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PROPERTIES OF FUNGAL 3-CARBOXYMUCONATE CYCLASES

by

Maria Teresa Ramalho

A thesis submitted to
The University of Kent at Canterbury
in part fulfilment for the degree of
Doctor of Philosophy

Faculty of Natural Sciences
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University of Kent

1987

This thesis presents an account of the work performed in the Biological Sciences Laboratory of the University of Kent at Canterbury between 1977 and 1982. All the experimental work is my own, except where indicated, and no part has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

M.T. Ramalho

March, 1987

To my Father

If he lived he would never tell me that I could not do it.

To my Mother

She consumed much of her last energies standing by my children in my absence

To Patricia and Cristina

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ABSTRACT

3-Carboxy-muconate cyclase [3-carboxymuconolactone lyase (decyclizing) EC.5.5.1.5] is a unique fungal enzyme involved in the dissimilation of protocatechuate *via* 3-oxoadipate. This enzyme mediates the lactonization of 3-carboxy-*cis*,*cis*-muconate to 3-carboxymuconolactone whereas in bacteria the same intermediate is lactonized to 4-carboxymuconolactone, by 3-carboxymuconate cycloisomerase [4-carboxymuconolactone lyase (decyclizing) EC.5.5.1.2].

The results of the detailed investigations on the bacterial enzymes of the 3-oxoadipate pathway have been the subject of extensive evolutionary discussions. The fungal 3-carboxymuconate lactonizing enzyme, which determines the divergence between the procaryotic and eucaryotic pathways has therefore raised a considerable interest. The *Aspergillus niger* L6 cyclase has been previously purified and some of its properties investigated. It has also been used to raise antiserum which cross-reacted with extracts of several fungi imperfectii, induced by growth on *p*-hydroxybenzoate or protocatechuate, but not with the analogous lactonizing enzyme from *Pseudomonas putida*.

In the present work an improved purification method of the *A. niger* L6 cyclase, involving ammonium sulphate fractionation, anion-exchange chromatography, gel filtration, hydrophobic chromatography and chromatofocusing achieved a 3380-fold purification of the enzyme. The native molecule behaved as a molecule with an apparent mol.wt. of 162 000 by calibrated gel permeation chromatography and its subunit size was 42 000 ± 1 000 by SDS-polyacrylamide gel electrophoresis. The enzyme appears therefore to be tetrameric. Its Michaelis constant at the optimum pH is 15μM. The product, 3-carboxymuconolactone, the substrate analogue 1-methyl 3-carboxy-*cis*,*cis*-muconate and several metallic ions are inhibitory to the enzyme. In the concentration range tested, *cis*,*cis*-muconate and the other isomeric muconic acids did not affect the enzyme activity. The isoelectric point determined by chromatofocusing is 4.85 ± 0.05.

Other fungal cyclases which are immunologically related to the *A. niger* enzyme appear also to be tetrameric, with subunit sizes in the range 38 500-43 000 and molecular sizes of 150 000-180 000, except the *Scopulariopsis candida* cyclase which behaved as a larger molecule by calibrated gel permeation chromatography. The Michaelis constants of these enzymes fall in a narrow range (12-17μM) but their isoelectric points vary between 4.2 and 6.0. Possible evolutionary implications of these results are discussed.

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CHAPTER 1

INTRODUCTION

1.1. The 3-Oxoadipate Pathway in the Degradation of Aromatic Compounds

Lignin is one of the most abundant natural polymers and one of the main sources of aromatic compounds in the environment. This fact alone would justify the research on aromatic catabolism as a major process in the overall recycling of carbon in nature (Dagley, 1978a). The practical implications of these studies are, however, no less important. Detailed knowledge of the degradative capabilities of microorganisms plays a role in the search for safer alternatives to hazardous synthetic aromatics which are increasingly appearing in the environment (Dagley, 1978a; Fewson, 1981).

The degradation of aromatic compounds, defined as their transformation into metabolites that can be directly used by the cell as carbon and energy sources involves a peripheral phase, leading from the primary aromatic substrates to key intermediates that can be directly dearomatized, followed by a restricted number of central pathways which transform the last aromatic intermediates into products that can be fed into the common metabolic pathways (Dagley, 1978a,b; 1981; Cain, 1980).

The solutions developed by aerobic microorganisms for the dearomatization step are mediated by dioxygenases. These enzymes, however, only accept benzenoid substrates carrying two hydroxyl substituents, either **ortho** or **para** to each other (Fewson, 1981; Dagley, 1982). The oxidative ring-cleavage and subsequent metabolic transformations of 1,4-diphenols are described elsewhere (c.f. reviews by Dagley). The **ortho**-diphenols may be cleaved by 1,2--dioxygenases (intradiol or **ortho** ring-cleavage) or by 1,6- or 2,3-dioxygenases (extradiol or **meta** ring-cleavage). The 1,2-dioxygenases specific for catechol and protocatechuate are the first enzymes of a central pathway in aromatic catabolism, the 3-oxoadipate pathway, inducible in a wide range of bacteria and fungi commonly found in soil or water (Cain, Bilton & Darrah, 1968; Stanier & Ornston, 1973; Ornston & Parke, 1977; Cain, 1980; Fewson, 1981). Two sets of converging metabolic transformations convert protocatechuate or catechol to succinate + acetyl-CoA, via 3-oxoadipate. The chemical reactions of the bacterial protocatechuate and catechol branches of the pathway are outlined in Fig.1.1 and those of the fungal variant of the protocatechuate branch in Fig.1.2. The routes for dissimilation of catechol via 3-oxoadipate are identical in bacteria and fungi.

3-Oxoadipate appears,however, to be a key metabolic intermediate since it may derive from other precursors by a different mechanism:

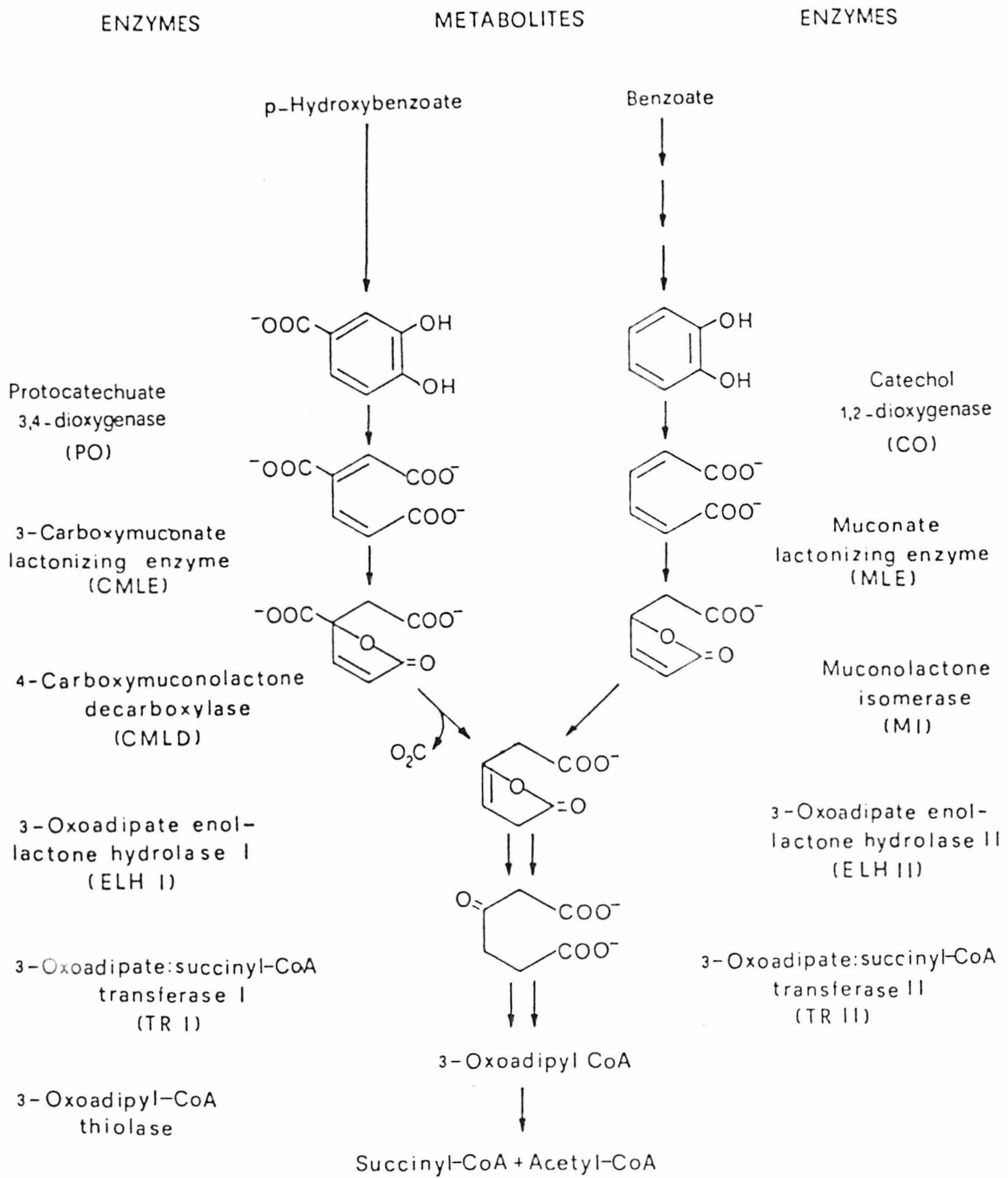


Fig.1.1. Intermediates and enzymes of the 3-oxoadipate pathway in bacteria. The abbreviations used in the text for the names of the enzymes are shown in brackets.

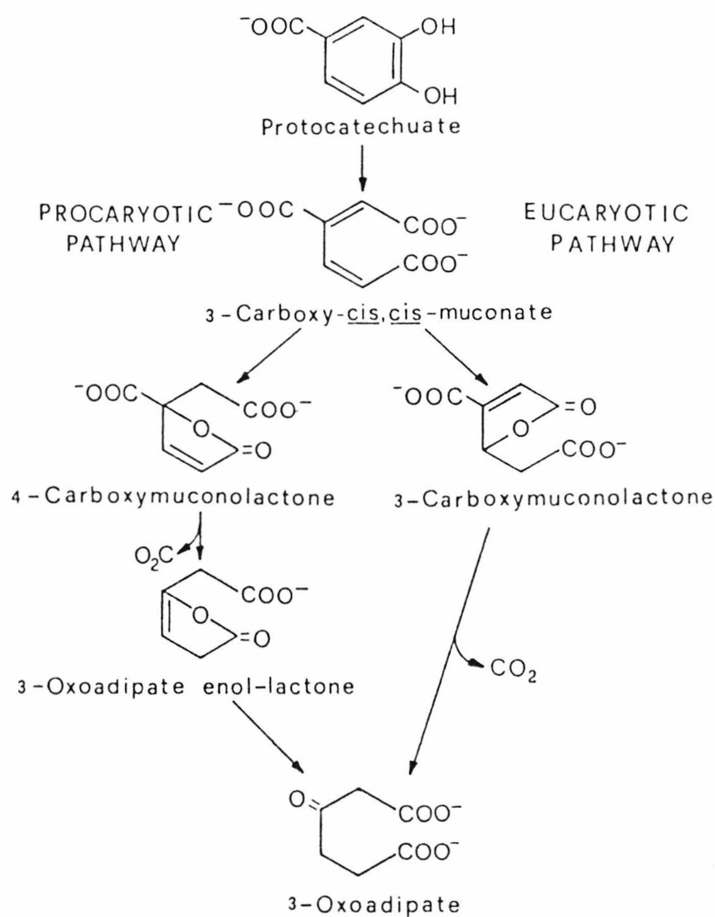
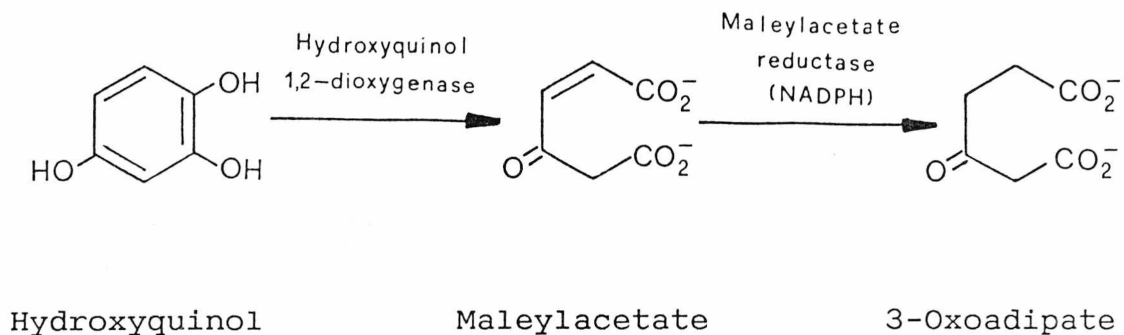


Fig.1.2. Comparison of the protocatechuate branch of the 3-oxoadipate pathway in bacteria and fungi. Procaryotes form an unstable 4-carboxymuconolactone, whereas eucaryotes yield 3-carboxymuconolactone.



Hydroxyquinol Maleylacetate 3-Oxoadipate

This sequence has been identified in the catabolism (i) of benzoate, gentisate and resorcinol in the soil yeast Trichosporon cutaneum (Anderson & Dagley, 1980; Gaal & Neujhar, 1979), (ii) of resorcinol in a P. putida strain (Chapman & Ribbons, 1976), (iii) of vanillate in the white-rot fungus Sporotrichum pulverulentum (Buswell & Eriksson, 1979; Ander, Eriksson & Yu, 1983) and also (iv) of salicylate, resorcinol and hydroxyquinol in A. niger (Shailubhai et al., 1983, and references therein).

1.2. The Bacterial 3-Oxoadipate Pathway

The earlier biochemical investigations which contributed for the elucidation of the 3-oxoadipate pathway in representatives of the genera Pseudomonas, Acinetobacter and Alcaligenes have been summarized by Stanier & Ornston (1973). The inducibility of the pathway in the actinomycete Nocardia opaca has equally been demonstrated (Cain,1980).

1.2.1. **Chemical and mechanistic features.** The first three steps of the catechol and protocatechuate branches (Fig.1.1) are chemically analogous reactions: oxidative intradiol ring-cleavage followed by lactonization and allylic rearrangement of the isomeric lactones. The later step is coupled to a decarboxylation in the protocatechuate intermediate, yielding 3-oxoadipate enol-lactone, the first metabolite common to both branches. In the subsequent reactions the enol-lactone is hydrolyzed to 3-oxoadipate and converted to 3-oxoadipyl-CoA, with succinyl-CoA as CoA donor; thiolytic cleavage of 3-oxoadipyl-CoA finally yields acetyl-CoA and succinyl-CoA.

Independent chemical investigations (Elvidge *et al.*, 1950a; Ainsworth & Kirby, 1968) have contributed to the unambiguous demonstration that ring cleavage of catechol and protocatechuate leads to the postulated *cis*, *cis*-isomers of muconate and 3-carboxymuconate, respectively. Avigad & Englard (1969) established that the intermediate lactone in the catechol branch was (4S)-(+)-muconolactone and demonstrated that the lactonization reaction occurred by a *syn*-addition across the 4,5-double bond. Because of the instability of the analogous 4-carboxymuconolactone, which decarboxylates spontaneously, its absolute configuration and the stereochemical course of the reaction catalyzed by the *P. putida* enzyme were only recently established (Chari *et al.*, 1985):the product of the lactonization was trapped by reduction to (4S)-homocitric lactone, corresponding to (4R)-4-carboxymuconolactone; when the reaction was performed

in D_2O the product was identified as (4R,5R)-4-carboxy-[5-^{2}H]-muconolactone, indicating that the reaction proceeded by an _anti_-addition across the 4,5-double bond.

Recent contributions to the elucidation of the mechanism of the reaction mediated by _cis_, _cis_-muconate cycloisomerase (muconate lactonizing enzyme) from _P. putida_ have been reported. Ngai, Ornston & Kallen (1983) demonstrated that the enzyme has a single Mn^{2+}-binding site per subunit and deduced the dissociation constant of the Mn^{2+}-enzyme complex by titration of the free enzyme with Mn^{2+}, monitored by EPR spectroscopy and steady-state kinetic measurements. Ngai & Kallen (1983) investigated the primary and secondary kinetic and equilibrium deuterium effects on the kinetic parameters of the reaction and have shown that they are consistent with a stepwise mechanism involving a proton abstraction at C(5) as the rate-limiting step in the conversion of the muconolactone to _cis_, _cis_-muconate.

1.2.2. Regulation. In some bacterial groups the reactions common to both branches of the pathway are catalyzed by isofunctional enzymes independently synthesized for specific operation in one of the branches. This is the case in _Acinetobacter calcoaceticus_ where protocatechuate induces one enol-lactone hydrolase (**ELH-I**) and one 3-oxoadipate: succinyl-CoA transferase (**TR-I**).The corresponding enzymes of the catechol branch (**ELH-II** and **TR-II**) are induced by _cis_, _cis_-muconate. The regulation mechanism is different in fluorescent _Pseudomonas_ species where

3-oxoadipate induces the synthesis of the carboxymuconate lactonizing enzyme (**CMLE**), carboxymuconolactone decarboxylase (**CMLD**), enol-lactone hydrolase (**ELH**) and of the transferase. In the catechol branch, cis, cis-muconate regulates the synthesis of the isomerase (**MI**). 3-Oxoadipate induces the subsequent enzymes (**ELH** and **TR**) so that **CMLE** and **CMLD** appear as gratuitously induced enzymes in the course of the catabolism of catechol by fluorescent Pseudomonas even though they have no physiological function in this branch of the pathway. In the genus Azotobacter only the protocatechuate branch is induced and catechol is dissimilated by a route involving **meta**-cleavage of the ring. Full details of the regulation mechanism in these and other bacterial genera have been reviewed by Stanier & Ornston (1973) and Cain (1980).

1.3. The Fungal 3-Oxoadipate Pathway

1.3.1 The protocatechuate branch. Two important differences distinguish the fungal and bacterial variants of the protocatechuate branch (Fig.1.2). Firstly, the formation of 3-carboxymuconolactone from 3-carboxymuconate and secondly, the conversion of this lactone to 3-oxoadipate. The formation of the fungal lactone was first demonstrated by Gross, Gafford & Tatum (1956) in Neurospora crassa and by Cain, Bilton & Darrah (1968) in a wide range of soil fungi. The participation of only one enzyme with both decarboxylase and hydrolase activities was first reported by Thatcher

(1972) and confirmed by independent genetic evidence (Cain, 1980).

The lactonization reaction catalyzed by the *N. crassa* enzyme is a *syn*-addition across the 4,5-double bond as demonstrated by Kirby, O'Loughlin & Robins (1975) who identified the product formed from 3-carboxy-[5-^{2}H]-*cis*, *cis*-muconate as the (4S,5S)-3-carboxy-[5-^{2}H]-muconolactone. The participation of a histidine residue in the catalytic reaction of the *A. niger* lactonizing enzyme was suggested by the experiments of photo-inactivation in the presence of Rose Bengal (Thatcher & Cain, 1975). The presence of a thiol group at or near the active site has been demonstrated by the inhibitory effect of *p*-chloromercuribenzoate (Halsall, 1971) and also by titration of the enzyme with 5, 5'-dithiobis-(2-nitrobenzoic acid), as described by Thatcher & Cain (1975).

The next step of the fungal protocatechuate branch involves a 1,3-suprafacial proton shift, as shown by Hill, Kirby & Robins (1977) using an enzyme preparation from *N. crassa*. This mechanism is consistent with the formation of an intermediate enol-lactone which, however, has never been isolated (see section 7.6).

The regulatory mechanisms of the protocatechuate branch are very similar in most fungal genera so far examined. Protocatechuate induces all the enzymes in the pathway, including the transferase but only the lactonizing enzyme and the hydrolase/decarboxylase are coordinately induced (Cain, 1980).

1.3.2. The catechol branch. The catechol branch, if inducible, is chemically identical in procaryotes and eucaryotes. Some details of its regulation mechanism are not fully elucidated but the available evidence suggests that in most fungi, with the reported exception of Aureobasidium pullulans, one single 3-oxoadipyl: succinyl -CoA transferase is common to the catechol and protocatechuate branches (Cain, 1980).

Most of the enzymes mediating the specific reactions of the catechol branch in eucaryotes have not been investigated in detail. Gaal & Neujhar (1980) however, reported some properties of the muconate cyclase from Trichosporum cutaneum. The enzyme exists in two forms, separable by isoelectric focusing. Both are apparently monomeric (mol.wts.48 000 $\pm$ 2 000) but have different contents of essential thiol groups. Unlike the bacterial form, this enzyme does not require Mn^{2+} for activity. The yeast enzyme appears also to be slightly less substrate-specific than the Pseudomonas enzyme.

The catechol-1,2-dioxygenase from A. niger has also been partially purified (Ninnekar & Vaidyanathan, 1981) but little information is available since the purified enzyme preparation appeared to be highly unstable.

1.4. Comparative Studies of Enzymes of the 3-Oxoadipate Pathway in Bacteria

Most of the experimental evidence concerning the enzymology of the 3-oxoadipate pathway has been extensively reviewed by Ornston & Yeh (1982) and will therefore be only briefly summarized to illustrate major conclusions, except where further information is available.

1.4.1. Evolutionary homology of isofunctional enzymes from different sources. The 3-oxoadipate pathway is an example of a metabolic route regulated by different mechanisms in different microbial groups. As stressed by Stanier & Ornston (1973) this situation may reflect either the independent evolution of its enzyme complement or, alternatively, the divergent evolution of the enzymes and the independent acquisition of the regulation mechanisms. Evidence derived from serological studies supported the second hypothesis for the following isofunctional enzymes: (a) protocatechuate dioxygenases from A. vinelandii, P. putida and A. calcoaceticus (Durham et al., 1980), (b) carboxymuconate cycloisomerases from A. vinelandii and P. putida and (c) P. putida tranferase and A. calcoaceticus transferase-I (Yeh & Ornston, 1981).

Some of the isofunctional enzymes from P. putida and A. calcoaceticus were, however, serologically unrelated: the muconate cycloisomerases, muconolactone isomerases, carboxymuconate cycloisomerases and carboxymuconolactone

decarboxylases (Stanier et al., 1970; Patel & Ornston, 1976).

Amino acid sequencing data subsequently reinforced the conclusion of evolutionary homology for some of these enzymes and detected further relationships. The decarboxylases from A. vinelandii and A. calcoaceticus shared approximately 83% and 50% of identical amino acid residues, respectively, with the P. putida decarboxylase (Yeh, Fletcher & Ornston, 1980b; Yeh et al., 1981), the muconolactone isomerases from P. putida and A. calcoaceticus were identical in approx. 50% of the compared NH2-terminal amino acid residues (Yeh et al., 1978) and the P. putida enol-lactone hydrolase shared some amino acids and small peptides with both of the A. calcoaceticus hydrolases (Yeh, Fletcher & Ornston, 1980 a).

As a step in their characterization the subunit sizes and oligomeric compositions of the enzymes of the bacterial 3-oxoadipate pathway were also determined. As shown in Table 1.1 several isofunctional enzymes share similar molecular and (or) subunit sizes. It should be emphasized, however that these parameters of the protein molecules do not provide, per se, enough grounds to infer evolutionary relationships, as was demonstrated by London & Kline (1973) in their studies on frutose-1,6-bis-phosphate aldolases from lactic acid bacteria. Indeed, after the advent of X-ray crystallography of proteins it became apparent that the tertiary structures of divergent proteins were highly conserved in the course of evolution. This conservative feature is compatible, nevertheless, with many neutral amino

TABLE 1.1.

Oligomeric compositions of isofunctional enzymes in

P. putida and A. calcoaceticus

Enzyme(a)	P. putida	A. calcoaceticus	References
CMLD	6x13200		Parke(1979); Yeh, Fletcher & Ornston (1980 b)
		6x13300	Yeh, Fletcher & Ornston (1980 b)
MI	10(or 12)x11300		Parke (1979)
		10(or 12)x12500	Patel, Meagher & Ornston (1974)
ELH	3x11000 (b)		McCorckle et al. (1980)
ELH-I/II		2X12000	Patel, Mazundar & Ornston (1975)
TR	2x(25000+25000)		Yeh & Ornston (1982)
TR-I		2x(25000+25000)	Yeh & Ornston (1982)

(a) Abbreviations are: **CMLD**, carboxymuconolactone decarboxylase; **MI**, muconolactone isomerase; **ELH**, 3-oxoadipate enol-lactone hydrolase; **TR**, 3-oxoadipate succinyl-CoA transferase.

(b) More recent evidence based on amino acid sequence data (Yeh & Ornston, 1984) indicate that the enzyme is monomeric.

acid replacements, deletions or insertions which do not substantially alter the specificity of the proteins (Hartley, 1974; Dickerson, 1980).

The replacement of just one or a few key amino acid residues may, however, account for important changes in substrate specificity (Hartley, 1974) or in regulatory properties (Perutz, 1984) if the replacement are with ionically very different residues (e.g. glutamate for glycine or histidine, lysine for leucine, or histidine for phenylalanine).

One possible interpretation of the observed homologies between isofunctional enzymes of the 3-oxoadipate pathway is that intergeneric gene transfer has occurred. Ornston & Yeh (1982) however, deduced from the serological studies of Patel & Ornston (1976), involving several strains of fluorescent Pseudomonas, that this event was most unlikely. It seems, therefore, that the pathway probably had an ancient origin, preceeding the differentiation of the bacterial genera in which it has been investigated.

Another significant feature emerging from the experimental data is that different types of enzymes have diverged at different rates. Some of them, such as the protocatechuate dioxygenases, the carboxymuconate decarboxylases or the muconolactone isomerases displayed either considerable conservation of their immunological determinants or a high rate of amino acid homologies,whereas the enol-lactone hydrolases were extensively divergent. Assuming, as suggested by experiments on directed evolution

(cf. reviews by Clarke, 1974, 1978; Hartley, 1979) that selective pressure favours enzyme evolution, a high degree of conservation of enzyme structure may imply an approach to their optimum level of efficiency. Alternatively, their evolutionary divergence could have been a comparatively recent event but this condition certainly does not apply to the 3-oxoadipate pathway.

1.4.2. Evolutionary divergence of enzymes mediating parallel steps within a single cell line. Immunological studies involving the enzymes from a single cell line which mediate parallel steps in each branch of the pathway revealed without exception, their serological unrelatedeness. The *Pseudomonas* enzymes mediating lactonization and lactone isomerization (Stanier *et al.*, 1970) and the two isofunctional hydrolases in *A. calcoaceticus* (Patel, Mazundar & Ornston, 1975) illustrate this feature. Such findings were supported by comparisons of the NH_2-terminal sequences of decarboxylases and isomerases (Yeh, Fletcher & Ornston, 1980 b) and of *A. calcoaceticus* hydrolases (Yeh, Fletcher & Ornston, 1980 a) in which percentages of amino acid homologies of only 20-30% were revealed.

The muconate and carboxymuconate cycloisomerases from *P. putida* have similar subunit sizes of approx. 40 000 (Patel, Meagher & Ornston, 1973; Meagher & Ornston, 1973) but they probably differ in their oligomeric compositions. The carboxymuconate cycloisomerase has an apparent mol.wt. of 190 000 (Ornston, 1966 a) and the muconate cycloisomerase

has been described as an hexamer of mol.wt. 252 000 (Avigad et al., 1974). In contrast with the enzyme operating in the protocatechuate branch, the muconate cycloisomerase has an absolute requirement for Mn^{2+} and the stereochemical courses of the two reactions are opposite, as described in section 1.2. Analysis of molecular models of the substrates in plausible conformations at the active site of the respective enzymes (depicted in Fig.1.3) suggests widely different structural organizations of binding and catalytic residues. If the principle of selection for a suitable binding site has operated in the acquisition of these activities and if they had a common precursor it would be reasonable to expect conservation of the stereochemistry (Hanson & Rose, 1975). Indeed, substantial alterations in the structure of the active site might prevent the correct folding of the protein chains and their evolution through active forms (Hartley, 1979).

1.4.3. The analogous dioxygenases. The catechol dioxygenase from P. arvilla and the protocatechuate dioxygenase from P. aeruginosa are non-heme iron-containing proteins which have been extensively investigated by spectroscopic methods. They belong to the group of iron-tyrosinate proteins, as established by resonance Raman spectroscopy; X-ray absorption studies have shown that the active site Fe^{3+} is coordinated by two tyrosines, two histidines, one H_2O molecule and a weakly bound and unidentified residue (Roe et al., 1984 and references

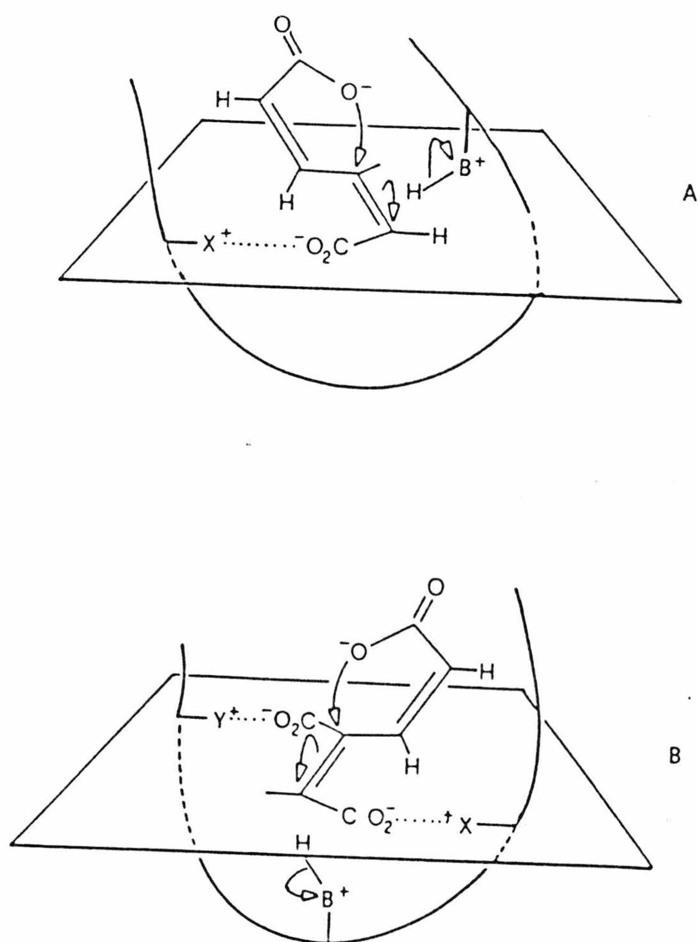


Fig.1.3. Putative modes of binding of cis, cis-muconate (A) and 3-carboxy-cis,cis-muconate (B) at the active sites of the respective bacterial lactonizing enzymes. The binding residues for the carboxyl groups in the substrates are represented by X^+ and Y^+. Nucleophilic attack by carboxyl-1 at C(4) leads to the formation of intermediate carbanions which are protonated at C(5) by the donor HB^+.

therein). The data on the composition of the two enzymes have been reviewed by Lauffer & Que (1982). The protocatechuate dioxygenase is an octamer of $\alpha_2\beta_2Fe^{3+}$ units, of mol.wt. 780 000, whereas the catechol dioxygenase has a composition $\alpha\beta Fe^{3+}$ and a mol.wt. 63 000. The same authors described the application of ^1H and ^2H n.m.r. spectroscopy to the study of the complexes formed by the dioxygenases with the substrate analogue 4-methyl-catechol. The results showed that while this analogue chelated the ferric ion in protocatechuate dioxygenase it formed a monodentate complex with catechol dioxygenase.

The fact that both enzymes are iron-tyrosinate proteins does not imply that they are evolutionary related since the transferrins and the purple acid phosphatases also belong to the same group (Hay, 1984). Que et al. (1977) have demonstrated, however, that catechol is attacked by protocatechuate dioxygenase at 0.4% of the rate of the natural substrate and that the carboxylate of protocatechuate interacts with a lysine residue in the enzyme through a salt bridge. The divergence of the two existing enzymes from a common precursor is therefore conceivable but mutations leading to altered association of the subunits and to the change of that lysine residue into a non-polar one, in catechol dioxygenase, would presumably be required.

1.4.4. Evolutionary relatedeness of enzymes with different specificities. The aligned sequences of P. putida transferase and of the enol-lactone hydrolases, carboxymuconolactone decarboxylases and muconolactone isomerases from P. putida and A. calcoaceticus are depicted in Fig.1.4, with data taken from Yeh & Ornston (1982) and Ornston & Yeh (1982). The partial amino-terminal sequence of the δ^5-3-ketosteroid isomerase from P. testosteroni was also included since Meagher (1977) detected significant homologies between this enzyme and the P. putida muconolactone isomerase. In this alignment an attempt was made to emphasize, firstly, which amino acid residues in each sequence were common to enzymes of different specificity and, secondly, those common to enzymes of the same specificity. The two types of residues, taken together, accounted for over 50% of the compared sequences. Particularly remarkable was the feature that only 5 out of 22 positions in the sequences of the transferase subunits were not represented in the other enzymes.

The sequencing data together with the demonstration of the serological relatedeness of decarboxylases and transferases (Yeh & Ornston, 1982) and the requirement for a free thiol group shared by transferases and hydrolases (Yeh & Ornston, 1984) strongly suggest a common ancestry of the structural genes for the enzymes mediating the terminal steps of the 3-oxoadipate pathway. Such an hypothesis had already been advanced by Ornston (in Stanier & Ornston, 1973) on the grounds of overlapping binding sites and appears even

Enzyme																						
P. putida	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Transferase (α, β)	Thr	ILE	Asn	LYS	THR	Tyr	Glu	ARG	Ile	Glu	MET	ALA	Gln	Glu	VAL	Ile	Ala	ASP	Ile	GLN	GLN	THR
P. putida	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Transferase (α, β)	MET	ILE	THR	LYS	Lys	LEU	Glu	ARG	Thr	Glu	MET	ALA	VAL	ARG	ARG	ALA	Ala	ASP	Ile	GLN	GLN	HIS
A. calcoaceticus	5	6	7	8	9		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Hydrolase - I	Ser	ILE	Met	Ile	THR	---	Asn	ARG	Gln	Gly	Lys	Thr	Leu	Ser	VAL	Glx	Ile	ASX	Tyr	Pro	GLX	Asx
P. putida		1	2	3	4	5		6	7	8	9		10	11	12	13	14	15	16	17	18	19
Hydrolase		Ala	His	Leu	Gln	LEU	---	Ala	Asp	Gly	Val	---	Leu	Asn	Tyr	Gln	Ile	ASP	Gly	Pro	Glu	Asn
A. calcoaceticus		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Hydrolase - II	---	Phe	Lys	Asp	THR	LEU	Thr	Ala	Gln	Asp	Val	ALA	Leu	Asp	Tyr	ALA	Thr	Phe	Gly	GLN	Ala	Asp
A. calcoaceticus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Decarboxylase	MET	Asn	Asp	Glu	Gln	Arg	Tyr	Lys	Gln	Gly	Leu	Glu	VAL	ARG	Thr	Glu	Val	Leu	Gly	Glu	Lys	HIS
P. putida	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Decarboxylase	MET	Asp	Glu	LYS	Gln	Arg	Tyr	Asp	Ala	Gly	MET	Gln	VAL	ARG	ARG	ALA	Val	Leu	Gly	Asp	Ala	HIS
A. calcoaceticus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		19	20	21
Isomerase	MET	Leu	Phe	Gln	Val	Arg	Met	Asp	Val	His	Leu	Pro	VAL	Ser	Met	Pro	Thr	ASP	---	GLN	Ala	Asn
P. putida	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		20	21
Isomerase	MET	Leu	Phe	His	Val	Lys	Met	Thr	Val	Lys	Leu	Pro	VAL	Asp	Met	Asp	Pro	Ala	Lys	---	Ala	THR
P. testosteroni	1	2	3	4	5	6	7	8	9	10	11	8	9	10	11	12		13	14		15	16
KS-Isomerase	MET	Asn	THR	Pro	Glu	His	Met	Thr	Ala	Val	Val	Thr	Ala	Val	Val	Gln	---	ARG	Tyr	---	Val	Ala

Fig.1.4. Aligned NH_2-terminal amino acids sequences of enzymes of the 3-oxoadipate pathway. The sequence data were taken from Yeh & Ornston (1982) for the *P. putida* transferase, from Ornston & Yeh (1982), for other *P. putida* and *A. calcoaceticus* enzymes and from Meagher (1977) for the *P. testosteroni* ketosteroid isomerase. Residues of the transferase represented in other enzymes are written in bold capital letters. Identical residues in isofunctional enzymes are indicated in bold type and residues which are common to enzymes of different specificities are underlined. Gaps in the sequences are indicated by dashes.

more plausible when the enzymes act by similar mechanisms. An example illustrating this possibility was described by Jeffcoat & Dagley (1973). In *Klebsiella aerogenes* and *Pseudomonas acidovorans* glucarate is dehydrated to 4-deoxy-5-oxoglucarate by glucarate hydrolyases. This product then undergoes aldol fission in *K. aerogenes* and dehydration-decarboxylation in *P. acidovorans*. The purified glucarate hydrolyase from *P. acidovorans* displayed, nevertheless, a residual aldolase activity. Since the enzyme-mediated formation and aldol fission of 4-deoxy-5-oxoglucarate both require electron sinks (Mg^{2+}) and involve similar electron shifts, the aldolase gene could have been derived from that encoding for glucarate hydrolyase.

