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THE BIOSYNTHESIS AND SOME PROPERTIES OF CYTOCHROME P-450 FROM SACCHAROMYCES CEREVISIAE

Being a Thesis presented in accordance with the Regulations governing the Award of the Degree of Doctor of Philosophy in the University of Surrey

by

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Department of Biochemistry,
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Surrey, England.

August, 1979
Summary

The intracellular concentration of cyclic AMP controls the biosynthesis of the microsomal cytochrome P-450 of Saccharomyces cerevisiae. The concentration of cyclic AMP in the cell is in turn related inversely to the extracellular concentration of glucose. When yeast was grown to the logarithmic phase in medium containing low concentrations of glucose and then transferred to high glucose medium, rapid biosynthesis of cytochrome P-450 occurred, essentially under conditions of glucose repression of the mitochondrial cytochromes.

The biosynthesis of cytochrome P-450 was studied in yeast protoplasts transferred to high glucose medium. This process was inhibited by actinomycin D and cycloheximide and therefore required de novo protein synthesis. Cyclic AMP added to protoplasts suspended in the high glucose medium caused partial repression of cytochrome P-450 biosynthesis. Conversely, all previous reports of cyclic AMP effects in microorganisms have involved a positive effect on enzyme biosynthesis due to the lifting of glucose repression.

The cytochrome P-450 produced by yeast was found to be capable of metabolising benzo(a)pyrene. The major metabolites, as identified by high-pressure liquid chromatography, were 3-hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene and
7,8-dihydro-7,8-dihydroxybenzo(a)pyrene. Pretreatment of the yeast with benzo(a)pyrene during growth decreased the Michaelis constant (Km) of the aryl hydrocarbon hydroxylase for benzo(a)pyrene compared with control yeast, using either NADPH or cumene hydroperoxide as the cofactor. A solubilised and a solubilised and immobilised cytochrome P-450 preparation was capable of benzo(a)pyrene hydroxylation using cumene hydroperoxide as the cofactor.

The interaction of benzo(a)pyrene with cytochrome P-450 was further investigated by means of an equilibrium gel filtration method using \[\text{[C-}^{3}\text{H]}\text{benzo(a)pyrene}. \] There appeared to be twenty binding sites for benzo(a)pyrene in both yeast and rat liver microsomes.

Benzo(a)pyrene was found to produce a modified type I spectral change with yeast and rat liver microsomes. Type II changes were obtained with imidazole and sodium phenobarbitone.

Several drug-metabolising assays of cytochrome P-450 activity were investigated but none proved to be satisfactory.
To

My Wife, Jenny and My Parents
Acknowledgements

I would like to express my thanks to Dr. Alan Wiseman for his help and guidance throughout the course of this work and to Dr. G.M. Cohen for his help with the high-pressure liquid chromatography of the benzo(a)pyrene metabolites.

I would also like to thank the University for its financial support, without which this work would not have been possible.
The White Rabbit put on his spectacles.
"Where shall I begin, please your Majesty?" he asked.

"Begin at the beginning," the King said gravely, "and go on till you come to the end; then stop."

Lewis Carroll
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CHAPTER 1.

General Introduction.
1. General Introduction

1.1. Introduction

It is the purpose of this General Introduction to review several major areas of research to which the work described in this thesis is related. These areas are the control of enzyme biosynthesis by means of cyclic AMP and several aspects of the action of cytochrome P-450, notably the metabolism of polycyclic aromatic hydrocarbons and some drugs. The mechanism of the action of cytochrome P-450 will be described in relation to this metabolism.

1.2. Cyclic AMP and Enzyme Biosynthesis

A number of studies have indicated that cyclic AMP plays a major role in the synthesis of inducible enzymes. The addition of an exogenous source of cyclic AMP enhanced the differential rate of β-galactosidase synthesis in Escherichia coli (Ullman & Monod, 1968) but did not affect the duration of the lag period before the onset of enzyme synthesis (Pastan & Perlman, 1968 and De Crombegghe et al., 1969). It has also been shown that glucose repression of many enzymes in E. coli can be decreased by cyclic AMP and this applied to β-galactosidase in many bacterial species (De Crombegghe et al., 1969 and Carpenter & Sells, 1973).

Cyclic AMP appears to stimulate the transcription of messenger RNA from the lac operon (Jacquet & Kepes, 1969,
Varmus et al, 1970a & b and De Crombagghe et al, 1971) and Chambers & Zubay (1969) observed a marked stimulation by cyclic AMP of the in vitro induced synthesis of β-galactosidase in a coupled system able to transcribe mRNA and to synthesise protein. The site of action of cyclic AMP appeared to be the promoter of the operon since point mutation within this region or deletion of most of the promoter markedly reduced the sensitivity to stimulation by cyclic AMP (Pastan & Perlman, 1968, Perlman et al, 1969 and Silverstone et al, 1969).

In contrast with these conclusions mutants effected in the operator and regulator genes showed similar responses to the wild-type strain (Pastan & Perlman, 1968, Perlman & Pastan, 1968a, Ullman & Monod, 1968 and Tyler & Magasanik, 1969).

Rickenberg (1974) has claimed that catabolite repression of the proteins coded by the lac operon as well as the reversal of catabolite repression by cyclic AMP, occurred at the level of translation. Dahl et al (1971) and Ramirez et al (1972) have suggested, however, that cyclic AMP exerts its effect predominantly at the level of transcription and Chao & Weathersbee (1974) reported that glucose, α-methyl glucoside, 2-deoxyglucose and pyridoxal 5'-phosphate repressed maltodextrin phosphorylase synthesis in E. coli and that this repression was reversed by cyclic AMP. Haggerty & Schleif (1975) reported that the addition of glucose, but not glycerol, to E. coli lowered the initiation frequency of lac mRNA within 10 sec and intracellular cyclic AMP concentrations responded
identically. It can be concluded therefore that cyclic AMP exerts its action at the level of transcription, this action is not directly on DNA but by means of the cyclic AMP receptor protein.

Cyclic AMP has been shown to control the biosynthesis of a number of enzymes in yeast and it has also been shown that the intracellular concentrations of cyclic AMP are in turn controlled by the concentration of glucose in the medium. Van Wijk & Konijn (1971) have demonstrated an increase in the intracellular concentration of cyclic AMP from 0.04μM to 0.32μM during the induction of α-glucosidase in yeast during transfer from 2%-glucose to 2%-maltose plus 0.1%-glucose medium. Montenecourt et al (1973) have shown that the concentrations of cyclic AMP correlated with the sensitivity of invertase synthesis to glucose repression and van der Plaat & van Solinger (1974) reported that trehalose synthesis was under the control of a system regulated by cyclic AMP during the lag phase of growth in Baker's yeast. An increase in the intracellular concentration of cyclic AMP was followed by a 6 to 8-fold increase in trehalose activity.

Schlanderer & Dellweg (1974) observed an inverse proportionality between glucose concentration and intracellular cyclic AMP in Schizosaccharomyces pombe. Watson & Berry (1977) and Hartig & Breitenbach (1977), in experiments in which cyclic AMP was measured during the cell cycle of
Saccharomyces cerevisiae, found an increase in cyclic AMP concentration during the induction of sporulation and a decrease before DNA synthesis and mitosis, indicating that cyclic AMP may play an important role in the control of cell division. It has also been shown that during the growth of S. cerevisiae the intracellular concentration of cyclic AMP is 2 to 3 times greater in 0.1%-glucose than in 20%-glucose medium (Lim, 1976). Sy & Richter (1972) have shown that glucose inhibits adenyl cyclase and this observation may indicate a possible mechanism by which glucose can influence the intracellular concentration of cyclic AMP.

1.3. Mammalian Cytochrome P-450

Several reviews of the mammalian mixed-function oxidase system, of which cytochrome P-450 is a part, have been published (Lu et al, 1976 for example). The metabolism of foreign compounds by mammalian tissues was first described by Mueller & Miller (1948 & 1953), Axelrod (1955 & 1956) and Brodie et al (1955). They established that the enzyme system was localised in liver microsomes and that the reaction required both NADPH and molecular oxygen. A feature of this system was that its action towards drugs and foreign compounds was enhanced by in vivo pretreatment of animals with a variety of compounds (Conney et al, 1956 & 1957a, Remmer, 1958 and Remmer & Alsleben, 1958).

The liver microsomal mixed-function oxidase system catalyses the following general reaction:
RH + NADPH + H⁺ + O₂ → ROH + NADP⁺ + H₂O

where RH is the substrate and ROH the product of the reaction. Although NADH may replace NADPH as the electron donor, the rate of the reaction is markedly reduced. Some substrates are normal body constituents such as steroids, fatty acids and bile acids but the majority are foreign compounds such as drugs, carcinogens, insecticides and herbicides (Conney, 1967 and Kuntzman, 1969). Depending on the chemical nature of the substrate, the reaction may be the oxidation of an aliphatic side chain, the hydroxylation of an aromatic ring, the dealkylation of a secondary or tertiary amine, or a deamination or dehalogenation reaction. In all of these reactions, the initial step can be visualised as an hydroxylation reaction (Gillette, 1966).

It is well-established that the microsomal mixed-function oxidase system consists of at least two protein components, a haemoprotein called cytochrome P-450 (EC 1.14.14.1) and a flavoprotein called NADPH-cytochrome c reductase or NADPH-cytochrome P-450 reductase (EC 1.6.2.4) (Gillette et al., 1972). Cytochrome P-450 is the substrate- and oxygen-binding site of the hydroxylation system (Omura et al., 1965, Imai & Sato, 1966 and Schenkman et al., 1967) while the reductase serves as an electron carrier shuttling electrons between NADPH and cytochrome P-450 (Gigon et al., 1969).
The haemoprotein, cytochrome P-450, was discovered in the liver microsomal fraction by Klingenberg (1958) and by Garfinkel (1958) and is so-called as a result of its property to exhibit an absorption maximum at 450nm when the reduced form is complexed with carbon monoxide (Omura & Sato, 1964) and this property forms the basis of the spectrophotometric method for its determination (Omura et al., 1965).

Conney et al. (1957b) showed that the microsomal oxidative demethylation of 3-methylaminocazobenzene was inhibited by carbon monoxide and Orrenius et al. (1964) subsequently demonstrated that the oxidative metabolism of other substrates by liver microsomes was also inhibited by CO. Although the mechanism of CO inhibition was not known, these results suggested the involvement of a haemoprotein in hydroxylation reactions and the first direct evidence establishing the involvement of cytochrome P-450 in these reactions was obtained by Cooper et al. (1965a). These workers established that the hydroxylation of acetonilide, the N-demethylation of monomethylaminopyrine and the O-demethylation of codeine by liver microsomes were inhibited by CO and that this inhibition could be relieved by monochromatic light at 450nm. The resultant photochemical action spectrum was identical to the spectrophotometrically determined spectrum of the CO derivative of the reduced microsomal CO-binding pigment, thus establishing cytochrome P-450 as the oxidase of the liver microsomal hydroxylation system.
The flavoprotein, NADPH-cytochrome c reductase, was first described by Horecker (1950) and subsequently studied by Williams & Kamin (1962), Phillips & Langdon (1962) and Kamin et al (1965). Several lines of evidence suggested that the enzyme was involved in microsomal hydroxylation. Firstly, cytochrome c, an artificial electron acceptor for this enzyme, inhibited the overall reaction for hydroxylation (Gillette et al, 1957) secondly, both the NADPH-cytochrome c reductase reaction and the overall hydroxylation reaction were competitively inhibited by NADP+ (Orrenius, 1965). Thirdly, the pretreatment of animals with inducers such as sodium phenobarbitone resulted in a parallel increase in the level of the overall hydroxylation reaction and NADPH-cytochrome c reductase (Orrenius et al, 1965). Finally, antibody prepared against NADPH-cytochrome c reductase inhibited both NADPH-cytochrome c reductase and overall hydroxylation (Masters et al, 1971).

In addition to the NADPH-dependent, cytochrome P-450-containing hydroxylation system, liver microsomes also contain an NADH-dependent electron transport system which functions in fatty acid desaturation (Marsh & James, 1962, Holloway et al, 1963 and Oshino et al, 1966). This NADH-dependent system consists of a flavoprotein (NADH-cytochrome b5 reductase), a haemoprotein (cytochrome b5) and a cyanide-sensitive factor (Shimakata et al, 1972). Lipid has also been shown to be essential in this system, but the exact
site(s) at which lipid is functioning has not been established (Jones & Wakil, 1967, Jones et al, 1969 and Holloway, 1971).

The components of the NADPH- and NADH-dependent electron transport pathway appear to interact with each other at several sites (Figure 1.1). Thus, both cytochrome P-450 and cytochrome b\textsubscript{5} can be reduced by either NADPH or NADH (Cooper et al, 1965\textsuperscript{b} and Sato et al, 1965). Hildebrandt & Estabrook (1971) have suggested that the first of the two electrons required for microsomal hydroxylation is donated by NADPH through NADPH-cytochrome c reductase, whereas the second electron is derived from either NADPH or NADH via cytochrome b\textsubscript{5}. Correia & Mannering (1973) have also concluded that cytochrome b\textsubscript{5} is an electron transport carrier in the NADPH-dependent mixed-function oxidase system.

However, the obligatory involvement of cytochrome b\textsubscript{5} in the NADPH-dependent hydroxylation reaction is very unlikely (Gillette et al, 1972, Levin et al, 1974, Lu et al, 1974 and Sasame et al, 1974) although it has been established that cytochrome b\textsubscript{5} is an obligatory component in NADH-dependent hydroxylation. The components of this system are NADH-cytochrome b\textsubscript{5} reductase, cytochrome b\textsubscript{5}, lipid and cytochrome P-450 (Sasame et al, 1973 and West et al, 1974).

The activity of the liver microsomal hydroxylation enzyme system is stimulated by the administration of a wide variety
of drugs, carcinogens, insecticides and other foreign compounds. This enhanced enzyme activity represents an increase in the concentration of the enzyme by de novo protein synthesis since the induction process can be blocked by inhibitors of protein synthesis (e.g., ethionine and puromycin) and nucleic acid synthesis (e.g., actinomycin D) (Conney, 1967). The marked effect of inducers on the synthesis of microsomal protein in vivo is also paralleled by increased in vitro incorporation of amino acids into microsomal protein (Kato et al., 1966). Recently, Fernandez et al. (1978) have shown that the induction of cytochrome P-450 by 2-diethylaminethyl-2,2-diphenylvalerate hydrochloride (SKF 525A) was by an increase in protein synthesis and a decrease in protein degradation.

One of the most widely studied inducers of this enzyme system is the barbiturate, sodium phenobarbitone. The chronic administration of this compound to animals has been shown to result in an increase in the concentrations of microsomal cytochrome P-450, NADPH-cytochrome c reductase and phospholipids, and an acceleration in the rate of metabolism of many compounds in vitro (Ernster & Orrenius, 1965). There are many other inducers of the liver microsomal hydroxylation system, the extent of induction is dependent not only on the inducer used but also on the substrates studied. The rate of metabolism of many substrates (e.g., aminopyrine, benzphetamine, sodium hexobarbitone and ethylmorphine) is
increased by sodium phenobarbitone-pretreatment, but unchanged or even decreased by compounds such as polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene and 3-methylcholanthrene). The metabolism of some other compounds (e.g. benzo(a)pyrene and acetaldehyde) may be enhanced by both types of inducer (Conney et al, 1960, Levin et al, 1969 and Ullrich, 1969).

Based on the specificity of induction, all inducers have been generally divided into two distinct categories. The first type of inducer, exemplified by sodium phenobarbitone, is characterised by its ability to stimulate the metabolism of a large range of substrates by the induction of cytochrome P-450. In contrast, the second type of inducer, exemplified by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, stimulate the metabolism of only a limited number of compounds by a form of the enzyme designated cytochrome P-448. This difference in induction specificity has led to the conclusion that sodium phenobarbitone and 3-methylcholanthrene (or benzo(a)pyrene) evoke their stimulatory effects through different induction mechanisms (Conney, 1967). Furthermore, when sodium phenobarbitone and 3-methylcholanthrene, each in a dose known to give a maximal inductive effect, are administered to rats simultaneously, microsomal mixed-function oxidase activity is increased to a level greater than that observed in rats treated with these compounds alone (Gillette, 1963). These observations are consistent with the idea that sodium phenobarbitone and 3-methylcholanthrene
induce different hydroxylation enzyme systems through different mechanisms.

Much evidence has accumulated in support of the idea that there are several forms of cytochrome P-450 in liver microsomes. Most of this evidence is derived from studies using microsomes prepared from sodium phenobarbitone- and 3-methylcholanthrene-pretreated rats. The increase in benzo(a)pyrene hydroxylase activity in rats after 3-methylcholanthrene-pretreatment is related to the formation of a new haemoprotein called cytochrome P-448 (Alvares et al., 1967). Changes in the substrate specificity of microsomes and in the spectral properties of cytochrome P-450 following 3-methylcholanthrene-pretreatment have been interpreted as indicating that cytochrome P-448 is a new and different haemoprotein from cytochrome P-450, produced in untreated and sodium phenobarbitone-pretreated rats (Sladek & Mannering, 1966, Alvares et al., 1968, Kuntzman et al., 1968, Kuntzman et al., 1969, Mannering et al., 1969 and Mannering, 1971). Other workers have proposed that the spectral properties of cytochrome P-448 might result from the binding of the hydrocarbon inducer or its metabolites to the native cytochrome P-450, rather than from the formation of a new haemoprotein (Hildebrandt & Estbrook, 1969 and Schenkmann et al., 1969), but there is considerable evidence which supports the view that cytochrome P-450 and P-448 are two different entities and not the same enzyme in two interconvertible forms.

More recent evidence has shown that there may be more than two forms of cytochrome P-450 in liver microsomes. For example, Mailman et al. (1977) have shown that in mouse liver microsomes a total of seven cytochromes would be necessary to explain results obtained using density subfractionation and spectral behaviour to measure the response to sodium phenobarbitone and 3-methylcholanthrene. Using immunochemical methods Thomas et al. (1976a & b) have presented evidence for the existence of six different cytochromes P-450 in the endoplasmic reticulum of rat liver, although these immunochemical differences may be due to the presence of different sugar moieties in the cytochrome P-450 glycoprotein. Using gel electrophoresis, Bell & Hodgson (1977) have demonstrated the existence of four cytochrome P-450 polypeptides in mouse liver microsomal preparations, one increased with 3-methylcholanthrene administration, two increased with sodium phenobarbitone and the fourth decreased following treatment with either of these inducing agents. This type of gel electrophoresis will not separate component polypeptides of a similar molecular weight and so a greater
number of different cytochromes than four is possible.
Anion-exchange chromatography of detergent-solubilised
cytochrome P-450 from the livers of untreated rats yielded
four fractions with 70 to 80% recovery, each of these fractions
was further resolved by electrofocussing into numerous proteins,
more than eight of which contained haem (Warner et al, 1978).

A number of cytochromes P-450 have been purified to
apparent homogeneity (Guengerich, 1978a). One fraction from
the liver microsomes of 3-methylcholanthrene-pretreated rats
and two fractions from sodium phenobarbitone-pretreated rats
have been purified to homogeneity as judged by five different
criteria. These homogeneous cytochrome P-450 proteins were
distinguished by different electrophoretic mobilities, amino
acid compositions, NH₂- and HOOC-terminal amino acid sequences
and immunochemical characteristics, although there was some
evidence that they were immunochemically related. Enzymic
hydrolysis followed by peptide mapping in polyacrylamide gel
confirmed that these three proteins are distinct (Guengerich,
1978b).

Having described the various components of the microsomal
mixed-function oxidase system it is now time to relate these
components to the mechanism of the action of this system.
As shown in Figure 1.1 the function of cytochrome P-450 is
of prime importance in this system and the mechanism may
be conveniently divided into six stages.

(i) the interaction of the ferric haemoprotein
with the substrate.

(ii) the reduction of this complex by an electron
donated by the NADPH-cytochrome c reductase flavoprotein.

(iii) the interaction of oxygen with the
haemoprotein to form an oxycytochrome P-450 complex to which
the substrate is still tightly bound.

(iv) the further reduction of the ternary complex
of oxygen, substrate and cytochrome P-450 by an electron
donated from NADPH-cytochrome c reductase or NADH-cytochrome
b5 reductase.

(v) the activation of oxygen for interaction
with the organic substrate and finally

(vi) the dissociation of the product with
regeneration of the ferric haemoprotein.

A closer look at the binding of substrate to the cytochrome
P-450 is perhaps worthwhile here. The technique of difference
spectrophotometry has been used widely for more than ten
years to study spectral changes which represent the binding
of drugs and other chemicals to liver microsomal cytochrome
P-450 (Remmer et al., 1966, Imai & Sato, 1966 and Schenkman
et al., 1967). Three fundamentally empirical types of spectra
have been well characterised with various drugs and other
chemicals although they are poorly understood. These
spectra are: "type I", with spectral maxima at 385-390nm and
minima at 420nm; "type II", with minima at 390-410nm and
maxima at 427-430nm and "reverse type I", which is almost
exactly the mirror image of the type I spectrum. This
Figure 1.1. The Electron-Transport Reactions in Microsomes.

( adapted from Coon et al, 1975, Hrycay et al, 1976 and Estabrook, 1978)
technique, however, reveals nothing in absolute terms about the changes occurring at or near the enzyme active site when the substrate interacts with the cytochrome.

The type I spectral change is thought to be due to the binding of a substrate to the apoprotein of cytochrome P-450 and is caused by an increase in the electronegativity of one ligand of the haem. Schenkman & Sato (1968) suggested that there may be two forms of cytochrome P-450, one unreacted enzyme and another which is bound to an endogenous type I substrate. Temple (1971) suggested that the type I interaction resulted by the replacement of this endogenous substrate by a more strongly bound exogenous substrate. The type II spectral change is associated with ferrirhaemochrome formation involving electron transfer between the sixth ligand of the haem iron and the nitrogen of the added compound (Schenkman et al, 1967).

laboratory (Kumaki et al, 1978) has shown that the pretreatment of several species of mammals with sodium phenobarbitone and 3-methylcholanthrene produced consistent detectable changes in the spin state of the cytochrome P-450 iron \textit{in vivo.} Simple liquids of relatively "pure" type I, type II and reverse type I character (cyclohexane, n-octylamine and n-butanol, respectively) produced detectable changes in the \textit{in vitro} spin state of the cytochrome P-450 iron. They also showed that the presence or absence of a spectral change depended largely on the \textit{in vivo} spin state of the cytochrome P-450 iron in freshly prepared microsomes at the start of the experiment.

In the cytochrome P-450 from \textit{Pseudomonas putida} induced by growing in the presence of $D$-$(+)$-camphor (Cytochrome P-450$_{cam}$) Sligar (1976) has shown that in the presence or absence of substrate there was a mixture of high-spin and low-spin haem iron which was temperature sensitive. In related studies, Rein et al (1977) reported the presence of a substrate-induced high-spin/low-spin equilibrium for rabbit liver microsomal cytochrome P-450 and that this equilibrium was temperature dependent. At the same time Pierson & Cinti (1977) found that the microsomal type I and reverse type I spectral changes could be produced without the addition of substrate simply by an alteration in the temperature, they also showed that the substrate-induced type I and type II spectral changes were affected by temperature.
More recently Cinti et al (1979) demonstrated that microsomal and solubilised cytochrome P-450 from rat liver existed as a mixture of two spectral forms which were interconvertible as the sample temperature was varied. Intact microsomal enzyme consisted of approximately equal amounts of high- and low-spin forms at 20°C but when lipid and endogenous substrates were removed by solubilisation and purification, the molecule was found to be in a predominantly low-spin state. Rein and other workers (Ristau et al, 1978) evaluated the equilibrium constants and thermodynamic parameters describing the spin transition and the substrate binding from temperature and substrate difference spectra. These two interacting equilibria were then presented in terms of a thermodynamic model, which provided a clear quantitative description of the properties of the cytochrome P-450-substrate system. From this model, the cause of the substrate difference spectra was explained and the importance of the spin shift in the presence of substrate with respect to the reduction rate was discussed. Cinti et al (1979) also used their data to derive various thermodynamic parameters for the system and in addition they also calculated the precise absolute extinction coefficients for pure high-spin and low-spin forms of cytochrome P-450.

