ANTIBODIES AGAINST THE SHIKIMATE PATHWAY ENZYMES

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This thesis is dedicated

to

my parents

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Abbreviations

The abbreviations used in this thesis are as set out in 'Instructions to Authors', Biochemical Journal (1989) 257, 1-21, except the following:

DAHP	3-deoxy-D-arabinoheptulosonate 7-phosphate
DCPIP	2,6-dichlorophenolindophenol
DTT	dithiothreitol
EPSP	5-enolpyruvylshikimate-3-phosphate
HRP	horseradish peroxidase
K	kilodalton
PMS	phenazine methosulphate
PMSF	phenylmethanesulphonyl fluoride
PQQ	pyrrolo-quinoline quinone
SDS PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylene diamine

ABSTRACT

1. Antibodies have been raised against the Neurospora crassa arom complex, which is a pentafunctional enzyme that catalyses steps 2 to 6 in the shikimate pathway, and against the monofunctional enzymes from Escherichia coli, which catalyse the same reactions. In the case of the E. coli enzymes, antibodies were raised against both native and denatured proteins.

2. Antibodies raised against the E. coli enzymes were tested for potency and specificity by enzyme inhibition assay, immunoprecipitation assay with protein A, ELISA and immunoblots against both native and denatured enzymes. Antibodies raised against a native protein were more active against that form than against the denatured form of the protein and vice versa. No cross reaction was detected between an antibody raised against any single enzyme and any of the other enzymes of the pathway.

3. The anti-N. crassa arom complex inhibits only the activities of 3-dehydroquinate synthase (E1) and 5-enolpyruvylshikimate 3-phosphate synthase (E5) in the complex and does not affect the other three activities. This suggests that the active sites of E1 and E5 are close to the surface of the arom complex and represent important surface epitopes while the active sites of 3-dehydroquinase (E2), shikimate dehydrogenase (E3) and shikimate kinase (E4) are buried.

4. Partial proteolysis of the arom complex yields two major fragments one of M_r 110K that contains the E1 and E5 activities

and the other M_r 68K which contains the E2 and E3 activities, and a minor fragment, of M_r 74K that contains the E5 activity (Smith & Coggins, 1983; Boocock, 1983). All of these fragments bind to the anti-N. crassa antibody. When enzyme activities are measured results similar to these observed with the complete complex are obtained. If the arom complex and arom proteolysate are immunoprecipitated by anti-arom, all activities are lost from the supernatant.

5. The anti-N. crassa arom complex cross reacts with the yeast arom complex by immunoblotting suggesting a greater than 60% sequence homology between these proteins. Since arom complex from yeast and Aspergillus nidulans are known to contain extensive primary sequence homology we would conclude that these three arom complexes are very similar.

6. No cross reaction could be detected between any of the antibodies raised against the shikimate pathway enzymes from E. coli and the N. crassa arom complex. This is consistent with the low levels of sequence homology known to exist between these proteins (yeast arom complex and equivalent E. coli enzymes have less than 30% overall sequence homology).

7. The anti-E. coli E3 binds and inhibits E3 from E. coli K12 (wild type), E. coli ML308, Salmonella typhimurium, Erwinia carotovora but can bind to only 50% of E3 from Acinetobacter calcoaceticus. Antibody titre studies in these gram negative microorganisms indicated considerable sequence homologies between these enzymes with E. coli K12 > E. coli ML308 > S. typhimurium >

E. carotovora > *A. calcoaceticus*. It was concluded that the E3 gene from these organisms had evolved from the same primitive ancestor. The results with *A. calcoaceticus* E3 suggest that there are two isoenzymes in this species.

No cross reactions were observed with E3 from gram positive bacteria, fungi and a plant species.

8. E3 has been purified from *A. calcoaceticus* and obtained in a form which gives a single band on SDS PAGE. The purified *A. calcoaceticus* E3 is a monomer which a subunit M_r of 31,000 and uses $NADP^+$ as a cofactor. A simple dye-linked assay procedure has been developed to assay E3. Unlike the *E. coli* enzyme, E3 for *A. calcoaceticus* shows significant levels of activities with the dye as an electron acceptor at pH 7.5. In addition the enzyme from *A. calcoaceticus* can use NAD^+ as a cofactor to a greater extent than the *E. coli* enzyme at pH 10.6.

9. Based on immunoprecipitation results, the presence of two E3 isoenzymes was proposed. Additional supporting evidence for isoenzymes was provided by polyacrylamide gel electrophoresis under non denaturing condition followed by activity stain and by immunoblotting after non denaturing gel electrophoresis which showed that anti-*E. coli* E3 antibody can bind only to the one of the postulated isoenzymes. Attempts to separate the two isoenzymes by immunoaffinity chromatography and isoelectric focusing electrophoresis were unsuccessful.

1 Introduction

1.1 Introduction to the shikimate pathway

1.1.1 The shikimate or aromatic biosynthetic pathway

In plants and microorganisms the biosynthesis of aromatic compounds proceeds via the shikimate pathway (Haslam, 1974; Weiss & Edwards, 1980). Other eukaryotic organisms lack this biosynthetic capability and require a dietary intake of ~~at least two of~~ the three aromatic amino acids tyrosine, phenylalanine and tryptophan (Davis, 1955; Sprinson, 1960).

The shikimate pathway, which contains seven steps, converts the intermediates of carbohydrate metabolism, erythrose-4-phosphate and phosphoenolpyruvate, via shikimate to chorismate (Fig. 1.1). Chorismate is probably one of the most versatile chemical intermediates in primary metabolism (Haslam, 1974; Dewick, 1989) (Fig. 1.2). Many pathways diverge from chorismate to yield not only the three aromatic amino acids but also ubiquinone, plastoquinone, folic acid and vitamins E and K. In plants phenylalanine is a precursor of the lignins which provide much of the tensile strength of woody tissues (Boudet et al., 1985). In recent years a number of new aromatic compounds derived from the shikimate pathway have been identified including a number of microbial antibiotics e.g. streptonigrin (Gould & Erickson, 1988), sarubicin A (Hillis & Gould, 1985), geldanamycin (Rinehart et al., 1982), pactamycin (Rinehart et al., 1981), mitomycin (Anderson et al., 1980), ansamycin and maytansinoid (Kibby et al., 1980).

In this thesis the work presented is exclusively on the five enzymes 3-dehydroquinate synthase, 3-dehydroquinate dehydratase (3-dehydroquinase), shikimate dehydrogenase, shikimate kinase and

Figure 1.1 The reactions of the shikimate pathway

The numbers refer to the enzymes of the pathway:

(1) 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase; EO (EC 4.1.2.15)

(2) 3-dehydroquinate synthase; E1 (EC 4.6.1.3)

(3) 3-dehydroquinase; E2 (EC 4.2.1.10, alternative name 3-dehydroquinate dehydratase)

(4) shikimate dehydrogenase; E3 (EC 1.1.1.25)

(5) shikimate kinase; E4 (EC 2.7.1.71)

(6) 5-enolpyruvylshikimate 3-phosphate synthase; E5 (EC 2.5.1.19 alternative name 3-phosphoshikimate 1-carboxyvinyltransferase)

(7) chorismate synthase; E6 (EC 4.6.1.4).

The arom multifunctional enzyme catalyses the reactions numbered 2 to 6 in the above scheme.

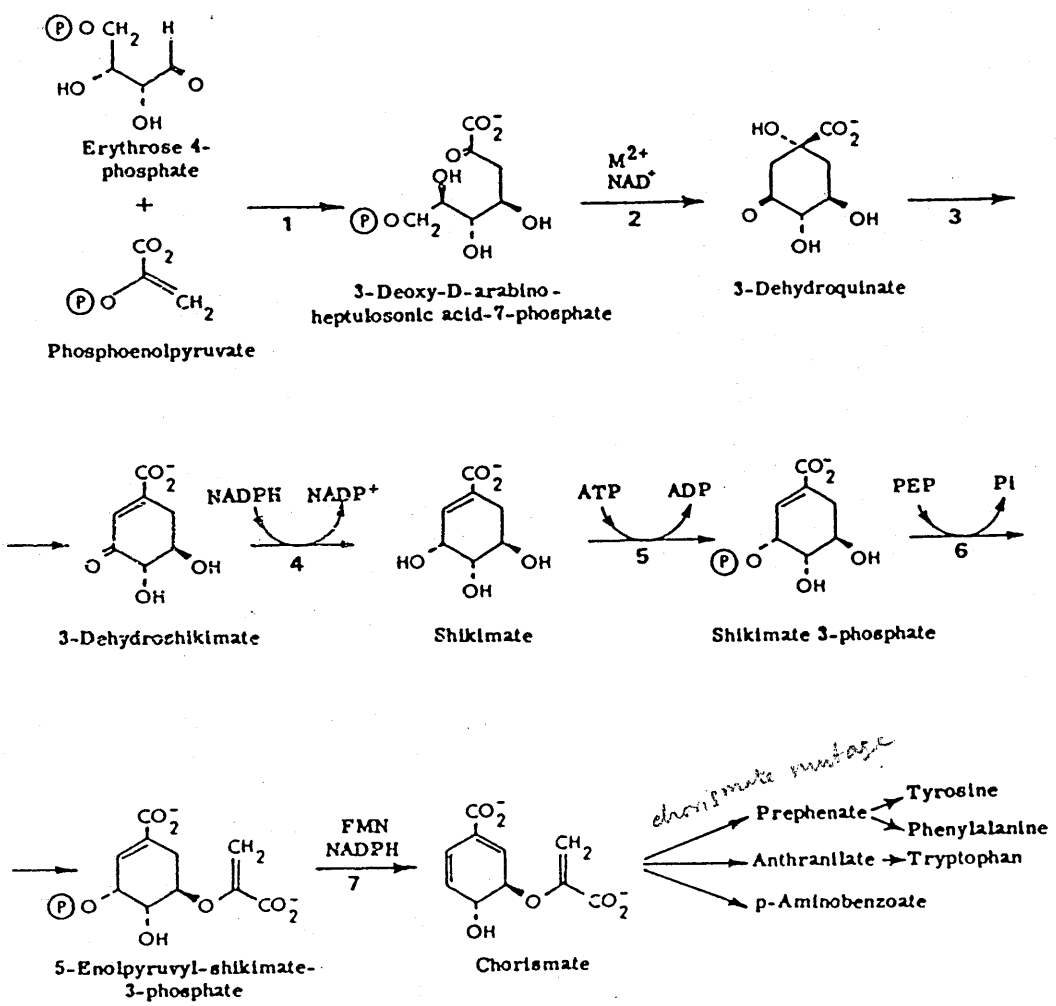
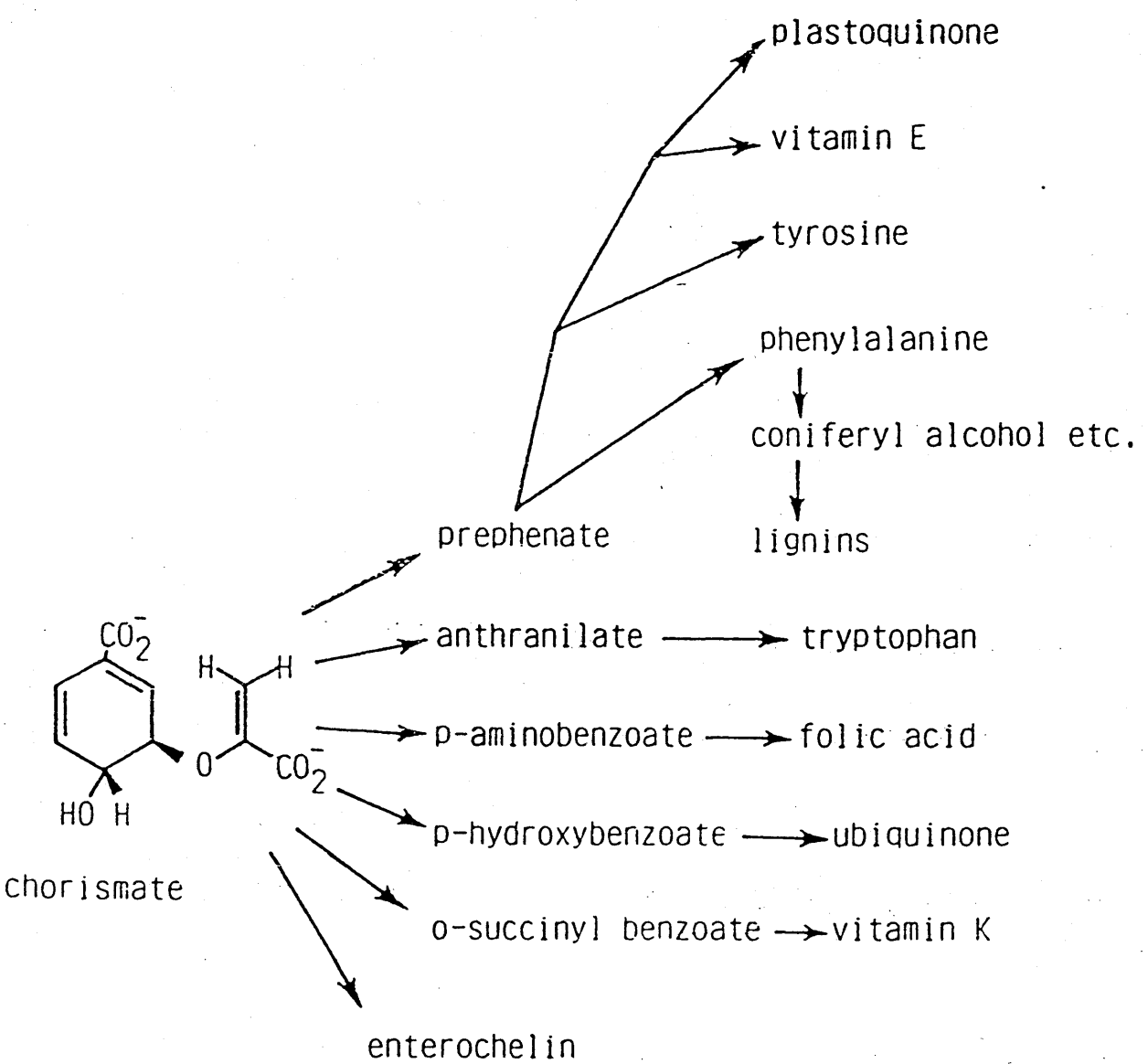


Figure 1.2 Some of the metabolic products derived from chorismic acid

Some of these products are found in both plants and microorganisms while others are found only in plants or only in microorganisms.



5-enolpyruvyl shikimate 3-phosphate synthase (Fig. 1.1). These enzymes catalyse steps 2 to 6 in the pathway respectively and in this thesis are designated as E1, E2, E3, E4 and E5. 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase) which catalyses the first step is designated E0 and chorismate synthase which catalyses the seventh step E6.

1.1.2 The organisation of the genes and enzymes in the shikimate pathway

The organisation of the genes and enzymes of this pathway in different organisms (prokaryotic and eukaryotic) is found to differ markedly, though the seven reactions of the pathway are the same in all organisms so far studied.

In the gram negative bacteria: Escherichia coli and Salmonella typhimurium (Bachmann, 1983, Sanderson & Roth, 1983) the genes encoding the shikimate pathway enzymes (the aro genes) are widely scattered around the chromosome (Table 1.1) and they encode separate enzymes with monofunctional polypeptide chains (Berlyn & Giles, 1969; Berlyn & Giles, 1973; Chaudhuri & Coggins, 1985; Coggins et al., 1985; Coggins et al., 1987a).

In the gram positive bacterium: Bacillus subtilis (Nakatsukaya & Nester, 1972; Henner & Hoch, 1980) there is some clustering of the aro genes (Table 1.1) and the enzyme organisation is very much more complex than in E. coli. The enzymes dehydroquinate synthase and chorismate synthase are found in a trifunctional multienzyme complex along with a flavin reductase (diaphorase) activity (Hasan & Nester, 1978a,b,c). A bifunctional polypeptide has also been identified, catalysing the first reaction of the shikimate pathway (DAHP synthase) and a later reaction in aromatic biosynthesis (chorismate mutase). This bifunctional

polypeptide occurs in non-covalent association with shikimate kinase (Huang et al., 1974a,b, 1975).

In lower eukaryotic organisms for example Neurospora crassa (Giles et al., 1967a; Catcheside et al., 1985) Aspergillus nidulans (Ahmed & Giles, 1969; Charles et al., 1986), Saccharomyces cerevisiae (de Leeuw, 1967; Larimer et al., 1983; Duncan et al., 1987), Schizosaccharomyces pombe (Strauss, 1979; Nakanishi & Yamamoto, 1984) and Euglena gracilis (Berlyn et al., 1970) (Table 1.1) the genes encoding steps 2 to 6 on the pathway are found to be clustered and in most of these cases it has been established that a single pentafunctional polypeptide chain, known as the arom complex or the arom multifunctional enzyme, catalyses all five reactions (Lumsden & Coggins, 1977; Gaertner & Cole, 1977; Boocock, 1983; Lambert et al., 1985; Coggins et al., 1987b; Duncan et al., 1987). This polypeptide contains five functional domains which show strong sequence homology with each of the corresponding monofunctional E. coli enzymes (Fig. 1.3) (Duncan et al., 1987; Coggins et al., 1987a; Charles et al., 1985, 1986)(see Appendix).

In plants five of the seven shikimate pathway enzymes are separable (Coggins, 1986) but two, 3-dehydroquinase and shikimate dehydrogenase, copurify and have been shown to occur as a bifunctional polypeptide (Table 1.1) (Polley, 1978; Koshiba, 1978; Coggins, 1986; Mousdale et al., 1987) (see section 1.2.8.2 below).

1.2 The individual enzymes of the shikimate pathway

1.2.1 3-Deoxy-D-arabino-heptulosonic acid 7-phosphate synthase (DAHP synthase)

DAHP synthase (EO, EC 4.1.2.15) catalyses the first committed step of the shikimate pathway which is the condensation of phosphoenolpyruvate and erythrose-4-phosphate to form the seven carbon compound DAHP (Fig. 1.1).

Table 1.1 Gene and enzyme organisation of the shikimate pathway in different organisms

Organism (gene organisation)	Enzyme organisation
Bacteria	
a) *E. coli* (no gene clustering)	separable
b) *B. subtilis* (some gene clustering)	trifunctional multienzyme complex (E1+E6+diaphorase) and bifunctional polypeptide or trifunctional complex [(EO/chorismate mutase)+E4]
Lower eukaryotes (*arom* cluster gene)	pentafunctional polypeptide (E1/E2/E3/E4/E5)
Plants	bifunctional polypeptide (E2/E3)

\+ = enzyme association
/ = enzyme resides on the same polypeptide

Figure 1.3 Homologies between the S. cerevisiae arom multifunctional enzyme and the five corresponding E. coli enzymes

The S. cerevisiae arom sequence is represented by the lower stippled box and the five corresponding E. coli monofunctional enzymes by the upper boxes. Each box represents a complete protein sequence. The E. coli enzymes are labelled with the letters of the aro genes which encode them:

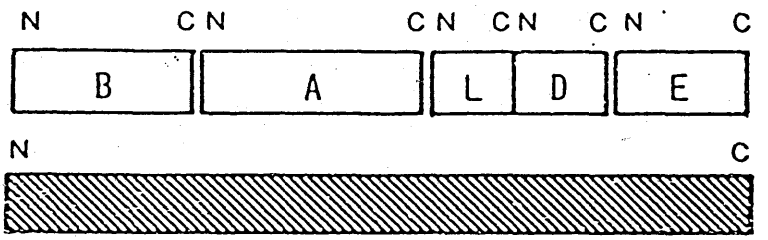
B, 3-dehydroquinate synthase;

A, EPSP synthase

L, shikimate kinase;

D, 3-dehydroquinase;

E, shikimate dehydrogenase.



Wild type E. coli contains three DAHP synthase isoenzymes: phenylalanine-sensitive DAHP synthase (Phe), tyrosine-sensitive DAHP synthase (Tyr) and tryptophan-sensitive DAHP synthase (Trp) (Doy & Brown, 1965), which are encoded by the unlinked genes aroG, aroF and aroH respectively. The DAHP synthases of E. coli are feedback inhibited by the aromatic end products and their genes are transcriptionally controlled by the tyrR (aroF and aroG) and the trpR (aroH) repressors. The three isoenzymes have all been purified to homogeneity (Hermann, 1983).

N. crassa, like E. coli, has three DAHP isoenzymes that are each inhibited by one of the three aromatic amino acids. The tryptophan-sensitive DAHP synthase has been purified to homogeneity (Nimmo & Coggins, 1981).

B. subtilis contains only a single DAHP synthase which occurs with chorismate mutase and shikimate kinase as described in 1.1.2 above.

1.2.2 Dehydroquinate synthase (DHQ synthase)

DHQ synthase (El, EC 4.6.1.3) catalyses the ring-closure of DAHP to form the saturated six-membered ring of dehydroquinate (Fig. 1.1) which is the precursor of the aryl group in the three aromatic amino acids and in the other primary and secondary metabolites derived from the pathway.

The E. coli enzyme was first characterised by Sprinson and his collaborators (Srinivasan et al., 1963; Maitra & Sprinson, 1978). The enzyme requires a divalent transition metal cation and catalytic amounts of NAD^+ for activity. The cloning of the E. coli aroB gene encoding DHQ synthase has allowed the construction of an overproducing strain for DHQ synthase (Duncan & Coggins, 1983; Frost et al., 1984; Millar & Coggins, 1986). The enzyme was first purified to homogeneity in Knowles' laboratory (Frost et al., 1984).

The B. subtilis enzyme, unlike the E. coli enzyme forms a trifunctional complex with chorismate synthase and a flavin reductase (diaphorase) as described in 1.1.2 above. The association with chorismate synthase is required for enzyme activity (Hasan & Nester, 1978c).

The DHQ synthase of N. crassa is a part of the pentafunctional arom complex. Like the E. coli enzyme it requires both NAD^+ and a metal cation (Zn^{2+}) for activity (Lambert et al., 1985).

1.2.3 Dehydroquinase

Dehydroquinase (E2, EC 4.2.1.10) catalyses the stereospecific dehydration of dehydroquinate to form dehydroshikimate (Fig. 1.1). The cloning of the gene (aroD) for this enzyme from E. coli (Kinghorn et al., 1981) and the construction of a dehydroquinase overproducing strain (Duncan et al., 1986) has facilitated the purification of this enzyme to homogeneity (Chaudhuri et al., 1986). It is a dimeric enzyme (Duncan et al., 1986) unlike the other four monofunctional E. coli enzymes with activities corresponding to the fungal arom protein which are monomeric (Frost et al., 1984a; Anton & Coggins, 1988; Millar et al., 1986a; Duncan et al., 1984a).

Dehydroquinase has been purified to homogeneity from peas (Pisum sativum) (Mousdale et al., 1987) and mung beans (Phaseolus mungo) (Koshiba, 1978). In both these higher plant species as well as in the moss (Physcomitrella patens) (Polley, 1978) the enzyme occurs on a single bifunctional polypeptide chain with shikimate dehydrogenase (section 1.2.4).

The biosynthetic dehydroquinases of N. crassa and E. coli are mechanistically similar. Both enzymes work via formation of a covalent intermediate with the substrate dehydroquinate. The keto

group of the substrate forms a Schiffs base (imine) with the epsilon amino group of a lysine side chain of the enzyme. This intermediate can be trapped by reduction with sodium borohydride (Butler et al., 1974; Chaudhuri et al., 1986).

1.2.4 Shikimate dehydrogenase

Shikimate dehydrogenase or dehydroshikimate reductase (E3, EC 1.1.1.25) catalyses the reduction of dehydroshikimate to shikimate (Fig. 1.1). It is an $NADP^+$ specific dehydrogenase and catalyses the transfer of a hydride ion from the A-side of the pyridine ring of NADPH (Dansette & Azerad, 1974). The E. coli shikimate dehydrogenase has been purified to homogeneity and is monomeric (Chaudhuri & Coggins, 1985). It is unusual in being the only monomeric, biosynthetic dehydrogenase reported to date (Anton & Coggins, 1988). The gene (aroE) which encodes shikimate dehydrogenase has been cloned from and overexpressed in E. coli (Anton & Coggins, 1988).

1.2.5 Shikimate kinase

Shikimate kinase (E4, EC 2.7.1.71) phosphorylates shikimate to give shikimate-3-phosphate (Fig. 1.1). Both E. coli (Berlyn & Giles, 1969) and S. typhimurium (Morell & Springson, 1968) contain two isoenzymes with this activity; shikimate kinase I and II. As already mentioned (1.2.1 above) in B. subtilis a single shikimate kinase occurs as a multienzyme complex with a bifunctional DAHP synthase and chorismate mutase; the kinase component has been purified to homogeneity and shown to be active only in the complex (Huang et al., 1975).

The E. coli shikimate kinase isoenzymes are differentially expressed. Kinase I is expressed constitutively while kinase II is transcriptionally regulated by the tyrR repressor (Ely & Pittard, 1979). Both of these isoenzymes coelute in gel filtration chromatography but can be separated by ion exchange chromatography (Ely & Pittard, 1979).

The E. coli aroL gene which encodes the tyrR-regulated shikimate kinase II has been cloned and an overproducing strain constructed (Millar et al., 1986a).

N. crassa has a single shikimate kinase as a part of the pentafunctional arom complex (see section 1.2.8). This activity is very readily inactivated by limited proteolysis with trypsin (Smith & Coggins, 1983; Boocock, 1983).

1.2.6 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase

EPSP synthase (E5, EC 2.5.1.19) converts shikimate 3-phosphate to EPSP (Fig. 1.1). The E. coli and P. sativum EPSP synthases have been purified and shown to be monomeric (Lewendon & Coggins, 1983; Mousdale & Coggins, 1984). The E. coli aroA gene encoding EPSP synthase has been cloned and used to construct an overproducing strain (Duncan & Coggins, 1983; Duncan et al., 1984b).

The discovery that glyphosate (N-phosphonomethylglycine), a potent, broad spectrum, post-emergence herbicide (sold as 'Round Up'), acts by inhibiting EPSP synthase (Steinrucken & Amrhein, 1980) has focussed a great deal of attention on this enzyme and its mechanism of action (Coggins, 1986; Amrhein, 1986; Kishore & Shah, 1988).

1.2.7 Chorismate synthase

Chorismate synthase (E6, EC 4.6.1.4) catalyses the seventh and final reaction in the shikimate pathway. This involves the elimination of orthophosphate from EPSP and introduces the second double bond of the aromatic ring to form chorismate (Fig. 1.1). All chorismate synthases studied so far require reduced flavin, either $FMNH_2$ or $FADH_2$, for activity (Morell et al., 1967; Boocock, 1983; Mousdale & Coggins, 1986). This reduced flavin is provided by a flavin reductase (Hasan & Nester, 1978a,b). The cloning and overexpression of the *E. coli aroC* gene encoding chorismate synthase has been achieved and chorismate synthase has been purified to homogeneity from a suitable overproducing strain (Millar et al., 1986b; White et al., 1988).

In *N. crassa* the chorismate synthase and flavin reductase (diaphorase) activities both reside on the same polypeptide chain (Welch et al., 1974; White et al., 1988). In *B. subtilis* the chorismate synthase subunit is part of a trifunctional complex also containing DHQ synthase and NADPH-dependent flavin reductase activities already described (Hasan & Nester, 1978a,b,c) (see section 1.2.1). The *E. coli* chorismate synthase is less specific in its reduced flavin requirements accepting $FADH_2$ or NADH but is sensitive to molecular oxygen and up until now has only been assayed under a N_2 atmosphere (Morell et al., 1967; White et al., 1987).

1.2.8 The multifunctional enzyme complexes

1.2.8.1 The arom complex

In fungi, as described in section 1.1.2 above, the five enzymes at the centre of the shikimate pathway (those catalysing step 2 to 6 in Figure 1.1) occur on a single, pentafunctional,

polypeptide chain (Lumsden & Coggins, 1977; Gaertner & Cole, 1977; Lambert et al., 1985; Duncan et al., 1987; Charles et al., 1985, 1986). Experiments involving genetics, limited proteolysis and chemical modification (reviewed in Coggins & Boocock, 1986) suggested that the N. crassa arom polypeptide consists of five essentially independent functional domains. This has now been confirmed by sequencing studies on the related A. nidulans and S. cerevisiae arom polypeptides which clearly establish that the arom polypeptides consist of five domains each of which is homologous to one of the corresponding monofunctional E. coli enzymes (Duncan et al., 1987; Charles et al., 1985, 1986) (see Appendix)

1.2.8.2 The bifunctional dehydroquinase/shikimate dehydrogenase polypeptide

In higher plant species, for example pea and mung bean, it has been shown that shikimate dehydrogenase and dehydroquinase occur as a single bifunctional polypeptide chain of M_r 60,000 (Mousdale et al., 1987; Koshiba, 1978). The co-purification of these two enzymes has also been demonstrated in other higher plant species (Boudet & Lecussan, 1974). This M_r of 60,000 is approximately the sum of the subunit M_r's of the two corresponding E. coli enzymes and of the corresponding domains located at the C-terminal end of the fungal arom multifunctional enzymes (Coggins et al., 1987a). Thus, it appears that the higher plant bifunctional enzymes represent an intermediate state of organisation in which two of the "bacterial" functional domains have become linked compared with the five that are linked in fungi. In this regard it is interesting to note that a functional C-terminal fragment of the N. crassa arom polypeptide carrying both dehydroquinase and shikimate dehydrogenase activity has been isolated following limited proteolysis (Smith & Coggins, 1983; Boocock, 1983; Coggins & Boocock, 1986).

In young plant tissue the bulk of the dehydroquinase/ shikimate dehydrogenase activity, in common with the other shikimate pathway activities, is chloroplastidic (Mousdale & Coggins, 1985; Mousdale et al., 1987). There is also an increasing amount of evidence that in many plant species there are two or more isoenzymes of dehydroquinase/shikimate dehydrogenase (Rothe et al., 1983; Fiedler & Schultz, 1985; Jensen, 1986; Wendel et al., 1988; Benedettelli & Hart, 1988). It has also been suggested that, at least in some species, one of the isoenzymes may be non-plastidic (Rothe et al., 1983; Mousdale et al., 1987; Wendel et al., 1988).

1.3 Introduction to the quinate pathway

1.3.1 The quinate catabolic pathway

Some organisms such as N. crassa (Ahmed & Giles, 1969) and Acinetobacter calcoaceticus (Canovas & Stanier, 1967) can readily degrade quinate, a compound which is structurally related to shikimate (Fig. 1.4), and which is found at high levels in many plant species (Boudet et al., 1985). Organisms which have the quinate pathway enzymes (see Fig. 1.5) can use quinate as a major source of carbon energy. Metabolism of quinate occurs via the aromatic intermediate, protocatechuate, which is subsequently converted into succinate and acetyl CoA via the β-ketoadipate pathway. The conversion of quinate into protocatechuate is carried out through the hydroaromatic pathway (Fig. 1.5). Clear relationships exist between the quinate pathway and the shikimate pathway. Three intermediates dehydroquinate, dehydroshikimate and shikimate and two enzymic reactions, shikimate dehydrogenase and dehydroquinase are shared by both pathways (Tresguerres et al., 1970a,b; Ingledew et al., 1971). In fungi the quinate pathway

Figure 1.4 Quinic acid and related compounds

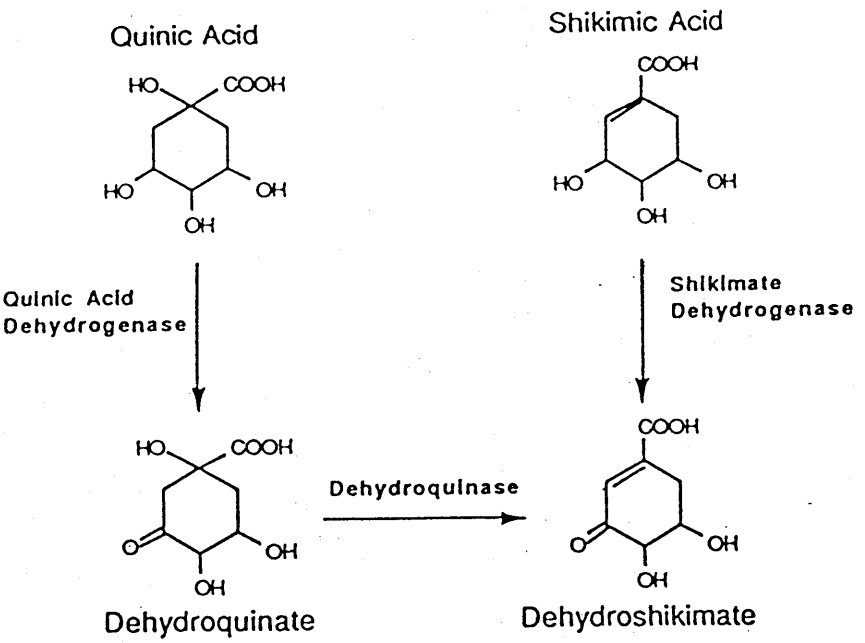
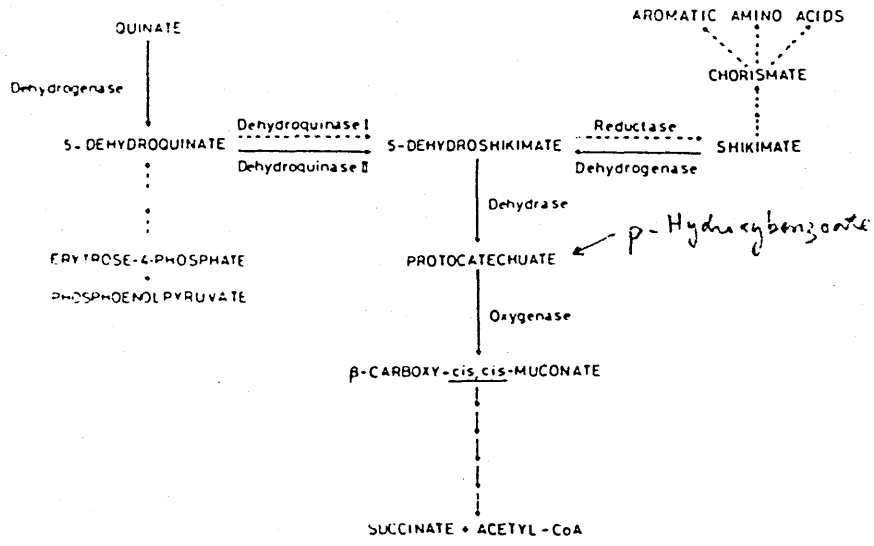


Figure 1.5 The reactions of the hydroaromatic pathway



A diagram to illustrate the conversion of quinate and shikimate into protocatechuate through the hydroaromatic pathway (full arrows). The metabolic interrelationships between this catabolic route and the aromatic biosynthetic pathway (dotted arrows) are also represented.

enzymes are inducible and there is the possibility of competition between the two pathways for intermediates. Giles has suggested that this competition for substrates would be alleviated if the biosynthetic intermediates were channelled within the arom complex (Giles et al., 1967a). In A. calcoaceticus there is a feedback mechanism involving protocatechuate, the last intermediate product of the pathway, which inhibits dehydroshikimate dehydrase (Tresguerres et al., 1972; Berlyn & Giles, 1973).

It is known that dehydroshikimate reductase or biosynthetic shikimate dehydrogenase, the enzyme responsible for the biosynthesis of shikimate (Yaniv & Gilvarg, 1955) has no role in its catabolism via the quinate pathway although it is able to oxidise this substrate 'in vitro' (Tresguerres et al., 1972). The catabolic function is performed by a dehydrogenase which also catalyses the oxidation of quinate (Tresguerres et al., 1970b, 1972; Ingledew et al., 1971). In fungi (Ahmed & Giles, 1969) there are two distinct dehydroquinases: one is an inducible catabolic enzyme which is involved in quinate utilisation, while the second is a distinct biosynthetic isoenzyme (part of the arom complex) which is involved in the biosynthesis of shikimate.

1.3.2 The organisation and regulation of the genes and enzymes of the quinate pathway

1.3.2.1 Organisation and regulation in fungi

In N. crassa the conversion of quinate and shikimate to protocatechuate is catalysed by three inducible enzymes which are the products of qa gene cluster (Giles et al., 1967b; Giles et al., 1985). Besides the three enzyme genes this cluster also contains two genes which regulate the inducible expression of the enzymes (Chaleff, 1974; Huiet, 1984).

The regulation of the three qa structural genes take place at the level of transcription (Patel et al., 1981). Huiet (1984) has shown that two regulatory genes, qa-1S and qa-1F encode a repressor and activator protein respectively, both of which are involved in the transcriptional control of qa gene expression.

The first of the inducible quinate pathway enzymes is the bifunctional quinate (shikimate) dehydrogenase (encoded by qa3, EC 1.1.1.24) which catalyses the NAD^+-dependent oxidation of quinate to dehydroquinate. This has been purified to homogeneity and is a monomer of M_r 41,000, with a single binding site for both substrate (Barea & Giles, 1978). The second enzyme, the catabolic dehydroquinase (encoded by qa2, EC 4.2.1.10) has also been purified to homogeneity and is a dodecamer M_r 20,000 (Chaudhuri & Coggins, 1982). The third enzyme dehydroshikimate dehydrase (encoded by qa4) has not yet been purified and characterised.

The inducible quinate pathway of Aspergillus nidulans is very similar to that found in N. crassa (Ahmed & Giles, 1969; da Silva et al., 1986).

1.3.2.2 Organisation and regulation in bacteria

The quinate pathway in A. calcoaceticus has been characterised more extensively than in other bacteria. As in N. crassa, there are two dehydroquinase isoenzymes but these differ from the N. crassa enzymes in thermostability (Ingledew et al., 1971; Berlyn & Giles, 1973). In A. calcoaceticus the inducible catabolic dehydroquinase is heat labile while the constitutive biosynthetic dehydroquinase is stable at 70°C for 10 min (Ingledew et al., 1971; Berlyn & Giles, 1973). The average M_r values of the degradative and biosynthetic isoenzymes based on sucrose gradients were 41,000 and 125,000 respectively (Berlyn & Giles,

1973). The other two enzymes involved in quinate breakdown are quinate dehydrogenase (dye-linked hydroaromatic dehydrogenase) which, as in N. crassa has shikimate dehydrogenase activity and dehydroshikimate dehydrase (Tresguerres et al., 1970b; Ingledew et al., 1971). Both are inducible.

In A. calcoaceticus, Pseudomonas putida and Pseudomonas aureofaciens the quinate/shikimate dehydrogenase is a $NAD(P)^+$-independent enzyme (EC 1.1.99.-) and is membrane associated (Tresquerres et al., 1970a,b; Ornston, 1971; van Kleef & Duine, 1988). The enzyme contains pyrrolo-quinoline quinone (PQQ or 2,7,9-tricarboxy-1H-pyrrolo [2,3-f] quinoline-4,5-dione) or methoxatin (Fig. 1.6) as a prosthetic group or cofactor, i.e. it is a quinoprotein (van Kleef & Duine, 1988). The physiological function and the biosynthetic pathway to PQQ have not yet been elucidated (Dokter et al., 1988; Goosen et al., 1989). It is possible that ubiquinone may act as a primary electron acceptor in this system (van Kleef & Duine, 1988) as is the case for the quinoprotein glucose dehydrogenase (Matsushita et al., 1989).

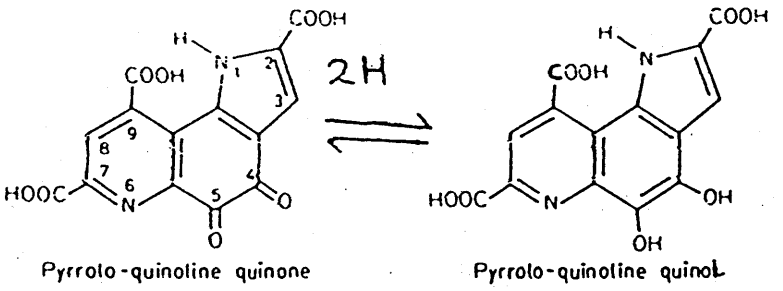
A particulate quinate dehydrogenase which does not require pyridine nucleotides has also been found in Acetomonas (Gluconobacter) oxydans, but in contrast with the enzyme of A. calcoaceticus, its synthesis is apparently constitutive (Whiting & Coggins, 1967). An NAD^+-dependent quinate dehydrogenase, similar to that found in N. crassa and other fungi, is also found in some bacteria, e.g. Aerobacter aerogenes (Mitsuhashi & Davis, 1954) and Pseudomonas aeruginosa (Ingledew & Tai, 1972).

1.3.2.3 Organisation and regulation in plants

Quinic acid and shikimic acid occur in many higher plants (Bohm, 1965; Boudet et al., 1985), often in appreciable concentrations. At the present time the enzymatic reactions

Figure 1.6 The structure of pyrrolo-quinoline quinone (PQQ)

The structures of PQQ and its reduction product are shown (from Duine et al., 1981; Geiger & Gorisch, 1989).



Pyrrolo-quinoline quinone Pyrrolo-quinoline quinol

involved in the synthesis and utilisation of quinic acid in plants have not been clearly identified but it has been suggested that quinic acid may be an intermediate on an alternative aromatic biosynthetic pathway which has developed during biochemical evolution (Boudet et al., 1985). Various studies have concluded that both quinic acid and shikimic acid are actively metabolised in plants (Haslam, 1974). Boudet and coworkers (Boudet et al., 1985) have found that there are two dehydroquinase isoenzymes in monocot species such as maize. Dehydroquinase-1 is associated with shikimate dehydrogenase and is probably involved in the shikimate pathway, whilst dehydroquinase-2 does not show any shikimate dehydrogenase activity but is activated by shikimate. This activation is very similar to that of the inducible dehydroquinase in fungi (Giles et al., 1967a) and bacteria (Ingledew et al., 1971; Berlyn & Giles, 1973) even though the plant enzyme differs from the fungal and bacterial enzymes in that it is constitutive and extremely heat labile. More detailed studies have shown that dehydroquinase-2 is associated with an NAD^+-dependent quinate dehydrogenase. These two enzyme activities co-migrate during purification, suggesting their association in an enzyme complex or on a bifunctional polypeptide chain (Graziana et al., 1980). The occurrence of the dehydroquinase-2/quinate dehydrogenase complex in addition to the biosynthetic dehydroquinase-1/shikimate dehydrogenase bifunctional enzyme found generally in higher plants (see 1.2.8.2 above), is almost certainly restricted to some families of monocots (Minamikawa, 1977; Boudet et al., 1985) and has not been found generally in plants.

