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UTILITY OF MONOCLONAL ANTIBODIES
IN THE CHARACTERISATION OF
ISOZYMES OF CYTOCHROME P-450

by

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

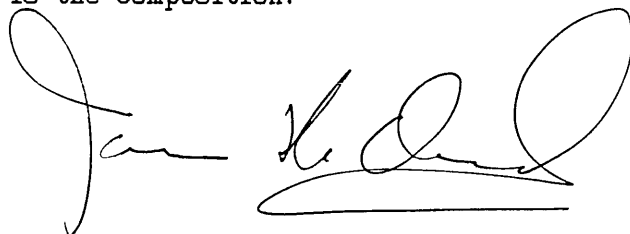
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DECLARATION

The work reported in this thesis was carried out under the supervision of Professor D.S. Davies of the Department of Clinical Pharmacology, Royal Postgraduate Medical School, London. The majority of the work presented was performed in the laboratories of that department. The project was funded by an ICI Joint Research Scheme Award, and some of the experimental work was performed in the laboratories of the ICI Corporate Bioscience Group, Runcorn, and the ICI Central Toxicology Laboratory, Alderley Edge, under the supervision of Drs. R.T. Robson and C.R. Elcombe respectively.

Unless stated in the acknowledgements section, all the results presented are the sole work of the author, as is the composition.

A handwritten signature in black ink, appearing to read 'James McQuade', with a large, stylized loop at the end.

James McQuade

August 1989

ABSTRACT

Cytochrome P-450 exists as families of isozymes within a tissue or cell. Each isozyme has a unique, but possibly overlapping, substrate and product specificity, and individual isozyme levels can change dramatically in response to environmental influences. These properties make the cytochrome P-450 isozymes extremely suitable for study by monoclonal antibody (MAB) directed methods.

Over twenty MAB's were prepared during the course of this project to isozymes of rat liver cytochrome P-450. Several were selected for further study on the basis of their cytochrome P-450 reactivity profiles generated by the ELISA, Western blotting, epitope binding and other screening programmes employed for their characterisation. One MAB, 3/4/2, raised against form c, was also found to react with a wide range of cytochrome P-450 isozymes, including forms from rat, rabbit and human. In addition, reactivity with a possibly unique form of cytochrome P-450 from PB treated rats was observed. Mab 3/4/2 was also found to cause a concentration dependant perturbation of the absolute spectrum of rat liver cytochrome P-450c. This MAB, together with MAB 12/2/3/2, reactive with rat forms c and d, and MAB 1/4, reactive with rat forms b and/or e, were selected for use in a variety of studies, in order to investigate the quantity, activity and localisation of, predominantly rat, cytochrome P-450 isozymes, as well as to purify cytochrome P-450 by means of a MAB affinity matrix.

MAB's were used to affect the activity of several model monooxygenase systems. Only one MAB, 12/2/3/2, was found to have any significant inhibitory effect. This inhibition was concentration dependant and was apparent in both microsomal and reconstituted assay systems, though it was more pronounced in the latter system. The effect of this MAB was also specific for activities catalysed by those forms of cytochrome P-450 recognised by it, namely forms c and d.

MAB 1/4 was successful in immunopurifying cytochrome P-450 forms b and/or e from solubilised liver microsomes from PB treated rats, as determined by SDS-PAGE analysis of the eluted protein. Several experimental systems were employed to immunopurify cytochrome P-450 during the course of this project. Initial studies concentrated on the use of MAB 3/4/2, due to its reactivity with cytochromes P-450 from a number of different species. Initial results with ¹²⁵I-labelled cytochrome P-450 were encouraging, but no eluted proteins could be detected on SDS-PAGE when unlabelled material was used.

A quantitative competitive ELISA assay was developed to investigate microsomal levels of MAB reactive cytochrome P-450 isozymes. Initial studies involved the use of ¹²⁵I- or ¹⁴C-labelled MAB's, but these proved to be ineffective in the proposed assay system. Using unlabelled MAB's, however, the specific content of MAB 12/2/3/2 reactive isozymes (forms c and d equivalent) were determined for a variety of liver microsomal preparations, including human.

The localisation of cytochrome P-450 forms c and d in liver and kidney sections from control, β NF and PB treated rats was determined using MAB's 3/4/2 and 12/2/3/2. A similar reactivity pattern was observed for both MAB's, indicative of the co-localisation of these two isozymes under the induction conditions used. In the liver, a preferential increase in staining was seen in the periportal region following treatment with β NF. In contrast, treatment with PB caused an increase in centrilobular staining, with an increase in levels over those seen in control liver. No such induction with PB was seen in the kidney, however, but treatment with β NF again caused an increase in Mab reactivity.

The results obtained from these studies highlight the utility of MAB's in the characterisation of isozymes of cytochrome P-450.

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Several colleagues provided reagents or advice used in this project: Ms. S. Baker supplied both purified and partially purified preparations of rat liver cytochrome P-450 isozymes, as well as purified NADPH cytochrome c reductase; Dr. A.R. Boobis made available a wide range of microsomal preparations; Dr. S. Plummer provided liver microsomes from SD and Fischer rats; Dr. E.F. Johnson kindly provided purified preparations of rabbit cytochromes P-450 LM2, LM3b, LM3c, LM4 and LM6; Mr. C. Hayward supplied the P3.NS1.1-Ag4-1 (NS1) cell line; Dr. C.R. Elcombe kindly supplied both resorufin and 7-ethoxyresorufin; Dr. A. Rees provided the control MAb used in the immunopurification studies, TB71; Mr. T.B. Seddon provided help and advice in determining the optimum conditions for the aldrin epoxidase assay, as did Drs. A.R. Boobis and C.R. Elcombe for the benzo[a]pyrene hydroxylase and 7-ethoxyresorufin-O-deethylase assays, respectively; Dr. J.R. Foster provided advice on the immunocytochemical detection of cytochrome P-450. Numerous other colleagues, both in the Department of Clinical Pharmacology, and in ICI laboratories, provided advice and encouragement throughout the project.

I would also like to acknowledge the contribution of several colleagues to the experimental work reported in this thesis. Dr. D. Sesardic provided excellent assistance with the Western blotting studies, and supplied advice and help to determine the experimental conditions that enabled the immunopurification of cytochrome P-450 to be achieved. Whilst ascites stocks for MAb's 3/4/2, 12/2/3/2, 1/4 and B3A3 were produced by me in the Department of Clinical Pharmacology, the hybridoma cell lines for these and all other anti-cytochrome P-450 MAb's were produced in the laboratories of the ICI Corporate Bioscience Group, under the direction of Mr. C. Hayward. Ascites for other MAb's discussed in Chapter 3 of this thesis were also provided by Mr. C. Hayward. Without his contribution, and that of his staff, no MAb reagents would have been available to perform the experimental work described in this thesis.

PUBLISHED WORK PRESENTED IN THIS THESIS

Portions of the work presented in this thesis have previously been reported in the following publications :

Boobis, A.R., McQuade, J., Sesardic, D., Robson, R.T., Hayward, C., Lock, E.A., Elcombe, C.R., Rose, M.S. and Davies, D.S.

'A monoclonal antibody raised to rat liver cytochrome P-448 (form c) which recognises an epitope common to many other forms of cytochrome P-450.'

Biochemical Pharmacology, 34, pp 1671-1681 (1985).

Boobis, A.R., Davies, D.S., McQuade, J. and Sesardic, D.

'Specificity of isozymes of cytochrome P-450 determined by inhibitory monoclonal antibodies'

British Journal of Pharmacology, 86, pp 511P (1985).

Sesardic, D., Boobis, A.R., McQuade, J., Baker, S., Lock, E.A., Elcombe, C.R., Robson, R.T., Hayward, C. and Davies, D.S.

'Inter-relatedness of some isozymes of cytochrome P-450 from rat, rabbit and human, determined with monoclonal antibodies.'

Biochemical Journal, 236, pp 569-577 (1986).

Foster, J.R., Elcombe, C.R., Boobis, A.R., Davies, D.S., Sesardic, D., McQuade, J., Robson, R.T., Hayward, C. and Lock, E.A.

'Immunocytochemical localisation of cytochrome P-450 in hepatic and extra-hepatic tissues of the rat with a monoclonal antibody against cytochrome P-450c.'

Biochemical Pharmacology, 35, pp 4543-4554 (1986).

CONTENTS

	<u>PAGE</u>
Declaration	I
Abstract	II
Acknowledgements	III
Published Work Presented in This Thesis	IV
Contents	V
List of Figures	XII
List of Tables	XV
Abbreviations Used	XVI
1. INTRODUCTION	1
1.1 The Importance of Drug Metabolising Systems	2
1.1.1 Introduction	2
1.1.2 Consequences of Drug Metabolism	3
1.2 Cytochrome P-450 and Mammalian Drug Metabolism	5
1.2.1 Introduction	5
1.2.2 Cytochrome P-450 : A Historical Perspective	6
1.2.3 Cytochrome P-450 Nomenclature	7
1.2.4 Methods of Studying Cytochrome P-450 Isozymes	7
1.2.5 The Mechanism of Cytochrome P-450 Action	10
1.2.6 Homology Studies of Cytochrome P-450 Isozymes	12
1.3 Monoclonal Antibody Technology	13
1.3.1 The Antibody Molecule	13
1.3.2 The Antibody Response	14
1.3.3 Production of Monoclonal Antibodies	15
1.3.4 Selection and Propagation of Required Hybridoma Cells	18
1.3.5 The Respective Merits of Polyclonal Antisera and Monoclonal Antibodies	20
1.3.6 Applications of Monoclonal Antibodies	22
1.4 The Application of Monoclonal Antibodies as Probes of Cytochromes P-450	23
1.4.1 Introduction	23
1.4.2 Specific Applications	24

	<u>PAGE</u>
2. MATERIALS AND GENERAL METHODS	33
2.1 Materials	34
2.1.1 Immunological and Associated Reagents	34
2.1.2 Electrophoresis and Associated Reagents	34
2.1.3 Tissue Culture Reagents	35
2.1.4 Radiochemicals and Associated Reagents	35
2.1.5 Chromatography Reagents	35
2.1.6 Animals	36
2.1.7 General Reagents	36
2.2 General Methods	37
2.2.1 Pretreatment of Animals and Preparation of Liver Microsomal Fractions	37
2.2.2 Generation of Monoclonal Antibodies to Isozymes of Rat Liver Cytochrome P-450	38
2.2.3 Culture of Hybridoma Cell Lines	40
2.2.4 Production of Ascites Tumours	40
2.2.5 Monoclonal Antibody Production by DEAE Affi-Gel Blue Chromatography	41
2.2.6 Enzyme Linked Immunosorbent Assay	41
2.2.7 Labelling of Proteins with ^{125}I	42
2.2.8 Polyacrylamide Gel Electrophoresis	43
2.2.9 Determination of Protein Concentration	43
2.2.10 Determination of Cytochrome P-450 Concentration	44
3. CHARACTERISATION OF MONOCLONAL ANTIBODIES	48
3.1 Introduction	49
3.2 Methods	50
3.2.1 Monoclonal Antibody Production	50
3.2.2 Solid Phase Immunoassays	50
3.2.2.1 Immunoglobulin Sub-Class Determination	50
3.2.2.2 Monoclonal Antibody Reactivity with Protein A	50
3.2.2.3 Enzyme Linked Immunosorbent Assay	51
3.2.2.4 Monoclonal Antibody Reactivity with other Haemoproteins	51
3.2.3 Immunoprecipitation of ^{125}I -Labelled Microsomal Proteins	51
3.2.4 Autoradiography	52
3.2.5 Western Blotting	52

3. continued	
3.2.6 Competitive Binding Assays	53
3.2.6.1 Double Antibody Binding Assays	53
3.2.6.2 Competitive Inhibition Assays	55
3.3 Results	56
3.3.1 Solid Phase Immunoassays	56
3.3.1.1 Immunoglobulin Sub-Class Determination	56
3.3.1.2 Monoclonal Antibody Reactivity with Protein A	56
3.3.1.3 Monoclonal Antibody Reactivity with Purified Isozymes of Cytochrome P-450	56
3.3.1.4 Monoclonal Antibody Reactivity with Liver Microsomal Fractions	57
3.3.1.5 Monoclonal Antibody Reactivity with other Haemoproteins	58
3.3.2 Immunoprecipitation of ¹²⁵ I-Labelled Microsomal Proteins	59
3.3.3 Western Blotting of Microsomal Proteins	60
3.3.3.1 Reactivity with Liver Microsomes from β NF and PB Treated Rats	60
3.3.3.2 Reactivity with other Liver Microsomal Preparations	61
3.3.4 Competitive Binding Assays	62
3.3.4.1 Double Antibody Binding Assay	63
3.3.4.2 Competitive Inhibition Assay	63
3.3.5 Binding Site Studies with MAb 3/4/2	63
3.3.5.1 Effect of MAb 3/4/2 on the Reduced Carbon Monoxide Difference Spectrum of Cytochrome P-450	64
3.3.5.2 MAb 3/4/2 Binding Spectrum	64
3.4 Discussion	66
3.4.1 Monoclonal Antibody Reactivity with Isozymes of Cytochrome P-450	66
3.4.1.1 ELISA Studies	66
3.4.1.2 Immunoprecipitation and Western Blotting Studies	67
3.4.2 Mab 3/4/2 Reactivity with Isozymes of Cytochrome P-450	70
3.4.2.1 ELISA and Western Blotting Studies	70
3.4.2.2 Mab 3/4/2 Binding Studies	75

3. continued	
3.4.3 Epitope Binding Studies	76
3.4.4 Conclusion	79
4. EFFECT OF MONOCLONAL ANTIBODIES ON CYTOCHROME P-450 MEDIATED MONOOXYGENASE ACTIVITIES	107
4.1 Introduction	108
4.2 Methods	111
4.2.1 Monoclonal Antibody Preparations	111
4.2.2 Benzo[a]Pyrene Hydroxylation Assay	111
4.2.3 Aldrin Epoxidation Assay	113
4.2.3.1 Incubation Conditions	113
4.2.3.2 Preparation of Dieldrin Standards	113
4.2.3.3 Quantitative Analysis of Dieldrin	114
4.2.4 7-Ethoxyresorufin-O-Deethylation Assay	114
4.2.4.1 Determination of Microsomal Activity	114
4.2.4.2 Determination of Reconstituted Activity	115
4.3 Results	116
4.3.1 Benzo[a]Pyrene Hydroxylase Activity	116
4.3.2 Aldrin Epoxidase Activity	116
4.3.3 7-Ethoxyresorufin-O-Deethylase Activity	117
4.4 Discussion	117
4.4.1 Non-inhibitory Monoclonal Antibodies	117
4.4.2 Inhibition of Monooxygenase Activity by MAb 12/2/3/2	121
4.4.2.1 Comparison of Microsomal B[a]P Hydroxylase and EROD Activities	121
4.4.2.2 Comparison of Microsomal and Reconstituted EROD Activities	124
4.4.3 Other Reported Studies with Inhibitory Monoclonal Antibodies	125
4.4.4 Conclusion	129
5. IMMUNOPURIFICATION OF CYTOCHROME P-450 ISOZYMES	139
5.1 Introduction	140
5.2 Methods	142
5.2.1 Monoclonal Antibody Preparations	142
5.2.2 Cytochrome P-450 Preparations	142
5.2.3 Screening Assay for MAb 3/4/2 Binding Conditions	142

5. continued

5.2.4	Screening Assay for MAb 3/4/2 Elution Conditions	143
5.2.5	Assay to Determine the Presence of Haem Containing Material	144
5.2.6	Binding of Antibodies to Protein A	145
5.2.7	Protein A-Sepharose Gel Matrix	145
5.2.7.1	Preparation of Monoclonal Antibody Coupled Protein A-Sepharose Gels	145
5.2.7.2	Estimation of the Amount of Mouse IgG Bound to Protein A-Sepharose Gels	147
5.2.7.3	Immunopurification Procedure with Protein A-Sepharose Gels	147
5.2.8	Polystyrene Bead Matrix	148
5.2.8.1	Preparation of Monoclonal Antibody Coupled Polystyrene Beads	148
5.2.8.2	Immunopurification Procedure with Polystyrene Beads	149
5.2.9	Cyanogen Bromide Activated Sepharose Gel Matrix	150
5.2.9.1	Preparation of Monoclonal Antibody Coupled Sepharose Gels	150
5.2.9.2	Immunopurification Procedure with Sepharose Gels	150
5.3	Results	151
5.3.1	Effect of Detergents on MAb 3/4/2 Binding	151
5.3.2	Effect of Eluting Agents on MAb 3/4/2 Binding	152
5.3.3	Assay for Haem Containing Material	152
5.3.4	Binding of Antibodies to Protein A	152
5.3.5	Estimation of the Amount of Mouse IgG Bound to Protein A-Sepharose Gels	153
5.3.5.1	Preparation of Standard Curves	153
5.3.5.2	Analysis of Antibody Coupled Protein A-Sepharose Gels	153
5.3.6	Analysis of Cytochrome P-450 Incubated Matrices	154
5.3.6.1	Determination of Haem Containing Material	154
5.3.6.2	Determination of Benzo[a]Pyrene Hydroxylase Activity	154
5.3.6.3	Determination of Reduced Carbon Monoxide Difference Spectra	154

5.	continued	
5.3.7	Analysis of Eluted Fractions	154
5.3.7.1	Elution Profile from Protein A-Sepharose Gels	154
5.3.7.2	SDS-PAGE Analysis	155
5.4	Discussion	156
6.	IMMUNOQUANTITATION OF MICROSOMAL CYTOCHROME P-450 ISOZYMES	182
6.1	Introduction	183
6.2	Methods	185
6.2.1	Monoclonal Antibody Iodination	185
6.2.2	Preparation of ¹⁴ C-Labelled MAb 12/2/3/2	185
6.2.3	Solid Phase Immunoassays	185
6.2.3.1	Enzyme Linked Immunosorbent Assay	185
6.2.3.2	Solid Phase Immunoassay with ¹²⁵ I-Labelled MAb 3/4/2	186
6.2.4	Quantitative Immunoassays	186
6.2.4.1	Immunoradiometric Assay	186
6.2.4.2	Enzyme Linked Immunoassay	187
6.2.4.3	Quantitative Enzyme Linked Immunoassay	187
6.3	Results	188
6.3.1	MAb 3/4/2 Binding Curves	188
6.3.2	Immunoradiometric Assay	188
6.3.3	Enzyme Linked Immunoassay	188
6.3.4	Quantitative Enzyme Linked Immunoassay	189
6.4	Discussion	190
7.	IMMUNOCYTOCHEMICAL STUDIES ON CYTOCHROME P-450 ISOZYME DISTRIBUTION	209
7.1	Introduction	210
7.2	Methods	211
7.2.1	Monoclonal Antibody Preparations	211
7.2.2	Tissue Samples and Cryostat Sections	211
7.2.3	Immunocytochemical Procedures	212
7.2.4	Analysis of Results	213
7.3	Results	213
7.3.1	Monoclonal Antibody Reactivity with Liver	213
7.3.2	Monoclonal Antibody Reactivity with Kidney	214
7.4	Discussion	215

	<u>PAGE</u>
8. OVERALL CONCLUSIONS	231
8.1 Introduction	232
8.1.1 The Rationale for using Monoclonal Antibodies in this Thesis	232
8.1.2 Production of Monoclonal Antibodies	234
8.2 Summary of Experimental Results	234
8.2.1 Structural Analysis of Cytochrome P-450 Isozymes	234
8.2.2 Inhibition of Cytochrome P-450 Mediated Monooxygenase Activities	237
8.2.3 Immunopurification of Cytochrome P-450 Isozymes	238
8.2.4 Immunoquantitation of Microsomal Cytochrome P-450 Isozymes	239
8.2.5 Immunocytochemical Localisation of Cytochrome P-450 Isozymes	242
8.3 Comparison of Monoclonal Antibody Results with those Possible with Desorbed Polyclonal Antisera	242
8.4 A Future Strategy for Generating Monoclonal Antibodies Against Isozymes of Cytochrome P-450	245
9. REFERENCES	246

LIST OF FIGURES

Figures referred to in the text of this thesis are listed below, together with their relevant page number. All figures are positioned at the end of the chapters in which they are referred.

<u>Figures and Titles</u>	<u>PAGE</u>
<u>Chapter 1</u>	
1.1 General Scheme of Drug Metabolism	28
1.2 Electron Pathway in the Microsomal Monooxygenase System	29
1.3 A Schematic Diagram of a Typical Immunoglobulin Molecule (Human IgG ₁)	30
<u>Chapter 2</u>	
2.1 Elution Profile of MAb 3/4/2 Purified on DEAE Affi-Gel Blue	46
2.2 SDS-PAGE Analysis of MAb 3/4/2 Fractions Following Chromatography on DEAE Affi-Gel Blue	47
<u>Chapter 3</u>	
3.1 Comparison of Protein A and Rabbit Anti-Mouse IgG Binding to Various Monoclonal Antibodies	82
3.2 Reactivity of Monoclonal Antibodies with Purified and Microsomal Rat Liver Cytochrome P-450c	83
3.3 Reactivity of MAb 3/4/2 with Purified Isozymes of Rabbit Cytochrome P-450 in a Solid Phase ELISA	84
3.4 Reactivity of Monoclonal Antibodies with Liver Microsomal Preparations	85
3.5 Monoclonal Antibody Reactivity with Various Haemoproteins	91
3.6 SDS-PAGE and Autoradiogram of ¹²⁵ I-Labelled Microsomal Proteins Immunoprecipitated by Monoclonal Antibodies	92
3.7 Western Blot Reactivity of Monoclonal Antibodies with Liver Microsomes from β NF Treated Rats	94
3.8 Western Blot Reactivity of Monoclonal Antibodies with Liver Microsomes from PB Treated Rats	95
3.9 Western Blot Reactivity of MAb 3/4/2 with Various Microsomal Preparations	96

Chapter 3 continued

3.10	Western Blot Reactivity of MAb 12/2/3/2 with Various Liver Microsomal Preparations	97
3.11	Western Blot Reactivity of MAb 1/4 with Various Liver Microsomal Preparations	98
3.12	Competitive Inhibition of ¹⁴ C-Labelled MAb 9/20/11 Binding to Liver Microsomes from β NF Treated Rats	100
3.13	Effect of MAb 3/4/2 on the Reduced Carbon Monoxide Difference Spectrum of Purified Rat Liver Cytochrome P-450c	101
3.14	Binding Spectrum Produced by Mab 3/4/2 Following Incubation with Liver Microsomes from β NF Treated Rats	102
3.15	Binding Spectrum Produced by MAb 7G1 Following Incubation with Liver Microsomes from β NF Treated Rats	104
3.16	Binding Spectrum Produced by Aniline Following Incubation with Liver Microsomes from β NF Treated Rats in the Presence of MAb 3/4/2	105
3.17	Binding Spectrum Produced by MAb 3/4/2 Following Incubation with Liver Microsomes from β NF Treated Rats in the Presence of Aniline	106

Chapter 4

4.1	The Primary Route of Hydroxylation of Benzo[a]Pyrene	131
4.2	The Epoxidation of Aldrin	132
4.3	The O-Deethylation of 7-Ethoxyresorufin	133
4.4	Effect of Monoclonal Antibodies on Liver Microsomal Benzo[a]Pyrene Hydroxylase Activity	134
4.5	Effect of Monoclonal Antibodies on Liver Microsomal Aldrin Epoxidase Activity	136
4.6	Effect of MAb 12/2/3/2 on 7-Ethoxyresorufin-O-Deethylase Activities	138

Chapter 5

5.1	Effect of Detergents on MAb 3/4/2 Binding to Hepatic Microsomes	165
5.2	Effect of Eluting Agents on MAb 3/4/2 Binding to Hepatic Microsomes	167
5.3	Absorbance Spectrum of 3,3',5,5' Tetramethylbenzidine Following Cytochrome P-450 Peroxidase Activity	169
5.4	Response Curve for Cytochrome P-450 Peroxidase Activity	170
5.5	Analysis of Antibody Binding to Protein A	171
5.6	Binding Capacity of Polystyrene Microtitre Wells	172
5.7	Response Curve for ¹²⁵ I-SAM F(ab') ₂ Binding to Adsorbed Mouse Immunoglobulin	173
5.8	Determination of Haem Containing Material on Cytochrome P-450 Incubated Polystyrene Beads	175
5.9	Reduced Carbon Monoxide Difference Spectra of Cytochrome P-450 Incubated Sepharose Beads	177
5.10	Elution Profile from Protein A-Sepharose Gels	179
5.11	SDS-PAGE Analysis of Eluted Material from Sepharose Gels	181

Chapter 6

6.1	MAb 3/4/2 Titration Curves	201
6.2	Competitive Immunoassay Titration Curves for ¹⁴ C-Labelled MAb 12/2/3/2	202
6.3	Competitive Immunoassay Titration Curves for MAb 1/4	203
6.4	Competitive Immunoassay Titration Curves for MAb 3/4/2	204
6.5	Competitive Immunoassay Titration Curves for MAb 12/2/3/2	205
6.6	Quantitative Immunoassay Standard Curve for MAb 12/2/3/2	207

Chapter 7

7.1	Cytochrome P-450 Localisation in Rat Liver Sections by MAb 3/4/2	224
7.2	Cytochrome P-450 Localisation in Liver Sections from βNF Treated Rats by MAb 12/2/3/2	226
7.3	Cytochrome P-450 Localisation in Liver Sections from βNF Treated Rats by MAb B3A3	227
7.4	Cytochrome P-450 Localisation in Rat Kidney Sections by MAb 3/4/2	228
7.5	Cytochrome P-450 Localisation in Kidney Sections from βNF Treated Rats by MAb 12/2/3/2	230

LIST OF TABLES

Tables referred to in the text of this thesis are listed below, together with their relevant page number. All tables are positioned at the end of the chapters in which they are referred.

<u>Tables and Titles</u>	<u>PAGE</u>
<u>Chapter 1</u>	
1.1 Monoclonal Antibodies Produced Against Isozymes of Cytochrome P-450	31
<u>Chapter 2</u>	
2.1 Principle Reactivities of Monoclonal Antibodies Described in this Thesis	45
<u>Chapter 3</u>	
3.1 Determination of Monoclonal Antibody Immunoglobulin Sub-Class	81
3.2 Competitive Binding of Monoclonal Antibodies to Liver Microsomes from β NF Treated Rats	99
<u>Chapter 5</u>	
5.1 Analysis of Mouse Immunoglobulin Bound to Protein A-Sepharose Gels	174
5.2 Benzo[a]Pyrene Hydroxylase Activity of Reconstituted Cytochrome P-450 Incubated Polystyrene Beads	176
<u>Chapter 6</u>	
6.1 Quantitative Immunoassay Results for MAb 12/2/3/2	208

ABBREVIATIONS USED

2-AAF	2-N-acetylaminofluorene
AHH	aryl hydrocarbon hydroxylase
AI	Additivity Index
B(a)A	benzo(a)anthracene
B(a)P	benzo(a)pyrene
β NF	β -naphthoflavone
BSA	bovine serum albumin
$^{\circ}$ C	degrees celsius
cm	centimetre
CNBr	cyanogen bromide
cpm	counts per minute
DAB	diaminobenzidine
DMSO	dimethylsulphoxide
(c)DNA	(complimentary)deoxyribonucleic acid
ECOD	7-ethoxycoumarin-O-deethylase
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EROD	7-ethoxyresorufin-O-deethylase
FCS	foetal calf serum
g	relative centrifugal force
GLC	gas liquid chromatography
HAT (media)	tissue culture media containing hypoxanthine, aminopterin and thymidine
HCB	hexachlorinatedbiphenyl
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HPLC	high pressure (performance) liquid chromatography
(m)IgG	(mouse) immunoglobulin G
i.p.	intra peritoneal

Abbreviations continued

IRMA	immunoradiometric assay
ISF	isosafrole
^{125}I -SAM	iodinated sheep anti-mouse IgG
M	molar
mA	milliamperes
MAB(s)	monoclonal antibody(ies)
MAB-Seph	monoclonal antibody coupled to CNBr-activated Sepharose gel
mCi	millicurie
mg	milligram
μg	microgram
ml	millilitre
μl	microlitre
mM	millimolar
mm	millimetre
NADH	reduced nicotinamide adenine dinucleotide
NADP ⁺	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nm	nanometre
nmoles	nanomoles
NRS	normal rat serum
OD 492nm	optical density of a solution at 492nm
OPD	o-phenylenediamine
PAGE	polyacrylamide gel electrophoresis
pA-HRP	protein A conjugated to horseradish peroxidase
pA-Seph	protein A coupled Sepharose gel
PB	phenobarbitone sodium
PBS	phosphate buffer saline (10mM potassium phosphate buffer, pH 7.4, containing 0.15M sodium chloride)

Abbreviations continued

PBS-BSA	PBS containing 1% (w/v) BSA
PBST	PBS containing 0.05% (v/v) Tween 20
PCB	polychlorinatedbiphenyl
PGE	0.1M potassium phosphate buffer, pH7.4, containing 30% (v/v) glycerol and 0.1mM EDTA
pmoles	picomoles
RAM	rabbit anti-mouse IgG
RAM-IgG ₁	rabbit anti-mouse IgG ₁
RAM-HRP	rabbit anti-mouse IgG conjugated to horseradish peroxidase
RIA	radioimmunoassay
(m)RNA	(messenger)ribonucleic acid
SAM	sheep anti-mouse IgG
SAM-HRP	sheep anti-mouse IgG conjugated to horseradish peroxidase
S.D.	standard deviation
SDS	sodium dodecyl sulphate
TCCD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TK	thymidine kinase
TMBZ	3,3',5,5'-tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethanesulphonic acid
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 The Importance of Drug Metabolising Systems

1.1.1

Introduction

The mammalian drug metabolising systems have evolved to produce, in general, metabolites that are more polar and less lipid soluble than the parent compound from which they are derived. Compounds with a high lipid/water partition coefficient readily diffuse back from the kidney tubules and are retained in the body's tissues. As a consequence, such compounds tend to have a low renal clearance and therefore, a long plasma half life. A more polar and less lipid soluble compound or metabolite on the other hand, with a lower partition coefficient, will tend to have a much greater renal clearance, with a concomitant lowering of its plasma half life (Goldstein *et al.*, 1974). The hydro-solubility of a compound is often enhanced by conjugation, or phase II, reactions (e.g. glucuronidation, sulphation, amidation or methylation) with a suitable agent. Conjugation can only occur if the conjugating agent is presented with a compound which it can readily react with. A few drugs meet this requirement as they are, and can be conjugated without further metabolism. More often than not, only their metabolites can participate in conjugation reactions. Some compounds or their metabolites can be so water soluble that they can be excreted without the requirement for any prior conjugation. This overall picture is summarised in Figure 1.1 .

The development of drug metabolism systems may be an evolutionary adaption to the terrestrial existence of mammals. Fish and other marine organisms commonly lack some of the mammalian drug metabolising systems. However, they can excrete lipid soluble compounds into the surrounding water, down an almost infinite concentration gradient (Brodie and Maickel, 1962).

Drug metabolising systems catalyse a large number of individual reactions, but the resulting reactions can be categorised under the three main headings of oxidation, reduction and hydrolysis. Whilst it may be convenient to describe these reactions separately, it should be noted that metabolism of a single compound may involve the sequential or simultaneous operation of several metabolic processes. Since each of these may result in the production of several different metabolites, each of which may be conjugated in several different ways, it can be seen that a single compound may give rise to many different and varied excretion products.

1.1.2

Consequences of Drug Metabolism

The decrease in lipid solubility of a drug metabolite does not necessarily mean that it will also have increased water solubility. The anti-bacterial sulfonamides, for example, are metabolised to more polar, less lipid soluble acetyl derivatives, but some of them are less water soluble than their parent compounds. Sulfathiazole, for example, was abandoned as an anti-bacterial agent due to the toxicity problems caused by its acetyl metabolite precipitating in the renal tubules of patients (Goldstein *et al.*, 1974).

Although the duration of drug action tends to be decreased by metabolic transformation, it would be incorrect to describe all forms of drug metabolism as detoxication. In many examples, the metabolites can have a greater biological activity than the parent compound itself. Indeed, the desirable pharmacological action of some drugs can be wholly attributed to their metabolites. Likewise, the toxic side effects of some drugs may be due in some way to their metabolites. The analgesic and antipyretic compounds phenacetin and acetanilide can both be metabolised to acetaminophen. Inhibition of phenacetin metabolism by SKF-525A abolished the antipyretic activity of the parent compound, indicating that its

pharmacological activity resided wholly in the alkylated metabolite (Conney *et al.*, 1966). The toxic side effects of acetanilid on red blood cells also illustrates the consequences of drug metabolism. Following administration of acetanilid, methaemoglobin is often seen, which can lead to destruction of red blood cells after large doses. This toxic action is not caused by acetanilid itself, nor by acetaminophen. Instead, it is caused through the metabolic formation of aniline, which itself is metabolised to the toxic compounds phenylhydroxylamine and nitrosobenzene, which then produce methaemoglobin (Kiese, 1965).

Drug metabolism systems can also be associated with the susceptibility of individuals to certain types of cancer (e.g. Ioannides and Parke, 1987). Two cytochrome P-450 polymorphisms that are associated with cancer susceptibility are those concerned with debrisoquine 4-hydroxylase activity (Idle *et al.*, 1981; Ayesb *et al.*, 1984) and aryl hydrocarbon hydroxylase activity (AHH, usually studied as B[a]P hydroxylation) (Kouri *et al.*, 1982). In the former case, the extensive debrisoquine metaboliser phenotype has a disproportionally higher risk of lung cancer amongst cigarette smokers, and of cancer of the liver and gastrointestinal tract. The poor metaboliser phenotype on the other hand, can experience severe hypotension following administration of the drug. Individuals of the high AHH inducibility phenotype have an increased risk of cigarette induced lung cancer.

These examples highlight the problems involved in drug metabolism. The phenotype that allows the development of an acute drug response need not be the same as that which predisposes to a chronic response, which can include cancer. In addition, the same chronic response can be apparent within two unrelated polymorphisms, such as debrisoquine 4-hydroxylase activity and AHH inducibility and their association with lung cancer.

1.2 Cytochrome P-450 and Mammalian Drug Metabolism

1.2.1

Introduction

The oxidative metabolism of many xenobiotics is mediated by the cytochrome P-450 dependent monooxygenase system, located in the endoplasmic reticulum of most mammalian cells (Gillette *et al.*, 1972). This system detoxifies a wide variety of xenobiotic compounds, but can also activate some to potentially toxic, or carcinogenic, metabolites (Jacoby, 1980). Endogenous compounds such as fatty acids can also be metabolised by this system (Kupfer, 1982). Purification of the monooxygenase system components, allied with kinetic, inhibitory and genetic studies have revealed the presence of families of cytochrome P-450 isozymes (e.g. Lu and West, 1980; Nebert *et al.*, 1987). Exposure of mammals to certain compounds of both environmental and experimental origin can induce the synthesis of individual, or groups of, cytochrome P-450 isozymes and may dramatically change the content, proportion and distribution of specific isozymes (Snyder and Remmer, 1982).

This enzyme multiplicity, coupled with a unique but overlapping substrate specificity has limited the determination of the cytochrome P-450 isozymes present in tissues and individuals. The presence of specific isozymes can influence the metabolic pathway of a xenobiotic, and may be important in determining the choice between the alternatives of detoxication or activation of the xenobiotic.

Whilst this thesis is concerned with the study of cytochrome P-450, it should be noted that several non-cytochrome P-450 dependent drug metabolism systems are also present in mammalian species. These include alcohol and aldehyde dehydrogenases (Gillette, 1959); purine oxidation (Elion *et al.*, 1963); monoamine and diamine oxidases (Blaschko, 1973; Zeller,

1973) and azo- and nitro-reduction (Gillette, 1971). However, the cytochrome P-450 dependent monooxygenase system still remains the most widespread and varied of the mammalian drug metabolising systems.

1.2.2

Cytochrome P-450: A Historical Perspective

Whilst it had been known for many years that compounds could be oxidised *in vivo*, it was not until the 1940's and '50's that the oxidative metabolism of xenobiotics began to be demonstrated *in vitro* using liver slices and sub-cellular fractions. These early *in vitro* studies used substrates such as azo dyes (Meuller and Miller, 1948) and amphetamines (Axelrod, 1955). Numerous drugs were also found to be metabolised by similar enzyme systems through several separate pathways (Brodie *et al.*, 1958). These studies concluded that the metabolising enzymes were present in the microsomal fraction of the cell, and required the presence of both NADPH and molecular oxygen for their activity.

At this time, Klingenberg (1958), reported that the total amount of protohaem present in liver microsomes could not be accounted for solely by the presence of cytochrome b₅. Liver microsomes, on reduction with either NADPH or sodium dithionite, were also found to bind carbon monoxide, and in such a state to exhibit a difference spectrum characterised by an absorption maximum at 450nm. In the same year, Garfinkel (1958) also reported on the presence of a pigment in liver microsomes, although he concluded that due to its spectral properties, this protein probably did not contain iron. The carbon monoxide binding pigment responsible for this spectrum was subsequently found to be a protohaem cytochrome and was named cytochrome P-450 (Omura and Sato, 1962; 1963; 1964a; 1964b).

Anaerobic solubilisation of liver microsomal membranes with *Trimeresures flavoviridis* snake venom or sodium deoxycholate to remove cytochrome P-450 caused a blue shift in the absorption maximum of the reduced carbon

monoxide binding spectrum, from 450nm to 420nm. This indicated that the membrane associated cytochrome P-450 had been changed in some way by the solubilisation procedures. This solubilised, and inactive, form of the haemoprotein was thus termed cytochrome P-420 (Omura and Sato, 1962).

Studies on metabolising enzymes by various groups have provided evidence that cytochrome P-450 dependent enzyme systems are also present in a wide range of non-hepatic mammalian tissues. These include adrenal cortex (Cooper *et al.*, 1965); placenta (Meigs and Ryan, 1968); kidney (Orrenius *et al.*, 1973); skin (Poland *et al.*, 1974); testes (Betz *et al.*, 1976); brain (Cohn *et al.*, 1977); intestinal mucosa (Jones *et al.*, 1980); lung (Philpot *et al.*, 1982), and, in fact, virtually all tissues of the body.

1.2.3

Cytochrome P-450 Nomenclature

As yet, there is no universally accepted nomenclature for the different cytochrome P-450 isozymes that have been reported to date, although an attempt has been made recently to rationalise the nomenclature of the genes involved (Nebert *et al.*, 1987). In this thesis, the term 'cytochrome P-450' is used in a generic sense to denote any or all forms of the haemoprotein under discussion. Specific forms of cytochrome P-450 from rat liver have been designated according to the convention of Ryan *et al.* (1984), and those forms from rabbit liver according to the convention of Johnson *et al.* (1984).

1.2.4

Methods of Studying Cytochrome P-450 Isozymes

A variety of methods have been used in the study of cytochrome P-450 isozymes. These have generally fallen into one of two categories depending on the source of cytochrome P-450 under study. Conventionally, these two sources are either microsomal fractions or purified isozymes.

That being the case though, many researchers have used similar study protocols regardless of the source of cytochrome P-450. Catalytic, kinetic and spectral studies of cytochrome P-450, coupled with inhibition studies, have provided valuable information on the number of different isozymes, together with their various substrate specificities. The spectral properties of cytochrome P-450 have long been used as analytical tools for determining the amount and relative purity of unfractionated or purified isozymes. In addition, the absorption maximum of various cytochrome P-450 isozymes can be used as an indication of the relative abundance of those isozymes in microsomal fractions.

Various reconstituted cytochrome P-450 isozymes have overlapping substrate specificities. The kinetics of these reactions may be very similar, as in the case of aniline hydroxylation (Haugen *et al.*, 1975), or they may vary by a considerable degree, as in the case of B[a]P hydroxylation and benzphetamine N-demethylation (Guengerich, 1977a; Ryan *et al.*, 1979). Many isozymes have also been shown through catalytic studies to exhibit stereo and positional selectivity (Deutsch *et al.*, 1978). Kinetic analysis of the metabolism of compounds such as aminopyrine were also instrumental in determining the existence of multiple forms of cytochrome P-450 (Pederson and Aust, 1970).

Various compounds can also be used to selectively affect the metabolism of a variety of substrates through their action on cytochrome P-450. The effects of metyrapone (Owens and Nebert, 1975) and SKF-525A (Sladek and Mannering, 1969) on the activities of PB inducible cytochrome P-450 isozymes, or the effect of α -naphthoflavone on the activities of 3MC inducible cytochrome P-450 isozymes (Wiebel *et al.*, 1971) are examples of this type of approach.

Many investigations, though, are more restrictive on the source of cytochrome P-450 that can be used. The effects of chemical and environmental inducers on the relative levels of cytochrome P-450 isozymes,

for example, can only be adequately performed with whole tissue preparations or microsomal fractions, rather than with purified isozymes. Similar cytochrome P-450 preparations are also required for developmental studies and investigations into the genetic evolution of cytochrome P-450 isozymes within an animal.

Purified isozymes can also be used in a variety of quite distinct investigations. SDS-PAGE analysis has been useful in the characterisation of cytochrome P-450 through the estimation of the purity and molecular weights of different purified isozymes.

Both spectral and SDS-PAGE analysis of cytochrome P-450 suffer from the drawback that they are very much dependent on the precise protocols followed by the investigator. Two dissimilar isozymes may, in different laboratories, give rise to the same spectral patterns and molecular weights. In a similar way, a single isozyme may appear to be two clearly distinct forms if studied by separate investigators. This can be overcome to some extent, though, by the exchange of preparations between researchers. The techniques of peptide mapping and amino acid sequencing performed on purified isozymes should, however, provide clear and unambiguous information as to the identity of the isozyme concerned.

Many immunological studies have also been performed on cytochrome P-450 isozymes, resulting in a large amount of information on both families and individual isozymes. The application of antibodies, and of MAb's in particular, in this area is discussed in more detail in section 1.4 of this chapter, as well as in subsequent chapters of this thesis. Today, though, an increasing number of studies are performed directly on cytochrome P-450 genes, through the use of genomic and cDNA libraries, together with genetic probes and restriction enzyme digestion profiles. Such studies can provide definitive results as to the identity of a particular isozyme and are discussed further in section 1.2.6 of this chapter.

1.2.5

The Mechanism of Cytochrome P-450 Action

Cytochrome P-450 isozymes are grouped together in families of closely related haemoprotein isozymes, which act as the terminal oxidase for a variety of oxidative reactions. This range of isozymes, each with a variable and sometimes overlapping substrate specificity, gives this system an unprecedented position as the foremost mammalian drug metabolising system. Various drugs can be oxidised *in vitro* in a system requiring microsomes, NADPH, molecular oxygen and possibly magnesium ions. Microsomes can also be replaced by the individual components of NADPH cytochrome c reductase, cytochrome P-450 and lipid in a reconstituted system (Lu *et al.*, 1968). In the oxidation of a xenobiotic, one molecule of molecular oxygen is consumed for each molecule of substrate oxidised. One atom of oxygen is introduced into the substrate, whilst the other is reduced to form water. The monooxygenase reaction therefore requires two electrons to complete the oxidation of one substrate molecule (Hildebrandt and Estabrook, 1971).

The electron pathway has been deduced and is represented in Figure 1.2. The electron transport chain involves the primary electron donor NADPH. This is oxidised by the flavoprotein, NADPH cytochrome c reductase to NADP^+ , and results in the transfer of an electron by the flavoprotein to the oxidised (Fe^{3+}) form of cytochrome P-450, which has already interacted with its substrate. The reduced (Fe^{2+}) cytochrome P-450 substrate complex can bind molecular oxygen to form a ternary complex. This breaks down on the addition of a second electron, to yield oxidised substrate, oxidised (Fe^{3+}) cytochrome P-450 and water. The second electron may also come from NADPH cytochrome c reductase, but the contribution of NADH and cytochrome b_5 in the transfer cannot be ruled out (Noshiro *et al.*, 1980). The cytochrome P-450 haem domain is partially exposed on the surface of

the protein and appears to be structured from several sections of its polypeptide chain (Dus, 1982).

Cytochrome P-450 and NADPH cytochrome c reductase are known to associate with each other in a 1:1 molar ratio, with an association constant of approximately $10^{-7}M$ when present as purified forms in a reconstituted enzyme system (French *et al.*, 1980). Aggregation of both cytochrome P-450 and NADPH cytochrome c reductase has also been shown to occur in the presence of detergents and lipids (Guengerich *et al.*, 1980). This suggests that such an association may also occur in the membrane environment. Different forms of cytochrome P-450 can compete with each other for the transfer of electrons from NADPH cytochrome c reductase. It is also known that the inactive form of cytochrome P-450, cytochrome P-420, does not compete with cytochrome P-450 for this electron transfer (Guengerich *et al.*, 1980).

Although it has not been fully determined at present, an understanding of the regulation and distribution of individual cytochrome P-450 isozymes may provide information which could be useful in determining the response of an individual to exposure of drugs and environmental pollutants. The metabolism of a drug is an important determinant of its intensity and duration of action. The detoxication, toxication and rate of excretion of a drug are fundamental to both its effect, and possible side effects. Allowing for variability in drug metabolism, both between and within species, is also of vital importance in effective drug usage. With animal studies conducted on all new drugs, variability between laboratory strains and man could have vital implications on drug safety. Similarly, differences between humans in rates of drug metabolism are important in the efficacy of a drug in the population as a whole. Examples of these would be the genetic polymorphisms of 'slow' and 'fast' acetylators (Hearse and Weber, 1970) and 'poor' and 'extensive' metabolisers of debrisoquine (Mahgoub *et al.*, 1977). Differences in drug metabolism within a species

may also be due to concomitant drug therapy (Schary *et al.*, 1975); smoking (Pantuck *et al.*, 1972); sex of the individual (Chao and Chung, 1982); age (Kato *et al.*, 1964) and diet, with the presence of cytochrome P-450 inducers in some foods (Conney *et al.*, 1976). All of these factors can have considerable influences on the efficacy of drug treatment, through modulation of the cytochrome P-450 mediated monooxygenase system.

1.2.6

Homology Studies of Cytochrome P-450 Isozymes

Major advances have been made in the last few years in elucidating the structure of several cytochrome P-450 isozymes. In some cases, most of the amino acid sequence has been obtained (e.g. Heinemann and Ozols, 1983; Yuan *et al.*, 1983; Haniu *et al.*, 1984; 1986). In other studies, the entire nucleotide sequence of the structural gene has been determined (e.g. Fujii-Kuriyama *et al.*, 1982; Sogawa *et al.*, 1984). Such studies have revealed that cytochrome P-450 isozymes belong to a number of different families, with one or more members of each family being present in each species (Fujii-Kuriyama *et al.*, 1984; Nebert *et al.*, 1987). All the members within a single family of cytochrome P-450 isozymes exhibit a considerable degree of structural homology with each other. Their response to the different classes of cytochrome P-450 inducers is also similar. There can be marked differences, however, in cytochrome P-450 sequences between members of different isozyme families (Black and Coon, 1987).

With homologies being apparent between individual isozyme families across different species, it has been possible to produce gene probes which cross-hybridise between species (Phillips *et al.*, 1985). Such probes can be used to determine the homology between various isozymes. They could also be used to locate, and to quantify, cytochrome P-450 mRNA levels in a variety of tissues, through *in situ* hybridisation studies. Although gene probes can provide valuable information, it is sometimes possible to

produce a probe which hybridises to a part of the gene sequence which does not code for part of the functional protein (Atchison and Adnesnik, 1983). There have also been reports of a lack of correlation between mRNA levels and specific cytochrome P-450 isozyme content or enzyme activity (Tuteja *et al.*, 1986; Iversen *et al.*, 1987; McClellan-Green *et al.*, 1987).

An alternative approach to DNA probes for investigating the structural and functional relationships between different cytochrome P-450 isozymes is in the application of polyclonal and monoclonal antibodies. Well characterised MAb's have proved invaluable in recent years in the study of cytochrome P-450 structure and function (e.g. see Gelboin and Friedman, 1985; Thomas *et al.*, 1986; Gelboin *et al.*, 1988). The utility of MAb's in the characterisation of cytochrome P-450 isozymes is the subject of this thesis. The production and characterisation of the MAb's used in this thesis, and the particular applications for which they were used, are discussed in subsequent chapters.

1.3 Monoclonal Antibody Technology

1.3.1

The Antibody Molecule

Antibodies, or immunoglobulins, are a highly heterogeneous family of proteins, capable of the specific recognition of thousands of separate antigen moieties. The total antibody repertoire of a single animal is unknown, but the best estimates put the figure in excess of one million separate antibody reactivities (Kunkel, 1970). In spite of this, all antibody molecules are similarly composed of 'heavy' and 'light' polypeptide chains. Each chain consists of a variable region of about 105 to 120 amino acids, and one or more constant regions of about 110 amino acids

(Milstein and Svasti, 1971). The exact number of constant regions depends on the the antibody class and type of chain involved, with light chains having only one constant region (Figure 1.3). Constant regions are associated with specific effector functions, such as cell surface binding or complement activation. Antibody specificity, on the other hand, resides within the combination of the light and heavy chain variable regions. To allow for an excess of one million different antibody specificities by light and heavy chain association alone would require at least one thousand light and heavy chain types. Sequence studies have revealed, however, that this is not the case, and that a more limited range of chains are available for association. Within each limited range of variable regions however, there are three clusters of hypervariable regions which are the main site of antibody variability, and hence variations in antigen binding (Wu and Kabat, 1970). These hypervariable regions occur at the site of variable region RNA splicing and can occur through inaccurate ligation or errors during repair. These mechanisms alone would still not be enough to provide the variability required for the total antibody repertoire, and somatic mutation within each antibody producing cell is believed to be a major contributor to antibody diversity (Munro, 1978).

1.3.2

The Antibody Response

When a foreign substance enters, or is experimentally introduced into, an animal, the host may mount an immune response against it. The type and scale of the response will be dependent on many factors. The antigenicity (or foreignness) of the substance, the extent of previous exposure, the time after this or previous exposures, the site and method of exposure as well as the immunocompetent state of the host all combine to regulate the immune response. Though the host can mount both non-specific cellular responses

and specific cell mediated responses, the most common response is humoral, or antibody, mediated.

The antibody response to a typical antigen is heterogeneous, with many diverse antibodies being directed against different antigenic sites on the foreign substance, and against different determinants on a single antigen. In addition, several different antibodies can be directed against a single antigenic determinant, or epitope, on the foreign substance, each with varying affinities. Given this complexity, it is almost impossible to separate the various antibodies present in the humoral response.

Conventional antisera therefore contain mixtures of antibodies, the proportions of each varying not only between animals, but also within the same animal from bleed to bleed. The unpredictable nature of the immune response coupled with this antibody heterogeneity means that any potentially useful antiserum is usually only available in limited amounts. Only where an antiserum has been affinity purified against specific epitopes to remove cross-reacting antibodies can reproducible preparations be prepared. The absolute amount of antibody that can be prepared in such a way is, however, also limited.

1.3.3

Production of Monoclonal Antibodies

Whilst an antiserum can contain many thousands of individual antibody molecules, it can be sub-divided into groups of identical antibodies, each possessing the same amino acid sequences. These distinct groups of antibodies are made by different lineages of antibody secreting cells, or B lymphocytes (Burnet, 1959). If it were possible to select only those cells secreting antibodies of interest and to propagate them indefinitely, a valuable source of antibody could be prepared. Unfortunately, primary antibody secreting cells cannot be maintained indefinitely in tissue culture. However, some antibody secreting lines can be maintained in

culture, and by passage through histocompatible animals. These are the myeloma cell lines, tumours which can be induced by the injection of mineral oil into a mouse (Potter and Lieberman, 1967). Each of these cell lines have been derived from a single B lymphocyte parent, and all the antibodies secreted by an individual myeloma cell line are identical. They are, in effect, true monoclonals. However, in the vast majority of examples it is not yet known what antigen these various MAb's are specific for. Neither has it been possible to induce myelomas that produce antibody to a specific antigen. What was required therefore, was some way to combine the specificity of antibody secreting cells with the ability of myeloma cells to grow indefinitely in culture.

Such a combination was achieved by Köhler and Milstein, when they mixed murine spleen cells, immunised against sheep red blood cells, with mouse myeloma cells in the presence of a fusion promoting agent, in this case Sendai virus (Köhler and Milstein, 1975). The resulting hybrid cells could be propagated both in culture and as ascites tumours in histocompatible mice. In addition, these hybridoma cells secreted specific anti-sheep red blood cell antibodies.

Some refinement of this simple protocol is required however to produce individual MAb secreting hybridoma cell lines. The first is to remove unfused myeloma cells from the culture. If left, these rapidly growing cells would quickly take over any mixed culture, thereby depriving the desired hybridomas of growth factors present in the culture medium. Their effective removal is achieved by the use of mutant myeloma lines. These cells possess genetic deficiencies which inhibit their growth under certain conditions. The most common myelomas used lack the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT) or thymidine kinase (TK). These mutants can be selected by their ability to grow in the presence of azaguanine or bromodeoxyuridine, respectively. Such mutants, lacking as they are the salvage pathway enzymes, are resistant to these DNA analogues.

For the same reason, they are unable to incorporate externally supplied hypoxanthine or thymidine. If endogenous synthesis of DNA precursors is blocked in these cells by the addition of the folic acid antagonist aminopterin to the culture medium, then the cells will die, even when hypoxanthine and thymidine are also included in the growth medium (HAT medium) (Littlefield, 1964).

Spleen cells will also die in HAT, or any other culture medium, as they do not contain the necessary metabolic pathways to maintain their growth under culture conditions. Myeloma cells however, can be rescued from HAT medium by fusion with spleen cells, as the latter contain the wild type salvage pathway enzymes. These hybrid cells can then be selected from both parental types as they will be the only cells that will grow and divide in HAT medium.

Initial fusions were performed with myeloma lines that still secreted their own antibody molecules (Köhler and Milstein, 1975; Köhler *et al.*, 1976). The resultant hybridomas were then capable of producing two sets of immunoglobulin molecules, whose chains could associate in a random manner. The MAb's that were produced in these studies did not always possess the desired specificity or affinity required. Subsequent fusion protocols utilised myeloma lines that were no longer able to synthesise either heavy or light immunoglobulin chains (Shulman *et al.*, 1978; Kearney *et al.*, 1979; Fazekas de St. Groth and Scheidegger, 1980). These subsequent hybridomas therefore produce only spleen cell derived antibodies.

1.3.4

Selection and Propagation of Required Hybridoma Cells

Myeloma - spleen cell fusions provide only the basic tool to generate MAb's. Further involved work is required before the desired MAb is available for widespread use.

Following fusion, hybridomas may be cultured under a variety of conditions, in 12 or 24 well plates or in smaller volumes in 96 well microtitre plates, for example. Culture medium is required to be changed during the initial culture stages before clusters of hybridomas can be detected. Stable hybridoma cultures are not, however, obtained following this initial culture period. As hybridomas grow at different rates, it is impossible to continue to propagate them all, once they outgrow their initial culture wells. The major prerequisite for MAb production, is the availability of rapid screening assays to identify the desired antibody reactivities in the culture supernatants. The most useful assays are those which have been developed for use in a 12 x 8 microtitre plate format, such as ELISA (Engvall and Pesce, 1978) or dot-blot assays (Smith *et al.*, 1984), which can utilise harvesting devices such as multi-channel pipettes.

Positive cultures can then be transferred to larger culture wells and eventually to flasks. To ensure the preservation of any potentially interesting hybridoma cultures, cells can be frozen in liquid nitrogen, to be re-cultured as and when required in the future.

It is important to ensure that all cells within a positive culture have been derived from a single parental cell. To achieve this, positive cultures are cloned, usually by limiting dilution or in soft agar (Campbell, 1984). The former method requires that cells from a positive culture are plated out into 96 well microtitre plates, such that there will be, on average, only one cell in every two or three wells. To achieve this, a range of cell dilutions is usually prepared. Culturing is carried out over

a period of ten to fourteen days, usually in the presence of feeder spleen cells from histocompatible mice to encourage growth. Wells in which growth and division of a single cell is observed are fed with fresh medium until large cell clusters are obtained. The culture supernatant can then be assayed and positive cultures expanded. The latter cloning method involves plating out cells in large well plates at higher cell concentrations than limiting dilution, in medium containing agar. The agar forms a soft gel within the culture well which allows individual cells to form small discrete clusters upon division. Feeder cells are usually incorporated in the wells as an initial separate layer of agar containing medium. Individual clones can be selected using a finely drawn pasteur pipette and expanded in normal liquid medium. Supernatants from these cultures can be screened for antibody reactivity and positive cultures selected.

Regardless of the cloning procedure used, positive cultures should be cloned at least twice to ensure that the MAb secreting cells are both clonal in nature and stable in culture. Once this has been achieved, the cells can be grown in mass culture and frozen stocks prepared. The culture supernatants obtained during this procedure can be used as a valuable source of MAb. In addition, cells may be injected i.p. into a histocompatible mouse, where they form an ascitic tumour and induce the formation of ascites fluid containing large amounts of the required MAb (Potter *et al.*, 1972). The concentration of MAb in tissue culture fluid is often in the region of 10 $\mu\text{g/ml}$, whilst in ascites fluid, yields of 10 mg/ml can be obtained. It is also possible to store hybridoma lines in liquid nitrogen for many years, so that a further supply of identical antibody can be made available at a later date.

1.3.5

The Respective Merits of Polyclonal Antisera and Monoclonal Antibodies

An antiserum, prepared by immunising an animal with a preparation of an antigen, will contain a wide spectrum of immunoglobulin molecules. Some of these will be directed against different epitopes on the antigen of interest, and will bind with different affinities. The reactivity of an antiserum, for example, its ability to specifically react with a protein in solution, can therefore be thought of as the average specificity of all of its component immunoglobulins. In a similar way, the ability of an antiserum to bind to protein A, to fix complement or to be resistant to degradation on long term storage, is also dependent on the average ability of the component immunoglobulins to perform these functions. MAb's are, however, individual immunoglobulins. As such, their antigen reactivity and other functions are not dependent on the contribution of any other immunoglobulins. This individuality enables MAb's to be exquisitely specific in their reactivities, and allows their reactivities to be accurately defined. However, this individuality does not allow the usual latitudes available with polyclonal antisera to be readily applied to MAb's. If an undesirable cross-reactivity with another protein is observed with a polyclonal antiserum, then it can be removed from the preparation by the removal of the cross-reactive immunoglobulins. Such an approach is not possible with a MAb, however, as removal of an unwanted cross-reactivity, due to the presence of a similar epitope on another protein, cannot be effected without the removal of the desired MAb reactivity as well.

The individuality of MAb's can also cause problems in their purification. Some MAb's are not easily purified due to their low tolerance to extremes of pH, or to high salt concentrations. The ability of immunoglobulins to bind to protein A has been used for many years as a rapid method for their purification from an antiserum. This reactivity, like all others, is manifest as the average reactivity of the immunoglobulins present. A MAb

will therefore either possess or lack the ability to bind to protein A. This reactivity can, of course, be screened for during MAb production by the use of enzyme conjugated, or ^{125}I -labelled, protein A, instead of anti-immunoglobulin in the initial screening assays. However, the use of protein A as a screening reagent may allow a non-protein A binding, but otherwise potentially useful MAb, to be missed.

An ideal MAb could be described as one which was highly specific, which showed no undesirable cross-reactivity with other proteins, which was stable both under storage and under the conditions in which it would be most likely used, which bound protein A, and which possessed the desired affinity with its epitope. This last characteristic is pertinent to the intended usage of the MAb. A MAb intended for use in an immunoassay, for example, may benefit from having a high affinity for its epitope, as this could allow for reduced incubation times, more suitable in a clinical situation. On the other hand, a MAb which will be used for the immunopurification of a protein would be more suitable if it allowed the easy elution of the bound protein. It is more usual, however, to initially select MAb's on the basis of their reactivity profile, and then to determine their suitability in the desired applications at a later date.

