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THE PRODUCTION, PURIFICATION AND CATALYTIC UTILITY
OF LIGNIN PEROXIDASE FROM SPOROTRICHUM PULVERULENTUM.

by

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This thesis is presented for the Degree of Doctor of
Philosophy, in the Department of Biological Sciences,
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DEDICATION

TO MY MOTHER AND FATHER.

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Lastly, I would like to thank the S.E.R.C. Biotechnology Directorate for funding the project.

DECLARATION

The work contained in this thesis was the result of original work conducted by myself under the supervision of Professor H. Dalton. All sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

STEPHEN ALEXANDER CHAMBERS.

SUMMARY.

Production, purification and catalytic utility of lignin peroxidase (LiP) from Sporotrichum pulverulentum.

The study of LiP has been hampered by the difficulty in producing this enzyme in sufficient quantities. Several strains of Phanerochaete chrysosporium and Sporotrichum pulverulentum were screened for LiP expression under different culture conditions to find a method of producing adequate supplies of the enzyme for the proceeding work in this thesis. A reliable method of LiP production was achieved using 750ml agitated cultures of S.pulverulentum containing the detergent tween 80.

LiP from S.pulverulentum was purified by HPLC and was found to consist of up to 14 isozymes which varied in molecular weight, pH optimum and specific activity for veratryl alcohol. However, their catalytic spectra were similar. The isozymes from S.pulverulentum had higher molecular weights and lower pI values than those published for LiP from P.chrysosporium, which suggested that they were not as closely related as had been assumed.

LiP from S.pulverulentum was able to oxidise a range of methoxy-substituted benzyl alcohols to their respective aldehydes. The susceptibility of benzyl alcohol oxidation by LiP depended upon the amount and position of methoxyl group substitution. LiP oxidation of these substrates was dependent upon how electron-rich the molecular π -orbitals of the substrates were, but steric effects may also have been important. LiP oxidation of benzyl alcohols under aerobic conditions led to additional products such as quinones, ring-cleavage products and chloro-substituted aromatics. These latter products provided evidence for the existence of LiP-derived aryl radical cations for a range of benzyl alcohol substrates, which is consistent with the peroxidative one-electron oxidation theory of LiP degradation of lignin.

In addition LiP was shown to catalyse the peroxidative one-electron oxidation of phenolics such as p-cresol and catechol to produce dimers and polymers.

Lyophilised LiP was shown to be catalytically active in organic solvents such as ether and propyl acetate. An increase in enzyme stability of up to 30 times of that in water and a broadening of its catalytic spectrum was observed.

LiP was also found in C.versicolor demonstrating that LiP may be a common constituent of ligninolytic white-rot fungi. In addition, other extracellular peroxidases were present in this fungus. These peroxidases were novel compared to the extracellular peroxidases from P.chrysosporium since at least one of these could not oxidise veratryl alcohol and neither of these peroxidases were manganese-dependent.

ABBREVIATIONS.

A	Absorbance.
APPL	Acid-precipitable polyphenolic polymeric lignin
β -1	diaryl alkane
β -O-4	beta-guaiacyl ether
bp	Base pairs
$^{\circ}$ C	Degrees Celsius
CBQase	Cellobiose:quinone oxidoreductase
CVP	<u>Coriolus versicolor</u> Peroxidase
DDT	1,1,1,-trichloro-2-2-bis (paarchlorophenyl)ethane
DEAE-	Diethylaminoethyl-
DHP	Dehydropolymerisate
DMHB	Dimethoxyhydrobenzoin
DNA	Deoxyribose nucleic acid
ESR	Electron spin resonance
FAD	Flavin adenine dinucleotide
GC	Gas chromatography
GC/MS	Gas chromatogram / Mass spectroscope
H ₂ O ₂	Hydrogen peroxide
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
hrs	Hours
IEF	Isoelectric focusing
Kd	Kilodalton
Km	Michaelis constant

KTBA	2-Keto-4-thiomethyl butyric acid
L	Litres
LiP	Lignin peroxidase
Log P	The logarithm of the partition coefficient in an octanol/water 2-phase system
M	Molar
mins	minutes
mg	milligrams
MnP	Manganese-dependent peroxidase
MWL	Milled wood lignin
NAD(H)	Nicotinamide adenine dinucleotide (reduced form)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced form)
O ₂	Oxygen
PAGE	Polyacrylamide gel electrophoresis
PCB	Polychlorinated biphenyls
pI	Isoelectric point
PPL	Porcine pancreatic lipase
rpm	Revolutions per minute
U	Units of activity
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume

C H A P T E R 1.

1. INTRODUCTION

1.1. The physiological and chemical properties of lignin.

Next to cellulose, lignin is the most abundant renewable carbon source on earth. As much as 40% of the carbon content of higher plants consists of lignin. It forms a recalcitrant barrier around much of the plants' cellulose and hemi-cellulose. The degradation of lignin is thus of primary importance in biological carbon recycling.

Lignin is present in ferns and other higher plants, but it is absent from liverworts, mosses and algae. It serves to maintain the structural integrity of the plant by providing mechanical strength. The water permeation-reducing property of lignin plays an important role in the internal transport of water, nutrients and metabolites in the plant by preventing leakage. Also, lignification is a common response to injury and infection. Such lignified tissue is associated with an increased resistance to infection.

Lignin is an amorphous, water-insoluble three-

dimensional aromatic polymer formed from the cinnamyl alcohol precursors; p-sinapyl (I), p-coniferyl (II) and p-coumaryl (III) alcohols (fig 1.1) which differ only in their degree of ring methoxylation. Their relative proportions are species and tissue specific.

Gymnosperm lignin is made from coniferyl alcohol and angiosperm lignin is made from mixtures of coniferyl and sinapyl alcohol. Both types of lignin contain small amounts (less than 10%) of units derived from coumaryl alcohol.

Lignin is formed by the oxidative polymerisation of the cinnamyl alcohol precursors by peroxidase-mediated single electron oxidation of the p-hydroxyl groups. The resultant radicals, which exist in a number of mesomeric forms, couple randomly with each other in the growing polymer lattice to form a heterogenous phenylpropanoid structure.

Fig 1.2 shows the schematic structural formula for lignin. Certain intralignin linkages predominate, particularly the arylglycerol- β -aryl ether (β -O-4) linkage, which consists of over 50% of the interphenylpropanoid bonds. There are, in addition, at least ten inter-unit carbon-carbon and aryl ether bonds. There is also evidence for covalent linkages between lignin and hemi-cellulose (based on reviews by; Brauns & Brauns, 1960; Adler, 1977; Eriksson et.al., 1980; Kirk and Farrell, 1987).

The very complexity of lignin presents a major

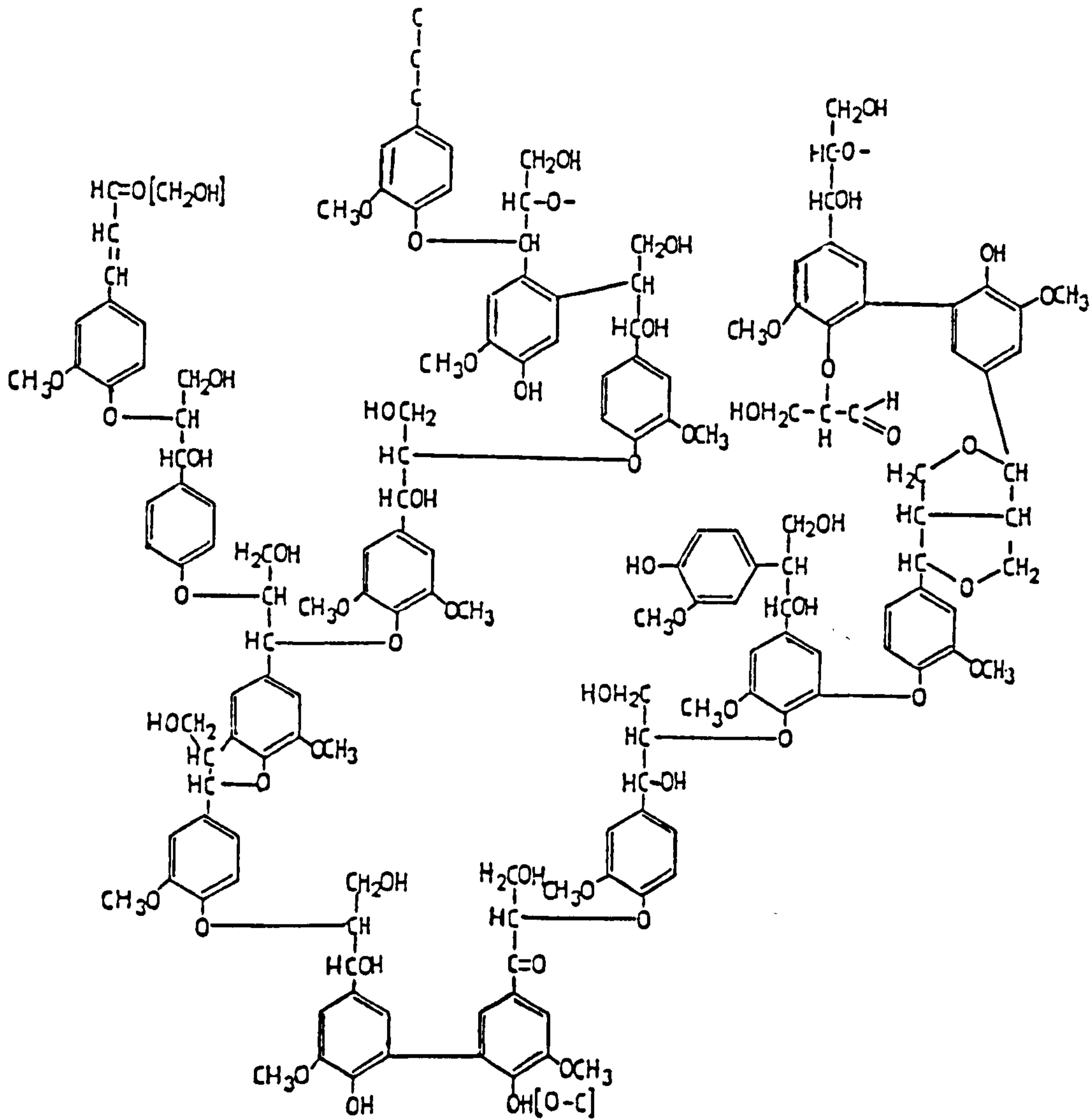


Figure 1.1. Prominent structures in softwood lignin (Adler, 1977).

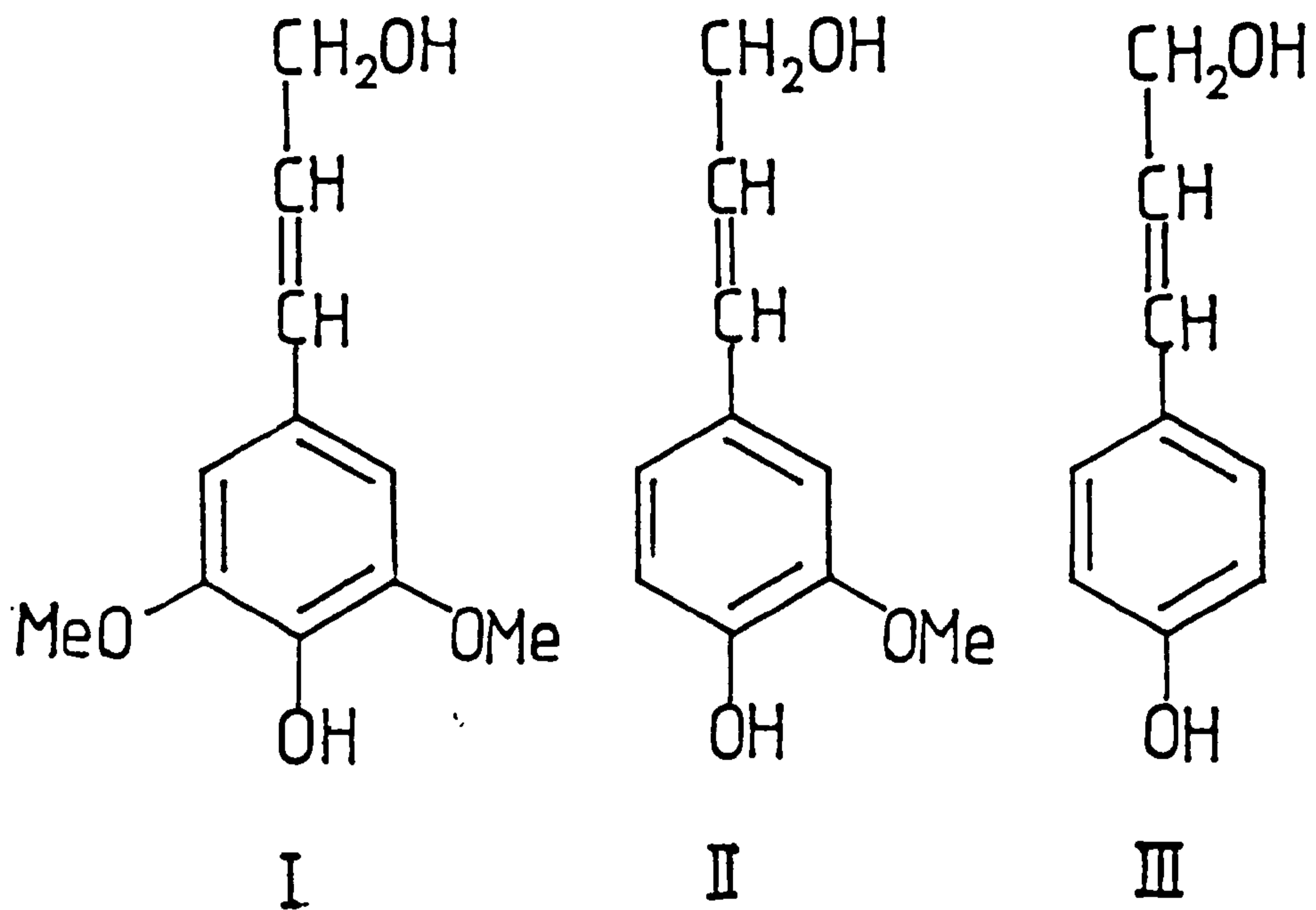


Figure 1.2. Cinnamyl alcohol precursors of lignin (Adler, 1977): sinapyl alcohol (I), coniferyl alcohol (II) and p-coumaryl alcohol (III).

challenge to biodegrading organisms faced with its' degradation not previously seen in other polymer degrading systems such as those for starch, cellulose, and proteins. These latter polymers are principally two-dimensional covalent structures consisting of regular repeatable units that are easily cleaved by hydrolysis. The very size, randomness and three-dimensional nature of the lignin structure necessitates the use of an extra-cellular, non-hydrolytic system which is either unusually non-specific, or it consists of a particularly complex array of enzymes.

1.2. Methodology used in the study of lignin biodegradation.

In order to study lignin biodegradation, the problems in obtaining purified, unmodified lignin preparations and the development of a valid assay system must be overcome.

1.2.1. Lignin as a microbial substrate.

Lignin is physically associated with cellulose and hemi-cellulose within plants. Extraction of the lignin to obtain pure samples can be achieved by physical (milling) and chemical methods. However, such harsh

treatments can result in modifications to the abstracted lignin such as alteration of structure or reduced molecular weight. It may also contain impurities such as sugars and sulphur-containing substances (for further details of extracted lignin preparations and their characteristics, see Buswell & Odier, 1987).

Not only do the different extraction techniques affect the resultant lignin preparations in different ways, but the sources of lignocellulose are often diverse and therefore heterogenous with respect to the chemical structure of lignin and its physical association with other plant materials. This lack of standardisation must be taken into account, particularly when comparing the degradative capacity of different organisms by different authors.

1.2.2. Assay procedures used for the measurement of lignin biodegradation.

To determine the nature and extent of lignin biodegradation, it is first necessary to develop suitable assay procedures. Several assay systems have been utilised, depending upon whether screening, rate, or nature of lignin degradation is under investigation.

If particular labelled precursors (e.g. ^{14}C -phenylalanine or ^{14}C -cinnamic acid) are added to growing plants, they are primarily incorporated into

lignin structures. The extracted ^{14}C -lignin can then be incubated with the organism of interest. The release of $^{14}\text{CO}_2$ or other ^{14}C -fragments demonstrates the specific degradation of ^{14}C -lignin (Haider and Trojanowski, 1975; Crawford et.al., 1977). The extracted ^{14}C -lignin however must be free of ^{14}C low molecular weight contaminants and ^{14}C -protein.

An alternative substrate is dehydropolymerisate (DHP)-lignin (Haider and Trojanowski, 1975; Kirk et.al., 1975) produced by horseradish peroxidase (HRP)-catalysed oxidation of cinnamyl alcohols. This is a useful substrate because the cinnamyl alcohol precursors can be labelled on either the ring, carbon chain or the methoxy groups, thus allowing an insight into the method of degradation, as well as the rate. However, the synthetic nature of this polymer does cast some doubt on its relevance to lignin degradation.

Coloured dyes such as Remazol brilliant blue R have been used, particularly for the rapid colorimetric screening of possible ligninolytic organisms. Although these aromatic compounds are structurally different from lignin compounds their colour loss is often closely correlated with lignin degradation (Glenn & Gold, 1983; Ulmer et.al., 1984).

Dimeric lignin model compounds such as β -O-4 aryl ether, or β -1 diarylpropane structures (Adler et.al., 1952) have been particularly useful for the

determination of the actual mechanisms of ligninolysis (see 1.8.2.).

1.3. Bacterial degradation of Lignin.

1.3.1. Lignin mineralisation by Actinomycetes.

Actinomycetes have the same filamentous nature as fungi and are often found in association with rotting wood. To date, they are the most well-studied bacteria implicated in lignin degradation.

The actinomycete Norcadia autotrophica can release $^{14}\text{CO}_2$ from ^{14}C -DHP. After 15 days, 14.1% of methoxyl groups, 9.5% of propyl side chains and 7.6% of aromatic ring label was released (Haider et.al., 1978).

Norcadia DSM 1069 was also found to oxidise lignin and structural elements characteristic of lignin (Trojanowski et.al., 1977).

Antai & Crawford (1981) found that Streptomyces viridosporus and Streptomyces setonii caused hard and soft wood lignin losses of about one third over a period of twelve weeks. In the same period, weight loss of grass lignin was 44% and 39% respectively. Crawford et.al. (1982) showed that S.viridosporus attacked lignin in a similar fashion to white-rot, causing demethylation, ring cleavage and oxidative attack upon phenylpropanoid side-chains. McCarthy &

Broda (1984) noted that in contrast to white-rot fungi, $^{14}\text{CO}_2$ evolution from ^{14}C -lignin occurred during the primary growth phase and it was not inhibited by agitation.

Of all the Streptomyces that have been found to oxidise ^{14}C -lignin; Streptomyces, Micromonospora, Thermomonospora, Saccharomonospora, Norcadia and Rhodococcus, the highest $^{14}\text{CO}_2$ evolution (generated by Thermomonospora fusca) was 8% in 14 days (McCarthy & Broda, 1984). This is still very low compared to the white rot basidiomycete Sporotrichum pulverulentum which released 50% of ^{14}C as $^{14}\text{CO}_2$ in 15 days (McCarthy et.al., 1984) using the same substrate of ^{14}C -lignin wheat lignocellulose.

1.3.2. Lignin solubilisation by Actinomycetes.

Crawford et.al. (1983) found that S.viridosporus could modify lignins to produce acid-precipitable polyphenolic polymeric lignin (APPL). APPL, a water soluble polymer, was the main degradative constituent (>30%). Pometto & Crawford (1986) discovered that lignin mineralisation in S.viridosporus was optimal at pH 6.5, but the optimum for APPL formation was at pH 8.5. The precise structure of APPL and the reason for its formation was not clear. However, Pometto & Crawford (1986) suggested that lignin solubilisation by

actinomycetes may play an important role in the metabolism of lignin in neutral and alkaline soils where ligninolytic fungi are not highly competitive.

Recently, Mason et.al. (1988) have partially characterized an enzyme responsible for ^{14}C -lignin solubilisation in Streptomyces cyaneus. It was an extracellular enzyme with an apparent molecular weight of 20kd and it is thought unlikely to be a cellulase or a xylanase.

Indirect evidence for the role of extracellular peroxidases, esterases and endoglucanases in lignin degradation by S.viridosporus was found by Ramachandra et.al. (1987) who found that an APPL-overproducing strain of S.viridosporus T7A had higher levels of these enzymes than the wild-type. Subsequently, Donnelly & Crawford (1988) found that extracellular esterase activity, consisting of seven esterase-active proteins, from S.viridosporus was capable of releasing p-coumaric acid and vanillic acid from lignocellulose. However, this esterase activity may preferentially hydrolyse aromatic ester bonds within hemi-cellulose rather than lignin, since MWL and APPL were poor substrates for the enzymes. These esterases may therefore only have a peripheral role in lignin degradation.

The most promising evidence for the direct role of extracellular enzymes from Streptomyces in lignin degradation has been reported by Ramachandra et.al.

(1988). They discovered a heme-containing 'ligninase' which was capable of cleaving Ca-C β bonds in lignin model compounds. This enzyme contrasts with the Lignin peroxidase (LiP) from the white-rot fungi P.chryso sporium (see 1.7.) in that it is expressed during primary metabolism (see 1.6.4.) and it has a molecular weight of only 17.8 Kd which is less than half the size of LiP (see 1.7.1.).

1.3.3. Lignin degradation by Eubacteria.

Eubacteria have also been implicated in lignin degradation. Odier et.al. (1981) found that several Gram-negative bacteria (Pseudomonas, Xanthomonas and Acinebacter) could degrade mild acidolysis lignin (lignin extracted from lignocellulose by dioxane in the presence of HCl) and poplar milled wood lignin (MWL). Kerr et.al. (1983) found a bacterium (tentatively identified as an Arthrobacter) capable of mineralising 2.9% of ¹⁴C-lignin-labelled lignocellulose from cord-grass in 10 days, as well as utilising peanut hull lignin preparations as a sole carbon source. Kern (1984) found a Xanthomonas species that could utilise ¹⁴C-DHP-lignin as a sole carbon source. They detected ¹⁴C in the form of ¹⁴CO₂ and as ¹⁴C-biomass indicating that this species was able to fully utilise at least

some of the DHP-lignin.

Pseudomonas species have been shown to degrade dimeric lignin models such as arylglycerol β -aryl ether (Vicuna et.al., 1987) and biphenyls (Katayama et.al., 1988). Bacterial consortia have utilised dimeric (Pellinen et.al., 1984) and tetrameric lignin model compounds (Jokela et.al., 1985; Jokela et.al., 1987) as sole carbon sources. These bacteria may have an important role in the degradation of low molecular weight lignin breakdown products, but substrate size limitation prevents them from effectively degrading polymeric lignin, presumably due to the lack of an extracellular degradative system.

1.4. Yeast degradation of lignin.

Glanser et.al. (1986) demonstrated a two-step biodegradation of lignin by a mixed culture of yeasts in which Pachysolen tannophilus fermented xylose, in cornstover hydrolysate, to ethanol which was then utilised by Trichosporon fermentans. The growth of T.fermentans resulted in depolymerisation of lignin. Although 50% of the lignin in the hydrolysate was mineralised to CO₂, the average molecular weight of the original lignin preparation was only 200-600d. Lignin degradation by yeasts may well be limited in a similar fashion to that of many of the eubacteria.

1.5. Fungal degradation of lignin.

1.5.1. White-rot fungi.

White-rot fungi consist mainly of basidiomycetes. They can degrade all three components of wood (cellulose, hemi-cellulose and lignin) rapidly. In addition, many are able to fully metabolise lignin to CO₂ given the right conditions.

Investigations using scanning electron microscopy have shown that certain white-rot fungi can selectively remove lignin from wood (Blanchette, 1980; Otjen & Blanchette, 1982; Blanchette, 1984a; Blanchette, 1984b; Otjen & Blanchette, 1985; Blanchette & Reid, 1986).

Blanchette & Reid (1986) found that during selective degradation of aspen and birchwood by Phlebia tremellosus, the cell wall was degraded from the lumen inwards towards the middle lamella. The degradation appeared to be spatially distinct from the fungal hyphae, suggesting that extracellular enzymes may be involved. Once the secondary wall layers were altered the middle lamella was destroyed between the cells, leaving just the cell corner regions persisting. In conjunction with these ultrastructural observations, they found a 70% lignin loss compared to only a 30% wood weight loss after 12 weeks. Reid (1985) also demonstrated the degradative selectivity of Merulius

tremellosus on aspen wood. They found a 52% loss in lignin content compared with only a 12% loss in wood weight in 8 weeks.

Blanchette (1984b) indicated that hemi-cellulose was removed in addition to lignin during selective degradation. Kirk & Highly (1973) also demonstrated that at least one of the wood polysaccharides must be concomitantly degraded with lignin.

During simultaneous-rot (degradation of all three major wood components) the ultrastructural changes were different from those during selective degradation of lignin and hemi-cellulose. Degradation of the cell wall was localised around the immediate area surrounding the fungus. All white-rot fungi that cause selective degradation can probably also cause simultaneous-rot. Where both types of degradation occur at the same time, but are spatially distinct from one another, this is classified as 'white mottled rot' (Otjen & Blanchette, 1986).

Yang et.al. (1980) found that lignin degradation by the white rot Phanerochaete chrysosporium was not necessarily slow compared to its ability to degrade cellulose. They measured a rate of 2.9mg lignin degraded per mg of fungal protein per day, which they crudely estimated to be approximately 25% of the rate of cellulose degradation, given optimum conditions. Ulmer et.al. (1983) demonstrated the rapidity of white-

rot degradation of various lignins to small molecular weight fragments (>1kd). They found that 1g per litre of P.chryso sporium mycelia degraded 90-100% of lignin (1g per litre) in 2-3 days.

1.5.2. Brown-rot fungi.

Brown-rot fungi are basidiomycetes that mainly degrade wood polysaccharides, but also partially decompose lignin to produce a brown residue. In a comparison of the white rot Phanerochaete chryso sporium to the brown-rot Gleophyllum trabeum, the percentage loss of wood dry weight is similar (approx 15% in 600hrs). However, the $^{14}\text{CO}_2$ liberated from side chain-, aromatic ring- and methoxyl-labelled groups was four times higher in cultures of P.chryso sporium compared to G.trabeum (Kirk et.al., 1975). The major effect of brown-rot on lignin appeared to be the demethylation of aryl methoxyl groups (Kirk & Adler, 1970). Side chain carbon was poorly oxidised and aromatic carbon was almost untouched. Brown-rot fungi would appear not to have the full ligninolytic system possessed by the closely related white-rot basidiomycetes.

1.5.3. Soft-rot fungi.

A number of ascomycetes and fungi imperfecti termed 'soft-rot fungi' are known to cause appreciable carbohydrate degradation of water-soaked wood. Haider & Trojanowski (1975) found that four soft-rot species had a comparable capacity to that of white-rot in the degradation of labelled phenol monomers. They were also found to release $^{14}\text{CO}_2$ from specifically labelled ^{14}C -coniferyl alcohol DHP (up to 7.8% in 15 days). It was concluded that soft-rot fungi could degrade methoxyl groups, depolymerise side chain α -linkages and even degrade ring structures. Although conditions have not been optimised as extensively as the white-rot fungi, it would appear that soft-rot degradation is much slower and it may not be as complete as that undergone by the white-rot fungi.

1.6. Physiology of lignin degradation by Phanerochaete chrysosporium.

1.6.1. Phanerochaete chrysosporium, the model system.

Over the last decade the white-rot basidiomycete P.chrysosporium has been chosen as a model for lignin degradation. It is easy to grow, it conidiates well and it rapidly and extensively degrades lignin. As a result the following description of the nutritional and physiological factors involved in ligninolysis are primarily based upon this fungus, but parallels with other organisms are chosen where appropriate.

1.6.2. Requirement for growth substrate.

Kirk et.al. (1976) found that the growth of P.chrysosporium and Coriolus versicolor on ^{14}C -labelled synthetic lignin was negligible unless a co-substrate such as cellulose or glucose was added. The rate and extent of $^{14}\text{CO}_2$ evolution depended on the amount of co-substrate present (see fig 1.3.). $^{14}\text{CO}_2$ evolution stopped upon exhaustion of the co-substrate. Reid (1979) and Ulmer et.al. (1983) found similar results with P.chrysosporium. Leatham (1986) found that

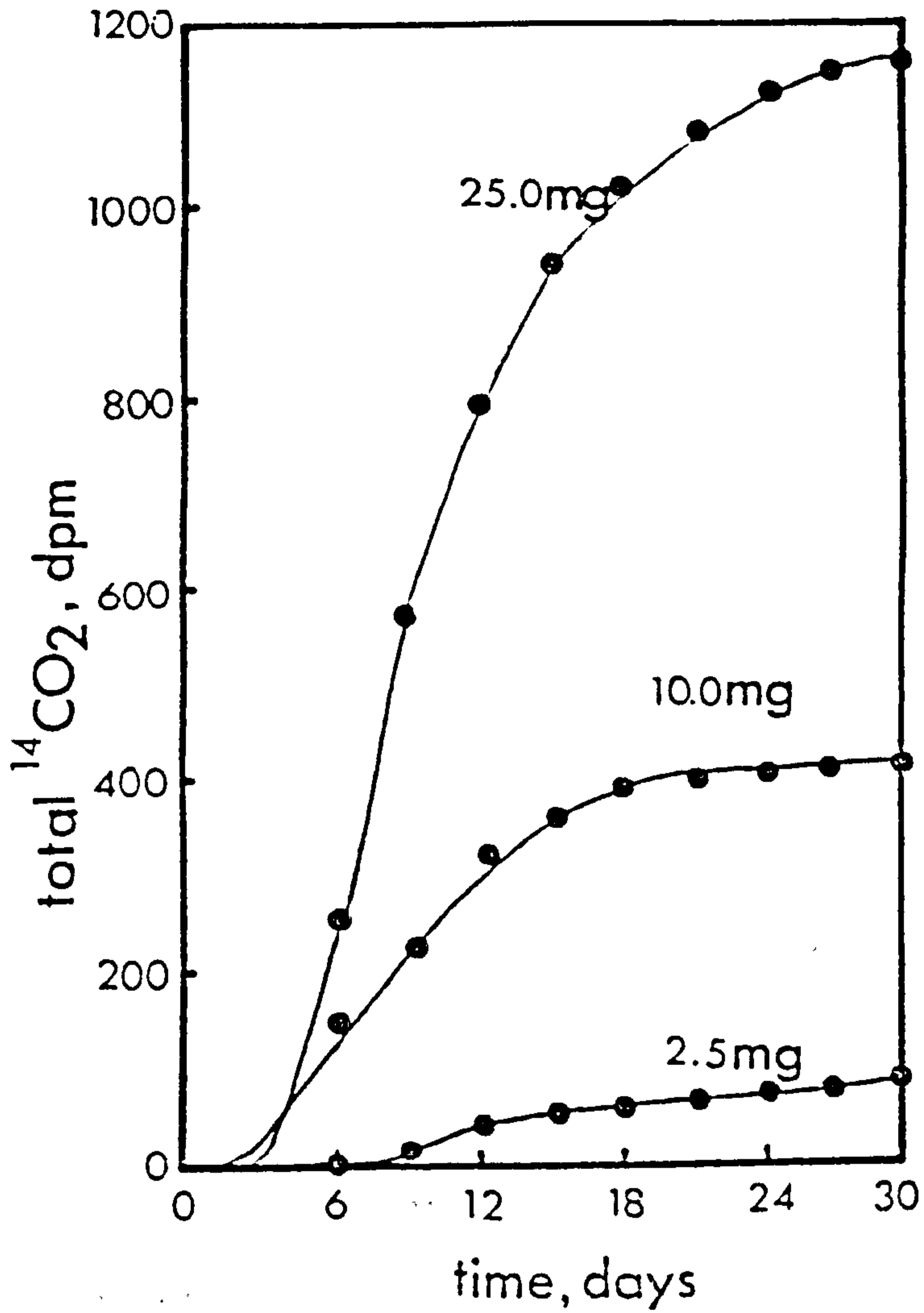


Figure 1.3. Effect of cellulose addition (mg) on rate of ^{14}C -lignin degradation by *P.chrysosporium* (Kirk et.al., 1976).

Lentinus edodes also required an exogenous carbon source.

1.6.3. Effect of Oxygen

Ligninolysis appears to be very limited, if present at all in anaerobic environments. Zeikus et.al. (1982) and Colberg & Young (1985) showed that only low molecular weight lignins were decomposed in anoxic sediments. Holt & Jones (1983) found only limited erosion of Scots pine blocks in anaerobic muds after 18 months. Odier & Monties (1983) found no conversion of ^{14}C -lignin to $^{14}\text{CO}_2$ or $^{14}\text{CH}_4$ after 6 months in anaerobic conditions. Contrary to most reports, some slow anaerobic degradation of ^{14}C -lignin was noted by Benner and Hodson (1985) at 55°C using a thermophilic enrichment culture. However, it is possible that this degradation may mainly be attributable to the degradation of radiolabelled non-lignin components such as protein.

It is clear therefore, that oxygen is a mandatory requirement for effective lignin degradation. Interestingly, elevated levels of oxygen above ambient appear to enhance lignin degradation by wood-rotting fungi (Kirk et.al., 1978; Bar-lev & Kirk, 1981; Reid & Seifert, 1982). For instance, Kirk et.al., (1978) found that P.chrysosporium would not degrade lignin in

a 5% oxygen atmosphere, but found a 2-3 fold stimulation at 100% oxygen, compared to air (20%, see fig 1.4.). They found a similar effect with C.versicolor and suggested that this may be a general effect for white-rot fungi.

Bar-lev & Kirk (1981) showed that increasing the oxygen tension in cultures of P.chrysosporium had a dual effect. Firstly, preceding the appearance of the ligninolytic system, oxygen levels determined the amount of ligninolytic activity that developed. Secondly, once the system had developed, oxygen tension affected the rate of mineralisation. However, whether this was a direct effect upon the lignin-degrading system or upon the generation of required components was not clear.

As well as lignin degradation as a whole, a number of individual aspects of the lignin-degrading system are enhanced by oxygen. H_2O_2 , which is required for the ligninolytic enzyme lignin peroxidase (LiP, see 1.7) was enhanced by high oxygen titres (Faison & Kirk, 1985). Lignin peroxidase itself was enhanced by high oxygen titres (Faison & Kirk, 1985). In addition, Shimada et.al. (1981) showed that an increase in oxygen levels enhanced the synthesis of veratryl alcohol, a secondary metabolite of P.chrysosporium which has been considered to positively affect the LiP system in a number of ways (see 1.6.9.)

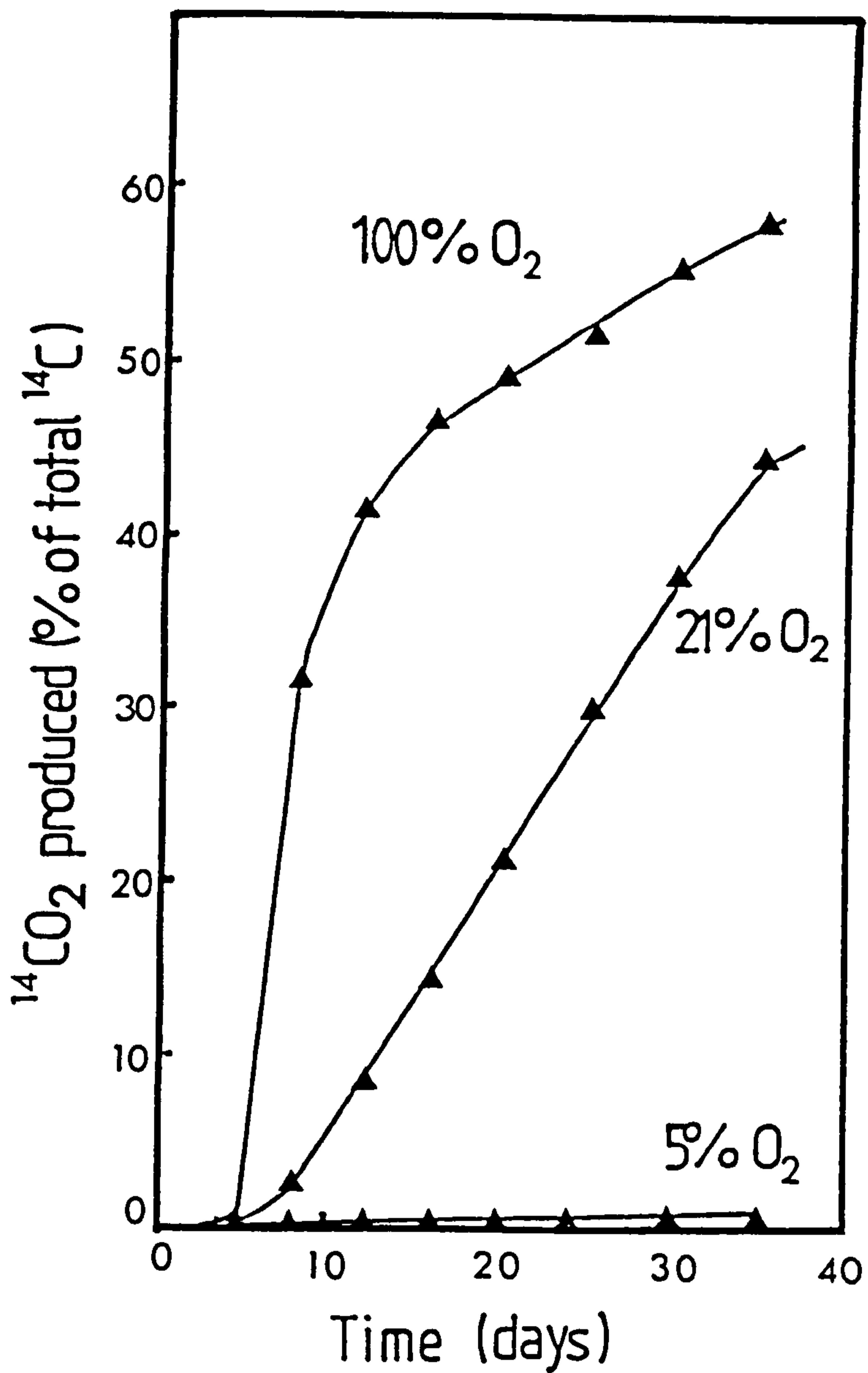


Figure 1.4. Effect of O_2 concentration on the production of $^{14}\text{CO}_2$ from ^{14}C -lignin by P.chrysosporium cultured at 39°C without shaking (Kirk et.al., 1978).

1.6.4. Effect of Nitrogen.

Lignin degradation by P.chrysosporium is essentially an idiophasic event which can be triggered by limitation of nitrogen (Kirk et.al., 1978; Keyser et.al., 1978, fig 1.5; Reid, 1983a; Reid, 1983b), carbon or sulphur (Kirk et.al., 1978), but not phosphorus (Jeffries et.al., 1981).

Regulation of ligninolysis in nature may be controlled in part by nitrogen limitation, since wood is naturally low in nitrogen (Cowling & Merrill, 1966). However, Leatham & Kirk (1983) showed that nitrogen limitation was not necessarily a universal factor in lignin degradation by white-rot basidiomycetes. Phlebia brevispora, Coriolus versicolor and Pholiota mutabilis were also effected by nitrogen levels, but there was some limited ligninolytic activity under nitrogen excess. Pleurotus ostreatus was not inhibited at all by high nitrogen levels whereas lignin degradation by Lentinus edodes was in fact stimulated by high levels of nitrogen. In the case of bacterial degradation, Barder & Crawford (1981) showed that lignin degradation by Streptomyces was actually enhanced by high nitrogen concentrations.

During the transition between nitrogen depletion and lignin degradation, the intracellular amino acid pool (particularly glutamate) in P.chrysosporium increases then rapidly drops. The addition of

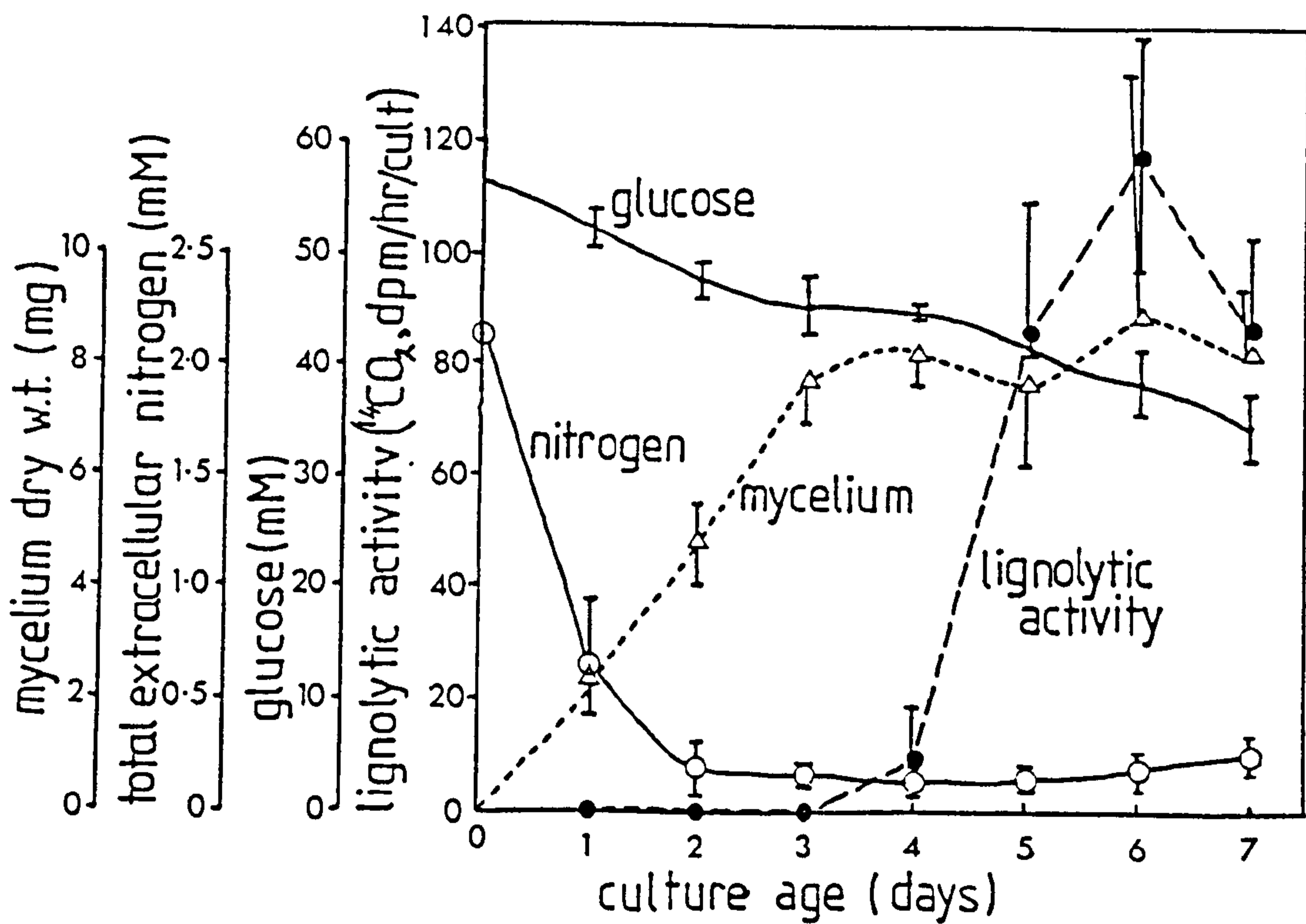


Figure 1.5. Relationship between nitrogen levels and other culture parameters with ligninolytic activity in P.chrysosporium over a 7 day growth period (Keyser et.al., 1978).

nitrogen, especially in the form of glutamine, glutamate or histidine suppressed secondary metabolic events such as veratryl alcohol production, lignin degradation (Fenn & Kirk, 1981) and LiP production (Faison & Kirk, 1985). Nitrogen suppression of the key ligninolytic enzyme LiP (see 1.7) could be responsible for suppression of lignin degradation.

Under nitrogen-limiting conditions (i.e. actively ligninolytic), high levels of the NADP-dependent glutamate dehydrogenase (a biosynthetic enzyme) were present, coupled with low levels of NAD-dependent glutamate dehydrogenase (a degradative enzyme). This further supports the suggestion by Fenn & Kirk (1981) that glutamate metabolism has an important role in the control of lignin degradation.

1.6.5. Agitation.

Early investigations showed that agitation strongly suppressed the degradation of synthetic DHP-lignin (kirk et.al., 1978) and reduced by up to 50% the degradation of dimeric lignin models (Weinstein et.al., 1980) to CO₂. Culture agitation also inhibited the synthesis of veratryl alcohol (Shimada et.al., 1981) and the appearance of LiP (Faison & Kirk, 1985). The suppression of the ligninolytic system was considered to be due to low oxygen tension within mycelial pellets

(formed under agitation) compared to mycelial mats (formed under stationary conditions) due to restricted diffusion.

The problem of ligninolysis suppression was overcome by two strategies. Firstly, strains were isolated that could degrade lignin, independently of agitation effects (Reid et.al., 1985; Gold et.al., 1984b). Secondly, the addition of certain reagents to cultures were found to overcome suppression. For example, Jager et.al. (1985) found lignin degradation and relatively high LiP titres in agitated cultures supplemented with non-ionic surfactants. Also Leisola and Fiechter (1985) found LiP activity under agitation when either veratryl alcohol or veratrylaldehyde was added to four-day old cultures. The physiological basis for overriding suppression of ligninolysis by these reagents is however, not understood.

1.6.6. pH.

The pH value for optimum lignin degradation by P.chrysosporium is critical. Little or no mineralisation occurred below pH 3.5 or above pH 5.5. Maximal activity occurred at pH 4.5 (Kirk et.al., 1978) which is in contrast to that observed in actinomycetes where mineralisation of lignin occurred at neutral and alkaline pH values.

1.6.7. Trace nutrients.

Thiamine is the only vitamin required for growth, and lignin degradation in P.chrysosporium (Kirk et.al., 1978).

Jeffries et.al. (1981) measured the effect of varying the concentration of divalent metal ions (zinc, molybdenum, iron, calcium, magnesium and manganese) on lignin degradation. Only manganese exerted a significant effect. At 10 times basal concentration (i.e. 30 μ M), manganese strongly inhibited lignin degradation, but not growth. Elevated levels of calcium or magnesium countered the inhibitory effect by the manganese. This contradicts Weinberg (1977) who, in his summary of the powerful and often crucial influences of trace metals on secondary metabolism, found that zinc and iron are usually the most influential divalent metal ions.

1.6.8. Veratryl alcohol as a function of secondary metabolism.

Veratryl alcohol (3,4,dimethoxybenyl alcohol) is produced de novo from glucose in cultures of P.chrysosporium (Lundquist & Kirk, 1978). It is synthesised via phenylalanine, 3,4-dimethoxycinnamyl alcohol and veratryl glycerol (fig 1.6, Shimada et.al.,

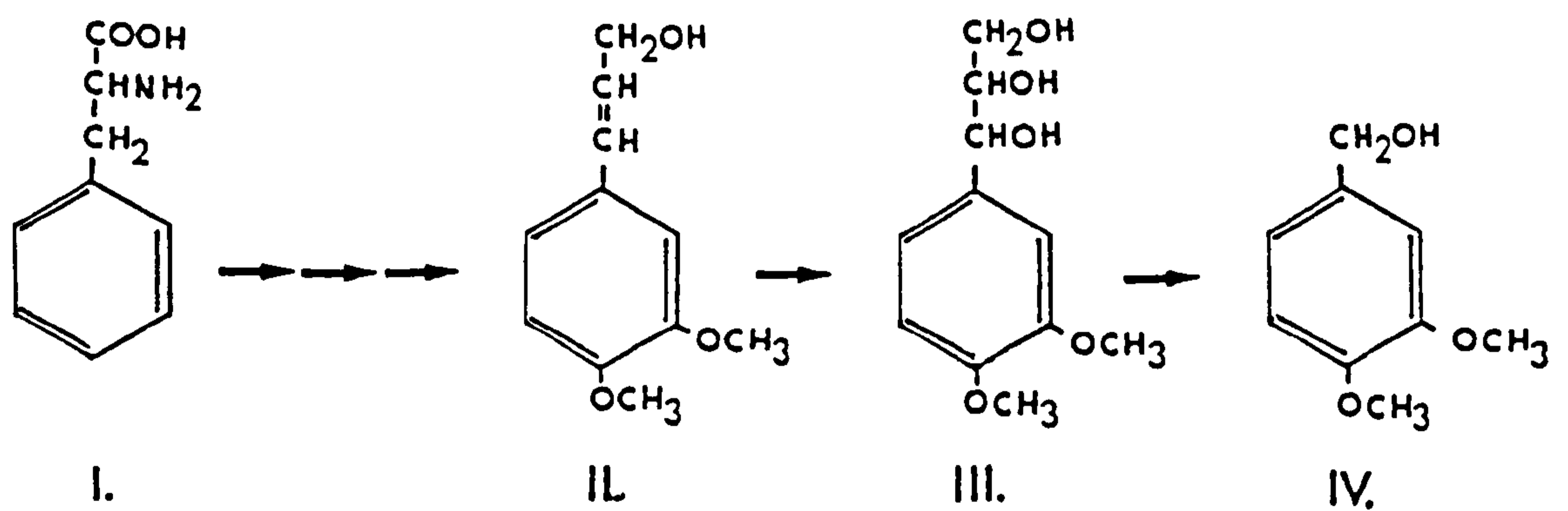


Figure 1.6. Conversion of phenylalanine (I) to veratryl alcohol (IV), via 3,4-dimethoxycinnamyl alcohol (II) and veratrylglycerol (III) in 6-day old cultures of P.chrysosporium (Shimada et.al., 1981).

1981). Production of veratryl alcohol occurs during the transition between primary and secondary metabolism in nitrogen-limited (Keyser et.al., 1978) and carbon-limited cultures (Jeffries et.al., 1981).

The link between secondary metabolism and veratryl alcohol production was further substantiated by Gold et.al. (1982) who found a mutant of P.chrysosporium that was unable to degrade lignin was also unable to produce veratryl alcohol. The whole biosynthetic pathway for veratryl alcohol production appeared to be switched off since the three intermediate reactions were not expressed. Liwicki et.al. (1985) also found that some (but not all) mutants of P.chrysosporium incapable of lignin degradation were also incapable of veratryl alcohol synthesis.

1.6.9. The role of veratryl alcohol in lignin degradation.

The presence of the secondary metabolite veratryl alcohol appears to enhance the lignin degrading system in a number of ways. Faison & Kirk (1985) found that the addition of veratryl alcohol to idiophasic cultures of P.chrysosporium produced a 4.5 fold stimulation of LiP titre (table 1.1). It also allowed a significant enhancement in H₂O₂ production and lignin oxidation.

Faison et.al. (1986) suggested that veratryl

Culture addition	LiP activity	H ₂ O ₂ production
Lignins		
Birch	4.0	1.4
Spruce	3.1	NA
Coniferyl (synthetic)	2.2	NA
Lignin dimers		
β -O-4 (CaHOH)	5.2	0.8
β -O-4 (Ca=O)	2.6	1.4
β -1	0.4	1.4
Lignin monomers		
Syringyl alcohol	1.5	NA
Vanillyl alcohol	1.1	NA
4-hydroxybenzyl alcohol	0.2	NA
Anisyl alcohol	0.9	NA
3,4,5-trimethoxybenzyl alcohol	0.9	NA
Fungal metabolites		
Veratryl alcohol	4.5	1.4
Veratraldehyde	3.7	1.0
Veratrylglycerol	3.0	1.1

Table 1.1. Effect of lignin-related compounds on ligninolytic activities (Faison et.al., 1986).

alcohol acted as an inducer rather than a stabiliser for LiP since the normal rapid decrease in LiP titre was not affected in any way by the addition of the alcohol. However, Tonon & Odier (1988) found that veratryl alcohol protected one of the LiP isozymes from H₂O₂ inactivation when using a 10 fold concentration (2mM) of veratryl alcohol above that used by Faison et.al. (1986). Recently, Wariishi & Gold (1989) have substantiated the claim of Tonon & Odier that veratryl alcohol acts to protect the enzyme (see 1.8.8.).

In vitro studies have shown that the presence of veratryl alcohol enhanced LiP oxidation of aromatic substances. Harvey et.al. (1986) suggested that veratryl alcohol works as a redox mediator (see 1.8.8.).

1.6.10. Induction of the ligninolytic system.

Initially, it was reported that ligninolytic activity in nitrogen-limited cultures of P.chrysosporium appeared in the absence of lignin and that addition of lignin neither affected the titre of expression nor the rate of ligninolysis (Keyser et.al., 1978). However, Ulmer et.al. (1984) found an enhanced rate of Remazol brilliant blue R decolourisation and lignin degradation by pre-incubating cultures of P.chrysosporium with lignin for 48hrs. Duran et.al.

(1987) noticed a similar effect on dye decolourisation by the addition of lignin to cultures of the white rot Chrysonila sitophila.

Individual components of the ligninolytic system have also been affected by lignin and lignin-related compounds. H₂O₂ production has been enhanced by the addition of lignocellulosic materials (Greene & Gould, 1983). Faison & Kirk (1985) found that elevated ligninase, and in some cases H₂O₂ titres, occurred when cultures of P.chrysosporium were spiked with lignin, lignin model dimers, monomers and fungal metabolites (table 1.1).

Lignin itself is an unlikely candidate as an inducer/enhancer because of its size and insolubility. However, soluble low molecular weight lignin-derived compounds may effect ligninolysis. For instance, veratryl alcohol, which was reported to have the highest inducible effect on LiP titre by Faison & Kirk (1985, Table 1.1.) may be produced indirectly from lignin biodegradation by P.chrysosporium.

In nitrogen-starved cultures of P.chrysosporium, the role of lignin and lignin-related compounds have been shown to have a significant effect on enhancing ligninolysis and individual components of the degradative system. However, no de novo induction has been reported in nitrogen-excess cultures, suggesting that lignin degradation is primarily a secondary metabolic event rather than a directly inducible event.

1.7. Lignin peroxidase (LiP).

1.7.1. Discovery and physical properties of LiP.

Evidence for the direct involvement of enzymes in ligninolysis was demonstrated by Tien & Kirk (1983) and Glenn et.al. (1983). Both groups discovered a H₂O₂-requiring enzyme in the supernatant of nitrogen-limited cultures of P.chrysosporium that was able to degrade lignin substructure model compounds and partially depolymerise spruce and birch lignins.

Further characterisation of the purified lignin peroxidase was carried out by Tien & Kirk (1984) and Gold et.al. (1984b). The enzyme was a glycoprotein (13% carbohydrate by weight) which contained one protoheme IX per molecule, a molecular weight of 42Kd and a pI value of 3.5. The purified enzyme exhibited absorption maxima at 409nm and 502nm which shifted to 420nm and 544nm respectively upon oxidation by H₂O₂.

The enzyme has been found to be made up of a number of similar isozymes (see 4.1). The major LiP isozyme has recently been crystallised by Troller et.al. (1988) into orthorhombic brown crystals with a space group of P2,2,2, and four LiP molecules per cell.

Recently, LiP genes have been cloned and sequenced

(Zhang et.al., 1986; Tien & Tu, 1987a,b; Smith et.al., 1988; Asada et.al., 1988). Cloning and sequencing of these genes is the first step in obtaining a greater understanding of lignin degradation at the molecular level. In addition, expression of the cloned gene(s) in suitable hosts may allow economic large-scale production of LiP for commercial use (see 1.10).

Asada et.al. (1988) cloned an LiP gene from a P.chrysosporium genomic DNA library containing 1116bp of protein-encoding sequence, of which 84bp encoded the signal peptide. The sequence was interrupted by eight introns and putative regulatory sequences of the 'CAAT' and 'TATA' box-type were present in the 5' flanking region.

1.7.2. Measurement of LiP activity.

LiP activity has been assayed by H₂O₂-dependent generation of ethylene from 2-keto-4-thiomethyl butyric acid (KTBA, Glenn et.al., 1983) and the quantitation of ¹⁴C veratraldehyde produced on the cleavage of 1,2-bis-(3-methoxy-4-[¹⁴C] methoxyphenyl) propane-1,3-diol (Tien & Kirk, 1984). However, the H₂O₂-dependent oxidation of veratryl alcohol to veratraldehyde at pH 3 and 37°C, measured spectrophotometrically at 310nm (Tien & Kirk, 1984; fig 1.7.) has been adopted as the standard assay system for LiP because of its simplicity. It requires up to 5 minutes per assay as

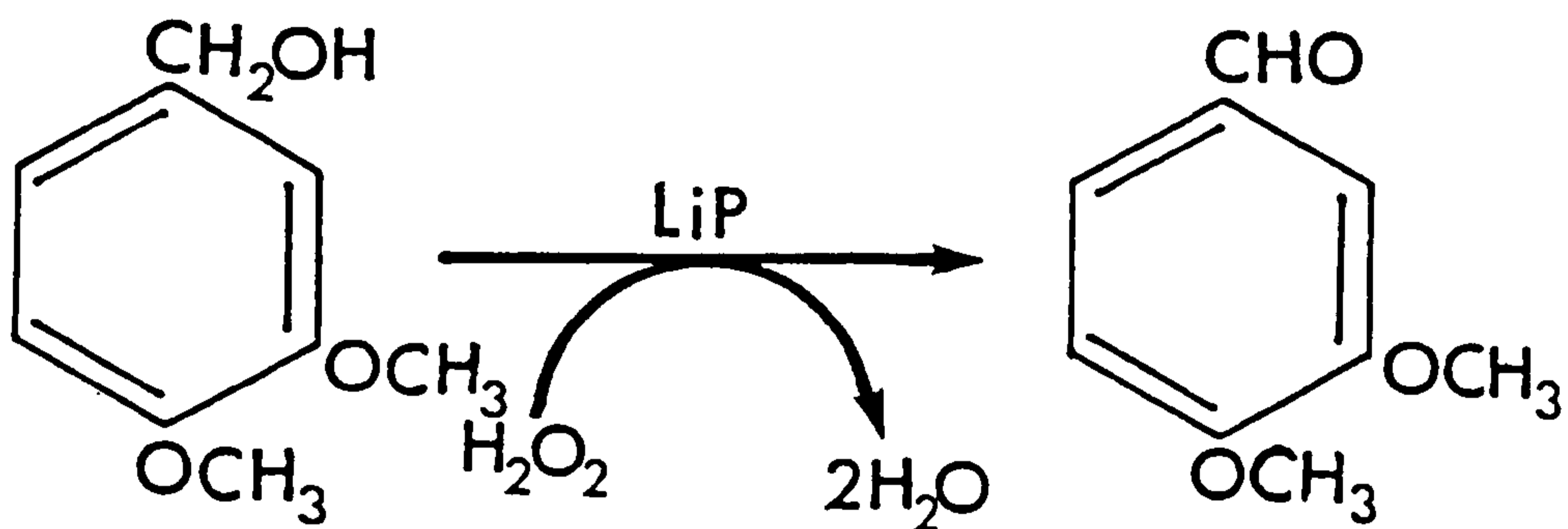


Figure 1.7. H_2O_2 -dependent oxidation of veratryl alcohol to veratraldehyde by LiP (Tien & Kirk, 1984).

opposed to over 1 hour for the method of Glenn et.al. (1983) and it doesnot require the long and costly synthesis of ^{14}C -labelled substrate (Tien & Kirk, 1984).

1.7.3. LiP from other fungi.

LiP activity as measured by H_2O_2 -dependent veratryl alcohol oxidation has recently been observed in cultures of other white-rot fungi, namely: Chrysonilia sitophila (Duran et.al., 1987); Coriolus versicolor (Dodson et.al., 1987; Waldner et.al., 1988); Trametes versicolor (Jonsson et.al., 1987); Chrysosporium pruinoseum (Waldner et.al., 1988) and Phlebia radiata (Kantelinen et.al., 1988).

LiP has been purified and partially characterised from Trametes versicolor (Jonsson et.al., 1987); Coriolus versicolor (Dodson et.al., 1987) and Phlebia radiata (Kantelinen et.al., 1988). These LiPs were similar in size to LiP from P.chrysosporium (43-45Kd) except for that from C.versicolor which was slightly larger (50Kd). All three LiPs were present as isozymes. In addition, Kantelinen et.al. (1988) found that LiP from P.chrysosporium and LiP from Phlebia radiata cross-reacted with each others antibodies. LiP from C.pruinoseum and C.versicolor also reacted with antibodies against LiP from P.chrysosporium (Waldner

et.al., 1988). These results suggest therefore, that there is a strong similarity between the LiP enzymes from different white-rot fungi.

However, it is also evident that white-rot fungi undergoing ligninolysis do not necessarily require or even possess LiP. Leatham et.al. (1986) failed to detect H₂O₂-dependent veratryl alcohol oxidation in ligninolytic cultures of Lentinus edodes in either a wood, or concentrated liquid medium despite the ability of L.edodes to convert synthetic ¹⁴C-lignin to ¹⁴CO₂ at about one-third of the rate of P.chryso sporium. In addition, Waldner et.al. (1988) found that ligninolytic cultures (¹⁴C-lignin → ¹⁴CO₂) of Pleurotus ostreatus, Bjerkandera adusta, Fomes lignosus and Trametes cingulata did not possess LiP as assayed by veratryl alcohol oxidation either.

Leatham et.al. (1986) suggested that L.edodes either possessed very low titres of LiP or its ligninase complement had differing substrate specificities. Regardless of the reason for the lack of veratryl alcohol oxidation, the work by Leatham et.al. (1986) and Waldner et.al. (1988) illustrate that there are limitations in the veratryl alcohol assay as a general assay for ligninolytic enzymes since other ligninolytic mechanisms must exist in these fungi.

1.8. Catalysis by LiP.

1.8.1. Reactions catalysed by LiP.

Ca-C β bonds that are present in β -O-4 and β -1 model compounds are representative of approximately 50% and 7% respectively of the intralignin linkages (see 1.1). The cleavage of these bonds is necessary if any extensive degradation of lignin were to take place. Before the discovery of LiP, nitrogen limited static cultures of P.chrysosporium had been shown to cleave Ca-C β bonds in β -1-diarylpropane/ethane compounds (Gold et.al., 1984a and Enoki & Gold, 1982) and β -O-4-aryl ether compounds (Goldsby et.al., 1980; Enoki et.al., 1980; Enoki et.al., 1981a; Enoki et.al., 1981b; Umezawa & Higuchi, 1985a). More recently, aromatic ring cleavage of β -O-4 compounds has been demonstrated in cultures of P.chrysosporium (Umezawa & Higuchi, 1985b).

These key reactions of lignin biodegradation have subsequently been shown to be part of the catalytic repertoire of LiP. Tien & Kirk (1983) were the first to find that crude LiP, in the presence of H₂O₂, could cleave Ca-C β bonds in β -1 and β -O-4 structures (fig 1.8.). The β -1 model compound 1,2-bis-(3,4-dimethoxyphenyl)-propane-1,3-diol (I) was cleaved to form vanillin methyl ether (II) and 1-(3',4'-dimethoxyphenyl)ethane-1,2-diol (III). The diol product was further oxidised to form the aldehyde (II)

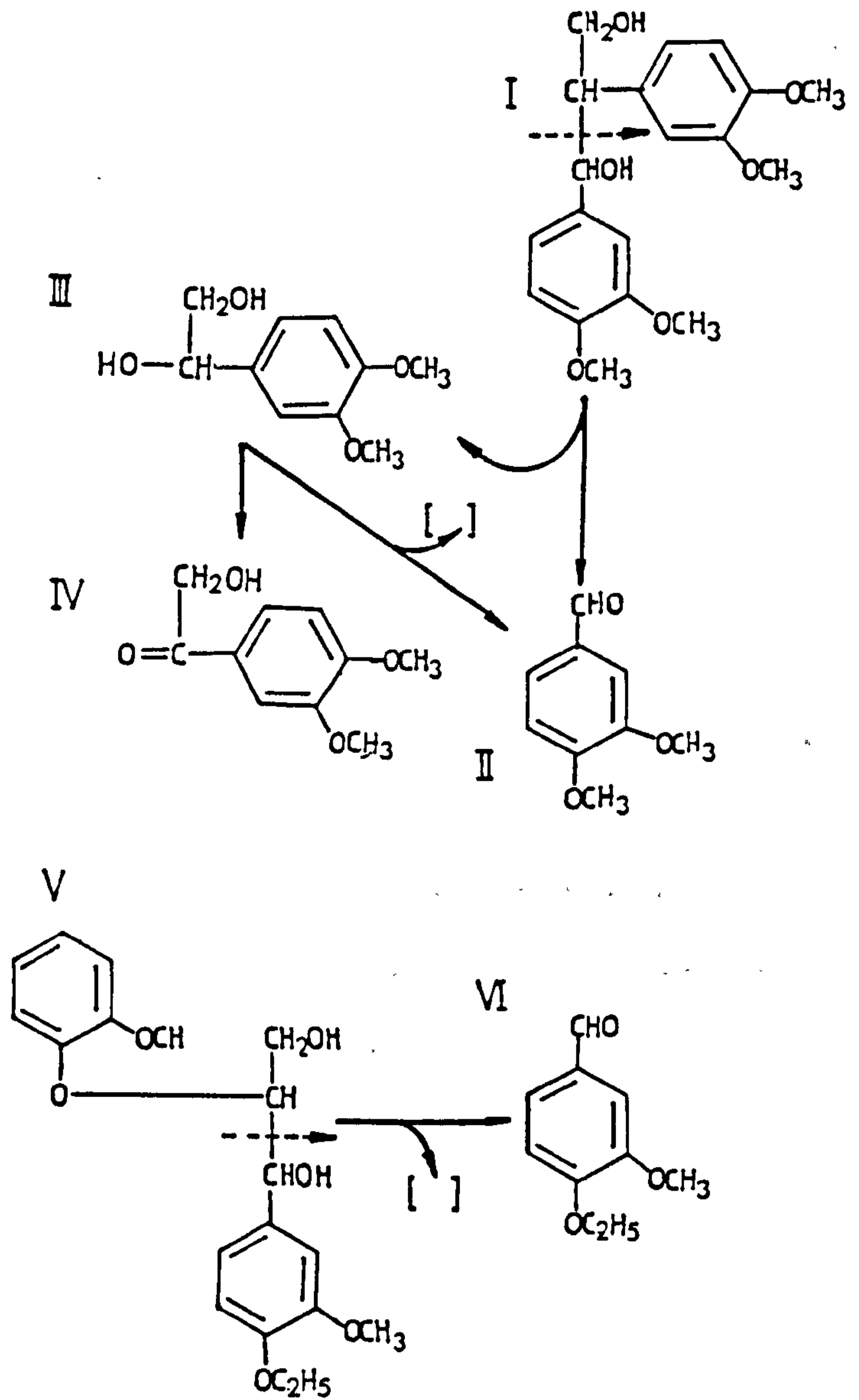


Figure 1.8. H_2O_2 -dependent Ca-C β cleavage of β -1 (I) and β -O-4 (V) model compounds by LiP (Tien & Kirk, 1983).

and the ketol (IV). The β -O-4 model 1-(4-ethoxy-3-methoxyphenyl)-2-(o-methoxyphenoxy)propane-1,3-diol (V) differs from (I) in having an aryl ether substituent at C-2. Like (I) this compound was cleaved between Ca and C β , with the formation of an aromatic aldehyde (in this case, vanillin ethyl ether, VI).

Subsequently, the demonstration of Ca-C β cleavage of diarylpropane and arylglycerol- β -aryl ether model compounds by LiP has been well documented (Glenn et.al., 1983; Tien & Kirk, 1984; Gold et.al., 1984b; Hammel et.al., 1985; Kirk et.al., 1986; Miki et.al., 1986b).

In addition to Ca-C β cleavage, LiP was found to catalyse a number of apparently disparate reactions (reviewed in table 1.2.). However, from the relatively non-specific nature of LiP catalysis, a unifying theory based on radical chemistry began to emerge.

1.8.2. The Mechanism of LiP action.

Several groups proposed a similar general mechanism to explain the products of enzymatic lignin degradation (Harvey et.al., 1985; Schoemaker et.al., 1985; Kersten et.al., 1985; Hammel et.al., 1985; Renganathan et.al., 1986). Fig 1.9. shows the example given by Schoemaker et.al. (1985) as exemplified below:

The heme group of LiP is first oxidised by H₂O₂ to

Cleavage of Ca-C β bonds in lignin-model dimers
(1,2,3,4,5,6,7,).

Intradiol cleavage in phenylglycols (2,3).

Oxidation of benzyl alcohols to aldehydes or ketones (2).

Oxidation of methoxybenzenes and benzyl alcohols to quinones
(13,17).

Oxidation of o-arylpropane models to quinones and
alcohols (8).

Aromatic ring cleavage of β -O-4 models and veratryl
alcohol (9,10,11,13,15,16).

Rearrangements in β -aryl ether monomers (12).

Hydroxylation of Ca=C β olefinic bonds in styryl
structures (2,3,4)

Hydroxylation of benzylic methylene groups (2)

oxidation of phenols (2)

Oxidation of pollutants (see 1.10.2.2.)

Table 1.2. Reactions catalysed by LiP. (1)Tien & Kirk(1983); (2)Tien & Kirk(1984); (3)Gold et.al.(1984); (4)Glenn et.al.(1983); (5)Hammel et.al.(1986); (6)Kirk et.al.(1986); (7)Miki et.al.(1986b); (8)Huynh et.al.(1986); (9)Umezawa et.al.(1986); (10)Miki et.al.(1987); (11)Umezawa & Higuchi (1987); (12)Miki et.al.(1986a); (13)Haemmerli et.al.(1987); (14)Umezawa & Higuchi(1986); (15)Shimada et.al.(1987); (16)Leisola et.al.(1985); (17)Kersten et.al.(1985).

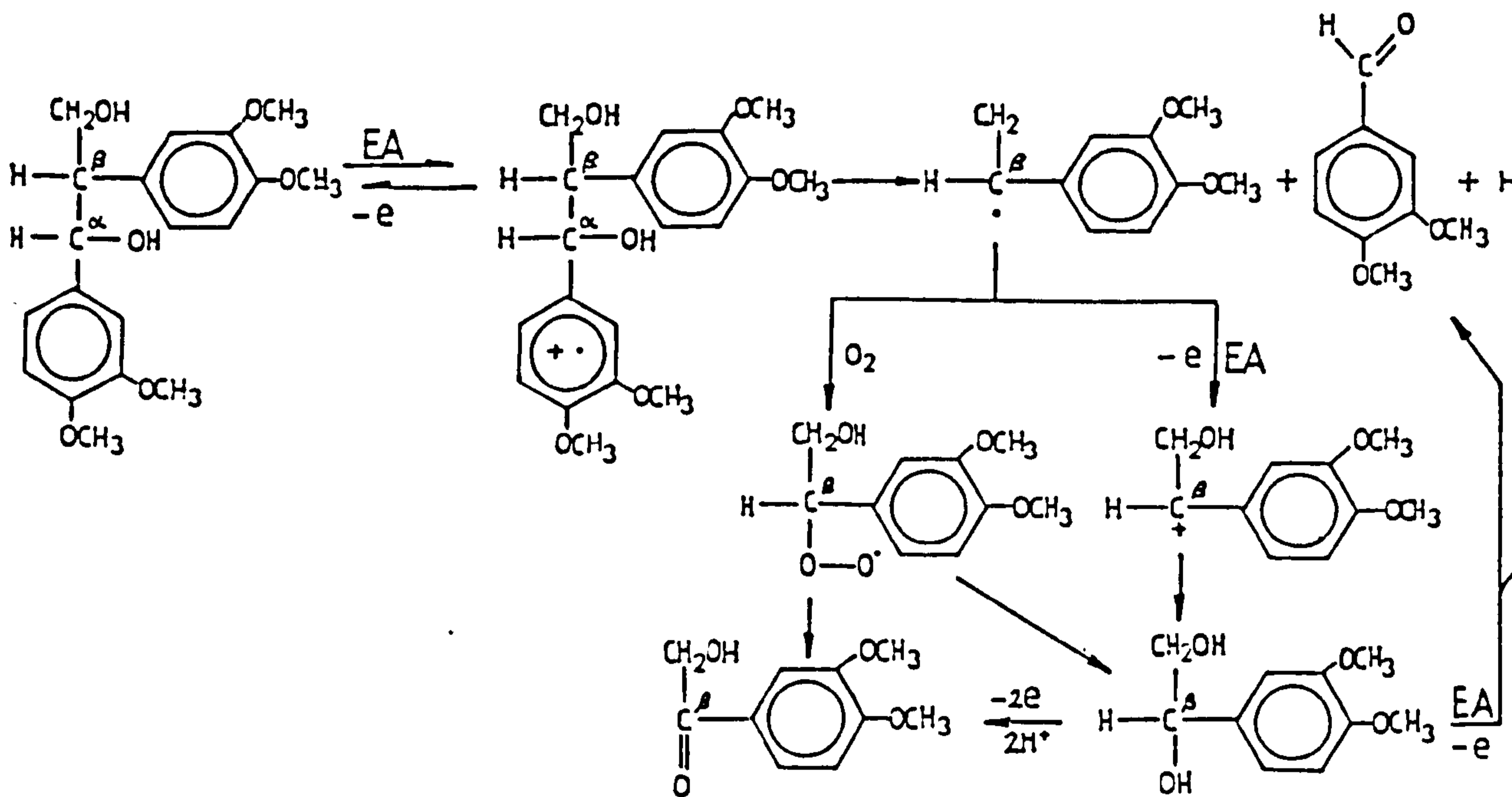


Figure 1.9. The proposed mechanism for LiP-mediated one-electron oxidation of model lignin compounds via radical cation intermediates (EA = electron acceptor, e.g. LiP; Schoemaker et.al., 1985).

produce compound I (Fe-IVO[•]) which, in turn, can oxidise the substrate (in this example, a diarylpropane compound) by initial one electron transfer to give a substrate cation radical and LiP compound II (Fe-IV)^{••}. The unstable radical cation intermediate undergoes C α -C β bond cleavage yielding two fragments, a cation which then deprotonates, and a radical. The radical may be subjected to two possible fates:

- i) It can undergo further one electron oxidation (either by LiP compound II or a second LiP compound I) to yield a cation, which upon hydration forms a glycol. The glycol is itself a substrate for one electron oxidation by LiP and in a similar fashion to the original diarylpropane substrate, the resultant radical cation may either undergo C α -C β cleavage or deprotonate to yield a ketol.
- ii) Dioxygen may attack the carbon centered radical to form a peroxy radical, which, after disproportionation, gives rise to either the ketol or the glycol.

