A STUDY OF THE *AROM* ENZYME COMPLEX

BY LIMITED PROTEOLYSIS

by

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To my parents (for putting up with me)

ACKNOWLEDGEMENTS

I would like to thank Professor R.M.S. Smellie for providing facilities to enable me to perform the research reported in this thesis. I am indebted to Dr. J.R. Coggins for both help and patience throughout. Thanks are also due to Miss A.A. Coia and Miss B. Brodie for growing the *N. crassa* from which the *arom* complex was isolated. Finally thanks are due to Mrs. J. Greenwood for typing this thesis.

ABBREVIATIONS

The following abbreviations, in addition to those recognised in the Biochemical Journal, are used in this work:

BAPNA	-	DL-N^{α}-benzoyl-L-arginine-*p* nitroanilide
DAHP	-	3-deoxy-D-*arabino*-heptulosonate 7-phosphate
DTT	-	dithiothreitol
EPSP	-	5-enoylpyruvoylshikimate 3-phosphate
PMSF	-	phenylmethylsulphonyl fluoride
SDS	-	sodium dodecyl sulphate
TPCK	-	*p*-tosyl-L-phenylalanyl chloromethyl ketone

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SUMMARY

An improved method of purifying the arom complex from N. crassa has been devised. This procedure takes advantage of the tight binding of the arom complex to blue-dextran sepharose and yields around 3mg of pure enzyme from 100g of lyophilised N. crassa mycelia (30-35%).

Limited proteolysis has been used to probe the domain structure of the arom complex. The shikimate kinase activity of the complex is very susceptible to inactivation by proteolysis and is probably located on the surface of the arom complex. A 69000 molecular weight domain containing both the shikimate dehydrogenase and dehydroquinase activities of the arom complex has been characterised by a combination of gel electrophoresis in the presence of 1M urea, 8M urea and SDS and by substrate-labelling of the dehydroquinase active-site. The shikimate dehydrogenase-dehydroquinase proteolytic fragment of the arom complex can regain shikimate dehydrogenase activity after polyacrylamide gel electrophoresis in the presence of 8M urea. This domain of the arom complex can refold in the absence of the remainder of the arom polypeptide. Under the same conditions shikimate dehydrogenase activity was not regained from the arom complex after polyacrylamide gel electrophoresis in the presence of 8M urea. Evidence has been obtained that a proteolytic fragment of molecular weight 110000 is produced from the N-terminal region of the arom polypeptide and may catalyse both the dehydroquinate synthetase and EPSP synthetase reactions of the complex.

Chemical modification of the arom complex with formaldehyde/ sodium borohydride inactivated both the shikimate dehydrogenase and

dehydroquinase activities of the complex. Shikimate protected the shikimate dehydrogenase but not the dehydroquinase activity against inactivation by reductive alkylation. This and other evidence indicated that the shikimate dehydrogenase and dehydroquinase active-sites are spatially distinct.

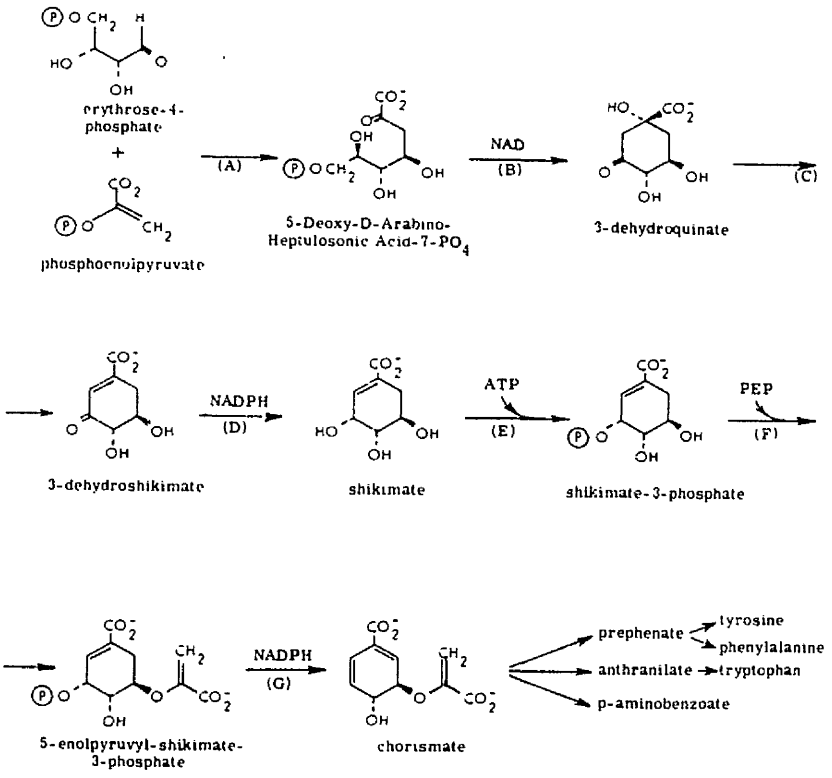


Figure 1 The reactions of the early common pathway of aromatic amino-acid biosynthesis. The arom complex catalyses the five reactions B to F responsible for converting DAHP into EPSP.

CHAPTER 1

INTRODUCTION

1. The organisation of the enzymes of the early common pathway of aromatic amino-acid biosynthesis

In bacteria and in plants aromatic amino-acids are synthesised from phosphoenolpyruvate and D-erythrose 4-phosphate via the common intermediate chorismic acid (Fig. 1). The state of aggregation of five of the enzymes of this early common pathway of aromatic amino-acid biosynthesis, those responsible for the overall conversion of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate into 5-enoylpyruvoyl-shikimate 3-phosphate, was studied in a number of species of bacteria and fungi by ultracentrifugation in sucrose density gradients (Giles *et al*, 1967; Berlyn and Giles, 1969; Ahmed and Giles, 1969). In crude extracts of six different bacteria (Table 1) these activities were found to be separable by sucrose density gradient centrifugation (Berlyn and Giles, 1969) whereas in six species of fungi the five activities were recovered in the same ammonium sulphate fraction and sedimented together in sucrose density gradients (Ahmed and Giles, 1969). The enzyme activities in a further species of fungus, *Neurospora crassa*, had previously been shown to sediment together in sucrose density gradients (Giles *et al*, 1967) the sedimentation coefficients for all the fungal species being similar (Table 2) indicating a molecular weight around 230,000 for all of these *arom* enzyme aggregates.

Genetic studies of the organisation of the genes coding for the five associated activities of the early common pathway showed them to occur as a gene cluster in *N. crassa* (Gross and Fein, 1960; Giles *et al*, 1967) and to be transcribed as a single polycistronic mRNA

Table 1 The molecular weights of aromatic synthetic enzymes of different bacterial species studied by Berlyn and Giles (1969). Where more than one value is tabulated more than one peak of activity was detected.

Bacterium	Dehydroquinate synthetase	Dehydroquinase	Dehydroshikimate reductase	Shikimate kinase	EPSP synthetase
E. coli	56000	40000	25000	17000 21000 12000	38000
S. typhimurium	56000	42000	24000	18000 25000	33000
A. aerogenes	56000	76000	27000	18000	35000
P. aeruginosa	69000	159000	27000	15000	45000
S. coelicolor	50000	134000	23000	10000	42000
B. subtilis	49000	39000	44000	1o000	52000 78000 39000

Table 2 The sedimentation coefficients ($S_{20,w}$) and molecular weights of the aromatic synthetic enzyme of fungi. Data of Ahmed and Giles (1969) except that for N. crassa (Giles et al, 1967)

Fungus	$S_{20,w}$	molecular weight $\times 10^{-3}$
Rhizopus stolonifer	11.3	232
Phycomyces nitens	11.2	228
Absidia glauca	11.6	242
Aspergillus nidulans	10.8	217
Coprinus lagopus	10.9	220
Ustilago maydis	10.6	211
Neurospora crassa	11.3	200

Table 3 The order of the genes of the arom gene cluster. The genes are listed from the 5' end of the cluster

Gene	Enzyme	Step in pathway (see Fig. 1)
Arom 1	Shikimate dehydrogenase	D
Arom 9	Dehydroquinase	C
Arom 5	Shikimate kinase	E
Arom 4	EPSP synthetase	F
Arom 2	Dehydroquinate synthetase	B

(Case and Giles, 1968). The order of the genes in the cluster (Table 3) was finalised by Rhines et al (1969).

2. Purification of the arom complex from Neurospora crassa

The first reported purification of the arom complex of N. crassa was that of Burgoyne et al (1969). Equilibrium centrifugation gave a molecular weight of 231,000 for the complex and, although gel electrophoresis in the presence of sodium dodecyl sulphate indicated a single subunit size of around 100,000, equilibrium centrifugation in guanidine hydrochloride revealed a heterogeneous preparation with species of molecular weights from 29,000 to 120,000.

Jacobson et al (1972) studied the conditions under which the arom aggregate dissociated and found dissociation to be favoured by high pH, low ionic strength and high temperature. Jacobson et al (1972) purified an arom aggregate of molecular weight 230,000 which could be dissociated to yield a number of lower molecular weight active species. On the basis of their results on the dissociation of the arom aggregate they estimated that a minimum of four polypeptide chains were present in the complex.

A different method of purifying the arom complex was devised in another laboratory (Gaertner, 1972). The molecular weight of the complex purified by their procedure was measured by sedimentation equilibrium centrifugation and found to be 290,000. The purified complex, although homogeneous on non-denaturing gel electrophoresis, showed four 'subunits' of molecular weights 54,000, 63,000, 84,000 and 95,000 on SDS gel electrophoresis.

In the same laboratory Gaertner and Cole (1976) isolated the arom complex by a different procedure and showed that the purified complex was proteolytically degraded on storage. Immediately after purification the complex was homogeneous on non-denaturing gel electrophoresis but showed two major bands, at molecular weights of 150,000 and 50,000, and many less prominent bands on SDS gel electrophoresis. On storage of the complex SDS gel electrophoresis showed the loss of the 150,000 molecular weight polypeptide and the production of a number of lower molecular weight species. The single band observed on non-denaturing gels of freshly purified complex was also lost and replaced by a number of more rapidly migrating proteins. The previous observations of multiple subunits of the arom complex could then be explained as a result of proteolysis during purification, however, the actual structure of the arom complex, whether a 'true' multienzyme complex or a multifunctional protein, remained unclear.

Lumsden and Coggins (1977) devised a rapid method for purifying the arom complex which paid due heed to the problems of proteolysis during purification. Precautions, the use of a DEAE-cellulose column under conditions in which the arom complex does not bind but a number of active N. crassa proteases do and the maintenance in the buffers of protease inhibitors at all times, were taken to minimise proteolysis. During the preparation three of the enzymes of the arom complex, shikimate dehydrogenase, shikimate kinase and dehydroquinase, were shown to co-purify with a constant activity ratio. The purified complex showed a single band on both non-denaturing gel electrophoresis and SDS gel electrophoresis, the subunit molecular weight being 165,000. From the results of cross-linking and glycerol density

gradient centrifugation experiments it was concluded that the enzyme was a dimer of similar, and probably identical, polypeptide chains.

Gaertner and Cole (1977), using the purification procedure of Gaertner and Cole (1976) but including protease inhibitors in the buffers used, concluded that the arom complex was a dimer of polypeptide chains of 150,000 molecular weight.

Both sets of authors (Lumsden and Coggins, 1977 and Gaertner and Cole, 1977) indicated that genetic evidence favoured the presence of only one type of polypeptide chain in the arom complex.

The difference in the subunit molecular weight reported by the two groups (Lumsden and Coggins, 1977; Gaertner and Cole, 1977) may be due either to differences in the SDS gel system and marker proteins used or to proteolysis of the enzyme prepared by Gaertner and Cole (1977). In either case the higher estimate (Lumsden and Coggins, 1977) of 165,000 seems more reliable.

In a subsequent paper Lumsden and Coggins (1978) proved the arom complex to be composed of a single type of polypeptide chain. Purified complex showed a single band on polyacrylamide gels containing 8M urea and peptide mapping of arom complex labelled with ^{14}C on either cysteine or methionine residues revealed the predicted number of spots in each case.

3. Potential advantages of organised enzyme complexes

A number of potential advantages of organised enzyme systems when contrasted with sequences of non-interacting enzymes have been proposed (G.R. Welch, 1977; F.H. Gaertner, 1978). The association of two or more enzymes may lead to conformational changes occurring

in the proteins such that the catalytic efficiencies of the associated enzymes are increased relative to the unassociated enzymes. If the activities of the enzymes in a multienzyme complex or in a multifunctional protein are sequential on a metabolic pathway the product of one reaction may be channelled into the active-site of the next enzyme. Substrate channelling may have a number of functions. Potentially reactive intermediates could be protected whilst passing from one active-site to the next by being retained within the enzyme complex. Different metabolic pathways may utilise the same intermediate, in these cases it may be advantageous to compartmentalise the pathways thus preventing competition for a common pool of substrate. Such compartmentalisation could be effected if one, or both, pathways were catalysed by enzyme complexes and substrate-channelling between the enzymes of the complexes occurred. A third proposed effect of substrate channelling is to reduce the time elapsing on shifting from one steady state rate to another, the transient time. The transient time is reduced since channelling removes the necessity for each substrate in the reaction sequence to come to its new equilibrium concentration in the solution by preventing exchange between enzyme-complex-bound and solution populations of substrate.

Coordinate control, either activation or inhibition, of more than one activity of an enzyme complex may occur, such control requires effector molecule binding to a single site and is mediated by conformational changes transmitted through the complex.

A final possibility, applying predominantly to multifunctional proteins, is that the coordination of synthesis and aggregation of the enzymes of the complex may be simplified if the enzyme complex is

encoded in a single gene, transcribed as a single mRNA and translated as a single polypeptide.

4. Properties of the arom enzyme complex

The arom enzyme complex has been claimed to exhibit a number of novel properties proposed for enzyme complexes (Gaertner et al, 1970; Welch and Gaertner, 1975; Welch and Gaertner, 1976). When considering the results of these studies it must be borne in mind that the arom complex used had been only partially purified and had probably been subject to proteolytic attack. It is not clear whether these properties are shared by intact purified arom complex.

Gaertner et al (1970) studied the rate of production of anthranilate from erythrose 4-phosphate and phosphoenolpyruvate and from the intermediate shikimate under conditions in which the arom complex was rate-limiting. Their results showed the arom complex to more rapidly catalyse the formation of EPSP from DAHP than from shikimate. It also appeared that, whereas the addition of shikimate to assays containing sub-saturating concentrations of erythrose 4-phosphate had little or no effect on the rate of production of anthranilate, the addition of sub-saturating concentrations of erythrose 4-phosphate to assays containing saturating shikimate concentrations greatly increased the rate of production of anthranilate. Gaertner et al (1970) suggested that these observations were best explained if substrates were channelled through the complex and the rate of reaction of 'complex-bound' substrates was higher than that of external substrates due to a slow rate of entry of external substrates into the active-sites of the complex.

Welch and Gaertner (1975) compared the rate of the overall reaction catalysed by the arom complex (conversion of DAHP to EPSP) with the predicted rate of reaction for a sequence of five unassociated enzymes, with the same kinetic constants, derived from a computer model of such a system. It was claimed that the arom complex significantly reduced the transient time, a measure of the delay between initiation of the reaction and the attainment of a constant rate of reaction, when contrasted with the computer simulation. In addition to the cautionary note above concerning the purity and structural integrity of the arom complex used in this work it should be noticed that the kinetic parameters used in the computer model of Welch and Gaertner (1975) were those obtained with substrates which were supplied 'externally' to the complex; if, as suggested by Gaertner et al (1970), the rate of entry of these substrates into the complex may be slow, a comparison between the experimental data and the computer simulation may be invalid.

A further novel kinetic property claimed for the arom complex is that of coordinate activation by the first substrate of the complex, DAHP (Welch and Gaertner, 1976). These authors studied the effect of pre-incubation of the arom complex with DAHP on the kinetic properties of the enzymes of the complex. In general the V_{max} values of the enzymes were unaltered by preincubation, however, the K_m's of all the enzymes except shikimate kinase were lowered on pre-incubation with DAHP. The concentration of DAHP required for half-maximal activation was around 1mM and so did not appear to be caused by DAHP binding to the active-site of the first enzyme. The coordinate activation of the arom complex was discussed in the light of previous proposals (Giles et al, 1967; Gaertner and De Moss, 1969) that the complex might exist to channel substrates down the biosynthetic

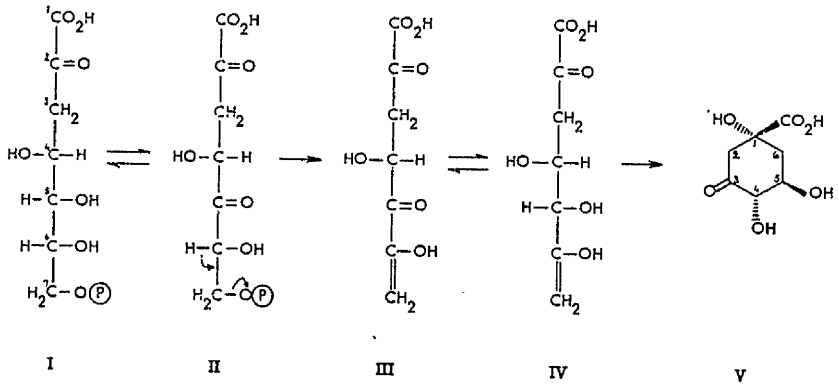


Figure 2 The mechanism proposed by Maitra and Sprinson (1978) for the dehydroquinate synthetase reaction. The modern convention for numbering the dehydroquinic acid ring (and those of dehydroshikimic and shikimic acids) is shown in structure V.

pathway thus compartmentalising the anabolic, arom complex, pathway and a catabolic pathway in N. crassa in which a dehydroquinase reaction step also occurs. Coordinate activation caused the shikimate kinase activity to become rate-limiting for the overall reaction catalysed by the arom complex and could lead to a breakdown of channelling of shikimate which would then become available to the catabolic pathway.

A short report (Vitto and Gaertner, 1978) has been made concerning the susceptibility of the arom complex to proteolysis. The shikimate kinase activity of the complex was the most susceptible to proteolysis by trypsin, chymotrypsin or a N. crassa protease preparation. DAHP protected each activity against proteolysis to some extent. These results will be discussed more fully later in relation to date presented in this thesis.

5. The mechanisms of the reactions of the aromatic synthetic enzymes

Dehydroquinate Synthetase

The dehydroquinate synthetase activity present in extracts of E. coli was studied by Srinivasan et al (1963). NAD was shown to be a cofactor for this enzyme since no activity was detected in the absence of NAD or in the presence of NAD'ase, Co^{++} was also required for catalytic activity. A hypothetical reaction scheme was suggested by Srinivasan et al (1963) and subsequent work analysed in the light of this mechanism (Adlersberg and Sprinson, 1964; Rotenberg and Sprinson, 1970). Rotenberg and Sprinson (1970) found that tritium incorporated at C-7 of DAHP was fully retained in 3-dehydro-quinate thus at no point in the reaction were the protons on C-7 of

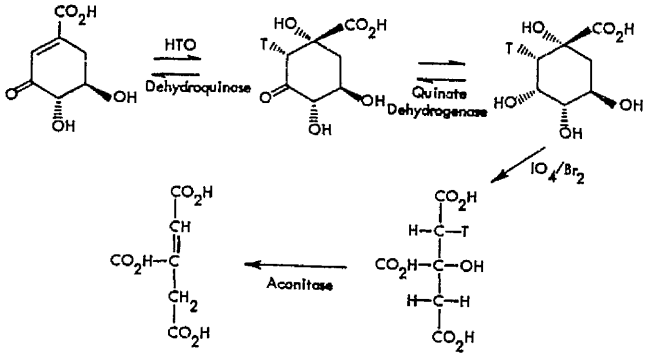


Figure 3 The reactions used by Hanson and Rose (1963) to define the stereochemistry of the dehydroquinase reaction.

DAHP subject to exchange with the solvent excluding ketonisation of the postulated enol intermediate IV (see Fig. 2). Further studies of the mechanism of the dehydroquinate synthetase reaction were made by chemical characterisation of intermediates formed during the reaction using large amounts of pure enzyme (Maitra and Sprinson, 1978). Maitra and Sprinson (1978) proposed the mechanism shown in Figure 2, this scheme is consistent with the observations of kinetic isotope effects on incorporation of tritium at C-5 of DAHP (Maréchal and Azerad, 1976; Rotenberg and Sprinson, 1978). A further point concerning the stereochemistry of the reaction is known. The hydride transfers to NAD and from NADH occur with the same stereospecificity (Maréchal and Azerad, 1976) since tritium incorporated at C-5 of DAHP is retained at C-4 of dehydroquinate.

3-Dehydroquinase

The stereochemistry of the 3-dehydroquinate hydrolyase (dehydroquinase) reaction was determined by Hanson and Rose (1963). 3-Dehydroshikimate was converted enzymatically to quinic acid by an extract of *A. aerogenes* containing dehydroquinase and quinate dehydrogenase in tritiated water. The tritiated quinic acid formed was chemically converted to citric acid and this then treated with aconitase (Fig. 3). After incubation with aconitase essentially all the tritium present in the citric acid was removed into the water and, since the stereospecificity of the aconitase reaction and the stereochemistry of quinic acid were known, the dehydroquinase reaction was deduced to involve a syn elimination of water.

The mechanism of the *E. coli* dehydroquinase was studied by Butler *et al* (1974). These authors showed that the enzyme was

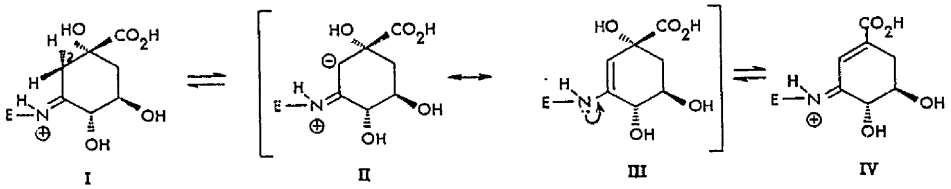


Figure 4 The mechanism of the dehydroquinase reaction involving a Schiff's base intermediate.

inhibited by sodium borohydride only in the presence of the substrate, dehydroquinate, indicative of a Schiff's base intermediate in the reaction. A mechanism accounting for the stereochemistry of the elimination was proposed on the basis of the Schiff's base intermediate (Fig. 4). Abstraction of the hydrogen from C-2 of dehydroquinate is facilitated by the electron-withdrawing properties of the imine intermediate (I) and the carbanion (II) formed stabilised by formation of an eneamine intermediate (III). From this intermediate a facile pathway to the product imine (IV) exists. Hydrolysis of the imine between product and enzyme then yields free dehydroshikimate and enzyme.

Shikimate Dehydrogenase

Shikimate dehydrogenase from both E. coli (Dansette and Azerad, 1974) and Pisum sativum (Davies et al, 1972) transfer hydrogen to and from the A-face of the nicotinamide ring of NADP.

On the basis of studies with competitive inhibitors of shikimate dehydrogenase, which were potential analogues of shikimate, and of the variation of kinetic parameters with pH (Dennis and Balinsky, 1972) it was proposed that a group of pK_a 9.6, probably a lysine residue, binds the carboxylate of shikimate and that a group with a pK_a of 8.6 binds the 4-hydroxyl function of shikimate at the active-site of the pea enzyme. Further kinetic and isotope exchange studies of the enzyme from Pisum sativum showed the enzyme to exhibit an ordered mechanism binding NADP prior to shikimate (Cleland et al, 1971).

EPSP Synthetase

Levin and Sprinson (1964) demonstrated that 5-enolpyruvyl shikimate

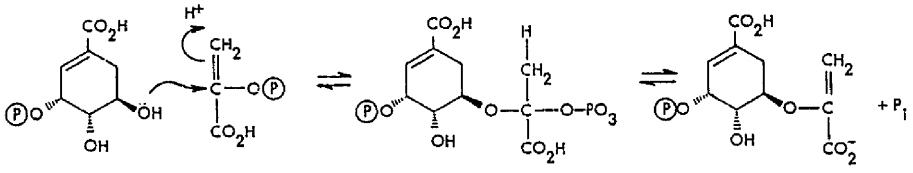


Figure 5 The mechanism of the EPSP synthetase reaction proposed by Levin and Sprinson (1964).

3-phosphate (EPSP) was formed when shikimate 3-phosphate and phosphoenolpyruvate were incubated with an extract of E. coli. A mechanism for the addition of the enolpyruvyl residue to shikimate phosphate was proposed (Levin and Sprinson, 1964) (Fig. 5).

The EPSP synthetase reaction was studied using phosphoenolpyruvate labelled with ^{18}O in the enol ester oxygen and by performing the reaction in tritiated and in deuterated water (Bondinell et al, 1971) The phosphate released from the labelled phosphoenolpyruvate contained all the ^{18}O present showing C-O cleavage of the phosphate ester to occur. Following incubation of shikimate phosphate and phosphoenolpyruvate with enzyme in tritiated water it was found that the EPSP formed had incorporated tritium. No tritium was incorporated into phosphoenolpyruvate on incubation with enzyme in the absence of shikimate phosphate. After reaction in deuterated water NMR spectroscopy of the resulting EPSP showed that the deuterium incorporated was localised in, and equally distributed between, the two vinyl methylene positions. Bondinell et al (1971) noted that these results were consistent with the proposed mechanism of Levin and Sprinson (1964) and further suggested that the methyl group of the intermediate was free to rotate in order to explain the equal incorporation of deuterium in the vinyl methylene positions of EPSP.

6. The structure and evolution of multifunctional proteins

Current theories of protein structure are based on the concept of the domain. The domain is a folding unit of protein structure and each domain performs a function, for example, nucleotide binding or catalysis. Domains have been recognised in the pyridine-nucleotide

linked dehydrogenases (Liljas and Rossman, 1974) and it has been proposed that the very similar nucleotide-binding domains of these dehydrogenases may have evolved from a simple ancestral nucleotide-binding protein. Similarly multifunctional proteins are believed to be composed of domain structures (Kirschner and Bisswanger, 1976).

Multifunctional enzymes may have evolved by a process of gene fusion (Bonner et al, 1965) such that the catalytic activities which were previously properties of separately isolable enzymes came together on a single polypeptide chain. If this is the case then the conformation of that region of the multifunctional enzyme catalysing the same reaction as the previously monofunctional enzyme is presumably similar. Multifunctional enzymes can be thought of as a number of individual proteins linked by sequences of polypeptide coded for by the previously inter-cistronic regions of the genome (Kirschner and Bisswanger, 1976; Stark, 1977). If multifunctional enzymes do indeed arise in this manner that the activities thus associated are often sequential in a metabolic sequence, as in the arom and fatty acid synthetase complexes, is not directly explicable. It seems likely that other processes as well as gene fusion are at work in these cases. Such a process could be that of gene duplication. The substrates of enzymes in a metabolic pathway are similar and similar binding processes to each enzyme may occur thus each enzyme could have evolved from an ancestral 'substrate-binding protein'. The structural gene for the ancestral protein would be duplicated a number of times and each copy of the gene would evolve into one coding for an enzyme in the currently observed metabolic route. If the duplicated genes remained linked the evolved enzymes would be carried on a multifunctional

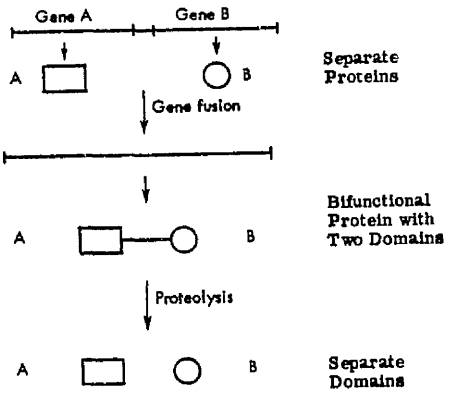


Figure 6 Scheme illustrating the evolution of a bifunctional enzyme by gene fusion and its subsequent study by limited proteolysis.

polypeptide. This model is consistent with the idea of the multifunctional enzyme being constructed from a number of domains each catalysing one of the reactions of the complex and joined by spacer regions of polypeptide which are not necessarily structured. Such a gene-duplication model of multifunctional enzyme evolution is analogous to that proposed by Buehner et al (1973) for the evolution, from an ancestral nucleotide-binding protein, of the nucleotide binding domains of several NAD^+-linked dehydrogenases.

7. The study of multifunctional enzymes by limited proteolysis

Native enzymes tend to be more resistant to proteolysis than denatured proteins, thus the tightly folded domains of multifunctional enzymes may be considerably more resistant to proteolysis than the 'spacer' sequences of polypeptide and may, therefore, be separable from the remainder of the protein by proteolysis (Fig. 6). After separation of the domains of a multifunctional enzyme by proteolysis they may retain the specific folding characteristics they had within the native enzyme, becoming analogous to normal monofunctional enzymes, and it may be possible to ascribe any change in their characteristics, for example the response to effector molecules, to a loss of interaction between the domains of the multifunctional enzyme.

