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TOXICOLOGICAL STUDIES IN YEAST SPECIES: PRODUCTION OF CYTOCHROME P450 AND EVALUATION OF CARBON TETRACHLORIDE TOXICITY

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A thesis submitted in partial fulfilment of the requirements of the University of North London for the degree of Doctor of Philosophy

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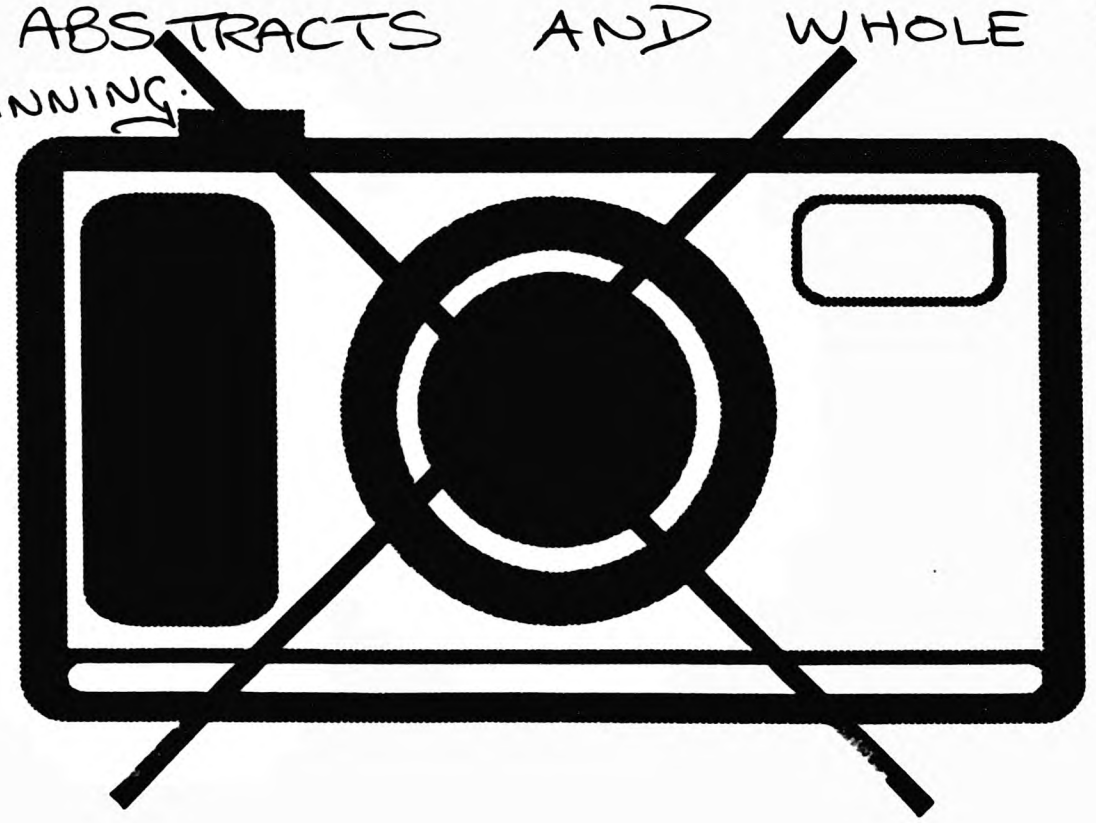
POSTER ABSTRACTS AND WHOLE PAPER AT BEGINNING.



TOXICOLOGICAL STUDIES IN YEAST SPECIES: PRODUCTION OF CYTOCHROME P450 AND EVALUATION OF CARBON TETRACHLORIDE TOXICITY

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ABSTRACT

In this work a number of yeast species were assessed for their potential in testing putative toxic and genotoxic compounds. Carbon tetrachloride (CCl_4) was used as a model compound. Exposure of cells of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* to carbon tetrachloride showed that the compound was toxic to the cells in a dose-dependent manner. *Sch. pombe* was more sensitive to the effects of carbon tetrachloride than *S. cerevisiae*.

Administration of the anti-oxidant α-tocopherol (vitamin E) to cells of *Sch. pombe* treated with a toxic dose of carbon tetrachloride appeared to increase cell survival. These data are consistent with work carried out on mammalian cells, and indicate that the toxic effects of carbon tetrachloride are brought about via the generation of free-radical species.

Investigation of the genotoxicity of carbon tetrachloride in *Sch. pombe* and *S. cerevisiae* revealed that the compound induced mutations to chloramphenicol resistance at concentrations which were toxic to the cells. Carbon tetrachloride also induced forward mutations in *Sch. pombe ade6*. Genetic analysis of chloramphenicol resistant mutants indicated non-Mendelian inheritance.

The drug-metabolising enzyme cytochrome P450 is thought to metabolise carbon tetrachloride to toxic species. The ability of carbon tetrachloride to act as a substrate for the haemoprotein was indicated by the type 1 binding spectrum produced in *Sch. pombe*. α-Tocopherol was found to increase levels of cytochrome P450. The production of cytochrome P450 was investigated in the three yeast species *Sch. pombe*, *S. cerevisiae* and *Candida parapsilosis*. The enzyme was optimally produced under conditions of glucose repression during the late logarithmic phase of growth. Levels of the enzyme were consistently found to be greater in respiratory deficient (*petite*) mutants of the yeast *S. cerevisiae* than in wild type strains.

The enzyme was isolated from microsomal preparations of *C. parapsilosis*. SDS-PAGE analysis revealed a major band in the solubilised fraction possessing a molecular mass of approximately 48 kDa which was tentatively identified as cytochrome P450.

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CONTENTS

LIST OF PLATES

LIST OF TABLES

ABBREVIATIONS

A	absorbance
AMPS	ammonium persulphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5'-monophosphate
CAP	chloramphenicol
cfu	colony forming units
dry wt.	dry cell weight
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMM2	Edinburgh minimal medium number 2
EMS	ethyl methanesulphonate
g	gravity
GSVB	Giese's salts vitamin buffer
kDa	kilodalton
Mol wt	molecular weight
mRNA	messenger ribonucleic acid
MEA	malt extract agar
MOPS	morpholinopropanesulphonic acid
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ND	not determined
P420	cytochrome P420
P450	cytochrome P450
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
R (superscript)	resistant
R_f	relative mobility
S (superscript)	sensitive
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N' tetramethylethylenediamine
TET	triethylenetetraminehydrochloride
UV	ultraviolet
YEA	yeast extract agar
YEL	yeast extract liquid
YEPD	yeast extract peptone dextrose
YEPG	yeast extract peptone glycerol
wet wt.	wet cell weight
IARC	International Agency for Research on Cancer

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The enzyme was isolated from microsomal preparations of *C. parapsilosis*. SDS-PAGE analysis revealed a major band in the solubilised fraction possessing a molecular mass of approximately 48 kDa which was tentatively identified as cytochrome P450.

In memory of my Father and
to Iain and my Mother.

Jeremy Bentham, Philosopher, 1748-1832, on animals:

The question is not, can they reason? Nor, can they talk? But can they suffer?

CHAPTER 1

INTRODUCTION

1.1 Cancer and carcinogenesis

In industrialized societies cancer has become the second major cause of death after cardiovascular disease. WHO statistics from the early 1980s indicate that 19% of all deaths in these societies are caused by cancer (Lohman *et al.* 1992). British figures show that in 1991 145,355 people in England and Wales were diagnosed as having malignant or benign neoplasms (Central Statistical Office, 1992).

A cancer or neoplasm can be defined as a mass of cells which has undergone irreversible modification in physiology leading to unrestrained proliferation; this is often referred to as a tumour. A malignant tumour has the ability to metastasize and become established in other parts of the body (Groenwald *et al.*, 1992).

Some cancers have been shown to be familial e.g. chronic myelogenous leukaemia (Bodmer, 1986), Wilms tumour and retinoblastoma (Knudson, 1985). However at least 80 % of cancer incidence can be attributed to environmental factors (Bodmer, 1986). These factors have been highlighted by localised high incidences of specific cancers. The earliest recorded incidence is that of scrotal cancer in chimney sweeps (Pott 1775); this was related to exposure to soot. More recent observations include the induction of angiosarcomas in members of the population exposed to vinyl chloride (IARC, 1979) and high occurrences of

oesophageal cancer in the population of areas of China exposed to nitrosamines (Poirier *et al.*, 1987). Epidemiological studies of lung cancer incidence have shown a correlation with habitual cigarette smoking (Cairns, 1978).

Striking evidence for environmentally induced cancers can also be inferred from a comparison of the levels of specific cancers in different countries. In Japan for example, stomach cancer has a high prevalence whereas the incidence of breast cancer has been found to be high among Bombay Parsis and that of oesophageal cancer high among Iranians (Nery, 1986). Investigations of stomach cancer and large bowel cancer in the populations of Europe, Latin America, Japan and the North American continent suggested that both are associated with dietary factors (Higginson and Muir, 1982). Furthermore, migrants have been shown to suffer similar rates of cancer development to the indigenous population of their host countries. This can be illustrated by the fact that Japanese migrants to the United States exhibit a low incidence of stomach cancer and high incidences of colon and breast cancer, which are all characteristic of the United States population rather than the native Japanese population (Nery, 1986).

Among the environmental factors which are important in the aetiology of cancer are chemical carcinogens. Carcinogens are agents that may transform cells to a malignant or cancerous state. Carcinogens have often been found to be mutagens (*viz* agents which can produce a heritable genetic change). However potent mutagens are not always

strong carcinogens (Douglas *et al.*, 1988).

The somatic mutation theory of cancer proposed by Boveri (1914), stated that mutations are able to precipitate malignancy. However, a two or multistage theory of carcinogenesis has subsequently been developed (Rous and Kidd, 1941). This suggests that a carcinogen may first initiate the carcinogenic process by causing a stem cell mutation; tumours are thus monoclonal in origin. Malignancy may then be promoted by the action of compounds causing a disruption in cell division. Evidence for this process includes work by Berenblum (1941), who discovered that the application of carcinogens in certain solvents to mouse skin produced a higher incidence of tumours than the carcinogen alone. These solvents or tumour promotors may increase the growth of initiated cells. Thus there is a strong rationale for screening environmental chemicals for their ability to initiate mutagenic events and also for their tumour promoting ability.

1.2 Cytochrome P450 and carcinogenesis

Some chemical carcinogens are 'direct-acting' compounds, e.g. the nitrogen mustards and nitrosamides. These compounds are generally alkylating agents which, being strongly electrophilic, may react with cellular macromolecules (Miller and Miller, 1971). However many compounds e.g. aromatic amines and polycyclic hydrocarbons are pro-carcinogens i.e. inactive until metabolised to an active form (Miller and Miller, 1976). Activation may involve the formation of intermediate or proximate carcinogens which cannot react directly with cellular

macromolecules until conversion to ultimate carcinogens (Becker, 1975). Procarcinogens thus exist in the environment in a stable form until activated by the host. These chemicals may therefore prove to be extremely hazardous to living organisms.

An enzyme system which plays a central role in the activation of promutagens is the monooxygenase system. Cytochromes P450, a family of haemoproteins, participate as monooxygenases in certain biotransformation reactions (Lu, 1976). These proteins were first detected by Klingenberg (1958) and Garfinkel (1958) in mammalian liver microsomes where their difference peak at 450 nm was observed on binding with carbon monoxide. They have been found to play an important role in the metabolism of a wide range of compounds from endogenous sources, such as steroid hormones and prostaglandins (Alexander *et al.*, 1974) and also of exogenous compounds (xenobiotics), in some cases generating toxic and genotoxic metabolites (Callen and Philpot, 1977).

Foreign compounds or xenobiotics may be toxic if they are not excreted rapidly. As many xenobiotics tend to be lipid-soluble the role of the cytochrome P450 system is to render them water-soluble so that they may be excreted rapidly. This is mainly achieved by hydroxylation or dealkylation reactions (Astrom and DePierre, 1986). In hydroxylation the insertion of one oxygen atom from molecular oxygen into the organic substrate is catalysed while the other is used for the formation of water. This can be shown by the general equation where RH is the organic

substrate and NADPH is the electron donor:

$$H^+ + RH + NADPH + O_2 \longrightarrow ROH + NADP^+ + H_2O$$

The terminal oxidases require two electrons for the reduction of the oxygen atom to water. The electron donor is usually NADPH via the flavoprotein NADPH-cytochrome P450 reductase or cytochrome c reductase (Lu, 1976). The second electron may also be transferred from cytochrome b_5 (Taniguchi *et al.*, 1984). As another substrate is needed to donate electrons for the reduction of the second oxygen atom to water the monooxygenases are often called mixed-function oxidases. Fig. 1.1. shows the catalytic cycle of cytochrome P450. Cytochromes P450 carry out phase I metabolic reactions which involve activation of the xenobiotic. The second phase of metabolism catalysed by phase II enzymes involves conjugation with a hydrophilic moiety such as sulphate or glucuronate often resulting in inactivation (Astrom and DePierre, 1986). The activation of xenobiotics by cytochrome P450 catalysed reactions can give rise to products more toxic than the parent compound. These products may react with nucleophilic groups in protein, RNA and DNA (Miller and Miller, 1971) and the mutagenic effects which can result from DNA damage may then, in turn, give rise to carcinogenic or teratogenic events (Pelkonen and Nebert, 1982).

Cytochromes P450 may be broadly divided into groups according to

Fig. 1.1.

Scheme of the catalytic cycle of cytochrome P450 (from Ortiz de Montellano, 1986).

I. Binding of substrate to cytochrome P450 to give a high-spin ferric complex.

II. One-electron reduction of the iron to the iron(II) state.

III. Generation of the oxy form $SFe^{3+}O_2^-$ from the binding of dioxygen.

IV. Generation of the iron peroxo species $Fe^{3+}O^2{}_2^-$ from a second one-electron reduction.

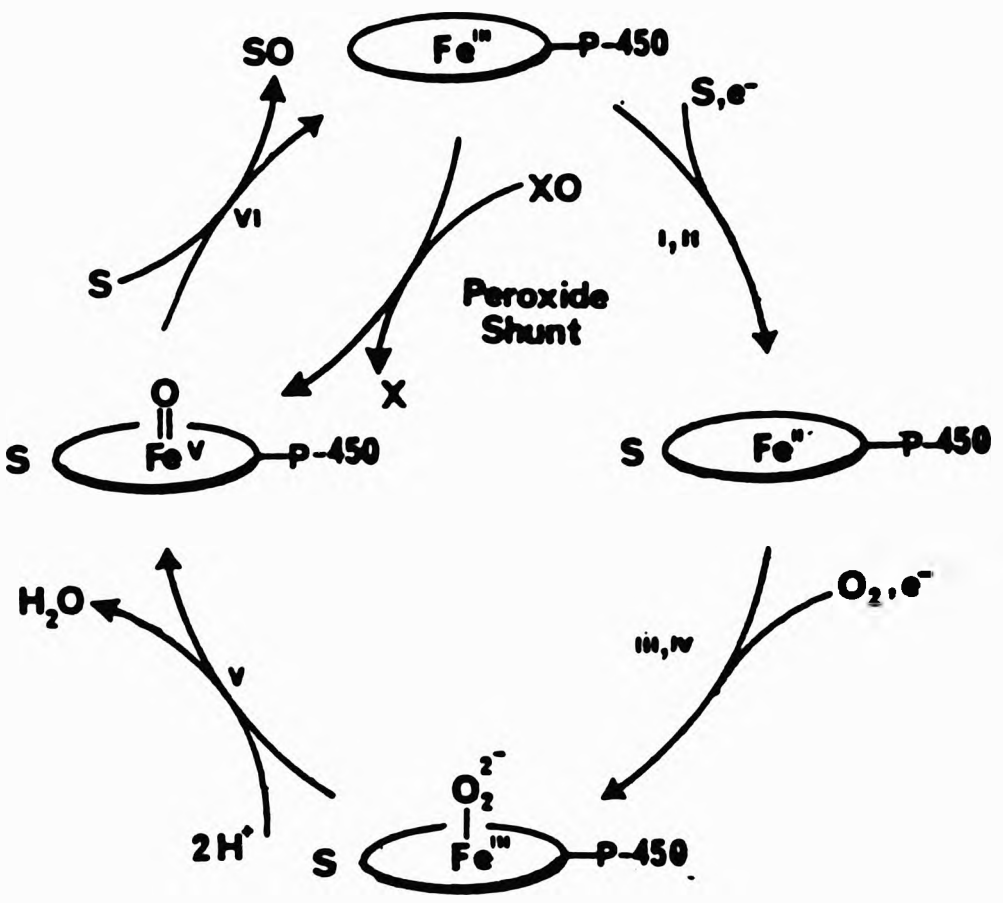
V. Generation of $[FeO]^{3+}$ and a molecule of water after heterolysis of the O-O bond.

VI. Production of SO and regeneration of the ferric state of the enzyme after a two-electron oxidation of the substrate.

Abbreviations:

S= substrate.

Fig. 1.1.


SO
Fe^III—P-450
S,e⁻
XO
VI
I, II
S
Peroxide
Shunt
X
O
S
Fe^V—P-450
S
Fe^II—P-450
H₂O
V
III, IV
O₂, e⁻
O₂²⁻
2H⁺
S
Fe^III—P-450

substrate specificity: CYP1A (alternatively known as cytochrome P448) and CYP2B. These two groups of isozymes can be categorised by their inducibility either by 3-methylcholanthrene in the case of the former or phenobarbital in the case of the latter group. The two groups of enzymes can also be distinguished by the structure of their substrates. Substrates of CYP1A have been categorised as thin, planar molecules, whereas CYP2B substrates have been described as ellipsoid and globular. Generally substrates of cytochromes CYP2B contain aliphatic groups; those of CYP1A contain aromatic rings. Another major difference between the two categories of haemoprotein is the position of amino acid residues at the active site of the molecule. Phenylalanine residues in CYP1A lie three residues apart, whereas in CYP2B they lie adjacent to each other (Lewis *et al.*, 1987). For the purposes of this document, however, both groups of enzymes will generally be referred to as cytochromes P450.

Cytochromes P450 have been detected and characterised in mammalian liver microsomal fractions (Omura and Sato, 1964). These authors also reported the presence of a species showing absorption at 420 nm in reduced carbon monoxide difference spectra. This haemoprotein has been designated cytochrome P420 and is the soluble form of cytochrome P450 located in the cytosol.

Additionally cytochromes P450 have been found in a wide variety of other species, including microorganisms. In 1968 a soluble cytochrome P450 (CYP101) was discovered in *Pseudomonas putida* strain c1. This

enzyme is specific in the hydroxylation of camphor (Katagiri *et al.*, 1968). Since then cytochrome P450 has also been found in *Bacillus megaterium,* where it is involved in the hydroxylation of steroids and fatty acids, and *Corynebacterium* species, where it has a role in the oxidation of *n*-octane (Cardini and Jurtshuk, 1968). However, the cytochromes P450 detectable in bacteria have shown narrow substrate specificity (Sariaslani, 1991). The cytochrome P450 system possessed by prokaryotes is termed Type II and is also present in the mitochondria of eukaryotes. Type II systems utilise two proteins in electron transfer to cytochrome P450. The first is a reductase containing an FAD prosthetic group, the second an iron-sulphur protein, ferredoxin (fig. 1.2). This arrangement differs from the Type I cytochrome P450 system located in the endoplasmic reticulum of eukaryotic cells. In this case electron transfer occurs via NADPH-cytochrome P450 reductase which contains both FAD and FMN as prosthetic groups (fig. 1.2). This allows the sequential transfer of electrons to FAD and then from FAD to FMN and subsequently to cytochrome P450 which accepts a single electron at a time (Ortiz de Montellano, 1986). In general the haemoprotein is not prevalent among bacteria, however some eukaryotic organisms, such as helminths are thought not to possess cytochrome P450 (Precious and Barrett, 1989).

1.3 Bioassays of genotoxicity

Animals have been traditionally used for testing potential carcinogens. However such studies tend to be both time consuming and costly to complete. Increasing pressure against animal experimentation has

Fig. 1.2.

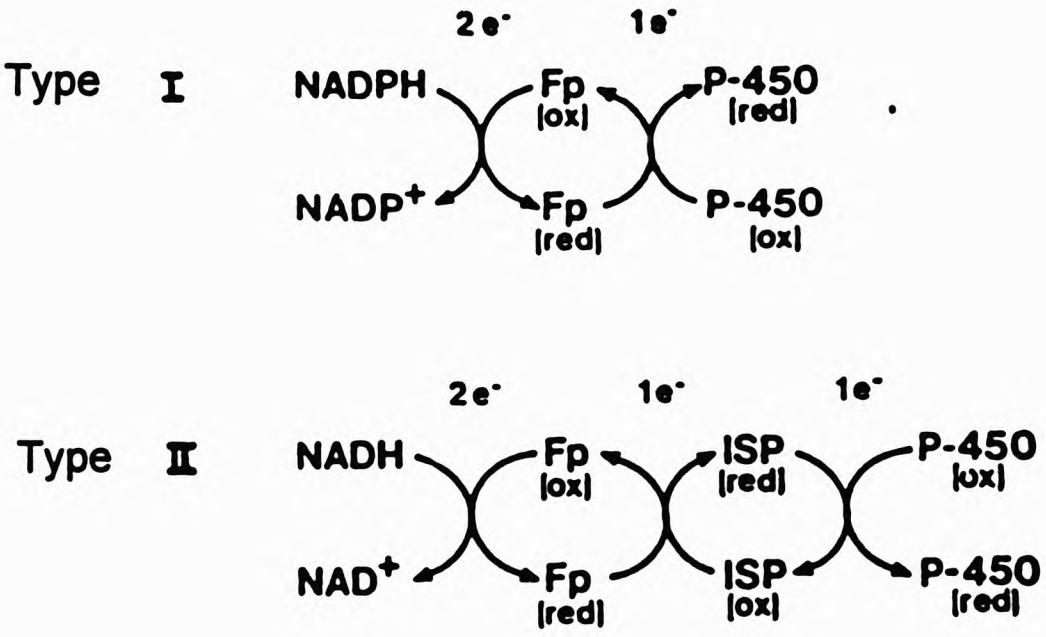
Electron transport to cytochrome P450 in Type I and Type II systems (from Ortiz de Montellano, 1986).

Abbreviations:

Fp : flavoprotein

ISP: iron-sulphur protein.

Fig. 1.2.


Type I
2e⁻
1e⁻
NADPH
Fp
[ox]
P-450
[red]
NADP+
Fp
[red]
P-450
[ox]
Type II
2e⁻
1e⁻
1e⁻
NADH
Fp
[ox]
ISP
[red]
P-450
[ox]
NAD+
Fp
[red]
ISP
[ox]
P-450
[red]

resulted in the decreased use of animal testing in general. For example in the cosmetics industry the use of the Draize eye test in 1990 was only 10% of its level in 1979 (Balls *et al.*, 1991). These facts together with the need for rapid and reproducible assays have encouraged the development of *in vitro* test systems. Some of these systems such as those using specific cell lines may be problematic due to dedifferentiation. Cultures of hepatocytes, for example, may lose cytochrome P450 enzymes in the *in vitro* environment. Genetic instability may also push cells towards a malignant state (Phillips, 1989).

Many short-term test systems have been designed using bacteria as indicator organisms. Such systems have confirmed that most carcinogenic compounds are also mutagenic (Ames *et al.*, 1975, Maron and Ames, 1983). The Ames test utilises as an end-point the reversion of histidine-requiring *Salmonella typhimurium* strains to prototrophy (Maron and Ames, 1983). Other bacterial systems include the fluctuation test, developed by Green *et al.* in 1976, which uses as an end-point reversion to prototrophy in auxotrophic mutants of *S. typhimurium* or *Escherichia coli* and the *Bacillus subtilis* rec-assay system (Kada *et al.*, 1972), which distinguishes mutagens by their cytotoxic effects on DNA repair deficient cells.

1.4 Disadvantages of short-term bacterial tests

Most *in vitro* test systems for mutagenicity which utilise bacteria require mammalian hepatic post-mitochondrial fractions (S9 mix) as a source of monooxygenase activity for the metabolic activation of pro-carcinogens.

The S9 fraction is usually prepared from rats pretreated with the inducing agent Aroclor-1254. However this polychlorinated biphenyl mixture has been found to destroy certain forms of cytochrome P450 such as CYP2E1, the enzyme reputed to be responsible for the activation of halogenated hydrocarbons in human cell lines (Crespi, 1991). The hazardous nature of Aroclor-1254 has led to investigations of alternative inducing agents such as phenobarbitone/β-naphthoflavone (Elliott *et al.*, 1992).

The necessity of S9 fraction incorporation in bacterial test systems yields additional disadvantages. Firstly, the varying sources of metabolic activation may produce discrepancies between bioassays. Secondly, the balance between activating and deactivating enzymes may be incorrect. These variations may arise due to loss of cytosolic deactivating enzymes during preparation of the S9 fraction (Douglas *et al.*, 1988). Further problems may occur in connection with the proximity of the generated active metabolites to the target molecules. This is particularly critical when unstable metabolites are monitored (Douglas *et al.*, 1988). In bacterial tests, where such metabolites are usually generated outside the cell, the permeability of the cell membrane to the chemical species must also be considered (Callen *et al.*, 1980).

It may in part be due to the above factors that some known human carcinogens e.g. arsenic salts, benzene and oestradiol 17-B, cannot be detected as mutagens in bacterial test systems. Furthermore many compounds classified as tumour promoting agents such as carbon

tetrachloride (tetrachloromethane, CCl_4) have also been found to be non-mutagenic in these tests (Mohn, 1981). The inability of *Salmonella* to detect some classes of potential mutagens has been highlighted. In particular the chlorinated hydrocarbons may yield low numbers of mutants in the *Salmonella* assay (Haroun and Ames, 1981).

1.5 Yeast test systems

Yeast test systems share many of the advantages of short-term bacterial mutagenicity tests. These include the fact that i) they are economical, ii) a wide range of chemicals can be screened in a relatively short time, and iii) accurate measurements may be made of exposure levels of the test chemical. The structure of yeast cells, which is similar to that of the higher eukaryotes, however, makes them more desirable organisms to use in *in vitro* tests than the morphologically simpler bacteria. In yeasts the target sites of toxic and genotoxic species are more likely to be pinpointed. Both nuclear and mitochondrial mutations may be monitored and, unlike bacterial systems, chromosome aberrations and changes in chromosome number may be investigated. The versatility of yeast cells is illustrated by the range of genetic endpoints which may be monitored such as mitotic recombination and mitotic and meiotic aneuploidy (Zimmerman, 1975).

1.6 Cytochrome P450 in yeast

The cytochrome P450 system is widely distributed among yeasts and filamentous fungi where, as in other eukaryotic cells, it has a microsomal location. This property makes yeasts desirable models for testing

putative toxic or mutagenic compounds as it negates the need for exogenous monooxygenase activity. Furthermore, experiments by Kelly and Parry (1983) have shown that yeast cells have the capability of metabolising substrates of both CYP2B and CYP1A.

The production of cytochrome P450 in yeasts has been well documented (Kappeli, 1986). Cytochromes P450 were first detected in the yeast *Saccharomyces cerevisiae* by Lindenmayer and Smith (1964). In normal endogenous metabolism they are involved in the catalysis of the 14 α-demethylation of lanosterol to ergosterol, the major sterol in yeast membranes (Aoyama *et al.*, 1984). The enzyme CYP51 is required for the first step in this biosynthetic pathway. The haemoprotein is readily inhibited by azole derivatives. Such interactions produce type II binding spectra and also result in low levels of cellular ergosterol and the formation of 14α-methylsterols which lead ultimately to impaired membrane function (Vanden Bossche, 1985). Type II binding spectra are generally exhibited by inhibitors of cytochrome P450. These compounds bind to the haem of the haemoprotein as opposed to substrates of cytochrome P450 which bind to the active site of the molecule generating type I binding spectra (Jefcoate, 1979).

Extensive research has been carried out on cytochrome P450 in the budding yeast *S. cerevisiae* (Wiseman and King, 1982). However, comparatively little work has been carried out on the haemoprotein in the fission yeast *Schizosaccharomyces pombe* (Bligh and Kelly, 1988). Although the cytochrome P450 system of some species of *Candida* have

been studied (Hitchcock *et al.*, 1989a), no documentation exists on cytochrome P450 in *Candida parapsilosis*.

Previous work on the genus *Candida* has shown these yeasts to be hydrocarbon-degrading. Hydroxylation of alkane substrates is via the cytochrome P450-dependent monooxygenase system (Wiseman and Woods, 1977), the cytochrome P450 content being induced by the substrate and linearly related to its uptake (Gmunder *et al.*, 1981). Some reports have suggested that cytochrome P450 has only been detected in *Candida* in the presence of the hydrocarbon substrate (Lebeault *et al.*, 1971). However, Sanglard *et al.* (1984) reported that in *C. tropicalis* cells, different monooxygenases were detected according to the carbon source employed. CYP2B were reputed to be present following growth in media enriched with *n*-alkanes, whereas CYP1A could be detected when the cells were grown in media enriched with glucose.

1.7 *Petite* mutants

Increased yields of the haemoprotein have been observed in cells of *S. cerevisiae* grown under conditions of glucose repression (Woods and Wiseman, 1980), or with cells grown in the presence of inhibitors of mitochondrial protein synthesis (Wiseman and King, 1982). However there appear to have been few previous studies of the levels of cytochrome P450 in *petite* mutants.

Petite and respiratory deficient mutants (ρ^- or rho^-) fail to grow on non-fermentable carbon sources such as glycerol or lactate. These

substrates must be respired aerobically and thus can only be utilised by cells with fully functional mitochondria. All *petite* mutants form small colonies on glucose media but they may vary in the amount of mitochondrial DNA they possess. The frequency of spontaneously occuring *petites* (approximately 1-2%) may be increased by exposure to various physical and chemical agents including chemical mutagens. Some treatments may eliminate all detectable mitochondrial DNA, thereby generating 'ρ^0' *petites* (Nagley and Linnane, 1972).

Petite mutants may only be induced in some yeast species. Yeast species have been classified as '*petite*-positive' or '*petite*-negative' (Bulder, 1964) according to whether respiratory deficient mutants could be isolated by acriflavine treatment. *S. cerevisiae* has been found to belong to the former group while *Sch. pombe* and *C. parapsilosis* belong to the latter (Evans, 1983). Nuclear or segregational *petites*, which were first discovered by Sherman (1963) have, however, been isolated in *Sch. pombe* (Heslot *et al.*, 1970). These latter mutants have been found to show Mendelian inheritance. A certain category of *petites*, however, when crossed with wild type cells produce a higher number of diploid *petites* than that arising from crossing the p^+ parent of the *petite* and the wild type (Ephrussi *et al.*, 1955). This characteristic known as suppressivity is thought to arise from the possession of a replicative advantage due to the presence of *ori/rep* sequences (Evans, 1983). Other *petites* categorised by the frequencies of their *petite* diploid progeny are neutral *petites*. In crosses with wild-type cells these mutants produce the same frequency of *petite* diploid progeny as

crosses with the p^+ parent.

Yeast species have been described as glucose-sensitive or glucose-insensitive according to whether the addition of glucose to the growth medium affects the respiration rate. Glucose-sensitive yeasts such as *S. cerevisiae* exhibit suppression of the respiration rate upon glucose addition, while glucose-insensitive yeasts are unaffected (De Deken, 1966; Fiechter *et al.*, 1981). The former category of yeasts are subject to the glucose and Pasteur effect. *Sch. pombe* however, has been described by some authors as glucose-sensitive (Fiechter *et al.*, 1981). An example of a glucose-insensitive yeast is *C. parapsilosis* which is *petite*-negative (Niimi *et al.*, 1988).

1.8 Yeast genotoxicity test systems

Although most yeast genotoxicity assays utilise *S. cerevisiae*, test systems have also been developed with *Sch. pombe*. As certain of the characteristics of *Sch. pombe* show greater similarity to mammalian cells than do those of the budding yeast, *Sch. pombe* is increasingly being used as a model system. An example is in cell cycle research as the *Sch. pombe* cell cycle is thought to be similar to that of higher eukaryotes (Nurse, 1985). Furthermore *Sch. pombe* possesses only three linkage groups facilitating easier genetic analysis than in *S. cerevisiae*. Comparison of the repair pathways of *Sch. pombe* and *S. cerevisiae* show the former yeast to be more efficient (Phipps *et al.*, 1985). *Sch. pombe* has been utilised in a variety of short-term genotoxicity tests using both forward and reverse mutational systems to elucidate the

effects of acute exposure to chemicals (Loprieno *et al.*, 1983). A widely used test system monitors mutations in the adenine biosynthetic pathway (table 1.1). Forward mutation in red *ade6* mutants results in double adenine-requiring mutants which are white in colour and have acquired a further mutation at the *ade1*, *ade3*, *ade4*, *ade5* or *ade9* locus (Loprieno *et al.*, 1983). In addition white prototrophic *ade6*$^{+}$ revertants may be monitored. *Sch. pombe* has also been found to be suitable for monitoring chronic chemical exposure using forward mutation to antibiotic resistance as an end-point (McAthey and Patel, 1987).

1.9 Carbon tetrachloride

Carbon tetrachloride is a colourless non-flammable liquid, solidifying at -23 °C , with a boiling point of 76.7 °C. The compound is miscible with compounds such as alcohol, benzene, chloroform and ether. Carbon tetrachloride is extensively used as a solvent for oils, fats and waxes and as a dry cleaning agent. It is also used in the manufacture of freon, in agriculture for the destruction of pests such as grain weevils and in medicine as an antihelmintic agent. The United States demand for carbon tetrachloride in 1976 was 385,000 metric tons (Kirk and Othmer, 1978).

The compound has been cited as a water contaminant and has been found in effluent from industries such as paper production. Studies by the National Cancer Institute (U.S.A.) have established that carbon tetrachloride and some other chlorinated hydrocarbons are carcinogenic (Weisburger, 1977). The WHO International Agency for Research on

Abbreviations used: PRPP, 5-phosphoribosyl-1-pyrophosphate. PRA, phosphoribosylamine. GAR, phosphoribosyl-glycineamide. FGAR, phosphoribosyl-formylglycineamide. FGAM, phosphoribosyl-formylglycineamidine. AIR, phosphoribosylaminoimidazole. CAIR, phosphoribosyl-aminoimidazolecarboxylate. SAICAR, phosphoribosyl-aminoimidazole succinocarboxamide. AICAR, phosphoribosyl-aminoimidazole carboxamide. FAICAR, phosphoribosyl-formamido-imidazole carboxamide. SAMP, adenylosuccinate.

[a] Pigment forming intermediates.

(From Fluri *et al.*, 1976).

Table 1.1 The adenine biosynthetic pathway in *Sch. pombe*

Pathway	Gene	Gene Product
PRPP		
↓	*ade4*	PRPP amidotransferase
PRA		
↓	*ade1A*	GAR synthetase
GAR		
↓	*ade5*	GAR formyltransferase
FGAR		
↓	*ade3*	FGAR amidotransferase
FGAM		
↓	*ade1B*	AIR synthetase
AIR[a]		
↓	*ade6*	AIR carboxylase
CAIR[a]		
↓	*ade7*	SAICAR synthetase
SAICAR		
↓	*ade8*	Adenylosuccinate lyase
AICAR		
↓	*ade10*	AICAR formyltransferase
FAICAR		
↓	*ade10*	IMP cyclohydrolase
IMP		
↓	*ade2*	SAMP synthetase
SAMP		
↓	*ade8*	Adenylosuccinate lyase
AMP		

Cancer (IARC) has also classified carbon tetrachloride as a "Ia" compound exhibiting carcinogenic properties. The IARC (1979) found carbon tetrachloride to be hepatocarcinogenic in rats and mice. It has also been postulated that this compound may act as a tumour promoter (Mohn, 1981).

In some studies the carcinogenicity of the halogenated aliphatic hydrocarbons has been said to be related to their metabolic activation by the microsomal monooxygenase system (Callen *et al.*, 1980). In the case of certain halogenated alkanes the production of free-radical species has been implicated in their carcinogenicity during metabolism (Bonse and Henschler, 1976).

Data on the mutagenic potential of carbon tetrachloride has been found to be inconclusive (IARC, 1972-1980). This data was collected from DNA damage and gene mutation assays in a variety of biological systems. Several halogenoalkanes tested in *in vitro* systems such as the Ames test have been shown to be mutagenic (Brem *et al.*, 1974). However tests with carbon tetrachloride have given negative results (McCann *et al.*, 1975). Other submammalian test systems have also been found to yield negative results for the mutagenicity of carbon tetrachloride (Heddle, 1982). However, work carried out by Callen *et al.*, (1980) has suggested that the compound is mutagenic in *S. cerevisiae*.

Carbon tetrachloride has been found to be toxic to mammalian cells and to cause extensive liver injury in mammals (Slater, 1978). The toxic

effects of the compound, however, have not been widely investigated in yeasts. Consequently it is desirable carry out further work in this area.

1.10 Aim of current work

The aim of this work is to evaluate the potential toxicity and genotoxicity of carbon tetrachloride utilising yeasts as a test system. Studies were also carried out to maximise the efficiency of this test system. The main objectives of this study were:

i) The analysis of cytochromes P450 in strains of *S. cerevisiae, Sch. pombe* and *C. parapsilosis* and the maximisation of endogenous levels of this haemoprotein by manipulation of environmental conditions. Analyses were carried out of cytochromes P450 production in respiratory deficient mutants of the yeast *S. cerevisiae* and a comparison made with wild type strains of the same species.

ii) The evaluation of the toxicity of carbon tetrachloride using *Sch. pombe* and *S. cerevisiae* cells and preliminary attempts to establish the mechanism of toxicity.

iii) The evaluation of the genotoxicity of carbon tetrachloride using *Sch. pombe*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

All chemicals (AnalaR grade) were purchased from Sigma Chemical Co unless otherwise stated.

2.2 Yeast strains

Schizosaccharomyces pombe strains used were haploid and were: the wild type *Sch. pombe* 972 h^- (NCYC 1827) and the following auxotrophs *Sch. pombe ade6*-704 h^- (NCYC 1859), *Sch. pombe his*1-102 h^- (NCYC 1896), *Sch. pombe lys*1-131 h^- (NCYC 1921), *Sch. pombe lys*1-131 h^+ (NCYC 1922), *Sch. pombe leu*2-120 *lys*3-37 h^- (NCYC 2136) and *Sch. pombe leu*1-32 *pho*1-44 h^-. The latter was donated by Dr. S.L. Kelly, (Sheffield University).

Saccharomyces cerevisiae strains used were haploid except for *S. cerevisiae* 188 (diploid) and were the following: the wild types *S. cerevisiae* α-159 (NCYC 858) and *S. cerevisiae* 188 (Philip Harris Scientific). *Petite* strains used were *S. cerevisiae* NCYC 673 (generated from bakers' yeast by X-ray irradiation) and *S. cerevisiae* 188 *petite* isolate no. 4. This latter strain was generated from *S. cerevisiae* 188 by treatment of 0.1 ml of a growing culture with 200 μg ethidium bromide for 24 h at 30°C. Cells were harvested, grown for a further 48 h and plated out on Magdala red agar (Philip Harris Scientific). Small, dark red colonies were picked and replated on YEPG and YEPD agar (see 2.3.1) to confirm the *petite* phenotype. The auxotrophic *S. cerevisiae*

strain used was *S. cerevisiae ade2* (strain collection, University of North London).

Candida parapsilosis, a laboratory isolate, was identified by API-bioMerieux (UK) Ltd., Basingstoke, Hants.

2.2.1 Strain maintenance

Strains were stored on silica gel for long-term maintenance (Gutz *et al.*, 1974). For short-term storage, strains were subcultured at three monthly intervals on appropriate solid media slopes or at two weekly intervals on appropriate plates and stored at 4°C. Cells were routinely grown at 30°C.

2.3 Microbiological media

2.3.1 Media for growth and maintenance

S. cerevisiae and *C. parapsilosis* strains were routinely grown in yeast extract peptone dextrose (YEPD) liquid containing 1 % (wt/vol) yeast extract, 2 % (wt/vol) peptone (Oxoid Ltd.) and 2 or 20% (wt/vol) D-glucose. *Sch. pombe* strains were grown either in YEPD or yeast extract liquid (YEL) containing 1 % (wt/vol) yeast extract and 2 or 20% (wt/vol) D-glucose. These media were solidified using 2% (wt/vol) agar (Difco) to give yeast extract peptone dextrose agar and yeast extract agar (YEA) respectively. A complex medium containing a non fermentable carbon source (glycerol) was made up using YEA with 2 % (vol/vol) glycerol (YEPG). For the isolation of chloramphenicol resistant mutants, YEPG plates were supplemented with 1 g l^{-1} chloramphenicol (CAP) for *Sch. pombe* (McAthey and Patel, 1987) and 4 g l^{-1} CAP for *S. cerevisiae*

(Pinto *et al*, 1982).

Edinburgh minimal medium number 2 (EMM2), (see table 2.1), was used for experiments with auxotrophic strains and supplemented with 50 mg l^{-1} of the appropriate amino acid when necessary (Gutz *et al.*, 1974). EMM2 was prepared according to Mitchison (1970) and solidified using 2% (wt/vol) agar (Difco) when appropriate (see table 2.1). Malt extract agar was used as sporulation medium and contained 50 g l^{-1} malt extract agar powder (Oxoid Ltd).

2.3.2 Microbiological buffers

For toxicity and mutagenicity assessments $^1/_4$ strength Ringers solution (Oxoid) was used for *S. cerevisiae*. Giese's salts vitamins buffer (GSVB), modified as described by Clarke (1963), was used for *Sch. pombe* strains, its composition was as shown in table 2.2. For cytochrome P450 work in *Sch. pombe*, *S. cerevisiae* and *C. parapsilosis* buffers were used as detailed in 2.5.

2.3.3 Sterilisation of media and chemicals

Sterilisation of all growth media and buffers was carried out at 121°C, 103.5 KPa for 15 min. Antibiotics and test chemicals used in toxicity and genotoxicity assays were dissolved in 99% (vol/vol) ethanol before incorporation into sterile media.

2.4 Growth curve construction

Strains were grown in complete medium containing 20% (wt/vol) D-

Table 2.1

Composition of Edinburgh minimal medium Number 2 (pH 5.5)

modified from Mitchison (1970)

carbon source:	
glucose	5 g
nitrogen source:	
NH_4Cl	5 g
phosphate source:	
NaH_2PO_4	300 mg
salts:	
CH_3COONa	1 g
KCl	1 g
$MgCl_2$	500 mg
Na_2SO_4	10 mg
$CaCl_2$	10 mg
vitamins	
Inositol	10 mg
Nicotinic acid`	10 mg
Calcium pantothenate	1 mg
Biotin	10 mg
trace elements	
H_3BO_3	500 mg
$MnSO_4.H_2O$	400 mg
$ZnSO_4.7H_2O$	400 mg
$FeCl_3.6H_2O$	200 mg
$H_2MoO_4.H_2O$	160 mg
KI	100 mg
$CuSO_4.5H_2O$	40 mg
Citric acid	1 mg
distilled water (to final volume)	1 litre

Salts, vitamins and trace elements were stored as concentrated stock solutions at 4°C until use, (salts 50 x, and vitamins and trace elements 100 x working strength).

