STUDIES ON THE AROM MULTIENZYME COMPLEX OF NEUROSPORA CRASSA.

A thesis presented for the degree of

Master of Science

By

Janet M. Jamieson.

June

1977

Department of Biochemistry

University of Glasgow.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements

I would like to thank all the members of the Biochemistry Department, University of Glasgow, who have helped me during the course of this work, especially my supervisor, Dr. J.R. Coggins for his advice and encouragement.

Professor R.M.S. Smellie and Professor A.R. Williamson for making available the facilities of the Biochemistry Department.

Dr. J. Lumsden for helpful discussion and advice.

Finally I am grateful to Miss Sheila McLaren for typing this thesis.

Abbreviations

All abbreviations used in this thesis are as recommended by the Biochemical Journal "Policy of the Journal and Instructions to Authors" (1976); with the exception of the following:-

DAHP	3-deoxy-D-arabinoheptulosonic acid-7-phosphate.
DHQ	3-dehydroquinate
DTNB	5,5' dithiobis (2-nitrobenzoic acid)
Tris	Tris (hydroxymethyl) aminomethane.

CONTENTS

PAGE

List of Figures.

List of Tables PAGE

Table

SUMMARY

Practical conditions were developed for growing Neurospora crassa on a scale from which substantial amounts of arom multienzyme complex could be extracted. 100 ml seed cultures were inoculated with 1 x 10^8 spores and grown for 20 to 24 hours at 30°C. Large scale cultures (three litres) were inoculated with two 100 ml seed cultures and grown at 30°C for between 16 and 24 hours. Arom multienzyme complex was extracted from large scale cultures.

Attempts to purify the arom multienzyme complex following published methods were unsuccessful. The amount of protein and shikimate dehydrogenase activity in crude extracts was found to vary greatly during different purifications. Furthermore large amounts of enzyme activity were lost during the initial purification steps, especially after ammonium sulphate precipitation.

It was realised that crude extracts of N.crassa contained very active proteases and it was necessary to use extra precautions to protect the arom complex from proteolysis. A new, more rapid, purification was used in which the addition of extra protease inhibitors was shown to give a more reproducible purification in terms of total amount of shikimate dehydrogenase extracted. After cellulose phosphate chromatography, it was shown that a substantial purification of arom was achieved: simple polyacrylamide gel electrophoresis showed a single band of protein.

Sodium dodecyl sulphate polyacrylamide gel electrop...

of purified arom gave a single band of protein and the molecular weight was estimated as being 175,000. In some preparations fainter bands of lower mol. wt. were also observed.

The effect of sulphydryl reagents, namely iodoacetate iodoacetamide and p-mercuribenzoate upon the activity of shikimate dehydrogenase was studied. Iodoacetate and iodacetamide did not initially significantly affect enzyme activity. However on inhibition of the enzyme with p-mercuribenzoate total inhibition of activity occurred after twenty minutes. Shikimate was shown to protect against inhibition of dehydrogenase by p-mercuribenzoate, suggesting that the inhibitor may be reacting as a substrate analogue of shikimate.

CHAPTER I

INTRODUCTION

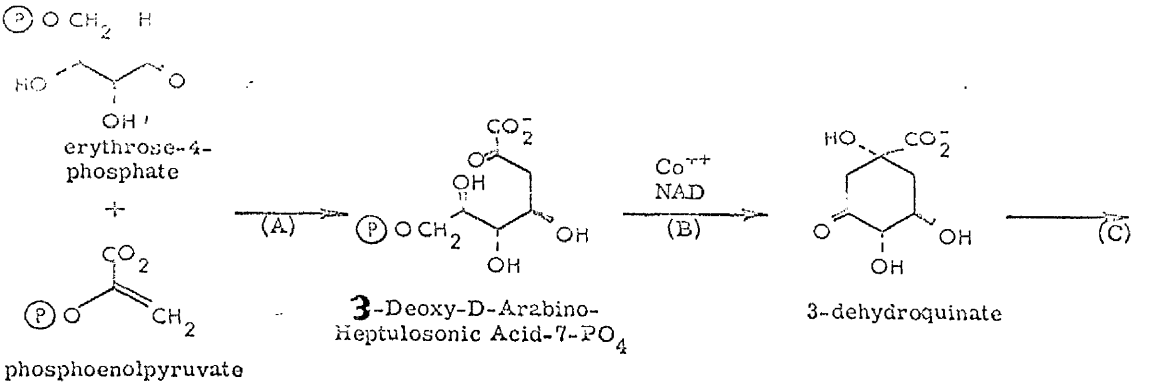
1.1. General outline of aromatic amino acid biosynthesis in micro-organisms.

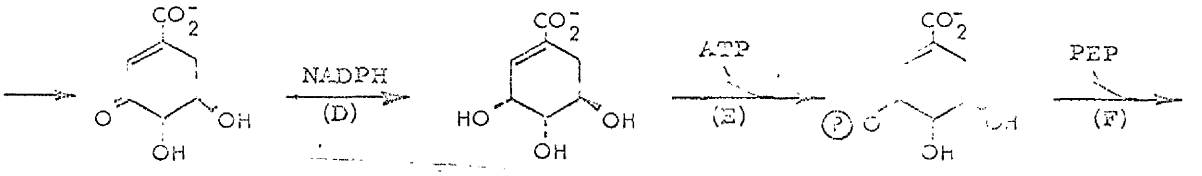
The aromatic amino acids, L-phenylalanine, L-tyrosine and L-tryptophan can be synthesised from carbohydrates by a number of micro-organisms and higher plants, but not by animals. The path of aromatic biosynthesis is one of the longest and most complicated of the pathways involved in amino acid synthesis. More than ten enzymes are required to form the aromatic amino acids and some of the intermediates also serve for the formation of other aromatic substances, such as 4-aminobenzoic acid, ubiquinone and vitamin K. In higher plants, the aromatic amino acids can be converted into a number of secondary plant metabolites such as lignins, alkaloids and flavonoids.

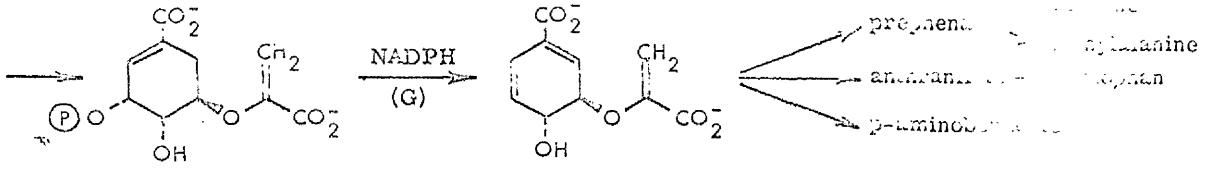
The enzymes and intermediates of the pathway, which is commonly known as the shikimate pathway (Fig. 1.1.), have been well established in micro-organisms primarily due to the work of B. D. Davis and colleagues in the 1950's (Davis, 1955). Using aromatic auxotrophs of Escherichia coli and Aerobacter aerogenes, Davis showed that shikimic acid could replace the growth requirement in certain mutants for the aromatic amino acids and para-aminobenzoic acid; other mutant strains accumulated shikimic acid in the culture medium. This established shikimic acid as an intermediate of the pathway (Davis, 1951).

In 1963, Gibson and Jackman, using an auxotrophic mutant of A. aerogenes, established the branchpoint intermediate, chorismic acid, which is formed from 5-enolpyruvylshikimic acid-3-phosphate (EPSP) and showed that it could be enzymically converted to prephenic acid and anthranilic acid (Gibson & Jackman, 1963; Gibson, M & Gibson F, 1964).

Elucidation of the initial steps in the sequence resulting







(A) 3-Dehydroquinate synthetase

(B) 3-Dehydroquinase

(C) 3-Dehydroshikimate reductase

(E) Shikimate kinase

(F) 5-Enolpyruvl shikimate-3-phosphate sythetase

(G) Chorismate synthetase

from the use of isotopic tracer studies and experiments at the enzyme level.

Sprinson, using E.coli mutants converted variously labelled [14C] derivatives of D-glucose into shikimic acid and analysed the distribution of the label by chemical degradation. His results indicated that shikimic acid was synthesised from a three carbon fragment derived from glycolysis and a four carbon sugar from the pentose phosphate pathway. Studies using cell free extracts of E.coli 83 - 24 led to the identification of these two precursors, namely phosphoenolpyruvate and erythrose - 4 - phosphate, together with the probable condensation product, 3-deoxy-D-arabinoheptulosonic acid - 7 - phosphate, (DAHP), which was assumed to cyclise directly to 3-dehydroquinate, (DHQ). (Sprinson, 1960). This assumption was supported by using chemically synthesised DAHP which was converted quantitatively to DHQ by bacterial extracts (Sprinson et al., 1963).

Further evidence for the pathway was obtained by the isolation and characterization of enzymes from bacterial mutants, which were capable of catalysing individual steps in the pathway.

Purification of similar enzymes and intermediates in plants together with the use of isotopic tracer experiments showed that the route of biosynthesis of aromatic amino acids was similar if not identical with the Shikimate Pathway in microorganisms (Yoshida, 1969).

1.1. The DAHP multienzyme complex of Neurospora crassa.

In the fungus Neurospora crassa the five enzymes catalysing the conversion of DAHP to EPSP in the common part of the shikimate pathway have been found by Burgoyne et al (1969) to occur in a multienzyme aggregate. Furthermore the genes controlling the

synthesis of this multienzyme aggregate, which was named the aroM complex, were found to be clustered together on the [illegible] genome.

The gene cluster was first detected in N.crassa by Gross and Pittard (1964) when the discovery of a mutant (G162) which was capable of catalysing the conversion of DAHP to EPSP. Genetic studies showed that at least four of the five enzymes mapped in a tight cluster on Linkage Group II of the N.crassa genome. Until this time, gene clustering had been considered a characteristic of procaryotes, while in eucaryotes functionally related genes were thought to be dispersed throughout the genome. Thus, this finding of Gross and Pittard (1964), presented a new concept at the time. In later years however, other examples of gene clustering emerged, in particular, in [illegible] and Aspergillus nidulans a cluster of three genes involved in histidine biosynthesis was found (Fink, 1966). This cluster possessed a number of properties which were characteristic of bacterial operons and it prompted N.H. Giles and colleagues to begin similar investigations of the aroM cluster of N.crassa.

Giles et al established the organisation of the genes [illegible] the aroM cluster by genetic and biochemical analyses of [illegible] polyaromatic auxotrophs (Giles et al, 1967). Furthermore they showed that the gene cluster possessed properties which were characteristic of bacterial operons; such as biochemical pleiotropy, polarity effects and the asymmetrical genetic localisation of completely non-complementing mutants.

The major classes of polyaromatic mutants were detected. The first category, designated single gene mutants, were each defective in a single enzymatic activity but possessed high levels of the other four activities and had a mol. wt. comparable

to wild type. (In these early experiments the mol. wt. of wild type was estimated as being 200,000; later work by Burgoyne and Giles (1969) indicated it to be larger). On the [illegible] these results, single gene mutants were interpreted [illegible] from multiple mutations.

A class of single gene mutants, defective in dehydroquinase, which catalyses the conversion of 3-dehyroquinate to 3-dehydrochikimate was not detected in these early experiments. However it was later realised that 3-dehydroquinate could induce another enzyme, an inducible dehydroquinase, which could mask the effect of a mutant which lacked the dehydroquinase of the aroM complex (Giles et al, 1968).

The second class of mutants established by Giles et al (1968) were designated pleiotropic or polarity mutants, and characterised by their inability to complement with mutants in two or more of the single gene groups. These mutants lacked more than one of the aroM activities and possessed greatly reduced levels of the other activities.

Some of these mutants were attributed to nonsense or chain terminating mutations which gave rise to the production of either incomplete polypeptide chains or partial enzyme aggregates. In support of this suggestion it was found that some of these mutations could be suppressed by known suppressors of nonsense mutations in E. coli. Furthermore biochemical characterisation of the aroM activities in the suppressed mutants indicated that all five activities occurred in an aggregate with a mol. wt. indistinguishable from wild type and the levels of each activity were approximately 70% of wild type. In contrast, single gene mutants were not suppressed by nonsense suppressors. These results were important since they indicated, particularly in the case of suppression of totally non-complementing mutants, that transcription occurred in a polarised fashion via a single polycistronic mRNA

(Case & Giles, 1968).

Mol. wt. determinations by zone gradient centrifugation, of the aggregates produced by pleiotropic mutants indicated that these mutants produced partial aggregates ranging in mol. wt. from 60,000 to 80,000. These results could also be explained by the mutations giving rise to incomplete polypeptide chains. Furthermore some information was obtained from these studies that there is a correlation between aggregate size and the genetic mapping position of the mutant, since more distal mutants produce larger aggregates (Case & Giles, 19?.)

In pleiotropic mutants, the levels of the arom activities, which were present, were all markedly reduced. Perhaps the reason for this effect, is the need for the formation of a wild type aggregate for the full expression of enzyme activity.

Giles noted in these studies, that certain arom 1 mutants (lacking shikimate dehydrogenase), exhibited a pleiotropic character in that they formed arom aggregates of about half the size of wild type (mol. wt. of 400,000) and had reduced levels of all five activities. When a heterocaryon was produced from these arom 1 mutants an aggregate with a mol. wt. of 200,000, equivalent to wild type, was produced.

Furthermore this strain possessed increased levels of all five arom activities when compared with the parental types. It was suggested that such a mutant could result from a missense mutation which affects aggregation of the complex, perhaps one of the reasons for the failure of nonsense suppressors to suppress all the pleiotropic mutants of the arom complex.

Other reasons, however, such as mutations giving rise to frameshifts, or mutations resulting from different nonsense codons, could also be the reason for this effect.

1.3. Comparison between the aro enzymes of fungi and bacteria.

The multienzyme aggregates have also been shown to occur in other species of fungi and also in the yeast, Saccharomyces cerevisiae. Sucrose density gradient centrifugation of partially purified extracts from six other species of fungi, i.e. Rhizopus stolonifer, Phycomyces nitens, Absidia glauca, Aspergillus nidulans, Neurospora sagovus and Ustilago maydis, indicate that all five aro enzymes sediment together. Furthermore, the sedimentation coefficients for these enzymes are very similar in the six species studied, and also are comparable to that of the arom multienzyme complex of N.crassa (Ahmed & Giles, 1969).

Genetic evidence regarding the organisation of the arom genes is not yet available for these organisms and so it is not yet known if these aggregates are encoded by a gene cluster. However genetic studies using the yeast S.cerevisiae, which also possesses an arom multienzyme complex have shown the presence of an aro gene cluster (A. de Leeuw, unpublished results). Thus it seems probable that in the above mentioned species of fungi an arom gene cluster will be found.