It can be seen from the above comments that MAb's can be very specific reagents. However, this specificity, coupled with their individuality, can lead to problems in interpreting some of the results obtained in their usage. This is especially true when various MAb's are produced by several groups against similar proteins, as is the case with cytochrome P-450.

1.3.6

Applications of Monoclonal Antibodies

MAb technology has been applied to a wide variety of areas in the last decade. In many cases they have become a standard laboratory reagent due to their high specificity and reproducibility.

In the area of clinical diagnostics, MAb's are generally replacing conventional antisera in commercial kits and laboratory protocols. Tumour immunotherapy is another clinical area that has been influenced by the arrival of MAb's. The removal of T lymphocytes from bone marrow allografts to reduce graft versus host disease (Janossy *et al.*, 1982) or the removal of leukemic cells from bone marrow autografts (Kemshead *et al.*, 1983) are two such examples of the therapeutic use of MAb's. Other studies involving MAb's to tumour cell surface antigens though, have not been as successful. Reasons for this lack of success may involve the host's reactivity against the mouse MAb, modulation of the tumour cells to produce unreactive subpopulations, or inactivation of the MAb by its reactivity with free antigen released from the tumour cell. MAb's in these studies have been used either alone, or to transport a specific reagent to the tumour cell. Examples of such reagents would include cytotoxic drugs, toxins, or immunomodulating agents, such as interferon.

The specificity of MAb's has also been put to use in the preparation of antigen from a crude mixture. One of the most successful applications of this technique was in the purification of alpha interferon (Secher and Burke, 1980).

These applications of MAb's are expanded upon, and others described, in several reviews which cover this topic in more detail than can be discussed in this thesis (e.g. see Albertini and Ekins, 1981; Eisenbarth, 1981; Yelton and Scharff, 1981; McMichael and Fabre, 1982; Kennet *et al.*, 1984; Vora, 1985).

1.4 The Application of Monoclonal Antibodies as Probes of

Cytochromes P-450

1.4.1

Introduction

Cytochromes P-450 are present as multiple forms within a tissue or cell, and at varying concentrations. They have substrate and product specificities which sometimes overlap, and their levels can change in response to environmental, hormonal or nutritional influences. These properties make the cytochrome P-450 isozymes extremely suitable for study by antibodies, and immunochemical approaches have been used to reveal the distinct nature of some of these isozymes, as well as some features that are more commonly found amongst them (e.g. Thomas *et al.*, 1976a; 1981; Guengerich *et al.*, 1982; Reik *et al.*, 1982). Antibodies, in turn, are also ideally suited as probes of the structure and function of cytochrome P-450 isozymes due to their inherent specificity. However, the heterogeneity of conventional polyclonal antibodies poses several problems in their use as cytochrome P-450 probes. A polyclonal antiserum is directed against many different epitopes on an antigen, with each epitope being recognised by a heterogeneous population of antibodies with diverse binding affinities. The development of monoclonal antibody technology by Köhler and Milstein in 1975 overcame the problem of antibody heterogeneity by allowing the production of MAb's which have a discrete binding affinity for a single epitope. That epitope may be unique to a particular protein, such as a cytochrome P-450 isozyme, for example, thereby making the MAb monospecific. However, it is equally possible that the epitope may be common to a number of proteins, or isozymes, in which case the MAb would then be cross-reactive.

This cross-reactivity might at first thought appear to be a disadvantage in the use of MAb's to study the cytochrome P-450 system. However, this

need not necessarily be the case. A comprehensive library of MAb's could contain ones that are specific for unique forms of cytochrome P-450. Alternatively, MAb's could be produced that react with two or more forms, or even with all forms of cytochrome P-450. This library could then be used to classify cytochromes P-450 as either unique forms, or as belonging to specific classes, as defined by their MAb reactivity pattern. Cytochrome P-450 classification by MAb's can therefore be seen as an alternative system to the more conventional methods of isozyme classification such as isozyme purification and/or SDS-PAGE gel migration profile, spectral properties, induction profile and substrate specificity. Such a system would be limited only by the number of MAb's in the MAb library. A number of MAb's have now been produced against isozymes of cytochrome P-450. The publications referring to these MAb's are listed in Table 1.1, together with the species and cytochrome P-450 induction regimes used by the authors in preparing the MAb's. These MAb's have been used in a variety of studies on cytochrome P-450 function, localisation, quantification and purification. Individual MAb application areas are discussed below, and in the separate chapters of this thesis.

It is important to realise that before any MAb classification of cytochrome P-450 isozymes can proceed, individual MAb's have to be characterised fully for their cytochrome P-450 reactivity profile. The characterisation methods employed for the MAb's used in this project are described and discussed in Chapter 3 of this thesis.

1.4.2

Specific Applications

As indicated above, MAb's can be used in a variety of different studies on the cytochrome P-450 system. One of the most studied problems in the cytochrome P-450 field is the determination of the relative contribution

each unique form or class of cytochrome P-450 makes to the total metabolism of an individual substrate in different tissues and species. An understanding of the substrate and product specificity of cytochrome P-450 isozymes conventionally relies on the purification of individual isozymes, or on specific induction or inhibition studies. The latter methods have been unable to supply much useful information due to the lack of inducers or inhibitors that are specific for a single unique form of cytochrome P-450. The former method though, has provided useful information on the substrate specificity of individual cytochrome P-450 isozymes. However, it has not contributed to any understanding of microsomal or, indeed, *in vivo*, cytochrome P-450 reactivity, as the use of purified isozymes requires that reconstitution studies are performed, which may not accurately reflect those situations. This approach is also limited by the requirement to purify individual forms of cytochrome P-450. This is severely restrictive when constitutive, minor or extrahepatic forms are under study. MAb's that inhibit the enzyme activity of the cytochrome P-450 isozymes to which they bind are therefore extremely useful reagents for investigating the substrate and product specificity of those isozymes.

Since MAb's usually bind to only a single epitope on an antigen, they tend not to form precipitates on binding. There have been reports that MAb's can precipitate cytochrome P-450 isozymes following incubation (Park *et al.*, 1982b). However, this may have been due in part to the instability in solution of the cytochrome P-450 preparations used, rather than the specific action of the MAb's in question. Thus, it would be expected that MAb's would not inhibit cytochrome P-450 activity by non-specific immunoprecipitation. It could be possible, however, that the MAb reactive epitope on the cytochrome P-450 isozyme is at, or near, the enzyme active site. Specific inhibition of cytochrome P-450 activity may then be possible on MAb binding.

The technique of MAb inhibition to map cytochrome P-450 substrate specificity (or to 'reaction phenotype' cytochrome P-450 isozymes) has been

used extensively by Gelboin and his co-workers (see review by Friedman *et al.*, 1985f), as well as by many others in the cytochrome P-450 field (e.g. Reubi *et al.*, 1984a; 1984b; Boobis *et al.*, 1985a; 1985c; Sugano *et al.*, 1985; Ding *et al.*, 1986; Thomas *et al.*, 1986; Ko *et al.*, 1987; Brady *et al.*, 1988). This particular approach in the use of MAb's is expanded upon in Chapter 4 of this thesis.

As indicated above, the purification of individual cytochrome P-450 isozymes has severe limitations as a method for studying their substrate specificity. Purification of isozymes is, however, also required for amino acid sequence studies, or as a source of antigen for polyclonal and monoclonal antibody production and screening. Although purification of individual isozymes have been reported (see Boobis *et al.*, 1985b), the yields have remained low, a problem compounded when extrahepatic tissues are used as starting material. The specificity of MAb's offers a way of purifying the multiple forms of cytochrome P-450. MAb's covalently linked to Sepharose have been successfully used to purify a variety of cytochrome P-450 isozymes for further analysis (e.g. Lewis *et al.*, 1982; Reubi *et al.*, 1984a; 1984b; Mendelson *et al.*, 1985; Ding *et al.*, 1986; Friedman *et al.*, 1986; Robinson *et al.*, 1986; Wrighton *et al.*, 1986; Finlayson *et al.*, 1987). This particular application of anti-cytochrome P-450 MAb's is further discussed in Chapter 5 of this thesis.

The specificity of MAb's in general has been utilised in many studies for both the qualitative and quantitative measurement of antigen in a variety of situations. In the field of cytochrome P-450 study, MAb's have been used to quantify the microsomal content of MAb epitope specific cytochrome P-450 isozymes by a variety of different techniques (e.g. Paye *et al.*, 1984; Reubi *et al.*, 1984a; 1984b; Beaune *et al.*, 1985; Rothwell *et al.*, 1985; Andersson and Jörnvall, 1986; Ding, *et al.*, 1986; Friedman *et al.*, 1986; Park *et al.*, 1986c; Wrighton *et al.*, 1986; Kloeppler-Sams *et al.*, 1987). In

addition, MAb's have proved their worth in immunocytochemical studies of cytochrome P-450 localisation in tissues and individual cells (e.g. Sugano *et al.*, 1985; Forkert *et al.*, 1986; Foster *et al.*, 1986; Koliada, 1986; Anderson *et al.*, 1987; Finlayson *et al.*, 1987; Murray *et al.*, 1987). The use of MAb's in such procedures is discussed in more detail in, respectively, Chapters 6 and 7 of this thesis.

Figure 1.1 General Scheme of Drug Metabolism

The relationship between metabolism and conjugation of a drug is illustrated with respect to its excretion from the body.

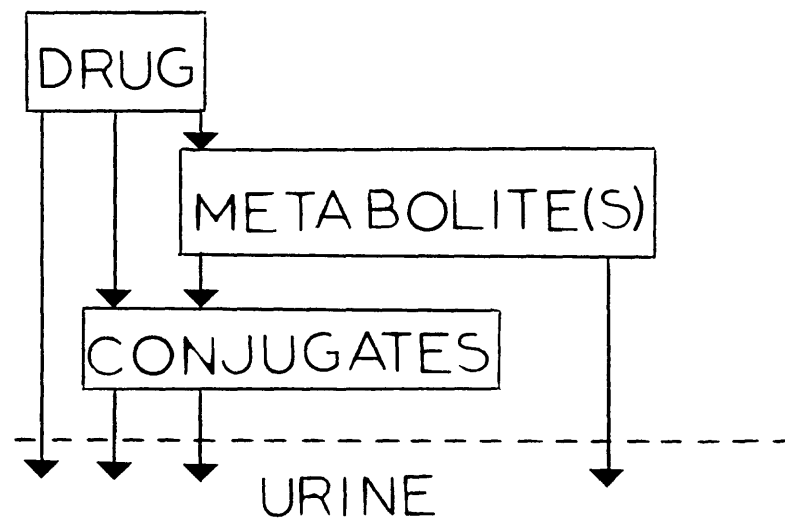


Figure 1.2 Electron Pathway in the Microsomal Monooxygenase System

The electron pathway for the microsomal monooxygenase system is illustrated (from Hildebrandt and Estabrook, 1971).

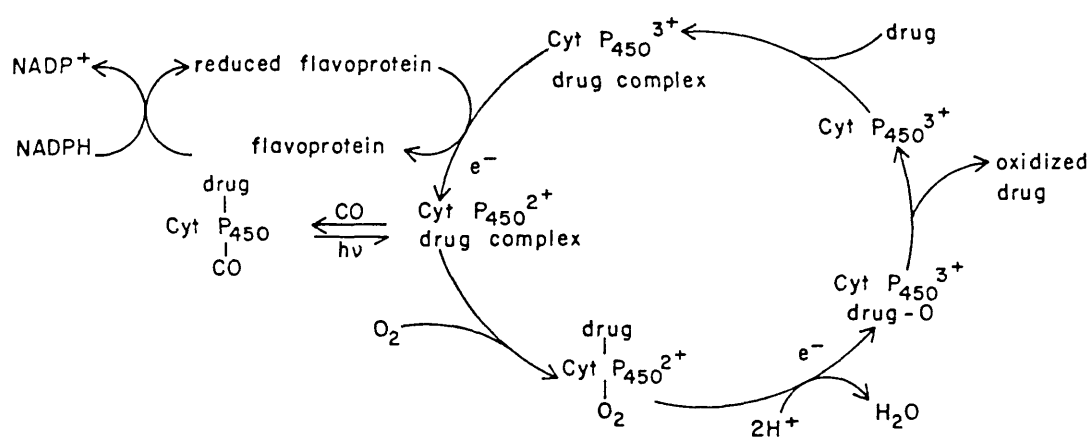


Figure 1.3 A Schematic Diagram of a Typical Immunoglobulin Molecule
(Human IgG₁)

The abbreviations represent:

Fab antibody combining site
F(ab')₂ divalent antibody combining site
Fc C terminal region of heavy chains following papain cleavage
Fd N terminal region of heavy chain following papain cleavage

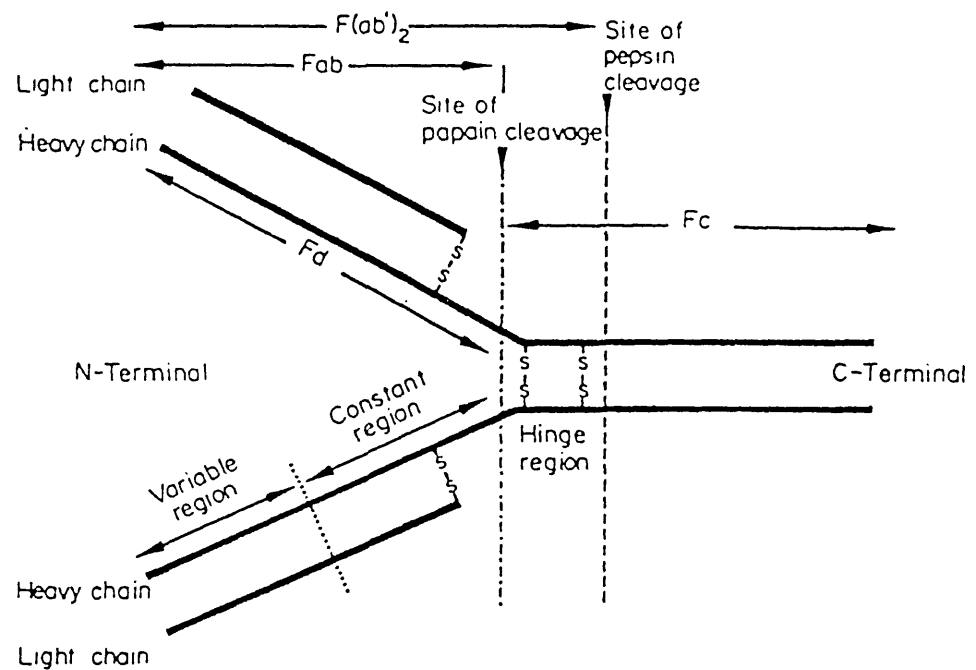


Table 1.1 Monoclonal Antibodies Produced Against Isozymes of
Cytochrome P-450.

Below is a listing of the published MAb's produced against isozymes of cytochrome P-450. In all cases, the MAb's were of murine origin.

A) Rat Cytochrome P-450 Isozymes

Initial specificity of MAb's (or inducing agent used in MAb production)	References
P-450c/d	Park <i>et al.</i> , 1982b
P-450c/d	Thomas <i>et al.</i> , 1984a
(PB)	Letawe-Goujon <i>et al.</i> , 1984
(PB)	Paye <i>et al.</i> , 1984
(PB)	Park <i>et al.</i> , 1984
(PB)	Bonkovsky <i>et al.</i> , 1985
P-450c	Boobis <i>et al.</i> , 1985a
P-448 _H	Hashimoto <i>et al.</i> , 1985
P-450b	Reik <i>et al.</i> , 1985
P-450b	Rothwell <i>et al.</i> , 1985
P-450 forms a, b, c, d and P-450 PB/PCN-E	Koliada, 1986
P-450 2a/PCN-E	Park <i>et al.</i> , 1986b
P-450c/d & b/e	Sesardic <i>et al.</i> , 1986
P-450 _{2B}	Andersson and Jörnvall, 1986
P-450 _{mt}	Park <i>et al.</i> , 1986a
P-450 _{mt}	Ko <i>et al.</i> , 1987
P-450 _{15B} & P-450 _{16α}	Morgan <i>et al.</i> , 1987
P-450p	Hostetler <i>et al.</i> , 1987

Table 1.1 continued

B) Rabbit Cytochrome P-450 Isozymes

Initial specificity of MAb's (or inducing agent used in MAb production)	References
P-450 LM2	Park <i>et al.</i> , 1980
P-450 LM4	Boobis <i>et al.</i> , 1981
P-450 LM2 & LM4	Park <i>et al.</i> , 1982a
P-450 3b	Reubi <i>et al.</i> , 1984a
P-450 1	Reubi <i>et al.</i> , 1984b
P-450 3a (P-450 _{ALC})	Ding <i>et al.</i> , 1986

C) Other Cytochrome P-450 Isozymes

Initial specificity of MAb's (or inducing agent used in MAb production)	References
Human P-450 _{AROM}	Mendelson <i>et al.</i> , 1985
Human P-450	Beaune <i>et al.</i> , 1985
Human P-450	Barnes <i>et al.</i> , 1987
Human P-450 HLx	Wrighton <i>et al.</i> , 1987
Fish P-450E	Stegeman <i>et al.</i> , 1985
Fish P-450E	Park <i>et al.</i> , 1986c
Bovine P-450 _{11B} and P-450 _{SCC}	Sugano <i>et al.</i> , 1985

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 Materials

2.1.1

Immunological And Associated Reagents

Miles Laboratories Ltd. (Slough, Berks.) supplied immunoglobulin fractions of rabbit anti-mouse IgG (RAM), rabbit anti-mouse IgG₁ (RAM-IgG₁) and horseradish peroxidase conjugated rabbit anti-mouse IgG (RAM-HRP), as well as horseradish peroxidase conjugated protein A (pA-HRP). Mouse IgG subclass-specific sheep anti-mouse IgG's (SAM), conjugated with horseradish peroxidase, were purchased from Serotec Ltd. (Bicester, Oxford.). Sheep anti-mouse IgG, both ¹²⁵I-labelled and ¹²⁵I-labelled F(ab')₂ fragment, and donkey anti-rabbit IgG (DAR), ¹²⁵I-labelled F(ab')₂ fragment, were supplied by Amersham International plc (Lt. Chalfont, Bucks.). Freund's complete adjuvant, normal mouse IgG, normal rat and rabbit sera, protein A and o-phenylenediamine (OPD) were supplied by Sigma Chemical Co. (Poole, Dorset). Microtitre plates and individual microtitre wells were purchased from Dynatech Ltd. (Billingham, Sussex).

2.1.2

Electrophoresis and Associated Reagents

Bio-Rad Laboratories Ltd. (Watford, Herts.) supplied acrylamide, bisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, SDS, Amido Black 10B and 4-chloro-1-naphthol. Bromophenol blue, Coomassie Brilliant Blue R-250 and protein molecular weight standards were purchased from Sigma. Hybond-C nitrocellulose membranes were provided by Amersham International plc, whilst 2-mercaptoethanol was supplied by BDH Ltd. (Dagenham, Essex). Materials for autoradiography were obtained from Kodak Ltd. (Hemel Hempstead, Herts.).

2.1.3

Tissue Culture Reagents

All tissue culture plastics were supplied by Becton Dickinson (Cowley, Oxford.). RPMI 1640 media (Dutch modification), L-glutamine, penicillin, streptomycin, sodium pyruvate and foetal calf serum (FCS) were obtained from Gibco Ltd. (Uxbridge, Middx.). Trypan blue and dimethylsulphoxide (DMSO) were purchased from Sigma, whilst Koch-Light Ltd. (Haverhill, Suffolk) supplied polyethylene glycol 1540. The myeloma cell line P3.NS1.1-Ag4-1 was a gift of Dr. Richard Robson and Mr. Christopher Hayward of the Corporate Bioscience Group, ICI plc.

2.1.4

Radiochemicals and Associated Reagents

Amersham International plc supplied L-[U-¹⁴C]-leucine, >300 mCi/mmol, in sterile aqueous solution and carrier free ¹²⁵I for protein iodination, >100 mCi/ml. Bolton and Hunter reagent was obtained from NEN Ltd. (Southampton, Hants.). Instagel was purchased from Packard Instruments Ltd. (Pangbourne, Bucks.), whilst chloramine T was obtained from Sigma.

2.1.5

Chromatography Reagents

DEAE Affi-Gel Blue was purchased from Bio-Rad. Sephadex G-50 (fine), cyanogen bromide activated Sepharose CL-4B and protein A coupled Sepharose CL-4B were obtained from Pharmacia Fine Chemicals Ltd. (Milton Keynes, Bucks.). Polystyrene beads (15.8µm average diameter) were supplied by Sigma, whilst dimethylpimelimidate was obtained from Aldrich Chemical Co. (Gillingham, Dorset). Chromosorb (AW-DMCS) was supplied by Chrompack U.K. Ltd. (London).

2.1.6

Animals

Wistar rats and Balb/c mice were supplied by Olac (1976) Ltd. (Bicester, Oxford.). Alp/Alp rats, a Wistar derived strain, were supplied from breeding stocks held by the Central Toxicology Laboratories, ICI plc.

2.1.7

General Reagents

Sigma provided β -naphthoflavone (β NF), benzo[*a*]pyrene (B[a]P), sodium cholate, bovine serum albumin (fraction V) (BSA), purified haemoproteins, sodium deoxycholate, aniline, NADPH (tetrasodium salt, type 1), phosphatidylcholine and diaminobenzidine (DAB). BDH supplied Triton X-100, Nonidet NP-40, sodium dithionite, hydrogen peroxide, Tween 20, ammonium sulphate, 3,3',5,5'-tetramethylbenzidine (TMBZ), *p*-benzoquinone, Harris' haematoxyline and DPX mountant. Pristane (2,6,10,14-tetramethylpentadecane) was supplied by Aldrich, whilst OCT mounting fluid was obtained from Raymond Lamb Ltd. (London). Isosafrole (*cis* and *trans*) (ISF) was purchased from Eastman-Kodak Ltd. (Liverpool, Merseyside), whilst phenobarbitone sodium (PB) was obtained from McArthur's (London). Aldrin and dieldrin were kindly provided by Shell U.K. Ltd., whilst resorufin and 7-ethoxyresorufin were gifts of Dr. Clifford Elcombe of the Central Toxicology Laboratory, ICI plc. Renex 690 was a gift from Atlas Chemical Industries Ltd.

All other chemicals and reagents were of the highest quality available commercially.

2.2 General Methods

2.2.1

Pretreatment of Animals and Preparation of Liver Microsomal Fractions

Male Wistar rats (150-220 grams) were allowed free access to food and water. Isozymes of cytochrome P-450 were induced by the administration of β NF, ISF, or PB. Induction by β NF was achieved by two doses of 80 mg/kg of the compound in corn oil, by i.p. injection, 48 hours and 24 hours prior to killing the animals. Induction by ISF was achieved using a similar injection regimen, with the ISF also dissolved in corn oil. Four daily doses of 80 mg/kg of PB in saline were administered by i.p. injection, the last one being given 24 hours before the animals were killed.

Rats were killed by cervical dislocation and hepatic microsomal fractions prepared by the method of Boobis *et al.* (1980). In brief, the livers were excised from the peritoneal cavity and cut into small (0.1cm³) pieces in 0.15M potassium phosphate buffer, pH 7.4, containing 0.15M potassium chloride and 1mM EDTA. The tissue was then washed several times in the same buffer and homogenised using a motorised homogeniser. The resulting homogenate was centrifuged at 9,000 g for 20 minutes at 4°C and the supernatant decanted and centrifuged at 105,000 g for 60 minutes at 4°C. The resulting microsomal pellet was resuspended in 0.1M potassium phosphate buffer, pH 7.4, containing 30% (v/v) glycerol and 0.1mM EDTA (PGE buffer), using a glass/teflon hand-operated homogeniser and stored at -70°C. Aliquots of the preparations were taken for determination of protein and cytochrome P-450 concentrations.

Microsomes were prepared from human liver samples, obtained with Local Research Ethics Committee permission, as described by Boobis *et al.* (1980). Hepatic microsomal fractions from Fischer and DA rats were gifts of Dr. Simon Plummer, of the Department of Clinical Pharmacology, RPMS, London. Other liver microsomal preparations used in this thesis, from pretreated

rats, mice and rabbits, and control rats, mice, guinea pigs, hamsters, pigs and baboons, were as described by Pacifici *et al.* (1981).

Partially purified and homogeneous preparations of rat liver cytochrome P-450 form c from BNF treated rats were kindly supplied by Ms. Susan Baker, of the Department of Clinical Pharmacology, RPMS, London. Specific contents for these preparations were 5.1 and 17.1 nmoles cytochrome P-450/mg protein respectively. A partially purified preparation of hepatic cytochrome P-450 forms b and/or e (with a specific content of 6.2 nmoles cytochrome P-450/mg protein) from PB treated rats was also provided by Ms. Baker.

Samples of homogeneous rabbit cytochromes P-450 LM2, LM3c, LM4 and LM6 were gifts from Dr. Eric Johnson, of the Scripps Clinical Research Foundation, La Jolla, CA, U.S.A.

2.2.2

Generation of Monoclonal Antibodies to Isozymes of Rat Liver

Cytochrome P-450

The Mab's described in this thesis were produced in conjunction with Dr. Richard Robson and Mr. Christopher Hayward, of the Corporate Bioscience Group, ICI plc, by the methods described by Boobis *et al.* (1985a). In brief, male Balb/c mice were immunised with 0.4mg of partially purified preparations of rat liver cytochromes P-450, in conjunction with Freund's complete adjuvant, followed by two i.p. boosts of 0.4mg in PBS at 3-weekly intervals. Four days after the final injection, the mice were killed, their spleens aseptically removed, and a monodispersed cell suspension prepared. Fusion of immunised spleen cells and the myeloma cell line P3.NS1.1-Ag4-1 (NS1) was facilitated by the presence of polyethylene glycol, of average molecular weight 1540. Screening of the resulting cell cultures was by a solid phase immuno-radiometric assay (IRMA) using the same immunising

preparations of cytochromes P-450 and ^{125}I -SAM. Cloning of positive cultures was by the technique of limiting dilution.

Using these methods, a number of hybridomas were generated which produced MAb's reactive with partially purified isozymes of rat liver cytochrome P-450 in a solid phase immunoassay. A list of the MAb's described in this thesis, together with their determined rat cytochrome P-450 reactivities is presented in Table 2.1 .

All MAb's produced during this project were given a unique identification code. These codes were derived initially from the MAb's primary fusion experiment number. Subsequently, wells from these fusions which were found to be positive in solid phase IRMA's were selected, and individual cell colonies cloned in sequentially numbered wells. Positive wells from these primary clonings were reselected and individual clones picked. These were then taken for secondary cloning, again in sequentially numbered wells. Positive clones at this stage were either expanded directly in liquid culture, as described in section 2.2.3 below, or were repeatedly cloned to remove any ambiguity over their clonality. Thus, the majority of MAb's produced can be identified by a 3 or 4 digit number, encoding the fusion experiment and identifying the primary cloning well number. In the case of MAb's identified by 4 digit codes, the secondary cloning well number is also identified. A final digit then encodes the total number of times that the particular MAb has been cloned. In most cases this figure indicates that MAb's were cloned twice.

Some variations in MAb designation were introduced during the time course of this project. Specifically, with the '7' series of fusions (see Table 2.1 for list of MAb's), the primary cloning wells were identified by a letter rather than by a number. In addition, the digit encoding the cloning cycles experienced by these MAb's was also not used routinely in the laboratory. This was also the case with MAb's 1/4 and 9/20/11. In all of these cases, the MAb's had been cloned twice.

2.2.3

Culture of Hybridoma Cell Lines

Hybridoma cell lines were routinely maintained in RPMI 1640 medium buffered by sodium bicarbonate and HEPES, and supplemented with L-glutamine, penicillin, streptomycin, sodium pyruvate and 10% (v/v) FCS. Cells were seeded at a density of 10^4 cells/ml and incubated at 37°C in a humidified atmosphere of 95 : 5 (by volume) air : carbon dioxide. Culture medium was changed as required. Cells were harvested by centrifugation at 150 g for 10 minutes at 4°C, for further culture, freezing or initiation of ascites tumours. Cell viability was determined by trypan blue exclusion. Frozen stocks of the hybridoma cell lines were maintained by freezing 5×10^6 viable cells in 1ml aliquots of 50 : 40 : 10 (by volume) of FCS : RPMI 1640 : DMSO, and storing them under liquid nitrogen.

2.2.4

Production of Ascites Tumours

10 week old female Balb/c mice were pre-treated with 0.5ml of pristane i.p., 14 days prior to the injection of 10^7 viable hybridoma cells i.p. (Potter *et al.*, 1972). After approximately 2 weeks, ascites fluid was collected by syringe and centrifuged at 2000 g for 10 minutes at 4°C to obtain a clear supernatant. This was fractionated by salt precipitation by taking the supernatant to 50% (w/v) saturation with ammonium sulphate, the precipitate being collected by centrifugation at 5000 g for 20 minutes at room temperature. The resulting pellet was dissolved in PBS, and extensively dialysed against several changes of PBS at 4°C.

For some procedures, MAb's prepared by the ammonium sulphate fractionation of ascites fluid were further purified by DEAE Affi-Gel Blue chromatography, as described in section 2.2.5 below. Unless otherwise stated, however, all MAb's were used as ammonium sulphate fractions of ascites fluid.

2.2.5

Monoclonal Antibody Purification by DEAE Affi-Gel Blue Chromatography

For some procedures, MAb's were purified before use by the method of Bruck *et al.* (1982). In brief, ammonium sulphate fractionated ascites fluid was dialysed against several changes of 25mM Tris hydrochloride buffer, pH 7.2, (column buffer) for a total of 24 hours at 4°C. The dialysis residue was ultracentrifuged at 100,000 g for 30 minutes at 4°C to remove particulate material, and 100mg of protein was loaded onto a 110ml (2.2cm x 30cm) column of DEAE Affi-Gel Blue resin, previously equilibrated with column buffer. The column was washed with 5 bed volumes of column buffer, and the IgG fraction eluted by the use of a 2 step elution procedure using column buffer containing either 25mM or 50mM sodium chloride. The column flow rate was 25 ml/hour and all procedures were performed at 4°C. 2ml fractions were collected and their optical density determined at 280nm (OD 280nm). The fractions were also analysed by SDS-PAGE, as described in section 2.2.8 below, and the IgG containing fractions pooled. A representative column elution profile and the corresponding SDS-PAGE analysis are shown in Figures 2.1 and 2.2 respectively for the purification of MAb 3/4/2.

2.2.6

Enzyme Linked Immunosorbent Assay

Solid phase immunoassays were performed by the ELISA method of Boobis *et al.* (1981). Wells of polystyrene microtitre plates were coated with 100µl of liver microsomes at a protein concentration of 100 µg/ml in PBS. Adsorption of the protein was achieved by incubating the plates for 60 minutes at 37°C and then for 18 hours at 4°C before use. Appropriate dilutions of the MAb's under investigation were prepared in PBS and the plates incubated with 100µl of these solutions per well, for 2 hours at 37°C. A 0.001% (v/v) solution of RAM-HRP in PBS containing 10% (v/v)

glycerol and 1% (v/v) normal rat serum (NRS) was used as second antibody at 100 μ l per well, with an incubation regime of 2 hours at 37°C. The RAM-HRP solution incorporated NRS to reduce non-specific binding of the conjugate to the adsorbed rat microsomes. After each incubation period in the assay, the plates were extensively washed with PBS containing 0.05% (v/v) Tween 20 (PBST), to remove unbound reagents. The presence of bound RAM-HRP was detected with 100 μ l per well of a 400 μ g/ml solution of OPD in a 51 : 49 (by volume) solution of 0.2M disodium hydrogen phosphate : 0.1M citric acid, at a final pH of 5.0, with hydrogen peroxide added to 0.04% (v/v) final concentration just before use (Voller *et al.*, 1979). Colour development was quantified from the optical density at 492nm (OD 492nm) using a Flow Laboratories Titretrek Multiskan spectrophotometer, following 30 minutes incubation in the dark, at room temperature.

2.2.7

Labelling of Proteins with ^{125} I

Both rat liver microsomes and partially purified isozymes of cytochrome P-450 from rat liver were labelled with ^{125} I by the chloramine T method of McConahey and Dixon (1966). Microsomal fractions were solubilised with sodium cholate and centrifuged at 105,000 g for 60 minutes at 4°C. 2mg of solubilised microsomal proteins or 0.5mg of partially purified cytochrome P-450 were iodinated with 1mCi of ^{125} I. The reaction was stopped after 60 seconds by the addition of sodium metabisulphite. Unreacted ^{125} I was removed from the reaction mixture by gel filtration on Sephadex G-50, previously equilibrated with PBS and blocked with 0.1% (w/v) BSA in PBS to prevent non-specific binding of the labelled proteins. Fractions of 250 μ l were collected and the activity present in each determined by gamma counting. The peak fractions were pooled to form the ^{125} I labelled protein preparation.

2.2.8

Polyacrylamide Gel Electrophoresis

Discontinuous SDS-PAGE was performed by the method of Laemmli (1970), with modifications described by Boobis *et al.* (1977). Separating slab gels were prepared with a 10% (w/v) acrylamide content and dimensions of 100 x 140 x 1.5 mm (height x width x thickness). Stacking gels, of dimensions 10 x 140 x 1.5 mm (height x width x thickness), contained 3% (w/v) acrylamide. All samples were prepared in 65mM Tris hydrochloride buffer, pH 6.8, containing 1% (w/v) SDS, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue as tracking dye. Samples were denatured by the addition of 0.05% (v/v) 2-mercaptoethanol, followed by boiling for 5 minutes.

Electrophoresis was performed in a model 220 cell (Bio-Rad) with approximately 20µg of sample being applied to the gel. Gels were stained for 1 hour, in 35 : 10 : 55 (by volume) methanol : acetic acid : water, containing 0.2% (w/v) Coomassie Brilliant Blue R-250. Gels were destained for 18 hours in 25 : 10 : 65 (by volume) methanol : acetic acid : water, using a model 222 charcoal diffusion destainer (Bio-Rad), and then dried under vacuum at 60°C on a model 224 gel drier (Bio-Rad).

Molecular weight standards were included in all electrophoresis runs. The standards used (with their subunit molecular weights) were as follows: BSA (69,000); catalase (58,000); glutamic dehydrogenase (53,000); ovalbumin (43,000); aldolase (40,000).

2.2.9

Determination of Protein Concentration

Protein concentrations were determined by the method of Lowry *et al.* (1951), using BSA in appropriate buffers as the protein standard.

2.2.10

Determination of Cytochrome P-450 Concentration

Cytochrome P-450 content was assayed by carbon monoxide difference spectroscopy of sodium dithionite reduced samples, using the method of Omura and Sato (1963). A Perkin-Elmer model 555 split beam scanning spectrophotometer with microprocessor-controlled background correction was used in the assay . An extinction coefficient of $91\text{mM}^{-1}\text{ cm}^{-1}$ was assumed for the carbon monoxide-bound form of reduced cytochrome P-450 between 450nm and 490nm wavelength (Omura and Sato, 1963).

Table 2.1 **Principle Reactivities of Monoclonal Antibodies**
Described in this Thesis

The principle reactivities of the MAb's described in this thesis with isozymes of rat liver cytochrome P-450 are listed below.

Mab	Rat Liver Cytochrome P-450 Specificity
1/4	Form b and/or e
3/4/2	Form c
3/9/2	Form c
3/23/2	Form c
3/26/2	Form c
7B1	Form c
7G1	Form c
7G2	Form c
7H1	Form c
7L2	Form c
7L6	Form c
8/9/2/2	Form c
9/20/11	Forms c and d
12/2/3/2	Forms c and d
12/2/3/5	Forms c and d
12/5/2/2	Form c

Figure 2.1 Elution Profile of Mab 3/4/2 Purified on DEAE Affi-Gel Blue

Mab 3/4/2 was purified on DEAE-Affi Gel Blue as described in section 2.2.5 . Fractions were analysed for their absorbance at 280nm. Arrows indicate where different buffers were applied during the chromatography procedure:

- A - Sample applied in column buffer (25mM Tris hydrochloride buffer, pH 7.2)
- B - 25mM sodium chloride in column buffer
- C - 50mM sodium chloride in column buffer

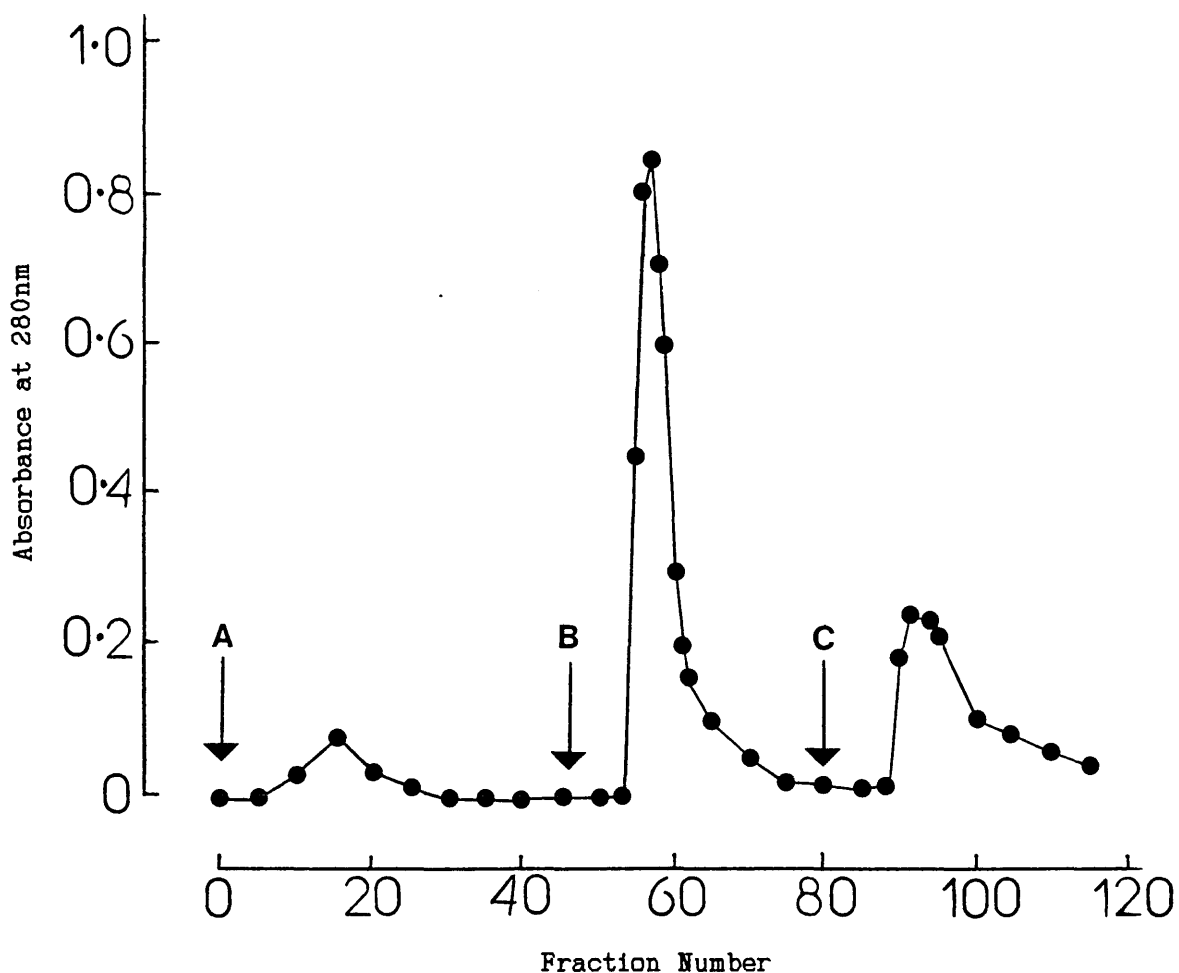


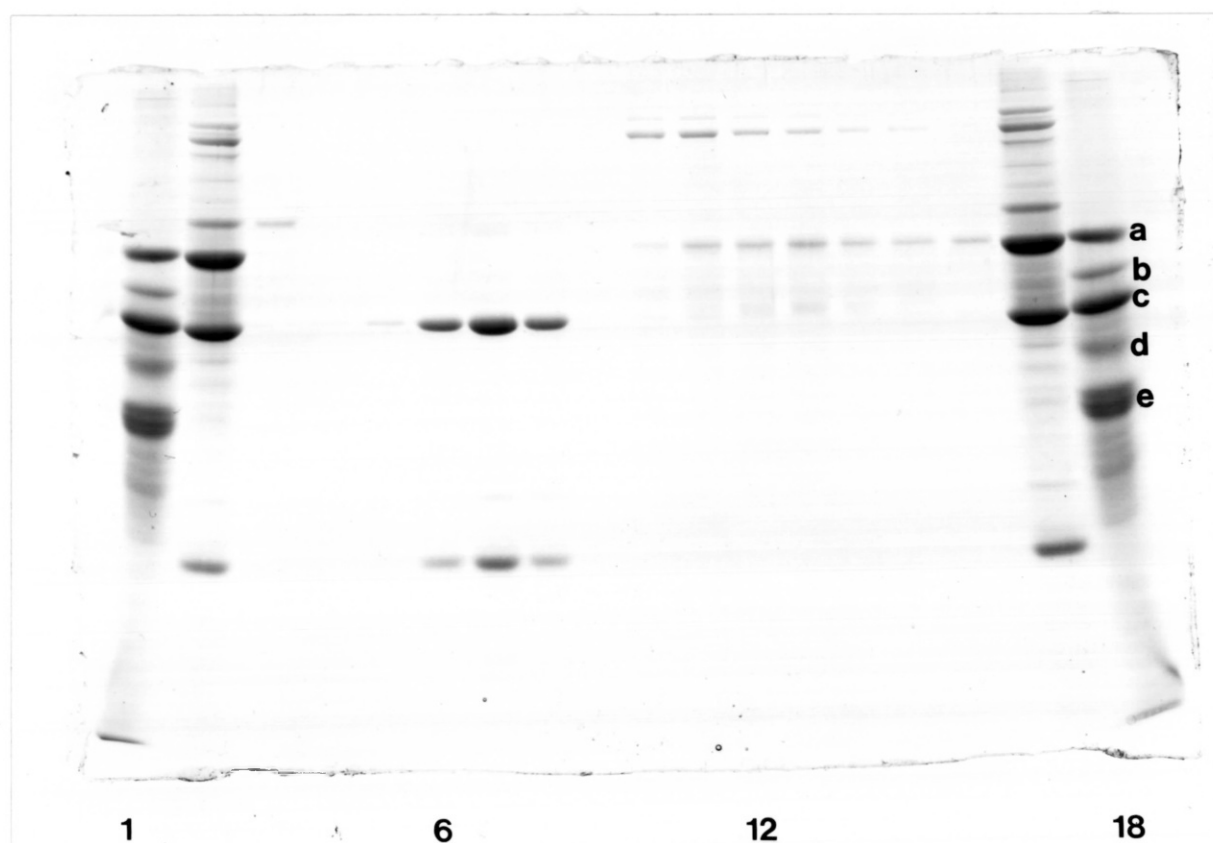
Figure 2.2 SDS-PAGE Analysis of MAb 3/4/2 Fractions Following
Chromatography on DEAE Affi-Gel Blue

Selected fractions collected during the MAb 3/4/2 purification procedure illustrated in Figure 2.1 were analysed by SDS-PAGE, as described in section 2.2.8. Tracks were loaded as follows:

1 and 18 - Molecular weight standards (a-69,000, b-58,000, c-53,000, d-43,000, e-40,000)

2 and 17 - Ammonium sulphate preparation of MAb 3/4/2

3 - Fraction 14	8 - Fraction 59	13 - Fraction 95
4 - Fraction 53	9 - Fraction 82	14 - Fraction 97
5 - Fraction 54	10 - Fraction 90	15 - Fraction 99
6 - Fraction 55	11 - Fraction 91	16 - Fraction 113
7 - Fraction 57	12 - Fraction 93	



CHAPTER 3

CHARACTERISATION OF MONOCLONAL ANTIBODIES

3.1 Introduction

There are now a wide range of murine MAb's raised against isozymes of cytochrome P-450, and Table 1.1 in Chapter 1 of this thesis summarises these MAb's with respect to their predominant cytochrome P-450 specificity. In the rat, MAb's to cytochromes P-448_H, P-450 PCN-E, P-450_p, P-450_{2E}, P-450_{1t}, P-450_{1EB}, P-450_{1εα} and P-450 forms a, b, c, d and e have been produced. In the rabbit, MAb's have been raised to cytochrome P-450 LM forms 1, 2, 3a, 3b and 4, whilst in other species, production of MAb's to several human forms of cytochrome P-450 have been reported, as have MAb's to fish cytochrome P-450E and bovine cytochromes P-450_{11B} and P-450_{SCC}. The references for these MAb's are also listed in Table 1.1 . These reports of cytochrome P-450 MAb production have been mirrored by an increasing number of publications describing the various properties and applications of these MAb's. These have included the inhibition of cytochrome P-450 isozyme activities, the immunopurification, localisation and structural analysis of cytochromes P-450, the quantitation of cytochromes P-450 by a variety of techniques and the screening of cytochrome P-450 cDNA libraries. References to such applications have previously been described in section 1.4 of this thesis.

The high specificity of MAb's makes them ideal as probes of cytochrome P-450 structure and function. However, the usefulness of any MAb can only be as good as the selection and characterisation procedures used in their production and evaluation. A MAb is directed against a single epitope on the cytochrome P-450 molecule. That epitope, however, may or may not be shared amongst other cytochrome P-450 isozymes. It is therefore vital that the reactivity profile of each MAb is determined as fully as possible, before its inclusion in any further studies. The techniques and procedures used to determine the reactivity profiles of the MAb's produced during the course of this project, together with the results and conclusions reached, are discussed in the proceeding sections of this chapter.

3.2 Methods

3.2.1

Monoclonal Antibody Production

MAB's were produced against partially purified preparations of rat liver cytochromes P-450 form c and forms b and/or e, as described in section 2.2.2 .

3.2.2

Solid Phase Immunoassays

A number of solid phase immunoassays were used in the characterisation of the MAB's produced.

3.2.2.1 Immunoglobulin Sub-Class Determination

The proteins present in supernatants from cultured hybridoma cell lines were adsorbed onto microtitre plates at a concentration of 100 µg/ml in PBS, under the same conditions as for ELISA (section 2.2.6). After washing with PBST, a 0.001% (v/v) solution of mouse immunoglobulin sub-class specific SAM-HRP in PBS, containing 10% (v/v) glycerol, was added to the coated wells, which were then incubated for 2 hours at 37°C. Binding of SAM-HRP to adsorbed MAB's was determined by the addition of 100µl of a 400 µg/ml solution of OPD, as for ELISA. The OD 492nm of the resulting solutions was determined using a Titretek Multiskan spectrophotometer.

3.2.2.2 Monoclonal Antibody Reactivity with Protein A

The proteins present in supernatants from cultured hybridoma cell lines were adsorbed onto microtitre plates as described in section 3.2.2.1 above. After washing with PBST, 100µl of either a 0.001% (v/v) solution of RAM-HRP or a 0.01% (v/v) solution of protein A-HRP (pA-HRP), both in PBS containing 10% (v/v) glycerol, were added, and the plates incubated for 2 hours at

37°C. The binding of enzyme conjugated RAM or protein A to the adsorbed MAb's was then determined as described in section 3.2.2.1 above.

3.2.2.3 Enzyme Linked Immunosorbent Assay

ELISA's were performed as described in section 2.2.6 . Microsomal preparations were adsorbed onto microtitre plates at a concentration of 100 µg/ml in PBS. Purified isozymes were used at a concentration of 5 µg/ml in PBS.

3.2.2.4 Monoclonal Antibody Reactivity with other Haemoproteins

A solid phase immunoradiometric assay (IRMA) was performed to investigate MAb reactivity with other haemoproteins. This assay was carried out in conjunction with Mr. Christopher Hayward of the Corporate Bioscience Group, ICI plc, using the same incubation regimen as for ELISA (section 2.2.6). Purified haemoproteins were adsorbed onto individual microtitre wells at a concentration of 5 µg/ml in PBS. After washing with PBST, wells were incubated with various MAb's at a concentration of 100 µg/ml in PBS. Following washing with PBST, each well was incubated with 10⁵ cpm of ¹²⁵I-SAM. After thorough washing with PBST, the counts remaining in each well were determined by gamma counting.

3.2.3

Immunoprecipitation of ¹²⁵I-Labelled Microsomal Proteins

Solubilised microsomes or partially purified isozymes of cytochrome P-450 were labelled with ¹²⁵I, as described in section 2.2.7 . MAb's under investigation were diluted in PBS to various concentrations, and incubated with 2 x 10⁵ cpm of the ¹²⁵I-labelled proteins for 18 hours at 4°C, with gentle agitation. RAM, previously affinity purified using a mouse IgG-Sepharose CL-4B column (Hudson and Hay, 1980), was added in excess, with the incubation continuing for a further 4 hours at room temperature.

Any immunoprecipitates formed were collected by centrifugation at 10,000 g for 15 minutes. The resulting pellet was washed three times with 0.1M Tris hydrochloride buffer, pH 7.4, containing 0.1M sodium chloride, 0.5% (w/v) sodium deoxycholate and 1% (v/v) Triton X-100. The pellet was then washed once with 10mM Tris hydrochloride buffer, pH 7.4, containing 50mM sodium chloride, and finally resuspended in SDS-PAGE sample preparation buffer. Preparations were then subjected to SDS-PAGE, as described in section 2.2.8, and the resulting gel autoradiographed.

3.2.4

Autoradiography

Samples for autoradiography, such as the immunoprecipitates collected in section 3.2.3 above, were resolved by SDS-PAGE, as described in section 2.2.8. After separation of the proteins, the gels were stained and destained. Gels were dried under vacuum at 60°C onto a card support using a Bio-Rad model 224 gel drier. Dried gels were exposed to X-Omat R8 X-ray film for 3 days at -70°C, in X-Omatic cassettes fitted with intensifying screens. The films were developed using DX-80 developer and fixed in FX-40 X-ray film liquid fixer.

3.2.5

Western Blotting

In some experiments, proteins separated by SDS-PAGE (section 2.2.8) were electrophoretically transferred to Hybond-C nitrocellulose membranes using a model 160/1 Trans-Blot cell (Bio-Rad). The methods used were those of Towbin *et al.* (1979) and Burnette (1981). Transfer conditions were standardised and consisted of a 4 hour transfer at 250mA current. Proteins transferred by this method were visualised by staining with 1% (w/v) Amido Black 10B in 50 : 45 : 5 (by volume) methanol : water :

acetic acid. Membranes were destained with a solution of the same composition, without stain.

The reactivity of MAb's with such transferred proteins was investigated by the following protocol. The unstained nitrocellulose membrane was incubated in a solution of 3% (w/v) BSA in PBS for 18 hours at 4°C, to block unreacted protein binding sites. Portions of the membrane were then incubated with various concentrations of MAb's in PBS, for 2 hours at room temperature, with gentle agitation. Membrane strips were then extensively washed with PBST. A 0.001% (v/v) solution of RAM-HRP in PBS, containing 10% (v/v) glycerol and 1% (v/v) NRS, was then applied, again with agitation, for a further 2 hours at room temperature, after which the membranes were again extensively washed with PBST. The presence of bound RAM-HRP was visualised by the use of a substrate system composing 2.2mM 4-chloro-1-naphthol in 25mM Tris hydrochloride buffer, pH 7.4, containing 20% (v/v) methanol, 0.14M sodium chloride and 0.03% (v/v) hydrogen peroxide (Nakane, 1968). The colour reaction was terminated after 10 minutes by placing the membranes into distilled water.

3.2.6

Competitive Binding Assays

To identify those MAb's which compete with each other for binding to their antigen, two different competitive binding assays were utilised.

3.2.6.1 Double Antibody Binding Assay

In the first series of experiments, the double antibody binding assay of Friguet *et al.* (1983) was used, modified by the use of ¹²⁵I-SAM instead of enzyme conjugated RAM. For this technique, two MAb's, or one MAb and buffer, were incubated together for 2 hours at 37°C in individual microtitre wells, previously coated under ELISA coating conditions with liver

microsomes from β NF treated rats at 1 μ g/ml in PBS. The concentrations of MAb's used were determined from previous titration experiments with ^{125} I-SAM, such that maximum binding to the microsome coated wells was achieved. The wells were then washed thoroughly with PBST and incubated with 5×10^4 cpm of ^{125} I-SAM for 18 hours at 4°C. The wells were then again washed with PBST, and the counts remaining determined by a gamma counter.

The results of these experiments were expressed in terms of an additivity index (AI), defined as follows:

$$AI = \frac{C_{1+2} - \left(\frac{C_1 + C_2}{2} \right)}{C_1 + C_2 - \left(\frac{C_1 + C_2}{2} \right)} \times 100\%$$

This equation can be simplified to:

$$AI = \left(\frac{2 C_1 + 2}{C_1 + C_2} - 1 \right) \times 100\%$$

In these equations, C_1 , C_2 and $C_1 + 2$ are the counts remaining on microsome coated wells following incubation with, respectively, the first MAb alone, the second MAb alone, and the two MAb's together (Friguet *et al.*, 1983).

If the two MAb's are incubated together and compete with each other for binding, then C_{1+2} will tend towards the mean value of C_1 and C_2 , and the AI in this case will be less than 50%. If, however, the two MAb's bind independently to separate epitopes on the molecule, then C_{1+2} will tend towards the sum of C_1 and C_2 , and the AI will be greater than 50%.

3.2.6.2 Competitive Inhibition Assay

In a second series of experiments, the competitive inhibition assay of Hewitt *et al.* (1982) was used to determine the competition in binding, if any, between a ^{14}C -labelled MAb with unlabelled MAb's. MAb 9/20/11 was internally labelled by growing the hybridoma cells in media supplemented with [^{14}C] leucine. The MAb was partially purified from the tissue culture supernatant by ammonium sulphate precipitation (section 2.2.4). The resulting preparation had a specific activity of 3×10^3 cpm/ μg of total protein.

For this assay, individual microtitre wells were coated with liver microsomes from βNF treated rats at a concentration of $100 \mu\text{g}/\text{ml}$ in PBS, under standard ELISA conditions (section 2.2.6). After washing the coated wells with PBST, serial dilutions of unlabelled MAb's in PBS were added to the wells, starting at 1mg of total protein/ ml , with the addition of a constant amount (10^4 cpm in PBS) of ^{14}C -labelled MAb 9/20/11. After incubation for 2 hours at 37°C and thorough washing with PBST, the wells were allowed to dry and placed in glass scintillation vials, to which was added 10ml of Instagel. Upon dissolution of the microtitre wells, the radioactivity present was determined by liquid scintillation spectrometry. Specific counts were calculated by subtracting those counts obtained from wells incubated with only PBS from those of the test wells. Results were expressed as a percentage of the maximum observed counts obtained, that is, ^{14}C -labelled MAb 9/20/11 incubated only with PBS.

3.3 Results

3.3.1

Solid Phase Immunoassays

3.3.1.1 Immunoglobulin Sub-Class Determination

Results are illustrated in Table 3.1 for those MAb's studied. A high OD 492nm reading obtained (1.1 - 1.3) was taken to be indicative of reactivity between a MAb and the corresponding sub-class specific SAM. Non reactivity with a particular SAM was seen as background (0.1 - 0.2) OD 492nm readings.

As can be seen, all of the MAb's produced against isozymes of rat liver cytochrome P-450 were class G immunoglobulins, and the majority were of sub-class 1 (IgG₁).

3.3.1.2 Monoclonal Antibody Reactivity with Protein A

The results illustrated in Figure 3.1 indicate that only one of the MAb's tested, namely MAb 12/5/2/2, reacted with protein A as effectively as it did with RAM.

3.3.1.3 Monoclonal Antibody Reactivity with Purified Isozymes of Cytochrome P-450

MAb's were tested for their reactivity with purified rat liver cytochrome P-450c (17.1 nmoles/mg protein) in a solid phase ELISA. In the same experiment, the reactivity of these MAb's with liver microsomes from β NF treated rats was determined for comparison. Results are shown in Figure 3.2. All of the MAb's illustrated reacted with both the purified preparation of rat liver cytochrome P-450c and with the microsomal preparation, in which this form is induced by pretreatment with β NF.

MAb 3/4/2 was studied further for its cross-reactivity with several purified isozymes of rabbit liver cytochrome P-450 in a solid phase ELISA.

From the results illustrated in Figure 3.3, it is apparent that MAb 3/4/2 shows some reactivity with all of the rabbit liver isozymes tested, when compared to the appropriate buffer only controls. However, it would appear to react more with the hydrocarbon inducible forms, namely forms 4 and 6.

3.3.1.4 Monoclonal Antibody Reactivity with Liver Microsomal Fractions

The reactivity of several MAb's with various liver microsomal preparations was determined by a solid phase ELISA. MAb's were incubated with microsomes from various control and treated animals from a number of species. The results obtained from these experiments, with MAb's raised against a partially purified preparation of rat liver cytochrome P-450c, are illustrated in Figure 3.4 .

The reactivity profiles obtained varied for most of these MAb's. Only with MAb's 7L2 and 7L6 was a similar profile observed. This similarity was, in fact, not unexpected, as these two MAb's were derived from hybridomas produced in the same initial culture well following fusion with myeloma cells. Their similar reactivities may therefore indicate that these MAb's were in fact derived from the same fusion event. These two MAb's showed a higher reactivity with microsomes from β NF treated rats than with any of the other microsomal preparations used. Only with liver microsomes from 3MC treated mice was there any other appreciable increase in binding seen with these MAb's. Whilst MAb's 3/9/2, 3/23/2, 7G1 and 7H1 all showed less binding to liver microsomes from control and PB treated rats than to those from β NF treated rats, MAb 3/4/2 produced similar results with all three of these microsomal preparations. In contrast, MAb's 3/26/2 and 7B1 showed a similar degree of binding to liver microsomes from both β NF and PB treated rats, but less binding to liver microsomes from control rats.

Similar incubations were also performed for all of the MAb's described above with liver microsomes from control, PB and β NF treated Fischer and

DA rats. For all of these MAb's, the reactivity was similar to that observed with the corresponding microsomal preparation from Wistar rats. Reactivity of the MAb's studied with rat liver microsomes would therefore appear not to be strain dependent.

The reactivity profiles of MAb's 3/9/2, 3/23/2, 7G1 and 7H1 with mouse liver microsomes were very similar to those obtained with the corresponding preparations from rat. However, in all cases the degree of binding to mouse microsomes appeared to be less than to the corresponding rat microsomal preparations. MAb 3/4/2, on the other hand, bound to mouse liver microsomes to a similar extent as it did to the rat liver microsomal preparations. Little reactivity with mouse liver microsomes was observed for MAb's 3/26/2 and 7B1. When liver microsomal preparations from other laboratory animals were tested, each MAb was found to have its own reactivity profile. This varied from the non-reactivity of MAb's 3/26/2, 7L2 and 7L6, through the variable binding of MAb's 3/4/2, 3/9/2, 7B1, 7G1 and 7H1, to the relatively high degree of binding observed with MAb 3/23/2.

With the microsomal preparations from human liver samples, only MAb 3/4/2 reacted to a degree that was comparable with the reactivities observed with liver microsomes from β NF treated rats.

3.3.1.5 Monoclonal Antibody Reactivity with other Haemoproteins

Several MAb's were studied for their ability to bind to haemoproteins other than cytochrome P-450, to determine if they bound to a common epitope, such as reductase binding site. The results obtained from a solid phase IRMA are illustrated in Figure 3.5. None of the MAb's tested showed any reactivity with any of the studied haemoproteins, other than with cytochrome P-450c. This would indicate that the epitopes the MAb's recognise on cytochrome P-450 do not appear to be present on these other haemoproteins.