The following supplements were added as required per litre (Gutz *et al.*, 1974) :

adenine	50 mg
lysine	50 mg
leucine	50 mg

Table 2.2

Giese's Salts Vitamin Buffer (GSVB)

(modified as described by Clarke, 1963)

Salts	
$(NH_4)_2SO_4$	3 g
$MgSO_4 . 6H_2O$	700 mg
NaCl	500 mg
$Ca(NO_3)_2$	400 mg
KH_2PO_4	6.8 g
Vitamins	
Inositol	10 mg
Nicotinic acid	10 mg
Calcium pantothenate	1 mg
Biotin	10 mg
Distilled water (to final volume)	1 litre

Stock solutions of salts (10 x) and vitamins (100 x working strength) were stored at 4°C until use.

glucose. For the auxotrophic mutant *Sch. pombe ade6 h⁻* this medium was supplemented with 50 mg l^{-1} adenine (Gutz *et al.*, 1974). Growth was at 30°C without shaking. Time zero was taken as the time of inoculation into the growth medium (100 ml in 250 ml conical flasks). The growth of the organisms was followed by measuring absorbance (A) at 540 nm using a CE 292 UV spectrophotometer.

2.5 Methods of spectral analysis

Precultures of yeast strains were routinely grown at 30°C in 10ml YEPD or YEL containing 2 % glucose (wt/vol) until late logarithmic phase and were inoculated into 95 ml YEPD containing 2 or 20% (wt/vol) D-glucose or 20% (wt/vol) sodium acetate for cytochrome P450 determinations and 2% (wt/vol) D-glucose for mitochondrial cytochrome determinations. The cultures were then grown at 30°C to late logarithmic phase. Cultures grown with aeration were shaken at 120 rpm. The cells were harvested by centrifugation (MSE Europa 24 with swingout rotor) at 1400 x *g* for 15 minutes, washed twice in ice-cold 0.1 M- phosphate buffer (pH 7.2) containing 20% (vol/vol) glycerol and 1 mM- ethylenediaminetetraacetic acid (EDTA) as described by Bligh and Kelly (1988) and resuspended to one-tenth of the original volume. Dry weights were determined by heating 1 ml of resuspended whole cells at 95°C.

Samples were scanned for the presence of cytochrome P450 in the 400-500 nm region using a Philips PU 8740 UV/VIS spectrophotometer. Difference spectra were recorded between cells reduced with 20 mg sodium dithionite and the same sample after the cuvette had been

sparged with carbon monoxide for 30 s. Cytochrome P450 levels were determined using an extinction coefficient of 91 mM^{-1} cm^{-1} by the method of Omura and Sato (1964). Cytochrome P420 levels were determined using an extinction coefficient of 111 mM^{-1} cm^{-1} (Azari and Wiseman, 1980). Samples were scanned between 500 and 630 nm for the presence of mitochondrial cytochromes. Difference spectra were recorded between oxidised cells after the addition of 20 µl hydrogen peroxide (30 % vol/vol) to the cuvette and reduced cells after the addition of 20 mg sodium dithionite using the method modified from Williams (1964).

Binding spectra were elucidated using a Perkin-Elmer lambda 5 UV/VIS double-beam spectrophotometer. Carbon tetrachloride was added in 0.1 ml ethanolic solution to the test cuvette and 0.1 ml 99% (vol/vol) ethanol to the reference cuvette before scanning between 350 and 500 nm. Baselines were taken before the addition of carbon tetrachloride and scanning between 350 and 500 nm was carried out after its addition (Jefcoate, 1978).

Sample calculation of cytochrome P450

Using the Lambert-Beer law the cytochrome P450 content of the cells was calculated as in the following example:

$$A = \varepsilon c l$$

A = absorbance

ε = extinction coefficient

c = concentration of absorbing species

l = length of cell

$$\varepsilon = \frac{A}{c \times l}$$

Absorbance difference from reduced CO spectra = 0.010 (typical value)

Extinction coefficient = 91 mM^{-1} cm^{-1}

Cuvette path length = 1 cm

Dry cell weight of 1 ml of sample = 34.9 mg

The cytochrome P450 concentration is given by

$$\frac{0.010 \times 1000}{91} = 0.1099 \text{ nmol } ml^{-1} \text{ sample.}$$

$$\frac{0.1099}{34.9} = 0.00315 \text{ nmol } mg^{-1} \text{ dry cell weight of culture.}$$

Therefore specific content of cytochrome P450 = 3.15 nmol g^{-1} dry cell weight of culture.

2.6 Methods for partial purification of cytochrome P450

2.6.1 Preparation of yeast microsomes

For the preparation of *Sch. pombe* and *S. cerevisiae* microsomes cells from eight 100 ml cultures were prepared as described in section 2.5. For the preparation of *C. parapsilosis* microsomes a batch culture of 9 litres was grown in YEPD containing 20% (wt/vol) glucose with aeration at 30°C by inoculating an exponentially growing 1 litre culture into 8 litres of culture medium. Whole cells of each strain were resuspended to one-tenth of their original volume in 0.25 M- mannitol (pH 7.4) containing 0.025 M- morpholinopropanesulphonic acid (MOPS) and 0.02 mM- triethylenetetraminehydrochloride (TET). Homogenisation was carried out in a Braun homogeniser MSK (B. Braun, Melsungen AG, Melsungen, Germany) for 3 x 30s bursts with 15s intermittent cooling using liquid carbon dioxide. Cell debris was removed by centrifugation at 1900 x *g*. Microsomes were precipitated by the method of Albro *et al.* (1987) diluting the supernatant with 4 volumes of 0.0125 M- mannitol (pH 7.5) containing 0.1 mM- TET and 8 mM- calcium chloride, stirring with a glass rod for 30 s and centrifuging for 10 minutes at 1000 x *g*. A second method attempted was as described by Wright and Honek (1989). Microsomes in this instance were precipitated by the addition of 7.5 % (wt/vol) polyethylene glycol (mol wt 8,000) to the cell-free extract and centrifugation at 40,000 x *g* for 20 minutes.

2.6.2 Solubilisation of cytochrome P450 from yeast microsomes

To allow extraction of solubilised cytochrome P450 using the method of Wright and Honek (1989), a 10 ml sample of *C. parapsilosis* microsomes

was stirred at 4°C for 1 h with 25 ml of medium. The composition of the medium was as follows: 100 mM- KH_2PO_4, 1 mM- dithiothreitol (DTT), 1 mM- EDTA, 200 mM- phenylmethylsulfonyl fluoride (PMSF), 1 % sodium cholate (wt/vol), 20 % glycerol (vol/vol), pH 7.5. The suspension was then centrifuged at 100,000 x *g* for 1 h. Both the supernatant fraction containing the cytochrome P450 and the pelleted fraction were subjected to spectral analyses.

2.6.3 Protein estimation

Protein estimations were performed on yeast cell-free fractions using the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as the standard.

2.6.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell extracts

The apparent molecular mass of tentatively identified cytochrome P450 in cell extracts of *C. parapsilosis* was estimated using SDS-PAGE [10% (wt/vol) acrylamide slab gels] using the method of Laemmli (1970). A series of standards with known molecular weights were electrophoresed concurrently with the unknown samples.

Composition of stock solutions

Acrylamide/bis acrylamide:

30% (wt/vol) acrylamide, 0.8% (wt/vol) NN^1 bis acrylamide.

Sample buffer:

0.78 ml of 1.875 M Tris buffer, pH 8.8, 0.8 g SDS, 0.4 g DTT, 0.2 ml 0.5% (wt/vol) bromophenol blue, 0.8 g sucrose in 7.02 ml distilled water.

Stacking buffer:

726.8 mg Tris base in 1 litre distilled water, pH 6.8.

Gel buffer:

1.875 M Tris buffer, pH 8.8.

Tank buffer (5x strength):

30.3 g Tris base, 144.15 g glycine and 5 g SDS were dissolved in 1 litre distilled water, pH 8.3.

AMPS:

10% (wt/vol) ammonium persulphate was prepared daily and used fresh.

Gel casting

The running gel was composed as follows:

Stock solutions	Volume of additions (ml)
gel buffer	13.2
acrylamide/bis acrylamide	8.0
distilled water	18.0
SDS (10% wt/vol)	0.4
TEMED*	0.04
AMPS (added last)	0.2

*N,N,N',N' tetramethylethylenediamine

After polymerisation the stacking gel (5% [wt/vol] acrylamide) was overlaid on the running gel and was composed as follows:

Stock solutions	volume of additions (ml)
stacking buffer	2.0
acrylamide/bis acrylamide	3.4
distilled water	14.4
SDS	0.2
TEMED	0.015
AMPS (added last)	0.1

Sample preparation

Proteins were dissolved in sample buffer. The molecular weight standards and unknown samples were each mixed with sample buffer and incubated at 100°C for 2 minutes. Samples were then cooled and mixed before pipetting into the wells. The protein standards used were as follows: carbonic anhydrase from bovine erythrocytes, molecular mass 29 kDA; ovalbumin, molecular mass 45 kDa; bovine plasma albumin, molecular mass 66 kDa; phosphorylase b from rabbit muscle, molecular mass 97.4 kDa; β-galactosidase from *Escherichia coli*, molecular mass 116 kDa.

Samples were loaded as follows:

Gel no. 1:standards, 150 μg protein; unknown samples, 50 μg protein.

Gel no. 2 :standards, 60 μg protein; unknown samples, 100 μg protein.

Running of gels

The cassette was placed in the electrophoresis tank and the gel cooled to 4°C. The tanks were filled with tank buffer. The appropriate protein sample was loaded into each well. A current of 40 mA was supplied until the leading boundary had reached 1 cm from the base of the cassette.

Staining of gels

Routinely protein was fixed in the gel with methanol/water/acetic acid (4:5:1, by vol.) for at least 1 h. Protein bands were visualised by staining overnight with the above solution (5:4:1, by vol.) containing 0.2% (wt/vol) Coomassie brilliant blue. Gels were subsequently destained with three washes of methanol/water/acetic acid (5:5:1, by vol.) containing 10% (vol/vol) glycerol for at least 1 h.

Calculation of molecular weights

The distance travelled by each band was measured from the loading site and the relative mobility of each band calculated as follows:

$$\text{relative mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}}$$

The molecular weights of the unknown samples were estimated from plots of $\log_{10}$ molecular weight versus relative mobility for the known protein standards.

2.7 Methods for toxicity and genotoxicity assays

2.7.1 Chemicals

Carbon tetrachloride (supplied by Rose chemicals Ltd., 83 Darenth Road, London N16) ethyl methane sulphonate (EMS) and α-tocopherol (vitamin E) were used in these assays. Stock solutions of these chemicals were prepared by dissolving in 99% (vol/vol) ethanol. EMS is a chemical mutagen and consequently appropriate safety precautions were taken. The chemical was stored in a 'poisons cabinet' at 25°C when not in use. Disposable gloves were used when handling the compound and apparatus and media in contact with EMS was neutralised with 10% (wt/vol) sodium thiosulphate for at least 24 h before cleaning or disposal.

2.7.2 Treatment conditions

Stock solutions of carbon tetrachloride, α-tocopherol and the positive control EMS were prepared by dissolving in 99% (vol/vol) ethanol to give a range of concentrations. The final concentration of ethanolic stock solutions used in the growth medium was 1% (vol/vol). The concentration of carbon tetrachloride was maintained as far as possible by the use of sealed containers. Solvent controls were set up by administering a final concentration of 1% (vol/vol) ethanol to the growth medium. Cultures were initiated from single colonies isolated from solid medium and cells were grown in 10ml YEL containing 20% (wt/vol) D-glucose for 18 h at 30°C without shaking in the presence of the test compounds.

2.7.3 Initial growth and toxicity measurements

To determine the toxic range of the chemical the absorbance of cultures

treated with different concentrations of carbon tetrachloride was measured using a CE404 colorimeter at 540 nm.

2.7.4 Isolation of mutants

Cells were harvested by centrifugation at 1400 x *g* in an MSE Centaur centrifuge. *Sch. pombe* cells were washed twice in GSVB whereas *S. cerevisiae* cells were washed twice with Ringers solution ($^1/_4$ strength). Both species were then resuspended in the appropriate buffer to the original volume, which was taken as the 10^o concentration. Aliquots of 0.1 ml of this concentration were used for the enumeration of prototrophic revertants on EMM2. The enumeration of 'double' adenine requiring mutants isolated from Sch. pombe *ade6* and adenine requiring auxotrophs isolated from *Sch. pombe* 972 was carried out by plating 0.1 ml aliquots of 10^o concentration on YEA plates and scoring on the basis of colony colour. Cells at the 10^o concentration were also used for the enumeration of antibiotic resistant mutants. Aliquots of 0.1 ml were plated on YEPG agar containing 1 g l^{-1} CAP for the isolation of *Sch. pombe* mutants and 4 g l^{-1} for the isolation of *S. cerevisiae* mutants and scored after 10 days incubation at 30°C.

Viable counts were taken by plating 0.1 ml aliquots of appropriate dilutions on unsupplemented complete media or EMM2 supplemented with the appropriate amino acid or adenine for auxotrophic mutants and scoring after 48 h incubation at 30°C.

Mutation frequencies were expressed as the number of mutants per 10^6

viable cells. Percentage survival was tabulated and plotted against carbon tetrachloride concentration to determine the toxic effects of the compound.

2.7.5 Evaluation of mutagenicity

The criteria used were according to Mehta and von Borstel (1985) as shown in table 2.3.

Table 2.3

Criteria for the determination of the mutagenic potential of test compounds

Criteria	+/-
2-fold increase in mutation frequency, increase in mutation yield and dose-dependent response	+
2-fold increase in mutation frequency without increase in mutation yield.	+
2-fold increase in mutation frequency and mutation yield without dose-dependent response	+
No increase in mutation frequency or mutation yield.	-

+ = positive response indicating mutagenic potential.

- = negative response indicating lack of mutagenic potential.

Strong evidence for the mutagenicity of a compound is shown by a minimum 2-fold increase in mutation frequency and an increase in mutation yield coupled with a dose-related response. Good evidence for mutagenicity of a compound is indicated by a minimum 2-fold increase in mutation frequency without an increase in mutation yield. This is especially applicable if a compound exhibits toxicity. A positive response is shown by compounds which do not show a dose-dependent response, but at least a 2-fold increase in mutation frequency and mutation yield is observed. A compound is regarded as negative if no increase in mutation frequency or mutation yield is observed.

2.7.6 Mutant analysis

White mutants isolated from experiments with *Sch. pombe ade6* were tested for their ability to grow on minimal media with and without adenine supplementation. Isolates retaining the inability to grow on minimal media were scored as 'double adenine' requiring mutants possessing an additional mutation at the *ade1*, *ade3*, *ade4*, *ade5* or *ade9* locus (Gutz *et al.*, 1974). Isolates which had gained the ability to grow on unsupplemented minimal media were scored as prototrophic.

Antibiotic resistant mutants were isolated, plated on complete media without the antibiotic and then replated on antibiotic containing media to confirm the stability of the isolates.

2.7.7 Genetic analysis

Crosses were performed according to Gutz *et al.* (1974).

The parental types crossed were as follows:

h^+ cap^S lys^- X h^- cap^R lys^+

Newly isolated cap^R h^- mutants and the known lysine auxotroph lys^- h^+ were grown on separate YEA slopes for 48 h at 30°C. Growth from the slopes was washed off with 4-5 ml of 0.85% (wt/vol) NaCl. Haploid cells of opposite mating type (h^+ and h^-) were encouraged to form diploid pairs by placement on the sporulation medium MEA. Equal volumes of saline suspensions of h^+ cap^S lys^- and h^- cap^R lys^+ cells were mixed and 0.2 ml aliquots of the mixture were transferred to the surface of MEA slopes. The slopes were incubated for at least 7 days at 25°C to allow meiosis, and the subsequent development and disruption of unordered asci. The released ascospores were then subjected to random spore analysis.

Growth from each MEA slope was washed off by adding 2.5 ml of 50% (vol/vol) ethanol and the suspension was then incubated at 18°C for 10 mins. This treatment allows selective killing of the vegetative cells. The suspension was subsequently diluted to 10^{-4} using GSVB. 0.1 ml aliquots of appropriate dilutions were plated on YEA and incubated for 48 h at 30°C. Cells from individual colonies, which had each developed from germinated ascospores, were tested for their ability to grow on selective media by aseptic transferance to i) EMM2 , ii) EMM2 containing

50 mg l^{-1} lysine, iii) YEPG containing 1 g l^{-1} CAP and iv) YEA. 180 Transfers were attempted per cross. The plates were incubated at 30°C for 24 h.

A segregation of 1:1 between the *lys*$^{+}$ and *lys*$^{-}$ progeny indicated that the cross had been successful. The proportion of antibiotic resistant progeny to antibiotic sensitive progeny could be calculated by comparing the colony count on the antibiotic supplemented plates with that on the YEA plates. A segregation of 1:1 indicated that the mutant phenotype was derived from nuclear mutation(s), while a segregation of 0:1 indicated that the mutant phenotype was derived from mitochondrial mutation(s).

2.8 Statistical analysis

Analysis of variance (ANOVA) was carried out using Minitab (IBM) on data from cytochrome P450 experiments. The least significant difference (LSD) was calculated and using this criterion significant differences between values could be determined. ANOVA was also applied to data from toxicity and genotoxicity experiments. The LSD was calculated and represented as an error bar on the graphed data. The difference between two given values when shown graphically or in tabulated form could be concluded as being significant if it was calculated to be greater than the LSD. Statistical analysis of data is shown in Appendix 1.

CHAPTER 3

ANALYSIS OF CYTOCHROME P450 PRODUCTION IN *SCH. POMBE, S. CEREVISIAE* AND *C.PARAPSILOSIS*

3.1 INTRODUCTION

To investigate the production of cytochrome P450 and determine conditions required to maximise yields, a number of yeast strains was screened for the presence of the haemoprotein including *Sch. pombe* 972, *ade6*, *leu*1-32 *pho*1-44 and *lys*1-131 and *Saccharomyces cerevisiae* 188, 159, 673 and *ade2*. The cells were analysed under varying conditions including differing carbon sources, length of culture growth and aeration. The cytochrome P450 content of respiratory deficient strains of the yeast *S. cerevisiae* was determined. *S. cerevisiae* 673 and a *petite* derivative of *S. cerevisiae* 188 were the mutants employed and comparisons were made with concentrations of the enzyme in the wild-type strains *S. cerevisiae* 159 and *S. cerevisiae* 188.

The evaluation of the cytochrome P450 content of whole cells is potentially subject to interference from other cytochromes such as aa3 which has an absorbance minimum at 441 to 445 nm. The true cytochrome P450 content of the cells may thus be disguised (Karenlampi and Hynninen, 1981). As the intracellular location of cytochrome P450 in eukaryotic cells has been reported to be microsomal (Ishidate *et al.*, 1969), these organelles were analysed for the haemoprotein. Measurements were attempted in *Sch. pombe*, *S. cerevisiae* and *C.*

parapsilosis. The fact that there is no literature precedent for cytochrome P450 analysis in *C. parapsilosis* prompted the latter investigation.

The complete purification of cytochrome P450 has rarely been reported in yeast. The protein has been isolated from *S. cerevisiae* by King *et al.* (1984) and Yoshida and Aoyama (1984). King *et al.* (1984) reported a molecular mass of 55 kDa, whereas Yoshida and Aoyama reported a mass of 58 kDa. Cytochrome P450 was also purified from *Lodderomyces elongisporus* (Riege *et al.*, 1981) and found to have a molecular mass of 53 kDa.

The sulphonylurea herbicide inducible cytochrome P450 enzymes CYP105A1 and CYP105B1 from *Streptomyces griseolus* were each found to have a molecular mass of about 46 kDa (O'Keefe *et al.*, 1988). A molecular mass of 45 kDa was reported in another bacterium *Pseudomonas putida* for CYP101 (Katagiri *et al.*, 1968).

3.2 RESULTS AND DISCUSSION

3.2.1 Time course of cytochrome P450 production

The growth of several strains of *Sch. pombe* and *S. cerevisiae* was measured in complete medium containing 20% (wt/vol) glucose at 30 °C. Representative growth curves showed that the cultures underwent logarithmic phase from approximately 3 to 24 h, (figs. 3.1a-c). Late logarithmic phase was observed to be approximately 18-24 h, with stationary phase approaching 24 h after inoculation. *Sch. pombe ade6,* an adenine requiring auxotroph, exhibited poor growth in complete medium (data not shown), however when 50 mg l^{-1} adenine was added to the medium the growth of the strain improved (fig. 3.1b). The measurement of the absorbance of *S. cerevisiae* 188 cultures was impaired by clumping or flocculation of the cells (data not shown).

The growth kinetics observed in these experiments were in broad agreement with those obtained with *S. cerevisiae* D6 and JD1 during growth in 20% (wt/vol) glucose (Kelly and Parry, 1983). In the latter study the cells also approached stationary phase after 24 h. The work of Bligh and Kelly (1988) showed the growth of *Sch. pombe leu*1-32 *pho*1-44 to have a similar profile under these conditions. The cells were found to decrease in growth rate after 16 h and reached stationary phase 27 h after inoculation.

Time course analysis of the production of cytochrome P450 was carried out in several strains. Spectral analysis of *Sch. pombe ade6* and 972 showed no detectable levels of the haemoprotein after 8 h growth.

Fig. 3.1.

Determination of the growth of a) *Sch. pombe* 972 , b) *Sch. pombe ade6* and c) *S. cerevisiae* 159. Cells were grown in YEPD containing 20% (wt/vol) glucose without aeration.

Fig. 3.1a

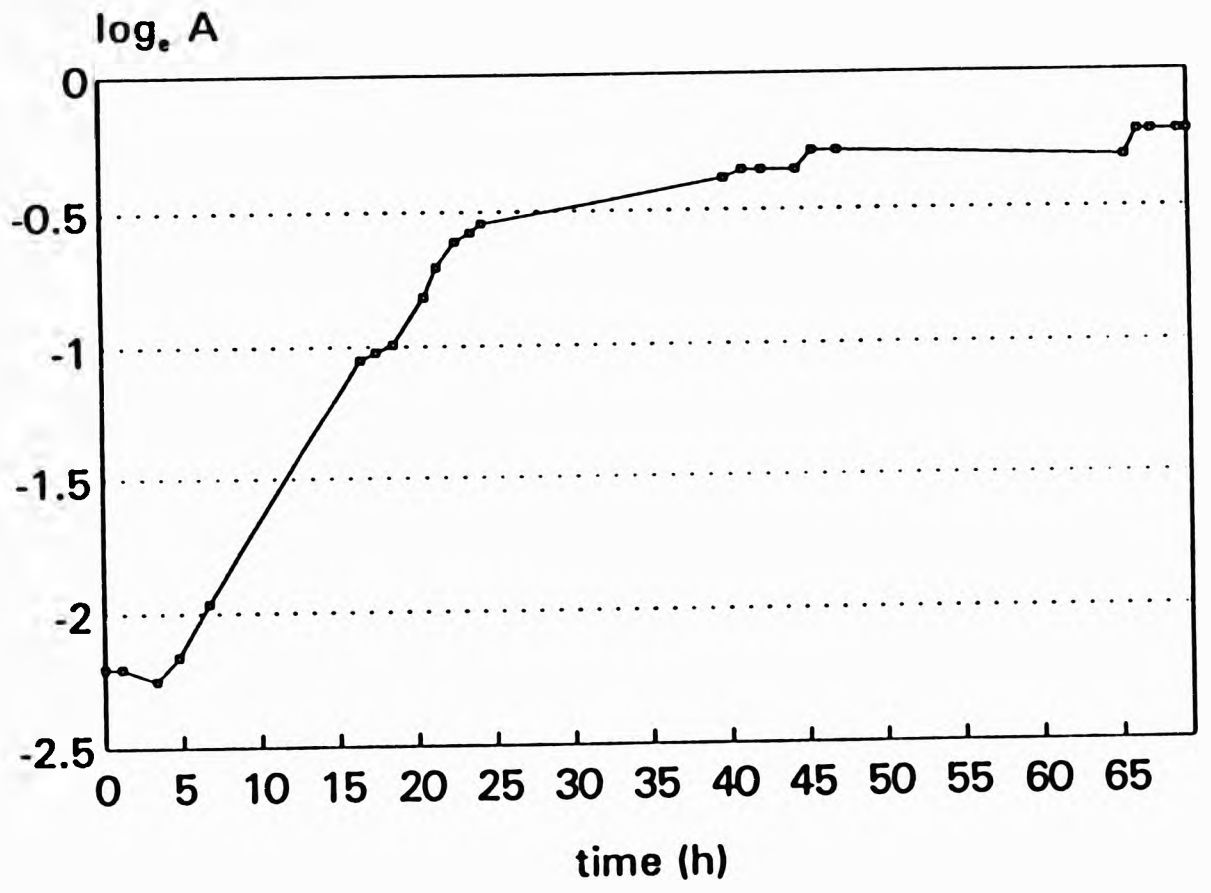
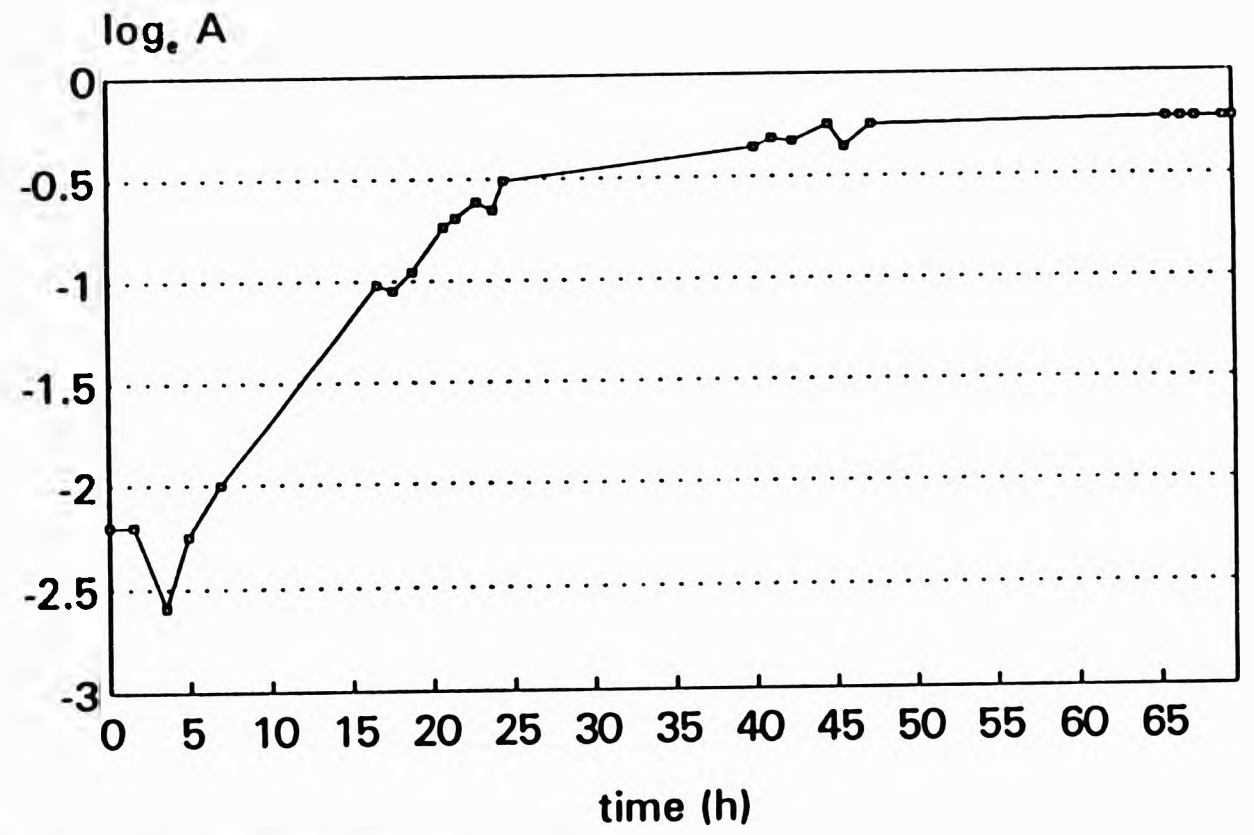
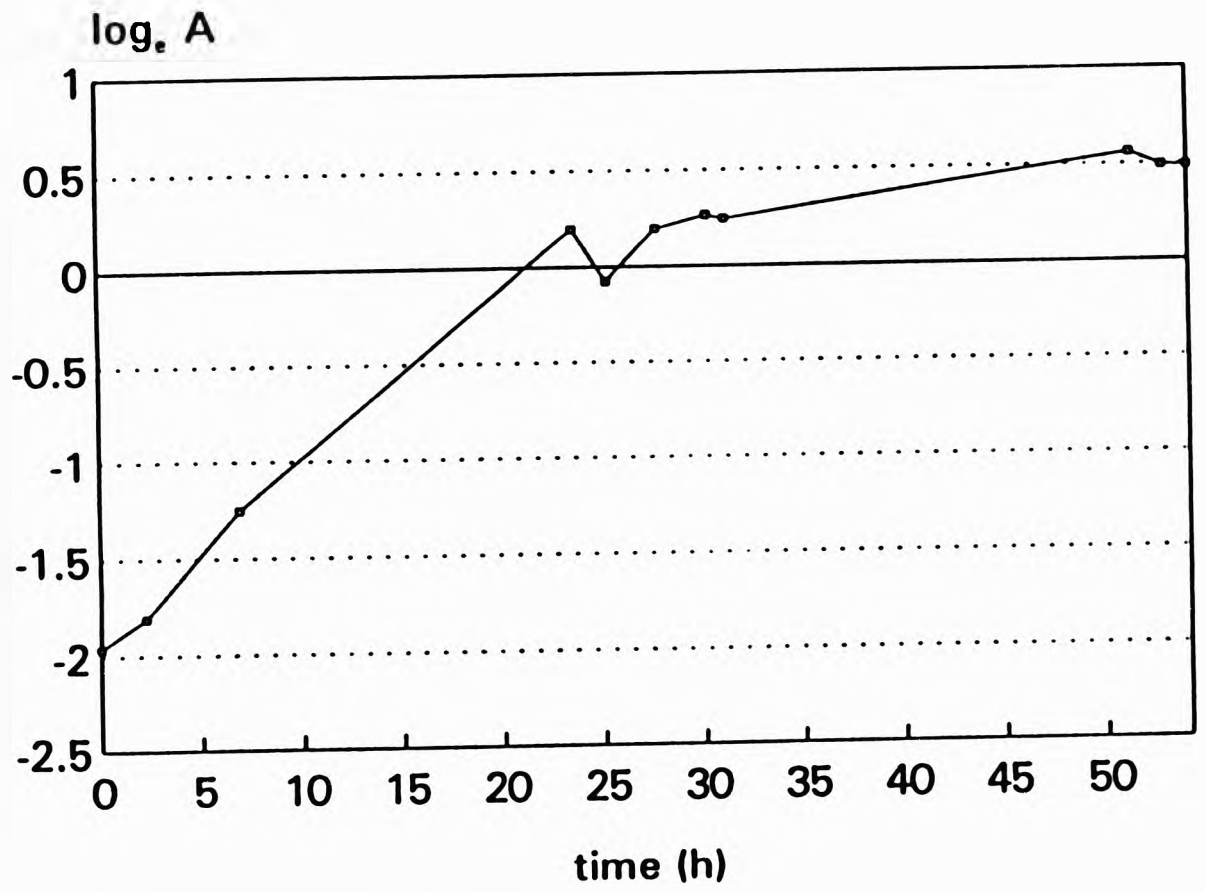


Fig. 3.1b



`Media supplemented with adenine

Fig. 3.1c



However a gradual increase in the production of cytochrome P450 was seen during the late logarithmic phase (fig. 3.2). Levels of the haemoprotein rose in *Sch. pombe ade6* to 0.82 nmol g^{-1} dry cell weight after 16 h of growth and reached a plateau after 17 h growth (table 3.1). A similar pattern of development of the haemoprotein was seen in *Sch. pombe* 972. Detectable levels of the enzyme were seen after 17 h growth giving 0.47 nmol cytochrome P450 g^{-1} dry cell weight.

Table 3.1

Time course of cytochrome P450 and P420 production in non-aerated cultures of *Sch. pombe* 972 and *ade6* grown in YEL containing 20% (wt/vol) glucose

strain	growth time (h)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
Sch. pombe ade6	8	0	0
Sch. pombe ade6	16	0.59	0.82
Sch. pombe ade6	17	0.63	1.03
Sch. pombe ade6	19	0.50	0.97
Sch. pombe 972	8	0	0
Sch. pombe 972	16	1.26	0
Sch. pombe 972	17	0.78	0.47
Sch. pombe 972	19	1.13	0.40

Data represents one determination for each time period. For the growth of *Sch. pombe ade6* YEL was supplemented with 50 mg l^{-1} adenine.

A value of 0.4 nmol cytochrome P450 g^{-1} dry cell weight was observed after 19 h growth, showing that in this strain also, of the time periods sampled maximum production of the enzyme was between 17 and 19 h

Fig. 3.2.

Difference spectra of non-aerated cells of *Sch. pombe ade6* after growth in YEL containing 20% (wt/vol) glucose for a) 8 h, b) 16 h, c) 17 h and d) 19 h yielding a) 0, b) 0.82, c) 1.03 and d) 0.97 nmol P450 g^{-1} dry cell weight and a) 0, b) 0.59, c) 0.63 and d) 0.50 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.2a.

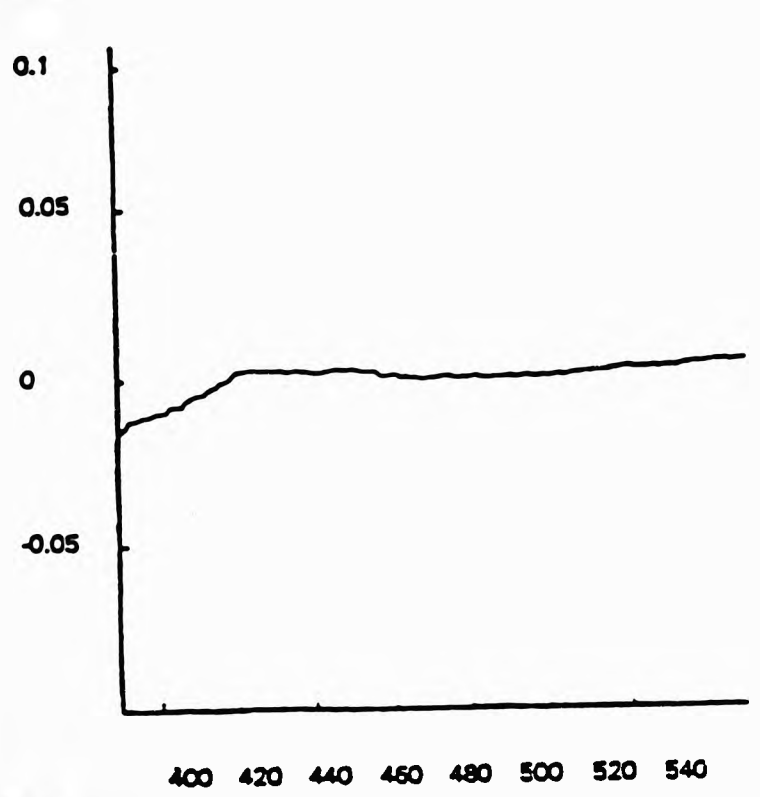


Fig. 3.2b.

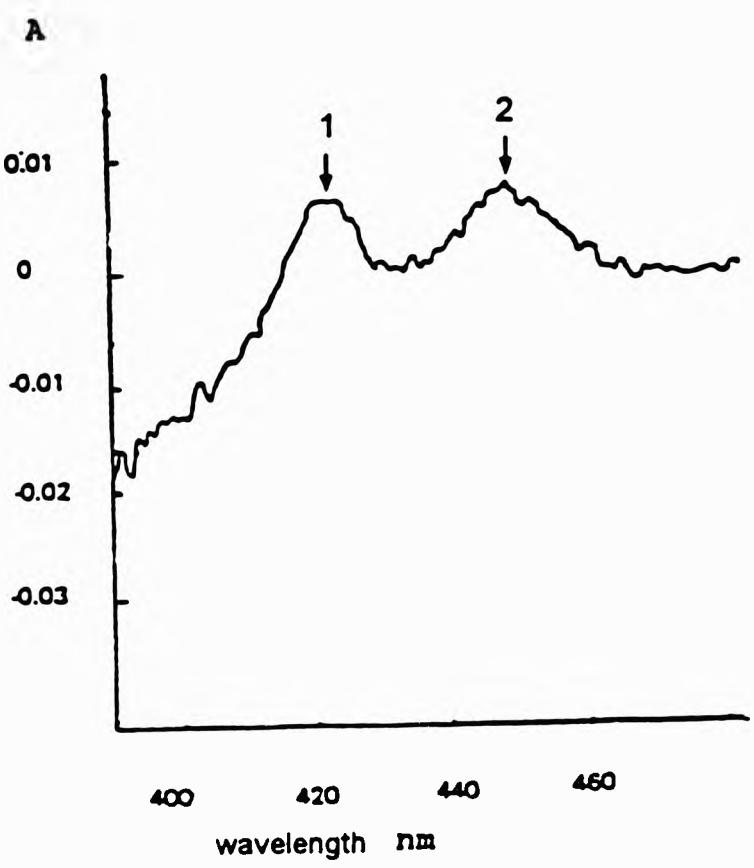


Fig. 3.2c.

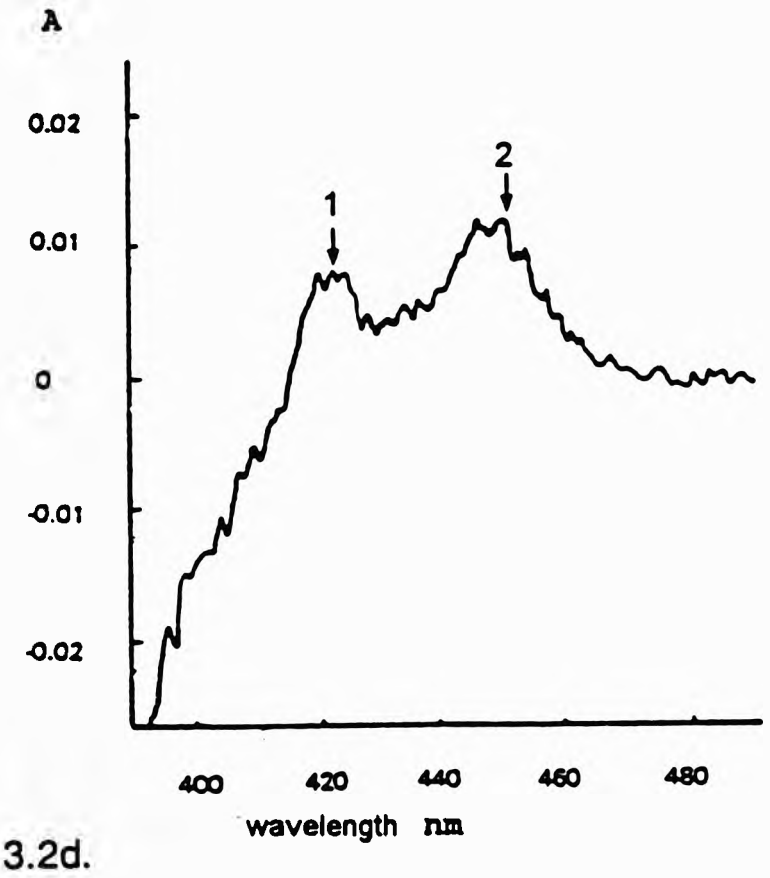


Fig. 3.2d.

after inoculation (table 3.1).

In the strain *Sch. pombe leu*1-32 *pho*1-44, a higher level of cytochrome P450 (12.52 nmol g^{-1} dry cell weight) was detected after 18 h of growth in complete medium with aeration than at later times in the growth phase. After 24 h growth 7.02 nmol cytochrome P450 g^{-1} dry cell weight was detected (table 3.2).

Table 3.2

Time course of cytochrome P450 and P420 production in aerated cultures of *Sch. pombe leu*1-32 *pho*1-44 grown in YEPD containing 20% (wt/vol) glucose

time (h)	A at 420 nm	A at 450 nm	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
7	0	0	0.4	0	0
18	0.167	0.060	52.7	28.55	12.51
20	0.146	0.050	77.0	17.08	7.14
24	0.087	0.046	72.0	10.89	7.02

Data represents one determination for each time period.

Since results in this chapter indicate the extreme sensitivity of cytochrome P450 to changes in factors such as carbon source and aeration all experiments were carried out while adhering to a strict protocol to minimise any variation in cytochrome P450 levels that may have been incurred during preparation of the samples.

The observed maximum cytochrome P450 production was thus found to

be between 17 and 19 h after inoculation during the late logarithmic phase of growth. This agrees with work by Wiseman *et al.* (1975) who reported that cytochrome P450 was produced in aerobic cultures of yeast during the logarithmic phase of growth. This was also the reported phase of growth for highest cytochrome P450 production by Kelly and Parry (1983) with *S. cerevisiae* D6 and Del Carratore *et al.* (1983) with *S. cerevisiae* D7. Von Borstel *et al.* (1985) found that in the D5 strain of *S. cerevisiae* increased absorbance at 450 nm was observed during the logarithmic growth phase. In the present study a time course determination of cytochrome P450 in *Sch. pombe leu*1-32 *pho*1-44 showed that the yield of cytochrome P450 peaked at 18 h, while Bligh and Kelly (1988) found the maximal production in this strain was 16 h after inoculation. However, in the present study cytochrome P450 determinations were not made in this strain at 16 h growth. In both investigations the level of the haemoprotein was beginning to decline 24 h after inoculation.

Further determinations of cytochrome P450 yields in *Sch. pombe leu*1-32 *pho*1-44, 972 and *lys*1-131 showed that levels declined between 18 and 24 h growth (fig. 3.3, table 3.3). In the case of *Sch. pombe* 972 almost a one third loss in enzyme level was observed. This suggests that degradation of the haemoprotein was occurring as stationary phase approached. This is supported by the work of von Borstel *et al.* (1985) who showed that loss of the haemoprotein occurred during stationary phase.

Fig. 3.3.

Difference spectra of aerated cells of Sch. pombe *leu*1-32 *pho*1-44 after growth in YEPD containing 20% (wt/vol) glucose for a) 18 h and b) 24 h yielding a) 8.14 nmol P450 and 15.57 nmol P420 g^{-1} dry cell weight and b) 5.68 nmol P450 and 7.76 nmol P420 g^{-1} dry cell weight respectively. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.3a.

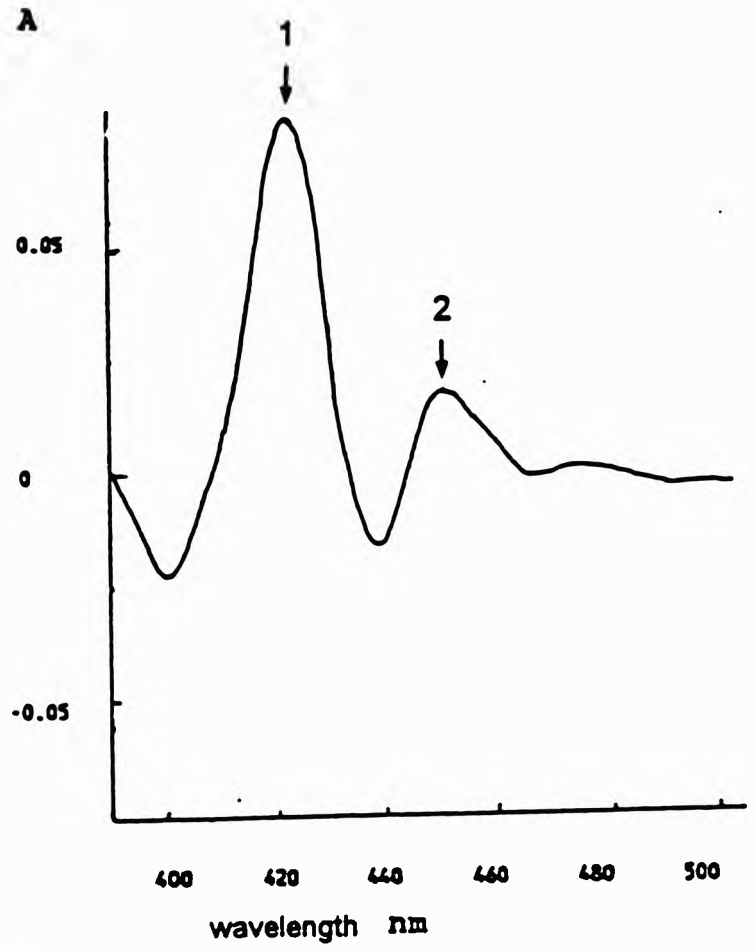


Fig. 3.3b.