In contrast comparative studies in a number of species of bacteria, namely, Escherichia coli, Salmonella typhimurium, Aerobacter aerogenes, Bacillus subtilis, Pseudomonas aeruginosa and Streptomyces coelicolor have indicated, on the basis of sedimentation of crude extracts in sucrose density gradients that the five arom enzymes are not physically associated in an enzyme aggregate (Boylyn & Giles, 1969). Also in those species of bacteria for which genetic information about the organisation of the arom genes is available, namely in E.coli (Pittard & Wallace, 1966) and in S.typhimurium (Nishioka et al, 1967), they are widely dispersed throughout the bacterial genome. Thus the

clustering of the *aroM* genes in *N.crassa*, *S.cerevisiae* and other fungi does not appear to be observed in bacteria. This indicates that the *arom* gene cluster fulfills a function in fungi which is not necessary in bacteria.

The clustering of *aroM* genes does not appear to function in genetic regulation since the enzymes are synthesised constitutively and no evidence has been obtained for the presence of any regulatory genes or for an operator gene in the cluster. Berlyn and Giles (1969) suggest, that, in at least *N.crassa* and *S.cerevisiae*, that the gene cluster is a necessary prerequisite for the formation of the *arom* multienzyme complex. In bacteria, where the genes are separated in the genome, a multienzyme aggregate is not found.

Giles *et al* (1968) suggest that one function of the *arom* multienzyme complex, at least in *N.crassa*, is to segregate two metabolic pathways, each of which have one substrate in common, namely 3-dehydroquinate (Fig. 1.2.). They showed that two dehydroquinases were present in *N.crassa*. Each had quite different physical properties as based on ammonium sulphate precipitation and thermolability studies. Zone centrifugation indicated that the mol. wts. of these enzymes were different. One of the dehydroquinases was induced in the presence of either quinate or dehydroquinate, was stable to heat treatment, with an estimated mol. wt. of 154,000. The other dehydroquinase was produced constitutively, was labile to heat treatment and had a mol wt. of c.200,000. This constitutive dehydroquinase sedimented with the *arom* enzymes in sucrose density gradients. (Later work by Lumsden *et al* (1969) indicated the *arom* as having a mol wt. of 1[illegible],000) Rines *et al* (1969) detected a mutant, *cr-1*, by ultraviol. radiation and complementation studies, which lacked the inducible dehydroquinase. This mutant was used to detect single gene [illegible] in constitutive dehydroquinase and gave final proof that [illegible]

[illegible] and constitutive dehydroquinases

of [illegible].

(A) Quinate dehydrogenase

(B) 3-Dehydroquinase

(C) 3-Dehydroshikimate dehydrase

(1) 3-Dehydroquinate synthetase

(2) 3-Dehydroquinase

(3) 3-Dehydroshikimate reductase

encoded by the arom region.

The inducible dehydroquinase functions in an inducible quinic acid catabolic pathway. The arom enzyme aggregate is thought to [illegible] dehydroquinate flow to the synthetic pathway, preventing its utilisation by catabolic enzymes. In support of this theory, Giles et al. (1968) have shown that in "normal" wild type N.crassa the catabolic enzymes are not induced, thus indicating that the complex does efficiently segregate both pathways. However in the six species of fungi, mentioned above, all of which possess arom multienzyme aggregates, only in one, A.nidulans and U.reydia, was the catabolic shunt [illegible] quinase activity detected. In U.reydia the catabolic [illegible] was produced constitutively, i.e. even in the absence of quinate or dehydroquinate (Ahmed & Giles, 1969). This indicates that further studies are necessary before generalisations between species of fungi regarding the function of the arom complex can be made.

In bacteria, however, which do not possess arom [illegible] is no evidence for an inducible (or constitutive) [illegible] distinct from the synthetic dehydroquinase and characteristic of a quinic acid catabolic pathway. This indicates that since catabolic enzymes are not found in bacterial species, no selective advantage would be conferred upon them, by possessing [illegible] multienzyme complex (Berlyn & Giles, 1969).

1.4. Physical properties of the arom multienzyme complex of N.crassa.

Most of the work performed on the arom system in [illegible] concerned with establishing the arrangement of the [illegible] the arom gene cluster. Until 1969, studies on the arom complex themselves had been confined to crude extracts of wild type and various mutants of N.crassa. Sucrose density gradient centrifugation

[illegible] that the arom enzymes were a multienzyme complex; a [illegible] 230,000 was obtained for wild type enzyme in these [illegible] (Giles et al., 1967).

[illegible] by highly purified protein would have to be [illegible] before a detailed examination of the physical properties of the arom multienzyme could be undertaken. Giles and colleagues have described purification procedures for the arom complex from two different strains of N.crassa; strain 87, which lacked chorismate synthetase activity (Burgoyne et al, 1969), but had an increased level of arom activities with respect to wild type (Burgoyne et al, 1969), and wild type strain 74A (Jacobson et al, 1972). Both strains produced arom complexes with similar properties.

The purified aggregate, containing all five activities, had a mol. wt. of 230,000 as estimated by sucrose density gradient centrifugation. It could be easily dissociated into a half molecular weight fragment of 115,000, by treatment with dissociating agents, such as urea. This agreed with earlier genetic experiments by Case & Giles (1971) where allelic complementation between arom mutants (lacking shikimate dehydrogenase) [illegible] produced wild type aggregates, although these mutants formed arom aggregates of half the normal molecular weight. It was concluded from these results that the arom consisted of identical or very similar half complexes, held together by non-covalent bonds.

Using harsher conditions to promote dissociation, such as alkaline pH, storage in low ionic strength buffers or exposure to high temperatures, Jacobson et al, 1972, obtained [illegible] with molecular weights ranging from 20,000 to 80,000.

In another laboratory, Chertner, (1972), using an alternative method of purification isolated an arom aggregate from [illegible]

[illegible], which had different physical properties. The mol. wt. was estimated by sedimentation equilibrium analysis to be [illegible]. It could be dissociated by sodium dodecyl sulphate [illegible] gel electrophoresis [illegible] fully shared [illegible] with subunit molecular weights totalling [illegible].

However there was clearly a need for further study of the aroM complex. In particular information is required about which subunit possesses which activity and also about the number of copies of each subunit present in the intact aggregate. This prompted us to begin a detailed study of the aroM complex with a view to determining its quaternary structure and eventually studying the properties of the individual enzymes. Obviously this was an ambitious goal and the scope of the work undertaken for this thesis was more limited.

1.5. The scope of the thesis

1. To find a method for growing N.crassa on a convenient scale.
2. To develop a method for isolating and purifying the aroM multienzyme complex.
3. To begin a physical and chemical characterisation of the complex.
4. To make a preliminary study of the shikimate dehydrogenase activity.

CHAPTER 3

DEVELOPMENT OF GROWTH CONDITIONS FOR

M. GRASSA

2.1. [illegible] solutions.

(i) [illegible] solution (Davis & de Serres, 1970).

5 g biotin in 100 ml of 50% (V/V) of ethanol was prepared and stored at 4°C. Biotin solutions were prepared like this every [illegible] months.

(ii) [illegible] element solution (Davis & de Serres, 1970).

The following compounds were added to 95 ml of distilled water:-

5.00g of citric acid. $4H_2O$.

5.00g of $ZnSO_4.7H_2O$.

1.00g of $Fe(NH_4)_2(SO_4)_2.6H_2O$.

0.25g of $CuSO_4.5H_2O$.

0.05g of $MnSO_4.4H_2O$.

0.05G of H_3BO_3.

0.05G of $NaMoO_4.2H_2O$.

The final volume was adjusted to 100 ml and the solution stored at room temperature. 2 ml of chloroform was added as a preservative.

(iii) Vogels medium - N. (Davis & de Serres, 1970).

This salt solution is stored as a 50-strength solution. The following compounds were added to 75 ml of distilled water:-

150g of NA_3 citrate. $5H_2O$.

250g of KH_2PO_4.

100g of NH_4NO_3.

10g of $MgSO_4.7H_2O$.

5g of $CaCl_2.2H_2O$.

5ml of Biotin solution

5ml of trace element solution

$CaCl_2.2H_2O$ should be predissolved in 20 ml of distilled water

2.2. Stock culture.

Freeze-dried stock culture of N.crassa strain 74-OR23-1A was obtained from the Fungal Genetics Stock Centre, Humboldt State University Foundation, Arcata, California.

2.3. Growth of Spores and Preparation of Silica Gel Stocks.

Freeze-dried stock culture was suspended in sterile distilled water and used to inoculate slants. The slants consisted of 250 ml conical flasks containing 50 ml of medium made up of 2% agar, 2% sucrose and containing single strength Vogels medium -N. Stocks of spores were preserved on silica gel according to the method of Davis and de Serres, (1970). These silica gel stocks were used to inoculate slants for the growth of spores for seeding small (100 ml) shaking cultures. The secondary slants were grown in batches of 20 for 5 days at 30° and then stored at 4°. The spores were viable for several months. Spores were harvested from each slant by adding 30 ml of sterile distilled water and gently shaking. The resulting spore suspension was withdrawn with a sterile pipette and aliquots immediately used for the inoculation of the shaking cultures. It was assumed that a solution containing 10^9 spores/ml had an A_{600} of 1.0.

2.4. Small Scale Shaking Cultures.

250 ml Erlenmeyer flasks, containing 100 ml of liquid growth medium were inoculated with spores and then shaken on an orbital shaking table at a speed of 180 r.p.m. at 30°C. The liquid culture medium contained 2% sucrose, single strength Vogels medium -N, and was autoclaved before use.

2.5. Large Scale Cultures.

These were grown on 3 L of liquid growth medium (see 2.4. above) in 10 L flat bottomed, boiling flasks at 30°C. The cultures were stirred magnetically at 100 r.p.m. with a 1.5 inch bar magnet and aerated at a rate of 400 ml/min. Growth was assisted by

[illegible] with two 100 ml shake cultures. Mycelia were harvested by vacuum filtration using Whatman No. 1 paper; the mycelial pads were washed with distilled water and freeze-dried overnight. The dried pads were stored at 20°C.

2.6. Measurement of Growth Curves for the small shake cultures.

The object of this study was to find conditions which gave a high yield of cells but avoided the cultures reaching stationary phase. The effect of varying the time of growth and the size of inoculum was studied.

Spores were grown as described in 2.3. above. Aliquots containing from 0.1 x 10^8 to 20 x 10^8 spores were added to a series of small shake cultures. (see 2.4. above). After set time intervals the flasks were harvested by vacuum-filtration and the pH of the growth medium measured. The cells were dried to constant weight in a 100° oven in preweighed glass vials. The results of two separate experiments are shown in Figs. 2.1. and 2.2.

Near logarithmic growth was obtained for up to 30 hours with inocula in the range 0.1 x 10^8 spores to 1 x 10^9 spores (Fig 2.1.). Not surprisingly higher yields of cells were obtained with the larger inocula. When the inocula were increased further (Fig 2.2.) the cells began to reach stationary phase before 30 hours. The aim of these experiments was to achieve a simple practical means of growing H.crassa which could be used to inoculate larger scale, (3L) cultures, from which the arom multienzyme complex could be extracted. Although more detailed experiments would be required to determine the best growth conditions, these results show clearly that a good yield of rapidly growing cells suitable for inoculating large culture could be obtained by using a spore inoculum of 1 x 10^8 spores and continuing for 20 to 30 hours.

In later experiments a growth time of 20 to 24 hours with an inoculum of 1 x 10^8 spores was used for growing all seed cultures.

Fig 2.1.

EFFECT OF SIZE OF INOCULA ON GROWTH IN SMALL SHAKE CULTURES.

100 ml shaking cultures were set up as described in section 2.4. and inoculated with spores in the range of $0{\cdot}1 \times 10^8$ to 1×10^8. Flasks were harvested at set time intervals by vacuum filtration. The cells were dried to constant weight in a 100° oven in preweighed glass vials.

—○— $0{\cdot}1 \times 10^8$ spores

—×— $0{\cdot}5 \times 10^8$ spores

—△— $1{\cdot}0 \times 10^8$ spores

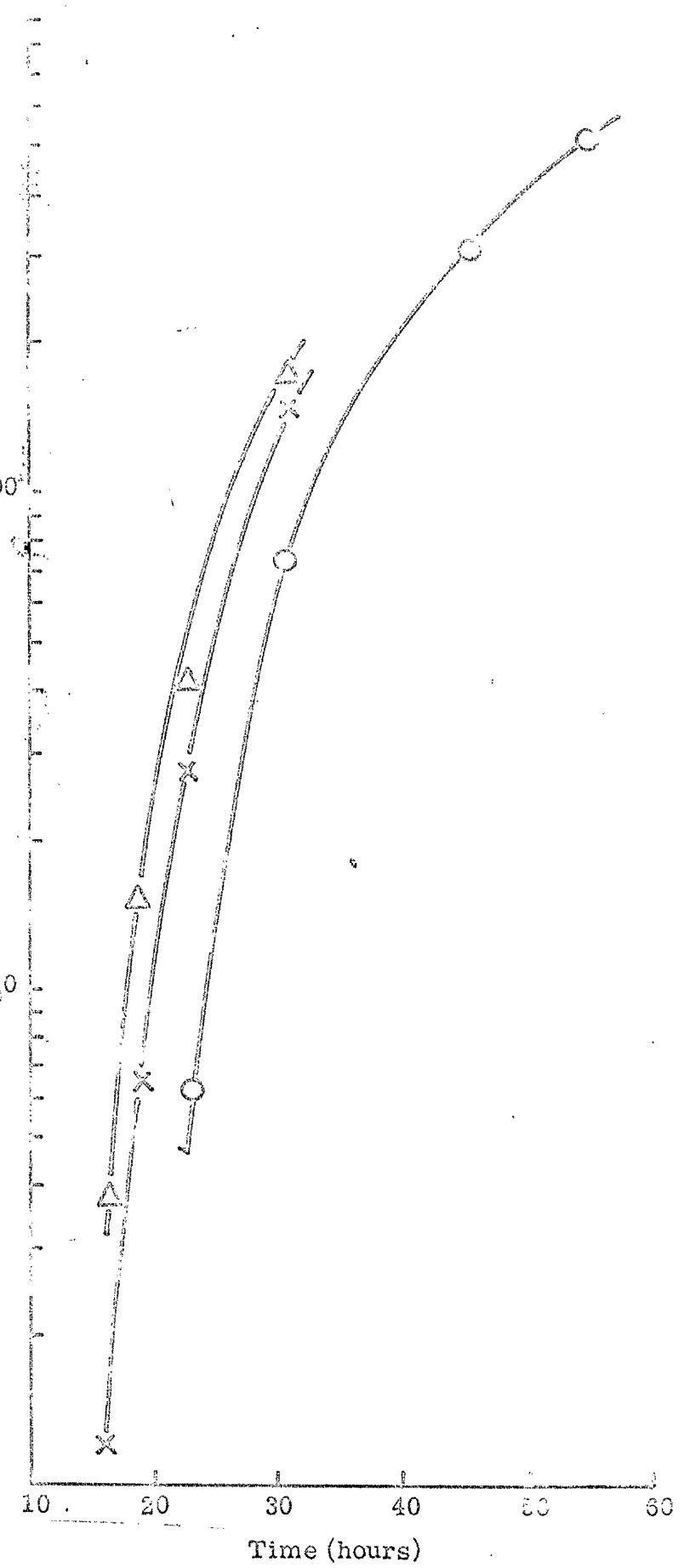

GROWTH EXPRESSED AS DRY WEIGHT (MGS)
100
10
10
20
30
40
50
60
Time (hours)

Fig 2.2.