These conditions appear to apply to the decarboxylases, isomerases, hydrolases and transferases. Indeed,the reactions mediated by these enzymes can be described within similarly organized active sites (Figs.1.5 to 1.8). All of them presumably require a binding residue for the C(6) carboxylate, and a basic residue to act as a proton exchange group or activator of a water molecule. The thiol group, which is nucleophilic in the hydrolase reaction, with a pH optimum of 8-10 (Ornston, 1966 a) and also in the transferase reaction, is probably involved in the formation of intermediate thioesters. In the reactions mediated by the decarboxylase and isomerase the participation of a thiol group has not been demonstrated but it could, if present, assist the stabilization of the intermediate carbanions. It is interesting to note that the pH optima of the isomerase

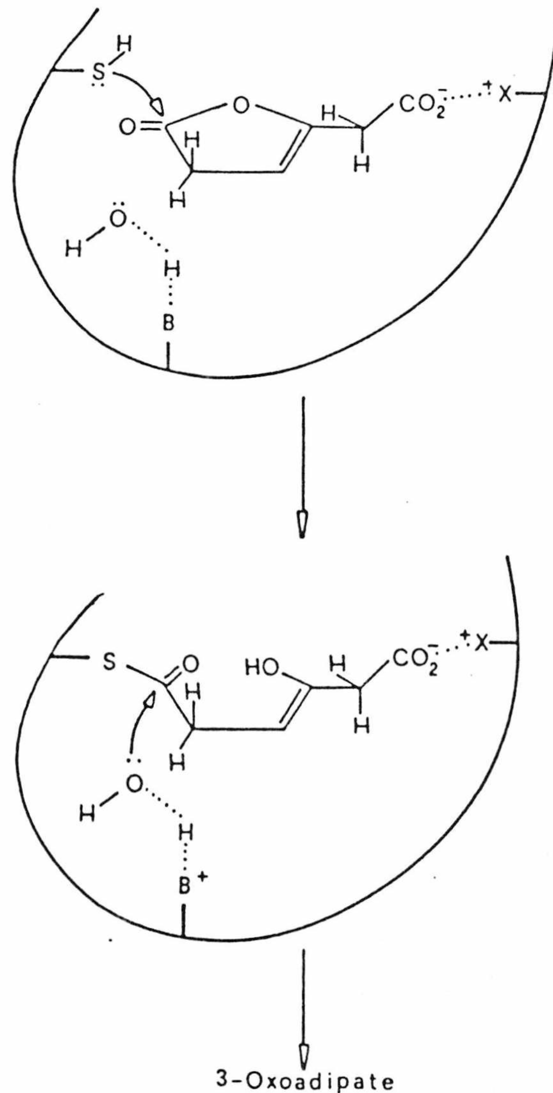


Fig.1.5. A possible reaction mechanism for the bacterial 3-oxoadipate enol-lactone hydrolase. Formation of an intermediate thioester is assumed. The interaction of a water molecule with a basic residue in the enzyme would increase its nucleophilicity and facilitate the hydrolysis of the thioester.

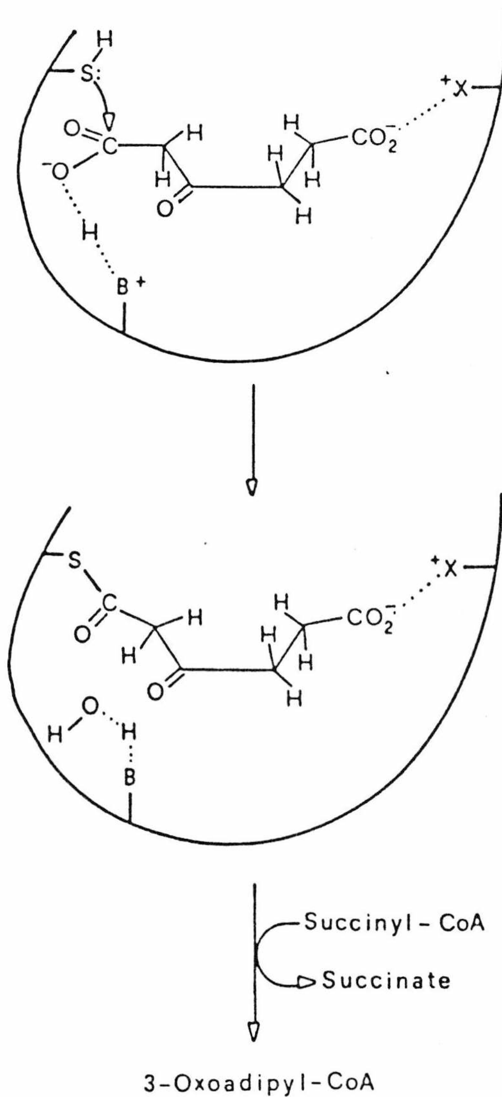


Fig.1.6. A possible mechanism for the formation of an intermediate thioester in the reaction of the 3-oxoadipate: succinyl-CoA transferase, facilitated by a proton-donating group, HB^+, at the active site of the enzyme. Thioester exchange would then account for the products.

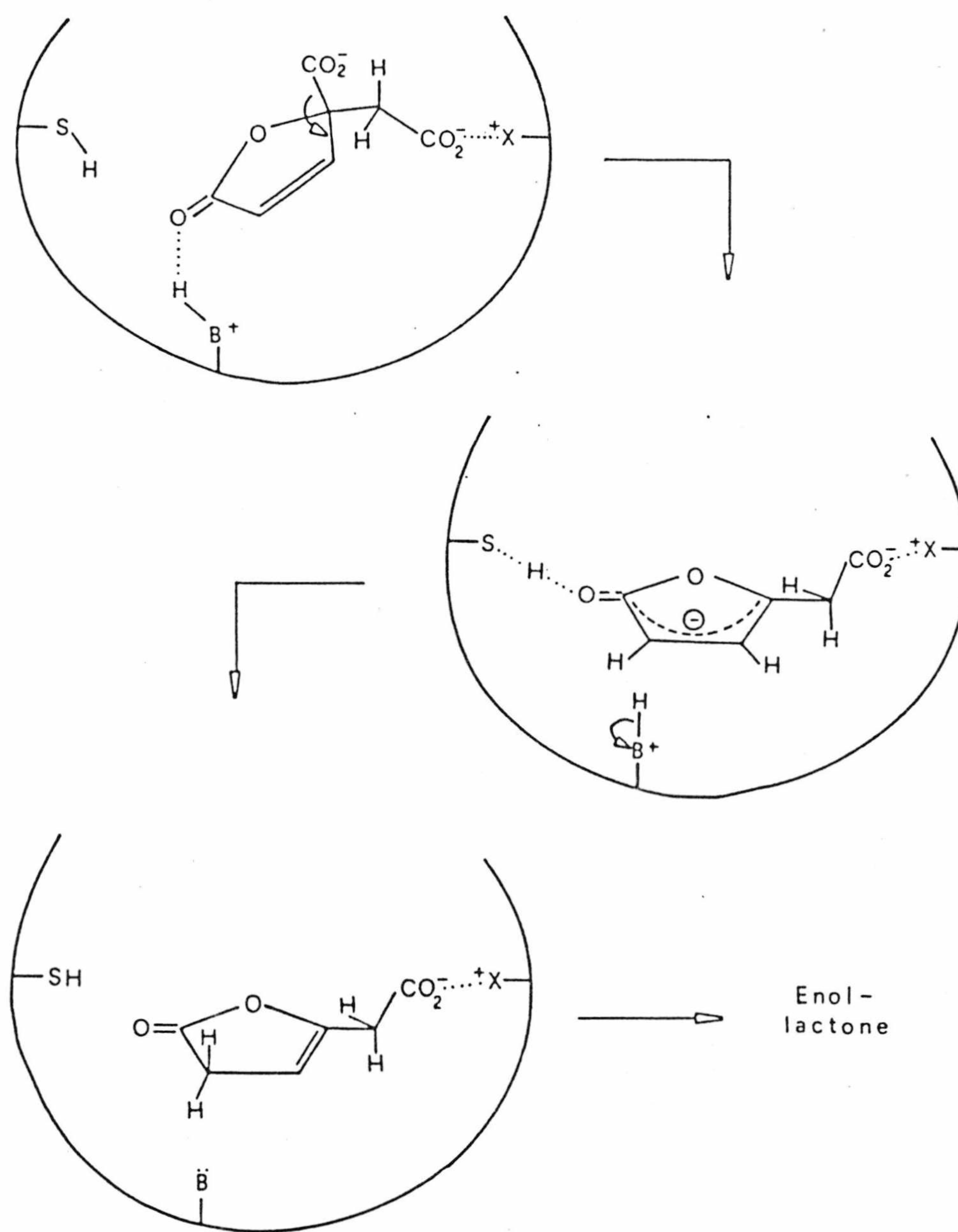


Fig.1.7. A possible reaction mechanism for 4-carboxymucono-lactone decarboxylase, assisted by a proton donor (HB^+) at the active site. A thiol group, if present, could participate in the stabilization of the intermediate carbanion.

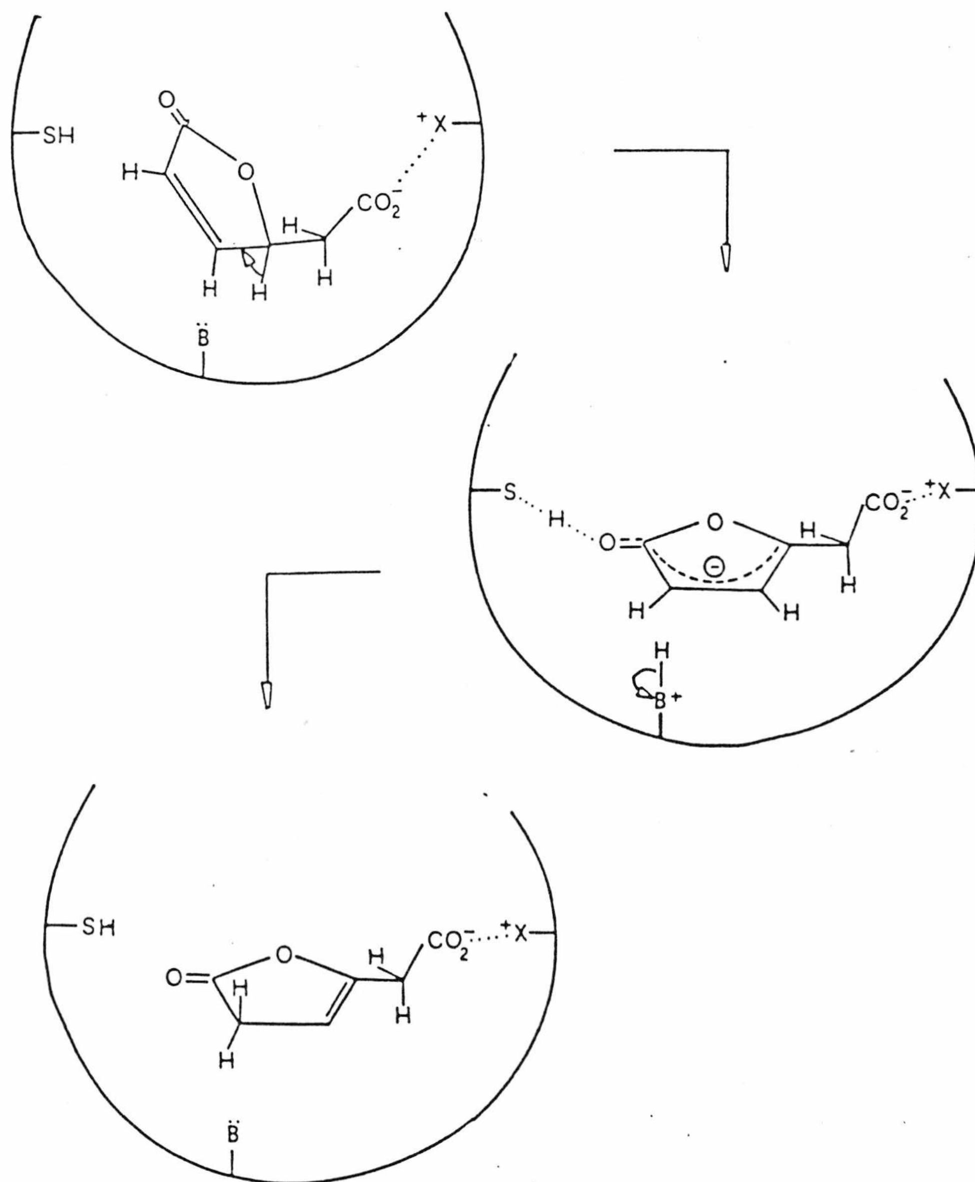


Fig.1.8. A possible reaction mechanism for muconolactone isomerase. A proton is removed from C(4) by a basic residue of the enzyme (B) and returned to C(5) with or without exchange with the solvent. A thiol group, if present, could assist the stabilization of the intermediate carbanion.

and of the decarboxylase are 8.5 (Ornston, 1966 b) and 6.5-7 (Ornston, 1966 a), respectively. These data are consistent with the possible involvement of an histidine residue which would be initially protonated in the decarboxylase reaction and unprotonated in the isomerase reaction.

1.5. Research Aims

The existence of the bacterial and fungal variants of the protocatechuate pathway is primarily determined by the different specificities of their lactonizing enzymes which yield isomeric carboxymuconolactones from 3-carboxy-*cis,cis*-muconate. According to Stanier & Ornston (1973) this observation strongly suggested the independent evolution of the pathway in bacteria and fungi. Further observations on the properties of the *A. niger* enzyme reported by Thatcher & Cain (1974a,b; 1975) and the serological investigations of Cook & Cain (1977) provided evidence supporting the same hypothesis (Ornston, 1982; Ornston & Yeh, 1982).

The direction in which the studies on the enzymes of the bacterial pathway developed, however, revealed the evolutionary homology of isofunctional or analogous enzymes even when they were serologically unrelated or their subunit and molecular sizes were different (Section 1.4). It was therefore decided to prepare a sample of homogeneous *A. niger* cyclase,through the improvement of its purification method, for eventual amino acid sequence analysis and comparison with the sequences of bacterial cycloisomerases

which were expected to be determined.

As a unique fungal enzyme, the 3-carboxymuconate cyclase has also been used as a marker of serological relatedeness between representatives of several fungal genera. Using antiserum raised against the A. niger enzyme a broad correlation was detected, within aspergilli and penicillia, between the immunological cross-reaction pattern and taxonomic sub-divisions based on morphological characters (Cook & Cain, 1977). Representatives of the genera Paecilomyces, Gliocladium, Monascus and Scopulariopsis produced more distantly related cyclases and the enzymes from the basidiomycetes Shyzophyllum commune and Rhodotorula mucilaginosa did not cross-react with the reference antiserum. A preliminary investigation of physical and kinetic properties of serologically-related cyclases was therefore undertaken to confirm whether other features of the enzymes further supported the correlation revealed by the extent of cross-reaction with the antiserum raised against the A. niger enzyme.

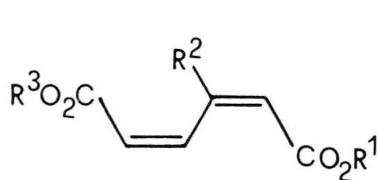
C H A P T E R 2

CHEMICAL SYNTHESES

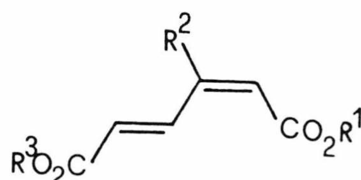
2.1. Nomenclature

Muconic acid is the name currently used in the literature for 2,4-hexadienedioic acid. The trivial names of related compounds with the same backbone structure are based on the positions of the substituents on the butadiene portion of the parent muconic acid, as in 3-carboxymuconic acid.

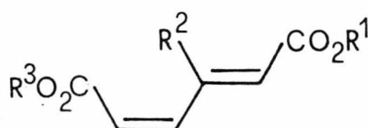
According to the classical view that each particular arrangement of the substituents in a double bond defines a particular configuration, 3-substituted muconates exist as four distinct stereoisomers. Their names are formed with the prefixes _cis_ and _trans_ to specify the configurations around the double bonds:



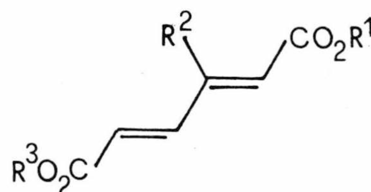
cis,cis



cis,trans

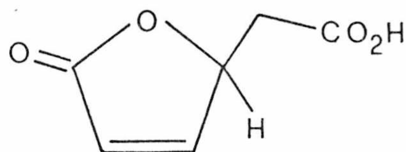


trans,cis

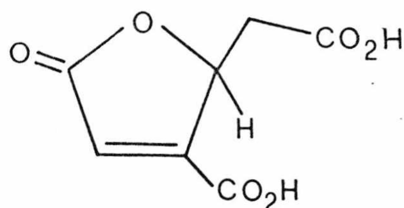


trans,trans

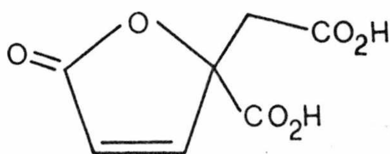
The lactones derived from muconic acids are also trivially named as muconolactones. A few examples are:



Muconolactone

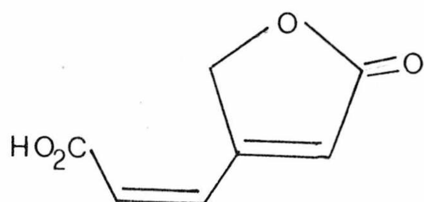


3-Carboxymuconolactone



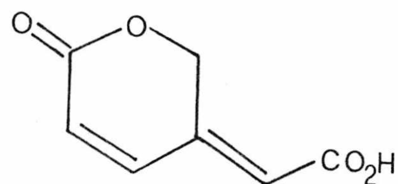
4-Carboxymuconolactone

According to the IUPAC nomenclature rules for organic compounds, alternative names for these lactones are as follows (rule C-472): 4-carboxymethyl-2-buten-4-olide (muconolactone), 3-carboxy-4-carboxymethyl-2-buten-4-olide (3-carboxymuconolactone) and 4-carboxy-4-carboxymethyl-2-buten-4-olide (4-carboxymuconolactone). 3-Hydroxymuconic acids or their monomethyl esters also exist as lactones. The γ- and δ-lactones of the _trans_, _cis_-acid can be named as 3-hydroxymethyl-_trans_, _cis_-1,3-muconolactone and 3-hydroxymethyl-_trans_, _cis_-6,3-muconolactone, respectively:



3-Hydroxymethyl-

trans,cis-1,3-muconolactone



3-Hydroxymethyl-

trans,cis-6,3-muconolactone

2.2. Objectives

The major objectives of the syntheses described in this chapter were, in summary :(i) synthesis of 3-carboxy-cis, cis-muconate (ii) trial of alternative synthetic methods for substrate analogues which had already been used in previous investigations and (iii) search for inhibitors potentially useful in the preparation of an affinity adsorbent for the purification of the cyclase.

In this search it has been assumed that the three isomeric muconic acids and 3-carboxy-cis, trans-muconate were all good inhibitors of the cyclase, according to observations reported by Thatcher & Cain (1975). Such results suggested that the recognition of the butadiene-1,4-dicarboxylate chain was an important factor in the interaction enzyme-substrate analogue, independently of the relative positions of carboxylate groups, which vary widely in the different isomers. It was therefore expected that some other analogue with a reactive substituent at C(3) and a cis, trans configuration of the muconate chain would

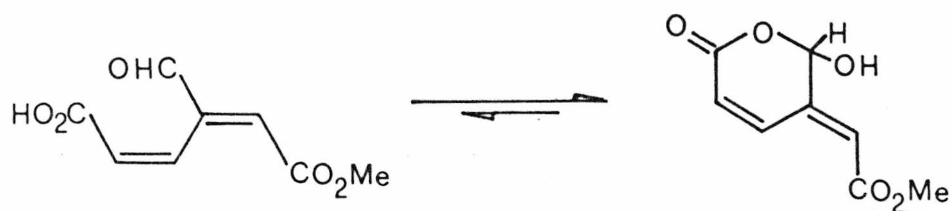
be an inhibitor of similar effectiveness.

2.2.1. Trial of alternative synthetic methods for muconate derivatives. The substrate analogues 1-methyl 3-carboxy-*cis*, *cis*-muconate and the isomeric muconic acids had been previously tested as cyclase inhibitors (Thatcher & Cain, 1975). For the synthesis of the monomethyl ester no alternative method could be found in the literature. The *cis*, *cis*-isomer of muconic acid,which is readily converted to the other isomers (Elvidge *et al.*, 1950a, b), is usually prepared by the oxidation of phenol with peracetic acid. Elvidge *et al.*, (1950a) report a yield of 35%, which is not objectionable, but the reaction takes 15 days. An attractive alternative seemed to be the method described by Tsuji & Takayanagi (1974), who reported that catechol reacted with molecular oxygen activated by cuprous chloride in pyridine containing methanol, to produce monomethyl *cis*, *cis*-muconate. A reinvestigation of this reaction by Rogic, Demmin & Hammond (1976) showed, however, that catechol was oxidized by the pyridine complex of cupric methoxychloride ($PyCuOCH_3Cl$), in anaerobic conditions, thus ruling out the participation of molecular oxygen in the reaction.

When the conditions described by Tsuji & Takayanagi (1974; 1978) were reproduced the result was, invariably, the formation of a brown material from which no crystalline product could be isolated. Under the conditions described by Rogic *et al.* (1976) and starting with 5mmol catechol, a few milligrams of crystalline product (m.p.79-80°) were isolated. According to these authors, monomethyl *cis*, *cis*-

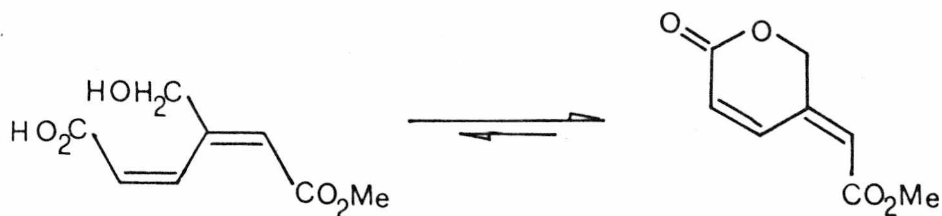
muconate has m.p.80-80.5°. Because of the small yield, this product was not further investigated and the method was abandoned.

2.2.2. Search for alternative potential inhibitors. The search for other potential cyclase inhibitors was essentially directed to muconates with a reactive substituent at C(3) and, if possible, a _cis_, _cis_ configuration. Two obvious candidates were 3-formyl- and 3-hydroxymethyl-_cis_, _cis_-muconate. The stable forms of their 1-methyl esters, in aqueous acidic solution and in aprotic solvents are the lactol and the δ-lactone ester, respectively. These compounds can readily be synthesized: the lactol by the chlorite oxidation of vanillin (Husband _et al._, 1955) and the δ-lactone as described by Ainsworth & Kirby (1968) or Dence, Gupta & Sarkanen (1962). The structures of these muconate derivatives are shown below:



1-Methyl 3-formyl-
cis,_cis_-muconate

Lactol



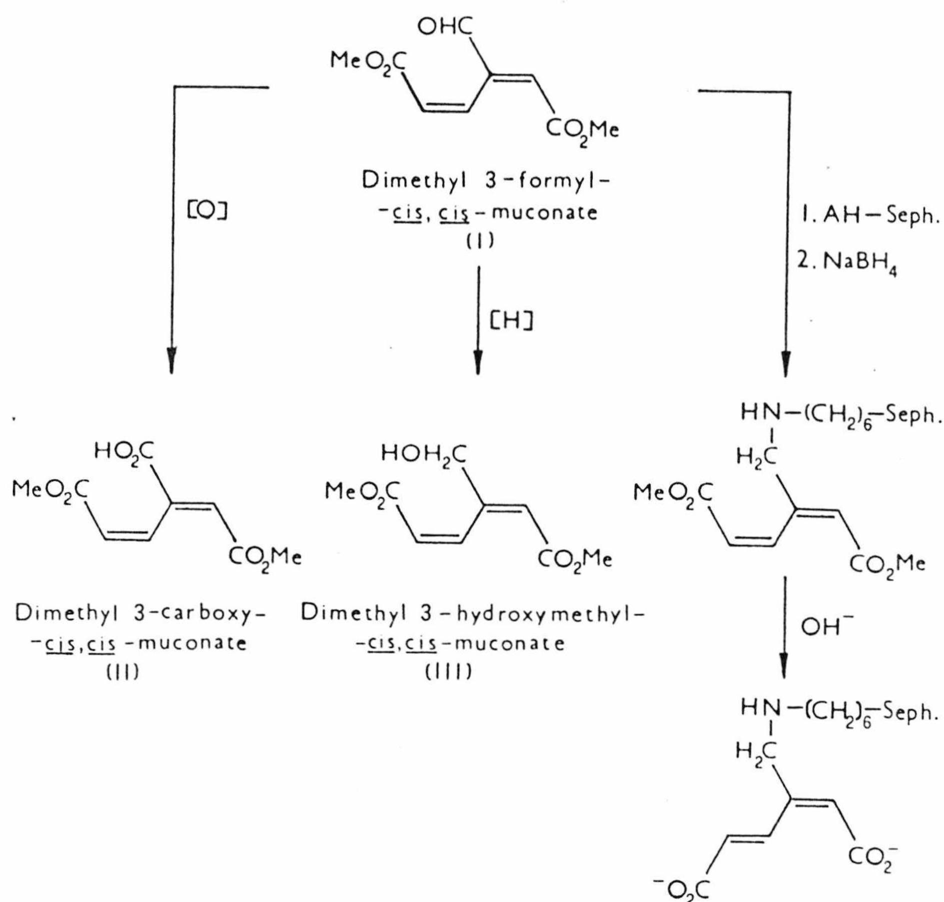
1-Methyl-3-hydroxymethyl-
cis,_cis_-muconate

δ-Lactone ester

Starting either from the lactol or the δ-lactone, the first attempts were directed to the isolation of their disodium salts, by hydrolysis with NaOH, followed by the slow addition of propan-2-ol. These conditions were similar to those described for the isolation of trisodium 3-carboxy-*cis*, *cis*-muconate except for the use of excess of NaOH, in an attempt to prevent the complete isomerization of the 2,3-double bond. Isomerization of the 4,5-double bond would however be expected, as discussed by Ainsworth & Kirby (1968). Both attempts failed, however. In the reaction of the lactol, no precipitate was formed, and the solution turned quickly to an orange colour during the addition of propan-2-ol. With the δ-lactone, the solid which separated was a very crude mixture as judged from ^{1}H n.m.r. analysis.

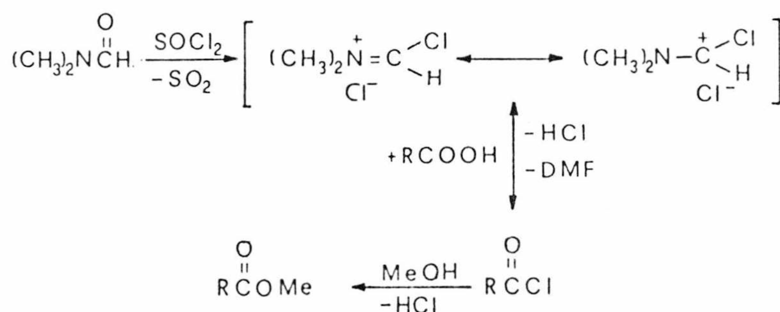
The possibility of esterification of the C(6)-carboxyl in the lactol, after ring opening, was next considered. This esterification, if possible without inversion of the 4,5-double bond, would yield dimethyl 3-formyl-*cis*, *cis*-muconate, a derivative which could be useful in several ways, as summarized in Scheme 1. The dimethyl ester **II** could, theoretically, be coupled to aminohexyl-Sepharose (AH-Sepharose), *via* a carbodiimide; the dimethyl ester **III** could be coupled, through the hydroxymethyl group to epoxy-activated Sepharose. Subsequent hydrolysis of the ester groups would produce muconate derivatives, probably with *cis*, *trans* configurations, attached through the 3-substituent to the spacer arms of the derivatized Sepharoses. In addition to that, compound **II** could be compared with the dimethyl 3-carboxy-*cis*, *cis*-muconate,

prepared as described by Husband et al. (1955), in which the identity of the second esterified carboxyl could not be established (Ainsworth & Kirby, 1968). Whether they were identical or not, the structure of that dimethyl ester would be unambiguously defined. The direct coupling of I through the formyl group to AH-Sepharose, followed by reduction with $NaBH_4$, in conditions similar to those described by Ryan & Fottrel (1972) was also considered. Alternatively, reduction of the intermediate Schiff base with sodium cyanoborohydride might be attempted (Borch, Bernstein & Durst,1971).



SCHEME 1

The esterification of the lactol was attempted with the Vilsmeir reagent (dimethylformamide-thionyl chloride), in conditions adapted from those described in Fieser & Fieser (1967). The reactive species in the mixture DMF-$SOCl_2$ is the dimethylchloroformiminium chloride, which reacts with carboxylic acids producing acyl chlorides. Subsequent reaction with methanol yields methyl esters. This route is summarized in Scheme 2.



SCHEME 2

The isolated product was, however, the dimethyl 3-(dimethoxymethyl)-cis, trans-muconate, (section 2.6) showing that methylation at the formyl group had also occurred. Ettlinger & Jaroszewsky (1980) described the synthesis of a dimethyl 3-formylmuconate dimethylacetal (configuration unspecified), from which they isolated a dimethyl 3-formylmuconate of configuration also unspecified.

As at the stage of the experimental work in which the dimethyl acetal was identified, the evidence from preliminary inhibitor studies with isomeric muconic acids (Section 5.7) did not fully support the findings of Thatcher & Cain (1975) the use of the dimethyl acetal was not further explored.

2.3. Sources

Laboratory reagents and solvents were purchased from BDH Chemicals (Poole, England) and were, whenever possible, of the "Anala R" grade.

3-Carboxymuconolactone, used in inhibitor assays, had been synthesized by Dr. E.F.Ahlquist by incubating _p_-hydroxybenzoate with mycelia of a mutant of _A. niger_ genetically blocked in the 3-carboxymuconolactone hydrolase.

2.4. Synthesis of 3-Carboxy-_cis_,-_cis_-Muconate Derivatives

2.4.1. 1-Methyl 3-carboxy-_cis_, _cis_-muconate was synthesized by the oxidation of vanillin with sodium chlorite in aq. sulphuric acid (pH 0.5) as described by Husband _et al._ (1955), in 10-15% yields. The isolated crystalline product showed a pale yellow colour which was completely removed by washing with several portions of cold dry ether. The compound, which was not further purified, had m.p. 142 -144° [lit:143-144° (Husband _et al._,1955); 142-144° (Ainsworth & Kirby, 1968)] and its ^{1}H n.m.r. spectrum (60MHz,D_2O, without internal standard) showed

absorptions at δ 3.63($-OCH_3$,s), 6.06[HC(5),d,J=16Hz], 6.62 [CH(2),s], 7.05[HC(4),d,J=16Hz], in good agreement with the data reported by Ainsworth & Kirby (1968).

2.4.2. **Dimethyl 3-carboxy-*cis*, *cis*-muconate** was prepared in 40% yield from the monomethyl ester by reaction with methanol-sulphuric acid, as described by Husband *et al.* (1955). After recrystallization from diethyl ether-petroleum ether (40-60°) the product had m.p. 124-125° [lit: 124-125° (Ainsworth & Kirby, 1968); 125-126° (Husband *et al.*, 1955)].

2.4.3. **Trisodium 3-carboxy-*cis*, *cis*-muconate** was quantitatively prepared from the monomethyl ester by hydrolysis with sodium hydroxide in aq. methanol, followed by precipitation of the salt with propan-2-ol (Ainsworth & Kirby,1968). The stereochemical purity of the final preparation was assessed by enzymatic analysis: an enzyme assay was performed in the standard conditions of temperature and buffer composition (section 3.2) using a partially purified enzyme extract in which the absence of delactonizing activity had been previously checked. 3-Carboxymuconate (100µM) was added to the assay cuvette and the absorbance change at 260nm was compared with the initial absorbance of a 100µM solution of 3-carboxymuconolactone in assay buffer containing the same amount of enzyme extract. The results showed that the synthetic 3-carboxymuconate contained, at least, 95% of the *cis*, *cis*-isomer.

2.5. Synthesis of the Isomeric Muconic Acids

2.5.1. _cis_, _cis_-Muconic acid was prepared by oxidation of phenol with peracetic acid, as described by Elvidge _et al._, 1950 a). The solid product which precipitated from the reaction mixture was recrystallized from hot ethanol. The product (final yield 24%) melted at 181-182° [lit. 184° (Elvidge _et al._ (1950 a)]. Analysis of the product by thin layer chromatography on a silicagel plate, developed with ethanol-ammonia-H_2O (20:1:4,v/v), together with an authentic specimen of _cis_, _cis_-muconic acid, showed one spot (R_f=0.44) for both samples, after visualization with iodine vapour. Its **uv** spectrum in potassium-sodium phosphate buffer (pH 7.0) had λ_{max}257nm, in agreement with the value reported by Sistrom & Stanier (1954) for the _cis_, _cis_-isomer.

2.5.2. _cis_, _trans_-Muconic acid was formed by briefly boiling a sample (30mg) of _cis_, _cis_-muconic acid, dissolved in distilled water (Elvidge _et al._, 1950 b). The compound was not isolated but, in solution, in the conditions described above for the _cis_, _cis_-isomer, it showed both bathochromic and hyperchromic shift (new λ_{max} 260nm) indicating that inversion of the 4,5-double bond had occurred (Sistrom & Stanier, 1954).