As indicated in Figure 1.1 the mammalian mixed-function oxidase system is capable of using cofactors other than NADPH. Kadlabur et al (1973) found that cytochrome P-450 was able
to catalyse the C-oxidation of several amine substrates when NADPH was replaced by a variety of organic hydroperoxides, the maximum rate was obtained when cumene hydroperoxide (I) was used, oxygen was not required for the reaction.

\[
\text{H}_3\text{C} - \text{CH}_2\text{COOH} \\
\text{H}_2\text{C}
\]

(I)

Hrycay & O'Brien (1974) and Hrycay & Prough (1974) found that several hydroperoxides, progesterone 17\(\alpha\)-, pregnenolone 17\(\alpha\)- and cumene hydroperoxide, were effective as substrates in a NADH-cytochrome b\(5\) reductase and cytochrome P-450 dependent microsomal NADH-peroxidase activity. Rahimtula & O'Brien (1974) showed that cumene hydroperoxide could support the hydroxylation of a number of aromatic compounds such as aniline, benzo(a)pyrene, coumarin and biphenyl and Rahimtula et al (1974) have reported a unique spectral change when cumene hydroperoxide is added to rabbit liver microsomes. This spectrum slowly disappeared with time and was explained, along with a number of other observations, as being due to the formation of higher valence states of the haem iron (e.g. Fe\(^{3+}\text{O}_2\) from Figure 1.1 is equivalent to Fe\(^{5+}\text{O}_2\)).

Cumene hydroperoxide will also support the cytochrome P-450-linked fatty acid hydroxylation in liver microsomes (Ellin & Orrenius, 1975), in these experiments there appeared to be
two hydroxylase enzymes, an ω- and an (ω-1)-hydroxylase. Rahimtula & O'Brien (1977) demonstrated that cumene hydroperoxide would support the oxidation of ethanol to acetaldehyde in the presence of catalase, purified cytochrome P-450 or rat liver microsomes, other haemoproteins such as horseradish peroxidase, cytochrome c and haemoglobin were ineffective, in addition to ethanol higher alcohols were also oxidised.

In addition to hydroperoxides, other compounds which can replace NADPH in the mixed-function oxidase system have been found. Hrycay et al (1975) found that in the microsomal hydroxylation of a number of steroids, sodium periodate could replace NADPH with an increased reaction rate. Sodium chlorite could also replace NADPH but gave a decreased reaction rate. Lichtenberger et al (1976) found that in the presence of iodosobenzene, liver microsomes could catalyse the O-deethylation of 7-ethoxycoumarin and several iodosylbenzene derivatives have been shown to be oxygen donors in cytochrome P-450-catalysed steroid hydroxylations (Gustafsson et al, 1979). Iwasaki et al (1977) found that reduced methyl viologen could be used as the electron donor in a reconstituted system containing cytochrome P-450 and NADPH-cytochrome c reductase in the reduction of tiaramide N-oxide, the reduction proceeded at a much higher rate if NADPH-cytochrome c reductase was omitted from the system.
The cumene hydroperoxide-supported aromatic hydroxylation reaction in liver microsomes has been compared with the NADPH-dependent reaction (Rahimtula et al., 1978). It was found that these two reactions had comparable "NIH shifts" and therefore proceeded via a common intermediate, an arene oxide. The NIH shift is an important part of the mechanism of the hydroxylation reaction and was discovered when an assay for the enzyme phenylalanine hydroxylase, based on the release of tritium from \( \text{[4-}^3\text{H]} \) phenylalanine during conversion to tyrosine, showed that greater than 95% of the tritium migrated and was retained at the 3-position of the tyrosine, only 5% was lost to the medium (Figure 1.2) (Guroff et al., 1966). Such intramolecular migrations with concomitant retentions of aryl substituents during hydroxylation by monoxygenases were termed the NIH shift (Guroff et al., 1967), over a hundred examples of this reaction, catalysed by plant, animal and microbial enzymes, have been observed (Daly et al., 1972). As shown in Figure 1.3, keto tautomers of phenols are key intermediates in the mechanism of the NIH shift. The migration of the isotope occurs at step a and the ratio of tritiated to untritiated product is determined by the isotope effect in step b and the participation of a minor direct-loss pathway in step c.
Figure 1.2. The NIH Shift in Phenylalanine Hydroxylase.
Figure 1.3. The Key Intermediates in the Mechanism of the NIH Shift.
l.4. Microbial Cytochrome P-450

The microbial cytochrome P-450 which has been studied in most detail is the so-called cytochrome P-450Cam from the bacterium Pseudomonas putida grown in the presence of D-(+)-camphor. Its biological role is the selective hydroxylation of the 5-methylene carbon of D-(+)-camphor to form the exo-5-alcohol, the ferrous cytochrome P-450Cam forms a CO complex with a Soret band at 446nm (O'Keeffe et al., 1978). The various redox states of the bacterial cytochrome P-450Cam and of its associated electron transport system, putidaredoxin, has been investigated by systematic low-temperature studies in fluid solvents (Lange et al., 1977) and the oxy-ferrous complex has been stabilised and investigated in detail (Eisenstein et al., 1977). Single-electron transfers involved in the reaction mechanism have also been studied at low temperatures (Hoa et al., 1978) and spin transitions of the camphor-bound cytochrome P-450Cam have been investigated (Lange & Debey, 1979 and Lange et al., 1979).

In eukaryotic microorganisms, cytochrome P-450 has been found mostly in connection with specific metabolic capacity such as alkaloid biosynthesis or aryl hydrocarbon hydroxylation in fungi. In certain yeasts it is a component of an alkane-induced monoxygenase system catalysing the first step in alkane metabolism.
There are three cytochrome P-450-containing systems with different physiological functions in the fungi. Cytochrome P-450 has been shown to be involved in the production of alkaloids by a strain of *Claviceps purpurea*, both cytochrome P-450 and alkaloid synthesis could be induced by sodium phenobarbitone and 3-methylcholanthrene (Ambike et al, 1970 and Ambike & Baxter, 1970). A monoxygenase system in *Cunninghamella bainieri* has been shown by Ferris et al (1973 and 1976) to resemble the hepatic microsomal system in that it was able to catalyse the oxidative N-demethylation of aminophenazone, the O-demethylation of 4-nitroanisole and anisole, the reduction of nitro- and azo-groups as well as the hydroxylation of anisole, aniline and naphthalene. This enzyme was also inhibited by CO, SKF 525A and metyrapone, was NADPH- and oxygen-dependent and had a pH optimum of 7.8. NADPH-cytochrome c reductase was also shown to be an essential part of this system. The third fungal cytochrome P-450 was found in *Rhizopus nigricans* by Breškvar & Hudnik-Plevnik (1977) where it was shown to be involved in the 11α-hydroxylation of progesterone, the reaction was inhibited by CO, inhibition being reversed by irradiation with light at 450nm.

The terminal alkane degradation for many microorganisms and especially yeasts proceeds by the formation of the primary alcohol followed by further oxidation via the aldehyde to the fatty acid (Nyns et al, 1969, Klug et al, 1971, Liu et al
1971, Gallo et al 1976 and Hammer & Liemann, 1976). The involvement of cytochrome P-450 as the terminal oxidase of the alkane monooxygenase system has been demonstrated for bacteria (Cardini & Jurtschuk, 1968 and 1970) and was first reported for yeasts by Tulloch et al (1962) and later by Heinz et al (1970) where it was found that it catalysed the \( \omega \)-hydroxylation of fatty acids in Torulopsis sp. In Candida tropicalis grown on tetradecane, cytochrome P-450 has been found to take part in two different reactions, the conversion of \( n \)-alkanes to primary alcohols and the \( \omega \)-hydroxylation of fatty acids (Gallo et al, 1971 and Lebeault et al, 1971).

The complete Candida system consisted of cytochrome P-450, NADPH-cytochrome P-450 reductase and a heat-labile fraction (Duppel et al, 1973) as does the mammalian system (Lu & Coon, 1968). These latter components from the yeast system could be replaced by the corresponding fractions from rat liver in a reconstituted system (Ellin et al, 1973). Cytochrome P-450 has also been demonstrated in strains of Candida guilliermondii (Riege et al, 1977 and Tittelbach et al, 1976) and in Endomyces lipolytica (Delaissé & Nyns, 1974) grown on alkanes as the sole carbon source.

The presence of a cytochrome P-450-like CO-binding pigment in cells of Saccharomyces cerevisiae was first reported by Lindenmeyer & Smith (1964) and later by Ishidate et al (1969), it has also been found in Saccharomyces carlsbergensis (Cartledge et al, 1972) and in Schizosaccharomyces pombe.
(Poole et al., 1974). The production of cytochrome P-450 appears to be due to a particular physiological state which occurs when the cellular metabolism switches from respiration to fermentation. This state results either from a low supply of oxygen i.e. semi-anaerobic conditions or by aerobic fermentation conditions at high glucose concentrations which leads to repression of the respiratory chain. The influence of oxygen on cytochrome P-450 content was demonstrated by Ishidate et al. (1969) where the enzyme was not formed at low glucose concentrations under completely aerobic or completely anaerobic conditions. An inverse correlation between cytochrome P-450 and cytochrome oxidase was reported by Wiseman et al. (1975a) and is confirmed in this thesis under conditions of high glucose concentrations.

As to the function of cytochrome P-450 in yeast, it has been postulated that the enzyme may be involved in the demethylation of lanosterol (II) to zymosterol (III), as this conversion was inhibited by CO by 57\% in a preparation of disrupted yeast cells (Alexander et al., 1974).
The group of Yoshida et al have succeeded in isolating and purifying cytochrome P-450 (Yoshida et al, 1974a, Yoshida & Kumaoka, 1975 and Yoshida et al, 1977), cytochrome b5 (Yoshida et al, 1974b), NADH-cytochrome b5 reductase (Kubota et al, 1977a) and NADPH-cytochrome P-450 reductase (Kubota et al, 1977b and Aoyama et al, 1978) from the microsomal fraction of S. cerevisiae, demonstrating the presence of an electron-transport system analogous to that of liver microsomes. These workers also reported that this system was capable of the hydroxylation of aniline and the demethylation of aminopyrine. Subsequent to these investigations Aoyama & Yoshida (1978a) showed that a reconstituted cytochrome P-450 system was capable of the 14α-demethylation of lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) which is one of three demethylations necessary for conversion to zymosterol (5α-cholesta-8,24-dien-3β-ol). The other demethylations proceed via a cyanide-sensitive mixed function oxidase (Ohba et al, 1978) which involves three enzymic steps (Moore & Gaylor, 1968, Miller & Gaylor, 1970 and Miller et al, 1971). A lanosterol-induced type I spectral change has also been
described for the purified cytochrome P-450 (Aoyama & Yoshida, 1978b).

Cytochrome P-450 has also been investigated in S. cerevisiae by Callen & Philpot (1977) in the context of the activation of promutagens. This activation was detected by measuring the number of trp5 and ade2 convertants of the D4 strain and it was found that five promutagens, aflatoxin B, dimethyl-nitrosamine, ethyl carbamate, α-naphthylamine and cyclophosphamide, were metabolised to active mutagens. More recently, Simmon (1979) has suggested a similar system as an alternative to the Ames test for mutagenicity, the recombinogenic activity of chemical carcinogens was measured using S. cerevisiae D3, an activation system using Aroclor-pretreated rat liver homogenates was also used. In this system, 100% of the ultimate carcinogens tested gave positive results although only 38% of the procarcinogens used were positive.
1.5. **Benzo(a)pyrene Metabolism**

A number of studies have demonstrated the carcinogenic properties of polycyclic aromatic hydrocarbons in different species, including man, and in different tissues, including the lung and skin (Blacklock, 1957, Auerbach et al, 1970, Inui & Takayama, 1971, Feron, 1972, Hirao et al, 1972, Saffiotti et al, 1972, Shubik, 1972, Henry et al, 1973 and Little & O'Toole, 1974). Benzo(a)pyrene (IV) seems to be either the most active carcinogenic agent or at least the best indicator of the carcinogenicity of the complex residues resulting from the incomplete combustion of organic fuels (Sterling & Pollack, 1972)

![Diagram of Benzo(a)pyrene](IV)

The major pathways for the metabolism of benzo(a)pyrene and other polycyclic aromatic hydrocarbons in mammalian tissues involve the aryl hydrocarbon monoxygenase (hydroxylase) system (EC 1.14.14.2), epoxide hydrolase and glutathione S-epoxide transferase (Conney, 1967 and Oesch, 1973). There is a large body of evidence that aryl hydrocarbon hydroxylase, the first of these enzymes, introduces an oxygen atom into its substrates to produce epoxides (arene oxides) (Oesch
et al., 1972, Oesch, 1973, Heidelberger, 1973, Jerina & Daly, 1974, Sims & Grover, 1974 and Heidelberger, 1975). Jerina et al. (1970a) have shown that naphthalene oxide is an obligatory intermediate in the metabolism of naphthalene to naphthol, diol and the glutathione conjugate. Oxides of pyrene, benzo(a)pyrene and dibenz(a,h)anthracene have been detected (Selkirk et al., 1971 and Grover et al., 1972). Tissues in which the aryl hydrocarbon hydroxylase system has been demonstrated to metabolise polycyclic aromatic hydrocarbons to epoxides or dihydrodiols, which arise from the further metabolism of the epoxide, include rat liver, lung and skin, mouse skin, and human liver, lung, lymphocytes, monocytes, adrenal gland and placenta (Gelboin et al., 1975, Pelkonen, 1975 and Sims, 1975).

The arene oxides formed from polycyclic aromatic hydrocarbons can rearrange non-enzymically to form phenols (Swaisland et al., 1973) or they can be further metabolised by two main pathways (Mandel, 1971 and Oesch, 1973). Epoxide hydrase may add water to the arene oxides to yield dihydrodiols (Figure 1.4) (Pandov & Sims, 1970 and Oesch et al., 1971) or alternatively, the arene oxides may be conjugated by glutathione S-epoxide transferase (Boyland & Sims, 1965a and b, Boyland & Williams, 1965 and Keysell et al., 1975). This conjugation with glutathione may also occur non-enzymically, arene oxides may also react directly with proteins and nucleic acids. It has been found that arene oxides may
Figure 1.4. The Metabolism of Polycyclic Aromatic Hydrocarbons in Rat Liver. (R=remainder of ring system)
be reduced back to the parent hydrocarbon by a NADPH-dependent reductase which is inhibited by oxygen (Booth et al., 1975 and Kato et al., 1976a and b).

The metabolism of the dihydrodiols formed from polycyclic aromatic hydrocarbons has been extensively studied and it has been found that these can either be conjugated with glucuronic acid or dehydrogenated to catechols by a soluble dehydrogenase (Ayengar et al., 1959, Jarina et al., 1967 and 1970 and Nemoto & Gelboin, 1976). It has also been reported that phenols of benzo(a)pyrene can be conjugated with glucuronic acid (Nemoto & Gelboin, 1976) and sulphate (Cohen et al., 1976). In addition, the catechols may be meta- or para-O-methylated by a non-particulate (Creveling et al., 1970, Creveling et al., 1972 and Meyer & Scheline, 1972) or a microsomal (Inscoc et al., 1965 and McCormick et al., 1972) hepatic catechol O-methyltransferase. Glutathione conjugates are thought to be metabolised further leading to loss of the glutamate and glycine residues, followed by acetylation of the resulting free amino acid group of the cysteinylin residue to produce a mercapturic acid, which is then excreted (Boyland & Chasseaud, 1969).

It has been believed for some time that the aryl hydrocarbon hydroxylase system and the microsomal mixed-function oxidase system are the same (Conney et al., 1956 and Conney, 1967). Both these systems are localised in the
endoplasmic reticulum of rat liver and require NADPH and molecular oxygen. Carbon monoxide also inhibits both systems and the isolated components of the cytochrome P-450 system can be reconstituted to give aryl hydrocarbon hydroxylase activity. In addition, benzo(a)pyrene and other polycyclic aromatic hydrocarbons induce increased levels of both aryl hydrocarbon hydroxylase and cytochrome P-450 (P-448) in various tissues (Sladek & Mannering, 1966; Silverman & Talalay, 1967; Alvares et al., 1967 and Gram et al., 1967).

The isolation and characterisation of multiple forms of cytochrome P-450 from rabbit liver microsomes has been described (van der Hoeven & Coon, 1974, van der Hoeven et al., 1974, Haugen et al., 1975 and Haugen & Coon, 1976). The sodium phenobarbital-inducible form, cytochrome P-450IM2, and the 3-naphtho-flavone-inducible form, cytochrome P-450IM1, have been shown to be distinct proteins and additional forms of cytochrome P-450IM have been isolated in a partially purified state (Coon et al., 1977 and Dean & Coon, 1977). The overall hydroxylation of benzo(a)pyrene has been demonstrated in reconstituted enzyme systems containing purified cytochrome P-450IM from 3-methylcholanthrene-pretreated rats (Lu et al., 1972) and from 3-naphtho-flavone-pretreated mice of both inducible and non-inducible strains (Nebert et al., 1973b). Using a number of inbred strains of mice, Nebert et al. have demonstrated that induction of cytochrome P-448 by polycyclic aromatic hydrocarbons is genetically associated with the
induction of 7-ethoxycoumarin 0-deethylase activity, as well as a number of other microsomal monoxygenase activities, in addition to aryl hydrocarbon hydroxylase (Nebert et al., 1973b, Robinson & Nebert, 1974, Atlas et al., 1975 and Thorgierson et al., 1975).

The introduction of high-pressure liquid chromatography for the separation of benzo(a)pyrene metabolites (Selkirk et al., 1974a) significantly advanced the potential for metabolic studies of the polycyclic aromatic hydrocarbons, and it has since been used by many workers in this field (Freudenthal et al., 1975, Vadi et al., 1975, Huberman & Sachs, 1976 and Hundleby & Freudenthal, 1977). Those benzo(a)pyrene metabolites separated by this method include the 7,8-, 9,10- and 4,5-dihydrodiols, the 3-, 7- and 9-phenols and the 1,6-, 3,6- and 6,12-quinones (Selkirk et al., 1974a and b, Selkirk et al., 1975, Selkirk et al., 1976 and Yang et al., 1977) while under special conditions benzo(a)pyrene-4,5-oxide can also be isolated (Selkirk et al., 1975).

A variety of parameters have been calculated for polycyclic aromatic hydrocarbons by quantum mechanical methods in anticipation that one would correlate with the carcinogenicity in vivo or with products formed in vitro. Among these theories, the "K-region" theory of Pullman & Pullman (1955) has attracted much attention. This theory asserted that a hydrocarbon must possess a "K-region" with high electron
density in order to be carcinogenic, while a highly reactive "L-region" would mean decreased carcinogenic activity. The theory implies some form of metabolic activation at the "K-region" to form a reactive metabolite, such as an arene oxide, as opposed to some detoxication mechanism such as quinone formation at an "L-region". The work of Miller (1970) and Miller & Miller (1973) has brought wide acceptance to this concept that chemical carcinogens, which themselves are not chemically active, exert their carcinogenic effect through metabolites which are sufficiently active to modify cellular macromolecules such as DNA, RNA and protein.

Several approaches to the mechanism of action of benzo(a)pyrene, including covalent binding to nucleic acid, mutation of bacterial and mammalian cells, transformation of cells in culture and in vivo carcinogenicity studies, have all indicated that the benzo ring of benzo(a)pyrene is of key importance in the carcinogenicity of this compound. Borgen et al (1973) found that liver microsomal metabolism of benzo(a)pyrene-7,8-dihydrodiol resulted in more extensive binding to DNA than did metabolism of benzo(a)pyrene or several other benzo(a)pyrene metabolites. Sims et al (1974) provided evidence that a 7,8-diol-9,10-epoxide was the specific metabolite responsible for this binding to DNA and Levin et al (1978) have described the mutagenic and carcinogenic activity of 33 benzo(a)pyrene metabolites and derivatives and concluded that a 7,8-diol-9,10-epoxide was
indeed an "ultimate carcinogen". The formation of the 7,8-diol-9,10-epoxides is illustrated in Figure 1.5.

The theory of the carcinogenesis of polycyclic aromatic hydrocarbons used today is the "bay-region" theory of Jerina et al (Jerina et al, 1976, Jerina et al, 1977a, Jerina & Lehr, 1977 and Jerina et al, 1978) which postulates that in the case of benzo(a)pyrene the critical structural feature is an epoxide on a saturated benzo-ring which forms part of a "bay-region". The simplest example of a bay-region is the hindered region between the 4- and 5-positions of phenanthrene (V). Figure 1.6 also shows bay-regions for benzantracene (VI), chrysene (VII) and benzo(a)pyrene. Thus 1,2,3,4-tetrahydrophenanthrene-3,4-epoxide is much more reactive than the isomer 1,2,3,4-tetrahydrophenanthrene-1,2-epoxide. Similarly the 9,10-epoxide of 7,8,9,10-tetrahydrobenzo(a)pyrene is 10-fold more reactive than the 7,8-epoxide (Wood et al, 1976). Another example of the reactivity of the bay-region in 7,8,9,10-tetrahydrobenzo(a)pyrene is the highly selective attack of lead tetraacetate at the 10-position (Kon & Roe, 1945 and Yagi & Jerina, 1975). The bay-region theory appears to be consistent with existing carcinogenicity data on methyl- and fluorine-substituted hydrocarbons (Jerina & Daly, 1977 and Jerina et al, 1977b) while attempts to demonstrate that K-region arene oxides are proximate or ultimate carcinogenic metabolites have been unsuccessful. The K-region arene oxides of benzantracene,
Figure 1.5. The Metabolic Formation of the Diastereomeric Benzo(a)pyrene-7,8-diol-9,10-epoxides (from Jerina et al, 1978).
Figure 1.6. The Positions of the Bay-Regions of a Few Polycyclic Aromatic Hydrocarbons.
7-methylbenz(a)anthracene, 3-methylcholanthrene and benzo(a)pyrene have low carcinogenic activity when compared with the parent compound (Burki et al., 1974, Levin et al., 1976 and Slaga et al., 1976). Interestingly, polycyclic aromatic hydrocarbons with a K-region often possess a bay-region and this may help to explain the correlation noted by Pullman & Pullman (1955).
CHAPTER 2.

The Regulation of the Biosynthesis of Cytochrome P-450 in *Saccharomyces cerevisiae.*
2. The Regulation of the Biosynthesis of Cytochrome P-450 in *Saccharomyces cerevisiae*

2.1. Introduction

Good yields of cytochrome P-450 can be obtained by growing yeast under conditions of mitochondrial repression, for example in 20% glucose medium (Wiseman et al. 1975a). A rapid production of cytochrome P-450 can be brought about by transferring yeast which has previously been grown to the end of the exponential phase in growth medium containing as little as 0.5% glucose to a relatively small volume of 20% glucose medium. The production of this enzyme, over at least 8h, is accompanied by a failure to resynthesise cytochrome oxidase despite a 4-fold growth of the yeast (Wiseman & Woods, 1977).

Experiments using yeast protoplasts have shown that cyclic AMP has a negative effect on this biosynthesis of cytochrome P-450, likely to be at the level of transcription.

2.2. Materials and Methods

2.2.1. Growth of the Yeast

*Saccharomyces cerevisiae* (N.C.Y.C. No 240) was maintained on slopes of Sabouraud-Dextrose agar and transferred using a platinum wire to growth medium containing sodium chloride (5g/l), yeast extract (10g/l) and bacteriological peptone (20g/l) autoclaved at 15psi for 15min. In addition, the medium contained glucose at concentrations of 5 to 200g/l autoclaved separately to prevent the formation of interfering breakdown...
pigments. The yeast was grown in 100ml of medium contained in a 250ml conical flask shaken in a Mickle shaking water bath (Mickle Laboratory Engineering Co., Godshall, Surrey) at 30°C, shaking at 50rpm with a stroke of 5cm and was harvested by centrifugation in a M.S.E. bench centrifuge (Measuring and Scientific Equipment Ltd., London).

2.2.2. The Preparation of Protoplasts

Protoplasts were prepared by a modified method of Eddy & Williamson (1950). Yeast was grown in 0.5% glucose growth medium for 48h, harvested by centrifugation and washed once in 100mM-citrate/phosphate buffer, pH 5.8. The yeast was then resuspended to 100mg/ml in 100mM-citrate/phosphate buffer, pH 5.8 containing 0.14M-2-mercaptoethanol and 0.04M-EDTA and incubated at 30°C for 30min. After centrifugation in a Piccolo centrifuge (Hereaeus Christ, GmbH), the yeast was washed three times in buffer without additives and resuspended to 1g/ml.