Limited proteolysis may also be used as a probe for conformational changes in enzymes (Citri, 1973) since on binding small molecules the protein may become more or less susceptible to proteolysis. It is usually more reasonable to ascribe changes in susceptibility to proteolysis to conformational changes in the protein than to physical protection of labile bonds by the small molecule ligand (Markus, 1965).

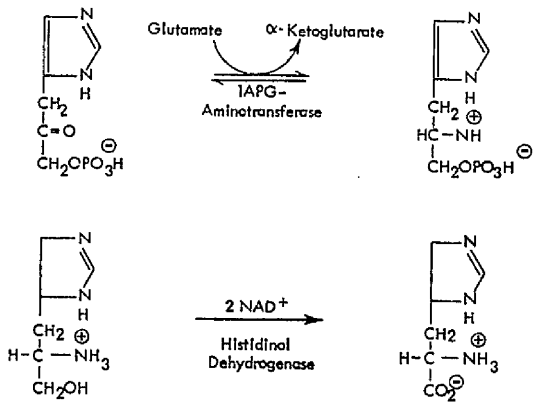


Figure 7 The reaction catalysed by imidazolylacetolphosphate: L-glutamate aminotransferase and histidinol dehydrogenase.

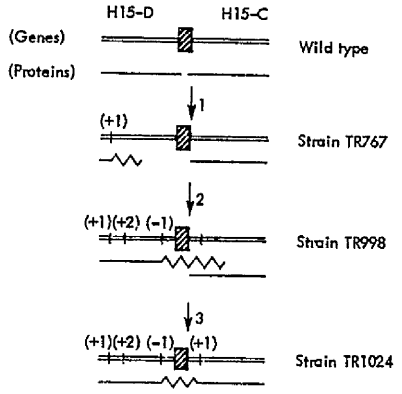


Figure 8 The genetic events responsible for the fusion of the his D and his C of S. typhimurium. A straight line in the 'proteins' position represents in-phase reading, a pleated line out-of-phase reading. The deletion (-1) in step 2 causes the intercistronic region to be read out-of-phase and the stop codon is not recognised, the insertion (+1) then corrects the reading frame in step 3.

It was asserted above that one potential mechanism for the evolution of multifunctional enzymes was by gene fusion. Such a process has been observed involving two of the genes of the histidine operon in Salmonella typhimurium (Yourno et al, 1970). The his D gene of S. typhimurium codes for the enzyme histidinol dehydrogenase (HDH) and the his C gene for imidazolylacetolphosphate : L-glutamate amino-transferase (AT) (Fig. 7). A mutant strain, TR 1024, was found in which the his D and his C genes had become fused by two frameshift mutations near to, but on either side of, the intercistronic region causing the normal punctuation signals between the two genes to be read out of frame and thereby not recognised (Fig. 8). The product of the fused genes was characterised (Yourno et al, 1970). On purification of the HDH activity it was found that the AT activity copurified, only one band, of molecular weight 88,000, being present in SDS gels of the purified enzyme. Neither gel filtration nor chromatography on DEAE-sephadex resolved the HDH and AT activities, although in both cases more than one peak of each activity was detected. The subunit molecular weights of wild-type HDH and AT are, respectively, 49,000 and 40,000 so the molecular weight of 88,000 determined for the bifunctional HDH-AT enzyme agreed will with that expected of the product of fusion of the two adjacent genes. Further proof that the bifunctional HDH-AT enzyme was a product of fusion of the genes for each activity was sought by peptide mapping of the purified protein. Tryptic peptide maps of the bifunctional enzyme were the sum of maps of the individual wild-type enzymes. Amino-acid analyses of peptides derived from the HDH (four peptides) and the AT (four peptides) enzymes were compared with the relevant peptides derived from the bifunctional enzyme, in all cases the peptides were of identical amino-acid composition. No gross

difference in primary structure is present in the bifunctional enzyme when compared with the sum of the two individual wild-type enzymes.

The occurrence of this mutant bifunctional enzyme demonstrates that gene fusion is a plausible mechanism for the evolution of at least some multifunctional enzymes. The mutant enzyme not only retained the ability to catalyse both reactions, and so must be able to fold in such a way as to form the two active-sites, but could also aggregate to form high molecular weight oligomers.

An interesting extension of the work on the mutant HDH-AT bifunctional enzyme was a study of its structure by limited proteolysis (Kohno and Yourno, 1971). The bifunctional enzyme shows multiple bands on non-denaturing gel electrophoresis, reflecting different states of aggregation, which are not readily interconvertible; a purified form of one of these was studied by limited proteolysis with trypsin. The AT activity of the bifunctional enzyme was very sensitive to tryptic proteolysis, the HDH activity being considerably more stable in this respect. This result paralleled work on the native monofunctional enzymes suggesting the conformation of the domains of the multifunctional enzyme to be similar to those of the individual monofunctional enzymes. Limited proteolysis of the bifunctional enzyme produced a main fragment of molecular weight 45,000 on SDS gel electrophoresis, close to the subunit size of native HDH (49,000). Gel filtration of trypsin-proteolysed bifunctional enzyme gave a molecular weight of 125,000 for the HDH fragment, considerably greater than that of native enzyme indicating the presence of non-covalently bound peptides remaining associated with the HDH fragment after proteolysis.

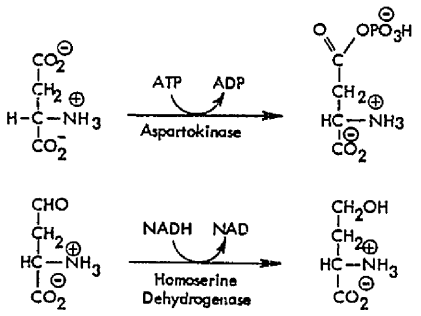


Figure 9 The reactions catalysed by aspartokinase I - homoserine dehydrogenase I. The reactions are respectively the first and third in the series leading from aspartate to threonine.

The results of limited proteolysis of this mutant bifunctional enzyme are consistent with the presence of a two domain structure, one domain catalysing the HDH reaction and the other the AT reaction. Each domain folds in a conformation similar to that of the native, monofunctional, enzymes.

A study of the bifunctional enzyme, aspartokinase I - homoserine dehydrogenase I (AK - HSDH) (Fig. 9) of E. coli by limited proteolysis yielded information on both the domain structure of the enzyme and the location of the binding site for the inhibitor, threonine (Véron et al, 1972). In this work four proteases, chymotrypsin, papain, trypsin and subtilisin, were found to give the same results. On proteolysis the AK activity was lost whereas the HSDH activity was relatively stable, moreover, although at all times the AK activity remaining was inhibitable by threonine, the HSDH activity was gradually desensitised implying that the inhibitory threonine binding site is located in the AK domain of the intact enzyme. Gel electrophoresis of enzyme after different times of proteolysis showed, on non-denaturing gels, the loss of the native enzyme with formation of a faster-running, HSDH active, protein. SDS gel electrophoresis showed the conversion of the native 86,000 molecular weight subunit to a subunit size of 55,000. Gel filtration on sephadex G.200 was used to further characterise the products of proteolysis. After gel filtration a HSDH fragment devoid of AK activity was obtained with a molecular weight of 110,000. The molecular weight of this fragment was confirmed both by sucrose-density gradient centrifugation which gave a sedimentation coefficient of 6.0 S, corresponding to a molecular weight of 109,000 and by sedimentation equilibrium centrifugation

which gave a molecular weight of 108,000. The fragment was therefore a dimer of two 55,000 molecular weight polypeptides. Protein chemistry was used to show that the HSDH domain was located at the C-terminal end of the polypeptide chain. C-terminal amino-acid analysis using carboxypeptidase A showed both the HSDH fragment and the native enzyme to have valine, glycine and leucine as the three amino-acids nearest the carboxyl terminus (sequence -leu,-gly-val-CO_2). N-terminal analysis of the HSDH fragment gave two amino-acids, isoleucine and alanine, neither of which corresponded to the N-terminus of the native enzyme (methionine).

An interesting mutant, Gif 108, was found which had only AK activity. On peptide mapping of the native AK-HSDH bifunctional enzyme, the HSDH fragment derived by limited proteolysis of the bifunctional enzyme and the mutant enzyme carrying only the AK activity the maps of the HSDH fragment and the mutant AK were found to be complementary. The AK domain was, therefore, the N-terminal region of the bifunctional enzyme. The mutant enzyme was threonine inhibitable showing the threonine binding site to be located in the AK domain.

These results showed the AK-HSDH bifunctional enzyme to be composed of two domains both of which were catalytically active when isolated from each other. Since threonine inhibits both activities in the native enzyme but not the HSDH fragment activity and the threonine binding site is located in the AK domain the two domains must interact in the native enzyme to transmit a conformational change from the AK domain to the HSDH domain on threonine binding to the inhibitory site.

Limited proteolysis of bifunctional enzymes can lead to the isolation

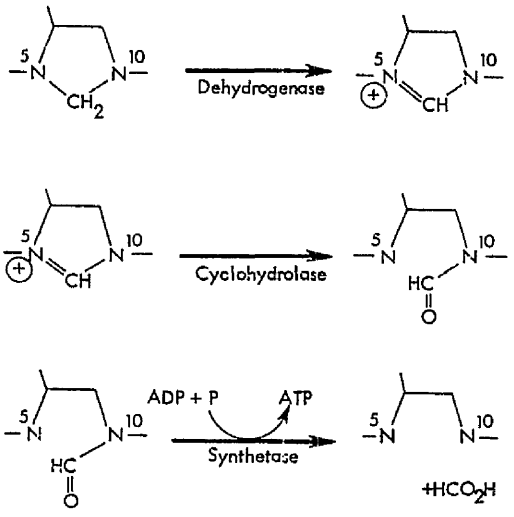
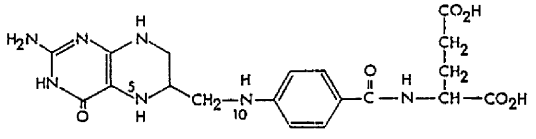


Figure 10 The reactions catalysed by the trifunctional enzyme methylenetetrahydrofolate dehydrogenase - methenyltetrahydrofolate cyclohydrolase - formyltetrahydrofolate synthetase and, below, the structure of the tetrahydrofolic acid molecule.



of active fragments and thus give information on the domain structure of such enzymes. When multifunctional enzymes with more than two activities per polypeptide are considered proteolytic fragments carrying more than one activity may result, that is, the fragments may themselves be multifunctional. In these cases the multifunctional fragments may still be regarded as domains since they are presumably tightly packed regions of polypeptide and if 'lower-order' domain structure exists within them it may require X-ray diffraction analysis for its elucidation.

The enzyme activities methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase and formyltetrahydrofolate synthetase (Fig. 10) are associated as a trifunctional enzyme in porcine liver (Tan et al, 1977). Proteolysis of this enzyme with trypsin in the presence of NADP led to the loss of synthetase activity with little change in either the dehydrogenase or cyclohydrolase activities (Tan and MacKenzie, 1977). Affinity chromatography on $NADP^+$-sepharose with substrate elution using an $NADP^+$ gradient was used to purify a bifunctional fragment carrying both the dehydrogenase and cyclohydrolase activities. The purified bifunctional fragment had a subunit molecular weight of around 33,000 as opposed to 100,000 for the native trifunctional enzyme. It was proposed that the small size of the bifunctional fragment reflected a close association between the two active-sites and substrate channelling was later observed between these two activities in the native enzyme (Cohen and MacKenzie, 1978).

Chymotryptic proteolysis of this trifunctional enzyme gave complementary results. The dehydrogenase and cyclohydrolase activities were lost more rapidly than the synthetase activity. Following

chymotryptic proteolysis it was possible to purify a synthetase fragment by passing the enzyme through a 2'-5'-ADP-sepharose column to which the remaining unproteolysed enzyme, carrying the dehydrogenase active-site, bound whereas the synthetase fragment was washed through. The subunit molecular weight of the fragment was 67,000 as measured by SDS gel electrophoresis. Immunochemical techniques showed no identify between the two proteolytic fragments implying they were derived from two distinct sections of the native protein. Amino-terminal sequence analysis of the dehydrogenase-cyclohydrolase fragment showed it to be cleaved from the N-terminal end of the polypeptide chain.

The results of proteolysis of this trifunctional enzyme are consistent with a two-domain structure, the C-terminal domain carrying the synthetase activity and the N-terminal domain containing both the dehydrogenase and cyclohydrolase activities (Tan and MacKenzie, 1979).

The fatty acid synthetase complex has been studied by limited proteolysis with trypsin and chymotrypsin (Agradi et al, 1976) and a thioesterase domain purified following proteolysis with trypsin (Smith et al, 1976) and with elastase (Guy et al, 1978).

Agradi et al (1976) studied the effect of proteolysis with trypsin or chymotrypsin on the seven reactions catalysed by fatty acid synthetase and on the overall reaction of the complex. As proteolysis with either protease increased the overall fatty acid synthetase activity decreased and the product specificity shifted, the only component reaction to be inhibited was that catalysed by the thioesterase. It was observed that on ammonium sulphate precipitation of proteolysed fatty acid synthetase significant amounts of thioesterase remained in the supernatant after precipitation of all the residual fatty acid

synthetase activity. Following trypsin proteolysis of fatty acid synthetase a thioesterase fragment was purified by ammonium sulphate; precipitation and gel filtration (Smith et al, 1976). The molecular weight of this fragment was estimated, by gel filtration, to be 32,000 and it cross-reacted with anti-(fatty acid synthetase) γ-globulin, it was therefore immunologically similar to the thioesterase in the native complex and probably had a similar tertiary structure. Further studies of the isolated thioesterase fragment by Dileepan et al (1979) showed that trypsin further cleaved the fragment into two smaller polypeptides each of 17,500 molecular weight. The thioesterase domain of fatty acid synthetase can also be released by digestion with elastase (Guy et al, 1978). During the course of elastase digestion the overall fatty acid synthetase activity was lost while the thioesterase activity increased. A polypeptide of molecular weight 35,000 was formed rapidly and could be isolated as an active thioesterase fragment by ammonium sulphate precipitation. Elastase did not affect the other activities of the complex since fatty acid synthetase activity could be recovered after proteolysis by the addition of medium-chain thioesterase.

Proteolysis with both trypsin and elastase released thioesterase domains of similar molecular weight from fatty acid synthetase suggesting that at some point on the complex there is an exposed loop of peptide sensitive to proteolytic attack and showing that limited proteolysis can be used to gain information on the structure of large multifunctional proteins.

CHAPTER 2

MATERIALS AND METHODS

1. Chemicals

The chemicals used in this work were obtained from the following sources: DTT, thioglycollic acid, phenylmethyl sulphonylfluoride and DL-N^{α}-benzoyl-L-arginine-p nitroanilide from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; Nitro Blue Tetrazolium, phenazine methosulphate, sodium borohydride, guanidine hydrochloride (aristar grade), hydrogen peroxide (100 vols) and all chemicals for polyacrylamide gel electrophoresis from BDH Chemicals, Poole, Dorset, U.K.; benzamidine hydrochloride and shikimic acid from Aldrich Chemical Co., Gillingham, Dorset, U.K.; phosphoenolpyruvate, NAD, NADH, NADP, NADPH, ADP and ATP from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.; Coomassie Brilliant Blue G.250 and Coomassie Brilliant Blue R.250 from Serva Feinbiochemica, Heidelberg, West Germany; sodium boro-[^{3}H]-hydride from The Radiochemical Centre, Amersham, Bucks, U.K.; Sephadex G-25 (medium grade) from Pharmacia (G.B.) Ltd., London, W.5, U.K.; DEAE-cellulose (DE52) from Whatman Biochemicals, Maidstone, Kent, U.K.; ammonium dehydroquinate, 3-deoxy-D-arabino-heptulosonate 7-phosphate and dimethyl suberimidate dihydrochloride were gifts from Dr. J.R. Coggins and were prepared as previously described (Lumsden and Coggins, 1977). Shikimate phosphate was also a gift from Dr. J.R. Coggins. Urea was obtained from BDH and recrystallised from absolute alcohol before use. Blue dextran-sepharose was a gift from Dr. P.A. Lowe.

2. Proteins and Enzymes

Myoglobin and elastase were obtained from BDH; rabbit muscle aldolase from Boehringer; trypsin (TPCK treated), chymotrypsin and

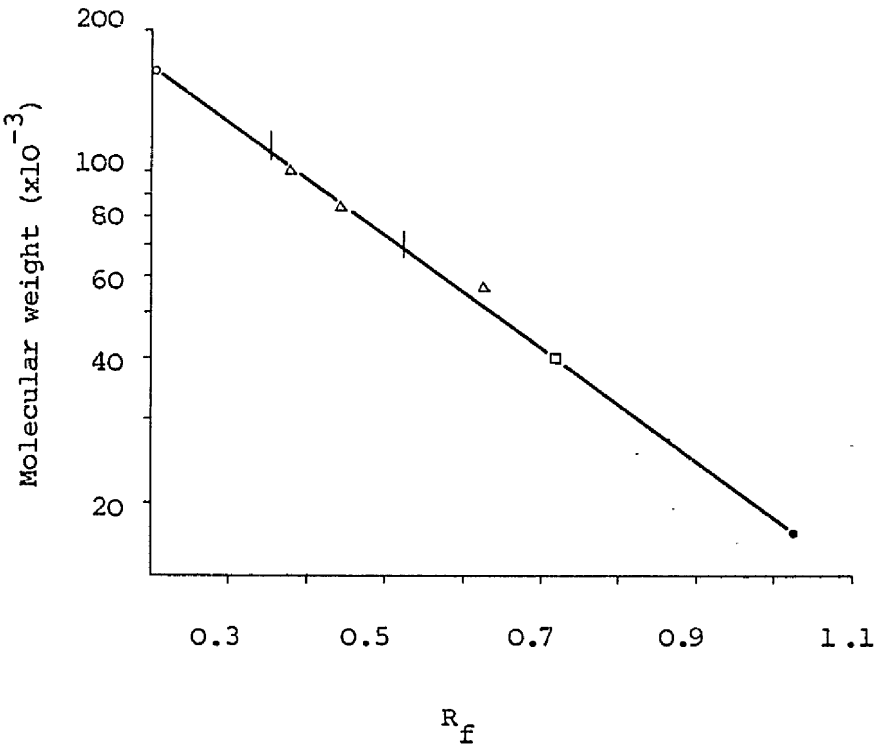


Figure 11 A typical standard line for the estimation of molecular weights of polypeptides separated by SDS polyacrylamide gel electrophoresis. The standard proteins used were, arom complex (165000); pyruvate dehydrogenase multienzyme complex (E1, 100000; E2, 83000 and E3, 56500); aldolase (40000) and myoglobin (17200). The vertical bars indicate the mobilities of the two major polypeptides formed after trypsin proteolysis of the arom complex.

papain were from Worthington Biochemical Corp., Freehold, NJ 07727, U.S.A.; subtilisin carlsberg (Type VIII) and soy-bean trypsin inhibitor (Type I-S) were from Sigma. Agarose-trypsin inhibitor (capacity 3mg trypsin/ml settled resin) was obtained from Research Products, Miles Laboratories Inc., Elkhart, Ind. 46514, U.S.A. Lima-bean trypsin/chymotrypsin inhibitor was a kind gift from Dr. J. Kay, Department of Biochemistry, University College, Cardiff CF1 1XL, Wales, U.K. and pyruvate dehydrogenase from Escherichia coli was a gift from Dr. J.R. Coggins. Pyruvate kinase-lactate dehydrogenase mixture was obtained from Boehringer.

3. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Weber and Osborn (1969). Protein samples for electrophoresis were made 1% (w/v) in SDS, 1% (v/v) in 2-mercaptoethanol and 20% (v/v) in glycerol, and immediately placed in a boiling water bath for 2 min. Bromophenol Blue (0.002% w/v) was added to the sample and electrophoresis at 7mA/gel performed in 5% polyacrylamide gels in 0.1M sodium phosphate pH6.5 containing 0.1% SDS. After electrophoresis the position of the dye was marked with a wire. A typical molecular weight estimation is shown in Figure 11.

Electrophoresis in 5% (w/v) polyacrylamide gels containing 1M urea was by the method of Davis (1964) as modified by Hayes and Wellner (1969) except that the gels contained 1M urea. Gels were pre-run for 30 min at 2mA per gel prior to electrophoresis of the protein at the same current. After electrophoresis in this manner gels could be stained for enzyme activity as well as for protein.

Electrophoresis in 4% (w/v) polyacrylamide gels containing 8M urea was by the method of Davis (1964) as modified by Hayes and Wellner (1969) except that the upper well buffer contained 0.02% (w/v) thioglycollic acid in place of DTT. Samples were made 8M in urea by the addition of solid recrystallised urea and incubated for 20 min at 25°C prior to electrophoresis. Gels were pre-run for 30 min at 3mA/gel prior to electrophoresis at the same current for 135 min.

For two-dimensional polyacrylamide gel electrophoresis the first dimension tube gels, either containing 1M urea or 8M urea, were run in gel tubes of internal diameter 0.4cm. The first dimension gels were equilibrated with 0.1M sodium phosphate pH6.5, 0.1% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol before electrophoresis in second dimension 5% polyacrylamide slab gels (6cm x 6cm x 0.5cm), containing SDS overnight at 12mA in 0.1M sodium phosphate pH6.5, 0.1% SDS.

4. Polyacrylamide gel staining

Polyacrylamide tube gels were stained for protein by incubation for 45 min at 40°C in 0.1% (w/v) Coomassie Brilliant Blue G.250 in methanol/acetic acid/water (5:1:4, by volume) and destained, also at 40°C, in methanol/acetic acid/water (1:1:8, by volume) (Lumsden and Coggins, 1977).

Polyacrylamide slab gels were stained for protein for 110 min either as above or by incubation, with shaking at 25°C in 0.1% (w/v) Coomassie Brilliant Blue R.250 in methanol/acetic acid/water (5:1:4, by volume). Gels were destained in methanol/acetic acid/water (1:1:8, by volume) at 40°C.

Shikimate dehydrogenase activity was detected by a modification

of the general method of Gabriel (1971) as described by Lumsden and Coggins (1977). Gels were soaked for 30 min in 0.025M Tris/HCl pH9.0 to remove DTT then in a reaction mixture which contained 0.25M Tris/HCl pH9.0, 0.5mM NADP, 0.5mM shikimic acid, 0.5mg/ml Nitro Blue Tetrazodium and 5µg/ml phenazine methosulphate.

EPSP synthetase activity was detected by a method devised by Dr. G.A. Nimmo (unpublished work). Gels were soaked for 30 min in 0.1M glycine/KOH pH10.0 then in a reaction mixture which contained, 0.1M glycine/KOH pH10.0, 8mM $CaCl_2$, 1mM shikimate phosphate, 2mM phosphoenol-pyruvate. Active species were detected by the appearance of white bands of precipitated calcium phosphate.

Polyacrylamide tube gels stained for protein or for shikimate dehydrogenase activity were scanned at 600nm in a Gilford spectrophotometer fitted with a model 252 gel scanner.

For the estimation of the areas of peaks on gel scans data were digitised using a Summagraphics ID-CTR data tablet digitizer and processed using a Digital PDP 11/34 computer with an RSX 11M operating system programmed in Fortran.

5. Detection of Radioactivity in Polyacrylamide Gels

Radioactivity was detected in polyacrylamide gels as described by Bates *et al* (1975). Gel slices containing protein, or 1mm gel discs if the entire gel was sliced, were placed in plastic scintillation vials and dried at 40°C. The dried gels were solubilised by incubation in 0.3ml of 100 vol hydrogen peroxide either overnight at 40°C or 60°C. Scintillant (3ml of toluene triton, 2:1 by volume, containing 5g/l 2,5-diphenyloxazole and 20g/l 1,4-bis (5-phenyloxazol-2-yl) benzene)

was added and the samples counted in an Intertechnique SL 4000 scintillation counter.

6. Enzyme Assays

Enzyme assays were performed using a Unicam SP8000 spectrophotometer with a slave recorder attachment. All assays of the enzymes of the arom complex were performed in a total volume of 1ml at 37°C, except for certain specified kinetic studies which were performed at 25°C.

Dehydroquinase (3-dehydroquinate hydrolyase, E.C. 4.2.1.10) was assayed as described by Lumsden and Coggins (1977). The reaction mixture contained 0.1M Tris/HCl pH7.5 and 0.33mM ammonium dehydroquinate; production of 3-dehydroshikimate was followed at 234nm (ε = 1.2 x 10^4 litre mol^{-1} cm^{-1}).

Shikimate dehydrogenase (the reverse reaction of enzyme 3 of the complex, 3-dehydroshikimate reductase E.C. 1.1.1.25) was assayed by following the reduction of NADP at 340nm (ε = 6.18 x 10^3 litre mol^{-1} cm^{-1}) in a reaction mixture containing 0.1M Na_2CO_3 pH10.6, 4mM shikimic acid and 2mM NADP (Lumsden and Coggins, 1977).

Shikimate kinase (E.C. 2.7.1.71) was assayed by coupling to pyruvate kinase and lactate dehydrogenase (G.A. Nimmo, unpublished work). The reaction mixture contained 1mM shikimic acid, 1mM phosphoenolpyruvate, 0.2mM NADH, 2.5mM ATP, 0.1M KCl, 20mM $MgCl_2$, 50mM triethanolamine hydrochloride pH7.2, pyruvate kinase and lactate dehydrogenase. The oxidation of NADH was followed at 340nm.

Dehydroquinate synthetase was assayed by coupling to the dehydroquinase and dehydroshikimate reductase activities of the arom complex. The assay solution contained 50mM triethanolamine hydrochloride pH7.2,

0.5mM NAD, 0.1mM NADPH, 0.45mM DAHP. The oxidation of NADPH was monitored at 340nm.

Trypsin was assayed by the method of Erlanger et al (1961). The assay was performed at 25°C in a volume of 3ml containing 0.1mM BAPNA and 50mM Tris/HCl pH8.2. The production of p-nitroaniline was followed at 410nm (ε = 8800 litre mol^{-1} cm^{-1}).

For all the enzyme activities of the arom complex one unit is defined as the amount of enzyme that catalyses the conversion of 1μmole of substrate/min at the stated temperature. One unit of trypsin is defined as the amount of enzyme that catalyses the formation of 1nmole of p-nitroaniline/min. The specific activity of the trypsin was 720 units/mg.

7. Substrate Labelling of the Dehydroquinase Active-Site

To proteolysed arom complex at 0°C in 50mM sodium phosphate pH7.5, 0.4mM DTT was added ammonium dehydroquinate to a final concentration of 0.1mM. Successive aliquots of sodium boro-[^{3}H]-hydride, dissolved in ice-cold 10mM sodium hydroxide, were added. The dehydroquinase activity was measured between each addition. When the dehydroquinase activity was reduced to around 15% of the starting value the reaction solution was stirred for a further 30 min at 0°C to allow volatile tritium to escape. Excess tritium was then removed by gel filtration through sephadex G.25 (medium) and the labelled protein recovered.

8. Growth of Cells

Neurospora crassa strain 74-OR 23-1A (F.G.S.C. No. 987 obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA 95521, U.S.A.) was grown as described by Lumsden and Coggins (1977). Cells were routinely grown by Miss A.A. Coia or Miss B. Brodie.

CHAPTER 3

RESULTS

1. Purification of the Arom Complex from Neurospora crassa

The method of purification of the arom complex reported by Lumsden and Coggins (1977) has been revised and improved for use on a larger scale. 100 grams of powdered, lyophilised N. crassa cells were stirred into 1500ml of 0.1M Tris/HCl pH7.5, 0.4mM DTT, 1.2mM PMSF, 5.0mM EDTA, stirred for 1 h at 4°C then centrifuged for 30 min at 25000g. The supernatant conductivity was adjusted to $4mn^{-1}$ by the addition of 1M KCl and then applied to a column of DEAE-cellulose (1.5 x 6.5cm) pre-equilibrated with 50mM Tris/HCl, 75mM KCl, 0.4mM DTT, 1.2mM PMSF. The eluate containing arom complex, which did not bind to this column, was collected and incubated for 90 min at 37°C. After a further centrifugation for 45 min at 25000g benzamidine was added to the supernatant to a concentration of 1mM. The protein precipitating between 40% and 50% saturation with ammonium sulphate was collected, resuspended in 20ml of 0.1M Tris/HCl pH7.5, 0.4mM DTT, 1.2mM PMSF and dialysed overnight against 2 litres of 50mM Tris/HCl pH7.5, 0.4mM DTT, 1.2mM PMSF. The enzyme was then applied at 100ml/h to a column of DEAE-cellulose (1.3 x 2.5cm) pre-equilibrated with the same buffer and the column then washed with buffer containing 30mM KCl at 200ml/h until the A_{280} of the eluate was less than 0.1. Protein was then eluted from the column, at a flow-rate of 100ml/h collecting 6 min fractions, with a linear 1000ml gradient (30-300mM KCl) in the same buffer. The fractions containing arom complex were pooled and applied directly to a column of blue dextran-sepharose (4cm x 1cm), this was routinely done overnight and the flow-rate adjusted accordingly. After the sample was loaded the column was washed with 0.1M Tris/HCl pH7.5, 0.5M KCl, 0.4mM DTT, 1.2mM PMSF at 10ml/h collecting 12 min fractions until the A_{280} of the eluate was

Table 4b The results of some typical purification of the arom complex

Preparation	Yield: shikimate dehydrogenase activity (%)	Protein (mg)	Specific activity (units/mg)
1	31.5	2.4	91.0
2	31.7	3.1	78.5
3	26.0	3.1	71.5
4	32.1	4.0	61.0
5	30.3	3.7	77.0
6	31.5	2.9	75.2

The ratio of the shikimate dehydrogenase/dehydroquinase/ shikimate kinase activities of the arom complex is 6.45/1.22/1.00

Table 4 Purification of the arom complex from 100g N. crassa. The activity measured is that of the shikimate dehydrogenase component of the arom complex.