2.5.3. _trans_, _trans_-Muconic acid was prepared by irradiation of a solution of _cis_, _cis_-muconic acid (100mg) in ethanol containing a trace of iodine, with a 100W lamp,

placed at 40cm from the solution, for 15 min (Elvidge et al., 1950 a). The material which precipitated from the solution had m.p.299° [lit. 301° (Elvidge et al., 1950 a)]; its **uv** spectrum had λ_{max} 263nm, in phosphate buffer (pH 7.0) in agreement with the value reported by Sistrom & Stanier (1954).

2.6. Synthesis of 3-Formylmuconate Derivatives

2.6.1. 1-Methyl 3-formyl-cis, cis-muconate, in the lactol form (see Section 2.2), was prepared by the oxidation of vanillin with sodium chlorite, in a phosphate-citrate buffer (pH 3.8). The method described by Husband et al. (1955) produced variable yields (10-20%) of lactol. The following conditions produced, more consistently, yields approaching 20%. The buffer contained, per litre, 51.6g (0.246mol) of citric acid monohydrate and 54.9g(0.308mol) of disodium hydrogen phosphate dihydrate. Vanillin (10g;0.066mol) was suspended in 500ml of this buffer, at 4-5°C; technical grade sodium chlorite, containing approx. 80% $NaClO_2$ (approx. 0.26mol) was dissolved in 100ml cold water and added to the vanillin suspension with vigorous stirring, while the reaction vessel was kept in an ice bath. The temperature increased to 25-28°C and chlorine dioxide evolution became apparent shortly afterwards. In the course of 2h, the reaction mixture changed from dark-orange to green-yellow and finally to golden yellow. At this stage, excess chlorine dioxide was removed under vacuum. The mixture was extracted with 5x150ml of diethyl ether. The

lactol was isolated from the ether extracts as described by Husband *et al.* (1955). The crude product was washed with several portions of cold dry ether and recrystallized from chloroform by "freezing-out". The m.p. was 101-103° (dec.) [lit.: 104-105° (Husband *et al.*, 1955); 103-105° (Ainsworth & Kirby, 1968)]. The ^{1}H n.m.r. spectrum (CDCl , TMS as internal standard) showed absorptions at δ 3.80(-OCH ,s), 8.23[HC(4),d,J=10Hz] and a broad signal at 5.3-5.9(HO); the spectrum did not resolve the signals of HC(2), HC(5) and HC(OH).O, wich appeared as two peaks of similar intensities at 6.14 and 6.28, integrating for three protons.

2.6.2. Dimethyl 3-(dimethoxymethyl)-*cis*, *trans*-muconate. Lactol (184mg; 1mmol) was dissolved in chloroform (5ml) containing 5 drops of DMF; methanol (0.33ml, 8mmol) was added to the cold solution (-10°C), followed by thionyl chloride (0.09ml; 1.1mmol). After reaching room temperature, the mixture was kept in a bath at 40-45°C for 1h, and then evaporated to dryness. The residue was dissolved in ether and the ethereal solution was washed with 2 portions of 5% aq. $NaHCO_3$ and 2 portions of distilled water. After drying overnight over anhydrous sodium sulphate, the ether was evaporated leaving a pale yellow oily residue (200mg); its ^{1}H n.m.r. spectrum ($CDCl_3$, TMS as internal standard) showed absorptions at δ 3.30[-CH(OC$\underline{H}_3$)$_2$, s,6H], 3.80[-(CO_2CH_3)$_2$,s,6H], 5.08[-C$\underline{H}$(OCH_3)$_2$,s,1H] and 8.40[HC(4),d,J=16Hz]; two peaks centered at δ 6.48, with a more intense upfield signal, accounted for the absorptions of HC(5),d,J=16Hz and HC(2),s. The coupling constant, $J_{4,5}$ =

=16Hz was consistent with the *trans* configuration of the 4,5-double bond.

2.7. Synthesis of 3-Hydroxymethylmuconate Derivatives

2.7.1. 1-Methyl 3-Hydroxymethyl-*cis*, *cis*-muconate, in the δ-lactone form (see section 2.2) was prepared: (i) from vanillin, by reduction with sodium borohydride to vanillyl alcohol, followed by oxidation of the alcohol with sodium chlorite, in aq. sulphuric acid (pH 1.0) (Dence *et al.*, 1962); (ii) from the lactol (prepared as described in Section 2.6), by reduction with sodium borohydride, in aq. methanol (Ainsworth & Kirby, 1968). The ^1H n.m.r. spectrum (100MHz, in $CDCl_3$, TMS as internal standard) showed absorptions at δ 3.76($-OCH_3$,s), 4.95[$-OCH_2$,**d**,J=2Hz], 5.90 [HC(2), unresolved multiplet], 6.10[HC(5),**dd**,$J_{4,5}$ =10.6Hz, $J_{2,5}$ =1.8Hz] and 8.21[HC(4),**d**,$J_{4,5}$ =10.6Hz].

2.7.2. 3-Hydroxymethyl-*trans*, *cis*-1,3-muconolactone was synthesized by treatment of the lactol, in aq. methanol, with a small excess of sodium borohydride, in conditions otherwise similar to those which led to the formation and isolation of the δ-lactone ester (section 2.7.1). The crude product had m.p. 176-179° [**lit.**: 177-179° (Ainsworth & Kirby, 1968)] and was identified by its ^1H n.m.r. spectrum, in DMSO, with TMS as internal standard, which showed absorptions at δ 5.18[$-OCH_2$,s], 6.20[HC(5),**d**,$J_{4,5}$ =13Hz], 6.48[HC(2),s], and 6.90[HC(4),**d**,$J_{4,5}$ =13Hz], in good agreement with the values reported by Ainsworth & Kirby (1968). The

formation of this lactone (γ-lactone) resulted from the exposure of the δ-lactone ester, which forms very quickly, to the alkaline conditions generated by excess sodium borohydride. These conditions led to ring opening, hydrolysis of the ester and inversion of the 2,3-double bond; upon acidification, cyclisation in the alternative sense produced the γ-lactone, as demonstrated by Ainsworth & Kirby (1968).

CHAPTER 3

BIOLOGICAL AND BIOCHEMICAL METHODS

3.1. Organisms and Growth Conditions

3.1.1. Source of the organisms. The organisms used are listed in Table 3.1 and were obtained (a) from the Commonwealth Mycological Institute, Kew, Surrey (U.K.) and are identified by the CMI reference number; (b) *Aspergillus niger* L6 was originally isolated from soil by Dr. B. A. Halsall at the University of Newcastle upon Tyne and was the same strain used by Thatcher & Cain (1974 a); (c) several mutants of L6, blocked in steps of the protocatechuate and catechol branches of the 3-oxoadipate pathway were produced by E. F. Ahlquist at the University of Kent (Alhquist, 1977); strain **PL30** was a β-propiolactone-induced mutant in which the 3-carboxymuconate cyclase was temperature-sensitive. Normal activities of the enzyme were produced when the mutant was grown at incubation temperatures of 25°C or below, but at 30°C and 32°C the fungus grew very poorly, the cyclase activity was grossly impaired and 3-carboxy--muconate accumulated in the growth medium.

3.1.2. Maintenance of the organisms. Fungal cultures were kept in the dark, at 4°C, in slopes formed in 28ml McCartney bottles, with rubber lined screw caps; they were

subcultured every 2-3 months. For most fungi, the medium used was potato dextrose agar (**Difco**), prepared according to the manufacturer's instructions. The same medium was used to recover cultures which had been purchased from the Commonwealth Mycological Institute, either freeze-dried or in slopes of solidified medium.

TABLE 3.1

Source of the Organisms

Organism	Source
Aspergillus flavus, CMI 910196	(a)
Aspergillus niger, **L6**	(b)
Aspergillus flavus **L6** mutant **PL30**	(c)
Aspergillus sydowii, **CMI** 63904	(a)
Penicillium chrysogenum, CMI 24314	(a)
Penicillium crustosum **P6**, CMI 154731	(a)
Penicillium terrestre, CMI 111867	(a)
Gliocladium deliquescens, CMI 101525	(a)
Monascus ruber, CMI 138218	(a)
Paecilomyces variotii, CMI 108007	(a)
Scopulariopsis candida, CMI 129578	(a)

3.1.3. Growth media for liquid cultures. All fungi were grown in a basal salts medium, adjusted to pH 5.5 with NaOH containing, per litre, $(NH_4)_2SO_4$ (0.5g), KH_2PO_4 (1.0g), $MgSO_4.7H_2O$ (0.05g) and 1ml of trace elements solution. The trace elements solution was a modified version of Barnett & Ingram's (1955) solution containing, per litre, $FeSO_4.4H_2O$ (400mg), $MnSO_4.4H_2O$ (400mg), $ZnSO_4.7H_2O$ (200mg), $CuSO_4.5H_2O$ (40mg), $CoCl_2.6H_2O$ (40mg), KI (300mg), $NaMoO_4.2H_2O$ (50mg) and NaCl (10mg).

The basal salts medium was supplemented by the addition (i) of glucose (20g/l), yeast extract (1g/l) and mycological peptone (1g/l), for the growth of non-induced mycelium (**medium A**), (ii) of glucose (1g/l), yeast extract (0.2g/l), mycological peptone (0.2g/l) and p-hydroxybenzoate (2g/l of the free acid), for the growth of induced inocula (**medium B**), and (iii) of glucose (0.5g/l), yeast extract (0.1g/l), mycological peptone (0.1g/l) and p-hydroxybenzoate (2g/l) of the free acid), for the bulk growth of induced mycelia (**medium C**). Yeast extract, mycological peptone and p-hydroxybenzoate were added to the basal salts solution before adjusting the pH and autoclaving. Glucose was prepared as a 40% (w/v) solution, autoclaved separately for 15 min at 15 psi (121°C) and added aseptically to the sterile media in the required amounts.

3.1.4. **Bulk growth of induced mycelium.** Inocula for liquid cultures were obtained from freshly-grown slopes. With sporulating species, 4ml of sterile distilled water were added to the slope and the bottle vigorously shaken; 0.5ml of the resulting spore suspension was used to inoculate 50ml of sterile **medium A**. With non-sporulating species, a portion of mycelium was removed from the agar slope with a sterile scalpel, transferred to a 100ml sterile homogenizer vessel containing 50ml of **medium A** and homogenized for a few seconds; 250ml indented conical flasks fitted with non-absorbent cotton-wool plugs were used at this stage. The cultures were then incubated, at 30°C, in an orbital incubator shaker, at 180-200 rev./min. The temperature sensitive A.niger mutant, **PL30**, and S. candida were grown at 25°C. When an abundant mycelium had developed, usually after 36-48h incubation, the cultures were transferred to sterile homogenizing vessels and macerated for 1.5-2 min at **medium** speed in a high speed blender (**Virtis 45**). This step was essential to ensure a faster growth of the mycelium in the presence of the inducer, p-hydroxybenzoate, particularly in species which tended to grow in pellet form, like the aspergilli.

The homogenized mycelium was added, in 50ml batches, to 2 l indented conical flasks containig 500ml of **medium B**. The flasks were shaken at the required temperature, as described above, and the p-hydroxybenzoate disappearance monitored at intervals. When 70-80% of the inducer had been utilized, the cultures were homogenized again and

transferred directly to 20 l fermenter pots containing 18 l of **medium C.** The pots were fitted with rubber bungs through which the air tube, sampling port and exhaust tube passed. The air was supplied by a compressed air line, filtered through a glass fiber air filter and delivered to the medium through two sintered glass spargers. The air flow was adjusted to allow a vigorous agitation of the medium. The pots were incubated at constant temperature for the required periods. Samples of the liquid cultures were aseptically collected at intervals and monitored for p-hydroxybenzoate disappearance. When only 20-25% of the original p-hydroxybenzoate remained in the medium (usually after 24-36h periods of incubation) the cultures were harvested by pouring the contents of the pot through a double layer of muslin. The retained mycelium was washed with tap water and then distilled water, and squeezed to remove as much as possible of surplus water. If not used immediately, the mycelium was wrapped in aluminium foil and stored frozen at $-24^{o}C$.

3.1.5. Monitoring of growth in the presence of p-hydroxybenzoate. Samples of medium taken from the growing cultures were filtered or the mycelium allowed to settle out. From the clear filtrate or supernatant, 10µl were added to 3.0ml of distilled water, in a quartz cuvette, and its absorbance read at 245nm against a blank of distilled water. Incubation was stopped when the absorbance reached 20-25% of the initial value.

3.2. Enzyme Assays

All enzymes, except the PL30 cyclase, were assayed spectrophotometrically at 30°C in 3ml quartz cuvettes of 10mm light path. The spectrophotometers used were either a Unicam SP800 or a Pye-Unicam SP1800, both fitted with Pye-Unicam AR25 recorders. The spectrophotometers had constant temperature cuvette holders through which water was circulated from a constant temperature bath (model SB1 from Grant Instruments, Cambridge Ltd.). One unit of enzyme activity is defined as the amount of enzyme catalysing the disappearance of 1 μmol substrate per minute under the conditions of the assay.

4-Hydroxybenzoate monooxygenase [4-hydroxybenzoate NADH: oxygen oxidoreductase (3-hydroxylating) EC.1.14.13.2] was assayed by measuring the substrate dependent oxidation of NADH at 340nm (ε_{340}=6220 $M^{-1}.cm^{-1}$). The reaction mixture in the assay cuvette contained (μmol): Tris-HCl (pH 7.5), 150; FAD, 0.02; NADH, 0.3; *p*-hydroxybenzoate, 1.0; enzyme extract and water to a final volume of 3ml. The reaction was started by the addition of *p*-hydroxybenzoate and the rate of NADH oxidation corrected for endogenous oxidation in the absence of substrate. The oxidation of 1 μmol of NADH was equivalent to the hydroxylation of 1 μmol of *p*-hydroxybenzoate.

Protocatechuate 3,4-dioxygenase [protocatechuate: oxygen 3,4-oxidoreductase (decyclizing), EC.1.13.11.3] was assayed by recording the decrease in absorbance of protocatechuate at 290nm (ε_{290}=3890 $M^{-1}.cm^{-1}$). The product of reaction, 3-carboxy-*cis*, *cis*-muconate, did not accumulate when crude extracts were used. The reaction mixture in the assay cuvette contained (μmol): Tris-HCl (pH 7.5) 150; protocatechuate, 0.5; extract and water to a final volume of 3ml. The reference cuvette lacked the aromatic substrate, which was added to start the reaction.

3-Carboxymuconate cyclase [3-carboxymuconolactone lyase (decyclizing) EC.5.5.1.5] was assayed by the decrease in absorbance, at 260nm, of 3-carboxy-*cis*, *cis*-muconate (ε_{260}=8150 $M^{-1}.cm^{-1}$) with time. The product of this reaction and subsequent products do not absorb appreciably at this wavelength. Routine assays were performed in 0.1M acetate buffer (pH 6.0), (Thatcher & Cain, 1974 a). The reaction mixture in the assay cuvette contained (μmol): acetate buffer (pH 6.0), 250; 3-carboxymuconate, 0.3, extract and water to 3ml. The mixture was pre-incubated for 5 min at 30°C before addition of the substrate. The reference cuvette lacked substrate. Enzyme assays at pH values other than 6.0 were performed in the following buffers: 0.1M acetate buffers for pH values between 4.5 and 6.0 and 0.1M histidine-HCl buffers for pH values between 6.0 and 7.5; Kinetic parameters were not altered by change of buffer, as checked by performing duplicate determinations in the two different buffers in the overlapping pH zones.

The following enzymes were assayed as described in Bergmeyer (1974): catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC.1.1.1.6), fumarase (L-malate hydrolyase EC.4.2.1.2), lactate dehydrogenase (L-lactate: NAD oxidoreductase EC.1.1.1.27); and malate dehydrogenase (L-malate: NAD oxidoreductase EC.1.1.1.37).The following modification was introduced in the assay of malate dehydrogenase: a freshly prepared solution of neutralized oxaloacetate was used, instead of being generated *in situ* by the reaction between L-aspartate and 2-oxoglutarate in the presence of L-aspartate: 2-oxoglutarate aminotransferase.

3.3. Protein Assays

Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin (**fraction V**, from **Sigma**) as standard. Ferritin and myoglobin concentrations were monitored by their absorbance at 405nm.

3.4. Kinetic Assays

3.4.1. Estimation of Michaelis parameters. When the kinetic course of a particular enzyme reaction obeys the Michaelis-Menten equation, $v=V_{max}.S/(K_m+S)$, the kinetic parameters K_m and V_{max} may be estimated through a linear transformation of that equation. Linear transformations currently used are:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}}$$ (Lineweaver & Burk, 1934)

$$\frac{S}{v} = \frac{1}{V_{max}} \cdot S + \frac{K_m}{V_{max}}$$ (Hanes, 1932)

$$v = -K_m \cdot \frac{v}{S} + V_{max}$$ (Eadie, 1942; Hofstee, 1952)

Each set of experimental results should, ideally, fit a straight line, whichever linear transformation of the Michaelis-Menten equation were used. When, however, the results are affected by experimental errors, the choice is not indifferent, as has been shown by computer simulation, by Dowd & Riggs (1965) who introduced random errors in a set of ideal values. When the resulting data were plotted by each of the three methods, and the straight line fitted by eye, it was found that: (i) the Lineweaver-Burk plot gave the worst estimates of K_m and V_{max}; (ii) the other two methods were almost equivalent. However, for reasons related to the distortion of the error bars associated with experimental values (Johansen & Lumry, 1961) the Hanes plot is the best of those three methods, and was therefore adopted.

Another possible method for the estimation of K_m and V_{max} is the direct linear plot of Eisenthal & Cornish-Bowden (1974); according to Atkins & Nimmo (1975) it compares favourably with any linear transformation of the Michaelis-Menten equation; its only disadvantage arises from the difficulty of determining, by eye, the intercepts of the straight lines generated by each pair (S,v) of experimental data, specially when those intercepts lie very close together.

Unless stated otherwise, the results of the experimental assays were expressed as μmoles of substrate transformed per minute per mg protein. Each value of the initial velocity, v, was the average obtained from duplicate experiments differing less than 5%. Usually, substrate concentrations in the range 5-100μM were used. The best-fitting straight line and its parameters (intercept, slope and correlation coefficient) were determined by linear regression analysis in a programmed calculator; K_m and V_{max} were calculated from those parameters.

3.4.2. **Inhibitor constants.** Inhibitor constants (K_i) were determined from Dixon plots of 1/v <u>vs</u>.inhibitor concentration (Dixon, 1953), at two different substrate concentrations. The intercept of the best-fit straight lines provided an estimate of K_i. As this type of plot does not unambiguously distinguish between competitive and mixed inhibitors a plot of S/v. <u>vs</u>.inhibitor concentration was also done (Cornish-Bowden, 1974): competitive inhibitors

generate parallel lines whereas mixed inhibitors generate intercepting lines.

3.5. Centrifugation

Refrigerated centrifuges and rotors previously cooled down to 5°C were used throughout. Large volumes of cell homogenates or cell-free extracts were centrifuged in the 6x300ml rotor at 14 000 rev./min (21 000g, av.) of a MSE centrifuge (model **HS21**), for 60-70 min; smaller volumes were centrifuged for 30 min in a 8x50ml rotor, in the **HS21** centrifuge, at 20 000 rev./min (33 000g, av.) or in a **MSE** (model **HS50**) centrifuge, for 15 min at 35 000 rev./min (110 000g, av.). Sedimentation of precipitates in very small volumes (up to 1ml) was performed in a bench centrifuge (**Beckman Microfuge**, model **152**), for periods of 30-120s.

3.6. Preparation of Cell-Free Extracts

Frozen mycelium, in batches of 200-250g was broken in small pieces and thawed in 0.05M potassium-sodium phosphate buffer (pH 6.8) (1-1.5ml/g wet mycelium) except where stated otherwise. The cell suspension was homogenized at maximum speed in the **Virtis** high-speed blender, for a period of 15min. Blending was interrupted several times to keep the temperature of the slurry at 10-15°C. The operation was performed in the cold room and the ice surrounding the homogenizing vessel was frequently renewed. The slurry was

transferred to balanced centrifuge bottles and centrifuged as described in section 3.5. The pellet was discarded and the supernatant kept for ammonium sulphate fractionation.

Occasionally, the mycelium was ground to a fine powder, under liquid nitrogen as described by Cook & Cain (1977) and then thawed in buffer. This method, however, was too time-consuming to be useful when large-scale preparations of cell-free extracts were being performed.

3.7. Ammonium Sulphate Fractionation

The reported purification procedures of 3-carboxymuconate cyclases and cycloisomerases refer to ammonium sulphate fractions in terms of percent saturation; to allow an easier comparison of results, this was also the method followed in this work. The required amount of solid ammonium sulphate was calculated from a table published in Ferdinand (1976), based on the data of Kunitz (1952), and slowly added to the cold protein solution (2-5°C) with constant stirring. The pH was monitored and readjusted to the initial value by dropwise addition of 2M NH_3. When the addition was complete, the mixture was kept in the cold for 30-60min, to allow complete precipitation of the protein. Centrifugation was performed in the conditions described in section 3.5 and the pellet was discarded or redissolved in buffer, as required.

3.8. Dialysis

Dialysis was performed whenever a buffer change in a cyclase solution was necessary, usually before ion-exchange chromatography, hydrophobic interaction chromatography or chromatofocusing. The dialysis tubing was treated as described by McPhie (1971) and kept in the cold in distilled water containing sodium azide (0.01%), if stored for more than a few days. Dialysis was usually carried out overnight (12-16h) in the cold. The dialysis buffer was used in a volume 200 to 300-fold the volume of the dialysed solution. Occasionally, dialysis was performed over a 6h period with 2 changes of buffer, under gentle agitation.

3.9. Concentration of Protein Samples

Concentration of cyclase solutions was best performed by precipitation with ammonium sulphate at 65% saturation, with a virtually quantitative recovery from solutions with protein concentrations 0.2mg/ml or higher. Occasionally, protein solutions were concentrated in Millipore cells, operated under air pressure, using **PM10 Diaflo** ultrafiltration membranes (mol.wt. cut-off 10 000), but with much poorer recovery yields; **PM50 Diaflo** membranes were unsuitable for the concentration of cyclase solutions as approx. 15% of the recovered activity was found in the ultrafiltrate.

3.10. Column Chromatography

The columns used in chromatography were from **Pharmacia** (K25/45), **LKB** (26x100; 16x50; 16x30) and **Wright** (GA 16x45; GA 16x30; GA 10x15). All columns, except the K25/45 had adjustable end-stops. Unless stated otherwise, the complete assembly for a column chromatography experiment was as follows: a three-way valve connected the column inlet to the buffer and sample reservoirs; the column eluate was pumped by an **LKB 10200** Perpex peristaltic pump through an **LKB 8300 Uvicord II** detector system with a 280nm light source; the detector system was connected to an **LKB 6520** chopper bar recorder, which continuously recorded the eluate transmittance; an **LKB UltroRac 7000** was used to collect the fractions.

3.10.1. Gel permeation chromatography. Sephadex G-200, Sephacryl S-300 SF and Ultrogel AcA34 were used as gel permeation media. Sephadex G-200, available in the dry beads form, was swollen in buffer for 72h before packing. Sephacryl S-300 and Ultrogel AcA34 were purchased in the pre-swollen form and directly packed in the columns.

Sephadex G-200 was packed in a K25/45 column, to a bed height of approx. 40cm under 12cm hydrostatic pressure, and washed with three bed volumes of the buffer used in the experiment. The protein sample (2-3ml), made 5% (w/v) in sucrose, was carefully overlayered on the gel surface with a

pipette, under the eluting buffer. The column inlet was, in this case, connected directly to the buffer reservoir. The column was eluted at a flow rate of 8-10ml/h, under hydrostatic pressure and 2-3ml fractions were collected.

Ultrogel AcA34 and Sephacryl S-300 were packed in 2.6x100cm LKB columns, to a bed height of 80-90cm by pump-induced flow at 30ml/h for Ultrogel and 40-45ml/h for Sephacryl S-300. Samples (3-5ml) were applied to the columns through the three-way valves. Both Ultrogel and Sephacryl were eluted by downward flow, at flow rates of 20-25ml/h. Fractions of 2.5-3.5ml were collected. In calibrated gel permeation chromatography experiments, the void volume of each column was determined as the elution volume of **Blue Dextran 2000** (**Pharmacia**) applied to the column as a 1% solution (2ml). The Blue Dextran concentration in the eluate was monitored by its absorbance at 605nm.

3.10.2. **Anion-exchange chromatography.** DEAE-Celluloses (**Whatman DE22 and DE34**), which require lengthy recycling and fines removal procedures,were used only in early trial experiments, after pre-treatment according to the manufacturer's instructions. Later, DEAE-Sepharose CL-6B and DEAE-Sephacel, both from **Pharmacia**, replaced those materials because of their greater simplicity and advantages in use.

Both DEAE-Sepharose and DEAE-Sephacel were first extensively washed, in a sintered glass funnel, with the starting buffer of the particular experiment, until the pH of the washings was identical to the starting buffer. The material was then packed in a column of the appropriate dimensions and again washed with the starting buffer, (4-6 bed volumes) until the conductivities of the washings and of the starting buffer were identical.

The sample was equilibrated with starting buffer by dialysis. After loading the sample, the column was washed with starting buffer (3-4 bed volumes) to remove unbound proteins and elution was performed with a linear concentration gradient of NaCl in starting buffer (5-6 bed volumes) at a flow rate of 20-25ml/h. Eluted fractions were monitored for protein, activity and conductivity. The conductivities were measured in a conductivity meter model **WPA CMD400, Saffron Walden,** England, previously calibrated with 0.1M KCl. The starting buffers in gradient anion-exchange chromatography were 0.05M phosphate (pH 6.8) or 0.1M Tris-HCl (pH 7.2).

3.10.3 Hydrophobic chromatography. Preliminary experiments revealed that a purification method involving ammonium sulphate fractionation, one or more steps in ion-exchange chromatography and gel permeation chromatography did not yield an homogeneous cyclase preparation. Other chromatographic methods, exploiting different properties of the protein molecules were then

tried. One of them was hydrophobic chromatography, which developed from the observation that **n**-alkyl and **n**-aminoalkyl-substituted agaroses displayed adsorbing properties towards some proteins (Er-el, Zaidenzaig & Shaltiel, 1972). Those authors have unequivocally shown that an hydrophobic interaction was the main factor involved in this process since protein retention was dependent on the length of the agarose substituent.

The application of this technique to the cyclase purification was tested with 1ml samples of **n**-alkyl and **n**-aminoalkyl agaroses (**Miles Laboratories**) with an even number of carbon atoms in the alkyl chains from zero (control) to ten. The gel samples were equilibrated with imidazole-HCl buffers (pH 6.8); with a 0.025M buffer, the cyclase was retained by ethyl and aminoethyl-agaroses, and higher homologues; in the 0.1M buffer, binding was only observed with butyl-and aminobutyl-agaroses and higher homologues. Approximate elution conditions were established by washing the columns, in which cyclase binding was observed, with starting buffer containing NaCl in increasing concentrations from 0.025M to 0.25M. Following the principle that the agarose with the shortest alkyl chain would be the most selective, ethyl-agarose was chosen as a possible step in the A. niger cyclase purification (see also sections 4.6 and 4.9).

3.10.4. Chromatofocusing. Chromatofocusing was performed in Polybuffer exchanger PBE94 with Polybuffer PB74 as eluent (both from **Pharmacia**). In most experiments, a column of PBE94 with a bed volume of 10ml was eluted with a pH gradient from 7 to 4. Following the manufacturer's instructions, the starting buffer (0.025M imidazole-HCl) was adjusted to pH 7.4; Polybuffer 74 was diluted 1:8 and adjusted to pH 4.0. A total volume of 120ml (12 bed volumes) was used. The packed column was extensively washed with degassed starting buffer. Before sample application, 5ml of degassed Polybuffer 74, (pH 4.0), were passed through the column. After loading the sample the column was eluted with Polybuffer at flow rates of 10-12ml/h. Fractions of 1.5-2.5ml were collected and monitored for protein, activity and pH.

Fractions containing cyclase activity, which were to be analysed by SDS-polyacrylamide gel electrophoresis, were kept separate, adjusted to pH 6.8 and concentrated by ultrafiltration as described in section 3.9. Fractions from chromatofocusing were not usually assayed for protein since Polybuffer interferes with the protein assay of Lowry et al. (1951). The Coomassie blue reagent for protein assay (Bradford, 1976) did not produce reliable results and other methods normally used to remove polybuffer, such as ammonium sulphate precipitation or hydrophobic chromatography, were not tried because of the limited amounts of cyclase present in those fractions.

3.10.5. Concanavalin A-Sepharose chromatography. Concanavalin A-Sepharose adsorbs certain polysaccharides (Aspberg & Porath, 1970) and previous investigations on the A. niger cyclase (Thatcher & Cain, 1974b) suggested the presence of carbohydrate in the native molecule. Concanavalin A-Sepharose was then tested for its possible use in the cyclase purification. The gel (5ml) was packed in a small column and equilibrated, in different experiments, with 0.01M or 0.02M phosphate buffers (pH 6.8), containing NaCl, to prevent unspecific adsorption; NaCl concentrations of 1M, 0.1M and 0.02M were used. The commercial preparation of Concanavalin A-Sepharose (**Pharmacia Fine Chemicals**) contained the divalent cations Mn^{2+} and Ca^{2+}, which are essential for the binding activity of Concanavalin A (Agrawal & Goldstein, 1968). Cyclase samples equilibrated with each starting buffer were passed through the column and fractions were collected during sample loading and subsequent washing with starting buffer. All the recovered activity (38-40%) eluted sharply, without noticeable retardation, in the first fractions collected. No activity was recovered when the column was washed with 0.1M-α-methyl-D-glucopyranoside in starting buffer.

Other attempts, using a cationic buffer, 0.1M Tris-HCl (pH 7.0), containing NaCl (1M and 0.1M) produced essentially the same results.

3.11. SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was performed in slab polyacrylamide gels, approx. 14.5 cm wide and 20 cm long, of the composition described by Laemmli (1970), using an apparatus of the model described by Studier (1973). Gels were cast in a mould formed by a pair of glass plates, separated at the bottom and side edges by 0.15cm-thick perspex spacers sealed with vaseline. A perspex comb was used to form the sample wells in the stacking gel.

3.11.1. Gel preparation. Separating gels with a constant acrylamide concentration were prepared by mixing 10ml of the **separating gel buffer stock solution** [1.5M Tris-HCl (pH 8.8), containing 0.4% (w/v) SDS], with the required volume of the **acrylamide stock solution** [30% (w/v) acrylamide plus 0.8% (w/v) N,N'-methylene-bis-acrylamide] and water to 40ml. The mixture was degassed on a water pump and its polymerization was initiated by the addition of 40µl of **TEMED** (N,N,N,N'-tetramethylethylenediamine) and 200µl of a 10% (w/v) freshly prepared solution of ammonium persulphate. The mixture was immediately poured into the gel mould and overlayered with 2-butanol.

The stacking gel mixture, with 4 or 5% (w/v) acrylamide, was prepared by mixing 2.5ml of the **stacking gel buffer solution** [0.5M Tris-HCl (pH 6.8), containing 0.4% (w/v) SDS] with the required volume of the **acrylamide stock solution** and water to 10ml. After degassing, 10µl of **TEMED** and 50µl of the ammonium persulphate solution were added.

The surface of the polymerized separating gel was washed with a small portion of this mixture, and the remaining was poured into the gel mould. The well-forming comb was immediately introduced and the gel was allowed to set for at least 1h (or overnight, in the cold, and protected from light).

Separating gels with a concentration gradient of acrylamide were also prepared, using a gradient mixer. The vessel of the gradient mixer to which the outlet was connected was filled with 20ml of a gel mixture containing the higher concentration of acrylamide; the mixture with the lower acrylamide concentration (20ml) was placed in the second vessel. The gel mould was placed at approx. 30° to the horizontal and the mixture was pumped into the mould through a thin silicone tube. The tube was progressively raised in the course of the operation, which had to be completed in 5-8 min to avoid the setting of the acrylamide solutions. The subsequent procedures were identical to those used for gels of constant acrylamide concentration.

The mould with the polyacrylamide gel was placed in the electrophoresis apparatus, using vaseline as sealing agent, after removal of the bottom spacer and of the well-forming comb. The upper and lower electrode compartments were filled with **electrode buffer** [0.025M Tris-0.191M glycine (pH 8.3), containing 0.1% (w/v) SDS]. Air bubbles trapped at the bottom of the gel were removed by flushing with electrode buffer, from a syringe with a bent needle, introduced between the glass plates of the gel mould.

3.11.2. Sample preparation and gel electrophoresis. The samples, in **sample buffer** [0.0625M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1% (w/v) SDS and 0.001% (w/v) bromophenol blue, as tracking dye] were prepared mixing 3 volumes of protein sample with 1 volume of the **sample buffer stock solution**, prepared with a concentration 4-fold the final concentration. Protein denaturation and inactivation of any proteolytic enzymes which might be present was achieved by heating the samples, in a boiling water bath, for 3-10 min (Pringle, 1970). Samples containing high salts concentration were previously dialysed against distilled water.

After this treatment, the samples were introduced in the gel wells, with a microsyringe. Volumes of 5-50µl were usually loaded, according to the protein concentration; from very dilute samples, volumes up to 150µl could be loaded in a well provided that each sample portion was allowed to enter the stacking gel, by switching on the current for a few minutes, before adding another portion.

Electrophoresis was carried out at a constant current of 37mA for 5-7h (according to the gel concentration) until the tracking dye approached the bottom of the gel. After electrophoresis, the gel was removed from the mould and fixed in methanol-acetic acid-water (5:1:5, by volume) or in 30% aq. methanol for a minimum of 1h with two changes of fixing solution.

3.11.3. Detection of protein bands. Gels were routinely stained in a 0.25% (w/v) Coomassie brilliant blue R250 solution in 50% aq. methanol, for 2h, and destained in 30% aq. methanol or methanol-acetic acid-water (3:1:6, by volume) by diffusion with gentle shaking. The gel was kept in the destaining solution for 3h and, after that period, the solution was changed several times until the bands became visible and the background clear. This method allows the detection of 0.1-0.5 μg of protein (Weber & Osborn, 1975). Stained gels were kept in plastic boxes in 7% aq. acetic acid.

In the experiments involving the detection of the cyclase digestion products the method of silver-staining described by Wray et al. (1981) was used.

The detection of glycoprotein was also attempted, following a method adapted from that described by Phillips (1981). SDS was removed from the gel by diffusion overnight, with gentle shaking, in isopropanol-acetic acid-water (25:10:65, by volume) in the presence of a mixed bed ion-exchange resin (prepared by mixing equal amounts of BioRad, AG1-X8 anion exchanger, in the OH^- form, and BioRad 50W-X8 cation exchanger in the H^+ form). The gel was then treated as follows: (i) incubation with 1% (w/v) periodic acid solution in 3% aq. acetic acid, for 1h, in the dark, at room temperature; (ii) incubation with 0.5% (w/v) sodium arsenite solution in 5% aq. acetic acid, for 20 min; (iii) washing with 10% aq. acetic acid, in the presence of the mixed bed ion-exchange resin, for 3-4h; (iv) incubation with

Schiff's reagent, for 2h, in the dark; (v) washing with 0.1% (w/v) sodium metabisulphite in 0.01M HCl (with 3 changes of the solution). This method produced inconclusive results, as described in section 4.10.