Snail gut enzyme (0.4ml/g yeast) was then added and the mixture incubated at 30°C for 45min. The protoplasts were harvested by centrifugation and resuspended in 1ml of medium/50mg original yeast.

2.2.3. The Measurement of the Mitochondrial Cytochromes and Cytochrome P-450

The concentration of the mitochondrial cytochromes in whole yeast and in protoplasts was determined by a method modified from that of Williams (1964). Yeast suspension was placed (usually 0.1g wet weight/ml) in each of two
spectrophotometer cuvettes of 1cm light path and the sample cuvette reduced by the addition of a few grains of sodium dithionite, the reference cell being oxidised by addition of 50µl of 20 volume hydrogen peroxide. The difference spectrum between 500 and 640nm was then recorded by means of a Pye Unicam SP1800 spectrophotometer (Figure 2.1.) and the concentrations of the mitochondrial cytochromes (cytochrome oxidase, cytochrome b, cytochrome c₁ and cytochrome c) derived from the differences in absorbance at four wavelength pairs which were then used to solve four simultaneous equations in four unknowns, this process being speeded up considerably by the use of a computer.

The concentration of cytochrome P-450 was then determined using the same sample by a method modified from that of Omura et al. (1965). The reference cell, previously oxidised with H₂O₂ was reduced by the addition of excess sodium dithionite and a baseline between 410 and 490nm recorded. Carbon monoxide was then bubbled through the test cuvette for 30secs and the scan repeated. The peak height at 450nm above the baseline was then used to calculate the concentration of cytochrome P-450, assuming an extinction coefficient of 91cm⁻¹.mM⁻¹ (for spectrum see Figure 2.2.)

Interference by cytochrome oxidase with the cytochrome P-450 assay has been described (Pohl et al, 1976) but was ruled out by measuring the cytochrome P-450 content of a microsomal suspension in the presence and absence of a mitochondrial suspension, there was no difference in the size
Figure 2.1. The Oxidised-Reduced Difference Spectrum of Yeast Suspension.
Figure 2.2. The Carbon Monoxide-Difference Spectrum of Yeast Suspension.
of the peak at 450nm.

2.2.4. Materials

Saccharomyces cerevisiae (No 240) was obtained from the National Collection of Yeast Cultures, Nutfield, Surrey. Powdered yeast extract and Sabouraud-dextrose agar were obtained from Oxoid Co. Ltd., Basingstoke, Hants and bacteriological peptone from Difco. Adenosine 3':5'-phosphate, adenosine 5'-phosphate, adenosine 2'-(3')-phosphate (mixed isomers), actinomycin D and chloramphenicol were obtained from the Sigma Chemical Corp. (London) Ltd., 2-mercaptoethanol was obtained from Koch-Light, Colnbrook, Bucks. D-glucose and all other chemicals were supplied by BDH Chemicals Ltd, Poole, Dorset.

2.3. Results and Discussion

2.3.1. The Effect of Glucose on the Mitochondrial Cytochromes and Cytochrome P-450 in Yeast

The biosynthesis of cytochrome P-450 in yeast may be promoted by firstly growing in 0.5%-glucose medium and transferring to 20%-glucose medium. Yeast was grown as described above in 0.5%-glucose medium and harvested by centrifugation after 48h and washed in 100mM-citrate/phosphate buffer, pH 5.8. The yeast was divided into 500mg portions and each portion resuspended in 15ml of medium containing various concentrations of glucose in a 25ml conical flask and shaken at 30°C as before. At various times up to 24h the yeast suspension was centrifuged and the pressed (wet) weight determined. The yeast was then resuspended in 5ml of distilled water and half was put into
each of two 3ml spectrophotometer cuvettes. The amounts of the mitochondrial cytochromes and cytochrome P-450 were determined as described above.

Thus Figures 2.3 and 2.4 show that transfer of yeast to 1%-glucose medium resulted in a small increase in yeast weight and an induction of some cytochrome P-450, there was also a decrease in the amount of cytochrome oxidase. These effects were more marked when 5% and 20%-glucose media were used (Figures 2.5, 2.6, 2.7 and 2.8). In the case of 20%-glucose medium there was a short lag phase before cytochrome P-450 production started which was absent at lower glucose concentrations, but there was no lag phase in the growth of the yeast under any of these conditions. The decrease in cytochrome oxidase was maximal in 20%-glucose growth medium, probably due to dilution of the enzyme by growth of the yeast, since under conditions of high glucose concentrations it is not synthesised. There does, however, appear to be synthesis of the other mitochondrial cytochromes after a lag phase since their concentrations decreased and then increased again to a level somewhat higher than that at which they started.

Studies of the energy requirements of this process have shown that energy production by mitochondria is not required. Thus transfer of yeast to 20%-glucose medium containing 1mM-KCN (Figures 2.9 and 2.10) which inhibits cytochrome oxidase by 98% (Slater, 1950), had no effect on the growth of the yeast, production of cytochrome P-450 or the changes in cytochromes.
Figure 2.3. The Effect of Transfer of Yeast from 0.5% to 1%-glucose Medium on Cytochrome P-450 and Amount of Yeast.
Figure 2.4. The Effect of Transfer of Yeast from 0.5% to 1% glucose Medium on the Mitochondrial Cytochromes.
Figure 2.5. The Effect of Transfer of Yeast from 0.5% to 5% glucose Medium on Cytochrome P-450 and Amount of Yeast.
Figure 2.6. The Effect of Transfer of Yeast from 0.5%-

 to 5%-glucose Medium on the Mitochondrial Cytochromes.
Figure 2.7. The Effect of Transfer of Yeast from 0.5%-
to 20%-glucose Medium on Cytochrome P-450 and Amount of Yeast.
Figure 2.8. The Effect of Transfer of Yeast from 0.5\% to 20\%-glucose Medium of the Mitochondrial Cytochromes.
Figure 2.9. The Effect of Transfer of Yeast from 0.5% glucose to 20% glucose Medium Containing 1mM-KCN on Cytochrome P-450 and Amount of Yeast.
Figure 2.10. The Effect of Transfer of Yeast from 0.5%-glucose to 20%-glucose Medium Containing 1mM-KCN on the Mitochondrial Cytochromes.
already described. However, transfer to 20%-glucose medium containing 50mM-iodoacetate, which inhibits glycolysis at hexokinase (Jones et al., 1975), glyceraldehyde-3-phosphate dehydrogenase (Levitski, 1974) and alcohol dehydrogenase (Hasilik, 1973) completely stopped any growth of the yeast or production of cytochrome P-450 (Figures 2.11 and 2.12). The concentrations of the mitochondrial cytochromes remained constant for 6-8h but after 24h had decreased, especially cytochrome b and this was likely to be due to death of the yeast as a result of a lack of energy production. It would therefore appear that under conditions of high glucose concentrations energy production is solely by glycolysis, a not-unexpected result.

Yeast was also transferred to 20%-glucose non-growth medium i.e. 20%-glucose buffered to pH 4.5 without any yeast extract, bacteriological peptone or sodium chloride. Under these conditions there was very little growth of the yeast, that which did occur was probably due to intermediates present in the yeast when it was transferred, a small amount of cytochrome P-450 was also formed (Figures 2.13 and 2.14).

The transfer of yeast to growth medium containing 20%-galactose also resulted in little growth of the yeast and no cytochrome P-450 was produced. There was also little change in the concentrations of the mitochondrial cytochromes which would seem to indicate that this process is glucose dependent (Figures 2.15 and 2.16).
Figure 2.11. The Effect of Transfer of Yeast from 0.5\%-glucose to 20\%-glucose Medium Containing 50mM-iodoacetate on Cytochrome P-450 and Amount of Yeast.
Figure 2.12. The Effect of Transfer of Yeast from 0.5\%-glucose to 20\%-glucose Medium Containing 50mM-iodoacetate on the Mitochondrial Cytochromes.
Figure 2.13. The Effect of Transfer of Yeast from 0.5% glucose Growth Medium to 20% glucose Non-Growth Medium on Cytochrome P-450 and Amount of Yeast.
Figure 2.14. The Effect of Transfer of Yeast from 0.5\%-glucose Growth Medium to 20\%-glucose Non-Growth Medium on the Mitochondrial Cytochromes.
Figure 2.15. The Effect of Transfer of Yeast from 0.5%-glucose to 20%-galactose Growth Medium on Cytochrome P-450 and Amount of Yeast.
Figure 2.16. The Effect of Transfer of Yeast from 0.5%-glucose to 20%-galactose Growth Medium on the Mitochondrial Cytochromes.
2.3.2. The Effect of Glucose and Cyclic AMP on Cytochrome P-450 Production by Yeast Protoplasts

Several workers have shown that there is a reciprocal relationship between the intracellular concentration of cyclic AMP and the concentration of glucose in the growth medium of yeast. Also, addition of cyclic AMP is known to remove the catabolite repression of β-galactosidase biosynthesis by glucose in *Escherichia coli* (Perlman & Pastan, 1968b and Ullman & Monod, 1968) by its action at the promoter of the *lac* operon causing stimulation of transcription (De Crombugghe *et al.*, 1971). Pang & Butow (1970) have shown that reversal of mitochondrial repression can be produced by incubating yeast protoplasts in 10%-glucose medium containing cyclic AMP but not by 10%-glucose medium alone and it has been observed that cyclic AMP removes catabolite repression of α-glucosidase biosynthesis by glucose in yeast protoplasts (Wiseman & Lim, 1974).

If protoplasts made from yeast which had previously been grown in 0.5%-glucose medium for 48h were incubated in a medium containing a higher concentration of glucose (e.g. 5%) they produced cytochrome P-450 in a similar manner to whole yeast cells transferred to 20%-glucose medium as described above. This production of cytochrome P-450 was accompanied by growth of the protoplasts and a decrease in the concentration of cytochrome oxidase (Figures 2.17 and 2.18). Incubation of protoplasts in 5%-glucose medium containing 10mM-cyclic AMP resulted in a decrease in the amount of cytochrome P-450
Figure 2.17. The Effect of Incubation of Yeast Protoplasts in 5%-glucose Medium on Cytochrome P-450 and Amount of Protoplasts.
Figure 2.18. The Effect of Incubation of Yeast Protoplasts in 5%-glucose Medium on the Mitochondrial Cytochromes.
produced although growth of the protoplasts and the changes in the mitochondrial cytochromes were unaffected (Figures 2.19 and 2.20). This effect appears to be specific for cyclic AMP since incubation of protoplasts in 5%-glucose medium containing 10mM-adenosine 5'-phosphate (5'-AMP) (Figures 2.21 and 2.22) or 10mM-adenosine 2'-(3')-phosphate (mixed isomers) (2'-(3')-AMP) (Figures 2.23 and 2.24) did not produce any significant effect on cytochrome P-450 production.

Cycloheximide (10mg/l) or actinomycin D (80mg/l) added at zero time prevented the appearance of cytochrome P-450. The action of actinomycin D was presumably to prevent the transcription of the genes for cytochrome P-450 synthesis, although it cannot be ruled out that this may be an indirect effect through haem synthesis if that were to be limiting.

A comparison of the effects of cyclic AMP on cytochrome P-450 production with the control experiment is given in Figure 2.25, the difference between these results after 6h of incubation was significant according to the Student's t-test (P < 0.01).

If the concentration of glucose in the medium was increased to 20% then the amount of cytochrome P-450 produced was less than that produced in 5%-glucose medium (Figures 2.26 and 2.27). This was probably due to an osmotic effect of the high glucose concentration on the protoplasts.
Figure 2.19. The Effect of Incubation of Yeast Protoplasts in 5% glucose Medium Containing 10mM-cyclic AMP on Cytochrome P-450 and Amount of Protoplasts.
Figure 2.20. The Effect of Incubation of Yeast Protoplasts in 5% glucose Medium Containing 10mM-cyclic AMP on the Mitochondrial Cytochromes.
Figure 2.21. The Effect of Incubation of Yeast Protoplasts in 5%-glucose Medium Containing 10mM-5'-AMP on Cytochrome P-450 and Amount of Protoplasts.
Figure 2.22. The Effect of Incubation of Yeast Protoplasts in 5%-glucose Medium Containing 10 mM-5'-AMP on the Mitochondrial Cytochromes.
Figure 2.23. The Effect of Incubation of Yeast Protoplasts in 5% glucose Medium Containing 10mM-2′-(3′)-AMP on Cytochrome P-450 and Amount of Protoplasts.
Figure 2.24. The Effect of Incubation of Yeast Protoplasts in 5% glucose Medium Containing 10mM-2'-(3')-AMP on the Mitochondrial Cytochromes.
Figure 2.25. A Comparison of the Effect on Cytochrome P-450 of Incubation of Yeast Protoplasts in 5% glucose Medium in the Presence (○) and Absence (●) of 10mM-cyclic AMP. Bars indicate standard deviation.
Figure 2.26. The Effect of Incubation of Yeast Protoplasts in 20% glucose Medium on Cytochrome P-450 and Amount of Protoplasts.
Figure 2.27. The Effect of Incubation of Yeast Protoplasts in 20% glucose Medium on the Mitochondrial Cytochromes.
It would therefore appear that the production of cytochrome P-450 in yeast is controlled by the intracellular concentration of cyclic AMP and that this is in turn dependent on the glucose concentration outside the cell. It should be pointed out that this is a negative effect of cyclic AMP on the transcription of mRNA prior to the synthesis of protein, which is unique. But it should be noted, however, that there is the possibility that some promoters may have the reverse requirements for transcription start and stop operations, compared with the classical lac operon of E. coli. Other negative-type effects have been reported, for example, the production of sex pili is inhibited in E. coli K-12 carrying de-repressed sex factors (Harwood & Meynell, 1973). This need not be a direct effect on the transcription of the genes involved in pilus production however, but could be an indirect effect of cyclic AMP of the bacterial cell envelope. Cyclic AMP can also, under some conditions, suppress the induction of antibody synthesis in male spleen-cell cultures (Bosing-Schneider, 1975).

Cyclic GMP has been reported to inhibit the biosynthesis of β-galactosidase and tryptophanase in E. coli growing in minimal medium on glucose or glycerol via transcription by binding to the promoter of the cyclic AMP-binding protein, in competition with cyclicAMP (Artman & Werthemer, 1974) and is antagonistic with cyclic AMP in some mammalian systems. Experiments in the present study in which protoplasts were incubated in a medium containing 0.5%-glucose and 10mM-
cyclic GMP did not however consistently show an antagonistic relationship between cyclic AMP and cyclic GMP in this system since under these conditions production of cytochrome P-450 could not be reproducibly stimulated.
CHAPTER 3.

The Metabolism of Benzo(a)pyrene

by Saccharomyces cerevisiae.
3. The Metabolism of Benzo(a)pyrene by Saccharomyces cerevisiae

3.1. Introduction

The metabolism of benzo(a)pyrene by the aryl hydrocarbon hydroxylase from Saccharomyces cerevisiae was investigated using two different techniques. The fluorometric assay for the formation of the highly fluorescent metabolite, 3-hydroxybenzo(a)pyrene, was used to measure the Michaelis-Menten parameters of the enzyme after various pretreatments and also to measure other parameters, such as the pH optimum, thermal stability, time course and the effects of changing protein and cofactor concentrations. It was also found that NADPH could be replaced as cofactor by cumene hydroperoxide. A solubilised and immobilised enzyme preparation was able to metabolise benzo(a)pyrene with cumene hydroperoxide as the cofactor.

The second technique used to investigate the metabolism of benzo(a)pyrene was that of thin-layer and high-pressure liquid chromatography. These methods showed that besides 3-hydroxybenzo(a)pyrene other major metabolites produced were 9-hydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-dihydrodiol).
3.2. Materials and Methods

3.2.1. The Preparation of Yeast 5,000xg Supernatant and Microsomes

Yeast was grown in 20%-glucose medium containing sodium chloride (5g/l), yeast extract (10g/l) and bacteriological peptone (20g/l) as described above for 48h. At the end of this time the yeast was harvested by centrifugation for 10min at full speed in a MSE bench centrifuge and transferred to a water-cooled Vibromill disrupter (Edmund Bühler, Tübingen, Germany) where it was shaken with approximately 3 volumes of glass beads of diameter 1.00 to 1.05mm (B. Braun, Melsungen, Germany) for a total of 6min, the disrupter being stopped after 1min to top up with glass beads. At the end of this time the glass beads were washed with ice-cold 0.1M-Tris/HCl buffer, pH 7.0 and the washings centrifuged at 6,000rpm (5,000xg) for 10min in a MSE High Speed 18 centrifuge.

In a typical experiment 100ml of medium would yield 5g of yeast and the washings would have a volume of approx. 12ml, after centrifugation this would give 10ml of supernatant. If microsomes were required this 10ml of supernatant was centrifuged in a Beckman L5-65 Ultacentrifuge at 50,000rpm (160,900xg_{av}) for 40min, the microsomal pellet was then re-suspended in buffer by hand using a Potter-Elvehjem homogeniser.

The method of calcium precipitation of microsomes as described by Schenkman & Cinti (1972) was investigated as a possible rapid method for preparing yeast microsomes.
The 5,000xg supernatant was made 8mM with respect to calcium ions and spun at 15,000xg (11,000rpm) in a MSE High Speed 18 centrifuge for 10min. Only approx. 50% of the cytochrome P-450 present was found to be in the pellet, the rest remaining in the supernatant. Addition of more calcium followed by another centrifugation brought down more cytochrome P-450 into the pellet but some enzyme was still present in the supernatant. This method was therefore not employed as a means of making yeast microsomes.

Cytochrome P-450 was measured by the method of Omura et al (1965), the spectrum produced by yeast microsomes was different to that produced by whole yeast in that there was not a peak at 420nm (Figure 3.1). Protein was measured by the method of Lowry et al (1951).

3.2.2. The Measurement of Mitochondrial Cytochrome Oxidase

Mitochondrial cytochrome oxidase was measured by a method modified from that of Yonetani (1967). Cytochrome c (100mg) was first reduced by dissolving in 10ml of 0.01M-phosphate buffer, pH 7.0 containing 1mM-EDTA and adding a solution of ascorbic acid neutralised with KOH to give a pale pink colour. This solution was then dialysed in visking tubing against 100ml of buffer for 24h, the buffer changed and the solution dialysed for a second 24h period. The reduced cytochrome c solution was then saturated with nitrogen and stored in the cold. To measure cytochrome oxidase activity 100µl of the reduced cytochrome c solution was put into each
Figure 3.1. The Carbon Monoxide-Difference Spectrum of Yeast Microsomal Fraction.
of two spectrophotometer cuvettes containing 3ml of 0.01M-phosphate buffer, pH 6.0 containing 1mM-EDTA and placed in a Pye Unicam SP1800 spectrophotometer. The reaction was initiated by the addition of 50µl of the enzyme sample and the increase in absorbance at 550nm recorded. The measurement was carried out at 37°C and the differential extinction coefficient used between reduced and oxidised cytochrome c was 19.6mM⁻¹.cm⁻¹. At least six determinations were made for each sample.

3.2.3. The Measurement of Aryl Hydrocarbon Hydroxylase Activity

Aryl hydrocarbon hydroxylase activity was determined by a method modified from that of Dehnen et al. (1973). Each incubation contained 0.5ml of 5,000xg supernatant, 0.5ml of 0.1M-Tris/HCl buffer, pH 7.0 and an NADPH-regenerating system giving final concentrations of 4mM-NADP, 20mM-D-glucose-6-phosphate and 8 units D-glucose-6-phosphate dehydrogenase. When NADPH was replaced by cumene hydroperoxide the latter was used at a concentration of 2.4mM. The final concentration of cytochrome P-450 was usually approx. 0.2µM.

The reaction was started by the addition of benzo(a)pyrene from a stock solution of 2mg/ml in acetone to give a final concentration up to 316µM and incubated at 37°C for 1h. The reaction was stopped by adding 1ml of ice-cold acetone and the precipitated protein removed by centrifugation in a Piccolo centrifuge (Heraeus Christ, GmbH) at full speed for
5min. A 0.6ml sample of this 50%-acetone solution was then placed with 1.4ml of 10.7%-triethylamine (v/v) solution in a fluorimeter cuvette and scanned from 500 to 560nm emission (467nm excitation) in a Perkin Elmer MPF3 fluorescence spectrophotometer to find the peak height at 520nm. The fluorescence was calculated relative to 10μg quinine sulphate/ml 2M-sulphuric acid, which was in turn calibrated against a standard 3-hydroxybenzo(a)pyrene solution.

The direct fluorometric assay for benzo(a)pyrene hydroxylase reported by Yang & Kicha (1978) was attempted but this method was not successful in the yeast system, probably because it was not sufficiently sensitive.

3.2.4. The Solubilisation and Immobilisation of Yeast Cytochrome P-450

The method used to solubilise yeast cytochrome P-450 was modified from that of Yoshida et al (1977); sodium cholate, EDTA and dithiothreitol were dissolved in 5,000xg supernatant to give final concentrations of 1%, 0.05% and 0.03% (w/v) respectively. The solution was then mixed on a roller-mixer in a stoppered test-tube at 4°C for 1h and then spun at 160,900xg for 40min. In a typical experiment, approx. 50% of the original cytochrome P-450 was recovered, all of it being in the supernatant.

Various materials were tried as supports for immobilising cytochrome P-450 once it had been solubilised, those which
were found to be unsuitable were carboxymethyl- and DEAE-cellulose, DEAE-Sephadex and titanium oxide beads. Cytochrome P-450 could be successfully immobilised onto microcrystalline cellulose as has been shown previously (J. Gondal, unpublished work). A typical example of an immobilisation is as follows: 10ml of 5,000xg supernatant was solubilised as described above but not centrifuged, 1.5g of cellulose was added to the solution and then mixed for 1h on a roller-mixer. Cross-linking was accomplished by adding 300μl of 0.25M-glutaraldehyde solution (final concentration was 7.5mM) and mixing for a further 1h after which time the cellulose was separated by centrifuging in a MSE High Speed 18 centrifuge at 12,000xg (10,000rpm) for 10min. From 1.5g of dry cellulose approximately 6g of immobilised enzyme was produced, the concentration of cytochrome P-450 was 0.45nmole/g cellulose, which represented 74% of the original amount of the enzyme.

Entrapment in polyacrylamide gel was also attempted but this was unsuccessful, the method used to make the gel was that of Hicks & Updike (1966). Polymerisation was initiated by means of either potassium persulphate and riboflavin or potassium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED). Unfortunately no cytochrome P-450 could be detected in the gel.
3.2.5. The Separation and Identification of the Benzo(a)pyrene Metabolites Formed by Yeast Aryl Hydrocarbon Hydroxylase

Preliminary experiments were performed using thin-layer chromatography to separate the metabolites formed by the yeast enzyme. The incubation consisted of 0.5ml of 5,000xg supernatant (final cytochrome P-450 concentration was 0.3nmole/ml), 0.5ml of 0.1M-Tris/HCl buffer, pH 7.0, a NADPH-regenerating system comprising of 4mM-NADP, 21mM-D-glucose-6-phosphate and 8 units of D-glucose-6-phosphate dehydrogenase and 10μCi of [9-3H]-benzo(a)pyrene in 2μl of toluene. The specific activity of the benzo(a)pyrene was 24Ci/mmele and 5mCi/ml. The control consisted of the above except that boiled 5,000xg supernatant was used. The mixtures were incubated at 37°C for 1h and then extracted three times with 2ml of ice-cold ethyl acetate, this extract was evaporated to dryness in a rotary evaporator and the residue dissolved in 50μl of acetone. This solution was spotted onto a GL500 Silica Gel plate (Schleicher and Schüll, W. Germany) and run in benzene:ethanol (9:1). The radioactivity on the plate was detected by means of a Dunnschicht Scanner II LB 2723 (Berthold, Germany) scanning at 300mm/h on range lcps and time constant 10sec (Figure 3.14).