The occurrence of these parallel enzyme complexes and isoenzymes in some plant species has led to the suggestion that the

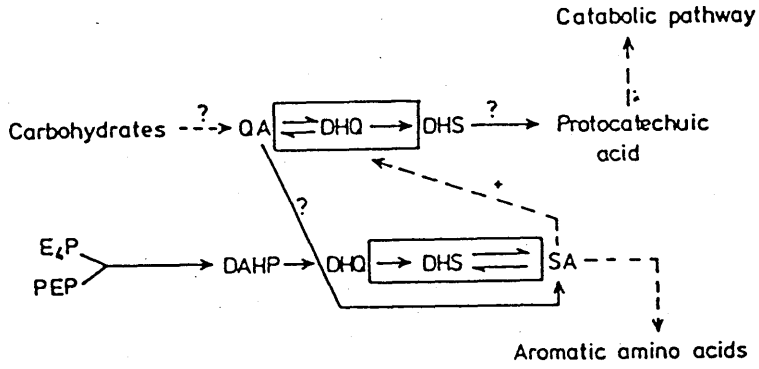
major role of the bifunctional enzymes may be to channel intermediates (Boudet et al., 1985). This would allow the independent functioning of the biosynthetic pathways and the catabolic pathways even though they involve many intermediates in common. Work on the maize has led to the following specific suggestion about the molecular organisation of the shikimate and quinate pathway enzymes in this species (Boudet et al., 1985) (Fig. 1.7). Quinic acid and shikimic acid originate independently from central metabolism and quinate can be directly converted to shikimate (Fig. 1.7). The quinate dehydrogenase/ dehydroquinase-2 complex produces dehydroshikimic acid from quinic acid and this is preferentially converted to protocatechuic acid and metabolised through a catabolic pathway. The dehydroquinase-1/ shikimate dehydrogenase bifunctional enzyme is part of the 'normal' shikimate pathway and produces dehydroshikimic acid which is used preferentially for biosynthesis of shikimate and the aromatic amino acids. Shikimate activates dehydroquinase-2 and this may serve to channel the carbon flow from quinic acid to protocatechuic acid and its metabolites (Boudet et al., 1985).

1.4 Evolution of shikimate pathway

1.4.1 Evolution of individual enzymes in bacteria

Complete amino acid sequences have been deduced from gene sequence for all seven enzymes of the shikimate pathway of E. coli (section 1.2). Many of these sequences have been determined for more than one species. For example, the sequences of Erwinia carotovora and E. coli shikimate kinase are known (Minton et al. 1989; Millar et al., 1986a). The sequence information shows that each enzyme is not closely related at the primary structure level to any of the other pathway enzymes and it appears at this stage unlikely that all the pathway enzymes have diverged from a single common ancestor.

Figure 1.7 Possible molecular organisation of the shikimate and the quinate pathways and their interrelations in maize (from Boudet et al., 1985)



QA = quinic acid

DHQ = dehydroquinate

DHS = dehydroshikimate

E4P = erythrose-4-phosphate

PEP = phosphoenolpyruvate

SA = shikimic acid

However, comparison of the sequences of each individual enzyme from several species show very clear homologies (see Duncan et al., 1987). There is also clear evidence that three of the enzymes, dehydroquinate synthase, shikimate dehydrogenase and shikimate kinase contain regions that are clearly homologous to the characteristic nucleotide binding regions of other synthases, dehydrogenases and kinases, for example, (Walker et al., 1982; Finch & Emmerson, 1984; Millar & Coggins, 1986; Millar et al., 1986a; Anton & Coggins, 1988; Minton et al., 1989) (see Appendix).

1.4.2 Evolution of the arom complex in fungi

The mosaic structure of the fungal arom polypeptides described in section 1.2.8 suggests that these pentafunctional polypeptides may have arisen by a series of gene fusions. The gene fusion hypothesis is particularly attractive as a model for the origin of the entire arom polypeptide because the subunit M_r values of the five corresponding E. coli polypeptide add up to 159,686 (Coggins et al., 1987a) while the Neurospora and yeast arom subunits M_r are 165,000 (Coggins et al., 1987b) and 174,555 (Coggins et al., 1987a) respectively and the sequence homologies with the five monofunctional E. coli enzymes are very convincing (Table 1.2) (Duncan et al., 1987; Charles et al., 1985, 1986). Gene fusion leading to multifunctional polypeptide chains have been reported for other enzymes involved in amino acid biosynthesis, for example, for E. coli anthranilate synthase (Miozzari & Yanofsky, 1979) and for yeast tryptophan synthase (Zalkin & Yanofsky, 1982).

1.5 Aims of this project

As described above, structural and evolutionary relationships between enzymes can be evaluated from amino acid and

Table 1.2

The polypeptide chain size and the subunit organisation of the five central shikimate pathway enzymes in *E. coli* and *S. cerevisiae*. The data are taken from Millar & Coggins, 1986; Duncan *et al.*, 1986; Chaudhuri *et al.*, 1986; Chaudhuri & Coggins, 1985; Anton, 1985; Millar *et al.*, 1986a; Lewendon & Coggins, 1983; Duncan *et al.*, 1984b; Duncan *et al.*, 1987). The *N. crassa* and *A. nidulans arom* polypeptide chains are approximately the same length as the *S. cerevisiae arom* chain (K. Duncan and J.R. Coggins, unpublished work; Charles *et al.*, 1986).

Enzyme	Polypeptide chain length (amino acids)	Calculated M_r	Quaternary structure	Number of residues conserved between the *E. coli* monofunctional enzymes and the *S. cerevisiae* multifunctional enzyme
3-Dehydroquinate synthase	362	38,880	monomer	130 (36%)
3-Dehydroquinase	240	26,377	dimer	162 (38%)
Shikimate dehydrogenase	272	29,380	monomer	39 (23%)
Shikimate kinase	173	18,937	monomer	50 (21%)
EPSP synthase	427	46,112	monomer	68 (25%)
Total	1474	159,689		449 (30%)
Yeast *arom*	1588	174,555	dimer	

nucleotide sequences. Alternatively antibodies raised against the enzyme from one organism can be used to detect structural similarities with the same enzyme isolated from other organisms by analysis of cross reactions. The degree of cross reaction can be related to similarities of amino acid sequences and can in addition give information on structural similarity. The aims of this project were three fold.

1) to use antibodies to detect sequence and structural similarities between the enzymes of the shikimate pathway within one species;

2) to use antibodies to detect sequence and structural similarities between the shikimate pathway enzymes of different species;

3) to purify the enzymes which were shown by immunological techniques to be interesting, for further study.

It was expected that the immunological studies would help to throw further light on evolutionary relationships between the shikimate pathway enzymes of different species.

2. Materials & Methods

2.1 Materials

2.1.1 Fine chemicals

All reagents used were the best grade available commercially. With the exceptions of the materials listed below, reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Bovine serum albumin (Fraction V)-Armour Pharmaceuticals Co. Ltd., Eastbourne, Sussex, U.K.

HRP color development reagent (4-chloro-1-naphthol) - Bio-Rad Laboratories Ltd., Watford, Hertfordshire, U.K.

Freund's complete and incomplete adjuvant - Difco Laboratories, Central Avenue, West Molesey, Surrey, U.K.

HRP donkey anti-rabbit IgG, normal rabbit serum, normal donkey serum - Scottish Antibody Production Unit, Law Hospital, Carluke, U.K.

ADP, ATP, protein A *S. aureus* (Cowan strain) crude cell suspension, PMSF, Coomassie Brilliant Blue G-250, Deoxyribonuclease I (DNaseI), SDS PAGE calibration proteins, Tween 20 - Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

NAD^+, $NADP^+$, NADPH, NADH, phosphoenolpyruvate, DTT, Tris, lactate dehydrogenase/pyruvate kinase - Boeringer Corp., Lewes, East Sussex, U.K.

Shikimic acid, DCPIP, Benzamidine.HCl - Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.

DAHP, dehydroquinate, EPSP - gifts from Professor J.R. Coggins, Department of Biochemistry, University of Glasgow.

Partial purified yeast *arom* complex - gift from Ms. A. Stuart, Department of Biochemistry, University of Glasgow.

Partial purified S. coelicolor shikimate dehydrogenase - gift from Dr. P.J. White, Department of Biochemistry, University of Glasgow.

Crude extract of R. graminis shikimate dehydrogenase - gift from Mr. M. Yasin, Department of Biochemistry, University of Glasgow.

2.1.2 Chromatographic media

Prepacked Mono Q, Phenyl superose and Superose 12 columns (f.p.l.c. system) - Pharmacia Ltd., London, U.K.

CNBr-activated Sepharose 4B, DEAE-cellulose, DEAE-Sephacel, Blue dextran Sepharose, Sephacryl S-200, ADP-Sepharose - Pharmacia Ltd., London, U.K.

Ultrogel AcA44 - LKB Instruments Ltd., South Croydon, Surrey, U.K.

Red Sepharose CL-6B (Procion Red HE3B), DEAE Affi-Gel Blue (Cibacron Blue F3GA) - Amicon Ltd., Upper Mill, Stonehouse, U.K.

Trypsin inhibitor immobilised on agarose - Miles-Yeda Ltd., Slough, Berkshire, U.K.

2.2 Organisms

The organisms used in this study are shown in Table 2.1. Some were obtained from stocks maintained in the Department of Biochemistry and Genetics but were ultimately derived from culture collection strains. Others were isolated in the Department of Biochemistry, University of Glasgow. The source of each organism is also indicated in the Table 2.1.

2.3 Sterilisation

2.3.1 Moist heat

Heat-stable solutions were autoclaved. All solutions were autoclaved at 109^{o}C (5 psi) for times established by Fewson

*1. American Type Culture Collection

2. Millar & Coggins, 1986

3. Duncan et al., 1986

4. Anton & Coggins, 1988

5. Millar et al., 1986a

6. Duncan et al., 1984b

7. National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland

8. SCRI. Invergowrie, Scotland

9. National Collection of Type Cultures, PHLS, London, England

10. Dr. I.S. Hunter, Department of Genetics, University of Glasgow

11. Fungal Genetics Stock Center, Arcata, California, U.S.A.

12. Genex Corp., Gaithersburg, Maryland, U.S.A.

13. Dr. A.J.P. Brown, Department of Genetics, University of Glasgow.

14. Clause (U.K.), Charvill, Reading, Berkshire.

Table 2.1 Organisms used in this study

Organism	Genotype with respect to aromatic amino acid biosynthesis	Source*
Bacteria		
Escherichia coli K12 (ATCC 14948)	wild type	1
Escherichia coli K12: AB2826/pGM107	E1 overproducing strain	2
Escherichia coli K12: AB2848/pKD201	E2 overproducing strain	3
Escherichia coli K12: AB2834/pIA321	E3 overproducing strain	4
Escherichia coli K12: HW1111/pGM450	E4.II overproducing strain	5
Escherichia coli K12: AB2829/pKD501	E5 overproducing strain	6
Escherichia coli ML308 (ATCC 15224)	wild type but lac i^-	1
Salmonella typhimurium (NCIB 10255)	wild type	7
Acinetobacter calcoaceticus (NCIB 8250)	wild type	7
Erwinia carotovora ssp. *atroseptica* 1034	wild type	8
Staphylococcus aureus Oxford (NCTC 6571)	wild type	9
Bacillus subtilis (NCTC 3610)	wild type	9
Streptomyces rimosus (NCIMB 4018)	wild type	7
Streptomyces coelicolor GLW209	wild type	10
Fungi		
Neurospora crassa 74 OR23-1A	wild type	11
Rhodotorula graminis KGX 39	wild type	12
Saccharomyces cerevisiae 5228C	wild type	13
Plant		
Pisum sativum (variety Onward)	wild type	14

(unpublished results) as adequate for sterilisation. Efficiency of sterilisation was verified by using Browne's tube (type one, A. Browne Ltd., Chancery Street, Leicester).

2.3.2 Dry heat

Pipettes were wrapped in either Kraft paper or placed in metal cannisters before sterilisation. Each sterilisation (160^{o}C, 1.75 h) was checked by including a Browne's tube (type three). Where necessary glassware was also sterilised by dry heat at 160^{o}C for 1.75 h.

2.3.3 Ethylene oxide

Plastic pipettes and other disposable apparatus were sterilised with ethylene oxide. All items were sealed with polythene film and exposed to ethylene oxide (Anprolene) for 12 h in a sterilising box (AN74; H.W. Anderson Products Ltd., Clacton-on-Sea, Essex). Sterilisation was verified by an Anprolene exposure indicator (AN85) or by a steritest unit (AN80). All apparatus was aired for at least 24 h prior to use to remove residual gas.

2.4 Media

2.4.1 Nutrient broth

Nutrient broth (Oxoid CM1; 13g/l) was prepared, sterilised by autoclaving and used for culture maintenance, for preparation of inocula and for both small and large scale growth of *E. coli*, *S. typhimurium*, *E. carotovora*, *S. aureus* and *B. subtilis*.

2.4.2 Nutrient agar plates

The nutrient agar (Oxoid CM3; 28g/l) was boiled, stirred to ensure even suspension of agar and then autoclaved. The sterile molten medium was mixed well, allowed to cool to 55°C and poured into petri dishes. After the agar had solidified the plates were left inverted for 24 h at 37°C to dry the surface of the agar.

2.4.3 Soy broth

Soy broth (BBL and Trypticase; 30g/l) was used for growing inocula and large scale growth of S. rimosus.

2.4.4 Complex medium

The complex medium (Allison et al., 1985) contained (g/l) :

Oxoid No. 1 nutrient broth	26.0
KH_2PO_4	2.0
$(NH_4)_2SO_4$	1.0
L-glutamic acid.HCl	0.9
$MgSO_4.7H_2O$	0.4

The medium was adjusted to pH 7.0 with 5 M-NaOH, made to the correct volume with distilled water and autoclaved. This medium was used for preparation of inocula and for large scale growth of A. calcoaceticus.

2.4.5 Minimal medium I

This medium was adapted from Canovas & Stanier (1967) for induction the quinate dehydrogenase and shikimate dehydrogenase in A. calcoaceticus. The medium was prepared as several components which were separately sterilised by autoclaving.

1) Chel metals

Chel metals was prepared as follows:- Nitrilotriacetic acid (Chel) (80g) was dissolved in 1 M-NaOH (625ml) and the solution was adjusted to pH 7.0 with 5 M-HCl. Salts were then added in the following order ensuring that each component was completely dissolved before the next was added:

$FeSO_4.7H_2O$	1.1g
$Na_2MoO_4.2H_2O$	50mg
$MnSO_4.4H_2O$	50mg
$ZnSO_4.7H_2O$	50mg
$CuSO_4.5H_2O$	25mg
$CoCl_2.6H_2O$	25mg

The solution was made up to 1 l with distilled water, autoclaved and stored at room temperature until required (Beggs & Fewson, 1977).

2) Minimal medium I containing carbon source

This minimal medium contained (g/l):

KH_2PO_4	2.0
$(NH_4)_2SO_4$	1.0
Carbon source	(10mM quinic acid or 10mM p-hydroxybenzoic acid or 20mM succinic acid)

The pH of the medium was adjusted to 7.0 with 5 M-NaOH, made to the correct final volume with distilled water and autoclaved.

3) 2% (w/v) $MgSO_4.7H_2O$ was prepared and sterilised by autoclaving.

Immediately before inoculation complete medium was prepared by adding aseptically 2ml chel metals and 20 ml 2% $MgSO_4.7H_2O$ to each litre of minimal medium.

2.4.6 Minimal medium II

Minimal medium II was prepared as four components which were autoclaved separately:

1) P medium contained 5.44g KH_2PO_4 per litre, adjusted to pH 7.0 with 5 M-NaOH.

2) MgNS contained 9.86g $MgSO_4.7H_2O$ and 105.7g $(NH_4)_2SO_4$ per litre.

3) $FeSO_4$ contained 0.22g $FeSO_4.7H_2O$ per litre, and was adjusted to pH 2.0 using 5N-HCl before sterilisation.

4) Carbon source (stock solutions) (pH 7.0)

   a) glucose 1 M

   b) glycerol 1 M

   c) sodium succinate 1 M

Before inoculation, 2ml MgNS and 2ml $FeSO_4$ were added to a flask containing 140ml P medium. Carbon source (glucose or glycerol or succinate) was then added to give the desired final concentration (20mM) and the volume made to 160ml with sterile H_2O. This minimal medium was used for growth of *E. coli* and *E. carotovora*.

2.5 General methods

2.5.1 pH measurements

The pH of solutions was measured using a direct reading pH meter and combination electrode. Calibration of the electrode was carried out immediately before use using a pre-prepared solution of known pH (BDH Buffer tablets).

2.5.2 Conductivity measurements

The conductivities of solutions were measured at $4^{o}C$ using a Radiometer conductivity meter type CDM2e (Radiometer, Copenhagen, Denmark).

2.5.3 Protein estimation

Protein estimation was made by the method of Bradford (1976) using bovine serum albumin as standard.

Protein elution from column was followed by measuring the A_{280} of the eluant using elution buffer for standardisation of A_{280}.

2.5.4 Acid-washed glassware

All glassware used for protein chemistry (e.g. amino acid analysis, enzyme denaturation) was soaked overnight or boiled for 15 min in 30% (v/v) nitric acid. The acid-washed glassware was rinsed extensively with distilled water and dried in an oven before use.

2.5.5 Glassware

2.5.5.1 General glassware

Glassware was washed routinely by soaking in approximately 1% (w/v) Haemosol solution (Meinecke and Co., Baltimore, U.S.A.) then rinsed thoroughly in tap and glass-distilled water and dried in oven.

2.5.5.2 Pipettes

Before use all pipettes were washed by soaking in Haemosol solution (1% w/v) followed by thorough rinsing in tap and deionised water and drying in an oven. All pipettes were plugged with non-absorbent cotton wool.

2.5.6 Dialysis

Dialysis was carried out using Visking tubing (Scientific Instruments Centre Ltd., London), which had been immersed in boiling 1% (w/v) EDTA solution, pH 7.0 for 15 min and then washed with distilled water. If not used immediately the prepared dialysis tubing was stored in 20% (v/v) ethanol and rinsed thoroughly in distilled water before use.

2.5.7 Lyophilisation

Samples were frozen over the surface of a suitable vessel by placing them into methanol/dry ice. The top of the vessel was covered with nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan) and punctured with a needle. Frozen samples were placed in a dessicator connected to a Flexi-dry (FTS Systems Inc., Stone Ridge, U.S.A.) and maintained under vacuum by a high vacuum pump (Javac PTY Ltd., Farnham, U.K.) until completely dry.

2.5.8 Concentrations of protein samples

Samples of protein of less than 10ml volume were concentrated using Centricon 30 microconcentrators (Amicon Ltd., Stonehouse, U.K.).

2.6 Maintenance and production of biological material : preparation of cell free extracts

2.6.1 Microbiological safety

The recommendations as outlined in "Guidelines for Microbiological Safety" were followed.

2.6.2 Bacterial strain storage

Wild type strains were stored on nutrient agar slants or on sealed plates of nutrient agar at 4°C. Overproducing strains were maintained in nutrient agar containing suitable drugs [ampicillin (50μg/ml) or tetracycline (20μg/ml)] to keep selective pressure for plasmid retention during storage. For short term storage all strains were maintained in 10ml nutrient broth, stored at 4°C. For mutant strains drugs were added to the medium, as necessary, for plasmid maintenance.

2.6.3 Bacterial growth

All bacterial growth was carried out in temperature controlled rooms. Cultures of *E. coli*, *S. typhimurium*, *S. aureus* and *B. subtilis* were grown at 37°C, of *A. calcoaceticus* and *S. rimosus* at 30°C and of *E. carotovora* at 23°C.

Small scale growth of cells was carried out by inoculating into 500ml of the appropriate sterile medium in 2 litre conical flasks which were stoppered with polystyrene foam bungs (A. & J. Beveridge Ltd., 5 Bonnington Road Lane, Edinburgh, U.K.). Cultures were grown on a rotary shaker at 120 rpm at the appropriate temperature.

For larger scale growth of cells 4 litres of appropriate medium in 10 litre flat-bottomed flasks were used. These flasks were fitted with plugs of non-absorbent cotton wool through which passed a plugged 10ml glass pipette. After inoculation the flasks were vigorously mixed using a 45mm polypropylene-coated magnetic bar on apparatus built in Glasgow (Harvey *et al.*, 1968). Adequate aeration was ensured by passing air (at 400ml/min) through the glass pipette in the bung.

Bacterial growth was monitored by optical density at 420nm using an LKB ultrospec spectrophotometer (LKB/Pharmacia, Milton Keynes, U.K.). All samples were read within the linear range of this spectrophotometer for proportionality of optical density by light scattering to bacterial density (optical density values of less than 0.6). All samples above this range were diluted in the appropriate blank medium as necessary.

2.6.4 Harvesting of bacteria

Cultures at late exponential stage of growth were harvested by centrifugation in 750ml polypropylene bottles (maximum volume 500ml) at 6000g for 30 min at 4°C in an MSE Mistral 6L centrifuge. Each pellet was resuspended in about 50ml ice-cold sterile 50mM-Tris/HCl, pH 7.5 and the centrifugation repeated. Supernatants were decanted and the bacterial pellets were stored at -20°C until required.

2.6.5 Preparation of crude extracts

2.6.5.1 Bacterial crude extracts

Pelleted cells were thawed and resuspended in an appropriate volume of chilled 50mM-Tris/HCl, pH 7.5 containing 0.4mM-DTT and 1.2mM-PMSF. Cells were broken by three passages through a pre-chilled French pressure cell at 98 MPa (14,300 psi) (internal pressure). The homogenate was then digested with 0.5mg DNaseI at 4°C for 1 h and centrifuged at 100,000g for 2 h at 4°C in Beckman L5-65 ultracentrifuge. The supernatant was kept on ice and used for enzyme inhibition assay (section 2.13), immunoprecipitation assay with protein A (section 2.15) and preparation of purified enzyme (section 2.10).

2.6.5.2 Fractionation of plant crude extracts

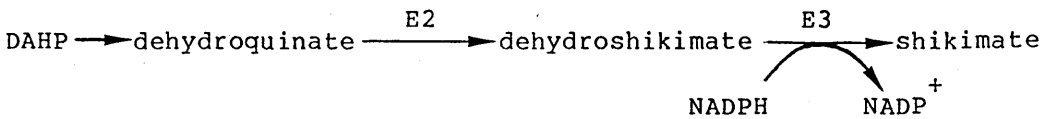
Seeds of pea (P. sativum) were germinated and grown as described by Mousdale et al. (1987). Shoot tissue was taken from 9 to 12-day old plants. 250g of fresh shoot tissue was homogenized in 500ml of chilled 50mM-Tris/HCl, pH 7.5, containing 0.4mM-DTT, 1mM-EDTA, 1mM-Benzamidine.HCl and 1mM-PMSF. The chilled brei was filtered through muslin and centrifuged at 80,000g for 1 h. The supernatant was decanted, brought to 35% saturation with solid $(NH_4)_2SO_4$ and centrifuged at 12,000g for 20 min at 4°C. The supernatant was again decanted, $(NH_4)_2SO_4$ added to 55% saturation and the precipitated protein collected by centrifugation. The precipitate was dissolved in a small amount of chilled 20mM-tris/HCl, pH 7.5, containing 0.4mM-DTT, 1mM-Benzamidine.HCl and 1mM-PMSF and dialysed overnight at 4°C against 2 l of this buffer. The extract was used for enzyme inhibition assay (section 2.13) and immunoprecipitation assay with protein A (section 2.15).

2.7 Enzyme assay

Enzymes were generally assayed spectrophotometrically in plastic 1 cm light-path cuvettes in a Ultrospec spectrophotometer at 25°C. The reaction components were added to the cuvettes and preincubated to the correct temperature by standing in a temperature controlled cuvette holder. The components of the reaction mixture were mixed and the reaction initiated by the addition of enzyme and further mixing. The rate of change in absorbance was measured over a period of 5 min during which time the assay was linear. One unit of enzyme activity is defined as the amount of enzyme required to catalyse the conversion of 1μmole of substrate to product per min under the condition of the assay.

2.7.1 DHQ synthase (E1)

DHQ synthase activity was measured by coupling the release of dehydroquinate using dehydroquinase (E2) and shikimate dehydrogenase (E3) to dehydroshikimate then shikimate with oxidation of NADPH as shown in diagram below.



This method was modified from Coggins et al. (1987b). The final assay contained : 0.1 M-glycine/KOH (pH 8.4), 0.2mM-$CoCl_2$, 0.2mM-NADPH, 0.4mM-DAHP, 50µM-NAD^+, 0.4 units/ml E. coli dehydroquinase and 0.4 units/ml E. coli shikimate dehydrogenase. The oxidation of NADPH was monitored at 340nm and converted to enzyme unit using the molar extinction coefficient of NADPH ($\varepsilon 340 = 6.18 \times 10^3 M^{-1} cm^{-1}$).

2.7.2 Dehydroquinase (E2)

Dehydroquinase activity was measured by coupling the release of dehydroshikimate with NADPH using shikimate dehydrogenase to produce $NADP^+$ and was modified from Coggins et al. (1987b). The assay contained 0.1 M-dehydroquinate, 50mM-potassium phosphate buffer, pH 7.0, 0.2mM-NADPH and 0.4 units/ml E. coli shikimate dehydrogenase. The oxidation of NADPH was monitored at 340nm and converted to enzyme units as under 2.7.1.

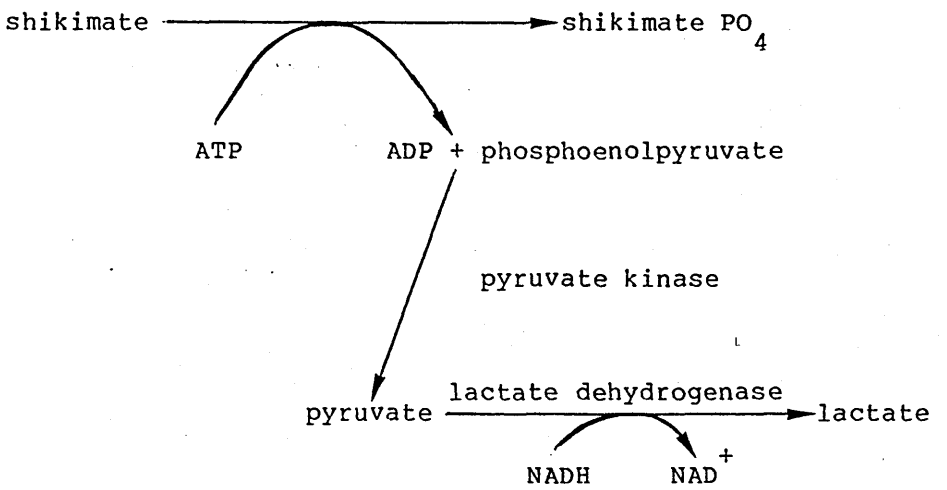
2.7.3 Shikimate dehydrogenase (E3)

Shikimate dehydrogenase activity was measured in the reverse direction by monitoring the reduction of $NADP^+$ at 340nm (Coggins et al., 1987b). The assay mixture contained 100mM-sodium carbonate

buffer, pH 10.6, 4mM-shikimic acid and 2mM-$NADP^+$. Shikimate dehydrogenase can also be assayed in the forward direction using the following assay mixture: 0.15 mM-dehydroshikimate, 0.17mM-NADPH and 100mM-potassium phosphate buffer, pH 7.0 and monitoring loss of absorption at 340nm. Enzyme units were calculated using the molar extinction coefficient of NADPH as above (section 2.7.1).

2.7.4 Shikimate kinase (E4)

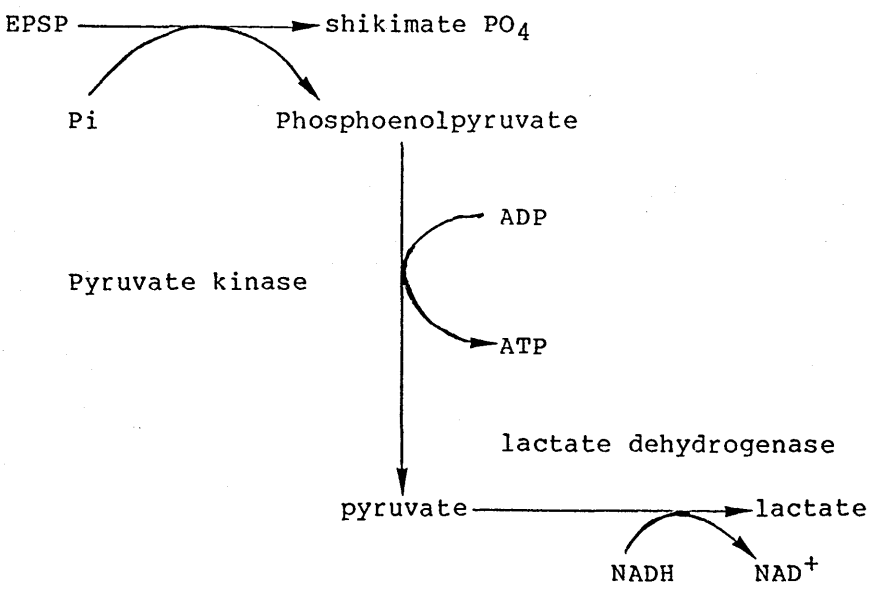
Shikimate kinase was assayed spectrophotometrically at 340nm by monitoring the disappearance of NADH (Coggins et al., 1987b). This was achieved by coupling the release of ADP produced by the reaction to the pyruvate kinase and lactate dehydrogenase reactions as shown in diagram below.



The final assay contained 50mM-triethanolamine.HCl/KOH pH 7.0, 50mM-KCl, 2.5mM-$MgCl_2$, 0.1mM-NADH, 1mM-phosphoenolpyruvate (neutralised with KOH), 2.5mM-ATP (neutralised with KOH), 1mM-shikimic acid (neutralised with KOH) and pyruvate kinase (3 units/ml)/lactate dehydrogenase (2.5units/ml). The oxidation of NADH was converted to enzyme units using the molar extinction coefficient of NADH ($\varepsilon_{340} = 6.22 \times 10^3 M^{-1} cm^{-1}$).

2.7.5 EPSP synthase (E5)

EPSP synthase was also assayed in the reverse direction by coupling the release of phosphoenolpyruvate from the reactions to the pyruvate kinase and lactate dehydrogenase reactions as shown in diagram below (Coggins et al., 1987b).



Oxidation of NADH was monitored at 340nm. The assay contained (final concentrations) 0.05mM-EPSP, 2.5mM-ADP, 0.1mM-NADH, 2.5mM-$MgCl_2$, 100mM-potassium phosphate buffer, pH 7.0, 3 units/ml of pyruvate kinase and 2.5 units/ml of lactate dehydrogenase. Enzyme units were calculated using the molar extinction coefficient of NADH as in section 2.7.4.

2.7.6 Quinate dehydrogenase

Quinate dehydrogenase was assayed by the method of Tresguerres et al. (1970a) and van Kleef & Duine (1988) using DCPIP as an electron acceptor. The reaction mixture contained (final concentrations) 100mM-potassium phosphate buffer pH 6.0, 10mM-quinate (neutralised with KOH) and 0.4mM-DCPIP. The decrease

of absorbance at 600nm was measured and converted to enzyme units using molar-extinction coefficient of DCPIP (ε_{600} = 22.0 x 10^{3} M^{-1} cm^{-1}).

2.8 Polyacrylamide gel electrophoresis (PAGE)

2.8.1 SDS PAGE (discontinuous system)

SDS PAGE was performed by the method of Laemmli (1970) in a slab gel apparatus built in the Department of Biochemistry, University of Glasgow.

2.8.1.1 Stock solutions

Solution A (separating gel buffer): 36.6g Tris and 0.23ml TEMED. The pH was adjusted to 8.8 with concentrated HCl and the volume made to 100ml with distilled water. The solution was stored at room temperature.

Solution B (electrophoresis buffer): 6g Tris; 28.8g glycine and 10ml 20% (w/v) SDS were made to 2 litres with distilled water and stored at room temperature.

Solution C: 28g acrylamide and 0.735g N,N'-methylene bisacrylamide were made to 100ml with distilled water. This solution was stored at room temperature and used within one month.

Solution D (stacking gel buffer): 1.16g Tris; 4ml 20% (w/v) SDS and 0.25ml TEMED. The pH was adjusted to 6.8 with concentrated HCl and the volume made to 100ml with distilled water. The solution was stored at room temperature.

Solution E: 20% (w/v) SDS. The solution was stored at 37°C to prevent the SDS coming out of solution.

2.8.1.2 Gel plates

Glass plates (9.5cm x 20.0cm x 10.0cm) were washed in Decon 75 to ensure they were free of silicon grease, then rinsed with distilled water. The plates were assembled using 1.5mm teflon spacers and silicon grease and placed into the custom-built casting box suitable for making up to 4 gels at a time.

2.8.1.3 Preparation of gels

1) Separating gel

Gels were prepared from the volumes (mls) of stock solutions shown on Table 2.2. Solution A, C and H_2O were mixed and degassed; SDS and ammonium persulphate were added and the gel was poured into the casting box. The layer of propan-2-ol was carefully placed on the top of the gel and the gel allowed to polymerise for 30 min. The layer of propan-2-ol was then washed off with distilled water and the top of the gels blotted dry with filter paper immediately before addition of stacking gel.

2) Stacking gel

The stacking gel was prepared as shown in Table 2.2 using solutions C, D and H_2O. After mixing the solution was degassed before addition of ammonium persulphate. This mixture was then poured on the top of the separating gel. The gel was allowed to polymerise around a well-forming teflon template for 30-60 min. After polymerisation the gels were wrapped in absorbent paper moistened in electrophoresis buffer and clingfilm then stored at $4^{o}C$. Gels were used within one week of preparation.

2.8.1.4 Sample preparation and electrophoresis conditions

Protein solutions were mixed with sample buffer (1.5g SDS, 8g sucrose and 100µg pyronin Y in 10ml of 0.5M-Tris/HCl, pH 8.8) in the following way: 160µl sample, 100µl 0.2M-DTT and 40µl sample

Table 2.2 Ratio of polyacrylamide gel preparation (mls)

Stock solutions	Separating gel				Stacking gel
	8%	10%	12.5%	16.5%	5.6%
	(w/v)				
solution A	25	25	25	25	-
solution C	57.2	71.5	89.3	118	17.5
solution D	-	-	-	-	10.0
20% SDS*	1	1	1	1	-
H_2O	114.3	100	82.2	53.5	55
solid ammonium persulphate (mg)	150	150	150	150	150

* For native gels omit the 20% SDS and add H_2O

buffer. The sample was then denatured by boiling for 2 min before application to the gel. The amounts of loading are defined in the legend in particular figures. Electrophoresis was carried out in solution B at 90 mA per slab gel. While the electrophoresis was taking place, the gel was cooled continuously by circulating water from an ice/water mixture through a chamber which was built into the electrophoresis apparatus. The electrophoresis was stopped when the dye (pyronin Y) had almost reached the bottom of the gel. The gel was then stained (section 2.9.1) or immunoblotted (section 2.17.1).

2.8.2 Non-denaturing PAGE

This was carried out in 10% (w/v) polyacrylamide gel by the method of Davis (1964) as modified by Hayes & Wellner (1969).

2.8.2.1 Stock solutions

Solution A (separating gel buffer): 36.6g Tris and 0.23ml TEMED, the pH was adjusted to 8.8 with concentrated HCl and the volume was made to 100ml with distilled water.

Solution B (electrophoresis buffer): 6g Tris and 28.8g glycine were made to 2 l with distilled water and stored at room temperature. Before use 0.01% (v/v) 2-mercaptoethanol was added.

Solution C: 28g acrylamide and 0.735g N,N'-methylene bisacrylamide were made to 100ml with distilled water.

Solution D (stacking gel buffer): 1.16g Tris and 0.25ml TEMED, the pH was adjusted to 6.8 with concentrated HCl and the volume was made to 100 ml with distilled water.

2.8.2.2 Gel plates

These were prepared as described in Section 2.8.1.2.

2.8.2.3 Preparation of gels

Gels were prepared as described in Section 2.8.1.3 except that SDS was omitted from all solutions.

2.8.2.4 Sample preparation and electrophoresis condition

Protein solutions were mixed with 5µl tracking dye [0.02% (w/v) Bromophenol blue in 50% (v/v) glycerol] and layered into each well. The gel was pre-electrophoresed for 1 h at 20mA at 4°C in solution B before application of the protein solution. Electrophoresis was then run at 20mA, 4°C until the tracking dye approached the bottom of the gel. The gel was then activity stained (section 2.9.2) or immunoblotted (section 2.17.2).

2.9 Staining

2.9.1 Protein staining

2.9.1.1 Coomassie Blue staining

Routinely, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G250 in methanol/acetic acid/H_2O (50:10:40 by volume) for 1 h at 60°C. Gels were destained overnight at room temperature in methanol/acetic acid/H_2O (10:10:80 by volume).

2.9.1.2 Silver staining

This was a sensitive method, based on that described by Wray et al. (1981). Before staining gels were soaked overnight in 50% (v/v) methanol. Staining solution was prepared by adding silver nitrate solution (0.8g $AgNO_3$ in 4ml distilled water) dropwise with vigorous stirring to an alkaline ammonia solution [1.4ml of 14.8M-NH_4OH added to 21ml 0.36% (w/v) NaOH]. Distilled water was

added to give 100ml. The gel was gently agitated in this staining solution for 15 min and then rinsed with many changes of distilled water for 1 h. The stain was developed by immersing the gel in a solution containing 2.5ml 1% (w/v) citric acid and 0.25ml 38% (v/v) formaldehyde in 500ml distilled water. Staining was stopped when the protein bands appeared by washing the gel immediately in distilled water.

2.9.2 Activity staining

Enzyme activity of shikimate dehydrogenase was detected after non denaturing gel electrophoresis by the method of Lumsden & Coggins (1977). Gels were soaked at 4°C in 4 changes of 0.25 M-Tris/HCl, pH 9.0 for 30 min to remove 2-mercaptoethanol. The gels were then transferred to the staining solution containing 0.25 M-Tris/HCl, pH 9.0, 0.5mM-$NADP^+$, 0.5mM-shikimic acid, nitroblue tetrazolium (0.5mg/ml) and phenazine methosulphate (5µg/ml). This was kept in the dark at 23°C with gentle shaking until shikimate dehydrogenase was indicated by a purple precipitate of formazan.

2.10 Enzyme preparations

These enzymes were prepared for raising antibodies against native configuration of the enzymes.

2.10.1 Purification of arom complex from N. crassa

The *N. crassa arom* complex was purified to homogeneity as described by Coggins *et al.* (1987b). The progress of the purification was followed by monitoring the E2 activity of *arom* complex. The results of the purification are shown in Table 2.3.

Table 2.3 Purification scheme of the arom complex of N. crassa

Starting material was 100g of freeze-dried mycelia.

Purification step	Vol (ml)	Protein (mg/ml)	total protein (mgs)	E_2 activity (u/ml)	total activity (units)	specific activity (u/mg)	purification (fold)	yield (%)
1: crude extract	1300	15.9	20,000	.087	113	0.0056	1	100
2 : DEAE-Cellulose	1030	10.6	10,900	.104	108	0.0099	1.8	96
3 : 40-50% $(NH_4)_2SO_4$	100	14.6	1,460	.87	87	0.060	10.7	77
4 : DEAE-Sephacel	120	0.96	115	.58	70	0.61	108.9	62
5 : Blue dextran-sepharose	18	1.21	21.8	2.5	45	2,06	367.9	40

2.10.2 Purification of E3 from E. coli AB2834/pIA321 (E3 overproducing strain)

E3 from E. coli was purified to homogeneity as described by Chaudhuri et al. (1987). The purification steps and results of the purification are shown in Table 2.4.

Other purified enzymes used (e.g. E1, E2, E4 and E5, from E. coli overproducing strains) were gifts from Mr. J.J. Greene, Department of Biochemistry, University of Glasgow.

2.11 Enzyme denaturation

To increase the number of epitopes available as antigens the five purified proteins (E1 to E5) from the E. coli shikimate pathway were denatured by reduction and carboxymethylation. The carboxymethylated proteins were used as antigens.

2.11.1 Dialysis

Before denaturation approximately 1mg/ml of each of the purified proteins used were exhaustively dialysed against 0.5% (w/v) ammonium bicarbonate, with 6 changes each of 2.5 l for 24 h at 4°C then lyophilised to dryness.

2.11.2 Reduction and carboxymethylation

Carboxymethylation of cysteine residue was carried out as described by Lumsden & Coggins (1978). Lyophilised samples were resuspended in 2ml 0.1 M-Tris/HCl, pH 8.2 containing 8M urea and 2mM-DTT and incubated in the dark for 1 h at room temperature under an atmosphere of N_2. Iodoacetate was then added to the solution to give a final concentration of 15mM, and the solution incubated for a further 1 h in the dark under N_2. The reaction was terminated by addition of excess DTT (30mM, final concentration).

Table 2.4 Purification scheme of E3 from E. coli AB2834/pIA321

The results presented are for a typical purification starting from 20g of cells.

Purification step	Vol (ml)	Protein (mg/ml)	total protein (mgs)	activity (u/ml)	total activity (units)	specific activity (u/mg)	purification (fold)	yield (%)
1 : crude extract	69	23	1,587	615	42,435	26.73	1	100
2 : 30-55% $(NH_4)_2SO_4$	62.5	20.5	1,281	582	36,375	28.39	1.06	86
3 : 1st DEAE-Sephacel	77	4.2	323	453	34,881	107.99	4.04	82
4 : 2nd DEAE-Sephacel	5	21	105	4,272	21,360	203.43	7.61	50
5 : Sephacryl S-200	12	3.125	37.5	1,359	16,308	434.88	16.27	38
6 : ADP-Sepharose	12	1.43	17.2	841	10,092	586.74	21.95	24

The carboxymethylated (alkylated) proteins were dialysed against 0.5% (w/v) ammonium bicarbonate with 4 changes each of 2.5 l for 24 h at 4°C. The samples were then lyophilised to dryness.

2.11.3 Analysis of amino acid composition

The amino acid compositions of the native proteins and carboxymethylated proteins were determined after acid hydrolysis using an LKB 4400 amino acid analyser operated by Mr. J. Jardine, Department of Biochemistry, University of Glasgow. It was found that all cysteines present in the original proteins had been changed into carboxymethylcysteine (results not shown).