Stage	Vol.(ml)	Activity(u/ml)	Total Activity (units)	Yield(%)
Extraction	1300	0.53	689	100
First DEAE-cellulose	1180	0.48	566	82.1
Heat step	1155	0.50	579	83.9
$(NH_4)_2SO_4$	31	12.3	381	55.3
Second DEAE-allulase	78	3.6	280	40.6
Blue Dextran Sepharose	7.6	28.5	217	31.5

The specific activity of the purified arom complex was 75.2 units/mg.

zero, usually about 4 h. The arom complex was then eluted directly from the column with a step of 0.1M Tris/HCl pH7.5, 1.5M KCl, 0.4mM DTT, 1.2mM PMSF. The fractions containing the arom complex were pooled, dialysed overnight against 500ml of 50mM sodium phosphate pH7.5, 0.4mM DTT, 1.0mM benzamidine, 50% (v/v) glycerol and stored at -15°C.

Arom complex purified in this way was obtained in 30-35 per cent yield with a specific activity of 70-80 units of shikimate dehydrogenase per mg (Table 4 and Fig. 12).

Lumsden and Coggins (1977) employed cellulose phosphate, a possible affinity adsorbent for the arom complex (Cole and Gaertner, 1975), as the last stage in the purification of the arom complex. When substrate elution with either ATP or shikimate was attempted no enzyme activity was eluted from the cellulose phosphate column by either substrate. Similarly no arom complex was eluted from blue-dextran sepharose by 5mM ATP nor by 15% (v/v) glycerol in the presence or absence of 5mM NADPH nor by 10mM NADPH in the absence of glycerol.

2. The Activity-Ratio Plot

Following trypsin proteolyses which were terminated by passage of the solution through a column of immobilised soy-bean trypsin inhibitor (Chapter 3, Section 3.1) it was desirable to estimate the degree of proteolysis which had occurred. A graphical method was devised which relied only on assays of the proteolysed material and was independent of the amount of trypsin used or the duration of proteolysis.

This plot depends on the fact that the shikimate kinase activity is lost rapidly relative to the shikimate dehydrogenase and

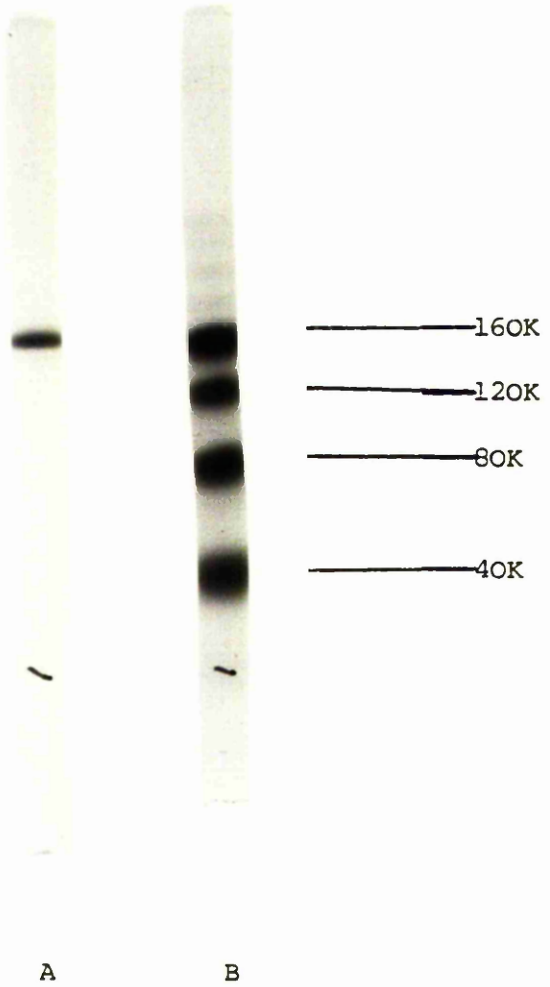


Figure 12 3% SDS polyacrylamide gel electrophoresis of A, purified arom complex and B, cross-linked aldolase. The gels were run as described by Lumsden and Coggins (1977).

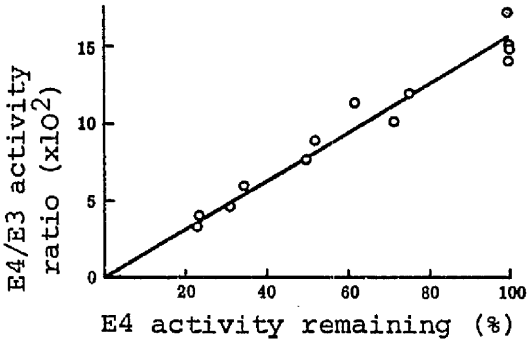


Figure 13 Activity-ratio plot relating the shikimate kinase/shikimate dehydrogenase (E4/E3) activity ratio to the shikimate kinase (E4) activity remaining after proteolysis.

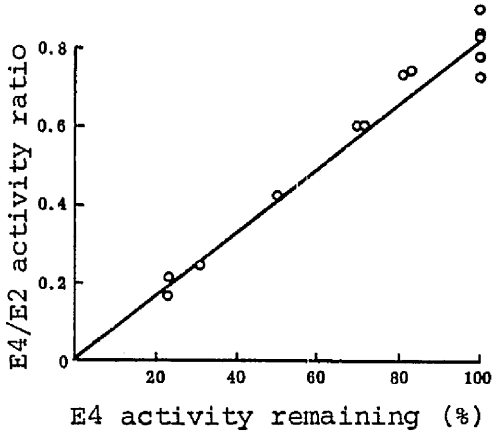


Figure 14 Activity-ratio plot relating the shikimate kinase/dehydroquinase (E4/E2) activity ratio with the shikimate kinase (E4) activity remaining after proteolysis.

dehydroquinase activities of the arom complex during proteolysis with trypsin. To a good approximation the shikimate dehydrogenase activity is unaffected by proteolysis. If a graph of the ratio of the shikimate kinase activity to the shikimate dehydrogenase versus the amount of shikimate kinase activity remaining after proteolysis (expressed as a percentage) is plotted it is found to vary from zero, when no shikimate kinase activity remains, to 0.155, the activity ratio of the arom complex when no proteolysis has occurred. Accumulated experimental data fit the predicted line well (Fig. 13). This so called 'activity-ratio' plot provides a convenient method of monitoring the extent of proteolysis. A similar activity-ratio plot relating the shikimate kinase/dehydroquinase activity ratio with the shikimate kinase activity remaining after proteolysis may be constructed (Fig. 14).

Limited Proteolysis of the Arom Enzyme Complex

3.1 Limited Proteolysis of the Arom Enzyme complex with Trypsin

Proteolysis of the arom complex was performed at 25°C in 50mM sodium phosphate pH7.5, 0.4mM DTT, 1mM EDTA to which was added any required protecting ligands and the desired amount of trypsin. The arom complex to be proteolysed was either dialysed against this buffer overnight prior to the experiment or, in the case of some smaller scale experiments when only the loss of activity was being monitored, the glycerol stock solution of arom complex was diluted into this buffer. In the cases in which the arom complex was diluted prior to proteolysis the rate of loss of activity was lower than when arom complex was dialysed prior to the experiment since benzamidine, a competitive inhibitor of trypsin (Mares-Guia and Shaw, 1965), is present in the

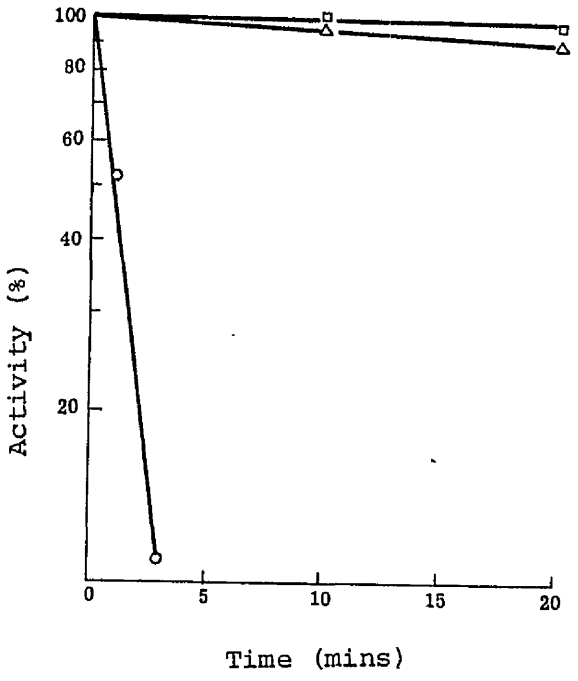


Figure 15 The effect of proteolysis with 6.96 units/ml trypsin on the shikimate kinase (O), shikimate dehydrogenase (Δ) and dehydroquinase (□) activities of the arom complex.

glycerol containing buffer in which the arom complex is stored. Proteolysis was stopped in one of the following three ways: (1) by dilution into an assay cuvette when studying the rate of loss of enzyme activity alone; (2) by the addition of soy-bean inhibitor, a three times weight excess over trypsin, when gel electrophoresis was to be performed or; (3) by passage through a small column of immobilised soy-bean trypsin inhibitor, approximately 1ml bed volume, poured on top of a sephadex G.25 column (10cm x 1cm or 20cm x 1cm depending on the volume of the sample) allowing removal of trypsin and desalting in one operation.

3.2 Proteolysis in the Absence of Substrates of the Arom Complex

Arom complex was incubated with 6.96 units/ml trypsin and at intervals after the addition of trypsin samples were withdrawn into soy-bean trypsin inhibitor to stop the proteolysis (method (2) above). A control experiment was performed to which no trypsin was added. The shikimate kinase, shikimate dehydrogenase and dehydroquinase activities of the samples were tested and SDS polyacrylamide gel electrophoresis of aliquots from each sample performed.

Shikimate kinase activity was quickly lost under these conditions (Fig. 15) whilst the shikimate dehydrogenase and dehydroquinase activities were scarcely affected. SDS polyacrylamide gels of material proteolysed for increasing lengths of time showed the rapid disappearance of the 165000 molecular weight arom band and the appearance of a number of lower molecular weight polypeptides (Fig. 16). After proteolysis for 1 min, by which time the shikimate kinase activity was reduced to 52% of the control, two major bands were present with molecular weights of 69000 and 110000 and a further minor species of molecular weight

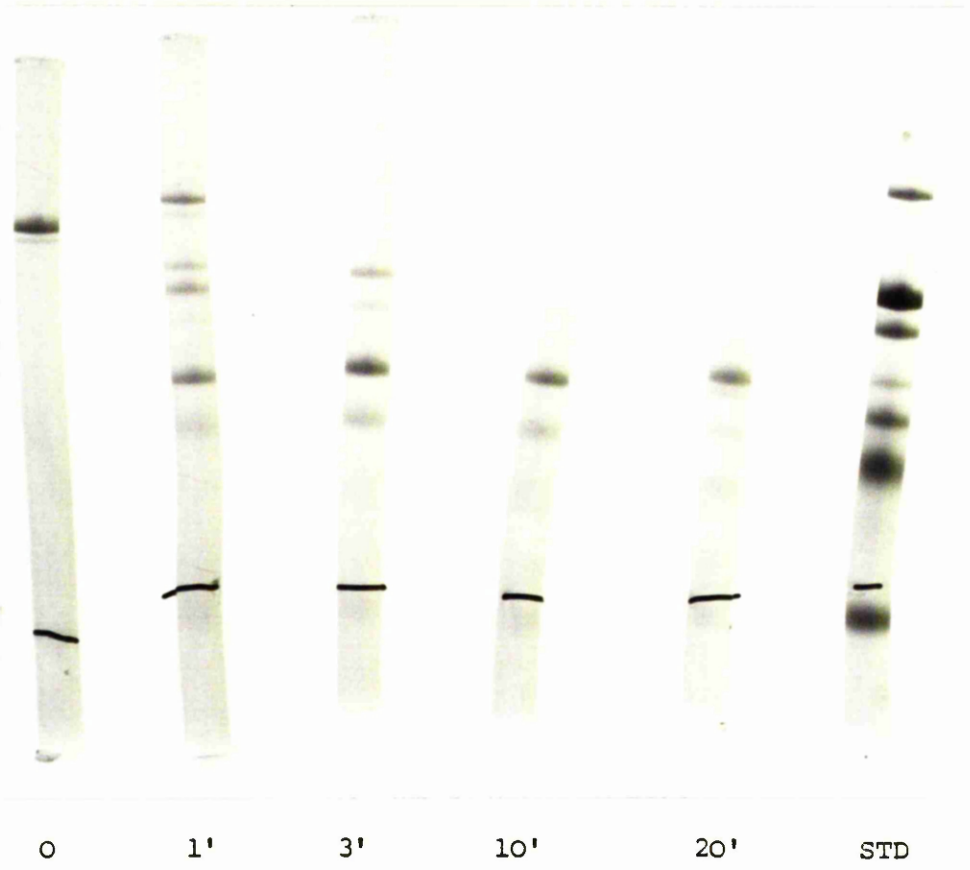


Figure 16 SDS polyacrylamide gel electrophoresis of arom complex proteolysed for increasing times (given here, as in other figure legends, in minutes) with 6.96 units/ml trypsin in the absence of arom complex substrates. Proteolysis was stopped by method (2).

Table 5 The effect of substrates of the arom complex on the time for fifty per cent inactivation ($t_{\frac{1}{2}}$) of the shikimate kinase activity by trypsin

Protecting ligand(s)	$t_{\frac{1}{2}}$ (min)
None	3.6
$MgCl_2$ + ATP	5.5
$MgCl_2$ + ATP + Shikimate	16
$MgCl_2$ + ADP + Shikimate	43.5
Shikimate	11
NADP	4.4
NADP + Shikimate	26
DAHP	6.2

The concentration of the protecting ligands are as given in the legend to Fig. 17.

124000 was also present. After 3 min incubation the arom polypeptide was almost completely lost and the shikimate kinase activity had dropped to 11% of the control. The main polypeptides present were those of molecular weight 69000 and 110000; the 124000 band had disappeared. On further proteolysis the 110000 molecular weight protein was degraded whereas the polypeptide of molecular weight 69000 was relatively stable.

3.3 Protection of the Shikimate Kinase Activity by Substrates of the Arom Complex

In order to investigate the protective effects of substrates of the arom complex on the rate of loss of the shikimate kinase activity during proteolysis with trypsin a series of experiments were performed. In these experiments twenty microlitres of arom complex were diluted to 0.2ml with the usual buffer used for proteolysis to which had been added the substrate, or combination of substrates, to be tested. Trypsin was added to a concentration of 7.2 units/ml and aliquots removed at various times and assayed for shikimate kinase activity by dilution into assay cuvettes. A number of substrates and combinations of substrates protected the shikimate kinase activity against proteolysis by trypsin (Fig. 17 and Table 5). Protection of the shikimate kinase activity by these substrates is most reasonably attributable to conformational changes induced in the arom complex by substrate binding (see Discussion Section).

3.4 Proteolysis in the Presence of Shikimate Phosphate

To arom complex in the phosphate buffer used for proteolysis was added shikimate phosphate to a concentration of 1.9mM. Trypsin,

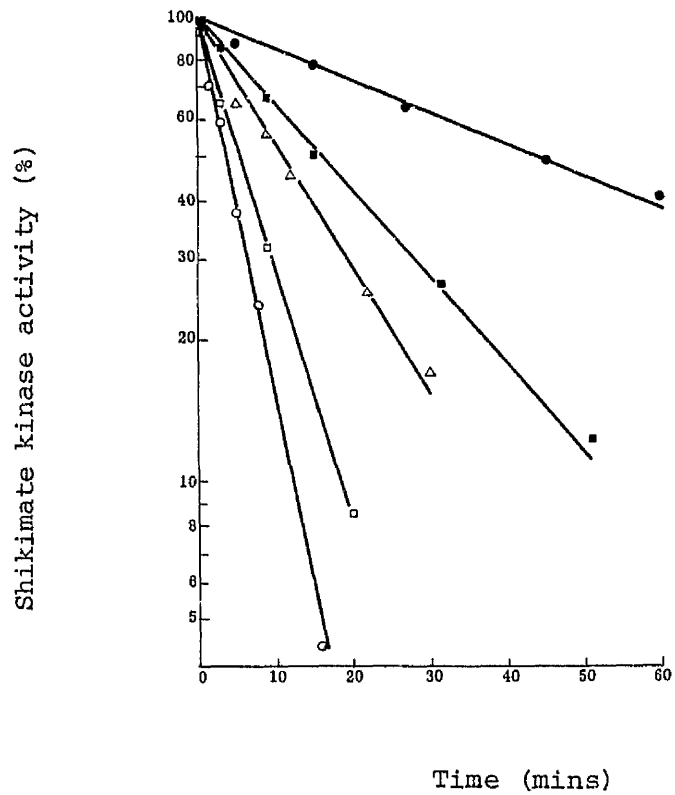


Figure 17a Inactivation of the shikimate kinase activity by proteolysis with 7.2 units/ml trypsin in the absence of substrates of the arom complex (O) and in the presence of 10mM $MgCl_2$ + 1mM ATP (□); 1mM shikimate (Δ); 10mM $MgCl_2$ + 1mM ATP + 1mM shikimate (■); 10mM $MgCl_2$ + 1mM ADP + 1mM shikimate (●). Assays were performed by diluting samples into assay cuvettes after various times.

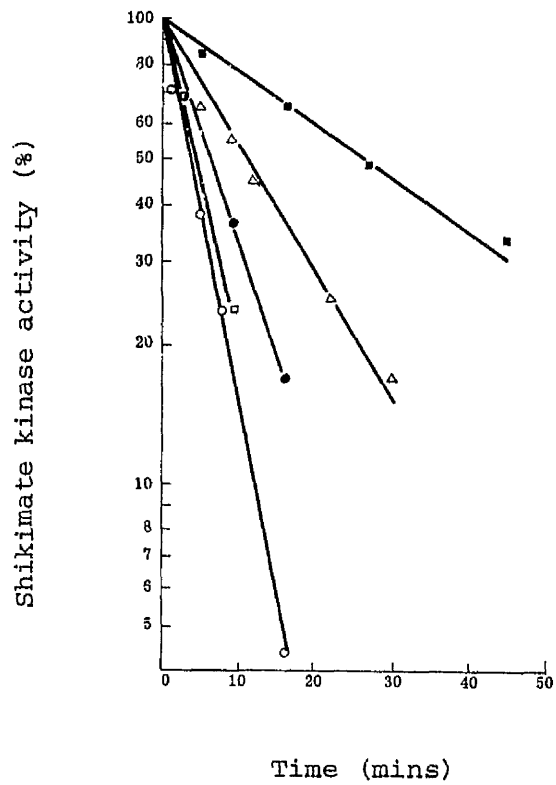


Figure 17b Inactivation of the shikimate kinase activity as in the legend to Figure 17A in the absence of substrates (O) and in the presence of 5mM NADP (□); 1mM DAHP (●); 1mM shikimate (Δ); 5mM NADP + 5mM shikimate (■).

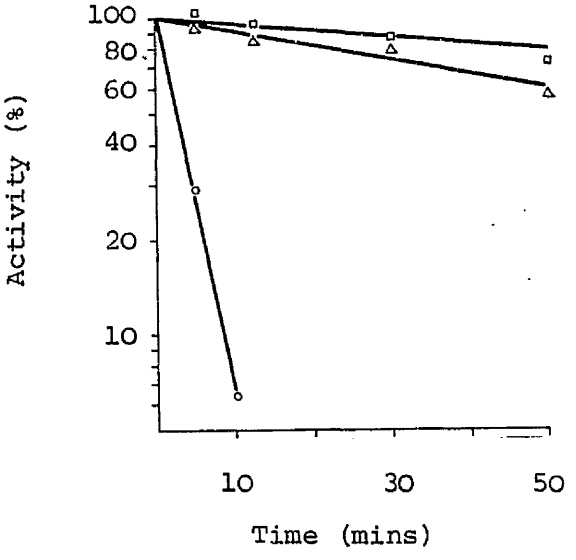


Figure 18 The inactivation of the shikimate kinase (O), shikimate dehydrogenase (Δ) and dehydroquinase (□) activities of the arom complex by proteolysis with 7.6 units/ml trypsin in the presence of 1.9mM shikimate phosphate. Assays were performed on samples treated with soy-bean trypsin inhibitor at various times after the addition of trypsin (method (2) in text).

7.6 units/ml, was added and at intervals aliquots were treated with soy-bean trypsin inhibitor (method (2) above). The samples were assayed (Fig. 18) and analysed by SDS polyacrylamide gel electrophoresis (Fig. 19). The shikimate kinase activity was rapidly inactivated and the arom band on SDS polyacrylamide gels quickly lost. The main bands formed on SDS polyacrylamide gels had molecular weights of 110000 and 70000. The former band was rather unstable and disappeared on prolonged exposure to trypsin whereas the latter, 70000, band was more stable.

3.5 Proteolysis in the Presence of Magnesium, ADP and Shikimate

A study of the proteolysis of the arom complex by trypsin in the presence of a mixture of magnesium, ADP and shikimate, which could potentially form a dead-end complex at the shikimate kinase active-site, was carried out. The arom complex was dialysed overnight against 50mM sodium phosphate pH7.5 containing 0.4mM DTT, 10mM $MgCl_2$ and 5% (v/v) glycerol. Prior to proteolysis ADP and shikimate were each added to a final concentration of 1mM. The proteolysis was performed with 7 units/ml trypsin, samples withdrawn after 15, 45 and 90 min and proteolysis stopped with soy-bean trypsin inhibitor (method (2) above). The shikimate kinase, shikimate dehydrogenase and dehydroquinase activities of the proteolysed samples and of the control, incubated in the absence of trypsin, were determined (Fig. 20) and SDS polyacrylamide gels run (Fig. 21). The shikimate kinase activity was again lost more rapidly than either the shikimate dehydrogenase or dehydroquinase activities. SDS polyacrylamide gel electrophoresis showed the loss of the 165000 molecular weight arom polypeptide and the appearance of major polypeptides of molecular weight 69000 and 125000. A minor band was present with a molecular weight of 110000.

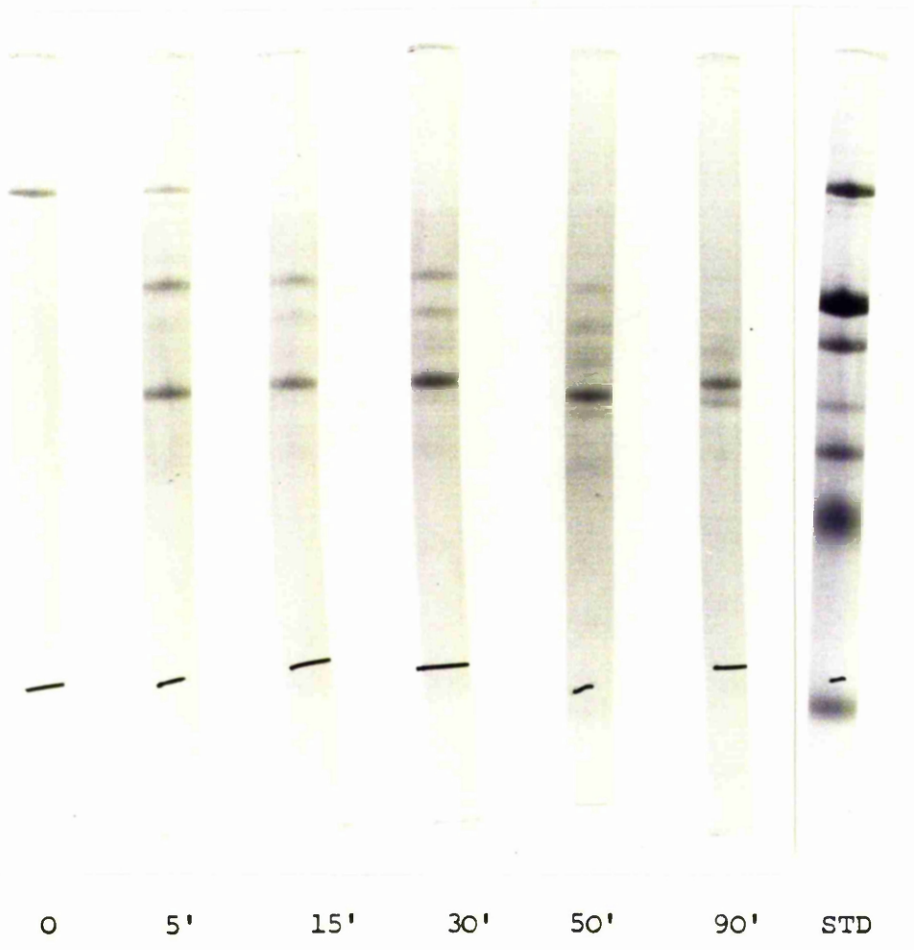


Figure 19 Time-course of proteolysis of the arom complex with 7.6 units/ml trypsin in the presence of 1.9mM shikimate phosphate monitored by SDS polyacrylamide gel electrophoresis. Proteolysis was stopped at different times by withdrawing samples into soy-bean trypsin inhibitor (method (2) in text).

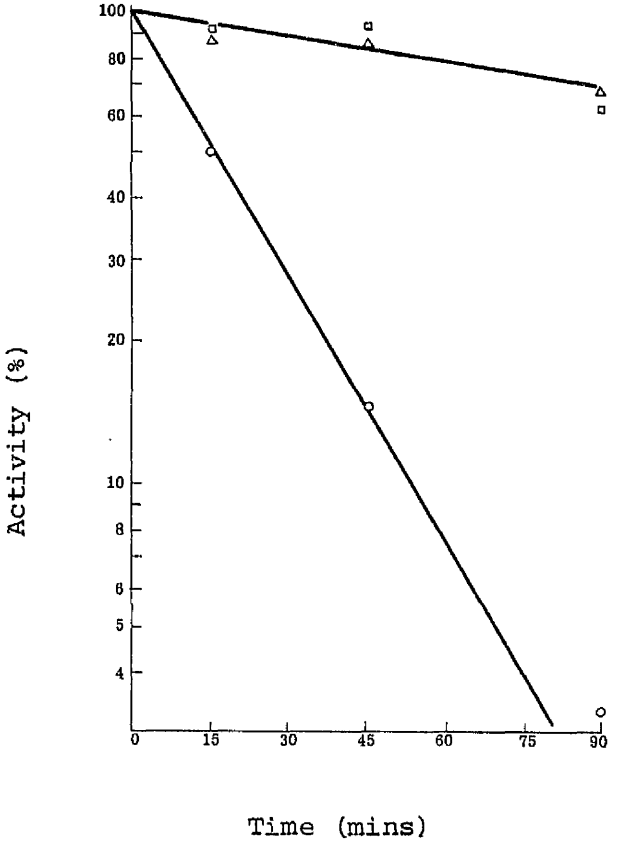


Figure 20 The effect of proteolysis of the arom complex with 7 units/ml trypsin in the presence of 10mM $MgCl_2$, 1mM ADP and 1mM shikimate on the shikimate kinase (O), shikimate dehydrogenase (Δ) and dehydroquinase (□) activities of the complex. Proteolysis was stopped at various times after the addition of trypsin using soy-bean trypsin inhibitor (method (2) in text).