After these initial experiments using thin-layer chromatography the metabolites were identified more reliably using high-pressure liquid chromatography. Each incubation consisted of 2ml of 5,000xg supernatant, a NADPH-regenerating system comprising of 2mM-NADP, 10mM-D-glucose-6-phosphate
and 8 units of D-glucose-6-phosphate dehydrogenase and 10μCi of \([3^{-3}H]\)-benzo(a)pyrene. Incubation was for 2.5h at 37°C after which time the metabolites were extracted twice with 5ml of ice-cold ethyl acetate which was then filtered through a 5μm Millipore filter and rotary evaporated to dryness, the residue being redissolved in 50μl of acetone. This extract was then applied to a Phase Separations ODS column in a Laboratory Data Corporation (Riviera Beach, Florida, USA) high-pressure liquid chromatograph. The metabolites were eluted with a methanol:water gradient linear from 50% to 80% in 50min, the flow rate was 1ml/min. Samples were collected every half-minute in a LKB 7000 fraction collector and counted in a Packard Tri-Carb Scintillation Spectrometer using toluene: metabol (2:1) scintillant containing 0.5% (w/v) 2,5-diphenyloxazole (PPO) and 0.02% (w/v) 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP). The column was simultaneously calibrated with unlabelled standards which were detected by their absorbance at 254nm using a UV detector (Figure 3.15).

3.2.6. Materials

Bacteriological peptone was obtained from Difco Labs, Detroit, Michigan, USA and cytochrome c was supplied by The Boehringer Corporation (London) Ltd., Lewes, E. Sussex. Nicotinamide adenine dinucleotide phosphate (NADP), D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase, microcrystalline cellulose, TEMED, potassium persulphate, riboflavin and unlabelled benzo(a)pyrene were obtained from The Sigma
Chemical Company (London) Ltd., Poole, Dorset. Cumene hydroperoxide (80% in cumene) was supplied by Merck, Munich. Carboxymethyl- and DEAE-cellulose were supplied by Whatman Lab Sales Ltd., Maidstone, Kent, DEAE-Sephadex by Pharmacia (Great Britain) Ltd., London and titanium oxide beads by BTP Tioxide Ltd., Billingham, Cleveland. Glutaraldehyde (25% for electron microscopy) was supplied by BDH Chemicals Ltd., Poole, Dorset. \([3^3\text{H}]\)-benzo(a)pyrene was obtained from The Radiochemical Centre, Amersham, Bucks. PPO and dimethyl POPOP were supplied by The Packard Instrument Co. Inc., Illinois, USA and metapolo was obtained from Durham Chemicals Distributors Ltd., Birtley, Tyne & Wear.

3.3. Results and Discussion

3.3.1. The Fate of Cytochrome P-450 During Yeast Disruption and Subcellular Fractionation

Figure 3.2 shows the procedure for a typical disruption of the yeast from 100ml of 20%-glucose growth medium after growing for 48h. The yields of cytochrome P-450 and protein from such a procedure are given in Table 3.1, the overall recovery of cytochrome P-450 from that in the whole yeast was 24% with the greatest loss being at the disruption stage, presumably as a result of localised heating during this process. The recovery during the centrifugation procedures was 41%. The table also gives values for the specific activity of cytochrome P-450 in the various fractions and it can be seen that this increases by approximately three times as a result of making the microsomal pellet.
Figure 3.2. A Typical Procedure For The Disruption and Subcellular Fractionation of Yeast
<table>
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<th>Fraction</th>
<th>Total P-450 (nmole)</th>
<th>% Original P-450</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmole/mg)</th>
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<td>-</td>
<td>-</td>
</tr>
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<td>250</td>
<td>0.048</td>
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<td>0.033</td>
</tr>
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<td>31</td>
<td>147</td>
<td>0.045</td>
</tr>
<tr>
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<td>0</td>
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<td>-</td>
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</table>

Table 3.1. The Fate of Cytochrome P-450 During Yeast Disruption and Subcellular Fractionation
3.3.2. The Fate of Cytochrome Oxidase During Yeast Disruption and Subcellular Fractionation

Cytochrome oxidase, a mitochondrial enzyme marker, was measured during the same disruption procedure as above in order to show whether mitochondria were brought down during the 5,000xg centrifugation step, thus ruling out the possibility that the cytochrome P-450 was mitochondrial. Table 3.2 shows the results from such an experiment and it can be seen that a large amount of the cytochrome oxidase was present in the 160,900xg supernatant which implies that this enzyme, which is normally bound to the mitochondrial membrane, has in some way been released from it. The most likely explanation for this is that the disruption procedure used, which is necessarily very vigorous to break the yeast cell walls, has also disrupted a large proportion of the mitochondria resulting in the cytochrome oxidase being present in the soluble fraction of the cell.

3.3.3. Yeast Aryl Hydrocarbon Hydroxylase

(a) NADPH-Supported Aryl Hydrocarbon Hydroxylase

NADPH-supported aryl hydrocarbon hydroxylase was characterised as indicated in the accompanying diagrams using a benzo(a)pyrene concentration of 316μM. The time course (Figure 3.3) shows that the production of fluorescent metabolites occurs linearly over a period of approximately 30 min and then the reaction stops. In the case of cumene hydroperoxide-supported aryl hydrocarbon hydroxylase (Figure 3.9) the reaction was linear for 45 min and since there was little decrease in the amount
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cytochrome Oxidase (nmole/min/mg)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (nmole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Disruptate</td>
<td>2.6 (0.4)</td>
<td>250</td>
<td>650</td>
</tr>
<tr>
<td>5,000xg Pellet</td>
<td>1.4 (0.2)</td>
<td>48</td>
<td>67</td>
</tr>
<tr>
<td>5,000xg Sup.</td>
<td>3.5 (0.4)</td>
<td>147</td>
<td>515</td>
</tr>
<tr>
<td>160,900xg Pellet</td>
<td>2.0 (0.2)</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>160,900xg Sup.</td>
<td>5.3 (0.4)</td>
<td>70</td>
<td>371</td>
</tr>
</tbody>
</table>

Table 3.2. The Fate of Cytochrome Oxidase During Yeast Disruption and Subcellular Fractionation. The figures in parentheses are standard deviations (n = 6)
pmole 3-hydroxybenzo(a)pyrene/
nmole cytochrome P-450

Figure 3.3. The Time Course of the NADPH-Supported Yeast Aryl Hydrocarbon Hydroxylase.
of metabolites formed after 1h, incubation time was usually for this period. These results compare favourably with the
time course published by Nebert & Gelboin (1968) in which
metabolite production tailed off after 45min.

The pH optimum was also determined (Figure 3.4) and yeast
aryl hydrocarbon hydroxylase was found to have a broad pH
optimum in the region from 6.5 to 7.0 which is lower than
that found in mammalian systems. For example, Nebert & Gelboin
(1968) found the pH optimum in mammalian cell culture to
be pH 7.5 and this pH was also used by Robie et al (1976)
in experiments using rat liver microsomes. Alvares et al
(1967) and Gurtoo et al (1968) used pH 7.4 for their
experiments with rat liver microsomes. Cumps et al (1977)
showed that the pH optimum for rat liver microsomes was 7.8
and the pH-activity curve had a similar shape to Figure 3.4.

Changing the protein (cytochrome P-450) concentration
in the incubation mixture resulted in the graph shown in
Figure 3.5. The degree of metabolism of benzo(a)pyrene was
linear for cytochrome P-450 concentrations up to 0.2μM after
which point the degree of metabolism remained constant,
presumably as a result of some other factor, such as cofactor
concentration, becoming limiting.

Figure 3.6 shows a similar result when the concentration
of the cofactors was changed. The optimum concentrations
were 4mM-NADP, 21mM-D-glucose-6-phosphate and 8 units of
D-glucose-6-phosphate dehydrogenase and these were routinely
Figure 3.4. The Effect of pH on the NADPH-Supported Yeast Aryl Hydrocarbon Hydroxylase
Figure 3.5. The Effect of Cytochrome P-450 Concentration on the NADPH-Supported Yeast Aryl Hydrocarbon Hydroxylase
Figure 3.6. The Effect of Cofactor Concentration on the NADPH-Supported Yeast Aryl Hydrocarbon Hydroxylase
used. Any increase above these values resulted in no further increase in the extent of benzo(a)pyrene metabolism, presumably since the enzyme concentration was limiting.

The thermal stability of the aryl hydrocarbon hydroxylase was compared with that of cytochrome P-450 as measured spectrophotometrically by the carbon monoxide-binding spectrum. Figure 3.7 shows that plotting log10 of the activities of aryl hydrocarbon hydroxylase and cytochrome P-450 against time at 50°C gave two straight lines, in fact, within the limits of experimental error, these points can be combined to give the dotted line. This means that aryl hydrocarbon hydroxylase and cytochrome P-450 have the same thermal stability and therefore may be the same enzyme. The thermal stability of the yeast aryl hydrocarbon hydroxylase was very similar to that of the aryl hydrocarbon hydroxylase measured in mammalian cell culture by Nebert & Gelboin (1968). In their experiments approximately 10% of the enzyme activity remained after 20 min at 45°C and from Figure 3.7 it can be seen that at 50°C there was approximately 10% of yeast aryl hydrocarbon hydroxylase activity remaining after 20 min.

The kinetics of the yeast aryl hydrocarbon hydroxylase were investigated by means of double-reciprocal Lineweaver-Burk plots of rate of metabolite formation against benzo(a)pyrene concentration as shown in Figure 3.8. Experiments were performed using the cytochrome P-450 from yeast grown in normal 20%-glucose growth medium (control) and also from
Activity of Aryl Hydrocarbon Hydroxylase (○) and Cytochrome P-450 (●) 
($\log_{10}$ % zero time activity)

Figure 3.7. The Thermal Stability of Yeast Aryl Hydrocarbon Hydroxylase and Cytochrome P-450 at 50°C
Figure 3.8. Lineweaver-Burk Plots of NADPH-Supported Yeast Aryl Hydrocarbon Hydroxylase After Pretreatment with Sodium Phenobarbitone (O), Benzo(a)pyrene (●) and Control (■)
that grown in medium containing, in addition, either 0.15%-sodium phenobarbitone (6mM) or 5µg/ml-benzo(a)pyrene (20µM). Table 3.3 gives the values for the Michaelis constant (Km) and maximum rate (Vmax) obtained from these graphs by means of least squares regression analysis. The correlation coefficient which is a measure of how good the points fit the regression line is also given, the best fit possible would have a coefficient of 1.000.

It can be seen that pretreatment of the yeast with benzo(a)pyrene caused a decrease in the Km from 0.45 to 0.17mM (a reduction of approx. three times) whereas sodium phenobarbitone-pretreatment did not have any significant effect on the Km. In an experiment in which yeast was pretreated with 1µg/ml-benzo(a)pyrene (4µM) no difference in the Km was produced compared with the control. A decrease in the Km after pretreatment with a polycyclic aromatic hydrocarbon has been described by Alvares et al (1968) and by Kuntzman et al (1969) in experiments in which the aryl hydrocarbon hydroxylase activity in control rat liver microsomes gave a value for the Km some seven times greater than that for 3-methylcholanthrene-pretreated rats, sodium phenobarbitone had no effect on the Km (Table 3.4). Rickert & Fouts (1970) showed that pretreatment of rats with benzo(a)pyrene resulted in an increase in the Km of the aryl hydrocarbon hydroxylase but this was subsequently explained as being due to problems associated with the protein concentration used during the measurement of the enzyme activity and Hansen & Fouts (1972)
<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Km (mM)</th>
<th>Vmax *</th>
<th>Vmax **</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.45</td>
<td>15.1</td>
<td>0.68</td>
<td>0.988</td>
</tr>
<tr>
<td>Sodium Phenobarbitone</td>
<td>0.53</td>
<td>19.7</td>
<td>0.89</td>
<td>0.999</td>
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<tr>
<td>Benzo(a)pyrene</td>
<td>0.17</td>
<td>26.1</td>
<td>1.17</td>
<td>0.989</td>
</tr>
</tbody>
</table>

* - pmoles 3-HO-benzo(a)pyrene/nmole cytochrome P-450/h  
** - pmoles 3-HO-benzo(a)pyrene/mg protein/h

Table 3.3. The Values of the Michaelis-Menten Parameters for NADPH-Supported Aryl Hydrocarbon Hydroxylase
<table>
<thead>
<tr>
<th>Reference</th>
<th>Pretreatment</th>
<th>Km (uM)</th>
<th>Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebert &amp; Gelboin (1968)</td>
<td>Control</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>Alvares et al (1968)</td>
<td>Control</td>
<td>14.0</td>
<td>3.00*</td>
</tr>
<tr>
<td>Kuntzman et al (1969)</td>
<td>3-MC</td>
<td>2.0</td>
<td>10.80</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>14.0</td>
<td>7.30</td>
</tr>
<tr>
<td>Rickert &amp; Fouts (1970)</td>
<td>Control</td>
<td>23.0</td>
<td>8.40**</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>52.0</td>
<td>27.60</td>
</tr>
<tr>
<td>Hansen &amp; Fouts (1972)</td>
<td>Control</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BP</td>
<td>20.0</td>
<td>-</td>
</tr>
<tr>
<td>Robie et al (1976)</td>
<td>Control</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3-MC</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>Cumps et al (1977)</td>
<td>Control</td>
<td>2.5</td>
<td>0.63***</td>
</tr>
<tr>
<td></td>
<td>3-MC</td>
<td>0.2</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>3.5</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* - μmoles hydroxybenzo(a)pyrene formed/g liver/h  
** - μmoles 8-HO-benzo(a)pyrene formed/mg protein/h  
*** - μmoles 3-HO-benzo(a)pyrene formed/mg protein/h

Table 3.4. A Comparison of the Literature Values of the Michaelis-Menten Parameters of Mammalian Aryl Hydrocarbon Hydroxylase
and Robie et al (1976) were able to show that 3-methylcholanthrene- and benzo(a)pyrene-pretreatment resulted in a decrease in the Km when aryl hydrocarbon hydroxylase activity was measured under conditions of lower protein concentration.

From Table 3.4 it can be seen that while it is generally agreed that pretreatment with a polycyclic aromatic hydrocarbon will decrease the Km of aryl hydrocarbon hydroxylase, the actual numerical value of the Km seems to vary according to which group of workers measure it. The most likely explanation for this is that it is due to strain differences in the rats used or possibly variations in diet.

Table 3.4 also gives various literature values for the Vmax of the mammalian aryl hydrocarbon hydroxylase. It can be seen that these are in the region of $10^{-6}$ to $10^{-9}$ moles of benzo(a)pyrene metabolite formed per hour per mg protein (or g of liver) and these values are much higher than for the yeast aryl hydrocarbon hydroxylase given in Table 3.3. Table 3.1 gives a typical value of the specific activity for cytochrome P-450 in the 5,000xg supernatant as 0.045nmoles cytochrome P-450/mg protein and this was used to calculate the Vmax values derived from Figure 3.7 in units of pmoles 3-hydroxybenzo(a)pyrene/mg protein/h (Table 3.3). Thus it can be seen that the Vmax of the mammalian enzyme is in the region of $10^4$ to $10^6$ times greater than that of the yeast enzyme.
Table 3.3 also shows that pretreatment of the yeast with benzo(a)pyrene caused an increase in the Vmax, an effect which is in agreement with the observations of many workers, since pretreatment of rats with benzo(a)pyrene or 3-methylcholanthrene usually stimulates aryl hydrocarbon hydroxylase activity (Wattenberg et al, 1968, Gnosspeilus et al, 1969, Gurtoo et al, 1970 and Schlede et al, 1970), the exception being Cumps et al (1977) (see Table 3.4). This overall stimulation of aryl hydrocarbon hydroxylase activity after pretreatment of animals with polycyclic aromatic hydrocarbons is related to the appearance of cytochrome P-450.

An experiment in which yeast aryl hydrocarbon hydroxylase was inhibited by 43% by the presence of carbon monoxide demonstrated the involvement of cytochrome P-450 in this reaction. This observation supports the thermal stability data presented above.

(b) Cumene Hydroperoxide-Supported Aryl Hydrocarbon Hydroxylase

It was found that cumene hydroperoxide, an oxygen and electron donor, which has been previously used as a cofactor in steroid hydroxylations (Hrycay et al, 1975), could replace the NADPH-regenerating system used above. A number of experiments were performed in order to compare the cumene hydroperoxide-supported aryl hydrocarbon hydroxylase with the NADPH-supported reaction.
Figure 3.9 shows that the time course was similar to that of the NADPH-supported reaction as previously stated. Figure 3.10 shows a Lineweaver-Burk plot obtained by varying the concentration of cumene hydroperoxide, the Km for the cofactor was 7.1mM (correlation coefficient was 0.998). The concentration of cumene hydroperoxide routinely used to measure aryl hydrocarbon hydroxylase activity was 2.4mM since it was found that any increase in cumene hydroperoxide concentration above 5mM resulted in a decrease in enzyme activity due to destruction of the enzyme by cumene hydroperoxide (Figure 3.11).

Figure 3.12 shows the Lineweaver-Burk plots obtained by varying the concentration of benzo(a)pyrene in the incubation mixture, the values of the Km and Vmax derived from these plots by least squares linear regression analysis are given in Table 3.5. There was an overall increase in the value of the Km compared with the NADPH-supported reaction (Table 3.3) which may be due to the effect of cumene hydroperoxide on the enzyme. As described above, high concentrations of cumene hydroperoxide caused destruction of enzyme activity and it may be that at low concentrations it reacted with the enzyme to chemically modify the active site or to produce conformational changes in the protein thus changing the Km.

It is of interest to note that the values for the Vmax were greater than those for NADPH-supported aryl hydrocarbon hydroxylase by a factor of up to seventeen times and this
Figure 3.9. The Time Course of the Cumene Hydroperoxide-Supported Yeast Aryl Hydrocarbon Hydroxylase
Figure 3.10. Lineweaver-Burk Plot of Cumene Hydroperoxide-Supported Yeast Aryl Hydrocarbon Hydroxylase

1/Aryl Hydrocarbon Hydroxylase

\[
\frac{1}{\text{pmole 3-hydroxybenzo(a)pyrene/}}
\frac{1}{\text{pmole cytochrome P-450/h}}
\]

1/Cumene Hydroperoxide (mM\(^{-1}\))
Figure 3.11. The Destruction of Yeast Aryl Hydrocarbon Hydroxylase by Cumene Hydroperoxide
Figure 3.12. Lineweaver-Burk Plots of Cumene Hydroperoxide-Supported Aryl Hydrocarbon Hydroxylase for Control (■), Sodium Phenobarbitone (○) and Benzo(a)pyrene (●) Pretreated Yeast
<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Km (mM)</th>
<th>Vmax *</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.00</td>
<td>286</td>
<td>0.974</td>
</tr>
<tr>
<td>Sodium Phenobarbitone</td>
<td>3.00</td>
<td>357</td>
<td>0.993</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>0.36</td>
<td>63</td>
<td>0.999</td>
</tr>
</tbody>
</table>

* - pmoles 3-HO-benzo(a)pyrene/nmole cytochrome P-450/h

Table 3.5. The Values of the Michaelis-Menten Parameters for Cumene Hydroperoxide-Supported Aryl Hydrocarbon Hydroxylase
may be due to the fact that cumene hydroperoxide by-passes the electron transport chain which must be used when NADPH is the cofactor thus increasing the efficiency of the overall reaction. Whereas pretreatment of the yeast with benzo(a)pyrene caused a slight increase in the Vmax for the NADPH-supported reaction, in the case of the cumene hydroperoxide-supported reaction this pretreatment resulted in a decrease in the Vmax. It would therefore seem that the form of cytochrome P-450 produced after benzo(a)pyrene-pretreatment cannot use cumene hydroperoxide as efficiently as the other forms.

In other words, the ability of the enzyme to use NADPH is determined by the electron transport system (cytochrome P-450 reductase) and since this is unchanged by pretreatment the Vmax observed for the NADPH-supported reaction is only slightly altered. Cumene hydroperoxide, however, interacts directly with the cytochrome P-450 molecule and therefore changes in it caused by pretreatment have a much greater effect on the ability of the enzyme to use cumene hydroperoxide and hence the Vmax.

Other cofactors which were tried unsuccessfully as replacements of NADPH were sodium periodate, methyl viologen and hydrogen peroxide.
3.3.4. Solubilised and Immobilised Yeast Aryl Hydrocarbon Hydroxylase

The yeast from 100ml of 20%-glucose growth medium (approximately 5g) was disrupted to give 10ml of 5,000xg supernatant and solubilised as described above. This solubilised enzyme was then used in experiments to measure the Km of the aryl hydrocarbon hydroxylase for benzo(a)pyrene by incubating 0.5ml of the enzyme (final concentration was 0.08μM cytochrome P-450) with 0.5ml of Tris/HCl buffer, pH 7.0, cumene hydroperoxide (final concentration was 2.4mM) and benzo(a)pyrene in the range 0 to 0.20mM at 37°C for 1h. Figure 3.13 shows the Lineweaver-Burk plot resulting from this experiment and Table 3.6 gives the values of the Michaelis-Menten parameters of this reaction.

The solubilised enzyme was immobilised onto cellulose as described above and the cellulose resuspended in 10ml of 0.1M-Tris/HCl buffer, pH 7.0 containing 20%-glycerol. Benzo(a)pyrene, to give a final concentration in the range 0 to 0.32mM and cumene hydroperoxide (final concentration was 4.8mM) were added to 1ml of this suspension in order to obtain the Lineweaver-Burk plot shown in Figure 3.13. The values of the Michaelis-Menten parameters obtained from this graph are given in Table 3.6.

It should be pointed out that benzo(a)pyrene hydroxylation could not be achieved with the solubilised enzyme using NADPH as the cofactor, but only with cumene hydroperoxide.
Figure 3.13. Lineweaver-Burk Plots of Cumene Hydroperoxide-Supported Aryl Hydrocarbon Hydroxylase for Solubilised (○) and Immobilised (⊗) Enzyme
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Km (mM)</th>
<th>Vmax *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilised</td>
<td>0.11</td>
<td>56.2</td>
</tr>
<tr>
<td>Immobilised</td>
<td>0.20</td>
<td>39.4</td>
</tr>
</tbody>
</table>

* = pmoles 3-HO-benza(a)pyrene formed/nmole Cytochrome P-450/h

Table 3.6. The Values of the Michaelis-Menten Parameters for Cumene Hydroperoxide-Supported Solubilised and Immobilised Aryl Hydrocarbon Hydroxylase
This would lead to the conclusion that the electron transport system necessary for NADPH utilisation was disrupted during the solubilisation procedure and that cumene hydroperoxide worked as a cofactor because it by-passed this system.

The value of the Km of the solubilised aryl hydrocarbon hydroxylase was much less (approximately thirty times) than that of the same enzyme when bound to the microsomal membrane (Table 3.5) and this was probably due to steric hindrance of the enzyme by the membrane in which it was embedded. Immobilisation of the enzyme resulted in an increase in the Km but not to the value of the membrane-bound enzyme. This probably reflects the relative degree of hindrance between the membrane-bound and immobilised aryl hydrocarbon hydroxylases.

The value of the Vmax of the solubilised aryl hydrocarbon hydroxylase was less than that of the membrane-bound enzyme and this was in agreement with the previous argument that it was changes in the enzyme molecule which effected the Vmax for the cumene hydroperoxide-supported reaction. It would seem likely that the solubilisation process, by removing the enzyme from the membrane, resulted in conformational changes in the enzyme. Subsequent immobilisation increased the amount of conformational change and thus brought about a further decrease in Vmax. The use of glutaraldehyde, for example, may have not only produced cross-links between the enzyme and its support but also within the enzyme itself thus resulting in the changes in the Michaelis-Menten parameters.
3.3.5. The Benzo(a)pyrene Metabolites Formed by Yeast

The results of a preliminary experiment in which the benzo(a)pyrene metabolites formed by yeast 5,000xg supernatant were separated by means of thin-layer chromatography are given in Figure 3.14. The figure is the result of scanning the TLC plate for radioactivity as described in Section 3.2.5 and it can be seen that the result is difficult to interpret since the size of the peaks is small. The main conclusion which can be drawn from this experiment is that the metabolites produced were 3-hydroxybenzo(a)pyrene, a dihydrodiol and a quinone.

