A Study of Cytochrome P-448, a Component of the Monooxygenase System from Saccharomyces cerevisiae: Purification and Characterization

This Thesis is Presented in Accordance with the Regulations Governing the Award of the Degree of Doctor of Philosophy in the University of Surrey

by

Mahmood Rezazadeh Azari, M.Sc.

February 1984

Department of Biochemistry
University of Surrey
Guildford, GU25XH
Surrey, England
Summary

The effect of glucose concentration in growth medium on biosynthesis of cytochrome P-450/P-448 under aerobic and anaerobic conditions was examined. High glucose concentration was required in both cases.

The cytochrome P-450 enzyme from yeast *Saccharomyces cerevisiae* showed a Soret peak in the reduced CO-difference spectrum at 448nm, was purified to homogenity and had a molecular weight of 55,500. Cytochromes c(P-450) reductase, b$_5$, b$_5$ reductase were also purified from the same microsomal preparation.

Amino acid analysis of yeast cytochrome P-448 revealed the presence of 407 residues per molecule. The sum of molecular weight of the polypeptides and other components was in good agreement with the molecular weight value obtained from SDS-polyacrylamide gel electrophoresis.

A reconstituted system of purified yeast cytochrome P-448, NADPH:cytochrome c(P-450) reductase and phospholipid showed benzo(a)pyrene hydroxylase activity. The spin state of substrate-free and benzo(a)pyrene-bound purified yeast
cytochrome P-448 were 94\% and 82\% low spin at 22°C respectively.

Equilibrium gel filtration analysis of the number of benzo(a)pyrene binding sites per mole of enzyme monomer showed a value of 1 for purified yeast cytochrome P-448 and 6 for this enzyme in microsomal form.

In addition to benzo(a)pyrene, lanosterol, ethylmorphine, dimethylnitrosamine and sodium phenobarbitone showed Type I binding spectra with yeast cytochrome P-448.

A more specific and efficient form of benzo(a)pyrene hydroxylase was induced by the addition of benzo(a)pyrene to the yeast growth medium at zero time.

Based on the effect of modification of various amino acids on parameters of yeast cytochrome P-448, participation of -SH group(s) and tyrosyl residue(s) in the active site of this enzyme is suggested.
Various supports were evaluated for immobilization of yeast cytochrome P-448. Calcium alginate was found to be especially useful.

Cytochrome P-448 from *Saccharomyces cerevisiae* is identified as a distinct enzyme of the cytochrome P-450 family. This enzyme, however, has many properties in common with cytochrome P-448 from mammalian sources.
Acknowledgements

I would like to express my thanks to my supervisor, Dr. Alan Wiseman, for his teaching and encouragement throughout the course of this work, and to Dr. Gordon Gibson for sharing with me his vast experience in various aspects of cytochrome P-450.

I would like to thank my fellow post-graduate students: David King for his discussion, Paul Tamburini for his help in spin state studies, and Caroline Phillipson for performing the Ames test.

I would also like to express my appreciation to the parents of my wife, Mr. and Mrs. Edwin Dyer of California, U.S.A., and my brother, Mr. Hadi R. Azari of Oregon, U.S.A. for their support and encouragement during this effort.

INTRODUCTORY NOTE

WORK ON BIOSYNTHESIS AND INDUCTION WAS CARRIED OUT WITH D.J. KING ON AN EQUALLY SHARED BASIS

David King
Dedication

To My Parents and My Wife, Sarah
The Creed of a Chemist

The chymists are a strange class of mortals impelled by an almost insane impulse to seek their pleasure among smoke and vapour, soot and flame, poisons and poverty, yet among all these evils I seem to live so sweetly, that may I die if I would change places with the Persian King.

-- Johann Joachim Becker
"Acta Laboratorii Chymica Monacensis, seu Physica Subterranea" (1669)
(vii)

CONTENTS
# Contents

1. Introduction ........................................ 2  
   1.1. Cytochrome P-450: Historical Aspects ............ 2  
   1.2. Distribution of Cytochrome P-450 ................. 3  
   1.3. Structure of Cytochrome P-450 .................... 6  
   1.4. Spectral Changes of Cytochrome P-450 ............ 9  
   1.5. Mechanism of Cytochrome P-450 Linked Oxidation 17  
   1.6. The Induction of Mammalian Cytochrome P-450 and the Presence of Multiple Forms 23  
   1.7. Cytochrome P-450 from Bacteria Species .......... 34  
   1.8. Cytochrome P-450 from Yeast .................... 40  
   1.9. Metabolism of Benzo(a)pyrene by Cytochrome P-450-Dependent Mixed-Function Oxidase System 50  

2. Studies on the Biosynthesis of Cytochrome P-450 in *Saccharomyces cerevisiae* .......... 56  
   2.1. Introduction .................................. 56  
   2.2. Methods and Materials ......................... 58  
   2.2.1. Growth of Yeast ............................ 58  
   2.2.2. Measurement of Cytochrome P-450 in Yeast Cells 59  
   2.2.3. Measurement of Mitochondrial Cytochromes .......... 61  
   2.2.4. Measurement of Cyclic AMP in Yeast Cells .... 63  
   2.2.5. Materials .................................. 64
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.</td>
<td>Results and Discussion</td>
<td>64</td>
</tr>
<tr>
<td>2.3.1.</td>
<td>Growth Conditions for Cytochrome P-450 Biosynthesis in <em>Saccharomyces cerevisiae</em></td>
<td>64</td>
</tr>
<tr>
<td>2.3.2.</td>
<td>Genetic Analysis of Cytochrome P-450 Production in <em>Saccharomyces cerevisiae</em></td>
<td>75</td>
</tr>
<tr>
<td>3.</td>
<td>Solubilization, and Purification of Cytochrome P-450/P-448 and Other Mixed-Function Oxidase Proteins from <em>Saccharomyces cerevisiae</em></td>
<td>92</td>
</tr>
<tr>
<td>3.1.</td>
<td>Introduction</td>
<td>92</td>
</tr>
<tr>
<td>3.2.</td>
<td>Methods and Materials</td>
<td>93</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Growth of Yeast</td>
<td>93</td>
</tr>
<tr>
<td>3.2.2.</td>
<td>The Preparation of Yeast Microsomes</td>
<td>94</td>
</tr>
<tr>
<td>3.2.3.</td>
<td>The Measurement of Cytochrome P-450/P-448 and Cytochrome P-420</td>
<td>92</td>
</tr>
<tr>
<td>3.2.4.</td>
<td>Solubilization of Yeast Mixed-Function Oxidase Proteins</td>
<td>96</td>
</tr>
<tr>
<td>3.2.5.</td>
<td>Purification of Yeast Mixed-Function Oxidase Proteins</td>
<td>98</td>
</tr>
<tr>
<td>3.2.6.</td>
<td>Further Purification of Cytochrome c(P-450) Reductase, Cytochrome b₅ and Cytochrome b₅ Reductase</td>
<td>102</td>
</tr>
<tr>
<td>3.2.7.</td>
<td>Purification Cytochrome P-450/P-448 from Rat Liver</td>
<td>103</td>
</tr>
<tr>
<td>3.2.8.</td>
<td>Protein Determination</td>
<td>104</td>
</tr>
<tr>
<td>3.2.9.</td>
<td>Measurements of NADPH: Cytochrome c(P-450) Reductase Activity</td>
<td>105</td>
</tr>
<tr>
<td>3.2.10.</td>
<td>Measurement of Cytochrome b₅</td>
<td>106</td>
</tr>
</tbody>
</table>
3.2.11. Measurement of NADH: Cytochrome b5 Reductase Activity ........................................ 107
3.2.12. SDS-Polyacrylamide Gel Electrophoresis of Yeast Cytochrome P-448 .................. 108
3.2.13. Detection of Proteins on Polyacrylamide Gel .................................................. 111
3.2.14. Spectrophotometry for Thermostability and Spectral Studies ............................. 111
3.2.15. Removal of Emulgen 911 from Yeast Cytochrome P-448 .................................. 112
3.2.16. Lyophilization of Cytochrome P-448 ................................................................. 112
3.2.17. Materials .................................................. 113
  A: Preparation and Regeneration
     8-Amino-n-octyl-Sepharose 4B ................................................................. 113
  B: Other Materials ............................................................................ 115
3.3. Results and Discussion .............................................................................. 116
3.3.1. Storage and Disruption of Yeast ............................................................... 116
3.3.2. Storage of Microsomes and Solubilization of Yeast Cytochrome P-450/P-448 .......... 118
3.3.3. Thermal Stability of Yeast Cytochrome P-448 ............................................. 126
3.3.4. Purification of Yeast Cytochrome P-448 .................................................. 131
3.3.5. Further Purification of Other Mixed-Function Oxidase Proteins of Yeast ............. 139
3.3.6. Spectral Properties of Yeast Cytochrome P-448 ........................................ 144
3.3.7. Molecular Weight of Yeast Cytochrome P-448 ........................................... 146
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Time Dependency of Carbon Monoxide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Difference Spectrum of Reduced</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P-448 from <em>Saccharomyces cerevisiae</em>: Slow Reduction Caused</td>
<td></td>
</tr>
<tr>
<td></td>
<td>by Presence of Triton X-100 and Emulgen 911</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>152</td>
</tr>
<tr>
<td>4.2</td>
<td>Methods and Materials</td>
<td>154</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Preparation Microsomal Fraction from <em>Saccharomyces cerevisiae</em></td>
<td>154</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Measurement of the Reduction Rate of</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>Cytochrome P-448 by Sodium Dithionite</td>
<td></td>
</tr>
<tr>
<td>4.2.3</td>
<td>Determination of Mid-Point Redox Potential</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>of Cytochrome P-448</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>Results and Discussion</td>
<td>157</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Time Dependency of Cytochrome P-448 Assay</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>in the Presence of Triton X-100</td>
<td></td>
</tr>
<tr>
<td>4.3.2</td>
<td>Mid-Point Redox Potential and Spin State Equilibrium</td>
<td>164</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>The Binding of Compounds to Cytochrome P-448</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>from <em>Saccharomyces cerevisiae</em></td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>173</td>
</tr>
<tr>
<td>5.2</td>
<td>Methods and Materials</td>
<td>174</td>
</tr>
<tr>
<td>5.2.1</td>
<td>The Spectral Interactions of Compounds with</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>Yeast Cytochrome P-448</td>
<td></td>
</tr>
<tr>
<td>5.2.2</td>
<td>Equilibrium Gel Filtration of Benzo(a)pyrene</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>Yeast Cytochrome P-448 Complex</td>
<td></td>
</tr>
<tr>
<td>5.2.3</td>
<td>Materials</td>
<td>181</td>
</tr>
<tr>
<td>5.3</td>
<td>Results and Discussion</td>
<td>182</td>
</tr>
</tbody>
</table>
5.3.1. The Spectral Interactions of Benzo(a)pyrene with Yeast Cytochrome P-448 182
5.3.2. The Spectral Interactions of Other Compounds with Yeast Cytochrome P-448 190
5.3.3. The Equilibrium Gel Filtration of Benzo(a)pyrene Yeast Cytochrome P-448 Complex 201

6. The Induction and Characterization of Benzo(a)pyrene-3-Monooxygenase Activity of Cytochrome P-448 from Saccharomyces cerevisiae 210
6.1 Introduction 210
6.2. Methods and Materials 212
6.2.1. Preparation of Microsomal Fraction of Induced Yeast 212
6.2.2. Preparation of Purified Yeast Cytochrome P-448 213
6.2.3. Measurement of NADPH-Supported Benzo(a)pyrene-3-Monooxygenase Activity of Yeast Cytochrome P-448 213
6.2.4. Measurement of Cumene Hydroperoxide-Supported Benzo(a)pyrene-3-Monooxygenase Activity of Yeast Cytochrome P-448 215
6.2.5. Measurement of Hydrogen Peroxide-Supported Benzo(a)pyrene-3-Monooxygenase Activity of Yeast Cytochrome P-448 216
6.2.6. Attempt to Detect the Mutagenic Metabolites of Benzo(a)pyrene Using the Ames Test, after Activation with Yeast Cytochrome P-448 216
6.2.7. Materials 220
6.3. Results and Discussion 221
### 6.3.1. The Induction of Cytochrome P-448 Dependent Benzo(a)pyrene-3-Monooxygenase in *Saccharomyces cerevisiae*  221

### 6.3.2. Reconstitution of NADPH-Supported Benzo(a)pyrene-3-Monooxygenase Activity of Purified Yeast Cytochrome P-448  231

### 6.3.3. Parameters of Yeast Benzo(a)pyrene-3-Monooxygenase Activity  236

### 6.3.4. The Inhibition of Yeast Benzo(a)pyrene-3-Monooxygenase Activity  255

### 6.3.5. The Detection of Mutagenic Metabolites of Benzo(a)pyrene Using the Ames Test after Activation with Yeast Cytochrome P-448  258

### 6.3.6. Attempted Hydroxylation of Other Compounds such as Lanosterol, Aminopyrene, Aniline, Dimethylnitrosamine, Ethylmorphine, Benzphetamine and Ethoxyresorufin with Yeast Cytochrome P-448  261

### 7. Structural Analysis of Cytochrome P-448 from *Saccharomyces cerevisiae*  268

#### 7.1. Introduction  268

#### 7.2. Methods and Materials  269

##### 7.2.1. Amino Acid Analysis of Yeast Cytochrome P-448  269

##### 7.2.2. Heme Analysis of Yeast Cytochrome P-448  273

##### 7.2.3. Carbohydrate Analysis of Yeast Cytochrome P-448  274

##### 7.2.4. Measurement of Phospholipid Content of Yeast Cytochrome P-448  274

#### 7.2.5. Materials  275
### 7.3. Results and Discussion

#### 7.3.1. Structural Analysis of Yeast Cytochrome P-448

### 8. Chemical Modification of Amino Acid Residues in Cytochrome P-448 from *Saccharomyces cerevisiae*: The Effect on its Reduced CO-Difference Spectrum, Benzo(a)pyrene-3-Monooxygenase Activity, Binding Parameters and Temperature-Induced Spin State Equilibrium

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1. Introduction</td>
<td>282</td>
</tr>
<tr>
<td>8.2. Methods and Materials</td>
<td>284</td>
</tr>
<tr>
<td>8.2.1. Preparation of Cytochrome P-448 for Chemical Modification</td>
<td>284</td>
</tr>
<tr>
<td>8.2.2. Preparation of Sulphydryl Reagents</td>
<td>285</td>
</tr>
<tr>
<td>8.2.3. Modification of Sulphydryl Groups in Yeast Cytochrome P-448</td>
<td>285</td>
</tr>
<tr>
<td>8.2.4. Modification of Histidyl Residues of Yeast Cytochrome P-448</td>
<td>286</td>
</tr>
<tr>
<td>8.2.5. Modification of Arginyl Residues of Yeast Cytochrome P-448</td>
<td>287</td>
</tr>
<tr>
<td>8.2.6. Modification of Tryptophyl Residues of Yeast Cytochrome P-448</td>
<td>288</td>
</tr>
<tr>
<td>8.2.7. Modification of Tyrosyl Residues of Yeast Cytochrome P-448</td>
<td>289</td>
</tr>
<tr>
<td>8.2.8. Spin Equilibrium Analysis of Yeast Cytochrome P-448</td>
<td>290</td>
</tr>
<tr>
<td>8.2.9. Determination of Benzo(a)pyrene-3-Monooxygenase Activity and Apparent Binding Parameters of Modified Yeast Cytochrome P-448</td>
<td>292</td>
</tr>
<tr>
<td>8.2.10. Materials</td>
<td>293</td>
</tr>
</tbody>
</table>
8.3. Results and Discussion 293

8.3.1. Titration of Sulfhydryl Group in Yeast Cytochrome P-448 293

8.3.2. Effect of Sulfhydryl Modification on Benzo(a)pyrene-3-Monoxygenase Activity and Binding Parameters of Yeast Cytochrome P-448 297

8.3.3. Effect of Modification of Histidyl, Arginyl, Tryptophyl, Tyrosyl Residues on Benzo(a)pyrene-3-Monoxygenase Activity and Binding Parameters of Yeast Cytochrome P-448 302

8.3.4. Effect of Modification of Cysteinyl, Histidyl, Arginyl, Tryptophyl and Tyrosyl Residues on Spin State Equilibrium of Yeast Cytochrome P-448 308

8.3.5. Tyrosine, A Possible Sixth Ligand of Yeast Cytochrome P-448 310

9. Evaluation of Immobilized Cytochrome P-448 from Saccharomyces cerevisiae Using Permeabilized Whole Cell, Microsomal Fraction and Purified Reconstituted Forms, with Benzo(a)pyrene-3-Monoxygenase Activity 317

9.1. Introduction 317

9.2. Methods and Materials 318

9.2.1. Permeabilization of Yeast Cells 318

9.2.2. Immobilization of Yeast Cytochrome P-448 on Calcium Alginate 319

9.2.3. Immobilization of Yeast Cytochrome P-448 in Polyacrylamide, by Co-Polymerization with Acrylamide Monomer 319

9.2.4. Immobilization of Cytochrome P-448 on BrCN-Activated Sepharose 4B 320
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.2.5. Immobilization of Yeast Cytochrome P-448 by Microcrystalline</td>
<td>321</td>
</tr>
<tr>
<td>Cellulose, Cross-Linking by Use of Glutaraldehyde</td>
<td></td>
</tr>
<tr>
<td>9.2.6. Immobilization of Cytochrome P-448 by Agarose-Concanavalin A,</td>
<td>322</td>
</tr>
<tr>
<td>Cross-Linking by Use of Glutaraldehyde</td>
<td></td>
</tr>
<tr>
<td>9.2.7. Measurement of Benzo(a)pyrene-3-Monooxygenase Activity of</td>
<td>323</td>
</tr>
<tr>
<td>Immobilized Cytochrome P-448</td>
<td></td>
</tr>
<tr>
<td>9.2.8. Materials</td>
<td>324</td>
</tr>
<tr>
<td>9.3. Results and Discussion</td>
<td>324</td>
</tr>
<tr>
<td>9.3.1. Evaluation of Immobilized Yeast Cytochrome P-448 Using</td>
<td>324</td>
</tr>
<tr>
<td>Permeabilized Whole Cell</td>
<td></td>
</tr>
<tr>
<td>9.3.2. Evaluation of Immobilized Yeast Cytochrome P-448 Using Microsomal</td>
<td>329</td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
</tr>
<tr>
<td>9.3.3. Evaluation of Immobilized Yeast Cytochrome P-448 Using Purified</td>
<td>329</td>
</tr>
<tr>
<td>Reconstituted System</td>
<td></td>
</tr>
<tr>
<td>9.3.4. Possible Applications of Immobilized Cytochrome P-450/P-448</td>
<td>332</td>
</tr>
<tr>
<td>10. Final Discussion</td>
<td>335</td>
</tr>
<tr>
<td>References</td>
<td>354</td>
</tr>
<tr>
<td>Appendices</td>
<td>381</td>
</tr>
<tr>
<td>Publications</td>
<td>385</td>
</tr>
</tbody>
</table>
CHAPTER 1
1. Introduction

1.1. Cytochrome P-450: Historical Aspects

Cytochrome P-450 is the collective name for a distinct group of protoheme-containing proteins which show a Soret absorption band at around 450nm (446 to 454nm) in the CO-difference spectrum of dithionite-reduced samples. The occurrence in liver microsomes of this CO-binding pigment was first reported independently by Klingenberg (1958) and by Garfinkel (1958). This led to the characterization of this unique hemoprotein by Omura and Sato (1962, 1964a, 1964b). The first evidence for the participation of cytochrome P-450 in monooxygenase reactions was reported by Estabrook et al. (1963). Using Warburg's arrangement for the photochemical action spectrum, these authors demonstrated that light of 450nm was optimal for the reversal of carbon monoxide inhibition of steroid C-21 hydroxylation. By the late 1960s it was realized that cytochrome P-450 was of major importance in the metabolism of many foreign chemicals including drugs, carcinogens, insecticides and in the metabolism of endogenous compounds including steroids and fatty acids.

Much of the early knowledge about the properties of cytochrome P-450 came from studies on purified preparations
of the NADH-dependent soluble cytochrome P-450\textsubscript{cam} isolated from \textit{Pseudomonas putida} (Katagiri \textit{et al}, 1968; Peterson, 1971). Initial attempts to solubilize mammalian cytochrome P-450 using different types of detergent resulted in conversion to inactive cytochrome P-420 (Omura and Sato, 1964a). This was overcome by glycerol (Ichikawa and Yamano, 1967). Solubilization of the membrane bound enzyme led to the resolution of three components: cytochrome P-450, NADPH:cytochrome c(P-450) reductase and phospholipid, which when combined would catalyze the hydroxylation of lauric acid (Lu and Coon, 1968).

1.2. Distribution of Cytochrome P-450

Cytochrome P-450 was described in mammalian liver microsomes and was originally thought to be a curiosity occurring only in restricted biological systems. However, subsequent studies have revealed that hemoproteins with similar spectral properties are distributed very widely in nature; they can be detected in almost all forms of life.

In mammals, cytochrome P-450 is found at varying concentrations in microsomes (endoplasmic reticulum) of liver (Klingenberg, 1958; Garfinkel, 1958; Omura and Sato, 1964a, 1964b), kidney (Elin \textit{et al}, 1972), small intestine (Takesue and Sato, 1968), lung (Matusubara and Tochino,
1971), adrenal cortex (Estabrook et al., 1963), skin (Poland et al., 1974), testis (Betz et al., 1976), placenta (Meigs and Ryan, 1968), and several other tissues. However, microsomes from other organs such as brain, muscle and thyroid gland appear to be devoid of this type of hemoprotein. Cytochrome P-450 has also been detected in microsomes of various tissues, notably the liver, of non-mammalian vertebrates, i.e. birds, reptiles, amphibians and fish (Garfinkel, 1963; Strittmatter and Umberger, 1969).

Mitochondria of mammalian endocrine glands such as the adrenal cortex (Harding et al., 1964), testis (Puris et al., 1973) and corpus luteum (Yohro and Horie, 1967) which synthesize and excrete steroid hormones, invariably contain cytochrome P-450. Although, the mitochondrial localization of this group of hemoproteins is not restricted to steroid hormone-producing glands. The occurrence of cytochrome P-450 in mitochondria of chicken kidney (Henry and Norman, 1974; Ghazarian et al., 1974) and rat liver (Taniguchi et al., 1973) has been confirmed, although the concentrations in such mitochondria are rather low.

Besides microsomes and mitochondria, the nuclear envelope of rat liver has been reported to contain cytochrome P-450 (Khaudwala and Kasper, 1973). This does
not appear to be due to microsomal contamination, since the cytochrome, unlike its microsomal counterpart, cannot be induced by phenobarbital treatment of the animals, although it is normally inducible by 3-methylcholanthrene administration (Khaudwala and Kasper, 1973).

Studies on the distribution of cytochrome P-450 in invertebrates have not been extensive, but in connection with the work on insecticide metabolism it has been shown that microsomal fractions from whole insects such as the housefly and Drosophila contain significant amounts of this type of hemoprotein (Morello et al, 1971; Plapp and Casida, 1970; Capdevila et al, 1975).

In higher plants, cytochrome P-450 has been discovered during attempts to elucidate the pathways involved in specialized metabolisms such as the biosynthesis of phyto-hormones, lignins, and alkaloids. To date, the occurrence of cytochrome P-450 has been reported in such sources as sorghum seedlings (Potts et al, 1974), cauliflower buds (Rich and Bendall, 1975), castor bean endosperm (Young and Beevers, 1976), Catharanthus roseus seedlings (Madyastha et al, 1976), and avocado mesocarp (Markham, 1976). In all these plant sources the hemoproteins were reported to be localized in the microsomal fraction.
The occurrence of cytochrome P-450 in yeast \textit{Saccharomyces cerevisiae} was first reported by Lindenmayer and Smith (1964). This hemoprotein can particularly be found in microsomal fraction of cells grown semi-anaerobically (Yoshida et al, 1974a) or under the condition of glucose repression (Wiseman et al, 1976) (read on). Eukaryotic microorganisms such as \textit{Candida tropicalis} grown on tetradecane as carbon source (Duppel et al, 1973) have, however, been reported to possess cytochrome P-450 that are apparently not membrane-bound and can be recovered in the soluble fraction.

Localization in the soluble cytoplasmic fraction seems to be a general feature of bacterial cytochrome P-450. All cytochromes of this type so far identified in bacteria, e.g. in \textit{Pseudomonas putida} grown on camphor as carbon source (Katagiri et al, 1968).

1.3. \textbf{Structure of Cytochrome P-450}

Cytochromes are classified into three types: a, b, c, according to their absorption maxima. Cytochrome P-450 is classified as a b-type cytochrome, and in common with other b-type cytochromes, contains an iron protoporphyrin IX prosthetic group. Cytochrome P-450 differs from other b-type cytochromes in that it can react with oxygen, cyanide
and carbon monoxide, and also in that it has an intense red-shifted absorption peak at 450nm when the reduced hemoprotein is complexed with carbon monoxide. Treatment of cytochrome P-450 with various agents brings about conversion to cytochrome P-420, which has the spectrum of a typical b-type cytochrome, with a Soret band at 420nm. The nature of these agents, e.g. detergents or lipase, indicates that is the heme of cytochrome P-450 bound in a highly lipophilic environment (Omura and Sato, 1964a; Imai and Sato, 1967a). However, proteases and denaturants such as urea which primarily alter the native conformation of hemoprotein, also cause the conversion of cytochrome P-450 to cytochrome P-420 (Mason et al, 1965a). Thus protein structure and lipid environment appear to be important for maintenance of the usual spectral properties of cytochrome P-450.

The iron atom of cytochrome P-450 heme has four planar ligand interactions with nitrogen atoms of the proto-porphyrin ring system, and has two axial ligand positions available for further binding. Electron spin (paramagnetic) resonance spectroscopy (ES(P)R) has been widely used in conjunction with optical spectroscopy to study cytochrome P-450 and in particular the nature of the fifth and sixth ligands. EPR is used to detect unpaired electrons and is particularly useful for the study of transition metal
complexes such as hemoproteins. Cytochrome P-450 has been shown to exist in both high spin (5 unpaired electrons) and low spin states (Jefcoate and Gaylor, 1969a; Hill et al., 1970) (read on).

Following the observation of a loss of low spin character after treatment of hepatic microsomes with p-chloromercuribenzoate, Mason et al (1965a) suggested that the sulfur atom was liganded to the heme of cytochrome P-450. Subsequent studies using model systems with thiol agents and hemoglobin or metamyoglobin showed similar low spin EPR spectra (Jefcoate and Gaylor, 1969a; Hill et al., 1970; Blumberg and Peisach, 1971; Peisach et al., 1973; Collman et al., 1975; Tang et al., 1976) to those obtained with cytochrome P-450 from mammalian (Jefcoate and Gaylor, 1969a; Peisach et al., 1973; Stern et al., 1973) and bacterial (Tsai et al., 1970) sources. Stern and Peisach (1974) demonstrated the formation of an absorption maximum at 450nm following the addition of carbon monoxide to a model system of reduced heme, thiol and a strong base. From this study they concluded that a thiolate anion is required to produce the characteristic spectrum of cytochrome P-450.

Although cytochrome P-450 has been shown to have a heterogeneous ligands field in the vicinity of heme
(Peisach et al, 1973), the nature of the sixth ligand is still undecided. Various proposals have been made which include the imidazole nitrogen of a histidine residue (Jefcoate and Gaylor, 1969a; Tang et al, 1976; Chevion et al, 1977), a hydroxyl group (Nebert et al, 1976; Kumaki and Nebert, 1978) and water (Peterson and Griffin, 1973; Griffin and Peterson, 1975) (read Chapter 8).

1.4 Spectral Changes of Cytochrome P-450

The first report of a cytochrome P-450 dependent spectral change was made by Narasimhulu et al (1965), using 17-hydroxyprogesterone in microsomes from adrenal cortex. Similar spectra were later described by Remmer et al (1966), Imai and Sato (1966) and Schenkman et al (1967) following the addition of various compounds to microsomal suspensions from liver.

These spectral changes were divided into three categories by Schenkman et al (1967): type I, type II and reverse type I (type RI). Type I spectral changes are characterized by an absorption maximum at 385-390nm and a minimum at about 420nm; type II are characterized by an absorption maximum at 420-435nm and a minimum at 390-405nm. The type RI spectrum is essentially a mirror image of a
type I spectral change and is typified by a minimum at 385-390nm and a maximum at about 420nm.

Schenkman et al (1967) suggested that these spectral changes were due to interaction between oxidized cytochrome P-450 and the added compound. These authors devised a method of measuring an apparent affinity constant for a given compound analogous to the Michaelis constant ($K_m$) for enzyme substrate complexes. A double reciprocal plot (Lineweaver and Burk, 1934) of spectral change versus substrate concentration produced an intercept on the $X'$ axis which the authors termed the spectral dissociation constant or $K_s$. This constant is defined as the concentration of reactant that results in 50% of the maximal spectral change, and is by definition independent of protein concentration. The magnitude of the spectral change is dependent not only on the substrate concentration, but also on the concentration of cytochrome P-450 in the microsomal suspension (Estabrook et al, 1972). The maximal absorbance change ($\Delta A_{\text{max}}$) is analogous to the maximal velocity ($V_{\text{max}}$) of enzyme reactions.

The type I spectral change is elicited by a wide range of endogenous and exogenous substrates of cytochrome P-450. These include steroids, fatty acids, barbituates and
polycyclic aromatic hydrocarbons. In general, compounds which elicit the type I spectral change are lipophilic. Schenkman and Sato (1968) suggested that type I compounds interact with apoprotein protein of cytochrome P-450 rather than with heme. The role of phospholipids in the type I interaction is uncertain. Treatment of microsomes with phospholipase C (Chaplin and Mannering, 1970; Eling and DiAugustine, 1971) or extraction with isooctane (Leibman and Estabrook, 1971) which removed about 70% of the phospholipids present, decreased or eliminated type I binding spectra. In contrast, Vore et al (1974) reported that extraction of microsomes with butan-1-ol and acetone removed about 80% of the phospholipids present yet increased the binding of the same type I substrates. Various other authors (Narasimhulu, 1975; Levin et al, 1974) have reported that lipid is not essential for type I binding. A correlation between $K_s$ values and lipid solubility for some type I ligands has been demonstrated by Kitigawa et al (1972) and Al-Gailany (1975).

Type II spectral changes are brought about by interaction of amines with cytochrome P-450 (Schenkman et al, 1967) Jefcoate et al, 1969; Schenkman, 1970). Other compounds containing nitrogen atoms with a lone pair of electrons such as pyridine and imidazole also produce the type II (Temple, 1971). This type of spectral change is
thought to be caused by ferrihemochrome formation, involving electron transfer between the nitrogen atom of the added compound and the sixth ligand position of the heme iron (Schenkman et al., 1967), and in which the nitrogen atom is primarily an sp$^2$ or sp$^3$ hybrid, with a spatially accessible lone electron pair (Kulkarni et al., 1974; Mailman et al., 1974). Aniline has been reported to compete with carbon monoxide for reduced cytochrome P-450 indicating interaction at the heme iron (Schenkman et al., 1967). In addition, aniline produces two Soret peaks at 425 and 455 (Imai and Sato, 1967b) similar to those produced by ethyl isocyanide which has been shown to compete with carbon monoxide (Omura and Sato, 1964a).

Few compounds which give rise to type II spectrum are metabolized by cytochrome P-450. However, aniline, a compound which acts as a substrate for cytochrome P-450 and produces a type II spectrum, has been shown to have a type I component (Schenkman, 1970). This type I component is thought to be related to aniline metabolism.

The type RI (inverse type I or modified type II) has often been likened to the type II interaction (Whysner et al., 1970). However, whereas type II compounds can displace carbon monoxide, type RI compounds cannot, nor do
they alter aniline binding to the oxidized hemoprotein (Schenkman et al., 1973). A diverse range of compounds produce the type RI spectral change including phenacetin, acetanilide, short monohydric alcohols, warfarin and several allyl-containing barbiturates. It has been suggested that type RI spectrum is a reversal of the type I spectral change caused by displacement of bound endogenous substrates (Diehl et al., 1970; Schenkman et al., 1969, 1972, 1973). Diehl et al. (1970) showed that butanol, when added to microsomes containing sufficient cyclohexane to saturate the type I binding site, produced a difference spectrum which was the exact mirror image of the cyclohexane (type I) binding spectrum. Schenkman et al. (1973) found that on solvent extraction of microsomes, the type RI spectrum induced by phenacetin was reduced. Powis et al. (1977) found that the addition of bovine serum albumin to microsomal suspensions resulted in a type RI binding spectrum, which the authors concluded was due to displacement of endogenous substrates from cytochrome P-450.

In contrast to the results of Schenkman et al. (1973), Vore et al. (1974) found increased type RI binding for phenacetin and ethanol using butanol-acetone extracted microsomes. Al-Gailany (1975) proposed type RI binding is due to binding at a specific site of cytochrome P-450 rather
than simply displacing endogenous substrates. This hypothesis was based on data using fluorescent probes, which suggested that type I, type II and type RI sites were in differing environments with respect to their lipophilicity.

It has been suggested that since many compounds which give a type RI interaction contain oxygen, the oxygen atoms act much like nitrogen atoms as nucleophiles and displace the sixth ligand of cytochrome P-450 (Mailman et al., 1974). Yoshida and Kumaoka (1975b) also concluded from binding spectral studies that type RI binding is due to a heme ligand interaction between high spin cytochrome P-450 and the type RI compound. Kumaki et al (1970) reached a similar conclusion from binding spectra and EPR studies.

The causes of the spectral changes observed on binding are now interpreted in terms of spin state changes. The active cytochrome P-450 molecule contains a heme group with iron in the ferric form. This means that the iron atom has five electrons in the d-orbitals and depending on the extent of spin-pairing of these, can exist in two spin states, high spin (total spin of \( S = 5/2 \)) and low spin (total spin of \( S = 1/2 \)). The low spin form results when four of five d-electrons are paired and correspond to a six coordinated heme iron. The high spin form results when the five
electrons are in separate energy levels and not paired, and corresponds to a five coordinated heme iron. In intact microsomal membranes, a mixture of the two spin states occurs, with approx. equal amounts of each, the ratio of the two forms being in a temperature-dependent equilibrium (Cinti et al, 1979). Temperature-dependency of spin state of cytochrome P-450 is further discussed in Chapter 8.

The interaction of a type I compound with cytochrome P-450 is associated with conversion of the hemoprotein iron from low to high spin (Jefcoate and Gaylor, 1970; Tsai et al, 1970; Kumaki et al, 1978). This increase in high spin character is thought to be due to displacement of the sixth ligand of cytochrome P-450 heme, which in turn decreases the ligand field strength of the heme, and results in a hypsochromic shift. The formation of type II spectrum is associated with the production of a low spin iron species which produces a red-shifted absorbance peak. This is thought to be caused by an increase in ligand field strength. The exact nature of type RI spectrum has yet to be fully understood, although it seems likely that the mechanism of interaction between cytochrome P-450 and type RI compounds may involve one or more of the mechanisms mentioned earlier, depending on the nature of the compound and the cytochrome P-450 species present. Multiple
interactions between compounds and various sites of cytochrome P-450 are possible (Yoshida and Kumaoka, 1975b). It seems that type RI interaction involves the conversion of a high spin cytochrome to a low spin cytochrome species, and the sixth ligand position of the iron becomes occupied by a weak ligand. Whether this weak ligand is the oxygen atom of the type RI compound or the intrinsic sixth ligand of the protein is a matter for speculation.

It has been shown that the spin state of cytochrome P-450 controls the redox potential of the molecule, with the high spin form having a less negative redox potential (Sligar, 1976). Type I binding (generated by compounds that are usually substrates) thus not only results in a transition from low to high spin, but also a change in the redox potential, making it less negative and hence allowing electrons to flow to the cytochrome P-450 molecule more easily (Sligar et al, 1979). Thus the substrate induced spin state change results in an acceleration of reduction rate of the cytochrome (Misselwitz et al, 1980). This reveals a mechanism whereby the substrate facilitates the electron flow to cytochrome P-450, hence enabling the reaction to proceed. The role of lipids in the membrane may also be important in holding the enzyme in a high spin
configuration and facilitating the monooxygenase reaction (Gibson et al., 1980).

1.5. Mechanism of Cytochrome P-450 Linked Oxidation

A wide variety of oxidative reactions mediated by cytochrome P-450 occurs in microsomes and all reactions (aromatic hydroxylation, aliphatic hydroxylation, N-dealkylation, o-dealkylation, deamination, sulphoxidation and N-oxidation) can be considered essentially as hydroxylations. The source of the hydroxyl group for microsomal hydroxylation has been shown to be molecular oxygen:

\[
RH + \frac{1}{2}O_2 \rightarrow ROH + \frac{1}{2}O_2
\]

Such a hydroxylation reaction in which one atom of each oxygen molecule is transferred to the substrate is a "monooxygenase" as opposed to the "dioxygenase" reaction in which both atoms of the oxygen molecule are inserted into the substrate.

Microsomal cytochrome P-450 linked monooxygenases require an external source of electrons such as NADPH or NADH for the insertion of oxygen into the substrate and the reaction is thought of as a mechanism whereby NADPH reduces a
component (A) in microsomes, which reacts with molecular oxygen to form an "active oxygen" intermediate which is then transferred to the substrate. The overall reaction has been envisaged by Gillete et al (1968) as follows:

\[ (1) \text{NADPH} + A + H^+ \rightarrow \text{AH}_2 + \text{NADP}^+ \]

\[ (2) \text{AH}_2 + O_2 \rightarrow \text{"active oxygen"} \]

\[ (3) \text{"Active oxygen"} + RCH_3 \rightarrow RCH_2OH + A + H_2O \]

(overall reaction) \[ \text{NADPH} + H^+ + RCH_3 + O_2 \rightarrow RCH_2OH + \text{NADP}^+ + H_2O \]

Since this complex enzymatic reaction involves the oxidation of NADPH to NADP\(^+\), the reaction has been termed a "mixed-function oxidase." Cytochrome P-450 linked enzymes are therefore frequently referred to as either monooxygenases or mixed-function oxidases (E.C.1.14.14.1). The term terminal oxidases is also used to denote these enzymes. The key microsomal enzymes in the overall reaction are NADPH:cytochrome c(P-450) reductase (a flavoprotein containing one FAD and one FMN group) involved in NADPH oxidation and the reduction of oxidized cytochrome P-450, and cytochrome P-450, which is generally considered to be component A. These components have been purified and reconstituted into an active monooxygenase system by many workers. This was first achieved by Lu and Coon (1968) who
reconstituted a system capable of oxidizing fatty acids, steroids, drugs, and other xenobiotics from components isolated from rabbit liver microsomes. These authors reported that phospholipid is also required for a fully functional system, where it may have a structural role in associating the protein components together, although the actual role of phospholipid is unknown.

The mechanism of the cytochrome P-450 monooxygenase reaction is not fully understood, but a major sequence of events is known and is shown in Figure 1.1 (Schenkman and Gibson, 1981). The mechanism involves firstly binding of substrate to the ferric enzyme, followed by one electron transfer from NADPH via NADPH:cytochrome c(P-450) reductase. The ferrous cytochrome P-450-substrate complex reacts with oxygen producing an oxygenated reduced cytochrome P-450-substrate complex. The ocytochrome P-450 complex then undergoes a further one electron reduction. The nature of the active oxygen species is currently the subject of extensive research, but is thought to involve a number of radical species (White and Coon, 1980; Castro, 1980). After transfer within the complex of one oxygen atom and uptake of two protons, the complex dissociates to give oxidized cytochrome P-450, water and hydroxylated substrate.
Figure 1.1. The Cytochrome P-450 Reaction Cycle. $e_1^-$ is transferred from NADPH via NADPH:cytochrome c(P-450) reductase without/with cytochrome b$_5$ as a mediator or from NADH via NADH:cytochrome b$_5$ reductase and cytochrome b$_5$. 

$b_5$ = cytochrome $b_5$

$F_{P_a}$ = NADPH:cytochrome c(P-450) reductase

$F_{P_b}$ = NADH:cytochrome b$_5$ reductase

SH = substrate
Several studies have indicated that the second reduction can be mediated by NADH:cytochrome b$_5$ reductase (containing FAD as a prosthetic group) and cytochrome b$_5$ (Hildebrandt and Estabrook, 1971; Estabrook, 1971; Cohen and Estabrook, 1971; Sasame et al., 1973), providing an explanation for the synergistic effect of NADH on mono-oxygenation. Studies by Lu et al. (1974) indicated that involvement of cytochrome b$_5$ in the NADPH-dependent reaction varies with both the substrate and the species of cytochrome P-450 present. Imai and Sato (1977) have shown that, although cytochrome b$_5$ is not obligatory for this reduction step, if NADH, cytochrome b$_5$ and NADH:cytochrome b$_5$ reductase are included in a reconstituted system of cytochrome P-450, NADPH:cytochrome c(P-450) reductase, sodium cholate and NADPH with benzphetamine or dimethylaniline as substrate, the second electron is supplied almost exclusively by NADH via cytochrome b$_5$.

As it was mentioned above, the nature of the active oxygen intermediate involved in the substrate hydroxylation reaction remains uncertain. Hamilton (1964) suggested that monooxygenases catalyze their reactions by means of an oxygen atom transfer or oxenoid mechanism, because of their similarity to nitrene and carbene reactions. Support for this mechanism has been provided by the observation of "NIH
"shift" (Guroff et al., 1967) or the displacement of an existing ring substituent to an adjacent position by the incoming hydroxyl group. Hrycay and O'Brien (1971a, 1971b) provided evidence that cytochrome P-450 could function as a peroxidase with organic hydroperoxides. Following the report by Kadlubar et al. (1973) the organic peroxides could support the oxidative N-dealkylation of a variety of amine substrates in rat liver microsomes. Rahimtula and O'Brien (1974, 1975) showed that cumene hydroperoxide and other hydroperoxides could sustain the hydroxylation of a variety of substrates in rabbit liver microsomes in the absence of NADPH and molecular oxygen. Other "active oxygen" containing compounds such as sodium periodate and sodium chlorite have been shown to support the hydroxylation of various steroids and fatty acids in the absence of NADPH and molecular oxygen (Hrycay et al., 1975a, 1975b, 1976). During the course of reaction supported by cumene hydroperoxide, is consumed for every mol of substrate hydroxylated, and the major reaction product is phenyl-2-propanol (cumenol) (Rahimtula and O'Brien, 1974). The "NIH shift" also occurs during hydroperoxide-dependent aromatic hydroxylation (Rahimtula et al., 1978). The appearance of a free radical signal as determined by EPR and distinctive optical spectral changes led to the proposal that a ferryl ion complex (in resonance with a ferric oxene complex
Fe$^{4+}$O$_2$($\rightarrow$Fe$^{3+}O$) was the active oxygen species involved in cytochrome P-450 mediated reactions (Rahimtula et al., 1974).

1.6. **The Induction of Mammalian Cytochrome P-450 and the Presence of Multiple Forms**

The activity of the liver microsomal monooxygenase system is increased by the treatment of the animal with a wide variety of drugs, pesticides, food additives, carcinogens and other compounds. This increased activity results from an increase in the concentration of the cytochrome P-450 enzyme involved, by de novo protein synthesis. This was shown to be by de novo synthesis as it can be blocked by inhibitors of nucleic acid synthesis such as actinomycin D (Conney, 1967).

Two of the most widely studied inducers of the mammalian liver cytochrome P-450 system are phenobarbital and 3-methylcholanthrene. After induction with these compounds, different forms of the enzyme are observed in each case, with different substrate specificities. Phenobarbital induces a form of the enzyme with a very wide substrate specificity, the rate of metabolism of many substrates (e.g. aminopyrine, benzphetamine and ethylmorphine) being increased. However, these activities are
unchanged or decreased by 3-methylcholanthrene pretreatment, which induces an enzyme with a relatively narrow substrate specificity with high activity towards carcinogens such as benzo(a)pyrene. The metabolism of some other compounds (e.g. acetanilide) is increased by both inducers. These observations led to the classification of inducers into two broad categories. The first group of these, exemplified by phenobarbital, induced cytochrome P-450 with a very wide substrate specificity with a peak in the reduced carbon monoxide difference spectrum at 450nm (cytochrome P-450). The second group, including 3-methylcholanthrene and other polycyclic hydrocarbons, induced an enzyme with relatively narrow specificity, particularly aryl hydrocarbon hydroxylase activity. This narrow specificity enzyme had a Soret peak in the induced carbon monoxide difference spectrum at 448nm, and so was termed cytochrome P-448. Initially, it was thought that cytochrome P-450 and cytochrome P-448 might be interconvertible forms of the same enzyme (Schenkman et al., 1969), but it was quickly established that these two enzymes arose from distinct gene products (Lu et al., 1973; Conney et al., 1973; Levin et al., 1974).

Several hundred inducers of cytochrome P-450 enzymes are now known and some examples are listed in Table 1.1. As further work on the induction of microsomal enzymes was...
<table>
<thead>
<tr>
<th>Inducer</th>
<th>Description</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>Sedative</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>3-methylcholanthrene</td>
<td>Carcinogen</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>Carcinogen</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>β-naphthoflavone</td>
<td>Hydrocarbon analog</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>Polychlorinated biphenyls (aroclor)</td>
<td>Insulators, lubricants, heat exchange fluids</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td>Safrole</td>
<td>Carcinogen (formerly a flavouring agent)</td>
<td><img src="image6" alt="Structure" /></td>
</tr>
<tr>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)</td>
<td>Herbicide impurity</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
<tr>
<td>Pregnenolone 16α-carbonitrile</td>
<td>Steroidal derivative</td>
<td><img src="image8" alt="Structure" /></td>
</tr>
</tbody>
</table>

Table 1.1. Mammalian Cytochrome P-450 Inducing Agents
carried out, it became apparent that more newly discovered inducers were neither phenobarbital-like nor 3-methylcholanthrene-like. For example, ellipticine induced its own cytochrome P-450 mediated metabolism better than phenobarbital or 3-methylcholanthrene (Lesca et al., 1977). Similar observations have now been made with more than 60 inducers (including safrole and pregnenolone 16α-carbonitrile, see Table 1.1).

These observations are now understood in terms of multiple forms of cytochrome P-450. It has become well-established that more than two forms of the enzyme exist with different yet overlapping substrate specificities. The evidence for this has come from several types of study, including studies with inducers, isolation and purification of different forms, immunological differences and kinetic and substrate binding studies (Guengerich, 1979). Also, sequencing studies have recently revealed distinct amino acid differences between cytochrome P-450 isozymes (Coon et al., 1982; Mizukami et al., 1983). The discovery of multiple forms of cytochrome P-450 has led to considerable confusion in the literature. Both cytochrome P-450 and cytochrome P-448 are now known to exist as a mixture of isoenzymes, and are thus general terms for a range of enzyme species.
The effect of inducers is now thought to be in inducing a range of cytochrome P-450 enzymes, the combined properties of which make up the characteristic properties of the 'induced enzyme.' Some of these enzyme species may be present in uninduced animals. Ryan et al (1979) purified three forms of cytochrome P-450 from rat liver microsomes, designated cytochrome P-450a, b and c. These three enzymes were distinguished by differences in molecular weight, immunological properties and peptide mapping. In addition, partial amino acid sequences revealed that these enzymes were distinct gene products and not due to post-translational modifications of one primary gene product (Bothelo et al, 1979). Cytochrome P-450a and c were found after induction with 3-methylcholangthrene. Cytochrome P-450b is the major form induced by phenobarbital and cytochrome P-450c, the major form induced by 3-methylcholangthrene. After induction with aroclor 1254 (a mixture of polychlorinated biphenyls, known to induce both cytochrome P-450 and cytochrome P-448 activities), cytochrome P-450a, b and c were all present. Cytochrome P-450a has a Soret peak in the reduced carbon monoxide difference spectrum at 452nm, cytochrome P-450b at 450nm and cytochrome P-450c at 447nm. These enzymes were observed to have different yet overlapping substrate specificities. Aniline was hydroxylated equally well by all three enzymes.
Cytochrome P-450s hydroxylated testosterone at the 7α position but had low activity towards benzphetamine, benzo(a)pyrene and 7-ethoxycoumarin. Cytochrome P-450b hydroxylated testosterone at the 16α position and was very active towards benzphetamine demethylation. Cytochrome P-450c hydroxylated testosterone preferentially at the 6β position, had low activity towards benzphetamine demethylation but had very high activity for benzo(a)pyrene metabolism and 7-ethoxycoumarin O-dealkylation (Ryan et al, 1979).

Colbert et al (1979) showed that cytochrome P-450b, induced by phenobarbital, was produced as a result of an increased level of mRNA specific for this protein, as early as 6h after administration. Similarly, Bresnick et al (1981) showed an increased level of cytochrome P-450c mRNA 7h after treatment with 3-methylcholanthrene, showing that these enzymes are coded by distinct mRNA species which are increased on induction.

The mechanism of induction by phenobarbital has been studied by several groups. The administration of phenobarbital has been shown to result in an increased level of mRNA specific for inducible cytochrome P-450 forms (Colbert et al, 1979; Kuman et al, 1980). Adesnick et al (1981)
found the mRNA level for phenobarbital inducible cytochrome P-450 increased 30 fold on induction. Fujii-Kuriyama et al (1982) have sequenced the coding nucleotides of copy DNA to mRNA of phenobarbital inducible cytochrome P-450 from rat liver, and have used this to predict the amino acid sequence of the cytochrome. The predicted molecular weight and amino acid composition agreed with those of the purified enzyme. These workers sequenced three copy DNA's to phenobarbital induced mRNA and one of these, although similar, was not identical to the other two. Several amino acid substitutions occur in a limited portion of the sequence, perhaps indicating a 'variable region.'

Vlasuk et al (1982) have shown that four immunochemically identical forms of phenobarbital induced rat liver cytochrome P-450 exist in unique combinations, which characterize different strains of rats and may partially explain the well-known strain differences in cytochrome P-450 enzymes (Hodgson, 1979). Walz et al (1982) have characterized the mRNA from two of these different cytochrome P-450 isozymes and found that they are different species. When translated in vitro, they give rise to distinct isozymes corresponding exactly to the enzyme characterizing the group of rats from which they were isolated.
The group of Coon and co-workers have worked extensively on the multiple forms of cytochrome P-450 in rabbit liver microsomes (Haugen and Coon, 1976; Coon et al., 1978, 1980; Koop et al., 1981, 1982). These have labeled their cytochrome P-450 enzymes with numbers, LM1-7 (LM = liver microsomes) based on their relative electrophoretic mobilities, with the lower number given to those forms with highest mobility towards the anode. Cytochrome P-450LM2 is the major form isolated from phenobarbital treated animals and has a peak in the reduced carbon monoxide difference spectrum at 451nm and a molecular weight of 48,000 (Haugen and Coon, 1976). Cytochrome P-450LM4 is the major form induced by 3-methylcholanthrene, benzo(a)pyrene, β-naphthoflavone and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and has also been detected in small amounts in untreated animals (Coon et al., 1978, 1980). The enzyme from all of these sources is indistinguishable by several criteria, has a molecular weight of 54,000 and a Soret peak in the reduced carbon monoxide spectrum at 448nm.

The major cytochrome P-450 proteins present in uninduced rabbit liver are LM3b and LM3c, a small amount of LM3a and LM4 also being detectable (Koop et al., 1981). LM3c is also found alongside LM2 in phenobarbital induced rabbits
Cytochrome P-450LM3b and LM3c have molecular weights of 52,000 and 53,000 and Soret peaks at 450nm and 449nm respectively. Other workers have also described LM3 enzymes in uninduced rabbit liver (Ingelman-Sundberg et al, 1980; Johnson, 1980). Cytochrome P-450LM3a has recently been shown to be the major form present after induction with ethanol (Koop et al, 1982). This enzyme has a molecular weight of 51,000, a Soret peak at 452nm and is very active in oxidation of ethanol and the p-hydroxylation of aniline.

Cytochrome P-450LM6 has been found as a minor component after induction with TCDD in adult rabbits, but is the major component after TCDD induction of neonatal rabbits (Norman et al, 1978). This enzyme has a molecular weight of 57,000 and a Soret peak in the induced carbon monoxide spectrum at 448nm, and is also the major form after induction with isosafrole (Koop et al, 1982). Cytochrome P-450LM1 and LM7 have also been observed, but as yet have not been purified and are poorly characterized. The characteristics of the major rabbit liver cytochrome P-450 enzymes are summarized in Table 1.2. These enzymes have also been shown to have different but overlapping substrate specificities.
Table 1.2. Major Rabbit Liver Cytochrome P-450 Enzymes

<table>
<thead>
<tr>
<th>Cytochrome P-450</th>
<th>Molecular Weight</th>
<th>Soret peak (carbon monoxide bound)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM 2</td>
<td>48,000</td>
<td>451</td>
<td>Haugen &amp; Coon (1976)</td>
</tr>
<tr>
<td>LM 3a</td>
<td>51,000</td>
<td>452</td>
<td>Koop et al (1982)</td>
</tr>
<tr>
<td>LM 3b</td>
<td>52,000</td>
<td>450</td>
<td>Koop et al (1981)</td>
</tr>
<tr>
<td>LM 3c</td>
<td>53,000</td>
<td>449</td>
<td>Koop et al (1981)</td>
</tr>
<tr>
<td>LM 6</td>
<td>57,000</td>
<td>448</td>
<td>Norman et al (1978)</td>
</tr>
</tbody>
</table>
Although extensive work has already gone into characterization of forms of cytochrome P-450, the exact number of isozymes is not known. Current estimates range from 12-20 enzymes present in rabbit liver microsomes (Koop et al., 1982) and a similar number has been postulated for rat liver microsomes and other microsomal systems (Guengerich, 1982). Some workers believe that hundreds or even thousands of cytochrome P-450 molecules may exist, although not all at one time, produced in a manner analogous to antibody synthesis (Nebert et al., 1981). At any one time, an organism may be exposed to 10-20 important inducers, so that 10 or 20 forms of induced cytochrome P-450 would exist in quantities sufficient to be detected. At a later time, the organism might be exposed to a different range of inducers and thus a different profile of cytochrome P-450 enzymes may be produced (Nebert et al., 1981). For this system it is necessary to postulate induction of cytochrome P-450 enzymes in control organisms, presumably by endogenous substrates such as fatty acids and steroids. Nebert and co-workers have also worked extensively on the mechanism of induction of cytochrome P-450 by polycyclic hydrocarbons. This occurs via the 'Ah locus'; the inducer binds to a cytosolic receptor which is translocated to the nucleus where cytochrome P-450 structural gene is switched on. This model is discussed more fully in Chapter 2.
1.7. **Cytochrome P-450 from Bacteria Species**

Cytochrome P-450 has been found in several species of bacteria. These are listed in Table 1.3 along with the reported role of the enzyme in each case. By far the best characterized of these enzymes is from *Pseudomonas putida*. This organism produces a cytochrome P-450 enzyme when grown on D(+)camphor which metabolizes camphor, hence enabling growth of the organism on this compound as sole carbon and energy source (Yu et al, 1974). This enzyme (termed cytochrome P-450$_{\text{cam}}$) hydroxylates the 5-methylene carbon of camphor to form the exo-5-alcohol (Gunsalus et al, 1975). Studies on cytochrome P-450$_{\text{cam}}$ have been greatly facilitated as, unlike mammalian cytochrome P-450 enzymes, it is a soluble enzyme and not membrane bound. This enzyme has been purified to homogeneity and crystallized; it has a molecular weight of 44-46,000 and has a Soret peak in the reduced carbon monoxide difference spectrum at 446nm (O'Keefe et al, 1978).

The electron transport chain of cytochrome P-450$_{\text{cam}}$ supported by NADPH, contains an extra protein component, putidaredoxin, a small iron-sulphur protein. Also the flavoprotein reducase contains only one flavin group, an FAD (Dus, 1975). In these respects, this system differs from microsomal cytochrome P-450 but is very similar to mammalian
<table>
<thead>
<tr>
<th>Organism</th>
<th>Role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>camphor hydroxylation</td>
<td>Gunsalus <em>et al</em> 1975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>O'Keefe <em>et al</em> 1978</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>steriod</td>
<td>Berg &amp; Rafter 1981</td>
</tr>
<tr>
<td>ATCC 13368</td>
<td>15 β-hydroxylation</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>hydroxylation of fatty acids, alcohols and</td>
<td>Matson <em>et al</em> 1977</td>
</tr>
<tr>
<td>ATCC 14581</td>
<td>amides</td>
<td></td>
</tr>
<tr>
<td><em>Corynebacterium</em> sp.</td>
<td>hydroxylation of alkanes</td>
<td>Cardini &amp; Jurtchuk 1970</td>
</tr>
<tr>
<td>7E1C</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nocardia</em> NHI</td>
<td>p-O-dealkylation</td>
<td>Broadbent &amp; Cartwright</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1974</td>
</tr>
<tr>
<td><em>Rhizobium japonicum</em></td>
<td>Role in N₂ fixation?</td>
<td>Appleby</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1978</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>p-nitroanisole</td>
<td>Edelson &amp; McMullen 1977</td>
</tr>
<tr>
<td><em>Photobacterium</em></td>
<td>aliphatic hydroxylation</td>
<td>Ismailova <em>et al</em> 1981</td>
</tr>
<tr>
<td><em>fischeri</em></td>
<td>(in bacterial luminescence)</td>
<td>Danilov <em>et al</em> 1982</td>
</tr>
</tbody>
</table>

Table 1.3. Bacterial Cytochrome P-450 Systems
mitochondrial cytochrome P-450 systems, which also contain an extra iron-sulphur protein and reductase (a soluble enzyme) with one flavin group (Suhara et al, 1978). Dus et al (1980) have compared the immunochemical properties of cytochrome \( P-450_{\text{cam}} \) with cytochrome \( P-450_{\text{scc}} \), the mitochondrial cytochrome P-450 from adrenal cortex of mammals responsible for side chain cleavage of cholesterol. Antibodies to one of these proteins could inhibit the activity of the other, and the two antibodies showed a high degree (approx. 75%) of cross-reactivity, indicating one or more antigenic determinants in common. In these experiments, cytochrome LM2 from phenobarbital induced rabbit liver microsomes also showed 60% cross-reactivity with antibody to cytochrome \( P-450_{\text{cam}} \). Recently, the primary structure of cytochrome \( P-450_{\text{cam}} \) has been completely elucidated (Haniu et al, 1982). The enzyme consists of a chain of 412 amino acid residues, and detailed comparisons with other cytochrome P-450 enzymes will now be possible once their sequence becomes available.

Two cytochrome P-450 species with different specificities have been reported in different strains of \( \text{Bacillus megaterium} \). The strain ATCC 14581 produces a cytochrome P-450 with \( \omega-2 \) hydroxylase activity towards fatty acids (with some \( \omega-1 \) and \( \omega-3 \) activity) which can also
hydroxylate corresponding amides and alcohols (Matson et al., 1977). This enzyme can also catalyze the epoxidation of unsaturated fatty acids (Ruettinger and Fulco, 1981). Substrates of this enzyme were unable to induce the enzyme to higher levels, but when phenobarbital was tested, a 28 fold induction was observed (Narhi and Fulco, 1982).