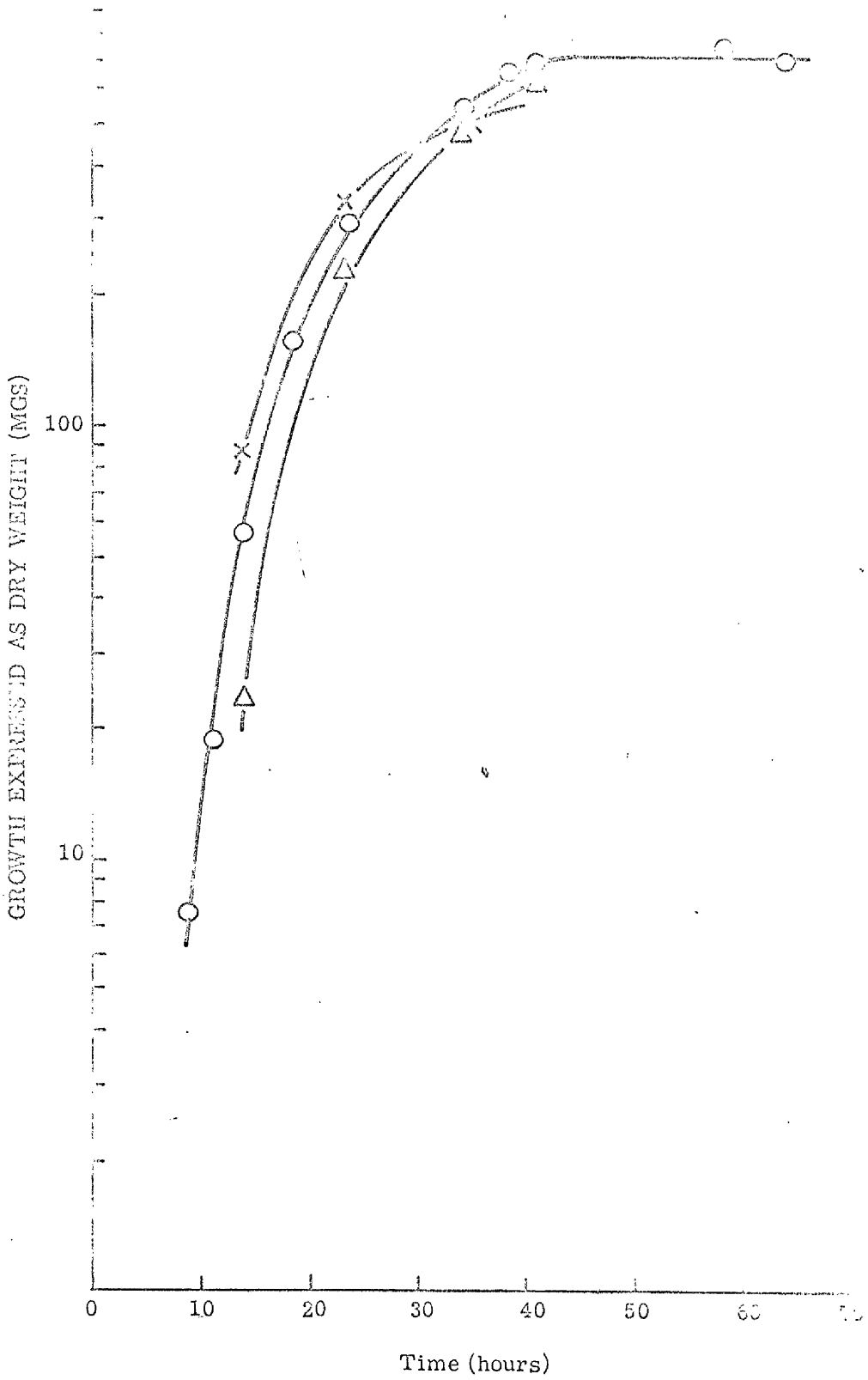
EFFECT OF FURTHER INCREASING THE SIZE OF INOCULUM ON GROWTH IN SMALL SHAKE CULTURES.

100 ml shaking cultures were set up as described in section 2.4 and inoculated with spores in the range of 5×10^6 to 20×10^8. Flasks were harvested at set time intervals by vacuum filtration. The cells were dried to constant weight in a 100° oven in preweighed glass vials.

—△— 5×10^8 spores

—○— 10×10^8 spores

—×— 20×10^8 spores


GROWTH EXPRESSED AS DRY WEIGHT (MGS)
100
10
0
10
20
30
40
50
Time (hours)

2.7. [illegible] conditions for the growth of large [illegible] cultures.

Attempts were made to find conditions for growing N. crassa in large scale cultures and also to try to establish if rate of growth had an effect on the amount of aroM complex that could be extracted.

Large scale cultures were grown as described in 2.5. above. The flasks were inoculated with two 100 ml shake cultures. Mycelial pads were harvested by vacuum filtration at various times. The pH of the growth medium was measured and the cells freeze-dried and weighed. The amount of aroM complex was estimated by assay of shikimate dehydrogenase activity in crude extracts by the method of Gaertner and DeMoss (1970).

Enzyme was extracted from 2g of freeze-dried N. crassa harvested at various time intervals. Cells were pondered dry in a Waring Blender resuspended in 0.1M - potassium phosphate buffer, pH 7.5, containing 0.4mM-dithiothreitol 0.1mM-phenylmethane sulphonyl fluoride and stirred slowly at 4°C for an hour. The suspension was centrifuged, at 12K (rpm) for 30 min at 4°C. Deoxyribonuclease was added to the supernatants to give a final concentration of 5 μg/ml and left for 2 hours at 37°C with occasional shaking and then recentrifuged. 2 ml fractions of the supernatants were dialysed against two changes of two litres of the extraction buffer described above. Protein content was estimated by the method of Lowry et al (1951), using bovine serum albumin (Armour pharmaceutical Co. Ltd., Eastbourne, Essex, U.K.) as a standard. The results of these experiments are shown in Fig 2.3., Fig 2.4. and Fig 2.5.

Growth appears to be linear in these experiments (Fig 2.3.). However the pH of the growth medium decreases to a minimum value

value [illegible] about 3·6 after 15 hours and then seems to rise slowly [illegible] (Fig 2.3b). The amount of shikimate dehydrogenase activity extracted per gram dry weight of N.crassa reaches a peak value between eight to ten hours of growth and then decreases slowly over the times measured (Fig 2.4). The total amount of shikimate dehydrogenase extracted at each harvesting does not seem to change significantly after an initial steady increase to twelve hours of growth (Fig 2.4).

The amount of protein extracted per gram dry weight of N.crassa levels off between twelve to fourteen hours, however the total amount of protein extracted at each harvesting continues to increase linearly to at least 20 hours (Fig 2.5).

The effect of growth on the pH of the growth medium is probably due to the accumulation of acidic waste products in the medium. The increase in pH after 15 hours is likely to be due to the breakdown of cellular material caused by the low pH of the medium and also perhaps because the cells have used up all the sucrose in the growth medium. Thus, although growth appears to be linear after 15 hours, perhaps some change in the composition of the cells begins to occur at this time.

However the aim of these experiments was mainly to determine practical conditions for growing N.crassa in large scale cultures from which substantial amounts of the arom complex could be extracted. The situation can be seen most clearly in Fig 2.6. The yield of arom activity from a given culture reaches a maximum value at about 12 hours. The total amount of activity then remains approximately constant until 24 hours (Fig 2.6) although the specific activity falls, as total extractable protein, still increases until 32 hours in these experiments (Fig 2.5). For reasons of practical convenience arom was usually isolated from cells grown for between 16 and 24 hours at 37°C.

Pag 2.3

GROWTH OF LARGE SCALE CULTURES OF N.CRASSA

Large scale cultures were set up as described in section 2.5 and each inoculated with two 100 ml shaking cultures. Mycelial pads were harvested at set time intervals by vacuum filtration. The pH of the growth medium measured and the cells freeze-dried and weighed.

(a) Dry weight of cells as a function of time.

(b) Effect of growth on the pH of the growth medium.

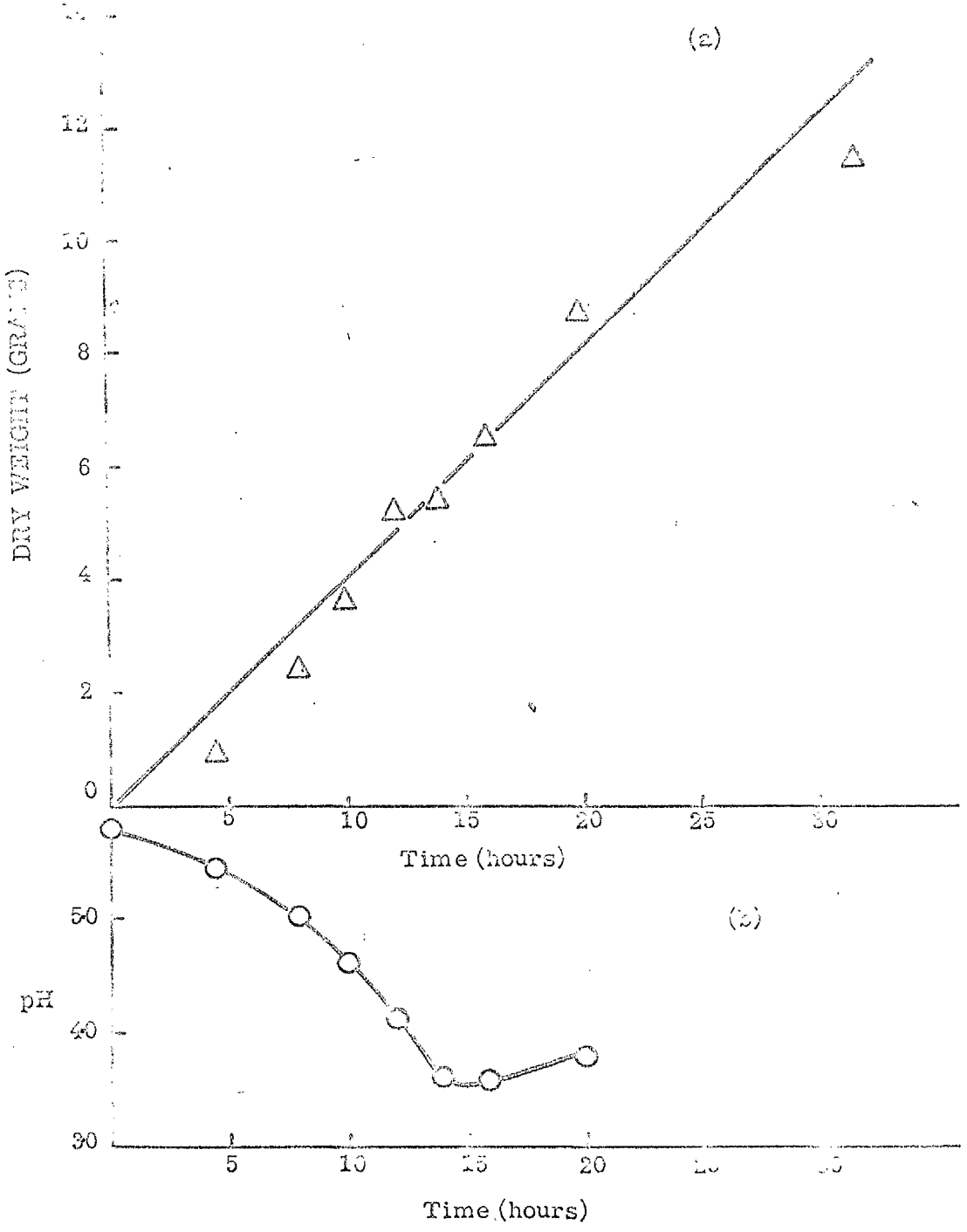

(a)
DRY WEIGHT (GRAMS)
0
2
4
6
8
10
12
5
10
15
20
25
30
Time (hours)
(b)
pH
50
40
30
5
10
15
20
Time (hours)

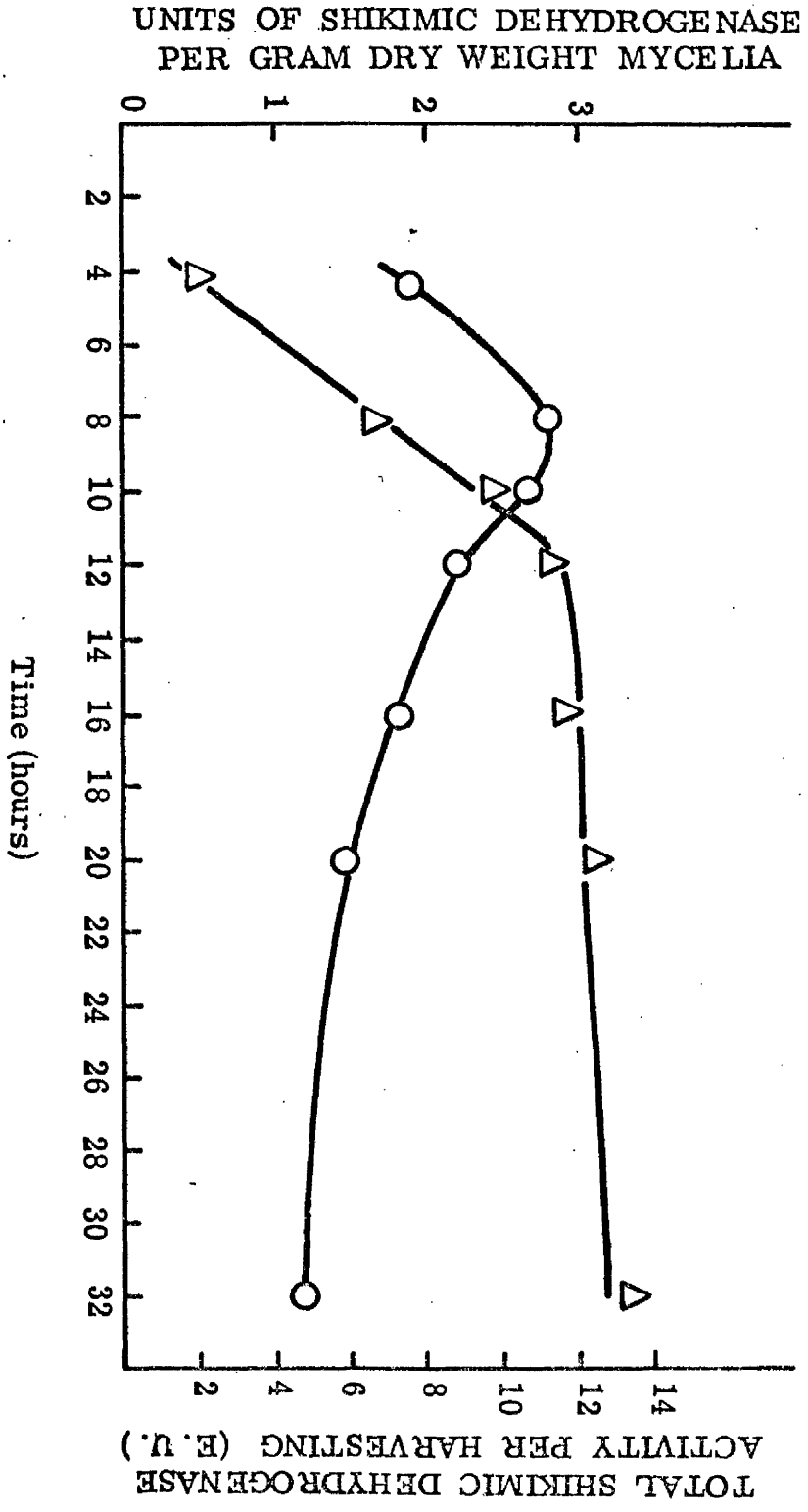
Fig 2.4.