High-pressure liquid chromatography of the benzo(a)pyrene metabolites was next used to identify them more reliably and the result of such an experiment is given in Figure 3.15. It can be seen that the metabolites formed were the 7,8-dihydrodiol, 9-hydroxybenzo(a)pyrene, 3-hydroxybenzo(a)pyrene, a quinone and an unidentifiable metabolite. These results are similar to those from mammalian systems, for example, a purified rabbit liver cytochrome P-450 (fraction IM2), separated from solubilised extracts using polyacrylamide gel electrophoresis showed the same range of products (Deutsch et al, 1978). However this preparation did not contain epoxide hydrolase which is presumably present in the yeast preparation and this may be the reason why it had this metabolite profile.
Figure 3.14. A Scan of the Radioactivity of a Thin-Layer Chromatogram of the Benzo(a)pyrene Metabolites Produced by the NADPH-Supported Yeast Aryl Hydrocarbon Hydroxylase.
Figure 3.15. The Benzo(a)pyrene Metabolites Formed by Yeast 5,000xg Supernatant
(a) 9,10-dihydriodiol, (b) 4,5-dihydriodiol, (c) 7,8-dihydriodiol,
(d) 9-hydroxybenzo(a)pyrene, (e) 3-hydroxybenzo(a)pyrene,
(f) benzo(a)pyrene, (g) quinone and (h) unknown.
Experiments in other mammalian systems have shown that in the rat liver the 4,5-, 7,8- and 9,10-dihydrodiols were produced by homogenates or microsomes from normal animals and the 7,8- and 9,10-dihydrodiols were formed by animals pretreated with 3-methylcholanthrene (Sims & Grover, 1974). In short-term organ cultures of hamster lung, benzo(a)pyrene was metabolised to the 9,10- and 7,8-dihydrodiols and benzo(a)pyren-3-yl hydrogen sulphate, with little or no 3-hydroxybenzo(a)pyrene or 4,5-dihydrodiol being formed (Cohen & Moore, 1977). Schmaltz et al (1978) have investigated the metabolite profiles of benzo(a)pyrene in primary cell cultures of rat liver and found that the metabolites formed were qualitatively similar to those formed by rat liver microsomal fraction.

It would seem, however, that it is the formation of the 7,8-dihydrodiol which is significant in mammalian systems since this is further metabolised in cells in culture and in model systems in vitro to the 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene-9,10-oxide and this diol-epoxide is thought to be the ultimate carcinogen, reacting directly with DNA (Sims et al, 1974).

Experiments in which 14C-benzo(a)pyrene was incubated with suspensions of plant cells from Chenopodium rubrum showed that plant cells produce more polar metabolites than hepatic microsomes, the metabolites which were identified were benzo(a)pyrene-3,6-quinone, benzo(a)pyrene-1,6-quinone
and the 9,10-dihydrodiol, no 7,8-dihydrodiol was formed (Harms et al, 1977). Other non-mammalian systems which have been studied include Beijerinckia strain B-836 which was found to metabolise benzo(a)pyrene to cis-9,10-dihydro-9,10-dihydroxybenzo(a)pyrene (Gibson et al, 1975). Cell suspensions of parsley and soybean have also been incubated with benzo(a)pyrene (van der Trenck & Sandermann, 1978), although the metabolites were not identified it was found that total conversion was up to 2.2% in the case of parsley cells and 28% in the case of soybean cells.

Figure 3.16 shows that pretreatment of yeast with sodium phenobarbitone or benzo(a)pyrene did not have any effect on the metabolite profile produced by yeast. A comparison of this result with mammalian systems is complicated by the fact that in mice, for example, there appear to be some strains in which 3-methylcholangrene-pretreatment causes changes in the metabolism of benzo(a)pyrene (responsive) and some strains in which this pretreatment has no effect (non-responsive). Holder et al (1975) found that there were no significant differences either in the metabolite profiles or in the total conversions of benzo(a)pyrene after 3-methylcholangrene-pretreatment of non-responsive mice (strain DBA/2J) but that the same pretreatment of responsive mice (strain C57B/6J) increased the benzo(a)pyrene metabolism and this increase was in the conversion to the dihydriodols (mainly the 7,8-dihydriodiol), there being no increase in the amounts of the
Figure 3.16. High-Pressure Liquid Chromatography of the Benzo(a)pyrene Metabolites Formed by 5,000xg Supernatant from Control (a), Sodium Phenobarbitone- (b) and Benzo(a)pyrene- (c) Pretreated Yeast
quinones and phenols produced. The same workers (Holder et al, 1974) had previously found that pretreatment of immature Long-Evans rats with 3-methylcholanthrene and sodium phenobarbitone produced different metabolite profiles than in control rats, there being an increase in the amount of the 9,10-dihydriodiol after this pretreatment and an increase in the 4,5-dihydriodiol after sodium phenobarbitone pretreatment. Epoxide hydrase was found to be involved in dihydriodiol formation in this system.

Rasmussen & Wang (1974) were also able to show differences in the metabolite profile in rats pretreated with a variety of polycyclic aromatic hydrocarbons and sodium phenobarbitone. They found, though, that these differences in metabolism were in part due to differences in the induced levels of epoxide hydrase. Oesch (1973) has also suggested the existence of a coupled multienzyme system consisting of mixed-function oxidase (aryl hydrocarbon hydroxylase) and epoxide hydrase which catalyses the conversion of naphthalene to the 1,2-dihydriodiol via an oxide. Oesch & Daly (1972) showed that the 3-methylcholanthrene-pretreatment of the animals increased the total conversion of naphthalene to 1,2-dihydriodiol by inducing this coupled enzyme system whereas sodium phenobarbitone induced the uncoupled epoxide hydrase so that the conversion of naphthalene to the 1,2-dihydriodiol was unaffected but the rate of conversion of the oxide to the dihydriodiol was increased. Oesch also suggested that since
epoxide hydrolase has a broad substrate specificity it may 
consist of a number of different enzymes in the same way 
as cytochrome P-450. It has also been shown that there are 
at least two forms of aryl hydrocarbon hydroxylase in rabbit 
liver (Johnson & Muller-Eberhard, 1977). Thus it seems that 
in mammalian systems the different metabolite profiles 
produced after various pretreatments are the result of a 
complex induction of different forms of both epoxide hydrolase 
and aryl hydrocarbon hydroxylase, it is not surprising that 
the situation is simpler in the yeast.

The kinetic data presented above suggest that pretreatment 
of yeast with benzo(a)pyrene produced a different form of 
aryl hydrocarbon hydroxylase by virtue of the change in Km 
but the metabolite profiles contrast with the majority of 
the work carried out in mammalian systems since no difference 
was seen after pretreatment. A possible explanation for 
this may be that since the formation of the dihydrodiols and 
phenols requires the production of an epoxide by aryl 
hydrocarbon hydroxylase, it is possible that in the yeast, 
although pretreatment with benzo(a)pyrene changed the kinetics 
of the reaction, the same epoxides were formed. This would 
mean that the subsequent non-enzymic and epoxide hydrolase- 
catalysed steps would produce the same metabolites as in 
control yeast.
CHAPTER 4.

The Metabolism of Other Compounds
by Cytochrome P-450 from *Saccharomyces cerevisiae*.
4. The Metabolism of Other Compounds by Cytochrome P-450 from Saccharomyces cerevisiae

4.1. Introduction
In addition to those techniques described elsewhere, a number of other methods were investigated in order to study the properties of yeast cytochrome P-450 in greater detail. Unfortunately these methods were found not to work in the yeast system, in particular the hydroxylation of biphenyl was investigated using several different methods and in all cases negative results were obtained. Other widely used mammalian drug metabolising assays also proved to be unsatisfactory.

4.2. Materials and Methods
4.2.1. The Measurement of Biphenyl Hydroxylation
(a) The Fluorometric Measurement of Biphenyl Hydroxylation
The method of Creaven et al (1965) was used to measure the production of the fluorescent metabolites, 2- and 4-hydroxybiphenyl from biphenyl (I).
(b) The High-Pressure Liquid Chromatography of the Biphenyl Metabolites

The method of Burke et al (1977) was used to investigate the metabolism of biphenyl by yeast microsomes by means of high-pressure liquid chromatography. The microsomes (final concentration of cytochrome P-450 was 0.22µM) were incubated with the usual amount of biphenyl and a NADPH-regenerating system which gave final concentrations of 4mM-NADP, 20mM-D-glucose-6-phosphate and 8 units of D-glucose-6-phosphate dehydrogenase for 1h at 37°C in a final volume of 2ml. A control experiment using boiled microsomes was carried out simultaneously. The reaction was stopped by the addition of 1ml of 4M-HCl and this was extracted with 7ml of iso-octane containing 1.5% iso-amyl alcohol after the precipitated protein had been removed by centrifugation. The organic phase was then filtered in a 5µm Millipore filter and evaporated to dryness in a rotary evaporator, the residue being redissolved in 100µl of HPLC solvent, 30µl of which were injected onto the column.

(c) The Metabolism of $[^{14}C]$Biphenyl

To 2ml of a microsomal suspension (final concentration of cytochrome P-450 0.35µM) was added 50µl of $[^{14}C]$biphenyl (unknown specific activity) and 100µl of NADPH-regenerating system. This mixture was incubated at 37°C for 30min and extracted into 8ml of ice-cold ethyl acetate which was then evaporated to dryness in a rotary evaporator. The residue
was redissolved in 50μl of ethyl acetate and spotted onto a Silica Gel plate (Schleicher & Schull, W. Germany). A control experiment using boiled microsomal suspension was performed simultaneously. Standard 2- and 4-hydroxybiphenyl and biphenyl were also spotted onto the thin-layer chromatography plate which was then developed in toluene: ethanol (95:5) and the position of the standards determined by their fluorescence under UV light. The radioactivity on the plate was detected by autoradiography using Kodak BB5 X-Ray Film developed in Kodak Universal developer and Kodafix fixer.

4.2.2. The 0-Deethylation of 7-Ethoxycoumarin

The method used to measure the 0-deethylation of ethoxy-coumarin (II) to 7-hydroxy-coumarin (umbelliferone) was essentially that of Ullrich & Weber (1972). This direct fluorometric assay was performed in a cuvette in a Perkin Elmer MPF3 Fluorescence Spectrometer using excitation 355nm and emission 460nm. The cuvette contained 2ml of yeast 5,000xg supernatant, 100μl of 2.4mM-7-ethoxy-coumarin solution (final concentration 0.12mM) and either 1mM-NADPH or 10mM-cumene hydroperoxide as cofactor.

![Chemical structure](image)
A static method for 7-ethoxycoumarin O-deethylation was investigated, tubes containing microsomal suspension, cofactor and 7-ethoxycoumarin were incubated at 37°C for 30min and the 7-ethoxycoumarin removed by extracting into n-hexane. The aqueous phase was then extracted with ether containing 1% iso-amyl alcohol, this ether extract was back-extracted into 0.1M glycine/NaOH buffer, pH 10.4 and the fluorescence of this solution was measured in the fluorimeter at 355nm excitation and 460nm emission.

4.2.3. The O-Deethylation of Ethoxyresorufin

The method of Burke & Mayer (1974) was used to investigate the O-deethylation of ethoxyresorufin (7-ethoxyphenoxyazone, III) to resorufin by yeast cytochrome P-450.

\[
\begin{array}{c}
\begin{array}{c}
\text{O} \\
\text{N} \\
\text{O} \\
\text{O} \\
\text{C}_2\text{H}_5 \\
\rightarrow \\
\text{O} \\
\text{N} \\
\text{O} \\
\text{OH}
\end{array}
\end{array}
\]

(III)

4.2.4. The O-Demethylation of p-Nitroanisole

The direct spectrophotometric method for measuring the O-demethylation of p-nitroanisole (IV) to p-nitrophenol as described by Netter & Seidel (1964) was used to investigate this reaction in yeast microsomes.

\[
\begin{array}{c}
\begin{array}{c}
\text{NO}_2 \\
\text{OCH}_3 \\
\rightarrow \\
\text{NO}_2 \\
\text{OH}
\end{array}
\end{array}
\]

(IV)
4.2.5. The N-Demethylation of Aminopyrine

The formation of formaldehyde as a result of the N-demethylation of aminopyrine (V) was measured by the method of Nash (1953). Yeast 5,000xg supernatant (1ml) was added to 1ml of 0.1M-Tris/HCl buffer, pH 7.0 containing 100μl of aminopyrine solution (final concentration 10mM) and 1mM-NADPH. The mixture was incubated at 37°C for 15min and the reaction stopped by the addition of 0.5ml of 10% trichloroacetic acid (w/v). The precipitated protein was removed by centrifugation and 2ml of the supernatant added to 2ml of Nash reagent (0.4% acetylacetone (w/v) in 4M-ammonium acetate) which was incubated at 37°C for 40min and the colour read at 412nm in a Cecil CE272 UV Spectrophotometer (Cecil Instruments, Cambridge, England).

![Chemical Structure]

4.2.6. NADPH Oxidation by Yeast Microsomes in the Presence of Substrates

Yeast microsomal suspension (0.5ml) was placed in a quartz spectrophotometer cuvette in a Pye Unicam SP1800 spectrophotometer containing 2.5ml of 0.1M-Tris/HCl buffer, pH 7.0 and 50μl of 5mg/ml NADPH (final concentration 0.2mM). The
substrate to be tested was then added to the sample cuvette and the change in absorbance at 340nm recorded, in this way any endogenous NADPH oxidation was automatically subtracted. Compounds tested by this method were biphenyl, aminopyrine, ethoxyresorufin, ethoxycoumarin, p-nitroanisole and ethylmorphine.

4.2.7. The Measurement of Oxygen Uptake by Means of an Oxygen Electrode

A Clark-type oxygen electrode (Rank Bros, Bottisham, Cambridge) was used to investigate the oxygen uptake that occurred as a result of reactions catalysed by cytochrome P-450. The electrode consisted of a gold electrode separated from the reaction mixture by a Teflon membrane and was calibrated using distilled water in the presence (anaerobic) and absence (aerobic) of sodium dithionite. The reaction mixture was stirred by means of a small magnetic stirrer. Compounds investigated by this method were biphenyl, p-nitroanisole, benzo(a)pyrene, benzo(e)pyrene, benzantracene, dimethylaniline, ethylmorphine and oleic acid (methyl ester).

4.2.8. The Demethylation of Lanosterol

The possibility of the demethylation of lanosterol was investigated by measuring formaldehyde formation by the method of Nash (1953). Incubates consisted of 1ml of yeast 5,000xg supernatant in 0.1M-Tris/HCl buffer, pH 7.0 containing 50μl of 5mg/ml lanosterol and NADPH or cumene hydroperoxide as
cofactors. The reaction was stopped by the addition of 1ml of 10%-trichloroacetic acid (w/v) after incubating at 37°C for 30min and the protein precipitate removed by centrifugation. The supernatant (1ml) was then added to 1ml of Nash reagent and the colour measured at 412nm after incubation at 37°C for 40min.

4.2.9. The Spectrophotometric Measurement of Cytochrome b

Cytochrome b was measured by the method of Omura & Sato (1964) from the reduced/oxidised spectrum by measuring the trough to peak height (409 to 426nm) and using a millimolar extinction coefficient of 17lM\(-1\).cm\(^{-1}\).

4.2.10. Materials

Benzo(a)pyrene, benzo(e)pyrene, benzantracene, dimethylaniline and 7-ethoxycoumarin were obtained from The Sigma Chemical Corp. (London) Ltd. Trichloroacetic acid, biphenyl, acetylacetone and ammonium acetate were supplied by BDH Chemicals Ltd., Poole, Dorset. Ralph N. Emanuel supplied the p-nitroanisole and aminopyrine while the 2- and 4-hydroxy-biphenyl standards were supplied by Hopkin & Williams Ltd., Chadwell Heath, Essex. Ethylmorphine was obtained from May & Baker Ltd., Dagenham and ethoxyresorufin from Pierce, Rockford, Illinois, USA. Lanosterol was obtained from Steraloids Ltd., Croydon, Surrey.
4.3. Results and Discussion

4.3.1. The Measurement of Biphenyl Hydroxylation

The metabolism of biphenyl has been extensively studied in various animal species, the major metabolite in rat, hamster and guinea pig being 4-hydroxybiphenyl while minor products include 2-hydroxybiphenyl, 3-hydroxybiphenyl, dihydroxy-, trihydroxy-, hydroxymethoxy- and dihydroxymethoxybiphenyls (Raig & Ammon, 1970 and 1972, Raig et al, 1976, Meyer & Scheline, 1976, Meyer et al, 1976a and b and Meyer, 1977). The selective enhancement of biphenyl 2-hydroxylase activity by carcinogens both in vivo and in vitro has been described and it has been suggested that this enhancement could provide a basis for a preliminary in vitro screening system for testing potential chemical carcinogens (McPherson et al, 1974 and 1976).

The assay for the formation of the fluorescent metabolites, 2- and 4-hydroxybiphenyl was attempted under several different conditions but it was not possible to obtain a positive result. Both NADPH and cumene hydroperoxide were used as cofactors and the assay was attempted for an extended incubation time (1h), the extraction procedure was modified in order to increase the efficiency of extraction and 5,000xg supernatant from yeast pretreated with sodium phenobarbitone and benzo(a)pyrene was also used, in all these cases no product could be detected. Different pHs were used, the usual pH of 8.1 was replaced by 7.6 and a reduced
temperature (25°C) was used in case the lack of activity was due to thermal denaturation of the enzyme. Microsomes were also used in place of 5,000xg supernatant but again no activity could be detected. As a positive control an experiment was performed using rat liver microsomes as well as yeast microsomes. The results showed no activity in the yeast microsomes for hydroxylation in the 2- or 4-position while the rat liver microsomes showed both activities, the rates being 3.4 nmoles 4-hydroxybiphenyl/nmole cytochrome P-450/min and 0.2 nmoles 2-hydroxybiphenyl/nmole cytochrome P-450/min. These results are in contrast with those of Wiseman et al. (1975) where the NADPH-supported 4-hydroxylation of biphenyl by yeast microsomal fraction was described.

The technique of high-pressure liquid chromatography is as sensitive as the fluorometric method but also permits the detection of other biphenyl metabolites such as 2,2', 3,3' and 4,4'-dihydroxybiphenyls. The experiment was performed as described above but no difference could be detected between the control (boiled microsomes) and test incubations, the conclusion being that no metabolism of biphenyl had occurred.

After thin-layer chromatography and autoradiography several bands were seen in addition to \([^{14}\text{C}]{\text{biphenyl}}\), unfortunately these bands were also present in the control and were therefore thought to be formed by auto-oxidation.
and not by an enzymic reaction. The positions of the unlabelled standards were detected by means of UV light and the Rf's were as follows: 4-hydroxybiphenyl - 0.30, 2-hydroxybiphenyl - 0.45 and biphenyl - 0.73.

4.3.2. The O-Deethylation of 7-Ethoxycoumarin

Increases in fluorescence were observed when NADPH and cumene hydroperoxide were used as cofactors, but it was found subsequently that this increase occurred even in the absence of substrate and therefore was not associated with the O-deethylation of 7-ethoxycoumarin. The static method gave negative results confirming that these observations were not due to 7-ethoxycoumarin metabolism.

4.3.3. The O-Deethylation of Ethoxyresorufin

No metabolism of ethoxyresorufin could be detected when NADPH was the cofactor, an increase in fluorescence was observed in the presence of cumene hydroperoxide but this was subsequently shown to be non-enzymic.

4.3.4. The O-Demethylation of p-Nitroanisole

An increase in absorbance at 420nm was observed but this was shown to occur even in the absence of p-nitroanisole and it must therefore be concluded that yeast microsomes do not catalyse the O-demethylation of p-nitroanisole.
4.3.5. The N-Demethylation of Aminopyrine

The Nash method for determining acetaldehyde showed that there was more acetaldehyde in the control than in the test incubate. A time course was also attempted but there was no difference between the 0min and 60min samples. It must be concluded, therefore, that there was no N-demethylation of aminopyrine.

4.3.6. NADPH Oxidation by Yeast Microsomes in the Presence of Substrates

The results from this experiment were difficult to interpret since a number of compounds produced an increase in absorbance at 340nm, these compounds were ethylmorphine, codeine, p-nitroanisole, 7-ethoxycoumarin and aniline. This increase was presumably as a result of these compounds inhibiting the endogenous rate of NADPH oxidation. As a result of this, the method was abandoned as a possible method for the determination of the metabolism of compounds by yeast cytochrome P-450.

4.3.7. The Measurement of Oxygen Uptake by Means of an Oxygen Electrode

This was another technique which it was hoped could be used to screen a variety of compounds to see whether they were metabolised by the yeast enzyme. Unfortunately, this method is rather insensitive and requires a rate of product formation of some 4nmole/min/mg protein and this criterion
is fulfilled by only a small number of compounds even in the much more active rat liver system. As a result of this, the method was found to be unsatisfactory.

4.3.8. The Demethylation of Lanosterol

The 14α-demethylation of lanosterol by a reconstituted cytochrome P-450 system from yeast has been demonstrated (Aoyama & Yoshida, 1978) and it was hoped that the Nash reaction for measuring formaldehyde could be used to show this demethylation in the system presently under investigation. However, this method was not sufficiently sensitive to detect any formaldehyde formation.

4.3.9. The Spectrophotometric Measurement of Cytochrome b5

Figure 4.1 shows the reduced/oxidised spectrum obtained from yeast 5,000xg supernatant. The concentration of cytochrome b5 was calculated to 0.88μM compared with a cytochrome P-450 concentration of 0.75μM which indicates that these two cytochromes are present in approximately equal amounts.
Figure 4.1. The Reduced/Oxidised Spectrum of Yeast
5,000xg Supernatant
CHAPTER 5.

The Binding of Compounds to Cytochrome P-450 from Saccharomyces cerevisiae.
5. The Binding of Compounds to Cytochrome P-450 from Saccharomyces cerevisiae

5.1. Introduction

It was found that several compounds were able to produce characteristic binding spectra when added to suspensions of yeast microsomes. The most interesting of these compounds, benzo(a)pyrene, was found to give a spectrum which had the normal type I shape and also an additional peak.

The binding of benzo(a)pyrene with yeast microsomes was further investigated by means of an equilibrium gel filtration method for measuring the association constant and number of binding sites. The results from this experiment were compared with the results from similar experiments performed with rat liver microsomes.

5.2. Materials and Methods

5.2.1. The Spectral Interaction of Compounds with Yeast Cytochrome P-450

Spectral changes resulting from the addition of compounds to microsomal suspensions were recorded in one of two spectrophotometers, either a Perkin-Elmer 356 Two-Wavelength Double Beam Spectrophotometer (Split-Beam Mode) or a Varian Cary 219 Spectrophotometer. When benzo(a)pyrene was the compound added a double-cell technique similar to that described by Goujon et al. (1972) was employed in order to remove interference from the benzo(a)pyrene which absorbs
in the wavelength range used. In this method one compartment of each double-cell contained either 3ml of Tris/HCl buffer, pH7.0 or 2.5ml of buffer plus 0.5ml of microsomal suspension (usually diluted x10 to give a final concentration of approximately 0.01μM but higher when the interaction was weaker e.g. for imidazole the conc. was 0.1μM). Benzo(a)pyrene (2mg/ml) was added a microlitre at a time to both the sample microsomal fraction and reference buffer compartments and an equal volume of acetone was added to the sample buffer and reference microsomal fraction compartments. The difference spectrum was then recorded between 500 and 350nm.

Spectral titrations at fixed wavelengths were subjected to double-reciprocal plots, derived from the kinetic equation

\[
E + S \xrightleftharpoons[k_2]{k_1} C \xrightleftharpoons[k_3]{k_2} E + P
\]

where \( E \) is enzyme, \( C \) is the enzyme-substrate complex and \( S \) is the substrate. Titrations were performed at room temperature and in the absence of cofactor, hence the reaction does not proceed to product \( P \). The reversibility of the first step in such a reaction has been demonstrated by Schenkman et al (1967).

The dissociation constant, \( K_S \), termed the "Spectral Dissociation Constant" (Schenkman et al, 1967) is

\[
K_S = \frac{([E] - [C]) \cdot [S]}{[C]}
\]
where \( E_t \) is the total amount of enzyme. By manipulation

\[
K_s = \frac{[E_+] [S] - [S]}{[C]}
\]

and

\[
[C] = \frac{[E_+] [S]}{K_s + [S]}
\]

The reciprocal of this equation

\[
\frac{1}{[C]} = \frac{K_s}{[E_+] [S]} \left( \frac{1}{[S]} + \frac{1}{[E_t]} \right)
\]

is the equation of a straight line, when the reciprocal of the spectral change \( 1/[C] \) is plotted against the reciprocal of the substrate concentration \( 1/[S] \), the Y intercept is equal to \( 1/[E_t] \) or the reciprocal of \( [C]_{max} \) and the X intercept is equivalent to \(-\frac{1}{K_s}\).