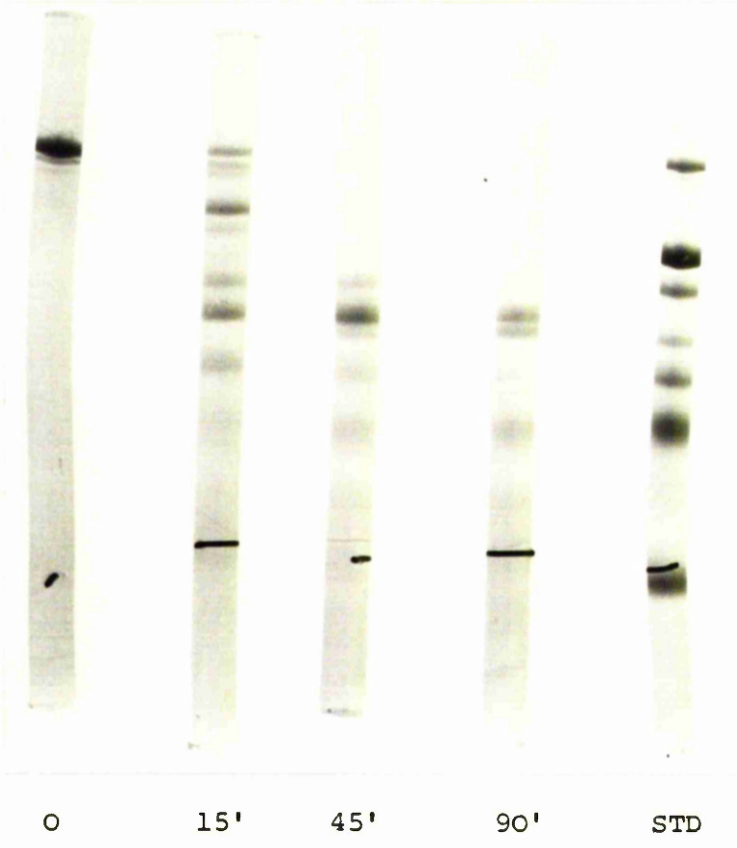


Figure 21 SDS polyacrylamide gel electrophoresis of arom complex proteolysed for increasing times with 7 units/ml trypsin in the presence of 1mM ADP and 1mM shikimate. Proteolysis was stopped at various times using soy-bean trypsin inhibitor (method (2)).

After 45 min proteolysis the predominant band was that of molecular weight 69000 while after 90 min exposure to trypsin polypeptides with molecular weights 69000, 64000 and 37000 were present.

3.6 Proteolysis in the Presence of Shikimate and NADP

Arom complex was subjected to proteolysis in the presence of shikimate and NADP, both at a final concentration of 5mM, and the reaction stopped by the addition of soy-bean trypsin inhibitor (method (2) above). The rate of loss of the shikimate kinase activity increased as the concentration of trypsin used for the proteolysis was raised (Fig. 22). SDS polyacrylamide gel electrophoresis of a time-course of proteolysis with 6 units/ml trypsin in the presence of NADP and shikimate showed a progressive loss of the native arom polypeptide with the formation of lower molecular weight polypeptides (Fig. 23).

After a prolonged exposure to trypsin (240 min, 5.34 units/ml) most of the shikimate dehydrogenase activity was present in a fast-moving band on polyacrylamide gels containing 1M urea stained for this activity (Fig. 24). At this stage of proteolysis two main bands were present on SDS polyacrylamide gels with molecular weights of 68000 and 63000 (Fig. 25).

The molecular weights of the principal polypeptides formed by proteolysis with 7.2 units/ml trypsin in the presence of NADP and shikimate were determined on a sample of arom complex proteolysed until the shikimate kinase activity was around 50% of the starting value and from which trypsin was removed using immobilised trypsin inhibitor (method (3) above). SDS polyacrylamide gel electrophoresis showed three main bands to be present (Fig. 26), residual arom

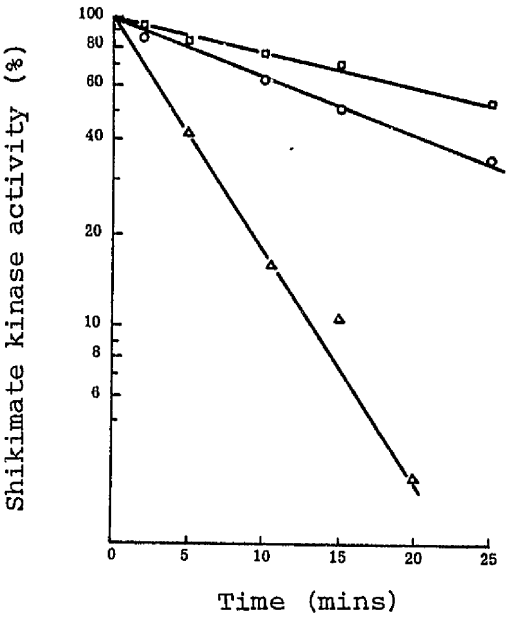


Figure 22 The rate of loss of the shikimate kinase activity of the arom complex on proteolysis in the presence of 5mM NADP and 5mM shikimate with trypsin at concentration of 2.58 units/ml (□); 5.91 units/ml (O) and 14.1 units/ml (Δ).

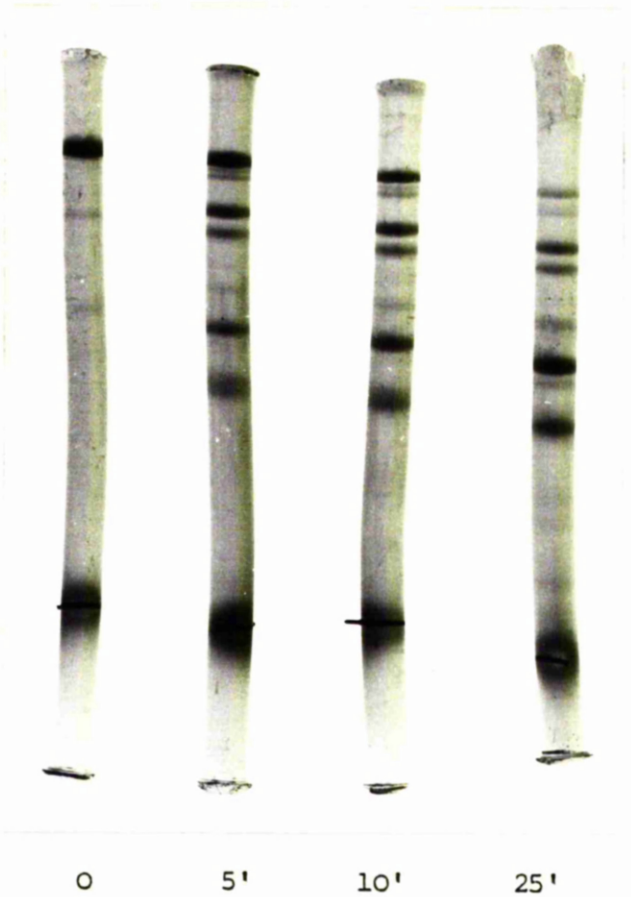


Figure 23 Time-course of proteolysis of the arom complex with 6 units/ml trypsin in the presence of 5mM NADP and 5mM shikimate studied by SDS polyacrylamide gel electrophoresis. Proteolysis was stopped using soy-bean trypsin inhibitor (method (2) in text).

Figure 24 Shikimate dehydrogenase activity stains of A, arom complex proteolysed with 5.34 units/ml trypsin for 240 min in the presence of 5mM NADP and 5mM shikimate and B, unproteolysed arom complex after polyacrylamide gel electrophoresis in the presence of 1M urea.

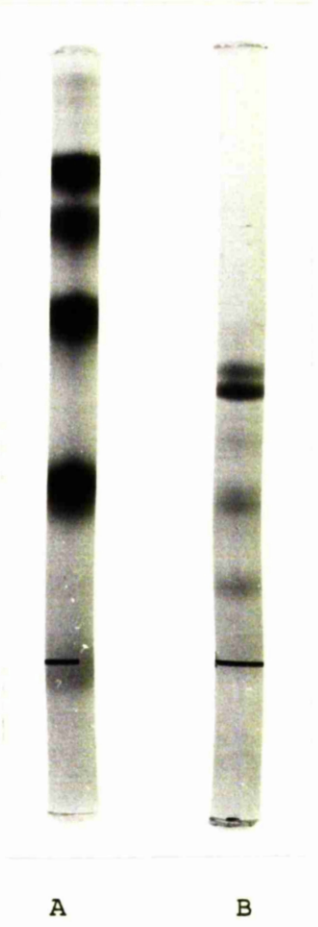


Figure 25 SDS polyacrylamide gel electrophoresis of A, cross-linked aldolase and B, arom complex proteolysed for 240 min with 5.34 units/ml trypsin in the presence of 5mM NADP and 5mM shikimate. Proteolysis was stopped using soy-bean trypsin inhibitor (method (2) in the text).

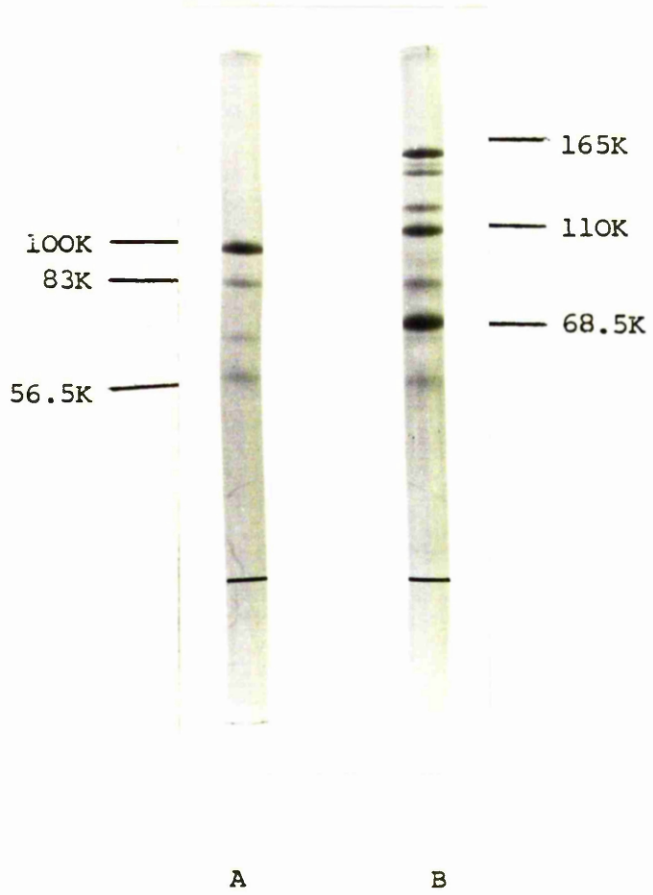


Figure 26 SDS polyacrylamide gels of A, pyruvate dehydrogenase multienzyme complex marker and B, arom complex proteolysed with trypsin in the presence of 5mM NADP and 5mM shikimate. Proteolysis was stopped using immobilised trypsin inhibitor (method (3) in text).

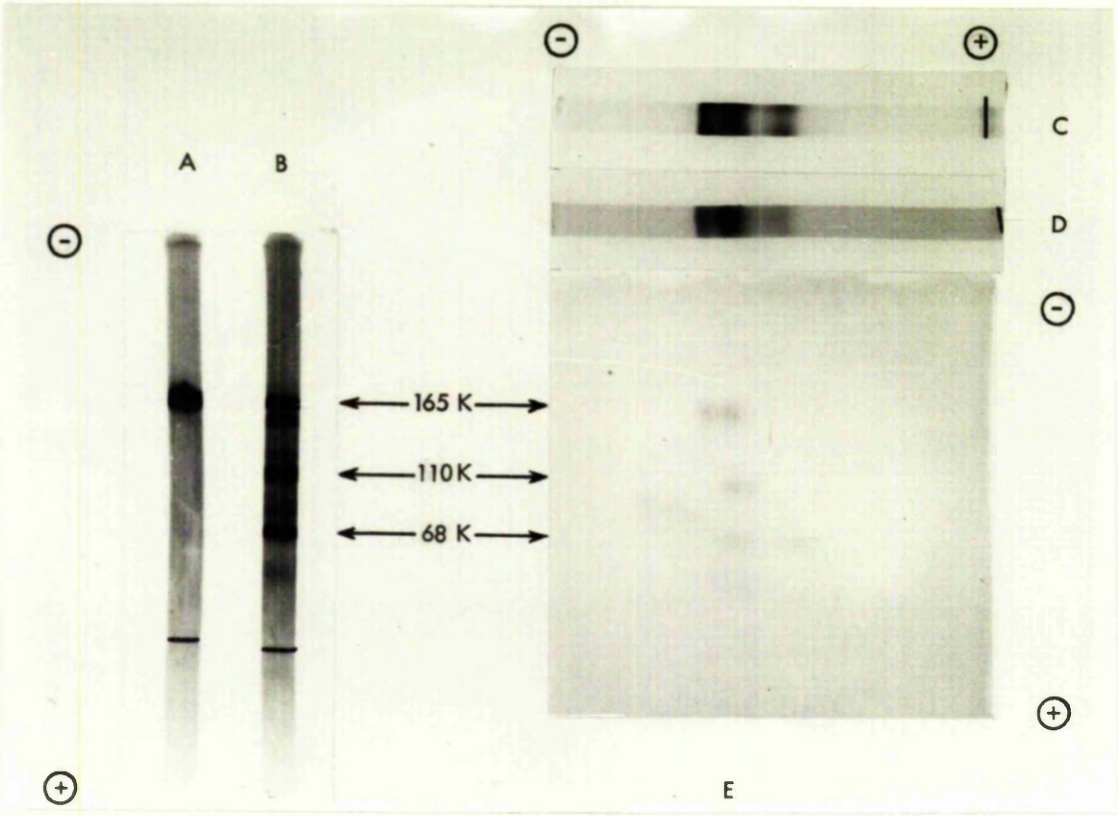


Figure 27 Characterisation of a shikimate dehydrogenase domain of the arom complex obtained by trypsin proteolysis. Gels A and B are SDS polyacrylamide gels stained for protein. A is a control sample of arom complex, the protein in B had been treated with 7.5 units/ml trypsin for 15 min at 25°C in the presence of 5mM NADP and 5mM shikimate. Proteolysis was stopped by passing the solution through immobilised trypsin inhibitor. Gels C and D are polyacrylamide gels containing 1M urea of protein prepared as for gel B. Gel C was stained for shikimate dehydrogenase activity and gel D for protein. Gel E in a second dimension SDS polyacrylamide slab gel run after first dimension gel electrophoresis as for gels C and D.

polypeptide, a fragment of 110000 molecular weight and a second fragment of molecular weight 68500.

3.7 The Characterisation of a Bifunctional Proteolytic Fragment of the Arom Complex

In order to characterise the products of trypsin proteolysis of the arom complex a large-scale proteolysis experiment was performed. 1.75mg of arom complex were proteolysed with 7.5 units/ml trypsin in the presence of 5mM NADP and 5mM shikimate. After 15 min the reaction was stopped by passing the solution through immobilised trypsin inhibitor and gel filtration into 50mM sodium phosphate pH7.5, 0.4mM DTT. Dehydroquinase active-sites were then labelled by reductive alkylation of the Schiff's base intermediate with sodium boro-[^{3}H]-hydride (specific activity 555mCi/mmole). After gel filtration to remove excess tritium the labelled, proteolysed arom complex was dialysed against 50mM sodium phosphate pH7.5, 0.4mM DTT, 1mM benzamidine, 50% (v/v) glycerol for storage at -15^{o}C.

Polyacrylamide gel electrophoresis in the presence of 1M urea separated three bands of protein and of shikimate dehydrogenase activity (Fig. 27 gels C and D). Second dimension SDS polyacrylamide gel electrophoresis showed the most slowly migrating 1M urea gel band to contain arom polypeptides only, the centre band to contain all three of the major sizes of polypeptide present and the fastest moving 1M urea gel band to contain only the smallest, 68000 molecular weight, of the major polypeptides present. Since the 68000 molecular weight polypeptide stained for shikimate dehydrogenase activity it must carry the shikimate dehydrogenase component of the arom complex.

ble 6 The incorporation of tritium into, and the specific radioactivity of, the major polypeptides present after trypsin proteolysis of the arom complex

Molecular ight ($x10^{-3}$)	Tritium c.p.m.	% Total c.p.m.	*Amount of Protein in gel band (P)	Specific Radio-activity ($\frac{cpm}{P}$)
165	850	39.7	1.400	607
110	63	2.9	0.929	67.8
68	1228	57.4	1.962	626

The amount of protein in each gel band is expressed as the product of the area under the peaks of coommassie blue stained gels and $\frac{68000}{M}$, where M is the molecular weight of the band under consideration.

Determination of the radioactivity in slices of SDS polyacrylamide tube gels was used to locate the dehydroquinase active-sites. Tritium was present in the 165000 molecular weight arom polypeptide and in the 68000 molecular weight fragment (Fig. 28). By estimating the amount of protein present in each gel band by integration of the peak areas of gel scans of coommassie blue stained SDS polyacrylamide gels a specific radioactivity was calculated for the arom polypeptide and for each of the two major fragments (Table 6).

3.8 Gel Electrophoresis of Trypsin Proteolysed Arom Complex in the Presence of 8M Urea

Gel electrophoresis in the presence of 8M urea combined with second dimension SDS polyacrylamide gel electrophoresis was used to study the products of trypsin proteolysis of the arom complex. Arom complex proteolysed in the presence of 5mM NADP and 5mM shikimate with 7.5 units/ml trypsin, and from which the trypsin was removed by passage through immobilised trypsin inhibitor (method (3) above), containing predominantly unproteolysed arom polypeptides and polypeptides of molecular weights 106000 and 69000 was analysed in this system. The 165000, 106000 and 69000 molecular weight polypeptides were completely separated by gel electrophoresis in 8M urea and, as shown by carrying out two dimensional electrophoresis using a 5% SDS polyacrylamide slab gel as the second dimension, only one species of molecular weight 69000 was present (Fig. 29).

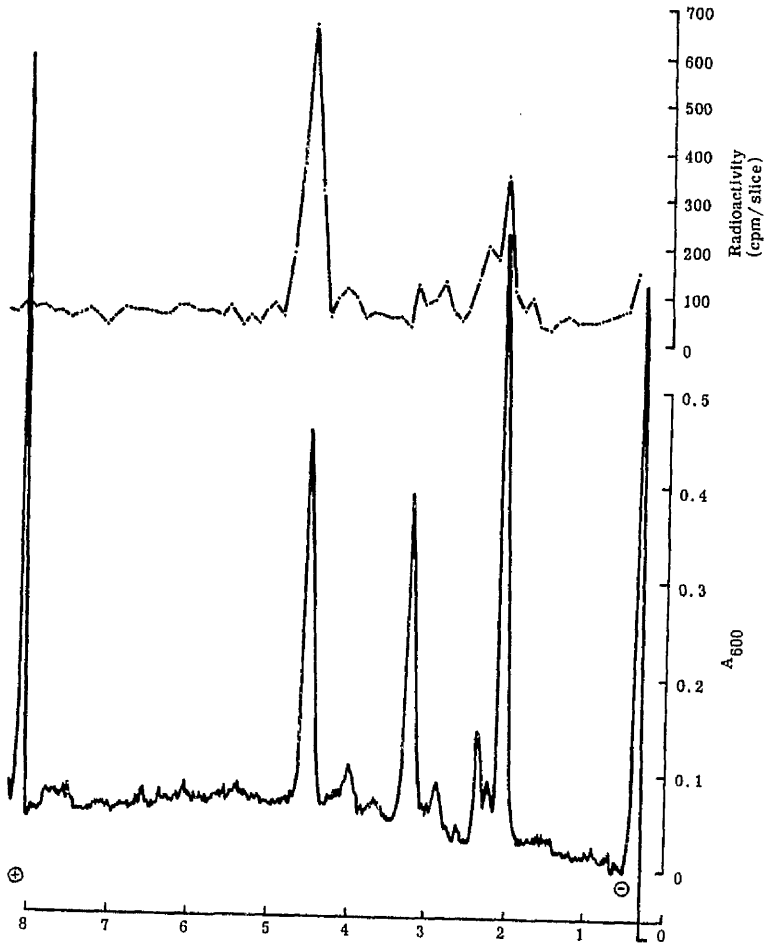


Figure 28 Location of dehydroquinase active-sites on the polypeptides of trypsin-proteolysed arom complex prepared as described in the text. SDS polyacrylamide gel electrophoresis was used to separate the polypeptides of the proteolysed complex. The bottom trace is a densitometer scan of a coommassie blue stained gel showing unproteolysed arom complex and two major fragments of molecular weights 110000 and 68000. The upper trace shows the tritium present in 1mm discs of a sliced gel.

3.9 Renaturation of Shikimate Dehydrogenase Activity of Trypsin Proteolysed Arom Complex after Gel Electrophoresis in the Presence of 8M Urea

Following polyacrylamide gel electrophoresis in the presence of 8M urea as above shikimate dehydrogenase activity could be detected after washing the gel for 50 min in 100ml of 25mM Tris/HCl pH9.0, 0.5mM DTT with shaking and then staining for shikimate dehydrogenase activity as usual (see Materials and Methods). Shikimate dehydrogenase activity was detected only in the region of the gel containing the 69000 molecular weight proteolytic fragment (Fig. 29).

3.10 Cross-Linking of Trypsin Proteolysed Arom Complex with Dimethyl suberimidate

Arom complex which had been treated with 7.2 units/ml trypsin for 15 min in the presence of 5mM NADP and 5mM shikimate was subsequently cross-linked with dimethyl suberimidate. The cross-linking reaction was performed in 0.1M triethanolamine /HCl pH8.2 for 1 h at 25^{o}C with 20mM dimethyl suberimidate. After this time 0.1M-ammonium bicarbonate was added and samples run on SDS polyacrylamide gels. Two major bands were evident on 3% SDS polyacrylamide gels indistinguishable from those observed on cross-linking native arom complex (Fig. 30).

3.11 Kinetic Studies of Trypsin Proteolysed Arom Complex

The proteolysed arom complex used to determine the K_m for NADP and shikimate consisted mainly of the 69000 molecular weight bifunctional fragment. This proteolysed complex was produced by incubation with 7.1 units/ml trypsin for 90 min in the presence of 5mM NADP and 5mM

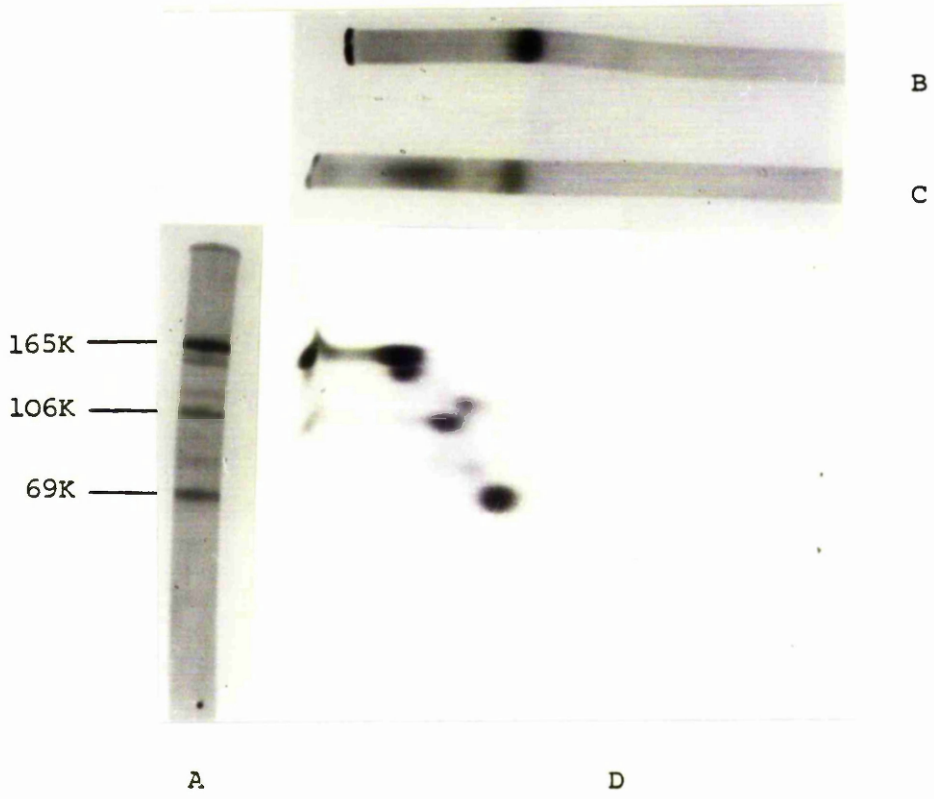


Figure 29 Polyacrylamide gel electrophoresis of trypsin-proteolysed arom complex in the presence of SDS and 8M urea. Gel A is an SDS polyacrylamide gel; gels B and C are 8M urea polyacrylamide gels, gel B was stained for shikimate dehydrogenase activity and gel C for protein. Gel D in a second dimension SDS polyacrylamide slab gel loaded from a gel run in the same manner as gels B and C.

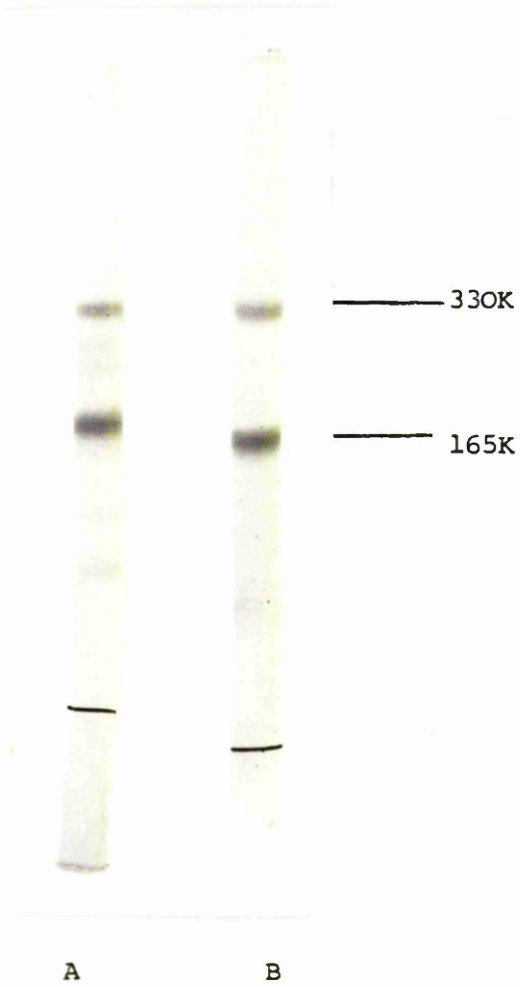


Figure 30 3% SDS polyacrylamide gel electrophoresis of A, cross-linked, trypsin-proteolysed arom complex and B, cross-linked arom complex.

Table 7 The K_m values of the substrates of the shikimate dehydrogenase and dehydroquinase components of native and of trypsin proteolysed *arom* complex

Substrate	K_m (μM) Native *arom* complex	Proteolysed *arom* complex
Shikimate	20.1	16.7
NADP	41.4	25.8
dehydroquinate	6.4	9.8

Table 8 The Arrhenius activation energies of the shikimate dehydrogenase and dehydroquinase components of native and of trypsin proteolysed *arom* complex

Activity	E_A (kcal.mol^{-1}) Native *arom* complex	Proteolysed *arom* complex
Shikimate dehydrogenase	14.0	18.2
dehydroquinase	8.3	9.6

shikimate, after this time the trypsin was removed by passing the solution through a column of immobilised soy-bean trypsin inhibitor and gel filtration into 50mM sodium phosphate pH7.5, 0.4mM DTT. Also present in the proteolysed sample were polypeptides of molecular weight 63000, 84000 and 110000 and very small amounts of unproteolysed arom polypeptide (Fig. 31). For both native and proteolysed arom complex the K_m for NADP in the presence of 2mM shikimate was determined (Fig. 32 and Table 7) as was the K_m for shikimate in the presence of 2mM NADP (Fig. 33 and Table 7). These were found from assays at 25°C in 0.1M potassium phosphate pH7.0. The K_m for dehydroquinate was also found under the same conditions for native and for proteolysed arom complex (Fig. 34 and Table 7).

From the variation of rates of reaction with temperature, in 0.1M potassium phosphate pH7.0, the Arrhenius activation energies of the shikimate dehydrogenase and dehydroquinase reactions catalysed by both the native (Fig. 35) and proteolysed (Fig. 36) arom complex were calculated (Table 8).

3.12 The Effect of Salt and Denaturants on the Shikimate Dehydrogenase Activity of Native and of Trypsin Proteolysed Arom Complex

In the course of active-band centrifugation experiments it was observed that the shikimate dehydrogenase activity of the arom complex was increased in the presence of 0.3M sodium chloride, this effect was further investigated. The shikimate dehydrogenase activity of native arom complex and of trypsin-proteolysed arom complex (7.2 units/ml trypsin for 15 min in the presence of 5mM NADP and 5mM shikimate) at both low substrate concentrations (2mM NADP and 4mM shikimate) and high substrate concentrations (10mM NADP and 10mM shikimate)

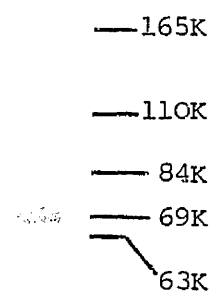


Figure 31 SDS polyacrylamide gel electrophoresis of the proteolysed arom complex used in the kinetic studies.