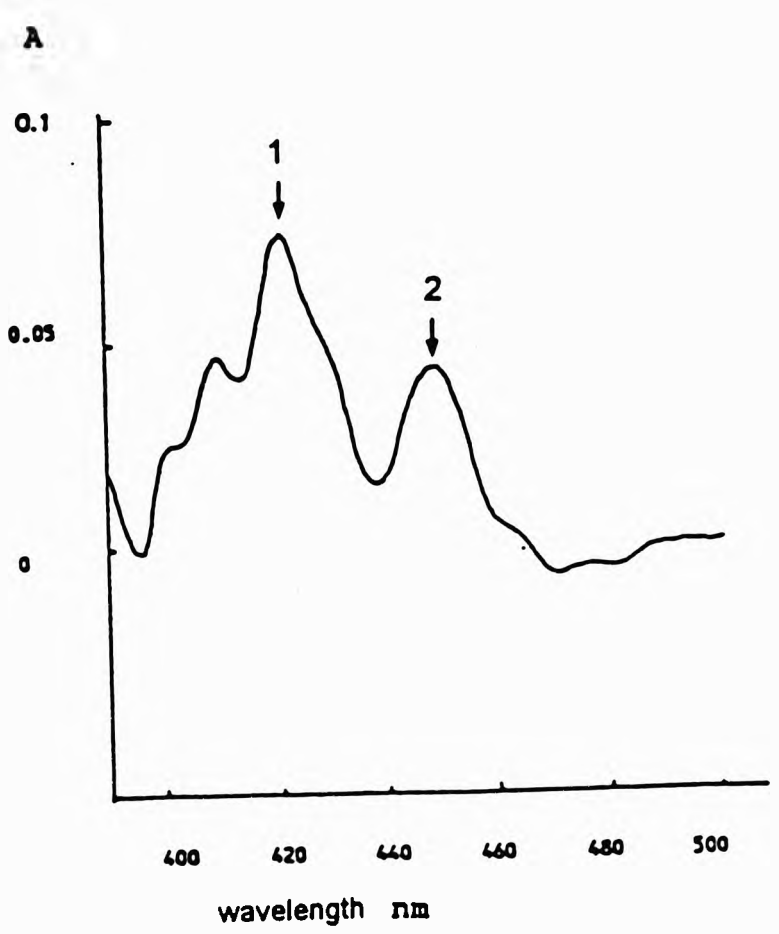


Table 3.3

Production of cytochrome P450 and P420 in aerated cultures of *Sch. pombe* at the late logarithmic phase of growth in YEPD containing 20% (wt/vol) glucose

strain	time (h)	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
Sch.pombe leu 1-32 *pho* 1-44	18	56.7	15.57	8.14
Sch.pombe leu 1-32 *pho* 1-44	24	87.1	7.76	5.68
Sch.pombe 972	18	86.9	8.40	6.96
Sch.pombe 972	24	108.4	5.07	4.36
Sch.pombe lys 1-131	18	67.5	12.70	8.14
Sch.pombe lys 1-131	24	75.5	14.32	7.71

Data represents one determination for each time period.

In whole cells of most cultures analysed absorbance was seen at 420 nm. Such absorbance has been attributed to cytochrome P420. This haemoprotein was first discovered by Omura and Sato (1964) in liver microsomes and by Lindenmayer and Smith (1964) in yeast. It is thought to be the soluble form of cytochrome P450 and is located in the cytosol, cytochrome P450 normally being located in the microsomal membrane.

In the species used in the present work absorbance at 420 nm could be detected at the late logarithmic phase of growth (fig. 3.2). Von Borstel *et al.* (1985) suggested that the role of cytochrome P420 was as important as cytochrome P450 in the metabolism of xenobiotic compounds.

3.2.2 Effect of type and concentration of carbon source on cytochrome P450 production

Levels of cytochrome P450 were found to vary in yeasts according to the type and concentration of substrate utilised. Experiments with *C. parapsilosis* showed that cytochrome P450 could not be detected when the cells were grown with the non-fermentable carbon source sodium acetate (fig. 3.4b, table 3.4), although a large peak at 420 nm could be seen. This could have been due to conversion of cytochrome P450 to cytochrome P420 under these conditions. Certain carbon sources such as galactose have been shown to give a low level of cytochrome P450 in yeast cells (Callen and Philpot, 1977). As a consequence under these conditions fewer toxic metabolites were produced from halogenated hydrocarbons.

Table 3.4

Production of cytochrome P450 and P420 in aerated cultures of *C. parapsilosis* after growth for 18 h in YEPD containing different substrates.

strain	medium	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
C.parapsilosis	20% (wt/vol) glucose	16.29 ± 1.73	11.79 ± 2.35
C. parapsilosis	20% (wt/vol) sodium acetate	74.03 ± 5.73	0

Data represents average ± SEM of determinations from three separate experiments.

A role for glucose in the production of cytochrome P450 was indicated

Fig. 3.4.

Difference spectra of aerated cells of *C. parapsilosis* after growth in YEPD for 18 h containing a) 20% (wt/vol) glucose and b) 20% (wt/vol) sodium acetate yielding a) 12.14 nmol P450 and 16.94 nmol P420 g^{-1} dry cell weight and b) 0 nmol P450 and 75.6 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.4a.

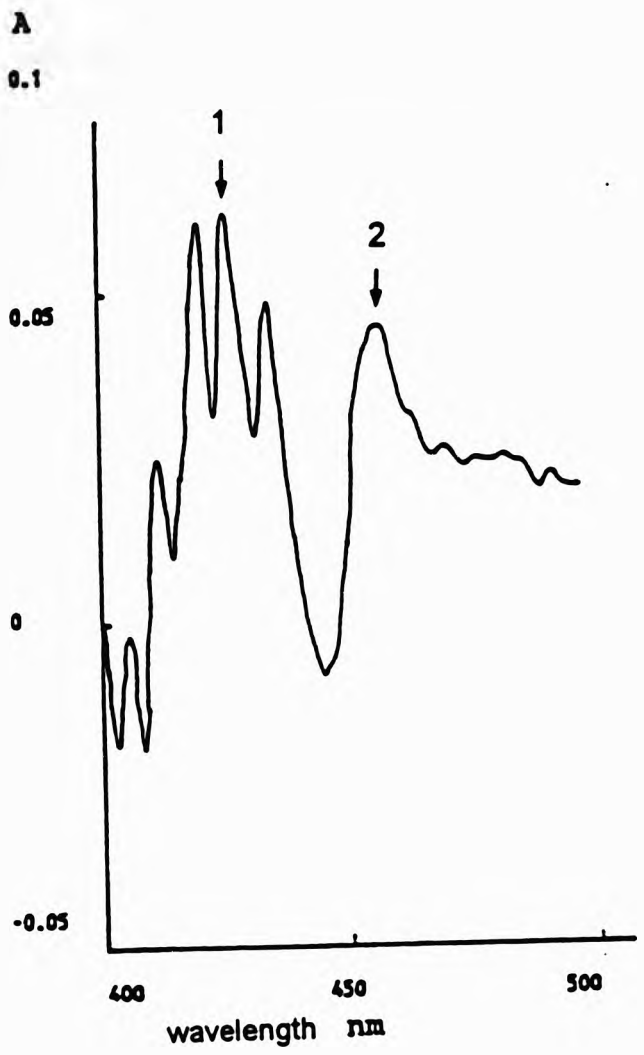
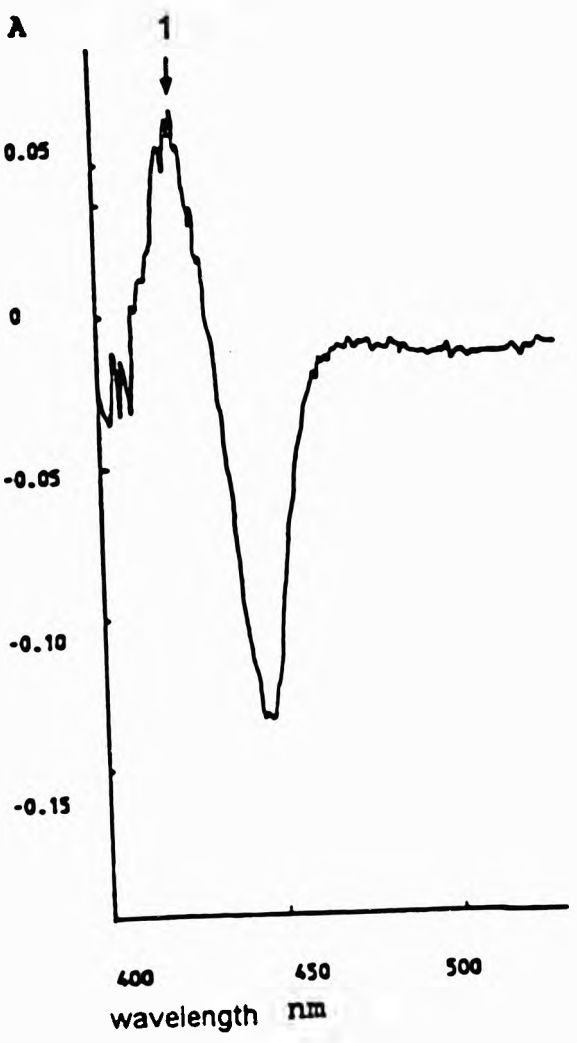


Fig. 3.4b.



by the fact that in the glucose-insensitive yeast *C. parapsilosis* production of the haemoprotein was glucose-dependent.

It has been reported that the production of cytochrome P450 in yeasts of the genus *Candida* requires the presence of a hydrocarbon substrate (Lebeault *et al.*, 1971). Detectable levels of the haemoprotein could not be found in cells grown under conditions of glucose repression. Glucose has actually been found to act as a repressor of cytochrome P450 production in *C. tropicalis* (Gmunder *et al.*, 1981).

In these species oxygen was also found to be inhibitory to cytochrome P450 levels (Mauersberger *et al.*, 1980). However, Sanglard *et al.* (1984) observed the species *C. tropicalis* to produce cytochrome P450 when grown on glucose. These workers have suggested that different haemoproteins are produced according to the carbon source present. Glucose grown cells are reputed to exhibit a P448 type enzyme, whereas cells grown on *n*-alkanes produce an alkane hydroxylase system showing characteristic spectra with a peak at 450 nm.

No data, however, appear to exist on cytochrome P450 production in *C. parapsilosis.* This species has been reported as being glucose-insensitive (Niimi et al., 1988), which suggests that it cannot undergo fermentation and depends upon oxidative metabolism for growth.

Where glucose was provided as the sole carbon source, the concentration present in the growth medium was found to affect cytochrome P450 levels. In *S. cerevisiae* 673, 159, 188 and 188 *petite*

isolate 4 (see section 2.2.1) the haemoprotein could not be detected when the cells were grown on 2% (wt/vol) glucose (fig. 3.5). However, under these conditions, a peak at 420 nm was present.

When the same strains were grown on 20% (wt/vol) glucose, absorbance could be seen at 450 nm giving average values of 17.33 nmol cytochrome P450 g^{-1} dry cell weight for *S. cerevisiae* 673 and 7.85 nmol cytochrome P450 g^{-1} for *S. cerevisiae* 188 *petite* isolate 4. The respiratory proficient strains *S. cerevisiae* 188 and 159 produced on average 1.80 and 4.28 nmol cytochrome P450 g^{-1} dry cell weight respectively (see section 3.2.3).

Growth of *Sch. pombe* in 20% (wt/vol) glucose produced high levels of cytochrome P450, in 972 with a maximum yield of 22.35 nmol g^{-1} dry cell weight being recorded (fig. 3.6, table 3.5) and in *ade6* when adenine was present in the medium (fig. 3.7, table 3.5). Cytochrome P450 was also found to be present in *S. cerevisiae* 188 (fig. 3.8, table 3.6) and *ade2* grown in 20% (wt/vol) glucose and when adenine was present in the medium in the case of the latter strain (fig. 3.9, table 3.6).

The results in all three species are thus in line with the observation that cytochrome P450 is produced in yeast cells grown using high concentrations of glucose as carbon source. Increased yields of the haemoprotein have been documented in cells when grown under conditions of glucose repression (Woods and Wiseman, 1980). Work on the genus *Saccharomyces* by Kelly and Parry (1983) showed that under high concentrations of glucose (20% wt/vol), strain D6 and the

Fig. 3.5.

Difference spectra of aerated cells of a) *S. cerevisiae* 673, b) 188 *petite*, c) 159 and d) 188 after growth in YEPD containing 2% (wt/vol) glucose for 18 h. Arrows indicate peaks at 420 nm (1).

Fig. 3.5a.

Fig. 3.5b.

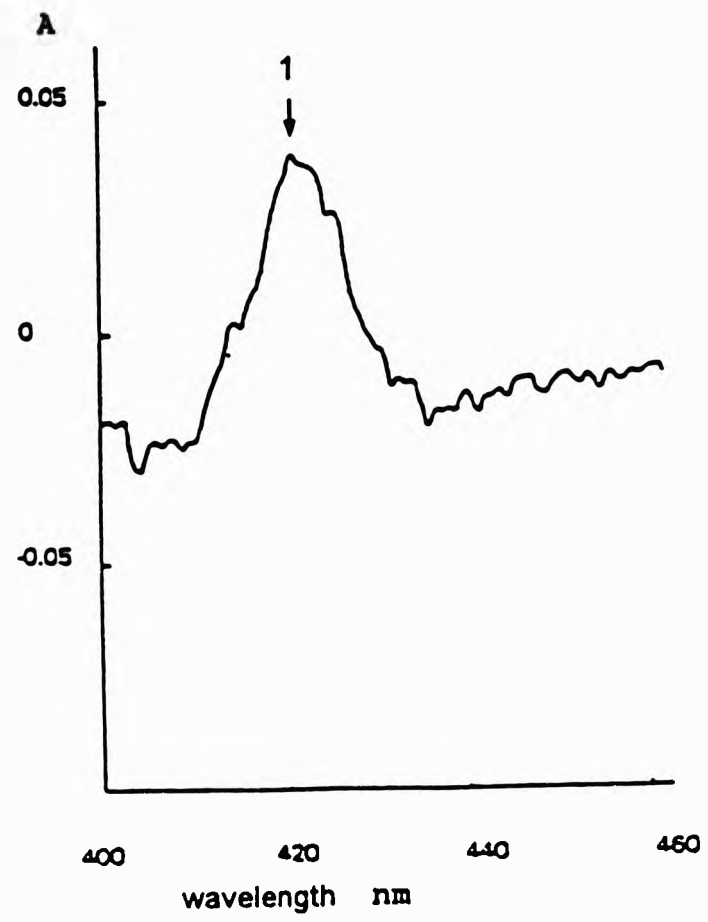


Fig. 3.5c.

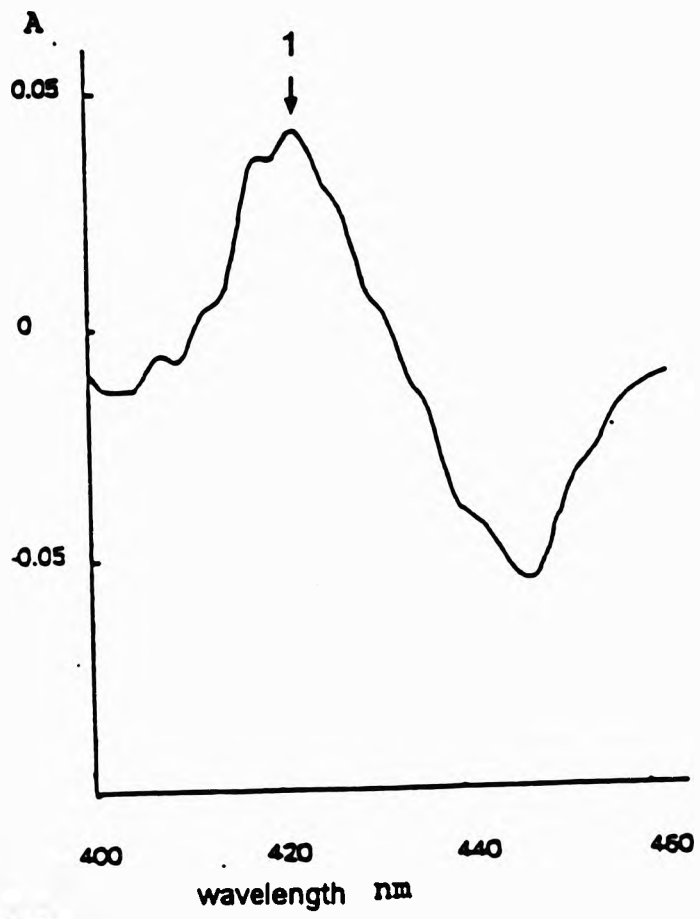


Fig. 3.5d.

Fig. 3.6. Difference spectrum of non-aerated cells of *Sch. pombe* 972 after growth in YEL containing 20% (wt/vol) glucose for 18 h yielding 22.35 nmol P450 and 19.85 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.6.

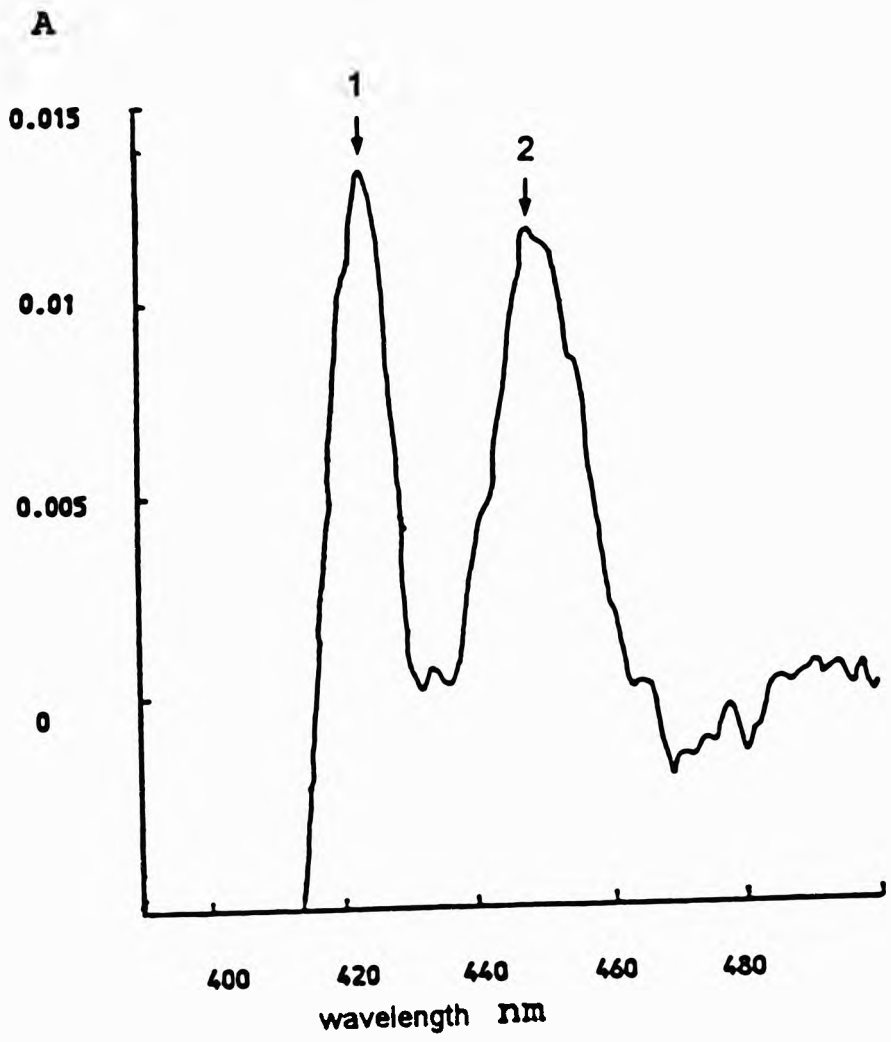


Fig. 3.7.

Difference spectra of non-aerated cells of *Sch. pombe ade6* after growth for 18 h in YEL containing 20% (wt/vol) glucose a) without adenine and b) with 50 mg l^{-1} adenine yielding a) 0 P450 and P420 and b) 6.73 nmol P450 and 9.19 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.7a

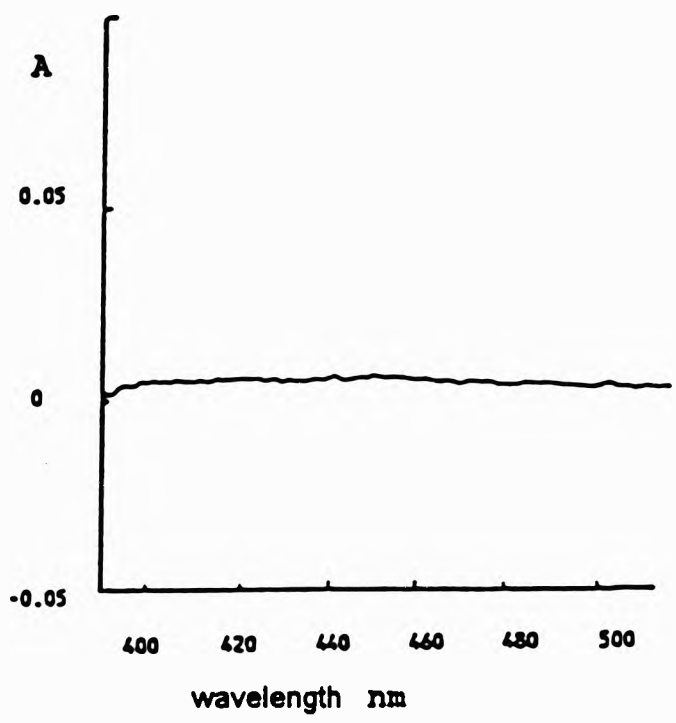


Fig. 3.7b.

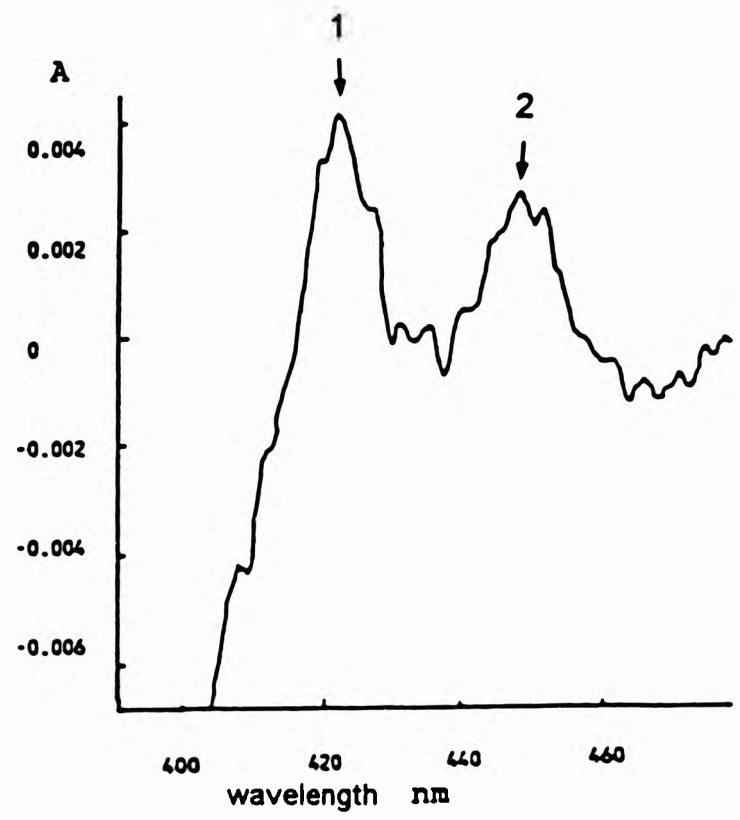


Fig.3.8.

Difference spectrum of non-aerated cells of *S. cerevisiae* 188 after growth in YEPD containing 20% (wt/vol) glucose for 18 h yielding 5.13 nmol P450 and 3.60 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.8.

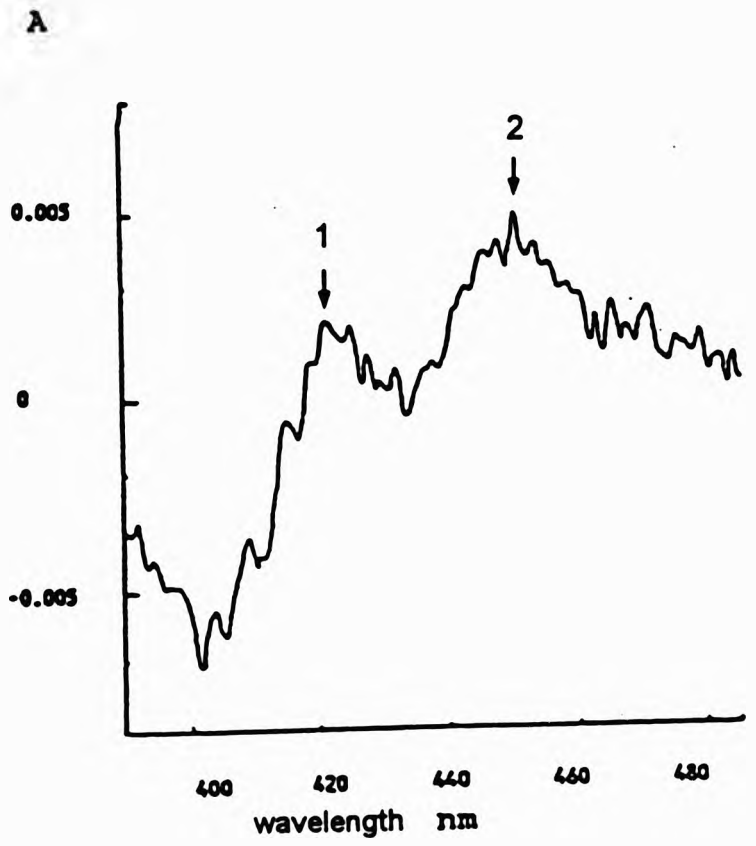
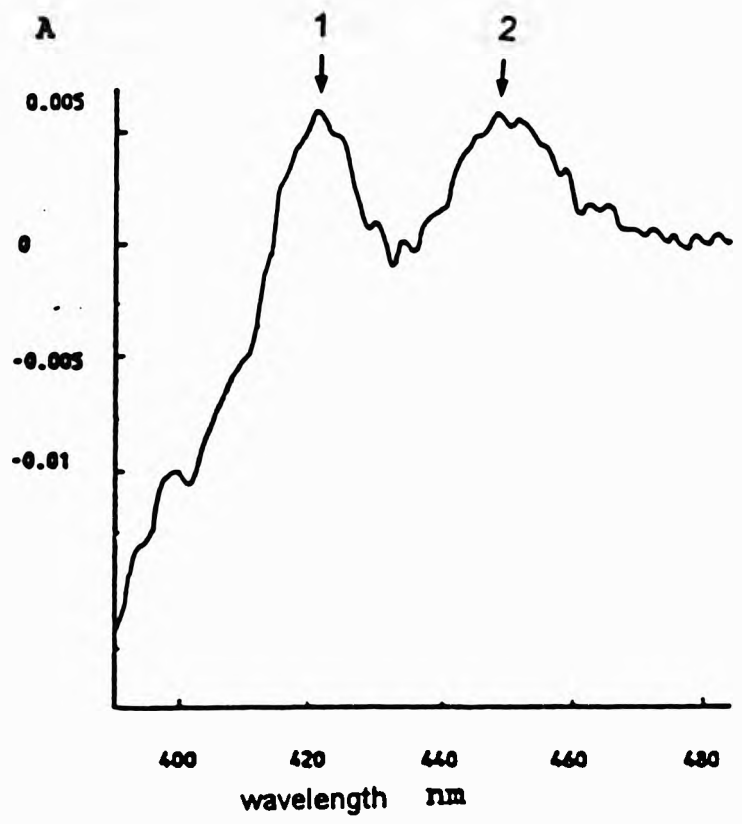


Fig. 3.9.

Difference spectrum of non-aerated cells of *S. cerevisiae ade2* after growth in YEPD containing 20% (wt/vol) glucose and 50 mg l^{-1} adenine for 18 h yielding 9.99 nmol P450 and 10.65 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.9.



petite strain JD1 exhibited the presence of cytochrome P450 at earlier growth times and for longer durations. Cells grown in 4% (wt/vol) glucose medium showed a lower level of cytochrome P450.

Table 3.5

Production of cytochrome P450 and P420 in non-aerated strains of *Sch. pombe* after 18 h growth in YEL containing 20% (wt/vol) glucose.

strain	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
Sch. pombe 972	14.22 ± 2.84	14.04 ± 4.99
Sch. pombe ade6	9.19	6.73

Data represents determinations from three separate experiments for *Sch. pombe* 972 ± SEM and from one experiment for *Sch. pombe ade6*. YEL was supplemented with 50mg l^{-1} adenine in the case of *Sch. pombe ade6*.

Table 3.6

Production of cytochrome P450 and P420 in non-aerated strains of *S. cerevisiae* after 18 h growth in YEPD containing 20% (wt/vol) glucose

strain	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
S. cerevisiae 188	5.5 ± 1.75	4.79 ± 0.34
S. cerevisiae ade2	10.73 ± 0.08	9.39 ± 0.60

Data represents determinations from three separate experiments in *S. cerevisiae* 188 and two separate experiments in *S. cerevisiae ade2* ± SEM. YEPD was supplemented with 50 mg l^{-1} adenine in the case of *S. cerevisiae ade2*.

3.2.3 Effect of respiratory deficiency on cytochrome P450 production

Work on *Saccharomyces* has shown that cytochrome P450 is produced under certain physiological conditions involving a reduction in respiration and an increase in fermentation i.e. semi-anaerobic growth conditions. Such conditions also dictate that mitochondrial repression is in effect (Wiseman and King, 1982). To investigate this phenomenon further, a comparative analysis of cytochrome P450 levels in *petite* (respiratory deficient) mutants and wild-type cells was carried out.

When grown with 20 % (wt/vol) glucose, detectable levels of cytochrome P450 could be seen in two respiratory deficient strains *S. cerevisiae* 673 and *S. cerevisiae* 188 *petite* isolate no. 4 and two respiratory proficient strains *S. cerevisiae* 159 and 188 (fig. 3.10). Under these conditions the *petite* strain *S. cerevisiae* 673 consistently produced greater levels of cytochrome P450 than the respiratory proficient strains *S. cerevisiae* 188 and 159; yielding, after five separate determinations, an average of 17.33 nmol P450 g^{-1} dry cell weight. This is approximately 9.5 times the yield in *S. cerevisiae* 188 and 4 times that of *S. cerevisiae* 159 (fig. 3.11). Furthermore, the newly isolated *petite* S. cerevisiae 188 isolate 4, produced on average 7.85 nmol g^{-1} dry cell weight after five separate determinations. This value is approximately 4.4 times the level of cytochrome P450 than in the strain from which it was derived, (fig. 3.11; Atchia *et al.* 1991a). The cytochrome P450 values from the *petite* strain *S. cerevisiae* 673 were found to be significantly higher than those of the wild type strains tested at $P<0.01$ using the LSD criterion from ANOVA.

Fig. 3.10.

Difference spectra of aerated cells of a) *S. cerevisiae* 673, b) 188 *petite*, c) 188 and d) 159 after growth in YEPD containing 20% (wt/vol) glucose for 18 h yielding a) 11.13, b) 4.91, c) 3.06 and d) 0 nmol P450 g^{-1} dry cell weight and a) 15.08, b) 11.18, c) 0 and d) 9.60 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.10a.

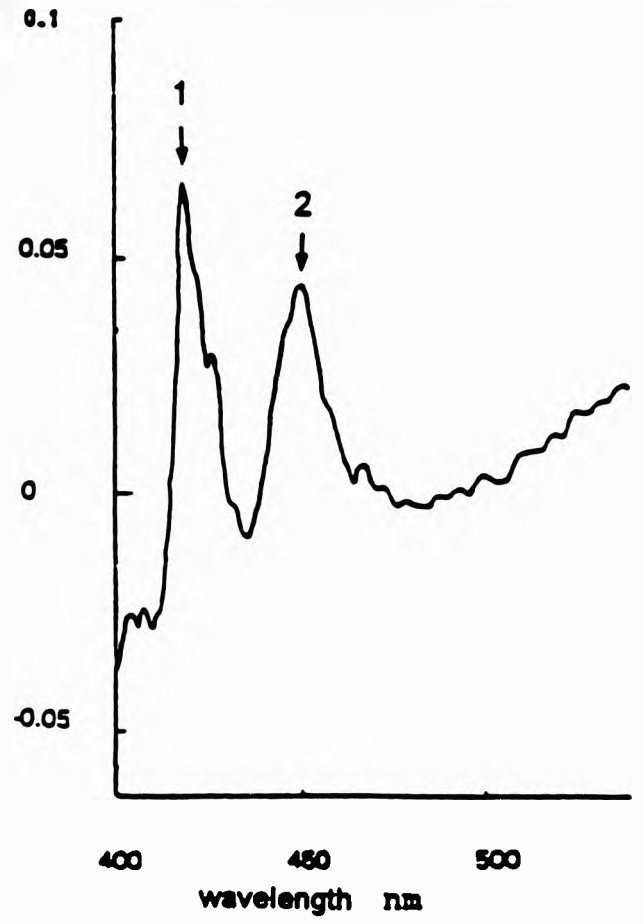


Fig. 3.10b.

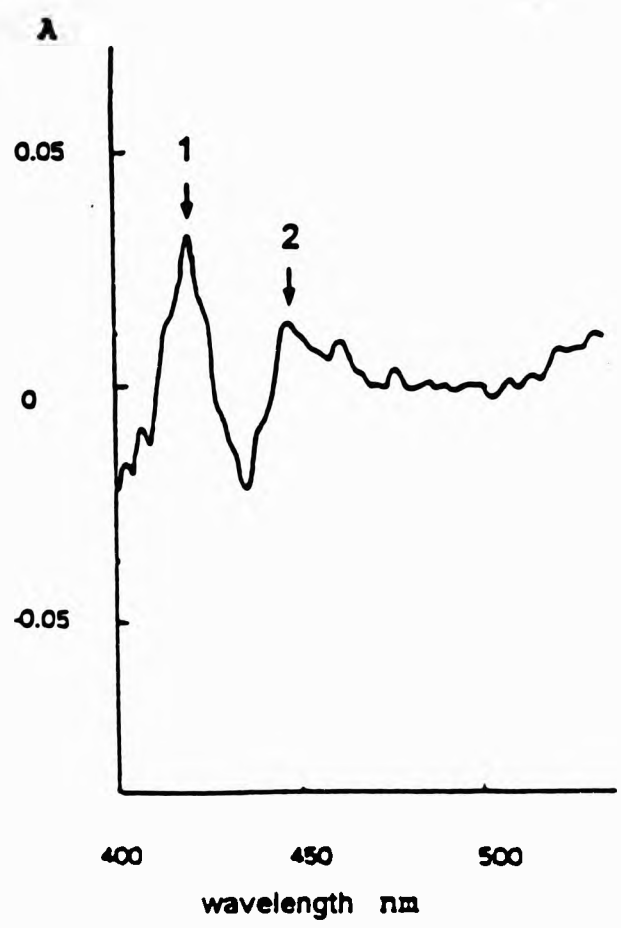


Fig. 3.10c.

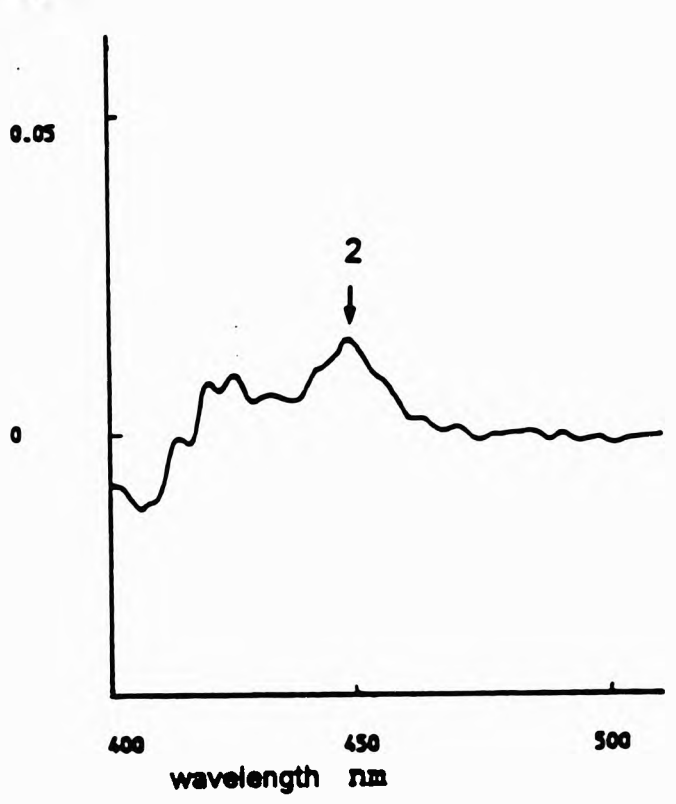


Fig. 3.10d.

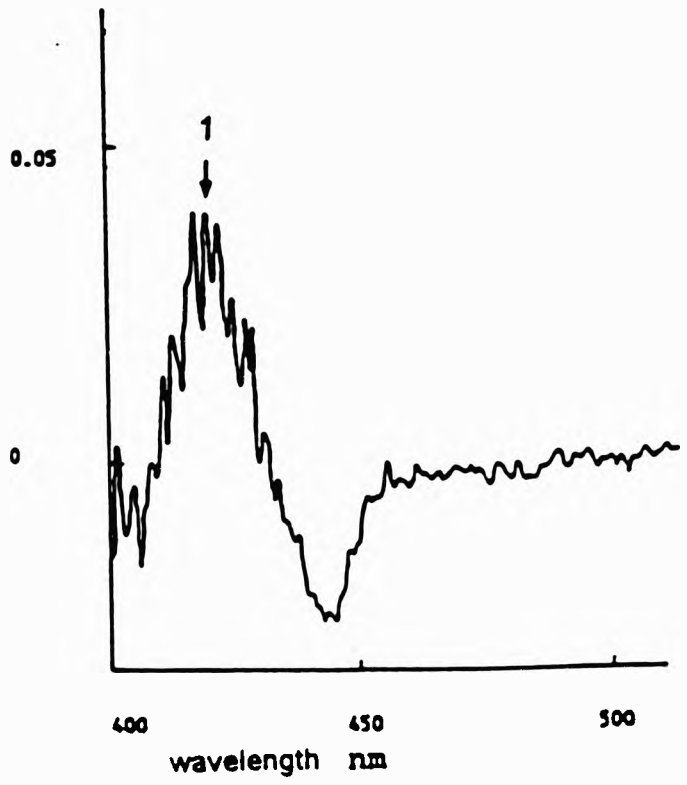
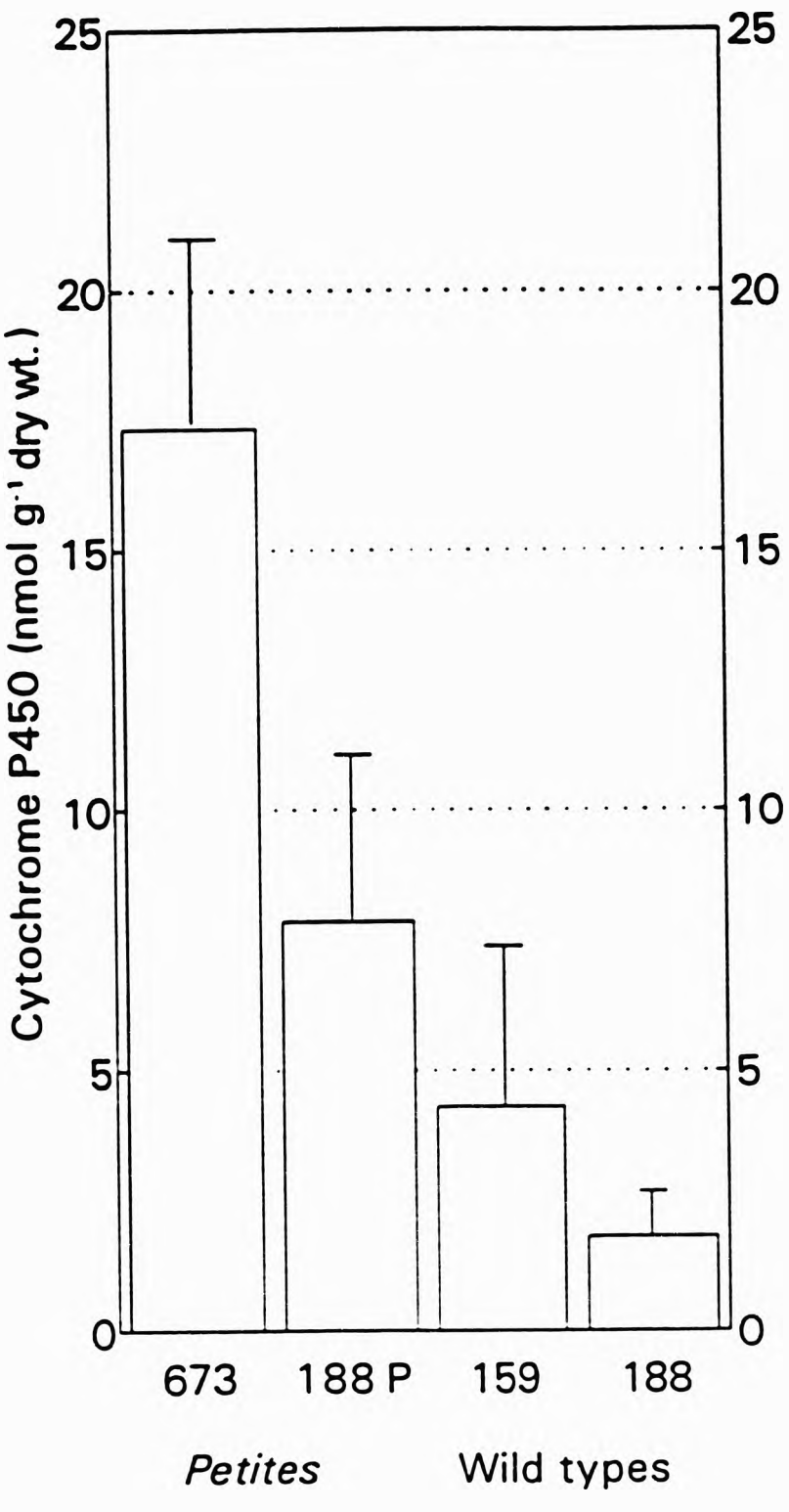


Fig. 3.11.