CHANGES IN [illegible] ACTIVITY AS ESTIMATED BY SHIKIMATE DEHYDROGENASE ASSAYS, AS A FUNCTION OF GROWTH OF MYCELIA IN LARGE SCALE CULTURES.

Crude extracts of N.crassa were prepared as described in section 2.7 and shikimate dehydrogenase activity estimated by the method of Gaertner and DeMoss (1970). One unit of activity is defined as the amount of enzyme which catalyses the formation of 1umole of NADPH/min at 37°C.

—△— Total shikimate dehydrogenase activity extracted.

—○— Units of shikimic dehydrogenase extracted per gram dry weight of mycelia.


UNITS OF SHIKIMIC DEHYDROGENASE
PER GRAM DRY WEIGHT MYCELIA
0
1
2
3
Time (hours)
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
TOTAL SHIKIMIC DEHYDROGENASE
ACTIVITY PER HARVESTING (E.U.)
2
4
6
8
10
12
14

-- 2.5

[illegible] EXTRACTABLE PROTEIN FROM GROWTH

OF MYCELIA IN LARGE SCALE CULTURES.

Crude extracts of M.smegma were prepared as described in section 2.7 and amount of protein present was estimated by the method of Lowry et al (1951).

—○— total protein extracted per harvesting.

—△— amount of protein extracted per gram dry weight mycelia.

AMOUNT OF PROTEIN (MGS) EXTRACTED PER GRAM DRY WEIGHT MYCELIA
0
50
100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
TIME (HOURS)
TOTAL AMOUNT OF PROTEIN (MGS) EXTRACTED PER HARVESTING
500
1000

CHAPTER 3

PURIFICATION OF THE AROM MULTIENZYME COMPLEX FROM N.CRASSA.

3.1. General outline.

A modification of the method described by Jacobson et al (1972) was used in attempts to purify the arom multienzyme complex from wild type N.crassa strain 74A. The purification was followed at each stage by assaying shikimate dehydrogenase activity, according to the method of Gaertner and DeMoss (1970). Protein content was estimated by the method of Lowry et al (1951), using bovine serum albumin as a standard.

3.2. Growth of cells

N.crassa was grown in large scale culture and harvested after 24 hours as described in section 2.5.

3.3. Preparation and nuclease treatment of crude extracts

In general, 20g of freeze-dried mycelia were powdered dry in a Waring blender at 4°C. The powder obtained was suspended by slowly stirring in 300 ml of 0·1M potassium phosphate buffer, pH 7·5, containing 0·4mM-dithiothreitol and 0·1mM-phenylmethane sulphonyl fluoride, at 4°C for 30 min. The suspension was then centrifuged at 25,000 g for two hours at 4°C. Deoxyribonuclease was added to the supernatant solution to give a final concentration of 5µg/ml and the extract kept at room temperature for one hour. The resulting precipitate was removed by centrifugation at 12,000 g for 20 minutes at 4°C. The results of three separate experiments are shown in Table 3.1.

3.4. Ammonium sulphate precipitation.

Powdered $(NH_4)_2SO_4$ was added to the extract to give 25% saturation (144 g/l). The solution was then stirred for 10 min at 4°C. The resulting precipitate was removed by centrifugation at 12,000 g for 30 min at 4°C. Powdered $(NH_4)_2SO_4$ was added to the supernatant to give 50% saturation (158 g/l). The solution was stirred for 10 min at 4°C, and the precipitate, which contained

the arom aggregate, was collected by centrifugation at 18,000 g for 30 min at 4°C. The precipitate was dissolved in 50mM Tris/HCl, pH 7·5, 0·4mM - dithiothreitol, 0·1mM - phenylmethanesulphonyl fluoride, and dialysed overnight against 1L. of the same buffer. The results of two separate experiments are shown in Table 3.2.

3.5. Chromatography on DEAE - cellulose.

After dialysis, the extract was applied to a column (13cm x 2·8cm) of DEAE - cellulose, which had been pre-equilibrated with 50mM -Tris/HCl, pH 7·5, 0·4mM-dithiothreitol 0·1mM-phenylmethane-sulphonyl fluoride. The column was washed with the same buffer, containing 30mM-KCl, until the A_{280} was low (about 0·05). The column was then eluted with a linear gradient in the range 30mM to 0·5M-KCl, in the same buffer. Fractions containing shikimate dehydrogenase activity were pooled. The elution pattern of a typical DEAE-cellulose gradient experiment is shown in Fig. 3.1. The activities of the pooled fractions from three separate experiments are shown in Table 3.3.

3.6 The lack of reproducibility in these attempts at purification of the arom multienzyme complex.

The amount of protein and shikimate dehydrogenase activity obtained in crude extracts varies greatly during different purifications (Table 3.1). After ammonium sulphate treatment large amounts of shikimate dehydrogenase activity were lost (Table 3.2). In some preparations, the results of which are not shown, all shikimate dehyrogenase activity was lost at this step.

The results obtained on DEAE - cellulose were more satisfactory (Table 3.3). A reproducible elution pattern was obtained and as shikimate dehydrogenase eluted ahead of the main protein peaks it was possible to effect a substantial separation from the bulk of the protein in the sample (Fig 3.1). However when the enzyme

Table 3.1.

Amount of protein and shikimate dehydrogenase activity found in crude extracts of N.crassa.

Experiment	Total extractable protein (mg)	Total extractable shikimate dehydrogenase	Specific activity (E.U. per mg protein)
1	882	142.6	0.162
2	3975	4.3	0.001
3	2605	29.1	0.011

Crude extracts were prepared from 20g dry weight of powdered mycelia, as described in section 3.3. Protein estimations were made according to the method of Lowry et al (1951) and shikimate dehydrogenase activity assayed by the method of Gaertner and DeMoss (1970). One enzyme unit (E.U.) was defined as the amount of enzyme that catalyses the formation of 1 μ mol of NADPH/min at 37°C.

Table 3.2.

Amount of protein and shikimate dehydrogenase activity in ammonium sulphate precipitates of of crude extracts.

Ammonium sulphate treatment of crude extracts was performed as described in section 3.4. Shikimate dehydrogenase and protein estimations were made upon the redissolved precipitate produced from 50% saturation.

Experiment	Total Protein (mg)	Total Shikimate dehydrogenase (E.U.)	Specific activity (E.U. per mg protein)	Percentage of initial extractable protein remaining	Percentage of initial extractable dehydrogenase remaining
1	272.0	2.4	0.009	30.84	1.70
3	740.2	19.4	0.030	28.45	66.60

Table 3.3.

The amount of protein and shikimate dehydrogenase activity found in enzyme preparations after chromatography on DEAE - cellulose.

Extract was applied to a column (13 x 2.8cm) of DEAE - cellulose previously equilibrated with 50mM-Tris/HCl, pH 7.5, 0.4mM-dithiothreitol, 0.1mM-phenylmethanesulphonyl fluoride. The column was washed with the same buffer, containing 30mM-KCl, until the A_{280} was low and then eluted with a linear gradient, 30mM - 500mM-KCl in the same buffer. Shikimate dehydrogenase and protein estimations, of the pool of fractions containing activity, were made.

Table 3.3.

Experiment	Total protein (mg)	Total shikimate dehydrogenase (E.U.)	Specific activity	Percentage of initial extractable protein remaining	Percentage of initial dehydrogenase remaining
1	71.8	1.64	0.023	8.14	1.15
2	5.8	0.96	0.166	0.14	22.80
3	47.3	17.87	0.380	1.82	61.30

Fig 3.1.

Chromatography of arom multienzyme aggregate on DEAE - cellulose using the initial purification scheme.

Enzyme was applied to a DEAE - cellulose column (13 x 2.8cm) previously equilibrated with 50mM-Tris/HCl, pH 7.5, 0.4mM-dithiothreitol, 0.1mM-phenylmethanesulphonyl fluoride and the column then washed with the same buffer containing 30mM-KCl, until the A_{280} was low. The column was then eluted with a linear salt gradient 30mM to 500mM-KCl in the same buffer.

—△— conductivity (mmho)

—X— specific activity (units/mg protein)

—○— A_{280} (nm)

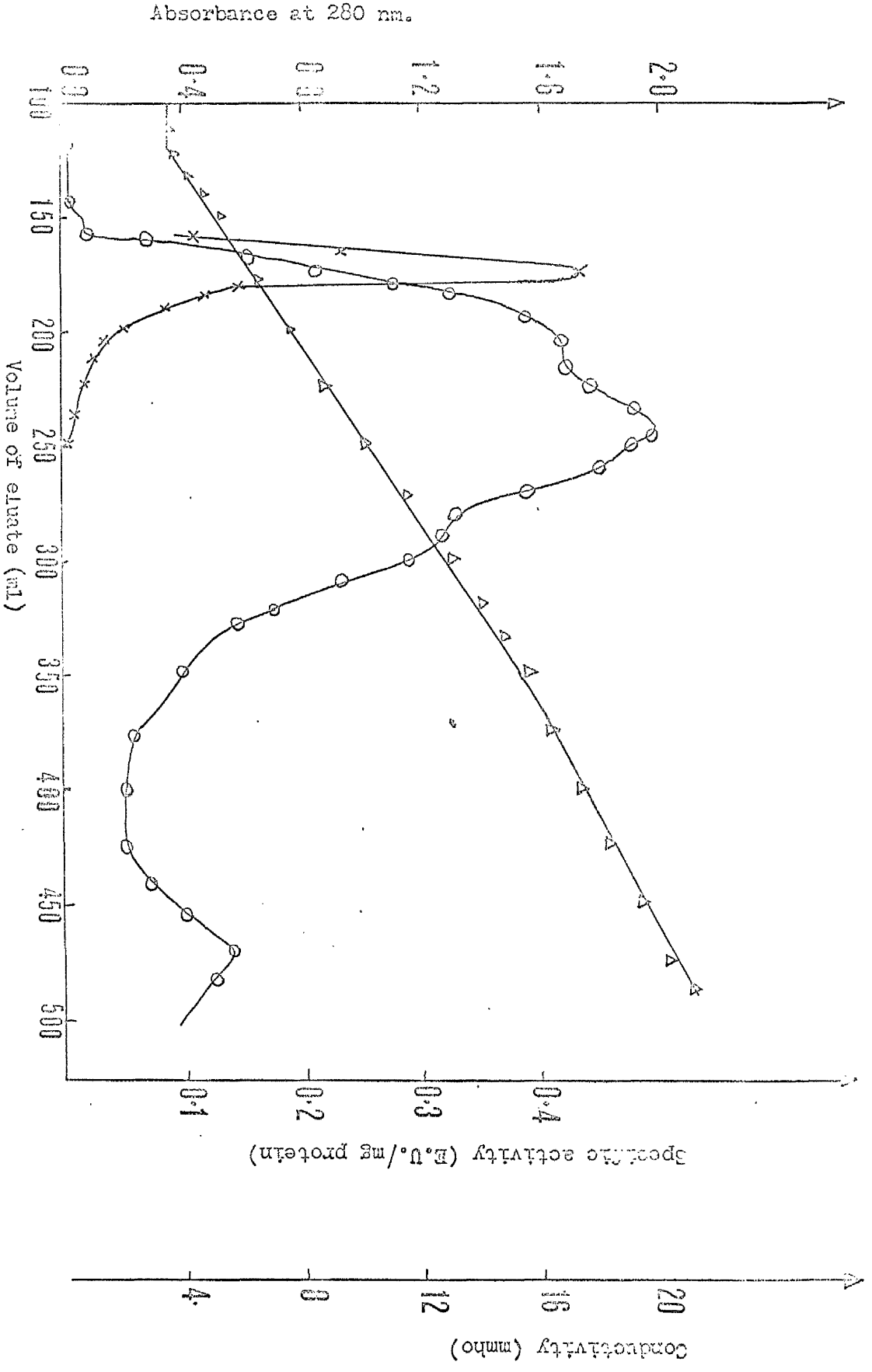

Absorbance at 280 nm.
0.0
0.4
0.8
1.2
1.6
2.0
100
150
200
250
300
350
400
450
500
Volume of eluate (ml)
0.1
0.2
0.3
0.4
Specific activity (E.U./mg protein)
4
8
12
16
20
Conductivity (mmho)

Table 3.4.

The specific activities of shikimate dehydrogenase during three stages of purification.

Attempts were made to purify arom aggregate on two separate occasions, following the procedures described in sections 3.3, 3.4 and 3.5. Shikimate dehydrogenase activity was used to monitor the purification and was estimated according to the method of Gaertner and DeMoss (1970). Protein was determined by the method of Lowry et al (1951). Specific activity was calculated and expressed as enzyme units per mg protein. An enzyme unit was defined as the amount of enzyme that catalyses the formation of 1 μ mol of NADPH/min at 37°C.

	Specific Activity (experiment 1)	Specific Activity (experiment 3)
Crude extract	0.162	0.011
$(NH_4)_2SO_4$ precipitate	0.009	0.030
DEAE-cellulose Pooled fractions	0.023	0.380

activity and protein concentration were determined, there was little improvement in specific activity (Table 3.4). This must be due to the inactivation of the enzyme during the earlier purification steps.

Parallel studies in this laboratory, by Lumsden and Coggins, have also shown that the purification was unreproducible. It was realised that extra precautions had to be taken to protect the arom multienzyme complex from the effects of proteases, some of which were already known to become activated during the purification (Pringle, 1975). Lumsden and Coggins developed a new more rapid purification. In it the concentration of phenylmethanesulphonyl fluoride was increased twelve-fold and because of its instability was added to buffers immediately before use (Gold, 1967). Another protease inhibitor, benzamidine (Mares-Guia and Shaw, 1965) was also added in the ammonium sulphate step of the purification, since it had been realised that phenylmethane-sulphonyl fluoride is salted out of solution at high concentrations of $(NH_4)_2 SO_4$.