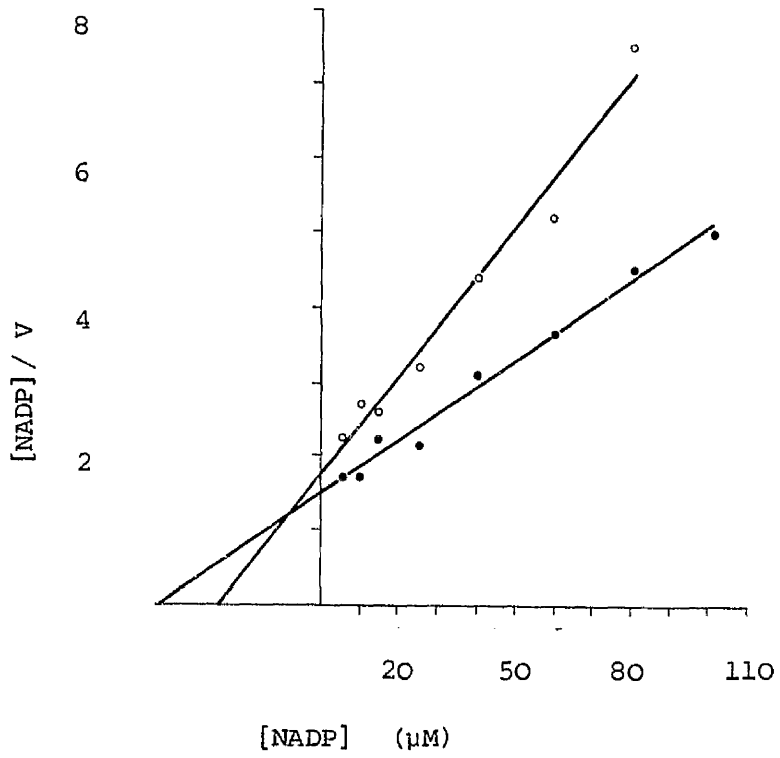


Figure 32 Determination of the K_m for NADP of native arom complex (●) and of proteolysed arom complex (O). Assays were performed at 25°C in 0.1M potassium phosphate pH7.0 in the presence of 2mM shikimate. Lines were fitted to the experimental data by the method of least squares.

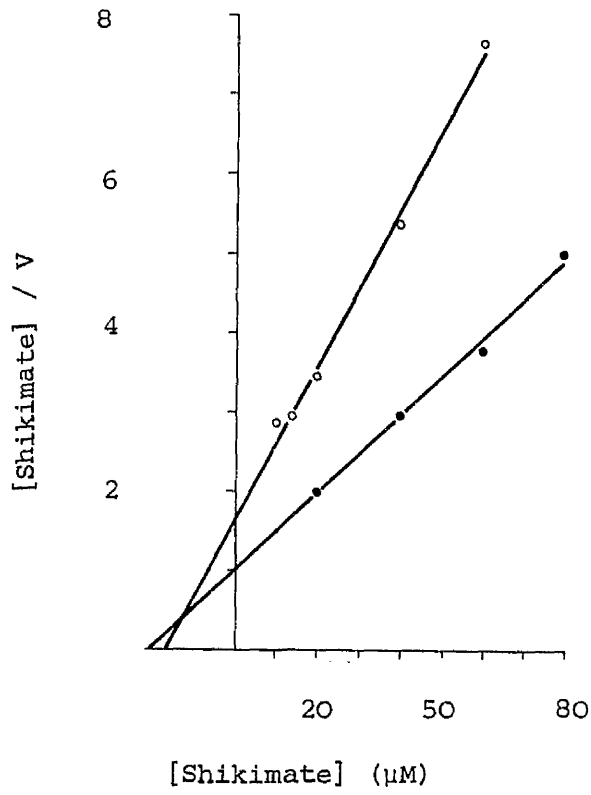


Figure 33 Determination of the K_m for shikimate of native (●) and of proteolysed (O) arom complex. Assays were performed at 25°C in the presence of 2mM NADP in 0.1M potassium phosphate pH7.0.

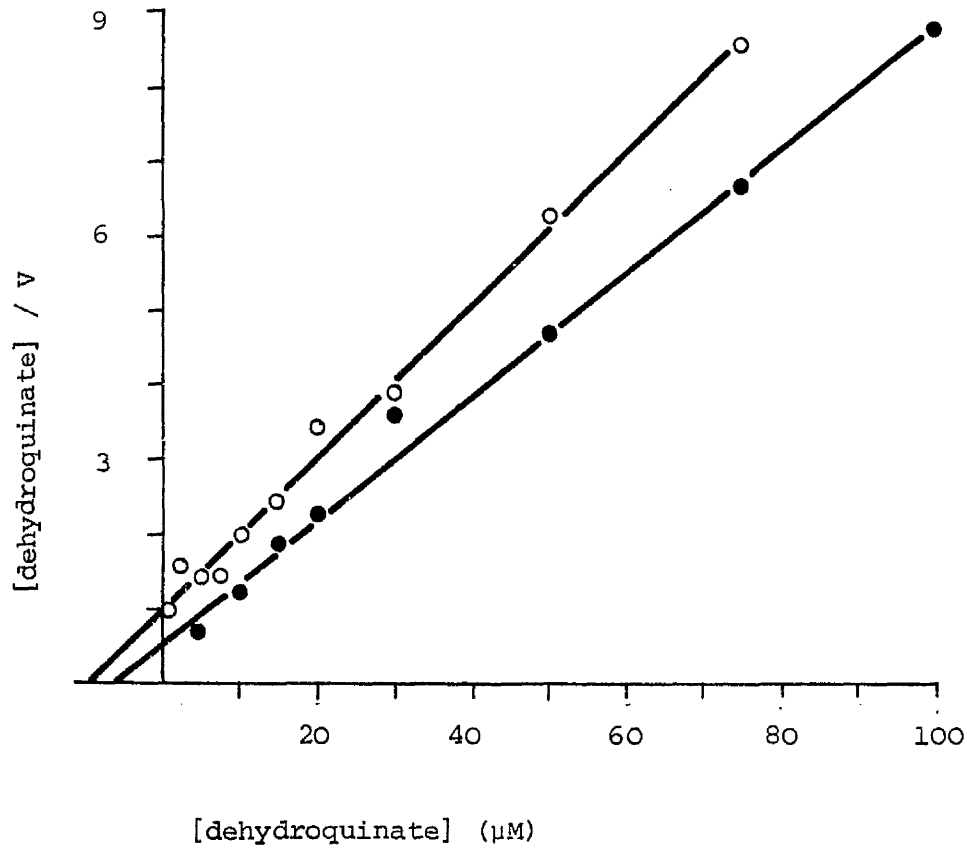


Figure 34 Determination of the K_m for dehydroquinate of native arom complex (●) and of proteolysed arom complex (O). Assays were performed at 25°C in 0.1M potassium phosphate pH7.0.

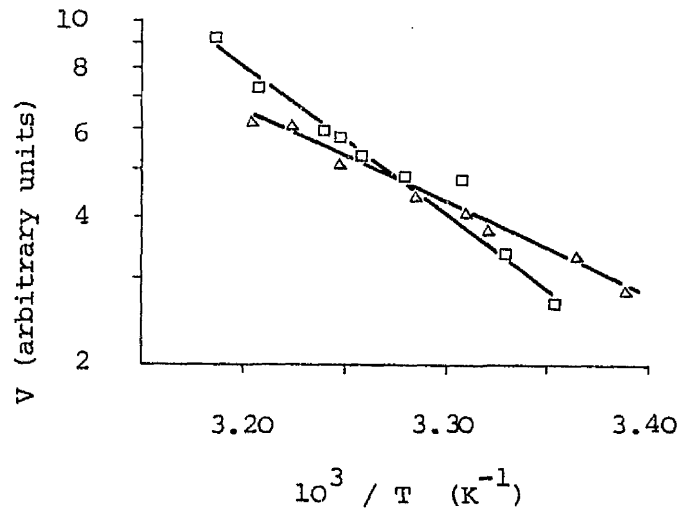


Figure 35 Arrhenius plots for the shikimate dehydrogenase (□) and dehydroquinase (Δ) reactions of the arom complex. Assays were performed in 0.1M potassium phosphate pH7.0.

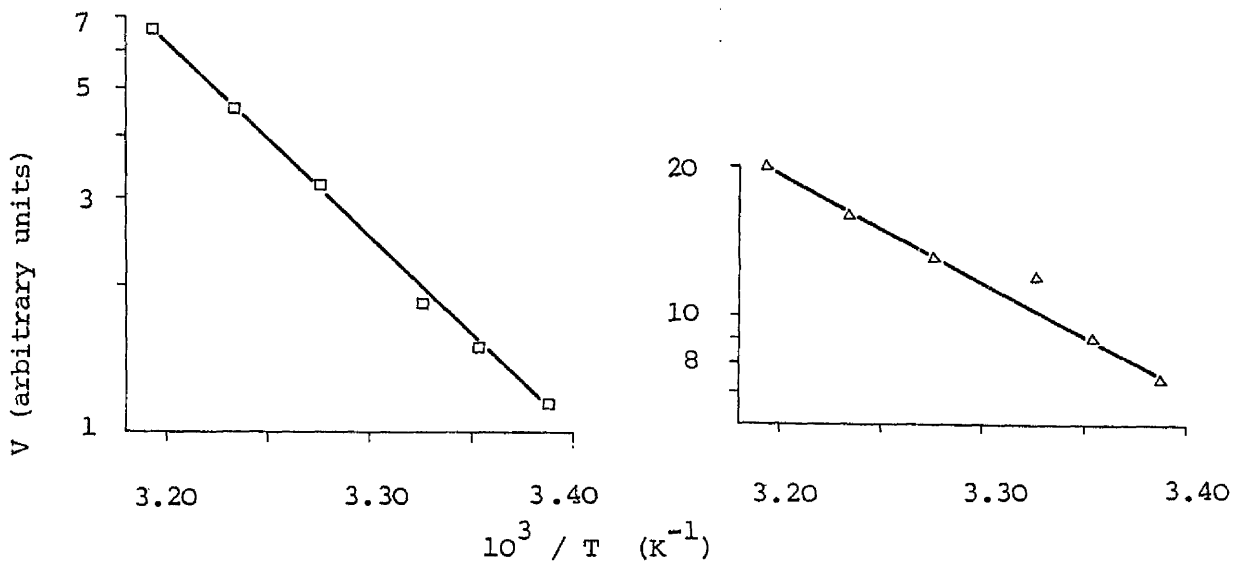


Figure 36 Arrhenius plots for the shikimate dehydrogenase (□) and dehydroquinase (Δ) activities of proteolysed arom complex.

in the presence of various concentrations of sodium chloride in 0.1M Tris/HCl pH8.85 was determined. The effects of sodium chloride concentration on the shikimate dehydrogenase activity of native and of proteolysed arom complex were similar (Fig. 37).

The effects of urea and guanidine hydrochloride concentration on the shikimate dehydrogenase activity of native and of proteolysed arom complex when assayed in 0.1M Tris/HCl pH7.5, 2mM NADP, 4mM shikimate are shown in Figures 38 and 39, again the effects on both native and proteolysed arom complex were similar.

3.13 The Effect of Trypsin Proteolysis on the Dehydroquinate Synthetase Activity of the Arom Enzyme Complex

The inactivation of the dehydroquinate synthetase component of the arom complex by proteolysis with trypsin was studied in the same manner as the substrate protection of the shikimate kinase activity. Twenty microlitres of arom complex was diluted to 0.2ml with the usual buffer used for proteolysis and the proteolysis performed with 7.2 units/ml trypsin. At various times after the addition of trypsin samples were taken and the dehydroquinate synthetase activity assayed by dilution into assay cuvettes. The experiment was performed in the absence of substrates and in the presence of 1.9mM DAHP, the substrate of the dehydroquinate synthetase reaction. Figure 40 shows the inactivation observed and, for comparison, the rate of loss of the shikimate kinase activity in the absence of DAHP.

3.14 Polyacrylamide Gel Electrophoresis of Arom Complex Proteolysed in the Presence of DAHP

DAHP, the substrate of the first enzyme of the arom complex,

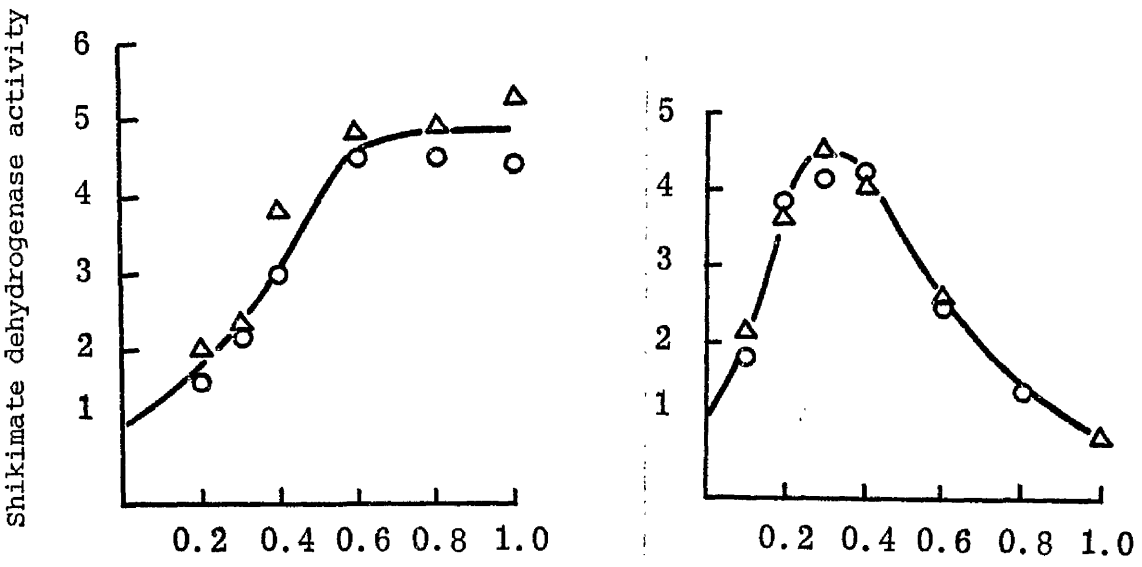


Figure 37 The response of the shikimate dehydrogenase activity of native (O) and of proteolysed (Δ) arom complex in the presence of low (right) and high (left) substrate concentrations (see text for details) to the concentration of NaCl present during assays.

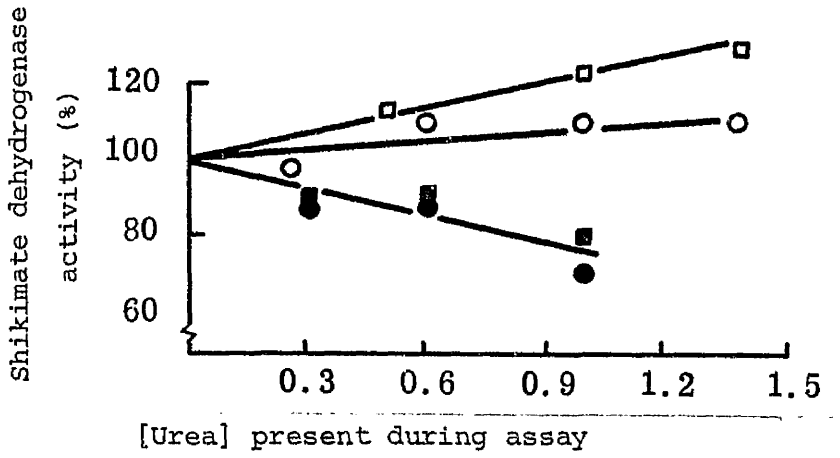


Figure 38 The response of the shikimate dehydrogenase activity of native arom complex to urea concentration in the absence (O) and presence (⊙) of 0.3M NaCl and also the response of proteolysed arom complex in the absence ( ) and presence (⊛) of 0.3M NaCl.

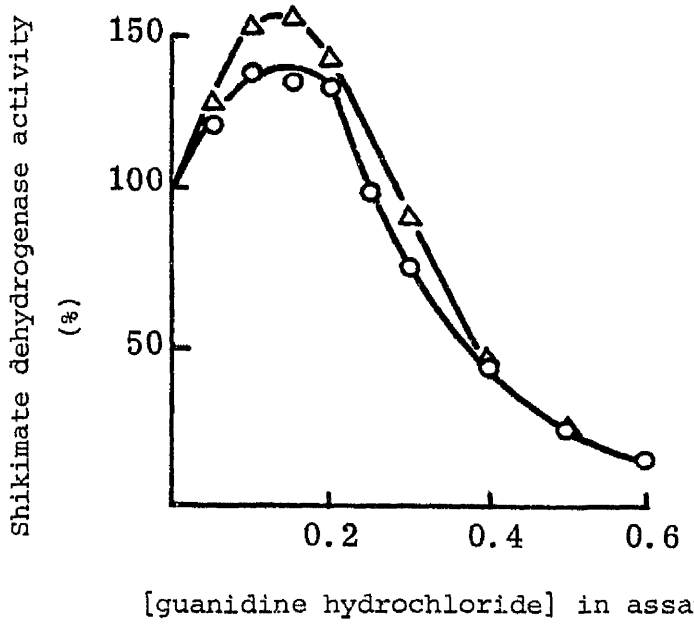


Figure 39 The effect of guanidine hydrochloride concentration on the the shikimate dehydrogenase activity of native (O) and of proteolysed (Δ) arom complex.

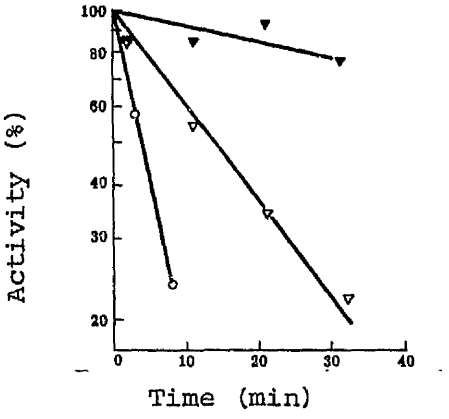


Figure 40 The effect of proteolysis of the arom complex with 7.2 units/ml trypsin on the shikimate kinase (o) and dehydroquinate synthetase (▽) activities in the absence of substrates and on the dehydroquinate synthetase activity when proteolysis was performed in the presence of 1.9mM DAHP (▼).

Table 9 The time for 50% inactivation ($t_{\frac{1}{2}}$) of the enzyme activities of the arom complex on proteolysis with trypsin in the presence of DAHP

Activity	$t_{\frac{1}{2}}$ (min)
shikimate kinase	7.5
dehydroquinate synthetase	60
shikimate dehydrogenase	>> 60
dehydroquinase	>> 60

dehydroquinate synthetase, protected both the dehydroquinate synthetase and the shikimate kinase activities somewhat against proteolysis by trypsin (Figs. 40 and 17). Arom complex was dialysed overnight against 50mM sodium phosphate pH7.5, 0.4mM DTT, 1mM EDTA, DAHP was added to a concentration of 1.9mM followed by trypsin to a concentration of 7.2 units/ml, during the reaction samples were periodically taken and proteolysis stopped with soy-bean trypsin inhibitor (method (2) above). The samples were assayed for shikimate dehydrogenase, shikimate kinase, dehydroquinate synthetase and dehydroquinase activities and also analysed by SDS polyacrylamide gel electrophoresis. The shikimate kinase activity was lost most rapidly, the shikimate dehydrogenase and dehydroquinase activities were relatively unaffected and the dehydroquinate synthetase activity lost at an intermediate rate (Table 9). SDS gel electrophoresis showed two main proteolytic fragments to be formed (Fig. 41), one of molecular weight 110000 the other of 68000 molecular weight. The amount of protein present in the arom band and as each of the major fragments was estimated from measurements of the areas under the peaks of scans of the coommassie blue stained SDS polyacrylamide gels. The rate of loss of protein from these bands was correlated with the rate of loss of the activities of the arom complex (Fig. 42). The stability of the shikimate dehydrogenase and dehydroquinase activities correlated well with the stability of the 68000 molecular weight fragment and the loss of the shikimate kinase activity was reasonably correlated with the loss of the arom band. The 110000 molecular weight band was lost more rapidly than the 68000 molecular weight band and less rapidly than the arom band. The rate of loss of the 110000 band did not correspond precisely with the rate of loss of the dehydroquinate synthetase activity.

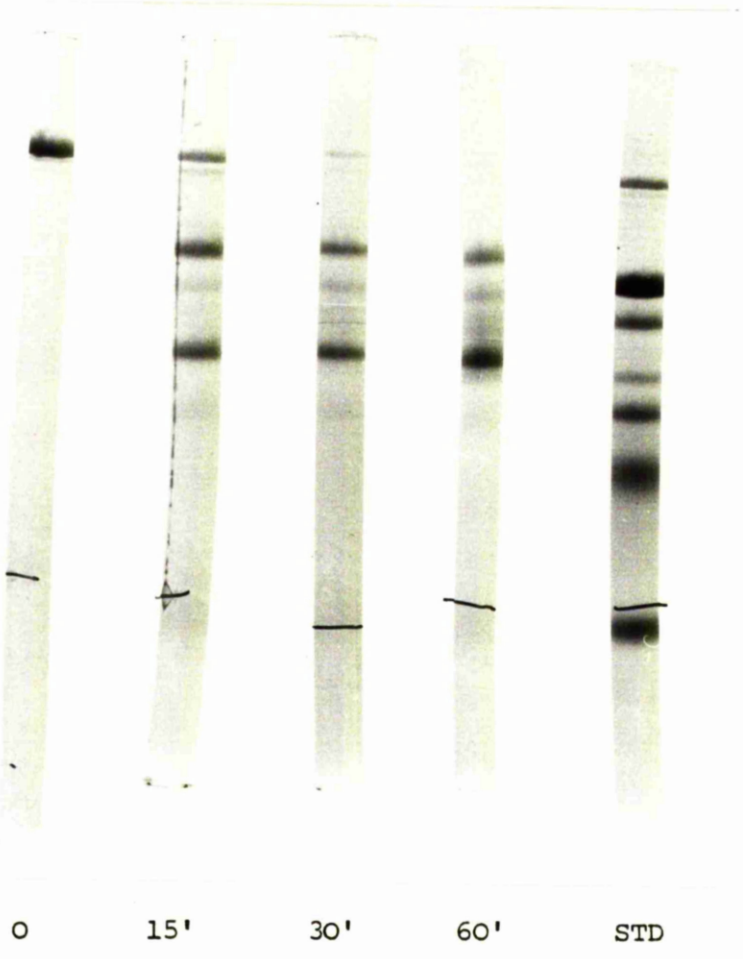


Figure 41 SDS polyacrylamide gel electrophoresis of samples of arom complex proteolysed with 7.1 units/ml trypsin in the presence of 1.9mM DAHP for various times. The proteolysis was stopped by the addition of soy-bean trypsin inhibitor (method (2)).

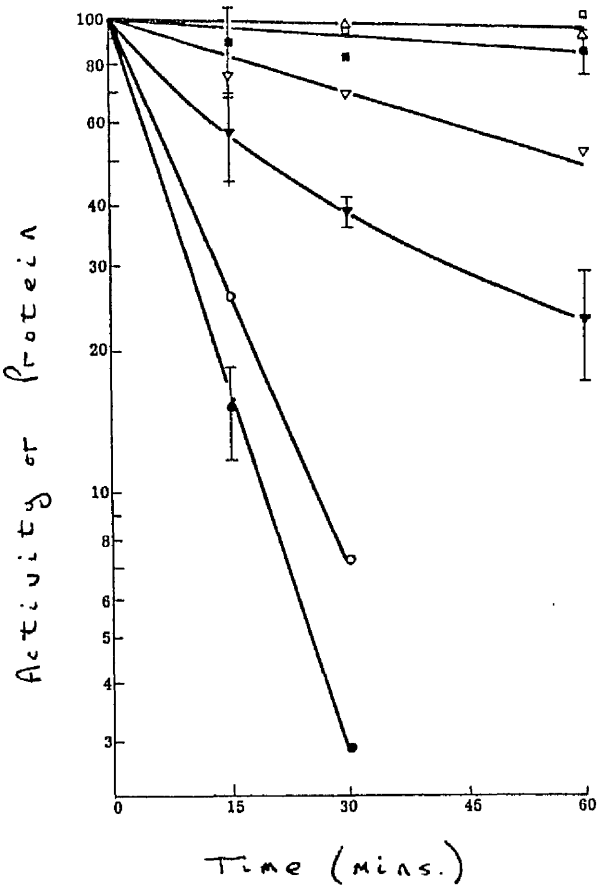


Figure 42 The effect of trypsin proteolysis in the presence of 1.9mM DAHP on the shikimate kinase (O), dehydroquinate synthetase (∇), shikimate dehydrogenase (Δ) and dehydroquinase (□) activities of the arom complex and on the amount of protein in the arom bound (●), the sum of the arom and 110000 bands (▼) and the sum of the arom and 69000 bands (■) on SDS polyacrylamide gels. The amount of protein in each gel band was calculated as the product of the area under the peak of the gel scan of coomassie blue stained gels and $\frac{69000}{M}$, where M is the molecular weight of the polypeptide under consideration. Protein was estimated in two gels. The extremities of the bars are at the values found from each gel, the points are at the mean of the two values.

Table 10 The R_f values in polyacrylamide gels containing 1M urea of native *arom* complex and of the fragments of the *arom* complex produced by proteolysis with trypsin in the presence of DAHP

	R_f		
	Protein stain	shikimate dehydrogenase stain	EPSP synthetase stain
Native *arom* complex	0.20	-	0.22
Proteolysed *arom* complex	0.32	-	0.35
(experiment 1)	0.42	0.44	-
Proteolysed *arom* complex	-	0.29	0.29
(experiment 2)	-	0.41	-

Polyacrylamide gel electrophoresis in the presence of 1M urea and in the presence of SDS were used to study arom complex proteolysed in the presence of DAHP. Arom complex was dialysed overnight into the buffer used for proteolysis experiments. DAHP was added to a concentration of 1.9mM and proteolysis performed with 7.2 units/ml trypsin for 30 min at 25°C. After this time proteolysis was stopped by the addition of soy-bean trypsin inhibitor (method (2) above). Polyacrylamide gel electrophoresis in the presence of 1M urea showed that protein migrating faster than native arom complex had been formed during the proteolysis (Fig. 43). Polyacrylamide gels of native arom complex and of proteolysed arom complex were stained for shikimate dehydrogenase activity and for EPSP synthetase activity (Fig. 43). The R_f values for native arom complex and of the bands present on gels of proteolysed arom complex were calculated (Table 10), the results of a similar experiment in which better resolution of shikimate dehydrogenase components of proteolysed arom complex was achieved (Fig. 44) are also presented in Table 10. Gel electrophoresis in the presence of 1M urea was not wholly successful in separating the polypeptides present in the proteolysed sample of arom complex. It appears that the more slowly migrating band of shikimate dehydrogenase activity, which also stains for EPSP synthetase, contains polypeptides of molecular weights 109000, 68000 and 51000 while the more rapidly migrating band of shikimate dehydrogenase activity contains only polypeptides of 68000 molecular weight (Fig. 44).

3.15 Characterisation of an Anomalous Trypsin Proteolysis Experiment

In one experiment in which arom complex was proteolysed with 7 units/ml trypsin in the presence of 5mM shikimate and 5mM NADP the molecular weights of the polypeptides found on SDS gels were not as

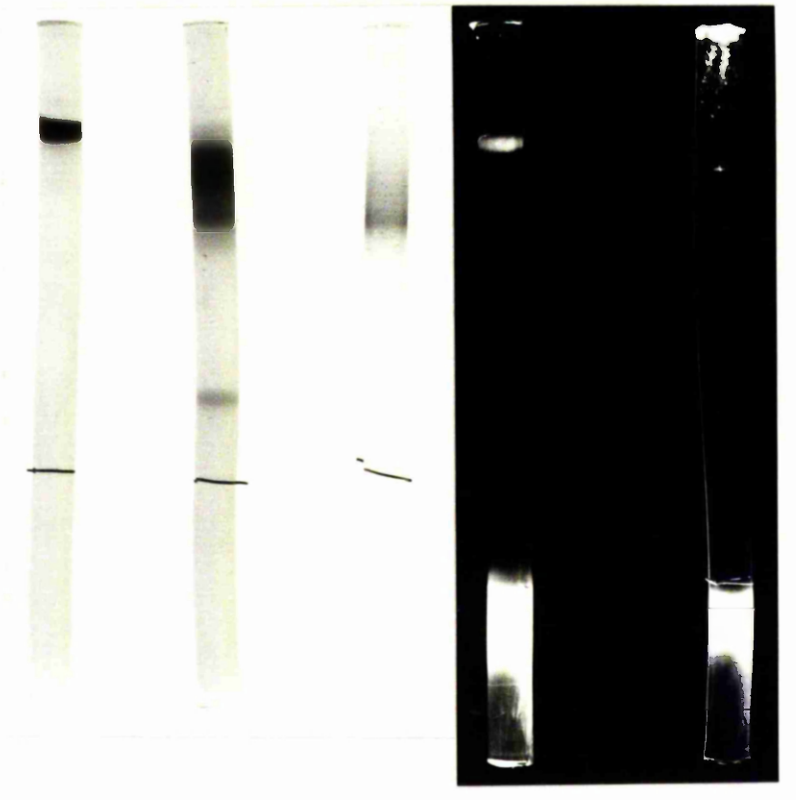


Figure 43 Polyacrylamide gel electrophoresis in the presence of 1M urea of arom complex proteolysed in the presence of DAHP. Gels A and D are of unproteolysed arom complex stained for protein and EPSP synthetase activity respectively. Gels B, C and E are of proteolysed arom complex and stained for protein, shikimate dehydrogenase activity and EPSP synthetase activity. The heavy bands below the wire on gels D and E are due to the presence of phosphate in the sample loaded onto the gels.