**Bacillus megaterium** ATCC 13368 also contains a cytochrome P-450 monooxygenase system, which has very different properties to that of ATCC 14581 described above. This enzyme is mainly a 15 $\beta$-hydroxylase (with some 6 $\beta$-hydroxylase activity) for 3-oxo-$\Delta^4$-steroids such as progesterone (Berg et al., 1977). This cytochrome P-450 (termed cytochrome P-450$_{meg}$) is also a soluble enzyme and has been purified to homogeneity and found to have a molecular weight of 52,000 and an amino acid composition similar to that of cytochrome P-450$_{cam}$. The reduced carbon monoxide difference spectrum reveals a peak at 450nm. This system also contains a three-protein component electron transport chain similar to that in *P. putida*, as do all the bacterial cytochrome P-450 monooxygenase systems investigated so far. One difference in the cytochrome P-450$_{meg}$ system is that the reductase contains FMN and not FAD as the prosthetic group (Gustafsson et al., 1980). Studies on the small iron sulphur protein (ferridoxin) component have
revealed that its molecular weight and amino acid composition closely resemble those of putidaredoxin and adrenodoxin, the corresponding proteins in P. putida and mammalian adrenal cortex mitochondria (Berg, 1982). It has been found that in common with other bacterial systems (and mammalian mitochondrial systems), the substrate specificity of this enzyme is very narrow, with only 3-oxo-Δ^4-steroids acting as substrate (Berg and Rafer, 1981). In addition, these workers found that no induction of cytochrome P-450 could be detected with either substrates or classical inducers of mammalian cytochrome P-450.

Appleby (1978) purified three cytochrome P-450 enzymes from Rhizobium japonicum grown symbiotically on soybean root nodules. The three enzymes designated cytochrome P-450a, b and c, had Soret peaks in the reduced carbon monoxide difference spectrum at 449nm, 449nm and 447nm for cytochrome P-450a, b and c respectively. The role of these R. japonicum enzymes is unknown, but they may have a role in the removal of oxygen in this strictly anaerobic system, or in electron transport at very low oxygen tension to generate ATP required for nitrogenase activity (Bergersen and Turner, 1975).
Edelson and McMullen (1977) examined *Escherichia coli* for cytochrome P-450 activity. This organism contained an enzyme, inhibited by carbon monoxide, which could dealkylate p-nitroamisole. This activity could be induced by phenobarbital, as is the case for the fatty acid hydroxylase from *B. megaterium* described above.

Cytochrome P-450 has also been reported to be involved in the bacterial luminescence of *Photobacterium fischeri* (Ismailova et al, 1981). The luciferase complex of this organism is thought to consist of four protein components in a multienzyme complex, one of which is cytochrome P-450. The role of cytochrome P-450 in this process is thought to be in hydroxylation of aliphatic aldehydes, substrates of luminescence reaction (Ismailova et al, 1981). Danilov et al (1982) have shown that the cytochrome P-450 from *Photobacterium fischeri* gives rise to a type I binding spectrum with camphor, and that this compound can inhibit the luminescence system by competitive inhibition of cytochrome P-450.

In addition, the presence of cytochrome P-450 has been reported in *Corynebacterium* species, grown on n-octane which can hydroxylate n-octane (Cardini and Jurtchuk, 1970) and *Norcardia* species, grown on iso-vanillate, which can
O-dealkylate p-alkylphenyl ethers (Broadbent and Cartwright, 1974).

In all of the bacterial cytochrome P-450 systems characterized so far, the cytochrome P-450 is soluble and operates via an electron transport chain of three protein components.

1.8. Cytochrome P-450 from Yeast

A cytochrome P-450 has been demonstrated in several yeast species when grown on n-alkanes as sole carbon source. These organisms use cytochrome P-450 as an alkane hydroxylase catalyzing the first step in the degradation of alkanes for growth, in a fashion similar to the bacterial system, in Corynebacterium mentioned in section 1.7. The first report of an alkane grown yeast containing cytochrome P-450 was for Candida tropicalis grown on n-tetradecane as sole carbon source (Gallo et al., 1971). This enzyme catalyzes the ω-hydroxylation of alkanes and of lauric acid, and is also capable of N-demethylation of aminopyrine, hexobarbital, benzphetamine and ethylmorphine (Lebeault et al., 1971). This enzyme is dependent on oxygen and NADPH, is inhibited by carbon monoxide, is bound to microsomal membrane and requires two protein components (cytochrome P-450 and its reductase) and lipid for full activity.
Surprisingly, both the reductase and the lipid components could be replaced by the corresponding fractions from rat liver microsomes (Duppel et al., 1973). Hexadecane has also been shown to be a potent inducer of this system, as have all n-alkanes tested with more than 10 carbon atoms, and also long chain alkanes and alcohols (Gilewicz et al., 1979). The components of the electron transport chain from C. tropicalis have been purified and reconstituted into an active hydroxylase system (Bertrand et al., 1979a, 1979b). Mansuy et al. (1980) have shown that cytochrome P-450 from C. tropicalis is in a spin state equilibrium similar to that of mammalian microsomal cytochrome P-450 and that this enzyme can also undergo spectral changes on binding.

A similar enzyme has been described in Candida guilliermondii grown on n-alkanes which hydroxylates long chain alkanes (preferably hexadecane to octadecane) to their primary alcohols (Muller et al., 1974). This microsomal enzyme also produces binding spectra with several compounds including a type I spectrum with the substrate hexadecane (Muller et al., 1979). It was also shown that C. guilliermondii could not produce cytochrome P-450 during growth on glucose, nor when glucose was added together with the hydrocarbon inducer. Mauersberger et al. (1980) have
shown that the level of alkane-induced cytochrome P-450 in
*C. guilliermondii* is higher when the oxygen level is low. These workers suggested that this effect might be due to oxygen limitation causing a decreased alkane hydroxylation rate, to which the yeast responds by increasing biosynthesis of the monooxygenase.

Mauersberger and Matyashova (1980) reported the occurrence of cytochrome P-450 also in *Candida lipolytica* grown on n-alkanes and Il'chenko *et al* (1980) demonstrated a similar system in *Torulopsis candida*. *Lodderomces elongisporus* is also capable of producing a cytochrome P-450 when grown on n-alkanes, particularly tetradecane (Mauersberger *et al*, 1981). This enzyme and its reductase have recently been purified and reconstituted into an active hydroxylating system which requires phospholipid (Riege *et al*, 1981; Muller *et al*, 1982). *Saccharomycopsis lipolytica* also produces a cytochrome P-450 alkane hydroxylase when grown on n-alkanes, which is very active towards lauric acid hydroxylation (Marchal *et al*, 1982).

The occurrence of cytochrome P-450 in *Saccharomyces* yeasts is now well established. Lindenmayer and Smith (1964) were the first to demonstrate the occurrence of this hemoprotein in *Saccharomyces cerevisiae* grown anaerobically
in a medium containing 4% glucose and to a lesser extent when grown aerobically in the same medium. Cytochrome P-450 has now been demonstrated in several species of *Saccharomyces* and related species of yeast (Table 1.4).

Ishidate *et al* (1969a) showed that cytochrome P-450 was located in the microsomal fraction of *S. cerevisiae*, and that its spectral properties were similar to those of mammalian microsomal cytochrome P-450. These findings were later verified by Yoshida *et al* (1974a). Ishidate *et al* (1969b) also found that cytochrome P-450 was present in yeast grown semi-anaerobically but was lost on exposure to aerobic conditions. This loss paralleled the formation of active mitochondria within the yeast cells and could be prevented by high concentrations of glucose or chloramphenicol, both of which also prevented the development of mitochondrial respiration. Rogers and Stewart (1973) showed that no cytochrome P-450 could be detected in yeast grown under strictly anaerobic conditions, but found a maximum level of cytochrome P-450 was reached at a low oxygen concentration (0.25μM) when in 4% glucose media, which decreased at higher oxygen concentrations. Subsequent work has established that cytochrome P-450 production in *S. cerevisiae* is associated with the exponential growth phase under conditions of repression of mitochondrial...
## Table 1.4. Saccharomyces and Related Species of Yeast which Contain Cytochrome P-450 during Growth on Glucose Media.

<table>
<thead>
<tr>
<th>Organism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Lindenmayer &amp; Smith, 1964;</td>
</tr>
<tr>
<td></td>
<td>Wiseman et al, 1975</td>
</tr>
<tr>
<td>Saccharomyces uvarum</td>
<td>Cartledge et al, 1972;</td>
</tr>
<tr>
<td>formerly S. carlsbergensis</td>
<td>Sauer et al, 1982</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>Poole et al, 1974</td>
</tr>
<tr>
<td>Saccharomyces bayanus</td>
<td>Karenlampi et al, 1980</td>
</tr>
<tr>
<td>Saccharomyces chevalieri</td>
<td>&quot;</td>
</tr>
<tr>
<td>Schizosaccharomyces japonicus</td>
<td>&quot;</td>
</tr>
<tr>
<td>Pichia fermentans</td>
<td>&quot;</td>
</tr>
<tr>
<td>Debaryomyces hansenii</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hansenula anomala</td>
<td>&quot;</td>
</tr>
<tr>
<td>Kluuyveromyces fragilis</td>
<td>&quot;</td>
</tr>
<tr>
<td>(formerly S. fragilis)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Brettanomyces anomalous</td>
<td>&quot;</td>
</tr>
<tr>
<td>Torulopsis dattila</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

---
cytochromes, and the enzyme is rapidly lost during stationary phase, or when formation of active mitochondria can occur (Woods, 1979; Wiseman, 1980). Thus cytochrome P-450 is produced in *S. cerevisiae* under conditions which lead to repression of mitochondrial cytochromes, such as semi-anaerobic conditions or at a high glucose concentration in aerobic conditions.

Wiseman *et al* (1978) have shown that de novo synthesis of cytochrome P-450 in *S. cerevisiae* is controlled by the intracellular concentration of cyclic AMP by repression. The level of cyclic AMP in the yeast cell is determined by the glucose concentration in the medium in an inverse relationship (Sy and Richter, 1972). Therefore under conditions of glucose repression of mitochondrial cytochromes, the cyclic AMP concentration is low and the repressive effect of cyclic AMP is removed, allowing de novo synthesis of cytochrome P-450 to occur (Wiseman *et al*, 1978). As expected, cytochrome a+a₃ and cyclic AMP show a direct relationship to each other and an inverse relationship to cytochrome P-450 levels at various glucose concentrations (Qureshi *et al*, 1980). This inverse relationship between cytochrome a+a₃ and cytochrome P-450 has also been noted in *S. cerevisiae* grown semi-anaerobically (Schunk *et al*, 1978).
The production of cytochrome P-450 during growth of *S. cerevisiae* on a range of sugars has been studied by Karenlampi *et al.* (1981). These workers found that high concentrations of cytochrome P-450 were produced during growth of yeast on high concentrations of a strongly fermentable sugar such as glucose, fructose and sucrose. Cytochrome P-450 was also produced to a lesser extent during growth on galactose or maltose where fermentation and respiration occurred concomitantly. However, when a non-fermentable carbon source was used for growth (such as glycerol, lactate or ethanol), no cytochrome P-450 was produced. These workers thus linked cytochrome P-450 synthesis of conditions of rapid growth and fermentation, though not necessary to the repression of mitochondrial cytochromes. As heme is synthesized during both fermentative and respirative conditions (Lebbe-Bois and Volland, 1977), it is probable that apoprotein synthesis is regulated in these different conditions.

The presence of a complete electron transport chain in *S. cerevisiae* analogous to that in liver microsomes (containing only two protein components: a reductase with two flavin groups, one FAD, one FMN and a cytochrome P-450) has been demonstrated by Yoshida *et al.* (1977). However, these workers in addition to purification of yeast
cytochrome P-450 (Yoshida et al, 1977) and NADPH:cytochrome 
c(P-450) reductase (Kubota et al, 1977; Aoyama et al, 1978),
have isolated cytochrome b₅ (Yoshida et al, 1974b) and 
NADH:cytochrome b₅ reductase (Kubota et al, 1977) from this 
species. The purified cytochrome P-450 had a molecular 
weight of 51,000 and a reduced carbon monoxide at 447-448nm 
(Yoshida et al, 1977). These workers also suggested that 
most of yeast cytochrome P-450 is in the high spin state in 
vivo and may therefore be substrate bound (Yoshida and 
Kumaoka, 1975b). However, the function of cytochrome P-450 
in S. cerevisiae is still uncertain.

S. cerevisiae cytochrome P-450 is thought to be 
involved in the demethylation of lanosterol to zymosterol, 
which is an intermediate in the conversion of lanosterol to 
ergosterol, the major sterol in yeast membranes. This 
process involves three demethylation steps and was inhibited 
57% by carbon monoxide (Alexander et al, 1974). It was also 
shown that partially purified low spin yeast cytochrome 
P-450 could be partially converted to high spin by adding 
lanosterol (Yoshida and Kumaoka, 1975b). Aoyama and Yoshida 
(1978a) showed that lanosterol stimulated the oxidation of 
NADPH by purified yeast cytochrome P-450 and reductase and 
demonstrated a type I binding spectrum for lanosterol with 
purified yeast enzyme, indicative of a substrate
relationship. It was subsequently shown that cytochrome P-450 is involved in the 14α-demethylation of lanosterol, the other two demethylation steps proceeding via a cyanide-sensitive monooxygenase (Ohba et al; Aoyama et al, 1981b). This 14α-demethylation step is inhibited by antibodies to yeast cytochrome P-450, and a reconstituted system of purified cytochrome P-450 and NADPH:cytochrome c(P-450) reductase could 14α-demethylate lanosterol in the presence of NADPH and oxygen (Aoyama and Yoshida, 1978b). The postulation of this endogenous role for cytochrome P-450 posed the question of how ergosterol was synthesized under conditions of aerobic growth at low glucose concentrations when no cytochrome P-450 could be detected. Recently, however, Aoyama et al (1981a) have shown that a small amount of cytochrome P-450, not detectable by usual methods, may be present under these conditions and be responsible for ergosterol biosynthesis.

A second role of \textit{S. cerevisiae} cytochrome P-450 in ergosterol biosynthesis has recently been reported by Hata et al (1981). These workers claimed that yeast cytochrome P-450 may also be involved in $\Delta^{22}$-desaturation of ergosta-5,7-dien-3β-ol to form ergosterol, a step later in the same pathway. This role was postulated on the basis of studies
with the cytochrome P-450 inhibitors carbon monoxide and metyrapone.

The metabolism of benzo(a)pyrene (read on) by the cytochrome P-450 monooxygenase system from *S. cerevisiae* is now well established (Wiseman and Woods, 1979; Woods and Wiseman, 1980). Benzo(a)pyrene is hydroxylated by this enzyme to a range of products, predominantly 3-hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (Woods and Wiseman, 1979). The involvement of yeast cytochrome P-450 in this activity has been demonstrated by thermal stability experiments and inhibition of benzo(a)pyrene hydroxylase by carbon monoxide (Woods, 1979). Studies using tritium NMR on substrate–heme interactions in a partially pure enzyme preparation also supported the involvement of cytochrome P-450 in benzo(a)pyrene hydroxylation (Libor et al., 1980). The metabolites of benzo(a)pyrene produced by the yeast enzyme are more similar to those produced by a mammalian cytochrome P-448 than by a mammalian cytochrome P-450 (Gozukara et al., 1981).

Cytochrome P-450 from *S. cerevisiae* has also been claimed to activate certain promutagens to active mutagenic compounds, as shown by an increased yeast mutation rate
(Callen and Philpot, 1977). These compounds include dimethylnitrosamine, aflatoxin B, β-naphthylamine, ethyl carbamate and cyclophosphamide. A later report by these workers presented spectral evidence for the involvement of cytochrome P-450 in these activations (Callen et al, 1980).

1.9. Metabolism of Benzo(a)pyrene by Cytochrome P-450-Dependent Mixed-Function Oxidase System

A number of studies have demonstrated the carcinogenic properties of cyclic aromatic hydrocarbons in different species, including man, and in different tissues, including the lung and skin (Saffioti et al, 1972; Shubik, 1972; Henry et al, 1973; Little and O'Toole, 1974). Benzo(a)pyrene 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 and Pollack, 1972).

The major pathways for the metabolism of benzo(a)pyrene involve aryl hydrocarbon monooxygenase (hydroxylase) system, epoxide hydrase and glutathione S-epoxide transferase (Oeseh, 1973).

It has been believed for sometime that the aryl hydrocarbon hydroxylase system and microsomal mixed-function
oxidase system are the same (Conney, 1967). Both these systems are localized 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 hydrocarbon hydroxylase and cytochrome P-450/P-448 in various tissues (Alvares et al, 1967; Gram et al, 1967).

There is a large body of evidence that aryl hydrocarbon hydroxylase (mixed-function oxidase), the first of the enzymes mentioned above, introduces an oxygen into its substrates to produce epoxides (arene oxides) (Oeseh et al, 1973; Heidelberger, 1975). Tissues in which the aryl hydrocarbon hydroxylase system has been demonstrated to metabolize 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, adrenal gland and placenta (Gelbion et al, 1975; Sims, 1975).

The oxidative metabolism of benzo(a)pyrene (Figure 1.2) catalyzed by the mixed-function oxidase proceeds initially
Figure 1.2. The Metabolism of Benzo(a)pyrene by Microsomal Mixed-Function Oxidase System
through the formation of reactive epoxides, as was mentioned above. Oxidation may occur at the 4,5-position, the K-region position, the 7,8-position, or at the 'bay region' 9,10-position (Jerina and Dally, 1974; Yang et al, 1978). These epoxides are reactive species which undergo further metabolism. They may be deactivated by the cytosolic glutathione S-epoxide transferase to yield glutathione conjugates which are excreted in the bile, or further metabolized to mercapturates which are excreted in the urine. The epoxides can also undergo non-enzymatic intramolecular rearrangement into phenols (NIH shift mechanism) which are conjugated with glucuronic acid, or sulfate, and excreted. Both of these routes lead to the deactivation of the epoxides. Phenols and quinones may also form by mixed-function oxidase system by direct hydroxylation.

All three epoxides are also substrates of the microsomal enzyme, epoxide hydrase, which catalyzes the hydration of the epoxides to corresponding trans-dihydrodiols. The 9,10- and 7,8-diols, unlike the K-region 4,5-diol, still contain olefinic bonds which undergo mixed-function oxidation to give rise to the diol-epoxides, the ultimate carcinogens, of benzo(a)pyrene and of several other polycyclic aromatic hydrocarbons.
The bay region 9,10-epoxide is more readily formed from the benzo(a)pyrene-7,8-diol than is the 7,8-epoxide from 9,10-diol (Thakker et al, 1978). This product, the benzo(a)pyrene-7,8-diol-9,10 epoxide is considered to be the major ultimate carcinogen of benzo(a)pyrene (Levin et al, 1976; Yang et al, 1976).

The principal RNA and DNA adducts isolated from tissues of animals treated with benzo(a)pyrene arise through the interaction of the anti isomer of the 7,8-diol-9,10-epoxide (diol epoxides of polycyclic hydrocarbons could exist as two geometrical isomers, denoted syn and anti) with the interacellular macromolecules (Sims et al, 1978; King et al, 1976). The formation of the diol-epoxides comprises only a minute fraction of total metabolism of the benzo(a)pyrene, and administration of this compound to animals leads primarily to its detoxication to phenols and dihydrodiols which are subsequently conjugated and excreted (Herbst et al, 1971).
CHAPTER 2
2. Studies on the Biosynthesis of Cytochrome P-450 in Saccharomyces cerevisiae

2.1 Introduction

Since the presence of cytochrome P-450 was first shown in the yeast Saccharomyces cerevisiae, several studies have been made to investigate the conditions required for its biosynthesis. The initial report of the presence of cytochrome P-450 in a baker's yeast strain of Saccharomyces cerevisiae found that this enzyme was present under anaerobic growth conditions and to a lesser extent when grown aerobically at a high glucose concentration (4%, w/v) (Lindenmayer and Smith, 1964). The production of yeast cytochrome P-450 under semi-anaerobic growth conditions was confirmed by Ishidate et al. (1969a) with very little cytochrome P-450 (approx. 10% of the semi-anaerobic value) produced under fully aerobic conditions. During aerobic growth at low glucose concentration (1%, w/v) no cytochrome P-450 was observed. These workers also found that cytochrome P-450 in semi-anaerobically grown yeast was rapidly lost on exposure to aerobic conditions (Ishidate et al., 1969b). This loss paralleled the formation of active mitochondria within the yeast cells and could be prevented by high concentrations of glucose or chloramphenicol, both of which also prevented the development of mitochondrial
respiration. Essentially similar findings were made by Rogers and Stewart (1973), who found a maximum cytochrome P-450 level in yeast grown in a medium containing 4% (w/v) glucose at the low oxygen concentration of 0.25μ M. The cytochrome P-450 level declined at both lower and higher oxygen concentrations.

Since these early studies, work on cytochrome P-450 from Saccharomyces cerevisiae has been done largely on the enzyme from yeast grown under two sets of conditions. Yoshida et al (1972, 1975a, 1975b, 1977) have worked on cytochrome P-450 produced from a baker's yeast strain grown semi-anaerobically in a medium containing 6% (w/v) glucose, whereas Wiseman et al (1975, 1976, 1978) have worked on the enzyme produced in brewer's yeast grown aerobically in a medium containing 20% (w/v) glucose. Both of these sets of conditions achieve repression of mitochondrial cytochromes and production of a high level of cytochrome P-450.

It has become well established that cytochrome P-450 production in yeast is rapid during the exponential phase of growth but ceases at the beginning of the stationary phase (Woods, 1979; Karenlampi et al, 1981). It has also been shown that Tween 80 is capable of increasing the level of cytochrome P-450 in yeast grown for 48h or more, presumably
due to the prevention of degradation rather than to increased biosynthesis after this time (Wiseman et al, 1976).

In this study biosynthesis of cytochrome P-450 growing in a range of different glucose concentrations under aerobic conditions are examined and the results are compared with those grown under a semi-anaerobic atmosphere (as reported by King, 1983; Blatiak et al, 1983 who used the same strain yeast in experiments carried out in the same laboratory at the University of Surrey). Also, a study of genetic regulation of cytochrome P-450 production in *S. cerevisiae* is presented. Although cytochrome P-448 is the dominant cytochrome P-450-type enzyme in yeast (Chapter 3), for simplicity when it is studied in yeast cells in this chapter, it is referred to by its generic name cytochrome P-450.

2.2. Methods and Materials

2.2.1. Growth of Yeast

Strains of *Saccharomyces cerevisiae* were maintained on slopes of Sabouraud-Dextrose agar which were subcultured at regular intervals. The strain *S. cerevisiae* NCYC No. 240 was used unless otherwise described. Liquid cultures were inoculated directly from slopes with a wire loop. The growth medium consisted of yeast extract (1%, w/v),
mycological peptone (2%, w/v), sodium chloride (0.5%, w/v) and glucose at various concentrations. The cultures were grown at 30°C in a shaking water bath at constant speed. Normally, yeast was grown in 250ml flasks, each containing 100ml of medium. The yeast growth medium was previously autoclaved at 15 p.s.i. for 15min with glucose being autoclaved separately to prevent the formation of breakdown products which interfere with the cytochrome P-450 assay (Lim, 1976).

2.2.2. Measurement of Cytochrome P-450 in Yeast Cells

Cytochrome P-450 levels in yeast cells were measured by a modification of the difference spectrum method of Omura et al (1965). Yeast was suspended to a concentration of 0.1g wet weight/ml in 0.1M potassium phosphate buffer pH 7.2, and the sample divided into two cuvettes. Sodium dithionite was added to reduce each cuvette and a baseline was drawn in the range 390-500nm using either a SP 1800 (Pye Unicam, U.K.) or Cary 219 (Varian, U.S.A.) spectrophotometer. The sample cuvette was removed and carbon monoxide bubbled through it for 30s. The difference spectrum was then recorded again between 390-500nm. A typical yeast cytochrome P-450 assay is shown in Figure 2.1. The concentration of cytochrome P-450 was calculated from the difference between 450 and 490nm with reference to the
Figure 2.1. A Typical Yeast Reduced Carbon Monoxide Difference Spectrum. A yeast suspension of 0.1g/ml (wet weight) was used.
baseline, assuming the same extinction coefficient as for mammalian cytochrome P-450 of 91mM$^{-1}$cm$^{-1}$.

2.2.3. Measurement of Mitochondrial Cytochromes

Mitochondrial cytochromes were assayed by a modification of the method of Williams (1964). Yeast was suspended at a concentration of 0.1g wet weight/ml in 0.1M potassium phosphate buffer pH 7.2, and divided into two cuvettes. The sample cuvette was reduced with sodium dithionite and the reference cuvette oxidized by the addition of 50µl of hydrogen peroxide (6%, w/v). The spectrum was then recorded between 500-640nm using a SP 1800 Pye Unicam spectrophotometer. A typical spectrum obtained is shown in Figure 2.2. The absorbance between four wavelength pairs was measured and used to calculate the concentrations of the individual cytochromes, cytochrome c, c$_1$, and b and a+a$_3$, using four simultaneous equations which allow for the spectral overlap of the cytochromes.

The four wavelength pairs were:

- $a_{15} = 550-535\text{nm}$
- $a_{25} = 554-540\text{nm}$
- $a_{35} = 563-577\text{nm}$
- $a_{45} = 605-630\text{nm}$
Figure 2.2. A Typical Yeast Reduced-Oxidized Difference Spectrum, showing Mitochondrial Cytochromes. A yeast suspension of 0.1g/ml (wet weight) was used.
The cytochromes $c(x_1), c_1(x_2), b(x_3)$ and $a+a_2(x_4)$ were then calculated from the simultaneous equations below.

\[
\begin{align*}
21.0x_1 + 10.3x_2 - 3.12x_3 + 0.63x_4 &= a_{15} \\
6.51x_1 + 18.8x_2 + 2.55x_3 + 0.95x_4 &= a_{25} \\
-1.16x_1 + 0.91x_2 + 14.3x_3 - 0.3264 &= a_{35} \\
-0.22x_1 - 0.59x_2 + 0x_3 + 12.0x_4 &= a_{45}
\end{align*}
\]

The calculations were considerably speeded up by the use of a computer program (Appendix I).

2.2.4. **Measurement of Cyclic AMP in Yeast Cells**

Considerable difficulty was found in obtaining a reproducible sample for cyclic AMP measurement. After extensive tests, the following method was found to give the most reproducible results. The yeast sample (200mg wet weight) was suspended in 2ml of 10% (w/v) trichloroacetic acid and sonicated for 1min to help the release of cyclic AMP. The mixture was shaken for five min and the yeast spun down on a bench centrifuge (10min, full speed). The supernatant was retained and the yeast was re-extracted with a further 1ml of 10% (w/v) trichloroacetic acid. The supernatants were combined, 1M hydrochloric acid (1/10 vol) and 2 volumes of dimethyl ether were added and the solution was shaken in a cold room (8°C) for 10min. The ether phase
was discarded and the solution was re-extracted with ether as before, five times. The aqueous phase was then evaporated to dryness at 55°C under a stream of nitrogen and the residue re-dissolved in 0.05M Tris-HCl buffer pH 7.5 containing 0.004M EDTA. The cyclic AMP content was measured using a competition protein binding assay with tritium labeled cyclic AMP as provided in an assay kit from Amersham International Ltd.

2.2.5. Materials

*Saccharomyces cerevisiae* strains NCYC 240, 239 were obtained from the National Collection of Yeast Cultures, Agricultural Research Council (Norwich, U.K.). Yeast extract was obtained from Oxoid Ltd. Mycological peptone was from London Analytical and Bacteriological Media Ltd. The Cyclic AMP assay kit was from Amersham International Ltd. Glucose, hydrogen peroxide and all other chemicals were from BDH Chemicals Ltd.

2.3. Results and Discussion

2.3.1. Growth Conditions for Cytochrome P-450 Biosynthesis in *Saccharomyces cerevisiae*

Figure 2.3 shows the results of an initial experiment to determine the best *in oculo* method for cytochrome P-450 production in the yeast *Saccharomyces cerevisiae* NCYC 240 in
Figure 2.3. Cytochrome P-450 Production in *S. cerevisiae* Grown in 20% Glucose Media after Inoculation by (a) Slope Method and (b) Liquid Sub-Culture. Values are the mean of 8 determinations. Bars indicate standard deviations.
growth media containing 20% (w/v) glucose as carbon and energy source. The two methods looked at were direct inoculation from a Sabouraud-Dextrose agar slope with a wire loop and inoculation with 1ml of a yeast culture grown for 24 hours in 0.5% (w/v) glucose containing media. Very little difference was observed in the maximal production of cytochrome P-450 (which was approx. 3nmol/g Wet weight of yeast) or the position of the peak in the growth. However, the yeast grown from slope appeared to give a slightly broader peak with more consistent results, and this method was therefore used for all subsequent experiments. It should be noted here that in later experiments, it was found that the yield of cytochrome P-450 produced was variable depending on the age of the slopes; as far as possible, experiments were conducted on freshly grown slopes.

In an attempt to study the interaction of high glucose concentration with aerobic/semi-anaerobic conditions, the level of cytochrome P-450 produced at various times of growth was measured during growth under aerobic conditions at glucose concentrations of 0.1%, 0.5%, 1%, 5% and 20% (w/v), and the results were compared with those obtained by King (1983) in this laboratory, in which the same strain of yeast was grown under semi-anaerobic conditions at the same glucose concentrations. When yeast was grown under
semi-anaerobic conditions by King (1983), the flasks were completely filled with growth medium, inoculated with yeast and bubbled through with nitrogen for 10 min, after which the flasks were sealed and growth was achieved by putting each flask into a 30°C room and stirring gently with a magnetic stirrer. Semi-anaerobic conditions rather than strictly anaerobic conditions were used by King (1983), as this was directly comparable with earlier work (Ishidate et al, 1969a, 1969b; Yoshida et al, 1974a, 1974b). Also a small amount of oxygen is required for several biosynthetic reactions during yeast growth and other workers have reported that cytochrome P-450 is not produced under strictly anaerobic conditions but at a low oxygen concentration (Rogers and Stewart, 1973).

At glucose concentrations of 0.1% and 0.5% (w/v) no cytochrome P-450 was produced under either aerobic or semi-anaerobic conditions. At 1% (w/v) glucose, no cytochrome P-450 was found after growth in aerobic conditions but under semi-anaerobic conditions cytochrome P-450 was produced to a maximal concentration at 2.6nmol/g wet weight of yeast after approx. 62h. The relatively late appearance of the peak is probably due to the slow growth rate which occurs under these conditions, and also to the slowing of degradation of the cytochrome which requires oxygen. The lack of any
cytochrome P-450 produced aerobically in 1% (w/v) glucose media is in disagreement with Wiseman et al (1975, 1976), who have described a fast initial synthesis of cytochrome P-450 under these conditions which was rapidly lost. In the experiments reported here, the first time point is at 24h and it is possible therefore that some cytochrome P-450 is produced early in growth and lost before 24h.

Figure 2.4 shows the production of cytochrome P-450 in yeast growth at 5% (w/v) glucose concentration. Wiseman et al (1976) reported that 5% (w/v) glucose was sufficient to produce 'high-glucose behavior' at which the cytochrome P-450 level was induced maximally after approx. 32h to a level of 3.5nmol/g wet weight of yeast. The results obtained here show a peak level of cytochrome P-450 of only 1.2nmol/g wet weight of yeast after approx. 24h in aerobic conditions. The apparent discrepancy of these results may reflect a higher initial growth rate being achieved here. This would result in the cytochrome P-450 reaching the maximal value, which occurs at the end of the exponential growth, earlier. Under semi-anaerobic conditions at 5% (w/v) glucose, King (1983) reported that the growth is much slower and the cytochrome P-450 peak value is reached later in growth at approx. 48h and reaches a higher level. Also the semi-anaerobically grown yeast loses its cytochrome
Figure 2.4. Time Course of Cytochrome P-450 Production in Yeast Grown Aerobically or Semi-Anaerobically in 5% Glucose Media. Values are the mean of 8 determinations. Bars indicate standard deviations.
P-450 at a slower rate than when under aerobic conditions. This probably reflects the oxygen requirement for cytochrome P-450 degradation in yeast which has been demonstrated by Blatiak et al (1980). These growth conditions (semi-anaerobic, 5% (w/v) glucose medium) are similar to those used by other workers to produce cytochrome P-450 in Saccharomyces cerevisiae. Ishidate et al (1969a, 1969b) used a medium of 4% (w/v) glucose under semi-anaerobic conditions as did Lindenmayer and Smith (1964) and Rogers and Stewart (1973), whereas Yoshida et al (1974a, 1974b) have worked extensively on yeast grown semi-anaerobically at 6% (w/v) glucose.

When yeast was grown aerobically at 20% (w/v) glucose, a peak in cytochrome P-450 level was found after 36-40 hours at approx. 3nmol/g wet weight of yeast (Figure 2.5). In later experiments it was found that with fresher yeast slopes a peak level of approx. 4nmol/g wet weight of yeast could be reached (results not shown). However, the older results are used here to enable a strict comparison with the other results presented. These results agree closely with those of Wiseman et al (1975, 1976). Under semi-anaerobic conditions at 20% glucose, very little difference was observed in the time course of cytochrome P-450 production.
Figure 2.5. Time Course of Cytochrome P-450 Production in Yeast Grown Aerobically or Semi-Anaerobically in 20% Glucose Media. Values are the mean of 8 determinations. Bars indicate standard deviations.
with a slightly higher level of cytochrome P-450 (approx. 3.5nmol/g wet weight) being observed (King, 1983).

As a check that mitochondrial repression was occurring in high glucose media, the levels of mitochondrial cytochromes were measured after 44h aerobic growth in 20% and 0.5% (w/v) glucose media (Table 2.1). In the high glucose medium, a very low concentration of cytochrome a+a₃ was observed compared to the value observed in 0.5% (w/v) glucose medium. This confirms that this yeast was glucose repressed.

These results support the theory of cytochrome P-450 being synthesized in yeast only under conditions of repression of mitochondrial cytochromes. Two ways of achieving this repression are by a high glucose concentration and by anaerobic conditions. From the results observed at 20% (w/v) glucose it seems that these effects are not additive, which is consistent with both sets of conditions eliciting cytochrome P-450 synthesis through a similar mechanism. This mechanism is thought to be regulated by the level of intracellular cyclic AMP (Wiseman et al, 1978). Intracellular cyclic AMP levels are inversely related to the glucose concentration in the growth medium; thus at high glucose concentrations when the cytochrome
Concentration of Glucose in Yeast Growth Medium | Level of Cytochromes (nmol/g wet weight of yeast)  
--- | --- | --- | --- | --- | ---  
 | a+a_3 | b | c | P-450 |  
20% | 7.8 | 14.8 | 2.3 | 9.4 | 2.3  
0.5% | 20.4 | 14.7 | 5.3 | 8.8 | 0  

Table 2.1. Level of Mitochondrial Cytochromes in *S. cerevisiae* Grown Aerobically for 40h.
P-450 level is high, the cyclic AMP concentration is low. This has led to the suggestion that cyclic AMP has a repressive effect on cytochrome P-450 synthesis (Wiseman, et al., 1978). These workers also showed a direct effect of cyclic AMP in repressing cytochrome P-450 synthesis in yeast protoplasts, although 5'-AMP and 2'-(3')-AMP had no effect. This means that under conditions of mitochondrial repression by a high glucose concentration in the growth medium, the cyclic AMP level is low, therefore the repressive effect of cyclic AMP is removed and de novo synthesis of cytochrome P-450 can occur. As expected, cytochrome α+α₃ and cyclic AMP show a direct relationship to each other and an inverse relationship to the cytochrome P-450 level at various glucose concentrations (Qureshi et al., 1980). An inverse relationship between cytochrome α+α₃ and cytochrome P-450 has also been shown in *S. cerevisiae* grown semi-anaerobically (Schung et al., 1978).

It has been explained above that semi-anaerobic conditions are not able to elicit production of cytochrome P-450 at 0.1% or 0.5% (w/v) glucose. It is possible that some cytochrome P-450 is produced at times earlier than 24h, the first time point in these experiments. However, the small amount of growth which can occur under these conditions makes any measurements of cytochrome P-450 before
24h extremely difficult. The conclusion must be drawn that semi-anaerobic conditions alone are not enough to produce cytochrome P-450 without a higher glucose concentration in the medium. This may mean that this enzyme is only produced at the faster growth rates observed at higher glucose concentration. It is also possible that a higher glucose concentration is required in order to repress cyclic AMP levels sufficiently for cytochrome P-450 synthesis to occur.

Figure 2.6 shows the time course of cytochrome P-450 production in *S. cerevisiae* NCYC 239 grown in a growth medium containing 20% (w/v) glucose. This was observed to reach a maximal value of approx. 6nmol/g wet weight of yeast at 38-40h of growth. Although the yield of cytochrome P-450 is high, it does not approach the level of 11nmol/g reported by Qureshi *et al* (1980). Also, the peak occurs at 38-40h, which is similar to NCYC 240, and not at 110h as reported by Qureshi *et al* (1980).

2.3.2. Genetic Analysis of Cytochrome P-450 Production in *Saccharomyces cerevisiae*

In a study looking at the genetic factors controlling cytochrome P-450 biosynthesis in *S. cerevisiae*, initial experiments were performed to screen 18 haploid strains of *S. cerevisiae* for cytochrome P-450 production using the
Figure 2.6. Time Course of Cytochrome P-450 Production in *S. cerevisiae* NCYC 239 Grown on 20% Glucose Media. Values are the mean of 8 determinations. Bars indicate standard deviations.
reduced carbon monoxide difference spectrum method. Of these strains, only 3 were found to produce significant quantities of cytochrome P-450 under the growth conditions used (growth for 44h at 30°C). From these haploid strains, two were selected for tetrad analysis, one of which was capable of producing a high level of cytochrome P-450 and one which produced no cytochrome P-450. A cross was made between these two strains and the resulting diploid isolated. This diploid was then transferred to a sporulation medium and allowed to sporulate. Four resulting asci were isolated and the spores of each tetrad were separated by micro-dissection and allowed to grow. (Crossing the yeast strains and isolating the tetrad yeast strains was performed by Dr. Wilkie of University College, London, whom I gratefully acknowledge.) The resulting yeasts were analyzed for cytochrome P-450 content; the results are shown in Table 2.2. These results show that the cross of a haploid cytochrome P-450 producing strain with a haploid non-producing strain resulted in a diploid non-producing strain. When the diploid sporulates and produces haploid spores, two of these are cytochrome P-450 producers and two are non-producers. Of the two tetrad segregants which produce cytochrome P-450, one appears to produce a higher level of cytochrome P-450 than the other. It should be noted here that the levels of cytochrome P-450 reported in
<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Cytochrome P-450 (nmol/g wet weight of yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/B</td>
<td>0.7</td>
</tr>
<tr>
<td>D22</td>
<td>0</td>
</tr>
<tr>
<td>Diploid</td>
<td>0</td>
</tr>
<tr>
<td>Tetrad 1:</td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>0.6</td>
</tr>
<tr>
<td>1b</td>
<td>0.7</td>
</tr>
<tr>
<td>1c</td>
<td>0</td>
</tr>
<tr>
<td>1d</td>
<td>0</td>
</tr>
<tr>
<td>Tetrad 2:</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>1.35</td>
</tr>
<tr>
<td>2b</td>
<td>0</td>
</tr>
<tr>
<td>2c</td>
<td>0.44</td>
</tr>
<tr>
<td>2d</td>
<td>0</td>
</tr>
<tr>
<td>Tetrad 3:</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>0.9</td>
</tr>
<tr>
<td>3b</td>
<td>0</td>
</tr>
<tr>
<td>3c</td>
<td>1.75</td>
</tr>
<tr>
<td>3d</td>
<td>0</td>
</tr>
<tr>
<td>Tetrad 4:</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>0</td>
</tr>
<tr>
<td>4b</td>
<td>0.95</td>
</tr>
<tr>
<td>4c</td>
<td>1.1</td>
</tr>
<tr>
<td>4d</td>
<td>0.9</td>
</tr>
<tr>
<td>NCYC 240</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Table 2.2. Tetrad Analysis of Cytochrome P-450 Distribution. Values are the average of three runs in duplicate, with yeast grown for 24 hours in a rotary shaker (30°C).
Table 2.2 are not maximal values for each yeast strain but are the level obtained after growth for 24h on a fast rotary shaker at 30°C to reduce yeast flocculation. This was necessary as several of the isolated strains flocculated easily during growth and this flocculation made it impossible to record a value for the cytochrome P-450 level due to settling of the yeast during the spectral assay. The yeast *S. cerevisiae* NCYC 240 gave a yield of 0.95nmol/g under these conditions compared to its optimal yield of approx. 3nmol/g. The only exception to this 2:2 distribution of cytochrome P-450 producers to non-producers was in tetrad 4 where a 3:1 ratio was observed.

To confirm the difference in cytochrome P-450 levels found, one tetrad of yeast strains was subjected to a time course of cytochrome P-450 production analysis along with the parent strains. The results obtained are shown in Figure 2.7. It can be seen that the difference observed in the cytochrome P-450 yields of the producing strains of the tetrad are real and not due to experimental error. The yeasts are observed to have peak levels of cytochrome P-450 at different times due to the growth characteristics of the individual yeast strains. Each yeast reaches a peak cytochrome P-450 level at about the end of the exponential growth phase. These results for the segregation of
Figure 2.7. Time Course of Cytochrome P-450 Production in Yeast Strains B/B, 2a & 2c. Values are the mean of 8 determinations. Bars indicate standard deviations.
cytochrome P-450 production amongst the tetrad yeast strains lead to the conclusion that production of cytochrome P-450 is controlled by a single nuclear gene. This gene is obviously under the control of one or more modifying factors which result in a small or a larger amount of cytochrome P-450 being produced. The fact that the diploid yeast produced from the two parents (one of which was a cytochrome P-450 producer and one a non-producer) did not produce cytochrome P-450 shows that these results do not reflect segregation of the cytochrome P-450 structural gene but of a regulatory gene. If all that was necessary for cytochrome P-450 production was the possession of the structural gene, then almost certainly the diploid would produce cytochrome P-450. These conclusions lead to the proposal of a genetic model for the segregation of a regulatory gene for cytochrome P-450 production as shown in Figure 2.8. This is the most simple model which could be proposed to account for observations made. In this model, the presence of the regulatory gene allowing cytochrome P-450 to be produced results in production of cytochrome P-450 yet does not determine the level produced. This is determined by the presence of a modifier gene (more than one may be involved) which gives rise to one of the tetrad yeasts having a small amount of cytochrome P-450 and one having a larger amount.
\[ \text{Figure 2.8. Model for the Segregation of a Gene Controlling Cytochrome P-450 Production in } S. \text{ cerevisiae.} \]
The identity of the regulatory gene shown to exist in these experiments is unknown. However, since cyclic AMP regulates the biosynthesis of cytochrome P-450 (Wiseman et al., 1978), the levels of this compound were examined in each of the parent, diploid and tetrad yeasts. The results are shown in the Table 2.3. The levels of cyclic AMP measured are consistent with the general levels obtained in Saccharomyces yeasts under similar growth conditions by Sy and Richter (1972). In general, it was observed that a higher level of cyclic AMP was detected in those yeasts not producing cytochrome P-450. This work is consistent with that of Wiseman et al. (1978) which showed a higher cyclic AMP level present under conditions which do not allow cytochrome P-450 biosynthesis to occur. As all of these yeasts were grown under identical conditions for the same time, the difference probably reflects a genetic difference between the yeast strains studied. The yeast 2a gave a low result which seems out of the pattern observed with the other yeasts. These results must therefore be viewed with some caution. However, it seems possible that the regulatory gene which is involved in controlling the presence/absence of cytochrome P-450 may also have an effect in regulating the level of cyclic AMP. Indeed it is possible that the function of the gene may be to regulate cyclic AMP levels which in turn control cytochrome P-450.
<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Cytochrome P-450 Level (nmol/g wet weight of yeast)</th>
<th>Cyclic AMP Level (pmol/200mg yeast) (wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D22</td>
<td>0</td>
<td>6.25</td>
</tr>
<tr>
<td>B/B</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Diploid</td>
<td>0</td>
<td>5.2</td>
</tr>
<tr>
<td>2a</td>
<td>3.0</td>
<td>3.4</td>
</tr>
<tr>
<td>2b</td>
<td>0</td>
<td>3.6</td>
</tr>
<tr>
<td>2c</td>
<td>0.5</td>
<td>3.75</td>
</tr>
<tr>
<td>2d</td>
<td>0</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Table 2.3. The Level of Cyclic AMP in Yeast Strains from Tetrad Analysis.
levels. In this respect, the gene may be coding for adenylate cyclase or phosphodiesterase or some other protein involved in regulating the intracellular cyclic AMP levels. In an attempt to look at this further, theophylline, an inhibitor of cyclic AMP phosphodiesterase was added to growing cultures of the tetrad yeasts in an attempt to alter the cytochrome P-450 level produced in the producer or non-producer strains. However, no effect on enzyme level was observed after this treatment (Table 2.4). The inability of theophylline to influence cyclic AMP levels in yeast has been observed before (Mahler and Lin, 1978) and may reflect either a property of the yeast enzyme or poor transport of the compound into the cell.

It has been shown that multiple forms of cytochrome P-450 exist in *Saccharomyces cerevisiae*, and that an altered profile of these forms can be induced by treatment of growing yeast with benzo(a)pyrene and several other compounds (see Chapter 6). In this respect it was thought that the difference observed in the amount of cytochrome P-450 produced in the tetrad strains may be due to differences in the forms of cytochrome P-450 present. This was investigated by attempting to alter the levels of cytochrome P-450 in the tetrad yeasts by inducing with benzo(a)pyrene (Table 2.4). No cytochrome P-450 could be
<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Cytochrome P-450 Level (nmol/g wet weight of yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>5.70±.34</td>
</tr>
<tr>
<td>2b</td>
<td>0</td>
</tr>
<tr>
<td>2c</td>
<td>1.10±.22</td>
</tr>
<tr>
<td>2d</td>
<td>0</td>
</tr>
<tr>
<td>2a + theophylline (1% w/v)</td>
<td>6.15±144</td>
</tr>
<tr>
<td>2b + theophylline (1% w/v)</td>
<td>0</td>
</tr>
<tr>
<td>2a + benzo(a)pyrene (95μM)</td>
<td>5.14±119</td>
</tr>
<tr>
<td>2b + benzo(a)pyrene (95μM)</td>
<td>0</td>
</tr>
<tr>
<td>2c + benzo(a)pyrene (95μM)</td>
<td>2.26±.06</td>
</tr>
<tr>
<td>2d + benzo(a)pyrene (95μM)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.4. Attempted Induction of Cytochrome P-450 in Tetrad 2 Yeast Strains. Yeast grown 40 hours at 30°C in a shaking water bath. Benzo(a)pyrene and theophylline were added at the beginning of the growth period. Values are the mean of 8 determination ± standard deviation.
induced in the non-producing strains (2b and 2d) after benzo(a)pyrene treatment. It would seem therefore that the regulatory gene has an on/off mechanism which cannot be altered by benzo(a)pyrene treatment. Yeast 2a showed no significant difference in cytochrome P-450 level after benzo(a)pyrene treatment, although this does not rule out the possibility that the profile of forms of the enzyme has been altered (see Chapter 6). Yeast 2c which produces a low level of cytochrome P-450 gave a significant increase in cytochrome P-450 level after growth in the presence of benzo(a)pyrene, though still only to less than half of the value observed for yeast 2a. It is unlikely that any effect of benzo(a)pyrene is occurring at the regulatory gene, though benzo(a)pyrene may be altering the effect of the modifier gene(s). The fact that 2c could not be induced to produce the same level cytochrome P-450 as 2a implies that a modifier gene is involved in this regulation. Much further work is required before the mechanisms involved are discovered.

The absence of cytochrome P-450 in the non-producer strains raises an interesting question as to how these yeasts carry out lanosterol demethylation to produce egosterol, necessary for growth as mentioned earlier. If indeed lanosterol demethylation is catalyzed by cytochrome
P-450, then it is possible that the absence of the regulatory gene allowing cytochrome P-450 production (or the presence of a gene not allowing cytochrome P-450 production) does not stop its biosynthesis completely. Therefore, a small amount of the enzyme not detectable by normal techniques is present in a situation analogous to that in aerobically grown yeast in low glucose media described earlier. It is also possible that other enzymes may take the role of lanosterol 14α-demethylation proposed for cytochrome P-450. In this respect it is of interest that several other monooxygenase enzymes have been shown to be involved in the lanosterol to ergosterol conversion in *S. cerevisiae* (Ohba *et al*, 1978; Aoyama *et al*, 1981b).

No previous work has been attempted on the genetics of cytochrome P-450 production in yeast. However, the group of Nebert *et al* has worked extensively on the genetic regulation of cytochrome P-450 activities in the mouse (Nebert *et al*, 1981; Nebert, 1979). These workers have looked at the forms of cytochrome P-450 induced in the mouse by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, β-naphthoflavone and benzo(a)pyrene. They have demonstrated that induction by these compounds is mediated through the 'Ah locus' which is a combination of regulatory, structural and possibly temporal genes which may
or may not be linked. These workers have evidence that the process of induction by these compounds occurs by binding of the inducer, which passively crosses the cell membrane, to a cytosolic Ah receptor protein, regarded as the major regulatory gene product (Okey et al, 1979). This complex is then translocated into the nucleus and results in the activation of structural genes coding for forms of cytochrome P-450. A simplified system drawn from the results of Nebert et al on mouse cytochrome P-450 is shown in Figure 2.9. It is of great interest that this scheme requires the presence of at least one regulatory gene giving rise to a specific regulatory gene product, in this case the binding receptor for polycyclic aromatic hydrocarbons. In the results described in this chapter it has been shown that a regulatory gene product is involved in the biosynthesis of yeast cytochrome P-450. Although the nature of the regulatory gene product is unknown in the case of yeast cytochrome P-450 biosynthesis, it may be involved in cyclic AMP regulation. Clearly, much further work is needed to characterize this system more fully.
Figure 2.9. The Ah Complex as Proposed by Nebert et al. Adapted from Nebert et al. (1981).
CHAPTER 3
3. Solubilization, and Purification of Cytochrome P-450/P-448 and Other Mixed-Function Oxidase Proteins from Saccharomyces cerevisiae

3.1. Introduction

Cytochrome P-448, cytochrome c(P-450) reductase, cytochrome b$_5$ and cytochrome b$_5$ reductase from Saccharomyces cerevisiae, grown under conditions of glucose repression, were isolated, solubilized, separated and purified. A novel combination of purification steps were used to purify cytochrome P-448 and other above-mentioned proteins. Precipitation by polyethylene glycol is proposed for large scale isolation of these enzymes without recourse to high g centrifugation. The stability of cytochrome P-448 in microsomal fraction (in storage and through solubilization) and in purified form was studied and optimized. Purified cytochrome P-448 proved to be homogenous by SDS-polyacrylamide gel electrophoresis and was free of other known components of mixed-function oxidase. Spectral properties of purified cytochrome P-448 were examined and as the Soret peak of reduced CO complex was situated at 447-448nm (not at 450nm) this cytochrome is called cytochrome P-448 hereafter throughout the rest of this study. The molecular weight of cytochrome P-448 was estimated also by SDS-polyacrylamide gel electrophoresis and along with
specific content were compared with values for cytochrome P-450 and P-448 from yeast, bacteria and mammalian sources reported in literature. Based upon this molecular weight and the specific content (nmol of cytochrome P-448, determined by reduced CO-compound, per mg of protein) the degree of purity for cytochrome P-448 was determined. The expression cytochrome P-450/P-448 (P-450 or P-448) is used in this chapter to refer to both forms of the enzyme in the microsomal fraction. This expression also used to denote this enzyme in general term.

3.2. Methods and Materials

3.2.1 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 (5g/L), yeast extract (10g/L) and mycological peptone (20g/L) autoclaved at 15 p.s.i. for 20min. This medium in addition contained glucose at a concentration of 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., U.K.) at 30°C, shaking at 80rpm with a stroke of 5cm and was harvested after 44h by centrifugation
at 5000g for 10min in a MSE bench centrifuge (Measuring and Scientific Equipment Ltd., U.K.).

When yeast was grown on a large scale the medium was formulated and autoclaved as described above and yeast was grown in a 5 or 10L fermentor (L. H. Engineering, U.K.) from an inoculum of 20 grams. The duration of this fermentation was 24h while the temperature was maintained at 30°C and the medium was stirred at 500rpm. The oxygen concentration and pH were set and controlled at minimum and 5.5 respectively. Yeast was harvested by centrifugation at 2800g for 15min in a M.S.E. Coolspin centrifuge (M.S.E. Ltd., U.K.).

3.2.2. The Preparation of Yeast Microsomes

Harvested yeast was disrupted according to the method of Wiseman et al (1975b) in which it was transferred to a disrupter (Edmund Buhler Tubingen, W. Germany) where it was shaken with approximately 3 volumes of glass beads of diameter 1.00 to 1.05mm (B. Braun, Melsungen, W. 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 a minimum volume of ice-cold 0.1M potassium phosphate buffer, pH 7.2 containing 0.001M ethylenediamine tetra-acetic acid (EDTA), 0.001M dithiothreitol and 20% (v/v) glycerol. After removal of the cell
debris and nuclear material by centrifugation in a MSE High Speed 18 centrifuge (M.S.E. Ltd., U.K.) at 7500g for 15min, yeast microsomal fraction was obtained by centrifugation at 160,900g for 1h in a Beckman L5-65 ultracentrifuge (Beckman, U.S.A.). In a typical experiment 100ml of medium would yield 3g of yeast and from this 0.6g of microsomes would be obtained. Microsomal pellet was then resuspended in an equal quantity of the above-mentioned buffer by hand using a Potter-Elvehjem homogeniser.

In large scale preparation, yeast was suspended in an equal volume of 0.1M potassium phosphate buffer pH 7.2 containing 0.001M-EDTA, 0.001M dithiothreitol and 0.65M sorbitol. This suspension was cooled down to 2°C in an acetone-ice bath and passed through an APV Gaulin homogenizer (A.P.V. Ltd., U.K.) at 300-400kg/cm² twice as is described by Yoshida et al (1974).

3.2.3. The Measurement of Cytochrome P-450/P-448 and Cytochrome P-420.

Cytochromes P-448 and P-420 levels were measured by the method of Omara et al (1965). Microsomal suspension or solubilized solution was placed in both reference and sample cuvettes. The content of both cuvettes was reduced by addition of solid sodium dithionite (1mg/ml) and a baseline
of equal light absorbance between 390 and 500nm was recorded using either a SP1800 (Pye Unicam, U.K.) or a Cary 219 (Varian, U.S.A.) spectrophotometer. Carbon monoxide was bubbled at a rate of one bubble per second for 30 seconds through the contents of the sample cuvette and a difference spectrum was recorded (Figure 3.1). The concentration of cytochrome P-450/P-448 and P-420 were determined assuming a value of 91mM$^{-1}$cm$^{-1}$ for the molar extinction increment between 450/448 and 490nm for cytochrome P-448 (Omura et al, 1965) and a value of 110mM$^{-1}$cm$^{-1}$ between 420 and 490nm for cytochrome P-420 (Omura and Sato, 1964a).

3.2.4. **Solubilization of Yeast Mixed-Function Oxidase Proteins**

Microsomal suspension was diluted to 30mg protein/ml with 0.1M potassium phosphate buffer pH 7.2 containing 0.001M-EDTA, 0.001M dithiothreitol, 20% (v/v) glycerol. A number of ionic and non-ionic detergents were used to solubilize cytochrome P-450/P-448. Cytochrome P-450/P-448 was finally solubilized for purification by selecting one of these, the ionic detergent sodium cholate at a final concentration of 1% and always in presence 0.1% Triton X-100 or 0.1% reduced glutathione. Microsomal suspension plus sodium cholate and a stabilizer were stirred at 4°C for 1h under $N_2$ atmosphere and was centrifuged at 160,000g for
Figure 3.1. The Carbon Monoxide-Difference Spectrum of Yeast Microsomal Fraction. ----, baseline; ---, CO-difference spectrum of reduced form.
50min and the supernatant, the solubilized protein was collected.

In large scale preparation, the disrupted yeast was diluted the same way and 0.1% (w/v) reduced glutathione and 1% (w/v) sodium cholate were added. After stirring in 4°C under N₂ for 1h it was diluted further (3 folds) with the above-mentioned phosphate buffer and polyethylene glycol (M.W. 6000) from a 50% (w/v) solution was added to this to give a final concentration of 16% in the microsomal suspension. Protein from 16% polyethylene glycol precipitation was collected by centrifugation at 7500g using a MSE High Speed 18 centrifuge for 60min and was dissolved in 0.1M potassium phosphate buffer pH 7.2 containing 0.001M-EDTA, 0.001M dithiothreitol and 20% (v/v) glycerol.