3.12. Attempted Electrophoretic Elution of Cyclases from Gel Slices

The electrophoretic elution of cyclase bands from polyacrylamide gel slices was considered as a possible method for the isolation of homogeneous samples which would be necessary for N-terminal amino acid analysis. The method followed was similar to those described by Walker et al. (1982) and by Braatz & McIntire (1977). Gel slices were introduced on the top of stick gels, approx. 6cm high, cast in glass tubes 7.5cm long; those tubes were cut from standard glass Pasteur pipettes, with one end tapered to 4mm. A piece of dialysis tube, fitting the glass tube tightly, was forced down the tapered end, filled with buffer in excess of the amount required to cover the gel slices, and tied with string. The gel tubes were introduced, with the dialysis bags on the bottom (anode compartment), in a **Shandon** disc gel electrophoresis apparatus; electrophoresis was performed for 12h, in the cold, at 3mA per gel. The buffer used in the gel preparation and as electrode buffer was 50mM sodium bicarbonate, containing 0.1% (w/v) SDS; the same buffer was used to cover the gel slices and fill the dialysis tube. After electrophoresis, the solution in the dialysis bag was collected and treated with n-octanol, to

remove Coomassie blue (Gibson & Gracy, 1979). The aqueous phase was then freeze-dried.

3.13. Attempted N-terminal Group Determination

The freeze-dried samples obtained as described in section 3.12 were dansylated and hydrolyzed following the method described by Gray (1972). The hydrolysates were analysed by two-dimensional chromatography, as described by Woods & Wang (1967), using polyamide-coated sheets, containing fluorescence indicator (**Eastman chromagram**); the solvent systems used were 1.5% (v/v) aq. formic acid and toluene-glacial acetic acid (10:1, by volume) as described by Perham (1978). The developed sheets were examined under **uv** light (365 nm), but only the blue fluorescence of Dns-OH could be detected.

3.14. Fingerprinting in SDS-Polyacrylamide Gels

Since no homogeneous preparations were available, digestions of cyclase bands cut from SDS-polyacrylamide gels were performed. The method described by Nikodem & Fresco (1979) was used for the CNBr digestion of gel bands. Digestions with chymotrypsin and Staphylococcus aureus V8 protease were performed as described by Cleveland et. al. (1977). The gels used in the analysis of the digestion fragments contained a concentration gradient (10-22.5%, w/v) of acrylamide in the separating gel and a 5cm high, 4% (w/v) acrylamide stacking gel. The Laemmli (1970) buffer system

was used throughout, as described in section 3.11. The gels were silver-stained by the method of Wray _et al._ (1981).

CHAPTER 4

THE PURIFICATION OF THE A. NIGER CYCLASE

4.1. Conditions for Cell Disruption

Fungal mycelia are usually disrupted by mechanical methods such as high-speed blending of a mycelial suspension in buffer (Thatcher & Cain, 1974 a), grinding under liquid nitrogen, followed by thawing in buffer (Cook & Cain, 1977) or grinding with sand, as an abrasive agent (Ahlquist, 1977). These three methods were compared on several portions of the same batch of A. niger mycelium and, in addition, the optimum blending time in the high-speed homogenizer used in this work (**Virtis 45**) was also established. The buffer used in all extractions was 0.05M potassium-sodium phosphate, pH 6.8 (1.5ml per gram wet-weight mycelium). The samples of cell homogenates collected after blending or grinding were centrifuged as described in Section 3.5 and the supernatants were assayed for protein and activity. The results listed in Table 4.1 showed that the amount of cyclase extracted was a constant fraction of the total protein in the crude extract and that the extension of the blending time was not deleterious to the enzyme, provided that the temperature of the slurry was kept at 5-15°C, which were the conditions of temperature in this experiment. A blending period of 15 min was then

adopted for mycelial disruption, after comparison of the amounts of cyclase extracted by this method and by the alternative methods involving grinding. The comparative results are summarized in Table 4.2.

TABLE 4.1.

Influence of the blending time in the amount of cyclase in the cell-free extract

Blending time (min)	Protein ($mg.ml^{-1}$)	Activity ($Units.ml^{-1}$)	Specific activity [Units(mg protein)$^{-1}$]
2	2.3	0.49	0.22
4	2.6	0.54	0.21
6	2.9	0.62	0.21
8	3.1	0.66	0.21
10	3.4	0.72	0.21
12	3.6	0.79	0.22
15	3.6	0.80	0.22

TABLE 4.2

Comparison of alternative methods of mycelial disruption

Method	Protein ($mg.ml^{-1}$)	Specific activity [Units(mg protein)$^{-1}$]
Blending (15 min)	3.6	0.22
Grinding with sand	2.8	0.21
Grinding under liq. N_2	2.6	0.22

4.2. Effect of the Buffer on the Specific Activity of Cell-Free Extracts.

Potassium-sodium phosphate buffers (pH 6.8), were used in the preparation of cell-free extracts of the A. niger cyclase and in several purification steps of the method described by Thatcher (1972). It is known, however, that phosphate ions are inhibitory to 3-carboxymuconate lactonizing enzymes both from A. niger (Thatcher, 1972) and P. putida (Ornston, 1966 b). A cationic buffer might then be advantageous in the preparation of cell-free extracts and in the course of purification. The influence of buffer composition on the specific activity of the cyclase in

cell-free extracts was therefore examined, using several samples of the same batch of A. niger mycelium. The results of this experiments (Table 4.3) were not conclusive at this stage, since the small differences found in specific activities were within experimental error.

TABLE 4.3

Influence of the buffer on the specific activities of cell-free extracts

Buffer	pH	Specific activity [Units(mg protein)$^{-1}$]
0.05M Phosphate (Na^+,K^+)	6.8	0.33
0.1M Imidazole (Cl^-)	6.8	0.32
0.05M Tris (Cl^-)	7.2	0.34
0.1M Ethylenediamine (Cl^-)	7.0	0.31

4.3. Ammonium Sulphate Fractionation

Preliminary experiments revealed that ammonium sulphate added to 50% saturation, as previously used by Thatcher (1972) did not precipitate all the cyclase from cell-free extracts in which protein concentrations were in the range 2.8-3.6(mg protein)ml^{-1}. Because precipitation by ammonium

sulphate is a convenient method for volume reduction in protein solutions, the conditions for maximum recovery of the cyclase were investigated. The distribution of cyclase activities in the protein fractions precipitating after each stepwise increase in the ammonium sulphate concentration is indicated in Table 4.4.

TABLE 4.4

Fractionation of cell-free extracts with ammonium sulphate

The cell-free extract (90ml;288mg protein) in 0.05M potassium-sodium phosphate (pH 6.8), contained 75 Units of *A. niger* cyclase. Each fraction was redissolved in 2ml of the same buffer.

Ammonium sulphate (% saturation)	Cyclase activity (Units)	Yield (%)	Protein (mg)	Enrichment (-fold)
0-30	0	0	5	N.D.
30-40	3.8	5	14	
40-50	43.5	58	40	4.4
50-60	24.7	33	31	
60-65	1.5	2	11	N.D.

N.D.: Not determined

As an alternative to this process, another sample of cell-free extract was brought to 65% saturation by the addition of ammonium sulphate. Precipitated protein was collected by centrifugation and resuspended in the buffer used in the previous experiment (2-3ml/100ml of cell-free extract). Undissolved protein was removed by centrifugation and the clear solution was subjected to a second fractionation with ammonium sulphate. The results are listed in Table 4.5.

TABLE 4.5

Second ammonium sulphate fractionation of cell-free extracts

The bulk of the cyclase activity in 100ml of cell-free extract (80 Units, 320 mg protein) in 0.05M potassium-sodium phosphate, pH 6.8, was precipitated by ammonium sulphate at 65% saturation. The precipitated protein fraction was redissolved in the same buffer and fractionated again by stepwise addition of ammonium sulphate.

Ammonium sulphate (% saturation)	Cyclase activity (Units)	Yield (%)	Protein (mg)	Enrichment (-fold)
0-30	2	2.5	N.D.	N.D.
30-40	51	64	29	10.5
40-50	25	31		
50-60	0	0	N.D.	N.D.

N.D.: Not determined

A considerably higher enrichment of the cyclase preparation was achieved by the second method which was therefore adopted for the fractionation of cell- free extracts. Similar experiments of ammonium sulphate fractionations when cell-free extracts were prepared in cationic buffers (0.1M imidazole-HCl, pH 6.8 or 0.05M Tris-HCl, pH 7.2) produced, consistently, recoveries of cyclase activity in the range 75-80%.

4.4. Comparison of Different Gel Permeation Media

In the course of the various attempts to improve the A. niger cyclase purification method, several gel permeation media were tried. Sephadex G-200 has a good resolution in the range of interest and was used in earlier experiments. The native molecular weights of the cyclase from A. niger (wild type) and from the strain **PL30** (temperature-sensitive mutant) were both determined in calibrated columns of this gel (Section 5.1). In other trial experiments Ultrogel AcA34 and Sephacryl S-300, with improved flow properties, were also used. The elution profiles of typical experiments with these gels (Figs. 4.1 and 4.2) indicated that Ultrogel AcA34 had a better resolution in the zone of interest. This conclusion was confirmed by comparison of the experimental selectivity curves of both gels (section 6.3.). The yields and enrichments factors achieved in these experiments are indicated in Table 4.6 and also favoured the choice of Ultrogel AcA 34.

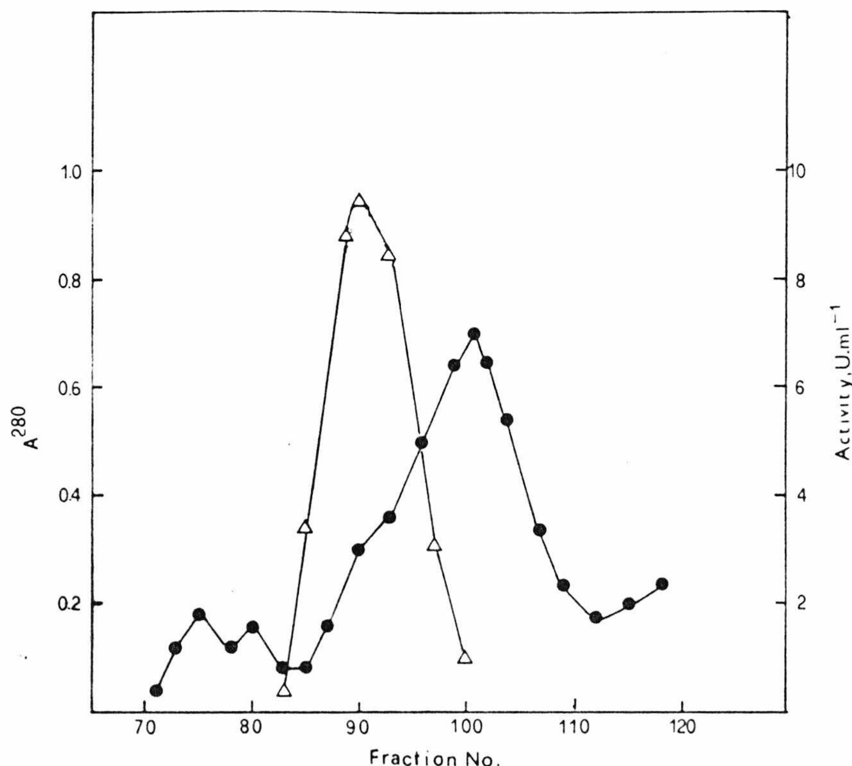


Fig.4.1. Gel permeation chromatography of the *A. niger* cyclase in Ultrogel AcA34. The cyclase sample (238 Units, 40mg protein), had been subjected to two ammonium sulphate fractionation steps, as described in section 4.3. The column was eluted with 0.05M potassium-sodium phosphate (pH 6.8) and fractions of approx. 2.6ml were collected. This step produced a 6-fold enrichment in 95% yield. The absorbance at 280nm (●) and the activity (△) were plotted against fraction number.

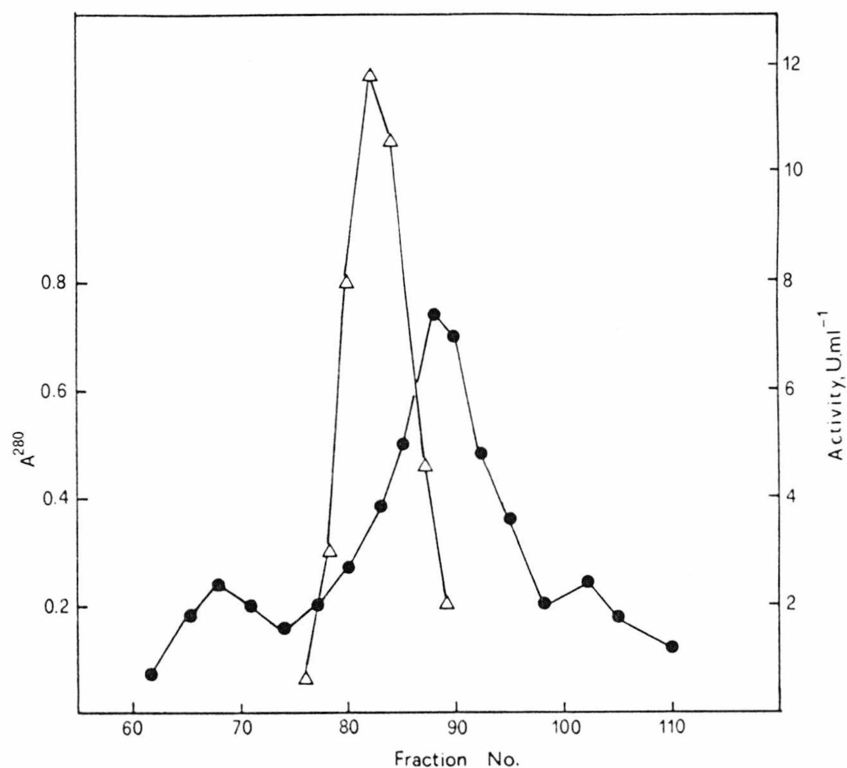


Fig.4.2 Gel permeation chromatography of the A. niger cyclase in Sephacryl S-300. The cyclase sample (330 Units, 46mg protein), had been subjected to two ammonium sulphate fractionation steps, as described in section 4.3. The column was eluted with 0.05M Tris-HCl (pH 7.2) and fractions of approx. 2.8ml were collected. This step produced a 3.7-fold enrichment in 76% yield. The absorbance (●) and the activity (△) of a number of a fractions were plotted againsts fraction number.

TABLE 4.6

Comparison of Ultrogel AcA34 and Sephacryl S-300 in the purification of the *A. niger* cyclase

Gel	Buffer	Yield (%)	Enrichment (- fold)
Ultrogel AcA34	0.05M Phosphate (pH 6.8)	95	6.0
Sephacryl S-300	0.05M Tris-HCl (pH 7.2)	76	3.7

4.5. Anion-Exchange Chromatography

The introduction of anion-exchangers, such as DEAE-Sepharose CL-6B and DEAE-Sephacel, easier to use and performing better than the DEAE-celluloses, previously included by Thatcher (1972) in the purification of the *A. niger* cyclase, required the re-investigation of the optimum conditions for this step.

The results from small-scale tests, which allowed the choice of the convenient pH range for the starting buffer, are summarized in Table 4.7. For each pH value, 1ml of DEAE-Sephacel, packed in a syringe, was equilibrated with 0.02M potassium-sodium phosphate buffer; the cyclase sample (10 Units), equilibrated with the same buffer, was passed

through the column, which was then eluted with the starting buffer made 0.2M in NaCl, in 1ml portions, until no activity was detected in the eluate.

TABLE 4.7

Cyclase activities eluted from DEAE-Sephacel at several pH values

In each test, 10 Units of cyclase were applied to the anion-exchanger, previously equilibrated with 0.02M potassium-sodium phosphate buffer (pH 6.8).

pH	Volume of eluate (ml)	Cyclase activity (units)
6.0	3	8.2
6.4	3	8.5
6.8	3	9.2
7.0	3	9.2
7.2	3	9.1
7.6	5	6.5

The results in Table 4.7 showed that the cyclase, at pH 7.6, was strongly bound to the anion-exchanger, and the comparatively low yield was interpreted as the consequence of partial irreversible binding. Since this effect was not observed in the other buffers, the optimum pH range (6.8-7.2) was adopted on the grounds of the recovery yields.

The same starting buffers and the same elution conditions produced similar results when DEAE-Sepharose was used as anion-exchanger.

Both anion-exchangers were also tested with 0.05M Tris-HCl (pH 6.8, 7.2 and 7.6) as starting buffers. The results supported the choice of the optimum pH range deduced from the experiments with phosphate buffers.

In another series of small-scale experiments, the anion-exchangers were equilibrated with buffers of increasing concentration and cyclase binding was tested. In potassium-sodium phosphate buffer (pH 6.8) cyclase binding was observed at concentrations up to 0.05M; with Tris-HCl buffer (pH 7.2) the activity was fully retained in the column at buffer concentrations up to 0.1M.

The capacities of DEAE-Sephacel and DEAE-Sepharose for crude cyclase preparations (2-3 units/mg protein) in 0.05M potassium-sodium phosphate (pH 6.8) and 0.1M Tris-HCl (pH 7.2) as starting buffers were estimated as 150-200 mg protein/ml packed exchanger. In subsequent fractionation experiments, columns with a capacity at least 10-fold the minimum required for complete cyclase binding were used.

The elution profiles of typical fractionations performed on DEAE-Sepharose and DEAE-Sephacel are illustrated in Figs. 4.3 and 4.4. Each sample was previously dialysed against starting buffer; after sample loading the columns were washed with 3-4 bed volumes of the same buffer, and eluted with a concentration gradient of NaCl, as described in each legend. Yields and enrichment factors for both experiments are listed in Table 4.8.

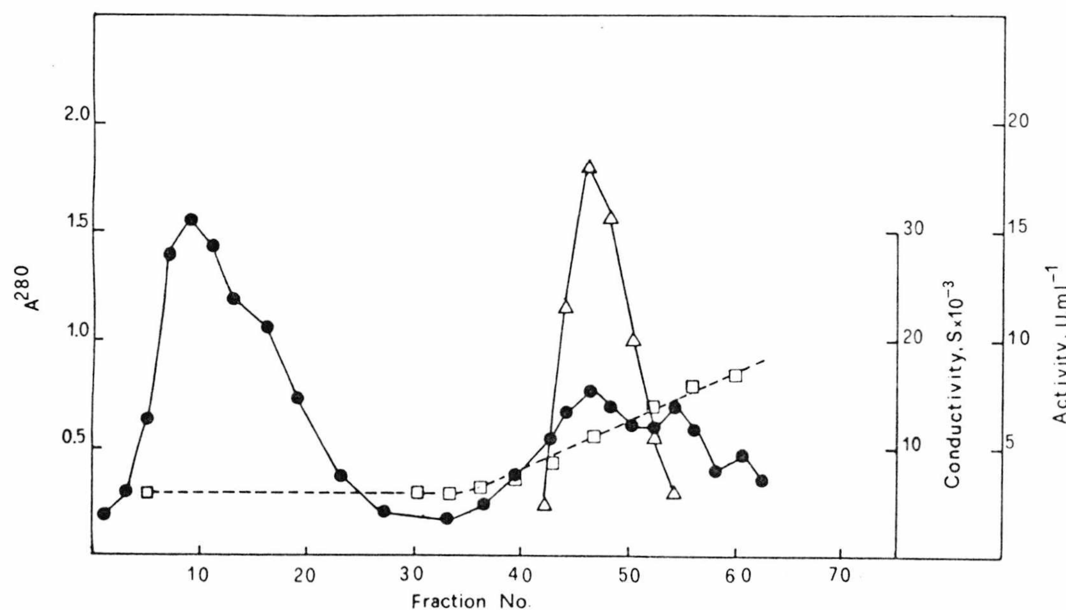


Fig.4.3. Anion-exchange chromatography of the *A. niger* cyclase in DEAE-Sepharose. The cyclase sample (450 Units, 96 mg protein) had been subjected to two ammonium sulphate fractionation steps, as described in section 4.3, and dialyzed against starting buffer, (0.1M Tris-HCl, pH 7.2). The column had a bed volume of 20ml (1.6x10cm) and was eluted with 120ml of a 0-0.25M gradient of NaCl in starting buffer. Fractions of 2.6ml were collected. This step produced a 5.1-fold enrichment, in 60% yield. The absorbance (●) the activity (△) and the conductivity (□) of a number of fractions were plotted against fraction number.

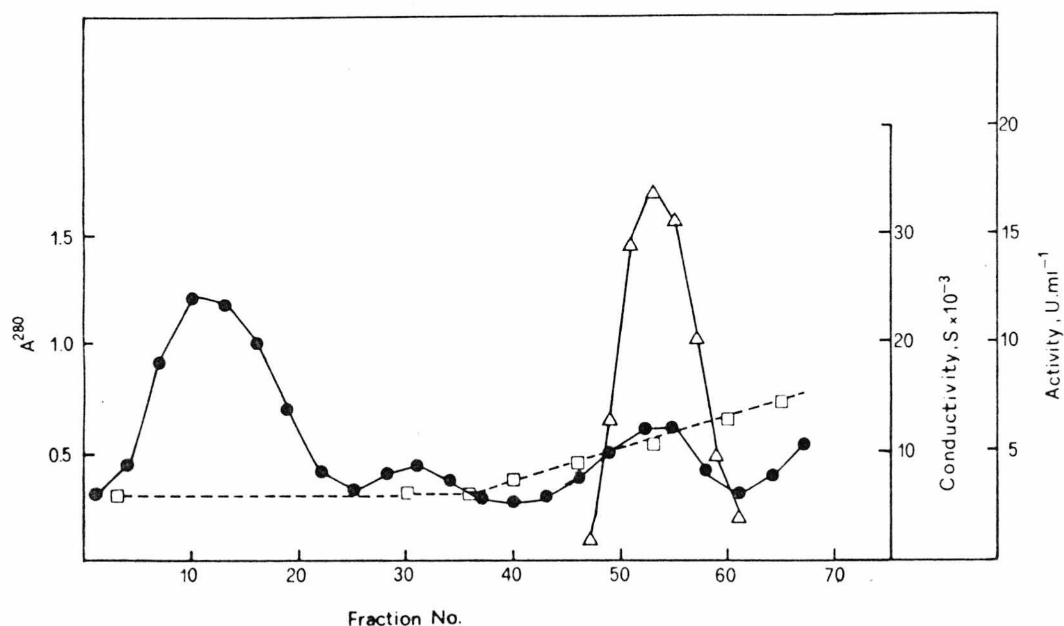


Fig.4.4. Anion-exchange chromatography of the *A. niger* cyclase in DEAE-Sephacel. The cyclase sample (300 Units, 72 mg protein) had been subjected to two ammonium sulphate fractionation steps, as described in section 4.3, and dialyzed against starting buffer, (0.05M potassium-sodium phosphate, pH 6.8). The column had a bed volume of 20ml (1.6x10cm) and was eluted with 100ml of a 0-0.15M gradient of NaCl in starting buffer. Fractions of 2.5ml were collected. This step produced a 7.8-fold enrichment, in 90% yield. The absorbance (●), the activity (△) and the conductivity (□) of a number of a fractions were plotted against fraction number.

TABLE 4.8

Comparison of two anion-exchangers in the cyclase fractionation

Exchanger	Starting buffer	Yield (%)	Enrichment (- fold)
DEAE-Sepharose	0.1M Tris-HCl (pH 7.2)	60	5.1
DEAE-Sephacel	0.05M Phosphate (pH 6.8)	90	7.8

4.6. Hydrophobic Chromatography

The conditions for hydrophobic chromatography in alkyl- or aminoalkyl-substituted agaroses were established folowing a method similar to that described above for anion-exchange chromatography. In terms of recovery yields, ethyl-agarose and aminohexyl-agarose seemed more promising, as judged from the preliminary experiments. Accordingly, those gels were tested in two separate experiments. As described in section 3.10.3, the cyclase was adsorbed by ethyl-agarose in 0.025M imidazole-HCl buffer and by aminohexyl-agarose at concentrations up to 0.1M of the same buffer. The elution profiles of fractionations in each of these two adsorbents are illustrated in Figs. 4.5 and 4.6. The cyclase samples had been partially purified by ammonium sulphate fractionation,

and gel permeation chromatography; the eluate from the last step was concentrated and equilibrated, by dialysis, with the starting buffer of the hydrophobic chromatography step. Both columns had bed volumes of approx. 10ml and were eluted with linear concentration gradients of NaCl in starting buffer. Other experimental details are described in each legend. The results from both experiments are compared in Table 4.9.

TABLE 4.9

Comparison of two hydrophobic chromatography adsorbents

Adsorbent	Starting buffer	Yield (%)	Enrichment (- fold)
Ethyl-agarose	0.025M Imidazole-HCl(pH 6.8)	64	2.2
Aminohexyl-agarose	0.1M Imidazole-HCl(pH 6.8)	66	1.6

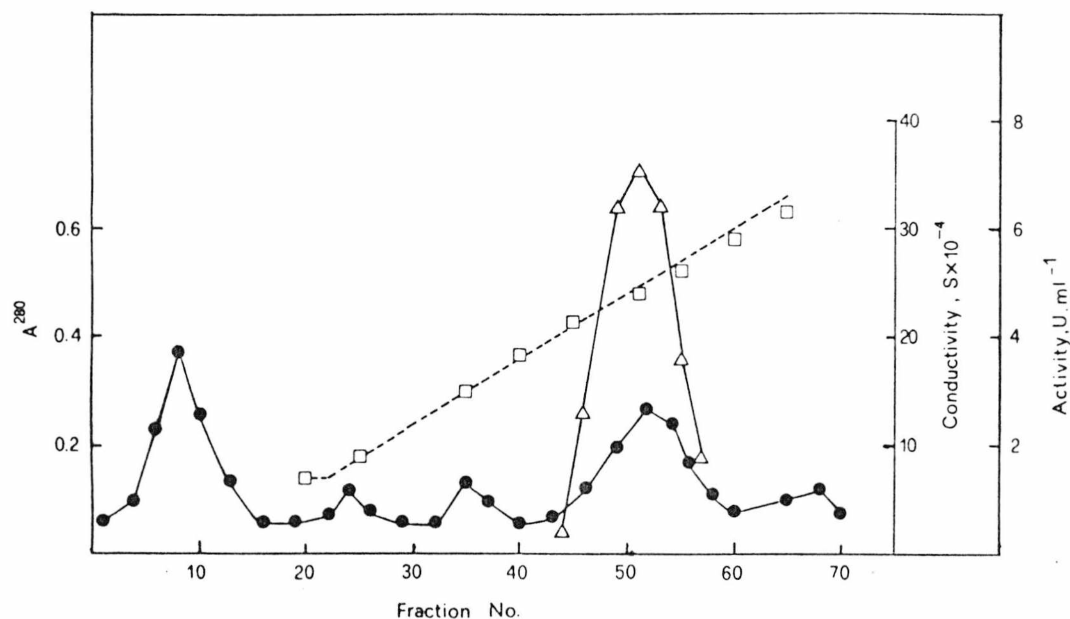


Fig.4.5. Hydrophobic chromatography of the A. niger cyclase in ethyl-agarose. The cyclase sample (160 Units, 7mg protein) had been partially purified by ammonium sulphate fractionation and gel permeation chromatography and dialyzed against the starting buffer (0.025M imidazole-HCl, pH 6.8). The column had a bed volume of 10ml (1x12.5cm) and was eluted with 100ml of a 0-0.05M gradient of NaCl in starting buffer. Fractions of 2ml were collected. This step produced a 2.2-fold enrichment in 64% yield. The absorbance (●), activity (△) and conductivity (□) of a number of fractions were plotted against fraction number.

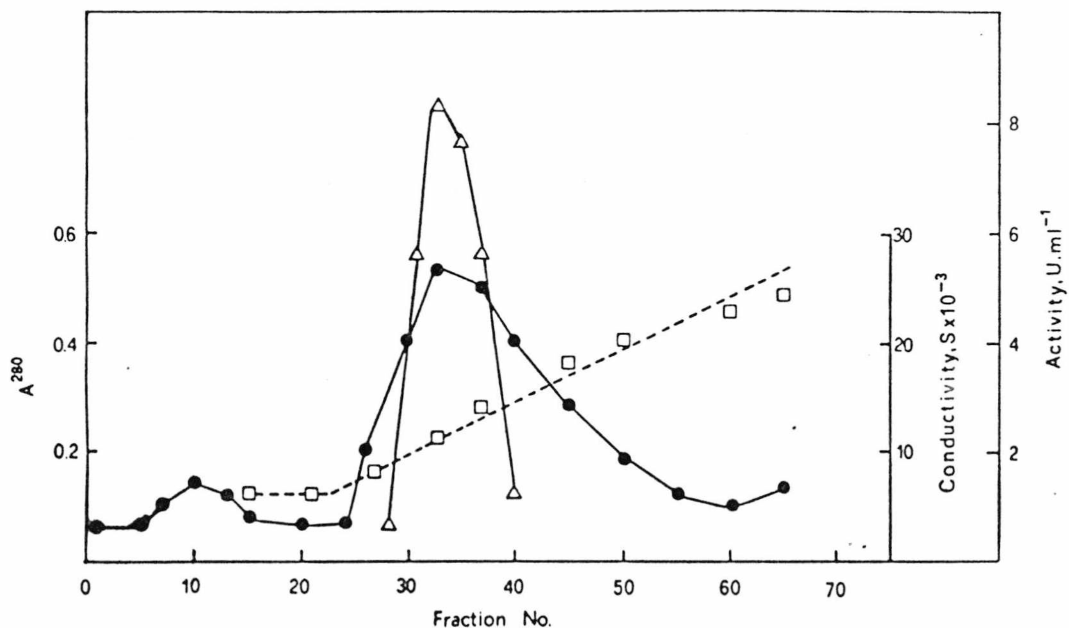


Fig.4.6. **Hydrophobic chromatography of the A. niger cyclase in aminohexyl-agarose.** The cyclase sample (154 Units, 10.5mg protein) had been partially purified by ammonium sulphate fractionation and gel permeation chromatography and dialyzed against the starting buffer (0.1M imidazole-HCl, pH 6.8). The column had a bed volume of 10ml (1x12.5cm) and was eluted with 100ml of a 0-0.25M gradient of NaCl in starting buffer. Fractions of 2ml were collected. This step produced a 1.6-fold enrichment in 66% yield. The absorbance (●), activity (△) and conductivity (□) of a number of fractions were plotted against fraction number.

4.7. Chromatofocusing

The inclusion of chromatofocusing in the purification of A. niger cyclase was determined by the non-homogeneity of the enzyme samples subjected to the various purification steps described in this chapter (see Fig. 5.3). Trial chromatofocusing experiments demonstrated that it was possible to recover active enzyme in the eluate, in yields approaching 65% and the analysis of a typical elution profile (Fig. 4.7) suggested a considerable enrichment of the enzyme preparation. This conclusion was further supported by comparative electrophoretic analysis of cyclase samples before and after chromatofocusing.

4.8. Additional Dialysis Experiments

Similar purification techniques, when performed in phosphate buffer and cationic buffers (Tris, imidazole) invariably yielded much higher activity recoveries whenever the first of those buffers was involved. Even dialysis, wich might be anticipated as a mild process, produced considerable activity losses when cationic buffers were used. To confirm the assumption of the comparative cyclase instability in this type of buffer, several cyclase samples were dialysed for 6h, at 4°C, against buffers of different compositions. The results are summarized in Table 4.10, showing that the cyclase was considerably more stable in phosphate buffer, at pH 6.8, and that EDTA could, with some advantage, be added to that buffer.

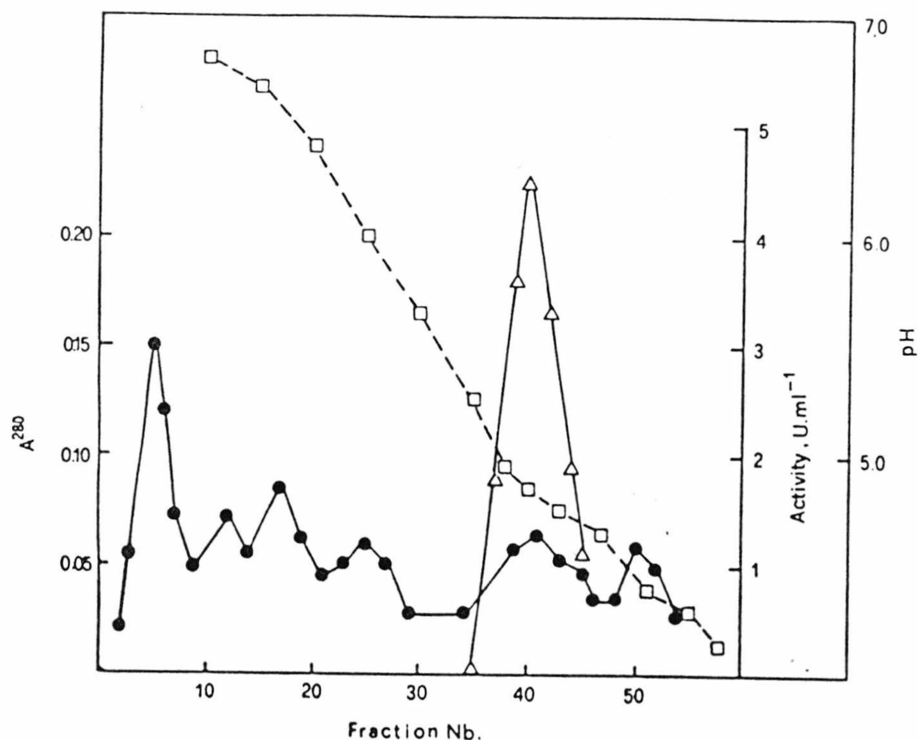


Fig.4.7. Chromatofocusing of the A. niger cyclase in Polybuffer exchanger with a 7-4 pH gradient. The partially purified cyclase sample (62 Units, 3mg protein) had been subjected to ammonium sulphate fractionation and DEAE-Sephacel chromatography. The column had a bed volume of 10ml (1x12.5cm) and the experimental conditions were as described in section 3.10.4. The recovery of activity in this step was 65%. The absorbance (●), activity (△) and pH (□) of a number of fractions were plotted against fraction number.

TABLE 4.10

Recoveries of cyclase activity after dialysis against buffers of different compositions

The dialysed samples were aliquots (2ml) of a cyclase preparation containing 7.5Units/ml.

Buffer composition	pH	Cyclase activity (Units)
0.05M Phosphate (Na^+,K^+)	6.8	14.2
0.05M Phosphate (Na^+,K^+),1mM EDTA	6.8	14.7
0.1M Imidazole (Cl^-)	6.8	9.8
0.1M Imidazole (Cl^-),4mM phosphate	6.8	12.0
0.1M Imidazole (Cl^-),4mM phosphate, 1mM EDTA	6.8	13.0
0.1M Tris (Cl^-)	7.2	10.0

4.9. Final Purification Method

The results of the last attempted purification sequence of the A. niger cyclase are summarized in Table 4.11. The different steps in that sequence are detailed below.

Step 1: Preparation of the cell-free extract

Frozen mycelium (3.5 kg) was homogenized in the presence of 0.05M phosphate buffer (pH 6.8), containing 1mM EDTA (**buffer A**), as described in section 3.6. The resulting

cell homogenate was centrifuged for 60 min. at 21 000g, producing 4790ml of cell-free extract.