2.11.4 Comparison by SDS PAGE

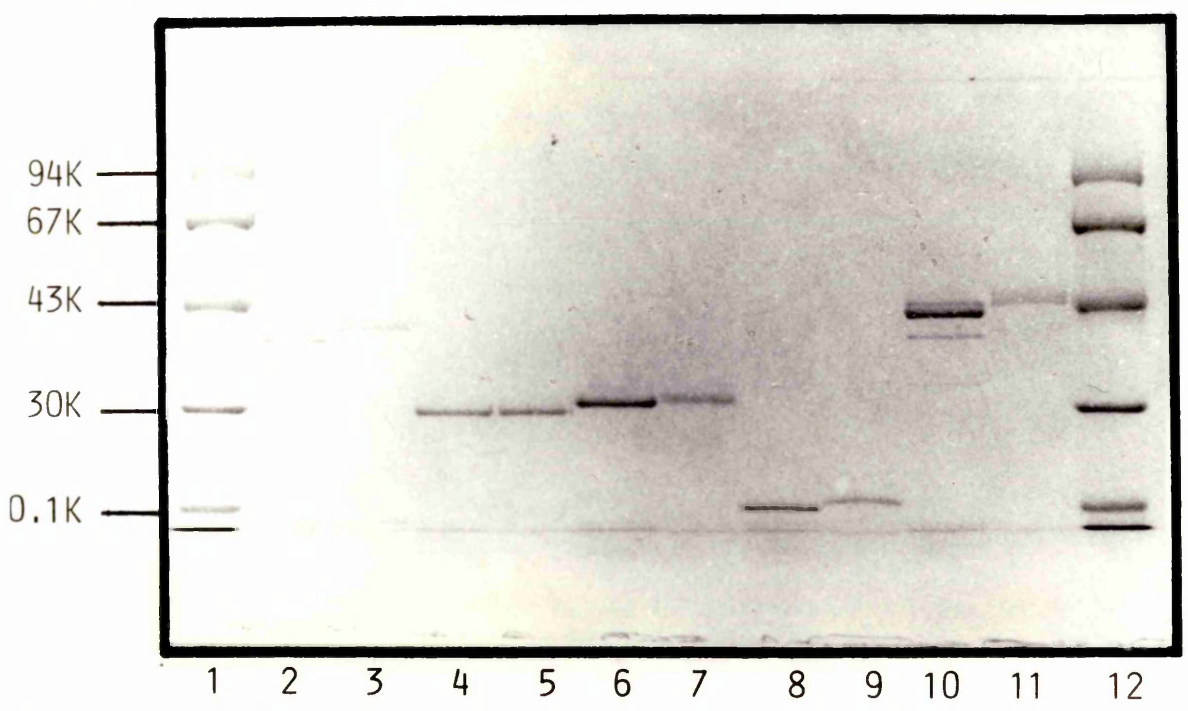
The carboxymethylated and non-carboxymethylated proteins were compared by SDS PAGE (section 2.8.1). It was found that all of the carboxymethylated proteins moved more slowly than the corresponding non-carboxymethylated proteins (Bretscher, 1962) with the exception of E2 (Fig. 2.1). Although, it had been reported that E2 contains cysteine (Duncan et al., 1986) it is now known that this enzyme contains no cysteine residues (E. Borthwick, L.D. Graham & J.R. Coggins, unpublished results) and consequently could not be carboxymethylated by this procedure.

2.12 Preparation of antisera

Antibodies were raised in the Department of Biochemistry, University of Glasgow against many of the enzymes of the aromatic amino acid biosynthetic pathway. Both native and denatured proteins (see section 2.10 and 2.11) were used as antigens to ensure that antibodies could be produced against as many epitopes as possible. All antisera were raised in New Zealand white rabbits of

Figure 2.1 Separation of non-carboxymethylated and carboxymethylated proteins on SDS PAGE

Approximately 3ug of protein was loaded on each track of the 10% SDS PAGE; after electrophoresis protein bands were located by staining with Coomassie Blue (section 2.9.1.1). Tracks 1, 12, M_r standard proteins (section 2.18.1); tracks 2, 4, 6, 8, 10, non-carboxymethylated samples of E1, E2, E3, E4 and E5 respectively; tracks 3, 5, 7, 9, 11, carboxymethylated, denatured samples of E1, E2, E3, E4 and E5 respectively.



bodyweight about 2.5 Kg at the start of the immunisation procedure. All procedures involving animals were carried out by Dr. I.D. Hamilton under whose licence the procedures were authorised.

2.12.1 Preparing immunogens

A general procedure was used for preparation of material for immunisation and the details of amounts used are given in Table 2.5. In general, approximately 0.5mg immunogenic protein was dissolved in 1ml of buffer solution and then emulsified, either by repeatedly passing through a fine bore needle or by sonication, with an equal volume of Freund's complete adjuvant. The emulsion was injected subcutaneously at many sites (0.2ml/site) on the back and sides of the rabbit.

After 6 weeks a second (boost) immunisation was given. For this approximately 0.25mg protein was emulsified in Freund's incomplete adjuvant and injected as before. Animals were first bled two weeks after the boost injection.

In some cases rabbits were given more extensive boost injection either to produce more antisera or to produce antisera of higher potency. In particular anti-shikimate dehydrogenase antibody was used extensively for development work and antiserum against this protein was raised over a period of 3.25 years and required repeated boosting and bleeding of the rabbit. Details of the immunisation procedure for this rabbit are given in Figure 3.2.

2.12.2 Preparing antisera

Blood was removed from rabbits at the ear vein. At any one time about 25ml of blood was removed for antisera production. Blood samples were allowed to clot at room temperature, reamed to prevent the clot sticking to the side of the container and placed at

Table 2.5 Immunogens used in this study

This table shows the type and amount of protein injected into rabbits for raising antibodies.

Types of immunogen	Amount for initial immunisation (µg)	Amount for boost immunisation (µg)
E1	500	250
E2	500	250
E3*	500	250
E4	500	250
E5	500	250
arom	gel preparation	250
denatured-E1	400	200
" -E2	400	200
" -E3	400	200
" -E4	400	200
" -E5	400	200

*see Figure 3.2

4°C overnight. The following morning the serum was separated from the clot and centrifuged at 15,000g for 10 min to remove residual clotted material. Antisera were then stored at -20°C, either as 1ml aliquots or in larger quantities, until required.

Individual preparations of antisera were kept and used separately with no two batches being mixed. Consequently some antisera may have slightly different properties from others. To uniquely identify a particular preparation of antisera it was labelled with both the identification marker for the rabbit and the number of the antisera preparation. The identification code 159:8 therefore indicates the 8th batch of antisera prepared from rabbit 159. Where it is relevant the code of the particular preparation of antisera is given in the figure legend.

2.13. Antibody enzyme inhibition studies

Purified enzymes or enzyme containing extracts (crude extracts) were diluted in 50mM-Tris/HCl, pH 7.5 to give appropriate concentrations and then incubated with an equal volume (usually 20µl) of an appropriate dilution of antisera (diluted in normal rabbit serum) or purified IgG (diluted in 50mM-Tris/HCl, pH 7.5) for 1 h with gentle rotation on a rotating wheel at room temperature. Enzyme samples were also incubated with normal rabbit serum or Tris buffer as controls. After the incubation period aliquots (20µl) were removed and assayed for enzyme activity.

2.14 Immunoprecipitation assay

The same procedure as in 2.13 was used except that at the end of the incubation period the samples were centrifuged for 5 min in an Eppendorf 3200 centrifuge (12,000g). Then aliquots (20µl) were removed from the supernatant and assayed for enzyme activity.

2.15 Immunoprecipitation studies with S. aureus protein A

2.15.1 Preparation of concentrated protein A

1ml of a crude S. aureus cell suspension containing protein A (10% w/v) was centrifuged for 2.5 min in an Eppendorf 3200 centrifuge and the pellet washed twice with 50mM-Tris/HCl, pH 7.5 then resuspended in 50mM-Tris/HCl, pH 7.5 containing 50% (v/v) glycerol to a final volume of 200µl. The concentrated protein A S. aureus preparation was stored at -20°C until required.

2.15.2 Immunoprecipitation assay with protein A

Appropriate dilutions (20µl) of antisera (in normal rabbit serum) or purified IgG (in 50mM-Tris/HCl, pH 7.5) were incubated with an equal volume of appropriate concentration (diluted in 50mM-Tris/HCl, pH 7.5) of purified enzymes or enzyme containing extracts for 15 min at room temperature with gentle rotation on a rotating wheel. Concentrated protein A (20µl) was added and the samples were further incubated for 1 h with gentle rotation at room temperature. Enzyme samples were also incubated with normal rabbit serum or with 50mM-Tris/HCl, pH 7.5 containing protein A, as described above, for use as controls. Protein A-immunocomplexes were sedimented by centrifugation for 5 min in an Eppendorf 3200 centrifuge. Then aliquots (30µl) from the supernatants were removed and assayed for enzyme activity.

2.16 Enzyme-linked immunosorbent assay (ELISA)

2.16.1 Stock solutions

Coating buffer : 50mM-sodium carbonate buffer, pH 9.6.

Washing solution : 9g NaCl supplemented with 0.5ml Tween 20 per litre.

Incubation buffer : 8.5g NaCl; 1.28g Na_2HPO_4; 0.156g $NaH_2PO_4.2H_2O$; 22.5µl Tween 20 per litre.

Substrate solution : 0.1mg/ml o-phenylene diamine.diHCl with 0.03% (v/v) H_2O_2 in citrate-phosphate buffer (25mM-citric acid in 50mM-Na_2HPO_4, pH 5.0). The substrate was prepared immediately before use from reagents preincubated at 23°C.

Stopping solution : 6N-H_2SO_4.

2.16.2 ELISA procedure

ELISA was performed in 96-well microtitre plates (Dynatech MicroELISA Systems). The plates were washed with distilled water, then incubated overnight at 4°C with 100ng antigen/well in 50µl of coating buffer to coat the bottom surface of the well. Plates were rinsed 5 times with washing solution which, because it contains Tween 20 will also block the plate surface and so prevent further adsorption of proteins onto the plate. 100µl of a serial dilution of antiserum or of purified IgG in the incubation buffer was added to each well and the plates incubated at room temperature for 1 h. Samples of normal rabbit serum and 50mM-Tris/HCl, pH 7.5 buffer were also diluted with incubation buffer and included as controls on each plate. After incubation plates were washed 5 times with washing solution and 100µl of peroxidase-conjugated antibody (HRP donkey anti-rabbit IgG) (1:1,000 dilution of conjugated antibody in incubation buffer) was added to each well. Plates were again incubated at room temperature for 1 h and subsequently washed 5 times with washing solution. 100µl of substrate solution was added to each well and the plates were incubated for 30 min at 23°C. The reaction was terminated by addition of 50µl stopping solution to each well and the colour measured at 492nm in a Titretek Multiskan Spectrophotometer.

2.17 Immunoblotting method

This method is based on that described by Towbin et al. (1979) as modified by Batteiger et al. (1982). This modified technique has been used by Nimmo et al. (1986).

2.17.1 Immunoblot from SDS PAGE

2.17.1.1 Stock solutions

Transfer (blotting) buffer : 0.19 M-glycine; 0.025 M-Tris; 0.02% (w/v) SDS and 20% (v/v) methanol.

Blocking buffer : 0.02 M-Tris/HCl, pH 7.2; 0.15 M-NaCl and 0.5% (v/v) Tween 20.

Washing buffer : 0.02 M-Tris/HCl, pH 7.2 and 0.15 M-NaCl.

Development solution : 30mg HRP colour development reagent (containing chloronaphthol) in 10ml methanol was mixed with 50ml 10mM-Tris/HCl, pH 7.4 and 150µl 4% (v/v) H_2O_2 immediately before use.

2.17.1.2 Immunoblotting conditions

When SDS PAGE was complete, the protein was transferred to 0.45µ nitrocellulose paper using a Bio-Rad Trans-blot cell. The transfer was carried out for 4 h in transfer buffer at 360-400mA (0.70 V, constant voltage) with the temperature maintained constant by cooling with tap water. After transfer the nitrocellulose paper was placed in blocking buffer for at least 16 h at 4°C. Under these conditions the unoccupied binding sites were blocked by Tween 20 (Batteiger et al., 1982). Specific antigens were detected on the membranes by incubating the nitrocellulose paper in blocking buffer containing 5% (v/v) normal donkey serum and 0.5% (v/v) specific antisera or purified IgG at 23°C for 90 min with gentle

agitation on an orbital shaker. The paper was washed four times in blocking buffer to remove unbound antibodies and once in washing buffer to reduce the Tween 20 concentration. The paper was then incubated in washing buffer containing 5% normal donkey serum and 0.1% HRP donkey anti-rabbit IgG at 23°C for 90 min with gentle agitation. After being washed a further five times in washing buffer, the blot was developed in freshly prepared development solution at 23°C for 5 min. The developed blot was then washed thoroughly in distilled water and dried at room temperature between sheets of filter paper.

2.17.2 Immunoblot from non-denaturing PAGE

2.17.2.1 Stock solutions

Stock solutions are the same as 2.17.1.1 except transfer buffer contained neither SDS nor methanol.

2.17.2.2 Immunoblotting conditions

Every step was carried out at 4°C but was otherwise the same as 2.17.1.2. The development step was performed at 23°C as in 2.17.1.2.

2.18 Molecular weight determinations

M_r estimations were performed either by SDS PAGE or gel filtration chromatography. Standard curves were obtained from proteins of known M_r.

2.18.1 Subunit molecular weight determination

The subunit M_r's of the purified proteins were estimated using SDS PAGE followed by Coomassie Blue or silver staining of the gels. The electrophoretic mobilities of the proteins were obtained

from the distance that each protein migrated through the separating gel. The following standard proteins (from Sigma) were used for calibration:

Protein	Subunit M_r
Phosphorylase b	94,000
Bovine serum albumin	67,000
Ovalbumin	45,000
Carbonic anhydrase	30,000
Soybean trypsin inhibitor	20,100
∝-lactalbumin	14,400

2.18.2 Native molecular weight determination

The native M_r values of purified enzymes were estimated by gel permeation chromatography at room temperature on a Superose 12 column in a Pharmacia f.p.l.c. apparatus. The column was eluted with 50mM-Tris/HCl, pH 7.5 containing 0.4mM-DTT (flow rate 0.5ml/min, fraction size 0.5ml). The eluate was monitored at 280nm. The column was calibrated with the following proteins from the shikimate pathway of *E. coli* whose molecular weights are known.

Protein	Native M_r
dehydroquinate synthase	39,000
dehydroquinase	52,000
shikimate dehydrogenase	30,000
shikimate kinase	20,000
EPSP synthase	47,000

3. Cross reaction within five central enzymes of the shikimate pathway

3.1 Introduction to immunology

3.1.1 Antigens and immunogens

The traditional meaning of antigen is any molecule which evokes a specific immune response but is also used more broadly to denote mixtures of molecules, whole microorganisms or cells used as an immunizing entity or as a complex target for antibody binding in immunoassays; thus red cells may be described as an immunizing antigen or as the antigen in agglutination tests. To distinguish between molecules that evoke antibody production and those that are the targets for antibody binding, it is the modern convention to use the term immunogen for the former and antigen for the latter (Walter, 1986; Catty, 1988). Natural immunogens are usually macromolecules, with a molecular weight greater than 1,000 and normally above 5,000. Smaller structures are usually less immunogenic but it is possible to increase their immunogenicity if they are covalently coupled to larger carrier molecules where they act as haptens on constructed immunogens (Catty, 1988). Often, bovine serum albumin or keyhole limpet haemocyanin are used as carriers (Scheidtmann, 1989). Such coupling also decreases the rate of degradation of haptens in the animal (Scheidtmann, 1989).

3.1.2 Antigen(ic) determinants: epitopes

These are structurally-defined sites of three-dimensional composition in both immunogens and antigens. Each determinant has a size corresponding to 4-6 amino acid or sugar residues, but the combination of shapes and charge distributions that can be offered

by determinants is nevertheless very large. It is common to divide determinants into two major categories (Benjamin et al., 1984; Berzofsky, 1985; van Regenmortel, 1987):

1. Segmental (continuous or sequential or linear) epitopes recognise the structural features that reside in a single short segment of the polypeptide chain.
2. Assembled topographic (conformational) epitopes recognise the amino acid residues that may be quite distant within the amino acid sequence but are brought into proximity during folding of the protein.

It is apparent that recognition by antibodies directed against assembled topographic epitopes would depend greatly on the preservation of requisite secondary and/or tertiary structural features with denatured proteins showing greatly decreased or totally abolished antigenicity, while epitopes of the segmental type should be reactive, if accessible, in both native and denatured forms of the enzyme. Considering that the adjuvants commonly used as vehicles for injection of immunogen would hardly be selected as the solvents of choice for maintaining native protein structure, and considering all the trauma that must assault a protein's structure as it proceeds from being injected to actually evoking an immune response, so immunisation with native protein can give rise to antibodies specific for the denatured form. It also follows that reactivity with antibodies directed against epitopes of the assembled topographic type might be influenced by perturbation of the tertiary structure, such as conformational changes induced by ligand binding, whereas this seems less likely with segmental epitopes (Wilson, 1987).

3.1.3 Antibody combining sites: paratopes (antigen binding sites)

These are the sites on antibody molecules which have stereochemical specific binding activity for complementary antigen determinants. They are constructed from the folding of variable domains of light and heavy chains of antibody molecules to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to these features of antigen determinants. It is estimated that the diversity-generating potential of the immune system offers in the range 10^6-10^7 different specificities to the antibody response (Catty, 1988).

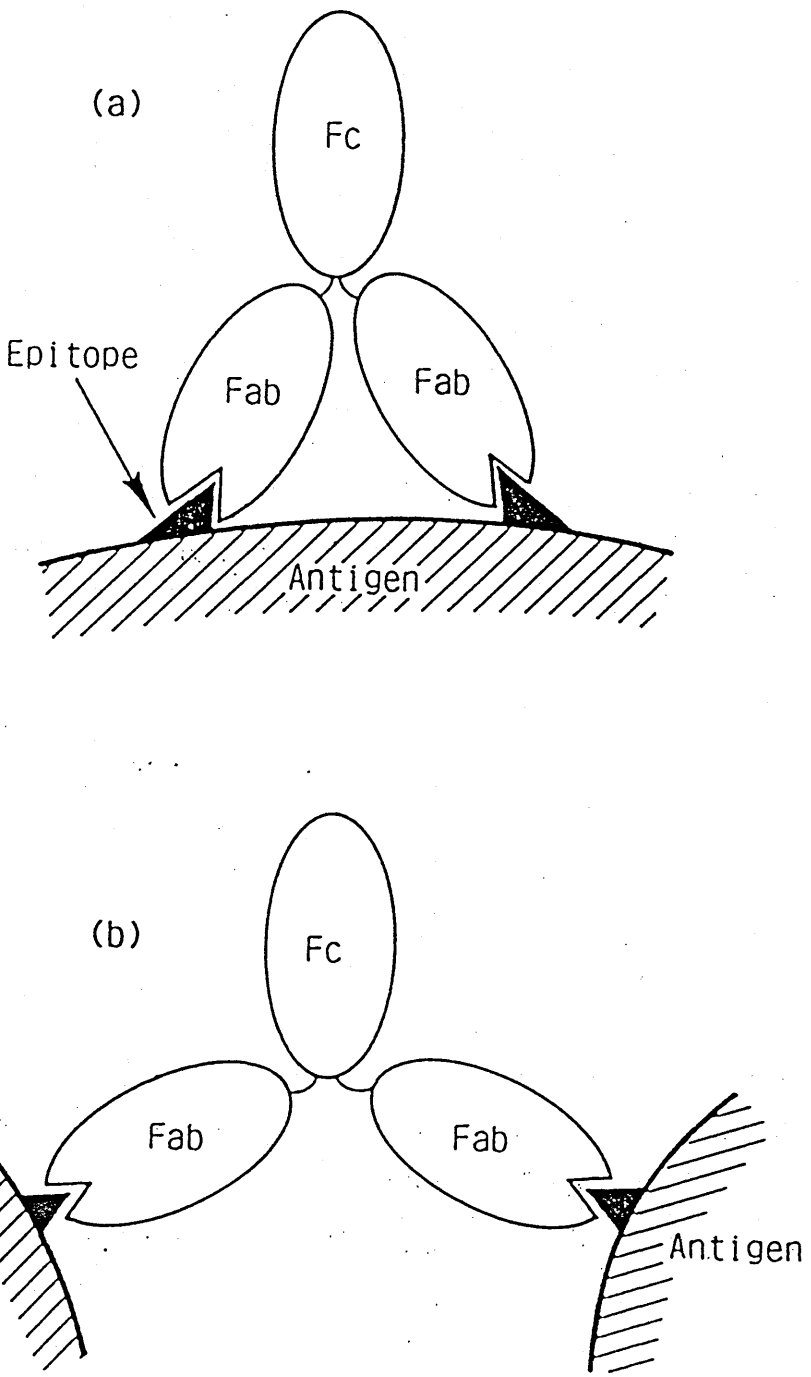
Most antibodies possess more than one functional binding site. For example, IgG molecules have two combining sites that can 'bridge' two determinants either within an antigen molecule or between two antigen molecules (Catty, 1988; Steward, 1984) (Fig. 3.1A). As a result, when interacting with a macromolecular antigen with more than one epitope, large complexes can form between antibody and antigen molecules.

3.1.4 Antibody specificity: cross reactivity

Antibody-antigen interactions can show a high degree of specificity. This means that the antigen combining sites of antibodies directed towards determinants on one particular antigen are not complementary to determinants on a second antigen. The specificity of an antiserum, by contrast, reflects the many specificities of the constituent antibodies . An antiserum may bind exclusively to one antigen if the range of its constituent antibody specificities extends to determinants exclusive to that antigen. Commonly, however, some antigen determinants are shared between molecules, especially if these are similar molecules of related species. In this case some antibodies induced in response

Figure 3.1 A Diagrammatic representation of antibody combining site

(a) binding of single antibody to two determinants on a single antigen; (b) binding of antibody to two antigens (adapted from Steward, 1984).



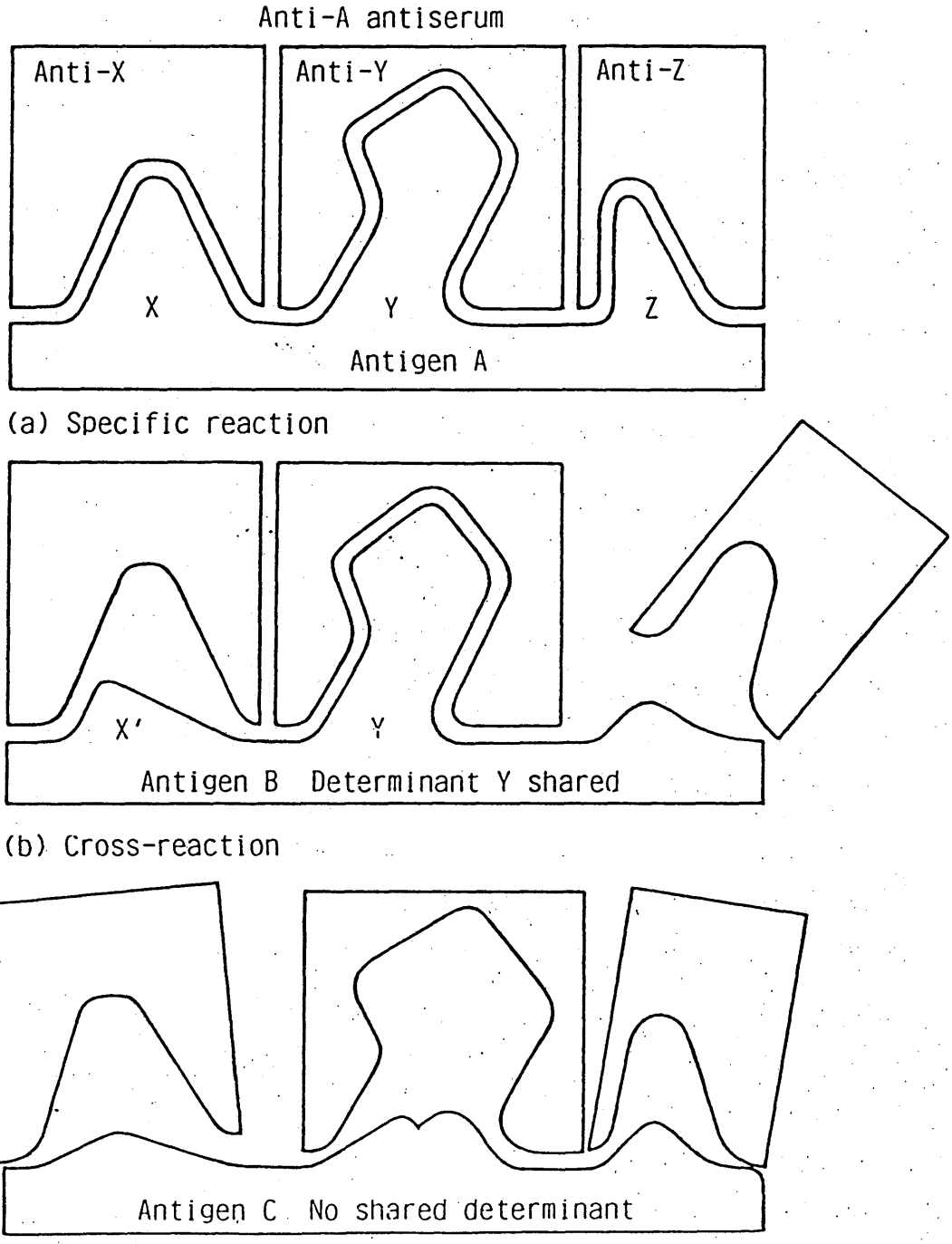
to one antigen may bind to another antigen and are then said to be cross-reacting and the containing antiserum is said to lack specificity or be cross-reactive. Cross-reactive antisera are shown in Figure 3.1B. Each antibody molecule within the antiserum to antigen A reacts with a different part of the same antigenic determinants (e.g. determinant X, Y and Z), and the combined reactivity of all the antibody molecules in the serum gives the antiserum its specificity (Fig. 3.1Ba). However, when some of the determinants on antigen A are shared by a second antigen B (e.g. determinant Y) (Fig. 3.1Bb) then a proportion of the antibody molecules directed to antigen A will also react with antigen B. Furthermore if the antiserum to A also weakly recognises another determinant on B (e.g. X') then this will also contribute to this cross-reactivity of antiserum to X'. Obviously, if no antigenic determinants are shared between antigen A and antigen C, there will be no reaction of anti-A with antigen C (Fig. 3.1Bc) (Steward, 1984; Catty, 1988).

3.1.5 Antibody affinity and avidity

The ability of antibodies to precipitate antigens from solution involves the formation of a lattice of cross-linked antibody-antigen complex which is sufficiently large to be easily sedimented from the solution by centrifugation. An immunoprecipitate will however not always be formed when antibody encounters antigen. The concentration of both antibody and antigen are very important and immunoprecipitation will only occur within a limited range close to the equivalence point (Johnstone & Thorpe, 1987). Outside of this range conditions of either antibody or antigen excess exist and only small (soluble) complexes of antibody-antigen are formed. The affinity of antibody for antigen

Figure 3.1 B The principle of antibody specificity and cross reactivity

(a) antiserum to antigen A (anti - X Y Z) reacts specifically with antigen A; (b) anti-A cross reacts with antigen B through recognizing determinant Y and partial recognition of X'; (c) anti-A shows no reaction with antigen C which has no shared determinants (from Steward, 1984).



is important in immunoprecipitation; usually only high affinity antibodies give good precipitation. Affinity relates to the exactitude of stereochemical fit of an antibody combining site to its complementary antigen determinant. Affinity is defined as the equilibrium constant when a monovalent antibody reacts with a monovalent antigenic determinant. In this case, where we use antisera of multiple specificity, to many determinants on a multivalent antigen, affinity cannot be assessed. Avidity can be considered and is defined as a combination of the individual affinities of each antibody combining site (antigenic determinant interaction). The terms affinity and avidity are sometimes replaced by 'intrinsic affinity' and 'functional affinity' respectively (Catty, 1988; Johnstone & Thorpe, 1987).

3.1.6 Properties of polyclonal antisera and monoclonal antibodies and their use in assessing homology between proteins

3.1.6.1 Polyclonal antisera

A polyclonal antiserum is the conventional serum product of an immunized animal, usually a rabbit, sheep or goat (Johnstone & Thorpe, 1987; Catty, 1988). It contains many different antibody specificities to the various epitopes of the structurally complex immunogen. The major advantage of polyclonal antisera lies in their capacity to form large insoluble immune complexes with antigen. It does so because the antiserum is the product of many clones of cells responding to the numerous antigenic sites present on the protein surface. Hence, the resulting polyclonal antibodies are extremely heterogeneous in their specificities, titre and affinity, and represent a collection of antibody molecules capable of simultaneously interacting with several different epitopes on the

proteins. Consequently extensive cross reaction may occur amongst antibodies and proteins which have similar determinants (Campbell, 1984; van Regenmortel, 1987). For all their value, animal antisera have certain limitations for exploitation in immunoassays, the main one being their heterogeneity in specificity, even when reacting to small antigens and their variability between animals and batches. In one antiserum there may be antibodies to many discrete antigens (multispecific or polyspecific), to a few antigens (oligospecific) or to a single antigen (unispecific), but even in the latter case the reagent is not homogeneous as single immunogens are still multi-determinant structures which stimulate a polyclonal, multi-determinant-specific response (Catty, 1988). In addition, the response to individual epitopes may be clonally diverse and antibodies of different affinity may compete for the same epitope. The significance of such heterogenicities within and between polyclonal antisera in practice means that each product is unique in specific antibody composition, in optimal binding conditions and in performance and requires to be separately assessed for its suitability in any particular immunoassay. Without affinity purification specific antibodies in antisera, or in the immunoglobulin fraction, are represented by, at most, 20-30% of the immunoglobulin in most cases. This reduces their efficiency in some procedures and may lead to high background readings in others.

Because of their polyclonal, multispecific nature, conventional antisera cannot be prepared easily or routinely to the degree of specificity needed to determine the structure and antigenic differences between molecules at the individual epitope level (Catty, 1988; Wilson, 1987). Nevertheless, polyclonal antisera are still useful for assessing the extent to which proteins possess common features because firstly they are easy to produce and

secondly because the broad range of determinants they can recognise ensures that any common features, whose actual structure is unknown, can be identified. Polyclonal antisera can be used to probe for common sequences in the primary structure before further analysis by protein or gene sequencing (Benyamin et al., 1986), for locating functional domains or detecting conformational changes in protein structures (Hue et al., 1989), to detect structurally similar regions in isoenzymes (Schwartz et al., 1980; Hirabayashi et al., 1987), for immunological quantification or purification of protein and peptide mapping (Sibbald & White, 1987).

The ability of antisera to detect cross reactivity immunologically is related to the degree of amino acid sequence similarity with cross reactivity between proteins only being observed if the level of sequence homology is above 60% of the primary sequence (Prager & Wilson, 1971a,b; ~~Schwarz et al., 1989~~). The cross reactivity will be influenced by the immunogenicity of the common epitopes and so epitopes which contain amino acids or structures which are highly immunogenic will promote detection of cross reaction. In this regard the amino acids histidine, lysine, alanine, leucine, aspartate and arginine, which are found most often in epitopes (Scheidtmann, 1989) will be important.

Disruption of the structure of a protein can be useful because it exposes new epitopes on the surface of the protein and so alters the range of features which are immunogenic. Denaturation by reduction and carboxymethylation has been shown in some cases, to generate an immunogen which produces an antisera which will recognise common features in proteins which cannot be detected if the native proteins are used as immunogens (Arnon & Maron, 1971; Arnheim et al., 1971; Zakin et al., 1978; Pekkala-Flagan &

Ruoslahti, 1982). In contrast to native proteins, a sequence homology of only 40% is required for cross reaction to be detected in these proteins (Arnon & Maron, 1971; Arnheim et al., 1971; Pekkala-Flagan & Ruos lahti, 1982). Denaturation by detergents, such as SDS, results in the production of an immunogen quite different from the native protein in the sense that antisera raised against the SDS treated protein will not recognise the native protein despite a 100% sequence homology (Schwartz, 1980). Whether this is due to SDS covering or partially covering all native antigenic sites is not clear. Similar results have been obtained when antisera were raised against peptides corresponding to portions of the primary sequence of the protein. In this case antisera against peptides did not recognise any features of the native protein but could bind to the protein after denaturation by reduction and carboxymethylation (Walter, 1986).

Hence antibodies have been used to look for common features in protein but their usefulness is influenced by the degree of sequence homology between the proteins, by the nature of the antigenic sites on the proteins and by the way the proteins are treated before use as an immunogen.

3.1.6.2 Monoclonal antibodies

Monoclonal antibodies have been made by immunising an animal with the protein, and subsequently, lymphocytes are prepared from the spleen or lymph nodes (Catty, 1988; Wilson, 1987). These cells possess the capacity for producing antibodies but are not capable of being propagated in culture. However, if these cells are fused with a suitable myeloma cell line, the resulting 'hybridoma' possesses two highly desirable properties: they are capable of producing antibodies, a capability inherited from their lymphocyte precursor, and they can be propagated indefinitely in culture, a characteristic conferred by the myeloma (Wilson, 1987).

The secreted antibody is homogeneous in specificity, affinity and isotype and each monoclonal product is specific to a single antigen determinant of the immunogen (monospecific) (Catty, 1988). The major disadvantage of monoclonal antibodies is that, due to their monospecificity, they lack or have only poor antigen precipitation properties as single reagents (Campbell, 1984). Monoclonal antibodies active against a particular epitope are ideal for determining if an identical or even comparable epitope is present on another protein. However, in this case, monoclonal antibodies are unsuitable for looking at common features on proteins because the nature of the epitopes are unknown. It would be necessary to raise a complete family of monoclonal antibodies before any meaningful comparative study could be carried out and this would only be worthwhile for doing detailed structural studies on proteins (van Regenmortel, 1987).

3.2 Production and characterisation of polyclonal antibodies

3.2.1 Production of antibodies.

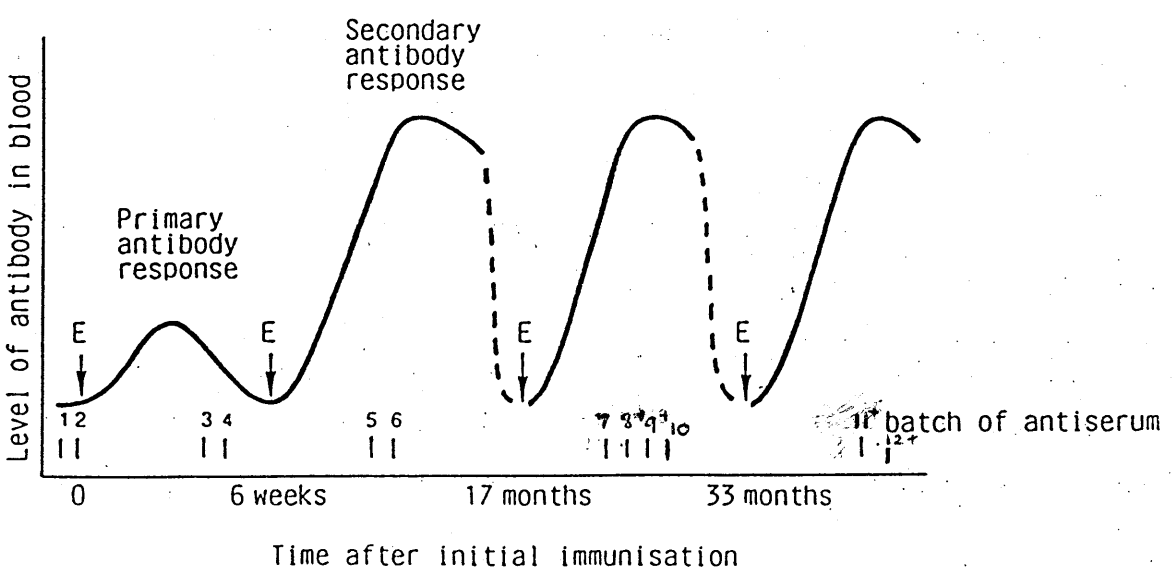
Antibodies were raised against particular antigens as described in section 2.12. Figure 3.2 represents an idealised picture of antibody levels against shikimate dehydrogenase during the immunisation of rabbit No. 159 with the enzyme shikimate dehydrogenase from *E. coli*. In addition to showing antibody levels, the diagram illustrates when blood for antisera production was removed from the rabbit and explains the nomenclature used in defining particular batches of antisera against an immunogen. Normally when an animal is first injected with an immunogen, there is a period of several days when no antibody can be detected in the blood. After a period of 7-10 days, antibodies appear and reach a

Figure 3.2 The immune response to E. coli shikimate dehydrogenase

The figure provides a diagramatic representation of the response of an animal to injection by immunogen. Injection of immunogen (E) stimulates antibody production. The initial injection produces a smaller response than subsequent (booster) injections. Samples of blood were taken at intervals, and antisera prepared as described in section 2.12.2.

The diagram illustrates the production of antibodies against shikimate dehydrogenase from E. coli where 12 batches of antisera were taken over a period of 36 months. The + indicates antisera used in this study.

For production of antibodies against other immunogens only a single boost injection was given and consequently the diagram is only applicable over the first two peaks. The antisera used for these immunogens would be equivalent to samples 5 and 6 on the diagram.



peak at about 14-21 days after which the level falls. The first response to the immunogen is termed the primary antibody response. When the animal is given a second injection of the immunogen, the levels of antibody in the blood begin to rise within two or three days and within about 10 days reach a level which is constant for approximately 6 weeks and is in excess of that achieved in the primary response. This is termed the secondary antibody response. This accelerated response is the result of the stimulation by the immunogen of antibody-forming cells of the immune system which had been sensitized by the earlier injection of antigen. Subsequent immunisations result in responses like the secondary response although both the level and nature of the antibody molecules in the blood can vary after additional immunisation injections. After preparation, each batch of antisera is tested to measure its titre and specificity. In the work described here the only immunisation that was examined in any detail was that produced by rabbit 159 against shikimate dehydrogenase from E. coli. The results available (Fig. 3.3) suggest that this immunisation is consistent with Figure 3.2.

3.2.2 Antisera titre and specificity

Three distinct assays were used to assess antisera. Initially the ability of the antisera to inhibit enzyme activity was used to show that an active antiserum had been produced. This was supplemented by an ELISA which used purified enzyme protein and gave a measure of binding. Thirdly the antisera was used in a blotting assay both to measure binding but more importantly to determine how specifically the antisera could recognise a particular protein in a crude cell extract.

3.2.2.1 Anti-shikimate dehydrogenase antisera

Several batches of anti-shikimate dehydrogenase antisera were assessed. These were all raised in a single rabbit with two batches (159:8 and 159:9) being prepared after one particular boost and the other two (159:11 and 159:12) after a later boost (Fig. 3.2). Antisera batch 159:10 was also assessed but gave a poor inhibition of enzyme activity and was not tested further. All four batches of antisera tested give very high levels of inhibiton of enzyme activity (approximately 100%) when mixed undiluted with enzyme. On assessing the titre of these antisera it was found that two of the batches 159:11 and 159:12 give a significantly higher titre than the others (Fig. 3.3). Similar results are obtained irrespective of whether a crude extract from a shikimate dehydrogenase overproducing strain of E. coli or a sample of pure shikimate dehydrogenase is used for the assay of titre (Fig. 3.3A,B). These results were confirmed by the ELISA using purified shikimate dehydrogenase as antigen. Batches 159:11 and 12 give significantly higher titres than the other two antisera (Fig. 3.4).

Immune blots were used to assess how specific the antisera were and the results are shown in Figure 3.5. Blotting, with all four batches of antisera, of both crude extracts of E. coli containing high shikimate dehydrogenase activity and samples of purified shikimate dehydrogenase, give similar results with crude extract samples showing the presence of a major band of the same molecular weight as purified shikimate dehydrogenase. When the gel is overloaded with enzyme subsidiary bands are obtained with samples of both pure enzyme and crude extract.

It was therefore concluded that the antisera raised against shikimate dehydrogenase all contain a high level of antibody against this protein and that overall the antisera are sufficiently specific

Figure 3.3 Assay of anti-shikimate dehydrogenase antisera by enzyme inhibition assay

Samples of anti-shikimate dehydrogenase (rabbit No. 159) were diluted in normal rabbit serum and each dilution mixed with an equal volume of shikimate dehydrogenase (0.045eu/assay). Samples were gently mixed at room temperature for 1 h then assayed for shikimate dehydrogenase activity as described in section 2.7.3. A control using normal rabbit serum was included to confirm the stability of shikimate dehydrogenase over the period of incubation. The ability of antisera to inhibit enzyme activity was assessed using both purified shikimate dehydrogenase (Fig. A) and crude extract from the E. coli shikimate dehydrogenase overproducing strain (Fig. B).

	dilution at 50% inhibition	
	A	B
O:159:8	1:4	1:4
□:159:9	1:2	1:2
●:159:11	1:32	1:32
●:159:12	1:32	1:32

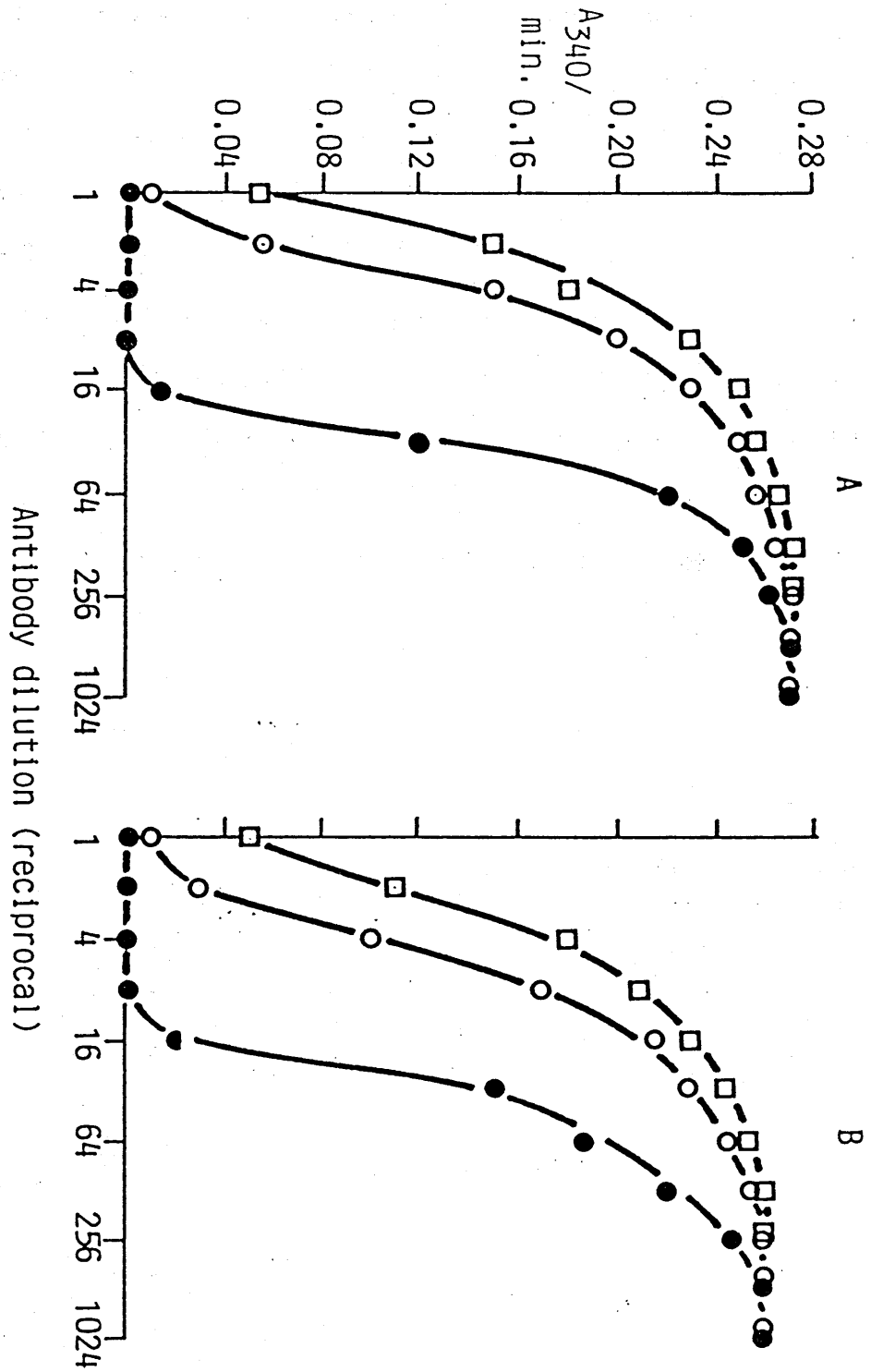

A
B
A_{340}/ min.
0.04
0.08
0.12
0.16
0.20
0.24
0.28
1
4
16
64
256
1024
Antibody dilution (reciprocal)

Figure 3.4 Assay of anti-shikimate dehydrogenase antisera by ELISA

An aliquot of native, purified shikimate dehydrogenase from *E. coli* (0.06 eu; 100ng protein/well) was added to each of the wells as a duplicate of a microtitre plate. After allowing binding to occur overnight at 4°C in coating buffer, the unbound material was washed from the plates and the plates used for ELISA as described in section 2.16.2.

