAHP synthase (Tyr)	Tyrosine sensitive DAHP synthase
DAHP synthase (Phe)	Phenylalanine sensitive DAHP synthase

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SUMMARY

1. A method for the purification of EPSP synthase of E. coli K12 has been developed. The purification procedure consisted of ammonium sulphate fractionation, ion-exchange chromatography and hydrophobic chromatography. The final step involved substrate elution from a phosphocellulose column. EPSP synthase was purified 843-fold and in 22% yield over the $(\text{NH}_4)_2\text{SO}_4$ fraction.
2. E. coli EPSP synthase has been shown to be a monomeric enzyme. The subunit M_r was estimated to be 49,000 by SDS PAGE, and native M_r values of 42,000 and 55,000 were determined by gel filtration. Kinetic parameters for E. coli EPSP synthase are reported. The enzyme was inhibited by the herbicide glyphosate, inhibition was competitive with respect to phosphoenolpyruvate.
3. EPSP synthase has also been purified from an overproducing strain, E. coli AB2829/pKD501. The overproduced enzyme was purified 50-fold and in 30% yield over the crude extract fraction. EPSP synthase can be purified in milligram quantities from the overproducing strain.
4. The overproduced enzyme has been shown to be identical in its physical and kinetic properties to EPSP synthase purified from E. coli K12. The amino acid composition and N-terminal amino acid sequence of E. coli EPSP synthase are reported.
5. Chemical modification of EPSP synthase by 3-bromopyruvate has been examined. Although substrate protection against inactivation was observed, bromopyruvate did not appear to be an/

an active-site-directed reagent for E. coli EPSP synthase.

6. Phosphoserine aminotransferase has been purified from the overproducing strain, E. coli AB2829/pKD501. The purification procedure was similar to that developed for EPSP synthase; $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange chromatography and hydrophobic chromatography. The final step was ion-exchange chromatography on a mono-Q column. PSAT was purified approximately 7-fold over the crude extract fraction.
7. The subunit M_r of E. coli PSAT has been shown to be 39,000 and this enzyme appeared to be dimeric. The amino acid composition and N-terminal amino acid sequence of PSAT are reported. Some kinetic properties of this enzyme are also described.

CHAPTER 1

INTRODUCTION.

1.1 Introduction

Bacteria, plants and fungi are able to synthesise aromatic compounds from simple intermediates derived from carbohydrate metabolism while other organisms must rely on exogenously supplied aromatic rings. The early steps in aromatic biosynthesis are common to all organisms that synthesise aromatic compounds. This common pathway, which consists of the seven step synthesis of chorismic acid (figure 1.1), is often called the shikimate pathway after the central intermediate, shikimic acid. Chorismate is the precursor for the synthesis of the aromatic amino acids and a host of other aromatic compounds (figure 1.2).

This thesis is largely concerned with studies on the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase) of Escherichia coli. EPSP synthase is the sixth enzyme activity of the shikimate pathway (figure 1.1). It catalyses the addition of the enolpyruvyl moiety of phosphoenolpyruvate to shikimate 3-phosphate and results in the formation of EPSP and inorganic phosphate.

This chapter provides an introduction to EPSP synthase and the shikimate pathway, and chapters 3, 4 and 5 describe studies on E. coli EPSP synthase. Chapter 6 describes studies on phosphoserine aminotransferase of E. coli, the background to these studies is given at the beginning of chapter 6.

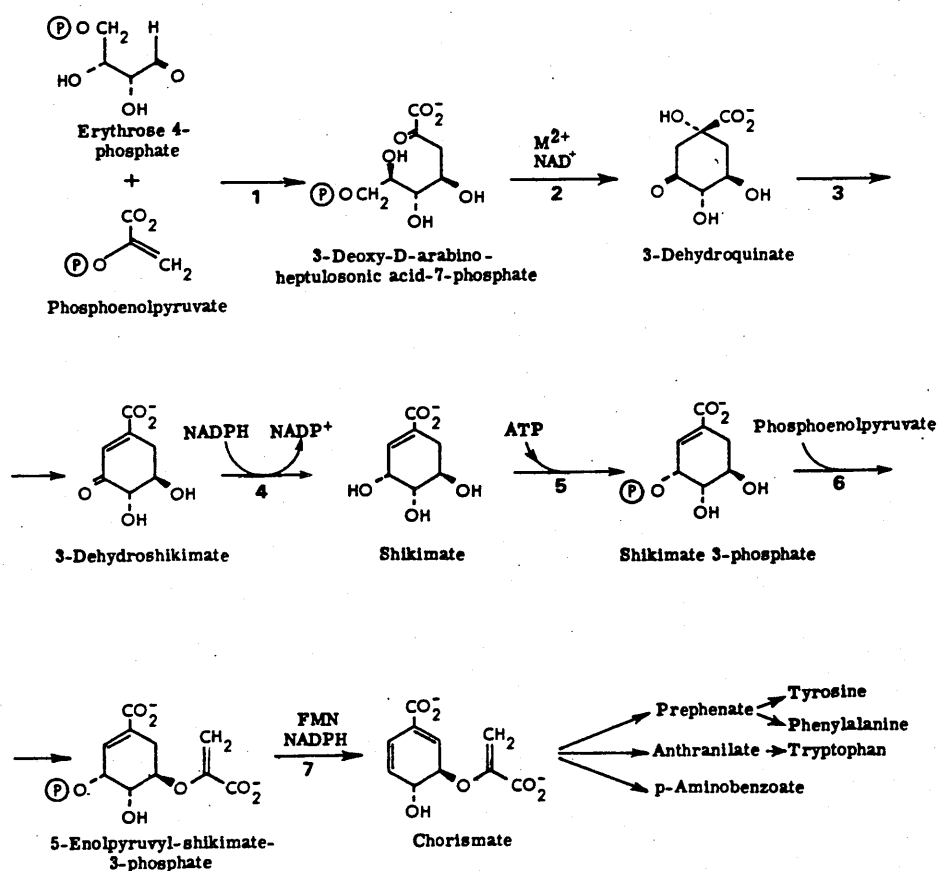
1.2 The shikimate pathway and aromatic amino acid biosynthesis.

The sequence of the steps and the intermediates in the biosynthesis of chorismate and the aromatic amino acids were elucidated using bacterial auxotrophs largely by the groups of Davis (1955) and Sprinson (1960).

The/

FIGURE 1.1

The shikimate pathway.



Pathway step

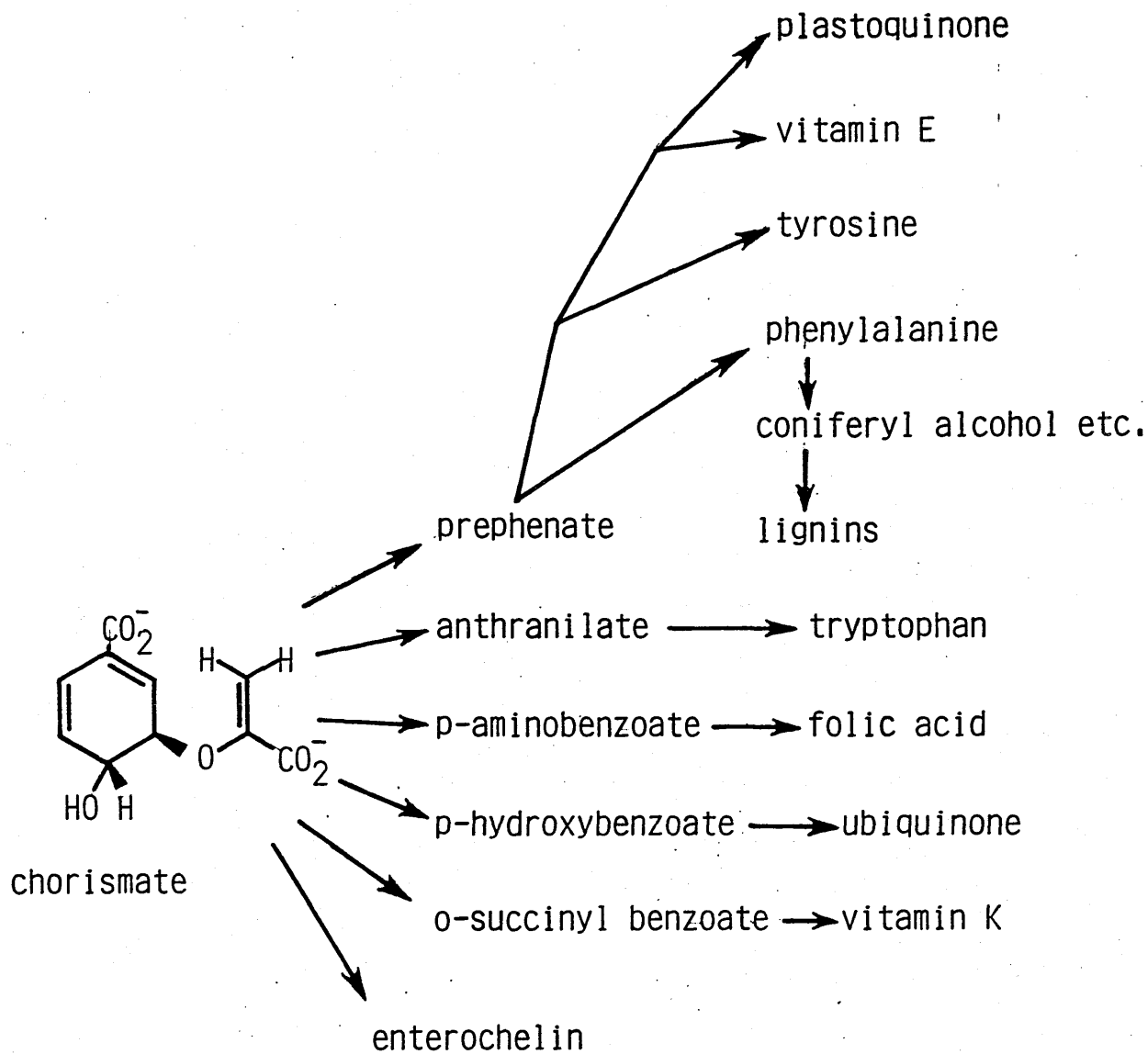
Enzyme

1
2
3
4
5
6
7

DAHP synthase
DHQ synthase
Dehydroquinase
Shikimate dehydrogenase
Shikimate kinase
EPSP synthase
Chorismate synthase

FIGURE 1.2

Utilisation of chorismate.



The shikimate pathway (figure 1.1) is a major biosynthetic route to chorismate. The carbon atoms of chorismate are derived from one molecule of erythrose 4-phosphate and two molecules of phosphoenolpyruvate (PEP). The first committed step of aromatic biosynthesis is the conversion of erythrose 4-phosphate and PEP to the seven carbon compound, 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP). DAHP is cyclised and transformed into chorismate in six further steps. The second molecule of PEP is incorporated in the sixth step of the shikimate pathway, that catalysed by EPSP synthase.

The seven steps of the shikimate pathway are common to the biosynthesis of the majority of aromatic compounds. However, after chorismate, the biosynthetic pathways diverge to give many different products (figure 1.2). Aromatic biosynthesis has been reviewed by Haslam (1974).

Separate pathways exist for the biosynthesis of each aromatic amino acid from chorismate (figure 1.3). In phenylalanine and tyrosine biosynthesis, chorismate is re-arranged to give prephenate which is then either oxidised or dehydrated to yield 4-hydroxyphenylpyruvate or phenylpyruvate respectively. The final step is catalysed by an aminotransferase and yields tyrosine or phenylalanine respectively. In some organisms, transamination precedes decarboxylation and aroenate is a precursor of phenylalanine and tyrosine.

Biosynthesis of tryptophan from chorismate requires five further steps. The first step is synthesis of anthranilic acid with loss of the enolpyruvyl group of chorismate. The other carbon atoms/

FIGURE 1.3

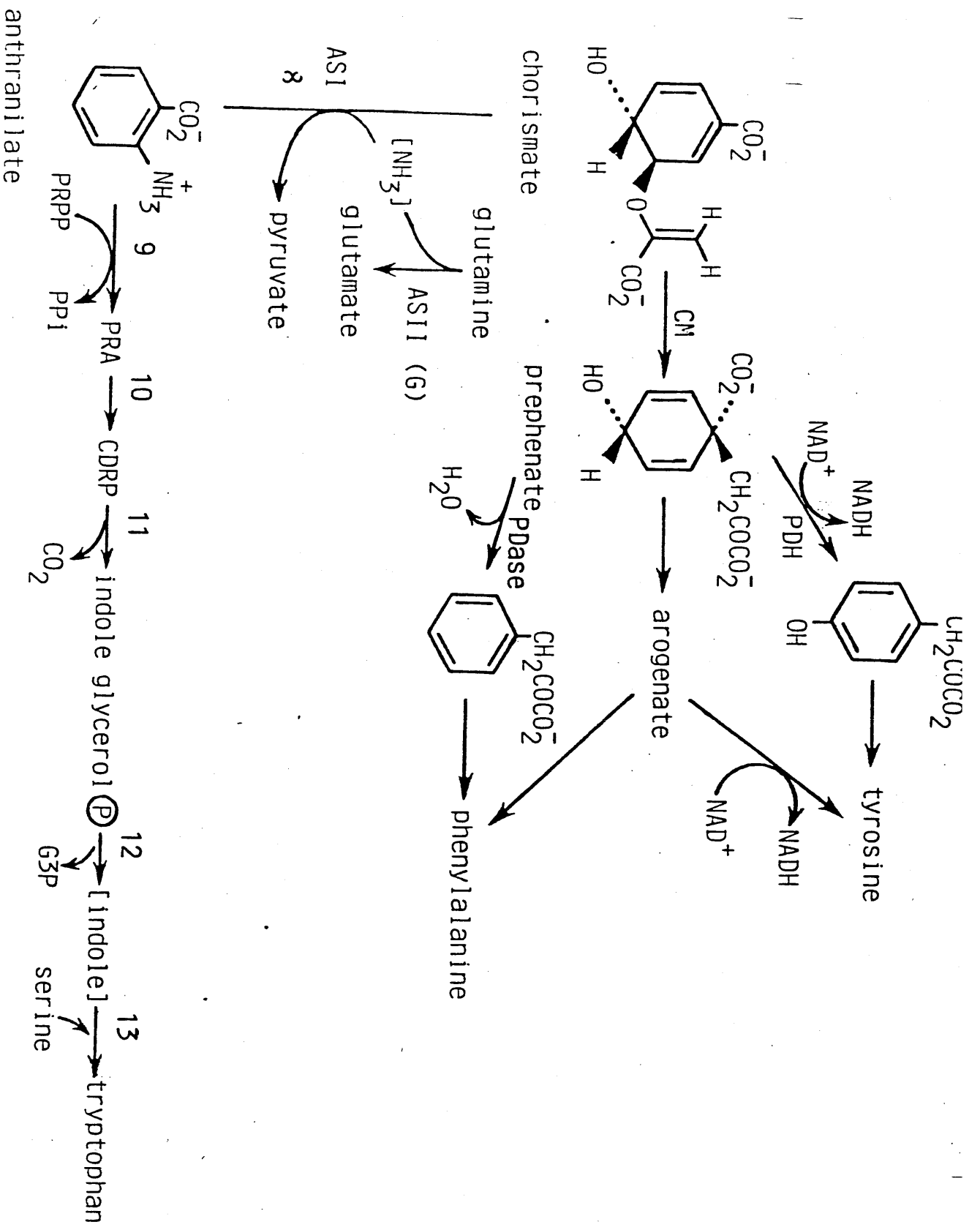
Biosynthesis of the aromatic amino acids from chorismate.

Enzymes:

CM	chorismate mutase
PDH	prephenate dehydrogenase
PDase	prephenate dehydratase
ASI (8)	anthranilate synthase catalytic subunit
ASII (G)	anthranilate synthase glutaminase subunit
9	anthranilate phosphoribosyl transferase
10	phosphoribosyl anthranilate isomerase
11	indole glycerol phosphate synthase
12	tryptophan synthase step 1
13	tryptophan synthase step 2

Intermediates:

PRA	phosphoribosyl anthranilate
CDRP	(O-carboxyphenylamino)-1-deoxyribulose 5-phosphate
PRPP	phosphoribosyl pyrophosphate
G3P	glyceraldehyde 3-phosphate



atoms of tryptophan are derived from phosphoribosyl pyrophosphate and serine (figure 1.3).

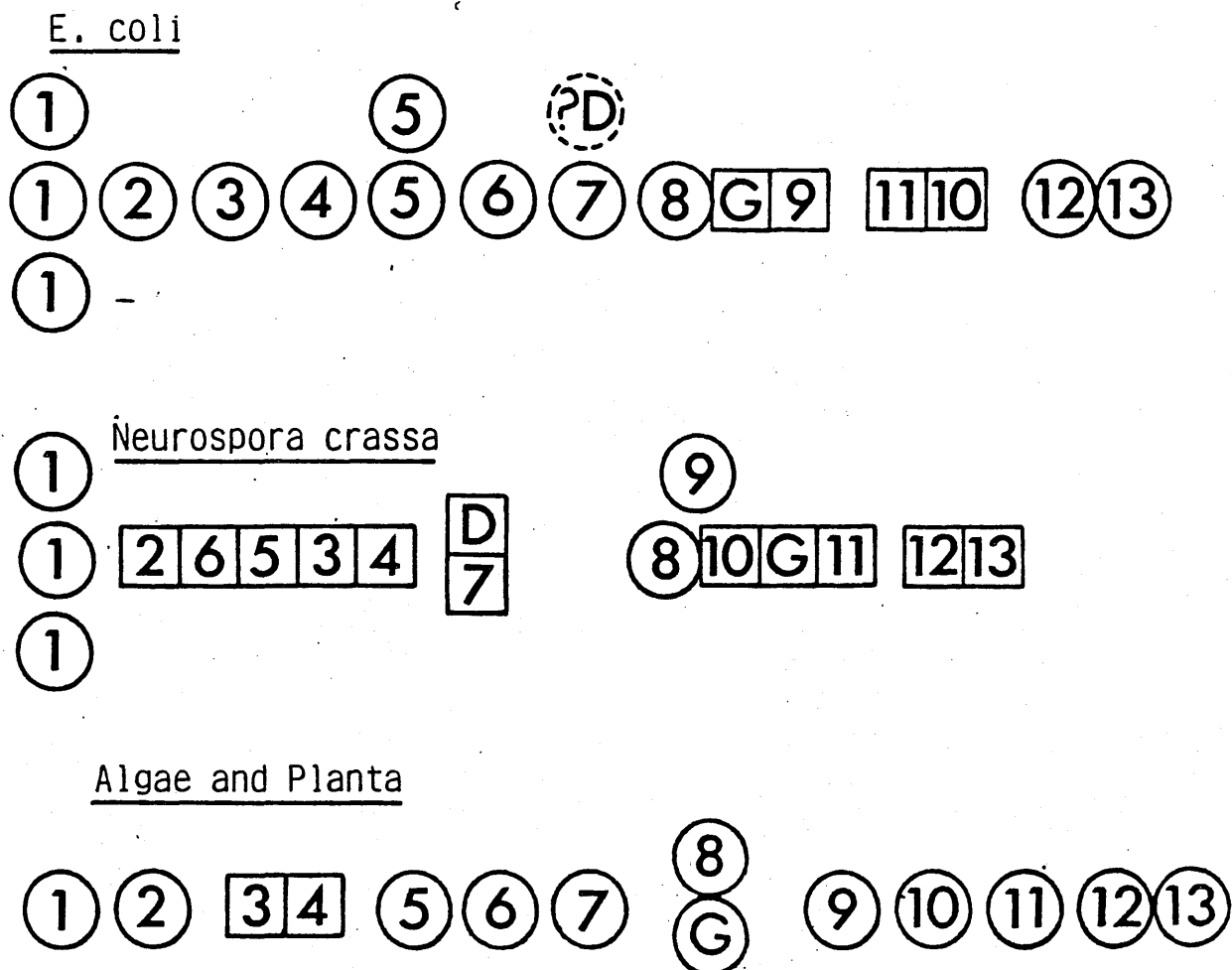
1.3 The organisation of the enzymes of the shikimate pathway.

Studies on the enzymology of the shikimate pathway have tended to concentrate on bacteria and fungi, with E. coli and the bread mould Neurospora crassa as examples because of the ease with which genetic studies can be carried out in these organisms. However, photosynthetic organisms have recently received more attention. The organisation of the enzymes of the shikimate pathway in E. coli, N. crassa and plants is strikingly different (figure 1.4).

Historically, the organisation of enzymes 2 to 6 of the shikimate pathway in N. crassa attracted attention by the discovery that the "genes" coding for these five enzymes were closely linked (Giles et al, 1967a). It was subsequently demonstrated that these activities were associated as a multifunctional enzyme, the arom enzyme complex of N. crassa (Lumsden & Coggins, 1977; Gaertner & Cole, 1977). The arom enzyme complex consists of two identical M_r 165,000 polypeptides (Lumsden & Coggins, 1978). Thus steps 2 to 6 of the shikimate pathway are catalysed by one polypeptide chain in N. crassa. It appears probable that other species of fungi possess a similar enzyme complex (Ahmed & Giles, 1969). Genetic studies on Saccharomyces cerevisiae (de Leeuw, 1967) and Schizosaccharomyces pombe (Strauss, 1979) showed that these yeasts contain gene clusters which appear to be similar to the arom locus of N. crassa. The ARO1 cluster of S. cerevisiae has been cloned and appears to produce a polypeptide/

FIGURE 1.4

Structural organisation of the enzymes of the shikimate pathway and tryptophan biosynthesis.



The enzymes are numbered as in figures 1.1 and 1.3. Rectangles represent multifunctional proteins, circles represent mono-functional proteins and joined circles represent multienzyme complexes (Crawford, 1980). Multiple circles represent isoenzymes: DAHP synthases (①) of *E. coli* and *N. crassa* and shikimate kinases (⑤) of *E. coli*.

Additional abbreviations:

G - glutaminase component of anthranilate synthase.

D - diaphorase or flavin reductase component of chorismate synthase.

polypeptide of M_r 150,000 (Larimer et al, 1983).

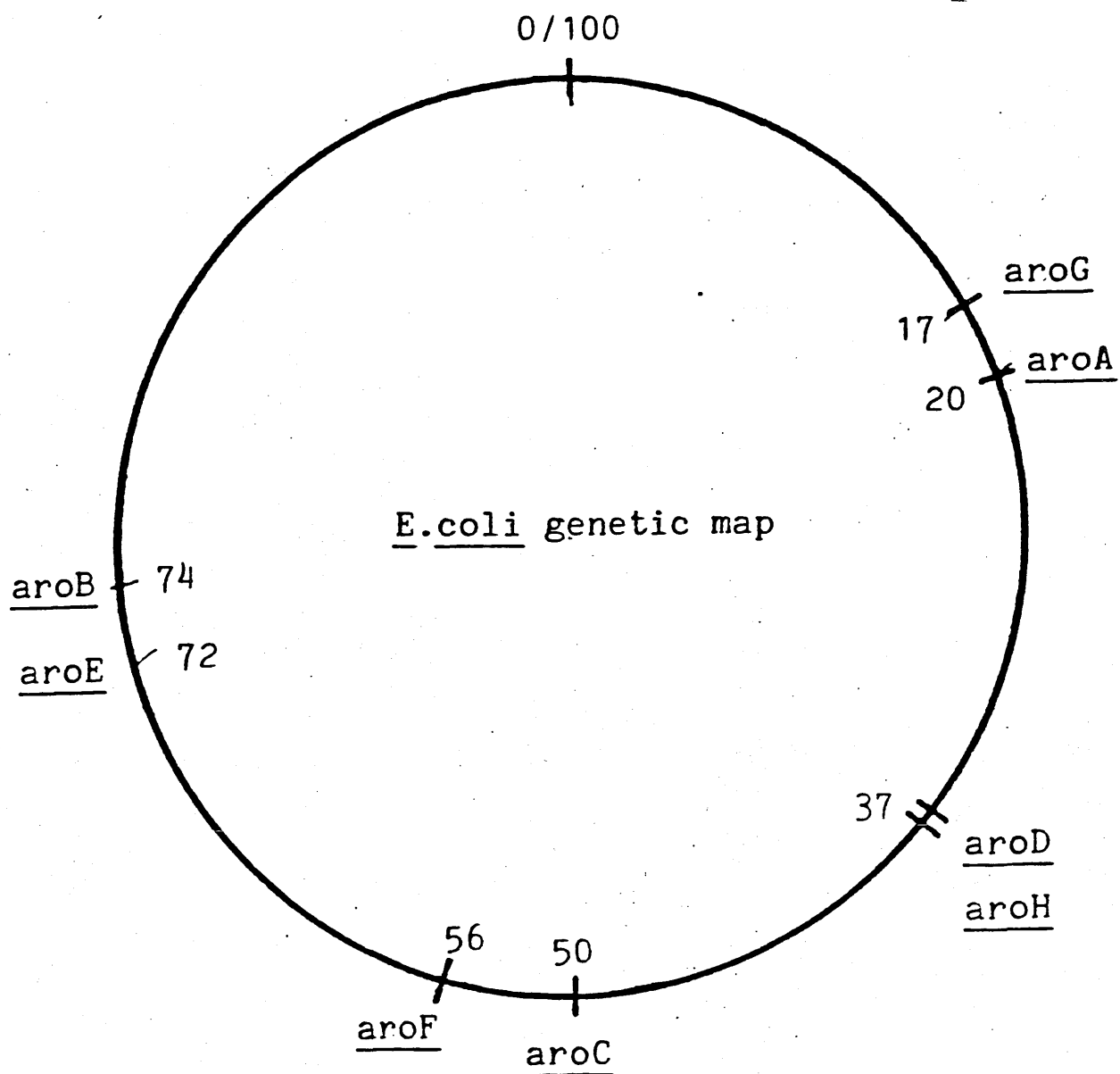
In bacteria, the enzyme organisation is very different (figure 1.4). The genes coding for the shikimate pathway enzymes are widely scattered around the E. coli chromosome (figure 1.5; Pittard & Wallace, 1966; Bachmann, 1983). It was demonstrated that enzyme activities 2 to 6 of the shikimate pathway were separable by density gradient centrifugation in several species of bacteria (Berlyn & Giles, 1969) and not aggregated as these activities are in fungal species (Ahmed & Giles, 1969). Many of the separable E. coli enzymes have recently been purified to homogeneity (Frost et al, 1984; Chaudhuri & Coggins, 1984 and unpublished results; Lewendon & Coggins, 1983).

In many photosynthetic organisms, the organisation is of a yet different pattern. Plants appear to have separable enzymes with the exception that the enzymes catalysing steps 3 and 4 appear to occur as a complex (Berlyn et al, 1970; Boudet & Lecussan, 1974; Polley, 1978; Koshiba, 1979a). This complex has been shown to be a bifunctional enzyme complex in the moss, Physcomitrella patens (Polley, 1978) and in pea seedlings, Pisum sativum (M.S. Campbell, unpublished results). However, this type of enzyme organisation is not the rule for all photosynthetic organisms as Euglena gracilis, a green alga, contains an enzyme complex similar to the N. crassa arom enzyme complex (Patel & Giles, 1979).

The enzyme organisation of the tryptophan biosynthetic pathway is also shown in figure 1.4, again different levels of enzyme organisation are found in E. coli, N. crassa and plants. It is evident that multifunctional proteins in aromatic amino acid biosynthesis are not limited to one organism or pathway.

FIGURE 1.5

Genetic map of E. coli.



Pathway step	Gene	Enzyme encoded
1	<u>aroF</u>	DAHP synthase (Tyr)
1	<u>aroG</u>	DAHP synthase (Phe)
1	<u>aroH</u>	DAHP synthase (Trp)
2	<u>aroB</u>	DHQ synthase
3	<u>aroD</u>	Dehydroquinase
4	<u>aroE</u>	Shikimate dehydrogenase
5		Shikimate kinase
6	<u>aroA</u>	EPSP synthase
7	<u>aroC</u>	Chorismate synthase

1.4 The enzymes and genes of the shikimate pathway.

This section provides a brief summary of the information currently available on each of the shikimate pathway enzymes with the exception of EPSP synthase which is discussed in detail later.

E. coli and N. crassa are again the main examples used.

1.4.1 DAHP synthase.

Carbon flow into the shikimate pathway appears to be regulated by the first enzyme of the pathway, DAHP synthase. This enzyme catalyses the condensation of erythrose 4-phosphate and PEP.

E. coli and N. crassa each contain three DAHP synthase isoenzymes (figure 1.4), the activity of each of these is regulated by feedback inhibition by one of the three aromatic amino acids (Brown, 1968; Nimmo & Coggins, 1981a). In E. coli, the amount of DAHP synthase activity is also regulated at the level of transcription (Herrmann, 1983). This pattern of DAHP synthase activity is not universal, Bacillus subtilis contains a single DAHP synthase which is inhibited by prephenate (Llewellyn et al, 1980). Plant DAHP synthases also appear to be feedback inhibited by aromatic amino acids although these systems have not been fully characterised (Herrmann, 1983).

The properties of many purified DAHP synthases have been reported. DAHP synthase (Trp) of N. crassa obeys a rapid-equilibrium ordered mechanism where PEP is the first-binding substrate and DAHP the last product released (Nimmo & Coggins, 1981b). Salmonella typhimurium and E. coli DAHP synthases (Tyr) also obey sequential mechanisms with the same order of substrate binding (DeLeo et al, 1973; Schoner & Herrmann, 1976). E. coli DAHP synthase (Phe) appears to contain iron/

iron (McCandliss & Herrmann, 1978), this isoenzyme is inactivated by EDTA as is DAHP synthase (Trp) of N. crassa (Nimmo & Coggins, 1981b).

The E. coli aroG (Davies & Davidson, 1982) and aroF (Schultz et al, 1984) genes have been sequenced. These genes code for the phenylalanine- and tyrosine-sensitive DAHP synthases respectively, the amino acid sequences of these two proteins are 53% homologous (Schultz et al, 1984). The aroF gene occurs in an operon together with the tyrA gene which codes for the bifunctional enzyme chorismate mutase : prephenate dehydrogenase (figure 1.3; Wallace & Pittard, 1967).

1.4.2 Dehydroquinate synthase.

3-dehydroquinate (DHQ) synthase converts DAHP to DHQ. The mechanism of ring closure is unclear, catalytic amounts of NAD^+ and a metal ion are required for activity (Srinivasan et al, 1963). The N. crassa arom complex DHQ synthase activity has been shown to be a zinc-dependent enzyme (J.M. Lambert, Boocock, M.R. & Coggins, J.R., unpublished results). DHQ synthases from mung bean, Phaseolus mungo (Yamamoto, 1980) and E. coli (Maitra & Sprinson, 1978; Frost et al, 1984) have been purified, these enzymes are reported to require Cu^{++} and Co^{++} respectively for activity. DHQ synthase of B. subtilis has also been purified, this enzyme occurs as part of a multienzyme complex together with chorismate synthase and a flavin reductase activity (Hasan & Nester, 1978c).

The E. coli aroB gene has been cloned and DHQ synthase purified from an overproducing strain of E. coli. DHQ synthase appears to be a monomeric protein of M_r 40,000 to 44,000 (Frost et al, 1984).

The/

The nucleotide sequence of the aroB gene has been determined (G. Millar, unpublished results).

1.4.3 Dehydroquinase.

This enzyme catalyses the dehydration of DHQ to yield 3-dehydroshikimate thus introducing the first double bond of the aromatic ring system. Dehydroquinase has been purified from E. coli K12 and an overproducing strain of E. coli. It is a dimeric enzyme of subunit M_r 29,000 (S. Chaudhuri & Coggins, J.R., unpublished results). The E. coli aroD gene has been cloned (Kinghorn et al, 1981) and the nucleotide sequence of this gene has been determined (Duncan, 1984).

As discussed in section 1.3, in plants dehydroquinase occurs in association with shikimate dehydrogenase, the next enzyme of the shikimate pathway. The purified dehydroquinase: shikimate dehydrogenase of P. patens appears to be a single polypeptide of M_r 48,000-49,000 (Polley, 1978).

1.4.4 Shikimate dehydrogenase.

Shikimate dehydrogenase catalyses the reduction of 3-dehydroshikimate to shikimic acid; this enzyme is specific for NADPH. Shikimate dehydrogenase has been purified to homogeneity from E. coli, it appears to be a monomeric enzyme of M_r 31,000 (Chaudhuri & Coggins, 1984). The E. coli aroE gene has been cloned and the nucleotide sequence of this gene has been determined (I.A. Anton, unpublished results).

1.4.5 Shikimate kinase.

This enzyme catalyses the phosphorylation of shikimate to give shikimate/

shikimate 3-phosphate (Shik 3-P). Shikimate kinase has been partially purified from two plant species, P. mungo (Koshiba, 1979b) and Sorghum bicolor (Bowen & Kosuge, 1979). A shikimate kinase activity has been purified to homogeneity from B. subtilis strain 168; it is a small polypeptide of M_r 10,000 which is active only in association with the DAHP synthase: chorismate mutase complex found in this strain (Huang et al, 1975).

E. coli appears to contain two shikimate kinase isoenzymes as single step mutants lacking shikimate kinase activity could not be isolated and two different forms of shikimate kinase activity were resolved by ion-exchange chromatography (Ely & Pittard, 1979). The level of shikimate kinase activity in E. coli appears to be under transcriptional control; there is some evidence that only one shikimate kinase isoenzyme is repressed (Ely & Pittard, 1979). The possible function of control of shikimate kinase activity in E. coli is unclear.

1.4.6 EPSP synthase.

This enzyme catalyses the formation of EPSP from shik 3-P and PEP and will be discussed in detail in section 1.5.

1.4.7 Chorismate synthase.

Chorismate synthase catalyses the formation of the branch point intermediate, chorismic acid. Reduced flavin (FMN or FAD) appears to be necessary for activity. E. coli chorismate synthase requires a reduced flavin-regenerating system and an O_2 free atmosphere (Morell et al, 1967), this enzyme has been little studied. B. subtilis and N. crassa chorismate synthases have been purified to homogeneity, both/

both enzymes appear to be associated with a flavin reductase activity. The B. subtilis enzyme occurs as part of a chorismate synthase : DHQ synthase : flavin reductase multienzyme complex (Hasan & Nester, 1981b, c) whereas N. crassa chorismate synthase activity appears to occur as a bifunctional enzyme complex together with a flavin reductase activity (Boocock, 1983). Both of these flavin reductase activities appear to utilise NADPH as the reducing agent (Welch et al, 1974; Hasan & Nester, 1981a; Boocock, 1983). The reduced flavin requirement for chorismate synthase activity is not understood.

1.4.8 Catabolic dehydroquinase and quinate dehydrogenase.

If N. crassa is grown on quinate as a carbon source, enzymes necessary for the catabolism of quinate are induced. These enzymes are quinate (shikimate) dehydrogenase, a catabolic dehydroquinase and 3-dehydroshikimate dehydratase (figure 1.6). These enzymes are coded for by the qa-3, qa-2 and qa-4 genes (Chaleff, 1974). These three genes are part of the qa gene cluster of N. crassa which consists of five structural and two regulatory genes (Tyler et al, 1984).

Under conditions where the qa gene cluster is induced, N. crassa contains two dehydroquinase activities (Giles et al, 1967b), the catabolic activity and the biosynthetic arom complex dehydroquinase (figure 1.6). The catabolic dehydroquinase has been purified to homogeneity (Hawkins et al, 1982; Chaudhuri & Coggins, 1981), this enzyme appears to be a dodecamer of subunit M_r 20,000 (Chaudhuri & Coggins, 1981). The inducible quinate dehydrogenase has also been purified, it is a monomeric enzyme of M_r 41,000. This enzyme activity appears/

FIGURE 1.6

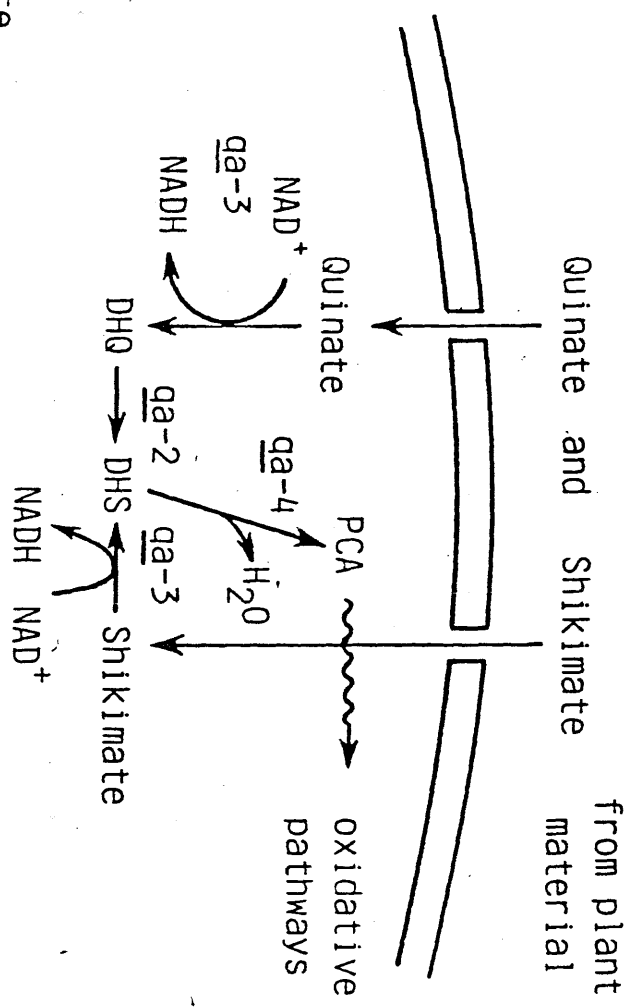
The catabolic quinate pathway and biosynthetic shikimate pathway of N. crassa.

<u>Gene</u>	<u>Enzyme</u>
<u>qa-2</u>	catabolic dehydroquinase
<u>qa-3</u>	catabolic quinate (shikimate) dehydrogenase
<u>qa-4</u>	catabolic dehydroshikimate dehydratase

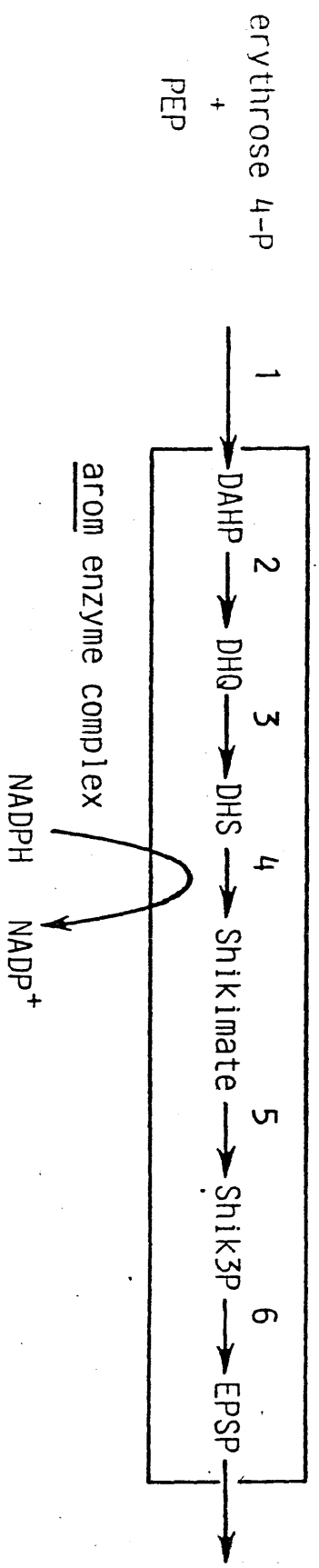
Intermediates:

DHQ	3-dehydroquinate
DHS	3-dehydroshikimate
PCA	protocatechuate

Catabolic quinate pathway



Biosynthetic shikimate pathway



appears to be NAD^+ -dependent and can utilise shikimate although less well than quinate (Barea & Giles, 1978). The nucleotide sequences of the qa-2 and qa-3 genes have been determined (N.H. Giles & Co-workers, unpublished results). Preliminary searches for sequence homology (Maizel & Lenk, 1981) indicated that the deduced amino acid sequence of quinate (shikimate) dehydrogenase may show some homology with the sequence of E. coli shikimate dehydrogenase (I.A. Anton, unpublished results), however, no homology was detected between the catabolic dehydroquinase and the E. coli dehydroquinase amino acid sequences (K. Duncan, unpublished results).