Cytochrome P450 content (nmol g^{-1} dry cell weight) of the four strains of *S. cerevisiae* grown in 20% (wt/vol) glucose. The values shown are means (bars:SEM) of 5 experiments, with whole cells. Significant differences between values were calculated using the LSD criterion from ANOVA. LSD calculated for data $P< 0.01= 12.44$.

Fig. 3.11. Cytochrome P450 values of *S. cerevisiae petites* and wild types



Mitochondrial cytochrome production was analysed in all four strains (fig. 3.12). Cytochromes b (detected as a shoulder at 562 nm) and c (absorption maximum 552 nm) were found to be present in both wild-type strains; cytochrome aa3 (absorption maximum 603 nm), the terminal acceptor in the electron transport chain, was present in *S. cerevisiae* 159 and a small peak was also indicated in *S. cerevisiae* 188. Cytochrome c, a nuclear encoded protein (Schatz and Mason, 1974), was also detected in *S. cerevisiae* 673. This cytochrome can be unaffected or overproduced by the *petite* mutation, (Egilsson *et al.*, 1979). However the mitochondrial gene products cytochromes aa3 and b (Borst and Grivell, 1978) were not detectable in the two *petite* mutants, indicating that they do not possess functional mitochondria. Respiratory deficiency was further confirmed in *S. cerevisiae* 673 and 188 isolate 4 by their inability to grow on glycerol, a non-fermentable carbon source (data not shown).

These observations are consistent with the fact that cytochromes associated with oxidative metabolism decrease as cytochrome P450 production increases (Trinn *et al.*, 1982). This further indicates that cytochrome P450 is produced in greatest amounts under conditions of mitochondrial repression.

The postulation of adenylate cyclase activity in the *S. cerevisiae* mitochondrial membrane (Wiseman, 1980) established a link between functioning mitochondria and cyclic AMP (cAMP) production. As the intracellular concentration of cAMP is known to be inversely proportional

Fig. 3.12.

Difference spectra showing mitochondrial cytochromes in aerated cells of a) *S. cerevisiae* 673, b) 188 *petite*, c) 188 and d) 159 after growth in YEPD containing 2% (wt/vol) glucose for 18 h. Arrows indicate peaks at 552 nm (1), 562 nm (2) and 603 nm (3).

Fig. 3.12a.

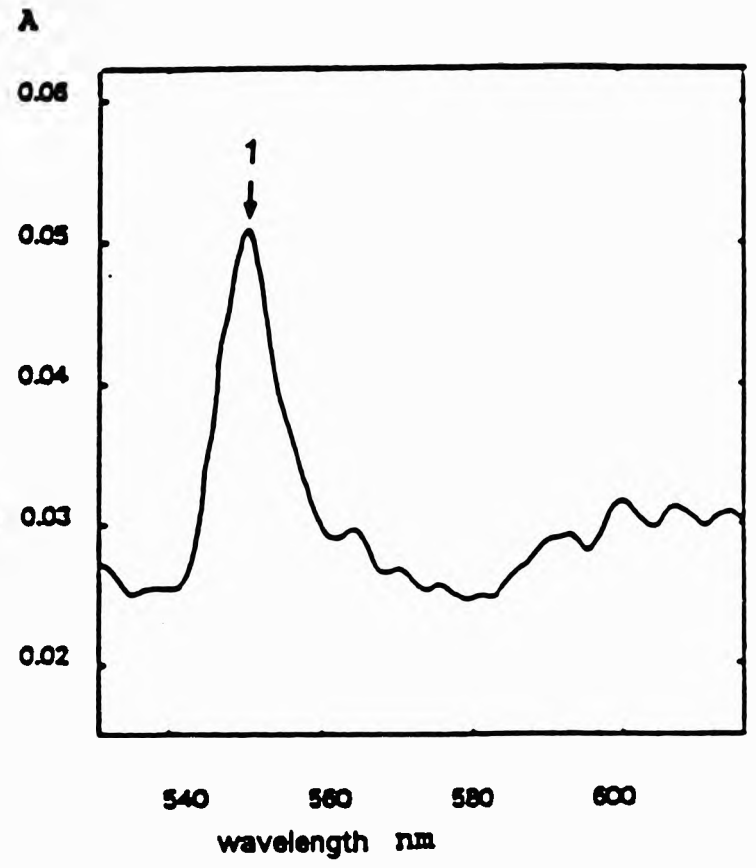


Fig. 3.12b.

Fig. 3.12c.

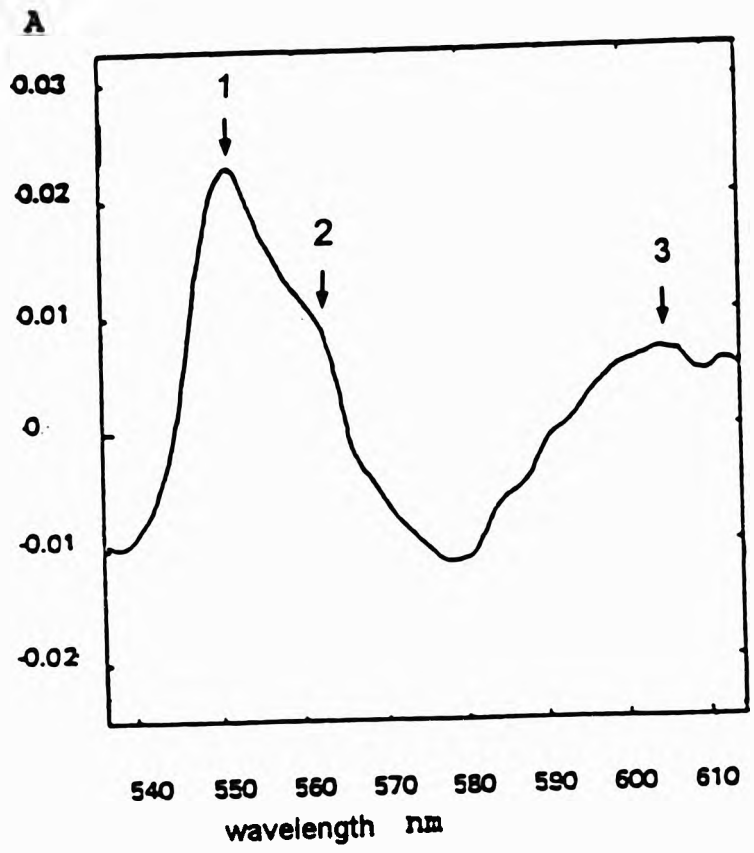
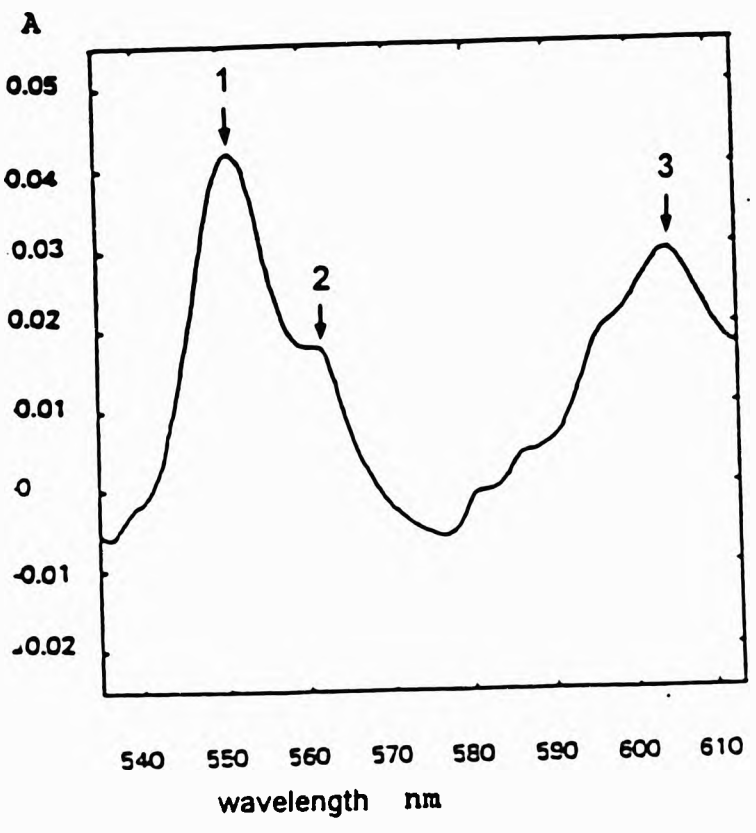


Fig. 3.12d.



to that of cytochrome P450 (Qureshi *et al.*, 1980), this could indicate how abolition of mitochondrial function may enhance the level of the haemoprotein. It has been postulated that this cAMP control which itself is glucose repressible, operates via repression of the transcription of cytochrome P450 mRNA (Wiseman *et al.*, 1978). Furthermore, mitochondrial DNA levels have been reported to be reduced in glucose-repressed cells (Goldthwaite *et al.*, 1974). Therefore in *petite* cells under these conditions, levels may be expected to be greatly reduced.

In operon systems of *E.coli* cAMP has been found to promote transcription of the regulated genes (Pastan and Perlman, 1969). It has been suggested that the mitochondrial system of yeasts may be involved in cAMP-mediated glucose derepression (Evans, 1983).

However other researchers have detected high levels of cAMP during glucose repression in some yeasts (Eraso and Gancedo, 1984) and have concluded that cAMP is not involved in catabolite repression in these organisms. This has been confirmed by the use of mutants lacking adenylate cyclase (Gancedo, 1992).

A slower rate of degradation of the haemoprotein in *petites* may give an alternative explanation for the elevated levels of cytochrome P450 in the latter mutants, since the degradation of this enzyme is thought to require functioning mitochondria (Blatiak *et al.*, 1980).

The amount of cytochrome P450 produced by the two *petite* strains used

in the present study differed considerably. Each also differed from that reported by von Borstel *et al.* (1985) in *petites* which had been spontaneously derived from *S. cerevisiae* D5. These variations in cytochrome P450 contents may be due to differences in the amount of mitochondrial DNA present in the *petites*. Evans (1983) has reported that up to 80% mitochondrial DNA may be retained by spontaneous *petites*, whereas mutagens such as ethidium bromide and X-rays, which were used in the respective generation of *S. cerevisiae* 188 isolate 4 and *S. cerevisiae* 673, are likely to cause larger deletions.

Flocculation, a characteristic which is thought to be mitochondrially linked due to its frequent loss in *petite* mutants (Egilsson *et al.*, 1979) was present in both *S. cerevisiae* 188 and its newly isolated respiratory deficient derivative. This indicates that a considerable amount of mitochondrial DNA may have persisted in the *petite* derivative of the latter strain.

3.2.4 Effect of aeration on cytochrome P450 production

Several strains of yeast including *Sch. pombe* 972, *leu*1-32 *pho*1-44, *S. cerevisiae* 673, 188 *petite* isolate 4, 188, 159 and *C. parapsilosis* were grown with and without aeration and analysed for cytochrome P450 content (figs. 3.13-3.14, tables 3.7-3.9). The effect of oxygen levels upon cytochrome P450 production gave somewhat equivocal results between strains and also between determinations.

In experiments with the wild-type strains *Sch. pombe* 972, *S. cerevisiae*

188, and the auxotrophic strains *Sch. pombe ade6* and *S. cerevisiae ade2* no cytochrome P450 could be detected and growth was poor when the cells were grown in medical flat bottles without shaking (data not shown). This was probably due to the limited amounts of oxygen present suggesting that the haemoprotein is not produced under anaerobic conditions. This is in agreement with work by Rogers and Stewart (1973) who could not detect cytochrome P450 in yeast grown under these conditions.

However, when the same strains were grown in 250 ml flasks without shaking, cytochrome P450 was present (figs. 3.6-3.9, tables 3.5, 3.6). Under these conditions high levels of the enzyme were achievable, giving a maximum yield in *Sch. pombe* 972 of 22.35 nmol P450 g^{-1} dry cell weight and 5.13 nmol g^{-1} in *S. cerevisiae* 188. In these flask cultures semi-anaerobic conditions were thought to prevail. Trinn *et al.* (1982) reported yields of cytochrome P450 of 3-5 pmol mg^{-1} dry cell weight in *S. cerevisiae* grown under glucose-repressed conditions below a dissolved oxygen tension of approximately 15%. However, under conditions of glucose-derepression, cytochrome P450 was only produced when oxygen limitation resulted in glucose repression.

Flask cultures of *C. parapsilosis* grown without shaking, however, possessed no detectable cytochrome P450 (fig. 3.13b). This could have been due to the fact that this species is an example of a glucose-insensitive yeast which requires aeration for growth and thus does not exhibit fermentation or ethanol production. In these cells oxygen may not

Fig. 3.13.

Difference spectra of cells of *C. parapsilosis* after growth in YEPD containing 20% (wt/vol) glucose for 18 h a) with aeration and b) without aeration yielding a) 15.66 nmol P450 and 18.92 nmol P420 and b) 0 nmol P450 and P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.13a.

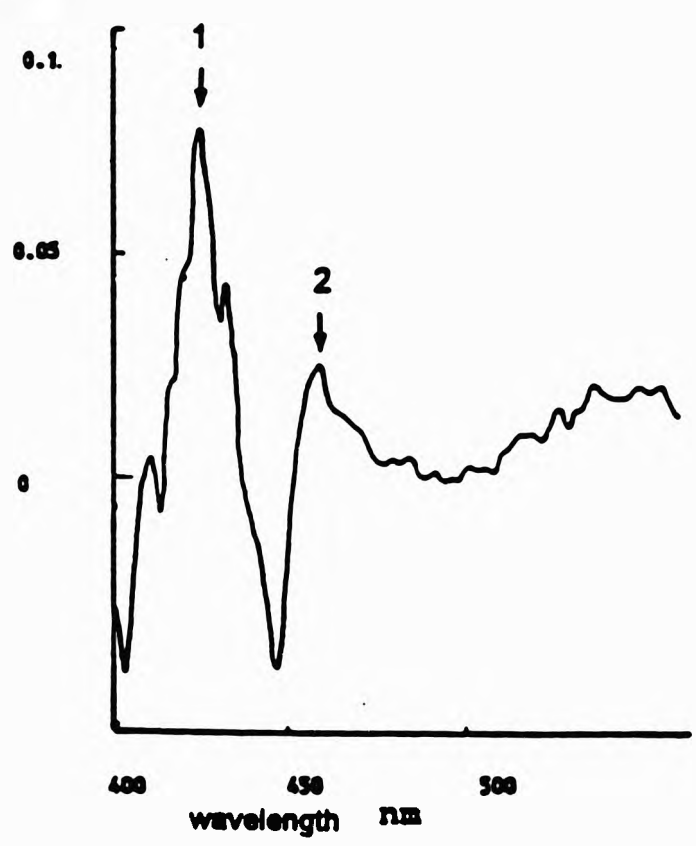
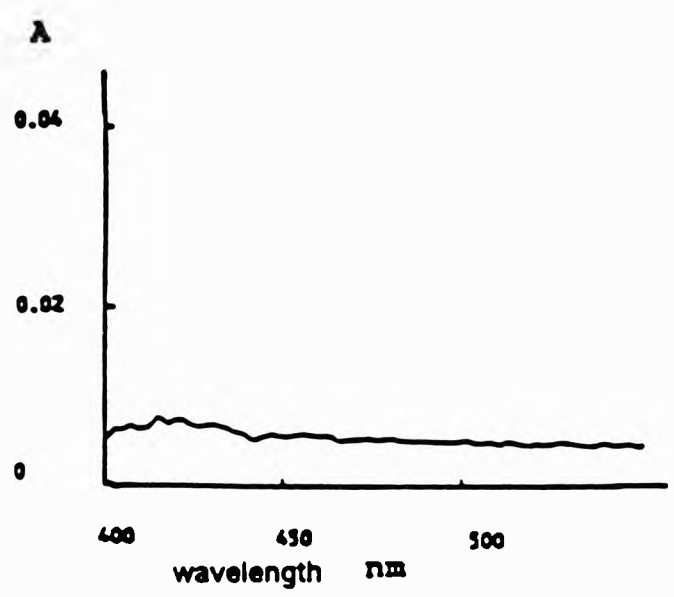


Fig. 3.13b.



have been sufficiently plentiful for growth and subsequent production of the haemoprotein. *C. parapsilosis*, however, produced good yields of the enzyme when the cultures were shaken, giving a maximum yield of 15.66 nmol P450 g^{-1} dry cell weight (fig. 3.13, table 3.7).

Table 3.7

Cytochrome P450 and P420 production in aerated and non-aerated cultures of *C. parapsilosis* after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

strain	conditions	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
C. parapsilosis	aerated	40.0	16.29 ±1.73	11.79 ± 2.35
C. parapsilosis	non-aerated	10.0	0	0

Data represents average ± SEM of determinations from three separate experiments.

Shaken cultures of other strains tested were consistently found to contain cytochrome P450, giving a maximum yield of 7.6 nmol g^{-1} dry cell weight in *Sch. pombe* 972 (table 3.8) and 3.15 nmol g^{-1} in *S. cerevisiae* 188 (table 3.9).

Table 3.8

Cytochrome P450 and P420 production in aerated and non-aerated cultures of *Sch. pombe* after growth in YEPD containing 20% (wt/vol) glucose for 18 h

strain	conditions	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
Sch. pombe 972	aerated	10.62 ± 2.31	5.78 ± 0.98
Sch. pombe 972	non-aerated	5.05 ± 0.60	4.87 ± 1.36
Sch. pombe *leu*1-32 *pho*1-44	aerated	4.92	2.40
Sch. pombe *leu*1-32 *pho*1-44	non-aerated	0.29	0.39

Data represents determinations from three separate experiments ± SEM for *Sch. pombe* 972 and one determination for *Sch. pombe leu*1-32 *pho*1-44. No significant difference was calculated between the data groups for P450 values ($P > 0.05$) using ANOVA.

Comparisons between non-shaken and shaken cultures showed that in the former the production of cytochrome P420 was reduced (fig. 3.14). This could have been due to the slowing down of the conversion of cytochrome P450 to P420, a process which is thought to require oxygen (Blatiak *et al.*, 1980). These workers discovered that protection of cytochrome P450 from degradation was afforded by anaerobic conditions such as glucose repression or by inhibition of mitochondrial protein synthesis. In shaken cultures a contribution to the high absorbance at 420 nm was also possibly made by cytochrome c peroxidase (cytochrome aa3) as suggested by Ishidate *et al.* (1969).

Fig. 3.14.

Difference spectra of cells of *Sch. pombe leu*1-32 *pho*1-44 after growth in YEPD containing 20% (wt/vol) glucose for 18 h a) with aeration and b) without aeration yielding a) 2.4 nmol P450 and 4.92 nmol P420 and b) 0.39 nmol P450 and 0.29 nmol P420 g^{-1} dry cell weight respectively. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.14a.

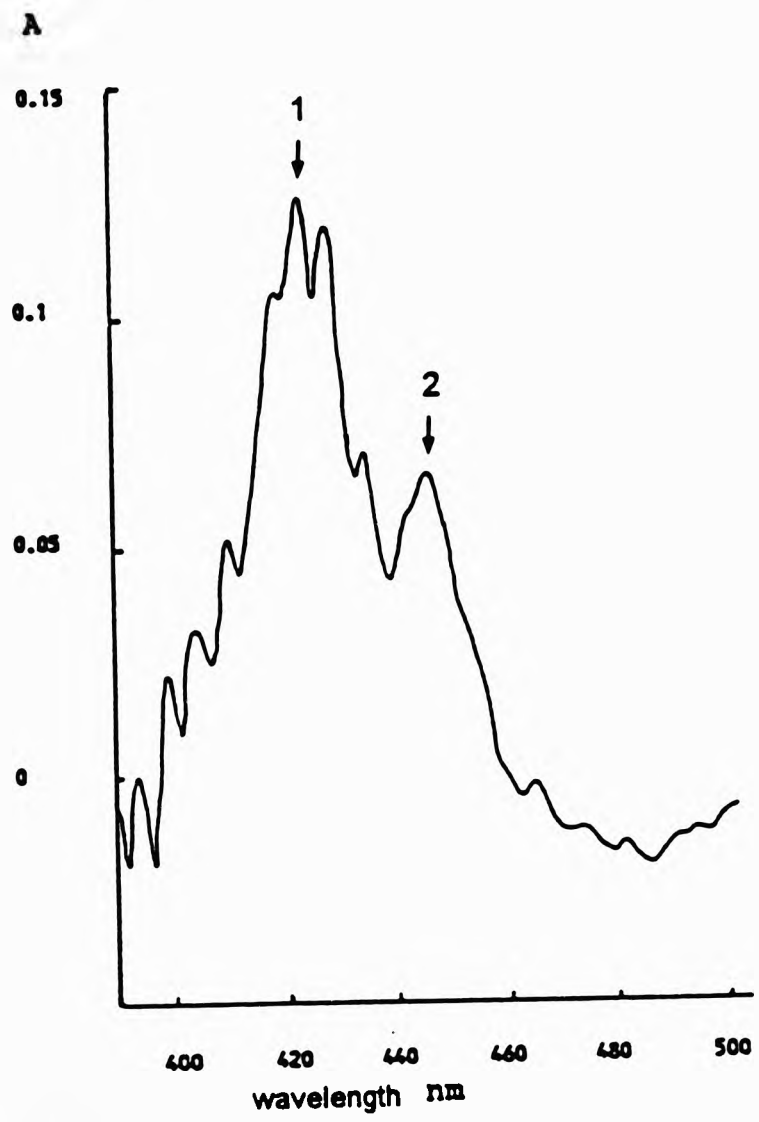


Fig. 3.14b.

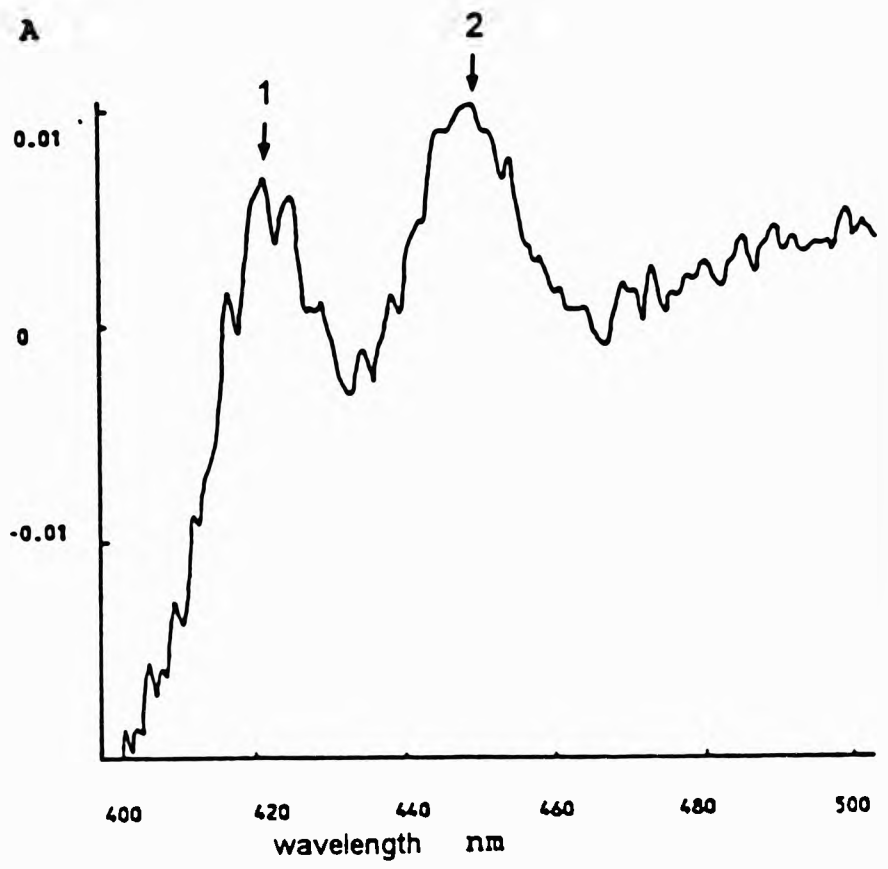


Table 3.9

Cytochrome P450 and P420 production in aerated and non-aerated cultures of *S. cerevisiae* after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

strain	conditions	A at 420 nm	A at 450 nm	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
S. cerevisiae 673	aerated	0.180	0.082	45.8	35.41	19.67
S. cerevisiae 673	non-aerated	0	0.010	12.1	0	9.08
S. cerevisiae 188 *petite*	aerated	0	0.010	22.8	0	4.82
S. cerevisiae 188 *petite*	non-aerated	0	0	10.5	0	0
S. cerevisiae 188	aerated	0.016	0.010	34.9	4.13	3.15
S. cerevisiae 188	non-aerated	0.019	0	15.4	11.12	0
S. cerevisiae 159	aerated	0.082	0	63.3	11.67	0
S. cerevisiae 159	non-aerated	0	0	0	0	0

Data represents determinations from one experiment. No significant difference was calculated between the data groups for P450 values ($P > 0.05$) using ANOVA.

Direct comparison of the cytochrome P450 content of aerated and non-aerated cultures in *Sch. pombe* 972, *Sch. pombe leu*1-32 *pho*1-44, *S. cerevisiae* 188, 188 *petite*, 673 and 159 showed the aerated cultures to give slightly higher values (fig. 3.14, tables 3.8, 3.9). However these differences were found not to be statistically significant ($P>0.05$) using ANOVA. These results indicate that cytochrome P450 can be produced

in both shaken and non-shaken cultures in *Sch. pombe* and *S. cerevisiae*.

The relative yields of cytochrome P450 from shaken and non-shaken cultures i.e. under aerobic and semi-anaerobic conditions respectively suggests a role for oxygen in the production of the haemoprotein. This has also been suggested by Blatiak *et al.*, (1983) who monitored cytochrome P450 production in aerobically growing cultures switched to anaerobic conditions. When this was achieved during the exponential phase of growth, the biosynthesis of cytochrome P450 was inhibited with production of the haemoprotein being less than 10% of the aerobic culture.

The effect of oxygen on cytochrome P450 production may be related to the endogenous role of the enzyme in the 14 α-demethylation of lanosterol, in the metabolic pathway which ultimately produces ergosterol, the prevalent sterol in yeast membranes (Yoshida and Aoyama, 1980). As the biosynthesis of this sterol requires oxygen, cytochrome P450 may only be produced under these conditions.

Another explanation for the role of oxygen in cytochrome P450 production has been given by Morichetti *et al.* (1989). In this work the diploid strain *S. cerevisiae* D7 and DNA repair deficient strains were subjected to UV and X-ray irradiation. Cytochrome P450 and catalase production were monitored and were found to increase after these treatments, indicating that the enzymes are involved in a protective role against damage by the reactive oxygen species produced.

In bacteria a mechanism operates for protection against active oxygen species induced by UV and H_2O_2 (Tyrrell, 1985). In yeast also, a number of proteins are produced after UV and X-ray irradiation treatments (Schwencke and Moustacchi, 1982). The generation of oxygen radicals by UV irradiation and during aerobic respiration has been reported by Vuillaume (1987). This in turn leads to the production of other toxic species such as peroxides which perpetuate cell damage. Various enzymes such as catalase and superoxide dismutase are involved in the detoxification of these species (Christman *et al.*,1985). That cytochrome P450 may also fulfill this role is additionally supported by the fact that cells exposed to inhibitors of the haemoprotein were more sensitive to H_2O_2 than control cultures (Morichetti *et al.*, 1989). Cytochrome P450 has also been shown by other researchers to have peroxidase activity (Kappeli, 1986). Morichetti *et al.* (1989) concluded that during conditions of catabolite repression cytochrome P450 is active in cellular protection, whereas under aerobic conditions catalase may be dominant in this role.

Oxygen can therefore be seen to be a critical factor in cytochrome P450 production in yeasts. A delicate balance appears to operate between dissolved oxygen tension and cytochrome P450 production, the optimum conditions prevailing when oxygen is present but at low enough concentrations for semi-anaerobic conditions to operate.

3.2.5 Effect of subculturing on cytochrome P450 production

The method of culture storage and maintenance was also found to have an effect on the levels of cytochrome P450 in the strains of yeasts tested.

Comparisons were made between cells of *C. parapsilosis*, *S. cerevisiae* 673 and *Sch. pombe leu*1-32 *pho*1-44 after subculturing the cells 2-3 times on solid medium and after storage on silica gel without subculturing. Routine subculturing was found to lead to reductions in the amount of enzyme present, in the case of *C. parapsilosis* from 12.14 to 7.56 nmol P450 g^{-1} and in *S. cerevisiae* 673 from 15.25 to 13.32 nmol P450 g^{-1} dry cell weight (tables 3.10, 3.11).

Table 3.10

The effect of subculturing on cytochrome P450 production in *C. parapsilosis* after growth in YEPD with aeration containing 20% (wt/vol) glucose for 18 h.

subcultured on solid medium	stored on silica gel
nmol P450 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
7.56	12.14

Data represents determinations from one experiment. Original sample contained 12.14 nmol P450 g^{-1} dry wt.

Subculturing of *Sch. pombe leu*1-32 *pho*1-44 led to a total loss of the haemoprotein in the cells (table 3.12). However in all the subcultured cells analysed absorbance at 420 nm was detectable and appeared to correlate with the production of cytochrome P420 (table 3.12). This could mean that conversion from the membrane-bound cytochrome P450 to the cytosolic form P420 was occurring.

Table 3.11

The effect of subculturing on cytochrome P450 production in *S. cerevisiae* 673 after growth in YEPD with aeration containing 20% (wt/vol) glucose for 18 h.

subcultured on solid medium	stored on silica gel
nmol P450 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
13.32	15.25

Data represents determinations from one experiment. Original sample contained 15.25 nmol P450 g^{-1} dry wt.

Table 3.12

The effect of subculturing on cytochrome P450 production in *Sch. pombe leu*1-32 *pho*1-44 after growth in YEPD with aeration containing 20% (wt/vol) glucose.

	subcultured on solid medium	subcultured on solid medium	stored on silica gel	stored on silica gel
growth time (h)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
7	0	0	0	0
18	66.81	0	28.55	12.51
20	69.89	0	17.08	7.14
24	73.74	0	10.89	7.02

Data represents determinations from one experiment. Original samples before storage/subculturing contained the same values of P450 as samples after storage on silica gel.

When cultures were maintained by long-term storage on silica gel without routine subculturing, cytochrome P450 levels appeared to be unaffected (tables 3.10-3.12). To allow maximum expression of the haemoprotein

all strains were subsequently stored on silica gel prior to cytochrome P450 determinations.

3.2.6 Determination of the cytochrome P450 content of microsomal fractions of *Sch. pombe*, *S. cerevisiae* and *C. parapsilosis*

In this study subcellular fractionation and isolation of the microsomes of *Sch. pombe* 972, *S. cerevisiae* 673 and *C. parapsilosis* was attempted. The cell wall structures of these yeasts differ greatly. *S. cerevisiae* contains β-(1- 3)-linked and β-(1- 6)-linked glucans; glucans in *Candida parapsilosis* have also been found to contain β-(1- 3)- and β-(1- 6)- linked residues (Fleet, 1991). However *Sch. pombe* cell walls contain α-(1- 3)-glucan linkages as well as β-glucan (Dickinson and Isenberg, 1982). The widely used method to degrade enzymatically the cell walls of yeasts using the gastric juice of *Helix pomatia* which contains β-glucanase activity is thus limited in *Sch. pombe*. In order to use the same method of cell breakage for each species analysed, all cells were disrupted mechanically by a Braun homogeniser MSK. Cell breakage was confirmed by microscopical examination.

Differential centrifugation was then employed to obtain the microsomal fraction. To minimise damage to the microsomes by long periods of centrifugation, attempts were made to precipitate the fraction using centrifugation steps requiring a shorter length of time. A calcium chloride aggregation method for isolating the microsomes was investigated (Albro *et al.*, 1987), but with *Sch. pombe* this proved unsuccessful (data not shown). The polyethylene glycol precipitation method of Wright and

Honek (1989), involving low spin speed (40,000 x *g*) for a short duration (20 minutes) was also attempted.

Table 3.13

Cytochrome P450 and P420 content of microsomes of *S. cerevisiae* 673

sample	mg protein $(ml)^{-1}$	pmol P420 (mg of protein)$^{-1}$	pmol P450 (mg of protein)$^{-1}$
microsomes	35	28.31	24.50

Cells were grown in YEPD containing 20% (wt/vol) glucose for 18 h and contained 23.98 nmol P450 and 37.54 nmol P420 g^{-1} dry wt. Microsomes were prepared using the method of Wright and Honek (1989).

Sch. pombe still gave poor results with this method (data not shown), however microsomes could be successfully prepared from *S. cerevisiae* 673 and *C. parapsilosis* (fig. 3.15, tables 3.13, 3.14).

Yoshida and Aoyama (1984) reported the cytochrome P450 content of *S. cerevisiae* microsomes to be 63 pmol mg^{-1} protein. In the present study *S. cerevisiae* 673 microsomes were found to contain a maximum of 24.5 pmol cytochrome P450 mg^{-1} protein (fig. 3.15, table 3.13). Total protein was determined by Lowry assay as shown in fig. 3.16. The maximum cytochrome P450 content of *C. parapsilosis* was found to be 74 pmol mg^{-1} microsomal protein (table 3.14). This compares favourably with the previously published values for *C. albicans* of 74-100 pmol mg^{-1} protein (Vanden Bossche *et al.*, 1987). While a lower value of 20 pmol mg^{-1} was

Fig. 3.15.

Difference spectra of microsomes of *S. cerevisiae* 673 yielding 24.50 pmol P450 and 28.31 pmol P420 (mg of protein)$^{-1}$. Cells were grown in YEPD containing 20% (wt/vol) glucose for 18 h with aeration. Microsomes were prepared using the method of Wright and Honek (1989). Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.15

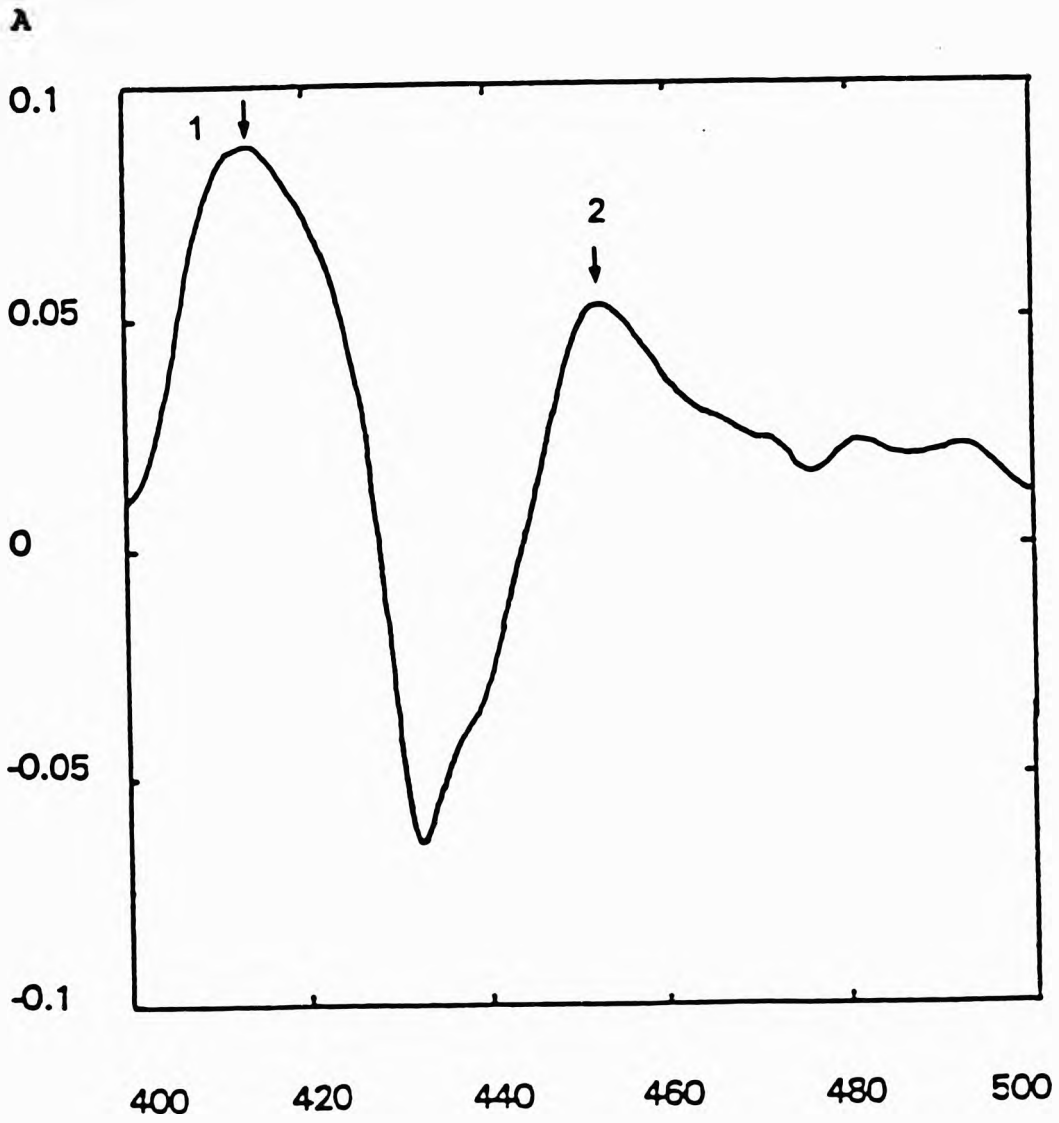
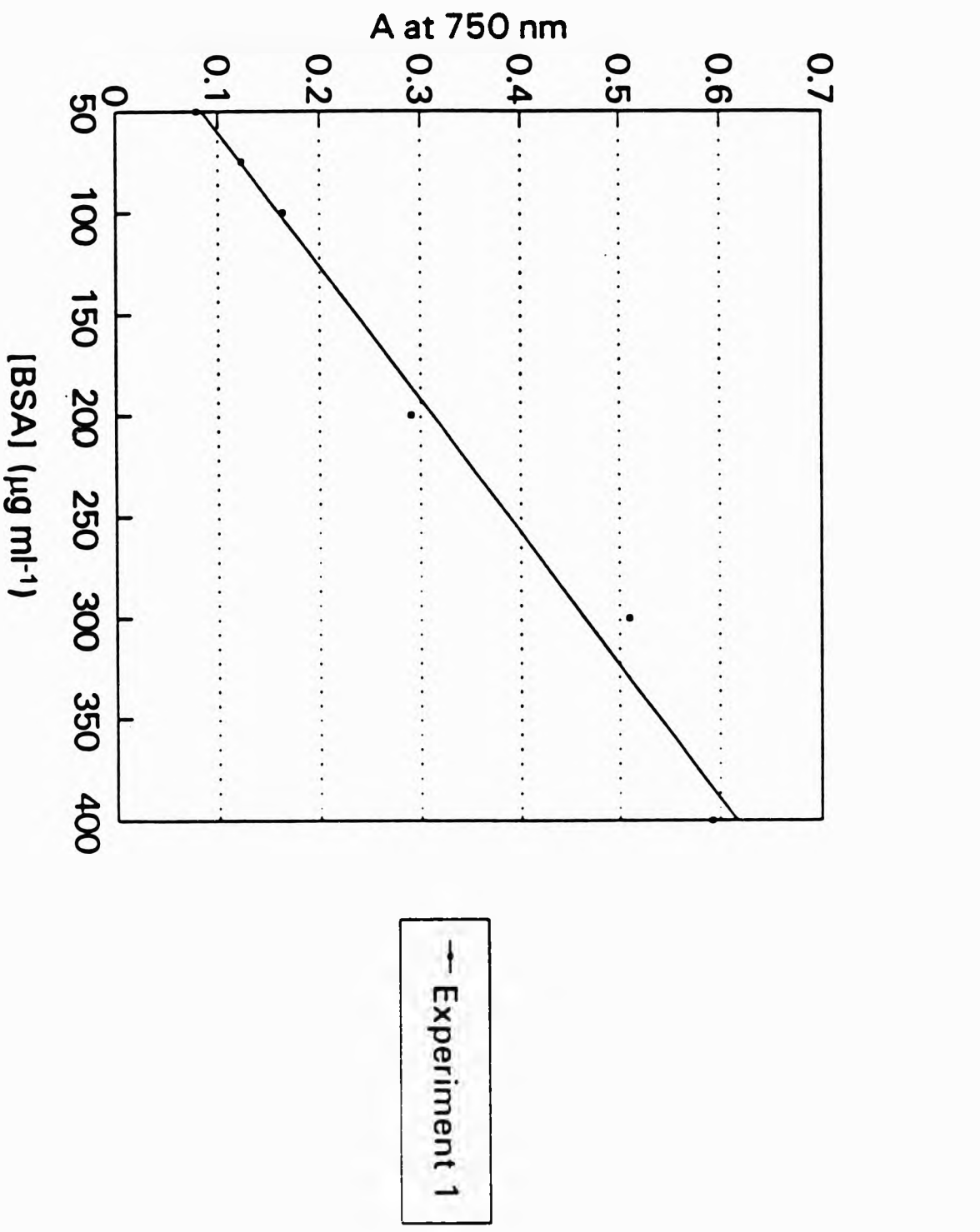


Fig. 3.16.

Standard curve for protein determination by Lowry *et al.* (1951) assay.



noted by Hitchcock *et al.* (1989a) in *C. albicans* strain 3153.

Table 3.14

Cytochrome P450 and P420 content of subcellular fractions of *C. parapsilosis*

sample	mg protein $(ml)^{-1}$	pmol P420 $(mg\ of\ protein)^{-1}$	pmol P450 $(mg\ of\ protein)^{-1}$
microsomes (expt 1)	10.63	77.12	74.43
microsomes (expt 2)	5.00	43.24	21.98
microsomes (expt 3)	10.00	37.84	0
solubilised microsomes (from expt 2)	2.00	0	17.58
solubilised microsomes (from expt 3)	2.00	0	0
pellet fraction of microsomes (from expt 2) after sodium cholate treatment	4.95	27.30	0

Cells were grown in YEPD containing 20% (wt/vol) glucose for 18 h and contained 19.32 nmol P450 and 6.13 nmol P420 g^{-1} dry wt. Microsomes were prepared and cytochrome P450 solubilised by sodium cholate treatment using the method of Wright and Honek (1989).

Some loss of cytochrome P450 was observed during preparation of the microsomes (fig. 3.15). This was likely to be due to conversion of the haemoprotein to cytochrome P420. The use of mechanical disruption methods has been found by other researchers to cause damage to cytochrome P450 (Kappeli, 1986). The presence of an absorbance peak at 450 nm in other cell fractions may also be attributed to the extreme mechanical forces used for cell breakage. This agrees with observations

by Duppel *et al.* (1973).

3.2.7 Extraction and purification of cytochrome P450 from *C. parapsilosis*

Due to the fact that cytochrome P450 is a membrane-bound enzyme it was necessary to solubilise the haemoprotein using detergents. Sodium cholate was used to release the enzyme from its membrane-bound state and the conversion to P420 was minimised by the use of glycerol in the solubilising medium to stabilise the enzyme (Ichikawa and Yamano, 1967).

Solubilisation of the membrane-bound enzyme from isolated microsomes of *C. parapsilosis* followed by differential centrifugation showed that the solubilised fraction (or supernatant) contained a cytochrome species absorbing at 450 nm, whereas the 420 nm absorbing species remained in the pelleted fraction (fig. 3.17). This agrees with observations by Honek (1993). The solubilised fraction yielded a value of 17.58 pmol cytochrome P450 mg^{-1} protein.