Step 2: First ammonium sulphate fraction

The cell-free extract was brought to 65% saturation in ammonium sulphate, by addition of the solid salt. Precipitated protein was collected by centrifugation at 21 000g for 60 min. and the pellet was resuspended in **buffer A**. Undissolved protein and remaining cell debris were removed by centrifugation at 21 000g for 30 min.. The clear protein solution was the first ammonium sulphate fraction (170ml).

Step 3: Second ammonium sulphate fraction

The solution from step 2 was again fractionated with ammonium sulphate and the protein precipitating between 30 and 50% saturation in ammonium sulphate was collected. This fraction was redissolved in **buffer A** and dialysed for 12h against **buffer A** made 0.075M in NaCl (**buffer B**). After dialysis, the precipitated protein was removed by centrifugation. The clear supernatant (25ml) was the second ammonium sulphate fraction.

Step 4: Batch DEAE-Sepharose chromatography

The sample from the previous step was applied to a column (1.6x12cm) of DEAE-Sepharose equilibrated with **buffer B**; the column was washed with the same buffer until no activity was detected in the eluate. The fractions

containing activity were pooled and concentrated by precipitation with ammonium sulphate at 65% saturation. The pellet was redissolved in **buffer A** and dialysed against the same buffer for 12h. The conditions used in this step minimized the interaction between the enzyme and the ion-exchanger, and the activity loss was negligible. The enrichment observed was not substantial but it had the advantage of easily removing those contaminant proteins which bound more strongly to the ion-exchanger than the cyclase, thus allowing the use of a shorter column in the next step.

Step 5: Gradient DEAE-Sephacel Chromatography

The dialysed solution from the previous step was passed through a column (1.6x16cm) of DEAE-Sephacel equilibrated with **buffer A.** The column was washed with 100ml of the same buffer, to remove unbound protein, and then eluted with 150ml of a linear gradient concentration (0-0.15M) of NaCl in starting buffer. Fractions of 4.4ml were collected, and those containing the bulk of the enzyme activity were pooled (57ml). The conductivity and A^{280} of a number of fractions were measured. The elution profile is illustrated in Fig.4.8.

Step 6: Gel Permeation Chromatography

The eluate from the previous step was concentrated as described in step 4. The concentrated extract (4ml) was applied to a column of Ultrogel AcA34 (480ml bed volume) equilibrated with **buffer A.** The column was eluted at a flow rate of $24.6ml.h^{-1}$. Fractions of 4.1ml were collected and

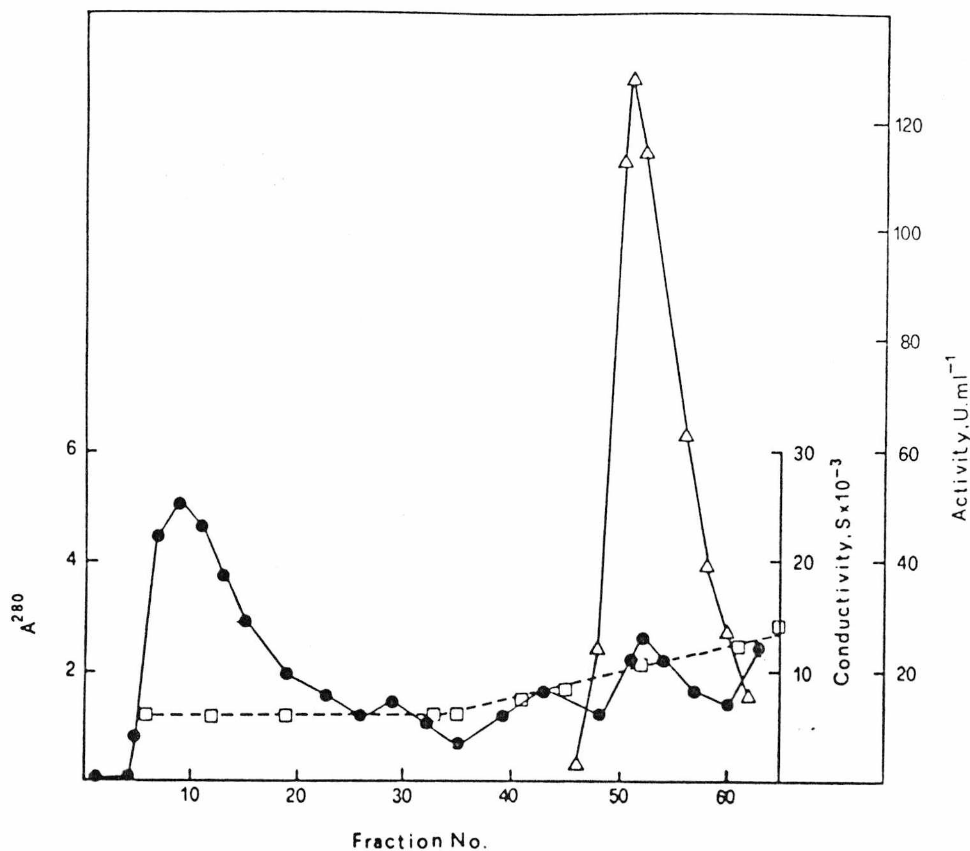


Fig.4.8. Gradient DEAE-Sephacel chromatography in the large-scale purification of the A. niger cyclase (Step 5).The experimental conditions are described in section 4.9. The absorbance at 280nm (●), the activity (△) and conductivity (□) of a number of fractions were plotted against fraction number.

those containing activity were pooled. The elution profile for this experiment is illustrated in Fig.4.9.

Step 7: Ethyl-agarose chromatography

The Ultrogel AcA34 eluate was precipitated with ammonium sulphate, as described above. The pellet was dissolved in 2ml of 5mM phosphate, 1mM EDTA, pH 6.8 (**buffer C**) and dialysed against the same buffer for 12h. The dialysed solution (3.8 ml) was applied to a column (1.6x10cm) of ethyl-agarose equilibrated with **buffer C**, and eluted with 100ml of a linear gradient of phosphate buffer, pH 6.8 (5-25mM) containing 1mM EDTA. When all the activity had been collected, the column was washed with 50mM phosphate (pH 6.8) to remove the more strongly bound proteins. Fractions of 2.5ml were collected and those containing activity were pooled (22ml). Results from this step are shown in Fig.4.10.

Step 8: Chromatofocusing

A column of Polybuffer exchanger PBE94 with 10ml bed volume was equilibrated with 0.025M histidine-HCl, pH 6.2 (**buffer D**), according to the manufacturer's instructions for a pH gradient from 5.5 to 4.0. The eluate from the previous step was concentrated and equilibrated with **buffer D** and applied to the column. Elution was performed with 120ml of Polybuffer 74 adjusted to pH 4.0 with HCl. Fractions of 2.4ml were collected.

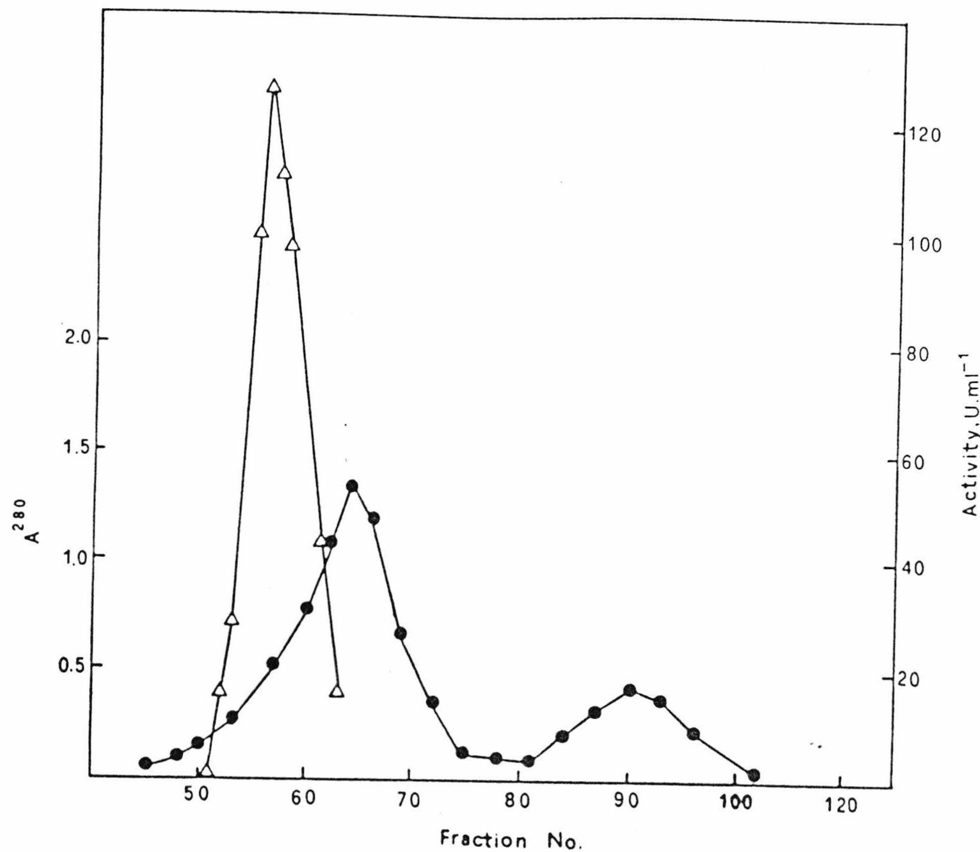


Fig.4.9. Gel permeation chromatography in Ultrogel AcA34, in the large-scale purification of the A. niger cyclase (Step 6). The experimental conditions are described in section 4.9. The absorbance at 280nm (●), and the activity (△) of a number of fractions were plotted against fraction number.

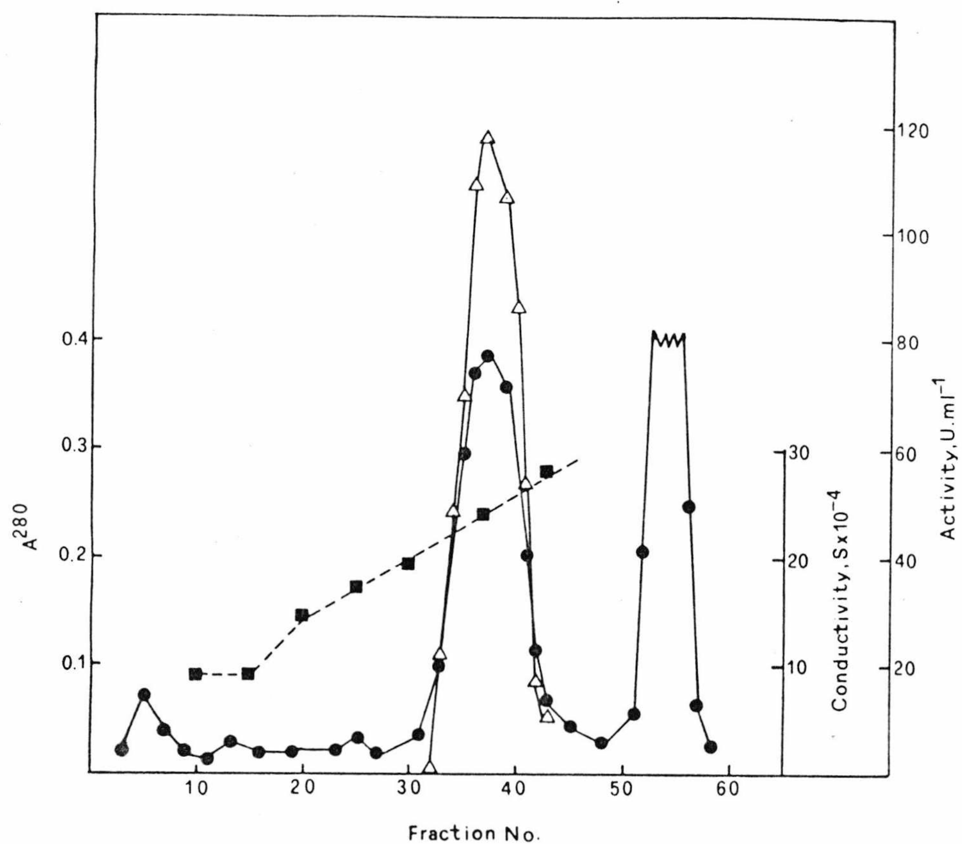


Fig.4.10. Ethyl-agarose chromatography in the large-scale purification of the A. niger cyclase (Step 7). The experimental conditions are described in section 4.9. The absorbance at 280nm (●), activity (△) and conductivity (■) of a number of fractions were plotted against fraction number.

Given the elution profile (Fig.4.11), fractions 27-30 (**batch 1**) and 31-34 (**batch 2**) were separately pooled and assayed for activity. The recovered activity was 950 Units in batch 1 and 680 Units in batch 2.

Step 9: Batch butyl-agarose chromatography

Batches 1 and 2 from Step 8 were adjusted to pH 6.8, diluted 1:2 with distilled water and passed through a 1ml column of butyl-agarose, equilibrated with 0.075M imidazole-HCl (pH 6.8). All the activity was retained in the column. After washing with the same buffer, the activity was eluted with 0.1M imidazole-HCl (pH 6.8) containing 0.025M NaCl. The recovered activity was 682 Units from batch 1 and 270 Units from batch 2. This step was intended to remove polybuffer and allow protein determination by the method of Lowry et al. (1951). The results from the protein assays were the following:

Batch 1 (7ml): 60 μg/ml

Batch 2 (7ml): 40 μg/ml

4.10. Analysis of Purified Cyclase Samples by SDS-Polyacrylamide Gel Electrophoresis.

Cyclase samples from the last purification steps of the process described in section 4.9 were analysed by SDS-polyacrylamide gel electrophoresis. The patterns for the concentrated Ultrogel eluate, batch 1 from chromatofocusing and batches 1 and 2 after butyl-agarose chromatography are shown in Fig.4.12. Aliquots from fractions of the ethyl-

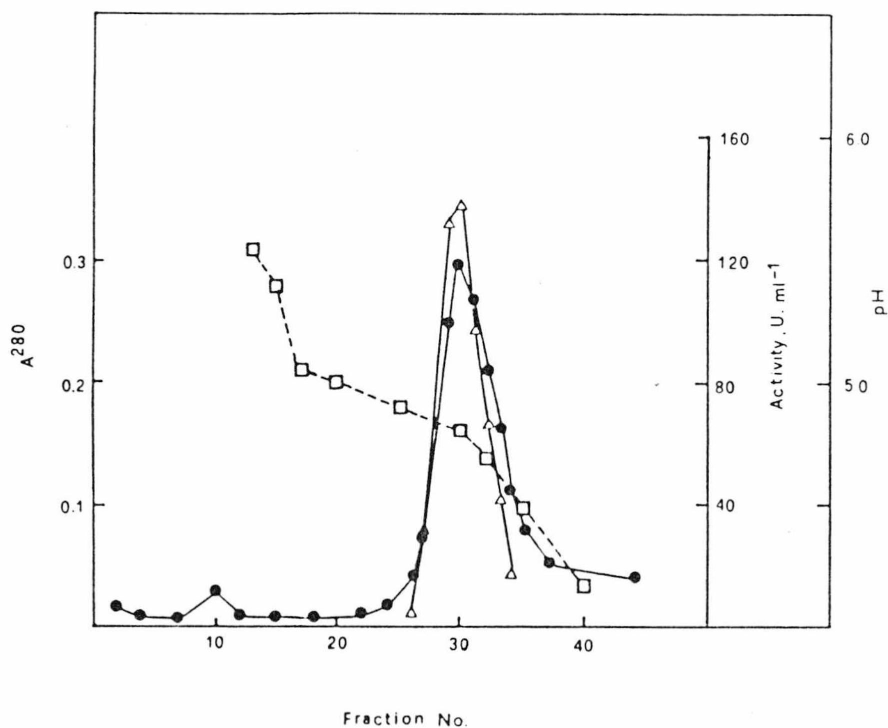


Fig.4.11. Chromatofocusing in the large-scale purification of the A. niger cyclase (Step 8). The experimental conditions are described in section 4.9. The absorbance at 280nm (●), activity (△) and the pH (□) of a number of fractions were plotted against fraction number.

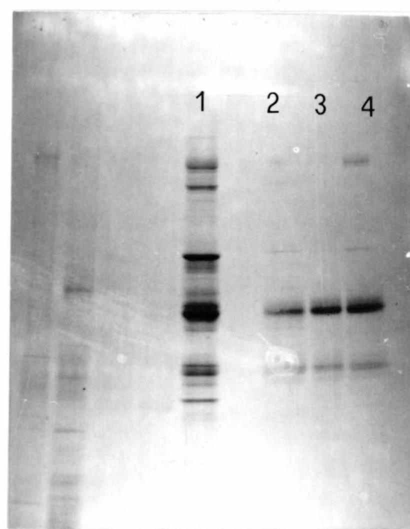


Fig.4.12. SDS-Polyacrylamide gel of purified cyclase samples. The wells contained samples from: (1) concentrated Ultrogel eluate; (2) chromatofocusing eluate (batch 1); (3) butyl-agarose eluate (batch 1); (4) butyl-agarose eluate (batch 2). The gel contained a gradient concentration of acrylamide (7.5-15%,w/v).

agarose eluate, run together with marker proteins, were used in one determination of the subunit mol.wt. of the cyclase (see Fig. 5.3). The observation of these gels revealed that the purest cyclase sample was batch 1, after butyl-agarose chromatography, and that its content in pure cyclase could be roughly estimated as, at least, 85% of the total protein. Comparison of the band patterns of the eluates from Ultrogel, ethyl-agarose, chromatofocusing and butyl-agarose revealed also that the last steps produced a significant reduction of the contaminant proteins.

Another gel was run with the pooled fractions of the ethyl-agarose-eluate and stained for glycoprotein, as described in section 3.11. Samples of ovalbumin and glycoprotein (bovine, fraction VI, from **Sigma**) were used as positive controls and bovine serum albumin as a negative control. The positive controls were the first to appear, as pink bands after staining. The contaminating band of lower mobility in the cyclase sample appeared next but, even after the washing process, all of the more intense bands in the cyclase sample and the negative control became visible.On the grounds of the time course of band appearance it was provisionally concluded that the contaminating band of lower mobility, in the cyclase sample, contained carbohydrate but the final observation made this experiment inconclusive and the gel was not photographed.

TABLE 4.11

Purification of the *A. niger* cyclase

Experimental details of this process are described in Section 4.9.

Step	Volume (ml)	Protein (mg)	Total activity (Units)	Recovery of activity (%)	Specific activity [Units(mg protein)$^{-1}$]	Enrichment (-fold)
Cell-free extract	4 790	15 800	7 664	-	0.48	-
First $(NH_4)_2SO_4$ fraction	170	3 550	7 434	97	2.1	4.3
Second $(NH_4)_2SO_4$ fraction	25	1 400	7 280	95	5.2	10.8
Batch DEAE-Sepharose	27	1 050	7 154	93	6.8	14.2
Gradient DEAE-Sephacel	57	102	5 600	74	56	116
Ultrogel AcA 34	47	16.5	4 175	54	253	527
Ethyl-agarose	22	3.8	1 999	26	526	1 096
Chromatofocusing (batch 1)	9.5	-	950	12	-	-
Butyl-agarose (batch 1)	7.0	0.42	682	9	1 624	3 383

CHAPTER 5

STRUCTURE AND PROPERTIES OF THE A. NIGER 3-CARBOXYMUCONATE CYCLASE

5.1. Native Molecular Weight

The native molecular weight of the A. niger cyclase was determined by gel permeation chromatography in Sephadex G-200. A Pharmacia column (K25/45) was packed with the swollen gel and equilibrated with 0.05M phosphate buffer, (pH 6.8). The void volume, determined with Blue Dextran (section 3.10.1) was 58.5±1ml and the bed volume was approx. 216±2ml. A partially purified cyclase sample (2ml; 30 Units; 7.5 Units/mg) was mixed with the following marker proteins: 50μl **ferritin** (horse spleen), 100 Units each of **lactate dehydrogenase** (rabbit muscle) and **malate dehydrogenase** (porcine heart), 1 000 Units **catalase** (bovine liver), 20 Units **fumarase** (porcine heart) and 2mg **myoglobin** (equine heart). The mixture was applied to the column which was then eluted with the equilibrating buffer at 12ml/h, under hydrostatic pressure; 2ml fractions (average volume) were collected. The elution volumes of the cyclase and of the marker proteins were determined volumetrically, and a plot of **log (mol.wt.)** vs. K_{av} (Fig.5.1) was constructed. In this figure the heights of the bars correspond to the mol.wt. range reported in the literature for each marker:

(Gel Filtration: Theory and Practice, Pharmacia Fine Chemicals; Andrews, 1965; Mantle, 1978; Klotz _et al._, 1975). From this experiment the native mol.wt. of the _A. niger_ cyclase was determined as 162 000.

In a separate experiment, a mixture of the cyclases from the wild type and the temperature-sensitive mutant **PL30** (Ahlquist, 1977) was loaded on the same Sephadex G-200 column. Cyclase activity eluted as a broader peak than in the previous experiment. Fractions containing activity were placed in water-bath at 30°C and assayed at intervals. After 45 min it was observed that the peak activity was found at V_e=98ml (K_{av}=0.25) whereas in the next fraction (V_e=100ml;K_{av}=0.26) the activity decreased more rapidly (Fig.5.2). This was taken as evidence that the temperature-sensitive cyclase had a slightly lower mol.wt. (150 000), than the wild-type enzyme,as deduced from the calibration line in Fig.5.1.

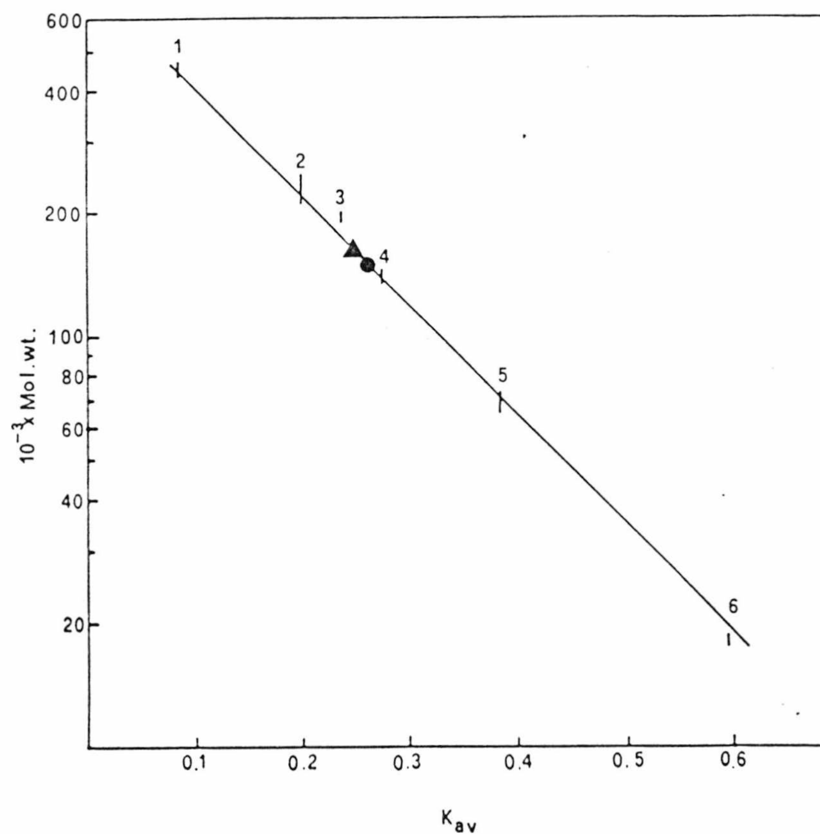


Fig.5.1. Molecular weights of the cyclase from A. niger L6 and strain PL30 by calibrated gel permeation chromatography in Sephadex G-200. The column had V_0=58.5 ± 1ml and V_t=216 ± 2ml. The marker proteins were ferritin (1), catalase (2), fumarase (3), lactate dehydrogenase (4), malate dehydrogenase (5) and myoglobin (6). The mol.wts. read off the line were 162 000 ± 8 000 for the wild-type (▲) and 150 000 ± 8 000 for the temperature-sensitive mutant (●).

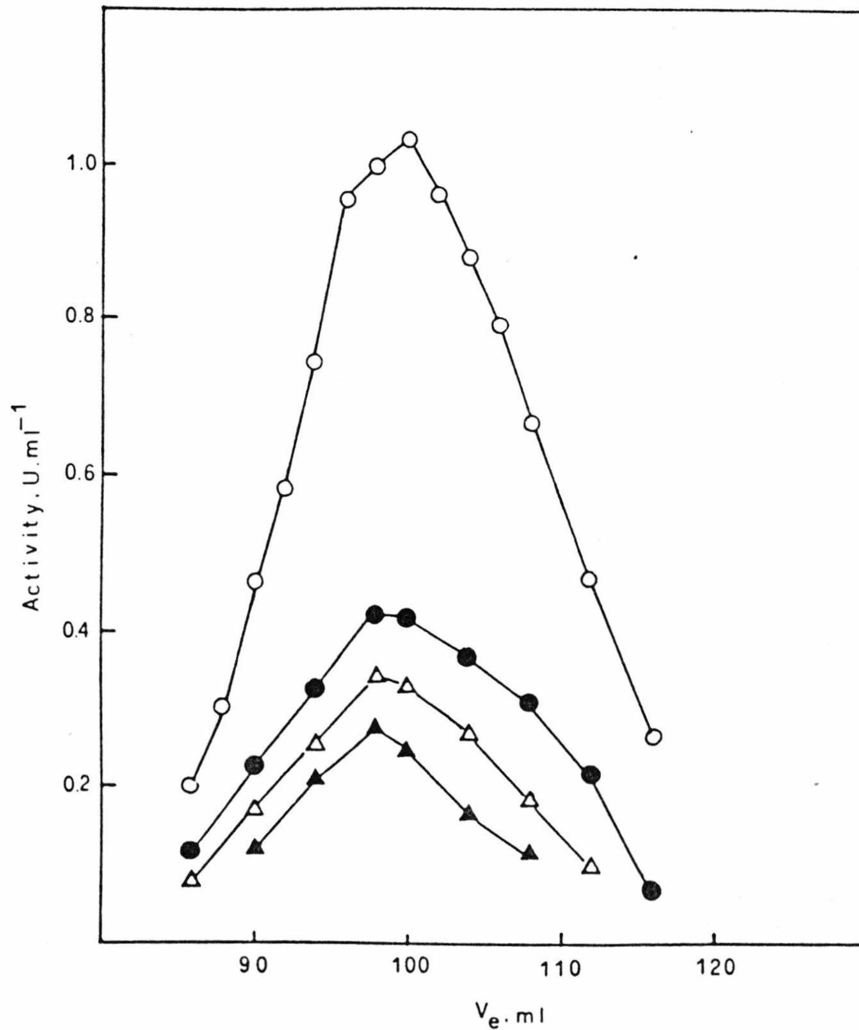
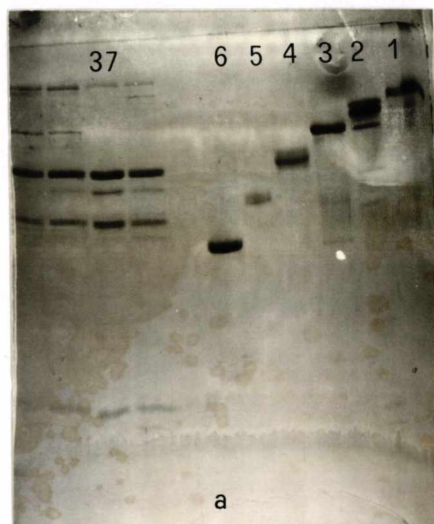


Fig.5.2. Thermal inactivation of a mixture of the cyclase from A. niger and strain PL30 subjected to gel permeation chromatography in Sephadex G-200. The activities of a number of fractions were plotted against elution volume after heating periods of 20min (●), 30min (△) and 45min (▲) at 30°C. The initial activities are represented by the upper curve (O).

5.2. Subunit Molecular Weight

In the presence of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol most oligomeric proteins dissociate into subunits and bind 1.4g SDS/g protein; the electrophoretic migration of the SDS-bound peptides in a porous medium, such as a polyacrylamide gel, is proportional to the log of its mol.wt. (Shapiro et al., 1967; Weber & Osborn, 1969). This technique provides a reliable means for the mol.wt. estimation of denatured polypeptides providing that SDS-binding is not impaired by structural factors such as a carbohydrate moiety or a high electric charge (Pitt-Rivers & Impiombato, 1968; Tung & Knight, 1971). Deviations observed with glycoproteins can, however, be overcome using gels with a gradient concentration of polyacrylamide (Lambin, 1978)

Calibrated SDS-PAGE was performed in the conditions described in section 3.11. A gel run with cyclase samples and markers, and the corresponding calibration line are shown in Fig.5.3. The cyclase samples were taken from fractions of the ethyl-agarose eluate (section 4.9, step 7). The molecular weights of the marker proteins were taken from Weber & Osborn (1969): bovine serum albumin, 68 000; pyruvate kinase, 57 000; glutamate dehydrogenase, 53 000; ovalbumin, 43 000; lactate dehydrogenase, 36 000 and chymotrypsinogen A, 25 700. The patterns of the different cyclase samples loaded in this gel exhibited a band with an apparent mol.wt. of 42 000; this was the major component in every fraction, and was also stronger in the peak activity fraction when compared with the homologous bands from the other fractions.



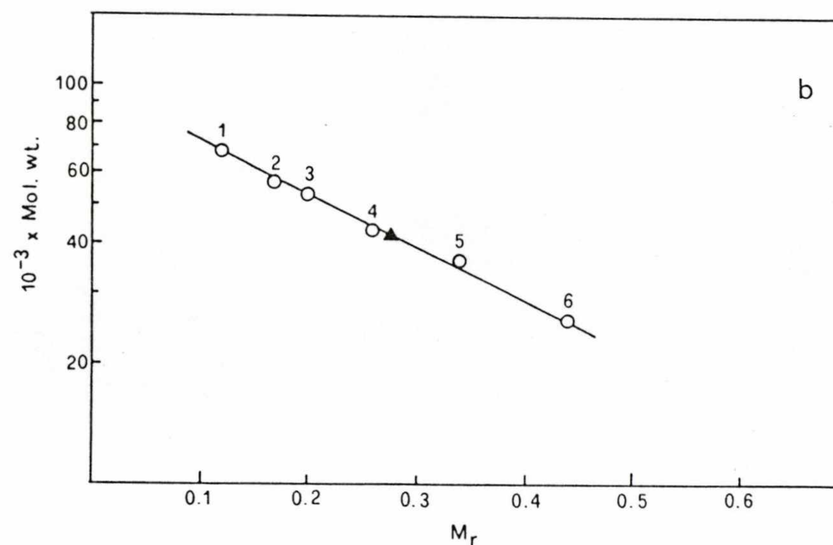


Fig.5.3. Determination of the subunit molecular weight of the A. niger cyclase. (a) A SDS-polyacrylamide gel, containing 15% (w/v) acrylamide, was run with aliquots of fractions of the ethyl-agarose eluate (section 4.9), including the fraction with maximum activity (37) and with the marker proteins bovine serum albumin (1), pyruvate kinase (2), glutamate dehydrogenase (3), ovalbumin (4), lactate dehydrogenase (5) and chymotrypsinogen A (6). (b) The calibration line was constructed by plotting log (mol.wt.) of the marker proteins against their relative mobilities (M_r). The mol.wt. of the cyclase (▲) read off the line was **42 000**.

5.3. Oligomeric Structure

The oligomeric structure of one enzyme is usually established by independent estimates of the native and subunit(s) molecular weight. In the simplest case where only one type of component polypeptide is detected, the number of subunits is the closest integral to the ratio native mol.wt./subunit mol.wt.. If this criterion is applied to the *A. niger* cyclase it is found, from the results in section 5.1 and 5.2 that 162 000/42 000=3.86 and it can be deduced that the native enzyme is probably tetrameric.

In the course of this work it was observed that SDS-PAGE was highly reproducible with the differences between replicate experiments on different batches of enzyme within 2.5% of the average. On these grounds, another estimate for the native mol.wt can be obtained:

$$(42\ 000 \pm 1\ 000) \times 4 = 168\ 000 \pm 4\ 000)$$

If an uncertainty of 5% is assumed in the estimate obtained by calibrated gel permeation chromatography (162 000 ± 8 000) it can be concluded that both estimations are consistent within experimental error.

5.4. Isoelectric Point

Chromatofocusing of *A. niger* cyclase preparations performed on different occasions, in pH gradients from 7 to 4 invariably produced a maximum of the recovered activity in a fraction with pH 4.85 ± 0.05.

A typical elution profile is represented in Fig.4.7. The same result was also observed in step 8 (Fig.4.11) of the large-scale purification of the *A. niger* cyclase, where a pH gradient from 5.5 to 4 was used.

5.5. Kinetic Properties

Kinetic assays with the *A. niger* cyclase at different pH values showed that the optimum pH for the lactonization reaction was 6.0, in agreement with previous observations reported by Thatcher & Cain (1975). A significant difference was detected, however, in the K_m value of the enzyme estimated from preliminary assays at the optimum pH. Further experiments were therefore required for a more accurate estimation of this kinetic parameter.

The standard buffer (section 3.4.1) was used in the assays. The amount of enzyme was adjusted so that the values of ΔA/min were within the range 0.02-0.10 when substrate concentration was varied between 5 and 100 μM. Because of the low value of K_m given by the preliminary estimates (14-16μM) it has been necessary to use very low enzyme concentrations in order to allow reasonable measurements of the initial velocity at low substrate concentrations. The experimental data were analyzed using the Hanes plot (Hanes, 1932). One of those plots is illustrated in Fig.5.4. The assay cuvettes received 10 μl (1.93 μg protein) of a partially purified cyclase solution. The Michaelis constant (K_m=14.8 μM) was deduced from the parameters of the best-fitting line. The maximum velocity in

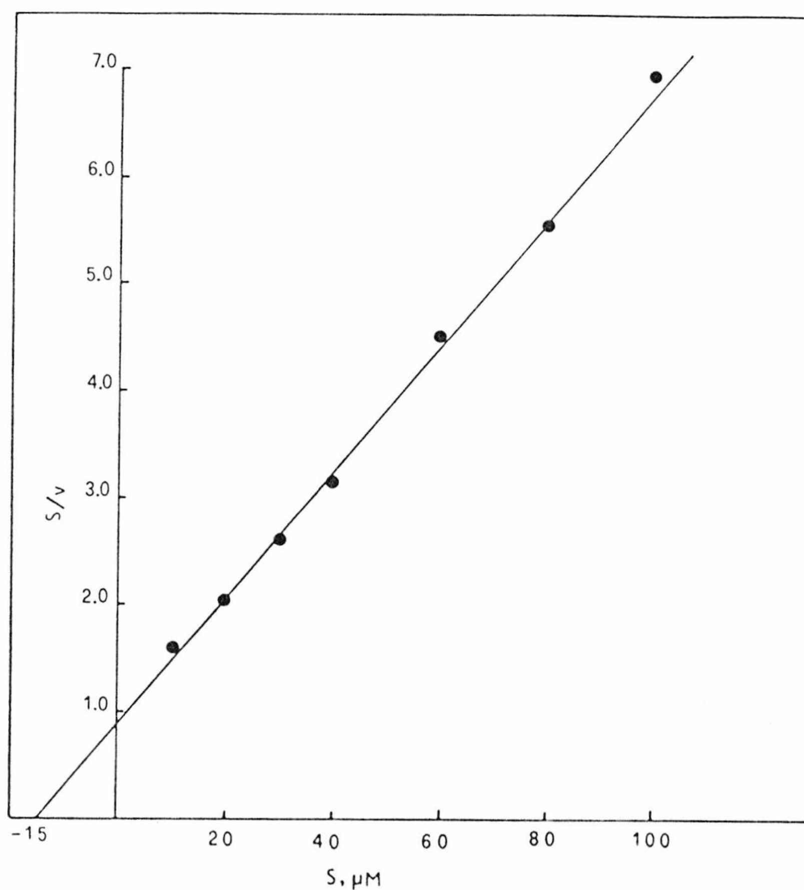


Fig.5.4. Determination of the K_m of the *A. niger* cyclase from a Hanes plot. Initial velocities (v) were measured at several substrate concentrations (S). The K_m (14.8μM) was estimated as described in Section 3.4, from the parameters of the line (Corr=0.997):

Slope=0.0595 Intcp=0.879

this particular assay was 16.8 μmoles. min^{-1}. (mg protein)$^{-1}$.