3.3.2

Immunoprecipitation of ^{125}I -Labelled Microsomal Proteins

Having established by solid phase immunoassays that the MAb's described in section 3.3.1 above reacted with proteins present in liver microsomal fractions, initial immunoprecipitation experiments with ^{125}I -labelled microsomal proteins were performed to determine the molecular weights of those immunoreactive proteins. The results obtained from a representative experiment are illustrated in Figure 3.6. In this experiment, constant amounts of a ^{125}I -labelled partially purified rat liver cytochrome P-450c preparation (5.1 nmoles/mg protein) were incubated with varying amounts of several MAb's. The amount of RAM added was also kept constant. On an SDS-PAGE gel (Figure 3.6A) a heavy 50,000 molecular weight precipitation band could be observed when larger amounts of MAb (40 μg total protein and above) were used. The corresponding tracks on the autoradiogram (Figure 3.6B) are greatly distorted in this region, with the ^{125}I -labelled proteins having an apparent molecular weight of 45,000. Incubations with decreased amounts of MAb do not show this anomaly. This distortion is most likely due to the large amounts of antibody heavy chains at 50,000 molecular weight having a 'snowplough' effect on the labelled microsomal proteins, causing them to have an apparently lower molecular weight. When the amount of MAb in the incubation was reduced, this effect was eliminated, and a more accurate determination of the molecular weight of the precipitated proteins could be obtained.

In some tracks, namely 3, 5 and 8 (i.e. MAb 7B1 at 40 μg and 1 μg per incubation, and MAb 7G1 at 1 μg per incubation respectively), a band of approximately 69,000 molecular weight was observed on the SDS-PAGE gel (Figure 3.6A). This band was not radiolabelled, as seen from the corresponding tracks on the autoradiogram (Figure 3.6B), and was therefore not derived from the ^{125}I -labelled partially purified cytochrome P-450c preparation. The presence of these bands is most likely due to ascites

fluid derived proteins being trapped in the collected immunoprecipitates, and not subsequently removed due to insufficient washing of these particular precipitates.

From the autoradiographic results obtained using decreased amounts of MAb (Figure 3.6B), it is apparent that MAb's 3/4/2, 3/9/2, 7B1, 7G1, 7H1 and 7L6 all precipitated two proteins from the ¹²⁵I-labelled partially purified rat liver cytochrome P-450c preparation. These two proteins, of approximately 55,000 and 52,000 molecular weight, are in the correct molecular weight range for isozymes of rat liver cytochrome P-450. However, these results were only preliminary, and more definitive Western blotting experiments were performed to determine the molecular weights of immunoreactive proteins in various liver microsomal preparations.

3.3.3

Western Blotting of Microsomal Proteins

Western blotting studies were performed with various liver microsomal preparations to determine the number and molecular weights of those microsomal proteins recognised by the MAb's described in the above sections.

3.3.3.1 Reactivity with Liver Microsomes from SNF and PB Treated Rats

Western blotting of liver microsomal proteins from SNF treated rats was performed, and representative results are shown in Figure 3.7 . MAb's 9/20/11, 12/2/3/2 and 12/2/3/5 all reacted with two proteins of approximately 56,000 and 54,000 molecular weight. In the same experiment, MAb's 3/4/2, 3/9/2 and 7L6 reacted with only the higher molecular weight protein. In Figure 3.8, results with liver microsomal proteins from PB treated rats are shown. MAb's 3/4/2 and 1/4 each reacted with a single, but different, protein of approximately 56,000 and 54,000 molecular weight respectively. In the same experiment, however, MAb's 7L6, 3/9/2 and

9/20/11 did not react with any protein. Overall, though, all of these MAb's react with proteins of the same molecular weight range as isozymes of rat liver cytochrome P-450.

3.3.3.2 Reactivity with other Liver Microsomal Preparations

Several MAb's were investigated for their reactivity with liver microsomes from a variety of sources. The results obtained for MAb's 3/4/2, 12/2/3/2 and 1/4 are illustrated in Figures 3.9, 3.10 and 3.11 respectively. From these results, it is clear that both MAb's 3/4/2 and 12/2/3/2 react with liver microsomal preparations from more than one source. MAb 3/4/2 reacted with a protein of approximately 56,000 molecular weight from BNF, ISF and PB treated rats. With liver microsomes from ISF treated rats, reactivity was also observed with a protein of approximately 47,000 molecular weight. On incubation with blotted liver microsomes from 3MC treated rabbit, this MAb reacted with two proteins, of approximately 57,000 and 54,000 molecular weight respectively. MAb 3/4/2 was also observed to react with a protein of approximately 54,000 molecular weight, present in electroblotted human liver microsomes. No reactivity, however, was observed for this MAb with control rat liver microsomes.

MAb 3/4/2 would therefore appear to react with a protein, of the same molecular weight, present in liver microsomes of rats treated with three types of chemical inducer. This protein, by virtue of its molecular weight, is most probably cytochrome P-450c. The reactivity with the 47,000 molecular weight protein present in ISF treated rats is most probably due to the presence of a degraded form of the ISF inducible cytochrome P-450c isozyme. The immunoreactive proteins at 57,000 and 54,000 molecular weight present in 3MC treated rabbits are most probably the hydrocarbon inducible cytochrome P-450 isozymes, forms LM6 and LM4 respectively.

In contrast, MAb 12/2/3/2 reacted with proteins present only in liver microsomes from β NF and ISF treated rats. In both cases, reactivity with two proteins of approximately 56,000 and 54,000 molecular weight was observed, indicative of reactivity with cytochromes P-450, forms c and d respectively. There was more reactivity with form d than form c in liver microsomes from ISF treated rats. In contrast though, the reactivity with form c was greater in liver microsomes from β NF treated rats, when compared to ISF treated rats. Whilst the amount of immunoreactive form c in ISF treated rats was less than that in β NF treated rats, it was still more than that present in the control untreated animal, which showed no reactivity with MAb 12/2/3/2.

MAb 1/4 reacted with a protein of approximately 54,000 molecular weight in liver microsomes from PB treated rats. The molecular weight of this protein, and the induction regime of the animals concerned, would indicate that this immunoreactive protein corresponded to cytochrome P-450, forms b and/or e. The similarity in the molecular weights of forms b and e, precludes the identification of the immunoreactive protein on the basis of its molecular weight. However, form b is the major isozyme of rat liver cytochrome P-450 induced by PB treatment, and it is therefore more likely that this 54,000 molecular weight immunoreactive protein is, at least in part, form b.

3.3.4

Competitive Binding Assays

The possibility that the MAb's described in the sections above were binding to the same epitope on cytochrome P-450 was investigated for two separate groups of MAb's, using two different assay techniques.

3.3.4.1 Double Antibody Binding Assay

The results obtained for this assay are shown in Table 3.2 . These results would indicate that MAb 12/5/2/2 does not compete for binding with any of the other MAb's tested. However, the remaining MAb's, namely 3/4/2, 3/9/2, 7G2, 7L2 and 7L6, all appear to compete, both with themselves and with each other, for binding to the adsorbed liver microsomes from β NF treated rats.

3.3.4.2 Competitive Inhibition Assay

The results obtained from this assay are shown in Figure 3.12 . As would be expected, unlabelled MAb 9/20/11 inhibited the binding of ^{14} C-labelled MAb 9/20/11. Similar results were also obtained with MAb 12/2/3/2, indicating that this MAb competes with MAb 9/20/11 for binding. However, MAb 3/4/2 did not compete with the ^{14} C-labelled MAb 9/20/11, indicating that these two MAb's bind to independent epitopes on cytochrome P-450c.

3.3.5

Binding Site Studies with MAb 3/4/2

From the results obtained in the studies described above, it was apparent that MAb 3/4/2 reacts with different isozymes of cytochrome P-450 from the livers of various species, including human. Presumably then, this MAb binds to an epitope common to these isozymes. One such site could be where the haem moiety is attached to the apoprotein. This possibility was investigated by studying the effects of this MAb on some spectral properties of rat liver cytochrome P-450c.

3.3.5.1 Effect of MAb 3/4/2 on the Reduced Carbon Monoxide Difference Spectrum of Cytochrome P-450

A purified preparation of rat liver cytochrome P-450c (25 μ g of a 17.1 nmoles/mg protein preparation) was incubated with 2mg of DEAE Affi-Gel Blue purified MAb 3/4/2, for 30 minutes at 4°C. A control incubation of rat liver cytochrome P-450c, incubated under the same conditions with DEAE Affi-Gel Blue column buffer only, was also performed. The reduced carbon monoxide difference spectrum of the cytochrome P-450 was then determined, and the results obtained are shown in Figure 3.13 . It can be seen that MAb 3/4/2 binds to rat liver cytochrome P-450c at a site which does not inhibit the binding of carbon monoxide to the reduced isozyme.

3.3.5.2 MAb 3/4/2 Binding Spectrum

Although MAb 3/4/2 did not affect the formation of the reduced carbon monoxide difference spectrum of cytochrome P-450c, it was observed to cause some perturbation of this isozyme, as indicated by its effect on the oxidised difference spectrum of the haemoprotein. Pre-incubating 6mg of liver microsomes from β NF treated rats with various amounts of MAb 3/4/2 for 2 hours at 4°C produced a binding spectrum, when compared to a reference cuvette containing microsomes pre-incubated with buffer only (Figure 3.14). This spectrum required that MAb 3/4/2 was pre-incubated with the microsomes for at least 1 hour to develop fully, and was found to be MAb concentration dependent, with respect to the peak height at 425nm, relative to the baseline (Figure 3.14).

The specificity of the interaction between MAb 3/4/2 and the cytochrome P-450c present in the microsomes was investigated further, to determine if the spectrum was produced by specific antibody - antigen binding, or by non-specific protein - protein interactions. To determine this, liver microsomes from β NF treated rats were incubated as described above, with 5mg of MAb 7G1. The difference spectrum obtained is shown in Figure 3.15 . Thus, it is apparent that the trough at 405nm, relative to the

baseline, produced by MAb 3/4/2 is due to non-specific interaction with cytochrome P-450c, as a similar trough was observed with MAb 7G1. However, the peak at 425nm, relative to the baseline, obtained on incubation with MAb 3/4/2 was not observed with MAb 7G1, and would therefore appear to be due to a specific interaction of MAb 3/4/2 with rat liver cytochrome P-450c.

The binding spectrum produced by MAb 3/4/2 was similar to that produced by a type II compound, such as aniline. It was therefore possible that both MAb 3/4/2 and aniline were producing their respective binding spectra by interacting with the same site on the rat liver cytochrome P-450c molecule. This hypothesis was investigated further, by incubating liver microsomes from β NF treated rats with MAb 3/4/2 in a sample cuvette, and with buffer only in a reference cuvette, to produce the characteristic binding spectrum of MAb 3/4/2. Addition of aniline to the sample cuvette only, produced a type II binding spectrum (Figure 3.16). One possible explanation of this would be that aniline displaced MAb 3/4/2 from a common binding site. However, the addition of aniline to the reference cuvette revealed that a MAb 3/4/2 binding spectrum could still be observed in the sample cuvette (Figure 3.17). This therefore indicates that both MAb 3/4/2 and aniline can bind to cytochrome P-450c at the same time, and do not compete with each other for binding. MAb 3/4/2 and aniline can therefore produce similar binding spectra with isozymes of rat liver cytochrome P-450 which are not mutually exclusive in their production. This would imply that both produce a similar effect on the cytochrome P-450, but that this effect is mediated through two different sites on the molecule. It is therefore unlikely that MAb 3/4/2 binds to immunoreactive isozymes of cytochrome P-450 at their type II binding site.

3.4 Discussion

The aims of the experiments described in this chapter were to characterise as fully as possible the reactivity of a series of MAb's generated against isozymes of rat liver cytochrome P-450.

The majority of the MAb's produced were class G immunoglobulins, of sub-class 1 (IgG₁), which is possibly not suprising as this sub-class is the major one present in the serum of many animals, including mice (McConnell *et al.*, 1981). This predominance of immunoglobulin sub-class IgG₁ is also reflected in the general inability of the MAb's produced to bind to protein A, which is a common characteristic of mouse IgG₁ immunoglobulins (Ey *et al.*, 1978). The consequence of these findings was that purification of these MAb's had to be performed by means other than by the relatively fast and simple method of protein A affinity chromatography. The method chosen was that of ion-exchange chromatography using DEAE Affi-Gel Blue as the column matrix (Bruck *et al.*, 1982).

3.4.1

Monoclonal Antibody Reactivity with Isozymes of Cytochrome P-450

3.4.1.1 ELISA Studies

Initial experiments established that MAb's raised against rat liver cytochrome P-450c reacted with purified form c in a solid phase ELISA. Microsomal reactivity in ELISA varied, however, depending on the source of the microsomes used. Each of the MAb's in the microsomal reactivity study, with the exception of MAb's 7L2 and 7L6, had a unique reactivity with the microsomes tested. All of the MAb's reacted strongly with liver microsomes from hydrocarbon treated rats, which contain, of course, the immunising antigen. Some of the MAb's showed a similar, but reduced, reactivity with liver microsomes from hydrocarbon treated mice (MAb's 3/23/2, 7G1 and 7H1). However, MAb 3/4/2 showed greater cross-reactivity,

giving positive results with liver microsomes from the majority of the species tested, including human.

Reactivity of a MAb generated against a preparation of form c with microsomes from other induction regime or species may be due to the presence of endogenous form c in those rat microsomes tested, or to the existence of cross-reactive epitopes on other cytochrome P-450 isozymes. Such conserved regions have been identified by amino acid (Haniu *et al.*, 1984; 1986) and nucleotide (Fujii-Kuriyama *et al.*, 1984) sequencing of cytochrome P-450 isozymes. MAb reactivity with endogenous form c and cross-reactivity with conserved epitopes cannot, however, be determined by ELISA alone. The methods of immunoprecipitation of ^{125}I -labelled microsomal proteins and Western blotting of unlabelled microsomal proteins, both allow determination of the number of immunoreactive proteins in the antigen preparation, together with their molecular weights.

3.4.1.2 Immunoprecipitation and Western Blotting Studies

For several MAb's, the results of immunoprecipitation experiments disagreed with those obtained by Western blotting. The determination of the molecular weights of the immunoreactive proteins by the former technique is influenced by the presence of high concentrations of RAM heavy chains in the electrophoresed samples. However, this problem can be overcome by decreasing the amount of MAb in the primary incubation with the ^{125}I -labelled proteins. An additional discrepancy between the two techniques was in the number of immunoreactive proteins detected. In immunoprecipitation experiments, all of the MAb's in one series appeared to react with two proteins of 55,000 and 52,000 molecular weight. When the same MAb's were used in Western blotting studies, however, only a single protein of 56,000 molecular weight was detected. This discrepancy may be due to differences in the conditions to which the different preparations of cytochrome P-450 were subjected to in the two techniques. In the former case, cytochrome P-450 preparations were labelled with ^{125}I by the

chloramine T method. This technique is highly oxidising and may have partially degraded the cytochrome P-450. Such degraded species would be of a lower molecular weight than the original isozyme, but may still contain the MAb reactive epitope. Subsequent immunoprecipitation with MAb and RAM would therefore reveal more than one immunoreactive band. In Western blotting however, although the cytochrome P-450 preparation is incubated with the disulphide reducing agent 2-mercaptoethanol prior to incubation with MAb, the actual molecular weight of the isozyme remains the same. Thus, if the MAb reactive epitope is present on only one isozyme, then only a single band will be observed in Western blotting. However, it is still possible that the epitope is present on more than one isozyme, in which case bands of more than one molecular weight might be observed, provided that the different isozymes can be resolved by SDS-PAGE. Thus, Western blotting offers a more reliable and accurate means of determining the number and molecular weights of MAb reactive cytochrome P-450 isozymes. However, the technique is not without its own problems. As with immunoprecipitation, it is difficult to resolve proteins detected by a single MAb if they differ by less than approximately 1000 in their molecular weight. In addition to this, if the MAb reactive epitope consists of a group of non-contiguous amino acids brought into close proximity to each other by the tertiary structure of the protein, then the MAb will not react with the linearised protein on Western blotting. All of the MAb's described in this thesis, however, reacted with electroblotted homologous antigen preparations (that is, liver microsomes from β NF or PB treated rats), indicating that their MAb reactive epitopes are linear sequences of amino acids. This may indicate that the cytochrome P-450 preparations used for immunisation were in some way degraded, thereby exposing linear sequences of amino acids that would otherwise not be seen by the immunised mouse.

On Western blotting, some of the MAb's raised against a rat cytochrome P-450c preparation (9/20/11, 12/2/3/2 and 12/2/3/5) reacted with two proteins of 56,000 and 54,000 molecular weight with liver microsomes from β NF treated rats, corresponding to cytochrome P-450 forms c and d respectively (Iida et al., 1983). Other MAb's raised against the same preparation (3/4/2, 3/9/2 and 7L6) reacted with only the 56,000 molecular weight (form c) protein. Thus, the former group of antibodies recognise individual epitopes common to cytochrome P-450 forms c and d, whilst the latter group recognise epitopes present on form c, but not on form d. The reactivities of MAb's 3/4/2 and 12/2/3/2 with forms c and forms c and d respectively, were confirmed in Western blots with liver microsomes from ISF treated rats. When this same series of MAb's was incubated with electroblotted liver microsomes from PB treated rats, only one MAb, 3/4/2, produced any reaction. The immunoreactive protein had a molecular weight of 56,000, the same as that of form c. The molecular weight of this protein is different from that of the major isozyme induced by treatment with PB, namely form b, which has an approximate molecular weight of 54,000 (Ryan et al., 1982). This form, and possibly also form e, a minor PB inducible form with a similar molecular weight to that of form b (Ryan et al., 1982), was the only isozyme detected by MAb 1/4. This MAb did not react with any protein in electroblotted liver microsomes from β NF treated rats. In addition, none of those MAb's tested reacted with electroblotted liver microsomes from control rats.

The reactivity of any particular MAb, with the exception of MAb 3/4/2, in Western blotting experiments would appear to be restricted to microsomal preparations containing the immunising antigen. MAb's raised against a preparation of form c reacted only with microsomes from β NF or ISF treated rats, whereas MAb 1/4, raised against a preparation of form b, only reacted with microsomes from PB treated rats. There would appear to be limited cross-reactivity, therefore, between hydrocarbon (and ISF) inducible and PB

inducible forms of cytochrome P-450, at least for the range of MAb's described here.

Cross-reactivity of MAb's raised against isozymes of cytochrome P-450 has been reported by others. Reubi *et al.* (1984a) described a MAb raised against rabbit cytochrome P-450 LM1 which also reacted with form LM3b in ELISA. Thomas *et al.* (1984a) described three MAb's raised against rat liver cytochrome P-450c which, like MAb's 9/20/11, 12/2/3/2 and 12/2/3/5, also reacted with form d. The authors in that paper made the interesting observation that the cross-reacting MAb's all possessed lambda light chains, whilst the remaining, non cross-reactive, MAb's contained kappa light chains. Light chain determination was not performed on the MAb's described in this thesis, and so no such comparison could be made here. This same group reported on the production of several MAb's to the PB inducible cytochrome P-450 forms b and e (Reik *et al.*, 1985). In that range of MAb's, only two out of twelve reacted solely with form b. Interestingly, all MAb's in that report were found to possess kappa light chains. Several reports have also been published by Gelboin and his co-workers on the inter-species cross-reactivity of their MAb, 1-7-1, which, like MAb's 9/20/11, 12/2/3/2 and 12/2/3/5 above, react with cytochrome P-450 forms c and d (see review by Friedman *et al.*, 1986).

3.4.2

MAb 3/4/2 Reactivity with Isozymes of Cytochrome P-450

3.4.2.1 ELISA and Western Blotting Studies

The restricted cytochrome P-450 reactivity observed with the majority of MAb's described in this chapter was not, however, apparent with MAb 3/4/2. In addition to reacting with cytochrome P-450c in Western blots of microsomes from β NF and ISF treated rats, this MAb also reacted with a protein at 54,000 molecular weight in Western blots of a human liver microsomal preparation, and with a protein of the same molecular weight as

rat liver form c in Western blots of liver microsomes from PB treated rats. However, form c is not induced in rat livers following treatment of the animals with PB (Thomas *et al.*, 1983). In addition, if this protein was form c, then MAb 12/2/3/2 should also react with it. The molecular weights of rat liver cytochrome P-450 forms a, b and d - j are all less than that of form c (Ryan *et al.*, 1984). In work performed subsequent to this thesis, MAb 3/4/2 was found to have different Western blot reactivity profiles with proteolytically digested liver microsomes from 3MC and PB treated rats, indicative of its reactivity with different proteins (Sesardic *et al.*, 1986). Thus, the protein at 56,000 molecular weight induced by treatment of the rats with PB appears to be an isozyme of cytochrome P-450 as yet not characterised by the major laboratories. This isozyme shares the MAb 3/4/2 reactive epitope with form c, but not the MAb 1/4 reactive epitope with form b, and therefore more resembles a hydrocarbon inducible form of cytochrome P-450 than a PB inducible form.

MAb 3/4/2 does not, however, react in Western blots with liver microsomes from control rats. This is at odds with its observed reactivity with the same preparation in ELISA. As discussed above, MAb 3/4/2 reacts with forms of cytochrome P-450 present in liver microsomes from β NF, ISF and PB treated rats. The levels of these induced isozymes would be expected to be at constitutive levels in liver microsomes from control rats. Reduced or abolished reactivity would therefore be expected, and such a situation was observed in Western blots. In ELISA, however, the reactivity observed for MAb 3/4/2 with liver microsomes from control rats was comparable to that seen with microsomes from β NF or PB treated rats. This discrepancy may be due to differences in sensitivity between the two techniques, a situation also observed for MAb 3/4/2 in its reactivity with rabbit cytochrome P-450 isozymes, and discussed further below. In contrast to MAb 3/4/2, neither MAb 1/4 nor MAb 12/2/3/2 showed any reactivity with liver microsomes from control rats, either in ELISA or

Western blotting studies (Sesardic *et al.*, 1986). For these MAb's, therefore, Western blot sensitivity does not appear to be an issue. Reactivity of MAb 3/4/2, and possibly other MAb's, with microsomes from control rats may have been observed if the peroxidase substrate incubation time had been increased in the Western blotting studies. Such a step, however, would have undoubtedly increased the non-specific background staining, which would have made identification of specifically stained bands difficult.

It may be that MAb 3/4/2 does react with one or more constitutive isozymes of cytochrome P-450, due to their possession of an immunoreactive epitope, and that it is this reaction which is detected in ELISA studies. If that is the case, then it must be presumed that the MAb 3/4/2 reactive epitopes on these isozymes are denatured in some way as to render them non-reactive with the MAb in Western blotting studies. This would be the case if the epitope in these constitutive isozymes was composed by the spatial arrangement of non-contiguous amino acids. Denaturation of the isozymes prior to SDS-PAGE would effectively destroy the epitope, rendering the isozyme non-reactive in subsequent Western blot studies. Such epitopes would, however, be at variance to those postulated on isozymes reactive with MAb 3/4/2 in Western blotting studies, such as that present on form c, which are not denatured by SDS-PAGE. The extent of any epitope denaturation is likely to be variable, and may well be dependent on the actual amino acids which comprise the epitope. The specific MAb 3/4/2 reactive epitope present on cytochrome P-450c, for example, would conceivably require a greater degree of denaturation to occur before immunoreactivity was abolished. In contrast, a MAb 3/4/2 cross-reactive epitope, such as that postulated for one or more constitutive isozymes, would require less denaturation to abolish its immunoreactivity. The same procedure performed on both epitopes, such as preparation for SDS-PAGE,

could therefore produce different immunoreactivity results, such as those observed with MAb 3/4/2.

The reasons for the variant reactivity of MAb 3/4/2 with liver microsomes from control rats cannot be conclusively determined from the experimental studies described in this thesis. The arguments above, however, go some way in explaining the experimental results obtained. Further work is obviously required to determine fully the immunoreactive pattern of MAb 3/4/2 with liver microsomes from control rats. Such studies would probably require access to purified isozymes of rat cytochrome P-450 for use as reference standards in ELISA and Western blotting studies.

MAb 3/4/2 also reacted in ELISA with purified rabbit cytochrome P-450 forms LM6 and LM4, the major hydrocarbon inducible forms in this species. On Western blotting, this MAb reacted with two proteins of 57,000 and 55,000 molecular weight, present in liver microsomes from 3MC treated rabbits. The molecular weights of these proteins corresponds to cytochrome P-450 forms LM6 and LM4 respectively (Johnson, 1979). In ELISA, MAb 3/4/2 also showed some cross-reactivity with several other purified rabbit isozymes, namely forms LM2, 3b and 3c. Reactivity with these proteins was not detected in Western blots. However, the ELISA was performed with the purified isozymes, whereas the Western blotting studies were performed with liver microsomes from 3MC treated rabbits, in which the predominant forms of cytochrome P-450 present are LM4 and LM6 (Johnson *et al.*, 1979). Reactivity of MAb 3/4/2 with these other isozymes may therefore be below the detection level of this assay. The extent to which the MAb 3/4/2 epitope on forms LM2, 3b and 3c resembles that on forms LM4 and LM6 has yet to be determined, but from the reduced reactivity in ELISA it would appear to be only partial.

Such cross-species reactivity can be determined during initial MAb screening assays. Boobis *et al.* (1981) generated MAB's against rabbit

cytochrome P-450 LM4, and screened the initial fusion cultures against liver microsomes from both 3MC treated rabbits and control rats. Only those MAb's reacting exclusively with the rabbit microsomal preparation were selected for cloning and further study. However, some cross-reactivity with liver microsomes from control and PB treated rabbits was still observed for these MAb's, possibly due to the presence of constitutive amounts of form LM4.

MAb 3/4/2 appears to react, therefore, with an epitope common to several hydrocarbon inducible isozymes of cytochrome P-450. These include rat liver form c, rabbit forms LM4 and LM6, and a 54,000 molecular weight protein present in human liver, possibly analogous to rat liver form c. In addition, it also reacts with a 56,000 molecular weight protein present in liver microsomes from PB treated rats, which may be a unique form of cytochrome P-450. MAb 3/4/2 did not, however, react with rat liver cytochrome P-450d. This lack of reactivity is interesting given the reactivity of this MAb with rabbit form LM4. Sequence analyses of these two proteins shows about 70% homology between them (Kawajiri *et al.*, 1984). It is interesting to consider, therefore, that given the reactivity of MAb 3/4/2 with rat form c and rabbit forms LM4 and LM6, but not with rat form d, it is conceivable that rat form d has diverged from form c to a greater extent than rabbit form LM4 has from form LM6. MAb 3/4/2 will react with only a specific region of the cytochrome P-450 molecule, consisting of a few amino acids. However, a substitution or deletion of any of these amino acids may severely reduce the ability of MAb 3/4/2 to bind to that epitope. Such a mutation may be of no consequence to other functions of form d, and indeed it can be speculated that if only certain rat strains contained this mutation, the only apparent difference between each of the form d's present in these strains could be their ability, or lack of it, to bind to MAb 3/4/2. It would be of interest therefore, to incubate MAb 3/4/2 with Western blotted liver microsomes from several

hydrocarbon treated strains of rat, to determine if any differences in form d reactivity were apparent. In this case, the preferred treatment would be with ISF, given that it preferentially induces form d (Thomas *et al.*, 1983).

3.4.2.2 MAb 3/4/2 Binding Studies

Since MAb 3/4/2 reacts with an epitope common to several isozymes of cytochrome P-450, the possibility that such an antibody was reacting with an epitope common to other haemoproteins, such as a reductase binding site, was considered. This was examined by performing an IRMA with a range of other haemoproteins. None of the MAb's tested reacted with any haemoprotein, other than a cytochrome P-450c preparation included for comparison. Although the MAb's do not react with other haemoproteins it was still possible that they could bind at or near the site of attachment of the haem moiety to the cytochrome P-450 apoprotein, as this region is quite distinct from that of other haemoproteins (Schenkman *et al.*, 1982). The nature of the binding site of MAb 3/4/2 on cytochrome P-450c was therefore investigated. It was established that MAb 3/4/2 did not affect the reduced carbon monoxide-binding spectrum of purified rat liver cytochrome P-450c. However, it did cause a perturbation of the absolute spectrum of rat liver cytochrome P-450c, as indicated by the production of a difference spectrum with the oxidised haemoprotein. The formation of this binding spectrum was specific, concentration dependent and resembled that produced by type II compounds, such as aniline. However, MAb 3/4/2 and aniline did not compete with each other for binding.

MAb 3/4/2, therefore, binds at a site on cytochrome P-450c which, whilst not influencing the binding of carbon monoxide to the haem, causes sufficient perturbation of the haem moiety to produce a characteristic binding spectrum.

Similar studies have been reported by Thomas *et al.* (1984a) for a MAb specific for rat liver cytochrome P-450c. Like MAb 3/4/2, this MAb did

not influence the binding of aniline or carbon monoxide to rat liver cytochrome P-450c. However, unlike MAb 3/4/2, this particular MAb did not produce any spectral change when incubated with the oxidised haemoprotein, and hence presumably binds to a different epitope from MAb 3/4/2.

3.4.3

Epitope Binding Studies

The minimum number of epitopes involved in the binding of MAb's to isozymes of cytochrome P-450 was determined by competitive binding studies. Two different solid phase immunoassays were used. The first of these was the double antibody binding assay of Friquet *et al.* (1983). This technique does not require the previous purification or labelling of the MAb's studied. It thus saves on time and materials, and provides a high degree of flexibility in the choice of MAb's for study. However, it is important that MAb's are added in sufficient excess to achieve saturation of available binding sites, otherwise even when the MAb's react with the same epitope, they will bind additively. In contrast, the other competitive binding technique used, that of Hewitt *et al.* (1982), requires some initial MAb labelling and partial purification. This may therefore limit the number of MAb's that can be studied with ease at any one time. Saturated binding of the labelled MAb is not important in this assay, however, as the unlabelled MAb's are initially added in large excess, together with a constant amount of the labelled MAb.

Of the MAb's studied in the first assay, namely 3/4/2, 3/9/2, 7G1, 7L2, 7L6 and 12/5/2/2, only one, MAb 12/5/2/2 did not compete for binding with the others. All of the competing antibodies in this series reacted with cytochrome P-450 form c, but not form d in Western blots of liver microsomes from BNF treated rats. MAb 12/5/2/2 on the other hand reacted with both forms c and d. Thus, this MAb must bind to a different epitope than the other MAb's, which, from the binding assay results, is spatially

distinct from the epitope to which the other MAb's bind. However, competitive binding does not necessarily imply reactivity with an identical epitope. Several implications are possible. The MAb's may indeed recognise the same epitope on the cytochrome P-450c molecule, or they may recognise different epitopes that share common or overlapping determinants. It is also possible that two MAb's may compete with each other for binding, even though they are specific for distinct epitopes, because the proximity of the epitopes is such that it causes steric hindrance in the binding of the MAb's. Such epitopes do not have to be in a linear arrangement on the antigen. Hence, it would be more appropriate to define competitive MAb binding as the binding of two MAb's to the same spatial region of the molecule in question.

Whilst it was observed that MAb 3/4/2 competed for binding with MAb's 3/9/2, 7G1, 7L2 and 7L6, it must be specific for a separate, but possibly overlapping, epitope to that recognised by the other MAb's. This must be the case because of the different reactivities of these MAb's in both ELISA and Western blotting. Whilst MAb 3/4/2 reacts with a wide range of microsomal preparations, the other MAb's react predominantly with microsomes from hydrocarbon treated rats. This indicates that the epitope recognised by MAb 3/4/2 is not identical to the epitope recognised by the other MAb's. Therefore, these epitopes must either overlap, or be separate but in such close proximity as to allow steric hindrance of MAb binding to occur.

As discussed above, MAb 3/4/2 belongs to a series of MAb's that react only with cytochrome P-450c in Western blots of liver microsomes from β NF treated rats, and which compete with each other for binding to this preparation. MAb 12/5/2/2 represents a different series of MAb's, reacting with cytochrome P-450 forms c and d, and binding additively with the MAb's of the first series to form c. Two further members of this second series were identified using the competitive binding assay of Hewitt *et al.* (1982).

These MAb's, 9/20/11 and 12/2/3/2, competed with each other for binding, whereas MAb 3/4/2 did not. This was confirmed by work performed subsequent to this thesis, on the Western blot reactivities of these MAb's with proteolytically digested cytochrome P-450c (Sesardic *et al.*, 1986). The reactivity profile obtained with MAb 3/4/2 was totally different to those obtained with MAb's 9/20/11 and 12/2/3/2. These two MAb's gave very similar profiles, but ones which were sufficiently different to indicate that they recognised overlapping, rather than distinct, epitopes.

These epitope binding studies, together with the MAb proteolytic digestion reactivity profiles, indicate that there are a minimum of four MAb reactive epitopes on cytochrome P-450c, split into two groups, each containing two overlapping epitopes. One of the overlapping epitope groups is also present on cytochrome P-450d. The epitope recognised by MAb 12/5/2/2 may represent another epitope class present on both cytochrome P-450 forms c and d. However, no competitive binding studies were performed with it and either MAb 9/20/11 or 12/2/3/2 to determine whether these epitopes were the same or similar. In addition to its presence on rat cytochrome P-450c, the epitope recognised by MAb 3/4/2 is also present on cytochrome P-450 isozymes from a range of species, including human. The epitope reactivity of MAb's 3/4/2 and 12/2/3/2 were further studied subsequent to this thesis. These investigations used computer algorithm analysis of reported hydrocarbon inducible cytochrome P-450 sequences, coupled with isozyme denaturation and MAb binding studies (Edwards *et al.*, 1987). The results of this investigation concluded that the binding site for MAb 3/4/2 lay between amino acids 380-385, whilst that for MAb 12/2/3/2 lay between amino acids 174-182. Epitope mapping studies by Song *et al.* (1985b) indicated that with their series of MAb's, at least three distinct classes of epitopes could be distinguished on cytochrome P-450 isozymes induced by treatment with 3MC. No distinction was made as to whether this was for form c, form d or both. Work

reported by Thomas *et al.* (1984a) indicated that at least six MAb reactive epitopes are present on cytochrome P-450c, with one of the epitopes also being present on cytochrome P-450d. Subsequent Western blotting experiments by this group (Thomas *et al.*, 1984b) indicate that a number of these cytochrome P-450c MAb reactive epitopes are conserved amongst several species. Similar studies on a range of MAb's to cytochrome P-450 isozymes induced by treatment with PB have been reported by Reik *et al.* (1985). In that study, results indicated that there are at least six spatially distinct epitopes present on cytochrome P-450b, together with evidence for a further three overlapping ones.

It is impossible to determine if any of the reported cytochrome P-450 forms c and d epitopes are identical to those identified in this chapter, without comparative epitope binding studies being performed with the various MAb's. However, it is apparent that the range of MAb's described in this chapter which react with cytochrome P-450c do not fully cover the range of identified MAb reactive epitopes.

3.4.4.

Conclusion

Several series of MAb's were generated against partially purified preparations of rat liver cytochromes P-450. The reactivity of these MAb's to various preparations of cytochrome P-450 was investigated by a number of techniques, including solid phase immunoassays, Western blotting and competitive binding assays. Several MAb's reacted with cytochrome P-450 preparations from a number of sources and species. MAb 3/4/2, however, showed the widest reactivity profile, reacting with liver cytochrome P-450 isozymes from β NF, ISF and PB treated rats, 3MC treated rabbit, and control human, as well as with liver microsomal preparations from a variety of other species. Other MAb's, such as 1/4 and 12/2/3/2,

were found to react with cytochrome P-450 preparations to which they had been raised, or which contained homologous antigen.

On the basis of their observed cytochrome P-450 reactivities, some of the MAb's produced during this project were selected for further study. These MAb's were 3/4/2 and 12/2/3/2, raised against a preparation of cytochrome P-450c and MAb 1/4, raised against a preparation of cytochrome P-450b. At this stage in the project, a control MAb, B3A3, was selected for inclusion in these further studies as a negative control. This MAb, an IgG₁ sub-class immunoglobulin, was raised against bovine growth hormone and was a gift from Dr. Richard Robson and Mr. Christopher Hayward, of the Corporate Bioscience Group, ICI plc. It did not react with any cytochrome P-450 preparation when tested in a variety of assays. In the few specific experiments in which MAb B3A3 was not included, other control antibody preparations were used. The use of these are indicated in the relevant method sections.

The use of these MAb's in the immunopurification, quantification and tissue localisation of isozymes of cytochrome P-450, and their affect on cytochrome P-450 dependent monooxygenase activities, is described in the following chapters of this thesis.

Table 3.1 Determination of Monoclonal Antibody Immunoglobulin Sub-Class

MAb sub-class determination was performed as described in section 3.2.2.1 . The results for the following MAb's are expressed as the average of duplicate OD 492nm determinations. A high OD 492nm reading (1.1 - 1.3) is indicative of reactivity between a MAb and the corresponding sub-class specific SAM. Non reactivity with a particular SAM is seen as background (0.1 - 0.2) OD 492nm readings.

A - MAb 3/4/2	C - MAb 9/20/11	E - MAb 1/4	G - MAb 7G1
B - MAb 3/9/2	D - MAb 12/2/3/2	F - MAb 7B1	H - MAb 7L2

The following sub-class specific SAM-HRP's were used :

- 1 - Anti mouse IgG, IgM and IgA (heavy and light chains)
- 2 to 6 - Anti mouse IgG₁, IgG_{2a}, IgG_{2b}, IgM, IgA respectively

MAb SAM	A	B	C	D	E	F	G	H
1	1.30	1.22	1.25	1.29	1.22	1.20	1.26	1.25
2	1.20	0.20	1.30	0.13	0.15	1.25	1.23	1.20
3	0.15	1.16	0.20	1.18	1.20	0.15	0.14	0.17
4	0.20	0.18	0.15	0.16	0.20	0.17	0.18	0.20
5	0.20	0.16	0.14	0.19	0.14	0.15	0.17	0.15
6	0.20	0.15	0.17	0.13	0.15	0.15	0.18	0.17

Figure 3.1 Comparison of Protein A and Rabbit Anti-Mouse IgG Binding to Various Monoclonal Antibodies

Monoclonal antibody reactivity with protein A-HRP (▨) or RAM-HRP (▧) was determined as described in section 3.2.2.2 . Results are expressed as the average OD 492nm of duplicate determinations.

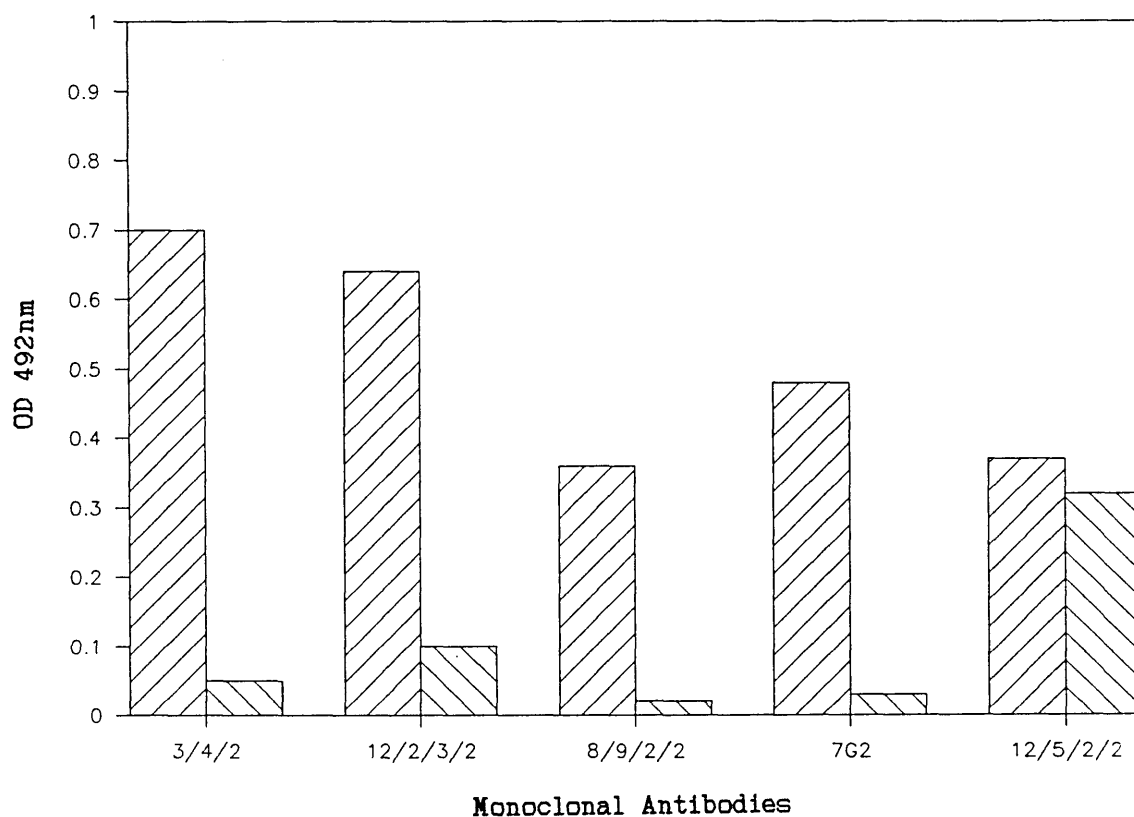


Figure 3.2 Reactivity of Monoclonal Antibodies with Purified and
Microsomal Rat Liver Cytochrome P-450c

Microtitre plates were coated with either purified rat liver cytochrome P-450c (5 µg/ml) (▨) or liver microsomes from βNF treated rats (100 µg/ml) (▧) . MAb's were tested at a concentration of 100 µg/ml. Conditions were otherwise as described in section 3.2.2.3. The results are expressed as the average OD 492nm of duplicate determinations.

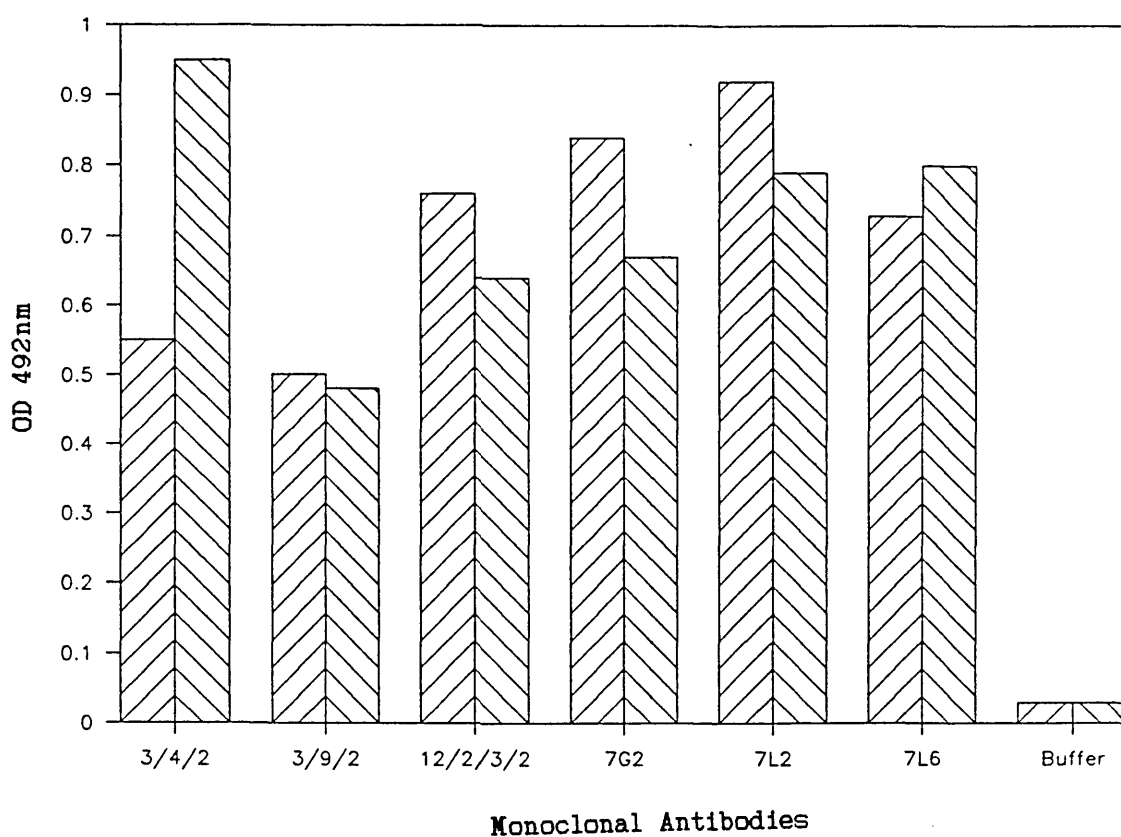


Figure 3.3 Reactivity of MAb 3/4/2 with Purified Isozymes of Rabbit
Cytochrome P-450 in a Solid Phase ELISA

Purified isozymes of rabbit liver cytochrome P-450 were coated onto microtitre plates at 5 µg/ml, whilst liver microsomes from β NF treated rats (indicated as 'Control' in figure) were coated at 100 µg/ml. MAb 3/4/2 was used at a concentration of 100 µg/ml. Conditions were otherwise as described in section 3.2.2.3. The results are expressed as the average OD 492nm of duplicate measurements.

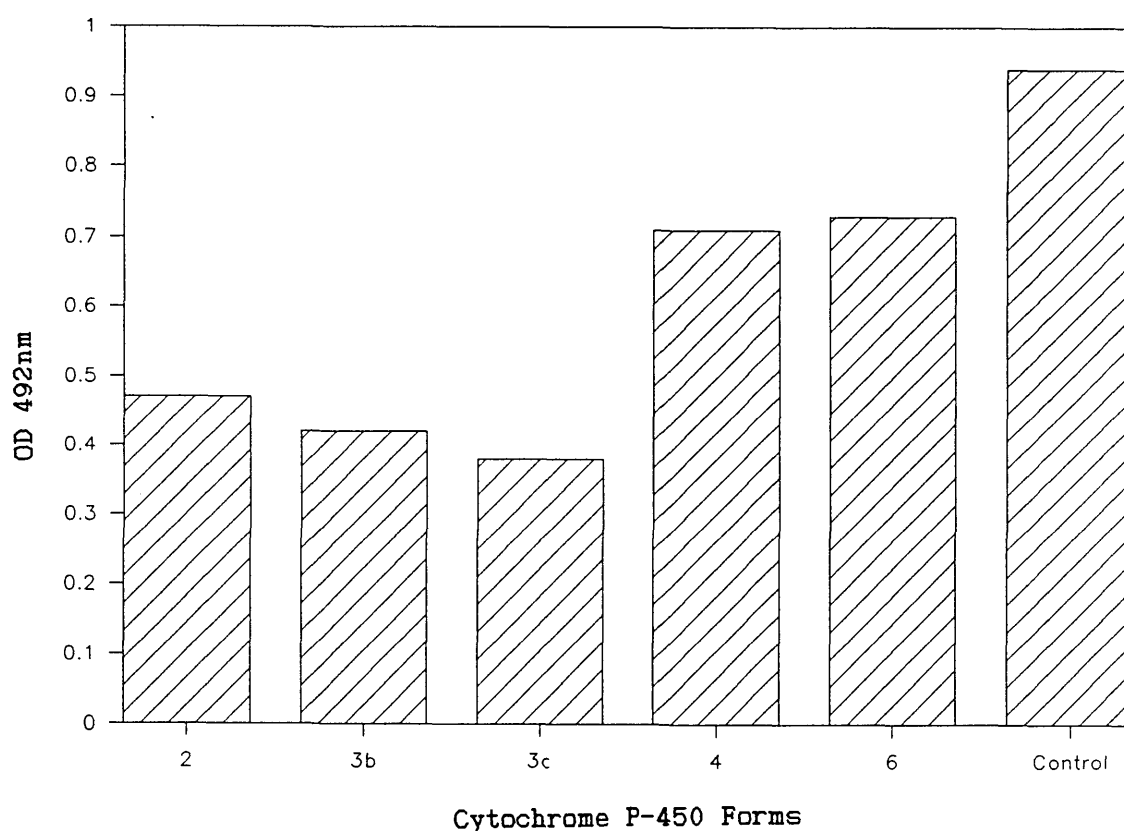


Figure 3.4 Reactivity of Monoclonal Antibodies with Liver Microsomal Preparations

MAb's, raised against rat liver cytochrome P-450c, were tested for their reactivity with liver microsomes from various species and pretreatment regimes, using the solid phase ELISA described in section 3.2.2.3 . Microsomes were coated at 100 µg/ml, and MAb's were tested at a concentration of 100 µg/ml. Results are expressed as the average OD 492nm of duplicate determinations.

The following liver microsomal preparations were used with each MAb :

- 1 - βNF treated Wistar rat
- 2 - PB treated Wistar rat
- 3 - Control Wistar rat
- 4 - Control Syrian hamster
- 5 - Control Dunkin-Hartley guinea pig
- 6 - Control baboon
- 7 - Control pig
- 8 - 3MC treated NZW rabbit
- 9 - 3MC treated Balb/c mouse
- 10 - PB treated Balb/c mouse
- 11 - Control Balb/c mouse
- 12 - Control human
- 13 - Blank (no microsomes present)

Figure 3.4 continued

The following MAb's were tested in this assay :

- A - MAb 3/4/2
- B - MAb 3/9/2
- C - MAb 3/23/2
- D - MAb 3/26/2
- E - MAb 7B1
- F - MAb 7G1
- G - MAb 7H1
- H - MAb 7L2
- I - MAb 7L6

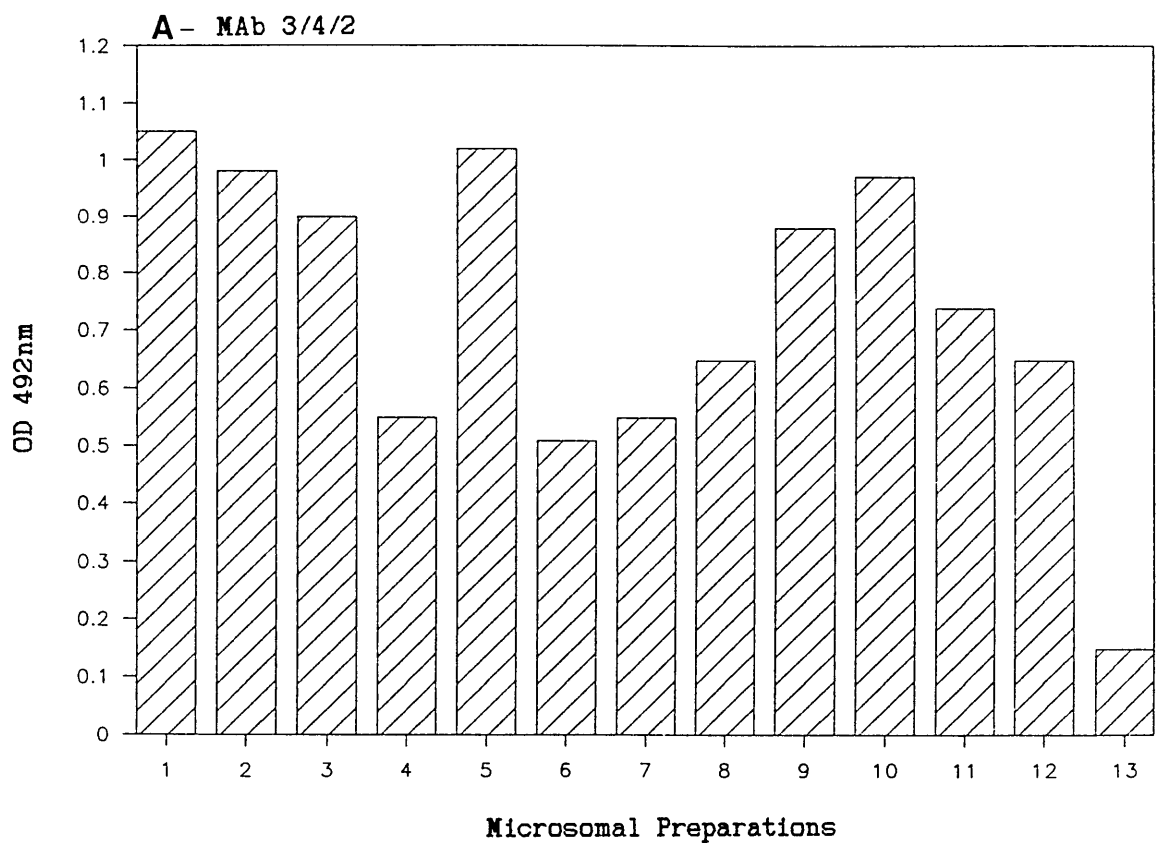


Figure 3.4 continued

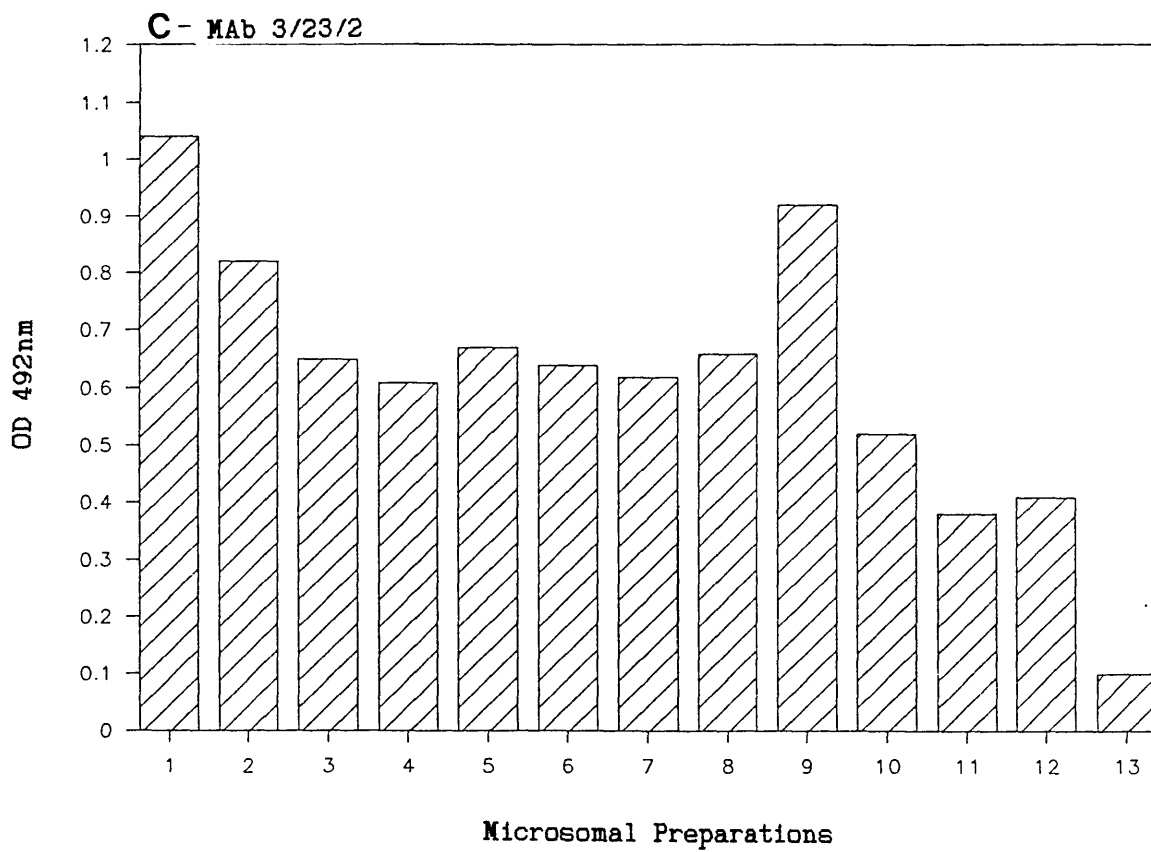
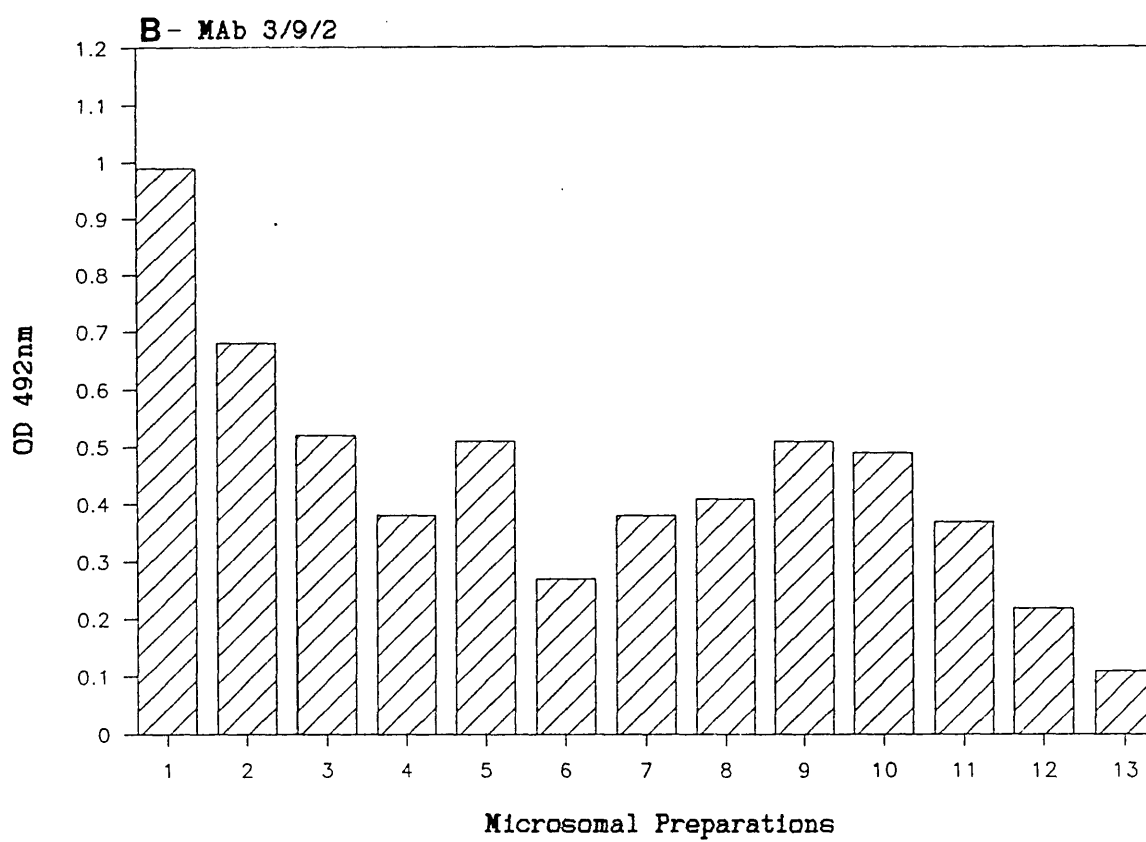