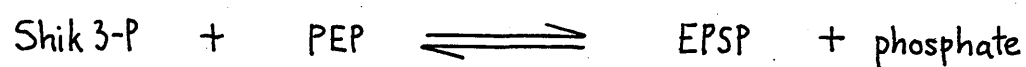
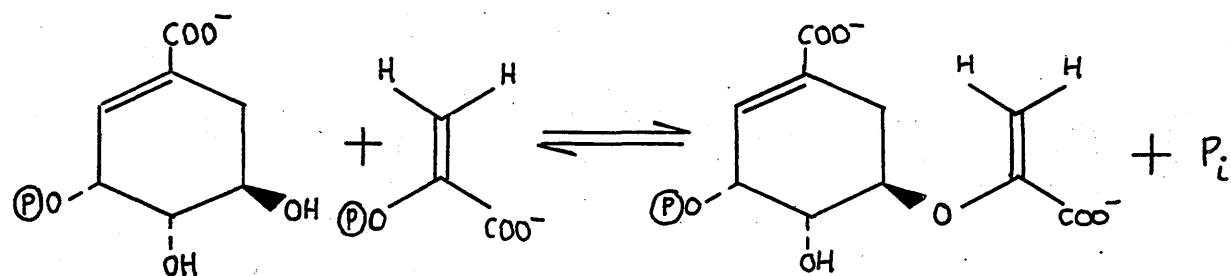
NAD^+ -dependent quinate dehydrogenase activities have been detected in plants (Gamborg, 1967; Minimakawa, 1979) although it is not yet clear whether these enzymes play a biosynthetic or degradative role. Both free and esterified forms of quinic acid can be found in large amounts in many plant species. Zea mays appears to contain a quinate dehydrogenase that occurs as a complex with a dehydroquinase activity, this is probably not a universal situation (Graziana et al, 1980). The activity of carrot cell quinate dehydrogenase has been reported to be controlled by reversible phosphorylation (Refeno et al, 1982) although the significance of this is not clear.

1.5 EPSP synthase : introduction.

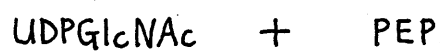
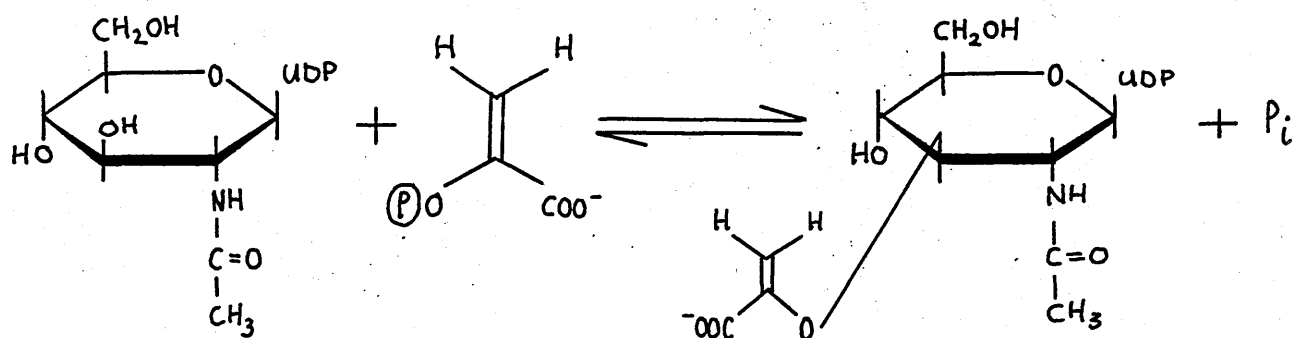
EPSP synthase catalyses the reversible transfer of the enolpyruvyl moiety of PEP to the 5-hydroxyl of shik 3-P to give EPSP and inorganic phosphate (figure 1.7a; Levin & Sprinson, 1964). This type of reaction is somewhat unusual and only one other similar biosynthetic reaction is known. This is the condensation of UDPGlcNAc and/

FIGURE 1.7

(a) The reaction catalysed by EPSP synthase.



(b) The reaction catalysed by UDPGlcNAc enolpyruvyl transferase.



and PEP which is catalysed by the enzyme UDPGlcNAc enolpyruvyl transferase. This latter reaction is the first step of peptidoglycan biosynthesis (figure 1.7b; Gunetileke & Anwar, 1968). EPSP synthase and UDPGlcNAc enolpyruvyl transferase are distinct from other PEP-utilising enzymes in that they preserve the enolpyruvyl part of PEP (Davies, 1979). Because EPSP synthase catalyses an unusual reaction, the mechanism of this reaction has been studied by a number of groups, particularly those of Sprinson (Bondinell et al, 1971) and Knowles (Grimshaw et al, 1982).

1.6 Aspects of the organisation of EPSP synthase.

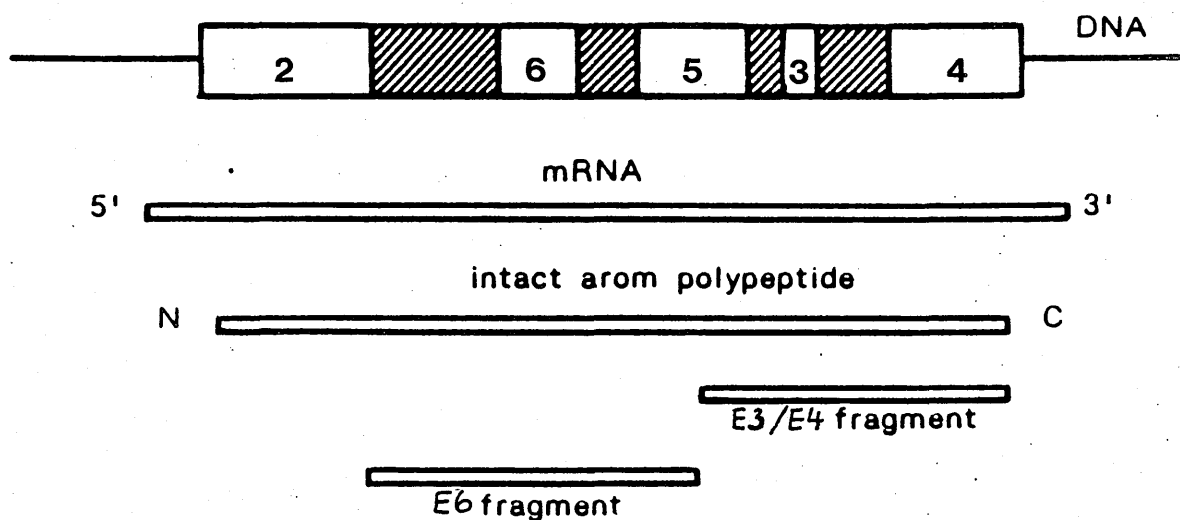
EPSP synthase in E. coli and other bacteria and in plants appears to be a separable, monofunctional enzyme (Berlyn & Giles, 1969; Berlyn et al, 1970) whereas in N. crassa and other fungi, EPSP synthase is one of the activities of the pentafunctional arom enzyme complex (see figure 1.4; Lumsden & Coggins, 1977; Ahmed & Giles, 1969).

There is evidence from genetic data and from protein studies that many multifunctional proteins are composed of domains, where each domain may correspond to one particular function of the multifunctional protein (Kirschner & Bisswanger, 1976).

Genetic studies of the N. crassa arom gene cluster have elucidated the order of the enzyme activities along the polypeptide chain (Giles et al, 1967a; Rines et al, 1969). Figure 1.8 shows that mis-sense mutations which lead to the absence of one of the enzyme activities of the arom complex map in five discrete regions along the gene and therefore along the polypeptide chain. Further evidence in favour of a domain structure for the N. crassa arom enzyme/

FIGURE 1.8

The arom locus of N. crassa.



The unhatched areas on the genetic map indicate that mis-sense mutations which lead to the absence of one of the enzyme activities of the arom complex have been mapped in this area (Giles et al, 1967a; Rines et al, 1969). The enzyme activities are numbered as in figure 1.1. The mRNA and polypeptide products of the arom locus are shown. The approximate sections of the arom polypeptide which correspond to the EPSP synthase (E6) and dehydroquinase/shikimate dehydrogenase (E3/E4) fragments isolated after limited proteolysis of the arom complex (Boocock, 1983; Smith & Coggins, 1983) are indicated.

enzyme complex comes from studies involving limited proteolysis of pure arom complex. After limited proteolysis it has proved possible to isolate two non-overlapping fragments, one of which carries dehydroquinase and shikimate dehydrogenase activities (Smith & Coggins, 1983; Boocock, 1983), while the other carries only EPSP synthase activity. The EPSP synthase fragment has an M_r of 74,000 under denaturing conditions and appears to have a dimeric structure (Boocock, 1983).

The simplest model for the origin of multifunctional proteins must be that involving fusion of adjacent genes coding for monofunctional proteins. There is good evidence from DNA sequencing studies that two multifunctional proteins of tryptophan biosynthesis, the bifunctional anthranilate synthase component II : anthranilate-5-phosphoribosylpyrophosphate phosphoribosyl transferase of E. coli and the bifunctional tryptophan synthase of S. cerevisiae (figures 1.3 and 1.4) have been produced by gene fusion (Miozarri & Yanofsky, 1979; Zalkin & Yanofsky, 1982). The question of whether the fungal arom enzyme complexes have been produced by gene fusion is likely to be answered at the DNA sequence level. It is encouraging that many of the monofunctional E. coli genes have recently been sequenced (G. Millar, unpublished results; Duncan, 1984; I.A. Anton, unpublished results; Duncan et al, 1984b), and also that the arom gene clusters of S. cerevisiae and S. pombe have been cloned (Larimer et al, 1983; Nakanishi & Yamamoto, 1984).

1.7 Glyphosate.

Interest in EPSP synthase has increased greatly since Amrhein and/

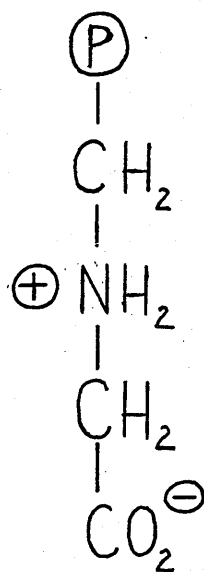
and co-workers identified EPSP synthase as the site of action of the herbicide glyphosate (Amrhein et al, 1980; Steinrucken & Amrhein, 1980). Glyphosate (N-[phosphonomethyl] glycine; figure 1.9) is a broad-spectrum, post-emergence herbicide, it has low mammalian toxicity and is widely used in agriculture, glyphosate has been very successful commercially. Glyphosate was shown to interfere with aromatic biosynthesis (Jaworski, 1972), this inhibition was subsequently traced to inhibition of the shikimate pathway and EPSP synthase (Amrhein et al, 1980). It was demonstrated that EPSP synthase in extracts of Aerobacter aerogenes (= Klebsiella pneumoniae) was very sensitive to inhibition by glyphosate (Steinrucken & Amrhein, 1980).

Since then, a number of EPSP synthase activities have been shown to be sensitive to glyphosate inhibition. A detailed study of glyphosate inhibition of N. crassa EPSP synthase has been reported, glyphosate was shown to be a competitive inhibitor with respect to PEP, that is, glyphosate appears to act as an analogue of PEP (Boocock & Coggins, 1983). Glyphosate has been reported to inhibit both bacterial (Anton et al, 1983; Duncan et al, 1984a; Steinrucken & Amrhein, 1984b) and plant (Amrhein et al, 1982; Mousdale & Coggins, 1984a; Rubin et al, 1984) EPSP synthases in the same manner.

Glyphosate inhibition of EPSP synthase is very specific, structural analogues of glyphosate do not inhibit EPSP synthase and glyphosate does not inhibit other PEP-utilising enzymes at concentrations similar to those required to inhibit EPSP synthase (Boocock, 1983; Steinrucken & Amrhein, 1984b).

FIGURE 1.9

The structure of glyphosate.



1.8 Studies on the mechanism of EPSP synthase.

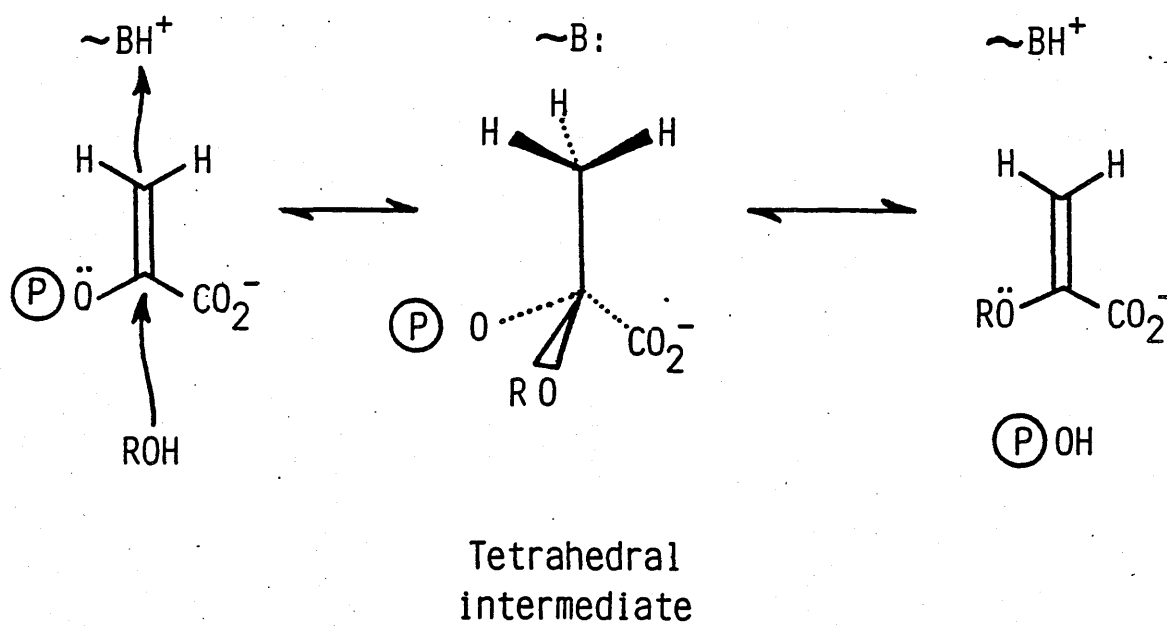
1.8.1 Isotope exchange studies.

An addition-elimination mechanism was proposed for EPSP synthase by Levin & Sprinson (1964), such a mechanism is shown in figure 1.10. Addition of shik 3-P to C-2 of PEP yields a tetrahedral intermediate and elimination of phosphate gives EPSP. The findings of elegant isotope exchange studies that have been carried out on EPSP synthase are outlined below. Partially purified bacterial extracts were used as a source of EPSP synthase activity for these studies.

- (i) PEP labelled with ^{18}O in the C-O-P position released essentially all the label during synthesis of EPSP, thus elimination of phosphate occurred with C-O bond cleavage (Bondinell et al, 1971).
- (ii) EPSP synthesised in $^2\text{H}_2\text{O}$ was labelled, the label was equally distributed between the E and Z positions of the carboxyvinyl group. This indicated that a freely rotating methyl group is formed at C-3 of PEP during the course of the reaction. No incorporation of label into PEP was seen in the absence of shik 3-P (Bondinell et al, 1971).
- (iii) PEP labelled with ^2H and/or ^3H at C-3 was used to demonstrate that there is discrimination against heavy isotopes in both the addition and elimination steps of the reaction (Ife et al, 1976; Grimshaw et al, 1982).
- (iv) Stereospecifically labelled [$3\text{-}^2\text{H}$, ^3H] PEP was used to synthesise EPSP, because of the observed discrimination against heavy isotopes (Grimshaw et al, 1982) the EPSP produced was also/

FIGURE 1.10

An addition-elimination mechanism for EPSP synthase
(Levin & Sprinson, 1964).



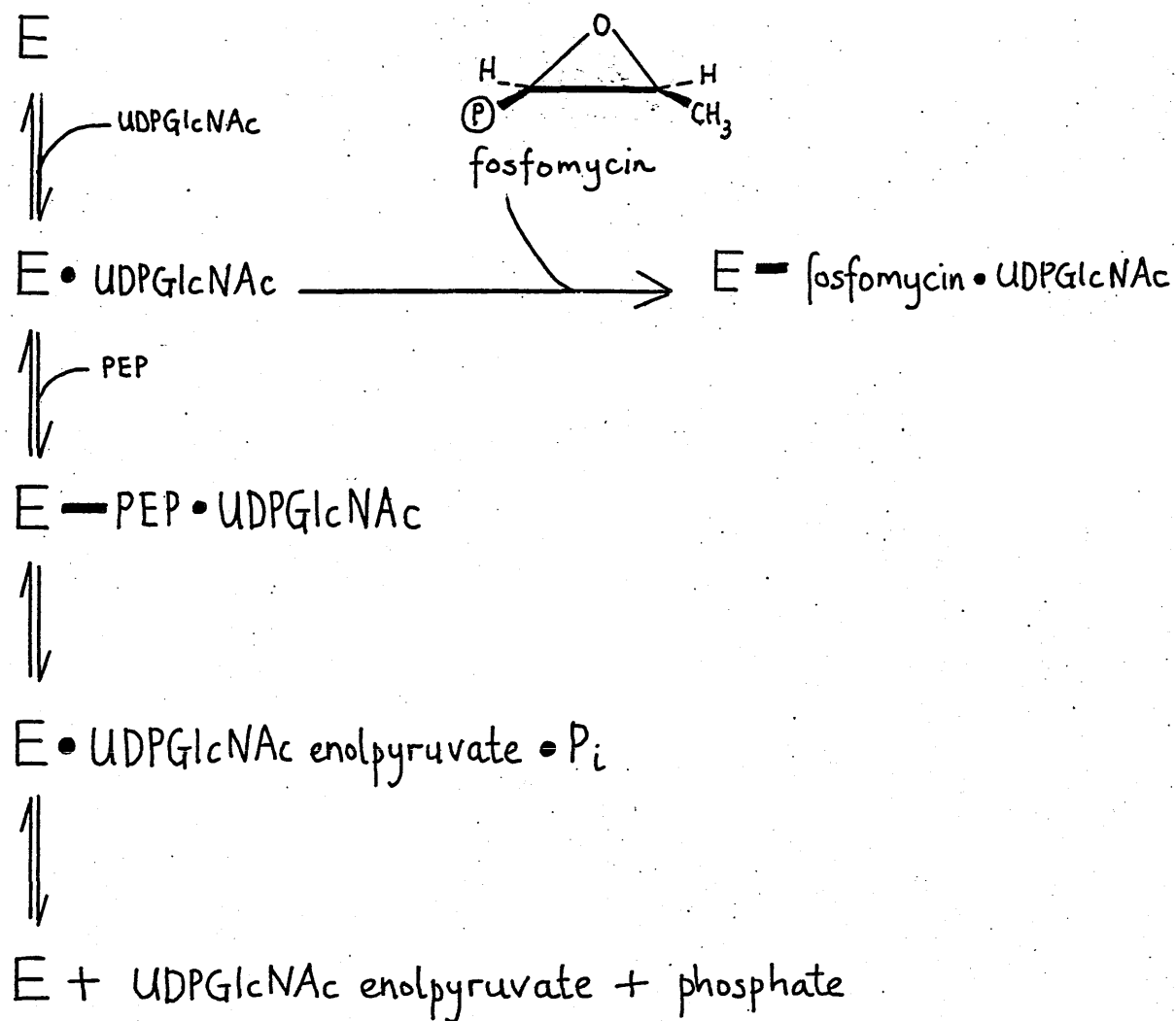
also stereospecifically labelled. It was deduced that the addition and elimination steps have opposite stereochemical courses, i.e. if addition is syn, elimination is anti or vice versa (C.E. Grimshaw, Sogo, S.G., Copley, S.D. & Knowles, J.R., unpublished results).

The results of the isotope exchange studies are in agreement with the proposed addition-elimination mechanism (figure 1.10). It should be noted that this mechanism differs somewhat from the mechanisms of most other PEP-utilising enzymes, e.g. the pyruvate kinase, PEP carboxylase and PEP carboxykinase reactions proceed via cleavage of the O-P rather than the C-O bond at C-2 of PEP (Davies, 1979). DAHP synthase (section 1.4.1) also cleaves the C-O bond (DeLeo et al, 1973), however the mechanism of this reaction is different from that of EPSP synthase as it involves stereospecific addition of erythrose 4-phosphate to C-3 of PEP (Floss et al, 1972; DeLeo et al, 1973).

Isotope exchange studies of Micrococcus lysodeikticus UDPGlcNAc enolpyruvyl transferase have shown that a freely rotating methyl group is formed during the course of this reaction (Cassidy & Kahan, 1973). The mechanisms proposed for UDPGlcNAc enolpyruvyl transferase, on the basis of substrate labelling experiments, involve the formation of an enzyme-PEP or enzyme-enolpyruvyl covalent intermediate (figure 1.11). Formation of the covalent intermediate requires the initial presence of UDPGlcNAc (Cassidy & Kahan, 1973; Zemell & Anwar, 1975). A cysteine residue has been implicated in the formation of the enzyme-PEP covalent intermediate. UDPGlcNAc enolpyruvyl transferase is inactivated by the antibiotic fosfomycin owing to covalent modification/

FIGURE 1.11

The covalent intermediate mechanism proposed for UDPGlcNAc enolpyruvyl transferase (Cassidy & Kahan, 1973; Zemell & Anwar, 1975).



- E = UDPGlcNAc enolpyruvyl transferase
 $-$ = covalent linkage
 \bullet = Michaelis complex

modification of a cysteine residue by this compound (figure 1.11). Fosfomycin was thought to act as a PEP analogue as inactivation required the prior binding of UDPGlcNAc (Kahan et al, 1974). Fosfomycin appears not to inactivate EPSP synthase (Boocock, 1983; Steinrucken & Amrhein, 1984b), similarly glyphosate does not inhibit UDPGlcNAc enolpyruvyl transferase (Steinrucken & Amrhein, 1984b).

It has been pointed out that the isotope exchange studies on EPSP synthase do not rule out the possibility of a mechanism involving a covalent enzyme-PEP intermediate for this enzyme (Cassidy & Kahan, 1973; Ganem, 1978; Boocock, 1983). In an attempt to gather evidence for such an intermediate, Anton et al (1983) used 4, 5-dideoxyshikimate 3-phosphate as an analogue of shik 3-P. This group observed some exchange of the hydrogen atoms at C-3 of PEP with the solvent in the presence of partially purified EPSP synthase and 4, 5-dideoxyshikimate 3-phosphate. As yet, no reports of successful substrate labelling of EPSP synthase have appeared.

1.8.2 Steady-state kinetic studies.

Kinetic studies on N. crassa EPSP synthase (Boocock, 1983; Boocock & Coggins, 1983) and K. pneumoniae EPSP synthase (Steinrucken & Amrhein, 1984a, b) have been reported. Different kinetic mechanisms have been proposed for these EPSP synthase activities.

Detailed steady-state kinetics including inhibition by glyphosate and the use of arsenate as a pseudo-substrate have led to the proposal of the following kinetic mechanism for N. crassa EPSP synthase (Boocock, 1983; Boocock & Coggins, 1983).

- (i) Shik 3-P is the first substrate to bind to EPSP synthase.
- (ii) The enzyme•shik 3-P complex can bind either PEP or glyphosate.
- (iii)/

(iii) Release of EPSP and phosphate from the enzyme is in random order. Glyphosate inhibition of EPSP synthase would be due therefore to formation of an enzyme•shik 3-P•glyphosate dead-end complex (Boocock & Coggins, 1983).

However, Steinrucken & Amrhein (1984a) have proposed a random sequential mechanism for K. pneumoniae EPSP synthase in the forwards direction. In this case, glyphosate would inhibit EPSP synthase by binding to the free enzyme as well as to the enzyme•shik 3-P complex (Steinrucken & Amrhein, 1984b).

It is impossible to say whether the bacterial and fungal EPSP synthases do indeed have different kinetic mechanisms as rather different assay methods and conditions were used in each study (Boocock, 1983; Steinrucken & Amrhein, 1984a).

1.9 Introduction to E. coli EPSP synthase and the objectives of the present study.

When this study commenced, no purification scheme for a separable EPSP synthase had been reported, only the EPSP synthase activities of the arom enzyme complexes of N. crassa (Lumsden & Coggins, 1977) and E. gracilis (Patel & Giles, 1979) had been purified to homogeneity. The amount of information in the literature on separable EPSP synthases at that time was very limited.

It was decided therefore to purify and characterise a separable, monofunctional EPSP synthase. The source of separable EPSP synthase selected was E. coli. Bacteria were easier to obtain than the other possible source of separable EPSP synthases, plant material. Plants also have the disadvantage that their extracts generally contain low protein/

protein concentrations. A factor behind the selection of E. coli as a source of EPSP synthase was that Mr. K. Duncan in our laboratory was engaged in cloning and sequencing the gene coding for EPSP synthase (the aroA gene) from E. coli. Thus protein and DNA studies would complement each other.

It was known from genetic studies that E. coli EPSP synthase was coded for by a single gene (Pittard & Wallace, 1966) and also that this gene was constitutively expressed (Tribe et al, 1976).

EPSP synthase activity had been first demonstrated in extracts of E. coli where the reaction was shown to be reversible (Levin & Sprinson, 1964). Some characterisation of E. coli EPSP synthase that had been purified 5-fold by ammonium sulphate fractionation was carried out. It was reported that the enzyme did not have any co-factor requirements and that it was most active between pH5.4 and pH6.2. Michaelis constants for PEP and shik 3-P were reported to be 0.24 mM and 0.34 mM respectively (Levin & Sprinson, 1964). The greatest degree of purification reported for a bacterial EPSP synthase was in the case of S. typhimurium EPSP synthase which was purified 80-fold by $(\text{NH}_4)_2\text{SO}_4$ fractionation and ion-exchange chromatography (Bondinell et al, 1971). This 80-fold purified EPSP synthase was not characterised but was used for mechanistic studies (section 1.8.1). The native M_r of EPSP synthase activity in extracts of E. coli had been estimated as 38,000 by sucrose density gradient centrifugation (Berlyn & Giles, 1969).

Therefore, the objectives of this study were:

1. To develop a purification procedure for E. coli EPSP synthase.
2. To characterise the purified enzyme as fully as possible.
- 3./

3. To adapt the purification procedure so that large amounts of homogeneous, monofunctional EPSP synthase would be available for structural and mechanistic studies.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and biochemicals.

(Grade I, free acid) Tris, Triethanolamine HCl (TEA·HCl), ADP^(Na⁺ salt), PEP^(K⁺ salt), NADH^(Grade II, Na⁺ salt), NADPH^(Grade II, Na⁺ salt), NAD⁺, dithiothreitol (DTT) and phenylmethanesulphonyl fluoride (PMSF) were obtained from Boehringer Corp. (London), Lewes, Sussex.

FMN^(Na⁺ salt), 3-hydroxypyruvate^(free acid), 3-phosphohydroxypyruvate^(H⁺ salt), 3-phosphoserine^(free acid), 3-bromopyruvate^(free acid) and bis-Tris (bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane) were obtained from Sigma(London) Chemical Co., Poole, Dorset.

Glutamate^{HCl}, nitroblue tetrazolium, phenazine methosulphate and reagents for polyacrylamide gel electrophoresis (PAGE) were obtained from BDH Chemicals, Poole, Dorset.

All other reagents used were of the highest grade commercially available.

Glyphosate was a gift from Dr. S. Ridley, Plant Protection Division, ICI plc.

2.1.2. Chromatography media.

DEAE-Sephacel, Sephadex G-25 (medium grade), phenyl-Sepharose CL-4B and Sephacryl S200 superfine were obtained from Pharmacia (GB) Ltd., London, W5. Phosphocellulose (P11) and DEAE-cellulose (DE52) were obtained from Whatman Biochemicals, Maidstone, Kent. Amberlite CG400 was obtained from BDH Chemicals. Blue dextran Sepharose was a gift from Dr. S. Chaudhuri.

Pre-packed columns for gel filtration (TSK G2000SW and TSK G3000SW) were obtained from L.K.B. Ltd., South Croydon, U.K. A mono-Q (HR 5/5) column was obtained from Pharmacia (GB) Ltd, the mono-Q column is a prepacked anion exchange column.

2.1.3/

2.1.3 Enzymes and proteins.

The following enzymes were obtained from Boehringer Corp.:

aldolase (EC 4.1.2.13) from rabbit muscle,
alkaline phosphatase (EC 3.1.3.1) from calf intestine,
carbonic anhydrase (EC 4.2.1.1) from bovine erythrocytes,
catalase (EC 1.11.1.6) from beef liver,
deoxyribonuclease I (EC 3.1.4.5) from bovine pancreas,
glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
from rabbit muscle,
glutamate dehydrogenase (EC 1.4.1.3) from beef liver,
isocitrate dehydrogenase (EC 1.1.1.42) from pig heart,
lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle,
malate dehydrogenase (EC 1.1.1.37) from pig heart,
3-phosphoglycerate kinase (EC 2.7.2.3) from yeast,
pyruvate kinase (EC 2.7.1.40) from rabbit muscle.

The following proteins were obtained from Sigma Chemical Co.;

horse heart cytochrome c,
bovine serum albumin,
chicken ovalbumin.

Staphylococcus aureus V8 protease was obtained from Miles,
Slough, U.K.

Bovine pancreatic trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.4.5)
were obtained from Worthington Biochemical Corp., Freehold, New
Jersey, U.S.A.)

2.1.4 E. coli and N. crassa.

(a) E. coli

E. coli/

E. coli K12 was obtained from the American Type Culture collection, catalogue number ATCC 14948 (F⁻, λ lysogenic derivative of K12; Lederberg strain W3100).

E. coli AB2829/pKD501 (Duncan et al., 1984a) was obtained from

Mr. K. Duncan, Department of Biochemistry, Glasgow University.

E. coli AB2829 (aroA354, λ^- , supE42) is an aroA⁻ derivative of E. coli K12.

(b) N. crassa

N. crassa strain 74-OR23-1A (F.G.S.C. No. 987) was obtained from

the Fungal Genetics Stock Centre, Arcata, CA, U.S.A. N. crassa

was grown, harvested and stored as described by Lumsden & Coggins (1977).

2.1.5 Miscellaneous materials.

Coomassie Brilliant Blue G250 was from Serva Feinbiochemica, Heidelberg, West Germany. Nutrient broth was obtained from Oxoid, Basingstoke, U.K. Bacto-tryptone and yeast extract were from Difco, Detroit, Michigan, U.S.A.

2.2 General Methods

2.2.1 pH measurements.

The pH of solutions was measured using a Radiometer pH electrode at room temperature.

2.2.2 Conductivity measurements.

The conductivity of solutions was measured at 4°C using a Radiometer conductivity meter type CDM2e (Radiometer, Copenhagen, Denmark).

2.2.3 Acid-washed glassware.

Glassware for protein chemistry was immersed overnight in conc. nitric acid, then rinsed extensively with water. The acid-washed glassware was dried by baking in an oven.

2.2.4/

2.2.4 Protein estimation.

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.2.5 Preparation of chromatography media.

Sephacryl S200, phenyl-Sepharose and DEAE-Sephacel were equilibrated with the appropriate buffer before use. DEAE-Sephacel was recycled by washing with 50 mM Tris-HCl pH7.5, 2M NaCl, and phenyl-Sepharose was recycled by washing with water.

DEAE-cellulose (DE52) was prepared as in the manufacturer's instructions. Phosphocellulose was pre-cycled by washing in dilute alkali and acid, de-fined and resuspended in either 10 mM potassium citrate pH6.0 (for EPSP synthase preparations) or 50 mM potassium phosphate pH6.5 (for chorismate synthase preparations). Phosphocellulose and DEAE-cellulose were discarded after being used once.

2.3 Polyacrylamide gel electrophoresis.

The ratio of acrylamide:bis-acrylamide in all PAGE experiments was 30:0.8. Polymerisation was induced by 0.03% (v/v) N,N,N',N'-tetramethyl diamine and 0.05% ammonium persulphate.

2.3.1 Non-denaturing PAGE.

The discontinuous gel system of Davis (1964) was used, electrophoresis was performed at 4°C in 7% polyacrylamide tube gels. The gel buffer was 0.375 M Tris-HCl pH8.9 and the well buffer was 10 mM Tris/76 mM glycine, the upper well buffer contained 0.1 mM DTT. Gels were pre-electrophoresed for 1 h before enzyme samples were applied.

Samples for non-denaturing PAGE were mixed with 5 μ l 0.05% (w/v) bromophenol blue and made 10% (v/v) glycerol if necessary before electrophoresis.

2.3.2/

2.3.2 SDS PAGE

SDS PAGE was carried out at room temperature as described by Laemmli (1970) in a slab gel apparatus (Raven Scientific Ltd., Haverhill, Suffolk). Slab gels were generally 20 cm x 15 cm x 1.2 mm. Separating gels were 10% or 15% polyacrylamide, the gel buffer was 0.375 M Tris-HCl pH8.8, 0.1% SDS. Stacking gels were 3% polyacrylamide and the gel buffer was 0.125 M Tris-HCl pH6.8, 0.1% SDS. The well buffer was 25 mM Tris/190 mM glycine, 0.1% SDS.

Samples for SDS PAGE were prepared by adding an equal volume of 3% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue, 10% (v/v) glycerol. The samples were then heated for 2 min at 100°C prior to electrophoresis.

2.3.3 Staining.

(a) Protein staining

Unless otherwise stated, gels were stained for protein using Coomassie Brilliant Blue G250. Gels were immersed in 0.1% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid for 1 h at 40°C and then destained in 10% (v/v) methanol, 10% (v/v) acetic acid also at 40°C.

Gels were stained for protein by the silver method as described by Wray et al (1981) with some modifications. After SDS PAGE, the gel was soaked for 2 to 3 d in 50% (v/v) methanol. The staining solution was prepared by adding 0.8 g AgNO_3 dissolved in 4 ml water to 1.4 ml 14.8 M NH_4OH and 21 ml 0.36% (w/v) NaOH with stirring, the volume was then increased to 100 ml with water. The gel was soaked in the staining solution for 15 min with gentle agitation, it was then rinsed with water and soaked in water/

water for at least 1 h with occasional agitation. The gel was then placed in developing solution which consisted of 2.5 ml 1% (w/v) citric acid and 250 μ l 38% formaldehyde in 500 ml water. Staining was stopped by transferring the gel to 10% (v/v) methanol, 10% (v/v) acetic acid.

(b) Activity staining.

(i) EPSP synthase

The method of Nimmo & Nimmo (1982) was used to stain gels for EPSP synthase activity after non-denaturing PAGE. After electrophoresis, gels were soaked in 50 mM glycine-KOH pH10.0 for 30 min, and then transferred to 50 mM glycine-KOH pH10.0, 10 mM CaCl_2 , 2 mM PEP, 1 mM shik 3-P. EPSP synthase activity was detected as a white band of precipitated calcium phosphate.

(ii) Phosphoserine aminotransferase

After non-denaturing PAGE gels to be stained for phosphoserine aminotransferase (PSAT) activity were soaked in 0.1 M Tris-HCl pH8.2 for 30 min to remove DTT. The gels were then transferred to staining solution which was kept in the dark. The staining solution contained 0.1 M Tris-HCl pH8.2, 12.5 mM 2-oxoglutarate, 0.6 mM nitroblue tetrazolium, 10 mM serine, 1 mM NAD^+ , 0.2 mM phenazine methosulphate and 48 units glutamate dehydrogenase per 8 ml staining solution. PSAT activity was detectable as a purple precipitate.

2.3.4 One-dimensional peptide mapping.

This was performed as described by Cleveland et al (1977). SDS PAGE was carried out as described in section 2.3.2 except that all gel solutions contained 1 mM EDTA.

The protein samples (approximately 2.5 μ g) for one-dimensional peptide/

peptide mapping were contained in gel slices that had been cut out of a 10% polyacrylamide SDS gel stained with Coomassie blue. The gel slices were soaked for 30 min in 0.125 M Tris-HCl pH6.8, 0.1% (w/v) SDS, 1 mM EDTA, and then pushed in to the wells of a 15% polyacrylamide SDS gel. The gel slices were first overlaid with 10 μ l of the above buffer containing 20% (v/v) glycerol, and then with 10 μ l of the same buffer containing 10% glycerol and protease. The protease concentrations were: S. aureus V8 protease, 100 μ g/ml; trypsin, 100 μ g/ml; chymotrypsin, 50 μ g/ml. The current was switched off for 30 min after the samples had reached the bottom of the stacking gel to give time for proteolysis within the gel.

2.4 Preparation and standardisation of substrates.

2.4.1 EPSP synthase

Shik 3-P was isolated from culture filtrates of an Aerobacter aerogenes aromatic auxotroph, and was a gift from Dr. G.A. Nimmo. Shik 3-P was standardised by conversion to chorismate using EPSP synthase and chorismate synthase activities.

PEP was standardised by conversion to lactate using pyruvate kinase and lactate dehydrogenase.

EPSP was prepared enzymatically from shik 3-P and PEP using the N. crassa arom enzyme complex (3 units EPSP synthase activity in the reverse direction), and was purified essentially as described by Knowles et al (1970). The purified barium salt was converted to potassium EPSP by the addition of a 5-fold excess of potassium sulphate. EPSP was standardised by conversion to chorismate using chorismate synthase activity.

2.4.2/

2.4.2 Phosphoserine aminotransferase.

Glutamate was standardised by conversion to 2-oxoglutarate using glutamate dehydrogenase. Treatment with alkaline phosphatase was used to convert 3-phosphohydroxypyruvate to 3-hydroxypyruvate, 3-hydroxypyruvate solutions were standardised by conversion to glycerate using lactate dehydrogenase.

2.5 Enzyme assays.

Spectrophotometric assays were performed at 25°C in a total volume of 1.0 ml. A Gilford Unicam SP500 spectrophotometer equipped with a Gilford photoelectric detector and recorder was used.

One unit of enzyme activity is defined as the amount of enzyme catalysing the conversion of 1 μmol of substrate per minute.
Lines in kinetic plots were fitted by eye.

2.5.1 EPSP synthase

EPSP synthase was routinely assayed in the forwards direction by coupling to chorismate synthase. Chorismate ($\epsilon_{275} = 2630 \text{ M}^{-1} \text{ cm}^{-1}$; Gibson, 1970) formation was monitored at 275 nm. The routine assay mixture contained 50 mM TEA·HCl-KOH pH7.0, 50 mM KCl, 2.5 mM MgCl_2 , 0.5 mM shik 3-P, 0.5 mM PEP, 20 μM NADPH, 10 μM FMN and 8 m units partially purified N. crassa chorismate synthase.

EPSP synthase was assayed in the reverse direction by coupling the release of PEP to the pyruvate kinase and lactate dehydrogenase reactions. Oxidation of NADH ($\epsilon_{340} = 6200 \text{ M}^{-1} \text{ cm}^{-1}$) was monitored at 340 nm. The assay mixture contained 100 mM potassium phosphate pH7.0, 2.5 mM MgCl_2 , 2.5 mM ADP, 0.1 mM NADH, 50 μM potassium EPSP, 3 units pyruvate kinase and 2.5 units lactate dehydrogenase.

Unless otherwise stated, units of EPSP synthase activity quoted have been/

been determined using this assay procedure.

-- For kinetic studies of EPSP synthase in the-forwards direction, the assay mixture contained 50 mM bis Tris-HCl pH7.0, 50 mM KCl, 2.5 mM MgCl_2 , 10 μM FMN, 20 μM NADPH, 10 m units partially purified N. crassa chorismate synthase, PEP, shik 3-P and where appropriate, glyphosate. In the reverse direction the assay mixture contained 50 mM bis Tris-HCl pH7.0, 50 mM KCl, 2.5 mM MgCl_2 , 0.1 mM NADH, 2.5 mM ADP, 3 units pyruvate kinase, 2.5 units lactate dehydrogenase, phosphate and EPSP.

2.5.2 Phosphoserine aminotransferase.

This enzyme was routinely assayed by a modification of the method of Hirsch & Greenberg (1967) using 3-hydroxypyruvate and glutamate as substrates. PSAT activity was monitored at 340 nm by coupling the release of 2-oxoglutarate to glutamate dehydrogenase. The routine assay mixture contained 50 mM Tris-HCl pH8.2, 32 mM ammonium acetate, 2 mM glutamate, 2.5 mM 3-hydroxypyruvate, 0.2 mM NADH and 12 units glutamate dehydrogenase.

Modifications to this routine assay were introduced in order to assay PSAT activity with other pairs of keto acids/amino acids.

(i) To determine PSAT activity with other keto acids,

3-hydroxypyruvate was replaced with the appropriate substrate at a concentration of 1 mM.

(ii) Glutamate was replaced with 1 mM aspartate, and glutamate dehydrogenase with 6 units malate dehydrogenase in order to determine PSAT activity with aspartate as an amino group donor; ammonium acetate was omitted.

PSAT was also assayed in the reverse direction with either serine or 3-phosphoserine as amino donor.