3.2.5. Purification of Yeast Mixed-Function Oxidase Proteins

Solubilized protein was diluted with 0.1M potassium phosphate buffer pH 7.2 containing 0.001M-EDTA, 0.001M dithiothreitol and 20% (v/v) glycerol to adjust the sodium cholate concentration to 0.33%. A precipitate at 35-65% sat. (NH₄)₂ SO₄ was prepared while the pH was maintained at 7.0 by addition 0.1M NH₄OH and centrifugation was performed at 7500g for 40min by MSE High Speed 18 centrifuge. The
precipitate was then suspended in a minimum volume of 0.02M potassium phosphate buffer pH 7.0 containing 0.001M-EDTA, 0.001M dithiothreitol and 20% (v/v) glycerol and 0.3% (w/v) sodium cholate. In a typical preparation run from 500 grams of yeast, 100 grams microsomes were isolated and from this 266nmol of cytochrome P-450/P-448 (along with other proteins) was solubilized which after (NH₄)₂SO₄ fractionation was readily suspended in the above-mentioned buffer to give a final volume of 100ml. This suspension was dialyzed overnight against 30 volumes of 0.01M potassium phosphate buffer containing 0.001M-EDTA, 0.001M dithiothreitol and 0.3% (w/v) sodium cholate. It was subsequently centrifuged at 160,900g and the supernatant was collected. When Triton X-100 was used for stabilization purposes through solubilization, to eliminate the residual amount of this detergent still present in preparation, 10g of Bio-Bead SM-2 (prewashed with methanol and H₂O) was mixed with the supernatant and stirred for 30min before addition to the top of a 2.0x4.0-cm packed Bio-Bead column. Cytochrome P-450/P-448 and other proteins were eluted from the column by washing the column with the dialysis buffer. This eluate was applied to a 2.4x12.0-cm, 8-amino-n-octyl-Sepharose 4B column which had been preequilibrated with 0.01M potassium phosphate buffer pH 7.0 containing 0.3% (w/v) sodium cholate, 0.001M-EDTA and 20% (v/v) glycerol (equilibration
buffer). After washing the column with the same buffer, cytochrome P-450/P-448 was eluted with the same buffer also containing 0.1% (v/v) Emulgen 911. For elution of NADPH: cytochrome c(P-450) reductase the aminooethyl-Sepharose 4B column was washed with this buffer also containing 0.2% (w/v) sodium deoxycholate and 2μM flavin mononucleotide (FMN). For elution of cytochrome b₅ the 0.001M potassium phosphate buffer pH 7.0 containing 0.4% (w/v) sodium deoxycholate, 0.2% (w/v) sodium cholate, 0.001M-EDTA and 20% (v/v) glycerol. Fractions rich in cytochrome P-450/P-448 were applied to a 1.6x6.0-cm hydroxylapatite-cellulose (1:1, w/w) column preequilibrated with 0.01M potassium phosphate buffer pH 7.0 containing 20% (v/v) glycerol. This column was washed with 0.03M potassium phosphate buffer, pH 7.0 containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911, and NADH:cytochrome b₅ reductase was eluted by 0.05M potassium phosphate pH 7.0 containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911. Cytochrome P-450/P-448 was eluted by 0.1M potassium phosphate pH 7.0 containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911. Cytochrome P-450/P-448 was eluted from the column and were combined and diluted 10 fold with a solution of 20% glycerol containing 0.2% (v/v) Emulgen 911. This was then applied to a 1.0x5.0-cm carboxymethyl-Sephadex C-50 column preequilibrated with 0.01M potassium phosphate buffer pH 7.0 containing
20% (v/v) glycerol and 0.2% (v/v) Emulgen 911. After washing the column with 3 volumes of 0.04M potassium phosphate buffer pH 7.0 containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911, cytochrome P-450/P-448 was eluted by passing through 0.1M potassium phosphate buffer pH 7.0 containing 20% (v/v) glycerol and 0.2% Emulgen 911. Cytochrome P-450/P-448 was dialyzed twice against 30 volumes of 0.005M potassium phosphate buffer pH 7.7 containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911, each time 12h, diluted 2 fold with a solution of 20% (v/v) glycerol containing 0.2 (v/v) Emulgen 911, adjusted to pH 7.7 and then applied to an 0.85x7.0-cm DEAE-Sephacel column preequilibrated with the above dialysis buffer. Most of the cytochrome P-448 did not bind to the column and was eluted by washing the column with some of the equilibration buffer. Cytochrome P-450 form bound to the column and was eluted with 0.035M potassium phosphate buffer pH 7.7 containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911. Both cytochromes P-448 and P-450 were concentrated by ultrafiltration using an Amicon ultrafiltration apparatus with PM-30 membrane (Chem. Lab., U.K.). Cytochrome P-448 (with or without Emulgen 911) in 0.1M potassium phosphate buffer pH 7.0 containing 20% (v/v) glycerol was stored at -70°C before use in this study.
3.2.6. **Further Purification of Cytochrome c(P-450) Reductase, Cytochrome b_5 and Cytochrome b_5 Reductase**

Cytochrome c(P-450) reductase fractions from the amino-n-octyl-Sepharose 4B column was applied directly to 1.2x5-cm column of 2',5'-ADP-Agarose which was subsequently washed with 0.3M potassium phosphate buffer pH 7.7 containing 0.001M-EDTA, 20% (v/v) glycerol and 0.1% (v/v) Emulgen 911, until no more extraneous protein was eluted. This column was further washed with 0.03M potassium phosphate buffer pH 7.7 containing 0.001M-EDTA, 20% (v/v) glycerol and 0.2% (w/v) sodium deoxycholate (the intermediate buffer). Cytochrome c(P-450) reductase was eluted with the intermediate buffer to which 2'-AMP had been added up to 0.005M in concentration. Those fractions rich in cytochrome c(P-450) reductase were pooled, dialyzed twice (16h each time) against 20 volume of 0.1M potassium phosphate buffer, pH 7.0 containing 20% (v/v) glycerol and was concentrated using an Amicon ultrafiltration cell (PM-30 membrane) and was stored in -70°C.

Cytochrome b_5, eluted from amino-n-octyl-Sepharose 4B, was further purified by gel filtrations on a 2.4x48.0-cm Sephadex G-75 column (twice) using 0.01M Tris-acetate buffer pH 8.1 containing 0.001M-EDTA and 0.47 (w/v) sodium deoxycholate. In these gel filtrations, the red, hemoprotein
was partially included and eluted behind a front-running, brownish bank of excluded material. After the second gel filtration the cytochrome b5 fraction was dialyzed twice (24h each time) against 30 volumes of 0.02M Tris-acetate pH 8.1 containing 0.001M-EDTA and was stored in -70°C. NADH:cytochrome b5 reductase, eluted from hydroxylapatite column, was concentrated by an Amicon ultrafiltration cell (PM-10 membrane) 20 folds and was diluted 5 fold with water containing 0.2% (v/v) Emulgen 911. It was applied to a 2.4x10.0-cm DEAE-Sephacel column preequilibrated with 0.01M potassium phosphate buffer pH 7.0 containing 0.2% (v/v) Emulgen 911. After washing the column with 0.01M and 0.03M potassium phosphate buffer pH 7.0 both containing 0.2% (v/v) Emulgen 911 cytochrome b5 reductase was eluted by 0.1M potassium phosphate buffer pH 7.0 containing 0.2% (v/v) Emulgen 911 and was stored in -70°C.

3.2.7. Purification Cytochrome P-450/P-448 from Rat Liver

Cytochrome P-450 from phenobarbital-treated and cytochrome P-448 from β-naphthoflavone-treated rat livers were isolated and purified in the Department of Biochemistry (using a method described by Guengerich, 1978). These enzymes were gifted by Mr. P. Tamburini, whom I gratefully acknowledge.
3.2.8. **Protein Determination**

Protein was determined by a modified method of Lowry *et al.* (1951) using bovine serum albumin as standard.

**Reagents**

- 15% Trichloroacetic acid (TCA)
- 1% (w/v) Hydrated copper sulfate
- 2% (w/v) Sodium potassium tartrate
- 2% (w/v) Sodium carbonate in 0.1M NaOH
- Folin-Ciocalteau phenol reagent diluted with water (1:1, v/v)
- Bovine serum albumin in water (10mg/ml)

**Method**

One ml of 15% TCA solution was added to an equal volume of enzyme samples (microsomal or purified). Precipitate was collected by centrifugation at 9000rpm for 15min by a MSE bench centrifuge and was suspended in water to give appropriate protein concentrations. The protein standard was precipitated with 15% TCA in an identical way and was suspended in water to give concentrations of 50, 100, 200, and 250 μg/ml. Copper sulfate, sodium potassium tartrate and sodium carbonate were mixed in ratio 1:1:100 by volume immediately before use (this mixture is called sodium carbonate reagent). The protein standards, samples and
blanks (0.5ml) were mixed with the sodium carbonate reagent (2.5ml) and allowed to incubate for 10min at room temperature. 0.25ml of diluted Folin-Ciocalteau phenol reagent was added and the solutions were mixed immediately. After at least 30min at room temperature, the absorbance was recorded at 750nm using a Cecil CE272 spectrophotometer (Cecil Instrument, U.K.).

3.2.9. Measurements of NADPH: Cytochrome c(P-450) Reductase Activity

This enzyme is thought by Kamataki et al. (1979) to be the same as NADPH: cytochrome c reductase (EC 1.6.2.4). As the direct measurement of cytochrome P-450 reduction is difficult to perform, cytochrome c is used as an alternative electron acceptor. The reduction of cytochrome c was monitored spectrophotometrically (Pye Unicam SP1800 spectrophotometer) using a method modified from that of Williams and Kamin (1962).

Reagents
- 0.1M Tris-HCl buffer pH 7.5
- 0.1mM Cytochrome c in 0.1M Tris-HCl buffer pH 7.5
- 0.025M-NADPH in 0.1M Tris-HCl buffer pH 7.5
Method
Enzyme suspension (0.1ml), cytochrome c (1ml) and 0.1M Tris-HCl buffer pH 7.5 were placed in each of two cuvettes to give a final volume of 2.975ml in the sample and 3.0ml in the reference. The cuvettes were preincubated at 22°C for 5min, and the reaction was started by addition of NADPH (0.025ml) the sample cuvette. The reduction of cytochrome c was measured by monitoring the absorbance change of 550nm and an extinction coefficient of 19.6mM⁻¹cm⁻¹ (Yonetani, 1965) was used to calculate the rate of reduction.

3.2.10. Measurement of Cytochrome b₅
Cytochrome b₅ was measured by reduction with NADH in microsomal and solubilized fractions.

Reagents
- 0.1M Tris-HCl buffer pH 7.5
- 0.01M-NADH in 0.1M Tris-HCl buffer pH 7.5

Method
Microsomal of solubilized enzyme solution was diluted with 0.1M Tris-HCl buffer pH 7.5 to give a final concentration of 1mg protein/ml and was placed in each of the sample and reference cuvettes (2.96ml). A baseline of equal light absorbance was established between 400 and
440nm. NADH (0.04ml) was added to the sample cuvette and the spectrum was recorded. The concentration of cytochrome b\textsubscript{5} was determined by measuring the difference in absorbance between 424 and 409nm, using an extinction coefficient of 164 mM\textsuperscript{−1} cm\textsuperscript{−1} (Yoshida et al, 1974b).

3.2.11. Measurement of NADH:Cytochrome b\textsubscript{5} Reductase Activity

NADH:cytochrome b\textsubscript{5} reductase is shown by Mihara and Sato (1975) to be the same as NADH:ferricyanide reductase (EC 1.6.2.2.) which was measured by a method modified from that of Strittmatter and Velick (1957).

Reagents

- 0.1M Tris-HCl buffer pH 7.5 containing 0.2mM potassium ferricyanide
- 0.03M-NADH in 0.01M NaHCO\textsubscript{3}

Method

To each of the sample and reference cuvettes 2.955ml of 0.1M Tris-HCl buffer pH 7.5 containing 0.2mM potassium ferricyanide and 0.02ml of enzyme solution were added. Both cuvettes were incubated in 22°C for 5min and NADH (0.025ml) solution was added to the sample cuvette. Absorbance changes at 420nm were measured as a function of time and
cytochrome b₅ reductase activity was determined using 1.02 mM⁻¹cm⁻¹ as an extinction coefficient (Schellenberg and Hellerman, 1958).

3.2.12. **SDS-Polyacrylamide Gel Electrophoresis of Yeast Cytochrome P-448**

The method of Laemmli (1970) was used for analysis of purified cytochrome P-448. The enzyme sample was initially treated with sodium dodecyl sulfate (SDS) and then processed on polyacrylamide gel (10%) using a discontinuous buffer system.

**Reagents**

- Gel Stock: 30% (w/v) acrylamide containing 0.8% (w/v) N,N'-methylenebis-acrylamide.
- Buffer 1: 1.5M Tris-HCl pH 8.8, containing 0.4% (w/v) SDS.
- Buffer 2: 0.5M Tris-HCl pH 6.8 containing 0.4% (w/v) SDS.
- Buffer 3: 0.025M Tris-HCl pH 8.3, containing 0.192M glycine and 0.1% (w/v) SDS.
- Buffer 4: 0.062M Tris-HCl pH 6.8, containing 2.3% (w/v) SDS, 15% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue.
- Ammonium persulfate solution 10% (w/v).
Method

A vertical slab gel apparatus made in the workshop of the Biochemistry Department was used. The gel was contained in a glass and perspex cuvette, with internal dimensions of 120mm x 100mm x 1.5mm. Electrophoresis was carried out at room temperature.

The glass plates of the gel cuvette were washed in detergent, rinsed with tap water, distilled water, methanol and acetone, and allowed to dry. The cuvette was assembled and sealed with 1.5% (w/v) molten agar and then clamped in vertical position. The lower (running) gel was prepared by mixing Buffer 1 (10ml), gel stock (13.3ml), water (16.6ml), and polymerization was initiated by addition of N,N'-tetramethylethylenediamine (0.02ml) and freshly prepared ammonium persulfate solution (0.24ml). The solution was poured into the glass cuvette to a height of 80mm. A layer of distilled water was introduced above the gel mixture to insure a flat interface between the separating gel and stacking gel after polymerization. When polymerization was complete the water layer was removed and the upper (stacking) gel, consisting of a mixture of Buffer 2 (2.5ml), gel stock (1.0ml), water (6.5ml), ammonium persulphate solution (0.06ml), and N,N'-tetramethylethylenediamine (0.02ml) was added to the cuvette above the running gel.
A perspex comb was introduced in the stacking gel before polymerization was complete and a small amount of Buffer 3 was introduced into the sample wells to keep them separate. The lower spacer was removed from the cuvette, and the cuvette was then placed in an electrophoresis tank. Buffer 3 was added to the upper and lower reservoirs, and air bubbles trapped underneath the gel were removed using a syringe.

Purified cytochrome P-448 was diluted with Buffer 4 to a final concentration of 0.1mg protein/ml. A solution of marker proteins consisting of bovine serum albumin (M.W. 68000), catalase (monomeric M.W. 60000), ovalbumin (M.W. 45000), aldolase (monomeric M.W. 39500), 0.1mg of each in 1ml of Buffer 4, was prepared. Cytochrome P-448 sample and marker protein mixture were placed in boiling water for 3min. Both solutions were cooled and applied to the stacking gel by placing various volumes of cytochrome P-448 and marker proteins mixture in alternating wells. A constant current of 20mA was used until the bromophenol blue entered the separating gel, and then this was increased to a constant current of 40mA until the bromophenol blue was within 5mm of the end of the gel. The cuvette was removed from the tank when electrophoresis was complete. The glass
plates were separated, the stacking gel was removed and the gel was stained as described below.

3.2.13. Detection of Proteins on Polyacrylamide Gel

The principal means used for the detection of proteins was the Coomassie Blue stain.

Reagents

- Propan-2-ol/acetic acid/water (5:2:13 by volume)
- Propan-2-ol/acetic acid/water (1:1:8 by volume)

Method

Staining of the proteins was achieved by immersing the gel overnight in propan-2-ol/acetic acid/water (5:2:13 by volume) containing 0.05% (w/v) Coomassie Blue G-250. The gel background was destained by immersing in progressive changes of propan-2-ol/acetic acid/water (1:1:8 by volume) over a period of 48h. The gel was stored in 3% (v/v) glycerol at 4°C once the background was cleared.

3.2.14. Spectrophotomery for Thermostability and Spectral Studies

The thermostability of microsomal fraction and purified cytochrome P-448 was measured by a fluorescence
spectrophotometric technique based on the "normalization" of the absorption parameters of tryptophyl residues in denatured unhydrolyzed protein. A Perkin-Elmer MPF3 fluorescence spectrophotometer (Hitachi Ltd., Japan) was used for fluorescence measurement of tryptophan at 295 and 330 nm excitation and emission wavelengths respectively.

Absolute and various difference spectra of purified cytochrome P-448 were recorded using a Pye Unicam SP1800 or a Varian Cary 219 spectrophotometer.

3.2.15. Removal of Emulgen 911 from Yeast Cytochrome P-448

After the final step of the purification procedure, Emulgen 911 was removed from cytochrome P-448 (72 nmol) by dialysis against 60 volumes of 0.01 M potassium phosphate buffer pH 7.25 containing 0.005 M dithiothreitol and 20% glycerol and was applied to a hydroxylapatite column (1.5 x 8 cm). The column was washed with the same buffer until no absorption at 276 nm was detectable in the eluate and the enzyme was eluted with 0.5 M potassium phosphate buffer pH 7.25 containing 0.005 M dithiothreitol and 20% glycerol.

3.2.16. Lyophilization of Cytochrome P-448

A purified cytochrome P-448 solution free of Emulgen 911, with protein concentration of 0.4 mg per ml was used for
lyophilization. Reduced glutathione was added to this
solution to give a final concentration 0.1% (w/v). This
solution was precipitated by ammonium sulfate (65% of
saturation), pellet was collected by centrifugation at
27,750g for 30min and was dissolved in 0.005M potassium
phosphate buffer pH 7.0 containing 10% sorbitol. It was
then processed by gel filtration on a Se-phadex G-25 column
(1.5x8.0cm) prewashed with the same buffer. To this
cytochrome P-448 solution which was free of glycerol and
ammonium sulfate, reduced glutathione was added to give a
final concentration of 0.1% (w/v). It was lyophilized by a
high vacuum lyophilizer (Edwards, U.K.) overnight. The
lyophilized enzyme was stable in storage at 4°C for 3
months.

3.2.17. Materials
A: Preparation and Regeneration 8-Amino-n-octyl-
Sepharose 4B

8-Amino-n-octyl-Sepharose 4B was prepared according to
the method of Cuatrecacas (1970) in the following manner in
a ventilated fume hood. A volume of 400ml of wet Sepharose
4B, previously washed with 6L distilled water, was stirred
in 1L of distilled water, with a thermometer and the
electrode of a pH meter immersed in the suspension. CNBr
(100g) was dissolved in 300ml 1,4-dioxane and was added
drop-wise to the stirred Sepharose 4B over 10min. During this time the pH was maintained at 11 by the addition of 8N NaOH and ice was added to keep the temperature between 20 and 25°C. After an additional 10min of stirring at pH 11 and 20-25°C, the reaction was quenched by the addition of 600ml of crushed ice. The suspension was poured into a sintered glass funnel placed over a Buchner flask which was attached to an aspirator. The gel was washed with 4L of cold distilled water (4°C) and was added to a solution of 114g of 1,8-diaminooctane dissolved in 400ml of distilled water. The pH of the resulting suspension was adjusted to 10 using 6N HCl and the mixture was stirred gently for an additional 16h at 40°C. The gel was washed in the sintered glass funnel successively with a total of 10L of distilled water, 2L of 0.1M potassium phosphate buffer pH 7.25, and 10L of distilled water. The resulting gel was stored at 4°C in a 0.02% (w/v) NaN₃ solution.

8-Amino-n-octyl-Sepharose 4B was reused after being washed in a batch-wise manner with the following solutions (2L of each) as described by Guengerich (1977a).

1 - 0.01M Potassium phosphate buffer pH 7.0 containing 0.4% (w/v) sodium deoxycholate, 0.2% (w/w) sodium cholate and 0.001M-EDTA.
2 - 0.1M Potassium phosphate buffer pH 7.2 containing 0.4% (w/w) Emulgen 911 and 0.4% (w/w) sodium deoxycholate
3 - Distilled water
4 - 10% (v/v) Solution of 1,4-dioxane
5 - Distilled water
6 - 0.5M Potassium phosphate buffer pH 7.2 containing 0.5M KCl an 0.001M-EDTA
7 - Distilled water
8 - 0.1M Potassium phosphate buffer pH 7.2 containing 0.7% (w/w) sodium cholate and 0.001M-EDTA

It was stored in an equal volume of 0.01M potassium phosphate buffer pH 7.0 containing 0.3% (w/w) sodium cholate, 0.001M-EDTA, 20% glycerol and 0.02% sodium azide.

B: Other Materials
Sodium cholate, sodium deoxycholate, sodium dodecyl sulfate, dithiothreitol, NADH, NADPH, flavin mononucleotide, sodium potassium tartarate, albumin, reduced glutathion were obtained from Sigma Chemical Ltd. 2',5'-ADP-Agarose and 2'-AMP were supplied by P-L Biochemicals. Powdered cellulose was supplied by Whatman Lab. Ltd. and 2-mercaptoethanol was obtained from Koch-Light Lab. Ltd. DEAE-Sephacel, CM-Sephadex C-50, Sepharose 4B, Sephadex
G-75, protein marker kit were purchased from Pharmacia Fine Chemical. Potassium persulfate, acrylamide, N,N'-methylenebis-acrylamide, bromophenol blue, Coomassie Blue G-250, N,N'-tetramethylethlenediamine, hydroxylapatite, were obtained from Bio-Rad Lab. Emulgen 911 and 913 and Renex 698 were supplied by Honeywell Atlas Ltd. Sodium azide and cyanogen boromide were obtained from Aldrich Chemical Co. and cytochrome c was purchased from Calbiochem-Behring Co. Triton X-100, Triton N-101, Tween 80, ethylenediamine tetra-acetic acid, polyethylene glycol, potassium ferricyanide and other chemicals were supplied by BDH Chemicals Ltd.

3.3. Results and Discussion

3.3.1. Storage and Disruption of Yeast

Various solutions were used to stabilize cytochrome P-448 in yeast cells while in Storage (Table 3.1). Storage of yeast cells at 4°C in 0.1M potassium phosphate buffer pH 7.2 containing 0.001M-EDTA, 0.001M 2-mercaptaethanol, 20% (v/v) glycerol and 15% (w/v) glucose, prevented the disappearance and conversion to cytochrome P-420 of cytochrome P-450/P-448. Triton X-100 did not show any stabilizing effect on cytochrome P-450/P-448 in yeast cells; perhaps due to its large molecular size, it did not penetrate through the cell wall. Glucose as a carbon source for
<table>
<thead>
<tr>
<th>Yeast Storing Condition</th>
<th>Cytochrome P-450/P-448 (%) of recovery</th>
<th>Cytochrome P-450 or P-448/Cytochrome P-420</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Harvested yeast cells</td>
<td>58</td>
<td>1.10</td>
</tr>
<tr>
<td>II. In the phosphate buffer</td>
<td>35</td>
<td>0.44</td>
</tr>
<tr>
<td>III. In the phosphate buffer plus additive and DTT</td>
<td>69</td>
<td>1.41</td>
</tr>
<tr>
<td>IV. In the phosphate buffer plus additives and 0.1% Triton X-100</td>
<td>39</td>
<td>0.48</td>
</tr>
<tr>
<td>V. In the phosphate buffer plus additives, DTT and 15% glucose</td>
<td>95</td>
<td>1.90</td>
</tr>
<tr>
<td>VI. In the phosphate buffer plus additives, mercaptoethanal and 15% glucose</td>
<td>100</td>
<td>2.00</td>
</tr>
<tr>
<td>VII. In the phosphate buffer plus additives, reduced glutathione, 15% glucose</td>
<td>96</td>
<td>1.91</td>
</tr>
</tbody>
</table>

Table 3.1. Stabilization in Storage of Cytochrome P-450/P-448 in Yeast Cells. One gram of yeast after harvest contained 3.0 and 2.0 nmol of cytochrome P-450/P-448 and cytochrome P-420 respectively. In all cases yeast cells were stored in 4°C for a period of one week. 0.1M Potassium phosphate pH 7.2 containing 0.001M-EDTA and 20% glycerol was denoted as the phosphate buffer plus additives. 2-Mercaptoethanol, dithiothreitol(DTT) and reduced glutathione were used 0.001M in concentration.
survival of yeast cells and sulfhydryl compounds (dithiothreitol, reduced glutathione, 2-mercaptoethanol) were essential in stabilizing cytochrome P-450/P-448 in yeast cells while in storage.

Disruption of yeast cells by water-cooled Vibro Mill produced a great loss in cytochrome P-450/P-448. This loss could be the result of localized heat generated during this process.

3.3.2. Storage of Microsomes and Solubilization of Yeast Cytochrome P-450/P-448

When yeast was grown in small scale, it was necessary to store microsomes to be solubilized later in bulk. Table 3.2 shows the stabilization of cytochrome P-450/P-448 in microsomal fraction by various compounds. Triton X-100 in 0.1M potassium phosphate pH 7.2 containing 0.001M-EDTA, 0.001M dithiothreitol and 20% (v/v) glycerol had a positive effect on protecting microsomal cytochrome P-448 in storage at -70°C. The disadvantage of Triton X-100 as a stabilizing or solubilizing agent is that it has a tight interaction with cytochrome P-448 and its complete separation (necessary since chromatography on basis of hydrophobic interaction is one of the purification steps) involved an extra step, e.g. Bio Beads SM-2 (Melcher et al., 1975) or Biogel A-50
## Table 3.2. Stabilization in Storage of Cytochrome P-450/P-448 in Yeast Microsomal Fraction.

One gram of microsomes contained 2.6mol of cytochrome P-450/P-448 and 1.1nmol of cytochrome P-420. In all cases microsomal fraction was stored in -70°C for a three-week period. 0.1M potassium phosphate pH 7.2 containing 0.001M-EDTA and 20% glycerol was denoted as the phosphate buffer plus additives. 2-Mercaptoethanol, dithiothreitol and reduced glutathione were used 0.001M in concentration.

<table>
<thead>
<tr>
<th>Microsomes Storage Condition</th>
<th>Cytochrome P-450/P-448 (% of recovery)</th>
<th>Cytochrome P-450 or P-448/Cytochrome P-420</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Microsomes (pellet)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II. In the phosphate buffer plus additives and 0.1% Triton X-100</td>
<td>87</td>
<td>2.43</td>
</tr>
<tr>
<td>III. In the phosphate buffer plus additives and DTT</td>
<td>55</td>
<td>1.54</td>
</tr>
<tr>
<td>IV. In the phosphate buffer plus additives and cysteine</td>
<td>10</td>
<td>0.70</td>
</tr>
<tr>
<td>V. In the phosphate buffer plus additives and mercaptoethanol</td>
<td>55</td>
<td>3.85</td>
</tr>
<tr>
<td>VI. In the phosphate buffer plus additives and reduced glutathione</td>
<td>91</td>
<td>2.55</td>
</tr>
</tbody>
</table>
(Loach et al., 1970). Reduced glutathione has been reported to make a small reconversion of cytochrome P-420 to cytochrome P-450/P-448 possible (Franklin, 1972a). Stabilized cytochrome P-448 in microsomal fraction and solubilization by sodium cholate (Tables 3.2 and 3.3).

The main reasons for the difficulty in purification lay in the high instability of the cytochrome P-450/P-448 through solubilization and in that separating the integral proteins of microsomal membranes from one another (these are likely to associate by hydrophobic binding) without denaturation. Unsaturated fatty acids involved in the phospholipid as constituent of microsomal membranes are oxidizable with air to produce lipid peroxides, accompanied by destruction of the heme (Omura and Sato, 1964a). Thus, most of cytochrome P-450/P-448 disappears gradually without corresponding formation of cytochrome P-420, when solubilization is carried out aerobically without the proper stabilizers being present. On anaerobic treatment the majority of cytochrome P-450/P-448 is converted to cytochrome P-420 in most solubilization treatments especially when stabilizers are not present. Since cytochrome P-450/P-448 is firmly bound in microsomal membranes and is one of the least solubilizable proteins in microsomes against the general procedures used for
<table>
<thead>
<tr>
<th>Detergent</th>
<th>Source of Microsomes</th>
<th>Cytochrome P-450 or P-448 (% of recovery)</th>
<th>Cytochrome P-450 or P-448/Cytochrome P-420 (%)</th>
<th>Cytochrome P-450 or P-448 (% of recovery)</th>
<th>Cytochrome P-450 or P-448/Cytochrome P-420 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulphate (0.5%)</td>
<td>Yeast</td>
<td>21</td>
<td>1</td>
<td>36</td>
<td>0.3</td>
</tr>
<tr>
<td>Triton X-100 (1%)</td>
<td>Yeast</td>
<td>100</td>
<td>22</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Emulgen 911 (1%)</td>
<td>Yeast</td>
<td>50</td>
<td>10</td>
<td>57</td>
<td>4</td>
</tr>
<tr>
<td>Sodium cholate (1%)</td>
<td>Yeast</td>
<td>36</td>
<td>1</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>Triton X-100 (1%)</td>
<td>Rat liver</td>
<td>93</td>
<td>7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Sodium cholate (1%)</td>
<td>Rat liver</td>
<td>80</td>
<td>6</td>
<td>87</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3.3. Solubilization of Cytochrome P-450/P-448 by Various Detergents in Presence and Absence of Stabilizers. Yeast microsomes contained initially 0.7nmol cytochrome P-450/P-48 and 0.3nmol of cytochrome P-420 per ml and liver microsomes from phenobarbital-treated rats contained 1.5nmol cytochrome P-450 and 0.1nmol of cytochrome P-420 per ml. 0.1M Potassium phosphate buffer pH 7.2 containing 0.001M-EDTA, 0.001M dithiothreitol (except when reduced glutathione was present) and 20% glycerol was denoted as the phosphate buffer plus additives.
solubilizing membrane proteins, it often is solubilized by treatments which cause disruption of the hydrophobic interactions accompanying denaturation i.e. conversion to cytochrome P-420. Along with disruptions of the hydrophobic interactions, attack on natural ligand (a sulfhydryl group) induces the conversion of cytochrome P-450/P-448 to cytochrome P-420. The destruction of hydrophobic interactions, which protect the integrity of heme environment, also causes the displacing of the natural ligand.

Since the beginning attempts have been made to solubize cytochrome P-450/P-448 from microsomal fraction under conditions in which the lipid peroxidation is prevented and the natural ligand is not destroyed or removed or replaced. Under these conditions almost total recovery of cytochrome P-450/P-448 is possible and the ratio of cytochrome P-450/P-448 to cytochrome P-420 could actually be increased through this process. The cytochrome P-450/P-448 to cytochrome P-420 ratio during solubilization was also dependent on the pH of the medium. The highest ratio was obtained at pH 7.2. The presence of EDTA and a sulfhydryl compound (reduced glutathione in solubilization process) protected the cytochrome P-448 and increased the ratio of cytochrome P-448 to cytochrome P-420. This may be interpreted as a result of an increased aggregation of
cytochrome P-420 induced by EDTA in addition to the protection offered by a sulfhydryl compound (Autor et al., 1973). Amongst sulfhydryl compounds, reduced glutathione proved to be the best protector of cytochrome P-448 in the storage of microsomal fractions (Table 3.3). Dithiothreitol which is used most commonly as a stabilizer of cytochrome P-450 (Capdevila et al., 1975) was not as effective as reduced glutathione for yeast microsomal cytochrome P-448, but proved to be just as effective (and was used) later in the purification process. Ichikawa and Yamano (1967) found that glycerol and other polyols are able to stabilize cytochrome P-450 by preventing its conversion to cytochrome P-420. Glycerol is used most commonly in all handling of cytochrome P-450/P-448 and in this study it was replaced with sorbitol only in large scale disruption of yeast and in lyophilization.

For storage of microsomes in -70°C, 0.1% (v/v) Triton X-100 in 0.1M potassium phosphate buffer pH 7.2 (plus additives as described in Table 3.2) produced results similar to those of reduced glutathione (0.1%) in the above potassium phosphate buffer. In comparison cytochrome P-450 of rat liver microsomal fraction was very stable at -70°C stored in the phosphate buffer (containing 20% (v/v) glycerol and 0.001M dithiothreitol) alone.
Microsomes in the phosphate buffer (plus additives), containing 0.1% Triton X-100 and also in the phosphate (plus additives) and 0.1% reduced glutathione were solubilized by various detergents with the release of cytochrome P-450/P-448 (Table 3.3). Good yields in the enzyme were obtained with 1% (v/v) Triton X-100 in the presence or absence of 0.1% (w/v) reduced glutathione and by 1% (w/v) sodium cholate in the phosphate buffer (plus additives) and 0.1% (v/v) Triton X-100 only. In both cases 100% yield were obtained for yeast microsomal fraction, but for rat liver cytochrome P-450 the recoveries were 93% (1% Triton X-100) and 87% (1% sodium cholate in presence of 0.1% Triton X-100). When cytochrome P-450/P-448 was solubilized from yeast microsomal fraction by 1% sodium cholate in phosphate buffer (plus additives) containing 0.1% (w/v) reduced glutathione the yield (85%) was lower but the solubilization by or in presence of Triton X-100 produced problems in chromatography by hydrophobic association, unless its separation from soluble enzyme solution was accomplished thoroughly. Sodium cholate alone gave a good yield (80%) for rat liver, but only 36% for solubilization of yeast cytochrome P-450/P-448. Sodium dodecyl sulfate (0.5%, w/v) gave a very poor yield of yeast cytochrome P-450/P-448 (Table 3.3). The highest cytochrome P-450 or P-448/cytochrome P-420 ratios were obtained for solubilization of yeast cytochrome P-448 by or
in presence of Triton X-100 in the range 22-26, compared with only 7 for the rat liver enzyme. 1% (v/v) Emulgen 911 gave about 50% yield from the yeast microsomal fraction, regardless of the presence of 0.1% (v/v) Triton X-100. Renex 698, Tween 80, Triton N-101, Lubrol WX all in 1% (v/v) concentration, had no (or very little) solubilization effect on cytochrome P-450/P-448 from yeast microsomes.

Triton X-100 changes the spin state of cytochrome P-450 from high to low spin (Tamura et al., 1976), and has been shown to stabilize some enzymes (Takeda et al., 1972), by formation of micelles, which protect the hydrophobic environment of the enzyme. Conversely it has been reported that sulfhydryl reagents (Murakami and Mason, 1967) or high ionic strength of medium (Yoshida and Kumaoka, 1972), cause the spin state to change from low to high state and also prompt the cytochrome P-450 to cytochrome P-420 conversion by destroying or removing the sulfhydryl ligand. This suggests that Triton X-100, by changing the spin state from high to low state and retaining it in that state, may protect the sulfhydryl ligand and make it more accessible to heme iron, thus exerting a stabilizing effect on the cytochrome P-450/P-448.
3.3.3. Thermal Stability of Yeast Cytochrome P-448

Yeast cytochrome P-450/P-448 was more stable toward thermal denaturation when in the form of microsomes than when solubilized but not purified. After incubating microsomal enzymes even at 50°C for 5min only 50% of the yeast cytochrome P-448 was lost (Figure 3.2). For rat liver microsomal cytochrome P-450 no detectable loss was observed after 5min at 50°C. This again shows the liver enzyme to be more stable than the yeast enzyme as shown by Wiseman et al (1975b). When yeast microsomes cytochrome P-450/P-448 were treated with 0.1% (v/v) Triton X-100 although the critical temperature (temperature at which 50% of the enzyme is lost) remained the same, the temperature at which 5min incubation caused 100% loss of cytochrome P-448 increased from 55° to 60°C.

Solubilized yeast cytochrome P-450/P-448 is far less resistant to thermal denaturation than microsomal fraction (having a critical temperature of 35°C) as is expected from the loss of the lipid membrane. Triton X-100 at 0.1% was not able to restore the thermal stability of soluble enzyme to that of the microsomal fraction. A possible reason for the relative instability of the solubilized enzyme is the presence of 1% sodium cholate used for solubilizing the enzyme.
Figure 3.2. Thermal Stability of Cytochrome P-450/P-448 in Yeast Microsomal Fraction. Cytochrome P-448 (x), fluorescence (●), cytochrome P-420 (▲). Samples were incubated at various temperatures for 5min.
Surprisingly, purified cytochrome P-448 was more stable than solubilized enzyme (even when studied at lower protein concentration) as the temperature at which 50% of cytochrome P-448 is lost, was 45°C (Figure 3.3). This may have been because of absence of ionic detergent (sodium cholate) and the presence of a residual amount of non-ionic detergent (Emulgen 911) which was used during purification. This residual Emulgen may have a stabilizing effect due to protecting hydrophobic regions of the enzyme from the aqueous environment.

Fluorescence spectrophotometry (Figures 3.2 and 3.3) showed, only in the case of purified cytochrome P-448 the fluorescence increased as the result of a classical denaturation of protein, with apoprotein unfolding and heme being lost as the temperature was increased. Unfolding should have resulted in conversion of cytochrome P-448 to cytochrome P-420, but as the temperature was increased this conversion was not stoichiometric, probably due to loss of heme from the protein completely. Figure 3.4 shows when microsomal fraction was incubated in 45°C for more than 10 min there is a sharp disappearance in both cytochrome P-448 and cytochrome P-420.
Figure 3.3. Thermal Stability of Purified Cytochrome P-448 from Yeast. Cytochrome P-448 (x), fluorescence (●), cytochrome P-420 (▲). Samples were incubated at various temperatures for 5 min. Only residual amount of Emulgen 911 may be present.
Figure 3.4. Effect of Incubation at 45°C on Microsomal Cytochrome P-450/P-448 (x), Cytochrome P-420 (▲) and Fluorescence (◆).
More results on thermal stability of yeast cytochrome P-448 as measured by benzo(a)pyrene-3-monoxygenase activity are shown in Chapter 6.

3.3.4. Purification of Yeast Cytochrome P-448

Ammonium sulfate fractionation (at 35-65% of sat.) increased the specific content of yeast cytochrome P-450/P-448 nearly 10 fold with 86% yield. This fractionation was best performed at the enzyme pH optimum (pH 7.0) as reported by Woods and Wiseman (1979). At lower pH, \((\text{NH}_4)_2\text{SO}_4\) denatured cytochrome P-448, and cytochrome P-420 was formed. The denaturation of cytochrome P-450 by a high concentration of neutral salt has been reported by Imai and Sato (1967b), without specifying the pH used. They attributed this conversion to the disturbance of the hydrophobic environment around the heme moiety, either by primary action of the neutral salts or by secondary effects due to conformational changes in the cytochrome P-450 molecule. When ammonium sulfate fractionation was carried out with a sodium cholate concentration of protein solution higher than 0.33% the \((\text{NH}_4)_2\text{SO}_4\)-protein precipitate floated and did not separate from the solution by centrifugation. Therefore the sodium cholate-solubilized enzyme solution had to be diluted before the addition of ammonium sulfate.
Another limitation of ammonium sulfate fractionation was that even at pH 7.0 the cytochrome P-448 precipitate had to be collected and redissolved and dialyzed within three hours after the addition of the salt, otherwise denaturation occurred. This limitation made the large scale fractionation of this enzyme very difficult and therefore it was replaced by polyethylene glycol precipitation. Polyethylene glycol was also necessary to precipitate out the cytochrome P-450/P-448 and other mixed-function oxidase proteins (solubilized but still mixed with membrane material), by performing the centrifugation at a low speed (7500g).

Ammonium sulfate fractionation has been used mostly in purification of microbial cytochrome P-450/P-448. Yoshida et al (1977) in their purification steps to prepare cytochrome P-448 from semi-anaerobically grown Saccharomyces cerevisiae (baker's yeast) have included an ammonium sulfate fractionation (at 35-60% sat.) without having to maintain the pH at 7.0. Duppel et al (1973) also have used this fractionation to purify cytochrome P-450 from Candida tropicalis at 27-45% saturation. In purification of cytochrome P-450/P-448 from mammalian systems ammonium sulfate fraction is not used as commonly as in purifications, from yeast systems. Only Takemori et al (1975) in
purification of cytochrome P-450 from bovine adrenocortical mitochondria and Dialameh (1978) in preparation of this enzyme from untreated rat liver have utilized ammonium sulfate fractionation. In both cases the degree of purification achieved by use of this step was not as high as the ones reported for the yeast system, although because of the lower molecular weight of these two particular cytochrome P-450 the precipitation was completed at lower ammonium sulfate concentration.

Fractionation with polyethylene glycol, a nonionic hydrophilic polymer, was more widely used in earlier attempts to purify highly hydrophobic amino acids containing cytochrome P-450/P-448 (Chapter 7) especially when purifications were carried out only by ion exchange and absorption chromatographies (Coon et al, 1978; Johnson, 1980; Elshourbagy and Guzelian, 1980; Miki et al, 1980; Koop et al, 1981; Saito and Strobel, 1981). In all these cases to achieve the best results in selective fractionation, polyethylene glycol is added in step-wise manner and precipitate is collected after each addition.

Figure 3.5 shows the purification pathway mixed-function oxidase proteins from a single solution of yeast (Saccharomyces cerevisiae) microsomal fraction. It is shown
Figure 3.5. Purification Pathway of Cytochromes P-450/P-448, b<sub>5</sub>, NADPH:cytochrome c(P-450) Reductase and NADH:cytochrome b<sub>5</sub> Reductase from the same Preparation of Microsomes.
in this summary that hydrophobic absorption chromatography by n-aminooctyl Sepharose 4B fractionates cytochrome P-448, NADPH:cytochrome c(P-450) reductase, cytochrome b5 very effectively. Chromatography by this material is used by many workers in this field in recent years. In earlier stages of this study attempts were made to use the commercially available n-aminohexyl Sepharose 4B (Yoshida et al., 1977). Chromatography with this material gave a considerably lower yield (40%) for cytochrome P-448 than chromatography with n-aminooctyl Sepharose 4B (70%).

Table 3.4 presents data on purification steps of cytochrome P-448 from microsomal fraction of Saccharomyces cerevisiae. A novel combination of chromatographic techniques were used for the purification of cytochrome P-448. Besides the use of n-aminooctyl Sepharose 4B instead of low yielding n-aminohexyl derivative, the fast running hydroxylapatite-cellulose column, CM-Sephadex C-50 and finally DEAE-Sephacel were involved in the purification procedure. Hydroxylapatite-cellulose chromatography separated NADH:cytochrome b5 reductase from cytochrome P-448 successfully and DEAE-Sephacel was found to increase the purity of cytochrome P-448. By DEAE-Sephacel chromatography cytochrome P-450 was separated from cytochrome P-448. Although the amount of cytochrome P-450 (which bound to
<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total Amount (nmol)</th>
<th>Specific Content (nmol/mg)</th>
<th>Stage Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal fraction</td>
<td>7200</td>
<td>266</td>
<td>0.036</td>
<td>100</td>
</tr>
<tr>
<td>Solubilized</td>
<td>6100</td>
<td>266</td>
<td>0.043</td>
<td>100</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4) Fractionation</td>
<td>600</td>
<td>230</td>
<td>0.38</td>
<td>86</td>
</tr>
<tr>
<td>Aminoocetyl Sepharose 4B</td>
<td>20</td>
<td>160</td>
<td>8.00</td>
<td>70</td>
</tr>
<tr>
<td>Hydroxylapatite-cellulose</td>
<td>10</td>
<td>120</td>
<td>12.00</td>
<td>75</td>
</tr>
<tr>
<td>CM-Sephadex C-50</td>
<td>6.9</td>
<td>100</td>
<td>14.50</td>
<td>83</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>4.1</td>
<td>72</td>
<td>17.60</td>
<td>72</td>
</tr>
</tbody>
</table>

Table 3.4. Summary of Purification of Cytochrome P-448 from Yeast Microsomal Fraction. Cytochrome P-450 content of preparation was 1.5 nmol separated from cytochrome P-448 by DEAE-Sephadex step.
DEAE-Sephacel) was eluted to be only 2% of cytochrome P-448 eluate, this was an evidence for existence of yeast cytochrome P-450/P-448 in multiform.

Purified cytochrome P-448 had a specific content of 17.6 nmol per mg of protein, and overall yield of cytochrome P-448 from microsomal fraction was 27%. This highly purified enzyme was not found to contain any NADPH:cytochrome c(p-450) reductase, cytochrome b$_5$, NADH:cytochrome b$_5$ reductase and cytochrome P-420.

Table 3.5 presents a comparison between specific content and degree of purity of cytochrome P-448 purified from *Saccharomyces cerevisiae* for this study and other recent preparations. Specific content of 17.6 nmol/mg presents 97% purity in respect to molecular weight of this enzyme determined by SDS-polyacrylamide gel electrophoresis (read on).

Other purification methods such as chromatography with known cytochrome P-450/P-448 specific affinity materials were examined in order to simplify the procedure shown on Table 3.4, but none produced any significant result. Laurate-aminohexyl Sepharose 4B used by Gibson and Schenkman (1978) did not retain cytochrome P-448 from
| Cytochrome P-448 of *S. cerevisiae* (brewer's yeast) in this study | 17.6 | 55500 | 97 |  
| Cytochrome P-448 from *S. cerevisiae* (baker's yeast) | 14.7 | 51000 | 75 | *Yoshida* et al., 1977 |
| Cytochrome P-448 (β-naphthoflavone-treated rat liver) | 18.1 | 55000 | 99 | *Saito* and *Strobel*, 1981 |
| Cytochrome P-448 (3-methylcholanthrene-treated rat liver) | 17.9 | 54000 | 96 | *Elshaharbey* and *Guzelian*, 1980 |
| Cytochrome P-448 (3-methylcholanthrene-treated rabbit liver) | 17.9 | 54000 | 96 | *Imai* et al., 1980 |
| Cytochrome P-450 (phenobarbital-treated rat liver) | 15.1 | 50000 | 75.5 | *Elshaharbey* and *Guzelian*, 1980 |
| Cytochrome P-450 (isosafrol-treated rat liver) | 17.0 | 52900 | 90 | *Fisher* et al., 1981 |
| Cytochrome P-450 (clofibrate-treated rat liver) | 17.2 | 50000 | 86 | *Gibson* et al., 1982 |
| Cytochrome P-450 (2,3,7,8-tetrachloro-dibenzo-p-dioxin-treated rabbit liver) | 19.8 | 51500 | 100 | *Johnson*, 1980 |
| Cytochrome P-450 (phenobarbital-treated rabbit liver) | 13.2 | 49000 | 64 | *Guengerich*, 1977b |
| Cytochrome P-450 (β-naphthoflavone-treated rabbit liver) | 13.3 | 53000 | 70 | *Guengerich* 1977b |
| Cytochrome P-450 (untreated rabbit liver) | 19 | 52000 | 98 | *Koopand Coon* 1979 |
| Cytochrome P-450 (pseudomonas putida) | 16 | 46000 | 73 | *Katagiri* et al., 1968 |
Saccharomyces cerevisiae. Aniline Sepharose 4B suggested by Takemori et al (1975) bound to this enzyme but the recovery was very low due to denaturation of cytochrome P-448 by high concentration of KCl in the elution buffer. When immobilized cytochrome b5 on Sepharose 4B (Miki et al, 1980) was used as chromatography the retention was insignificant.

3.3.5. Further Purification of Other Mixed-Function Oxidase Proteins of Yeast

NADPH:cytochrome c(P-450) reductase eluted from n-aminooctyl Sepharose 4B was further purified by 2',5'-ADP-agarose column. Table 3.6 presents the purification summary of NADPH:cytochrome c(P-450) reductase as a by-product of cytochrome P-448. The specific activity of purified NADPH:cytochrome c(P-450) reductase obtained from Saccharomyces cerevisiae by this procedure was 48 U/mg (one unit of NADPH:cytochrome c(P-450) reductase is the amount of enzyme that reduces 1 μmol of cytochrome c per min at 22°C). NADPH:cytochrome c(P-450) reductase content of microsomal fraction (0.08 U/mg) was in agreement with 0.081 U/mg reported for Saccharomyces cerevisiae (baker's yeast) by Yoshida et al (1974b). Although the specific activity of purified NADPH:cytochrome c(P-450) reductase obtained in this study is lower than 60 U/mg and 56 U/mg reported for purified enzyme from rat liver (phenoarbital-treated) by
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total Activity (μmol min⁻¹, 22°C)</th>
<th>Specific Content (μmol min⁻¹ mg⁻¹)</th>
<th>Stage Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal fraction</td>
<td>7200</td>
<td>600</td>
<td>0.08</td>
<td>100</td>
</tr>
<tr>
<td>Solubilized</td>
<td>6100</td>
<td>552</td>
<td>0.09</td>
<td>92</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Fractionation</td>
<td>600</td>
<td>420</td>
<td>0.70</td>
<td>76</td>
</tr>
<tr>
<td>Aminoocytly Sepharose 4B</td>
<td>32</td>
<td>360</td>
<td>11.25</td>
<td>69</td>
</tr>
<tr>
<td>2',5'-ADP-Agarose</td>
<td>4</td>
<td>192</td>
<td>48.00</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 3.6. Summary of Purification of NADPH:Cytochrome c(P-450) Reductase from Yeast. Microsomal Fraction.
Guengerich (1977), Gum and Strobel (1979) respectively, it was free of cytochrome P-448, cytochrome b_5 and NADH:cytochrome b_5 reductase. Overall yield of 26% (from microsomal fraction) is in line with yields reported by the above workers on rat hepatic enzyme, despite the fact that ammonium sulfate fractionation was optimized only for cytochrome P-448.

Tables 3.7 and 3.8 show the steps performed to purify cytochrome b_5 and NADH:cytochrome b_5 reductase from Saccharomyces cerevisiae again as by-products of cytochrome P-448. The specific content of cytochrome b_5 was 74.8 nmol/mg which is slightly higher than the value (74 nmol/mg) obtained for this cytochrome from anaerobically grown Saccharomyces cerevisiae (baker's yeast) by Yoshida et al (1974a). These workers purified cytochrome b_5 exclusively by ammonium sulfate fractionation, gel filtration on Sephadex G-75 column and two DEAE-cellulose chromatographies with an overall yield of 14% in comparison with 21% obtained in this study.

The presence of NADH:cytochrome b_5 reductase in Saccharomyces cerevisiae was reported by Yoshida et al (1974a). This enzyme has been solely purified from bovine liver microsomes by Schafer and Hultquist (1980) with
<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total Amount (nmol)</th>
<th>Specific Content (nmol/mg protein)</th>
<th>Stage Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal fraction</td>
<td>7200</td>
<td>295</td>
<td>0.04</td>
<td>100</td>
</tr>
<tr>
<td>Solubilized</td>
<td>6100</td>
<td>290</td>
<td>0.05</td>
<td>98</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Fractionation</td>
<td>600</td>
<td>280</td>
<td>0.46</td>
<td>96</td>
</tr>
<tr>
<td>Aminooctyl Sepharose 4B</td>
<td>5</td>
<td>110</td>
<td>22.00</td>
<td>39</td>
</tr>
<tr>
<td>First Sephadex G-75</td>
<td>2.70</td>
<td>80</td>
<td>29.60</td>
<td>73</td>
</tr>
<tr>
<td>Second Sephadex G-75</td>
<td>0.82</td>
<td>62</td>
<td>74.80</td>
<td>78</td>
</tr>
</tbody>
</table>

Table 3.7. Summary of Purification of Cytochrome b⁵ from Yeast Microsomal Fraction.
<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total Activity (mol min⁻¹, 22°C)</th>
<th>Specific Content (mol min⁻¹ mg⁻¹)</th>
<th>Stage Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal fraction</td>
<td>7200</td>
<td>670</td>
<td>0.09</td>
<td>100</td>
</tr>
<tr>
<td>Sodium cholate extract</td>
<td>6100</td>
<td>625</td>
<td>0.10</td>
<td>93</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Fractionation</td>
<td>600</td>
<td>612</td>
<td>1.02</td>
<td>97</td>
</tr>
<tr>
<td>Aminooctyl Sepharose 4B</td>
<td>20</td>
<td>515</td>
<td>25.75</td>
<td>84</td>
</tr>
<tr>
<td>Hydroxylapatite-cellulose</td>
<td>6</td>
<td>142</td>
<td>23.70</td>
<td>28</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>1.8</td>
<td>126</td>
<td>70.00</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 3.8. Summary of Purification of NADPH: Cytochrome b₅ Reductase from Yeast Microsomal Fraction.
specific activity of 102 U/mg in comparison to the value of 70 U/mg (Table 3.8) obtained for yeast enzyme in this study (one unit of NADH:cytochrome b$_{5}$ reductase is the amount of enzyme that reduces 1μmol of ferricyanide per min at 22°C). This enzyme was also purified to homogeneity from rabbit liver microsome by an extensive purification procedure (Mihara and Sato, 1975). The specific activity of this enzyme in homogenous state was shown by these workers to be 1245 U/mg. In this study cytochrome b$_{5}$ and NADH:cytochrome b$_{5}$ reductase were separated from cytochrome P-448 by n-aminooctyl Sepharose 4B and hydroxylapatite chromatography respectively. Each of these proteins were free of other components of mixed-function proteins.

3.3.6. **Spectral Properties of Yeast Cytochrome P-448**

The absorption spectrum shown in Figure 3.6, further indicate that the purified yeast cytochrome P-448 is devoid of both cytochromes P-420 and b$_{5}$. The spectra are essentially identical with that of solubilized cytochrome P-448. The differences between these spectra with that reported by Yoshida et al (1977) were locations of α- and β-bands at 565, 578nm rather than 540, 575nm and the fact that the Soret band (at 418nm) for the cytochrome P-448 obtained in this study did not shift to 412nm in reduced form. Reduction of cytochrome P-448 was found to fuse the
Figure 3.6. Absorption Spectra of Yeast Cytochrome P-448. Cytochrome P-448 was in 0.005M potassium phosphate buffer, pH 7.0, containing 20% glycerol. ____, oxidase form; ______, reduced form; ______, reduced CO-difference spectrum.
α- and β-bands into a single absorption at 565nm. It was noted that the spectrum of oxidized form in addition to have a Soret Peak at 418nm, showed no absorption in the 650nm region which is characteristic of a low-spin cytochrome P-450/P-448 (Yoshida, 1978). As mentioned the Soret peak of reduced CO-difference spectrum of yeast is situated at 447-448nm. In this respect, therefore, yeast enzyme resembles the cytochrome purified from liver microsomes of 3-methylcholanthrene-pretreated mammals (Kawalek et al., 1975; Hashimoto and Imai, 1976), although the latter preparation exhibits a high-spin type absorption spectrum in the oxidized state.

The spectral parameters of cytochrome P-448 from *Saccharomyces cerevisiae* are shown in Table 3.9. Here the values of the extinction coefficient were calculated on the basis of one mol of cytochrome contains one mol of protoheme (Chapter 7).

### 3.3.7. Molecular Weight of Yeast Cytochrome P-448

SDS-polyacrylamide gel electrophoresis showed the homogenous nature of the cytochrome P-448 (Figure 3.7). By use of several marker proteins the molecular weight of cytochrome P-448 was estimated to be 55,500 (Figures 3.7 and 3.8). This is similar to the molecular weight of cytochrome
<table>
<thead>
<tr>
<th>Cytochrome P-448 form</th>
<th>Absorption Maxima (nm)</th>
<th>(mM$^{-1}$.cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidised</td>
<td>417, 565, 578</td>
<td>120, 14, 12</td>
</tr>
<tr>
<td>Reduced</td>
<td>418, 565</td>
<td>78, 12</td>
</tr>
<tr>
<td>CO-complex of reduced form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(absolute spectrum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>446, 560</td>
<td></td>
</tr>
<tr>
<td>CO-difference spectrum of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reduced form</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>448, 562</td>
<td>92 (447-490nm), 20</td>
</tr>
</tbody>
</table>

Table 3.9. Spectral Parameters of Cytochrome P-448 from Yeast.
Figure 3.7. Electrophoresis of Purified Cytochrome P-448 from Yeast. In wells 1, 3, 5, 7, 9, 11 various quantities of cytochrome P-448 and in wells 2, 4, 6, 8, 10, 12 various quantities of protein markers were used.
Figure 3.8. Molecular Weight Estimation of Purified Cytochrome P-448. Mobility of cytochrome P-448 on SDS-polyacrylamide gel (10%) was compared with the mobility of bovine serum albumin (A) M.W. = 68000, catalase (B) monomeric M.W. = 60000, ovalbumin (C) M.W. = 45000 and aldolase (D) monomeric M.W. = 39500.
P-448 from liver microsomes of 3-methylcholanthrene or β-naphthoflavone treated mammals, 54000-55000 but is larger than the molecular weight of cytochrome P-448 from semi-anaerobically grown baker's yeast, 51000 (Table 3.6). This molecular weight value determines the specific content of a 100% pure cytochrome P-448 as 18nmol per mg of protein. Therefore, the cytochrome P-448 obtained in this study is 97% pure.

Considering the homogeneous state of this preparation shown by SDS-polyacrylamide gel electrophoresis, three possibilities may be cited to account for this 3% discrepancy. First, a considerable amount of apo-cytochrome P-448 may be present in the purified preparation. Secondly, the SDS-gel electrophoresis method may have given a smaller molecular weight because of the strongly hydrophobic nature of cytochrome P-448 (Chapter 7). Finally, the purified cytochrome may have been contaminated with an impurity or impurities having the same or nearly the same molecular weight as the yeast cytochrome P-448.
4. Time Dependency of Carbon Monoxide Difference Spectrum of Reduced Cytochrome P-448 from *Saccharomyces cerevisiae*: Slow Reduction Caused by Presence of Triton X-100 and Emulgen 911

4.1 Introduction

Triton X-100, added to microsomal fraction of *Saccharomyces cerevisiae* for the purpose of stabilization or solubilization, affects the carbon monoxide difference spectrum of reduced cytochrome P-448 and consequently the measurement of cytochrome P-448. It has been shown the cytochrome P-450/P-448 from *Saccharomyces cerevisiae* in the presence of Triton X-100 has an absolute spectrum identical to that of low-spin state i.e. Soret peak at 418nm (Yoshida and Kumaoka, 1972). Yeast cytochrome P-450/P-448 is known to exist in the high and low-spin states and that the equilibrium between the two states depends on the environmental conditions (Mitani and Horie, 1969a and 1969b). High spin and low-spin cytochrome P-450/P-448 corresponds to the Soret peaks of the absolute spectrum at 390 and 418nm respectively. Triton X-100 is shown here to behave as a type II binding compound (absorption maximum at 418nm and minimum at 390nm) and to modulate the spin state of yeast cytochrome P-448 from high to low form. Low-spin yeast cytochrome P-448 having lower mid-point redox potential, is
reduced by sodium dithionite more slowly than the high spin form.

Although sodium dithionite is routinely used as the reagent to reduce the heme iron in the spectrophotometric assay of cytochrome P-450/P-448, little has been reported about the kinetics or mechanism of this reaction (Omura and Sate, 1964b; Peterson, 1971). In the course of studies directed toward the determination of extinction coefficients for membrane-bound and solubilized cytochrome P-450 and P-420, Schenkman et al. (1973) noted that complete reduction of the cytochromes by sodium dithionite required 2 min. In this chapter experiments were performed to investigate the reduction rate by sodium dithionite of cytochromes P-448 and P-450 from yeast and phenobarbital-treated rat liver in the presence and absence of Triton X-100. As it was observed in Chapter 3, cytochrome P-448 accounts for the majority of type of enzyme in yeast and therefore is denoted as P-448, in microsomal fraction and otherwise, in this chapter and thereafter.
4.2. Methods and Materials

4.2.1. Preparation Microsomal Fraction from *Saccharomyces cerevisiae*

Brewer's yeast *Saccharomyces cerevisiae* (NCYC No. 240) was grown and microsomal fraction was obtained as described in Chapter 3. The microsomal fraction was suspended in 0.1M potassium phosphate buffer, pH 7.2, containing 0.001M-EDTA, 0.001M dithiothreitol and 20% (v/v) glycerol. Triton X-100 was added to this suspension with a final concentration of 0.1%. The control samples did not contain any Triton X-100.

Solubilization of cytochrome P-448 by Triton X-100 was achieved by adding 1% (v/v) of this detergent to the microsomal suspension, stirring for 1h, centrifuging at 160,900g for 1h and collecting the supernatant, which contained the solubilized cytochrome P-448. For determination of mid-point redox potential of cytochrome P-448, purified enzyme, prepared as described in Chapter 3, was used.

4.2.2. Measurement of the Reduction Rate of Cytochrome P-448 by Sodium Dithionite.

The carbon monoxide difference spectrum of yeast cytochrome P-448 was recorded by a spectrophotometric method.
as described in Chapter 3. Spectrophotometry was carried out at 22°C unless mentioned otherwise.

The rate of reduction was measured by placing cytochrome P-448 (microsomal fraction) in both reference and sample cuvettes. Carbon monoxide was bubbled through the sample cuvette at the rate of one bubble per second for 30 seconds, one mg/ml of sodium dithionite was added to the sample cuvette and mixed quickly, and absorbance changes at 448nm were recorded. In some cases carbon monoxide was not bubbled through the solution and the rate of reduction was measured at 440nm.