Figure 3.4 continued

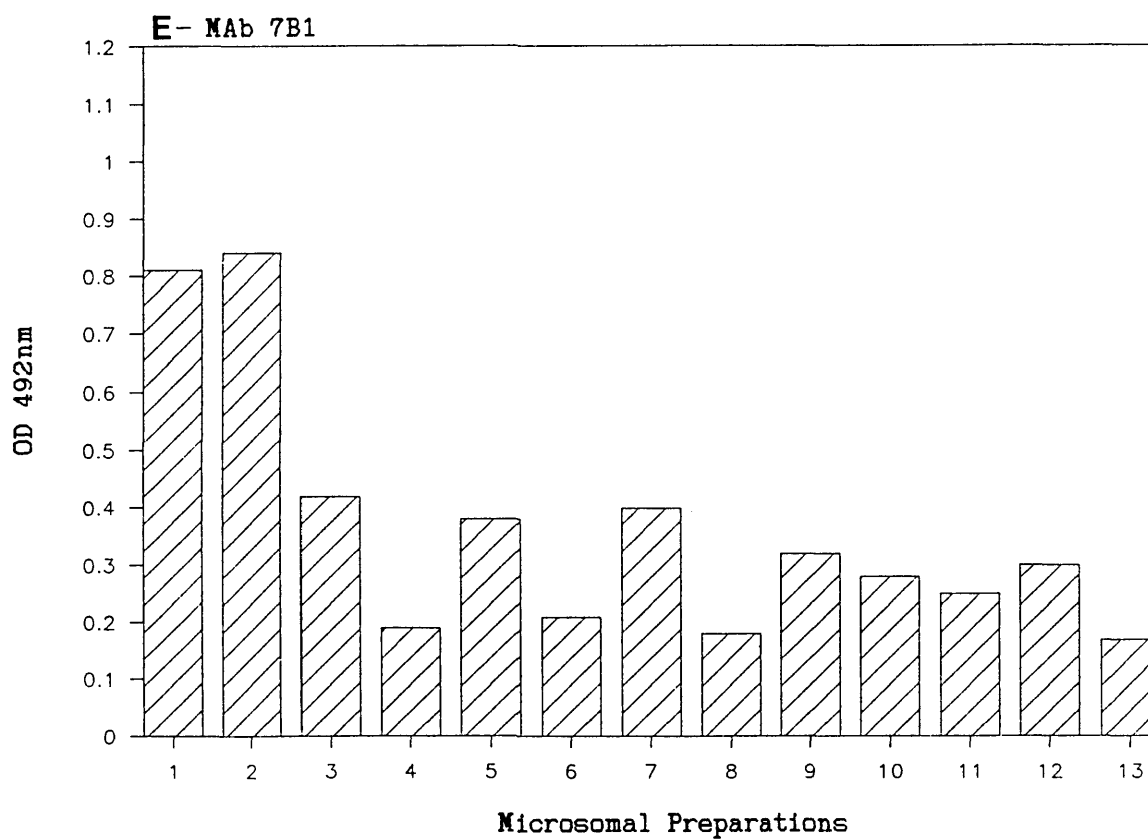
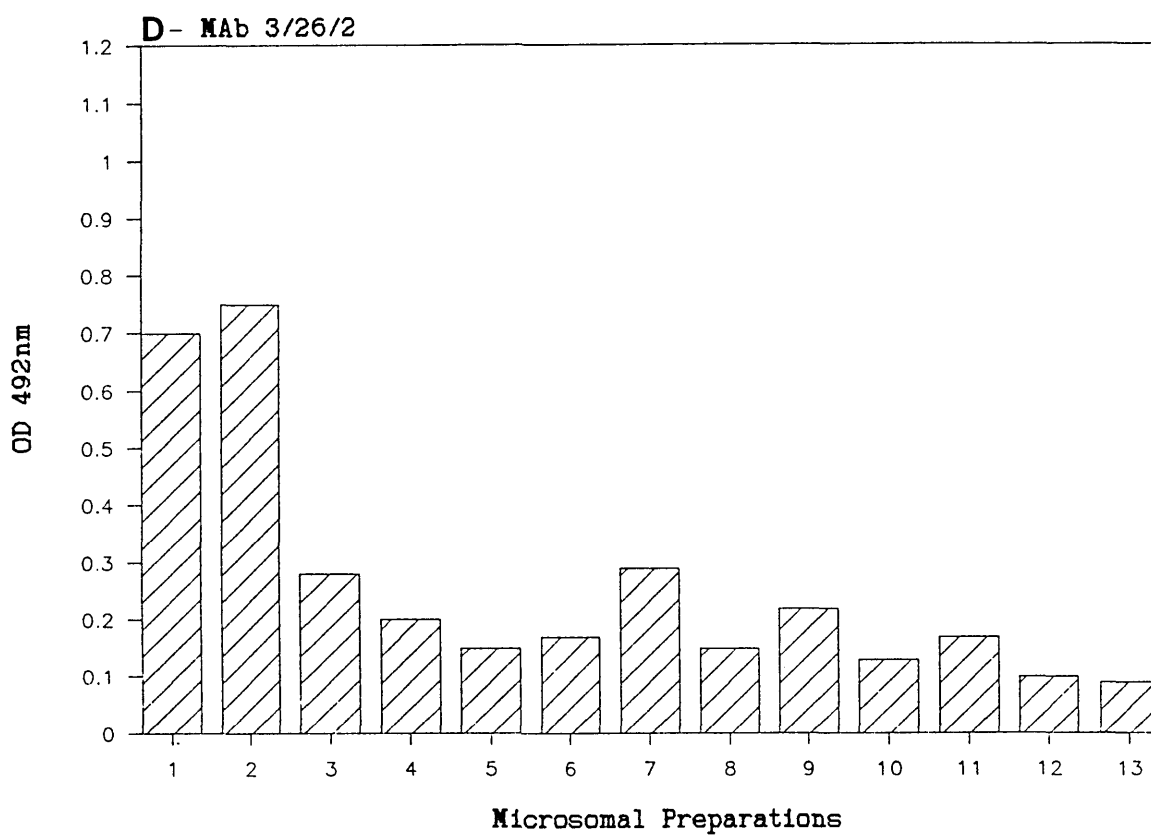


Figure 3.4 continued

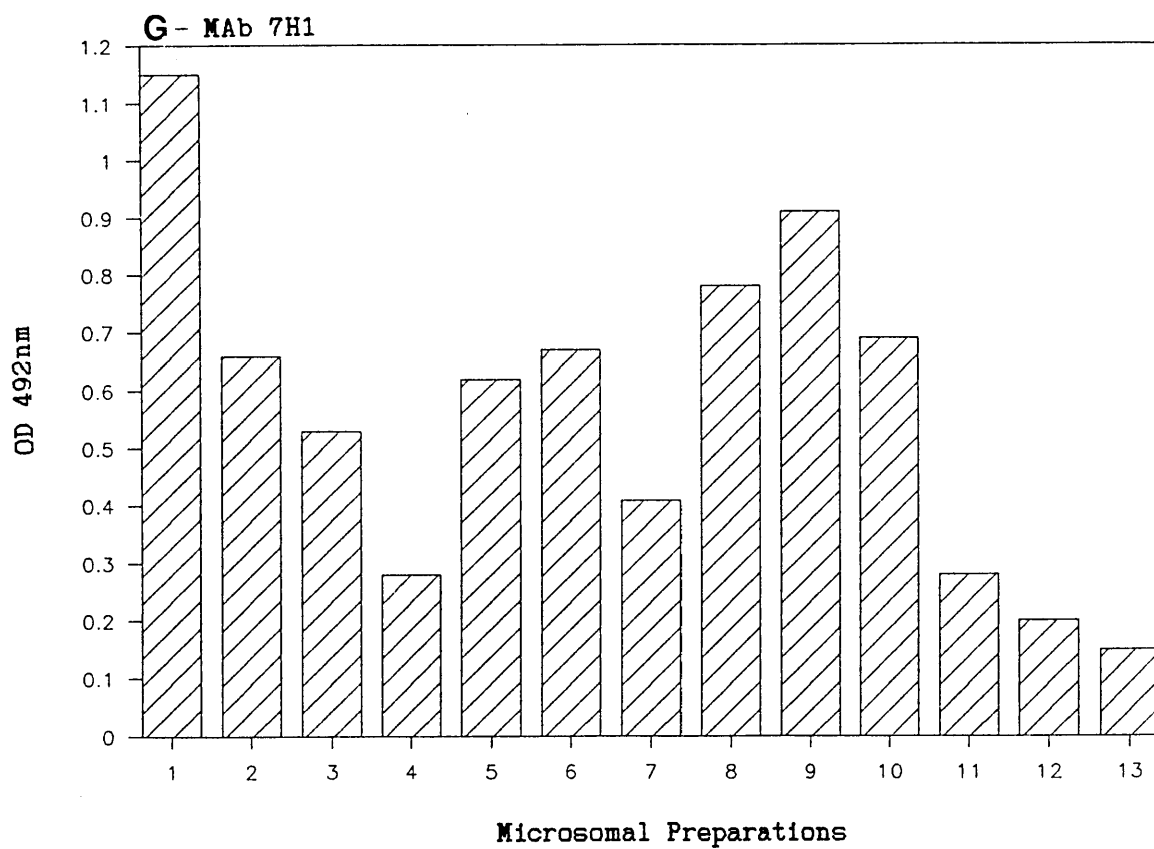
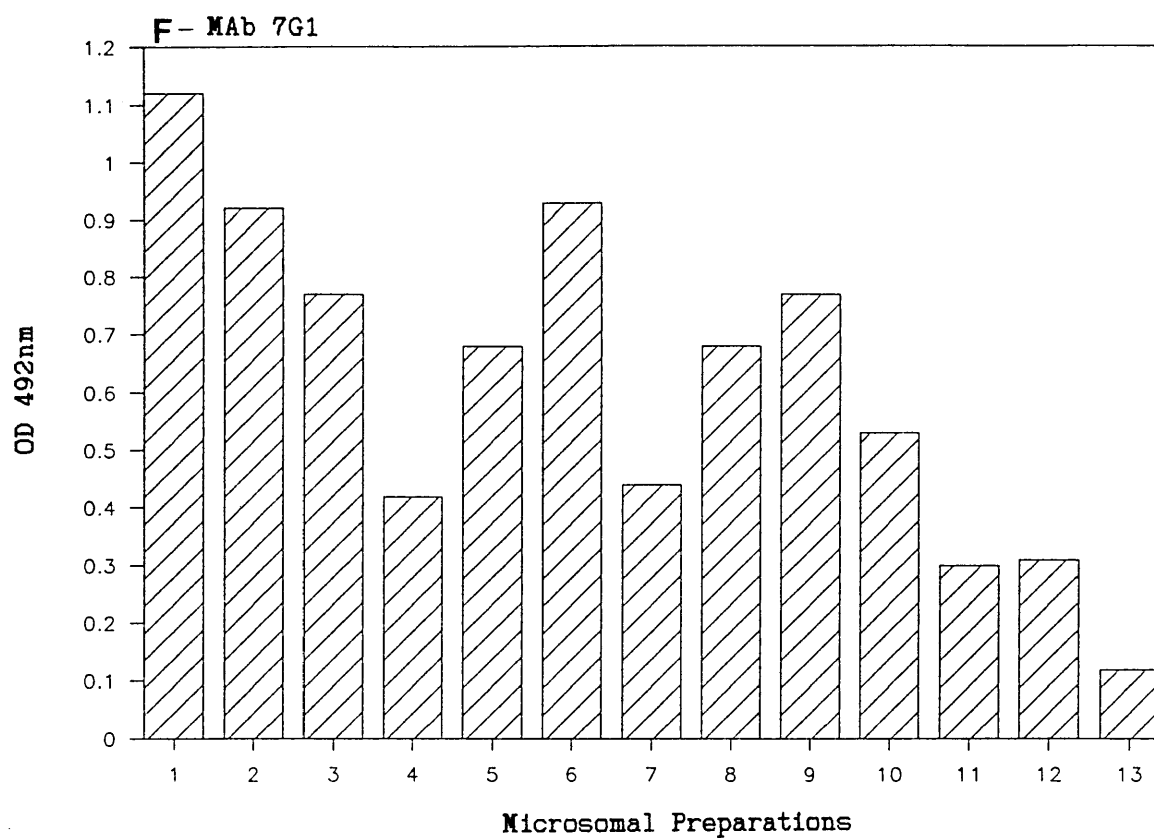


Figure 3.4 continued

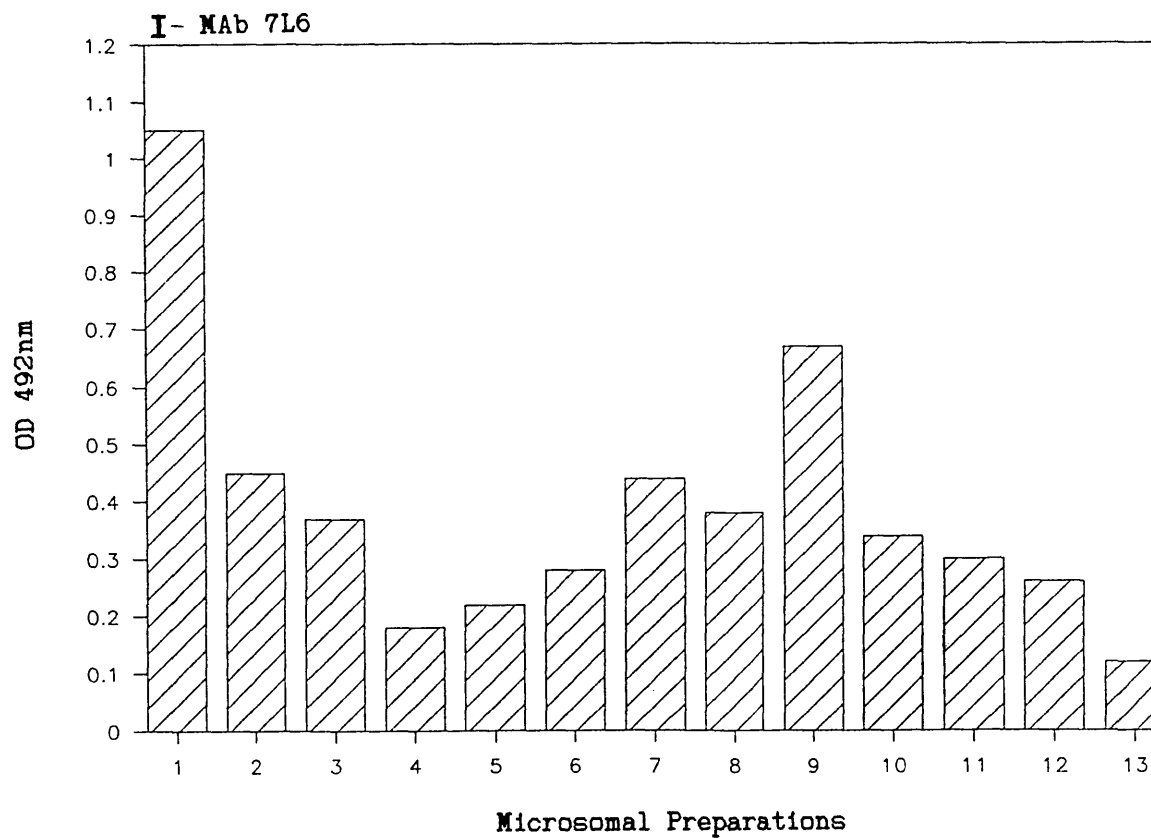
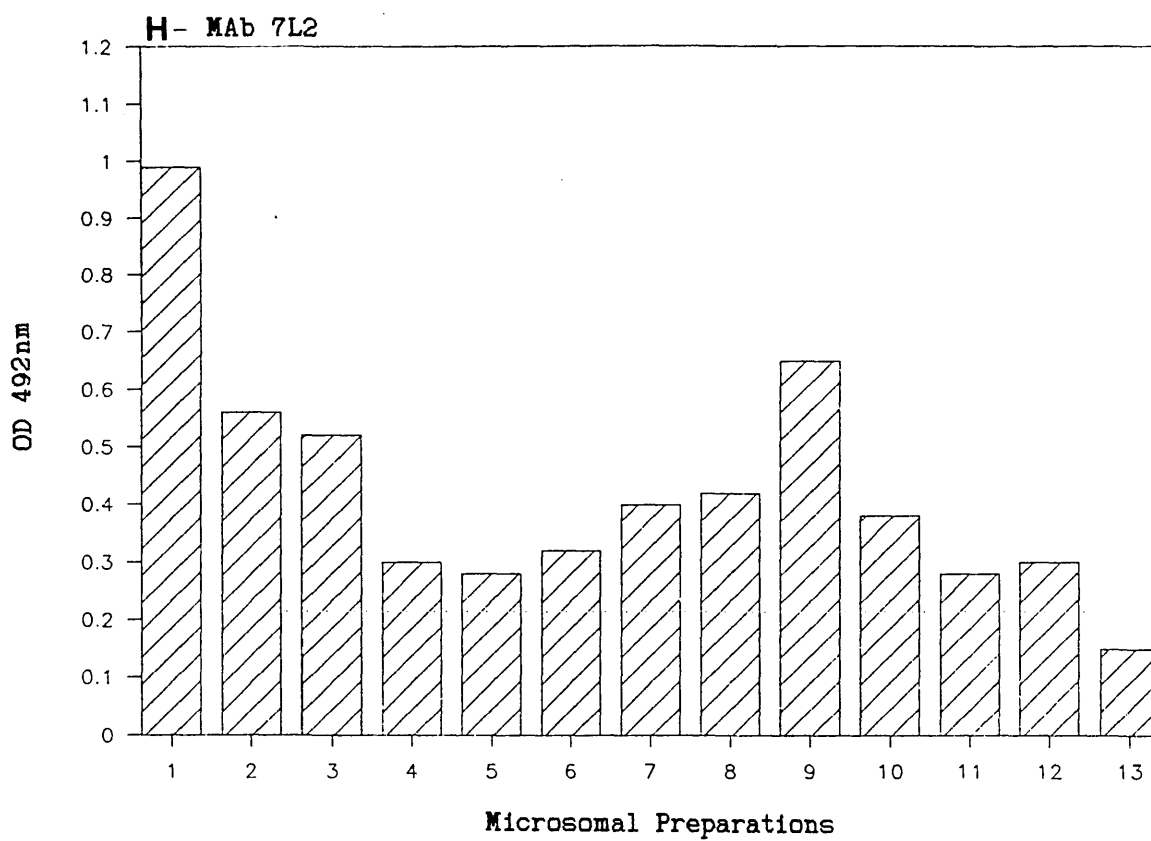


Figure 3.5 Monoclonal Antibody Reactivity with Various Haemoproteins

Purified haemoproteins (5 µg/ml) were adsorbed onto individual microtitre wells. A partially purified preparation of rat liver cytochrome P-450c (25 µg/ml) was used as a comparison. MAb's 3/4/2 (▤) , 3/9/2 (▥) and 3/26/2 (▧) were used at a concentration of 100 µg/ml, and assays were performed as described in section 3.2.2.4 . The results are expressed as cpm of ¹²⁵I-SAM remaining bound to wells, and are the average of duplicate determinations.

The following haemoprotein preparations were used :

- | | |
|--|----------------------------|
| 1 - Partially purified rat liver cytochrome P-450c | |
| 2 - Horse Type IV cytochrome c | 6 - Human myoglobin |
| 3 - Pigeon Type XIV cytochrome c | 7 - Human haemoglobin |
| 4 - Tuna Type XI cytochrome c | 8 - Bovine catalase |
| 5 - Keyhole limpet haemocyanin | 9 - Horseradish peroxidase |

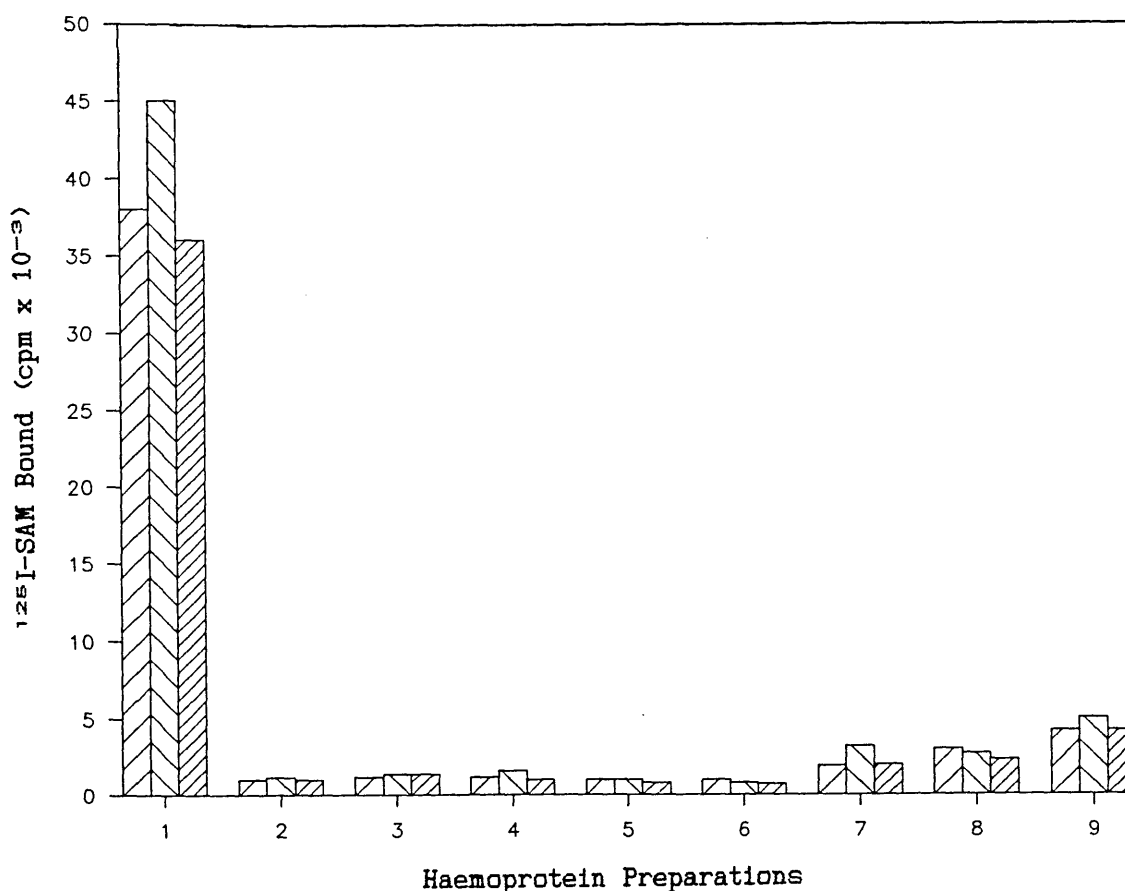


**Figure 3.6 SDS-PAGE and Autoradiogram of ^{125}I -Labelled Microsomal
Proteins Immunoprecipitated by Monoclonal Antibodies**

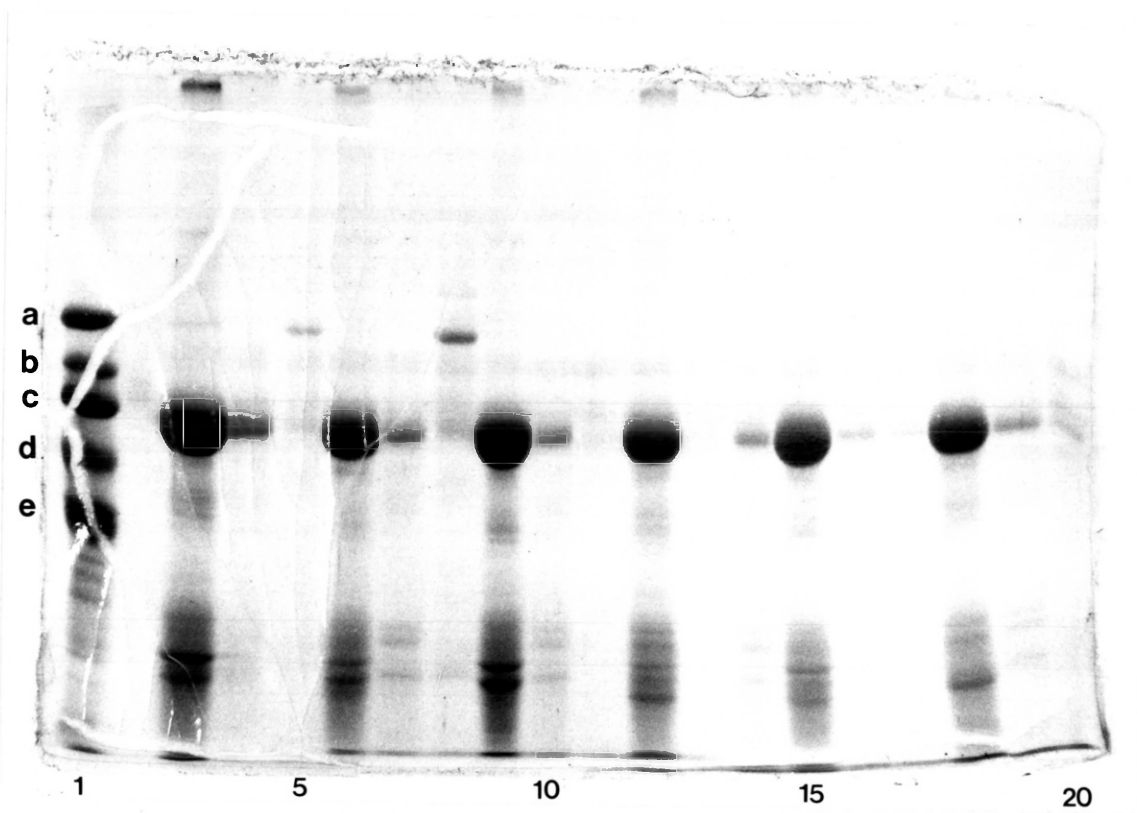
A partially purified preparation of rat liver cytochrome P-450c was labelled with ^{125}I as described in section 2.2.7 . Aliquots containing 2×10^6 cpm were incubated with various concentrations of MAb and precipitated by the addition of excess RAM as described in section 3.2.3. The washed precipitates were analysed by SDS-PAGE (A) as described in section 2.2.8 , and an autoradiogram (B) was produced as described in section 3.2.4 .

Tracks were loaded as follows :

- 1 - Molecular weight standards (a-69,000, b-58,000, c-53,000, d-43,000, e-40,000)
- 2 - ^{125}I labelled microsomal proteins not incubated with any MAb's
- 3-20 - Immunoprecipitates produced by various amounts of the following MAb's :
 - 3-5 - MAb 7B1, at 40, 10 and 1 μg respectively
 - 6-8 - MAb 7G1, at 40, 10 and 1 μg respectively
 - 9-11 - MAb 7H1, at 60, 10 and 1 μg respectively
 - 12-14 - MAb 7L6, at 40, 1 and 10 μg respectively
 - 15-17 - MAb 3/9/2, at 45, 10 and 1 μg respectively
 - 18-20 - MAb 3/4/2, at 80, 10 and 1 μg respectively

Figure 3.6 continued

A SDS-PAGE



B Autoradiogram

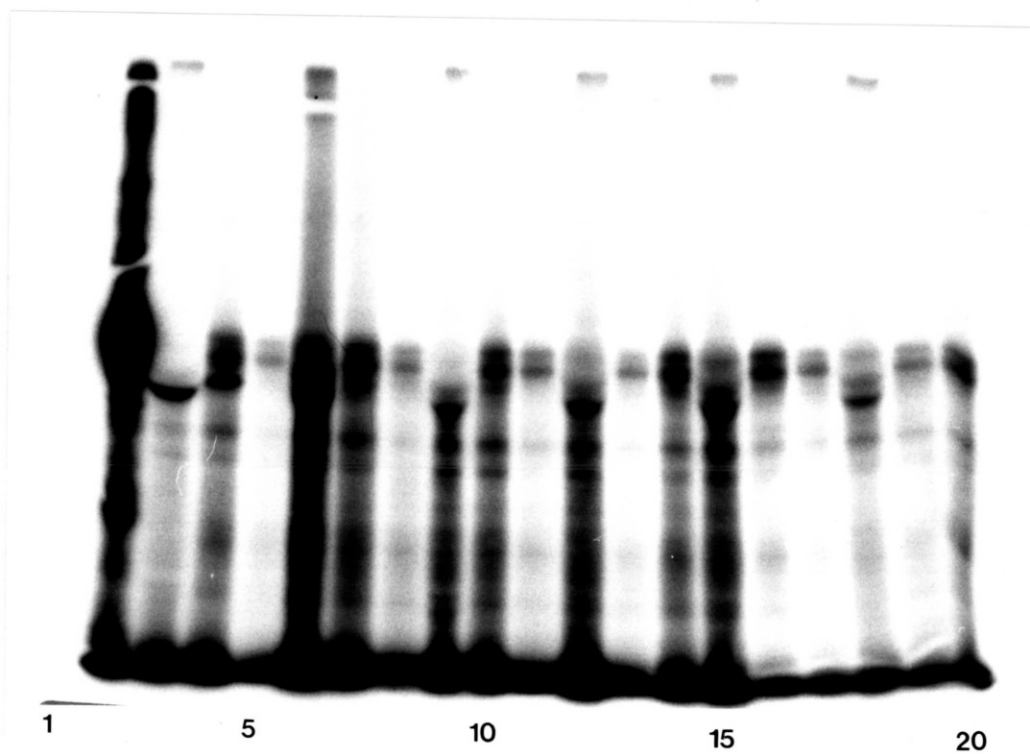


**Figure 3.7 Western Blot Reactivity of Monoclonal Antibodies with Liver
Microsomes from BNF Treated Rats**

Liver microsomes from BNF treated rats were subjected to SDS-PAGE as a single track across the width of the gel, and subsequently electroblotted onto Hybond-C nitrocellulose membrane as described in section 3.2.5 . The membrane was cut into strips and the reactivity of various MAb's (at a concentration of 100 µg/ml) determined as described in section 3.2.5 .

Strips were incubated with the following MAb's :

- | | |
|--------------|--------------|
| 1 - 3/4/2 | 4 - 12/2/3/2 |
| 2 - 9/20/11 | 5 - 3/9/2 |
| 3 - 12/2/3/5 | 6 - 7L6 |

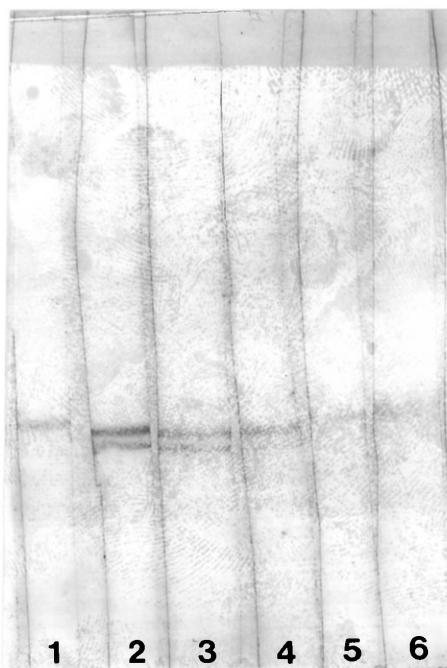


**Figure 3.8 Western Blot Reactivity of Monoclonal Antibodies with
Liver Microsomes from PB Treated Rats**

Liver microsomes from PB treated rats were subjected to SDS-PAGE and electroblotting using the conditions described for Figure 3.8 .

The following MAb's were tested for their reactivity at a concentration of 100 µg/ml :

- | | |
|-----------------|---------------|
| 1 - MAb 1/4 | 4 - MAb 3/9/2 |
| 2 - MAb 3/4/2 | 5 - MAb 7L6 |
| 3 - MAb 9/20/11 | |

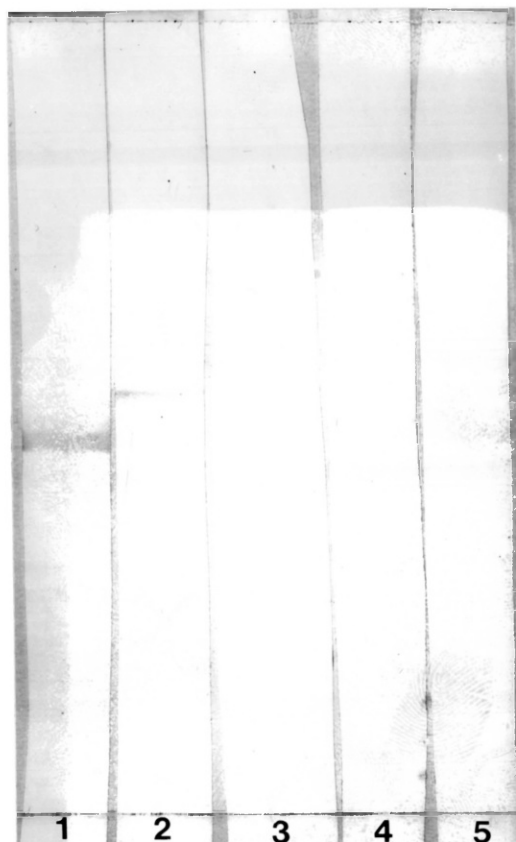


Figure 3.9 Western Blot Reactivity of MAb 3/4/2 with Various
Liver Microsomal Preparations

Various liver microsome preparations were subjected to SDS-PAGE and electroblotted as described in section 3.2.5 . The nitrocellulose sheet was then incubated with MAb 3/4/2 at a concentration of 100 µg/ml and immunoreactivity determined as described in section 3.2.5 .

The following microsomal preparations were tested :

- | | |
|----------------------------------|------------------------------|
| 1 - β NF treated rat liver | 4 - Control rat liver |
| 2 - ISF treated rat liver | 5 - 3MC treated rabbit liver |
| 3 - PB treated rat liver | 6 - Control human liver |



**Figure 3.10 Western Blot Reactivity of MAb 12/2/3/2 with Various
Liver Microsomal Preparations**

Various liver microsomal preparations were subjected to SDS-PAGE and electroblotted as described in section 3.2.5 . The nitrocellulose sheet was then incubated with MAb 12/2/3/2 at a concentration of 100 µg/ml and immunoreactivity determined as described in section 3.2.5 .

The following microsomal preparations were tested :

- | | |
|----------------------------|------------------------|
| 1 - β NF treated rat | 4 - Control rat |
| 2 - ISF treated rat | 5 - 3MC treated rabbit |
| 3 - PB treated rat | 6 - Control human |



**Figure 3.11 Western Blot Reactivity of MAb 1/4 with Various
Liver Microsomal Preparations**

Various liver microsomal preparations were subjected to SDS-PAGE and electroblotted as described in section 3.2.5 . The nitrocellulose sheet was then incubated with MAb 1/4 at a concentration of 100 µg/ml and immunoreactivity determined as described in section 3.2.5 .

The following microsomal preparations were tested :

- | | |
|----------------------------|------------------------|
| 1 - β NF treated rat | 4 - Control rat |
| 2 - ISF treated rat | 5 - 3MC treated rabbit |
| 3 - PB treated rat | 6 - Control human |

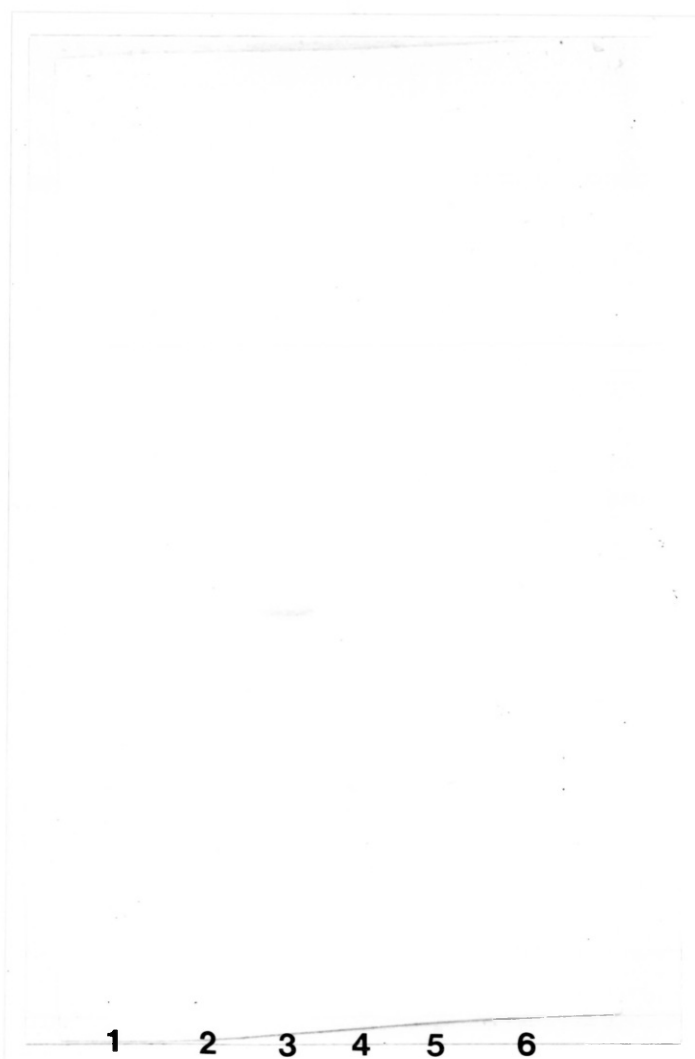


Table 3.2 Competitive Binding of Monoclonal Antibodies to Liver

Microsomes from SNF Treated Rats

The competitive binding of the MAb's below was determined as described in section 3.2.6.1 . Results are expressed as the additivity index derived from the means of triplicate determinations.

MAb	3/4/2	3/9/2	12/5/2/2	7G2	7L2	7L6
3/4/2	6.7	33.4	81.7	11.6	12.7	24.1
3/9/2	-	8.4	80.3	8.1	16.7	13.2
12/5/2/2	-	-	2.6	54.9	64.6	71.2
7G2	-	-	-	10.3	0.4	9.2
7L2	-	-	-	-	6.9	16.5
7L6	-	-	-	-	-	4.5

Figure 3.12 Competitive Inhibition of ^{14}C -Labelled MAb 9/20/11 Binding to Liver Microsomes from BNF Treated Rats

Competitive binding of MAb's 3/4/2 (▲), 9/20/11 (■) and 12/2/3/2 (●) with ^{14}C -labelled MAb 9/20/11 to liver microsomes from BNF treated rats was determined as described in section 3.2.6.2 . Results are expressed as the percentage binding of ^{14}C -labelled MAb 9/20/11 to that obtained in the absence of competing antibody, and are the average of duplicate determinations.

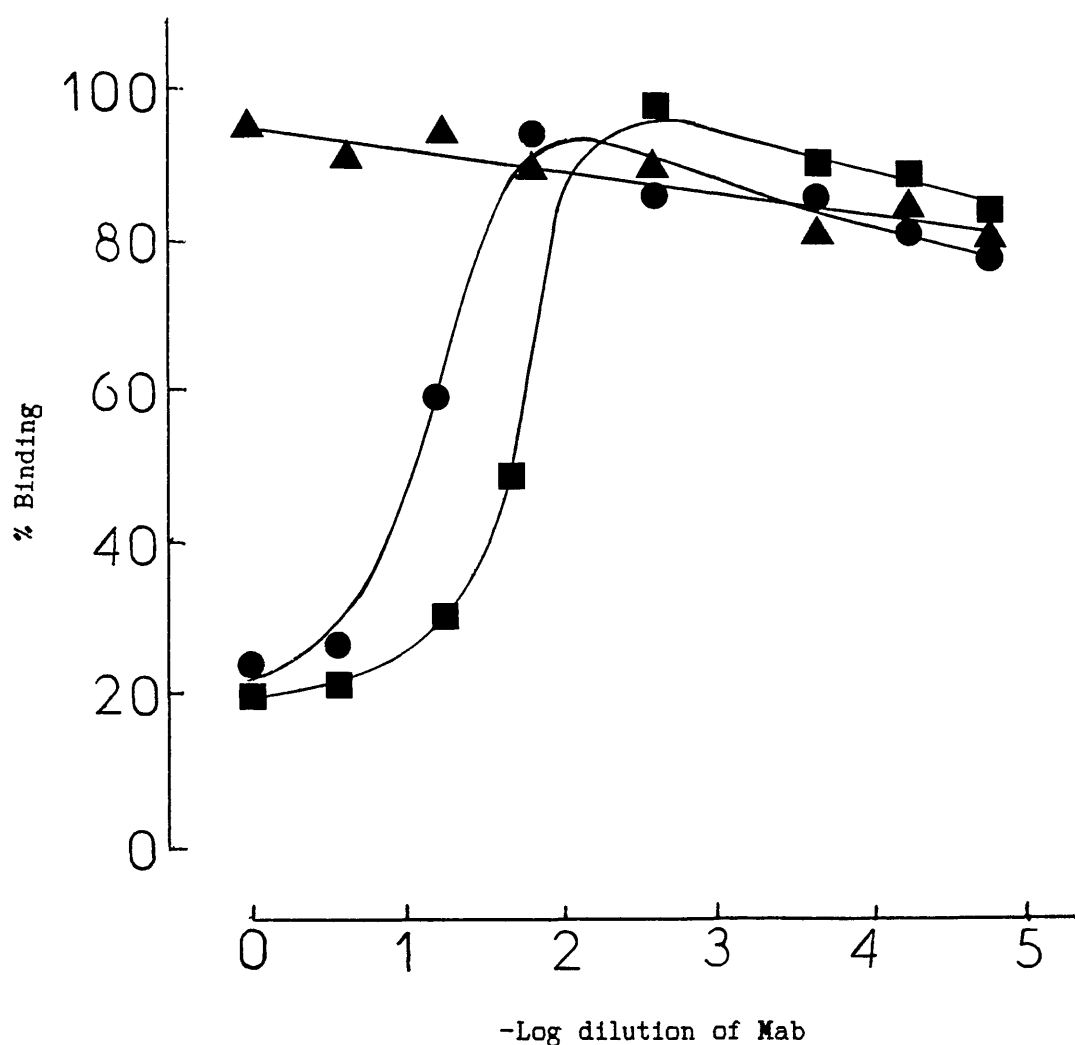


Figure 3.13 Effect of MAb 3/4/2 on the Reduced Carbon Monoxide
Difference Spectrum of Purified Rat Liver Cytochrome P-450c

The reduced carbon monoxide difference spectrum produced by 25 μ g of a purified preparation of rat liver cytochrome P-450c following incubation with (A) 2mg of MAb 3/4/2 or (B) buffer only was determined, as described in section 3.3.5.1 .

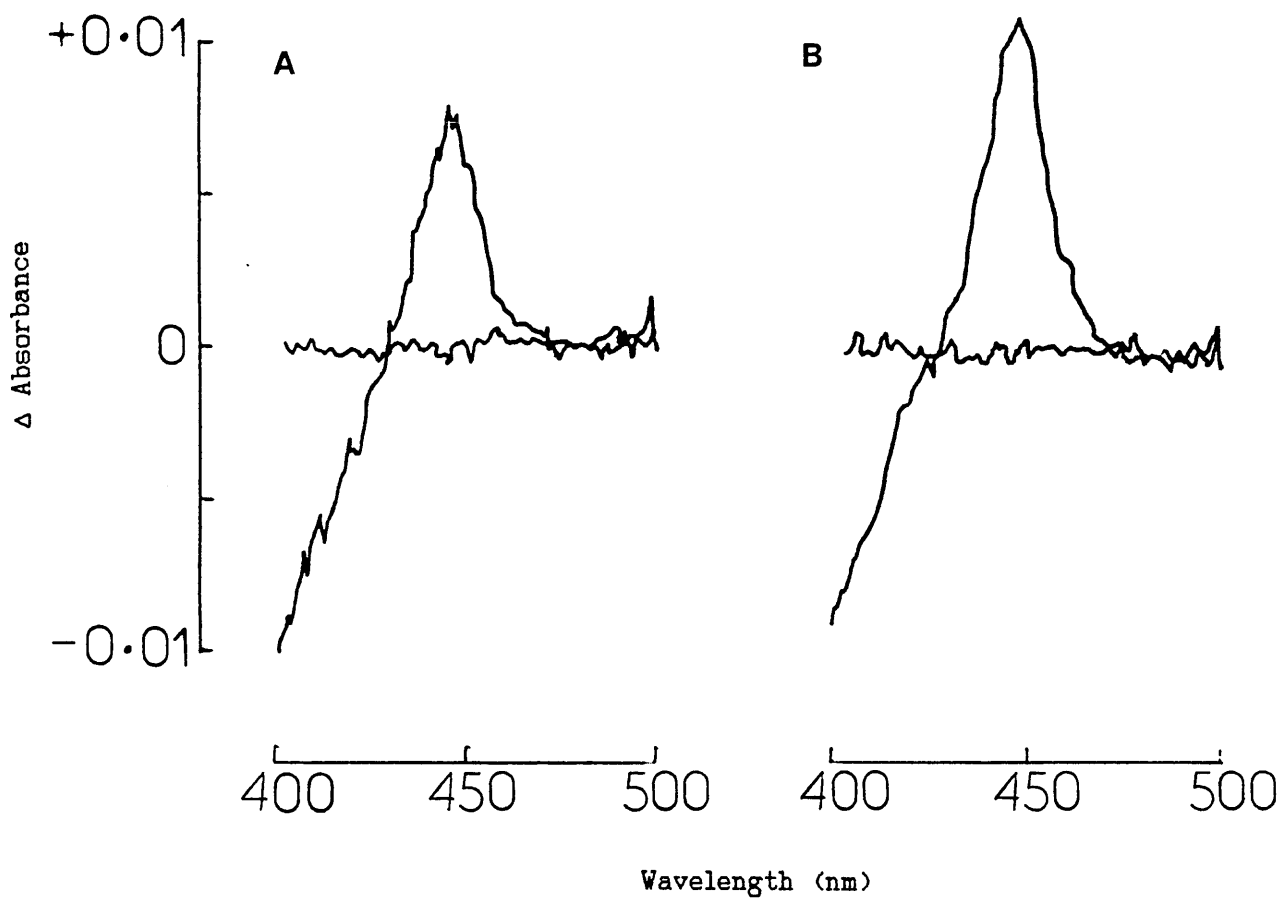


Figure 3.14 Binding Spectrum Produced by Mab 3/4/2 Following Incubation with Liver Microsomes from β NF Treated Rats

The binding spectrum produced by incubating 6mg of liver microsomes from β NF treated rats with (A) 4mg and (B) 20mg of Mab 3/4/2 was determined, as described in section 3.3.5.2 .

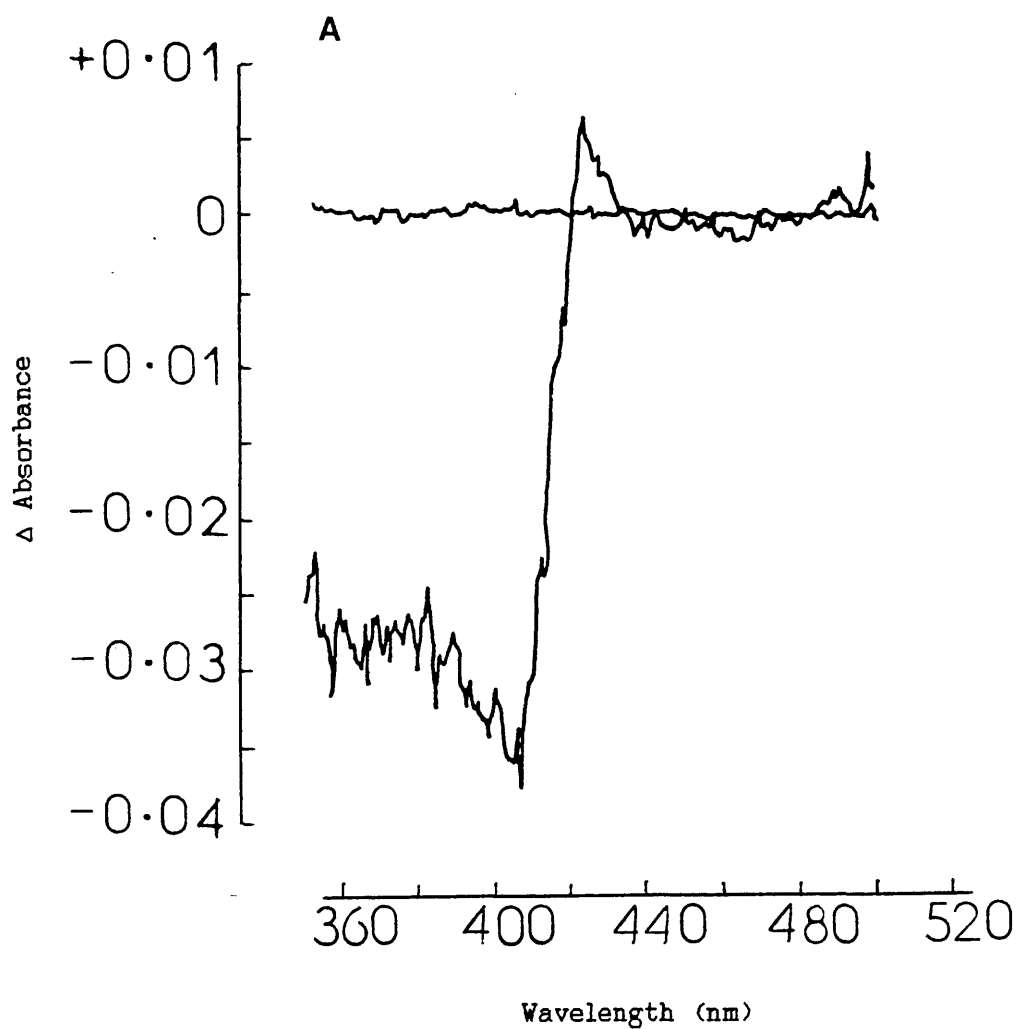


Figure 3.14 continued

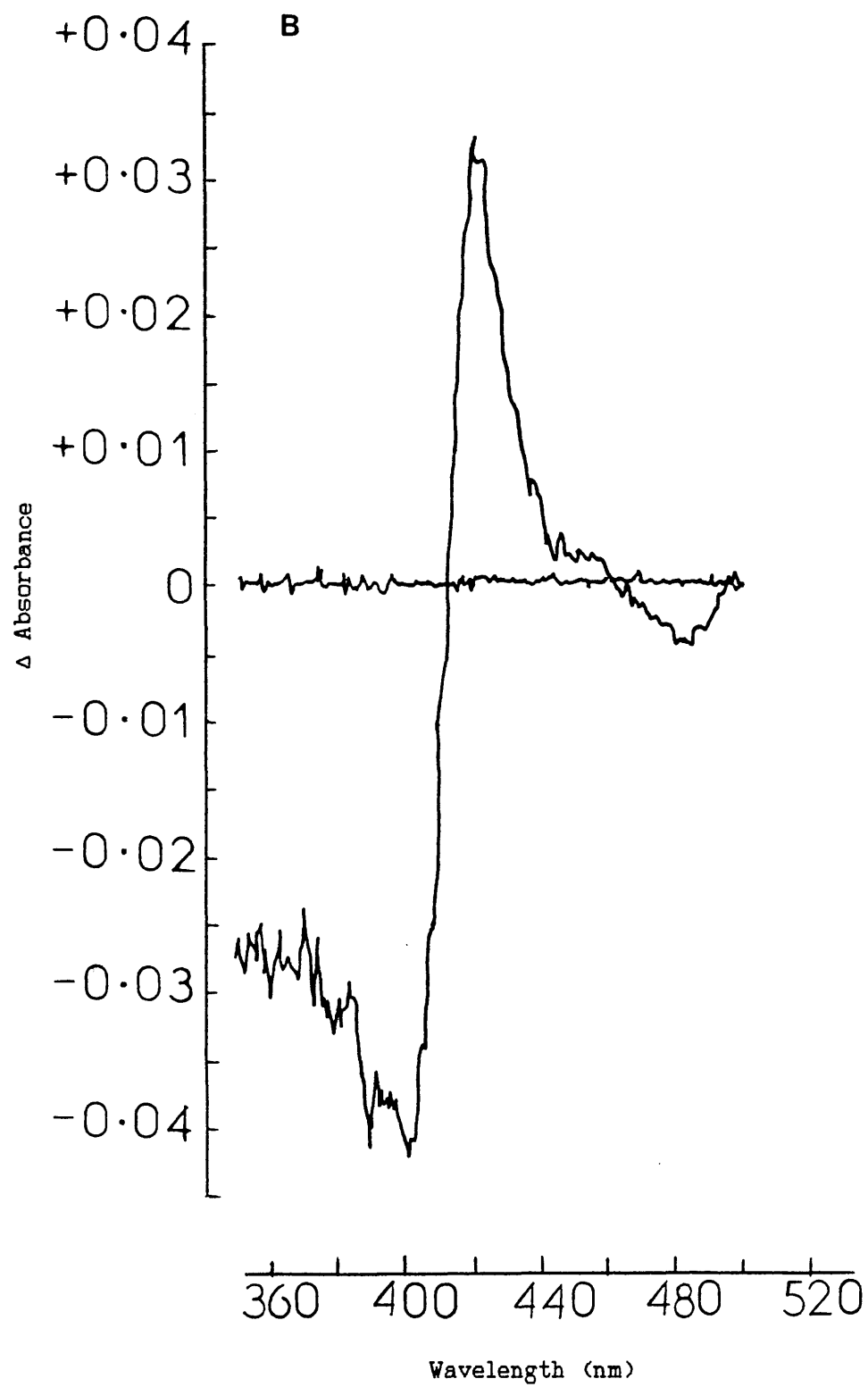


Figure 3.15 Binding Spectrum Produced by MAb 7G1 Following Incubation with Liver Microsomes from β NF Treated Rats

The binding spectrum produced by incubating 6mg of liver microsomes from β NF treated rats with 5mg of MAb 7G1 was determined, as described in section 3.3.5.2 .

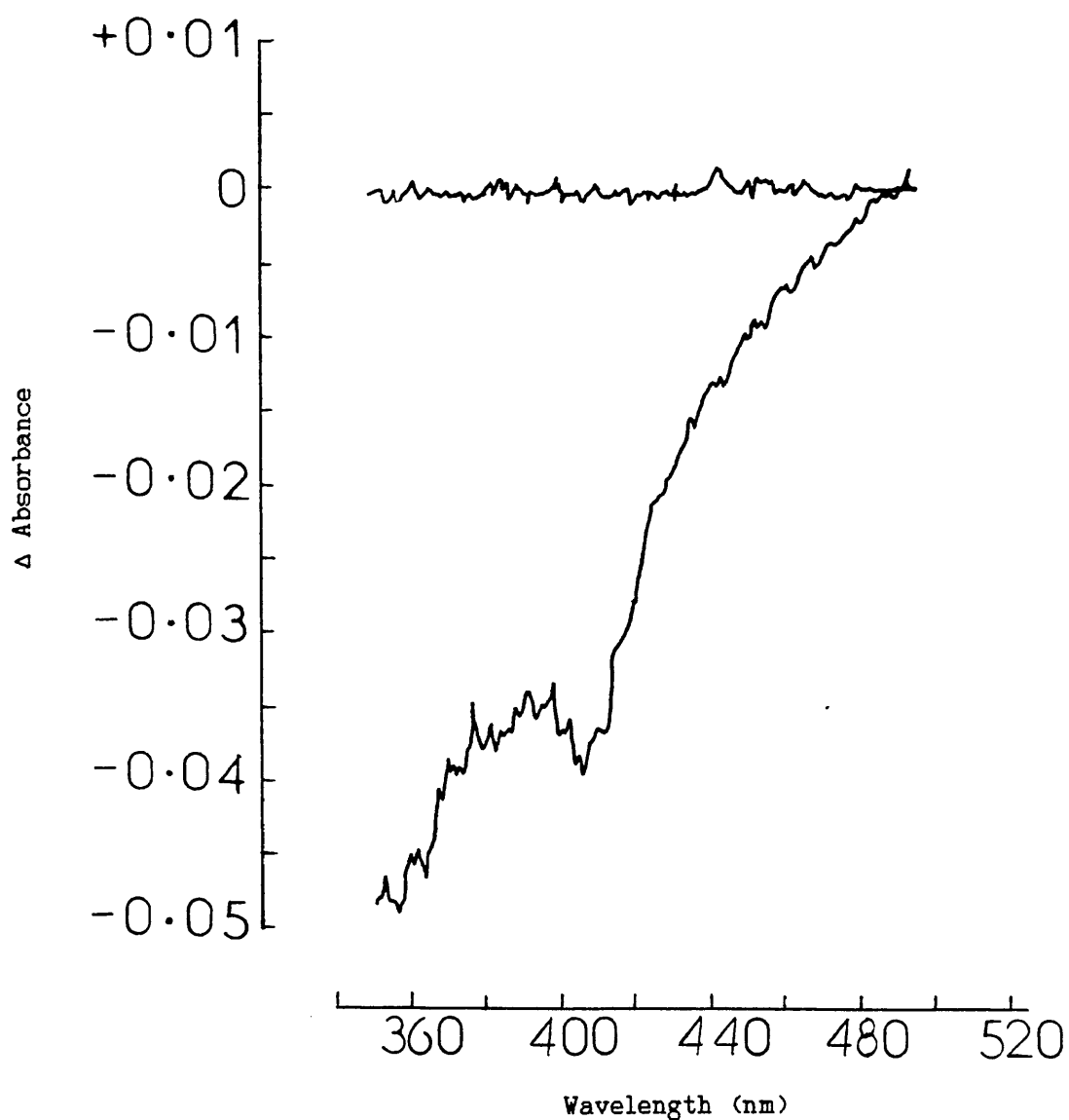


Figure 3.16 Binding Spectrum Produced by Aniline Following Incubation
with Liver Microsomes from SNF Treated Rats in the Presence
of MAb 3/4/2

The binding spectrum produced by incubating 6mg of liver microsomes from SNF treated rats with 5mM aniline, in the presence of 10mg of MAb 3/4/2, was determined, as described in section 3.3.5.2 .