In summary, an initial peroxidative one electron oxidation of a susceptible substrate by LiP produces unstable aryl radical cations which undergo a variety of abiotic reactions, leading to a wide array of products.

Certain techniques have been employed to provide evidence for this general theory:

1.8.3. Biomimetic studies.

The use of simple inorganic complexes to mimic enzyme-catalysed reactions can be useful in determining the mechanism of enzymatic action. Biomimetic studies have been used to implicate the mechanism of one electron oxidation in LiP-catalysed lignin degradation. One electron oxidants such as tetraphenylporphyrinatoiron(III) chloride (Shimada et.al., 1984); ceric ammonium sulphate; tris(phenanthroline) iron(III) (Shoemaker et.al., 1985) and copper peroxydisulfate (Huynh, 1986) have been shown to cause Ca-C β cleavage in lignin model dimers, giving rise to a product spectrum similar to that obtained when using LiP and H₂O₂. In addition, ceric ammonium nitrate has been used to oxidise veratryl alcohol to veratraldehyde, two quinones and two ring cleavage lactones. These are essentially the same products as derived from LiP oxidation of veratryl alcohol.

1.8.4. Spectral studies.

Peroxidases mediate their reactions by the oxidation of the heme group with H_2O_2 to create a high redox potential oxyferryl centre ($Fe(IV)=O$) which can then extract electrons from the substrate via two single electron oxidation steps. Spectral studies have detected the formation of an oxyferryl centre in oxidised LiP (Renganathan & Gold, 1986; Harvey et.al., 1985; Kuila et.al., 1985). Transient-state kinetics revealed spectral changes in the Soret region, which by analogy to horseradish peroxidase (HRP), were consistent with compounds I and II (the two-electron and one-electron oxidised forms respectively, Tien et.al., 1986; Andrawis et.al., 1988) adding weight to the evidence that LiP was capable of peroxidative one-electron oxidation.

1.8.5. The demonstration of cation radical formation using lignin model compounds.

Demonstration of the formation of LiP-catalysed cation radicals in the substrate was important because, not only would it demonstrate that an electron had been removed from the substrate but it would show that Ca-C β cleavage fragments were a direct result of LiP oxidation.

Camaioni & Franz (1984) showed that one electron oxidation of 1,2-diarylethanes and 1-phenyl-2-arylethanes by peroxydisulphate produced radical cations which fragmented either by benzylic proton loss or C α -C β cleavage.

Kersten et.al. (1985) were the first group to demonstrate that LiP was capable of generating aryl cation radicals. They were detected by ESR spectroscopy during the LiP-catalysed oxidation of methoxybenzenes to quinones and methanol (fig 1.10.). Voltametric generation of radical cations from methoxybenzenes led to the formation of the same products.

Subsequent work concentrated on the detection of cation radicals in lignin model dimer compounds catalysed by LiP. For example, Kirk et.al. (1986) detected a cation radical by ESR spectroscopy during LiP oxidation of the β -O-4 model lignin compound 1-(3,4-dimethoxyphenyl)-3-hydroxy-2-(4-methoxyphenoxy)propane-1-one. Hammel et.al. (1986a) used the spin trap nitrosobenzene to detect carbon-centred radicals (cation radical cleavage products) from dimeric β -1 linked model compounds. They also found radical coupling dimers produced stoichiometrically.

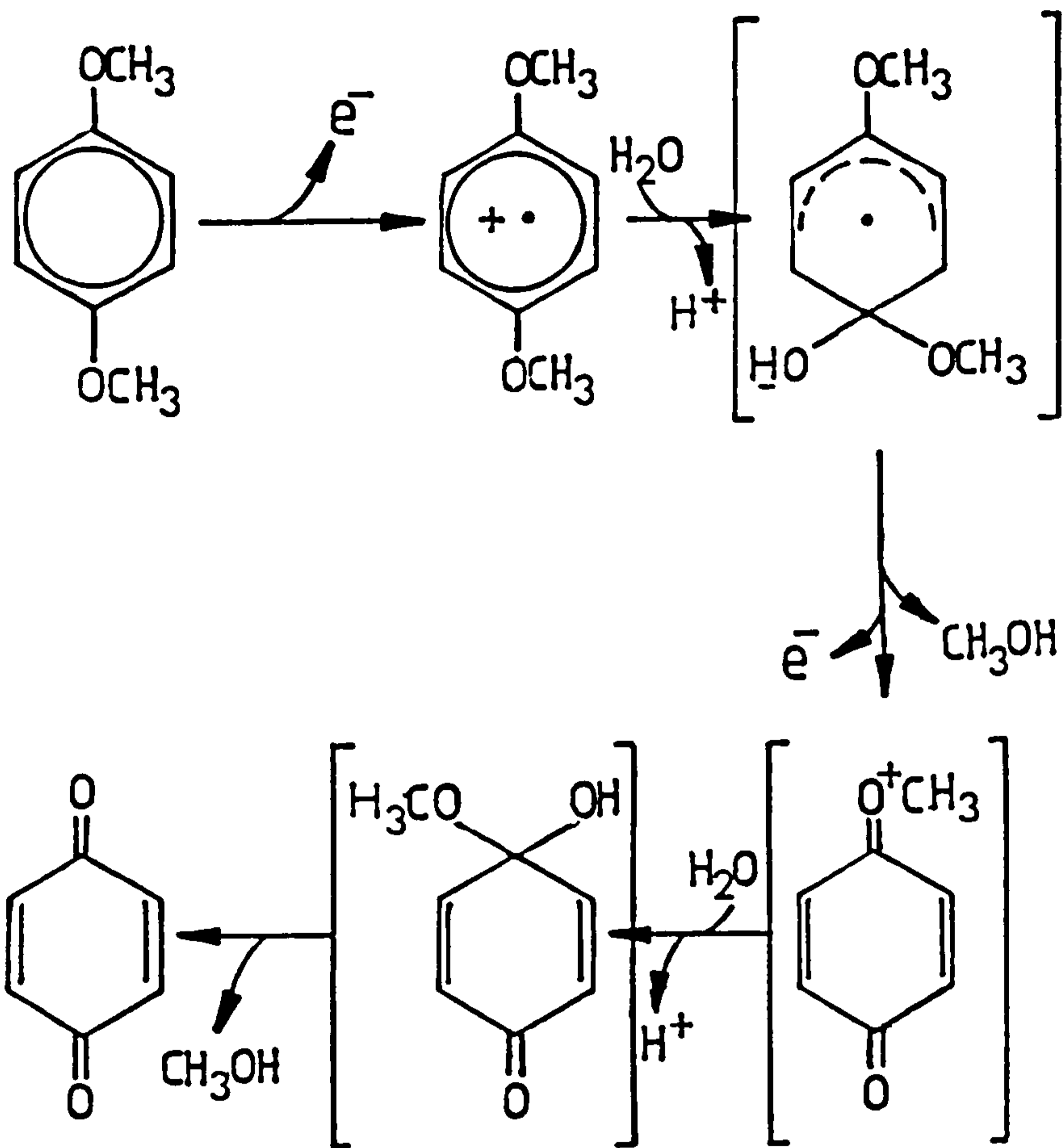


Figure 1.10. Hypothetical scheme showing two sequential one-electron oxidations of 1,4-dimethoxybenzene by LiP. The intermediate cation radical and the products p-benzoquinone and methanol have been identified (Kersten et.al., 1985).

1.8.6. The determination of the peroxidative action of LiP under aerobic conditions.

Sub-stoichiometric amounts of H_2O_2 were required for β -1 model cleavage under aerobic conditions (Hammel et.al., 1985). This needed to be explained if the peroxidative one-electron mechanism of LiP was to be fully accepted.

Hammel et.al. (1985) chose to study the lignin model dimer 1-(3,4-dimethoxyphenyl)-2-phenylethanol (dimethoxybenzoin or DMHB) because, upon C α -C β cleavage by LiP, a simple stoichiometry was obtained which allowed a much clearer interpretation of results. Upon oxidation by LiP the cation radical could either cleave to produce benzaldehyde and a hydroxydimethoxybenzyl alcohol radical or 3,4-dimethoxybenzaldehyde and a hydroxybenzyl radical (fig 1.11.). Both hydroxybenzyl radicals were detected with ESR spectroscopy. The radicals could be oxidised further to produce the corresponding aldehydes, hence the simple stoichiometry. Under anaerobic conditions, the mechanism appeared to be singularly peroxidative. Under aerobic conditions, O_2 could attack the carbon-centred radicals as shown by the stoichiometric uptake of O_2 and the replacement of the 5,5-dimethyl-1-pyrroline-N-oxide-centred ESR signal with an O_2 -centred signal. The resultant hydroxybenzylperoxy radicals would then

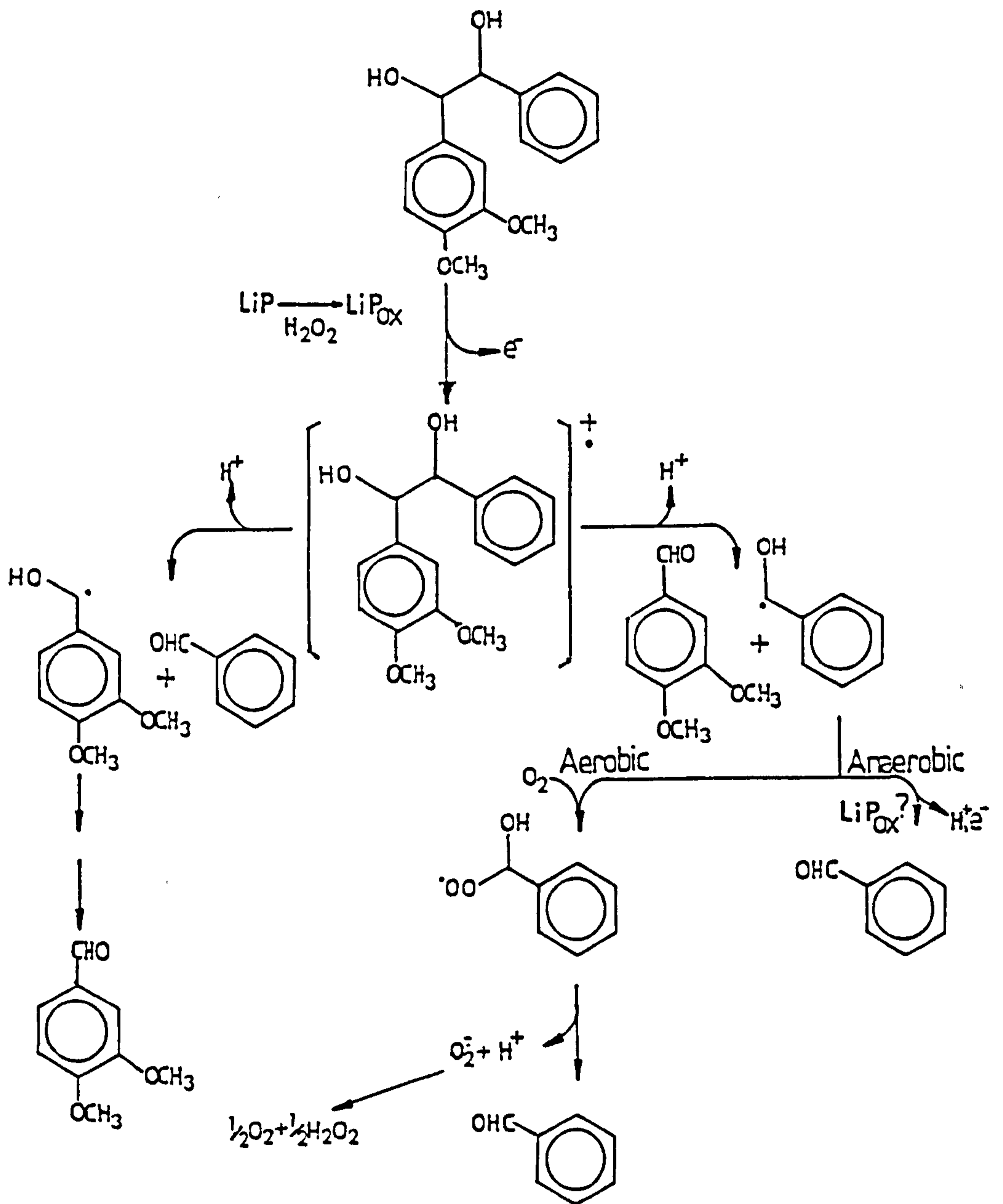


Figure 1.11. Proposed scheme for aerobic and anaerobic cleavage of DMHB by LiP from *P. chrysosporium* (Hammel et.al., 1985).

disproportionate to form the aldehyde and a superoxide radical. The resultant superoxide radical dismutates to H_2O_2 and O_2 . This regeneration of H_2O_2 explains why only catalytic amounts of H_2O_2 were required for cleavage, so that even under aerobic conditions, the mechanism can still be regarded as peroxidative.

1.8.7. Aromatic ring cleavage by LiP.

In addition to Ca-C β cleavage, H_2O_2 -dependent LiP oxidation of veratryl alcohol and β -O-4 model compounds has resulted in the detection of ring cleavage products under aerobic conditions (Umezawa & Higuchi, 1986; Umezawa et.al., 1986; Haemmerli et.al., 1987; Shimada et.al., 1987; Umezawa & Higuchi, 1987; Miki et.al., 1987).

Shimada et.al. (1987) proposed that ring cleavage by LiP was in accord with the one-electron transfer theory. Using H_2^{18}O studies, they found that one atom of oxygen from water and one from dioxygen were incorporated into cleavage products. They suggested that peroxidative oxidation of veratryl alcohol to the radical cation was followed by attack by H_2O and subsequently O_2 resulting in ring cleavage.

Haemmerli et.al. (1987) found that oxidation of veratryl alcohol by the one-electron oxidant cerium(IV) gave the same ring-cleavage lactone products as its oxidation by LiP. They considered that the perhydroxyl

radical formed during peroxidative oxidation of veratryl alcohol to veratraldehyde, is in addition to water, responsible for the ring cleavage of cation radicals to form lactones (fig 1.12.). They further suggested that the involvement of activated oxygen in LiP-catalysed aromatic ring cleavage and quinone formation may be of importance in the degradation of lignin itself.

1.8.8. LiP oxidation of veratryl alcohol and its role in lignin degradation.

The H_2O_2 -dependent oxidation of veratryl alcohol to veratraldehyde yielded a stoichiometry of 1 mol H_2O_2 consumed to 1 mole of aldehyde formed (Tien & Kirk, 1984; Tien et.al., 1986). During steady state catalysis, a ping-pong mechanism for the reaction has been observed, i.e. the enzyme formed an intermediate with H_2O_2 , followed by a reaction with veratryl alcohol returning the enzyme to its resting state (Tien et.al., 1986).

Schoemaker et.al. (1985) postulated that veratryl alcohol oxidation proceeds via two consecutive one-electron oxidation steps with distinct intermediates, namely, the cation radical and, following deprotonation, the radical. Subsequently, Renganathan & Gold (1986) corroborated this idea by finding that

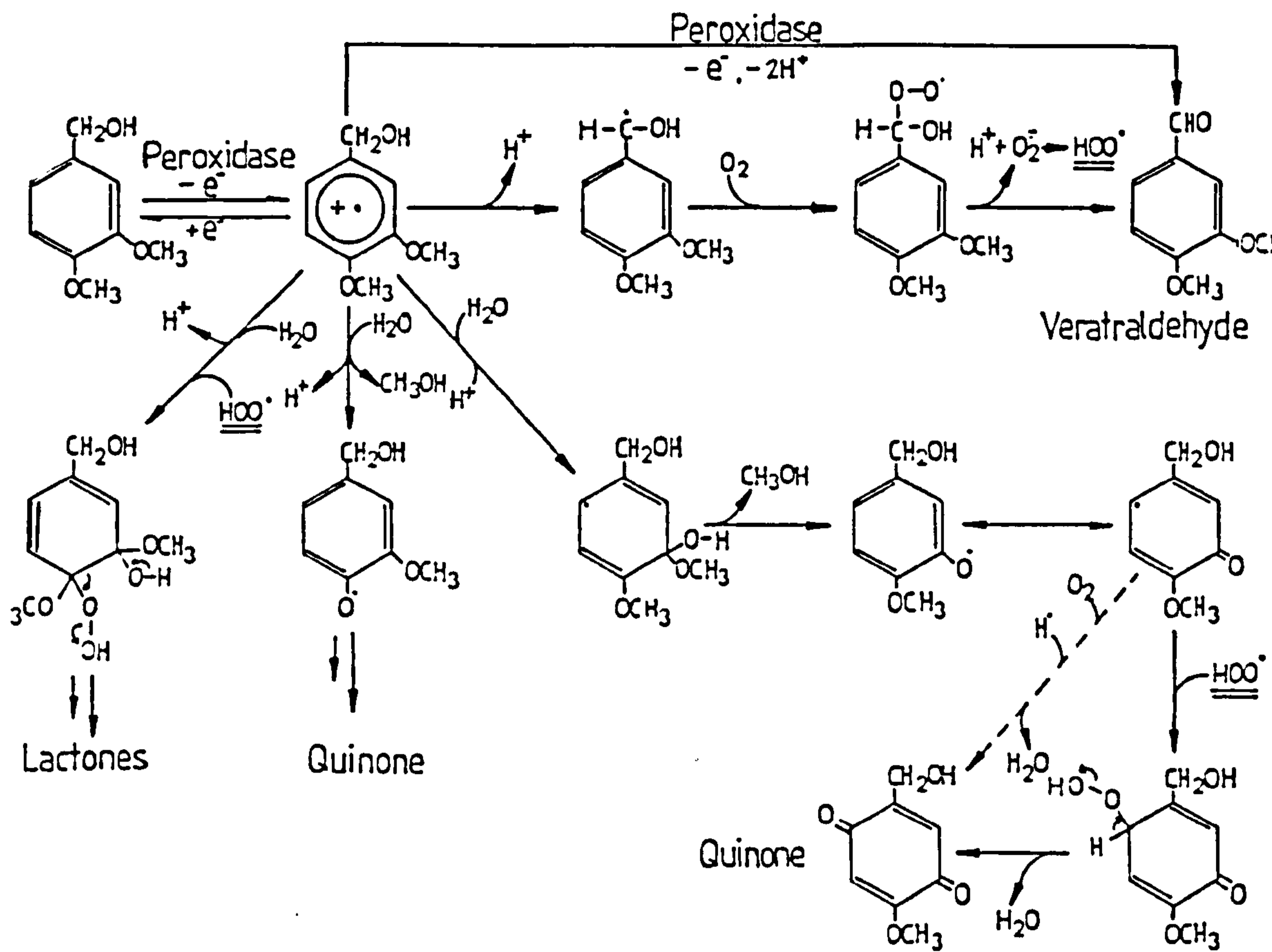


Figure 1.12. Possible mechanisms of ring-cleavage of LiP-produced veratryl alcohol cation radicals via addition of H₂O and HOO[•] (Haemmerli et.al., 1987).

LiP I could be converted to LiP II using a 0.5 molar equivalent of veratryl alcohol, which suggested that two single-electron oxidation steps had taken place.

However, Tien et.al. (1986) found that no LiP compound II (one electron oxidised form) was observed during the oxidation of the two-electron substrate veratryl alcohol and spin-trapping experiments failed to detect the one-electron oxidised intermediate, the veratryl alcohol cation radical. This led them to conclude that veratryl alcohol was oxidised by direct oxygenation, or perhaps via two-rapid and consecutive one-electron oxidations.

Nevertheless, there have been observations which have inferred the presence of free veratryl alcohol cation radicals. Renganathan et.al. (1985) showed that KTBA was oxidised by LiP to ethylene only in the presence of veratryl alcohol. This suggested that the veratryl alcohol cation radical was the reacting species. Harvey et.al. (1986) found that the addition of catalytic amounts of veratryl alcohol or 1,4-dimethoxybenzene greatly enhanced the oxidation of unfavourable substrates such as anisyl alcohol and 4-methoxy mandelic acid. They proposed that the veratryl alcohol radical cation, which is relatively stable in acidic conditions, acted as a mediator between the enzyme and the substrate. Veratryl alcohol would be regenerated upon oxidation of the substrate (fig

1.13.). Finally, and in contradiction to the results of Tien et.al. (1986), Marquez et.al. (1988) used transient state kinetics to demonstrate the formation of LiP compound I (and compound II) during veratryl alcohol oxidation, which was consistent with one-electron oxidation of veratryl alcohol to an aryl cation radical by LiP.

Haemmerli et.al. (1986b) found a 15-fold increase in the oxidation of benzo(a)pyrene in the presence of veratryl alcohol. The same three quinone products were formed in each case. In contrast to Harvey et.al. (1986) they suggested that veratryl alcohol was protecting the enzyme against over oxidation by H_2O_2 rather than it acting as a mediator. This was substantiated by Wariishi & Gold (1989) who found that LiP compound II could be readily and irreversibly oxidised to an inactive compound III ($Fe^{III}O_2^{\cdot-}$ complex). They found that veratryl alcohol not only protects inactivation by the reduction of LiP compound II to the native enzyme, but also veratryl alcohol was capable of displacing $O_2^{\cdot-}/HO_2^{\cdot}$ from LiP III, converting the enzyme back into the native state.

Veratryl alcohol may therefore have a dual effect in the enhancement of LiP catalysis. Firstly, as an electron mediator and secondly, as a stabiliser of LiP.

An important question that has often been neglected is 'how does LiP actually oxidise such a large insoluble polymer?' Even studies on dimeric

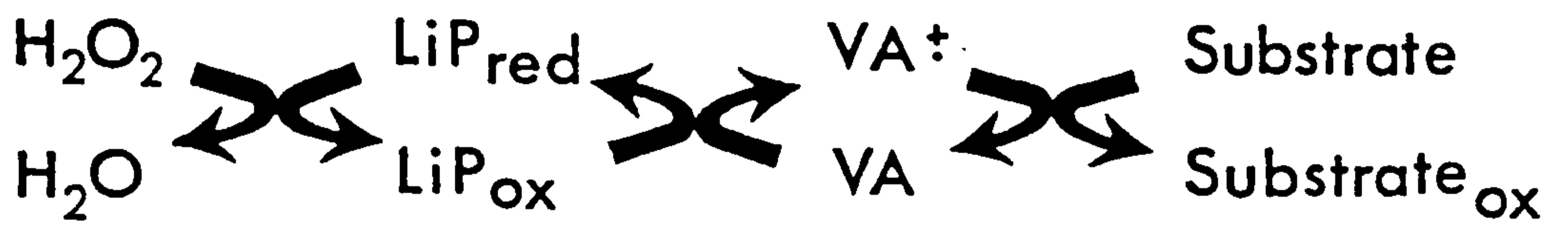


Figure 1.13. Degradation of substrate by LiP with veratryl alcohol (VA) acting as a mediator (Harvey et.al., 1986).

lignin model compounds have necessitated the use of N, N-dimethylformamide, albeit in low concentrations (up to 0.05%, Enoki et.al., 1980) to allow solubilisation of the substrates. Despite the growing evidence for the formation of the veratryl alcohol cation radical by LiP oxidation, their existence as 'free' molecules, spatially separate from the active site of LiP has not been proven. Nevertheless, the proposal by Harvey et.al.(1986) that the veratryl alcohol cation radical acts as a mediator between the enzyme and substrate (in this case lignin) is not only eloquent but it is the only realistic explanation that has been put forward for the effective oxidation of such an insoluble polymer. Indeed a direct effect upon lignin was observed by Haemmerli et.al. (1986a) who found that the addition of veratryl alcohol to LiP, H₂O₂ and lignin enhanced lignin polymerisation.

An analogous enzyme-mediator-lignin system (although not involving cation radicals) was proposed for the Manganese-dependent peroxidase using Mn(II)-lactate as an intermediate (see 1.9.3.).

1.8.9. LiP catalysis: Conclusions.

LiP mimics intact nitrogen-limited ligninolytic cultures of P.chrysosporium in that it has been shown to cleave Ca-C β bonds of aryl ether, Ca-C β bonds

diarylpropane structures and methoxylated aromatic rings. The LiP-catalysed cleavage of these structures, all of which are vital to the integrity of the lignin molecule, can be explained by a single mechanism, namely, peroxidative one-electron oxidation of susceptible structures to form cation radicals. The subsequent chemical breakdown of these radical species starts a number of chemically- and possibly enzymatically-oxidised miscellaneous and divergent reactions including further $\text{C}\alpha\text{-C}\beta$ cleavage and aromatic ring cleavage. This led Kirk & Farrell (1987) to coin the phrase 'Enzymatic combustion' for the mechanism of LiP-catalysis, which aptly describes the non-specific oxidation of the polymer initiated by LiP.

1.9. Other enzymes implicated in lignin degradation.

1.9.1. Hydrogen peroxide-producing enzymes.

The importance of H_2O_2 in lignin degradation has become increasingly apparent. Forney et.al. (1982) used cytochemical staining to determine the site of fungal H_2O_2 production which was found to be localised in periplasmic microbodies, present in actively ligninolytic cultures. Later, Tien & Kirk (1983) found that H_2O_2 was required for LiP activity. This has led to further investigations concerning the source of H_2O_2 .

Forney et.al. (1982) found that H_2O_2 was produced upon the addition of glucose to stationary phase cell extracts of P.chrysosporium, suggesting that glucose oxidase may play a part in H_2O_2 production. Subsequently a glucose oxidase from P.chrysosporium was purified and characterised by Kelley & Reddy (1986a) as a 180kd flavoprotein capable of oxidising a range of sugars. The implication that glucose oxidase was important in the degradation of lignin was substantiated by Kelley & Reddy (1986b) who found that nutritional factors affecting lignin degradation had a parallel effect on glucose oxidase. This was also true of a glucose-2-oxidase, which was discovered and characterised by Eriksson et.al. (1986), from carbon-limited cultures of P.chrysosporium.

Further evidence was put forward by Ramesay et.al. (1986) who found that glucose oxidase mutants (Gox^-) do not produce H_2O_2 , LiP or degrade lignin. Some idiophasic properties were still present, such as the formation of conidia and the production of veratryl alcohol, but only at a low level. Gox^+ revertants were able to degrade lignin.

Despite the evidence in favour of glucose oxidase as a primary source of H_2O_2 , this intracellular enzyme has not been shown to directly influence the extracellular H_2O_2 pool. For this reason, other oxidases were investigated.

Greene & Gould (1984) suggested that the presence of fatty acyl-CoA oxidase activity in peroxisomes of P.chrysosporium may be an extracellular source of H_2O_2 . Eriksson (1987) found a methanol oxidase and H_2O_2 produced in cultures of P.chrysosporium during secondary metabolism. From this observation, he suggested that the demethoxylation of lignin (Ander & Eriksson, 1985) provided the methanol for H_2O_2 production.

The extracellular manganese(II)-dependent peroxidase (MnP) found in cultures of P.chrysosporium possessed oxidase activity (Paszczyński et.al., 1985; Glenn et.al., 1986). However, although NAD(H), and NADP(H), have been reported in intact cultures (Kuwahara et.al., 1984), it is not known how the

organism may regenerate the reduced cofactor necessary for oxidase activity. There may be an extracellular substrate yet to be found that will support MnP oxidase activity.

Kersten & Kirk (1987) found an extracellular oxidase capable of oxidising glyoxyl and methylglyoxyl. Along with its temporal appearance with LiP and its broad substrate range, they suggested that the glyoxyl oxidase may be an important contributor to the extracellular H₂O₂ pool.

Although several oxidases, both intracellular and extracellular, have been implicated in H₂O₂ production during ligninolysis, the individual importance of each has yet to be determined.

1.9.2. Laccase

The majority of white rot fungi produce extracellular phenol oxidases, in particular, laccase (p-diphenol:oxygen oxidoreductase EC 1.10.3.2.), which is an enzyme that has long been associated with lignin biodegradation. There is however, a conflict of views concerning the actual role that laccase plays.

Ander & Eriksson (1976) found that phenol oxidase (Pox⁻) mutants of Sporotrichum pulvurulentum would not degrade lignin, but Pox⁺ revertants or the addition of exogenous laccase would allow lignin degradation.

Haars & Hutterman (1980) found that the removal of

laccase activity from Fomes annosus by copper chelation did not prevent lignosulfate degradation. In the presence of active laccase, polymerisation actually took place. Evans (1985) repeated both findings of Haars & Hutterman using antibodies to inhibit laccase activity in Coriolus versicolor.

Laccase, like HRP and LiP, will catalyse the single electron oxidation of phenols (see 5.3.). This almost certainly explains the observation that laccase will polymerise lignin.

Higuchi (1986) and Kawai et.al. (1988) subjected diarylpropane diol lignin models to purified laccase from C.versicolor. They found that the enzyme oxidised phenolic diarylpropane models to phenoxy radicals, but non-phenolic models were not oxidised. The phenoxy radicals, once formed, undergo various non-enzymatic reactions; Ca-C β cleavage, Ca oxidation, or disproportionation followed by hydration, causing alkyl-aryl cleavage. Both LiP and laccase appear to be able to cleave model dimers, but only LiP can degrade both phenolic, and non-phenolic dimers.

Lignin contains approximately 10-20% phenolic hydroxyl groups. Ca-C β cleavage by LiP and laccase followed by hydrolysis will increase the phenolic content. Higuchi (1986) suggested that lignin degradation may proceed by the cooperation of LiP and laccase. Earlier results showing the ability of

laccase inhibited cultures of white-rot to degrade lignin (Haars & Hutterman, 1980; Evans, 1985) may be due to the presence of an LiP-type enzyme since whole cultures of C.versicolor have been shown to metabolise non-phenolic dimers (Kamaya & Higuchi, 1984; Kawai et.al., 1985; Kawai et.al., 1987) and subsequently LiP-type activity has been detected in nitrogen-limited cultures of this fungus (see 7.2). This does not however explain the possible cooperative effect between LiP and Laccase.

1.9.3. Manganese-dependent Peroxidase (MnP).

Kuwahara et.al. (1984) found, in addition to LiP, a Mn(II)-dependent, lactate-activated peroxidase in the extracellular growth media of P.chrysosporium. This enzyme was capable of oxidising a number of aromatic dyes, including phenol red, o-dianisidine and Poly R-481. It could also decarboxylate vanillic acid without the prerequisite Mn(II), but it would not oxidise veratryl alcohol. A similar Mn(II)-dependent peroxidase was also found by Huynh & Crawford (1985), but it did not require lactate nor did it oxidise phenol red. This difference may be a result of the two different strains used.

Glenn & Gold (1985); Paszczynski et.al. (1985) and Paszczynski et.al. (1986) purified and further characterised MnP which was shown to be 46kd

glycoprotein with a high spin protoporphyrin IX prosthetic group.

Like LiP, MnP has subsequently been shown to exist as multiple molecular forms, as demonstrated by Leisola et.al. (1987) who found six MnP isozymes in nitrogen-limited cultures of P.chrysosporium. Their reaction with specific antibodies suggested that they may all be products of one gene. This enzyme may be common amongst other white-rot fungi since two isozymes of a Mn(II)-dependent lactate-requiring peroxidase have recently been found in the white-rot Trametes versicolor (Johansson & Nyman, 1987).

Although the H₂O₂-MnP complex had similar spectral properties to HRP, MnP differed in that it had high a specificity for Mn(II) oxidation. This high specificity led Glenn et.al. (1986) to suggest that Mn(II) was the natural substrate of MnP and that upon its' oxidation the resultant Mn(III) complexed with lactate or some other suitable ligand. This Mn(III)-lactate species could then act as a diffusible oxidant allowing the oxidation of phenolic substrates within wood that are inaccessible to enzymes (fig 1.14, Glenn et.al., 1986). MnO₂(MnIV), which is slowly formed in the absence of lactate has been found in dark patches on wood decayed by several white rot fungi (Blanchette, 1984a) which led Glenn et.al. (1986) to suggest that its presence may be a result of MnP activity.

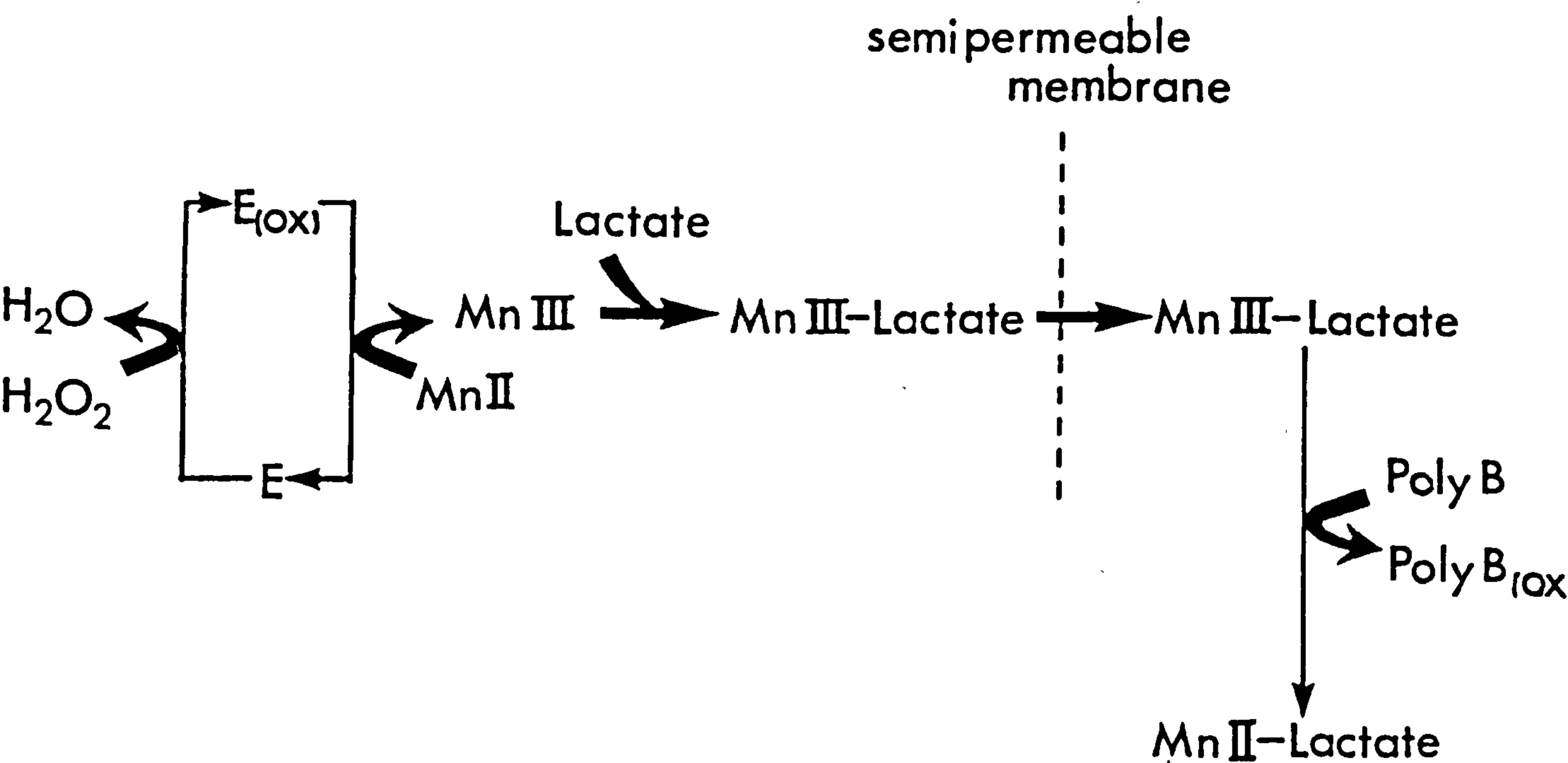


Figure 1.14. Scheme for the indirect oxidation of the polymeric dye Poly B by Mn-peroxidase (E) showing Mn-lactate as an obligatory intermediate (Glenn et.al., 1986).

1.9.4. Cellobiose:quinone oxidoreductase (CBQase).

In vitro attack on lignin by LiP has been reported to cause further polymerisation (Haemmerli et.al., 1986a), which is probably caused by the oxidative polymerisation of the resultant phenoxy radicals and quinones. Since a net depolymerisation of lignin occurs in vivo, part of the ligninolytic system must be involved in removing phenoxy radicals, either by reduction to phenols (which are themselves susceptible to re-oxidation by the phenol oxidising capacity of LiP and laccase), or by further oxidation.

One possible candidate was the extracellular enzyme found by Westermarck & Eriksson (1974a) in Polyporus versicolor (Coriolus versicolor) which was capable of reducing quinones in the presence of cellobiose. Purification and characterisation of a similar enzymic activity in S.pulverulentum showed it to be a glycosylated 58kd flavoprotein with a FAD prosthetic group (Westermarck & Eriksson, 1975). CBQase reduced ortho and para quinones as well as phenoxy radicals which led to the suggestion that CBQase offsets undesirable laccase activity (fig 1.15, Westermarck & Eriksson, 1974b).

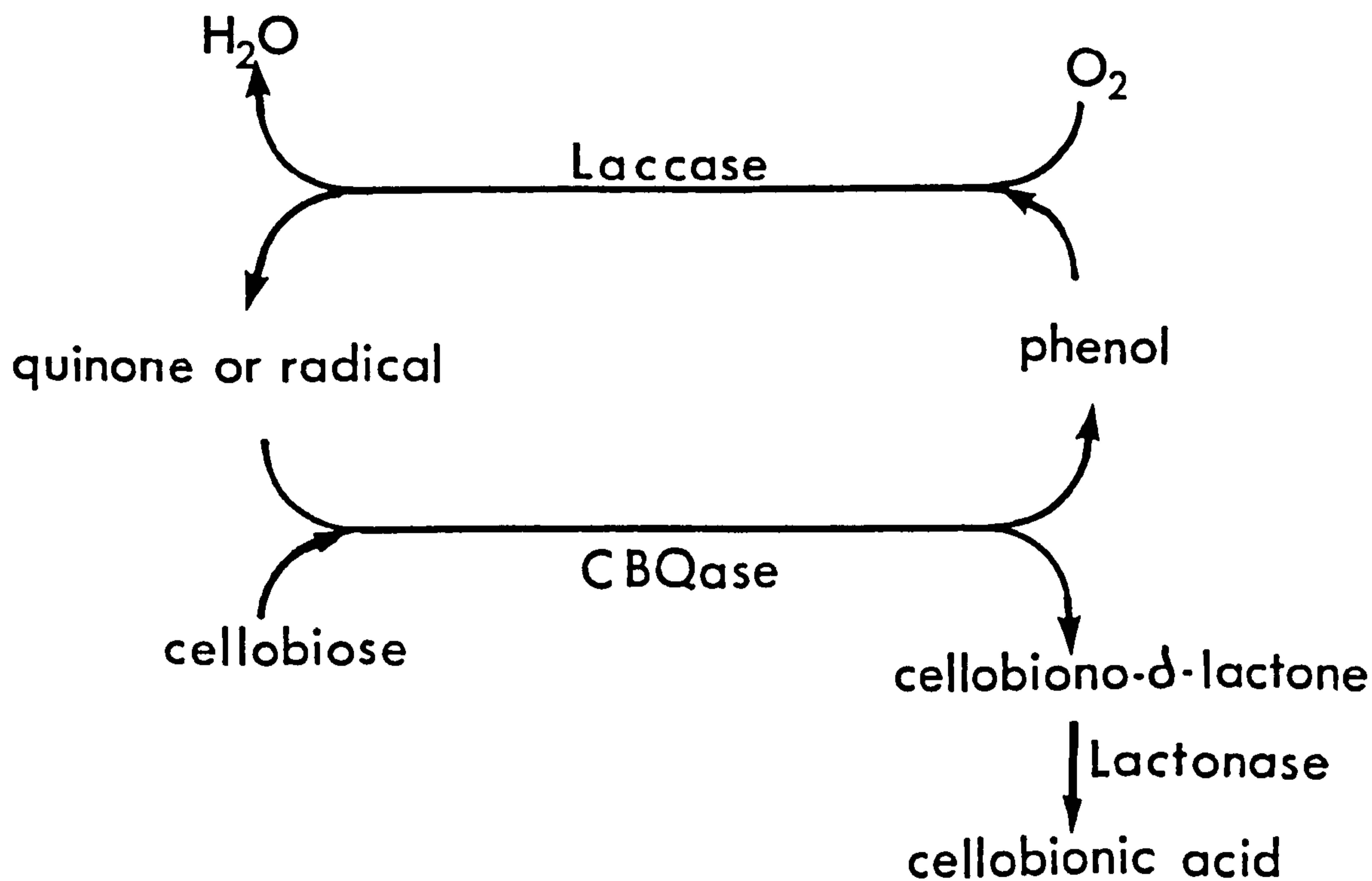


Figure 1.15. Possible mechanism of CBQase in the regulation of laccase activity (Westermarck & Eriksson 1974b).

1.10. The Commercial Applications of Lignin-biodegrading Organisms.

The potential commercial applications for biological lignin-degradation have largely been responsible for the current surge of interest in the physiology of ligninolytic organisms. A review of lignin biodegradation would therefore not be complete without discussing the present applications and future potential for lignin-degrading organisms and their enzymes.

1.10.1. The development of biocatalysts for the pulp and paper industry: Biopulping and biobleaching.

The pulp and paper industry convert wood to pulp which is subsequently used to manufacture paper and paperboard products. Pulp production necessitates the specific removal of the lignin matrix to allow the cellulose fibres to associate with one another. Lignin is currently removed chemically by solubilisation followed by bleaching to remove residual lignin to increase the brightness of the pulp, making it suitable for the manufacture of high grade paper. Alternatively, pulp can be made mechanically by physically shredding the wood (thermomechanical pulping)

However, these physico-chemical methods are unsatisfactory from several viewpoints since they cause cellulose fibre damage, are corrosive and, in particular, are costly. For instance, a typical pulping plant in Sweden uses the equivalent energy in mechanical pulping as the domestic demand of the town of Malmo (population 300,000) in southern Sweden (personal communication Eriksson).

Since the discovery of LiP (Tien & Kirk, 1983; Glenn et.al., 1983) interest has been concentrated on ligninolytic enzymes as possible alternatives to traditional pulping and bleaching methods. RepliGen (1986) are investigating the use of lignin degrading enzymes to partially but specifically delignify coarse thermomechanical pulp (TMP) to reduce energy costs by 25-30% and to act as an alternative to chlorine-based oxidising agents in bleaching, with the aim of reducing cellulose fibre damage, plant corrosion costs and the release of polluting organochlorides.

As an alternative to using enzymes, Karl-Erik Erikssons group in Stockholm have concentrated on the development of cellulase-deficient strains of various white-rot fungi which can specifically remove the lignin, leaving the cellulose fibres intact (Eriksson & Goodell, 1974; Eriksson et.al., 1983; Johnsrud & Eriksson, 1985). Original studies on Sporotrichum pulverulentum showed that a 0.5-1% reduction in lignin

by Cell⁻ strains could save 25% of the energy costs of pulping, but the incubation time of 4 weeks was far too long to be economically viable. The lack of a readily assimilable carbon source was probably the major reason for such slow growth. Improvements have since been made using xylose-utilising strains or using the wild-type on glucose (2%) impregnated wood chips to repress cellulase and hemi-cellulase action but not ligninolysis (Eriksson personal communication).

Future developments are more likely to involve whole organisms rather than enzymes. The mycelial nature of white-rot fungi allows dense colonisation of wood resulting in extensive attack (see 1.5.1.). Although enzymes do not require the prerequisite incubation time of several days that is needed to establish ligninolytic fungal cultures, they could only thoroughly process wood chips that were pre-shredded. In addition, a costly cocktail of enzymes including ring-cleavage enzymes would be necessary to prevent re-polymerisation of lignin via LiP-produced aromatic radical species and to prevent the formation of quinones which appear to be the chemical units that cause brightness reversion (Farrell, 1986).

1.10.2. Waste treatment.

1.10.2.1. Decolourisation.

One of the biggest single costs in the pulp and paper industry is the treatment of bleach plant waste effluents to reduce the pollution in water courses. These brown coloured effluents can be treated by passing them through aerated lagoons or activated sludge systems to reduce the BOD (Biological oxygen demand) and COD (Chemical oxygen demand) , but this type of treatment will not remove colour (Livernoche et.al., 1983). The use of coagulants such as lime or alum to remove colour are uneconomic. This problem has led to the suggestion of using white-rot fungi to decolourise bleach effluent (Ek & Eriksson, 1980; Livernoche et.al., 1983; Belsare & Prasad, 1988).

Livernoche et.al. (1983) screened 15 strains of white-rot fungi on sucrose supplemented kraft effluent. Coriolus versicolor proved to be the most effective at decolourisation. It removed 60% of the colour from the effluent in 6 days. This was further improved by the immobilisation of C.versicolor in calcium alginate, allowing up to 80% decolourisation within 3 days. An immobilised system would offer the additional advantage of being able to recycle the biomass/ligninolytic system. However, Livernoche et.al. (1983) recognised

the need to find a much cheaper method of immobilisation if such a process were to attain economic viability.

Ek & Eriksson (1980) suggested that the nutritious white-rot fungus Sporotrichum pulverulentum (Thomke et.al., 1980) could be used to decolourise the effluent and then be harvested as single cell protein for domestic animals. They argued that the process would be viable because the pulp effluent substrate required costly treatment before it could be disposed of. Johnson & Carlson (1978) used the same economic argument when they proposed that mycelia harvested from waste effluent could be incorporated into wood fibre (5-10% w/w) to produce 'mycelial paper'.

Recently, Belsare & Prasad (1988) demonstrated the very efficient treatment of effluent by Schizophyllum commune from bagasse-based pulp mills. Colour was reduced by 90%, BOD and COD were reduced by 70% and 72% respectively in 2 days.

In addition to cultures of white-rot fungi, enzymes have been considered for decolourisation processes. Paice and Jurasek (1984) used horseradish peroxidase (HRP) to decolourise effluent from the caustic extraction stage of a bleach plant. Decolourisation was initially rapid, but it was not as complete as that obtained by C.versicolor. This is understandable since, other enzymes apart from peroxidases are likely to be involved in complete

biological lignin degradation (see 1.9). In addition, HRP is not as powerful an oxidant as LiP (Hammel et.al., 1986b) which has recently been found in ligninolytic cultures of C.versicolor (Dodson et.al., 1987; Waldner et.al., 1988).

RepliGen (1986) have considered the use of LiP enzymes in the decolourisation process. A program of U.V. mutagenesis of P.chrysosporium BKM followed by screening for enhanced decolourisation of alkali extracted bleach pulp effluent has produced the LiP-overproducing strain SC26. However, the large-scale production of LiP from P.chrysosporium SC26 is unlikely to be cost-effective because of the 6-8 day culture time required. Further research at RepliGen has concentrated on the production of a constitutive mutant of SC26 and the cloning and expression of the recombinant-LiP gene in Escherichia coli and other organisms that lack cellulases and are suitable for industrial production (Farrell, 1987).

1.10.2.2. Detoxification.

Bumpus et.al. (1985) demonstrated that nitrogen-limited cultures of P.chrysosporium could degrade DDT [1,1,1-trichloro-2,2-bis(parachlorophenyl)ethane], polychlorinated biphenyls (PCBs), chlorobenzodioxin, lindane and benzo(a)pyrene to CO₂. This led to the

proposal that various aerobic waste treatment processes (activated sludge, trickling filters, rotating biological contactors and aerobic composts) could be inoculated with P.chrysosporium where there was a demand for the disposal of hazardous chemical wastes. An example of this being pulp effluent itself which contains mutagenic chlorinated compounds derived from the bleaching process (Kringstad et.al., 1981).

Bumpus et.al. (1985) found that the degradation of ^{14}C -DDT followed a similar pattern to that of ^{14}C -lignin degradation under the same conditions. It also required the same nutritional parameters, which suggested that LiP may be involved in the degradation of halo-aromatic pollutants.

Subsequently, several toxic aromatic pollutants have been found to be at least partly degraded by LiP (fig 1.16 I-V). Haemmerli et.al. (1986b) demonstrated the oxidation of benzo(a)pyrene (I) to quinones by purified LiP. Similar studies by Hammel et.al. (1986b) found that pyrene (II) could be oxidised by LiP to quinones and that the oxygen in the quinone products was derived from H_2^{18}O . In addition, dibenzo(p)dioxin (III) and 2-chlorodibenzo(p)dioxin were also oxidised by LiP. Noteably, the dibenzo(p)dioxin cation radical was found to be an intermediate (see 1.8.2.).

Bumpus & Brock (1988) found that purified LiP would decolourise crystal violet (N,N,N',N',N'',N''-hexamethylparasosaniline, IV) and six other

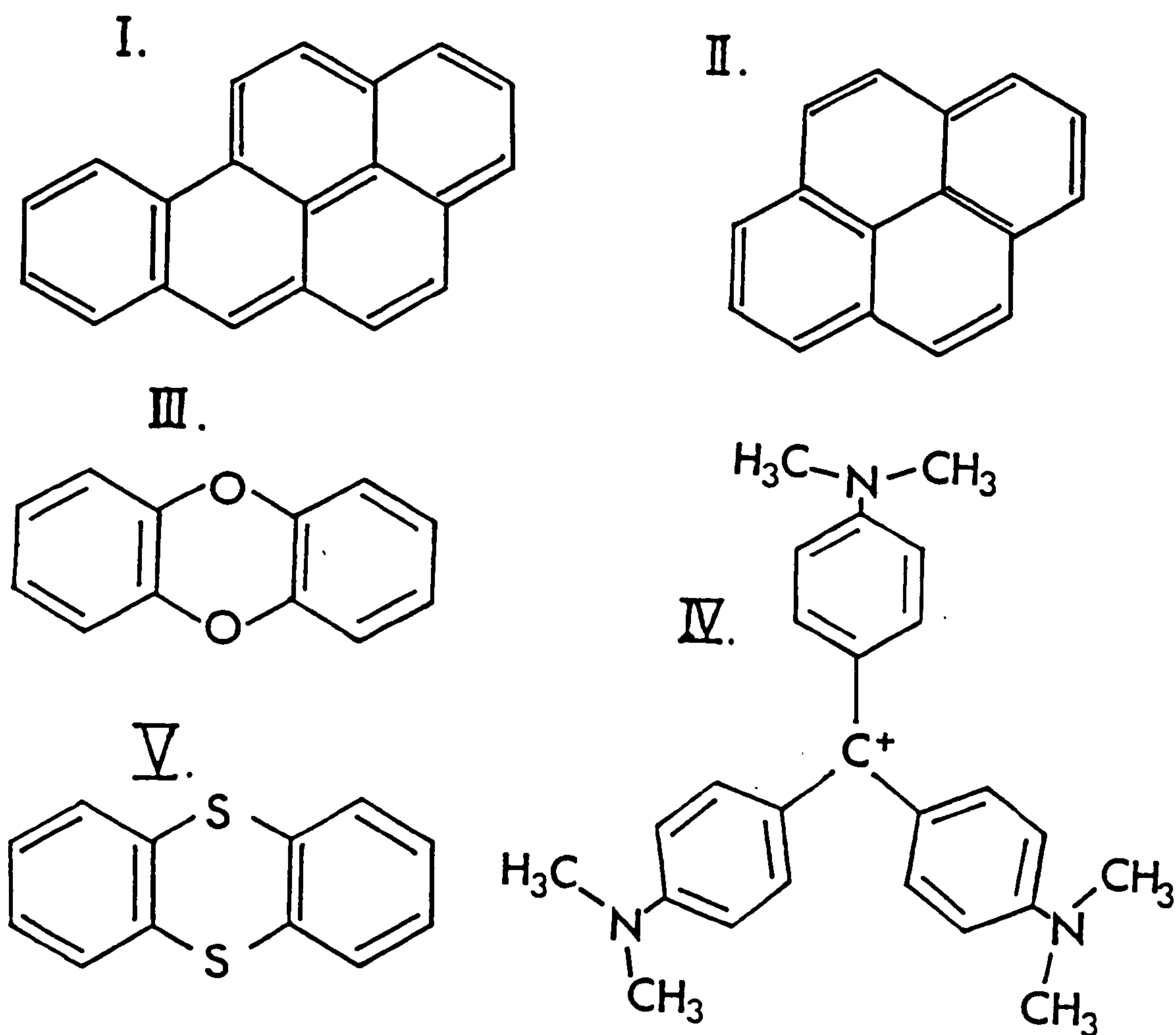


Figure 1.16. LiP-oxidisable toxic aromatic pollutants: benzo(a)pyrene (I; Haemmerli *et.al.*, 1986b), pyrene (II), dibenzo(p)dioxin (III; Hammel *et.al.*, 1986b), crystal violet (IV; Bumpus & Brock, 1988) and thianthrene (V, Schreiner *et.al.*, 1988).

triphenylmethane dyes via N-demethylation.

Interestingly, N-demethylation has also been reported for HRP by Kedderis and Hollenberg (1983).

Schreiner et.al. (1988) found that thianthrene (V), a constituent of coal, could be oxidised to thianthrene monosulphoxide by LiP. They suggested that LiP may be useful in the desulphurisation of coal since the monosulphoxide is more soluble, and therefore more amenable to leaching from coal supplies.

Since lignin does not even contain nitrogen or sulphur, these examples (Bumpus & Brock, 1988; Schreiner et.al., 1988) are further demonstrations of the non-specificity of this enzyme. The fact that Hammel et.al. (1986b) found a cation radical intermediate during the oxidation of benzo(p)dioxin, suggests that LiP may be able to attack a wide range of aromatic compounds (lignin related or not) via the same method of one electron oxidation leading to the formation of a cation radical and its subsequent chemical breakdown products.

Whole cultures of white-rot fungi rather than purified LiP would probably be the best system for detoxification since LiP does not necessarily detoxify compounds but may convert them into other, equally toxic compounds. For example, benzo(a)pyrene was oxidised to CO₂ by whole cultures of P.chrysosporium (Bumpus et.al., 1985) but it was only oxidised to

benzo(a)pyrene 1,6-, 3,6-, and 6,12-quinones by the H₂O₂/LiP system (Haemmerli et.al., 1986b). However, the enzyme may be more useful in a situation where the effluent to be treated was too toxic for fungal growth.

1.10.3. The use of white-rot fungi/LiP in the production of chemical feedstocks from lignocellulose wastes.

Lignin is an excellent natural depository of aromatic and polyaromatic compounds. The possible applications of products derived from lignin are extensive (Table 1.3.). Despite this, the most common use of lignin is for fuel because of the high separation costs, structural variability, complexity and low reactivity (Janshekar & Fiechter, 1983).

The processing of lignin with white-rot fungi remains a long-term prospect mainly because of the wide variety (100+) of small molecular weight products that have been identified, most of which appear in low yields. In addition, only limited types of monomeric compounds (3,4 di- and 3,4,5, tri-substituted phenylpropane, phenylethane and phenylmethane compounds) are produced, which further restricts the number of useful compounds that can be recovered (Ribbons, 1987).

The production of heteroaromatic polymers using

Energy

Polymers and modified polymers

Filters, rubber reinforcement, carriers for controlled release in fertilizers and pesticides, dispersants, emulsion stabilizers, complexing agents, precipitants, coagulants, stabilizer applications e.g. as antioxidants, ion exchange.

Prepolymers

Polyphenolics, resins and extenders, foams, adhesives.

Fragmentation and chemical conversion

Hydrogenation → phenols, hydrocarbons, substituted mononuclear phenols.

Hydrolysis → phenols, catechols, substituted phenols.

Oxidation → vanillin, dimethyl sulfide, methyl mercaptan.

Alkali fission → phenolic acids, catechols.

Pyrolysis → acetic acid, phenols, substituted mononuclear phenols, CO,
CO₂, CH₄, and H₂.

Thermolysis → acetylene, ethylene.

Table 1.3. Alternative uses for lignin (Janshekar & Fiechter, 1983).

chemically modified polyphenolic intermediates of biodegradation (for example APPLs produced by Streptomyces species from corn stover, Crawford et.al., 1983) may be a more realistic shorter-term application for microbial-processed lignin (Crawford & Crawford, 1980).

1.10.4. The use of white-rot fungi in the production of animal feedstocks from lignocellulose wastes.

Low value lignocellulosic materials such as bagasse, aspen wood and forestry by-products are potentially good sources of polysaccharides for feeding ruminants. However, such materials are poorly digested because of the inability of ruminant microflora to liberate cellulose and hemi-cellulose from the lignin matrix. Unfortunately, ruminants appear to be able to degrade lignin on a very limited scale. For example, Akin & Benner (1988) , using selective antibiotic studies on microbial population groups in ruminal fluid, found that ruminal fungi utilised lignin poorly from bermuda grass leaf blades. Although some solubilisation of lignin occurred, lignin was not further degraded to gaseous end products. This is in accordance with observations that ligninolysis is low or even non-existent in anaerobic environments (see 1.6.3.).

If lignocellulosic wastes were to be used for animal feedstocks, their nutritional value needs to be improved by the removal of some or all of the lignin present. This has led to a number of authors investigating solid-state delignification of lignocellulosic wastes by white-rot basidiomycetes (Platt et.al., 1984; Agosin & Odier, 1985; Reid, 1985; Rolz et.al., 1986; Kewalramani et.al., 1988).

The economics of such a process depend not only on the rate of delignification, but the specificity of lignin breakdown since the improvement of lignocellulose digestibility is dependent on the selective removal of lignin and not total degradation (Reid, 1985; Zadrazil & Brunnet, 1982).

Strong candidates for upgrading lignocellulose digestibility based upon their selectivity in lignin degradation include Pleurotus ostreatus which degraded 60% of the lignin from cotton straw in 21 days with a resultant four fold increase in exogenous cellulase digestibility (Platt et.al., 1984). Merulius tremellosus degraded 52% of Aspen wood lignin in 8 weeks, causing a drop of only 12% total wood weight which coincided with a three fold increase in cellulose digestibility. Supplementation of the latter solid-state fermentation with salts, thiamine and trace elements had little effect. However, a restriction of nitrogen supply not only increased the rate of

delignification, but also increased the specificity for delignification (Reid, 1985).

Rolz et.al. (1986) screened 12 white-rot fungi grown on solid-state cultures of lemon grass and citronella bagasse. They concluded that the nature of degradation is very fungus-substrate specific and that a general trend was difficult to define. This is in agreement with Blanchette (1984b) who found that lignin degradation of the wood of different trees varied since they may be degraded in different ways by the same fungus. Therefore, screening for a rapid and specifically ligninolytic white-rot fungi must be taken into account with the desired substrate.

1.10.5. The production of fungal fruiting bodies.

The cultivation of fungal fruiting bodies for human consumption is certainly not a new idea. The edible Shi-ta-ke mushroom is thought to have been grown in China 2,000 years ago by brushing logs with a spore suspension in the hope that colonisation would take place.

Today, mushroom production from lignocellulosics is a major industry, particularly in the Far East. The world market for edible fungi is large and ever expanding (Zadrazil & Grabbe , 1986 see table 1.4.). The industry today is essentially a low technology

concern and efforts to introduce intensive cultivation on artificially prepared substrates has met with cultural opposition. Nevertheless, the process has been improved by keeping the system as sterile as possible, by the induction of fructification and by classic strain selection (Zadrazil & Grabbe, 1986).

In recent years, lignocellulosic wastes as well as logs have been utilised. For instance, Pleurotus ostreatus is now commonly grown on cereal straw and other plant wastes (Darmycel, U.K., 1988). Tree bark (Daugulis and Bone, 1978) and sawdust (Pamment et.al., 1978) have also been considered as substrates.

Species	Common name	Disribution	Quantity(000tons)
<u>Lentinus edodes</u>	Shii-ta-ke	Japan/Far East	180
<u>Pleurotus</u>	Oyster mushroom	Worldwide	40
<u>Pholotia nameko</u>	Nameko	Japan	20
<u>Auricularia polytricla</u>			12
<u>Auricularia auricula-judae</u>	Jews ear		
<u>Tremella</u> sps		Taiwan/Worldwide	3

Fig 1.4 World production of edible fungi, 1981 (excluding Agaricus bisporus).

1.11. The Present work.

Since the discovery of LiP by Tien & Kirk in 1983, research on lignin biodegradation has increased rapidly. Much of this research was concerned principally with the expression and function of LiP and other enzymes in lignin degradation.

The present work was aimed at initiating an investigation into the catalytic utility of LiP without necessarily considering its role in biodegradation. The long-term purpose would be to find a use for this novel peroxidase as a commercial biocatalyst principally as an agent for biotransformations of a variety of aromatic compounds.

Studies in the literature prior to 1985 had indicated that LiP was only produced in relatively small quantities in culture. The initial requirement was therefore to find a method aimed at producing sufficient quantities of LiP for further study.

Secondly, a high resolution purification of LiP was required since this enzyme was thought to consist of multiple molecular forms (see 4.1) which may be catalytically heterogenous. Individual isozymes must therefore be used to validate individual biotransformations unless it can be shown that these different molecular forms have the same catalytic spectrum. Also, high resolution purification of LiP

could unveil new forms of the enzyme with different catalytic properties.

In addition to LiP from P.chrysosporium, other white-rot fungi were to be investigated and screened for LiP-type enzymes to show whether or not the ligninolytic system from P.chrysosporium was a common method of Lignin biodegradation. If other LiP-type enzymes were to be found, they could be investigated for useful catalytic properties like LiP.

C H A P T E R 2

2. Materials and methods.

2.1. Organisms.

The following fungi were used in this work:

Sporotrichum pulverulentum Novobrabova CMI 174 727(T).

Phanerochaete chrysosporium Burdsall CMI 74 691.*

Phanerochaete chrysosporium Burdsall CMI 74 693.*

Phanerochaete chrysosporium Burdsall ATCC 24725 (BKM-1767).

Phanerochaete chrysosporium Burdsall ATCC 34541 (ME-446).

Coriolus versicolor CMI 79 126.

(* These strains were a gift from Dr A. Wiseman, University of Surrey, Guilford, U.K.).

2.2 Growth conditions for static cultures.

The following defined growth medium was used (based upon Kirk et.al., 1978) except where stated otherwise in the text:

In 1L distilled water, the following were added: 0.2g KH_2PO_4 ; 0.05g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.01g CaCl_2 ; 10g Glucose; 0.2g ammonium tartrate(1.2mM); 1.46g 2,2-dimethylsuccinate(10mM buffer); 1ml stock mineral solution and 0.5ml stock vitamin solution.

1L of stock mineral solution contained the following:

1.5g nitrilotriacetate; 3g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 1g NaCl ; 100mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 100mg CoSO_4 ; 82mg CaCl_2 ; 100mg ZnSO_4 ; 10mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 10mg $\text{AlK}(\text{SO}_4)_2$; 10mg H_3BO_4 and 10mg NaMoO_4 .

1L of stock vitamin solution contained the following: 2mg biotin; 2mg folic acid; 5mg thiamine.HCl; 5mg riboflavin; 10mg pyroxidine.HCl; 0.1mg cyanocobalamine; 5mg nicotinic acid; 5mg DL-calcium pantheonate; 5mg p-aminobenzoic acid and 5mg thioctic acid.

The pH of the medium was corrected to 4.5 with potassium hydroxide (5M) and then autoclaved. After cooling, the stock vitamin and mineral solutions were added.

Spore suspensions used for the culture inoculation

of S.pulverulentum and P.chryso sporium were prepared by flushing 3-4 week old malt agar-grown cultures with 0.1% tween 80. The resultant spore suspension was added at 1ml per litre of medium.

For culture inoculations of C.versicolor, 5 x 10ml two-week old liquid cultures were drained and homogenised with 25g x 3mm dia ballotini and the resultant homogenate was added to 10 L of medium.

After inoculation 10ml aliquots of medium were added to 100ml Erlenmeyer flasks which were then sealed with suba-seals. Cultures were incubated at 37°C and flushed with O₂ for 1 minute on days 3 and 6.

2.3. Growth conditions for agitated cultures.

The culture medium used was the same as that described above, except that 0.1% tween 80 and 0.4mM veratryl alcohol were added in addition. Cultures consisted of 30ml medium aliquots in 100ml Erlenmeyer flasks, or 750ml aliquots in 2L Erlenmeyer flasks and incubated at 30°C at 180 r.p.m. on an orbital shaker.

2.4. Fermentation studies.

Batch cultures of S.pulverulentum were grown using the medium described in 2.3 in a LH series 2000 fermentor (L.H. Engineering Ltd, Stoke Poges, Bucks,

U.K.) with a working volume of 2.5 L. Cultures were stirred at 200 r.p.m. and the temperature was maintained at $30^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. The pH was maintained 4.5 ± 0.2 either by the automatic addition of 1 M KOH/1M HCl or by the use of 2,2-dimethylsuccinate as an internal buffer.

2.5. Spectrophotometric procedures.

Routine spectrophotometric determination of protein and ammonia concentrations were carried out on a Pye Unicam SP1800 U.V./Vis recording spectrophotometer. All enzyme assays were performed at 30°C in 0.7ml reaction volumes using 1cm light-path quartz cuvettes in a Pye-Unicam SP8-200 double beam spectrophotometer with integral chart recorder.

2.5.1. Protein.

Protein was assayed at 595nm using commercially available Bio-rad reagent (Bio-rad Ltd, Watford, Herts, U.K.) and bovine serum albumin as a protein standard. Samples containing tween 80 were first treated with SM2-Biobeads (Bio-rad Ltd, Watford, Herts, U.K.) at $0.5\text{gml}^{-1}\text{hr}^{-1}$ at 4°C to remove detergent which is a major cause of interference with all dye-based protein

assays.

2.5.2. Ammonia.

Ammonia was assayed at 625nm using a commercially available Chaney & Marbach (1962) modification (BDH Chemicals, Poole, Dorset, U.K.).

2.5.3. Lignin peroxidase (LiP).

LiP activity was measured using a modified version of that proposed by Tien & Kirk (1984). The enzyme preparation was incubated with 0.4mM veratryl alcohol, 0.1M sodium tartrate buffer pH3 and 0.25mM H₂O₂ which was added to start the reaction. The formation of veratraldehyde ($E_{310} = 9,300 \text{ M}^{-1}\text{cm}^{-1}$) from veratryl alcohol (fig 1.7.) was measured by following the increase in absorbance at 310nm.

This spectrophotometric method was also used to measure the oxidation by LiP of the benzyl alcohols 2,4-dimethoxybenzyl alcohol (E_{310} 2,4-dimethoxybenzaldehyde = $11,200 \text{ M}^{-1}\text{cm}^{-1}$) and 2,5-dimethoxybenzyl alcohol (E_{350} 2,5-dimethoxybenzaldehyde = $4,500 \text{ M}^{-1}\text{cm}^{-1}$).

Aerobic oxidation of benzyl alcohols by LiP was achieved by using 20ml reaction volumes in 100ml Erlenmeyer flasks flushed with oxygen, sealed with Suba-Seals and incubated in a orbital shaker/water bath

at 200 r.p.m. Further aliquots of H_2O_2 were added periodically, when the reaction rate (as measured by an increase in absorbance at 300-350nm) slowed down.

2.5.4. Lyophilised LiP.

For the determination of lyophilised LiP activity, a modification of the spectrophotometric assay as described above was applied. LiP in 50mM sodium tartrate buffer pH3 was lyophilised onto the bottom of 50ml Erlenmeyer flasks and 20ml of solvent was added. Stock solutions of veratryl alcohol and H_2O_2 were made up in the chosen solvent. 1ml aliquots were taken out and assayed at 310nm every 1 minute and then replaced in the reaction vessel.

2.5.5. Peroxidative iodide oxidation.

The enzyme preparation was incubated with 1.7mM KI, 50mM sodium acetate buffer pH3 and 0.27mM H_2O_2 which was used to start the reaction. The formation of I_3^- was measured by following the change in absorbance at 353nm ($I_3^- E_{353} = 22,900M^{-1}.cm^{-1}$).

2.6.1. Polyacrylamide gel electrophoresis.

Analytical polyacrylamide gel electrophoresis (PAGE) was carried out on vertical 5-20% linear gradient gels (2mm x 150mm x 180mm) using a 5% stacking gel. A discontinuous buffer system was used consisting of 0.375M tris/HCl pH 8.8 resolving gel buffer, 0.125M tris/HCl pH 6.8 stacking gel buffer and 0.025M tris/glycine pH 8.3 reservoir buffer. All buffers were supplemented with 0.1% sodium dodecyl sulphate (SDS) and protein samples were treated with α -mercaptoethanol and 0.2% SDS for 2 mins at 100°C prior to loading onto the gel.

For the determination of molecular weights, protein samples were run against Dalton Mk VII molecular weight standards (Sigma Chemical Co Ltd, Poole, Dorset, U.K.), which consisted of the following proteins: Bovine albumin (66,000Kd), egg albumin (45,000Kd), glyceraldehyde-3-phosphate dehydrogenase (sub-unit 36,000Kd), carbonic anhydrase (29,000Kd), trypsinogen (24,000Kd), trypsin inhibitor (20,100Kd) and β -lactalbumin (14,200Kd).

Gels were run at 40mA constant current. On completion of electrophoresis, gels were then stained.

2.6.2. Staining of Polyacrylamide gels.

Coomasie blue staining.

The staining solution consisted of 45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) Coomasie Brilliant Blue R250. Gels were immersed in stain (500ml/gel) on a shaking platform for up to 5 hours. Destaining was carried out using a 45% (v/v) methanol/10% glacial acetic acid (v/v) mix until the background was acceptable. The gels were then preserved in 7% glacial acetic acid.

Silver staining.

The method of Wray et.al. (1981) was chosen because of its simplicity and rapidity. Briefly, the method was as follows:

- 1) Soak overnight in 50% (v/v) Analar methanol followed by five half hour changes in 50% (v/v) Analar methanol.
- 2) The stain solution was prepared as follows:
1.6g of silver nitrate (Johnson Matthey Chemicals, Royston, Hertfordshire, U.K.) was dissolved in 8ml of deionised water to produce solution A. 21ml of 0.36% (w/v) sodium hydroxide solution was mixed with 2.8ml of 14.8 M ammonium hydroxide solution to produce solution B. Solution A was added dropwise to solution B with constant vortexing and finally made up to 200ml with

deionised water.

3) The gel was soaked in the stain solution for 15 minutes.

4) The gel was thoroughly washed in deionised water 3 x 5 minutes.

5) The gel was then soaked in developer solution until bands appeared (usually up to 15 minutes). The developer solution was prepared by mixing 2.5ml of 1% (w/v) citric acid and 0.15ml of 37% (w/v) formaldehyde solution and making the volume up to 500ml.

2.6.3. Photography of gels.

Gels were photographed on a light box using Panatomic X (32 ASA) 35mm monochrome film which was developed in Kodak D19 developer for 3 minutes and fixed in kodafix for 5 minutes. A yellow filter was used to improve the contrast with Coomassie Blue stained gels.

2.7. Gas chromatography.

LiP oxidation of benzyl alcohols was quantified using a Pye series 104 gas chromatograph with a flame ionisation detector (Pye Unicam, Cambridge, U.K.) connected to a Hewlett-Packard 3380A integrator

(Hewlett-packard, Avondale, Pa., U.S.A.).

5 μ l aqueous or ether-based samples were injected through an inlet onto a 4mm x 1.5m column packed with Cellite 545S (Simadzu Corporation, Kyoto, Japan). Nitrogen was used as a carrier gas at a flow rate of 30ml.min⁻¹. The oven temperature was maintained at 150°C and the flame ionisation detector was heated to 215°C.

2.8. Gas chromatography / Mass spectroscopy.

Lip oxidation products were determined using a MFC 500 Carlo Erba high resolution capillary gas chromatograph linked to a Kratos MS25RFA mass spectrometer.

Samples in an aqueous reaction mixture were extracted with ethyl acetate, concentrated on a Buchi RE111 Rotavapor rotary evaporator, then dried using desiccated magnesium sulphate before loading onto the GC column.

The gas chromatograph was fitted with a 25QC3/BP1 2.0 non-polar column or a 25QC3/BP20 0.5 polar column, depending on the substances to be resolved. The injection volume was 1.8 μ l, with a split ratio of 1:2 and a flow rate of 1mlmin⁻¹.

The mass spectrometer was set to the following conditions: Source 70eV, source temperature 200°C,

injector temperature 240°C, mass range 28-800, resolution 1000, source pressure 10⁻⁵ torr.

2.9. High Performance Liquid Chromatography (HPLC)

Fine resolution of LiP into isozymes was determined by ion-exchange HPLC using a Beckman HPLC system consisting of a 421A controller, two 114M solvent delivery modules and a 160 absorbance detector linked up to a Pharmacia FRAC-300 fraction collector.