3.7. The revised purification of the arom multienzyme complex of N.crassa.

(i) Preparation of crude extract

20 g of freeze-dried N.crassa were powdered in a Waring blender and added slowly with stirring at 4°C to 300 ml of 0·1M-Tris/HCl, pH 7·5, containing 0·4mM-dithiothreitol, 1·2mM-phenylmethanesulphonyl fluoride, 5·0mM-EDTA. Stirring was continued for one hour and then the extract was centrifuged at 12,000 g for 30 min. at 4°C. The conductivity of the supernatant was adjusted to 4·0mmho with a few ml. of 1M-KCl.

(ii) Passage through DEAE-cellulose.

The crude extract was then applied to a column of DEAE-cellulose (2·8cm x 14·0cm), previously equilibrated with 50mM-Tris/HCl, pH7·5,

75mM-KCl, 0·4mM-dithiothreitol, 1·2mM-phenylmethanesulphonyl fluoride, at a flow rate of 200 ml/h collecting 12·5 ml fractions. All fractions containing shikimate dehydrogenase activity were pooled.

(iii) Deoxyribonuclease treatment.

Deoxyribonuclease to give a final concentration of 5µg/ml was added to the pooled fractions and the mixture incubated for 2h at 37°C in a water bath and then centrifuged at 15,000 g for 45 min.

(iv) Ammonium sulphate fractionation.

Benzamidine was added to the supernatant to give a final concentration of 1mM and powdered $(NH_4)_2SO_4$ added to give 40% saturation. The solution was stirred for 15 mins and the precipitate removed by centrifugation at 15,000g for 20mins. More $(NH_4)_2SO_4$ was added to the supernatant to give 50% saturation. After stirring for 15 min, the precipitate was collected by centrifugation at 15,000g for 20 min. The precipitate was dissolved in 50mM-Tris/HCl, pH 7·5, containing 0·4mM-dithiothreitol, 1.2 mM-phenylmethanesulphonyl fluoride and dialysed overnight against 500 ml of the same buffer.

(v) Chromatography on DEAE-cellulose.

The dialysed extract was applied to a column (13cm x 0·9cm) of DEAE-cellulose, previously equilibrated with 50mM-Tris/HCl, pH 7·5, 0·4mM-dithiothreitol, 1·2 mM-phenylmethanesulphonyl fluoride. The column was washed with this buffer containing 30mM -KCl until the A_{280} of the eluate was less than 0·05. The enzyme was eluated with a linear gradient (150 ml) of 30mM to 300mM-KCl in the same buffer. The flow rate was 15 ml/h and the eluate was collected in 6 min fractions. Those fractions containing shikimate dehydrogenase activity were pooled. The

typical type of elution curve obtained is shown in Fig. 3.2.

(vi) Cellulose phosphate chromatography. Cole and Gaertner (1975)

The extract was dialysed for 1 - 2h against 10mM - potassium phosphate, pH 6·5, 0·4mM - dithiothreitol, 1·2mM-phenylmethanesulphonyl fluoride and then applied to a column (42cm x 0·9 cm) of cellulose phosphate, pre-equilibrated with 10mM-potassium phosphate, pH 6·5, and washed freshly with the same buffer, containing 0·4mM-dithiothreitol, 1·2 mM- phenylmethanesulphonyl fluoride. The column was washed with this buffer until the A_{280} of the eluate was zero; then the enzyme was eluted with 0·2M-potassium phosphate, pH 6·5, 0·4mM-phenylmethanesulphonyl fluoride. 1·5 ml fractions were collected and the flow rate of the column was 7·5 ml/h. Fractions containing the highest specific activity were pooled and dialysed against 50mM-sodium phosphate, pH 7·5, 0·4mM-dithiothreitol, 1·0mM-benzamidine. The enzyme was stored at -20°C in 50% glycerol. A typical elution pattern obtained is shown in Fig. 3.3.

(vii) Determination of shikimate dehydrogenase activity.

Assays were performed in the reverse direction of the normal synthetic reaction, following the reduction of $NADP^+$ at 37°C. The reaction mixture contained 0·1M - sodium carbonate buffer, pH 10·6, 4mM - shikimate and 2mM- $NADP^+$ in a final assay volume of 1·0 ml. The reduction of $NADP^+$ was followed at 340nm in a Unicam SP 8000 spectrophotometer with a chart recorder attachment. The reaction was started by addition of an aliquot of enzyme.

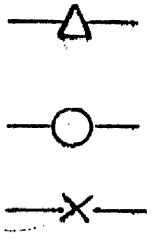
(viii) Polyacrylamide Gel Electrophoresis.

Disc electrophoresis was performed according to a modified method of Davis (1964) by Hayes and Wellner (1969). Electrophoresis was carried out at 2mA per gel after an initial pre-run of 1 h. An aliquot of sample (50 µl) containing 10 µl of

Fig 3.2.

DEAE - cellulose chromatography of arom multienzyme aggregate using the revised purification proceedure.

Dialysed enzyme obtained after ammonium sulphate precipitation was applied to a DEAE - cellulose column and eluted with a linear salt gradient as described in section 3.7.(v). Shikimate dehydrogenase assays were performed as described in section 3.7. (vii).



A_{280}(nm)

conductivity (mmho)

Activity (units/ml)

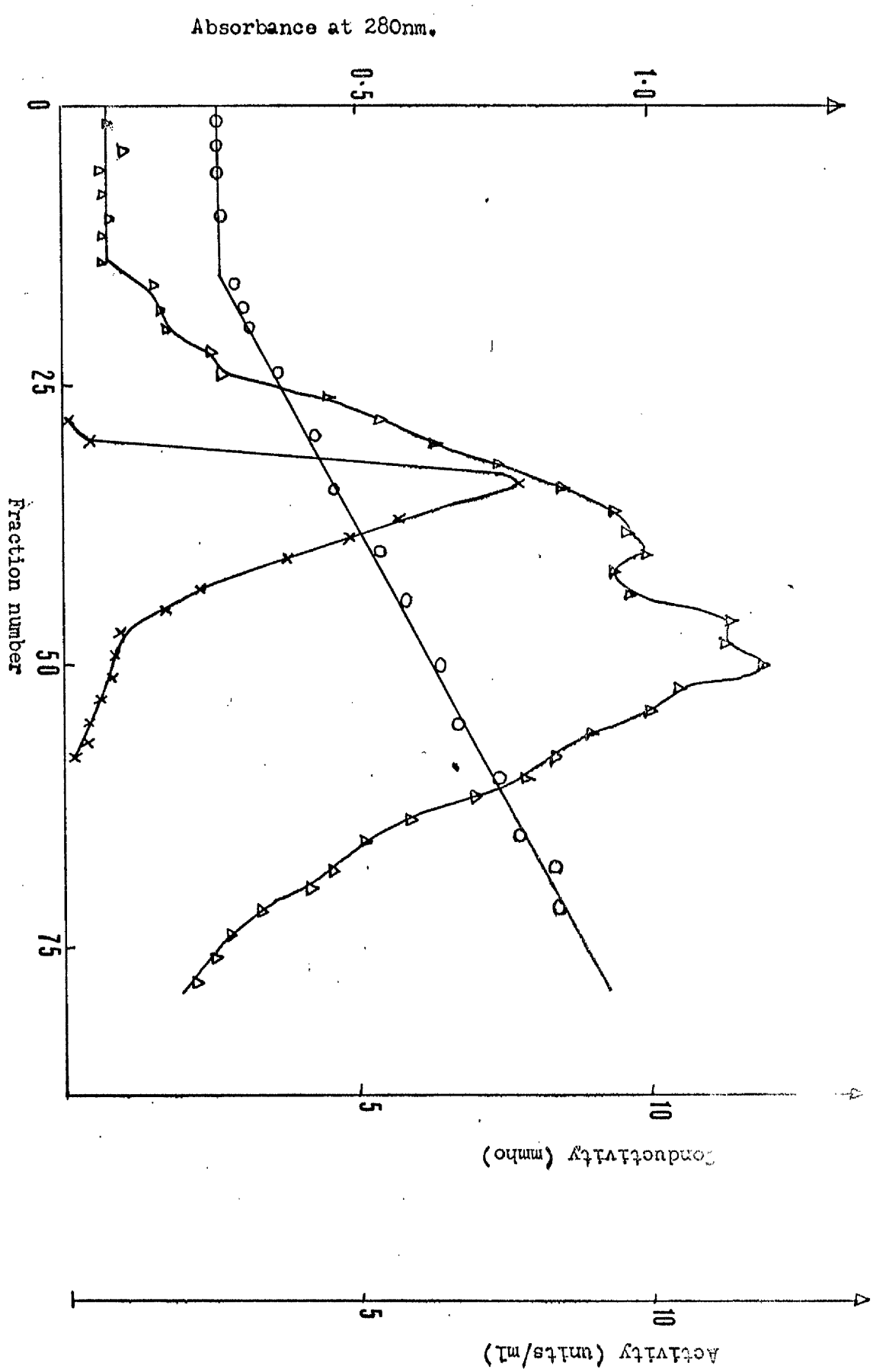

Absorbance at 280nm.
0
0.5
1.0
25
50
75
Fraction number
5
10
Conductivity (mmho)
5
10
Activity (units/ml)

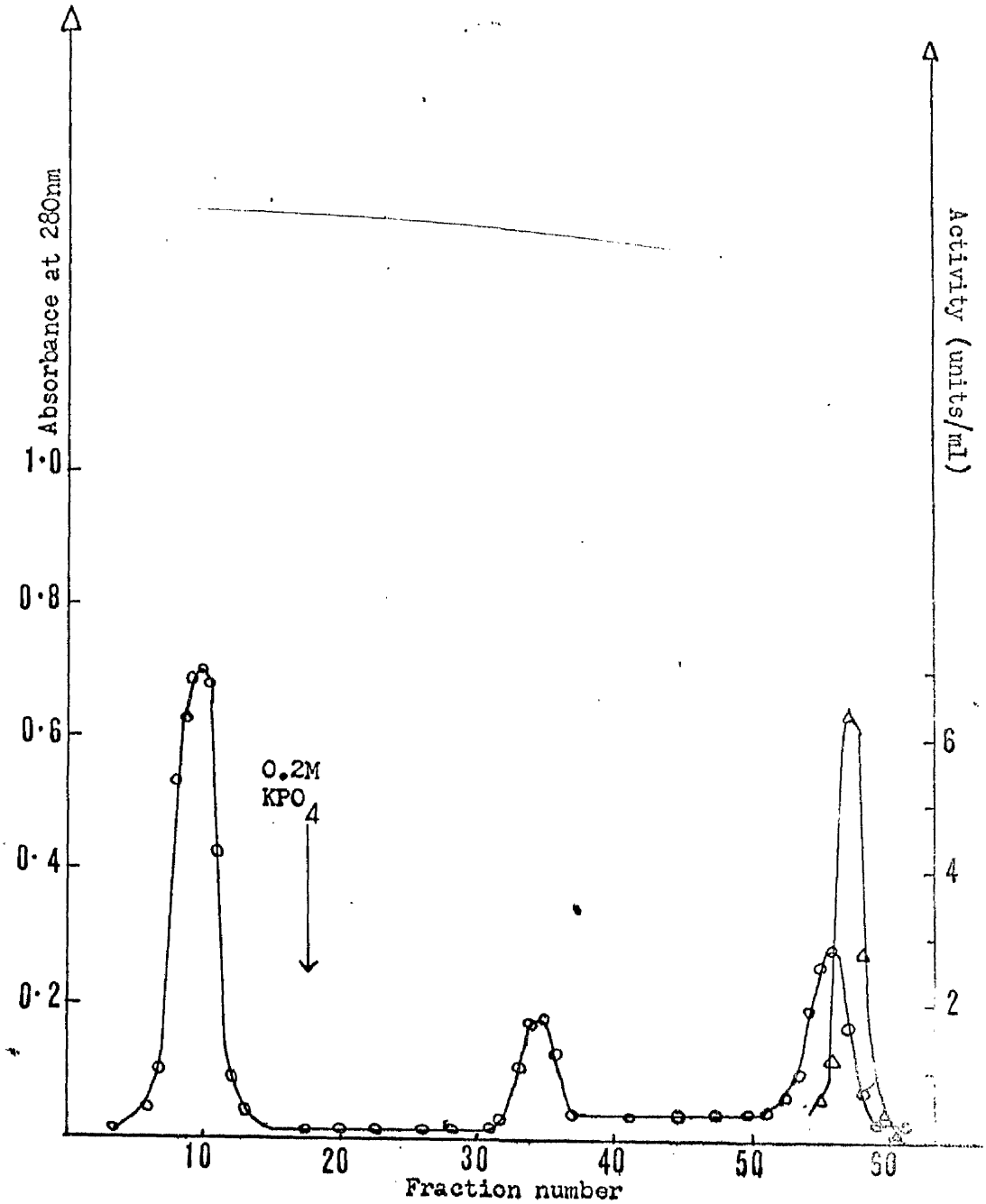
Fig 3.3.

Cellulose phosphate chromatography of arom multienzyme aggregate.

Enzyme obtained after DEAE - cellulose chromatography was dialysed against 10mM-KPO_4, pH 6.5, 0.4mM-dithiothreitol, 1.2mM-phenylmethanesulphonyl fluoride and then applied to a column of cellulose phosphate pre-equilibrated with the same buffer. The column was eluted as described in section 3.7 (vi), the A_{280} and shikimate dehydrogenase activity of the eluate were determined.

—○— A_{280} (nm)

—△— Activity (enzyme units/ml)


Absorbance at 280nm
1·0
0·8
0·6
0·4
0·2
0.2M
KPO4
Activity (units/ml)
6
4
2
10
20
30
40
50
60
Fraction number

0·05% (w/v) Bromophenol Blue and 10 µl of glycerol was layered on the top of each gel before electrophoresis. Gels were stained for protein and shikimate dehydrogenase activity according to the method of Lumsden and Coggins (1977). Rf values were calculated relative to the marker band. Gels were scanned at 600nm in a Gilford spectrophotometer fitted with a model 252 gel scanner.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to Shapiro and Maizel (1969). Gels were usually 5% in acrylamide.

3.8. The success of the revised purification proceedure.

The results of a typical purification following the methods outlined in section 3.7. are shown in Table 3.5. Simple polyacrylamide gel electrophoresis photographs, of various stages of the purification, where gels have been stained both for protein and shikimate dehyrogenase activity are shown in Fig. 3.4. A gel stained for activity and also a scan of a gel stained for protein of a sample obtained after cellulose phosphate chromatography are shown in Fig. 3.5.

In the revised purification scheme the total amount of shikimate dehydrogenase, obtained from different preparations, is not only extracted in reproducible quantities but is also increased over that obtained in most of the earlier purification attempts. This must be due to the increased amount of phenylmethanesulphonyl fluoride which was added to the extraction buffer, and strongly indicates that highly active proteases are present in crude extracts of mycelia.