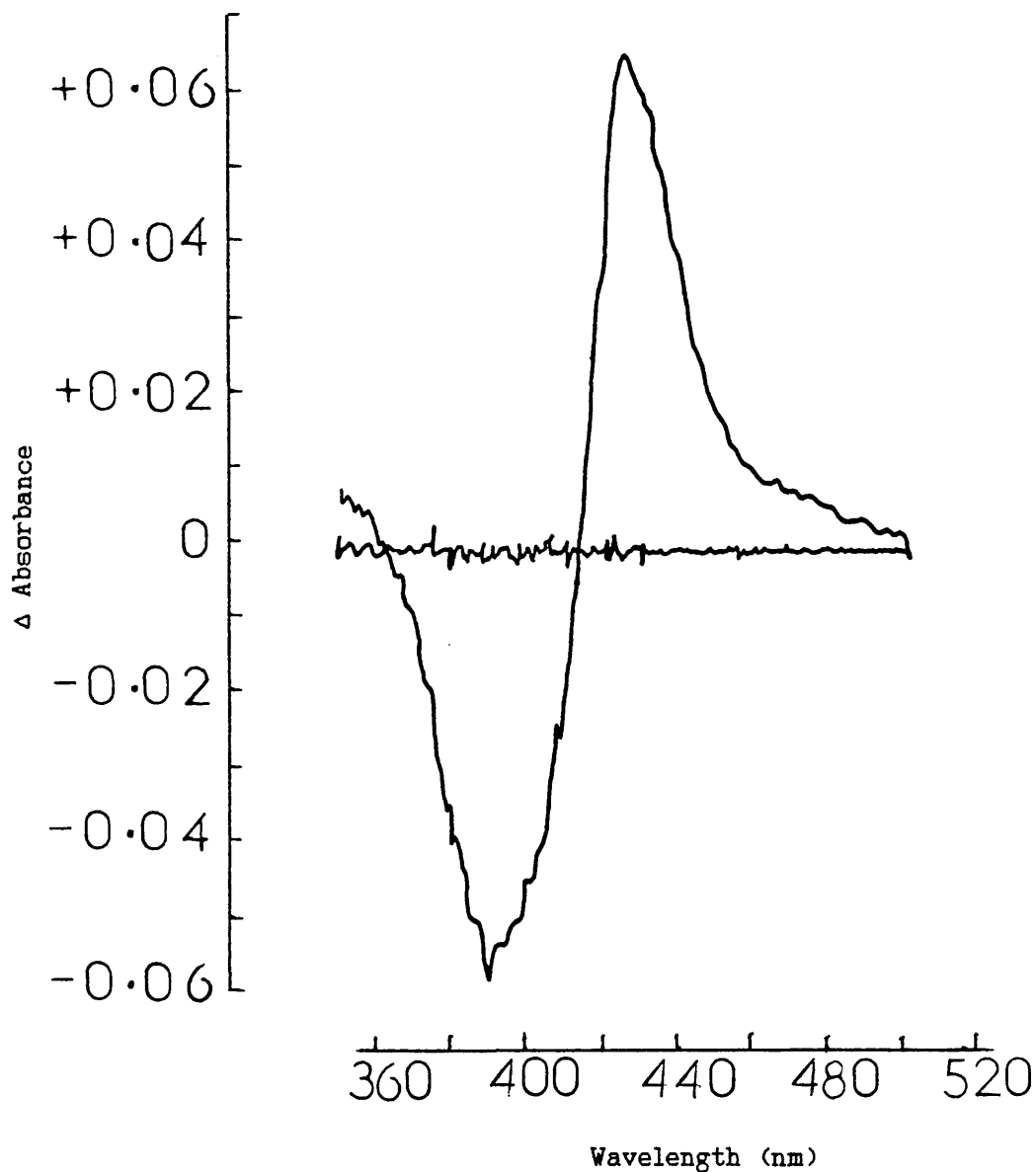
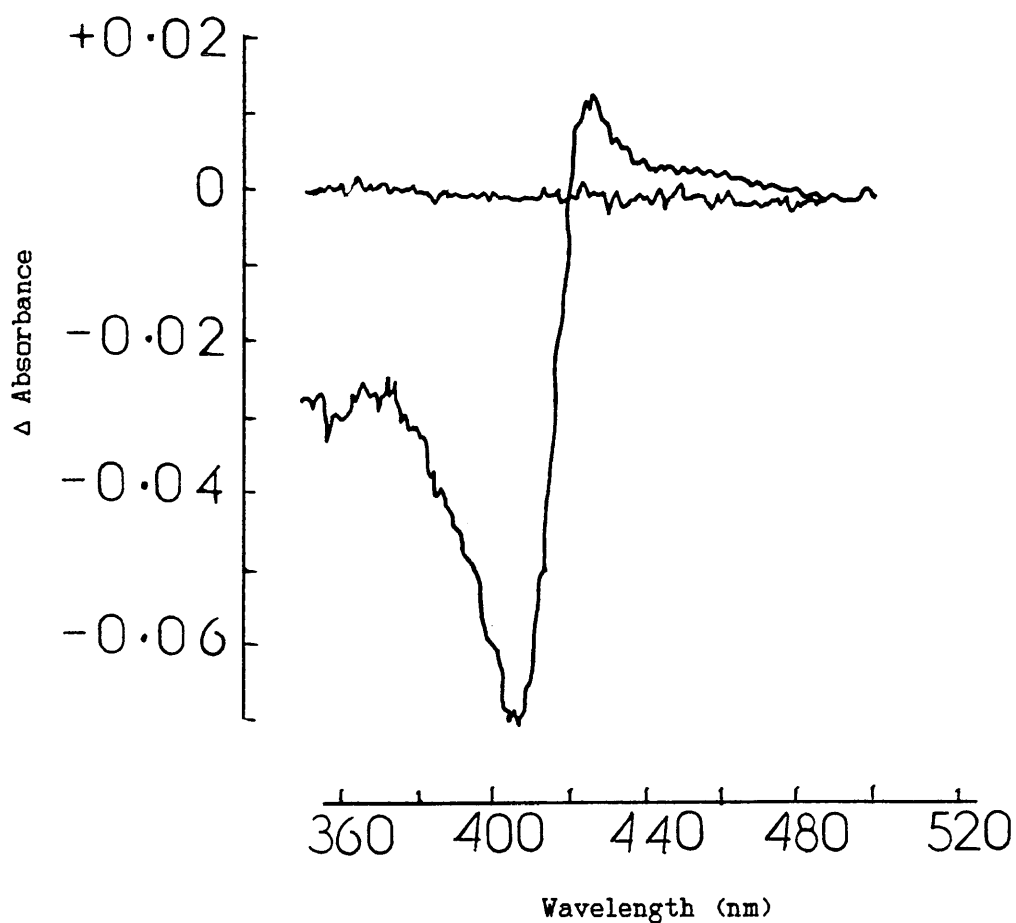


Figure 3.17 Binding Spectrum Produced by MAb 3/4/2 Following Incubation with Liver Microsomes from BNF Treated Rats in the Presence of Aniline

The binding spectrum produced by the addition of 5mM aniline to the reference cuvette, following the production of the binding spectrum illustrated in Figure 3.19, was determined, as described in section 3.3.5.2 .



CHAPTER 4

EFFECT OF MONOCLONAL ANTIBODIES ON CYTOCHROME P-450 MEDIATED MONOOXYGENASE ACTIVITIES

4.1 INTRODUCTION

Cytochrome P-450 exists as families of isozymes, each with a unique but overlapping substrate specificity. Such a system does not permit the contribution of an individual isozyme to the overall metabolism of a given xenobiotic to be easily determined. It might be possible to obtain such information by the purification to homogeneity of individual isozymes of cytochrome P-450. The activity of these isozymes could then be determined in a reconstituted system. Such purified isozymes exhibit a range of specificities consistent with results obtained with microsomal fractions. As such, they have provided valuable information on the potential metabolism of xenobiotics (Guengerich *et al.*, 1982; Ryan *et al.*, 1982).

There are, however, several limitations to this approach. The complete characterisation of the cytochrome P-450 system in any tissue would require the purification to homogeneity of every cytochrome P-450 isozyme in that particular tissue. Although extensive progress in this direction has been made with liver from rats and rabbits, it is probable that at the present time, not all of the isozymes of any tissue from any species have yet been purified to homogeneity. Only the complete sequencing of cytochrome P-450 genes would allow the total number of isozymes present in any species to be determined. However, even then, if individual isozymes are subject to post-translational modifications, gene sequencing will not permit the total number of cytochrome P-450 isozymes to be estimated. In any event, the amino acid sequence of a purified isozyme, or the nucleotide sequence of its gene, will be required to determine which isozyme has been purified. An additional problem in such studies is that the specificity of an isozyme may change during purification. Finally, to predict the contribution of any such purified isozyme to the overall microsomal metabolism of a xenobiotic, the K_m of that, and of all the other isozymes present, would also have to be determined.

However, in determining the relative contribution of an isozyme to the overall metabolism of a xenobiotic, the purification of individual cytochrome P-450 isozymes need not be performed. One alternative which potentially could be more effective, would be to selectively inhibit isozymes within the microsomal fraction of the tissue or organ under study.

Chemical inhibitors of the monooxygenase system have been used for many years to obtain information of the substrate specificity of cytochrome P-450 isozymes. The effects of α -naphthoflavone on the activity of liver microsomes from 3MC treated rats (Wiebel *et al.*, 1971), or of metyrapone (Owens and Nebert, 1975) and SKF-525A (Sladek and Mannering, 1969) on the activity of liver microsomes from PB treated rats, are examples of this type of approach. However, these inhibitors have a relatively broad specificity. Antibodies on the other hand, particularly MAB's, can be highly specific. As such, they are ideal candidates for such an approach.

Inhibition studies with polyclonal antibodies have provided valuable information on microsomal and reconstituted cytochrome P-450 systems. Evidence for the existence of multiple forms of cytochrome P-450 was provided through the differential effects of polyclonal antibodies on microsomal rat liver B[a]P hydroxylation and benzphetamine N-demethylation activities (Welton *et al.*, 1975; Thomas *et al.*, 1976a). The antibody preparations produced by Thomas and co-workers above were also used to inhibit liver cytochrome P-450 mediated reactions by control or PB, 3MC or PCN treated rats (Thomas *et al.*, 1977). Whilst the specificity of each antibody preparation was found to be similar in both microsomal and reconstituted systems, the extent of microsomal activity inhibition was, in general, less than that observed with the corresponding reconstituted system. This decreased inhibition was obviously not due to the use of less inhibitory antibody preparations. It may be, therefore, that antibody reactive epitopes on some cytochrome P-450 isozymes may not be readily

accessible when microsomes are used. Alternatively, as the antibodies were raised against purified isozymes of cytochrome P-450, those isozymes present in microsomes, but lost during the purification procedures, may not be inhibited in microsomal assays, and may therefore contribute to the activity under study.

The heterogeneity of conventional polyclonal antisera does, however, present problems for their use as specific probes of the diversity and activity of cytochrome P-450. This may be due in part to the heterogeneity of the cytochrome P-450 preparations used in some studies to prepare the polyclonal antibodies. Improvements in cytochrome P-450 purification protocols have resolved this to some extent, but for many isozymes, the amounts available for such studies are still limited. The problem of antibody heterogeneity can also be resolved to some extent by removing undesirable cross-reactivities through adsorption of the antiserum against heterologous cytochrome P-450 preparations (Thomas *et al.*, 1977; 1981; Reik *et al.*, 1982; Kelley *et al.*, 1987). Such preparations are finite in their availability, however, and can only be prepared when suitable heterologous cytochrome P-450 preparations are available. Reactivity with minor or constitutive cytochrome P-450 forms are therefore still possible. MAb's, however, are directed against a single epitope, which may, or may not, be present on one or more cytochrome P-450 isozymes. This defined specificity, together with a potentially unlimited supply, makes them ideal as potential inhibitors of specific cytochrome P-450 isozymes.

Several groups have now reported on the inhibitory effects of MAb's on cytochrome P-450 dependent monooxygenase activities. These have included studies on rat liver microsomal activities from animals treated with hydrocarbons (e.g. Thomas *et al.*, 1984a; Boobis *et al.*, 1985a; 1985c; Friedman *et al.*, 1985f), PB (e.g. Letawe-Goujon *et al.*, 1984; Reik *et al.*, 1985; Friedman *et al.*, 1985f) and ethanol (e.g. Ko *et al.*, 1987). In the rabbit, studies on cytochrome P-450 forms LM1 (e.g. Finlayson *et al.*, 1987),

LM2 (e.g. Park *et al.*, 1980), LM3a (e.g. Ding *et al.*, 1986), LM3b (e.g. Reubi *et al.*, 1984a) and LM4 (e.g. Boobis *et al.*, 1981; 1985c; Park *et al.*, 1982a) have been performed. Other species have also been investigated, with samples from fish (e.g. Kloepper-Sams *et al.*, 1987), bovine (e.g. Sugano *et al.*, 1985) and human (e.g. Friedman *et al.*, 1985f; Pelkonen *et al.*, 1986) species having been studied.

In this chapter, the effects of MAb's 1/4, 3/4/2, 12/2/3/2 and B3A3 on three different monooxygenase activities were determined. These activities were selected as representative of activities mediated by different isozymes of cytochrome P-450. The activities chosen were B[a]P hydroxylase (Nebert and Gelboin, 1968), aldrin epoxidase (Wolffe *et al.*, 1979) and 7-ethoxyresorufin O-deethylase (EROD) (Burke and Mayer, 1974). The metabolic profiles of these three compounds are shown in Figures 4.1, 4.2 and 4.3 respectively.

4.2 Methods

4.2.1

Monoclonal Antibodies Preparations

MAb's 1/4, 3/4/2, 12/2/3/2 and B3A3 were purified before use by ion-exchange chromatography on DEAE Affi-Gel Blue, as described in section 2.2.5 .

4.2.2

Benzof[a]Pyrene Hydroxylation Assay

B[a]P hydroxylase activity was determined by a modification of the method described by Nebert and Gelboin (1968). Rat liver microsomes were diluted to 1 mg/ml with PGE buffer and 50µl (50µg protein) incubated with

various amounts of MAb for 30 minutes at room temperature. The volume of MAb solution added was kept constant at 700 μ l by making up the volume with DEAE Affi-Gel Blue column buffer. Controls containing buffer only were also included.

Following incubation with MAb, 100 μ l of 0.5M Tris hydrochloride buffer, pH7.4, 25 μ l of 0.125M magnesium chloride, 25 μ l of a 12 mg/ml solution of NADPH and 50 μ l of a 10 mg/ml solution of BSA were added, with mixing, and the samples incubated for a further 5 minutes at 37°C. The reaction was initiated by the addition of 50 μ l of 2mM B[a]P in methanol, to a total incubation volume of 1ml. Samples were incubated in a shaking water bath in indirect lighting for 10 minutes at 37°C, and in unsealed vials to provide access to air. The reaction was terminated by the addition of 4.25ml of an ice cold hexane : acetone solution (3.25 : 1 by volume). Blank (zero time) samples were prepared by the addition of microsomes to the sample vials after the addition of the hexane : acetone solution.

B[a]P and its metabolites were extracted into the organic phase by incubating the samples in a shaking water bath for 10 minutes at 37°C. Extraction was completed by vortex mixing the samples for 30 seconds and then centrifuging them at 5,000 g for 10 minutes, to separate the aqueous and organic phases. The polar metabolites of B[a]P produced during the incubation were back-extracted into alkali by the addition of 0.5ml of 1M sodium hydroxide to 3ml of the separated organic phase. Following a further stage of vortex mixing and centrifugation, the fluorescence of the aqueous phase was determined with an Aminco Bowman SP-500 spectrophotofluorimeter. The excitation wavelength was 396nm, whilst the emission wavelength was 522nm. Both light paths had a 5mm slit width, and the power setting was 750 volts.

Results were calculated directly as the relative fluorescence intensity obtained, as compared to a quinine sulphate standard, following subtraction of the blank (zero time) values. Activities in the presence of MAb were expressed as a percentage of those obtained in the buffer only controls.

4.2.3

Aldrin Epoxidation Assay

Aldrin epoxidase activity was determined by a modification of the method described by Wolff *et al.* (1979).

4.2.3.1 Incubation Conditions

Rat liver microsomes were diluted to 2.5 mg/ml in PGE buffer, and 20 μ l (50 μ g protein) incubated with various amounts of MAb for 30 minutes at room temperature. The volume of MAb solution added was kept constant at 150 μ l by making up the volume with DEAE Affi-Gel Blue column buffer. Controls containing buffer only were also included.

Following incubation with MAb, 50 μ l of 0.5M Tris hydrochloride buffer, pH7.4, 10 μ l of 0.125M magnesium chloride and 5 μ l of 2.5mM aldrin in DMSO were added, with mixing, and the samples incubated for a further 5 minutes at 37°C. The reaction was initiated by the addition of 15 μ l of a 30 mg/ml solution of NADPH, to a total incubation volume of 250 μ l. Samples were incubated in a shaking water bath for 10 minutes at 37°C, and in unsealed vials to provide access to air. The reaction was terminated by the addition of 2.5ml of ice cold hexane.

Aldrin and its epoxide metabolite, dieldrin, were extracted into the hexane by vortex mixing for 30 seconds. The organic and aqueous phases were separated by centrifugation at 5,000 g for 10 minutes. An aliquot of the organic phase was removed and stored at -20°C until subsequent analysis by GLC.

4.2.3.2 Preparation of Dieldrin Standards

External dieldrin standards were prepared by the substitution of aldrin in the incubation mixture by 5 μ l of dieldrin standards at 2.5, 5.0, 7.5, 10.0 or 12.5 ng/ml in DMSO. No MAb's were included in the incubation mixture, only DEAE Affi-Gel Blue column buffer. Incubations were performed as

described in section 4.2.3.1 above, without microsomes, which were added after the addition of the ice cold hexane. Subsequent extraction of the dieldrin standards was as described in section 4.2.3.1 above.

4.2.3.3 Quantitative Analysis of Dieldrin

The quantitative analysis of dieldrin was performed with a Perkin Elmer F17 gas-liquid chromatograph, coupled to a [^{63}Ni] electron capture detector. The stationary phase was Chromosorb (AW-DMCS), 80-100 mesh, on 5% SE-30 silicone rubber. The volume of hexane injected was 5 μl , which, for the dieldrin standards, corresponded to the injection of 100, 200, 300, 400 or 500pg of dieldrin on column. Extracts from samples incubated with MAb's were diluted with hexane, such that the amount of dieldrin injected onto the column fell within the range of the prepared standards.

Results were calculated as ng of dieldrin produced over the total incubation period. Activities obtained in the presence of MAb were expressed as a percentage of those obtained with the buffer only controls.

4.2.4

7-Ethoxyresorufin-O-Deethylation Assay

EROD activity was determined by a modification of the method described by Burke and Mayer (1974).

4.2.4.1 Determination of Microsomal Activity

Rat liver microsomes were diluted to 2.5 mg/ml in PGE buffer, and 20 μl (50 μg protein) incubated with varying amounts of MAb for 30 minutes at room temperature, in a fluorimeter cuvette. The volume of the MAb solution was kept constant at 850 μl by making up the volume with DEAE Affi-Gel Blue column buffer. Controls containing buffer only were also included.

Following incubation with MAb, 100 μl of 0.5M Tris hydrochloride buffer, pH7.4., and 10 μl of 1mM 7-ethoxyresorufin in ethanol were added, with

mixing. The samples were further incubated for at least 2 minutes in a 37°C maintained cuvette holder in an Aminco Bowman SP-500 spectrophotofluorimeter, coupled to a chart recorder, until a steady baseline recording was obtained. The excitation wavelength was 510nm with a 3mm slit width, whilst the emission wavelength was 586nm with a 5mm slit width. The power setting of the machine was 750 volts. The reaction was initiated by the addition, with mixing, of 20µl of a 40 mg/ml solution of NADPH, to a total incubation volume of 1ml. Samples were incubated further for several minutes at 37°C with access to air provided, until a suitable recording was obtained. The spectrophotofluorimeter was then calibrated by the addition of 50µl of 10.5µM resorufin in ethanol to the reaction cuvette.

Results were calculated as pmoles of resorufin produced during the first minute of the reaction. Activities obtained in the presence of MAb were expressed as a percentage of those obtained with the buffer only controls.

4.2.4.2 Determination of Reconstituted Activity

The method used to determine EROD activity in the reconstituted system was similar to that used for the microsomal fractions. A volume of 5µl (20pmoles) of a partially purified preparation of rat liver cytochrome P-450c (5.1 nmoles/mg protein) was incubated with varying amounts of MAb for 15 minutes at room temperature. The volume of MAb solution added was kept constant at 200µl by making up the volume with DEAE Affi-Gel Blue column buffer. Controls containing buffer only were also included.

The monooxygenase system was then reconstituted by the addition of 30µl (500 units) of NADPH cytochrome c reductase and 60µl of a 1 mg/ml solution of phosphatidylcholine to the samples, with mixing. These components were incubated together for 1 minute at 37°C before the addition, with mixing, of 100µl of 0.5M HEPES buffer, pH7.4, 150µl of 0.1M magnesium chloride, 425µl of water and 10µl of 1mM ethoxyresorufin in ethanol. Samples were

incubated for at least a further 2 minutes in the 37°C maintained cuvette holder, until a stable baseline recording was obtained.

The reaction was then initiated, and results calculated, as described for the microsomal assay in section 4.2.4.1 above.

4.3 Results

4.3.1

Benz[*a*]Pyrene Hydroxylase Activity

Of the MAb's tested, only MAb 12/2/3/2 had any inhibitory effect on B[a]P hydroxylase activity (Figure 4.4). Inhibition was apparent with microsomes from β NF treated rats only, and was concentration dependent, with a resulting maximum inhibition of 70% (i.e. 30% residual activity) at 500 μ g of MAb per incubation. Neither MAb B3A3 nor 3/4/2 had any effect on B[a]P hydroxylase activity of either control or β NF treated rats. Activity in the presence of these MAb's was consistently between 80 and 110% of that obtained in the buffer only control. MAb 1/4, however, caused a consistent increase in B[a]P hydroxylation when incubated with microsomes from either control or PB treated rats, with activities of between 120 and 160% of that obtained in the buffer only control.

4.3.2

Aldrin Epoxidase Activity

None of the MAb's tested inhibited the microsomal aldrin epoxidase activity of either control or PB treated rats (Figure 4.5). Activities with microsomes from PB treated rats were consistently between 90 and 110% of that obtained in the buffer only control. With microsomes from control rats, there appeared to be some stimulation of the aldrin epoxidase activity

with MAb's 3/4/2, B3A3 and 1/4, but not with MAb 12/2/3/2, to a maximum of 150% of that obtained in the buffer only control.

4.3.3

7-Ethoxyresorufin-O-Deethylase Activity

MAb 12/2/3/2 produced a concentration dependent inhibition of EROD activity in both microsomal and reconstituted systems (Figure 4.6). However, inhibition was greater in the reconstituted system, being consistently 40% more than that observed in the microsomal system. Maximum inhibition, at 500µg of MAb 12/2/3/2 per incubation, was 95% and 60% in the reconstituted and microsomal systems respectively (i.e. a residual activity of 5 and 40% respectively). MAb's 3/4/2, B3A3 and 1/4 had no effect on EROD activity in either assay system, with activities being consistently between 85 and 105% of that obtained in buffer only controls (results not shown).

4.4 Discussion

Of the MAb's tested in this study, only one, MAb 12/2/3/2, was inhibitory. The inhibition profile of this MAb was as would be expected from its immunoreactivity profile, in that it only inhibited BNF induced B[a]P hydroxylase and EROD activities and reconstituted cytochrome P-450c mediated EROD activity. No inhibition of monooxygenase activities of control rats, or aldrin epoxidase activity of PB treated rats was observed.

4.4.1

Non-inhibitory Monoclonal Antibodies

The majority of the MAb's tested in this study were non-inhibitory. Such MAb's have been described in previous monooxygenase inhibition

studies. In the earliest MAb inhibition studies, by Gelboin and co-workers (Park *et al.*, 1980), it was found that only some MAb's against rabbit cytochrome P-450 form LM2 inhibited B[a]P hydroxylase and 7-ethoxycoumarin O-deethylase (ECOD) activities catalysed by the homologous antigen in a reconstituted system. In addition, no MAb produced by the same group against rabbit cytochrome P-450 form LM4 was inhibitory (Park *et al.*, 1982a). Similarly, of the four MAb's against cytochrome P-450 LM4 reported by Boobis *et al.* (1981), only one was inhibitory for reconstituted B[a]P hydroxylase activity of the homologous antigen. Of the remaining MAb's, one had no effect on this activity, whilst the presence of either of the other two MAb's caused an increase in activity.

Several of the reports on MAb's to rat liver cytochrome P-450 isozymes have included reference to the production of non-inhibitory MAb's (e.g. Park *et al.*, 1982b; 1984; 1986a; Thomas *et al.*, 1984a). However, the production of such MAb's was not emphasised in any of these publications. In another paper, it was reported that only partially inhibitory MAb's had been produced (Letawe-Goujon *et al.*, 1984).

Thus, it appears from both the present study and from the reports cited above, that non-inhibitory MAb's are not uncommon. Indeed, when one considers that for a MAb to be inhibitory, it must react with a sensitive and accessible epitope on the cytochrome P-450 molecule, it is apparent that non-inhibitory MAb's are to be expected. This is especially true of microsomal monooxygenase activities, where the accessibility of an otherwise MAb sensitive epitope on a cytochrome P-450 isozyme may be restricted. It must be considered, therefore, that the lack of inhibition seen with MAb's 3/4/2 and 1/4 may be due to inaccessibility of the MAb sensitive epitopes on the microsomal cytochrome P-450 isozymes. However, inhibition of monooxygenase activity was observed for MAb 12/2/3/2 using the same microsomal preparations. This would infer that cytochrome P-450 forms c and d were, at least partially, accessible to MAb binding. If MAb

3/4/2 was inhibitory for cytochrome P-450c mediated monooxygenase activities, then some evidence should have been apparent using the same conditions as for MAb 12/2/3/2. However, no inhibition was observed. It may be that if instead of whole microsomes, solubilised or detergent treated microsomes had been used, the accessibility of cytochrome P-450 isozymes to the various MAb's may have been improved. However, when EROD activity was studied in a reconstituted system, thereby allowing direct access to cytochrome P-450c, only MAb 12/2/3/2 was found to be inhibitory. The contribution of cytochrome P-450 form d in EROD activity is discussed further in section 4.4.2 below. On the evidence obtained from the studies reported here, it must be concluded, therefore, that MAb 3/4/2 is non-inhibitory, at least towards cytochrome P-450c. Further, in addition to the investigations reported here, MAb 3/4/2 has also been studied for its effect on several other monooxygenase activities in a number of species (Boobis *et al.*, 1985a). Without exception, none of the activities tested, whether microsomal or reconstituted, were inhibited by this MAb.

MAb 1/4 was studied for its possible inhibitory effects on microsomal B[a]P hydroxylase and aldrin epoxidase activities using liver microsomes from control and PB treated rats. Whilst no inhibition was seen in these studies, an increase in B[a]P hydroxylase activity was observed for this MAb in both of these microsomal preparations. The fluorimetric assay for B[a]P hydroxylase activity used in these studies is not, however, specific, and will measure the production of the majority of the polar metabolites of B[a]P. As discussed in section 4.4.2 below, the production of B[a]P metabolites, as measured by the fluorimetric assay, is probably due to the action of several different isozymes of cytochrome P-450. It may be, therefore, that MAb 1/4 has actually inhibited a specific form of cytochrome P-450. In doing so, however, it may have allowed other, normally competing, isozymes to metabolise B[a]P to a higher degree than they are normally able to do. The overall effect of such a situation may

be an apparent increase in the observed B[a]P hydroxylase activity of the microsomes concerned. To resolve this situation, the differential inhibition pattern of MAb 1/4 would have to be established using a more specific assay for B[a]P metabolism, such as one utilising HPLC or GLC separation of metabolites. Such studies have been performed by Boobis *et al.* (1985c) to investigate the effect of rabbit cytochrome P-450 form LM4 specific MAb's on rabbit liver mediated hydroxylation of 2-AAF. Results from this study revealed three different MAb inhibition patterns, which could not have been detected using a non-specific assay for 2-AAF hydroxylase activity.

An increase in observed monooxygenase activity, similar to that obtained above with MAb 1/4 for B[a]P hydroxylation, was also observed for MAb's 1/4, 3/4/2 and B3A3 for aldrin epoxidase activity in microsomes from control rats. The specificity of the aldrin epoxidation assay, coupled with the fact that similar results were obtained with the control MAb B3A3, make it hard to postulate that the explanation discussed above for the effects of MAb 1/4 on B[a]P hydroxylase activity is also valid in this case. This observed effect is probably due to non-specific factors, especially as it was not directly related to the amount of MAb present in the assay. It is not clear, though, why MAb 12/2/3/2 did not show a similar pattern in this assay.

These results highlight some of the problems of inhibition studies performed with microsomal preparations. Reconstituted systems using purified isozymes may produce results which are easier to interpret, but, by their nature, they are more artificial and do not truly represent the situation found in the liver. They do, however, provide valuable information on individual isozymes. Perhaps the way forward lies in more specific detection systems for the monooxygenase activities under study.

4.4.2

Inhibition of Monooxygenase Activity by MAb 12/2/3/2

4.4.2.1 Comparison of Microsomal B[a]P Hydroxylase and EROD Activities

Only MAb 12/2/3/2 produced any directly observed inhibition of the monooxygenase activities studied. This MAb caused a concentration dependent inhibition of both B[a]P hydroxylase and EROD activities of microsomes from β NF treated rats. Whilst the maximum degree of inhibition was similar for both activities, the profile of inhibition was different, with EROD activity being more sensitive to MAb inhibition.

An inhibitory antibody, such as MAb 12/2/3/2, might inhibit cytochrome P-450 dependent monooxygenase activities by any one of several different means. It could, for example, bind to a sensitive epitope on the isozyme, thereby causing a conformational change which prevents binding of the substrate or integration of the isozyme with cytochrome c reductase. Alternatively, the antibody may interfere with the metabolism of bound substrate. Inhibition could also occur through direct competition between the antibody and substrate for binding to cytochrome P-450. The MAb could also interfere in the binding of cytochrome c reductase to the cytochrome P-450 molecule through steric interactions. With these mechanisms of inhibition, the affinity of the MAb sensitive cytochrome P-450 isozyme for the substrate, together with the substrate concentration, may affect the degree of inhibition achieved by a given concentration of an inhibitory antibody. It is clear, however, that from results obtained both in Chapter 3 and in this chapter, the inhibitory effects of MAb 12/2/3/2 are directed through the cytochrome P-450 isozyme, and not through NADPH cytochrome c reductase. Western blotting studies on MAb 12/2/3/2 described in Chapter 3 with a variety of rat microsomal preparations did not reveal any reactivity with proteins of the same molecular weight as NADPH cytochrome c reductase, namely 78,000. In addition, whilst

inhibition of both B[a]P hydroxylase and EROD activities could be obtained for MAb 12/2/3/2, no inhibition of microsomal aldrin epoxidase activity was observed for this MAb. These results would therefore counter the possibility that the inhibitory effects observed with MAb 12/2/3/2 are mediated through its reactivity with NADPH cytochrome c reductase.

In the microsomal studies performed with MAb 12/2/3/2 in which inhibition of monooxygenase activity was observed, the maximum degree of inhibition of B[a]P hydroxylase and EROD activities were similar, whilst the profile of inhibition was different, with EROD activity being more sensitive. It may be that the concentration of B[a]P used in the assay was able to compete with the MAb more effectively than the ten-fold lower concentration of 7-ethoxyresorufin, thereby explaining the different inhibition profiles obtained. In both cases, though, the substrate concentrations used should not have been limiting. Alternatively, this differential inhibition pattern could be due to these microsomal activities being mediated by different isozymes of cytochrome P-450, such as forms c and d. In such a case, one of the isozymes would have a higher activity for 7-ethoxyresorufin, whilst the other isozyme would predominantly metabolise B[a]P. The total contribution of the two isozymes to these monooxygenase activities would have to be the same to explain the same maximal inhibition of these two activities by MAb 12/2/3/2, although the affinity of the MAb for the two isozymes may differ, or indeed the relative amounts of the two isozymes may vary in the microsomal preparation used. As discussed in section 4.4.2 above, the fluorimetric assay for B[a]P hydroxylase activity is not specific for any one hydroxy metabolite. The inhibition observed for MAb 12/2/3/2 for this activity may, therefore, be obscured by the influence of another isozyme not inhibited by this MAb. MAb 12/2/3/2 reacts with both forms c and d, as determined from Western blotting studies. It would be expected, therefore, that it inhibited both forms, but at the same time, it is difficult to differentiate between the

relative effects of this MAb on both of these forms. For that reason, the inhibition of monooxygenase activities seen with MAb 12/2/3/2 must be ascribed to its effect on both forms c and d. It would have been interesting to repeat these studies into the effect of MAb 12/2/3/2 on B[a]P hydroxylation and EROD activities, with liver microsomes from ISF treated rats, given the higher specific content of form d in such preparations. Any differential effects of this MAb on the activities of forms c and d may well have become apparent in such a study.

It has been reported that cytochrome P-450c mediated B[a]P hydroxylase activity has been inhibited in a reconstituted system by a form c specific MAb (Thomas *et al.*, 1984a). With the MAb 12/2/3/2 inhibition of reconstituted cytochrome P-450c mediated EROD activity observed in this present study, it would seem likely that both B[a]P hydroxylation and EROD activities are mediated in the rat, at least to some extent, by the same isozyme, or isozymes, of cytochrome P-450. Further studies by Thomas and co-workers using this same MAb together with a form d specific polyclonal antibody preparation confirmed this hypothesis, and also indicated that microsomal EROD activity in ISF and 3MC treated rats is mediated, to some extent, by both forms c and d (Kelley *et al.*, 1987). Recent MAb inhibition studies by Pacifici *et al.* (1988) also indicate that microsomal EROD activity is mediated by more than one cytochrome P-450 isozyme. In liver microsomes from BNF treated rats, however, the major isozyme contributing to the metabolism of these substrates is probably form c, which, for B[a]P hydroxylation at least, has a higher catalytic activity than form d (Ryan *et al.*, 1980). The lack of inhibition observed for MAb 12/2/3/2 with liver microsomal B[a]P hydroxylase activity of control rats would also indicate that cytochrome P-450 forms c and d do not contribute, to any significant extent, to that activity in the uninduced animal. Similarly, the lack of inhibition on liver aldrin epoxidase activity of PB treated rats by this MAb indicates that these two isozymes do not participate to any appreciable extent in that reaction.

4.2.2.2 Comparison of Microsomal and Reconstituted EROD Activities

The inhibition curve of reconstituted cytochrome P-450c EROD activity with MAb 12/2/3/2 was parallel to that obtained with a liver microsomal fraction from β NF treated rats, but was shifted to the left, making it more MAb 12/2/3/2 sensitive. Although the ratio of MAb to cytochrome P-450c was very similar in the two assay systems, several other parameters may have been different. For example, the kinetics of cytochrome P-450 reduction may have been different in the two systems, or indeed, the affinity of cytochrome P-450c for 7-ethoxyresorufin may have been altered during the purification procedure. Alternatively, access of the MAb to cytochrome P-450c within the microsomal membrane may be restricted, such that higher concentrations of MAb 12/2/3/2 were required to produce the same degree of inhibition as that obtained with the reconstituted isozyme. Levin and co-workers also found that microsomal monooxygenase activity was not as sensitive to inhibition by a polyclonal antibody as that of the reconstituted isozyme (Thomas *et al.*, 1977). The authors attributed this difference to restricted access of the antibody to the isozyme within the membrane.

An alternative explanation for the differential effects of MAb 12/2/3/2 in the two systems is that there may be MAb 12/2/3/2 insensitive isozymes of cytochrome P-450 contributing to the EROD activity of the microsomal fraction. Certainly residual microsomal EROD and, for that matter, B[a]P hydroxylase activities, was still apparent with 500 μ g of MAb 12/2/3/2 per incubation. At this concentration of the MAb, reconstituted EROD activity was almost totally inhibited. These results suggest that MAb 12/2/3/2 insensitive isozymes of cytochrome P-450 could contribute up to 40% of the microsomal metabolism of these substrates. From the specificity of MAb 12/2/3/2, it can be presumed that these isozymes are ones other than forms c and d. Using a MAb specific for cytochrome P-450 forms c and d (MAb 1-7-1), Pacifici *et al.* (1988) have suggested that up to 65% of microsomal

EROD activity may be mediated by cytochrome P-450 isozymes other than forms c or d. Later studies with MAb 12/2/3/2 indicated that a residual 20% of B[a]P hydroxylase activity still remained after maximal MAb inhibition in a reconstituted system (Boobis *et al.*, 1985c). Differential inhibition of B[a]P hydroxylase activities of microsomal and reconstituted cytochrome P-450 by a MAb has also been reported by Levin and co-workers (Thomas *et al.*, 1984a). These authors concluded that reduced inhibition with the microsomal fraction was due to the presence of MAb insensitive isozymes contributing to the hydroxylase activity, rather than restricted MAb access. Further studies will be required, however, before a full explanation for the reduced inhibition of EROD activity by MAb 12/2/3/2 in microsomal fractions from β NF treated rats can be obtained.

4.4.3

Other Reported Studies with Inhibitory Monoclonal Antibodies

Extensive studies in this area have also been reported by Gelboin and co-workers using an inhibitory MAb, 1-7-1 (Park *et al.*, 1982b). This MAb, like MAb 12/2/3/2, reacts with cytochrome P-450 forms c and d in Western blots of liver microsomes from β NF treated rats, and has also been reported to immunopurify two proteins of the same molecular weights as forms c and d from solubilised liver microsomes from 3MC treated rats (Friedman *et al.*, 1983). The inhibitory effects of this MAb on several activities, including EROD, B[a]P hydroxylase, ECOD and testosterone hydroxylase, have been studied in a wide variety of tissues and species, including human samples (e.g. Fujino *et al.*, 1982; 1984a; 1984b; Park *et al.*, 1982b; Cheng *et al.*, 1984a; Wiebel *et al.*, 1984; Friedman *et al.*, 1984; 1985e; Hietanen *et al.*, 1986; 1987; Pelkonen *et al.*, 1986; Slusher *et al.*, 1987; Waxman *et al.*, 1987; Pacifici *et al.*, 1988). From these studies, the relative contributions of MAb 1-7-1 sensitive cytochrome P-450 isozymes to the metabolism of these xenobiotics were determined. In addition, the differential effects of this

MAb on the production of hydroxy metabolites of 2-AAF by cytochrome P-450 induced by treatment with 3MC were established (Thorgeirsson *et al.*, 1983).

Inhibition studies similar to those performed with MAb 12/2/3/2 have also been reported by Levin and co-workers (Thomas *et al.*, 1984a). In that report, the B[a]P hydroxylase activities of both 3MC treated rat liver microsomes and reconstituted rat liver cytochrome P-450c were inhibited by a cytochrome P-450c specific MAb. As previously mentioned, the reconstituted system in this study was more sensitive to MAb inhibition, with only 3% B[a]P hydroxylase activity remaining, compared to 20% remaining in the microsomal system at the same MAb - cytochrome P-450 molar ratio. This same MAb also inhibited reconstituted cytochrome P-450c mediated ECOD and zoxazolamine hydroxylase activities to the same extent as for B[a]P hydroxylase (Parkinson *et al.*, 1986). As previously described above, this MAb was also reported to inhibit microsomal EROD activities of both ISF and 3MC treated rats (Kelley *et al.*, 1987).

Further studies with MAb 12/2/3/2 have shown that it also inhibits phenacetin O-deethylase activity of liver microsomes from both control and 3MC treated rats, by approximately 80% and 70%, respectively (Boobis *et al.*, 1985c). Kinetic analysis revealed that the high affinity component of this activity was inhibited by 100% in both cases. Thus, this high affinity component is catalysed by the same forms of cytochrome P-450 in both control and 3MC treated rats. In control animals, this activity is therefore presumably catalysed by the constitutive levels of these inducible forms.

The influence of MAb's on the activities of PB inducible forms of cytochrome P-450 have been studied to a much lesser extent than for those forms inducible by hydrocarbon treatment. Gelboin and co-workers have reported on the inhibitory effects of a MAb specific for PB inducible forms

of cytochrome P-450 (e.g. Park *et al.*, 1984; Weibel *et al.*, 1984; Hietanen *et al.*, 1986; 1987; Slusher *et al.*, 1987). This MAb, 2-66-3, inhibited B[a]P hydroxylase, ECOD, aldrin epoxidase, aminopyrine N-demethylase and testosterone hydroxylase activities from a variety of preparations from rat, mouse and rabbit species. It was also found to inhibit aflatoxin B₁ mutagenicity by mouse liver S9 fraction. This MAb had no effect, however, on B[a]P hydroxylase or ECOD activities in microsomes prepared from the livers of either smoking or non-smoking individuals (Pelkonen *et al.*, 1986). This MAb has also been reported to inhibit 7, 12-dimethylbenz[*a*]anthracene metabolism in microsomal fractions from a variety of tissues from both control and PB treated rats (Christou *et al.*, 1987).

Other groups have also reported on inhibitory MAb's specific for PB inducible isozymes of cytochrome P-450. Those described by Letawe-Goujon *et al.* (1984) were only partially inhibitory for B[a]P hydroxylase, ECOD, aldrin epoxidase and pregnenolone 16- α -hydroxylase activities of liver microsomes from PB treated rats. In contrast, several of the MAb's reported by Reik *et al.* (1985) completely inhibited benzphetamine N-demethylase and testosterone hydroxylase activities of both a microsomal system prepared from PB treated rats and a reconstituted cytochrome P-450b system.

Other rat cytochrome P-450 systems which have been studied with inhibitory MAb's include those forms induced by ethanol and acetone treatment. MAb's have been reported that inhibit aniline hydroxylase and nitroso-dimethylamine demethylase activities of both a reconstituted cytochrome P-450 system prepared from acetone treated rats, and a microsomal system prepared from acetone, pyrazole, methylpyrazole and imidazole treated rats (Park *et al.*, 1986b; Ko *et al.*, 1987). Other studies have reported on the MAb inhibition of these forms, using ether deethylation as a model system (Brady *et al.*, 1988). Inhibition of testosterone metabolism by MAb's has also been reported (Morgan *et al.*,

1987). Results from this study highlighted the immunochemical similarity between the isozymes responsible for 15 β - and 16 α -hydroxylation of testosterone.

Several studies on inhibitory MAb's have also been reported with several rabbit cytochrome P-450 systems. Early studies were performed by Gelboin and co-workers with MAb's against cytochrome P-450 forms LM2 and LM4. Reconstituted B[a]P hydroxylation assays, comprised with either isozyme, could be inhibited by a form specific MAb. Cytochrome P-450 form LM2 mediated ECOD activity could also be inhibited by form LM2 specific MAb's (Park *et al.*, 1980; 1982a). Inhibitory MAb's against cytochrome P-450 form LM4 have also reported by Boobis and co-workers. Initial studies showed that inhibition of reconstituted cytochrome P-450 form LM4 mediated B[a]P hydroxylase activity could be achieved by a series of MAb's (Boobis *et al.*, 1981). Further studies revealed that these MAb's also produced an interesting inhibition profile in the rabbit liver microsomal mediated metabolism of 2-AAF (Boobis *et al.*, 1985c). Two of the MAb's in this series were found to inhibit both the 1- and N-hydroxylation of the amine, whilst having no effect on its 7-hydroxylation. Another MAb inhibited only the 7-hydroxylation reaction, whilst a fourth inhibited all three reactions. These results therefore revealed three different inhibition patterns. Such MAb's should prove useful in studies of the biological activity of 2-AAF and related compounds.

Johnson and co-workers have also used inhibitory MAb's, in this case against rabbit cytochrome P-450 form LM1, to inhibit liver microsomal B[a]P hydroxylase activity as well as liver microsomal and cytochrome P-450 form LM1 reconstituted progesterone 21-hydroxylase activity (Reubi *et al.*, 1984b; Johnson and Griffin, 1985; Raucy and Johnson, 1985). This group also found that progesterone 21-hydroxylase activity was not inhibited in kidney microsomes by an inhibitory MAb specific for cytochrome P-450 form LM1.

However, lauric acid 11-hydroxylase activity was inhibited in the same study by a MAb specific for a previously unidentified rabbit cytochrome P-450 form, termed cytochrome P-450 K (Finlayson *et al.*, 1987). In another publication, this same group reported on the use of a MAb, specific for cytochrome P-450 form LM3b, to characterise the participation of that isozyme in microsomal progesterone 6 β and 16 α -hydroxylation (Reubi *et al.*, 1984a). This MAb inhibited the latter activity in microsomes from either NZW or IIIVO/J strains, but only inhibited the former activity in microsomes prepared from NZW rabbits. This clearly indicates the presence of variant forms of cytochrome P-450 LM3b in different rabbit strains.

MAb inhibition of two other cytochrome P-450 systems have also been reported. In one, the inhibition of bovine adrenocortical mitochondrial mediated 11 β - and 18-hydroxylation of 11-deoxycorticosterone was achieved. These activities were also inhibited by the same MAb in a reconstituted bovine cytochrome P-450_{11 β} assay system (Sugano *et al.*, 1985). In the other cytochrome P-450 system studied, Gelboin and co-workers used MAb's against liver cytochrome P-450E from the marine fish *Stenotomus chrysops* (scup) to inhibit B[a]P hydroxylase and EROD activities of reconstituted cytochrome P-450E, as well as liver microsomal EROD and B[a]P hydroxylase activities (Stegeman *et al.*, 1985; Park *et al.*, 1986c; Kloepper-Sams *et al.*, 1987).

4.4.4

Conclusion

The possible inhibitory effects of MAb's 1/4, 3/4/2, 12/2/3/2 and B3A3 on B[a]P hydroxylase, aldrin epoxidase and EROD activities were investigated. Only one MAb, 12/2/3/2, inhibited any of the activities studied, and its inhibition profile was confined to those microsomal activities induced by BNF treatment of rats, or to that catalysed by partially purified rat liver

cytochrome P-450c. The studies described above for MAb 12/2/3/2, and for other MAb's elsewhere in the literature, indicate the feasibility for using such inhibitory MAb's to determine the contribution of individual cytochrome P-450 isozymes to the overall metabolism of a substrate. Unfortunately, MAb's 1/4 and 3/4/2 were found to be not suitable for the assay systems used in this chapter.

The use of MAb's in such inhibition studies has allowed investigators to assess the relative contribution of a number of different cytochrome P-450 isozymes to the metabolism of a variety of substrates. The use of a panel of inhibitory MAb's, together with assays for monooxygenase activities representative of the major classes of cytochrome P-450, can provide a greater depth of information than can be supplied by a much wider range of individual substrate assays. With the information provided by such an approach, it may be possible to predict the effects of, for example, exposure to environmental pollutants or xenobiotics, or the inheritance of particular genetic traits, on an individual's ability to metabolise certain classes of substrates. It may then be possible to determine the consequence of such a 'substrate profile' on that individual's susceptibility to xenobiotic toxicity, or even cancer.

Figure 4.1 The Primary Route of Hydroxylation of Benzo[a]Pyrene

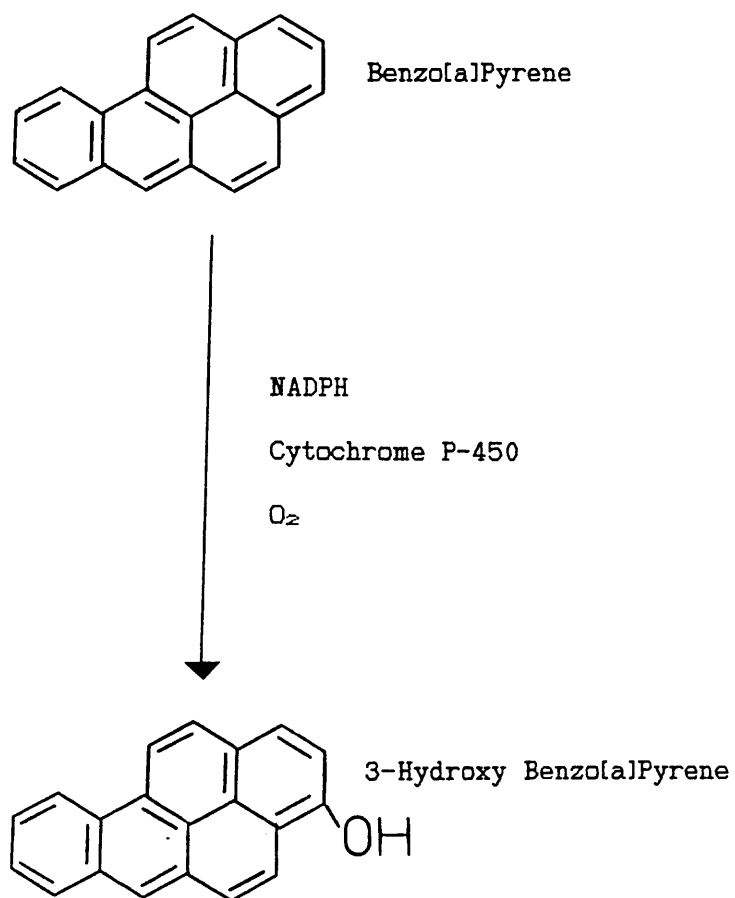


Figure 4.2 The Epoxidation of Aldrin

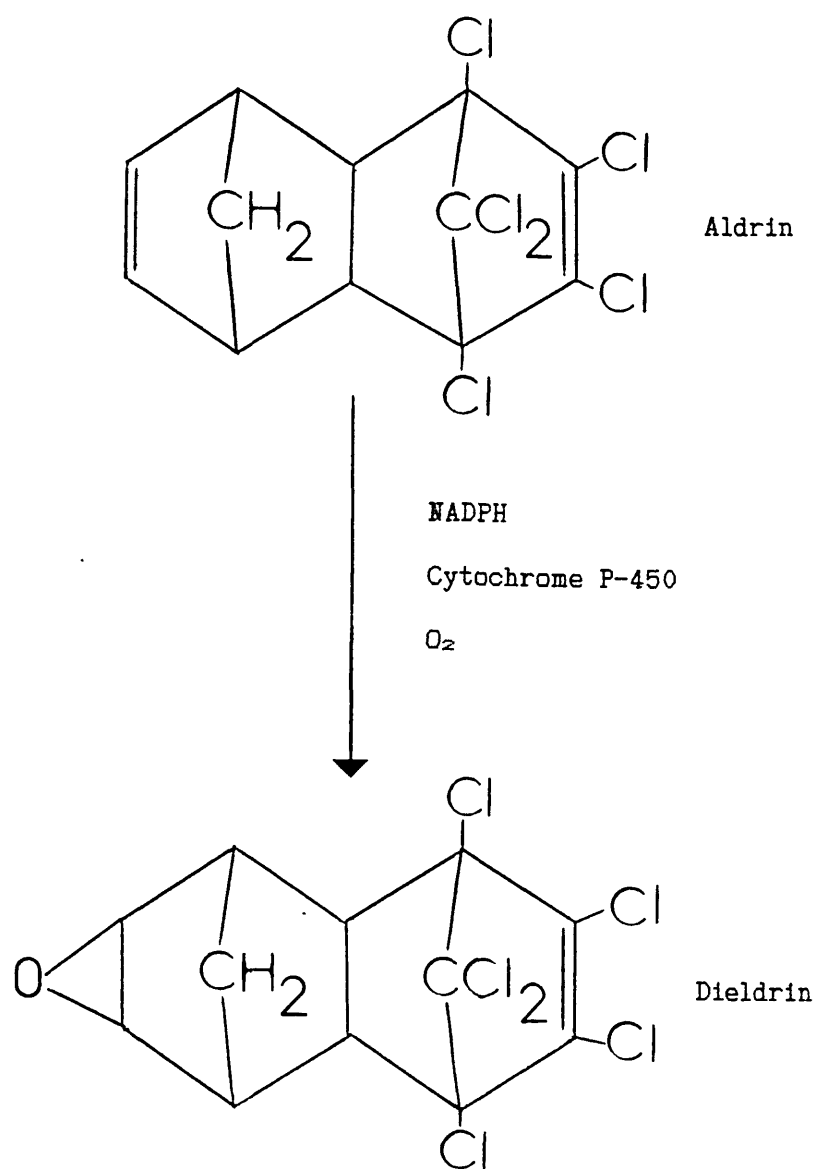


Figure 4.3 The O-Deethylation of 7-Ethoxyresorufin

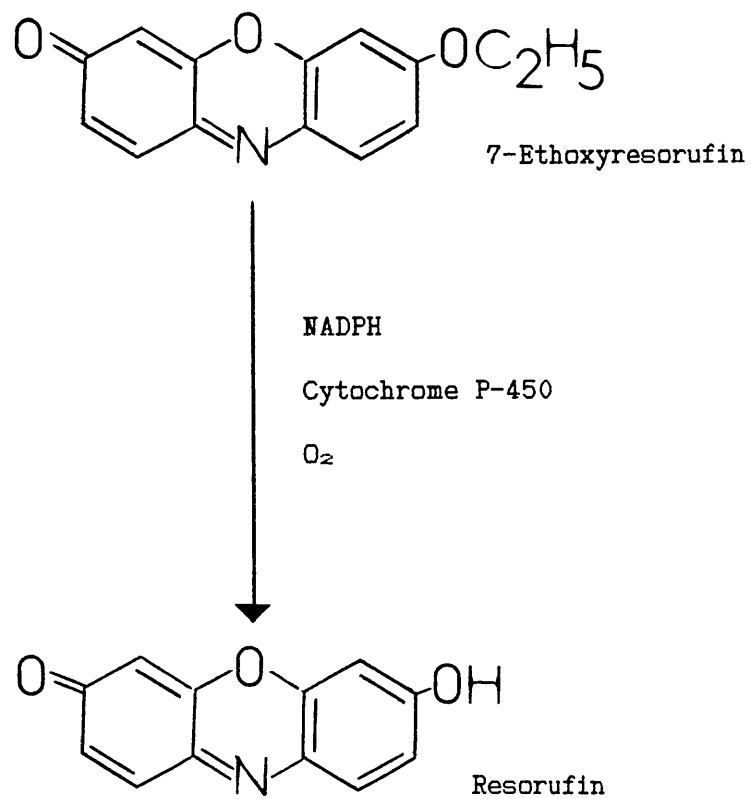


**Figure 4.4 Effect of Monoclonal Antibodies on Liver Microsomal
BenzofalPyrene Hydroxylase Activity**

The effects of MAb's 3/4/2 (▨), 12/2/3/2 (▧), B3A3 (▩) and 1/4 (▦) on liver microsomal B[a]P hydroxylase activities from (A) control and (B) βNF treated rats (PB treated rats for MAb 1/4) were determined as described in section 4.2.2 . The results are expressed as a percentage of the activity of the appropriate buffer only (i.e. no MAb) control, and are the average of duplicate determinations.

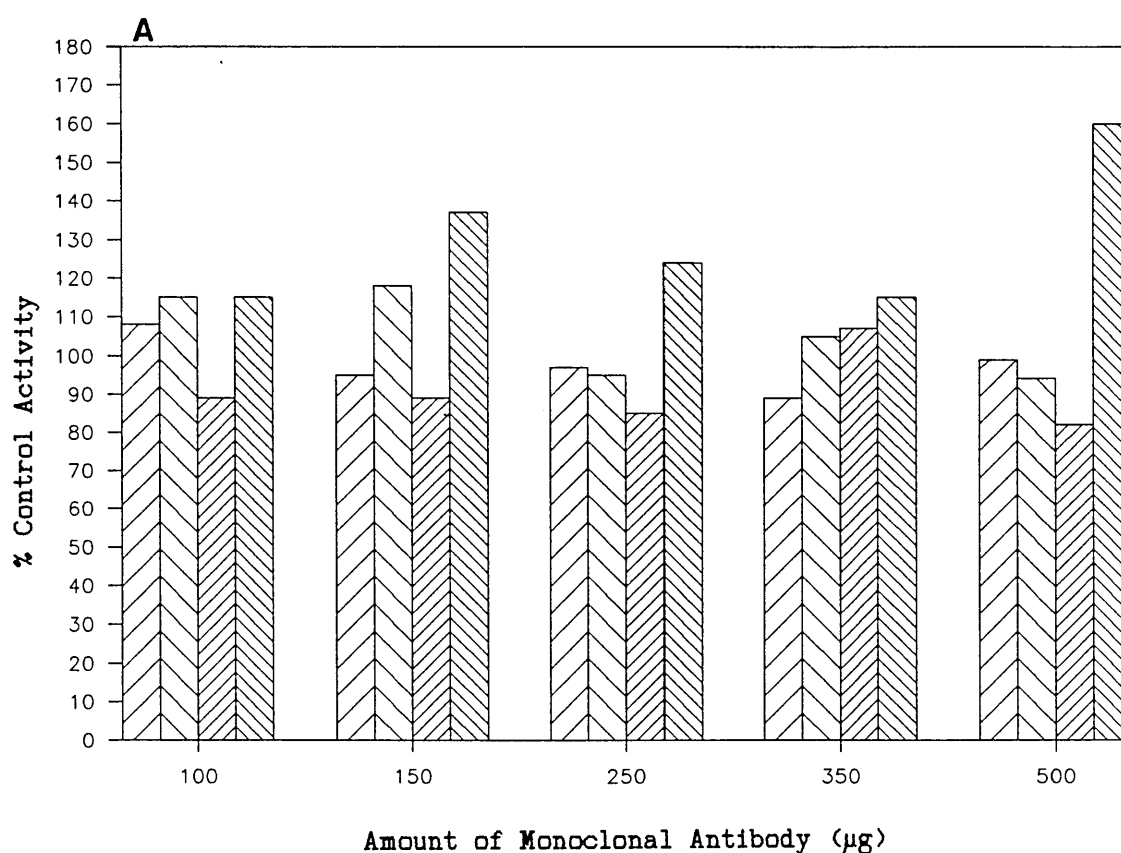


Figure 4.4 continued

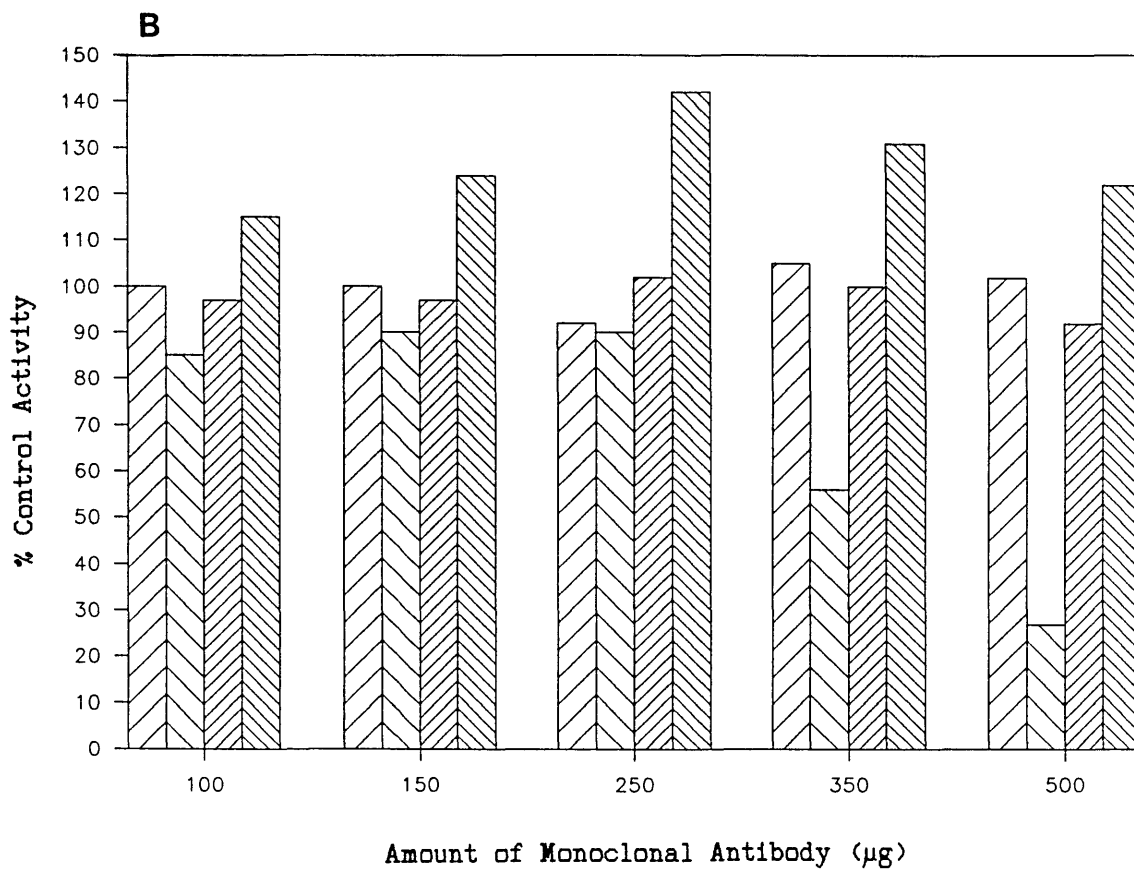


**Figure 4.5 Effect of Monoclonal Antibodies on Liver Microsomal
Aldrin Epoxidase Activity**

The effects of MAb's 3/4/2 (▨), 12/2/3/2 (▧), B3A3 (▩) and 1/4 (▦) on liver microsomal aldrin epoxidase activities from (A) control and (B) PB treated rats were determined as described in section 4.2.3 . Due to its protein concentration and the total incubation volume of the assay, MAb 3/4/2 could not be included at a concentration of 350µg per incubation. The results are expressed as a percentage of the activity of the appropriate buffer only (i.e. no MAb) control, and are the average of duplicate determinations.