Anti-shikimate dehydrogenase antisera were diluted in incubation buffer and added to the well. Normal rabbit sera, diluted in the same buffer, were included on each microtitre plates as controls.

After assay and reading at 492nm, the potency of the antisera was assessed as that dilution which gave an absorbance value of 0.5 above the normal rabbit sera control.

	dilution for A > 0.5
Δ:159:8	1:96,000
●:159:9	1:6,000
*:159:11	1:200,000
□:159:12	1:200,000
O:normal rabbit serum	1:250

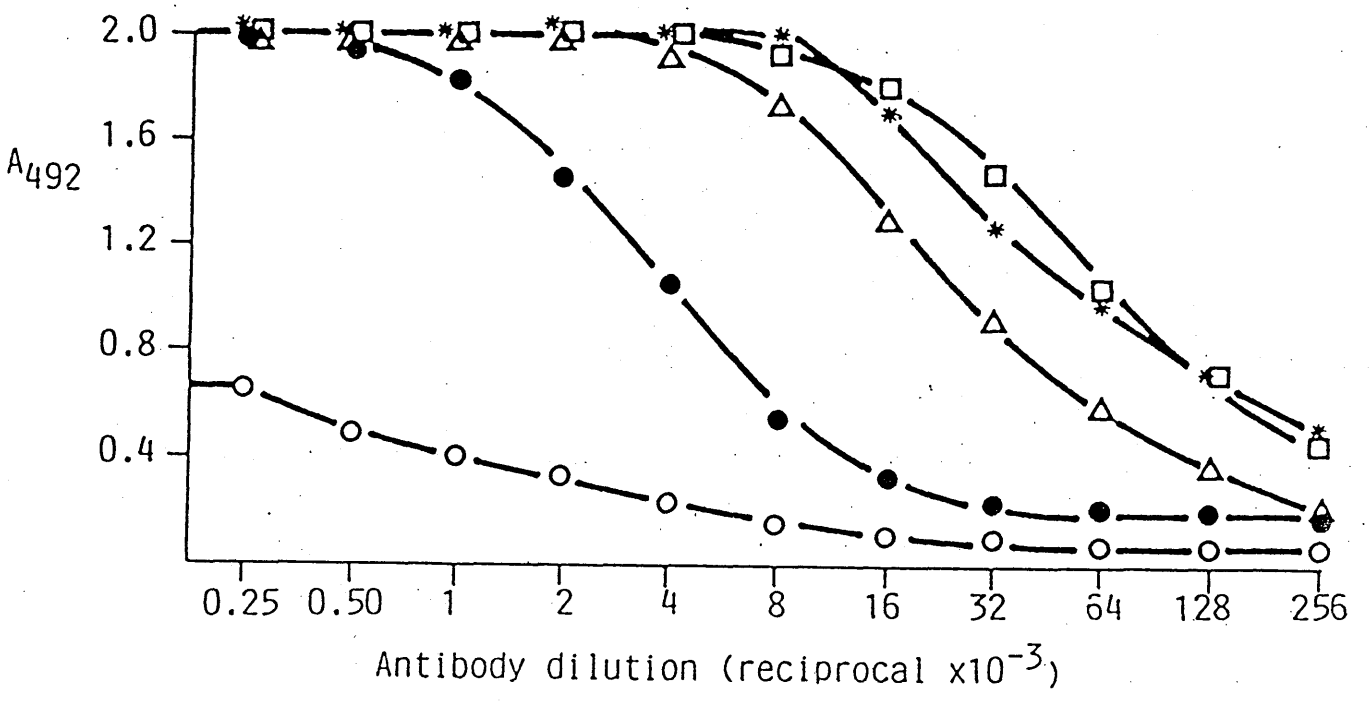


Figure 3.5 Immunoblotting of shikimate dehydrogenase from E. coli with anti-shikimate dehydrogenase antisera

Samples of both purified shikimate dehydrogenase from E. coli and of a crude extract from E. coli strain K12 : AB2834/pIA321 (shikimate dehydrogenase overproducer) were prepared and applied to a 12.5% SDS polyacrylamide gel and electrophoresis carried out as described in section 2.8.1.4.

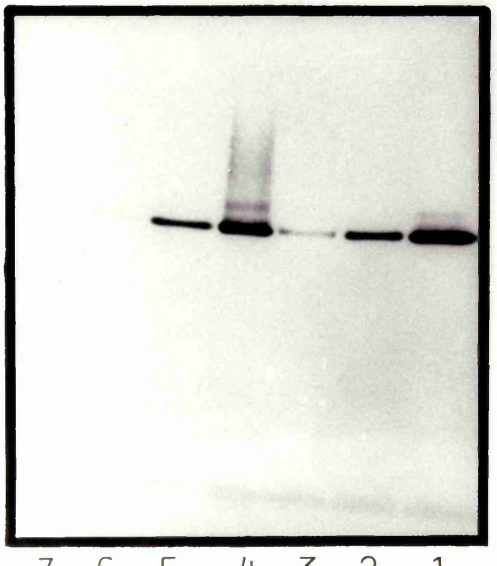
After electrophoresis the proteins were transferred to nitrocellulose and immunoblotted using several batches of anti-shikimate dehydrogenase antisera (see Fig. 3.2) as described in section 2.17.1.2. All antisera were used at a dilution of 1:200 in blocking buffer containing 5% normal rabbit sera and 0.5% Tween 20.

track No.	Sample applied (ng protein)
1	1000 (crude extract)
2	100 (crude extract)
3	10 (crude extract)
4	50 (pure E3)
5	5 (pure E3)
6	0.5 (pure E3)
7	M_r standard proteins

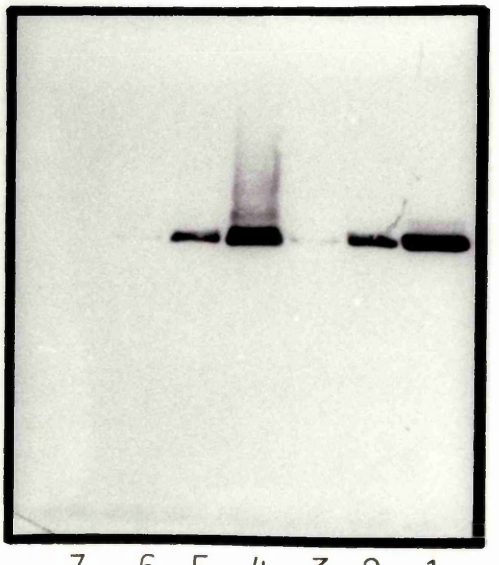
159:8

159:9

159:11



159:12



to be unable to detect any proteins, in crude extracts of E. coli, other than the immunogen against which they were raised. Antisera batches 159:11 and 159:12 were found to contain higher levels of antibodies than the other preparations and were the antisera chosen for further experiments.

3.2.2.2 Antisera raised against E. coli enzymes of the shikimate pathway

In total ten different antisera were raised against enzymes of the shikimate pathway. The E. coli enzymes which corresponded to the activities observed in the arom complex were used, in both their native form and in the form produced by reduction and carboxymethylation of the native form, as immunogens. The antisera produced were tested using the same tests as those used to test the anti-shikimate dehydrogenase antisera.

Assays, which depended on inhibition of enzyme activity, were used to assess the ability of antisera to bind to/inhibit the native form of the protein against which they were raised. The data show that antisera raised against the native form of the enzyme are effective in reducing the enzyme activity remaining in the assay (Table 3.1a and Fig. 3.6). Antisera raised against denatured protein have a lower titre in the enzyme inhibition assay (Table 3.1b) but in three cases (anti-E2, E3 and E4) they contain substantial levels of antibodies able to bind to the native form of the protein. In three cases out of five the addition of protein A S. aureus to the enzyme inhibition assay eliminates all enzyme activity from the supernatant (Table 3.1c). Since protein A S. aureus binds specifically to the Fc region of antibody (IgG) molecules (Langone, 1982), it will only remove enzyme activity from solution if the enzyme is bound to an antibody (IgG) molecule.

Table 3.1 Inhibition and immunoprecipitation of shikimate pathway enzymes by antisera

duplicate
A sample of pure native E1 (0.006 eu/assay) from E. coli overproducing strain was mixed with an equal volume of antisera raised against E1. The mixture was incubated at room temperature as described in section 2.13 then assayed for enzyme activity. Similar incubations using the pure native samples of the other shikimate pathway enzymes and their corresponding antisera were also set up, incubated as above and assayed for residual enzyme activity (section 2.7). The results are shown in Table a.

A second set of incubation was set up in which pure native enzyme was mixed with antisera directed against the corresponding denatured protein and the residual activity remaining after 1 h was assayed (Table b).

A third set of incubations similar to set 2 were set up but after incubation for 15 min protein A S. aureus was added as described in section 2.15.2. After centrifugation the activity remaining in the supernatant was assayed (Table c)

The results are expressed as the total activity lost (inhibition for enzyme inhibition assay and precipitation for immunoprecipitation assay) from the supernatant as a percentage of the control activity i.e.

$$\% \text{ inhibition or precipitated enzyme} = \frac{\text{activity in control S/N} - \text{activity in experimental S/N}}{\text{activity in control S/N}} \times 100$$

Table 3.1

a) Enzyme inhibition assay

Enzyme	anti-native enzyme	% inhibition
E1	310:2 (anti-E1)	100
E2	160:4 (anti-E2)	100
E3	159:11 (anti-E3)	100
E4	315:1 (anti-E4)	100
E5	163:2 (anti-E5)	100

b)

Enzyme	anti-denatured enzyme	% inhibition
E1	420:2 (anti-denatured-E1)	0
E2	421:2 (anti-denatured-E2)	92
E3	387:2 (anti-denatured-E3)	55
E4	423:2 (anti-denatured-E4)	60
E5	424:2 (anti-denatured-E5)	0

Immunoprecipitation assay with protein A

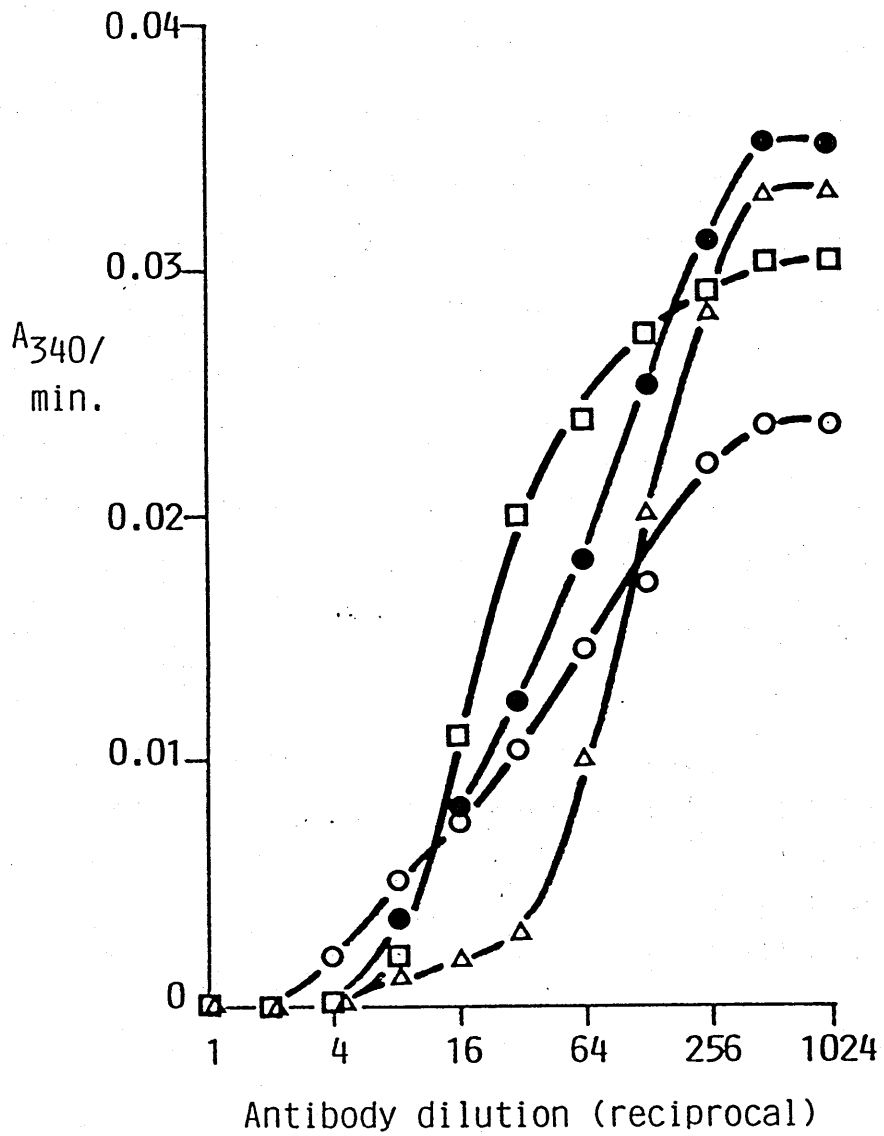
c)

Enzyme	anti-denatured enzyme	% precipitated enzyme
E1	420:2 (anti-denatured-E1)	0
E2	421:2 (anti-denatured-E2)	100
E3	387:2 (anti-denatured-E3)	100
E4	423:2 (anti-denatured-E4)	100
E5	424:2 (anti-denatured-E5)	0

Figure 3.6 Assay of antibody titre for antisera against aromatic pathway enzymes from *E. coli*

Antibodies, raised against the native enzymes of the shikimate pathway from *E. coli* (see Table 3.1a), were diluted in normal rabbit serum and each of the dilutions was incubated with an equal volume of a sample of the purified protein (approximately 0.006 eu/assay) against which it was raised. After incubation, with gentle mixing for 1 h, as described in section 2.13, the enzyme activity remaining was assayed as described in the methods section 2.7 for assay of each enzyme activity (section 2.7). A control in which each enzyme was incubated with a sample of normal rabbit serum, was included to measure the stability of the enzyme under the conditions of the incubation. No loss of activity in the controls was found. The titre was estimated as that concentration of antibody which gave 50% less of activity under the conditions used.

Enzyme	titre at 50% inhibition
●:E1 (dehydroquinate synthase)	1:32
O:E2 (dehydroquinase)	1:32
Δ:E4 (shikimate kinase)	1:64
□:E5 (EPSP synthase)	1:16


0.04
0.03
0.02
0.01
0
A_{340}/ min.
1
4
16
64
256
1024
Antibody dilution (reciprocal)

Two preparations of antisera, i.e. anti-denatured-E1 raised against denatured dehydroquinate synthase (E1) and anti-denatured-E5 raised against denatured EPSP synthase (E5), do not demonstrate a high antibody level against the native protein and even in the presence of protein A S. aureus are unable to remove enzyme activity from the supernatant (Table 3.1c).

It is clearly impossible to use an enzyme inhibition assay to assess the ability of antibody to bind to denatured enzyme and so an ELISA was used to assess the titre of antibody raised against denatured proteins to bind to both native and denatured proteins. As observed with the inhibition assay results, it was found that the antisera raised against denatured E1 and denatured E5 are of much lower titre against the native enzymes than the other antisera. All other antisera demonstrate substantial titres against both native and denatured enzymes (Table 3.2).

It was concluded from these results that, with the exception of anti-denatured-E1 and anti-denatured-E5, all of the antisera prepared contain high levels of antibodies against their immunogens and that antisera raised against denatured proteins contain substantial levels of antibodies which recognise the native protein. It was therefore thought likely that for each enzyme antibodies had been produced against a large number of the different epitopes present and that the antibodies would be able to recognise both assembled topographic and sequential epitopes of that enzyme.

All of these antisera were then tested in immunoblots, to assess their ability, firstly, to specifically identify particular proteins in crude extracts and, secondly, to recognise both the native and denatured forms of their own immunogen. The ability of the antisera to recognise specific proteins in crude extracts was carried out using extracts prepared from cultures which

Table 3.2 Assessment of antibody titre against both native and denatured enzymes by ELISA

ELISA plates were set up containing 100ng of purified protein/well and were used, as described in section 2.16, to measure the potency of antibody preparations. Antibodies were diluted in incubation buffer and the amount bound at each diluted measured by absorbance after processing at 492nm. A control of normal rabbit serum was included on each plate.

For each combination of enzyme and antiserum the titre was assessed as the greatest dilution which gave an absorbance of 0.5 above the control serum.

Table 3.2

Enzyme	Antiserum	dilution for A $\rangle$ 0.5
native enzyme	anti-native enzyme	titre
E1	310:2	1:51,200
E2	160:4	1:25,600
E3	159:11	1:200,000
E4	315:1	1:102,400
E5	163:2	1:38,400
native enzyme	anti-denatured enzyme	titre
E1	420:2	1:3,200
E2	421:2	1:102,400
E3	387:2	1:102,400
E4	423:2	1:51,200
E5	424:2	1:4,800
denatured enzyme	anti-native enzyme	titre
denatured-E1	310:2	1:204,800
denatured-E2	160:4	1:38,400
denatured-E3	159:11	1:51,200
denatured-E4	315:1	1:204,800
denatured-E5	163:2	1:51,200
denatured enzyme	anti-native enzyme	titre
denatured-E1	420:2	1:12,800
denatured-E2	421:2	1:51,200
denatured-E3	387:2	1:38,400
denatured-E4	423:2	1:51,200
denatured-E5	424:2	1:76,800

overexpressed one of the shikimate pathway enzymes. The results are shown in Figure 3.7 for each enzyme. In all cases it was found that the antibodies could identify, as a major band, the protein against which they were raised. There are three problems. Firstly one of the antisera (310:2; anti-E1) recognises one of the molecular weight markers. Secondly antisera 315:1 (anti-E4) recognises many proteins in the crude extract other than its own immunogen. Thirdly, the amount of enzyme required to be loaded onto the gel to ensure reliable detection (see legend to Fig. 3.7) is high and with the exception of E3, would be difficult to detect in an extract from a wild type strain. It was therefore concluded that in order to examine cross reactions it was essential to use purified enzymes (or at least partially purified enzymes) rather than crude extracts. Using purified proteins, applied to the gel at levels able to be detected by the antisera, it was found that antibody prepared against a particular immunogen is able to detect both the native and denatured form of the antigen. This result is true if either the native or denatured form of the protein is used as the immunogen (Figs. 3.8, 3.9, 3.10 and 3.11).

3.3 Immunological cross reactions between shikimate pathway enzymes

3.3.1 Antigenic cross reaction within the shikimate pathway enzymes of E. coli

Considerable attempts were made to demonstrate that the five enzymes of the shikimate pathway against which antisera had been prepared contained epitopes in common. Initially this was assessed using the enzyme activity assay and measuring the extent to which enzyme activity could be reduced by different antibody preparations.

Figure 3.7 Quantitative assessment of antigen detection by immunoblotting

Crude extracts were prepared from cells of each of the overproducing strains (section 2.6.5.1) and the specific activity of the overproduced enzyme measured. Samples, containing known amounts of enzyme activity, were run on 12.5% SDS slab gels (section 2.8.1.4) and blotted to nitrocellulose (section 2.17.1.2). A sample of pure enzyme (*) and molecular weight standards were run on each gel as markers.

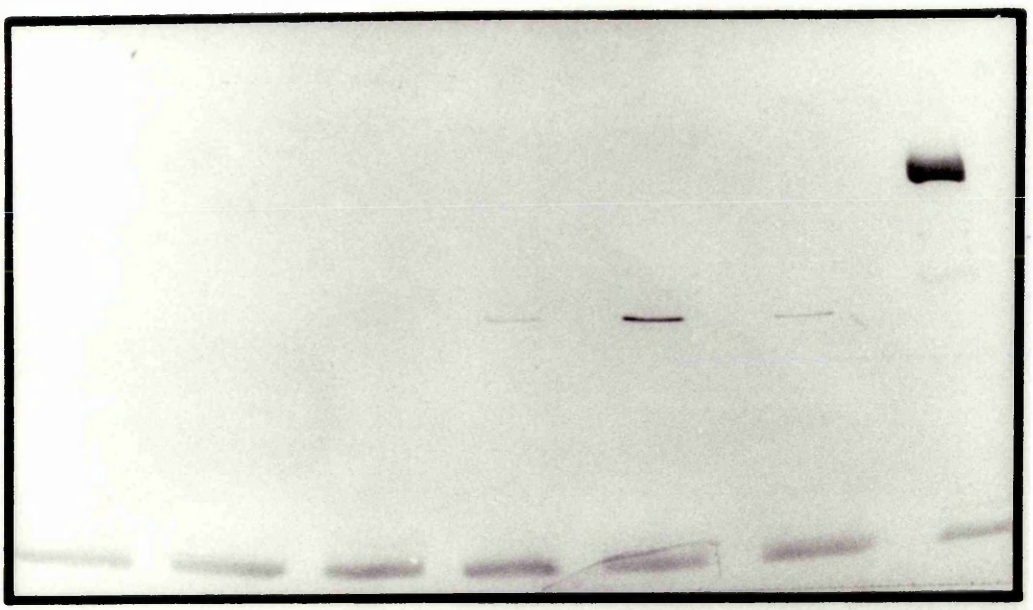
After blotting each enzyme was detected using antisera specific for that enzyme (see Table 3.1a).

The data are shown on Figure 3.7 (A to E) and are summarised below.

		detection limits/track	
		μunits	ngprotein
Figure A	E1 blotted with anti-E1	13	50
Figure B	E2 blotted with anti-E2	950	750
Figure C	E3 blotted with anti-E3	5450	250
Figure D	E4 blotted with anti-E4	400	1000
Figure E	E5 blotted with anti-E5	62	150

A.

anti-E1 (310:2)

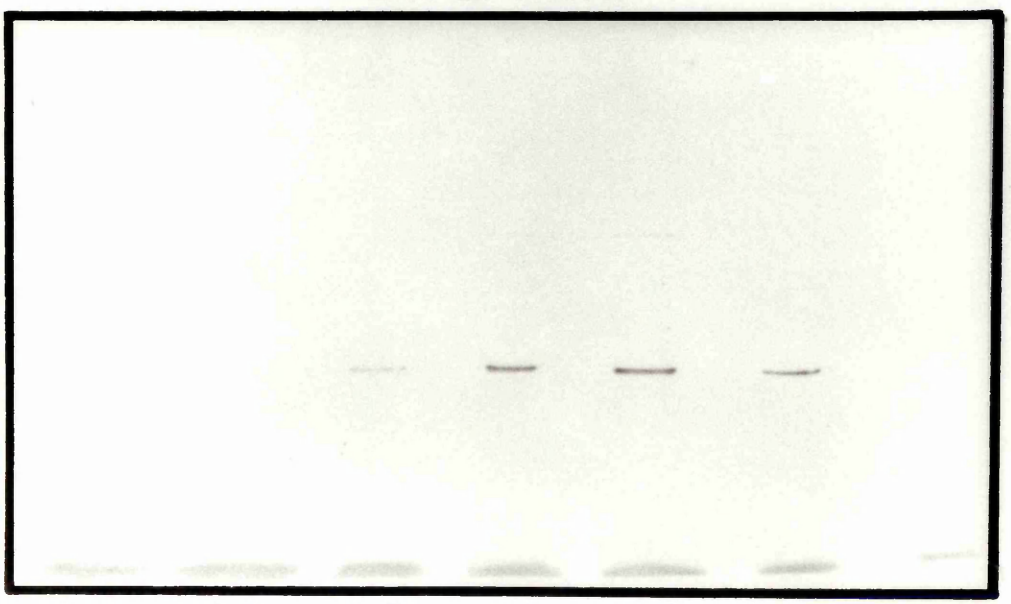


0.8 1.6 3.2 6.4 12.8 12.8* Markers

Amounts of enzyme (eu/track x 10^{-6})

B.

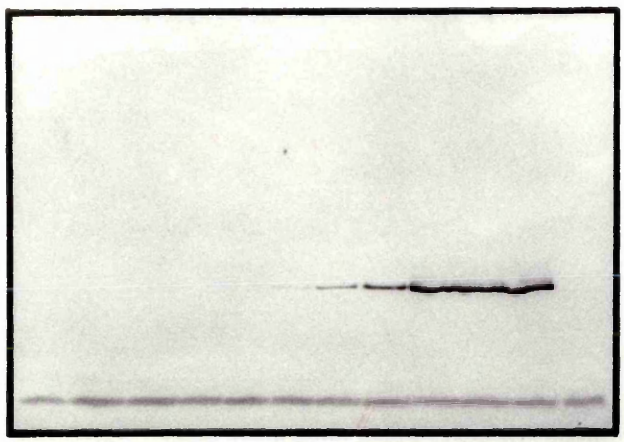
anti-E2 (160:4)



0.2 0.5 1.0 2.0 3.8 3.8* Markers

Amounts of enzyme (eu/track x 10^{-3})

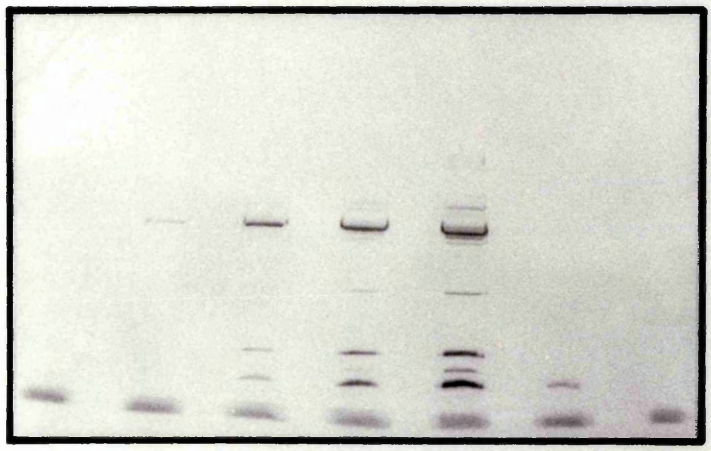
C. anti-E3 (159:11)



Markers 0.3 0.7 1.4 2.7 5.4 10.9 21.8 43.6 87.2 87.2* Markers

Amounts of enzyme (eu/track x 10^{-3})

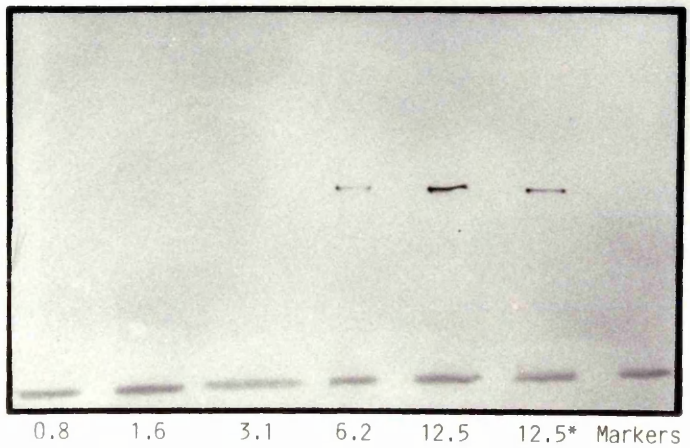
D. anti-E4 (315:1)



0.2 0.4 0.8 1.6 3.2 3.2* Markers

Amounts of enzyme (eu/track x 10^{-3})

E. anti-E5 (163:2)



0.8 1.6 3.1 6.2 12.5 12.5* Markers

Amounts of enzyme (eu/track x 10^{-5})

No reduction in activity is observed when an enzyme is mixed with any antisera other than the antisera raised against it (either as a native or denatured protein). The sensitivity of this assay was increased by using protein A S. aureus to precipitate all enzyme molecules bound to antibodies. After incubation in the presence of protein A S. aureus and centrifugation no loss of activity from the supernatant is observed suggesting no binding of enzyme to antibody occurs.

Similar results were obtained when immunoblotting was used as a technique to assess cross reaction. As with enzyme inhibition, antisera could only detect the protein that had been used as an immunogen in preparation of the antisera (Fig. 3.8, 3.9, 3.10, 3.11). The only consistent cross reaction that is observed is with the antisera raised against denatured dehydroquinate synthase (anti-denatured-E1) which is able to detect the denatured but not the native form of the enzymes E1, E3, E4 and E5 (Fig. 3.11, box 1). No cross reaction with denatured-E2 is observed (Fig. 3.11, box 1). The only group these four denatured proteins have in common is the carboxymethyl group derived from iodoacetate which is present as a result of the denaturation reaction. It was concluded that this group acted as a hapten or haptenic determinant on the dehydroquinate synthase protein molecules during immunisation and the antisera generated are able to detect this structure on all the denatured proteins. This conclusion was confirmed by the non cross reaction of denatured-E2 because this protein contains no cysteine residues (E. Borthwick, L.D. Graham & J.R. Coggins, unpublished results, see Appendix) and is therefore unable to be carboxymethylated. This cross reaction serves as a very good positive control for the assay system because the denaturation of shikimate dehydrogenase, for example, results in the insertion of only 3 carboxymethyl groups/molecule of enzyme. The result shows that the technique is

Figure 3.8 Antigenic cross reactions amongst five *E. coli* central enzymes corresponding to *N. crassa arom* complex

1) Native enzymes + antisera against native enzymes

Approximately 100ng samples of each of the purified shikimate pathway enzymes from *E. coli* which correspond to the *arom* complex and of the *N. crassa arom* complex were loaded onto 12.5% SDS polyacrylamide slab gels and were electrophoresed and blotted to nitrocellulose as described in section 2.17.1.2.

The blots were then incubated with antisera (see Table 3.1a) specific for one of the proteins on the gel and binding of antibody to the protein detected.

Each blot contained:

track 1: purified E1

track 2: purified E2

track 3: purified E3

track 4: purified E4

track 5: purified E5

track 6: purified *arom* complex

track 7: M_r standard proteins

The antisera used to detect the proteins are shown above each blot.

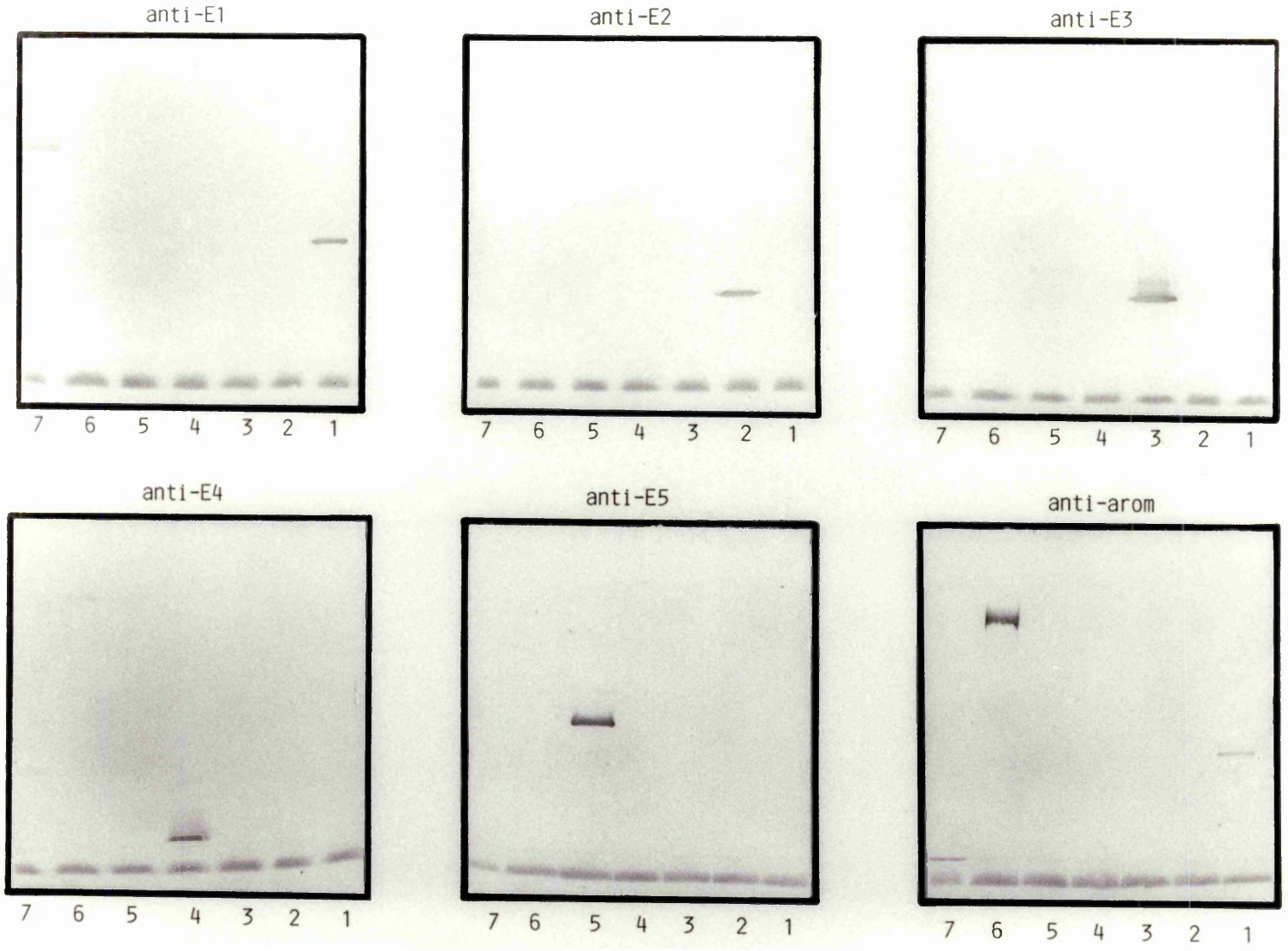

anti-E1
7 6 5 4 3 2 1
anti-E2
7 6 5 4 3 2 1
anti-E3
7 6 5 4 3 2 1
anti-E4
7 6 5 4 3 2 1
anti-E5
7 6 5 4 3 2 1
anti-arom
7 6 5 4 3 2 1

Figure 3.9 Antigenic cross reactions amongst five *E. coli* central enzymes corresponding to *N. crassa arom* complex.

2) Denatured enzymes + antisera against native enzymes

A second set of blots were set up as for Figure 3.8 except that the purified enzymes from *E. coli* were denatured by reduction and carboxymethylation (section 2.11.2) before loading to the gel. Electrophoresis, blotting and protein detection by antibody was carried out as for Figure 3.8.

Each blot contained:

track 1: carboxymethylated E1

track 2: carboxymethylated E2

track 3: carboxymethylated E3

track 4: carboxymethylated E4

track 5: carboxymethylated E5

track 6: M_r standard proteins

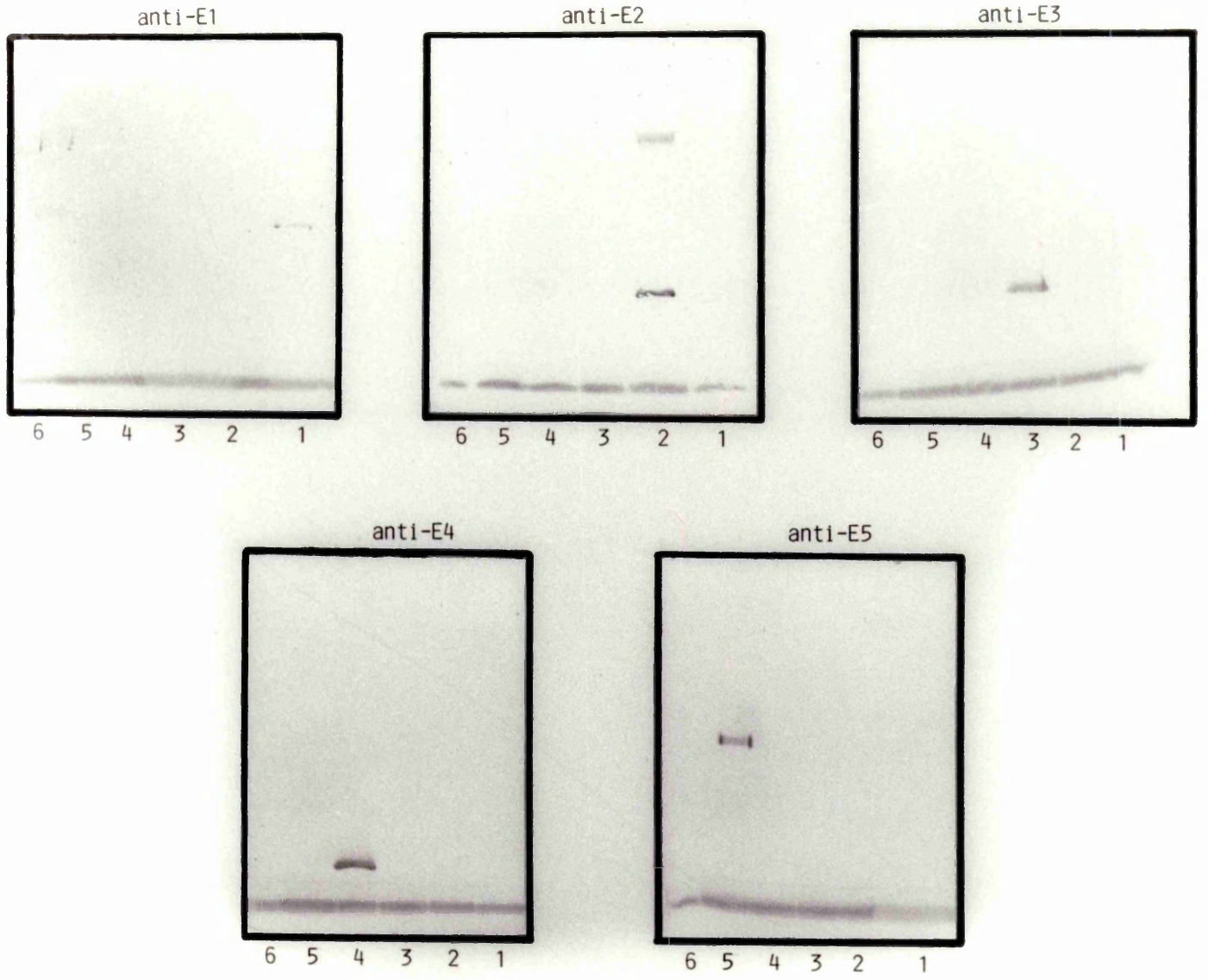

anti-E1
6 5 4 3 2 1
anti-E2
6 5 4 3 2 1
anti-E3
6 5 4 3 2 1
anti-E4
6 5 4 3 2 1
anti-E5
6 5 4 3 2 1

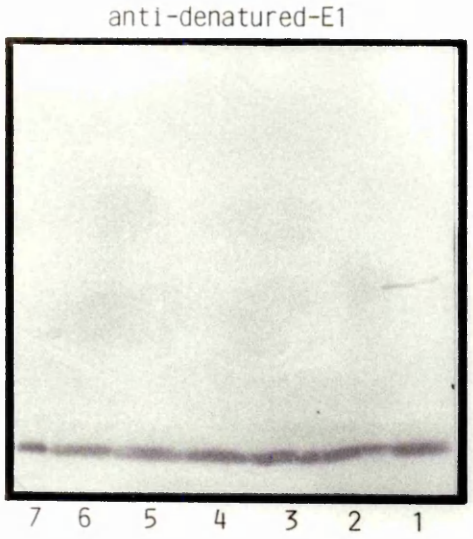
Figure 3.10 Antigenic cross reactions amongst five *E. coli* central enzymes corresponding to *N. crassa arom* complex

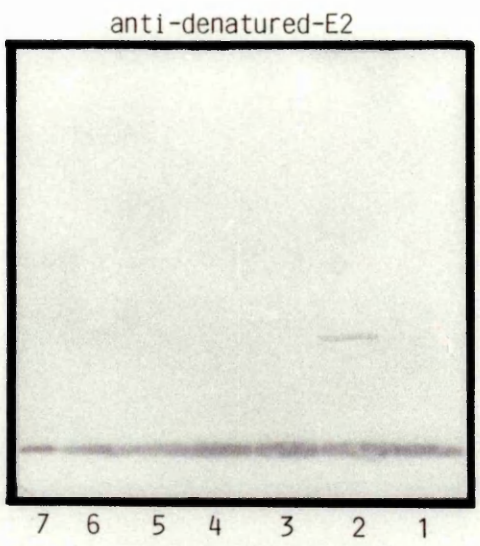
3) Native enzymes + antisera against denatured enzymes

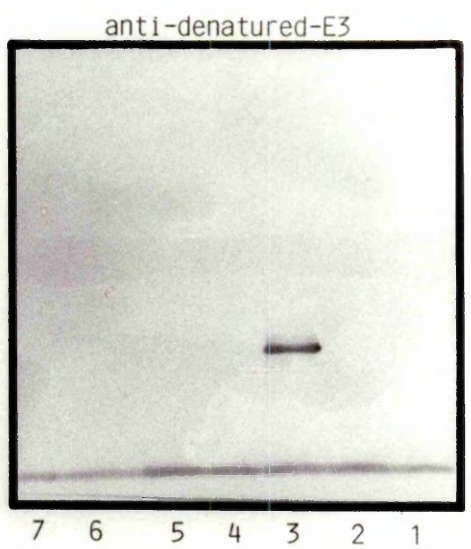
A third set of blots were set up as for Figure 3.8 except that the antisera used to detect proteins was raised against enzymes which had been denatured by reduction and carboxymethylation before use as an immungen (see Table 3.1b). All other conditions were as described in Figure 3.8.