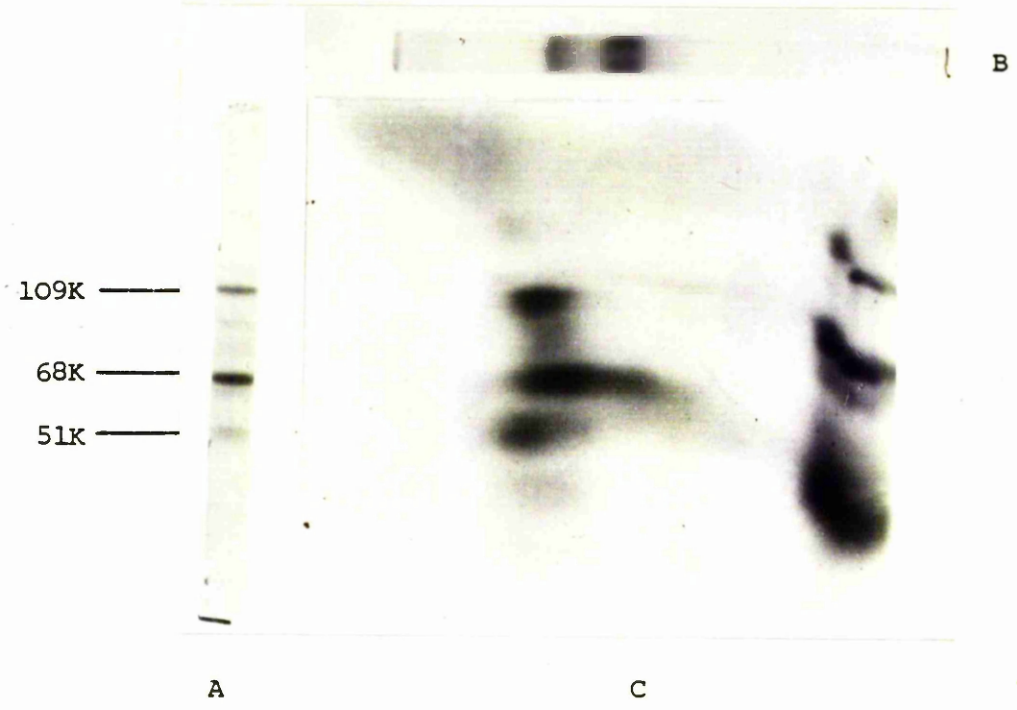


Figure 44 Polyacrylamide gel electrophoresis of arom complex proteolysed with 7.2 units/ml trypsin in the presence of 1.9mM DAHP for 30 min at 25°C. Proteolysis was stopped with immobilised soybean trypsin inhibitor (method (3) in the text). Gel A is an SDS polyacrylamide gel stained for protein, gel B a polyacrylamide gel containing 1M urea stained for shikimate dehydrogenase activity and gel C a second dimension SDS polyacrylamide gel loaded from a gel run as for gel B.

Table 11 The activity ratios measured before and after proteolysis with 7 units/ml trypsin for 15 min in the presence of 5mM shikimate and 5mM NADP

Activity Ratio Measured	Ratio prior to proteolysis	Ratio after proteolysis
$\frac{E4}{E2}$	0.1558	0.0535
$\frac{E4}{E2}$	0.7328	0.2866
$\frac{E4}{E1}$	4.878	2.518

E1 = dehydroquinate synthetase

E2 = dehydroquinase

E3 = shikimate dehydrogenase

E4 = shikimate kinase

expected on the basis of previous experiments. Proteolysis was performed at 25°C for 15 min and terminated by passing the reaction solution through immobilised soy-bean trypsin inhibitor and gel filtration into 50mM sodium phosphate pH7.5, 0.4mM DTT (method (3) above). The activity ratios were measured before and after proteolysis (Table 11) and indicated that around 35% of the shikimate kinase activity remained as did around 66% of the original dehydroquinate synthetase activity.

SDS polyacrylamide gel electrophoresis showed the main polypeptides present after proteolysis to have molecular weights of 122000, 68000, 62000 and 50000; no unproteolysed arom polypeptide remained (Fig. 45).

Polyacrylamide gel electrophoresis in the presence of 8M urea separated a number of protein bands. Second dimension SDS polyacrylamide gel electrophoresis established that each of these bands contained only one size of polypeptide chain (Fig. 45).

After polyacrylamide gel electrophoresis in the presence of 8M urea shikimate dehydrogenase activity could be detected in the gel after soaking for 50 min in 100ml of 25mM Tris/HCl pH9.0, 0.5mM DTT and then staining for activity as usual. Shikimate dehydrogenase activity was found associated with the polypeptides of molecular weights 122000, 68000 and 62000 but not with the most abundant polypeptide of molecular weight 50000 (Fig. 45). Other shikimate dehydrogenase staining bands are probably associated with a minor polypeptide of molecular weight around 48000 not resolved from the major band of molecular weight 50000 on single dimension SDS gel electrophoresis, and possibly with a polypeptide of molecular weight 37000.

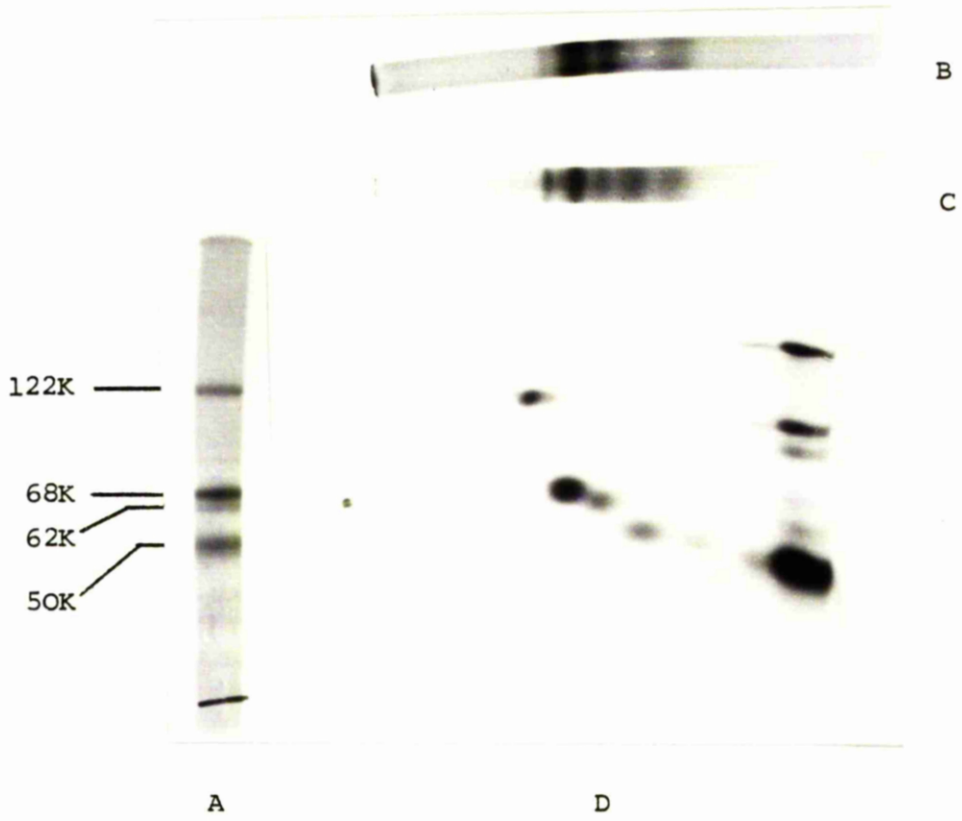


Figure 45 Polyacrylamide gel electrophoresis of trypsin-proteolysed arom complex. Gel A is an SDS polyacrylamide gel. Gels B and C are polyacrylamide gels containing 8M urea stained for shikimate dehydrogenase activity and protein respectively. Gel D is a second dimension SDS polyacrylamide slab gel loaded from a gel run as for gels B and C.

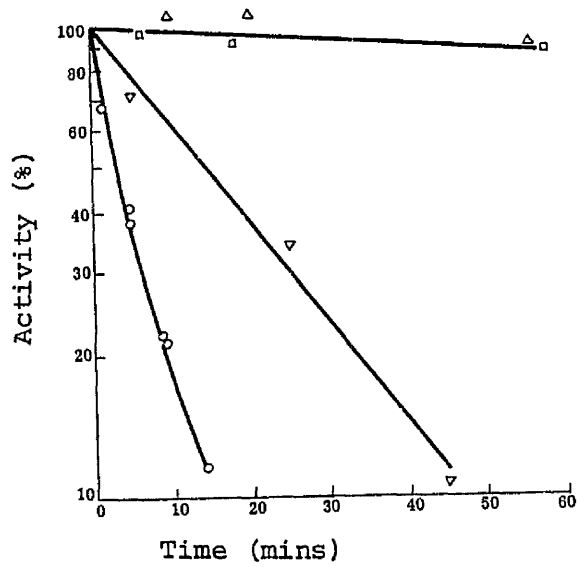


Figure 46 The effect of proteolysis of the arom complex with 3µg/ml subtilisin at 0°C on the shikimate kinase (O), dehydroquinate synthetase (∇), shikimate dehydrogenase (Δ) and dehydroquinase (□) activities of the complex. Assays were performed by diluting aliquots of the reaction solution into the appropriate assay cuvette at various times after the addition of protease.

4.1 Proteolysis of the Arom Enzyme Complex with Subtilisin

Proteolysis of the arom complex with subtilisin was performed in 50mM sodium phosphate pH7.5, 0.4mM DTT, 1mM EDTA at either 25°C or 0°C. The reaction was stopped either by diluting proteolysed arom complex into an assay cuvette for activity measurements or by adding a freshly prepared 6mg/ml solution of PMSF in 95% ethanol to a final concentration of 1.2mM.

4.2 The Susceptibility of the Shikimate Kinase, Dehydroquinate Synthetase, Shikimate Dehydrogenase and Dehydroquinase Activities to Proteolysis with Subtilisin

Small scale experiments were performed to test the stability of the enzyme activities of the arom complex toward proteolysis with subtilisin. In each case twenty microlitres of arom complex was diluted to 0.2ml with 50mM sodium phosphate pH7.5, 3µg/ml subtilisin added and the activity measured by dilution into assay cuvettes after various periods of incubation with protease at 0°C. Figure 46 shows the loss of activity with increasing length of incubation for the four activities measured, shikimate kinase, shikimate dehydrogenase, dehydroquinase and dehydroquinate synthetase. As was the case with trypsin the shikimate kinase activity was rapidly lost, the dehydroquinate synthetase activity was inactivated less rapidly and both the shikimate dehydrogenase and dehydroquinase activities were relatively stable.

4.3 Polyacrylamide Gel Electrophoresis of Subtilisin Proteolysed Arom Enzyme Complex

The sizes of the proteolytic fragments produced by cleavage of the arom complex with subtilisin were studied by SDS polyacrylamide gel

electrophoresis. Two methods were used to stop the reaction, boiling the reaction mixture or addition of PMSF as above. The latter method was used to investigate the molecular weights of the polypeptides produced by proteolysis at 0°C and at 25°C.

4.4 Termination of Subtilisin Proteolysis of the Arom Complex by Boiling

Arom complex was dialysed overnight into 50mM sodium phosphate pH7.5, 0.4mM DTT, 1mM EDTA at 4°C and then proteolysed with 3µg/ml subtilisin at 0°C. During the proteolysis samples were removed and assayed by dilution into assay cuvettes and at intervals during the reaction aliquots were taken, boiled for 1.5 min to inactivate the protease, and analysed by SDS gel electrophoresis (Fig. 47). A control incubation was run in the absence of protease. SDS polyacrylamide gel electrophoresis of arom complex proteolysed in this manner showed two major fragments of molecular weights 66000 and 50000. At early times during the reaction higher molecular weight polypeptides, 156000, 120000, 104000 and 79000, were observed.

4.5 Termination of Subtilisin Proteolysis with PMSF

Proteolysis of the arom complex at 0°C was performed as described in the previous section except that the reaction was stopped by treating samples with PMSF to a final concentration of 1.2mM. The samples were subjected to SDS polyacrylamide gel electrophoresis (Fig. 48). Proteolytic fragments of molecular weights 125000, 110000, 69000 and 52000 were produced. After 30 min the main bands were those of molecular weights 69000, 52000 and 110000.

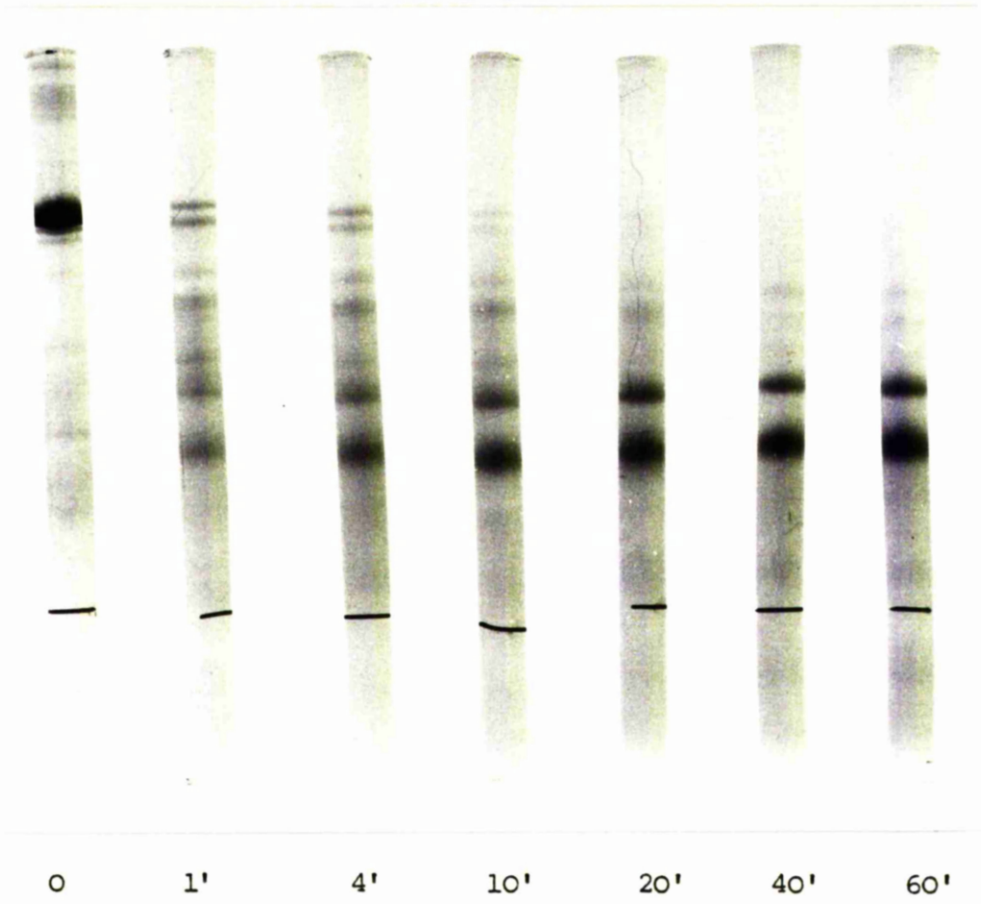


Figure 47 SDS polyacrylamide gel electrophoresis of a time-course of subtilisin proteolysis of the arom complex at 0°C. Proteolysis was stopped at various times by boiling.

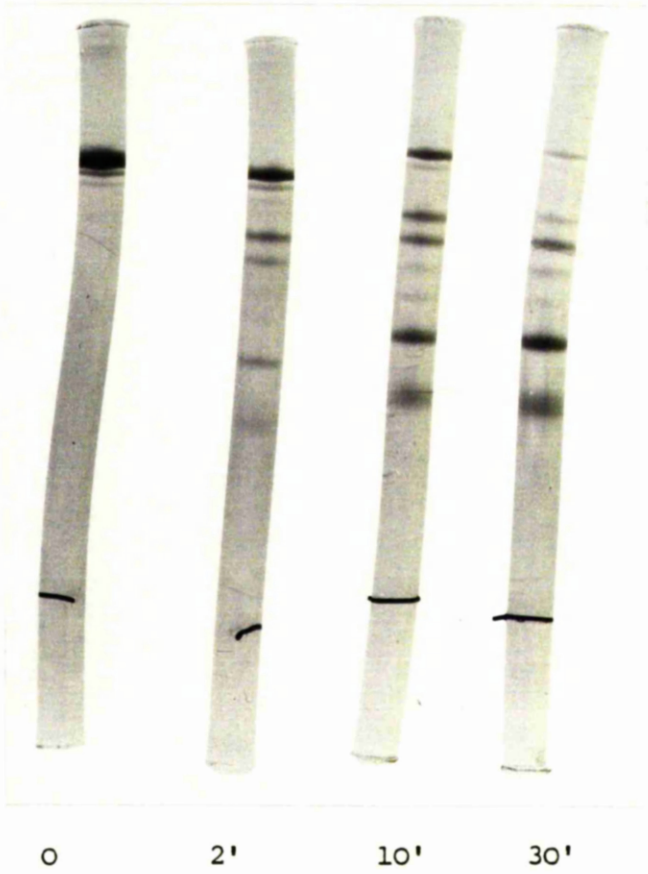


Figure 48 SDS polyacrylamide gel electrophoresis of a time-course of proteolysis of the arom complex at 0°C with subtilisin. Proteolysis was stopped by the addition of PMSF.

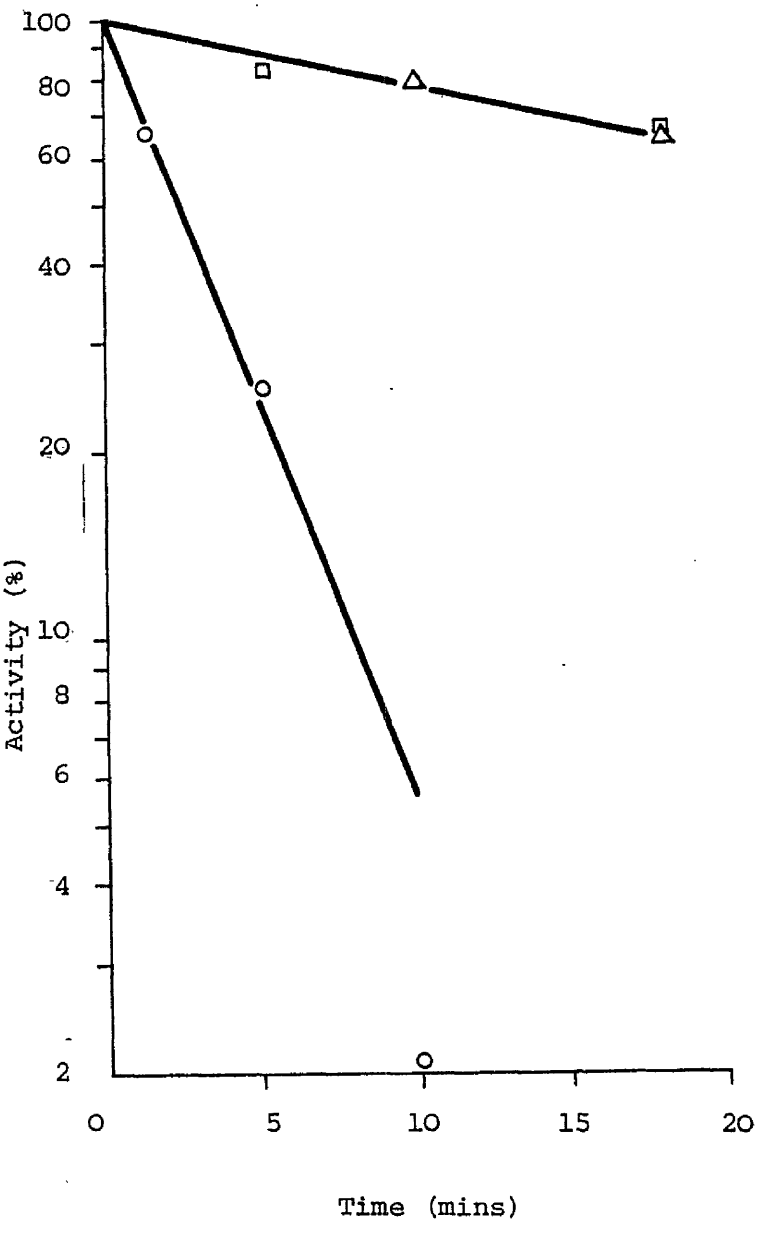


Figure 49 The effect of proteolysis of the arom complex with 3µg/ml subtilisin at 25°C on the shikimate kinase (O), shikimate dehydrogenase (Δ) and dehydroquinase (□) activities of the complex. Proteolysis was stopped with PMSF (see text).

4.5 Proteolysis of the Arom Complex at 25°C with Subtilisin

Proteolysis of the arom complex at 25°C was performed in the same manner as was proteolysis at 0°C. The concentration of subtilisin used was 3µg/ml and the reaction was stopped by mixing samples of the reaction solution with sufficient PMSF to give a final concentration of 1.2mM. The shikimate kinase, shikimate dehydrogenase and dehydroquinase activities of the samples were measured (Fig. 49) and SDS polyacrylamide gels run (Fig. 50). The molecular weights of the fragments produced by proteolysis at 25°C were the same as these found after proteolysis at 0°C. After 10 min proteolysis the only fragments present had molecular weights of 68000 and 50000.

A further subtilisin proteolysis experiment performed at 25°C again showed the shikimate kinase activity to be rapidly lost. In this case the fragment produced at a molecular weight around 50000 was resolved into two components, one of molecular weight 52000, the other of 50000 molecular weight (Fig. 51).

4.6 Location of Dehydroquinase Active-Sites after Proteolysis of the Arom Complex with Subtilisin

Arom complex was proteolysed at 0°C for 30 min with 3µg/ml subtilisin and then PMSF added to a concentration of 1.2mM to stop the reaction. The dehydroquinase active-sites of the proteolysed arom complex were then labelled by reductive alkylation with sodium boro-[^{3}H]-hydride (specific activity 500mCi/mmole) of the Schiff"s base formed with dehydroquinate during the dehydroquinase reaction. Excess tritium was separated from the labelled protein by gel filtration and the protein stored at -15°C in 50mM sodium phosphate pH7.5, 0.4mM DTT, 1mM EDTA, 50% (v/v) glycerol.

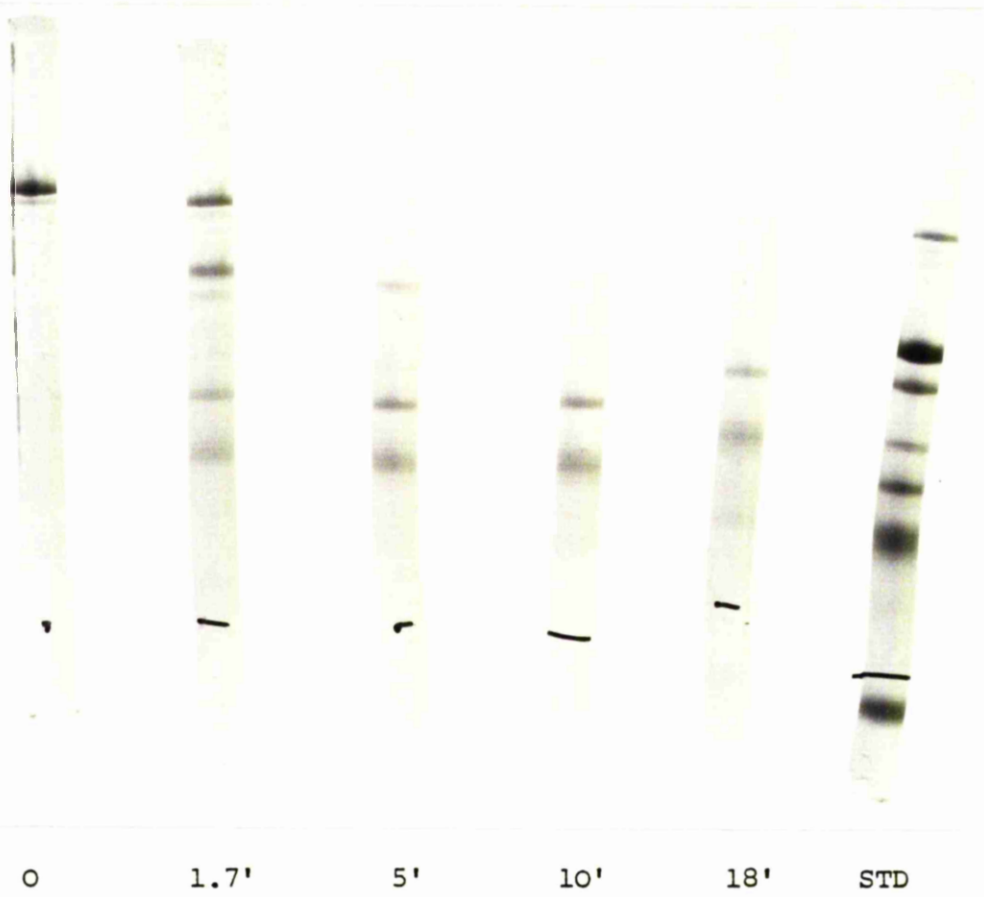


Figure 50 SDS polyacrylamide gel electrophoresis of arom complex proteolysed with subtilisin at 25°C. Proteolysis was stopped at various times by the addition of PMSF.

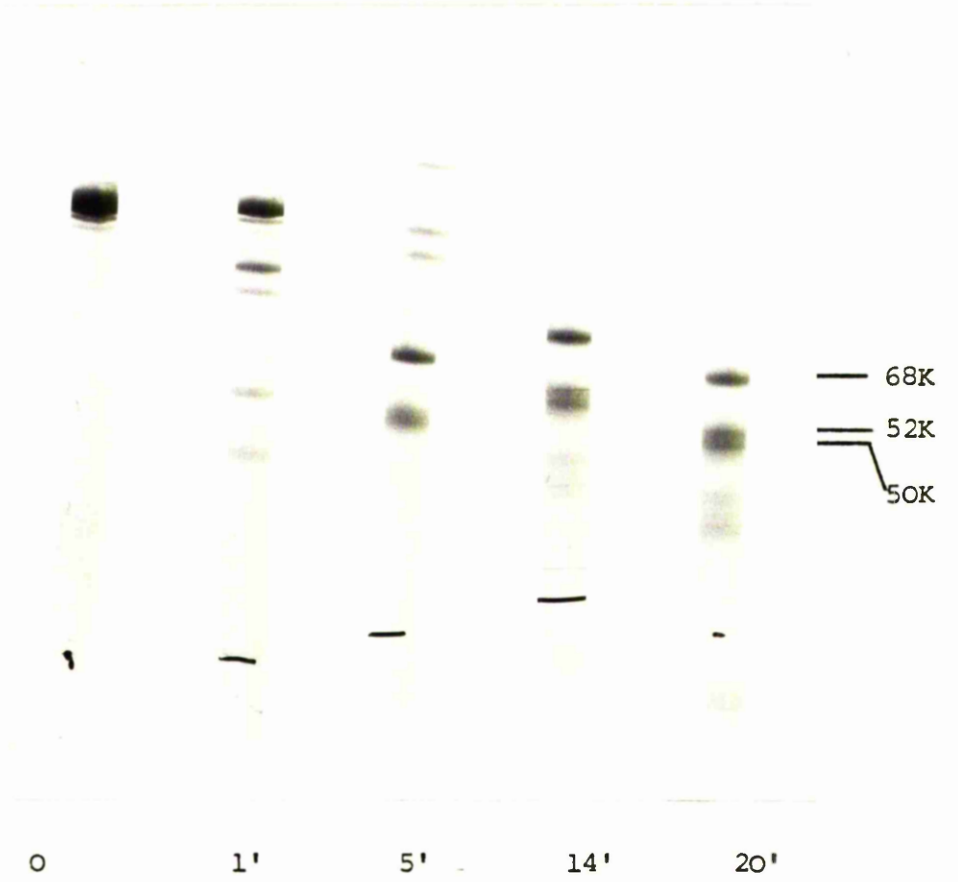


Figure 51 SDS polyacrylamide gel electrophoresis of arom complex proteolysed with subtilisin at 25°C. Proteolysis was stopped by the addition of PMSF. In this experiment two bands of molecular weights 52000 and 50000 were resolved.