Thus for benzo(a)pyrene, double-reciprocal plots of \( A_{415} + A_{385-375} \) against benzo(a)pyrene concentration were used to determine values of \( K_s \) by means of least-squares regression analysis. Different wavelengths were used for other compounds according to the shape of the binding spectrum, for simple spectra the peak to trough height was normally used. The other compounds examined were safrole, lanosterol, biphenyl, oleic acid (methyl ester), 7-ethoxycoumarin, hexobarbitone, cyclohexane, butanol, n-octylamine, aniline, carbon tetrachloride, naphthalene and camphor.
5.2.2. Equilibrium Gel Filtration

A method of equilibrium gel filtration derived from the principles outlined by Ferscht (1977) and by Kerridge & Tipton (1972) was used to investigate the binding of benzo(a)pyrene to microsomal fractions from yeast and rat liver. This technique was first described by Hummel & Dreyer (1962) and is analogous in principle to equilibrium dialysis. A protein, P, is dissolved in a solution of a compound I to which it binds. The concentration of free I is thereby reduced by an amount equivalent to the P-I complex formed. An aliquot of this solution is then placed on a suitable Sephadex column which has been equilibrated with the same solution of I as was used to dissolve the protein. The column is thereafter eluted with the I solution and the concentration of I in the eluate is measured. As the P-I complex emerges at the excluded volume of the column, the total amount of I in the eluate rises above the equilibrium level and correspondingly, at some point after the protein peak the concentration of I in the eluate is decreased below the base-line level to form a trough which extends to the salt volume of the column. The amount of free I removed from the solution as displayed by this trough is equal to the excess concentration of I found in the protein peak. Thus as the protein moves down the column in the excluded volume it continues to remove I from the equilibrium solution within the Sephadex until an equilibrium is reached at the base-line concentration of I. Inasmuch as P and P-I are in equilibrium with a constant
concentration of free I as they emerge, even weak interactions may be studied provided the concentration of I is sufficiently high.

If we let the initial and equilibrating concentration of benzo(a)pyrene be cmol/l and the amount of benzo(a)pyrene bound to n sites on the cytochrome P-450 in the microsomal fraction is pmoles then the concentration of the cytochrome P-450-benzo(a)pyrene complex is \((p/n)/V\) where \(V\) is the volume of the benzo(a)pyrene solution in litres. The concentration of free benzo(a)pyrene must be \((e-p/n)/V\), where \(e\) is the total amount of cytochrome P-450. If

\[
P-450 + BP \rightleftharpoons P-450-BP
\]

\[
\frac{e-p/n}{V} \rightleftharpoons \frac{p/n}{V}
\]

then the apparent association constant for each binding site is given by

\[
K = \frac{(p/n)}{(e-p/n)c}
\]

or

\[
Ke(e-p/n) = p/n
\]

therefore

\[
\frac{p}{nc} = Ke - \frac{Kp}{n}
\]

and

\[
\frac{1}{c} \cdot \frac{p}{c} = nK - Kc \cdot \frac{p}{c}
\]
If we put

\[ \gamma = p/e \]

Then

\[ \frac{\gamma}{c} = nK - K \]

Hence a plot of \( \gamma/c \) against \( \gamma \) (a Scatchard plot) is a straight line of slope \(-K\), the intercept of which on the \( \gamma \) axis is \( n \), when \( \gamma/c = 0 \).

A column was made from a Pasteur pipette by placing a plug of glass-wool in the bottom and packing with Sephadex G-25 (the column dimensions were 5 x 45mm). The column was equilibrated with 0.2M-phosphate buffer, pH 7.0 containing 10%-dioxan (v/v) and benzo(a)pyrene in a range of different concentrations. The benzo(a)pyrene in the buffer consisted of a fixed, known amount of \([G-\text{H}]-\text{benzo(a)pyrene}\) and various known amounts of cold benzo(a)pyrene, the specific activity of the benzo(a)pyrene in the buffer was determined by counting the radioactivity of a small volume.

Once the column was equilibrated with the benzo(a)pyrene-containing buffer, usually after passing 20 to 30ml, a small volume of microsomal suspension, usually 10μl, was applied to the top of the column and 2-drop samples were collected (the sample volume was 0.058ml). These samples were counted in a Packard Tri-Carb Scintillation Spectrometer after adding 4ml of toluene:metapal (2:1) scintillant containing 0.5%-PPO (w/v) and 0.02%-dimethyl POPOP (w/v). The binding of
the benzo(a)pyrene to the cytochrome P-450 in the microsomal fraction resulted in an increase in the radioactivity when the fraction was eluted, although no trough was formed. By using the known specific activity of the benzo(a)pyrene it was possible to calculate the amount bound. The experiment was repeated at increasing benzo(a)pyrene concentrations (decreasing specific activity) and by making a Scatchard plot it was possible to calculate the apparent association constant for each binding site and the number of binding sites per mole of cytochrome P-450.

5.2.3. Materials

Sephadex G-25 was obtained from Pharmacia (Great Britain) Ltd, Hounslow, Middlesex. Biphenyl, n-butanol, aniline and carbon tetrachloride were supplied by BDH Chemicals Ltd, Poole, Dorset. Sodium hexobarbitone was obtained from May & Baker Ltd, Dagenham and lanosterol from Staraloids Ltd, Croydon, Surrey. Cyclohexane, n-octylamine, (+)-camphor, oleic acid (methyl ester), 7-ethoxycoumarin and naphthalene were obtained from The Sigma Chemical Corp. (London) Ltd.
5.3. Results and Discussion

5.3.1. The Spectral Interactions of Compounds with Yeast Cytochrome P-450

The number of compounds which could produce spectral changes with yeast microsomal fraction was extremely limited when compared with hepatic microsomes. The only compounds, other than benzo(a)pyrene, which it was found could produce binding spectra were imidazole and sodium phenobarbitone, both of which gave type II spectra.

Figure 5.1 shows the difference binding spectrum obtained with imidazole in the concentration range 0 to 2.0mM and Figure 5.2 is the double-reciprocal plot of $A_{385-430 \text{nm}}$ against imidazole concentration, the spectral binding dissociation constant, as calculated by least squares regression analysis, was 0.35mM (correlation coefficient = 0.998). These results were similar to those published by Dickins et al (1975) in which a type II spectrum was obtained with rat liver microsomes having a peak at 428nm, a trough at 394nm and spectral binding dissociation constant of 0.17mM.

The interaction of sodium phenobarbitone with yeast microsomes was very weak, the spectrum had a peak at 420nm and a trough at 385nm (Figure 5.3) and the spectral binding dissociation constant was 5.8mM (Figure 5.4) (correlation coefficient = 0.999)
Figure 5.1. The Binding Spectrum of Imidazole with Yeast Microsomes.
Figure 5.2. The Double-Reciprocal Plot of the Spectral Binding of Imidazole to Yeast Microsomes.
Figure 5.3. The Binding Spectrum of Sodium Phenobarbitone with Yeast Microsomes.
Figure 5.4. The Double-Reciprocal Plot of the Spectral Binding of Sodium Phenobarbitone to Yeast Microsomes.
As already pointed out, the binding of benzo(a)pyrene to yeast microsomes resulted in a spectrum which had two peaks (365 and 385nm) and a trough at 415nm (Figure 5.5). As the spectrum was titrated with benzo(a)pyrene there was an increase in the base-line below 400nm and so double-reciprocal plots of $A_{415} + A_{385-375nm}$ were plotted against benzo(a)pyrene concentration in order to determine the spectral binding dissociation constant (Figure 5.6) which was calculated to be 32μM (correlation coefficient = 0.999).

Solubilisation of the yeast microsomes did not alter the binding spectrum obtained with benzo(a)pyrene nor was there much change in the value of the dissociation constant. Figure 5.7 shows the double-reciprocal plot for solubilised yeast microsomes and the dissociation constant was calculated as 55μM (correlation coefficient = 0.998). Thus it would seem that whereas solubilisation has a large effect on the kinetics of benzo(a)pyrene metabolism (see Chapter 3) the effect on the spectral binding dissociation constant is small and this probably reflects the very strong interaction of benzo(a)pyrene with cytochrome P-450 which occurs whether the enzyme is held in the microsomal membrane or solubilised from it.

When rat liver microsomes were used in place of yeast microsomes the same spectrum was observed and such a spectrum has been published (Estabrook et al., 1978). Figure 5.8 shows
Figure 5.5. The Binding Spectrum of Benzo(a)pyrene with Yeast Microsomes.
Figure 5.6. The Double-Reciprocal Plot of the Spectral Binding of Benzo(a)pyrene to Yeast Microsomes.
Figure 5.7. The Double-Reciprocal Plot of the Spectral Binding of Benzo(a)pyrene with Solubilised Yeast Cytochrome P-450.
the double-reciprocal plot obtained by using rat liver microsomes and the value of the spectral binding dissociation constant was 9µM (correlation coefficient = 0.978). This was lower than that for yeast microsomes and probably reflects the much lower values of Km for the liver enzyme compared with the yeast enzyme.

Benzo(e)pyrene (I) gave the same binding spectrum as benzo(a)pyrene with yeast microsomes (Figure 5.9) and the spectral binding dissociation constant (Figure 5.10) was also similar to that of benzo(a)pyrene being 49mM (correlation coefficient = 0.982).

It would seem therefore that the structural requirements for this interaction are met equally by both compounds.

It is possible to calculate from the binding spectrum of benzo(a)pyrene with microsomes the amount of cytochrome P-450 to which benzo(a)pyrene is bound since values for the extinction coefficient of the benzo(a)pyrene-cytochrome P-450 complex have been published. This value is a function of the haemoprotein and is not altered by the source of the enzyme.
Figure 5.8. The Double-Reciprocal Plot of the Spectral Binding of Benzo(a)pyrene with Rat Liver Microsomes.
Figure 5.9. The Binding Spectrum of Benzo(e)pyrene with Yeast Microsomes.
Figure 5.10. The Double-Reciprocal Plot of the Spectral Binding of Benzo(e)pyrene with Yeast Microsomes.
or the substrate used. Estabrook et al (1978) used a value of 57mM$^{-1}$ cm$^{-1}$ for the maximal change in absorbance observed from 420 to 500nm and Cinti et al (1979) used a value of 126mM$^{-1}$ cm$^{-1}$ for the change from 385 to 419nm. Table 5.1 illustrates the calculation of the amount of the benzo(a)-pyrene-cytochrome P-450 complex present during the titration of yeast and liver microsomes using these values. The percentage of liver cytochrome P-450 which was bound to benzo(a)pyrene was small and this was probably due to the fact that control microsomes were used, if 3-methylcholanthrene- or benzo(a)pyrene-pretreated microsomes had been used the percentage of the haemoprotein bound to benzo(a)pyrene would have been approximately 46% (Estabrook et al, 1978) reflecting the increased proportion of cytochrome P448 in the microsomes. The percentage of yeast cytochrome P-450 bound to benzo(a)-pyrene was in the region of 30% and this was higher than for the control rat liver microsomes suggesting that there was a larger proportion of cytochrome P-448-like enzyme in the untreated yeast microsomes.
<table>
<thead>
<tr>
<th></th>
<th>Yeast</th>
<th>Rat Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo(a)pyrene Conc. (μM)</td>
<td>20.8</td>
<td>7.8</td>
</tr>
<tr>
<td>Cytochrome P-450 Conc. (nM)</td>
<td>8.3</td>
<td>18.7</td>
</tr>
<tr>
<td>ΔA_{415nm}</td>
<td>0.155</td>
<td>0.051</td>
</tr>
<tr>
<td>ΔA_{peak-trough}</td>
<td>0.250</td>
<td>0.090</td>
</tr>
<tr>
<td>Complex Conc. (415) (nM)</td>
<td>2.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Complex Conc. (p-t) (nM)</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Percent of P-450 (415)</td>
<td>33.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Percent of P-450 (p-t)</td>
<td>24.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Table 5.1 The Calculation of the Percentage of the Cytochrome P-450 Bound as Cytochrome P-450-Benzo(a)pyrene Complex for Yeast and Rat Liver Microsomes.
5.3.2. The Equilibrium Gel Filtration of the Benzo(a)pyrene-Cytochrome P-450 Complex

Gel filtration experiments were carried out at increasing benzo(a)pyrene concentrations (c) using the same amount of cytochrome P-450 (e). The specific activity of the labelled benzo(a)pyrene was determined by counting a 50µl sample of the equilibrating buffer and this was used to calculate the amount of benzo(a)pyrene bound to the cytochrome P-450 (p). Figure 5.11 shows a typical result of one gel filtration experiment. From the values of p, e and c thus determined, p/e (δ) and δ/c were calculated and Scatchard plots were constructed (Figure 5.12). The values of the number of binding sites (n) and the apparent association constant (K) were then estimated by extrapolation. The experiment was performed using yeast microsomes (Table 5.2) and control rat liver microsomes (Table 5.3).

The values of n determined were 6.3 for yeast microsomes and 0.95 for rat liver microsomes, these values are 6 and 1 to the nearest whole numbers. Thus it would seem that six moles of benzo(a)pyrene were bound to each mole of yeast cytochrome P-450 present compared with one mole of benzo(a)pyrene for rat liver cytochrome P-450.

The values obtained for the association constants of the yeast and rat liver enzymes were 0.077nM⁻¹ and 0.064nM⁻¹ respectively and were very similar. This would suggest that
Figure 5.11. A Typical Result of the Gel Filtration of the Benzo(a)pyrene-Cytochrome P-450 Complex.
Figure 5.12. The Scatchard Plot of the Equilibrium Gel Filtration of the Benzo(a)pyrene-Cytochrome P-450 Complex from Yeast (○) and Rat Liver Microsomes (●).
Table 5.2. Data from the Equilibrium Gel Filtration of the Benzo(a)pyrene-Cytochrome P-450 Complex for Yeast Microsomes.

<table>
<thead>
<tr>
<th>c (nM)</th>
<th>e (nmoles)</th>
<th>p (nmoles)</th>
<th>p/e (δ)</th>
<th>δ/c (nM⁻¹)</th>
<th>K (nM⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>0.02</td>
<td>0.0117</td>
<td>0.59</td>
<td>0.472</td>
<td>0.0765</td>
<td>6.3</td>
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<tr>
<td>7.59</td>
<td>0.02</td>
<td>0.0422</td>
<td>2.11</td>
<td>0.278</td>
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</tr>
<tr>
<td>13.93</td>
<td>0.02</td>
<td>0.0690</td>
<td>3.45</td>
<td>0.248</td>
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<tr>
<td>20.27</td>
<td>0.02</td>
<td>0.0720</td>
<td>3.60</td>
<td>0.178</td>
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<tr>
<td>26.62</td>
<td>0.02</td>
<td>0.0900</td>
<td>4.50</td>
<td>0.169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c (nM)</td>
<td>e (pmoles)</td>
<td>p (pmoles)</td>
<td>p/e (ŋ)</td>
<td>8/c (nM⁻¹)</td>
<td>K (nM⁻¹)</td>
<td>n</td>
</tr>
<tr>
<td>-------</td>
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<td>----------</td>
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</tr>
<tr>
<td>2.5</td>
<td>3.2</td>
<td>0.35</td>
<td>0.108</td>
<td>0.043</td>
<td>0.0642</td>
<td>0.95</td>
</tr>
<tr>
<td>8.8</td>
<td>3.2</td>
<td>1.37</td>
<td>0.428</td>
<td>0.048</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>15.2</td>
<td>3.2</td>
<td>1.28</td>
<td>0.400</td>
<td>0.026</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>21.5</td>
<td>3.2</td>
<td>1.90</td>
<td>0.594</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 5.3. Data from the Equilibrium Gel Filtration of the Benzo(a)pyrene-Cytochrome P-450 Complex for Rat Liver Microsomes.
the affinity with which the benzo(a)pyrene is bound to the binding sites of these enzymes is approximately the same. In contrast with this, the value of the spectral binding dissociation constant was smaller for the liver enzyme than the yeast enzyme, which suggests a slightly higher affinity of the liver enzyme for benzo(a)pyrene than the yeast enzyme.

These relatively small differences in the values of the association and dissociation constants of benzo(a)pyrene for rat liver and yeast cytochrome P-450 are negligible however, when compared, for example, to the large differences between the published values of the Michaelis constants for benzo(a)pyrene metabolism by the rat liver enzyme and the values determined experimentally for the yeast enzyme. It may be concluded that these differences in the Km are not due to differences in the benzo(a)pyrene-protein interactions but must be due to some other differences in these enzymes.

For further discussion of these results see Chapter 6.
CHAPTER 6

Final Discussion
6. Final Discussion

The effect of cyclic AMP on the biosynthesis of cytochrome P-450 described here is one of the very few examples of a negative effect of this compound on protein synthesis. Recently, a positive effect of cyclic AMP has been described by Adhya & Miller (1979) when they reported that the gal operon of *E. coli* was controlled by two independent promoters, one was activated and the other inhibited by cyclic AMP and cyclic AMP receptor protein. These two promoters were modulated, however, by the same operator locus and repressor protein, in this way the promoters met the additional requirements of galactose enzymes for anabolic reactions under extreme physiological conditions, that is excess and deficiency of cyclic AMP.

That glucose effects the activities of cytochromes within the cell, particularly mitochondrial cytochromes, is well established. Polakis *et al* (1964) found that the concentration of the mitochondrial cytochromes in *S. cerevisiae* varied inversely with the glucose concentration of the medium and Jollow *et al* (1968) showed that the formation of mitochondria in this yeast depended on the availability of ergosterol and unsaturated fatty acids. Note that this requirement for ergosterol may be related to the proposed role of cytochrome P-450 in steroid metabolism, particularly since it has been shown in this thesis that this enzyme seems to
appear when the yeast is growing rapidly and would therefore have a large requirement for the constituents of the cellular membrane and also for mitochondria. In the cell-cycle of *Schizosaccharomyces pombe* grown aerobically in 1%-glucose (Poole et al) it was found that the amounts of cytochrome oxidase and cytochrome b oscillated in phase and that the amounts of cytochrome P-450 increased during the first three-quarters of the cell-cycle, cytochrome P-450 appeared to have a physiological function since it was reducible by glucose and endogenous substrates.

While evidence was presented in the Introduction that cyclic AMP brought about glucose-derepression in yeast, Law & Ferro (1977) have extrapolated similar findings to incorporate S-adenosylmethionine in a theory of cellular regulation. In *S. cerevisiae* it has been shown that the intracellular concentration of S-adenosylmethionine is dependent on the glucose concentration of the medium (Schlenk et al, 1965) and since the synthesis of S-adenosylmethionine requires the transfer of an adenosyl-moieties from ATP to methionine, these compounds share a metabolic precursor with cyclic AMP. Law & Ferro (1977) found that S-adenosylmethionine inhibited RNA synthesis during glucose-derepression of *S. cerevisiae* and that cyclic AMP stimulated RNA synthesis, indicating that these compounds were antagonists and therefore supporting the idea that S-adenosylmethionine functions as a regulatory molecule.
It has been shown in this thesis that the addition of exogenous cyclic AMP to a suspension of yeast protoplasts inhibited the formation of cytochrome P-450. Protoplasts were used in these experiments because it was thought that the nucleotide would not be able to pass through the intact cell wall. However, Mahler & Lin (1978) have demonstrated that cyclic AMP added to a suspension of yeast cells maintained in glucose medium was able to overcome the catabolite repression of the enzyme δ-aminolevulinate dehydrogenase in *S. cerevisiae* which implies that cyclic AMP can penetrate the intact cell wall.

As far as the metabolic activity of cytochrome P-450 from *S. cerevisiae* is concerned, this would seem to be severely limited when compared to the vast number of different substrates which have been reported for the hepatic microsomal enzyme. The only substrate which was found to be at all suitable was benzo(a)pyrene. The activity of the enzyme towards this compound was much less than has been reported for the mammalian system. It must be concluded that the endogenous substrate is metabolised at greater rates than those found for benzo(a)pyrene and that this substrate remains to be found. However, in contrast with this are the observations of the Japanese workers in this area who have succeeded in measuring the 14α-demethylation of lanosterol by a reconstituted system containing cytochrome P-450 from *S. cerevisiae* (Aoyama & Yoshida, 1978 a).
There is also the possibility that the method of preparation of the yeast enzyme is in some way selecting one enzyme from a mixture of enzymes, for example, by virtue of differences in thermal stability during the disruption procedure. If the enzyme was prepared from osmotically disrupted yeast protoplasts, for example, it may be that activity towards other compounds might be detected. An experiment in which protoplasts were made from yeast grown in 20%-glucose medium resulted in no cytochrome P-450 being recovered, presumably since the method of protoplast formation used requires a long period (1.5h) at 30°C and all the enzyme was denatured. The absence of other activities could also be due to the presence of endogenous substrates bound to the enzyme. However, experiments in which microsomes were pre-incubated with NADPH to remove endogenous substrate followed by attempts to obtain binding spectra with various compounds were unsuccessful.

With respect to benzo(a)pyrene, it will be noted that the values of the spectral binding dissociation constant (e.g. 32μM for yeast microsomal fraction compared with 9μM for rat liver microsomal fraction) and the Michaelis constant (0.45mM for the NADPH-supported reaction using control yeast enzyme compared with the published values of 0.6 to 100μM for control rat liver enzyme) are very different in the case of the yeast enzyme although they are approximately the same in the case of the rat liver enzyme. A close correlation
between $K_s$ and $K_m$ values has been found for some type I interactions. Ullrich et al. (1973) for example, found that in the case of the 0-deethylation of 7-ethoxycoumarin, the $K_s$ and $K_m$ values agreed exactly for sodium phenobarbitone- and 3-methylcholanthrene-pretreated animals, but there have also been a number of cases where $K_s$ and $K_m$ values have not agreed. Kratzer & Staudinger (1968) found that the $K_s$ value for coumarin was some 100 times greater than the $K_m$. Since $K_m$ values for microsomal drug metabolism encompass the individual kinetics of several components of a complex system, the poor correlation between $K_m$ and $K_s$ is not surprising especially as the optical changes defined by $K_s$ values result primarily from conformational changes in cytochrome P-450, which is only one component of the monooxygenase system. Differences between these two parameters are even more likely if only a proportion of the cytochrome P-450 is engaged in the formation of a spectrally apparent substrate complex (see Chapter 5).

Studies of the metabolism of benzo(a)pyrene have shown that in mammalian systems certain areas of the molecule are predominantly the site of hydroxylation, these are the 3-, 4- and 5-positions and the 7-, 8-, 9- and 10-positions and because of this observation it has been proposed (Estabrook et al., 1978) that the benzo(a)pyrene molecule interacts with the cytochrome P-450 in such a way that the area of the molecule interacting with oxygen is directly over the haem-iron, which is the site of oxygen binding. In the present
study it was observed that benzo(e)pyrene produced the same spectral interaction with yeast microsomes as did benzo(a)pyrene and this would suggest that it is the 7-, 8-, 9-, 10-, 11- and 12-positions of the benzo(a)pyrene molecule (I) and thus the 9-, 10-, 11-, 12-, 1- and 2-positions of benzo(e)pyrene (II) which are involved in this interaction. It would therefore be of interest to investigate the possible metabolism of benzo(e)pyrene in order to gain further insight into this interaction.

As has already been described, there are two major forms of cytochrome P-450 found in mammalian systems according to the inducers used, these are characterised by the absorption maxima of their carbon monoxide-binding spectra as either cytochrome P-450 or P-448. However, there is some doubt, since each of these enzymes may be a mixture of different forms, whether these wavelengths are a true indication of the actual absorption maxima or whether they represent average values. In microbial systems purified cytochrome P-450 from Rhizopus sp had three separate haemoproteins which had
absorption maxima at 449, 449 and 447nm (Appleby, 1978) while the cytochrome P-450 from Pseudomonas putida had absorption maximum of 446nm (O'Keeffe et al, 1978). In yeast, Yoshida & Kumaoka (1975) found the absorption maximum of a partially purified preparation to be at 448nm and it has been measured in the system under study in this thesis as being at 449nm. Thus the term cytochrome P-450 has been used throughout for the yeast enzyme in a purely generic form and does not mean that the absorption maximum of the CO-complex was at 450nm.