4.2.3. Determination of Mid-Point Redox Potential of Cytochrome P-448

Mid-point redox potentials of cytochrome P-448 in the presence and absence of Triton X-100 were quantified with the dye photoreduction technique as described by Sligar et al (1979) and Sligar et al (1974). Safranine T, with a mid-point potential of -289mV (Clark, 1960), was used as an indicator and mediator.

Reagents
- 0.05M Sodium phosphate buffer, pH 7.25 containing 0.01M-EDTA and 20% (v/v) glycerol
- 0.2M Glucose solution
- 0.15mM Safranine T
- Mixture of glucose oxidase and catalase, 100 I.U. of each per ml of mixture

Method
Buffer (1.4ml) and glucose solution (0.4ml) were placed in each of the reference and sample Thunberg cuvettes and baseline 390 to 540nm was recorded. Difference spectrum of fully oxidized enzyme and dye was recorded (390-450nm) after the addition of one ml of purified cytochrome P-448 (7.2nmol, with or without 1% Triton X-100), 0.06ml of Safranine T and 0.1ml of glucose oxidase-catalase mixture to the sample cuvette and 0.1ml of glucose oxidase-catalase mixture and 1.06 ml of buffer to reference cuvette. Before the spectrophotometric recording of the spectra, the enzyme and dye solution was made anaerobic by repeated evacuation and flushing of the Thunberg cell with argon that had been freed of oxygen using an apparatus described by Meites and Meites (1948). The system potential (E_D) was set by irradiation for 30s bursts with an 100 watt lamp, causing successive reductions. The fraction of cytochrome P-448 and dye reduced was determined by the optical density at 420nm and 520nm respectively (Sligar, 1976). Absorbance values were determined by scanning between these wavelengths and
were recorded when stable values were obtained. Finally 100% reduction of the enzyme and dye was accomplished by the addition of sodium dithionite and absorbances were recorded. Safranine T gave a negligible absorbance at 420nm and cytochrome P-448 was isosbestic (oxidized-reduced) at 518nm rendering deconvolution of cytochrome P-448 and dye absorbance at these wavelengths unnecessary. All absorbances were measured at 25°C and data were displayed as standard Nernst plots.

**Materials**

Safranine T was purchased from Sigma Chemical Ltd. Glucose oxidase and catalase were obtained from Calbiochem-Behring Co. Other chemicals were supplied by BDH Chemicals Ltd.

4.3. **Results and Discussion**

4.3.1. **Time Dependency of Cytochrome P-448 Assay in the Presence of Triton X-100**

The addition of Triton X-100 (a polyoxyethylene alkyl phenol) affected the CO-difference spectrum of yeast cytochrome P-448 (reduced CO cytochrome P-448 minus reduced cytochrome P-448) (Figure 4.1). It also affected the CO-difference spectrum of reduced cytochrome P-448 minus oxidized cytochrome P-448. The difference in absorbance at
Figure 4.1. Carbon Monoxide Difference Spectrum of Reduced Cytochrome P-448. (I) Microsomes treated with Triton X-100 (0.1%), curves 1-3 and 8 were recorded 1, 2, 3, and 8 min after initial scanning. (II) Control microsomes recorded 20 s after addition of CO. Cytochrome P-448 1 nmol/ml; sodium dithionite 1 mg/ml; 22°C.
448nm was low and eventually reached its maximum (real) value only after approximately 8min delay. The percentage of reduction was calculated as described by Gigon et al (1969), Ando and Horie (1971), and the maximal increase in absorbance in the presence of excess sodium dithionite was taken at 100%. The percentage of remaining oxidized cytochrome P-448 was plotted on a logarithmic scale (Figure 4.2), and shows that the rate of reduction of cytochrome P-448 in the presence of Triton X-100 is biphasic with complete reduction taking 8min.

The time dependency of the carbon monoxide difference spectrum of Triton X-100 treated yeast cytochrome P-448 could not be attributed to slow solubilization of CO and therefore its slow binding to the reduced cytochrome P-448. As was described by Ando and Horie (1971), carbon monoxide binding is a very rapid reaction. When sodium dithionite was introduced to the sample cuvette 4min after the addition of carbon monoxide, the CO-difference spectrum of reduced cytochrome P-448 was still time dependent. The same results were obtained in the absence of CO for Triton X-100 treated microsomes by measuring the rate of reduction at 440nm and, although the difference in absorbance was very small, it increased with time as before.
Figure 4.2. Effect of Triton X-100 on the Rate of Reduction by Sodium Dithionite in the Presence of Carbon Monoxide. (A) Microsomes treated with Triton X-100 (0.1%). (B) Control microsomes. The reaction was measured at 448 nm. Cytochrome P-448 1 nmol/ml; sodium dithionite 1 mm/ml; 22°C.
In another experiment the CO-difference spectrum of Triton X-100 treated yeast microsomes was recorded 4min after the addition CO. The sample cuvette was freed of CO by bubbling oxygen (1 bubble/s for 30s) through it, and it was oxidized by adding H$_2$O$_2$ (4mM in concentration). Then the Triton X-100-difference spectrum was recorded, and the difference spectrum showed a type II change, i.e., no peak at 390nm, a Soret peak at 418nm. This ruled out the slow displacement of Triton X-100 by the addition of CO, to which the time dependency of the CO-difference spectrum of Triton X-100 treated microsomes could have been related.

Other factors such as the temperature and the rate of CO bubbling through the samples were taken into consideration. At a low temperature (4°C) the height of 448nm peak in the carbon monoxide difference spectrum of cytochrome P-448 was smaller, and as the temperature was raised toward 22°C, the peak became larger. At a constant temperature, the spectrum was time dependent when the sample contained Triton X-100 and was not time dependent when free of Triton X-100 (control). If carbon monoxide was bubbling faster than 2 bubbles/s, a small portion of cytochrome P-448 was converted to cytochrome P-420, and as the time went by (5min) some of this cytochrome was reconverted to cytochrome P-448. But in the case of Triton X-100 treated microsomes,
their reconversion was completed in 5min and then another 5min was required for the 448nm peak to reach its maximum height. This showed that the rate of bubbling CO to the sample cuvette is a negligible factor and the time dependency of CO-difference spectrum of Triton X-100 treated microsomes is due only to the slow rate reduction.

Aniline, a type II substrate (Chapter 5), proved to have the same effect as Triton X-100 on the CO-difference spectrum of yeast microsomes. The 448nm peak decreased considerably in comparison with the control microsomes and it reached its real height after approximately 8min (Figure 4.3).

Emulgen 911, another non-ionic detergent (a polyoxyethylene alkylaryl ether), used in all the chromatography buffers in the purification process, also decreased the reduction rate of cytochrome P-448 by sodium dithionite. But Emulgen 911 treated microsomes or Emulgen 911 containing purified cytochrome P-448, needed only 3min for their reduction to be completed.
Figure 4.3. Carbon Monoxide Difference Spectrum of Aniline Bound Reduced Cytochrome P-448. Microsomes were treated with aniline (2mM). Curves 1-3 and 8 were recorded 1, 2, 3 and 8 min after initial scanning.
4.3.2. Mid-Point Redox Potential and Spin State Equilibrium

The Nernst equation which uses as a standard plot of oxidation-reduction equilibrium, is as follows:

\[ E_D = E_m \text{(dye)} - \frac{RT}{F} \ln \frac{f^D}{f^0_D} = E_m \text{(Protein)} - \frac{RT}{F} \ln \frac{f^R}{f^0_P} \]

where

- \( f_D \) is the fraction of dye oxidized (o) or reduced (r)
- \( f_P \) is the fraction of protein oxidized (o) or reduced (r)
- \( E_D \) = system potential
- \( E_m \) = mid-point redox potential
- \( R \) = gas constant
- \( T \) = absolute temperature
- \( F \) = Faraday constant

Tables 4.1 and 4.2 show the values of \( E_D \) determined from 420 and 518nm absorbances at different stage of oxidation-reduction in the absence and presence of Triton X-100 respectively.

Values of mid-point redox potential for yeast cytochrome P-448 in the presence and absence of Triton X-100 was determined by the standard plot of the Nernst equation to be -347mV and -308mV respectively (Figure 4.4). The decrease
<table>
<thead>
<tr>
<th>Oxidation-Reduction State</th>
<th>Absorbance</th>
<th>$f_p^R$</th>
<th>$f_p^O$</th>
<th>$\ln \frac{f_P^R}{f_P^O}$</th>
<th>$f_D^R$</th>
<th>$f_D^O$</th>
<th>$\frac{RT \ln f_D^O}{f_D^O}$</th>
<th>$E_D$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully oxidized</td>
<td>0.163</td>
<td>0.0554</td>
<td>-------</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>First Photoreduction</td>
<td>0.1597</td>
<td>0.0512</td>
<td>0.0873</td>
<td>0.9127</td>
<td>2.347</td>
<td>0.1239</td>
<td>0.8761</td>
<td>-50.2</td>
</tr>
<tr>
<td>Second Photoreduction</td>
<td>0.1572</td>
<td>0.0489</td>
<td>0.1534</td>
<td>0.8466</td>
<td>-1.708</td>
<td>0.1917</td>
<td>0.8083</td>
<td>-36.9</td>
</tr>
<tr>
<td>Third Photoreduction</td>
<td>0.1565</td>
<td>0.0461</td>
<td>0.1720</td>
<td>0.8280</td>
<td>-1.572</td>
<td>0.2743</td>
<td>0.7257</td>
<td>-25.0</td>
</tr>
<tr>
<td>Fully Reduced</td>
<td>0.1252</td>
<td>0.0215</td>
<td>-------</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 4.1. Determination of System Potential at Various Stages of Oxidation-Reduction for Cytochrome P-448 Free of Triton X-100.
<table>
<thead>
<tr>
<th>Oxidation-Reduction State</th>
<th>Absorbance</th>
<th>$f^r_p$</th>
<th>$f^o_p$</th>
<th>$\ln \frac{f^r_p}{f^o_p}$</th>
<th>$f^r_D$</th>
<th>$f^o_D$</th>
<th>$\frac{RT}{F} \ln \frac{f^r_D}{f^o_D}$</th>
<th>$E_D$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully oxidized</td>
<td>0.1545</td>
<td>0.0502</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>First Photoreduction</td>
<td>0.1496</td>
<td>0.0475</td>
<td>0.1307</td>
<td>0.8673</td>
<td>-1.895</td>
<td>0.1071</td>
<td>0.8929</td>
<td>-54.5</td>
</tr>
<tr>
<td>Second Photoreduction</td>
<td>0.1465</td>
<td>0.0396</td>
<td>0.2133</td>
<td>0.7867</td>
<td>-1.305</td>
<td>0.4206</td>
<td>0.5794</td>
<td>-8.2</td>
</tr>
<tr>
<td>Third Photoreduction</td>
<td>0.1442</td>
<td>0.0357</td>
<td>0.2747</td>
<td>0.7253</td>
<td>-0.971</td>
<td>0.5754</td>
<td>0.4246</td>
<td>7.8</td>
</tr>
<tr>
<td>Fourth Photoreduction</td>
<td>0.1409</td>
<td>0.0334</td>
<td>0.3627</td>
<td>0.6373</td>
<td>-0.564</td>
<td>0.6667</td>
<td>0.3333</td>
<td>17.8</td>
</tr>
<tr>
<td>Fifth Photoreduction</td>
<td>0.1395</td>
<td>0.0317</td>
<td>0.4000</td>
<td>0.6000</td>
<td>-0.405</td>
<td>0.7341</td>
<td>0.2659</td>
<td>26.1</td>
</tr>
<tr>
<td>Fully Reduced</td>
<td>0.1170</td>
<td>0.025</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 4.2. Determination of System Potential at Various Stages of Oxidation-Reduction for Cytochrome P-448 in the Presence of 1% Triton X-100.
Figure 4.4. Redox Potential Titration of Purified Yeast Cytochrome P-448 in the Presence and Absence (Control) of Triton X-100 (1%).
in mid-point redox potential in the presence of Triton X-100 could explain the slow rate of reduction in this case.

As was shown by Yoshida and Kumaoka (1975b) the peak at 394 nm of spectral high spin form of hepatic cytochrome P-450 is shifted to 416 nm (reverse type I spectral change), a spectral low spin form, upon binding with a compound containing a hydroxyl group. The Triton X-100 in this study used to stabilize or solubilize cytochrome P-448 in or from yeast microsomes, also contains hydroxyl groups.

The absolute spectra of Triton X-100 solubilized yeast microsomes gave direct evidence for this spectral spin state change (Figure 4.5). A major peak at 418 nm and no absorbance at 650 nm showed the existence of low spin cytochrome P-448, while the spectrum of the solubilized cytochrome P-448 with sodium cholate showed a shoulder at 390 nm, proving a spectral mixed spin form to be present. It was not possible to determine the spin content of Triton X-100 containing cytochrome P-448 by the temperature-dependent spin equilibrium technique (Chapter 8), due to the lack of temperature-induced spectral changes (specially in 4-22°C range).
Figure 4. Absolute Spectrum of Solubilized Cytochrome P-450 with Triton X-100 (1%); treated with sodium cholate (1%).
It has been clearly demonstrated that cytochrome P-450/P-448 redox equilibria vs. regulated via the spin state of the heme iron (Sligar et al, 1979; Gigon et al, 1969; Rein et al, 1979; Ristau et al, 1979). Therefore, it was suggested that the binding of the type I substrate to hepatic cytochrome P-450 could increase the rate of electron flow from NADPH to the cytochrome by modulating the spin state equilibrium of the electron accepting hemoprotein. Ando and Horie (1971) showed that reduction of cytochrome P-450 from adrenocortical mitochondria by sodium dithionite or by NADPH-dependent enzymatic system is not completely instantaneous. They reported substrates that produce the type II spectral changes such as pregnenolone, decrease the rate of reduction. They also indicated that when the spin state of heme iron is modulated from high to low, the rate of reduction is decreased and conversely, when it is changed from low to high the rate of reduction increases. These are in stark contrast with the Heintz and Peterson (1980) findings for cytochrome P-450cam that the factors controlling the reduction of this cytochrome by dithionite anion monomer are not simply a function of the spin state of the heme iron. They arrived at this conclusion by observing the rate constant for the reduction of camphor-free cytochrome P-450 (low spin) as being four times greater than
rate constant for the reduction of camphor-bound cytochrome P-450 (high spin).

It may be concluded that Triton X-100 can modulate the spin state of yeast cytochrome P-448 (the same as a type II or a reverse type I compound). This causes slow cytochrome P-448 reduction by sodium dithionite, making the measurement of cytochrome P-448 by the CO-difference spectrum method time dependent, which indicates the existence of a major error in the standard "immediate assay" procedure.

The measurement of the hepatic cytochrome P-450 in contrast, was not time dependent with or without the presence of Triton X-100. This may be caused by a higher mid-point redox potential in hepatic cytochrome P-450 (phenobarbital-treated rats) and/or due to the fact the Triton X-100 behaves like type I substrates (binding through its hydrophobic site to the cytochrome P-450 instead of via its hydroxyl group) upon addition to microsomal fraction of different animal species (Denk et al, 1971; Burke et al, 1975; Takeshige et al, 1972; Hutterer et al, 1970; Franklin, 1972b).
CHAPTER 5
5. The Binding of Compounds to Cytochrome P-448 from Saccharomyces cerevisiae

5.1. Introduction

The binding of several putative substances to highly purified cytochrome P-448 from yeast was studied. Type I spectral interactions were observed with lanosterol, ethylmorphine, sodium phenobarbitone, dimethylnitrosamine, perhydrofluorene and benzo(a)pyrene. 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, when interacted with microsomal fraction or purified cytochrome P-448 from yeast. Type II spectral changes were observed with imidazole, aniline and benzphetamine. The two well-known substrates of this enzyme, benzo(a)pyrene and lanosterol, showed high affinity (low spectral dissociation constants) for the enzyme.

The binding of benzo(a)pyrene with microsomal fraction and purified cytochrome P-448 from yeast, 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 cytochrome P-450 from phenobarbital-induced and cytochrome P-448 from β-naphthoflavone-induced rats.
5.2. Methods and Materials

5.2.1. The Spectral Interactions of Compounds with Yeast Cytochrome P-448

Spectral changes resulting from the addition of compounds to a solution of microsomal fraction or purified cytochrome P-450/P-448 (from yeast and rat liver) were recorded in 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 1ml of active cytochrome P-450/P-448 (usually 1M) and the other compartment contained 1ml of heat-denatured enzyme originally 1µM (devoid of cytochrome P-450/P-448 or of cytochrome P-420). Benzo(a)pyrene dissolved in dimethylformamide (2mg/ml) was added 1 microliter at a time to the active-enzyme compartment of the sample cuvette and the inactive-enzyme compartment of the reference cuvette. An equal volume of dimethylformamide was added to the sample's inactive-enzyme and reference's active-enzyme compartments. The difference spectrum was then recorded between 350 and 500nm.
Spectral titrations at fixed wavelengths were subjected to double-reciprocal plots, derived from the kinetic equation:

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

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, therefore, the reaction does not proceed to product \( P \). The reversibility of the first step in such a reaction had been demonstrated by Schenkman et al. (1967).

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

\[
K_s = \frac{([E_t] - [C]) \cdot [S]}{[C]}
\]

where \( E_t \) is the total amount of enzyme. By manipulation

\[
K_s = \frac{[E_t] [S]}{[C]} - [S]
\]

and

\[
[C] = \frac{[E_t] [S]}{K_s + [S]}
\]
The reciprocal of this equation

\[
\frac{1}{[C]} = \frac{K_S}{[E_t]} \cdot \frac{1}{[S]} + \frac{1}{[E_t]}
\]

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 \([C]\) and the X intercept is equivalent to \(-(1/K_S)\).

Thus for benzo(a)pyrene, double-reciprocal plots of \((\Delta A^{500-418nm})\) or \((\Delta A^{387-377nm} + \Delta A^{500-418nm})\) against benzo(a)pyrene concentration were used to determine values of \(K_S\) by means of least squares regression analysis. \((\Delta A^{387-377nm} + \Delta A^{500-418nm})\) was adopted instead of simply using \((\Delta A^{387-377nm})\) to allow for changes in base-line during the addition of benzo(a)pyrene. The double-cell technique was also used for spectral recording of ethylmorphine, lanosteryl and sodium phenobarbitone, but in all three cases a buffer solution (0.1M potassium phosphate pH 7.0 containing 20% glycerol) replaced the inactive enzyme. Different wavelengths were used for each compound according to the shape of the binding spectrum. For simple spectra the peak to trough height was normally used. The other compounds which showed spectral interaction were
aniline, benzphetamine, dimethylnitrosamine and perhydro-fluorene. The other compounds examined were sodium hexobarbitone, aminopyrene, ethoxyresorufin, β-naphthoflavone, lauric acid, ethoxycoomarin, benzo(a)pyrene, isosafrole and biphenyl.

5.2.2. Equilibrium Gel Filtration of Benzo(a)pyrene Yeast Cytochrome P-448 Complex

A method of equilibrium gel filtration derived from the principles outlined by Ferscht (1977) and by Kerridge and Tipton (1972) was used to investigate the binding of benzo(a)pyrene to purified cytochrome P-450/P-448 from yeast and rat liver. This technique was first shown by Hummel and 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 G-25 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 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 G-25 until an equilibrium is reached at the base-line concentration of I. So far as P and 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 the initial and equilibrating concentration of benzo(a)pyrene is c mol/L and the amount of benzo(a)pyrene bound to n sites on the cytochrome P-450/P-448 is p mol, then the concentration of the cytochrome P-450/P-448-benzo(a)pyrene complex is \( \frac{p}{n} \) mol/L, where V is the volume of the benzo(a)pyrene solution in liters. The concentration of free cytochrome P-450/P-448 must be \( \frac{e-p}{n} \) mol/L, where e is the total amount of cytochrome P-450/P-448. If

\[
P-448 + \text{BP} \xrightarrow{\text{P-450/P-448}} P-448-\text{BP}
\]

\[
\frac{e - \frac{p}{n}}{V} \quad c \quad \frac{p}{n} \quad \frac{1}{V}
\]
then the apparent association constant for each binding site is given by

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

or

\[ Kc \left( e - \frac{p}{n} \right) = \frac{p}{n} \]

therefore

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

and

\[ \frac{1}{c} \cdot \frac{p}{e} = nK - K \cdot \frac{p}{e} \]

If we put

\[ \delta = \frac{p}{e} \]

then

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

Hence a plot of \( \delta/c \) against \( \delta \) (a Scatchard plot) is a straight line of slope \(-K\), the intercept of which on the axis is \( n \), when \( \delta/c = 0 \).
Gel filtration was carried out on a small column (Pasteur pipette with dimensions of 45x5mm) packed with Sephadex G-25. The column was equilibrated with 0.2M potassium phosphate buffer pH 7.0, containing 10% dioxane (v/v) and benzo(a)pyrene, before each experiment. The benzo(a)pyrene in the buffer consisted of a fixed, known amount of [G-\(^3\)H] benzo(a)pyrene (10\(\mu\)Ci) and various known amounts of unlabeled benzo(a)pyrene for each experiment. In each experiment after the column was equilibrated with benzo(a)pyrene-containing buffer, a 50\(\mu\)l sample was taken and specific radioactivity was determined. In each experiment a small volume of purified cytochrome P-450/P-448 (usually 10\(\mu\)l) was applied to the column and 2-drop (0.06ml) samples were collected. These samples were counted for radioactivity in LKB1210 Ultrabeta scintillation counter (LKB, U.K.) after adding 4ml of toluene-metapol (2:1, v/v) scintilant containing 0.5% (w/v) 2,5-diphenyloxazole (PPO) and 0.02% (w/v) 1,4-bis (5-phenyloxazol-2-yl) benzene (POPOP). The binding of the benzo(a)pyrene to the cytochrome P-450/P-448 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 binding sites per mol of cytochrome P-450/P-448.

5.2.3. **Materials**

β-Naphthoflavone, aminopyrene, perhydrofluorene and benzo(a)pyrene was supplied by Aldrich Chemical Co. Imidazole, lanosterol, benzo(a)pyrene, 2,5-diphenyloxazole, isosafrol, ethoxycojiarin and lauric acid, were obtained from Sigma Chemical Corp. Ltd. Ethylmorphine and sodium hexobarbitone was supplied by May and Baker Ltd. [G-\(^3\)H]-benzo(a)pyrene was obtained from the Radiochemical Centre, Amersham (U.K.). Dr. G. G. Gibson of Department of Biochemistry, University of Surrey, supplied the benzphetamine. Dimethylnitrosamine was purchased from Phase Separation Ltd. (Scotland). Aniline, sodium phenobarbitone and biphenyl were supplied by BDH Chemical Ltd. 1,4-Bis-(5-phenyloxazol-2-yl) benzene was obtained from the Packard Instrument Co. Metapol was obtained from Durham Chemical Distributors Ltd. (U.K.)
5.3 Results and Discussion

5.3.1. The Spectral Interactions of Benzo(a)pyrene with Yeast Cytochrome P-448

When the binding spectrum of benzo(a)pyrene to microsomal cytochrome P-448 from yeast was recorded previously in this laboratory (Woods and Wiseman, 1979), a double-cell technique was adapted to remove the interference of benzo(a)pyrene, which absorbs in the wavelength range used (350-500nm). They used a buffer solution in one of the compartments of both the reference and the sample cuvettes to complement the enzyme solution in adjoining compartments. In order to determine the spectral binding dissociation constant ($K_s$), the results of spectral titrations at fixed wavelength at a range of benzo(a)pyrene concentrations were subjected to a double-reciprocal plot. In this study both spectral changes at peak plus trough ($\Delta A^{387-377nm} + \Delta A^{500-418nm}$) and at trough alone ($\Delta A^{418-500nm}$) were used in the double-reciprocal plot to establish an accurate method to determine $K_s$ and the extent of cytochrome P-448 binding to benzo(a)pyrene. Other investigators have used the spectral change in trough region ($\Delta A^{500-418nm}$) of the benzo(a)pyrene binding spectrum but with only a single-cell technique (Estabrook et al., 1978).

Non-specific binding, however, produces a maximum absorbance change in the 387-418nm region (determined from
from double-reciprocal plot of \((\Delta A_{387-377nm} + \Delta A_{500-418nm})\) vs. benzo(a)pyrene concentration), that is larger than 126 mM\(^{-1}\)cm\(^{-1}\), the theoretical maximum absorption coefficient, which is used to quantify the spectral change due to benzo(a)pyrene specific binding in the region (Ciniti et al., 1979).

The use of a heat-denatured enzyme (devoid of cytochrome P-448 or of cytochrome P-420) was investigated instead of the buffer in the double-cell method. In addition, another series of readings were made against denatured cytochrome P-448 and phospholipid (instead of active enzyme) with buffer in other compartments in the double-cell method. Results indicated that (1) non-specific binding is possibly the binding of benzo(a)pyrene to the phospholipid content of the enzyme, and (2) this non-specific binding to phospholipid increases only the absorbance changes in the 367-387nm region (i.e., at peak wavelength) and does not effect the spectral changes in the 500-418nm trough region. Therefore, if \(\Delta A_{\text{max}}\) at 418nm was taken to determine the concentration of cytochrome P-448-benzo(a)pyrene complex by using 57 mM\(^{-1}\)cm\(^{-1}\) as an absorption coefficient (Estabrook and Werringloer, 1978), it was not necessary to use denatured enzyme as a reference in a double-cell system to eliminate the effect of non-specific
binding. Spectra changes at this trough wavelength (418nm) were due only to the binding of cytochrome P-448 to benzo(a)pyrene.

Figure 5.1 shows the binding of benzo(a)pyrene (at various concentrations) with purified yeast cytochrome P-448. As previously pointed out, the binding of benzo(a)pyrene to yeast purified, resulted in a spectrum which had two peaks (367 and 387nm) and trough at 418nm. In contrast the peaks for yeast microsomal fraction were located at 365 and 385nm and the trough was at 415nm.

The spectral binding dissociation constant \( K_s \) was found to be the same whether \( \Delta A_{387-377nm} + \Delta A_{500-418nm} \) was used in the double-reciprocal plot against benzo(a)pyrene concentration or only the \( \Delta A_{500-418nm} \) values were employed. \( \Delta A_{500-418nm} \) was used for determination \( K_s \) values and the extent cytochrome P-450/P-448 binding to benzo(a)pyrene in all cases (Figure 5.2 and Table 5.1).

Table 5.1 shows the benzo(a)pyrene-binding properties of cytochrome P-450/P-448 (from yeast and rat liver). The values obtained for dissociation constants \( K_s \) were 18\( \mu \)M for microsomal fraction and 50\( \mu \)M for purified cytochrome P-448 from yeast. In contrast \( K_s \) values for microsomal fraction and purified cytochrome P-450 from phenobarbitol
Figure 5.1. The Binding Spectrum of Benzo(a)pyrene with Yeast Purified Cytochrome P-448. The benzo(a)pyrene concentration was in the range of 7.9-39.5 μM.
Figure 5.2. The Double-Reciprocal Plot of Spectral Binding Benzo(a)pyrene with Purified Cytochrome P-450/P-448. Phenobarbital-treated rat liver (x), β-naphthoflavone-treated (●) rat liver, yeast (▲).
Table 5.1. Comparison of the Benzo(a)pyrene-Binding Properties of Cytochrome P-450/P-448. Microsomal fraction (M) purified form (P).
(PB)-induced rats were 5\(\mu\)M and 31\(\mu\)M respectively. Corresponding \(K_g\) values were also determined for microsomal fraction and purified cytochrome P-448 from \(\beta\)-naphthofalalone (BNF)-induced rats to be 6\(\mu\)M, 38\(\mu\)M. These results should be compared with those reported previously, namely 0.8\(\mu\)M (Estabrook et al., 1978) and 9\(\mu\)M (Woods and Wiseman, 1979) for hepatic microsomal cytochrome P-450 from phenobarbital-induced rats and 36\(\mu\)M (Woods and Wiseman, 1979) for microsomal cytochrome P-448 from yeast.

The dissociation constant values obtained showed that, although the affinity of cytochrome P-450/P-448 for benzo(a)pyrene is relatively high (i.e., low \(K_g\) values), the unusual ability to achieve stoichiometric titration of cytochrome P-450 by benzo(a)pyrene that was demonstrated for rat liver microsomal fraction (Estabrook et al., 1978) is not achieved here (especially for purified enzymes). A stoichiometric titration (1:1molar ratio) would require \(K_g\) values of approximately 0.5\(\mu\)M, 0.26\(\mu\)M and 0.46\(\mu\)M for purified cytochromes P-448 (yeast), P-450 (phenobarbital-induced rat liver), P-448 (\(\beta\)-naphthofalalvone-induced rat liver), respectively.

It was also found that 100\% of yeast purified cytochrome P-448, 53\% (phenobarbital) and 92.5\%
(β-naphthoflavone) of rat liver purified cytochrome P-450/P-448 reacted with benzo(a)pyrene, as shown by comparison of benzo(a)pyrene-binding absorption with CO-binding absorption. When microsomal fractions were used instead of purified enzymes the values were 89%, 16% and 80% respectively. This value for microsomal fraction of 3-methylcholantherene-treated rat liver is 46% as reported by Estabrook et al (1978). The absorption coefficient used for determination of the concentration of the benzo(a)pyrene-cytochrome P-450/P-448 complex was 57mM cm$^{-1}$ for (ΔA$^{500-418nm}$) (Estabrook and Werringloer, 1978). The fact that a high percentage of cytochrome P-450/P-448 in purified form reacts with benzo(a)pyrene is not surprising as 94% of the purified yeast cytochrome P-448 (see Chapter 8) and 82% purified cytochrome P-450 from phenobarbital-induced rats (Gibson and Tamburini, 1980) were found to be in low spin state at room temperature (22°C), which readily combines with type I substrates such as benzo(a)pyrene.

Purified cytochrome P-448 from yeast showed less affinity for benzo(a)pyrene than microsomal fraction. The value of the dissociation constant for purified enzyme was calculated to be 50μM in comparison to 18μM for microsomal fraction. Thus it would seem that whereas solubilization has improved the benzo(a)pyrene metabolism by yeast
cytochrome P-448 (see Chapter 6) the effect is the reverse for spectral binding dissociation constant.

The percentage of purified hepatic cytochrome P-450, from phenobarbital-treated rats, which was bound to benzo(a)pyrene (53%) was small in comparison to 92.5% for purified cytochrome P-448 from β-naphthoflavone-treated rats, reflecting the increased proportion of benzo(a)pyrene-binding hemoprotein in spectrally P-448 dominant cytochrome. As for hepatic microsomal fractions, these percentages are smaller, but in case of cytochrome P-448 from β-naphthoflavone-treated rats is still high (80%). This is rather surprising in the light of the reported high content of the high spin form of hepatic microsomal cytochrome P-448 (Nebert and Kon, 1973).

5.3.2. The Spectral Interactions of Other Compounds with Yeast Cytochrome P-448

The binding spectra of yeast cytochrome P-448 with various compounds are shown in Figures 5.3-5.9. Table 5.2 describes the spectral binding type and parameters of the interaction of these compounds with highly purified yeast cytochrome P-448. The spectral interaction of lanosterol which is thought to be an endogenous substrate for cytochrome P-448 in *Saccharomyces cerevisiae* has previously
Figure 5.3. The Binding Spectrum of Lanosterol with Yeast Purified Cytochrome P-448. The lanosterol concentration was in the range 30–62μM.
Figure 5.4. The Binding Spectrum of Ethylmorphine with Yeast Purified Cytochrome P-448. The ethylmorphine concentration was in the range of 60-1000μM.
Figure 5.5. The Binding Spectrum of Sodium Phenobarbitone with Yeast Purified Cytochrome P-448. The sodium phenobarbitone concentration was in the range of 14-77μM.
Figure 5.6. The Binding Spectrum of Dimethylnitrosamine with Yeast Purified Cytochrome P-448. The dimethylnitrosamine concentration was in the range of 50–250 µM.
Figure 5.7. The Binding Spectrum of Imidazole with Yeast Purified Cytochrome P-448. The imidazole concentration was in the range of 400-1000μM.
Figure 5.8. The Binding Spectrum of Aniline with Purified Yeast Cytochrome P-448. The aniline concentration was in the range of 2-5mM.
Figure 5.9. The Binding Spectrum of Benzphetamine with Yeast Purified Cytochrome P-448. The benzphetamine concentration was in the range of 2-20mM.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Difference Spectrum Maxima (nm)</th>
<th>Difference Spectrum Minima (nm)</th>
<th>Type of Binding</th>
<th>K_s (µM)</th>
<th>ΔA_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole</td>
<td>429</td>
<td>407</td>
<td>II</td>
<td>8</td>
<td>0.06, (ΔA^{430-408nm})</td>
</tr>
<tr>
<td>Lanosterol</td>
<td>367, 387</td>
<td>416</td>
<td>I</td>
<td>80</td>
<td>0.019, (ΔA^{387-377nm} + ΔA^{500-416nm})</td>
</tr>
<tr>
<td>Ethylmorphine</td>
<td>355</td>
<td>412</td>
<td>I</td>
<td>166</td>
<td>0.06, (ΔA^{382-412nm})</td>
</tr>
<tr>
<td>Aniline</td>
<td>425</td>
<td>408</td>
<td>II</td>
<td>5</td>
<td>0.039, (ΔA^{426-408nm})</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>367, 387</td>
<td>417</td>
<td>I</td>
<td>66</td>
<td>0.021, (ΔA^{385-375nm} + ΔA^{500-416nm})</td>
</tr>
<tr>
<td>Benphetamine</td>
<td>422</td>
<td>406</td>
<td>II</td>
<td>1660</td>
<td>0.018, (ΔA^{423-405nm})</td>
</tr>
<tr>
<td>Dimethylnitrosamine</td>
<td>385</td>
<td>420</td>
<td>I</td>
<td>220</td>
<td>0.021, (ΔA^{385-420nm})</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>367, 387</td>
<td>418</td>
<td>I</td>
<td>50</td>
<td>0.057, (ΔA^{418-500nm})</td>
</tr>
</tbody>
</table>

Table 5.2. Binding Properties of Various Compounds to Yeast Cytochrome P-448. K_s and ΔA_{max} values were calculated by double reciprocal plots of concentrations of the compounds against absorbances of the enzyme-compound complex at the indicated wavelength.
been described by Aoyama and Yoshida (1978a). These authors also found that this compound gave rise to a type I interaction with purified cytochrome P-450/P-448 from baker's yeast grown semi-anaerobically although no value for the spectral dissociation constant ($K_s$) was reported.

In this study, in addition to benzo(a)pyrene and lanosterol, type I binding spectra were observed with ethylmorphine (reported as a type I substrate for mammalian cytochrome P-450 by Davies et al., 1969; Sasame and Gillette, 1969), sodium phenobarbitone (also reported as a type I substrate by Schenkman et al., 1969; Topham, 1970), dimethyl-nitrosamine and perhydrofluorine. Yeast cytochrome P-448 also showed type II spectral changes with imidazole (Imai and Sato, 1967a), aniline (Schenkman et al., 1967) and benzphetamine. Benzphetamine is a substrate of mammalian cytochrome P-450 which gives rise to a type I spectrum with the enzyme (Goujon et al., 1972; Hewick and Fouts, 1970). When exposed to yeast cytochrome P-448 it is possible that the amino group of this substrate binds to the heme, producing type II spectrum changes instead of hydrophobic interaction which evidently occurs between benzphetamine and mammalian cytochrome P-450 as reported.
Aminopyrine, which causes type I spectral changes with hepatic cytochrome P-450 from untreated rats and with hepatic enzyme from untreated rabbits, did not bind to yeast cytochrome P-448. Sodium hexobarbitone (Shenkman, 1967) β-naphthoflavone (Goujon et al., 1972), lauric acid (Orrenius and Thor, 1969), biphenyl (Burke and Bridges, 1975), and all known type I spectral-producing with untreated mammalian cytochrome P-450/P-448 did not produce any measurable spectral changes with yeast cytochrome P-448. 7-Ethoxyresorufin, ethoxycoumarin, and isosafrole were also tested with the yeast enzyme, but no interaction was detected with compounds.

As for benzo(a)pyrene difference spectrum with yeast cytochrome P-448 an extra peak was observed in experiments with ethylmorphine, lanosterol and sodium phenobarbitone.

Imidazole and aniline, both of which gave rise to type II spectral changes, had the highest affinity for yeast cytochrome P-448 with spectral dissociation constants (Kₜ) values of 8μM and 5μM respectively. The extent of binding of these compounds, as measured by the peakΔAₘₐₓ values, was lower than benzo(a)pyrene but higher than other type I binding compounds. The two known substrates of yeast cytochrome P-450/P-448, benzo(a)pyrene and lanosterol showed
high affinity for the enzyme, although the extent cytochrome P-448 binding to lanosterol was not very high (Table 5.2).

5.3.3. The Equilibrium Gel Filtration of Benzo(a)pyrene
Yeast Cytochrome P-448 Complex

Gel filtration experiments were carried out at increasing benzo(a)pyrene concentrations (c) using the same amount of cytochrome P-450/P-448 (e). The specific activity of 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-448 (p). Figure 5.10 shows a typical result of one gel filtration experiment. From the values of p, e and c thus determined, p/e (γ) and γ/c were calculated and Scatchard plots were constructed (Figure 5.11). The values of the number of binding sites (n) and the apparent association constant (K) were then estimated by extrapolation. In addition to microsomal fractions this experiment was performed using purified cytochrome P-450/P-448 from yeast (Table 5.3), liver of phenobarbital-treated rats (Table 5.4) and from liver of β-naphthoflavone-treated rats (Table 5.5).

The nearest whole values of n determine for yeast microsomal and purified cytochrome P-448 were six (as also
Table 5.3. Data from the Equilibrium Gel Filtration of the Benzo(a)pyrene Cytochrome P-448 Complex (Purified Enzyme from Yeast).

<table>
<thead>
<tr>
<th>c (nM)</th>
<th>e (nmol)</th>
<th>p' (nmol)</th>
<th>p/e (γ)</th>
<th>γ/c (nM⁻¹)</th>
<th>K (nM⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>0.01</td>
<td>0.00028</td>
<td>0.028</td>
<td>0.022</td>
<td>0.027</td>
<td>1</td>
</tr>
<tr>
<td>7.59</td>
<td>0.01</td>
<td>0.0019</td>
<td>0.19</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.93</td>
<td>0.01</td>
<td>0.0032</td>
<td>0.32</td>
<td>0.023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.27</td>
<td>0.01</td>
<td>0.0033</td>
<td>0.33</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.62</td>
<td>0.01</td>
<td>0.0042</td>
<td>0.42</td>
<td>0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.5</td>
<td>0.01</td>
<td>0.0051</td>
<td>0.51</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.10. A Typical Result of the Gel Filtration of Benzo(a)pyrene Cytochrome P-450/P-448 Complex.
Table 5.4. Data from the Equilibrium Gel Filtration of the Benzo(a)pyrene Cytochrome P-450 Complex (Purified Liver Enzyme from PB-Treated Rats).

<table>
<thead>
<tr>
<th>c (nM)</th>
<th>e (nmol)</th>
<th>p (nmol)</th>
<th>p/e</th>
<th>γ/c (nM⁻¹)</th>
<th>K (nM⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.0036</td>
<td>0.000126</td>
<td>0.045</td>
<td>0.0180</td>
<td>0.023</td>
<td>0.53</td>
</tr>
<tr>
<td>8.8</td>
<td>0.0036</td>
<td>0.00055</td>
<td>0.152</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.2</td>
<td>0.0036</td>
<td>0.000794</td>
<td>0.220</td>
<td>0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27.2</td>
<td>0.0036</td>
<td>0.00148</td>
<td>0.310</td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.5. Data from the Equilibrium Gel Filtration of the Benzo(a)pyrene Cytochrome P-448 Complex (Purified Liver Enzyme from BNF-Treated Rats).

<table>
<thead>
<tr>
<th>c</th>
<th>e</th>
<th>p</th>
<th>p/e</th>
<th>γ/c</th>
<th>K</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(nM)</td>
<td>(nmol)</td>
<td>(nmol)</td>
<td>(γ)</td>
<td>(nM⁻¹)</td>
<td>(nM⁻¹)</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>0.0036</td>
<td>0.0082</td>
<td>2.3</td>
<td>0.418</td>
<td>0.112</td>
<td>6.05</td>
</tr>
<tr>
<td>10.3</td>
<td>0.0036</td>
<td>0.0122</td>
<td>3.4</td>
<td>0.330</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.2</td>
<td>0.0036</td>
<td>0.0130</td>
<td>3.6</td>
<td>0.236</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.5</td>
<td>0.0036</td>
<td>0.0151</td>
<td>4.2</td>
<td>0.195</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33.5</td>
<td>0.0036</td>
<td>0.0170</td>
<td>4.7</td>
<td>0.140</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.11. The Scatchard Plot of the Equilibrium Gel Filtration of the Benzo(a)pyrene Cytochrome P-450/P-448 Complex. Purified yeast cytochrome P-448 (●), purified PB-treated rat liver cytochrome P-450 (○), purified BNF-treated rat liver cytochrome P-448 (▲).
found by Woods and Wiseman, 1980) and one respectively. Yeast microsomal and purified enzyme bind 100% to benzo(a)pyrene, so that the number of binding is six (microsomal) and one (purified) per enzyme molecule binding to benzo(a)pyrene. The number of binding sites rat liver enzyme (PB-treated) per molecule binding to benzo(a)pyrene (only 16% of microsomal cytochrome P-450 and 53% of purified form bound to benzo(a)pyrene) were six \((0.91 \times 100/16)\) and one \((0.53 \times 100/53)\) as the \(n\) values were 0.91 and 0.53 respectively. This is a revision of the value of 20 for microsomal fractions calculated by Woods and Wiseman (1980), which was based on the extent of benzo(a)pyrene binding of 33% for yeast and 5% for liver enzymes.

As for cytochrome P-448 from the liver of 8-naphthoflavone-treated rats, the number of binding sites was ten (microsomal) and six (purified). Multiple binding sites could be due to (1) multiple active site with other sites facilitating transfer of benzo(a)pyrene to the active site, (3) binding of benzo(a)pyrene to the hydrophobic part of the molecule with no connection with catalysis.

The apparent association constant \((K)\) obtained for yeast cytochrome P-448 was \(0.08\text{nM}^{-1}\) (microsomal fraction) and \(0.027\text{nM}^{-1}\) (purified form). These are equivalent to
dissociation constants \( (K_s) \) of \( 1.3 \times 10^{-8} \text{M} \) and \( 3.7 \times 10^{-8} \text{M} \) respectively. For rat liver cytochrome P-450 (PB-treated) the corresponding \( K \) values were \( 0.064 \text{nM}^{-1} \) and \( 0.024 \text{nM}^{-1} \) which are equivalent to dissociation constants \( (K_g) \) of \( 1.6 \times 10^{-8} \text{M} \) (microsomal fraction) and \( 4.3 \times 10^{-8} \text{M} \) (purified form). For rat liver cytochrome P-448 (BNF-treated) the values were \( 0.096 \text{nM}^{-1} \) (microsomal fraction) \( 0.112 \text{nM}^{-1} \) (purified form) which in turn give dissociation constants \( (K_g) \) values of \( 1.0 \times 10^{-8} \text{M} \) and \( 0.89 \times 10^{-8} \text{M} \) respectively.

Low values for all dissociation constant suggests that the binding affinity is very high. These \( K_s \) values are much smaller than the spectral \( K_s \) \( 18 \mu \text{M} \) (microsomal fraction) and \( 50 \mu \text{M} \) (purified form) for yeast and the corresponding values \( 5 \mu \text{M} \) and \( 3 \mu \text{M} \) for rat liver (PB-treated) and \( 6 \mu \text{M} \) and \( 38 \mu \text{M} \) for rat liver (BNF-treated).

The extra binding sites found for the bound form of the enzymes (microsomal fractions) is clearly due to the high-affinity binding of benzo(a)pyrene to the lipid present, as this affinity is lower in purified enzymes.
6. The Induction and Characterization of Benzo(a)pyrene-3-Monooxygenase Activity of Cytochrome P-448 from Saccharomyces cerevisiae

6.1 Introduction

The benzo(a)pyrene metabolites formed by action of yeast cytochrome P-448 (microsomal fraction) were previously identified by HPLC as 7,3-dihydro-7,8-dihydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene (Wiseman and Woods, 1979). 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 in both microsomal form after various pretreatments and reconstituted system using purified cytochrome P-448. The parameters were compared with those of hepatic cytochrome P-450/P-448 of mammalian system. Other parameters, such as the pH optimum, thermal stability, temperature activity, time course and the effect of enzyme concentration were also measured. It was also found that NADPH as a cofactor may be replaced by either cumene hydroperoxide and hydrogen peroxide.

The addition of benzo(a)pyrene to the yeast during growth caused only a small increase in cytochrome P-448
levels but resulted in a dramatic improvement in the apparent kinetics of benzo(a)pyrene hydroxylase activity of microsomal fraction as measured by a decrease in the Michaelis constant and an increase in maximal velocity. Dimethylnitrosamine, phenobarbital and 3-methylcholanthrene also induced this enzyme to various degrees. Yeast pretreatment with 8-naphthoflavone did not effect this enzyme, yet pretreatment with lanosterol resulted in a decreased affinity for benzo(a)pyrene. In this chapter the implications of these findings with regard to the presence of multiple forms of cytochrome P-448/P-450 in yeast are briefly discussed.

The Ames test was used to detect the mutagenic metabolites of benzo(a)pyrene after activation with yeast cytochrome P-448.

It was found the benzo(a)pyrene hydroxylase activity of yeast cytochrome P-448 (reconstituted system) is inhibited by compounds which bind to this enzyme. The best inhibition was observed with lanosterol and dimethylnitrosamine. These results confirm the identity of benzo(a)pyrene hydroxylase as a cytochrome P-448 dependent activity. This enzyme was also inhibited by the flavonoid compounds, flavone and 7,8-benzoflavone, in a non-competitive fashion.
Attempted hydroxylation with other compounds such as aniline, ethoxyresorufin, ethylmorphine, benzphetamine, aminopyrene with yeast cytochrome P-448 was not successful. Some degree of hydroxylation was observed with this enzyme when either lanosterol or dimethylnitrosamine were used as a substrate but the results were not consistent and reproducible.

6.2. Methods and Materials
6.2.1. Preparation of Microsomal Fraction of Induced Yeast
Saccharomyces cerevisiae (NCYC No. 240) was grown batchwise by a method described in Chapter 3. Growth was for 44 hours in a medium containing 1% yeast extract, 2% mycological peptone and usually 20% glucose. Inducing agents, dissolved in a small amount dimethylformamide, were added to the growth media at beginning of the growth period. An equivalent amount of dimethylformamide was added in control experiments.

Microsomal preparations were obtained by differential centrifugation after disruption of yeast in a Vibro Mill disruptor by a method also described in Chapter 3. Microsomal preparations were resuspended in 0.1M potassium phosphate buffer pH 7.2 containing 0.001M-EDTA and 0.001M
dithiothreitol and 20% (v/v) glycerol, by use of a hand held Potter type homogenizer.

6.2.2. Preparation of Purified Yeast Cytochrome P-448

The purified yeast cytochrome P-448 used for reconstitution of hydroxylation activity of this enzyme system, was solubilized from untreated yeast microsomes and purified by the method described in Chapter 3.

6.2.3. Measurement of NADPH-Supported Benzo(a)pyrene-3-Monooxygenase Activity of Yeast Cytochrome P-448

Benzo(a)pyrene hydroxylase activity of yeast microsomal fraction (benzo(a)pyrene-treated and control) and purified cytochrome P-448 was measured by the method of Dehnen et al (1973) as modified by Wiseman and Woods (1979). In this method one of the metabolites of benzo(a)pyrene by mixed function oxidase system (involving cytochrome P-450/P-448) namely 3-hydroxybenzo(a)pyrene is fluorimetrically measured. The term benzo(a)pyrene-3-monooxygenase is used interchangeably with benzo(a)pyrene hydroxylase to express this activity throughout this report. Each incubation contained 0.5ml of microsomal suspension (with cytochrome P-448 concentration of 2μM), 0.5ml of 0.1M Tris-Hcl buffer, pH 7.0, containing a NADPH-regenerating system giving final concentrations of 0.004M of NADP, 0.02M of
D-glucose-6-phosphate, 6µM MgCl₂ and 8 I.U. of glucose-6-phosphate dehydrogenase.

The reaction was started by the addition of benzo(a)pyrene from a stock solution of 2mg/ml in the dimethylformamide to give a final concentration in the range of 0-158µM and incubated at 37°C for 15min. The reaction was stopped by adding 1ml of ice-cold acetone and the precipitated protein removed by centrifugation in a Piccolo centrifuge (Heracus Christ, W. Germany) at full speed for 5min. A 0.6ml sample of this 50% acetone solution was then placed with 1.4ml of triethylamine solution (10.7%, v/v) 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 523nm. The fluorescence intensities were measured relative to a solution of quinine sulfate (10mg in 2M sulfuric acid) and were corrected against fluorescence intensities of blank solutions, prepared by the same method from equivalent reaction mixtures in all of which heat-denatured cytochrome P-448 were used. The concentration of 3-hydroxybenzo(a)pyrene was determined from a standard curve in which the fluorescence intensities of various quantities of 3-hydroxybenzo(a)pyrene (in 2ml solution of water,
acetone, triethylamine, 0.3:0.3:1.4) were plotted against the concentrations.

The NADPH-supported benzo(a)pyrene-3-monooxygenase activity of purified cytochrome P-448 was usually measured by using 0.5ml reconstituted system containing 1nmol of cytochrome P-448, 1 unit of cytochrome c(P-450) reductase (1 unit = 1μmol of cytochrome c reduced per min. at 22°C) and 30μg of dilauroyl phosphatidylcholine, in place of microsomal suspension in the above described assay mixture.

The direct fluorometric assay for benzo(a)pyrene hydroxylase reported by Yang and Kicha (1978) was attempted, but did not produce consistent results in the yeast system, probably because it was not sufficiently sensitive.

6.2.4. **Measurement of Cumene Hydroperoxide-Supported Benzo(a)pyrene-3-Monooxygenase Activity of Yeast Cytochrome P-448**

When NADPH was replaced by cumene hydroperoxide the latter was used at a concentration of 2.4mM. Cytochrome c(P-450) reductase and dilauryl phosphatidylcholine were not used for reconstitution of benzo(a)pyrene-3-monooxygenase activity, when this system was supported by cumene hydroperoxide.
6.2.5. Measurement of Hydrogen Peroxide-Supported
Benzo(a)pyrene-3-Monooxygenase Activity of Yeast Cytochrome P-448

When benzo(a)pyrene-3-monooxygenase activity was supported by hydrogen peroxide, this substance was generated by a system with final concentrations of 0.001M of D-glucose and 2.5 I.U. of glucose oxidase. Cytochrome c(P-450) reductase and dilauryl phosphatidylcholine were not required for reconstitution of benzo(a)pyrene-3-monooxygenase activity when it was supported by H$_2$O$_2$. Although the presence of a small amount of cytochrome c(P-450) reductase increased the activity.

6.2.6. Attempt to Detect the Mutagenic Metabolites of Benzo(a)pyrene Using the Ames Test, after Activation with Yeast Cytochrome P-448

The Ames test is a sensitive and simple bacterial test for detecting chemical mutagens (Ames et al., 1975). It utilizes especially constructed strains of *Salmonella typhimurium*, which may be reverted specifically from a histidine requirement back to prototrophy by a wide variety of mutagens (both frameshift and base-pair substitution). The test is carried out on Petri plates, where the chemical, bacteria and usually an activating enzyme system (i.e., mixed function oxidase system plus an NADPH generating
mechanism) are incubated together in a semi-solid agar medium at 37°C. After 48h the number of colonies (revertants) on both test and control plates are counted. In addition to the detection of mutagenecity of chemicals, the ability of the activating enzyme system to produce reactive metabolites from a known carcinogen/mutagen, is estimated by the Ames test. This is determined by the number of bacteria reverted back from the histidine-requiring mutant to the wild form. The inclusion of a trace amount of histidine in the agar allows the growth of a background lawn of bacteria, as several divisions are often necessary for mutagenesis to become evident (especially with frameshift mutagens, Ames et al, 1975) and also allows any cytotoxic effects to be noted.

Mixed-function oxidase systems examined by this test were in the form of microsomes from benzo(a)pyrene-treated yeast, solubized enzymes also from microsomes of benzo(a)pyrene-treated yeast (containing dilauroyl phosphatidylcholine) and finally a reconstituted system using purified cytochrome P-448, cytochrome c(P-450) reductase (1nmol/1 unit) and dilauroyl phosphatidylcholine. The phospholipid was used as much as 30μg per ml of test
solution, and all enzyme systems were sterile filtered on a 0.45μm microfilter before addition to the test system.

**Test Mixture**
- 0.4ml Of mixed function oxidase system (cytochrome P-448 concentration of 2μM in the test solution).
- 0.1ml Of NADPH generating system (NADP concentration of 0.004M in the test solution).
- 0.4ml Potassium phosphate buffer (0.1M) pH 7.4.
- 0.1ml Of an overnight-grown nutrient broth culture of bacterial tester strain TA100.
- 10μl Benzo(a)pyrene (2mg/ml, in dimethylformamide).

**Method**
The test mixture was incubated at 37°C in a water bath-shaker (Mickel Lab. Engineering, U.K.) for 90min before being added to 2ml of top agar solution (melted at 45°C) containing 0.05mM L-histidine and 0.05mM biotin. The contents were immediately mixed and poured onto minimal agar plates to ensure the activating enzyme system was not kept at the temperature of the melted agar for more than a few seconds. Uniform distribution of the top agar on the
surface of plates was accomplished by gently tilting and rotating the plates, before the agar had started to solidify. After 30 min of incubation at room temperature, the plates were incubated at 37°C in the dark for 48 h.

Control plates were set to examine the spontaneous mutation rate of bacteria in this test. These control plates contained buffer in place of mixed-function oxidase system. Both the complete and control systems were also tested on separate plates for effect of solvent on spontaneous mutation rate, where only dimethylformamide was used.

After 48 h, the colonies on both test and control were counted, and the presence of a light background lawn of bacterial growth was confirmed. If massive bacterial death had occurred, the background growth on test plates was sparse, and, as more histidine was available for the individual surviving bacteria, these appeared as small colonies. Care was taken not to mistake these as revertants. Ideally for Ames test to be considered positive for a particular system, the number of revertants (colonies) in the presence of carcinogen/mutagen should be at least twice as many as those caused by spontaneous mutation (Ames et al., 1975).
The number of bacteria present in this Ames test effect to some extent (though not by a single relationship) the number of revertants which may be formed from them. Therefore, it was important to determine the effect of components in the incubation mixture on this viability. At the end of each incubation 100μl samples were removed from one of triplicate mixtures of each test and control systems, were diluted 1:100 three times by nutrient broth and 100μl of final dilutions were added to sterile plastic tubes containing 1.6ml of nutrient agar, 0.2ml of 0.1M L-histidine and 0.2ml of 0.5mM biotin. These tubes were shaken and poured onto the minimal agar plates, which when solidified were incubated at 37°C for 24h. 100μl of broth culture of bacterial tester (TA100) was also diluted and grown by the same method to serve as a control for viability assessment.

6.2.7. Materials

Oxoid No. 2 nutrient broth (made up and autoclaved as directed) was purchased from Oxoid Ltd. Top agar (0.6% Difco-Bacto nutrient agar containing 0.5% NaCl, autoclaved) was obtained from Difco Lab. Ready-poured minimal agar plates (Vogel-Bonner E Plates) were obtained from Gibco Ltd. Nicotinamide adenine dinucleotide phosphate (NADP), D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase flavone, 7,8-benzoflavone, dilauroyl phosphatidylcholine,
3-methylcholanthrene, L-histidine and biotin were obtained from Sigma Chemical Co. Glucose oxidase was purchased from Calbiochem-Behring Co. Cumene hydroperoxide (80% in cumene) was supplied by Merck and Co. Inc. (W. Germany). 3-Hydroxybenzo(a)pyrene was a gift from Dr. Diane Bedford of the toxicology laboratory, University of Surrey. Quinine sulfate and other chemicals were obtained from BDH Chemical Ltd.

6.3. Results and Discussion

6.3.1. The Induction of Cytochrome P-448 Dependent Benzo(a)pyrene-3-Monooxygenase in Saccharomyces cerevisiae

The addition of benzo(a)pyrene to yeast growing in medium containing 20% glucose affected the apparent kinetics of benzo(a)pyrene hydroxylation (as determined by benzo(a)pyrene-3-monooxygenase activity) and also the level of cytochrome P-448 in the yeast (Table 6.1). The level of cytochrome P-448 as measured by carbon monoxide-induced difference spectra of yeast suspensions was slightly higher in yeast treated with benzo(a)pyrene at high concentrations. The kinetics of benzo(a)pyrene-3-monooxygenase activity as supported by NADPH were investigated in microsomal fraction by means of double-reciprocal Lineweaver-Burk plots (Figure 6.1). The results show clearly that benzo(a)pyrene pretreatment of yeast results in a lower Michaelis constant
<table>
<thead>
<tr>
<th>Benzo(a)pyrene Concentration (μM)</th>
<th>Cytochrome P-448 (nmol/g wet weight yeast)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (pmol 3-hydroxy-benzo(a)pyrene/min/nmol cytochrome P-448)</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.36 ± 0.24</td>
<td>111</td>
<td>11.1</td>
<td>0.9953</td>
</tr>
<tr>
<td>16</td>
<td>3.66 ± 0.41</td>
<td>100</td>
<td>14.66</td>
<td>0.9980</td>
</tr>
<tr>
<td>32</td>
<td>3.43 ± 0.31</td>
<td>95</td>
<td>22.2</td>
<td>0.9964</td>
</tr>
<tr>
<td>63</td>
<td>3.95 ± 0.14</td>
<td>56</td>
<td>33.33</td>
<td>0.9965</td>
</tr>
<tr>
<td>95</td>
<td>4.36 ± 0.23</td>
<td>42</td>
<td>29.6</td>
<td>0.9480</td>
</tr>
<tr>
<td>190</td>
<td>*</td>
<td>32</td>
<td>38.66</td>
<td>0.9013</td>
</tr>
</tbody>
</table>

Table 6.1. Induction of Benzo(a)pyrene-3-monooxygenase with Benzo(a)pyrene. Microsomal fractions were used for determination of the activity. Values of cytochrome P-448 are quoted ± standard deviation, n = 9. *This concentration of benzo(a)pyrene resulted in precipitation of benzo(a)pyrene which interfered with the cytochrome P-448 assay rendering them invalid. Kinetic parameters of benzo(a)pyrene hydroxylase determined from Lineweaver-Burk plots using a linear regression, n = 5.
Figure 6.1. Lineweaver-Burk Plot of Benzo(a)pyrene Induced Microsomal Benzo(a)pyrene Hydroxylase. Control microsomal activity (●) (no benzo(a)pyrene added to growth medium) is shown with plots for microsomal activity from yeast treated with benzo(a)pyrene at concentrations of 16 M(○), 32 M(●), 63 M(□), 95 M(▲) and 190 M(▲). The v values are expressed in pmol of 3-hydroxybenzo(a)pyrene/min/nmol of cytochrome P-448.
(Kₘ) i.e., higher affinity for benzo(a)pyrene and a higher maximal velocity (Vₘₐₓ). These effects were clearly dependent on the concentration of benzo(a)pyrene in the medium (Table 6.1, Figure 6.2).