(i)/

- (i) serine: the assay mixture contained 50 mM Tris-HCl pH8.2, 1 mM serine, 1 mM 2-oxoglutarate, 0.2 mM NADH and 2.8 units lactate dehydrogenase.
- (ii) 3-phosphoserine: the assay mixture contained 50 mM Tris-HCl pH8.2, 1 mM 2-oxoglutarate, 0.2 mM NAD⁺ and 60 units glutamate dehydrogenase.

2.5.3 Chorismate synthase.

N. crassa chorismate synthase was assayed spectrophotometrically at 275 nm. The assay mixture contained 50 mM TEA·HCl-KOH pH7.0, 50 mM KCl, 2.5 mM MgCl₂, 20 μM NADPH, 10 μM FMN and 50 μM potassium EPSP.

2.6 Growth of E. coli and preparation of cell extracts.

2.6.1 Media.

Minimal medium + glucose (MM + glc) consisted of M9 salts plus 0.2% (w/v) glucose and 100 μM CaCl₂.

M9 salts contained (per litre):

Na ₂ HPO ₄	6 g
KH ₂ PO ₄	3 g
NH ₄ Cl	1 g
MgSO ₄ ·7H ₂ O	0.13 g

Nutrient broth medium was 13 g oxoid nutrient broth per litre.

L-Broth medium contained (per litre):

bacto-tryptone	10 g
yeast extract	5 g
NaCl	10 g

and 0.1% (w/v) glucose.

All media were sterilised by autoclaving.

E. coli were grown at 37°C, in 250 ml conical flasks, with shaking on an orbital shaker unless otherwise specified.

2.6.2 Growth of E. coli K12.

E. coli K12 cells for enzyme preparations were grown by B.A. Brodie, A.A. Coia and J. Greene.

Working stock cultures which had been grown in nutrient broth were stored at 4°C.

E. coli K12 cells were grown as follows:

- (i) Nutrient broth (50 ml) was inoculated with 2 or 3 drops of stock culture and grown overnight. It was then stored at 4°C for several hours.
- (ii) A portion of this overnight culture (1 ml) was used to inoculate 50 ml MM + glc, this second culture was then grown overnight.
- (iii) Then 8 flasks containing 50 ml MM + glc were inoculated with 1 ml of the overnight MM + glc culture, and grown for 8 h. These cultures were stored at 4°C overnight.
- (iv) Large scale growth of E. coli K12 was in 3 l MM + glc in 10 l flasks, each 3 l of MM + glc was inoculated with 2 x 50 ml MM + glc cultures. The 3 l cultures were stirred constantly by rotating bar magnets and compressed air was introduced to each flask at a flow rate of 400 ml/min. Growth of the large scale cultures was monitored at 420 nm, the cells were harvested in the late logarithmic phase of growth when the A_{420} was between 3.5 and 5.0 (the growth time was approximately 8 h).
- (v) The cells were harvested by centrifugation at 6,000 g for 15 min at 4°C. The pellets were resuspended in a minimal volume of

100 mM Tris-HCl pH7.5, 0.4 mM DTT and stored at -20°C.

Typically, a yield of 25-30 g E. coli K12 cells (wet weight) was obtained from 12 l MM + glc.

2.6.3 Growth of E. coli AB2829/pKD501.

The stock culture of E. coli AB2829/pKD501 was in L-Broth containing 40% (v/v) glycerol at -20°C.

E. coli AB2829/pKD501 cells were grown for enzyme preparation as follows:

- (i) L-Broth (10 ml) containing 20 µg/ml tetracycline, was inoculated with E. coli AB2829/pKD501 and grown overnight.
- (ii) MM + glc (100 ml) was inoculated with 0.5 ml of the overnight L-Broth culture. This culture was grown for 8 to 10 h.
- (iii) The MM + glc culture was used to inoculate 18 x 500 ml batches of MM + glc in 2 l conical flasks to an A_{650} of 0.005. These large scale cultures were grown for 11 to 15 h until the A_{650} of the cultures was between 1.3 and 1.5.
- (iv) E. coli AB2829/pKD501 cells were then harvested and stored as described for E. coli K12 (section 2.6.2).

Approximately 20 g of E. coli AB2829/pKD501 were obtained from 9 l MM + glc.

2.6.4 Growth of E. coli for the preparation of 100,000 g supernatants.

Both E. coli K12 and E. coli AB2829/pKD501 were grown as described below for the preparation of 100,000 g supernatants.

- (i) A 10 ml overnight L-Broth culture was grown up, in the case of E. coli AB2829/pKD501 the culture contained 20 µg/ml tetracycline.
- (ii)/

- (ii) MM + glc (100 ml) was inoculated with 0.5 ml of this overnight culture, this MM + glc culture was grown until the A_{650} was approximately 1.0.
- (iii) The cells were collected by centrifugation at 7,000 g for 15 min at 4°C, then resuspended in 100 ml 0.2 M Tris-HCl pH7.5, 0.2 M KCl, 0.4 mM DTT. The cells were again collected by centrifugation and resuspended in 5 ml of the above buffer. At this stage cells were either stored at -20°C overnight or sonicated immediately.

2.6.5 Cell breakage.

(a) Sonication and the preparation of 100,000 g supernatants.

Sonication was used to break cells for the preparation of 100,000 g supernatants. A soniprobe type 1130 A (Dawe Instruments Ltd.) was used, sonication was at 3 amps for 3 x 30s with 30s rest intervals. The soniprobe housing was kept cool during sonication using an ice-water slurry.

The sonicated cells were centrifuged at 100,000 g for 2 h at 4°C, the supernatant was retained for enzyme assays.

(b) French pressure cell.

E. coli cells for enzyme purification (approximately 20 g wet weight) were resuspended to a final volume of 30 to 40 ml in 0.1 M Tris-HCl pH7.5, 1 mM EDTA, 0.4 mM DTT at 4°C. The cells were broken by two passages through a French pressure cell at 8 tons/in². The body of the French pressure cell was cooled in ice prior to use. (The cell has a 1 in diameter piston with manual fill and a capacity/

TABLE 2.1

Purification scheme for chorismate synthase from N. crassa.

Step	Stage	Volume (ml)	Protein concentration (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (-fold)
1	Crude extract	1290	8.2	0.038	49.0	100	0.0046	1
2	-ve DEAE-cellulose	1290	5.8	0.038	49.0	100	0.0066	1.4
3	40-50% (NH ₄) ₂ SO ₄	66	24.8	0.578	38.1	78	0.023	5.0
4	DEAE-cellulose	186	2.9	0.208	38.7	79	0.072	15.6
5	-ve blue dextran sepharose	195	2.6	0.182	35.5	72	0.07	15.2
6	phosphocellulose I	22	0.8	1.26	27.7	56	1.58	343
7	phosphocellulose II	24	0.31	0.59	14.2	29	1.9	413

The results presented are for a typical purification from 100 g of N. crassa mycelia. Chorismate synthase was assayed as described in section 2.5.3.

capacity for 40 ml, Cat. no. J43398A, American Instruments Company, Silver Spring, Maryland, U.S.A.).

2.7 Enzyme Preparation.

All Tris-HCl buffers used during enzyme purification were prepared by dilution of a stock solution of 1 M Tris adjusted to pH7.5 with HCl at room temperature. No further adjustment of pH was made on dilution.

2.7.1 N. crassa arom enzyme complex.

The arom enzyme complex was purified by the method of Smith & Coggins (1983) as adapted by Lambert et al (Lambert, J.M., Boocock, M.R. & Coggins, J.R., unpublished results).

2.7.2 N. crassa chorismate synthase.

Chorismate synthase was purified from N. crassa for use as a coupling enzyme. The purification procedure was essentially that described by Boocock (1983), and is summarised in Table 2.1.

All steps were carried out at 0-4°C, and all buffers contained 1.2 mM PMSF and 0.4 mM DTT unless otherwise specified.

1. Crude extract: N. crassa mycelia (100 g) were stirred for 1 h in 1.5 l 100 mM potassium phosphate pH7.0, 5 mM EDTA. The extract was centrifuged at 10,000 g for 30 min, the supernatant or crude extract was retained.

2. Negative DEAE-cellulose chromatography: The crude extract was pumped through a column (30 cm x 10 cm) of DEAE-cellulose (DE52) equilibrated in 100 mM potassium phosphate pH7.0.

Chorismate synthase does not bind to DEAE-cellulose under these/

these conditions.

3. $(\text{NH}_4)_2\text{SO}_4$ fractionation: The chorismate synthase pool from step 2 was fractionated with $(\text{NH}_4)_2\text{SO}_4$. The 40-50% saturated fraction was obtained, resuspended in 100 mM potassium phosphate pH7.0 and then dialysed overnight against 2 l 50 mM Tris-HCl pH7.5. The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was centrifuged at 15,000 g for 15 min to remove precipitated protein.
4. DEAE-Sephacel chromatography: The 40-50% $(\text{NH}_4)_2\text{SO}_4$ fraction was subjected to ion-exchange chromatography on a column of DEAE-Sephacel (12 cm x 5 cm). Chorismate synthase was bound to DEAE-Sephacel in 50 mM Tris-HCl pH7.5, the column was washed with this buffer containing 30 mM KCl. A gradient of 30-300 mM KCl in 50 mM Tris-HCl pH7.5 (total volume, 1 l) was applied to elute chorismate synthase activity.
5. Negative blue dextran Sepharose chromatography: The pool of chorismate synthase activity from step 4 was pumped through a column of blue dextran Sepharose (vol = 3 ml) equilibrated in 50 mM Tris-HCl pH7.5, 50 mM KCl. Chorismate synthase does not bind to this column but the arom enzyme complex does, therefore N. crassa EPSP synthase is removed at this step. Chorismate synthase was dialysed against 2 x 2 l 10 mM potassium phosphate pH6.5, 0.2 mM DTT, 0.6 mM PMSF.
6. Phosphocellulose chromatography I: Chorismate synthase from step 5 was loaded on to a column of phosphocellulose (25 cm x 1 cm) equilibrated in 10 mM potassium phosphate pH6.5, 0.2 mM DTT, 0.6 mM PMSF, the column was washed with the same buffer./

buffer. Chorismate synthase was eluted with a gradient (total volume, 400 ml) of 10 mM to 400 mM potassium phosphate pH6.5 containing 0.6 mM PMSF, 0.2 mM DTT. Chorismate synthase was dialysed against 2 l 40 mM potassium phosphate pH6.5, 0.2 mM DTT.

7. Phosphocellulose chromatography II: Chorismate synthase activity was loaded on to a column of phosphocellulose (vol = 3 ml) equilibrated in 40 mM KP_i pH6.5, 0.2 mM DTT, the column was washed with the same buffer. Chorismate synthase was eluted with a step of 250 mM potassium phosphate pH6.5, 0.2 mM DTT, and then dialysed against 50 mM Tris-HCl pH7.5, ^{50% (v/v) glycerol,} 0.4 mM DTT and stored at -20°C.

N. crassa chorismate synthase was purified 413-fold by this procedure and was free of activities that interfered with its use as a coupling enzyme.

2.8 Characterisation of EPSP synthase and phosphoserine amino-transferase.

2.8.1 Performic acid oxidation and amino acid analysis.

Samples of EPSP synthase and PSAT were treated with performic acid (Hirs, 1967) prior to amino acid analysis.

The protein samples were extensively dialysed against 0.5% (w/v) ammonium bicarbonate at 4°C, and then lyophilised.

The performic acid reagent was prepared by mixing 95 volumes of formic acid with 5 volumes of 30% (w/v) H_2O_2 , this mixture was incubated at 25°C for 2 h. Lyophilised protein (approximately 1 mg) was resuspended in 420 μ l 70% formic acid plus 60 μ l methanol. Both the protein sample and the performic acid reagent were cooled to between/

between -5 and 0°C, then 500 μ l of reagent was added to the protein and incubation at -5°C was continued for 2.5 h. The reaction was stopped by adding 10 ml ice-cold water, and the sample was lyophilised.

Samples of performic acid oxidised protein for amino acid analysis (approximately 200 - 250 μ g) were hydrolysed in 500 μ l 6 N HCl, 0.1% (v/v) 2-mercaptoethanol in vacuo for 24, 48, 72 and 96 h at 105°C. The samples were then lyophilised.

Duplicate analyses were carried out on an LKB 4400 amino acid analyser operated by Mr. J. Jardine at the Department of Biochemistry, University of Glasgow.

2.8.2 Carboxymethylation and protein sequencing.

(a) Carboxymethylation.

Carboxymethylation was carried out as described by Lumsden & Coggins (1978). Protein samples were dialysed against 0.5% (w/v) ammonium bicarbonate and then lyophilised. The lyophilised protein was resuspended in 2 ml 0.1 M Tris-HCl pH8.2, 8 M Urea, 2 mM DTT and incubated in the dark for 1 h at room temperature under an atmosphere of N₂. The solution was then made 15 mM in iodoacetate and incubated for a further 1 h. The reaction was terminated by addition of DTT to a final concentration of 30 mM. The carboxymethylated protein was dialysed against 0.5% (w/v) ammonium bicarbonate and lyophilised.

(b) Protein sequencing.

The N-terminal amino acid sequences of carboxymethylated EPSP synthase and PSAT were determined using a Beckman Model 890 liquid phase sequencer (Smith et al, 1982) operated by Mr. B. Dunbar of Aberdeen University. The phenylthiohydantoin samples were analysed by/

by chromatography on a Waters Resolve C₁₈ reverse phase column with a pH5.0 acetate-acetonitrile buffer system (Carter et al, 1983).

Amino acid analysis after hydrolysis with HI (Smithies et al, 1971) was used to confirm doubtful residues.

The automatic protein sequencing was carried out on the SERC funded protein sequencing facility at Aberdeen University with assistance from Mr. B. Dunbar, Professor J.E. Fothergill and Dr. L.A. Fothergill.

2.8.3 Molecular weight determinations.

Standard molecular weight proteins were taken from the list of proteins and enzymes given in section 2.1.3.

(a) Subunit M_r.

SDS PAGE (section 2.3.2) was used to determine the subunit M_r of purified proteins. The proteins used to produce a standard curve of R_f vs log M_r are listed below together with their subunit molecular weights (Weber & Osborn, 1969):

<u>Protein</u>	<u>Subunit M_r</u>
bovine serum albumin	68,000
catalase	60,000
glutamate dehydrogenase	53,000
aldolase	40,000
glyceraldehyde 3-phosphate dehydrogenase	36,000
carbonic anhydrase	29,000

(b) Native M_r.

Gel filtration was used to determine the native M_r of proteins. Two different gel filtration media were used.

(i)/

(i) Sephacryl S200.

A column (56 cm x 1.1 cm) of Sephacryl S200 was equilibrated in 50 mM Tris-HCl pH7.5, 50 mM KCl, 0.4 mM DTT at 4°C. The flow rate was 2.5 ml/h and 0.9 ml fractions were collected. The column void volume (V_o) was measured using blue dextran. The elution volumes (V_e) of proteins of known M_r were determined and a standard curve of V_e/V_o constructed. The standard proteins used to calibrate this column are listed below:

<u>Protein</u>	<u>M_r</u>	<u>Reference</u>
lactate dehydrogenase	144,000	Castellino & Barker, 1968
alkaline phosphatase	100,000	Engström, 1961
malate dehydrogenase	70,000	Thorne & Kaplan, 1963
phosphoglycerate kinase	47,000	Krietsch & Blücher, 1970
cytochrome c	12,400	Margolish <u>et al</u> , 1961.

(ii) HPLC gel filtration. A Gilson model 303 pump and a Michrom model M300 variable wavelength UV. detector were used for HPLC gel filtration.

Both the TSK G2000SW and the TSK G3000SW gel filtration columns

(60 cm x 0.75 cm) were equilibrated with 0.067 M potassium phosphate pH6.8 at room temperature. The flow rate was 0.5 ml/min and the column eluate was monitored at 215 nm.

The calibration proteins for each column are listed below:

TSK G2000SW:

<u>Protein</u>	<u>M_r</u>	<u>Reference</u>
serum albumin	68,000	Tanford <u>et al</u> , 1967
ovalbumin	45,000	Castellino & Barker, 1968
carbonic anhydrase	29,000	Armstrong <u>et al</u> , 1966
myoglobin	17,200	Edmundson, 1965

TSK/

TSK G3000SW

<u>Protein</u>	<u>M_r</u>	<u>Reference</u>
alkaline phosphatase	100,000	Engström, 1961
serum albumin	68,000	Tanford <u>et al</u> , 1967
isocitrate dehydrogenase	61,000	Colman <u>et al</u> , 1970
phosphoglycerate kinase	47,000	Krietsch & Bülcher, 1970
carbonic anhydrase	29,000	Armstrong <u>et al</u> , 1966
myoglobin	17,200	Edmundson, 1965

2.8.4 Chemical modification of EPSP synthase by 3-bromopyruvate.

Samples of EPSP synthase for chemical modification were desalted by HPLC gel filtration on a TSK G2000SW column equilibrated in the appropriate buffer (50 mM potassium citrate pH6.8 or 50 mM bis Tris-HCl pH6.8, 50 mM KCl or 20 mM Hepes-NaOH pH6.8). Gel filtration was carried out essentially as described in section 2.8.3 except that the flow rate was 1 ml/min and the column eluate was monitored at 280 nm.

A fresh stock solution of 3-bromopyruvate (BrPyr) was prepared in the appropriate buffer. A portion of this BrPyr solution was added to a sample of EPSP synthase and this mixture was incubated at 25°C. Aliquots were removed at intervals and chemical modification was stopped by dilution (between 200-fold and 500-fold) into an assay mixture. The remaining EPSP synthase activity was then assayed immediately. The 100% value of EPSP synthase activity was obtained in a parallel experiment in which a sample of EPSP synthase was treated with substrates and/or inhibitors where appropriate, and a volume of buffer equivalent to the volume of BrPyr added in the chemical modification experiment. In all these experiments, EPSP synthase/

synthase was assayed in the forwards direction by coupling to chorismate synthase; the assay buffer was 50mM bis Tris-HCl pH7.0, 50 mM KCl, 2.5 mM MgCl_2 (section 2.5.1).

CHAPTER 3

THE PURIFICATION AND PRELIMINARY CHARACTERISATION OF EPSP SYNTHASE FROM E. COLI K12

3.1 Introduction

This chapter describes the simple and reproducible procedure that was developed for the purification of E. coli K12 EPSP synthase. Some properties of the purified enzyme are also described.

3.2 Purification Procedure

All steps in the purification procedure after cell breakage were carried out at 0-4°C.

(a) Preparation of crude extract

E. coli K12 cells (approximately 20 g) were passed two times through a French pressure cell as described in section 2.6.5. The broken cells were extracted with 60 ml of 100 mM Tris-HCl pH7.5, 0.4 mM DTT (buffer A) containing 1 mM EDTA, deoxyribonuclease I (0.5 mg) was added and the solution was stirred for 1 h. Centrifugation at 38,000 g for 30 min. produced a supernatant that was termed the crude extract.

(b) Ammonium sulphate fractionation

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the crude extract with stirring to give a final concentration of 291 g/l (50% saturation). This solution was stirred for 30 min, then centrifuged at 23,000 g for 30 min. The pellet was discarded and solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant with stirring to give a final concentration of 416 g/l (70% saturation). This solution was stirred for 30 min, and the precipitate was then collected by centrifugation at 23,000 g for 30 min. The precipitate was redissolved in a minimum volume of buffer A, and then dialysed overnight against 1 l 50 mM Tris-HCl pH 7.5, 50 mM KCl, 0.4 mM DTT (buffer B).

(c) DEAE-Sephacel chromatography

The dialysed 50-70% $(\text{NH}_4)_2\text{SO}_4$ fraction was loaded on to a column of DEAE-Sephacel (12 cm x 2.5 cm) equilibrated with buffer B. The column was washed with this buffer until the A_{280} of the eluate was less/

less than 0.2; the flow rate was 70 ml/h and 6 ml fractions were collected. A gradient of 50 to 250 mM KCl in 50 mM Tris-HCl pH7.5, 0.4 mM DTT (gradient volume, 400 ml) was applied to the column (see figure 3.1 for a profile). Fractions containing EPSP synthase activity were pooled and dialysed overnight against 1 l buffer A.

(d) Phenyl-Sepharose chromatography

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the dialysed DEAE-Sephacel pool, with stirring to give a final concentration of 164 g/l (30% saturation). This solution was stirred for 15 min, and then loaded on to a column of phenyl-Sepharose (12 cm x 2 cm) equilibrated with buffer A containing 0.8 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with a further 100 ml of this buffer, then a gradient (total volume, 300 ml) of 0.8 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in buffer A was applied. The flow rate throughout was 70 ml/h and 6 ml fractions were collected (see figure 3.2 for a profile). Fractions containing EPSP synthase activity were combined and dialysed overnight against 2 x 2 l 10 mM potassium citrate pH6.0, 0.4 mM DTT (buffer C).

(e) Phosphocellulose chromatography

The dialysed enzyme was applied to a column of phosphocellulose (bed volume, 5 ml) equilibrated in buffer C, the column was washed with this buffer until the A_{280} of the eluate was less than 0.01. The flow rate was 7.2 ml/h and 2.4 ml fractions were collected. EPSP synthase activity was eluted with buffer C containing 1 mM PEP and 1 mM shik 3-P (figure 3.3). Fractions containing EPSP synthase activity were pooled and desalted on a column (27 cm x 2.5 cm) of Sephadex G-25 equilibrated in buffer A. Solid $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 164 g/l (30% saturation) was added to the desalted enzyme which was then bound to/

TABLE 3.1

Purification scheme for EPSP synthase of E. coli K12

Stage	Volume (ml)	Protein concentration (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (-fold)
Crude extract	74	5.5	¹ ND	ND	-	-	-
50-70% (NH ₄) ₂ SO ₄	33	6.4	0.135	4.4	100	0.021	1
DEAE-Sephacel	52	0.22	0.058	3.0	68	0.264	12.6
Phenyl-Sephadex	38	0.022	0.066	2.5	57	3.0	143
Phosphocellulose ²	1.2	0.044	0.780	0.96	22	17.7	843

¹ Not determined.² Data for enzyme eluted from phosphocellulose are for gel filtered and glycerol-dialysed enzyme.

The results presented are for a typical purification from approximately 20 g of E. coli K12 cells. Enzyme activity was assayed in the reverse direction (assay buffer, potassium phosphate pH7.0).

to a small column of phenyl-Sepharose (bed volume, 2 ml) equilibrated in buffer A containing 0.8 M $(\text{NH}_4)_2\text{SO}_4$. EPSP synthase was subsequently eluted with buffer A and dialysed against buffer B containing 50% (v/v) glycerol before storage at -20°C .

(f) Summary of purification procedure

The results obtained from a typical purification procedure are shown in Table 3.1.

3.3 Purity

Non-denaturing PAGE of purified EPSP synthase showed a single band of R_f 0.48 (7% polyacrylamide gels) on staining for protein. This corresponded with a band of precipitated calcium phosphate on gels stained for EPSP synthase activity (figure 3.4). SDS PAGE of the purified enzyme also showed a single band on staining for protein (track E of figure 3.5).

3.4 Molecular weight

Measurements of native and subunit M_r were made in order to determine the quaternary structure of E. coli EPSP synthase.

The subunit M_r of the purified enzyme was determined by SDS PAGE. The mobilities of standard proteins of known subunit M_r were used to construct a standard curve of electrophoretic mobility against log subunit M_r (figure 3.6). Comparison of the mobility of EPSP synthase with these markers gave a subunit M_r of 49,000.

The native M_r of EPSP synthase was determined by gel filtration. Standard proteins of known M_r were used to calibrate a column of Sephacryl S200 and a standard curve of V_e/V_o against log M_r was constructed (figure 3.7). The measured V_e/V_o for EPSP synthase corresponded to an M_r of 55,000. Gel filtration produced identical values/

TABLE 3.2

Kinetic parameters of E. coli EPSP synthase

Substrate	K_m (co-substrate concentration)
PEP	15 μ M (200 μ M shik 3-P)
Shik 3-P	3.5 μ M (500 μ M PEP)
Phosphate	2.5 mM (50 μ M EPSP)
EPSP	2.5 μ M (50 mM phosphate)

Michaelis constants were determined at fixed concentrations of co-substrates, the assay buffer was 50 mM bis Tris-HCl pH7.0, 50 mM KCl, 2.5 mM $MgCl_2$ for all determinations. K_m values were derived from double reciprocal plots (e.g. Figure 3.9), these K_m values were obtained from single determinations. Duplicate determinations of K_m values differed by not more than $\pm 5\%$.

values of V_e/V_o for purified enzyme and for EPSP synthase activity in 100,000 g supernatants of E. coli K12 cell extracts.

The native M_r of EPSP synthase was also determined by HPLC gel filtration. A standard curve of V_e against $\log M_r$ was constructed from the V_e values measured for proteins of known M_r (figure 3.8). The measured V_e of E. coli EPSP synthase in this case corresponded to an M_r of 42,000.

These results indicate that E. coli EPSP synthase is a monomeric protein under the conditions used for the native molecular weight determinations.

3.5 Kinetic parameters

Michaelis constants for the substrates of the forward and reverse EPSP synthase reactions were determined at fixed concentrations of co-substrates. K_m values were estimated from double reciprocal plots, typical plots are shown in figure 3.9 (a) and (b) from which K_m values for PEP and phosphate were determined. The K_m values are listed in Table 3.2.

The herbicide glyphosate was found to be a potent inhibitor of E. coli EPSP synthase. The inhibition was competitive with respect to PEP (figure 3.10). A K_i for glyphosate of $1.0 \mu M$ was obtained under the conditions specified in the figure legend.

3.6 Discussion

A purification procedure has been developed that reproducibly gives homogeneous EPSP synthase from E. coli K12. This procedure consisted of four steps as outlined in Table 3.1. The specific activity of EPSP synthase in the crude extract is not reported.

This/

This is because there are high blank rates of NADH oxidase activity in the E. coli extracts which prevented accurate measurement of EPSP synthase activity. EPSP synthase activity can be determined in 100,000 g supernatants of E. coli extracts as the NADH oxidase activity sediments in this case. The specific activity in such an extract of E. coli K12 was 0.006 units/mg of protein. The specific activity of EPSP synthase in the crude extract fraction is likely to be somewhat lower than this as some protein is undoubtedly removed through sedimentation.

The NADH oxidase activity was removed by the first step in the purification procedure, ammonium sulphate fractionation. EPSP synthase activity was located in the 50-70% saturation fraction. This activity was stable after $(\text{NH}_4)_2\text{SO}_4$ fractionation provided that 0.4 mM DTT was present in all buffers.

Three chromatographic steps were required to produce homogeneous enzyme. The first of these was ion-exchange chromatography on DEAE-Sephacel (figure 3.1). The major proportion of EPSP synthase eluted by a salt gradient was pooled, and subjected to hydrophobic chromatography on phenyl-Sepharose. E. coli EPSP synthase bound to phenyl-Sepharose in 0.8 M $(\text{NH}_4)_2\text{SO}_4$ and was eluted in good yield by a decreasing gradient of $(\text{NH}_4)_2\text{SO}_4$.

The final step in the purification procedure involved substrate elution from a phosphocellulose column. Phosphocellulose has been reported to be an affinity chromatography medium for both the arom enzyme complex and chorismate synthase of N. crassa (Cole & Gaertner, 1975). A number of purification schemes reported for enzymes of the shikimate pathway include chromatography on phosphocellulose as one step in the procedure. Phosphocellulose has been used to purify the arom enzyme complex of N. crassa (Gaertner & Cole, 1976; Lumsden & Coggins, 1977) and also chorismate synthase of B. subtilis (Hasan & Nester/

Nester, 1978b). Both these enzymes were eluted from phosphocellulose by an increase in phosphate concentration.

Substrate elution from phosphocellulose has been successful in the purification of two shikimate pathway enzymes other than E. coli EPSP synthase. Homogeneous DAHP synthase (Trp) of N. crassa was eluted from phosphocellulose using PEP elution (Nimmo & Coggins, 1981a). PEP is the first substrate to bind to this enzyme which has been shown to have a rapid-equilibrium ordered mechanism (Nimmo & Coggins, 1981b). Chorismate synthase of N. crassa has also been purified by a scheme that involves substrate elution from phosphocellulose. In this case, NADPH, FMN and either EPSP or chorismate are required for efficient elution of the enzyme (Boocock, 1983; A. Lewendon, unpublished results).

E. coli EPSP synthase differs from the above examples in that the enzyme will not bind to phosphocellulose in phosphate buffer, therefore potassium citrate buffer was used for the substrate elution step. A lower pH (pH6.0 rather than pH6.5) was also required, however, EPSP synthase was quite stable under these conditions. The most useful method of substrate elution was with both PEP and shik 3-P in the eluting buffer. A step of phosphate (10 mM) eluted EPSP synthase as a peak of activity, but enzyme eluted in this way was not homogeneous. PEP applied alone did not elute EPSP synthase activity, shik 3-P alone did elute activity but as a very broad peak. A mixture of PEP and shik 3-P eluted a sharp peak of EPSP synthase activity (figure 3.3) that was homogeneous as judged by non-denaturing and SDS PAGE (figures 3.4 and 3.5). This elution pattern is consistent with an ordered mechanism in which shik 3-P is the first substrate to bind to EPSP synthase as has been demonstrated for the N. crassa EPSP synthase (Boocock & Coggins, 1983).

The/

The substrate elution step meant that it was necessary to remove substrates from the purified enzyme. EPSP synthase from phosphocellulose chromatography was desalted on a column of Sephadex G-25, the purified enzyme was concentrated on a small column of phenyl-Sepharose, followed by dialysis into buffer containing 50% glycerol. Purified EPSP synthase was stable for at least 4 months when stored at -20°C .

Figure 3.5 shows an SDS gel that monitors the purification at each stage in this procedure. The specific activity of purified E. coli EPSP synthase was 17.7 units/mg assayed in the reverse direction (phosphate assay buffer) and 62 units/mg when assayed in the forwards direction (triethanolamine assay buffer). EPSP synthase is purified 843-fold and in 22% yield over the $(\text{NH}_4)_2\text{SO}_4$ fraction (Table 3.1). If the specific activity of EPSP synthase in the crude extract is taken as 0.006 units/mg, the total purification achieved is approximately 3000-fold. The yield of pure enzyme was 53 μg from approximately 20 g E. coli K12 cells.

This purification scheme has proved useful in the purification of one other separable EPSP synthase, the enzyme from Pisum sativum has been purified to homogeneity by a very similar procedure (Mousdale & Coggins, 1984a).

Purification schemes for other bacterial EPSP synthases have recently been reported. Anton et al (1983) purified K. pneumoniae EPSP synthase more than 700-fold by $(\text{NH}_4)_2\text{SO}_4$ fractionation, DEAE-cellulose chromatography and gel filtration on Sephadex G-150. EPSP synthase was reported to be more than 90% pure after this procedure. Steinrucken & Amrhein (1984a) have purified K. pneumoniae EPSP synthase to homogeneity by a six step procedure of $(\text{NH}_4)_2\text{SO}_4$ fractionation, a heat precipitation step, and/

TABLE 3.3

Specific activities of purified EPSP synthases

Source	Reference	Assay conditions	Specific activity (units/mg)
<u>E. coli</u> K12	this study	0.5 mM PEP, 0.5 mM Shik 3-P 25°C, 50 mM TEA·HCl-KOH pH7.0, 50 mM KCl, 2.5 mM MgCl ₂ .	62
<u>K. pneumoniae</u>	Steinrucken & Amrhein (1984a)	30°C, 20 mM Hepes-NaOH pH6.8 5 mM PEP, 5 mM Shik 3-P	37.8
<u>K. pneumoniae</u>	Anton et al (1983)	37°C, 10 mM Tris-HCl pH7.6 2 mM PEP, 3 mM Shik 3-P	45
<u>P. sativum</u>	Mousdale & Coggins (1984a)	25°C, 100 mM TEA·HCl-KOH pH7.0 0.5 mM PEP, 0.5 mM Shik 3-P	11.5

The specific activities reported for three purified EPSP synthases are compared with the specific activity of E. coli EPSP synthase. In each case, EPSP synthase is assayed in the forwards direction.

and chromatography on DEAE-cellulose, Sephadex G-75, phosphocellulose (where EPSP synthase was eluted by a salt gradient) and finally chromatofocussing.

The specific activities reported for the other purified, monofunctional EPSP synthases are compared with the specific activity of the E. coli enzyme in Table 3.3. The specific activity of EPSP synthase purified in this study compares favourably with the specific activities of the other purified enzymes, although differences in the assay conditions must be considered.

The M_r values reported here indicate that E. coli EPSP synthase is a monomeric enzyme. As EPSP synthase in relatively crude extracts of E. coli K12 has the same native M_r as the purified enzyme, it is unlikely that quaternary structure is being lost during the purification procedure.

The different M_r values determined for E. coli EPSP synthase by gel filtration on Sephacryl S200 and by HPLC gel filtration on a TSK G2000SW column could be explained by differences in the properties of these gel filtration media. Discrepancies in M_r values obtained from these two types of column have been observed with other enzymes (Chaudhuri, S. & J.R. Coggins, unpublished results). The fact that different sets of standard M_r proteins were used to calibrate each of these columns must also be considered.

The other separable EPSP synthases that have been purified to homogeneity also appear to be monomeric enzymes. Table 3.4 shows a comparison of native and subunit M_r values determined for the K. pneumoniae (Steinrucken & Amrhein, 1984a) and P. sativum (Mousdale & Coggins, 1984a) EPSP synthases. The M_r estimates are rather similar for all three enzymes. M_r values have been reported for several unpurified EPSP synthase activities, a number of bacterial EPSP synthases were shown/

TABLE 3.4

Molecular weights of purified EPSP synthases

Source	Subunit M_r	Native M_r	Reference
<u>E. coli</u> K12	49,000	55,000 42,000	this study
<u>P. sativum</u>	50,000	44,000	Mousdale & Coggins (1984a)
<u>K. pneumoniae</u>	43,000	32,400	Steinrucken & Amrhein (1984a)

M_r values determined for E. coli EPSP synthase are compared with the molecular weights reported for the two other monofunctional EPSP synthases to have been purified to homogeneity. All subunit M_r values were determined by SDS PAGE, and all native M_r values by gel filtration.

shown to have molecular weights in the range 33,000 to 45,000 by sucrose density gradient centrifugation (Berlyn & Giles, 1969). The native M_r of partially purified EPSP synthase from mung beans, Phaseolus mungo (Koshiba, 1979a) and the fungus, Hansenula henricii (Bode & Birnbaum, 1981) were reported to be 44,000 by gel filtration. All these M_r estimates are in the same range as the M_r values determined for E. coli EPSP synthase.

Michaelis constants for E. coli EPSP synthase were determined using purified enzyme (Table 3.2). The K_m values reported in this study are much lower than those reported previously by Levin & Sprinson (1964), however they used a bioassay procedure ^{at a pH of 6.1} to assay EPSP synthase while a sensitive coupled assay ^{at a pH procedure} ~~was used~~ in this study.

Glyphosate is a potent inhibitor of E. coli EPSP synthase, inhibition is competitive with respect to PEP (figure 3.10). This type of inhibition has been reported for the EPSP synthases of N. crassa (Boocock & Coggins, 1983), P. sativum (Mousdale & Coggins, 1984a) and K. pneumoniae (Steinrucken & Amrhein, 1984b). K_i values for glyphosate for the K. pneumoniae and N. crassa EPSP synthases ($1 \mu M$ and $1.1 \mu M$ respectively) are similar to that reported here for the E. coli enzyme ($1.0 \mu M$). However, a K_i of 80 nM was reported for the P. sativum EPSP synthase which therefore appears to be more sensitive to glyphosate inhibition than the bacterial and fungal enzymes.

Further characterisation of EPSP synthase was limited by the rather small amounts of enzyme that could be readily purified from E. coli K12. This problem was solved by the use of a strain of E. coli that contained elevated levels of EPSP synthase, the use of this strain is described in the next chapter.

FIGURE 3.1

Ion-exchange chromatography on DEAE-Sephacel.

The dialysed 50-70% $(\text{NH}_4)_2\text{SO}_4$ fraction was chromatographed on a column of DEAE-Sephacel as described in Section 3.2 (c). (o), A_{280} ; (●), EPSP synthase activity; (□-□), conductivity.

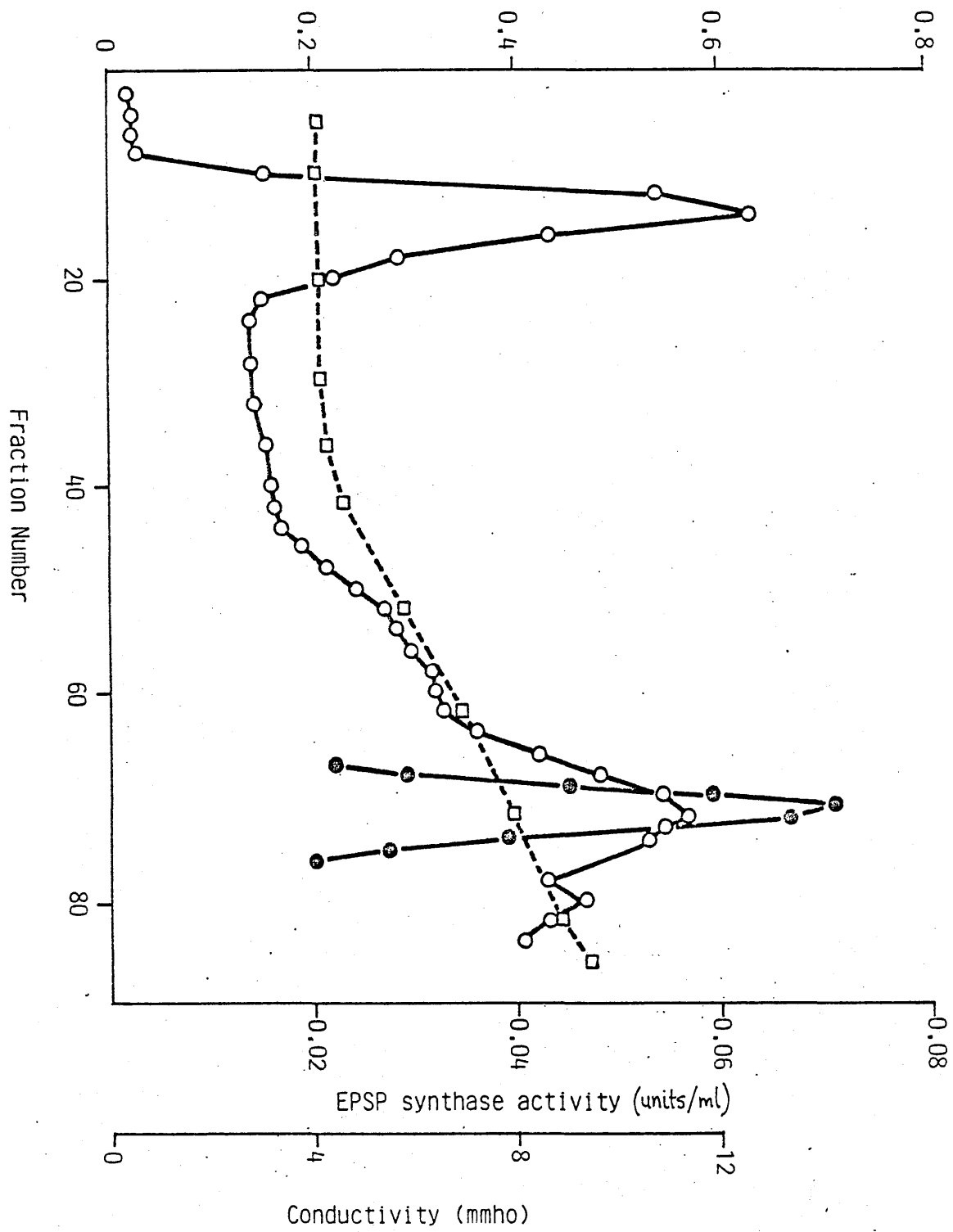


FIGURE 3.2

Chromatography on phenyl-Sepharose.

Enzyme from chromatography on DEAE-Sephacel was subjected to chromatography on phenyl-Sepharose as described in Section 3.2 (d).

(o), A_{280} ; (●), EPSP synthase activity, (---), conductivity.