Crude LiP was added to a TSK-Gel DEAE 2SW column equilibrated with degassed 20mM sodium tartrate buffer pH 4.5. Typically, a one- or two-step gradient of a 0-200mM MgCl₂ was applied with a flow rate of 1mlmin⁻¹. The detector was set to measure absorbance at 280nm and fractions were taken every minute.

Further isozyme resolution was achieved by applying partially-purified LiP to a Biogel HPHT (hydroxyapatite) column equilibrated with degassed 10mM potassium phosphate buffer pH 5.9. A gradient of 10-350mM potassium phosphate was used, with a flow rate of 0.5mlmin⁻¹ and a sampling time of 2 minutes.

2.10. Chromatofocusing.

Crude protein was dialysed overnight against 0.025M piperazine-HCl buffer pH5 and applied to the top of a 1 x 20 cm PBE 94 column (Pharmacia, Uppsala, Sweden) degassed and pre-equilibrated in 0.025M piperazine-HCl buffer pH5. To ensure even sample application, 2cm of Sephadex G-25 was layered on top of the bed. The column was eluted at 25mlmin^{-1} with polybuffer 74-HCl pH 2.6 for 12 column volumes giving a decreasing pH gradient of 4.5-2.8. The eluent was monitored for protein at 280nm (LKB 4, Bromma, Sweden). Individual fractions containing protein were assayed for LiP activity.

2.11. Gel filtration

The molecular weight of polycatechol was determined using an LH20 gel filtration column (V_t 80ml) equilibrated with 10mM borate buffer pH10. The following dye standards were used to calibrate the column: methyl orange (327 m.w.), indigo carmine (466 m.w.) and light green (792 m.w.).

The void volume was calculated by the use of blue dextran. To estimate the molecular weight, K_{av} (the fraction of the stationary gel volume which is available for a given solute species) was plotted

against log molecular weight.

$$K_{av} = \frac{(V_o - V_e)}{(V_t - V_o)}$$

Where V_o is the void volume , V_e is the elution volume and V_t the total column volume.

2.12. Ultrafiltration.

LiP-containing glass wool-filtered culture supernatant was concentrated down in a 200ml Amicon ultrafiltration chamber with a PM-10 (10kd cut-off) membrane under 40 psi nitrogen. The PM-10 membrane was removed and washed with distilled water after filtration of every 100ml of supernatant because of a marked reduction in flow rate due to polysaccharide build-up.

2.13. Ammonium sulphate precipitation.

50% (w/v) enzyme grade ammonium sulphate was added over a period of 10 minutes to a beaker of glass wool-filtered LiP-containing culture supernatant stirring in ice water. The pH was monitored and adjusted back to pH 4.5. Protein precipitation was removed by centrifugation at 10,000g for 20 minutes. A further 45% (w/v) ammonium sulphate was added to the

supernatant (making 95%, w/v in total) over a period of 10 minutes. After a further 20 minutes, the precipitation was removed by centrifugation. LiP was resuspended in 20mM sodium tartrate pH 4.5.

2.14. Chemicals.

Most compounds were obtained from the following suppliers: BDH Chemicals, Poole, Dorset, U.K.; Fisons Scientific Apparatus, Loughborough, Leics, U.K.; Sigma Chemical Co Ltd, Poole, Dorset, U.K.; Aldrich Chemical Company Co Ltd, Gillingham, Kent, U.K.

2.15. Gases.

Oxygen, helium, nitrogen, hydrogen and carbon monoxide were obtained from the British Oxygen Co Ltd, London, U.K. Ultra high purity oxygen (99.998%) was obtained from BDH Chemicals, Poole, Dorset, U.K.

C H A P T E R 3.

LIGNIN PEROXIDASE (LiP) PRODUCTION IN CULTURES OF PHANEROCHAETE CHRYSOSPORIUM AND SPOROTRICHUM PULVERULENTUM.

3.1 Introduction.

Research on LiP has been hindered by the difficulty in producing it in sufficient quantity to enable detailed studies on the enzyme to be undertaken. For example, Faison & Kirk (1985) showed that LiP was expressed transiently and rather frugally by P.chrysosporium ME-446 in shallow stationary cultures (see fig 3.1). In addition, the use of shallow stationary cultures caused problems in scale-up. If LiP is to be studied to any great extent and particularly if the enzyme were to be exploited commercially, then the problems of low titre and scale- must be overcome.

The primary aim of the work reported in this chapter was to develop a reliable method for the production of significant quantities of LiP.

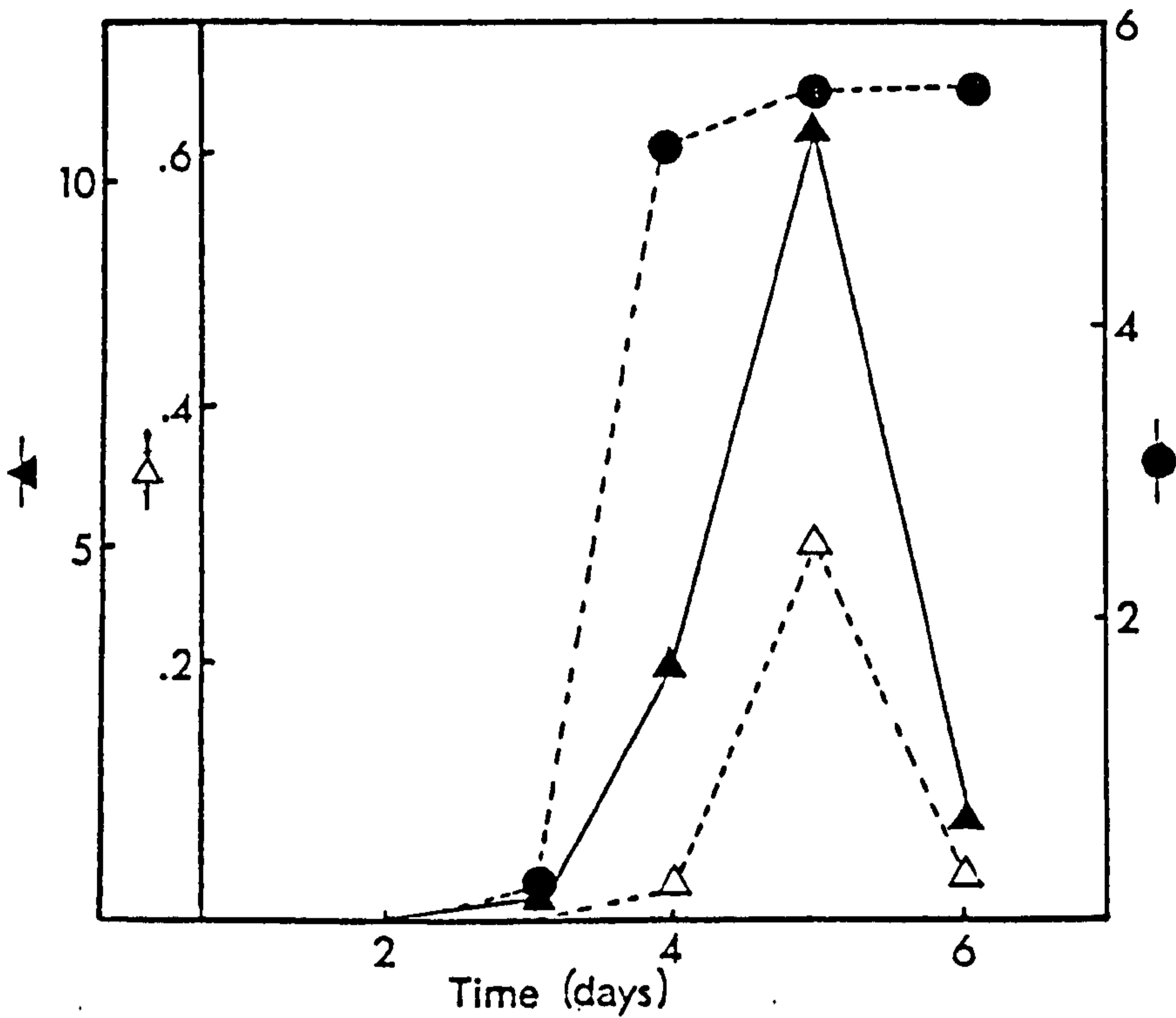


Figure 3.1. Time course for ligninolytic activities in nitrogen-limited cultures of P.chrysosporium (Faison & Kirk, 1985). ● Lignin oxidation to CO₂, (% label day⁻¹ culture⁻¹). ▲ Oxidation of veratryl alcohol (nmol min⁻¹ ml culture supernatant⁻¹). △ Cleavage of β-O-4 model compound (% label min⁻¹ ml culture supernatant⁻¹).

3.2. LiP expression in static cultures.

Initial optimisation studies were carried out based upon growth conditions required for ^{14}C -lignin oxidation to $^{14}\text{CO}_2$ by P.chrysosporium ME-446 developed by Kirk et.al. (1978).

Unless stated otherwise, the following procedure was adopted: The defined medium consisted of 1% glucose, 1.2mM ammonium tartrate, minimal salts and 10mM 2,2-dimethylsuccinate buffer. This medium was adjusted to pH 4.5 with 5M potassium hydroxide and autoclaved. Stock mineral and vitamin solutions (see 2.2) were then added, followed by inoculation with a conidial suspension in 0.1% Tween 80. 10ml aliquots of inoculated medium were added to 100ml Erlenmeyer flasks which were then sealed with Suba-Seals and incubated at 37°C . The flasks were flushed with oxygen on days 3 & 6. 0.3ml samples were taken by syringe from triplicate cultures and assayed for LiP activity (see 2.2 for further details).

3.2.1. Screening strains of P.chrysosporium for LiP activity.

Nitrogen-limited glucose-containing cultures of P.chrysosporium have been reported to possess LiP activity (Faison & Kirk, 1985, fig 3.1). These conditions were used as a positive control to establish

LiP production. Cultures of P.chryso sporium ME-446, BKM 1767, CMI 691, CMI 693 and, at a later date, S.pulverulentum were screened for LiP activity under these conditions. Generally, activity appeared in LiP positive strains on days 3-4 after inoculation, two days after nitrogen depletion. Activity reached a maximum on days 4-7 and disappeared within 2-3 days (see fig 3.2 a-d). The best strain for LiP production was S.pulverulentum novabranova whose LiP activity reached a maximum very early (day 4) with a titre of 15UL^{-1} . The LiP titre of this strain, unlike the other strains, very gradually disappeared after reaching peak activity (well over 6 days instead of 2-3 days). However, this strain was not investigated initially, so P.chryso sporium ME-446 (giving a maximum titre of 7.8UL^{-1}) was chosen for the following studies.

3.2.2. The effect of different carbon sources upon LiP production.

Nitrogen excess (24mM) cultures of P.chryso sporium ME-446 containing one of each of the following carbon sources: glucose, glycerol, mannitol, sorbitol, xylitol, ethane diol, propane diol and sodium acetate (all at 1% w/v) were screened for LiP activity.

The use of sugar alcohols as carbon sources was based upon the work of Buswell et.al. (1984) who found

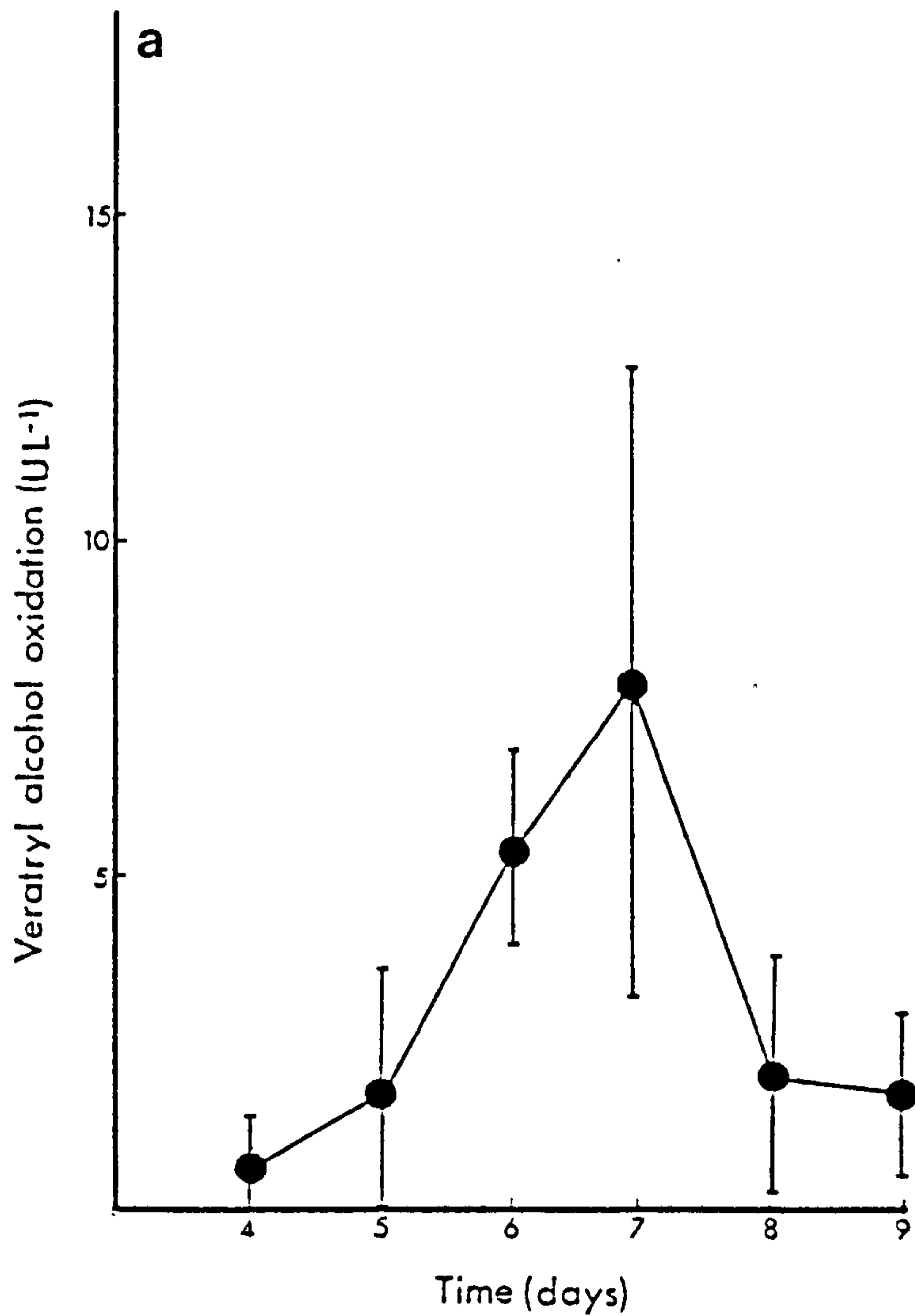


Figure 3.2. Time course for LiP activity in nitrogen-limited cultures of *P. chrysosporium* ME-446 (a), BKM 1767 (b), CMI 693 (c) and *S. pulverulentum* novabranova (d) utilising glucose (1%) as the carbon source.

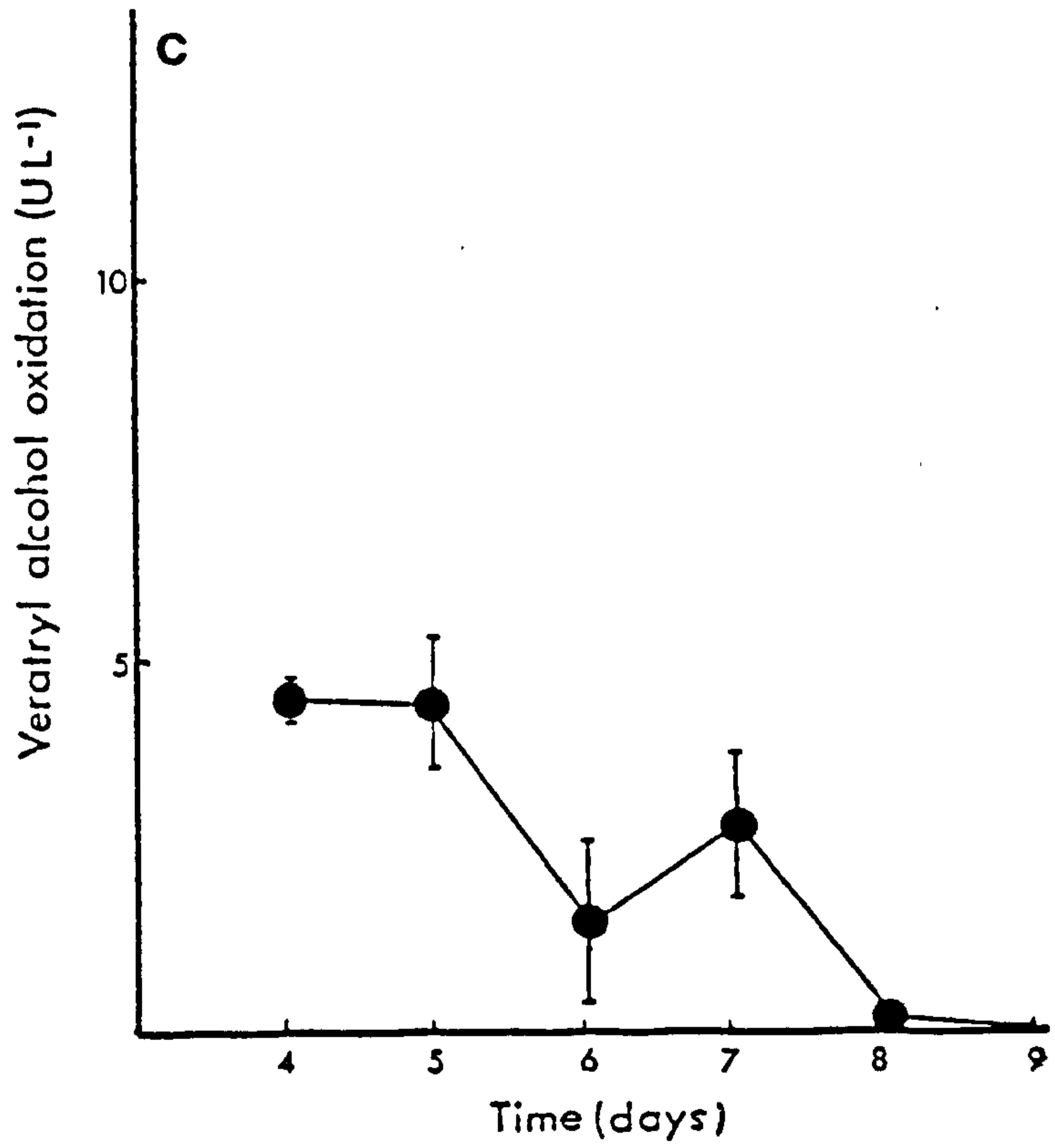
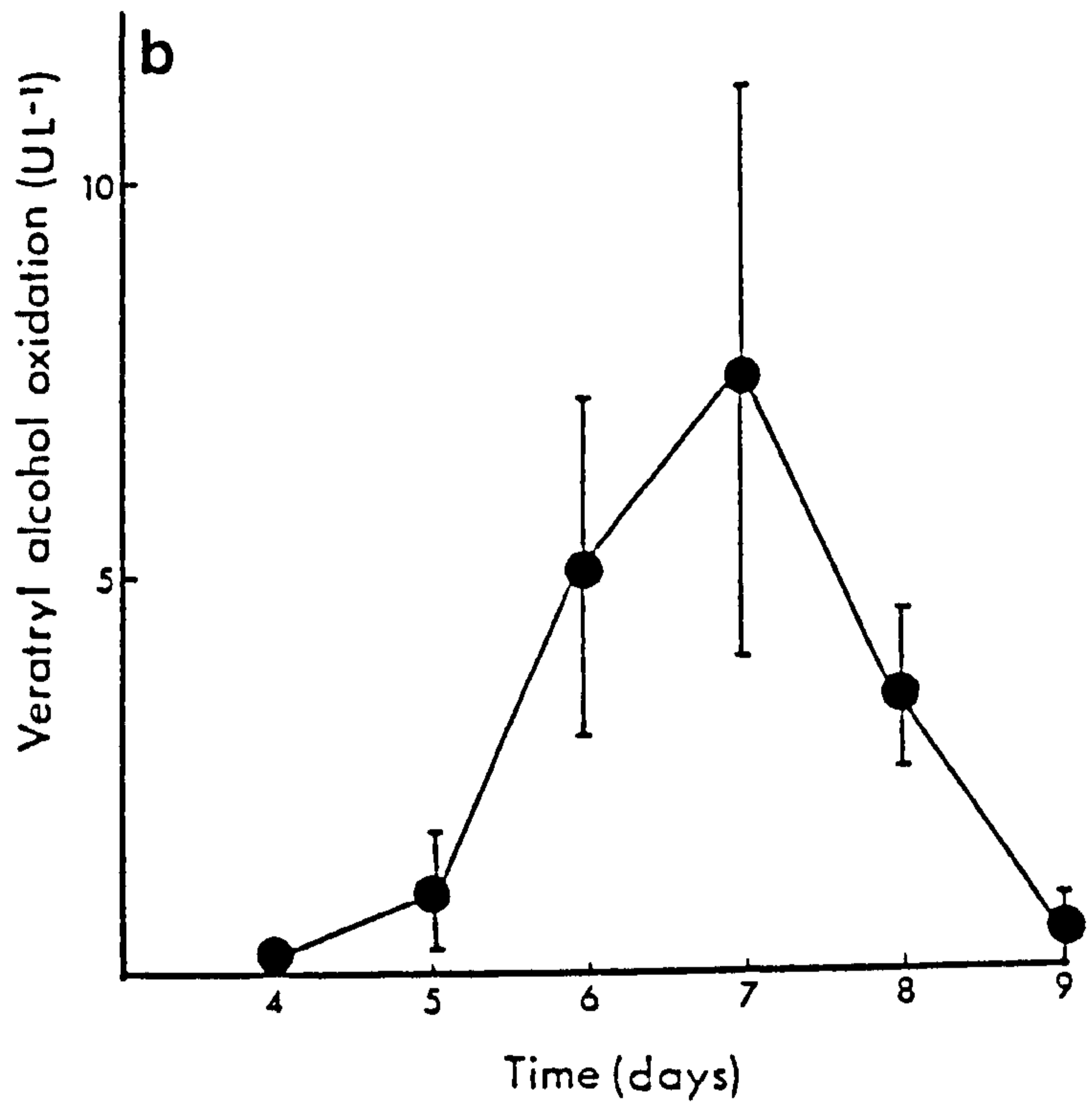


Figure 3.2. (Continued).

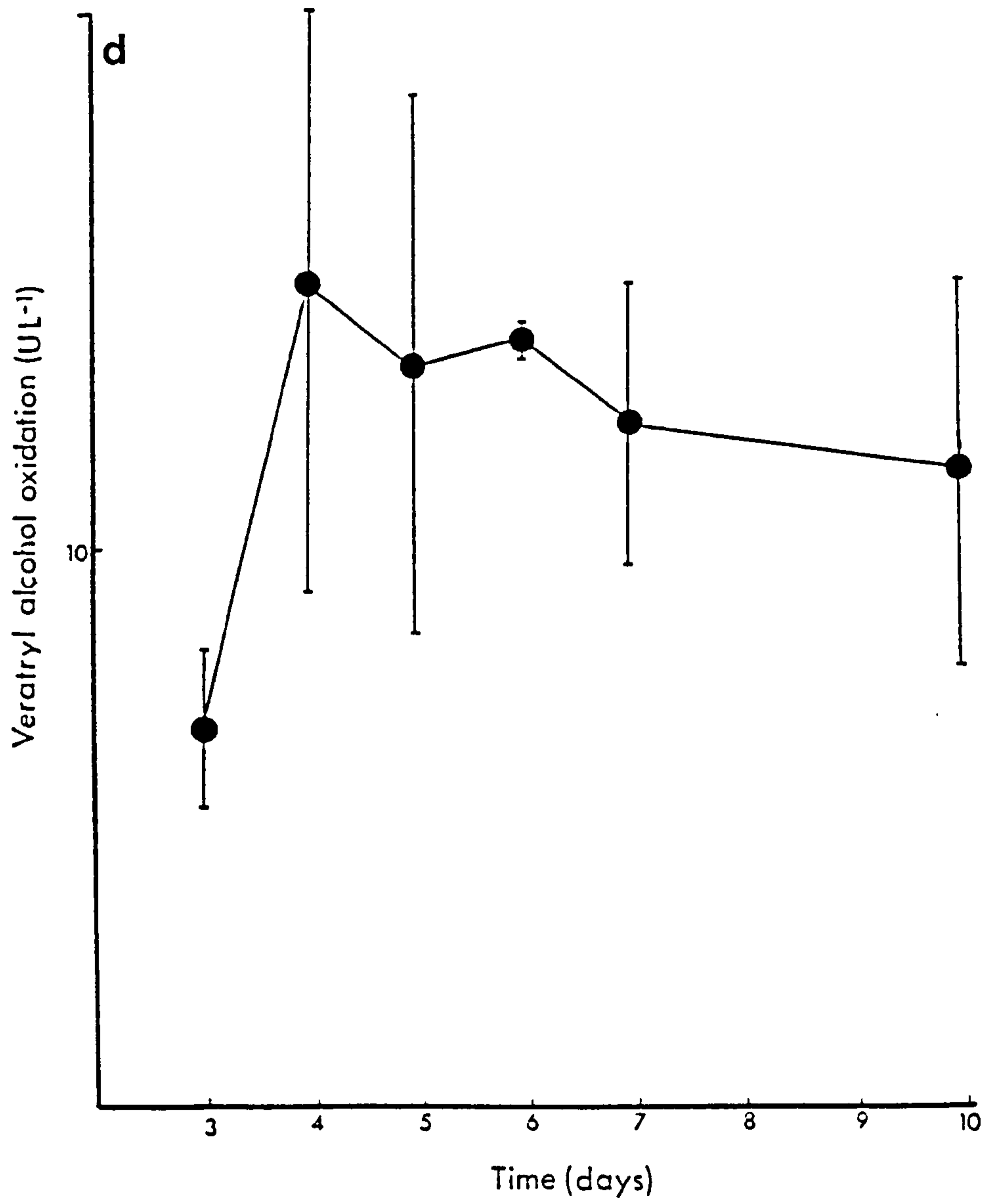


Figure 3.2. (Continued).

that P.chrysosporium INA-12 (a derivative of ME-446) produced high levels of LiP in nitrogen-sufficient cultures with glycerol as the carbon source.

Figures 3.3 (a-c) show that mannitol-containing cultures yielded the highest titres of LiP, 15.7 UL^{-1} compared to 4.9 UL^{-1} and 1.0 UL^{-1} for glycerol- and sorbitol-containing cultures respectively. In addition, maximal LiP titre using mannitol as the carbon source was achieved on day 4 (or earlier), at least 2 days before maximum LiP titre in glycerol or nitrogen-limited glucose containing cultures.

No LiP was produced in xylitol and sodium acetate based cultures, almost certainly due to the poor growth on these substrates. No growth at all was obtained with propanediol- and ethanediol-containing cultures.

Glucose-based cultures grown under nitrogen sufficient conditions (24mM) did not contain any LiP activity up to day 14. Faison & Kirk (1985) found that addition of nitrogen (in the form of NH_4^+ or glutamate) to cultures that were already producing LiP caused strong suppression of activity. This corresponds with the well established idea that lignin degradation is a secondary metabolic event which can be triggered by nitrogen or carbon limitation (Kirk et.al., 1978; Keyser et.al., 1978). The use of excess nitrogen in glycerol- or mannitol-based cultures did not prevent expression of LiP activity. Buswell et.al. (1984)

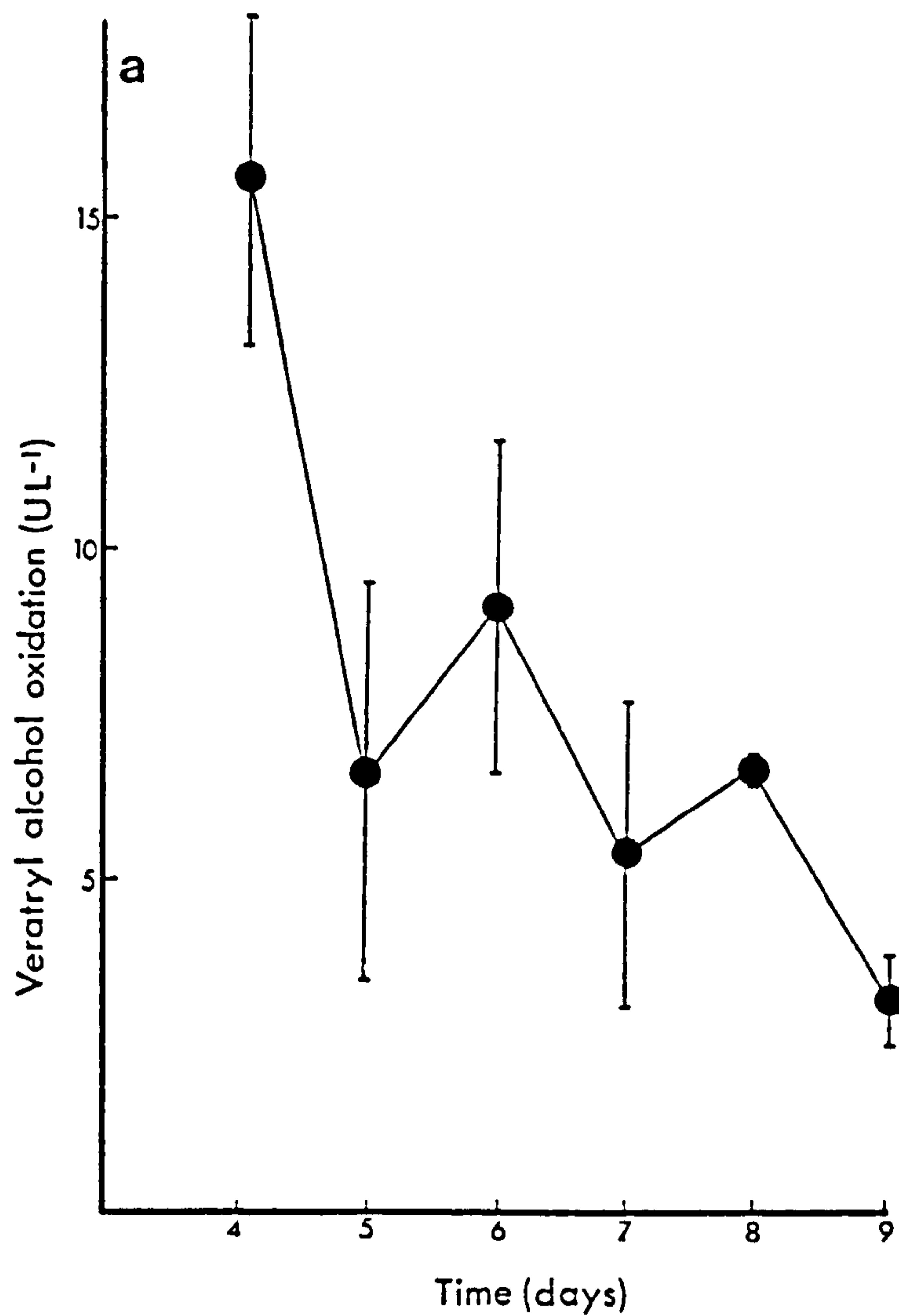


Figure 3.3. Time course for LiP activity in nitrogen-excess cultures of *P. chrysosporium* ME-446 containing mannitol (a), glycerol (b) and sorbitol (c) (all at 1%) as carbon sources.

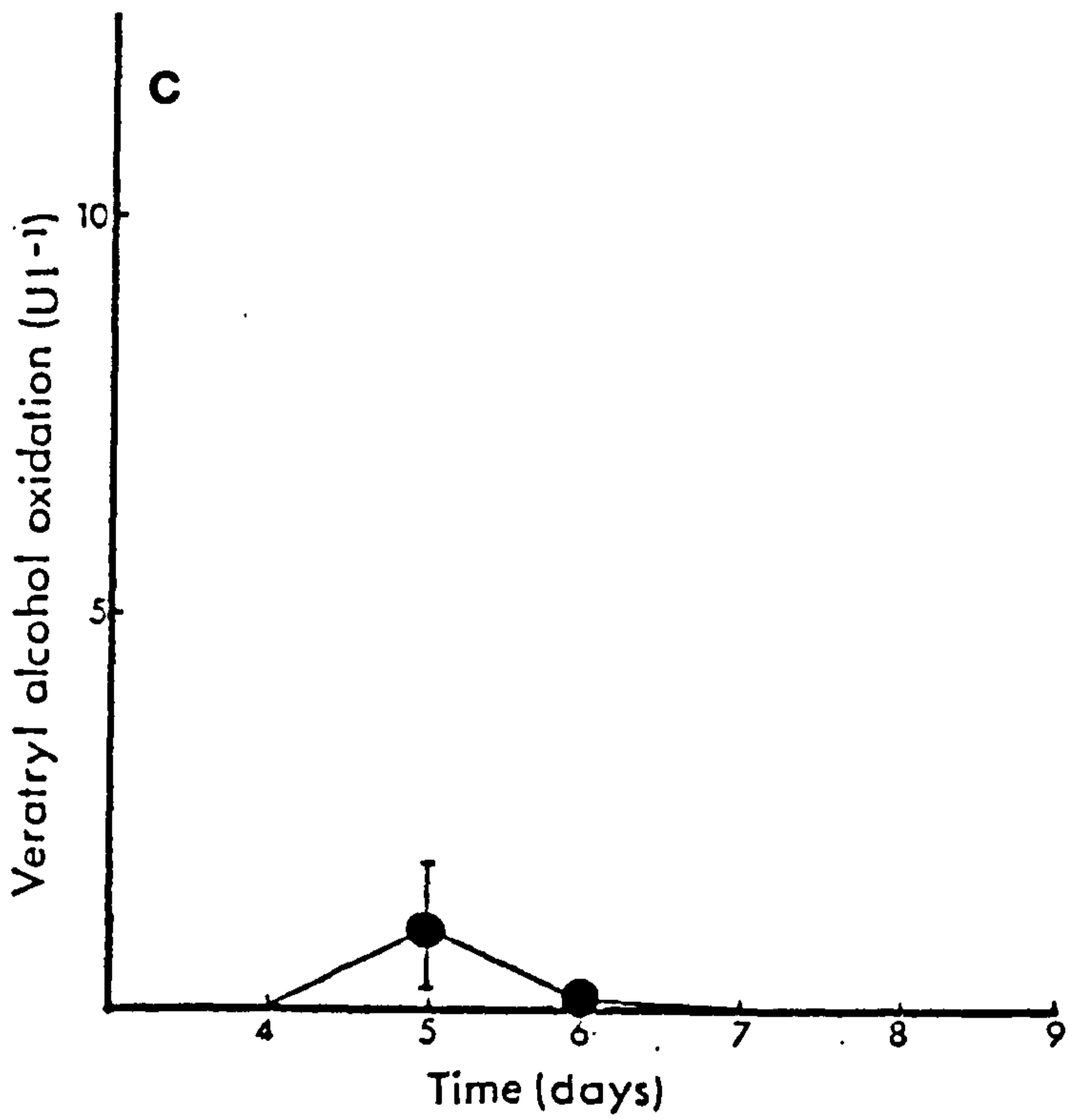
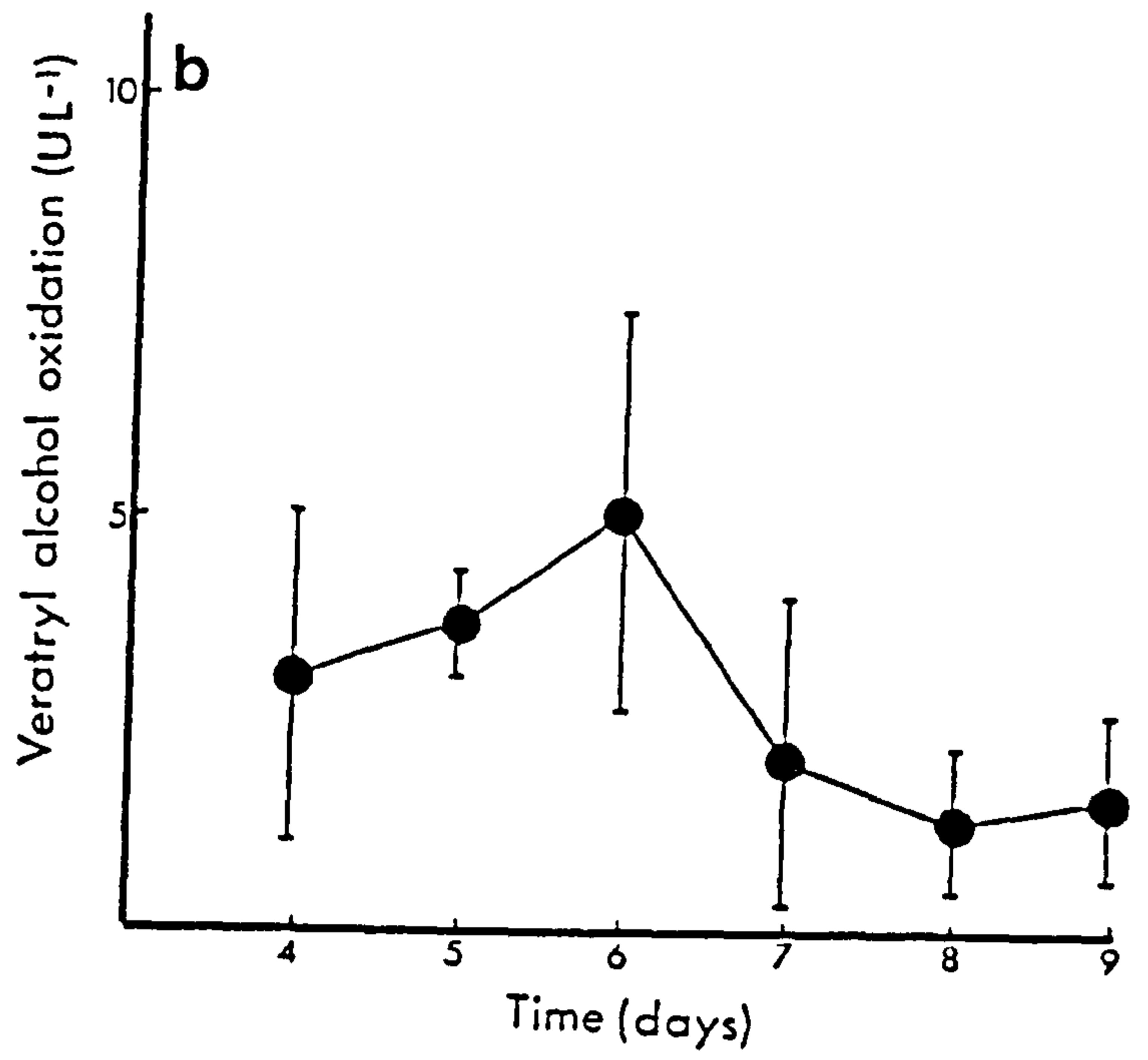


Figure 3.3. (Continued).

reported that the poor growth of INA-12 when glycerol served as a carbon source, linked with the appearance of the idiolite veratryl alcohol, suggested that such cultures were in idiophase. Therefore, cultures using mannitol, glycerol or sorbitol were probably carbon-limited because of their inability to efficiently assimilate carbon rather than because of insufficient carbon levels in the medium.

3.2.3. The effect of different nitrogen sources on LiP production.

The concentration and type of nitrogen source used for LiP production was investigated using static cultures of P.chrysosporium ME-446. The type of nitrogen source used (ammonium tartrate, ammonium chloride, urea, potassium nitrate, proline or casein hydrolysate all at 2.4mM with respect to nitrogen) did not affect the maximum LiP titre to any great extent. This makes an interesting comparison with the work done by Kirk et.al. (1978) who found that the source of nutrient nitrogen did not significantly influence the rate of ¹⁴C-lignin degradation, further implying that LiP production and lignin degradation are closely linked.

LiP activity was not found in cultures containing

proline or potassium nitrate until day 10 (3-4 days later than the other nitrogen sources). However, thick mycelial mats were not established until day 9 for cultures grown on these nitrogen sources, suggesting that the time lag was due to initial assimilation of nitrogen rather than some specific nitrogen source suppression of LiP production.

In addition to excess ammonium tartrate, excess (24mM) urea and ammonium chloride prevented LiP expression up to at least day 14. Excess casein hydrolysate did not delay the onset of LiP activity, but did cause a dramatic reduction in LiP titre (see fig 3.4).

The use of excess glutamate as the nitrogen source for mannitol-containing cultures caused complete suppression of LiP activity. The addition of exogenous nitrogen to ligninolytic cultures of P.chrysosporium, particularly in the form of glutamate has been shown to suppress ¹⁴C-ligninolytic activity (Fenn & Kirk, 1981; Fenn et.al., 1981, see 1.6.4.). In the case of mannitol-containing cultures, the use of glutamate even overrided the effect of carbon-limitation, which is further evidence to support the idea that glutamate metabolism plays a primary controlling role in LiP expression (see 1.6.4.).

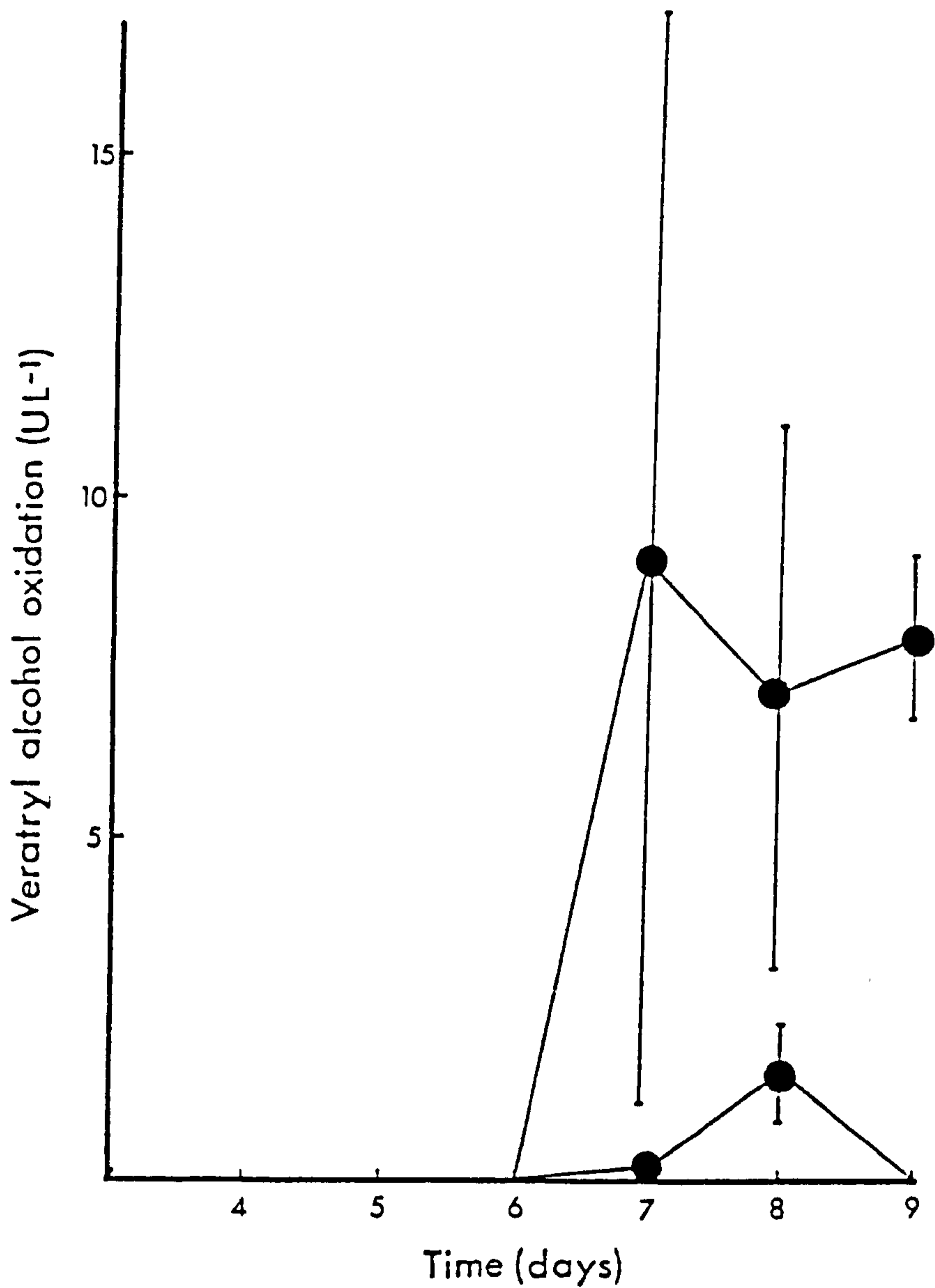


Figure 3.4. Time course for LiP activity in cultures of P.chrysosporium ME-446 using casein hydrolysate at 2.4mM (I) and 24mM (II) as the nitrogen source.

3.2.4. Problems associated with LiP production in static cultures.

A major hindrance in the development of a reliable method of obtaining LiP was the capricious nature of the organism. LiP production under a given set of conditions could not always be duplicated. Titres varied widely between identical cultures and some cultures would not produce any LiP. At one stage, five months passed without the detection of any LiP at all. During this time, practically every conceivable culture parameter was investigated:

- i) All LiP assay and medium stock solutions were systematically replaced with fresh stocks. However, no LiP activity was measured.
- ii) To check for any inhibitory effects that the medium components may have had, concentrations of various components were replaced with alternatives and/or their concentrations altered. For example: the concentration of 2,2-dimethylsuccinate buffer was lowered, firstly from 20mM to 15mM and then to 10mM; the buffer was also replaced by phthalate and then by tartrate buffer.

Reid et.al. (1985) found that some tanks of welding grade oxygen contain unspecified impurities that were toxic to P.chrysosporium, so standard grade oxygen was replaced by high purity (99.998%) oxygen. The nitrogen concentration was altered (5.6mM-1.2mM) and different nitrogen sources were used (ammonium chloride, urea,

potassium nitrate). The vitamin and mineral stock solution concentrations (see 2.2) were altered (0.1 - 10 fold) and different culture oxygenation patterns were used (e.g. cultures were not gassed until day 3, or cultures were gassed every day). In each instance when conditions were varied, no detectable levels of LiP were observed.

(iii) The original freeze-dried cultures were reinvestigated and used as the inoculum in case constant sub-culturing of the original strains resulted in degeneration of ligninolytic capacity. Again no LiP activity was detected.

A partial explanation for the variation in LiP levels amongst identical cultures and experiments was found to be the way in which samples were taken from the same cultures (in triplicate) every day. The LiP titres measured were systematically lower upon every re-sampling of a culture when compared with the control (once only sampled) cultures (see fig 3.5). Agitation of cultures is already known to suppress LiP production (see 3.3.1.). However, it was apparent from this experiment that even the slightest disturbance of a culture (unavoidable, even when carefully sampling the supernatant with a catheter) was enough to cause a reduction in LiP activity over the next 24hrs.

One reason for the loss in activity could be the

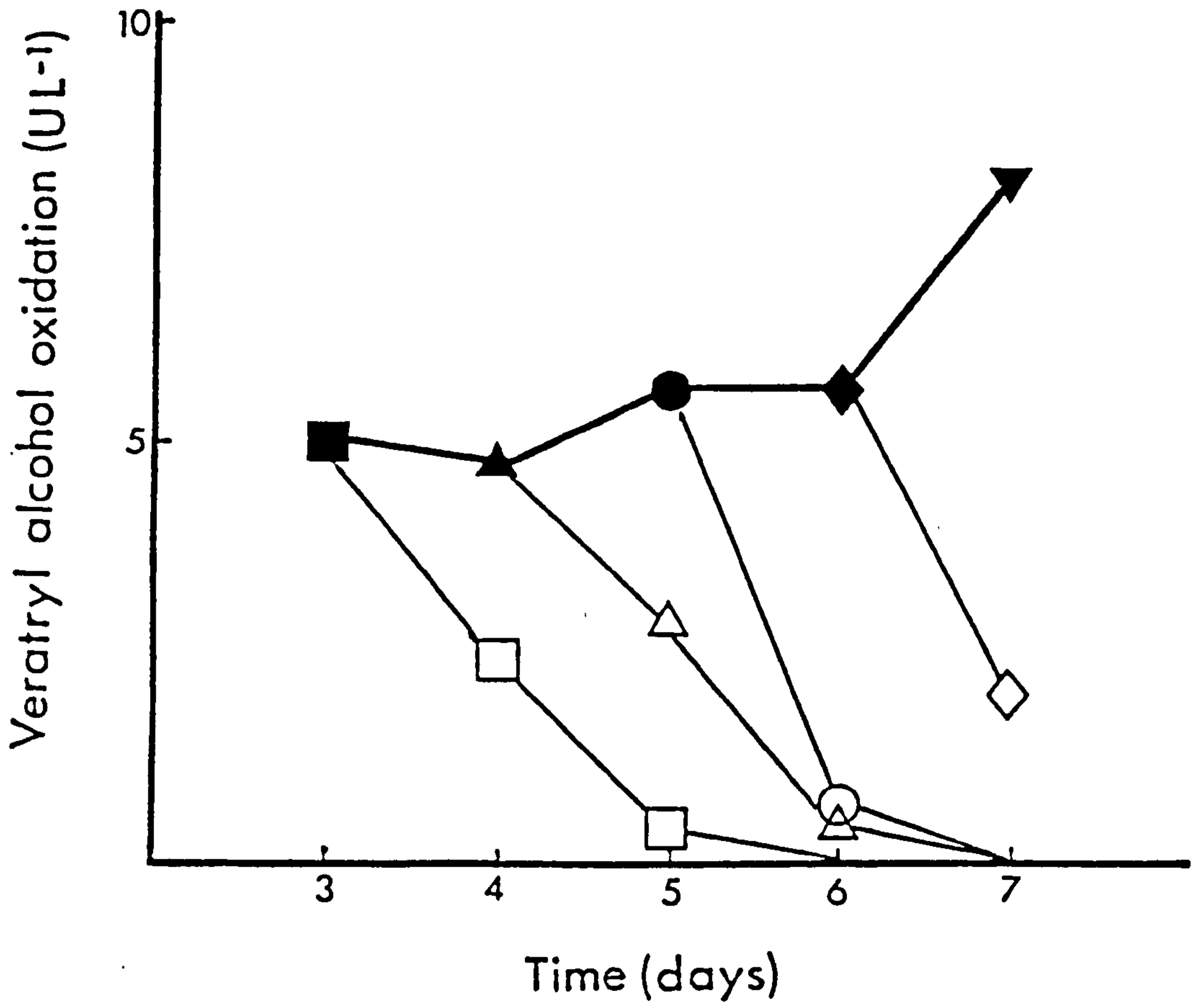


Figure 3.5. Time course for LiP activity in nitrogen-limited glucose(1%)-containing cultures of P.chrysosporium ME-446. Shaded shapes donate triplicate virgin-sampled cultures. Open shapes represent the same triplicate culture sets after being sampled more than once.

release of protease. The addition of the protease inhibitor phenylmethylsulfonylflouride (PMSF) to LiP-producing cultures did not decelerate the loss in LiP titre upon resampling, implying that protease release was not an important factor. The loss in LiP titre could be due to re-adsorption or the entrapment of the protein in the polysaccharide layer caused by the physical movement of the mycelial mat. Alternatively, culture movement may have affected secondary metabolism in general, preventing de novo LiP synthesis. The drop in existing LiP titre could subsequently be caused by protein-turnover primarily by thermal denaturation.

The change in sampling technique from continuous re-sampling of the same cultures to one-off sampling of individual cultures was certainly responsible for an improvement in reproducibility of LiP titres. However, it could not be wholly responsible for the five month period where no LiP could be detected.

Later, it was found that good titres of LiP could be achieved if de-ionised water was used instead of distilled water in the culture medium. One possible explanation was that compounds capable of affecting LiP activity or production were inefficiently removed from water during distillation. The lignin-degrading system appears to be very sensitive to small changes in the environment. High levels of nitrogen (Kirk et.al, 1978; Keyser et.al., 1978) and manganese (Jeffries et.al., 1981) have already been shown to prevent lignin

degradation, but not to affect vegetative growth. In addition, LiP activity can be affected by metal ions. 1mM manganese, molybdenum or iron almost completely inhibited LiP activity and zinc and nickel ions caused a marked reduction in LiP activity (table 3.1.).

If inhibitory compounds were present in the distilled water, they must have been present seasonally, since the LiP was detectable in cultures grown in autumn, but not spring-early summer. Nevertheless, the continued use of de-ionised water prevented the return of this problem, although there was always a small proportion of cultures in an identical batch (approximately 5-20%) that would not yield any LiP activity.

3.2.5. Summary of optimum conditions for LiP production in static cultures.

Cultures of P.chryso sporium ME-446 grown at 37°C in static cultures under nitrogen excess (24mM ammonium tartrate) with mannitol (1%) as the carbon source allowed high titres (up to 16 U L⁻¹) of LiP to be produced. The strategy for further research could involve increasing the specific titre by further alterations of the growth conditions, but it was decided to investigate methods of scale-up, since the former strategy was time-consuming and unpredictable.

Metal ion (1mM)	LiP activity. (relative)
-	1.0
Mg ²⁺	1.0
Cu ²⁺	1.0
Co ²⁺	1.0
Zn ²⁺	0.8
Ni ²⁺	0.6
Mn ²⁺	0.1
Fe ²⁺	0.1
MoO ₄ ²⁻	0.0

Table 3.1. Comparison of the inhibitory effect of metal ions on LiP activity.

3.3. Submerged agitated cultures.

3.3.1. Problems with scale-up of stationary cultures.

Research on LiP has been limited by the need to produce this enzyme in shallow stationary cultures. LiP titres in such cultures of P.chrysosporium are low (typically 5 UL^{-1}) and the alteration of growth conditions has only allowed a 2-3 fold increase in specific LiP titre (see 3.1,3.2). In addition, scale-up of shallow cultures is very labour intensive and it requires large surface areas for the mycelial mats to develop. Also, attempts to scale-up to large flasks or shallow trays have resulted in lower specific titres of LiP (Jager et.al., 1985).

One logical method of scale-up was to develop submerged cultures that could be oxygenated thoroughly by agitation. However, in agreement with Faison & Kirk (1985), agitation of cultures of P.chrysosporium (at 140 and 200 r.p.m. on a gyrorotatory shaker) completely suppressed expression of LiP. Kirk et.al. (1978) noted that lignin degradation itself was suppressed by agitation. They proposed that this was because the low surface area of the mycelial pellets formed under agitation meant that oxygen tensions within the pellets were far too low for expression of the ligninolytic

system. This explanation is unlikely since the following work showed that the maintenance of high surface areas under agitation still suppressed LiP formation:

- i) Static incubation for three days (allowing mycelial mats to be formed) followed by agitation did not allow LiP expression.
- ii) The addition of ballotini (2g x 1mm dia per culture) to break up the growing mycelia into much smaller pellets (>1mm dia instead of 2-5mm dia) did not result in the formation of LiP.
- iii) Slight physical disturbances of stationary cultures of P.chrysosporium ME-446 (see fig 3.5) and BKM 1767 resulted in reductions in LiP titre over the proceeding 24hrs.

3.3.2. LiP production in agitated cultures using an inert support.

Suppression of LiP by agitation was overcome by the inclusion of glass wool (0.2g per culture) as an inert support for the fungus grown at 140 r.p.m. on an orbital shaker. Fig 3.6 shows that maximum LiP titre was comparable with ordinary static cultures, except that the maximum titre occurred nearly three days earlier (compare with fig 3.2a).

Microscopic observations showed that the mycelia

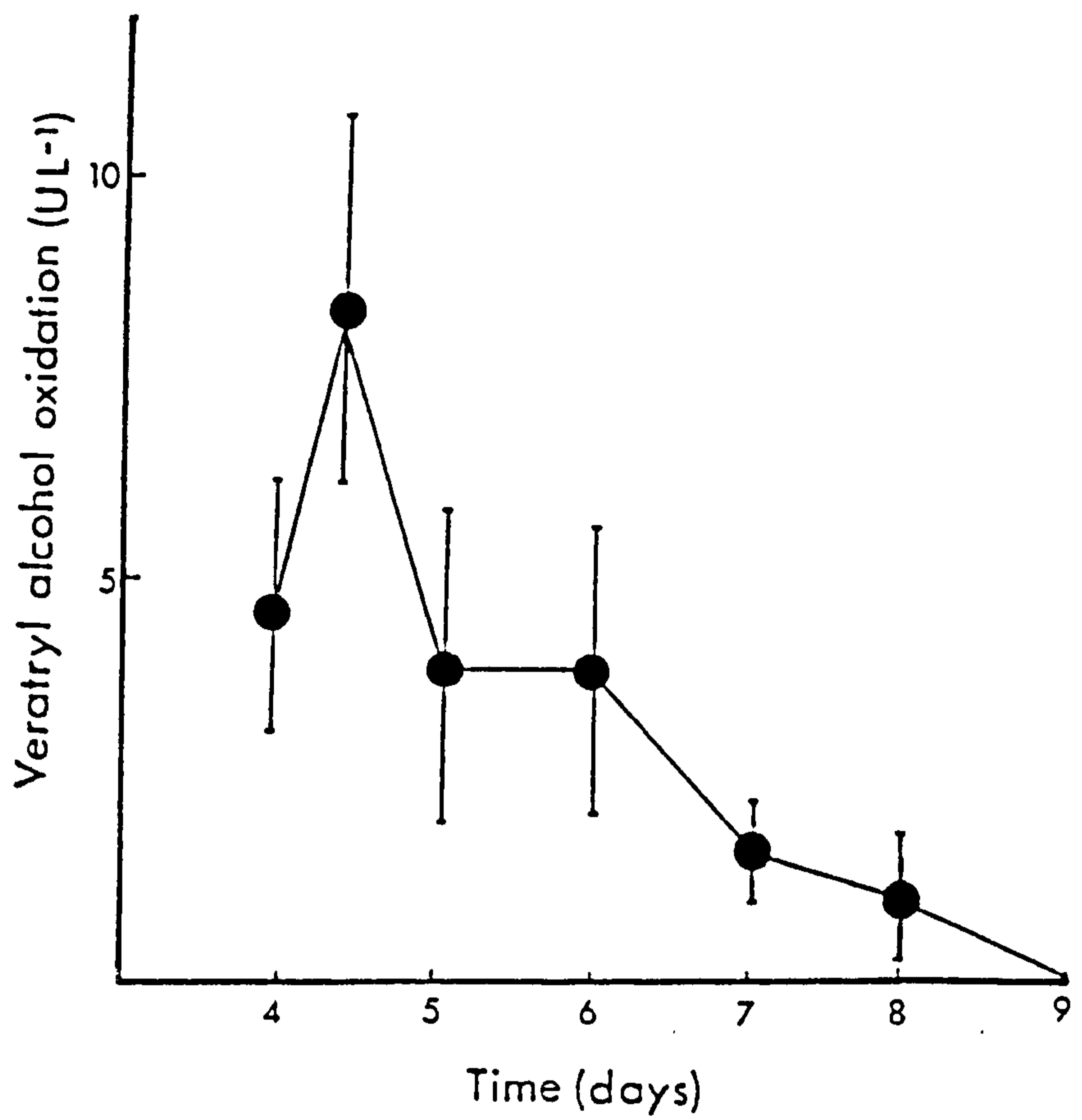


Figure 3.6. Time course for LiP activity in nitrogen-limited cultures of P.chrysosporium ME-446 immobilised onto glass wool (0.2g per 10ml culture) and agitated at 140 r.p.m. on an orbital shaker.

had grown rather diffusely (compared to free mycelial mats or pellets) on and around the glass fibres. There was no evidence of mycelial growth in the free supernatant.

The physical anchorage of the mycelia to the glass wool must be responsible for the production of ligninase under agitation. The diffuse growth of the mycelia could not be responsible for allowing LiP to readily disperse into the supernatant since the washings from ground up dense mycelial pellets did not reveal any LiP activity whatsoever. The nature of LiP production under agitation must therefore have a deeper physiological basis.

Although the potential for LiP production under agitation using immobilised mycelia was evident, no LiP activity was measured using larger cultures (200ml cultures in 2L flasks) containing the glass wool support. These scaled-up cultures contained large amounts of glass wool (2-5g) which clumped tightly together causing a reduction in the surface area available for fungal colonisation. Although an alternative support which could retain its spatial structural integrity could be the solution, it was decided at this stage to investigate LiP production in cultures grown in the presence of detergent (see 3.3.3.).

The potential of immobilised cultures of

P.chrysosporium has recently been realized by Kirkpatrick and Palmer (1987) who used agitated cultures of polyurethane foam-immobilised P.chrysosporium to achieve titres of nearly 32 UL^{-1} (original culture volume) of LiP on a semi-continuous basis.

3.3.3. Production of LiP in submerged agitated cultures containing detergents.

Jager et.al. (1985) reported that the addition of the non-ionic surfactants tween 80, tween 20 or 3[(3-colamidopropyl)dimethylammonio]1-propanesulphonate to agitated submerged cultures of P.chrysosporium permitted the development of LiP activity.

This method of LiP production was investigated further because:

- i) Relative titres of LiP were found to be much higher in these cultures than in ordinary static cultures, for example, P.chrysosporium ME-446 produced 40 UL^{-1} of LiP under agitation compared with 7 UL^{-1} under static conditions (Jager et.al., 1985).
- ii) Such a simple submerged system should facilitate scale-up, leading onto the use of stirred-tank fermentors.

A range of non-ionic and ionic surfactants (tween

80, Brij 35, Nonidet P40, triton X-100, Lubrol, SDS, and deoxycholate) were added at 0.05% v/v to 30ml cultures of P.chrysosporium ME-446. These were incubated at 30°C and agitated at 180 r.p.m. The lower temperature (30°C instead of 37°C) made a negligible difference to LiP titre and was chosen for logistical reasons.

Only the addition of tween 80 allowed good titres of LiP ($>12 \text{ UL}^{-1}$), but some LiP activity was found with Brij 35 (3 UL^{-1}) and triton X-100 (1 UL^{-1}). Growth occurred as small round pellets with all the detergents used except that the fungus grown with triton X-100 was in the form of long (1-3cm x 1-2mm) cylindrical pellets.

Now that a useful detergent for LiP production had been established, a range of P.chrysosporium strains (BKM 1767, ME-446, CMI 691 and CMI693) were screened for LiP production in submerged agitated cultures containing 0.1% tween 80.

Fig 3.7 a-c shows the relative capacities of the P.chrysosporium strains to produce LiP under agitation. CMI 691 failed to express LiP activity up to day 10. The other three strains show 2-3 fold increased maximal specific titres under agitation compared with static conditions (see table 3.2.). The highest activity was found in strain BKM 1767 (30 UL^{-1}). However, maximal titres generally occurred later under agitation (days

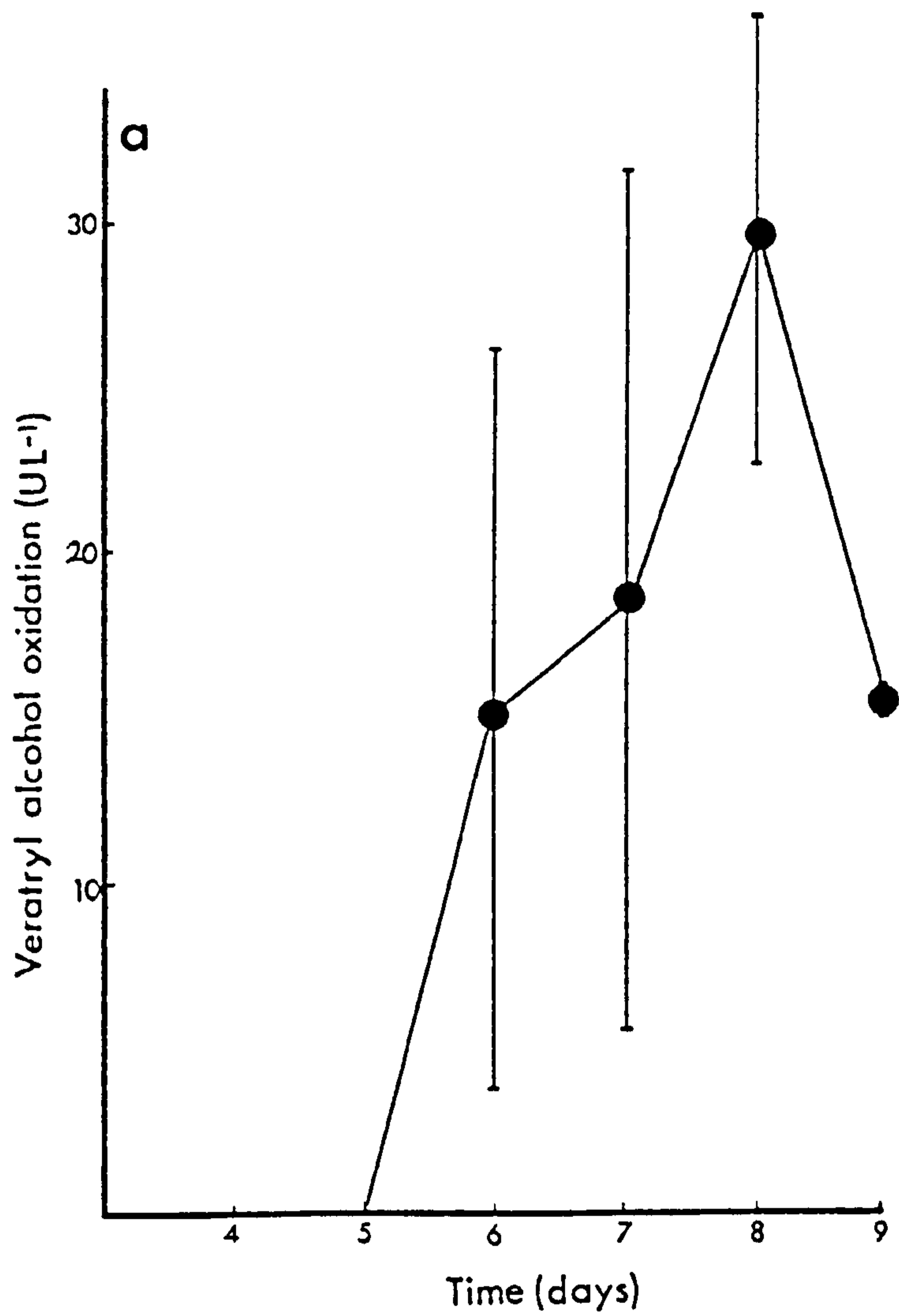


Figure 3.7. Time course for LiP activity in nitrogen-limited cultures of *P.chrysosporium* BKM 1767 (a), ME-446 (b) and CMI 693 (c) agitated at 180 r.p.m. and containing 0.1% tween 80.

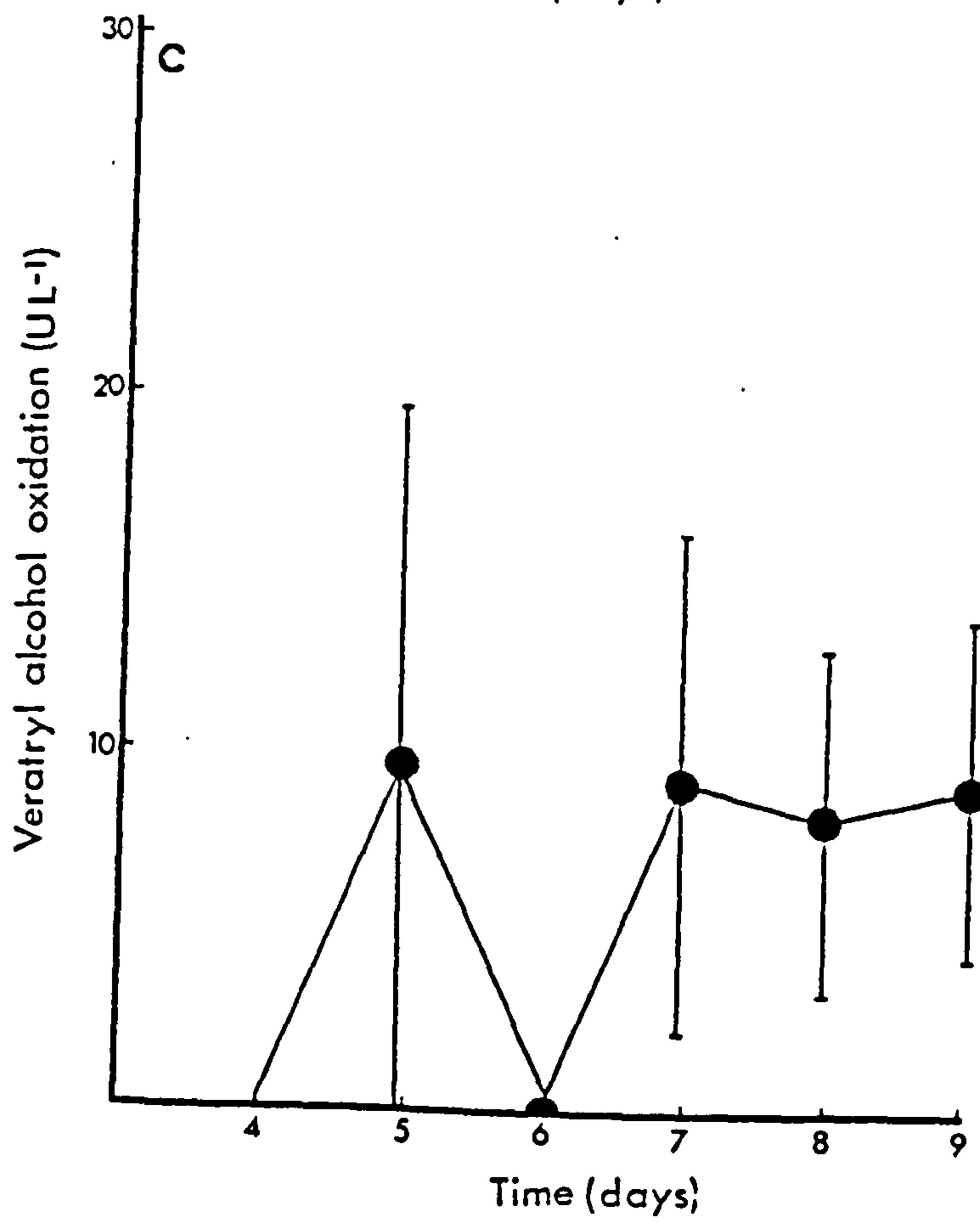
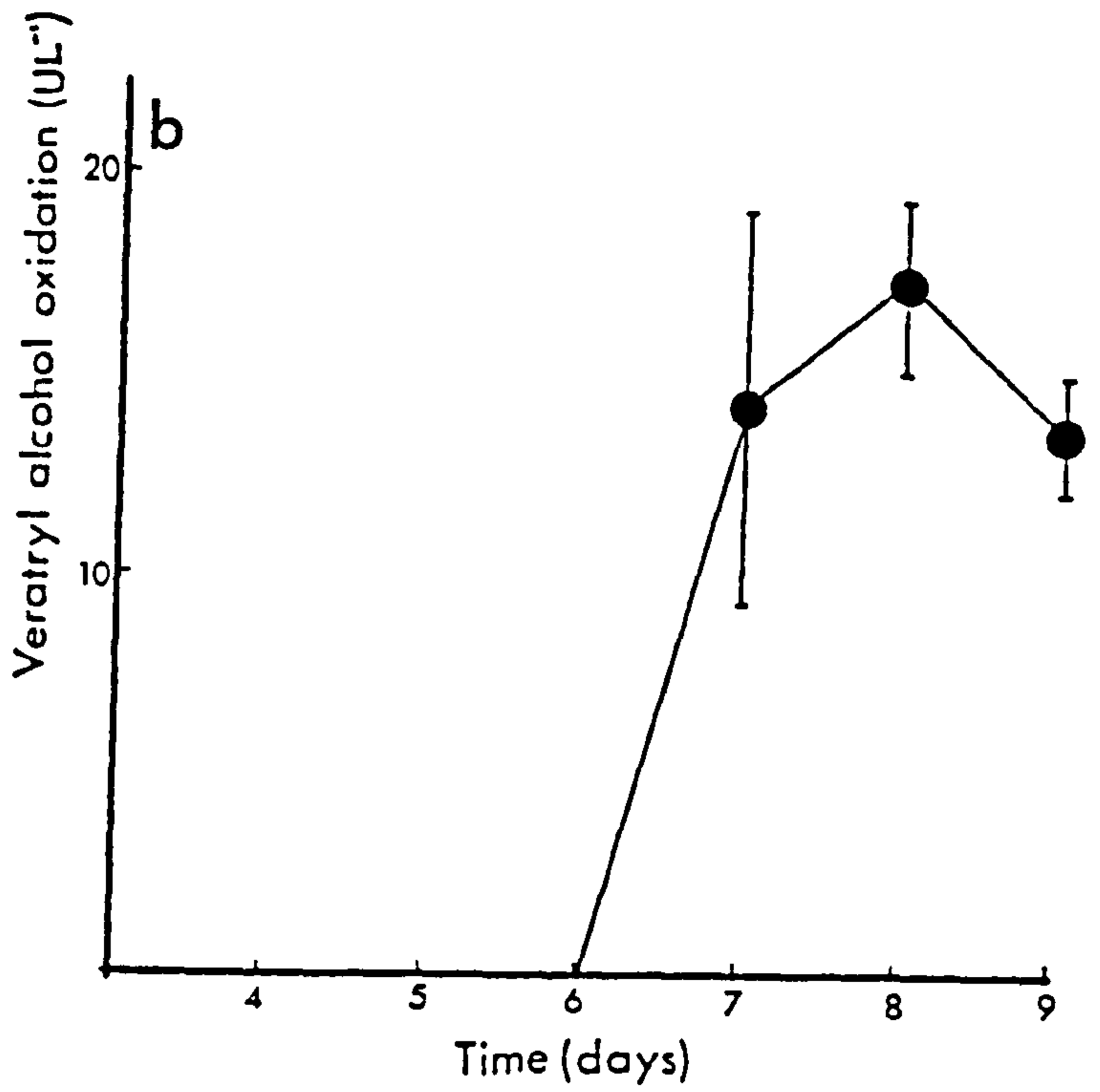


Figure 3.7. (Continued).