The initial velocity at S=100μM (substrate concentration in the standard conditions) was 14.6 μmoles. min^{-1}. (mg protein)$^{-1}$, as deduced from the Hanes plot. Assuming that the purest cyclase sample, obtained as described in section 4.9, contained 85% cyclase, the specific activity of the pure enzyme, determined at S=100μM would be approx. 1 620/0.85=1 900 Units (mg protein)$^{-1}$ and V_{max}=1 900x(16.8/14.6)=2 186 μmoles. min^{-1}. (mg enzyme)$^{-1}$.

5.6. Effect of the pH on Michaelis Parameters

The effect of pH on the apparent K_m and V_{max}, determined from Hanes plots of the initial velocities at several substrate concentrations (5-100μM) is summarized in Table 5.1. Fig.5.5 is a plot of **V_{max} vs. pH** in the range 4.5-7.5. In this series of assays the amount of enzyme was identical to that used in the experiment described in section 5.5; the assay buffers used were 0.1M acetate in the pH range 4.5-6.0 and 0.1M histidine-HCl in the range 6.0-7.5.

Theoretically it should be possible to estimate the ionization constants of groups which affect the enzyme activity and its affinity for the substrate by analysing the influence of pH on K_m and V_{max} (Dixon & Webb, 1979). The plot of **logV_{max} vs. pH**, with V_{max} values determined at saturating substrate concentrations, reflects the ionization constants in the enzyme-substrate complex only, since affinity effects derived from the ionization states of the free enzyme and substrate are thus eliminated. The ionization constants in

the free forms can be deduced from plots of **log** (V_{max}/K_m) <u>vs.</u> **pH** when the kinetic parameters are calculated from experimental measurements made at very low substrate concentrations ($S \ll K_m$). Under these conditions $v=(V_{max}/K_m).S$, <u>i.e.</u>, the reaction rate depends on the ionization constants of the free-enzyme and substrate, which only affect K_m, but not on the constants of the enzyme-substrate complex, which affect both V_{max} and K_m and are therefore cancelled out in the ratio V_{max}/K_m.

<u>TABLE 5.1</u>

Effect of pH on K_m and V_{max}

pH	K_m (μM)	V_{max} [μmoles.min^{-1}. (mg protein)$^{-1}$]
4.5	5.3	7.9
5.0	8.8	10.6
5.5	13.5	15.5
6.0	14.8	16.8
6.5	16.2	16.5
7.0	30.0	13.7
7.5	52.5	11.3

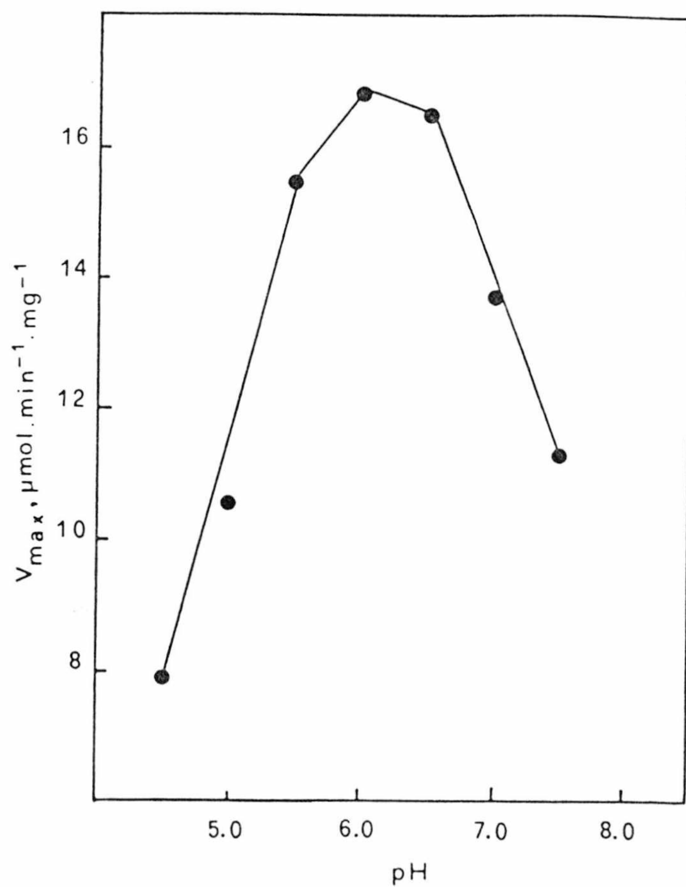


Fig.5.5. Determination of the optimum pH range of the A. niger cyclase. The values of V_{max} were determined from Hanes plots of kinetic data obtained at several pH values.

It is apparent that the conditions defined above for the kinetic assays did not satisfy either requirement, that being due to several experimental constraints. Firstly, the use of substrate concentrations much lower than K_m would not allow accurate rate measurements. Secondly, the high absorbance of the substrate and its inhibitory effect, which is noticeable, at the optimum pH, at concentrations higher than 100μM, prevented the use of substrate-saturated enzyme in the assays. It would therefore be pointless to attempt the interpretation of those plots. Even with reliable data that would probably be difficult considering that binding of 3-carboxy-cis, cis-muconate to the enzyme presumably involves electrostatic interactions between at least two of the three ionizable groups of the substrate (see section 5.7) and two ionizable residues at the active center, and that an additional protonated residue participates in the catalytic process. A minimum of five ionizable groups is therefore likely to affect the kinetics of the reaction as the pH is varied.

5.7. Inhibition by Metallic Ions and Organic Anions

The possible inhibitory effect of several metallic ions was tested in the standard assay mixtures where the ions had been added at final concentrations of 10 and 100 μM. Although without statistical meaning, the averages of two consistent assays were expressed as percentage of activity in the control, for an easier screening of the inhibitory ions (Table 5.2). Among the ions tested, Cu^{2+}, Fe^{2+} and Fe^{3+}

were found to be substantially inhibitory.

TABLE 5.2

Effects of several metallic ions on cyclase activity

The metallic ions were added as chlorides. The specific activity of the enzyme solution used in the assays was 5.5 Units/ml(control).

Metallic ion	Activity (% of control)	
	$[M^{n+}]$=10μM	$[M^{n+}]$=100μM
K^{+}	100	91
Mg^{2+}	100	94
Ca^{2+}	100	98
Fe^{2+}	94	73
Fe^{3+}	91	69
Co^{2+}	100	100
Cu^{2+}	72	60
Mn^{2+}	100	100
Zn^{2+}	100	91

Several of the substrate analogues and other organic dicarboxylic acids were also tested as potential inhibitors. The assays were performed at pH 6.0 in the standard assay mixture and the inhibitor constants ($K_{i,app}$) determined by plotting **1/v** vs. **inhibitor concentration** (Dixon, 1953), at two different substrate concentrations (10 and 50μM). With

those compounds which were inhibitory to the cyclase, the best-fitting lines intercepted above the X axis. As this pattern may occur with both competitive and mixed inhibitors, the ambiguity was resolved by replotting the data as **S/v** vs. **i** (Cornish-Bowden, 1974): competitive inhibitors produce parallel lines whereas with mixed inhibitors the lines intercept below the X axis. The Dixon plots for the experiments with 1-methyl 3-carboxy-cis, cis -muconate and 3-carboxymuconolactone are shown in Figs.5.6 and 5.7. A summary of the results obtained with the compounds tested as potential inhibitors is given in Table 5.3 where the slopes for the **S/v** vs. **i** plots are indicated. The small differences in the slopes for each pair of lines are within experimental error.

In the assays with cis, cis and cis, trans-muconate, concentrations higher than 100μM could not be used because the test compounds were added both to the blank and the assay cuvettes and their high absorbance, at 260nm, severely impaired the performance of the spectrophotometer.

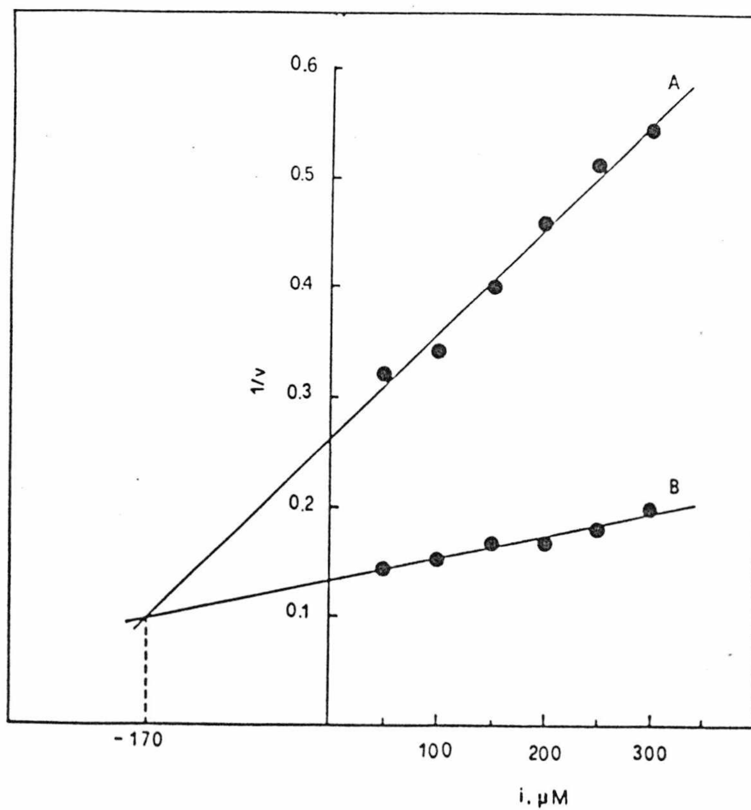


Fig.5.6 Inhibitor constant of 1-methyl 3-carboxy-*cis,cis*-muconate determined from a Dixon plot. The initial velocities were measured at fixed substate constrations of 10μM (line A) and 50μM (line B), and varying concentrations of inhibitor (i). Reaction velocities were expressed in $\mu moles.min^{-1}. ml^{-1}$.

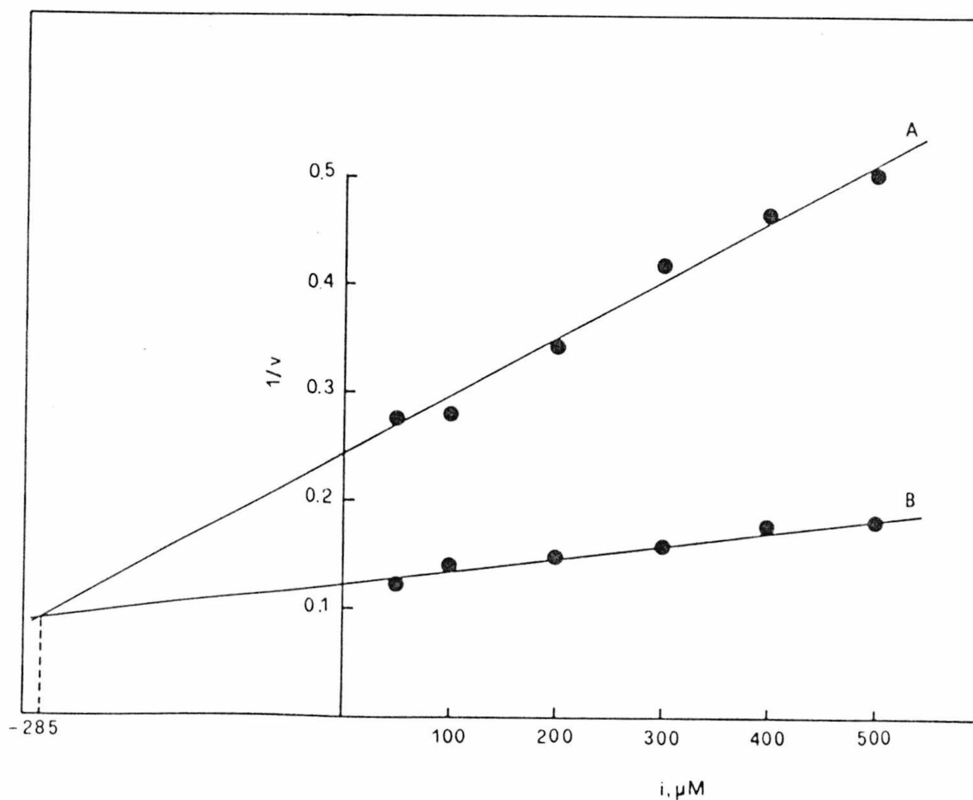


Fig.5.7. Inhibitor constant of 3-carboxymuconolactone, determined from a Dixon plot. The initial velocities were measured at fixed substrate concentrations of 10μM (line A) and 50μM (line B), and varying concentrations of inhibitor (i). Reaction velocities were expressed in $\mu moles.min^{-1}. ml^{-1}$.

TABLE 5.3

Data from kinetic assays with potential inhibitors

Test compound	$k_{i,app}$ (μM)	1/v *vs*. i plot (correlation)	S/v *vs*. i plot (slope)	Type of inhibition
1-Methyl 3-carboxy- -*cis*,*cis*-muconate	170	0.992(S=10μM) 0.993(S=50μM)	0.0095(S=10μM) 0.0094(S=50μM)	Competitive
3-Carboxymuconolactone	285	0.992(S=10μM) 0.981(S=50μM)	0.0054(S=10μM) 0.0060(S=50μM)	Competitive
Maleate	315	0.997(S=10μM) 0.978(S=50μM)	0.0047(S=10μM) 0.0042(S=50μM)	Competitive
cis,*cis*-Muconate	-			No effect
cis,*trans*-Muconate	-			No effect
Phthalate	-			No effect
Homophthalate	-			No effect

CHAPTER 6

COMPARATIVE STUDIES OF 3-CARBOXYMUCONATE CYCLASES

6.1. Specific Activities in Cell-Free Extracts

The specific activities of cyclases from different organisms in cell-free extracts prepared in 0.05M Tris-HCl (pH 7.2), following the procedure described in Section 3.6, varied between 1.0 and 3.2 Units/mg protein (Table 6.1).

When evidence accumulated about the instability of several cyclases in cationic buffers, cell-free extracts were prepared in 0.05M potassium-sodium phosphate buffer (pH 6.8). Significant differences in specific activities were observed in the cyclases from *Asp. flavus* (+80%), *Mon. ruber* (+117%) and *Pen. crustosum* (+30%), when compared with those in cell-free extracts, prepared in Tris buffer from the same batches of mycelium (figures in brackets, Table 6.1).

6.2. Ammonium Sulphate Fractionation

Cell-free extracts with average protein concentrations of 3 $\pm$ 0.5mg/ml were fractionated with ammonium sulphate following the method described in section 3.7. The pellet collected after each increment in ammonium sulphate concentration was resuspended in buffer and cleared by

TABLE 6.1

Specific activities of fungal cyclase in cell-free extracts

The cell-free extracts were prepared in 0.05M Tris-HCl (pH 7.2) or (figures shown in brackets) in 0.05M potassium-sodium phosphate (pH 6.8).

Cyclase source	Protein (mg/ml)	Activities (Units/ml)	(Units/mg protein)
Asp. flavus	3.6(3.2)	4.7(7.3)	1.3(2.3)
Asp. sydowii	3.5	7.4	2.1
Gl. deliquescens	3.6	9.8	2.7
Mon. ruber	2.5(2.8)	3.1(7.2)	1.2(2.6)
Paec. variotii	3.1(2.6)	3.3(3.3)	1.1(1.3)
Pen. chrysogenum	3.3	10.7	3.2
Pen. crustosum	2.0(2.9)	2.0(3.7)	1.0(1.3)
Pen. terrestre	2.5	3.7	1.5
Scop. candida	3.3	3.3	1.0

centrifugation, if necessary. The distribution of cyclase activity in the precipitated fractions and the enrichment factors observed in the two pooled fractions which contained maximum activity are listed in Table 6.2. The precipitated protein by addition of ammonium sulphate up to 30% saturation did not contain detectable activity.

TABLE 6.2

Fractionation of cell-free extracts with ammonium sulphate

The cell-free extracts were prepared in 0.05M Tris-HCl (pH 7.2) and the precipitated protein fractions were redissolved in the same buffer. Activity recoveries in each fraction are expressed as percentages of the initial activity. Enrichment factors were estimated in the two pooled fractions containing the bulk of enzyme activity.

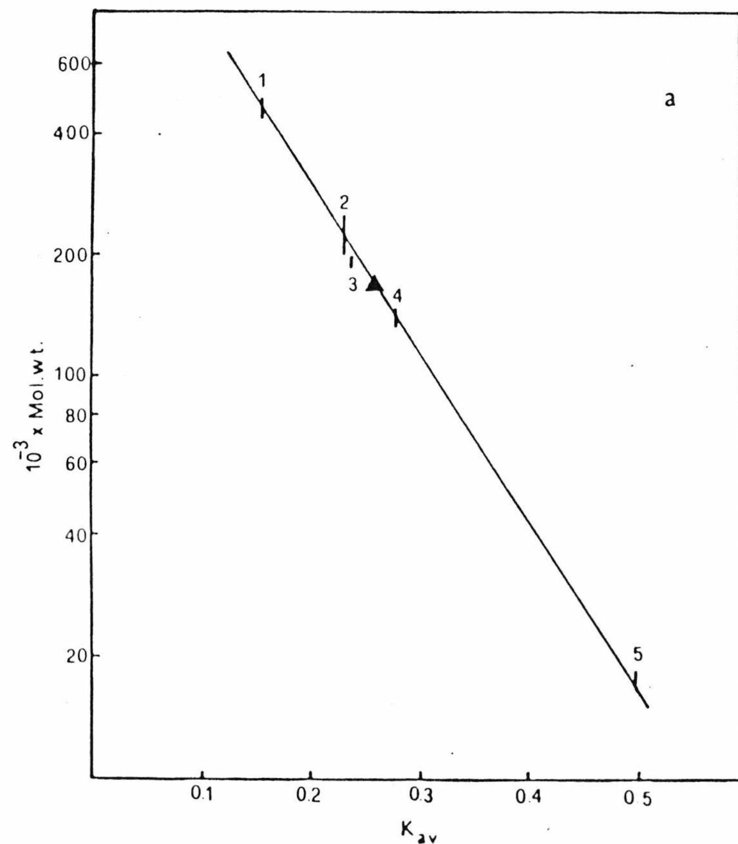
Cyclase source	Ammonium sulphate concentration % saturation				Enrichment (-fold)
	30-40	40-50	50-60	60-65	
Asp. flavus	0	10	65	9	2.8
Asp. sydowii	0	56	42	-	3.4
Gl. deliquescens	65	26	0	-	3.9
Mon. ruber	0	42	56	-	3.4
Paec. variotii	0	10	82	0	3.8
Pen. chrysogenum	2	68	31	-	3.5
Pen. crustosum	2	23	68	-	3.8
Pen. terrestre	2	67	13	0	2.7
Scop. candida	0	30	47	3	2.3

6.3. Native Molecular Weights

The apparent native molecular weights of several 3-carboxymuconate cyclases were determined by calibrated gel permeation chromatography. In all the experiments described in this section, a LKB column of 2.6x100cm with adjustable end-stops was used. The gel permeation medium was packed in

the column to a total bed volume (V_t) of 440-485ml. The void volume (V_0) of each column was determined as described in section 3.10.1.

The cyclase samples for calibrated gel permeation chromatography were the ammonium sulphate fractions containing the bulk of the enzyme activity (section 6.2). An aliquot of this fraction containing 50-150Units of enzyme and 10-30mg of protein was mixed with standards, in a total volume of 3-4ml, and applied to the column. The columns were eluted at flow rates of 22-26ml/h and fractions with an approximate average volume of 3ml were collected. Relevant fractions of the eluate were assayed for the markers and cyclase concentration. The elution volume (V_e) of each protein was the total volume flowing through the column from the start of sample application until collection of the fraction containing the maximum concentration of that protein. From the elution volumes of the markers, the values of K_{av} were estimated and calibration lines were constructed by plotting **log mol.wt.** vs. K_{av}. From these calibration lines the apparent molecular weights of the cyclases were determined with an estimated precision of $\pm$5%. When Sephacryl S-300 was used as gel permeation medium the eluting buffer was 0.05M Tris-HCl, (pH 7.2). Figs.6.1 and 6.2 illustrate the calibration lines obtained in experiments with extracts of *Asp. sydowii*, *Scop. candida* and *Gl. deliquescens*. In Fig.6.2(b) is shown the elution profile of the markers and of the *Gliocladium* cyclase in the corresponding experiment. The concentration of each marker



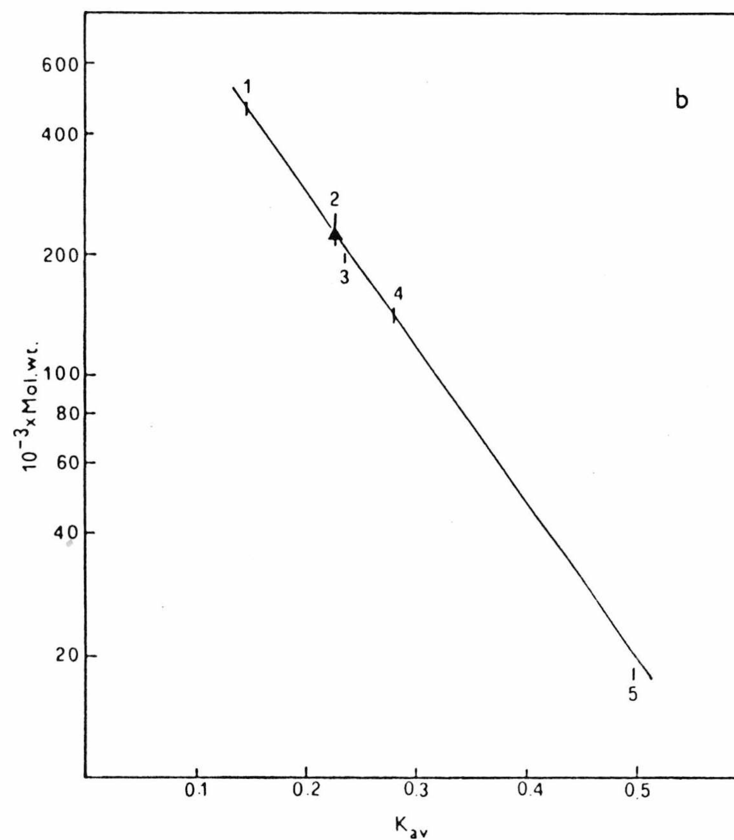
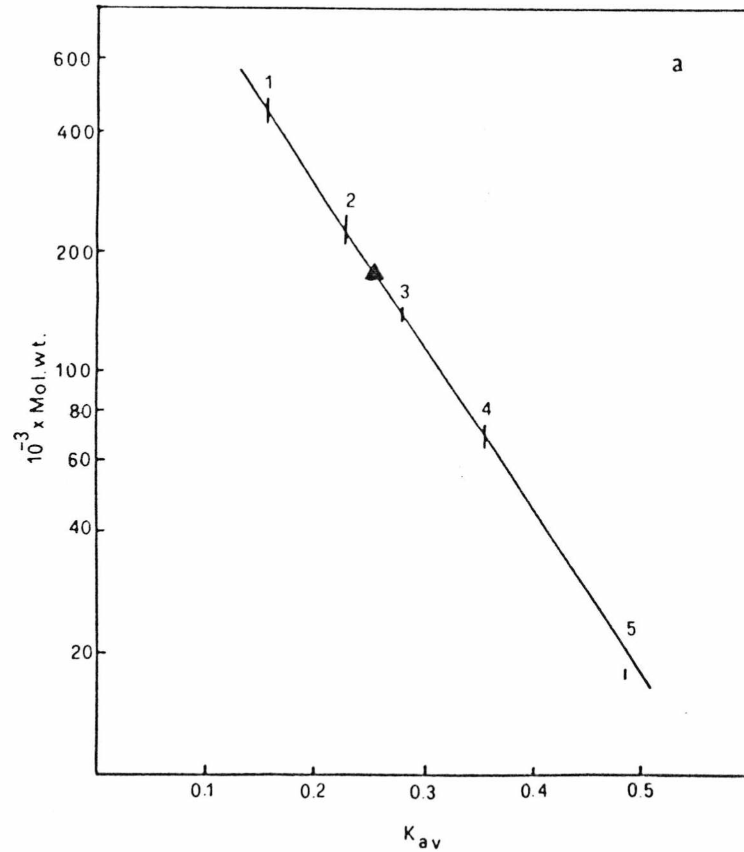


Fig.6.1. Molecular weights of the cyclase from Asp. sydowii (a) and Scop. candida (b) by calibrated gel permeation chromatography in Sephacryl S-300. The columns were eluted with 0.05M Tris HCl (pH 7.2) and the marker proteins were ferritin (1), catalase (2), fumarase (3), lactate dehydrogenase (4) and myoglobin (5). The estimated mol.wts. (▲) were 170 000 ± 8 000 and 220 000 ± 12 000, respectively, for the A. sydowii and S. candida cyclases.



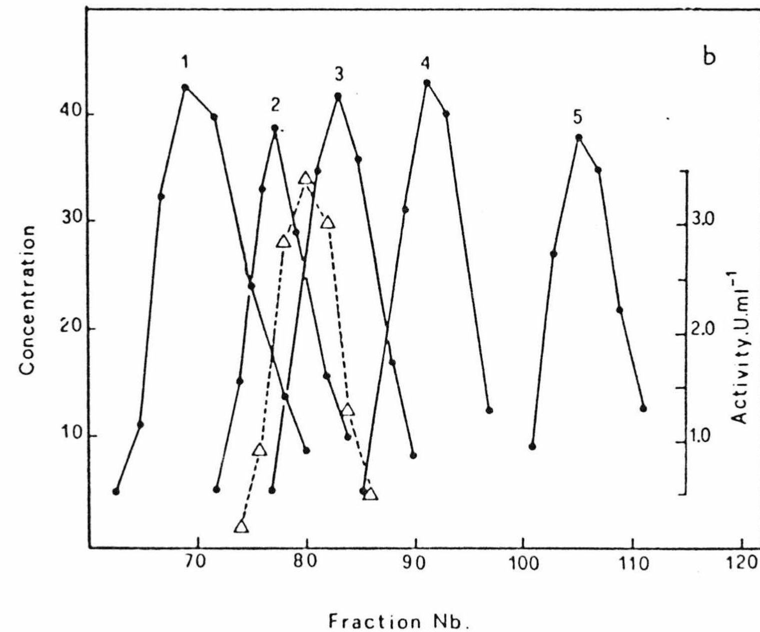


Fig.6.2. Molecular weight of the <u>Gl. deliquescens</u> **cyclase by calibrated gel permeation chromatography.** The gel was Sephacryl S-300 and the column (V_o=150ml, V_t=480ml) was eluted with 0.05M Tris-HCl (pH 7.2). Fractions with an average volume of 3.0ml were collected. The calibration line (a) was plotted from K_{av} values estimated from the elution profiles (b) of the cyclase (△) and of the marker proteins ferritin (1), catalase (2), lactate dehydrogenase (3), malate dehydrogenase (4) and myoglobin (5). The mol.wt. estimated from the calibration line (▲) was 180 000 ± 8 000.

is represented in arbitrary units proportional to ΔA/min/ml of enzyme or to A^{405} for ferritin and myoglobin. The cyclase concentration is expressed in Units/ml.

When extracts from Mon. ruber, Paec. variotii, Pen. chrysogenum, Pen. crustosum and Pen. terrestre were chromatographed under the conditions described above an abnormal elution behaviour of the cyclases was observed. The activity consistently eluted as a very broad peak, with the maximum appearing between lactate dehydrogenase and myoglobin; the observed recovery yields were in the range 30-40%. A typical elution profile for the Pen. chrysogenum cyclase is represented in Fig.6.3(a). The K_{av} values for the markers used in these experiments were within the average obtained in other experiments with Sephacryl S-300.

This problem was overcome by changing the gel permeation medium to Ultrogel AcA34 and using 0.05M potassium-sodium phosphate (pH 6.8) as eluting buffer. Fig.6.3(b) shows the comparable elution profile for the Pen. chrysogenum cyclase on an AcA34 column, and in Fig.6.4 is illustrated the respective calibration line. Similar improvements were obtained with extracts of Mon. ruber, Paec. variotii and Pen. crustosum. The final mol.wt. data for the native cyclases are listed in Table 6.3.

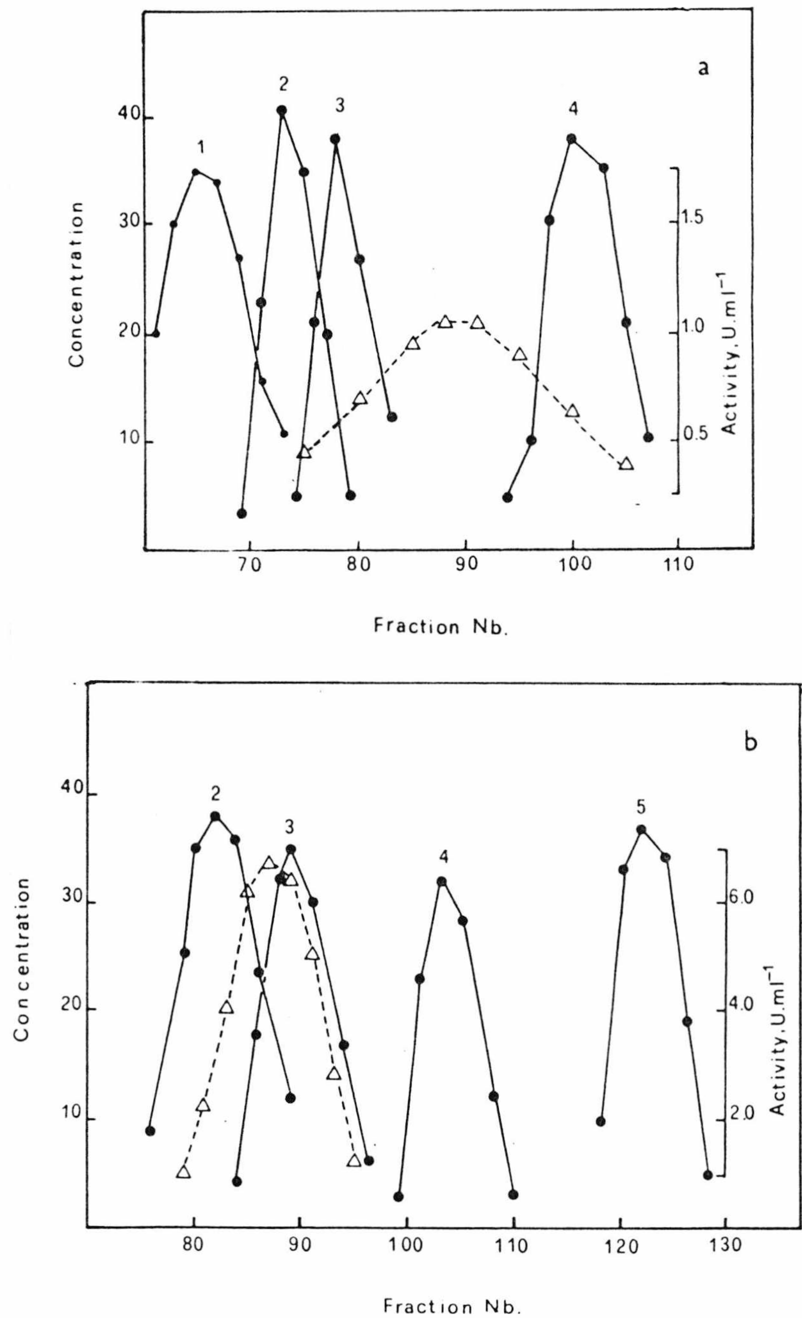


Fig.6.3. **Influence of the buffer and gel permeation medium on the elution profiles of the** **Pen. chrysogenum** **cyclase.** (a) The Sephacryl S-300 column (V_0=155 ml ; V_t=480 ml) was eluted with 0.05M Tris-HCl (pH 7.2). (b) The Ultrogel AcA34 column (V_0=164 ml; V_t=485 ml) was eluted with 0.05M potassium-sodium phosphate (pH 6.8). The protein markers were ferritin (1), catalase (2), lactate dehydrogenase (3), malate dehydrogenase (4) and myoglobin (5). The average fraction volumes were 3.1 ml and 3 ml in (a) and (b), respectively.

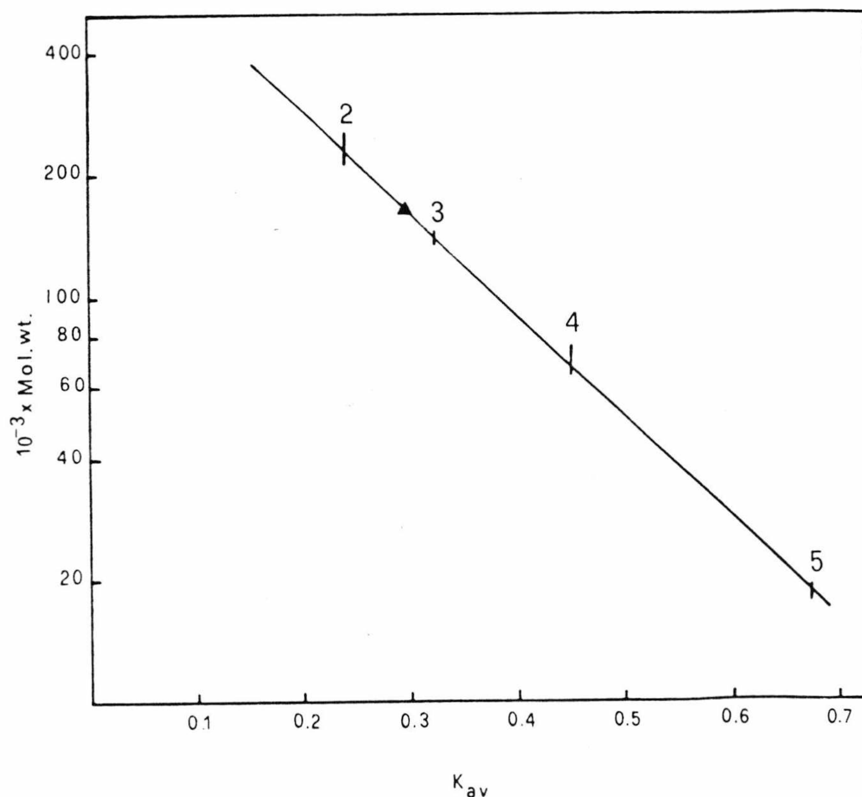


Fig.6.4. Molecular weight of the <u>Pen. chrysogenum</u> cyclase **by calibrated gel permeation chromatography in Ultrogel AcA34.** The experimental conditions were as described in Fig.6.3. The mol.wt. of the <u>Pen. chrysogenum</u> cyclase (▲) estimated from the calibration line was **165 000 ± 8 000.**

TABLE 6.3

Native molecular weights of several cyclases determined by calibrated gel permeation chromatography

Cyclase source	10^{-3}x Mol. weight (±5%)
Asp. flavus	170
Asp. sydowii	170
Gl. deliquescens	180
Mon. ruber	155
Paec. variotii	150
Pen. chrysogenum	165
Pen. crustosum	162
Scop. candida	220

6.4. Chromatofocusing and Isoelectric Points

Chromatofocusing revealed to be a valuable technique both in the purification of fungal cyclases and for the estimation of their isoelectric points (see Table 6.4). The sharpness of the cyclase elution peaks was an additional advantage because it made possible the identification of the cyclase band by comparative electrophoretic analysis of activity-containing fractions in the eluate (section 6.5).