Both fractions were subjected to SDS-PAGE analysis. Solubilised microsomal samples of *C. parapsilosis* showed a major band which was also consistently observed in the untreated microsomal samples (figs. 3.18, 3.19). From the absorption spectra data obtained (fig. 3.17) it may be postulated that the major band in the solubilised microsomes corresponds to cytochrome P450. Analysis of bands in gel number 2 (fig. 3.19) showed that the putative band containing cytochrome P450 found

Fig. 3.17.

Difference spectra of a) solubilised microsomes and b) pellet fraction after sodium cholate treatment of *C. parapsilosis* microsomes prepared in experiment 2. Cytochrome P450 content of the solubilised microsomes was 17.58 pmol (mg of protein)$^{-1}$. Cytochrome P420 content of the pelleted fraction was 27.30 pmol (mg of protein)$^{-1}$. Microsomes were prepared and solubilised according to the method of Wright and Honek (1989). Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 3.17a.

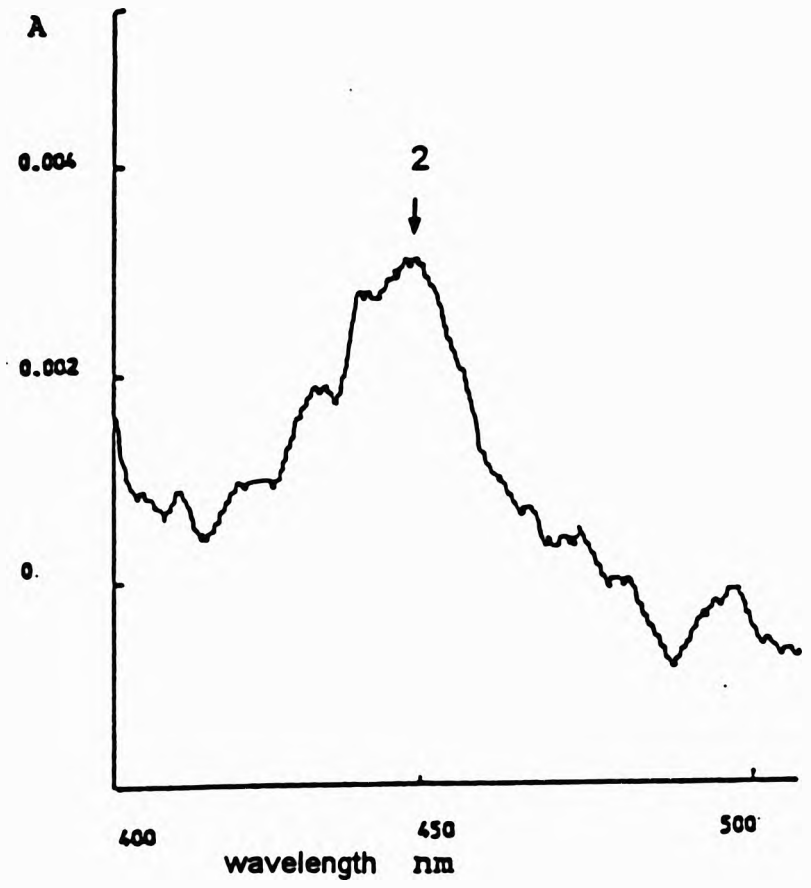


Fig. 3.17b.

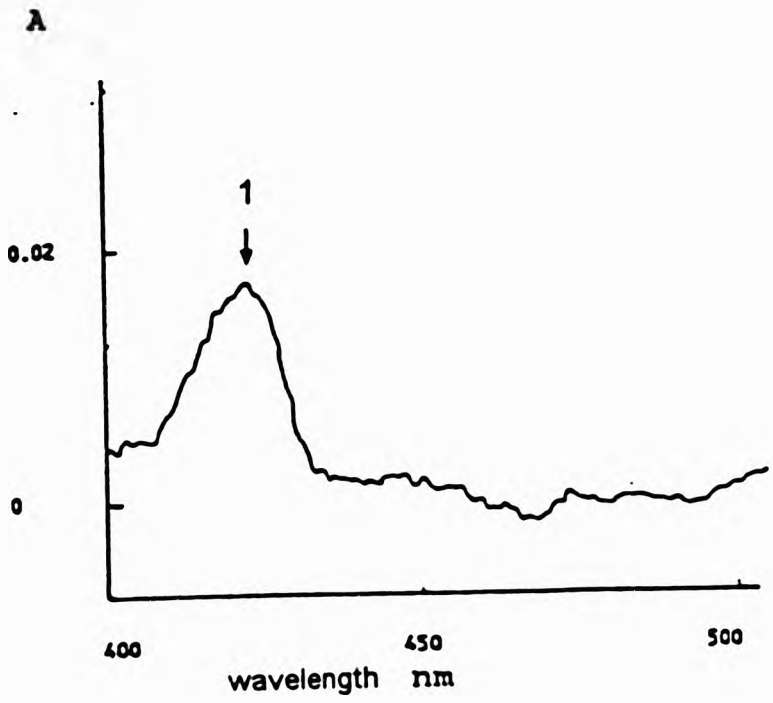


Fig. 3.18.

SDS-PAGE of solubilised microsomes of *C. parapsilosis*. 1: molecular mass standards, (β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa). 2: solubilised microsomes showing a major band migrating at 48 kDa (indicated by arrow). Microsomes from *C. parapsilosis* (prepared in experiment 2) were solubilised using the method of Wright and Honek (1989). Lane 1 contained 150 μg protein. Lane 2 contained 50 μg protein.

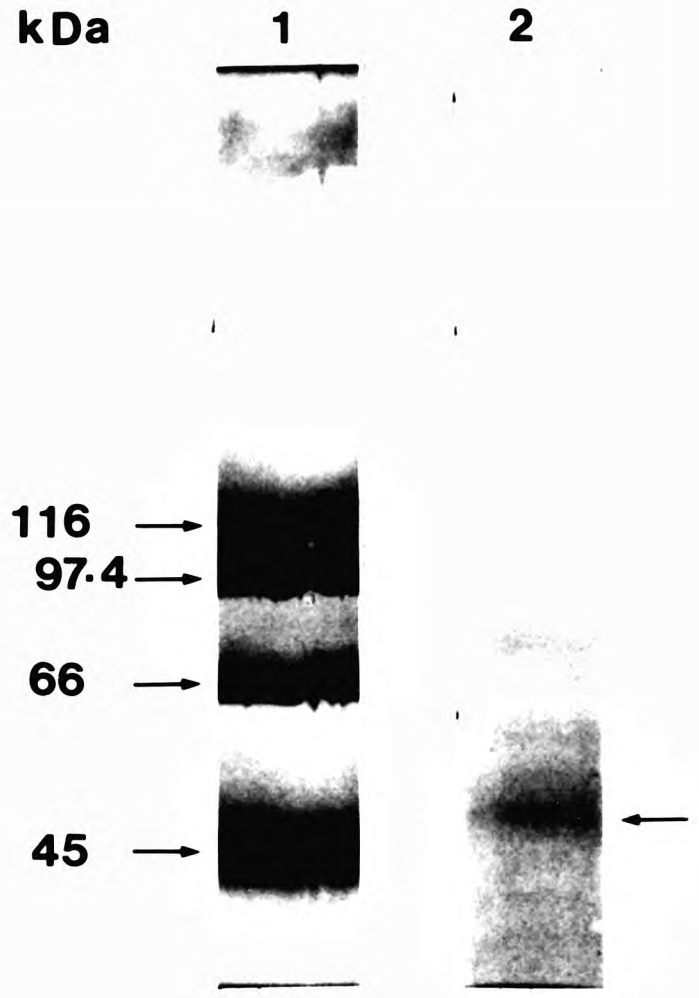
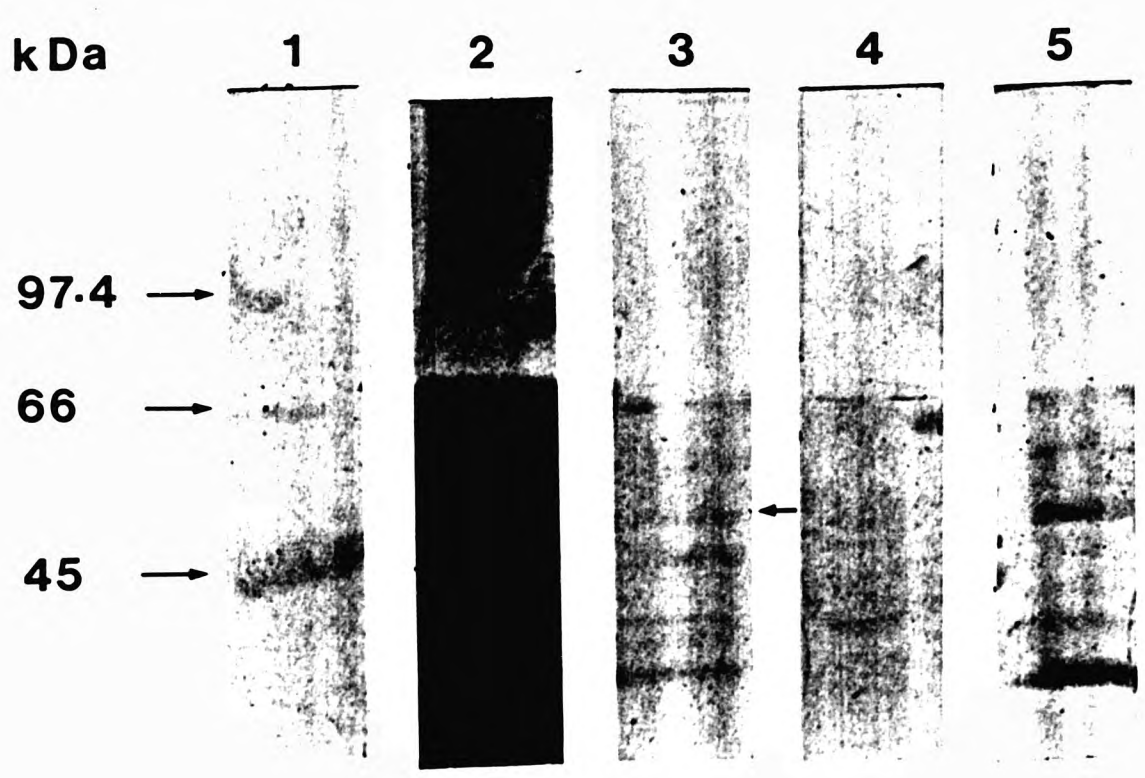

kDa
1
2
116
97·4
66
45

Fig. 3.19.

SDS-PAGE of microsomal fractions of *C. parapsilosis*. 1: molecular mass standards, (phosphorylase b, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa). 2: microsomes (prepared in experiment 2); 3: solubilised microsomes (from microsomes prepared in experiment 2); 4: solubilised microsomes (from microsomes prepared in experiment 3); 5: pelleted fraction from microsomes after treatment with sodium cholate. Microsomes were prepared and solubilised using the method of Wright and Honek (1989). Lane 1 contained 60 μg protein. Lanes 2, 3, 4 and 5 contained 100 μg of protein.


kDa
1
2
3
4
5
97.4
66
45

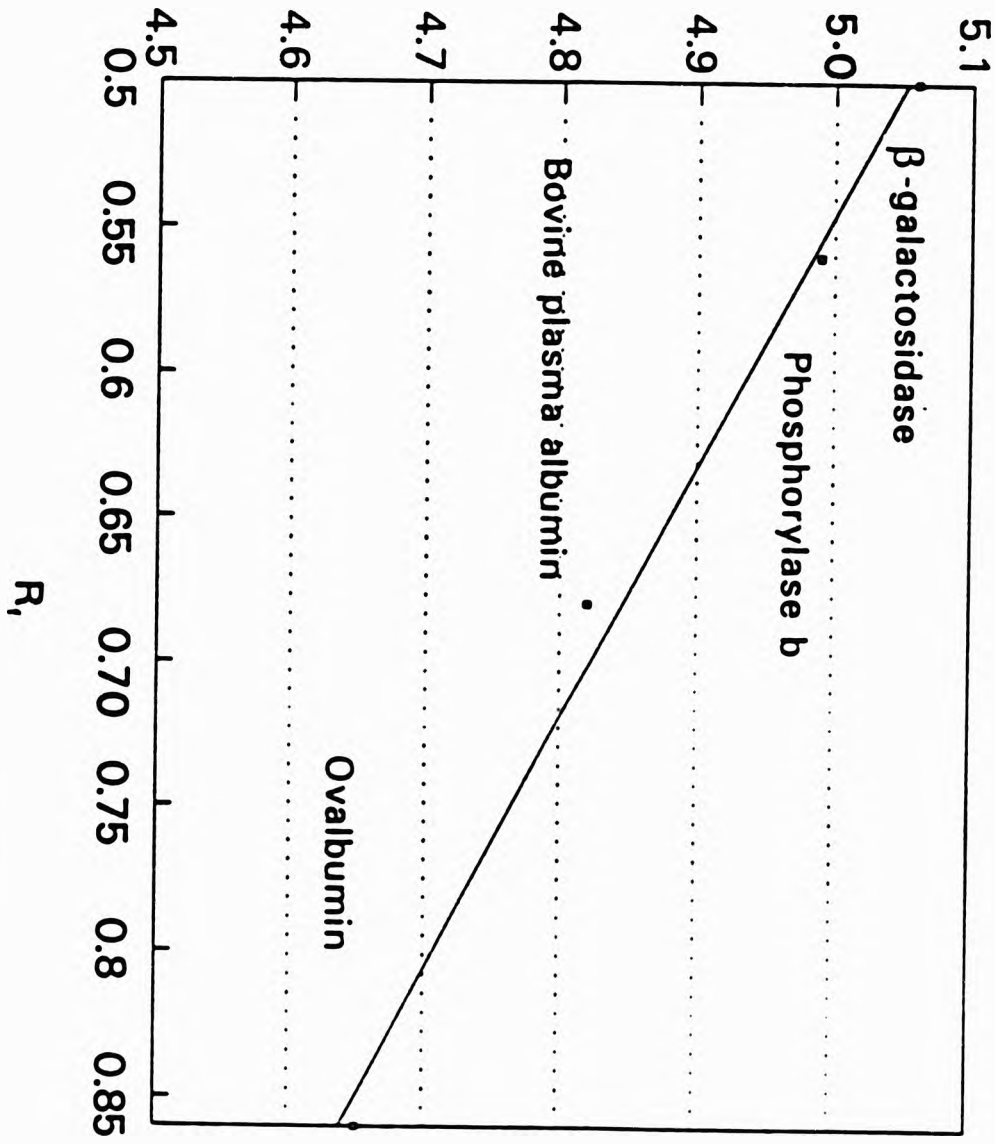
in the cholate treated microsomes prepared in experiment 2 was absent from the cholate treated microsomes prepared in experiment 3. This latter preparation was used as a negative control due to the observation that no species absorbing at 450 nm was present in this preparation.

Standard curves were constructed using the relative mobilities of proteins of known molecular mass calculated from SDS-PAGE analysis (fig. 3.20). From these plots the putative band corresponding to cytochrome P450 was estimated to be approximately 48 kDa. This value is similar to the reported molecular mass of 51 kDa for cytochrome P450-dependent 14α-sterol demethylase (CYP51) isolated from *C. albicans* (NCPF 3153) using SDS-PAGE in 9% gels. This microsomal cytochrome P450 has been found to perform the NADPH-dependent 14α-demethylation of lanosterol in the presence of dilauroyl-phosphatidylcholine and oxygen (Hitchcock *et al.*, 1989b).

Extensive research has been carried out on purified mammalian forms of cytochrome P450. Molecular masses of between 48 kDa and 56 kDa have been reported for different isozymes (Guengerich *et al.*, 1982; Ryan *et al.*, 1984). Since cytochrome P450 isozymes are sensitive to the varying conditions of SDS-PAGE, there may be a large variation in migration differences between isozymes and consequently in their calculated molecular weights (Guengerich *et al.*, 1982). Differences in molecular mass have been reported of up to 5 kDa for the same isozyme (Astrom and DePierre, 1986). Yeast cytochromes P450 have been concluded to be similar to mammalian ones in terms of their molecular weight (King *et al.*, 1984). Data have shown that the CYP51 from *S.*

Fig. 3.20.

Calibration curve for molecular mass determination of protein samples for SDS-PAGE gel shown in fig. 3.18. The calibration curve was fitted using least squares analysis on Harvard Graphics 3 (IBM).



cerevisiae exhibits more homology to its mammalian counterparts than to prokaryotic enzymes. Lai and Kirsch (1989) determined the nucleotide sequence of *C. albicans* CYPL1A1 and found a high degree of homology in size and amino acid sequence between *Saccharomyces* and *Candida* enzymes concluding that the genes from the two species were closely related.

CHAPTER 4

EVALUATION OF THE TOXICITY OF CARBON TETRACHLORIDE IN *SCH. POMBE* AND *S. CEREVISIAE*

4.1 INTRODUCTION

The toxicity of carbon tetrachloride has been well documented in mammalian cells. Carbon tetrachloride has been found to be toxic to liver cells *in vitro* and *in vivo* (Ugazio *et al.*, 1978). In mouse hepatocytes, Ruch *et al.* (1986) found that doses of 2.5 mM carbon tetrachloride caused up to 50% greater released lactate dehydrogenase activity, which was used as a measure of cytotoxicity.

There have, however, been only limited investigations of carbon tetrachloride toxicity in yeasts (Callen and Philpot,1977). This section is mainly concerned with the evaluation of the toxic effects of carbon tetrachloride in *Sch. pombe* 972 but data are also presented on work with *S. cerevisiae* 188. Carbon tetrachloride has been reported to produce free-radical metabolites in mammalian cells. This hypothesis was tested in *Sch. pombe* by the application of α–tocopherol, an anti-oxidant, to carbon tetrachloride-treated cells.

4.2 RESULTS AND DISCUSSION

4.2.1 Evaluation of the toxic action of carbon tetrachloride in yeast cells

Exposure of cells of *Sch. pombe* and *S. cerevisiae* to carbon tetrachloride gave results which demonstrated that the compound exhibited toxicity in a dose dependent manner in these species. A spectrophotometric method of determining inhibition of growth using absorbance measurements and a second method using viable cell counts indicated a difference in tolerance to the compound between the two yeasts (table 4.1, 4.2, figs. 4.1, 4.2). *Sch. pombe* can be seen to be slightly more sensitive to the effects of the compound, viability decreased sharply at 6.5 mM in both absorbance and viable count measurements.

Table 4.1

Toxicity of carbon tetrachloride in *Sch. pombe* 972 and *S. cerevisiae* 188 by absorbance measurement.

	Sch. pombe 972	*S. cerevisiae* 188
$[CCl_4]$ (mM)	Absorbance at 540 nm	Absorbance at 540 nm
0	0.39	0.62
0.065	0.38	0.58
0.32	0.36	0.67
0.65	0.37	0.70
6.5	0.015	0.49

Cells were grown for 18 h in YEPD containing 20% (wt/vol) glucose without aeration. Data represents determinations from one experiment.

Carbon tetrachloride is thought to exert cellular toxicity by the production

Fig. 4.1a.

Toxicity of carbon tetrachloride in *Sch. pombe* 972. Cells were grown in the presence of carbon tetrachloride for 18 h. Cell survival was evaluated by viable count. Data points represent the mean of four experiments ± SEM each comprising two replicates. Significant differences between values were calculated using the LSD criterion from ANOVA. Error bar shown represents LSD of $P< 0.01 = 28.80$.

Fig. 4.1a. Toxicity of carbon tetrachloride in *Sch. pombe* 972

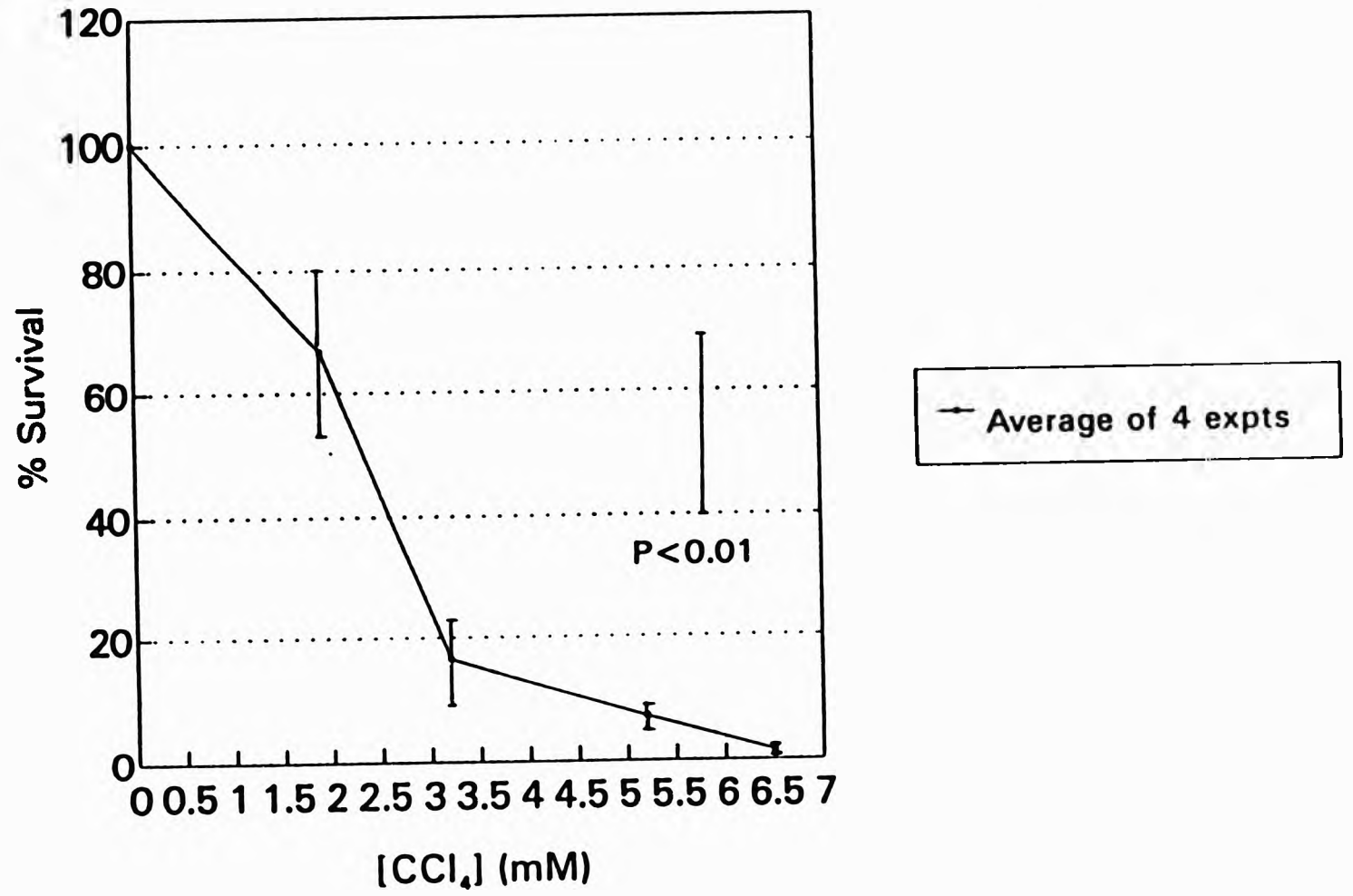


Fig. 4.1b.

Toxicity of carbon tetrachloride in *Sch. pombe* 972. Cells were grown in the presence of carbon tetrachloride for 18 h. Cell survival was evaluated by viable count determination. Data points represent the mean of four experiments ± SEM each comprising two replicates. Significant differences between values were calculated using the LSD criterion from ANOVA. Error bar shown represents LSD of $P < 0.01 = 45.27$.

Fig. 4.1b. Toxicity of carbon tetrachloride in *Sch. pombe* 972

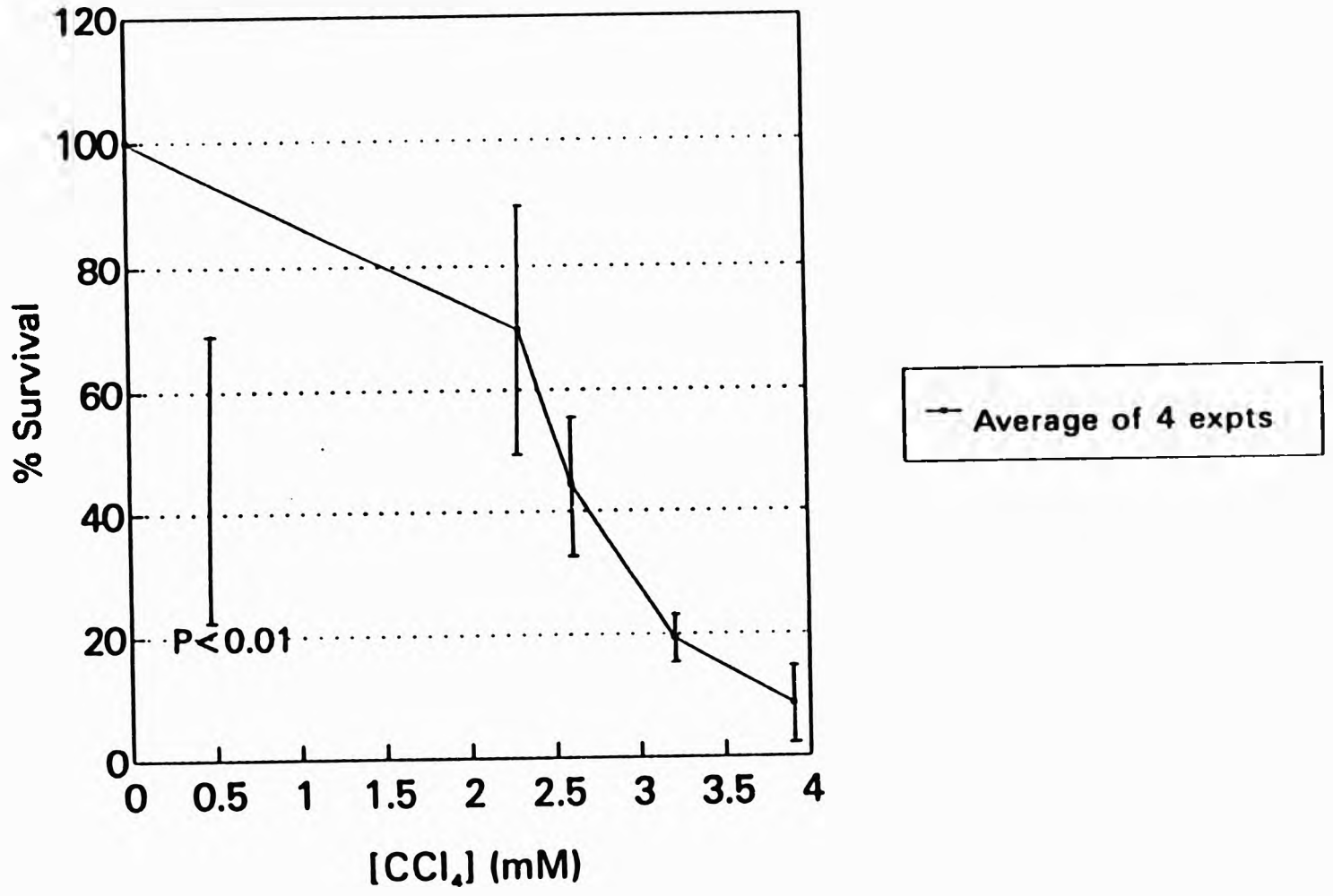


Fig. 4.1c.

Toxicity of carbon tetrachloride in *Sch. pombe leu*2-120 *lys*3-37. Cells were grown in the presence of carbon tetrachloride for 18 h. Cell survival was evaluated by viable count determination. Data points represent the mean of two replicates.

Fig. 4.1c. Toxicity of carbon tetrachloride in *Sch. pombe leu2-120 lys3-37*

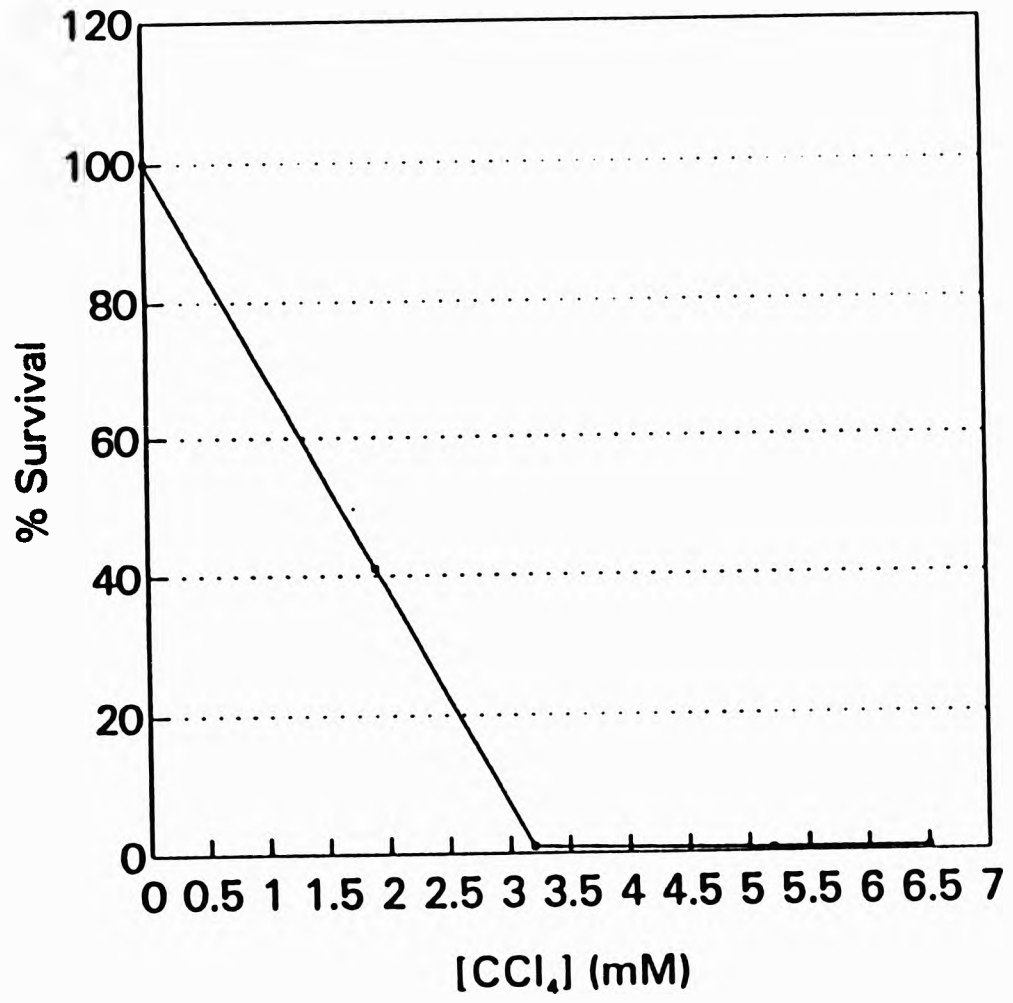


Fig. 4.1d.

Toxicity of carbon tetrachloride in *Sch. pombe lys*1-131. Cells were grown in the presence of carbon tetrachloride for 18 h. Cell survival was evaluated by viable count determination. Data points represent the mean of two replicates.

Fig. 4.1d. Toxicity of carbon tetrachloride in *Sch. pombe lys1-131*

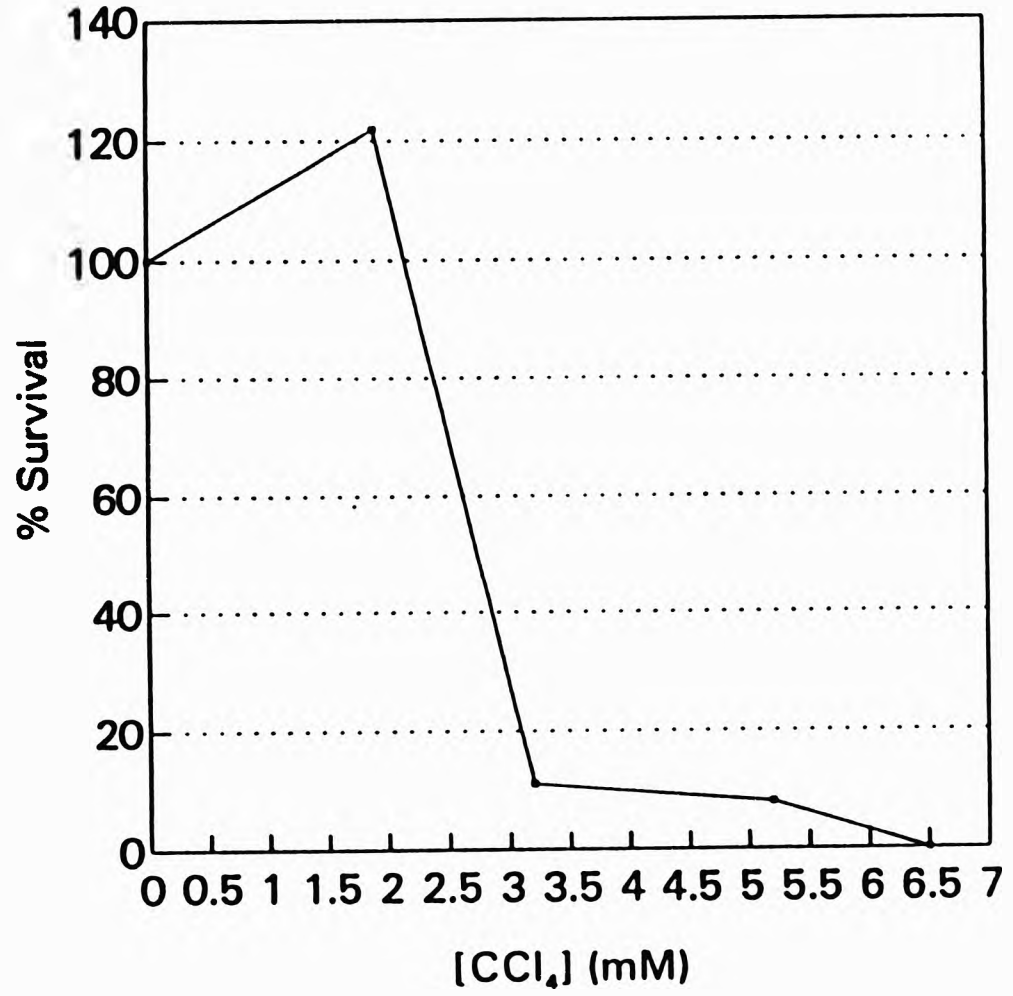


Fig. 4.1e.

Toxicity of carbon tetrachloride in *Sch. pombe ade6*. Cells were grown in the presence of carbon tetrachloride for 18 h. Cell survival was evaluated by viable count determination. Data points represent the mean of two experiments ± SEM each comprising two replicates. Significant differences between values were calculated using the LSD criterion from ANOVA. Error bar shown represents LSD of $P< 0.01 = 69.49$.

Fig. 4.1e. Toxicity of carbon tetrachloride in *Sch. pombe ade6*

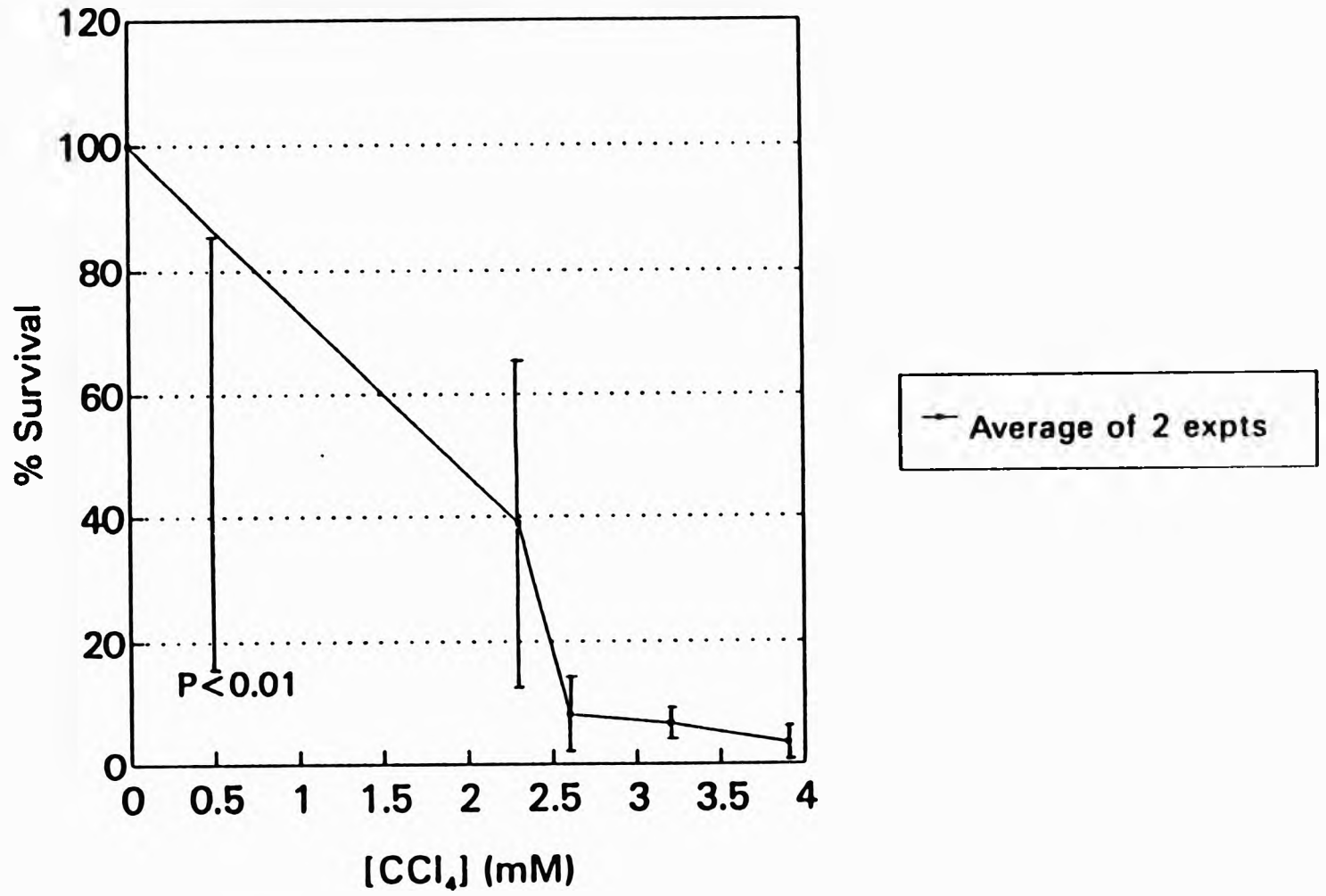


Fig. 4.2a.

Toxicity of carbon tetrachloride in *S. cerevisiae* 188. Cells were grown in the presence of carbon tetrachloride for 18 h. Cell survival was evaluated by viable count determination. Data points represent the mean of three experiments ± SEM each comprising two replicates. Significant differences between values were calculated using the LSD criterion from ANOVA. Error bar shown represents LSD of $P< 0.01 = 31.48$.

Fig. 4.2a. Toxicity of carbon tetrachloride in *S. cerevisiae* 188

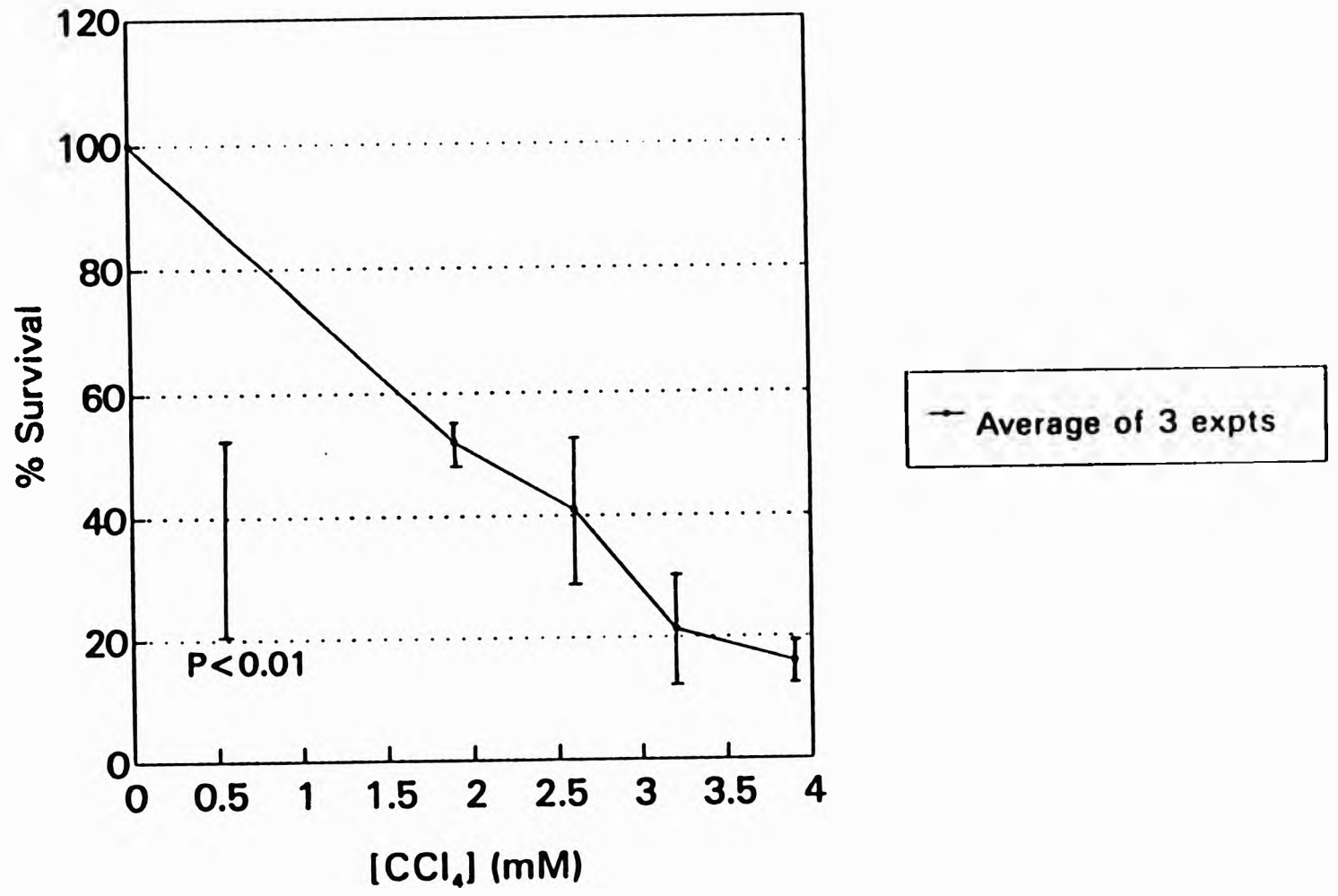
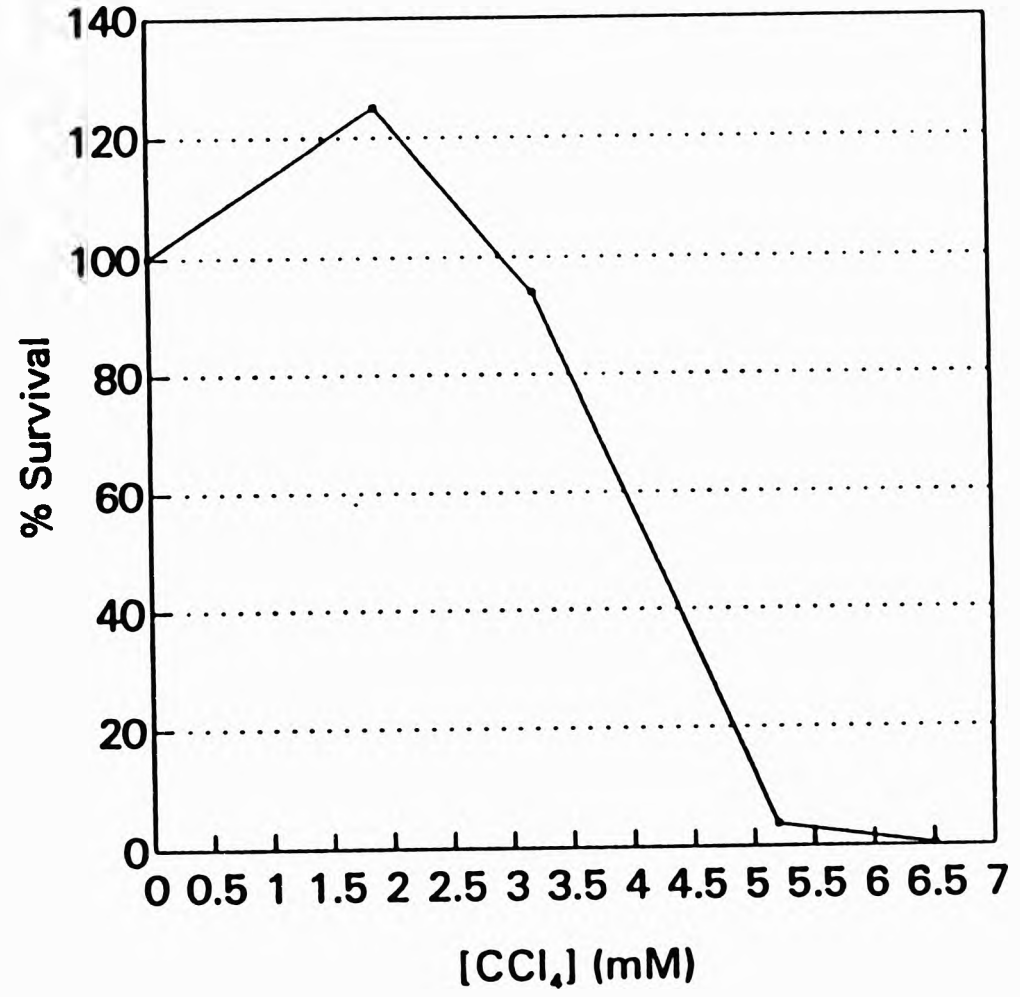


Fig. 4.2b.