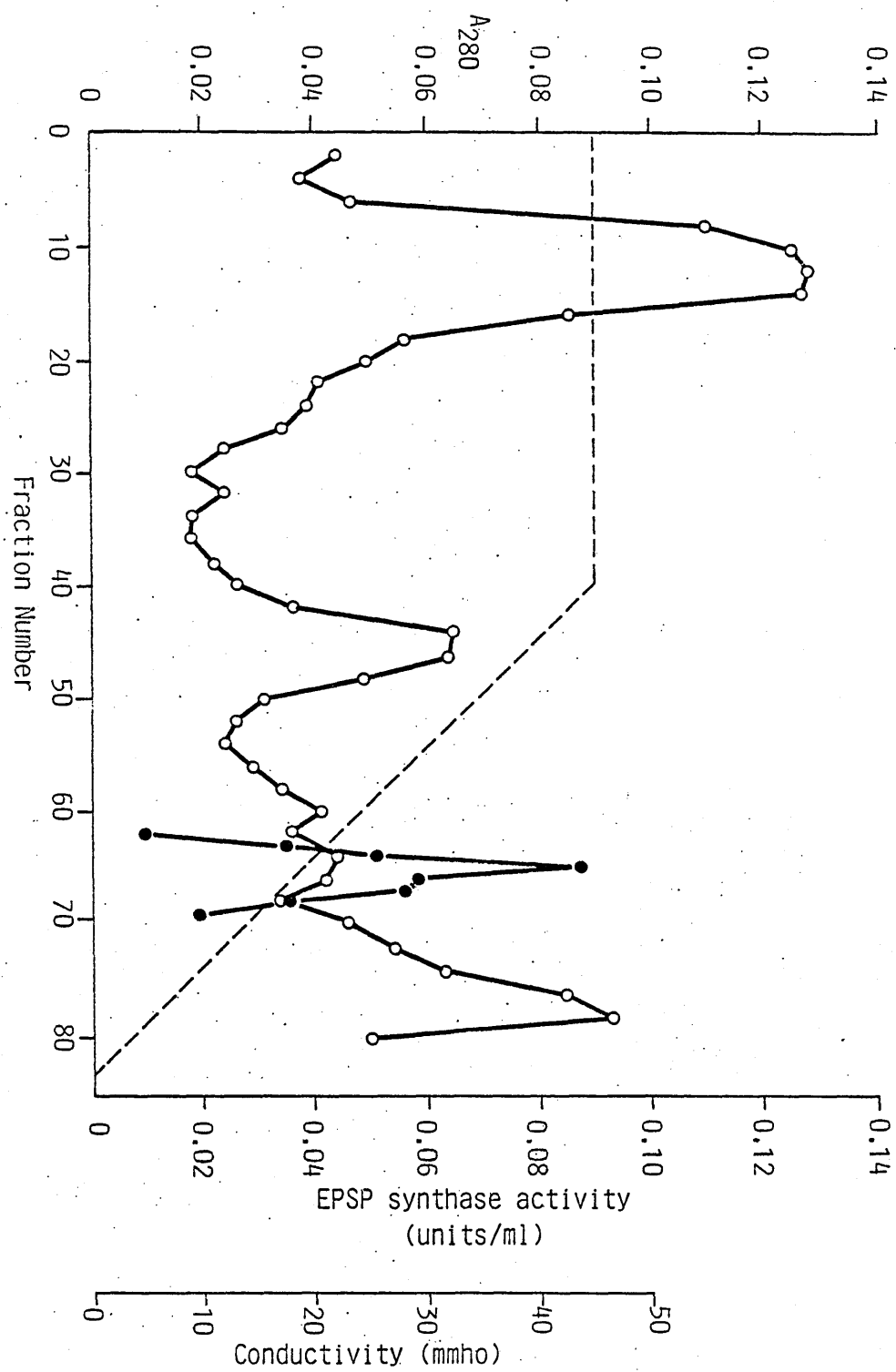


FIGURE 3.3

Chromatography of *E. coli* EPSP synthase on phosphocellulose.

Enzyme from chromatography on phenyl-Sepharose was loaded on to a column of phosphocellulose as described in Section 3.2 (e). The column was washed with buffer C and at the arrow buffer C containing 1 mM PEP and 1 mM shik-3-P was applied. (o), A_{280} ; (●), EPSP synthase activity.

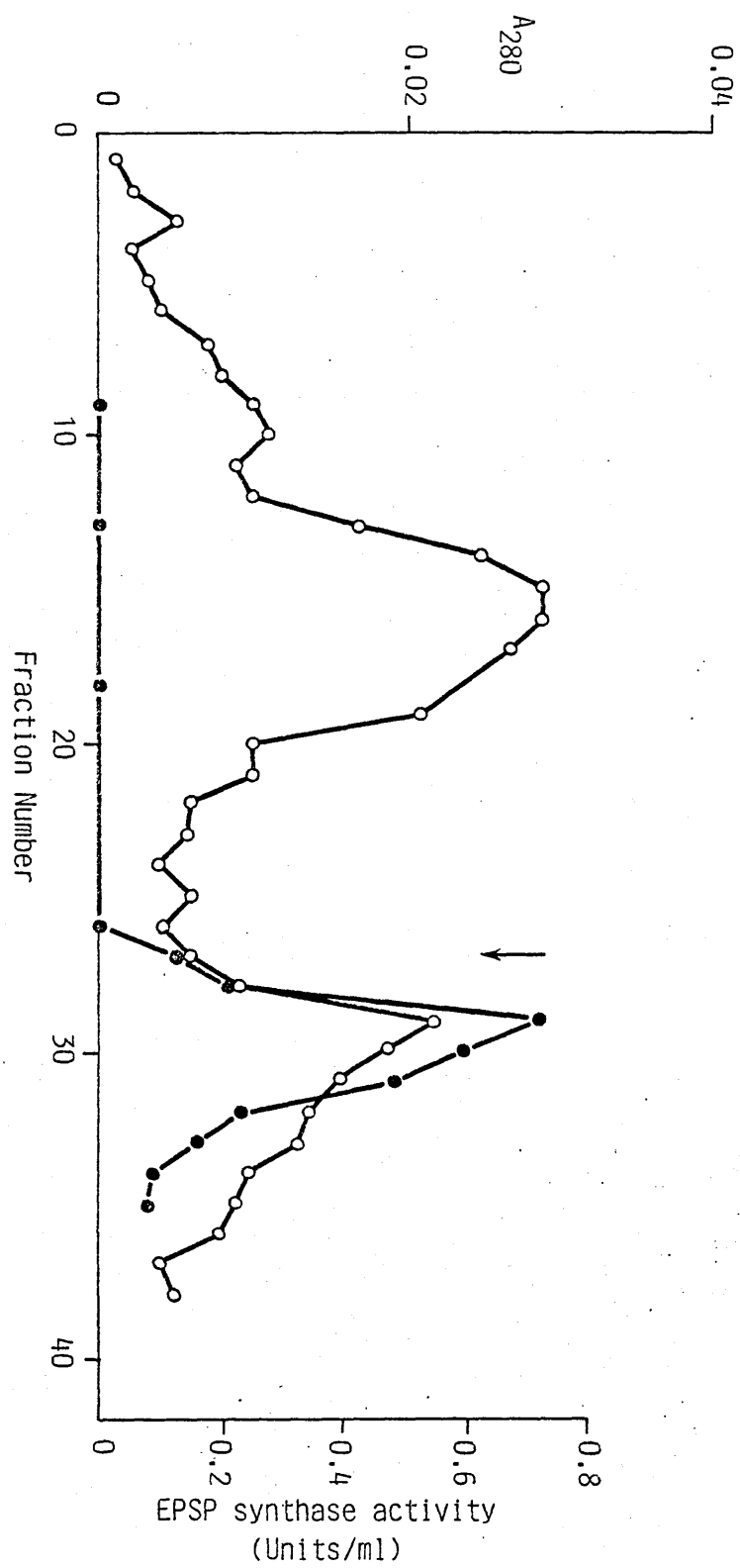
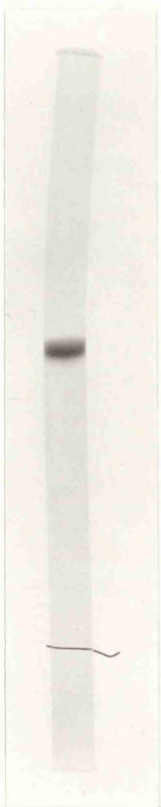


FIGURE 3.4

Non-denaturing PAGE of purified EPSP synthase.

Samples of purified EPSP synthase were subjected to non-denaturing PAGE (7% polyacrylamide gels) as described in Section 2.3.1. Gel A was stained for protein (2.2 μ g loaded) and gel B was stained for EPSP synthase activity (28.3 m units).



A

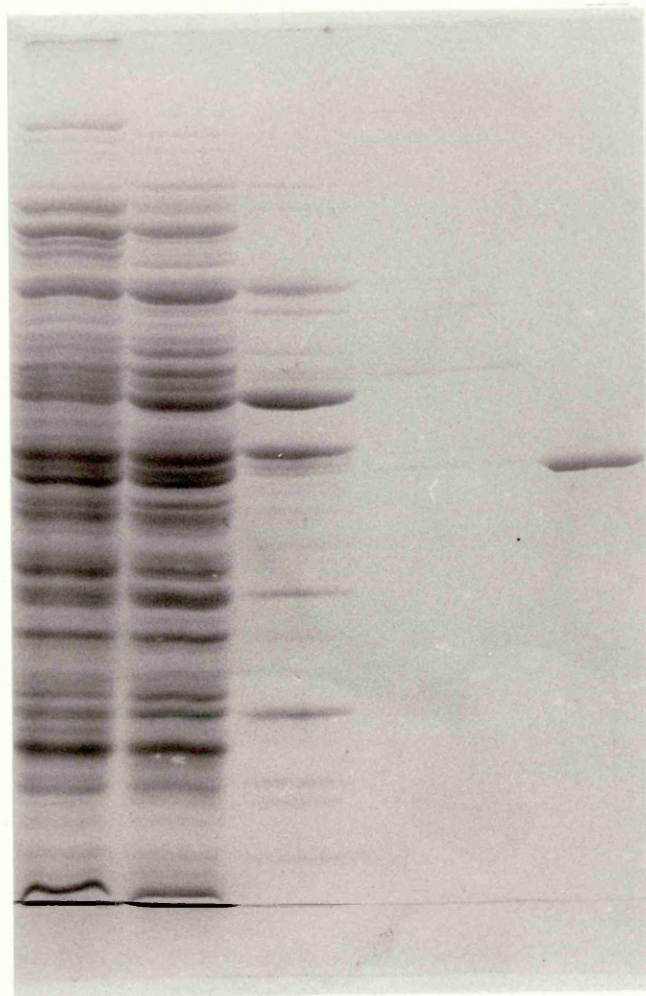


B

FIGURE 3.5

Purification of EPSP synthase from E. coli K12.

SDS PAGE (10% gel) monitoring the purification of EPSP synthase from E. coli K12. Track A, crude extract (200 μ g protein); track B, 50-70% $(\text{NH}_4)_2\text{SO}_4$ fraction (174 μ g); track C, DEAE-Sephacel pool (35 μ g); track D, phenyl-sepharose pool (4 μ g); track E, enzyme after chromatography on phosphocellulose (3.4 μ g).



A B C D E

FIGURE 3.6

Standard curve of R_f against $\log M_r$.

SDS PAGE (10% polyacrylamide gel) was carried out as described in Section 2.3.2, the standard proteins (●) are listed in Section 2.8.3, the R_f of EPSP synthase is shown (○).

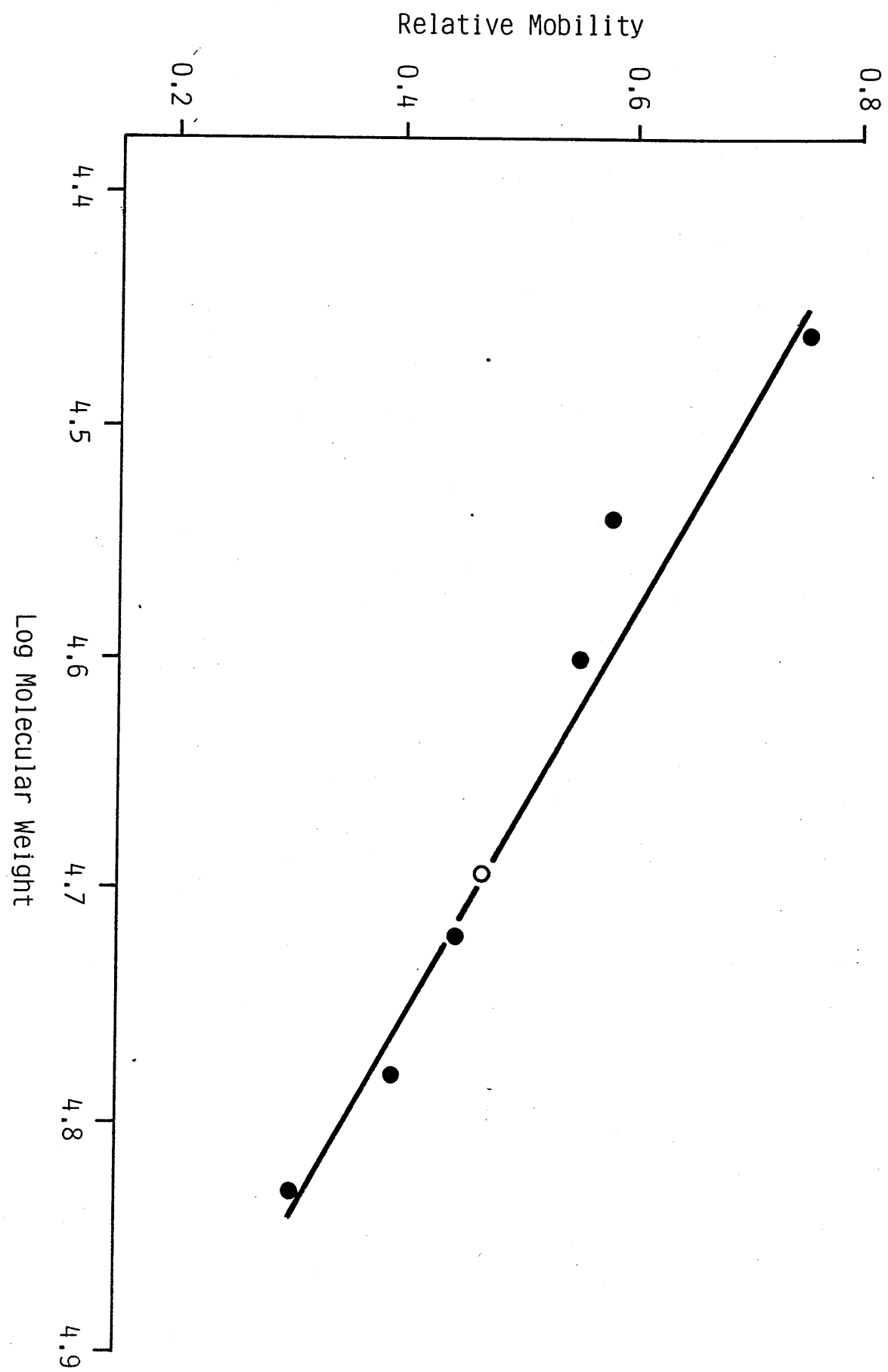


FIGURE 3.7

Standard curve of V_e/V_o against $\log M_r$.

A Sephacryl S200 column was calibrated with standard proteins (●) as described in Section 2.8.3. The V_e/V_o of E. coli EPSP synthase is shown (○).

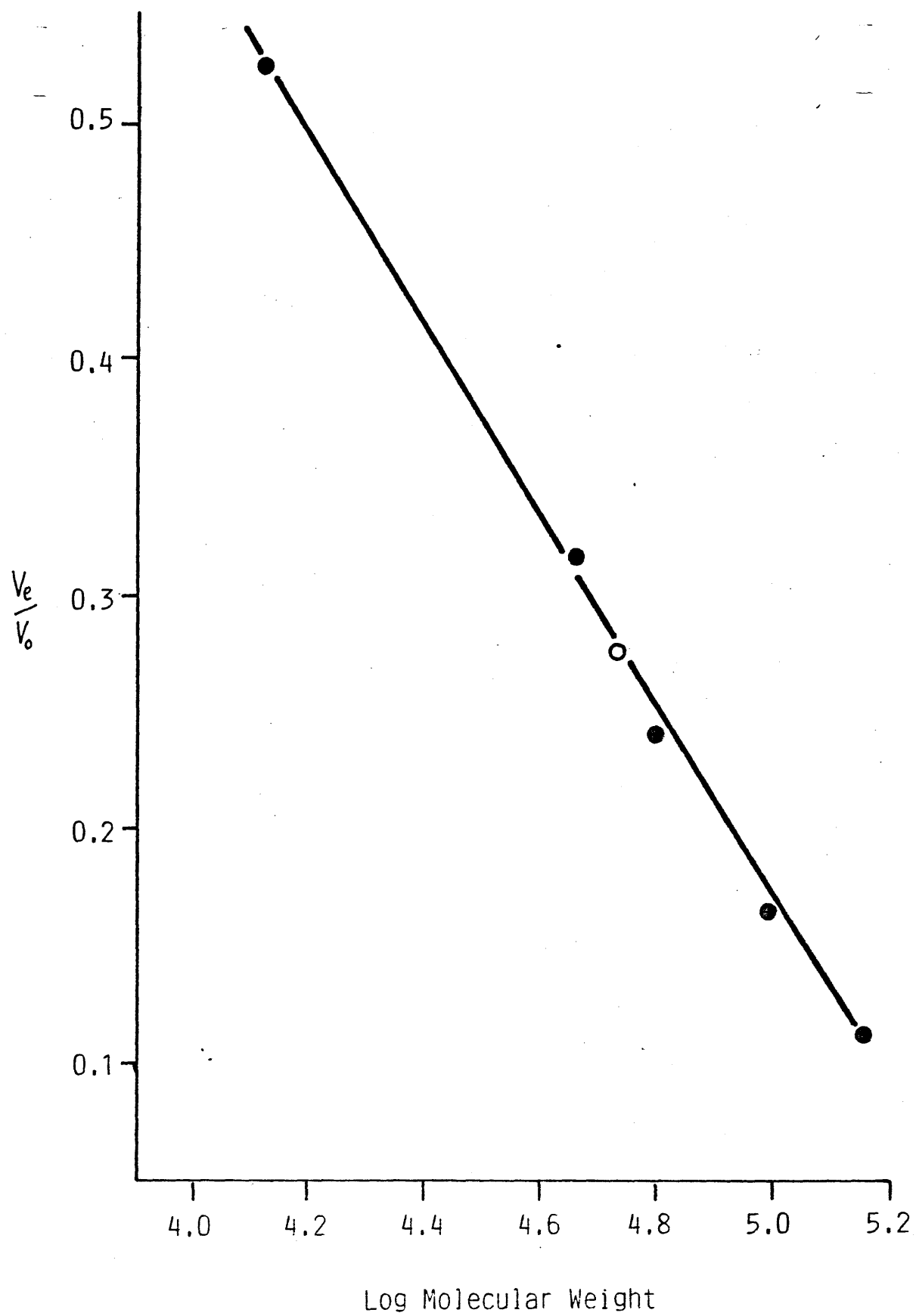


FIGURE 3.8

Standard curve of V_e against $\log M_r$.

An HPLC gel filtration TSK G2000SW column was calibrated with standard proteins (●) as described in Section 2.8.3. The V_e of EPSP synthase is shown (o).

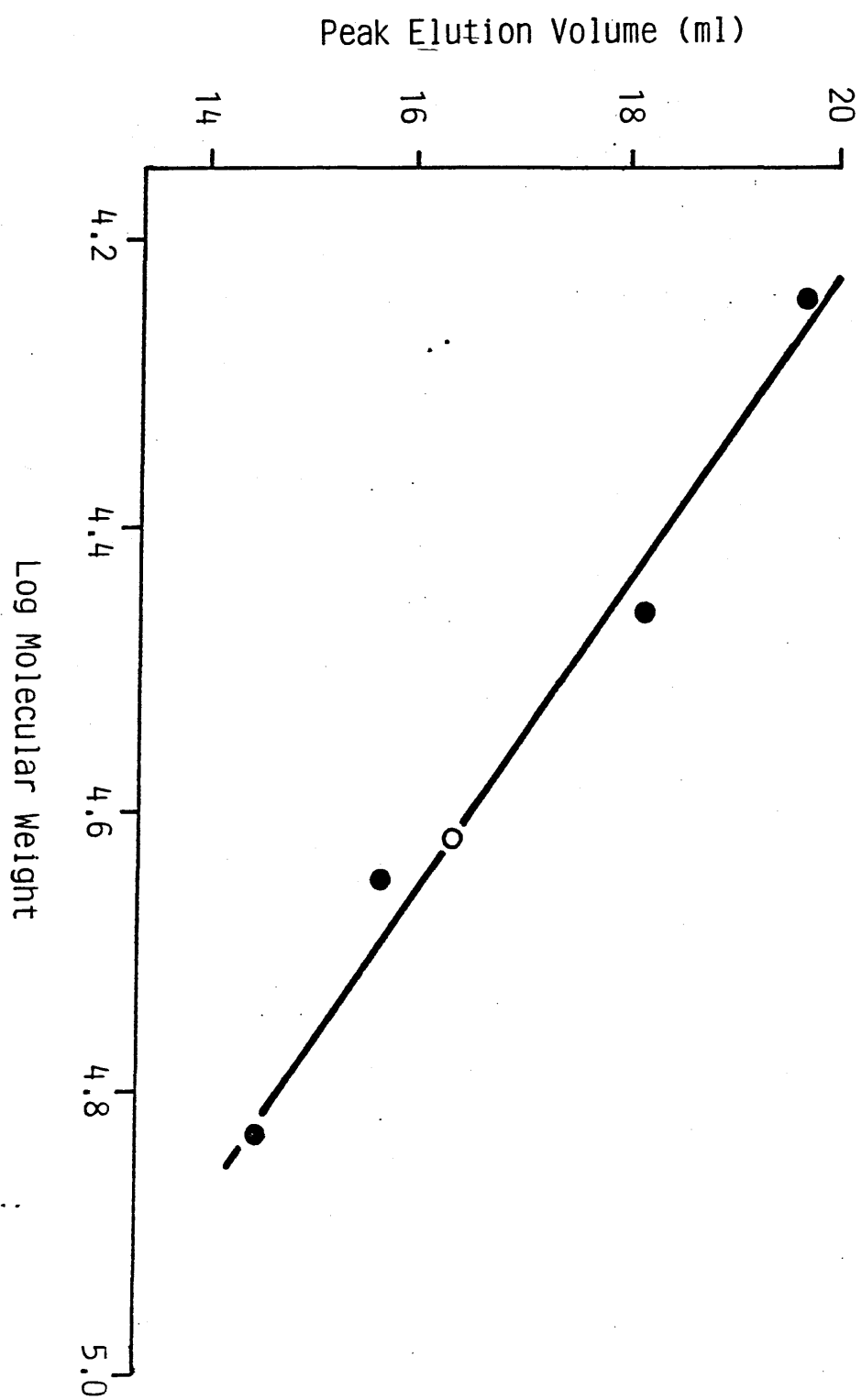


FIGURE 3.9

Double reciprocal plots of EPSP synthase.

(a) Double reciprocal plot of initial velocity against [PEP] at fixed 200 μM shik 3-P.

(b) Double reciprocal plot of initial velocity against [phosphate] at fixed 50 μM EPSP.

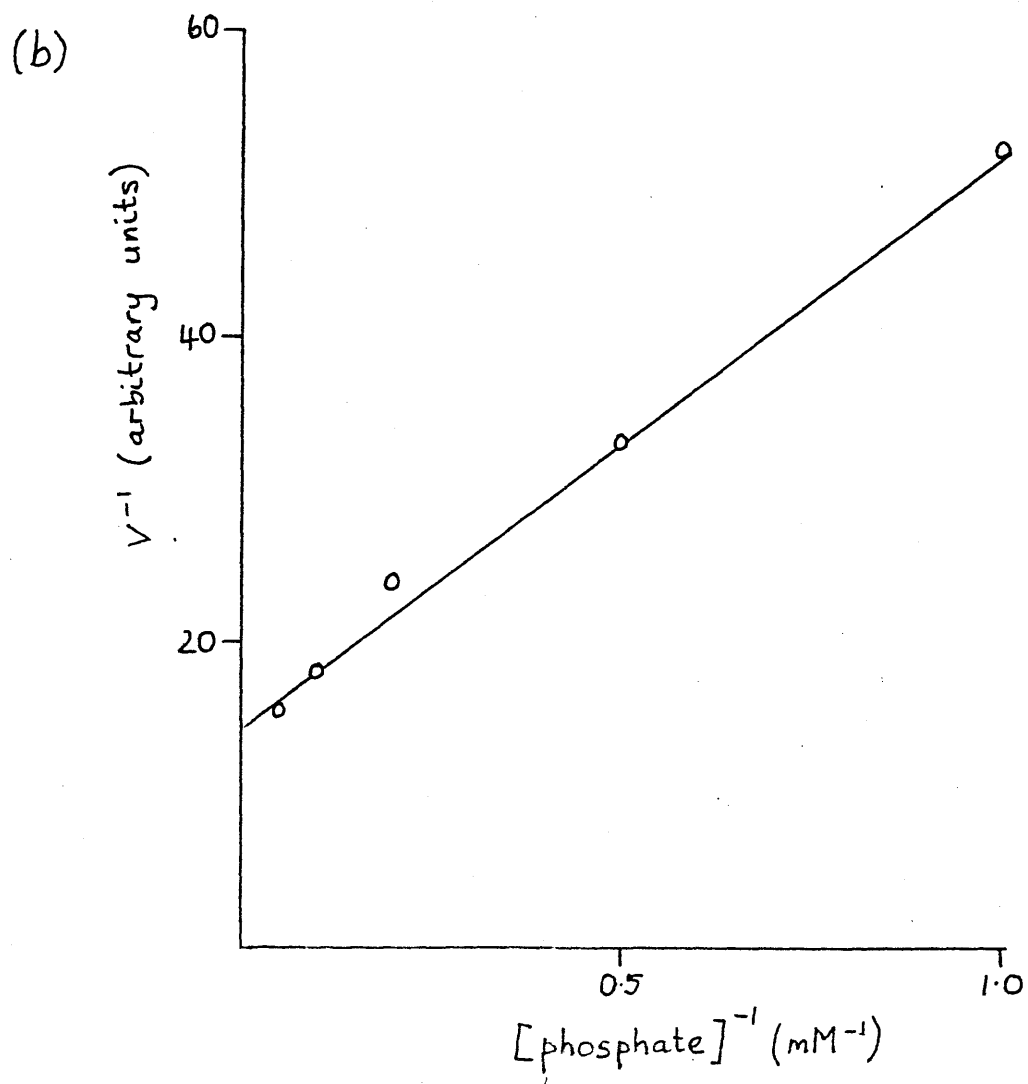
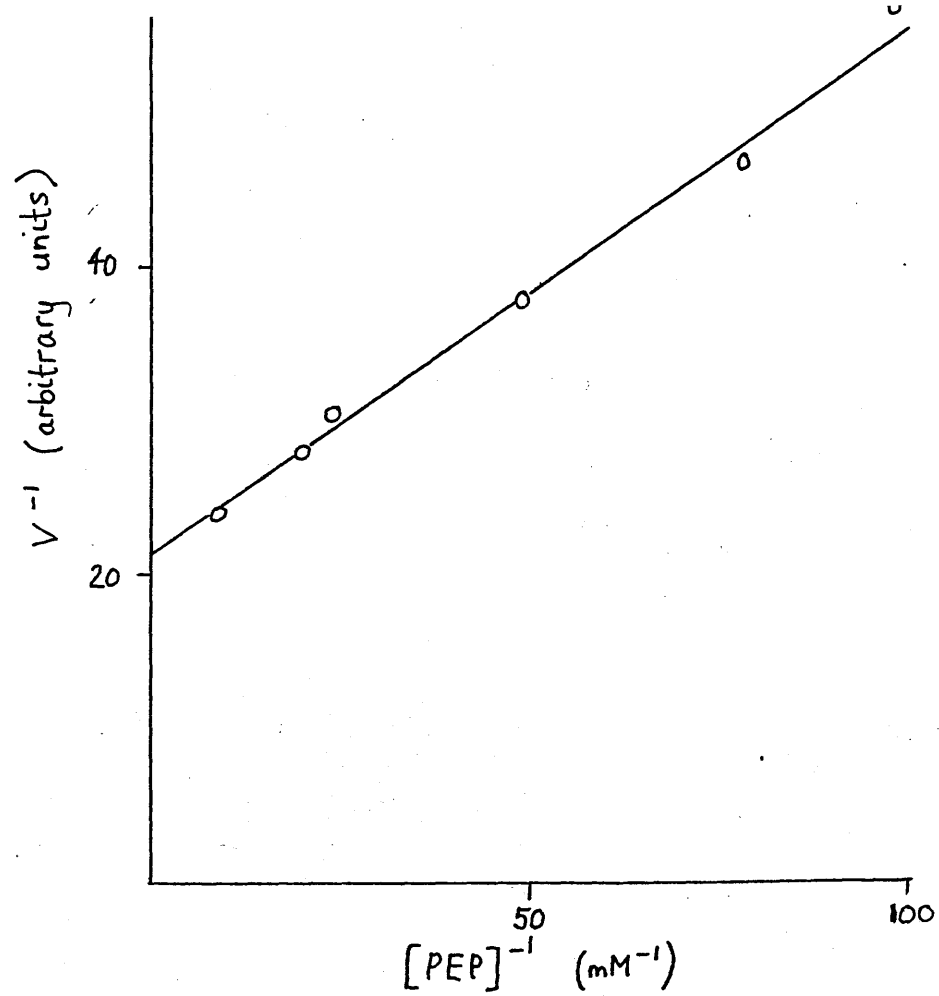


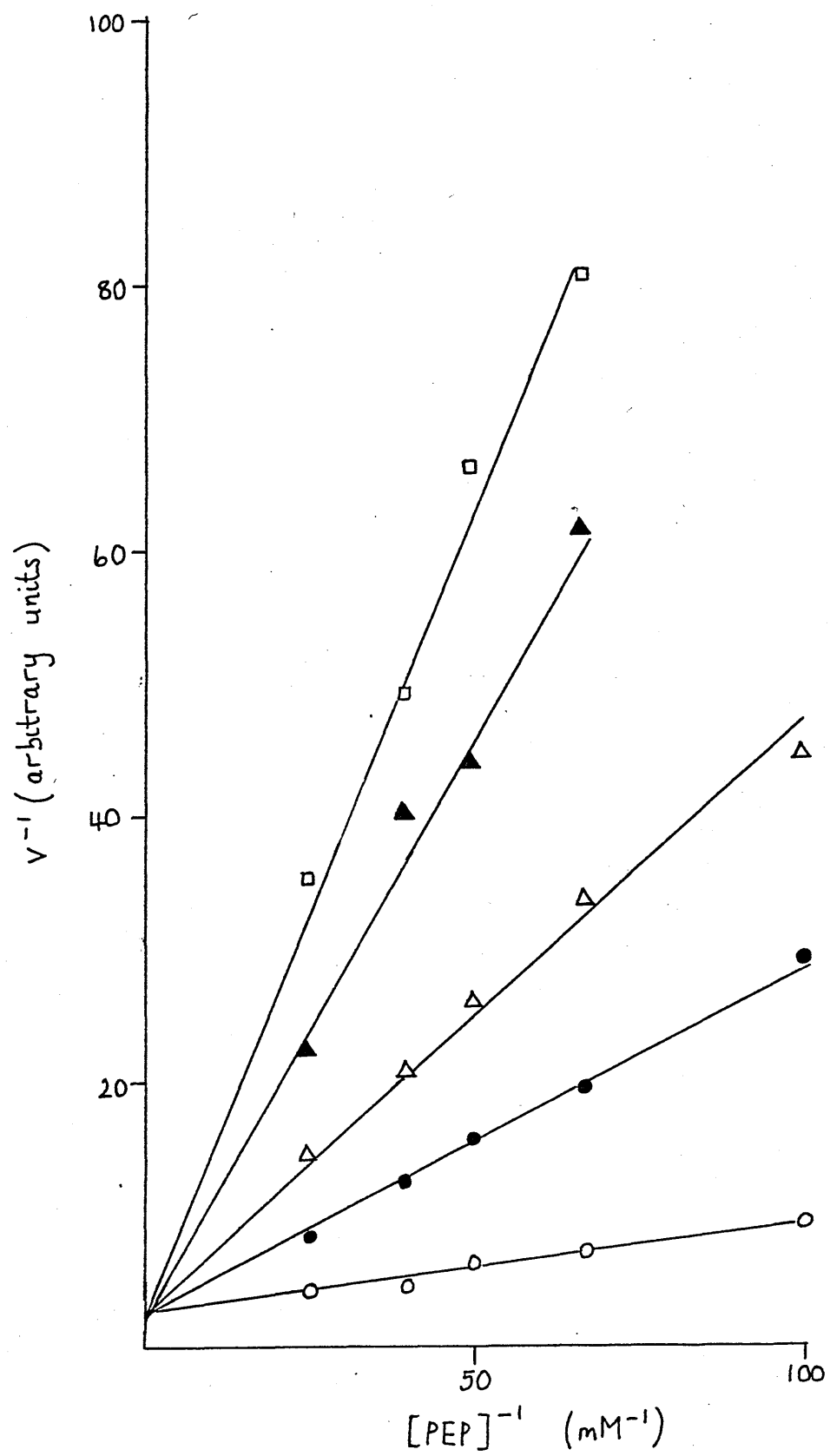
FIGURE 3.10

Inhibition of E. coli EPSP synthase by glyphosate.

Double reciprocal plot of initial velocity against [PEP] at fixed

200 μM shik 3-P and glyphosate at (o), 0 μM ; (\bullet), 0.5 μM ;

(Δ), 1 μM ; (\blacktriangle), 2 μM ; (\square), 5 μM .



CHAPTER 4

PURIFICATION OF EPSP SYNTHASE FROM AN OVERPRODUCING STRAIN OF E. COLI

4.1. Introduction

Characterisation of E. coli EPSP synthase was hampered by the limited amounts of homogeneous enzyme that could be readily purified from E. coli K12. This problem was solved by the cloning of the gene coding for EPSP synthase, aroA, from E. coli (Duncan & Coggins, 1984).

Two obvious advantages result from cloning the gene coding for a protein of interest: the DNA sequence of the gene can be readily obtained and it is possible to overproduce the protein. In the case of E. coli EPSP synthase the cloned gene has been used to construct an overproducing strain (Duncan et al., 1984a) and recently the complete nucleotide sequence of the gene has been determined (Duncan et al., 1984b).

The E. coli K12 aroA gene was first subcloned from the transducing phage, λ psrC (Duncan & Coggins, 1984). (Kitakawa et al.) on a 4.6kb Pst 1 fragment. This fragment was inserted into the multicopy plasmid pAT153 (Twigg & Sherratt, 1980), and the recombinant plasmid pKD501 (Duncan & Coggins, 1984) was used to transform an aroA⁻ strain of E. coli^{K12} called AB2829 (Pittard & Wallace, 1966). The transformed strain designated E. coli AB2829/pKD501 synthesised elevated levels of EPSP synthase (Duncan & Coggins, 1984). The insertion of cloned genes into vectors maintained at high copy number in E. coli has been found to be a satisfactory method of overproducing proteins.

This chapter describes the use of the overproducing strain, E. coli AB2829/pKD 501, as a source of EPSP synthase, and protein chemical studies with the overproduced enzyme. The results of the protein chemistry are compared with data obtained from DNA sequencing studies (Duncan et al., 1984b).

4.2. A comparison of the levels of EPSP synthase activity in E. coli K12 and E. coli AB2829/pKD501

EPSP synthase activity was assayed in 100,000g supernatants of E. coli K12 and E. coli AB2829/pKD501 cell extracts. The specific activities were 0.006 units/mg and 0.65 units/mg respectively (assayed in the reverse direction using the routine assay). E. coli AB2829/pKD501 is clearly a good source of EPSP synthase; this strain overproduces EPSP synthase by approximately 100-fold compared with E. coli K12. This level of overproduction is consistent with the presence of the aroA gene on a plasmid that has a copy number of approximately 100 per cell (Twigg & Sherratt, 1980).

4.3. Purification of EPSP synthase from E. coli AB2829/pKD501

The procedure was essentially that described in Chapter 3 for the purification of EPSP synthase from E. coli K12. Modifications to the procedure are described below.

(a) Preparation of crude extract and $(\text{NH}_4)_2\text{SO}_4$ fractionation

A crude extract was prepared from E. coli AB2829/pKD501 cells and the 50-70% $(\text{NH}_4)_2\text{SO}_4$ fraction obtained as described in section 3.2. (a) and (b). The $(\text{NH}_4)_2\text{SO}_4$ fraction was dialysed against buffer B (2.1) overnight.

(b) DEAE-Sephacel chromatography

The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was loaded on to a column of DEAE-Sephacel (15 cm x 4.5 cm) equilibrated with buffer B. The column was washed with buffer B (2 to 3 column volumes), and then a gradient of 50 to 250 mM KCl in 50 mM Tris-HCl pH7.5, 0.4 mM DTT was applied (gradient volume 1000 ml). The flow rate throughout was 100 ml/h and 9 ml fractions were collected. An elution profile is shown in figure/

figure 4.1. Fractions containing EPSP synthase activity were combined and the pool dialysed against buffer A (1.1) overnight..

(c) Phenyl-Sepharose chromatography

This was carried out as described previously (section 3.2 (d)), except that the flow rate was 60 ml/h and 7.5 ml fractions were collected. Fractions containing EPSP synthase activity eluted from this column (figure 4.2) were pooled and dialysed against buffer C (3 x 2 l) for a total of 24 h.

(d) Phosphocellulose chromatography

The dialysed enzyme was loaded on to a column of phosphocellulose (10 cm x 2 cm) equilibrated with buffer C and the column was washed with buffer C until the A_{280} of the eluate was less than 0.01. EPSP synthase was eluted with buffer C containing 1 mM shik 3-P and 1 mM PEP. An elution profile is shown in figure 4.3, the flow rate was 10 ml/h and 3.3 ml fractions were collected during loading and washing, 2 ml fractions were collected after application of the substrate elution buffer.

EPSP synthase eluted from the phosphocellulose column was concentrated by vacuum dialysis and then dialysed against buffer B containing 50% (v/v) glycerol before long term storage at -20°C.

4.4. Purity

EPSP synthase purified by this method was homogeneous as judged by giving one band on non-denaturing PAGE when stained for protein (figure 4.4 (a)) or EPSP synthase activity (not shown). Figure 4.5 shows an SDS gel that monitors the purification of EPSP synthase from E. coli AB2829/pKD501, tracks D and E show homogeneous enzyme eluted from the phosphocellulose column. EPSP synthase appears to be the major component of the phenyl-sepharose pool (track C), this can be compared/

TABLE 4.1

Purification scheme for EPSP synthase from E. coli AB2829/pKD501

Stage	Volume (ml)	Protein concentration (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (-fold)
Crude extract	77	10.4	4.35	335	100	0.42	1
50-70% $(\text{NH}_4)_2\text{SO}_4$	40	6.8	5.55	222	66	0.82	1.9
DEAE-Sephacel	81	0.65	2.12	171	51	3.2	4.0
Phenyl-Sepharose	60	0.14	2.25	135	40	16.1	38
Phosphocellulose	23	0.21	4.43	102	30	21.1	50

The results presented are for a typical purification starting from 18 g E. coli cells. Enzyme activity was assayed in the reverse direction (assay buffer, potassium phosphate pH7.0).

compared with track C of figure 3.5.

Details of the purification of EPSP synthase from E. coli AB2829/pKD501 are given in Table 4.1. The enzyme was purified 50-fold over the crude extract in 30% yield. Homogeneous EPSP synthase from E. coli K12 required 843-fold purification over the $(\text{NH}_4)_2\text{SO}_4$ fraction, this purification factor for EPSP synthase from the overproducing strain is only 26-fold. EPSP synthase can therefore be produced in milligram quantities from E. coli AB2829/pKD501. In the typical preparation reported in Table 4.1, 4.8 mg of homogeneous enzyme was obtained from 18 g of cells.

The specific activity of homogeneous, overproduced EPSP synthase of 21.1 units/mg is comparable with that of 17.7 units/mg for EPSP synthase purified from E. coli K12. The average specific activity of EPSP synthase purified from E. coli AB2829/pKD501 was 21.9 units/mg (7 preparations).

4.5. Aggregation of EPSP synthase

After storage of purified, overproduced EPSP synthase for several weeks at -20°C , additional bands that stain for protein and EPSP synthase activity are visible after non-denaturing PAGE (figure 4.4(b)). The number of additional bands increased upon storage up to a maximum of three. Each additional band migrated more slowly than the previous one.