Chromatofocusing was performed as described in Section 3.10, usually as the last step in a partial purification process of several cyclases which included ammonium sulphate

fractionation and gel permeation chromatography in Sephacryl S-300 or Ultrogel AcA34. Three to five fractions of the gel permeation eluate containing most activity (40-60% of the total eluted activity) were pooled, concentrated in an ultrafiltration cell and equilibrated, by dialysis, with the chromatofocusing starting buffer. Table 6.5 summarizes the data concerning the partial purification of the cyclases subjected to chromatofocusing. Figs.6.5 and 6.6 illustrate the elution profiles of cyclases which eluted at significantly different pH values.

TABLE 6.4

Isoelectric points of several cyclases determined by chromatofocusing

Cyclase source	pI ($\pm$ 0.05)
Asp. flavus	5.05
Asp. sydowii	5.25
Gl. deliquescens	5.50
Mon. ruber	5.90
Paec. variotii	6.05
Pen. chrysogenum	5.40
Pen. crustosum	5.50
Pen. terrestre	5.25
Scop. candida	4.60

TABLE 6.5

Partial purification of 3-carboxymuconate cyclases

Cell-free extracts of several fungi (step 1) prepared in (a) 0.05M Tris-HCl (pH 7.2) or (b)0.05M potassium-sodium phosphate (pH 6.8) were subjected to ammonium sulphate fractionation (step 2), gel permeation chromatography (step 3), concentration (step 4) and chromatofocusing (step 5). Steps 2-4 were performed in the buffer used in the preparation of the cell-free extract. Conditions for step 5 were as described in section 3.10.4 for a pH gradient from 7 to 4. Total cyclase (Units) and protein (mg) is indicated in each step, except for step 5, where protein was not assayed (see section 3.10.4).

Cyclase source	Step 1		Step 2		Step 3		Step 4		Step 5	Purification factor
	(Units)	(mg)	(Units)	(mg)	(Units)	(mg)	(Units)	(mg)	(Units)	(Step 4/ Step 1)
A. flavus (b)	230	98	217	25	83	2.3	36	1.4	15	11.0
A. sydowii (a)	337	162	328	46	144	5.2	79	2.6	38	14.6
G. deliquescens (a)	527	195	480	45	230	6.1	160	4.2	98	14.1
M. ruber (b)	145	56	138	14	-	-	74	6.0	46	15.3
P. variotii (b)	117	90	109	24	-	-	83	9.2	57	6.9
P. chrysogenum (b)	680	212	667	55	307	7.2	227	5.3	145	13.4
P. crustosum (a)	185	142	165	36	-	-	119	1.5	50	6.1
P. terrestre (a)	133	88	101	23	45	2.9	24	2.0	10	8.0
S. candida (a)	108	110	83	27	26	1.8	10	1.2	3	0.9

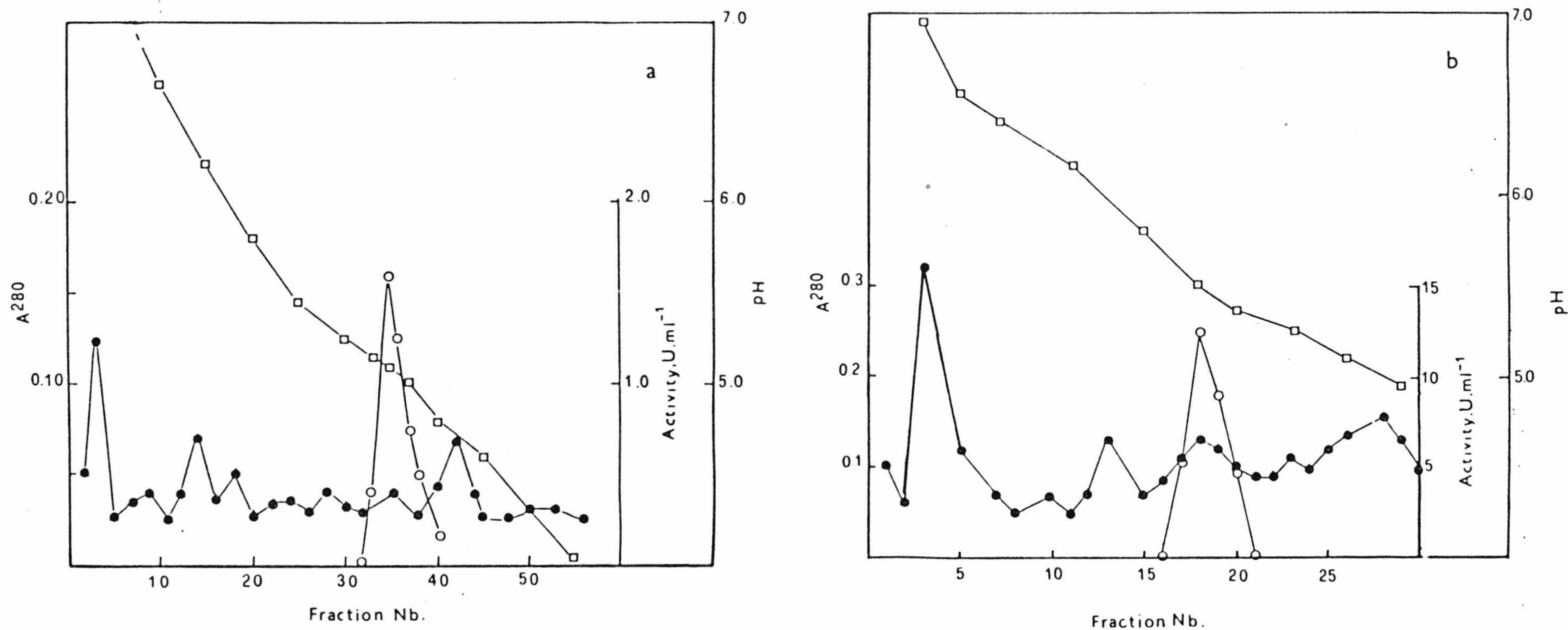


Fig.6.5. Chromatofocusing of the cyclases from Asp. flavus (a) and Gl. deliquescens (b). The experimental conditions were as described in section 3.10. The absorbance (●), pH (□) and activity (△) of a number of fractions were plotted against fraction number. The average fraction volume was 2.5ml in (a) and 3.0ml in (b). The isoelectric points read off the pH curve were 5.05 for the A. flavus cyclase and 5.50 for the G. deliquescens cyclase.

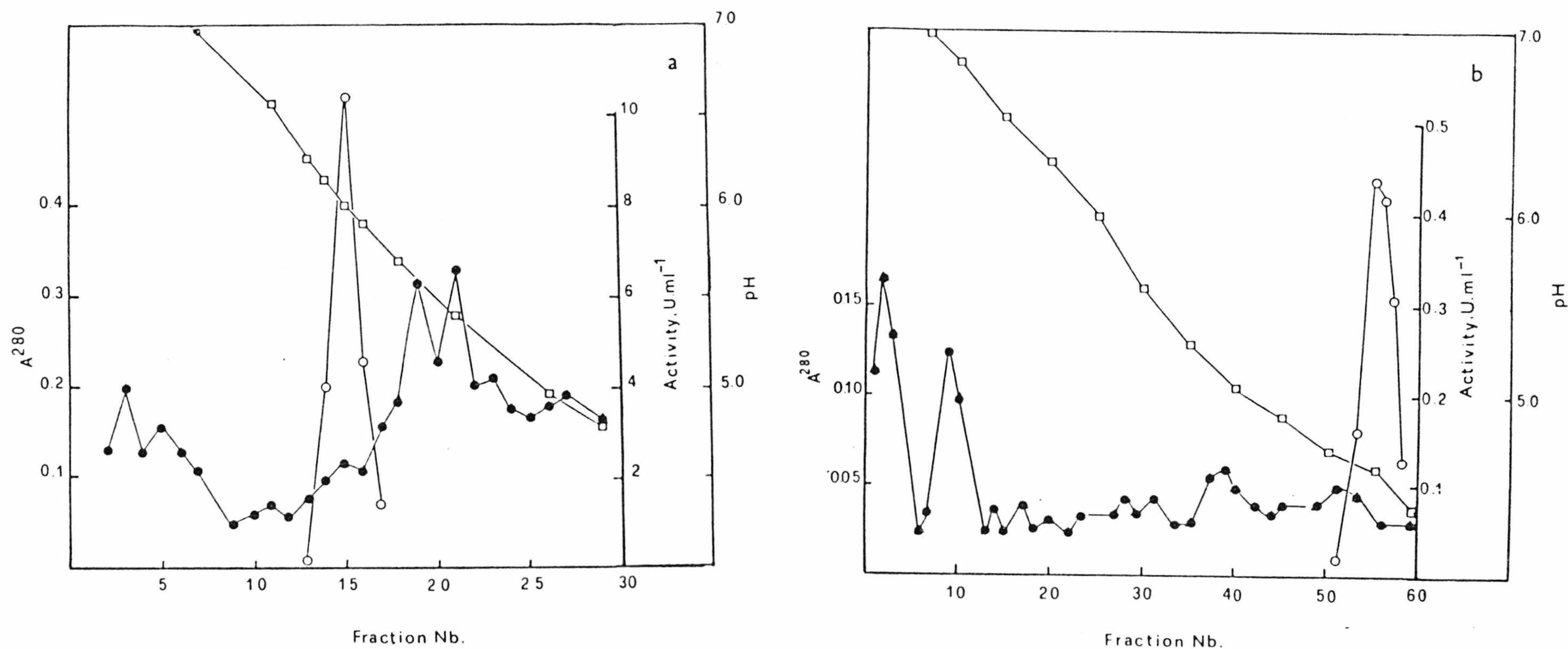


Fig.6.6. **Chromatofocusing of the cyclases from** Paec. variotii (a) and Scop. candida (b). The experimental conditions were as described in section 3.10. The absorbance (●), pH (□) and activity (○) of a number of fractions were plotted against fraction number. The average fraction volume was 2.5ml in (a) and 1.7ml in (b). The isoelectric points read off the pH curve were 6.05 for the Paec. variotii cyclase and 4.60 for the Scop. candida cyclase.

6.5. Subunit Molecular Weights

Subunit molecular weights were estimated by SDS-polyacrylamide gel electrophoresis, under reducing conditions, as described in section 3.11 using gels with acrylamide concentrations of 10, 12.5 or 15%. The cyclase samples applied to the gels had been subjected to a partial purification, as described in Table 6.5. Fractions of the chromatofocusing eluate containing cyclase activity were separately concentrated in a Millipore cell, using a PM10 Diaflo ultrafiltration membrane, to volumes of 0.2-0.3ml (approx. 10-fold). Samples from the fraction containing maximum activity were loaded on the polyacrylamide gel, together with aliquots from fractions which eluted before and after the activity maximum. Separate wells were used for marker proteins. After staining, the distances travelled by each protein were measured and relative mobilities (M_r) were calculated relatively to the overall length of the separating gel. Calibration lines were constructed by plotting **log mol.wt.** of the marker proteins against M_r. Inspection of the gels after staining revealed a band with a mol.wt. of approx. 40 000, which was intenser in the sample taken from the fraction containing maximum cyclase activity. The subunit size of the Scop. candida cyclase could not be obtained because no bands were detected in the gel, even after silver-staining.

The calibration line for the mol.wt. estimation of the Asp. sydowii cyclase subunit is shown in Fig.6.7. Photographs of some gels run with individual cyclases and of

one comparative gel with cyclases from several sources are shown in Fig.6.8. The latter gel shows that the Asp. flavus Asp. sydowii, Pen. chrysogenum and Pen. crustosum have almost identical apparent subunit sizes, slightly smaller than that of the Gliocladium enzyme and larger than that of the Paecilomyces enzyme. This observation is in reasonable agreement with the values estimated from the calibration lines for each individual cyclase which are summarized in Table 6.6.

TABLE 6.6

Subunit molecular weights of several cyclases determined by calibrated SDS-polyacrylamide gel electrophoresis

Cyclase source	Subunit mol.wt. ($x10^{-3}$)
Asp. flavus	43
Asp. sydowii	42.5
Gl. deliquescens	42.5
Mon. ruber	40
Paec. variotii	38.5
Pen. chrysogenum	42
Pen. crustosum	42
Pen. terrestre	42

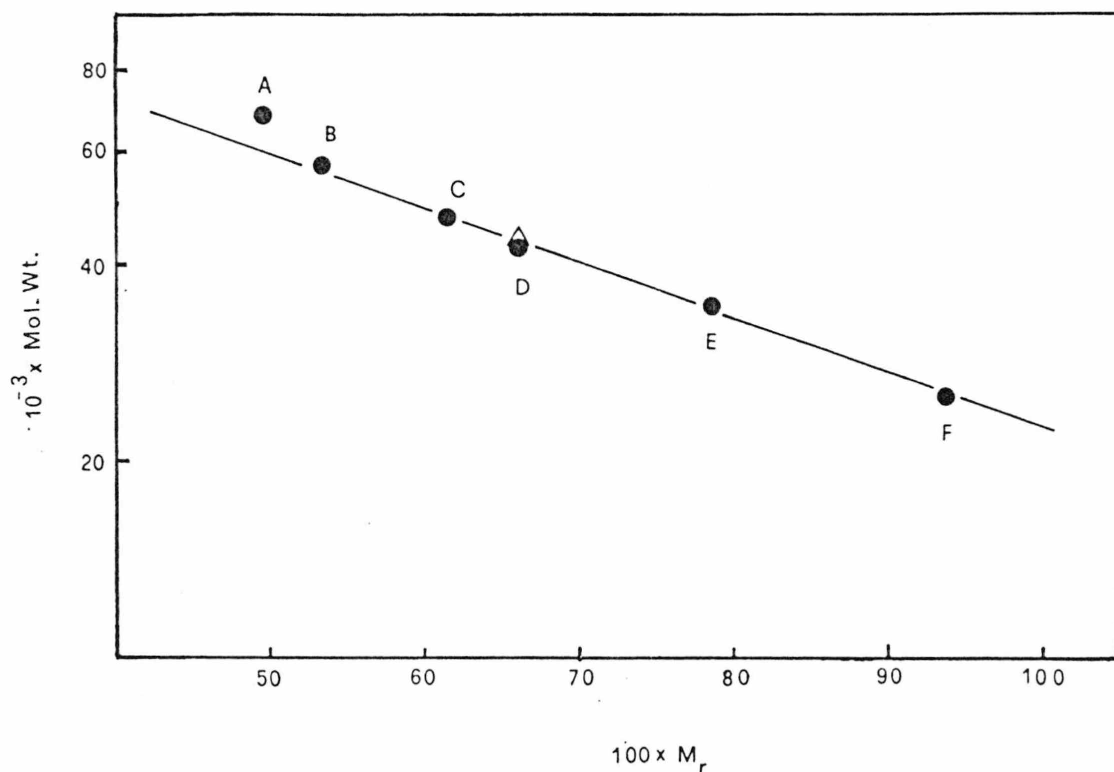
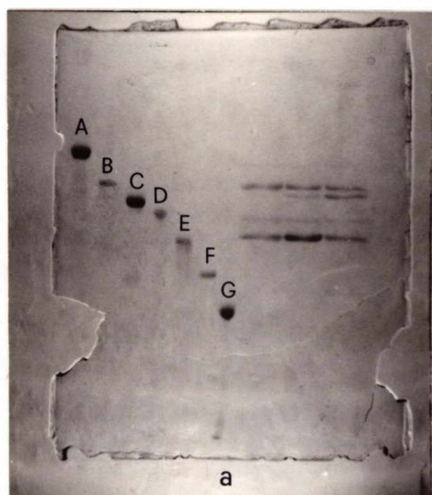
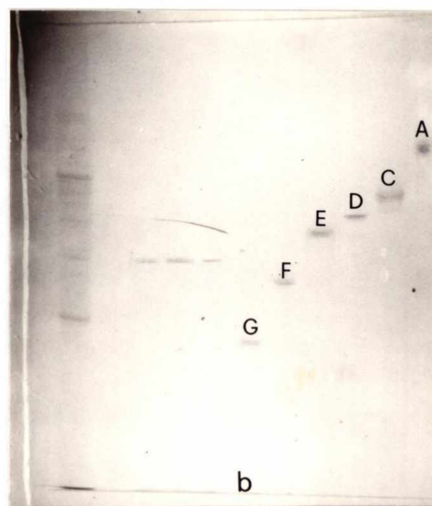
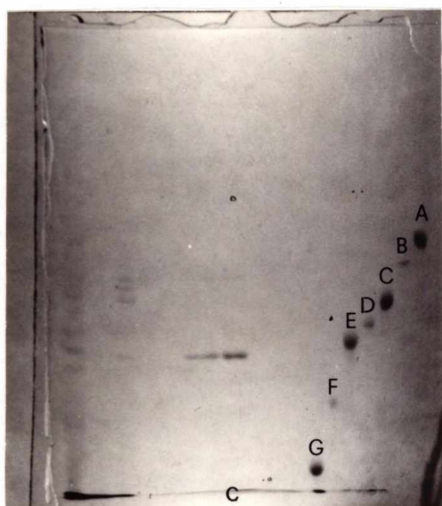


Fig.6.7. Subunit size of the cyclase from <u>Asp. sydowii</u> determined by SDS-polyacrylamide gel electrophoresis. The mol.wts. of the marker proteins were plotted against their relative mobilities, as described in Section 6.5. The marker proteins were bovine serum albumin (A), pyruvate kinase (B), fumarase (C), ovalbumin (D), lactate dehydrogenase (E) and chymotrypsinogen A (F). The gel was 10% (w/v) in acrylamide and was prepared and stained as described in Section 3.11. The cyclase mol.wt. read off the line was 43 000.







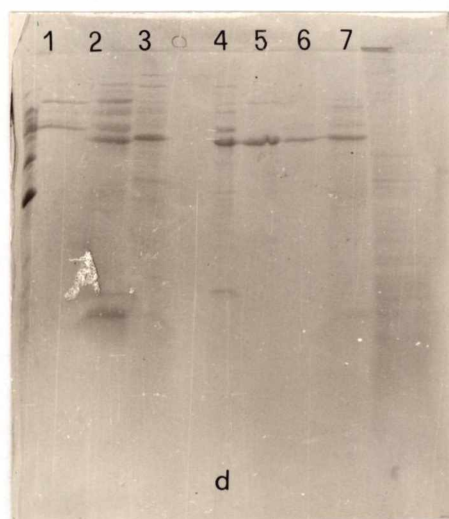


Fig.6.8. Calibrated SDS-polyacrylamide gels and comparative gel of several cyclases. The gels shown in photographs (a), (b) and (c) contained samples of the cyclases from Gl. deliquescens, Paec. variotii, and Pen. chrysogenum, respectively. The marker proteins were bovine serum albumin (A), pyruvate kinase (B), glutamate dehydrogenase (C), fumarase (D), ovalbumin (E), lactate dehydrogenase (F) and chymotrypsinogen (G). The comparative gel (d) contained samples of the cyclases from Gl. deliquescens (1), Paec. variotii (2), Mon. ruber (3), Pen. crustosum (4), Pen. chrysogenum (5), Asp. sydowii (6), and Asp. flavus (7). The cyclase samples were obtained as described in section 6.5.

6.6. Michaelis Constants

The Michaelis constants of several cyclases at the optimum pH (6.0) were estimated from Hanes plots (section 3.4) of experimental data obtained under the conditions described in section 5.5 and are listed in Table 6.7. Fig.6.9 shows a Hanes plot of the data for the K_m determination of the Asp. sydowii cyclase.

TABLE 6.7

Michaelis constants of several cyclases

Cyclase source	K_m (μM)
Asp. flavus	14
Asp. sydowii	12
Gl. deliquescens	14
Mon. ruber	17
Paec. variotii	16
Pen. chrysogenum	17
Pen. crustosum	17
Pen. terrestre	15
Scop. candida	12

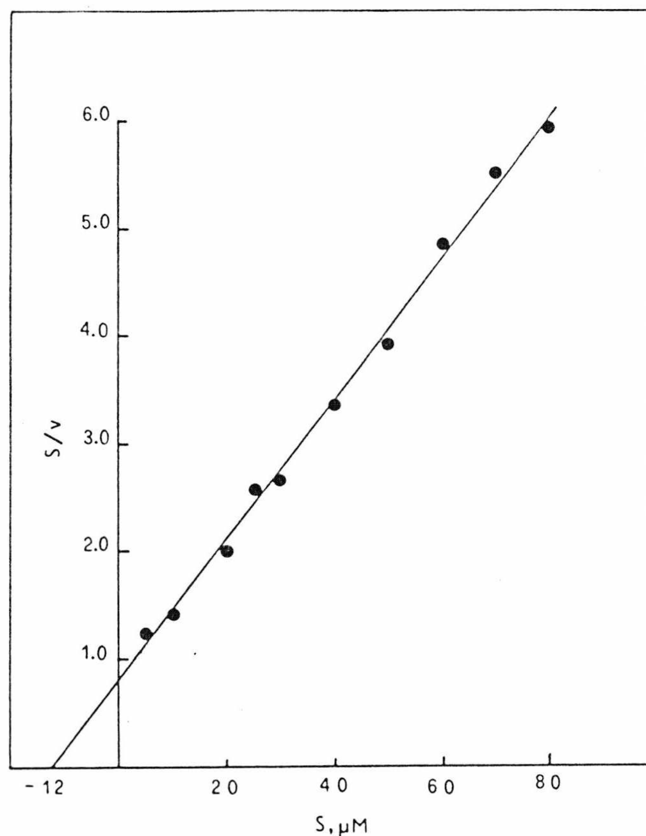


Fig.6.9. Determination of the K_m of the A. sydowii from a Hanes plot. Initial velocities [v, μmoles.min^{-1}. (mg protein)$^{-1}$] were measured at several substrate concentrations (S). The K_m (12.3 μM) was estimated as described in Section 3.4, from the parameters of the line (Corr=0.993): Slope=0.065 Intcp=0.80

6.7. Fingerprints of Cyclases by SDS-PAGE

Fingerprinting experiments under the conditions described in section 3.14 were partially sucessful. Indeed, as can be seen in Fig.6.10, the chymotrypsin preparation was very crude and some of its bands overlapped with cyclase fragments. The CNBr digest produced quite diffuse patterns of three or four barely detectable bands. The method may thus be useful for the isolation of large cyclase fragments but not for the detection of structural variability. In contrast, the digestion with _Staphylococcus aureus_ V8 protease yielded clear and distinct patterns with the four cyclases tested, suggesting widely different distributions of their acidic amino acid residues.

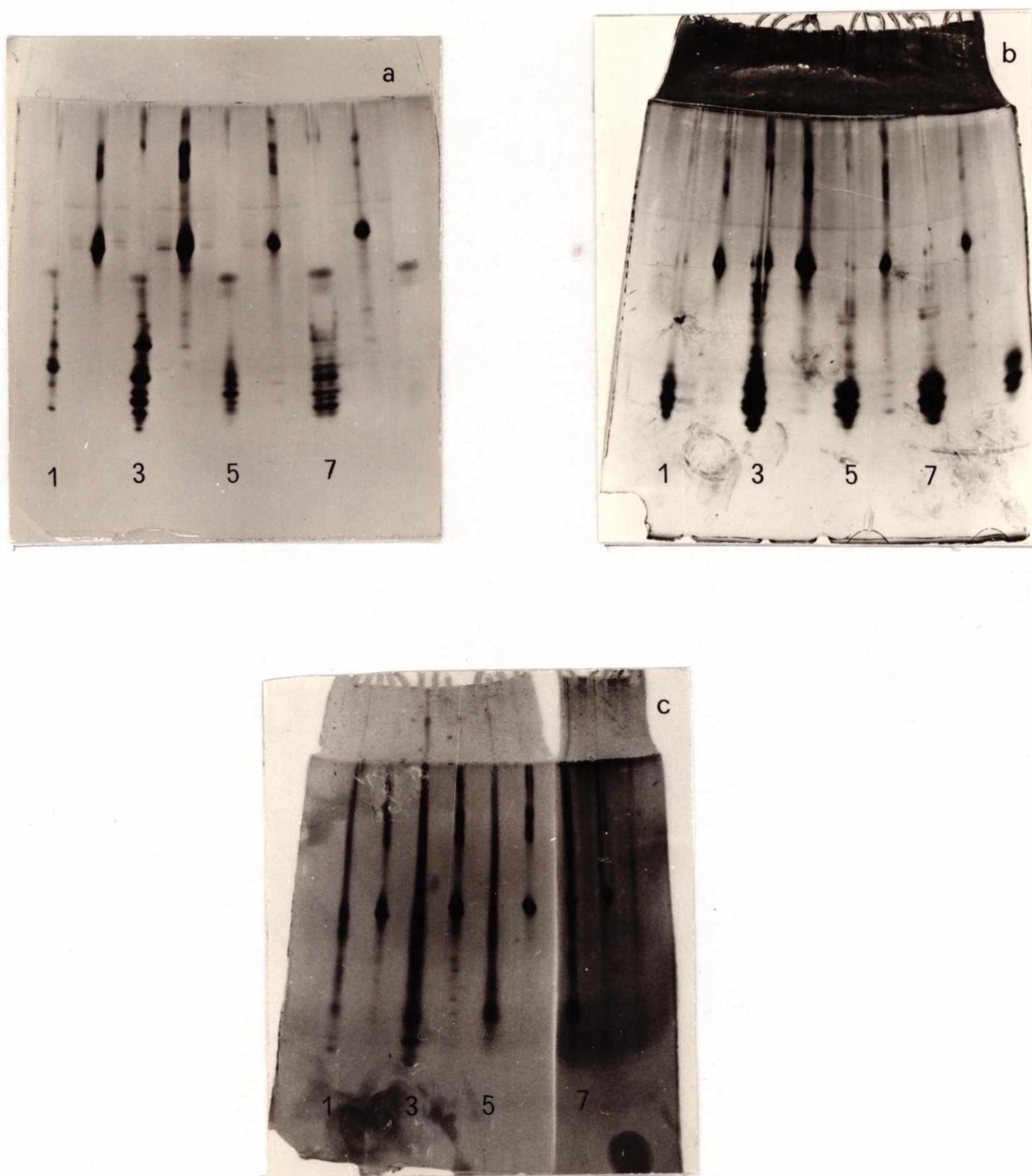


Fig.6.10. Fingerprints of cyclases. The digestions were performed with Staphylococcus aureus V8 protease (a), chymotrypsin (b) and CNBr (c) directly on gel slices, as described in section 3.14. The wells 1, 3, 5 and 7 contained the cyclases from Paec. variotii, Mon. ruber, Pen. crustosum and A. niger L6, respectively. Even numbered wells contained untreated slices. The last well to the right contained protease only.

CHAPTER 7

DISCUSSION

7.1. The Purification of the A. niger Cyclase

The eventual comparison of the amino acid sequences of bacterial and fungal 3-carboxymuconate lactonizing enzymes has been defined in the introductory chapter as one of the purposes of this work. Thatcher & Cain (1974 a) have previously reported a 543-fold purification of the A. niger enzyme, in 13% yield. The purified enzyme appeared electrophoretically homogeneous in polyacrylamide gels, using the Weber & Osborn (1969) buffer system and staining by Amido Black.

In the present work the Laemmli buffer system (Laemmli, 1970) and staining by Coomassie Blue were adopted for SDS-polyacrylamide gel electrophoresis, thus increasing the resolution and sensitivity of the technique (Weber & Osborn, 1975). With this method of analysis it became apparent, in early preliminary experiments, that a cyclase purification of approx. 500-fold did not yield homogeneous enzyme, as illustrated in Fig.4.12 which shows the still very complex band pattern of the Ultrogel eluate (step 6, Table 4.11) containing 527-fold purified cyclase. A more extensive purification, exploring additional properties of the protein molecules, would therefore be required.

The improved method described in section 4.9 involved ammonium sulphate fractionation of the cell-free extract, anion-exchange chromatography, gel filtration, hydrophobic chromatography and chromatofocusing and achieved a 3300-fold purification of the cyclase, in 9% yield. Surprisingly, however, the final preparation was not homogeneous. From the gel shown in Fig.4.12 the cyclase content in the purest sample has been estimated as 85% of the total protein.

Some difficulties were found in the application of this method, which can be related to the properties of the *A. niger* cyclase, particularly its decreased stability in cationic buffers, in buffers of low ionic strength and in extensively purified samples. The effects of the buffer composition on the stability of the enzyme are well illustrated in the preliminary experiments described in sections 4.4 to 4.8. The application of identical or equivalent separation techniques led to consistently lower recoveries of enzymatic activity when cationic buffers were used, comparatively to those performed in phosphate buffers. The extent of purification of the cyclase sample equally affected the yield of recovery of enzymatic activity. Step 6 (Table 4.11) was performed in 0.05M phosphate buffer with a 73% recovery which is significantly lower than the 95% yield in trial experiment (section 4.4) using the same buffer and gel permeation medium but a more crude cyclase sample.

The possible combined effects of sample and buffer composition and buffer ionic strength were particularly apparent in the later stages of the purification procedure.

Ethyl-agarose chromatography in 5mM phosphate buffer, chromatofocusing and batch butyl-agarose steps all led to considerable activity losses (section 4.9).

The increased stability of the enzyme in phosphate buffers may be due to a stabilization of the active-site conformation since phosphate is a competitive inhibitor of the A. niger cyclase (Thatcher & Cain, 1975). In contrast, organic buffering cations presumably interact with the negatively charged enzyme surface and affect its conformation and (or) its aggregation properties. Such effect may also occur at low ionic strength, with a consequent loss of enzymatic activity and altered elution behaviour.

The contaminants still present in the purest cyclase sample were well resolved by SDS-polyacrylamide gel electrophoresis (Fig.4.12). This observation suggests that preparative electrophoresis under denaturing conditions might be a satisfactory procedure for the isolation of homogeneous enzyme. However, a preliminary purification of approx. 1000-fold, equivalent to that achieved after ethyl-agarose chromatography, would probably be required since more crude preparations are still quite complex protein mixtures (Figs.4.12 and 5.3). Preparative SDS-PAGE is now a routinely used technique for the isolation of denatured proteins in adequate amounts for amino acid analysis and sequencing and for peptide mapping (Cleveland et.al., 1977; Walker et.al., 1982). The recovery of enzymatic activity from the inactive subunits, if possible,

would also constitute the unambiguous demonstration of the identity of the cyclase band in polyacrylamide gels. It should also be possible, in principle, to isolate native proteins and enzymes by gel electrophoresis. Doly & Petek (1977) described the use of gradient polyacrylamide gels, without SDS, for the isolation, on a milligram scale, of native proteins from mixtures containing 4 to 20 components, with yields of recovery of up to 60%, after electrophoretic elution.

An even better alternative for the isolation of homogeneous proteins from reasonably crude mixtures appears to be preparative isoelectric focusing in gel layers. The technique does not require denaturation of the samples, has a very high resolution and samples of up to 10g of protein mixtures can be applied to a single gel; an additional advantage is the simple elution procedure of the separated components (Blanicky, 1979).

If necessary, the technique of HPLC could also be explored in the cyclase purification. Recent reviews by Regnier & Gooding (1980) and Thatcher (1982) refer to a variety of media now available for the fractionation of proteins on the basis of different molecular properties as size, net charge, surface hydrophobicity and ion-pairing effects.

Affinity chromatography was another possibility that has been considered but, as described in Chapter 2, it has not been possible to find a compound with the properties of being, simultaneously, a good cyclase inhibitor and susceptible to covalent attachment to a derivatized gel.

This failure was the result of the chemical instability of the muconate derivatives and of the very high specificity of the cyclase for its substrate.

The absolute amount of cyclase finally isolated has also been severely limited by the low level of enzyme in the induced mycelium. From the data in Table 4.11 and assuming that the purest sample contained 85% of fully active enzyme, the cyclase amount in the cell-free extract was estimated as 0.03% of the total soluble protein. This result is in disagreement with earlier observations of Thatcher & Cain (1974a) who reported that enzyme yields of up to 30mg of enzyme could be isolated from 5kg of induced mycelium yielding cell-free extracts with a specific activity of 0,52 units (mg enzyme)$^{-1}$. According to previous investigations, a higher induction level of the enzymes of the protocatechuate pathway, particularly of the cyclase, appears possible. Ahlquist (1977) studied the regulation of the pathway in <u>A. niger</u> and observed that 3-hydroxy-4-methylbenzoate was a gratuitous inducer of the cyclase. The level of enzyme synthesis was increased 4.5-fold when the fungus was grown in mixtures of glucose (15mM) and inducer (2mM) relatively to mycelium grown in <u>p</u>-hydroxybenzoate only. Such an increase would greatly facilitate the purification procedure and allow the isolation of larger amounts of enzyme.

7.2. Subunit Composition of Fungal Cyclases

The subunit composition of oligomeric proteins is usually established from independent estimates of their native and subunit(s) mol.wts. The available methods for the estimation of these parameters differ in accuracy and precision and also in the kind of information they provide (Rowe, 1978). Factors other than accuracy and precision may, however, determine the choice of the method, e.g., limiting sample material and its non-homogeneity. Under these conditions, calibrated gel permeation chromatography and SDS-polyacrylamide gel electrophoresis are the most commonly used methods for the estimation of native and subunit mol.wts., respectively. The data obtained by the application of these techniques are best described as the **apparent native mol.wt.** by gel permeation chromatography and **apparent subunit mol.wts.** by SDS-polyacrylamide gel electrophoresis. If both values are consistent within experimental error they provide, in most cases, enough information for the determination of the subunit composition of the oligomeric protein. This appears to apply to most fungal cyclases examined in this work. Replicate experiments of SDS-polyacrylamide gel electrophoresis on different batches of the A. niger cyclase yielded values within the range 41 000 - 43 000. The **apparent** subunit mol.wt. was therefore estimated as 42 000±1 000. Similar estimates for other cyclases yielded values within the range 40 000-43 000 except for the Paecilomyces enzyme (Table 6.7) and the comparative gel shown in Fig. 6.8 revealed that the

mobilities of the cyclase bands from aspergilli, penicillia and Monascus ruber were virtually identical. Even though replicate experiments were not been performed for several of those cyclases it seems reasonable to assume that the reproducibility of these estimates was of the same order of magnitude as those for the A. niger enzyme. Furthermore, tetramers of these subunits should behave as molecules with **apparent** mol.wts. in the range 160 000-172 000, which is in good agreement with the data from calibrated gel permeation chromatography for the A. niger enzyme (section 5.1) and other cyclases (Table 6.4).