Toxicity of carbon tetrachloride in *S. cerevisiae* 159. Cells were grown in the presence of carbon tetrachloride for 18 h. Cell survival was evaluated by viable count determination. Data points represent the mean of two replicates.

Fig. 4.2b. Toxicity of carbon tetrachloride in *S. cerevisiae* 159



of an unstable trichloromethyl radical (Brattin *et al.*, 1985). This is

Table 4.2

Toxicity of carbon tetrachloride in *Sch. pombe* 972 by viable count measurement

$[CCl_4]$ (mM)	Average no. cfu ml^{-1}	% survival
0	4.2×10^6	100
0 (+ ETOH)	4.1×10^6	97.6
1.9	4.2×10^6	100
3.2	0.7×10^6	16.7
5.2	0.3×10^6	7.1
6.5	0.1×10^6	2.4

Determinations represent means of two replicates from one experiment (shown in fig. 4.1a experiment 2).

produced *in vivo* by the cytochrome P450 system metabolising carbon tetrachloride:

$$CCl_4 + e^- \longrightarrow \cdot CCl_3 + Cl^-$$

Cytochrome P450 was found to be present in both *Sch. pombe* 972 and *S. cerevisiae* 188 (see chapter 3), giving maximum values at 18 h growth of 22.35 and 5.13 nmol g^{-1} dry cell weight respectively. Toxicity assays were therefore performed after 18 h growth of the cultures in glucose medium when maximum levels of cytochrome P450 would be expected.

Addition of a final concentration of 6 mM carbon tetrachloride to cells of

Sch. pombe and microsomes of *S. cerevisiae* not previously exposed to the compound yielded a type I binding spectrum (fig. 4.3). This type of binding is indicated by a trough at 420 nm and a peak at 390 nm and is characteristic of binding to the active site of the enzyme. Type I binding spectra are therefore usually exhibited by substrates of cytochrome P450. This implies that carbon tetrachloride may have been acting as a substrate for yeast cytochrome P450. As the cells were not previously exposed to the compound it is possible that a constitutive cytochrome P450 species with broad substrate specificity was involved (Atchia *et al.*, 1991b). Carbon tetrachloride has previously been characterised as producing type I binding spectra in mammalian cells (McClean, 1967).

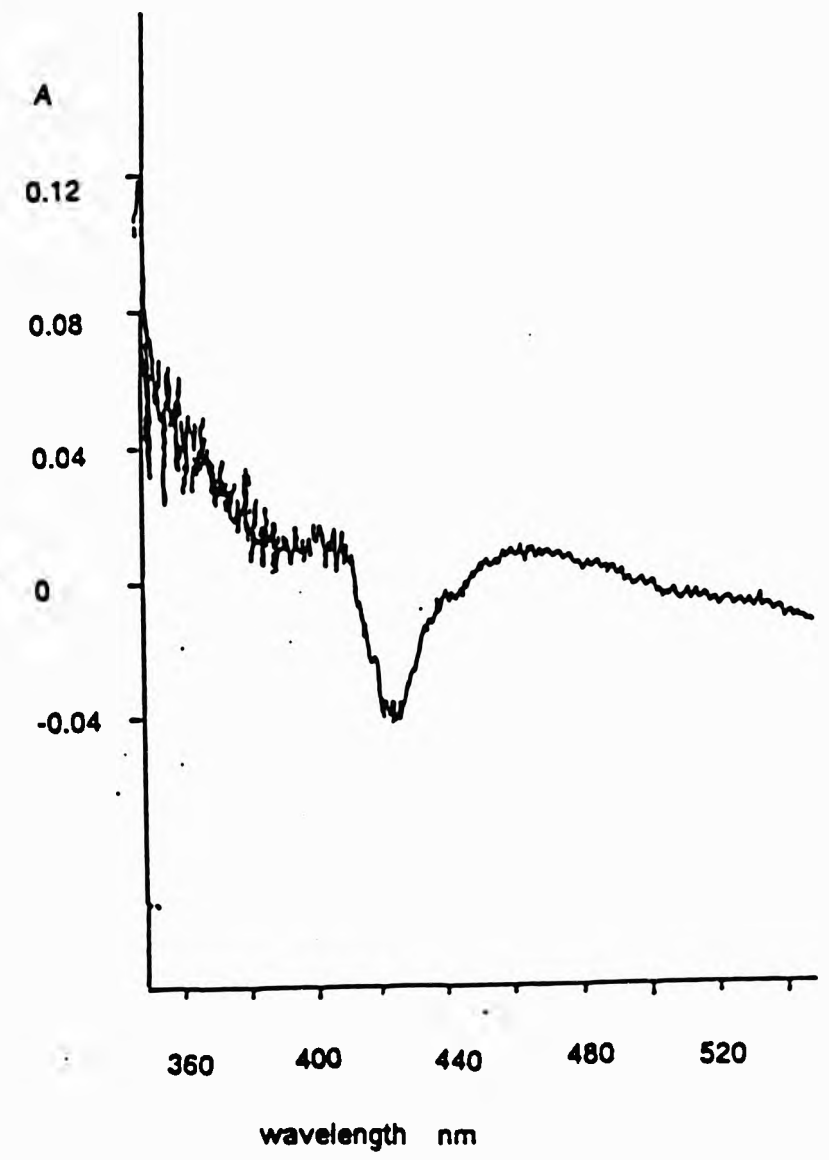
Carbon tetrachloride has been reported to be a substrate for the specific cytochrome P450 isozyme CYP2E1 in human cell lines (Crespi, 1991). In liver microsomes of phenobarbital-induced rats a 52 kDa form of cytochrome P450 was found to generate the trichloromethyl radical from carbon tetrachloride in a reconstituted system (Noguchi *et al.*, 1982).

Since ethanol was used for sterilising and dissolving carbon tetrachloride in this work, additional controls containing ethanol alone were run in many of the experiments. Cultures were grown with 1% (vol/vol) absolute ethanol in the growth medium and incubated for 18 h as with the carbon tetrachloride treated cultures. In the graphical representation of the data (figs. 4.1, 4.2) controls containing ethanol were taken as having 100% survival of the cells. Thus carbon tetrachloride toxicity could be seen over and above any toxic effects of the ethanol. Results showed

Fig. 4.3.

Type 1 binding spectrum produced after addition of 6 mM carbon tetrachloride to whole cells of *Sch. pombe* 972. Cells were grown in YEPD containing 20% (wt/vol) glucose for 18 h yielding 3.6 nmol cytochrome P450 g^{-1} dry cell weight.

Fig. 4.3.



that the presence of 1% ethanol in the cultures did not cause any significant additional toxic effects (table 4.3). In some cases the presence of the alcohol caused a slight rise in cell numbers, possibly due to utilisation of this compound as a substrate or it having a protective effect.

Table 4.3

Toxicity of 1% (vol/vol) ethanol in the growth medium of *Sch. pombe* 972

conditions	% survival
-ethanol	100%
+ethanol	110.9% ± SEM 13.8%

Data represents means ± SEM of seven experiments each comprising two replicates.

Work by Morita and Mifuchi (1984) showed that the presence of 1.5% ethanol in YPG medium increased the cytochrome P450 cellular content to 150% of that in control cells of *S. cerevisiae* D7. Ethanol has also been found to be an inducer of cytochrome P450 3a (Lewis *et al.*, 1987), which is additionally classified as CYP2E1 (Crespi, 1991, see also Chapter 5). It has been observed that this 'alcohol-inducible' form of cytochrome P450 has the ability to activate carbon tetrachloride (Ebel, 1989). However, in this present work any increase in cytochrome P450 levels induced by ethanol did not result in significant increases in cellular toxicity or genotoxicity (see chapter 5).

4.2.2 Effect of α–tocopherol on the toxic action of carbon tetrachloride.

Studies demonstrated some variability in the toxicity of carbon tetrachloride to *Sch. pombe* cells when concentrations of below 3.2 mM were utilised (fig. 4.1). This was taken into account when designing experiments to assess the effect of α–tocopherol on carbon tetrachloride toxicity. Carbon tetrachloride was consistently found to be toxic at concentrations of 5.2 and 6.5 mM (fig.4.1). These concentrations were thus utilised in subsequent experiments with α-tocopherol.

Preliminary experiments indicated that α–tocopherol had little effect on endogenous toxicity but increased recovery rates of the cells exposed to

Table 4.4

Effect of α–tocopherol on carbon tetrachloride toxicity in *Sch. pombe* 972.

$[CCl_4]$ (mM)	[α-tocopherol] (μM)	Average no. cfu ml^{-1}	% survival
0	0	16.7×10^6	100
0	5	16.9×10^6	101.2
0	50	14.6×10^6	87.4
5.2	0	3.9×10^6	23.4
5.2	5	3.8×10^6	22.8
5.2	50	7.1×10^6	42.5

Data represents determinations from one experiment comprising two replicates. Cells were grown in the presence of carbon tetrachloride and α-tocopherol for 18 h

carbon tetrachloride (tables 4.4, 4.5). The application of 50 μM α–tocopherol to cells treated with 5.2 mM carbon tetrachloride showed higher survival in comparison to carbon tetrachloride exposed cells without α–tocopherol treatment (table 4.4).

However although the highest concentration of α–tocopherol ameliorated the toxic effect of carbon tetrachloride in comparison to the control cultures, relatively little protection was afforded by the application of α–tocopherol to cells treated with 6.5 mM carbon tetrachloride, as this concentration caused greater cell death (table 4.5). *Sch. pombe* cells were subsequently exposed to 5.2 mM carbon tetrachloride and treated with varying concentrations of the anti-oxidant α–tocopherol (fig. 4.4).

Table 4.5

Effect of α–tocopherol on carbon tetrachloride toxicity in *Sch. pombe* 972.

[CCl_4] (mM)	[α-tocopherol] (μM)	Average no. cfu ml^{-1}	% survival
0	0	4.1×10^6	100
0	50	3.9×10^6	95.1
0	100	4.3×10^6	104.9
6.5	0	0.2×10^5	0.5
6.5	50	0.5×10^5	1.2
6.5	100	0.2×10^6	4.9

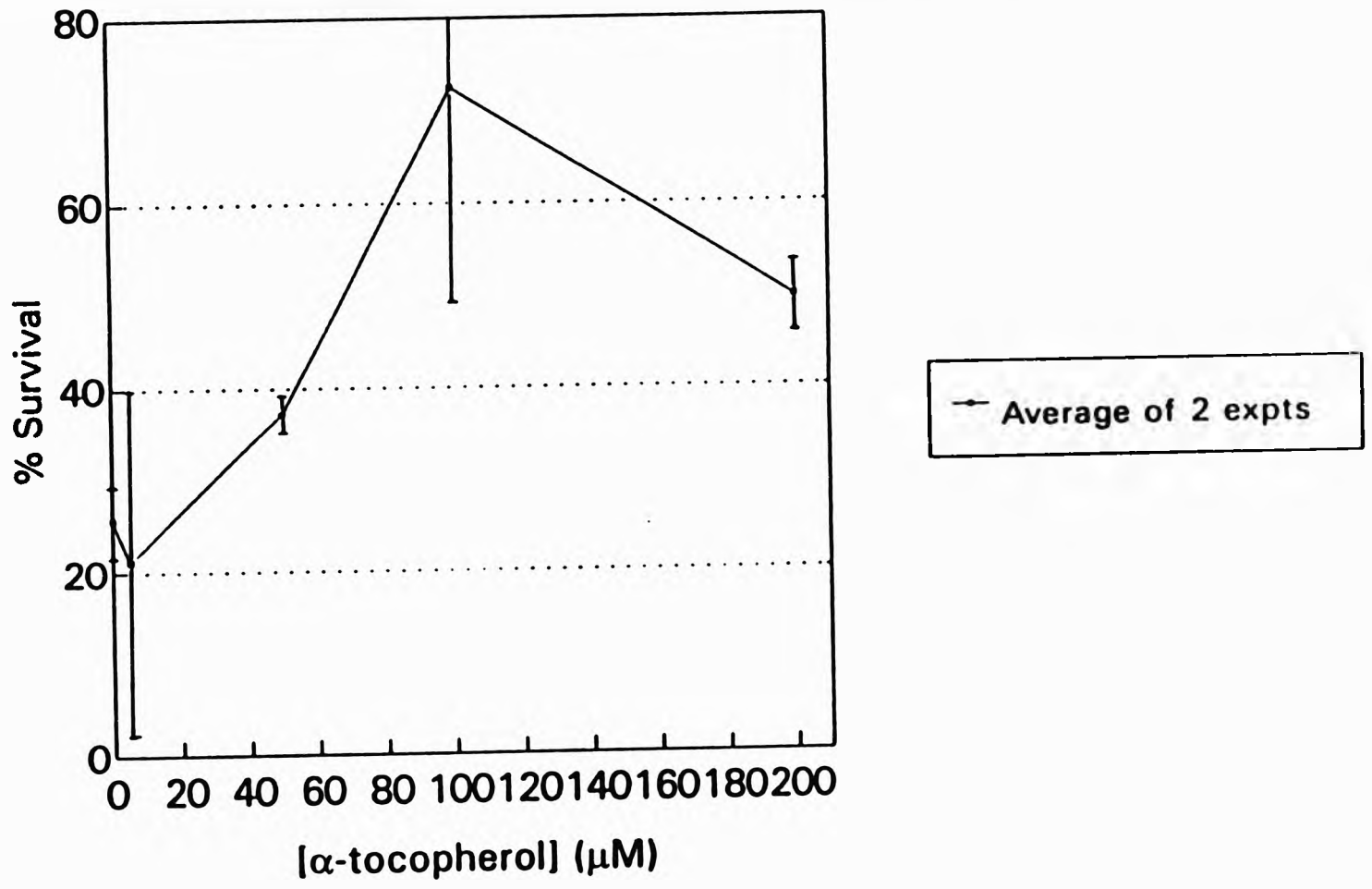
Data represents determinations from one experiment comprising two replicates. Cells were grown in the presence of carbon tetrachloride and α-tocopherol for 18 h

As the concentration of α–tocopherol increased above 5 μM, a trend was seen where the toxic

Fig. 4.4.

The effect of α-tocopherol on carbon tetrachloride toxicity in *Sch. pombe* 972. Cells were grown in the presence of 5.2 mM carbon tetrachloride and varying concentrations of α-tocopherol for 18 h. Cell survival was evaluated by viable count determination. Data points represent the mean of two experiments ± SEM each of which comprised two replicates.

Fig. 4.4. Effect of α-tocopherol on carbon tetrachloride toxicity in *Sch. pombe* 972



effect of carbon tetrachloride decreased. The protective effect was maximal at 100 μM (fig. 4.4). Without the anti-oxidant cell survival rates were approximately 22.7%. Cells exposed to 100 μM α–tocopherol showed a mean 72.5 % survival (fig. 4.4).

The optimum concentration of the anti-oxidant thus increased cell survival rates by approximately 50% over control cultures, (fig. 4.4; Atchia *et al.*, 1991b). These data compare with that of Ruch *et al.* (1986), who demonstrated that carbon tetrachloride-induced toxicity was reduced by the administration of 100 μM α–tocopherol acetate to mouse hepatocytes.

Slater and Sawyer (1970) found that administration of 5.5 μM α–tocopherol alleviated lipid peroxidation in carbon tetrachloride-treated rat microsomal suspensions. Miyazawa *et al.* (1990) observed a significant accumulation of phospholipid hydroperoxides, particularly phosphatidylcholine hydroperoxide (PCOOH), in the livers of carbon tetrachloride-treated rats and that the accumulation of this peroxidation product was inhibited by pre-administering α–tocopherol to the animals.

It is possible that in *Sch. pombe* the α–tocopherol may have been acting in the same way, *viz* preventing lipid peroxidation by scavenging free radicals generated from carbon tetrachloride. The involvement of lipid peroxidation in the toxicity of carbon tetrachloride was investigated using the thiobarbituric acid test on whole cells of *Sch. pombe* 972 exposed to varying doses of carbon tetrachloride. However no positive results could

be obtained (data not shown). This could have been due to the failure of thiobarbituric acid to penetrate the cells and reach the target molecules. However, as this test utilises the production of malonaldehyde as the end-point for the detection of lipid peroxidation, it could have been the case that significant amounts of this compound were not produced in this system. Alternatively the toxicity of carbon tetrachloride in *Sch. pombe* may not be related to lipid peroxidation.

The trichloromethyl radical generated from carbon tetrachloride metabolism is able to combine with molecular oxygen to form the trichloromethylperoxyl radical (Slater, 1982) as below:

$$\cdot CCl_3 + O_2 \longrightarrow CCl_3OO\cdot$$

This species is more electrophilic than the $\cdot CCl_3$ radical and may act predominantly by promoting lipid peroxidation (Slater, 1982). The mode of action of the trichloromethyl radical may also have other mechanisms such as covalent binding. The production of both radicals may contribute to the genotoxicity of the compound (see Chapter 5).

Lipid peroxidation is a process by which lipids are metabolised to form products such as lipid peroxides and hydroperoxides. The process, which proceeds by a series of chain reactions, occurs after the initial abstraction of a hydrogen atom from a fatty acid molecule by a radical species. When this occurs in a polyunsaturated fatty acid molecule, instability produces molecular rearrangements in the molecule generating

relatively stable compounds such as conjugated dienes (Holman, 1954). More oxygen uptake then occurs, propagating the process. This attack causes eventual decomposition of the peroxidised fatty acids (see fig. 4.5).

Damage caused by lipid peroxidation has been found to be reduced by the action of compounds with antioxidant properties such as α–tocopherol (fig. 4.6). The molecule acts by the donation of a hydrogen atom from the chromanol ring hydroxyl group to the lipid radical, generating a tocopheroxy radical. Since the reaction of α–tocopherol is very rapid and the tocopheroxy radical relatively unreactive the propagation of the peroxidation process ceases.

The phenomenon of lipid peroxidation has been reported not to occur in *S. cerevisiae* cells (Bilinski *et al.*,1989), due to their reputed inability to synthesise polyunsaturated fatty acids. However this report contrasts with the work of Gorlov *et al.* (1985), who detected diene conjugates in *S. cerevisiae* 746 and LL-20 after treatment with chelating agents. These conjugates are known to be products of lipid peroxidation.

4.2.3 Comparison of the fatty acid composition of *S. cerevisiae* and *Sch. pombe*

Despite the report of Bilinski *et al.* (1989), the presence of polyunsaturated acids in yeasts including *S. cerevisiae* has been the subject of much investigation.

Fig. 4.5.

Scheme to show the process of lipid peroxidation in a fatty acid containing three double bonds (from Halliwell and Gutteridge, 1989).

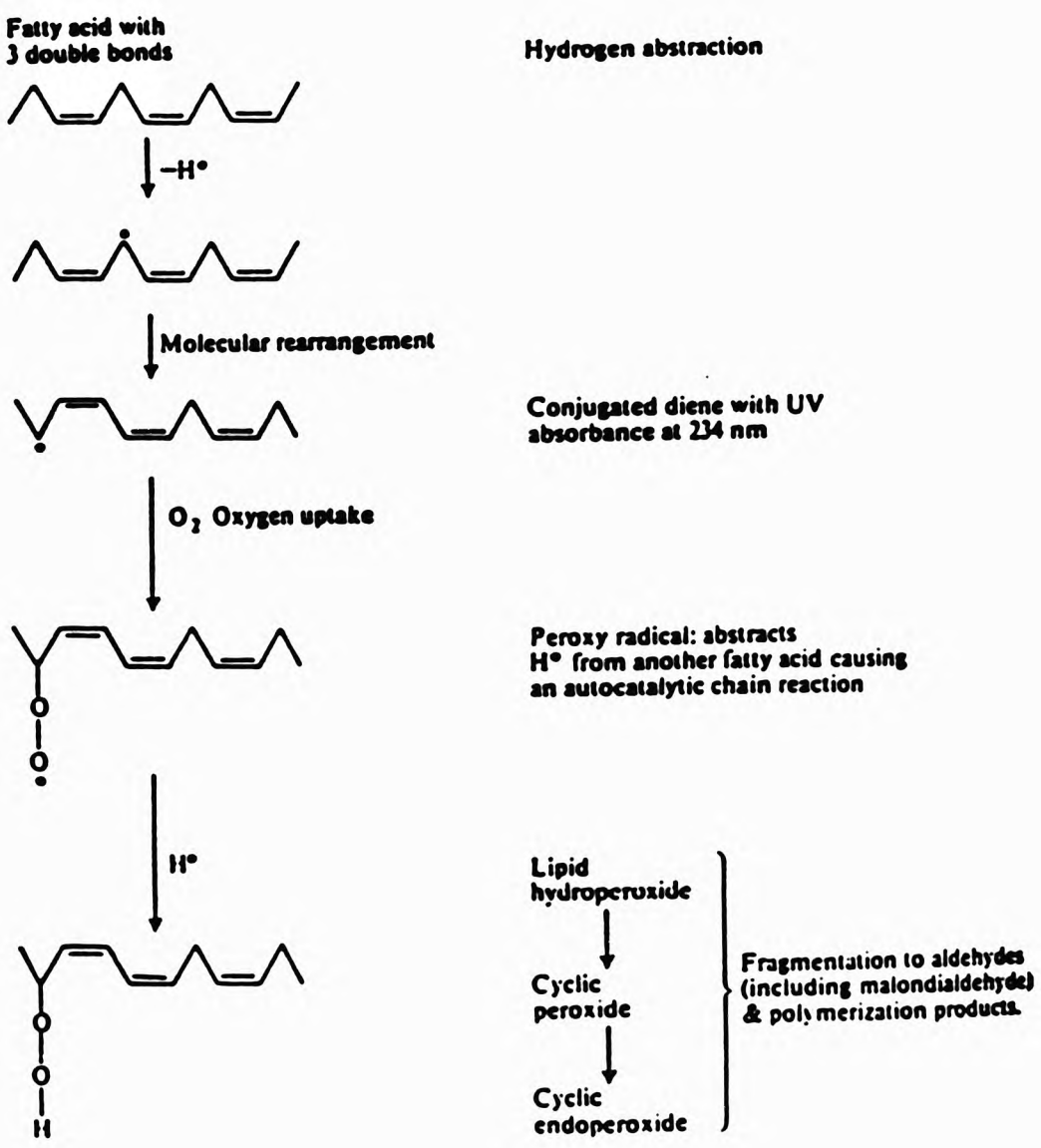
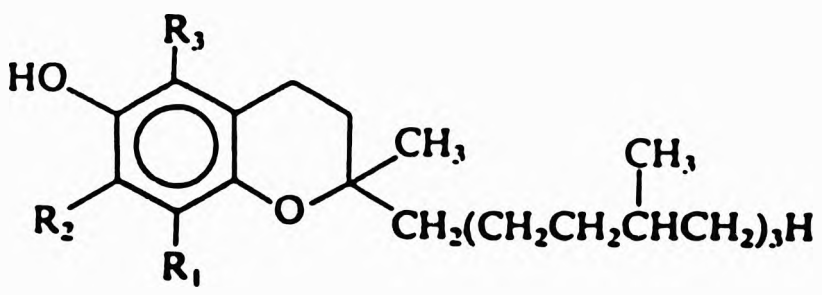


Fig. 4.6.

Structure of the tocopherols.



α-T = R_1, R_2, R_3, all CH_3
β-T = R_1, R_3, CH_3; R_2, H
γ-T = R_1, R_2, CH_3; R_3, H
δ-T = R_1, CH_3; R_2, R_3, H.

T= tocopherol.

In yeasts polyunsaturated fatty acids are generally limited to 18:2 and 18:3, resembling the fatty acids present in higher fungi. However these may vary with aeration and temperature (Rattray, 1988). Certain polyunsaturated acids however, are only present in specific species and strains. Johnson and Brown (1972) found that yeast species subject to glucose repression and *petite* formation generally do not possess 18:2 and 18:3 fatty acids. *S. cerevisiae* is recognised as a yeast with the above properties and lack of polyunsaturated fatty acids is common in this genus. This is possibly due to their ability to grow anaerobically, as such conditions have been found to give a low yield of unsaturated acids in *S. cerevisiae*. However, the presence of 18:2 fatty acid has been documented in some species of *Saccharomyces* and 16:2 fatty acid has been detected in *S. cerevisiae* (Wilson and McLeod, 1976). *Petite*-negative species however, due to their characteristically oxidative metabolism frequently contain these molecules. *Sch. pombe* is an example of this type of species and in certain determinations has been found to contain 18:2 and 18:3 fatty acids which each constitute 5.7% of the total fatty acid content (Bulder and Reinink, 1974). These studies suggest that *Sch. pombe* 972 may possess polyunsaturated fatty acids which are subject to lipid peroxidation by carbon tetrachloride. The possible absence of these molecules in *S. cerevisiae* 188 may explain why *Sch. pombe* 972 is more sensitive to the toxic effects of carbon tetrachloride than *S. cerevisiae* 188.

4.2.4 Effect of carbon tetrachloride on cytochrome P450 concentration

The growth of *Sch. pombe* 972 in the presence of carbon tetrachloride resulted in reduction in the levels of cytochrome P450 and P420. Incubation of the cells with 1.9 mM carbon tetrachloride, a sub-toxic dose of the compound, caused depletion of the haemoprotein under both conditions of aeration and non-aeration (fig. 4.7, table 4.6). This concentration produced a maximum reduction of 53% under non-aerated conditions.

Application of a toxic dose (3.2 mM) of carbon tetrachloride caused further reductions.

A trend was seen where increasing concentrations of carbon tetrachloride resulted in lower levels of cytochrome P450 in both aerated and non-aerated cells. Overall, non-aerated cells had lower P450 levels than aerated cells. The combination of no aeration and the highest carbon tetrachloride concentration used resulted in undetectable levels of P450 in the cells (table 4.6).

Fig. 4.7.

Difference spectra of a) aerated and b) non-aerated cells of *Sch. pombe* 972 after growth in YEPD containing 20% (wt/vol) glucose and 1.9 mM carbon tetrachloride for 18 h yielding a) 5.35 nmol P450 and 6.12 nmol P420 g^{-1} dry cell weight and b) 2.39 nmol P450 and 2.45 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 4.7a.

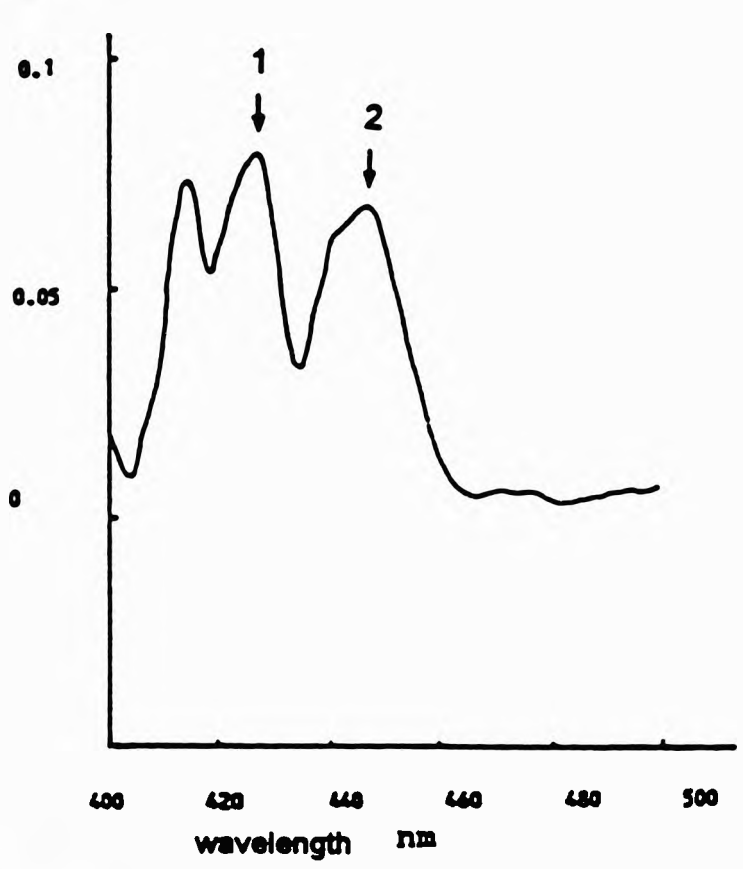


Fig.4.7b.

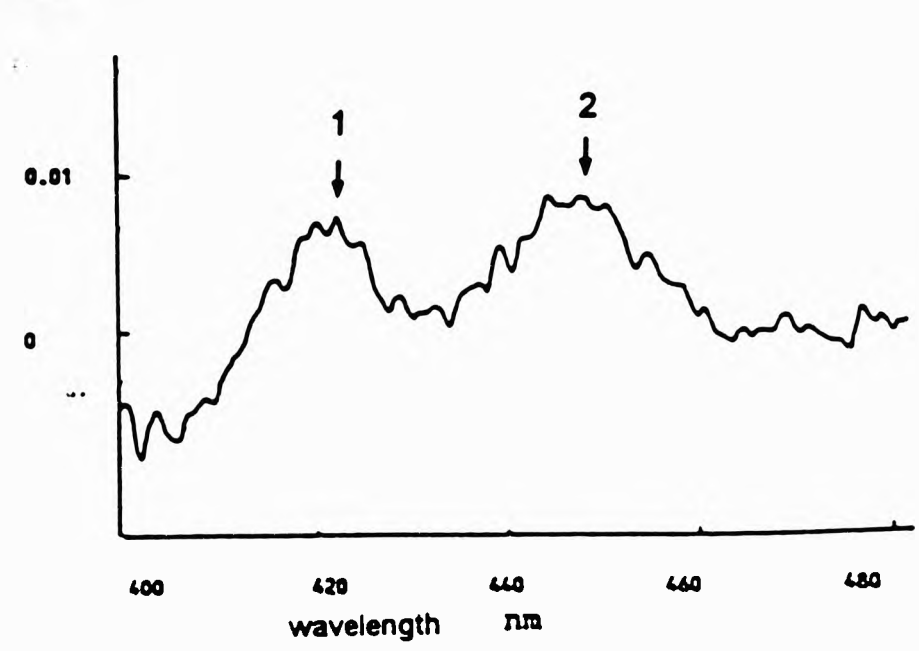


Table 4.6

The effect of carbon tetrachloride on cytochrome P450 levels in *Sch. pombe* 972 after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

[CCl_4] (mM)	conditions	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
0	aerated	49.1	15.23	7.61
1.9	aerated	88.3	6.12	5.35
3.2	aerated	96.8	5.77	5.22
0	non-aerated	25.8	5.94	5.11
1.9	non-aerated	36.8	2.45	2.39
3.2	non-aerated	18.9	0	0

Data represents determinations from one experiment. Cells were grown in the presence of carbon tetrachloride for 18 h. Significant differences between values were calculated using the LSD criterion from ANOVA. LSD calculated for data $P < 0.05 = 3.36$ for P450 values.

Similar results were also seen in *Sch. pombe leu*1-32 *pho*1-44 (table 4.7). A decrease in cytochrome P450 levels of 19% was seen in aerated cultures exposed to 1.9 mM carbon tetrachloride, whereas in non-aerated cultures cytochrome P450 levels dropped by 28% when exposed to the same concentration. These observations are in line with documentation that the actions of carbon tetrachloride may include destruction of cytochrome P450 (deGroot and Hass, 1981), resulting in a reduction in levels of the haemoprotein.

Table 4.7

The effect of carbon tetrachloride on cytochrome P450 levels in *Sch. pombe leu*1-32 *pho*1-44 after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

$[CCl_4]$ (mM)	conditions	A at 420 nm	A at 450 nm	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
0	aerated	0.100	0.040	4.92	2.40
1.9	aerated	0.066	0.050	2.10	1.94
0	non-aerated	0.010	0.011	0.29	0.39
1.9	non-aerated	0.0059	0.0069	0.20	0.28

Data represents determinations from one experiment.

Three chlorinated methanes, carbon tetrachloride, chloroform and methylene chloride were investigated for their ability to cause hepatic damage in rats by Kitchin and Brown (1989). The cytochrome P450 content of the liver cells was found to be decreased at sub-toxic and cytotoxic doses as in *Sch. pombe* cells. Carbon tetrachloride was also found to exhibit the highest level of cytotoxicity when compared with the other tested chlorinated methanes.

Sesardic *et al.* (1989) have found that treatment of 3-methylcholanthrene exposed hepatic microsomes with carbon tetrachloride decreased cytochrome P450 content by 60%. Cytochrome P450d was found to be most affected. Other researchers have stated that the specific metabolism of carbon tetrachloride is carried out by the phenobarbital inducible CYP2B isozyme (Frank *et al.* 1982). This species has been

found to have a molecular weight of 52 kDa. In work carried out by Head *et al.* (1981), rat microsomes showed a decrease in a 51.6 kDa form of cytochrome P450 after treatment with carbon tetrachloride. This indicates that a specific cytochrome P450 can be destroyed by the haloalkane. A loss of absorbance at 450 nm in carbon tetrachloride-treated rat microsomes has been reported by Noguchi *et al.* (1982) and a specific 52 kDa polypeptide was also observed by this group to be the first cytochrome P450 to disappear. In the latter study it was postulated that the mechanism of cytochrome loss might be by direct attack or lipid peroxidation. However de Groot and Haas (1980) found that cytochrome P450 destruction took place under anaerobic conditions which tends to suggest that lipid peroxidation is not involved in the process. These studies are supported by the present work on *Sch. pombe* where destruction of cytochrome P450 occurred more markedly in non-shaken cultures where hypoxic conditions would be expected to prevail than in shaken cultures where aerobic conditions would be expected to prevail. The effect of α–tocopherol on carbon tetrachloride damage to cytochrome P450 was also investigated (see 4.2.5). However, although small increases in cytochrome P450 levels occurred in the α–tocopherol treated cultures, these were not as great as increases observed in cells without exposure to carbon tetrachloride. The antioxidant may have given some protection against free-radical damage but this would be expected to be limited if mechanisms other than lipid peroxidation such as covalent binding are involved in damage to the haemoprotein (de Groot and Hass, 1981). Work by Manno *et al.* (1988) has indicated that the suicidal activation of carbon tetrachloride by cytochrome P450 occurs

at the haem site resulting in its destruction.

4.2.5 Effect of α–tocopherol on cytochrome P450

The presence of α–tocopherol was found to affect cytochrome P450 levels in cells grown without exposure to carbon tetrachloride. Cultures of *Sch. pombe* 972 grown with 50 μM α–tocopherol for 18 hours generated 5.55 nmol P450 g^{-1} dry cell weight. In these experiments the control cultures not exposed to the compound showed no detectable cytochrome P450 (table 4.8). Increasing the concentration of α–tocopherol to 100 μM gave a value of 8.52 nmol g^{-1} dry cell weight in comparison to undetectable levels in the control (fig. 4.8, table 4.9, Atchia *et al.*, 1991b). The control cultures in these experiments would have been expected to yield detectable levels of cytochrome P450, however the results illustrated the occasional difficulty of detecting cytochrome P450 in yeast cells even when grown under conditions to maximise levels of the haemoprotein.

The observed increase in endogenous levels of cytochrome P450 compares with work by Lokshina *et al.* (1988) where the cytochrome P450 content of the livers of vitamin E-deficient rats was elevated after administration of γ-tocopherol. It is probable in both cases that the tocopherols have a stabilising effect on cytochrome P450. Protection against free-radical damage may also occur as cytochrome P450 has been found to be a target for such damage (de Groot and Hass, 1981).

Fig 4.8.

Difference spectra of aerated cells of *Sch. pombe* 972 after growth for 18 h in YEPD containing 20% (wt/vol) glucose with a) 0 and b) 100 μM α-tocopherol yielding a) 0 P450 and 22.61 nmol P420 g^{-1} dry cell weight and b) 8.52 nmol P450 and 24.86 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 4.8a.

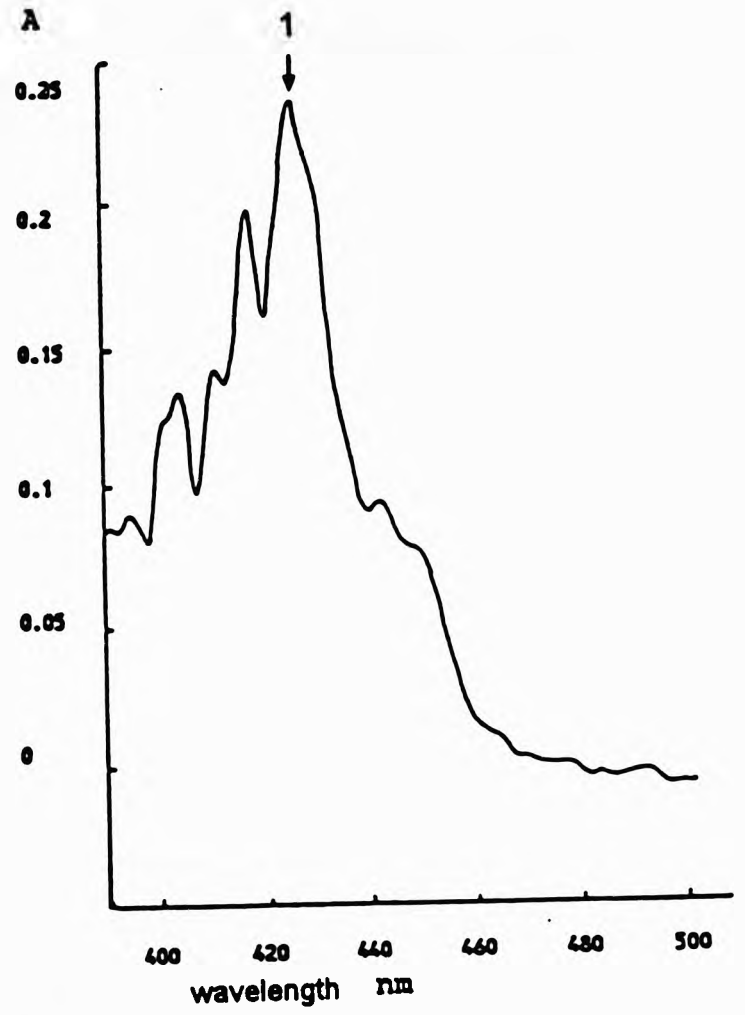


Fig. 4.8b.

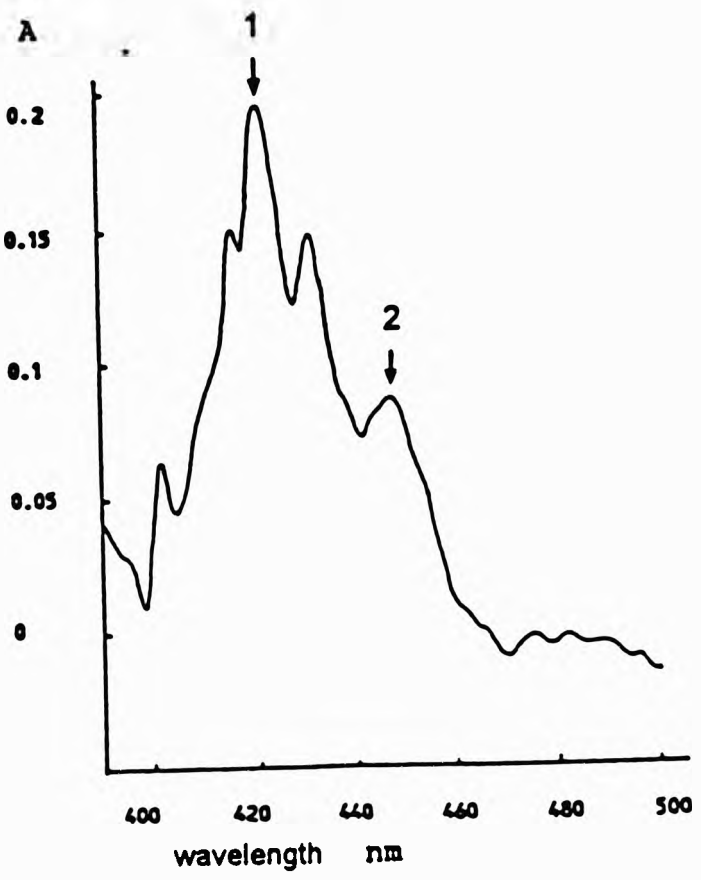


Table 4.8

The effect of α–tocopherol on cytochrome P450 levels in *Sch. pombe* 972 after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

conditions	[α-tocopherol] (μM)	A at 420 nm	A at 450 nm	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
aerated	0	0.141	0	74.9	17.00	0
aerated	50	0.150	0.036	71.3	18.95	5.55

Data is representative of one experiment (experiment was carried out twice). Cells were grown in the presence of α-tocopherol for 18h.