Yeast grown in 0.5% glucose containing medium usually contains no cytochrome P-448. In an attempt to induce benzo(a)pyrene-3-monooxygenase activity in yeast growing at low glucose concentration, benzo(a)pyrene was added. The production of cytochrome P-448 in this growth medium was not induced even in the presence of a high concentration (95μM) of benzo(a)pyrene.

Table 6.2 shows the effect of several other compounds on yeast cytochrome P-448 levels and the kinetics of microsomal benzo(a)pyrene-3-monooxygenase. Pretreatment with dimethylnitrosamine appeared to have a similar effect to pretreatment with benzo(a)pyrene in that the level of cytochrome P-448 slightly increased. The Kₘ for benzo(a)pyrene decreased and the Vₘₐₓ value increased. Higher concentrations of dimethylnitrosamine were necessary to achieve the same extent of induction as with benzo(a)pyrene. Yeast pretreatment with 3-methylcholanthrene was interesting as this resulted in no change in cytochrome P-448 levels and no change in the Vₘₐₓ.
Figure 6.2. Benzo(a)pyrene Induction of the Kinetic Parameters of Benzo(a)pyrene Hydroxylase
<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Cytochrome P-448 (nmol/g wet weight of yeast)</th>
<th>K (µM)</th>
<th>V (pmol 3-hydroxybenzo(a)pyrene/min/nmol cytochrome P-448)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.36 ± 0.24</td>
<td>111</td>
<td>11.1</td>
</tr>
<tr>
<td>Dimethylnitrosamine (108 µM)</td>
<td>3.98 ± 0.38</td>
<td>59</td>
<td>39.86</td>
</tr>
<tr>
<td>Dimethylnitrosamine (324 µM)</td>
<td>4.64 ± 0.38</td>
<td>40</td>
<td>31.73</td>
</tr>
<tr>
<td>3-Methylcholanthrene (30 µM)</td>
<td>3.48 ± 0.27</td>
<td>27</td>
<td>10.26</td>
</tr>
<tr>
<td>3-Methylcholanthrene (90 µM)</td>
<td>3.32 ± 0.35</td>
<td>20</td>
<td>11.6</td>
</tr>
<tr>
<td>Phenobarbital (32 µM)</td>
<td>3.50 ± 0.24</td>
<td>80</td>
<td>18.53</td>
</tr>
<tr>
<td>β-Naphthoflavone (30 µM)</td>
<td>3.80 ± 0.38</td>
<td>114</td>
<td>12.13</td>
</tr>
<tr>
<td>Lanosterol (19 µM)</td>
<td>3.62 ± 0.42</td>
<td>250</td>
<td>13.33</td>
</tr>
</tbody>
</table>

Table 6.2. Effect of Inducing Agents on Cytochrome P-448 and Benzo(a)pyrene-3-Monooxygenase. Microsomal fractions were used for determination of the activity. Values of cytochrome P-448 are quoted ± standard deviation, n = 8.
value yet a large decrease in $K_m$ for benzo(a)pyrene was observed. Phenobarbital also had no effect on yeast cytochrome P-448 levels but improved both $K_m$ and $V_{\text{max}}$ for this enzyme.

Pretreatment of yeast with β-naphthoflavone had no effect on either cytochrome P-448 levels or benzo(a)pyrene kinetics. The addition of lanosterol to the yeast growth medium resulted in a higher $K_m$ for benzo(a)pyrene than the control value and a slightly higher $V_{\text{max}}$. In this case the affinity for benzo(a)pyrene was reduced possibly due to induction of a different form of the enzyme to that induced by benzo(a)pyrene.

Benzo(a)pyrene is well known as an inducer of benzo(a)pyrene hydroxylase in mammals, where similar effects to those reported here on the kinetics of this enzyme have been shown (Gurtoo and Campbell, 1970; Schlede et al., 1970). This induction has been shown to be due to the selective induction of a form (or forms) of cytochrome P-450 with a high activity towards benzo(a)pyrene hydroxylation, cytochrome P-448. This form of cytochrome P-450, which is also induced by 3-methylcholanthrene, is known to have a relatively narrow substrate specificity and to activate carcinogens such as benzo(a)pyrene, to metabolically active products (results of the Ames test in this chapter).
The induction of mammalian cytochrome P-450/P-448 monooxygenase systems by various compounds has been widely studied and is now beginning to be explained in terms of multiple forms of the enzyme. Two of the most widely studied inducers, phenobarbital and 3-methylcholanthrene induce different forms of the enzyme. Phenobarbital induces a form with a very wide specificity whereas 3-methylcholanthrene as it was indicated above induces a form with a narrow specificity, cytochrome P-448 (Conney, 1967). It is now known that cytochrome P-450 exists in more than two different forms. The evidence for this comes, for example, from studies with inducers, isolation and purification of different forms, immunological studies and kinetics and binding studies (Guengerich, 1979). Multiple forms of cytochrome P-450/P-448 exist with different but overlapping substrate specificities. The results presented in this report suggest the induction by benzo(a)pyrene of a similar form of cytochrome P-448 to that of 3-methylcholanthrene induced enzyme in mammals, with a high activity towards benzo(a)pyrene in the yeast *Saccharomyces cerevisiae*.

The inability of benzo(a)pyrene to induce cytochrome P-448 in yeast grown in a low glucose medium shows that a high glucose concentration, or conditions leading to a
a similar physiological state such as anaerobic conditions, is a prerequisite for cytochrome P-448 production. This would be as expected if the production of cytochrome P-448 was being controlled by the presence of cyclic AMP as has been suggested (Wiseman et al, 1978; Chapter 2 in this report). It would seem therefore that a high glucose concentration lowers the level of cyclic AMP which allows cytochrome P-448 to be produced. The induction observed with benzo(a)pyrene is due to an effect on the amount of a particular form of cytochrome P-448 produced.

The addition of dimethylnitrosamine to yeast's growth media also showed considerable induction of benzo(a)pyrene-3-monooxygenase activity in a similar manner to benzo(a)pyrene, suggesting that this compound was inducing the same form of the enzyme as benzo(a)pyrene. β-naphthoflavone has been reported to induce a form of cytochrome P-450 in mammalian systems with a very high turnover number for benzo(a)pyrene which has an absorption maximum in the carbon monoxide-reduced difference spectrum at 446-448nm and has been hence termed cytochrome P-448 (Saito and Strobel, 1980). However, induction of benzo(a)pyrene-3-monooxygenase activity by β-naphthoflavone in Saccharomyces cerevisiae was not detected in this study.
Pretreatment of yeast with 3-methylcholanthrene, a classical inducer of the cytochrome P-448 system in mammalian liver, was expected to induce benzo(a)pyrene-3-monoxygenase also. A large decrease in $K_m$ was observed suggesting a higher affinity form of the enzyme yet no change in maximal velocity occurred. Phenobarbital, a classic inducer of the wide specificity cytochrome P-450 form in mammals induced benzo(a)pyrene-3-monoxygenase to some extent.

Lanosterol is the main endogenous substrate for cytochrome P-450/P-448 in *Saccharomyces cerevisiae* (Yoshida and Aoyama, 1980). The addition of this compound to yeast media during growth resulted in a decreased affinity for benzo(a)pyrene, showing that the induction of another form of cytochrome P-450/P-448 could be occurring.

The presence of multiple forms of cytochrome P-450/P-448 in *Saccharomyces cerevisiae* is previously unreported although some evidence from purification studies (Chapter 3) was given that at least two forms are present in uninduced yeast. Furthermore, the results of induction studies suggest that multiple forms of cytochrome P-450/P-448 exist in *Saccharomyces cerevisiae* and extend the list of
properties that the enzyme from this eukaryotic source shares with the mammalian system.

6.3.2. Reconstitution of NADPH-Supported Hydroxylase Activity of Purified Yeast Cytochrome P-448

Table 6.3 shows a heat stable factor, dilauroyl phosphatidylcholine, is needed to reconstitute full hydroxylase activity of this system. This heat stable factor is reported to cause the association of cytochrome c(P-450) reductase and cytochrome P-450/P-448 to form an active enzyme aggregate (Strobel et al., 1970). Table 6.3 also shows the residual non-ionic detergent Emulgen 911 content of cytochrome P-448 seemed to suffice for active enzyme reconstitution as had been reported by Ichihara, et al. (1971). The results shown in Table 6.3 clearly demonstrate an absolute requirement for cytochrome c(P-450) reductase in NADPH-mediated benzo(a)pyrene hydroxylation.

One complication in the hepatic microsomal system is that NADH, although much less effective than NADPH, can also act as an electron donor for cytochrome P-450/P-448-catalyzed monooxygenase reactions (Gillette et al., 1972). Reconstitution as well as immunochemical studies have indicated that the electron transfer in this case is mediated by a FAD-containing flavoprotein (NADH:cytochrome b5...
<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full system (Emulgen 911 as heat stable factor)</td>
<td>11.3</td>
</tr>
<tr>
<td>Full system (dilauroyl phosphatidylcholine as heat-stabilizing factor)</td>
<td>11.3</td>
</tr>
<tr>
<td>Minus cytochrome P-448</td>
<td>0.2</td>
</tr>
<tr>
<td>Minus cytochrome c(P-450) reductase</td>
<td>1.6</td>
</tr>
<tr>
<td>Minus NADPH-generating system</td>
<td>1.8</td>
</tr>
<tr>
<td>Minus dilauroyl phosphatidylcholine or Emulgen 911</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Table 6.3. Reconstitution of Hydroxylase Activity of Purified Yeast Cytochrome P-448. Concentration of benzo(a)pyrene used in all experiments was 79μM. V values are expressed in pmol 3-hydroxybenzo(a)pyrene/min/nmol cytochrome P-448. NADPH-generating system constructed a described in "Method." The dilauroyl phosphatidylcholine concentration was 30μg/ml of assay mixture and alternatively 0.1% (v/v) of Emulgen 911 was used in the assay mixture.
reductase) and a b-type cytochrome, cytochrome b₅ (Hrycay and Prough, 1974).

It has further been reported that NADH exerts a synergistic effect on NADPH-dependent monooxygenase reactions catalyzed by liver microsomes (Cohen and Estabrook, 1971; Gillette, 1971; Cinti et al, 1972). Hildebrandt and Estabrook (1971) have suggested that the first of the two electrons required for microsomal hydroxylation is donated by NADPH-cytochrome c(P-450) whereas the second electron is derived from either NADPH or NADH via cytochrome b₅. Correia and Mannering (1973) have also concluded that cytochrome b₅ is an electron transport carrier in the NADPH-dependent mixed-function oxidase system.

Although much remains unclear about the synergism, evidence from immunochemical and reconstitution experiments (Imai and Sato, 1977) suggest that the pathway through which an electron is transferred from NADH to cytochrome P-450 via cytochrome b₅ reductase and cytochrome b₅ is somehow involved in the phenomenon. However, the obligatory involvement of cytochrome b₅ in NADPH-dependent hydroxylation reaction is reported to be unlikely (Gillette et al, 1972; Leven et al, 1974; Liu et al, 1974 and Sasame et al, 1974).
This synergistic effect was examined in yeast's monooxygenase system using benzo(a)pyrene as the substrate (Table 6.4). Neither inclusion of NADH and other components of its pathway (cytochrome b$_5$, cytochrome b$_5$ reductase) nor just presence of either cytochrome b$_5$ or cytochrome b$_5$ reductase in NADPH-dependent reconstituted system had any effect on benzo(a)pyrene-3-monooxygenase activity. When NADH and other components of its electron transport pathway replaced NADPH generating system and cytochrome c(P-450) reductase some activity was observed and when cytochrome c(P-450) reductase was added back to this system the activity increased slightly. These experiments also showed that where NADPH is involved as electron donor cytochrome b$_5$ reductase and cytochrome b$_5$ may not replace cytochrome c(P-450) reductase (Table 6.4). In all, the results showed that NADH can also serve as an electron donor for the hydroxylation of benzo(a)pyrene by yeast's cytochrome P-448 although the rate of hydroxylation is considerably slower than with NADPH. When NADH was used as an electron donor it was necessary for cytochrome b$_5$ and cytochrome b$_5$ reductase to be present. The mechanism of the observed stimulation by cytochrome c(P-450) reductase in NADH-mediated yeast's benzo(a)pyrene-3-monooxygenase system was not well understood. Possibly, cytochrome c(P-450) reductase could somehow be enhancing the rate reduction of cytochrome P-448
### Table 6.4. Reconstitution of Hydroxylase Activity of Purified Yeast Cytochrome P-448 Supplemented with Components of NADH Electron Donating System. Full system consisted of cytochrome P-448, cytochrome c(P-450) reductase, NADPH generating system, dilauroyl phosphatidylcholine. Concentration of components of NADH electron donating system was 4mM (NADH), cytochrome b5 (one nmol per nmol of cytochrome P-448), cytochrome b5 reductase (one unit per nmol of cytochrome P-448). Concentration of benzo(a)pyrene used in all experiments was 79μM.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full system plus NADH, cytochrome b5, cytochrome b5 reductase</td>
<td>11.3</td>
</tr>
<tr>
<td>Full system plus cytochrome b5</td>
<td>11.3</td>
</tr>
<tr>
<td>Full system plus cytochrome b5 reductase</td>
<td>11.3</td>
</tr>
<tr>
<td>Full system with NADH, cytochrome b5, cytochrome b5 reductase in absence of NADPH</td>
<td>3.0</td>
</tr>
<tr>
<td>generation system and cytochrome c(P-450) reductase</td>
<td></td>
</tr>
<tr>
<td>Full system with NADH cytochrome b5, cytochrome b5 reductase replacing NADPH</td>
<td>5.0</td>
</tr>
<tr>
<td>generating system</td>
<td></td>
</tr>
<tr>
<td>Full system with NADH replacing NADPH</td>
<td>1.9</td>
</tr>
<tr>
<td>generating system</td>
<td></td>
</tr>
<tr>
<td>Full system with cytochrome b5, cytochrome b5 reductase replacing cytochrome c(P-450)</td>
<td>1.6</td>
</tr>
<tr>
<td>reductase</td>
<td></td>
</tr>
</tbody>
</table>
by NADH via cytochrome b5 reductase and cytochrome b5 or perhaps functioning as an effector rather than as an electron carrier, analogous to the proposal of Guengerich et al (1976) for the role of cytochrome b5 in the reaction of oxygen with reduced cytochrome P-450.

In a similar reconstitution study by West and Lu (1977) using hepatic cytochrome P-448 and other components from 3-methylcholanthrene-treated rats, the rate of NADPH-supported benzo(a)pyrene hydroxylation was observed to be 30-40 fold higher than the NADH-supported in contrast with approximately 4 fold difference shown here for the yeast system.

6.3.3. Parameters of Yeast Benzo(a)pyrene-3-Monoxygenase Activity

NADPH-Supported benzo(a)pyrene hydroxylase was characterized as indicated in the accompanying diagrams using a benzo(a)pyrene concentration of 79μM. The time course (Figure 6.3) shows the production of 3-hydroxybenzo(a)pyrene occurs linearly over a period of 15min, then for the next 30min the rate of this production decreases by a function of time and stops after 45min. In both the cumene hydroperoxide and in situ, hydrogen peroxide-supported systems the production of 3-hydroxybenzo(a)pyrene (fluorescent
Figure 6.3. The Time Course of the NADPH-Supported (●), Cumene Hydroperoxide-Supported (x) and Hydrogen Peroxide-Supported Yeast Reconstituted Benzo(a)pyrene Hydroxylase (▲). The v values are expressed in pmol of 3-hydroxybenzo(a)pyrene/nmol of cytochrome P-448 per various time intervals. Benzo(a)pyrene concentration was 79μM in all measurements.
emission peak at 523nm) reaches its maximum after 15min and a peak at 540nm begins to show. This 540nm peak is identified as a mixture of benzo(a)pyrene metabolites including phenols, diols and quinones (Rho, 1980). This agrees with results obtained upon using cytochrome P-450 of phenobarbital pretreated rats, described by Renneberg et al (1981). These authors reported that the production of quinones from peroxide-supported hydroxylase systems was much higher than the production of these metabolites in a NADPH-supported system. The results compare favourably with the time course published by Nebert and Gelboin (1968) in which the 3-hydroxybenzo(a)pyrene production on NADPH-supported system was completed after 45min.

The pH optimum was also determined (Figure 6.4) and yeast benzo(a)pyrene 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 and 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
Figure 6.4. pH-Stability (●) and pH-Activity (x) Reconstituted NADPH-Supported Benzo(a)pyrene Hydroxylase System of Yeast. In pH-activity studies incubations were performed at the given pH. In pH-stability studies cytochrome P-448 was exposed for 60 min at 22°C to the pH shown followed by determination of activity at pH 7.0. Benzo(a)pyrene concentration was 79μM in all measurements.
system often is assumed to be the same as the one for the microsomes. In situ hydrogen peroxide (glucose oxidase/glucose) supported system had a better rate at slightly higher (pH, 7.2) than the optimal pH for the cumene hydroperoxidase and NADPH-supported systems which was at 6.5-7.0. This is in spite of glucose oxidase having a pH optimum at 4-7 (Bright and Appleby, 1969) and G-6PDH, which was used in NADPH generating system having it at 9.2 (Domagk et al, 1969).

Changing the protein (cytochrome P-448) concentration in the incubation mixture resulted in the relationship shown in Figure 6.5. The degree of metabolism of benzo(a)pyrene was approximately linear for cytochrome P-448 concentration up to about 1μM after which point, the degree of metabolism remained mainly constant, presumably as a result of some other factor, such as cofactor concentration becoming limited.

The optimum concentrations of NADPH-generating components was measured (in this laboratory) by Woods and Wiseman (1979) to be 0.004M for NADP, 0.02M for D-glucose-6-phosphate and 8 I.U. for glucose 6-phosphate dehydrogenase in the system. These authors found that any increase in these values resulted in no further increase in the extent of benzo(a)pyrene metabolism, presumably since the enzyme
Figure 6.5. The Effect of Cytochrome P-448 Concentration on Reconstituted NADPH-Supported Benzo(a)pyrene Hydroxylase System of Yeast. The v values are expressed in pmol of 3-hydroxybenzo(a)pyrene/min per various concentration of cytochrome P-448. Benzo(a)pyrene concentration was 79µM in all measurements.
concentration was limiting. These concentrations were routinely used in all studies of benzo(a)pyrene metabolism.

The thermal stability of the benzo(a)pyrene hydroxylase was compared with that of cytochrome P-448 as measured spectrophotometrically by the carbon monoxide-binding spectrum (Table 6.5, Figure 6.6, Figure 3.3 in Chapter 3). The resemblance between the thermal stability of cytochrome P-448 and benzo(a)pyrene hydroxylase proved that these are the same enzymes and this activity is cytochrome P-448 dependent. The thermal stability of yeast benzo(a)pyrene hydroxylase was very similar to that of the same enzyme measured in mammalian microsomal fraction by Nebert and Gelboin (1968). Temperature-activity curve of yeast benzo(a)pyrene hydroxylase (Figure 6.6) gives 37°C as an optimal temperature. This was in spite of cytochrome P-448 not being fully stable at this temperature (also Figure 6.6).

In an attempt to increase the thermal stability of cytochrome P-448 dependent benzo(a)pyrene hydroxylase system of yeast, Triton X-100 and benzo(a)pyrene were examined using the least stable form of this enzyme, solubilized cytochrome P-448. As it is shown in Table 6.5, the
<table>
<thead>
<tr>
<th>Form of Yeast Cytochrome P-448</th>
<th>Temperature 50% of Cytochrome P-448 was lost (°C)</th>
<th>Critical Temperature at which 100% of Cytochrome P-448 was lost (°C)</th>
<th>Temperature at which 50% of Benzo(a)pyrene Hydroxylase was lost (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal</td>
<td>50</td>
<td>55</td>
<td>50</td>
</tr>
<tr>
<td>Solubilized</td>
<td>35</td>
<td>45</td>
<td>39</td>
</tr>
<tr>
<td>Solubilized + 0.1% Triton X-100</td>
<td>36</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>Solubilized + 80μM Benzo(a)pyrene</td>
<td>35</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>Purified</td>
<td>45</td>
<td>65</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 6.5. Thermal Stability of Different Types of Preparations of Yeast Cytochrome P-448 and its NADPH-Supported Benzo(a)pyrene Hydroxylase System. The enzyme preparations were pre-incubated for five minutes in a series of temperatures 0-75°C, cooled and assayed for cytochrome P-448 level and benzo(a)pyrene hydroxylase activity. Plots of activity and cytochrome P-448 level versus temperature were constructed. Protein contents were microsomal 26.5mg/ml, solubilized 23mg/ml, purified 0.05mg/ml. Cytochrome P-448 concentrations were the same in all the experiments.
Figure 6.6. Heat-Stability and Temperature-Activity Curves of Reconstituted NADPH-Supported Benzo(a)pyrene Hydroxylase System of Yeast. Activity of enzyme was measured at given temperatures (x). In heat-stability studies (●), the activity was measured at 37°C after 5 min incubation at the given temperature. Benzo(a)pyrene concentration was 79 μM in all measurements.
benzo(a)pyrene hydroxylase activity stabilizes to some degree in the presence of Triton X-100, but the addition of benzo(a)pyrene did not significantly affect the stability of this activity. The stabilization effect of Triton X-100 is probably due to the detergent substituting for lipid removed during solubilization and hence protecting the conformation of the enzyme.

The kinetics of yeast reconstituted benzo(a)pyrene hydroxylase system were determined by least squares linear regression analysis of double-reciprocal Lineweaver-Burk plots in which the rate of 3-hydroxybenzo(a)pyrene formation was plotted against benzo(a)pyrene concentration as is shown in Figure 6.7. This linear plot showed that the Michaelis Constant ($K_m$) for NADPH-supported of yeast reconstituted benzo(a)pyrene hydroxylase system was at 33µM (Table 6.6). This value is much smaller than 111µM for cytochrome P-448 measured in situ in the yeast microsomal fraction as shown in Table 6.1. This $K_m$ value is similar to that reported for liver enzyme (23µM) by Rickert and Fouts (1970) compared with the value of 1µM also for liver enzyme reported by Robie et al (1976). $V_{\text{max}}$ for the same system was found to be 16.7 pmol of 3-hydroxybenzo(a)pyrene per min per nmol of cytochrome P-448. This is greater than $V_{\text{max}}$ value of 11.1pmol 3-hydroxybenzo(a)pyrene/min/nmol of cytochrome
Figure 6.7. Lineweaver-Burk Plots of NADPH-Supported (○), Cumene Hydroperoxide-Supported (x) and Hydrogen Peroxide-Supported (▲) of Yeast Reconstituted Benzo(a)pyrene Hydroxylase System. The $v$ values are expressed in pmol of 3-hydroxybenzo(a)pyrene/min/nmol of cytochrome P-448.
### Table 6.6. The Values of the Michaelis-Menten Parameters of Yeast Benzo(a)pyrene Hydroxylase System. V<sub>max</sub> values are expressed as pmol of 3-hydroxybenzo(a)pyrene/min/nmol cytochrome P-448.

<table>
<thead>
<tr>
<th>Support System</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>16.7</td>
<td>33</td>
</tr>
<tr>
<td>Cumene Hydroperoxide</td>
<td>21.9</td>
<td>125</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>33.7</td>
<td>200</td>
</tr>
</tbody>
</table>
P-448 in microsomal fraction (Table 6.1), indicating the problem in using an immobilized enzyme (i.e., microsomal fraction) which includes a diffusion effect, when compared with a successfully reconstituted system.

The value determined here for $K_m$ agrees with dissociation constant ($K_s$) value of 50μM (presented in Chapter 5) which was determined for benzo(a)pyrene binding to yeast cytochrome P-448. The $K_s$ value for the microsomal cytochrome P-448 from yeast was 18μM. Even in purified systems, any disagreement would be due to the complexity of the benzo(a)pyrene-metabolizing system, and the $K_m$ values for this system are the aggregate of several components. In a case reported by Kratz and Staudinger (1968) the $K_s$ value for coumarin binding is found to be 100 times greater than the $K_m$ for its metabolism in an animal system.

Table 6.7 gives various literature values for $V_{max}$ and $K_m$ of the mammalian microsomal benzo(a)pyrene hydroxylase system. It can be seen that the values for $V_{max}$ are in the region of $10^{-6}$ to $10^{-9}$ mol of benzo(a)pyrene metabolite formed per h per mg protein (or g of liver). These $V_{max}$ values are higher than the that for NADPH-supported yeast microsomal fraction (6.0pmol 3-hydroxybenzo(a)pyrene/h/mg of protein).
Table 6.7. A Comparison of the Literature Values of the Michaelis-Menten Parameters of Mammalian Benzo(a)pyrene Hydroxylase.

* moles hydroxybenzo(a)pyrene formed/h/g liver
** nmoles 8-hydroxybenzo(a)pyrene formed/h/mg protein
*** moles 3-hydroxybenzo(a)pyrene formed/h/mg protein
From Table 6.7 it can also be seen that while it is generally agreed that pretreatment with a polycyclic aromatic hydrocarbon will decrease the $K_m$ of benzo(a)pyrene hydroxylase the actual numerical value of $K_m$ seems to vary according to which group of workers measure it. The most likely explanation for this is the strain differences in the rats used or possibly variations in diet.

The $K_m$ and $V_{max}$ values for reconstituted NADPH-supported benzo(a)pyrene hydroxylase systems of cytochrome P-448 from β-naphthoflavone-induced cytochrome P-450 from phenobarbital-induced rats were determined by least squares linear regression analysis of Lineweaver-Burk plots shown in Figures 6.8 and 6.9. In each of these experiments cytochrome c(P-450) reductase isolated and purified from the respective source, was used. These values for cytochrome P-450 from phenobarbital-induced rats were 46µM ($K_m$) and 21pmol of 3-hydroxybenzo(a)pyrene/min/nmol of cytochrome P-450 ($V_{max}$) roughly resembling the corresponding values, 33 and 16.7 for yeast cytochrome P-448. The turnover of benzo(a)pyrene metabolism by cytochrome P-448 of β-naphthoflavone-induced rats as determined from $V_{max}$ of 61pmol of 3-hydroxybenzo(a)pyrene per min per pmol of cytochrome P-448 was considerably greater than those of PB-induced and yeast. Gozukara et al (1982) reported more than
Figure 6.8. Lineweaver-Burk Plot of Reconstituted NADPH-Supported Benzo(a)pyrene Hydroxylase System of Phenobarbital-Induced Rat Liver. The $v$ values are expressed in pmol of 3-hydroxybenzo(a)pyrene/min/nmol of cytochrome P-450.
Figure 6.9. Lineweaver-Burk Plot of Reconstituted NADPH-Supported Benzo(a)pyrene Hydroxylase System of β-Naphthoflavone-Induced Rat Liver. The v values are expressed in pmol of 3-hydroxybenzo(a)pyrene/min/pmol of cytochrome P-448.
100 fold greater turnover for the BNF-induced cytochrome P-448 in comparison to PB-induced cytochrome P-450 as determined by the formation of 3-hydroxybenzo(a)pyrene from benzo(a)pyrene used only at 50µM concentration level. The value for $K_m$ of this system (BNF-induced cytochrome P-448) was 22.7µM which is slightly lower than the $K_m$ values of the other two systems. Saito and Strobel (1981) have reported a similar turnover (50pmol 3-hydroxybenzo(a)pyrene/min/pmol) value for benzo(a)pyrene metabolism by cytochrome P-448 from β-naphthoflavone-induced rats also using only one level of benzo(a)pyrene concentration (80µM).

Figure 6.7 also shows the Lineweaver-Burk plots of cumene hydroperoxide and hydrogen peroxide-supported yeast reconstituted benzo(a)pyrene hydroxylase system. The parameters of these hydroxylase systems are shown in Table 6.6. The $V_{max}$ values obtained for cumene hydroperoxide and hydrogen peroxide-supported systems (21.9 and 33.7pmol/min/nmol) are considerably higher than that of NADPH-supported system (16.7pmol/min/nmol). In spite of $V_{max}$ values, the comparison of $K_m$ values for systems showed that yeast cytochrome P-448 has a higher affinity for the substrate benzo(a)pyrene when hydroxylation is supported by NADPH and oxygen than those supported by peroxides. The higher $K_m$ values for cumene hydroperoxide and hydrogen
peroxide-supported systems may have been caused by the effect of these molecules on cytochrome P-448. It has been shown that a higher concentration of cumene hydroperoxide (Woods, 1979) or hydrogen peroxide (White and Coon, 1980) causes destruction of the enzyme and it may be that at low concentrations it reacts with the enzyme to chemically modify the active site or to produce conformational changes in the protein thus changing the $K_m$. In this study when hydrogen peroxide was generated in situ by glucose/glucose oxidase system the value of $K_m$ decreased in comparison to the direct addition of this substance to the hydroxylation system.

The higher $V_{max}$ values for cumene hydroperoxide and hydrogen peroxide-supported systems may be due to the fact that both these molecules by-pass the electron transport chain which must be used when NADPH is the cofactor thus increasing the efficiency of the overall reactions.

For microsomal enzymes from both PB- and BNF-treated rats the turnover of 3-hydroxybenzo(a)pyrene is reported to be higher for NADPH-supported system than that of cumene hydroperoxide (Capdevila et al, 1980). This may be due to the difference in metabolite profile when epoxide hydrase is present (microsomal fraction).
6.3.4. The Inhibition of Yeast

**Benzo(a)pyrene-3-Monooxygenase Activity**

Table 6.8 shows the percentage of inhibition of cytochrome P-448 (purified) dependent (NADPH-supported) benzo(a)pyrene hydroxylase activity by binding compounds and flavonoids along with the corresponding $K_s$ values. This table shows that dimethylnitrosamine and lanosterol result in a high degree of inhibition at 2.5mM and 77μM respectively. Both of these compounds result in type I interactions with purified yeast cytochrome P-448 with spectral dissociation constants ($K_s$ values) of 220μM and 80μM respectively. Dimethylnitrosamine is an inducer of cytochrome P-448 and binding studies (Chapter 5) showed evidence of its high affinity for this enzyme, although its rate of demethylation, using the Nash assay technique, was not reproducible (read on).

9-Hydroxyellipticine did not inhibit the yeast benzo(a)pyrene hydroxylase activity. This compound has been found to inhibit mammalian cytochrome P-448 in vitro (Delaforge et al., 1980).

The interaction of falvonoid compounds with benzo(a)pyrene hydroxylase system from mammalian liver has been studied by Huang (1981a, and 1981b). These workers found the flavone stimulated benzo(a)pyrene hydroxylase
Table 6.8. Inhibition of NADPH-Supported Yeast Benzo(a)pyrene Hydroxylase System. The one ml asays mixtures contained 1nmol of cytochrome P-448, 1 unit of cytochrome c(P-450) reductase and 30μg of dilauroyl phosphatidylcholine. Benzo(a)pyrene concentration was 79μM in all measurements.
activity of cytochrome P-450LM3c or LM4, yet inhibited the same activity catalyzed by cytochrome P-450LM2, LM3b and LM6. 7,8-Benzoflavone stimulated benzo(a)pyrene hydroxylation by cytochrome P-450LM3c, yet inhibited cytochrome P-450L6. It was found in this study that both 7,8-benzoflavone and flavone inhibit benzo(a)pyrene hydroxylase activity of yeast cytochrome P-448 strongly in a non-competitive fashion. 7,8-Benzoflavone is a more potent inhibitor than flavone. Double-reciprocal plots of velocity versus benzo(a)pyrene concentration at various concentrations of 7,8-benzoflavone showed that the inhibition by this compound is non-competitive, as the $K_m$ for benzo(a)pyrene is unchanged while maximal velocity is lowered. The inhibition constant, $K_i$ for 7,8-benzoflavone was determined as 176$\mu$M (benzo(a)pyrene was used in the range 40-158$\mu$M).

The inhibition of benzo(a)pyrene hydroxylase by cytochrome P-448 binding compounds once again confirms the identity of this enzyme as a cytochrome P-448 dependent activity. The difference between mammalian cytochrome P-450LM4 (a cytochrome P-448) and yeast cytochrome P-448 is shown by the effect of flavonoid compounds on benzo(a)pyrene hydroxylation by these systems. In this respect the yeast enzyme used in this study appears to resemble cytochrome
P-450LM6 which is produced in rabbit liver after induction with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

6.3.5. The Detection of Mutagenic Metabolites of Benzo(a)pyrene Using the Ames Test after Activation with Yeast Cytochrome P-448

Table 6.9 shows the result of the Ames test using solubilized cytochrome P-448 from benzo(a)pyrene-induced yeast. When the yeast microsomal fraction was used the test system became contaminated and sterilization was not easily possible due to the high concentrations of lipid and protein in this fraction.

The carcinogenic and mutagenic effects of many polycyclic aromatic hydrocarbons are generally believed to be mediated through epoxides (Jerina and Daly, 1974; Lehr et al, 1978; Gelboin et al, 1972). Microsomal cytochrome P-448-dependent mixed-function oxidase metabolizes benzo(a)pyrene, initially to the 7,8-oxide which is hydrated to the trans 7,8-dihydrodiol by the closely coupled epoxide hydratase (EC.4.2.1.63), as shown by Lu et al (1978). Mixed-function oxidase system then oxidizes the benzylic ring at the sterically-hindered bay region to give a diol epoxide with its oxirane ring in a position important in the production of active intermediate (Lehr et al, 1978). The
Table 6.9. The results of Ames Test. Crude solubilized cytochrome P-448 from benzo(a)pyrene-induced yeast was used in this test.

* As a result of 1:10 dilution of TA100 in the test systems the control viability counts should be 10 fold greater than that of blank and complete system.

** From which plates the viability samples were taken. For viability duplicate and for the test triplicate experiments were conducted. Dimethylformamide (DMF) is a solvent usually used to dissolve benzo(a)pyrene.

<table>
<thead>
<tr>
<th>Plate Content</th>
<th>Viability Counts</th>
<th>Test Counts (Revertants of TA100 per Plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA100 alone</td>
<td>125*</td>
<td>118*</td>
</tr>
<tr>
<td>Buffer + DMF</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Buffer + BP</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Complete system + DMF</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Complete system + BP</td>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>
Diol epoxide is believed to be a proximate carcinogen, as it reacts with DNA (Brookes, 1977) and is mutagenic (Wislocki et al., 1976; Huberman et al., 1976), a tumor initiator (Slaga et al., 1977) and a complete carcinogen (Kapitulnik et al., 1978). The mutagenicity of diol epoxide is not affected by the presence of epoxide hydratase, as it is a poor substrate of this enzyme (Wood et al., 1976; Levin et al., 1977).

The reconstituted yeast mixed-function oxidase system of yeast (benzo(a)pyrene hydroxylase) consisted of highly purified cytochrome P-448 and cytochrome c(P-450) reductase (both devoid of epoxide hydratase), did not show any effect in converting benzo(a)pyrene to its reactive metabolite in the Ames test. When this system was used the number of revertants in the test plates were not greater than that of blank plates. It is reported by Levin et al. (1977) that in the absence of epoxide hydratase, the reconstituted cytochrome P-448 containing mixed-function oxidase system metabolizes benzo(a)pyrene to phenols and quinones.

As it can be seen in Table 6.9 when solubilized cytochrome P-448 from microsomal fraction of benzo(a)pyrene-treated yeast was used in the Ames test, the number of induced revertants was higher than the blank (in this
The fact that the number of the induced revertants was not at least two fold larger than the spontaneous, could be due to the presence of benzo(a)pyrene bound cytochrome P-448 in the blank. This number in two of the test plates was two fold larger than the number counted for blank plates using buffer plus solvent or buffer plus benzo(a)pyrene.

In all, yeast cytochrome P-448 showed some mutagenic activation effect of benzo(a)pyrene in the Ames test and could be further tried in this test to examine the mutagenicity of other compounds.

6.3.6. Attempted Hydroxylation of Other Compounds such as Lanosterol, Aminopyrene, Aniline, Dimethylnitrosamine, Ethylmorphine, Benzphetamine and Ethoxyresorufin with Yeast Cytochrome P-448

The possibility of demethylation of lanosterol (I) was investigated by measuring formaldehyde formation by the method of Nash (1953). A NADPH-supported reconstituted system using purified cytochrome P-448 (as described in Section 6.2.3.) was used to achieve the demethylation of lanosterol. Incubates consisted of 0.5ml of reconstituted cytochrome P-448 system 50μl of 20mM lanosterol (in DMF), 1.95ml of 0.1M Tris-HCl buffer, pH 7.0 containing NADPH
generating system (also as described in Section 6.2.3.), 0.1M MgCl₂ and 0.02% (w/v) semicarbazide hydrochloride. The mixture was incubated at 37°C for 1h and the reaction was terminated by the addition of 1ml of 15% (w/v) ZnSO₄ followed by 1ml of 2:1 mixture of standard solutions of Ba(OH)₂ and Na₂B₄O₇. The precipitated protein was removed by centrifugation and 2ml of Nash reagent (0.4% acetylacetone (w/v) in 4M ammonium acetate) was added and the mixture was incubated at 37°C for 40min and the colour was read at 412nm in a Cecil CE272 spectrophotometer (Cecil Instruments, U.K.) and concentration of formaldehyde was calculated against a standard curve.

The 14 γ-demethylation of lanosterol by a reconstituted cytochrome P-450 system from yeast has been demonstrated (Aoyama and Yosida, 1978b) and it was hoped that the Nash reaction for measuring formaldehyde could be used to show this demethylation in the yeast system used in this study.
Although some demethylation activity was observed using the Nash reaction, this method was not sufficiently sensitive to show a productible rate of demethylation.

The same method was used to determine N-demethylation of aminopyrine (II), ethylmorphine (III), dimethyl-nitrosamine (IV) and benzphetamine (V) by yeast cytochrome P-448 reconstituted system.
The Nash method for determination of formaldehyde formed from N-demethylation of aminopyrine, ethylmorphine, benzphetamine showed that there was more formaldehyde in the blank than in the test incubates. A time course was attempted in all three cases but there was no difference between the zero min and 60min samples. It must be concluded, therefore, that there was no N-demethylation of these three substrates. Some N-demethylation activity was detected when dimethylnitrosamine was used as a substrate, but again as it was observed for lanosterol the results were not consistent and reproducible.

The ethoxyresorufin O-deethylation activity of reconstituted yeast cytochrome P-448 (VI) was measured by the method of Burke et al (1974), improved from that of Burke and Mayer (1971).

Ethoxyresorufin is O-deethylated to yield resorufin, these compounds are fluorescent with excitation maxima at 456 and 560nm and emission maxima at 570 and 586nm respectively. Therefore when the fluorimeter was set at
excitation and emission wavelengths of 510 and 586 nm respectively, the metabolism of the ethoxyresorufin can be measured by a progressive increase in fluorescence. This is proportionate to the concentration of product in the range 0.5-400 nM and there is no interference from amino acids and NADPH at these wavelengths.

The reaction mixtures consisted of 0.5 ml of reconstituted cytochrome P-448 system (as in Section 6.2.3.) 10 μl of 0.01 mM ethoxyresorufin and 1.5 ml of 0.1 M Tris-HCl buffer pH 7.8. The fluorimeter, set at 37°C, was first calibrated by adding 10 μl aliquots of resorufin (0.01 mM in ethanol) successively to 2 ml of buffer. The reaction mixture was then placed in the fluorimeter, the baseline was recorded, then the reaction was started with the addition of 10 μl of 50 mM NADPH solution and the initial rate was measured from the increase in fluorescence.

Neither reconstituted cytochrome P-448 system nor the microsomal fraction of benzo(a)pyrene-treated yeast showed any ethoxyresorufin O-deethylase activity.

Aniline is hydroxylated by mammalian hepatic cytochrome P-450 to p-aminophenol (VII) as reported by Imai and Sato (1974).
Aniline hydroxylase activity of reconstituted cytochrome P-448 system was determined by a method of Guarino et al (1969). Incubation mixture consisted of 0.5ml of reconstituted cytochrome P-448 system, 0.5ml of 0.04M aniline hydrochloride (pH 7.6) and 1ml of 0.1M Tris-HCl buffer, pH 7.6 containing NADPH generating system (as in Section 6.2.2.). After incubation for 15min, reaction was stopped by adding 1g of NaCl. 12ml Of peroxide free ether containing 1.5% (v/v) isoamylalcohol was added and P-aminophenol was extracted in a rotary shaker, for 20min. 10ml Of ether aliquots were subsequently added to 4ml of alkali phenol (0.5M tripotassium orthophosphate containing 1%, w/v, phenol) and extracted on a rotary mixer for 30min. The blue colour was measured at 620nm. Suitable blanks and standards (0.2-0.5mM in HCl) were carried through the same procedure. The colour in the test mixture was identified to that of blank, therefore no hydroxylation of aniline to P-aminophenol by reconstituted cytochrome P-448 system was detected.
CHAPTER 7
7. **Structural Analysis of Cytochrome P-448 from Saccharomyces cerevisiae**

7.1. **Introduction**

Structural components of cytochrome P-450/P-448 are predicted by Gunsalus and Coon (1980). In this chapter amino acid, heme carbohydrate and phospholipid content of yeast cytochrome P-448 is measured. The content of amino acid tryptophan in cytochrome P-448 was determined by an analytical ultraviolet fluorescence method in which the fluorescence intensity scale was calibrated using standard solutions of free tryptophan and a correlation coefficient between the fluorescence of cytochrome P-448 tryptophanyl residues and of free tryptophan was estimated in testing 10 well-characterized proteins. Cysteine content of this enzyme was also measured separately. Oxidation of this amino acid to cysteic acid (which contains sulfur in a stable oxidation state) made possible its accurate analysis in acid hydrolyzate of cytochrome P-448. The total amino acid residues present in yeast cytochrome P-448 was 407 which is less than those of mammalian cytochromes P-448 reported at this time. These values and the values for the other known structural components of various cytochrome P-450/P-448 are compared in this chapter.
7.2. Methods and Materials

7.2.1. Amino Acid Analysis of Yeast Cytochrome P-448

Highly purified cytochrome P-448 free of Emulgen 911 (7.2nmol) was twice dialyzed against 60 volumes of distilled water at 4°C and lyophilized in an Edwards High Vacuum lyophilizer (Edwards, U.K.). Three portions of this preparation were subsequently hydrolyzed by adding 2ml of 5.7-N HCl (under N₂) and incubating in sealed evacuated tubes at 100°C for a duration of 24, 48, 72h respectively. The tubes were then cooled and opened and their HCl content was removed by a Bucke rotary evaporator (Orem Scientific, U.K.). The amino acid content of these tubes was analyzed by a Beckman 121MB Amino Acid Analyzer (Beckman, U.S.A.). Final values for serine, threonine and methionine were obtained upon extrapolation to zero time hydrolysis.

The tryptophan content of cytochrome P-448 was measured by a fluorescence method shown by Pajot (1976). A 3ml solution of highly purified cytochrome P-448 free of Emulgen 911 but containing 0.03M of 2-mercaptoethanol was denatured by the addition of guanidine-HCl. The final concentrations of cytochrome P-448 and guainidine-HCl in the test solution were 1μM and 6M respectively. This solution was incubated at room temperature for 30min and its fluorescence was measured by a Perkin-Elmer MPF3 fluorescence

(269)
spectrophotometer (Hitachi, Japan) at 353nm emission wavelength (295nm excitation). A sample of quinine sulfate was used to adjust the fluorimeter to zero. Internal calibration was achieved by three successive additions of 45 μl of the stock solution of tryptophan (0.36mM). The internal calibration curve is constructed (Figure 7.1). This curve allows one to estimate by extrapolation the "free tryptophan fluorescence equivalent" of protein samples. In order to determine the best coefficient relating the actual tryptophan content of the protein sample to the observed fluorescence expressed in "free tryptophan fluorescence equivalent," measurements were carried out on 10 proteins, the amino acid compositions of which are all well-established (Figure 7.2.)

The cytoeine (or half-cytoeine) content of cytochrome P-448 was determined after performic acid oxidation of this amino acid to produce cysteic acid according to the method of Hirs (1967) modified from that of Moore (1953). Performic acid solution was prepared by mixing 5 volumes of 30% hydrogen peroxide and 95 volumes of 99% formic acid and was permitted to stand in a closed container at 25°C for 120min. To 2mg of cytochrome P-448 (35nmol), 2ml of the performic acid solution was added and the reaction mixture was permitted to stand at 0° for 4h. Then 0.3ml of a 48%
Figure 7.1. The Internal Calibration Curve for Determination of Tryptophan Content of Yeast Cytochrome P-448.
Figure 7.2. Correlation of the Tryptophan Content of Proteins with the Free Tryptophan Fluorescence Equivalent after Denaturation with Guanidine Hydrochloride. Proteins are cytochrome c (1), soybean trypsin inhibitor (2), β-lactoglobulin (3), bovine serum albumin (4), trypsin (5), pepsin (6), lysozyme (7), chymotrypsin (8), chymotrypsinogen (9), aldolase (10).
(v/v) HBr solution was added while swirling the reaction tube in the ice bath. The tube was attached to a rotary evaporator and 20ml of 1N NaOH was added to the condenser to absorb the bromine which distills over. It was then concentrated to dryness at a bath temperature of 40°C. Hydrolysis was achieved by adding 3ml of 5.7N HCL to the residue (under N2) and incubating in a sealed evacuated tube at 110°C for 18h. HCl was removed on the rotary evaporator. The residue was dissolved in 5ml of 0.2M sodium citrate buffer pH 2.2 and a 2ml aliquot was used for analysis by an analyzer equipped with a 150cm Amberlite IR-120 column. The integration constant used for cysteic acid was the same as that for aspartic acid (Hirs, 1967).

7.2.2. Heme Analysis of Yeast Cytochrome P-448

The heme content of the highly purified cytochrome P-448 was measured by a method described by Omura and Sato (1964b). The heme was converted to pyridine-hemochromogen by the addition of pyridine to cytochrome P-448 solution up to 20% (v/v) in final concentration in the presence of NaOH (0.1N). The sodium dithionite-reduced spectrum of pyridine hemochromogen was recorded at 500-575nm and its concentration was calculated by using an extinction coefficient of 32.2mM⁻¹cm⁻¹ between 557 and 575nm (Omura and Sato, 1964b).
7.2.3. Carbohydrate Analysis of Yeast Cytochrome P-448

For determination of the carbohydrate content of cytochrome P-448, a sample of this enzyme (20nmol) was hydrolyzed in 6N HCl in sealed tubes for 4h at 100°C (Spiro, 1973). The hydrolysate was taken to dryness at 30°C in Vacuo and the residue was dissolved in water, adjusted to pH 7.0, and applied to a column made by mixing equal amounts of Ag 50-X8 (H+ form) and Ag 1-X8 (OH− form) resins. The column was washed with water and then with methanol to elute neutral sugars and with 1N HCl to elute amino sugars. The acid eluate was taken to dryness, dissolved in 0.2M sodium citrate buffer, pH 2.2, and applied to the long column of an amino acid analyzer for characterization and quantitative determination of hexosamines.

For determination of neutral sugars, another sample of purified cytochrome P-448 was hydrolyzed and analyzed by gas-liquid chromatography using a Perkin-Elmer Gas-Liquid chromatograph (Hitachi, Japan) according to the method of Lehnhardt and Winzler (1968).

7.2.4. Measurement of Phospholipid Content of Yeast Cytochrome P-448

Following extensive dialysis of purified cytochrome P-448 (20nmol) against distilled water to remove phosphate
buffer, phospholipids were extracted by the method of Folch et al. (1957). In this method phospholipid was first extracted by a mixture of chloroform and methanol (2:1) and was washed by the same solvent solution twice, filtered and washed by the addition of KCl once and then by a mixture of chloroform, methanol, and water (3:48:47) three times. The phospholipid content of the protein then was estimated by determination of the phosphorus content according to the method described by Chen et al. (1956). The extracted phospholipid was dried down under N$_2$ in a water-bath at 30°C and was redissolved in distilled water (100µl). To this solution 0.5ml of perchloric acid (70%) and 0.2ml of hydrogen peroxide were added. The mixture was placed in a water-bath (25°C) and then was heated to 180-200°C for 20min and was cooled to 25°C immediately. To this 1ml of a reagent consisting of 6N sulfuric acid, water, 2.5% (w/v) ammonium molybdate, 10% (w/v) ascorbic acid (1:2:1:1:) was added and after 10min the absorbance was measured at 405nm against an appropriate blank. The concentration of phosphorus was determined in relation to a standard solution (disodium hydrogen phosphate).

7.2.5. Materials

Albumin (bovine), chymotrypsin (bovine), chymotrypsinogen (bovine), pepsin (porcine), were all obtained
from Mann Research Laboratories. Aldolase (rabbit muscle),
lysozyme (egg), cytochrome c (horseheart, type VI), trypsin
inhibitor (soybean), guanidine-HCl were purchased from Sigma
Chemical Ltd. Trypsin was obtained from Worthington
Biochemical Corp. β-Lactoglobulin was a Koch-Light
Laboratories product. Amberlite IR-120 was obtained from
Rohm and Haas Company. Ag 50-X8 and Ag 1-X8 were purchased
from Bio Rad Laboratories. Tryptophan, Pyridine, HBr and
other chemicals were supplied by BDH Chemicals Ltd.

7.3. **Results and Discussion**

7.3.1. **Structural Analysis of Yeast Cytochrome P-448**

The amino acid composition of yeast cytochrome P-448 is
shown in Table 7.1 along with the amino acid compositions of
cytochrome P-448 from untreated rat liver (Miki et al., 1981)
and cytochrome P-448 from β-naphthoflavone-pretreated rat
liver (Saito and Strobel, 1981). It was found that yeast
cytochrome P-448 contains 407 amino acid residues per
molecule in comparison to 469 for both mammalian cytochromes
P-448 (untreated and β-naphthoflavone-pretreated). Although
the number of amino acids is smaller than the total for the
two mammalian cytochrome P-448's mentioned above, the
hydrophobic residue content was almost identical with values
43%, 44% and 45% for yeast cytochrome P-448, rat liver
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Cytochrome P-448 Yeast</th>
<th>Cytochrome P-448 Rat Liver (Untreated)</th>
<th>Cytochrome P-448 Rat Liver (B NF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>41</td>
<td>33</td>
<td>41</td>
</tr>
<tr>
<td>Thr</td>
<td>21</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>Ser</td>
<td>24</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>Glx</td>
<td>37</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>Pro</td>
<td>23</td>
<td>36</td>
<td>28</td>
</tr>
<tr>
<td>Gly</td>
<td>42</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>Ala</td>
<td>27</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>Val</td>
<td>30</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>Met</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Ile</td>
<td>13</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>Leu</td>
<td>23</td>
<td>53</td>
<td>52</td>
</tr>
<tr>
<td>Tyr</td>
<td>15</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Phe</td>
<td>20</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td>Lys</td>
<td>27</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>His</td>
<td>17</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Arg</td>
<td>20</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Trp</td>
<td>12</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Cys</td>
<td>8</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>407</td>
<td>469</td>
<td>469</td>
</tr>
<tr>
<td>Polypeptide molecular weight</td>
<td>53,045</td>
<td>53,600</td>
<td>52,545</td>
</tr>
</tbody>
</table>

Table 7.1. Amino Acid Composition of Yeast Cytochrome P-448 Compared with Cytochrome P-448 from Untreated and β-Naphthoflavone Pretreated Rat Liver.
cytochrome P-448 (untreated) and rat liver cytochrome P-448 (β-naphthoflavone-pretreated) respectively.

The heme content of yeast cytochrome P-448 was, as expected, found to be one per polypeptide chain, arising from the experimental value of 1.09 nmol heme/nmol cytochrome P-448. Phospholipid was present at very low levels not exceeding 0.5 nmol/nmol cytochrome P-448. Phospholipid is reported to be present in β-naphthoflavone induced rabbit liver cytochrome P-450 enzymes as much as 0.3 nmol/nmol of cytochrome P-450LM2 and 0.5-1.6 nmol/nmol of cytochrome P-450LM4 (Haugen and Coon, 1976). The analysis for neutral sugars and amino sugars did not indicate the presence of these materials in appreciable amounts in yeast cytochrome P-448. Perhaps the method used for the determination of these compounds was not sensitive enough to measure neutral sugars and amino sugar in nmol scale.

Table 7.2 shows the structural analysis of yeast cytochrome P-448, as determined in this study, in comparison with those reported in literature for other cytochrome P-450/P-448 enzymes.

When the polypeptide chain molecular weight of 53,045 derived from the amino acid content is added to the
<table>
<thead>
<tr>
<th>Cytochrome P-450/P-448</th>
<th>Amino Acid</th>
<th>Heme</th>
<th>Neutral Sugar</th>
<th>Amino Sugar</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P-448 from yeast (in this study)</td>
<td>407</td>
<td>1.09</td>
<td>0.15</td>
<td>0.08</td>
<td>0.5</td>
</tr>
<tr>
<td>Cytochrome P-450 (Katagira and Suhara, 1980)</td>
<td>--</td>
<td>0.9</td>
<td>2.8</td>
<td>2.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Cytochrome P-450 (Katagiri and Suhara, 1980)</td>
<td>--</td>
<td>0.8</td>
<td>2.6</td>
<td>1.9</td>
<td>5.2</td>
</tr>
<tr>
<td>Cytochrome P-450LM2 (Haugan and Coon, 1976)</td>
<td>424</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>Cytochrome P-450LM4 (Haugan and Coon, 1976)</td>
<td>482</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table 7.2. A Comparison of the Known Structural Components of Various Cytochrome P-450/P-448. All data are expressed as mol/mol of cytochrome.
molecular weight of a ferro-protoporphrin IX group (approx. 1000) along with an estimated 2-4 hexose and heosamine (Dus et al., 1970; Katagiri and Suhara, 1980; Haugen and Coon, 1976) and molecular weight of phospholipid, the calculated molecular weight of cytochrome P-448 is in good agreement with the experimental value of 55,500 determined by SDS-polyacrylamide gel electrophoresis (Chapter 3).
CHAPTER 8
8. Chemical Modification of Amino Acid Residues in Cytochrome P-448 from Saccharomyces cerevisiae: The Effect on its Reduced CO-Difference Spectrum, Benzo(a)pyrene-3-Monooxygenase Activity, Binding Parameters and Temperature-Induced Spin State Equilibrium.

8.1. Introduction

Many attempts have been made to identify the nature of ligands of cytochrome P-450/P-448. One of these ligands, known as the fifth ligand is trans to the bound carbon monoxide in the ferrous carbonyl state of cytochrome P-450/P-448. Recent studies on some synthetic model heme complexes and on cytochrome P-450 itself, suggested that the unique spectrophotometric characteristic of this enzyme (showing a Soret band at around 450nm upon binding with CO in the ferrous state) is due to thiolate ligation at the fifth coordination position of heme iron (Shimiza et al, 1973). This ligand is probably present throughout the cycle of the enzyme. The more recent data on thiolate heme ligation and its spectral impact are reported by Hanson et al (1976), Champion et al (1982) and Hahn et al (1982).

Cytochrome P-450/P-448 in its native low-spin form undergoes spectral changes upon binding with a substrate (Imai and Sato, 1966; Murray et al, 1982; Sheets and
Vickery, 1983). Even in the absence of exogenous added substrate, temperature-induced spectral changes can be observed (Cinti et al., 1979). These spectral changes, indicative of a spin state shift, are caused by the removal of one of the native axial ligands in the active site of the free enzyme (Yoshida and Kumaoka, 1975a). This removable ligand may be the sixth ligand, trans to thiolate, where substrate binds. The removal of the bonding to thiolate may cause the conversion of the cytochrome to the inactive, cytochrome P-420 form. The nature of the ligand trans to thiolate (the sixth ligand) is not fully established yet. Two general types of ligands have been proposed, depending on whether coordination to iron occurs through a nitrogen or oxygen atom. A nitrogen for the sixth ligand is most frequently pictured as being from the side chain imidazole of a histidyl residue of the protein (Shimiza et al., 1973; Jefcoate and Gaylor, 1969b; Tang et al., 1976; Lipscom and Gunsalus, 1973; Chevion et al., 1977). The oxygen ligation would be derived from species such as serine, threonine (Jillrich et al., 1979), tyrosine (Ruckpaul et al., 1980) and water (Griffin and Peterson, 1975).

In this study the influence of -SH groups, and the candidates for the sixth ligand such as histidine, arginine, tryptophan and tyrosine on benzo(a)pyrene-3-monoxygenase
activity, binding parameters and the spin state equilibrium of yeast cytochrome P-448, is investigated. The results of chemical modification studies indicated the participation of -SH group(s) (other than the fifth ligand thiolate) in the catalytic ability of the enzyme and noted the possibility of a tyrosine residue as the ligand of this hemoprotein enzyme.

8.2. Methods and Materials

8.2.1. Preparation of Cytochrome P-448 for Chemical Modification

After the final step of the purification procedure, Emulgen 911 was removed from cytochrome P-448 (80nmol) by the method described in Chapter 3. When the storage time of this preparation at -70°C exceeded one week, the titratable sulfhydryl groups of the cytochrome P-448 preparation were decreased. To regenerate the free -SH group, the stored preparation prior to sulfhydryl modification was treated under N₂ at room temperature for 15min, with 10 fold in molar excess of sodium sulfite followed by the addition of 50 fold in molar excess of dithiothreitol as described by Kawalek et al (1977). The mixture was stirred under N₂ at room temperature for 15min, and the sulfite-dithiothreitol treated cytochrome P-448 was then dialyzed overnight against 200 volumes of 0.1M potassium phosphate buffer pH 7.4 containing 20% (v/v) glycerol followed by another 12-24h
dialysis with a fresh buffer to remove the sulfite and dithiothreitol completely.

8.2.2. **Preparation of Sulphydryl Reagents**

p-Chloromercuribenzoate (PCMB) was prepared in 0.33M sodium acetate buffer according to the method described by Boyer (1954) and was quantitated using $E_{233\text{nm}} = 17\text{mM}^{-1}\text{cm}^{-1}$.

5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) and 4,4'-dipyridinedisulfide (PDS) were dissolved in 0.01M potassium phosphate buffer pH 7.4 and standardized using the known extinction coefficients $E_{373\text{nm}} = 16.6\text{mM}^{-1}\text{cm}^{-1}$ for PDS; $E_{247\text{nm}} = 16.3\text{mM}^{-1}\text{cm}^{-1}$ for DTNB (Kawalek et al., 1977).

2-Nitro-5-thiocyanobenzoic acid (NTCB) was synthesized according to the method of Degani and Pathchornik (1971), dissolved in 0.1M potassium phosphate buffer pH 7.4 and was standardized using $E_{292\text{nm}} = 8\text{mM}^{-1}\text{cm}^{-1}$.