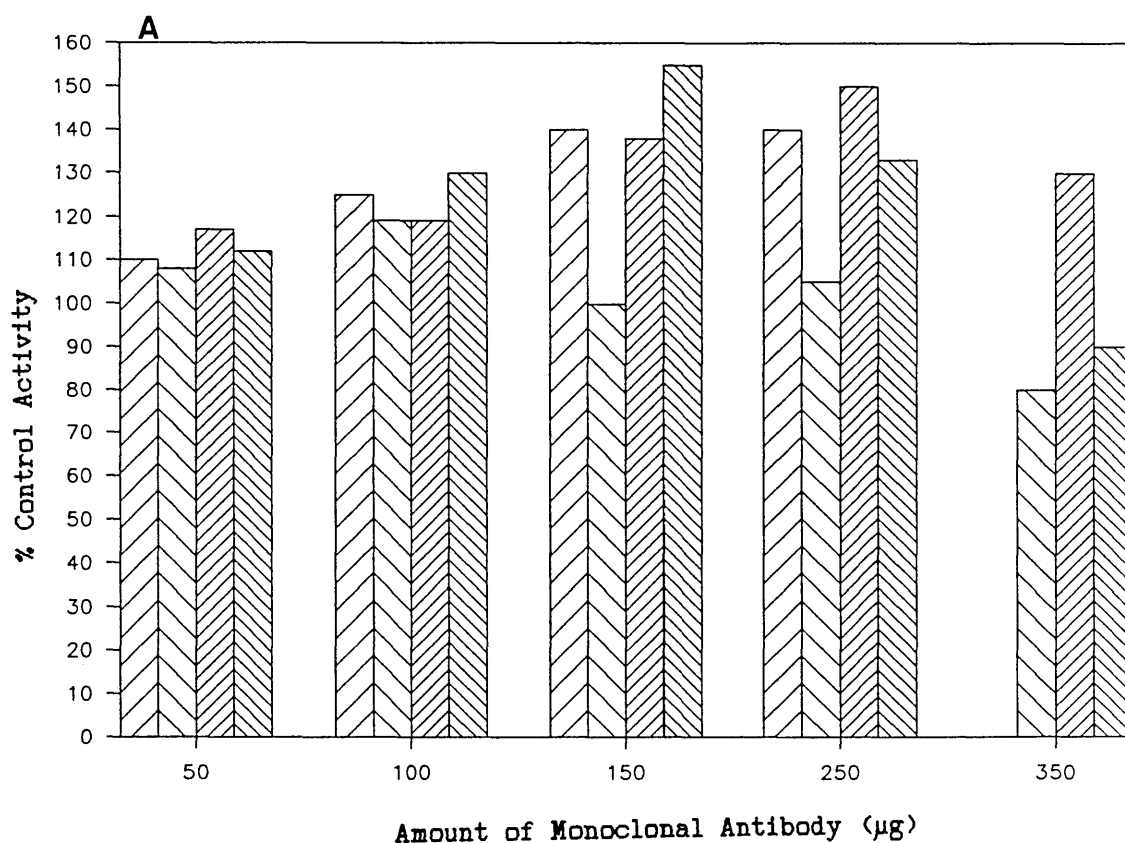
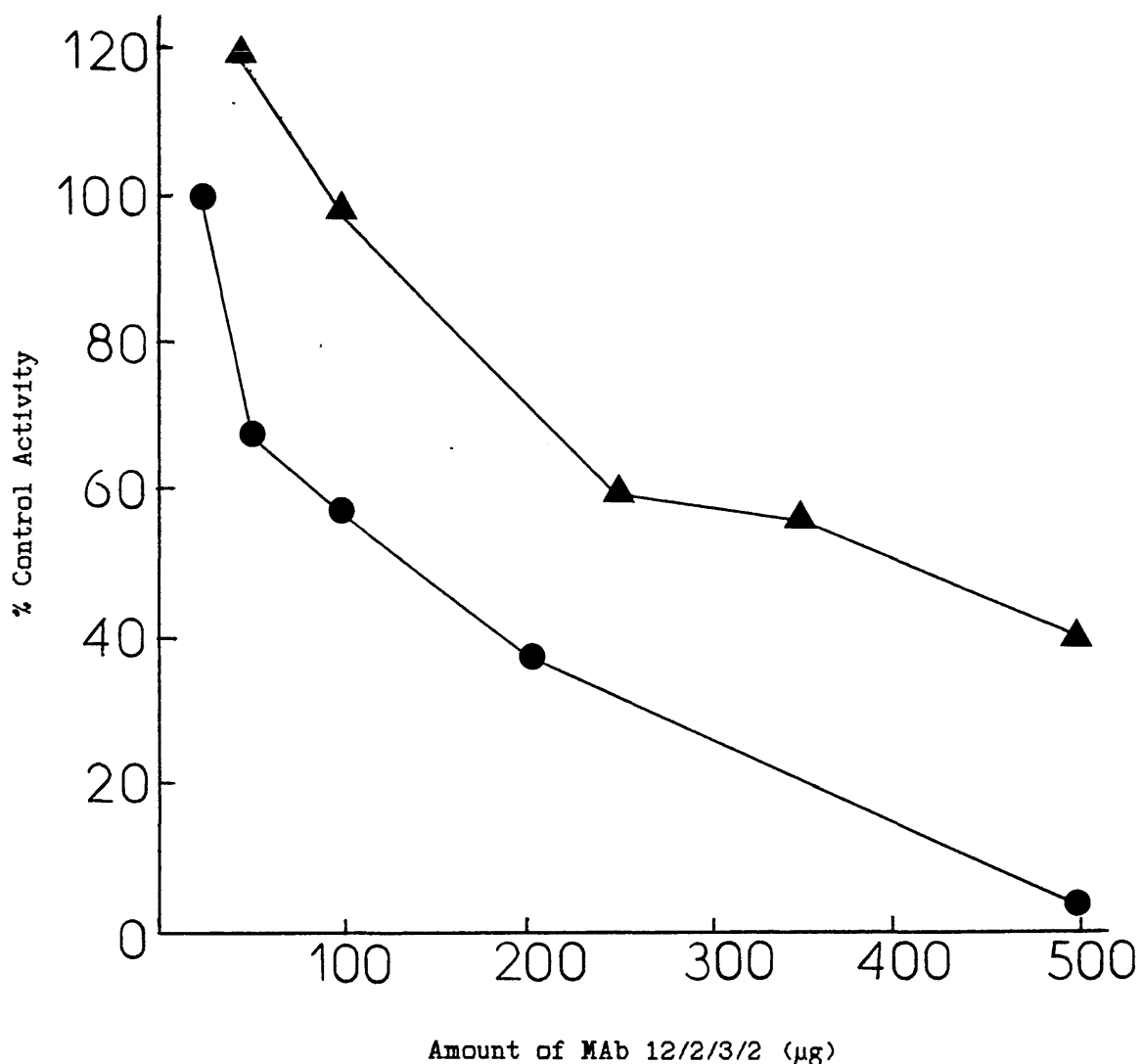


Figure 4.5 continued



**Figure 4.6 Effect of MAb 12/2/3/2 on 7-Ethoxyresorufin-O-Deethylase
Activities**

EROD activity was determined in the presence of MAb 12/2/3/2 with liver microsomes from β NF treated rats (▲) and with reconstituted cytochrome P-450c (●), as described in section 4.2.4 . The results are expressed as a percentage of the activity of the appropriate buffer only (i.e. no MAb) control, and are the average of duplicate determinations.



CHAPTER 5

IMMUNOPURIFICATION OF CYTOCHROME P-450 ISOZYMES

5.1 Introduction

Despite the increasing use of antibody and DNA reagents as specific probes for various cytochrome P-450 isozymes, for many applications, a source of the purified isozyme is still required. Such applications would include the determination of the full or partial amino acid sequence of the isozyme, peptide or epitope mapping, and the provision of isozyme standards in, for example, immunoassays or SDS-PAGE analysis. Whilst such purified isozymes could also be used in studies of substrate specificity, similar information can also be obtained with microsomal preparations using specific inhibitory MAb's, as discussed in Chapter 4 of this thesis. Classical procedures for cytochrome P-450 purification are often laborious and involve several chromatographic steps. Though successful purification has been reported from several laboratories (Lu and West, 1980; Sato and Kato, 1982; Boobis *et al.*, 1985b), the task has been generally slow, difficult and can result in low yields of the purified isozyme. For closely related isozymes or minor forms, such limitations are the norm, and even more so for extrahepatic forms. Individual isozymes in untreated animals, and for that matter in human tissue, are present in even smaller amounts and yields using standard techniques can be very poor.

One possible solution would be to capitalise on the high specificity that MAb's exhibit towards their corresponding epitopes. A series of MAb's will recognise specific epitopes which may or may not be unique to the cytochrome P-450 isozyme to which they were raised. In the latter case, such MAb's may be of use in the immunopurification of heterologous cytochrome P-450 isozymes, perhaps even from other species. One MAb produced during the course of this project, MAb 3/4/2, is specific for form c present in liver microsomes from β NF treated rats. However, it also exhibits a wide reactivity with microsomal fractions from several species, including man. In Western blotting studies with liver microsomal fractions it reacts with rat form c, rabbit forms 4 and 6, a human

cytochrome P-450 form and a unique PB inducible form of cytochrome P-450. Such a MAb would be an ideal candidate to use in immunopurification studies. The ability to purify isozymes of cytochrome P-450 from several species, including man, with a single MAb would open new areas of comparative cytochrome P-450 investigation.

Conventional methods of immobilising antibodies on to solid supports usually involve chemically coupling them to CNBr activated Sepharose. The use of this support can often produce affinity columns with low antibody activity due to multisite attachment and orientation of the antibody molecule, which can reduce the efficiency of its interaction with the antigen. An alternative method would be to utilise the specific interaction that exists between some immunoglobulin sub-classes and protein A from *Staphylococcus aureus*. Binding occurs at the Fc portion of the immunoglobulin molecule, and a matrix using protein A-Sepharose will therefore offer the optimal spatial orientation of the antibody, with the Fab portion being exposed to the liquid phase of the system. Not all MAb's bind to protein A with equal affinity, however, with mouse IgG₁ being recognised as having a poor binding ability (Kronvall *et al.*, 1970). A RAM intermediate layer may then need to be introduced to facilitate binding. Solid phase supports need not involve specific protein or chemical interactions for protein binding, however. Systems such as polystyrene microtitre wells, for example, rely on the physical attraction between the differently charged proteins and polystyrene for binding. Polystyrene beads could, therefore, also be used as a solid matrix for MAb immobilisation and subsequent immunopurification studies.

In this chapter, the effectiveness of these three types of solid matrices in the immunopurification of cytochrome P-450 isozymes is described, using as reagents for immunopurification, MAb's 1/4 and 3/4/2, together with control MAb's B3A3 and TB71.

5.2 Methods

5.2.1

Monoclonal Antibody Preparations

MAb's 1/4, 3/4/2 and B3A3 were purified before use by ion-exchange chromatography on DEAE Affi-Gel Blue, as described in section 2.2.5 . The control MAb TB71 was supplied by Dr. A. Rees of the MRC Tuberculosis Unit, RPMS, London, as an ion-exchanged purified preparation. It is directed against a surface antigen on *Mycobacterium tuberculosis* and did not show any cross-reactivity with any cytochrome P-450 preparation tested.

5.2.2

Cytochrome P-450 Preparations

Liver microsomal fractions from β NF and PB treated rats were prepared as described in section 2.2.1 . Microsomes were solubilised before use with sodium cholate, as described in section 2.2.7 .

5.2.3

Screening Assay for MAb 3/4/2 Binding Conditions

Polystyrene microtitre plates were coated with 100 μ l of a 100 μ g/ml suspension of liver microsomes from β NF treated rats in PBS, under normal ELISA conditions (section 2.2.6). Dilutions of MAb 3/4/2 covering the linear portion of the MAb's titration curve were prepared in the following detergent solutions:

- 1) PBS containing 0.2% (v/v) Renex 690
- 2) PBS containing 0.2% (w/v) sodium cholate
- 3) PBS containing 1.0% (w/v) SDS

Similar detergent solutions not containing MAb 3/4/2 were also prepared, as were identical dilutions of MAb 3/4/2 in PBS without the addition of any detergent.

Microtitre plates were then incubated sequentially with several combinations of the above reagents as follows:

Control)	Detergent alone, then MAb 3/4/2 in PBS only
Test)	PBS alone, then MAb 3/4/2 in detergent solutions
Max Binding)	PBS alone, then MAb 3/4/2 in PBS without detergents

Each incubation step was performed for 2 hours at 37°C, followed by extensive washing with PBST. RAM-HRP was then added and colour development initiated using standard ELISA conditions (section 2.2.6).

The control assay was included to determine whether the detergent solutions eluted adsorbed microsomal protein from the microtitre wells prior to the addition of MAb 3/4/2, which would have given a false indication of their effect on MAb 3/4/2 binding.

5.2.4

Screening Assay for MAb 3/4/2 Elution Conditions

Polystyrene microtitre plates were coated with 100µl of a 100 µg/ml suspension of liver microsomes from βNF treated rats in PBS, under normal ELISA conditions (section 2.2.6). Dilutions of MAb 3/4/2 covering the linear portion of the MAb's titration curve were prepared in PBS.

The following eluting agents were prepared to investigate their effect on MAb 3/4/2 binding:

- 1) 0.1M glycine/hydrochloric acid buffer, pH3.0
- 2) 50mM diethanolamine, pH 11.1
- 3) 4.0M urea, pH 9.1

Microtitre plates were incubated sequentially with several combinations of the above reagents as follows:

Control)	Eluting agent, then MAb 3/4/2
Test)	MAb 3/4/2, then eluting agent
Max Binding)	PBS alone, then MAb 3/4/2

Incubations with eluting agents or PBS were performed for 30 minutes at room temperature, whilst those with MAb 3/4/2 were for 2 hours at 37°C. These steps were followed by extensive washing with PBST. RAM-HRP was then added and colour development initiated using standard ELISA conditions (section 2.2.6).

The control incubation was included to determine that the eluting agents used were not affecting MAb binding by removing or altering adsorbed microsomal proteins.

5.2.5

Assay to Determine the Presence of Haem Containing Material

Assay methods utilised the peroxidase activity of cytochrome P-450, and were modified from those described by Thomas *et al.* (1976b). To 100µl of a cytochrome P-450 solution prepared in PBS, was added 850µl of assay reagent solution. This solution comprised 6.3mM 3,3',5,5'-tetramethylbenzidine (TMBZ) in methanol, diluted with an equal volume of 0.25M sodium acetate buffer, pH 5.0, before use (Bos *et al.*, 1981). Samples were incubated in the dark for 60 minutes at room temperature, before the addition of 50µl of 0.6M hydrogen peroxide in PBS, to a final concentration of 30mM. Samples were incubated in the dark for a further 30 minutes at room temperature, before their absorbance at 658nm was determined.

A standard curve was included in each series of determinations, constructed by preparing suitable dilutions, in PBS, of a partially purified preparation of cytochrome P-450c (5.1 nmoles/mg protein specific content).

The cytochrome P-450 content of this preparation was determined by the reduced carbon monoxide difference spectroscopy method described in section 2.2.10 .

5.2.6

Binding of Antibodies to Protein A

Individual polystyrene microtitre wells were coated with 100 μ l of a 1 μ g/ml solution of protein A in PBS, under normal ELISA conditions (section 2.2.6). The following antibodies were added at a concentration of 0.1 mg/ml in PBS, and incubated under normal ELISA:

- 1) affinity purified Ig fraction of RAM serum
- 2) rabbit anti-mouse IgG₁ whole serum (RAM-IgG₁)
- 3) DEAE Affi-Gel Blue purified MAb 3/4/2

The binding of MAb 3/4/2 to protein A was determined by the addition of 2×10^4 cpm of ^{125} I-labelled SAM F(ab')₂. Binding of the rabbit antibodies was determined by the addition of 2×10^4 cpm of ^{125} I-labelled donkey anti-rabbit IgG (DAR) F(ab')₂. Both preparations of ^{125} I-labelled antibodies were incubated in the microtitre wells for 2 hours at 37°C, followed by extensive washing in PBST. The amount of ^{125} I label remaining on the wells was determined using a gamma counter.

5.2.7

Protein A-Sepharose Gel Matrix

5.2.7.1 Preparation of Monoclonal Antibody Coupled

Protein A-Sepharose Gels

Portions (0.3g, equivalent to 1ml of swollen gel) of protein A-Sepharose (pA-Seph) were swollen and washed in 0.1M borate buffer, pH 8.2, and incubated for 18 hours at 4°C with various antibody preparations, also in borate buffer. RAM-IgG₁ serum was applied in buffer containing 0.5% (v/v)

Triton X-100, to minimise non-specific binding of serum proteins to the gel. Antibody gels were extensively washed with borate buffer followed by 0.2M triethanolamine buffer, pH 8.2. At this stage, some gels to which RAM-IgG₁ had been coupled were incubated with MAb 3/4/2 for 2 hours at room temperature. These gels were then further washed with borate and triethanolamine buffers.

All antibody pA-Seph gels were cross-linked to provide a stable gel matrix with minimal antibody loss on subsequent incubations. Cross-linking was achieved by gently mixing the gels for 45 minutes at room temperature in 20ml of 20mM dimethylpimelimidate in 0.2M triethanolamine buffer, pH 8.2 (Schneider *et al.*, 1982). Gels were extensively washed with triethanolamine buffer, and excess active sites blocked by centrifuging the beads and resuspending them in 20mM ethanolamine buffer, pH 8.2, for 5 minutes at room temperature. The cross-linked gels were then washed extensively with 0.1M borate buffer, pH 8.2, and stored in the same buffer containing 0.05% (w/v) sodium azide.

The following cross-linked pA-Seph gels were prepared:

- 1) pA-Seph + MAb 3/4/2
- 2) pA-Seph + RAM-IgG₁ + MAb 3/4/2
- 3) pA-Seph + RAM-IgG₁
- 4) pA-Seph + MAb TB71

Gel 4) was prepared as a negative control using MAb TB71. This MAb was included here in preference to the normal control MAb, B3A3, as it was found by its originator to bind well to protein A.

5.2.7.2 Estimation of the Amount of Mouse IgG Bound to Protein A-Sepharose Gels

The amount of mIgG bound to various pA-Seph gel matrices was determined using ^{125}I -SAM F(ab')_2 and previously calculated standard curves.

Individual microtitre wells were incubated with various protein concentrations of ^{125}I -labelled liver microsomes from βNF treated rats, under standard ELISA coating conditions (section 2.2.6). The amount of protein bound for each concentration used was then easily determined from the number of counts remaining on the microtitre wells. This allowed the construction of a standard curve for protein binding onto polystyrene. A second standard curve was then prepared by incubating individual microtitre wells with various concentrations of mIgG under standard ELISA conditions, followed by 18 hours incubation at 4°C with 7.5×10^5 cpm of ^{125}I -SAM F(ab')_2 . The number of counts remaining on each well allowed the relationship between the amount of mIgG bound per well and the level of ^{125}I -SAM F(ab')_2 binding to be determined.

The amount of mIgG bound to individual MAb coupled pA-Seph gels was then determined by preparing dilutions of each gel in PBS, each starting from the same initial amount of gel. Gels were incubated for 18 hours at 4°C with 7.5×10^5 cpm of ^{125}I -SAM F(ab')_2 , and then washed three times with PBST. The number of counts remaining on the gels was then determined and related to the mIgG standard curve previously prepared to estimate the amount of mIgG present on the gels.

5.2.7.3 Immunopurification Procedure with Protein A-Sepharose Gels

A partially purified preparation of rat liver cytochrome P-450c (5.1 nmoles/mg protein specific content) was radiolabelled with ^{125}I , as described in section 2.2.7. A 1ml portion of pA-Seph affinity gel 2) (pA-Seph + RAM-IgG₁ + MAb 3/4/2) was incubated with 2×10^6 cpm of the

^{125}I -labelled cytochrome P-450c preparation (approximately 0.8nmols of total cytochrome P-450). A 500 μl portion of affinity gel 4) (pA-Seph + MAb TB71) was incubated with 10^6 cpm of the labelled preparation (approximately 0.4nmols of total cytochrome P-450). Both gels were incubated for 2 hours at 4°C before being packed into a 2ml syringe barrel, and sequentially washed with the following buffers:

- A) 50mM Tris hydrochloride buffer, pH 8.2, containing 0.5M sodium chloride, 1mM EDTA and 0.5% (v/v) Nonidet NP-40
- B) 50mM Tris hydrochloride buffer, pH 8.2, containing 0.15M sodium chloride, 0.5% (v/v) Nonidet NP-40
- C) 0.15M sodium chloride containing 0.5% (w/v) sodium deoxycholate
- D) 50mM diethylamine, pH 11.5, containing 0.5% (w/v) sodium deoxycholate

Fractions eluted with elution buffer (buffer D) were immediately neutralised by the addition of 50 μl of 0.5M sodium dihydrogen phosphate buffer, pH 7.4 .

With all buffers, 500 μl fractions of eluate were collected and analysed for both the amount of ^{125}I -labelled protein present and, by the liquid phase assay described in section 5.2.5, for the presence of haem containing material.

5.2.8

Polystyrene Bead Matrix

5.2.8.1 Preparation of Monoclonal Antibody Coupled

Polystyrene Bead Matrix

Polystyrene beads, of 15.8 μm average diameter, were washed extensively with 50mM glycine/0.15M sodium chloride buffer, pH 8.2, (glycine/saline buffer). 1ml portions (at 10% (w/v) solid content) of washed beads were incubated, with mixing, for 18 hours at 4°C with either 5mg of purified MAb

3/4/2, or 5mg of control mouse IgG, made up to a total volume of 10ml with glycine/saline buffer. Beads were washed several times with glycine/saline buffer, and then washed twice with PBS. As the binding between proteins and polystyrene beads is due to the physical attraction of the two charged species, control mouse IgG was used in preference to MAb B3A3 in this experiment to maximise protein coupling to the beads, in order to obtain a satisfactory negative control.

5.2.8.2 Immunopurification Procedure with Polystyrene Beads

Antibody coated beads were incubated, with mixing, with solubilised liver microsomes from β NF treated rats for 18 hours at 4°C. MAb 3/4/2 coated beads were incubated with 8.5nmol of solubilised cytochrome P-450, whilst control mouse IgG coated beads were incubated with 20nmol. Total volume in both cases was made up to 20ml with PBS. Beads were then washed extensively with PBST

Portions (50 μ l) of cytochrome P-450 incubated beads, together with 100 μ l portions of the cytochrome P-450 preparation, unbound cytochrome P-450 and material which could be washed from the beads, were analysed for haem content using the liquid phase assay described in section 5.2.5 .

Portions (200 μ l) of both MAb coated matrices were reconstituted with NADPH cytochrome c reductase and lipid, as described in section 4.2.4.2, to determine the ability of any bound cytochrome P-450 to metabolise B[a]P, as described in section 4.2.2 .

To elute bound cytochrome P-450 from the beads, 200 μ l portions of washed beads were incubated for 10 minutes at room temperature with 2ml of 0.1M glycine/hydrochloric acid buffer, pH 3.0. Supernatants were neutralised by the addition of 20mg of Tris base, and subsequently analysed by SDS-PAGE, as described in section 2.2.8 .

5.2.9

Cyanogen Bromide Activated Sepharose Gel Matrix

5.2.9.1 Preparation of Monoclonal Antibody Coupled Sepharose Gels

The methods followed were modified from those described by Friedman *et al.* (1983). Purified MAb's were extensively dialysed against the gel coupling buffer, which was comprised of 0.2M sodium citrate buffer, pH 6.5, containing 0.2M sodium chloride. A total of 4mg of each purified MAb was reacted with 1 ml of CNBr activated Sepharose CL-4B gel for 18 hours at 4°C, using the protocol described by Pharmacia (1983). Excess active groups were blocked by the addition of 1M ethanolamine, pH 8.2, for 18 hours at 4°C. Coupled gels were then alternatively washed with 0.1M sodium acetate buffer, pH 4.0, and coupling buffer, for several cycles. MAb coupled Sepharose gels (MAb-Seph) were finally washed and stored in PGE buffer.

MAb coupled gels were prepared for MAb's 1/4, 3/4/2 and B3A3 using these methods.

5.2.9.2 Immunopurification Procedures with Sepharose Gels

Solubilised liver microsomes from β NF and PB treated rats were made up to a cytochrome P-450 concentration of 5 μ M with PGE buffer. Portions (1ml) of these preparations (5nmoles) were incubated, with mixing, for 1 hour at room temperature with 200 μ l (settled volume) of MAb B3A3-Seph to remove any material that might bind non-specifically to the MAb-Seph matrix. Supernatants were collected by centrifugation and then incubated, with mixing, for 1 hour at room temperature with 500 μ l (settled volume) of MAb 3/4/2-Seph for solubilised microsomes from β NF treated rats, or with MAb 1/4-Seph for solubilised microsomes from PB treated rats. In addition, both cytochrome P-450 preparations were further incubated with 500 μ l of MAb B3A3-Seph as control experiments.

Gels were then extensively washed with the following buffers to remove unbound material:

- E) 40mM phosphate buffer, pH 6.5, containing 2M sodium chloride and 20% (v/v) glycerol
- F) 40mM phosphate buffer, pH 7.4, containing 0.1mM EDTA, 1% (v/v) Triton X-100, 0.2% (w/v) sodium cholate and 20% (v/v) glycerol
- G) 40mM phosphate buffer, pH 7.4, containing 0.15M sodium chloride, 0.1mM EDTA, 1.5% (v/v) Renex 690 and 20% (v/v) glycerol
- H) PGE buffer

Washed gels were resuspended in PGE buffer, and 100µl portions were analysed for cytochrome P-450 content by the reduced carbon monoxide difference spectroscopy method, as described in section 2.2.10 .

To elute bound cytochrome P-450, 100µl portions of washed gels were treated with 100µl of 2% (w/v) SDS. The resultant supernatants were collected by centrifugation and analysed by SDS-PAGE, as described in section 2.2.8 .

5.3 Results

5.3.1

Effect of Detergents on MAb 3/4/2 Binding

The effects of several detergents on MAb 3/4/2 binding are illustrated in Figure 5.1 . Neither Renex 690 nor sodium cholate appeared to have any significant effect on the binding of MAb 3/4/2 to the adsorbed microsomal proteins, at the concentrations used. SDS, on the other hand, completely blocked the binding of MAb 3/4/2 when used as a 1.0% (w/v) solution. It can also be seen, however, that it also reduced MAb 3/4/2 binding when pre-

incubated with the adsorbed microsomes. This may be due to the introduction of a negative charge by the detergent on to the adsorbed proteins, which subsequently repelled MAb 3/4/2 in solution. It may also be possible that the detergent caused the loss of some protein from the surface of the well, thereby reducing that available for MAb 3/4/2 binding.

5.3.2

Effect of Eluting Agents on MAb 3/4/2 Binding

The effects of several eluting agents on MAb 3/4/2 binding are shown in Figure 5.2 . Glycine/hydrochloric acid buffer was successful in removing bound MAb 3/4/2 from adsorbed microsomes, whilst pre-incubation with this reagent had no effect on MAb 3/4/2 binding. In contrast, neither urea nor diethanolamine had any effect on the binding of MAb 3/4/2 to adsorbed microsomal proteins, at the concentrations used.

5.3.3

Assay for Haem Containing Material

The coloured complex formed by the action of cytochrome P-450 on TMBZ and hydrogen peroxide had a maximum absorbance at 658nm (Figure 5.3). The response curve for the reaction was linear within a cytochrome P-450 range of 5 to 80 pmoles/100µl sample, as seen for the standard curve illustrated in Figure 5.4 . Some deviation from linearity was however observed at cytochrome P-450 concentrations below 5 pmoles/100µl.

5.3.4

Binding of Antibodies to Protein A

It can be seen from the results illustrated in Figure 5.5, that whilst both RAM and RAM-IgG₁ showed very good protein A binding characteristics, MAb 3/4/2 produced very little protein A binding.

5.3.5

Estimation of the Amount of Mouse IgG bound to Protein A-Sepharose Gels

5.3.5.1 Preparation of Standard Curves

The results obtained for the percentage binding of ^{125}I -labelled microsomes to polystyrene microtitre wells are shown in Figure 5.6 . As the protein concentration decreased, the percentage of that protein which adsorbed onto the surface of the wells increased. Using this as a standard curve, it was possible to produce another standard curve for the relationship between the percentage of ^{125}I -SAM F(ab')_2 bound, against μg of mIgG adsorbed per microtitre well (Figure 5.7).

5.3.5.2 Analysis of Antibody Coupled Protein A-Sepharose Gels

From the prepared standard curves illustrated in Figures 5.6 and 5.7, the amount of mIgG bound per ml of antibody coupled pA-Seph gel was estimated from the dilution curves produced for the various gel matrices, when reacted with the same amount of ^{125}I -SAM F(ab')_2 . The estimates of mIgG coupled for the various gel matrices are given in Table 5.1 . Both gels 2 and 4 contained higher levels of mIgG bound per ml of gel, than either of gels 1 or 3. The results indicate that little MAb 3/4/2 bound directly to protein A, as would be expected from the results shown in Figure 5.5 . The results obtained for pA-Seph alone, when incubated with ^{125}I -SAM F(ab')_2 , gave values very similar to those obtained with gel 3. This presumably represents the non-specific background level due to reactivity of the labelled SAM with RAM-IgG, or protein A.

5.3.6

Analysis of Cytochrome P-450 Incubated Matrices

5.3.6.1 Determination of Haem Containing Material

Using the assay for haem determination, the presence of haem containing material on cytochrome P-450 incubated polystyrene beads was determined (Figure 5.8). From these results, it was estimated that 5% (i.e. 0.45nmoles) of the applied cytochrome P-450 preparation was bound to MAb 3/4/2 coated beads. In comparison, only 0.5% (i.e. 0.1nmoles) of the cytochrome P-450 preparation applied to mIgG coated beads bound.

5.3.6.2 Determination of BenzofalPyrene Hydroxylase Activity

The results obtained for cytochrome P-450 incubated polystyrene beads are shown in Table 5.2 . The MAb 3/4/2 coated, cytochrome P-450 incubated beads, had a much higher B[a]P hydroxylase activity associated with them, in comparison to the corresponding mIgG coated beads. This is indicative of a higher specific binding of cytochrome P-450 to the former beads.

5.3.6.3 Determination of Reduced Carbon Monoxide Difference Spectra

Reduced carbon monoxide difference spectra were obtained for MAb-Seph beads (Figure 5.9). MAb 1/4 coupled gel had a higher level of cytochrome P-450 associated with it than the corresponding MAb 3/4/2 coupled gel. It is also clear that a large proportion of bound cytochrome P-450 was present as degraded cytochrome P-420. MAb B3A3 coupled gels, in contrast, did not show the presence of any bound cytochrome P-450.

5.3.7

Analysis of Eluted Fractions

5.3.7.1 Elution Profile From Protein A-Sepharose Gels

The results obtained for both ¹²⁵I counts and haem content for the fractions eluted from both gel 2 and gel 4 are shown in Figure 5.10,

together with an indication of the different buffers used. Haem containing material was detected in all the fractions eluted during the initial washing of both gels. One of the fractions eluted from gel 2 with buffer D was also positive in the haem assay. This fraction also had the highest ^{125}I counts of all the fractions collected during the specific elution of that gel. The total number of cpm specifically eluted from gel 2 was 3.7×10^4 , representing approximately 2% (i.e. 16pmoles) of the material applied to the gel. This level of cytochrome P-450 was confirmed by the results obtained in the haem assay. In comparison, only 0.2% (i.e. 0.8pmoles) of the ^{125}I -labelled cytochrome P-450 preparation applied to gel 4 was specifically eluted using buffer D. Almost no radiolabelled material remained bound to either gel at the end of the elution procedures.

5.3.7.2 SDS-PAGE Analysis

Fractions eluted from MAb coated polystyrene beads with glycine/hydrochloric acid buffer was analysed. No protein bands were detected by Coomassie Blue staining.

With the MAb 1/4-Seph gel, elution with 2% (w/v) SDS specifically removed a 54,000 molecular weight protein, which corresponds in molecular weight to cytochrome P-450 forms b and/or e, whilst in contrast, no proteins were eluted from the MAb B3A3-Seph gel (Figure 5.11). Treatment of the MAb 3/4/2-Seph gel with 2% (w/v) SDS did not produce any protein bands detectable with Coomassie Blue staining.

5.4 Discussion

The use of CNBr activated Sepharose, together with MAb 1/4 as a solid phase matrix has enabled the purification of specific cytochrome P-450 isozymes to be performed to greater than 90% purity. The procedures used were simple and quicker than conventional methods of purification. However, such results were not achievable with all the MAB's studied.

With it's wide ranging microsomal reactivity, initial attempts at immunopurification concentrated on the use of MAb 3/4/2. In any such study, it would be of great value to be able to determine the conditions for eluting the antigen of interest from the antibody to be used. In addition, the influence of the major buffer components on antigen binding should also be considered. Studies performed with MAB 3/4/2 indicated that the presence of 0.2% (v/v) Renex 690 or 0.2% (w/v) sodium cholate in the cytochrome P-450 buffer did not interfere with antibody binding. Conversely, 1.0% (w/v) SDS completely negated any antibody binding. It should thus be possible to use routine cytochrome P-450 buffers containing Renex 690 and sodium cholate in immunopurification studies with MAB 3/4/2 without interference. Similar studies with MAB 3/4/2 also indicated that 0.1M glycine/hydrochloric acid buffer, pH 3.0, was an effective eluting agent for this MAB.

The poor binding of MAB 3/4/2 to protein A rendered the use of pA-Seph as a gel matrix inoperable. To circumvent that, a gel matrix was prepared using anti-mouse Ig as an intermediate protein A binding component. The use of this intermediate component ran counter to the main rationale for using pA-Seph, namely that it places the MAB in the optimal spatial orientation, with a free Fab region. However the use of an intermediate binding component, still allowed a high level of MAB to be immobilised. In addition, the use of RAM-IgG₁, with its specificity for mouse IgG₁ heavy chain, should still allow the MAB to bind cytochrome P-450 free in solution.

Covalent coupling of MAb's to CNBr activated Sepharose, whilst the most common matrix used for immunopurification studies, would not, in theory at least, allow for such free access of the MAb to the antigen containing solution. The results obtained with MAb's 1/4 with this system indicated that this was not a problem for this particular MAb.

Specific elution of labelled haem containing material was achieved with a pA-Sep_H + RAM-IgG₁ + MAb 3/4/2 matrix, following incubation with ¹²⁵I-labelled partially purified cytochrome P-450c. In all, 2% of the material applied to the column was specifically eluted. In comparison, a control MAb did not effectively remove any haem containing material from the same cytochrome P-450 preparation. Immunopurification with MAb 3/4/2 was also attempted using polystyrene beads as a solid matrix. In this case, haem containing material was detected bound to the beads, again with no comparable haem being present on a control matrix. In addition, B[a]P hydroxylase activity could be reconstituted on the gel by the addition of reductase and lipid. This type of study is only feasible, of course, with a non-inhibitory MAb (Lewis *et al.*, 1982). Unfortunately, fractions eluted with glycine/hydrochloric acid buffer from cytochrome P-450 incubated MAb 3/4/2 beads were not resolved by SDS-PAGE, an indication, perhaps, of the amount of protein which could be specifically eluted by the agents used. Similarly, with a MAb 3/4/2 coupled Sepharose matrix, no SDS eluted proteins could be detected by SDS-PAGE analysis. This was despite the fact that bound cytochrome P-450 could be detected from the reduced carbon monoxide difference spectrum of the gel. MAb 1/4, when coupled to activated Sepharose, was not only able to remove cytochrome P-450 from solution, as determined by the reduced carbon monoxide difference spectrum obtained, but also allowed specific elution, as determined from the protein molecular weights obtained by SDS-PAGE analysis.

These results indicate that methodologies for immunopurification are not as robust as to be readily transferable from one MAb to another. It may be that MAb 3/4/2 is unsuitable for immunopurification studies, despite the fact that it can bind cytochrome P-450 in the presence of detergents, and can be dissociated from cytochrome P-450 under relatively mild conditions. It is able to remove cytochrome P-450 from solution, as seen by haem determination, reconstitution of B[a]P hydroxylase activity and reduced carbon monoxide difference spectra. However, the elution of unlabelled cytochrome P-450 from the various matrices used could not be demonstrated. On the other hand, with MAb 1/4, purification of cytochrome P-450 forms b and/or c was achieved in a simple procedure using covalently coupled MAb-Sepharose matrix. It should be noted that no immunopurification studies were performed with MAb 12/2/3/2. The exclusion of this MAb was due to time constraints on the project as a whole, and its concurrent use in the immunoquantitation and immunocytochemical studies reported in Chapters 6 and 7 of this thesis, respectively, which limited its availability.

Immunopurification of cytochrome P-450 has been performed in several previous studies using polyclonal antibodies. Levin and co-workers have successfully purified various cytochrome P-450 isozymes including forms b and c from liver microsomal fractions prepared from PB and 3MC treated rats respectively (Thomas *et al.*, 1979). Purification of cytochrome P-450 form a was achieved from liver microsomes from 3MC treated rats (Thomas *et al.*, 1981), whilst form d was purified from liver microsomal preparations from ISF treated rats (Reik *et al.*, 1982). A human form of cytochrome P-450 homologous to rat liver cytochrome P-450d has also been isolated by immunopurification (Wrighton *et al.*, 1986). All the antibody preparations used in these studies were immunoabsorbed against heterologous cytochrome P-450 preparations to render them monospecific. Such procedures were necessary to eliminate cytochrome P-450 cross-reactivity present within each antibody preparation. Such cross-reactivities are common with

polyclonal antibodies due to their heterogeneous nature, and are the main reason that immunopurification studies are better performed with MAb's.

MAb immunopurification of cytochrome P-450 has also been extensively performed by other workers. Gelboin and co-workers have been successful in the immunopurification of several isozymes of cytochrome P-450 from different species. The MAb's used in those studies were very similar in their reactivity to those described in this chapter. MAb 1-7-1, like MAb's 9/20/11 and 12/2/3/2 described elsewhere in this thesis, recognises both forms c and d in liver microsomes from 3MC treated rats, but does not react with liver microsomes from control rats (Friedman *et al.*, 1983), whilst MAb 1-31-2, like MAb 3/4/2, recognises only form c in the same microsomal preparation (Cheng *et al.*, 1984a). MAb 2-66-3, like MAb 1/4, recognises forms b and/or e in liver microsomes from PB treated rats (Friedman *et al.*, 1983). Using MAb's 1-7-1 and 1-31-2, either individually or in tandem, the immunopurification of eight cytochrome P-450 isozymes has been achieved (Cheng *et al.*, 1984a). Whilst MAb 1-7-1 purified two forms of cytochrome P-450, at both 56,000 and 57,000 molecular weight, from both rat and C₅₇BL/6 mouse liver, only the latter molecular weight form was purified from rat lung, and hamster and DBA/2 mouse liver. A further form was also isolated from guinea pig liver which was of a different molecular weight (53,000) from other isozymes isolated. The isozymes purified from rat liver by MAb 1-7-1 are of the same molecular weights as would be expected from its isozyme reactivity. MAb 1-32-2, on the other hand, purified only a single 57,000 molecular weight form from rat lung, and rat and C₅₇BL/6 mouse liver. A 54,000 molecular weight isozyme was also successfully purified from rat liver using MAb 2-66-2 (Friedman *et al.*, 1983), this isozyme being the same molecular weight as the isozyme(s) isolated by MAb 1/4 in this study. It was observed by these authors that the forms of cytochrome P-450 purified by MAb's specific for isozymes induced by treatment with 3MC were associated more with B[a]P hydroxylase

activity, whilst isozymes purified from PB specific MAb columns possessed a greater ECOD activity, an observation previously noted by others (Lu and West, 1980). This group have also reported the purification of a 45,000 molecular weight protein from a human lymphoblastoid cell line which possessed monooxygenase activity (Friedman *et al.*, 1985c). This protein could be purified by three MAb's reactive with isozymes of rat liver cytochrome P-450 induced by treatment with either 3MC or PB, and may well be a human form of cytochrome P-450, though no catalytic or structural studies were performed on the purified protein. Mendelson *et al.* (1985) have also reported on the successful isolation of a human placental cytochrome P-450 isozyme. In that report, the isozyme was a 55,000 molecular weight species which was found to possess aromatase activity (cytochrome P-450_{AROM}). Interestingly, this protein could be eluted from the MAb-Sepharose matrix by both high salt (2M sodium chloride) and acid (0.2M glycine/hydrochloric acid buffer, pH 3.0) washes. Whether this reflects heterogeneity in the eluted protein, or in the MAb preparation used for purification is unclear.

It can be seen that acid elution of cytochrome P-450 can lead to preparations that still possess detectable catalytic activities. Investigation of catalytic activity is not possible, however, if an eluting agent such as SDS is used, as was performed for MAb 1/4 in the studies reported in this chapter.

This same eluting agent (SDS) was also used by Reubi *et al.* (1984b) and Finlayson *et al.* (1987) in studies involving MAb's to rabbit cytochrome P-450 form LM1. In the former report, the single step purification of rabbit isozyme LM1 by three separate MAb's was described using MAb-Sepharose as the immunopurification matrix. Some cross-reactivity with form LM3b was observed for one of the MAb's used, with that particular MAb co-purifying both these isozymes from rabbit liver microsomes. In the latter report, a novel form of rabbit kidney cytochrome P-450 was isolated, termed

cytochrome P-450 K, which was of a molecular weight between those of forms LM1 and LM3b. This new form of cytochrome P-450 appears to be involved in the lauric acid 11-hydroxylase activity, and to be inducible by PB treatment. In another publication, this same group also reported on the purification of rabbit cytochrome P-450 form LM3b (Reubi *et al.*, 1984a). Rabbit cytochrome P-450 LM3a has also been immunopurified from solubilised liver, kidney and nasal microsomes from ethanol treated rabbits, again using SDS as the eluting agent (Ding *et al.*, 1986).

Purification of specific isozymes has also been achieved by Gelboin and co-workers using antigen exchange, resulting in elution of catalytically active forms of cytochrome P-450 (Friedman *et al.*, 1985d). Cytochrome P-450 isozymes from 3MC and PB treated rats, which were purified from MAb-Seph columns by acid elution, retained their MAb sensitive epitope integrity, as determined by competitive radioimmunoassay (Friedman *et al.*, 1985a). In addition, these eluted isozymes still retained some catalytic activity, albeit at a low level, and were capable of displacing the corresponding catalytically active isozyme from fresh cytochrome P-450 incubated MAb-Seph matrix. This elution procedure is common in the affinity purification of anti-hapten antibodies or receptors from hapten or ligand coupled matrices. Its use, though with protein eluting agents is limited. The advantage of this approach is that catalytically active isozymes can be prepared using immobilised MAb's that are normally inhibitory in enzyme assays. Studies by Lewis *et al.* (1982), in which enzyme activity was reconstituted on a MAb-Seph column obviously requires the use of non-inhibitory MAb's, which may restrict the range of suitable MAb's available for study. Cross-reacting MAb's may also be used to prepare active isozymes. In such a case, the purification of a cross-reacting isozyme present in an extra-hepatic tissue may be possible using the acid eluted liver cytochrome P-450 to which the MAb was raised as the eluting agent. However, the presence of inactive acid eluted isozymes may

detract from the quality of the catalytic isozyme preparation, especially if enzyme activity is being correlated with the specific content of active isozyme. Nevertheless, as a technique it opens up many opportunities for investigating the catalytic repertoire of MAb specific cytochrome P-450 isozymes.

Structural investigations of MAb purified isozymes are also possible, with amino terminal and structural analysis of eluted isozymes having been performed successfully. Liver isozymes from 3MC treated animals, purified by MAb's 1-7-1 and 1-31-1, either singly or in tandem, have been analysed (Cheng *et al.*, 1984b; 1986). The 56,000 molecular weight forms purified from rats, and from C₅₇BL/6 and DBA/2 mice, had identical amino terminal sequences and similar peptide maps. The 57,000 molecular weight form purified from rats and C₅₇BL/6 mice were homologous to each other, but had little homology with the 56,000 molecular weight polypeptide. The 53,000 molecular weight isozyme purified from guinea pigs was found to have a unique amino terminal sequence, with no apparent homology with the other purified forms tested. The amino terminal sequence of the 57,000 molecular weight rat isozyme was found by the authors to be the same as that deduced for a cloned and sequenced cDNA for cytochrome P-450 from 3MC treated Sprague-Dawley rats (Yabusaki *et al.*, 1984). It was also noted that the 57,000 molecular weight polypeptide immunopurified from C₅₇BL/6 mice and Sprague-Dawley rats were largely homologous in the amino terminal regions with their possible counterparts in Long Evans rats (Haniu *et al.*, 1984), as well as with the amino acid sequences determined from the cDNA sequences reported for the mouse cytochrome P₁-450 isozyme (Kimura *et al.*, 1984a; 1984b). In this latter case, an additional proline-serine dipeptide insertion was identified in the immunopurified 57,000 molecular weight protein. Similarly, sequences for cytochrome P-450d from Long Evans rats (Botelho *et al.*, 1982) were found by the authors to be conserved in the 56,000 molecular weight polypeptide immunopurified from Sprague-Dawley

rats, C₅₇BL/6 and DBA/2 mice. This protein was also similar to the amino acid sequence determined from the cDNA sequence of the mouse cytochrome P₃-450 isozyme from C₅₇BL/6 mice (Kimura *et al.*, 1984b). A form of cytochrome P-450 induced by treatment with 3MC has also been isolated from pulmonary rat microsomes which has the same electrophoretic mobility, amino terminal sequence and peptide map as the 57,000 molecular weight form of cytochrome P-450 isolated from liver microsomes from 3MC treated rats (Robinson *et al.*, 1986).

Gelboin and co-workers have also reported on the characterisation of the 54,000 molecular weight form of cytochrome P-450 isolated from liver microsomes of PB treated rats (Friedman *et al.*, 1985b). The SDS-PAGE analysis of this protein showed a diffuse band at 54,000 molecular weight. In addition, the amino terminal sequence of the isolated protein displayed a microheterogeneity, indicative of the isolation of two forms of cytochrome P-450. Comparative analysis of other known cytochrome P-450 sequences were found to be inconclusive in identifying the proteins isolated.

The cytochrome P-450 LM3a proteins isolated from liver and kidney microsomes prepared from ethanol treated rabbits, and reported by Ding *et al.* (1986) above, were also analysed by peptide mapping and found to be identical.

The results obtained in the studies reported above by other workers in this area illustrate the potential for cytochrome P-450 immunopurification. The amino acid sequence and peptide maps of immunopurified proteins can be determined, as can some information on their catalytic activity. Isozymes from various tissues and species have also been isolated using this methodology and their structure and activity relationships investigated. The results presented in this chapter for MAb's 1/4 only touch on the possibilities in this field. Nevertheless, the successful purification of cytochrome P-450 forms b and/or e has been achieved. Further studies will be required to determine whether or not active isozymes could be prepared

using this MAb. Even if that were not possible, purification studies involving both extra-hepatic isozymes and other species could be attempted. Structural studies could also be performed to determine the relationship of the purified isozymes with respect to known isozyme sequences.

It was unfortunate that MAb 3/4/2 was not successful in purifying any cytochrome P-450 isozymes. Its wide microsomal reactivity with several species, including man, made it an ideal candidate for inclusion in such studies. Initial results obtained with both detergents and eluting agents indicated that this MAb would be suitable. Encouraging results were also obtained in studies involving incubations between ¹²⁵I-labelled partially purified cytochrome P-450c and a pA-Seph + RAM-IgG, + MAb 3/4/2 matrix. Similar incubations with unlabelled microsomal preparations though, did not fulfill these promising early results. However, this should not detract from other results obtained for this MAb and discussed elsewhere in this thesis.

In conclusion, MAb's can be used for the rapid immunopurification of cytochrome P-450 isozymes containing specific MAb reactive epitopes. They are potentially useful for the isolation of those cytochrome P-450 isozymes present at low levels in tissues whose purification by conventional methods may be hindered by low yields. Further studies are required, however, to ascertain the full potential of the MAb's described in this chapter.

**Figure 5.1 Effect of Detergents on MAb 3/4/2 Binding to Liver
Microsomes from BNF Treated Rats**

The effects of (A) Renex 690, (B) sodium cholate and (C) SDS on MAb 3/4/2 binding to liver microsomes from BNF treated rats was determined, as described in section 5.2.3 . Results are expressed as the average OD 492nm of duplicate determinations for maximum MAb binding (●), control binding (▲) and test binding (■).

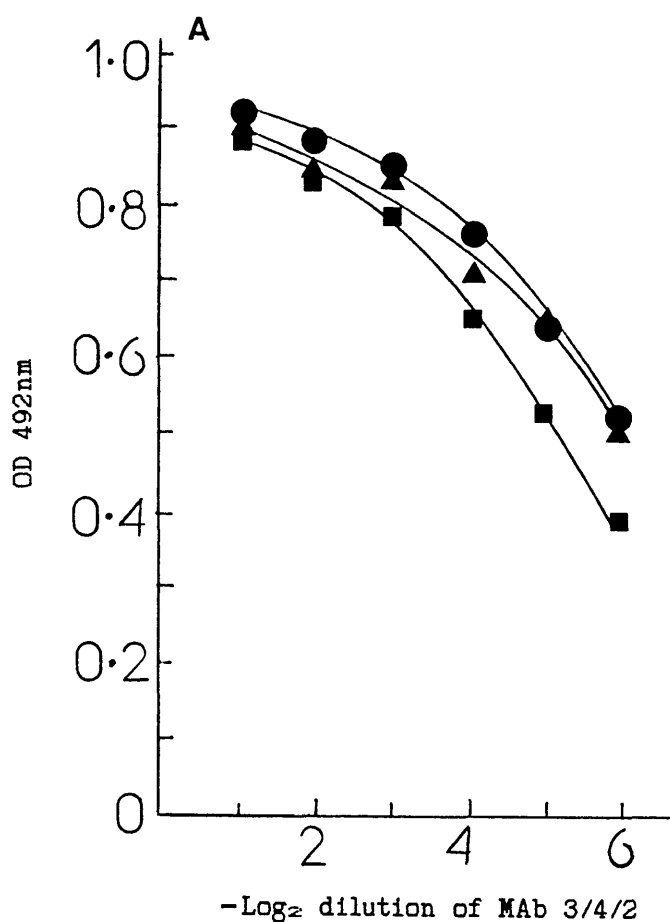


Figure 5.1 continued

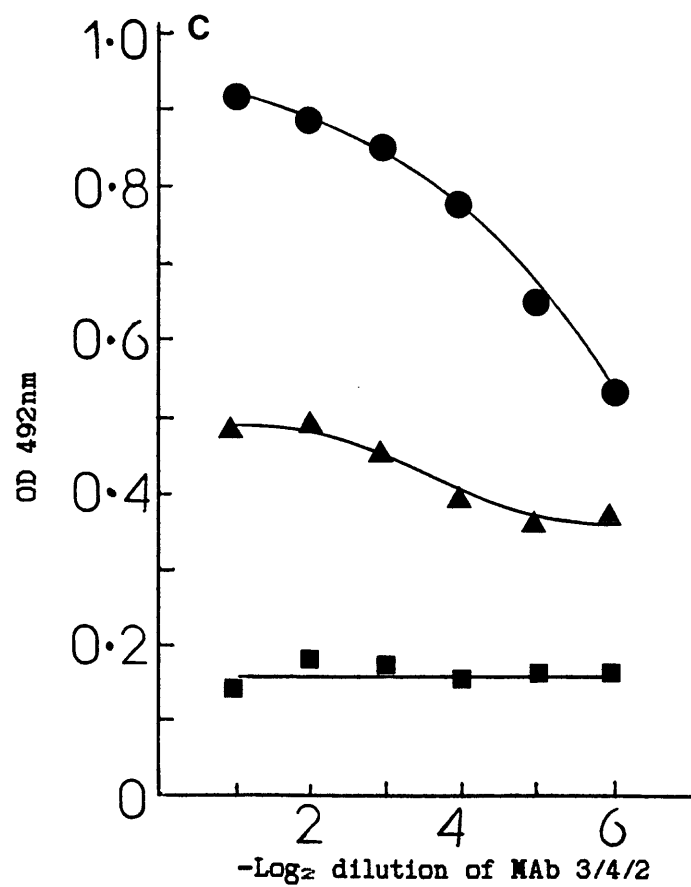
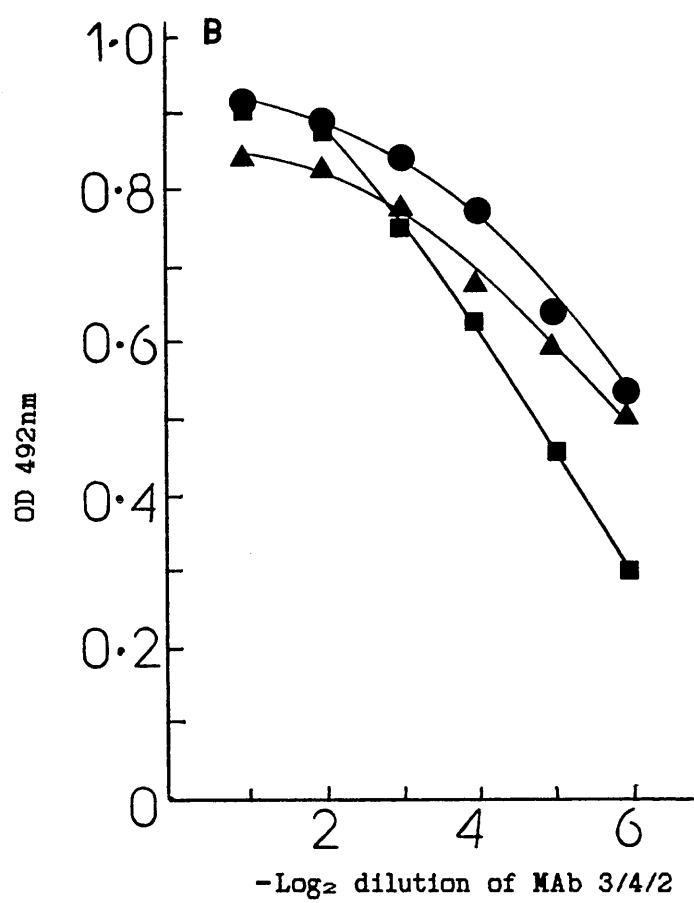


**Figure 5.2 Effect of Eluting Agents on MAb 3/4/2 Binding to Liver
Microsomes from BNF Treated Rats**

The effects of (A) glycine/hydrochloric acid buffer, (B) diethanolamine and (C) urea on MAb 3/4/2 binding to liver microsomes from β NF treated rats was determined, as described in section 5.2.4 . Results are expressed as the average OD 492nm of duplicate determinations for maximum MAb binding (●), control binding (▲) and test binding (■).

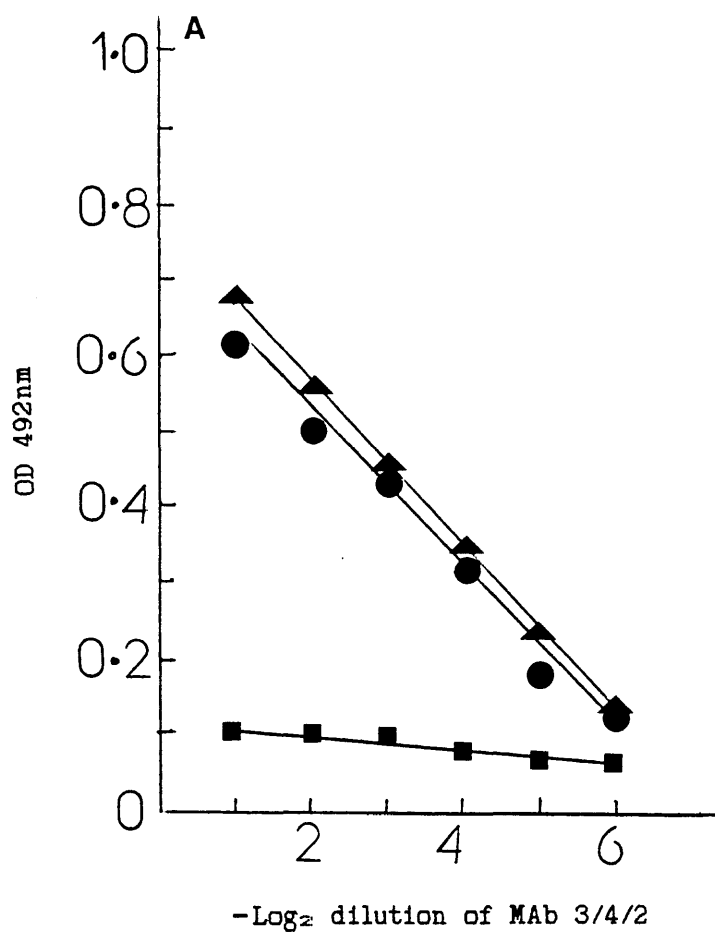


Figure 5.2 continued

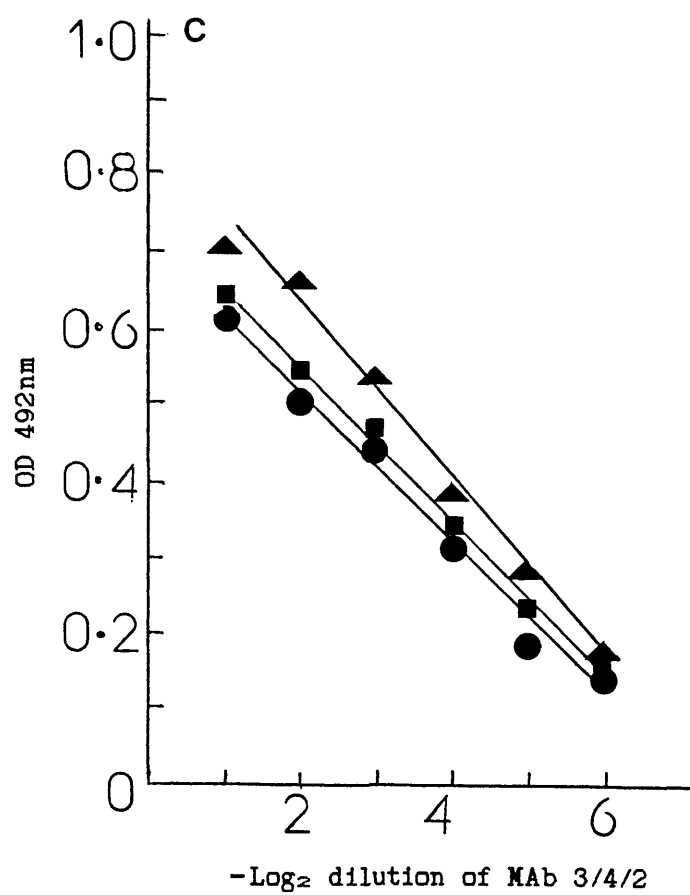
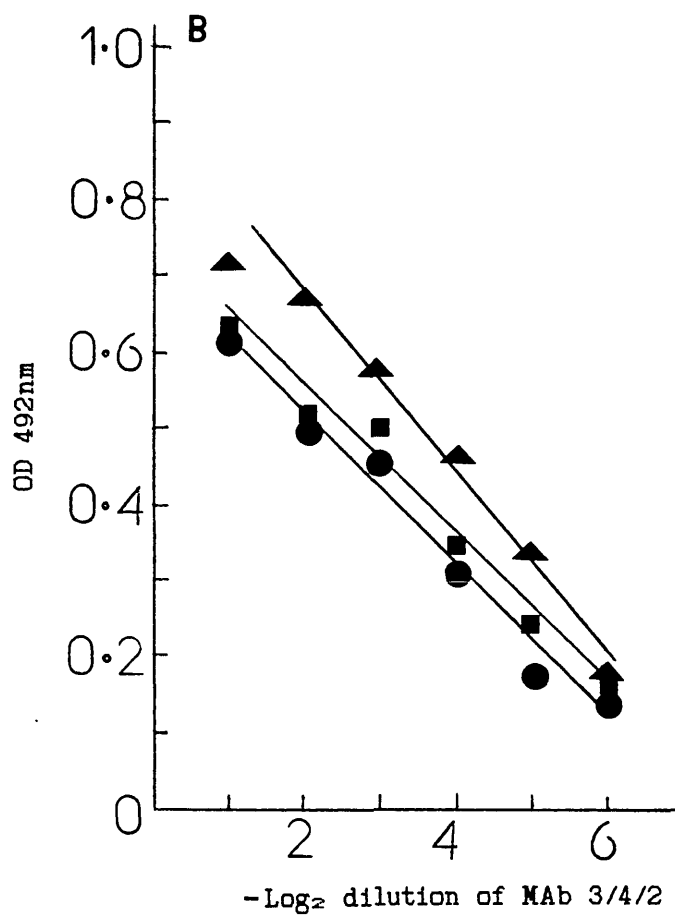


Figure 5.3 Absorbance Spectrum of 3,3',5,5' Tetramethylbenzidine
Following Cytochrome P-450 Peroxidase Activity

The coloured complex formed by the action of 50pmoles of cytochrome P-450 on hydrogen peroxide and TMBZ was analysed for its absorbance spectrum, as described in section 5.2.5 .