Gel filtration of this altered material on a TSK G2000SW column separated two peaks of protein, both of which coincided with peaks of EPSP synthase activity (figure 4.6 (a)). One of these peaks eluted at the same V_e as newly purified EPSP synthase, the other peak eluted earlier indicating that this form of EPSP synthase had a higher molecular/

molecular weight. Thus the additional bands seen on non-denaturing PAGE are due to aggregation of EPSP synthase rather than some other form of modification, e.g. proteolysis. This aggregation can be reversed by treatment of EPSP synthase with fresh reducing agent (1 mM DTT at 0°C overnight). After DTT treatment, both the additional bands on native gels and the higher M_r species on gel filtration (figure 4.6 (b)) are absent.

Non-denaturing PAGE of 100,000 g supernatants of extracts of E. coli AB2829/pKD 501 and E. coli K12 showed only one band of precipitated calcium phosphate when stained for EPSP synthase activity (figure 4.4 (c) and (d)). EPSP synthase activity in extracts had the same mobility as freshly purified EPSP synthase. This indicates that this aggregation is unlikely to be the native state of E. coli EPSP synthase.

As aggregation of EPSP synthase was reversed by the addition of fresh reducing agent, it seemed likely to be due to oxidation of sulphhydryl groups and perhaps to intermolecular disulphide bridge formation. Aggregation appeared to occur only at high protein concentrations, no aggregation of E. coli K12 EPSP synthase was observed even after storage for several months. The E. coli K12 enzyme was stored as more dilute solutions than the overproduced enzyme.

4.6. Evidence that EPSP synthases purified from E. coli K12 and E. coli AB2829/pKD501 are identical.

- (a) The homogeneous enzymes exhibit the same mobility on non-denaturing PAGE, $R_f = 0.48$ on 7% polyacrylamide gels.
- (b) SDS PAGE of the overproduced and "wild-type" EPSP synthase demonstrated/

TABLE 4.2

Comparison of the kinetic parameters of EPSP synthase purified from E. coli K12 and E. coli AB2829/pKD501

Kinetic parameter	<u>E. coli</u> K12 enzyme	<u>E. coli</u> AB2829/pKD501 enzyme
K_m for PEP	15 μM (200 μM shik 3-P)	16 μM (200 μM shik 3-P)
K_m for shik 3-P	3.5 μM (500 μM PEP)	2.5 μM (200 μM PEP)
K_m for phosphate	2.5 mM (50 μM EPSP)	2.5 mM (50 μM EPSP)
K_m for EPSP	2.5 μM (50 mM phosphate)	2.7 μM (50 mM phosphate)
K_i for glyphosate	1.0 μM (200 μM shik 3-P)	0.9 μM (200 μM shik 3-P)
Ratio of forwards:reverse rates	4.8	4.8

Kinetic parameters were determined at fixed concentrations of co-substrates (given in brackets), bis Tris-HCl pH7.0 was the assay buffer in all cases. The rates of the forward and reverse reactions of EPSP synthase were also determined in this buffer, the substrate concentrations were: forward reaction, 0.5 mM PEP, 0.5 mM shik 3-P; reverse reaction, 50 μM EPSP, 50 mM phosphate.

demonstrated that enzyme purified from both sources had the same subunit M_r (figure 4.7).

(c) Limited proteolysis and one-dimensional peptide mapping (Cleveland et al., 1977) of enzyme isolated from each source was carried out as described in section 2.3.4. Preliminary experiments had shown that it was difficult to obtain similar levels of proteolysis unless the protein samples were digested under identical conditions. EPSP synthase to be "mapped" was contained in slices cut out of an SDS gel. The protein samples were eluted electrophoretically into another SDS gel, and digestion occurred in the stacking gel of this second SDS gel. This ensured that both samples of EPSP synthase were digested under very similar conditions.

It proved difficult to digest E. coli EPSP synthase to give a number of readily separated fragments. Figure 4.8 shows one-dimensional peptide "maps" produced by digestion of EPSP synthase by three proteases of different specificity. The somewhat limited digestion patterns obtained for enzyme isolated from wild-type and overproducing E. coli are identical.

(d) Kinetic parameters for the overproduced EPSP synthase were determined. The K_m values and the K_i for glyphosate reported in Table 4.2 for the E. coli AB2829/pKD501 enzyme are essentially identical to the kinetic parameters of the E. coli K12 enzyme. The ratio of the rates of the forward:reverse EPSP synthase reactions are also compared in Table 4.2, identical values are found for enzyme from both sources.

The structural and kinetic parameters of EPSP synthase purified from E. coli AB2829/pKD501 are therefore identical to the properties of the wild-type enzyme. Once this had been established, E. coli AB2829/pKD501 was used as a source of EPSP synthase and all experiments/

experiments subsequently described in this thesis used enzyme purified from the overproducing strain.

4.7 Amino acid composition and N-terminal sequence of EPSP synthase.

(a) Amino acid composition.

The amino acid composition determined for E. coli EPSP synthase is shown in Table 4.3. The relative amino acid composition shown is based on 48 Leu residues per EPSP synthase. This composition can be easily compared with the amino acid composition derived from the DNA sequence of the aroA gene (Duncan et al., 1984b) which is also shown in Table 4.3. There is good agreement between the compositions derived at the protein and DNA levels.

(b) N-terminal sequence.

The N-terminal amino acid sequence of E. coli EPSP synthase was determined by automatic protein sequencing of the intact protein. The sequence is shown in figure 4.9, the sequence of residues 1 to 44 was determined before the build-up of background made unambiguous identification of PTH-amino acids impossible. AS PTH-His and PTH-Arg could not be definitely distinguished from each other by the HPLC analysis used, the assignment of residues 11, 27 and 36 was made by back-hydrolysis of the PTH-amino acids with HI (Smithies et al., 1971). Residues 1 to 12 were identified unambiguously, residue 13 could not be identified but back-hydrolysis showed that Asp was the most abundant amino acid present. The sequence was then unambiguous until residue 25 which could not be assigned, neither could residues 39 or 42. Residue 33 gave Ala:Val in a ratio of 2:1 and was tentatively assigned as Ala.

Figure 4.10 shows the DNA sequence of the E. coli aroA gene and the/

TABLE 4.3

The amino acid composition of E. coli EPSP synthase compared with the amino acid composition deduced for EPSP synthase from the E. coli aroA gene sequence.

Amino acid	Relative amino acid composition based on Leu = 48 residues	Theoretical amino acid composition predicted from the DNA sequence
Asp	41.9	44
Thr ¹	31.1	34
Ser ¹	19.7	21
Glu	38.8	34
Pro	18.1	18
Gly	42.8	37
Ala	44.1	46
Cys ²	4.9	6
Val	21.7	24
Met ³	13.6	14
Ile	24.2	26
Leu	48.0	48
Tyr	13.1	13
Phe	13.2	13
His	8.1	8
Lys	17.0	17
Arg	17.2	22
Trp	ND	2

¹ Experimental values were extrapolated to zero time.

² Determined as cysteic acid.

³ Determined as methionine sulphone.

Samples were analysed in duplicate after hydrolysis of performic acid oxidised-protein with 6M HCl at 105°C for 24, 48, 72 and 96 h (section 2.8.1). The 8 experimental values were simply averaged except where indicated in the footnotes.

the corresponding amino acid sequence of EPSP synthase (Duncan et al., 1984b). If the N-terminal sequence (figure 4.9) is compared with this sequence, it is evident that the sequence determined at the protein level agrees very well with that derived from the DNA sequencing studies, in fact, these sequences are identical for the first 22 residues.

4.8 Discussion

The good agreement between the amino acid compositions determined at the protein and DNA levels indicates that the sequence shown in figure 4.10 comprises the complete E. coli aroA gene. The known N-terminal sequence allowed the translation start of the aroA gene to be located rapidly and unambiguously.

The predicted amino acid sequence of the E. coli aroA gene corresponds to a 427 amino acid polypeptide (figure 4.10), the M_r of EPSP synthase calculated from the deduced sequence is 46112 (Duncan et al., 1984b). This value is in good agreement with the M_r values reported in Chapter 3 for E. coli EPSP synthase: the subunit M_r value of 49,000 by SDS PAGE and the native M_r value of 42,000 by HPLC gel filtration.

The protein studies described here and the cloning and DNA sequencing studies carried out by Mr. K. Duncan in our laboratory have complemented each other. Cloning the aroA gene (Duncan & Coggins, 1984) meant that relatively large amounts of EPSP synthase became available. This in turn made possible the protein chemical studies which have allowed the unambiguous location of the coding sequence of the aroA gene on the sequenced DNA (Duncan et al., 1984b).

FIGURE 4.1

Ion-exchange chromatography on DEAE-Sephacel.

The dialysed 50-70% $(\text{NH}_4)_2\text{SO}_4$ fraction was chromatographed on a column of DEAE-Sephacel as described in Section 4.3 (b). (o), A_{280} ; (●), EPSP synthase activity; (□-□), conductivity.

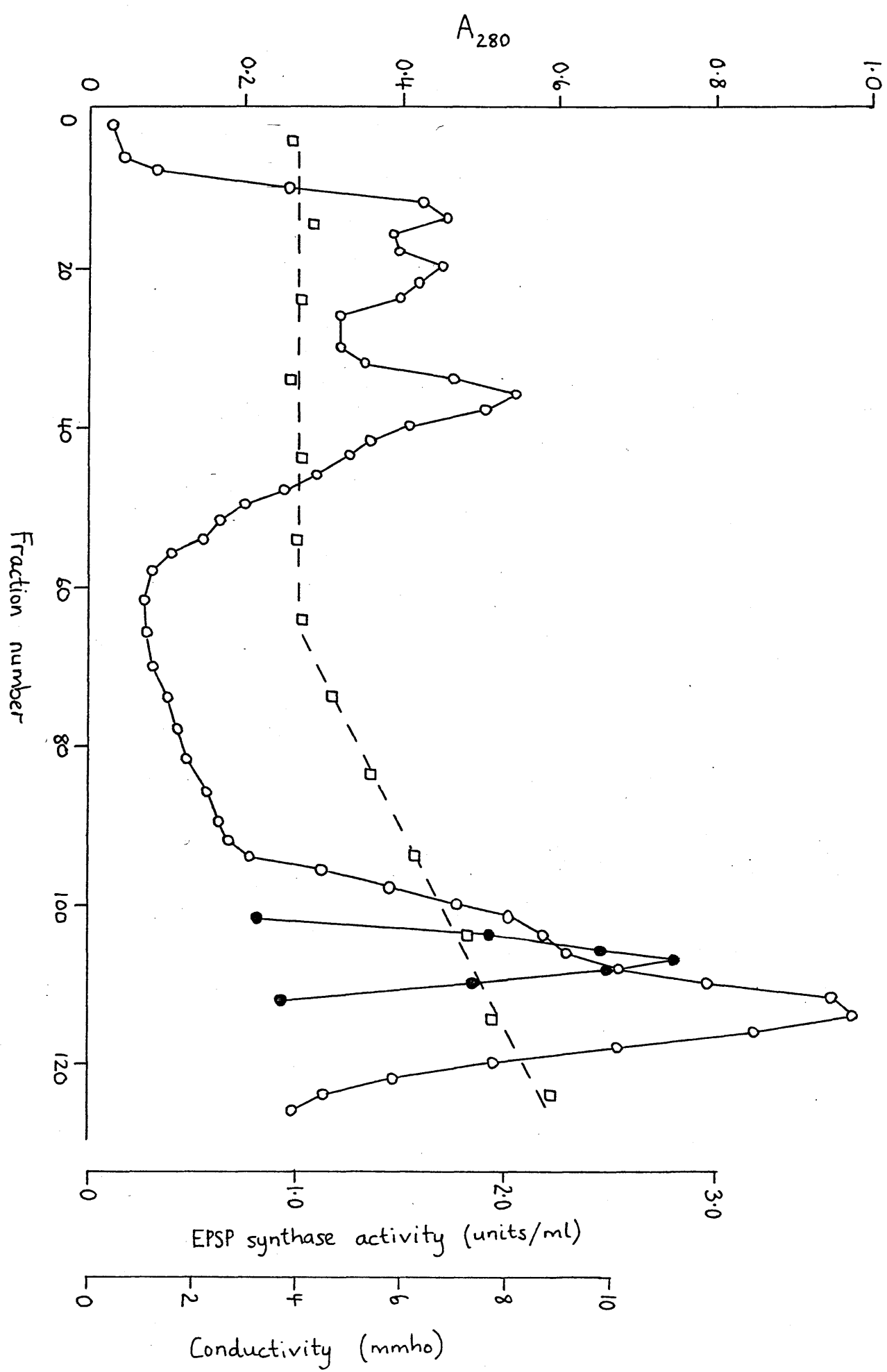


FIGURE 4.2

Chromatography on phenyl-Sepharose.

Enzyme from chromatography on DEAE-Sephacel was subjected to chromatography on phenyl-Sepharose as described in Section 3.2 (d). (o), A_{280} ; (●), EPSP synthase activity; (□-□), conductivity.

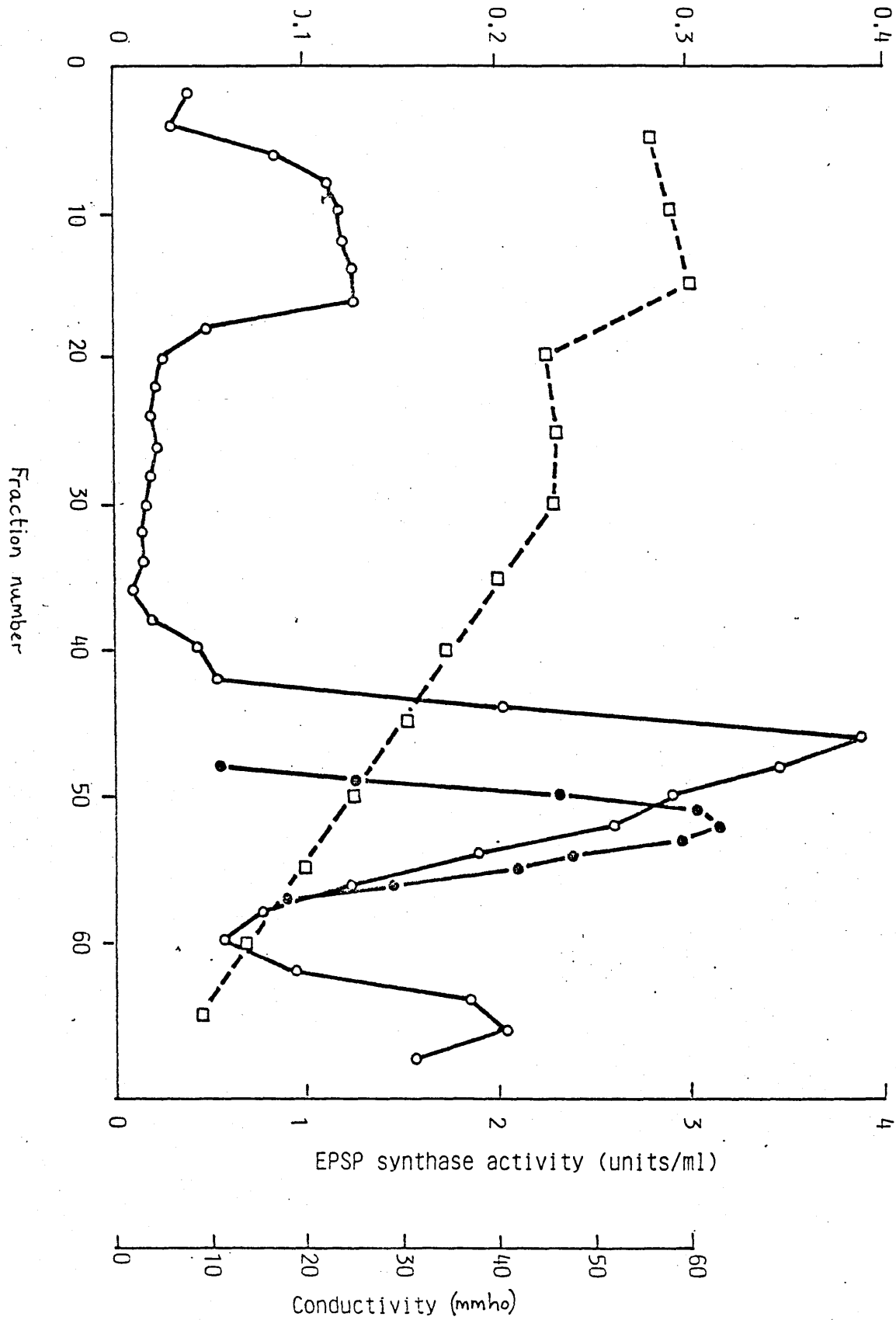


FIGURE 4.3

Chromatography on phosphocellulose.

Enzyme from chromatography on phenyl-Sepharose was loaded on to a column of phosphocellulose as described in Section 4.3 (d), the column was washed with buffer C. At the arrow, buffer C containing 1 mM PEP and 1 mM shik 3-P was applied, and the fraction size was decreased from 3.3 ml to 2 ml. (o), A_{280} ; (●), EPSP synthase activity.

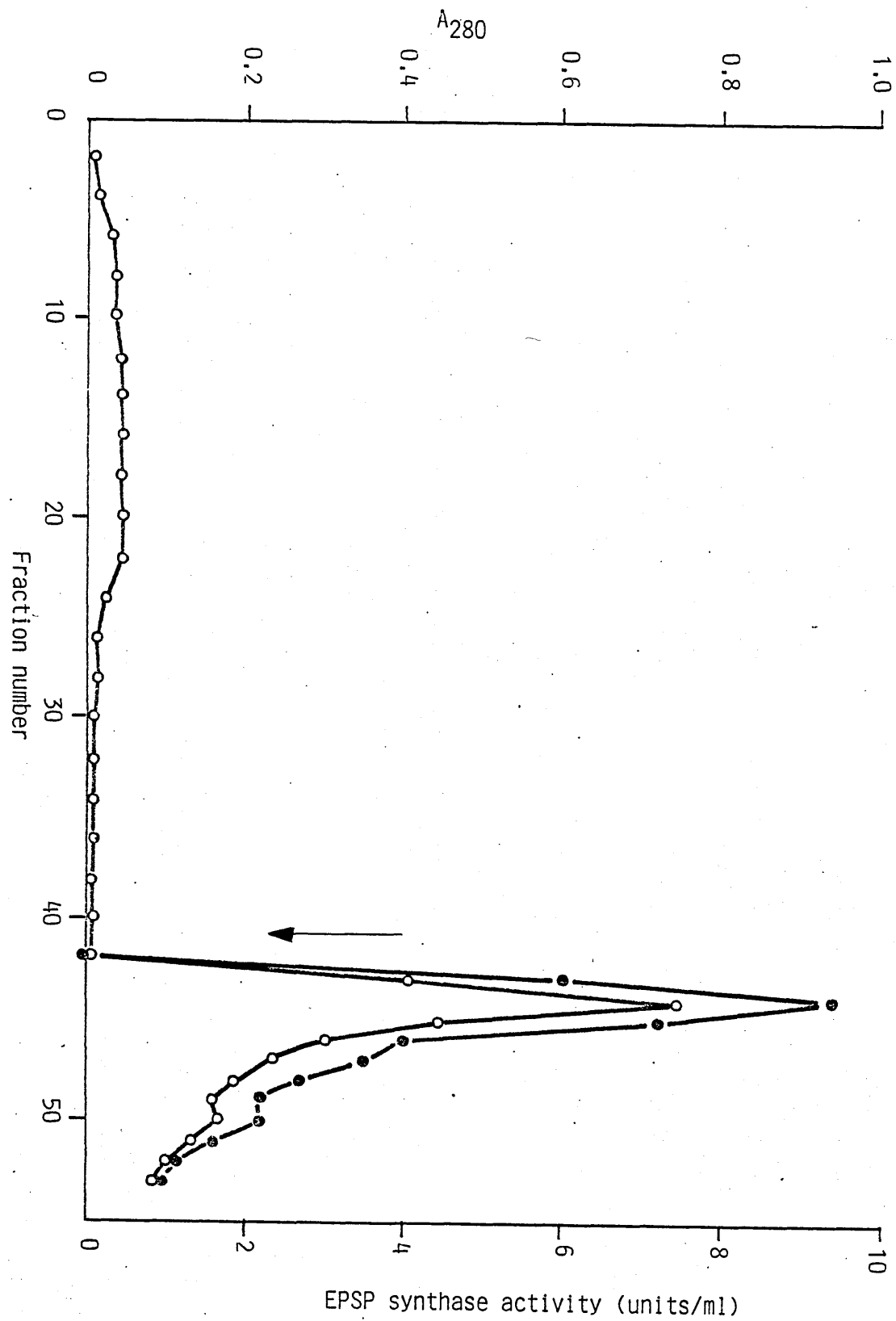


FIGURE 4.4

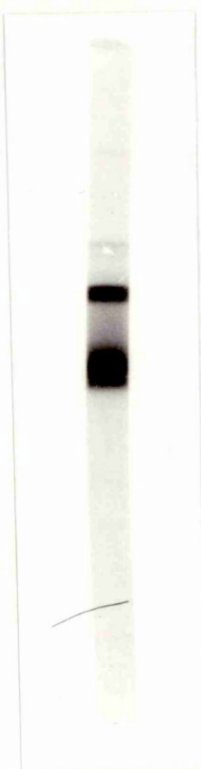
Non-denaturing PAGE of EPSP synthase.

Non-denaturing PAGE (7% polyacrylamide gels) of samples of EPSP synthase was carried out as described in Section 2.3.1. Gels A and B were stained for protein, and gels C and D were stained for EPSP synthase activity (Section 2.3.3).

- A. EPSP synthase purified from E. coli AB2829/pKD501 (2 μ g).
- B. EPSP synthase purified from E. coli AB2829/pKD501 (18 μ g), this enzyme preparation had been stored for 7 weeks prior to electrophoresis.
- C. EPSP synthase activity (0.46 m units) in a 100,000 g supernatant of E. coli K12 cell extract.
- D. EPSP synthase activity (6.6 m units) in a 100,000 g supernatant of E. coli AB2829/pKD501 cell extract.



A



B



C



D

FIGURE 4.5

Purification of EPSP synthase from E. coli AB2829/pKD501.

SDS PAGE (10% polyacrylamide gel) monitoring the purification of EPSP synthase from E. coli AB2829/pKD501. Track A, crude extract (104 μ g protein); track B, 50-70% $(\text{NH}_4)_2\text{SO}_4$ fraction (68 μ g); track C, DEAE-Sephacel pool (32 μ g); track D, phenyl-Sepharose pool (7 μ g); tracks E and F, phosphocellulose pool (4 μ g and 7.5 μ g).

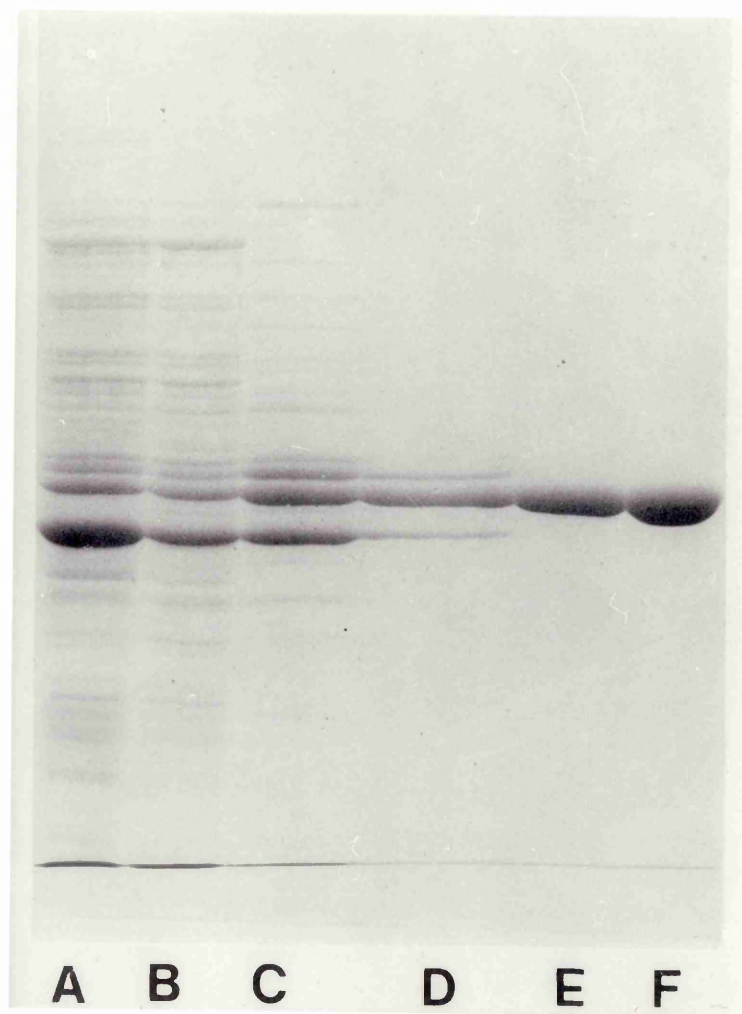


FIGURE 4.6

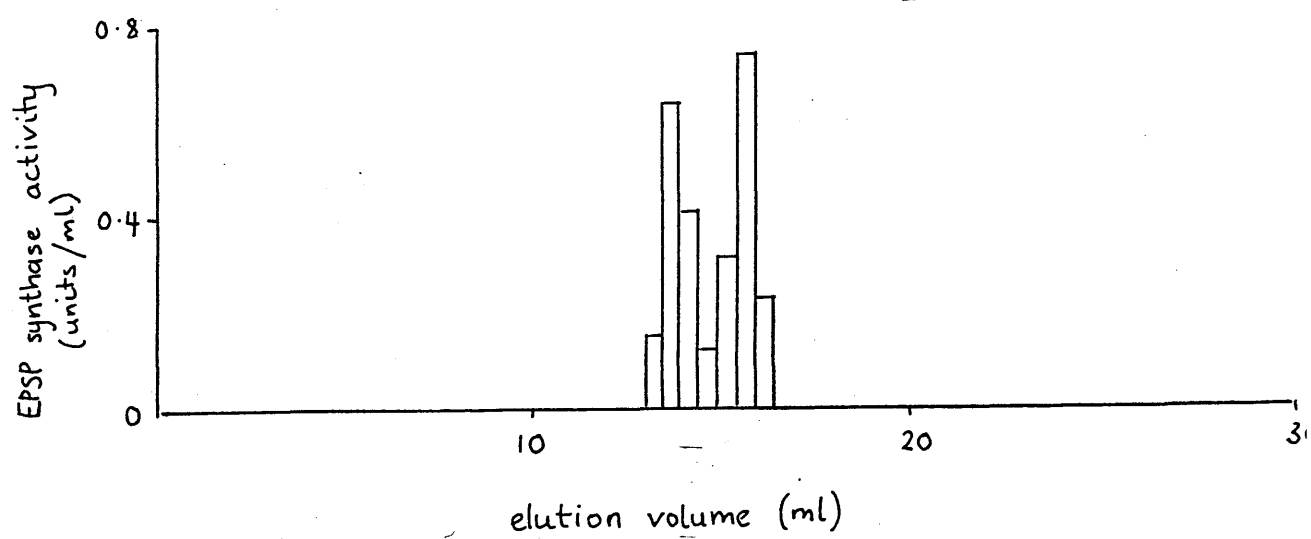
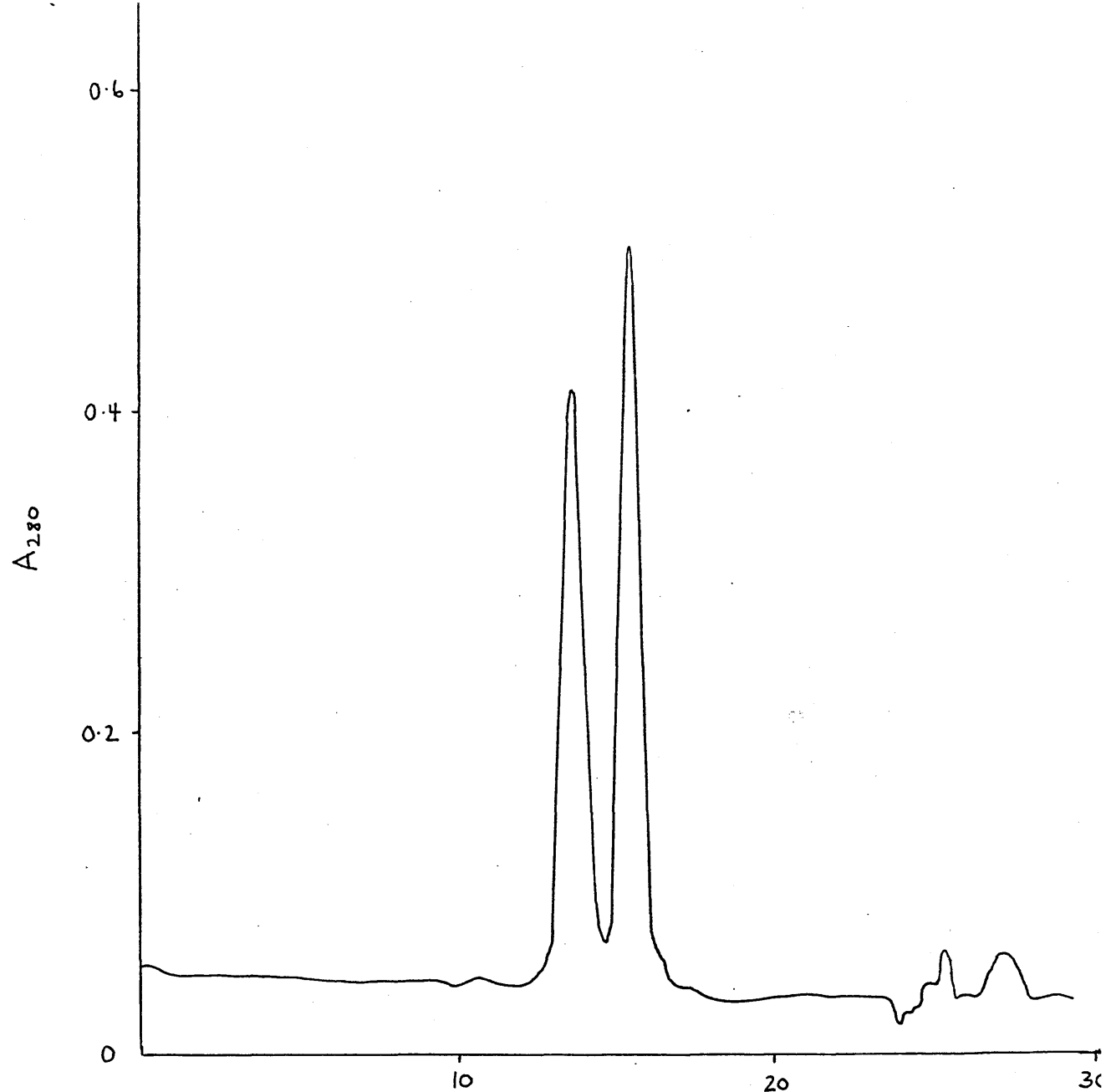
HPLC gel filtration of overproduced EPSP synthase.

HPLC gel filtration was carried out at room temperature on a

TSK G2000SW column equilibrated in 50 mM bis Tris-HCl pH6.8.

The flow rate was 1.0 ml/min , 0.5 ml fractions were collected and assayed for EPSP synthase activity. The column eluate was monitored at 280 nm with a full scale deflection equivalent to 0.1 absorbance units.

- (a) 70 μ g of purified, overproduced EPSP synthase that had been stored at -20°C for 20 weeks.
- (b) the sample was identical to (a) except that it had been incubated overnight at 0°C in the presence of 1 mM DTT.



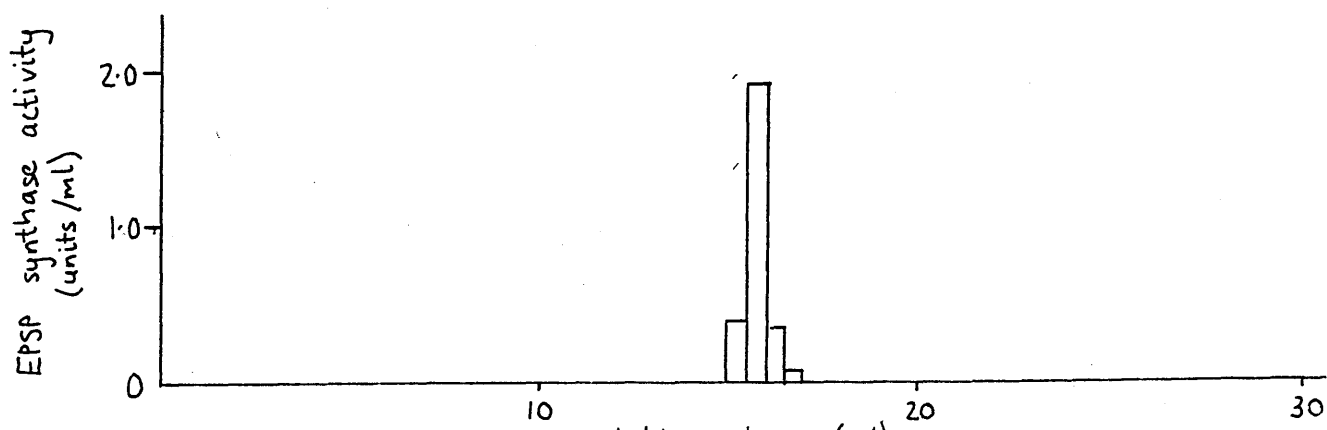
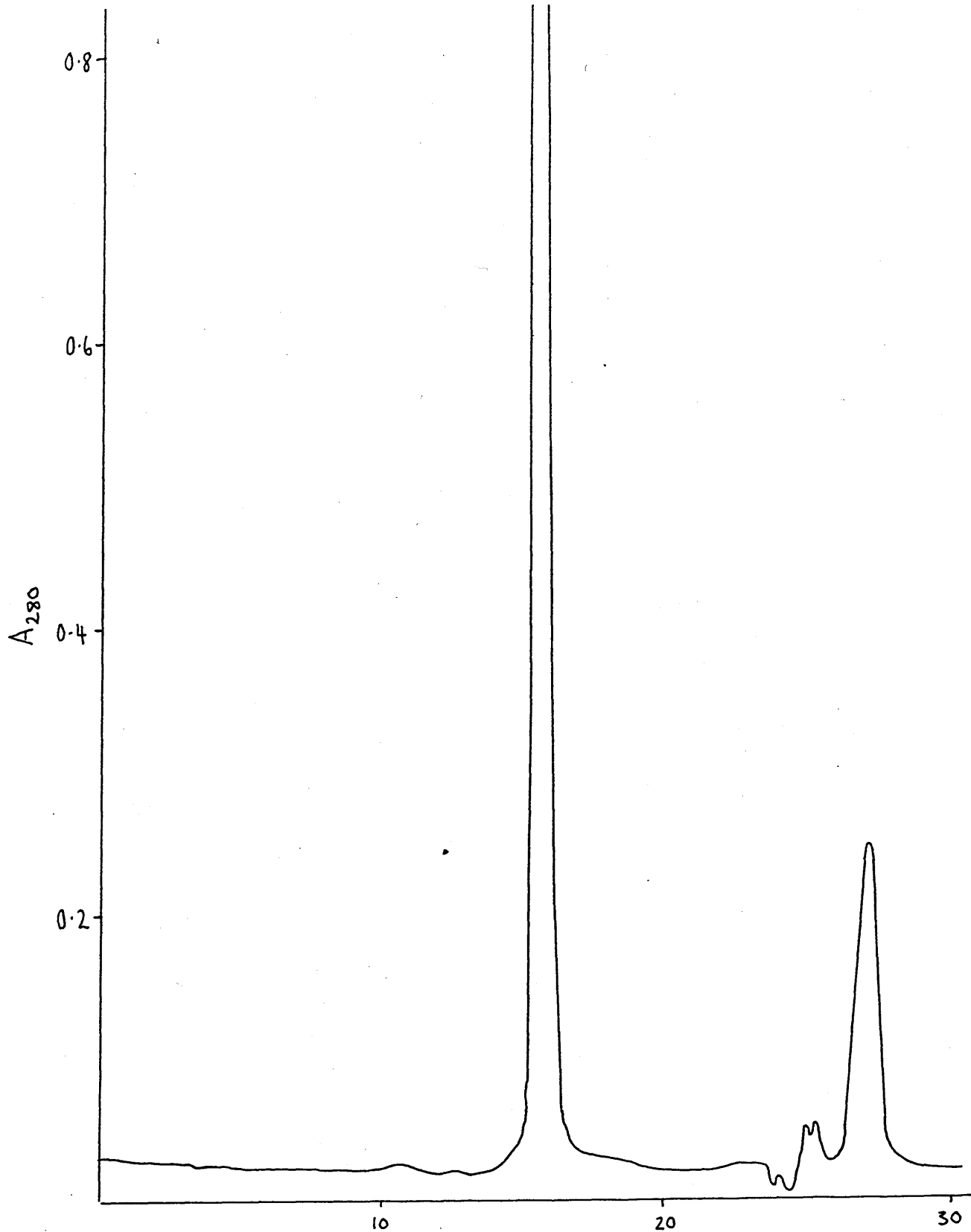


FIGURE 4.7

SDS PAGE of EPSP synthase purified from E. coli K12 and E. coli AB2829/pKD501.

SDS PAGE (15% polyacrylamide gel) of wild-type and overproduced EPSP synthase. This gel was stained for protein using the silver method.

- A. E. coli K12 EPSP synthase (0.3 μ g).
- B. E. coli AB2829/pKD501 EPSP synthase (0.7 μ g).

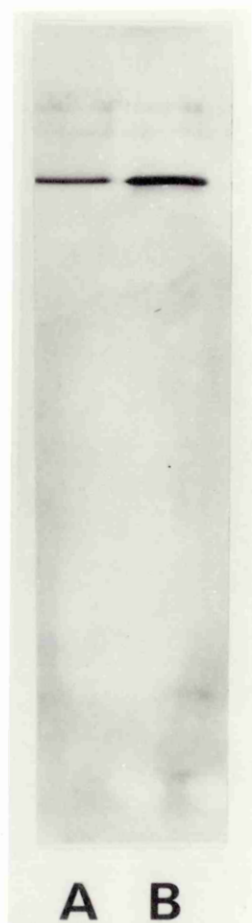
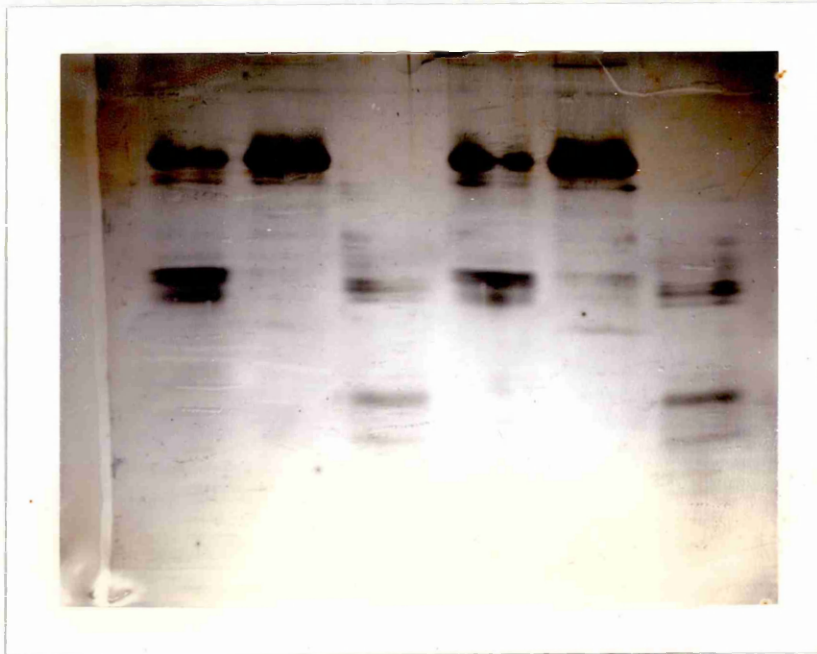


FIGURE 4.8

One-dimensional peptide maps of EPSP synthase purified from E. coli K12 and E. coli AB2829/pKD501.

Samples of EPSP synthase (2.5 μ g) were contained in bands cut out of a 10% polyacrylamide SDS gel. Limited proteolysis occurred during electrophoresis through the stacking gel of a 15% polyacrylamide SDS gel as described in Section 2.3.4. This gel was stained for protein by the silver method (Section 2.3.3).

- A. E. coli K12 EPSP synthase + chymotrypsin.
- B. E. coli K12 EPSP synthase + trypsin.
- C. E. coli K12 EPSP synthase + S. aureus V8 protease.
- D. E. coli AB2829/pKD501 EPSP synthase + chymotrypsin.
- E. E. coli AB2829/pKD501 EPSP synthase + trypsin.
- F. E. coli AB2829/pKD501 EPSP synthase + S. aureus V8 protease.