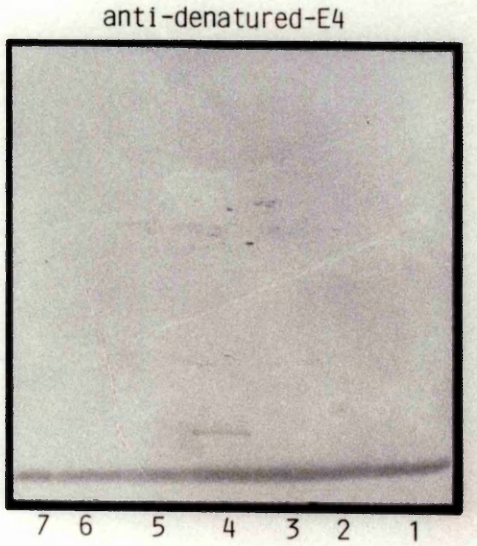
Each blot contained:

track 1:	purified E1
track 2:	purified E2
track 3:	purified E3
track 4:	purified E4
track 5:	purified E5
track 6:	purified *arom* complex
track 7:	M_r standard markers


anti-denatured-E1
7 6 5 4 3 2 1


anti-denatured-E2
7 6 5 4 3 2 1


anti-denatured-E3
7 6 5 4 3 2 1


anti-denatured-E4
7 6 5 4 3 2 1

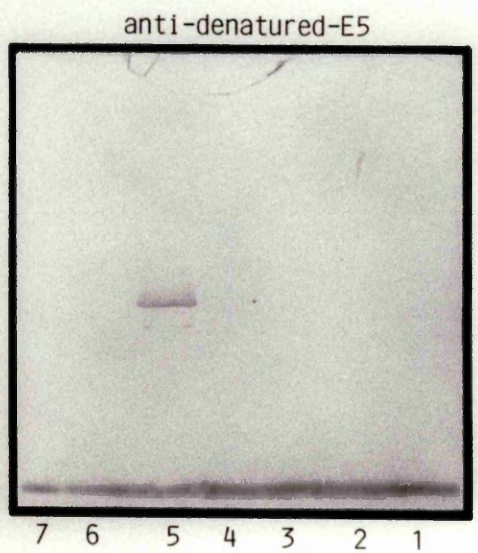

anti-denatured-E5
7 6 5 4 3 2 1

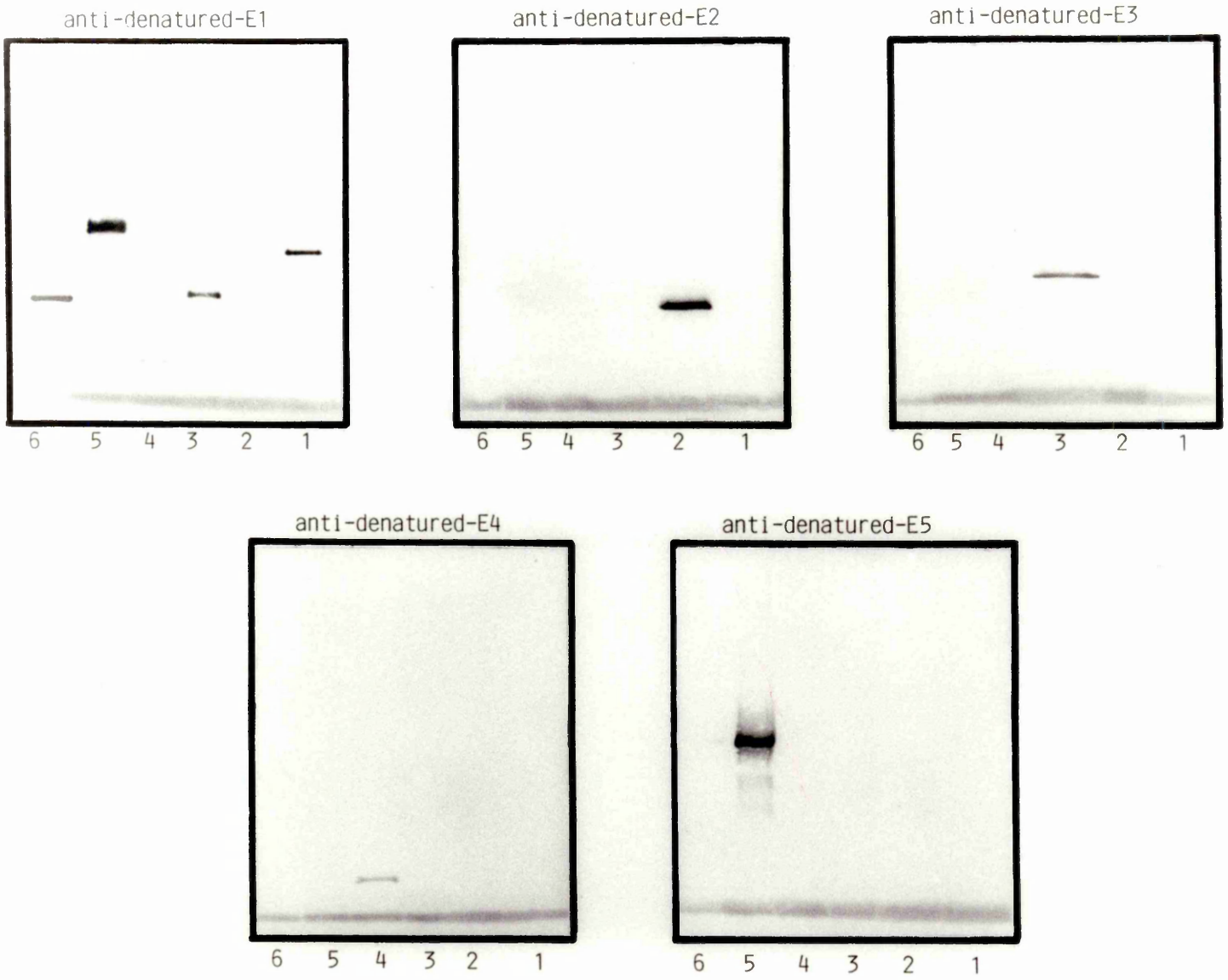
Figure 3.11 Antigenic cross reactions amongst five *E. coli* central enzymes corresponding to *N. crassa arom* complex

4) Denatured enzymes + antisera against denatured enzymes

A fourth set of blots were set up as for Figure 3.8 except that the purified enzymes were denatured by reduction and carboxymethylation before loading to the gel. As with Figure 3.10 the antisera used to detect the proteins was raised against enzymes denatured by reduction and carboxymethlation before use as an immunogen.

Each blot contained:

track 1:	carboxymethylated E1
track 2:	carboxymethylated E2
track 3:	carboxymethylated E3
track 4:	carboxymethylated E4
track 5:	carboxymethylated E5
track 6:	M_r standard proteins


anti-denatured-E1
6 5 4 3 2 1
anti-denatured-E2
6 5 4 3 2 1
anti-denatured-E3
6 5 4 3 2 1
anti-denatured-E4
6 5 4 3 2 1
anti-denatured-E5
6 5 4 3 2 1

able to demonstrate a cross reaction when a protein has only 3 epitopes able to bind antibody.

The conclusion from these experiments must therefore be that there is insufficient sequence or structural homology among these proteins of the shikimate pathway in E. coli to generate common antigenic determinants.

3.4 Antibody against arom complex from N. crassa

Antibody against arom complex was raised in rabbits using purified arom complex from N. crassa as an immunogen (section 2.12). The nature and titre of the antisera was determined using the enzyme inhibition assay.

3.4.1 Inhibition of activity of arom complex by anti-arom-antisera

Initially it was observed that the antibody preparation could only inhibit the E1 and E5 activities of the arom complex, with the E2, E3 and E4 activities being unaltered by incubation in the presence of antibody (Table 3.3a). All activities could however be precipitated by centrifugation of the arom complex/ antibody mixture even in the absence of protein A S. aureus (Table 3.3b).

Inhibition of E1 and E5 activities were dependent on the concentration of antisera added with a 1:8 dilution being sufficient to reduce both activities by 50% (Fig. 3.12). The potency of this antisera was therefore comparable to the other antisera prepared for this study.

Since it is known that all 5 activities are associated with the complex and are all precipitated by antibody, the inability of the antisera to inhibit E2, E3 and E4 activities suggests that the active site for these activities is buried within the structure of the complex and thus not susceptible to inhibition.

Table 3.3 Inhibition and precipitation of *N. crassa* *arom* complex by anti-*arom* antiserum

A sample of *arom* complex (6.6µg or 0.02eu of E3) was mixed with an equal volume of undiluted anti-*arom* antiserum (311:3) and incubated at room temperature with gentle mixing as described in section 2.13. After incubation, aliquots were taken from the mixture and assayed for each of the activities in the *arom* complex. Activities assayed were compared to control activity obtained by incubating *arom* complex with normal rabbit serum (Table a).

The sample was centrifuged at 12,000g for 5 min and the supernatant assayed for residual activity. As for Table a the activities assayed were compared with those from a control incubation containing normal rabbit serum (Table b).

a) enzyme inhibition

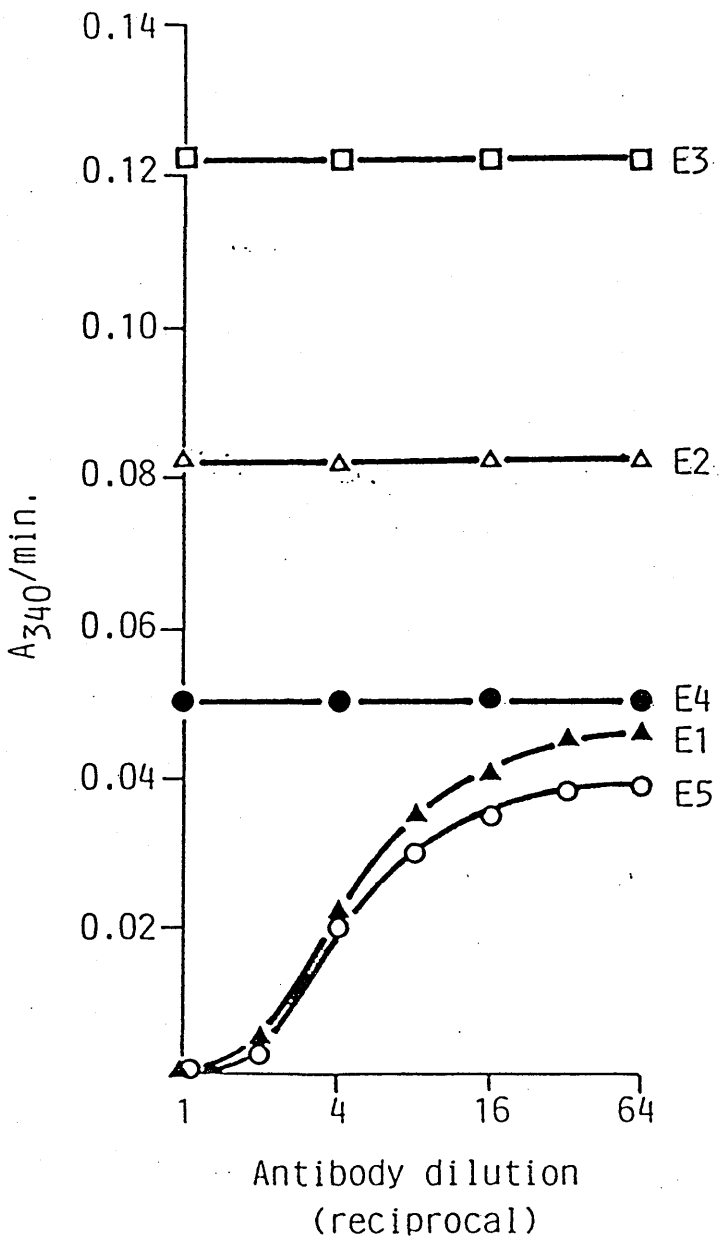
enzyme in *arom* complex	% inhibiton
E1	100
E2	0
E3	0
E4	0
E5	100

b immunoprecipitation

enzyme in *arom* complex	% precipitated enzyme
E1	100
E2	100
E3	100
E4	100
E5	100

Figure 3.12 Anti-arom antiserum titre by enzyme inhibition

Samples of antiserum raised against N. crassa arom complex (311:3) were diluted in normal rabbit serum and aliquots of each dilution were mixed with equal volumes of purified N. crassa arom (6.6µg or 0.02eu of E3). After incubation for 1 h at room temperature with gentle mixing (section 2.13) the activity remaining of the five enzymes of the complex was assayed. A control incubation with normal rabbit serum showed no change in activities from that observed with the 1:64 dilution of arom specific antiserum.



3.4.2 Proteolysis of arom complex

Limited proteolysis of the arom complex has been shown to generate 2 major and 1 minor fragments (Fig. 3.13). Of the two major fragments one of them (110K) contains the E1 and E5 activities while the other (68K) contains the E2 and E3 activities. The minor fragment (74K) contains only E5 activity (Coggins et al., 1985; Boocock, 1983). Limited proteolysis therefore represents a method of separating the E2/E3 activities from the complex and allows the ability of the antisera to bind to different parts of the complex to be ascertained.

Limited proteolysis was carried out as described by Boocock (1983). Purified arom complex was diluted in 50mm-potassium phosphate, pH 7.0, to a protein concentration of 100µg/ml. Trypsin (1mg/ml stock solution in 1mM-HCl) was added to a concentration of 100µg/ml and the mixture incubated at 25°C. The reaction was terminated after 2 min by passing the mixture through a 1ml column of immobilised trypsin inhibitor. 1ml fractions were collected and active fractions pooled.

The interaction of the antibody with the proteolysed arom complex was tested in two ways. Firstly the arom complex was run on SDS-PAGE and immunoblotted. The results (Fig. 3.14) showed that

a) unproteolysed arom complex binds antibody (track 1)

b) the proteolysis was complete with no unhydrolysed arom complex remaining after hydrolysis (track 2)

c) antibody recognises all three breakdown fragments produced by the hydrolysis.

The antiserum therefore contains antibodies which can bind to all of the fragments including the 68K fragment which contains E2 and E3 activities.

Figure 3.13 Proposal of mosaic model of N. crassa arom complex

Boocock (1983) has proposed the mosaic model for the structure of the M_r 165K arom complex based on data from limited proteolysis. The immediate products of an initial rapid cleavage by trypsin of the intact arom complex are two fragments of M_r 110K and 68K respectively. The 68K fragment or C-terminal domain contains the E2 and E3 activities while the 110K domain or N-terminal region contains the E1 and E5 activities. If proteolysis is continued, the 110K fragment disappears and a 74K is produced. E1 activity also disappears. The 74K fragment carries E5 activity and does not overlap the 68K fragments.

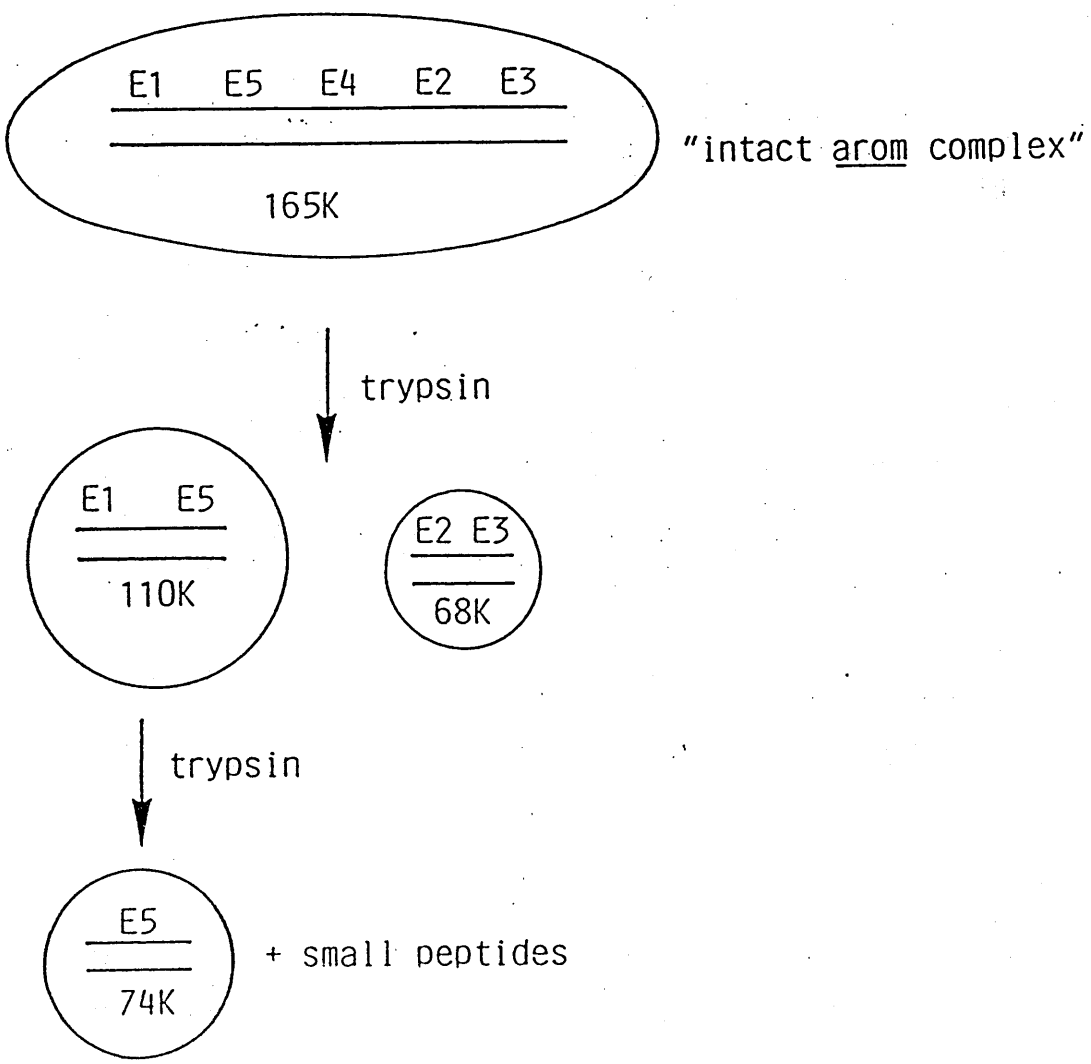
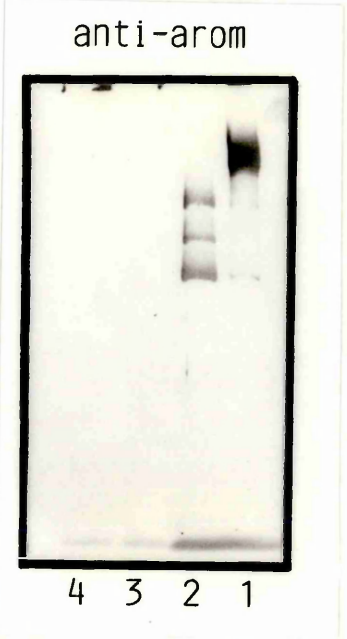


Figure 3.14 Immunoblot of *arom* proteolysate with anti-*arom* antiserum

N. crassa arom complex was digested with trypsin for 2 min and the digestion terminated by passing the mixture through an immobilised trypsin inhibitor column as described in section 3.4.2. The active fraction (ca. 50ng protein) was loaded onto a 12.5% SDS PAGE and then electrophoresed and immunoblotted as described in section 2.17.1.2. A sample of undigested *N. crassa arom* complex (ca. 100ng) was used as a control.

track no.	protein (ng)
1: undigested *arom*	100
2: digested *arom*	50
3: *E. coli* E3	100
4: M_r standard proteins	



The proteolysed arom complex mixture was then incubated with anti-arom antiserum. All five activities were measured. The results obtained (Table 3.4) were the same as those obtained using the unproteolysed arom complex (except for E4 which is destroyed by proteolysis). E1 and E5 activities are inhibited by antibody. E2 and E3 activities are not despite the fact that antibodies which bind to the E2/E3 arom fragment (Fig. 3.14) are present in the anti-arom antiserum. After centrifugation all activities are lost from the supernatant.

The antisera prepared against arom complex is therefore able to bind to many sites on the arom complex but binding is unable to inhibit activity of E2, E3 and E4 in the complex.

3.5 Cross reactions using enzyme preparations from different species

Antibodies were used to assess the presence of common epitopes between proteins with the same enzymic activity from different species using a combination of inhibition of activity and immunoblotting techniques. Three particular combinations were analysed:

a) comparison of arom complex from different species

b) comparison of arom complex from N. crassa with the corresponding enzyme activities from E. coli

c) comparison of E3 (shikimate dehydrogenase) activity from several organisms.

3.5.1 Common epitopes between the arom complex from N. crassa and yeast

The presence of common epitopes between arom complex prepared from both yeast and N. crassa was investigated by immune blotting. The results indicate that these two complexes contain

Table 3.4 Inhibition and immunoprecipitation by anti-*arom* antiserum of *arom* after trypsin digestion

Purified *arom* complex (100µg) was digested with trypsin and the hydrolysis terminated by passing the mixture through an immobilised trypsin inhibitor column (section 3.7.1). The fraction containing active *arom* fragments was mixed with an equal volume of anti-*arom* antiserum, incubated with mixing at room temperature for 1 h then assayed for the activities of the *arom* cmplex (section 2.13) (Table a). After assay the sample was centrifuged at 12,000g for 5 min and the activity remaining in the supernatant assayed (Table b).

A control incubation containing normal rabbit serum and proteolysed *arom* fragments was set up and assayed as above. Results presented here are activities found after incubation with *arom* specific antiserum compared to normal rabbit serum.

a) enzyme inhibition

enzyme in *arom* complex	% inhibiton
E1	100
E2	0
E3	0
E5	100

b immunoprecipitation

enzyme in *arom* proteolysate	% precipitated enzyme
E1	100
E2	100
E3	100
E5	100

common epitopes (Fig. 3.15). As far as can be assessed from the position of the bands on the SDS-PAGE the complexes from the two organisms have the same molecular weight.

3.5.2 Common epitopes between N. crassa arom complex and the corresponding enzymes in E. coli

Considerable attempts were made to demonstrate the presence of common epitopes between these activities. Samples of purified arom complex were incubated with each of the ten antibodies raised against the E. coli enzymes. No loss of activity of any of the component enzymes of the arom complex is found as a result of incubation. The sensitivity of this system was increased by use of protein A S. aureus to promote precipitation of any arom complex bound to antibody. No arom complex is precipitated by centrifugation. The reciprocal experiments were also carried out with pure samples of each of the five E. coli enzymes being incubated with anti-arom antiserum and the activity remaining in solution assayed. Again these incubations were repeated using protein A S. aureus to precipitate any active enzyme bound to antibody. In none of these incubations is the activity of any of the enzymes decreased as a result of the incubation with the antisera.

The analysis was extended by using ELISA to measure the amount of antibody bound to an antigen fixed to the ELISA plate. Figure 3.16 shows the results obtained for an ELISA in which pure arom complex from N. crassa was bound to the plate and the binding to this protein of antibodies raised against arom and against shikimate dehydrogenase from E. coli measured. No binding above background can be detected between the arom complex and either of the anti-shikimate dehydrogenase antisera. These binding studies were extended by using blotting rather than the ELISA method and the

Figure 3.15 Immunoblot of *N. crassa arom* and yeast *arom* using anti-*N. crassa arom* antiserum

50ng of *N. crassa arom* complex and yeast *arom* complex were loaded onto a 12.5% SDS PAGE, then electrophoresed and immunoblotted as described in section 2.17.1.2.

track no.

1: *N. crassa arom* complex

2: yeast *arom* complex

3: M_r standard proteins

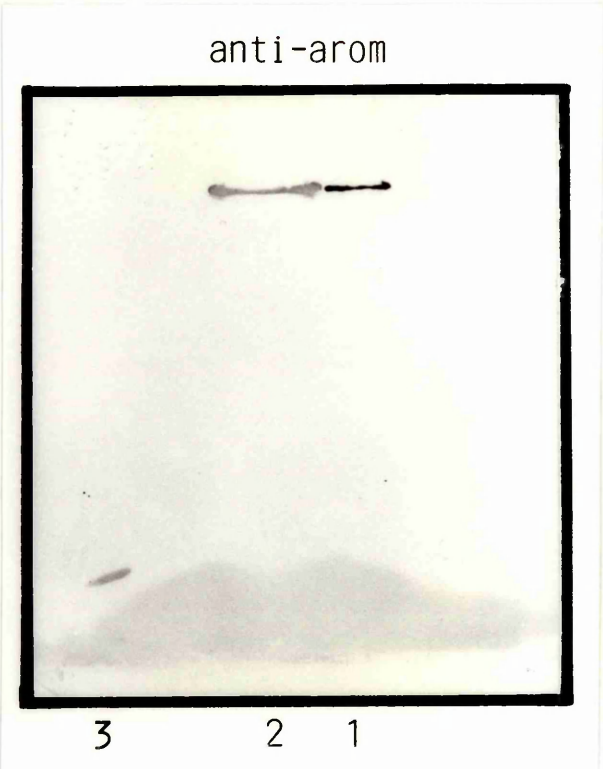


Figure 3.16 Binding of anti-*N. crassa arom* antiserum and anti-*E. coli* E3 antiserum to *N. crassa arom* complex by ELISA.

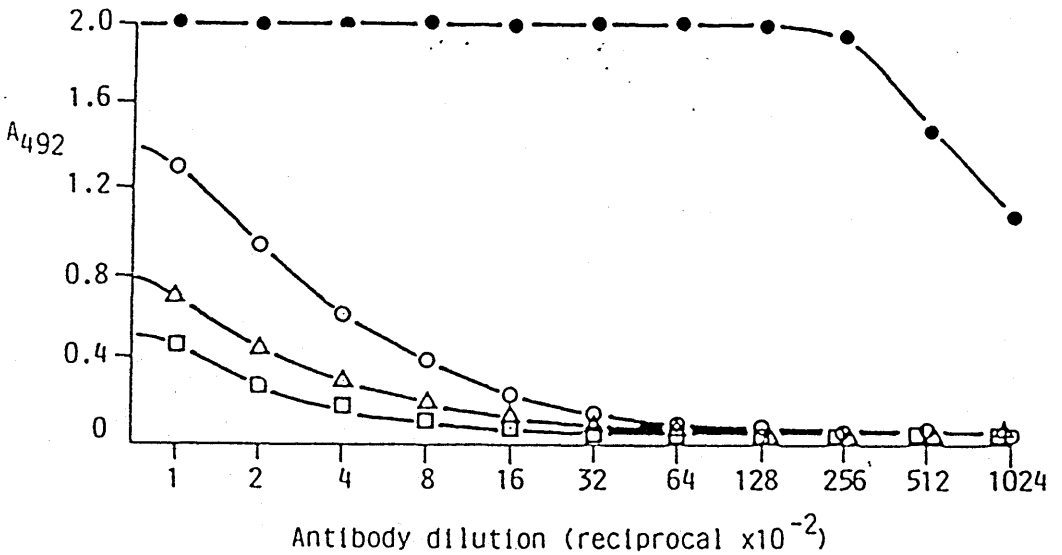
100ng of purified *arom* complex was bound to the surface of the wells of an ELISA microtitre plate and the ability of dilutions of antibody preparations in incubation buffer to bind under conditions as described in section 2.16.2 determined. All assayed were carried out in duplicate with controls, using normal rabbit serum, included on each plate.

●:311:3 anti-*N. crassa arom* complex

△:159:8 anti-*E. coli* E3 antiserum

□:159:11 anti-*E. coli* E3 antiserum

○: normal rabbit serum



results are shown in Figures 3.8 and 3.10. The blots shown on Figure 3.8 include arom complex in track 6. The first five boxes of this figure show that the presence of arom cannot be detected by any of the antisera raised against 'native' E. coli enzymes as immunogen and the final box shows that anti-arom antibody cannot bind to any of the E. coli enzymes. Figure 3.10 shows a similar experiment using antisera raised against the carboxymethylated E. coli enzymes. As before no binding of these antisera to arom can be detected. In some cases faint bands are seen on blots e.g. Figure 3.8, box 6 where an apparent cross reaction between anti arom antiserum and E1 is found. These cross reactions are due either to artifacts or to a low level of contamination of the pure proteins. In no cases where a band indicating a cross reaction is seen, does the band correspond to the location of the enzyme on the gel. These experiments show that no antigenic cross reaction can be detected between the arom complex and the corresponding enzyme activities in E. coli. It follows that if these proteins do have homologies in their amino acid sequence it must be less than 40% of the total sequence as this is the generally accepted minimum level of homology necessary to detect common antigenic features.

3.5.3 Common epitopes among shikimate pathway enzyme from various organisms

3.5.3.1 Shikimate dehydrogenase as a model system

Shikimate dehydrogenase has been chosen to investigate the degree to which common features occur and can be detected among the enzymes of the shikimate pathway in different organisms. It was selected because adequate supplies of antisera against the E. coli enzyme were available and because the ~~activity~~ assay is both sensitive and

easy to use even in crude extracts when E3 activity is only present in small amounts. The other activaties of the pathway suffer either because their assay procedure is less sensitive than the assay for shikimate dehydrogenase or because they are coupled to NADH as a cofactor. NADH oxidase activity in crude extracts is high and even after steps, such as ultracentrifugation, ammonium sulphate precipitation and cyanide inhibition, are taken the oxidase activity is still too high to allow easy assay of the NADH coupled enzymes.

Initial experiments were to be carried out using crude extracts and it was therefore essential to ensure that the extracts prepared contained the highest possible specific activity of shikimate dehydrogenase and that the method chosen to assess cross reaction was able to give a reliable answer.

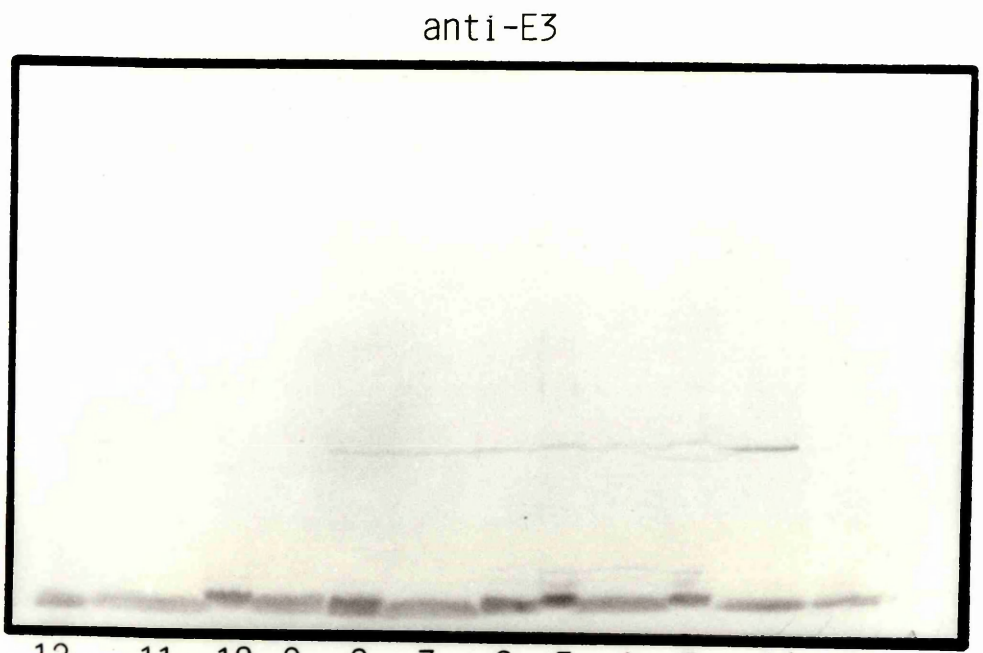
To produce extracts of high specific activity three organisms (two strains of E. coli and one of E. carotovora) were chosen and grown on media containing different carbon sources. Extracts prepared from cells grown on each of these media were prepared and assayed for shikimate dehydrogenase activity. The results, shown on Table 3.5, indicate that growth conditions have little affect on the specific activity of the enzyme and that growth on any medium would be acceptable.

These extracts were then used to assess the suitability of immune blotting as a technique for measuring cross reactions. The results (Fig. 3.17) show that immune blotting of crude extracts is not suitable. The antiserum is able to detect shikimate dehydrogenase protein in crude extracts from both strains of E. coli but as suggested when blots of the overproducing strain were being carried out (Fig. 3.7) the amount able to be loaded on the gel in extracts of wild type strains is close to the detection limit of the

Table 3.5 Shikimate dehydrogenase (E3) activity in bacteria grown on different carbon sources

E. coli (K12 and ML308) and *E. carotovora* were grown on nutrient broth (section 2.4.1) or on minimal medium II with one of glucose, glycerol and succinate as carbon source (section 2.4.6) and harvested at late exponential phase. The harvested cells were used to prepare crude cell extracts (section 2.6.5.1) and assayed for E3 activity (section 2.7.3) and protein (secton 2.5.3). The specific activity of E3 in each of the cell extracts was calculated.

Bacteria	C-source	specific activity (u/mg proteins)
E. coli K12	nutrient broth	0.044
	glucose	0.041
	glycerol	0.049
	succinate	0.055
E. coli ML308	nutrient broth	0.043
	glucose	0.044
	glycerol	0.030
	succinate	0.031
E. carotovora	nutrient broth	0.045
	glucose	0.055
	glycerol	0.068
	succinate	0.052


anti-E3
12
11
10
9
8
7
6
5
4
3
2+
1

technique. Immune blotting was not able to detect shikimate dehydrogenase from erwinia extracts (Fig. 3.17) and the blot background was showing many other proteins as faint bands. These bands, which were as dark as any positive band could, if the gel were overloaded further, give false positives.

Since the immunoblot could not detect shikimate dehydrogenase from E. carotovora, and the negative result could have been due to the amount being loaded onto the gel being below the detection level of the technique, no assessment of the level of common antigenic determinants between this enzyme and the E. coli enzyme could be made. It was therefore decided that blotting experiments would be carried out on preparations of pure or partially purified enzymes and that to decide which enzymes to purify, it was essential to use inhibition of enzyme activity as the method of screening extracts for antigenic cross reaction with antisera against the E. coli enzymes.

3.5.3.2 Immune inhibition of shikimate dehydrogenase activity in crude extracts

Specific antibody inhibition of shikimate dehydrogenase activity was carried out using antisera prepared against both the 'native' and 'denatured' forms of the enzyme. The ability of an antibody preparation to interact with the enzyme was assessed both directly by assaying loss of activity in the presence of antibody and by assessing the antibody-enzyme binding using protein A S. aureus as an immune precipitant. The results obtained are shown in Table 3.6 and those incubations which suggested interactions were occurring were examined in more detail (Fig. 3.18).

The results (Table 3.6) show that the enzyme from E. coli is the only one inhibited by both antibody preparations. The enzyme

Table 3.6 Inhibition and immunoprecipitation of shikimate dehydrogenase (E3) from various organisms by anti-*E. coli* E3 antisera

Three aliquots of crude extracts prepared as described in sections 2.6.5.1 and 2.6.5.2 from cells grown, as defined below, were mixed with equal volumes of each of normal rabbit serum, anti-*E. coli* E3 serum (anti-E3:159:11) and anti-carboxymethylated *E. coli* E3 (anti-denatured-E3:387:2) and incubated for 1 h with gentle mixing at room temperature. After incubation all mixtures were assayed for loss of E3 activity (section 2.7.3).

A second series of aliquots from the crude samples were similarily mixed with antisera as above and incubated for 15 min before addition of a protein A *S. aureus* suspension (section 2.15.2) and incubation for a further 1 h. After incubation the mixtures were centrifuged at 12,000g for 5 min and the activity of E3 remaining in the supernatants assayed.

All results are expressed as % inhibition or % precipitated enzyme as described for Table 3.1.

organisms	growth medium (section)
E. coli K12	2.4.1
E. coli ML308	2.4.1
S. typhimurium	2.4.1
E. carotovora	2.4.1
A. calcoaceticus	2.4.4
S. rimosus	2.4.3
S. aureus	2.4.1
B. subtilis	2.4.1
N. crassa	2.10.1
R. graminis	gift from M. Yasin
P. sativum	2.6.5.2

Table 3.6

Organism	anti-E3 % inhibition	anti-denatured-E3 % inhibition	anti-E3 + protein A % precipitated enzyme	anti-denatured-E3+protein A % precipitated enzyme
gram-				
E. coli K12	100	70	100	100
E. coli ML308	100	70	100	100
S. typhimurium	100	0	100	0
E. carotovora	70	0	100	0
A. calcoaceticus	0	0	50	0
gram+				
S. rimosus	0	0	0	0
S. aureus	0	0	0	0
B. subtilis	0	0	0	0
Fungi				
N. crassa	0	0	0	0
R. graminis	0	0	0	0
Plant				
P. sativum	0	0	0	0

from S. typhimurium is inhibited and that from E. carotovora partially inhibited by the antibody raised against the native E. coli enzyme. The enzyme from the erwinia strain is however totally precipitated from the incubation mixture of protein A S. aureus is added to precipitate immune complexes. The activity in the extract from A. calcoaceticus is not inhibited by either antibody preparation but does form immune complexes with the anti-native-enzyme antisera because 50% of the activity is lost from this incubation supernatant when protein A S. aureus is added (Table 3.6). In all the other extracts examined no interaction between the enzyme and antibodies against the E. coli enzyme could be detected.

For these incubations which demonstrated an enzyme antibody interaction, an inhibition titre was carried out. The results (Fig. 3.18) confirmed the inhibiton results and demonstrated that the 70% inhibition of the erwinia enzyme was not caused by limitation of antibody because a 1:4 dilution of the antibody gave the same result (Fig. 3.18D). The acinetobacter enzyme precipitation could have been due to an antibody limitation (Fig. 3.18E) but for the experiment (section 4.4.1) which showed that addition of more antisera to the purified enzyme did not increase the amount of enzyme able to be precipitated by protein A S. aureus.

As a result of these experiments, it was decided to partially purify the shikimate dehydrogenase (E3) enzymes from S. typhimurium, E. carotovora and A. calcoaceticus.

3.5.3.3 Partial purification of shikimate dehydrogenase from bacterial species

Shikimate dehydrogenase was purified from S. typhimurium, E. carotovora and A. calcoaceticus using a procedure developed for

Figure 3.18 Titration of inhibition and immunoprecipitation of shikimate dehydrogenase (E3) activity from bacteria with anti-*E. coli* E3 antisera

Anti-*E. coli* E3 (159:11) was serially diluted in normal rabbit serum then mixed with aliquots of crude extracts prepared as described for Table 3.6. As for Table 3.6 one series of mixtures were assayed for residual E3 activity after 1 h incubation while a second set was incubated for 15 min before addition of protein A *S. aureus* (section 2.15.2) followed by a further incubation of 1 h before centrifugation and assay of the E3 activity remaining in the supernatant.

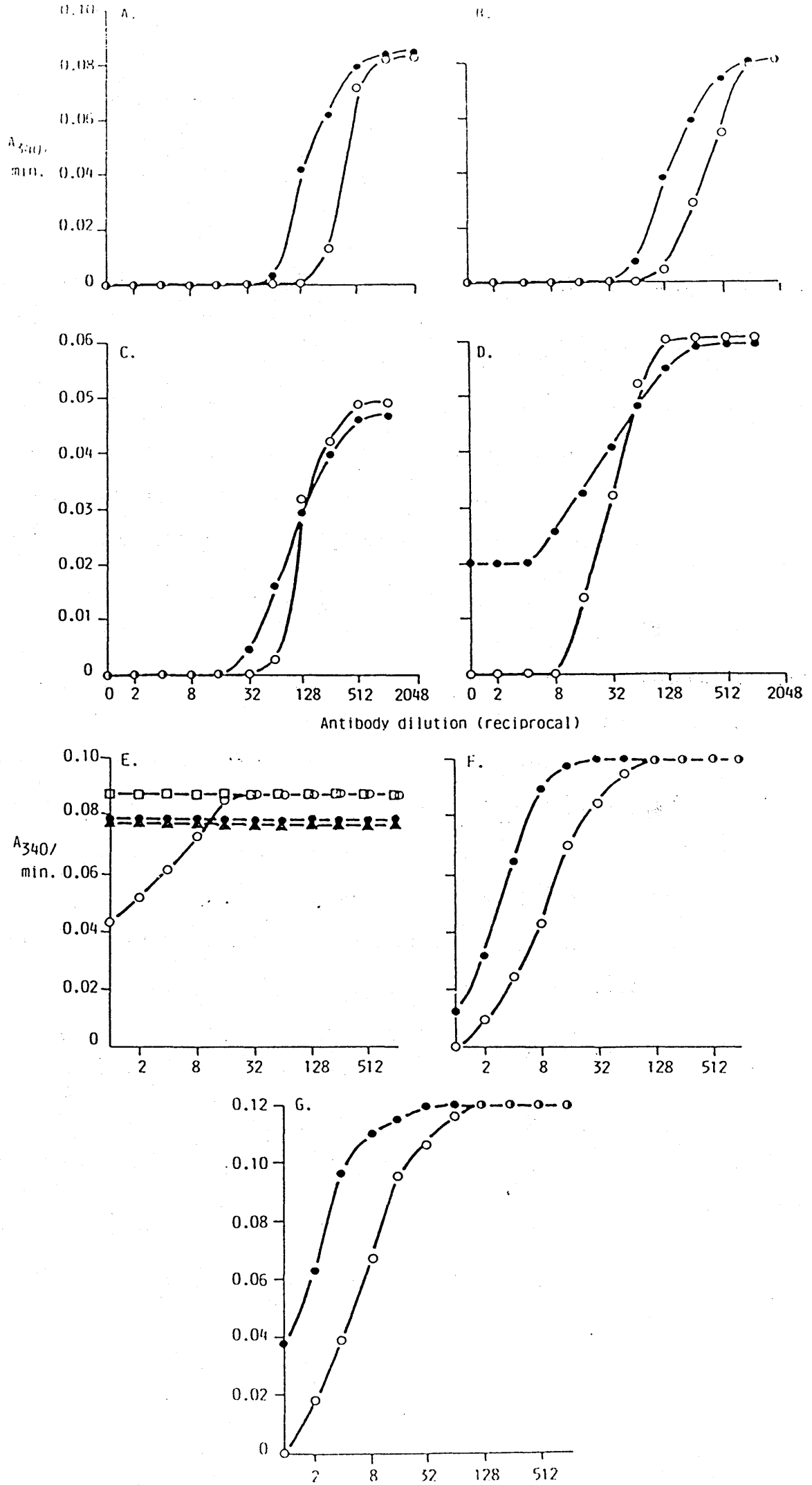
Enzyme activities were calculated as change in absorbance per min per 10ul of original extract used and the activity graphed against the antisera dilution.

As controls samples of crude extracts were mixed with normal rabbit serum and treated as experimental samples. In all cases controls gave results very similar to these obtained with the highest dilutions of active antisera used.

In Figure 3.18 E, F and G antisera raised against the carboxymethylated E3 (anti-denatured-E3) was used.

Figure No.	source of extract	antisera
A	*E. coli* K12	●,○ anti-E3
B	*E. coli* ML308	●,○ anti-E3
C	*S. typhimurium*	●,○ anti-E3
D	*E. carotovora*	●,○ anti-E3
E	*A. calcoaceticus*	●,○ anti-E3 ▲,□ anti-denatured-E3
F	*E. coli* K12	●,○ anti-denatured-E3
G	*E. coli* ML308	●,○ anti-denatured-E3

In all cases filled in symbols loss of activity in absence of protein A, opened symbols in presence of protein A.


A.
B.
C.
D.
E.
F.
G.
A_{340}/min.
0.10
0.08
0.06
0.04
0.02
0
0.12
0.05
0.03
0.01
0 2 8 32 128 512 2048
2 8 32 128 512
Antibody dilution (reciprocal)

the purification of this enzyme from E. coli. No attempt was made to optimise the procedure for use with each species.

Cells were grown either in nutrient broth (section 2.4.1) or in complex medium (section 2.4.4) to late exponential phase. Cells were harvested washed in 50mM-Tris/HCl, pH 7.5 then harvested by centrifugation at 6,000g for 30 min at 4°C and stored at -20°C until required.

Details of the purification procedure used are described in the following sections. All procedures, unless otherwise stated, were carried out at 4°C.

Step 1: Extraction and centrifugation

20g wet weight of cells were thawed, resuspended in 10ml of 50mM-Tris/HCl, pH7.5 containing 0.4mM-DTT and 1.2mM-PMSF (Buffer A) and broken by three passages through a French pressure cell. DNaseI (0.5mg) was added and the extract stirred for 1 h. The extract was then centrifuged at 100,000g for 2 h and the supernatant diluted to 70ml with buffer A.