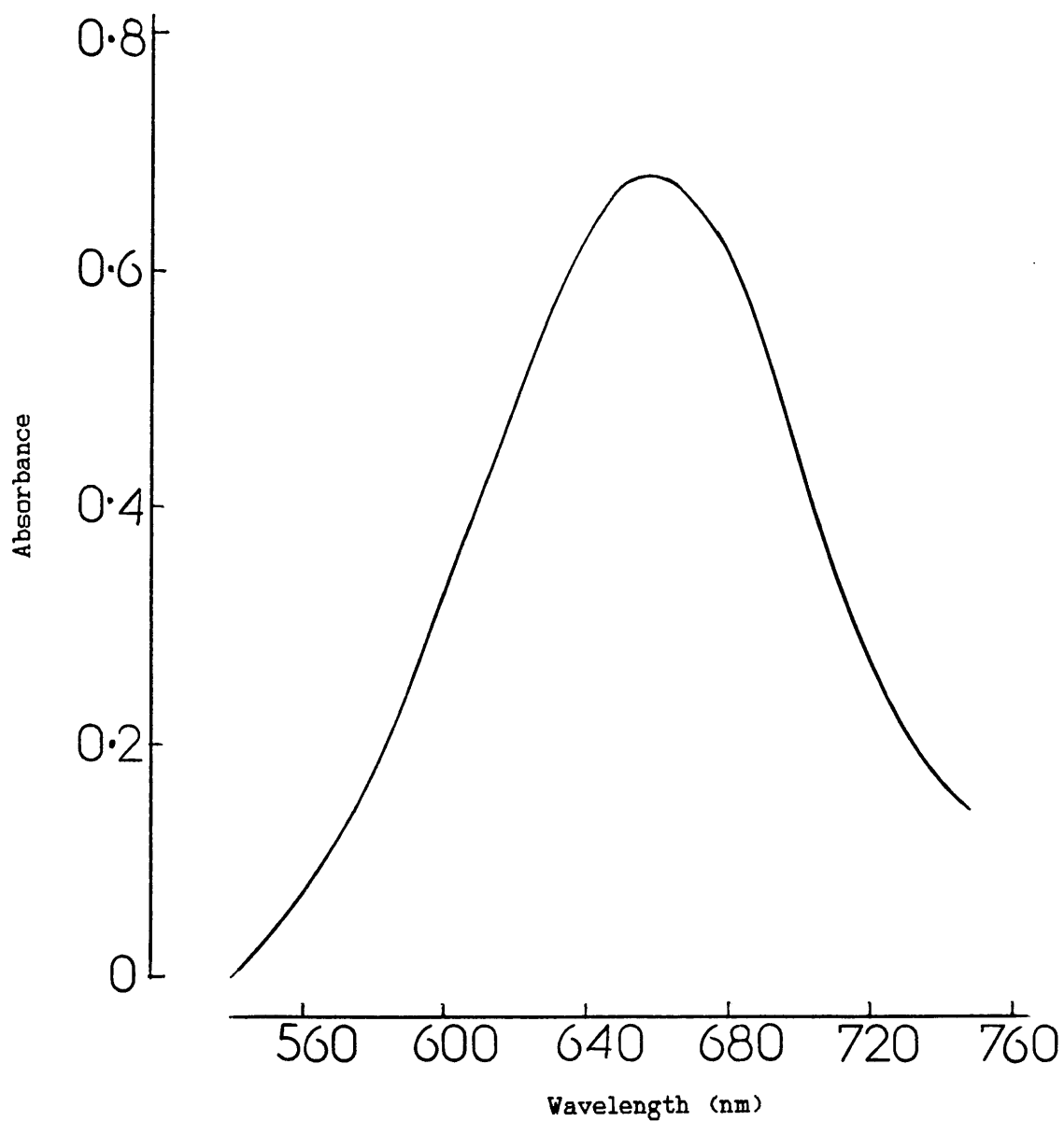


Figure 5.4 Response Curve for Cytochrome P-450 Peroxidase Activity

The coloured complex formed by the activity of cytochrome P-450 on hydrogen peroxide and TMBZ was analysed for its absorbance at 658nm for various cytochrome P-450 concentrations, as described in section 5.2.5 .

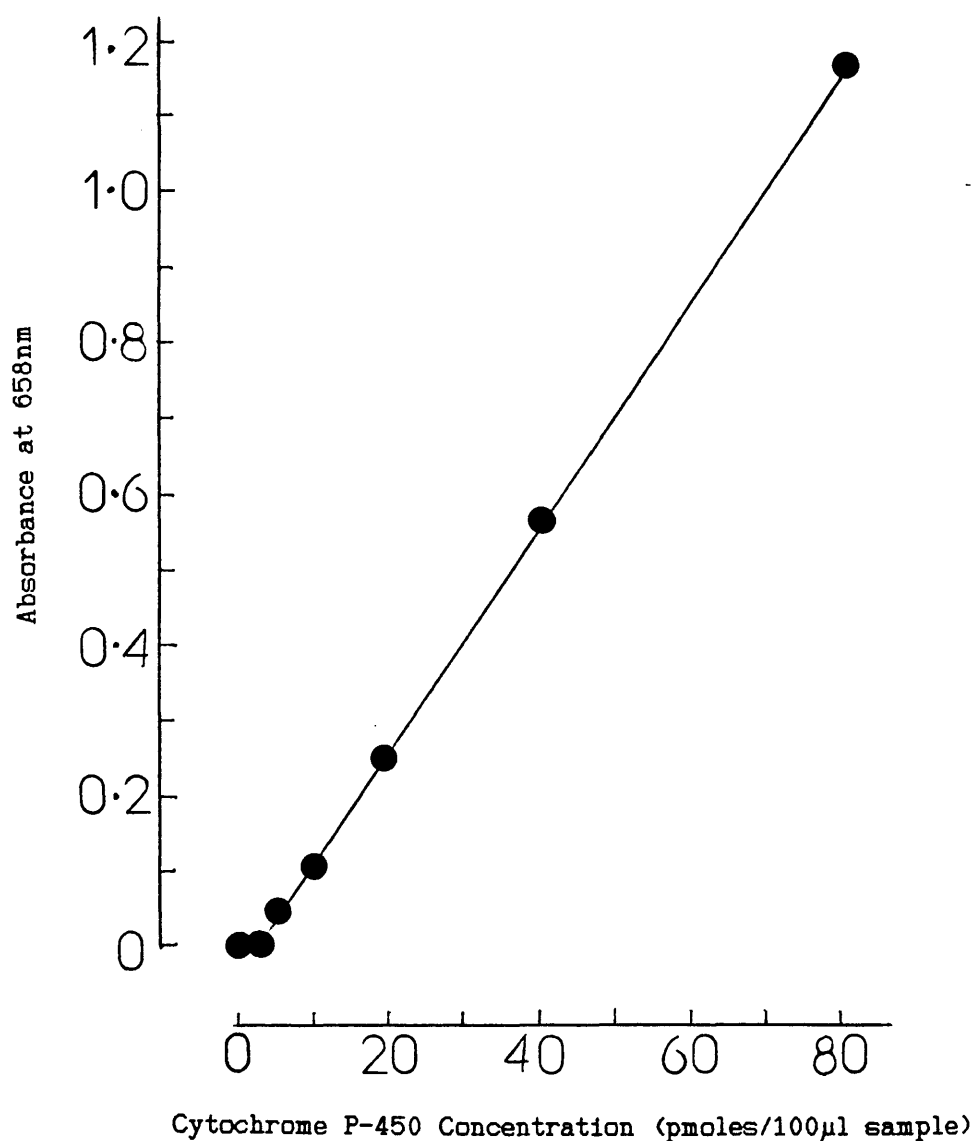


Figure 5.5 Analysis of Antibody Binding to Protein A

The ability of affinity purified RAM, RAM-IgG, and MAb 3/4/2 to bind to protein A was determined, as described in section 5.2.6 . Results are expressed as cpm of ^{125}I -labelled anti-mouse or anti-rabbit F(ab')_2 antibodies remaining bound, and are the average of duplicate determinations.

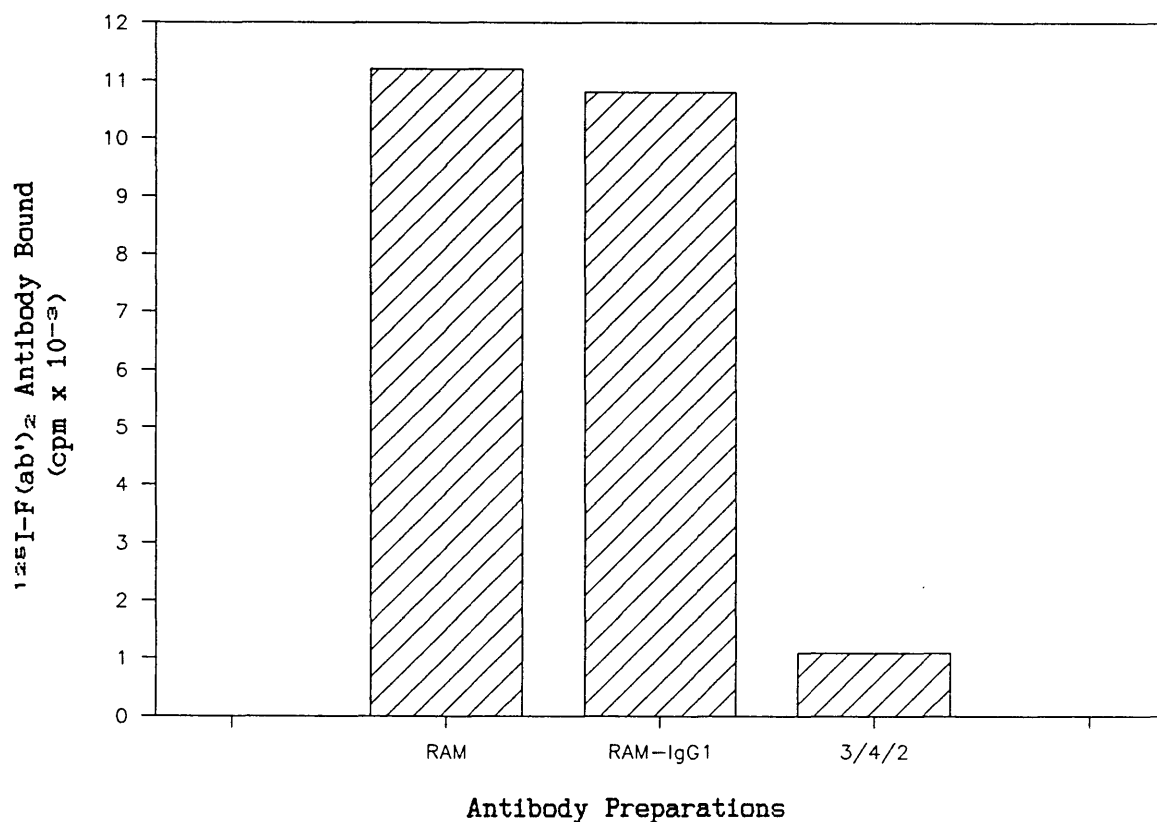


Figure 5.6 Binding Capacity of Polystyrene Microtitre Wells

The binding capacity of individual polystyrene microtitre wells for ^{125}I -labelled microsomes was determined, as described in section 5.2.7.2 . Results are expressed as the percentage of labelled microsomes remaining bound, for the microsomal protein concentrations indicated, and are the means of triplicate determinations, $\pm$ S.D. (where S.D. was greater than the size of the symbol).

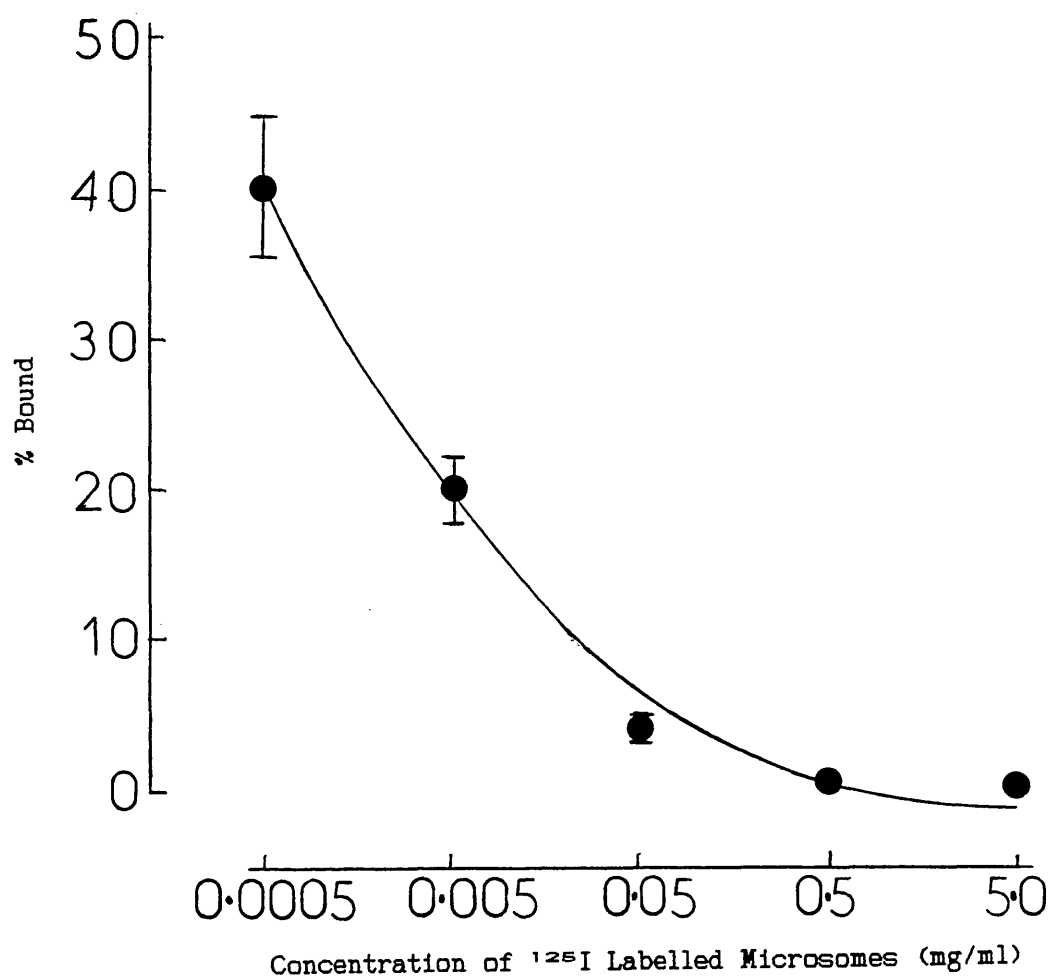


Figure 5.7 Response Curve for ^{125}I -SAM F(ab')₂ Binding to Adsorbed
Mouse Immunoglobulin

Using the binding capacity results from Figure 5.6 , the relationship between ^{125}I -SAM F(ab')₂ binding and the amount of mIgG adsorbed onto individual polystyrene microtitre wells was determined as described in section 5.2.7.2 . Results are expressed as the percentage of ^{125}I -SAM F(ab')₂ remaining bound, for the mIgG amounts indicated, and are the means of triplicate determinations, +/- S.D. (where S.D. was greater than the size of the symbol).

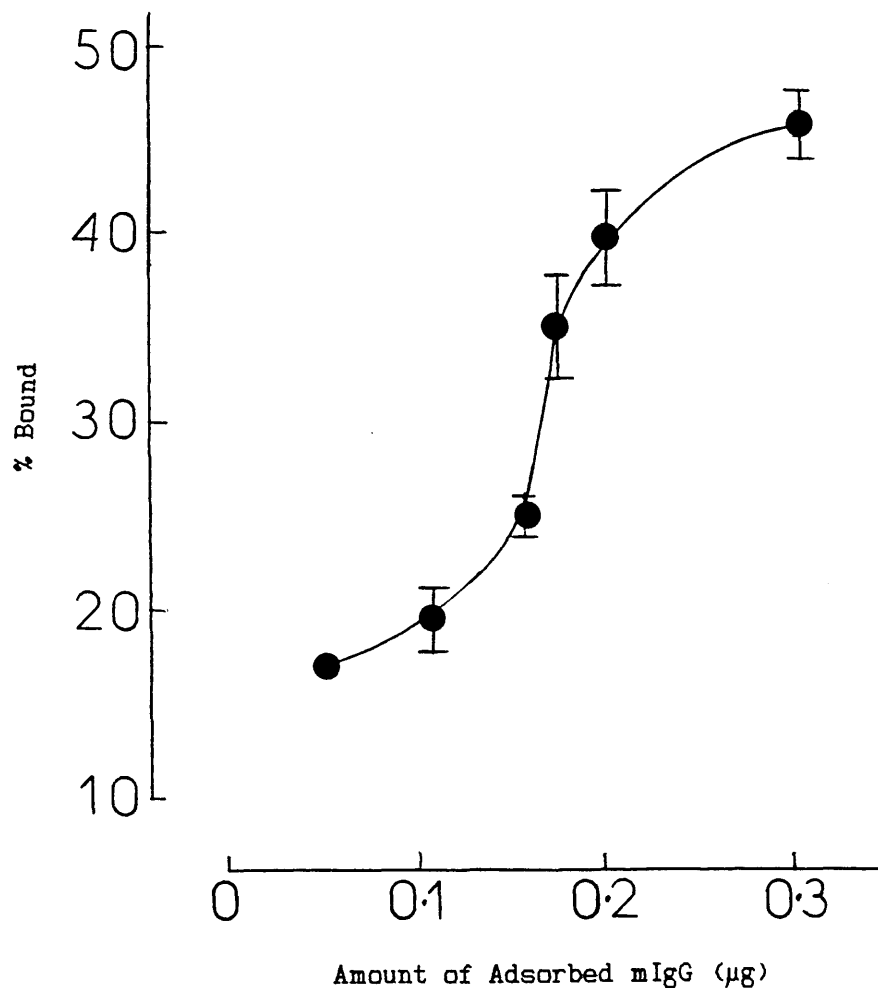


Table 5.1 Analysis of Mouse Immunoglobulin Bound to
Protein A-Sepharose Gels

From the standard curves illustrated in Figures 5.6 and 5.7, the amount of mIgG bound per ml (packed volume) of various antibody coupled pA-Seph gels was determined, as described in section 5.2.7.2 .

pA-Seph Gel	Description	mIgG Bound (mg/ml)
1	pA-Seph + MAb 3/4/2	0.5
2	pA-Seph + RAM-IgG ₁ + MAb 3/4/2	10.0
3	pA-Seph + RAM-IgG ₁	0.3
4	pA-Seph + MAb TB71	6.2

Figure 5.8 Determination of Haem Containing Material on Cytochrome P-450
Incubated Polystyrene Beads

Using the assay for haem determination, the presence of cytochrome P-450 bound to MAb 3/4/2 (▨) or mIgG (▩) coated polystyrene beads was determined, as described in section 5.2.8.2 . Results are expressed as the absorbance at 658nm of the coloured complex, for the following preparations :

- 1 - Solubilised liver microsomes from β NF treated rats
- 2 - Unbound material
- 3 - Material washed from beads
- 4 - Cytochrome P-450 incubated and washed beads

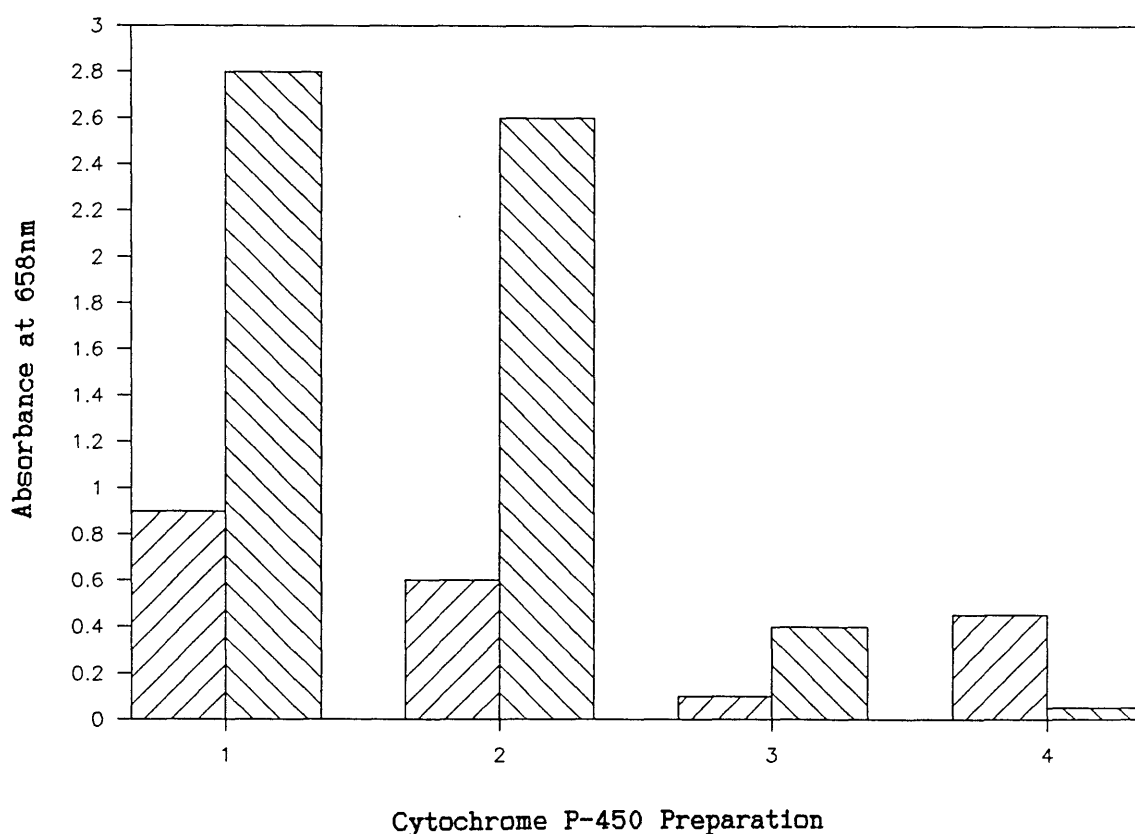


Table 5.2 Benzo[a]Pyrene Hydroxylase Activity of Reconstituted
Cytochrome P-450 Incubated Polystyrene Beads

The B[a]P hydroxylase activity of reconstituted cytochrome P-450 incubated MAb 3/4/2 and mIgG coated polystyrene beads was determined, as described in section 5.2.8.2 . Results have been normalised to give the MAb 3/4/2 coated beads a figure of 100%, which corresponds to a relative fluorescence intensity of 3183 (Nebert and Gelboin, 1968).

Components of Incubation	B[a]P Hydroxylase Activity (% MAb 3/4/2)
MAb 3/4/2 coated beads	100
MAb 3/4/2 coated beads (without reductase)	19
mIgG coated beads	0
reductase only	3

Figure 5.9 Reduced Carbon Monoxide Difference Spectra of
Cytochrome P-450 Incubated Sepharose Beads

The presence of cytochrome P-450 on (A) MAb 1/4, (B) MAb 3/4/2 and (C) MAb B3A3 coupled sepharose beads was determined, as described in section 5.2.9.2 , using the reduced carbon monoxide difference spectroscopy method.

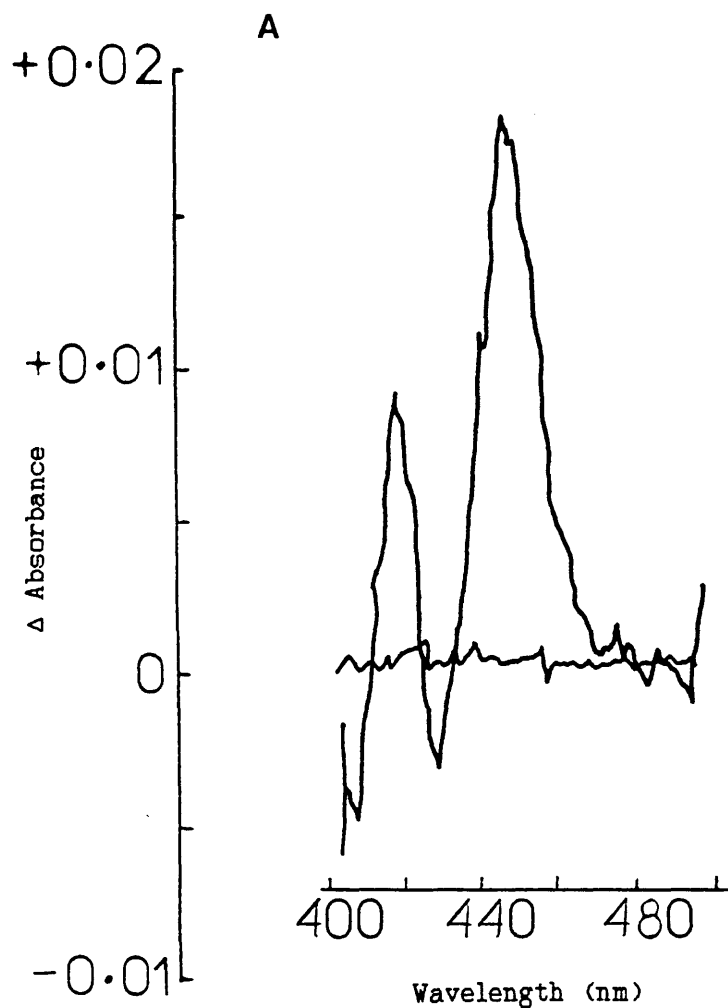


Figure 5.9 continued

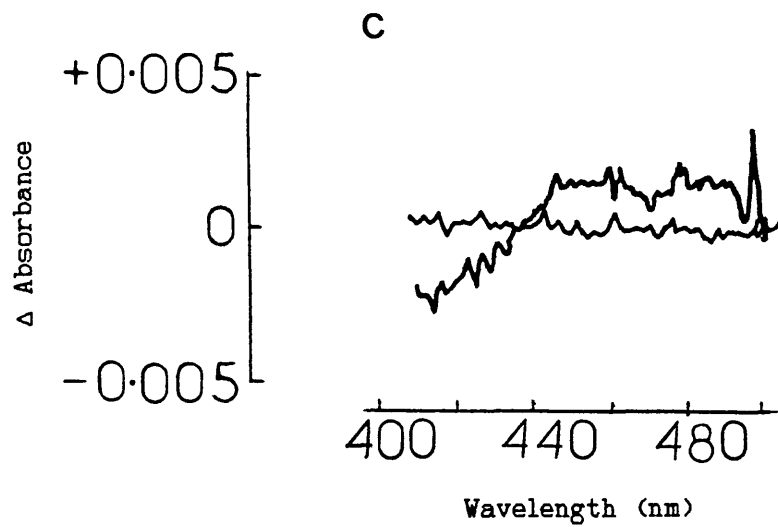
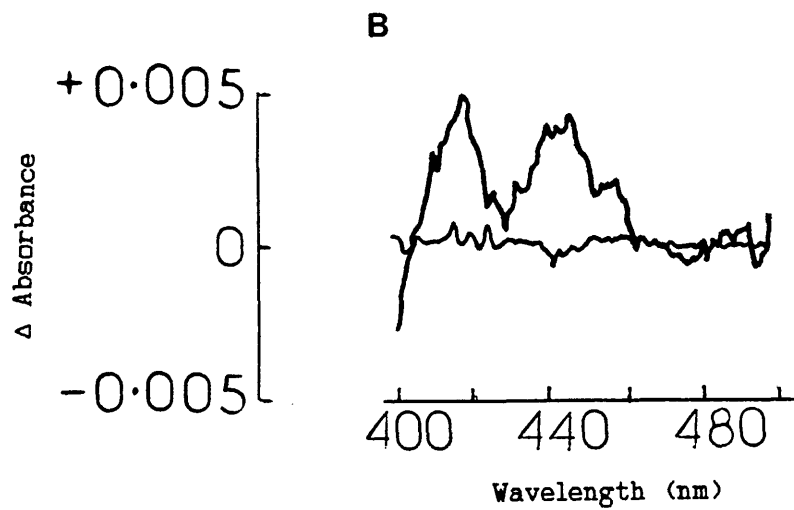


Figure 5.10 Elution Profile from Protein A-Sepharose Gels

Fractions eluted from (A) pA-Seph gel 4 (pA-Seph + MAb TB71) and (B) pA-Seph gel 2 (pA-Seph + RAM-IgG₁ + MAb 3/4/2) were analysed for both cpm present (●) and haem content (▲), as described in section 5.2.7.3 . Arrows indicate where different buffers were applied during the chromatography procedure, as indicated below :

A - 50mM Tris hydrochloride buffer, pH 8.2, containing 0.5M sodium chloride, 1mM EDTA and 0.5% (v/v) Nonidet NP-40

B - 50mM Tris hydrochloride buffer, pH 8.2, containing 0.15M sodium chloride, 0.5% (v/v) Nonidet NP-40

C - 0.15M sodium chloride containing 0.5%(w/v) sodium deoxycholate

D - 50mM diethylamine, pH 11.5, containing 0.5% (w/v) sodium deoxycholate

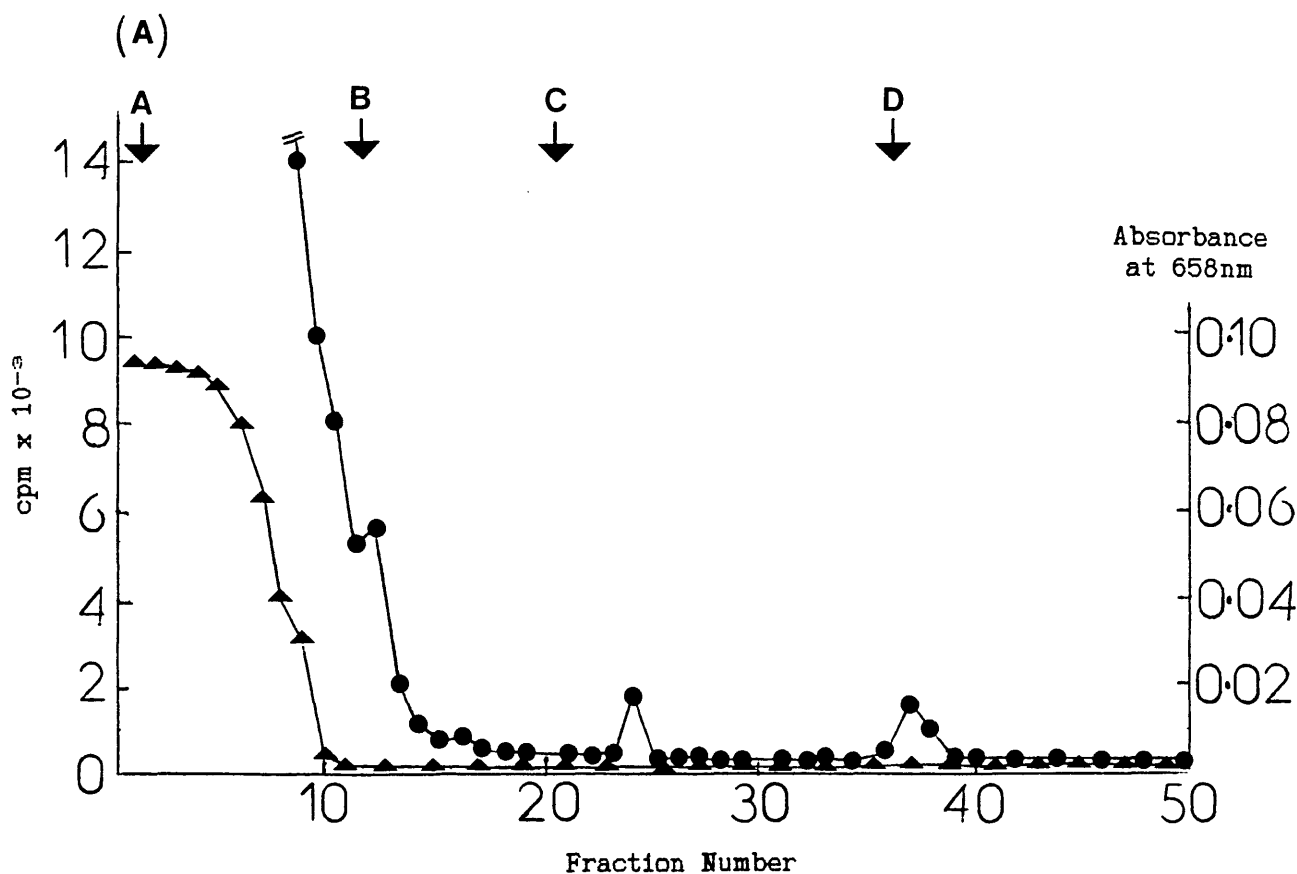


Figure 5.10 continued

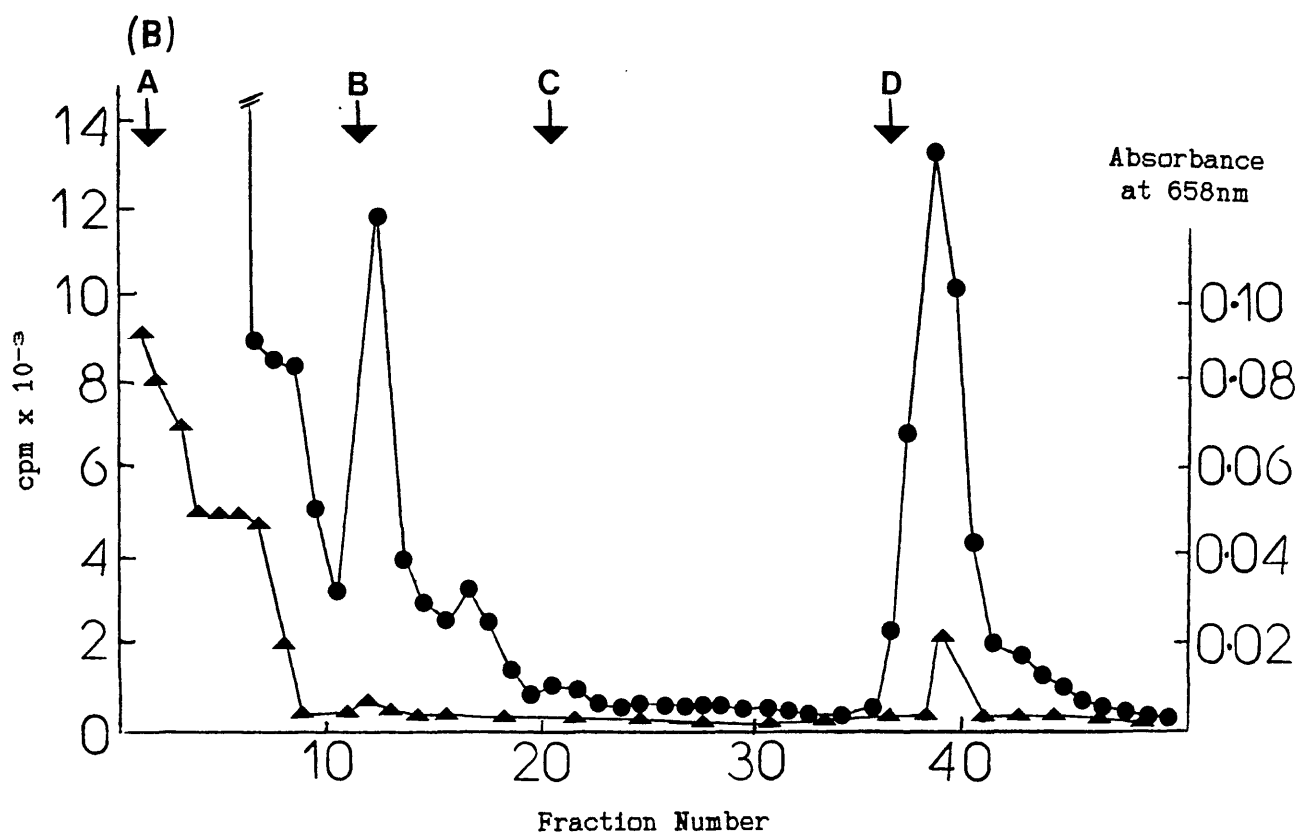
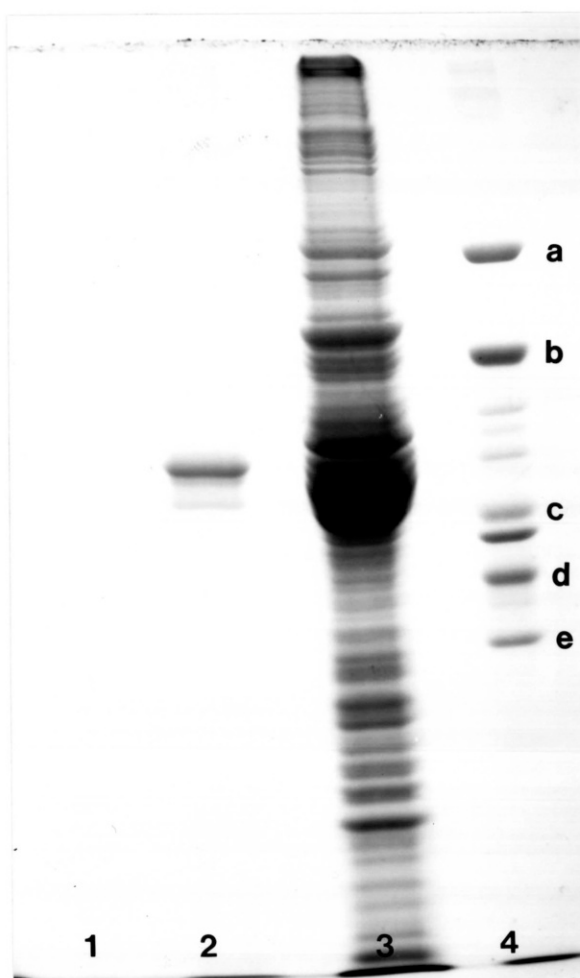


Figure 5.11 SDS-PAGE Analysis of Eluted Material from Sepharose Gels

Material eluted from MAb 1/4-Seph and MAb B3A3-Seph gels were analysed by SDS-PAGE, as described in section 2.2.8 . Tracks were loaded as follows :

- 1 - Material eluted from MAb B3A3-Seph gel
- 2 - Material eluted from MAb 1/4-Seph gel
- 3 - Solubilised hepatic microsomes from PB treated rats
- 4 - Molecular weight standards (a-69,000, b-58,000, c-53,000, d-43,000, e-40,000)



CHAPTER 6

IMMUNOQUANTITATION OF MICROSOMAL CYTOCHROME P-450 ISOZYMES

6.1 Introduction

It is well established that hepatic cytochrome P-450 exists as multiple isozymes, which are capable of metabolising a wide range of endogenous and exogenous compounds (Lu and West, 1980). The specific content of many of these isozymes can be induced by the administration of certain xenobiotics (Snyder and Remmer, 1982). In recent years, studies have been performed in animals to determine the molecular mechanisms which control cytochrome P-450 expression. Quantitation of the various cytochrome P-450 isozymes expressed has therefore become essential in determining the effects of, for example, age, sex, and xenobiotic exposure on the molecular control of cytochrome P-450 expression. The spectral, electrophoretic and catalytic properties of the various isozymes have been used in their quantitation. However, such methods are, by themselves, inadequate in many cases for completely determining the comparative effects of, for example, different induction regimes. Wavelength maxima of reduced carbon monoxide difference spectra of various cytochrome P-450 isozymes can vary by less than 5nm (Guengerich *et al.*, 1982). Several cytochrome P-450 isozymes are difficult to resolve from each other electrophoretically, for example, forms b and e (Ryan *et al.*, 1982). Many substrates for cytochrome P-450 are metabolised to varying extents by several isozymes (Johnson, 1979). Rates of metabolism are also usually not sufficiently specific to permit a certain catalytic activity to be used as characteristic of a given cytochrome P-450 isozyme. In addition, measurement of catalytic activity does not differentiate between cases where an activity is not apparent due to the lack of a particular isozyme and those where the isozyme is present but inhibited or inactivated. Thus, changes in the cytochrome P-450 content of microsomes can often fail to be detected.

During the last few years, immunoassays, both radioactive and enzymic in nature, have been developed and used to quantify a wide variety of

biological substances (Langone and Van Vunakis, 1982). Immunoassays involving competitive binding between a standard (either labelled or unlabelled) and an unknown for an antibody can provide a combination of sensitivity and ease of use that is not readily found in other quantitative procedures.

The production of both polyclonal and monoclonal antibodies to isozymes of cytochrome P-450 has allowed for a variety of such immunochemical studies. Polyclonal antibodies have been used to quantify specific forms of cytochrome P-450 by a number of techniques, including radial immunodiffusion (Ryan *et al.*, 1982; Parkinson *et al.*, 1983; Thomas *et al.*, 1983), immunoprecipitation (Newman *et al.*, 1982; Hara *et al.*, 1983), rocket immunoelectrophoresis (Pickett *et al.*, 1981), analysis of Western blots (Dannan *et al.*, 1983; Haaparanta *et al.*, 1983), ELISA (Seidal *et al.*, 1984) and conventional competitive radio-immunoassay (Luster *et al.*, 1983; Phillips *et al.*, 1983). In recent years, MAb's have also been used to quantify isozymes of cytochrome P-450 (e.g. see Beaune *et al.*, 1985; Friedman *et al.*, 1985f; 1986; Johnson and Griffin, 1985; Rothwell *et al.*, 1985; Hostetler *et al.*, 1987; Kloepper-Sams *et al.*, 1987; Wrighton *et al.*, 1987).

In this chapter, the development of an enzyme linked competitive immunoassay for the quantitation of cytochrome P-450 isozymes is described, using MAb's 3/4/2, 12/2/3/2 and 1/4 as specific probes of form c, forms c and d, and forms b and/or e, respectively.

6.2 Methods

6.2.1

Monoclonal Antibody Iodination

DEAE Affi-Gel Blue purified MAb 3/4/2 (15 μ g) was radiolabelled with 1mCi of ^{125} I by the use of the Bolton and Hunter reagent (Bolton and Hunter, 1972). The reaction was terminated by the addition of 0.2M glycine saline buffer, pH 8.5, and potassium iodide solution. Unreacted ^{125} I was removed from the reaction mixture by gel filtration on Sephadex G-50, previously equilibrated with PBS and blocked with 0.1% (w/v) BSA in PBS to prevent non-specific binding of proteins. Fractions of 250 μ l were collected and their activity determined in a gamma counter. Fractions with peak activity were pooled to form the ^{125} I-labelled MAb 3/4/2 preparation.

6.2.2

Preparation of ^{14} C-Labelled MAb 12/2/3/2

MAb 12/2/3/2 was internally labelled with ^{14} C by growing the hybridoma cells in medium supplemented with ^{14} C-leucine. The MAb was partially purified from the culture supernatant by ammonium sulphate precipitation, as described in section 2.2.4. The resultant preparation had a specific activity of 8×10^3 cpm/ μ g of total protein.

6.2.3

Solid Phase Immunoassays

6.2.3.1 Enzyme Linked Immunosorbant Assay

ELISA assays were performed as previously described in section 2.2.6. For direct binding assays using labelled MAb's and enzyme linked detection, individual microtitre wells, coated with microsomes as per ELISA, were used.

6.2.3.2 Solid Phase Immunoassay with ^{125}I -Labelled MAb 3/4/2

For those solid phase assays involving radioactive detection of ^{125}I -labelled MAb 3/4/2, individual microtitre wells were used, coated with liver microsomes from βNF treated rats as per ELISA. ELISA's involving ^{125}I -labelled MAb 3/4/2 were performed using microtitre plates coated with liver microsomes from βNF treated rats. Serial dilutions of unlabelled MAb 3/4/2 were added to three parallel sets of coated wells (two in microtitre plates and one in individual wells). To each well of two sets (one set performed in a microtitre plate and the other in individual wells) was added 6.5×10^4 cpm of ^{125}I -labelled MAb 3/4/2. Both plates and individual wells were incubated for 2 hours at 37°C . The incubations performed in individual wells were then used to determine binding of the ^{125}I -labelled MAb by gamma counting. The other two sets of incubations were analysed for total MAb 3/4/2 binding by standard ELISA procedures (section 2.2.6). Specific gamma counts were calculated by subtracting those counts obtained from blank wells (i.e. microsome coated only) from those of the test wells. Binding results obtained by gamma counting were expressed as a percentage of the maximum observed counts (i.e. counts obtained with coated wells incubated with ^{125}I -labelled MAb 3/4/2 only). Results obtained by ELISA were expressed as OD 492nm.

6.2.4

Quantitative Immunoassays

The solid phase quantitative immunoassay method of Song *et al.* (1984) was used with modification. Due to the nature of the assay method, it was not possible to include MAb B3A3 as a negative control.

6.2.4.1 Immunoradiometric Assay

^{14}C -Labelled MAb 12/2/3/2 (10^4 cpm) was incubated with varying amounts of liver microsomes from βNF treated rats, in a total volume of $200\mu\text{l}$.

Following 18 hours incubation at 4°C, 100µl of the incubation mixture was added to individual microtitre wells, previously coated with liver microsomes from βNF treated rats as per ELISA. Wells were incubated for 2 hours at 37°C, washed with PBST and dried. Dried wells were placed in glass scintillation vials, to which was added 10ml of Instagel. Upon dissolution of the wells, the radioactivity present was determined by liquid scintillation spectrometry. Specific counts were calculated by subtracting those counts obtained from blank (i.e. microsome coated only) wells from those of the test wells. Results were expressed as a percentage of the maximum observed counts (i.e. ¹⁴C-labelled MAb 12/2/3/2 incubated only with PBS before transfer to microsome coated wells).

6.2.4.2 Enzyme Linked Immunoassay

For those assay's involving enzyme detection, the initial incubations were as described in section 6.2.4.1 above. These were performed with liver microsomes from control, βNF treated or PB treated rats. A portion of the incubation mixture (100µl) was transferred into microtitre plates previously coated with liver microsomes from βNF treated rats (PB treated rats for MAb 1/4). Binding of MAb's to coated plates was determined by ELISA procedures, and the OD 492nm determined.

6.2.4.3 Quantitative Enzyme Linked Immunoassay

MAb 12/2/3/2 was incubated at 5 µg/ml, as per section 6.2.4.1 above, with various concentrations of a purified preparation of cytochrome P-450c (17.1 nmoles/mg protein). A standard curve was then determined using a curve fitting software programme (Flow Laboratories, Rickmansworth, Herts.). Incubations were also performed with a variety of liver microsomal fractions. The amounts of epitope specific isozymes present (corresponding to forms c + d equivalent forms) were then determined from the standard curve by the programme, and the specific contents of these isozymes calculated from the microsomal concentrations used in the assay.

6.3 Results

6.3.1

MAb 3/4/2 Binding Curves

The titration curves obtained for both ^{125}I -labelled and unlabelled MAb 3/4/2 are shown in Figure 6.1 . It is apparent that very little of the ^{125}I -labelled antibody bound to the microsome coated wells. However, the ELISA results obtained with serial dilutions of unlabelled MAb 3/4/2, spiked with ^{125}I -labelled 3/4/2, revealed that appreciable binding of the antibody present in the fraction obtained during the iodination procedure had occurred.

6.3.2

Immunoradiometric Assay

The results obtained for the ^{14}C -labelled MAb 12/2/3/2 competitive solid phase IRMA are shown in Figure 6.2 . It is clear that no competitive binding occurred with this MAb preparation, using radioactive detection. In comparison, the results obtained by ELISA detection using the same preparation of ^{14}C -labelled MAb (also shown in Figure 6.2) clearly show that some removal of MAb binding activity (i.e. competitive binding) had occurred, but only at the two highest microsomal concentrations.

6.3.3

Enzyme Linked Immunoassay

Due to the poor binding results obtained with the radio-labelled preparations of MAb's, the quantitative determination of cytochrome P-450 isozymes was attempted with unlabelled MAb's, coupled with enzyme linked detection. The results obtained from preliminary experiments with MAb's 1/4, 3/4/2 and 12/2/3/2, following incubation with liver microsomes from

β NF treated rats (PB treated for MAb 1/4), are shown in Figures 6.3, 6.4 and 6.5A respectively. From these results, it is clear that sufficient competition of binding was only achieved with MAb 12/2/3/2, and that at MAb concentrations of 50 and 5 μ g/ml total protein. When a heterologous antigen preparation (in this case liver microsomes from PB treated rats) was incubated with this MAb, sufficient competition was achieved only at a MAb concentration of 5 μ g/ml total protein (Figure 6.5B).

It is apparent from these results that, due to their poor performance when incubated with competing rat liver microsomes, MAb's 1/4 and 3/4/2 were unsuitable for inclusion in a quantitative immunoassay of this nature. MAb 12/2/3/2 was however suitable, and was subsequently used in a quantitative assay at a concentration of 5 μ g/ml total protein, a concentration judged as being optimum from the above results.

6.3.4

Quantitative Enzyme Linked Immunoassay

The results obtained from the quantitative immunoassay performed with MAb 12/2/3/2 are presented in Table 6.1, having first been derived from the standard curve obtained in the assay (Figure 6.6). The range of detection in this assay for the purified cytochrome P-450c preparation was between 1.25 and 2.5 pmoles per incubation. At concentrations outside this range, the OD 492nm readings obtained were outside the straight line portion of the standard curve. With an incubation volume of 100 μ l, the maximum sensitivity of the assay corresponds to an actual sensitivity of 12.5 pmoles of epitope specific isozymes of cytochrome P-450 per ml of microsomal preparation.

These results show that the highest specific content of forms c + d were present in liver microsomes prepared from hydrocarbon treated rats. Liver microsomes prepared from 3MC treated rats also had a higher specific content of these two isozymes, compared with the corresponding microsomal

preparation from BNF treated rats. Control rat liver microsomes were also shown to have a higher specific content of forms c + d than PB treated rat liver microsomes, although the specific content of both these preparations was much lower than that of liver microsomes from hydrocarbon treated rats. Liver microsomes from 3MC treated AKR mice had a higher specific content of epitope specific isozymes than those seen with control AKR mice, or liver microsomes from control or PB treated rats. Microsomal fractions from control guinea pig liver and one of the human liver preparations were also able to compete sufficiently for MAb 12/2/3/2 to permit the determination of their specific content of epitope specific isozymes. However, these results were obtained only at high microsomal protein concentrations, and were also at the limit of sensitivity for this assay. Nevertheless, it is apparent that some MAb 12/2/3/2 immuno-reactive isozymes were present in these microsomal fractions. Several of the liver microsomal fractions tested were, however, unable to compete sufficiently with MAb 12/2/3/2 to allow the specific content of their epitope specific isozymes to be determined, even at microsomal protein concentrations of up to 800 μ g per incubation.

6.4 Discussion

The specificity of MAb's to individual epitopes makes them extremely useful for detecting single isozymes, or classes of isozymes, within a multi-enzyme system, such as the cytochrome P-450 system.

One obvious application of MAb's against cytochrome P-450 is in the quantitation of isozymes in different microsomal preparations. In this study, initial attempts to achieve this by the use of radio-labelled MAb's were without success due to the difficulty in successfully labelling the antibody. It was readily apparent that although MAb 3/4/2 could be iodinated, this lead to a substantial reduction in its binding capacity, a

problem previously reported for other MAb's (Rothwell *et al.*, 1985). In addition, it also appeared that a considerable proportion of MAb 3/4/2 was not being labelled, and could therefore still bind to its antigen, although, of course, an unlabelled MAb is not of any use in an immunoradiometric assay. As a consequence, the use of iodination as a labelling technique was abandoned and other methods investigated. MAb 12/2/3/2 was successfully labelled internally with ^{14}C -leucine. However, the resultant preparation had a relatively low specific activity, and was found to be inadequate for competitive immunoassays.

Alternative MAb labelling methods are, of course, available. These include labelling with ^3H , using ^3H -sodium borohydride (Tack *et al.*, 1980), or the incorporation of high specific activity labels such as ^{35}S , as, for example, ^{35}S -methionine, to produce internally labelled MAb's. However, all of these methods produce radio-labelled MAb's. These therefore may have the possible problems of disruption of the MAb due to the label, as seen with ^{125}I ; incorporation of low specific activity labels such as ^{14}C or ^3H ; or rapid radio-active decay of the label, as seen with ^{125}I or ^{35}S . These potential problems, coupled with the limited success obtained with both ^{125}I - and ^{14}C -labelled MAb's, prompted the use of an enzyme-linked competitive immunoassay. With the ready availability of commercial preparations of enzyme conjugated second antibodies (RAM-HRP, for example), the use of the biotin/(strept)avidin system (Green, 1975), utilising biotin labelled MAb's, was not attempted.

Several MAb's were investigated for their usefulness in such an enzyme immunoassay. However, only one MAb preparation, namely MAb 12/2/3/2, was found to be suitable for inclusion in a quantitative assay. The remaining MAb's were found to give very poor response curves when incubated with a range of concentrations of the homologous antigen. MAb 12/2/3/2 on the other hand, produced good response curves, showing high maximum binding coupled with a complete inhibition of binding over a ten fold concentration range of the competing antigen, even with heterologous antigen preparations.

These characteristics allowed this MAb to be used in a quantitative assay involving several heterologous microsomal antigen preparations.

It was unfortunate that both MAb's 3/4/2 and 1/4 were unsuitable for use in the competitive assay. The assay described in this chapter is based on modulating the amount of enzyme, as RAM-HRP, remaining bound to the solid surface of the microtitre well, by competing MAb with free antigen. As illustrated by the results presented in this chapter, the sensitivity of this type of assay increases with decreased MAb concentration. This is because at low MAb concentrations a variation in the amount of competing cytochrome P-450 will have a larger impact on the subsequent interaction of MAb and solid phase cytochrome P-450 (Tijssen, 1985). Analysing the assay in terms of the law of mass action, the low MAb and cytochrome P-450 concentrations used will reduce the rate of MAb-cytochrome P-450 complex formation considerably. It should also be apparent that at a given concentration, a high affinity MAb will require less antigen to become 50% saturated than that required by a low affinity MAb. MAb affinity therefore, is vitally important in this type of assay system, since MAb's are not present in excess. The poor response observed with MAb's 3/4/2 and 1/4 is therefore most likely due to their low affinity. Good reactivity for both of these MAb's had previously been seen in ELISA and Western blot studies. Both those assay systems, however, rely on amplifying the response by maximising the amount of enzyme, as RAM-HRP, remaining bound to the solid phase used in the assay. Reactivity was therefore determined in MAb excess to obtain a maximum signal. The effect of MAb affinity on reactivity in such assay systems is therefore limited.

The utility of this type of quantitative immunoassay has been highlighted by the studies performed by Gelboin and co-workers. They have used a similar immunoassay method incorporating radio-labelled MAb's to quantitate the relative amounts of cytochrome P-450 isozymes in a variety

of tissues from control and chemically treated animals (Cheng *et al.*, 1984a; Song *et al.*, 1984). In addition, they have looked at the influence of smoking on human placental cytochrome P-450 content, as well as the influence of B[a]A on cytochrome P-450 content in cultured human lymphocytes, and found that both conditions dramatically increased the levels of cytochrome P-450 isozymes under investigation (Song *et al.*, 1985a). They have also recently used this type of immunoassay to determine the extent of MAb immuno-reactivity remaining on several cytochrome P-450 isozymes after their elution from MAb immuno-purification columns (Friedman *et al.*, 1985d), as well as determining cytochrome P-450 levels in a human cell line expressing monooxygenase activity (Friedman *et al.*, 1985e).

It can be seen, therefore, that this assay method has a wide range of applications. By substituting an ELISA based detection system however, the assay described in this chapter allows for the use of a greater variety of different MAb's, provided they are of sufficiently high affinity, as there is no need to purify or label a MAb before it can be used in the assay.

The interpretation of the results derived from the assay described in this chapter is complicated, however, by the fact that MAb 12/2/3/2 reacts with both cytochrome P-450 forms c and d. The derivation of the assay results therefore requires several assumptions to be made for the reactivity of MAb 12/2/3/2. The first is that MAb 12/2/3/2 binds with equal affinity to both forms c and d. The relevance of this lies in the fact that purified cytochrome P-450c was used to prepare the standard curve in the quantitative immunoassay. If MAb 12/2/3/2 reacts with forms c and d to different degrees, then the standard curve would not be valid if any form d was present in the competing microsomal preparations. Results obtained from Western blotting experiments with cytochrome P-450 preparations from β NF and ISF treated rats would, however, indicate that this assumption appears to be valid. Secondly, it must also be assumed

that if MAb 12/2/3/2 binds to isozymes present in the competing heterologous microsomal preparations, then it does so with the same affinity as for rat liver forms c and d. If this were not the case, then the calculated specific contents of the epitope specific isozymes present in those competing microsomes would be incorrect. It is not possible, however, to determine if this assumption is likely to be correct from the limited cross-species reactivity observed for this MAb by Western blotting. The final assumption is that MAb 12/2/3/2 reacts only with cytochrome P-450 forms c and d, and not with any other forms. If this were not the case, then the presence of other cross-reactive isozymes in the competing microsomal preparations would result in a higher calculated specific content for MAb 12/2/3/2 reactive isozymes. Results obtained from Western blotting experiments would indicate that this assumption is valid. If the arguments for these assumptions are accepted, it can be seen that any calculation of specific content for MAb 12/2/3/2 reactive isozymes present in the competing microsomal preparations must be expressed as the equivalent of forms c + d.

Having accepted these assumptions, the implications of the results obtained in the quantitative immunoassay with MAb 12/2/3/2 can now be considered. It can be seen that treatment of rats with either 3MC or β NF caused a dramatic increase in the specific content of cytochrome P-450 forms c + d, when compared to control rats. The specific content of forms c + d calculated for control rats was comparable to that previously determined by Thomas *et al.* (1983), whilst the figures for forms c + d following treatment by 3MC and β NF were slightly reduced in comparison to that study. Induction of cytochrome P-450 isozymes by hydrocarbons such as 3MC and β NF is well known, as shown by studies of enzyme activity (Haugen *et al.*, 1976) as well as by immunoassay (Thomas *et al.*, 1983). It is also apparent that the calculated specific content of forms c + d in liver microsomes from 3MC treated rats was greater than the corresponding

preparation from rats treated with β NF. This observation indicates that 3MC is a better inducer of these cytochrome P-450 isozymes than β NF. This difference between these two inducers has been previously noted using B[a]P hydroxylase activity as a determinant of induction (Boobis *et al.*, 1977). This difference in specific content is not likely to be due to differences in the ratio of form c to form d between the two microsome preparations, as studies have shown that the ratio of form c to form d does not alter between 3MC or β NF treatment, regardless of the level of induction achieved (Thomas *et al.*, 1983).

Also apparent from these results is the fact that the specific content of forms c + d was higher in liver microsomes from control rats than in liver microsomes from PB treated rats. This might indicate that treatment with PB suppresses the level of forms c + d expressed in the liver. Such findings have previously been noted by other workers using a similar assay method (Song *et al.*, 1984). That, and other immuno-quantitative studies (Phillips *et al.*, 1983), have shown that β NF induction can also cause a similar decrease in PB inducible isozymes expressed, when compared to control microsome preparations. It is therefore apparent that the expression of the various cytochrome P-450 isozymes is under dynamic control, the induction of individual isozymes having an effect on the expression of others.

It is also interesting to note, that although MAb 12/2/3/2 did not give a positive reaction in Western blots with liver microsomal fractions from either control or PB treated rats, or from a human liver preparation, it was possible to quantify the amount of forms c + d present in such preparations. It must be presumed, therefore, that the quantitative immunoassay is a more sensitive technique than Western blotting. However, this does not appear to be the case with liver microsomal fractions from 3MC treated rabbit. In that case, neither immuno-reactivity in Western blots nor competitive binding in the quantitative immunoassay was observed.

However, it should be noted that it is possible that the forms of cytochrome P-450 in the rabbit analogous to forms c and d in the rat (namely forms LM4 and LM6) do not contain the epitope recognised by MAb 12/2/3/2. For the other microsomal fractions tested, it is not possible to correlate the lack of competitive binding observed in the quantitative immunoassay with any reactivity with MAb 12/2/3/2 in Western blots as no blotting experiments were performed with these preparations.