Differences in the Michaelis-Menten parameters for aryl hydrocarbon hydroxylase between the NADPH- and cumene hydroperoxide-supported reactions were found in Chapter 3. Both the Km and the Vmax were greater for the cumene hydroperoxide-supported reaction. These two reactions have been compared in the liver (Yang & Strickhart, 1978) where other differences were found, for example, the cumene hydroperoxide-supported reaction was slower than with NADPH. The effects of inhibitors on these two reactions for sodium phenobarbitone, 3-methylcholanthrene and control microsomes was also different but it was concluded by the authors that these differences did not rule out the possibility that both reactions proceeded by the same cytochrome P-450 intermediate. The observed differences in the reactions might be due to differences between cytochrome P-450 and P-448 in the steps prior to this intermediate in the catalytic cycle.
Yang et al (1978) have also investigated the aryl hydrocarbon hydroxylase assay when the benzo(a)pyrene was added at 37°C or at lower temperatures (15-20°C) and found that measured activity was greater in the former method. They concluded that microsomes can exist in two states according to the ambient temperature, one form having greater activity than the other. It has already been pointed out that temperature can affect the spin state of cytochrome P-450 and it may be that such changes in spin state are of great importance in the catalytic activity of the enzyme. Temperature also influences membrane fluidity by means of its effect on lipid and this may present an alternative explanation of these observations.

It was shown in Chapter 5 that the stoichiometry of benzo(a)pyrene binding to rat liver microsomes was 1:1 (i.e. one binding site) whereas there were six binding sites in the case of yeast microsomes. These results represent a significant difference between these two enzymes, assuming that the greater binding by yeast microsomes was not due to non-specific binding. Work in collaboration with the Department of Chemistry (S. Libor, personal communication) in which tritium labelled benzo(a)pyrene was bound to yeast cytochrome P-450 purified to homogeneity according to gel electrophoresis, showed that 17.5nmoles of benzo(a)pyrene were bound to 3nmoles of cytochrome P-450, this approximates to a 6:1 stoichiometry. This confirms the results obtained
by equilibrium gel filtration but since the purified enzyme rather than the microsomal fraction was used this rules out the possibility of non-specific binding. This stoichiometry does, however, assume that all the binding sites on the cytochrome P-450 are occupied, whereas the results from spectral binding data presented in Chapter 5 showed that only some 30% of the yeast cytochrome P-450 present was bound to benzo(a)pyrene.

Thus the ratio of 6:1 which has been measured in these two experiments was only as a result of 30% of the cytochrome P-450 being bound to benzo(a)pyrene. In the spectral binding experiments, the excess of benzo(a)pyrene over cytochrome P-450 was 1000-fold and therefore it seems likely that the benzo(a)pyrene was bound to a maximum extent. This means that in order to obtain a 6:1 stoichiometry in the binding of benzo(a)pyrene to cytochrome P-450 the total number of binding sites in the 30% fraction of the cytochrome P-450 population which binds benzo(a)pyrene must be twenty.

The equilibrium gel filtration experiments also assumed 100% binding of benzo(a)pyrene to cytochrome P-450, but since in these experiments the excess of benzo(a)pyrene over cytochrome P-450 was also approximately 1000-fold there is no basis for this assumption. It seems likely, therefore, that the observed 1:1 stoichiometry for rat liver microsomes cannot be interpreted as meaning that there was only one
binding site. It was calculated that rat liver microsomes only bound benzo(a)pyrene to an extent of 5% and so by a similar process of deduction to that above it can be seen that this means that rat liver cytochrome P-450 consists of only 5% which can bind benzo(a)pyrene but that this must also have twenty binding sites (n.b. 5% of 20 is 1). Therefore it would seem that whereas it was initially thought that there was a difference between yeast and rat liver cytochrome P-450 with respect to their ability to bind benzo(a)pyrene, it may be that the actual protein molecules responsible for this interaction as situated in the lipid environment of the microsomal membrane are, in fact, very similar.
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Rapid and Economical Production of Microsomal Cytochrome P-450 in Yeast Resuspended in 20% Glucose Medium: Relationship to the Biosynthesis of Mitochondrial Cytochromes

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Cytochrome P-450 was originally found in anaerobically grown baker's yeast and to a lesser extent in yeast grown aerobically to the end of the exponential phase of growth in relatively high-glucose medium (4%, w/v) (Lindenmeyer & Smith, 1964). Good yields of cytochrome P-450 can be obtained under conditions of glucose repression of mitochondria, for example in 20% glucose medium, and the presence of cytochrome a+a3 (cytochrome oxidase) was noted to be reciprocal with cytochrome P-450 (Wiseman et al., 1975).

We now report a rapid and economical production of relatively large amounts of cytochrome P-450 when a heavy inoculum of yeast, previously grown to the end of the exponential phase in growth medium containing as little as 0.5% glucose, is transferred to a relatively small volume of 20% glucose medium (4-fold growth of the yeast occurs).

This synthesis, over at least 8h, is accompanied by a failure to resynthesize cytochrome a+a3 (unlike the resynthesis of cytochromes b and c that occurs after a 4h lag phase) despite growth. The concentration of cytochrome a+a3 in the yeast was measured by the method of Williams (1964) from the reduced-versus-oxidized difference spectrum. In this method, four simultaneous equations in four unknowns were derived from the knowledge of the contributions in absorbance of each cytochrome to the major maxima and minima of each of these cytochromes. Measurement of the difference in absorbance at four wavelength pairs between 500 and 640 nm enables the equations to be solved, this process being speeded up considerably by the use of a computer program.

Spectra similar to those obtained by Williams (1964) for isolated rat liver mitochondria have been reported for whole baker's yeast cells (Chance, 1957) and for Saccharomyces cerevisiae and Candida lipolytica (Skipton et al., 1973).

Saccharomyces cerevisiae (N.C.Y.C. no. 290) was grown from a slope culture as described previously (Wiseman et al., 1975), in 0.5% glucose medium. The yeast was harvested by centrifugation after 48h and washed once in 100 mM-citrate/phosphate buffer, pH5.8. The yeast thus obtained was divided into 500 mg amounts and each re-suspended in 15 ml of 20% glucose growth medium in a 25 ml conical flask and shaken at 30°C as before.

At various time intervals up to 24h the yeast suspension was centrifuged and the pressed (wet) weight of yeast was determined. The yeast was then re-suspended in 5 ml of distilled water, and 2.5 ml put into each of two 3 ml spectrophotometer cuvettes. A few grains of sodium dithionite were added to the sample cell and 50µl of 20-volume hydrogen peroxide was added to the reference cell. The difference spectrum between 500 and 640 nm was then measured in a Unicam SP. 1800 recording spectrophotometer. The concentration of cytochrome P-450 was measured as described by Wiseman et al. (1975) by reducing the contents of the reference cell with sufficient dithionite.

Fig. 1 shows rapid production, and maintenance of a high concentration of cytochrome P-450, especially in view of the 4-fold growth of the yeast with no apparent lag phase, in 20% glucose medium in about 10h. There is no synthesis of cytochrome a+a3 and therefore its concentration per g of yeast decreases in line with the dilution of the
existing enzyme with growth of the yeast (Fig. 2). The other cytochromes, especially $b$ and $c$, increase in concentration after the initial reduction, presumably as a result of their synthesis after an initial-lag phase (regardless of growth). Studies of the energy

![Graph](image-url)

**Fig. 1.** Production of cytochrome P-450 (a) and growth of yeast (b) after transfer of yeast from 0.5% to 20% glucose medium.

Each point is the mean of five to seven determinations±s.e.m.

![Graph](image-url)

**Fig. 2.** Concentrations (nmol/g wet wt. of yeast) of cytochromes $a$, $b$, $c_1$, and $c$ after transfer of yeast from 0.5% to 20% glucose medium.

Each point is the mean of five to seven determinations±s.e.m.
requirements of this process show that energy production in mitochondria is not required. Thus incubation in 20% glucose medium containing 1mM-KCN, which will inhibit cytochrome oxidase by 98% (Slater, 1950), had no effect on the growth of yeast or production of cytochrome P-450. However, incubation of yeast with 50µM-iodoacetate, which inhibits glycolysis at hexokinase (Jones et al., 1975), glyceraldehyde 3-phosphate dehydrogenase (Levitski, 1974) and alcohol dehydrogenase (Hasilik, 1973), completely stopped any growth of yeast or increase in cytochrome P-450. The changes in cytochromes shown in Fig. 2 were not altered by the presence of cyanide.

A decrease in cytochrome oxidase concentration as expressed per cell has been shown to occur in synchronous growth of *Saccharomyces cerevisiae* in 3% glucose (Cottrell & Avers, 1970). More recent work has shown that incubation of baker's yeast in 10% glucose produces catabolite repression of reduced coenzyme Q-cytochrome c reductase (complex III) and cytochrome oxidase (complex IV) activities of mitochondria via a mitochondrial translated repressor protein (Rouslin, 1976). Our results on the repression of cytochrome oxidase by glucose and the concomitant increase in cytochrome P-450 can probably be explained by the correlation with low concentrations of cyclic AMP in the cell under conditions of high glucose concentration (Van Wijk & Konijn, 1971). During the growth of yeast, the concentration of cyclic AMP is inversely related to that of cytochrome P-450 (A. Wiseman & T. K. Lim, unpublished work). The function of cytochrome P-450 in glucose-repressed yeast is not clear, although it may act as a lipid hydroxylase in the production of elements of the mitochondrial membrane (Wiseman et al., 1976).

Large quantities of cytochrome P-450 can be isolated by using this resuspension technique, and this will have important consequences, since it is required for pharmaceutical and medical applications (Wiseman, 1977). The rapid and economical production reported here should facilitate the application of the enzyme.

Partial Repression of Cytochrome P-450 by Adenosine 3':5'-Cyclic Monophosphate in Protoplasts of Saccharomyces cerevisiae

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Addition of cyclic AMP is known to remove the catabolite repression of β-galactosidase biosynthesis by glucose in Escherichia coli (Perlman & Pastan, 1968; Ullman & Monod, 1968), by its action at the promoter of the lac operon, causing stimulation of transcription (De Crombrugghe et al., 1971). We have noted a similar effect of added cyclic AMP on α-glucosidase biosynthesis in yeast protoplasts (Wiseman & Lim, 1974).

In the present study, we report the first negative effect of cyclic AMP, likely to be at the level of transcription, in the biosynthesis of cytochrome P-450 in yeast. We find this enzyme in brewer’s yeast under growth conditions of mitochondrial repression, i.e. at high glucose concentrations in aerated media, where the intracellular concentration of cyclic AMP is low (Wiseman et al., 1975; A. Wiseman & T.-K. Lim, unpublished work). Under these conditions the concentration of mitochondrial cytochrome oxidase is low (Wiseman & Woods, 1977).

Several workers have shown that there is a reciprocal relationship between the intracellular concentration of cyclic AMP and the concentration of glucose in the growth medium of yeast. For example, Van Wijk & Konijn (1971) have shown an increase in intracellular cyclic AMP from 0.04 nM to 0.32 nM during the induction of α-glucosidase in yeast during the transfer from 2% glucose to 2% maltose plus 0.1% glucose medium. Schlanderer & Dellweg (1974) observed an inverse proportionality between glucose concentration and intracellular cyclic AMP in Schizosaccharomyces pombe. Fang & Butow (1970) have shown that reversal of mitochondrial repression can be produced by incubating yeast protoplasts in 10% glucose containing cyclic AMP, but not by 10% glucose alone. Watson & Berry (1977a,b) and Hartig & Breitenbach (1977) in experiments in which cyclic AMP was measured during the cell cycle of S. cerevisiae have found a peak in cyclic AMP concentration during induction of sporulation and a decrease before DNA-synthesis and mitosis, indicating that cyclic AMP may play an important role in the control of cell division.

Previous work in this laboratory has shown that the drug-metabolizing enzyme cytochrome P-450 is produced in yeast when grown in medium containing high concentrations of glucose (Wiseman et al., 1976). This cytochrome P-450 may be recovered in the microsomal fraction after yeast disruption and has drug-metabolizing ability (Wiseman et al., 1975b). Also, the enzyme is inducible by phenobarbital (Wiseman & Lim, 1975).

Our results indicate that the appearance of cytochrome P-450 in yeast is due to synthesis de novo (for review, see Wiseman, 1975), as it is prevented by cycloheximide (10 μg/ml) in intact yeast.

Saccharomyces cerevisiae (N.C.Y.C. 240) was grown from a slope culture as described previously (Wiseman et al., 1975a) in 0.5% glucose medium. The yeast was
Fig. 1. Growth of yeast protoplasts
Yeast protoplasts were incubated in 5% glucose growth medium alone (●), or in medium containing 10 mM-cyclic AMP (■) or 80 μg of actinomycin D/ml (▲).
Cyclic GMP has been reported to inhibit the biosynthesis of β-galactosidase and tryptophanase in *E. coli* growing in minimal medium on glucose or glycerol. Its action was considered to be on transcription by binding to the promoter of the cyclic AMP-binding protein, in competition with cyclic AMP (Ariman & Werthamer, 1974). Experiments in the present study in which protoplasts were incubated in medium containing 0.5% glucose and 10mM-cyclic GMP have not, however, consistently shown an antagonistic relationship between cyclic AMP and cyclic GMP, since under these conditions production of cytochrome P-450 could not be reproducibly stimulated.

In conclusion, we suggest that the production of cytochrome P-450 in yeast is controlled by the intracellular concentration of cyclic AMP and this in turn is dependent on the glucose concentration outside the cell. There is some evidence that glucose inhibits yeast adenylate cyclase *in vitro* (Lim, 1976; Sy & Richter, 1972).

Other negative-type effects of cyclic AMP have been reported. The production of sex pilus is inhibited in *E. coli* K-12 carrying de-repressed sex factors (Harwood & Meynell, 1973). These authors commented that this need not be a direct effect on transcription of the genes involved in pilus production, but could be an indirect effect of cyclic AMP on the bacterial cell envelope. Also, under some conditions, cyclic AMP suppresses the induction of antibody synthesis in male spleen-cell cultures (Bosling-Schneider, 1975). A report by Khipongpan *et al.* (1975) has concluded that cyclic AMP promotes a co-ordinated inhibition of hepatic anabolic pathways, including protein synthesis, under conditions of enhanced gluconeogenesis. Studies on the cell-free system suggest a membrane-associated effect in translation. Effects on translation in our system cannot be ruled out, therefore, in view of the general doubt as to whether translational effects have been demonstrated for cyclic AMP in microbial (for review, see Wiseman, 1975) and animal systems.
Nevertheless, we note the possibility that some promoters may have the reverse requirements for transcription start or stop operations, compared with the classical lac operon of E. coli.

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Possible Removal of Benzo(a)pyrene from Some Foods Using Cytochrome P-450 from Brewer's Yeast

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Benzo(a)pyrene (BP) is a carcinogen whose metabolism has been extensively studied in relation to mammalian organs, in particular the liver and lung. The major metabolite, 3-hydroxybenzo(a)pyrene, was identified more than three decades ago,1-3 and the assay for the enzymatic formation of this highly fluorescent metabolite has been widely used in measuring microsomal mixed-function oxidases activity,4,5 and is generally known as aryl hydrocarbon hydroxylase (AHH) assay. Many other metabolites are produced, some of which are extremely reactive and constitute the ultimate carcinogenic molecular species.

The haemoprotein, cytochrome P-450, was first discovered in the microsomal fraction, isolated by ultracentrifugation of disrupted liver preparations, in 1958 by Klingenberg6 and by Garfinkel7 and was subsequently shown to be involved in hydroxylation and dealkylation reactions.4,5,6 The presence of a cytochrome P-450-like co-binding pigment in cells of brewer's yeast (Saccharomyces cerevisiae) was first reported by Lindenmeyer and Smith8 and later by Ishidate et al.9

A method modified from that of Dehnen et al.10 was used to measure the conversion of benzo(a)pyrene to 3-hydroxybenzo(a)pyrene by a 5000 x g supernatant fraction produced from disrupted brewer's yeast, triethylamine being used to quench the benzo(a)pyrene fluorescence. Our experiments have shown that the Km for BP with phenobarbitone-pretreated yeast and untreated yeast, cytochrome P-450 is 500 μM, which is larger than for the liver enzyme, which has been variously reported as 0.6 μM, 1 μM,10 and 23 μM.11 Pretreatment of yeast with BP (5 μg ml⁻¹ growth medium) results in a decrease in the Km to 160 μM presumably due to the induction of the BP-metabolising form of the enzyme, cytochrome P-448. A Km of this level would be quite suitable for an enzymic process for removal of fairly low concentrations of BP from foodstuffs.

The production of benzo(a)pyrene metabolites by microsomal enzymes from yeast or liver is dependent on the presence of NADPH and molecular oxygen12 but we have been able to make the process more amenable to large scale application with foods by replacing the expensive NADPH-regenerating system normally used in the AHH assay system by cumene hydroperoxide, an organic hydroperoxide which has been previously used in steroid hydroxylations13 and drug metabolism studies14 in mammalian systems.

The production of cytochrome P-450/P-448 in brewer's yeast has been studied15-20 and it is now possible to solubilise and immobilise the cytochrome P-450 system with retention of drug-metabolising ability. We suggest that production of yeast on a large scale, perhaps by continuous fermentation, followed by disruption and stabilisation (perhaps by immobilisation) of the cytochrome P-450 would permit its use in the removal of benzo(a)pyrene (and its highly reactive metabolites) from foodstuffs, such as smoked and toasted foods and some drinks.

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Short Paper Reading Meeting 1097

REGULATION OF THE BIOSYNTHESIS OF CYTOCHROME P-450 IN BREWER'S YEAST

ROLE OF CYCLIC AMP *

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Summary

The drug metabolising enzyme cytochrome P-450 has been studied in great detail in mammalian systems and its presence in microorganisms is also well established. However, neither its function nor its means of control in brewer's yeast, Saccharomyces cerevisiae, has been investigated. We demonstrate here using yeast protoplasts that it is the intracellular concentration of cyclic AMP which controls, by repression, the de novo synthesis of the enzyme, and also that cyclic AMP concentrations are in turn inversely related to the concentration of glucose in the yeast growth medium.

Introduction

The haemoprotein, cytochrome P-450 (EC 1.14.14.1.), was discovered in the liver microsomal fraction [1,2]. Subsequent investigation into carbon monoxide inhibition of microsomal oxidative reactions has shown that this CO-binding protein is involved in a large number of hydroxylation and dealkylation reactions [3—5]. In liver microsomes, the enzyme can catalyse reactions with drugs, steroids, fatty acids, hydrocarbons, insecticides, benzo(a)pyrene and other carcinogens [6—8].

The presence of a cytochrome P-450-like CO-binding pigment in cells of Saccharomyces cerevisiae was first reported by Lindenmeyer and Smith [9] and later by Ishidate et al. [10,11]. Cytochrome P-450 has also been found in many other micro-organisms and is, for example, inducible in Pseudomonas putida by D-(+)-camphor where it catalyses the hydroxylation of this compound to form the exo-5-alcohol [12]. The enzyme functions in Coryne-
bacterium sp. to oxidise n-octane [13,14] and has been found also in *Claviceps purpurea* [15], *Cunninghamella bainieri* [16] and in *Nocardia* sp. [17]. A cytochrome P-450-dependent fatty acid ω-hydroxylase has been found in *Bacillus megaterium* [18] and it is involved also in the hydroxylation of geraniol and neral by *Vinea rosea* [19].

Cytochrome P-450 has been found in yeasts other than *S. cerevisiae*; it is responsible, for example, for the ability of *Torulaspora* sp. to hydroxylate fatty acids [20,21] and may be induced in *Candida tropicalis* by growth on tetradecane [22,23] where it catalyses the ω-hydroxylation of fatty acids (especially lauric acid) and also the conversion of n-alkanes to primary alcohols. The complete *Candida* system consists of cytochrome P-450, NADPH-cytochrome P-450 reductase and a heat-labile fraction [24] as does the mammalian system [25]. These latter components from yeast can be replaced by the corresponding fractions from rat liver, which also catalyses fatty acid ω-hydroxylation [26].

**Experimental**

*Growth of yeast. Saccharomyces cerevisiae* (N.C.Y.C. No. 240) was maintained on slopes of Sabouraud-Dextrose agar and transferred using a platinum wire to growth medium containing sodium chloride (5 g/l), yeast extract (10 g/l) and bacteriological or mycological peptone (20 g/l) autoclaved at 15 lb/inch² for 15 min. In addition, the medium contained glucose at concentrations ranging from 1 to 200 g/l autoclaved separately to prevent the formation of interfering breakdown pigments. The yeast was grown in 100 ml medium contained in 250-ml conical flasks shaken in a Mickle shaking water bath (Mickle Laboratory Engineering Co., Gomshall, Surrey) at 30°C, shaking at 50 rev./min with stroke 5 cm for various times, and was harvested either by centrifugation in a M.S.E. bench centrifuge (Measuring and Scientific Equipment Ltd., London) or by filtering in a Büchner funnel using Whatman No. 1 filter paper.

*Preparation of protoplasts.* Protoplasts were prepared by a modified method of Eddy and Williamson [27]. Yeast was grown in 0.5% glucose growth medium for 48 h, harvested by centrifugation and washed once in 1.00 mM-citrate/phosphate buffer, pH 5.8. The yeast was then resuspended to 100 mg/ml in 100 mM-citrate/phosphate buffer, pH 5.8, containing 0.14 M 2-mercaptoethanol and 0.04 M-EDTA and incubated at 30°C for 30 min. After centrifugation in a Piccolo centrifuge (Hereaeus Christ, GmbH), the yeast was washed three times in buffer without additives and resuspended to 1 g/ml. Snail gut enzyme (0.4 ml/g yeast) was then added and the mixture incubated at 30°C for 45 min. The protoplasts were harvested by centrifugation and resuspended in 1 ml of medium/50 mg original yeast.

*Measurement of cyclic AMP.* The method used for the measurement of cyclic AMP depends on the competition between unlabelled cyclic AMP and a fixed quantity of tritium-labelled compound for binding to a protein which has a high specificity and affinity for cyclic AMP.

Blanks contained 160 µl 100 mM-Tris/EDTA buffer, pH 7.4, and 50 µl of a solution of [8-3H]adenosine 3',5'-phosphate. The standard curve and unknowns
contained 50 μl buffer, 50 μl cyclic [8-^H]AMP, 100 μl binding protein bound to charcoal in suspension and 50 μl standard cyclic AMP in the range 0—16 pmol or 50 μl unknown. Solutions were incubated and centrifuged to remove the charcoal and 200 μl supernatant were withdrawn and placed in 10 ml of scintillant in vials for counting. The scintillant contained 9 g PPO and 4.5 g POPOP in 1.51 toluene plus 750 ml Triton X-100. Vials were counted for 10 min in a Packard Tri-Carb Liquid Scintillation Spectrometer. The concentration of unknown cyclic AMP was calculated from a straight line standard curve of \( C_0/C_x \) against pmol cyclic AMP, where \( C_0 \) is the zero cpm and \( C_x \) is the cpm of the standards (or unknown) corrected for the blank.

To measure the concentration of cyclic AMP in yeast, cells (200 mg wet weight) were suspended in 2 ml 100 g/l trichloroacetic acid and incubated at room temperature for 5 min. The yeast was then separated in a bench centrifuge and re-extracted with 1 ml trichloroacetic acid solution and centrifuged again. To the combined supernatants, one tenth of their volume of 1 M hydrochloric acid and 2 volumes diethyl ether were added. The mixture was shaken in the cold for 10 min and the ether phase discarded. The aqueous phase was re-extracted five times with ether and then evaporated to dryness at 55°C under a stream of nitrogen. The dried samples were dissolved in 10 ml 100 mM Tris/EDTA buffer, pH 7.4, and 50 μl were used in the assay.

Measurement of cytochrome P-450 and mitochondrial cytochromes. Cytochrome P-450 in whole yeast and in protoplasts was determined by a method modified from that of Omura and Sato [28]. Yeast suspension (0.1 g wet wt/ml) was placed in each of two spectrophotometer cuvettes of 1-cm light path and reduced by the addition of a few grains of sodium dithionite. The baseline between 410 and 490 nm was then recorded using a Pye Unicam SP1800 spectrophotometer. Carbon monoxide was then bubbled through the test cuvette and the scan repeated. The peak height at 450 nm above the baseline was then used to calculate the concentration of cytochrome P-450, assuming an extinction coefficient of 91 cm\(^{-1}\)·mM\(^{-1}\).