8.2.3. **Modification of Sulphydryl Groups in Yeast**

**Cytochrome P-448**

In a typical titration, aliquots of standardized sulphydryl reagent (up to 50 l) in 0.01M potassium phosphate buffer were added to one ml of purified cytochrome P-448 (usually 1µM in 0.1M potassium phosphate buffer containing 20%, v/v, glycerol). The pH of buffers varied from 7.0 when PCMB to 7.4 when other sulphydryl reagents were used. To
measure the degree of modification the absorbance changes at
the selected wavelength pair were followed until there were
no changes, using a Perkin-Elmer 356 spectrophotometer
(Hitachi, Japan) in the split-beam mode. Appropriate
reagent blanks were used to account for any nonspecific
chemical breakdown of the reagents. The thiopyridine
released when PDS was used to modify the sulfhydryl groups,
was estimated from the absorbance change between 324 and
390nm using an extinction coefficient of 19.8 M⁻¹ cm⁻¹
(Grassetti and Murray, 1967). Thionitrobenzoate produced by
modification of -SH groups by DTNB and NTCB was determined
from absorbance changes between 412 and 500nm using an
extinction coefficient of 13.6 M⁻¹ cm⁻¹ (Ellman, 1959). When
PCMB was used to modify the cytochrome P-448, the protein-
mercuribenzoate complex was measured from the absorbance
change at 250nm in the difference spectrum, using an
extinction coefficient of 7.6 M⁻¹ cm⁻¹ (Boyer, 1954).

8.2.4. Modification of Histidyl Residues of Yeast

Cytochrome P-448

A solution of 10mM diethylpyrocarbonate in ethanol was
standardized by the addition of 5μl to one ml of 10mM
imidazole at pH 7.5 in a cuvette having a 1-cm length path.
The increase in absorbance at 230nm due to formation of N-
carbethoxyimidazole was determined using an extinction
coefficient of $3.0\text{mM}^{-1}\text{cm}^{-1}$ (Miles, 1977). To measure the extent of the modification of histidyl residues in yeast cytochrome P-448 by diethylpyrocarbonate, two cuvettes containing 1ml of aliquots of this enzyme (1μM in 0.1M potassium phosphate buffer pH 6.5 containing 20%, v/v, glycerol) were placed in the sample and reference compartments of a double-beam Pye Unicam SP 1800 spectrophotometer at 4°C. Difference spectra were recorded between 230 and 330nm before and at various time intervals after the addition of an amount of 10mM diethylpyrocarbonate in ethanol to the sample cuvette (an equal volume of ethanol to reference cuvette) to give a final concentration of 0.75mM. Absolute spectra are also recorded of the enzyme solution before and after treatment for 30min with 0.75mM diethylpyrocarbonate using a buffer blank. The number of histidyl residues modified at each time interval is calculated from the absorbance changes at 243nm using an extinction coefficient of $3.2\text{mM}^{-1}\text{cm}^{-1}$ (Miles, 1977).

8.2.5. Modification of Arginyl Residues of Yeast Cytochrome P-448

Arginyl residues were modified according to the method of Lu et al (1981). 2,3-Butadione was diluted to 1M in concentration with 0.05M sodium borate buffer, pH 8.0 and was added to the cytochrome P-448 (1μM in 0.05M sodium
borate buffer containing 20%, v/v, glycerol), up to 10,000 fold that of the enzyme on a molar basis. Excess 2,3-butanedione was removed by gel filtration on a Sephadex G-25 column preequilibrated with 0.05M sodium borate pH 8.0. The quantitation of modified arginyl residues by 2,3-butanedione were conducted by amino acid analysis as described in Chapter 7. The arginine content of the enzyme decreases upon modification, since 2,3-butanedione-arginine complex is degraded in acid hydrolysis, and thus the number of arginine residues modified is determined.

8.2.6. Modification of Tryptophyl Residues of Yeast Cytochrome P-448

The modification of tryptophyl residues in cytochrome P-448 (7μM in 0.05M sodium borate buffer pH 8.0 containing 20%, v/v, glycerol) was conducted according to the method described by Lu et al (1981). In this modification up to 25 equivalents of N-bromosuccinimide (0.01M in H₂O) per enzyme were added and the extent of the modification was determined as described by Spande and Witkop (1967) using 5.5mm⁻¹cm⁻¹ as an extinction coefficient. This method makes use of the fact that indole chromophore of tryptophyl, absorbing strongly at 280nm, on oxidation with N-bromosuccinimide, is converted to oxindole or an intermediate which are much weaker chromophores at this wavelength.
8.2.7. **Modification of Tyrosyl Residues of Yeast Cytochrome P-448**

Nitration of cytochrome P-448 with tetranitromethane (TNM) was conducted according to the method described by Yoshinega and Sano (1980). Cytochrome P-448 (3.5μM in 0.1M Tris-HCl pH 8.0 containing 20%, v/v, glycerol) was treated with TNM up to 50 fold that of enzyme on a molar excess. The extent of modification of tyrosyl residues was monitored by measuring an absorbance increase of nitroformate ion at 350nm (10-60min after each treatment) and using an extinction coefficient 14.4mM⁻¹cm⁻¹ for the determination of the concentration of this product (Riordan et al., 1966). At pH 8.0, nitration displaces two protons and produces one mol of nitroformate per mol of tyrosine, suggesting the formation of 3-nitrotirosine (Riordan et al., 1966). The reaction was stopped by the addition of β-mercaptoethanol up to 0.1M in final concentration. Then the solution was passed through a Sephadex G-25 column to remove both the nitroformate and β-mercaptoethanol. O-Acetylation of tyrosine with N-acetylimidazole was performed according to the method described by Riordan and Vallee (1972). Cytochrome P-448 (3.5μM in 0.1M potassium phosphate buffer pH 7.5 containing 20%, v/v, glycerol) was treated with N-acetylimidazole up to 200 fold that of enzyme on molar basis. The extent of tyrosine modification (10-60min after
each treatment) was calculated from the increase in absorbance at 278nm due to reversing the acetylation by 0.5M hydroxylamine (using extinction coefficient 11.6mM$^{-1}$cm$^{-1}$), after the excess N-acetylimidazole had been removed by gel filtration.

8.2.8. Spin Equilibrium Analysis of Yeast Cytochrome P-448

Spin equilibrium constant and subsequently spin content of various modified and control cytochrome P-448 were determined using a temperature-dependent spin equilibrium analysis method described by Cinti et al (1979). At any physiological temperature, the ferric cytochrome P-450/P-448 is considered to exist as a mixture of high spin (total spin, $S = 5/2$) or low spin ($S=1/2$) forms corresponding to the order of the five d-orbital electrons. Each of the high and low-spin forms correspond to a unique optical spectrum. Thus, at any wavelength the total absorbance may be expressed as

$$A = \varepsilon_{HS} [P-448_{HS}] + \varepsilon_{LS} [P-448_{LS}]$$

where $\varepsilon_{HS}$ and $\varepsilon_{LS}$ refer to the extinction coefficients and $[P-448_{HS}]$ and $[P-448_{LS}]$ to the concentrations of high and low spin forms respectively. In addition, at all wavelengths there exists a maximum, $A_{max}$, and a minimum, $A_{min}$. 

A_{min}, value of absorbance which correspond to the limiting cases when the cytochrome P-448 molecule is in either the complete high spin or complete low spin state. When cytochrome P-448 exists as a mixture of spin states, the absorbance will fall between the limiting values, A_{max} and A_{min}, with the distance from the upper and lower limits being directly proportional to the high and low spin fractions. For example, at 387nm the fraction of high spin material is given by the ratio \((A_{min}-A)/(A_{max}-A_{min})\) while the proportion of low spin is \((A-A_{max})/(A_{max}-A_{min})\). Defining an equilibrium constant for the processes cytochrome P-448_{LS} ⇌ cytochrome P-448_{HS} by

\[
K = \frac{[P-448_{HS}]}{[P-448_{LS}]}
\]

yields the following expression for spin equilibrium constant:

\[
K = \frac{A_{min} - A}{A - A_{max}}
\]

Here A is an observed absorbance difference at 387-418nm at a given temperature measured by the dual wavelength mode of a Perkin-Elmer 356 spectrophotometer. If this is assumed to follow the standard van't Hoff behavior it may be written:

\[
\ln K = \ln \left( \frac{A_{min} - A}{A - A_{max}} \right) = \frac{\Delta H}{RT} - \frac{\Delta S}{R}
\]
which describes the spin equilibrium constant as a function of temperature. Since the values of $A_{\text{max}}$ and $A_{\text{min}}$ cannot be determined prior to the experiment, they are used as parameters in a regression fit to the variation in the observed absorbance value as a function of temperature. Using the correlation coefficient as a convergence parameter for the least-squares straight line fit of

$$\ln \left( \frac{A_{\text{min}} - A}{(A - A_{\text{max}})} \right) \text{ vs.} \frac{1}{RT},$$

the values $A_{\text{max}}$ and $A_{\text{min}}$ which give the best fit to the observed absorbance as well as the slope $\Delta H$ and intercept $-\Delta S/R$ may be calculated. The best fit was determined by the use of a computer program presented in Appendix II.

8.2.9. Determination of Benzo(a)pyrene-3-Monooxygenase Activity and Apparent Binding Parameters of Modified Yeast Cytochrome P-448

Various modified and control samples of cytochrome P-448 (usually 1nmol) were used in one ml of assay mixture also containing 1 unit of cytochrome c(P-450) reductase and 30μg of dilauryl phosphatidylcholine. The NADPH-supported 3-hydroxbenzo(a)pyrene activity was determined as described in Chapter 6.

The apparent spectral dissociation constant ($K_s$) and maximum spectral interaction of cytochrome P-448 and benzo(a)pyrene in 387-418nm region in each case was
determined using denatured cytochrome P-448 as reference in a double-cell system as described in Chapter 5.

8.2.10. Materials

P-Chloromercuribenzoate (PCMB), 4,4'-dipyridine-disulfite (PDS), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 2,3-butadione and N-bromosuccinimide were purchased from Aldrich Co. Diethylpyrocarbonate and sodium sulfite were obtained from BDH Chemical Ltd. N-Acetylimidazole, tetranitromethane were purchased from Sigma Chemical Co.

8.3. Results and Discussion

8.3.1. Titration of Sulfhydryl Group in Yeast Cytochrome P-448

The sulfhydryl reagents were used to quantitate the -SH groups in freshly prepared cytochrome P-448 (Figure 8.1). PDS alkylated all the sulfhydryl groups present in cytochrome P-448 as determined by amino acid analysis. In the absence of 6M guanidine-HCl, 7.5, 7.6 and 7.75 sulfhydryl groups were titratable per mol of fresh cytochrome P-448 preparation by DTNB, PCMB and PDS respectively and the titrations were somewhat stoichiometric (Table 8.1 and Figure 8.2). Twenty fold excess of NTCB was required to titrate all eight of the sulfhydryl groups of cytochrome P-448. Normally four -SH groups were modified by
Figure 8.1. Reactions Between Free Sulfhydryl Groups in Protein and the Sulfhydryl Modifying Reagents.
<table>
<thead>
<tr>
<th>Reagents</th>
<th>nmol of -SH Group per nmol of Cytochrome P-448</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no Guanidine-HCl</td>
</tr>
<tr>
<td>PDS</td>
<td>7.75</td>
</tr>
<tr>
<td>DTNB</td>
<td>7.5</td>
</tr>
<tr>
<td>PCMB</td>
<td>7.6</td>
</tr>
<tr>
<td>Performic acid oxidation</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 8.1. Titration of Sulfhydryl Groups of Yeast Cytochrome P-448.
Figure 8.2. Titration of Yeast Cytochrome P-448 with Sulfhydryl Reagents: PDS (x), PCMB (•), DTNB (o).
this reagent (Table 8.2). Titratable -SH groups in stored yeast cytochrome P-448 (at -70°C) were treated with excess DTT and sodium sulfite followed by extensive dialysis all the sulfhydryl groups were titratable in the same mode as the fresh cytochrome P-448 preparation. Kawalek et al (1977) have indicated that the loss of titratable -SH groups in the stored preparation might be due to the formation of intramolecular and/or intermolecular disulfide bonds. Table 8.1 shows the number of sulfhydryl groups titrated by various reagents. The number of sulfhydryl groups in cytochrome P-448 as determined by the use of these reagents is in agreement with the results obtained by half-cystine analysis performed according to the method of Hirs (1967).

8.3.2. Effect of Sulfhydryl Modification on Benzo(a)pyrene-3-Monooxygenase Activity and Binding Parameters of Yeast Cytochrome P-448

When Cytochrome P-448 was titrated with PCMB all the benzo(a)pyrene-3-monooxygenase activity was lost when more than six -SH groups per mol of the enzyme were modified. In titration with PDS and DTNB, NTCB 70-80% of enzymatic activity was lost when four or more of sulfhydryl groups were modified. The apparent binding parameters ($K_s$ and $A_{max}$) were not altered appreciably upon these modifications, as it is shown for NTCB in Table 8.2. In all cases when benzo(a)pyrene was
<table>
<thead>
<tr>
<th>Form of Cytochrome P-448</th>
<th>Cytochrome P-448 content (% of control)</th>
<th>BP-3-Mono-oxygenase activity (% of control)</th>
<th>$K_s$ (µM)</th>
<th>$\Delta A_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>0.122</td>
</tr>
<tr>
<td>Four -SH Groups modified by NTBC</td>
<td>90</td>
<td>28</td>
<td>47</td>
<td>0.11</td>
</tr>
<tr>
<td>All histidyl residues modified</td>
<td>100</td>
<td>40</td>
<td>210</td>
<td>0.07</td>
</tr>
<tr>
<td>All arginyl residues modified</td>
<td>98</td>
<td>93</td>
<td>51</td>
<td>0.118</td>
</tr>
<tr>
<td>All tryptophyl residues modified</td>
<td>97</td>
<td>80</td>
<td>107</td>
<td>0.082</td>
</tr>
<tr>
<td>Five tyrosyl residues modified</td>
<td>100</td>
<td>15</td>
<td>54</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 8.2. Effect of Modification of Some Amino Acids on Catalytic Activity and Binding Parameters of Yeast Cytochrome P-448.
added to cytochrome P-448 solution prior to the modification, the loss of benzo(a)pyrene-3-monooxygenase was not prevented. If DTT was introduced into the modified cytochrome P-448 solution before addition of this enzyme to the monooxygenase assay mixture, 50% of its activity was restored. The loss of activity caused by modification of -SH groups by these reagents and the recovery of some of this activity upon addition of DTT suggests that sulfhydryl group(s) in yeast cytochrome P-448 may participate in catalysis. There is always a possibility of steric hindrance or conformational change in the enzyme molecule due to the introduction of a reagent with a bulky, charged functional group. This would also result in the loss of enzymatic activity. For this reason it was essential to use several sulfhydryl reagents with different substituents in this study. NTCB is one of the best reagents for the modification of sulfhydryl groups, since its -CN substituent which binds to the -SH group is smaller than the bulkier substituents of other reagents. Modification of the sulfhydryl groups by NTCB significantly inhibited the hydroxylation of benzo(a)pyrene which could then be reversed up to a certain degree by DTT. Since stoichiometric amounts of sulfhydryl reagent (except for NTCB) were used up to modify the cytochrome P-448 itself, NADPH: cytochrome c(P-450) reductase, the other component of benzo(a)pyrene
hydroxylase system, was not affected. As for NTCB-treated cytochrome P-448, the excess reagent was removed by gel filtration prior to reconstitution of the hydroxylase activity.

Modification of up to six -SH groups per mol of yeast cytochrome P-448 by PDS, NTCB, DTNB caused no conversion to cytochrome P-420 as determined by the CO-difference spectrum. As most of catalytic activity of cytochrome P-448 was lost after modification of four -SH groups by these reagents, it may be concluded that the loss of catalytic activity is not due to conformational change of cytochrome P-448 in the heme binding region.

In the case of PCMB, this conversion was parallel to the loss of benzo(a)pyrene-3-monooxygenase activity. Kawalek et al (1977) have indicated that modification of sulfhydryl groups in cytochrome P-448 causes extreme instability of this enzyme in reduced form. However, unmodified cytochrome P-450/P-448 is also less stable when it is fully reduced (Imai and Sato, 1974).

Earlier studies by Mason et al (1965b) have shown that modification by PCMB induces the conversion of cytochrome P-450/P-448 in oxidized and reduced form to cytochrome
P-420. These authors further concluded that the compound that displays a characteristic ESR signal is the sulfide of cytochrome P-420 and cytochrome P-450 is the phospholipid complex of sulfide of cytochrome P-420. Furthermore, CO displaced the sulfide but continues to interact with it and with phospholipid in the CO complex. Thus it is apparent that modification of all -SH (or half cystine residues) in cytochrome P-450/P-448 causes conversion to cytochrome P-420.

In all, when four -SH groups in cytochrome P-448 were modified, the binding parameters of benzo(a)pyrene to cytochrome P-448 was not impaired and a decrease in activity, except perhaps for modification with PCMB, was not due to conversion to cytochrome P-420 (i.e., modification of thiolate as a fifth ligand), thereby suggesting the existence of sulfhydryl groups in or near the active site of this enzyme in yeast. However, it was not determined in all cases whether loss of catalytic activity is caused by modification of -SH group(s), or indirectly, is due to the steric blockage (by added substitutent) and/or conformational changes in the protein. Furthermore, the generally accepted scheme of cytochrome P-450/P-448-catalyzed hydroxylase cycle does not clearly show the participation of sulfhydryl group(s) in the process of catalysis.
8.3.3. **Effect of Modification of Histidyl, Arginyl, Tryptophyl, Tyrosyl Residues on Benzo(a)pyrene-3-Monooxygenase Activity and Binding Parameters of Yeast Cytochrome P-448**

Figures 8.3 and 8.4, and Table 8.3 show the modification of these amino acids with respective reagents. Modification of histidyl residues in cytochrome P-448 was performed as described in "Method" and the carboxethoxylamination was completed when 0.75mM diethylpyrocarbonate was used for 30min. Analysis of data indicated that 15 histidyl residues were modified per mol of cytochrome P-448, two less than 17 histidyl residues present in the molecule as determined by amino acid analysis (Chapter 7). Modification of all the histidyl residues did not affect the CO-difference spectrum of the reduced cytochrome P-448. This modification decreased the benzo(a)pyrene-3-monooxygenase activity by 60%. It was apparent that modification of the histidyl residues interfered with benzo(a)pyrene binding to cytochrome P-448 since a significant decrease in affinity, an increase in apparent spectral dissociation constant ($K_s$) and a decrease in maximum spectral interaction ($\Delta A_{max}$), was also observed. The value of $K_s$ increased from 50 M to 200 M and $\Delta A_{max}$ decreased from 0.122 to 0.07.
Figure 8.3. Reaction Between Histidyl, Arginyl, Tyrosyl Residues in Protein and Modifying Reagents.
Figure 8.4. Oxidation Pathway of Tryptophyl Residue in Protein with N-Bromosuccinimide.
<table>
<thead>
<tr>
<th>Modified Amino Acid</th>
<th>Max. Quantity of Reagent Used Expressed as [Reagent]/[Enzyme]</th>
<th>Number of Amino Acids Modified</th>
<th>Number of Amino Acids Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>750</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Arginine</td>
<td>10,000</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>25</td>
<td>11.2</td>
<td>12</td>
</tr>
<tr>
<td>Tyrosine:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-acetylation</td>
<td>200</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Nitration</td>
<td>50</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table 8.3. Quantitation of Titration of Histidine, Arginine, Tryptophan and Tyrosine Residues of Yeast Cytochrome P-448 with Modifying Reagents.
It has been proposed that the initial product of the reaction of 2,3-butadione with the guanidino group is a 4,5-dimethyl-4,5-dihydroxy-2-imidazole that can be stabilized by esterification with borate (Lu et al, 1981). Amino acid analysis showed that only arginyl residues were affected by 2,3-butadione modification of cytochrome P-448. When 2,3-butadione in sodium borate, was added to cytochrome P-448, up to 10mM in concentration, all the arginyl residues were destroyed. The effect of this modification on benzo(a)pyrene-3-monoxygenase activity before and after the removal of borate by gel filtration was the same. The residual activities were 93% of the activity of the unmodified enzyme. Modification of arginyl residues did not have an appreciable effect on CO-difference spectrum of reduced cytochrome P-448.

When cytochrome P-448 was treated with N-bromosuccinimide, 25 equivalents of this reagent were needed to oxidize 11.2 out of 12 tryptophyl residues of this enzyme as determined by loss of absorption at 280nm. A proposed oxidation pathway of tryptophan by N-bromosuccinimide in acidic media is shown in Figure 8.4. It has been reported that N-bromosuccinimide cleaves the tryptophyl bonds of peptides in an acidic pH (Spande et al, 1970). However, at pH 8.0, although a considerable loss in
280nm absorption due to oxidation of tryptophyl residues were observed, there was no cleavage as determined by analytical gel filtration. It is possible that at pH 8.0 the oxidation pathway of tryptophan, in which the indole chromophore of this amino acid is converted to oxindole is not completed and only an intermediate is formed. However, in product and intermediates the electron configuration of nitrogen in the indole group is changed by this modification.

The titration of tryptophyl residues decreased the benzo(a)pyrene-3-monooxygenase activity of cytochrome P-448 by 20% while the effect on the CO-difference spectrum of reduced form was negligible. The elimination of borate by gel filtration did not increase the benzo(a)pyrene-3-monooxygenase activity of N-bromosuccinimide-modified cytochrome P-448. Both $K_s$ and $\Delta A_{\text{max}}$ were influenced when tryptophyl residues were modified progressively (Table 8.2). Amino acid analysis of the N-bromosuccinimide-modified cytochrome P-448 (when 11.2 tryptophyl residues were modified) indicated that 10-15% of cysteine and histidine residues were also destroyed.

Cytochrome P-448 was modified by N-acetylimidazole and tetranitromethane, which preferentially react with the tyrosyl residues, to identify other amino acids which are
involved in substrate binding and catalysis. Only five out of fifteen tyrosyl residues of cytochrome P-448 were accessible to be modified by both O-acetylation and nitration. O-Acetylation was successfully reversed by treatment with 0.5M hydroxylamine. No denaturation was observed (determined by CO-difference spectrum of reduced form) upon this modification, but 85% of benzo(a)pyrene-3-monooxygenase activity of the cytochrome P-448 was lost while $K_s$ and $\Delta A_{m\text{ax}}$ remained virtually unchanged (Table 8.2). When benzo(a)pyrene was added prior to the modification the loss of benzo(a)pyrene-3-monooxygenase activity was not prevented. In cytochrome P-448 treated with N-acetylindazole, no S-acetylation of -SH groups was observed as the titration of all 8 -SH groups of acetylated protein by PDS, was still possible.

8.3.4. **Effect of Modification of Cysteinyl, Histidyl, Arginyl,Tryptophyl and Tyrosyl Residues on Spin State Equilibrium of Yeast Cytochrome P-448**

When the recorded temperature-induced absorbance changes were fitted to an equilibrium constant, a van't Hoff plot was obtained (Figure 8.5). From the values of spin equilibrium constant the spin content of the control and each of the modified cytochromes P-448 were determined at various temperatures using the expression $P_{448} = \frac{K}{1 + K}$. 
Figure 8.5. Equilibrium Constant for Unmodified, Substrate-Free (x) and Tyrosine-Modified (●) of Yeast Cytochrome P-448. For unmodified and tyrosine-modified cytochrome P-448 correlation coefficients used as convergence parameters in the fitting analysis, were 0.99455 and 0.99998 respectively.
At 22°C the low spin content of unmodified substrate-free cytochrome P-448 was 94% (Table 8.4). As is demonstrated by the van't Hoff plot there was not an appreciable amount of spin state modulation by temperature changes after modification (acetylation) of five tyrosyl residues by N-acetylimidazole. When the -SH groups in cytochrome P-448 were modified the temperature-induced spin state shift was not observed due to total conversion of cytochrome P-448 to cytochrome P-420. Modification of four -SH groups did not result in any changes in the pattern of temperature-induced modulation from that of unmodified cytochrome P-448. For each of histidine-modified and tryptophan-modified cytochrome P-448 (maximum number of amino acid modified in both cases), low spin content differed slightly from that of the control at all temperatures. At 22°C the low spin contents were 95 and 97 percent for histidine-modified and tryptophan-modified cytochrome P-448 respectively. For arginine-modified cytochrome P-448 (all residues modified the spin state modulation was somewhat the same as the one of the control enzyme.

8.3.5. Tyrosine, A Possible Sixth Ligand of Yeast Cytochrome P-448

The amino acids with nitrogenous functional groups viewed as a possible sixth ligand of cytochrome P-448 (histidine, tryptophan, arginine) were modified as
Table 8.4. Spin State Equilibrium of Cytochrome P-448 at Various Temperatures in Presence and Absence of Benzo(a)pyrene. Benzo(a)pyrene was added to give final concentration 158μM. K is the spin equilibrium constant as defined in the text.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Substrate Free</th>
<th>In Presence of BP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Low Spin</td>
<td>K</td>
</tr>
<tr>
<td>18</td>
<td>95.8</td>
<td>0.044</td>
</tr>
<tr>
<td>22</td>
<td>94.0</td>
<td>0.064</td>
</tr>
<tr>
<td>26</td>
<td>91.7</td>
<td>0.091</td>
</tr>
<tr>
<td>30</td>
<td>88.6</td>
<td>0.128</td>
</tr>
<tr>
<td>34</td>
<td>85.7</td>
<td>0.166</td>
</tr>
<tr>
<td>38</td>
<td>81.5</td>
<td>0.226</td>
</tr>
</tbody>
</table>
described. Modification of histidine's imidazole side chain, which has been frequently pictured as the sixth ligand of cytochrome P-450/P-448 (Shimiza et al., 1973; Jefcoate and Gaylor, 1969b; Tang et al., 1976; Lipscomb and Gunsalus, 1973; Chevion et al., 1977), had an appreciable effect on benzo(a)pyrene-3-monooxygenase activity of cytochrome P-448. This impairment of the catalytic activity may have been caused by a decrease in binding affinity and the extent of interaction of benzo(a)pyrene with modified cytochrome P-448. The temperature-induced spin state modulation pattern of histidine-modified cytochrome P-448 was different from that of substrate-free unmodified enzyme, but the low spin content decreased with the rise of the temperature indicating the probable presence of a sixth ligand despite this modification.

The modification of arginyl residues did not alter the binding parameters or the catalytic activity of cytochrome P-448 with benzo(a)pyrene. The spin state equilibrium was not changed by this modification.

A slight decrease in benzo(a)pyrene-3-monooxygenase activity of tryptophan-modified cytochrome P-448 is possibly due to a decrease in binding affinity. The data indicates that this amino acid, along with histidine, is important in
substrate binding to the hemoprotein. The possibility of tryptophan being the sixth ligand is ruled out since spin state equilibrium of tryptophan-modified cytochrome P-448 was temperature dependent, although the low spin content at various temperatures differed from that of the unmodified hemoprotein.

Nitration and O-acetylation of tyrosyl residues of cytochrome P-448 revealed that only five of these residues are accessible to modification. O-Acetylation of these tyrosyl residues inhibited almost all of the cytochrome P-448 catalytic activity without denaturing the enzyme (450nm peak). The loss in activity was not caused by alteration of the binding site as there were no changes in the binding parameters of benzo(a)pyrene to tyrosine-modified enzyme. The data presented in this chapter indicates the yeast cytochrome P-448 possibly contains tyrosyl residue(s) in its active site.

According to current concepts (Gunsalus and Sligar, 1978; Yoshida et al, 1982) the high spin content of cytochrome P-450/P-448 is increased when the sixth ligand has been removed or replaced by ligand with a weaker field strength. In this study O-acetylation of the tyrosyl residues influenced greatly the temperature-induced spin
state modulation. As it is shown in Figure 8.5 there are virtually no changes in spin equilibrium constant K upon increasing the temperature. This indicates the possibility that modification of tyrosyl residues may have resulted in the removal of the sixth ligand.

Recently several workers have shown evidence that tyrosine is the sixth ligand in cytochrome P-448 (Ruckpaul et al, 1980; Janig et al, 1982), while others (Jillrich et al, 1979; Dawson et al, 1982) have simply indicated that the native sixth ligand might contain oxygen.

Ruckpaul et al (1980) have reported the pH-dependent decrease of the tyrosine absorbance bands in the medium ultraviolet region, with an increase in pH, is accompanied by a concurrent decrease of the heme absorbance in the Soret region. Based on this finding, they have postulated the existence of a heme-linked tyrosine as one of the axial heme iron ligands in cytochrome P-450.

In a study similar to the one presented here, Janig et al (1982) have indicated that N-acetylimidazole modification of tyrosyl residues in cytochrome P-450LM2 inhibits the N-demethylation of the benzphetamine, and "the spin equilibrium in the presence of benzphetamine exhibits a
more pronounced shift to the high spin state than the control."

The loss of benzo(a)pyrene-3-monooxygenase activity due to N-acetylation cytochrome P-448 (aimed at tyrosine residues) does not necessarily imply that tyrosine(s) is located at the active site. However, this loss of activity agrees with the view that this amino acid is the sixth ligand, since the oxygen atom incorporated into the substrate comes from a dioxygen molecule that is bound to the heme iron on the same side of the porphyrin as the sixth ligand. Therefore, this ligand could play an important role in the overall catalytic process.
CHAPTER 9
9. Evaluation of Immobilized Cytochrome P-448 from Saccharomyces cerevisiae Using Permeabilized Whole Cell, Microsomal Fraction and Purified Reconstituted Forms, with Benzo(a)pyrene-3-Monooxygenase Activity

9.1. Introduction

Immobilization prior to application of cytochrome P-450/P-448 has been attempted by various workers. Schubert et al (1980) have immobilized hepatic microsomal cytochrome P-450 by gelatin to construct an enzyme electrode for measurement of oxygen uptake by this enzyme system. Brunner et al (1980) have immobilized purified reconstituted hydroxylase enzyme system to BrCN-activated Sepharose 4B and also by co-polymerization in acrylamide for the purpose of increasing the stability. The simultaneous immobilization of cytochrome P-450 monooxygenase system and glucuronyl transferase to achieve the hydroxylation and subsequent glucuronidation of lipophilic compounds has been reported by Lehman et al (1981). Several other workers have also immobilized this enzyme in microsomal form on to solid supports (Sofer et al, 1975; Janig et al, 1977; Lu and West, 1978).

In this study some procedures for the immobilization of yeast cytochrome P-448 in permeabilized whole cell, in
microsomal fraction and in purified reconstituted mono-oxygenase system are reported. The feasibility of these methods is evaluated by comparison of the parameters of benzo(a)pyrene-3-monoxygenase activity and the stability in storage of these immobilized forms, which are derived from preparations at different levels of purification effort on this cytochrome P-448 from *Saccharomyces cerevisiae*.

9.2. **Methods and Materials**

9.2.1. **Permeabilization of Yeast Cells**

Harvested yeast cells were permeabilized using a method described by Murakami *et al* (1980), in which 1g of packed yeast suspended in 4ml 0.1M potassium phosphate buffer 7.4, containing 0.4M sorbitol, 15ml ml of toluene was added. The suspension was shaken at 40°C for 5min and then cooled in ice. The cells were collected by centrifugation at 10,000g for 10min, resuspended and washed in 0.02M sodium cacodylate buffer, pH 7.1, containing 0.4M sorbitol. The toluenized cells were finally suspended in the above buffer to a concentration corresponding to 200mg of original yeast per ml.
9.2.2. **Immobilization of Yeast Cytochrome P-448 on Calcium Alginate**

Permeabilized yeast cells, microsomal fraction and purified reconstituted system (the latter two prepared as described in Chapters 3 and 6) were entrapped by calcium alginate using a method modified from that of Kierstan et al (1977). In each case, 5ml of suspended cells, microsomes or purified reconstituted hydroxylase system (at 1nmol cytochrome P-448 per ml) was mixed with 15ml of 1% (w/v) sodium alginate and the total suspension was slowly extruded into a 0.05M CaCl₂ solution containing 10% (w/v) glucose using an Oxford Laboratories sample pipette with a tip having an orifice 1mm in diameter. The fibers formed were separated from the solution by filtration and were dried off before use. When this enzyme, in all three forms, was immobilized by calcium alginate, because of the quick settling of enzyme-calcium alginate beads, the retention yield could not be directly determined by CO-difference spectrum of reduced form. Therefore, retention was measured by the amount of cytochrome P-448 which remained free.

9.2.3. **Immobilization of Yeast Cytochrome P-448 in Polyacrylamide, by Co-Polymerization with Acrylamide Monomer**

Co-polymerization of cytochrome P-448 in all three forms was carried out according to the method of
Brunner et al. (1980). 4.5ml of enzyme solution (7.2nmol/ml) or yeast suspension (1nmol/ml) with 2.0ml of 0.2M Tris-HCl, pH 7.4 and N₂ was bubbled through for 20min at room temperature. The solution was then cooled down in an ice bath and 0.5g acrylamide and 0.034g N,N-methylene-bisacrylamide were added. The solution was stirred under N₂-atmosphere until these two compounds were completely dissolved. Co-polymerization was started by the addition of 0.25ml 5% (v/v) dimethylaminopropionitrile and 0.25ml of 5% (w/v) ammonium persulphate solution. The mixture was stirred under N₂-atmosphere at 4°C and, after the formation of the gel, was granulated or meshed.

9.2.4. Immobilization of Cytochrome P-448 on BrCN-Activated Sepharose 4B

Purified reconstituted monooxygenase system was immobilized by BrCN-activated Sepharose 4B by a method modified from that reported previously (Brunner et al., 1980; Cuatrecasas et al., 1968). Sepharose 4B (decanted) was mixed with an equal volume of water and cyanogen bromide (100mg per ml of settled Sepharose 4B) was added in an equal volume of water. The pH was immediately adjusted to, and maintained at, 11 by titration with 4N NaOH. When the reaction had ended, the Sepharose 4B beads were washed with approx. 20 volumes of cold 0.1M NaHCO₃ on a Buchner funnel.
under suction. The beads then were suspended in cold 0.1M potassium phosphate buffer, pH 7.5 containing 0.001M dithiothreitol and 20% (v/v) glycerol in a volume equal to that of the original Sepharose 4B, and the enzyme preparation (1nmol per ml) was added with a ratio of 6nmol cytochrome P-448 per g beads. The mixture was stirred overnight and beads were then washed thoroughly and suspended again in 0.1M potassium phosphate buffer, pH 7.5, containing 0.1M glycine, 0.001M dithiothreitol and 20% (v/v) glycerol. This buffer was used to deactivate all the remaining binding groups on the beads. The beads were finally stored until use in 0.1M potassium phosphate buffer, pH 7.0, containing 20% (v/v) glycerol.

9.2.5. Immobilization of Yeast Cytochrome P-448 by Microcrystalline Cellulose, Cross-Linking by Use of Glutaraldehyde

To 10ml of purified reconstituted hydroxylase system in 0.1M potassium phosphate buffer, pH 7.0, containing 20% (v/v) glycerol (1nmol per ml cytochrome P-448), 1.5g of cellulose (microcrystalline) was added and mixed on a roller-mixer for 1h. Cross-linking was accomplished by adding glutaraldehyde to give the final concentration of 0.5% (v/v) and stirring for another hour. Enzyme system bound to cellulose was then collected by centrifugation at
12000g for 10min and was washed and resuspended in 0.1M potassium phosphate buffer, pH 7.0 containing 20% (v/v) glycerol. Ethanolamine was added to the immobilized enzyme suspension up to 0.5M in concentration to deactivate the remaining functional groups of glutaraldehyde. The immobilized enzyme system was washed and resuspended in 0.1M potassium phosphate buffer, pH 7.0 containing 20% (v/v) glycerol.

9.2.6. Immobilization of Cytochrome P-448 by Agarose-Concanavalin A, Cross-Linking by Use of Glutaraldehyde

3.3ml of purified reconstituted hydroxylase system (1nmol per ml cytochrome P-448) in 0.2M potassium phosphate buffer, pH 7.4 containing 0.15M NaCl was stirred with 1g of packed Agarose-concanavalin A at 4°C for 30min. The gel was washed with 300ml 1M KCl. Cross-linking of the Agarose-concanavalin A cytochrome P-448 enzyme system conjugate with glutaraldehyde was accomplished by adding 0.01ml 0.1M potassium phosphate buffer, pH 8.0, and glutaraldehyde to give the final concentration of 0.5% (v/v), and stirring for 30min. The immobilized enzyme system was washed and resuspended in the 0.1M potassium phosphate buffer pH 8.0 and ethanolamine was added up to 0.5M in concentration to deactivate the remaining functional groups of
glutaraldehyde. The immobilized enzyme system was washed and resuspended in 0.1 potassium phosphate buffer pH 7.0 containing 20% (v/v) glycerol.

9.2.7. Measurement of Benzo(a)pyrene-3-Monoxygenase Activity of Immobilized Cytochrome P-448

This measurement was carried out by the method described in Chapter 6, modified for use of immobilized enzyme. A known amount of immobilized enzyme (usually 1.5 nmol cytochrome P-448) was placed in a magnetically-stirred flask at 37°C, and therefore approximates to a typical batch stirred tank reactor. Tris-HCl buffer, 0.1M, pH 7.0 containing NADPH-regenerating system was added to give a final concentration of 0.004M NADP, 0.02M D-glucose-6-phosphate, 6μM MgCl₂ and 8 IU of glucose-6-phosphate dehydrogenase. The reaction was started by the addition of benzo(a)pyrene from a stock solution of 2mg/ml in dimethylformamide, to give final concentrations in the range 0-158μM benzo(a)pyrene. Incubation was at 37°C with stirring for 15min. The reaction was stopped by separating the immobilized enzyme by centrifugation or filtration. The supernatant (or filtrate) was diluted 1:1 by addition of ice-cold acetone. A 0.6 ml aliquot of this 50% acetone solution was added to 1.4ml 10.7% (v/v) triethylamine solution in a fluorimeter cuvette and scanned on the range
500-560nm (emission) at 467nm (excitation) in a Perkin-Elmer MPF3 fluorescence spectrophotometer to find the peak at 523nm. Fluorescence was calculated relative to 10μg quinine sulfate/ml in 2M H₂SO₄, which was calibrated against a standard 3-hydroxybenzo(a)pyrene solution.

9.2.8. Materials

Sodium cacodylate and BrCN were purchased from Aldrich Chemical Co. Microcrystalline cellulose, dimethylamino-propionitrile, Agarose-concanavaline A were obtained from Sigma Chemical Co. Sodium alginate, glutaraldehyde and other chemicals if not mentioned in the "Material" section of previous chapters were supplied from BDH Chemicals Ltd.

9.3. Results and Discussion

9.3.1. Evaluation of Immobilized Yeast Cytochrome P-448 Using Permeabilized Whole Cell

The parameters (Kₘ, Vₘₐₓ) of benzo(a)pyrene-3-monoxygenase activity for all three forms of cytochrome P-448 immobilized by various supports were obtained by the Lineweaver-Burk plot (Figure 9.1) and shown in Table 9.1. The stability of immobilized cytochrome P-448 in storage at 4°C is shown in Figure 9.2.
Figure 9.1. Lineweaver-Burk Plot of the Benzo(a)pyrene-3-Monooxygenase Activity of Immobilized Cytochrome P-448 on Various Supports. Permeabilized yeast cells on calcium alginate (○), microsome on calcium alginate (■), highly purified reconstituted system on calcium alginate (○), highly purified reconstituted system on Sepharose 4B (x), highly purified reconstituted system on acrylamide (▲).
<table>
<thead>
<tr>
<th>Form of Enzyme</th>
<th>$V_{\text{max}}$ (pmol min$^{-1}$ nmol$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>Activity Remaining After 4 Weeks Storage at 4°C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permeabilized yeast cells immobilized on calcium alginate</td>
<td>11.1</td>
<td>222</td>
<td>20</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I &quot;Free&quot; enzyme</td>
<td>11.1</td>
<td>111</td>
<td>0</td>
</tr>
<tr>
<td>II Immobilized on calcium alginate</td>
<td>8.3</td>
<td>200</td>
<td>60</td>
</tr>
<tr>
<td>Purified reconstituted monooxygenase system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Free enzyme</td>
<td>16.7</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>II Immobilized on calcium alginate</td>
<td>12.3</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>III Immobilized on BrCN-Sepharose 4B</td>
<td>11.1</td>
<td>66</td>
<td>58</td>
</tr>
<tr>
<td>IV Immobilized on acrylamide</td>
<td>9.0</td>
<td>33</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 9.1. Parameters of Benzo(a)pyrene-3-Monooxygenase Activity in Immobilized Cytochrome P-448. $V_{\text{max}}$ values are expressed in pmol of 3-hydroxybenzo(a)pyrene per min per nmol of cytochrome P-448.
Figure 9.2. Storage Stability of Immobilized Cytochrome P-448 on Various Supports. Permeabilized yeast cells on calcium alginate (●), microsomes on calcium alginate (■), highly purified reconstituted system on calcium alginate (○), highly purified reconstituted system on Sepharose 4B (×), highly purified reconstituted system on acrylamide (▲). All immobilized enzymes were stored in 0.1M potassium phosphate buffer pH 7.0 containing 20% glycerol as stabilizer. In free enzymes stored at 4°C all the benzo(a)pyren-3-monooxygenase activity was lost for permeabilized yeast cells and microsomes after two weeks and for purified cytochrome P-448 after four weeks.
The permeabilization of the yeast cells resulted in a retention of 40% of the cytochrome P-448 peak, measured by CO-difference spectrum of reduced form, and 100% of this was retained upon immobilization. When these cells were immobilized in calcium alginate they showed almost normal benzo(a)pyrene-3-monooxygenase activity compared with the highly purified enzyme, with a $V_{\text{max}}$ of 11.1pmol of 3-hydroxybenzo(a)pyrene per min per nmol cytochrome P-448. Immobilized whole yeast cells or protoplast (prepared by incubation of cells in the presence of snail enzyme according to the method of Wiseman and Woods, 1978) did not show any benzo(a)pyrene-3-monooxygenase activity. Immobilization of permeabilized yeast cells in gelatin (Schubert et al., 1980) was not feasible as the gel depolymerized in a batch stirred tank reactor during incubation at 37°C. Permeabilized yeast cells lost their benzo(a)pyrene-3-monooxygenase activity when co-polymerized with acrylamide and they did not absorb onto cellulose or BrCN-activated Sepharose 4B. Permeabilized yeast cells immobilized by calcium alginate lost most of their benzo(a)pyrene-3-monooxygenase activity after 4 weeks storage at 4°C.
9.3.2. Evaluation of Immobilized Yeast Cytochrome P-448 Using Microsomal Fraction

Cytochrome P-448 in microsomal fraction is membrane-bound immobilized enzyme and is not easily immobilized to other supports. However, its entrapment by calcium alginate resulted in retention of all the cytochrome P-448 and much of the benzo(a)pyrene-3-monooxygenase activity ($V_{\text{max}}$ 8.3 pmol $\text{min}^{-1} \text{nmol}^{-1}$) and it lost only 40% of this activity after 4 weeks of storage at 4°C. The results (Table 9.1) indicated, however, a considerable diffusion limitation in this system, with lowered $V_{\text{max}}$ and raised $K_m$ values. Recently Schubert et al. (1982) have reported that the entrapment of rabbit liver microsomal cytochrome P-450 by calcium alginate, increases the stability of its aminopyrine demethylase activity (as compared with crude microsomes). These authors have employed a fixed bed recirculation reactor, where aminopyrine was continuously demethylated.

9.3.3. Evaluation of Immobilized Yeast Cytochrome P-448 Using Purified Reconstituted System

Purified reconstituted monooxygenase system was successfully immobilized by a number of supports. Entrapment by calcium alginate (with 90% retention yield) gave a high $V_{\text{max}}$ of 12.3 pmol $\text{min}^{-1} \text{nmol}^{-1}$ compared with the free purified reconstituted monooxygenase system, which had
V_{\text{max}} \text{ of } 16.7 \text{ pmol}^{-1} \text{ min}^{-1} \text{ nmol. The apparent } K_m \text{ (50 } \mu \text{M}) \text{ was also close to that of the free enzyme system (33 } \mu \text{M).}

Immobilization of the highly purified form on cellulose by absorption and cross-linking by glutaraldehyde gave the same results as previously obtained with the crude solubilized form of the enzyme (Woods and Wiseman, 1980). Eighty percent of the cytochrome P-448 did not bind to this support (or was denatured in the process of immobilization). The enzyme which bound to the cellulose lost some of its activity. Immobilization of the solubilized crude form of this enzyme on cellulose using the same procedure had resulted in a doubling of $K_m$ and a 35% decrease in $V_{\text{max}}$ (Woods and Wiseman, 1980).

Attempts were made to immobilize the purified reconstituted monooxygenase system through its carbohydrate side-chain to Agarose-concanavalin A. Cytochrome P-450 type enzymes are reported to be glycoproteins, containing 2-4 molecules of sugars such as mannose, glucose or glucosamine per molecule of the cytochrome (Haugen and Coon, 1976). As shown in Chapter 7, only 0.2mol of carbohydrate per mol of cytochrome P-448 was detected in this study. It was found here that only 50% of cytochrome P-448 was bound to Agarose-concanavalin A and most of the
benzo(a)pyrene-3-monooxygenase activity was lost. Glutaraldehyde seems to have a deleterious effect on cytochrome P-448, and it may impose a high degree of steric hindrance onto the active site of this enzyme in purified form.

Highly purified reconstituted monooxygenase system along with NADPH has been immobilized by BrCN-activated Sepharose 4B (Brunner et al., 1980; Breckenridge et al., 1977). The intended application was for use as an extracorporeal shunt for blood detoxication. This support proved to be the most effective for this highly purified enzyme. Almost all the cytochrome P-448 used was retained on these beads, although NADPH did not bind, and it was necessary to add this each time to the reaction mixture. \( V_{\text{max}} \) was relatively high (11.1 pmol min\(^{-1}\) nmol\(^{-1}\)) and availability of the enzyme for binding benzo(a)pyrene was demonstrated by the low \( K_m \) value obtained (50 \( \mu \)M). Immobilized highly purified reconstituted cytochrome P-448 on this support also showed a considerable degree of stabilization in storage at 4°C, and after 4 weeks only 42% of the enzyme activity was lost, instead of 93% for this enzyme preparation prior to immobilization.

The co-polymerization of purified reconstituted monooxygenase system with acrylamide resulted in the production
of an entrapped enzyme system (cytochrome P-448 retention, 60%) with a $K_m$ for benzo(a)pyrene the same as that of the free enzyme (33μM), but with a 46% decrease in $V_{max}$. The low $V_{max}$ (with unchanged $K_m$) might be due to the diffusional limitation imposed by this support on the 3-hydroxybenzo(a)pyrene availability in the assay mixture in the presence of the swollen gel that forms during the assay.

9.3.4. Possible Applications of Immobilized Cytochrome P-450/P-448

Immobilization of cytochrome P-448 highly purified reconstituted enzyme system using an effective support in a stirred tank and other enzyme reactors will improve the proposed applications of this enzyme, including the construction of an accurate enzyme electrode. Preliminary work in this direction was carried out during this study. The objective was to build an enzyme electrode for detection and measurement of benzo(a)pyrene and assess its viability for analytical application. Glucose oxidase and yeast cytochrome P-448 were co-immobilized by polyacrylamide gel, using the method described in this chapter. This immobilized bienzyme system was coupled to an oxygen electrode, in which the oxygen uptake for production of hydrogen peroxide by glucose/glucose-oxidase system, which ultimately is utilized for hydroxylation of benzo(a)pyrene
by cytochrome P-448, was measured. Although traces of benzo(a)pyrene (75 p.p.m.) were detected using this electrode, a linear response to benzo(a)pyrene concentration was not observed. An improved electrode as such could provide a rapid and specific assay for benzo(a)pyrene or other possible substrates of this enzyme which are present in foodstuffs and the environment.

Immobilization permeabilized yeast may be useful also in large scale application such as the removal of polyaromatic nuclear hydrocarbons from water and air. For some purposes (such as detoxication of blood), the use of the immobilized microsomal system may prove convenient and appropriate with high stability (but with diffusional limitation problems). Further studies using other types of enzyme reactors are required. The packed-bed type may prove to be more useful in the use of these enzymes for production of quantities of metabolites or for carcinogen removal, and the replacement of expensive cofactors by co-immobilized enzymes will be essential for large-scale applications.
CHAPTER 10
10. **Final Discussion**

The glucose effect on the activities of cytochromes within the cell, particularly mitochondrial cytochromes, is well established. Polakis *et al* (1964) found that the concentration of the mitochondria 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 depend 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/P-448 in steroid metabolism, particularly since it has been shown in this study 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.

Growth of yeast under semi-anaerobic conditions alone is not enough to produce cytochrome P-450/P-448 without a high glucose concentration in the medium. This may mean that this enzyme is only produced at the faster growth rates observed at higher glucose concentration. It is also possible that a higher glucose concentration is required in
order to repress cyclic AMP levels sufficiently for cytochrome P-450/P-448 synthesis to occur (Chapter 2).

Recently King (1983) showed that when a yeast culture growing exponentially is made semi-anaerobic, the biosynthesis of cytochrome P-450/P-448 is inhibited with an increase in cytochrome P-450/P-448 level between 24 and 48 hours of less than 10% of that in the aerobic control. This effect is not due to an altered growth rate, as very similar growth rates were observed under both sets of conditions, with sufficient glucose still present (at 24h) to ensure actively fermenting yeast cells under glucose repression. One effect of oxygen may be in acting as a substrate inducer of yeast cytochrome P-450/P-448. This may also explain why cytochrome P-450/P-448 cannot be detected in yeast grown under strictly anaerobic conditions (Rogers and Stewart, 1973). However, much further work is required to test this idea with experiments at a range of carefully controlled oxygen concentrations.

King (1983) has further shown that when yeast is grown aerobically to stationary phase (48h) and then made semi-anaerobic and incubated further, cytochrome P-450/P-448 does not appear to be lost as quickly. At stationary phase, the yeast cytochrome level is already falling and the removal of
oxygen at this time is unlikely to effect cytochrome P-450/P-448 biosynthesis. The slowing of cytochrome P-450/P-448 loss under semi-anaerobic conditions is due to protection of the enzyme from degradation as was described by Blatiak et al (1980).

The strain of Saccharomyces cerevisiae used for production of cytochrome P-450/P-448 in this study was NCYC No. 240. The strain NCYC No. 239 produced 6nmol of cytochrome P-450/P-448 per g of wet weight of yeast but due to lower yeast cells mass production, the overall yield of this enzyme was not higher than that from strain NCYC No. 240 (both strains grown in similar media). Recently it has been observed that strain NCYC No. 754 produced 6-7nmol of cytochrome P-450/P-448 per g wet weight of yeast in a pattern similar to that of NCYC No. 240 (D. King, personal communication). Unfortunately the purification of cytochrome P-450/P-448 from Saccharomyces cerevisiae was carried out before the genetic analysis (Chapter 2) and induction studies (Chapter 6). Therefore, cytochrome P-448 characterised in this study was from untreated Saccharomyces cerevisiae NCYC No. 240.

Cytochrome P-448, cytochrome b₅, NADPH:cytochrome c(P-450) reductase and NADH:cytochrome b₅ reductase were
purified from the same preparation of microsomes as shown in Chapter 3. Using this novel combination of purification steps, a small quantity of cytochrome P-450 (Soret peak of CO-difference spectrum of reduced form at 450nm) was separated from yeast's major cytochrome P-450-type isozyme, cytochrome P-448. Although cytochromes b$_5$, b$_5$ reductase and c(P-450) reductase purified by this method, did not show the homogeneity demonstrated by cytochrome P-448, they were free of each other and the cytochrome P-450/P-448.

Although cytochrome P-450/P-448 was first discovered in the yeast _Saccharomyces cerevisiae_ by Lindenmayer and Smith in 1964 this enzyme system was still poorly characterized and its role _in vivo_ is not even now fully understood. This study has established that yeast cytochrome P-448 is a distinct cytochrome P-450 isozyme but with many properties in common with cytochrome P-448 from mammalian sources. The molecular weight of the yeast enzyme (55,500) and its amino acid composition are closer to a mammalian hepatic cytochrome P-448 form than to a hepatic cytochrome P-450 form.

The amino acid composition of yeast cytochrome P-448 was determined and is shown in Chapter 7 along with the amino acid compositions of cytochrome P-448 from rat liver
(Miki et al., 1981) and cytochrome P-448 (Soret peak at 446nm but more commonly called P-448) from β-naphthoflavone pretreated rat liver (Saito and Strobel, 1981). It was found that yeast cytochrome P-448 contains 407 amino acid residues per molecule, with a content of hydrophobic residues of 43%. Although the number of amino acid residues is smaller than 469, the total for the two mammalian cytochromes mentioned above, the hydrophobic residue content was almost identical, with values of 43%, 44% and 45% for yeast cytochrome P-448, rat liver cytochrome P-448 and β-naphthoflavone-induced rat liver cytochrome P-448 respectively. Recently the amino acid sequence of phenobarbital-induced rat liver cytochrome P-450 has been deduced from the nucleotide analysis of cloned DNA giving a total of 491 residues (Mizukami et al., 1983). Lower proline and higher glycine contents of the yeast enzyme in comparison to the above mentioned mammalian enzymes may favour intermolecular packing.

In addition to benzo(a)pyrene, type I binding spectra to purified yeast cytochrome P-448 were observed with lanosterol, phenobarbitone, ethylmorphine, dimethyl-nitrosamine and perhydrofluorene. Of these, lanosterol, ethylmorphine, and phenobarbitone gave a similar extra peak at 367nm to that found in the binding spectrum of
benzo(a)pyrene (or in the case of ethylmorphine at 355nm). This double peak is not therefore specific for the interaction of benzo(a)pyrene with cytochrome P-450/P-448. Benzo(e)pyrene gives a type I binding spectrum with $K_s$ of 52μM (Woods and Wiseman, unpublished).

Finding that type I binding is observed with several other compounds, notably phenobarbitone, ethylmorphine and dimethylnitrosamine is of considerable interest. Of these, phenobarbitone and dimethylnitrosamine can also act as an inducer of a more efficient benzo(a)pyrene hydroxylase and all three result in some inhibition of benzo(a)pyrene hydroxylase activity (Chapter 6). In attempts to measure dimethylnitrosamine demethylation equivocal results were obtained with Nash assay. Yeast cytochrome P-448 activity towards aminopyrine has also been reported (Yoshida et al., 1977), although in this study neither binding nor any activity with this compound was demonstrated.

The highest affinity for the type I binding compounds tested was observed with benzo(a)pyrene, with a $K_s$ of 50μM. Benzo(a)pyrene bound to 100% of yeast cytochrome P-448, and this is a larger proportion than any other compound studied.
A type I spectral interaction for purified cytochrome P-448 from a baker's yeast strain of *S. cerevisiae* with lanosterol has previously been detected by Aoyama and Yoshida (1978a) although no $K_s$ value was quoted. Lanosterol is thought to be the endogenous substrate for yeast cytochrome P-448 but demethylation activity of the enzyme used in this study, towards this compound (using the rather insensitive Nash assay technique) was not detected.

In addition to showing a type I binding spectrum with yeast cytochrome P-448, lanosterol strongly inhibited the benzo(a)pyrene hydroxylase activity of this enzyme (Chapter 6). Recently, D. King has observed the lanosterol demethylation activity of this very enzyme, using an assay which involves radioactive sterols (D. King, personal communication). The postulation of an endogenous substrate role for lanosterol requires the presence of cytochrome P-448 under all yeast growth conditions because the synthesis of ergosterol for incorporation into membranes including endoplasmic reticulum itself, would always be necessary if no ergosterol is added to the growth medium. Recently, evidence has been presented for the presence of small amounts of cytochrome P-448 in yeast even under conditions of growth in anaerobic media at low glucose concentrations. This enzyme was reported to be capable of demethylation of lanosterol (Aoyama et al., 1981a).
King (1983) also has shown evidence, based upon the construction of second derivatives, of reduced carbon monoxide difference spectrum of this yeast cytochrome P-448, which allow the resolution of overlapping spectral bands, that a small amount of yeast cytochrome P-448 is present under these conditions.

Type II spectral interactions were observed for this enzyme with aniline, imidazole, and weakly with benzphetamine. Aniline and imidazole bind to yeast cytochrome P-448 with very high affinities ($K_s$ values of 5μM and 8μM respectively). These results show that the enzyme is also capable of undergoing a type II spectral change due to binding of a compound directly to the heme iron. Although aniline bound very tightly, the metabolism of this compound in a reconstituted system was not detected.

Equilibrium gel filtration studies of the binding of benzo(a)pyrene to purified yeast cytochrome P-448 and phenobarbital-induced rat liver cytochrome P-450 showed one binding site for each of these enzymes. In the case of the purified enzyme from β-naphthoflavone-induced rats the six binding sites may be due to some residual lipid or detergent in the purified preparation. However, it is also possible that this highly active enzyme molecule does indeed have six
benzo(a)pyrene binding sites per monomer, and is fundamentally different in its interaction with substrate and cytochrome c(P-450) reductase (Chapter 5).

It was established that benzo(a)pyrene hydroxylase activity from yeast is cytochrome P-448-dependent by several criteria including heat denaturation/deactivation studies (Chapters 3 and 6), inhibition studies with binding compounds (Chapter 6) and the reconstitution of an active benzo(a)pyrene metabolizing system from only cytochrome P-448, NADPH:cytochrome c(P-450) reductase and phospholipid (Chapter 6.) The omission of either of the enzymic components of this system results in loss of activity when NADPH is used as an electron donor. The omission of phospholipid also leads to a large drop in activity, although some hydroxylation still occurs. This requirement for phospholipid can also be fulfilled by the non-ionic detergent Emulgen 911, suggesting a structural role of phospholipid in associating the two enzymic components together. Including cytochrome b₅ and NADH:cytochrome b₅ reductase, in the presence and absence of NADH as a cofactor in the reconstituted system did not enhance the benzo(a)pyrene hydroxylase activity.
The NADPH-supported reaction using a reconstituted system (from untreated yeast) has a $V_{\text{max}}$ of 16.7 pmol of 3-hydroxybenzo(a)pyrene/min per nmol of cytochrome P-448 and a $K_m$ of 33μM. This value for Michaelis constant is somewhat similar to 22.7μM and 46μM for hepatic enzymes from β-naphthoflavone and phenobarbital-induced rats respectively. The rate of yeast enzyme is low compared with β-naphthoflavone (Chapter 6) and 3-methylcholanthrene-induced (Ryan et al, 1979) forms of cytochrome P-448 from rat liver but it is similar to cytochrome P-450 activities from uninduced (Hashimoto et al, 1977) and phenobarbital-induced rat liver (Chapter 6).

The $K_m$ of purified yeast enzyme, 33μM, was in good agreement with the spectral dissociation constant $K_s$, value of 50μM. The small difference in these values is presumably due to the complexity of the multicomponent benzo(a)pyrene hydroxylase system. Larger differences observed with microsomal enzyme are attributed to the diffusional limitation of this immobilized enzyme system.