Step 2: Fractionation with $(NH_4)_2SO_4$

The crude extract was made 1mM in benzamidine.HCl and adjusted carefully to 30% saturation with solid $(NH_4)_2SO_4$ (176g/l). After stirring for 30 min the precipitate was removed by centrifugation at 38,000g for 30 min. The supernatant was adjusted to 55% saturation (160g/l) and stirred for 30 min. The precipitated protein was collected by centrifugation at 38,000g for 30 min then resuspended in buffer A before dialysing against 2 l of 50mM-Tris/HCl, pH7.5 containing 0.4mM-DTT (Buffer B).

Step 3: Mono Q chromatography

This step was carried out at room temperature. The dialysed material was applied to a f.p.l.c. preparative scale Mono Q anion exchange column and eluted over 30 min with a linear gradient

of 0-0.5M-NaCl in buffer B (flow rate 4ml/min). 2.0ml fractions were collected and the active fractions pooled and dialysed overnight against 1 l of 50mM-Tris/HCl, pH 8.5 containing 0.4mM-DTT (Buffer C).

Step 4: Procion red chromatography

The dialysed extract was loaded, at 20ml/h, onto a Procion red column (20ml bed volume) which had been equilibrated with buffer C. The column was then washed with linear gradient of 0.1-1M-NaCl containing 0.1mM-NADPH in buffer C (flow rate 20ml/h). 2ml fractions were collected, the active fractions pooled and dialysed against buffer B containing 1M-$(NH_4)_2SO_4$.

Step 5: Phenyl superose chromatography

The enzyme from the previous step was applied onto a f.p.l.c. hydrophobic interaction phenyl superose column at room temperature and eluted over 25 min with a decreasing linear gradient of 0-1.0-M $(NH_4)_2SO_4$ in buffer B (flow rate 0.5ml/min). 0.5ml fractions were collected, active fractions pooled and dialysed against buffer B.

Details of the results obtained using this purification procedure from the three organisms are shown in Table 3.7, with monitoring of the progress of the purification, by SDS PAGE, shown on Figure 3.19.

Despite the large purification factors achieved, the final preparations did not always give a single band on SDS PAGE to indicate a pure sample of enzyme. The final sample of enzyme from E. carotovora gives a single band on SDS gel electrophoresis but the other two preparations still contain substantial quantities of contaminating proteins. Based on analysis by SDS-PAGE, gel filtration for the acinetobacter enzyme (section 4.2.5) and

Table 3.7 Purification scheme for shikimate dehydrogenase (E3) from bacteria

In all cases 20g wet weight of bacteria after growth on complex media (section 2.4.1 and 2.4.4) to late exponential phase was used as starting material and E3 purified from all three extracts by a procedure developed for purification of this activity from E. coli (section 3.5.3.3).

Table 3.7 (a) Purification table of S. typhimurium

Purification step	Vol (ml)	Protein (mg/ml)	total protein (mgs)	activity (u/ml)	total activity (units)	specific activity u/mg)	purification (fold)
1: crude extracts	55	16	880	.23	12.65	.014	1.0
2: 30-55% $(NH_4)_2SO_4$	12	34	408	.78	9.36	.023	1.6
3: MonoQ	8	10	80	.74	5.92	.074	5.3
4: Procion red	16	0.75	12	.18	2.88	.24	17.1
5: Phenyl superose	0.5	0.45	.225	1.22	0.61	2.7	192.8

Table 3.7(b) Purification Table of E. carotovora

Purification step	Vol (ml)	Protein (mg/ml)	total protein (mgs)	activity (u/ml)	total activity (units)	specific activity u/mg)	purification (fold)
1: crude extract	80	16.5	1,320	0.9	73	.055	1
2: 30-55% $(NH_4)_2SO_4$	10	55	550	8.0	80	.145	2.6
3: Mono Q	6	5.7	34.2	5.5	33	.965	17.5
4: Procion red	16	.045	.72	.56	9	12.5	227.2
5: Phenyl superose	1	.04	.04	2.3	2.3	57.5	1,045

Table 3.7(c) Purification Table of A. calcoaceticus

Purification step	Vol (ml)	Protein (mg/ml)	total protein (mgs)	activity (u/ml)	total activity (units)	specific activity u/mg)	purification (fold)
1: crude extract	70	12	860	.28	19.82	.023	1
2: 30-55% $(NH_4)_2SO_4$	19	41	680	.94	17.83	.028	1.2
3: Mono Q	8	3.8	30.4	1.68	13.46	.443	19.3
4: Procion red	13	.08	1.05	.10	1.29	1.23	53.4
5: Phenyl superose	1	.04	.04	.69	.69	17.18	747

Figure 3.19 Purification of shikimate dehydrogenase

Samples taken during the purification of shikimate dehydrogenase from S. typhimurium, E. carotovora and A. calcoaceticus were prepared (section 3.5.3.3) and run on 12.5% SDS PAGE as described in section 2.8.1.4.

Gels were stained either by Coomassie Blue (section 2.9.1.1) or by silver stain (section 2.9.1.2).

Gel A Purification from S. typhimurium (Coomassie blue stain)

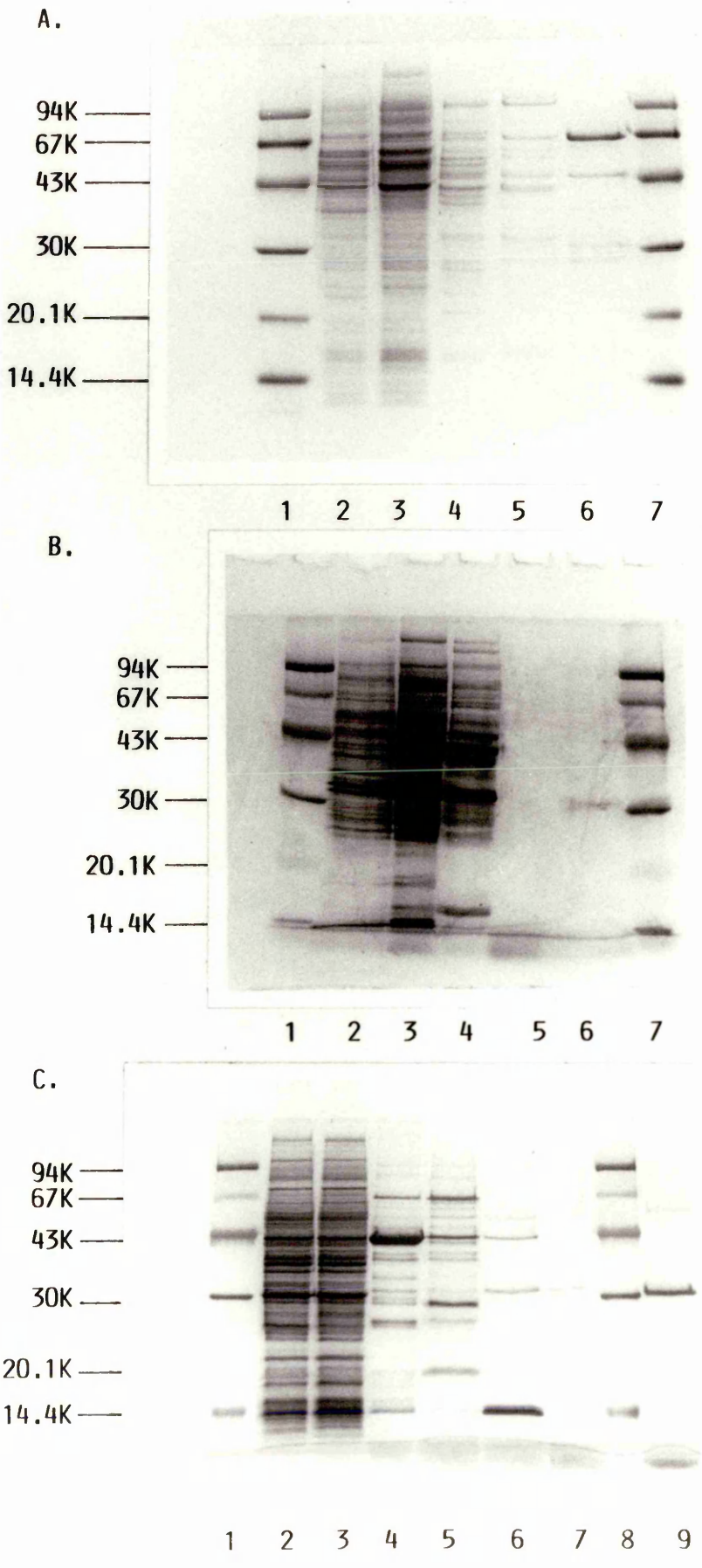
Track No.		amounts loaded (µg)
1 :	M_r standard proteins	-
2 :	crude extract	25
3 :	$(NH_4)_2SO_4$ fractionation	25
4 :	Mono Q	15
5 :	procion red	10
6 :	phenyl superose	7
7 :	M_r standard proteins	-

Gel B Purification from E. carotovora (silver stain)

Track No.		
1 :	M_r standard proteins	-
2 :	crude extract	10
3 :	$(NH_4)_2SO_4$ fractionation	10
4 :	Mono Q	7
5 :	procion red	5
6 :	phenyl superose	2
7 :	M_r standard proteins	-

Gel C Purification from A. calcoaceticus (silver stain)

Track No.		
1 :	M_r standard proteins	-
2 :	crude extract	10
3 :	$(NH_4)_2SO_4$ fractionation	10
4 :	Mono Q	7
5 :	procion red	5
6 :	phenyl superose	2
7 :	superose 12	1
8 :	M_r standard proteins	-
9 :	purified E. coli E3	2


A.
94K
67K
43K
30K
20.1K
14.4K
1 2 3 4 5 6 7
B.
94K
67K
43K
30K
20.1K
14.4K
1 2 3 4 5 6 7
C.
94K
67K
43K
30K
20.1K
14.4K
1 2 3 4 5 6 7 8 9

information from the literature for sucrose density analysis of the salmonella enzyme, it is possible to identify provisionally the bands on the gel which corresponds to the E3 activity.

Organisms	bands on SDS-PAGE	M_r of E3	Source for assignment
S. typhimurium	many	30,000	Berlyn & Giles, 1969
E. carotovora	1	30,000	Figure 3.19B
A. calcoaceticus	4	31,000	Figure 3.19C and section 4.2.5

Since the band on the gel associated with E3 activity could be assigned these preparations were used for blotting experiments.

3.5.3.4 Immunoblotting of partially purified shikimate dehydrogenases

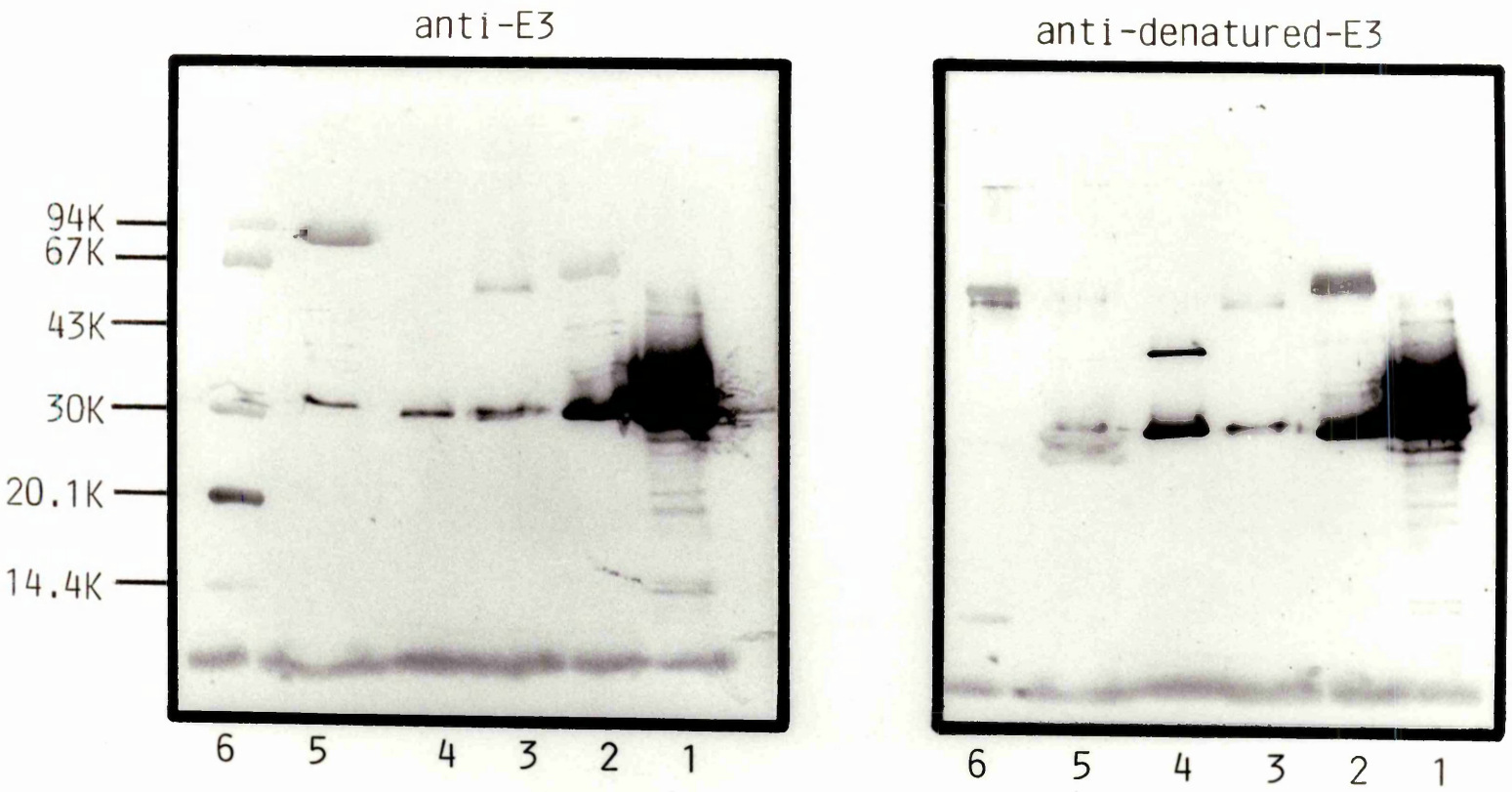
The partially purified shikimate dehydrogenases (phenyl superose fraction) from S. typhimurium, E. carotovora and A. calcoaceticus described in section 3.5.3.3 and a sample of partially purified enzyme from Streptomyces coelicolor (gift from P.J. White), which has also been reported to have a subunit M_r similar to that of the E. coli enzyme (Berlyn & Giles, 1969) were run on SDS gels and immunoblotted with both anti-native-E3 and anti-denatured-E3 from E. coli. The results (Fig. 3.20) show that both antisera can identify the enzyme from erwinia and that the antisera against the denatured enzyme binds strongly to the enzyme from acinetobacter. All other enzymes used show more than one faint bands on the gel. Thus, it could not be confirmed which one corresponded to shikimate dehydrogenase. These results indicate that the antisera can bind to the erwinia enzyme and may be able to bind to the streptomyces and salmonella enzymes.

Figure 3.20 Immunoblot of shikimate dehydrogenase (E3) acitvity from bacteria with antisera raised against native and carboxymethylated *E. coli* shikimate dehydrogenase

Samples of E3 from *S. typhimurium*, *E. carotovora* and *A. calcoaceticus*, purified as described (section 3.5.3.3, Table 3.6) and from *S. coelicolor* (gift from P. White) were applied to a 12.5% SDS polyacrylamide slab gel along with a sample of purified E3 from *E. coli* and M_r standards as markers.

After electrophoresis (section 2.8.1.4) and blotting to nitrocellulose (section 2.17.1.2) antibodies raised against native E3 (anti-E3) (159:11) and carboxymethylated E3 (anti-denatured-E3) (387:2) were used to blot the shikimate dehydrogenase protein.

track no.	Source of E3
1	*E. coli*
2	*S. typhimurium*
3	*E. carotovora*
4	*A. calcoaceticus*
5	*S. coelicolor*
6	M_r standard proteins


anti-E3
anti-denatured-E3
94K
67K
43K
30K
20.1K
14.4K
6
5
4
3
2
1
6
5
4
3
2
1

3.6 Discussion

Antibodies are proteins with specific binding sites able to recognise and bind small M_r compounds. With larger molecules the antibodies recognise a defined structure, within the larger structure, which complements the antibody binding site. In this way an antibody can be used to identify a specific feature of a molecule and to recognise features which are common to two molecules. Antibodies can therefore identify proteins that are structurally related and which may have evolved from common ancestor molecules. The use of antibodies to look for common features in molecules has been used with lysozyme from different species (Arnon & Maron, 1971; Arnheim et al., 1971), to demonstrate homology between the bifunctional aspartokinase/homoserine dehydrogenase isoenzymes in E. coli (Zakin et al., 1978) and to identify blood proteins which share common features (Benjamin et al., 1984; Rodda et al., 1986; Pekkala-Flagan & Ruoslahti, 1982).

These studies have indicated that for protein molecules to cross react antigenically they need to possess considerable amino acid sequence homology with an average value of 60% sequence homology being proposed as a minimum (Prager & Wilson, 1971a,b). This level of amino acid sequence homology can be reduced if the proteins are denatured before use as immunogens and denaturation by reduction and carboxymethylation can generate antisera able to cross react with proteins with only 40% amino acid sequence homology (Arnon & Maron, 1971; Arnheim et al., 1971; Pekkala-Flagan & Ruoslahti, 1982).

Antibodies, therefore, represent useful reagents for identifying molecules and antibodies, raised against one protein as an immunogen, can be used to detect fragments of that protein synthesised in mutants or related proteins synthesised in other systems. It was partly with these uses in mind, that antibodies

against the enzymes of the shikimate pathway were prepared. In the work described in this chapter antibodies against the enzymes of the shikimate pathway were investigated to determine the extent to which these antibodies would be useful reagents for investigations of the synthesis of aromatic compounds.

To maximise the ability of the antibody preparations to recognise features of proteins, antibodies were raised against those enzymes of the shikimate pathway in E. coli that could be prepared in sufficient quantity. Antisera was prepared against both the 'native' and 'denatured' (by reduction and carboxymethylation) forms of the enzymes. In this way it was hoped that the ability of antibodies to recognise common features between protein would be maximised and that proteins with amino acid homology as low as 40% could be found. These antisera were shown to possess high titres against their immunogen, usually in both native and denatured form, and to be able to specifically identify their immunogen in crude extracts containing high levels of the immunogen. In addition antisera was raised against arom complex from N. crassa and was shown to contain, at high titre, antibodies able to recognise not only the complete complex but also fragments produced by limited proteolysis (Boocock, 1983).

The antibodies were used to investigate the degree to which common epitopes could be detected using a range of techniques. The limit to which these techniques are able to detect common epitopes has not been determined but in theory a single common epitope should be able to be detected by the protein A S. aureus precipitation technique and the blotting technique is able to identify a protein, which if assessed by the carboxymethyl group antibody, has only three antibody binding sites.

Two different systems were investigated using antibodies

with varying degrees of success.

a) Common groups among the shikimate pathway enzymes in *E. coli*.

In this investigation all five enzymes were used and common epitopes were searched for using the antisera raised against both the native and denatured forms of the enzymes. No cross reactions were detected. It follows that these proteins have little structure in common and this is confirmed by the knowledge of the amino acid sequences. These enzymes show very little homology (Millar & Coggins, 1986; E. Borthwick, L.D. Graham & J.R. Coggins, unpublished results; Anton & Coggins, 1988; Millar *et al.*, 1986a; Duncan *et al.*, 1984b). (see Appendix) The proteins do not appear to share any common ancestry and the antisera are specific reagents only suitable for detection in *E. coli* of the protein or fragements of the protein against which they were raised.

b) Common groups between proteins with the same function but from different species.

Three different experiments were set up which fell into this category. In the first, in which the ability of antisera raised against *arom* complex from *N. crassa* was used, it was found that this antisera was able to detect, using blotting, the partially purified *arom* complex from yeast. Since only a single antisera raised against 'native' *arom* complex was used for this investigation there should be a greater than 60% amino acid sequence homology between these two proteins. In this system the antisera therefore would be useful, not just to detect *arom* complex from *N. crassa* but also to detect the corresponding protein from other species. In addition the ability of the antisera to detect fragments of *arom* complex generated by limited proteolysis shows that it would also be useful in detecting fragments of the *arom* complex which could be produced in mutants.

In the second series of experiments common epitopes were searched for between arom complex from N. crassa and the E. coli enzymes which catalyse the same reactions. No cross reactions were found. Since many antisera were used in this study, the conclusion must be that the extent to which these proteins have common features is low and the amino acid sequence homology should be less than 40%. This conclusion is consistent with what is known about the amino acid sequences of these proteins. The sequence of arom complex from yeast but not neurospora is known and it contains only 30% homology with the E. coli enzymes (Fig. 1.3 and Table 1.2). If the N. crassa enzyme is similar to the yeast enzyme, it is reasonable to assume that sequence homology between N. crassa arom and the E. coli enzymes would also be about 30% and so insufficient to show common antigenic determinants.

The third series of experiments was to examine the shikimate dehydrogenase from many organisms. In this case cross reactions indicating the presence of common epitopes were found using both enzyme inhibition and immune blotting techniques but the results obtained were dependent on the bacteria from which the enzyme was prepared. Common epitopes were only found among enzymes prepared from gram negative bacteria. All other extracts did not show any significant interaction. In enzyme inhibition assays a comparative sequence of E. coli > S. typhimurium > E. carotovora > A. calcoaceticus was suggested but in blotting assays, when enzyme denatured by SDS is being detected, the acinetobacter enzyme gave a strong reaction. It was concluded that the shikimate dehydrogenases from gram negative bacteria contain epitopes in common. S. typhimurium and E. coli have a number of groups in common, Erwinia E3 much less and these largely surface antigens because denatured protein does not give a strong signal. Acinetobacter has

many groups in common but these are based in the protein and are not exposed, for antibody binding, at the surface of the protein while it is active.

One interesting feature of the enzyme inhibition assays was that the enzyme from acinetobacter was not inhibited but could be precipitated, to 50% of the total activity by the addition of protein A S. aureus. This observation has been confirmed by studies described in Chapter 4. Since only 50% of the activity could be precipitated, only 50% of the enzyme molecules could bind antibody. There must therefore be at least two types of shikimate dehydrogenase present in extracts from acinetobacter. It was this observation and the postulate ~~of~~ that two shikimate dehydrogenases exist in acinetobacter that is investigated in Chapter 4.

In general the results from these studies was disappointing. It had been hoped that the antisera, such as the anti-E. coli-E3 preparations, would have been able to cross react much more extensively with this enzyme isolated from other species of bacteria. The results we obtained however show that the ability to cross react with the same enzyme from other species is very variable and generally gives a result which is at the limit of detection using the techniques used in this work. The antisera that have been prepared are essentially reagents specific for the proteins used as immunogens.

The only antiserum of more general applicability is the anti arom antiserum. This antiserum does cross react with the equivalent protein from other species and can be used to identify mutants which only express part of the whole complex. As such the antisera will be valuable for several types of experiments.

4. The purification and partial characterisation of shikimate dehydrogenase from A. calcoaceticus NCIB 8250

4.1 Introduction to A. calcoaceticus

4.1.1 A. calcoaceticus

A. calcoaceticus NCIB 8250, which grows at an optimal temperature of 30°C (Fewson, 1967), is unable to metabolise carbohydrates (Cook & Fewson, 1973) but can utilise a wide variety of organic compounds, including many aromatics, as sole sources of carbon and energy (Fewson, 1967).

All acinetobacters are aerobic, gram-negative, non-motile, catalase positive and oxidase negative organisms, with DNA compositions varying from approximately 38-47 mole % G + C (Henriksen, 1976; Juni, 1978). Most strains have an optimum growth temperature of 34-35°C (Breuil et al., 1975).

Acinetobacters have a ubiquitous distribution in nature as a result of their nutritional versatility and have been isolated from soil, sewage and many parts of the human body (Baumann, 1968; Warskow & Juni, 1972; Juni, 1972; Henriksen, 1973; Gaughan et al., 1979). Acinetobacters are also been found associated with oil pollution in aquatic environments and industrial effluents (Gutnick & Rosenberg, 1977; Bartha & Atlas, 1977) and are very important in biological phosphate removal from waste water (van Groenestijn et al., 1989). In addition A. calcoaceticus is of industrial importance since it can produce 'emulsans' which are extracellular polyanionic emulsifiers (Rubinovitz et al., 1982) and 'biodispersans' which are extracellular, non-dialysable polymers of monosaccharides that are able to disperse limestone (calcite) powders in water (Rosenberg et al., 1988a,b).

Recently, acinetobacters although not obligatory pathogens, have been implicated in various pathogenic conditions such as septicaemia, urinary tract infections (Glew et al., 1977; Retailliau et al., 1979; Lowes et al., 1980; Hoffman et al., 1982), endocarditis (Henriksen, 1973; Cohen et al., 1980; Rao et al., 1980), meningitis (Ghoneim & Halaka, 1980; Berk & McCabe, 1981; Kabayashi et al., 1983), pneumonia (Rudin et al., 1979; Holton & Shorvon, 1982; Markham & Telfer-Brunton, 1983), respiratory tract disease (O'Connell & Hamilton, 1981; Vila et al., 1989), skin and wound infections (Glew et al., 1977), peritonitis (Roxe & Santhanam, 1983; Larson, 1984), bacteremia (Biliau et al., 1989) and corneal ulcer (Zabel et al., 1989).

Acinetobacters are relatively resistant to many antibiotics (French et al., 1980) and also contain transferable plasmids (Hinchliffe & Vivian, 1980; Devaud et al., 1982; Goldstein et al., 1983). In addition they are relatively resistant to the ionising radiation (Kairiyama et al., 1979) which is used in food preservation and they can therefore be an economically important cause of food spoilage (Ito et al., 1976; Firstenberg-Eden et al., 1980).

The wide nutritional versatility, frequency of occurrence and possible economic and clinical importance of acinetobacters has stimulated researchers in the development of systems to study both their physiology and biochemistry. The recent developments of systems of genetic transformation (Juni, 1978); Ahlquist et al., 1980), conjugation (Towner, 1978) and transduction (Herman & Juni, 1974) including genetic mapping (Towner, 1983) should help to complement the biochemical work.

4.1.2 Plausibility of two E3 isoenzymes in A. calcoaceticus

As described in section 1.3, A. calcoaceticus can use both quinate and shikimate as sources of carbon and energy. It appears to have two dehydroquinase isoenzymes: one inducible and involved in quinate utilisation and the other constitutive and biosynthetic (Ingledew et al., 1971; Berlyn & Giles, 1973). It was also known that the biosynthetic shikimate dehydrogenase had no role in the degradation of shikimate (Yaniv & Gilvarg, 1955). This function is performed by a dye-linked hydroaromatic dehydrogenase which can also catalyse the oxidation of quinate (Tresguerres et al., 1970a,b, 1972). This suggested that both the degradative quinate dehydrogenase and shikimate dehydrogenase activities might rely on a single enzyme. However, recently evidence showed that quinate dehydrogenase is a membrane bound $NAD(P)^+$-independent enzyme and uses PQQ as a cofactor (van Kleef & Duine, 1988). This indicated that quinate dehydrogenase differed from shikimate dehydrogenase because shikimate dehydrogenase has been generally found to be a soluble $NADP^+$-dependent enzyme (Yaniv & Gilvarg, 1955) and raised the question of whether there might be two isoenzymes of shikimate dehydrogenase in A. calcoaceticus, one involved in biosynthesis and the other involved in the catabolic utilisation of shikimic acid. Recently, isoenzymes of shikimate dehydrogenase have been found in the cytosol of Triticum aestivum (Chinese spring wheat) (Benedettelli & Hart, 1988) and Zea mays (maize) (Wendel et al. 1988) by polyacrylamide and starch gel electrophoresis and by genetic linkage analyses. It has been suggested that these isoenzymes unlike the chloroplastidic isoenzymes which are biosynthetic, may be involved in the quinate catabolic pathway (see section 1.3.1.3). The experiments described in Chapter 3 showed that A. calcoaceticus may contain two E3 isoenzymes since anti-E. coli E3 can bind only 50% of

the total E3. To confirm the existence of isoenzymes and to try and establish their metabolic role it was decided to purify shikimate dehydrogenase from A. calcoaceticus. Of particular interest was the question whether one of the isoenzymes was both a quinate and a shikimate dehydrogenase. In this connection it is interesting to note that Mitsuhashi & Davis (1954) found, in A. aerogenes, an NAD^+-dependent quinate dehydrogenase which was unable to oxidise shikimic acid.

4.2 Purification of E3 from A. calcoaceticus NCIB 8250

4.2.1 The need for an improved purification scheme

The constitutive E3 from E. coli K12 wild type has already been purified to homogeneity and shown to be a monomeric protein of M_r 30,000 (Chaudhuri & Coggins, 1985). This purification had the major disadvantage that it involved many chromatographic steps and took over two weeks to complete and there were many opportunities to lose enzyme activity. Therefore a new and simplified purification procedure has been developed. This procedure has three advantages:

a) It takes only about three days as the conditions for eluting the columns have been speeded up.

b) Procion red (Dyematrix Red A) chromatography is used in a single step instead of DEAE Sephacel chromatography followed by ADP-Sepharose chromatography. Procion red is a combined anionic exchanger and affinity column and has proved very useful in the purification of $NADP^+$-linked enzymes ($NADP^+$-linked enzyme binds more strongly than NAD^+-linked enzymes) (Scopes, 1987).

c) a single range of buffer solutions was used.

The yield from this simplified protocol was better than the yields found previously for the E. coli enzyme purification.

4.2.2 Attempted induction of E3

It is much easier to purify an enzyme, if the amounts of the enzyme are very high in the cells. Tresguerres et al. (1970b) found that quinate and p-hydroxybenzoate are powerful inducers for increasing the quinate (shikimate) dehydrogenase activity in A. calcoaceticus strain 73; in comparison succinate is not a good inducer. However, they pointed out that the ability of the dehydrogenase to catalyse in vitro the oxidation of quinate and shikimate does not necessarily mean that this enzyme acts in vivo in the dissimilation of both compounds. To establish whether quinate and p-hydroxybenzoate were also inducers for A. calcoaceticus NCIB 8250 this organism was grown in the presence of quinate and of p-hydrozybenzoate. Cells were also grown in the presence of succinate as a control. When A. calcoaceticus NCIB 8250 was grown on the medium containing potential inducers, the E3 activity was found to be the same as in the cells grown in the presence of succinate and in cells grown on nutrient broth (Table 4.1). This suggested that the E3 in A. calcoaceticus NCIB 8250 is the constitutive enzyme which is found in all organisms. Presumably, selection for constitutivity may be due to shikimic acid being a normal metabolite, since it is an intermediate in the biosynthesis of aromatic compounds including the amino acids. Thus, shikimate can be available as source of carbon and energy all the time in the same way as intermediates of the glycolytic pathway. It should also be noted that these cells grow very well on succinate and nutrient broth but growth is slower on p-hydroxybenzoate and very slow on quinate. In addition, there is a transient accumulation of a polyphenol compound whose presence is detected by the typical blue colouring characteristic of the chelates of polyphenol and iron when cells were grown on quinate and p-hydroxybenzoate. These

Table 4.1 Effect of growth substrates on shikimate dehydrogenase activities in crude cell free extracts of A. calcoaceticus NCIB 8250

A. calcoaceticus was grown on nutrient broth (section 2.4.1) or on minimal medium I with different carbon sources (section 2.4.5) and harvested at late exponential phase. The harvested cells were used to prepare crude extracts (section 2.6.5.1) and assayed for E3 activity (section 2.7.3) and protein (section 2.5.3). The specific activity of E3 in each of the crude extracts was calculated.

growth substrate	specific activity (u/mg protein)
quinate	0.05
p-hydroxybenzoate	0.05
succinate	0.056
nutrient broth	0.048

observations were made under conditions which corresponded to Tresguerres et al.'s experiment (Tresguerres et al., 1972). All of the E3 from the cell free extracts of A. calcoaceticus grown on these carbon sources was still only 50% precipitated by treatment with protein A and anti-E. coli-E3.

4.2.3 Purification procedure

Unless otherwise stated all steps after the breaking of the cells were performed at 4°C.

Step 1 : Extraction and centrifugation

A. calcoaceticus NCIB 8250 was grown on complex medium as described in section 2.4.4. A 20g batch of cells (which had been stored as a frozen paste at -20°C) was suspended in 10ml of 50mM-Tris/HCl, pH 8.5, 0.4mM-DTT containing 1mM-EDTA and 1.2mM-PMSF (extraction buffer) and broken by three passages through a French pressure cell. DNaseI (0.5mg) was added and after 1 h stirring the resulting suspension was centrifuged at 100,000g for 2 h. The supernatant was diluted with extraction buffer and this was the crude extract from which the enzyme was purified.

Step 2 : Fractionation with $(NH_4)_2SO_4$

The crude extract was made 1mM in benzamidine.HCl and adjusted to 40% saturation with solid $(NH_4)_2SO_4$ (242g/l). After the mixture had been stirred for 30 min, the precipitate was removed by centrifugation at 28,000g for 30 min. The supernatant was adjusted to 60% saturation with solid $(NH_4)_2SO_4$ (130g/l) and stirred for 30 min. The precipitated protein was collected by centrifugation at 28,000g for 30 min, redissolved in a small volume of 50mM-Tris/HCl, pH 8.5 containing 0.4mM-DTT (buffer A).

Step 3 : Ultrogel AcA 44 chromatography

Concentrated enzyme from ammonium sulfate fractionation step was directly applied to a Ultrogel AcA 44 column (90cm x 2.1cm) that had

been pre-equilibrated with buffer A (flow rate 20ml/h). The enzyme was eluted with the same buffer (flow rate 20ml/h,5ml fractions) (Fig. 4.1). Fractions containing E3 activity were pooled.

Step 4 : Procion red chromatography

The enzyme from the previous step was applied directly to a Procion red column (10cm x 2.1cm) that had been washed with 10 column volumes of 8M-urea in 0.5M-NaOH, followed by copious amounts of water, then pre-equilibrated with buffer A. The column was washed with buffer A until the A_{280} was zero (flow rate 20ml/h). The protein was eluted with an 80ml linear gradient of NaCl (0.1-1.0M-NaCl) in buffer A containing 0.1mM-NADPH (flow rate 20ml/h, 2ml fractions) (Fig. 4.2). The fractions containing enzyme activity were pooled and dialysed overnight against 2 l of 50mM-Tris/HCl, pH 7.5 containing 0.4mM-DTT (buffer B) and 1mM-benzamine.HCl.

Step 5 : Chromatography on Mono Q

This step was carried out at room temperature with a Pharmacia f.p.l.c. system. The dialysed material from the previous step was loaded onto a Mono Q column (1ml bed volume). The enzyme was eluted with a linear gradient of 0-0.5M-NaCl in buffer B (flow rate 1ml/min, 0.5ml fractions in 30 min) (Fig. 4.3). The fractions containing E3 activity were pooled and dialysed overnight in buffer B containing 50% (v/v) glycerol before long-term storage at $-20^{o}C$.

4.2.4 Purity of the A. calcoaceticus E3

An SDS polyacrylamide gel showing the protein components at each stage is shown in Figure 4.4. The purified enzyme showed a single band with the silver staining (Fig. 4.4, track 6) and the subunit molecular weight was found to be a little larger than that of the purified *E. coli* E3 (Fig. 4.4, track 8).

Figure 4.1 Purification profile of A. calcoaceticus NCIB 8250 on Ultrogel AcA44

(Step 3 of purification scheme detailed in section 4.2.3)

Concentrated enzyme from Step 2 was loaded onto a column (2.1 x 90cm) of Ultrogel AcA 44 pre-equilibrated with buffer A. The flow rate was 20ml/h and 5ml fractions were collected. Fractions containing E3 activity were pooled.

●: A_{280} ; O: E3 activity

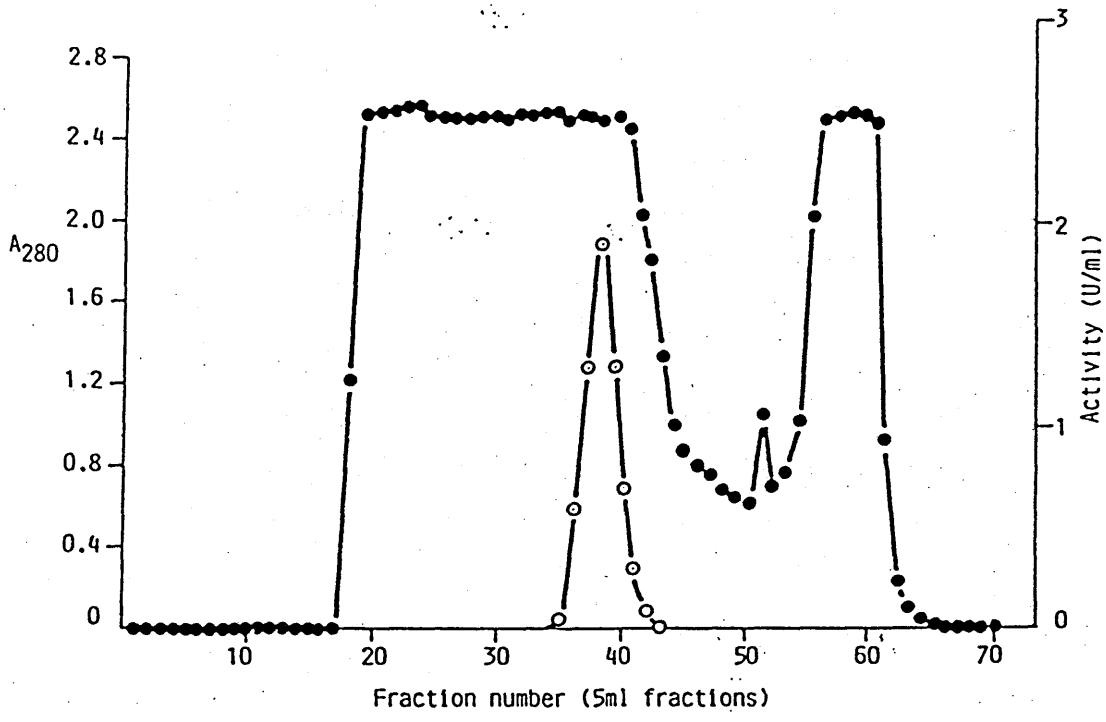


Figure 4.2 Purification profile of A. calcoaceticus NCIB 8250 on Procion Red

(Step 4 of purification scheme outlined in section 4.2.3)

The pooled active material from Step 3 was directly loaded onto a column of Procion red (2.1cm x 10cm) pre-equilibrated with buffer A. The column was washed with buffer A until the absorbance at 280nm was zero, then a linear gradient (total volume 80ml) of 0.1-1.0M-NaCl in buffer A containing 0.1mM-NADPH was applied. The flow rate was 20ml/h and 2ml fractions were collected.

○: protein; △: E3 activity; ●: conductivity

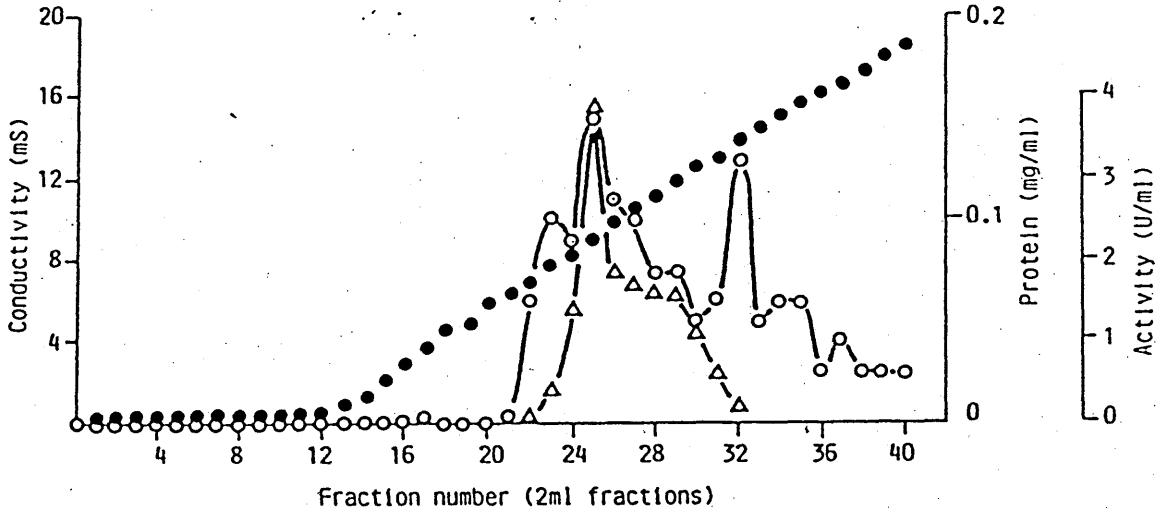


Figure 4.3 Purification profile of A. calcoaceticus NCIB 8250 on Mono Q

(Step 5 of purification scheme in section 4.2.3).

Enzyme from Step 4 was dialysed overnight against buffer B containing 1mM-benzamidine.HCl. The dialysed enzyme was applied to a Mono Q column equilibrated with buffer B. The enzyme was eluted with a linear gradient of 0-0.5 M-NaCl in buffer B. The flow rate was 1ml/min and 0.5ml fractions were collected. The purified enzyme was dialysed overnight against buffer B containing 50% (v/v) glycerol before long-term storage at -20°C.