A B C D E F

FIGURE 4.9

The N-terminal amino acid sequence of E. coli EPSP synthase.

1	10
Met - Glu - Ser - Leu - Thr - Leu - Gln - Pro - Ile - Ala -	
11	20
Arg - Val - (-) - Gly - Thr - Ile - Asn - Leu - Pro - Gly -	
21	30
Ser - Lys - Ser - Val - (-) - Asn - His - Ala - Leu - Leu -	
31	40
Leu - Ala - Ala - Leu - Ala - His - Gly - Val - (-) - Val -	
41	44
Leu - (-) - Asn - Leu -	

The sequence was determined on a liquid phase sequencer as described in section 2.8.2. The initial amount of protein sequencing was 19 nmol and the repetitive yield from residue 1 to 44, by least square regression analysis was 93% (correlation coefficient 0.95).

Continued over.

FIGURE 4.9 (continued)

Sequence number	PTH-amino acid	nmoles recovered	back hydrolysis
1	Met	15.8	
2	Glu	18.9	
3	Ser	3.7	
4	Leu	19.6	
5	Thr	4.4	
6	Leu	11.6	
7	Gln	13.3	
8	Pro	8.8	
9	Ile	7.4	
10	Ala	13.0	
11	Arg/His	9.2	Arg
12	Val	7.5	
13	-	-	Asp
14	Gly	7.4	
15	Thr	3.1	
16	Ile	5.3	
17	Asn	11.1	
18	Leu	5.6	
19	Pro	5.6	
20	Gly	9.1	
21	Ser	-	
22	Lys	4.5	
23	Ser	0.8	
24	Val	3.2	
25	-	-	
26	Asn	4.9	
27	His/Arg	3.4	His
28	Ala	4.0	
29	Leu	4.1	
30	Leu	2.9	
31	Leu	3.8	
32	Ala	1.8	
33	Ala + Val	3.1 + 1.7	
34	Leu	1.0	
35	Ala	1.8	
36	?His/Arg	-	His
37	Gly	1.0	
38	Val	1.6	
39	-	-	
40	Val	0.8	
41	Leu	1.1	
42	-	-	
43	Asn	1.1	
44	Leu	1.1	

FIGURE 4.10

The E. coli aroA gene.

The complete nucleotide sequence of the E. coli aroA gene and the corresponding amino acid sequence of E. coli EPSP synthase.

Nucleotides are numbered in the 5' to 3' direction beginning with the first residue of the ATG triplet encoding the N-terminal methionine.

The bracketed numbers refer to the amino acid positions in the sequence. This sequence is taken from Duncan et al (1984b).

MET GLU SER LEU THR LEU GLN PRO ILE ALA ARG VAL ASP GLY THR ILE ASN LEU PRO GLY
 [1] ATGGAATCCCTGACGTTACAAACCCATCGCTCGTGTCGATGGCACTATTAAATCTGCCCGGT
 10 20 30 40 50 60

SER LYS THR VAL SER ASN ARG ALA LEU LEU LEU ALA ALA LEU ALA HIS GLY LYS THR VAL
 [21] TCAAGACCGTTTCTAACCGCGCTTTATTGCTGGCGGCATTAGCACACGGCAAAACAGTA
 70 80 90 100 110 120

LEU THR ASN LEU LEU ASP SER ASP VAL ARG HIS MET LEU ASN ALA LEU THR ALA LEU
 [41] TAACCAATCTGCTGGATAGCGATGACGTGCGCCATATGCTGAATGCATTAAACAGCGTTA
 130 140 150 160 170 180

GLY VAL SER TYR THR LEU SER ALA ASP ARG THR ARG CYS GLU ILE ILE GLY ASN GLY GLY
 [61] GGGGTAAAGCTATACGCTTTCAGCCGATCGTACGCGTTGCGAAATTATCGGTAAACGGCGGT
 190 200 210 220 230 240

PRO LEU HIS ALA GLU GLY ALA LEU GLU LEU PHE LEU GLY ASN ALA GLY THR ALA MET ARG
 [81] CCATTACACGCAAGAGGTCCCTGGAGTTGTTCTCTCGGTACGCGCGGAACGGCAATGCGT
 250 260 270 280 290 300

PRO LEU ALA ALA ALA LEU CYS LEU GLY SER ASN ASP ILE VAL LEU THR GLY GLU PRO ARG
 [101] CCGCTGGCGCGCAGCTCTTTGTTCTGGGTAGCAATGATATTGTGCTGACCGGTGAGCGCGGT
 310 320 330 340 350 360

MET LYS GLU ARG PRO ILE GLY HIS LEU VAL ASP ALA LEU ARG LEU GLY GLY ALA LYS ILE
 [121] ATGAAGAAACGCCCGATTGGTTCATCTGGTGGATGCGCTGCGCCTGGGCGGGCGGAAGATC
 370 380 390 400 410 420

THR TYR LEU GLU GLN GLU ASN TYR PRO PRO LEU ARG LEU GLN GLY GLY PHE THR GLY GLY
 [141] ACTTACCTGGGAACAAGAAATTATCCGCCGTTGCGTTTACAGGGCGGGCTTTACTGGCGGC
 430 440 450 460 470 480

ASN VAL ASP VAL ASP GLY SER VAL SER SER GLN PHE LEU THR ALA LEU LEU MET THR ALA
 [161] AACGTTGACGTTGATGGCTCCGTTTCCAGCCCAATTCTCACCAGCACTGTTAAAGACTGCG
 490 500 510 520 530 540

PRO LEU ALA PRO GLU ASP THR VAL ILE ARG ILE LYS GLY ASP LEU VAL SER LYS PRO TYR
 [181] CCTCTTGCGCGCGGAAGATACGGTGATTGCTATTAAAGGCGGATCTGGTTTCTAAACCTTAT
 550 560 570 580 590 600

ILE ASP ILE THR LEU ASN LEU MET LYS THR PHE GLY VAL GLU ILE GLU ASN GLN HIS TYR
 [201] ATCGACATCACTCAATCTGATGAAGACGTTTGGTGTTGAATTGAATAACAGCACTAT
 610 620 630 640 650 660

GLN GLN PHE VAL VAL LYS GLY GLY GLN SER TYR GLN SER PRO GLY THR TYR LEU VAL GLU
 [221] CAACAATTTGTCTGTAAGAGGCGGGCAGTCTTATCAGTCTCCGGGTACTTATTTGGTCTGAA
 670 680 690 700 710 720

GLY ASP ALA SER SER ALA SER TYR PHE LEU ALA ALA ALA ALA ILE LYS GLY GLY THR VAL
 [241] GCGGATGCATCTTCGGCTTCTTACTTTCTGGCGAGCAGCAGCAATCAAGGCGGCACTGTA
 730 740 750 760 770 780

LYS VAL THR GLY ILE GLY ARG ASN SER MET GLN GLY ASP ILE ARG PHE ALA ASP VAL LEU
 [261] AAGTGACCGGTATTGGACGTAAACAGTATGCGAGGGTGATATTGCTTTGCTGATGTCTG
 790 800 810 820 830 840

GLU LYS MET GLY ALA THR ILE CYS TRP GLY ASP ASP TYR ILE SER CYS THR ARG GLY GLU
 [281] GAAAAAATGGGCGCGACCAATTGCTGGGGCGATGATTATTTCTCTGCACGCGTGCTGAA
 850 860 870 880 890 900

LEU ASN ALA ILE ASP MET ASP MET ASN HIS ILE PRO ASP ALA ALA MET THR ILE ALA THR
 [301] CTGAACGCTATTGATATGGATATGAACCATATTCCTGATGCGGCGGATGACCATTGCCACG
 910 920 930 940 950 960

ALA ALA LEU PHE ALA LYS GLY THR THR ARG LEU ARG ASN ILE TYR ASN TRP ARG VAL LYS
 [321] GCGGCGTTATTTGCAAAAGGCCACCACCAAGGCTGCGCAATATCTATAACTGGCGGTGTTAA
 970 980 990 1000 1010 1020

GLU THR ASP ARG LEU PHE ALA MET ALA THR GLU LEU ARG LYS VAL GLY ALA GLU VAL GLU
 [341] GAGACCGATCGCCTGTTTGGGATGGCAACAGAACCTGCTAAAGTCGGCGCGGAAGTGGA
 1030 1040 1050 1060 1070 1080

GLU GLY HIS ASP TYR ILE ARG ILE THR PRO PRO GLU LYS LEU ASN PHE ALA GLU ILE ALA
 [361] GAGGGGACGATTACATTCGTATCACTCTCTCCGGAAAACTGAACCTTTGCCGAGATCGCG
 1090 1100 1110 1120 1130 1140

THR TYR ASN ASP HIS ARG MET ALA MET CYS PHE SER LEU VAL ALA LEU SER ASP THR PRO
 [381] ACATACCAATGATCACCGGATGGCGAGTGTCTCTCGCTGGTGGCGTTGTTCAGATACACCA
 1150 1160 1170 1180 1190 1200

VAL THR ILE LEU ASP PRO LYS CYS THR ALA LYS THR PHE PRO ASP TYR PHE GLU GLN LEU
 [401] GTGACGATTCTTGATCCCAAAAGCAAGGCCAAACATTTCGGGATTATTTGAGGCAAGCTG
 1210 1220 1230 1240 1250 1260

ALA ARG ILE SER GLN ALA ALA ***
 [421] GCGCGGATTAGCCAGGCCGCTGA
 1270 1280

CHAPTER 5

CHEMICAL MODIFICATION OF E. COLI

EPSP SYNTHASE BY BROMOPYRUVATE.

5.1 Introduction.

As described in section 4.7, the complete amino acid sequence of E. coli EPSP synthase was available from DNA sequencing studies (Duncan et al, 1984b). If it were possible to identify the active site residues in this amino acid sequence, then the door would be opened for site-directed mutagenesis experiments (Winter et al, 1982) on EPSP synthase.

Chemical modification is a commonly used method for identifying active site residues. By modifying an enzyme with group specific reagents and examining the effect that modification has on enzyme activity, it is often possible to identify groups that have a role in catalysis or binding (Means & Feeney, 1971). However, even if loss of enzyme activity is associated with modification of a specific residue, this does not guarantee that this residue is located at or near the active site. The loss of activity could be due to steric hindrance of the active site or to a conformational change brought about by the covalent attachment of the modifying group.

Therefore although chemical modification can suggest that a particular residue is an active site residue, confirmation of this requires further information, for example, substrate protection experiments, and preferably knowledge of the three-dimensional structure of the enzyme.

Chemical modification of E. coli EPSP synthase was regarded as a potentially useful method for identifying active site residues. If modification of EPSP synthase by a particular group specific reagent resulted in loss of enzyme activity, it might be possible to correlate/

correlate loss of activity with modification of one specific amino acid residue. The stoichiometry of inactivation could be determined using radioactively labelled reagent, if more than one residue was modified, the residue(s) necessary for activity might be specifically labelled by modifying the enzyme first using unlabelled reagent in the presence of protecting ligands, and then treating the enzyme with labelled reagent in the absence of ligands. The location of specifically labelled residues on the amino acid sequence could be subsequently ascertained by standard protein chemical techniques.

This chapter describes preliminary chemical modification experiments that were carried out on E. coli EPSP synthase.

5.2 Chemical modification of EPSP synthase.

5.2.1 An active site thiol?

Some evidence was available that pointed to the importance of a thiol group in EPSP synthase activity.

- (i) E. coli EPSP synthase appears to be sensitive to oxidation, the presence of a reducing agent, for example, DTT, was found to be necessary for retention of enzyme activity during purification. The N. crassa EPSP synthase is also sensitive to oxidation (Boocock, 1983).
- (ii) N. crassa EPSP synthase can be inactivated by treatment with N-ethylmaleimide (M.R. Boocock, unpublished results), this reagent modifies cysteine residues.
- (iii) UDPGlcNAc enolpyruvyl transferase can be inactivated by the antibiotic fosfomycin, this compound has been shown to covalently modify a cysteine residue (Kahan et al, 1974). The similarity of/
of/

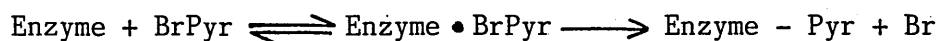
of some of the properties of UDPGlcNAc enolpyruvyl transferase to those of EPSP synthase was described in section 1.8.1.

5.2.2 Bromopyruvate.

A number of PEP and pyruvate utilising enzymes have been reported to be inactivated by the alkylating agent, 3-bromopyruvate (BrPyr). The PEP utilising enzymes include pyruvate kinase (Yun & Suelter, 1979); PEP carboxylase (Kameshita et al, 1979); PEP carboxykinase (Silverstein et al, 1980); DAHP synthase (Staub & Denes, 1969) and UDPGlcNAc enolpyruvyl transferase (Anwar & Vlaovic, 1980). BrPyr has been shown to modify cysteine residues in pyruvate kinase, PEP carboxykinase and PEP carboxylase.

In the cases of PEP carboxylase, DAHP synthase and pyruvate kinase, BrPyr appears to be acting as an analogue of PEP. This means that:

- (i) The enzyme can presumably bind BrPyr before irreversible modification occurs, i.e.



Therefore bromopyruvate inactivation shows saturation kinetics (Meloche, 1967).

- (ii) Since BrPyr binds to the active site of the enzyme, substrates afford protection against inactivation.

Because of its successful application to other enzymes, bromopyruvate has been investigated as a possible active-site-directed reagent for E. coli EPSP synthase.

5.3 Bromopyruvate inactivation of EPSP synthase.

E. coli/

E. coli EPSP synthase is inhibited by BrPyr and the degree of inhibition increases with time (figure 5.1). BrPyr inhibition is not reversible by dialysis. Therefore modification of EPSP synthase by BrPyr does result in inactivation. However, BrPyr does not completely abolish EPSP synthase activity, some residual activity remains (approximately 20-30% of the initial activity).

The rate of inactivation of EPSP synthase by BrPyr appears to be pseudo-first order for the first 50% of inactivation. It is evident from figure 5.1 that the rate of inactivation depends on the concentration of BrPyr. In order to determine whether or not inactivation showed saturation kinetics, the half-time ($t_{\frac{1}{2}}$) of inactivation was determined for a range of bromopyruvate concentrations and the pseudo-first order rate constant for inactivation, $k_{app} = \ln 2 / t_{\frac{1}{2}}$, was calculated. Figure 5.2 shows a plot of k_{app} vs [BrPyr], BrPyr inactivation does not appear to show saturation kinetics over the concentration range examined (25 to 500 μ M), the rate of inactivation appears to be proportional to [BrPyr].

5.4 Substrate protection against bromopyruvate inactivation.

The effect of the presence of substrates and glyphosate on the inactivation of EPSP synthase by BrPyr was examined (figure 5.3). In this experiment carried out with 1 mM BrPyr, it is evident that significant protection was afforded only by the presence of shik 3-P and glyphosate. When tested singly the substrates had a negligible protective effect.

At a lower concentration of BrPyr (0.1 mM) the combination of shik 3-P and glyphosate again gave the best protection (figure 5.4). However, both EPSP and glyphosate alone afforded some protection.
EPSP/

EPSP decreased the rate of inactivation whereas glyphosate appeared to decrease the extent of inactivation, as did PEP and shik 3-P to some extent.

5.5 Bromopyruvate: an active-site-directed reagent for EPSP synthase?

The somewhat preliminary results presented in sections 5.3 and 5.4 indicated that BrPyr did not appear to be a simple active-site-directed reagent for E. coli EPSP synthase. This statement is based largely on the absence of saturation kinetics for the inactivation. BrPyr does not appear to bind to EPSP synthase before modification occurs.

The observed substrate protection against inactivation implies that BrPyr inactivates EPSP synthase by modifying a residue close to the active site. However, the stoichiometry of inactivation by BrPyr has not been determined. It is possible that the observed substrate protection is due to stabilisation of the active conformation of the enzyme against modification by BrPyr at many sites.

It is also significant that EPSP synthase activity is not totally abolished by treatment with BrPyr. If this reagent does modify a cysteine close to the active site of the enzyme, it is unlikely to be a cysteine that is involved in formation of a putative Enzyme-PEP covalent intermediate. However, it is not yet known whether this residual activity is due to incomplete modification or to some retention of activity by completely modified enzyme. This question might perhaps be resolved by a comparison of the kinetic properties (i.e. Michaelis constants) of the native and modified enzymes but this has not been attempted.

5.6/

5.6 Inactivation of *K. pneumoniae* EPSP synthase by bromopyruvate.

BrPyr inactivation of *K. pneumoniae* EPSP synthase has been reported, including substrate protection studies (Anton et al, 1983; Steinrucken & Amrhein, 1984b). The kinetics of inactivation were not reported.

Anton et al (1983) observed substantial protection against inactivation by the combination of PEP and 4, 5-dideoxyshikimate 3-phosphate. This is analogous to the protection of *E. coli* EPSP synthase by shik 3-P and glyphosate. No significant protection by PEP or 4, 5-dideoxyshikimate 3-phosphate alone was reported.

However, Steinrucken & Amrhein (1984b) reported that good protection against inactivation was given by glyphosate alone. This result was part of the evidence presented (Steinrucken & Amrhein, 1984a, b) in favour of glyphosate binding to the free enzyme instead of binding only to an enzyme•shik 3-P complex as proposed by Boocock & Coggins (1983) for the *N. crassa* EPSP synthase.

The different patterns of substrate protection observed for the *E. coli* (figure 5.3 and 5.4) and *K. pneumoniae* (Steinrucken & Amrhein, 1984b) EPSP synthases may be due to differences in the conditions used for these studies. Steinrucken & Amrhein (1984b) conducted inactivation studies in 20 mM Hepes-NaOH pH6.8 whereas this study used 50 mM bis Tris-HCl pH6.8, 50 mM KCl (figure 5.4). The substrate protection pattern of *E. coli* EPSP synthase has been examined in 20 mM Hepes-NaOH pH6.8 and is shown in figure 5.5. The pattern observed is similar to that reported by Steinrucken & Amrhein (1984b) where glyphosate affords good protection, and PEP and EPSP protect to some extent.

Anton/

Anton et al (1983) carried out inactivation studies in 100 mM Tris-HCl pH7.6 and the substrate protection reported was analogous to that shown in figure 5.3. It is unlikely that the E. coli EPSP synthase differs significantly from the K. pneumoniae enzyme.

It is unclear why the substrate protection patterns obtained for E. coli EPSP synthase should be somewhat different in bisTris and Hepes buffers. E. coli EPSP synthase activity in 20 mM Hepes-NaOH pH6.8 was 65% of the activity in 50 mM bisTris-HCl pH6.8, 50 mM KCl. Steinrucken & Amrhein (1984a) reported that the activity of K. pneumoniae EPSP synthase was affected by the anion concentration.

5.7 Discussion.

It would be rash to draw conclusions about the order of substrate binding to E. coli EPSP synthase from the preliminary substrate protection studies presented in sections 5.4 and 5.6, steady-state kinetic studies would be a more reliable way of approaching this problem. The question of whether or not glyphosate can bind to the free enzyme would perhaps be best examined by a method that involved direct measurement of glyphosate binding, for example equilibrium dialysis experiments. It is interesting to note however that a glyphosate insensitive EPSP synthase, isolated from a glyphosate resistant strain of K. pneumoniae, is not protected by glyphosate against BrPyr inactivation (Sost et al, 1984).

As discussed in section 5.5, the stoichiometry of BrPyr inactivation must be examined before it can be decided if BrPyr will pinpoint a residue at or near the active site of E. coli EPSP synthase. It may be necessary to turn to other reagents so that chemical modification of residues other than cysteine residues can be/

be examined. Steinrucken & Amrhein (1984b) reported that K. pneumoniae EPSP synthase was inactivated by phenylglyoxal, a reagent that modifies arginine residues. Substrate protection against phenylglyoxal inactivation was also reported.

FIGURE 5.1

Bromopyruvate inactivation of EPSP synthase: time dependence.

A sample of EPSP synthase was desalted into 50 mM bisTris-HCl pH6.8, 50 mM KCl and treated with BrPyr as described in section 2.8.4. EPSP synthase activity was determined at intervals. Each incubation contained 64 m units EPSP synthase (assayed in the forwards direction).

(□), 0 mM BrPyr; (o), 0.025 mM BrPyr; (●), 0.1 mM BrPyr;
(Δ), 0.25 mM BrPyr; (▲), 0.4 mM BrPyr.

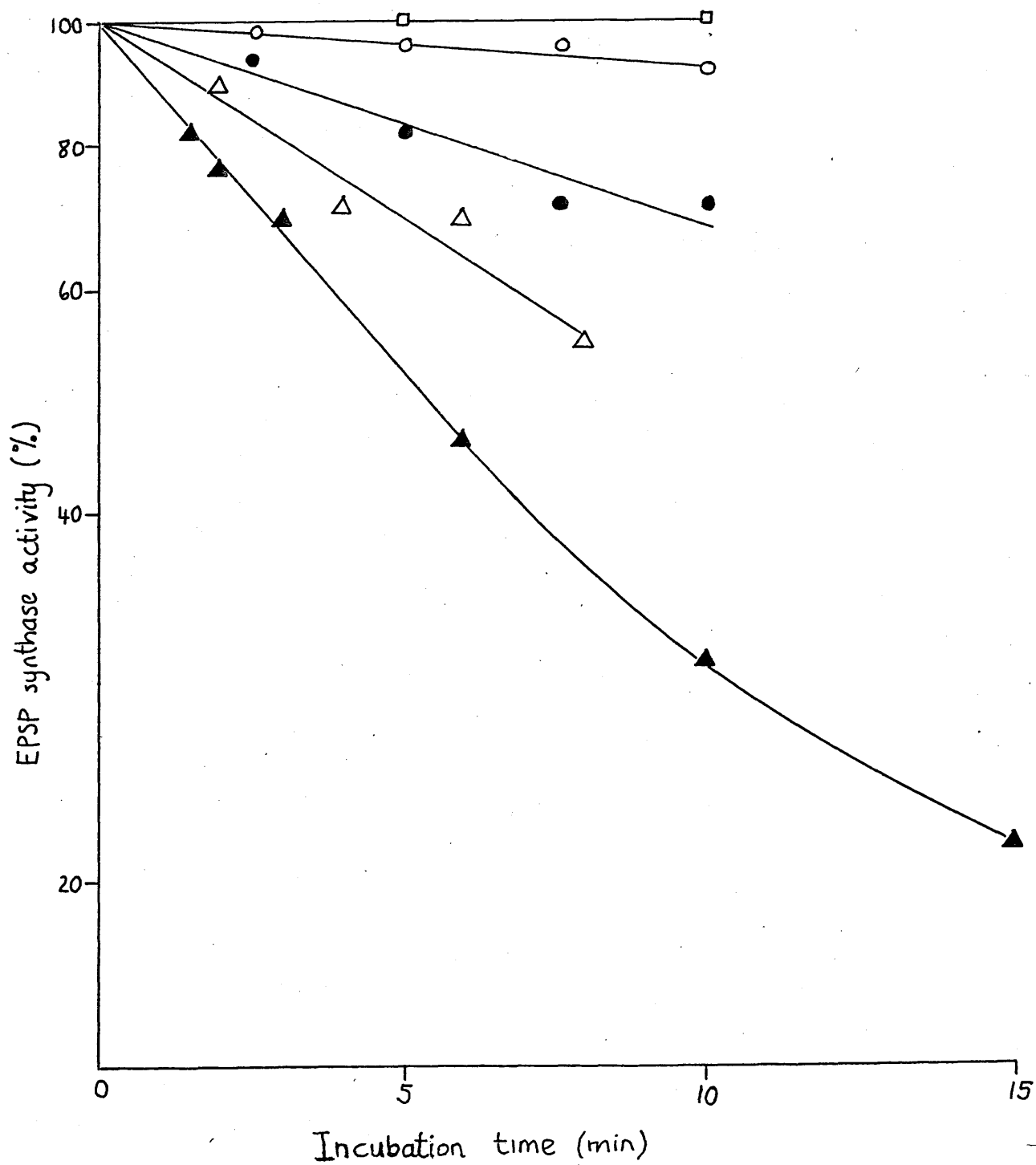


FIGURE 5.2

Bromopyruvate inactivation of EPSP synthase: kinetics of inactivation.

A sample of EPSP synthase was desalted into 50 mM bisTris-HCl pH6.8, 50 mM KCl and treated with BrPyr as described in section 2.8.4. Each incubation contained 64 m units E. coli EPSP synthase (assayed in the forwards direction). The pseudo-first order rate constant (k_{app}) at various concentrations of BrPyr was calculated and is plotted against [BrPyr].

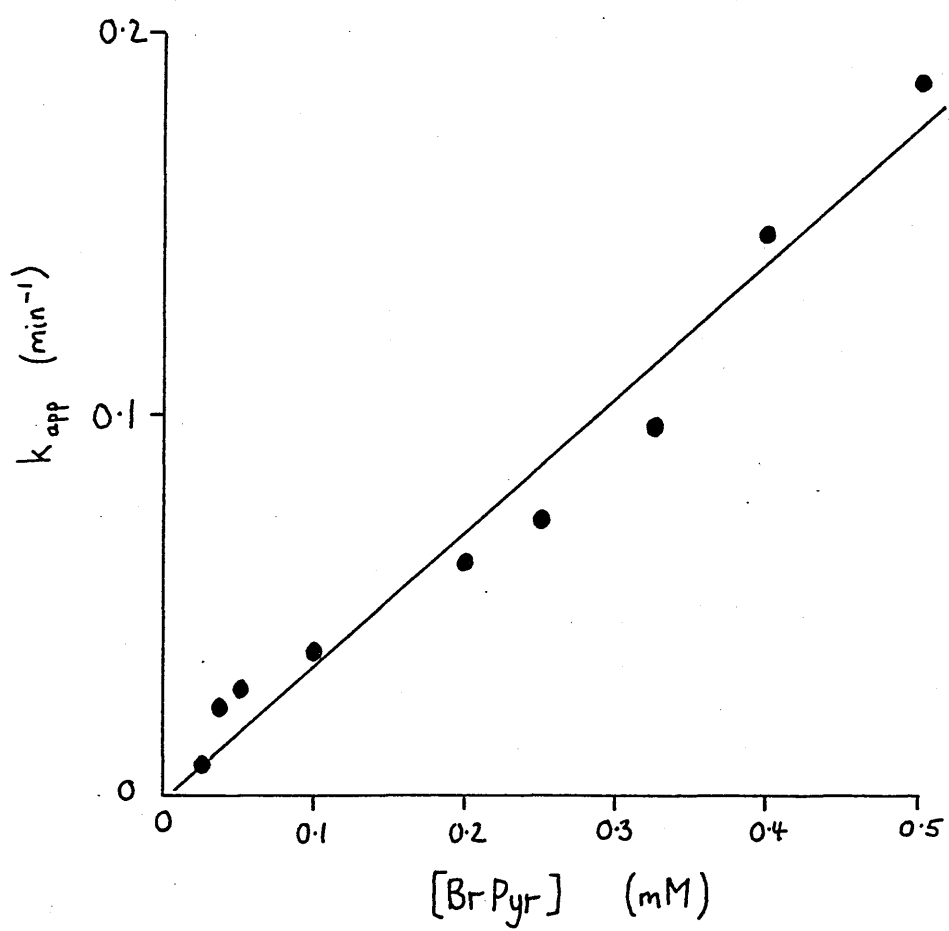


FIGURE 5.3

Bromopyruvate inactivation of EPSP synthase: protection by ligands I.

A sample of EPSP synthase was desalted into 50 mM potassium citrate pH6.8 and treated with BrPyr (1 mM) as described in section 2.8.4 in the presence and absence of various ligands. Each incubation contained 40 m units EPSP synthase activity (assayed in the forwards direction).

(●), no added ligands; (■), 0.2 mM shik 3-P and 0.2 mM glyphosate;

(▲), 0.2 mM shik 3-P; (Δ), 0.2 mM PEP; (o), 0.2 mM EPSP.

<u>protecting ligand</u>	<u>$t_{\frac{1}{2}}$ (min)</u>
none	9
shik 3-P + glyphosate	326
PEP	10
shik 3-P	12
EPSP	10.5

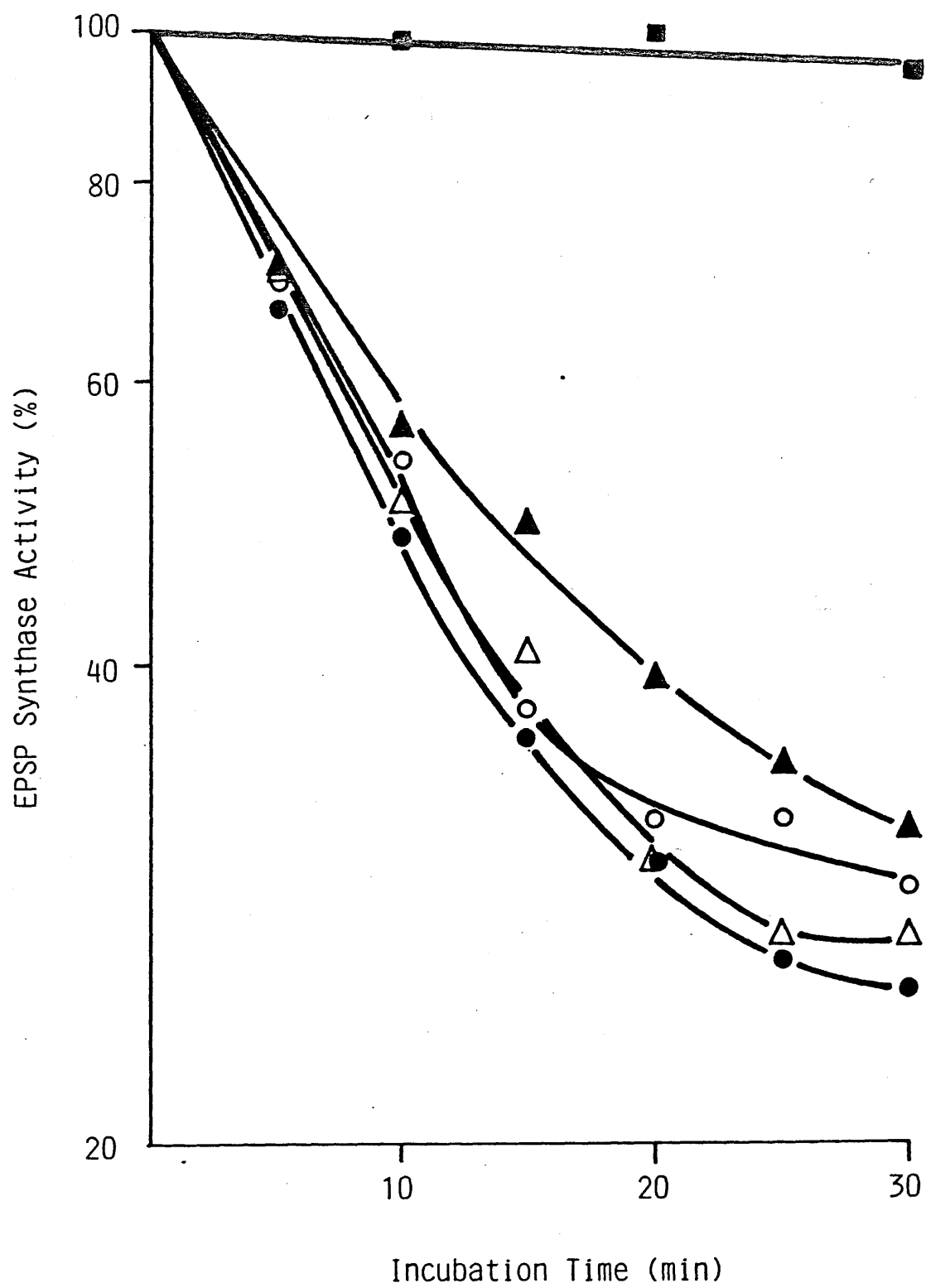


FIGURE 5.4

Bromopyruvate inactivation of EPSP synthase: protection by ligands II.

A sample of EPSP synthase was desalted into 50 mM bisTris-HCl pH6.8, 50 mM KCl and treated with BrPyr (0.1 mM) as described in section 2.8.4. in the presence and absence of various ligands. Each incubation contained 16 m units EPSP synthase (assayed in the forwards direction).

(●), no added ligands; (■), 0.2 mM shik 3-P and 0.2 mM glyphosate; (▲), 0.2 mM shik 3-P; (△), 0.2 mM PEP; (o), 0.2 mM EPSP; (□), 0.2 mM glyphosate.

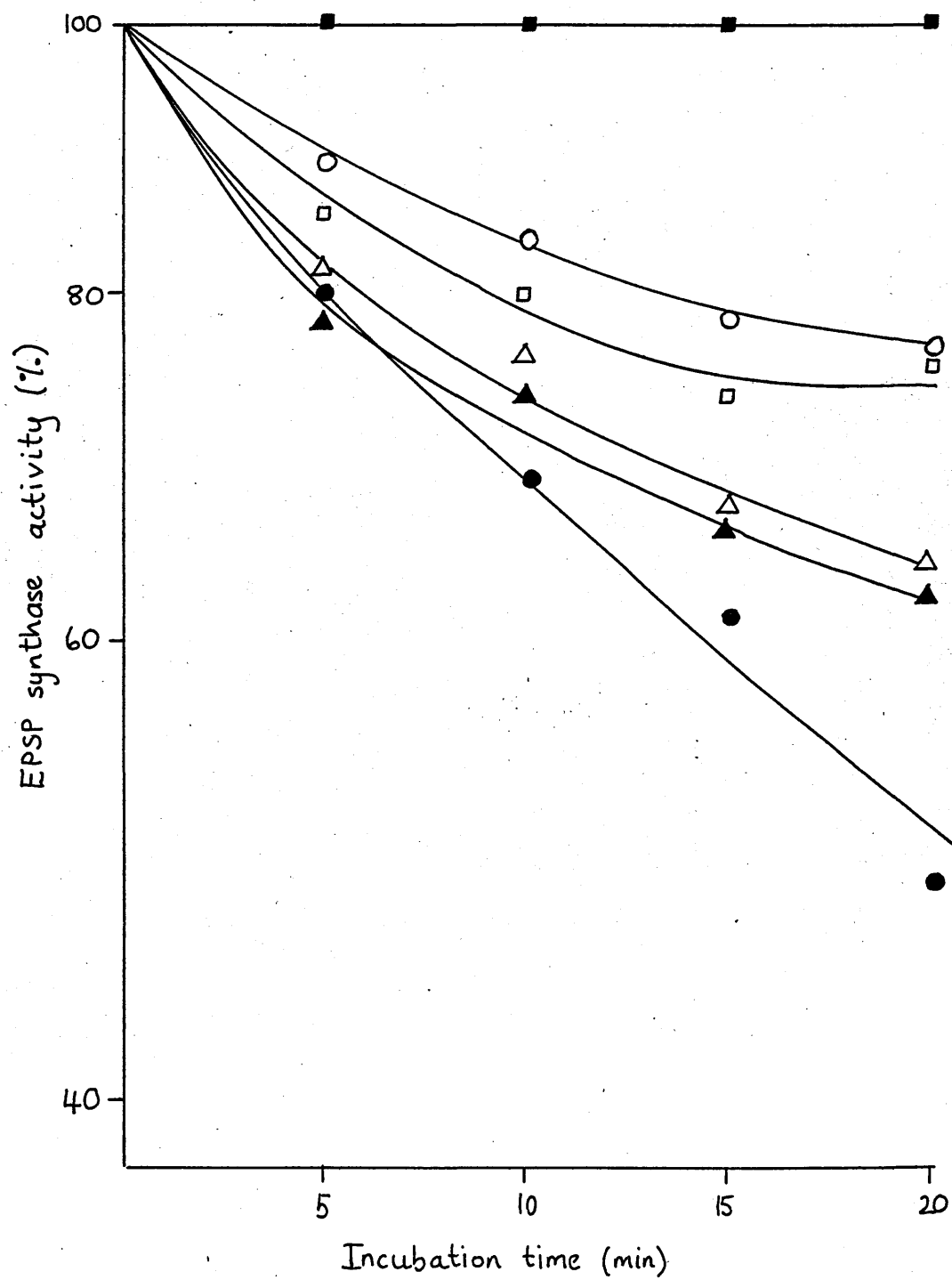
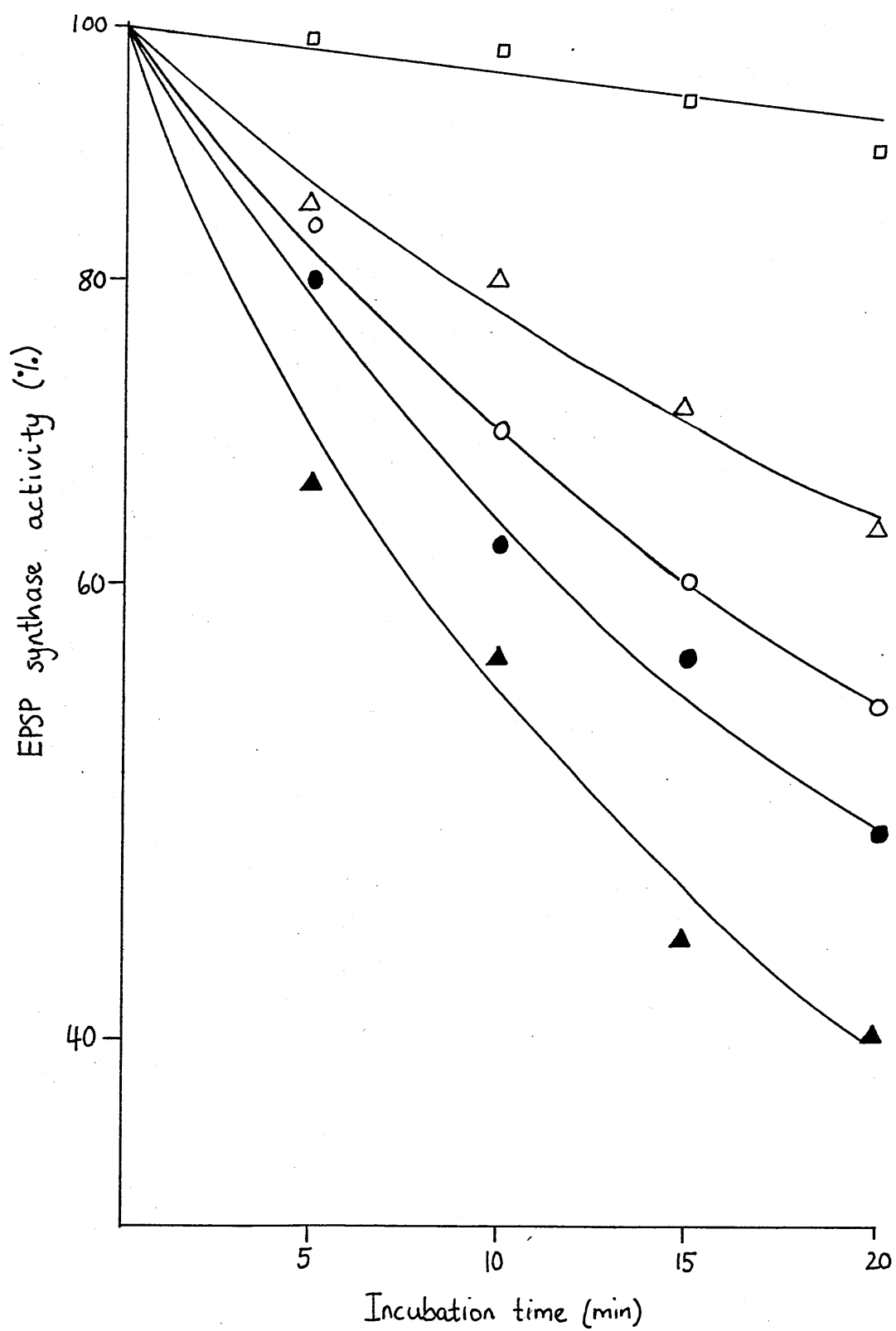


FIGURE 5.5

Bromopyruvate inactivation of EPSP synthase: protection by ligands III.

A sample of EPSP synthase was desalted into 20 mM Hepes-NaOH pH6.8 and treated with BrPyr (0.2 mM) as described in section 2.8.4 in the presence and absence of various ligands. Each incubation contained 104 m units EPSP synthase (assayed in the forwards direction). (●), no added ligands; (▲), 0.2 mM shik 3-P; (Δ), 0.2 mM PEP; (o), 0.2 mM EPSP; (□), 0.2 mM glyphosate.