The requirement of this system for NADPH as a cofactor could be replaced using cumene hydroperoxide or a glucose oxidase/glucose system generating hydrogen peroxide (Chapter 6). Both hydrogen peroxide generated from glucose
oxidase and glucose and the cumene hydroperoxide systems give higher maximal velocities than the NADPH-supported system. However, the apparent affinity of the enzyme for benzo(a)pyrene was lowered, as indicated by an increased $K_m$ value. In both the cumene hydroperoxide and in situ hydrogen peroxide-supported systems the production of 3-hydroxybenzo(a)pyrene (fluorescent emission peak at 523nm) is slowed after a few minutes and a peak at 540nm begins to show. This 540nm peak is identified as a mixture of benzo(a)pyrene metabolites including phenols, diols, and quinones (Rho, 1980). This agrees with results obtained using cytochrome P-450 of phenobarbital-induced rats, described by Renneberg et al (1981). These authors reported that the production of quinones from peroxide-supported cytochrome P-450 systems was much higher than the production of these metabolites in an NADPH-supported system. The optimal temperature benzo(a)pyrene hydroxylation was the same for all three systems (with yeast cytochrome P-448) at approximately 37°C, although the in situ hydrogen peroxide-supported system has a better rate at a slightly higher pH than the optimal pH for the cumene hydroperoxide- and NADPH-supported cases which is at 6.5-7.0.

The finding of inhibition in vitro of yeast P-448 by flavonoid compounds is unlike the finding for the major
rabbit liver cytochrome P-448 (LM4) in that this is stimulated by flavone. The pattern of flavonoid inhibition of yeast benzo(a)pyrene hydroxylase (Chapter 6) shows more resemblance to rabbit liver cytochrome P-450LM6, a form induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), particularly in neonatal rabbits, which also has a Soret peak at 448nm in the reduced carbon monoxide difference spectrum (Norman et al, 1978). It is of interest that TCDD induces both cytochrome P-450LM4 (major component) and cytochrome P-450LM6 (minor component) in adult rabbits, but only cytochrome P-450LM6 in neonatal rabbits. Cytochrome P-450LM6 has a molecular weight of 57,000 and a high activity in benzo(a)pyrene hydroxylation (Norman et al, 1978). In neonatal rabbits also, it has been shown that 3-methylcholanthrene induced a form of cytochrome P-450 which seems to be LM6 on the basis of its molecular weight and catalytic activities (Atlas et al, 1977). This form of the enzyme may therefore be more closely related to the yeast cytochrome P-448 (the subject of this study) than major adult rabbit liver cytochrome P-448.

The novel finding of a concentration dependent induction of more efficient forms of the benzo(a)pyrene hydroxylase should be pursued with other yeast strains and with other inducers. Discoveries of fundamental biochemical
interest seem likely in this area, as little is known of induction of these enzymes in yeast. In this study (Chapter 6) this was investigated by growing *S. cerevisiae* under conditions of glucose repression in the presence of several inducers of the mammalian system. When yeast was grown in the presence of benzo(a)pyrene only a small increase in cytochrome P-448 levels was observed (at the most 30%: at 95μM benzo(a)pyrene) which seems to be insufficient to account for the much more dramatic induction of benzo(a)pyrene hydroxylase found, as indicated by a greatly increased affinity for benzo(a)pyrene, with $K_m$ decrease 2-3 fold (also $V_{\text{max}}$ increased approx. 3 fold). Unfortunately the induction experiments were performed towards the end of this study and with the exception of the Ames test, cytochrome P-448 from benzo(a)pyrene-treated yeast was not applied in other various investigations. For example, immobilized cytochrome P-448 from BP-treated yeast could have produced the needed higher activity required for construction of a more sensitive enzyme electrode for detection of environmental benzo(a)pyrene (Chapter 9).

The induction of hydroxylase activity was also observed in a similar manner after growing yeast in the presence of dimethylnitrosamine and sodium phenobarbitone. 3-Methylcholanthrene was also found to decrease the $K_m$ for
benzo(a)pyrene yet in this case no effect on \( V_{\text{max}} \) was observed. \( \beta \)-Naphthoflavone, a compound which induces a cytochrome P-448 form of cytochrome P-450 with a very high turnover for benzo(a)pyrene in mammals had no effect on the yeast cytochrome P-448. The addition of lanosterol to yeast during growth resulted in no change in cytochrome P-448 concentration yet caused an enzyme to be produced with an increased \( K_m \) for benzo(a)pyrene.

The results indicate that at least one new form of enzyme was being produced in the presence of the inducing compound which seems likely to occur with compensating non-production of the existing form of the enzyme. By analogy with mammalian system it can be expected that this is due to the biosynthesis of a different form of cytochrome P-450 with a greater specificity for benzo(a)pyrene. Although induction of different forms of cytochrome P-450 in yeast has not been shown, some evidence from purification studies was obtained for the presence of at least two forms of cytochrome P-450 in uninduced yeast. The results from this induction study show that more than one form of cytochrome P-450 can exist in \textit{S. cerevisiae} and that the production of a more specific form for benzo(a)pyrene is likely to occur at the expense of the less specific form. Assuming that these effects occur at a transcriptional level, this leads
to a model whereby a high concentration of benzo(a)pyrene is responsible for switching off one gene and switching on another through binding to a repressor protein causing its activation or inhibition respectively. This would therefore represent both a positive and negative control exerted by the inducer over the different operators responsible for the functioning of the structural genes for synthesis of the corresponding forms of mRNA. Positive and negative effects on transcription have been reported previously in bacteria (Adhya and Miller, 1979) for effects of cyclic AMP on the promoter of the gal operon in E. coli. The partial repression of cytochrome P-450 synthesis in S. cerevisiae by cyclic AMP has been reported in studies on yeast protoplasts (Wiseman et al, 1978).

An alternative explanation might involve the effect of benzo(a)pyrene as a mutagenic agent, activated in situ by the cytochrome P-448 system in the yeast. It seems unlikely however that more efficient forms of the enzyme would be produced from mutant genes. Moreover, the efficient binding of benzo(a)pyrene, yet low catalytic activity, of the yeast cytochrome P-448 would tend to protect the yeast from the mutagenic effects associated with activation of benzo(a)pyrene.
The absolute spectrum of highly purified yeast cytochrome P-448 showed a characteristic low spin spectrum, i.e. with a Soret peak at 417nm and no absorbance in the 650nm region. This matter was pursued further by studying the temperature-dependent spin state equilibrium of this in the presence and absence of benzo(a)pyrene (Chapter 8). The purified enzyme was 94% low spin at 22°C. This compares with a value of 82% low spin at 22°C for purified hepatic cytochrome P-450 from phenobarbital pretreated rats (Gibson and Tamburini, 1980). The non-ionic detergents (Triton X-100 in particular) used for its stabilization and/or solubilization effects during the purification process, bound to yeast cytochrome P-448 possibly in same manner as a reverse type II binding compound and modulated the spin state to its low spin form. This resulted in lower midpoint redox potential and therefore the reduction of yeast cytochrome P-448 by sodium dithionite was time-dependent (Chapter 4). Although attempts were made to eliminate the non-ionic detergents prior to the spin state studies a residual amount may have resulted in the difference between the low spin contents at 22°C of yeast and hepatic enzymes. The hepatic cytochrome P-450/P-448 (from both phenobarbital- and β-naphthoflavone-induced rats) did not show any change in their spectra in the presence of Triton X-100 or any
other non-ionic detergent and their reduction by sodium dithionite was not made time-dependent by these compounds.

The binding of the substrate benzo(a)pyrene to yeast cytochrome P-448 results in a shift to a higher spin state, 18% high spin at 22°C. The modulation of the spin state, to high spin, by substrate is thought to be important in the catalytic mechanism of cytochrome P-450 enzymes by controlling the redox potential, hence allowing electrons to flow to the cytochrome P-450 molecule more easily in the reduction of its ferric form by the cytochrome c(P-450) reductase (Sligar et al., 1979). The change in mid-point redox potential of yeast cytochrome P-448 upon binding benzo(a)pyrene should be included in further studies on this subject. The mid-point redox potential of yeast cytochrome P-448 in the absence of substrate was reported in Chapter 4. Yeast cytochrome P-448 has previously been shown to be modulated from low to high spin state by the addition of lanosterol, although no quantitative measurement was attempted (Yoshida and Kumaoka, 1975b).

In attempts to improve the working characteristics of yeast cytochrome P-448 the cysteinyl, arginyl, histidyl, tryptophyl and tryrosyl residues were modified. Modification of four or more out of eight cysteinyl residues in
this enzyme resulted in the loss of more than 70% of benzo(a)pyrene hydroxylase activity, while the CO-difference spectrum peak of reduced form (at 448nm) remained unchanged. Modification of cysteinyl residues did not alter the binding parameters and temperature-induced spin state equilibrium. Therefore, participation of -SH group(s) (other than the one known as the 5th ligand and is responsible for forming the CO-reduced cytochrome P-450/P-448 complex) in the active site, is proposed.

Only five tyrosyl residues were accessible to chemical modification. O-Acetylation of these amino acid residues resulted in inhibition of benzo(a)pyrene hydroxylase activity and prohibition of temperature-induced spin state modulation, while the binding parameters of benzo(a)pyrene to cytochrome were not greatly altered. Recently tyrosyl modification results presented in this study have been confirmed by Janig et al. (1982 and 1983) (the results of modification studies presented in this thesis were obtained in the latter part of 1981 and early 1982). These authors reported that three tyrosyl residues in cytochrome P-450LM2 are accessible to chemical modification with tetra-nitromethane. They also reported that the nitration of two tyrosyl residues inactivates the enzyme to about 20% and that the modification in the presence of the inhibitor
metyrapone (which is coordinated via one of its pyridine nitrogen to the heme iron of cytochrome P-450) results in the protection of at least one tyrosyl residue from nitration, suggesting the location of one tyrosyl residue in the vicinity of heme iron (6th coordinate).

Based upon the results presented for modification of tyrosyl residues of yeast cytochrome P-448 in this study (Chapter 8), the participation of a tyrosyl residue in the active site of this enzyme (possibly as the 6th ligand) is suggested.


Ando, N. & Horie, S. (1971) J. Biochem. 70, 557-570


Burke, M. D. & Bridges, J. W. (1975) Xenobiotica 5, 357-376
Burke, M. D., Bridges, J. W. & Parke, D. V. (1975) Xenobiotica 5, 261-277


Edelson, J. & McMullen, J. P. (1977) Drug Metab1. Disp. 5, 185-190


Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70-77


Franklin, M. R. (1972b) Biolchem. Pharmacol. 21, 2697-2703


Guengerich, F. P. (1977b) J. Biochem. 252, 3970-3979


Guengerich, F. P. (1979) Pharmacol. Ther. 6, 99-121


Hodgson, E. (1979) Drug Metab. Rev. 10, 15-33


Kerridge, D. & Tipton, K. F. (1972) Biochemical Reasoning, pp. 7-9 and 236-30, W. A. Benjamin, Inc., California


Lehnhardt, W. F. & Winzler, R. J. (1968) J. Chromatog. 34, 471-479


Lipscomb, J. D. & Gunsalus, I. C. (1973) in Microsomes and Drug Oxidations (eds. Estabrook et al) pp. 1-5, Williams and Wilkins, Baltimore


Matusubara, T. & Tochino, Y. (1971) J. Biochem. 70, 981-991


Mitani, F. & Horie, S. (1969b) J. Biochem. 66, 139-149


Oeseh, F. (1973) Xenobiotica 3, 305-340


Omura, T. & Sato, R. (1964a) J. Biol. Chem. 239, 2370-2378

Omura, T. & Sato, R. (1964b) J. Biol. Chem. 239, 2379-2385


Peterson, J. A. & Griffin, B. W. (1973) Drug. Metab. Disp. 1, 14-19


Takeshige, K., Ito, A. & Minakami, S. (1972) J. Biochem. 72, 1361-1370

Takesue, Y. & Sato, R. (1968) J. Biochem. 64, 885-893


Temple, D. J. (1971) Xenobiotica 1, 507-520


Williams, J. N. (1964) Arch. Biochem. Biophys. 107, 537-543


Wiseman, A. & Woods, L. R. J. (1978) Biochem. Soc. Trans. 6, 397-400


Yang, S. K., McCourt, D. W., Roller, P. P. & Gelboin, H. V. (1976) Proc. Nat'l. Acad. Sci. 73, 2794-2798

Yohro, T. & Horie, S. (1967) J. Biochem. 61, 515-517


Yoshida, Y. & Kumaoka, H. (1975a) J. Biochem. 78, 455-468

Yoshida, Y. & Kumaoka, H. (1975b) J. Biochem. 78, 785-794


OK, BASICV

BASICV REV 18.2

>OLD SOLVE1

SOLVE1 THU, FEB 11 1982 15:30:09

30 PRINT "A PROGRAM TO SOLVE A SYSTEM OF FOUR LINEAR EQUATIONS"
100 DIM A(4,4),B(4,4),C(4,1),X(4,1)
110 DATA 21,10,3,-3,12,.63
120 DATA 6.5,18.8,2.55,.95
130 DATA -1.16,.91,14.3,-.326
140 DATA -.22,-.59,0,12
150 MAT READ A
160 MAT B=INV(A)
170 PRINT "TYPE IN DATA POINTS, ONE NUMBER PER ROW"
180 MAT INPUT C
190 MAT X=B*C
191 PRINT "THE SOLUTIONS, IN THE ORDER C, C1, B, A, ARE:"
200 MAT PRINT X
210 END

>RUN

SOLVE1 THU, FEB 11 1982 15:30:52
Appendix II

Return/Quit [PRINT Rev 2.0]

190 PRINT "QUART OF OIL AND SOME (MICRO) CHIPS!"
200 DIM A(40),X(40),T(40),Y(40)
210 PRINT "NUMBER OF DATA POINTS"
220 INPUT N
230 FOR K=1 TO N
240 READ A(K),T(K)
250 X(K)=1/(.002*(273+T(K)))
260 NEXT K
270 PRINT "INPUT EXTINCTION COEFF";
280 INPUT A3
290 LET P=10000
300 PRINT "INPUT STARTING VALUE FOR AMIN";
310 INPUT A2
320 LET A2=A2+.0005
330 A1=A2+A3
340 S1=0
350 S2=0
360 S3=0
370 S4=0
380 S5=0
390 FOR J=1 TO N
400 Y(J)=LOG((A(J)-A2)/(A1-A(J)))
410 S1=S1+X(J)
420 S2=S2+Y(J)
430 S3=S3+X(J)*Y(J)
440 S4=S4+X(J)*X(J)
450 S5=S5+Y(J)*Y(J)
460 NEXT J
470 D3=SQR((S4-S1*S1/N)/(N-1))
480 D4=SQR((S5-S2*S2/N)/(N-1))
490 H=(S3-S1*S2/N)/(S4-S1*S1/N)
500 R=H*D3/D4
510 B=(S2-H*S1)/N
520 IF P<R THEN 580
530 LET P=R
540 LET C=A2
550 LET M1=H
560 LET B1=B
570 GOTO 320
580 LET M2=-1*M1
Appendix II (continued)

590 LET B2=B1*1.98
600 PRINT "CORRELATION COEFF =";
610 PRINT P
620 PRINT "ENTHALPY=";H2;" KAL MOL-1"
630 PRINT "ENTROPY=";B2;" e.u"
640 A1=A2+A3
650 PRINT "1/RT Keq LnK Z HIGH SPIN"

660 FOR J=1 TO N
670 Y=(A(J)-C)/(A1-A(J))
680 L=LOG(Y)
690 Q=X(J)
700 T=1/(.002*X(J))-.273
710 P=(Y/(1+Y))*100
720 PRINT Q,Y,L,P
730 NEXT J
740 PRINT "TEMP A(390-420)"
750 FOR H=1 TO N
760 Z=T(H)
770 S=A(H)
780 PRINT Z,S

790 NEXT H
795 PRINT C
800 DATA .0205,5.5,.0357,19.2,.0425,25.5,.053,31.8,.061,36.8,.0707,42
820 END
OK, COMO -END
Table 1. Requirements for cytochrome P-450 biosynthesis in Saccharomyces cerevisiae

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Mitochondrial cytochrome P-450</th>
<th>Cycloheximide effect on mitochondrial protein biosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>High glucose concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

the high glucose concentrations or assisted ejection of cyclic AMP out through the cell membrane could account for the glucose effect in repression of many enzymes, and on the production of cytochrome P-450.

The subcellular location of adenylate cyclase in S. cerevisiae is dependent on the growth conditions of the yeast. Growth in 5% glucose-containing media was associated with the finding of adenylate cyclase in a microsomal fraction (Wheeler et al., 1974), rather than a cell envelope fraction (Londesborough & Nurminen, 1972). Location of this enzyme in a membrane fraction (unspecified) was reported recently (Varimo et al., 1978). A critical approach is essential to all studies after various disruption procedures on yeast. The available evidence suggests that there is room for doubt as to the subcellular location of various adenylate cyclases, although it does not seem to have been reported as associated with a classic mitochondrial fraction from S. cerevisiae. Location of some of the adenylate cyclase in the membrane of the limited number of large mitochondria present would be analogous to the position in bacteria where the location is in the membranes surrounding the cell. This would directly relate the mitochondrion in yeast to events in the cytosol through the regulation of cyclic AMP production. Many effects in the yeast cell are now thought to interrelate mitochondrial and other cell organelles, including effects at the cytoplasmic membrane related to uptake of nutrients, and the loss of flocculence in respiratory-deficient petite mutants (Eglioton et al., 1979).

We thank Dr. D. Wilkie, Department of Botany and Microbiology, University College London, for advice on whole-cell replicating effects of mitochondria in yeast.


Solubilization of cytochrome P-450 in high yield from Saccharomyces cerevisiae microsomal membranes by 1% Triton X-100: stabilization effect

MAHMood R. AZARI and ALAN WISEMAN

Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

Cytochrome P-450 from anaerobically grown baker's yeast has been solubilized and purified by Yoshida et al. (1974a,b, 1977). Our cytochrome P-450 from brewer's yeast (which has benzaldehyde hydroxylase activity: Woods & Wiseman, 1980) was mainly converted into cytochrome P-420 during the solubilization of microsomal fraction by sodium cholate by the method described by Yoshida et al. (1977). Attempts were made therefore to stabilize cytochrome P-450 in yeast microsomal fraction, during and after solubilization, in order to improve the yield for large-scale purification studies in progress.

Saccharomyces cerevisiae (N.C.Y.C. no. 240) was grown for 44 h under glucose repression in medium containing 20% glucose and mycological peptone by the method described previously (Wiseman & Lim, 1972). Yeast microsomal fraction was obtained by centrifugation at 100000x g for 1 h and collecting the supernatant, which contained the solubilized cytochrome P-450. The concentration of cytochrome P-450 and cytochrome P-420 was determined by a spectrophotometric method (Wiseman et al., 1978), modified from that of Omura et al. (1965), assuming extinction coefficients of 91 cm⁻¹·mM⁻¹ for cytochrome P-450 and 11 cm⁻¹·mM⁻¹ for cytochrome P-420.

Storage of the whole yeast containing the cytochrome P-450 was done in the phosphate buffer containing the additives described above and additionally made 15% to glucose. Other protective agents were needed to successfully store the frozen microsomal fraction from yeast. Triton X-100 in 0.1% concentration plus the additional use of 0.1% reduced glutathione in the phosphate buffer (plus additives) prevented the conversion of cytochrome P-450 into cytochrome P-420 in yeast microsomal fraction during storage at −40°C for several weeks. Rat liver microsomal fraction in comparison was very stable at −40°C stored in the phosphate buffer (plus additives) alone.

Microsomes in the phosphate buffer (plus additives) and also in the phosphate buffer (plus additives) containing 0.1% Triton X-100 were solubilized with various detergents with release of cytochrome P-450 (Table 1). Good yields in the enzyme were obtained with 1% Triton X-100 and 1% sodium cholate in the 0.1% Triton X-100-containing phosphate buffer (plus additives) only. Here 100% yield was obtained from yeast microsomal
fraction, whereas that from the rat liver microsomal fraction was 93% and 87% respectively. Sodium dodecyl sulphate (0.5%) gave a very poor yield of cytochrome P-450 from yeast microsomes (Table 1).

Table 1. Solubilization of cytochrome P-450 from microsomes by various detergents, with and without 0.1% Triton X-100.

<table>
<thead>
<tr>
<th>Detergents</th>
<th>Source of microsomes</th>
<th>Cytochrome P-450 (nmol/ml)</th>
<th>Cytochrome P-450/420 ratio</th>
<th>Cytochrome P-450 (nmol/ml)</th>
<th>Cytochrome P-450/420 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulphate 0.5%</td>
<td>Yeast</td>
<td>0.15</td>
<td>1</td>
<td>0.25</td>
<td>0.3</td>
</tr>
<tr>
<td>Triton X-100 1%</td>
<td>Yeast</td>
<td>0.65</td>
<td>22</td>
<td>0.70</td>
<td>26</td>
</tr>
<tr>
<td>Emulgen 911 1%</td>
<td>Yeast</td>
<td>0.35</td>
<td>10</td>
<td>0.40</td>
<td>4</td>
</tr>
<tr>
<td>Sodium cholate 1%</td>
<td>Yeast</td>
<td>0.25</td>
<td>5</td>
<td>0.70</td>
<td>18</td>
</tr>
<tr>
<td>Triton X-100 1%</td>
<td>Rat liver</td>
<td>1.40</td>
<td>7</td>
<td>1.40</td>
<td>7</td>
</tr>
<tr>
<td>Sodium cholate 1%</td>
<td>Rat liver</td>
<td>1.20</td>
<td>6</td>
<td>1.30</td>
<td>6</td>
</tr>
</tbody>
</table>

Enhancement of substrate-induced deactivation of penicillinase by cross-linking with dimethyl suberimidate

MARTYN FARRER and RICHARD VIRDEN
Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

Staphylococcal penicillinase (EC 3.5.2.6) undergoes slow reversible deactivation in the presence of cephaloridine or of certain other substrates, giving an exponential decrease in the rate of hydrolysis to a low value. This suggests that there is slow interconversion of active and less active (or inactive) conformational states (Virden et al., 1972; Pain & Virden, 1979). Covalent intramolecular cross-linking of penicillinase would be likely to affect the interconversion between these states by stabilizing certain conformational features.

Purified penicillinase (Robson & Pain, 1976), 4 mg/ml, in 0.43 M K₂SO₄ and sodium acetate buffer 10.5 mM EDTA (dissodium salt) and 50 mM sodium acetate/27 mM acetic acid, pH 5.0, was mixed with an equal volume of 0.1 M N-ethyl morpholine and then adjusted to pH 8.4 with acetic acid. Dimethyl suberimidate (0.5 mM) was added (final concn. 20 mM). After incubation at 20°C for 90 min a further portion of fresh reagent was added (total concn. 0.12 mM). In a control, dimethyl suberimidate was omitted. The reaction was stopped by lowering the pH (Coggins et al., 1969). Analysis of the product by electrophoresis in 12% (w/v) polyacrylamide gel containing 0.1% sodium dodecyl sulphate (Laemmli & Favre, 1973) showed no oligomers, although there was small decrease in mobility that was almost constant after 90 min of treatment. Subsequent results are for the control and 150 min-treated proteins, unless otherwise stated. The NH₂ group content/mmol of protein (Haberb, 1966) was 41 and 33 in the control and treated proteins respectively. These results are consistent with an average binding of four reagent molecules, assuming that each formed a cross-link.

Optical rotation (at 238 nm) of protein in 50 mM K₂SO₄ and a 2-fold dilution of acetate buffer, pH 5.0, decreased progressively when the temperature was raised from 24°C in increments of approx. 10°C and held at each temperature until rotation became constant. At 80°C, rotation in untreated and substituted protein was 73 and 82% respectively of the initial values. On cooling, the rotation of the treated protein increased to 103%.
Error in Assay due to Time Dependency of Carbon Monoxide Difference Spectrum of Reduced Yeast Cytochrome P-450: Slow Reduction Caused by Triton X-100 Present

MAHMOOD R. AZARI AND ALAN WISEMAN

Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, United Kingdom

Received May 11, 1981

Triton X-100, added to yeast Saccharomyces cerevisiae for the purpose of stabilization or solubilization affects the carbon monoxide difference spectrum of reduced cytochrome P-450 and consequently the measurement of cytochrome P-450. Eight minutes is needed for 450-nm peak to reach its maximum height. Triton X-100 is shown to behave as a Type II substrate (absorption maximum at 418 nm and minimum at 390 nm) and to modulate the spin state of cytochrome P-450 from high to low form. Low-spin yeast cytochrome P-450 is reduced more slowly than the high-spin form.

It has been reported by Yoshida and Ku- (5) on the hepatic cytochrome P-450 system (reduced enzymatically) and Sliger et al. (6) in which they have concluded that the binding of Type I substrate (absorption maximum at 390 nm and minimum at 418 nm) to hepatic cytochrome P-450 could increase the rate of electron flow from NADPH-reductase to cytochrome P-450 by modulating the spin state from low- to high-spin form.

EXPERIMENTAL PROCEDURES

Brewer’s yeast, Saccharomyces cerevisiae (NCYC NO. 240), was grown for 44 h under glucose repression in medium containing 20% glucose and mycological peptone by the method previously described by Wiseman and Lim (7).

Yeast microsomal fraction was obtained by centrifugation at 160,900g for 1 h, after disruption of the yeast by Vibromill, described by Wiseman et al. (8). The microsomal fraction was suspended in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol, 0.001 M EDTA, 0.001 M dithiothreitol. Triton X-100 was added to this suspension with a final concentration of
Carbon monoxide difference spectrum

0.20 R  A
0.18
0.18
0.14
0.12
0.12
0.10
420 440 460 480 500
Ylmmtricph
420 440 460 480 500

Fig. 1. Carbon monoxide difference spectrum of reduced cytochrome P-450. (A) Microsomes treated with Triton X-100 (0.1%), Curves 1-3 and 8 were recorded 1, 2, 3, and 8 min after initial scanning. (B) Control microsomes recorded 20 s after addition of CO. Cytochrome P-450 1 nmol/ml; sodium dithionite 1 mg/ml; 22°C.

0.1%. The control sample did not contain any Triton X-100.

In one case, solubilization was achieved by adding 1% Triton X-100 to the suspension of microsomes, stirring for 1 h, centrifuging at 160,900g for 1 h, and collecting the supernatant, which contained the solubilized cytochrome P-450.

The carbon monoxide difference spectrum of yeast cytochrome P-450 was recorded by a spectrophotometric method of Wiseman et al. (9), modified from that of Omura et al. (10). A 1-ml sample of solution was placed in each of two spectrophotometer cuvettes and reduced by 1 mg/ml sodium dithionite. The baseline between 390 and 500 nm was recorded using a Pye-Unicam SP 1800 spectrophotometer, carbon monoxide was then bubbled (at a rate of 1 bubble/s unless mentioned otherwise) through the sample cuvette for 30 s, and the scanning was repeated. The peak height at 450 nm above the baseline was used to calculate the concentration of cytochrome P-450 assuming an extinction coefficient of 91 cm⁻¹ mm⁻¹.

Spectrophotometry was carried out at 22°C unless mentioned otherwise. The rate of reduction was measured by placing cytochrome P-450 (microsomal fraction) in both reference and sample cuvettes. Carbon monoxide was bubbled through the sample cuvette at the rate of 1 bubble/s for 30 s, 1 mg/ml sodium dithionite was added to the sample cuvette and mixed quickly, and absorbance changes at 448 nm were recorded.

In some cases carbon monoxide was not bubbled through the solution and the rate of reduction was measured at 440 nm.

RESULTS AND DISCUSSION

The addition of Triton X-100 affected the carbon monoxide difference spectrum of yeast cytochrome P-450 (reduced CO cytochrome P-450 minus reduced cytochrome P-450) (Fig. 1). It also affected the difference spectrum of reduced CO cytochrome P-450 minus oxidized cytochrome P-450. The difference in absorbance at 448 nm was low and eventually reached its maximum (real) value only after approximately 8 min delay. The percentage of reduction was calculated as previously done by Gigon et al. (5) and Ando and Horie (4), and the maximal increase in absorbance in the presence of excess dithionite was taken at 100%. The percentage of remaining oxidized cytochrome P-450 was plotted on a logarithmic scale (Fig. 2), and shows that the rate of reduction of cytochrome P-450 in the presence of Triton X-100 is biphasic with complete reduction taking 8 min.

The time dependency of the carbon monoxide difference spectrum of Triton X-100 treated yeast cytochrome P-450 could not be attributed to slow solubilization of CO
and therefore its slow binding to the reduced cytochrome P-450. As was described by Ando and Horie (4), carbon monoxide binding is a very rapid reaction. When sodium dithionite was introduced to the sample cuvette 4 min after the addition of carbon monoxide, the CO difference spectrum of reduced P-450 was still time dependent. The same results were obtained in the absence of CO for Triton X-100 treated microsomes by measuring the rate of reduction at 440 nm and, although the difference in absorbance was very small, it increased with time as before.

In another experiment the CO difference spectrum of Triton X-100 treated yeast microsomes was recorded 4 min after the addition of CO. The sample cuvette was freed of CO by bubbling oxygen (1 bubble/s for 30 s) through it, and it was oxidized by adding H2O2 (4 mM in concentration). Then the Triton X-100 difference spectrum was recorded, and the difference spectrum showed a Type II change, i.e., no peak at 390 nm and a Soret peak at 418 nm. This ruled out the slow displacement of Triton X-100 by the addition of CO, to which the time dependency of CO difference spectrum of Triton X-100 treated microsomes could have been related.

Other factors such as the temperature and the rate of CO bubbling through the samples were taken into consideration. At a low temperature (4°C) the height of the 450-nm peak in the carbon monoxide difference spectrum of cytochrome P-450 was smaller, and as the temperature was raised toward 22°C, the peak became larger. At a constant temperature, the spectrum was time dependent when the sample contained Triton X-100. The sample was not time dependent when free of Triton X-100 (control). If carbon monoxide was bubbled faster than 2 bubbles/s, a small amount of cytochrome P-450 was converted to cytochrome P-420, and as the time went by (5 min) some of this P-420 reconverted to P-450. But in the case of Triton X-100 treated microsomes, their reconversion was completed in 5 min and then another 5 min was required for the 450-nm peak to reach its maximum height. This showed that the rate of bubbling CO to the sample cuvette is a negligible factor and the time dependency of CO difference spectrum of Triton X-100 treated microsomes is due only to the slow rate of reduction.
Aniline, a Type II substrate, proved to have the same effect as Triton X-100 on the CO difference spectrum of yeast microsomes. The 450-nm peak was decreased considerably in comparison with the control microsomes and it reaches its real height after approximately 8 min (Fig. 3).

As was shown by Yoshida and Kumaoka (11), the absorption peak at 394 nm of the spectral high-spin form of hepatic cytochrome P-450 is shifted to 416 nm (the modified Type II spectral change), a spectral low-spin form, upon binding with a compound containing a hydroxyl group. The Triton X-100 used to stabilize cytochrome in yeast microsomes (12) also contains hydroxyl groups.

The absolute spectra of Triton X-100 solubilized yeast microsomes gave direct evidence for this spectral spin state change (Fig. 4). A major peak at 418 nm and no absorbance at 650 nm showed the existence of low-spin P-450, while the solubilized P-450 with sodium cholate also had a peak at 390 nm, showing a spectral mixed spin form to be present.

It can be concluded that Triton X-100 can modulate the spin state of yeast cytochrome P-450 (the same as a Type II substrate). This causes slow P-450 reduction by sodium dithionite, making the measurement of cytochrome P-450 by the CO difference spectrum method time dependent, which indicates the existence of a major error in the standard "immediate assay" procedure.

The measurement of the hepatic cytochrome P-450, in contrast, was not time dependent with or without the presence of Triton X-100. This may be due to a higher midpoint redox potential in hepatic cytochrome P-450, which will be the subject of future studies.

REFERENCES
Purification and Characterization of the Cytochrome P-448 Component of a Benzo(a)pyrene hydroxylase from *Saccharomyces cerevisiae*

Mahmood R. Azari and Alan Wiseman

Division of Biochemistry, Department of Biochemistry, University of Surrey, Guildford GU2 5XH, Surrey, United Kingdom

Received December 15, 1981

Cytochrome P-448, a type of cytochrome P-450, from brewer's yeast (*Saccharomyces cerevisiae*) grown under conditions of glucose repression was isolated and purified. Triton X-100 in very low concentration proved to be very effective in stabilizing P-448 in the microsomal fraction and later prevented its conversion to cytochrome P-420 through solubilization with various ionic and nonionic detergents. Highest yields were obtained with 1% sodium cholate, in the presence of 0.1% Triton X-100 and reduced glutathione. A novel combination of hydrophobic adsorption and other chromatographic techniques was used for the purification of cytochrome P-448. These involve the use of amino octyl-Sepharose 4B, instead of the low-yielding aminohexyl derivative, followed by the fast-running hydroxyapatite-cellulose column. Finally, the use of DEAE-Sephalac was found to increase greatly the purity of the cytochrome P-448 obtained. The molecular weight of this preparation was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (M_, 55,500). Using the known molar extinction coefficient of the carbon monoxide-difference spectrum the estimate of degree of purity of cytochrome P-448 obtained by this purification procedure was between 88 and 97%. Electrophoresis also showed that this preparation was completely homogeneous and assays showed that it was also completely free of cytochrome b_, cytochrome c reductase and cytochrome P-420. Purified cytochrome P-448 reconstituted with cytochrome P-450 (cytochrome c) reductase, isolated from yeast, showed 10-fold higher aryl hydrocarbon hydroxylase activity with benzo[a]pyrene as a substrate than the corresponding microsomal fraction enzyme. Kinetics of benzo[a]pyrene hydroxylation were determined: K_ (33 μM) was comparable with that reported for purified hepatic cytochrome P-448. The number of binding sites of microsomal and purified cytochromes P-450 (from liver of phenobarbital-induced rats) and yeast cytochrome P-448 with benzo[a]pyrene has been determined using an equilibrium gel filtration method. There is one binding site in each case (contrast with six sites for microsomal enzymes). The Scatchard plot gives number of binding sites, apparent association constants (K), and the equivalent dissociation constants (K_s). Comparison is made with spectral dissociation constants for these enzymes and benzo[a]pyrene. Thus the proportion bound, dissociation constant (K_s), and stoichiometry of rat liver (phenobarbital induced) and yeast cytochrome P-448 with benzo[a]pyrene were compared with corresponding values for microsomal fractions of both systems. Purified enzymes had higher K_s values in both cases, and the proportion of enzyme that bound benzo[a]pyrene was high (53%) for liver and this value is 100% for purified enzyme from yeast, which is the same as the value obtained for the microsomal enzyme from yeast.

We have reported previously our preliminary studies on a cytochrome P-448-linked benzo[a]pyrene hydroxylase in membrane-bound form (microsomal fraction) from a disruptate of brewer's yeast, *Saccharomyces cerevisiae*, grown aerobically at 20% glucose concentration (1-3). Such conditions of mitochondrial repression are associated with low levels of cellular cyclic AMP in the yeast, and it is here that an endoplasmic reticulum-located enzyme of the cytochrome P-450 type is produced.

The enzyme in *Saccharomyces cerevisiae* is similar to the cytochrome P-448 of liver...
in the formation of metabolites of benzo[a]pyrene, especially the 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene formed via the corresponding epoxide (2). Assay of the enzyme was achieved by measurement of the rate of formation of the highly fluorescent metabolite, 3-hydroxybenzo[a]pyrene (4).

Previous attempts to solubilize yeast cytochromes P-450 which contain both the P-450 and P-448 forms of the enzyme have resulted in low yields and heterogeneous products. Preliminary purified enzyme from an aerobically grown baker's yeast were reported by Yoshida (5-7), involving a purification procedure using aminoexyl Sepharose 4B. We now report a method of solubilization and purification that involves a novel combination of hydrophobic adsorption and other chromatographic techniques. These use the much more efficient affinity material, aminoocetyl Sepharose 4B, followed by a fast-running hydroxyapatite-cellulose column. Much higher yields are obtained and much less conversion to the inactive form (cytochrome P-420) occurs in the rapid method employed. Preferential elution of the cytochrome P-448 form of the enzyme was obtained by the introduction of a final step in the purification using DEAE-Sephal. The cytochrome P-450 form of the enzyme remains bound to the column, and for its elution a buffer with higher ionic strength was needed. Greatly enhanced purity of the isolated cytochrome P-448 is thereby obtained.

**MATERIALS AND METHODS**

*Preparation of microsomes.* Brewer's yeast *S. cerevisiae* (NCYC 240) was grown at 30°C for 44 h under glucose repression in medium containing 20% glucose, 2% myceliological peptone, 1% yeast extract, and 0.5% NaCl using the method of Wiseman and Lim (8). Yeast cells were harvested by centrifugation at 2500g for 5 min. They were disrupted by a Vibro Mill (9) and then suspended in 0.1 M potassium phosphate buffer (pH 7.2), 20% glycerol, 0.001 M EDTA, and 0.001 M dithiothreitol (hereafter, all phosphate buffers are potassium phosphate and contain 20% glycerol, and 0.001 M EDTA plus 0.001 M DTT are called the additives). After removal of the cell debris and nuclear material by centrifugation at 7500g for 15 min, yeast microsomal fraction was obtained by centrifugation at 160,900g for 1 h. Microsomal fraction was suspended in 0.1 M phosphate buffer (pH 7.2), 0.001 M EDTA, and reduced glutathione was added to this suspension to 0.1% final concentration.

**Solubilization method.** Microsomal fraction was diluted to 30 mg protein/ml with phosphate buffer, pH 7.2, containing the additives. A number of ionic and nonionic detergents were used to solubilize cytochrome P-448 in the presence or absence of 0.1% Triton X-100. Cytochrome P-448 was finally solubilized for purification by selecting one of these, the ionic detergent sodium cholate at a final concentration of 1% and always in the presence of 0.1% Triton X-100. Microsomal fraction plus sodium cholate was stirred at 4°C for 1 h and was centrifuged at 160,900g for 50 min and the supernatant, the solubilized cytochrome P-448, was collected.

**Purification method.** Solubilized cytochrome P-448 was diluted (X3) with 0.1 M phosphate buffer (pH 7.2) plus the additives. The precipitate at 35-65% sat (NH₄)₂SO₄ (at pH 7.0) was prepared and then suspended in 100 ml of 0.02 M phosphate buffer (pH 7.0) plus the additives, 0.5% sodium cholate. This suspension was dialyzed overnight against 30 vol of 0.01 M phosphate buffer (pH 7.0) plus the additives and 0.3% sodium cholate. It was subsequently centrifuged at 160,900g and the supernatant was mixed with 10 g Bio-Bead SM-2 (prewashed with methanol and H₂O) and stirred for 30 min before addition to the top of a 2.0 X 4.0-cm packed Bio-Bead column. Cytochrome

1 Abbreviations used: DTT, dithiothreitol; SDS, sodium dodecyl sulfate.
P-448 was eluted from the column by washing it with 1 column vol of the dialysis buffer. The sodium cholate concentration in the cytochrome P-448 solution collected from the Bio-Bead column was adjusted to 0.5% and this was applied to a 2.4 X 12.0-cm, 8-amino-n-octyl-Sepharose 4B column which had been pre-equilibrated with 0.01 M phosphate buffer (pH 7.0) plus 0.3% sodium cholate, 0.001 M EDTA (equilibration buffer). After washing the column with the same buffer, cytochrome P-448 was eluted with this same buffer containing 0.1% Emulgen 911. For elution of cytochrome P-450 reductase the aminooctyl-Sepharose column was washed with this buffer also containing 0.2% deoxycholate and 2 μM FMN. Fractions rich in cytochrome P-448 were applied to a 1.6 X 6.0-cm hydroxylapatite-cellulose (1:1, w/w) column pre-equilibrated with 0.01 M phosphate buffer (pH 7.0). The following buffers all contained 0.2% Emulgen 911. This column was washed with 0.03 M phosphate buffer (pH 7.0) + Emulgen 911, and cytochrome P-450 was eluted with 0.1 M phosphate buffer (pH 7.0) + Emulgen 911. Cytochrome P-448 fractions eluted from the column were combined and diluted (×10) with 20% glycerol + 0.02% Emulgen 911. This was then applied to a 1.0 X 5.0-cm carboxymethyl-Sephadex C-50 column pre-equilibrated with 0.01 M phosphate buffer (pH 7.0) + Emulgen. After washing the column with 3 vol of the equilibration buffer and also with 3 vol 0.04 M phosphate buffer (pH 7.0) + Emulgen, cytochrome P-448 was eluted by passing through 0.1 M phosphate buffer (pH 7.0) + Emulgen. Cytochrome P-448 was dialyzed twice against 30 vol 0.005 M phosphate buffer (pH 7.7) + Emulgen, each time, for 12 h diluted (×2) with 20% glycerol + Emulgen, adjusted to pH 7.7, and then applied to an 0.85 X 7.0-cm DEAE-Sephascel column pre-equilibrated with the above dialysis buffer. Most of the cytochrome P-448 did not bind to the column and was eluted first by washing the column with some of the equilibration buffer. The cytochrome P-450 form bound to the column and could be eluted with 0.035 M phosphate buffer (pH 7.7) + Emulgen. Cytochrome P-448 was concentrated by ultrafiltration (Amicon PM-30 membrane). Emulgen 911 and 913 and Renex 698 were applied by Honeywill Atlas Ltd., Surrey, United Kingdom.

Assays and electrophoresis. Cytochrome P-448 and cytochrome P-420 were measured by a method modified from that of the Omura and Sato (10) using 91 mm and 111
### TABLE 2

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total amount (nmol)</th>
<th>Specific content (nmol/mg protein)</th>
<th>Stage yield (%)</th>
<th>Total cytochrome b₅ (nmol)</th>
<th>Total cytochrome P-450 reductase (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal fraction</td>
<td>7200</td>
<td>266</td>
<td>0.036</td>
<td>100</td>
<td>295</td>
<td>600</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>600</td>
<td>230</td>
<td>0.38</td>
<td>86</td>
<td>290</td>
<td>520</td>
</tr>
<tr>
<td>Aminooctyl Sepharose 48</td>
<td>20</td>
<td>160</td>
<td>8</td>
<td>70</td>
<td>2.5</td>
<td>4</td>
</tr>
<tr>
<td>Hydroxyapatite-cellulose</td>
<td>10</td>
<td>120</td>
<td>12</td>
<td>75</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>CM-Sephadex C-50</td>
<td>6.9</td>
<td>100</td>
<td>14.5</td>
<td>83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DEAE-Sephadel</td>
<td>4.1</td>
<td>72</td>
<td>17.5</td>
<td>72*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Overall yield 27% of microsomal fraction.

cm⁻¹ mm⁻¹ as extinction coefficients for cytochrome P-448 and cytochrome P-420, respectively.

Cytochrome b₅ and cytochrome c reductase were measured using the method of Yoshida et al. (6).

SDS-polyacrylamide gel electrophoresis (10% gel) was carried out by the method of Laemmli (11), and protein was determined by the method of Lowry et al. (12).

The measurement of aryl hydrocarbon hydroxylase was carried out by the method of Woods and Wiseman (1) modified from that of Dehnen et al. (4). Instead of microsomal fraction, a mixture of purified cytochrome P-448 and cytochrome P-450 reductase (1 nmol of cytochrome P-450 to 1 U of reductase) was used.

**Spectral studies on the binding of benzo[a]pyrene to cytochrome P-448.** Binding spectra of benzo[a]pyrene to microsomal and purified cytochrome P-450/P-448 were recorded using the double-cell technique (double-compartment cuvette) in order to remove interference from the benzo[a]pyrene, which absorbs in the wavelength range used (350-500 nm) (3). Spectral titrations at fixed wavelength were subjected to a double-reciprocal plot and the spectral dissociation constant (Kₛ) and maximum absorbance changes were determined. The absorbance changes, used for construction of double-reciprocal plots were those at the trough (ΔA₄15-₅00 nm).

**Equilibrium gel filtration of the benzo[a]pyrene cytochrome P-450 complex.** Gel filtration was carried out on a small column (Pasteur pipet) packed with Sephadex G-25. The column was equilibrated with 0.2 M phosphate buffer (pH 7.0), containing 10% dioxan (v/v) and benzo[a]pyrene, before each experiment. The benzo[a]pyrene in the buffer consisted of a fixed, known amount of [G-³H]benzo[a]pyrene and various known amounts of unlabeled benzo[a]pyrene for each experiment. In each experiment after the column was equilibrated with benzo[a]pyrene containing buffer, a 50-μl sample was taken and specific radioactivity was determined. In each experiment a small volume of microsomal suspension or purified cytochrome P-450/P-448 (usually 10 μl) was applied to the column and 2-drop (0.06-ml) samples were collected. These samples were counted for radioactivity in a LKB 1210 Ultrascan Scintillation Counter after adding 4 ml of toluene/metanol (2/1, v/v) scintillant containing 0.5% 2,5-diphenyloxazole and 0.2% 1,4-bis(5-phenyloxazol-2-yl)-benzene (w/v). From the specific radioactivity of benzo[a]pyrene, the amount bound to enzyme (p) was calculated. The same
PURIFICATION OF BREWER'S YEAST CYTOCHROME P-448

**Table 3**

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th><em>V</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Full system (Emulgen 911 as a heat stabilizing factor)</td>
<td>142</td>
</tr>
<tr>
<td>Full system (dilauroyl phosphatidylcholine as a heat-stabilizing factor)</td>
<td>138</td>
</tr>
<tr>
<td>Minus cytochrome P-448</td>
<td>10</td>
</tr>
<tr>
<td>Minus cytochrome P-450 (c) reductase</td>
<td>13</td>
</tr>
<tr>
<td>Minus NADPH-generating system</td>
<td>24</td>
</tr>
<tr>
<td>Minus dilauroyl phosphatidyl choline or Emulgen 911</td>
<td>75</td>
</tr>
</tbody>
</table>

**Note.** *V* values are expressed in pmol 3-hydroxybenzo(a)pyrene/h/nmol cytochrome P-448. NADPH-generating system consists of NADP, 4 mM; glucose 6-phosphate, 20 mM; plus 8 units of glucose-6-phosphate dehydrogenase/ml of the assay mixture. The dilauroyl phosphatidylcholine concentration was 30 μg/ml of assay mixture and alternatively 0.1% (v/v) of Emulgen 911 was used in the assay mixture.

**RESULTS AND DISCUSSION**

Approximately 1.3 kg yeast was used for isolation of purified enzyme. Cytochrome P-448 in harvested yeast kept in storage at 4°C in 0.1 M phosphate buffer (pH 7.2), 0.001 M EDTA, 0.001 M mercaptoethanol, and 15% glucose. This medium prevented disappearance and conversion of cytochrome P-448 to cytochrome P-420.

The greatest loss of cytochrome P-448 in the presentation of the microsomal fraction occurred during the disruption stage, which used the water-cooled Vibro Mill.

Cytochrome P-448 in microsomal fraction was kept in storage at −40°C for several weeks in 0.1 M phosphate buffer (pH 7.0), 0.001 M EDTA, 0.1% Triton X-100, 0.1% reduced glutathione. In comparison, cytochrome P-450/P-448 from rat liver microsomal fraction was very stable at −40°C, when stored only in 0.1 M phosphate buffer (pH 7.0) plus the additives.

The thermal stability of cytochrome P-448 was greatly improved in the presence of Triton X-100 such that its content was unchanged after incubation at 50°C for 10
min. Without Triton X-100, however, 50% of cytochrome P-448 was converted to cytochrome P-420 after incubation at 40°C for 10 min. Cytochrome P-450/P-448 content from rat liver microsomes, in phosphate buffer containing the additives, was almost unchanged after incubation at 60°C for 10 min.

Microsomes in phosphate buffer (pH 7.0) plus the additives, and also in phosphate buffer (pH 7.0) plus the additives and 0.1% Triton X-100, were solubilized by various detergents (13), with release of cytochrome P-448 as shown in Table 1. Good yields of enzyme were obtained with both 1% Triton X-100 and 1% sodium cholate in the 0.1% Triton X-100 containing phosphate buffer (pH 7.0) plus the additives only. Using this technique, a 100% yield was obtained from the yeast microsomal fraction, and the yield from the rat liver microsomal fraction was 93 and 87% with these two detergents, respectively. Sodium cholate (1%) alone gave a good yield (80%) from rat liver microsomal fraction, but only 36% from the yeast microsomal fraction. Emulgen 913 (1%) gave about 50% yield from the yeast microsomal fraction, regardless of the presence of 0.1% Triton X-100. Sodium dodecyl sulfate (0.5%) gave a very poor yield of cytochrome P-448 (Table 1). The highest cytochrome P-448/P-420 ratios in the range 22-26 were obtained from yeast microsomal fraction with 1% Triton X-100 compared with a ratio of only 7 for the rat liver. Renex 698, Tween 80, Triton N-101, all at 1% concentration, had no (or very little) solubilization effect on cytochrome P-448 from yeast microsomes.

Triton X-100 is known to change the spin state of cytochrome P-450 from high to low spin (14), and has been shown to protect some enzymes by formation of micelles, which protect the hydrophobic environment of the enzyme. Conversely, it has been reported that sulfhydryl reagents (15), or high ionic strength of medium (16), cause the spin state to change from low to high spin and also accelerate the cytochromes P-450/P-420.

### Table 4

**Data from the Equilibrium Gel Filtration of the Benzo(a)pyrene Cytochrome P-448 Complex (Purified Enzyme from Yeast)**

<table>
<thead>
<tr>
<th>c (nM)</th>
<th>e (nmol)</th>
<th>p (nmol)</th>
<th>p/c (γ)</th>
<th>γ/c (nm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>0.01</td>
<td>0.00028</td>
<td>0.028</td>
<td>0.022</td>
</tr>
<tr>
<td>7.59</td>
<td>0.01</td>
<td>0.0019</td>
<td>0.19</td>
<td>0.025</td>
</tr>
<tr>
<td>13.93</td>
<td>0.01</td>
<td>0.0032</td>
<td>0.32</td>
<td>0.023</td>
</tr>
<tr>
<td>20.27</td>
<td>0.01</td>
<td>0.0033</td>
<td>0.33</td>
<td>0.016</td>
</tr>
<tr>
<td>25.62</td>
<td>0.01</td>
<td>0.0042</td>
<td>0.42</td>
<td>0.015</td>
</tr>
<tr>
<td>29.50</td>
<td>0.01</td>
<td>0.0051</td>
<td>0.51</td>
<td>0.012</td>
</tr>
</tbody>
</table>

*Note. c is concentration of benzo(a)pyrene, e is the quantity of cytochrome P-450, and p is the concentration of the product.*
448-to-cytochrome P-420 conversion, by destroying or removing the sulfadryl ligand. This suggests that Triton X-100, by changing the spin state from high to low state and retaining it in that state, may protect the sulfadryl ligand and make it more accessible to heme iron, thus exerting a stabilizing effect on the cytochromes P-450/P-448.

(NH₄)₂SO₄ fractionation was carried out while maintaining the pH at the enzyme pH optimum (pH 7.0), as reported by Woods and Wiseman (1). At lower pH, (NH₄)₂SO₄ denatured cytochrome P-448, and cytochrome P-420 was formed. The denaturation of cytochrome P-448 by high concentration of neutral salt was reported by Imai and Sato (17); without specifying the pH used, they attributed this conversion to the disturbance of the hydrophobic environment around the heme moiety, either by primary action of the neutral salts or by secondary effects due to conformational changes in the cytochrome P-448 molecule.

Purified cytochrome P-448 had a specific content of 16–17.5 nmol/mg protein and overall yield of cytochrome P-448 from microsomal fraction was 27% (Table 2).

There were no changes in the 448-nm peak size of the reduced CO-difference spectrum after 6 weeks storage of this preparation at −40°C, and there was no loss of aryl hydroxylase activity after this time.

SDS-polyacrylamide gel electrophoresis showed the homogeneous nature of the cytochrome P-448. Using several marker proteins, the molecular weight of cytochrome P-448 was estimated to be 55,500. This is similar to the molecular weight of cytochrome P-450/P-448 from liver microsomes in 3-methylcholanthrene-induced animals, predominantly consists of a high-spin form. In our work, the cytochrome P-448 was initially in a mixed-spin state, but as has been reported by Yoshida et al., (21) and Fujita et al. (22), the treatment of cytochromes P-450 with Triton X-100 (or some other non-ionic detergent, such as Lubrol WX and Triton N-101) changes the spin state of cytochromes P-450 from high- to low-spin form.

A major metabolite of the benzo[a]pyrene hydroxylase system is 3-hydroxybenzo[a]pyrene (2). This enzyme system required two protein components: cytochrome P-450/P-448 and NADPH-cytochrome P-450 reduc-

---

**TABLE 5**

<table>
<thead>
<tr>
<th>c (nmol)</th>
<th>e (nmol)</th>
<th>p (nmol)</th>
<th>p/c (g)</th>
<th>γ/c (nmol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.0036</td>
<td>0.000126</td>
<td>0.045</td>
<td>0.018</td>
</tr>
<tr>
<td>8.8</td>
<td>0.0036</td>
<td>0.000550</td>
<td>0.152</td>
<td>0.017</td>
</tr>
<tr>
<td>15.2</td>
<td>0.0036</td>
<td>0.000794</td>
<td>0.220</td>
<td>0.013</td>
</tr>
<tr>
<td>27.9</td>
<td>0.0036</td>
<td>0.00148</td>
<td>0.310</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Note. c is concentration of benzo[a]pyrene, e is the quantity of cytochrome P-450, and p is the concentration of the product.
The heat-stable factor (phospholipid), which has been reported to associate with cytochrome P-450 and NADPH-cytochrome P-450 reductase to form an active enzyme aggregate (23), was not needed in our studies for the purpose of reconstitution (Table 3). The residual nonionic detergent (Emulgen 911) content of cytochrome P-448 seemed to suffice for active enzyme reconstitution (24). It was also necessary for flavine mononucleotide to be present in the buffer which was used to elute the cytochrome P-450 reductase from the aminooctyl-Sepharose 4B column, and this was present, therefore, in the hydroxylation reaction mixture, where it supplements the action of the reductase.

The benzo[a]pyrene concentration was used in the range 0-79 μM in the series of rate determinations in the derivation of the Lineweaver-Burk plot from these hydroxylations. Even high concentrations of benzo[a]pyrene dissolve in the lipid or detergent (0.1% Emulgen 911) present. This linear plot showed that the Michaelis constant ($K_m$) for benzo[a]pyrene hydroxylation of purified cytochrome P-450 was calculated at 33 μM. This value is much smaller than that of the cytochrome P-448 measured in situ in the yeast microsomal fraction reported by Woods and Wiseman (1). Our value is similar to that reported for liver enzyme (23 μM) by Rickert and Fouts (25) (compare the value for liver enzyme of 1 μM reported by Robie et al. (26)); we find that the $V$ (at 37°C) is 208 pmol 3-hydroxybenzo[a]pyrene formed/h/nmol of cytochrome P-450. This value is more than 10 times greater than that reported (13) for the yeast microsomal fraction, indicating the problem in using an immobilized enzyme (i.e., microsomal fraction) which includes a diffusion effect, when compared with a successfully reconstituted system free of lipid.

In the spectral titration of highly purified cytochrome P-450/P-448 from yeast and rat liver, the benzo[a]pyrene was used in a concentration range of 7.9-39.5 μM, and the binding spectra was recorded (Fig. 2). The spectral dissociation constant ($K_r$) was found to be 50 μM for the yeast purified enzyme. This value agrees with the $K_r$ value found by Woods and Wiseman (3) for crude solubilized yeast cytochrome P-448, and now in our purified enzyme there is excellent agreement between the values of $K_r$ (50 μM) and $K_m$ (33 μM). The $K_r$ value for the microsomal cytochrome P-448 from yeast was 18 μM. Even in purified systems, any disagreement would be due to the complexity of the benzo[a]pyrene-metabolizing system, because the $K_m$ values for this system are the aggregate of several components: in a case reported by Kratz and Staudinger (27) the $K_r$ value for coumarin binding is found to be 100 times greater than the $K_m$ for its metabolism in an animal system.

In equilibrium gel filtration of benzo[a]pyrene-cytochrome P-450/P-448 complex, from the values of $p$, $e$, and $c$, thus determined, $p/e$ ($\gamma$) and $\gamma/c$ were calculated (Tables 4 and 5). Using $\gamma$ and $\gamma/c$ as the $x$ and $y$ axes the Scatchard plots were constructed Fig. 3. The value of the number of binding sites ($n$) is the intercept of the $\gamma$ axis and the apparent average association constant ($k$) is the slope.

The nearest whole number values of $n$, determined for yeast microsomal and puri-
The successful isolation of these small quantities (4 mg, 72 nmol) of homogeneous cytochrome P-448 from *S. cerevisiae* (from 1.3 kg yeast) is the first step in a scale-up procedure using the large-scale fermentation and disruption facilities available. Several applications exist for xenobiotic-metabolizing cytochromes P-450 from microorganisms (see review by Wiseman (28)). Our immediate aims include a benzo[a]pyrene-specific enzyme electrode, employing this purified and highly specific cytochrome P-448 from *S. cerevisiae* No. 240. There is now considerable interest in the enzyme (29) and in yeast test systems for carcinogens using cytochrome P-450-containing yeasts (30,31).

ACKNOWLEDGMENTS

The authors thank Dr. G. G. Gibson and Mr. P. Tamburini for donation of the cytochrome P-450 (microsomal and purified) from phenobarbital-treated rats. This enzyme was purified by the method of Guengerich (32). We are also grateful for helpful discussion with these colleagues.

REFERENCES

Studies on the procedure to measure accurately the binding properties of benz[a]pyrene to cytochrome P-450/P-448

MAHMOOD R. AZARI and ALAN WISEMAN
Division of Biochemistry, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 7XH, U.K.

Problems in the spectral study of benz[a]pyrene binding to cytochrome P-450 (or P-448) have necessitated the search for an improved method. A single-cell method was used to measure the binding properties of benz[a]pyrene to cytochrome P-450 in rat liver microsomal fraction (Estabrook et al., 1978). The double-cell technique has been used previously by us for spectral investigation of benz[a]pyrene binding to cytochrome P-448 from yeast (Saccharomyces cerevisiae) (Woods & Wiseman, 1979).

Cytochrome P-448 was solubilized from yeast (Saccharomyces cerevisiae) microsomal fraction and purified by using methods previously described by Azari & Wiseman (1980) and Wiseman & Azari (1981) respectively. The microsomal fraction was obtained from yeast cells grown under conditions of glucose repression as before (Wiseman & Lim, 1973). When the binding spectrum of benz[a]pyrene to microsomal cytochrome P-448 from yeast was recorded previously in this laboratory, a double-cell technique was adapted to remove the interference of benz[a]pyrene, which absorbs in the wavelength range used (350-500nm). Only the buffer solution was used in one of the compartments of both the reference and the sample cuvettes to complement the enzyme solution in adjoining compartments. In order to determine the dissociation constant ($K_d$), the results of spectral titrations at fixed wavelength at a range of benz[a]pyrene concentrations were subjected to a double-reciprocal plot. In this method the absorbance changes were calculated by adding up the spectral changes at peak and trough regions of the binding spectrum ($\Delta A_{448} + \Delta A_{411}$) to allow for changes in base-line during...
The binding spectrum of benzololpyrene with microsomal and purified cytochrome P-450/P-448 was recorded by using a double-cell technique (Azari & Wiseman, 1982). Spectral titrations at fixed wavelength were subjected to a double-reciprocal plot, and the spectral dissociation constant \((K_d)\) and maximum absorbance changes were determined (Fig. 1). The absorbance changes used for construction of double-reciprocal plots were those at the trough \((\Delta A_{415-500})\) (Azari & Wiseman, 1982).