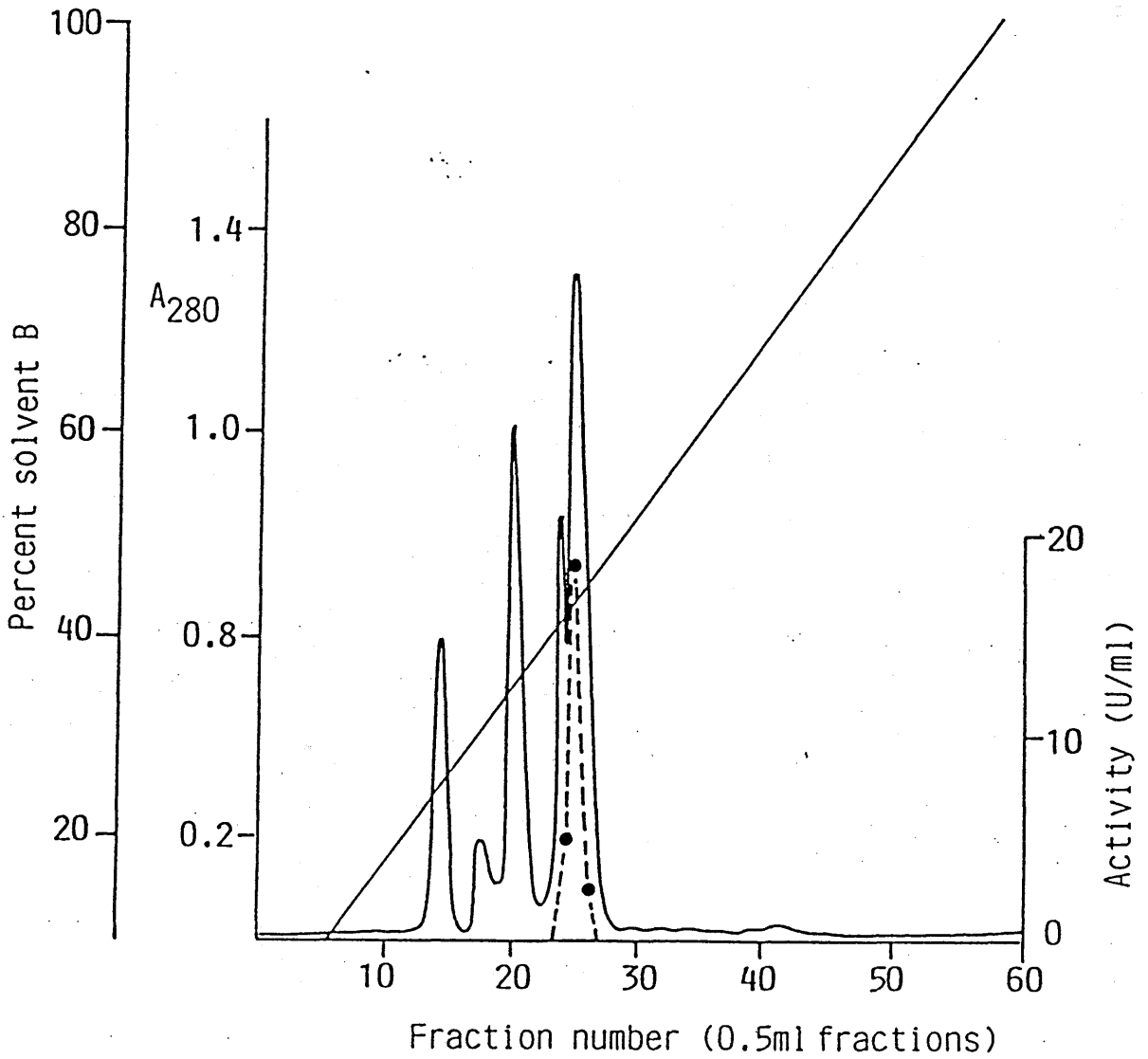
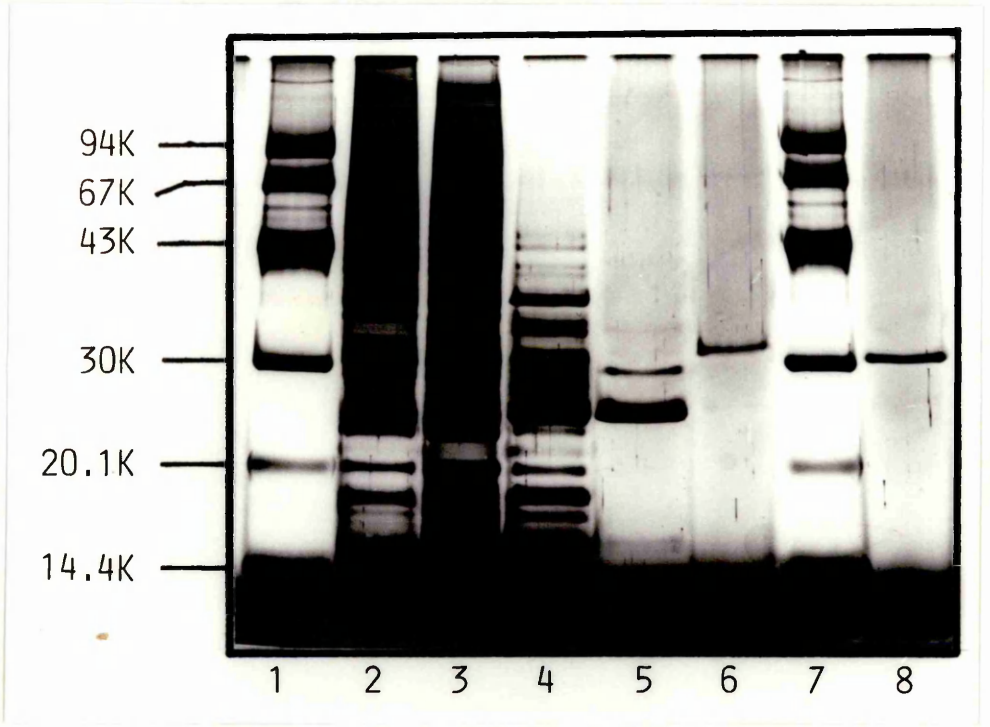


Figure 4.4 SDS PAGE showing the protein components at each step of the *A. calcoaceticus* shikimate dehydrogenase purification

The gel was a 12.5% gel and protein bands were visualised by silver staining (section 2.9.1.2).

Track No.			amounts loaded (µg)
1	:	M_r standard proteins	-
2	:	crude extract	10
3	:	Ammonium sulphate fractionation	10
4	:	ultrogel AcA44	5
5	:	Procion red	3
6	:	Mono Q	1
7	:	M_r standard proteins	-
8	:	purified *E. coli* E3	1



4.2.5 Molecular weight determination

The native M_r of A. calcoaceticus E3 was determined by gel permeation chromatography. The subunit M_r was determined by SDS PAGE.

4.2.5.1 Subunit M_r of the A. calcoaceticus E3

The mobility of the purified A. calcoaceticus E3 was compared with mobilities of standard proteins of known M_r using SDS PAGE (Fig. 4.5). Comparison with these markers gave a subunit M_r 31,000.

4.2.5.2 Native M_r of the A. calcoaceticus E3

The native M_r of the A. calcoaceticus E3 was determined by gel filtration on a Superose 12 column using a Pharmacia f.p.l.c. apparatus. The column was calibrated with 5 purified E. coli enzymes of known M_r and a standard curve of peak elution volume against M_r was constructed (Fig. 4.6). The measured peak elution volume for A. calcoaceticus E3 correspond to a M_r of 31,000.

4.3 Summary of the purification procedure

The purification of A. calcoaceticus E3 from a typical purification procedure is summarised in Table 4.2. To minimise the risk of proteolysis during the purification procedure, either PMSF or benzamidine.HCl was included in buffers (Lumsden & Coggins, 1977). After $(NH_4)_2SO_4$ fractionation the E3 activity was found in the 40-60% saturation fraction; the corresponding E. coli enzyme is found in the 30-55% saturation fraction. The enzyme was purified approximately 10,000 fold and in 32% overall yield by ammonium sulphate fractionation, gel filtration, chromatography on a Procion red column and ion exchange chromatography in Mono Q. The

Figure 4.5 Standard curve for 12.5% SDS PAGE

Migration distance of each protein marker from the top of the separating gel is plotted against log M_r.

The O indicates the average distance of migration of the A. calcoaceticus NCIB 8250 E3 subunit on the gels. This corresponds to an M_r of 31,000. The migration positions of the standard proteins are shown as ● .

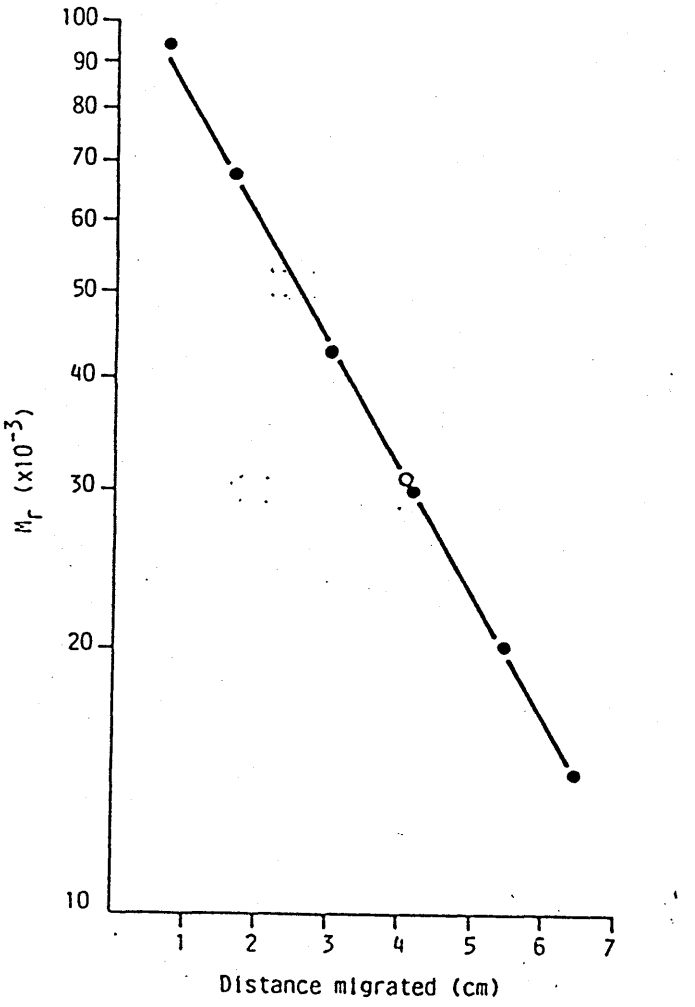


Figure 4.6 Standard curve for gel filtration on Superose 12

The elution volumes of the five purified E. coli shikimate pathway enzymes is plotted against log M_r.

The O indicates the elution volume of native E3 from A. calcoaceticus NCIB 8250. This corresponds to an M_r of 31,000. Further details are given in section 2.18.2 and 4.2.5.1.

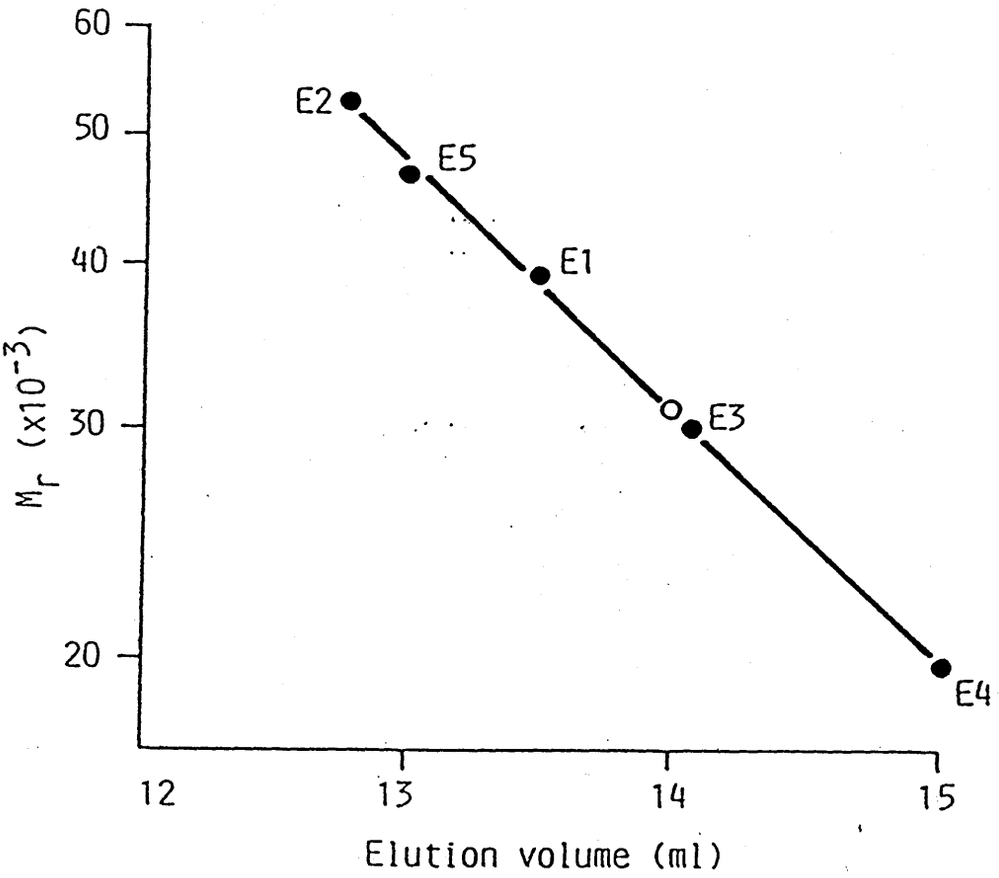


Table 4.2 Purification scheme for A. calcoaceticus shikimate dehydrogenase

The results presented are for a typical purification starting from 20g A. calcoaceticus NCIB 8250

purification step	Volume (ml)	protein (mg/ml)	total protein (mgs)	activity (u/ml)	total activity (u)	specific activity (u/mg)	purification (fold)	yield %
1:crude extract	70	21.5	1505	0.81	57	.038	1	100
2:40-60% $(NH_4)_2SO_4$	8	100	800	5	40	.05	1.3	70
3:Ultrogel AcA44	27	5.8	157	1.26	34	.216	5.7	60
4:Procion red	13	0.25	3.25	1.54	20	6.15	162	35
4:Mono Q	0.5	0.1	0.05	36	18	360	9473	32

pure enzyme, like the corresponding E. coli enzyme (Chaudhuri & Coggins, 1985), was monomeric and had a subunit M_r of 31,000.

4.4 Cross reaction with anti-E. coli E3

4.4.1 Immunoprecipitation assay with protein A

The purified A. calcoaceticus E3 was subjected to a cross reaction test with anti-E. coli-E3 as described for the crude enzyme preparation in section 3.5.3.2. It was still found that protein A precipitated only 50% of the enzyme activity as an immunocomplex and left 50% of the enzyme activity in the supernatant. After immunocomplex precipitation with protein A, an aliquot of the supernatant was again tested for cross reaction with anti-E3. There was no cross reacion (Table 4.3a). Similar experiments with crude extracts had ~~results had been obtained with crude extract of E3 (see section 3.5.3.2)~~ been ambiguous (see Tables 3.6 and 4.3b). This experiment suggested that A. calcoaceticus contained two isoenzymes, only one of which was precipitated by the anti-E. coli-E3 antibodies. One isoenzyme cross-reacted and gave an immune complex precipitate with protein A and the other did not. This suggests that one of the isoenzymes is homologous to, or at least has common antigenic sites with, the E. coli E3. Both of the postulated isoenzymes have the same M_r since they are not resolved on SDS-PAGE.

4.4.2 Antigen titration curve

To confirm the result obtained in section 4.4.1, an antigen titration curve was done on the purified enzyme. If either antigen or antibody is in excess in antigen-antibody binding experiments, the immunocomplex may not form or may not form completely (see section 3.1.5). To avoid artifacts of this kind various dilutions of antigen must be tried (Fig. 4.7). This antigen titration curve showed that the complex was not formed at either high or low

Table 4.3 Cross reaction of purified A. calcoaceticus shikimate dehydrogenase with anti-E. coli shikimate dehydrogenase was tested by immunoprecipitation assay with protein A

Purified A. calcoaceticus E3 or crude extract was treated with normal rabbit serum (NRS) and protein A or anti-E. coli E3 and protein A as described in section 2.15.2. After centrifugation aliquots of the supernatant were assayed for enzyme activity. the supernatant from experiment 2, which still contained 50% of the E3 activity was subjected to a second series precipitations (experiments 3 and 4).

(a)

	enzyme treated	activity u/ml	% precipitated enzyme
1.	E3 + NRS + protein A	0.38	0
2.	E3 + anti-E3 + protein A	0.19	50
	Aliquots taken from the supernatant of 2 and tested atain as above		
3.	$E3^+$ + NRS + protein A	0.09	0
4.	$E3^+$ + anti-E3 + protein A	0.09	0

+ = activity of E3 remaining in supernatant from experiment 2

(b)

	enzyme treated	activity u/ml	% precipitated enzyme
1.	CE3 + NRS + protein A	0.43	0
2.	CE3 + anti-E3 + protein A	0.21	50
	Aliquots taken from the supernatant of 1 and 2 and tested again		
3.	CE3 + NRS + protein A	0.16	0
4..	CE3 + anti-E3 + protein A	0.07	56

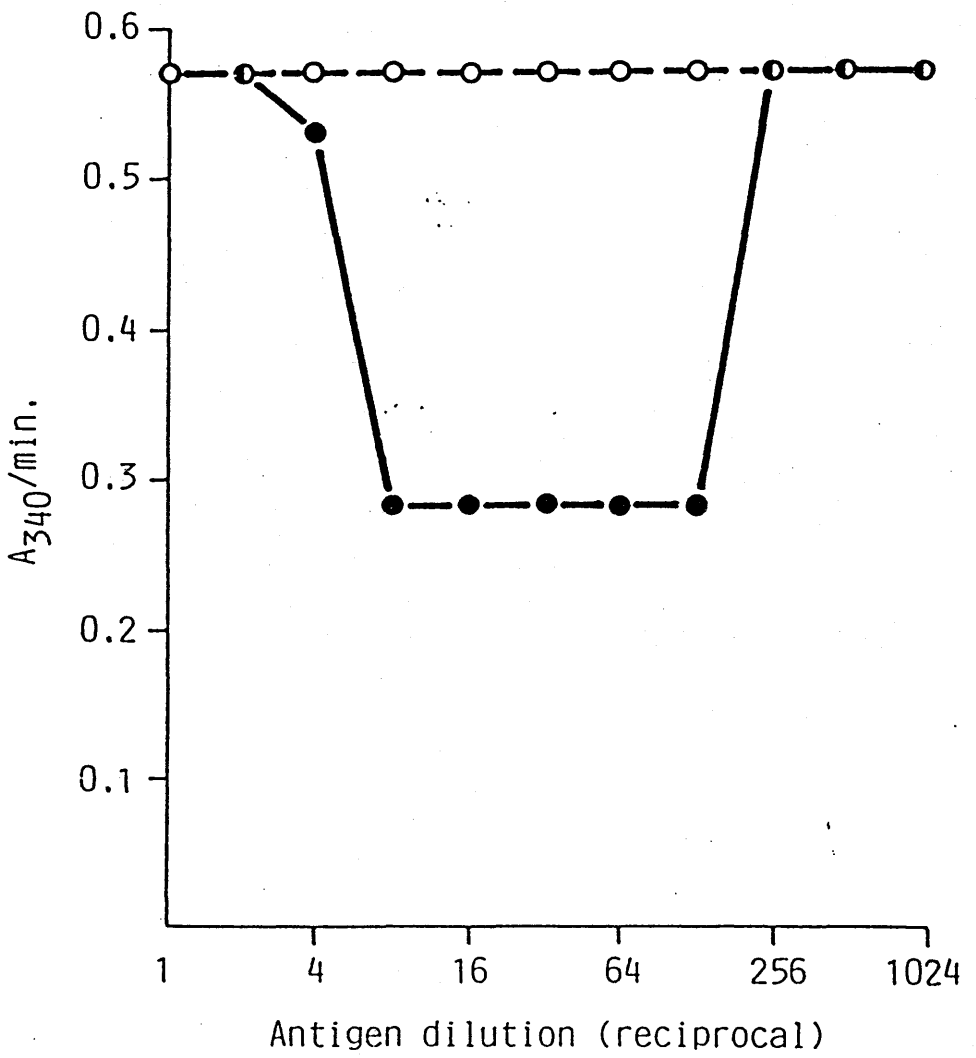
CE3 = crude extract containing E3

Figure 4.7 Antigen titration curve of purified A. calcoaceticus shikimate dehydrogenase

The purified enzyme was serially diluted with 50mM-Tris/HCl, pH 7.5 then 20ul samples were treated with an equal volume of anti-E. coli-E3 and protein A as described in section 2.15.2. The E3 activity was assayed in supernatant after centrifugation.

O: E3 + NRS + protein A

●: E3 + anti-E3 + protein A



concentration of antigen; it did form at suitable intermediate concentrations of antigen and antibody. However there were no conditions under which A. calcoaceticus E3 was wholly precipitated as an immunocomplex with anti-E. coli-E3 and protein A. It was found that at most only 50% of the A. calcoaceticus E3 was precipitated and 50% removed in the supernatant which was entirely consistent with the experiments described in sections 3.5.3.2 and 4.4.1. Nevertheless, when enzyme was in high dilution the difficulty of assay might have led to a false interpretation.

4.4.3 Non-denaturing polyacrylamide gel electrophoresis and immunoblotting

To try and provide further evidence for the existence of two isoenzymes of A. calcoaceticus E3 samples were electrophoresed on non-denaturing (native) gels and stained for activity. The A. calcoaceticus E3 gave two major bands and four minor bands while E. coli E3 showed only one band. One major band of A. calcoaceticus E3 had the same mobility as the E. coli E3 whereas the second major band had a lower mobility (Fig. 4.8). When these bands were immunoblotted with anti-E3 and anti-denatured-E3, only the upper band and minor band gave a cross reaction with anti-E3. Neither band cross-reacted with anti-denatured-E3 (Fig. 4.8). These experiments provided further evidence for the occurrence of two isoenzymes. The only obvious explanation for the multiple bands is proteolytic cleavage. While this is a reasonable explanation for the two faint, high mobility bands, it is a less likely explanation for the two strong bands.

4.4.4 Quinate dehydrogenase activity in purified E3

Tresguerres et al., (1970b) reported that the quinate dehydrogenase of A. calcoaceticus also possessed shikimate dehydrogenase activity as is found for the inducible quinate dehydrogenase of N. crassa (Giles et al., 1967b) (see section 1.3.2.2). It thus appeared that A. calcoaceticus had both a quinate (shikimate) dehydrogenase and a second enzyme which is simply a shikimate dehydrogenase. To investigate whether the

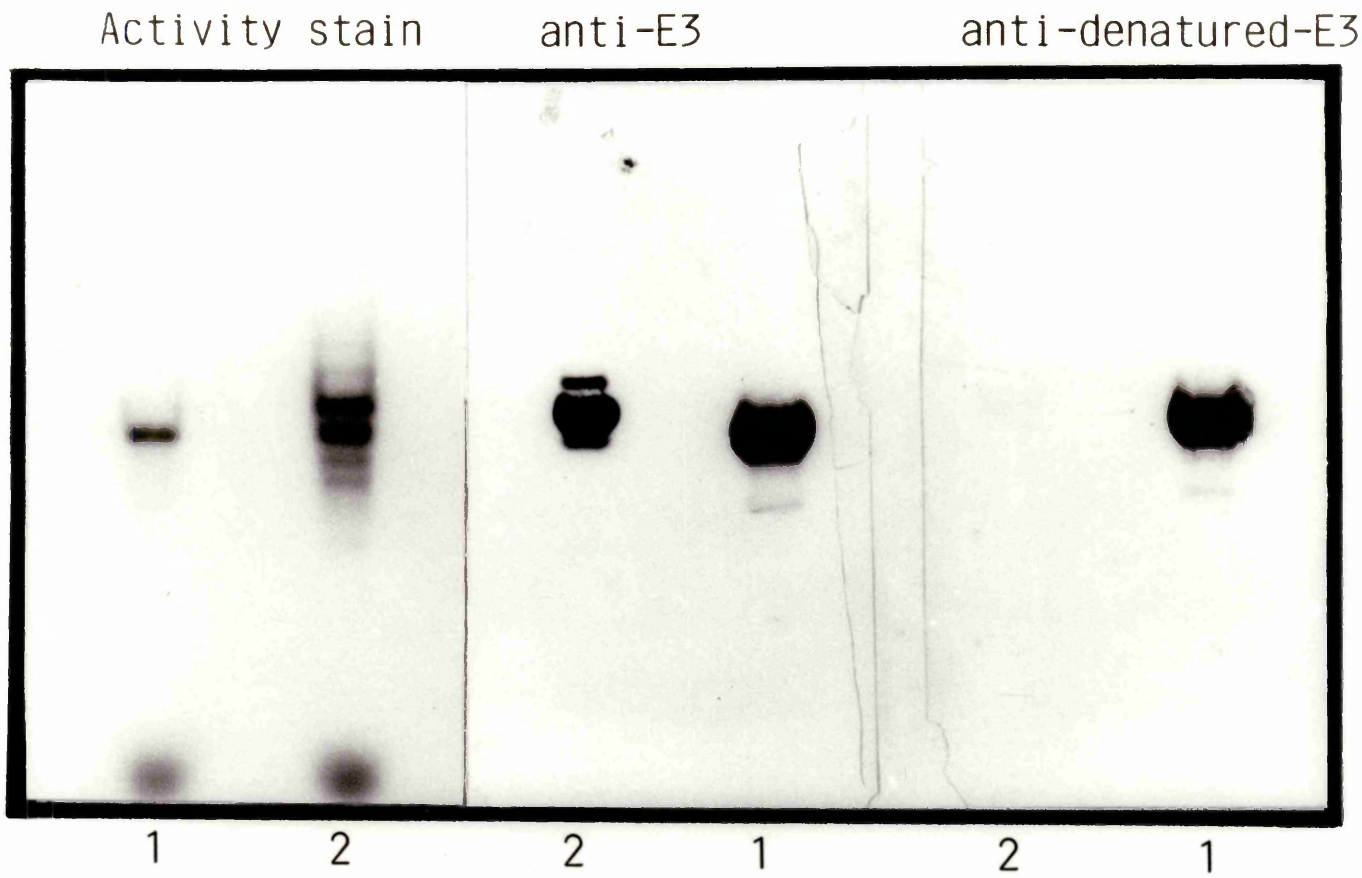
Figure 4.8 Non-denaturing gel electrophoresis of purified *A. calcoaceticus* shikimate dehydrogenase

A 10% polyacrylamide slab gel of purified E3 from both *E. coli* and *A. calcoaceticus* is shown. The gel was electrophoresed and immunoblotted with anti-*E. coli*-E3 and anti-*E. coli*-denatured-E3 as described in section 2.17.2.2. Bands of E3 were visualised by activity staining (left hand gel) (section 2.9.2) or by immunoblotting (centre and right hand gel). Only one of the two activity bands cross reacts with anti-*E. coli*-E3.

Track No.

1 : purified *E. coli* E3

2 : purified *A. calcoaceticus* E3


Activity stain
anti-E3
anti-denatured-E3
1
2
2
1
2
1

purified A. calcoaceticus E3 had quinate dehydrogenase activity assays were carried out at pH 6, 7.5 and 10.6 using NAD^+, $NADP^+$ and DCPIP as electron acceptors. Purified E. coli E3 was also assayed in the same way and the results are presented in Table 4.4. It was found that the A. calcoaceticus E3 did not have any detectable quinate dehydrogenase activity. This result is consistent with earlier work that showed that the quinate dehydrogenase of A. aerogenes had no shikimate dehydrogenase activity (Mitsuhashi & Davis, 1954). The data in Table 4.4 also shows that DCPIP was a good electron acceptor with an optimum at pH 7.5 which differs from the findings of Tresguerres et al. (1970a) who observed optimal activity at pH 6.0. At this pH A. calcoaceticus E3 gave a specific activity with this dye-linked assay 6-fold higher than that for E. coli E3 in the presence of 6μM PMS. It was also found that if the concentration of PMS was increased to 60μM, the specific activity of E3 both in A. calcoaceticus and E. coli increased approximately five times (from Table 4.4) but there was still no detectable quinate dehydrogenase activity. Both isoenzymes were able to use $NADP^+$ as a cofactor at all pH's while NAD^+ was only used by both enzymes at pH 10.6. The specific activity with $NADP^+$ is five times higher than NAD^+ for the A. calcoaceticus enzyme and around ten times higher for the E. coli enzyme at pH 10.6. In summary the shikimate dehydrogenases of A. calcoaceticus and E. coli have, like the corresponding A. aerogenes enzyme, no quinate dehydrogenase activity and are therefore distinct from the inducible quinate (shikimate) dehydrogenase of N. crassa and A. nidulans. Some very low levels of quinate dehydrogenase activity were detected in crude extracts of A. calcoaceticus by using DCPIP as an electron acceptor. Activity was found both in cells grown on minimal medium containing inducers and in cells grown only on nutrient broth.

Table 4.4 Comparison of shikimate/quinate dehydrogenase specific activities (u/mg protein) between A. calcoaceticus and E. coli with various electron acceptors

The electron acceptors used in this experiment were DCPIP, NAD^+, $NADP^+$ and DCPIP in the presence of 6μM electron carrier PMS. Quinate dehydrogenase was assayed as described in section 2.7.6. Exactly the same procedure was used for estimating the shikimate dehydrogenase activity but shikimic acid was used instead of quinic acid. The assays were performed at pH 6.0, 7.5 and 10.6 using 100mM phosphate buffer at pH 6.0, 100mM Tris/HCl buffer at pH 7.5, and 100mM sodium carbonate buffer at pH 10.6.

	A. calcoaceticus		E. coli
	Quinate dehydrogenase	Shikimate dehydrogenase	Shikimate dehydrogenase
pH 6.0			
DCPIP	nd	nd	nd
NAD^+	nd	nd	nd
$NADP^+$	nd	23.7	27.64
DCPIP + PMS	nd	2.2	0.67
pH 7.5			
DCPIP	nd	nd	nd
NAD^+	nd	nd	2.02
$NADP^+$	nd	129.4	120.68
DCPIP + PMS	nd	12.94	2.16
pH 10.6			
DCPIP	nd	nd	nd
NAD^+	nd	110.0	63.4
$NADP^+$	nd	504.8	660.7
DCPIP + PMS	nd	nd	nd

nd = no activity detected

Although no detailed studies were carried out it seems likely that the quinate dehydrogenase of A. calcoaceticus is a membrane-bound enzyme as proposed by Tresguerres et al. (1970a,b) and by van Kleef & Duine (1988) and will require emulsifiers, such as triton X-100, to extract it from the membrane.

4.5 Summary of the cross reaction experiments

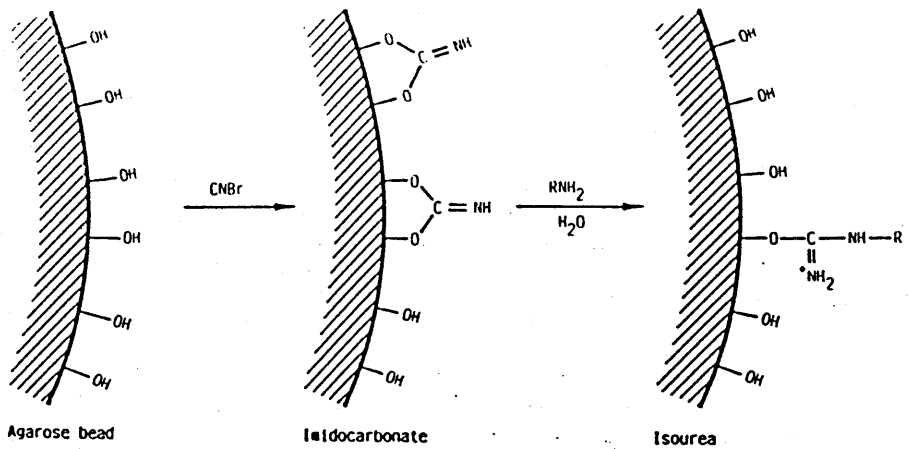
The experiments described in section 4.4, which confirm the preliminary experiments reported in section 3.5.3.2, provide strong evidence that A. calcoaceticus has two isoenzymes with shikimate dehydrogenase activity. Neither of these isoenzymes has quinate dehydrogenase activity. In vivo it is possible that these isoenzymes may have different functions: one may be a biosynthetic enzyme and the other may be a catabolic enzyme involved, for example, in quinate utilisation. To try and further characterise these isoenzymes an attempt was made to separate them by immunoaffinity chromatography as described in the next section.

4.6 Separation of isoenzymes by immuno-affinity chromatography

4.6.1 General introduction

Cyanogen bromide activated Sepharose is a convenient solid support which has been widely used for the preparation of antibody affinity columns. Cyanogen bromide reacts with the free hydroxyl groups of Sepharose or agarose at high pH to give imidocarbonates (activated products) which can then be reacted with any compound such as a protein, that contains primary amino groups. The final products are isoureas which carry a partial positive charge at neutral pH (Fig. 4.9) (Porath et al., 1967; Boegman & Crumpton, 1970; Cuatrecasas, 1970; Porath, 1974); the activated intermediates are probably imidocarbonates but this is still

Figure 4.9 Probable mechanism of CNBr coupling of protein to Sepharose (adapted from Johnstone & Thorpe, 1987)



uncertain (Johnstone & Thorpe, 1987). In the present study, the starting material used was commercially available CNBr-activated Sepharose 4B. The availability of this product made it unnecessary to handle free cyanogen bromide, an unpleasant and hazardous reagent (Plummer, 1987).

Cyanogen bromide activated Sepharose 4B is an ideal material for the preparation of immunoaffinity columns for the following reasons (Johnstone & Thorpe, 1987):

(a) It is suitable for column chromatography as it has a loose porous network and gives good flow rates.

(b) Non-specific absorption is minimal.

(c) It has a high capacity and the chemical linkages when formed are stable so that the ligand is rendered permanently insoluble and the columns can be reused.

Very high concentrations of ligand molecules per unit insoluble support can be obtained with Sepharose 4B. However it is important to avoid too high substitution since this may increase non-specific binding and steric hindrance of the ligand and make subsequent elution difficult. When coupling the ligand, in this case the antibody, it is also important to maintain it in its native conformation.

4.6.2 Purification of rabbit IgG

Before coupling to CNBr-activated Sepharose 4B the anti-E3 antibody was purified by chromatography on a DEAE Affi-Gel Blue column. The column was prepared and treated as recommended in the manufacturers instruction booklet. Crude rabbit serum (4ml) was dialysed against 1 l of 0.02 M-Tris/HCl, pH 8.0, 0.028M-NaCl, 0.02% NaN_3 (buffer A). The dialysed serum was applied to a column (15cm x 2.1cm) of DEAE Affi-Gel Blue which had been pre-equilibrated

with three bed volumes of buffer A. The column was eluted with three bed volumes of buffer A (flow rate 60ml/h, 2ml fractions). Fractions were collected and the A_{280} recorded (Fig. 4.10). For further use, the column was regenerated with two bed volumes of 2M-Guanidine.HCl in buffer A, followed by three bed volumes of buffer A. The fractions containing IgG were pooled and then lyophilised to dryness. The resulting solid was dissolved by adding small aliquots of buffer A to give finally the same total volume as the original crude antiserum. This solution was then dialysed overnight against 2 l of coupling buffer (0.1M-$NaHCO_3$, pH 8.3 containing 0.5M-NaCl). The purified antibody was checked for purity by SDS PAGE (Fig. 4.11); track 2 showed the IgG heavy chain and light chain of M_r 50,000 and 27,000 respectively. The purified antibody solution in coupling buffer was stored at -20°C until required.

When using new samples of DEAE Affi-Gel Blue, which had not been previously used for serum chromatography, the following pre-wash procedure was always used. The gel is transferred to a funnel and washed with at least five bed volumes of 0.10M-acetic acid, pH 3.0 containing 1.4M-NaCl and 40% (v/v) isopropanol. This wash elutes from new preparations of gel any residual dye which might otherwise be eluted in serum protein fractions. The gel is then washed with at least ten bed volumes of buffer A to reduce the ionic strength to that required for the serum chromatography.

4.6.3 Checking the properties of purified anti-E3

4.6.3.1 Enzyme inhibition assay and immunoprecipitation assay with protein A

Before coupling the purified anti-E3 to CNBr-activated Sepharose 4B, it was tested for its ability to bind and inhibit both

Figure 4.10 Purification of anti-E. coli-E3 on a DEAE Affi-Gel Blue column

The dialysed crude antiserum was applied to a DEAE Affi-Gel Blue column (2.1cm x 15cm) equilibrated with buffer A. The column was eluted with three bed volumes of buffer A as in the text. The flow rate was 60ml/h, 2ml fractions were collected and the absorbance at 280nm was recorded. The arrows indicate the pooled fractions.

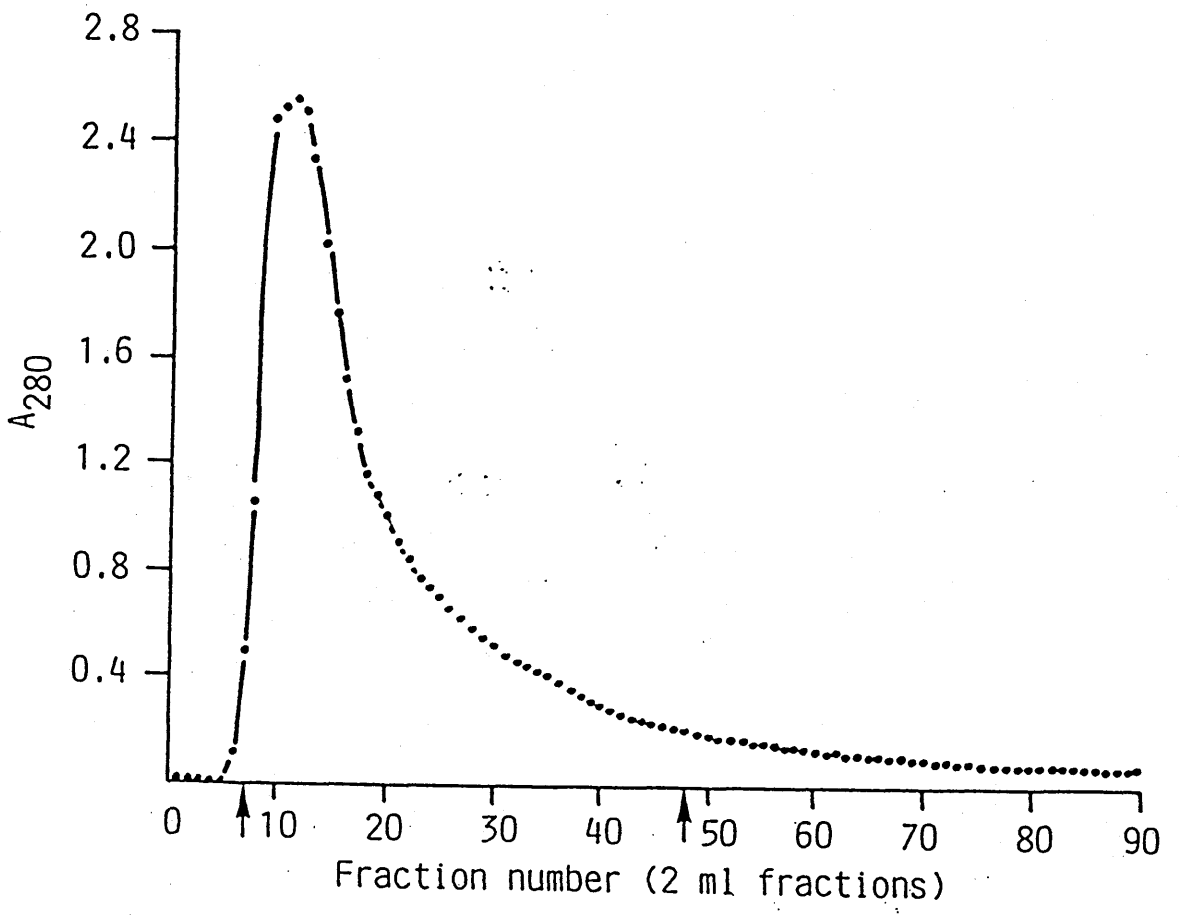
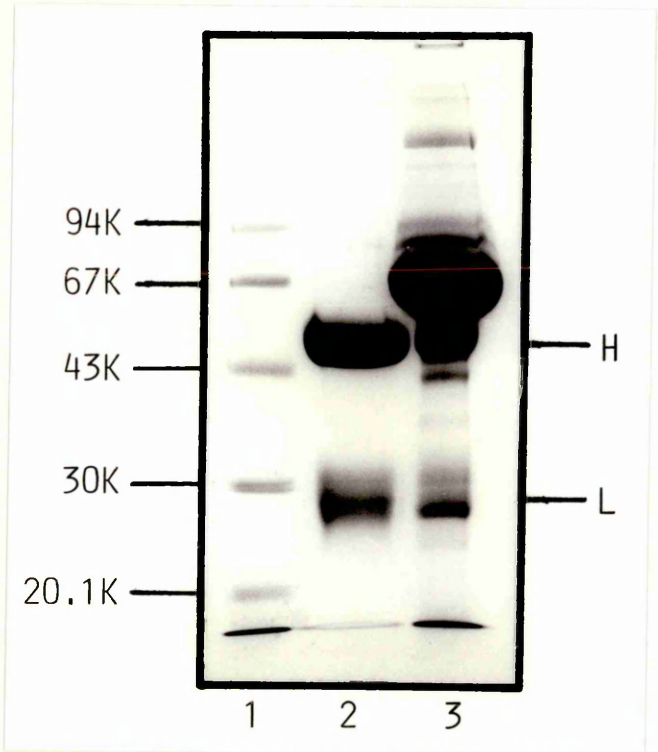


Figure 4.11 Purity of anti-E. coli-E3 purified on DEAE Affi-Gel Blue

This 10% SDS PAGE was stained with Coomassie Blue as described in section 2.9.1.1.

Track No.		amounts loaded (µg)
1	: crude antiserum	30
2	: purified IgG	15
3	: M_r standard proteins	-



native E. coli E3 and native A. calcoaceticus E3. It was found that purified IgG had the same activity as the crude antiserum i.e. it totally inhibited and precipitated E. coli E3 but only inhibited and precipitated, as an immunocomplex with protein A, 50% of the E3 activity of purified A. calcoaceticus E3.

To compare the efficiency of binding, antibody titration curves were measured against E. coli E3 for both the crude antiserum and the purified IgG fraction (Fig. 4.12). Under these conditions the purified IgG totally inhibited E. coli E3 activity at a dilution 1:8 while crude antiserum totally inhibited E. coli E3 at a dilution 1:16. Some IgG must have been lost during the purification since the efficiency of binding is less than for the crude antiserum.

4.6.3.2 ELISA

To further compare the efficiency of binding of the crude antiserum and the purified IgG fraction to native E. coli E3 an ELISA experiment was carried out. The titres above background of normal rabbit serum (0.5 O.D.) for the crude antiserum and purified IgG were 1:112,000 and 1:56,000 respectively (Fig. 4.13).

4.6.3.3 Immunoblots

The efficiency of binding to SDS-denatured E. coli enzyme was measured by immunoblotting both for the crude antiserum and the purified IgG (see Fig. 4.14). The efficiency of binding of the purified IgG is less than that found for crude antiserum; this can be seen by comparing the tracks corresponding to the low concentrations of antigen (Fig. 4.14).

4.6.4 Coupling purified IgG to CNBr-activated Sepharose 4B

The immunoaffinity column was prepared by coupling purified anti-E3 (approximately 15mg of IgG) to 3.5ml of CNBr-activated

Figure 4.12 Test of the efficiency of purified anti-E. coli-E3 in enzyme inhibition assay

Data are shown for crude antiserum (Δ) and purified IgG (□). The crude extract from the E. coli overproducing strain was serially diluted and then equal amounts of anti-E. coli-E3 was added and after incubation for 1 h enzyme activity was assayed (section 2.13).