CHAPTER 6

THE PURIFICATION AND PROPERTIES OF PHOSPHOSERINE

AMINOTRANSFERASE FROM E. COLI AB2829/pKD501

6.1 Introduction

6.1.1 A second protein is overexpressed by E. coli AB2829/pKD501.

The overproducing strain, E. coli AB2829/pKD501, appears to over-express another protein in addition to EPSP synthase. A comparison of SDS PAGE of crude extract fractions of E. coli AB2829/pKD501 (figure 4.5, track A) and of E. coli K12 (figure 3.5, track A) indicated that E. coli AB2829/pKD501 produces large amounts of a polypeptide of M_r 40,000 which does not appear to be produced to such an extent by E. coli K12.

When a systematic attempt was made by Mr. K. Duncan to reduce the size of the DNA insert in pKD501 (a 4.6 kb PstI fragment) to the minimum that would still permit overexpression of EPSP synthase, it was found that the level of EPSP synthase overproduction fell as soon as any of the upstream DNA sequence within 1.2 kb of the initiation codon of the aroA gene was deleted (Duncan, 1984). When this region was sequenced it was found to contain an open reading frame large enough to code for a 362 amino acid polypeptide with a calculated M_r of 39834 (Duncan, 1984).

The gene corresponding to this open reading frame has been identified as the serC gene. The serC gene codes for the enzyme, 3-phosphoserine: 2-oxoglutarate aminotransferase (PSAT), an enzyme of serine biosynthesis. The serC and aroA genes appear to be co-ordinately transcribed from a single promoter upstream of the serC gene. In E. coli strains harbouring pKD501, which carries both the serC and aroA genes, both PSAT and EPSP synthase are overproduced (Duncan, 1984).

As part of a collaborative effort with Mr. K. Duncan to confirm the identity of the M_r 40,000 polypeptide as PSAT, it was decided to attempt/

attempt the purification and characterisation of PSAT from E. coli AB2829/pKD501.

It is evident from figure 4.5 that the M_r 40,000 polypeptide believed to be PSAT was present in fairly large quantities in the phenyl-Sepharose pool of EPSP synthase activity purified from E. coli AB2829/pKD501. It therefore seemed likely that the purification scheme initially developed for EPSP synthase could be modified and used to purify the serC gene product. The purification scheme which was devised for E. coli PSAT is described in this chapter. Some properties of the purified enzyme are also described including protein chemical studies that were undertaken to demonstrate that the purified protein was the product of the serC gene.

6.1.2 Serine biosynthesis in E. coli.

Serine is synthesised in three steps from the glycolytic intermediate, 3-phosphoglycerate (figure 6.1). Pizer (1963) demonstrated that this pathway is a major route to serine in E. coli. Mutants lacking one of these enzyme activities require serine for growth.

The enzymes of serine biosynthesis are synthesised constitutively, carbon flow through this pathway appears to be controlled largely by feedback inhibition of the first enzyme, 3-phosphoglycerate dehydrogenase, by serine (McKittrick & Pizer, 1980). PSAT and 3-phosphoserine phosphatase do not appear to be controlled (Pizer, 1963).

6.1.3 Phosphoserine aminotransferase and pyridoxine biosynthesis.

The role of PSAT in serine biosynthesis is well-established. However, E. coli serC mutants require both serine and pyridoxine for growth (Dempsey, 1969a; Shimizu & Dempsey, 1978), serA and serB mutants/

mutants require serine only. It was suggested that PSAT was involved in pyridoxine biosynthesis as serC mutants do not synthesise pyridoxine (Dempsey, 1969b).

Shimizu & Dempsey (1978) demonstrated that E. coli K12 serC mutants revert readily to pyridoxine independence, this reversion is associated with elevated levels of serine: 2-oxoglutarate aminotransferase activity. The mutation resulting in pyridoxine independence was closely linked to aroA. The growth requirement of serC mutants for pyridoxine could be replaced by 3-hydroxypyruvate. Shimizu & Dempsey (1978) suggested that the role of PSAT in pyridoxine biosynthesis was the transamination of small amounts of serine to give 3-hydroxypyruvate. The importance of 3-hydroxypyruvate in pyridoxine biosynthesis is not understood.

6.1.4 Phosphoserine aminotransferases.

E. coli B PSAT has been purified to near homogeneity and some properties of the purified enzyme reported (Itoh & Dempsey, 1970). The native M_r was estimated to be 80,500 by sucrose density gradient centrifugation but the subunit M_r was not reported. The U.V. absorption spectrum of the isolated enzyme indicated that it contained pyridoxal phosphate.

PSAT has been purified to homogeneity from sheep brain. The native M_r of the mammalian enzyme was 96,000 determined by ultracentrifugation; again the enzyme was isolated with pyridoxal phosphate bound (Hirsch & Greenberg, 1967).

6.2 PSAT levels in E. coli K12 and E. coli AB2829/pKD501.

PSAT activity was assayed in 100,000 g supernatants of E. coli K12 and/

TABLE 6.1

Purification scheme for phosphoserine aminotransferase from E. coli AB2829/pKD501.

Stage	Volume (ml)	Activity (units/ml)	Protein concentration (mg/ml)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification (-fold)
Crude extract ¹	64	11.8	26.0	0.45	755	100	1
50-70% (NH ₄) ₂ SO ₄	51	9.8	16.2	0.60	500	66	1.3
DEAE-Sephacel	202	3.2	1.85	1.73	646	86	3.8
Phenyl-Sepharose							
pool I	103	2.14	0.97	2.21	220	29	4.9
pool II	139	1.86	0.82	2.27	258	34	5.0
Mono-Q ²	-	-	-	3.11	23.9	5.9	6.9

¹PSAT activity is not corrected for the OHPyr-dependent blank rate.

²Only one quarter of phenyl-Sepharose pool I was chromatographed on the mono-Q column. The specific activity reported is the average of 5 mono-Q pools.

The results presented are for the purification of PSAT from 27 g E. coli AB2829/pKD501 cells. PSAT was assayed as described in section 2.5.2 with OHPyr as substrate.

and E. coli AB2829/pKD501 by coupling the production of 2-oxoglutarate to the glutamate dehydrogenase reaction, the keto acid was 3-hydroxypyruvate (OHPyr) in the routine assay (section 2.5.2). PSAT activity was difficult to determine accurately owing to two interfering activities:

- (i) a OHPyr-independent NADH oxidase activity - this was corrected for by running an assay without OHPyr,
- (ii) a OHPyr-dependent NADH oxidation that was corrected for by running an assay without glutamate dehydrogenase.

The specific activity of PSAT in E. coli K12 extracts was approximately 0.011 units/mg and in E. coli AB2829/pKD501 extracts was 1.06 units/mg. Thus E. coli AB2829/pKD501 contains approximately 96-fold elevated levels of PSAT activity. This is very similar to the level of EPSP synthase overproduction by E. coli AB2829/pKD501 (section 4.2).

6.3 Purification of PSAT from E. coli AB2829/pKD501.

The early parts of the purification procedure are a modification of the method used to purify EPSP synthase from the overproducing strain. PSAT activity was monitored throughout the purification with OHPyr as substrate in the assay described in section 2.5.2.

Details of the purification are summarised in Table 6.1.

(a) Crude extract preparation and $(\text{NH}_4)_2\text{SO}_4$ fractionation.

These steps were carried out as described previously (section 3.2 (a) and (b)). The 50-70% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction contained a large proportion of the total PSAT activity (Table 6.1), it also contained the OHPyr-dependent NADH oxidase, however this was removed by/

by the next step in the purification procedure.

(b) DEAE-Sephacel chromatography.

Conditions were as described in section 4.3(b). PSAT was located in fractions following those containing EPSP synthase activity but the separation was incomplete and the accompanying protein peak overlapped the two peaks of activity (figure 6.2). The fractions corresponding to the protein peak were pooled and subjected to chromatography on phenyl-Sephadex.

(c) Phenyl-Sephadex chromatography.

The procedure was as described in section 3.2(d) except that a 400 ml gradient was used. The elution profile is shown in figure 6.3. PSAT activity was eluted from the phenyl-Sephadex column in two peaks, both of which were clearly resolved from the peak of EPSP synthase activity. The occurrence of two peaks of PSAT activity may have been due to overloading of the column. The two peaks of PSAT activity were pooled separately (figure 6.3) and the purity of the pools was examined by SDS PAGE. Only the M_r 40,000 polypeptide was present in both pools. Pool I also contained two other polypeptides (figure 6.4, track D), while pool II contained at least four polypeptides in addition to the one of M_r 40,000 (not shown). The polypeptide of M_r 40,000 was the major species in both pools.

PSAT present in pool I was subsequently purified to homogeneity by ion-exchange chromatography on a mono-Q column.

(d) Ion-exchange chromatography on mono-Q.

Pool I of PSAT activity from chromatography on phenyl-Sephadex was concentrated by vacuum dialysis until the final volume was approximately 2 ml. It was then dialysed against 1 l 25 mM Tris-HCl pH7.5/

pH7.5, 0.2 mM DTT overnight. Because of the limited capacity of the mono-Q column, ion-exchange chromatography was carried out on batches of this concentrated, dialysed material. The mono-Q column was equilibrated with 25 mM Tris-HCl pH7.5, 0.2 mM DTT, the flow rate throughout was 1.0 ml/min and the column eluate was monitored at 280 nm. The sample was loaded on to the column (5.3 mg protein in 200 μ l), and the column was washed with 25 mM Tris-HCl pH7.5, 0.2 mM DTT for 4 min. Then a linear gradient of 0 to 0.5 M KCl in 25 mM Tris-HCl pH7.5, 0.2 mM DTT was applied over 50 min, 0.5 ml fractions were collected during gradient application.

This procedure resulted in the resolution of two protein peaks (figure 6.5). PSAT activity was associated with the second major peak. Portions of fractions containing PSAT activity were subjected to SDS PAGE, those fractions containing only the polypeptide of M_r 40,000 were combined. Homogeneous PSAT was dialysed against 50 mM Tris-HCl pH7.5, 0.2 mM DTT, 50% (v/v) glycerol and stored at -20°C.

The specific activity of the purified enzyme was 3.11 units/mg (Table 6.1). The yield of homogeneous enzyme was low but only one quarter of phenyl-Sepharose pool I was subjected to ion-exchange chromatography on mono-Q. There must be some doubt about the measurement of PSAT activity in the crude extract and 50-70% $(\text{NH}_4)_2\text{SO}_4$ fractions because of the activities that interfere with the assay of PSAT activity described in section 6.2. Therefore, the purification factor given, 6.9-fold, must be regarded as tentative. However, PSAT comprises a very substantial fraction of the extractable protein from E. coli AB2829/pKD501.

6.4 Purity and molecular weight.

Figure 6.4 shows an SDS gel that monitors the purification of PSAT from E. coli AB2829/pKD501. After non-denaturing PAGE, purified PSAT showed only one protein band, which corresponded with an activity staining band (figure 6.6).

The subunit M_r of PSAT was determined to be 39,000 by SDS PAGE (figure 6.7). This agrees well with the calculated M_r of 39,834 derived from the DNA sequence (Duncan, 1984).

It is probable that E. coli PSAT is a dimeric enzyme. The native M_r of purified PSAT was estimated to be 68,000 by HPLC gel filtration on a TSK G3000SW column (figure 6.8). This estimate is somewhat lower than expected for a dimer of M_r 40,000 subunits. It was reported that highly purified PSAT of E. coli B had a native M_r of 80,500 (Itoh & Dempsey, 1970).

6.5 Amino acid composition and N-terminal sequence of PSAT.

(a) Amino acid composition.

The amino acid composition of E. coli PSAT was determined as described in section 2.8.1. The composition is shown in Table 6.2, it is based on 30 Leu residues per PSAT subunit so that the amino acid composition determined at the protein level can be easily compared with that derived from the DNA sequence of the serC gene (Duncan, 1984). It is evident that the agreement between the compositions shown in Table 6.2 is very good.

(b) The N-terminal amino acid sequence.

The N-terminal amino acid sequence of E. coli PSAT was determined by automatic protein sequencing of the intact protein as described/

TABLE 6.2

The amino acid composition of E. coli PSAT compared with the amino acid composition deduced for PSAT from the E. coli serC gene sequence.

Amino acid	Relative amino acid composition based on Leu = 30 residues	Theoretical amino acid composition predicted from the DNA sequence
Asp	39.6	45
Thr ¹	12.5	12
Ser ¹	20.2	20
Glu	33.3	30
Pro	15.7	15
Gly	31.4	28
Ala	43.2	41
Cys ²	4.7	4
Val ³	28.1	28
Met ⁴	10.4	11
Ile ³	18.8	19
Leu	30	30
Tyr	13.1	13
Phe	18.4	18
His	6.2	6
Lys	19.8	20
Arg	16.6	17
Trp	ND	5

¹ Experimental values were extrapolated to zero time.

² Determined as cysteic acid.

³ Average of 96 h experimental values.

⁴ Determined as methionine sulphone.

Samples were analysed in duplicate after hydrolysis of performic acid oxidised-protein with 6 M HCl at 105°C for 24, 48, 72 and 96 h. The 8

described in section 2.8.2. The N-terminal sequence is shown in figure 6.9, this sequence can be compared with that deduced from the DNA sequence of the E. coli serC gene (figure 6.10; Duncan, 1984). The N-terminal amino acid of PSAT is Ala, not Met, presumably the initiating Met is removed after synthesis of PSAT. It should be noted that the amino acid composition derived from the protein contains one less Met than the composition deduced from the DNA sequence.

Gaps in the sequence shown in figure 6.9 indicate residues where the PTH-amino acids could not be identified. Otherwise the N-terminal amino acid sequence agrees well with that derived from the DNA sequence. The exception is at residue 18. The protein sequence indicates Phe (figure 6.9) whereas the DNA sequence predicts Leu (figure 6.10). As the chromatographic conditions used to separate the PTH-amino acids (Carter et al, 1983) result in PTH-Phe and PTH-Leu being eluted very close together, it is possible that PTH-Leu may have been eluted at the position normally occupied by PTH-Phe and the chromatogram misinterpreted. The good agreement between the amounts of Leu and Phe in the experimentally derived and predicted compositions (Table 6.2) tends to support the view that residue 18 is Leu and not Phe.

6.6 Properties of E. coli PSAT.

6.6.1 Substrate specificity.

E. coli PSAT was assayed throughout the purification procedure using OHPyr as substrate. The physiological substrate for this enzyme was reported to be 3-phosphohydroxypyruvate (P-OHPyr; Pizer, 1963). The purified PSAT does transaminate this keto acid. Relative rates/

TABLE 6.3

Relative rates of PSAT activity.

keto acid	amino acid	Relative PSAT activity (%)
3-phosphohydroxypyruvate	Glu	172
pyruvate	Glu	151
hydroxyphenylpyruvate	Glu	0
phenylpyruvate	Glu	1.8
oxaloacetate	Glu	96
3-hydroxypyruvate	Glu	100
3-hydroxypyruvate	Asp	70
2-oxoglutarate	Ser	1.1
2-oxoglutarate	3-phosphoserine	0.9

Relative rates of E. coli PSAT activity with different pairs of substrates are compared, the rate with the substrate pair 3-hydroxypyruvate/Glu was taken as 100%. PSAT activity was assayed as described in section 2.5.2.

rates of PSAT activity with a number of keto acids and amino acids are shown in Table 6.3. Evidently PSAT can transaminate keto acids other than P-OHPyr and OHPyr as pyruvate and oxaloacetate are also substrates. Aromatic keto acids are not transaminated. A total description of the substrate specificity of E. coli PSAT would require the determination of kinetic parameters for each substrate and this has not so far been attempted.

6.6.2 Kinetic properties.

E. coli PSAT is inhibited by high concentrations of P-OHPyr. This keto acid is therefore a better substrate for PSAT than is evident from Table 6.3. Figure 6.11 shows a double reciprocal plot of initial velocity against [glutamate] at fixed concentrations of P-OHPyr. At low [P-OHPyr] the lines appear parallel but the slope is increased by increasing P-OHPyr. This kinetic pattern is typical of a ping-pong mechanism with competitive inhibition by the second substrate (Cleland, 1971). K_m values for P-OHPyr and glutamate were estimated from secondary plots to be $4.0 \mu\text{M}$ and 1.2 mM respectively.

The Michaelis constants determined at fixed [glutamate] (3 mM) for P-OHPyr and OHPyr were $2.5 \mu\text{M}$ and $167 \mu\text{M}$ respectively. This indicates that OHPyr is not as good a substrate as P-OHPyr.

It is evident from Table 6.3 that transamination is reversible although the observed rate is only approximately 1% of the rate in the forwards direction.

6.6.3 Pyridoxal phosphate and PSAT.

Purified E. coli PSAT is yellow coloured. The absorption spectrum of the purified enzyme (figure 6.11a) indicates that PSAT is isolated with pyridoxal phosphate (PLP) bound. Recovery of PSAT activity during the purification

TABLE 6.4

The effect of pyridoxal phosphate on PSAT activity.

Additions	Recovery of PSAT activity (m units)
none	26
50 μ M pyridoxal phosphate	25
100 μ M glutamate	13
100 μ M 3-hydroxypyruvate	24

Aliquots of purified E. coli PSAT (28 m units) were dialysed against 50 mM Tris-HCl pH8.2, 0.4 mM DTT with the additions specified above. After 24 h, PSAT activity was determined using the routine assay method (section 2.5.2).

was good although the buffers used in the purification procedure did not contain PLP. Addition of PLP to assay mixtures does not result in increased PSAT activity.

Table 6.4 shows that no increase in PSAT activity was observed after dialysis of purified enzyme against 50 μ M PLP. Dialysis against 100 μ M glutamate does decrease PSAT activity. This type of inactivation has also been observed for the highly purified PSAT of E. coli B. Itoh & Dempsey (1970) suggested that dissociation of pyridoxamine phosphate might occur more readily than PLP dissociation. The PSAT activity lost by dialysis against glutamate was not recovered by subsequent incubation of the inactivated enzyme in 50 μ M PLP, restoration of activity using pyridoxamine phosphate was not attempted.

6.6.4 Discussion.

The properties of E. coli PSAT described above can be regarded as properties expected of an aminotransferase.

- (i) PSAT appeared to be isolated with tightly bound PLP;
- (ii) the initial velocity kinetic patterns of E. coli PSAT were of the non-intersecting type;
- (iii) PSAT catalyses the transamination of a number of structurally related substrates.

Phosphoserine aminotransferases have also been purified from E. coli B (Itoh & Dempsey, 1970) and sheep brain (Hirsch & Greenberg, 1967). Both of these isolated enzymes contained tightly bound PLP. The E. coli B PSAT showed non-intersecting initial velocity kinetic patterns. This enzyme was assayed in the reverse direction with 3-phosphoserine and 2-oxoglutarate as substrates, the kinetics of the forward reaction were not described (Itoh & Dempsey, 1970). The mammalian PSAT differs from the E. coli AB2829/pKD501 enzyme in that/

that it does not transaminate OHPyr. Inhibition of the sheep brain PSAT was observed at high concentrations of P-OHPyr (Hirsch & Greenberg, 1967).

6.7 Comparison of E. coli PSAT with other aminotransferases.

As the amino acid sequence and some of the properties of E. coli PSAT have now been established it is interesting to compare PSAT with some of the other aminotransferase (AT) activities which have been characterised.

E. coli PSAT appears to be a dimeric protein of subunit M_r 40,000. The E. coli aspartate AT and aromatic amino acid AT have been shown to be dimeric enzymes of ^{subunit} M_r 43,000 and 46,000 respectively (Powell & Morrison, 1978). S. typhimurium imidazolylacetolphosphate:glutamate AT may also be a dimer of subunits of M_r approximately 30,000 (Martin et al, 1967) although this enzyme has been less well characterised. However, the E. coli branched chain amino acid AT appeared to be a hexameric protein of subunit M_r 31,000 (Lee-Peng et al, 1979).

Some sequence data have been reported for all of these aminotransferases. Limited N-terminal amino acid sequences of the imidazolylacetolphosphate:glutamate AT (13 amino acids) and the branched chain amino acid AT (81 amino acids) have been deduced from DNA sequencing studies of the S. typhimurium hisC gene (Riggs & Artz, 1984) and the E. coli ilvE gene (Lawther et al, 1978) respectively. The complete DNA sequence of the E. coli tyrB gene (which codes for the aromatic amino acid AT) has been determined (M. Edwards, unpublished results). The complete amino acid sequences of a number of aspartate aminotransferases have been determined by protein sequencing. These include/

include the E. coli enzyme (Kondo et al, 1984), pig heart mitochondrial (Kagamiyama et al, 1980) and cytosolic (Ovchinnikov et al, 1973) aspartate AT and the chicken cytosolic enzyme (Shlyapnikov et al, 1979). The sequence of the E. coli aspartate AT is 40% homologous with the pig heart isoenzymes.

The E. coli tyrB gene product shows homology with these aspartate aminotransferases. This is illustrated by the dot-plot (Maizel & Lenk, 1981) shown in figure 6.12 where a line of homology between the E. coli aromatic AT and the chicken cytosolic aspartate AT is evident. However, no homology between the tyrB gene product and E. coli PSAT was detected in the same type of test (figure 6.13; K. Duncan, unpublished results). Similarly, homology of the E. coli PSAT sequence with the limited N-terminal sequences of the imidazolylacetolphosphate:glutamate AT and the branched chain amino acid AT is not evident.

In view of the different substrate specificities of these aminotransferases, it is perhaps not surprising that homology between the amino acid sequence of E. coli PSAT and the sequences of the other aminotransferases cannot be readily detected. The homology observed between the aromatic amino acid AT and aspartate AT (figure 6.12) is explicable as aspartate AT can catalyse transaminations involving aromatic amino acid substrates and vice versa (Powell & Morrison, 1978).

6.8 Further discussion.

Phosphoserine aminotransferase has been purified to homogeneity from E. coli AB2829/pKD501, a strain which overproduces both PSAT and EPSP synthase. PSAT required approximately 7-fold purification from E. coli AB2829/pKD501, PSAT of E. coli B required 519-fold purification/

purification to near homogeneity (Itoh & Dempsey, 1970).

The purification factor for EPSP synthase from the overproducing strain was approximately 50-fold (Table 4.1), therefore it seems that E. coli AB2829/pKD501 cells contain more PSAT than EPSP synthase by mass. The serC-aroA operon appears to be transcribed from a single promoter, however, there is a transcriptional terminator between the serC and aroA genes which will reduce the number of complete serC-aroA messengers (Duncan, 1984). This provides a rationale for the different amounts of the two gene products although some form of translational control cannot be ruled out.

The protein chemical properties of purified PSAT confirm that it is the product of the gene upstream of aroA that had been identified as the serC gene. The amino acid composition and N-terminal amino acid sequence presented in section 6.5 agree well with the protein sequence deduced from the DNA sequence of the serC gene (Duncan, 1984). The kinetic properties of E. coli PSAT described in section 6.6 are consistent with the proposed physiological role of this enzyme in serine biosynthesis, the transamination of P-OHPyr to give 3-phosphoserine.

These studies on the isolated enzyme do not explain the pyridoxine requirement for growth of E. coli serC mutants (Dempsey, 1969a).

E. coli PSAT does catalyse the production of OHPyr from serine albeit at a somewhat low rate compared to the reverse reaction. Whether or not OHPyr production is one of phosphoserine aminotransferase's roles in vivo remains an open question.

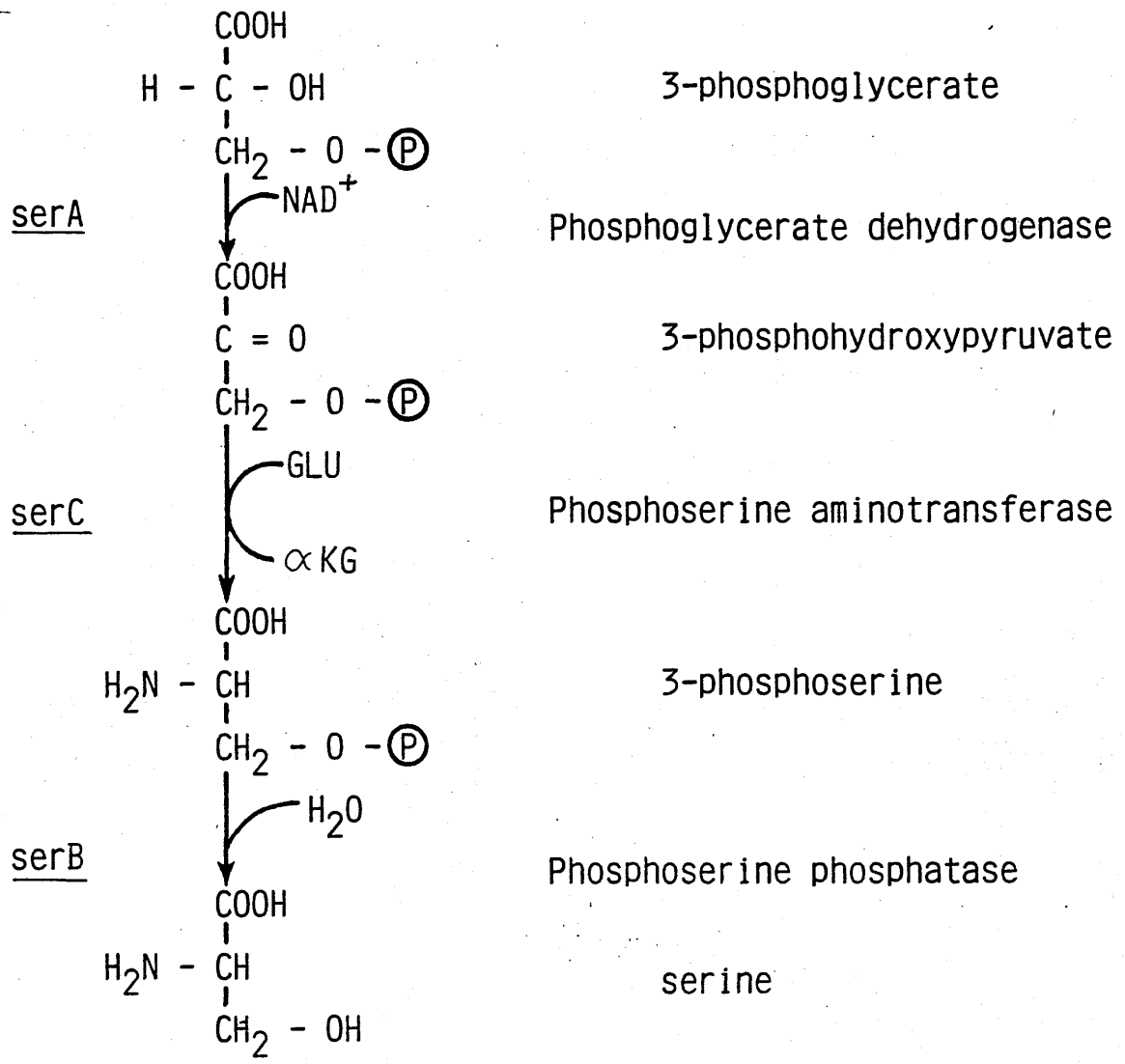


Figure 6.1 Serine biosynthesis in E. coli.

FIGURE 6.2

DEAE-Sephacel chromatography.

The 50-70% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction was loaded on to a column of DEAE-Sephacel and chromatographed as described in section 4.3(b).

(o), A_{280} ; (●), EPSP synthase activity; (Δ), PSAT activity;

(□ — □), conductivity.

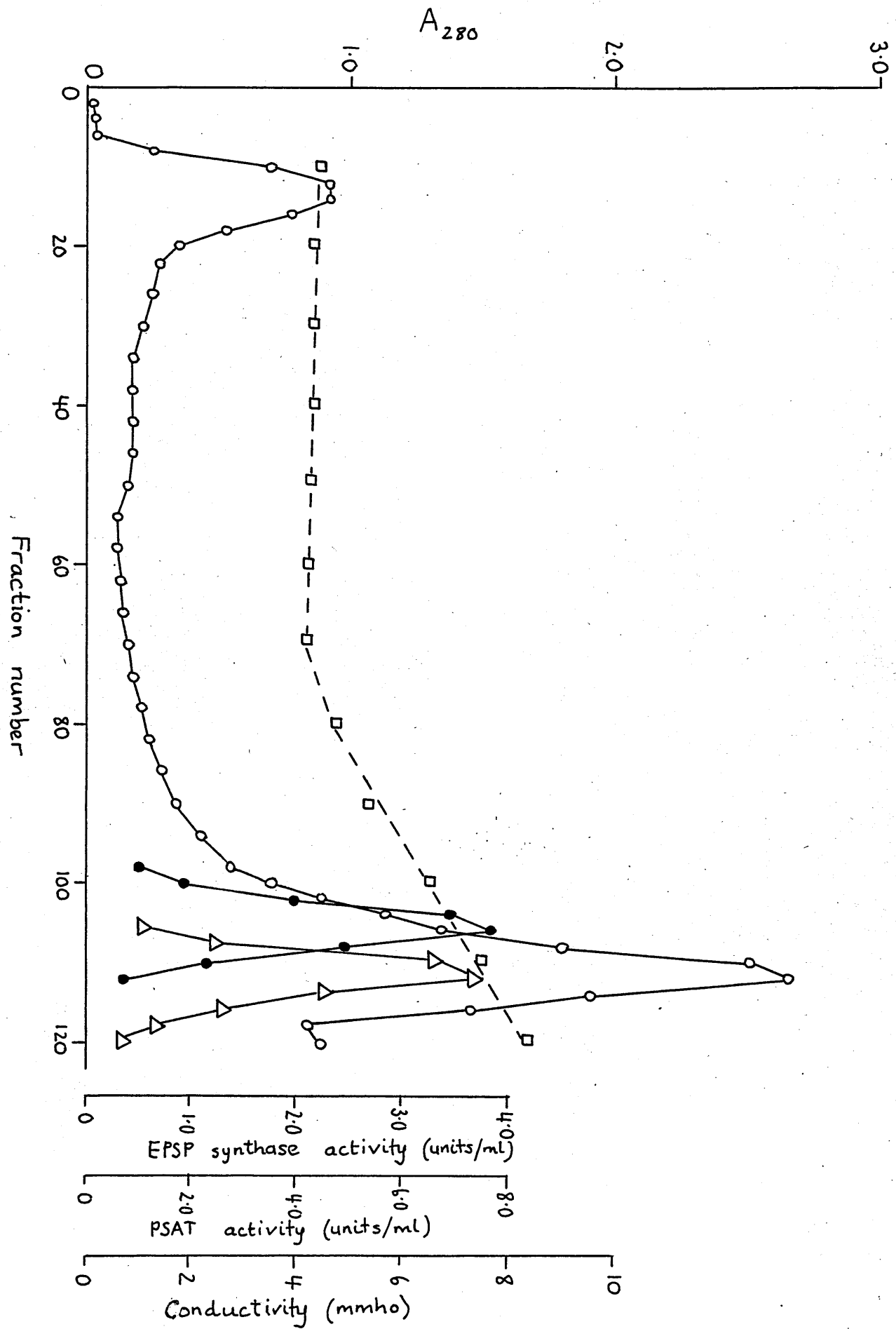


FIGURE 6.3

Chromatography on phenyl-Sepharose.

The pool of EPSP synthase and PSAT activities from chromatography on DEAE-Sephacel was treated with $(\text{NH}_4)_2\text{SO}_4$ (164 g/l) and loaded on to a column of phenyl-Sepharose equilibrated in buffer B containing 0.8 M $(\text{NH}_4)_2\text{SO}_4$ as described in section 3.2(d). A gradient (total volume, 400 ml) of 0.8 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in buffer B was applied. The flow rate was 80 ml/h and 8 ml fractions were collected. (o), A_{280} ; (●), EPSP synthase activity; (Δ), PSAT activity; ($\square-\square$), conductivity. The fractions included in pool I and pool II of PSAT activity are indicated.

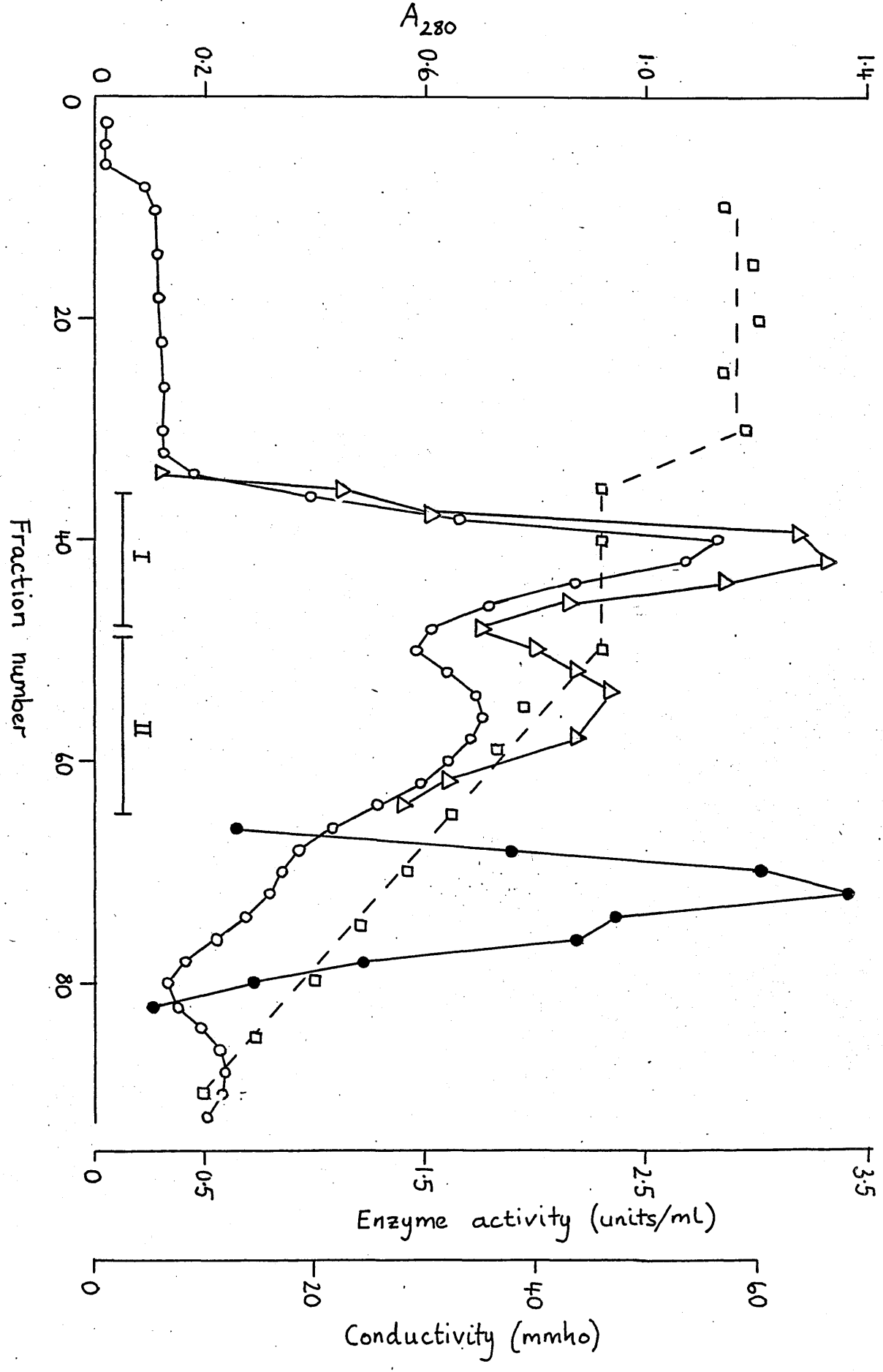
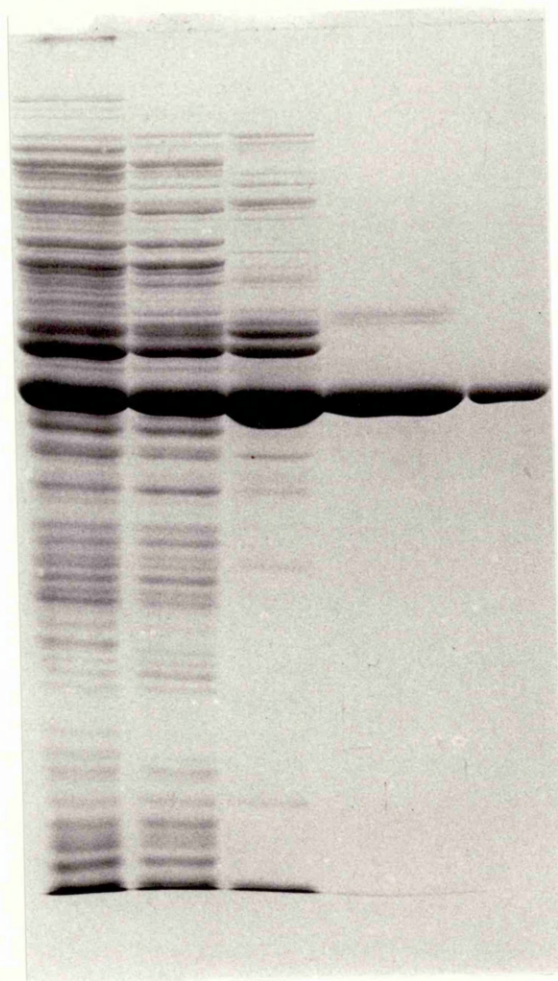


FIGURE 6.4

The purification of PSAT from E. coli AB2829/pKD501.

This 12% polyacrylamide SDS gel monitors the purification of PSAT from E. coli AB2829/pKD501. Track A, crude extract (141 μ g protein); track B, 50-70% $(\text{NH}_4)_2\text{SO}_4$ fraction (81 μ g); track C, DEAE-Sephacel pool (46 μ g); track D, phenyl-Sepharose pool I (24 μ g); track E, mono-Q pool (4 μ g).



A B C D E

FIGURE 6.5

Ion-exchange chromatography on mono-Q.

Ion-exchange chromatography was carried out as described in section 6.3 (d). A portion of concentrated PSAT pool I from phenyl-Sepharose chromatography (5.3 mg protein) was loaded on to the mono-Q column. A gradient of 0 to 0.5 M KCl in 25 mM Tris-HCl pH7.5 was applied. The column eluate was monitored at 280 nm. The section of gradient shown (0.2 M to 0.5 M KCl) is that in which peaks of material absorbing at 280 nm were eluted.

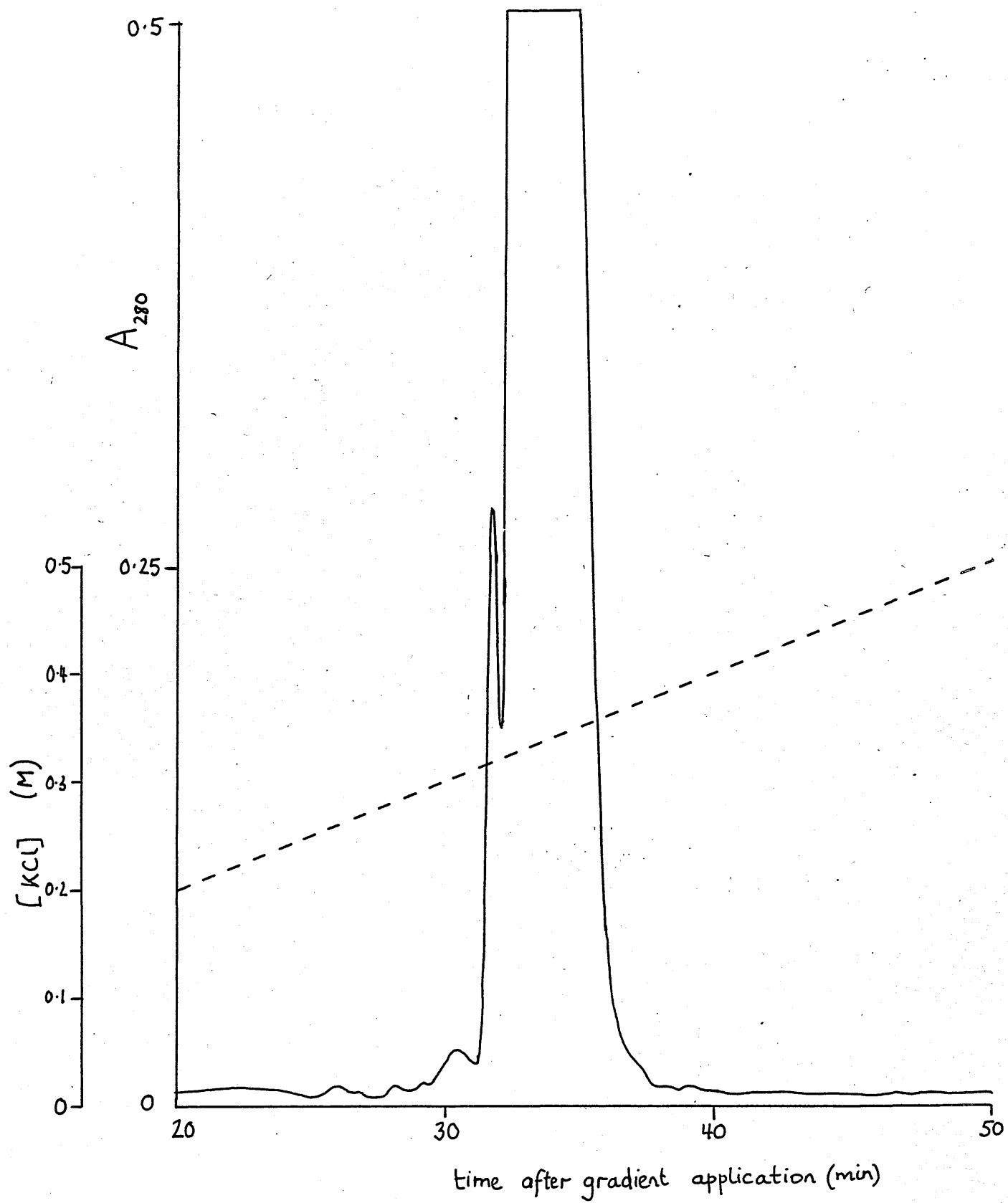
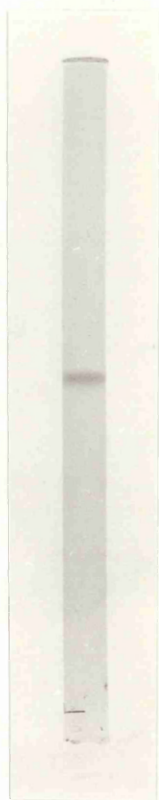


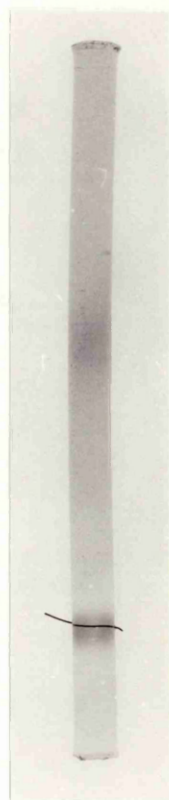
FIGURE 6.6

Non-denaturing PAGE of E. coli PSAT.