Purified enzymes from both systems produced type-I spectral changes with benzololpyrene, and the extra peak appeared at 367 nm instead of at 360 nm as with microsomal fractions.

The values obtained for dissociation constants were 5 \(\mu M\) for microsomal fraction and 31 \(\mu M\) for purified cytochrome P-450/P-448 from liver of phenobarbital-induced rat, and 18 \(\mu M\) for microsomal fraction and 50 \(\mu M\) for purified cytochrome P-448 from yeast (Fig. 1). These results should be compared with those reported previously, namely 0.8 \(\mu M\) (Estabrook et al. 1978) and 9 \(\mu M\) (Woods & Wiseman, 1979) for hepatic microsomal cytochrome P-450 from phenobarbital-induced rats, and 36 \(\mu M\) (Woods & Wiseman, 1979) for microsomal cytochrome P-448 from yeast.
A study of benzo[a]pyrene binding to microsomal and highly purified cytochrome P-450/P-448 by equilibrium gel filtration

ALAN WISEMAN and MAHMOOD R. AZARI

Division of Biochemistry, Department of Biochemistry, University of Surrey, Guildford, Surrey GU1 3XH, U.K.

A spectral study of benzo[a]pyrene binding to cytochrome P-450/P-448 has been reported by Woods & Wiseman (1979) and Azari & Wiseman (1982). We now report further investigation on benzo[a]pyrene interaction with microsomal and purified hepatic cytochrome P-450 (phenobarbital-induced rats) and cytochrome P-448 (Saccharomyces cerevisiae) by using the method of equilibrium gel filtration (Hummel & Dryer, 1962). This method has hitherto been used for investigation of such interaction only with the microsomal fractions by Woods & Wiseman (1980).

Cytochrome P-448 was purified from yeast grown under glucose repression (Wiseman & Azari, 1981). Gel filtration was performed on a small column (Pasteur pipette) packed with Sephadex G-25. The column was equilibrated with 0.2M phosphate buffer, pH 7.0, containing 10% (v/v) dioxan and benzo[a]pyrene before each experiment. The benzo[a]pyrene in the buffer consisted of a fixed known amount of [G-3H]benzo[a]pyrene and various known amounts of unlabelled benzo[a]pyrene for each experiment. In each experiment after the column was equilibrated with benzo[a]pyrene-containing buffer, a 50 µl sample was taken and the specific radioactivity was determined. The column was then washed with buffer and 2-µl (0.06 ml) samples were collected. These samples were counted for radioactivity in an LKB 1210 Ultratbeta scintillation counter after addition of 4 ml of toluene/Metapal (2:1; v/v) scintillant containing 0.5% 2,5-diphenyloxazole and 0.2% 1,4-bis(5-phenyloxazol-2-yl)benzene. From the specific radioactivity of benzo[a]pyrene, the amount bound to enzyme (p) was calculated.

It was found that 100% of yeast purified cytochrome P-448 and 53% of rat liver purified cytochrome P-450 reacted with benzo[a]pyrene, as shown by comparison of benzo[a]pyrene-binding absorption with CO-binding absorption. When microsomal fractions were used in place of purified enzymes, the corresponding values were 100% and 16% respectively. The absorption coefficient used for determination of concentration of benzo[a]pyrene-cytochrome P-450/P-448 complex was 57,500 M⁻¹cm⁻¹ for ΔA₄₅₀-nm (Estabrook & Werringloer, 1978). The fact that a high percentage of the cytochrome P-450/P-448 in purified form reacts with benzo[a]pyrene is not surprising, as much of the purified yeast cytochrome P-448 (M. R. Azari & A. Wiseman, unpublished work) and purified hepatic cytochrome P-450 from phenobarbital-induced rats (Wigram & Tamburini, 1980) is found to be in the low-spin state, which readily combines with type-1 substrates such as benzo[a]pyrene.

Purified and microsomal hepatic cytochrome P-450 from phenobarbital-induced rats was generously given by our colleagues Mr. P. Tamburini and Dr. G. G. Gibson. We thank them too for most helpful discussions and advice.


(404)

Fig. 1. Comparison of the benzo[a]pyrene-binding properties of cytochrome P-450/P-448 (M) microsomal fraction and (P) purified form

Benzo[a]pyrene final concentration range was 7.9-39.5 µM. The concentration of these enzymes was 0.7 µM, and specific contents were 17.5 nmol/mg (purified enzyme from yeast) and about 12 nmol/mg (Purified enzyme from rat liver). Six portions of benzo[a]pyrene (2 mg/ml, in acetone) were added successively.

The dissociation constant values obtained showed that, although the affinity of cytochrome P-450/P-448 for benzo[a]pyrene is relatively high (i.e. low Keq values), the unusual ability to achieve stoichiometric titration of cytochrome P-450 by benzo[a]pyrene that was demonstrated for rat liver microsomal fraction (Estabrook et al., 1978) is not achieved here (especially for purified enzymes). A stoichiometric titration (1:1 molar ratio) would require Keq values of about 0.2 µM and 0.4 µM for purified cytochromes P-450 (rat liver) and P-448 (yeast) respectively. Purified enzyme apparently has lost high-affinity binding capacity to benzo[a]pyrene and has only one binding site (Wiseman & Azari, 1982).
The nearest-whole-number values of $n$ determined for yeast microsomal and purified cytochrome $P-448$ were six (as found too by Woods & Wiseman, 1980) and one respectively. Yeast microsomal and purified enzyme bind 100% to benzololpyrene, so that the number of binding sites is six (microsomal) and one (purified) per enzyme molecule binding to benzololpyrene. The number of binding sites for rat liver enzyme per molecule binding to benzololpyrene, because only 16% of microsomal cytochrome $P-450$ and 53% of purified form bind to benzololpyrene (Azari & Wiseman, 1982), was six (0.9 x 100/16) and one (0.5 x 100/53) also (0 values were 0.9 and 0.5 respectively).

This is a revision of the value of 20 for microsomal frictions calculated by Woods & Wiseman (1980), which was based on underestimates (values of 33% for yeast; 5% for liver) of extent of benzololpyrene binding (see modified technique; Azari & Wiseman, 1982).

The apparent association constant ($K_a$) obtained for yeast cytochrome $P-448$ was 0.08 nm$^{-1}$ (microsomal fraction) and 0.027 nm$^{-1}$ (purified form). These are equivalent to dissociation constants ($K_d$) of $1.3 \times 10^{-2}$ m and $3.7 \times 10^{-4}$ m, respectively. For rat liver cytochrome $P-450$ the corresponding $K_a$ values are 0.064 nm$^{-1}$ and 0.024 nm$^{-1}$, which is equivalent to dissociation constants ($K_d$) of $1.6 \times 10^{-2}$ m (microsomal fraction) and $4.3 \times 10^{-4}$ m (purified form). The $K_a$ values for the corresponding form of enzyme in the two systems are similar. Low values for all dissociation constants suggest that the binding affinity is very high. These $K_a$ values are much smaller than the spectral $K_a$ values (0.1 nm$^{-1}$) or $K_D$ (0.01 nm$^{-1}$) for yeast cytochrome $P-450$ and rat liver (Azari & Wiseman, 1982).

Lipid-bound enzymes (the microsomal fractions in both systems) have higher affinity than purified enzyme for benzololpyrene (i.e. lower $K_d$ values) and give a stoichiometry of 6:1 (yeast) and 1:1 (rat liver). When the amount of cytochrome $P-450$ per P-448 that binds to benzololpyrene is considered, the number of binding sites found for microsomal fractions of both systems is six. The extra five binding sites found for the bound form of the enzymes is clearly due to high-affinity binding of benzololpyrene to the lipid present, as affinity is lowered in purified enzyme.

Poly(hexamethylenebiguanide hydrochloride), a novel cationic complexing agent for the assay of acidic polysaccharides

**JOHN F. KENNEDY, S. ALAN BARKER and IAN J. BRADSHAW***

Research Laboratory for the Chemistry of Bioactive Carbohydrates and Proteins, Department of Chemistry, University of Birmingham, Birmingham B15 2TT, U.K.

The lack of a standard assay method suitable for the quantitative determination of a range of acidic polysaccharides has been a continuing problem for a number of years. Colorimetric methods employing reagents such as phenol (Dubois et al., 1956), carbazole (Dötsch, 1947; Bitter & Muir, 1962) and m-hydroxydiphenyl (Blumenkrantz & Asboe-Hansen, 1973) have been used, but all suffer the disadvantage that various responses with different sugars are obtained. Methods based on the evolution of CO$_2$ have been developed in the past for carboxylated polysaccharides (National Research Council, 1972), but the procedure is laborious and preliminary purification of the sample is sometimes necessary before analysis. In the present work we set out to resolve the difficulties surrounding the aforementioned analytical problem.

Recently, we discovered (Kennedy et al., 1981) a novel cationic polymeric precipitating agent, PHMBH$^+$Cl$^-$, which was found to undergo a cross-linking reaction with a wide range of acidic polysaccharides in aqueous solution. In particular, this reaction was found to be suitable for the isolation of xanthan gum, the polysaccharide produced by Xanthomonas campestris. Further application of this same precipitating agent has resulted in the development of a rapid method for the assay of acidic polysaccharides.

The new assay is based on the addition of an excess amount of PHMBH$^+$Cl$^-$ to solutions of polysaccharide to quantitatively precipitate the sample and therefrom measuring the u.v. absorption of the residual complexing agent present in the supernatant. The procedure is carried out as follows. To a portion (5 ml) of sample, PHMBH$^+$Cl$^-$ solution (0.3%; 10 ml) in 1% sodium acetate is added and the mixture aspirated for 2 min to allow formation of the acidic polysaccharide-PHMBH$^+$ complex. After removal of the formed complex from solution

---

### Fig. 1. Comparison of the responses of various acidic polysaccharides to the PHMBH$^+$Cl$^-$ assay

**Symbols:**
- $\Delta$, xanthan;
- $\alpha$, carrageenan;
- $\Theta$, chondroitin 4-sulfate;
- $\bigcirc$, sodium alginate.

Values in parentheses in the Figure are the acidic group/sugar molecule ratios.

---

*Present Address: Department of Microbiology, University of Edinburgh, Edinburgh EH19 3JG, Scotland, U.K.

†Abbreviation: PHMBH$^+$Cl$^-$, poly(hexamethylenebiguanide hydrochloride).
THE INDUCTION OF CYTOCHROME P-448 DEPENDENT BENZO(a)PYRENE HYDROXYLASE IN SACCHAROMYCES CEREVISIAE

David J. King, Mahmood R. Azari and Alan Wiseman

Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey, U.K.

Received February 18, 1982

When grown in high concentrations of glucose, the yeast Saccharomyces cerevisiae produces a microsomal cytochrome P-450 monooxygenase system which is capable of hydroxylating benzo(a)pyrene. The addition of benzo(a)pyrene to the yeast during growth causes only a small increase in cytochrome P-448 levels but results in a dramatic improvement in the apparent kinetics of benzo(a)pyrene hydroxylation as measured by a decrease in the Michaelis constant and an increase in maximal velocity. Dimethylnitrosamine, phenobarbital and 3-methylcholanthrene also induce this enzyme to various degrees. Yeast pretreatment with 8-naphthoflavone did not affect this enzyme, yet pretreatment with lanosterol resulted in a decreased affinity for benzo(a)pyrene. The addition of benzo(a)pyrene to yeast growing at low glucose concentration does not induce cytochrome P-448. The implications of these findings with regard to the presence of multiple forms of cytochromes P-448/P-450 in yeast are briefly discussed.

INTRODUCTION

The yeast Saccharomyces cerevisiae possesses a microsomal cytochrome P-450 dependent monooxygenase system with many properties in common with that found in mammalian liver (1,2). This yeast enzyme system exhibits aryl hydrocarbon hydroxylase activity towards benzo(a)pyrene, the major metabolites being 3-hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (3). We have found the absorption peak of this enzyme in the reduced carbon monoxide difference spectrum to be at 448 nm.

The production of cytochrome P-448/P-450 in Saccharomyces cerevisiae occurs under fermentative conditions where mitochondrial cytochrome a+a₃ formation is repressed, for example anaerobic conditions or at high glucose concentrations (4,5). There is now evidence to suggest that the intracellular concentration of cyclic AMP controls the de novo synthesis of cytochrome P-448/P-450 in S. cerevisiae by repression, the level of cyclic AMP being determined by the glucose concentration in the growth medium.
in an inverse relationship (6). This is supported by the direct relationship between cyclic AMP and cytochrome a+a3 levels and their inverse relationship to cytochrome P-448/P-450 levels at various glucose concentrations (7). Karenlampi, et al. (8) have recently supported the need for fermentable sugar to be present before cytochrome P-448/P-450 is produced.

The induction of mammalian cytochrome P-450 monooxygenase systems by various compounds has been very widely studied and is now beginning to be explained in terms of multiple forms of the enzyme. Two of the most widely studied inducers, phenobarbital and 3-methylcholanthrene induce different forms of the enzyme. Phenobarbital induces a form with a very wide specificity whereas 3-methylcholanthrene induces a form with relatively narrow specificity, cytochrome P-448 (9). It is now known that cytochrome P-450 exists in more than two different forms. The evidence for this comes, for example, from studies with inducers, isolation and purification of different forms, immunological studies and kinetic and binding studies (10). Multiple forms of cytochrome P-450 exist with different but overlapping substrate specificities.

MATERIALS AND METHODS

Saccharomyces cerevisiae (NCYC No.240) was grown batch wise by the method previously described by Wiseman, et al. (6). Growth was for 44 hours in a medium containing 1% yeast extract, 2% mycological peptone and usually 20% glucose. Inducing agents were added at the beginning of the growth period dissolved in a small amount of dimethylformamide. An equivalent amount of dimethylformamide was added to control experiments.

Microsomal preparations were obtained by differential centrifugation after disruption of yeast in a Vibromill disrupter by a method already described (11). Microsomal preparations were resuspended in 0.1 M potassium phosphate buffer pH 7.2 containing 20% glycerol, 0.001 M EDTA and 0.001 M dithiothreitol by use of a hand held Potter type homogeniser.

Cytochrome P-448/P-450 was determined by the method of Omura, et al. (12) using an extinction coefficient of 91 mM⁻¹ cm⁻¹.

The measurement of benzo(a)pyrene hydroxylase was achieved by detecting the amount of 3-hydroxybenzo(a)pyrene produced by the fluorimetric method of Dehnen, et al. (13) as modified by Wiseman and Woods (3).

RESULTS

The addition of benzo(a)pyrene to yeast growing in a medium containing 20% glucose affected the apparent kinetics of benzo(a)pyrene hydroxylase and also the
TABLE 1: Induction of Benzo(a)pyrene Hydroxylase with Benzo(a)pyrene

<table>
<thead>
<tr>
<th>Benzo(a)pyrene Concentration</th>
<th>Cytochrome P-448 nmole/g wet weight yeast</th>
<th>Km pM</th>
<th>Vmax pmoles 3-hydroxybenzo(a) pyrene/hour/n mole P-448</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.36 ± .24</td>
<td>111</td>
<td>167</td>
</tr>
<tr>
<td>16</td>
<td>3.66 ± .41</td>
<td>100</td>
<td>220</td>
</tr>
<tr>
<td>32</td>
<td>3.43 ± .31</td>
<td>65</td>
<td>333</td>
</tr>
<tr>
<td>63</td>
<td>3.95 ± .14</td>
<td>56</td>
<td>500 *</td>
</tr>
<tr>
<td>95</td>
<td>4.36 ± .23</td>
<td>42</td>
<td>444</td>
</tr>
<tr>
<td>190</td>
<td>*</td>
<td>32</td>
<td>580</td>
</tr>
</tbody>
</table>

Values of cytochrome P-448 are quoted ± standard deviation, n = 8.

* This concentration of benzo(a)pyrene resulted in precipitation of benzo(a)pyrene which interfered with the cytochrome P-450 assays rendering them invalid.

level of cytochrome P-448/P-450 in the yeast (Table 1). The level of cytochrome P-448/P-450 as measured by carbon monoxide-reduced difference spectra of yeast suspensions was slightly higher in yeast treated with benzo(a)pyrene at high concentrations. The kinetics of benzo(a)pyrene hydroxylase activity as supported.
TABLE 2: Effect of Inducing Agents on Cytochrome P-448 and Benzo(a)pyrene Hydroxylase in Yeast

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Cytochrome P-448 nmoles/g wet yeast</th>
<th>Km μM</th>
<th>Vmax pmoles 1-hydroxybenzo(a) pyrene/hour/n mole P-448</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.36 ± 0.24</td>
<td>111</td>
<td>167</td>
</tr>
<tr>
<td>Dimethylnitrosamine 108 μM</td>
<td>3.98 ± 0.38</td>
<td>99</td>
<td>588</td>
</tr>
<tr>
<td>Dimethylnitrosamine 324 μM</td>
<td>4.64 ± 0.38</td>
<td>40</td>
<td>476</td>
</tr>
<tr>
<td>3-Methylcholanthrene 30 μM</td>
<td>3.48 ± 0.37</td>
<td>27</td>
<td>154</td>
</tr>
<tr>
<td>3-Methylcholanthrene 90 μM</td>
<td>3.32 ± 0.35</td>
<td>20</td>
<td>174</td>
</tr>
<tr>
<td>Phenobarbital 32 μM</td>
<td>3.50 ± 0.24</td>
<td>80</td>
<td>278</td>
</tr>
<tr>
<td>8-Naphthoquinone 30 μM</td>
<td>3.80 ± 0.38</td>
<td>114</td>
<td>182</td>
</tr>
<tr>
<td>Lanosterol 19 μM</td>
<td>3.62 ± 0.42</td>
<td>250</td>
<td>200</td>
</tr>
</tbody>
</table>

Values of cytochrome P-448 are quoted ± standard deviation, n = 8.

by NADPH were investigated in microsomal fraction by means of double-reciprocal Lineweaver-Burke plots. The results show clearly that benzo(a)pyrene pretreatment of yeast results in a lower Michaelis constant (K<sub>m</sub>) i.e. higher affinity for benzo(a)pyrene and a higher maximal velocity (V<sub>max</sub>). These effects were clearly dependant on the concentration of benzo(a)pyrene in the medium (Table 1, Fig.1).

Yeast grown in 0.5% glucose containing medium usually contains no cytochrome P-448/P-450. In an attempt to induce benzo(a)pyrene hydroxylase activity in yeast growing at low glucose concentration we added benzo(a)pyrene. We were unable to induce the production of cytochrome P-448/P-450 in this growth medium even in the presence of a high concentration (95 μM) of benzo(a)pyrene.

Table 2 shows the effect of several other compounds on yeast cytochrome P-448/P-450 levels and the kinetics of microsomal benzo(a)pyrene hydroxylase. Pretreatment with dimethylnitrosamine appears to have a similar effect to pretreatment with benzo(a)pyrene in that the level of cytochrome P-448/P-450 is
slightly increased, the \( K_m \) for benzo(a)pyrene is decreased and the \( V_{\text{max}} \) value increased. Higher concentrations of dimethylnitrosamine are necessary to achieve the same extent of induction as with benzo(a)pyrene. Yeast pretreatment with 3-methylcholanthrene is interesting as this results in no change in cytochrome P-448/P-450 levels and no change in the \( V_{\text{max}} \) value yet a large decrease in \( K_m \) for benzo(a)pyrene is observed. Phenobarbital also has no effect on yeast cytochrome P-448/P-450 levels but improves both \( K_m \) and \( V_{\text{max}} \) for this enzyme.

Pretreatment of yeast with \( \beta \)-naphthoflavone has no effect on either cytochrome P-448/P-450 levels or benzo(a)pyrene kinetics. The addition of lanosterol to the yeast growth medium results in a higher \( K_m \) for benzo(a)pyrene than the control value and a slightly higher \( V_{\text{max}} \). In this case the affinity for benzo(a)pyrene is reduced possibly due to induction of a different form of the enzyme to that induced by benzo(a)pyrene.

**DISCUSSION**

Benzo(a)pyrene is well known as an inducer of benzo(a)pyrene hydroxylase in mammals, where similar effects to those reported here on the kinetics of this enzyme have been shown (14,15). This induction has been shown to be due to the selective induction of a form (or forms) of cytochrome P-450 with a high activity towards benzo(a)pyrene hydroxylation, cytochrome P-448. This form of cytochrome P-450, which is also induced by 3-methylcholanthrene, is known to have a relatively narrow substrate specificity and to activate carcinogens such as benzo(a)pyrene, to metabolically active products. The results presented in this report suggest the induction by benzo(a)pyrene of a similar form of cytochrome P-450 with a high activity towards benzo(a)pyrene in the yeast *Saccharomyces cerevisiae*.

The inability of benzo(a)pyrene to induce cytochrome P-448 in yeast grown in a low glucose medium shows that a high glucose concentration, or conditions leading to a similar physiological state such as anaerobic conditions, is a prerequisite for cytochrome P-448 production. This would be as expected if the production of cytochrome P-448/P-450 was being controlled by the presence of cyclic AMP by repression as has been suggested (6). It would seem therefore that a
high glucose concentration lowers the level of cyclic AMP which allows cytochrome P-448 to be produced. The induction observed with benzo(a)pyrene is due to an effect on the amount of a particular form of cytochrome P-448 produced.

The addition of dimethylnitrosamine to yeast also showed considerable induction of benzo(a)pyrene hydroxylase in a similar manner to benzo(a)pyrene, suggesting that this compound was inducing the same form of the enzyme as benzo(a)pyrene. β-Naphthoflavone has been reported to induce a form of cytochrome P-450 in mammalian systems with a very high turnover number for benzo(a)pyrene which has an absorption maximum in the carbon monoxide-reduced difference spectrum at 446 nm and was hence termed cytochrome P-448 (16). However, we have been unable to demonstrate any induction of benzo(a)pyrene hydroxylase with this compound in Saccharomyces cerevisiae.

Pretreatment of yeast with 3-methylcholanthrene, a classical inducer of the cytochrome P-448 system in mammalian liver, was expected to induce benzo(a)pyrene hydroxylase also. A large decrease in $K_m$ was observed suggesting a higher affinity form of the enzyme yet no change in maximal velocity occurred. Phenobarbital, a classical inducer of the wide specificity cytochrome P-450 form in mammals did induce benzo(a)pyrene hydroxylase to some extent.

Lanosterol is thought to be an endogenous substrate for cytochrome P-448/P-450 in Saccharomyces cerevisiae (17). The addition of this compound to yeast media during growth resulted in a decreased affinity for benzo(a)pyrene, showing that the induction of another form of cytochrome P-450 could be occurring.

The presence of multiple forms of cytochrome P-448/P-450 in Saccharomyces cerevisiae is previously unreported although we have some evidence from purification studies of at least two forms present in uninduced yeast (18). The results of this study suggest that multiple forms of cytochrome P-448/P-450 exist in Saccharomyces cerevisiae and extends the list of properties that the enzyme from this eukaryotic source shares with the mammalian system.
Acknowledgement

This work was supported in part by an S.E.R.C. C.A.S.E. studentship award to D.J.K. with May & Baker Ltd., Dagenham, Essex under the supervision of Dr. C.J. Coulson and Dr. D.I. Dron.

REFERENCES

Evaluation of immobilized cytochrome P-448 from *Saccharomyces cerevisiae* using permeabilized whole cell, microsomal fraction and highly purified reconstituted forms, with benzopyrene-3-monoxygenase activity

Mahmood R. Azari and Alan Wiseman

*Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, UK*

(Received 17 March 1982; revised 3 June 1982)

Cytochrome P-448 from *Saccharomyces cerevisiae* in permeabilized whole cell, microsomal fraction and in a highly purified reconstituted benzopyrene-3-monoxygenase (EC 1.14.14.1) system have been immobilized on various supports. Calcium alginate was found to be especially useful and the kinetics of hydroxylation were close to that of the free enzyme system with all three forms of enzyme, even with permeabilized whole yeast cells (V_max of 664 pmol 3-hydroxybenzo(a)pyrene produced per h per nmol cytochrome P-448 compared with 1000 for free highly purified reconstituted enzyme system). Only the highly purified reconstituted form was successfully immobilized by BrCN-activated Sepharose-4B or by acrylamide. Both of these supports stabilized the highly purified reconstituted cytochrome P-448 benzopyrene-3-monoxygenase activity in prolonged storage at 4°C. Applications for various immobilized enzymes and cells are assessed.

Keywords: Chemical kinetics; cytochrome P-448; *Saccharomyces cerevisiae*; benzopyrene-3-monoxygenase; immobilized enzyme; immobilized cells

**Introduction**

The importance of cytochrome P-450/P-448 in the metabolism of drugs and xenobiotics is well known. We have already reported the occurrence of cytochrome P-448 in *Saccharomyces cerevisiae* along with a procedure for its solubilization and purification, and we have reported the metabolites and kinetics of its benzopyrene-3-monoxygenase (EC 1.14.14.1). We have attempted the immobilization of this enzyme by various workers. Schuber et al. have immobilized hepatic microsomal cytochrome P-450 by gelatin to construct an enzyme electrode for measurement of oxygen uptake by this enzyme system. Bruner et al. have immobilized purified reconstituted hydroxylase enzyme system to BrCN-activated Sepharose-4B and also by copolymerization in acrylamide for the purpose of increasing the stability. The simultaneous immobilization of cytochrome P-448 monooxygenase system and glucuronyl transferase to achieve the hydroxylation and subsequent glucuronidation of lipophilic compounds has been reported by Lehman et al. Several other workers have also immobilized this enzyme in microsomal form on to solid supports.

We report here some procedures for the immobilization of our yeast enzyme in permeabilized whole cell, microsomal fraction and in purified reconstituted monooxygenase system. The feasibility of these methods is evaluated by comparison of the kinetics of benzopyrene-3-monoxygenase activity and the stability in storage of these immobilized forms which are derived from preparations at different levels of purification effort on this cytochrome P-448 from *Saccharomyces cerevisiae*.

**Materials and methods**

Sodium cacodylate and BrCN were purchased from Aldrich Chemical Co, Dilauroyl phosphatidylcholine, dimethylamino-propionitrile, dithiothreitol, zagarose-concanavalin A, microcrystalline cellulose and sodium cholate were obtained from Sigma Chemical Co, Sodium azipate and glutaraldehyde were obtained from BDH Chemicals Ltd. Acrylamide, N,N-methylene-bisacrylamide and ammonium persulphate...
Papers were obtained from BioRad Lab. Sepharose-4B was from Pharmacia Fine Chemicals.

**Permeabilization of yeast cells**

Breuer’s yeast Saccharomyces cerevisiae (NCYC No. 240) was grown at 30°C for 44 h under glucose repression in a medium containing 20% glucose, 2% mycological peptone, 1% yeast extract and 0.5% NaCl, using the method of Wiseman and Lim. Yeast cells were harvested by centrifugation at 2500 g for 5 min and then cooled in ice. The cells were collected containing 20% glycerol, 0.001 M dithiothreitol. After removal of the cell debris and nuclear microsomal fraction was obtained by centrifugation at 10000 g for 10 min, resuspended and washed in 0.02 M sodium cacodylate buffer, pH 7.1, containing 0.4 M sorbitol. The toluenized cells were finally suspended in the above buffer to a concentration corresponding to 200 mg of original yeast/ml.

**Preparation of microsomes: solubilization and purification of cytochrome P-448**

Yeast cells were disrupted by a Vibro Mill, and then suspended in 0.1 M potassium phosphate buffer, pH 7.2, containing 20% glycerol, 0.001 M EDTA and 0.001 M dithiothreitol. After removal of the cell debris and nuclear material by centrifugation at 7500 g for 15 min, yeast microsomal fraction was obtained by centrifugation at 160900 g for 1 h. Cytochrome P-448 was solubilized by adding sodium cholate to microsomal fraction (30 mg protein/ml) up to 1% in concentration as previously described. Cytochrome P-448 and cytochrome P-450(c) reductase were purified to homogeneity using the method reported by Wiseman and Azari. 

**Immobilization of cytochrome P-448 on calcium alginate**

Permeabilized yeast cells, microsomal fraction and purified reconstituted hydroxylase system were entrapped by calcium alginate using a method modified from that reported previously. In Sepharose-4B (decanted) was mixed with an equal volume of water and cyanogen bromide (100 mg per ml of settled Sepharose-4B) was added in an equal volume of water. The pH was immediately adjusted to, and maintained at, 11 by titration with 4 N NaOH. When the reaction had ended, the Sepharose-4B beads were washed with ~20 volumes of cold 0.1 M NaHCO3 on a Buchner funnel under suction. The beads then were suspended in cold 0.1 M potassium phosphate buffer, pH 7.5, containing 0.001 M dithiothreitol and 20% glycerol (these are stabilizers, we have noted) in a volume equal to that of the original Sepharose-4B, and the enzyme preparation (1 nmol/ml) was added with a ratio of 6 nmol cytochrome P-448 per g beads. The mixture was stirred overnight and beads were then washed thoroughly and suspended again overnight in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 M glycine, 0.001 M dithiothreitol and 20% glycerol. This buffer was used to deactivate all the remaining binding groups on the beads. The beads were finally stored until use in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol.

**Immobilization of cytochrome P-448 on microcrystalline cellulose — crosslinking by use of glutaraldehyde**

To 10 ml of purified reconstituted hydroxylase system (1 nmol/ml cytochrome P-448) 1.5 g of cellulose (microcrystalline) was added and mixed on a roller-mixer for 1 h. Crosslinking was accomplished by adding 0.3 or 0.25 M glutaraldehyde solution and mixing for a further hour. Enzyme system bound to cellulose was then collected by centrifugation at 12000 g for 10 min and was stored in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol.

**Immobilization of cytochrome P-448 by acetone—concanavalin A — crosslinking by use of glutaraldehyde**

1.3 ml of purified reconstituted monooxygenase system (1 nmol/ml cytochrome P-448) in 0.02 M potassium...
phosphate buffer, pH 7.4, containing 0.15 M NaCl was stirred with 1 g of packed agarose–concanavalin A at 4°C for 30 min. The gel was washed with 300 ml 1 M KCl. Crosslinking of the agarose–concanavalin A–cytochrome P-448 enzyme system conjugate with glutaraldehyde was accomplished by adding 0.01 ml 0.1 M potassium phosphate buffer, pH 8, and glutaraldehyde to give the final concentration of 0.5%, and stirring for 30 min.

**Measurement of benzopyrene-3-monoxygenase activity of immobilized cytochrome P-448**

This measurement was carried out by a method modified from that of Woods and Wiseman. A known amount of immobilized enzyme (usually 1.5 nmol of cytochrome P-448) was placed in a magnetically-stirred flask at 37°C, and therefore approximates to a typical batch stirred tank reactor. Tris–HCl buffer, 0.1 M, pH 7.0 containing NADPH-regenerating system was added to give a final concentration of 0.001 M NADP, 0.02 M glucose-6-phosphate, 0.001 M MgCl₂ and 8 IU of glucose-6-phosphate dehydrogenase. The reaction was started by addition of benz[a]pyrene from a stock solution of 2 mg ml⁻¹ in dimethylformamide, to give a final concentration in the range 0–160 µM benz[a]pyrene. Incubation was at 37°C with stirring for 15 min. The reaction was stopped by separating the immobilized enzyme by centrifugation or filtration. The supernatant (or filtrate) was diluted 1:1 with addition of ice-cold acetone. A 0.6 ml aliquot of this acetone solution was added to 1.4 ml 10.7% (w/v) triethylamine solution in a fluorimeter cuvette and scanned on the range 500–560 nm (emission) at 467 nm (excitation) in a Perkin-Elmer MPF3 fluorescence spectrophotometer to find the peak at 523 nm. Fluorescence was calculated relative to 10 µg quinine sulphate/ml in 2 M H₂SO₄, which in turn was calibrated against a standard 3-hydroxybenz[a]pyrene solution.

**Results and discussion**

The kinetics ($K_m, V_{max}$) of benzopyrene-3-monoxygenase activity for all three forms of cytochrome P-448 immobilized by various supports were obtained by Lineweaver–Burk plot (Figure 1) and are shown in Table I. This stability of immobilized cytochrome P-448 in storage at 4°C is shown in Figure 2.

![Figure 1: Lineweaver-Burk plot of the benzopyrene-3-monoxygenase activity of immobilized cytochrome P-448 on various supports.](image)

![Figure 2: Storage stability of immobilized cytochrome P-448.](image)

The permeabilization of the yeast cells resulted in a retention of 40% of the cytochrome P-448 peak, measured by CO-difference spectrum of reduced form, and 100% of this was retained upon immobilization. When these cells were immobilized in calcium alginate they showed almost normal benzopyrene-3-monoxygenase activity compared with the highly purified enzyme, with a $V_{max}$ of 664 pmol 3-hydroxybenz[a]pyrene per h per nmol of cytochrome P-448. Immobilized whole yeast cells or protoplast (prepared according to the method of Wiseman and Woods) did not show any benzopyrene-3-monoxygenase activity. Immobilization of permeabilized yeast cells in gelatin was not feasible as the gel depolymerized in a batch stirred tank reactor during incubation at 37°C. Permeabilized yeast cells

**Table I: Kinetics of benzopyrene-3-monoxygenase activity in immobilized cytochrome P-448.** $V_{max}$ values are expressed in pmol of 3-hydroxybenz[a]pyrene per h per nmol of cytochrome P-448 (initial rate measured after 15 min of assay in batch stirred tank reactor).

<table>
<thead>
<tr>
<th>Form of enzyme</th>
<th>Apparent $V_{max}$ (pmol h⁻¹ µmol⁻¹)</th>
<th>Apparent $K_m$ (µM)</th>
<th>Activity remaining after 4 weeks at 4°C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free enzyme</td>
<td>668</td>
<td>111</td>
<td>0</td>
</tr>
<tr>
<td>Immobilized on calcium alginate</td>
<td>550</td>
<td>200</td>
<td>60</td>
</tr>
<tr>
<td>Purified reconstituted monooxygenase system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I: Free enzyme</td>
<td>1000</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>II: Immobilized on acrylamide</td>
<td>740</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>III: Immobilized on Sepharose-4B</td>
<td>664</td>
<td>66</td>
<td>58</td>
</tr>
<tr>
<td>IV: Immobilized on acrylamide</td>
<td>540</td>
<td>33</td>
<td>40</td>
</tr>
</tbody>
</table>

![Image 4](image)
lost their benzopyrene-3-monoxygenase activity when copolymerized with acrylamide and they did not absorb onto cellulose or BrCN-activated Sepharose-4B. Permeabilized yeast cells immobilized by calcium alginate lost most of their benzopyrene-3-monoxygenase activity after 4 weeks storage at 4°C.

Cytochrome P-448 in micromolar fraction is a membrane-bound immobilized enzyme and is not easily immobilized to other supports. However, its entrapment by calcium alginate resulted in retention of all the cytochrome P-448 and much of the benzopyrene-3-monoxygenase activity ($V_{max}$ 500 pmol h$^{-1}$ mmol$^{-1}$) and it lost only 40% of this activity after 4 weeks of storage at 4°C. The results (Table 1) indicate, however, a considerable diffusion limitation in this system, with lower $K_m$ and higher $V_{max}$ values.

Purified reconstituted monooxygenase system was successfully immobilized by a number of supports. Entrapment by cellulose (with 90% retention yield) gave a high $V_{max}$ of 740 pmol h$^{-1}$ mmol$^{-1}$ compared with the free purified reconstituted monooxygenase system, which had $V_{max}$ of 1000 pmol h$^{-1}$ mmol$^{-1}$. The apparent $K_m$ (50 μM) was also close to that of the free enzyme system (33 μM).

Immobilization of the highly purified form on cellulose by absorption and crosslinking by glutaraldehyde gave the same results as previously obtained with the crude soluble enzyme. Eighty per cent of the cytochrome P-448 did not bind to this support (or was denatured in the process of immobilization). The enzyme which bound to the cellulose lost some of its activity. Immobilization of the solubilized crude form of this enzyme on cellulose using the same procedure had resulted in a doubling of $K_m$ and a 25% decrease in $V_{max}$. Attempts were made to immobilize the purified reconstituted monooxygenase system through its carbohydrate side-chain to agarose-concanavalin A. Cytochrome P-450 type enzymes are reported to be glycoproteins, containing 1–2 molecules of sugars such as mannose, glucose or glucosamine per molecule of the cytochrome. We find that only 50% of cytochrome P-448 was bound to agarose-concanavalin A and most of the benzopyrene-3-monoxygenase activity was lost. Glutaraldehyde seems to have a deleterious effect on cytochrome P-448, and it may impose a high degree of steric hindrance onto the active site of this enzyme in purified form.

Highly purified reconstituted monooxygenase system along with NADPH has been immobilized by BrCN-activated Sepharose-4B beads. The intended application was for use as an extracorporeal shunt for blood during detoxication. This support proved to be the most effective for our highly purified enzyme. Almost all the cytochrome P-448 used was retained on these beads, although NADPH did not bind, and it was necessary to add this each time to the reaction mixture. $V_{max}$ was relatively high (664 pmol h$^{-1}$ mmol$^{-1}$) and availability of the enzyme system for binding benzo[a]pyrene was demonstrated by the low $K_{p}$ value obtained (50 μM). Immobilized highly purified reconstituted cytochrome P-448 on this support also showed a considerable degree of stabilization in storage at 4°C, and after 4 weeks only 42% of the enzyme activity was lost, instead of 93% for this enzyme preparation prior to immobilization.

The copolymerization of purified reconstituted monooxygenase system with acrylamide resulted in the production of an entrapped enzyme system (cytochrome P-448 retention, 60%) with a $K_{p}$ for benzo[a]pyrene the same as that of the free enzyme (123 μM), but with a 1.8-fold decrease in $V_{max}$. The low $V_{max}$ (with unchanged $K_{p}$) might be due to the diffusional limitation imposed by this support on the 3-hydroxybenzo[a]pyrene availability in the assay mixture in presence of the swollen gel that forms during the assay.

The immobilization of cytochrome P-448 highly purified reconstituted enzyme system using an effective support in stirred tank and other enzyme reactors will improve the proposed applications of this enzyme, including the construction of an accurate enzyme electrode. This could provide a rapid and specific assay for a substrate of this enzyme and substrates of related enzymes present in foods and the environment.

Im mobilized permeabilized yeast may be useful also in large-scale applications such as the removal of polynuclear aromatic nuclear hydrocarbons from water and air. For some purposes, the use of the immobilized microsomal system may prove convenient and appropriate, with high stability (but with diffusional limitation problems). We are developing methods for the large-scale rapid isolation of micromolecular fraction without recourse to high centrifugation.

Further studies using other types of enzyme reactor are in progress, and the packed-bed type may prove to be more useful in the use of these enzymes for production of quantities of metabolites or for carcinogen removal. The replacement of expensive cofactors by co-immobilized enzymes will be essential for large-scale applications, and some success has been achieved on this problem.

References

6 Lehman, J. P., Perret, L., Fajnice, C. and Yost, G. S. Drug Metab. Dispos. 1981, 9 (11), 15
9 Lu, A. Y. and West, S. B., Pharmacol. Ther. 1978, A2, 337
15 Mohara, K. and Sato, R., Biochem. J. (Tokyo) 1972, 71, 727
21 Haeven, D. and Coon, M. J., J. Biol. Chem. 1976, 251, 7919
CHARACTERIZATION OF CYTOCHROME P-448 FROM SACCHAROMYCES CEREVISIAE.

DAVID J. KING, MAHMOOD R. AZARI AND ALAN WISEMAN
Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey, GU2 5XH, U.K.

INTRODUCTION

The eucaryotic microorganism *Saccharomyces cerevisiae* contains a microsomal bound cytochrome P-448 which is part of a microsomal electron transport chain with many properties in common with that of mammalian liver (1, 2). This yeast cytochrome P-448 is produced under fermentative conditions (e.g. growth at high glucose concentration) which lead to the repression of mitochondrial cytochrome a + a formation (3, 4). Control of this process is thought to occur through cyclic AMP which exerts a repressive effect over *de novo* synthesis of cytochrome P-448, the level of cyclic AMP being determined by the glucose concentration in the growth medium in an inverse relationship (5).

This cytochrome P-448 system is capable of hydroxylation of benzo(a)pyrene to a range of metabolites, principally 3-hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (6). We have recently shown that this enzyme system can be induced with benzo(a)pyrene and several other compounds to produce only slightly higher levels of cytochrome P-448 but a great improvement in the efficiency of the enzyme at metabolizing benzo(a)pyrene, suggesting that more than one form of cytochrome P-450 can occur in this yeast (7). This finding has been backed up by the separation of two forms during purification studies (8). We have purified the major form of cytochrome P-448 from uninduced yeast to a high degree of purity (88-97% pure) and in this study we have undertaken to characterize this enzyme to allow a comparison with mammalian systems.

MATERIALS AND METHODS

*Saccharomyces cerevisiae* NCYC No. 240 was grown under glucose repression in a medium containing 1% yeast extract, 2% mycological peptone, 0.5% NaCl and 20% glucose for 44 hours at 30°C. Yeast was harvested and microsomes prepared from which cytochrome P-448 was purified as previously described (8). SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (9). Benzo(a)pyrene hydroxylase activity was determined by the fluorimetric method of Dehnen et al. (10) as modified by Woods and Wiseman (6). A reconstituted
enzyme system comprising of purified cytochrome P-448, purified NADPH:cytochrome P-450 reductase and dilauroylphosphatidylcholine (in mole cytochrome P-448 to IU of reductase).

RESULTS AND DISCUSSION

Cytochrome P-448 from uninduced Saccharomyces cerevisiae was purified to a specific content of 16–17.5nmole/mg protein. The homogeneity of this enzyme was shown by SDS-polyacrylamide gel electrophoresis, the molecular weight being determined as 55,500 using this method with several marker proteins. This molecular weight is similar to that of cytochrome P-448 from liver microsomes of 3-methylcholanthrene treated rats (11). Using this molecular weight the purity of our enzyme is 88–97%. Our preparation was free of NADPH:cytochrome P-450 reductase, cytochrome b₅ and cytochrome P-420. The soret peak of the reduced CO complex was at 448nm similarly to the form induced by polycyclic aromatic hydrocarbons in mammalian liver. A similarity to this enzyme is also suggested by the benzo(a)pyrene metabolite profile of the yeast enzyme (6).

The amino acid composition of yeast cytochrome P-448 was determined as shown in Table 1. This composition reveals 407 amino acid residues per molecule which leads to a molecular weight of 53,000 (a difference of 4.5% from the value determined by SDS-PAGE). The content of hydrophobic residues is 43% which is almost identical to the content of hydrophobic residues in induced and uninduced cytochromes P-450 from both rat (11) and rabbit liver (12, 13).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>No. Residues/Molecule</th>
<th>Amino Acid</th>
<th>No. Residues/Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>41</td>
<td>Phenylalanine</td>
<td>20</td>
</tr>
<tr>
<td>Threonine</td>
<td>21</td>
<td>Leucine</td>
<td>23</td>
</tr>
<tr>
<td>Serine</td>
<td>24</td>
<td>Isoleucine</td>
<td>13</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>37</td>
<td>Histidine</td>
<td>17</td>
</tr>
<tr>
<td>Proline</td>
<td>23</td>
<td>Methionine</td>
<td>7</td>
</tr>
<tr>
<td>Glycine</td>
<td>42</td>
<td>Lysine</td>
<td>27</td>
</tr>
<tr>
<td>Alanine</td>
<td>27</td>
<td>Arginine</td>
<td>20</td>
</tr>
<tr>
<td>Valine</td>
<td>30</td>
<td>Tryptophan</td>
<td>12</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>15</td>
<td>Cysteine</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total 470</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Benzo(a)pyrene gave rise to a type 1 binding spectrum with our purified cytochrome P-448 with an apparent spectral dissociation constant ($K_s$) of 50 uM. Lanosterol, ethylmorphine, phenobarbital, dimethylnitrosamine and perhydrofluorene also gave rise to type 1 binding spectra. Benzo(a)pyrene hydroxylase activity of a reconstituted system comprising of purified cytochrome P-448, purified NADPH:cytochrome P-450 reductase and dilauroylphosphatidylcholine showed a $K_m$ of 33 uM and a $V_{max}$ of 16.7 pmol 3-hydroxybenzo(a)pyrene/min/nmole P-448 when supported by NADPH. The requirement for cofactor could be replaced by cumene hydroperoxide or hydrogen peroxide generated from a glucose oxidase system, in each case the $K_m$ and $V_{max}$ are both increased (Table 2).

### TABLE 2

**BENZO(a)PYRENE HYDROXYLASE ACTIVITY OF CYTOCHROME P-448 IN A RECONSTITUTED SYSTEM**

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>$K_m$ (uM)</th>
<th>$V_{max}$ (pmol 3-hydroxybenzo(a)pyrene/min/nmole P-448)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH supported</td>
<td>33</td>
<td>16.7</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>125</td>
<td>21.9</td>
</tr>
<tr>
<td>Hydrogen peroxide (glucose oxidase)</td>
<td>200</td>
<td>33.7</td>
</tr>
</tbody>
</table>

A good agreement between the $K_m$ of the NADPH supported reaction (33 uM) and the benzo(a)pyrene $K_s$ (50 uM) was observed. The rate of benzo(a)pyrene hydroxylation observed with our enzyme is low compared to 3-methylcholanthrene induced (P-448) activities in rat liver, although it is comparable to forms isolated from uninduced mammalian sources (14, 15).

Yeast cytochrome P-448 is closely related to the family of enzymes from mammalian liver. The form of the enzyme which we have purified and studied in this report appears to resemble a cytochrome P-448 type of the mammalian enzyme rather than a cytochrome P-450 form, in its soret peak in the reduced CO spectrum, narrow substrate specificity and the range of benzo(a)pyrene metabolites formed. The molecular weight a amino acid composition are also closer to a P-448 form, although the activity towards benzo(a)pyrene hydroxylation is much lower than a mammalian cytochrome P-448.
ACKNOWLEDGEMENT

This work was supported in part by an S.E.R.C. CASE award to D.J.K. with May & Baker Ltd., Dagenham under the supervision of Dr. D.I. Dron and Dr. C.J. Coulson.

REFERENCES

Inhibition of highly purified benzo[α]pyrene hydroxylase from Saccharomyces cerevisiae by cytochrome P-448 binding compounds and by flavonoids

DAVID J. KING, MAHMOND R. AZARI and ALAN WISEMAN
Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

Cytochrome P-448 dependent benz[a]pyrene hydroxylase has been purified to homogeneity and characterized by Azari & Wiseman (1982). The kinetics of NADPH-supported benz[a]pyrene hydroxylation by this enzyme were reported for several putative substrates with this highly purified enzyme (Azari et al., 1982) and in this communication we report that flavone stimulated cytochrome P-448 and binding studies show evidence of high affinity interactions.

The mechanism of inhibition/activation of mammalian benz[a]pyrene hydroxylase by flavonoids has been studied by Huang et al. (1981a,b). We have examined the effect of two flavonoid compounds, flavone and 7,8-benzoflavone, both of which inhibit yeast cytochrome P-448 dependent benz[a]pyrene hydroxylase.

Yeast (Saccharomyces cerevisiae N.C.Y.C. no. 240) was grown under glucose repression and used to prepare microsomes from which cytochrome P-448 was purified to homogeneity (97% pure) as previously described (Azari & Wiseman, 1982). Benz[a]pyrene hydroxylase activity was assayed on a purified reconstituted system by the method of Dehnen et al. (1973) as modified by Woods & Wiseman (1979).

The percentage inhibition of benz[a]pyrene hydroxylation by binding compounds is shown in Table 1, along with the corresponding K values. This table shows that dimethyl nitrosamine and lanosterol result in a high degree of inhibition at 2.5 mM and 77 μM, respectively. Both of these compounds result in type 1 interactions with purified yeast cytochrome P-448 with spectral dissociation constants (K values) of 220 μM and 80 μM, respectively. Lanosterol has been reported to be an endogenous substrate of yeast cytochrome P-448 (P-450) by Ayasuma & Yoshida (1978). Dimethyl nitrosamine is an inducer of cytochrome P-448 and binding studies show evidence of high affinity for this enzyme, but so far we have not shown a reproducible rate of demethylation by using the Nash assay technique.

Our enzyme was not inhibited by 9-hydroxyellipticine. This compound has been found to inhibit mammalian cytochrome P-448 in vitro (Delforge et al., 1989).

The interaction of flavonoid compounds with benz[a]pyrene hydroxylase from mammalian liver has been studied by Huang et al. (1981a,b). These workers found that flavone stimulated benz[a]pyrene hydroxylase activity of cytochrome P-450 LM3c or LM4, yet inhibited the same activity catalyzed by cytochrome P-450 LM2, LM3b and LM6. 7,8-Benzoflavone stimulated benz[a]pyrene hydroxylase activity by cytochrome P-450 LM3c yet inhibited cytochrome P-450 LM6. We have found that both flavone and 7,8-benzoflavone inhibit our enzyme strongly, in a non-competitive fashion. 7,8-Benzoflavone is a more potent inhibitor than flavone. Double reciprocal plots of
Studied on the differences in thermal stability and critical temperature of cytochrome P-448 from Saccharomyces cerevisiae in microsomal, solubilized and highly purified form

MAHMOOD R. AZARI, DAVID J. KING and ALAN WISEMAN
Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

Studies on cytochrome P-448 from yeast microsomal fraction by Wiseman et al. (1975) revealed that this enzyme is less stable than that from rat liver toward thermal denaturation. This study also revealed that this enzyme from yeast or rat liver is considerably more stable toward thermal denaturation in the oxidized form than in the reduced form. We have previously reported the stabilization effect of Triton X-100 on yeast cytochrome P-448 in the oxidized form (Wiseman & Wiseman, 1982). We now report the differences in thermal stability of cytochrome P-448 from Saccharomyces cerevisiae in the form of microsomal, solubilized and purified enzyme as measured by both the content of cytochrome P-448 and the activity of benzaldehyde hydroxylase.

Yeast (Saccharomyces cerevisiae N.C.Y.C. no. 240) was grown and microsomes prepared as previously described (Wiseman et al., 1975). Microsomes were resuspended in 0.1 M-potassium phosphate buffer, pH 7.2, containing 20% (v/v) glycerol, 0.001 M-EDTA, 0.001 M-dithiothreitol and 0.1% glutathione. Cytochrome P-448 was stabilized by the addition of 1% cholate to the microsomes followed by stirring at 4°C under nitrogen for 1 h. This enzyme was purified to homogeneity by using the methods previously described (Azari & Wiseman, 1982). Cytochrome P-448 was assayed by the method of Omura et al. (1965) and benzaldehyde hydroxylase measured by the method of Dehnen et al. (1973) as modified by Woods & Wiseman (1979). Yeast cytochrome P-448 was more stable toward thermal denaturation when in the form of microsomes than when solubilized but not purified. After incubating microsomal enzymes even at 50°C for 5 min only, 50% of the yeast cytochrome P-448 is lost (see Table 1). In an equivalent experiment with rat liver microsomal cytochrome P-448 no detectable loss was observed after 5 min at 50°C. This again shows the liver enzyme to be more stable than the yeast enzyme, as shown by Wiseman et al. (1975). When yeast microsomes are treated with 1% Triton X-100 only 10% of cytochrome P-448 dependent benzaldehyde hydroxylase is lost after 10 min incubation at 40°C, whereas untreated microsomes lose 50% of activity under these conditions.

Solubilized yeast cytochrome P-448 is far less resistant to thermal denaturation than is microsomal fraction, as is expected from the loss of the lipid membrane (see Table 1). The stability of this solubilized enzyme was not significantly affected by the addition of benzaldehyde, which is a well-characterized substrate of this enzyme (Azari & Wiseman, 1982). The addition of 0.1% Triton X-100 had only a slight effect on the temperature at which 50% of the cytochrome P-448 was lost in 5 min incubation (Table 1a) yet was successful in preserving 100% loss of cytochrome P-448 in 5 min under an incubation temperature of 60°C is reached (Table 1b). Solubilized cytochrome P-448 is 100% lost after 5 min incubation at 45°C when no Triton X-100 is present. Triton X-100 at 0.1% was not able to return the stability of the soluble enzyme to that of the enzyme in microsomal fraction. The activity of benzaldehyde hydroxylase also shows a stabilization effect in the presence of Triton X-100 (Table 1c). This stabilization effect is probably

Vol. 10

(423)

Spectral binding studies on the interaction of some putative substrates with highly purified cytochrome P-448 from Saccharomyces cerevisiae

MAHMOOD R. AZARI, DAVID J. KING and ALAN WISEMAN
Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

Cytochromes P-450/P-448 undergo spectral changes due to their interaction with many different compounds (Schekman et al., 1981). Two major types of spectral interactions are observed, termed type I and type II. Type I interactions are thought to be due to the binding of cytochromes P-450/P-448 substrates to the protein moiety of the enzyme, possibly at the active site. This interaction results in difference spectra with an absorption peak at 385-390 nm and a trough at approx. 420 nm. Type II interactions are associated with the binding of a compound to the haem iron of cytochrome P-450/P-448, often via an amino group. The difference spectra resulting from type II interactions have an absorption maximum at 425-435 nm and a minimum at 390-405 nm. We report here the interaction of some compounds which are known to bind to mammalian cytochromes P-450/P-448 with highly purified cytochrome P-448 from Saccharomyces cerevisiae.

Cytochrome P-448 was solubilized from yeast (Saccharomyces cerevisiae N.Y.C. no. 240) microsomes and purified by using methods previously described by Azari & Wiseman (1980) and Azari & Wiseeman (1982a) respectively. The microsomes were obtained from yeast cells grown under conditions of glucose repression as before (Wiseman et al., 1975). The binding characteristics of each compound were determined by recording cytochrome P-448 difference spectra between 310 nm and 500 nm at various concentrations of the added compound. A double cell technique was used in several cases to eliminate interference, as previously described (Azari & Wiseman, 1982a).

Table 1 describes the spectral binding type and characteristics of the interaction of several compounds with highly purified yeast cytochrome P-448. We have previously described the binding of benzoapyrene to this enzyme (Azari & Wiseman, 1982c). The spectral interaction of lanosterol, which
Table 1. Binding properties of various compounds of yeast cytochrome P-448

<table>
<thead>
<tr>
<th>Compound</th>
<th>Difference spectrum maxima (nm)</th>
<th>Difference spectrum minima (nm)</th>
<th>Type of binding</th>
<th>$K_i$ (µM)</th>
<th>$A_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole</td>
<td>429</td>
<td>407</td>
<td>II</td>
<td>8</td>
<td>0.06</td>
</tr>
<tr>
<td>Lanosterol</td>
<td>367,387</td>
<td>416</td>
<td>I</td>
<td>80</td>
<td>0.019</td>
</tr>
<tr>
<td>Ethylmorphine</td>
<td>355,382</td>
<td>412</td>
<td>I</td>
<td>166</td>
<td>0.006</td>
</tr>
<tr>
<td>Aniline</td>
<td>425</td>
<td>408</td>
<td>II</td>
<td>5</td>
<td>0.039</td>
</tr>
<tr>
<td>Phenobarbnilone</td>
<td>367,387</td>
<td>417</td>
<td>I</td>
<td>66</td>
<td>0.021</td>
</tr>
<tr>
<td>Benzphetamine</td>
<td>422</td>
<td>406</td>
<td>I</td>
<td>1660</td>
<td>0.018</td>
</tr>
<tr>
<td>Dimethylnitrosamine</td>
<td>385</td>
<td>420</td>
<td>I</td>
<td>220</td>
<td>0.021</td>
</tr>
<tr>
<td>Perhydrofluorene</td>
<td>382</td>
<td>417</td>
<td>I</td>
<td>50</td>
<td>0.122</td>
</tr>
<tr>
<td>Benz[a]alpyrene</td>
<td>367,387</td>
<td>418</td>
<td>I</td>
<td>50</td>
<td>0.122</td>
</tr>
</tbody>
</table>

is thought to be an endogenous substrate for cytochrome P-448. In Saccharomyces cerevisiae, has previously been described by Aoyama & Yoshida (1978). These authors also found that this compound gave rise to a type I interaction with purified cytochrome P-448/P-448 from baker’s yeast grown semi-anerobically. Although no value for the spectral dissociation constant ($K_i$) was quoted.

Type II binding spectra were observed with benz[a]alpyrene, lanosterol, ethylmorphine, sodium phenobarbnilone, dimethylnitrosamine and perhydrofluorene. Benz[a]alpyrene bound to the greatest extent (almost 100% binding) and we have previously shown that this compound is metabolized by this enzyme, the major products being 3-hydroxybenzolalpyrene, 9-hydroxybenzolalpyrene and 7,8-dihydro-7,8-dihydroxybenzolalpyrene (Woods & Wiseman, 1979). So far we have been unable to detect the metabolism of any of the rest of these compounds using a reconstituted system. Type II spectral change was observed with imidazole, aniline and benzphetamine. Benzphetamine is a substrate of mammalian cytochrome P-450, but no interaction was detected with our purified cytochrome P-448. Type I binding spectra were observed with benzolalpyrene, lanostertol and phenobarbnilone. Benzolalpyrene gives rise to a type I spectrum with this enzyme (Goujon et al., 1972). We have also tested aminopyrine, 7-ethoxyresorufin, β-naphthoflavone, lauric acid, ethoxycoumarin, biphenyl, isoafoxinle and hexobarbnilone for a spectral interaction with our purified cytochrome P-448, but no interaction was detected with these compounds.

We have previously reported the presence of an extra peak at 365-375 nm in the difference spectrum with benzolalpyrene (Woods & Wiseman, 1980) and a similar ‘extra peak’ has been reported for the interaction of this compound with rat liver cytochrome P-450 (Estabrook et al., 1978). In these experiments a similar double peak was observed with ethylmorphine, lanosterol and phenobarbnilone.

Imidazole and aniline, both of which gave rise to type II spectral changes, had the highest affinity for yeast cytochrome P-448 with spectral dissociation constants ($K_i$) values of 8 µM and 5 µM respectively. The extent of binding of these compounds, as measured by the $A_{max}$ values, was lower than that of benzolalpyrene but higher than those of other type I binding compounds. The two known substrates of yeast cytochrome P-450/P-448, benzolalpyrene and lanosterol, show high affinity for the enzyme, although the extent of binding of lanosterol is not very high.

This work was supported in part by a S.E.R.C. CASE award to D. J. K. with May & Baker Ltd., Dagenham, under the supervision of Dr. C. J. Coulson and Dr. D. I. Dron.


ATPases associated with Golgi membranes from lactating rat mammary gland: high-affinity requirement for Ca++ and Mg2+ ions

RICHARD A. EASOM, DAVID W. WEST and ROGER A. CLEGG

Department of Biochemistry, Hannah Research Institute, Aye K46 SHL, Scotland, U.K.

In recent years high-affinity Ca++-stimulated Mg2+-dependent ATPases have been demonstrated in plasma membranes from number of tissues (see Lotersztajn et al., 1981, for references). Each is dependent on submicromolar concentrations of Ca++ and on low Mg2+ concentrations. We have reported (Easom & West, 1982) on the ATPase activities associated with a Golgi-membrane fraction from lactating rat mammary gland. We did not, however, distinguish between a Ca++-stimulated Mg2+-dependent ATPase and a simple Ca++-ATPase (Ca++-