Although purification of the enzyme does not seem to be effected during the three initial stages of the purification, these steps do appear to be necessary since the first DEAE-cellulose passage is thought to remove a number of proteases, which were present in crude extracts (Lumsden and Coggins, 1977, Siepen

Table 3.5.

The revised purification proceedure for the arom multienzyme aggregate.

Results are presented for a typical purification conducted as described in section 3.7. Protein was estimated according to Lowry et al (1951). Shikimate dehydrogenase activity estimated as described in section 3.7(vii). A unit of enzyme activity is defined as the amount of enzyme which catalyses the production of 1 μ mol of NADPH/min at 37°C.

Stage	Volume (ml)	Activity (Units/ml)	Total Activity (units)	Protein concentration (mg/ml)	Total Protein (mg)	Specific Activity	Purification (fold)
Crude extract	260	0.51	133.5	4.8	1250	0.11	1.0
First DEAE-cellulose	232	0.36	83.7	3.7	875	0.10	0.91
Deoxyribonuclease	222	0.41	91.9	3.9	862.5	0.11	1.0
Ammonium sulphate	6.5	10.8	70.3	21.1	137.5	0.51	4.6
Second DEAE-cellulose	15	2.77	41.6	0.86	13.0	3.20	29.1
cellulose phosphate	3	6.8	20.5	0.167	0.5	41.00	372.7

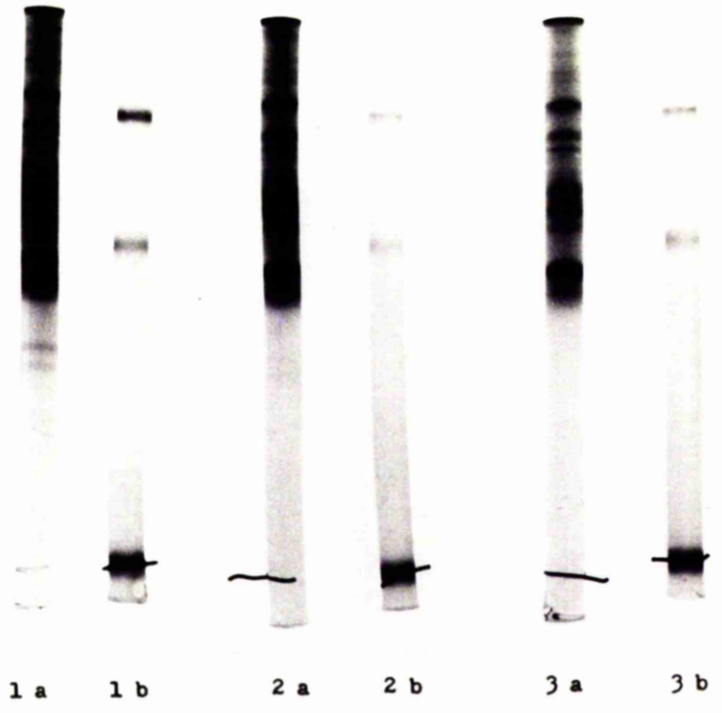
Fig 3.4.

Disc polyacrylamide gel electrophoresis of arom multienzyme complex at various stages of purification.

Disc electrophoresis was performed according to Hayes and Wellner (1969) and as described in section 3.7.(viii) Gels were stained for protein and shikimate dehydrogenase activity as according to Lumsden and Coggins, (1977).

(a) Gels stained for protein (b) Gels stained for activity.

1. Crude extract.
2. Deoxyribonuclease treatment.
3. First DEAE - cellulose chromatography.
4. Ammonium sulphate treatment.
5. Second DEAE - cellulose chromatography.


1 a
1 b
2 a
2 b
3 a
3 b

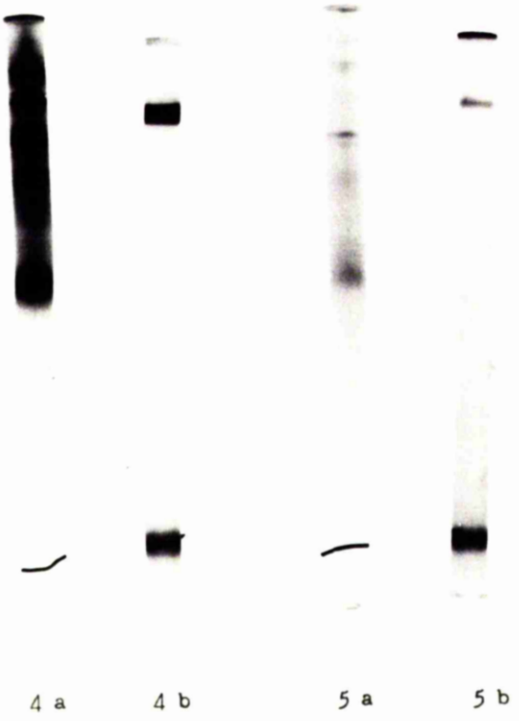
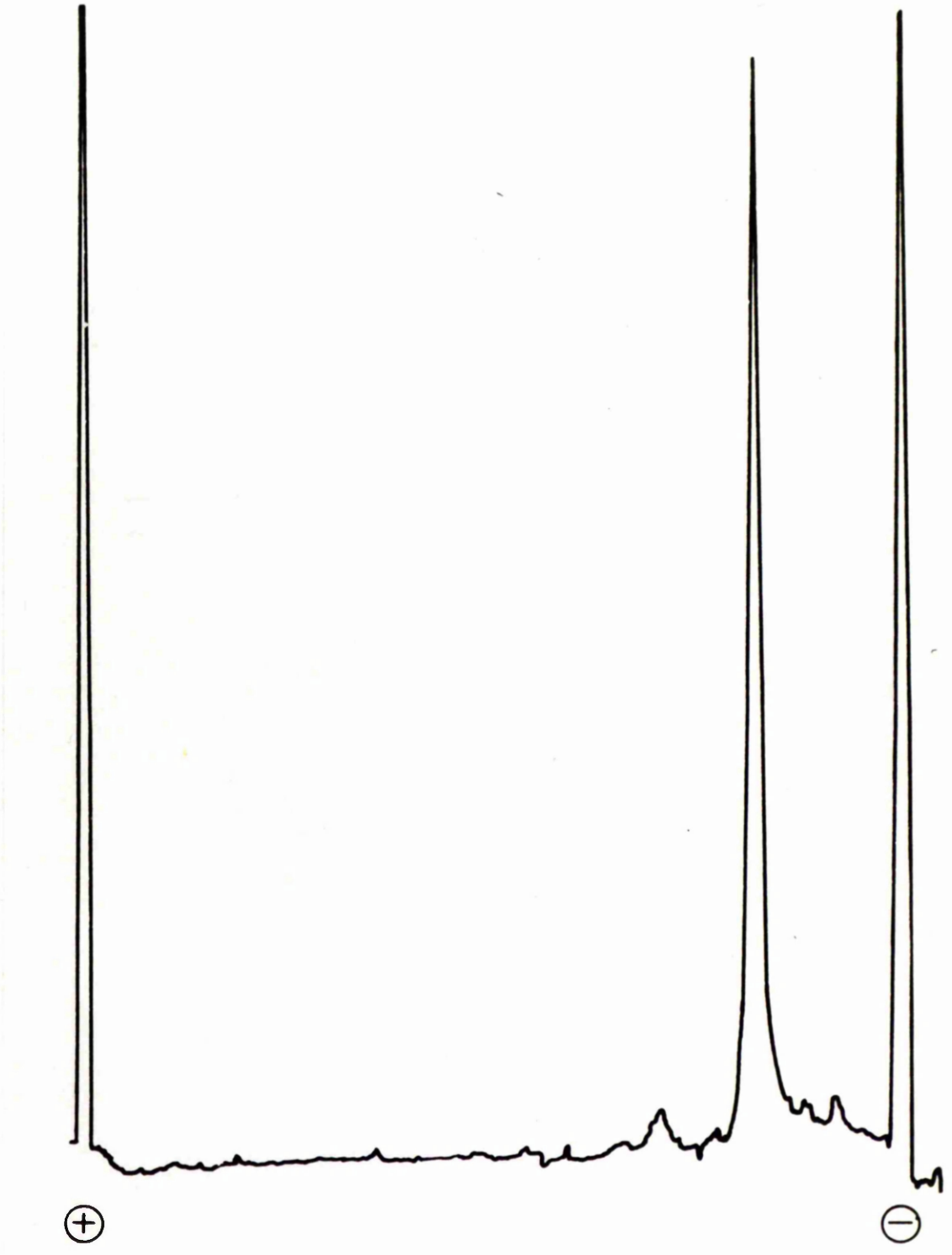
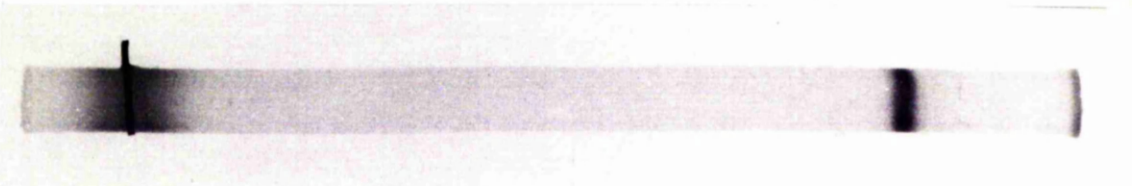

4 a
4 b
5 a
5 b

Fig 3.5.

Disc polyacrylamide gel electrophoresis of preparations of purified arom multienzyme complex.

Disc electrophoresis of purified arom complex was performed according to Hayes and Wellner and as described in section 3.7 (viii). Gels were stained for protein and shikimate dehydrogenase activity as according to Lumsden and Coggins, (1977). Protein stained gels were scanned at 600nm in a Gilford spectrophotometer fitted with a model 252 gel scanner. Activity stained gels were photographed.





et al, 1975). The nuclease step is useful, as unless DNA is hydrolysed, the $(NH_4)_2\ SO_4$ fraction was found to be very viscous, making it very difficult to use in the chromatography steps. From Table 3.5. it can be seen that there is an apparent slight increase in total units of activity after nuclease treatment.

The addition of benzamidine during ammonium sulphate precipitation does seem to prevent loss of activity during this step. A five fold purification of activity is also obtained at this stage. However it was not until the final two stages of the purification, especially until after cellulose phosphate chromatography, that a substantial purification was achieved. Lumsden and Coggins (1977) have obtained a slightly higher value for the final specific activity and yield of enzyme after the cellulose phosphate stages.

An analysis of the simple polyacrylamide electrophoresis gels at each stage of the purification, (Fig. 3.4.), showed that substantial purification of protein was not noted until after the second DEAE - cellulose treatment. After cellulose phosphate chromatography, there appeared to be only one band of protein (Fig. 3.5.). The presence of a faster moving activity band which was lost after ammonium sulphate precipitation was observed in gels stained for dehydrogenase activity (Fig. 3.4.). Lumsden and Coggins (1977) have also noted this faster moving band of activity in the initial stages of the purification.

CHAPTER 4

CHARACTERISATION OF THE AROM MULTIENZYME COMPLEX OF N.CRASSA

4.1. General Outline

A preliminary physical and chemical characterisation of the arom multienzyme aggregate, purified by the revised purification procedures of Lumsden and Coggins, (1977) and as described in section 3.7. was carried out. The mol. wt. was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

4.2. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

Electrophoresis was conducted as described by Shapiro and Maizel (1969). In general gels were made 5% in acrylamide. Protein samples were prepared by making them 1% (W/V) in sodium dodecyl sulphate and 1% (V/V) in 2-mercaptoethanol, then boiling them in a water bath for 5 min. This ensured that samples were fully denatured and sulphydryl groups completely reduced. Usually 50µl sample, a few µl of 0.05% (W/V) Bromophenol Blue and 20µl glycerol were applied to each gel. The dye front was marked after electrophoresis. Cross-linked aldolase, obtained from J.R. Coggins, was used as a molecular weight standard. Gels were stained for protein as described by Lumsden and Coggins (1977) and scanned at 600nm in a Gilford spectrophotometer fitted with a model 252 gel scanner. Rf values were calculated relative to the dye marker.

4.3. Estimation of the molecular weight of purified arom complex.

In early preparations of purified arom, sodium dodecyl sulphate polyacrylamide gel electrophoresis showed three bands after staining for protein: a major band with an Rf of 0·24 and two minor faster moving bands with Rf values of 0·54 and 0·63

respectively. A scan of such a gel is shown in Fig 4.1. Rf values were also calculated for cross-linked aldolase and a semi-logarithmic plot of mol.wt. against Rf drawn (Fig 4.2), from which mol. wts. were estimated for the arom bands. The mol. wt. of the major band was estimated as 175,000, the minor bands as being 82,000 and 65,000 respectively. Subsequent preparations of the arom complex gave either a single band of high mol. wt. or a predominant band of high mol. wt. with some faint bands of lower mol. wts. (Fig 4.3).

Lumsden and Coggins, using freshly purified arom complex have also obtained a single protein band on sodium dodecyl sulphate electrophoresis. By using a wider variety of markers, they estimated the mol. wt. of the single band to be 165,000. In older preparations, which had been stored at 4°C, they obtained a number of faster moving bands which they thought to be the result of proteolysis of native arom complex. This suggests that the minor faster moving bands of mol. wts. 82,000 and 65,000 are likely to be due to proteolysis of native arom complex.

Lumsden and Coggins have shown using sodium dodecyl sulphate gel electrophoresis that cross-linked arom complex contained a single slower-moving species with a mol. wt. of 330,000. Centrifugation on glycerol density gradients of native arom complex showed a single species which had an estimated mol. wt. of 282,000. These data and the subunit mol. wt. (see above) indicated that the arom multienzyme complex is a dimer.

The question then arises whether the arom consists of two identical or two non-identical subunits. Genetic evidence has already shown that two copies of some of the arom enzymes

Fig 4.1.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of early preparations of purified arom complex.

Disc electrophoresis was performed as described by Shapiro and Maizel (1969). Gels were stained for protein as described by Lumsden and Coggins (1977) and then scanned at 600nm in a Gilford spectrophotometer fitted with a model 252 gel scanner. Three protein bands can be seen: a large molecular weight species (Peak 1) and two faster moving lower molecular weight components (Peaks 2 and 3).