Strain.	LiP (U L ⁻¹) Static cultures.	LiP (U L ⁻¹) Agitated cultures.
ME-446	7.8	17.5
BKM 1767	7.6	29.0
CMI 693	4.5	10.0
CMI 691	0	0
<u>S.pulverulentum</u>	15	>15 (scale-up only).

Table 3.2. Comparison of maximum LiP titres produced in static and agitated cultures of selected strains of P.chryso sporium.

7-8) than under static conditions (days 4-6).

The standard deviation (for three replicate cultures) was noticeably larger for cultures under agitation than under static conditions (compare figs 3.2. with figs 3.7.). One explanation is that LiP was released in larger quantities and more suddenly under agitation than in static cultures, perhaps as a result of higher oxygen tensions which are known to enhance LiP titres (Faison & Kirk, 1985). Any loss therefore, in culture synchronicity from the time of inoculation would be more strongly emphasised in the standard deviation results. In addition, pellet formation was not always uniform. This could be argued as a major reason for differences in lignin degradation and LiP formation between identical cultures and different strains. However, observations in this work showed that pellet size was irrelevant to final LiP titre. As an extreme example, a 30ml culture containing one large round pellet (10mm dia) of P.chrysosporium BKM 1767 attained a similar maximum activity (20 UL^{-1}) as two identically inoculated cultures containing 30 - 40 x 1-1.5mm round pellets. Although pellet size may not be a reason for differences in maximal LiP titre, the observation that these originally identical cultures at the time of inoculum were no longer identical could be responsible for a marked difference in the timing of the release of LiP in agitated cultures compared with the apparently uniform mycelial mats produced under

static conditions.

To increase LiP yields still further, mannitol was used in agitated cultures as a carbon source under nitrogen excess since high titres of LiP were obtained using these conditions in static cultures (see fig 3.2a). However, despite a number of attempts growing P.chryso sporium ME-446, BKM 1767 and Sporotrichum pulverulentum under agitation, no LiP activity was found, which again suggested that physical agitation caused marked changes in the metabolism of the fungus.

3.3.4. The role of detergents in agitated cultures.

The actual role of detergents in extracellular enzyme secretion is still not fully understood. Reese & Maguire (1972) found that tween 80 stimulated the secretion of a range of enzymes including cellulases, amylases, β -glucanases and xylanases from different fungal cultures. They postulated that the surfactant acted at the cell membrane to promote the release of extracellular-bound enzymes. However, the effect of detergents upon cellulolytic fungi has been varied. For instance, Shewale & Sadana (1978) found a 2-2.5 fold increase in cellulase activity using 0.03% tween 80 in cultures of basidiomycete CPC 142. Tangnu et.al. (1981) found that 0.01-0.02% detergent had little effect but 0.1% caused a drop of 40% cellulase activity

(filter paper assay). Stutzenberger (1987) found that 0.1% tween 80 caused an increase in cellulase secretion, but this increase was largely due to the release of a specific enzymatic component.

The significant difference between enzyme release in agitated and static cultures has only been, to my knowledge, documented for P.chrysosporium. The suggestion that the surfactant simply allows the release of existing enzyme is unlikely in this system since Jager et.al.(1985) found that the addition of detergent to 6-day old agitated cultures did not result in the development of LiP activity. The detergent must therefore perform either a nutritional or some other physiological role.

Hulme & Stranks (1970) suggested that the promotion of cellulase activity in fungi was a result of the surfactant causing a reduction in oxygen transfer in liquid medium, effectively reducing metabolism by oxygen limitation. However, in the P.chrysosporium system, thorough oxygenation is vital to LiP production. Nevertheless, there have been reports that high oxygen tensions during the primary growth phase of static cultures are toxic (Faison 1985). Also, highly agitated cultures that were spiked with veratryl alcohol instead of detergent to aid LiP production, led to complete inhibition of LiP activity (Leisola & Fiechter, 1985). Preliminary work done in

this thesis indicated that LiP could be produced in agitated cultures of P.chrysosporium BKM containing tween 80, at 215 r.p.m. Therefore, it is possible that the detergent acts to protect the organism from oxygen toxicity during the initial growth phase, before LiP has been produced.

3.3.5. Scale-up of agitated submerged cultures containing Tween 80.

P.chrysosporium BKM1767 and ME-446 were scaled-up from 30ml cultures in 100ml flasks to 750ml cultures in 2L flasks, resulting in a loss in specific LiP titre of approximately 20 - 30% (maximum titres for both strains were typically 15-20 U L⁻¹ after 8-9 days). However, the 25 fold scale-up of LiP production easily offset this reduction.

S.pulverulentum, which produced the highest titres under static conditions was found to produce over 20U L⁻¹ in 750ml agitated cultures and was used as the organism of choice. However, the average titre under these conditions later dropped to 15-20 U L⁻¹. Nevertheless this system, using S.pulverulentum allowed a 2.5-fold increase in specific activity over the original strain (ME-446) and effectively a 10-fold.increase in the volume cultured at any one time.

3.3.6. Scale-up using stirred tank fermentors.

A 2.5 L culture of S.pulverulentum was grown in a 3L stirred tank fermentor (30°C, 200r.p.m., flushed with oxygen every 3 days). If the fungus could produce LiP in a fermentor, this could lead to increased scale-up to 100L volume cultures yielding ample supplies of the enzyme for further work. However, no LiP was produced after 15 days. The mycelia began to attach to the rotor blades and eventually 98% dry weight of the mycelia was present as a single large dense rubbery green/cream mass. The inside of this mass was watery and microscopic observations showed it to contain mycelial fragments suggesting that lysis had occurred, possibly due to oxygen starvation.

The fermentor work was suspended in favour of producing LiP in large shake flasks. Further work on a fermentor system would require a lengthy selection procedure to find a strain that would not agglomerate into large masses.

3.4. Conclusion.

LiP production in cultures of P.chrysosporium was, like lignin degradation itself, strongly controlled by nitrogen levels but the type of nitrogen source was not critical.

Suppression of LiP production under nitrogen excess could be overridden by using sugar alcohols as carbon sources. However, this effect was not observed for the nitrogen source glutamate which appeared to strongly inhibit LiP production.

Although the alteration of medium components or choice of strain allowed up to a 3-fold increase in original activity, volume scale-up using submerged agitated cultures was the only way to produce significant quantities of enzyme.

The use of Sporotrichum pulverulentum in 750ml agitated nitrogen-limited cultures containing 0.1% tween 80 effectively allowed a 25-fold increase in LiP production over the original method, developed from Faison & Kirk (1985). The eventual production figure of 50-100U of LiP per run was found to be an adequate amount of protein for the studies detailed in the following chapters.

PURIFICATION AND PROPERTIES OF LIP FROM SPOROTRICHUM PULVERULENTUM.

4.1. Introduction.

As early as 1984 there was evidence to suggest that the recently discovered LiP from P.chryso sporium consisted of more than one enzyme. Tien & Kirk (1984) found, during the purification of LiP, a second band possessing LiP activity. This second, relatively minor protein peak would not adhere to their DEAE Bio-Gel A column. They suggested that it was either a proteolytic fragment, or an isozyme.

Renganathan et.al. (1985) looked into this phenomenon of multiplicity in greater detail, resolving three molecular forms of LiP from agitated cultures of P.chryso sporium using a DEAE-Sepharose column (0-300mM NaCl in 20mM succinate, pH 5). The heme absorbance maxima of the native, reduced and ligand complexes of each of the three forms were essentially the same. All three forms oxidised several model compounds but their specific activities and Km values for veratryl alcohol were different ($8.5 - 19 \text{Umg}^{-1}$ and $55 - 95 \mu\text{M}$ respectively) as were the $K_{m_{app}}$ values for H_2O_2 ($39 -$

47 μ M) and their molecular weights (39 - 43Kd). In addition, all three isozymes were thought to be glycosylated (as determined by their affinity for concanavalin A-agarose).

Haemmerli et.al. (1986a) purified four veratryl alcohol-oxidising heme-proteins from an extracellular crude enzyme preparation from P.chryso sporium by isoelectric focusing. All four had an apparent molecular weight of 39Kd but their specific activities for veratryl alcohol were different (16 - 32 U mg^{-1}).

The complex nature of this system was further demonstrated by Kirk et.al. (1986) who resolved 6 LiP-type heme-proteins by H.P.L.C. from P.chryso sporium BKM-1767. A considerable degree of homology was found between these isozymes using Western blot analysis, peptide mapping and antibody cross reactivity.

Furthermore, Leisola et.al. (1987) found a total of 11 LiP isozymes, separated by isoelectric focusing in nitrogen-limited cultures of P.chryso sporium BKM-1767 and a total of 15 in both carbon- and nitrogen-limited cultures. Peptide mapping, amino acid analysis and reactivity against specific antibodies again demonstrated a large degree of homology.

4.2. Aims.

The aims of this chapter were to demonstrate whether S.pulverulentum also possessed a multiple LiP family. Also, if LiP isozymes did exist in this strain, they could then be purified and their respective catalytic abilities determined (a homogenous LiP preparation, with respect to substrate oxidation potential, was necessary for the work on catalytic utility in chapters 5 & 6).

Finally, despite the progressive research on the plurality of the LiP system of P.chrysosporium, no explanations have yet been forthcoming concerning the purpose of this apparently degenerate system. The third aim, therefore, was to physically compare and contrast the LiP isozymes in an attempt to elucidate this conundrum.

The use of the term isozyme in this text was based on the definition by Markert & Moller (1959), who simply proposed that isozymes refer to electrophoretically-distinguishable enzymes with similar substrate specificities.

4.3. Concentration of extracellular supernatant.

To investigate LiP, large volumes of culture must be grown in order to offset the low specific titre found in the extracellular supernatant of S.pulverulentum. This meant that an effective concentration technique was required as an initial step in the purification procedure. The technique had to be high yielding and rapid so as to maintain the integrity of the LiP system as much as possible.

Table 4.1 shows the concentration methods that were examined. Ultrafiltration gave very high yields of LiP and was a highly effective technique for small volumes (up to 500ml). With larger volumes there was a problem with speed, for example, 12 litres effectively took one week to concentrate 100-fold due to extracellular polysaccharide, found in the supernatant, blocking the membrane. Consequently, a further 20% drop in yield was found over this time period, probably due to a mixture of thermal denaturation and shear stress.

Ammonium sulphate precipitation was found to be a quick method of concentration (1-2 hrs) but it gave only moderate yields (approx 50%). This technique suffered from further disadvantages upon scale-up. Large volumes of culture supernatant would be costly to treat because complete precipitation of LiP requires

Method of concentration	LiP Yield(%)
Ultrafiltration (Amicon PM10, 10kd cut-off)	>90
Salt precipitation (50-95% NH ₄ SO ₄)	35-55
Affinity chromatography (Con-A Sepharose)	
elute with up to 0.5M - D methylglucoside	0
elute with up to 0.5M MgCl ₂	0
Chromatofocusing (On PBE-94, pH 6.5-2.8)	4-10
Ion-exchange (CM-Sephadex, elute with 0.4M MgCl ₂ pH3).	0
Ion-exchange (DEAE-cellulose, elute with 0.5M NaCl).	44
Ion-exchange (DEAE-cellulose, elute with 150mM MgCl ₂).	80-90

Table 4.1. Comparison of crude LiP concentration methods. (all crude LiP preparations were at pH 4.5 unless otherwise stated).

95% w/v ammonium sulphate, and, in addition, the yield dropped to 35% upon scale up from 50ml to 500ml of supernatant (probably due to the increased exposure time of the protein to entrapment by precipitated polysaccharide).

The use of an exchange matrix for adsorption of dilute protein, which could then be eluted in a small volume, offered a simple and fast method of concentration though choice of matrix and adsorption/elution conditions were found to be critical.

Con A-Sepharose and CM-Sephadex were effective in absorbing LiP but active enzyme could not be eluted. Chromatofocusing using PBE-94 gave good increases in specific activity but yields were poor (usually less than 10%).

DEAE-cellulose allowed a 95% adsorption of LiP at pH 6.5. Higher pH conditions produced a slight drop in final yield due to LiP instability at high pH values; below pH 6.5, there was a proportion of the LiP (over 10%) which would not bind despite the fact that the pH was still 3 units above the enzymes isoelectric points (see 4.5). Although overall yield was not affected much, failure to allow adsorption of this 10% may well have affected the integrity of the in vivo isozyme complement.

LiP bound strongly to DEAE-cellulose since

elution, even at pH 4, required 0.5M NaCl. Higher concentrations of NaCl caused the yield to drop, which suggested that even the bare minimum required for elution (0.5M) may itself be causing some enzyme inactivation and hence a poor yield (less than 50%). The use of divalent $MgCl_2$ as an eluent however, was found to give high yields (up to 90%) at 150mM. Thus the addition of dilute LiP at pH 6.5 to DEAE-cellulose, followed by elution with 150mM $MgCl_2$ combined speed with high yields and was consequently adopted as the standard preparatory procedure for LiP concentration.

4.4. Purification of LiP.

Fig 4.1 shows the stages of the initial purification (solid arrows). S.pulverulentum was grown in submerged nitrogen-limited agitated cultures (see 2.3 for details). The supernatant was harvested at the time when LiP titre was maximal (day 8) and immediately filtered through glass wool to remove the mycelial pellets and other hyphal fragments. At this stage there were 5.4L of supernatant containing 93U of LiP (specific activity $2.5Umg^{-1}$). The pH was re-adjusted to 6.5 using potassium hydroxide and 20ml of DEAE slurry was added and stirred at 50 revs min^{-1} on a

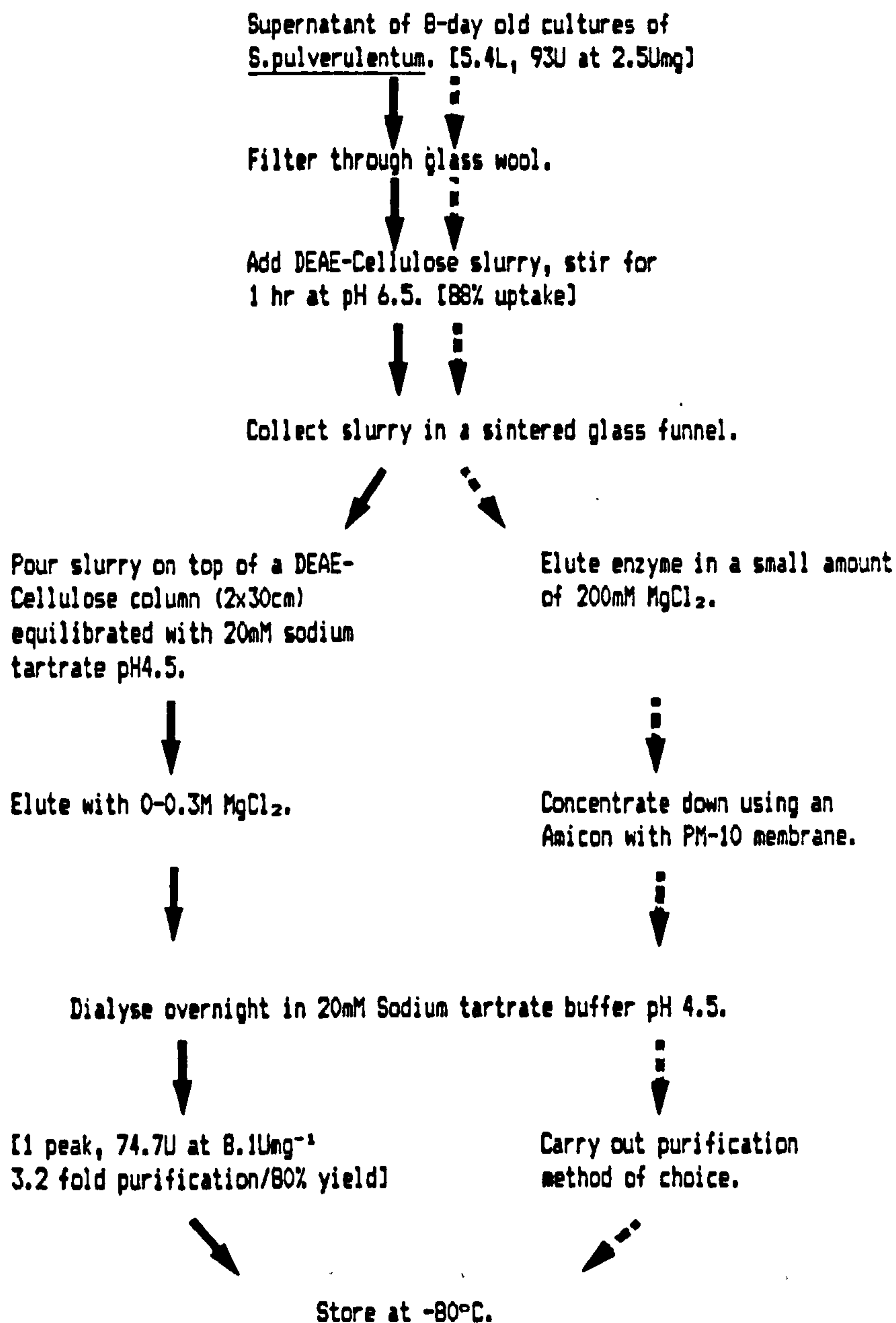


Fig 4.1. General scheme for LiP purification showing the preliminary purification procedure (, results in square brackets) and the standard preparatory procedure adapted thereafter ().

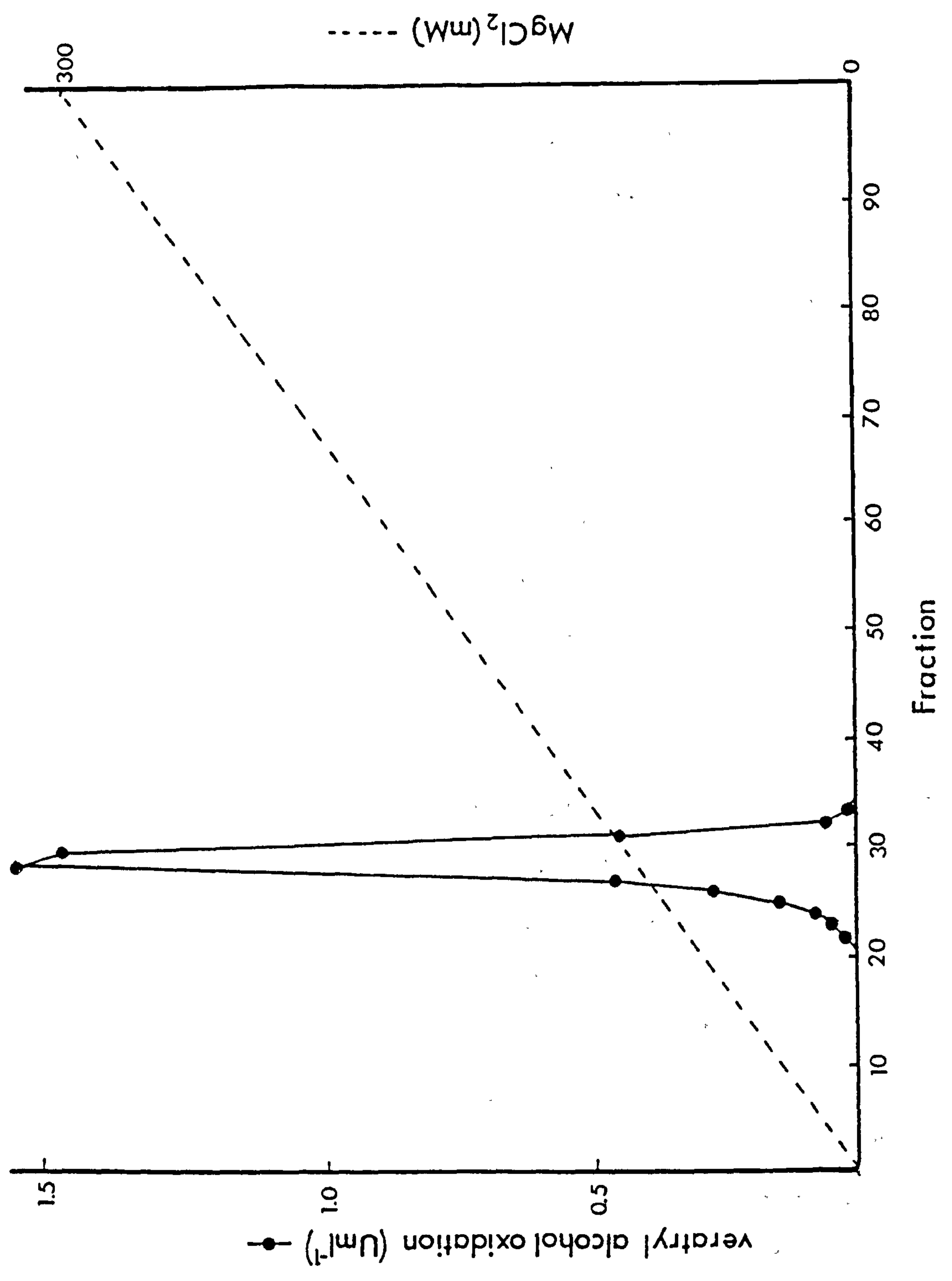
magnetic stirrer for 1 hr. The majority of the supernatant was then run off using a 1 litre sintered glass funnel to leave a thick brown slurry. This slurry was poured onto a DEAE-Sephadex column (2 x 30cm) and equilibrated with 20mM sodium tartrate buffer pH 4.5. A 0-300mM MgCl₂ gradient was run. LiP activity was eluted as a single brown band at 80 - 100mM MgCl₂ (fig 4.2). The pooled fractions were dialysed overnight in 20mM sodium tartrate buffer pH 4.5. After dialysis, LiP had a specific activity of 8.1Umg⁻¹ and a total activity of 74.7U, representing a 3.2-fold purification and an 80% yield.

The broken arrows (fig 4.1) indicate a modification of this method which was subsequently used as the preparatory procedure adopted, regardless of the final mode of purification.

4.5. Resolution of LiP by Chromatofocusing.

Standard DEAE ion-exchange chromatography failed to resolve crude LiP from agitated nitrogen-limited cultures of S.pulverulentum into more than one protein band (fig 4.2). Since at this stage it was not clear whether the LiP system of S.pulverulentum consisted of a single enzyme or whether the chromatographic technique used was too insensitive, a second method of resolution was required.

Figure 4.2. Elution profile of LiP from S. pulverulentum from a column of DEAE-Sephadex equilibrated with 20mM sodium tartrate buffer pH 4.5.



Successful resolution of LiP isozymes from P.chryso sporium on the basis of their isoelectric points (pI values) was achieved by Haemmerli et.al. (1986a) and Leisola et.al. (1987) using isoelectric focusing (IEF). Chromatofocusing is a column chromatographic method for the separation of proteins according to their isoelectric points. This was the method of choice, rather than IEF, because it was simple to use and it allowed relatively large samples (both in terms of volume and protein concentration) to be loaded.

2ml of crude LiP (5Uml^{-1}) was prepared by the method used in fig 4.1 (broken arrows), except that overnight dialysis was performed against 0.025M piperazine-HCl buffer pH 5. The sample was applied to a PBE 94 column (1 x 20cm) equilibrated with 0.025M piperazine-HCl buffer.

Chromatofocusing of crude LiP from S.pulverulentum was achieved by elution of the column with polybuffer 74-HCl pH 2.6. Two protein bands possessing LiP activity were resolved with pI values of 2.9 and 3.1 (fig 4.3). These were lower than any of the published pI values for LiP from P.chryso sporium BKM-1767 (Haemmerli et.al., 1986a; Leisola et.al., 1987). As a direct comparison, crude LiP from this latter strain was also resolved by chromatofocusing (fig 4.4). Two isozymes were separated with pI values of 3.25 and 3.45. These values were within the range found for the

Figure 4.3. Elution profile of LiP from *S. pulverulentum* from a PBE-94 chromatofocusing column equilibrated with 0.025M piperazine-HCl buffer pH 5.

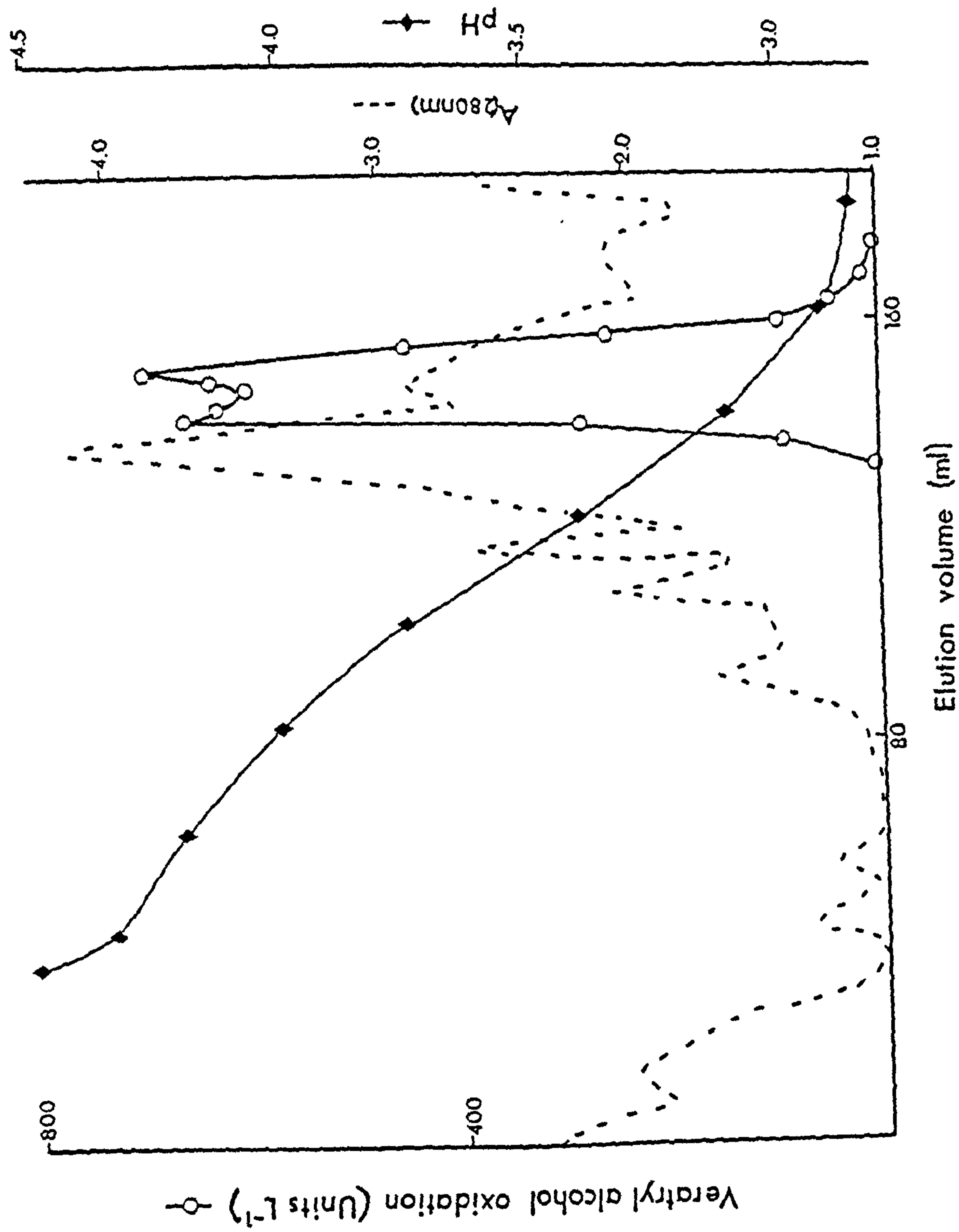
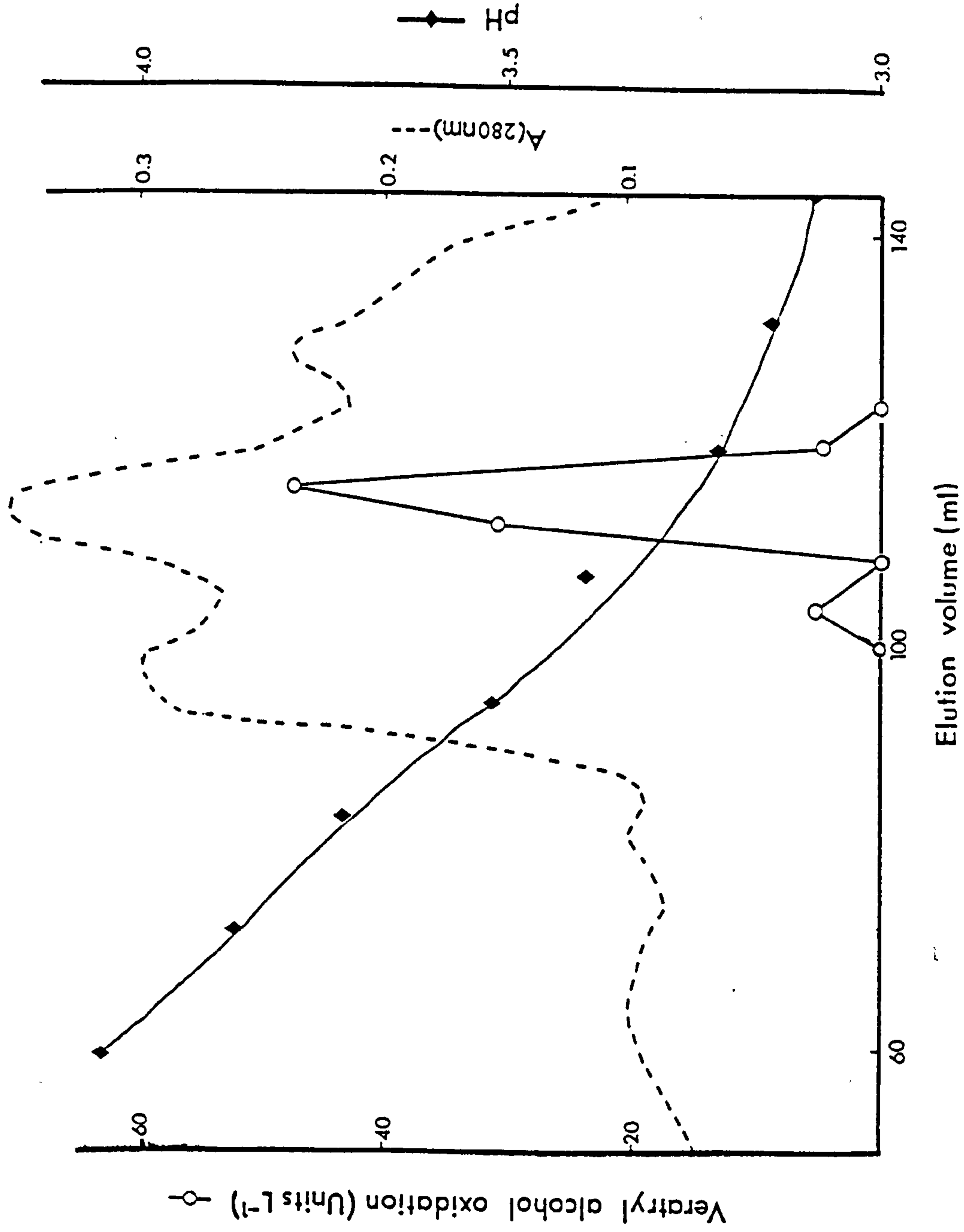


Figure 4.4. Elution profile of LiP from P. chrysosporium ME-446 from a PBE-94 chromatofocusing column equilibrated with 0.025M piperazine-HCl.



ten LiP isozymes (pI 3.2-4.9) resolved by IEF, from nitrogen-limited cultures of P.chryso sporium BKM-1767 by Leisola et.al.(1987).

Only two isozymes were isolated from the supernatant of S.pulverulentum. However, this does not imply that this LiP system was simpler than that from P.chryso sporium, since only two isozymes from the latter strain out of a possible eleven (Leisola et.al., 1987) were resolved. In addition, the 90%+ loss in yield of LiP during chromatofocusing would suggest that any of the isozymes that were present in small quantities would not even be detected.

Chromatofocusing has demonstrated that S.pulverulentum also possessed multiple molecular forms of LiP and that these isozymes had lower pI values than those found in P.chryso sporium. However, the limitations of chromatofocusing meant that a more sensitive chromatographic technique was needed.

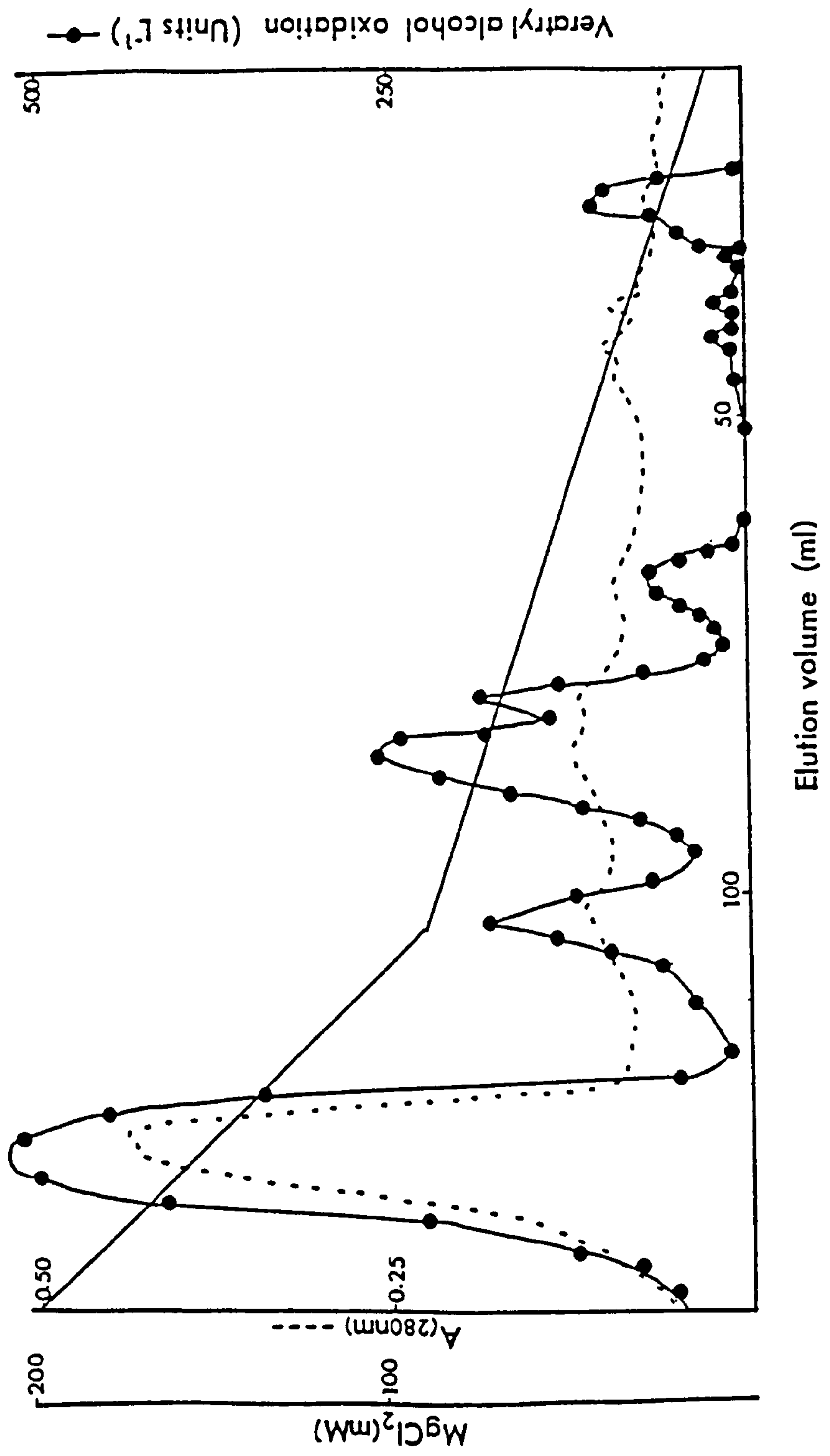
4.6. High performance liquid chromatography (H.P.L.C.) of crude LiP.

Ion-exchange H.P.L.C. was used as the chromatographic method of choice because of its' proven ability to effectively resolve proteins (Kirk et.al., 1986). Also, the speed of fractionation can be much higher using H.P.L.C. rather than standard ion-exchange

techniques (typically 1-2hrs as opposed to 8-10 hrs). This latter point was very important because a number of experiments were required, using the same 'fresh' LiP preparation, before the optimal conditions of pH and salt gradient were achieved, allowing maximal resolution of crude LiP into individual isozymes. 2.5ml (4Uml^{-1}) of crude LiP (as prepared in fig 4.1) was added to a TSK-Gel DEAE 2SW column equilibrated with 20mM sodium tartrate buffer pH4.5. The protein was eluted using a 0-200mM MgCl_2 two-step gradient with a flow rate of 1mlmin^{-1} . Samples were taken every minute.

Eight individual protein bands possessing LiP activity were resolved (see fig 4.5), demonstrating the existence of at least 8 isozymes. The largest peak (eluted at 150mM MgCl_2) contained at least half of the total recoverable LiP activity. Since such a large peak could be masking the presence of other LiP isozymes, this fraction was re-applied to the column and eluted with a much shallower salt gradient ($1\text{mM MgCl}_2\text{min}^{-1}$ instead of $2.5\text{mM MgCl}_2\text{min}^{-1}$) in an attempt to resolve any constituents which may have been present. The re-eluted LiP peak appeared to consist of three separate peaks, but they were not fully resolved. The failure to resolve this peak with a DEAE-cellulose column lead to the use of an alternative H.P.L.C. method, namely, the use of a hydroxyapatite column to

Figure 4.5. HPLC elution profile of LiP isozymes from S. pulverulentum from a TSK-Gel 2SW column equilibrated with 20mM sodium tartrate buffer pH 4.5.



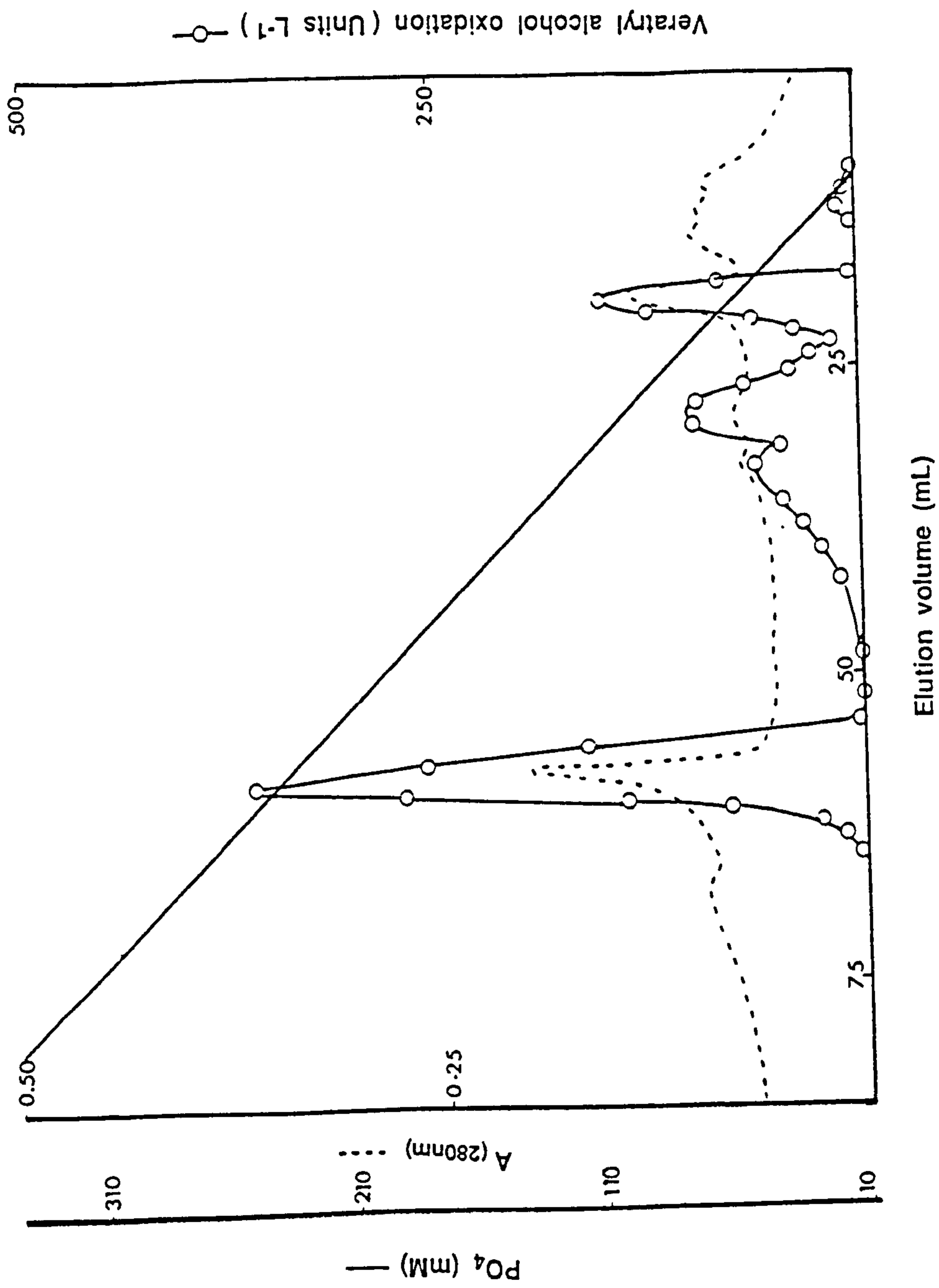
fully determine the presence of other isozymes. The LiP peak from the previous experiment was re-collected and dialysed overnight against 10mM Potassium phosphate buffer pH 5.9 and applied to a Biogel HPHT (hydroxyapatite) column equilibrated with the same buffer.

The protein was eluted with 10-350mM (2mMmin^{-1}) potassium phosphate buffer pH 5.9 with a flow rate of 0.5mlmin^{-1} and a sampling time of 2 minutes. The LiP peak was further separated into five individual peaks possessing LiP activity (fig 4.6).

Altogether, the original crude LiP preparation was resolved by H.P.L.C. into 12 electrophoretically-distinct protein bands, all of which were capable of veratryl alcohol oxidation. In addition, 5% of the crude enzyme (pH6.5) did not even bind to DEAE-cellulose during the preparative stage. This imported that another LiP isozyme, with a relatively high pI value, was also present in the original crude supernatant. Also the first LiP peak to be eluted from the TSK-Gel DEAE 2SW column had an asymmetric activity profile (fig 4.5) which was suggestive of two incompletely resolved isozymes.

These studies clearly revealed the presence of up to 14 electrophoretically-distinguishable LiP isozymes in S.pulverulentum. By comparison, the highest number of LiP isozymes found under a single set of culture conditions for P.chryso sporium was 11 (Leisola

Figure 4.6. HPLC elution profile of LiP peak 8 (fig 4.5.) from a Biogel HPHT column equilibrated with 10mM potassium phosphate buffer pH 5.9.



et.al.,1987). The LiP system of S.pulverulentum was therefore every bit as complex, if not more so, than the LiP system of P.chrysosporium.

4.7. Initial characterisation of LiP isozymes.

Preliminary investigations into the homology of the 12 purified isozymes showed that, in addition to oxidising veratryl alcohol, they were all sensitive to azide ($10^{-5}M$) and all possessed a Soret maximum at 408-409nm. However, their molecular weights, as measured by P.A.G.E. under denaturing conditions, varied from 47kd - 54kd (fig 4.7). The molecular weights of LiP isozymes from P.chrysosporium also vary, but in contrast to S.pulverulentum, the LiP isozymes were smaller (39 - 43Kd, Renganathan et.al., 1985).

This work has demonstrated the existence of LiP multiplicity and the partial characterisation of the resultant isozymes for S.pulverulentum. However, a more thorough investigation into the reasons for this multiplicity would require a large scale LiP purification so that sufficient quantities of at least some of the isozymes could be prepared.

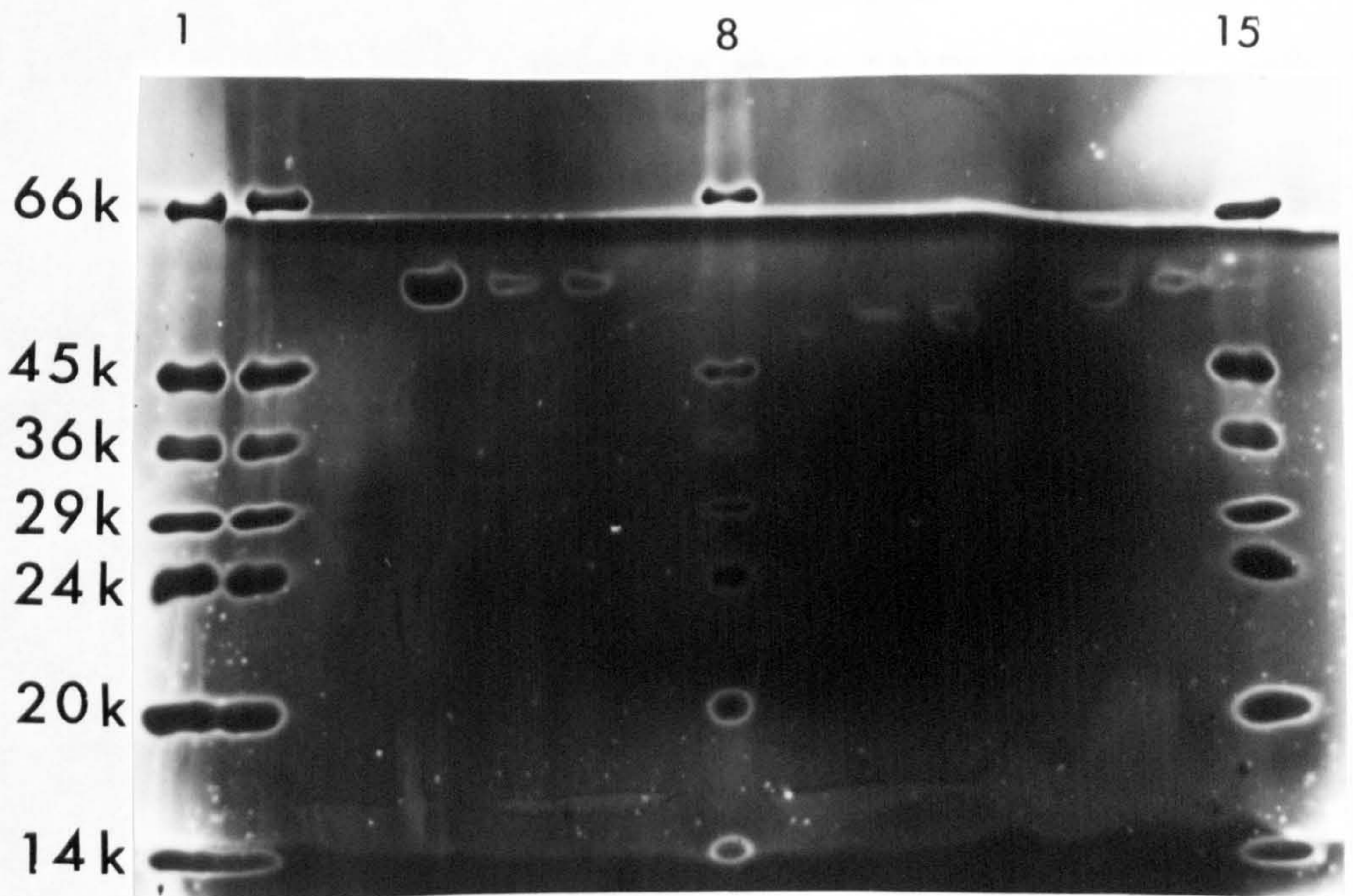


Figure 4.7. SDS-Polyacrylamide gel electrophoresis of LiP isozymes using Dalton Mk VII molecular weight markers (2.6.1.) in tracks 1,2,8 and 15. Molecular weights of LiP isozymes are as follows: Tracks 4,5,6, and 14, 54Kd; track 9, 47Kd; tracks 10 and 11, 48Kd; track 13, 52Kd. (The isozyme in track 12 was determined to be 51Kd from a repeat run of these samples). The gel was silver-stained.

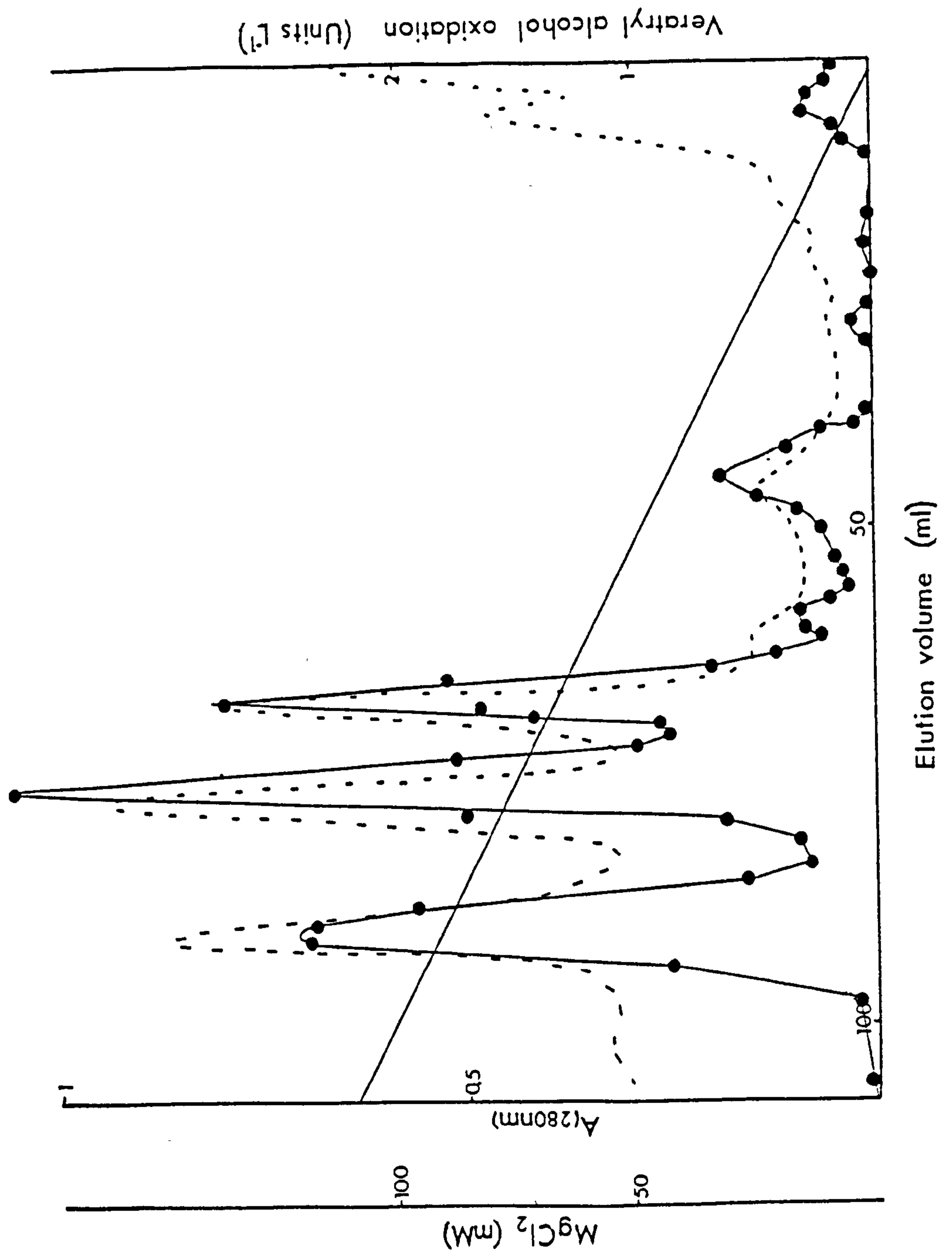
4.8. Large-scale LiP purification by H.P.L.C.

8.5ml (3.8Uml^{-1}) of crude LiP was prepared (see 4.1.) and added to the TSK-Gel DEAE 2SW column equilibrated with 20mM sodium tartrate pH 4.5. The protein was eluted using a 0-200mM MgCl_2 gradient with a flow rate of 1mlmin^{-1} . Samples were taken every minute.

Seven separate protein peaks possessing LiP activity were resolved (fig 4.8). These seven peaks correlated closely, but not identically with the seven smallest peaks in the smaller scale run (see fig 4.5). Noteably though, there was no large eighth peak eluted at around 150mM MgCl_2 . It was doubtful that the eighth peak had resisted elution from the column, since only an 18% reduction in concentrated LiP preparation occurred during the actual H.P.L.C. stage. However, during the crude LiP preparative procedure, 80 - 90% LiP yields were normally obtained from the DEAE-cellulose concentration step, but in this case, only a 55.3% yield was achieved. Although the reason for such a singularly low yield was not known, it may explain the 'disappearance' of the eighth peak.

Table 4.2 gives a summary of this purification run. Purification factors for LiP III - VII range from 2.5 - 4.3. This may seem low, but one must consider that P.A.G.E. of the crude culture supernatant

Figure 4.8. Large-scale HPLC resolution of LiP isozymes from S. pulverulentum on a TSK-Gel DEAE 2SW column equilibrated with 20mM sodium tartrate buffer pH 4.5.



(concentrated by ultrafiltration, 10kd cut off) showed the existence of only one major protein band, that of LiP (fig 4.9). In effect, there was very little other protein to purify LiP from.

The specific activity of LiP I was much lower than the value for the original crude extract (table 4.2). The high $A_{409}/280\text{nm}$ ratio (1.3:1) for LiP I suggested that LiP I had a poor specific activity with respect to veratryl alcohol. Alternatively, some or all of the denatured LiP present (equivalent to the 18% loss in yield) did not bind to the column and hence overlapped with the LiP I peak which was eluted in very low salt concentrations (<5mM).

LiP II also had a lower specific activity for veratryl alcohol than the crude extract. This could however, be because of the very low titre obtained and the subsequent inaccuracy in measuring the protein content (the Biorad protein microassay was insensitive to concentrations of less than 10ug). LiP II was available in insufficient quantities for detailed analysis. The following sections required a number of isozymes for comparison purposes and since an exhaustive list was not necessary, LiP II was consequently ignored.

In contrast to the investigations by Renganathan et.al. (1985); Kirk et.al. (1986) and Leisola et.al. (1987) on the LiP isozymes of P.chrysosporium, the following experiments were primarily designed to

Sample/Volume	Specific activity (U/mg)	Protein (mg)	A409/280	Total activity (U)	Purification factor.
Cell supernatant (6L)	0.87	67.8	-	60.0	1
DEAE concentrate (8.5ml)	1.13	27.2	-	30.7	1.3
LiP I (10ml)	0.14	3.8	1.3	0.53	0.16
LiP II (2.5ml)	0.44	0.04	0.2	0.02	0.5
LiP III (9ml)	2.33	0.54	0.9	1.23	2.7
LiP IV (2.5ml)	2.15	0.15	0.9	0.32	2.5
LiP V (6ml)	2.41	2.25	1.3	5.4	2.8
LiP VI (15.5ml)	2.87	4.26	0.9	12.2	3.3
LiP VII (9ml)	3.79	1.44	0.8	5.5	4.3

Table 4.2. HPLC LiP isozyme purification on DEAE-cellulose (section 4.8).

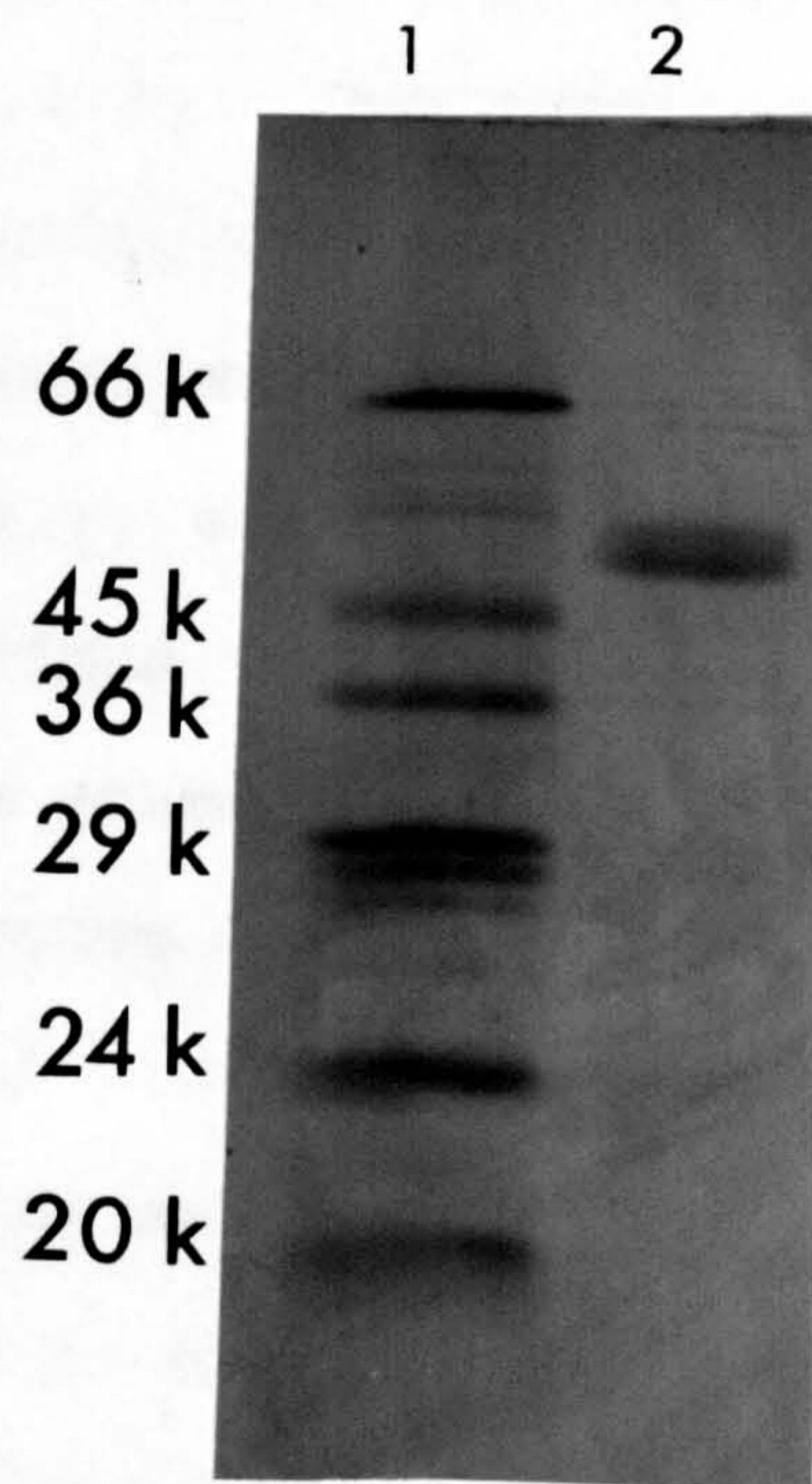


Figure 4.9. SDS-polyacrylamide gel electrophoresis of crude concentrated LiP (track 2) using Dalton Mk VII molecular weight markers (2.6.1.). The gel was stained with Coomassie blue.

investigate the possible physiological roles of the isozymes rather than the physical differences between them.

4.9. The effect of pH on LiP isozymes.

The pH profiles for the six isozymes were measured. Their respective pH optima varied from pH 3 - 3.5 (see table 4.3). Two questions could be asked of this result, firstly, can there be any physiological reasons for an organism to possess a family of isozymes? Secondly, even if there was, could the pH difference in optimum values of 0.5 be significant?

One possible answer to the first question is that such an enzyme system may allow the organism to extend its' environmental niches. LiP has an extracellular role to play in the metabolism of S.pulverulentum. Since external pH is a factor that cannot be readily be controlled directly by the fungus, it would be advantageous to the organism to utilise a battery of LiP enzymes each with a different pH optimum. The LiP isozymes could synergistically act as a buffer to allow continuous high rates of ligninolysis within the plant cell.

Recently, Holzbaur & Tien (1988) found that different LiP genes were transcribed in response to

LiP isozyme	pH optimum
I	3.5
III	3.5
IV	3.25
V	3.25
VI	3.0
VII	3.5

Table 4.3. pH optimum values for the LiP isozymes purified in section 4.8.

different conditions of nutrient deprivation i.e. LiP genes H2 and H8 were induced under nitrogen-limitation, but only H2 was induced under carbon-limitation. It may be possible that LiP isozyme expression could be controlled by other environmental factors such as pH, so that the extracellular pool of LiP consisted primarily of isozymes working under their optimal pH values.

The significance of such a narrow range of pH optimum values cannot be determined, but even a narrow range may have some contributory effect upon the efficiency of lignin degradation. Any slight improvement on such a low energy yielding system (lignin degradation requires a co-substrate, see 1.6.2.) may give the organism a disproportionately large advantage over its' competitors.

4.10. Comparison of the catalytic spectra of LiP isozymes.

The differences between the various forms of the enzyme may be manifest in their catalytic properties. It would be reasonable to hypothesise that catalytic specialisation of LiP isozymes may allow a more complete oxidation of such a complex molecule as lignin.

A range of mono-aromatic compounds (at

concentrations of 1 & 5 mM) were incubated with LiP isozymes I,III,IV,V,VI and VII(0.2Uml^{-1}) and H_2O_2 (50 μM) in 100mM sodium tartrate buffer pH 3. Positive oxidation by LiP was determined by observing a change in absorbance spectra between 300nm and 350nm.

Table 4.4 shows that there were no differences between the respective individual substrate ranges for each isozyme. One could consider that only a truly exhaustive list of possible substrates could really show the similarity of catalytic function between the molecular forms of LiP or indeed of any other isozyme system. However, looking at the closely related methoxybenzyl alcohol family, it can be seen that all 6 isozymes oxidised 3,4-, 2,3- and 2,4- dimethoxybenzyl alcohols but none of the 6 isozymes oxidised 3,5- dimethoxybenzyl alcohol or 3- and 4-methoxybenzyl alcohol. This would suggest that the oxidation potentials of the isozymes (in this case, the ability to oxidise a benzyl alcohol to a radical cation) are almost, if not absolutely, identical.

Substrate (1&5mM)	LiP isozyme					
	I	III	IV	V	VI	VII
3,4-dimethoxybenzyl alcohol	+	+	+	+	+	+
2,3-dimethoxybenzyl alcohol	+	+	+	+	+	+
2,4-dimethoxybenzyl alcohol	+	+	+	+	+	+
3,5-dimethoxybenzyl alcohol	-	-	-	-	-	-
4-methoxybenzyl alcohol	-	-	-	-	-	-
3-methoxybenzyl alcohol	-	-	n.a.	-	-	-
3,4-dimethoxyphenethyl alcohol	+	+	n.a.	+	+	+
3,4-dimethoxypropanol	+	+	n.a.	+	+	+
Trimethoxytoluene	-	-	n.a.	-	-	-
4-methylphenol*	+	+	n.a.	+	+	+
4-ethylphenol*	+	+	n.a.	+	+	+

Table 4.4. Comparison of LiP isozymes (purified in section 4.8) with respect to substrate range (all absorbances read at 310nm, except 250nm*).

4.11. The comparison of isozyme affinity for methoxybenzyl alcohols.

Although the substrate range of each of the isozymes were identical, differences in catalytic activity may be manifest in enzyme/substrate affinities. K_m values for four benzyl alcohols were determined for each of the 6 isozymes (table 4.5). These alcohols were chosen because of their wide range of K_m values for crude LiP: 3,4-dimethoxybenzyl alcohol and 3,4-dimethoxyphenethyl alcohol both had K_m values of approximately 0.1 mM; 2,4-dimethoxybenzyl alcohol and 2,3-dimethoxybenzyl alcohol had approximate K_m value of 1mM and 10mM respectively.

For all four of the alcohol substrates, the K_m values for each of the isozymes did not vary more than approximately two-fold. This was in agreement with Renganathan et.al. (1985) who found the K_m values for veratryl alcohol for their three LiP molecular forms from P.chrysosporium were 95, 71 and 55 μ M.

No general trends could be found where the K_m values for the alcohols for a particular isozyme were substantially different in relation to the other isozymes.

One of the problems associated with finding catalytic heterogeneity amongst LiP isozymes (if it indeed exists!) is that the precise physiological role of LiP is still unclear. Instead of using different

Substrate	Substrate Km values(mM) for LiP isozymes					
	I	III	IV	V	VI	VII
3,4-dimethoxybenzyl alcohol	0.11	0.20	0.10	0.13	0.19	0.10
2,4-dimethoxybenzyl alcohol	1.0	1.8	n.a.	0.74	0.53	1.3
2,3-dimethoxybenzyl alcohol	16.7	12.1	n.a.	11.8	7.1	9.5
3,4-dimethoxyphenethyl alcohol	0.11	0.25	n.a.	0.09	0.09	0.16

Table 4.5 Comparison of the Km values of methoxybenzyl alcohol substrates with LiP isozymes I,III,IV,V,VI and VII.

analogues of the probable natural substrate veratryl alcohol to try to find differences in the catalytic potential of LiP isozymes, future development of this work could be to screen the organism for other small aromatic extracellular fungal idiolites, which could be oxidised by LiP and could act, like veratryl alcohol as electron mediators.

4.12. Further discussion.

Plant peroxidases such as horseradish peroxidase (HRP) share many common features with LiP. They are glycosylated extracellular heme-peroxidases, and ironically, maize peroxidases have been found in association with cell wall fractions and may be involved in in vivo lignin polymerisation (Harkin & Obst, 1973).

Like LiP, HRP has also been found to consist of a large number of isozymes but there is no clear agreement upon the actual numbers of isozymes present: Shannon et.al. (1966) found seven HRP isozymes by ion-exchange chromatography; Klapper and Hackett (1965) resolved 11 isozymes by starch gel electrophoresis and Delincee & Radola (1970) found no less than 20 HRP isozymes using thin layer isoelectric focusing.

Although the physiological reasons for possessing

a large isozyme complement may still be obscure, mechanistic studies on the HRP and other plant peroxidase systems may shed some light upon the mechanisms that could be responsible for the existence of LiP plurality in S.pulverulentum.

4.12.1. Multiple loci.

Multiple loci can code for a structurally distinct series of isozymes. These multiple loci are thought to originate from gene duplications and subsequent divergence by point mutations. This mechanism is almost certainly responsible for at least some of the peroxidase isozymes found in plants. Rick et.al.(1974) found four peroxidase loci in red fruited tomatoes (Lycopersicon) and Brewbaker & Hasegawa (1974) found multiple peroxidase loci in Maize (Zea mays).

Recently, Zhang et.al. (1986) isolated four cDNA clones from a cDNA library representing 6 day old lignin degrading cultures of P.chrysosporium. Two of these clones did not hybridize with each other, suggesting there was at least two different genes for LiP.

4.12.2. Post-translational events.

Epigenetic modifications, such as disulfide bridging, hydrogen bonding and other interactions between chains or proteolysis and glycosylation may also be responsible for the formation of isozymes.

Liu (1975) found that HRP isozymes possessed different bouyant densities in a caesium chloride gradient and suggested this was due to the respective proportions of carbohydrate attached to each enzyme. LiP isozymes may themselves be heterologous with respect to glycosylation. LiP from S.pulverulentum, like that of P.chrysosporium is almost certainly glycosylated because of its ability to bind to Con A-Sepharose (see 4.3). However, LiP bound so strongly to Con A-sepharose that resolution of LiP by their respective carbohydrate moieties could not be achieved.

Modification of LiP by proteolysis is probably a minor factor. Culture supernatant containing LiP had a half-life of several weeks at room temperature, even if the mycelial pellets were not removed, suggesting that protease activity was very low, if present at all. Also, the stability of LiP at 37°C in static cultures was unaffected by the addition of proteases (see 3.2.4.). Variation due to proteolysis should not be completely ruled out since the small variation in molecular weight of the isozymes could infer that a

non-essential part of the protein chain was susceptible to either proteases or thermal hydrolysis. In addition, the affect of intracellular post-translational processing of LiP by proteases cannot be determined because no intracellular LiP activity was found at any stage of a 14-day culture incubation.

4.12.3. Physiological observations of peroxidase isozymes.

The number and relative concentration of HRP isozymes was noted to vary between different tissues and with the different developmental stages of the plant (Scandalios, 1969). Although differentiation and development of plants is a little difficult to relate to fungi, there is no reason to suggest that these factors may not have some role in the resultant LiP isozyme profile. For instance, not only is LiP production specifically a secondary metabolic event (Kirk et.al., 1978) but Kirk et.al. (1986) found that the relative titres of individual LiP isozymes varied, depending on the age of the culture.

A difference in HRP substrate specificity has been demonstrated by Liu (1975) who found that two plant peroxidase isozymes differed in their specific activity for the oxidation of homovanillic acid by nearly 40-fold, although no physiological reason was deduced.

Differences in specific activity for the oxidation of veratryl alcohol by LiP isozymes from P.chrysosporium have been reported (Renganathan et.al., 1985; Haemmerli et.al., 1986a; this work) but they appear to differ no more than 2-fold. However, this work showed that LiP VII had a specific activity for the oxidation of veratryl alcohol 27 times higher than LiP I (table 4.2). Although it cannot be totally ruled out that the LiP I fraction contained a high proportion of denatured LiP, there could be a physiological reason for this catalytic contrast. For example, in addition to veratryl alcohol, a second yet undetermined fungal idiolite could act as an electron mediator, allowing a more efficient electron transfer to certain lignin substructures. Such a mediator may be the natural substrate of LiP I, hence the low relative specific activity of this isozyme for veratryl alcohol.

4.12.4. Cytochrome P-450 isozymes.

Like HRP and LiP, cytochrome P-450 is also a hemoprotein oxidoreductase which has been found to consist of multiple molecular forms. Hepatic cytochrome P-450, in addition to being an oxidative agent for many endogenous compounds, is responsible for the oxidative conversion of many foreign compounds such as drugs and environmental pollutants.

Like LiP, multiple forms of P-450's can be distinguished by apparent minimum molecular weights (Coon & Vermillion, 1977). However, Dean & Coon (1977) found that antibodies prepared against the isozyme P-450_{LM2} (pheno-barbital inducible) did not cross-react with the isozyme P-450_{LM4} (polycyclic hydrocarbon inducible), which suggested that at least some P-450 isozymes are physically more fundamentally different than LiP isozymes, all of whose respective antibodies appear to cross react with one another (Leisola et.al., 1987).

The unusually wide substrate specificity of P-450's is thought to be at least partly due to multiple forms of the enzyme (Coon & Vermillion, 1977; Dean & Coon, 1977; Guengerich, 1977). Transcription of specific P-450 genes in rat liver appear to be inducible by particular xenobiotics. For instance, 3-methyl-cholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin induce the two P-450 isozymes P-450c and P-450d (Eisen 198X). Faison & Kirk (1985) found that some induction of LiP was possible, particularly with veratryl alcohol, veratraldehyde, veratrylglycerol and æ-O-4 dimers. Interestingly, Faison et.al. (1986) found that some, but not all LiP species in P.chryso sporium could be stimulated by veratryl alcohol.

Future work could involve the screening of

possible inducers for their effect upon the LiP isozyme profile if stringent enough conditions concerning the repetitively of isozyme type and quantity could be met under a given set of growth and purification conditions. Past experience has shown that both these conditions are very hard to repeat. It is not known whether the work of Faison et.al. (1986) was repeated until statistically relevant, but considering only three peaks possessing LiP activity were resolved in their experiments, a full picture of isozyme specific induction by veratryl alcohol could not have been accomplished.

4.12.5. The origins of the LiP system?

One of the reasons why the physiological relevance of LiP isozymes has been difficult to assess, is that the role of LiP has not been definitively answered. LiP cannot be induced de novo by lignin (see 1.6.10) and the mechanism of in vivo ligninolysis by LiP, including the actual substrates involved is still unclear.

It could be considered that its' role in lignin degradation is opportunistic. If this is the case, then what was its original role?

One possibility is that the LiP may have acted in a similar way to mammalian P-450's, i.e. it may have acted as a detoxification system. LiP is able to

oxidise a wide range of aromatic compounds, including recalcitrant aromatic pollutants such as benzo(a)pyrene (Haemmerli et.al., 1986b) and 2-chlorodibenzo(p)dioxin (Hammel et.al., 1986b). LiP is inducible, primarily because of some aspect of secondary metabolism, not because of the presence of lignin. One could hypothesise that the enzyme regulated the concentrations of fungal idiolites (including veratryl alcohol) to prevent toxicosis. Like many P-450 enzymes LiP may have once been a microsomal protein. It is not too difficult to imagine that at some stage in the evolution of a cellulolytic/hemicellulolytic organism, these LiP/H₂O₂/veratryl alcohol containing microsomes were directed to the cell surface. The possession of an extracellular system, capable of oxidising aromatic compounds, would allow such an organism to utilise lignin and lignin-protected polysaccharides, giving it an important competitive advantage over other polysaccharide-degrading organisms.