The mitochondrial cytochromes were determined by a method modified from that of Williams [29]. Yeast suspended as before is reduced only in the sample cuvette, the reference cell being oxidised by addition of 50 μl 20-vol. hydrogen peroxide. In this way it is possible to measure the mitochondrial cytochromes prior to cytochrome P-450 in the same sample, the hydrogen peroxide being subsequently reduced with dithionite. The difference spectrum between 500 and 640 nm is then recorded and the concentrations of the mitochondrial cytochromes (\( a + a_3, b, c_1 \) and \( c \)) derived from the difference in absorption at four wavelength pairs which are used to solve four simultaneous equations in four unknowns, this process being speeded up considerably by the use of a computer programme (for spectra see Fig. 1).

Materials. *S. cerevisiae* (No. 240) was obtained from the National Collection of Yeast Cultures, Nutfield, Surrey. Mycological peptone, powdered yeast extract and Sabouraud-dextrose agar were obtained from Oxo id Co. Ltd., Basingstoke, Hants. and bacteriological peptone from Difco.

Adenosine 3',5'-phosphate, adenosine-5'-phosphate, adenosine-2'-(3')-monophosphate (mixed isomers), actinomycin D and chloramphenicol were obtained from the Sigma Chemical Corp. (London) Ltd., 2-mercaptoethanol, 2,5-
diphenyloxazole (PPO) and 1,4-bis(5-phenyloxazolyl-2)-benzene (POPOP) were obtained from Koch-Light, Colnbrook, Bucks. The cyclic AMP assay kit was obtained from the Radiochemical Centre, Amersham. D-Glucose and all other chemicals were supplied by BDH Chemicals Ltd., Poole, Dorset.

Results and Discussion

The effect of glucose on cytochrome P-450 and the mitochondrial cytochromes

Fig. 2 shows the relationship of cytochrome P-450 accumulation to the growth phase of the yeast in media of different glucose concentrations. 'Low glucose behaviour', with early accumulation of the enzyme, is seen in 1% and 2.5% glucose media. No enzyme is produced in 0.1% glucose medium and 'high glucose behaviour', with late accumulation of cytochrome P-450, is seen in 5% glucose and above, with intermediate behaviour at 3% and 4% glucose.

The biosynthesis of cytochrome P-450 may be promoted in yeast by firstly growing in 0.5% glucose medium and transferring to 20% glucose medium. Yeast was grown as described above in 0.5% glucose medium and harvested by centrifugation after 48 h and washed once in 100 mM-citrate/phosphate buffer, pH 5.8. The yeast was divided into 500-mg portions and each portion resuspended in 15 ml 20% glucose medium in a 25-ml conical flask and shaken at 30°C as before. At various time intervals up to 24 h the yeast suspension was centrifuged and the pressed (wet) weight of yeast determined. The yeast was then resuspended in 5 ml distilled water and half was put into each of two 3-ml spectrophotometer cuvettes. The amounts of cytochrome P-450 and the mitochondrial cytochromes were determined as described above. Thus, Fig. 3 shows
the rapid production of cytochrome P-450 which occurs in about 10 h, at the same time as the yeast undergoes a 4-fold growth, with no apparent lag phase. There is no synthesis of cytochrome oxidase and therefore this concentration per g of yeast decreases in line with the dilution of the enzyme with growth of the yeast (Fig. 4). Of the other cytochromes, b and c increase somewhat in concentration, after the initial reduction, presumably as a result of their synthesis after an initial lag phase, which occurs regardless of growth.

Studies of the energy requirements of this process show that energy production by mitochondria is not required. Thus incubation in 20% glucose medium containing 1 mM KCN, which will inhibit cytochrome oxidase by 98% [30] had no effect on the growth of the yeast, production of cytochrome P-450 or the changes in cytochromes shown in Fig. 4. However, incubation of yeast in the presence of 50 mM iodoacetate which inhibits glycolysis at hexokinase [31], glyceraldehyde-3-phosphate dehydrogenase [32] and alcohol dehydro-
genase [33] completely stopped any growth of the yeast or increase in cytochrome P-450. These results indicate that the energy production under these conditions is solely by glycolysis.

The effect of glucose on cyclic AMP concentration
The intracellular concentrations of cyclic AMP in whole yeast were measured as described under Experimental and were found to be lower in yeast grown in high glucose medium (Table I) thus indicating an inverse relationship between glucose and cyclic AMP concentration.

These findings are in agreement with the results of other workers who found an increase in intracellular cyclic AMP from 0.04 μM to 0.32 μM during the induction of α-glucosidase in yeast during transfer from 2% glucose to 2% maltose plus 0.1% glucose medium [34]. An inverse proportionality has been observed between glucose concentrations and intracellular cyclic AMP in

<table>
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<th>Glucose in medium (%)</th>
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<td>0.1</td>
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Schizosaccharomyces pombe [35]. In experiments in which cyclic AMP was measured during the cell cycle of *S. cerevisiae*, a peak in cyclic AMP concentration has been observed during the induction of sporulation and a decrease prior to DNA synthesis and mitosis, indicating that cyclic AMP may have an important role in the control of cell division [36,37]. It has been shown [38] that glucose inhibits adenyl cyclase and this might indicate a possible mechanism whereby glucose can control the intracellular concentration of cyclic AMP.

The effect of glucose and cyclic AMP on cytochrome P-450 production by yeast protoplasts

Addition of cyclic AMP is known to remove the catabolite repression of β-galactosidase biosynthesis by glucose in *Escherichia coli* [39,40] by its action at the promoter of the *lac* operon causing stimulation of transcription [41]. Fung and Butow [42] have shown that reversal of mitochondrial repression can be produced by incubating yeast protoplasts in 10% glucose containing cyclic AMP, but not by 10% glucose alone, and we have noted that cyclic AMP removes catabolite repression of α-glucosidase biosynthesis by glucose in yeast protoplasts [43].

If protoplasts made from yeast previously grown in 0.5% glucose medium are transferred to a medium containing a higher concentration of glucose (5%) they produce cytochrome P-450 in a similar manner to whole yeast when transferred to 20% glucose medium as described above (see Fig. 5). The production of the enzyme by protoplasts is 60% repressed (at 6 h) by the presence of cyclic AMP (10 mM) in the medium although the same concentration of adenosine-5'-monophosphate or adenosine-2'-(3')-monophosphate (mixed isomers) did not produce any significant effect on cytochrome P-450 production. The difference between the results for cyclic AMP and controls was highly significant according to the Student's *t*-test (*P* < 0.01). Cycloheximide (10 mg/l) or actino-

![Fig. 5. Production of cytochrome P-450 by yeast protoplasts. Protoplasts were prepared as described in the Experimental section and incubated at 30°C in 5% glucose medium (•) containing cyclic AMP (10 mM) (*), 5'-AMP (10 mM) (○), 2'- (3')-AMP (10 mM (■) and actinomycin D (80 µg/ml) () and cycloheximide (10 µg/ml) (×). The results presented are means of up to six experiments, bars indicate S.D.](image-url)
mycin D (80 mg/l) added at zero time, prevented the appearance of cytochrome P-450. The action of actinomycin D is presumably to prevent transcription of genes for cytochrome P-450, although we cannot rule out an indirect effect through haem synthesis if that were to be limiting.

It would appear, therefore, that the production of cytochrome P-450 in yeast is controlled by the intracellular concentration of cyclic AMP and this in turn is dependent on the glucose concentration outside the cell. This effect is to our knowledge the first reported negative effect of cyclic AMP on the transcription of mRNA prior to the synthesis of protein, nevertheless, we note the possibility that some promoters may have the reverse requirements for transcription start or stop operations, compared with the classical lac operon of E. coli.

Other negative-type effects have been reported, for example, the production of sex pili is inhibited in E. coli K-12 carrying de-repressed sex factors [44]. This need not be a direct effect on transcription of the genes involved in pilus production, but could be an indirect effect of cyclic AMP on the bacterial cell envelope.

Cyclic GMP has been reported to inhibit the biosynthesis of β-galactosidase and tryptophanase in E. coli growing in minimal medium on glucose or glycerol. Its action was considered to be on transcription by binding to the promoter of the cyclic AMP-binding protein, in competition with cyclic AMP [45]. Experiments in the present study in which protoplasts were incubated in a medium containing 0.5% glucose and 10 mM cyclic GMP have not however consistently shown an antagonistic relationship between cyclic AMP and cyclic GMP, since under these conditions production of cytochrome P-450 could not be reproducibly stimulated.

As far as the function of cytochrome P-450 in yeast is concerned, it has been postulated that the enzyme may be involved in the demethylation of lanosterol to form zymosterol, as this conversion is inhibited by carbon monoxide by 57% in a preparation of disrupted yeast cells [46]. It has been reported also that most of the cytochrome P-450 in intact yeast is in a high-spin state and therefore thought to be substrate-bound. Furthermore, it was also observed that the low-spin type absorption spectrum of a purified preparation could be partially converted to a high-spin type by the addition of lanosterol [47]. Zymosterol is an intermediate in the formation of ergosterol, a membrane component in yeast which has a similar function to cholesterol in animals.

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Metabolism of Benzo[a]pyrene by the Cytochrome P-450/P-448 of Saccharomyces cerevisiae

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The major pathways for the metabolism of benzo[a]pyrene and other polycyclic hydrocarbons in mammalian tissue [for review, see De Pierre & Ernster (1978)] involve the aryl hydrocarbon hydroxylase system, epoxide hydrase and glutathione S-epoxide transferase (Conney, 1967; Oesch, 1973). The major metabolite of the aryl hydrocarbon hydroxylase system, which involves at least two protein components, cytochrome P-450/P448 and a flavoprotein, NADPH-cytochrome P-450 reductase, is 3-hydroxybenzo[a]pyrene, which was identified more than three decades ago (Chambers & Crowfoot, 1941; Berenblum et al., 1943; Berenblum & Schoental, 1955), and the fluorimetric assay for the enzymic formation of this highly fluorescent metabolite has been widely used.

The haemoprotein cytochrome P-450 (EC 1.14.14.1) was first discovered in the microsomal fraction, isolated by ultracentrifugation of disrupted liver preparation by Klingenberg (1958) and by Garfinkel (1958), and was subsequently shown to be involved in a large number of hydroxylation and dealkylation reactions (Conney et al., 1957; Orrenius et al., 1964; Cooper et al., 1965). The presence of a cytochrome P-450-like CO-binding pigment in cells of brewer’s yeast was first reported by Lindenmeyer & Smith (1964) and later by Ishidate et al. (1969). For a review of microbial cytochromes P-450 including those of yeasts, see Wiseman (1977).

We have been aware for some time of the presence of a cytochrome P-450/P448 aryl hydrocarbon hydroxylase system in Saccharomyces cerevisiae grown under conditions of mitochondrial repression, i.e. at high glucose concentrations. (A. Wiseman & J. Gondal, unpublished work) and report here some studies of this system.

Saccharomyces cerevisiae (Brewer’s yeast, N.C.Y.C. no. 240) was grown in medium containing 20% (w/v) glucose to effect the synthesis of cytochrome P-450/P-448 (Wiseman et al., 1973) and harvested by centrifugation after 48h. The yeast was then
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Fig. 1. Lineweaver-Burk plot of the NADPH-catalysed benzo[a]pyrene hydroxylation by yeast 5000g supernatant prepared from control (•), phenobarbital- (○) or benzo[a]-
pyrene-pretreated yeast (*)

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that the $K_m$ for benzo[a]pyrene decreased from 2.7 mM to 0.36 mM when yeast was pre-treated with benzo[a]pyrene. Pretreatment with phenobarbital again had no effect ($K_m = 2.8$ mM).

Solubilization of the cytochrome P-450/P-448 with sodium cholate resulted in a preparation that could metabolize benzo[a]pyrene when cumene hydroperoxide was the cofactor ($K_m = 95$ mM), but not with NADPH-regenerating system. This is presumably due to the disruption of the electron-transport system necessary for NADPH utilization, which is by-passed by cumene hydroperoxide. Addition of phosphatidylcholine did not enable us to restore the ability to use NADPH, and there was no stimulation of benzo[a]pyrene hydroxylation in contrast with the stimulation of NaIO$_4$-supported cytochrome P-450-catalysed steroid hydroxylation observed by Berg et al. (1976) in *Bacillus megaterium*.

Incubation (as above) of the yeast enzyme with [G$^3$H]benzo[a]pyrene and separation of the metabolites by high-pressure liquid chromatography has shown that besides 3-hydroxybenzo[a]pyrene, 9-hydroxy- and especially the 7,8-dihydrodiol benzo[a]pyrene are formed (the latter is characteristic of P-448 activity). Schenkman et al. (1969) and Powis et al. (1976) have reported that only the cytochrome P-450 induced by benzo[a]pyrene or 3-methylcholanthrene (usually called cytochrome P-448) is able to form a type-I spectral complex with benzo[a]pyrene. The yeast enzyme is able to produce type-I spectral changes with benzo[a]pyrene without any pretreatment, although the spectrum produced varies somewhat from the normal type-I spectrum in having an additional peak at 360 nm (Fig. 2). Experiments with untreated rat liver microsomal
fraction (cytochrome P-450/P-448) produced the same spectrum, including the extra peak at 360 nm. Double cells were used to eliminate the interference due to the colour of benzo[a]pyrene. The binding constant for yeast microsomal fraction was 36 µM ± 6 (for liver microsomal fraction we found it to be 9 µM). The binding constant for the solubilized yeast cytochrome P-450/P-448 was 53 µM ± 10. This value is similar to the $K_m$ value found (95 µM) with cumene hydroperoxide and solubilized enzyme (see above).

We gratefully acknowledge the help of Dr. G. M. Cohen, especially in the use of labelled benzo[a]pyrene and the high-pressure liquid chromatography of its metabolites.

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Benzo(a)pyrene Metabolites Formed by the Action of Yeast Cytochrome P-450/P-448

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Benzo(a)pyrene metabolites have been identified by high-pressure liquid chromatography after production by a cytochrome P-450 in brewer’s yeast disrupted. The major metabolites are 7,8-dihydro-7,8-dihydroxy-benzo(a)pyrene, 9-hydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene. Pretreatment of the yeast with benzo(a)pyrene, during its growth decreases the Michaelis constant \( K_m \) of the enzyme for benzo(a)pyrene, using either NADPH or cumene hydroperoxide as a cofactor.

1. Introduction

The ability of brewer’s yeast, \textit{Saccharomyces cerevisiae} (N.C.Y.C. No. 240) to produce a drug-metabolising enzyme similar to that of the cytochrome P-450/P-448 of mammalian liver when grown under conditions of mitochondrial repression i.e. at high glucose concentrations has been reported previously.\(^1\) This system is capable of demonstrating aryl hydrocarbon hydroxylase activity (A. Wiseman and J. Gondal, unpublished work) towards the carcinogen benzo(a)pyrene. The yeast is grown at a high glucose concentration in order to repress the formation of mitochondrial cytochrome oxidase and under these conditions the cytochrome P-450 is synthesised and subsequently isolated in the microsomal fraction \((105,000 \text{ g centrifugal sediment})\) after cell disruption. This enzyme (ferrous form) can be readily detected through its absorption at 450 nm in the presence of carbon monoxide, both in whole cells and extracts, by difference spectrophotometry.

We now report the finding that benzo(a)pyrene is a good substrate for this yeast enzyme system, in the presence of oxygen and NADPH, or alternatively in the presence of an oxygen and electron donor, such as cumene hydroperoxide. This enzyme, in free or immobilised form, could be useful therefore in the large scale removal of carcinogens such as benzo(a)pyrene from water, effluents and some foodstuffs or food additives (the highly reactive metabolites produced would be detoxicated \textit{in situ} by combination with other components or additives).

The major metabolite of the aryl hydrocarbon hydroxylase system, which involves at least two protein components, cytochrome P-450/P-448 and a flavoprotein, NADPH-cytochrome P-450 reductase, is 3-hydroxybenzo(a)pyrene. This was identified more than three decades ago\(^6\) and the fluorometric assay for the formation of this highly fluorescent compound is widely used.

The identification of the benzo(a)pyrene metabolites produced by the yeast enzyme by means of high-pressure liquid chromatography is described and comparison of the kinetics of benzo(a)pyrene metabolism by the enzyme from yeast pretreated with sodium phenobarbitone and benzo(a)pyrene, using either NADPH or cumene hydroperoxide as a cofactor.

2. Materials and methods

2.1. Preparation of yeast cytochrome P-450/P-448

The yeast was grown in 20% glucose medium containing 0.5% sodium chloride, 1% yeast extract and 2% bacteriological peptone (control) plus either 0.15% sodium phenobarbitone.

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2.2. Measurement of aryl hydrocarbon hydroxylase activity

Aryl hydrocarbon hydroxylase activity was measured by a method modified from that of Dehnen et al. Each incubation contained 0.5 cm$^3$ of 5000 g supernatant, 0.5 cm$^3$ of 0.1 M-Tris/HCl buffer, pH 7.0 and an NADPH-regenerating system giving final concentrations of 4 mM-NADP, 20 mM-D-glucose-6-phosphate and 8 units-D-glucose-6-phosphate dehydrogenase or 2.4 mM-cumene hydroperoxide. The reaction was started by the addition of benzo(a)pyrene from a stock solution of 2 mg cm$^{-3}$ in acetone to give a final concentration in the range 0.001-0.0316 $\mu$g cm$^{-3}$ and incubated at 310 K for 1 h. The reaction was stopped by the addition of 1 cm$^3$ of ice-cold acetone and the precipitated protein removed by centrifugation. A 0.6 cm$^3$ sample of this acetone solution was then added to 1.4 cm$^3$ of 10.7% (w/v) triethylamine solution in a fluorimeter cuvette and scanned from 500 to 560 nm emission (467 nm excitation) in a Perkin Elmer MPF3 fluorescence spectrophotometer to find the peak height at 520 nm. Fluorescence was calculated relative to 10 $\mu$g quinine sulphate cm$^{-3}$ in 1 M-H$_2$SO$_4$, which was in turn calibrated against a standard 3-hydroxybenzo(a)pyrene solution.

2.3. High-pressure liquid chromatography of benzo(a)pyrene metabolites

Each incubation contained 2 cm$^3$ of 5000 g supernatant, a NADPH-regenerating system comprising of 2 mM-NADP, 10 mM-D-glucose-6-phosphate and 8 units-D-glucose-6-phosphate dehydrogenase and 10 $\mu$Ci of (G-$^{3}$H)-benzo(a)pyrene in 2 $\mu$l benzene (specific activity was 24 Ci mm$^{-1}$ and 5 mCi cm$^{-3}$). Incubation was for 2.5 h at 310 K after which time the metabolites were extracted twice with 5 cm$^3$ ice-cold ethyl acetate which was filtered through a 5 pm Millipore filter and rotary evaporated to dryness, the residue being redissolved in 50 $\mu$l acetone. This extract was then applied to a Phase Separations ODS column in a Laboratory Data Corporation high-pressure liquid chromatograph. Metabolites were eluted with a methanol/water gradient linear from 50/50 to 80/20 in 50 min, the flow rate was 1 cm$^3$ min$^{-1}$. Samples were collected every half minute in an LKB 7000 fraction collector and counted in a Packard Tri-Carb scintillation spectrometer using toluene/metapal (2:1) scintillant containing 0.5% PPO and 0.02% dimethyl POPOP. The column was calibrated with unlabelled standards run simultaneously and detected by absorbance at 254 nm by means of a u.v. detector (see Figure 1).

2.4. Materials

(G-$^{3}$H)-benzo(a)pyrene was obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

3. Results and discussion

Growth of yeast in the presence of benzo(a)pyrene changes the composition of the mixture of isoenzymes produced in that it induces a form of the enzyme with higher affinity (lower $K_m$) for benzo(a)pyrene. We observed earlier an induction with 3-methylcholanthrene, associated with cytochrome P-448, that showed the characteristic increased O-de-ethylation of 7-ethoxyresorufin (A. Wiseman and J. Gondal, unpublished work).

The method described for measuring the production of the fluorescent metabolite, 3-hydroxybenzo(a)pyrene was used to compare the Michaelis constants ($K_m$) of the enzyme from yeast pretreated with either sodium phenobarbitone or benzo(a)pyrene. As can be seen from Table 1, pretreatment of yeast with sodium phenobarbitone had no significant effect on the $K_m$ for either NADPH- or cumene hydroperoxide-catalysed hydroxylation. Pretreatment with benzo(a)pyrene itself however caused a marked decrease in the $K_m$ as might be expected. Table 1 shows this decrease in $K_m$ after induction with benzo(a)pyrene only, which is observed with either cofactor. As with
Figure 1. High-pressure liquid chromatography of the benzo(a)pyrene metabolites from control yeast. (a), Emax: (i) 9, 10-dihydropyrone; (ii) 4,5-dihydropyrone; (iii) 7, 8-dihydropyrone; (iv) 9-hydroxybenzo(a)pyrene; (v) 3-hydroxybenzo(a)pyrene; (vi) benzo(a)pyrene. (b), The labelled metabolites produced by incubation with yeast 5000 g supernatant; (vii) quinone; (viii) unknown. For method see section 2.
Benzo(a)pyrene metabolites

the pretreatment of animals, the exposure of the yeast to benzo(a)pyrene is likely to increase the contribution of the cytochrome P-448 form of the enzyme known to activate the carcinogen in the body. Indeed the yeast enzyme (induced or uninduced) in its production of mainly the 7,8-dihydrodiol displays a predominantly P-448 activity and it displays a useful affinity for benzo(a)pyrene (see Table 1). Cytochrome P-450 activity is associated with the production, in a detoxication mechanism, of mainly the 4,5-dihydrodiol, and it is this form of the enzyme that is induced by pretreatment of animals with sodium phenobarbitone. The inability of phenobarbitone to affect benzo(a)pyrene metabolism has been reported previously in the rat. In these experiments benzo(a)pyrene pretreatment increased benzo(a)pyrene hydroxylase activity and cytochrome P-448 was formed.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>NADPH</th>
<th>Cumene hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.52</td>
<td>2.70</td>
</tr>
<tr>
<td>Sodium phenobarbitone</td>
<td>0.50</td>
<td>2.80</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>0.16</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Figure 1 shows the results of a high-pressure liquid chromatographic separation of the benzo(a)pyrene metabolites produced by yeast 5000 g supernatant obtained from control yeast, compared with standards run at the same time. The major metabolites produced were 7,8-dihydro-7,8-dihydroxy-benzo(a)pyrene (7,8-dihydrodiol), 9-hydroxybenzo(a)pyrene, 3-hydroxybenzo(a)pyrene, a quinone and an unidentified metabolite. Similar results were obtained in a preliminary investigation using thin-layer chromatography in benzene-ethanol (9:1).

These results are similar to those obtained with mammalian systems i.e. rat liver, with the exception of the dihydrodiols. In the rat the 4,5-, 7,8- and 9,10-dihydrodiols are formed by liver homogenates or microsomes from normal animals and the 7,8- and 9,10-dihydrodiols are formed from animals pretreated with 3-methylcholanthrene. In short-term organ cultures of hamster lung, benzo(a)pyrene is metabolised to the 9,10- and 7,8-dihydrodiols and benzo(a)pyrene-3-yl hydrogen sulphate with little or no 3-hydroxybenzo(a)pyrene or 4,5-dihydrodiol being formed. However it would seem that it is the formation of the 7,8-dihydridiol which is significant since this is further metabolised in cells in culture or in model systems in vitro to give the 7,8-dihydroxybenzo(a)pyrene-9,10-oxide and this diol-epoxide reacts directly with DNA. However, experiments in which 14C-benzo(a)pyrene was incubated with suspensions of plant cells from Chenopodium rubrum showed that plant cells produce more polar metabolites than liver microsomes, those metabolites which were identified were benzo(a)pyrene-3,6-quinone, benzo(a)pyrene-1,6-quinone and 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene, no 7,8-dihydrodiol was formed.

Pretreatment of yeast with sodium phenobarbitone or benzo(a)pyrene did not have any effect on the relative amount of the 7,8-dihydrodiol formed, these results are similar to those of Holder et al. in which there were no significant differences in the metabolic profiles or in the total conversions after 3-methylcholanthrene-pretreatment of 'non-responsive' mice. The major dihydrodiol formed was the 7,8-dihydridiol.

Acknowledgement

We gratefully acknowledge the help of Dr G. M. Cohen in the use of labelled benzo(a)pyrene and the high-pressure liquid chromatography of its metabolites.
References