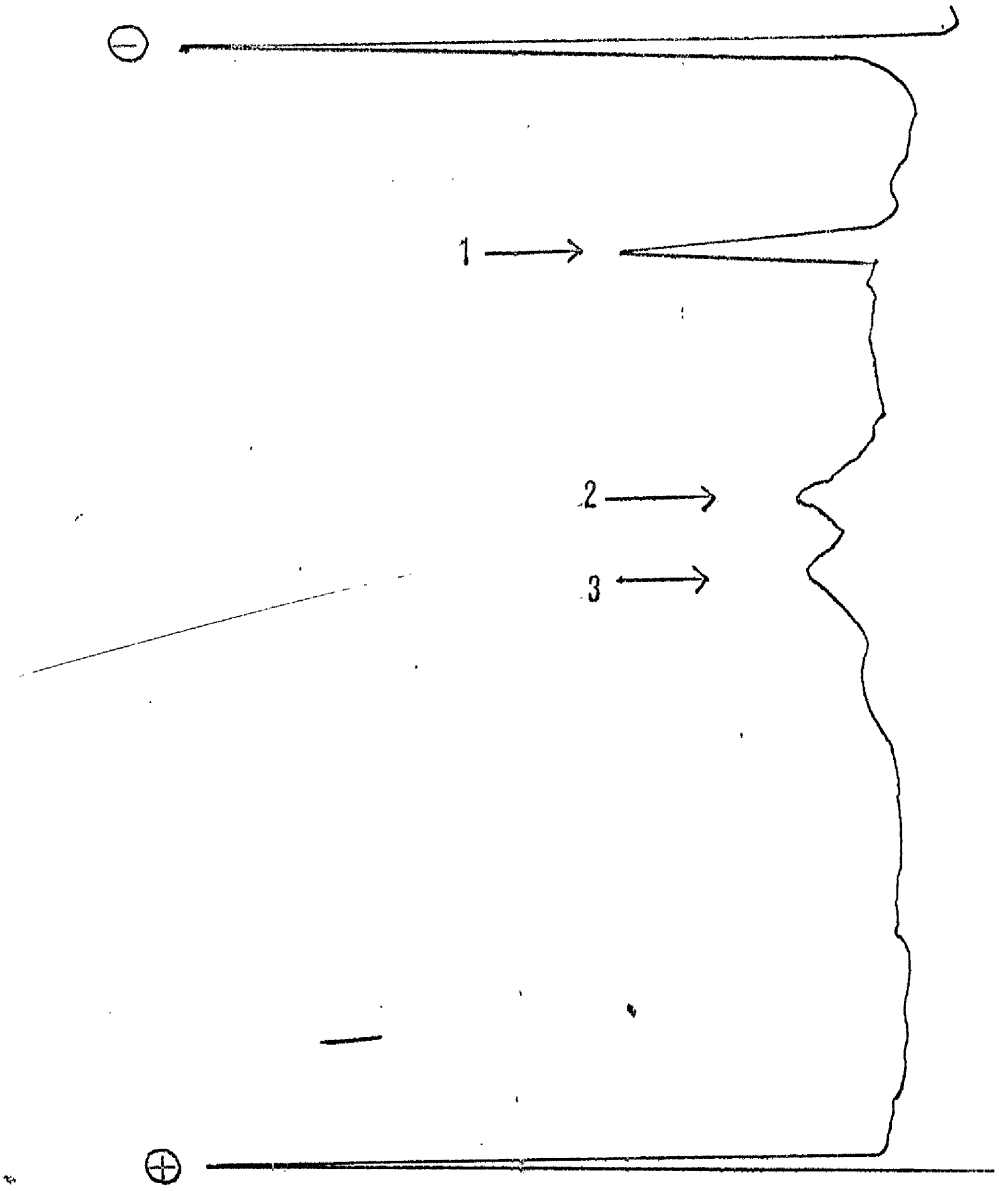
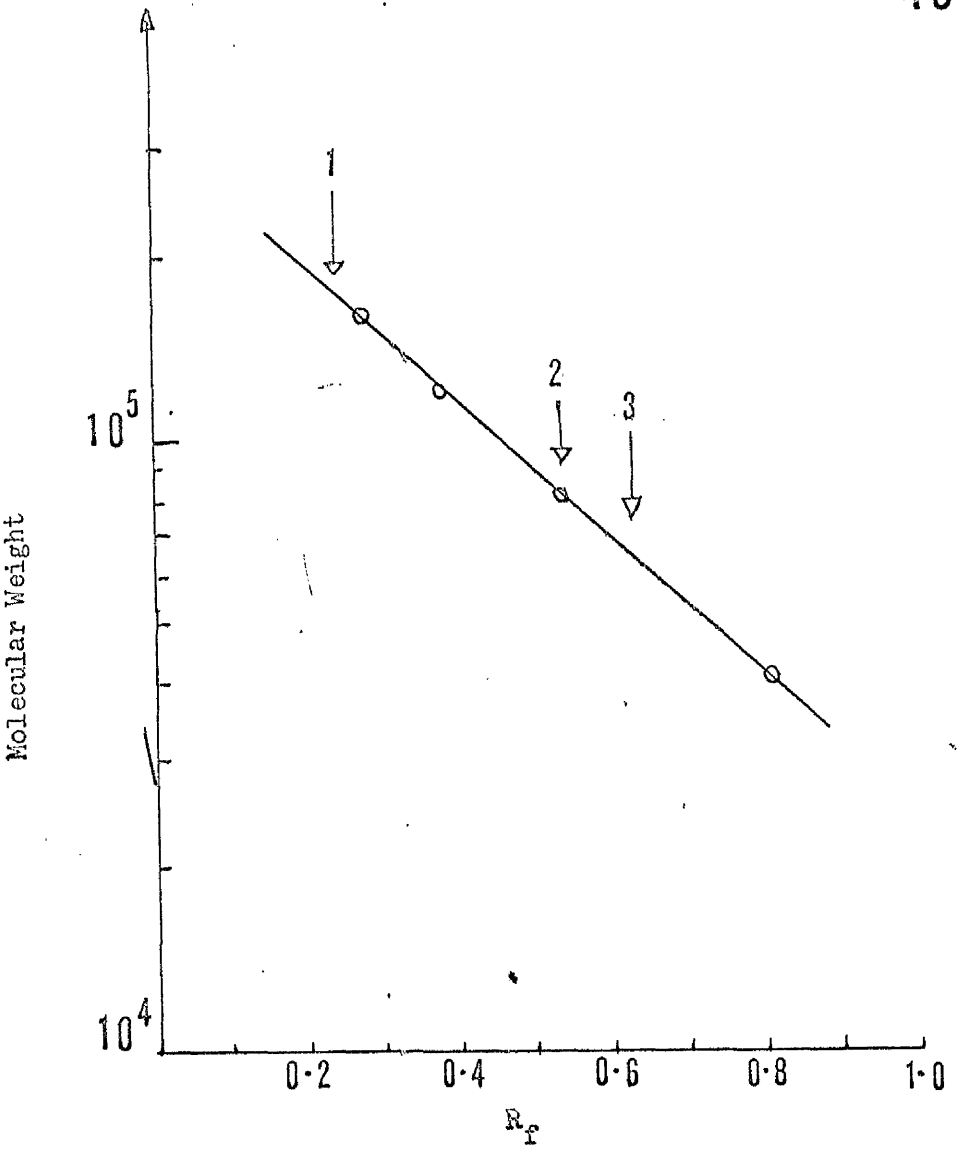

⊖
1
2
3
⊕

Fig 4.2.

Determination of molecular weight of purified arom aggregate by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

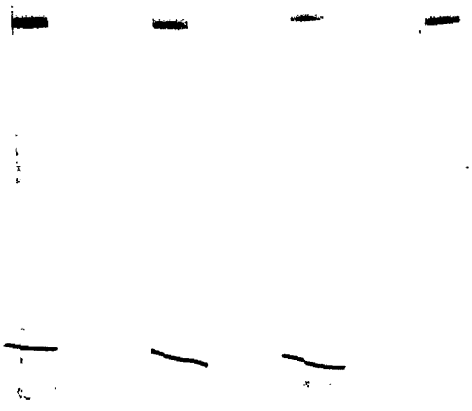
Disc electrophoresis was performed according to Hayes and Wellner (1969) and as described in section 3.7(viii). Gels were stained for protein as according to Lumsden and Coggins, (1977). Cross-linked aldolase was used as a molcular weight standard and a plot of logarithm of molecular weight against Rf drawn. This was used to calculate the molecular weights of purified arom (see Fig. 4.1.)



Protein Band	Rf	Mol. wt. Determination
1	0.24	175,000
2	0.54	82,000
3	0.63	65,000

Fig 4.3.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of later preparations of purified arom complex.



must be present in the arom complex (Giles et al, 1967; Case et al, 1969). It therefore seems likely that the arom complex consists of two identical subunits. However further protein chemistry is required before ascertaining that the subunits of the arom complex are identical.

Other examples of multifunctional polypeptides are known; many are enzymes which are involved in aromatic amino acid biosynthesis. For example, anthranilate synthase of N.crassa contains a trifunctional polypeptide (Hulett and DeMoss, 1975); DAHP synthetase - chorismate mutase of Bacillus subtilis (Huang et al, 1974) and indole - 3 - glycerol-phosphate synthase from E.coli (Creighton and Yanofsky, 1966) both contain bifunctional polypeptides.

In this project, it has been assumed that all five arom enzyme activities have co-purified with shikimate dehydrogenase activity. Lumsden and Coggins, (1977), have established quantitative assays for two of the other activities, namely dehydroquinase and shikimate kinase. They have shown that these activities do indeed co-purify with dehydrogenase activity in a constant ratio during the final three stages of the purification. The purified complex has also been shown to contain the other two activities.

4.4. Kinetic studies.

A preliminary kinetic analysis of shikimate dehydrogenase was performed.

(i) Effect of varying the concentration of shikimate.

Shikimate dehydrogenase assays were performed, at pH10.6, as described by Lumsden and Coggins (1977). The reaction was started by addition of purified arom complex. The effect of

varying shikimate concentration at three different constant concentrations of $NADP^+$ was studied. A reciprocal plot of reaction rate versus shikimate concentration is shown in Fig 4.4.

(ii.) Effect of varying the concentration of $NADP^+$.

The effect of varying the concentration of $NADP^+$ at three constant concentrations of shikimate was also studied. Assays on purified arom complex were performed as described in 4.4 (i) above. A reciprocal plot of reaction rate versus $NADP^+$ concentration is shown in Fig. 4.5.

CHAPTER 5

SHIKIMATE DEHYDROGENASE STUDIES.

5.1. Chemical modification of shikimate dehydrogenase using sulphydryl reagents.

The effects of iodoacetate, iodoacetamide and p - mercuribenzoate upon the activity of shikimate dehydrogenase were studied. Between pH 7 and pH 8 these reagents react with sulphydryl groups of proteins as shown in Fig 5.1.

Purified enzyme, containing only a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (Fig 4.3), was used in these experiments. In general, 20µg of protein in 0·1M Tris/HCl, pH 7·5, containing 0·4mM DTT, was incubated with 10mM sulphydryl reagent in a final volume of 0·5ml. The reaction was started by addition of inhibitor at 22°C and stopped by aliquoting a small volume (10µl) into a prepared assay cuvette. Shikimate dehydrogenase assays were conducted as described in section 3.7 (vii). Experimental results showing the effects of iodoacetate, iodoacetamide and p- mercuribenzoate upon activity are shown in Tables 5.1, 5.2, and 5.3 respectively.

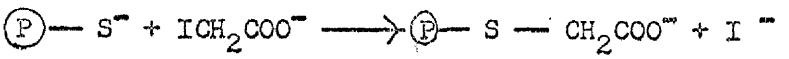
Initially, iodoacetate and iodoacetamide do not significantly affect the activity of shikimate dehydrogenase. However after a prolonged incubation of sixteen hours, a total loss of activity occurs when enzyme is incubated with iodoacetate and a 60% loss of activity results when incubated with iodoacetamide. It was thought that this loss of activity was probably the result of non-specific carboxymethylation of the protein, rather than due to a specific modification of the active site.

p-Mercuribenzoate produces a different effect when incubated with shikimate dehydrogenase. Total inhibition is effected after only twenty minutes incubation with shikimate dehydrogenase (Table 5.3). It was found that addition of dithiothreitol could restore come of the activity, suggesting that p-mercuribenzoate

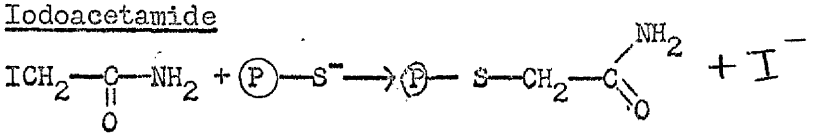
Fig 5.1.

The chemistry of the reactions of protein sulphydryl groups with various reagents at pH's between 7 and 9.

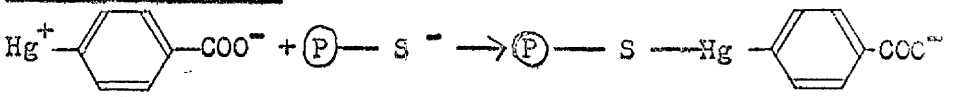
(a) Iodoacetate



(b) Iodoacetamide



(c) p-Mercuribenzoate



mercuribenzoate

Table 5.1.

Effect of iodoacetate on shikimate dehydrogenase activity.

Reaction Time (min)	Enzyme + Iodoacetate (Units/ml)	% activity remaining	Enzyme +buffer (units/ml)	%activity remaining
0	0.16	100	0.16	100
20	0.15	93.8	0.16	100
80	0.13	81.3	0.16	100
16 hrs	0.00	0	0.1	62.5

Table 5.2.

Effect of iodoacetamide upon shikimate dehydrogenase activity.

Reaction Time (min)	Enzyme + iodoacetamide (units/ml)	%Activity remaining	Enzyme +buffer (units/ml)	%activity remaining
0	0.23	1 00 %	0.23	100%
20	0.21	91.3%	0.24	104.3%
170	0.21	91.3%	0.22	91.7%
16 hrs	0.14	60.9%	0.12	52.2%

Table 5.3.

Effect of p-mercuribenzoate upon shikimate dehydrogenase actvity.

Reaction Time (min)	Enzyme + inhibitor (units/ml)	Enzyme + buffer (units/ml)
0	0.17	0.17
20	0.00	0.16

inhibited activity by reacting with sulphydryl groups of the protein. Thus p-mercuribenzoate must be able to react more rapidly, with the sulphydryl groups of shikimate dehydrogenase, than iodoacetate and iodoacetamide.

5.2. Effect of shikimate on inhibition of shikimate dehydrogenase by p-mercuribenzoate.

The experimental method, of section 5.1, was modified so that 20µg of protein was incubated with 3mM - p - mercuribenzoate and 3mM - shikimate. Blanks containing only protein and shikimate, and also protein and p-mercuribenzoate were prepared. Fig 5.2 shows the percentage of dehydrogenase activity remaining against time of incubation. Shikimate seems to afford some protection against inhibition of shikimate dehydrogenase by p-mercuribenzoate.

After forty minutes incubation of p-mercuribenzoate, with dehydrogenase, in the presence of shikimate 80% of activity remains, while in the absence of shikimate only 30% of the original activity was found.

5.3. Effect of iodoacetate on inhibition of shikimate dehydrogenase by p-mercuribenzoate.

The experimental method of section 5.1 was modified so that 20µg of protein was pre-incubated with 10mM-iodoacetate for 30 min at 22°C before addition of 10mM - p-mercuribenzoate. Blanks containing enzyme and enzyme plus p-mercuribenzoate were prepared. Results of a typical experiment are shown in Table 5.4. It can be seen that iodoacetate does not protect against inhibition of shikimate dehydrogenase by p-mercuribenzoate.