4.13. The comparison between LiP from P.chryso sporium and S.pulverulentum.

S.pulverulentum Novobranova has, on the basis of morphological comparison, been described as an imperfect form of P.chryso sporium (Eriksson &

Petterson, 1975). It has been shown to possess high DNA homology (59% GC content) with P.chryso sporium ME-446, but it differs with respect to restriction site positions (Raeder & Broda, 1984).

The use of isozyme analysis is fast becoming an established method in fungal taxonomy, for example, the differentiation of Peronosclerospora species (Micales et.al., 1986). Work in this chapter has demonstrated that S.pulverulentum and P.chryso sporium differ with respect to their LiPs. S.pulverulentum had a higher LiP isozyme complement (see 4.6.), the molecular weights of which were 5 - 15Kd larger (see 4.7.), and their pI values more acidic (see 4.5.) than the LiP isozymes from P.chryso sporium. In addition, LiP production in nitrogen-limited cultures of S.pulverulentum was two-fold higher than that by P.chryso sporium ME-446 and BKM-1767 (fig 3.2.).

Interestingly, S.pulverulentum novobranova (CMI 174 727,T) was originally supposed to be derived from ATCC 24725 (P.chryso sporium BKM-1767). However, the results of both the isozyme analysis and LiP production in static cultures suggest that either they are not the same strain or that CMI 174 727 (T) is a distant derivative of BKM-1767.

The absorption spectrum of the native LiP enzyme from S.pulverulentum (fig 4.10) showed a distinctive Soret maximum at 408nm, which suggested the presence of a heme group. This Soret maximum was in close

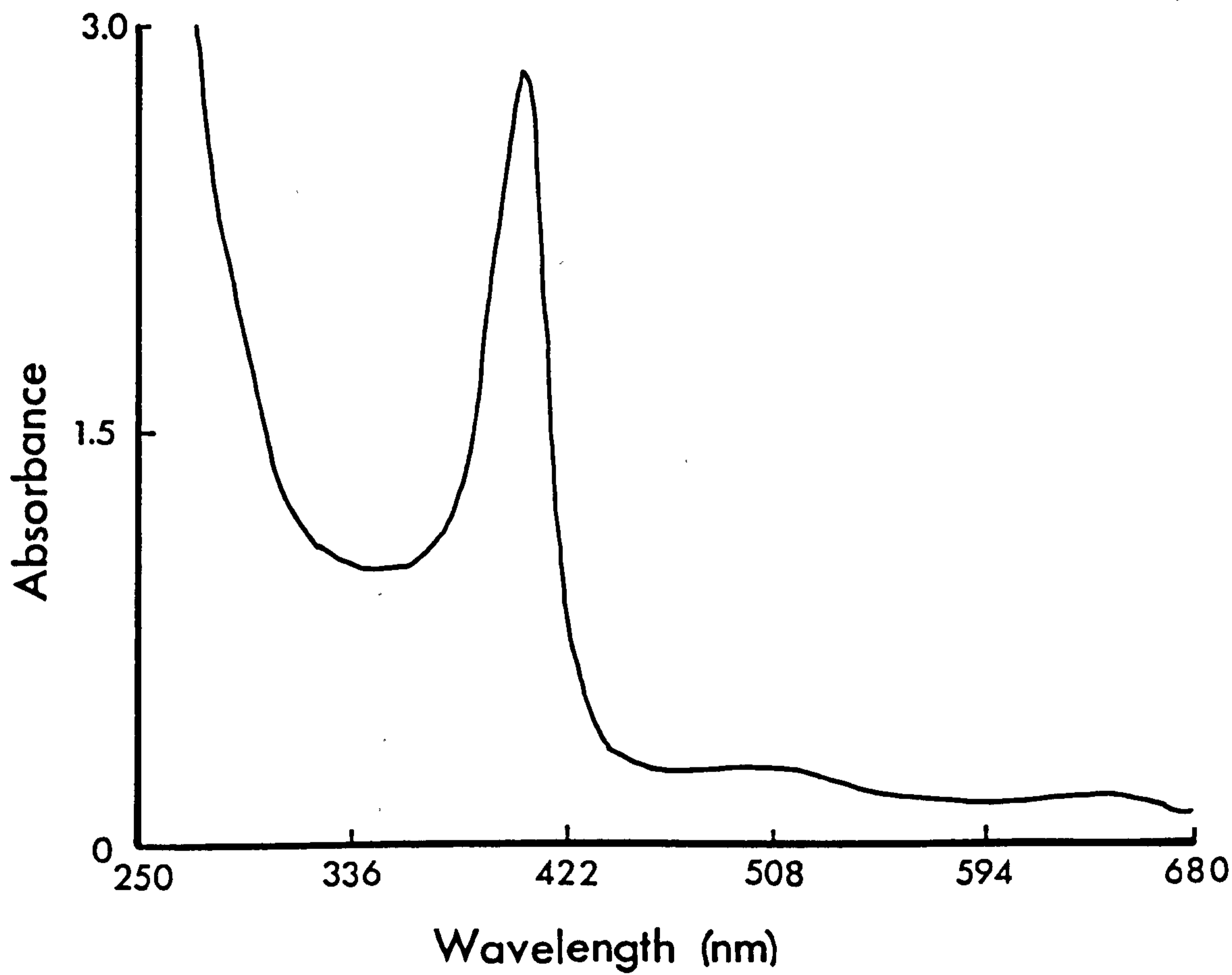


Figure 4.10. Absorption spectrum of the native LiP enzyme from *S.pulverulentum* in 10mM sodium tartrate pH 3.

correlation with the Soret maxima reported in P.chryso sporium BKM-1767 (409nm, Tien & Kirk, 1984) and a P.chryso sporium ME-446 derivative (407nm, Gold et.al., 1984; Renganathan et.al., 1985). The weak absorption maxima at 501nm and 635nm were also similar to those found in the two P.chryso sporium strains. The pyridine-hemochrome complex yielded an absorption maximum at 556nm (table 4.7), which was indicative of the presence of a protoporphoryin IX prosthetic group (Appelby & Martin, 1959; Gold et.al., 1984).

There were also similarities with other enzyme-ligand complexes of LiP from S.pulverulentum and LiP from P.chryso sporium (table 4.7.). The Soret maximum shifted to 435nm upon reduction by dithionite and the addition of H_2O_2 caused a shift in this maximum to 414nm. The Soret maximum shifts due to CN^- , N_3^- , and CO were also similar to those reported for an ME-446 derived-strain (Gold et.al., 1984). The fact that these ligands readily bound to the native enzyme led Gold et.al. (1984) to suggest the presence of an available 6th co-ordination site, or at least a loosely associated 6th ligand (such as H_2O). This, coupled with the similarities of the native enzyme to the visible spectrum of horseradish peroxidase (HRP) led them to consider that the ferric iron was high spin.

In conclusion there appeared to be no significant difference between the iron-centre part of the active

	<u>S.pulverulentum</u>	<u>P.chryso sporium</u>
	Max(nm)	Max(nm)
Native enzyme	408 501 635	407 500 632
Native enzyme + H ₂ O ₂	414 540 640	
Reduced enzyme (dithionite)	438 548 644	435 556
Reduced CO-complex	420 548 644	420 535 568
N ₃ ⁻ complex	414 540 642	418 540 575
CN ⁻ complex	423 540 642	360 425 540
Pyridine-hemochrome	420 524 556	419 472 525 557

Table 4.7 Spectral properties of LiP and LiP-ligand complexes from S.pulverulentum (this work) compared to those of a P.chryso sporium isolate (Gold et.al., 1984).

site of LiP from S.pulverulentum and that of the LiP's from the two P.chrysosporium strains based upon the spectral profiles of their native and ligand-bound prosthetic groups, suggesting that they are closely related.

4.14. General Summary.

- i) A preparatory procedure for the purification of LiP from S.pulverulentum was successfully developed, based upon efficient concentration of culture supernatant by DEAE-cellulose.
- ii) Two-stage H.P.L.C. purification of LiP from nitrogen-limited cultures of S.pulverulentum allowed the resolution of crude LiP into at least 12, possibly 14, isozymes.
- iii) These isozymes differed slightly in molecular weight, specific activity for veratryl alcohol oxidation and pH optima but they were catalytically speaking, very similar. Therefore, no determinate answer concerning the physiological role of LiP molecular multiplicity in lignin degradation could be concluded.
- iv) The use of partially purified LiP (see 4.4) should be sufficient for further catalytic studies (Chapters 5 & 6) because of the catalytic homology between the

isozymes. Such a simple purification method allows large quantities of consistent quality LiP to be made available.

v) The comparison of work done on plant peroxidase isozymes with that of LiP from S.pulverulentum insinuates that genetic and post-translational effects may be involved in the production of the LiP isozyme group.

vi) Similarities can be drawn between the LiP from S.pulverulentum and that from P.chryso sporium, namely, substrate range, multiplicity of isozyme family and spectral characteristics. They differed however in molecular weights and isoelectric points. The suggestion that S.pulverulentum was an anamorph of P.chryso sporium (Raeder & Broda, 1984) was therefore not entirely correct, on the basis of LiP heterogeneity.

C H A P T E R 5.

CATALYTIC UTILITY OF LiP: BENZYL ALCOHOL OXIDATION, PHENOL- OXIDATION, CO-OXIDATION.

5.1. Anaerobic LiP oxidation of benzyl alcohols.

5.1.1. Introduction.

Dimeric lignin model compounds of the β -1 and β -04 variety have been particularly useful in the implication of LiP as a major in vivo catalyst of lignin degradation as well as in determining the mechanism of LiP oxidation (see 1.8.). However, the following section deals with the question, 'What makes an aromatic compound susceptible to oxidation by LiP?'

This demands a rethink in terms of the type of compound that should be used in LiP oxidation experiments. The use of dimeric models can cause confusion because of the possibility that either ring A, or ring B of the substrate may be oxidised to radical cations, producing a complicated sequence of reactions and products (1.8.2.). To simplify the

process, the use of monomeric aryl compounds was necessary.

A range of substituted benzylic alcohols were chosen as substrates for the following reasons:

i) LiP oxidation of the 3,4-dimethoxylated derivative of benzyl alcohol, veratryl alcohol, has been well studied (Tien & Kirk, 1984; Schoemaker et.al., 1985; Tien et.al., 1986).

ii) LiP oxidation of this compound under anaerobic conditions has a simple stoichiometry in which 1 mole of veratraldehyde is produced per mole of veratryl alcohol and H₂O₂ consumed (Harvey et.al., 1985).

iii) The simple spectrophotometric assay first used by Tien & Kirk (1984) based on the absorbance difference between 3,4-dimethoxybenzyl alcohol and the corresponding aldehyde was found to be applicable to many benzyl alcohol derivatives.

iv) A wide range of benzyl alcohol/aldehyde pairs were commercially available, thus allowing the study of LiP oxidation on a basic class of compounds which can then be modified in terms of degree, position and type of ring substitution.

v) Direct comparison of substrate oxidation was made relatively easy because most of the alcohols used were readily soluble.

In the context of these experiments, it was not necessary to adhere rigidly to the substitution arrangements found in lignin such as syringyl (3,5-

dimethoxy,4-hydroxyaryl), or guaiacyl (3-methoxy,4-hydroxyaryl) compounds. However, the ability of LiP to oxidise these compound types has obvious implications in LiP oxidation of lignin.

A review of monomeric LiP substrates used by various authors (table 5.2) has been included for cross-referral and supplementation of the results in table 5.1 in an attempt to find an overall pattern in the catalytic specificity of LiP. For the sake of clarity, the results of this work (tables 5.1) are designated by small case letters in the text. The work done by other authors (table 5.2) is designated by roman numerals.

For the measurement of LiP oxidation of most substrates, the standard spectrophotometric assay, as used for veratryl alcohol was employed (for details see 2.6.3.). Where no aldehyde standards were available to aid in the determination of individual extinction coefficients of the alcohol substrates, gas chromatography was used to follow the disappearance of the alcohol.

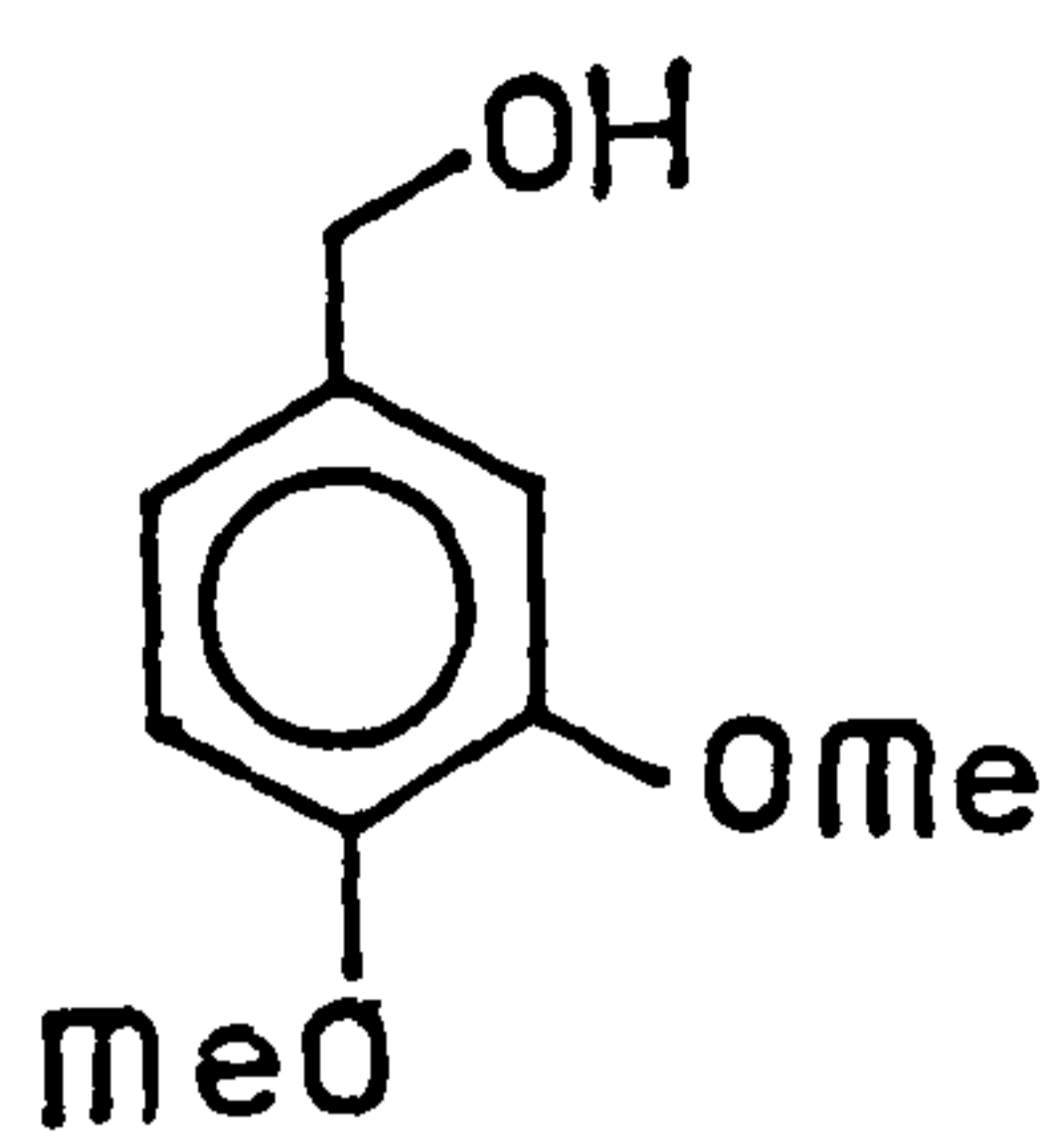
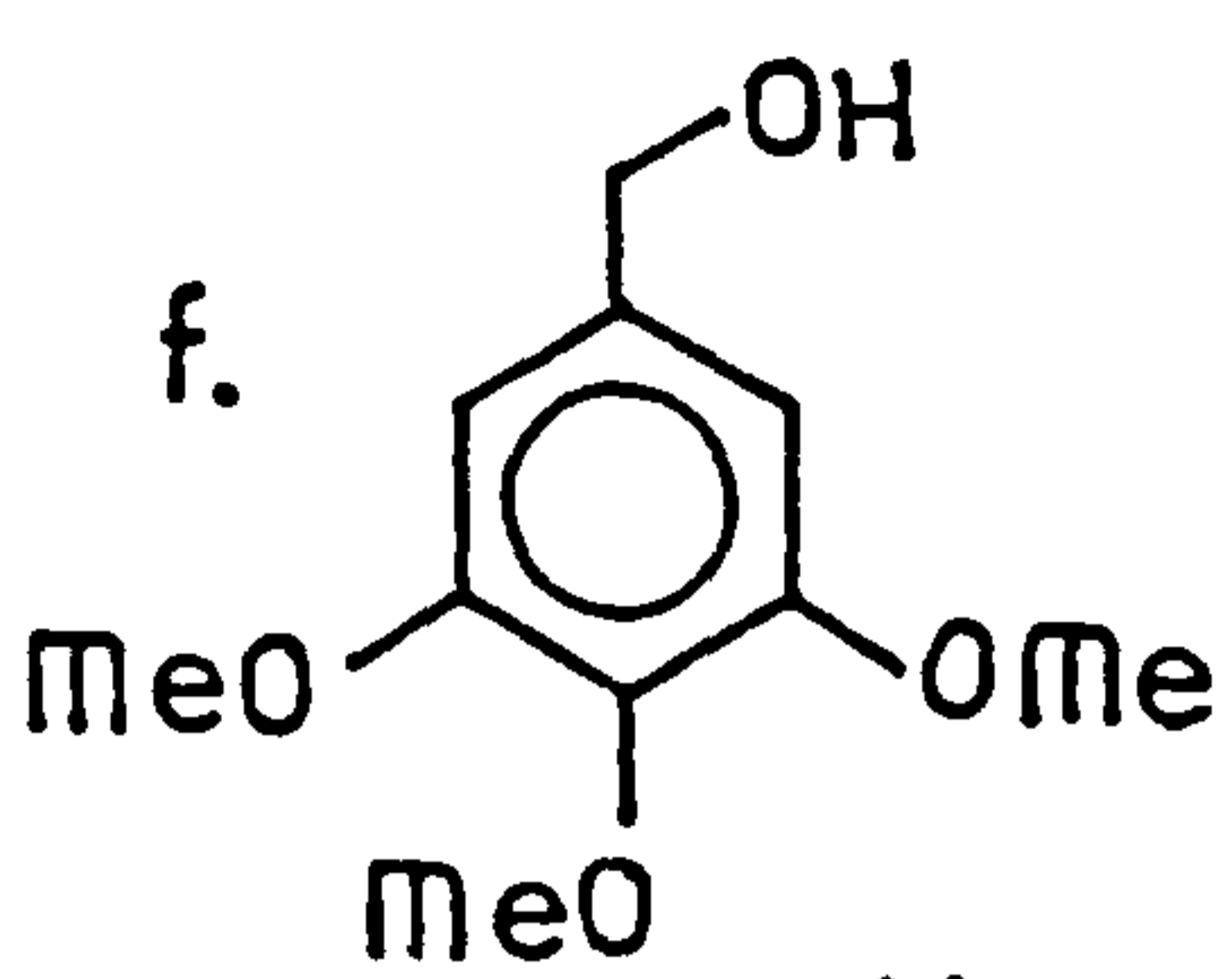
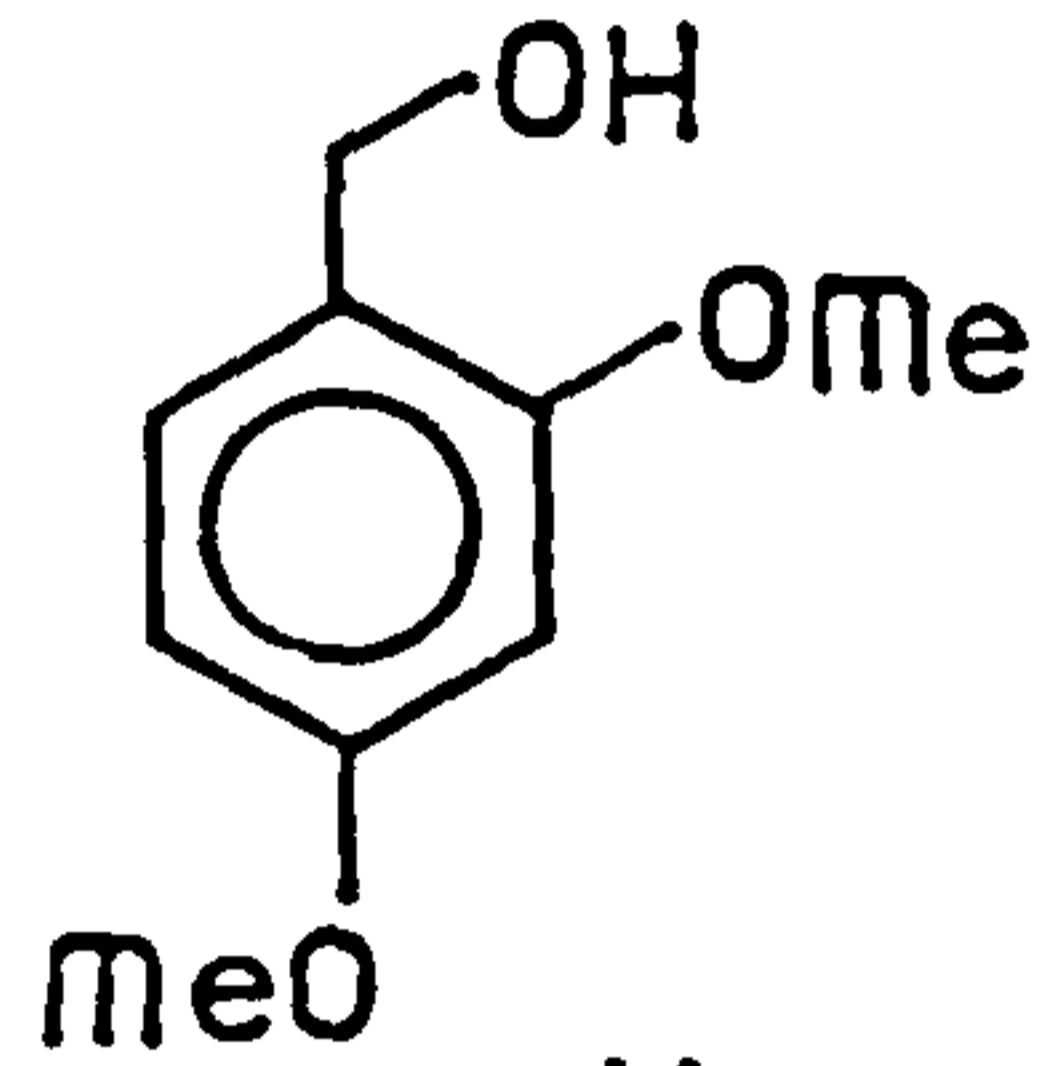
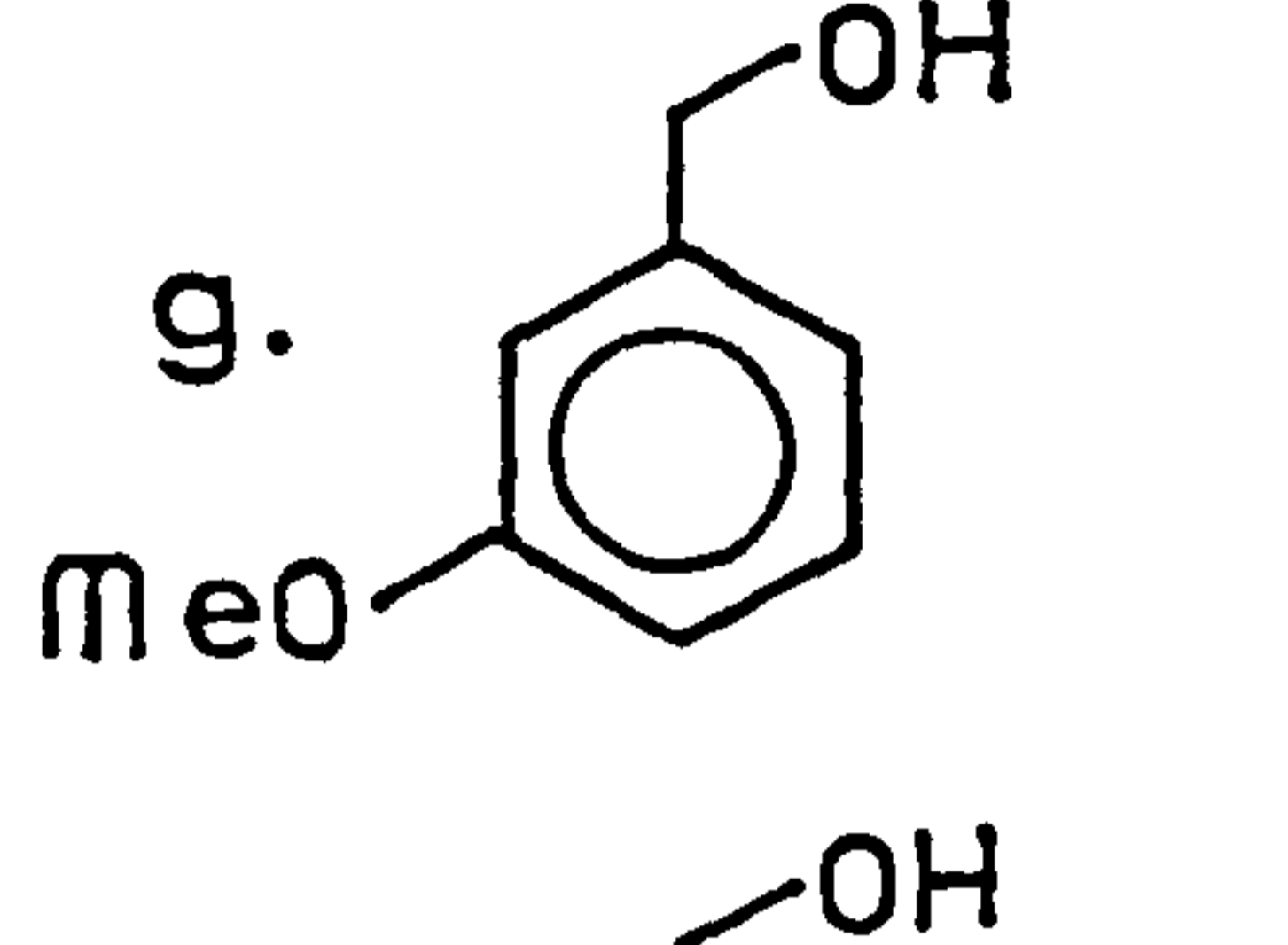
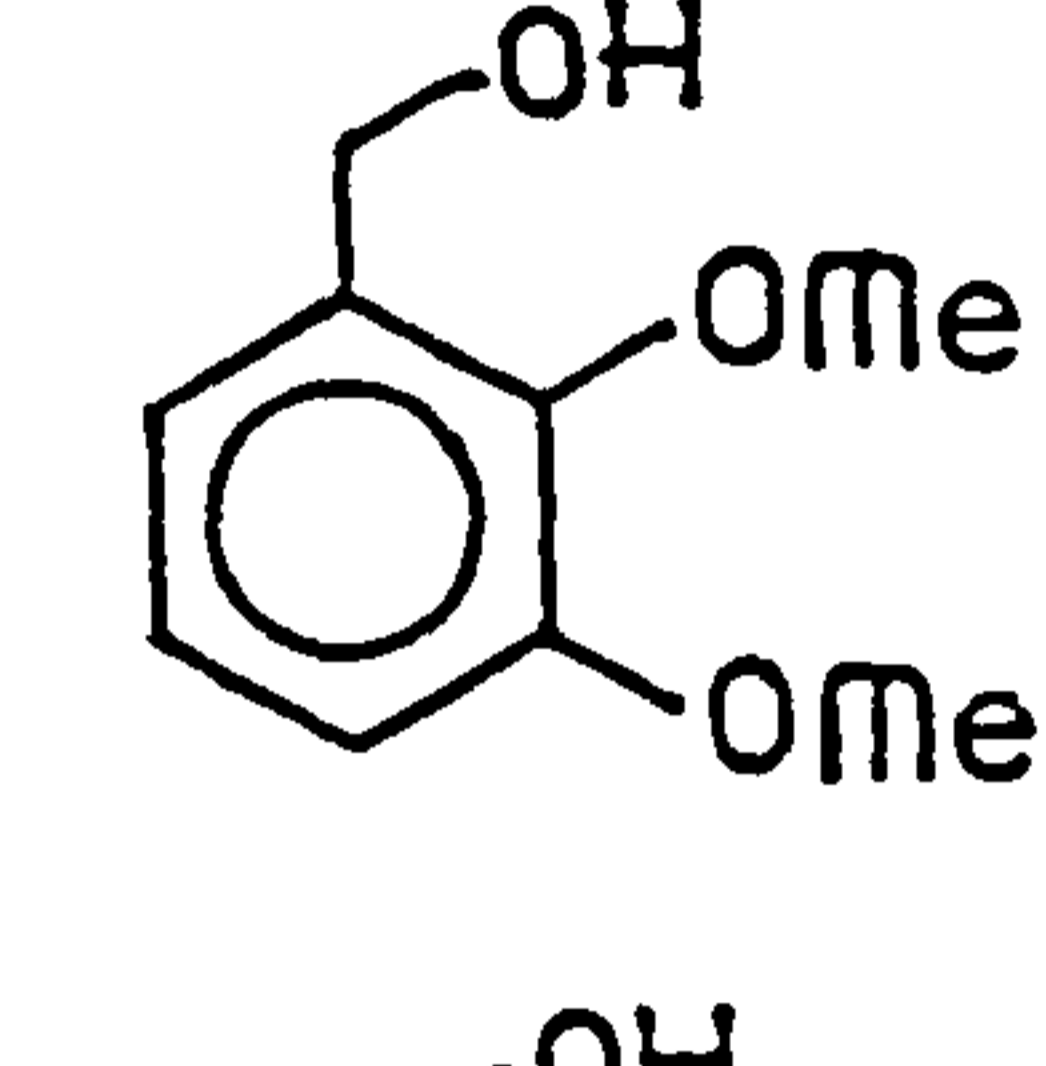
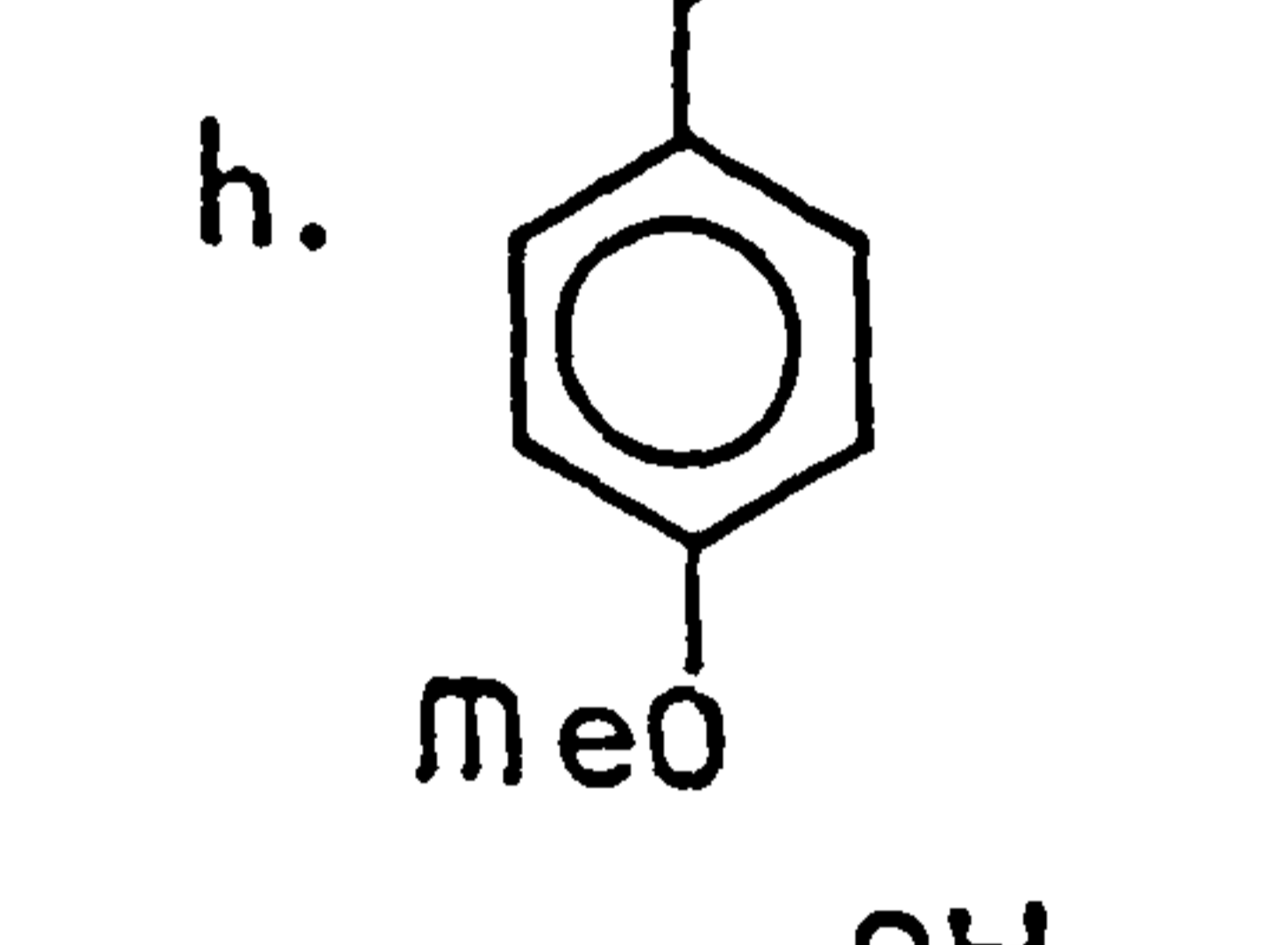
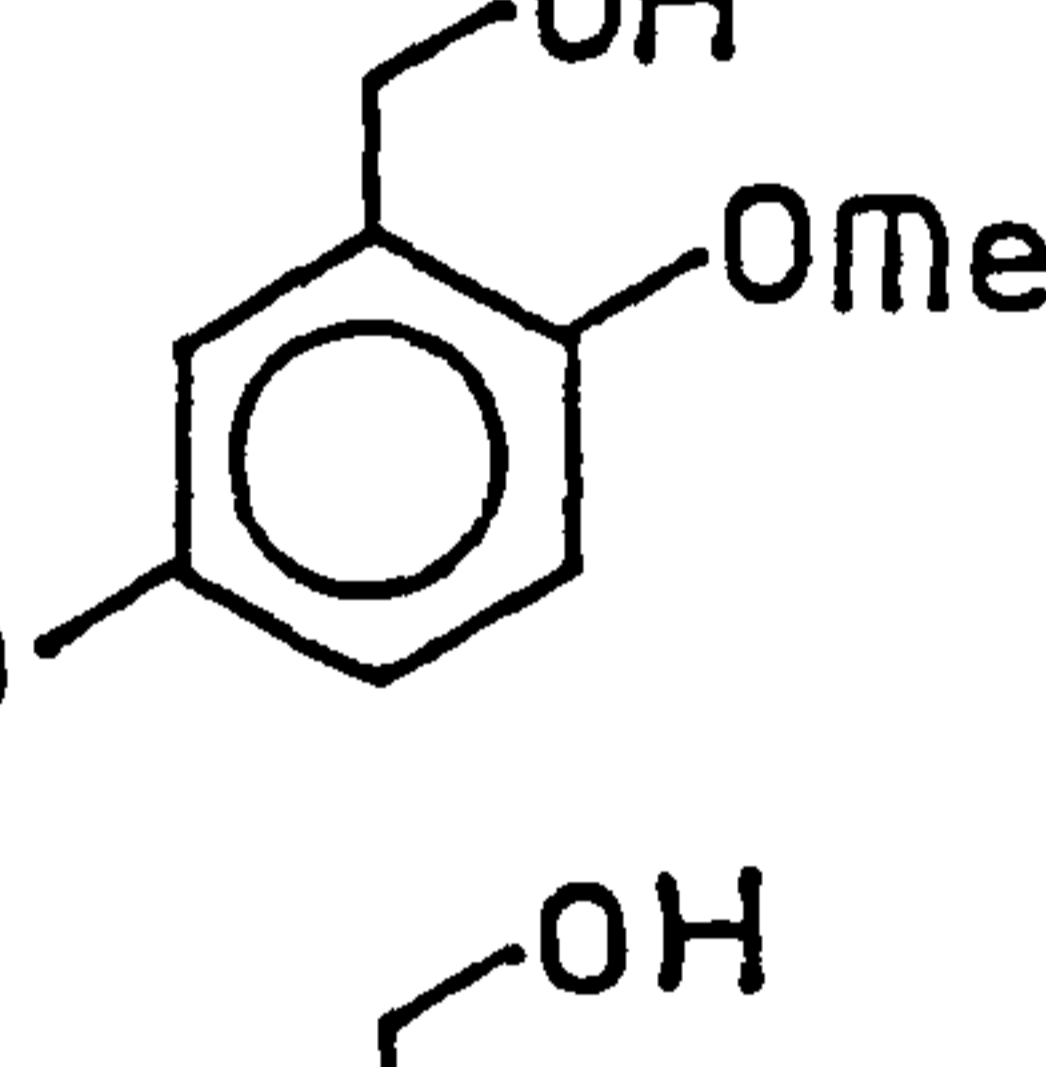
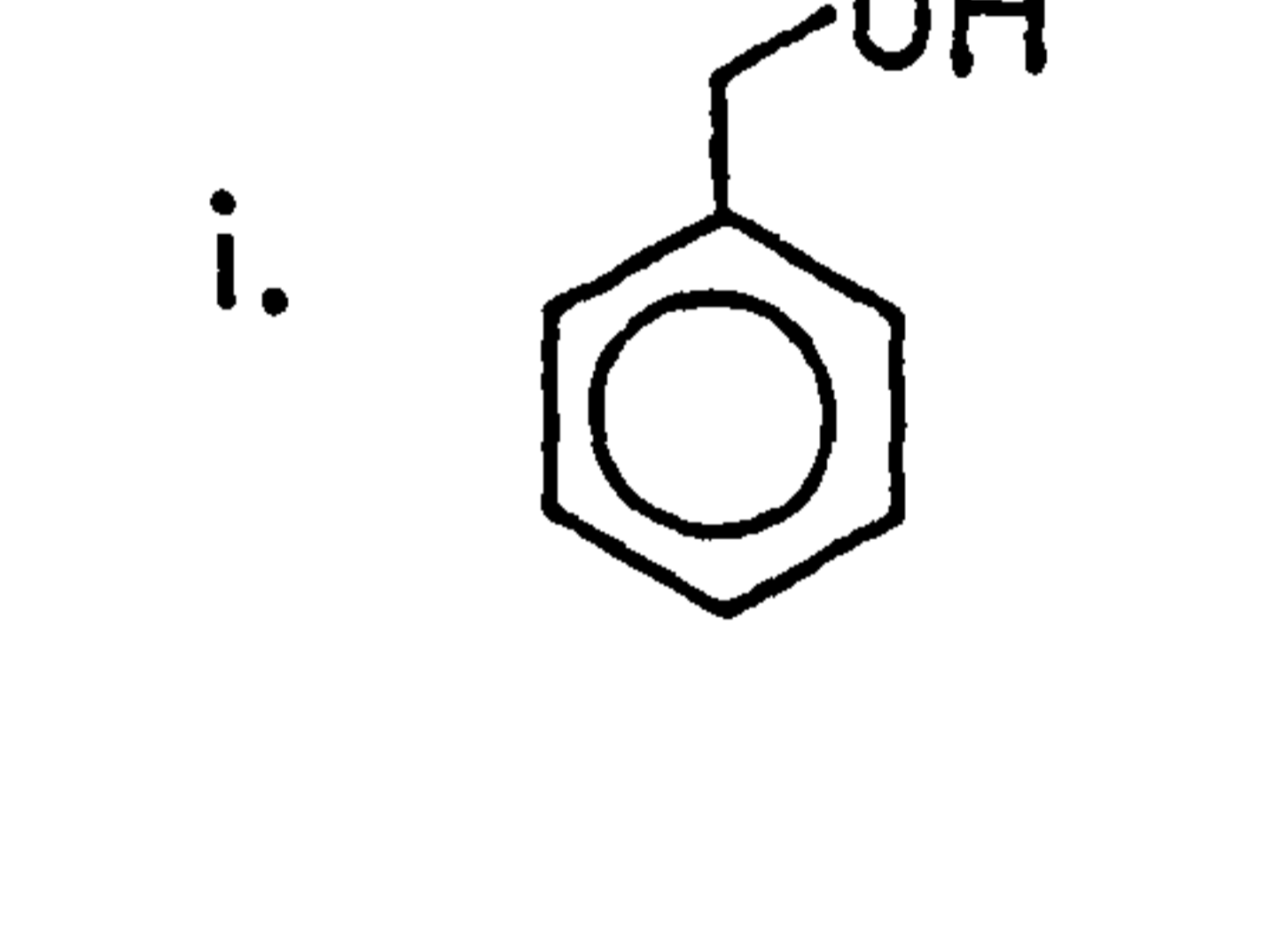
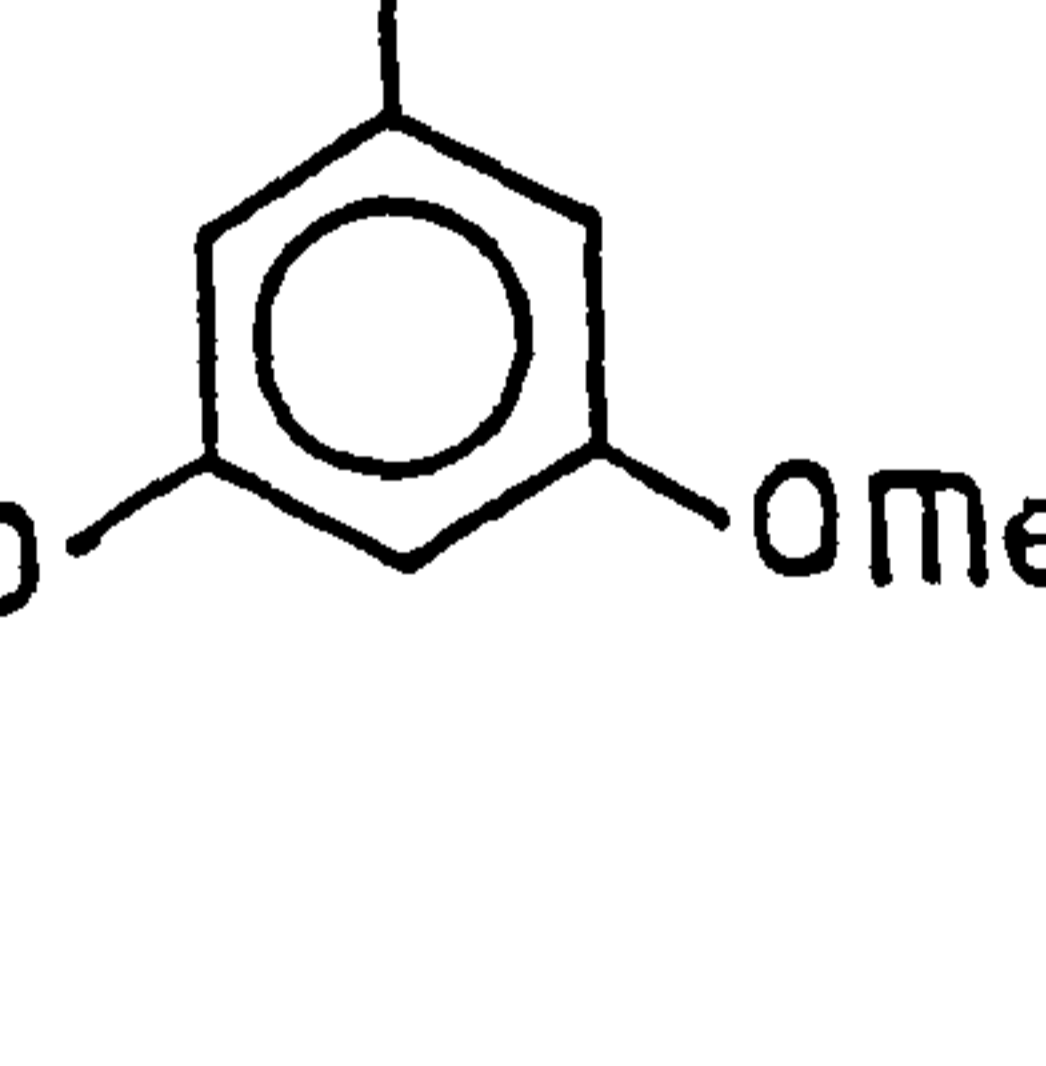
Compound	Rate of oxidation	Compound	Rate of oxidation
a. 	1.00 ($\equiv 0.30 \text{ U ml}^{-1}$)	f. 	0.35
b. 	0.58	g. 	0
c. 	0.43	h. 	0
d. 	0.17	i. 	0
e. 	0		

Table 5.1. Relative rates of LiP oxidation of various benzyl alcohols and other mono-aryl compounds (oxidation of veratryl alcohol, a, using LiP at 0.3 U ml^{-1} was taken as 1.00).

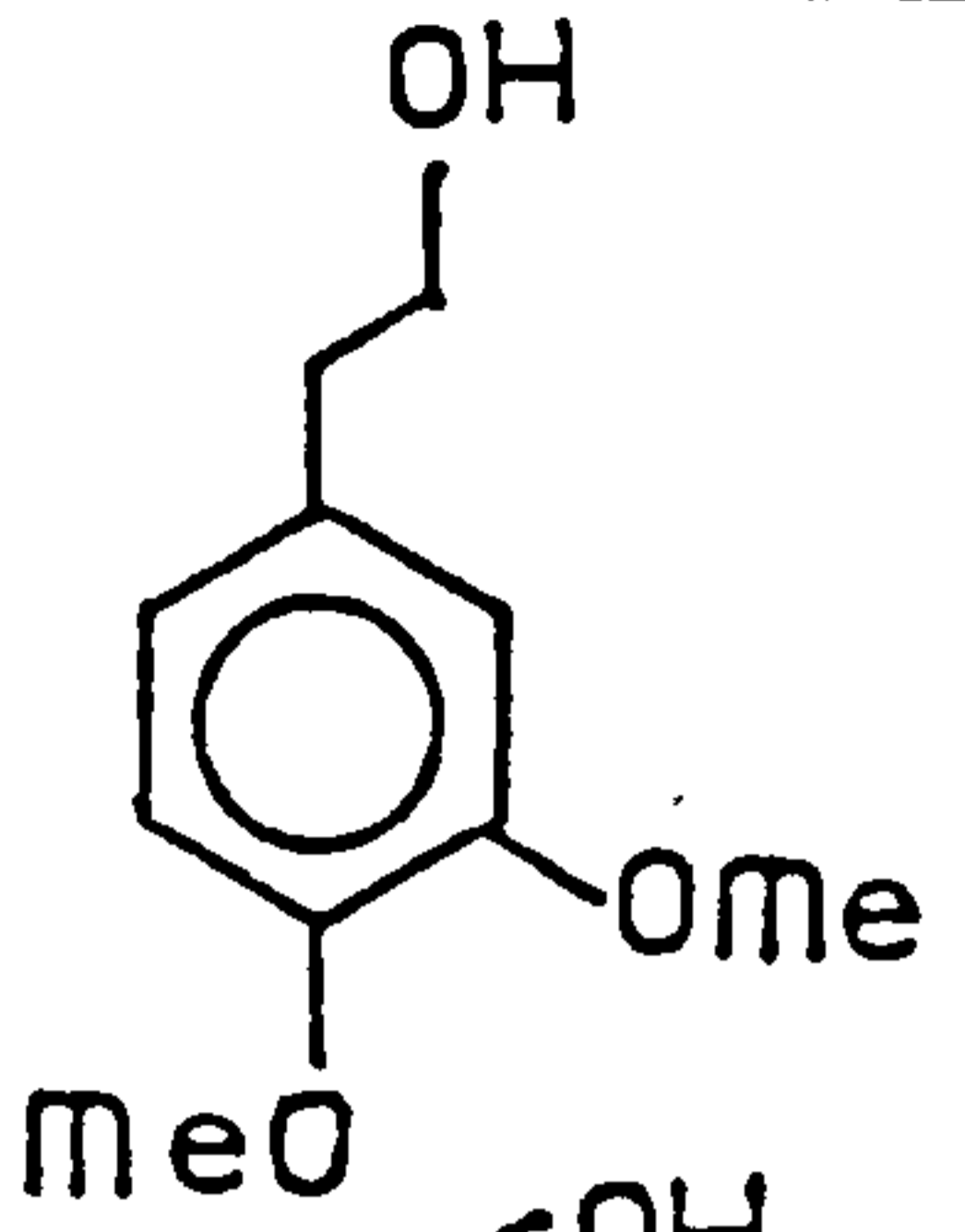
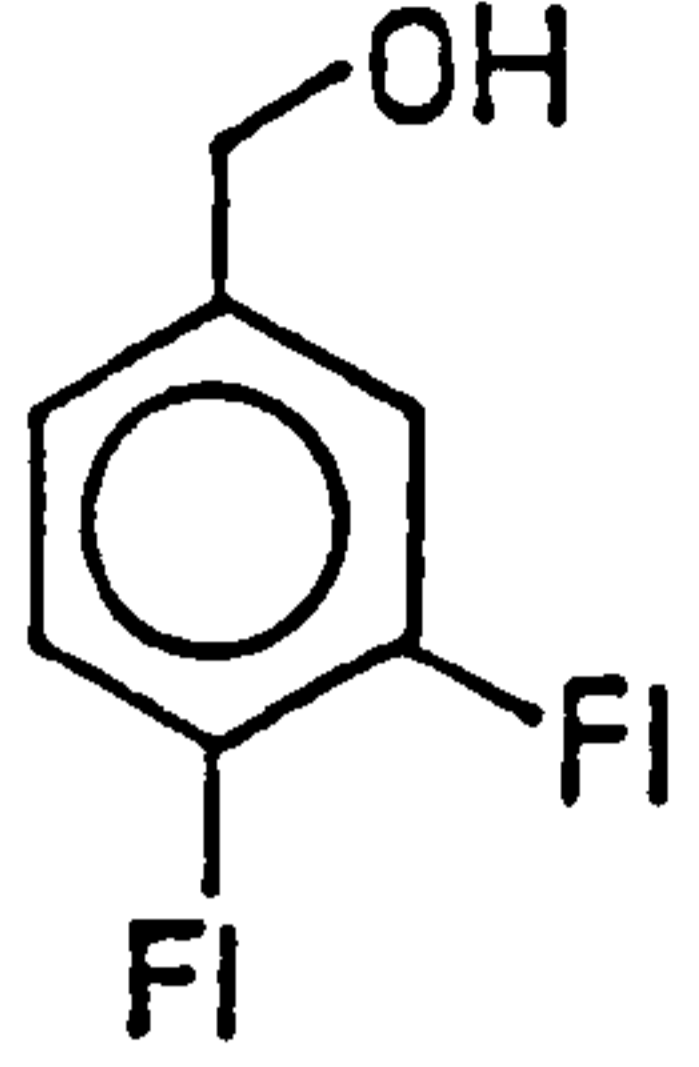
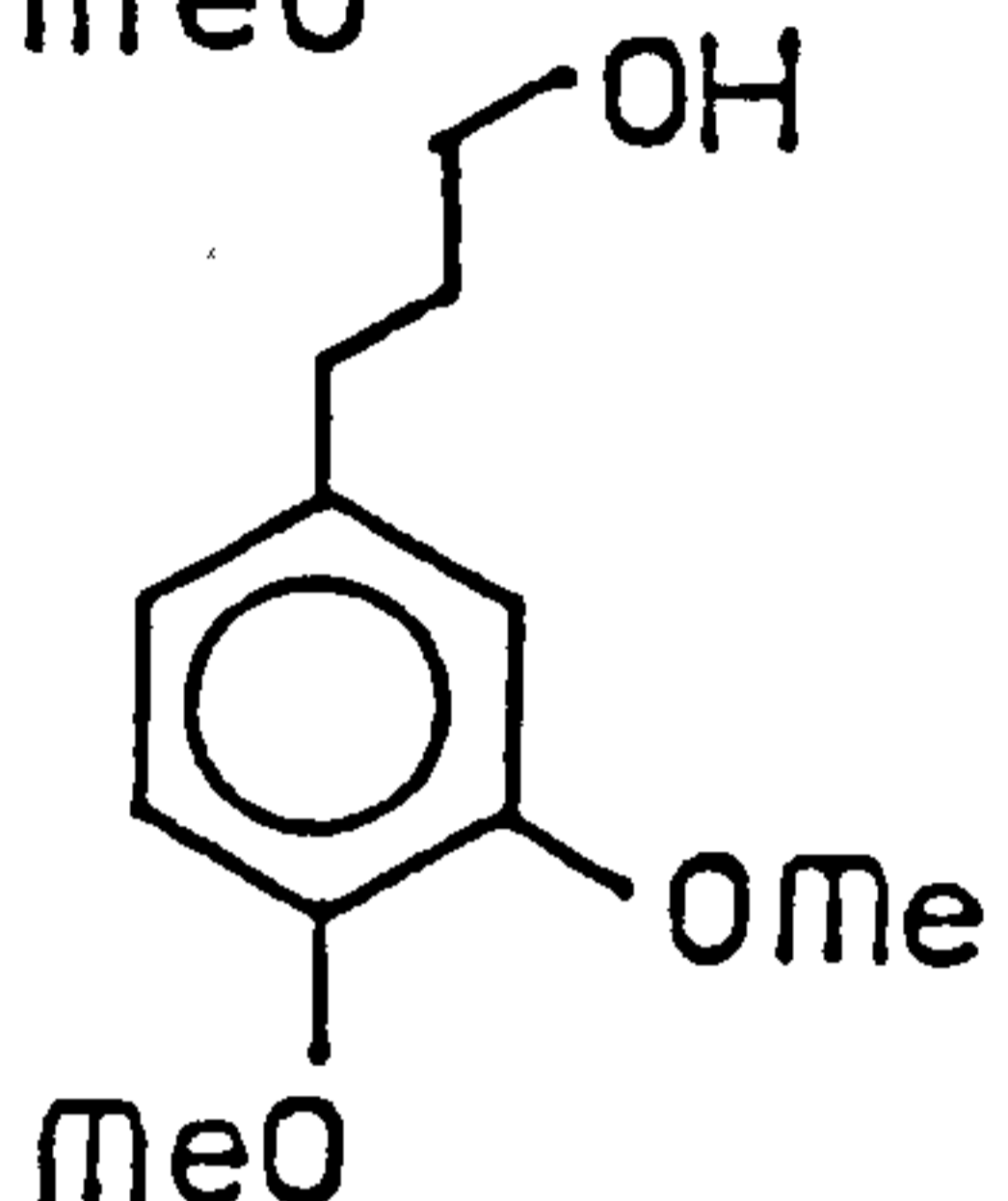
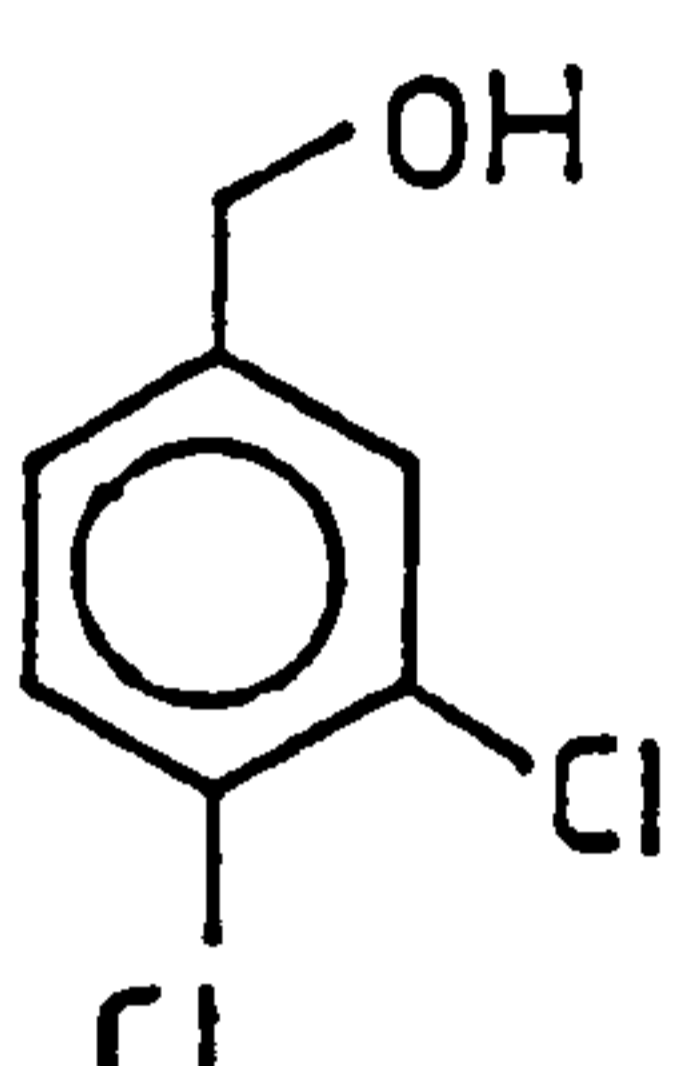
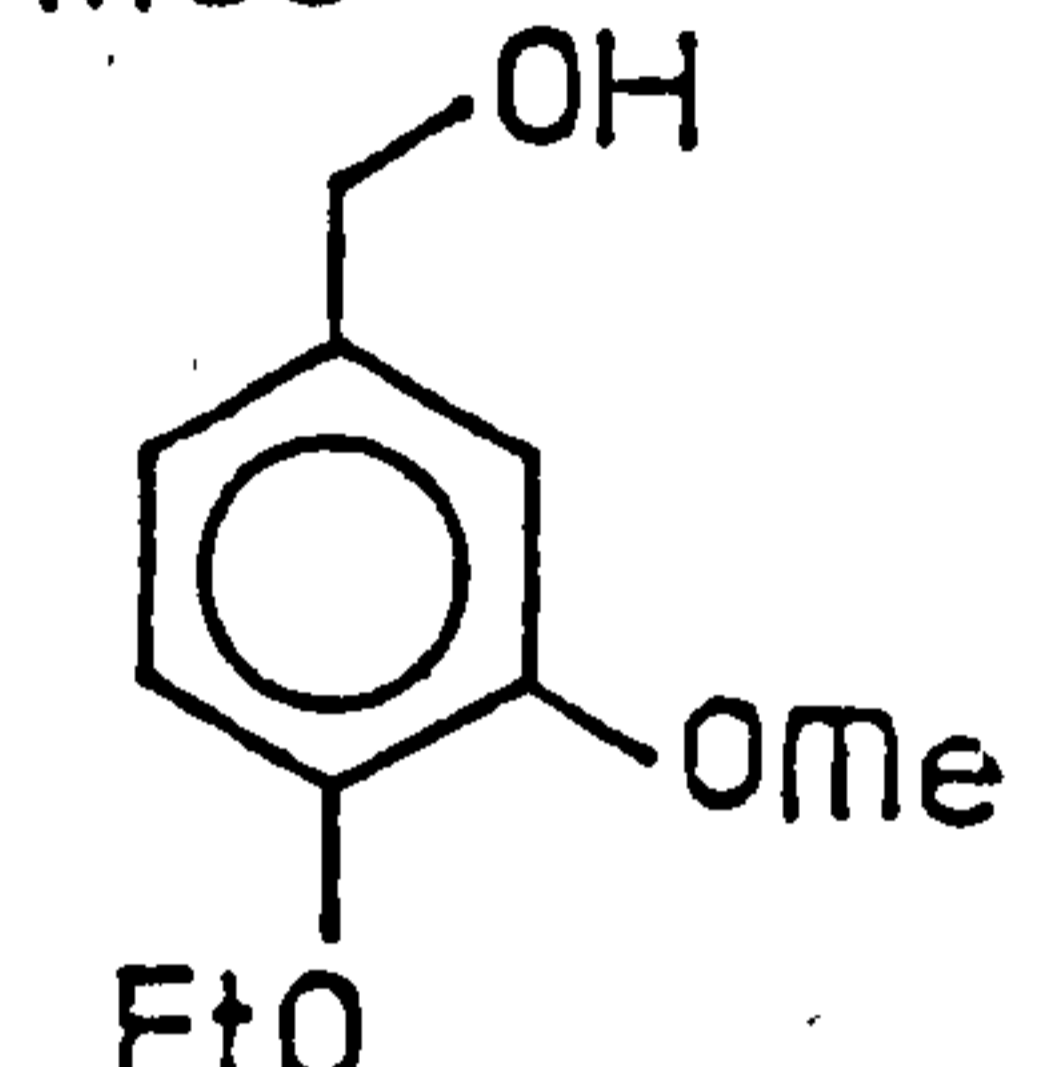
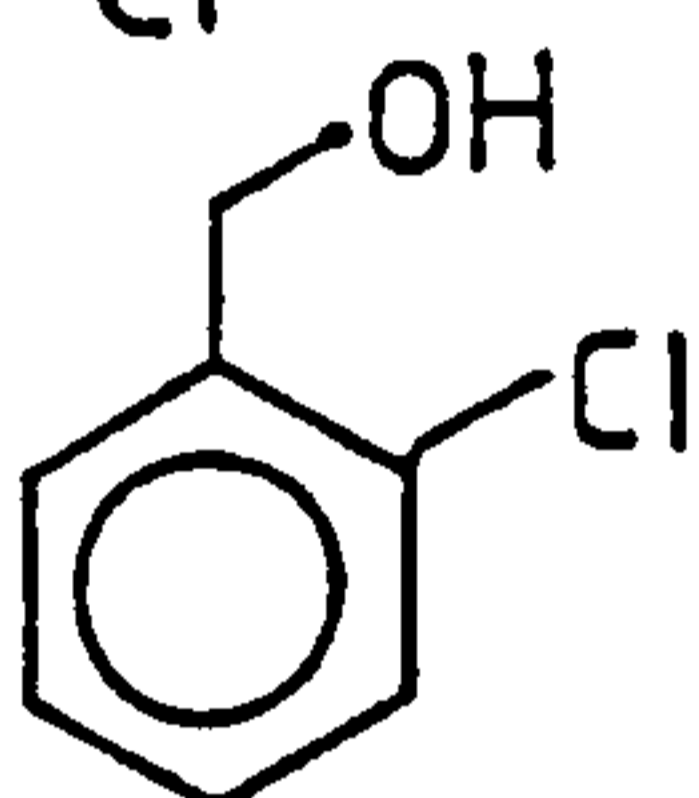
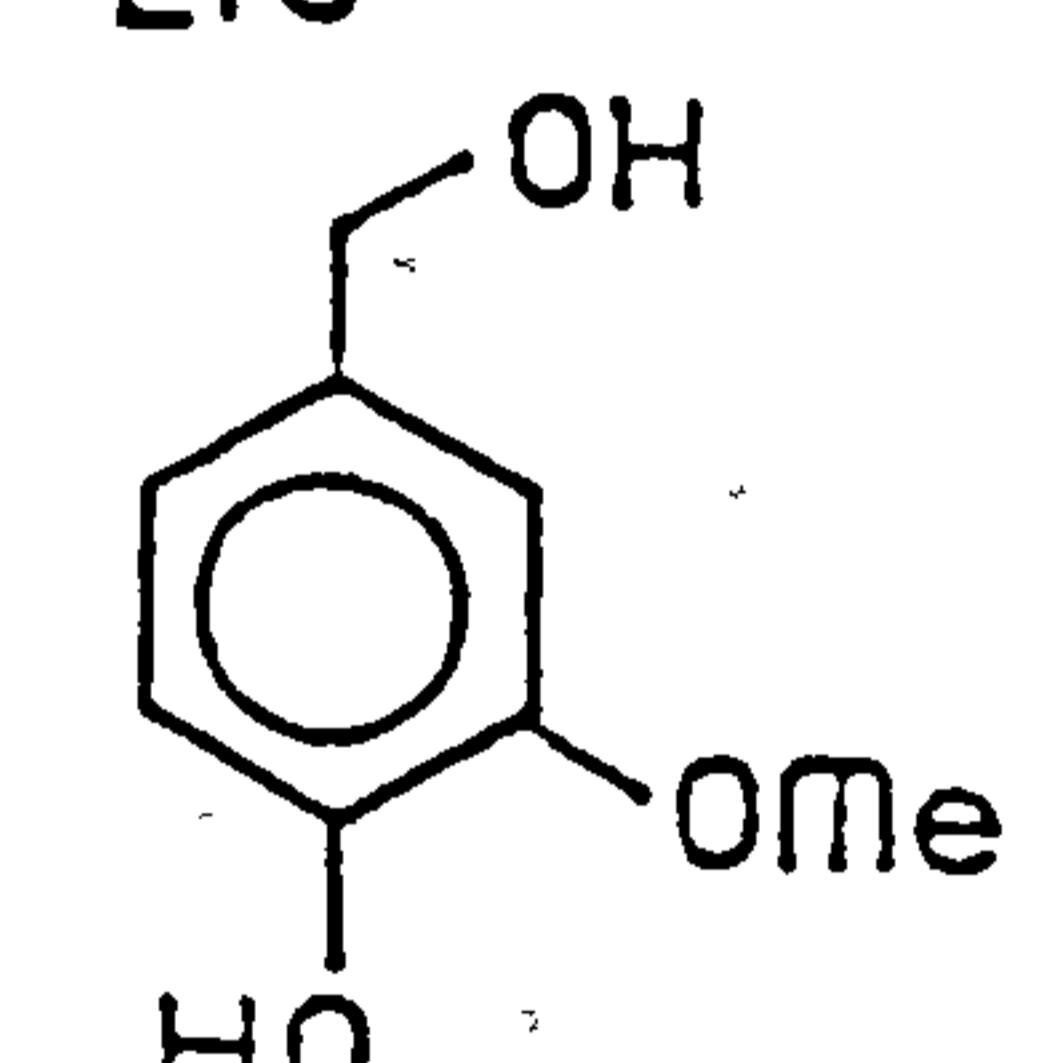
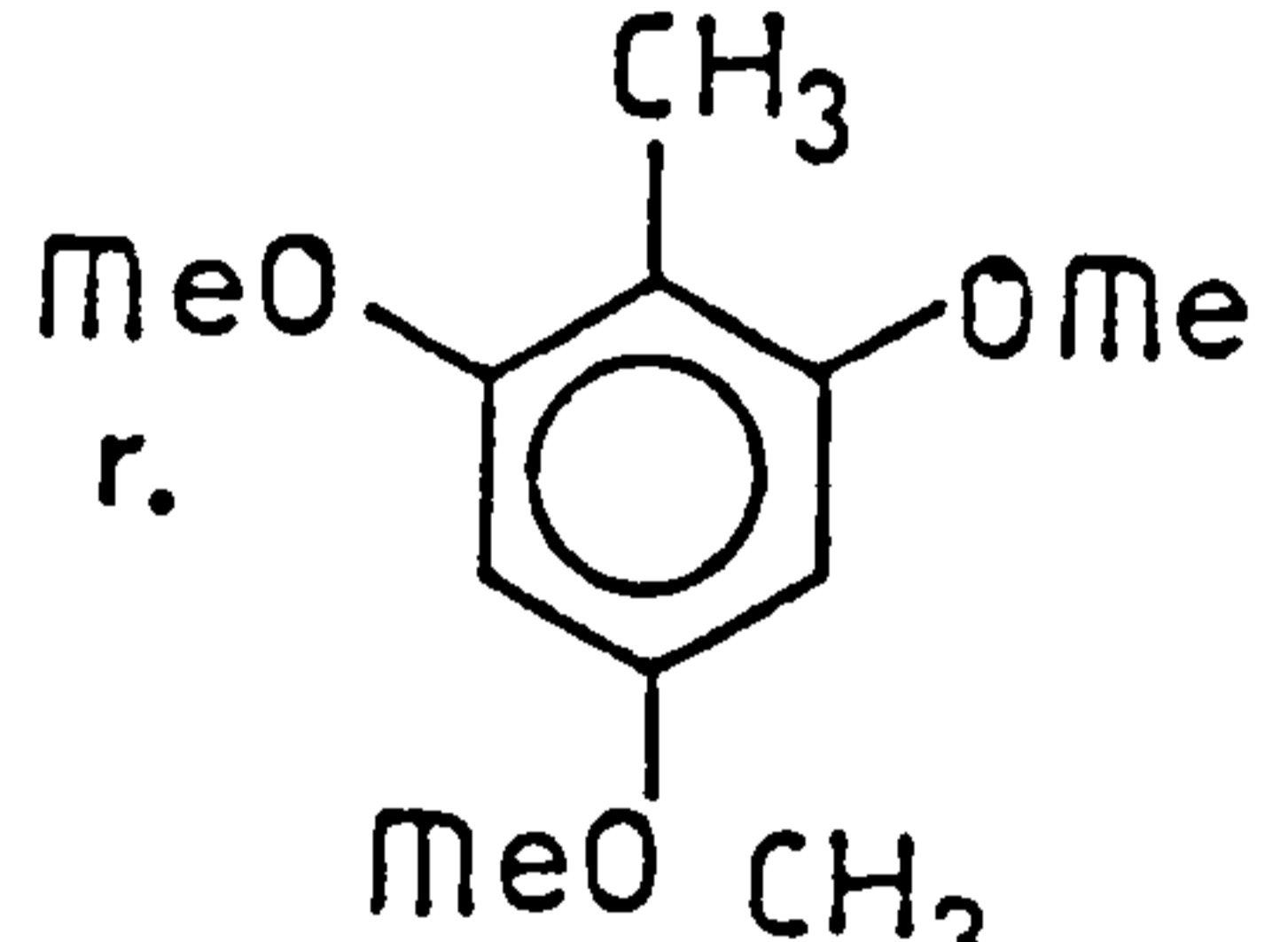
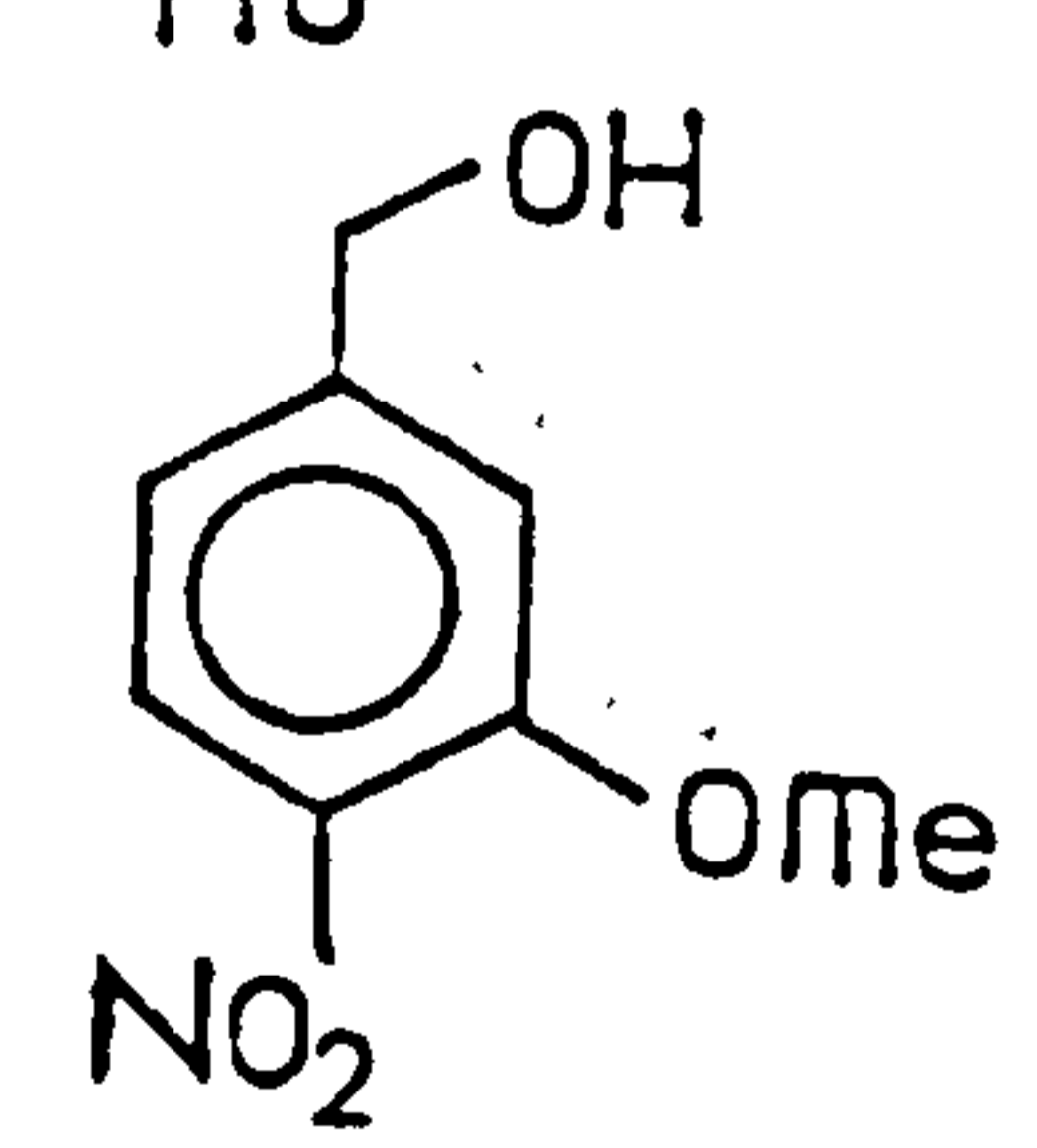
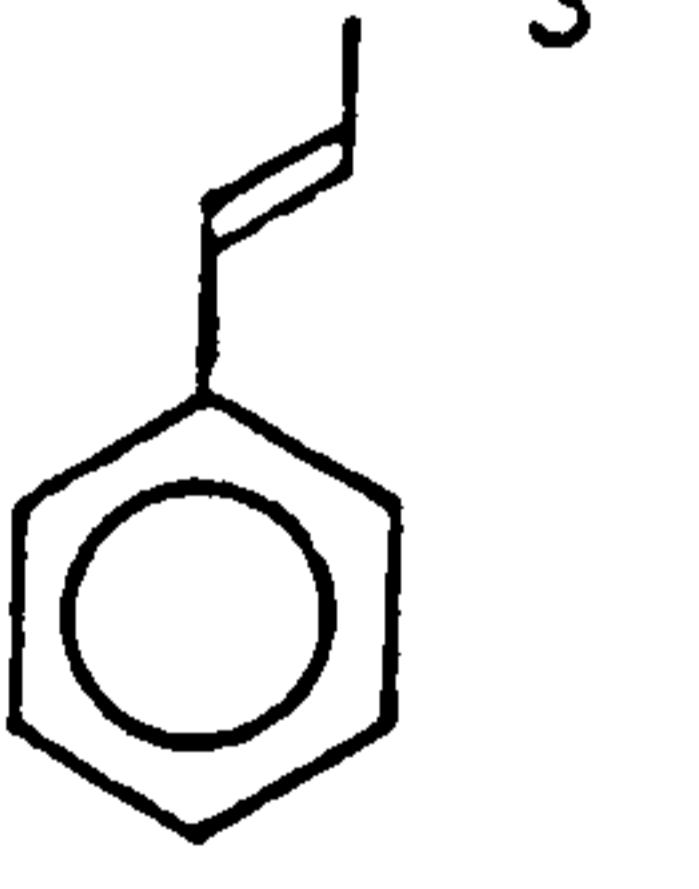
Compound	Rate of oxidation	Compound	Rate of oxidation
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k. 	0.10	p. 	0
l. 	0.80	q. 	0
m. 	0	r. 	0
n. 	0	s. 	0

Table. 5.1. (Continued).