Samples of purified PSAT were subjected to non-denaturing PAGE (section 2.3.1). Gel A was stained for protein (2.6 μ g loaded), gel B was stained for PSAT activity (9 m units loaded, section 2.3.3).



A



B

FIGURE 6.7

Standard curve of R_f against $\log M_r$.

SDS PAGE (10% polyacrylamide gel) was carried out as described in section 2.3.2. The R_f values of proteins of standard M_r (section 2.8.3) were calculated and are plotted against $\log M_r$ (●). The R_f value of purified PSAT is shown (o).

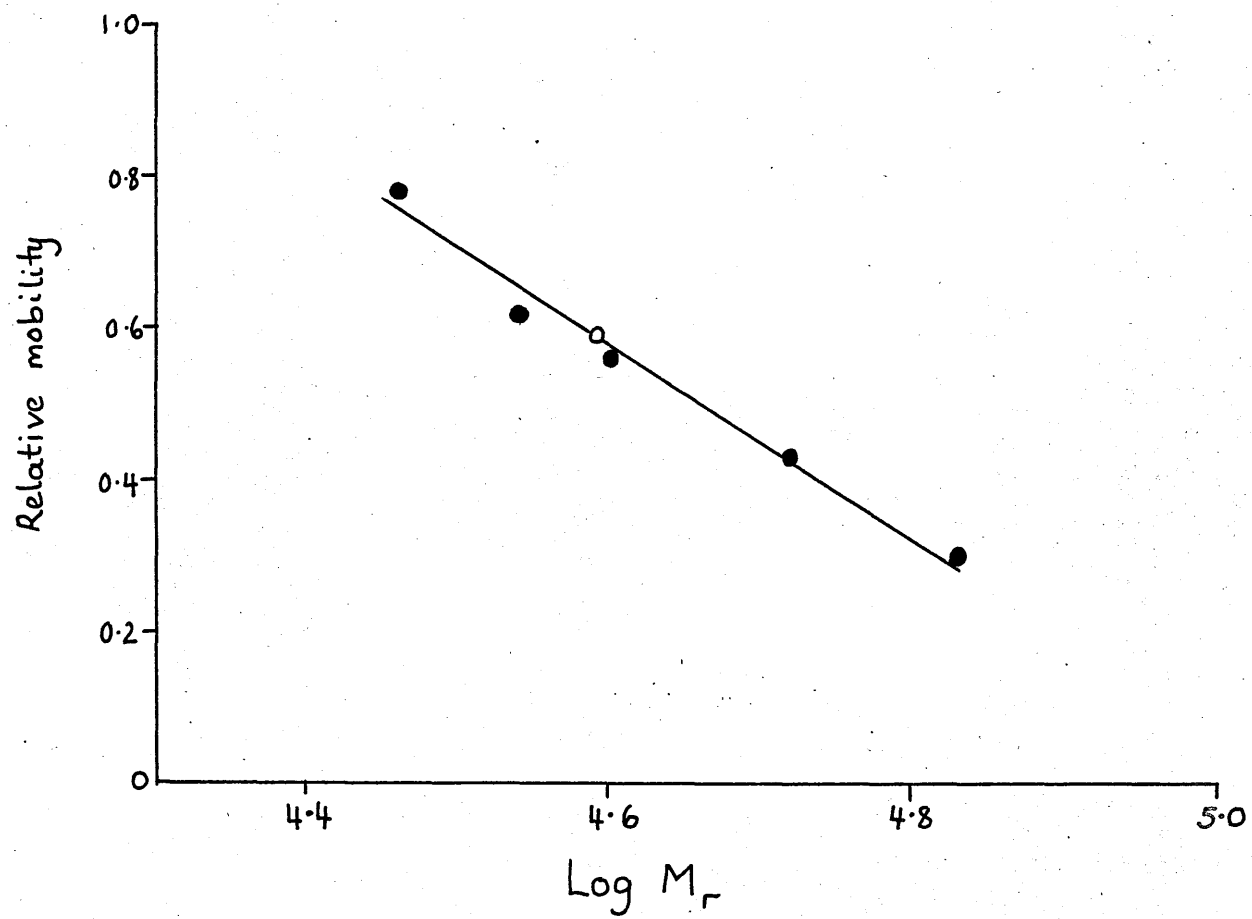


FIGURE 6.8

Standard curve of V_e against $\log M_r$.

A TSK G3000SW column was calibrated with the standard proteins (●) listed in section 2.8.3. The V_e of E. coli PSAT is shown (○).

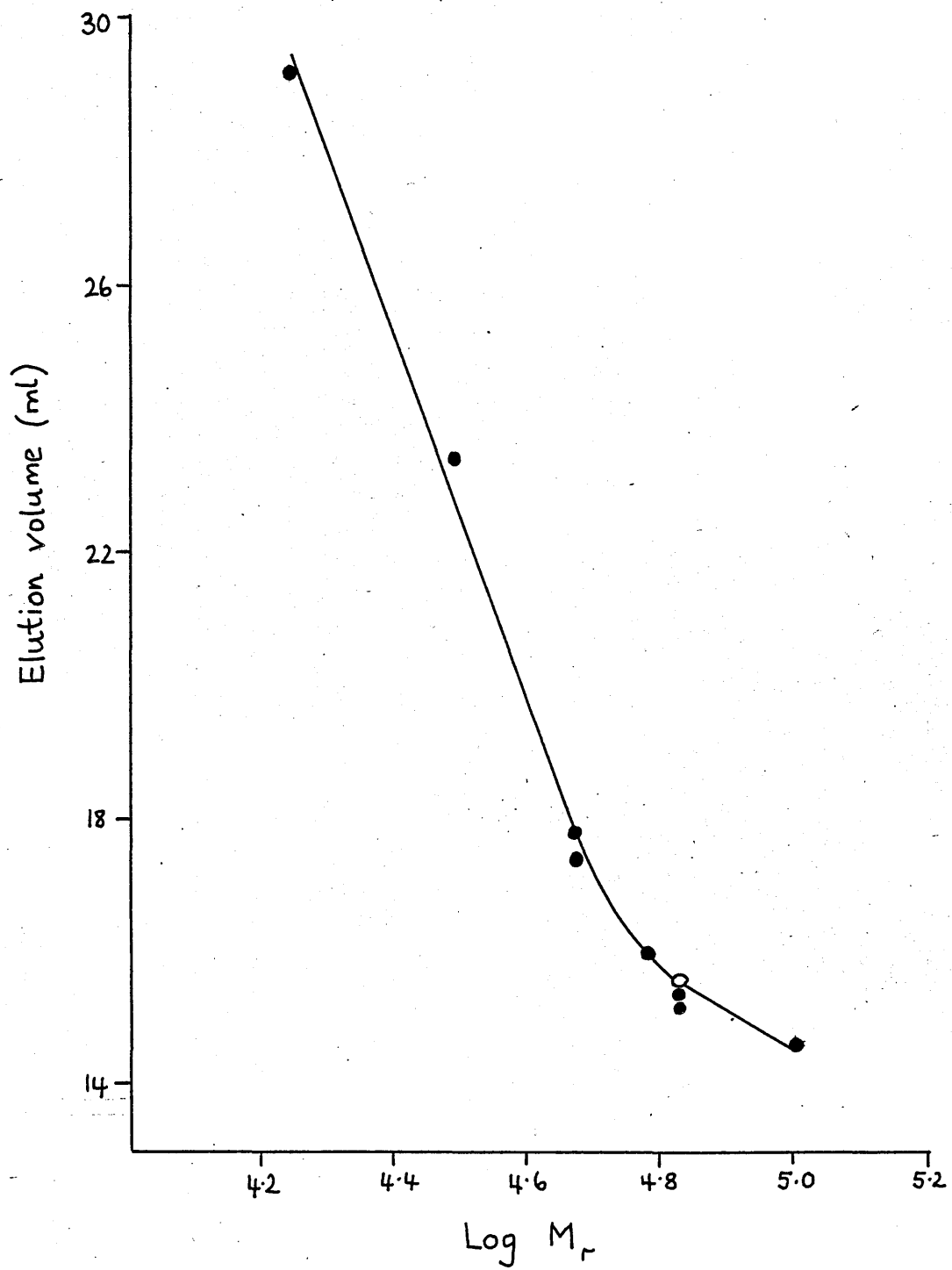


FIGURE 6.9

The N-terminal amino acid sequence of E. coli PSAT.

1	10
Ala - Gln - Ile - Phe - Asn - Phe - Ser - Ser - Gly - Pro -	
11	20
Ala - Met - Leu - Pro - (-) - Glu - Val - Phe - Lys - Gln -	
21	30
Ala - Gln - Gln - (-) - Leu - Arg - Asp - Trp - Asn - Gly -	
31	37
Leu - (-) - (-) - (-) - (-) - Met - Glu -	

The sequence was determined on a liquid phase sequencer as described in section 2.8.2. The initial amount of protein sequencing was 25 nmol and the repetitive yield from residues 1 to 37 was 91.7% (correlation coefficient 0.94).

Continued over.

FIGURE 6.9 (continued)

Sequence number	PTH-amino acid	nmoles recovered
1	Ala	22.5
2	Gln	25.1
3	Ile	13.0
4	Phe	23.8
5	Asn	22.4
6	Phe	21.0
7	Ser	-
8	Ser	-
9	Gly	7.0
10	Pro	16.5
11	Ala	12.6
12	Met	14.2
13	Leu	9.6
14	Pro	9.5
15	-	-
16	Glu	7.3
17	Val	12.9
18	Phe	10.3
19	Lys	6.4
20	Gln	5.5
21	Ala	6.0
22	Gln	5.5
23	Gln	5.4
24	-	-
25	Leu	2.9
26	Arg	2.7
27	Asp	5.3
28	Trp	1.5
29	Asn	2.0
30	Gly	1.1
31	Leu	1.8
32	-	-
33	-	-
34	-	-
35	-	-
36	Met	-
37	Glu	1.3

FIGURE 6.10

The DNA sequence of the E. coli serC gene.

The complete nucleotide sequence of the E. coli serC gene and the corresponding amino acid sequence of E. coli phosphoserine aminotransferase. Nucleotides are numbered in the 5' to 3' direction beginning with the first residue of the ATG triplet encoding the initiating methionine. The bracketed numbers refer to the amino acid positions in the sequence.

10 30 50
 ATGGCTCAAATCTTCAATTTTAGTTCTCGCTCCGGCAATGCTACCGGCAGAGCTGCTTAAA
 1 -----+-----+-----+-----+-----+
 MetAlaGlnIlePheAsnPheSerSerGlyProAlaMetLeuProAlaGluValLeuLys
 [1]

70 90 110
 CAGGCTCAACAGGAACTGCGCGACTGGAACGGTCTTGGTACGTCCGTGATGGAAGTGACT
 61 -----+-----+-----+-----+-----+
 GlnAlaGlnGlnGluLeuArgAspTrpAsnGlyLeuGlyThrSerValMetGluValSer
 [21]

130 150 170
 CACCGTGGCAAAGAGTTTCATTTCAGGTTGCAGAGGAAGCCGAGAAGGATTTTCGCGATCTT
 121 -----+-----+-----+-----+-----+
 HisArgGlyLysGluPheIleGlnValAlaGluGluAlaGluLysAspPheArgAspLeu
 [41]

190 210 230
 CTTAATGTCCCCTCCAACCTACAAGGTATTATTCTGCCATGGCGGTGGTCCGGTCAGTTT
 181 -----+-----+-----+-----+-----+
 LeuAsnValProSerAsnTyrLysValLeuPheCysHisGlyGlyGlyArgGlyGlnPhe
 [61]

250 270 290
 GCTGCGGTACCGCTGAATATTCTCGGTGATAAAACCACCGCAGATTATGTTGATGCCGGT
 241 -----+-----+-----+-----+-----+
 AlaAlaValProLeuAsnIleLeuGlyAspLysThrThrAlaAspTyrValAspAlaGly
 [81]

310 330 350
 TACTGGGCGGCAAGTGCCATTAAAGAAGCGAAAAAATACTGCACGCCTAATGTCTTTGAC
 301 -----+-----+-----+-----+-----+
 TyrTrpAlaAlaSerAlaIleLysGluAlaLysLysTyrCysThrProAsnValPheAsp
 [101]

370 390 410
 GCCAAAGTGACTGTTGATGGTCTCGCGCCGGTTAAGCCAATGCGTGAATGGCAACTCTCT
 361 -----+-----+-----+-----+-----+
 AlaLysValThrValAspGlyLeuArgAlaValLysProMetArgGluTrpGlnLeuSer
 [121]

Figure 6.10: Coding sequence of the *E.coli serC* gene.

```

      430              450              470
421  GATAATGCTGCTTATATGCATTATTGCCCCGAATGAAACCATCGATGGTATCGCCATCGAC
      +-----+-----+-----+-----+
      AspAsnAlaAlaTyrMetHisTyrCysProAsnGluThrIleAspGlyIleAlaIleAsp
      [141]

      490              510              530
481  GAAACGCCAGACTTCGGCGCAGATGTGGTGGTCGCCGCTGACTTCTCTTCAACCATTCTT
      +-----+-----+-----+-----+
      GluThrProAspPheGlyAlaAspValValValAlaAlaAspPheSerSerThrIleLeu
      [161]

      550              570              590
541  TCCCGTCCGATTGACGTCAGCCGTTATGGTGTAAATTTACCCTGCCGCCAGAAAAATATC
      +-----+-----+-----+-----+
      SerArgProIleAspValSerArgTyrGlyValIleTyrAlaGlyAlaGlnLysAsnIle
      [181]

      610              630              650
601  GGGCCGGCTGGCCTGACAATCGTCATCGTTTCGTGAAGATTTGCTGGGCAAAGCGAATATC
      +-----+-----+-----+-----+
      GlyProAlaGlyLeuThrIleValIleValArgGluAspLeuLeuGlyLysAlaAsnIle
      [201]

      670              690              710
661  GCGTGTCGGTCCGATTCTCGATTATTCCATCCTCAACGATAACGGCTCCATGTTTAAACACG
      +-----+-----+-----+-----+
      AlaCysProSerIleLeuAspTyrSerIleLeuAsnAspAsnGlySerMetPheAsnThr
      [221]

      730              750              770
721  CCGCCGACATTTGCCTGGTATCTATCTGGTCTGGTCTTTAAATGGCTGAAAGCGAACGGC
      +-----+-----+-----+-----+
      ProProThrPheAlaTrpTyrLeuSerGlyLeuValPheLysTrpLeuLysAlaAsnGly
      [241]

      790              810              830
781  GGTGTAGCTGAAATGGATAAAATCAATCAGCAAAAAGCAGAACTGCTATATGGGGTGATT
      +-----+-----+-----+-----+
      GlyValAlaGluMetAspLysIleAsnGlnGlnLysAlaGluLeuLeuTyrGlyValIle
      [261]

```

Figure 6.10 (cont.)

```

      850      870      890
841  GATAACAGCGATTTCTACCGCAATGACGTGGCGAAACGTAACCGTTCGCGGATGAACGTC
      -----+-----+-----+-----+-----+
      AspAsnSerAspPheTyrArgAsnAspValAlaLysArgAsnArgSerArgMetAsnVal
      [281]

      910      930      950
901  CCGTTCCAGTTGGCGGACAGTGGCCTTGACAAATTGTTCCCTTGAAGAGTCTTTTGCTGCT
      -----+-----+-----+-----+-----+
      ProPheGlnLeuAlaAspSerAlaLeuAspLysLeuPheLeuGluGluSerPheAlaAla
      [301]

      970      990      1010
961  GGCCTTCATGCACTGAAAGGTCACCGTGTGGTCCGCCGAATGCCCGCTTCTATTTATAAC
      -----+-----+-----+-----+-----+
      GlyLeuHisAlaLeuLysGlyHisArgValValGlyGlyMetArgAlaSerIleTyrAsn
      [321]

      1030      1050      1070
1021 GCCATGCCCGCTGGAAGGCGTTAAAGCGCTGACAACTTCATGGTTGAGTTCCAACGCCGT
      -----+-----+-----+-----+-----+
      AlaMetProLeuGluGlyValLysAlaLeuThrAspPheMetValGluPheGluArgArg
      [341]

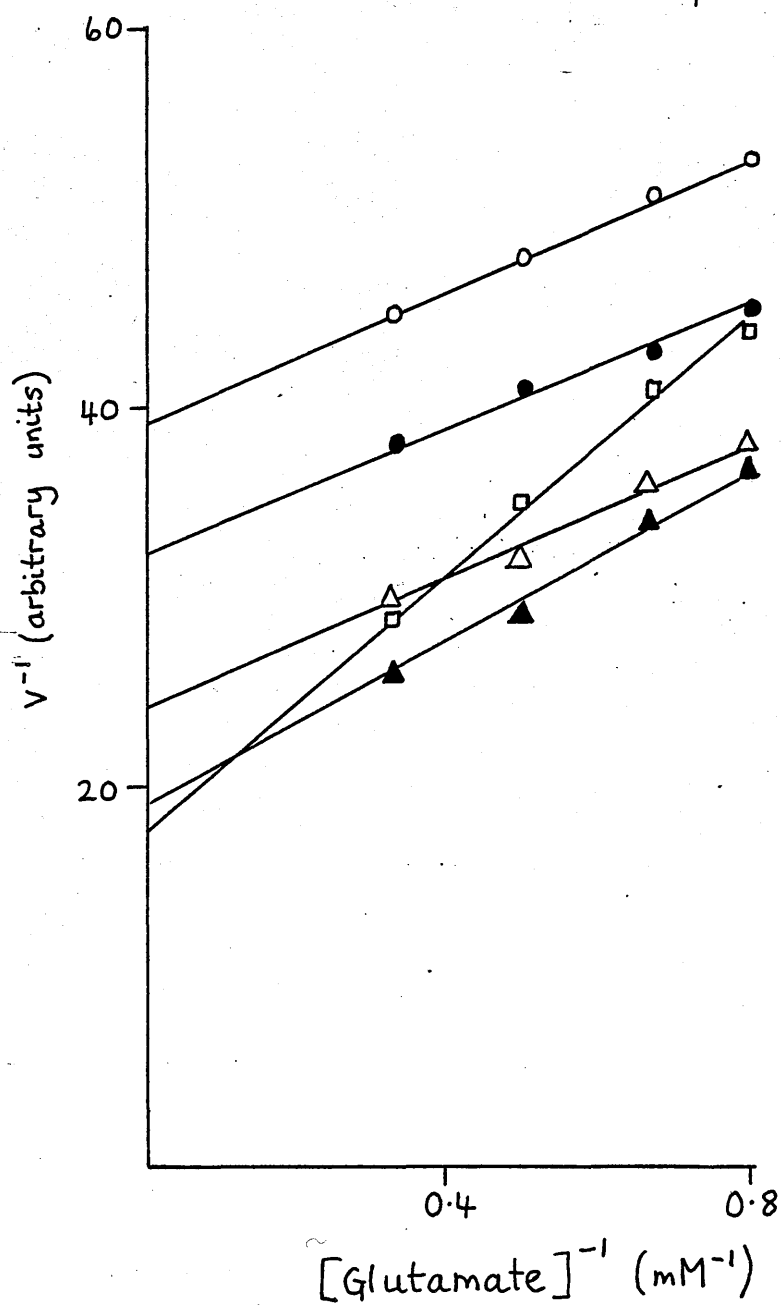
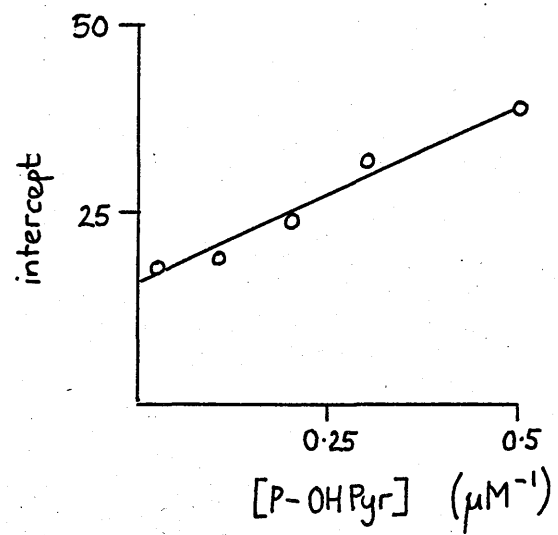
      CACCGTTAA
1081 ----- 1089
      HisGlyEnd
      [361]

```

FIGURE 6.11

Double reciprocal plot of E. coli PSAT.

A double reciprocal plot of initial velocity vs [glutamate] at a series of fixed P-OHPyr concentrations: (\square), 50 μM ; (\blacktriangle), 10 μM ; (\triangle), 5 μM ; (\bullet), 3.3 μM ; (\circ), 2 μM . Inset: replot of intercepts as a function of [P-OHPyr].



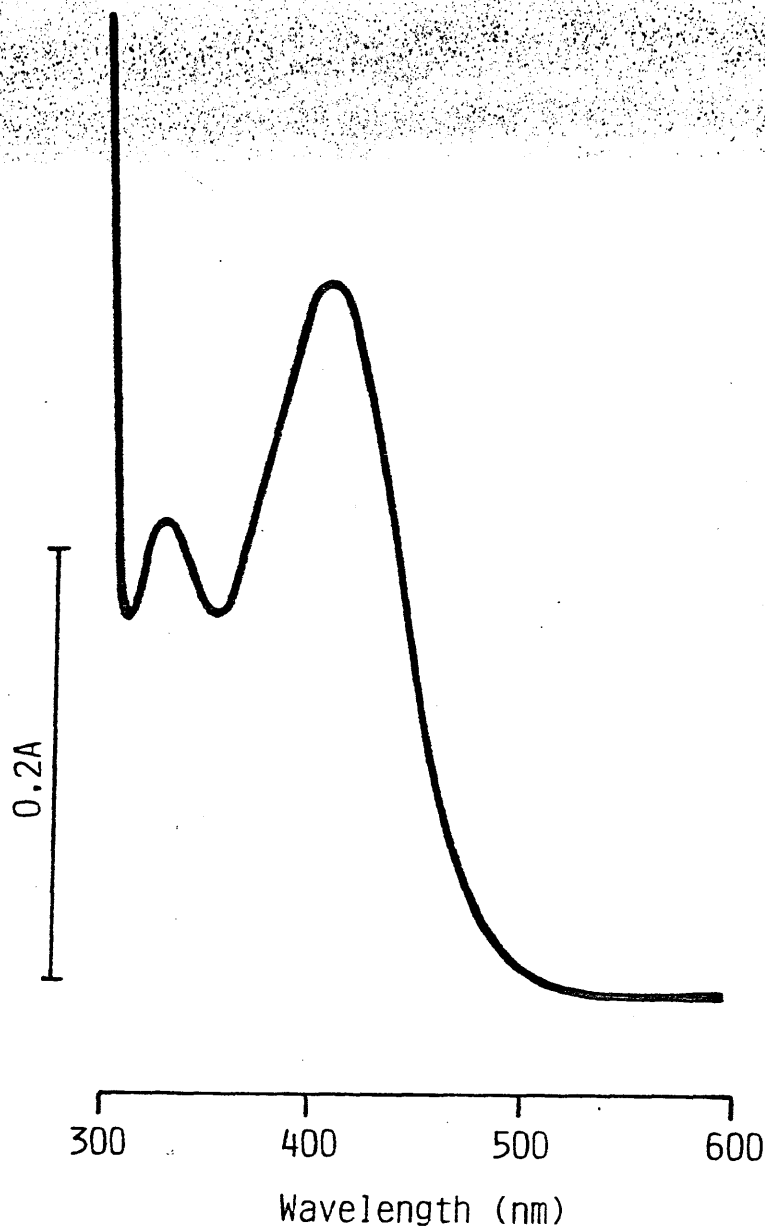


Figure 6.11a The absorption spectrum of *E. coli* PSAT.

The absorption spectrum of PSAT in the wavelength range 300 to 600 nm was obtained using a Pye Unicam SP 8-100 recording spectrophotometer at room temperature. The spectrum shown is of 4.8 mg PSAT in a total volume of 1.0 ml of 50 mM Tris-HCl pH 7.5, 50 mM KCl, 0.4 mM DTT. The bar indicates 0.2 absorbance units.

Absorption maxima at approximately 330 nm and 405 nm were recorded.

FIGURE 6.12

A comparison of the amino acid sequences of E. coli aromatic amino acid AT and chicken cytosolic aspartate AT.

A dot-plot produced by a search for sequence homology (Maizel & Lenk, 1981; Devereux et al, 1984) between the amino acid sequences of E. coli aromatic amino acid AT (M. Edwards, unpublished results) and chicken cytosolic aspartate AT (Shlyapnikov et al, 1979). A dot indicates that a match of two out of three residues occurs between the sequences.

Aspartate Aminotransferase (Chicken)

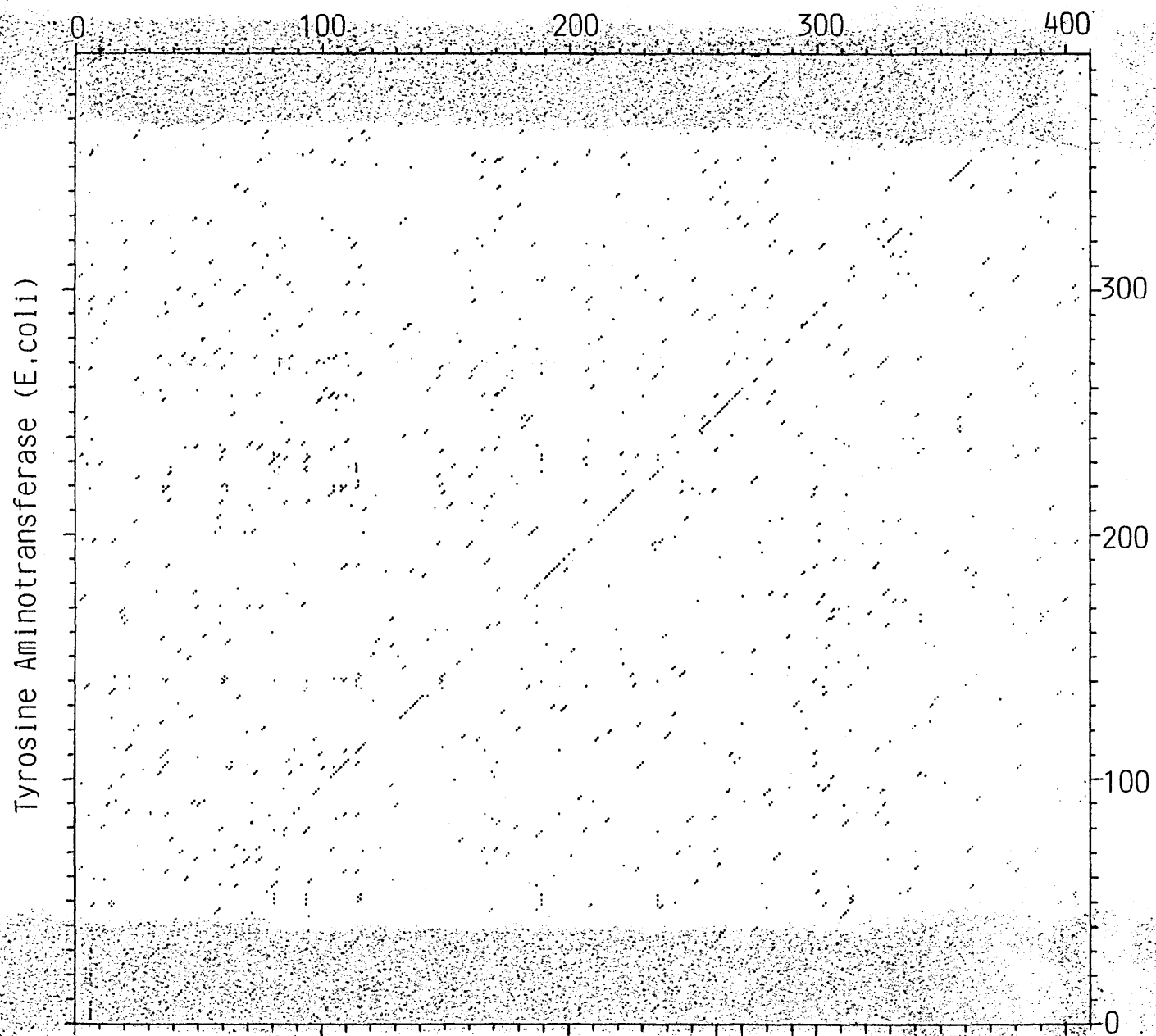
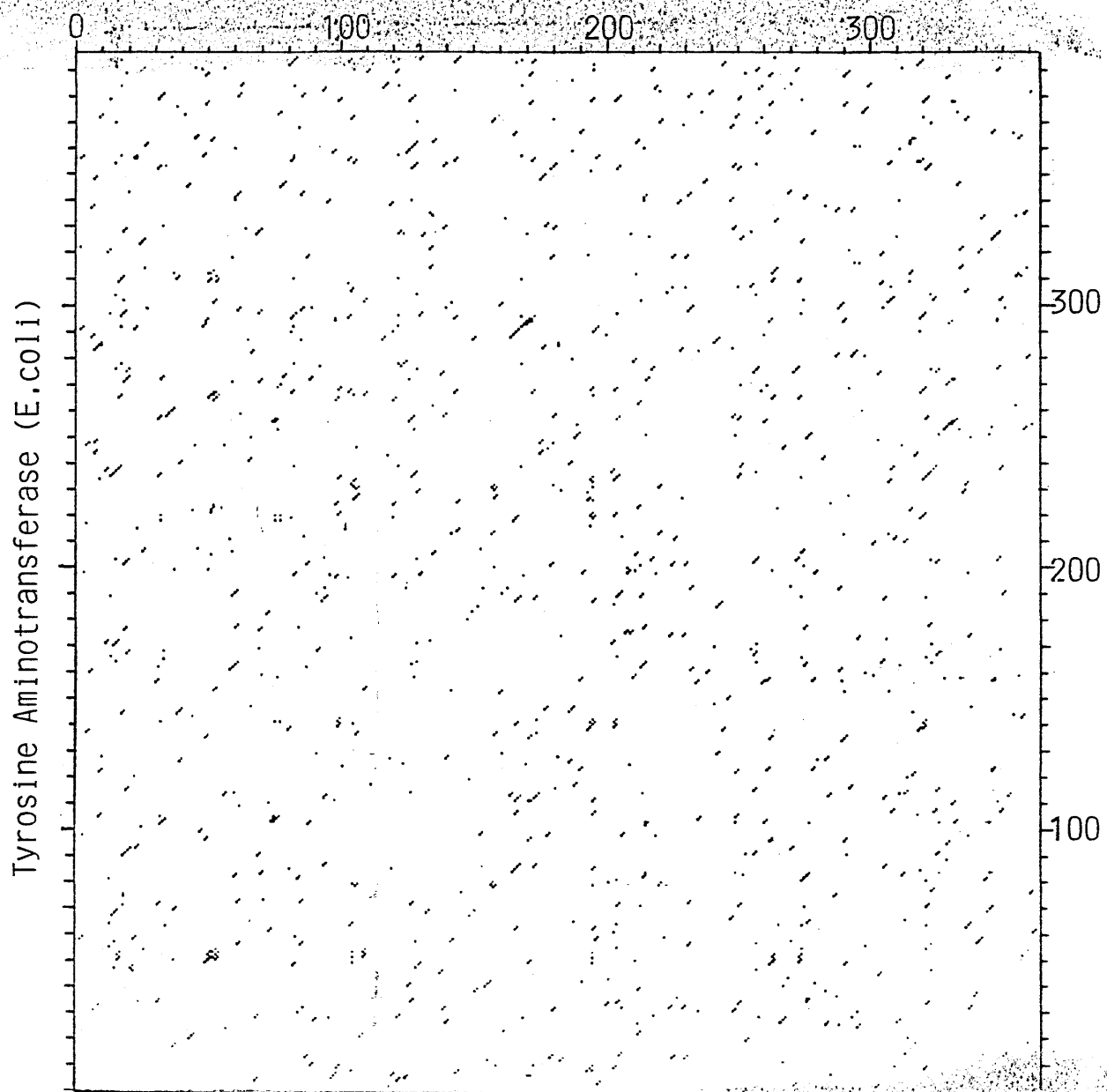


FIGURE 6.13

A comparison of the amino acid sequences of E. coli aromatic amino acid AT and E. coli PSAT.

A dot-plot produced by a search for sequence homology (Maizel & Lenk, 1981; Devereux et al, 1984) between the amino acid sequences of E. coli aromatic amino acid AT (M. Edwards, unpublished results) and E. coli PSAT. A dot indicates that a match of two out of three residues occurs between the sequences.

Phosphoserine Aminotransferase (E.coli)



CHAPTER 7

GENERAL DISCUSSION AND FUTURE PROSPECTS

7.1 EPSP synthase.

A method for the purification of EPSP synthase from E. coli and some properties of the purified enzyme have been described. Milligram quantities of homogeneous enzyme can be readily prepared from the overproducing strain of E. coli. It is likely that this purification procedure could be further "scaled up" in order to obtain even larger amounts of EPSP synthase. The complete amino acid sequence of E. coli EPSP synthase is available from DNA and protein sequencing studies (Duncan et al, 1984b). This combination of protein and DNA studies means that the E. coli enzyme is a good choice of monofunctional EPSP synthase on which to perform structural studies.

Structural studies which can now be considered include:

- (i) The determination of the three-dimensional structure of E. coli EPSP synthase by X-ray crystallography - this would depend on success in obtaining suitable protein crystals;
- (ii) the identification of possible active site residues in the amino acid sequence - preliminary attempts at chemical modification of E. coli EPSP synthase were described in chapter 5.

One aim of structural studies on EPSP synthase must be to determine how glyphosate interacts with the enzyme. It is interesting to note that glyphosate-resistant strains of bacteria have been isolated recently. Glyphosate resistance appears to be due either to overproduction of EPSP synthase (Rogers et al, 1983; Duncan et al, 1984a) or to production of a glyphosate-insensitive EPSP synthase (Comai et al, 1983; Schulz et al, 1984; Sost et al, 1984). The glyphosate resistance of a strain of S. typhimurium has been shown to be/

be due to a single mutation (Pro \rightarrow Ser) in the gene coding for EPSP synthase (D. Stalker, Hiatt, W. & Comai, L., unpublished results). It would be interesting to examine the effects of such mutations on the structural and kinetic properties of EPSP synthase.

The properties of E. coli EPSP synthase described here appear to be similar to those of other recently purified, monofunctional EPSP synthases (Anton et al, 1983; Mousdale & Coggins, 1984a; Steinrucken & Amrhein, 1984a). In particular, the pea seedling EPSP synthase resembles the E. coli enzyme in terms of the purification procedure that can be used, subunit structure, M_r values (Table 3.4) and kinetic parameters with the exception of the greater sensitivity of the P. sativum enzyme to glyphosate inhibition (Mousdale & Coggins, 1984a). The readily obtained E. coli enzyme may therefore be used as a model for the plant enzyme in studies that require large amounts of protein. As yet, only very limited amounts of pea seedling EPSP synthase are available (Mousdale & Coggins, 1984a).

The subcellular localisation of P. sativum EPSP synthase has recently been reported. EPSP synthase and three other shikimate pathway enzyme activities (DAHP synthase and the dehydroquinase/shikimate dehydrogenase complex) appear to be located in chloroplasts (Mousdale & Coggins, 1984b).

Monofunctional E. coli EPSP synthase is somewhat smaller than the polypeptide carrying EPSP synthase activity (subunit M_r 74,000) isolated after limited proteolysis of the N. crassa arom enzyme complex (figure 1.8; Boocock, 1983). It is possible that the M_r 74,000 fragment contains some polypeptide chain that is unnecessary for EPSP synthase activity. It should be noted that the sum of the subunit/

TABLE 7.1

M_r values of the E. coli shikimate pathway enzymes.

Step	Enzyme	M_r value	Reference
2	DHQ synthase	40,000	Frost <u>et al</u> , 1984
3	dehydroquinase	28,000	Duncan, 1984
4	shikimate dehydrogenase	29,000	I.A. Anton, unpublished results
5	shikimate kinase	20,000	Ely & Pittard, 1979
6	EPSP synthase	46,000	Duncan <u>et al</u> , 1984b.

The steps of the shikimate pathway are numbered as in figure 1.1. All M_r values are subunit M_r values with the exception of the M_r of shikimate kinase which was estimated after gel filtration of unpurified enzyme. The M_r values for dehydroquinase, shikimate dehydrogenase and EPSP synthase were calculated from the deduced amino acid sequences from the respective gene sequences. The M_r value for DHQ synthase was obtained by SDS PAGE of purified enzyme.

subunit M_r values of the monofunctional E. coli shikimate pathway enzymes 2 to 6 (figure 1.1) equals 163,000 (Table 7.1). This is similar to the subunit M_r of 165,000 determined for the N. crassa arom enzyme complex (Lumsden & Coggins, 1977). This suggests that the multifunctional arom complex may have been produced by gene fusion events.

7.2 Phosphoserine aminotransferase.

The purification to homogeneity and some properties of PSAT from the overproducing strain of E. coli have been described. Protein chemical studies have demonstrated that the purified enzyme is the product of the E. coli serC gene.

The purification procedure for E. coli PSAT could be improved. In particular, the last step, ion-exchange chromatography on the mono-Q column was found to be somewhat time-consuming due to the fact that the phenyl-Sepharose pool had to be chromatographed in batches because of the limited capacity of this column. The preliminary finding that E. coli PSAT is a dimeric enzyme requires further investigation, for example, cross-linking experiments should be carried out in order to confirm this subunit structure.

The kinetic properties of E. coli PSAT could be studied further, especially with regard to the conversion of serine to OHPyr by this enzyme. However, the role of PSAT in pyridoxine biosynthesis is likely to become clear only after the biosynthetic pathway for pyridoxine has been elucidated.

As with EPSP synthase, large amounts of E. coli PSAT can be readily purified and the complete amino acid sequence of this enzyme is known (Duncan, 1984). Structural studies on E. coli PSAT are therefore/

therefore possible. One functional residue of an aminotransferase that may be readily identified is the lysine residue to which pyridoxal phosphate is covalently bound via an imine linkage. It should be possible to identify this residue in E. coli PSAT by standard protein chemical techniques after reduction of the isolated enzyme with [³H] NaBH₄. Given the lack of obvious sequence homology between E. coli PSAT and other aminotransferases (section 6.7), it would be interesting to compare the sequence around the PLP-binding lysine in PSAT with corresponding sequences in other aminotransferases, e.g. in aspartate aminotransferases where this lysine residue has been identified (Kondo et al, 1984).

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