Table 4.9

The effect of α–tocopherol on cytochrome P450 levels in *Sch. pombe* 972 after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

conditions	[α-tocopherol] (μM)	A at 420 nm	A at 450 nm	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
aerated	0	0.136	0	54.2	22.61	0
aerated	100	0.146	0.041	52.9	24.86	8.52

Data is representative of one experiment (experiment was carried out twice). Cells were grown in the presence of α-tocopherol for 18 h.

Table 4.10

The effect of α–tocopherol on cytochrome P450 levels in cells of *Sch. pombe* 972 treated with 1.9 mM carbon tetrachloride after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

[α-tocopherol] (μM)	conditions	A at 420 nm	A at 450 nm	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
0	aerated	0.141	0.039	64.9	19.57	6.60
50	aerated	0.140	0.040	66.4	18.99	6.62

Data is representative of one experiment (experiment was carried out twice). Cells were grown in the presence of carbon tetrachloride and α-tocopherol for 18 h.

Table 4.11

The effect of α–tocopherol on cytochrome P450 levels in cells of *Sch. pombe* 972 treated with 1.9 mM carbon tetrachloride after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

[α-tocopherol] (μM)	conditions	A at 420 nm	A at 450 nm	dry wt. (mg)	nmol P420 g^{-1} dry wt.	nmol P450 g^{-1} dry wt.
0	aerated	0.124	0.040	52.9	21.12	8.31
100	aerated	0.106	0.037	45.1	21.17	9.02

Data is representative of one experiment (experiment was carried out twice). Cells were grown in the presence of carbon tetrachloride and α-tocopherol for 18 h.

In cultures which had been exposed to 1.9 mM carbon tetrachloride very small increases in cytochrome P450 concentration were seen with administration of the anti-oxidant suggesting that α-tocopherol could not alleviate the toxic effects of carbon tetrachloride to the enzyme. As

discussed in section 4.2.4 this may indicate that mechanisms other than lipid peroxidation are involved in the carbon tetrachloride-mediated destruction of cytochrome P450 (fig. 4.9, tables 4.10, 4.11).

Fig. 4.9.

Difference spectra of aerated cells of *Sch. pombe* 972 after growth in YEPD containing 20% (wt/vol) glucose and 1.9 mM carbon tetrachloride for 18 h with a) 0 and b) 50 μM α-tocopherol yielding a) 6.60 nmol P450 and 19.57 nmol P420 g^{-1} dry cell weight and b) 6.62 nmol P450 and 18.99 nmol P420 g^{-1} dry cell weight. Arrows indicate peaks at 420 nm (1) and 450 nm (2).

Fig. 4.9a.

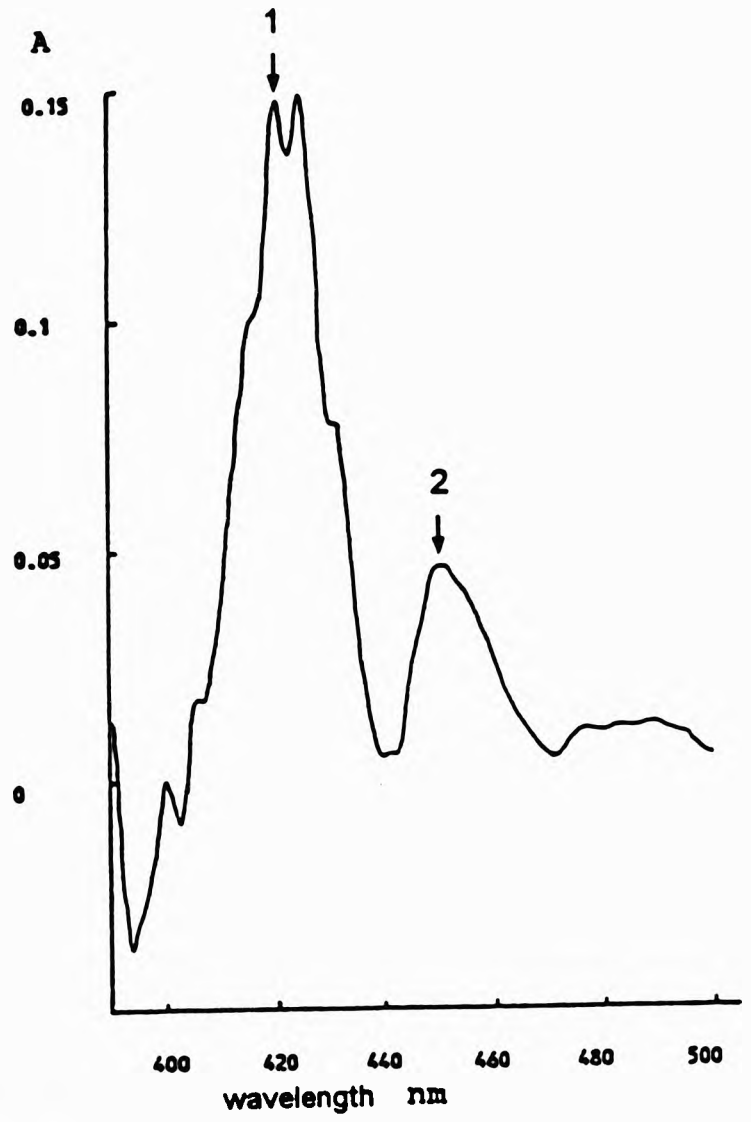
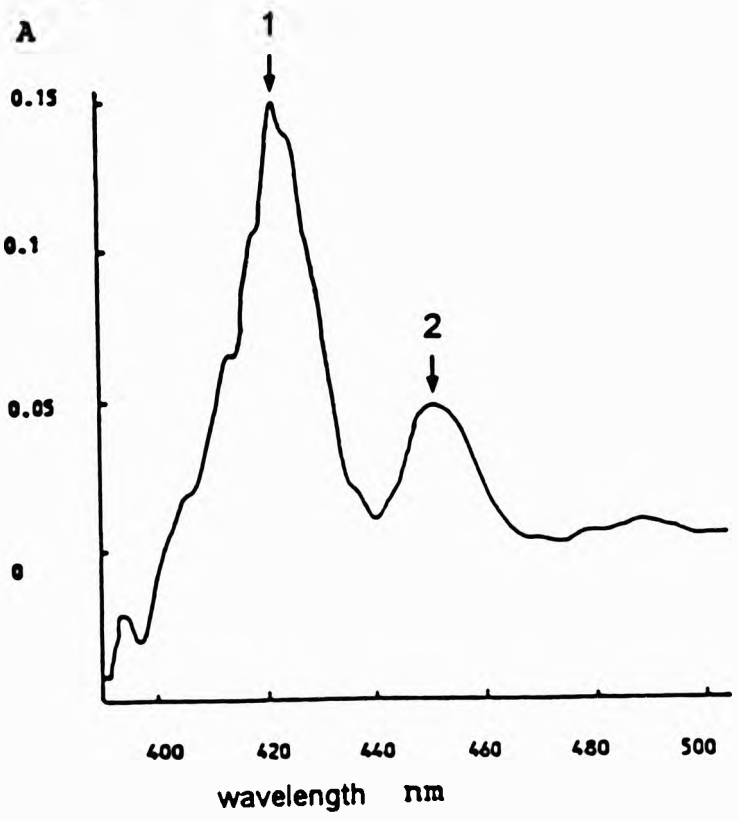


Fig. 4.9b.



CHAPTER 5

EVALUATION OF THE GENOTOXICITY OF CARBON TETRACHLORIDE IN *SCH. POMBE* AND *S. CEREVISIAE*

5.1 INTRODUCTION

The potential genotoxicity of carbon tetrachloride was examined in *Sch. pombe*. Forward and reverse mutation at the *ade6* locus and reversion to lysine and leucine prototrophy were monitored. Mutation to chloramphenicol (CAP) resistance was also measured. Chloramphenicol inhibits protein synthesis by binding to the 50S subunit of 70S ribosomes found in the cytoplasm of bacteria and in mitochondria. The compound affects chain elongation by inhibiting peptidyl transferase preventing peptide bond formation (Gale *et al.*, 1981). Data on *S. cerevisiae* are presented for comparison.

5.2 RESULTS AND DISCUSSION

5.2.1 Determination of spontaneous mutation frequencies in yeast cells

In the mutagenicity assays used in this work mutation to chloramphenicol resistance was one of a variety of end-points utilised. The frequency of spontaneous CAP^R mutants was found to vary between strains. The figure was approximately 3.13 $\pm$ 1.42 SEM mutants per 10^6 viable cells for *Sch. pombe* 972 as determined from 6 experiments. Patel (1986) observed the spontaneous mutation frequency to this end-point in *Sch. pombe* 972 to be 0.34 mutants per 10^6 viable cells in continuous cultures at a sampling time of 103 h. The spontaneous mutation frequency in *Sch. pombe lys*1-131 *h*$^-$ was found to be 0.71 mutants per 10^6 viable cells but was higher for *Sch. pombe leu*2-120 *lys*3-37 at approximately 6.4 mutants per 10^6 viable cells. For *S. cerevisiae* 159 and 188 the frequencies were 9.84 mutants and 15.33 mutants per 10^6 viable cells respectively.

Variations were observed in the spontaneous mutation frequencies to CAP^R. Variations between the species *Sch. pombe* and *S. cerevisiae* may be expected due to the differences between these yeasts including the structure of their mitochondrial DNA (see 5.2.4.1). However, variations in frequencies between experiments in the same species were also observed. This could have been due to differences in viable counts arising from slight variations in culture conditions such as temperature. Other possibilities could include the different susceptibilities of these strains to the action of chloramphenicol.

Sch. pombe ade6 was monitored for the production of phenotypically white colonies. Such colonies arise either by reversion to prototrophy or by a further mutation at a point earlier in the adenine biosynthetic pathway than the *ade6* blockage, thereby producing a 'double' adenine requiring mutant. The spontaneous mutation frequency to *ade6⁻adex⁻* was determined as 1 mutant per 10^5 viable cells by Chrysoglou (1991) and 11 mutants per 10^4 viable cells in the present study. As this latter value is high in comparison to other documented values, it is possible that the strain used in this work contained a mutator gene. The spontaneous mutation frequency to white adenine requiring cells was found to be 1 mutant per 10^4 viable cells in a mutator strain carrying the *rad10-198* mutation (Loprieno *et al.*, 1983).

5.2.2 Evaluation of the mutagenicity of ethanol

The use of ethanol for sterilising and dissolving carbon tetrachloride and ethyl methanesulphonate (EMS) necessitated its inclusion as a solvent control in most of the mutagenicity assessment experiments. The compound induced on average a less than two-fold increase in the frequency of mutants observed over the corresponding spontaneous levels indicating that ethanol was not itself mutagenic (see 2.7.5, table 5.1).

Occasional rises in numbers of induced mutants were observed on

incubation with ethanol, however these rises were found not to be statistically significant (table 5.1).

Table 5.1

Mutagenicity of ethanol in *Sch. pombe* 972.

conditions	No. CAP^R mutants/10^6 cells
- ethanol	2.22 ± 0.82
+ ethanol	2.45 ± 0.49

Data represents means of four separate experiments ± SEM. Each experiment comprised three replicates. No significant difference was calculated between the data groups ($P > 0.05$) using ANOVA.

Ethanol has been reputed to be a substrate of cytochrome P450 2El (cytochrome P450 3a), (Lewis *et al.*, 1987) and has also been found to increase cytochrome P450 concentrations in the yeast *S. cerevisiae* D7 (Morita and Mifuchi, 1984). Ethanol has been implicated as a causative agent in certain human cancers including breast cancer (Willett *et al.*, 1987). This may be related to mutagenic activity.

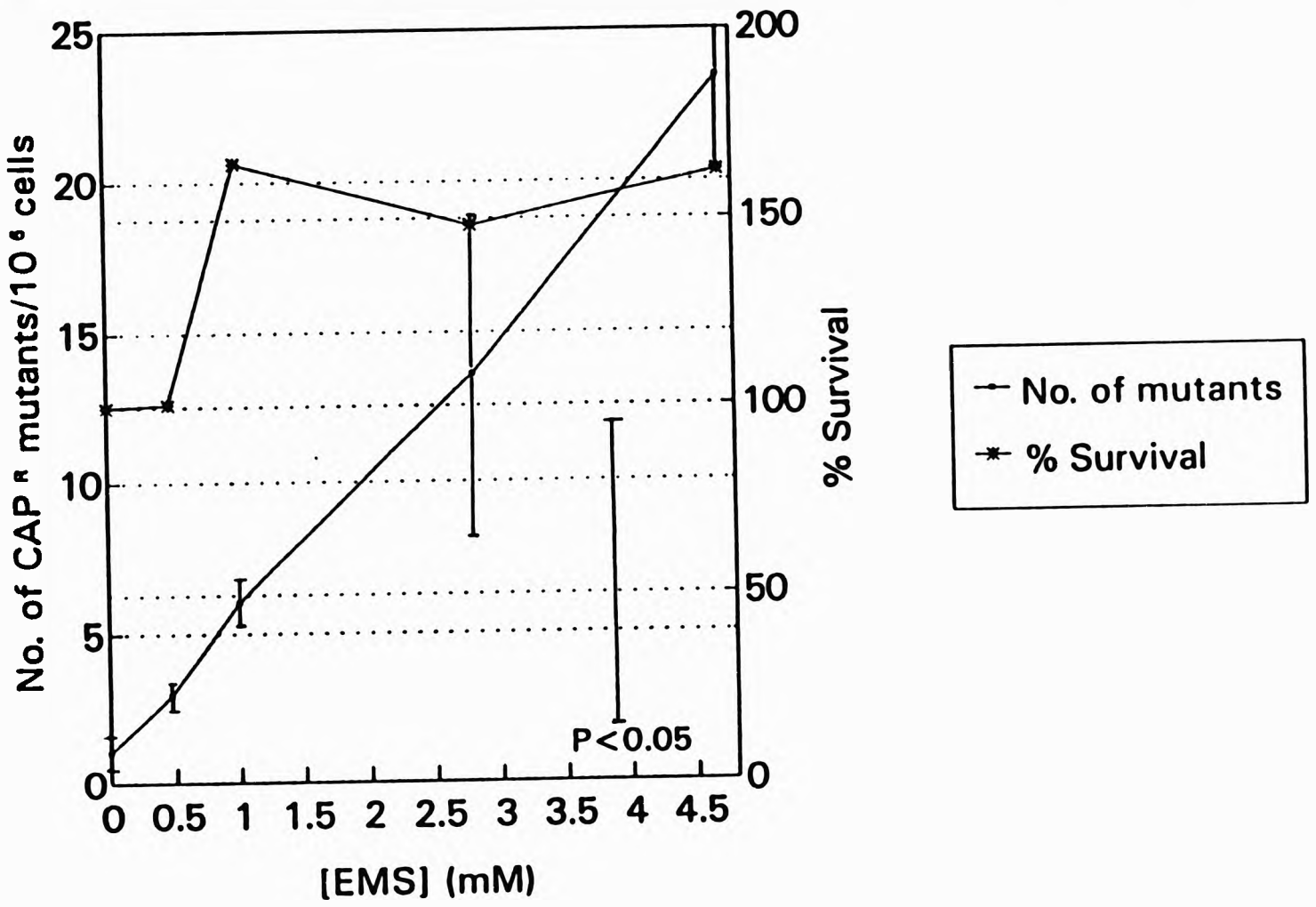
5.2.3 Evaluation of the mutagenicity of ethyl methanesulphonate

Ethyl methanesulphonate (EMS) was used as a positive control in the genotoxicity assessment experiments. The administration of EMS to cultures of *Sch. pombe* 972 brought about a dose-dependent increase in chloramphenicol resistant mutants (table 5.2, fig. 5.1a). Concentrations

Fig. 5.1a.

The mutagenicity of EMS in *Sch. pombe* 972. Cells were grown in the presence of EMS for 18 h. Data points are the mean values of two experiments ± SEM each comprising three replicates. The average % survival of both experiments is shown. Significant differences between values were calculated using the LSD criterion from ANOVA. Error bar represents LSD of $P< 0.05 = 10.41$ for mutant values.

Fig. 5.1a. Mutagenicity of ethyl methanesulphonate in *Sch. pombe* 972



of 0.47 mM and above were considered to be mutagenic, causing over twice the number of mutational events as in the presence of ethanol alone. Doses of 4.7 mM generated on average approximately twenty two times the number of chloramphenicol resistant mutants per 10^6 viable cells compared with the observed spontaneous frequency.

Table 5.2

Mutagenicity of EMS in *Sch. pombe* 972

[EMS] (mM)	Average no. cfu ml^{-1}	Average no. CAP^R mutants ml^{-1}	Average no. CAP^R mutants/10^6 cells
0	1.8×10^7	7	0.39
0.47	1.3×10^7	43	3.31
1.00	1.6×10^7	83	5.19
2.80	1.3×10^7	247	19.00
4.70	1.2×10^7	320	26.67

Determinations represent means of three replicates from one experiment (shown in fig. 5.1a, expt 2). Cells were grown in the presence of EMS for 18 h. Significant differences between values were calculated using the LSD criterion from ANOVA. LSD calculated for data $P < 0.05 = 10.41$.

Patel (1986) discovered 1mM (120 μg ml^{-1}) EMS to be mutagenic in continuous cultures of *Sch. pombe* 972 when mutation to chloramphenicol resistance was used as an end-point. The spontaneous mutation frequency in continuous cultures was found by Patel to be 0.34 CAP^R mutants per 10^6 viable cells at a sampling time of 103 h, while 11.94 mutants per 10^6 viable cells were detected at 101 h in continuous cultures exposed to 120 μg ml^{-1} EMS. Studies by Dodd (1990) also support this work. Concentrations of 120 μg ml^{-1} EMS in

continuous cultures of *Sch. pombe* 972 induced about one hundred times the number of CAP^R mutants than in control cultures at a sampling time of approximately 113 h. Statistical analysis using ANOVA indicated that in the present work a significantly higher number of CAP^R mutants than in the control were produced in cultures exposed to 2.80 and 4.70 mM EMS, ($P<0.05$). The induction of CAP^R mutants by EMS was also observed in *S. cerevisiae* 159 (fig. 5.1b), however the increase in induced over spontaneous mutants was calculated to be approximately 11-fold for the maximum concentration of EMS used.

Some of the *Sch. pombe* mutants isolated following induction by EMS treatment were subsequently subjected to genetic analysis and the origin of their mutations designated as mitochondrial or nuclear (see 5.2.4.1).

EMS was also found to induce mutants in *Sch. pombe ade6*, at 1 mM and 2.8 mM, inducing respectively approximately 3.4 and 15.5 times the number of phenotypically white mutants in *Sch. pombe ade6* than in the absence of EMS (fig. 5.1c). The mutants were found to retain their adenine auxotrophy. It is likely therefore that these EMS induced mutants had a double adenine requirement resulting from a further mutation at a point earlier in the adenine biosynthetic pathway than the *ade6* blockage, (*viz* in the *ade1, ade3, ade4, ade5,* or *ade9* locus). This was not unexpected as mutational events in an auxotrophic strain are more likely to result in a second forward mutation than in the production of a prototrophic revertant. Reversion, on the other hand, usually involves a second site mutation at a suppressor locus (Loprieno *et al.*, 1983).

Fig. 5.1b.

The mutagenicity of EMS in *S. cerevisiae* 159. Cells were grown in the presence of EMS for 18 h. Data points are the mean values of three replicates.

Fig. 5.1b. Mutagenicity of ethyl methanesulphonate in *S. cerevisiae* 159

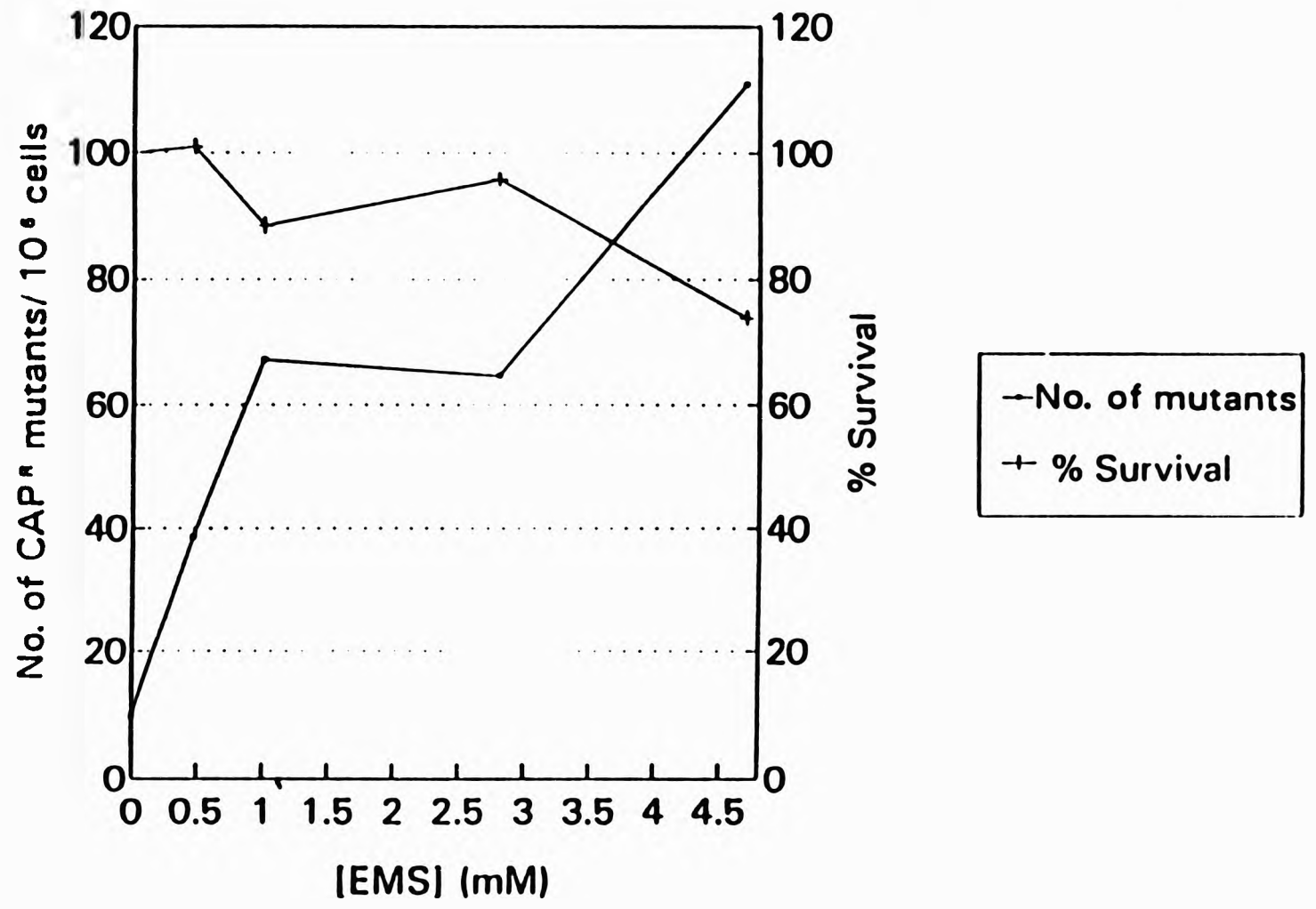
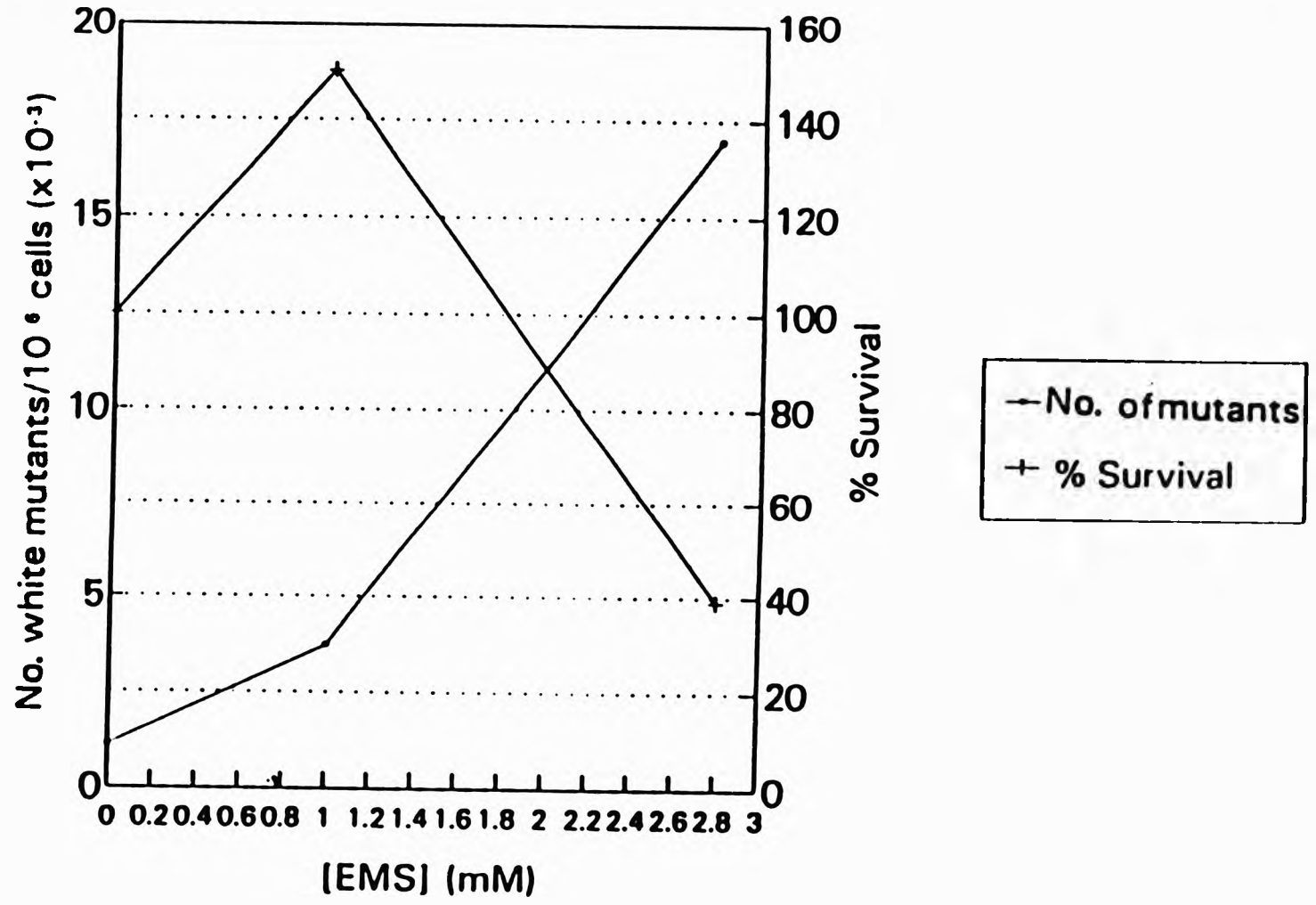


Fig. 5.1c.

The mutagenicity of EMS in *Sch. pombe ade6*. Cells were grown in the presence of EMS for 18 h. Data points are the mean values of three replicates.

Fig. 5.1c. Mutagenicity of ethyl methanesulphonate in *Sch. pombe ade6*



The genes *ade1*, *ade3*, *ade4*, *ade5* and *ade9* have been found to be chromosomally located (Loprieno, 1984). This suggests that EMS is also capable of causing mutations in nuclear DNA.

No forward mutations could be detected at the *ade6 or ade7* locus of *Sch. pombe* 972 even after treatment with EMS (table 5.3). The forward mutation frequency at these loci has been found to be approximately 4 mutants per 10^7 cells for the wild-type strain (Loprieno *et al.*, 1983). Cell numbers screened in this present work were thus probably too low to detect such mutation events.

Table 5.3

Mutagenicity of EMS in *Sch. pombe* 972

[EMS] (mM)	Average no. cfu ml^{-1}	Average no. red mutants ml^{-1}	Average no. red mutants /10^6 cells
0	4.0×10^6	0	0
1.00	7.8×10^6	0	0
2.80	1.0×10^6	0	0

Determinations represent means of three replicates from one experiment. Cells were grown in the presence of EMS for 18 h.

EMS is a known mutagen and carcinogen (Schalet, 1978) which has been found to act by alkylation, i.e. transfer of an alkyl group such as methyl (CH_3) or ethyl (CH_2CH_3) to a nucleotide. The most common reactions of EMS are with guanine. Alkylation has been found to occur mainly at the N-7 position; a tautomeric shift from the 1 to the 6 position of guanine may then occur. Alkylation at this position has been found to cause less mutational events than alkylation at the O-6 atom (Loveless and Hampton, 1969). The most common transition caused by this compound is therefore from a G-C base pair to an A-T base pair.

5.2.4 Evaluation of the mutagenicity of carbon tetrachloride

5.2.4.1 Induction of mitochondrial mutations

The induction of chloramphenicol resistant mutants was monitored in *Sch. pombe* 972, *lys*1-131 and *leu*2-120 *lys*3-37 and in *S. cerevisiae* 188, after exposure to carbon tetrachloride. A threshold was seen below which no significant increase in CAP^R mutants over the control frequency could be detected (table 5.4, figs. 5.2, 5.3). However a large increase in mutant numbers occurred at concentrations of the compound causing significant cell death (figs. 5.2, 5.3). The increase in mutation yields in *Sch. pombe* 972 for example, at doses of 3.2, 5.2 and 6.5 mM carbon tetrachloride was on average 3.8, 7.3 and 12.6-fold respectively. Since an increase of two-fold or more indicates a positive response (see 2.7.5), this suggests that at the cytotoxic doses tested, carbon tetrachloride was mutagenic in *Sch. pombe* 972. Statistical analysis indicated that at doses of 5.2 mM and 6.5 mM the number of CAP^R mutants was significantly higher than in the control, ($P<0.05$, table 5.4). Similar results

Fig. 5.2a.

The mutagenicity of carbon tetrachloride in *Sch. pombe* 972. Cells were grown in the presence of carbon tetrachloride for 18 h. Data points are the mean values of three experiments ± SEM each comprising three replicates. The average % survival of all three experiments is shown. Significant differences between values were calculated using the LSD criterion from ANOVA. Error bar shown represents LSD of $P< 0.05$ = 13.97 for mutant values.

Fig. 5.2a. Mutagenicity of carbon tetrachloride in *Sch. pombe* 972

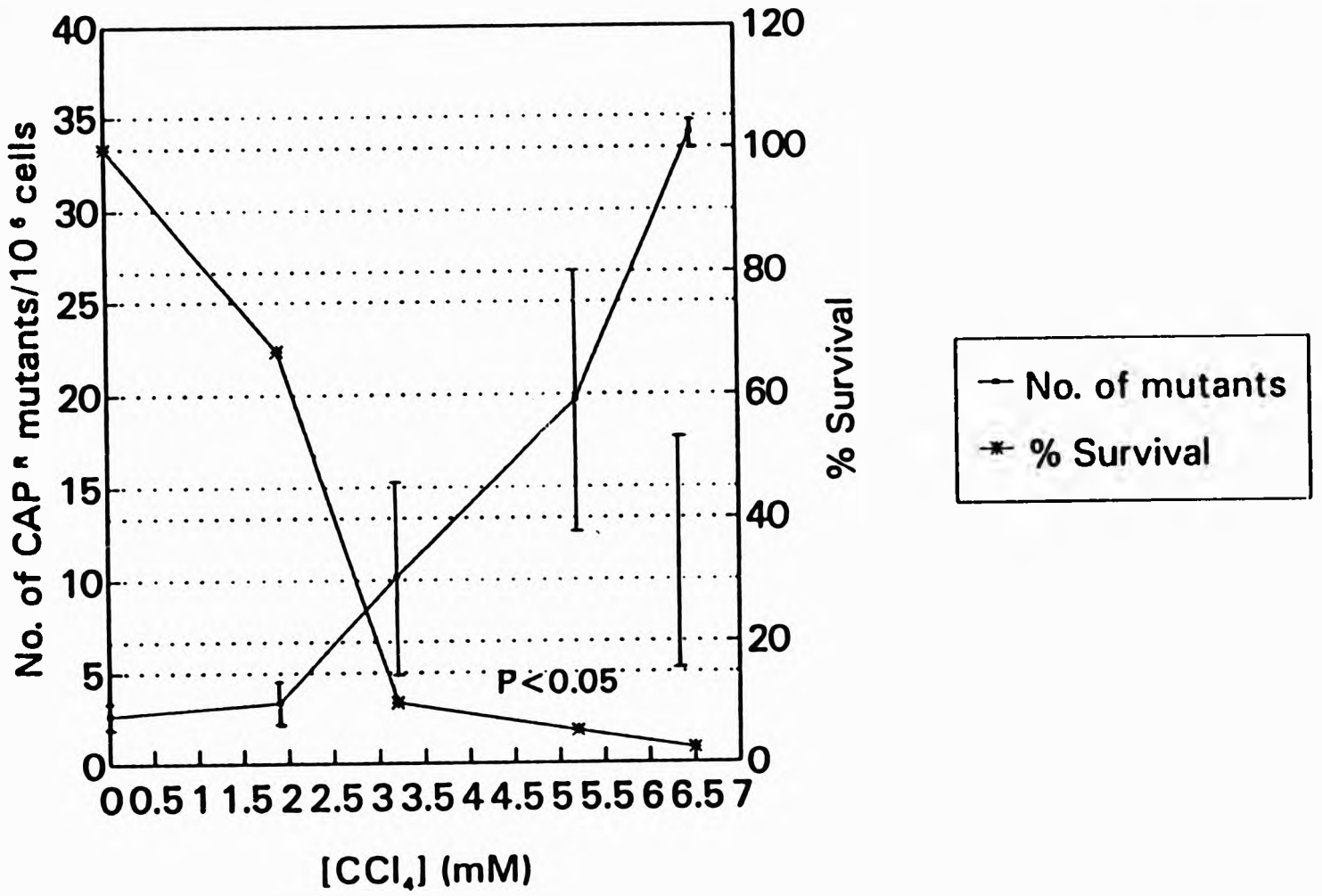


Fig. 5.2b.

The mutagenicity of carbon tetrachloride in *Sch. pombe* *lys*1-131. Cells were grown in the presence of carbon tetrachloride for 18 h. Data points are the mean values of three replicates.

Fig. 5.2b. Mutagenicity of carbon tetrachloride in *Sch. pombe lys*1-131

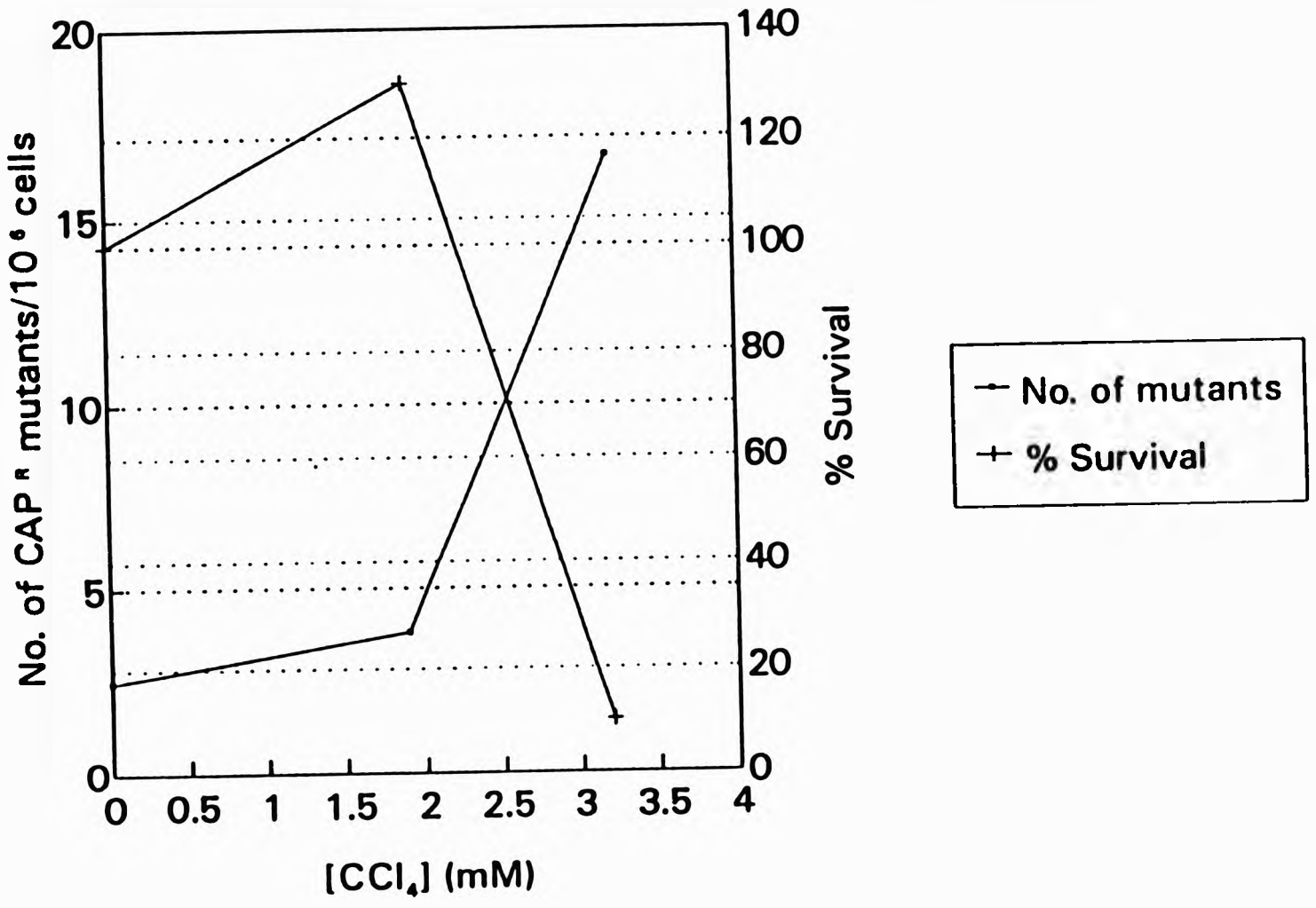


Fig. 5.2c.

The mutagenicity of carbon tetrachloride in *Sch. pombe leu*2-120 *lys*3-37. Cells were grown in the presence of carbon tetrachloride for 18 h. Data points are the mean values of three replicates.

Fig. 5.2c. Mutagenicity of carbon tetrachloride in *Sch. pombe leu2-120 lys3-37*

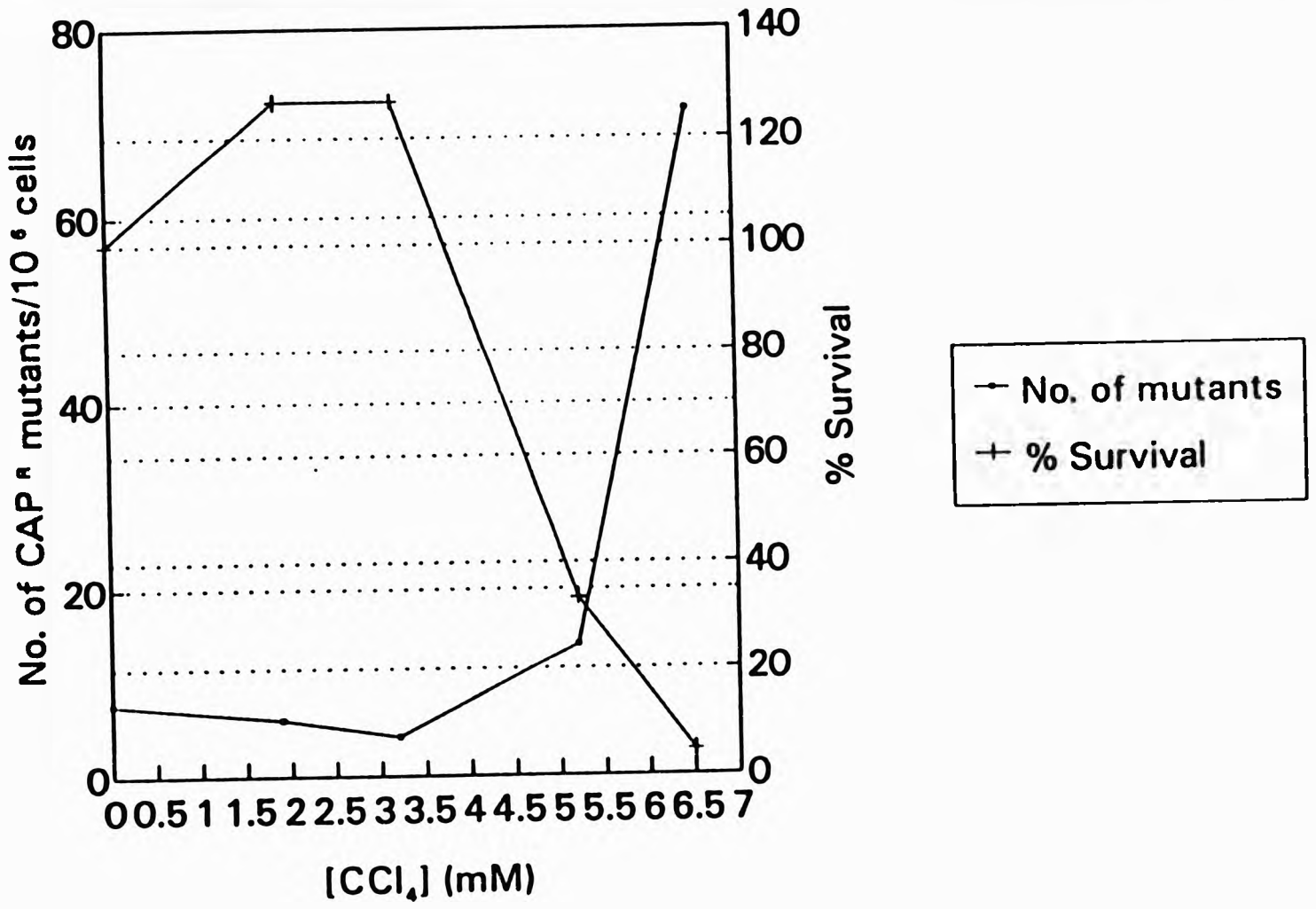
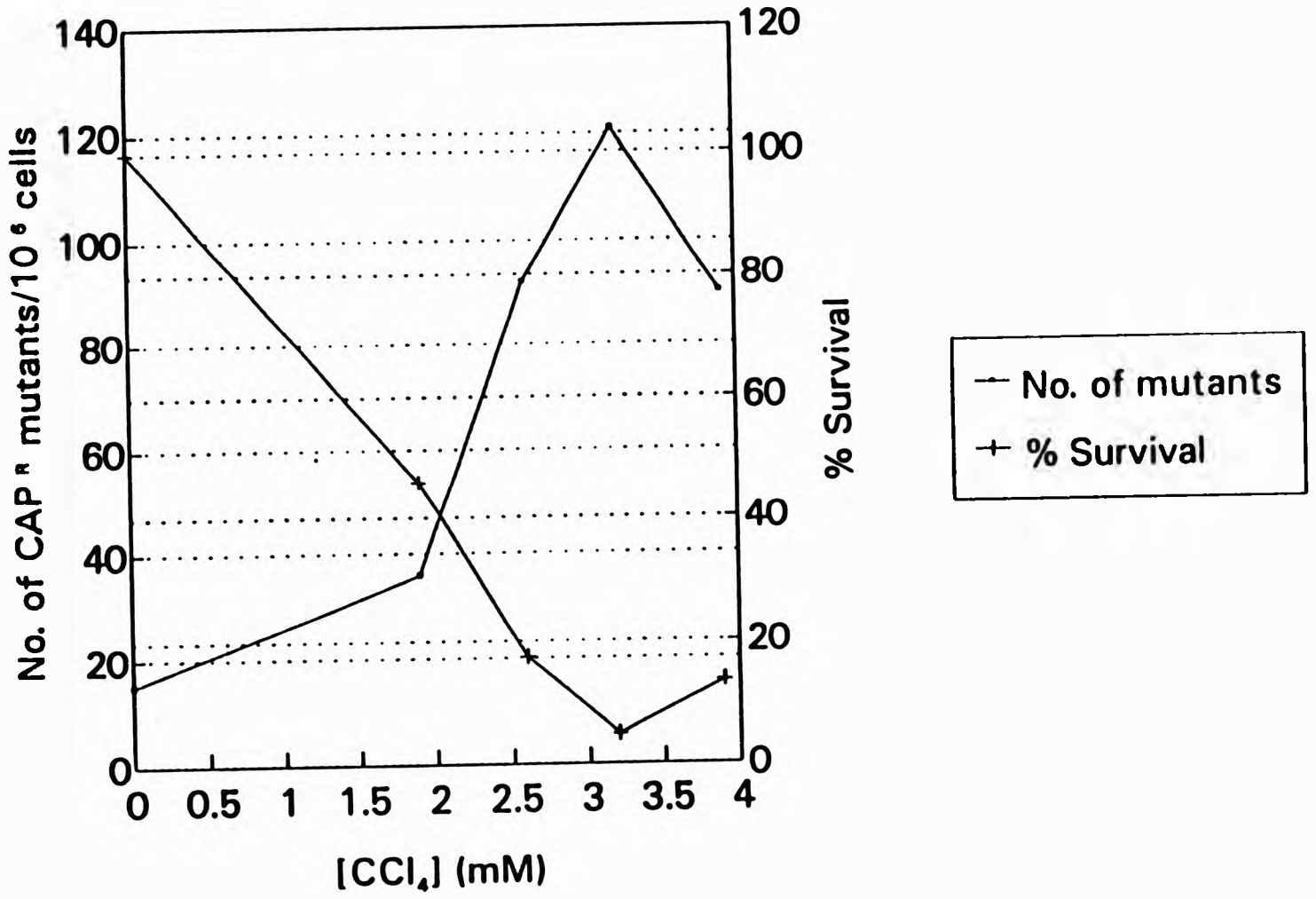


Fig. 5.3.