Substrate

Oxidation (refs.)

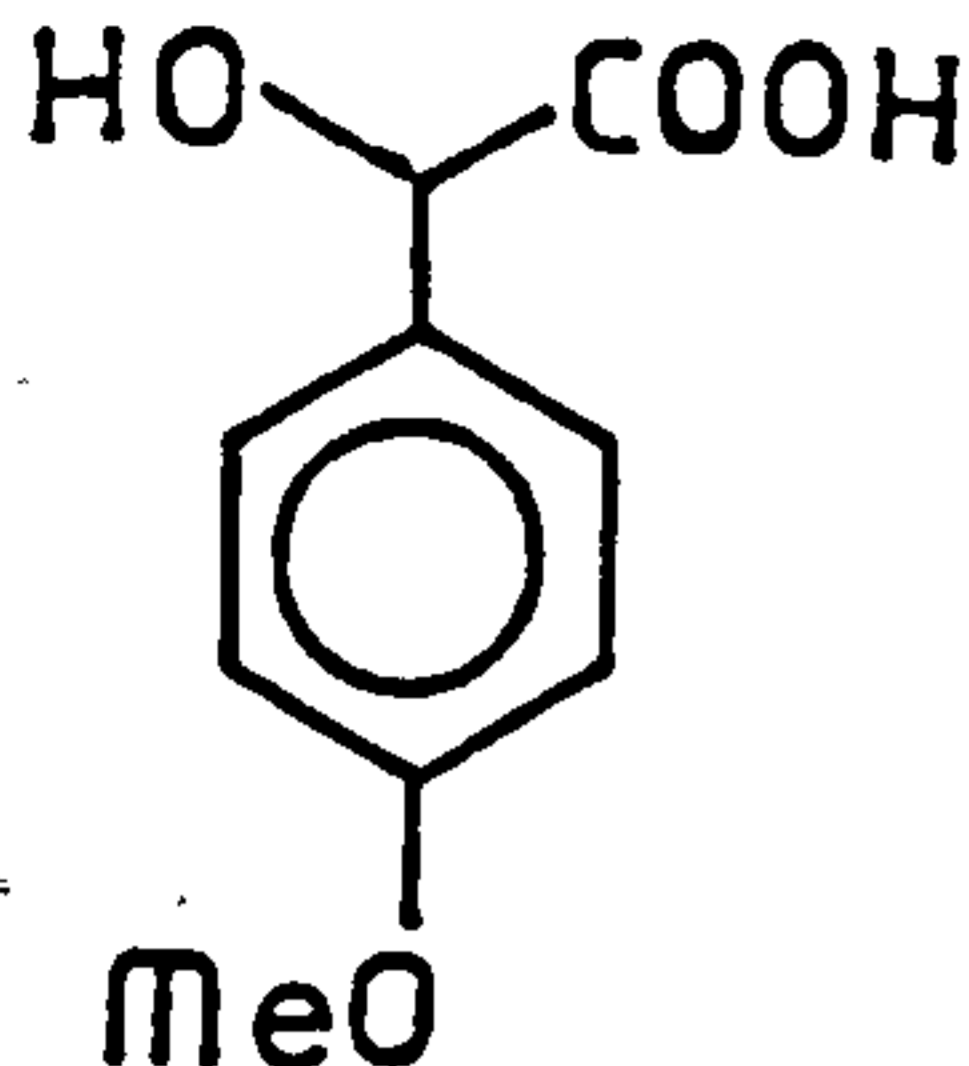
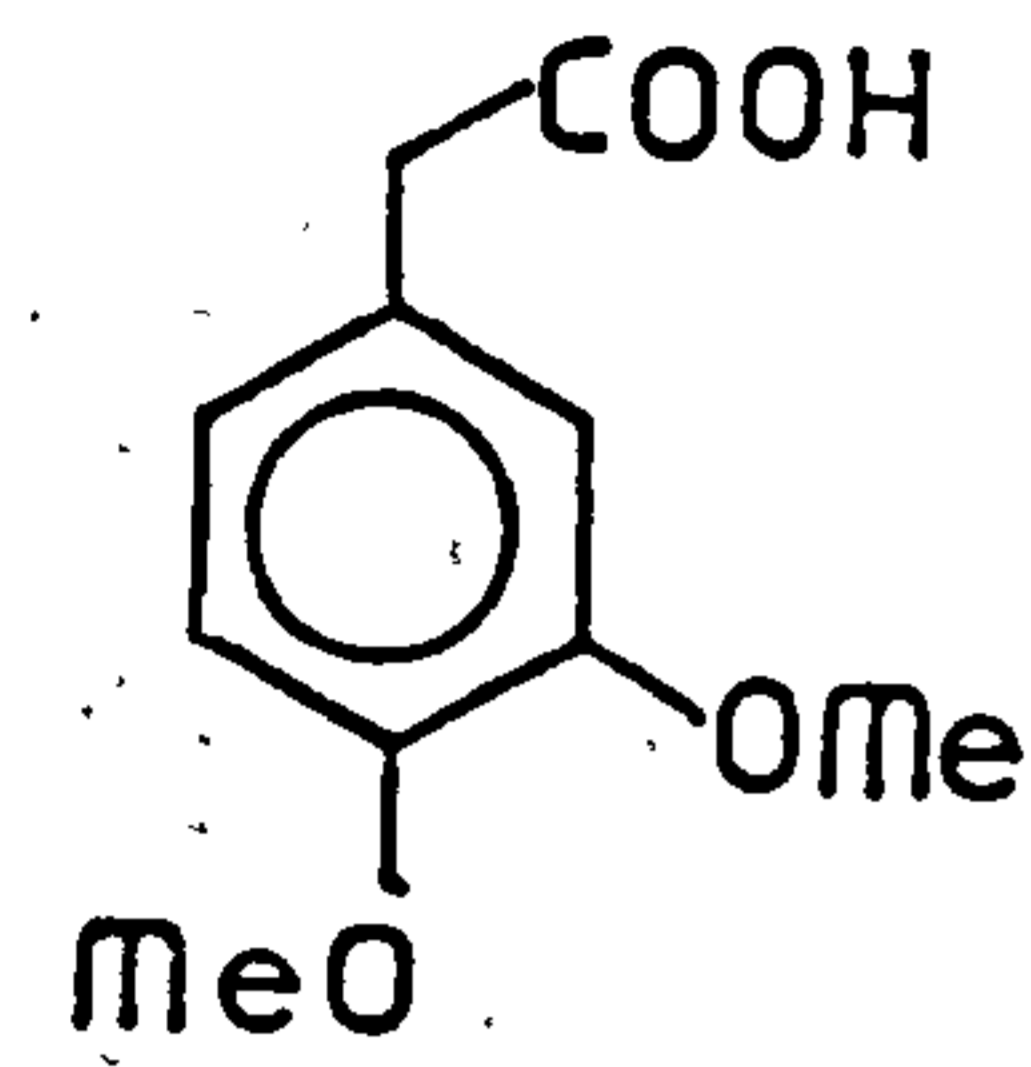
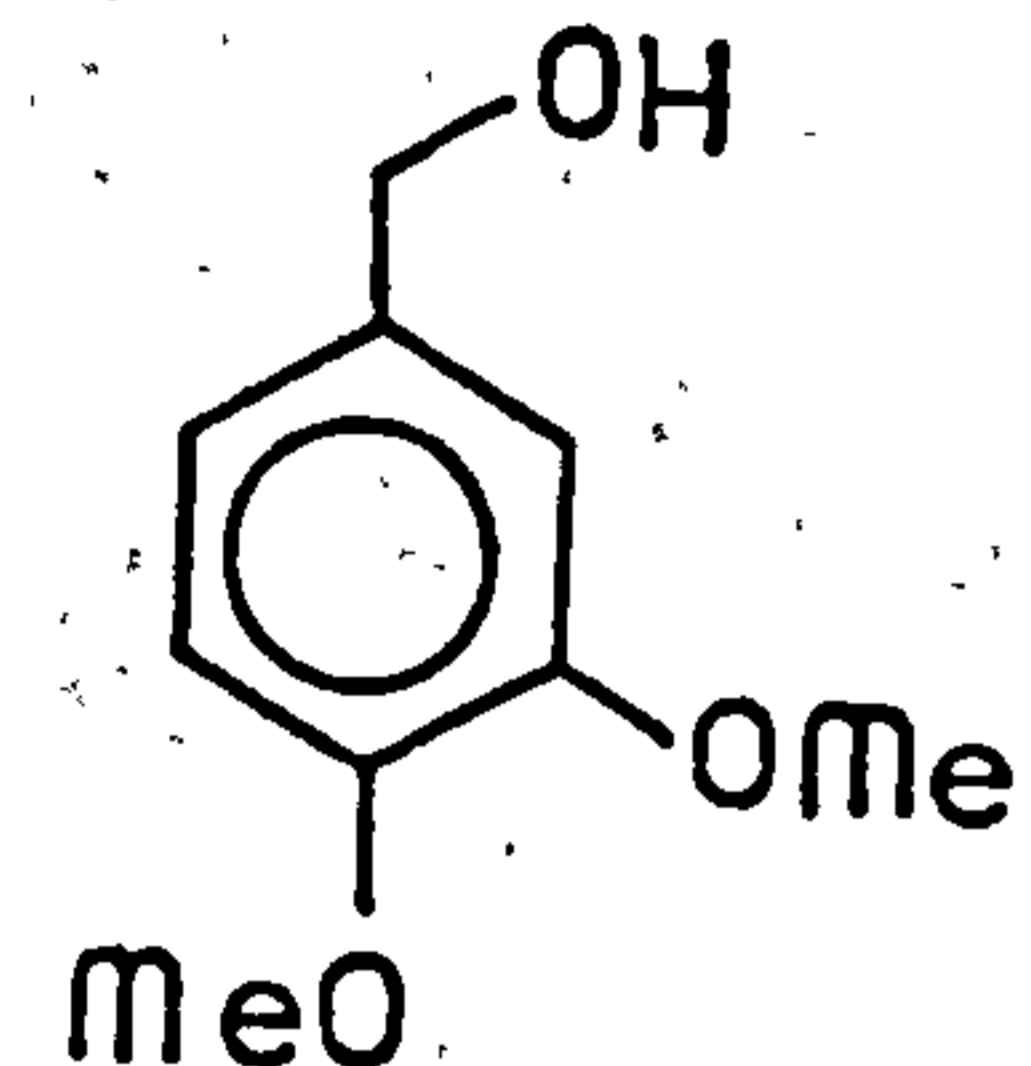
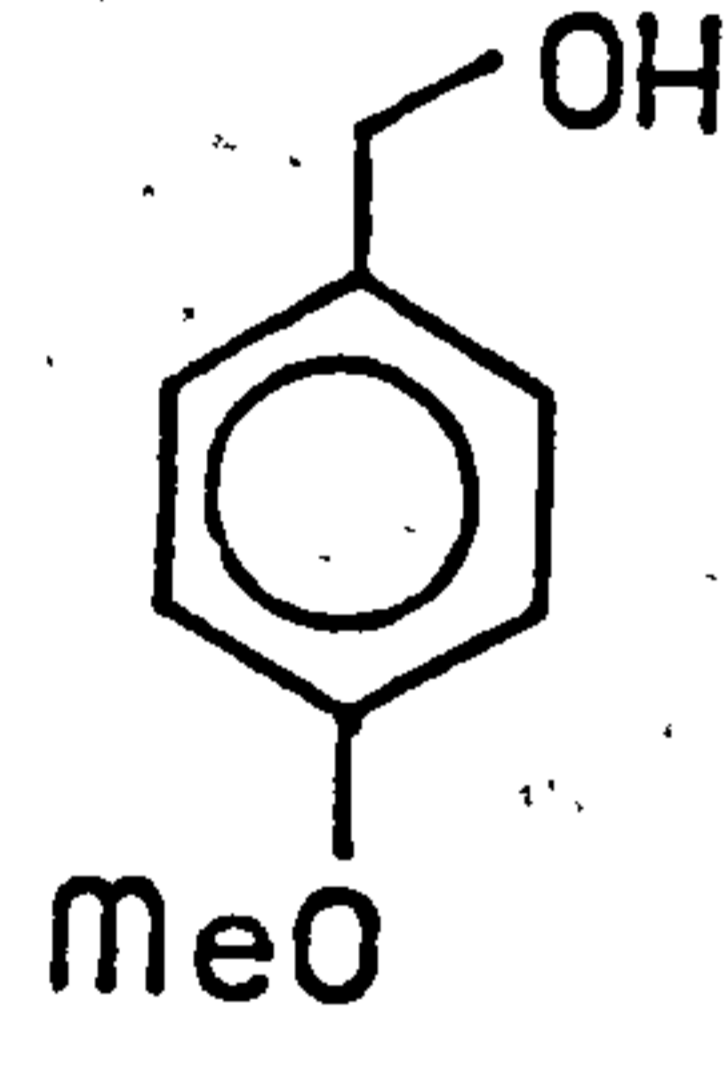
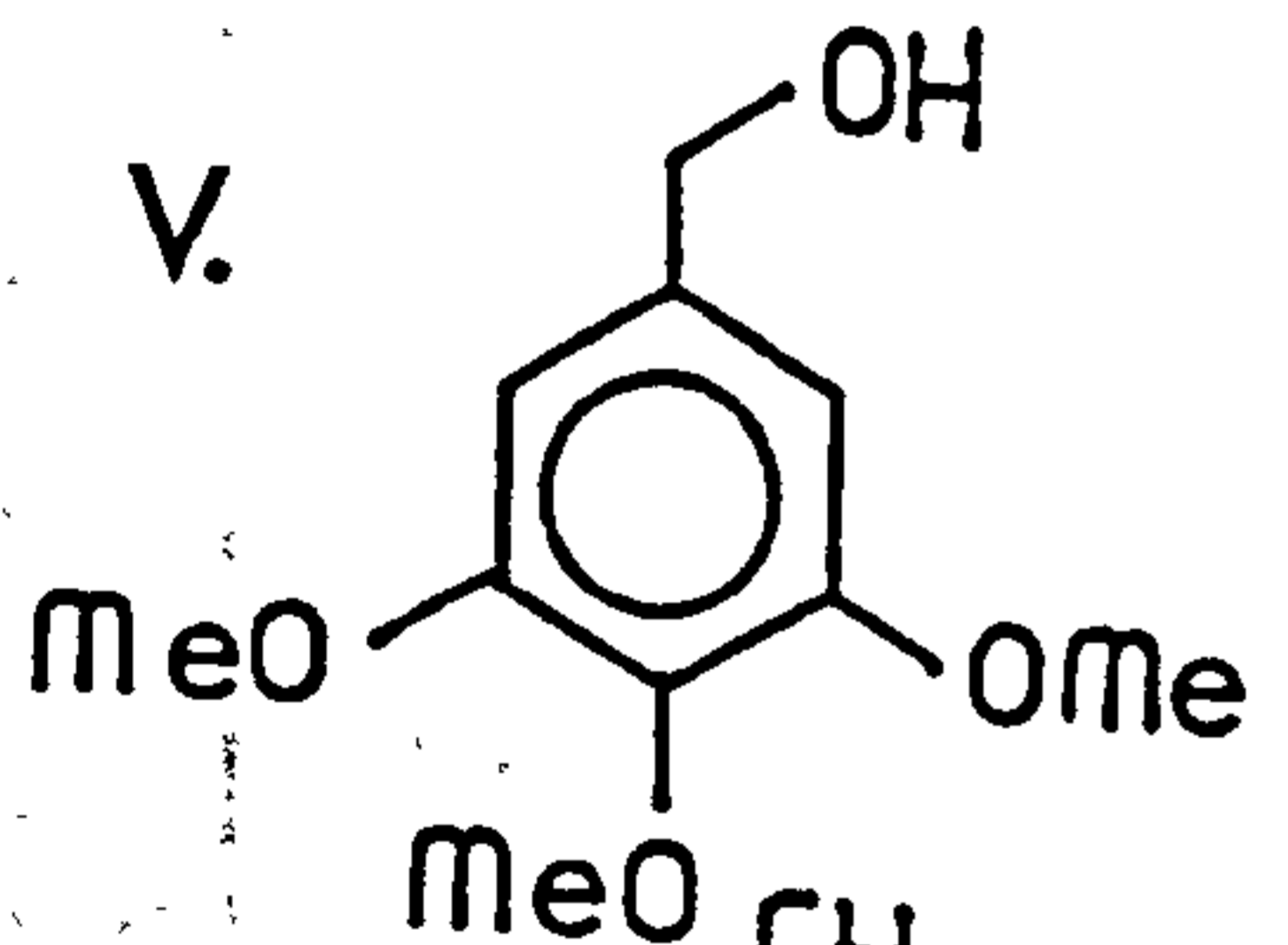
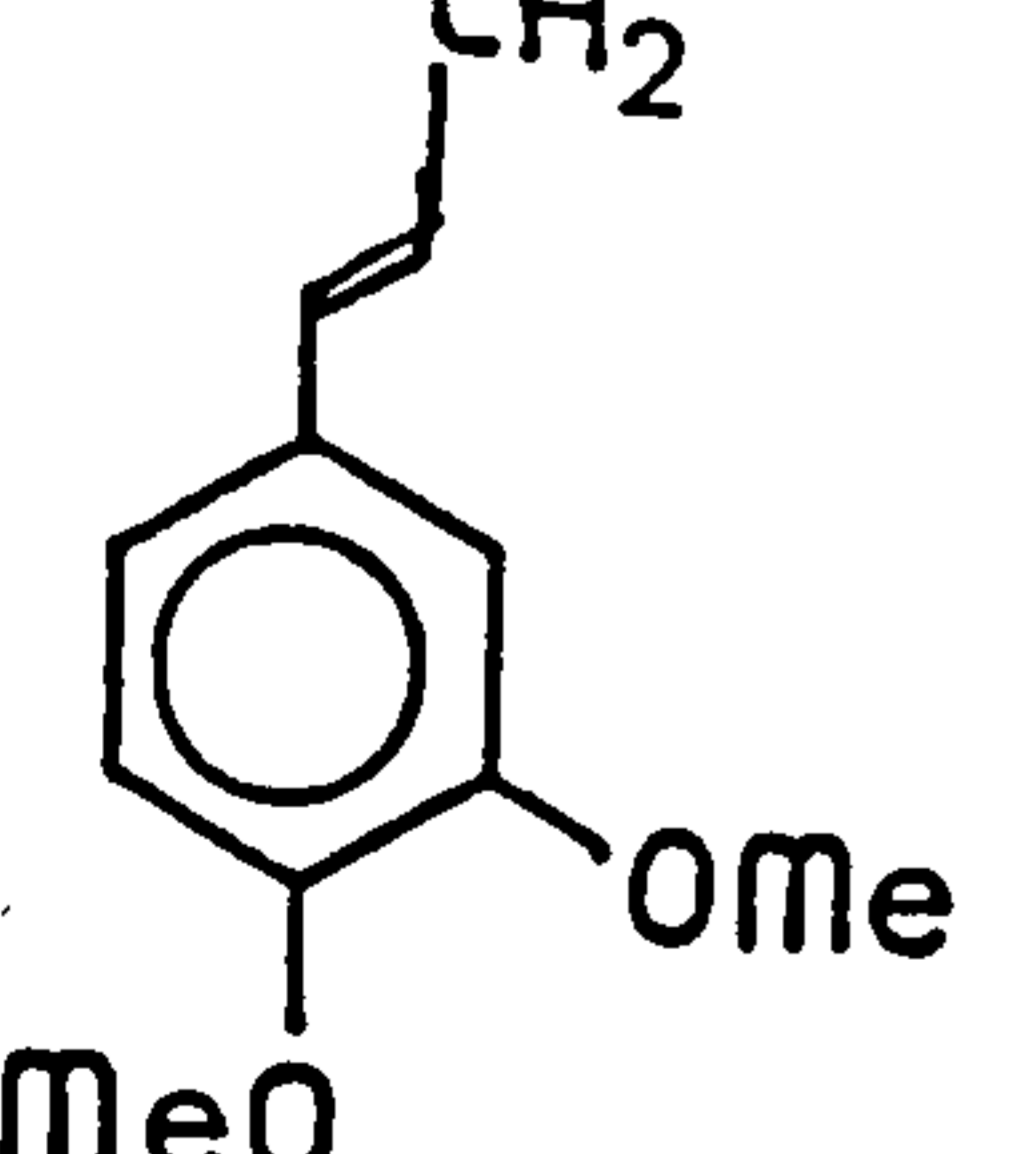
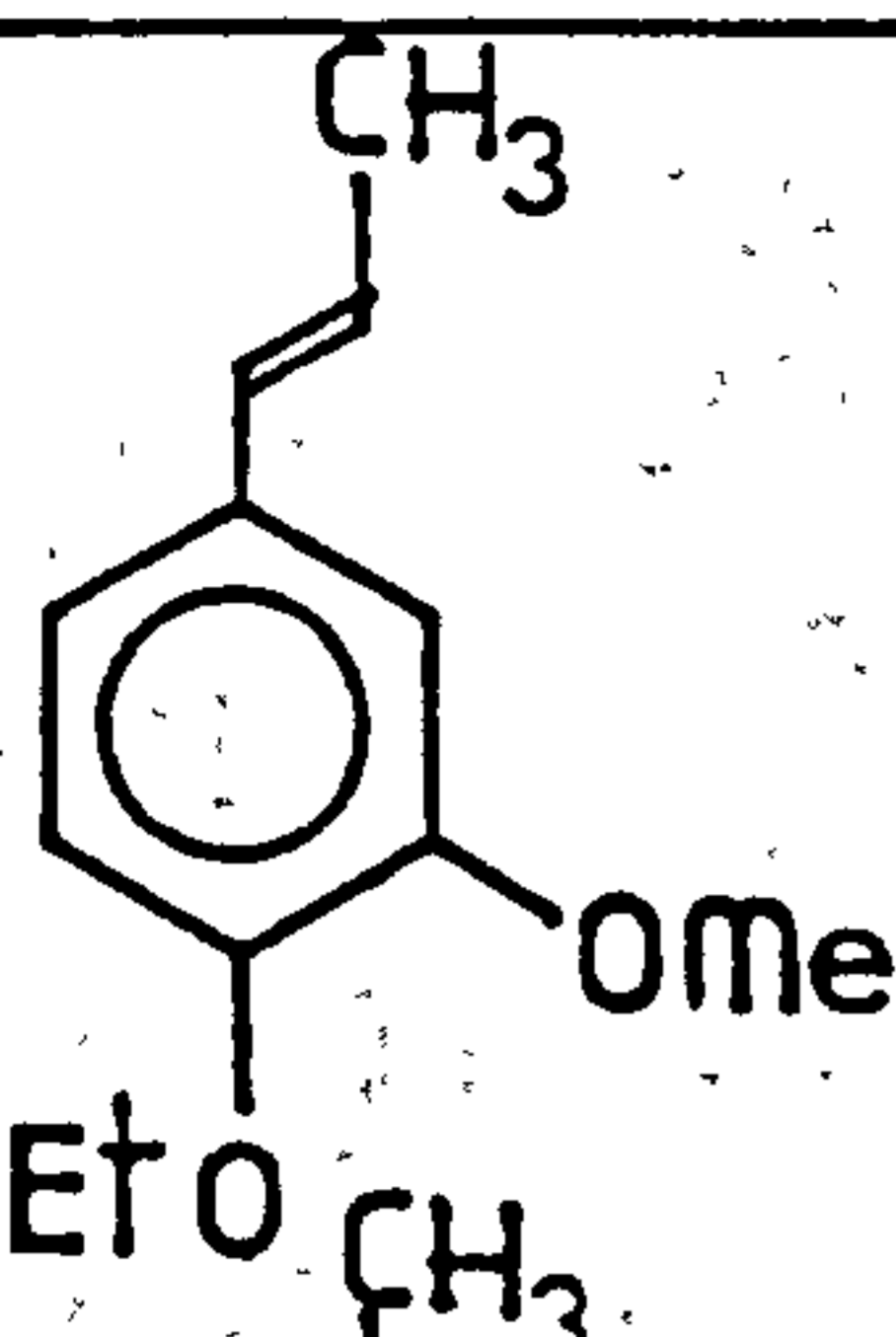
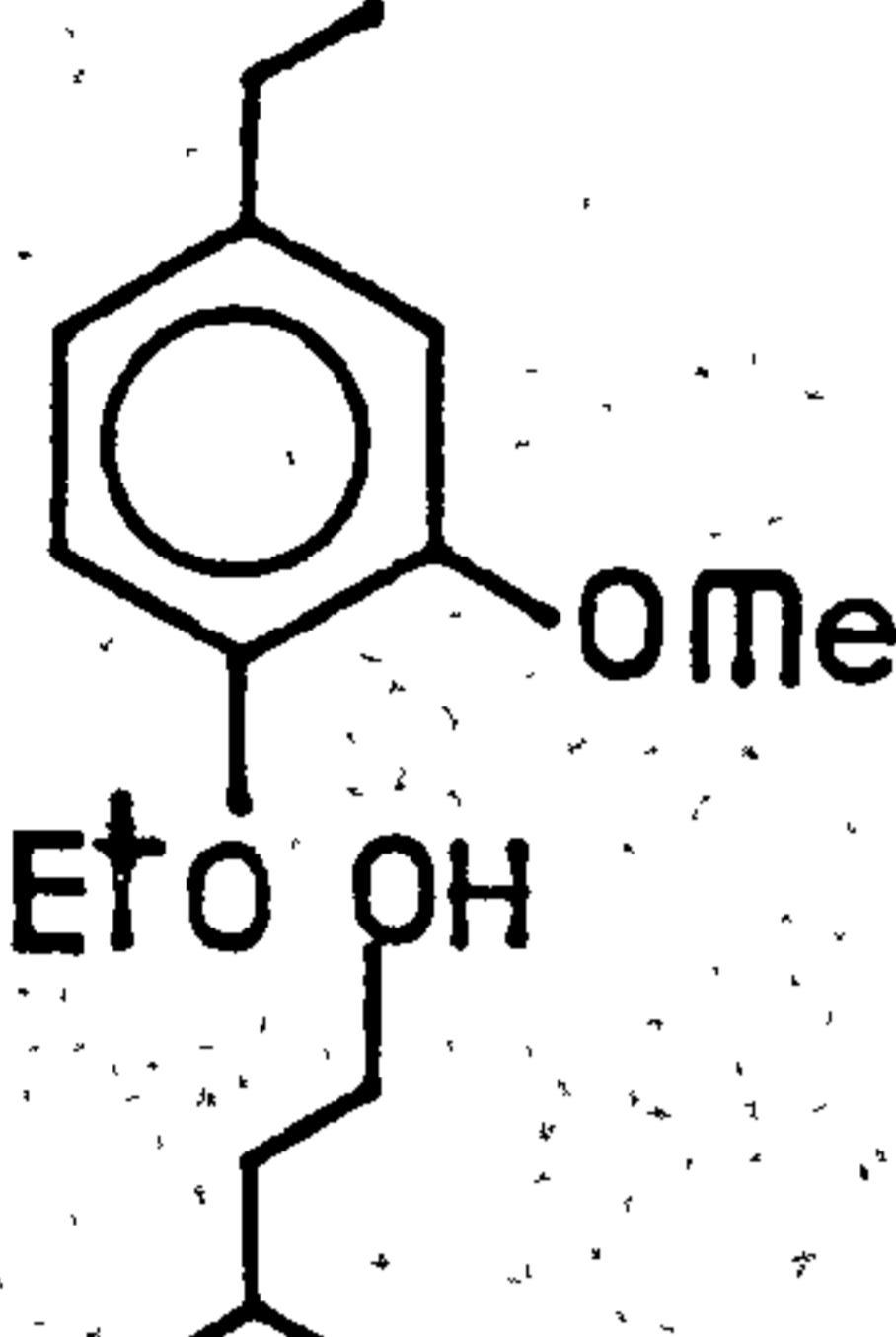
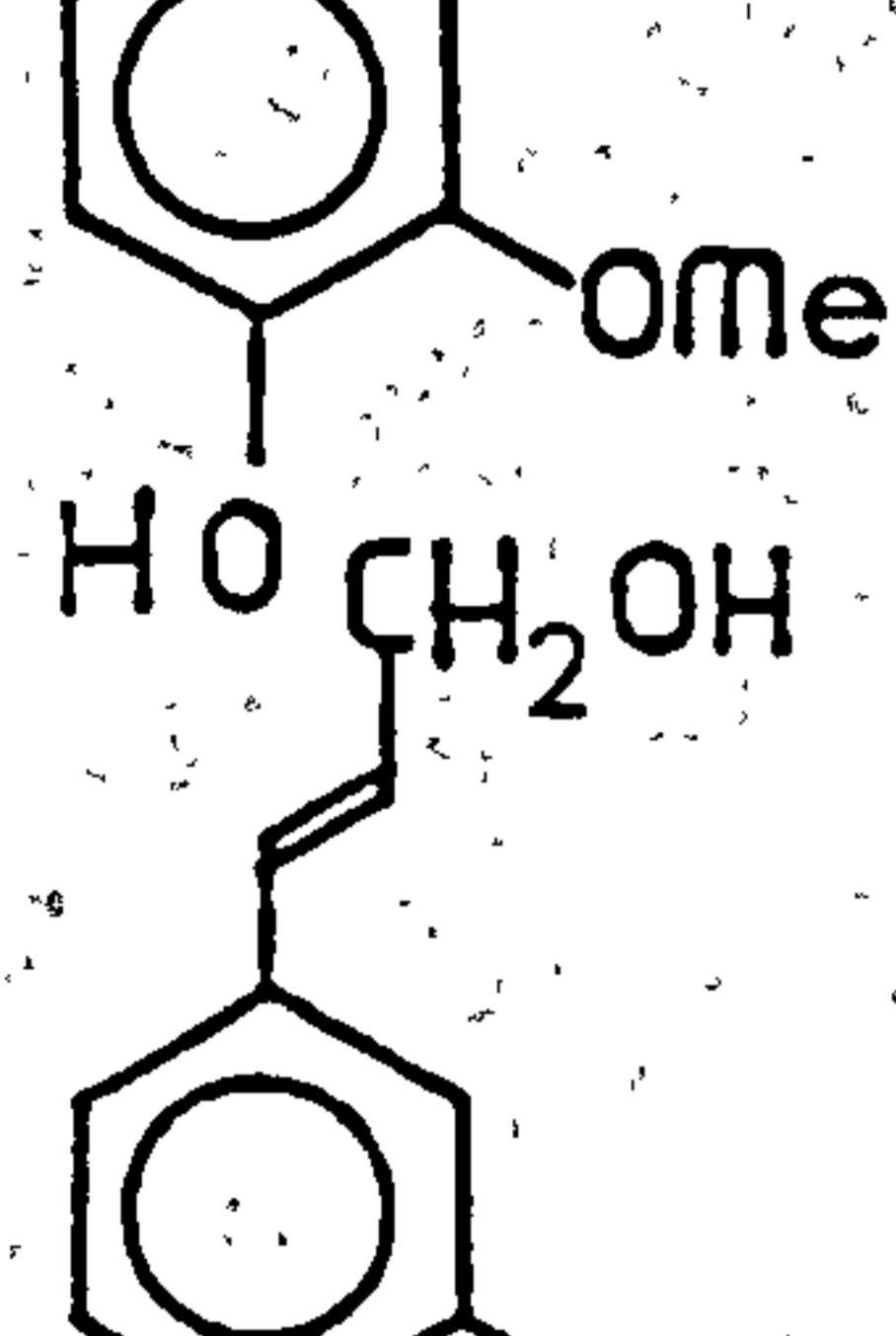
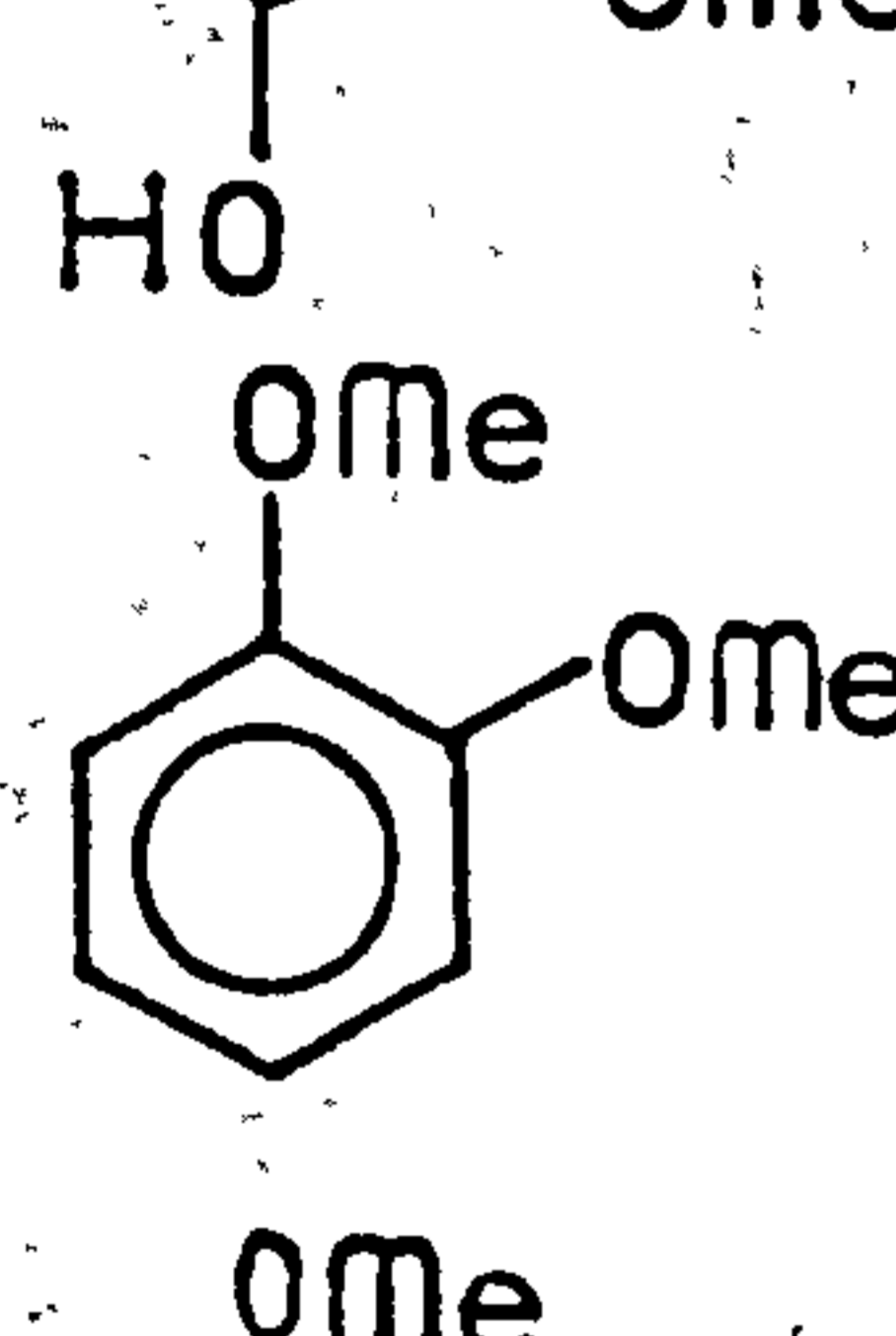
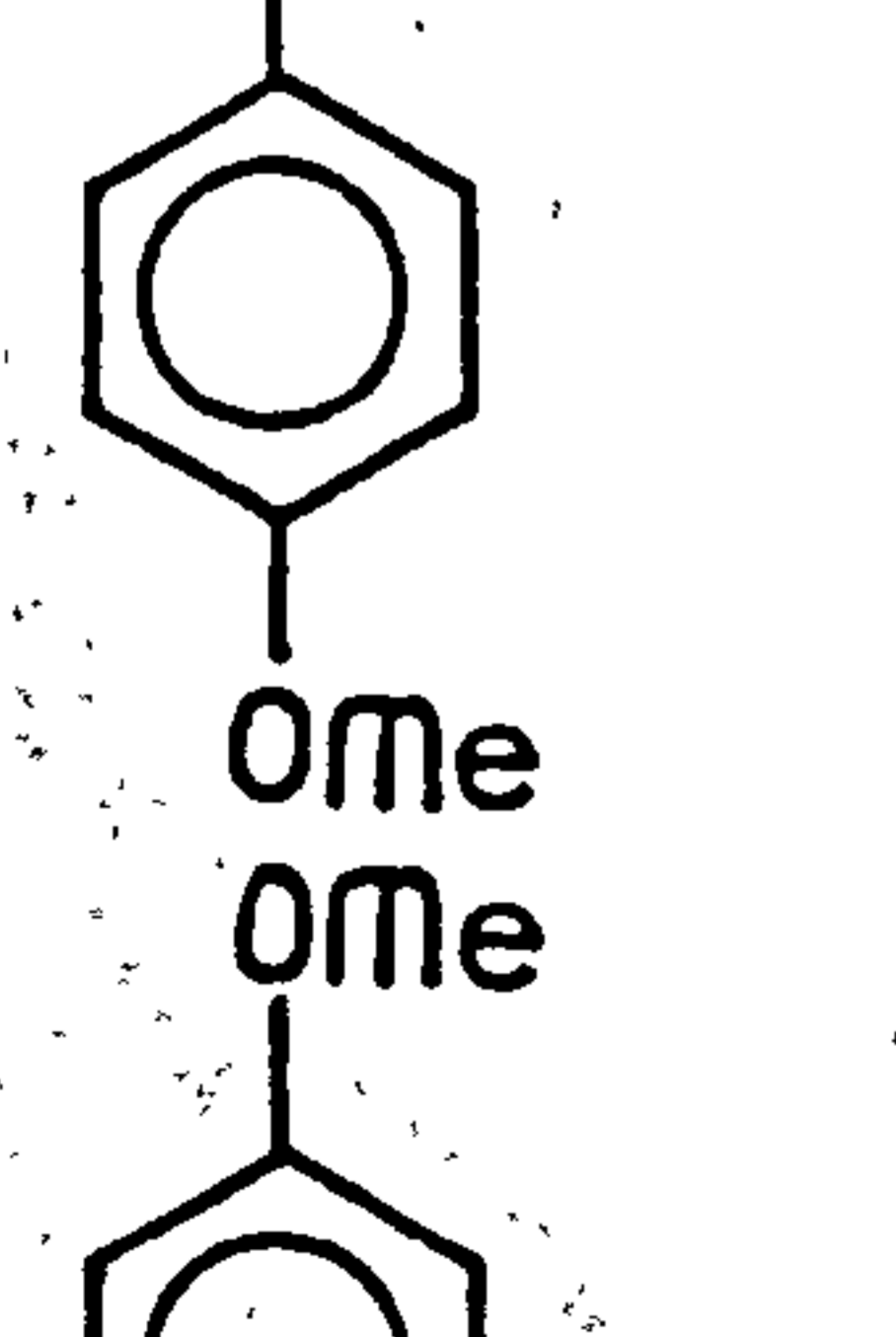
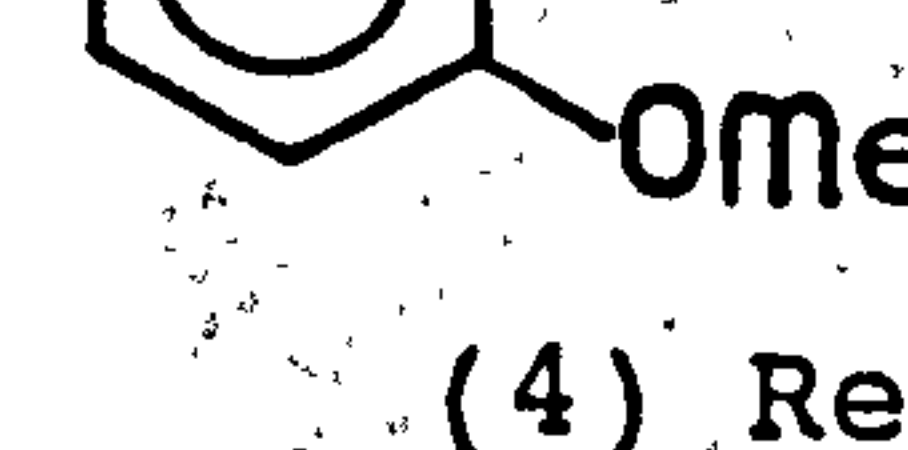
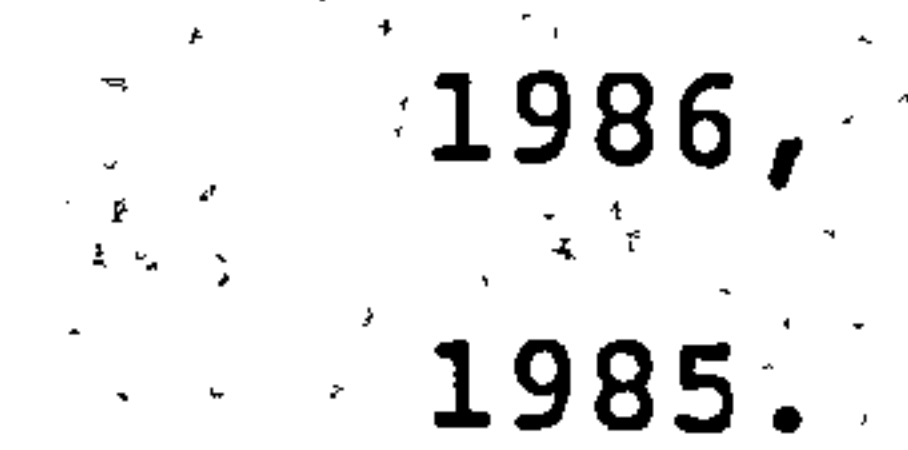
I.		4-methoxyphenyl, α -hydroxy acetic acid	+/- (v.slow)	(1)
II.		3,4-dimethoxyphenyl acetic acid	+	(1)
III.		veratryl alcohol	+	(1,2)
IV.		anisyl alcohol	+/- (v.slow)	(1,2)
V.		3,4,5-trimethoxybenzyl alcohol	+	(2)
VI.		3,4-dimethoxy styrene	+	(2)

Table 5.2. Methoxylated mono-aryl compounds that have been screened for susceptibility to LiP oxidation (1) Harvey *et.al.*, 1986, (2) Tien & Kirk, 1984, (3) Gold *et.al.*, 1984b ...

VII.		3-methoxy,4-ethoxy styrene	+	(3,4)
VIII.		3-methoxy,4-ethoxyphenyl propane	+	(4,5)
IX.		3-methoxy,4-hydroxyphenethyl alcohol	-	(6)
X.		3-methoxy,4-hydroxy cinnamic alcohol	+	(6)
XI.		1,2-dimethoxy benzene	+	(7)
XII.		1,4-dimethoxy benzene	+	(7)
XIII.		1,3-dimethoxy benzene	-	(7)

(4) Renganathan et.al., 1985, (5) Renganathan et.al., 1986, (6) Paszczynski et.al., 1986. (7) Kersten et.al., 1985.

5.1.2. The effect of methoxy substituents on LiP oxidation of benzyl alcohols.

Methoxy groups are significant in that they are commonly found in lignin at the 3, and 3,5 positions on aryl units (Adler, 1977). They are also present in the 'natural' substrate veratryl alcohol at the 3,4 positions.

Table 5.1. shows that both degree and position of methoxy substituents can have a profound effect on oxidation by LiP. Benzyl alcohol (i) itself was not oxidised, neither were the 3, and 4, positioned monomethoxylated derivatives (g,h).

Harvey et.al. (1986) found some oxidation of 4-methoxybenzyl alcohol (anisyl alcohol, IV), but it was only 1% of the rate found for the oxidation of 3,4-dimethoxybenzyl alcohol (III). They also found a similar low level for the decarboxylation of 4-methoxyphenyl α -hydroxy acetic acid (I) compared to 3,4-dimethoxyphenyl acetic acid (II).

Tien & Kirk (1984) found that 4-methoxybenzyl (anisyl) alcohol (IV) could be oxidised, but they did not mention to what extent relative to 3,4-dimethoxybenzyl alcohol. Despite repetitive attempts and the use of LiP specific activities of up to four times higher than preparations used by Tien & Kirk (1984), 4-methoxybenzyl alcohol (g)

could not be oxidised.

One possible reason for the difference between the results in this study and those observed by Tien & Kirk is that, in some of the Aldrich commercial preparations of methoxybenzyl alcohols, it was noticed that trace amounts of other methoxylated derivatives could be found. For example, commercial preparations of 2,4-dimethoxybenzyl alcohol and 3,5-dimethoxybenzyl alcohol used in this work contained small amounts of 2,3-dimethoxybenzyl alcohol and a mono-methoxylated benzyl alcohol respectively. If a catalytic amount of a methoxylated benzyl alcohol capable of being oxidised by LiP was present in the 4-methoxybenzyl alcohol used by Tien & Kirk, a false positive result may have been recorded because such a contaminant could have acted as an electron mediator (see 1.8.8.) between the enzyme and the monomethoxylated substrate.

Another possible explanation for the difference in results for 4-methoxybenzyl alcohol oxidation is that LiP from P.chrysosporium (Tien & Kirk, 1984) can oxidise substrates with higher ionisation potentials (i.e., the energy required to remove an electron from the substrate to form a cation radical) than the analogous enzyme from S.pulverulentum (this work).

5.1.3. The effect of di-, and tri-methoxy substitution on LiP oxidation of benzyl alcohols.

The position of the methoxy group on dimethoxylated benzyl alcohols was shown to exert a significant influence on the rate of substrate oxidation. 3,4-dimethoxybenzyl alcohol (a) was oxidised the fastest, followed by 2,4,; 2,3,; and 2,5, dimethoxybenzyl alcohols (b,c,d, respectively). 3,5-dimethoxybenzyl alcohol (e) was not oxidised at all.

Specificity of LiP oxidation of benzyl alcohols may be due to a combination of enzyme affinity for the substrate and the ionisation potential of the substrate. The degree of methoxylation influences the ionisation potential of a compound. Methoxylated aromatics are compounds with electron rich molecular π -orbitals. The greater the degree of methoxylation, the easier it should be for LiP to extract an electron from the π -orbital. This is probably the main reason why methoxy deficient substrates such as benzyl alcohol and the mono-methoxylated benzyl alcohols were poorly oxidised, if at all.

Methoxy group positioning may have a smaller but still significant effect on ionisation potential. This was shown by the difference in LiP oxidation of different dimethoxylated compounds. For example, 3,4-dimethoxybenzyl alcohol (a) was oxidised at 6 times the

rate of 2,5-dimethoxybenzyl alcohol (d), whereas 3,5-dimethoxybenzyl alcohol (e) was not oxidised at all.

Enzyme/substrate affinity should not be a confusing factor in this work, since the concentrations of those substrates that could be oxidised by LiP were well above their respective K_m values (see 4.12.). Compounds such as 3,5-dimethoxybenzyl alcohol which could not be oxidised by LiP may be invalid substrates because of steric factors rather than their high ionisation potentials. Particular aryl methoxy group positioning may prevent the formation of the enzyme/substrate complex in these compounds.

Kersten et.al. (1985), found some selectivity in LiP oxidation of dimethoxylated benzenes. These compounds were oxidised to benzoquinones and methanol by LiP via a radical cation. 1,4- and 1,2-dimethoxybenzene (XI,XII) were oxidised, but not 1,3-dimethoxybenzene (XIII). They also noted that tri, tetra, and pentamethoxy-substituted benzenes were not substrates.

In agreement with this, the highly substituted trimethoxytoluene (r) could not be oxidised. 3,4,5-trimethoxybenzyl alcohol (f) was a substrate of LiP but it was oxidised at only one third the rate of 3,4-dimethoxybenzyl alcohol (a). These highly methoxy-substituted compounds should be easily oxidised due to their electron rich molecular π -orbitals. It would

appear therefore, that substrate ionisation potential was not an important factor in these cases. More importantly, the high level of methoxy-substitution may cause steric hindrance, thus preventing the molecule (which thermodynamically speaking could be oxidised easily by LiP) associating with the active site of the enzyme.

One important point concerning the negative results of LiP oxidation of highly substituted methoxybenzenes by Kersten et.al. (1985) is that the substrates were introduced to the reaction mixtures at a concentration of 1mM. This could be well below their K_m values for LiP oxidation, since one of the methoxybenzyl alcohols used in this study (2,4-dimethoxybenzyl alcohol) had a K_m for LiP oxidation of 20mM. No oxidation of this alcohol by LiP was observed when it was used at a concentration of 2mM. If LiP has a comparatively low affinity for any of the highly-substituted methoxybenzenes used by Kersten et.al., then the subsequent negative results could be misinterpreted.

5.1.4. The effect of other substituents on LiP oxidation of benzyl alcohols.

The replacement of a 4-methoxy group with a 4-ethoxy group appeared to make little difference to rate of reaction. Oxidation of 3-methoxy,4-ethoxy benzyl alcohol (l) was only slightly slower than the oxidation of 3,4-dimethoxybenzyl alcohol (a). 3-methoxy,4-ethoxy derivatives of styrene (VII, Gold et.al.,1984b; Renganathan et.al.,1985) and phenylpropane (VIII, Renganathan et.al., 1985; 1986) have also been shown to be oxidised by LiP, but no comparisons were demonstrated with their 3,4-dimethoxy derivatives.

The replacement of a 4-methoxy group with a 4-hydroxy group prevented oxidation by LiP. 3-methoxy,4-hydroxy(guaiacyl)benzyl alcohol (m) was not oxidised. By analogy, 3,4-dimethoxy phenethyl alcohol was found to be a good substrate (j), but Paszczynski et.al. (1986) found that 3-methoxy,4-hydroxy phenethyl alcohol (IX) could not be oxidised. The inability of LiP to oxidise 3-methoxy,4-hydroxybenzyl alcohol may partly be due to its poor solubility. Nevertheless, this inability of LiP to oxidise guaiacyl-type compounds is surprising since this type of unit is very common in lignin. However, the presence of other constituent groups on the 3-methoxy,4-hydroxy ring may be significant. For instance, 3-methoxy,4-hydroxy cinnamic alcohol (X; Paszczynski et.al., 1986) was

oxidised (perhaps hydroxylated and cleaved like styryl structures?). Therefore one cannot necessarily extrapolate these results to include lignin

The replacement of a 4-methoxy group with a 4-nitro group also prevented oxidation by LiP. This, like 3-methoxy,4-hydroxybenzyl alcohol may also be due to its poor solubility.

Styryl structures are also substrates for LiP. 3-methoxy,4-ethoxy styrene (VII) was hydrated to the glycol, which was itself oxidised (probably via a radical cation) resulting in intradiol cleavage, giving the alkoxyated aldehyde (Glenn et.al., 1983; Gold et.al., 1984b; Renganathan et.al., 1985).

3,4-dimethoxystyrene (VI) was also hydroxylated (Tien & Kirk, 1984), but styrene (s), could not be oxidised.

Like the benzylic alcohols, styryl compounds appear to be oxidised by LiP only if they are sufficiently methoxy-substituted. Therefore, given a suitable range of compounds, it should be possible to predict which ones will be oxidised by LiP.

The ability of LiP to oxidise benzyl alcohols was restricted by the length of the alcohol side chain. 3,4-dimethoxybenzyl alcohol was oxidised the fastest (a) followed by 3,4-dimethoxyphenethyl alcohol (j). The slowest to be oxidised being 3,4-dimethoxypropanol (k). Lengthening the alcohol chain should impart very little effect on the π -electron cloud, so the

differences in oxidation potential between the three substrates is probably very small. However, it must be significant enough to affect the rate of oxidation since it is unlikely that longer chains would cause a reduction in this rate simply through steric effects, since many of the dimeric model compounds that have been used (see 1.8.1.) have the much larger, aryl propane-type group attached to ring position 1.

None of the halogenated benzyl alcohols were oxidised by LiP (o,p,q). Chloro and fluoro groups are electron withdrawing, leaving the aromatic ring electron deficient and therefore probably unavailable for oxidation by LiP.

5.1.5. Conclusion.

LiP oxidised some, but not all of the methoxybenzyl alcohols that were tested. In particular, it would not oxidise those substrates that possessed low methoxy-substitution. Since these substrates are relatively electron deficient in their π -orbitals, their ionisation potentials are probably too high for LiP oxidation.

Dimethoxylated benzyl alcohols were good substrates for LiP, but more highly methoxy-substituted alcohols were not. In this latter case, steric hindrance due to high methoxy-substitution was

probably the major factor preventing oxidation.

The oxidation of 3,4-X-substituted benzyl alcohols was primarily dependent on the solubility of the particular compound. For instance, the poorly soluble 3-methoxy,4-nitrosobenzyl alcohol and 3-methoxy,4-hydroxybenzyl alcohol were not oxidised by LiP, but the readily soluble 3,4-dimethoxybenzyl alcohol and 3-methoxy,4-ethoxybenzyl alcohol were oxidised.

Substitution of the alcohol chain with longer alcohol chains caused a reduction in the rate of LiP oxidation. Here, neither compound solubility, steric hindrance, nor ionisation potential would appear to be individually involved to any great extent.

5.2. Aerobic oxidation of benzylic alcohols.

Tien & Kirk (1984) showed that the oxidation of veratryl alcohol by LiP followed a simple stoichiometry: 1 mol alcohol + 1 mol H₂O₂ gave 1 mol aldehyde + 1 mol water. However, in 1987, Haemmerli et.al. found that under aerobic conditions the stoichiometry was much more complex. Four products in addition to veratrylaldehyde were found, namely two quinones, and two ring-cleavage lactones (see fig 1.13.). This was substantiated and added to by Shimada et.al. (1987) who in addition, found other lactone products.

This section describes the LiP oxidation of a range of benzyl alcohols under aerobic conditions in an attempt to determine, by the use of G.C./ mass spectroscopy, whether benzaldehydes are the major products and also what other products, if any, are present.

i) 3,4-dimethoxyphenethyl alcohol(fig 5.1a): Aerobic oxidation by LiP of this compound produced a yellow coloured solution. Separation by G.C. resolved this solution into three product peaks. Although no aldehyde standard was available, peak I was almost certainly the aldehyde peak since it was the major product (Haemmerli et.al.,1987 found that

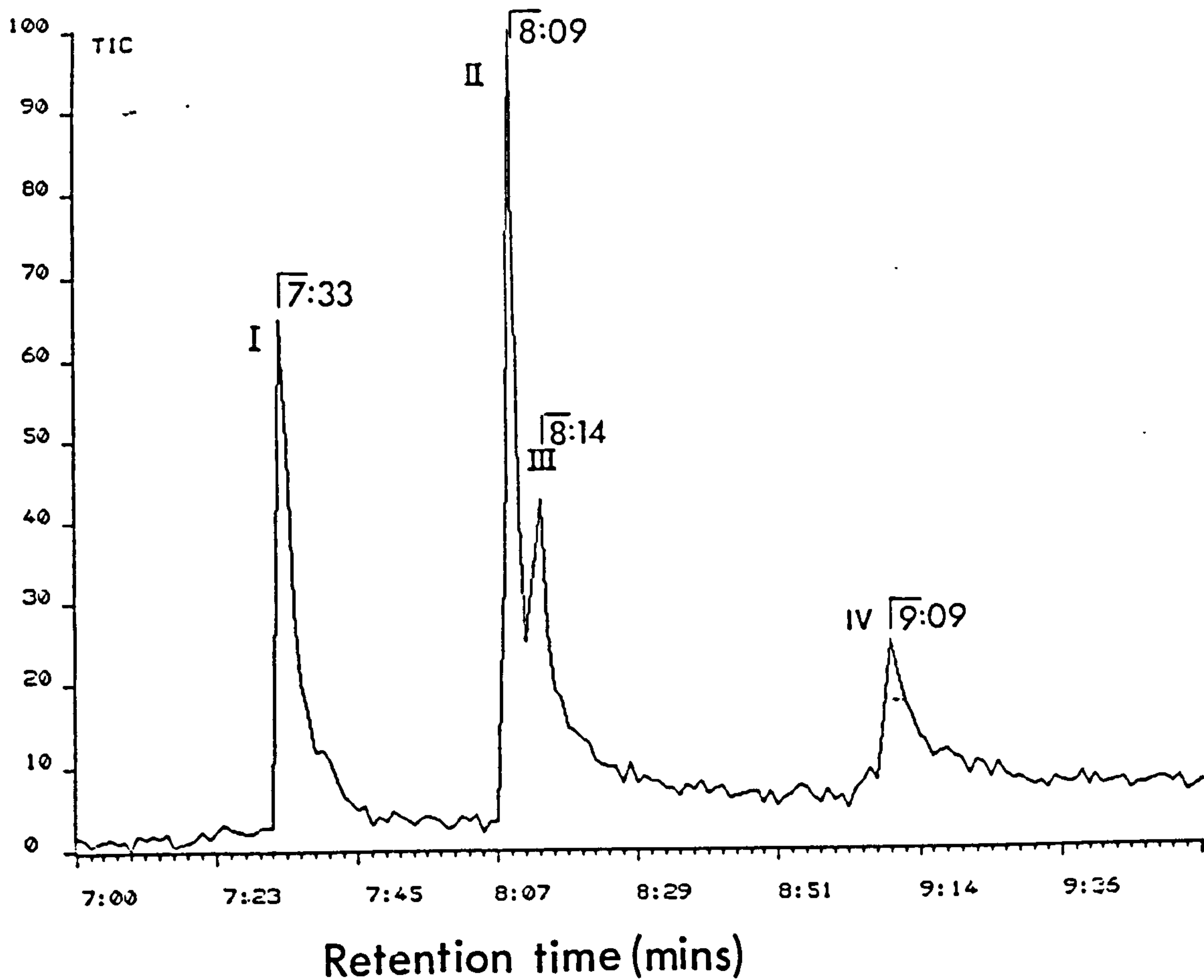


Figure 5.1.a. GC resolution of the products (I, III, IV) of LiP-oxidised 3,4-dimethoxyphenethyl alcohol (II) using a non-polar column (see 2.8).
 Overleaf: Mass fragmentogram of peaks I, II and IV, the products of LiP oxidation of 3,4-dimethoxyphenethyl alcohol.

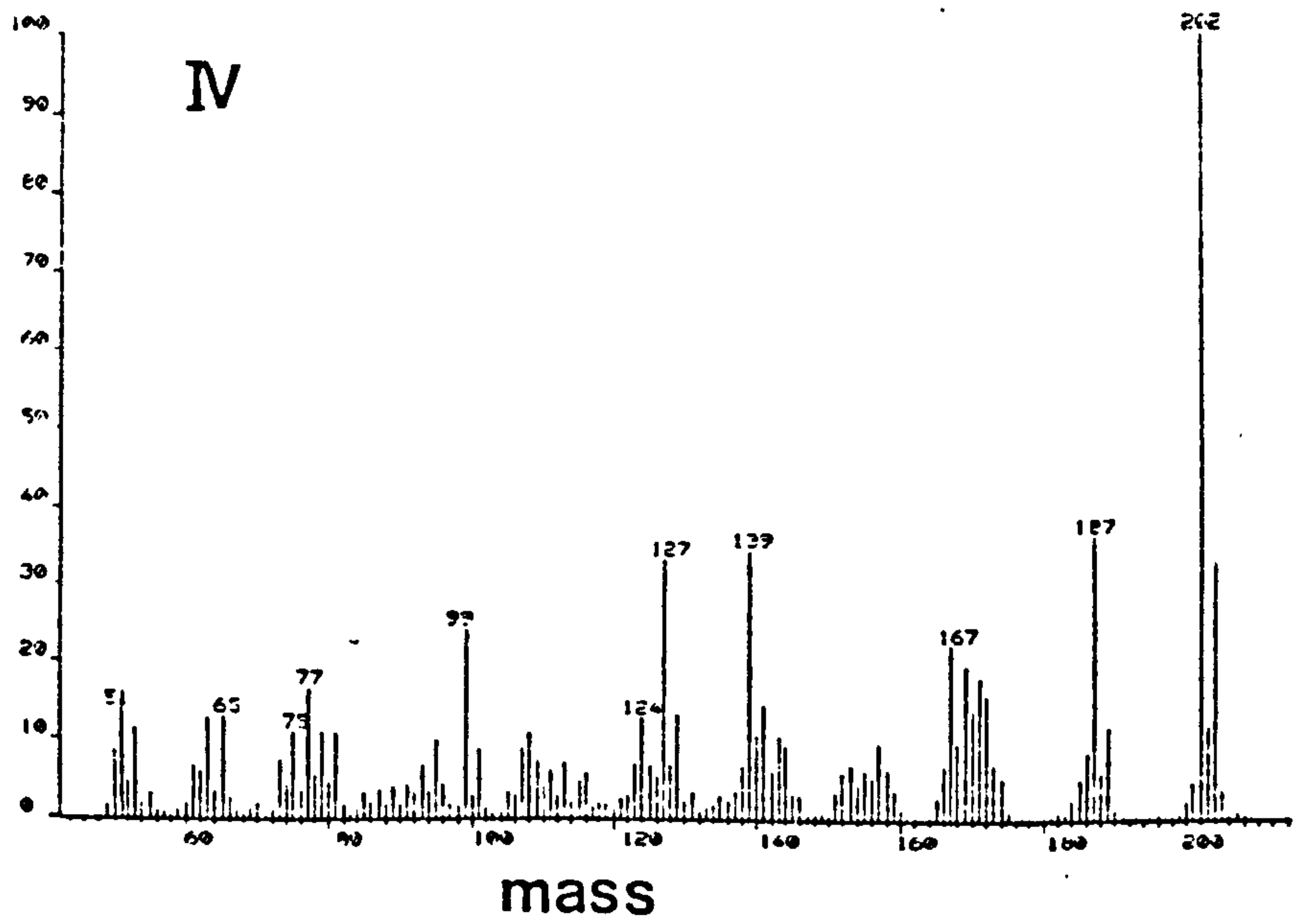
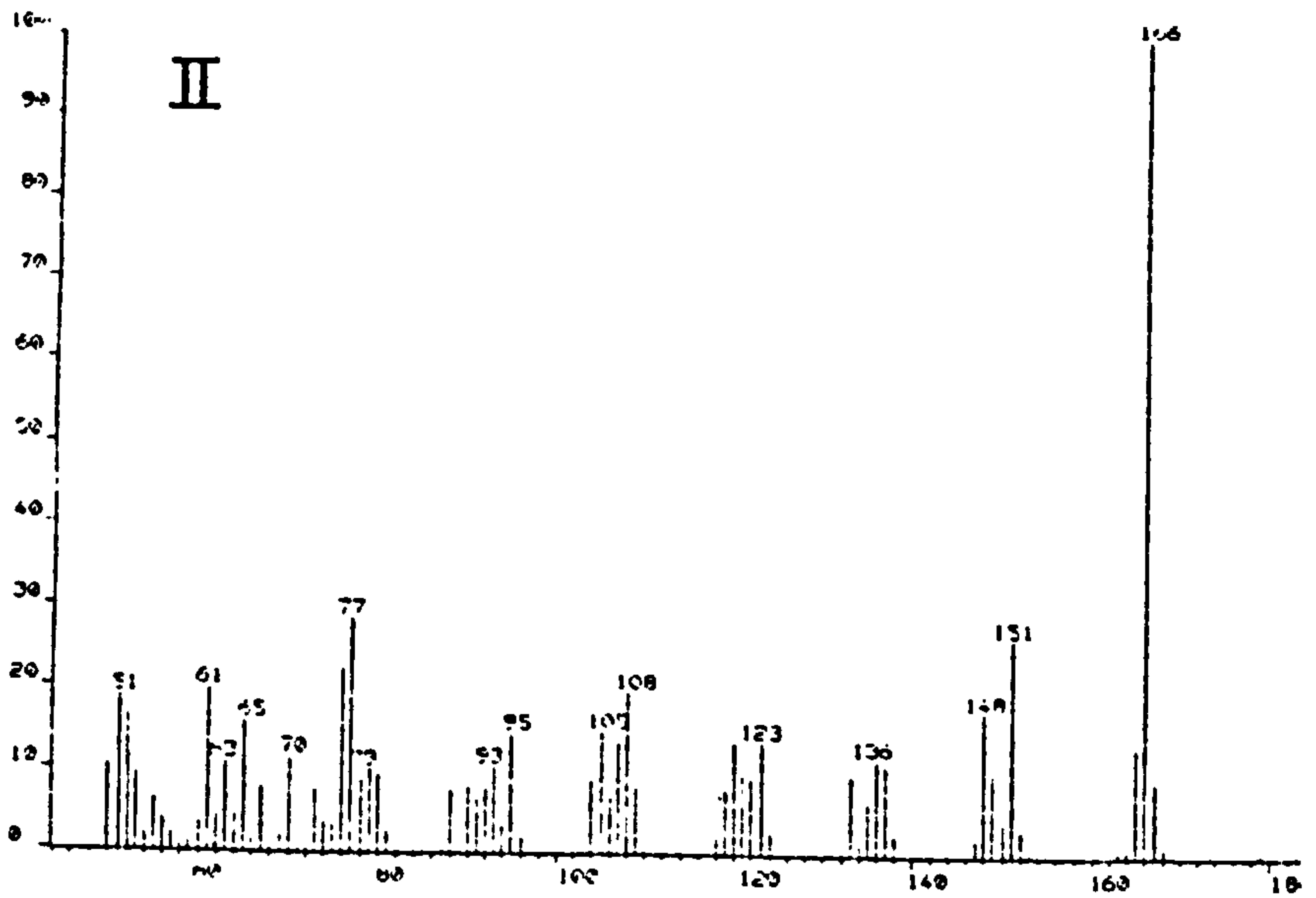
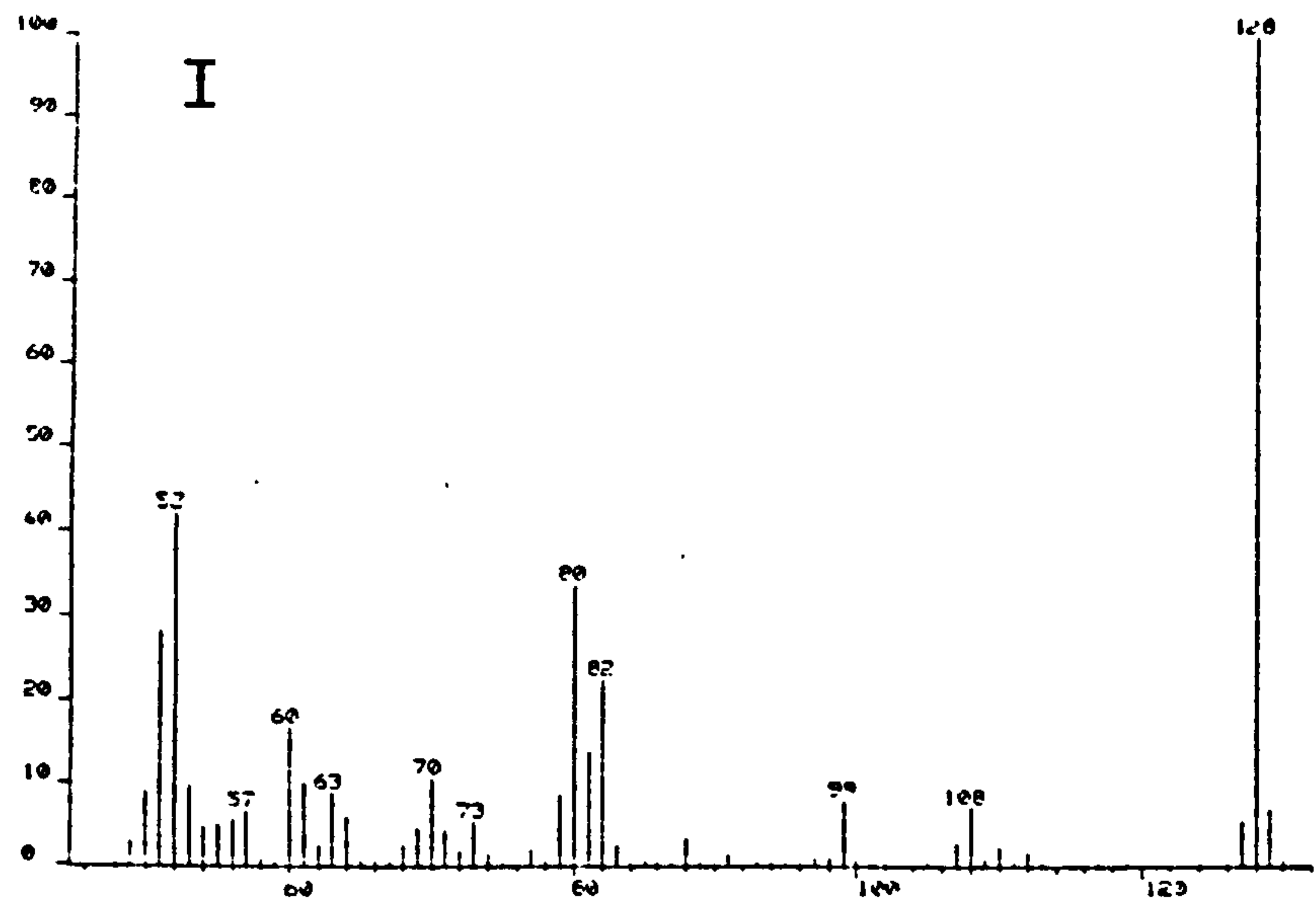


Figure 5.1.a. (Continued).

veratraldehyde was the major product of the aerobic oxidation of veratryl alcohol by LiP). It had a molecular ion peak of 181 and, in accordance with the general observation for methoxybenzyl alcohol/aldehyde pairs, the G.C. retention time was less than that of the alcohol.

Peak III had a very similar mass fragmentogram to that of the alcohol, but was not present in the starting solution. Both peak III and IV, with molecular ion peaks of 182 and 180 respectively, could have been quinone products. The yellow coloration was suggestive that at least one quinone had formed, (probably via demethoxylation followed by addition of molecular oxygen and water loss, Kersten et.al., 1985). This colouration was much less pronounced under anaerobic conditions suggesting that molecular oxygen was necessary for quinone formation. Haemmerli et.al. (1987) found that less quinone was produced upon the aerobic oxidation of veratryl alcohol when the pH value was increased from 3.5 to 4.5, or when Mn(II) was added to the reaction. They suggested from this that quinone/lactone formation required the perhydroxyl radical. This radical was thought to be formed via the one-electron oxidised veratryl alcohol radical cation, which subsequently deprotonates. Under anaerobic conditions, the hydroxy-substituted veratryl alcohol radical would undergo further one-electron oxidation to veratryl aldehyde but under aerobic conditions dioxygen

may attack the radical, forming a superoxide anion (see fig 1.13). This activated oxygen species would then protonate at low pH values to a perhydroxy radical. Hence, the reduction of quinone formation at higher pH values. Mn(II) chelates superoxide anions (and thus perhydroxy radicals) and reduces them to H₂O₂. Although no absolute proof is evident concerning the actual activated oxygen species involved in quinone formation, molecular oxygen itself is an obvious requisite.

ii) 3-methoxy,4-ethoxybenzyl alcohol (fig 5.1b): Aerobic oxidation of this compound by LiP gave rise to only one product. Judging by its relative retention time when compared to the alcohol and its molecular ion peak(180), it was very likely to be the corresponding aldehyde.

iii) 2,3-dimethoxybenzylalcohol(fig 5.1c): Here, three oxidation product peaks were found. Peak I had a molecular ion at 128 and may indicate a ring cleavage product. Peak II was 2,3-dimethoxybenzaldehyde as determined by comparing the mass fragmentogram with the library standard.