After the proteolysis 92% of the original shikimate dehydrogenase activity remained as did 79% of the original dehydroquinase activity. Substrate labelling of the dehydroquinase active-site reduced the residual dehydroquinase activity to 10% of the starting value but did not affect the shikimate dehydrogenase activity.

SDS polyacrylamide gel electrophoresis was used to separate the polypeptides produced by proteolysis (Fig. 52). The major band on the gels was the fragment of molecular weight 69000. Also present was a trace amount of unproteolysed arom complex and fragments of molecular weights 109000, 96000, 54000 and 51000. In order to find which polypeptides contained the dehydroquinase active-site SDS polyacrylamide gels were sliced into 1mm discs, solubilised with hydrogen peroxide and the tritium present in each determined by liquid scintillation counting (Fig. 53).

Dehydroquinase active-sites were located on the 69000 molecular weight polypeptide and also in the bands of 54000 and 51000 molecular weight.

5.1 Proteolysis of the Arom Enzyme Complex with Chymotrypsin

Proteolysis of the arom complex with chymotrypsin was carried out in the same buffer as proteolysis with trypsin. Proteolysis was ended either by dilution of samples into assay cuvettes or by the addition of a freshly prepared solution of lima-bean trypsin/chymotrypsin inhibitor in the same buffer to a concentration ten times that of the protease by weight.

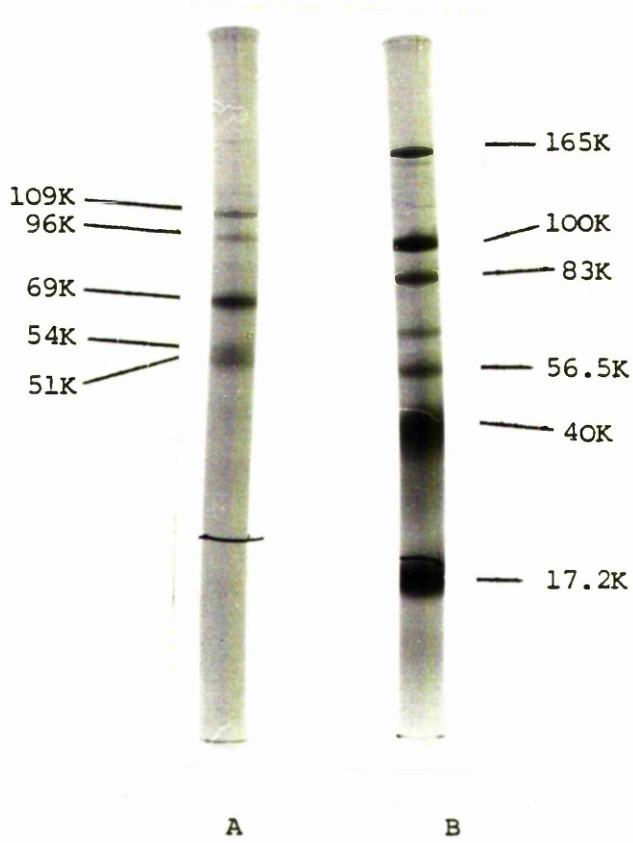


Figure 52 SDS polyacrylamide gel electrophoresis of A, arom complex proteolysed at 0°C with 3µg/ml subtilisin for 30 min prior to labelling of the dehydroquinase active-sites with dehydroquinate/sodium boro-[^{3}H]-hydride and B, standard proteins for molecular weight determinations.

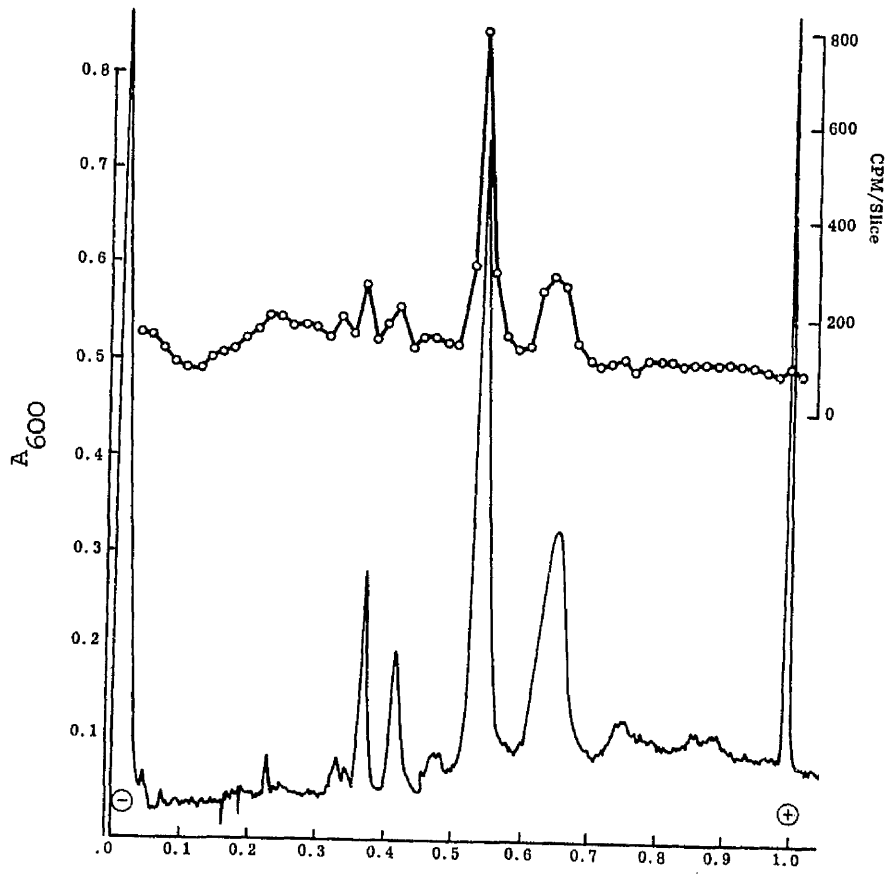


Figure 53 Location of the dehydroquinase active-sites of arom complex proteolysed with subtilisin at 0°C. The lower trace is a densitometer scan of a coommassie blue stained SDS polyacrylamide gel. A similar gel was sliced into 1mm discs and the radioactivity present in each determined, this is shown in the upper trace.

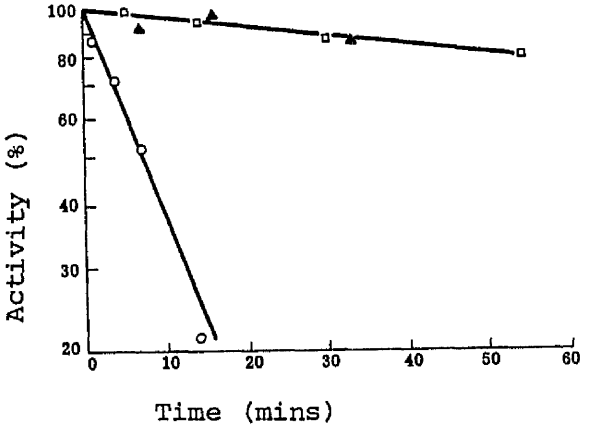


Figure 54 The effect of proteolysis of the arom complex with 1.25µg/ml chymotrypsin on the shikimate kinase (O), shikimate dehydrogenase (▲) and dehydroquinase (□) activities. Assays were performed by diluting samples into the appropriate assay cuvette at various times after the addition of protease.

5.2 The Stability of the Enzyme Activities of the Arom Complex to Proteolysis with Chymotrypsin

The stabilities of the shikimate kinase, shikimate dehydrogenase and dehydroquinase activities of the arom complex toward proteolysis with chymotrypsin were tested. Twenty microlitres of a stock solution of arom complex was diluted to 0.2ml with 50mM sodium phosphate pH7.5, proteolysis was then performed with 1.25µg/ml chymotrypsin at 25°C, at times after the start of the incubation samples were taken and assayed by dilution into assay cuvettes. The shikimate kinase activity was more susceptible to proteolysis than either the shikimate dehydrogenase or dehydroquinase activities (Fig. 54). The shikimate kinase activity was lost more rapidly when higher concentrations of chymotrypsin were used in the incubation.

5.3 SDS Polyacrylamide Gel Electrophoresis of Arom Complex Proteolysed with Chymotrypsin

SDS polyacrylamide gel electrophoresis of samples of arom complex proteolysed for 2, 5, 15 and 30 min with 1.45µg/ml chymotrypsin was performed. The proteolysis was stopped using lima-bean trypsin/chymotrypsin inhibitor and a control incubation performed in the absence of protease.

With increasing times of proteolysis the arom polypeptide was lost (Fig. 55) and polypeptides of molecular weights 124000, 107000, 69000 and 51000 formed. After 30 min incubation the 69000 and 51000 polypeptides were the major products.

6. Proteolysis of the Arom Complex with Elastase and with Papain

The time-courses of proteolysis of the arom complex with elastase

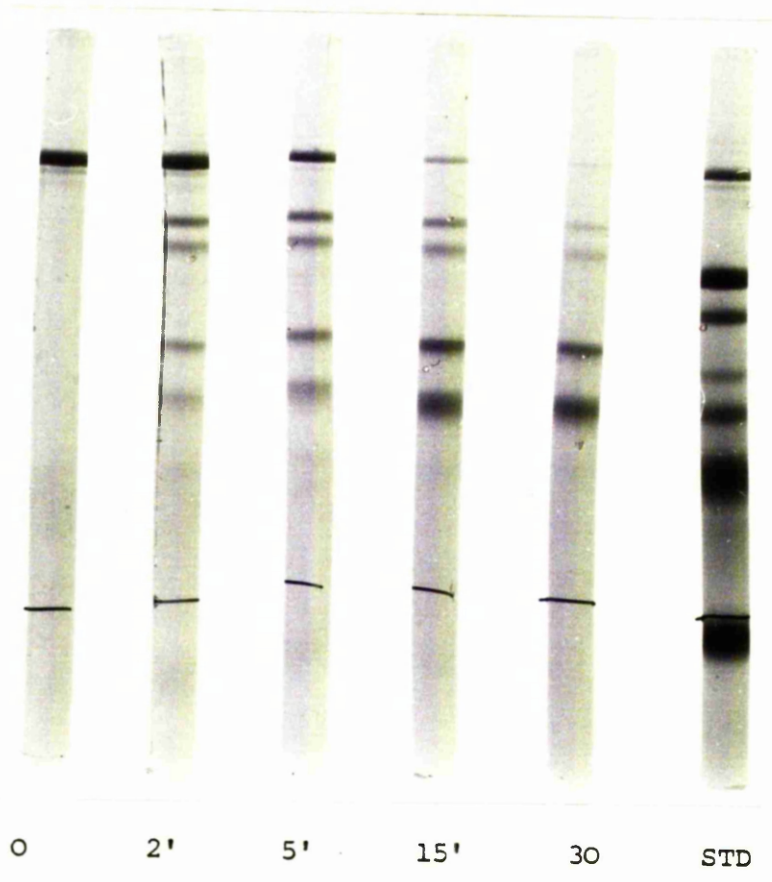


Figure 55 SDS polyacrylamide gel electrophoresis of a time-course of proteolysis of the arom complex with 1.45µg/ml chymotrypsin.

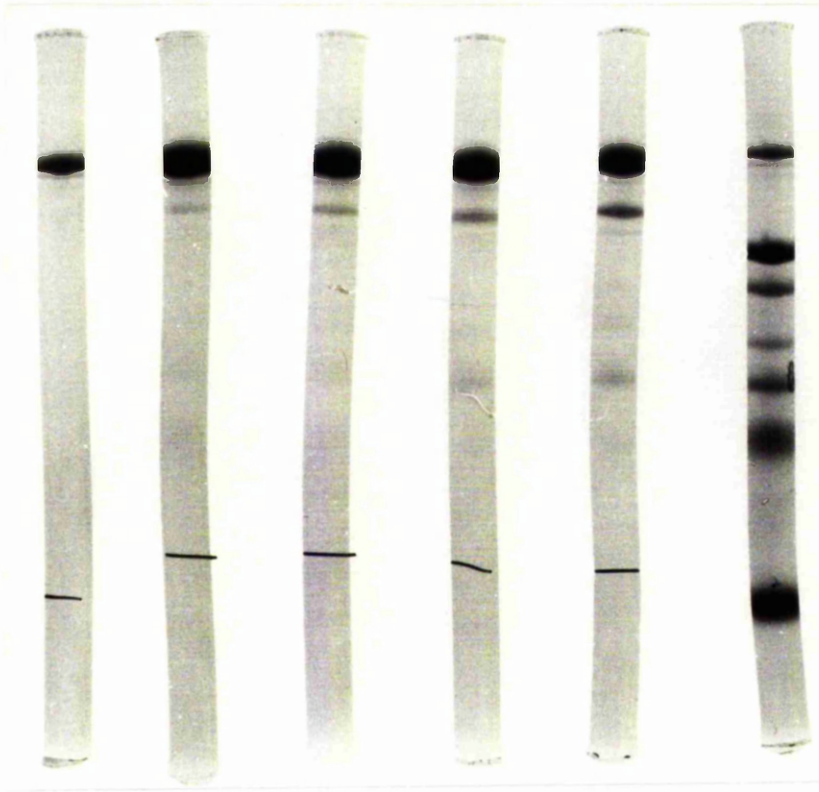


Figure 56 SDS polyacrylamide gel electrophoresis of a time-course of proteolysis of the arom complex with 5µg/ml elastase.

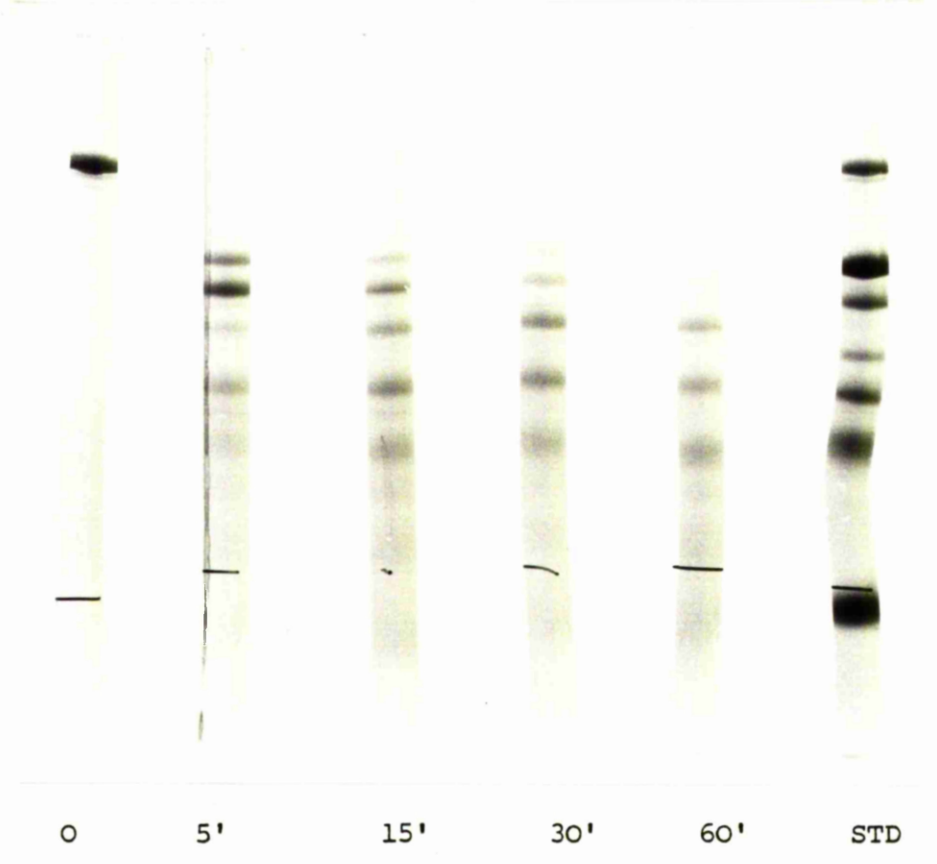


Figure 57 Time-course of proteolysis of the arom complex with 5µg/ml papain monitored by SDS polyacrylamide gel electrophoresis.

Table 12 The effect on the activities of the *arom* complex of cross-linking in the presence and absence of the substrates of the shikimate dehydrogenase component of the complex

	Shikimate dehydrogenase activity (%)	Dehydroquinase activity (%)	Shikimate kinase activity (%)
Control	100	100	100
Cross-linked *arom* complex	163	96	55
Arom complex cross-linked in the presence of NADP and shikimate	148	33	71

and with papain were followed by SDS polyacrylamide gel electrophoresis. Arom complex was proteolysed with either 5µg/ml elastase or papain in 50mM sodium phosphate pH7.5, 0.4mM DTT, 1mM EDTA. At intervals after the addition of protease samples were removed and boiled to stop further proteolysis.

The arom complex was degraded only slightly by elastase in 1 h under these conditions (Fig. 56). The molecular weights of the polypeptides formed after this time being 123000 and 52000.

In contrast to the low amount of proteolysis caused by elastase, papain rapidly attacked the arom complex (Fig. 57). After 5 min no native arom polypeptide remained, proteins of molecular weights 98000, 84000, 68000 and 50000 having been formed. After 1 h further proteolysis had occurred and polypeptides with molecular weights 68000, 50000 and 36000 were observed.

7. Chemical Studies of the Arom Enzyme Complex

7.1 The Effect of Cross-Linking on the Activities of the Arom Complex

The effect of cross-linking the arom complex with dimethyl suberimidate on the activities of the shikimate dehydrogenase, shikimate kinase and dehydroquinase components of the complex was studied. Cross-linking was performed with 20mM dimethyl suberimidate at 25°C for 1 h in 0.1M triethanolamine/HCl pH8.0, 0.4mM DTT in both the presence and absence of 5mM shikimate and NADP. A control incubation was performed to which no cross-linking reagent was added. After 1 h the cross-linking reaction was terminated by adding ammonium bicarbonate to a final concentration of 0.1M. The three enzyme activities were assayed.

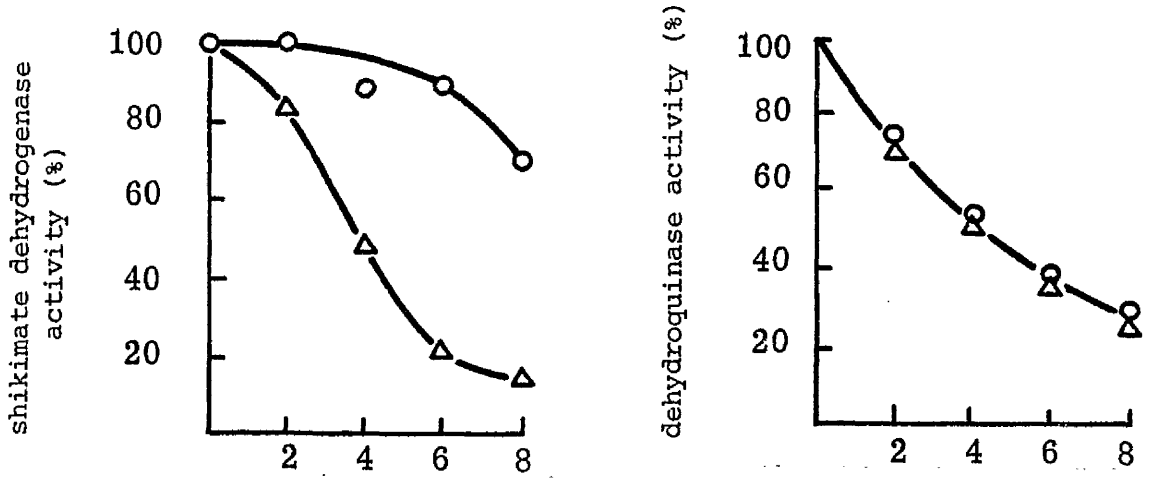


Figure 58 The effect of reductive alkylation of the arom complex with formaldehyde/sodium borohydride on the shikimate dehydrogenase (left) and dehydroquinase (right) activities in the absence (Δ) and in the presence of 5mM shikimate (O).

Cross-linking both in the presence and absence of NADP and shikimate affected the activities of the arom complex (Table 12).

7.2 Reductive Alkylation of the Arom Complex with Formaldehyde plus Sodium Borohydride

The effect of reductive alkylation of the arom complex by formaldehyde and sodium borohydride on the shikimate dehydrogenase and dehydroquinase enzyme activities was examined. To arom complex in 0.4ml 50mM sodium phosphate pH7.5 was added successive aliquots of 2μmoles of formaldehyde followed by 0.66μmoles of sodium borohydride. The enzyme activity was measured between each addition of formaldehyde and sodium borohydride. The effect of including 5mM shikimate in the reaction solution was investigated. Shikimate dehydrogenase and dehydroquinase activities were inactivated by reductive alkylation, the shikimate dehydrogenase activity being protected by shikimate (Fig. 58).

CHAPTER 4

DISCUSSION

1. Purification of the arom enzyme complex

The arom enzyme complex of N. crassa was purified to homogeneity by Lumsden and Coggins (1977). Despite the reasonable yield of the purification procedure (around 25%) less than 1mg of enzyme was obtained from 20g of lyophilised N. crassa mycelia. When the procedure was scaled-up to allow the extraction of 100g of mycelia in each preparation it became less reliable. In particular the final step of the purification, chromatography on cellulose phosphate, did not always yield pure arom complex. The enzyme used in this study of the arom complex was purified by a method based on that of Lumsden and Coggins (1977) but using chromatography on blue-dextran sepharose rather than on cellulose phosphate as the final purification step. The arom complex bound tightly to blue-dextran sepharose allowing the contaminant proteins still present at this stage to be eluted prior to elution of the purified arom enzyme complex with salt. This procedure readily allows the isolation of around 3mg of pure arom complex from 100g of lyophilised N. crassa mycelia.

The arom complex of N. crassa contains both a dehydrogenase and a kinase enzyme activity. Many kinases and pyridine-nucleotide-linked dehydrogenases contain homologous regions of three-dimensional structure, the dinucleotide binding fold, and it was suggested that chromatography on cibacron blue columns may be a general method for recognising and purifying proteins containing this super-secondary structural unit (Thompson et al, 1975). Attempts to elute the arom complex from blue-dextran sepharose with substrates of the shikimate kinase or shikimate dehydrogenase components of the complex were unsuccessful. Chromatography on blue-dextran sepharose is probably not a form of affinity chromatography of the arom complex.

Chromatography on blue-dextran sepharose has proved invaluable not only in the purification of the arom complex but also for obtaining preparations of anthranilate synthetase and chorismate synthetase which are free of arom complex. These preparations are necessary for use as coupling enzymes in the fluorimetric assay of the last enzyme of the arom complex, EPSP synthetase. Neither chorismate synthetase nor anthranilate synthetase bind to blue-dextran sepharose. The material which washed through the blue-dextran sepharose column during purifications of the arom complex is a useful source of both of these enzymes which may subseuqently be separated by chromatography on cellulose phosphate (Coia and Coggins, unpublished results).

2. The effect of proteolysis on the enzyme activities of the arom complex and the molecular weights of the polypeptides formed

A study of the arom complex by means of limited proteolysis was undertaken. It has been observed in investigations of multifunctional enzymes that one or more of the enzyme activities may be unaffected by proteolysis (see Chapter 1, section 7 for examples). In keeping with the domain theory of protein structure these protease-resistant regions are thought to be independent structural domains of the multifunctional enzymes and our goal was to attempt to isolate at least one such domain of the arom complex.

Limited proteolysis of the arom complex was achieved in a number of ways. The proteolytic enzymes trypsin, subtilisin, chymotrypsin, papain and elastase were used. In the case of proteolysis with trypsin the effect of performing the proteolysis in the presence of a number of substrates of the arom complex was investigated. When a number of proteases are shown to produce similar proteolytic fragments of a

Figure 59 The order of the genes of the arom gene cluster (Rhines et al, 1969) and the corresponding order of the enzymes on the arom polypeptide. The genes are numbered according to Rhines et al, (1969) and the enzymes according to their order on the metabolic chain.

3'	Arom 2	Arom 4	Arom 5	Arom 9	Arom 1	5'
NH_2	E1	E5	E4	E2	E3	CO_2H

E1 = dehydroquinate synthetase
E2 = dehydroquinase
E3 = shikimate dehydrogenase
E4 = shikimate kinase
E5 = EPSP synthetase

large enzyme the assignment of these fragments as domains of protein structure, linked to the remainder of the protein by exposed loops of polypeptide, can be more confidently made than on the basis of results with a single protease. The order of the genes coding for the five activities of the arom complex has been deduced from genetic studies (Rhines et al, 1969). From a knowledge of the order of the genes the order of the enzyme activities on the arom polypeptide can be derived (Fig. 59). The dehydroquinate synthetase activity is located at the N-terminus of the polypeptide and the EPSP synthetase is adjacent to it. The shikimate kinase is the central enzyme of the complex with, toward the C-terminus of the arom polypeptide, the dehydroquinase and, finally, the shikimate dehydrogenase. As will be demonstrated below the results of our limited proteolysis studies of the arom complex are consistent with this order of the enzymes on the arom polypeptide.

The shikimate kinase activity of the arom complex was susceptible to inactivation caused by proteolysis with trypsin (Fig. 15), subtilisin (Fig. 46) and chymotrypsin (Fig. 54). In each case the shikimate dehydrogenase and dehydroquinase activities were relatively resistant to proteolysis whilst, on proteolysis with trypsin or subtilisin, the dehydroquinate synthetase activity was lost more rapidly than the shikimate dehydrogenase or dehydroquinase activities but less quickly than the shikimate kinase activity (Figs. 40 and 46). These results are most easily explained by a model of the arom complex in which the shikimate dehydrogenase and dehydroquinase activities are contained in protease-resistant domains, the dehydroquinate synthetase activity is catalysed by a somewhat less stable domain and sequences of the arom polypeptide necessary for the shikimate kinase activity are exposed to proteolytic attack on the surface of the complex.

SDS polyacrylamide gel electrophoresis of arom complex proteolysed for increasing times with trypsin showed the rapid loss of the 165000 molecular weight arom polypeptide and the appearance of two major bands of molecular weights 110000 and 69000 (Fig. 16). The shikimate kinase activity is rapidly lost on proteolysis with trypsin (Fig. 15) and is located in the centre of the arom polypeptide (Fig. 59). Trypsin proteolysis of the arom complex thus appears to involve cleavage of the arom polypeptide in the region responsible for the shikimate kinase activity resulting in the production of two large proteolytic fragments, one derived from the region of the arom complex toward the N-terminal end of the polypeptide and the other from the region of the arom complex toward the C-terminal end of the arom polypeptide.

A number of substrates of the arom complex were shown to decrease the rate of loss of the shikimate kinase activity caused by proteolysis with trypsin (Table 5). Substrates of not only the shikimate kinase component of the arom complex but also of the shikimate dehydrogenase and dehydroquinate synthetase components (Table 5) slowed the rate of inactivation of the shikimate kinase activity. Vitto and Gaertner (1978) also found the shikimate kinase activity to be inactivated by proteolysis and suggested that protection by DAHP was caused by conformational changes of the arom polypeptide although no physical-chemical evidence was presented to support this hypothesis.

SDS polyacrylamide gel electrophoresis was used to investigate the molecular weights of the proteolytic fragments of the arom complex produced by proteolysis with trypsin in the presence of shikimate phosphate, DAHP, NADP plus shikimate and also ADP plus shikimate. These combinations of substrates protected the shikimate kinase activity against proteolysis to different extents (Table 5). The molecular weights of the main fragments observed on SDS polyacrylamide gels are

Table 13 The molecular weights of the major polypeptides formed by trypsin proteolysis of the arom complex in the presence of various substrates of the arom complex

Substrates present during proteolysis	Molecular weight ($\times 10^{-3}$) of major polypeptides formed
None	110
	69
Shikimate phosphate	110
	70
Shikimate + NADP	110
	68.5
DAHP	110
	68
Shikimate + ADP	125
	69

The molecular weight of the intact arom polypeptide is 165000 (Lumsden and Coggins, 1977)

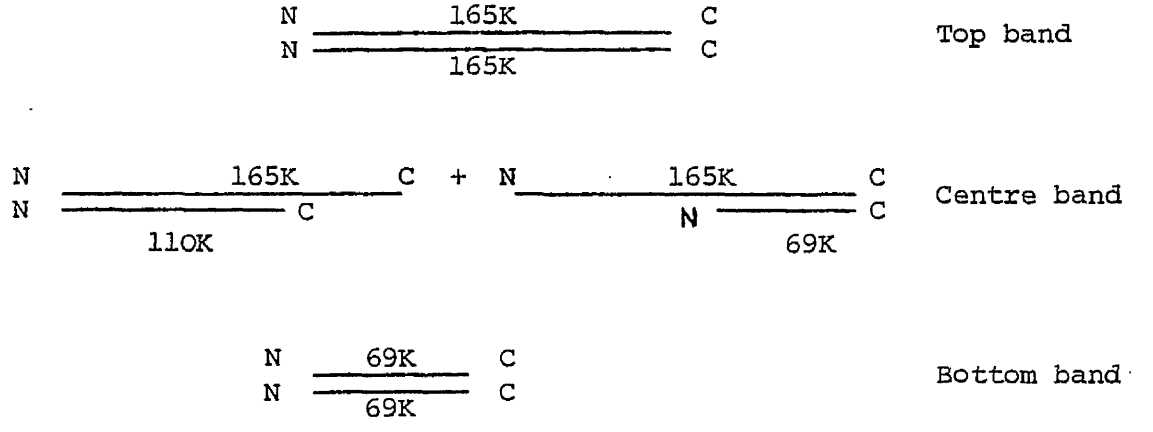
given in Table 13. In all cases a polypeptide of molecular weight around 69000 was produced and, except in the case of proteolysis in the presence of ADP plus shikimate, this was accompanied by a band at about 110000 molecular weight. After prolonged proteolysis a 64000 molecular weight polypeptide was formed and since the formation of this band correlated with the loss of the 69000 molecular weight band it seemed likely that this band was derived from the 69000 molecular weight band. The peptide bonds cleaved by trypsin in the absence of substrates and in the presence of shikimate phosphate, DAHP, and shikimate plus NADP appear to be in similar positions on the polypeptide chain. The presence of ADP plus shikimate, the most effective combination of ligands for protecting the shikimate kinase activity against proteolysis by trypsin, produced a slightly different pattern of fragments. In particular a polypeptide of molecular weight 125000 was formed and the 110000 band was present in low amounts (Fig. 21).