The mutagenicity of carbon tetrachloride in *S. cerevisiae* 188. Cells were grown in the presence of carbon tetrachloride for 18 h. Data points are the mean values of three replicates.

Fig. 5.3. Mutagenicity of carbon tetrachloride in *S. cerevisiae* 188



were observed in other strains of *Sch. pombe* and in *S. cerevisiae* (figs. 5.2, 5.3).

Table 5.4

Mutagenicity of carbon tetrachloride in *Sch. pombe* 972

$[CCl_4]$ (mM)	Average % survival	Average no. CAP^R mutants/ 10^6 cells ± SEM
0	100	2.87 ± 0.70
0 (+ ETOH)	117.1	2.70 ± 0.60
1.9	80.1	3.39 ± 1.17
3.2	9.7	10.18 ± 5.94
5.2	5.0	19.71 ± 7.8
6.5	2.6	34.12 ± 0.79

Determinations represent the mean values of three experiments ± SEM each comprising three replicates (fig. 5.2a). Cells were grown in the presence of carbon tetrachloride for 18 h. Significant differences between values were calculated using the LSD criterion from ANOVA. LSD calculated for data $P<0.05= 13.97$.

Comparing the action of carbon tetrachloride and EMS on a molar basis the increase in mutant yields was lower in carbon tetrachloride-treated cells. This can be seen at the highest concentrations of the compounds utilised (4.7 mM in the case of EMS and 6.5 mM in the case of carbon tetrachloride) where increases in mutant yields were approximately 1.7 times lower in carbon tetrachloride treated cells. This implies that carbon tetrachloride is a weaker mutagen than EMS in these test systems.

The antibacterial antibiotic chloramphenicol inhibits protein synthesis by interfering with the peptidyl transferase activity of the 70S type ribosome

(Gale *et al.*, 1981). Since mitochondrial ribosomes are of the 70S type, this antibiotic also acts as a specific inhibitor of eukaryotic mitochondrial protein synthesis. The induction of chloramphenicol resistant mutants by carbon tetrachloride therefore suggests that this compound might interact with those regions of mitochondrial DNA which code for components of mitochondrial ribosomes. Mutations in these genes may lead to an altered sensitivity to the antibiotic in the affected cells. The CAP^R mutants isolated were subsequently subjected to genetic analysis to characterise their origin as nuclear or mitochondrial.

Representative samples of chloramphenicol resistant mutants of *Sch. pombe* 972 induced by EMS and by carbon tetrachloride were replated on YEPD agar and then on YEPG agar containing 1 g l^{-1} chloramphenicol to confirm their CAP^R phenotype. Genetic analysis was performed by random spore analysis (section 2.7.7).

All germinated ascospores grew on YEA. Transference to selective media showed the proportion of *lys*$^-$ and *lys*$^+$ progeny was 1:1. This suggested that the crosses had been successful. The segregation of CAP^R and CAP^S progeny in a ratio of 0:1 indicated that resistance was inherited in a non-Mendelian manner and thus was probably mitochondrially derived (table 5.5). CAP^R mutants analysed by Patel (1986) and Dodd (1990) showed similar results. CAP^R mutants have also been reported to arise in *S. cerevisiae* by mutations in mitochondrial DNA (Coen *et al.*, 1970).

Table 5.5

Growth of ascospores on selective media

Isolate no.	EMM2	EMM2 + 50 mg l^{-1} lysine	YEPG + 1 g l^{-1} CAP	YEA
1	39	180	0	180
2	119	180	0	179
3	109	180	0	180
4	112	180	0	180
5	121	180	0	180
6	79	90	0	89
7	49	75	0	77
8	49	84	0	84
Total	677	1149	0	1149

The mitochondria of all eukaryotic cells including yeasts contain a single circular chromosome. In *Sch. pombe* this chromosome is approximately 17 x 10^6 daltons (Tabak and Weijers, 1976) whereas the mitochondrial genome of *S. cerevisiae* is approximately three times the size, being about 50 x 10^6 daltons (Hollenberg *et al.*, 1970). The GC to AT ratio also differs between the two yeasts; *Sch. pombe* possesses a high content of GC (33%) whereas *S. cerevisiae* contains 17% GC (Seitz *et al.*, 1977). Several authors have noted the high percentage of spacer DNA in the *S. cerevisiae* mitochondrial genome, which may constitute 50% of the genome, however the function of these intervening sequences is still unclear (Prunell and Bernardi, 1974). Because of the differences in the mitochondrial DNA of *Sch. pombe* and *S. cerevisiae*, the two species of yeast might be expected to react in different ways to the toxic and genotoxic effect of chemicals.

Research has indicated that some carcinogens exhibit greater activity on mitochondrial DNA than on nuclear DNA (Egilsson *et al.*, 1979). It has been suggested that increased sensitivity of mitochondrial DNA to putative mutagens may be due to the greater vulnerability of this DNA.

5.2.4.2 Induction of nuclear mutations

Sch. pombe ade6 was found to be highly sensitive to the toxic action of carbon tetrachloride (see chapter 4). This provided some difficulties in assessing mutation frequencies to *ade6+* or *ade6-adex-* due to the limited cell numbers which could be analysed.

The spontaneous mutation frequency to *ade6⁻adex⁻* in *Sch. pombe ade6* was calculated to be approximately 11 mutants per 10^4 viable cells. Administration of carbon tetrachloride to cultures of *Sch. pombe ade6* resulted in a greater than two-fold increase in these mutants over the corresponding spontaneous frequency (fig. 5.4, plates 5.1 and 5.2). The increase in mutation frequency was comparable with that induced by EMS. At low concentrations of the chemicals tested i.e. 1.9 mM carbon tetrachloride and 1 mM EMS the average increase in mutation frequency was 3 and 3.4-fold respectively over the spontaneous level. At 3.2 mM carbon tetrachloride the increase was on average 11.7 times and at 2.8 mM EMS the increase was approximately 15.5 times the spontaneous level. These results indicate that carbon tetrachloride can induce mutations in nuclear DNA.

When the carbon tetrachloride induced white mutants were tested for

Fig. 5.4.

The mutagenicity of carbon tetrachloride in *Sch. pombe ade6*. Cells were grown in the presence of carbon tetrachloride for 18 h. Data points are the mean values of three replicates.

Fig. 5.4. Mutagenicity of carbon tetrachloride in *Sch. pombe ade6*

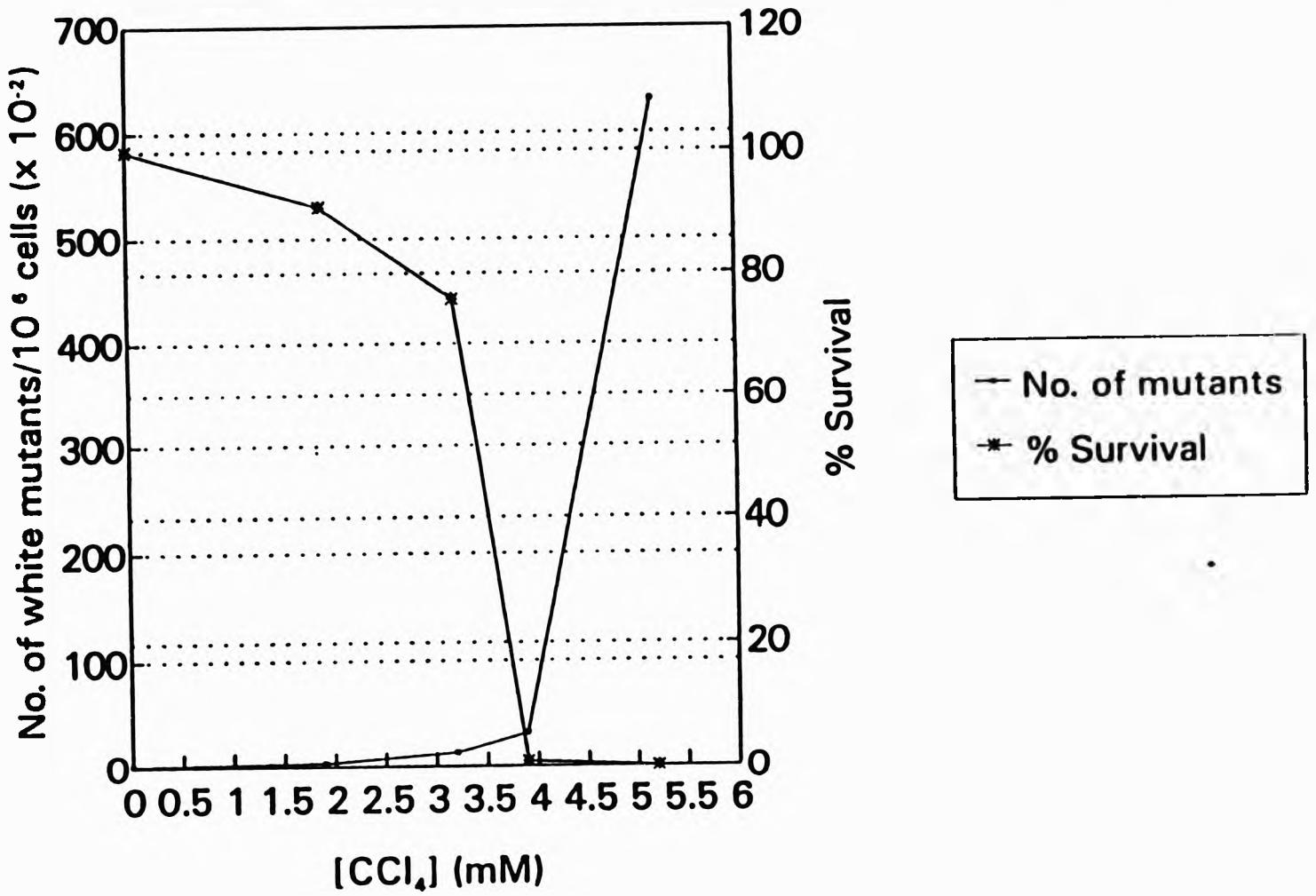


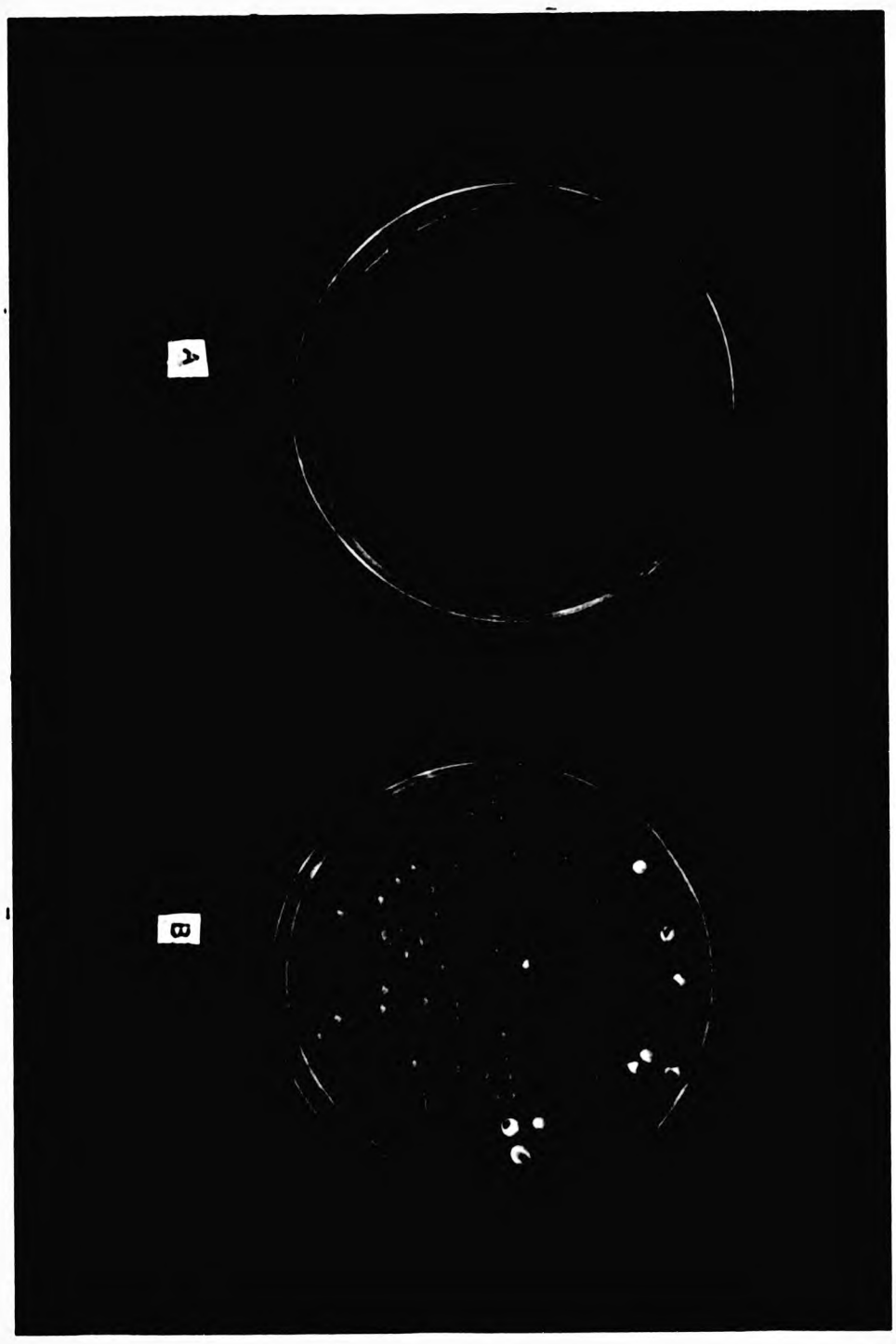
Plate 5.1.

YEA plates showing occurrence of white mutants of *Sch. pombe ade6* in a) control cultures and b) cultures exposed to 1.9 mM carbon tetrachloride.


A
B

Plate 5.2.

YEA plates showing occurrence of white mutants of *Sch. pombe ade6* in a) control cultures and b) cultures exposed to 5.2 mM carbon tetrachloride.


A
B

their nutritional requirements, they were found to retain their adenine auxotrophy. As in the case of the EMS-induced mutants, this indicated that the mutations were nuclear in origin and arose during a stage in the adenine biosynthetic pathway before the *ade6* blockage (see 5.2.3).

The auxotrophic mutants *Sch. pombe lys*1-131 and *leu*2-120 *lys*3-37 showed no reversion to prototrophy on exposure to carbon tetrachloride. It is possible that these strains contain mutations such as deletions which are not easily revertable. Reversion to prototrophy in Sch. pombe *leu*2-120 *lys*3-37 would in any case be expected to be rare as it requires two separate mutation events. Thus the frequency of such revertants would probably be too low to be detected by the assay performed in this study.

No forward mutations were observed in *Sch. pombe* 972 at the *ade6* or *ade7* locus (as detected by the production of red pigment) after treatment with carbon tetrachloride (data not shown). The forward mutation frequency at these loci has been found to be approximately 4 mutants per 10^7 cells in a wild-type strain and 4 mutants per 10^5 cells in a strain carrying the *rad10-198* mutation (Loprieno *et al.*, 1983). Therefore in this assay, cell numbers were probably too low for the detection of mutations at these loci.

The indication that carbon tetrachloride is mutagenic agrees with work carried out by Callen *et al.* (1980). The yeast *S. cerevisiae* D7 was exposed to carbon tetrachloride and an increase in the frequency of gene conversion at the *trp5* locus and in reversion at the *ilv1* locus were both

recorded. The most sensitive indicator of genetic damage was, however, the increase in frequency of mitotic recombination at the *ade2* locus. In all cases genetic alteration was seen most markedly at concentrations of carbon tetrachloride which were highly toxic to the cells. This was also the case in the present work. The concentrations of the compound to which D7 was exposed, however, were quite high (34mM being the maximum) in comparison with the concentrations used in this work. *Sch. pombe* can therefore be seen in the present study to be sensitive to the detection of carbon tetrachloride mutagenicity at low concentrations.

Schiestl (1989) tested carbon tetrachloride for possible mutagenic activity in the diploid strain *S. cerevisiae* RS112. Both deletion (DEL) and interchromosomal recombination (ICR) events were screened. While the concentration range of 4000-8000 $\mu g\ ml^{-1}$ gave positive responses in the ICR assay, there was a threshold below which no response could be detected. In this work, the author contrasted the dose-response curves of non-mutagenic carcinogens with those of carcinogens (such as EMS) detectable in the Ames test and proposed carbon tetrachloride to be a nonmutagenic carcinogen. In the present work also, the dose-response curves of EMS and carbon tetrachloride were seen to differ: carbon tetrachloride showing thresholds below which no response could be detected (figs. 5.2-5.4). Mirsalis *et al.* (1985) have also suggested that carbon tetrachloride acts by non-genotoxic means during the induction of liver tumours. The role of carbon tetrachloride as a tumour promoter has been discussed by Mohn (1981), see also Chapter 6.

Carbon tetrachloride was one of eight out of eleven chlorinated compounds tested to have genotoxic activity in *Aspergillus nidulans* (Crebelli *et al.*, 1988). Positive results were seen with the compound utilising induction of mitotic segregation as the end-point. Studies with the related compound tetrachloroethylene in yeast gave negative results (Bronzetti *et al.*, 1983). In this latter work the diploid strain *S. cerevisiae* D7 was tested using mitotic gene conversion, mitotic crossing-over and reverse point mutation at the *ilv* locus as endpoints. This is in contrast to studies carried out by Chrysoglou (1991) on *Sch. pombe ade6* where tetrachloroethylene was found to be mutagenic at concentrations from 0.008 to 0.8 mM, while higher concentrations were highly toxic to the cells.

Comparisons have been made of the mutagenicity of chlorinated hydrocarbons in yeast and bacteria. While 84% of the chemicals tested were mutagenic in yeast (Nestmann and Lee, 1983), only 53% were mutagenic in bacteria (Nestmann *et al*, 1980). Haroun and Ames (1981) have suggested that this difference is due to the lack of sensitivity of Salmonella to the mutagenic potential of chlorinated compounds.

5.2.5 Effect of α–tocopherol on mutation

Administration of the anti-oxidant α–tocopherol appeared to have little effect on the spontaneous mutation frequency in *Sch. pombe* 972 (table 5.6). However a dose-dependent reduction was seen in the number of induced mutants in cultures exposed to carbon tetrachloride. With the application of 50 μM α–tocopherol the mutation frequency in the

presence of 5.2 mM carbon tetrachloride was reduced to that of the spontaneous value (table 5.6).

As treatment of carbon tetrachloride-exposed cultures of *Sch. pombe* with α–tocopherol also appeared to decrease cytotoxicity (see Chapter 4), this suggests that toxic and genotoxic events induced by carbon tetrachloride may be related. It may be postulated, for example, that toxic free radical metabolites of carbon tetrachloride (such as the trichloromethyl radical $\cdot CCl_3$) may contribute to genotoxicity at high concentrations.

Table 5.6

Effect of α-tocopherol on genotoxicity in *Sch. pombe* 972

$[CCl_4]$ (mM)	[α-tocopherol] (μM)	Average no. cfu ml^{-1}	% survival	Average no. CAP^R mutants/ 10^6 viable cells
0	0	16.7×10^6	100	0.66
0	5	16.9×10^6	101.2	1.20
0	50	14.6×10^6	87.4	0.32
5.2	0	3.9×10^6	23.4	2.17
5.2	5	3.8×10^6	22.8	1.03
5.2	50	7.1×10^6	42.5	0.65

Determinations represent means of three replicates from one experiment. Cells were grown in the presence of carbon tetrachloride and α-tocopherol for 18 h.

Free-radical-producing agents have been implicated in genotoxicity and cancer induction in several systems (Miller and Miller, 1971). Carbon tetrachloride was found to induce somatic segregation in *Aspergillus*

nidulans (Gualandi, 1984), although gene mutation could not be detected in a haploid strain of this organism. The genotoxic action of the compound was explained by the formation of free-radicals and evidence was presented that the free-radical scavenger cysteamine but not α–tocopherol, could reduce the genotoxicity of carbon tetrachloride. However, α–tocopherol was found to be one of a series of compounds able to inhibit the mutagenic effects of 3,2'-dimethyl-4-aminobiphenyl in the Ames tester strains TA98 and TA100 (Reddy *et al.*, 1983). The anti-oxidant was effective in the dose range 5-50 mg per plate.

The relationship between carbon tetrachloride toxicity and genotoxicity has been examined in mammals. Exposure of rats to carbon tetrachloride only induced liver tumours at doses which were necrotic to this organ (Reuber and Glover, 1970). Shank and Barrows (1985) have shown that administration of carbon tetrachloride to rat liver caused the methyl group of s-adenosylmethionine to migrate to guanine. This may be a result of cytotoxic action and, in turn, lead to genotoxicity.

The involvement of cytochrome P450 in the metabolism of carbon tetrachloride has been discussed (see Chapter 4). Cells used in all genotoxicity studies were grown under conditions shown to maximise cytochrome P450 levels (see Chapter 3). Through its action on carbon tetrachloride this haemoprotein may thus contribute to the generation of genotoxic species.

CHAPTER 6

CONCLUSIONS

In the utilisation of yeasts as model systems for monitoring toxic and mutagenic events, an understanding of the role of their microsomal monooxygenase system is vital. In tested strains the factors found to influence production of cytochrome P450 included oxygen levels and carbon source. As cytochrome P450 was produced when cells were grown under conditions of glucose repression, high concentrations of glucose are required for maximum production of the haemoprotein. Manipulation of other physiological conditions such as high oxygen tension also resulted in induction of the enzyme, but only when glucose repression was in effect. Since glucose-repressed cells whether aerated or non-aerated contained the haemoprotein, glucose repression appeared to be the overriding factor for high yields of cytochrome P450.

This hypothesis was confirmed when conditions of glucose repression were mimicked genetically by the use of *petite* mutants. In comparison to wild-type cells these mitochondrially deficient mutants were found to produce high concentrations of the enzyme. The repression of mitochondrial function was thought to be a key factor in cytochrome P450 production and is in agreement with work carried out by Wiseman on the role of cAMP in production of this protein (Wiseman, 1980). The use of *petite* mutants could therefore have important applications in the production of high concentrations of cytochrome P450, for example as a non-animal derived replacement for S9 mix in the Ames test. Short-term

genotoxicity tests utilise rat liver S9 fractions from animals pre-treated with Aroclor-1254 (Elliott *et al.*,1992). However the high toxicity of this compound makes it undesirable for widespread use and thus yeast cytochrome P450 may be seen as a viable alternative.

The fission yeast *Sch. pombe* was found to produce good yields of cytochrome P450, with the wild-type strain *Sch. pombe* 972 in particular producing up to 22.35 nmol P450 g^{-1} dry cell weight when grown under conditions of glucose repression. Both this yeast and the budding yeast *S. cerevisiae* produced detectable levels of the haemoprotein when grown either with or without aeration. However the glucose-insensitive yeast *C. parapsilosis* only produced detectable levels of cytochrome P450 when grown with aeration in the presence of high concentrations of glucose. Solubilised microsomes prepared from this organism when subjected to SDS-PAGE analysis showed a major band migrating at 48 kDa. The solubilised microsomes were shown to contain cytochrome P450. This 48 kDa protein may be a strong candidate for cytochrome P450 and is close to published values for CYP51 isolated from *C. albicans* (Hitchcock *et al.*, 1989b).

The importance of *C. parapsilosis* as a nosocomial pathogen is being increasingly recognised. In favourable conditions this yeast may cause candidiasis, where symptoms include thrush, vaginitis and bronchocandidiasis, and in systemic infections septicaemia may also occur. Many infections of *C. parapsilosis* are linked to the use of contaminated devices used in medical treatment (Weems, 1992). The

pathogenicity of this species may be related to a growth advantage in high concentrations of glucose (Weems, 1992). The production of cytochrome P450 could therefore have important applications as an anti-fungal target due to the fact that azole compounds may interfere with the cytochrome P450 dependent demethylation of lanosterol to ergosterol (Vanden Bossche, 1985).

Attention should be drawn to the difficulties encountered in obtaining reproducible values of cytochrome P450 in yeasts. As discussed in Chapter 3, fluctuation in levels of the haemoprotein were minimised by strictly adhering to methods used for the growth and preparation of the cells and for the measurement of cytochrome P450. The problems associated with the measurement of turbid samples when using whole cells were minimised by the use of a spectrophotometer which reduced light scattering by positioning the cuvette in close proximity to the light source.

Sch. pombe was found to act as a good model organism for investigating the toxicological effects of carbon tetrachloride. This species appeared to be more sensitive to the effects of the compound than *S. cerevisiae*, an observation which could perhaps be related to the differing fatty acid content of the two yeast species (Johnson and Brown, 1972). The partial alleviation by α–tocopherol of carbon tetrachloride induced toxicity in *Sch. pombe* was comparable to published results with mammalian cells, and indicated a role for free radical metabolites of carbon tetrachloride in the causation of cell death.

From the mutagenicity data on carbon tetrachloride obtained in the various yeast assays employed, it can be inferred that this compound acts as a weak mutagen. Mutation is an important factor in the carcinogenic potential of many chemicals but is only involved in initiation.

The two-stage theory of carcinogenesis is a widely accepted view (Rous and Kidd, 1941), *viz* cells transformed by an initiating agent remain latent until a further event, brought about by a promoting agent induces tumourigenesis. Promotion may be brought about by many mechanisms including disruption of the mitotic apparatus and damage to the cell membrane, thereby interfering with intercellular communication (Ames, 1983). Carbon tetrachloride has been determined by some researchers to act as a promoting agent (Mohn, 1981).

The non-genotoxic mechanisms of action of carcinogenic compounds has been discussed (Douglas *et al.*, 1988). These mechanisms may also contribute to the action of carbon tetrachloride (Douglas *et al.*, 1988). The mechanisms by which such compounds act range in complexity. Many of them have been classified as 'epigenetic' and their common characteristics with respect to cancer induction have been summarised by Shank and Barrows (1985) :

i. They appear to induce cancer only at exposure levels which are near lethal doses.

ii. Many increase the incidence of common spontaneous tumours but do

not induce formation of those tumours which are rarely seen in control populations.

iii. They induce cancers only after long exposures relative to the life span of the test species.

iv. They do not form detectable levels of DNA adducts in *in vivo* tests.

In the present study, carbon tetrachloride was observed to induce mutation only at near lethal concentrations. This is in agreement with the work of Shank and Barrows (1985) who found the compound to be carcinogenic only after inducing extensive cytotoxicity. The alleviation of carbon tetrachloride-induced toxicity and genotoxicity by α–tocopherol suggests a link between toxic species such as $\cdot CCl_3$ and $CCl_3OO\cdot$ generated by carbon tetrachloride metabolism and the genotoxicity of the compound at high doses. The mutagenic potential of these species has been discussed by Halliwell and Gutteridge (1989). Other authors have cited a relationship between free-radical species (including those generated by lipid peroxidation) and mutagenicity. Kappus and Sies (1981) made reference to reactive oxygen species generated during O_2 metabolism and to reactive species produced by compounds including carbon tetrachloride as sources of mutagenic activity. It has been postulated that many 'complete' carcinogens and promoters exert their activity by the generation of oxygen radicals (Ames, 1983). In addition to cancer, free-radical formation has been implicated in the cause and propagation of conditions such as rheumatoid arthritis, Alzheimer's

disease and Parkinson's disease (Halliwell and Gutteridge, 1990). The present work has indicated that yeasts could serve as model systems for the detection of these species.

Antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been found to reduce carcinogenicity induced by compounds such as benzo(a)pyrene. The mechanism of action of these compounds has been related in part to an increase in detoxification mechanisms such as the glutathione-S-transferase system (Halliwell and Gutteridge, 1989).

Some cancers may originate as a result of faulty repair following DNA damage by free radicals (Chellman *et al.*, 1986). Useful organisms for investigating this process could therefore include yeasts deficient in DNA repair systems.

In the assessment of the mutagenicity of carbon tetrachloride, yeasts have been found in this work to give positive responses not observed in the Ames test. As previously discussed by Nestmann *et al* (1981) this has highlighted the need to include both yeasts and bacteria in any battery of genotoxicity test systems. However, for evaluation of the potential non-genotoxic mechanisms of carcinogenesis and tumour promoting abilities of compounds, the development of new test systems are required.

Many possibilities exist for further work in the development of yeasts for

toxicological and genotoxicological studies. Firstly in the elucidation of the action of carbon tetrachloride in *Sch. pombe* other antioxidants (e.g. propyl gallate) may be investigated for their activity. A comparison may also be carried out between the action of these antioxidants on *Sch. pombe* and *S. cerevisiae* which are thought to differ in fatty acid composition (Johnson and Brown, 1972).

Further work would also be useful in assessing cytochrome P450 production in *petite* mutants by screening a wide range of wild type strains and their *petite* derivatives.

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APPENDIX 1

STATISTICAL ANALYSIS OF DATA USING MINITAB (IBM)

Fig. 3.11. Cytochrome P450 values of *S. cerevisiae petites* and wild types

Raw Data

ROW	C1	
1	1	19.67
2	2	4.82
3	3	3.15
4	4	0.00
5	1	31.47
6	2	21.97
7	3	0.00
8	4	14.65
9	1	11.13
10	2	4.91
11	3	2.79
12	4	2.19
13	1	9.14
14	2	3.00
15	3	3.06
16	4	0.00
17	1	15.25
18	2	4.57
19	3	0.00
20	4	4.63

```
MTB> table   c1;
SUBC> means c2;
SUBC> stdev   c2.
```

ROWS: C1

	C2 MEAN	C2 STD DEV
1	17.332	8.878
2	7.854	7.929
3	1.800	1.648
4	4.294	6.096
ALL	7.820	8.651

MTB> oneway c2 c1

ANALYSIS OF VARIANCE ON C2

SOURCE	DF	SS	MS	F	P
C1	3	695.8	231.9	5.11	0.011
ERROR	16	726.3	45.4		
TOTAL	19	1422.0			

LEVEL	N	MEAN	STDEV
1	5	17.332	8.878
2	5	7.854	7.929
3	5	1.800	1.648
4	5	4.294	6.096

POOLED STDEV = 6.737
MTB> nooutfile

LEAST SIGNIFICANT DIFFERENCE (LSD) CALCULATIONS:

$$LSD = t_{\text{error df}} \sqrt{MS_{error}\,(1/n_1 + 1/n_2)}$$

MS = mean square

n = number of observations.

(2-tailed test)

LSD ($P < 0.01$) = 2.921 X 4.26 = 12.44.

Tables 3.8, 3.9. Cytochrome P450 and P420 production in aerated and non-aerated cultures of *Sch. pombe* and *S. cerevisiae*

ROW	C1	C2
1	1	4.30
2	2	2.30
3	1	7.60
4	2	5.10
5	1	5.44
6	2	7.10
7	1	2.40
8	2	0.39
9	1	19.60
10	2	9.00
11	1	4.80
12	2	0.00
13	1	3.14
14	2	0.00
15	1	0.00
16	2	0.00

```
MTB   > table C1 ;
SUBC > mean C2;
SUBC > stdev  C2.
```

ROWS: C1

		C2 MEAN	C2 STD DEV
Aer	1	5.9100	5.9670
Non-aer	2	2.9862	3.6158
	ALL	4.4481	4.9997

```
MTB > oneway C2 C1
```

ANALYSIS OF VARIANCE ON C2

SOURCE	DF	SS	MS	F	P
C1	1	34.2	34.2	1.40	0.256
ERROR	14	340.8	24.3		
TOTAL	15	375.0			

LEVEL	N	MEAN	STDEV
1	8	5.910	5.967
2	8	2.986	3.616

POOLED STDEV = 4.934
MTB > nooutfile

P value not significant.

Fig. 4.1a. Toxicity of carbon tetrachloride in *Sch. pombe* 972

MTB > PRIN C1 C2

ROW	C1	C2
1	1	100.0
2	2	36.8
3	3	10.8
4	4	7.7
5	5	2.3
6	1	100.0
7	2	102.5
8	3	17.1
9	4	7.3
10	5	2.5
11	1	100.0
12	2	65.6
13	3	36.0
14	4	12.1
15	5	0.0
16	1	100.0
17	2	62.5
18	3	2.6
19	4	1.6
20	5	1.6

MTB > TABLE C1 ;
SUBC > MEAN C2 ;
SUBC > STDEV C2.

ROWS: C1

	C2 MEAN	C2 STD DEV
1	100.000	0.000
2	66.850	27.046
3	16.625	14.216
4	7.175	4.306
5	1.600	1.134
ALL	38.450	41.352

MTB > ONEWAY C2 C1

ANALYSIS OF VARIANCE ON C2

SOURCE	DF	SS	MS	F	P
C1	4	29629	7407	38.85	0.000
ERROR	15	2860	191		
TOTAL	19	32490			

LEVEL	N	MEAN	STDEV
1	4	100.00	0.00
2	4	66.85	27.05
3	4	16.63	14.22
4	4	7.17	4.31
5	4	1.60	1.13

LSD (P < 0.01) = 28.80

POOLED STDEV = 13.81

Fig. 4.1b. Toxicity of carbon tetrachloride in *Sch. pombe* 972

MTB > PRIN C1 C3

ROW	C1	C3
1	1	100.0
2	2	57.1
3	3	42.8
4	4	14.3
5	5	0.0
6	1	100.0
7	2	21.2
8	3	14.5
9	4	28.9
10	5	0.6
11	1	100.0
12	2	84.6
13	3	51.3
14	4	12.8
15	5	28.2
16	1	100.0
17	2	116.0
18	3	68.1
19	4	21.7
20	5	5.9

MTB > TABLE C1 ;
SUBC > MEAN C3;
SUBC > STDEV C3.

ROWS : C1

	C3 MEAN	C3 STD DEV
1	100.000	0.000
2	69.725	40.318
3	44.175	22.403
4	19.425	7.419
5	8.675	13.284
ALL	48.400	39.262

MTB > ONEWAY C3 C1

ANALYSIS OF VARIANCE ON C3

SOURCE	DF	SS	MS	F	P
C1	4	22211	5553	11.77	0.000
ERROR	15	7077	472		
TOTAL	19	29288			

LEVEL	N	MEAN	STDEV
1	4	100.00	0.00
2	4	69.72	40.32
3	4	44.17	22.40
4	4	19.42	7.42
5	4	8.68	13.28

POOLED STDEV = 21.72

LSD (P < 0.01) = 45.27.

Fig. 4.1e. Toxicity of carbon tetrachloride in *Sch. pombe ade6*

MTB > PRIN C1 C2

ROW	C1	C2
1	1	100.00
2	2	65.30
3	3	2.20
4	4	8.70
5	5	0.87
6	1	100.00
7	2	12.60
8	3	14.20
9	4	4.70
10	5	6.30

MTB > TABLE C1;
SUBC > MEANS C2

ROWS : C1

	C2
	MEAN
1	100.000
2	38.950
3	8.200
4	6.700
5	3.585
ALL	31.487

MTB > ONEWAY C2 C1

ANALYSIS OF VARIANCE ON C2

SOURCE	DF	SS	MS	F	P
C1	4	13370	3342	11.27	0.010
ERROR	5	1483	297		
TOTAL	9	14853			

LEVEL	N	MEAN	STDEV
1	2	100.00	0.00
2	2	38.95	37.26
3	2	8.20	8.49
4	2	6.70	2.83
5	2	3.59	3.84

POOLED STDEV = 17.22

LSD (P < 0.01) = 69.49

Fig. 4.2a. Toxicity of carbon tetrachloride in *S. cerevisiae* 188

```
MTB > PRINT C4  C5

ROW       C4          C5

1          1         100.0
2          2          45.9
3          3          17.4
4          4           4.7
5          5          13.3
6          1         100.0
7          2          53.8
8          3          51.9
9          4          36.5
10         5          23.1
11         1         100.0
12         2          56.1
13         3          53.6
14         4          22.6
15         5          11.5

MTB  > TABLE  C4;
SUBC >  MEANS C5;
SUBC >  STDEV  C5.


ROWS:  C4

                C5          C5
                MEAN        STD DEV

1             100.000       0.000
2              51.933       5.350
3              40.967      20.427
4              21.267      15.942
5              15.967       6.243
ALL            46.027      32.677

MTB > ONEWAY C5  C4
```

ANALYSIS OF VARIANCE ON C5

SOURCE	DF	SS	MS	F	P
C4	4	13471	3368	22.79	0.000
ERROR	10	1478	148		
TOTAL	14	14949			

LEVEL	N	MEAN	STDEV
1	3	100.00	0.00
2	3	51.93	5.35
3	3	40.97	20.43
4	3	21.27	15.94
5	3	15.97	6.24

POOLED STDEV = 12.16
MTB > NOOUTFILE

LSD (P < 0.01) = 31.48

Table 4.6. The effect of carbon tetrachloride on cytochrome P450 levels in *Sch. pombe* 972 after growth in YEPD containing 20% (wt/vol) glucose for 18 h.

MTB > PRINT C1 C2 C5

ROW	C1	C2	C5
1	1	1	7.61
2	1	2	5.35
3	1	3	5.22
4	2	1	5.11
5	2	2	2.39
6	2	3	0.00

MTB > ANOVA C5 = C1 C2

Factor	Type	Levels	Values		
C1	fixed	2	1	2	
C2	fixed	3	1	2	3

Analysis of Variance for C5

Source	DF	SS	MS	F	P
C1	1	21.546	21.546	23.51	0.040
C2	2	14.217	7.108	7.75	0.114
Error	2	1.833	0.917		
Total	5	37.596			

MTB > NOOUTFILE

P value significant for aerated vs. non-aerated (0.04); not significant for CCl_4 treatment (0.114). However LSD calculated for data ($P < 0.05$) = 3.36. Therefore significant difference between some values.

Table 5.1. Mutagenicity of ethanol in *Sch. pombe* 972.

MTB > PRINT C5 C6

ROW	C5	C6
1	1	1.72
2	1	2.85
3	1	3.65
4	1	1.59
5	2	0.25
6	2	3.57
7	2	3.57
8	2	1.47

MTB > ONEWAY C6 C5

ANALYSIS OF VARIANCE ON C6

SOURCE	DF	SS	MS	F	P
C5	1	0.11	0.11	0.06	0.812
ERROR	6	10.96	1.83		
TOTAL	7	11.07			

LEVEL	N	MEAN	STDEV
1 (+ ETOH)	4	2.453	0.979
2 (- ETOH)	4	2.215	1.642

POOLED STDEV = 1.352
MTB > NOOUTFILE

P value not significant.

Fig. 5.1a. Mutagenicity of ethyl methanesulphonate in *Sch. pombe* 972

```
MTB > PRINT  C1  C2
ROW        C1          C2

1          1           1.72
2          2           2.66
3          3           6.85
4          4           8.15
5          5           20.20
6          1           0.38
7          2           3.30
8          3           5.18
9          4           19.00
10         5           26.66

MTB  > TABLE  C1;
SUBC > MEANS C2;
SUBC > STDEV  C2.

ROWS:  C1

                C2          C2
                MEAN        STD DEV

1               1.050       0.948
2               2.980       0.453
3               6.015       1.181
4               13.575      7.672
5               23.430      4.568
ALL              9.410      9.162

MTB > ONEWAY C2  C1

ANALYSIS OF VARIANCE ON C2
SOURCE    DF          SS          MS      F       P
C1        4           673.3       168.3   10.24   0.013
ERROR     5            82.2        16.4
TOTAL     9           755.6
```

LEVEL	N	MEAN	STDEV
1	2	1.050	0.948
2	2	2.980	0.453
3	2	6.015	1.181
4	2	13.575	7.672
5	2	23.430	4.568

POOLED STDEV = 4.055

LSD (P < 0.05) = 10.41

Fig. 5.2a. Mutagenicity of carbon tetrachloride in *Sch. pombe* 972

```
MTB >  PRINT C4  C6

ROW        C4          C6

1          1           2.85
2          2           5.32
3          3           10.00
4          4           8.00
5          5           33.33
6          1           3.65
7          2           3.57
8          3           20.55
9          4           34.48
10         5           35.71
11         1           1.59
12         2           1.27
13         3           0.00
14         4           16.67
15         5           33.33

MTB   >  TABLE C4;
SUBC >  MEAN C6;
SUBC >  STDEV C6.
ROWS: C4

                C6          C6
                MEAN        STD DEV

1               2.697       1.039
2               3.387       2.031
3               10.183      10.276
4               19.717      13.500
5               34.123      1.374
ALL             14.021      13.804

MTB >  ONEWAY C6  C4
```

ANALYSIS OF VARIANCE ON C6

SOURCE	DF	SS	MS	F	P
C4	4	2077.8	519.5	8.81	0.003
ERROR	10	589.9	59.0		
TOTAL	14	2667.7			

LEVEL	N	MEAN	STDEV
1	3	2.697	1.039
2	3	3.387	2.031
3	3	10.183	10.276
4	3	19.717	13.500
5	3	34.123	1.374

POOLED STDEV = 7.681
MTB > NOOUTFILE

LSD (P < 0.05) = 13.97

TITLE

TOXICOLOGICAL STUDIES IN YEAST SPECIES: PRODUCTION OF CYTOCHROME P450 AND EVALUATION OF CARBON TETRACHLORIDE TOXICITY

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