Peak IV possessed a molecular ion peak of 202, which was a molecular weight of 34 higher than that of the substrate alcohol. This ion peak showed a distinctive 3:1 ratio, which was strongly indicative of the presence of chlorine. Chlorine consists of two

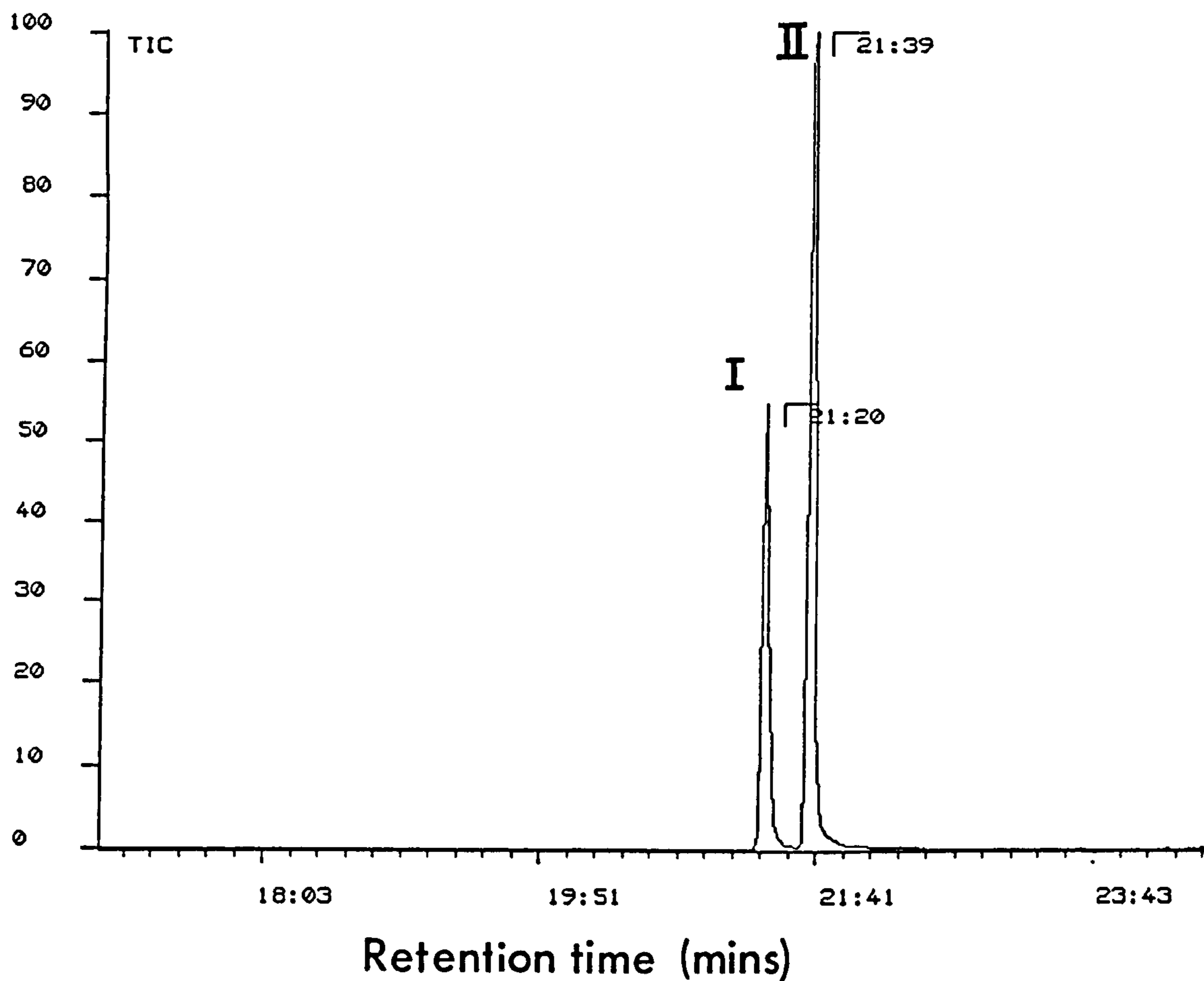


Figure 5.1.b. GC resolution of the product (I) of LiP-oxidised 3-methoxy,4-ethoxyphenethyl alcohol (II) using a non-polar column (see 2.8).

Overleaf: Mass fragmentogram of (I), the LiP oxidation product of 3-methoxy, 4-ethoxybenzyl alcohol.

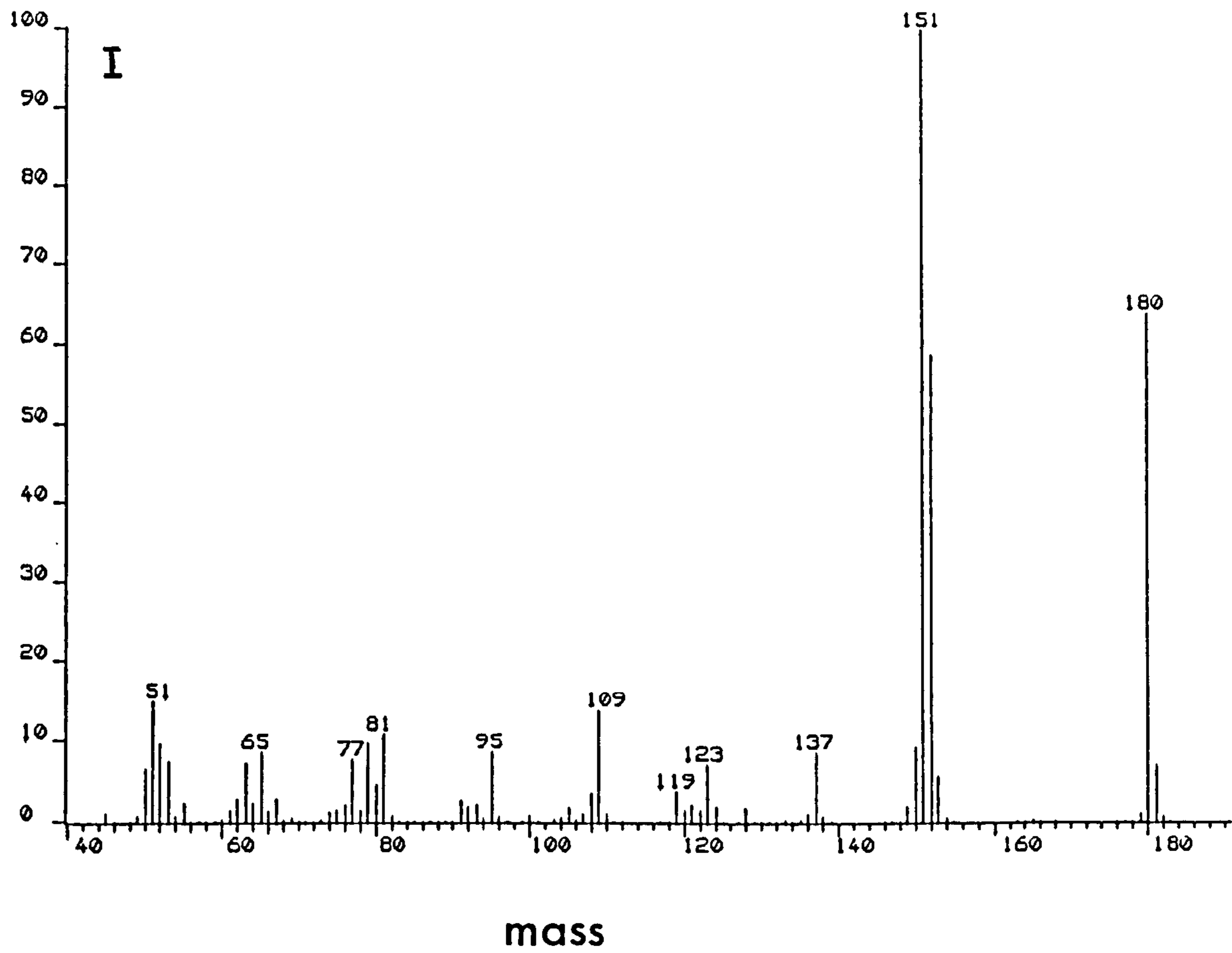


Figure 5.1.b. (Continued).

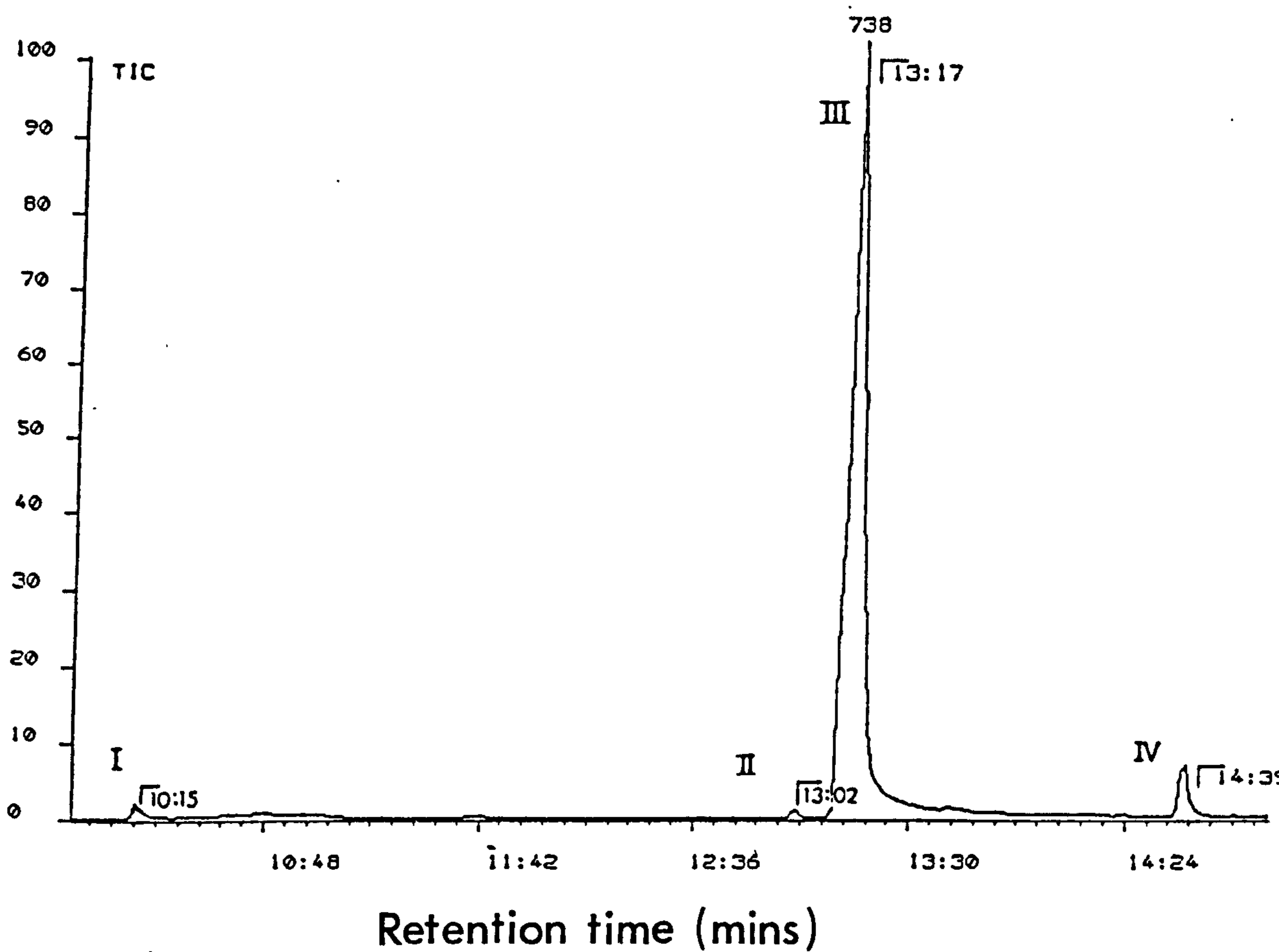
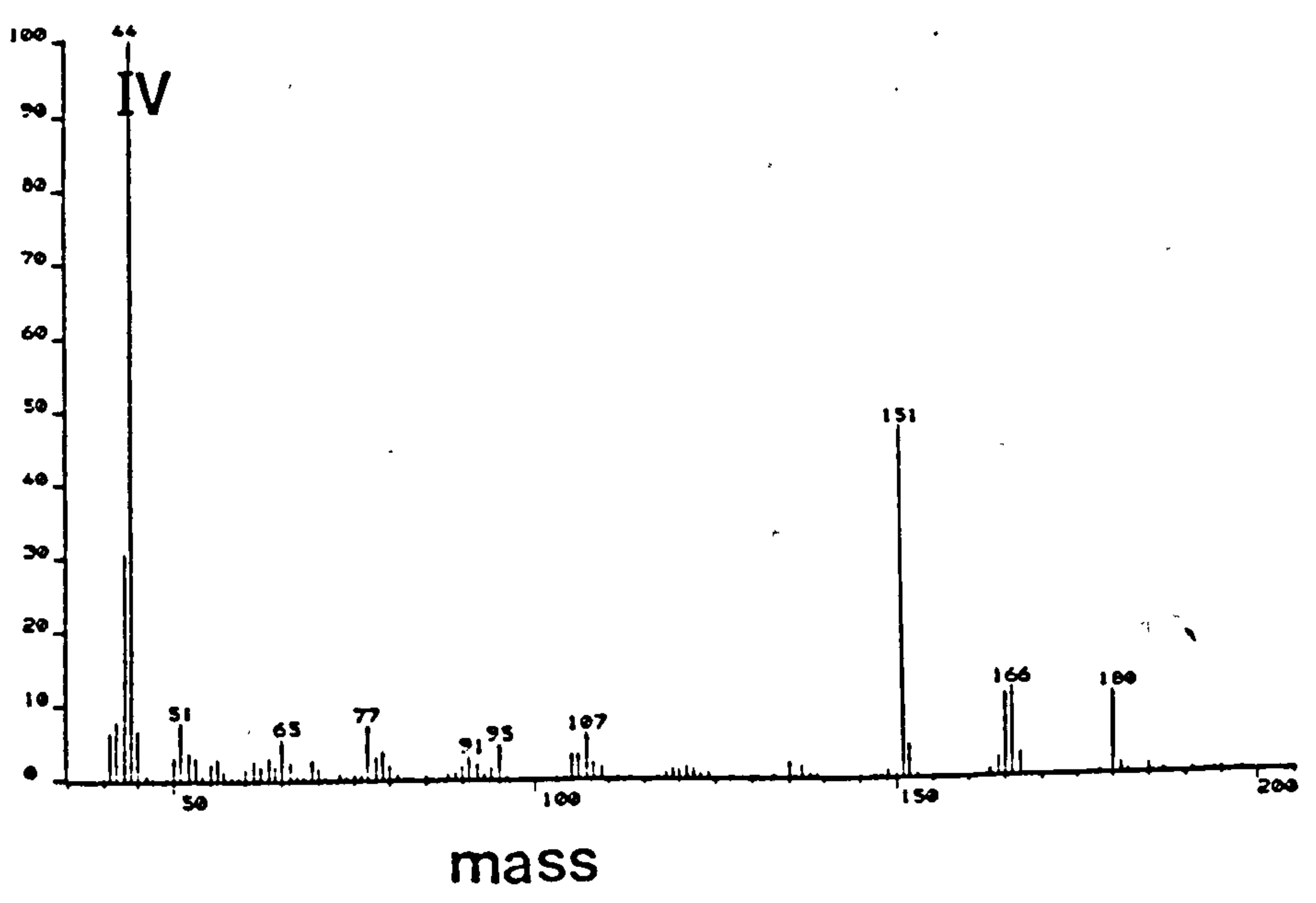
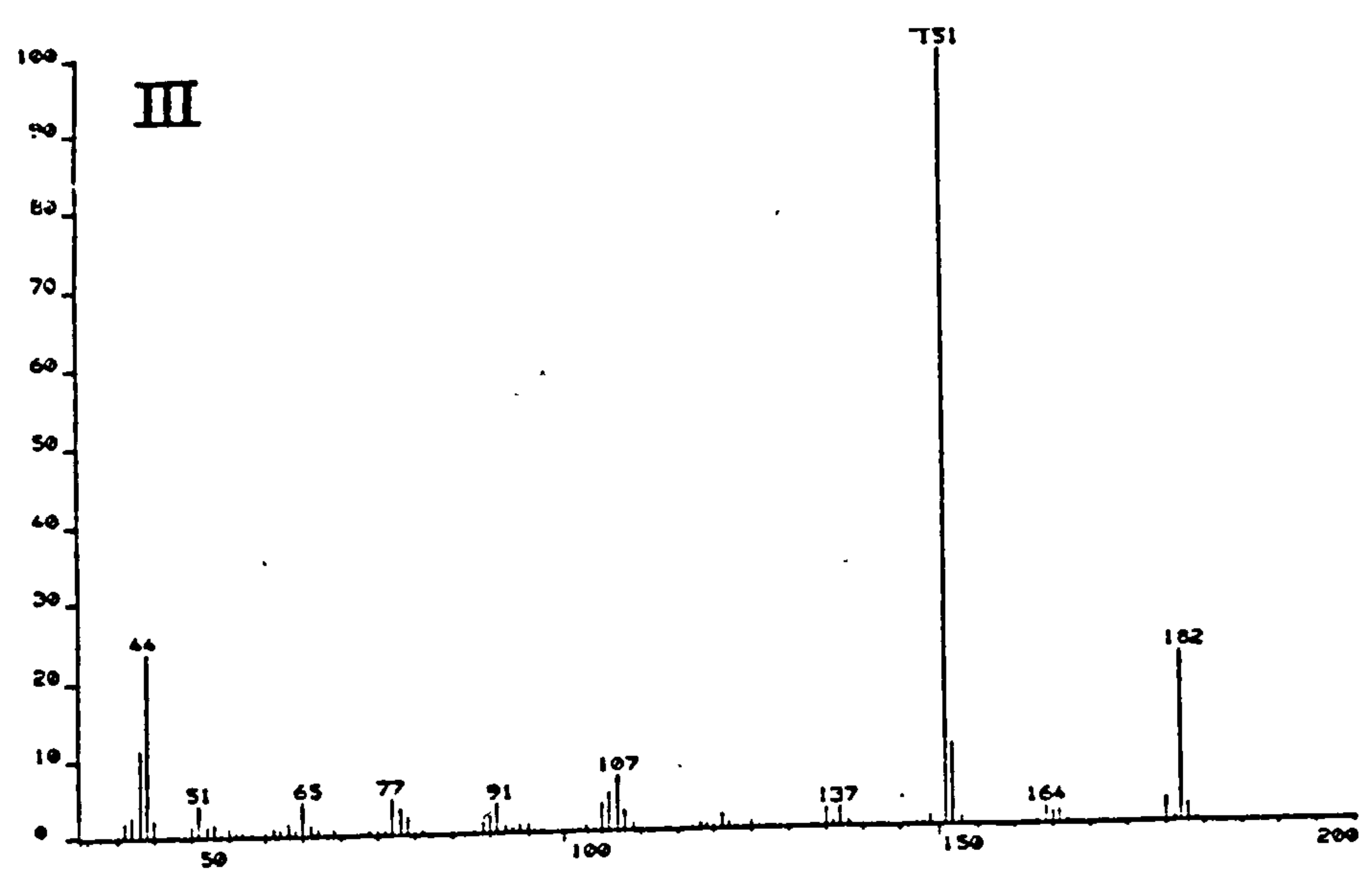
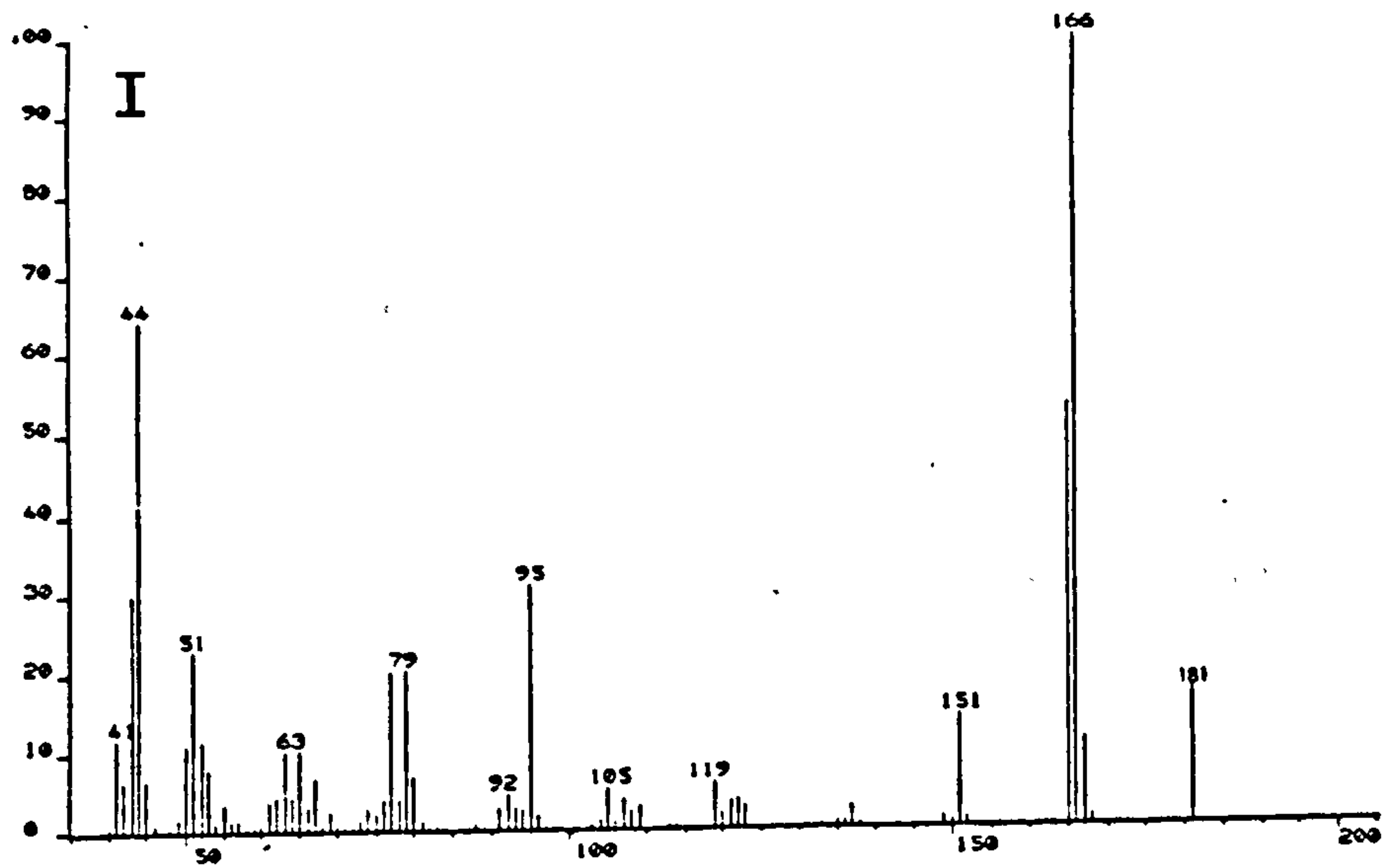


Figure 5.1.c. GC resolution of the products (I, II and IV) of LiP-oxidised 2,3-dimethoxybenzyl alcohol (II) using a non-polar column (see 2.8).

Overleaf: Mass fragmentogram of Peaks I, II and IV, the liP oxidation products of 2,3-dimethoxybenzyl alcohol.



mass

Figure 5.1.c. (Continued).

naturally occurring isotopes, ^{35}Cl and ^{37}Cl whose relative abundances are 75.4% and 24.6%, hence the 3:1 ratio in the ion peak. Chloride was a major impurity in reagent grade sodium tartrate (0.01% w/w) and it was also used in the form of HCl to pH the sodium tartrate buffer. The presence of chloride, in connection with the evidence from the mass fragmentogram suggested that the radical cation of 2,3-dimethoxybenzyl alcohol may be susceptible to attack by a chlorine species (unknown) to form a chlorinated benzyl compound. For instance, HCl may attack the deprotonated alcohol radical and upon subsequent homolytic fission, a free chlorine radical would be produced which may then undergo addition to another alcohol radical. Direct addition of chlorine is more likely than substitution with a methoxyl group since the 'chlorinated' product had a molecular weight of 34 higher than the original substrate suggesting that no methoxyl group loss occurred.

iv) 3,4,5-trimethoxybenzyl alcohol (fig 5.1d): Oxidation of this compound caused the formation of a deep red colouration. Eleven product peaks were formed. 3,4,5-trimethoxybenzaldehyde (Peak V) was determined by cross-reference to a standard. Peak XI possessed a molecular ion peak at 232, which showed an isotope ratio of 3:1. This compound was probably analagous to the chloro-compound found upon the oxidation of 2,3-dimethoxybenzyl alcohol.

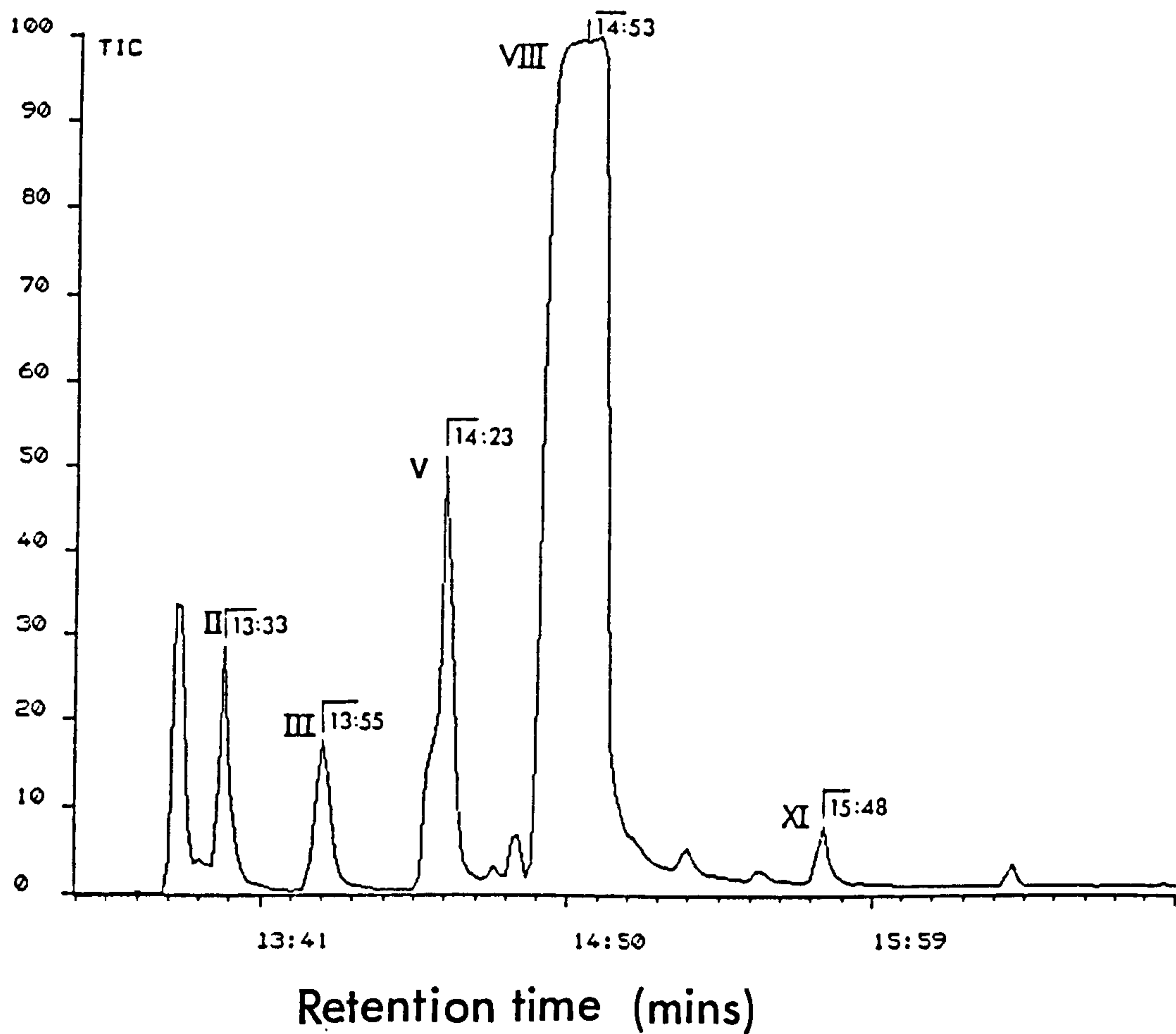


Figure 5.1.d. GC resolution of the products (I - VII and IX - XII) of LiP-oxidised 3,4,5-trimethoxybenzyl alcohol (VII) using a non-polar column (see 2.8).
 Overleaf: Mass fragmentograms of peaks II, III, V and XI, selected LiP oxidation products of 3,4,5-trimethoxybenzyl alcohol.

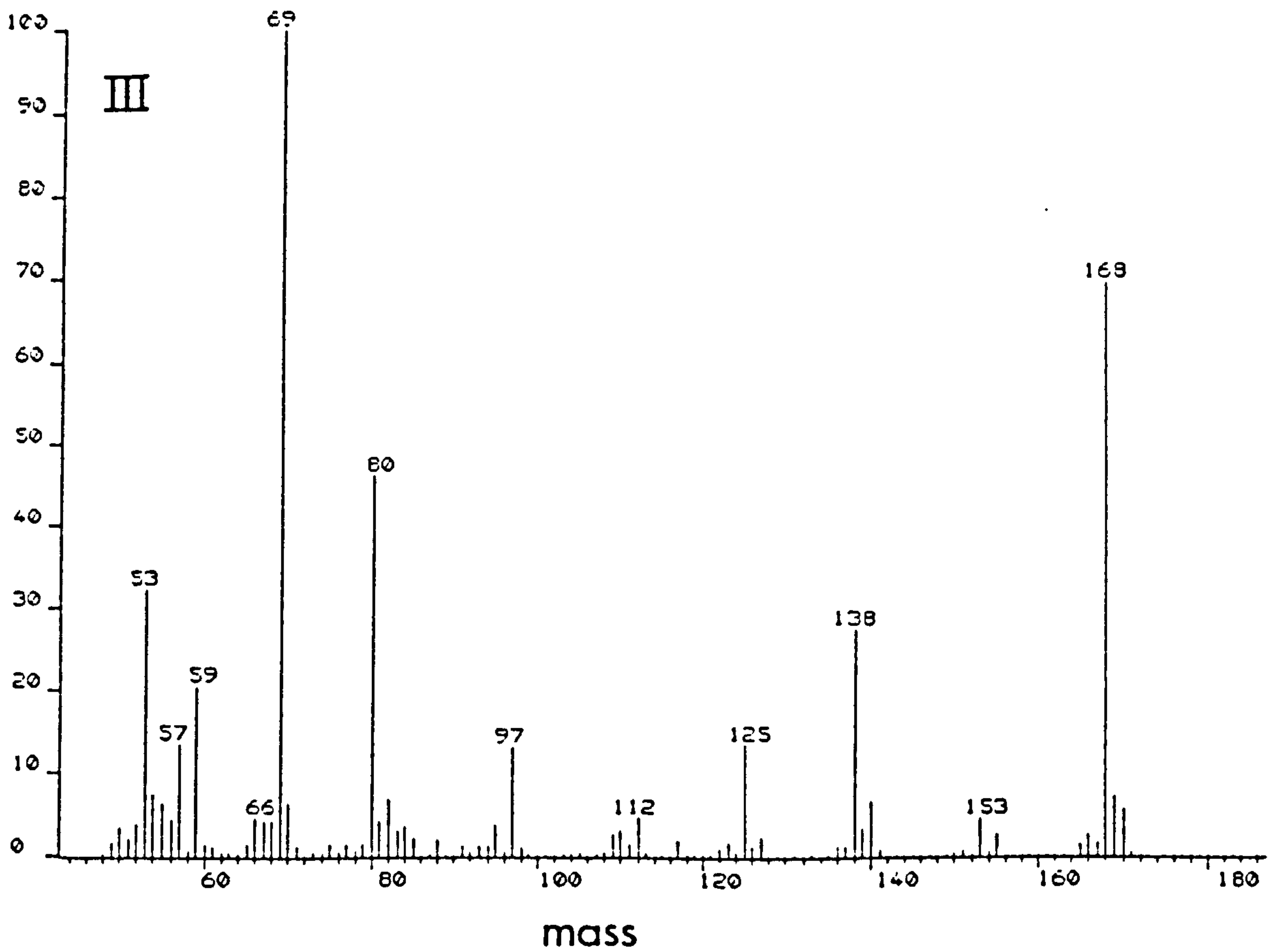
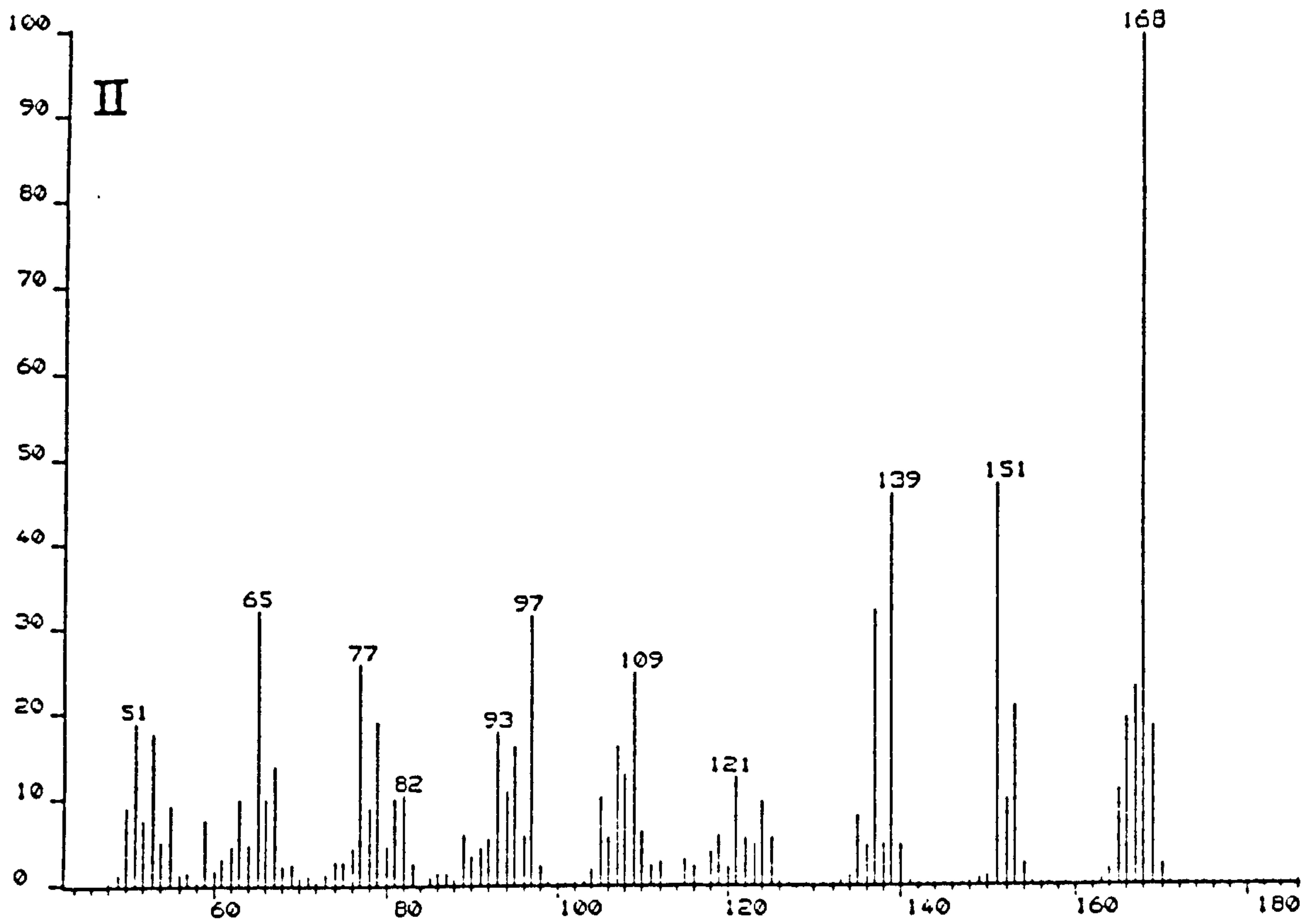


Figure 5.1.d. (Continued).

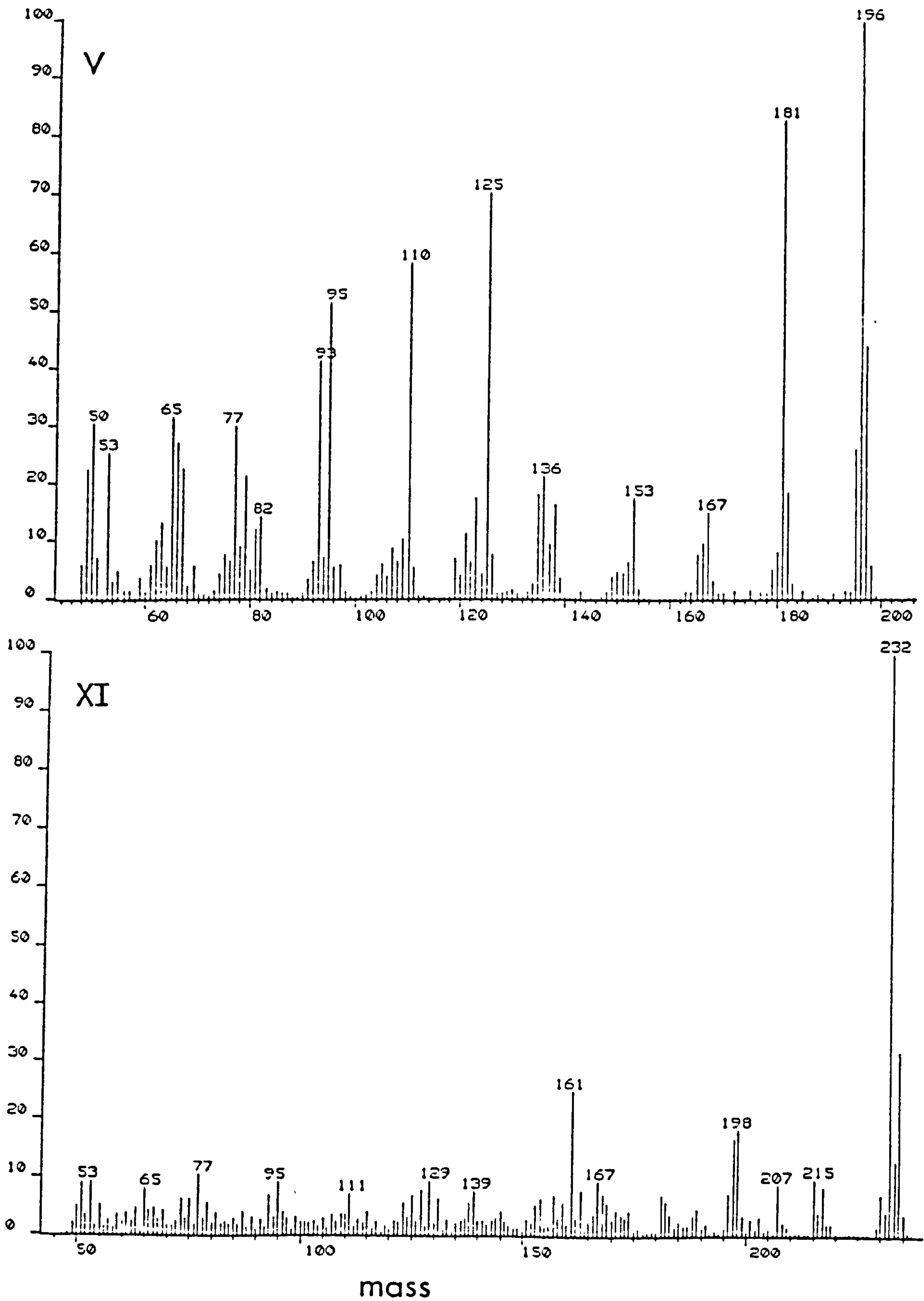


Figure 5.1.d. (Continued).

The deep red colour of the final solution suggested that quinones may have been produced. Peaks II and III have molecular ions at 168, which could have arisen by di-demethoxylation followed by quinone formation.

From these results, it is clear that benzaldehyde formation is the major product of LiP oxidation of benzyl alcohols under aerobic conditions as well as anaerobic conditions. The difference is that in the former, the aldehyde is the sole product. Under aerobic conditions a number of other products (probably quinones and ring cleavage lactones) were found, the number of which appeared to depend upon the initial alcohol substrate.

3-methoxy,4-ethoxybenzyl alcohol was oxidised to the aldehyde only. If it was oxidised by two rapid one electron steps (a mechanism put forward for veratryl alcohol oxidation, see 1.8.8.) then no side reactions due to intervening attacking species would have had time to take place. In sharp contrast to this substrate is the oxidation of 3,4,5-trimethoxybenzyl alcohol, which was oxidised to no less than eleven different compounds. This could be explained if the oxidation proceeded via two single electron-withdrawing steps, with a distinct intermediacy between them. If the subsequent one-electron oxidised alcohol radical cation, or after deprotonation the alcohol radical, had

a sufficiently long lifetime (whether through inherent stability or recalcitrance to further LiP oxidation) then it may be subjected to further attack by agents such as water, dioxygen, or even a chlorine species.

The other two alcohols tested here each formed three different oxidation products. The number and variation of reaction products under aerobic conditions therefore appears to be a function of benzylic alcohol substitution, which may be directly connected to stability and/or ease of LiP oxidation of the one electron oxidised radical cation species.

5.3. The phenol oxidising activity of LiP.

5.3.1. Introduction.

H_2O_2 -dependent phenol oxidising activity has been associated with lignin degradation and has been found in several lignin-degrading fungi (Lyr, 1956). Tien & Kirk (1984) first discovered that LiP possessed phenol-oxidising activity. They found that LiP oxidised 4-tert-butyl guaiacol to the 6-6'-dihydrodimer. Hammel et.al. (1986a) found the radical coupling dimer 2,3 diphenylbutane in the LiP catalysed degradation products of 1,(3,4-dimethoxyphenyl)-2-phenylpropanol. It consisted of 23% of the end products under anaerobic conditions. Also Haemmerli et.al. (1986a) found that LiP could polymerise lignin and suggested this was due to phenol oxidase activity.

Research reported in this section considers the LiP oxidation of simple aromatic compounds in an attempt to substantiate the claims that the enzyme possesses phenol oxidase activity.

5.3.2. LiP oxidation of p-cresol.

LiP oxidation of p-cresol led to the formation of the dimer 4a,9b dihydro-8,9b-dimethyl-3(4H)-dibenzofuranone (Pummerers' ketone fig 5.2), as determined by the correlation of mass spectroscopy data of the products with that of the chemically synthesised ketone (Chen et.al., 1969).

This ketone has also been synthesised by HRP oxidation of p-cresol (Hewson & Dunford, 1976). One-electron oxidation of the p-cresol molecule by HRP causes the formation of a p-methylphenol radical which, subsequently, electrophilically attacks another p-cresol molecule. Further one electron oxidation results in the ketone dimer (fig 5.3).

A second LiP oxidation product of p-cresol with a molecular ion peak at 214 was detected on the G.C. about thirty seconds after the ketone. This may be the ortho-linked dimer (fig 5.2) which can be formed by the coupling of two p-cresol radicals (Hewson & Dunford, 1976). A much smaller amount of this product relative to the ketone is expected since p-cresol serves as a radical scavenger.

4-ethylphenol was also found to be a substrate for LiP. Like the 4-methyl derivative, dimers were formed as determined by the detection of a molecular ion peak at 242 in the mass spectrum of the LiP oxidised

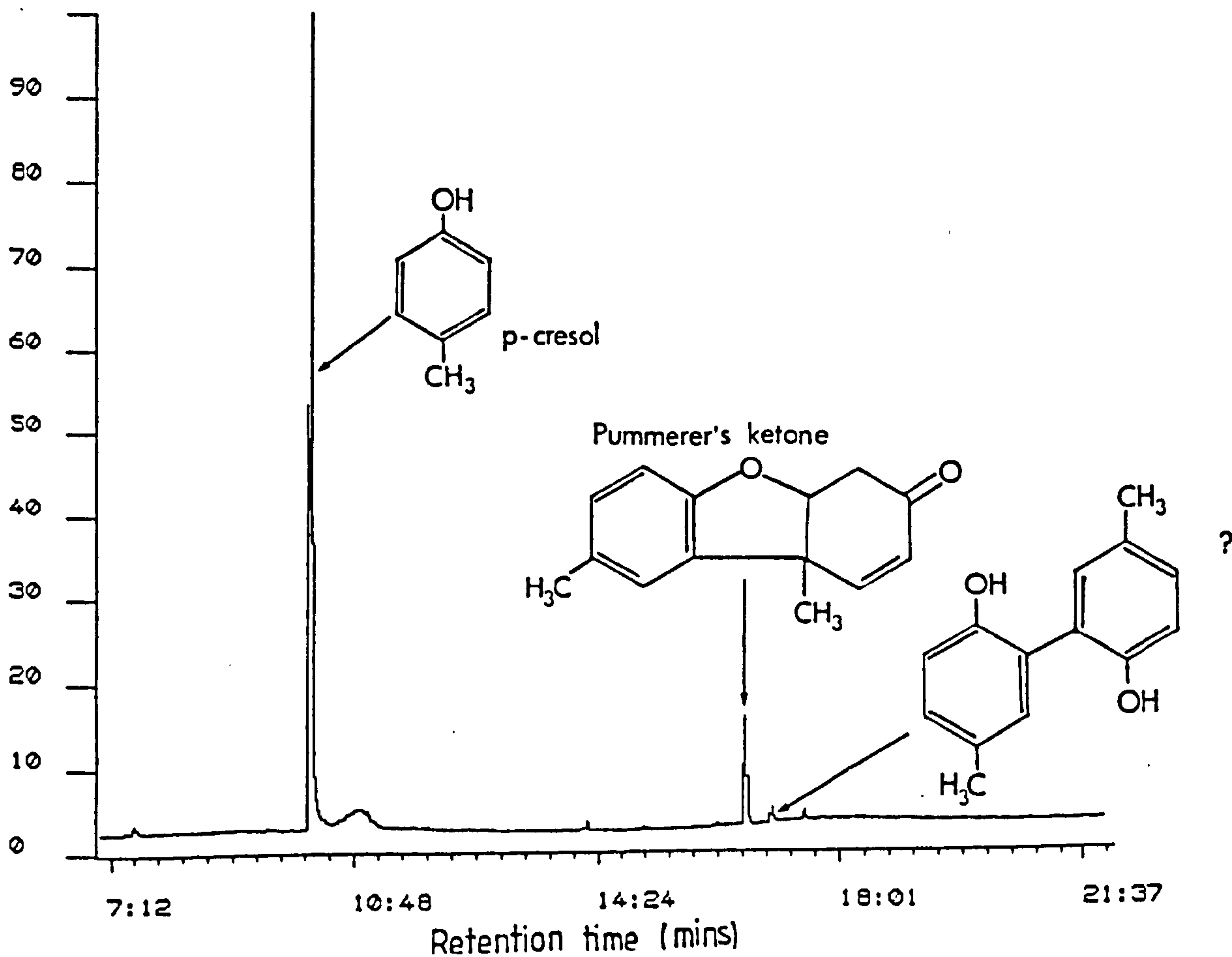


Figure 5.2. GC resolution of the products of LiP-oxidised p-cresol, Pummerers' ketone and the putative p-cresol ortho-linked dimer, using a non-polar column (see 2.8).

Overleaf: Mass fragmentograms of Pummerers' ketone (I) and the putative p-cresol ortho-linked dimer (II), the oxidation products of p-cresol.

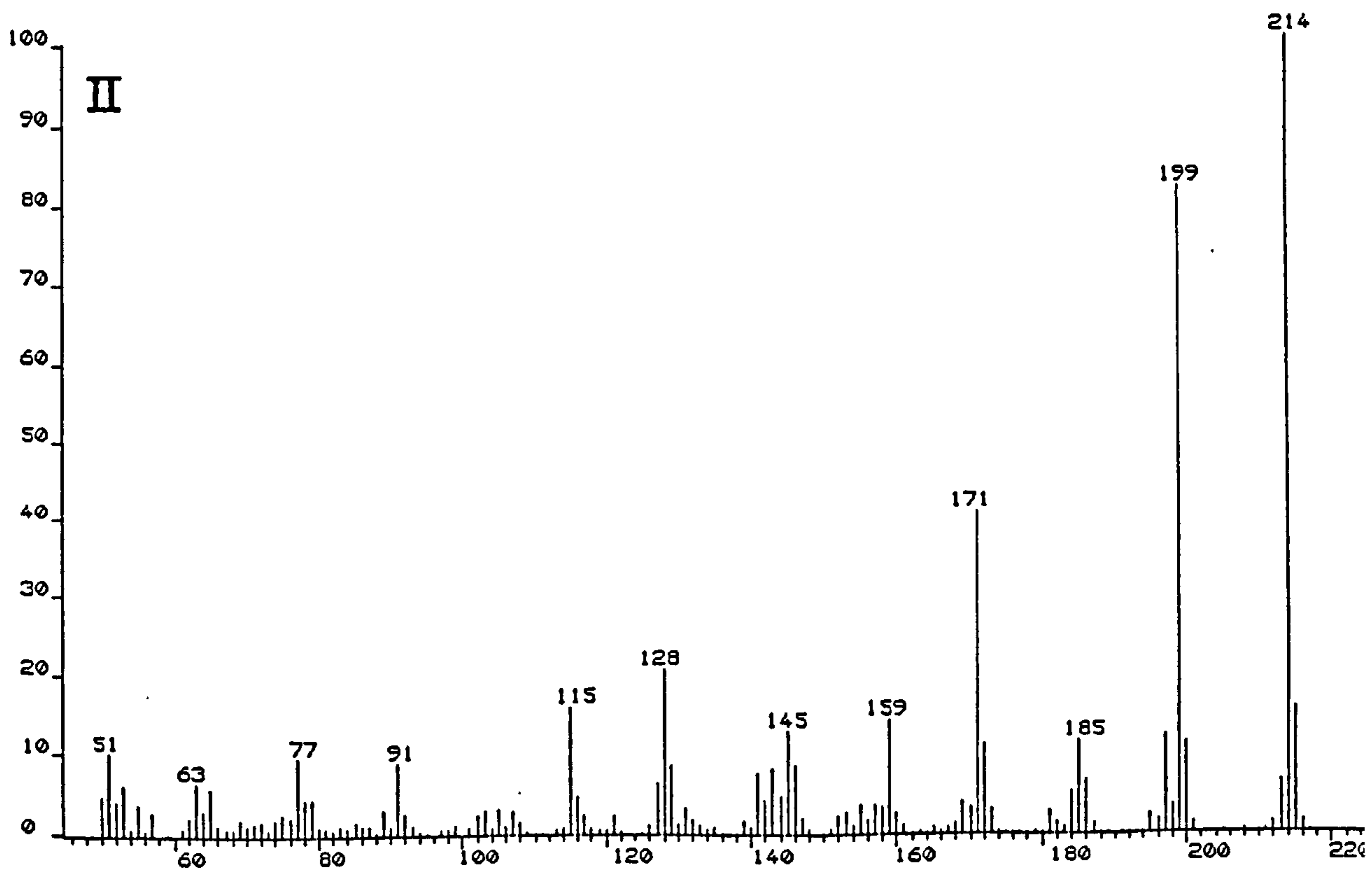
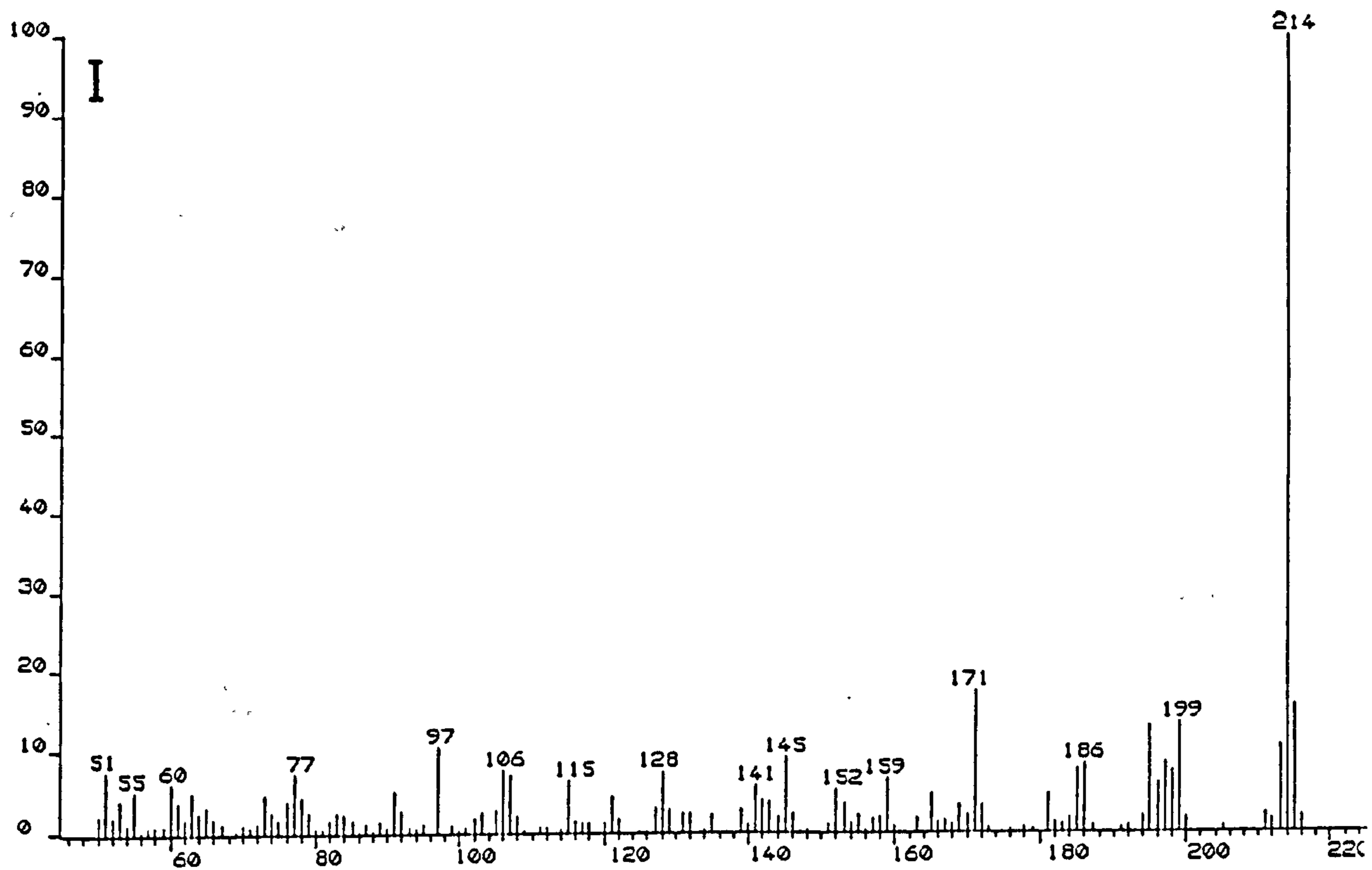


Figure 5.2. (Continued).

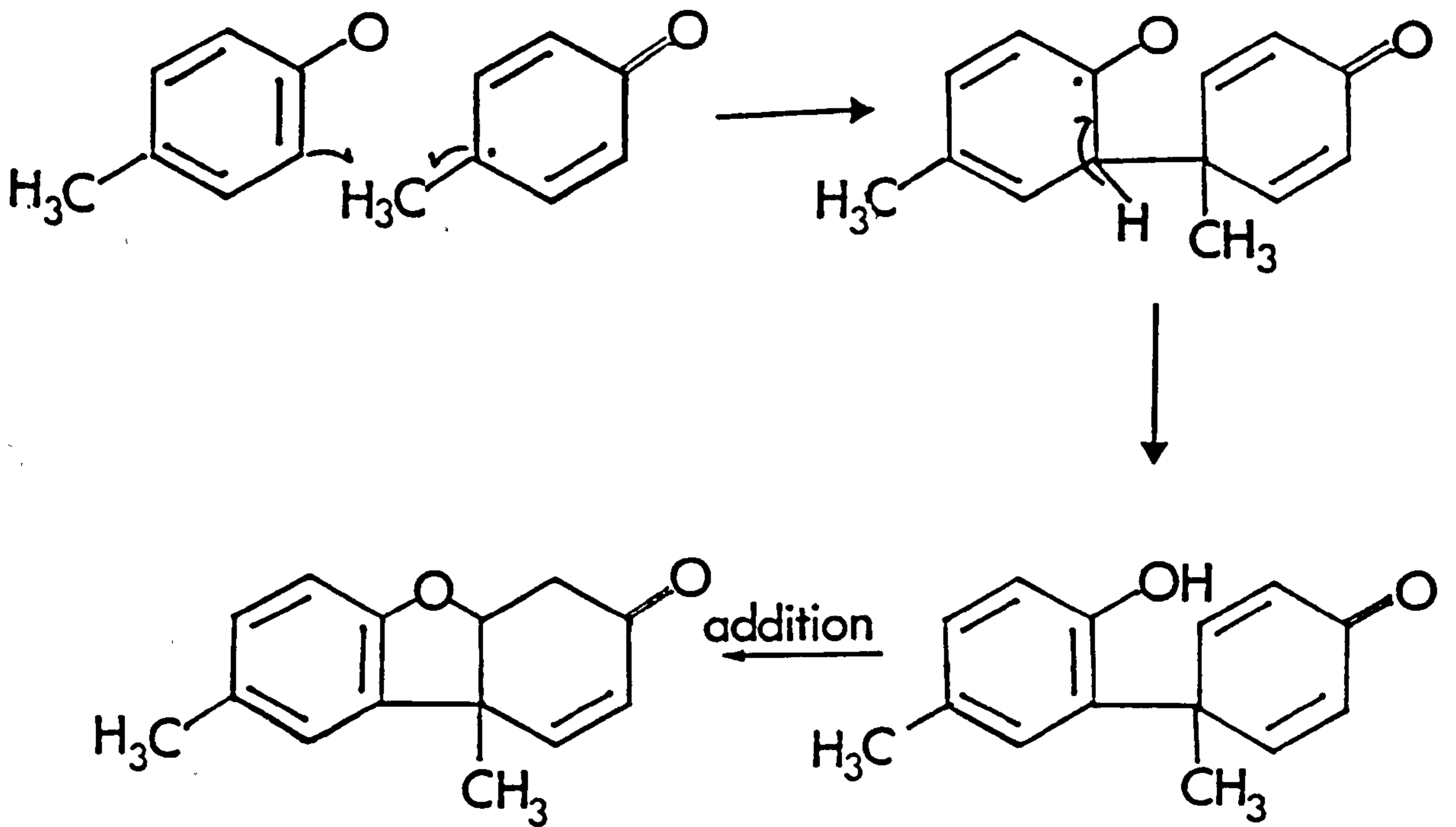


Figure 5.3. Formation of Pummerers' ketone via electrophilic attack of p-cresol by the HRP-produced p-methylphenoxy radical (Hewson & Dunford, 1976).

product.

5.3.3. LiP oxidation of catechol.

LiP oxidation of catechol produced a dark brown suspension. No free catechol was found in the supernatant, indicating that the oxidation was complete. The suspension was dissolved in 10mM borate buffer pH10 and the molecular weight of the oxidised catechol product determined on a LH20 gel filtration column. The brown band eluted off the column almost in the void volume, demonstrating that the molecular weight must be at least 1500d (fig 5.4). A sephadex G-75 column was used to determine more accurately the molecular weight. However, the brown 'polycatechol' band could not be eluted. This indicated that the polymer is either smaller than the recommended low cut off point for G-75 of 3kd, or that aromatic compounds strongly bind to G-75. Nevertheless, the important point is that catechol was polymerised by LiP. The actual molecular weight is more likely to be a function of polymer solubility rather than any inherent property of the enzyme since once a catechol molecule has been oxidised by LiP to the radical, polymerisation will only be terminated when the growing polymer chain falls out of solution.

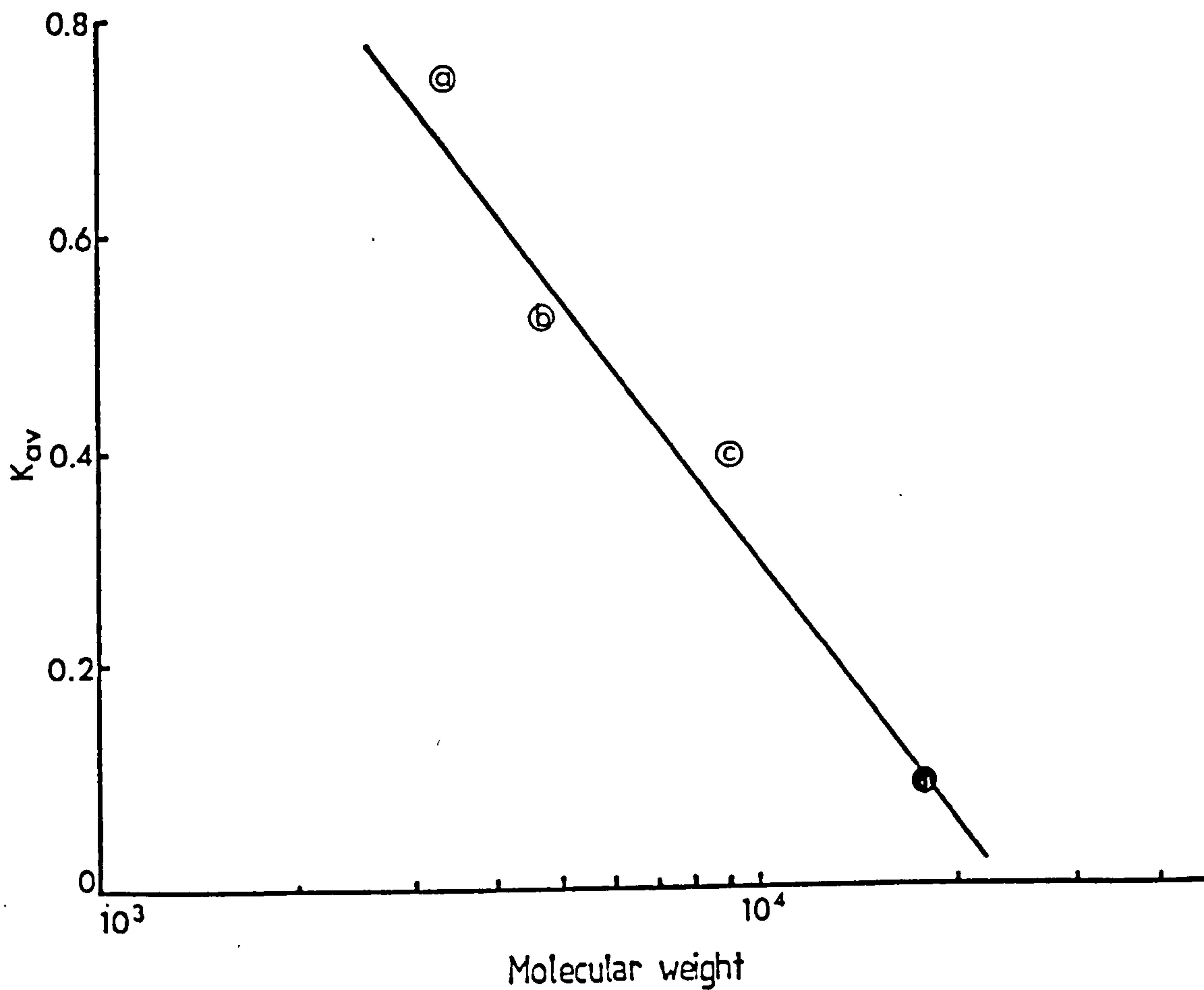


Figure 5.4. Molecular weight determination of polycatechol by Gel filtration, using an LH20 column ($V_t=80\text{mls}$) equilibrated with 10mM borate buffer pH 10 (standards: Methyl orange (a, 327 m.w.), indigo carmine (b, 466 m.w.) and BDH light green (c, 792 m.w.).

5.4. Co-oxidation by LiP.

5.4.1. Introduction

Enzymic cooxidation is a process in which, firstly, an enzyme oxidises a co-substrate. This activated co-substrate in turn oxidises a second substrate, the target substrate. The second substrate will only be oxidised if the co-substrate as well as the enzyme is present.

Harvey et.al.(1986) suggested that LiP degradation of lignin was itself a type of co-oxidation, mediated by veratryl alcohol (see 1.8.8.). The ability of LiP to produce radical species from low molecular weight aromatic compounds, which may then subsequently attack chosen target molecules could open up new areas of catalytic utility. For example, one could use other low molecular weight aromatic compounds as target molecules in order to produce heteropolymers (see 6.1 for the uses of phenolic polymers). Also, the enzymatically formed radical species could be altered by the addition of, for example, dioxygen or hydrogen bromide to form hydroperoxy or free bromine radicals respectively. These latter species could be used to attack unsaturated hydrocarbons to form oxygenated or brominated molecules.

5.4.2. Incorporation of molecular oxygen into unsaturated carbon structures by LiP.

The latter suggestion involved the modification of enzymatically produced radicals before the target molecule was attacked. One such area of possible exploitation in this respect is the epoxidation of styryl and other unsaturated carbon structures. HRP has been shown to oxidise styrene to styrene epoxide in the presence of H_2O_2 and p-cresol (Ortiz de Montellano & Grab, 1987; fig 5.5). HRP oxidises p-cresol to a radical, which is then attacked by dioxygen to produce a hydroperoxyradical. It is this latter species that actually effects the epoxidation of styrene. LiP should be a valid catalyst in such a system since its' ability to dimerise p-cresol almost certainly involves radical formation, although at present, no direct EPR evidence is available for this particular reaction.

The use of LiP in the p-cresol/styrene system of Ortiz de Montellano & Grab, resulted in no detectable formation of styrene epoxide or styrene glycol (the acid hydrolysed product), even though p-cresol dimers were produced. This suggests that p-cresol radicals existed, but they were too short lived to react with dioxygen and/or styrene, probably due to the immiscibility of styrene with water. The two-phase nature of the system, even under vigorous mixing meant

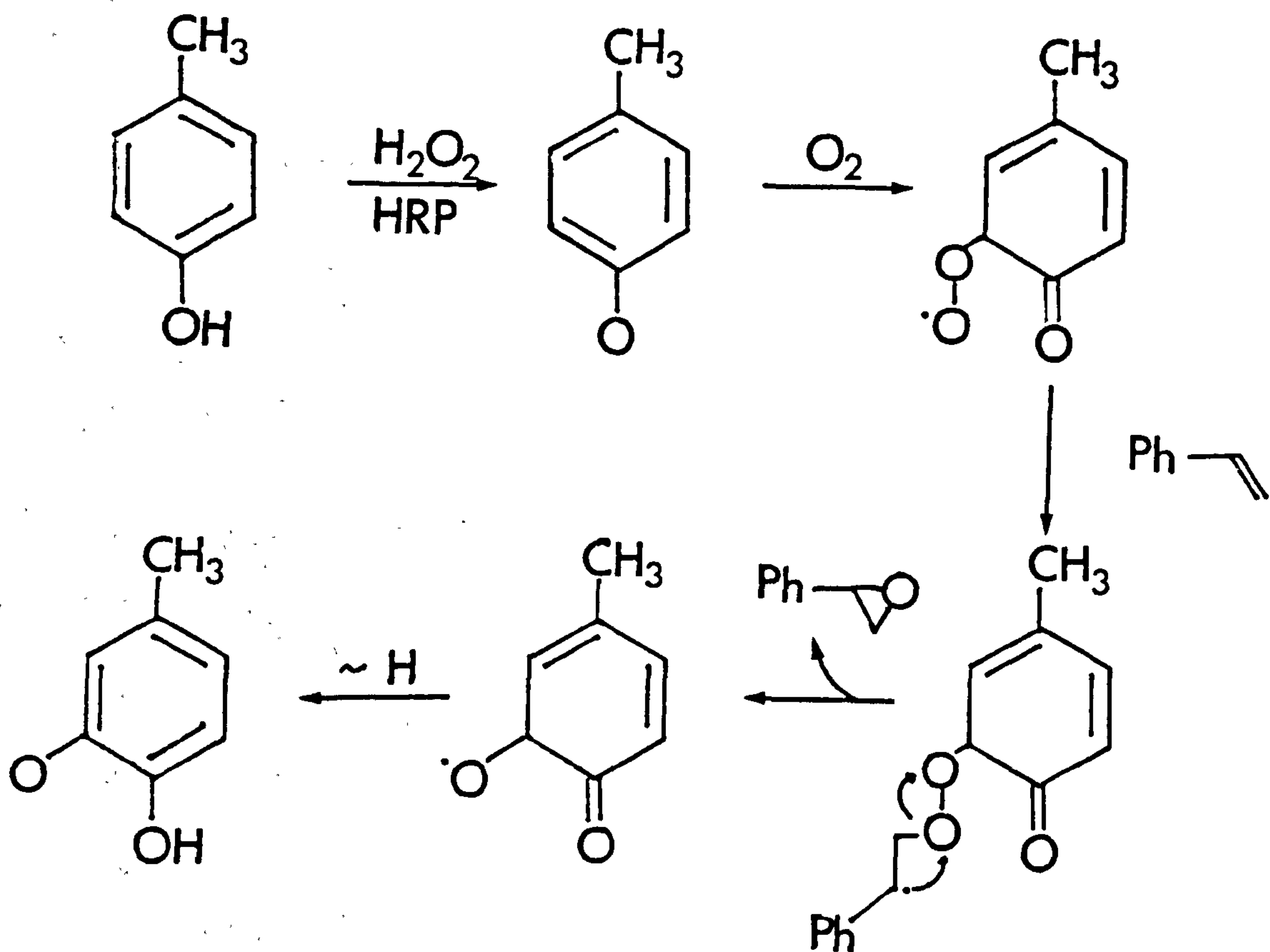


Figure 5.5. Mechanism proposed for the p-cresol-mediated cooxidation of styrene (Ortiz de Montemmano & Grab, 1987).

that the vast majority of the enzymatically formed p-cresol radicals would most likely have been scavenged by p-cresol molecules in the aqueous phase, before reaching any styrene molecules.

Since the initial attempts using the methods of Ortiz de Montellano & Grab (1987) to epoxidise styrene with LiP failed, a number of other methods were used to try to bring the short-lived radicals and the styrene together:

i) A 95% organic phase of ethylacetate, propyl acetate, or pentane was used to keep the p-cresol concentration in the aqueous phase to a minimum. The complete absence of dimer formation suggested that the enzyme was inactivated by the solvents, although oxidation of veratryl alcohol had been demonstrated in ethyl and propyl acetate (see 6.4.1.).

ii) 4-ethylphenol was used as a slightly more hydrophobic mediator in the hope that any radicals formed would more readily pass into the styrene phase. Although 4-ethylphenol dimers were formed, no oxidised styrene products could be detected.

iii) The surfactant Tween 80 (0.05% w/v) was added to allow the formation of a very fine aqueous/styrene emulsion in an effort to cut down on the time it takes for any radicals to diffuse into the styrene phase. Again, no styrene epoxide or glycol product was detected.

iv) A 10% emulsion of styrene with added LiP was entrapped in alginate beads and incubated on an orbital shaker containing a H₂O₂/p-cresol solution, in an effort to reverse the LiP localised substrate concentration in favour of styrene. Still only p-cresol dimers were formed.

A similar attempt to produce cyclohexane diol from cyclohexene also failed. The general failure to achieve epoxidation cannot lie with the enzyme since p-cresol was oxidised to form Pummerers' ketone. It is possible that most of the dimerisation occurred before the addition of dioxygen to the radical since no other major product, which could have been the subsequent result of a hydroperoxy p-cresol radical, could be detected. Oxygen saturation of the reaction mixture before the addition of LiP had no beneficial effect.

Apart from the particular enzyme used, the only physical difference between this set of experiments and those of Ortiz de Montellano & Grab (1987), is that of pH. The respective operating pH values of LiP and HRP were 3.5 and 7.4. However, Hammel et.al. (1986b) showed that during the degradation of dimethoxybenzoin by LiP at pH 3.0, hydroxybenzylperoxyl radicals were almost certainly intermediates. Therefore, it is unlikely that a low pH value has a negative effect on hydroxyperoxy radical formation but it may have an effect on the stability of the radical. If this

radical species is short lived, then the major problem of reaction with the water immiscible styrene molecules before it dismutates is still present.

5.5 General conclusion.

i) For the most part, aromatic compounds that possess electron rich molecular π -orbitals were good substrates for LiP. For example, dimethoxylated benzyl alcohols. Certainly, compounds that possess comparatively electron poor π -orbitals were not oxidised. For instance, mono-methoxylated or halogenated benzyl alcohols.