To interpret these patterns of proteolytic fragmentation it was necessary to try and establish which activities were associated with which fragments. Since the shikimate dehydrogenase and dehydroquinase activities of the arom complex were essentially unaffected by limited proteolysis it was decided to concentrate on establishing which of the proteolytic fragments contained these two activities.

3. Evidence that the 69000 molecular weight fragment is a bifunctional domain of the arom complex containing the shikimate dehydrogenase and dehydroquinase activities

Simple polyacrylamide gel electrophoresis of proteolysed arom complex in the absence of denaturants failed to resolve components of the proteolysed material, nor were any low-molecular-weight shikimate dehydrogenase-active fragments separated by active-band

Figure 60 The proposed structure of the components of proteolysed arom complex separated by polyacrylamide gel electrophoresis in the presence of 1M urea (Fig. 27)



ultracentrifugation. The inclusion of 1M urea in polyacrylamide gels perturbed the structure of the proteolysed complex sufficiently to allow the separation of different partially-aggregated forms of the protein (Fig. 27).

The mechanism of the dehydroquinase reaction involves a Schiff's base intermediate (Butler et al, 1974; Lumsden and Coggins, MS in preparation) providing a convenient method of specifically labelling the active-site by reduction of this intermediate with sodium boro-[^{3}H]-hydride. Dehydroquinase-containing proteolytic fragments of the arom complex could be located after polyacrylamide gel electrophoresis by the detection of tritium in gel slices. Shikimate dehydrogenase-containing fragments could be located after polyacrylamide gel electrophoresis by staining for this activity (Chapter 2, section 4).

Second dimension SDS polyacrylamide gel electrophoresis of proteolysed arom complex separated by polyacrylamide gel electrophoresis in the presence of 1M urea, which resolved three bands of protein coincident with three bands of shikimate dehydrogenase activity, showed that (a) the most slowly migrating band contained 165000 molecular weight polypeptides, (b) the centre band contained polypeptides of molecular weights 165000, 110000 and 69000 and (c) the fastest moving activity band contained only 69000 molecular weight material (Fig. 27). These were interpreted as being (a) unproteolysed arom complex, which probably migrates as a dimer under these conditions, (b) a mixture of two species of proteolysed arom complex one containing one unproteolysed polypeptide and one 110000 polypeptide and the other containing one unproteolysed arom polypeptide and one 69000 polypeptide and (c) a 69000 molecular weight shikimate dehydrogenase fragment derived from arom complex in which both polypeptides had been cleaved by trypsin (Fig. 60).

When proteolysed arom complex in which the dehydroquinase active-sites were labelled as above was analysed by SDS polyacrylamide gel electrophoresis the dehydroquinase active-sites were located on the 165000 and 69000 molecular weight polypeptides (Fig. 28). Proteolytic fragments of the arom complex of molecular weight 69000 stained for shikimate dehydrogenase activity and carried the dehydroquinase active-site. Either two polypeptides, each of molecular weight 69000, were produced, each catalysing one of the reactions, or the 69000 fragment was a bifunctional domain of the arom complex. The shikimate dehydrogenase and dehydroquinase activities of the arom complex are adjacent on the arom polypeptide, on the C-terminal section of the protein, as can be deduced from the order of the genes (Fig. 59). It seemed probable from the size of the fragment that it was bifunctional.

Since the arom complex is a dimer of identical polypeptide chains (Lumsden and Coggins, 1977 and 1978) each polypeptide contains the dehydroquinase active-site and can be labelled with dehydroquinate/sodium boro-[^{3}H]-hydride (Lumsden and Coggins MS. in preparation). Calculation of the specific radioactivity (cpm/mole) of bands separated by SDS polyacrylamide gel electrophoresis showed that the specific radioactivity of the arom band was very similar to that of the 69000 band (Table 6). This strongly suggested that most, if not all, the 69000 polypeptides carried the dehydroquinase active-site and were, therefore, bifunctional domains of the arom complex. SDS polyacrylamide gel electrophoresis in a Tris-glycine system failed to separate the 69000 band into more than one component.

Further evidence for the homogeneity of the 69000 fragment was obtained by polyacrylamide gel electrophoresis in 8M urea. Under these conditions all the polypeptides of trypsin-proteolysed arom complex are resolved. The fastest moving band, after removal of urea and renaturation, was found to contain shikimate dehydrogenase

activity (Fig. 29). Second dimension SDS polyacrylamide gel electrophoresis showed that only polypeptides of molecular weight 69000 were present in this band. This material must correspond to that already shown to contain the shikimate dehydrogenase and dehydroquinase activities after proteolysis.

SDS polyacrylamide gel electrophoresis of arom complex proteolysed with chymotrypsin (Fig. 56), papain (Fig. 57) and subtilisin (Fig. 48) showed that a fragment of molecular weight 69000 was produced. Proteolysis with subtilisin was stopped either by boiling or by inactivation of the protease with PMSF prior to SDS polyacrylamide gel electrophoresis. When proteolysis was stopped by the addition of PMSF (Fig. 48) a polypeptide of 69000 molecular weight was formed. When proteolysis was stopped by boiling the higher molecular weight polypeptides were lost more rapidly than when PMSF was used to stop the reaction (Figs. 47 and 48). This is probably caused by regions of the arom complex denaturing more rapidly than subtilisin and being rapidly degraded.

Thus the 69000 molecular weight proteolytic fragment of the arom complex produced by treatment of the complex with subtilisin or with trypsin under several different sets of conditions, and probably also with chymotrypsin and papain, is a structural domain of the arom complex. This domain contains the shikimate dehydrogenase and dehydroquinase activities of the complex and must be, on the basis of genetic evidence, derived from the C-terminal region of the polypeptide (Fig. 59).

4. Comparison of the properties of the shikimate dehydrogenase and dehydroquinase activities of native and of proteolysed arom complex

Comparison of the properties of the activities of multifunctional

proteins with those of catalytically-active domains may give some insight into how incorporation into an organised system modifies the enzyme activities.

The properties of the shikimate dehydrogenase and dehydroquinase components of proteolysed arom complex were compared with those of native arom complex. Little change in the activation energy for either reaction, or in the K_m for shikimate or dehydroquinate was found (Tables 7 and 8). The K_m for NADP of the shikimate dehydrogenase activity decreased from 41μM to 26μM after proteolysis indicating a slight change in protein structure enhancing somewhat the binding of NADP. The response of proteolysed arom complex to the concentration of sodium chloride (Fig. 37), urea (Fig. 38) or guanidine hydrochloride (Fig. 39) in assays was very similar to that of native arom complex. The activity changes observed are probably caused by structural changes within the shikimate dehydrogenase domain of the arom complex. The similar properties of proteolysed and of native arom complex indicate that proteolysis does not much change the structure of the shikimate dehydrogenase-dehydroquinase domain of the arom complex.

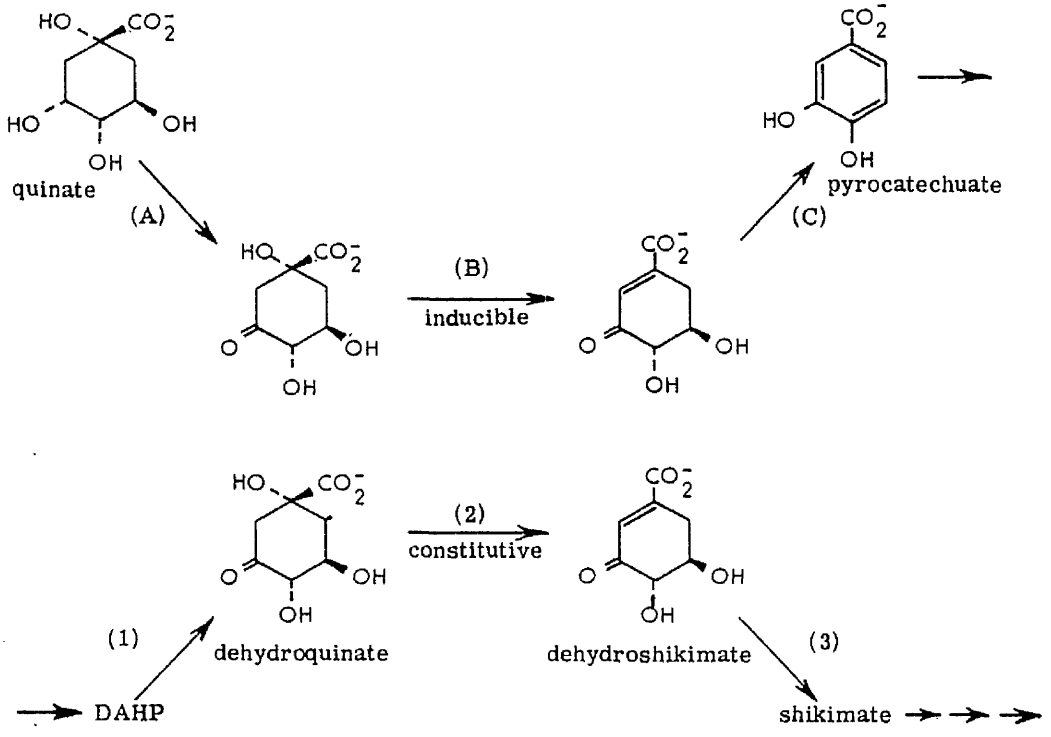
Domains of protein structure may be not only structural and functional units of the protein in its final folded state but also separate units of folding of the polypeptide chain. That is, although the primary structure of a protein dictates its final tertiary structure, for large multi-domain proteins it may be that each domain folds reasonably independently. Following polyacrylamide gel electrophoresis in the presence of 8M urea shikimate dehydrogenase activity could be recovered associated with the 69000 molecular weight trypsin-generated fragment of the arom complex (Fig. 29). The shikimate dehydrogenase enzyme of the arom complex is located at the

C-terminus of the polypeptide and is, therefore, the last region of the protein to be synthesised. The recovery of activity of the isolated domain of the arom complex means that the folding of this domain is not dependent on the previous folding of the remainder of the arom polypeptide. The 69000 domain may represent an independent folding unit of the arom complex.

Shikimate dehydrogenase activity was not found associated with 165000 molecular weight arom polypeptides after polyacrylamide gel electrophoresis in 8M urea (Fig. 29). The folding pathway of unproteolysed arom complex leading to the recovery of shikimate dehydrogenase activity may be different from, and is slower than, that for isolated domains of the arom complex. Shikimate dehydrogenase activity was recovered from a proteolytic fragment of molecular weight around 122000 (Fig. 45), the difference in folding pathways between arom complex and isolated domains of the arom complex may be due to interaction between the N-terminal and C-terminal regions of the arom polypeptide.

Something of a parallel with these results on the refolding of domains of the arom complex may be found in work on aspartokinase II - homoserine dehydrogenase II of E. coli (Dautry-Varsat and Garel, 1978). The kinetics of the refolding of both the native bifunctional enzyme and a proteolytic fragment containing homoserine dehydrogenase activity were studied. The refolding of the proteolytic fragment was much faster than that of the corresponding region of the intact enzyme suggesting that, although the homoserine dehydrogenase domain of the enzyme could refold when isolated as a proteolytic fragment, the refolding of the same region of the intact enzyme involved interaction with the remainder of the protein.

Figure 61 The reactions catalysed by the two dehydroquinases of N. crassa. The upper part of the scheme shows the reaction of the inducible catabolic pathway and the lower section the constitutive biosynthetic pathway. The enzymes labelled (β), (2), (3) are the first three activities of the arom complex.



5. Comparison of the 69000 molecular weight bifunctional domain with other multifunctional enzymes

Multifunctional enzymes are considered to be constructed from domains and each domain is usually thought of as catalysing a single reaction. A bifunctional domain has been isolated from the trifunctional enzyme methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase by limited proteolysis with trypsin (Tan and MacKenzie, 1977). As described in Chapter I, Section 7, this bifunctional fragment contained the dehydrogenase and cyclohydrolase activities of the trifunctional enzyme. The dehydrogenase and cyclohydrolase activities were later shown to be functionally integrated, preferentially channelling the product of the dehydrogenase reaction through the cyclohydrolase (Cohen and MacKenzie, 1978). The close structural relationship between the dehydrogenase and cyclohydrolase reactions may be a consequence of a functional requirement for efficient substrate-channelling between the two active-sites. The shikimate dehydrogenase and dehydroquinase activities of the *arom* complex are contained in a bifunctional domain. Substrate-channelling between the shikimate dehydrogenase and dehydroquinase active-sites has been proposed as one function of the *arom* complex (Giles *et al*, 1967); channelling between these two active-sites would prevent competition for substrates between the dehydroquinase activity of the *arom* complex and a second, catabolic, dehydroquinase present in *N. crassa* (Fig. 61). The close structural relationship of the shikimate dehydrogenase and dehydroquinase components of the *arom* complex may reflect a close functional relationship.

The question of the organisation of the shikimate dehydrogenase and dehydroquinase enzymes of the shikimate pathway in plants has been addressed by a number of workers (Boudet, 1971; Boudet and Lecussan,

1974; Boudet et al, 1975; Koshiba, 1977; Polley, 1978). A variety of techniques was used to attempt to separate the activities, mainly in crude extracts. In all cases studied, the shikimate dehydrogenase and dehydroquinase activities appeared to be associated as a complex of native molecular weight around 55000. In one species, Zea mays, a second dehydroquinase free of shikimate dehydrogenase was detected (Boudet et al, 1975). It was suggested that the shikimate dehydrogenase-dehydroquinase complex might exist to channel substrates through the shikimate pathway without competition from other metabolic pathways, an analogous role to that proposed for the arom complex of N. crassa (Giles et al, 1967) (Fig. 61). Polley (1978) purified shikimate dehydrogenase and dehydroquinase from Physcomitrella patens. His preparation contained only one size of polypeptide chain, molecular weight 48000, and sucrose density gradient centrifugation gave a molecular weight of 49500 for both activities. The shikimate dehydrogenase and dehydroquinase activities are probably catalysed by a bifunctional polypeptide.

In several species of plants shikimate dehydrogenase and dehydroquinase may occur as a bifunctional enzyme similar to the bifunctional domain of the arom complex of N. crassa. If, as has been suggested, one function of both the plant enzymes and the N. crassa arom complex is that of substrate channelling it would be of interest to discover if the requirement for this function had led to the evolution of similar protein structures.

6. Evidence that the 110000 fragment is formed from the N-terminal region of the arom polypeptide

Proteolysis of the arom complex with trypsin under a variety of conditions produced a polypeptide of around 110000 molecular weight

(Table 13). The order of the active-sites on the arom polypeptide (Fig. 59), deduced from the genetic studies of Rhines et al (1969) which established the order of the genes in the gene cluster, indicates that dehydroquinate synthetase is at the N-terminus of the arom polypeptide and EPSP synthetase is adjacent to it. A number of results suggest that the 110000 fragment may be formed from this N-terminal region of the arom polypeptide.

DAHP, the substrate of the dehydroquinate synthetase reaction, protects the dehydroquinate synthetase activity against inactivation (Fig. 40) and also stabilises the 110000 molecular weight proteolytic fragment (compare Figs. 16 and 41).

After polyacrylamide gel electrophoresis in the presence of 1M urea to separate different forms of proteolysed arom complex the EPSP synthetase activity was located in a region of the gel containing polypeptides of molecular weights 110000, 69000 and 51000 (Table 10 and Fig. 44). As shown above the 69000 polypeptide contains the shikimate dehydrogenase and dehydroquinase activities. The EPSP synthetase activity must therefore be associated with either or both of the polypeptides of 110000 and 51000 molecular weight.

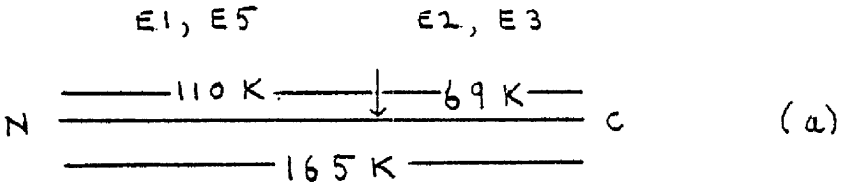
After polyacrylamide gel electrophoresis in the presence of 8M urea shikimate dehydrogenase could be recovered and detected in the gel. When a sample of arom complex containing predominantly 165000, 110000 and 69000 molecular weight polypeptides was run in this system no shikimate dehydrogenase activity was associated with the 110000 molecular weight polypeptide (Fig. 29). This could mean that either the 110000 polypeptide does not contain the shikimate dehydrogenase active-site, and is not derived from the C-terminal end of the arom complex, or that it did not refold into an active conformation. In

another experiment shikimate dehydrogenase activity was detected in a polypeptide of molecular weight 122000 (Fig. 45). For this reason it would be expected that the 110000 fragment, if it did catalyse the shikimate dehydrogenase reaction, would have refolded in such an experiment.

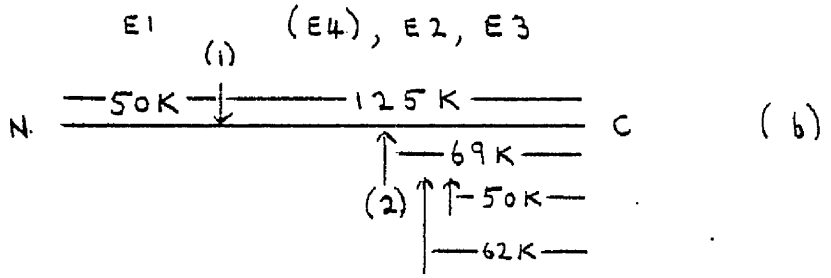
When proteolysed arom complex in which the dehydroquinase active-sites had been labelled with dehydroquinate/sodium boro-[^{3}H]-hydride was run in SDS polyacrylamide gels no radioactivity was found in the 110000 band (Fig. 28). Therefore the 110000 band did not contain the dehydroquinase active-site. Since the 69000 polypeptide contained both the shikimate dehydrogenase and dehydroquinase active-sites but the 110000 molecular weight polypeptide did not contain either the dehydroquinase or the shikimate dehydrogenase active-site it seems very likely that the two proteolytic fragments must be formed from different regions of the arom polypeptide. It follows that the 110000 molecular weight proteolytic fragment must be derived from the N-terminal region of the arom polypeptide and should therefore contain the dehydroquinate synthetase and EPSP synthetase active-sites.

The rate of loss of the activities of the arom complex could be correlated with the rate of loss from SDS polyacrylamide gels of the polypeptides supposed to catalyse each reaction (Fig. 42). The loss of the shikimate kinase activity could be reasonably attributed to the loss of the arom band. The stability of the shikimate dehydrogenase and dehydroquinase activities was reflected by the stability of the 69000 molecular weight fragment. The rate of loss of the dehydroquinate synthetase activity was much slower than the rate of loss of the 165000 arom band and was also slower than the loss of the 110000 band. The discrepancy between the rate of loss of the dehydroquinate synthetase

Figure 62 The proposed locations of the protease-sensitive regions of the arom polypeptide. Diagram (a) illustrates the predominant proteolysis proposed when trypsin is used in the absence of substrates or in the presence of DAHP, shikimate phosphate or NADP plus shikimate. Diagram (b) illustrates the predominant proteolysis suggested to occur with trypsin in the presence of ADP plus shikimate. Diagram (b) also shows the possible locations of the proteolytic events responsible for forming the 62000 and 50000 molecular weight shikimate dehydrogenase-active polypeptides seen in Fig. 45.



(a)



(b)

E1 = DHQ synthetase
E2 = dehydroquinase
E3 = shikimate dehydrogenase
E4 = shikimate kinase
E5 = EPSP synthetase

activity and that of the 110000 band is explicable if proteolysis increases the activity of the dehydroquinate synthetase activity approximately two-fold. From these results it thus seems likely that the 110000 fragment is derived from the N-terminal region of the arom polypeptide and contains both the dehydroquinate synthetase and EPSP synthetase active-sites.

7. The location of protease-sensitive regions of the arom polypeptide

The shikimate kinase activity of the arom complex is rapidly lost on proteolysis with trypsin (Fig. 15), subtilisin (Fig. 46) or chymotrypsin (Fig. 53) suggesting the shikimate kinase contains a protease-sensitive sequence on the surface of the arom complex necessary for activity. Proteolysis with trypsin in the presence of a number of combinations of protecting ligands produces polypeptides of molecular weights 110000 and 69000 (Table 13). Trypsin proteolysis of the arom complex probably involves cleavage of the polypeptide in the region of the chain responsible for the shikimate kinase activity producing a 110000 molecular weight fragment from N-terminal side of the cut and a 69000 fragment from the C-terminal end of the chain (Fig. 62). This is consistent with the retention of shikimate dehydrogenase and dehydroquinase activities by the 69000 molecular weight proteolytic fragment, with the rapid loss of shikimate kinase activity during proteolysis (Fig. 15) and with the observations indicating that the 110000 fragment comes from the N-terminal region of the arom polypeptide. The discrepancy between the molecular weight of the unproteolysed arom polypeptide (165000) and the sum of the molecular weights of the proteolytic fragments (179000) may indicate that the intact arom polypeptide runs anomalously fast on SDS polyacrylamide gels.

In one trypsin proteolysis experiment a considerable amount of shikimate kinase activity remained despite the absence of any 165000 arom band. Dehydroquinate synthetase activity was also present in the absence of any 110000 molecular weight polypeptides. Polyacrylamide gel electrophoresis in the presence of 8M urea separated the polypeptides present in this sample of proteolysed arom complex (Fig. 45). Shikimate dehydrogenase activity could be recovered in the bands containing the 122000, 69000 and 62000 polypeptides but was not associated with the major polypeptide of molecular weight 50000. Proteolysis in this case probably involved first the cleavage of the dehydroquinate synthetase from the N-terminus of the arom polypeptide as the proteolytic fragment of molecular weight 50000. The remainder of the polypeptide, molecular weight 122000, would contain the shikimate kinase activity. From this region, on further proteolysis, would be formed the 69000 molecular weight fragment (Fig. 62). The proteolytic fragments produced in this experiment were of the same molecular weight as those produced by trypsin proteolysis of the arom complex in the presence of ADP and shikimate, the pathway of proteolysis in both cases may be the same, as may the pathway of proteolysis with elastase.

The production of a polypeptide of molecular weight 50000 possibly catalysing the dehydroquinate synthetase reaction suggests that the bifunctional 110000 molecular weight fragment might contain a 50000 molecular weight domain catalysing the dehydroquinate synthetase activity.

Proteolysis of the arom complex with chymotrypsin (Fig. 55) and with subtilisin (Fig. 48) in which polypeptides of molecular weights around 125000, 110000, 69000 and 50000 are formed may involve the simultaneous occurrence of the two mechanisms of proteolysis illustrated in Figure 62.

The proposed locations of the protease-sensitive regions of the arom complex predict certain relationships between the polypeptides produced on proteolysis. Peptide mapping could be used to test these proposals, for example, the peptide maps of the 69000 and 110000 fragments should contain no common peptides and when combined should yield a map similar to that of unproteolysed arom complex.

8. Evidence for non-covalent interactions between different regions of the arom complex

After limited proteolysis of a multifunctional enzyme any large fragments formed may either be released free of the remainder of the protein, the system then becoming analogous to a mixture of separable enzymes, or they may remain non-covalently bound to the remainder of the protein, the system then being more akin to a multienzyme complex. To test these possibilities trypsin proteolysed arom complex was cross-linked with dimethyl suberimidate and also subjected to active-band ultracentrifugation during which the shikimate dehydrogenase activity was monitored. Cross-linking with dimethyl suberimidate produced predominantly two species with molecular weights very similar to the arom monomer and dimer on SDS polyacrylamide gels indicating that the polypeptides of trypsin proteolysed arom complex remain associated (Fig. 30). Active-band centrifugation confirmed this result, no slowly sedimenting shikimate dehydrogenase activity being observed. Since the polypeptides produced after proteolysis of the arom complex remain associated then non-covalent interactions must occur between different regions of the arom complex. Such interactions could allow conformational changes initiated in one domain of the complex to be transmitted to other domains.

9. Comparison of the results of limited proteolysis of the *arom* complex with earlier artifactual quaternary structures

Early purifications of the *arom* complex were dogged by the problem of proteolysis of the complex during the preparation. In two cases (Gaertner, 1972; Gaertner and Cole, 1976) the 'subunit' structure of the *arom* complex was studied by SDS polyacrylamide gel electrophoresis. In the latter study (Gaertner and Cole, 1976) the complex was shown to be progressively degraded on storage. Some differences in the apparent molecular weights of the fragments due to the different SDS polyacrylamide gel systems and marker proteins used might be expected between this work and the work of Gaertner (1972) and Gaertner and Cole (1976). For instance intact *arom* complex in the hands of Gaertner and Cole (1976 and 1977) has an apparent molecular weight of only 150000 as opposed to 165000 (Lumsden and Coggins, 1977). The molecular weights observed by Gaertner (1972) and Gaertner and Cole (1976) are remarkably similar to those of fragments produced by limited proteolysis of the *arom* complex (Table 14). This correspondence may be caused by chance, due to the number of fragments present, or may indicate that endogenous *N. crassa* proteases cleave the *arom* polypeptide in similar positions to the proteases studied here. The latter interpretation is consistent with there being a limited number of exposed bonds on the surface of the *arom* complex predominantly between domains of the complex.

10. Chemical modification studies of the *arom* complex

After cross-linking of the *arom* complex with dimethyl suberimidate at least three of the enzymes of the complex, shikimate dehydrogenase, dehydroquinase and shikimate kinase, were active (Table 12). The shikimate dehydrogenase activity of the *arom* complex increased after cross-linking (Lumsden and Coggins, 1977 and Table 12), the shikimate kinase activity decreased (Table 12) and the dehydroquinase activity

was relatively unaffected (Table 12). Performing the cross-linking reaction in the presence of NADP and shikimate caused inactivation of the dehydroquinase activity while the shikimate kinase activity was protected against inactivation (Table 12).

The dehydroquinase component of the arom complex, in common with the E. coli dehydroquinase (Butler et al, 1974), can be inactivated by reductive alkylation of a Schiff's base intermediate (Lumsden and Coggins, MS in preparation) and therefore contains an essential lysine residue at the active-site. Shikimate dehydrogenase also contains an essential lysine residue since it can be inactivated by both methyl acetimidate and formaldehyde/sodium borohydride (Lumsden and Coggins, MS in preparation).

The dehydroquinase component of the arom complex is inactivated by formaldehyde/sodium borohydride (Fig. 59). Protection experiments with shikimate showed that shikimate protected the shikimate dehydrogenase against inactivation by formaldehyde/sodium borohydride but did not protect the dehydroquinase (Fig. 59). The shikimate dehydrogenase and dehydroquinase active-sites are, therefore, spatially distinct and the lysine residues modified by formaldehyde/sodium borohydride at the two active-sites are different. Methyl acetimidate does not inactivate the dehydroquinase component of the arom complex (Lumsden and Coggins, MS in preparation) whereas formaldehyde/sodium borohydride does. The lysine residue modified by formaldehyde/sodium borohydride is probably that involved in Schiff's base formation with dehydroquinate. The environment of this residue precludes reaction with methyl acetimidate perhaps due to the charge of this reagent. The bifunctional fragment of the arom complex therefore contains two distinct active-sites and the interesting question is whether the product of the dehydroquinase reaction is channelled into the shikimate dehydrogenase active-site.

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