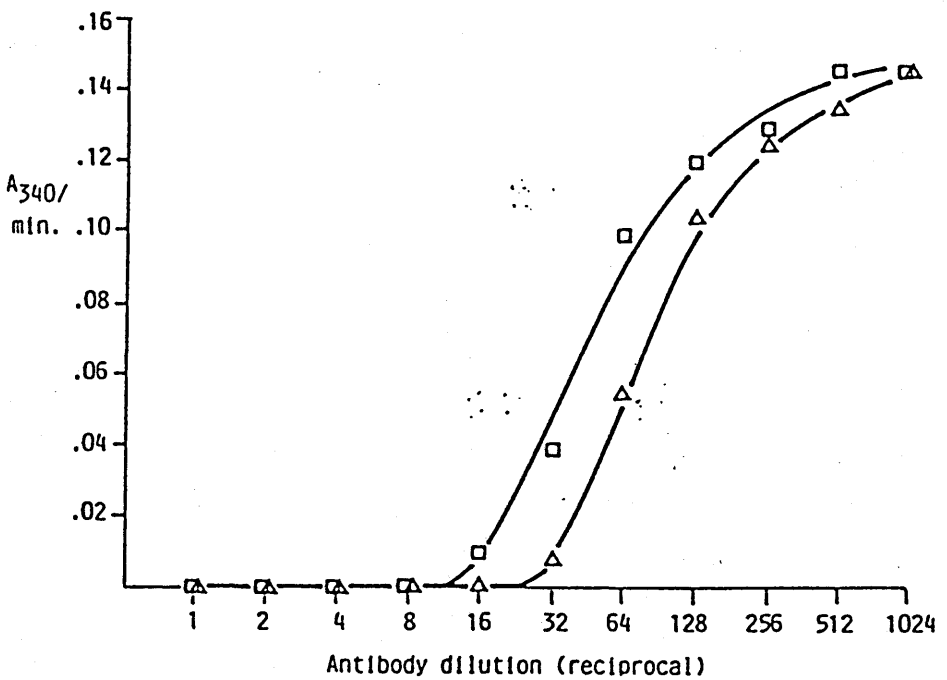


Figure 4.13 Test of the efficiency of binding of the purified anti-*E. coli*-E3 to native *E. coli* E3 by ELISA

Data are shown for control normal rabbit antiserum (O), crude anti-*E. coli* E3 (□) and the purified IgG (Δ). 100ng of purified *E. coli* E3 was coated onto the microElisa plate and left overnight at 4°C. 100µl aliquots of serial dilutions of antiserum or purified IgG were added. After incubation the peroxidase-conjugated antibody was added. The reaction was terminated after adding substrate solution by addition of 6N-H_2SO_4. The colour was monitored at 492nm as described in detail in section 2.16.2.

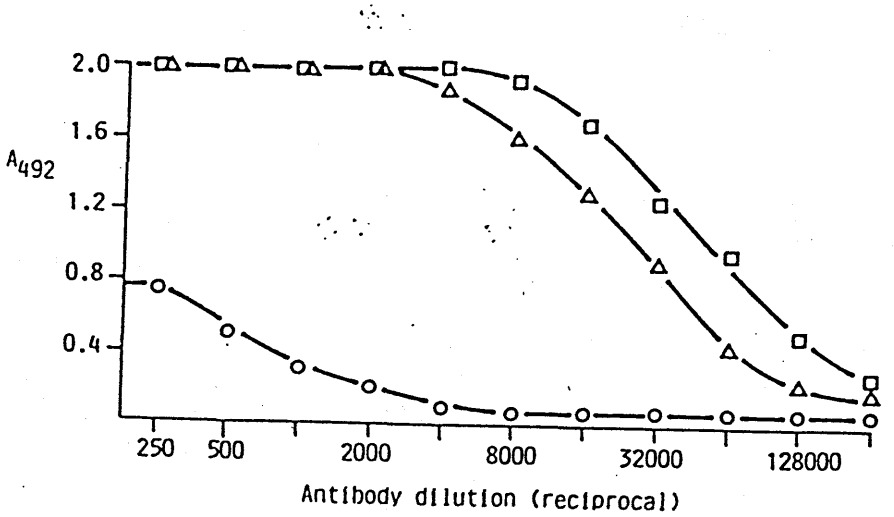


Figure 4.14 Test of the efficiency of binding of the purified anti-*E. coli* E3 to denatured E3 by immunoblotting

Various concentrations of purified enzyme and crude extract were investigated. The crude extract and purified enzyme from the *E. coli* overproducing strain was serially diluted, then electrophoresed on 10% SDS PAGE and immunoblotted with crude antiserum and purified IgG as described in section 2.17.1.2.

Track No.			amounts loaded (ng)
1	:	crude extract	1,000
2	:	crude extract	100
3	:	crude extract	10
4	:	purified E3	50
5	:	purified E3	5
6	:	purified E3	0.5
7	:	M_r standard proteins	-

Sepharose 4B. The coupling condition employed were those recommended by the manufacturers. 1g of the freeze dried (about 3.5ml swollen gel) CNBr-activated Sepharose 4B was suspended in 10ml 1mM-HCl (pH 2-3). The gel swelled immediately and was then washed for 15-20 min with 1mM-HCl (200ml) on a sintered glass filter (G3). The washing of the gel at low pH preserves the ligand binding of the Sepharose better than washing at pH 7.4. The CNBr-activated Sepharose 4B contains dextran and lactose to preserve its activity during freeze drying and these are removed by the washing procedure. Activated Sepharose was then washed with coupling buffer (500 ml) to raise the pH. The washed gel was collected by filtration on the sintered glass funnel and transferred to a 20ml universal tube containing purified antibody (15mg) in coupling buffer (12ml). The bottle was closed tightly and mixed continually by end-over-end rotation on a mechanical rotator for 2 h at room temperature and then overnight (16 h) at 4°C. Coupling appeared to be complete since all the A_{280} absorbing material (IgG) had bound to the gel. The gel was washed with 100ml coupling buffer and any remaining reactive groups were blocked by treatment with 12ml 0.1M-Tris/HCl, pH 8.0 for 2 h at room temperature with continual mixing on the rotator. The resulting gel was washed with three cycles of solutions of alternating pH. Each cycle consisted of 100ml 0.1M-acetate buffer, pH 4 containing 0.5M-NaCl followed by 100ml 0.1M-Tris/HCl, pH 8 containing 0.5M-NaCl. The final product was packed into a column and stored in phosphate buffer saline containing 0.05% NaN_3 at 4°C.

4.6.5 Enzyme purification by immunoaffinity chromatography

The general procedure used for immunoaffinity chromatography is outlined below. All steps should be carried out at 4°C.

Step 1: Column equilibration

The column with immobilised antibody is first washed with 10ml 50mM-Tris/HCl, pH7.5 followed by 5ml 0.5M-ammonium hydroxide containing 3M-potassium thiocyanate (chaotropic salt) and then with 20ml 50mM-Tris/HCl, pH 7.5. The flow rate should be 5ml/h.

Step 2: Sample loading

The enzyme (antigen) sample is then applied to the pre-equilibrated immunoaffinity column and the column washed with 50mM-Tris/HCl, pH 7.5 until no activity of the enzyme and no absorbance at 280nm can be detected in the effluent (flow rate 1ml/h, 1ml fractions).

Step 3: Elution

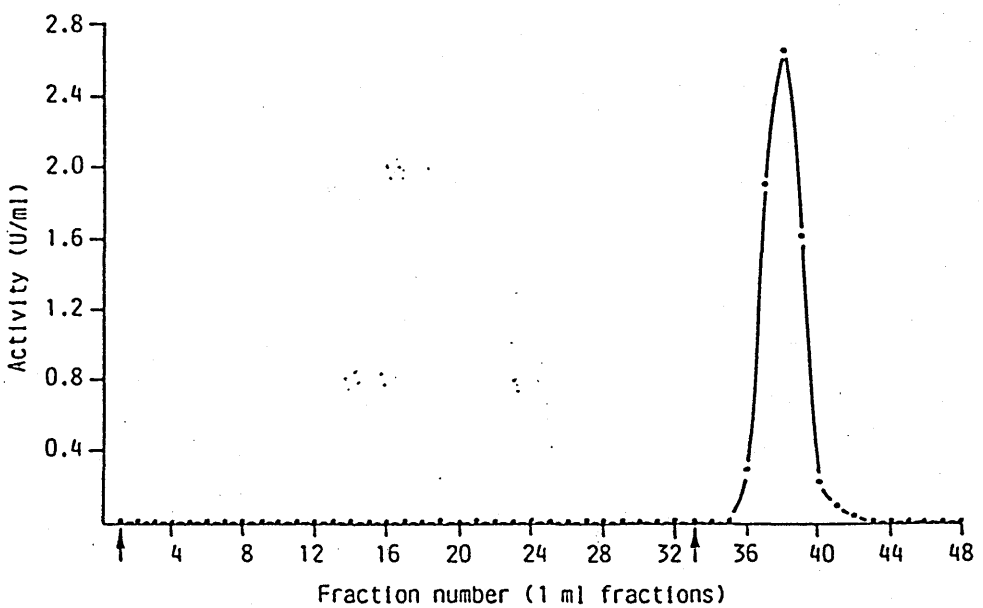
The column is then eluted with 5ml 0.5-M ammonium hydroxide containing 3M-potassium thiocyanate, followed by 10ml 50mM-Tris/HCl, pH 7.5 (flow rate 5ml/h, 1ml fractions). Fractions containing enzyme activity and/or protein are pooled as required.

4.6.5.1 Purification of E. coli E3 by immunoaffinity chromatography

Partially purified E. coli E3 (approximately 10 units) was loaded onto the immunoaffinity column and after washing the column was eluted with thiocyanate solution as described above. All of the E3 activity bound to the column and was subsequently eluted with the thiocyanate solution. The overall yield was 80% (Fig. 4.15).

Figure 4.15 Purification of E. coli E3 by immunoaffinity chromatography

Approximately 10 units of E. coli E3 were loaded onto the column; the flow rate was 1ml/h and 1ml fractions were collected. The column was washed with 50mM-Tris/HCl, pH 7.5 applied at the first arrow. The bound E3 was eluted with 0.5M ammonium hydroxide containing 3M-potassium thiocyanate applied at the second arrow. The flow rate was 5ml/h and 1ml/fractions were collected as described in section 4.6.5. E3 activity was monitored in all fractions.



4.6.5.2 Separation of A. calcoaceticus isoenzymes by immunoaffinity column

Purified A. calcoaceticus E3 (approximately 10 units) was loaded onto the immunoaffinity column as in section 4.6.5.1. Only 0.2 units bound to the column (Fig. 4.16) which indicated that the binding capacity of the anti-E. coli-E3 column for the A. calcoaceticus E3 was very low when compared with its capacity to bind the E. coli enzyme. The bound enzyme was then eluted with thiocyanate.

4.7 The antigenic properties of the immunoaffinity purified enzymes

4.7.1 Enzyme inhibition assay and immunoprecipitation assay with protein A

After elution with thiocyanate the bound material from the immunoaffinity column was dialysed overnight against 2 l of 50mM-Tris/HCl, pH 7.5 and was then tested in the enzyme inhibition assay and in the immunoprecipitation assay with protein A. The immunoaffinity purified E. coli E3 was totally inhibited and precipitated whilst the immunoaffinity purified A. calcoaceticus E3 was only 80% precipitated; 20% of the enzyme activity remained in the supernatant. In comparison a control sample of A. calcoaceticus E3 which had not been immunoaffinity purified, was only 49% precipitated by protein A with 51% of the activity remaining in the supernatant (Table 4.5).

These immunoaffinity chromatography results were disappointing. The column appears to bind selectively the A. calcoaceticus isoenzyme which is precipitated by the anti-E. coli-E3 but the binding capacity was too low for this to be practically useful.

Figure 4.16 Attempted separation of the isoenzymes of A. calcoaceticus E3 by immunoaffinity chromatography

Approximately 10 units of A. calcoaceticus E3 were loaded onto the column with flow rate 1ml/h and 1ml fractions were collected. The column was washed with 50mM-Tris/HCl, pH 7.5 applied at the first arrow. The bound material was eluted with potassium thiocyanate in alkaline solution applied to the second arrow. The flow rate was 5ml/h and 1ml fractions were collected as described in section 4.6.5. The E3 activity was monitored in all fractions.

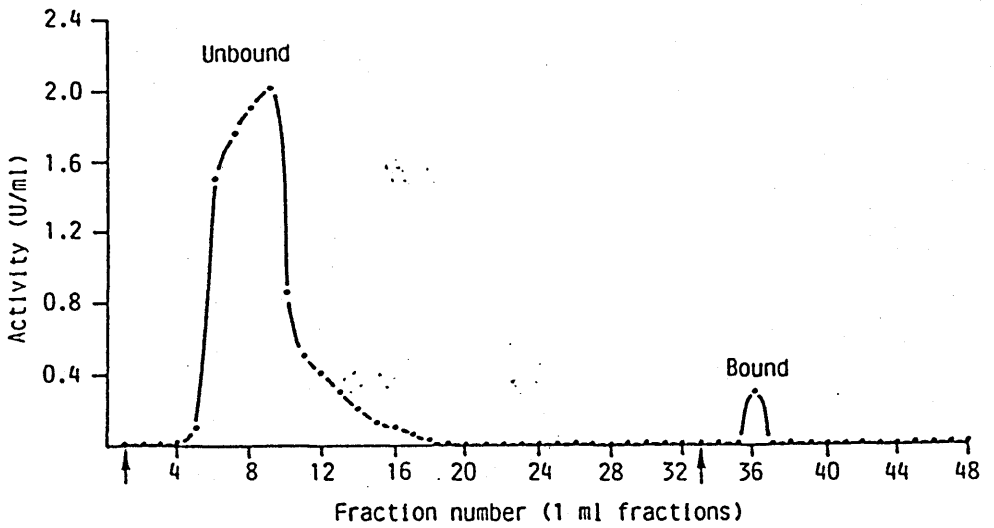


Table 4.5 Antigenic properties of enzyme which either failed to bind or bound to the immunoaffinity column

The immunoaffinity column was prepared by reacting purified anti-*E. coli*-E3 with cyanogen bromide activated Sepharose as described in the text. The material which failed to bind to the column and the material which bound and was subsequently eluted with potassium thiocyanate was tested by immunoprecipitation with anti-*E. coli*-E3 and protein A.

Enzyme samples and reagents	% precipitated enzyme	% enzyme in solution
1. bound E3 (*E. coli*) + anti-E3 + protein A	100	-
2. bound E3 (*A. calcoaceticus*) + anti-E3 + protein A	80	20
3. unbound E3 (*A. calcoaceticus*) + anti-E3 + protein A	49	51

4.7.2 Immunoblots

Although the yield from the immunoaffinity chromatography of the A. calcoaceticus enzyme was very low, the purified material was still tested by immunoblotting to confirm the binding assay results. Both the immunoaffinity purified enzyme and enzyme that failed to bind to the immunoaffinity column gave positive results both with crude antiserum and with purified IgG (Fig. 4.17A,B). This confirmed the immunoprecipitation results which indicated that the immunoaffinity purified A. calcoaceticus E3 was still a mixture of the two isoenzymes.

4.8 Separation of isoenzymes by isoelectric focusing

4.8.1 General introduction

Isoelectric focusing is one of the most powerful techniques for the resolution of protein mixtures. The components are separated according to their isoelectric point (pI) which is the pH at which they possess no net electric charge.

In isoelectric focusing electrophoresis, a pH gradient is formed in a gel between cathode and anode, and proteins migrate (due to the attraction of their charged groups for anode and cathode) until they reach their pI. As the protein is not charged in this position it remains stationary. Isoelectric focusing is therefore a steady state process and components should theoretically never run off the end of a gel containing the correct pH gradient.

A pH gradient will form automatically if a potential difference is applied to an H^+ containing ionizable solvent (H^+ ions will be displaced towards the cathode). However, such gradients are unstable and so gradient stabilizing molecules are added. These are normally purpose-made amphoteric compounds known

Figure 4.17 Immunoblots of shikimate dehydrogenase samples after immunoaffinity chromatography.

A 12.5% SDS PAGE immunoblotted with crude antiserum (159:11)

Track No.

1 : M_r standard proteins

2 : purified *E. coli* shikimate dehydrogenase

3 : purified *A. calcoaceticus* shikimate dehydrogenase

4 : purified shikimate dehydrogenase from *A. calcoaceticus* which failed to bind to immunoaffinity column

5 : immunoaffinity purified *A. calcoaceticus* shikimate dehydrogenase

B Immunoblot of 12.5% SDS PAGE with crude antiserum (left panel) and purified IgG (right panel) (159:11)

Track No.

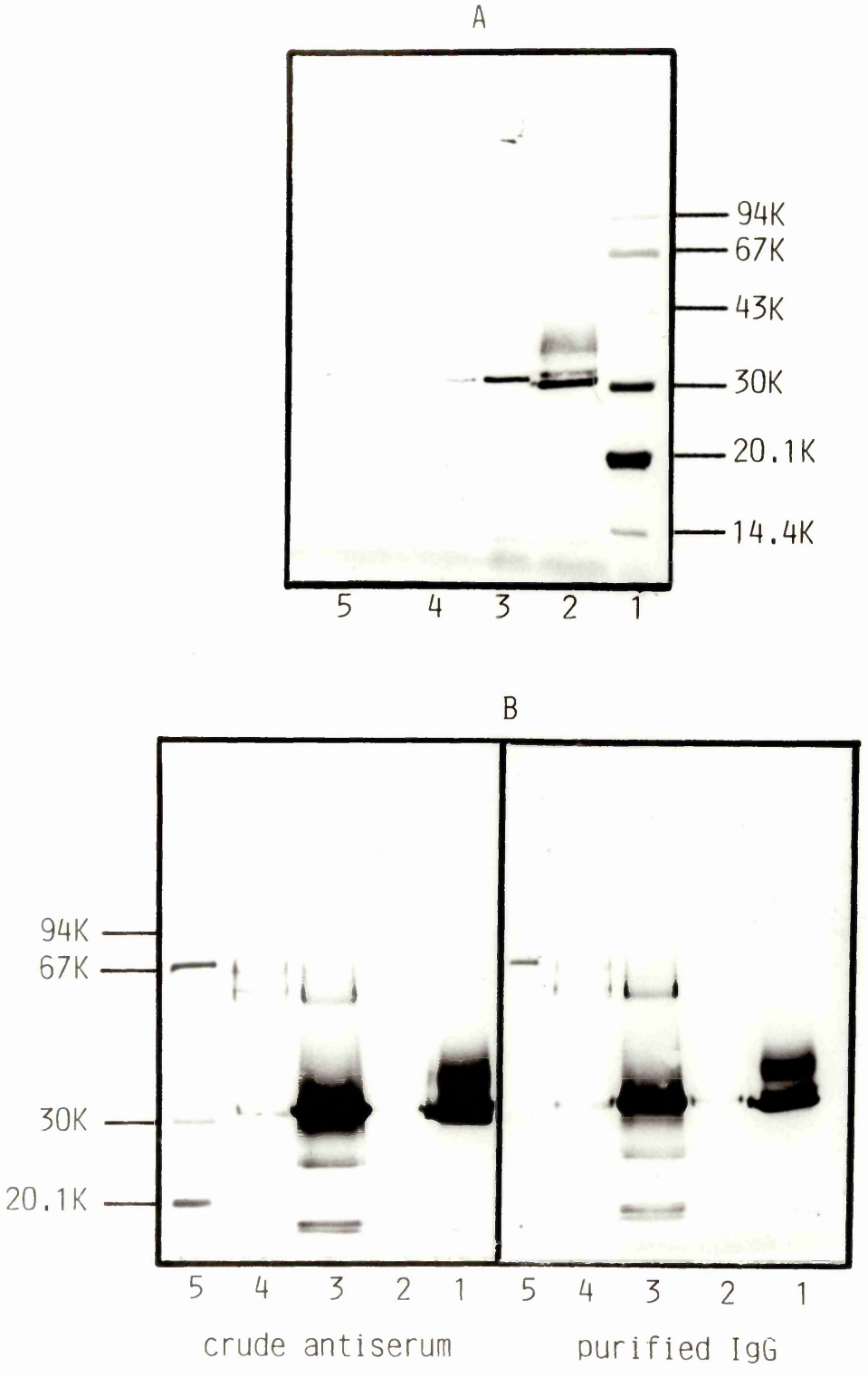
1 : *E. coli* E3 after elution from the column

2 : *A. calcoaceticus* E3 after elution from the column

3 : *E. coli* E3 before passage through the column

4 : *A. calcoaceticus* E3 before passage through the column

5 : M_r standard proteins


A
94K
67K
43K
30K
20.1K
14.4K
5 4 3 2 1
B
94K
67K
30K
20.1K
5 4 3 2 1 5 4 3 2 1
crude antiserum
purified IgG

as 'carrier ampholytes'. These zwitterions have sharply defined pI's and the mixture is chosen to have pI values evenly distributed over the desired pH range. Such mixtures are commercially available.

Polyacrylamide gel is an ideal anticonvection medium for isoelectric focusing because it is robust, permeable to most proteins, easy to prepare and shows very little electroendosmosis. This latter property is very important in isoelectric focusing as electroendosmosis will cause the whole pH gradient to move toward the cathode and thus disrupt the steady state process (Johnstone & Thorpe, 1987).

Recently Pharmacia have introduced a chromatographic version of isoelectric focusing. In this f.p.l.c. technique Mono P chromatofocusing chromatography is carried out on a Mono P column. A pH gradient is formed using polybuffer which is a commercially available mixture of ampholytes.

4.8.2 Separation of A. calcoaceticus isoenzymes by isoelectric focusing electrophoresis

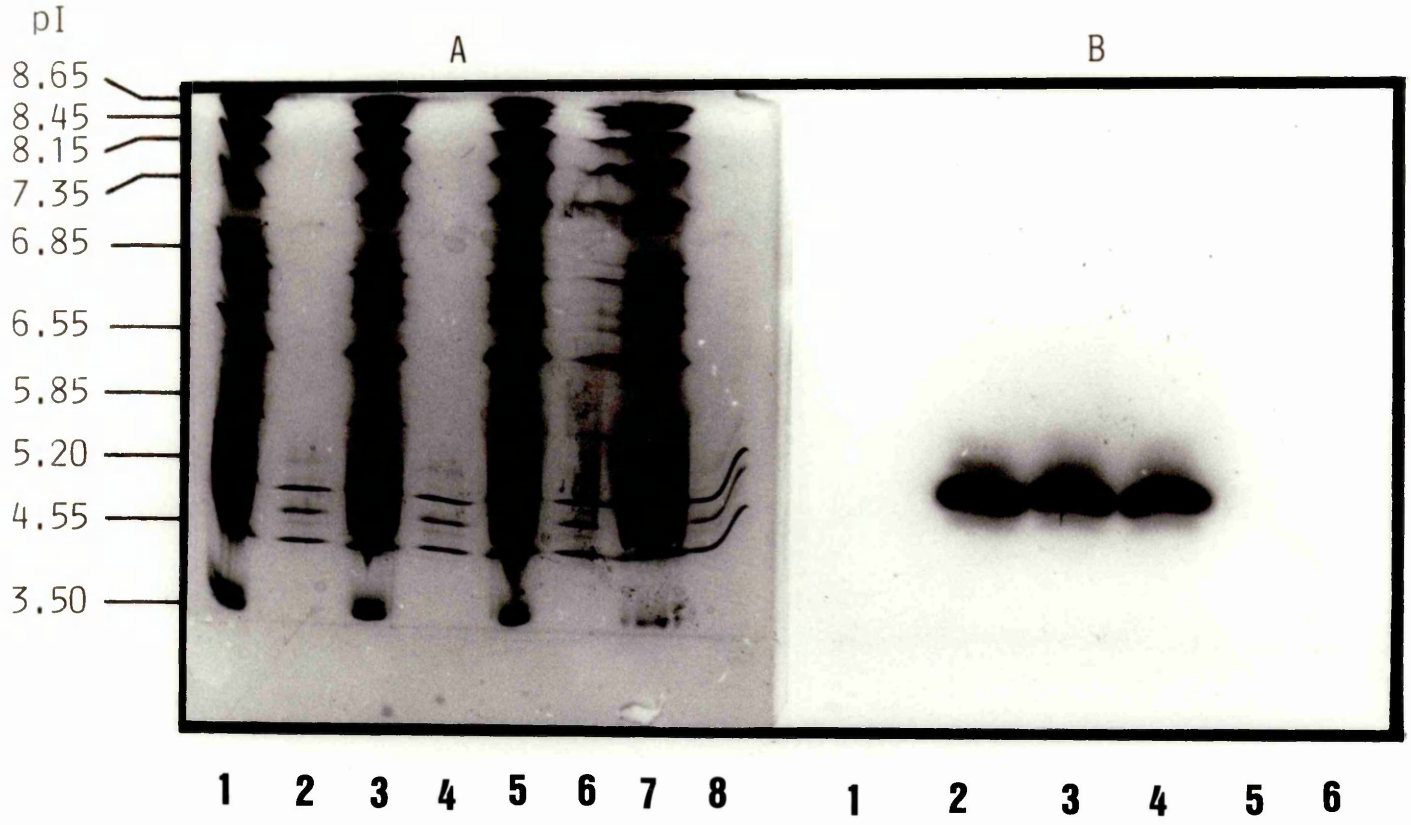
Partial purified A. calcoaceticus E3 was loaded onto a Phast Gel (Pharmacia) isoelectric focusing gel designed to operate in the pH range 3 to 9. The gel was run on a PhastSystem apparatus under the conditions recommended by the manufacturers (Pharmacia). The gels were stained for activity and for protein using the sensitive silver stain. The isoenzymes were not resolved and so they must have the same pI of 5.5 (estimated by comparing with the pI calibration Kit proteins) (Fig. 4.18). This confirms that the isoenzymes are acidic proteins, a property in accordance with their chromatographic behaviour. This experiment provides further evidence that the two isoenzymes are very similar. Again activity staining showed three minor bands which could arise from proteolysis.

Figure 4.18 Isoelectric focusing of A. calcoaceticus E3

The partially purified A. calcoaceticus E3 was loaded onto a PhastGel isoelectric focusing gel (pH range 3 to 9) as described in section 4.8.2. (A) silver stained gel (section 2.9.1.2) : track 1, 3, 5, 7, pI calibration kit proteins; track 2, 4, 6, 8, partially purified E3. (B) activity stained gel (section 2.9.2) : track 2, 3, 4, partially purified E3. The band which has enzyme activity has a pI of 5.2.

(A) silver stain

(B) activity stain


pI
A
B
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.50
1 2 3 4 5 6 7 8
1 2 3 4 5 6

4.9 Discussion

4.9.1 Purification of A. calcoaceticus E3

Three fast chromatographic steps were required to produce homogeneous A. calcoaceticus shikimate dehydrogenase in comparison with the four slow steps in the purification procedure developed for the E. coli enzyme (Chaudhuri & Coggins, 1985). At the ammonium sulphate fractionation step the A. calcoaceticus shikimate dehydrogenase activity was found in the 40-60% saturation fraction while the E. coli E3 was found in the 30-55% fraction. The first chromatographic step was ultrogel AcA44 which could be run overnight after the first day. This step eliminated a lot of unwanted proteins. The pooled active fractions could then be passed directly onto the Procion red column on the second day. Procion red is very useful for purifying $NAD(P)^+$-binding proteins since it acts as both an anion exchange and an affinity column (Scopes, 1987). The E3 activity was eluted at a salt concentration of 416mM in the presence of 0.1mM-NADPH. The material from this step had only five protein components when analysed by SDS PAGE (Fig. 4.4, track 5). The pooled active fractions from this step were then dialysed overnight in Tris buffer and the final purification step was chromatography on a high performance, strong anion exchange column (Mono Q). E3 was eluted free from other contaminating polypeptides at a salt concentration of 225mM and showed a single band of protein of M_r 31,000 on SDS PAGE (Fig. 4.4, track 6). Purified A. calcoaceticus E3 was stable for many months when stored in 50mM-Tris/HCl buffer, pH 7.5 in the presence of 50% glycerol at $-20^{o}C$.

The native molecular weight value obtained from f.p.l.c. gel filtration indicated that A. calcoaceticus E3 is a monomeric protein like the E. coli enzyme (Chaudhuri & Coggins, 1985).

4.9.2 Cross reaction with anti-E. coli-E3

Immunoprecipitation experiments on the purified A. calcoaceticus E3 were in agreement with those on crude preparations; only 50% of the enzyme activity was precipitated by anti-E. coli-E3 and protein A. This suggested that the two putative isoenzymes had the same molecular weight but somehow differed in the exposed amino acid sequences on the surface of the molecule. These differences affect the ability of the enzyme to interact with antibody; one isoenzyme must have epitopes in common with the E. coli enzyme and the other must lack the epitope, recognised by the anti-E. coli-E3 antibodies. To confirm the existence of isoenzymes antigen and antibody titration curves were determined and enzyme inhibition assays carried out. These experiments all provided evidence for the existence of two isoenzymes. The strongest evidence for two isoenzymes came from the native gel electrophoresis; both activity staining and immunoblotting confirmed the existence of two enzyme species one of which bound to the anti-E. coli-E3 while the other did not. Unfortunately isoelectric focussing failed to separate the two isoenzymes and although immunoaffinity chromatography resulted in some enrichment of the species which could be immunoprecipitated the yields were too low to be practically useful.

4.9.3 Specificity and role of the purified A. calcoaceticus E3

The question whether quinate dehydrogenase and shikimate dehydrogenase activities were associated with the same protein in A. calcoaceticus has also been resolved by this study. The purified A. calcoaceticus shikimate dehydrogenase had no detectable quinate dehydrogenase activity when NAD^+, $NADP^+$ or DCPIP were used as electron acceptors at various pH's. The enzymic properties

of the purified A. calcoaceticus enzyme with regard to preference for phosphorylated pyridine nucleotide and pH optimum were very similar but not identical to those of the well characterised E. coli biosynthetic enzyme. The A. calcoaceticus enzyme has a specific activity with NAD^+, at pH 10.6, which is 22% of the specific activity obtained with $NADP^+$ while the E. coli enzyme has a specific activity with NAD^+ of only 10% of that found with $NADP^+$. Although the specific activities with $NADP^+$ at pH 7.5 for both the A. calcoaceticus enzyme and the E. coli enzyme are very similar the A. calcoaceticus enzyme shows a specific activity 6-fold higher than the E. coli enzyme when DCPIP and PMS are used as electron acceptors. This relatively high level of dye-linked shikimate dehydrogenase activity found with the A. calcoaceticus enzyme distinguishes it from the E. coli enzyme.

Since it was impossible to separate completely the two postulated A. calcoacetius isoenzymes it was not possible to establish whether they differed in their specific activities or substrate binding properties.

The very low levels of quinate dehydrogenase activity found in crude extracts of A. calcoaceticus was at first surprising in the light of the findings that other microbial species which, like A. calcoaceticus can utilise quinic acid, have quite high levels of NAD^+-dependent quinate dehydrogenase (Ahmed & Giles, 1969; Ingledew & Tai, 1972). However the recent studies of van Kleef and Duine (1988) provide an explanation. These authors have shown that A. calcoaceticus has an NAD^+-independent, membrane associated quinate dehydrogenase which utilises PQQ as a co-factor. This enzyme which appears to be the dye-linked hydroaromatic dehydrogenase originally reported by Tresguerres et al. (1970a,b) is

the enzyme which enables A. calcoaceticus to utilise quinate. All the crude extracts of A. calcoaceticus used in this study were high speed supernatants (samples centrifuged at 100,000g for 2 h) and this would have effectively sedimented all membrane-associated enzymes. This accounts for the very low levels of quinate dehydrogenase activity found in the extracts.

4.9.4 Separation of the isoenzymes

Although there was good immunochemical evidence for two isoenzymes of shikimate dehydrogenase in A. calcoaceticus their quantitative separation was not achieved. The two isoenzymes were not separated by ion exchange and dye columns, by gel filtration, by SDS PAGE and by isoelectric focusing. They were resolved by PAGE under native conditions but this is not a practical preparative method. Since the two isoenzymes were immunochemically distinct the best idea for their separation appeared to be immunoaffinity chromatography. Although this method gave some enrichment for the isoenzyme that bound to the anti-E. coli-E3 column the yields were very low and quantitative separation was not achieved.

The major difficulty with the immunoaffinity column was its very low affinity for the A. calcoaceticus enzyme. One possible reason for this is that specific A. calcoaceticus enzyme binding antibody was only a minor fraction of the Ig on a column. The antibodies used were polyclonal and must have contained many different molecular species each with different specificities for the various epitopes of the structurally complex immunogen, E. coli shikimate dehydrogenase. Comparison of the antibody titres between E. coli E3 and A. calcoaceticus E3 highlights the problem. For the E. coli enzyme the titre was 1:128 while for the A. calcoaceticus enzyme the titre was only 1:1 (see Fig. 3.18). This indicates that

the IgG which is specific for the A. calcoaceticus enzyme is only a minor fraction of the mixture of IgG species present. The chemically coupling procedure required to attach the IgG molecules to the Sepharose column inevitably reduces the number of intact antigen binding sites and this is particularly damaging to column capacity when the number of sites available is already very small.

It is also likely that other factors such as low affinity and non-specific binding also contributed to the failure of the immunoaffinity chromatography. Better results might have been achieved if a high affinity, polyclonal antibody preparation had been available.

5. General discussion

The major objective of the work presented in this thesis was to prepare antibodies against the five E. coli enzymes that catalyse the reactions at the centre of the shikimate pathway and to use these reagents to detect sequence and structural similarities between the enzymes of the pathway within and between species.

Polyclonal antibody preparations were obtained against all five enzymes and against the pentafunctional arom protein of N. crassa which has a single, five domain, polypeptide chain that catalyses the same five reactions.

With the antibodies for the E. coli enzymes no cross reaction was detected between an antibody raised against any single enzyme of the pathway and any other enzymes of the pathway. This is consistent with sequence studies for the enzymes which initially revealed no homologies between the pathway enzymes (Duncan et al., 1987). More recent work has revealed one short region of homology between dehydroquinate synthase (E1) and dehydroquinase (E2) but the region showing homology represents only about 5% of the sequence (K. Kleanthous and J.R. Coggins, manuscript in preparation) and the chances of picking up such a short region of homology using antibody techniques are very low.

The antibodies raised against the E. coli enzymes proved to be of limited use for detecting the appropriate shikimate pathway enzymes in other species. Some success was obtained with studies of extracts of gram negative bacteria. Antibodies raised against the E. coli shikimate dehydrogenase (E3) cross reacted well with extracts of S. typhimurium and E. caratovora which suggested that

there was considerable conservation of the sequence of this enzyme between these species. There is very little comparative sequence data for bacterial shikimate pathway enzymes. In the case of EPSP synthase (E5) it is known that the sequences of the E. coli and S. typhimurium enzymes are 90% identical (Duncan et al., 1987); similar levels of homology have been reported for the dehydroquinases (E2) of E. coli and S. typhimurium (I.G. Charles, unpublished results) and the shikimate kinases (E4) of E. coli and E. caratovora (Minton et al., 1989). It seems very likely that there is a similar degree of homology between the shikimate dehydrogenases of these species.

The cross-reactions did not extend outside the gram negative species. Generally no cross reaction was found with gram positive bacterial extracts or with fungal or plant extracts. Again there is very limited comparative sequence information for the shikimate pathway enzymes in these species. For EPSP synthase it is known that the fungal and gram negative bacterial enzymes are only about 30% identical (Duncan et al., 1987) while the plant and gram negative bacterial enzymes are approximately 50% identical (Kishore & Shah, 1988; S.P. Granger and J.R Coggins, unpublished results). Only one shikimate pathway enzyme has been sequenced from a gram positive organism and this enzyme, dehydroquinase from S. coelicolor, is completely different from the corresponding E. coli enzyme. Preliminary sequence information indicates that this enzyme resembles the inducible fungal dehydroquinases (P.J. White and J.R. Coggins, unpublished results).

The cross reaction results confirm the value of antibodies for detecting enzymes from closely related species where the overall amino acid sequences are 90% or more identical. However where the species divide is wider so that the match of amino acid sequence falls to 50% or less antibodies are no longer useful for detecting homologous enzymes.

The most detailed studies reported in this thesis involved anti-E. coli shikimate dehydrogenase. Extracts of the gram negative soil bacteria A. calcoaceticus cross reacted weakly with the anti-E. coli-E3 and revealed that A. calcoaceticus had two forms of shikimate dehydrogenase. This was interesting beacause of the central position played by shikimate dehydrogenase in the metabolism of this type of organism.

It is well known that some organisms, which normally grow on living or dead plants or in the soil, such as N. crassa and A. calcoaceticus, have both a shikimate biosynthetic pathway which allows them to synthesise essential aromatic compounds and a quinate catabolic pathway which allows them to use abundant plant metabolites, such as quinic acid, as carbon sources. The shikimate pathway enzymes have so far only been studied in detail in E. coli and in the two filamentous fungi N. crassa and A. nidulans. The two fungi have both the biosynthetic shikimate and the catabolic quinate pathways. E. coli, in contrast only has the shikimate biosynthetic pathway; it lacks the quinate pathway (Mitsuhashi & Davis, 1954).

It was therefore of considerable interest to look at the comparative aspects of this pathway in a bacterial species, A. calcoaceticus which has the quinate pathway enzymes.

The results presented in detail in chapter 4 showed clearly that A. calcoaceticus differs totally from N. crassa in the way its shikimate and quinate pathway enzymes are organised. The five central shikimate pathway enzymes are separable like the corresponding E. coli enzymes; there is no pentafunctional polypeptide as found in N. crassa. A. calcoaceticus has no bifunctional $NAD(P)^+$-dependendent quinate (shikimate) dehydrogenase analogous to the inducible quinate pathway enzyme found in N. crassa. The purified shikimate dehydrogenase of A. calcoaceticus has no detectable quinate dehydrogenase activity. The quinate dehydrogenase of A. calcoaceticus appears to be a membrane associated, PQQ-dependent enzyme, which has been partially characterised by van Kleef & Duine (1988). Quinate dehydrogenase is therefore completely different in N. crassa and A. calcoaceticus.

While it is clear that A. calcoaceticus has both a biosynthetic shikimate pathway and the ability to utilise quinate the precise relationship between these two pathways remains unresolved. The enzymes required for the conversion of dehydroquinate to protocatechuate have not been fully characterised; it does appear that there are two dehydroquinases, as is found in N. crassa. The observation that there are two forms of shikimate dehydrogenase raises the possibility that one of these may be the shikimate pathway biosynthetic enzyme, while the other may be part of the catabolic pathway, which would allow the organism to use shikimate and products that can be converted to shikimate

as carbon sources.

Further work is undoubtedly necessary to complete the characterisation of both the shikimate and quinate pathway enzymes in A. calcoaceticus. This will be especially interesting because of the major differences reported in this thesis between the enzymes of A. calcoaceticus and N. crassa.

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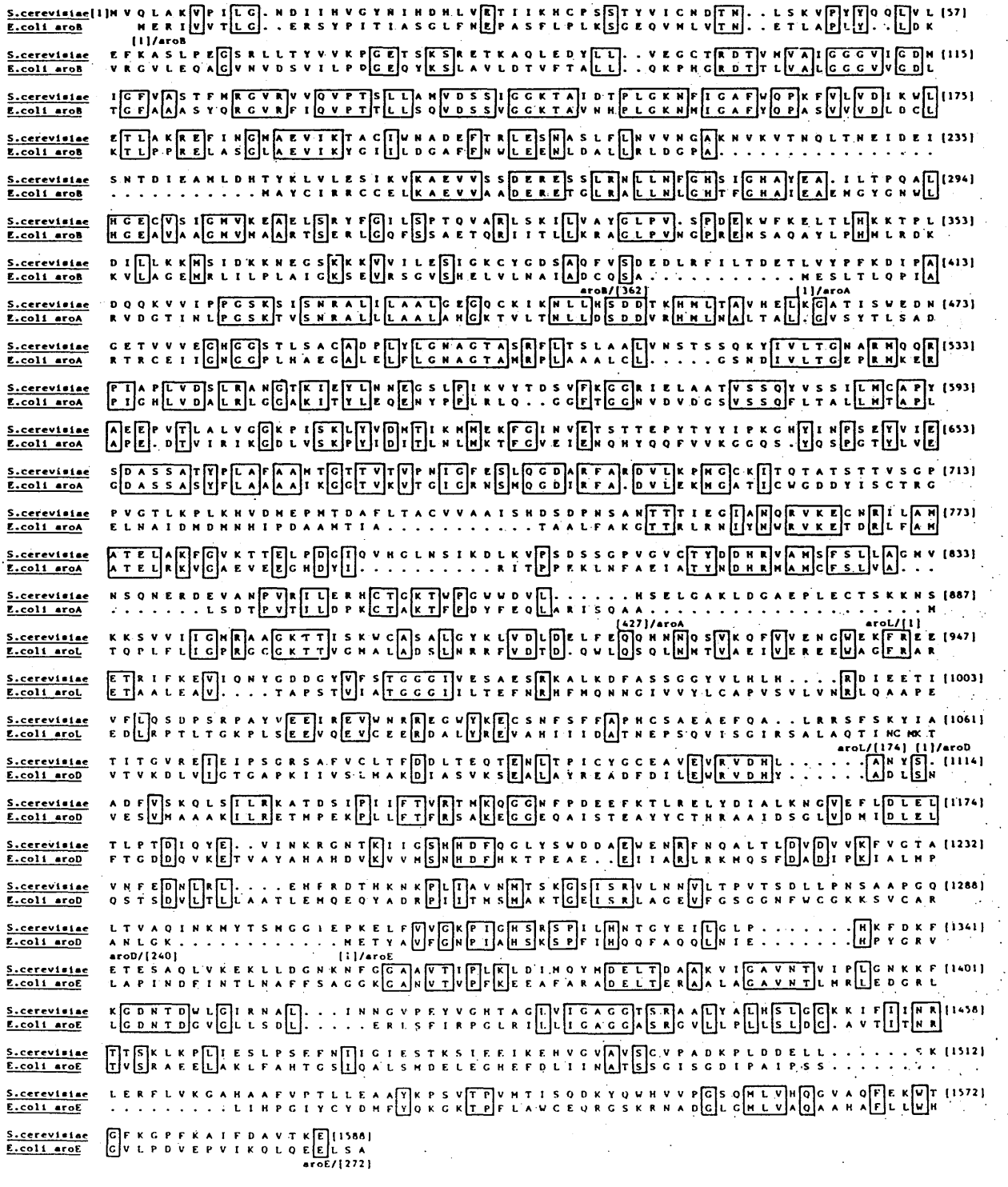
APPENDIX

Amino acid homologies between the S. cerevisiae arom multifunctional enzyme and the corresponding monofunctional E. coli enzymes (taken from Duncan et al., 1987). The sequence give in this figure for E. coli dehydroquinase (the aroD gene product) has recently been revised as shown on p. 184 (L.D. Graham, E. Borthwick and J.R. Coggins, unpublished results).



Revised sequence for *E. coli* dehydroquinase (L.D. Graham, E. Borthwick and J.R. Coggins, unpublished results).

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  1  MKTVTVKDLV IGTGAPKIIV SLMAKDIASV KSEALAYREA DFDILEWRVD
 51  HYADLSNVES VMAAAKILRE TMPEKPLLFT FRSAKEGGEQ AISTEAYIAL
101  NRAAIDSGLV DMIDLELFTG DDQVKETVAY AHAHDVKVVM SNHDFHKTPE
151  AEEIIARLRK MQSFDADIPK IALMPQSTSD VLTLLAATLE MQEQYADRPI
201  ITMSMAKTGV ISRLAGEVFG SAATFGAVKK ASAPGQISVN DLRTVLTILH
251  QA
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