However, other factors such as steric considerations may complicate the picture. Not only was there a wide variation in the stability of LiP to oxidise various methoxylated compounds, but further substitution (for instance, tri-methoxy) resulted in poorer oxidation rates.

ii) Under aerobic conditions the oxidation of benzyl alcohols to aldehydes appeared to be a general phenomenon, but there were, in addition, other products (probably quinones and ring-cleavage lactones). The number and type of these additional products depended on the nature of the substrate alcohol. The chemical stability of the subsequent radical cations or their further oxidation by LiP may determine the degree of

ring cleavage and substitution by attacking species such as H_2O , O_2 or chloride ion.

iii) LiP also possessed phenol oxidising activity, as demonstrated by its' ability to mimic classic HRP reactions such as dimer formation from p-cresol and the polymerisation of catechol. However, despite the ability of LiP to produce phenoxy radicals, cooxidation of styrene and cyclohexene with LiP, p-cresol and H_2O_2 to produce epoxides and glycols could not be demonstrated.

C H A P T E R 6.

THE CATALYTIC UTILITY OF Lip IN ORGANIC SOLVENTS.

6.1. Introduction.

In recent years there has been a growing interest in the use of enzymes and whole cells that are useful in preparative chemistry, to operate in organic solvents instead of an aqueous environment. The major reasons are as follows:

i) Many organic substrates are insoluble in water, but may be soluble in a range of organic solvents. For example, the use of alkenes in epoxidation processes (Brink & Tramper, 1985) catalysed by Mycobacterium Sp.

ii) Water can be the causative agent of undesirable side reactions. For example, lipases can be used to catalyse transesterifications in organic solvents but when water is present lipase catalysed hydrolysis becomes the main reaction (Klibanov, 1986).

iii) Some reactions, such as esterifications are thermodynamically unfavourable in water, but are thermodynamically favourable in organic solvents. For example, the esterification of butyric acid and

heptanol by Porcine pancreatic lipase (PPL) can take place in hexane and toluene, but not in water (Zaks & Klibanov, 1985).

iv) The stability of enzymes in nearly anhydrous media is often increased. For example, dry PPL in a tributyrin/heptanol mix can remain catalytically active after being at 100°C for several hours, whereas it is almost immediately inactivated in water (Zaks & Klibanov, 1984).

v) The insolubility of enzymes in organic media allows for their easy recovery and reuse (Klibanov, 1986).

The use of enzymes in organic solvent systems could therefore augment the field of biocatalysis by overcoming many of the previous restrictions and limitations. The use of LiP in organic solvents is potentially no exception. The high solubility of aromatic substrates in organic solvents compared to water may allow the spectrum and rate of LiP catalysed reactions to be increased. For example, lignin itself is poorly soluble in water but it is soluble in 95% dioxane (Klibanov, 1986). It may be possible, therefore, to achieve rapid degradation of lignin by LiP in organic solvents which could lead to the economic production of low molecular weight aromatic feedstock chemicals. Also, the degradation of recalcitrant halogenated aromatics by LiP (see 1.10.2.2.) may be enhanced by such a system due to the

increased solubility of such substrates.

Furthermore, an increase in the solubility of the product may also lead to further exploitation. In particular, one could consider the phenol-oxidising capability of LiP to produce aromatic polymers (see 5.3.).

Phenol-formaldehyde resins are polymers which have found use in fields such as wood composites, foundry resins, abrasives, friction and moulding materials, coatings and adhesives. However, due to the concern over toxicity of formaldehyde, alternative methods of polymer production have been sought. Enzymic polymerisation by peroxides is limited because of the relative insolubility of phenols, whose dimers and trimers fall out of solution, terminating further polymerisation.

Dordick et.al. (1987) found that horseradish peroxidase (HRP) would polymerise phenols to molecular weights of up to 2.6Kd in water/dioxane mixtures. LiP could well be an alternative to phenol-formaldehyde resin formation by HRP since it is relatively stable and it will operate under a lower pH regime than HRP.

In order to realise these potential applications, the ability of LiP to survive and operate in organic systems must be determined.

6.2. Stability of LiP in different organic solvents.

A range of organic solvents were tested (at 95% v/v) for their effect on the stability of LiP (table 6.1). Agitation (180 r.p.m. on a rotary shaker) was carried out in order to maximise contact between the continuous organic phase and the aqueous interphase, resulting in the formation of a fine emulsion.

Solvents were chosen over a wide range of Log P values. Log P is defined as "the logarithm of the partition coefficient in an octanol-water two-phase system" (Hilhorst et.al., 1984), and it is, in effect a measure of solvent polarity. Laane et.al. (1987) laid down general rules concerning biocatalysts in organic solvents. They suggested that biocatalysis in solvents with log P values of less than 2 is minimal, and that those solvents with log P values between 2 and 4 allow moderate activity. Highly apolar solvents possessing log P values above 4 allow the highest activity. They argue that the more polar the molecule, the more likely it is to distort or even strip the essential water layer around the enzyme. This water layer is necessary for maintaining the native catalytically active enzyme conformation.

In this work, no obvious relationship between solvent log P values and LiP stability was found (table 6.1). However, it should be noted that since the

Solvent	Log P	Half life (min)
Water		60
Methyl acetate	0.16	170
Butanone	0.29	33
Ethyl acetate	0.68	42
Ether	0.85	1800
Propyl acetate	1.2	960
Chloroform	2.0	6
Toluene	2.5	20
Octanol	2.9	80
Tetrachloromethane	3.0	7
Cyclohexane	3.2	8
Octane	4.5	96
Tetradecane	7.6	33
FC40	n.a.	15

Table 6.1. Stability of LiP in different solvents. (FC40 is a high molecular weight fluorocarbon with a water solubility of <0.0003 ppm; Log P values taken from Laane et.al., 1987) at 30°C.

spectrophotometric determination of veratraldehyde production could not be executed out on an emulsion, the activity of LiP was measured only after the removal of the organic phase. It was therefore a change in activity between the addition and extraction of LiP from the solvent which was being measured, rather than direct activity in situ. Any disruption caused by the solvent on the 'dormant' enzyme may be reversible upon its re-introduction into a fully aqueous environment.

This may partly explain the discrepancies between these results and the theories put forward by Laane et.al. (1987), but it does not, for instance, explain why LiP stability is so poor in halogenated carbons. Chloroform, tetrachloromethane and FC40 allowed stability of only 10 - 30% of that of the standard aqueous system. In each case, a precipitation formed at the interphase within 10 minutes resulting in irreversible inactivation. These solvents are relatively hydrophobic, so their ability to quickly inactivate LiP must be due to some other overriding factor. All three of these solvents have two obvious properties in common. Namely, they are all halogenated and they are all denser than water, but whether this has any bearing on their ability to quickly inactivate LiP is not clear. One possible reason for protein inactivation is aggregation (Baldwin, 1975). Since the precipitate was found at the water/halocarbon

interphase then some property of that interphase, perhaps high surface tension due to the density of the halocarbon, may have caused LiP to aggregate there. Nevertheless, it can be concluded that halocarbons, despite their hydrophobicity, are unacceptable solvents for LiP biocatalysis.

The range of LiP stability was wide, ranging from 10% - 3000% of that afforded by the aqueous system. The most effective solvents were ether and propyl acetate, which allowed an enhancement of 30 and 16-fold respectively over that of the aqueous system. An increase in thermal stability of enzymes in organic solvents has been shown for PPL in a tributyrin-heptanol mixture (Zaks & Klibanov, 1984). The lipase was almost instantly inactivated in 0.1M phosphate buffer pH8 at 100°C. However, with a water content of only 0.8%, the half life was increased to approximately 15 minutes. Reducing the water content further to 0.015% gave the lipase a half life of over 12hrs.

Ahern and Klibanov (1985) explained that irreversible thermoinactivation was due to a number of water requiring reactions: the enzyme unfolds and refolds to form invalid random structures; S-S bonds can be destroyed via β -elimination; asparagine and glutamine residues can be deamidated and lastly that peptide bonds can be hydrolysed at aspartic acid residues. These reasons may be valid in a system which contains 0.8% water, however the LiP system contained a

small (5%) but significant aqueous phase. Therefore the reduction of water-induced thermoinactivation probably played only a minor role in the stabilisation of LiP.

Ether itself is slightly soluble in water (0.8% v/v). The aqueous phase was ether-saturated but whether this imparted any change in the chemical nature of the essential water layer around the enzyme, leading to a reduction in thermoinactivation by the prevention of hydrolysis, is speculative.

6.3. LiP activity in Ether.

LiP oxidised 3,4-dimethoxybenyl alcohol to the corresponding aldehyde in a system with a continuous ether phase (95% v/v), albeit at a reduced rate in comparison to the aqueous system (fig 6.1). One possible reason for this reduction in activity was the inhibitory nature of the co-substrate hydrogen peroxide. Laane et.al. (1987) suggested that optimisation of biocatalysis in organic solvents can be increased when the polarity of the microenvironment of the biocatalyst ($\log P_i$) and the continuous phase ($\log P_{cph}$) are tuned to the polarities of both substrate ($\log P_s$) and product ($\log P_p$). More importantly in this reaction system $\log P_{cph} - \log P_s$ H_2O_2 and $\log P_i$

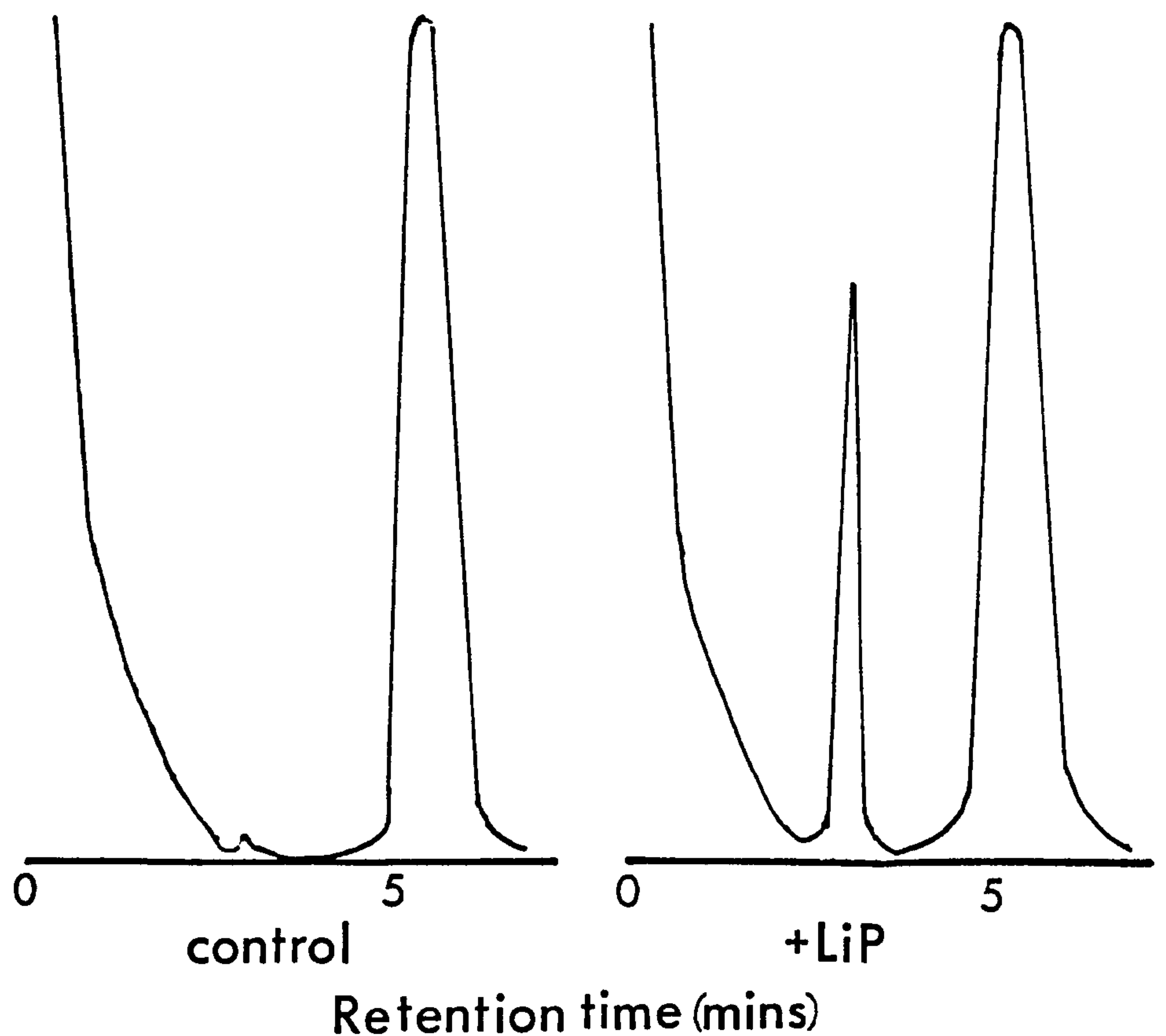


Figure 6.1. GC Elution profile from a Cellite 545S column showing the result of LiP oxidation of veratryl alcohol to veratraldehyde in 95% (v/v) ether (veratryl alcohol retention time, 5.13 mins; veratraldehyde retention time, 2.95 mins; oven temperature 150°C; flame ionisation detector 215°C).

LiP relative activity = 0.46 compared to activity in water.

- log Ps H₂O₂ must be as similar as possible. If log Pcph - log Ps H₂O₂ is too high, then LiP will be inactivated by a localised high concentration of hydrogen peroxide unless impractically small sequential additions of hydrogen peroxide are added. It follows that in this system, there was an obvious advantage in using a solvent with a low log P. Hydrogen peroxide was soluble in ether but, although there was room to optimise its sequential addition, it should not present an overriding practical problem.

Phenol-oxidising activity was also present in the 95% v/v ether system. A rate of catechol polymerisation of up to 30% of that found in an equivalent aqueous system was achieved.

6.4.1. Lyophilised LiP in organic solvents.

The water phase was reduced further by lyophilising LiP on to a glass surface. Ether was chosen as a suitable solvent since it imparted the greatest stability on LiP (table 6.1.). Klibanov (1986) stated that water saturation "quenches the thirst" of the more hydrophylic, but water immiscible solvents for the enzyme's essential water layer. Water-saturated ether (1.2% v/v) was thus chosen for the continuous phase.

Definitive production of 3,4-dimethoxybenzaldehyde

was demonstrated by the use of G.C./mass spectroscopy (fig 6.2.), but routine monitoring of veratryl alcohol oxidation was followed by the standard spectrophotometric assay. Veratryl alcohol oxidation only occurred in the original glass vessel that contained the lyophilised enzyme. The lyophilised powder would not dissolve even under vigorous agitation, but a suspension could be achieved by physically scraping the powder off the glass surface. In effect, without any further modification, the enzyme was immobilised onto the glass surface.

This has implications in a commercial scenario. For instance, in many industrial immobilised enzyme processes, such as the production of L-amino acids by aminoacylases, the major cost savings over the free-enzyme based batch reactors comes from reductions in the cost of enzymes and labour (Klibanov, 1983). Lyophilised LiP could easily be extracted and recycled if necessary.

Fig (6.3.) shows the comparison between the oxidation of 3,4-dimethoxybenzyl alcohol in a standard aqueous system to that of lyophilised enzyme in water-saturated ether and propyl acetate. Activity in the aqueous system was ten-fold higher than that in the solvent system. This may be due to the freeze-drying technique causing a reduction in catalytic area, due to obstruction by the crystallised buffer salts. Possible methods to get around this problem include reducing

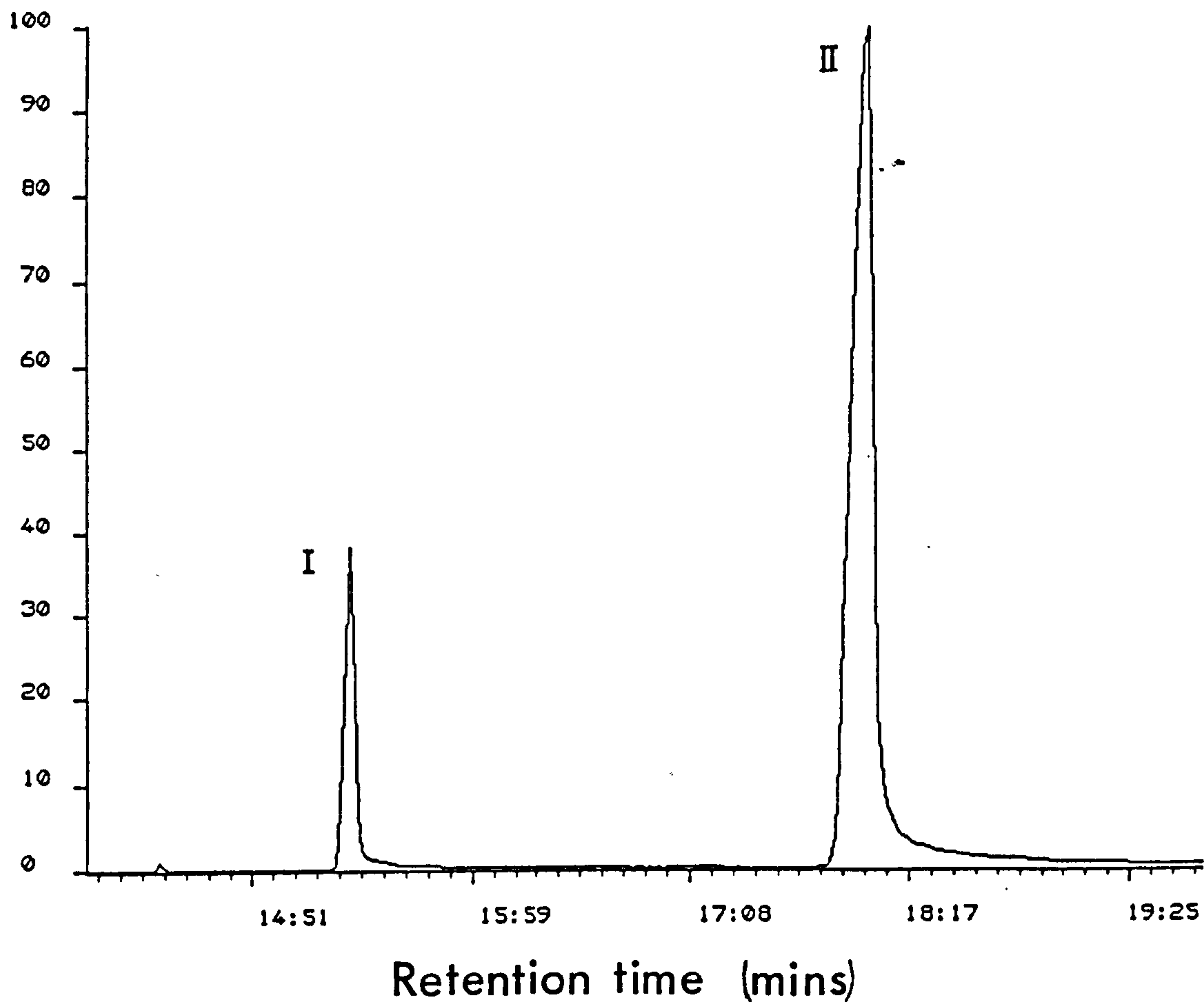


Figure 6.2. GC resolution of the product veratraldehyde (I) of LiP-oxidised veratryl alcohol (II) using a polar column (see 2.8).

Overleaf: Mass fragmentogram of veratraldehyde (I).

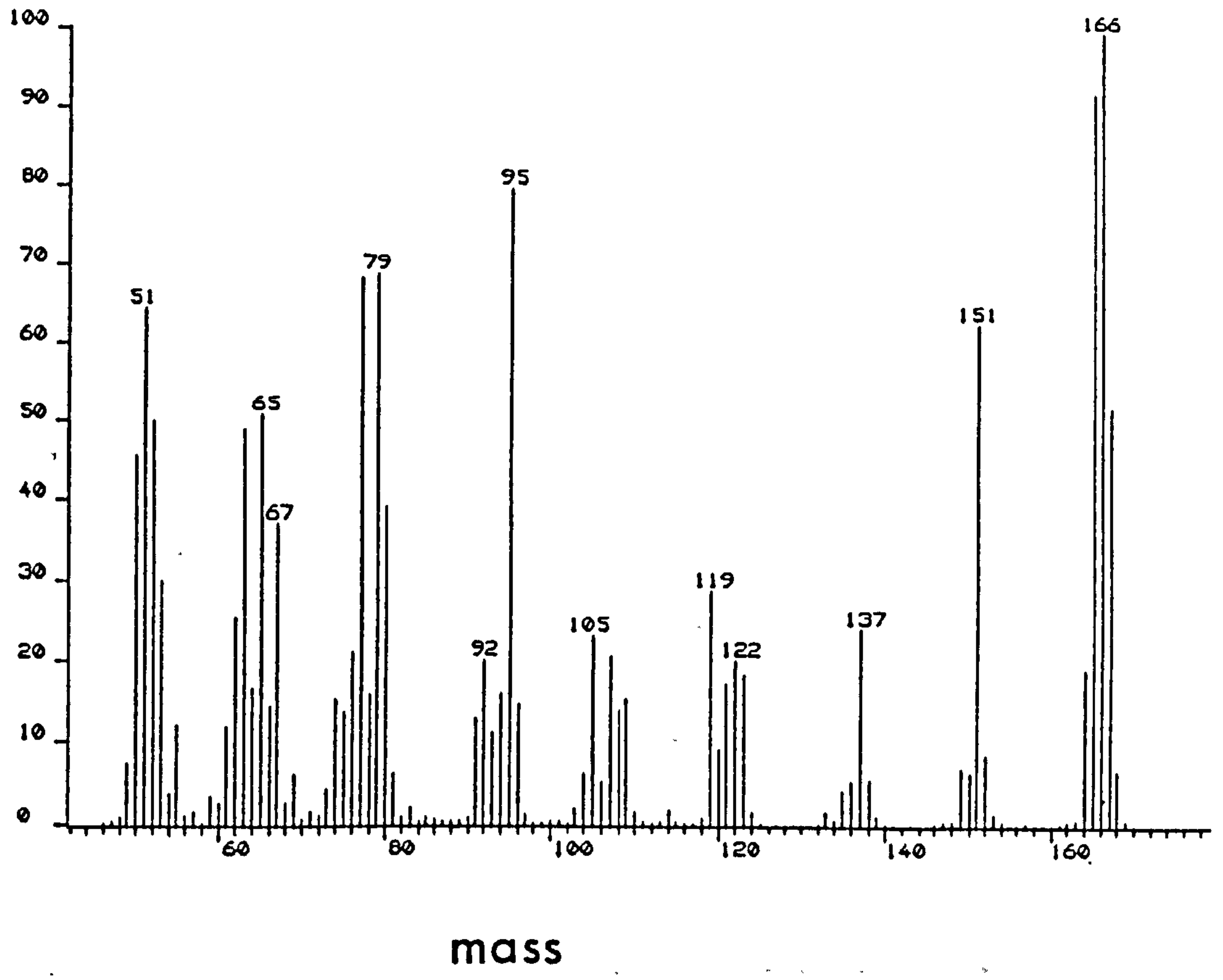


Figure 6.2. (Continued).

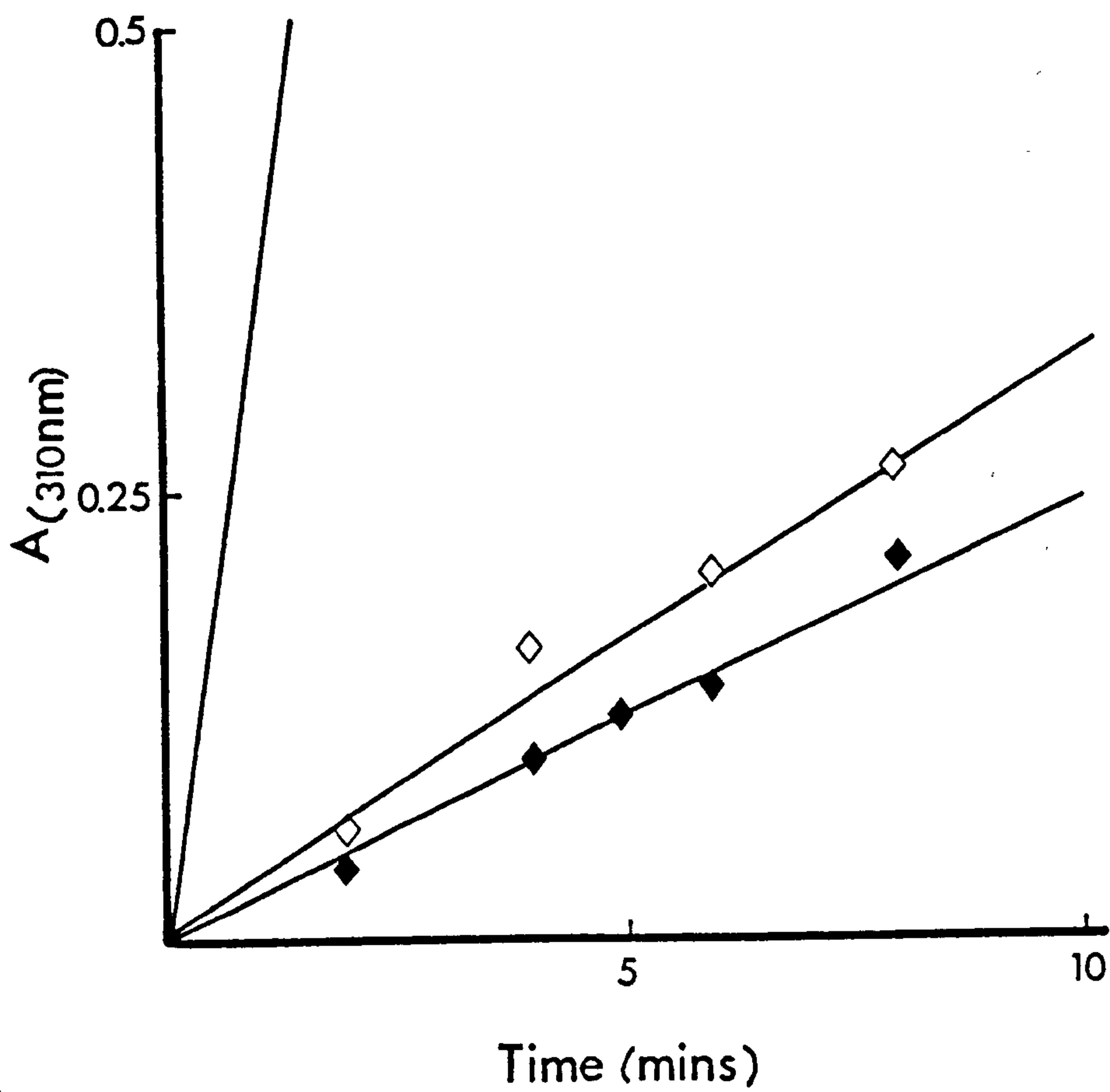


Figure 6.3. Comparison of LiP activity in — water, —◇— ether and —◆— propyl acetate (see 2.5.3. and 2.5.4.).

buffer concentration and increasing glass surface area or using a porous support instead. The use of free lyophilised enzyme in an agitated vessel was not practical since it was difficult to achieve a fine enough powder. In addition, it tended to agglomerate into even larger particles which greatly reduced the catalytic surface area. Also, by analogy to the 95% (v/v) ether system (6.3), hydrogen peroxide concentration and the frequency of its addition was important and perhaps even more critical in the water-saturated ether system where there is even less water to buffer the enzyme from peroxide inhibition if too much H_2O_2 were to be added.

Although ether has been shown to be a useful solvent it is a difficult chemical to work with since it is highly volatile and can spontaneously ignite under dry conditions. This would certainly be a problem for large scale applications. One possible alternative is propyl acetate, the log P value of which is similar to ether and it has already been shown to afford a high stability to LiP (table 6.1.). In the lyophilised system propyl acetate performs only slightly worse than ether (fig 6.3).

No observable oxidation of 3,4-dimethoxybenzyl alcohol was noted using water-saturated ethyl acetate, tetrachloromethane or octanol. This corresponded well with the poor stability of LiP in these solvents (table

6.1.).

6.4.2. pH in the Lyophilised LiP/Water saturated Ether system.

Enzyme reactions in aqueous solutions are pH-dependent. The role of pH in a system almost free of water is more obscure.

In fig 6.4., the pH values correspond to the pH of the enzyme in 10mM sodium tartrate buffer prior to freeze-drying. The specific activity of LiP is not only dependent on the original pH value but the optimal activity at pH 3.5 closely corresponds to the pH optimum in the standard aqueous environment (see 4.9).

Zaks & Klibanov (1984) found similar results with PPL in 2-pentanone. They suggested that the ionisable groups of the enzyme acquire the corresponding ionisation state from the immediately previous aqueous solution, and that these states are 'remembered' through the dehydration stage and introduction into organic solvent.

Zaks & Klibanov precipitated the lipase with acetone prior to lyophilisation. This would have the effect of removing the vast bulk of the buffer salts. In the LiP preparation, all the buffer salts were present in the dried powder. Although this may have an effect on overall specific activity, the massive increase in ionic strength during freeze-drying does

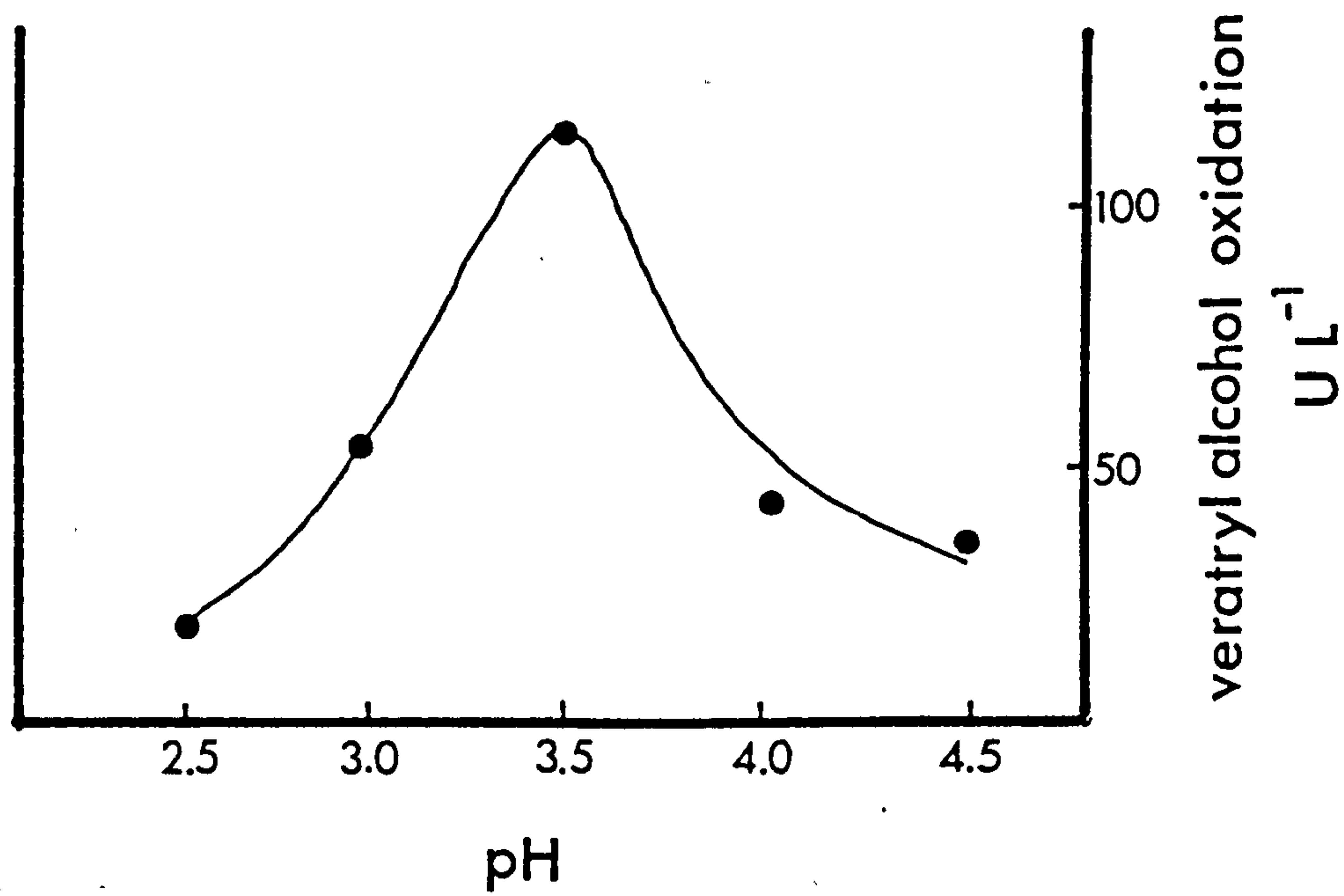


Figure 6.4. The pH profile of veratryl alcohol oxidation by Lyophilised LiP in water-saturated ether.

not appear to affect the pH optimum.

6.4.3. Catalytic specificity of LiP in the Water-saturated Ether system.

A range of benzylic alcohols were tested for oxidation in a lyophilised LiP/water saturated ether system. There was a marked difference in the catalytic spectrum of LiP under these conditions compared to the aqueous environment (table 6.2). Lyophilised LiP oxidised 3-methoxy,4-nitrosobenzyl alcohol; 3-methoxy,4-hydroxybenzyl alcohol and 3,4-dichlorobenzyl alcohol, all of which were not oxidised in the aqueous system.

The water environment in which an enzyme resides allows conformational flexibility. Lyophilised enzyme is considered to possess a greater degree of rigidity, which may cause a change in substrate specificity. For instance, Zaks & Klibanov (1984) found that PPL did not react with bulkier substrates such as tertiary alcohols under 'anhydrous' conditions. However, the results with LiP show an enhancement not a restriction in substrate range.

Solubility of the three alcohols mentioned above was poor in water but they were readily soluble in ether. Substrate solubility may be the primary reason for this difference in LiP catalytic activity. It is

Substrate	Aqueous	Ether
3,4-dimethoxybenzyl alcohol	+	+
3-methoxy,4-hydroxybenzyl alcohol	-	+
3-methoxy,4-nitrobenzyl alcohol	-	+
3,4-dichlorobenzyl alcohol	-	+
2,5-dimethoxybenzyl alcohol	+	+/-
3-methoxybenzyl alcohol	-	-

Table 6.2. Comparison of the catalytic utility of LiP in an aqueous system to that of an ether based solvent system.

worth noting that those compounds that were soluble in both ether and water were either oxidised in both solvents (e.g. 3,4-dimethoxybenzyl alcohol), or they were oxidised in neither solvent (e.g. 3-methoxybenzyl alcohol).

Product solubility may also positively affect the increase in catalytic potential of LiP in ether. The difference in solubilities of reactants and products can shift the reaction equilibrium of a process (Martinek & Semenov, 1981). In the case of aromatic alcohol oxidation by LiP, the corresponding aldehyde products are more hydrophobic and are therefore more likely to be removed from the aqueous/enzyme phase to the ether phase, thus shifting the equilibrium in favour of aldehyde production.

3,4-dichlorobenzyl alcohol possesses an electron deficient aromatic ring due to the electron-withdrawing effect of the chlorine atoms. According to the electron-withdrawing mechanism of LiP oxidation, (see 1.8) it should be a poorer substrate than the relatively electron rich 3-methoxy benzyl alcohol. The former was oxidised but the latter was not. Perhaps steric considerations may play a more important part in this case. The chlorinated alcohol has the same substitution arrangement as the substrate 3,4-dimethoxybenzyl alcohol. However no single-substituted mono-aromatic rings have been shown to be effective substrates for LiP (see tables 5.1., 5.2).

The ability of LiP to oxidise a chlorinated aromatic compound in ether that cannot be oxidised in water, may have implications in its use as a mineralisation agent of halogenated carbon compounds(see 1.10.2.2.).

6.5. General conclusion.

This chapter demonstrates that catalysis by LiP in an organic phase opens up new possibilities in bioconversions and biodegradative tasks. The high solubility of organic compounds in non-aqueous media allows greater catalytic flexibility. LiP will oxidise a greater range of benzyl alcohols in organic media than in an aqueous environment, including 3,4-dichlorobenzyl alcohol. Also, the presence of LiP phenol-oxidising activity in ether (95% v/v) has implications in the production of long chain phenolic polymers.

Although relative activity of LiP in organic solvents is low, it has no inevitable disadvantage since there is room for further optimisation. Even so, given the right solvent, LiP stability can actually be increased 30-fold (in the case of ether as the solvent) which more than offsets a low specific activity. In addition, increased thermal stability of LiP may allow

the use of operating temperatures much higher than originally envisaged, giving even greater flexibility to its 'catalytic potential by overcoming thermodynamic restrictions. Finally, the insolubility of LiP in organic media would allow easy recovery and re-use, removing the need for expensive immobilisation procedures.

C H A P T E R 7.

DISCOVERY AND PARTIAL PURIFICATION OF PEROXIDATIVE ENZYMES (CVP'S) FROM THE EXTRACELLULAR CULTURE FLUID OF CORIOLUS VERSICOLOR.

7.1. Introduction

Since the discovery of lignin peroxidase in the extracellular supernatant of P.chrysosporium (Gold et.al., 1984b; Tien & Kirk, 1984), research has been concentrated (perhaps a little too heavily) upon this particular white rot basidiomycete. The difficulty in producing high titres of LiP (see 3.1) from P.chrysosporium, coupled with the possible commercial potential of this enzyme (see 1.10) is perhaps reason enough to promote the mass screening of wood-rotting fungi in order to find strains that produce high yields of LiP type enzymes. In addition, to fully establish the importance of LiP as a lignin-degrading enzyme it must be shown to be a common, if not a universal constituent of the ligninolytic armoury of the white-rot basidiomycetes.

This chapter describes the attempt to find extracellular peroxidase activity from the white rot basidiomycete Coriolus versicolor.

7.2 Choice of organism.

There were a number of reasons for choosing C.versicolor as a candidate to screen for LiP-type enzymes. Next to P.chrysosporium, C.versicolor was perhaps the most widely studied white-rot basidiomycete. It has been shown to degrade effectively and simultaneously all three major wood components (Rosenburg, 1980). The physiological conditions necessary for lignin degradation in this fungus have already been worked out and have been found to be very similar to those required for P.chrysosporium (Kirk et.al., 1978), i.e. carbon or nitrogen limitation, low pH and high oxygen tension.

Studies involving the degradation of non-phenolic β -O4 (Kawai et.al., 1985) and non-phenolic β -1 model compounds (Kamaya & Higuchi, 1984) in cultures of C.versicolor have shown that, like in vitro studies with LiP from P.chrysosporium (see 1.8), the main degradative pathways involve Ca-C β cleavage,, Ca-oxidation and aromatic ring cleavage. This is strongly suggestive of the presence of an LiP type enzyme in the extracellular culture medium of C.versicolor.

In addition to lignin and lignin model compound degradation, a range of extracellular enzymes connected with lignin mineralisation have been found to be produced by this organism. For instance, Farmer

et.al. (1960) discovered a primary alcohol oxidase in the culture supernatant of C.versicolor. Extracellular oxidases have more recently been implicated in the production of H₂O₂ necessary for LiP activity (see 1.9.1). Westermarck & Eriksson (1974b) discovered an extracellular cellobiose:quinone oxidoreductase. This enzyme may be responsible for the prevention of repolymerisation of phenolics during lignin degradation (see 1.9.4). Fahraeus & Reinhammar (1967) discovered two laccases (polyphenol oxidases) which were secreted into the medium of Polyporus(Coriolus)versicolor. Laccases have since been implicated in the degradation of phenolic lignin substructures (see 1.9.2). Evans et.al. (1984) found a heme-peroxidase capable of producing oxygen radicals in the presence of H₂O₂. Since activated oxygen species have been considered to be important agents in lignin degradation (see 1.8.7), this enzyme could be potentially ligninolytic.

Recently, and in accordance with this work, Dodson et.al. (1987) found a 50kd veratryl alcohol-oxidising extracellular heme-protein (i.e. an LiP-type peroxidase) in carbon limited cultures of C.versicolor.

7.3. Choice of assay procedure.

The H₂O₂-dependent oxidation of iodide to triiodide (Banerjee et.al.,1985) was used as a simple colorimetric assay in the initial screening procedure for extracellular peroxidase (CvP) activity in C.versicolor. This assay correlated very well with veratryl alcohol-oxidising activity in cultures of P.chryso sporium (fig7.1). Although iodide oxidation was not specific for peroxidative veratryl alcohol-oxidising activity, it was found to be more sensitive than veratryl alcohol oxidation when assaying cultures of P.chryso sporium for LiP. Low titres of LiP were found in P.chryso sporium during initial screening studies (see 3.2), therefore the use of a highly sensitive assay was necessary to increase the possibility of peroxidase detection in cultures of C.versicolor.

Definitive detection of LiP-type activity was still determined by the oxidation of veratryl alcohol (see 1.7.2).

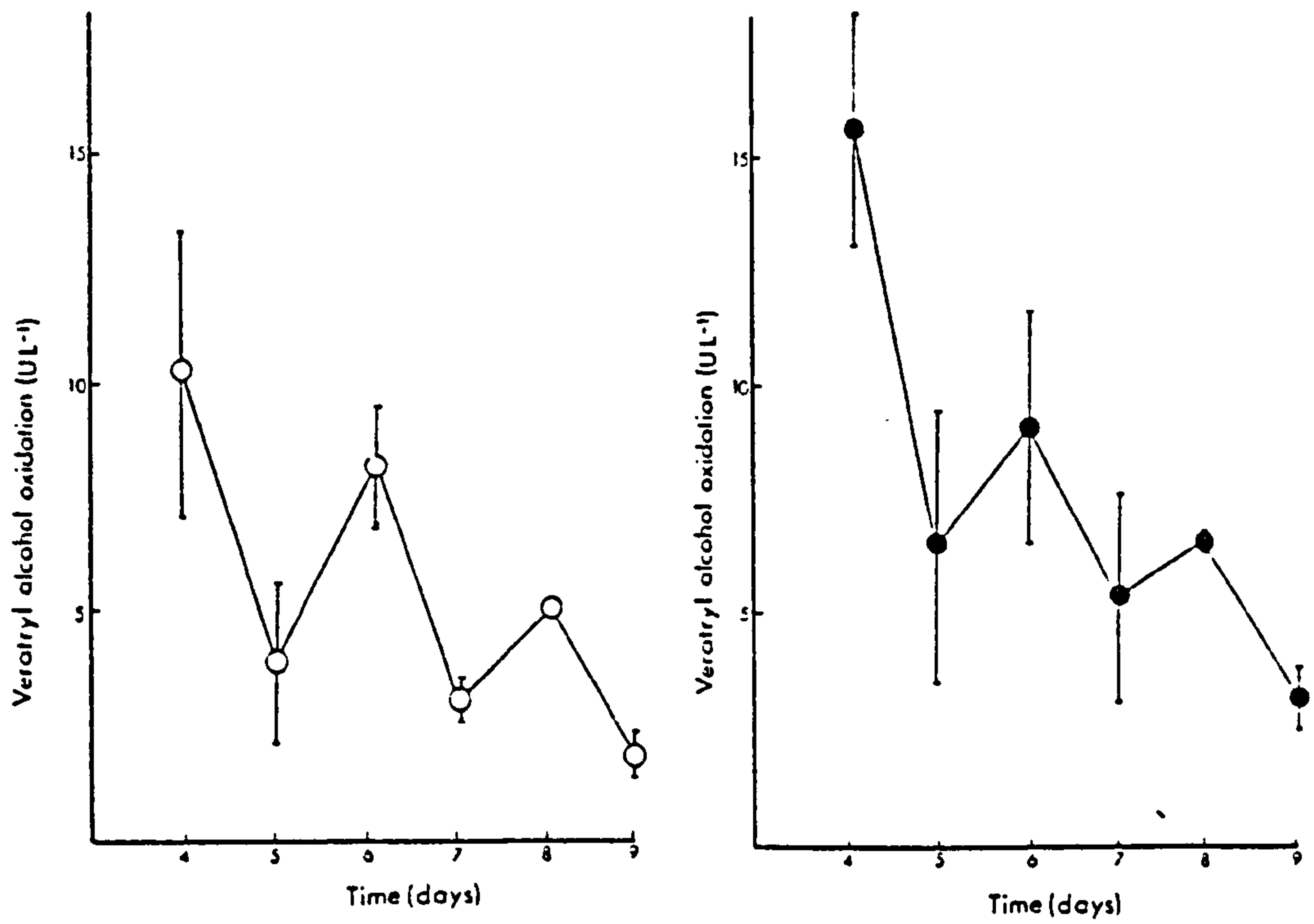


Figure 7.1. Comparison of iodide oxidation (○) with veratryl alcohol oxidation (●) during a time course for extracellular peroxidase activity in nitrogen-limited cultures of S.pulverulentum.

7.4. Growth conditions used for the detection of extracellular CvP activity.

According to Kirk et.al. (1978) the physiological conditions required for the mineralisation of ^{14}C - lignin to $^{14}\text{CO}_2$ in cultures of C.versicolor were similar to those required for P.chrysosporium. The defined medium initially chosen was that used by Tien & Kirk (1984) for P.chrysosporium BKM-1767 (see 2.2), except that the inoculum used was a partially homogenised liquid culture (previously grown in nitrogen-limited medium) instead of a spore suspension. This was necessary because unlike P.chrysosporium, a spore suspension of C.versicolor from agar slopes could not be made with Tween 80, nor could it be made with a range of other detergents (Tween 20; Brij 35; Nonidet P40; Lubrol and Triton X-100).

7.5. Detection and partial titre optimisation of CvP.

Extracellular CvP activity as measured by iodide oxidation was found to be optimal at 30°C or lower (table 7.1). Maximal activity attained at this temperature was 0.6UL^{-1} after two weeks. No LiP-type activity was found even after concentrating the crude extract to give 3.4UL^{-1} (iodide oxidation activity). As well as CvP secretion, sporulation was also poor

Temperature ($^{\circ}\text{C}$)	Maximum CvP activity (UL^{-1})
r.t.	0.8 (in 4 weeks)
30	0.6 (in 2 weeks)
37	ngl.
45	0

Table 7.1. The effect of temperature on CvP production in nitrogen limited static cultures of C.versicolor CMI 79 126.

above 30°C suggesting that elevated temperatures suppressed secondary metabolic events in general. Certainly there was no reason to believe that crude peroxidase from C.versicolor was significantly less stable than LiP from P.chrysosporium since both enzymes had half-lives of several weeks at room temperature. The temperature chosen for further studies was 30°C, which was a compromise between high CvP titre and fast mycelial growth.

Cultures that had been inoculated at pH3.5 and pH5.5 showed no CvP activity after two weeks. The original culture pH value of 4.5 was therefore chosen for further studies.

7.6. CvP activity in submerged cultures.

The successful strategy of using submerged agitated cultures (containing tween 80 and the inducer, veratryl alcohol) to facilitate scale-up and increase specific activity of LiP in Sporotrichum pulverulentum (see 3.3.3) was also used for C.versicolor. There were two reasons for this. Firstly, a much higher titre of the crude enzyme was necessary to study CvP further. Secondly, those physiological conditions that allowed a higher specific LiP titre (in particular the use of the inducer veratryl alcohol) may allow the successful determination of LiP-type activity in C.versicolor.

The use of agitated cultures of C.versicolor not only allowed an increase in maximum specific activity of CvP to 1.9UL^{-1} (fig 7.2), but also the maximum was achieved at day nine, five days before the maximum in static cultures. In addition, a low level of LiP-type activity was found in concentrated supernatant (see 7.7).

The time/activity profile of iodide oxidation by CvP in cultures of C.versicolor was very similar to that of veratryl alcohol oxidation by LiP in cultures of S.pulverulentum under the same conditions (see fig 3.2.d). The only major difference was that, unlike LiP from S.pulverulentum, CvP production does not require the gassing of cultures with 100% O_2 . The titre however, can be enhanced 2-fold by gassing with oxygen. Although crude CvP, unlike LiP, could not oxidise veratryl alcohol, the similarity of response by the two organisms in secreting their respective peroxidases under a particular set of growth conditions that allows ligninolysis suggests that CvP may have a role in lignin degradation by C.versicolor. In addition, the inability of extracellular peroxidases such as the Mn(II)-dependent peroxidases in P.chrysosporium to oxidise veratryl alcohol has not excluded them from being implicated in ligninolysis (Glenn et.al., 1986, see 1.9.3).

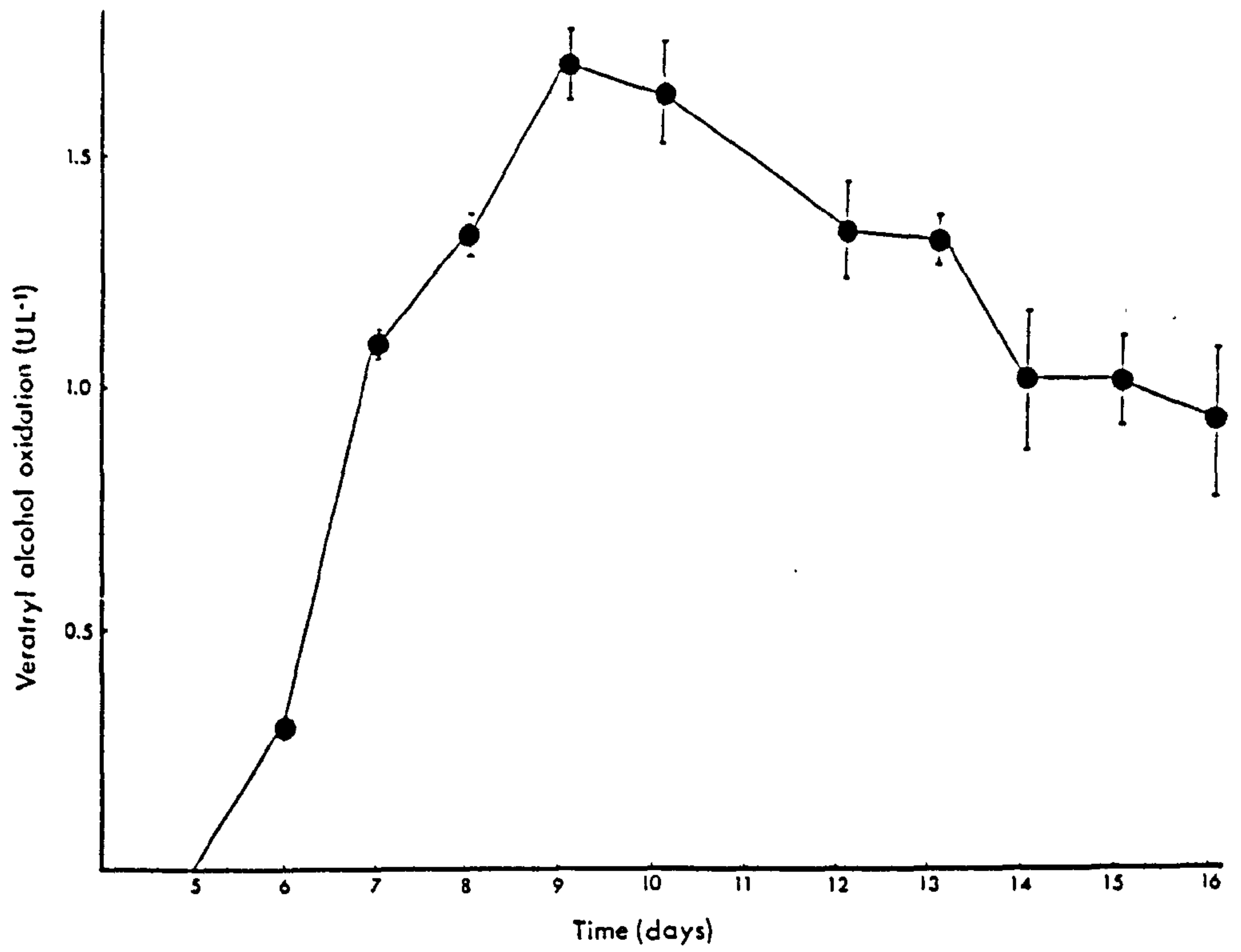


Figure 7.2. CvP activity in agitated submerged cultures of C.versicolor.

7.7. Partial purification of CvP activity.

There are two principle reasons for purifying CvP. Firstly, to fully implicate CvP in the degradation of lignin, the enzyme must be able to cleave lignin model compounds. The supernatant from agitated cultures of C.versicolor has been shown to contain small amounts of LiP-type (and presumably lignin model dimer cleavage) activity. Purification will determine whether this LiP-type activity is an intrinsic property of CvP, or whether it is due to another, as yet unresolved protein

Secondly, the LiP system of S.pulverulentum has been shown to consist of a large family of isozymes (see 4.6). By analogy, CvP may consist of not one, but several similar enzymes.

3 L of 14 day old extracellular culture fluid medium from C.versicolor (CvP activity = 0.05 Umg^{-1} protein) was filtered through glass wool to remove mycelial fragments. 5ml of a DEAE-Sephadex slurry was then added. After gently stirring the medium for one hour, 20% of CvP activity still remained unbound. The DEAE-Sephadex was removed by filtration through a glass sintered filter funnel and then poured onto the top of a 1 x 20cm DEAE-Sephadex column. After equilibration with 10mM dimethylsuccinate buffer (pH4.5), CvP was eluted with a 0 - 300mM MgCl_2 gradient and collected in 5ml fractions.

Peroxidase activity (as determined by H_2O_2 -dependent oxidation of iodide to triiodide) was associated with two separate peaks, CvP I and CvP II. These were eluted at 75mM MgCl_2 and 100mM MgCl_2 respectively (fig 7.3.).

CvP I, with a specific activity of 0.082 U mg^{-1} was purified 1.8 fold. CvP II, with a specific activity of 0.047 U mg^{-1} was not effectively increased by purification. The low purification factors may be partly due to the low total yield of only 32% (21% as CvP I and 11% as CvP II). Also, the low specific activities of CvP I and II, and the unusual absorbance spectrum of CvP I (see 7.8) implied that the enzymes were still relatively impure. However, the small amount of CvP present meant that it was impractical to further purify the enzymes.

7.8. Absorbance spectrum of CvP I.

Low protein yields allowed only the spectrum of CvP I to be determined (fig 7.4). There was a Soret maximum at 408nm. Oxidation of the enzyme by the addition of 200 μM H_2O_2 resulted in a shift of this maximum to 425nm. Addition of dithionite to the oxidised form resulted in a shift back to the original spectrum. These spectral properties are similar to LiP and other heme-containing enzymes (Tien & Kirk, 1984; Chance, 1952). In addition, CvP was inactivated by the

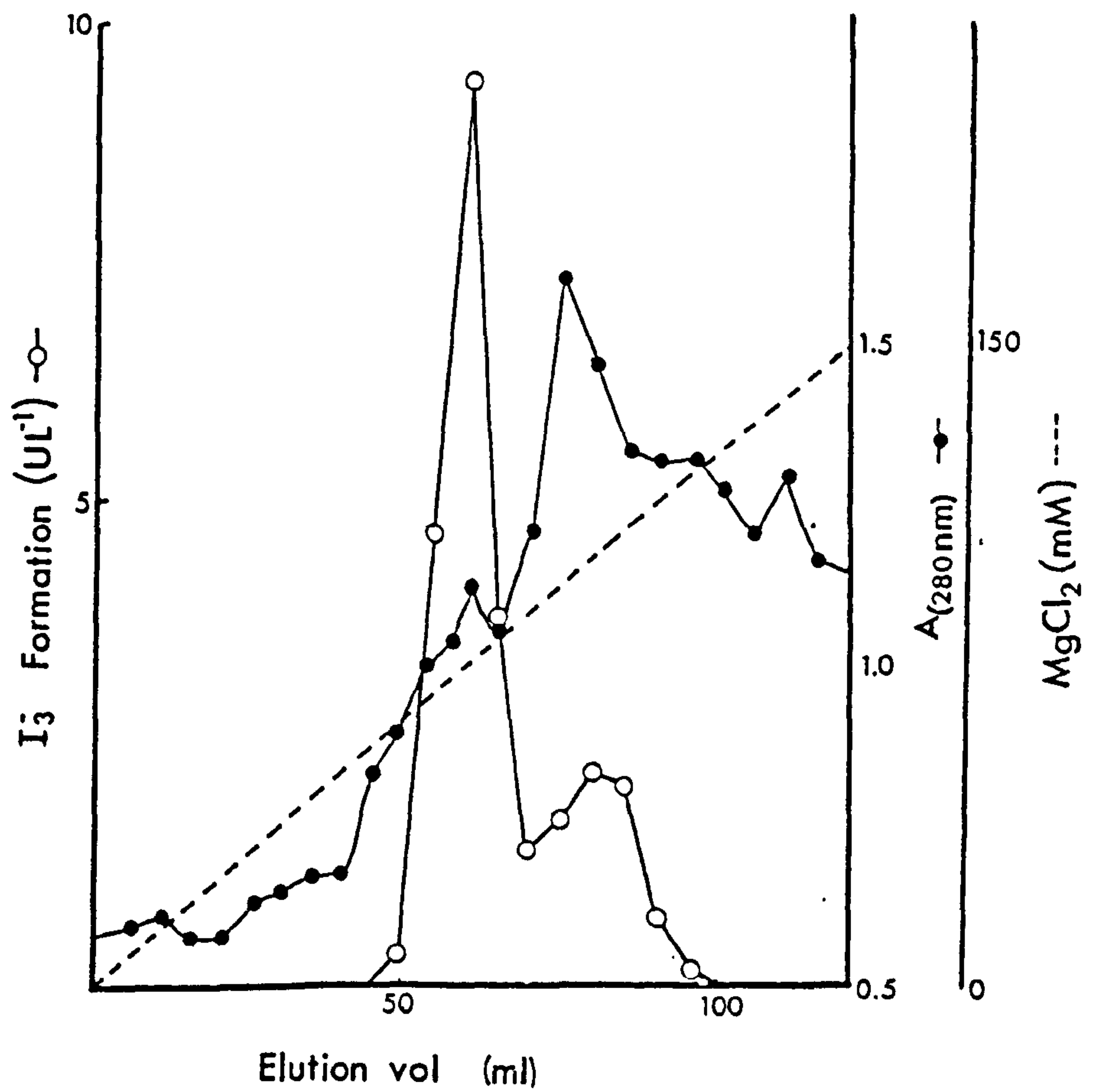


Figure 7.3. Elution profile of CvP from 14-day old cultures of C.versicolor from a column of DEAE-Sephadex equilibrated with 10mM dimethylsuccinate buffer pH4.5.

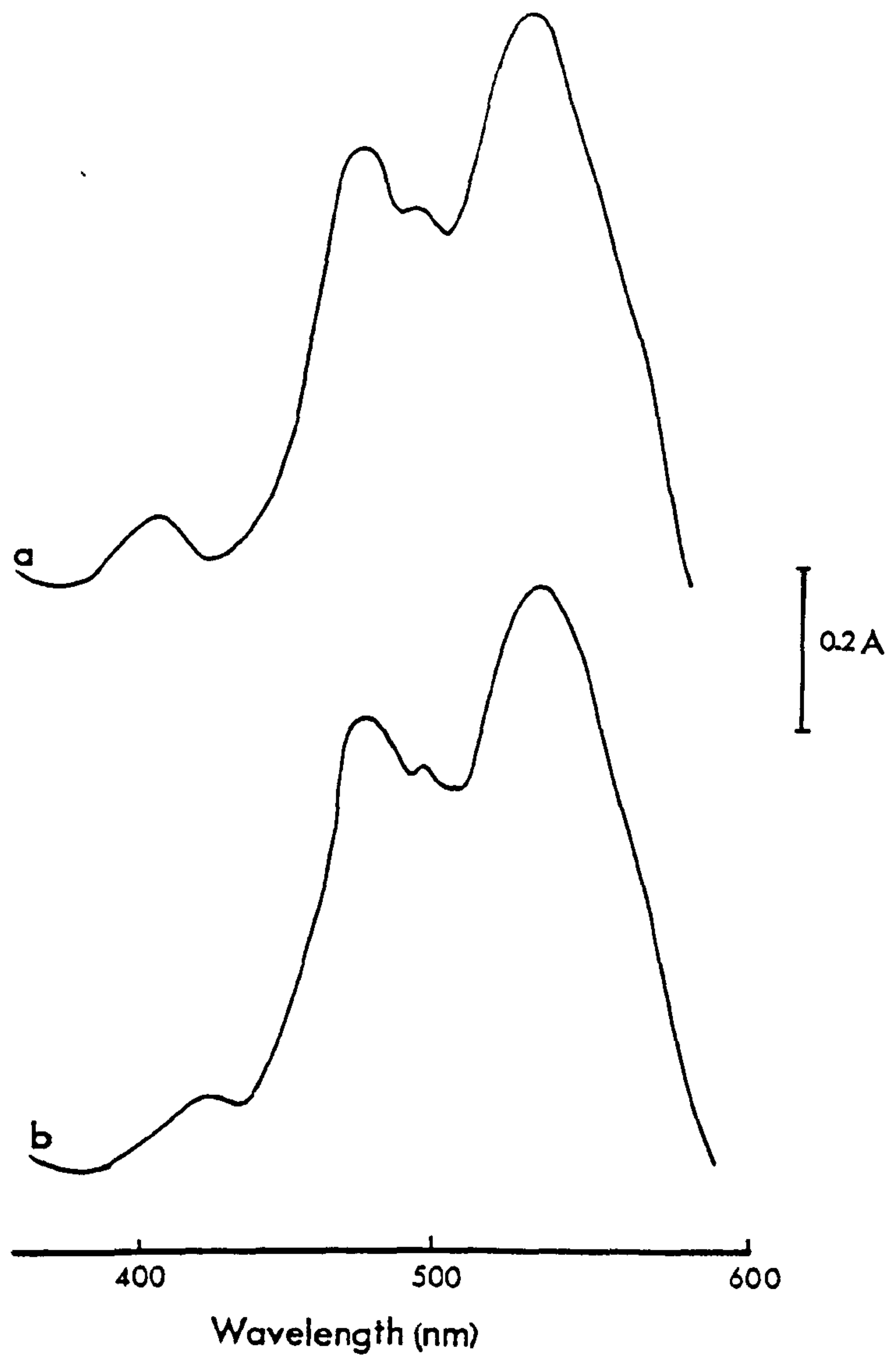


Figure 7.4. Absorption spectra of CvP I in 10mM dimethylsuccinate buffer pH4.5. before (a) and after (b) oxidation with 200 μ M H₂O₂.

same low levels of azide (10^{-5}M) that inactivated the haem-peroxidases LiP and HRP, further indicating the likelihood that CvP was a heme-protein. Unlike LiP there was no obvious reduction in absorbance of the oxidised maximum (425nm) relative to the base state (408nm).

Absorbance maxima also occurred at 470-500nm and 525nm. Both of these peaks were uncharacteristic of the spectrum of heme-proteins. They were much larger than the Soret peak which is normally the dominant maximum, and they were not affected by oxidation with H_2O_2 . The wide maximum at 470-500nm did however disappear upon the addition of dithionite. The nature of these other absorbance peaks, in conjunction with the low specific activity of CvP I, indicated that these other maxima may be due to the presence of a co-eluted contaminating protein. Laccase, although a major extracellular copper containing protein in cultures of C.versicolor can be ruled out as the contaminant since its spectrum possesses strong and weak maxima at 600nm and 450nm respectively (Falk & Reinhammar, 1972).

7.9. The determination of LiP-type peroxidase activity in CvP I and II.

Table 7.2 shows that neither the pooled fractions of CvP I nor those of CvP II were capable of oxidising veratryl alcohol to veratraldehyde. Concentrating the two enzymes approximately ten-fold using polyethylene glycol resulted in a very low, but detectable level of veratryl-alcohol oxidising activity with CvP II. The specific activity of CvP II for iodide oxidation was thirty times higher than that for veratryl alcohol oxidation. This could mean that its role in ligninolysis is primarily concerned with other degradative tasks, such as peroxidative degradation of phenolic lignin structures rather than the non-phenolic structures that are readily degraded by LiP from P.chrysosporium (see 1.8.1).

Alternatively, rather than CvP possessing a relatively low specific activity for veratryl alcohol oxidation, this activity could be due to the presence of a small quantity of an unresolved third enzyme. This would not be surprising when one considers the complexity of the LiP isozyme profile from S.pulverulentum (see 4.6). In addition, the recent discovery and purification of LiP in C.versicolor by Dodson et.al.(1987) would also suggest that the LiP-activity was due to a separate enzyme.

Enzyme	Activity in pooled fractions (UL ⁻¹).		Activity in concentrate (UL ⁻¹).	
	Iodide	Veratryl alcohol	Iodide	Veratryl alcohol
CvP I	7.4	0	6.2	0
CvP II	2.6	0	22	0.7

Table 7.2. Relative activities of CvP I and CvP II with respect to iodide and veratryl alcohol oxidation.

7.10. Future strategy for increasing LiP-type activity in cultures of C.versicolor.

Although this work demonstrated that LiP activity is present in C.versicolor, it is still very low compared to the recent reports of LiP in C.versicolor by Dodson et.al.(1987) and Waldner et.al.(1988). Dodson et.al. found that carbon-limited stationary cultures of C.versicolor(28-A PRL) gave a yield of LiP twice as high (6UL^{-1}) as that in nitrogen-limited cultures. However, Waldner et.al., 1988 achieved a titre of 20UL^{-1} in nitrogen-limited stationary cultures of C.versicolor(ATCC 42530). It would appear that the choice of strain can affect the LiP titre. Any future work should involve the screening of a number of strains of this organism.

The amount and timing of addition of the inducer, veratryl alcohol, should also be carefully investigated. Waldner et.al., 1988 found that degradation of ^{14}C -lignin to $^{14}\text{CO}_2$ in cultures of the white-rot Bjerkandera adusta was reduced by 30% upon the addition of 0.6mM veratryl alcohol at the time of inoculation. Veratryl alcohol may therefore act as a toxic agent that affects the ligninolytic system as well as an inducer of LiP. This work involved the addition of 0.5mM veratryl alcohol on the day of inoculation (as used for S.pulverulentum, see 2.3). Dodson et.al., 1987 added double this concentration of

veratryl alcohol, but to cultures that were 7 days old. To determine optimum conditions for LiP production, a fine balance between concentration and time of addition of veratryl alcohol must be optimised.

7.11. Conclusion.

1) Under conditions of nitrogen limitation and the use of veratryl alcohol as an inducer, two extracellular peroxidases (CvP I and II) were found in cultures of C.versicolor.

Although they may be involved in lignin degradation their role is obscure, since their ability to oxidise the methoxylated aromatic compound veratryl alcohol is negligible. Unlike the other peroxidase system of P.chrysosporium, they are not manganese-requiring.

2) LiP-type activity has been found (albeit at low levels) associated with CvP II, indicating that LiP-type activity is not unique to P.chrysosporium.

3) CvP I and II are probably contaminated with other protein; indeed the LiP activity of CvP II may be due to a separate enzyme. Given the complexity of the LiP system from S.pulverulentum the peroxidases themselves could be a mixture of isozymes. The LiP system from S.pulverulentum was only fully resolved by the use of

HPLC in conjunction with high titres of crude enzyme.

The low titre of CvP used, coupled with the emerging possibility that the CvP system is complex and may involve at least one LiP enzyme, means that further determination of this system could only be realistically achieved by strain screening and optimisation of culture conditions.

FINAL CONCLUSIONS AND FUTURE WORK.

8.1. Summary.

1) A reliable method of LiP production has been developed involving large (750ml) agitated cultures of S.pulverulentum containing the detergent tween 80.

2) The LiP-system of S.pulverulentum consists of up to 14 isozymes which vary in molecular weight, pH optimum and specific activity for veratryl alcohol, but they do not differ in their catalytic spectra.

The isozymes from S.pulverulentum have higher molecular weights and lower isoelectric points than LiP from P.chrysosporium but they appear to have similar heme-ligand spectra and they are catalytically similar.

3) LiP from S.pulverulentum will oxidise a range of methoxy-substituted benzyl alcohols to methoxybenzaldehydes and, under aerobic conditions, a variety of additional compounds including quinones, ring-cleavage products and chloro-substituted aromatics. The detection of these latter compounds provide evidence for the existence of LiP-derived aryl

radical cations from a range of benzyl alcohol substrates, which is consistent with the one-electron theory of lignin degradation by LiP.

The ability of LiP to oxidise these substituted benzyl alcohols appears to be primarily a function of how electron-rich the aromatic π -orbitals are, but steric effects may also be important.

In addition, LiP will initiate classical peroxidative one-electron oxidation of phenolics to produce dimers and polymers.

4) The use of LiP in organic solvents such as ether and propyl acetate allows an increase in enzyme stability of up to 30 times of that in water and a broadening of its catalytic spectrum.

5) LiP was also found in C.versicolor, demonstrating that LiP may be a common constituent of ligninolytic white-rot fungi. In addition, other extracellular peroxidases were present in this fungus, but their role in ligninolysis is more obscure, since they were unable to oxidise veratryl alcohol.

8.2. Future proposals.

The development of a process for LiP production in 750ml submerged cultures containing 0.1% Tween 80 (see 3.3) allowed adequate titres of the enzyme to be made available for the proceeding work in this thesis and for much of the projected work stated below. However, if the commercial potential of LiP is to be realised, high-yielding continuous methods of production need to be elaborated.

Although semi-continuous methods of LiP production using immobilised P.chrysosporium have been developed (Kirkpatrick & Palmer, 1987; Linko, 1988; Leisola personal communication) only a large-scale fully continuous process could be commercially justified. In this respect RepliGen are involved in the cloning and expressing of recombinant-LiP in organisms suitable for production in large stirred-tank fermentors and the development of a mutant of P.chrysosporium which constitutively produces high yields of LiP (Farrell, 1987).

LiP has been considered to be a potentially useful agent for biotransformations in the pulp and paper industry (see 1.10). However, the novel properties of LiP may make it a valuable biotransformant of aromatic compounds in areas unassociated with lignin degradation directly.

The peroxidase LiP is unique in that its H_2O_2 -oxidised states are more electropositive than the analogous compounds of classical peroxidases (Hammel et.al., 1986b). As a consequence, LiP can oxidise aromatic substrates that HRP is incapable of oxidising. For example, di- and tri-substituted methoxybenzyl alcohols (see 5.1.), 1,2- and 1,4-dimethoxybenzene (Kersten et.al., 1985) and lignin (Klibanov, 1986). In addition, LiP is capable of oxidising substrates of classic peroxidases, but at acidic rather than neutral pH values. For example, dimerisation of p-cresol, polymerisation of catechol (see 5.1.2.), oxidation of potassium iodide to triiodide (see 7.3.) and oxidation of pyrogallol to purpurogallin (additional work).

The unique catalytic properties of LiP can be further enhanced by the use of a two-phase organic solvent/aqueous system (see 6.). LiP suspended in ether not only allows a further increase in catalytic spectrum (see 6.4.3.), but the stability of the enzyme can be greatly increased in the solvent environment (see 6.2.). Future work should consist of refining LiP catalysis in organic solvents by optimising H_2O_2 addition so as to increase stability by preventing LiP inhibition by H_2O_2 in the aqueous phase. As an alternative to direct addition of H_2O_2 , an oxidase enzyme could be used in situ which would allow a small but constant supply of H_2O_2 to the enzyme.

LiP has been shown to oxidise 3,4-chlorobenzyl

alcohol in an ether/aqueous system (see 6.4.3.). Further research could consist of an investigation into the potential of LiP to oxidise chloro-aromatic pollutants in organic solvents with a view to develop a detoxification system for toxic effluents. This would involve large scale screening of aromatic pollutants, such as 2,4-di- and 2,4,5-trichlorophenoxy acetic acid, DDT, PCBs and lindane for susceptibility to LiP oxidation, followed by G.C./mass spectrometry determination of the products to see whether they have been fully mineralised, or whether they have been transformed into toxic or non-toxic products.

LiP has been shown to dimerise and polymerise phenols (see 5.3.). However, the main limitation to commercial peroxidative production of aromatic polymers in water is that the polymer chains are too short because polymerisation is terminated once the growing polymer falls out of solution (Dordick et.al., 1987). Initial investigations demonstrated that LiP can polymerise catechol in ether (see 6.3.). LiP oxidation of different aromatic compounds to produce long-chain homo- and hetero-aromatic polymers could be tested by looking at LiP catalysis in a range of organic solvents to find an optimum between LiP activity/stability and polymer chain length.

The screening of other white-rot fungi for LiPs has recently come to fruition (see 1.7.3; 7.). These

LiPs may be worth investigating as biocatalysts, however, they appear to be closely related (1.7.3.) and, by definition, all oxidise veratryl alcohol and may therefore be catalytically very similar. More importantly, one or more of these LiP⁺ species of white-rot fungi may be more amenable to industrial LiP production than LiP from P.chrysosporium if they can produce the enzyme constitutively, and/or in high quantities.

Despite the apparently common existence of LiP in white-rot fungi, there are white-rot fungi that are capable of significantly degrading lignin, but do not appear to possess LiP, for example, L.edodes (Leatham et.al., 1986), B.adusta, T.cingulata and F.lignosus (Waldner et.al., 1988). Other mechanisms may therefore exist to degrade the lignin polymer. Future research should determine whether other enzymatic systems, perhaps peroxidative or even oxygenative, exist in these fungi. They may have previously been overlooked because of their particular specificity towards certain lignin-derived compounds such as guaiacyl units that are not oxidised by LiP (see 5.1.4.).

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