5.4. Possible explanations of results and future work suggested by these experiments.

The results obtained using sulphydryl reagents to chemically modify shikimate dehydrogenase and the effect of modification upon activity can probably be explained in several ways. A

Fig 5.2.

Effect of shikimate upon the inhibition of shikimate dehydrogenase by p-mercuribenzoate.

20 µg of shikimate dehydrogenase was incubated with 3mM-shikimate and 3mM-p-mercuribenzoate as described in section 5.2. Blanks containing only enzyme and also enzyme plus p-mercuribenzoate were also prepared. Dehydrogenase assays were performed at set time intervals.

—○— Blank containing enzyme only.

—X— Blank containing enzyme plus p-mercuribenzoate.

—△— Enzyme plus shikimate plus p-mercuribenzoate.

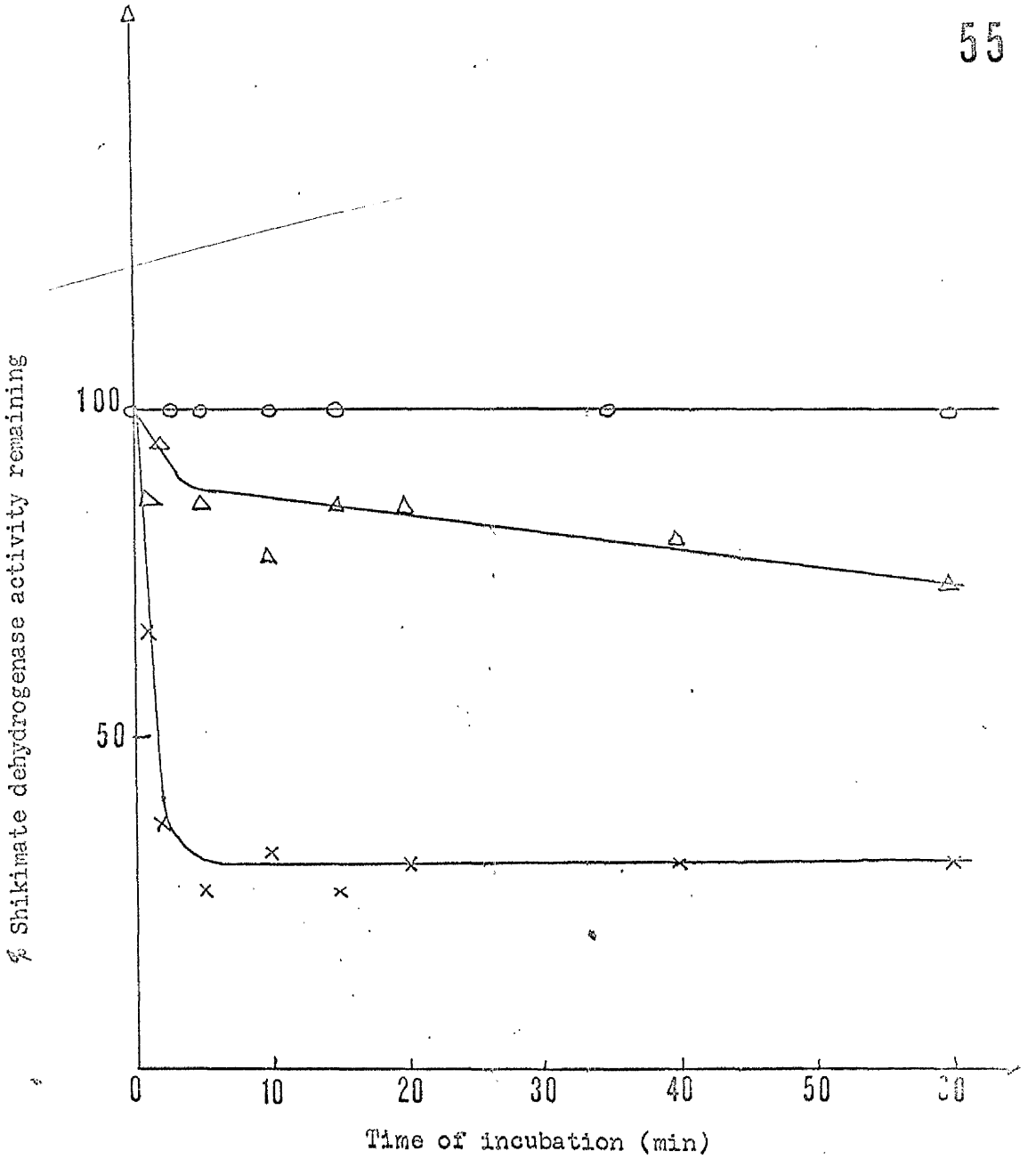

% Shikimate dehydrogenase activity remaining
100
50
0
10
20
30
40
50
Time of incubation (min)

Table 5.4.

Effect of pre-incubation with iodoacetate upon inhibition of shikimate dehydrogenase by p-mercuribenzoate.

Enzyme only	Enzyme only	Enzyme + iodoacetate	
% activity	%activity	%activity	
100%	100%	100%	After 30 min pre-incubation (no mercurial added) (+mercurial) (+mercurial)
100%	20%	20%	After addition and 20min incubation with mercurial.

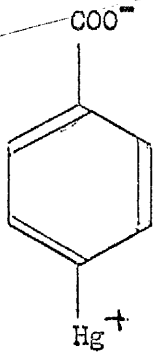
comparison of the structures of shikimate and p-mercuribenzoate could lead to one possible interpretation of these results. Both compounds possess carboxyl groups attached to a six carbon ring structure, although the conformation of the ring is different in each substance (Fig 5.3). By acting as a substrate analogue of shikimate, p-mercuribenzoate may be able to enter the active site, where it could inhibit the enzyme by reacting with a sulphydryl group which is essential for activity. Alternatively p-mercuribenzoate could react with a non-essential sulphydryl group in the active site and may only cause inhibition because its size prevents shikimate entering the active site.

Pre-incubation of the enzyme with shikimate does seem to protect against inhibition by p-mercuribenzoate (Fig 5.2). This does suggest that p-mercuribenzoate is reacting with a sulphydryl group in the active site of the enzyme. If p-mercuribenzoate is acting as a substrate analogue of shikimate then other substrate analogues, such as shikimate methyl ester and trihydroxybenzoic acid, should protect against inhibition by p-mercuribenzoate: an experiment of this nature would provide more evidence that p-mercuribenzoate was acting as a substrate analogue of shikimate.

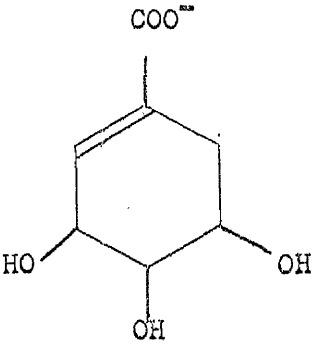
The failure of iodoacetate and iodoacetamide to inhibit shikimate dehydrogenase may be because these reagents cannot enter the active site: perhaps the active site is buried inside the molecule and in order to enter it the sulphydryl reagent must be more hydrophobic than the haloacetates. However, if the haloacetates can enter the active site, another possible explanation for their failure to inhibit activity could be that they are reacting with non-essential sulphydryl groups: being much smaller than p-mercuribenzoate, the haloacetates may not

Fig 5.3.

Comparison of the structures of shikimate and p-mercuribenzoate.



p-mercuribenzoate



shikimate

prevent shikimate entering the active site. If the haloacetates are reacting with sulphydryl groups in the active site, they must be reacting with different groups from p-mercuribenzoate, as pre-incubation of the enzyme with iodoacetate does not protect against inhibition by p-mercuribenzoate.

The experiments described in sections 5.1, 5.2, and 5.3 are only of a preliminary nature and hence attempts to explain the results obtained are very speculative. Further studies are necessary to test these hypotheses and also to quantitate the above results.

It has been assumed that p-mercuribenzoate inhibits shikimate dehydrogenase by reacting with sulphydryl groups. Addition of dithiothreitol can cause some reversal of inhibition which does suggest that p-mercuribenzoate is reacting with sulphydryl groups. However it would be useful to establish the total number of sulphydryl groups in the protein. One difficulty in accurately quantitating the number of sulphydryl groups in a protein is that extremely pure protein is required for such analysis. It was only towards the end of this project that pure arom was becoming readily available. A preliminary amino acid composition analysis suggests that there are sixteen sulphydryl groups per subunit (Lumsden and Coggins, unpublished results).

Another method of determining the total number of sulphydryl groups would be to treat denatured protein with 5, 5' - dithiobis (2 - nitrobenzoic acid), (DTNB), the number of sulphydryl groups reacting can be determined spectrophotometrically by measuring the change in extinction at 410mμ (Means and Feeney, 1971). It is also possible to determine the number of "buried" sulphydryl groups by reacting DTNB with native protein.

It would be interesting to find out if p-mercuribenzoate is reacting with a specific sulphydryl group. Cosson, Gros and

Talbot (1976) have developed a method for the selective replacement of p-mercuribenzoate bound to glutamate dehydrogenase by radioactive iodoacetate. It may be possible to adapt this method for the aroM complex and hence label the active site of shikimate dehydrogenase.

Balinsky and Davies (1961) have shown that p-mercuribenzoate inhibits shikimate dehydrogenase obtained from etiolated pea seedling; this inhibition is reversible by cysteine.

MATERIALS

Chemicals used in this work were mainly Analar grade and obtained from either Sigma (London) Chemical Co., or BDH Chemicals, particularly:-

Sigma (London) Chemical Co.	Deoxyribonuclease-1(type DN-CL)
Kingston-upon-Thames,	p-Hydroxymercuribenzoate
Surrey, U.K.	iodoacetamide
	iodoacetate
	Trizma-base
BDH Chemicals,	Biotin
Poole,	Bromophenol Blue
Dorset, U.K.	

Other sources of chemicals were as follows:-

Aldrich Chemical Co.,	benzamidine hydrochloride
Gillingham,	shikimic acid
Dorset, U.K.	
Boehringer Corp. (London) Ltd.	$NADP^+$
Lewes,	
Sussex, U.K.	
Eastman - Kodak,	phenylmethanesulphonyl fluoride.
Kirkby,	
Liverpool, U.K.	
Koch-Light Laboratories Ltd.	2-mercaptoethanol
Colnbrook,	
Bucks, U.K.	
Whatman Biochemicals,	DEAE-cellulose (DE52)
Maidstone,	cellulose phosphate(P11)
Kent, UK.	

REFERENCES.

Ahmed, S.I., and Giles, N.H., (1969) J. Bacteriol. 99 231 - 237

Balinsky, D., and Davies, D.D, (1961) Biochem. J. 80 292 - 296

Berlyn, M.B., and Giles, N.H., (1969) J. Bacteriol. 99 222 - 230

Burgoyne, L., Case, M.E., and Giles, N. H., (1969)
Biochem. Biophys. Acta. 191 452 - 462

Case, M.E., Burgoyne, L. and Giles, N.H., (1969) Genetics 63 581-588

Case, M.E., and Giles, N.H., (1968) Genetics 60 49 - 58.

Case, M.E, and Giles, N.H.,
(1971) Proc. Nat. Acad. Sci (USA) 68 58 - 62.

Cosson, M.P., Gros, C., and Talbot, J.C.,
(1976) Biochem. Biophys. Res. Commun. 72 1304 - 1310.

Creighton, T.E., and Yanofsky, C., (1966) J. Biol. Chem 291 4616-4624

Davis, B.D., (1951) J. Biol. Chem. 191 315 - 325

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

Davis, B.D., (1955) Adv. Enzymol 16 287 - 295

Davis, R.H., and De Serres, F.J., (1970)
in "Methods in Enzymology," Vol 17A, pp 79 - 143
(Tabor, H., and Tabor, C.W.,ed.) Academic Press, New York.

Fink, G.R., (1966) Genetics 53 445 - 459

Gaertner, F.H., (1972) Arch. Biochem. Biophys. 151 277 - 284

Gaertner, F.H., and DeMoss, J.A., (1970) in "Methods in
Enzymology" Vol 17A, pp 387 (Tabor, H., and Tabor, C.W., ed.)
Academic Press, New York.

Gibson, F., and Jackman, L.M., (1963) Nature 198 338 - 339

Gibson, M., and Gibson, F., (1964) Biochem. J. 90 248 - 253

Giles, N.H., Case, M.E., Partridge, C.W.H., and Ahmed, S.I.,
(1967) Proc. Nat. Acad. Sci (U.S.A.) 58 1453 - 1460

Giles, N.H., Partridge, C.W.H., Ahmed, S.I., and Case, M.

(1968) Proc. Nat. Acad. Sci. (U.S.A.) 58 1930 - 1937

Gold, A.M., (1967) in "Methods in Enzymology" Vol 11, pp 706-711 (Hirs, C.H.W., ed), Academic Press, New York.

Gross, S.R., and Fein, A., (1960) Genetics 45 885-904

Hayes, M.B., and Wellner, D., (1969) J. Biol. Chem. 244 6636-6444

Huang, L., Montoya, A.L. and Nester, E.W., (1974) J. Biol Chem. 249 4473 - 4479.

Hulett, F.M. and DeMoss, J.A., (1975) J. Biol. Chem 250 6648-6652

Laemmli, U.K., (1970) Nature (London), 227 680 - 685

Lowry, O.H., Rosenborough, W.J., Farr, A.L., and Randall, R.J., (1951) J. Biol. Chem. 193 265 - 275

Lumsden, J., and Coggins, J.R., (1977), Biochem. J. 161 599-607

Means, G.E, and Feeney, R.E., (1971) in "Chemical Modication of Proteins" pp 220 (Ed) Holden-Day, Inc., 500, Sansome St., San Francisco, California 94111.

Nishioka, Y., Demerec, M., Eisenstark, A., (1967) Genetics 56 341 - 351

Pittard, J., and Wallace, B.J., (1966) J. Bacteriol. 91 1494-1508

Pringle, J.R., (1975) Methods Cell Biol. 12 149 - 184

Rines, H.W., Case, M.E., and Giles , N.H., (1969) Genetics 61 789 - 800

Shapiro, A.L., and Maizel, J.V., (1969) Anal. Biochem 29 505-514

Siepen, D., Yu, P.-H., and Kula, M.-R., (1975) Eur. J. Biochem. 56 271 - 281.

Sprinson, D.B., (1960) Advances in Carbohydrate Chem. 15 235-270

Sprinson, D.B., Rothschild, J., and Sprecher, M., (196_. J. Biol. Chem. 238 3170 - 3175

Yoshida, S., (1969) Ann. Rev. Plant Physiol 20 41 - 62.

Jacobson, J.W., Hart, B.A., Doy, C.H., and Giles, N.H., (1972) Biochem. Biophys. Acta. 289 1-12.

Mares-Guia, M., and Shaw, E., (1965) J. Biol. Chem. 240 1579-1585.

Cole, K.W., and Gaertner, F.H., (1976) Biochem. Biophys. Res. Commun. 67 170-175.