The two exceptions detected in the comparative gel (Fig.6.8) were the cyclases from Paecilomyces and Gliocladium. The higher mobility of the Paecilomyces enzyme was consistent with the independent estimate of 38 500 for its subunit mol.wt. and also with the apparently smaller size of the native molecule (mol.wt. 150 000). The Gliocladium enzyme behaved as a larger molecule in calibrated gel permeation chromatography (mol.wt. 180 000) and also displayed a lower mobility in the comparative gel relatively to other cyclases. The less consistent data refer to the enzyme from Monascus ruber. Its mobility in the comparative gel did not significantly differ from those of most cyclases but the estimate of its native mol.wt. yielded a lower value (155 000). This result is, nevertheless, within 8% of the average mol.wts. of cyclases with apparently similar subunit sizes. The Scopulariopsis candida cyclase is not discussed because the estimate of its subunit size was not possible (section 6.6).

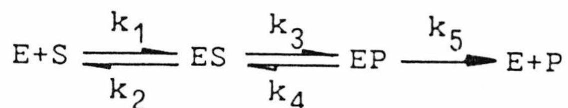
These conclusions, particularly those concerning the *A. niger* cyclase do not fully support previous findings (Thatcher & Cain, 1974a,b). The enzyme had been described as an octamer of subunits with a mol.wt. in the range 22 500-25 000, estimated by SDS-polyacrylamide gel electrophoresis and sedimentation equilibrium in guanidinium chloride, under reducing conditions. The native mol.wt. had been estimated as 200 000 by gel permeation chromatography and 191 000 by high-speed sedimentation equilibrium according to the method of Yphantis (1964). The description of the experimental conditions suggests that these determinations were performed with the purest cyclase sample (543-fold purified). This sample had been subjected to ammonium sulphate fractionation, ion-exchange and gel permeation chromatography and freeze-drying. The overall process was equivalent to steps 2 to 6 described in Section 4.9 and summarized in Table 4.11. The heterogeneity of a sample subjected to a purification of this order of magnitude is well documented in the gel shown in Fig.4.12. An erroneous assignment of the cyclase band by Thatcher & Cain (1974b) was therefore possible, particularly because of their less sensitive detection method.

The estimations from sedimentation equilibrium experiments, if performed with a non-homogeneous sample, yield a **weight-average** mol.wt., as discussed by Van Holde (1975) who also stressed that a linear plot of **ln c** *vs.* r^2 does not necessarily mean the homogeneity of the protein sample.

The divergences between the mol.wt. estimates by calibrated gel permeation chromatography could be accounted for by the different experimental conditions. In this work (section 5.1), the sample had not been extensively purified and markers of mol.wt. close to that of the cyclase have been included (catalase, fumarase and lactate dehydrogenase). In the experiment described by Thatcher & Cain (1974a), the marker proteins of mol.wt. closer to that of the cyclase were ferritin and lactate dehydrogenase and a process like freeze-drying, which they used, could have caused conformational alterations and an anomalous elution behaviour.

7.3. Kinetic Properties

The mechanism of uni-substrate enzymes can be described by the scheme:



$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_5} E+P$$

SCHEME 7.1

Making the assumption of Briggs-Haldane of a steady-state concentration for **ES** and **EP**, the following expression for the overall velocity of the reaction can be derived (Dixon & Webb, 1979):

$$v = \frac{k_3.k_5[Eo][S]}{(k_3 + k_4 + k_5)} / \{[S] + \frac{(k_2.k_4 + k_2.k_5 + k_3.k_5)}{k_1(k_3 + k_4 + k_5)}\} \quad (7.1)$$

Equation 7.1 is usually written in the form of the Michaelis-Menten equation:

$$v=k_{cat}[Eo][S]/\{[S] + K_m\} \qquad (7.2)$$

At very low substrate concentrations ($[S]<<K_m$) equation 7.2 can be written as:

$$v=k_{cat}[Eo][S]/K_m \qquad (7.3)$$

At very high substrate concentrations ($[S]>>K_m$) the reaction is zeroth order in respect to substrate concentration and its velocity reaches the maximum value:

$$v=V_{max}= k_{cat}[Eo] \qquad (7.4)$$

Equations 7.3 and 7.4 show that two different parameters must be used to assess the catalytic efficiency of enzymes: k_{cat}/K_m at low substrate concentrations and k_{cat} at high substrate concentration. Which of these parameters more adequately reflects the in vivo enzyme efficiency depends on the intracellular substrate concentration. It appears, however, that most enzymes of intermediary metabolism operate at low substrate concentrations; this fact may reflect evolutionary pressure on the kinetic parameters so that enzymes can more easily respond to varying substrate fluxes (Hall & Kohen, 1983).

The experimental determination of $k_{cat}=V_{max}/[Eo]$ requires homogeneous and fully active enzymes or, at least, samples where the amount of the enzyme can be quantified. These conditions could be met for the A. niger cyclase, for which the V_{max} was estimated (section 5.5) but not for other

fungal cyclases. It was not possible, therefore, to assess their relative catalytic efficiencies either at high or low substrate concentrations. Under an evolutionary perspective, however, it is also informative to compare the kinetic parameters of the bacterial and fungal 3-carboxymuconate lactonizing enzymes. The 3-carboxymuconate cycloisomerase from P. putida has been crystallized to a constant specific activity of 800 Units (mg enzyme)$^{-1}$, as described by Patel, Meagher & Ornston (1973). Given the conditions of the enzyme assay and the Michaelis constant (75μM) reported by Ornston (1966a) and assuming that the reaction follows the Michaelian kinetics, the V_{max} for the bacterial enzyme should be approx. 1 170 Units (mg enzyme)$^{-1}$. Assuming that the mol.wts. of the fungal and bacterial enzymes are 162 000 (section 5.3) and 190 000 (Ornston, 1966 a), respectively, and that the V_{max} for the A.niger cyclase is 2 180 Units (mg enzyme)$^{-1}$, as estimated in section 5.5, the fungal enzyme is approx. 1.6-fold as efficient as the bacterial one, at high substrate concentrations. At low substrate concentrations, however, the ratio of their catalytic efficiencies increases to 8.

Even though the kinetic parameters k_{cat}/K_m of fungal cyclases could not be determined, the experimental Michaelis constants, which all fell in the range 12-17 μM (section 5.5 and Table 6.8) suggest a few provisional conclusions. If scheme 7.1 adequately describes the mechanism of the reaction mediated by these enzymes, the K_m values will be determined by the nature of binding residues, which are likely to affect k_1, k_2 and k_5, by the catalytic residues

which more directly influence k_3 and k_4 and also by the active-site conformation which may affect all of the rate constants. Close K_m values in unrelated enzymes may occur by chance but this is not the case of the cyclases examined in this work. They are all serologically related (section 1.5) and close K_m values are thus consistent with the conservation of their active site structures, as expected in evolutionary related isofunctional enzymes (Hanson & Rose, 1975). It is apparent, however, that further evidence is required in support of this conclusion, particularly the determination of the k_{cat} for the various enzymes, the analysis of the effect of pH on the Michaelis parameters and the identification of the essential residues.

The considerations based on the K_m values may equally apply to the Neurospora crassa enzyme, for which a K_m of 18μM has been reported, but not to the cyclase from Rhodotorula mucilaginosa, which has a K_m of 67μM (data from Cain, Bilton & Darrah, 1968). This enzyme and those from Schyzophyllum and Sporobolomyces differ from other fungal cyclases in the instability of their oligomeric forms, which dissociate into smaller subunits, in freshly prepared cell-free extracts, without loss of total enzymic units (Cain, 1980). The cyclases from Rhodotorula and Schyzophyllum are also serologically distant from the A. niger enzyme (Cook & Cain, 1977).

The inhibitor studies described in section 5.7, involving the A.niger cyclase, and some of those performed in previous investigations (Thatcher & Cain, 1975) are

summarized in Table 7.1. These experimental data provide some indications on the structural requirements of the active site. The inhibition of the enzyme by 3-carboxymuconolactone and by the α-methyl ester of 3-carboxy-*cis*, *cis*-muconate and the non-inhibitory effect of *cis*, *cis*-muconate suggest that both the β- and δ-carboxyl groups in the substrate are essential for binding. The inhibitory effect of other di- or tricarboxylate ions further suggests that a protonated carboxyl group in the substrate is involved in the interaction with the enzyme. Indeed, carboxylic acids in which one of the ionization constants (pK_a) is 6 or higher are significantly more inhibitory than other analogues with lower ionization constants.

One of the essential residues of the *A. niger* cyclase is, presumably, histidine, as shown by the Rose Bengal photo-sensitized inactivation of the enzyme (Thatcher & Cain, 1975). This residue is expected to be predominantly protonated at (pH 6.0) and could thus be one of the binding residues. Most likely, however, histidine is directly involved in catalysis, since a proton-donation step is required in the conversion of 3-carboxymuconate to 3-carboxymuconolactone.

TABLE 7.1

Inhibitory effect of some organic anions on the *A. niger* cyclase

Anion	K_i (μM)	pK_a's
cis-Aconitate	500(a)	2.0 ;4.5 ;6.6
trans-Aconitate	5 000(a)	2.8 ;4.45;5.0
Maleate	360(a);315(b)	2.0 ;6.26
Fumarate	8 800(a)	3.0 ;4.47
Citraconate	620(a)	3.06;6.16
Mesaconate	2 800(a)	3.09;4.45
Citrate	5 000(a)	3.06;4.47;5.4
cis,*cis*-Muconate	65(a);N.I(b)	Unknown
1-Methyl 3-carboxy--*cis*, *cis*-muconate	170(b)	Unknown
3-carboxymuconolactone	285(b)	Unknown
Phthalate	N.I(b)	2.95;5.41
Homophthalate	N.I(b)	Unknown

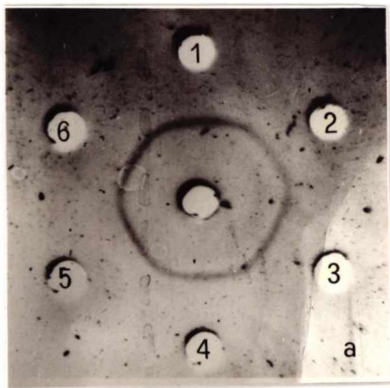
(a) Data from Thatcher & Cain (1975); (b) Data from section 5.7; N.I.: non-inhibitory.

7.4. Structural Relationships in Fungal Cyclases

Serological studies have been extensively used in the detection of taxonomic relationships within selected microbial groups (London & Kline, 1973 and references therein; Cook & Cain, 1977 and references therein). The observation of serological cross-reaction between homologous proteins or enzymes is usually taken as evidence of their evolutionary relatedeness and therefore of the phylogenetic relationship between organisms from which they were extracted. The major limitation of the method is that a negative result does not necessarily mean that such a relationship is non-existent since the replacement of a few amino acid residues may lead to the loss of serological cross-reaction.

The conclusions derived from positive serological cross-reactions are justified by the available information on tertiary structures of homologous enzymes (Section 1.4). Indeed, the probability of common immunological determinants, which are located precisely in the most variable region of the protein molecules, must be vanishingly small in the products of convergent evolution.

Some of the results obtained by the application of the Ouchterlony double-diffusion method to fungal cyclases are illustrated in Fig.7.1. Full homologous cross-reaction was only observed between induced extracts of several *A. niger* strains and antiserum raised against the *A. niger* L6 cyclase (Fig.7.1a; data from Cook & Cain, 1977).



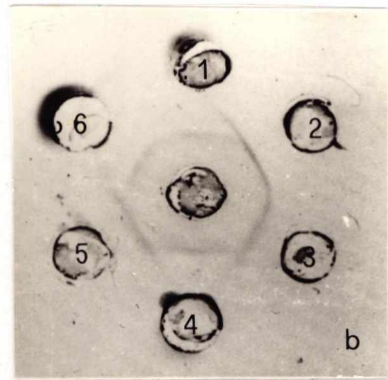


Fig.7.1. Ouchterlony double diffusion plates. Outer wells are numbered 1 to 6 begining with the uppermost and the centre well contained purified anti-cyclase IgG to A. niger L6. In plate (a) well 1 received purified A. niger L6 cyclase and wells 2 to 5 contained extracts of A. niger CMI 45551, A. niger (Birkbeck strain), A. niger L6 and A. niger (Newcastle strain), respectively, all induced by growth on p-hydroxybenzoate; well 6 received a crude extract of A. niger L6 grown on protocatechuate. In plate (b) well 2 received purified A. niger L6 cyclase and the other wells contained the extracts of p-hydroxybenzoate-grown P. crustosum (1), A. niger L6 (3), M. ruber (4), A. sydowii (5) and P. terrestre (6).

Extracts of other species, induced by growth in p-hydroxybenzoate, produced heterologous cross-reactions (Fig.7.1b; R.B. Cain, unpublished results). These observations suggest a certain degree of structural variability in the various cyclases which was indeed expected even though their molecular and subunit sizes and Michaelis constants were similar.

The isoelectric point is another molecular parameter which reflects compositional differences in charged superficial amino acid residues. The values listed in Table 6.6 covered a range of 1.45 pH units suggesting significantly different ratios of superficial acidic and basic residues. The extreme examples were the cyclases from Scop. candida (pI=4.60) and those from Paec. variotii (pI=6.05) and Mon. ruber (pI=5.90).

A linear correlation between the pI's of the various enzymes relative to the pI of the A. niger cyclase and their percentages of cross-reaction with the antiserum raised against the same enzyme was not expected because the immunological properties of the proteins are determined by a comparatively small fraction of the superficial residues. Table 7.2 reveals, however, a broad correlation between those two parameters. The only obvious exception was the Scop. candida cyclase. This enzyme, however, had already displayed an exceptional behaviour in gel permeation chromatography, presumably due to a different oligomeric composition (section 6.3). If this conclusion is correct, the structural factors responsible for the different association properties of the subunits in this enzyme,

relative to other cyclases, may equally be responsible for the considerable loss of antigenic determinants shared with the _A. niger_ enzyme.

TABLE 7.2.

Differences in isoelectric points relative to the pI of the _A. niger_ L6 cyclase and percentage immunological cross-reaction with the _A. niger_ L6 anti-cyclase IgG observed for the isofunctional enzymes from several fungi

Enzyme Source	ΔpI(a)	Cross-reaction (b) (%)
A. flavus	+0.20	61
A. sydowii	+0.40	56.8
P. terrestre	+0.40	37.3
P. chrysogenum	+0.55	43.6
P. crustosum	+0.65	40.0
Mon. ruber	+1.05	13.8
Paec. variotii	+1.20	9.2
Scop. candida	-0.25	3.9

(a) Data from sections 5.4 and 6.4

(b) Data from Cook & Cain (1977)

Further evidence for some structural differences between the cyclases was provided by the protease digests (Fig.6.10). The digest patterns in polyacrylamide gels contained several fragments of identical or similar mobilities. It must be considered, however, that similar mobilities do not signify identical amino acid compositions. Furthermore, the differences in the relative intensities of the bands suggest different rates of hydrolysis of the peptide bonds susceptible to the Staphylococcus aureus V8 protease. Since the polypeptide chains were fully denatured the different rates of hydrolysis were determined presumably, by distinct amino acid compositions in the vicinity of the hydrolyzed bonds.

The experimental evidence discussed above suggests, in summary, that the cyclases from representatives of a number of taxonomically close genera illustrate the structural variability in homologous enzymes which is, nevertheless, compatible with conservative features as the immunological properties, molecular and subunit sizes and, presumably, active site structures. It remains to be established, however, how far some of the conservative properties extend to other cyclases which are serologically unrelated to the A. niger enzyme.

7.5. Evolutionary Speculations

The 3-oxoadipate pathway is an important catabolic route for the dissimilation of aromatic compounds which can be converted to catechol or protocatechuate (Stanier & Ornston, 1973; Ornston & Parke, 1977; Cain, 1980). Although most of those aromatics can be related to lignin breakdown the emergence of the bacterial pathway is likely to have preceded the lignification process by some 1.5×10^9 years (Cain, 1980). In the presumable absence of exogenous aromatics the evolution of a pathway for the utilization of aromatic amino acids or some of their biosynthetic precursors could have represented an earlier step in the establishment of the 3-oxoadipate pathway, followed by later acquisition of the peripheral activities and transport systems. This possibility is suggested by the formation of catechol in one of the possible oxidative routes for tryptophan in bacteria and of protocatechuate from shikimate and dehydroquinate (Stanier & Ornston, 1973; Haslam, 1974).

The alternative hypothesis of evolution of the pathway for the dissimilation of some of its aliphatic intermediates would require their availability in the environment, which seems unlikely on chemical grounds. A few points should be particularly emphasized.

1. The syntheses of the correct stereoisomers of muconate and 3-carboxymuconate from aromatic precursors require strongly oxidizing conditions, which hardly appear to be reproducible in any conceivable environment (Elvidge *et*

al., 1950a; Husband et al., 1955; Schultz & Hecker, 1973; Tsuji & Takayanagi, 1974; Pandell, 1976; Rogic, Demmin & Hammond, 1976).

2. The chemical lactonization of cis, cis-muconate requires concentrated acid and leads to the racemic muconolactone (Eisner, Elvidge & Linstead, 1950).

3. 4-Carboxymuconolactone has only been synthesized enzymatically and has a half-life of 30min. at pH 6.0, 30°C, and 3-oxoadipate-enol lactone is either the product of the enzymatic isomerization of (+)-muconolactone or of the spontaneous decarboxylation of 4-carboxymuconolactone (Ornston & Stanier, 1966).

4. The intermediate which could eventually accumulate in the environment is 3-carboxymuconolactone, although as a racemic mixture, since this lactone slowly accumulates in aqueous acidic solutions of 3-carboxy-cis, cis-muconate (Ainsworth & Kirby, 1968). cis, cis-Muconate would probably isomerize to the more stable cis, trans-isomer (Elvidge et al., 1950b) and 3-oxoadipate enol-lactone spontaneously hydrolyzes to 3-oxoadipate in mildly alkaline conditions (Ornston & Stanier, 1966). The alternative formation of 3-oxoadipate by β-oxidation of adipate would be conceivable but, as a 3-oxoacid, its most likely fate in the environment would be decarboxylation, which is know to occur easily, catalyzed by metallic ions or even spontaneously (Walsh, 1979).

The chemical evidence summarized above shows that the only known precursors to the intermediate lactones are either cis, cis-muconate or 3-carboxy-cis, cis-muconate, the

formation of which is a major chemical barrier. Furthermore, the stability of most of the intermediates apparently precludes their accumulation in the environment. These data are consistent with the observation that the aliphatic intermediates do not usually support growth of either bacteria or fungi, although a few exceptions have been reported. P. acidovorans and P. testosteroni can grow on 3-carboxy-cis, cis-muconate and cis, cis-muconate, and a transport system for 3-oxoadipate is inducible in P. putida (Ornston & Parke, 1977). Fungal mycelia, induced for the enzymes of the protocatechuate branch slowly incorporate labelled 3-carboxymuconate but not 3-carboxymuconolactone or 3-oxoadipate (Cain, 1980). These metabolic capabilities could, however, represent a later evolutionary development.

Independently of the selective factors which determined the evolution of the 3-oxoadipate pathway, the available evidence suggests that the emergence of the specific dioxygenases has been a key evolutionary step.

1. The protocatechuate and catechol dioxygenases are unrelated to other iron-containing proteins like the ferredoxins (Barnabas, Schwartz & Dayhoff, 1981) or the cytochromes (Dickerson, 1980), of more remote origin.

2. The non-oxidative transformations of the pathway can be interpreted in terms of rather common mechanisms of acid or base catalysis or of acyl exchange (section 1.4).

3. The muconolactone isomerases, carboxymuconolactone decarboxylases, enol-lactone hydrolases and tranferases all appear to be evolutionary related, as suggested by their

amino acid sequences (section 1.4). A possible common precursor could have been some acyl-CoA transferase, since most of the transferase residues are represented in other classes of enzymes of the pathway. This hypothesis is supported by additional observations: (a) an acetoacetate acetyl-CoA transferase from E. coli, in which the 3-oxoadipate pathway is uninducible, is compositionally related to the 3-oxoadipate succinyl-CoA transferases from P. putida and A. calcoaceticus and has a similar oligomeric composition (Yeh & Ornston, 1981); (b) Nocardia opaca grown on adipate and other 3-oxoadipate homologues becomes induced for all the enzymes of the protocatechuate branch (Cain, 1980).

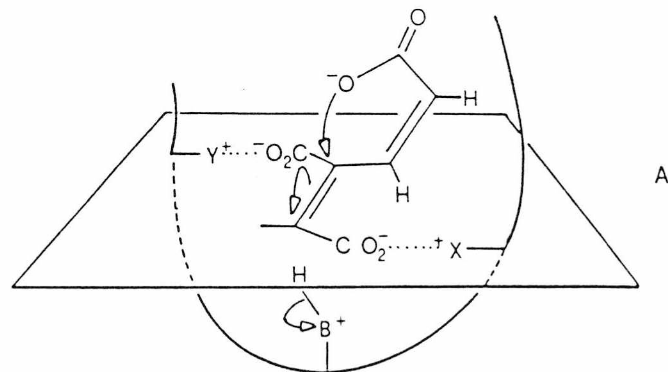
4. Representatives of P. acidovorans and P. testosteroni capable of growth on 3-carboxymuconate metabolize this substrate via 3-oxoadipate even though protocatechuate is dissimilated through the **meta**-cleavage pathway (Ornston & Parke, 1977).

The pattern of coordinate product induction observed in Pseudomonas and Nocardia is equally consistent with the evolution of some precursor 3-oxoacyl-CoA transferase towards other specificities of the pathway. The different enzymes could have arisen by initial gene duplication followed by divergence and still remain organized in a single operon controlled by 3-oxoadipate, through mutation in a regulatory gene. Coordinate substrate induction in Acinetobacter could have been established by mutation to constitutive enzyme synthesis followed by evolution of a regulatory gene controlled by protocatechuate.

The arguments developed above can also be applied to the evolution of the protocatechuate branch of the eucaryotic pathway. There is no immediate answer, however, for the problem of the evolutionary divergence of the lactonizing enzymes, which appears to be supported by the stereochemical evidence. Indeed the formation of different lactones from the same substrate and the stereochemical courses of the two reactions can only be interpreted assuming different structural arrangements of binding and catalytic residues (Fig.7.2), which is more consistent with their evolution from different precursors (see section 1.4.2). The a posteriori conclusion is that the independent evolution of the eucaryotic enzyme endowed this pathway with an increased efficiency not only because of the improved kinetic properties of the lactonizing enzyme (section 7.3) but also in result of the reduction in the number of enzymes required for the conversion of 3-carboxy-cis,cis-muconate to 3-oxoadipate.

7.6. Perspectives for Further Studies

As noted by Ornston (1982) many of the evolutionary problems related to the 3-oxoadipate pathway remain to be answered, particularly those which more closely concern the possible relationship between the procaryotic and eucaryotic pathways or between the eucaryotic catechol and protocatechuate branches. The chemical transformations of the catechol branch are identical in bacteria and fungi and



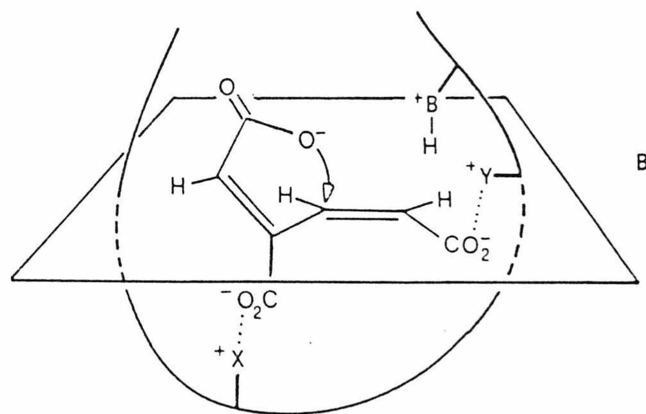


Fig.7.2. Possible conformations of 3-carboxy-*cis*, *cis*-muconate at the active sites of the bacterial (A) and fungal (B) lactonizing enzymes. The proton-donor groups are represented by BH^{+} and the binding residues by X^{+} and Y^{+}.

there is, therefore, the possibility of enzymatic homology, despite the observed differences in the regulatory mechanisms (Cain, 1980). This aspect has been neglected, so far, except for some preliminary investigations on the catechol dioxygenase from A. niger and cis,cis-muconate cyclase from Trichosporon cutaneum (section 1.3.2). The yeast and bacterial muconate lactonizing enzymes (section 1.4.2) have significantly different properties, reflected in the length of their polypeptide chains and in the catalytic residues but any conclusion will depend on a direct comparison of amino acid sequences and on the mechanism and stereochemistry of the eucaryotic reaction. The same data would equally be useful in a comparison of the muconate and carboxymuconate cyclases which are known to share a common requirement for reduced thiol groups.

The independent origin of the bacterial and fungal protocatechuate pathways, postulated on the grounds of the different courses of the chemical transformation of 3-carboxymuconate to 3-oxoadipate appears to be confirmed by the available evidence relating the two types of lactonizing enzymes (section 7.5). The procaryotic and eucaryotic routes share, however, isofunctional dioxygenases and transferases which eucaryotes could have inherited from their procaryotic precursors. The investigation of the fungal protocatechuate dioxygenase appears to be particularly relevant since the complete sequences of both subunits of the P. aeruginosa enzyme have been determined (Kohlmiller & Howard, 1979; Iwaki, Kagamiyama & Nozaki, 1979) as well as structural details of its active site

(section 1.4.3). Some available data (Cain, 1980) indicate that the A. niger and Rhodotorula mucilaginosa dioxygenases are much smaller molecules than their bacterial counterpart but a possible homology is not excluded because the association properties of protein subunits are known to be substantially affected by relatively minor modifications in the primary structure.

The relatedeness of the bacterial and fungal transferases is also possible since the 3-oxoacid CoA-transferase (EC. 2.8.3.5) acting with succinyl-CoA as co-substrate is an enzyme of broad specificity (Dixon & Webb, 1979). Their convergent evolution, however, would equally be conceivable because the formation of 3-oxoadipate appears to be the most direct metabolic route for the dissimilation of 3-carboxymuconate.

A final point deserving further investigation is that of the relationship between 3-carboxymuconate cycloisomerase and 3-carboxymuconolactone hydrolase. 3-Carboxymuconolactone is a stable metabolite and the enzymatic activity required for its conversion to 3-oxoadipate is therefore a necessary component of the pathway. This reaction has been investigated by Hill Kirby & Robins (1977) which have shown, using deuterium-labelled substrate, that an allylic isomerization of the substrate must occur at some stage of the catalytic mechanism, in result of a 1,3-suprafacial hydrogen shift (Fig.7.3). Another conceivable step in the same reaction is the formation of an intermediate thioester, considering that its hydrolysis would be an energetically favoured reaction and that the enzyme activity depends on a reduced thiol

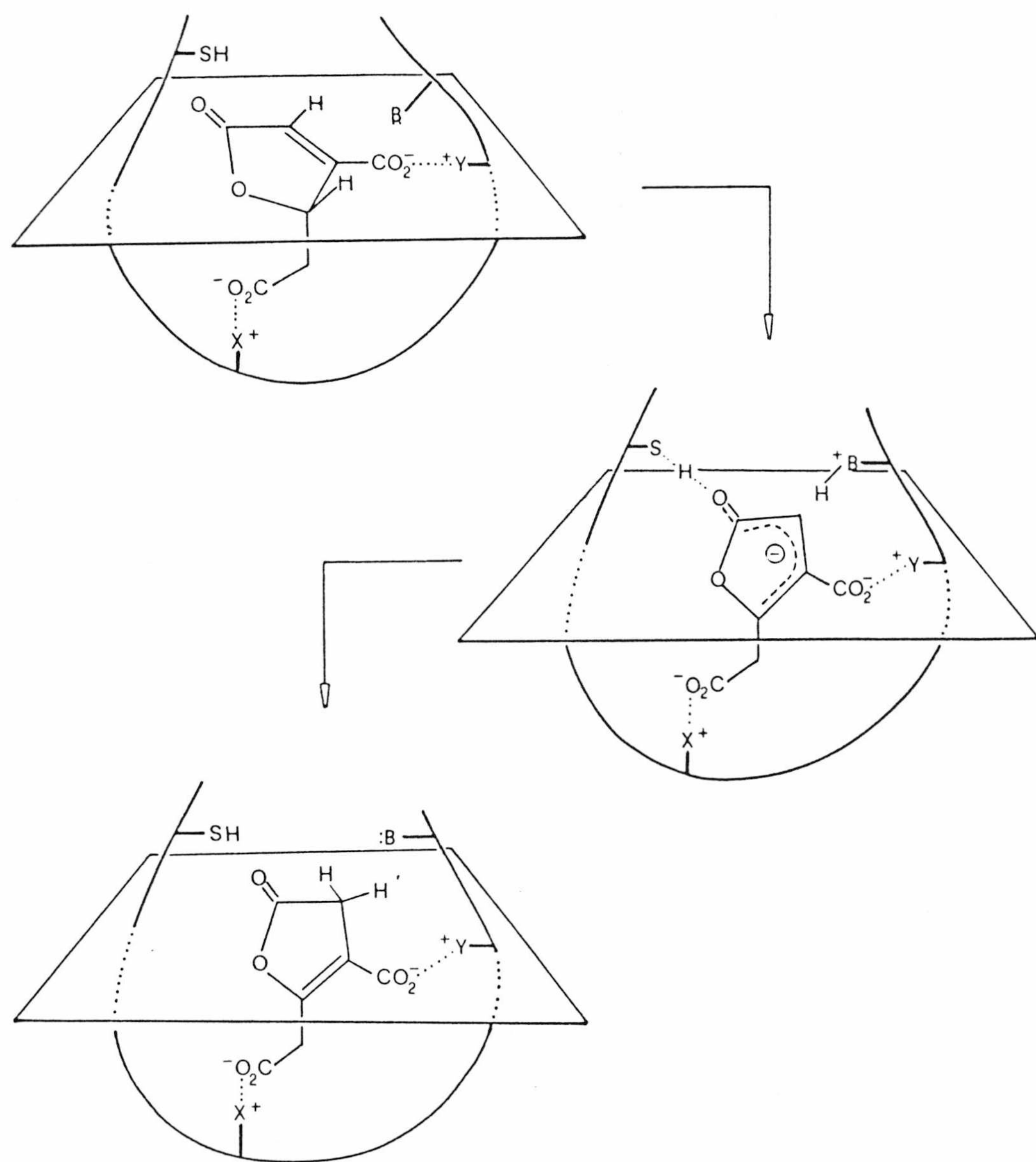


Fig.7.3. Allylic isomerization of 3-carboxymuconolactone as a possible step in its conversion to 3-oxoadipate.

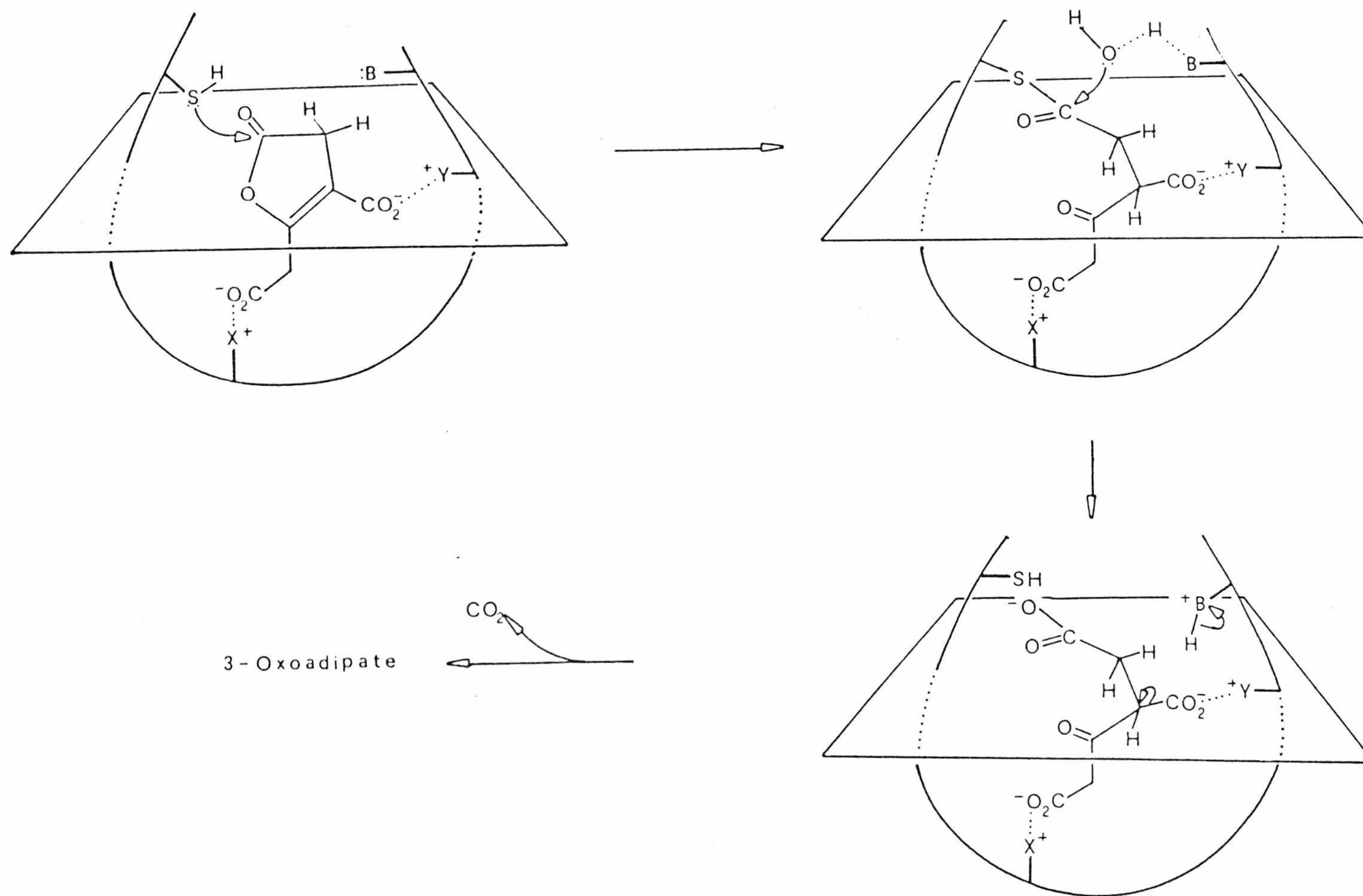


Fig.7.4. A possible mechanism for the formation of a thioester, followed by hydrolysis and decarboxylation, in the conversion of 3-carboxymuconolactone to 3-oxoadipate (see Fig.7.3).

group (Thatcher, 1972). If this step follows the isomerization of the lactone, the covalently bound enol intermediate could spontaneously rearrange to the corresponding oxo form; decarboxylation and thioester hydrolysis, either spontaneous or enzyme-mediated, would then follow (Fig.7.4).

The essential catalytic features of the enzyme mediating this type of reaction do not appear very different from those of the lactonizing enzyme, including the binding site (see Fig.7.2), a basic residue which, in the protonated form, acts as proton-donor in the conversion of 3-carboxymuconate to 3-carboxymuconolactone and the reduced thiol (Thatcher & Cain, 1974b).

This evidence and the observation that the synthesis of the two enzymes is coordinately induced in all fungi examined so far (Cain, 1980) are consistent with their evolution from a common precursor. Important clues for an answer to this problem could be provided by a more extensive investigation of the essential residues in the two enzymes and by the isolation of active site peptides, where amino acid conservation or replacements would more easily be interpreted.

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