Several groups in addition to Gelboin and co-workers have now reported on the use of MAb's in the quantification of cytochrome P-450 isozymes. The assay techniques in these reports are concentrated into either analysis of Western blots, or some form of competitive or non-competitive solid phase immunoassay.

In the former assay classification, work on the expression of cytochrome P-450_{2B} in rat liver microsomes has been reported (Anderson and Jörnvall, 1986). The specific content of this form of cytochrome P-450 was found to decrease on PB treatment from a level of about 60% of the total cytochrome P-450 present, whilst the absolute levels actually remained constant. This isozyme was also found to be male specific. The influence of a variety of cytochrome P-450 inducers on the levels of glucocorticoid inducible cytochrome P-450 forms in the rat has also been reported (Hostetler *et al.*, 1987). Results from that study indicate that this family of cytochrome P-450 isozymes consists of at least three forms which are structurally related, but appear to be under distinct regulatory control.

Analysis of rabbit cytochrome P-450 form LM3a has also been described (Ding *et al.*, 1986). The specific content of this isozyme was found to be increased in kidney microsomes following chronic ethanol treatment, but to be unchanged in nasal microsomes from the same animals. Several reports on the identification of human forms of cytochrome P-450 have also been published. In one, the quantification of human cytochrome P-450₁ in a

variety of liver samples was described (Beaune *et al.*, 1985). The mean specific content of this form was calculated to be 0.34 nmoles/mg protein, which was approximately 70% of the total detectable cytochrome P-450 content of the liver samples studied. Wrighton *et al.* (1986) reported on the identification of a human cytochrome P-450 form homologous to rat liver cytochrome P-450d. Relative levels of this form were determined in several human liver samples, though no absolute levels were calculated. This same group also reported on the quantitation of a human cytochrome P-450 form, termed HLx, in a variety of human liver microsomal preparations (Wrighton *et al.*, 1987). Although no correlation was observed between cytochrome P-450 HLx levels and the clinical state of the human subjects, a bimodal distribution of form HLx expression levels was suggested from their data.

The levels of fish cytochrome P-450E were also determined by analysis of Western blots (Klopper-Sams *et al.*, 1987). The content of this isozyme in liver microsomes was found to rise following treatment of fish with PCB and β NF, and to a greater extent following HCB treatment. The content of this form of cytochrome P-450 in liver microsomes from control fish varied considerably, and is thought to reflect the extent to which each individual fish was in contact with environmental pollutants, such as HCB's and PCB's.

Using a non-competitive, two-site solid phase immunoassay method, Johnson and co-workers have investigated the level of rabbit cytochrome P-450 form LM1 in control animals (Reubi *et al.*, 1984b; Johnson and Griffin, 1985; Raucy and Johnson, 1985). The microsomal content of this isozyme varied between 0.05 and 0.5 nmoles/mg protein in individual control animals, and correlated well with the microsomal B[a]P and progesterone 21-hydroxylase activities expressed by each animal. Whilst TCDD treatment of rabbits was found to increase microsomal B[a]P activity in these studies, no correlation with microsomal levels of cytochrome P-450 LM1 were performed. Two reports have also been published on the quantitation of PB

inducible forms of rat liver cytochrome P-450. Paye *et al.* (1984) reported on the use of an ELISA method to quantitate form PB-B in control and treated animals. Levels of this form were much higher in animals treated with PB, about 2.0 nmoles/mg protein, than in β NF treated animals, about 0.1 nmoles/mg protein. In the latter case, levels were comparable with those seen in control animals. Rothwell *et al.* (1985) described a radiometric assay for the quantitation of cytochrome P-450b. This form constituted about 10% of the total cytochrome P-450 content of liver microsomes from PB treated rats, whilst in control animals, no levels of this isozyme could be detected. Whilst no studies were performed with hydrocarbon treated animals, it was determined that 3% of the total mitochondrial protein present in the livers of PB treated rats was cytochrome P-450b. Again, no levels could be detected in liver preparations from control animals.

From the results obtained in this chapter, the immunoassay technique described appears to be suitable for determining the concentration of specific cytochrome P-450 isozymes in a range of microsomal preparations. The performance of the assay is however very much dependent on the antibody used. As seen with MAb's 1/4 and 3/4/2 for instance, not every MAb is suitable for this type of assay. When a suitable MAb is available however, such as MAb 12/2/3/2, the method allows for a high throughput of samples, coupled with a simple protocol. Other immunoassay techniques that have been used to quantitate cytochrome P-450 isozymes suffer from several drawbacks. The major drawback of early methods was in their use of polyclonal antibodies which, unless affinity purified, may not compare with the specificity of MAb's. That aside however, some polyclonal antibody assays such as radial immunodiffusion (Ryan *et al.*, 1982; Parkinson *et al.*, 1983; Thomas *et al.*, 1983), require several days to obtain a result. Other assays applicable to MAb's, such as the analysis of Western blots (Beaune *et al.*, 1985; Andersson and Jörnvall, 1986;

Ding *et al.*, 1986; Wrighton *et al.*, 1986; Kloepper-Sams *et al.*, 1987), can use relatively large amounts of antibody and are not suitable for a high throughput of various concentrations of different microsomal samples. In addition, when using MAb's in non-competitive solid phase assays, low antigen concentrations result in low measurements observed, be they absorbance (Paye *et al.*, 1984), or radioactive counts present (Reubi *et al.*, 1984b; Johnson and Griffin, 1985; Raucy and Johnson, 1985; Rothwell *et al.*, 1985). With competitive assays however, the opposite occurs (e.g. Friedman *et al.*, 1985f; 1986). Thus the quantitative enzyme immunoassay described in this chapter is suitable for the detection of low concentrations of epitope specific cytochrome P-450 isozymes present in heterologous microsomal preparations.

For those microsomal fractions tested in this chapter which could be quantified for their content of epitope specific cytochrome P-450 isozymes, the majority produced competitive binding within the range of the standard curve at a concentration of between 1 and 50 μ g of microsomal protein per incubation. This was added in a volume of 100 μ l, and therefore represents a microsomal protein concentration of between 10 and 500 μ g/ml. As the protein concentration of microsomal fractions is usually in the range of 10 to 30 mg/ml, this allows for a dilution of between 20 and 3,000 in the protein concentration of the microsomal preparations. Low concentrations of immuno-reactive cytochrome P-450 isozymes can therefore be accommodated by the use of a lower dilution of the microsomal fraction concerned. Examples of this were seen with the liver microsomal fractions from control Dunkin-Hartley guinea pig and one of the human liver preparations, which were used at 300 μ g and 800 μ g of microsomal protein per incubation, respectively. Some liver microsomal preparations on the other hand, such as those from control pig, 3MC treated NZW rabbit and some of the human liver preparations, did not produce any competitive binding, even at

concentrations up to 800 μ g per incubation (i.e. a microsomal protein concentration of 8 mg/ml).

In conclusion, immunoassay methods such as those described in this chapter will complement existing methods of cytochrome P-450 quantitation, and will allow a more accurate determination of the amount of specific isozymes present in microsomal fractions to be determined.

Figure 6.1 **MAb 3/4/2 Titration Curves**

Titration curves for MAb 3/4/2 were determined, as described in section 6.2.3 . Results are expressed as OD 492nm for unlabelled MAb 3/4/2, incubated either alone (●) or with ^{125}I -labelled MAb 3/4/2 (■), and also as % bound for unlabelled MAb 3/4/2 co-incubated with ^{125}I -labelled MAb 3/4/2 (▲). All results are the means of triplicate determinations, +/- S.D. (where S.D. is greater than the size of the symbol).

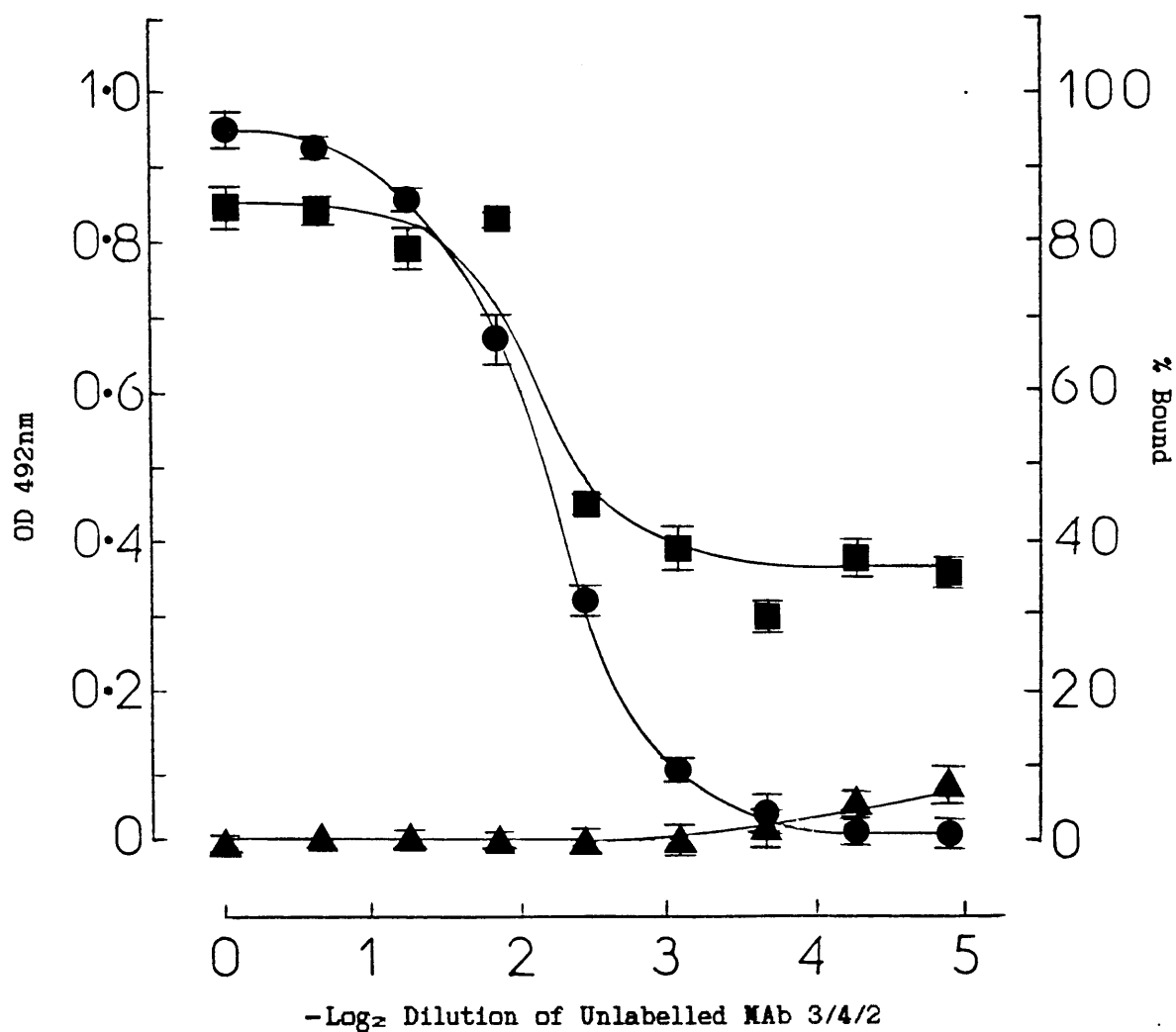


Figure 6.2 Competitive Immunoassay Titration Curves for ^{14}C -Labelled
MAB 12/2/3/2

Titration curves for ^{14}C -labelled MAB 12/2/3/2 were determined in a competitive immunoassay, as described in section 6.2.4, using both liquid scintillation spectrometry and enzyme linked detection methods. Results are expressed both as OD 492nm (●) and % bound (■) for this MAb preparation. All results are the means of triplicate determinations, +/- S.D. (where S.D. is greater than the size of the symbol).

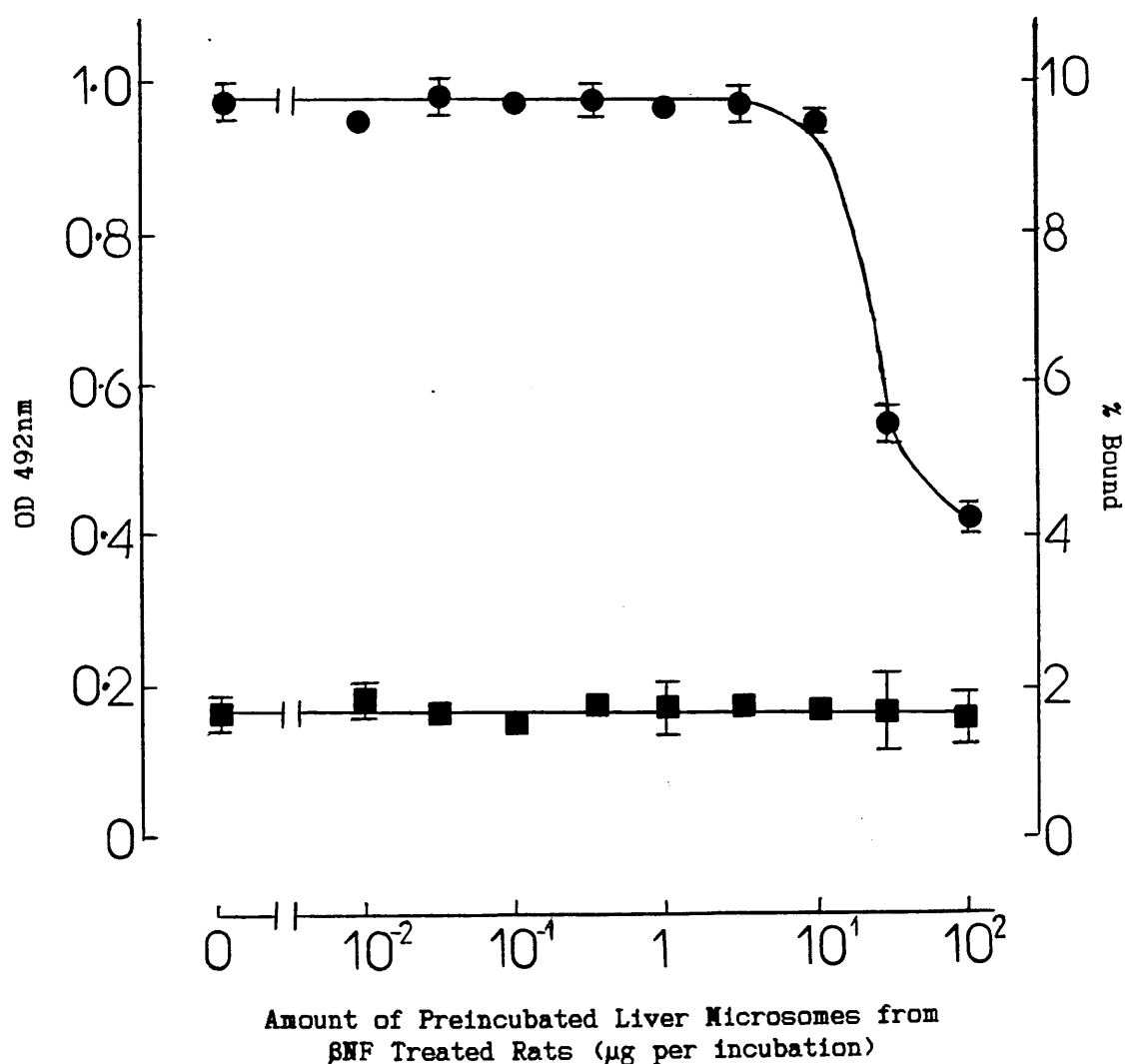


Figure 6.3 Competitive Immunoassay Titration Curves for MAb 1/4

Titration curves for MAb 1/4 were determined in a competitive immunoassay with liver microsomes from PB treated rats, as described in section 6.2.4.2 . Results are expressed as OD 492nm for MAb 1/4, at concentrations of 0.5 mg/ml (●), 50 μ g/ml (■) and 5 μ g/ml (▲), and are the means of triplicate determinations, $\pm$ S.D. (where S.D. is greater than the size of the symbol).

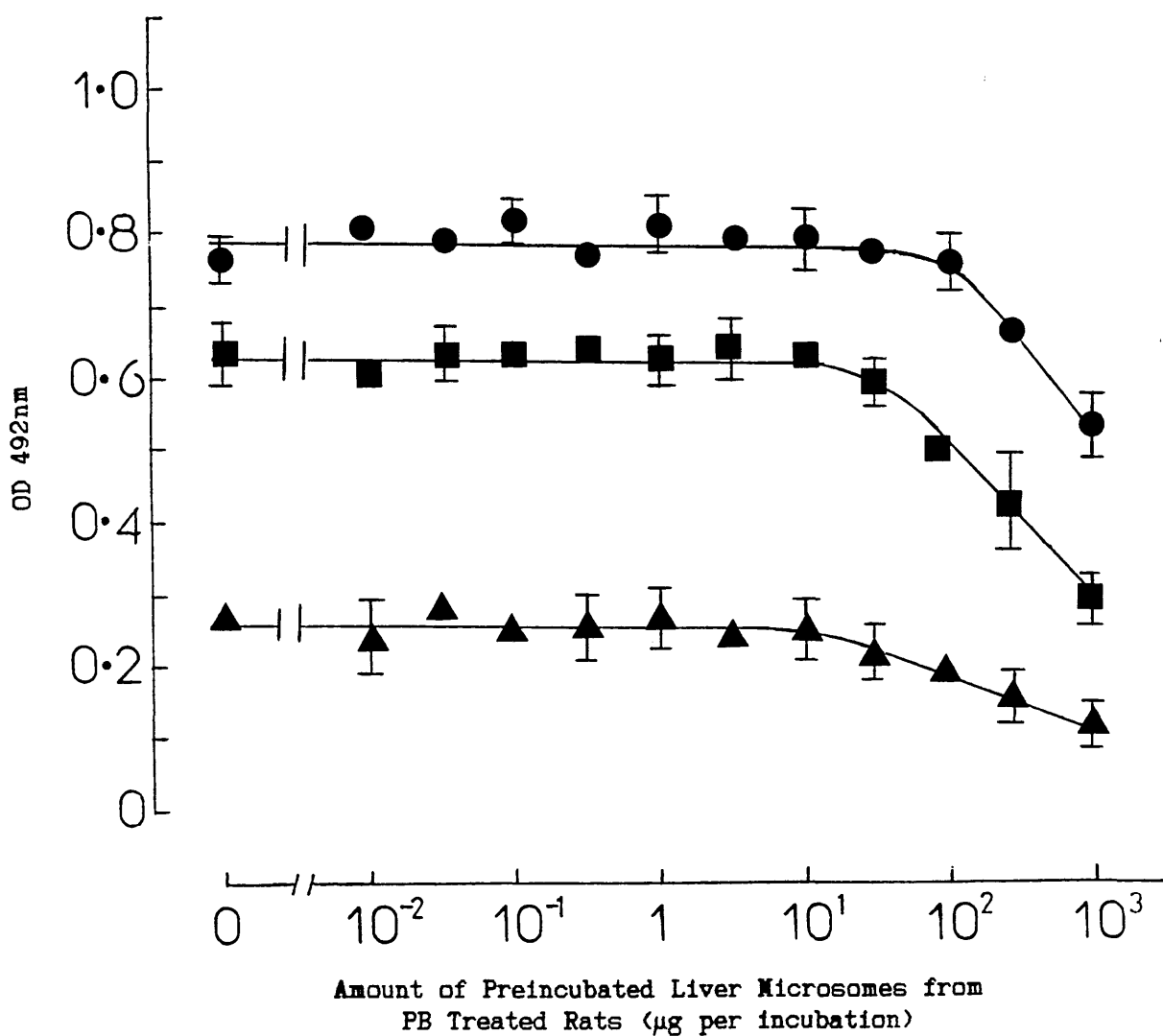


Figure 6.4 Competitive Immunoassay Titration Curves for MAb 3/4/2

Titration curves for MAb 3/4/2 were determined in a competitive immunoassay with liver microsomes from β NF treated rats, as described in section 6.2.4.2 . Results are expressed as OD 492nm for MAb 3/4/2, at concentrations of 0.5 mg/ml (●), 50 μ g/ml (■) and 5 μ g/ml (▲), and are the means of triplicate determinations, $\pm$ S.D. (where S.D. is greater than the size of the symbol).

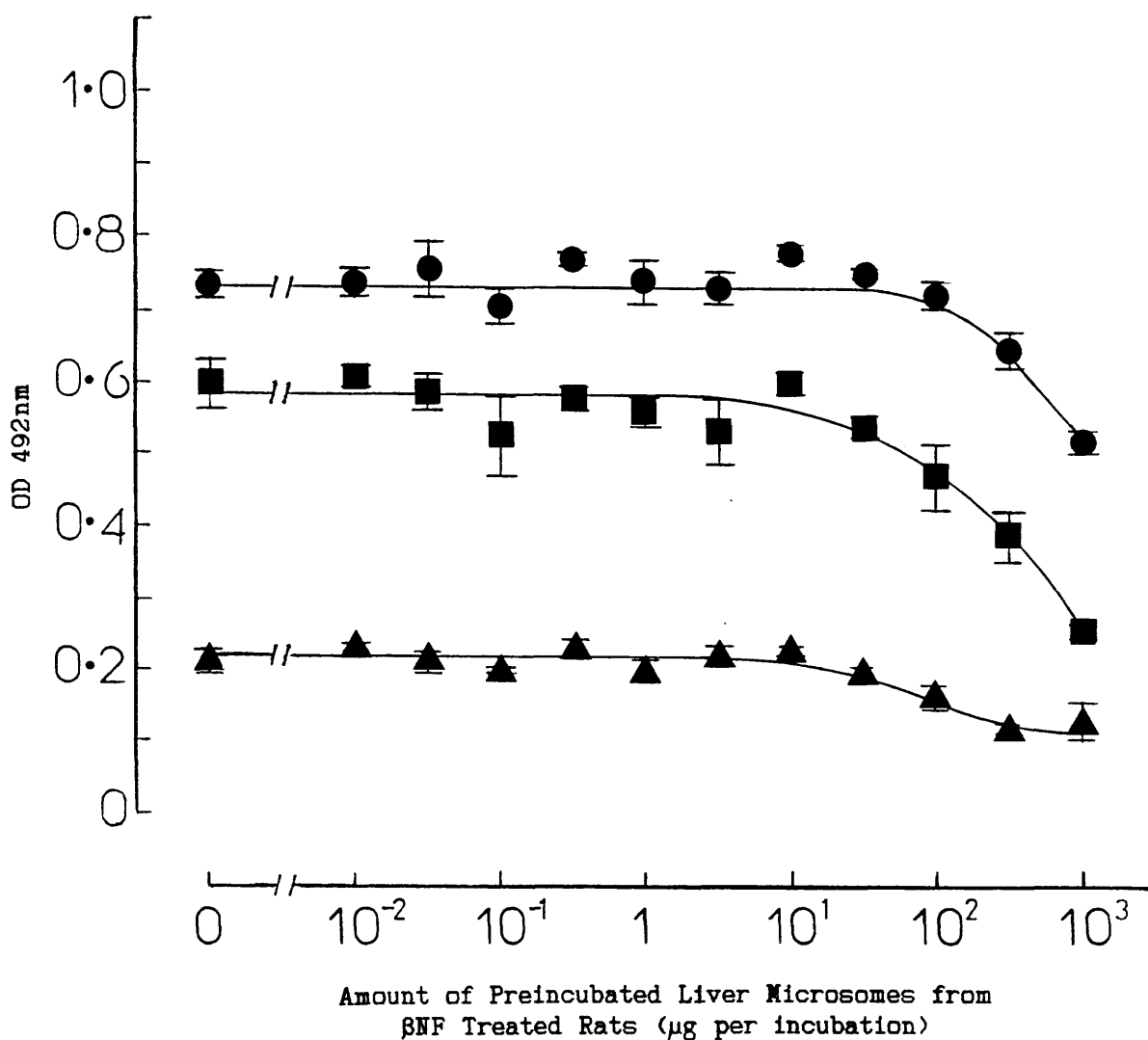


Figure 6.5 Competitive Immunoassay Titration Curves for MAb 12/2/3/2

Titration curves for MAb 12/2/3/2 were determined in a competitive immunoassay with liver microsomes from (A) β NF treated rats or (B) PB treated rats, as described in section 6.2.4.2 . Results are expressed as OD 492nm for MAb 12/2/3/2, at concentrations of 0.5 mg/ml (●), 50 μ g/ml (■) and 5 μ g/ml (▲), and are the means of triplicate determinations, $\pm$ S.D. (where S.D. is greater than the size of the symbol).

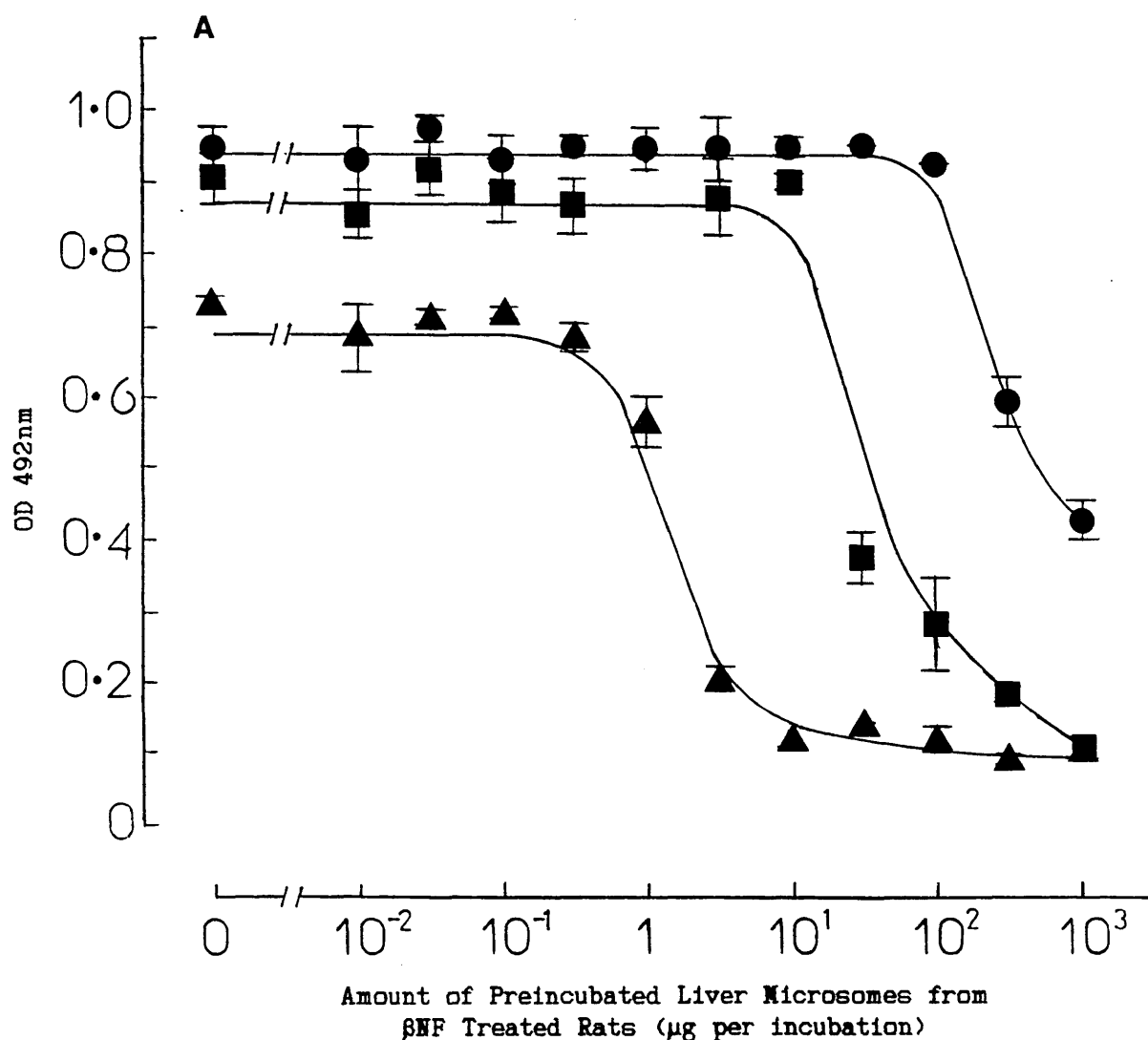


Figure 6.5 continued

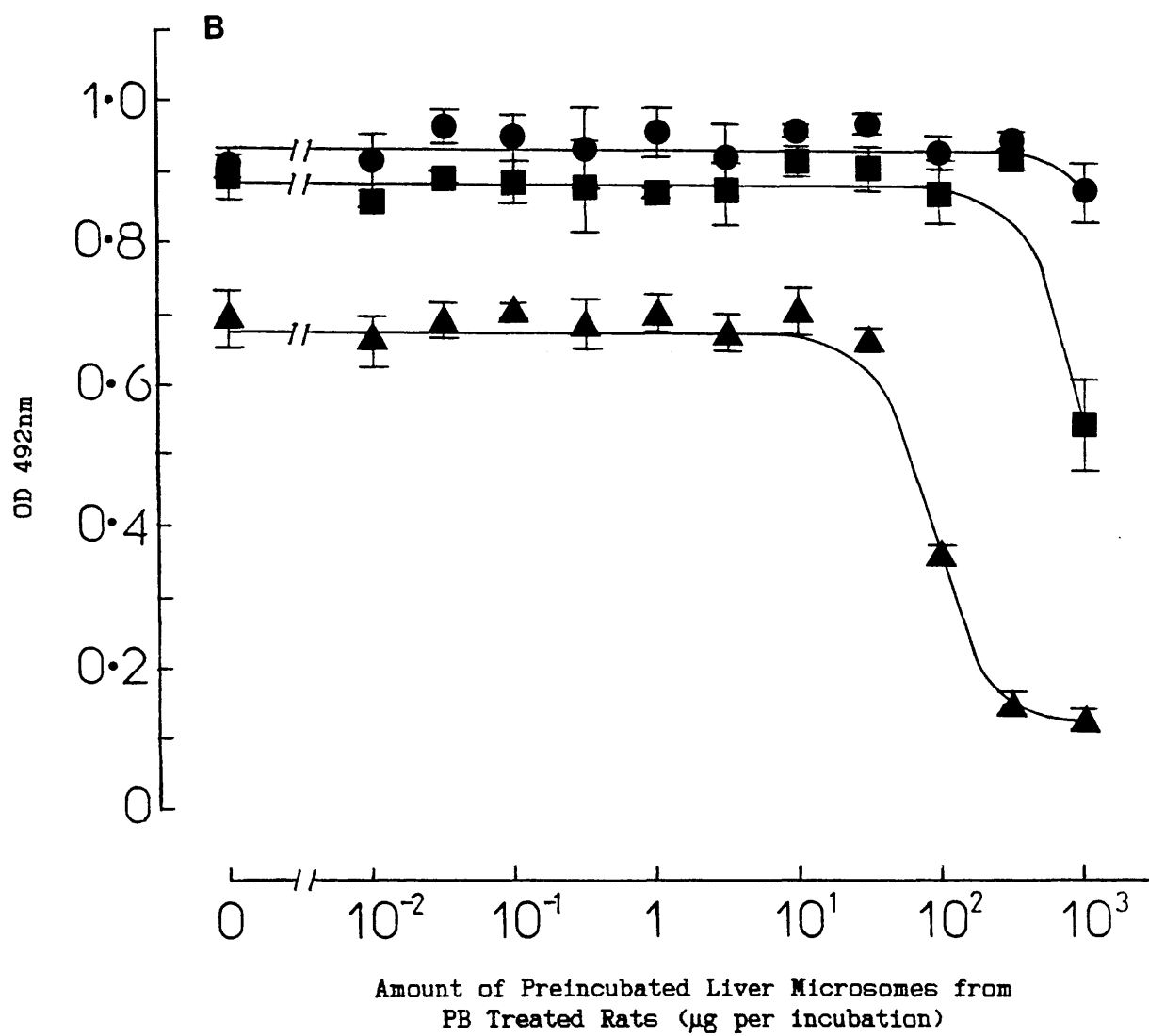


Figure 6.6 Quantitative Immunoassay Standard Curve for MAb 12/2/3/2

The standard curve for MAb 12/2/3/2 was determined in a quantitative competitive immunoassay with purified cytochrome P-450c, as described in section 6.2.4.3 . Results are expressed as OD 492nm for the various amounts of cytochrome P-450 preincubated, and are the means of triplicate determinations, $\pm$ S.D. (where S.D. is greater than the size of the symbol).

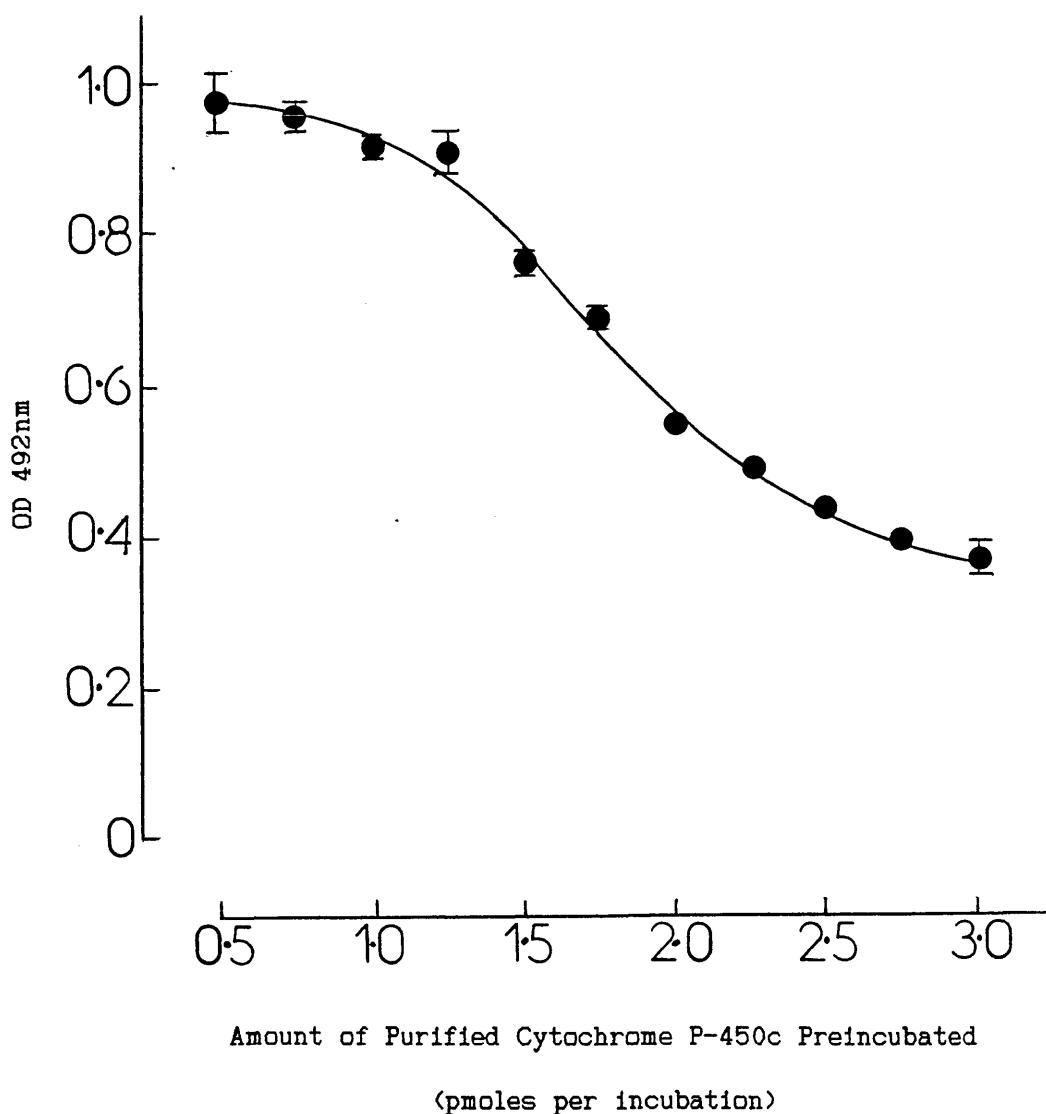


Table 6.1 Quantitative Immunoassay Results for MAb 12/2/3/2

Using the standard curve illustrated in Figure 6.6, the specific content of cytochrome P-450 forms c + d in various liver microsomal preparations was determined, as described in section 6.2.4.3 . Results were derived from the means of triplicate determinations.

Source of Liver Microsomes	Preincubated Amount (µg)	Mean OD 492nm Obtained	Equivalent Amount of Form c (pmoles)	Specific Content of Forms c + d (nmoles/mg protein)
BNF Wistar Rat	1.5	0.671	1.79	1.193
3MC Wistar Rat	1.5	0.588	2.00	1.333
PB Wistar Rat	50	0.791	1.46	0.029
Control Wistar Rat	40	0.635	1.88	0.047
3MC AKR Mouse	10	0.549	2.10	0.210
Control AKR Mouse	25	0.761	1.55	0.062
Control Guinea-Pig	300	0.645	1.85	0.006
Control Human (T5)	800	0.821	1.37	0.002
Control Human (T6)	800	0.924	-	-
Control Human (T7)	800	0.931	-	-
Control Pig	800	0.926	-	-
3MC NZW Rabbit	100	0.940	-	-

CHAPTER 7

IMMUNOCYTOCHEMICAL STUDIES ON CYTOCHROME P-450 ISOZYME DISTRIBUTION

7.1 Introduction

Significant advances in the understanding of the structural, functional and regulatory properties of cytochrome P-450 isozymes have been obtained from numerous studies performed in recent years (see Boobis *et al.*, 1985b). One area which has not received as much attention, however, is the distribution of individual cytochrome P-450 isozymes within a whole organ, and the influence of xenobiotics upon this distribution. Although the liver is the major site of xenobiotic transformation, other tissues such as the lung (Guengerich, 1977b), kidney (Grundin *et al.*, 1973) and small intestine (Takesue and Sato, 1968) also exhibit monooxygenase activity, albeit often at lower levels. Cytochrome P-450 isozymes can sometimes be induced in these non-hepatic tissues, in addition to induction in the liver.

One method by which the distribution of various isozymes within tissues can be investigated, is through the use of immunocytochemical techniques. The successful use of antibody-enzyme conjugates, peroxidase anti-peroxidase methods and biotin-streptavidin systems has allowed the identification of tissue antigens in histological sections (see review by Tubbs *et al.*, 1981). Such investigations provide a permanent record of antigen distribution, together with good morphological detail of the tissue concerned. The use of electron dense labels, such as ferritin or gold, can also enable the localisation of antigen at the sub-cellular level (Roth, 1982).

Immunocytochemical studies utilising polyclonal antibodies have mapped the distribution of certain cytochrome P-450 isozymes in liver (Baron *et al.*, 1981; Dees *et al.*, 1982; Ohnishi *et al.*, 1982; Moody *et al.*, 1983, 1985; Wolf *et al.*, 1984), kidney (Dees *et al.*, 1982), lung (Devereux *et al.*, 1981; Dees *et al.*, 1982) and adrenal (Baron *et al.*, 1978; Mitani *et al.*, 1982). However, cross-reactivity of polyclonal antibodies with other isozymes often makes interpretation of such studies difficult. MAb's, reacting as they do with individual epitopes, are ideally suited to such

studies, and offer a more precise reagent for the localisation of specific isozymes of cytochrome P-450. Several publications on the use of MAb's in investigating the localisation of cytochrome P-450 isozymes have recently been reported (Sugano *et al.*, 1985; Forkert *et al.*, 1986; Foster *et al.*, 1986; Koliada, 1986; Murray *et al.*, 1986; 1987; Anderson *et al.*, 1987; Finlayson *et al.*, 1987). The mapping of the intra- and inter-organ distribution of cytochrome P-450 isozymes may enable the identification of those tissues which could be at risk from metabolic activation of compounds to cytotoxic metabolites. It may also be possible to study cytochrome P-450 induction in tissues where levels of the isozyme under study are either too small or too focally distributed to be easily characterised by other techniques.

In the studies reported in this chapter, the distribution of cytochrome P-450 forms c and d in liver and kidney from control, β NF and PB treated rats was investigated using MAb's 3/4/2, 12/2/3/2 and B3A3.

7.2 Methods

7.2.1

Monoclonal Antibody Preparations

MAb's 3/4/2, 12/2/3/2 and B3A3 were purified before use by DEAE Affi-Gel Blue chromatography, as described in section 2.2.5, and diluted to a protein concentration of 1 mg/ml in DEAE Affi-Gel Blue column buffer.

7.2.2

Tissue Samples and Cryostat Sections

Tissue samples were prepared from male Alp/Apk rats (a Wistar derived strain), weighing 100-150g. Groups of animals were used either untreated, or following treatment with β NF in corn oil (at 100mg/kg/day, for four days

by i.p. injection), or with PB in saline (at 80mg/kg/day, for five days by i.p. injection). Animals were killed by cervical dislocation and their livers and kidneys removed. Tissue blocks of 0.1cm³ were rapidly prepared, snap frozen in isopentane previously cooled by liquid nitrogen, and stored at -70°C until required. Frozen tissue blocks were mounted in OCT fluid and 7-10µm thick sections were cut at -20°C. Sections were placed onto acetone cleaned glass slides, allowed to air dry for 18 hours at room temperature, and then stored at -70°C until required.

7.2.3

Immunocytochemical Procedures

Tissue sections were allowed to warm to room temperature before use, and were then treated according to the method of Wolf *et al.* (1984), with modifications as described by Foster *et al.* (1986). All procedures were performed at room temperature, unless otherwise stated. In brief, sections were fixed in 0.5% (w/v) p-benzoquinone in 0.2M sodium cacodylate buffer, pH 7.4, containing 20mM calcium chloride, for 5 minutes, and then washed with PBS. Endogenous peroxidase activity was blocked by incubating the sections in 95% (v/v) methanol, containing 0.01% (v/v) hydrogen peroxide, for 30 minutes. Sections were again washed with PBS, and to reduce non-specific protein binding during subsequent incubations, sections were incubated with a 5% (v/v) solution of non-immune rabbit serum in PBS, for 10 minutes. Sections were drained, but not washed, and MAb's were applied at various dilutions in PBS containing 1% (w/v) BSA (PBS-BSA), and incubated at 4°C for 18 hours in a humid atmosphere to prevent the sections drying out. Following this, the sections were washed with PBS, and then incubated with a 0.001% (v/v) solution of RAM-HRP in PBS-BSA for 30 minutes. Sections were again washed with PBS and then incubated in indirect lighting for 10 minutes, with 0.05% (w/v) DAB in 50mM Tris hydrochloride buffer, pH 7.6, containing 0.03% (v/v) hydrogen peroxide.

Sections were then washed with PBS, counterstained in Harris' haematoxylin for 30 seconds, 'blued' in running tap water for several minutes, dehydrated through graded ethanols and xylene, and mounted using DPX mountant. Stained sections were viewed under bright field illumination. Negative controls were also included for each set of MAb incubations, in which the MAb was omitted and the section incubated with PBS-BSA only.

7.2.4

Analysis of Results

The reactivity of the MAb's was assessed qualitatively from the intensity and distribution of the staining observed with each tissue. Some degree of quantification was obtained by determining the highest dilutions of the MAb's which gave positive reactivity with each tissue.

For each individual MAb, the whole section of at least two tissue blocks from at least two different animals of the same experimental group, were examined to determine the intensity and distribution of the staining obtained. In all cases, the results obtained for an individual MAb with animals from the same experimental group were qualitatively similar.

7.3 Results

7.3.1

Monoclonal Antibody Reactivity with Liver

Staining with MAb 3/4/2 in normal rat liver was observed to be evenly distributed across the liver lobule, but could only be visualised at a MAb dilution of 1 in 10 (Figure 7.1A). Treatment of rats with β NF caused an overall increase in the intensity of staining, but with a preferential increase in the periportal region (Figure 7.1B). In addition, a positive reaction was detected at a MAb dilution of 1 in 500, indicating that β NF

treatment had induced immunoreactive cytochrome P-450. In contrast, treatment of rats with PB caused an increase in staining of the centrilobular region, with very low levels of staining in the periportal area (Figure 7.1C). Positive staining could be achieved at a MAb dilution of 1 in 50, indicating that, in comparison to untreated rats, some induction of immunoreactive cytochrome P-450 had occurred, though not to the same extent as with β NF.

The pattern of staining observed with MAb 12/2/3/2 was qualitatively the same as that obtained with MAb 3/4/2 (Figure 7.2). However, there was some reduction in the intensity of staining observed with this MAb, such that a positive reaction with sections from β NF and PB treated rats was apparent at MAb dilutions of 1 in 250 and 1 in 25, respectively.

No staining was observed with MAb B3A3 for any of the rat liver preparations used in this study (Figure 7.3).

7.3.2

Monoclonal Antibody Reactivity with Kidney

With kidney sections from untreated rats, MAb 3/4/2 reacted strongly with a small proportion of cells (Figure 7.4A). The localisation and morphology of the stained cells indicated that they belonged to the S_3 (*pars recta*) portion of the proximal convoluted tubule (Trump *et al.* 1984). A similar pattern of staining was also observed in kidney sections prepared from rats treated with PB (Figure 7.4B). In both cases, positive reactivity was apparent at a MAb dilution of 1 in 10, indicating that no induction of immunoreactive cytochrome P-450 had occurred in the kidney following PB treatment. With sections prepared from β NF treated rats, however, staining was observed throughout the entire length of the proximal tubule in the cortex (Figure 7.4C). This was visible at a MAb dilution of 1 in 250, indicating that induction of immunoreactive cytochrome P-450 had

occurred. The glomeruli and distal tubules of the cortex and medulla were not stained with this MAb.

As with liver, the pattern of staining with MAb 12/2/3/2 was qualitatively similar to that observed with MAb 3/4/2 (Figure 7.5). Positive staining of sections from untreated or PB treated rats required the same MAb dilution as for MAb 3/4/2. However, with sections from BNF treated rats, a reduction in the MAb dilution to 1 in 200 was required for positive staining.

MAb B3A3 was again negative with all of the rat kidney preparations used (results not shown).

7.4 Discussion

Several methods have been reported in the literature for determining the tissue and cellular localisation of cytochrome P-450 isozymes present in a variety of tissues. The histochemical analysis of both fluorescent polycyclic aromatic hydrocarbon metabolites (Wattenberg and Leong, 1962) and cytochrome P-450 dependent aniline hydroxylase activity (Gangolli and Wright, 1971) have been applied to sections from rat liver. Microspectrophotometric analysis of cytochrome P-450 in unfixed rat liver sections has also provided information on the lobular distribution of cytochrome P-450 (Gooding *et al.*, 1978). Microdissection of kidney nephrons and the subsequent enzymatic quantification of cytochrome P-450 content has been performed with both control and treated rats and rabbits (Endou, 1983). Similarly, the cytochrome P-450 content of total (Heitanen *et al.*, 1985) and sub-fractionated intestinal epithelial cells (Bonkovsky *et al.*, 1985) has been determined in both control and treated rats.

Investigations involving tissue fractionation are, by their nature, open to error due to the mechanical manipulations involved in these procedures. The influence of various xenobiotics on the distribution of cytochrome

P-450 may therefore be masked by variability due to the techniques themselves. Additionally, those procedures which involve the *in situ* analysis of the metabolism of a substrate by cytochrome P-450 are necessarily confined to substrates, and/or metabolites, which can be easily detected by some means, such as fluorescence. Immunological methods of investigation, when coupled to the specificity of MAb's, can provide information on the tissue localisation of individual cytochrome P-450 isozymes down to the cellular level (Sugano *et al.*, 1985; Forkert *et al.*, 1986; Foster *et al.*, 1986; Koliada, 1986; Murray *et al.*, 1986; 1987; Anderson *et al.*, 1987; Finlayson *et al.*, 1987). Serial sections of the same tissue can be used in conjunction with several MAb's to produce information on the spatial relationship between different isozymes of cytochrome P-450. This could also be achieved on the same tissue section by multiple labelling techniques. Using electron dense detection reagents, investigators have also studied the localisation of cytochrome P-450 isozymes at the sub-cellular level (Sugano *et al.*, 1985).

In the studies reported in this chapter, the immunocytochemical localisation of cytochrome P-450 forms c and d has been performed on rat liver and kidney sections using MAb's 3/4/2 and 12/2/3/2. The results obtained for both these MAb's were qualitatively similar in all the tissue preparations studied. It must therefore be assumed that form d co-localises with form c under the induction conditions used in this study. However, form c may be expressed at sites different from form d, but, due to the reactivity of both MAb's with form c, this would not be apparent solely from their reactivity patterns. Both MAb's reacted more strongly with sections from liver, and in particular, from livers of rats treated with β NF, as would be expected from their reactivity profiles. Reactivity also occurred with livers from both control and PB treated rats. In both cases however, reactivity was apparent only at much lower MAb dilutions than with sections from β NF treated animals. The immunocytochemical

localisation of several forms of rat liver cytochrome P-450 by MAb's has been previously reported by Koliada (1986). Unfortunately, little information is forthcoming from that publication, other than that the studies were performed, since only a very brief English abstract is provided.

The localisation of cytochrome P-450 in control livers by both MAb's is similar to that reported by Baron *et al.* (1981) using a polyclonal antibody against 3MC induced cytochrome P-450. In that report, however, the centrilobular region staining reported appeared to be slightly more intense than that observed for MAb's 3/4/2 or 12/2/3/2 in this study. This may be due to the increased specificity enjoyed by the MAb's used in this study. Similar studies with polyclonal antibodies have also been reported by Wolf *et al.* (1984), in which low intensity staining was achieved with a polyclonal antibody against 3MC induced cytochrome P-450, with slightly more intense staining in the periportal region. A similar pattern of diffuse staining throughout the lobule has also been reported for control rabbit liver with polyclonal antibodies against cytochrome P-450 forms LM2 and LM3 (Dees *et al.*, 1982). Unlike rat forms c and d, rabbit form LM2 is a PB inducible form (Domin *et al.*, 1984). Rabbit form LM3, on the other hand, was originally reported to be a constitutive and non-inducible form (Johnson, 1980). Form LM3 has now been shown to comprise three different isozymes, namely LM3a, LM3b and LM3c, and forms LM3a and LM3c have been shown to be inducible by exposure to ethanol/acetone and erythromycin, respectively (Coon *et al.*, 1985).

The localisation of staining obtained with both MAb's with liver sections from β NF treated rats was very similar to that reported by Wolf *et al.* (1984). The antibody used in that study was a polyclonal raised against 3MC induced liver cytochrome P-450. It is therefore likely that both it and MAb's 3/4/2 and 12/2/3/2 react with a common group of cytochrome P-450 isozymes. The large increases in the MAb dilutions possible for positive reactivity following treatment of the rats with β NF

indicate that substantial induction of cytochrome P-450 forms c and/or d had occurred, as would be expected (Ryan *et al.*, 1982).

In liver sections from PB treated rats, the areas of reactivity of both MAb's 3/4/2 and 12/2/3/2 were localised to the centrilobular region. This reactivity pattern is similar to that reported by Wolf *et al.* (1984) using polyclonal antibodies against PB induced cytochrome P-450. Thus, although different isozyme preparations were used to prepare the antibodies used in these two studies, the staining pattern observed for the antibodies following PB treatment was very similar. It should be remembered though that MAb 3/4/2 also reacts in Western blots with a 56,000 molecular weight protein present in liver microsomes from PB treated rats. On the basis of it's molecular weight, it's non-reactivity with other MAb's and the differential reactivity of MAb 3/4/2 with proteolytically digested liver microsomes from PB and β NF treated rats, this protein would appear to be a previously unidentified cytochrome P-450 isozyme (Sesardic *et al.*, 1986). It must be assumed that MAb 3/4/2 will react with this protein in liver sections from PB treated rats, together with any form c also present. That the reactivity obtained for MAb 3/4/2 was qualitatively similar to that observed for MAb 12/2/3/2 indicates that if this MAb 3/4/2 reactive protein is indeed a cytochrome P-450 isozyme, then it must have the same lobular distribution as forms c and d following PB treatment. Induction of immunoreactive proteins in liver following PB treatment was certainly not as effective as that achieved with β NF, from the MAb dilutions required for reactivity. It must therefore be assumed that the MAb 3/4/2 reactive protein is only weakly induced by PB treatment.

A paradox still remains, however, in the results obtained with MAb 12/2/3/2 with liver sections from PB treated rats. Previous studies with this MAb indicated that it did not react in Western blots with any protein present in liver microsomes from PB treated rats (see Chapter 3 of this

thesis, and Sesardic *et al.*, 1986). From the studies reported here, it is apparent that this MAb does react with proteins present in rat liver following PB treatment. This difference in reactivity pattern of MAb 12/2/3/2 may be due to differences in the sensitivity of the two techniques. It must also be noted though, that reactivity with liver sections from PB treated rats could only be achieved, for both MAb's in this study, at, approximately, a ten fold increase in MAb concentration to that required for reactivity with liver sections from β NF treated rats. Western blotting experiments, on the other hand, were performed with the same MAb dilution, regardless of the microsomal preparation studied. This may have allowed immunoreactive proteins to be undetected in Western blot studies with liver microsomes from PB treated rats. The difference in MAb dilutions required, though, by both MAb's for reactivity with liver sections from control and PB treated animals, would still indicate that some degree of immunoreactive protein induction had occurred following PB treatment. However, this conclusion should be regarded in the context of the quantitative enzyme immunoassay results obtained with MAb 12/2/3/2, and discussed in Chapter 6 of this thesis. In that study, it was apparent that, in comparison to control rats, the total hepatic content of cytochrome P-450 forms c + d decreased in rats treated with PB. It would therefore seem apparent that, whilst PB treatment can induce, or possibly concentrate, cytochrome P-450 isozymes in the centrilobular regions that react with MAb 12/2/3/2, the remaining areas of the liver must have the levels of these isozymes suppressed to a much greater extent to account for the overall decrease seen for these forms in liver, following PB treatment.

Work on MAb localisation of cytochrome P-450 in normal human liver sections has been reported by Murray *et al.* (1986). In that study, a marked zonal distribution of staining was observed, which was predominantly centrilobular, with little mid zone staining and no apparent periportal reactivity. This zonal distribution may be important to the morphological pattern of liver injury caused by hepatotoxins that undergo metabolic

activation by cytochrome P-450 isozymes, such as the forms recognised by the MAb's used in that study. Further studies by this group demonstrated cytochrome P-450 localisation in human foetal liver (Murrey *et al.*, 1987). In this case, all foetal hepatocytes were found to contain immunoreactive cytochrome P-450, and no zonal distribution was observed.

No difference was observed between MAb's 3/4/2 and 12/2/3/2 in their reactivity profile with kidney sections from either control or PB treated rats. Neither the localisation of staining, nor the dilution of MAb required for reactivity, showed any alteration following PB treatment of the rats. This indicates that PB treatment does not induce the production of these immunoreactive forms of cytochrome P-450 in the kidney. This finding is in agreement with previous studies on both rat (Guengerich and Mason, 1979) and rabbit (Dees *et al.*, 1982). No Western blotting experiments were performed with MAb 3/4/2 on kidney microsomes from PB treated rats. It is therefore not known if a similar PB induced MAb 3/4/2 immunoreactive protein is present in the kidney, as it is in liver microsomes from PB treated rats. It is interesting to note, however, that biochemical studies have provided evidence that PB treatment of rats does not induce any renal cytochrome P-450 (Rush *et al.*, 1983).

In contrast to the lack of induction following PB treatment, the MAb reactivity with kidney sections from rats treated with β NF would indicate that a marked induction of renal cytochrome P-450 content had been achieved. This effect of β NF has been shown previously by several studies (Dees *et al.*, 1982; Endou, 1983; Rush *et al.*, 1983). As with liver, the similarity in reactivity pattern of the two MAb's would indicate that cytochrome P-450 forms c and d are co-localised in the liver, both in the control animal and following treatment with β NF.

Localisation studies similar to those reported here for MAb's 3/4/2 and 12/2/3/2 have also been performed on rabbit kidney sections by Finlayson *et al.* (1987). In that study, a new isozyme of cytochrome P-450 was

identified, termed cytochrome P-450 K, which localised to the S₂ and S₃ segments of the proximal tubule in the renal cortex, and, contrary to the result described above by Rush *et al.* (1983), was induced by PB treatment. In contrast, no localisation of rabbit cytochrome P-450 forms LM1 and LM3b were observed in any kidney sections. MAb staining was not performed on rabbit liver sections in that study to determine the localisation of cytochrome P-450 K. However, reactivity with a protein of molecular weight between that of cytochrome P-450 forms LM1 and LM3b was observed in Western blots of liver microsomes. It is suggested that this new form of rabbit cytochrome P-450 may be involved in lauric acid 11-hydroxylation.

Further immunocytochemical studies on rat tissues have been performed with MAb 3/4/2, in which the effect of clofibrate treatment on immunoreactive cytochrome P-450 induction was additionally investigated, together with a wider range of extra-hepatic tissues (Foster *et al.*, 1986). Reactivity was observed with this MAb in the enterocytes of the small intestine, in the Clara cells of the lung, in the olfactory epithelium and in the Bowmans glands of the olfactory tissue, in addition to the reactivity reported above for liver and kidney. No reactivity was observed, however, with sections prepared from either the testes or the adrenal gland, even following β NF treatment of the rats. Treatment of rats with β NF was found to induce immunoreactive cytochrome P-450 in the lung and small intestine, as well as in the liver and kidney. No induction was observed in the olfactory tissue however. Treatment with PB or clofibrate failed to induce any MAb 3/4/2 immunoreactive cytochrome P-450 in any of the tissues studied, with the exception of PB in the liver as discussed above.

Other studies on MAb localisation of cytochrome P-450 isozymes have also been reported. In one particular study the localisation of bovine cytochromes P-450_{11 β} and P-450_{sec} in adrenal tissue was described (Sugano *et al.*, 1985). It was determined that both forms were predominantly

localised in cortex parenchymal cells of the zona fasciculata and zona reticularis, rather than to the zona glomerulosa. Electron microscopical studies confirmed that both these forms were located intra-cellularly to mitochondria. These results confirmed previous studies that utilised polyclonal antibodies. Gelboin and co-workers have also reported on the tissue localisation of cytochrome P-450 isozymes. In one study, the MAb localisation of PB and 3MC inducible forms of cytochrome P-450 in mouse lung was described (Forkert *et al.*, 1986). A MAb specific for 3MC inducible forms of cytochrome P-450 (Mab 1-7-1) reacted strongly with parenchymal cells of 3MC treated rats, including Type II alveolar cells, but not with bronchiolar epithelial cells. No reactivity was observed with this MAb with control or PB treated rat lung tissue. Conversely, a MAb specific for PB inducible forms of cytochrome P-450 (Mab 2-66-3) reacted with both parenchymal, again including Type II alveolar cells, and bronchiolar epithelial cells, including Clara cells, in lung tissue from control, PB and 3MC treated mice. The non-inducibility of this particular MAb reactivity is perhaps indicative of it's reactivity with a constitutive form of cytochrome P-450. Further work on cytochrome P-450 localisation has also been described for Mab 1-7-1 (Anderson *et al.*, 1987). In that study, the inducibility phenotype of several inbred strains, and their F₁ and F₂ progeny crosses, were studied, in part, by immunocytochemical methods. A good correlation was observed between Mab 1-7-1 reactivity with liver sections, and the inducibility of cytochrome P-450 by 3MC, as determined by the metabolism of 3MC by liver homogenates.

The results obtained both in this study for MAb's 3/4/2 and 12/2/3/2, and in other MAb studies, highlight the applicability of MAb's as reagents for studying the localisation of cytochrome P-450 in both hepatic and extra-hepatic tissues. In addition, MAb's can be used to assess the inducibility of isozymes *in situ*, especially in those tissues where the

levels of the isozyme are too low, or where the distribution is too focal, to make other techniques practical.

Studies with MAb's such as 3/4/2 and 12/2/3/2, could be extended in the future to look at the sub-cellular localisation and induction of cytochrome P-450 isozymes by the use of electron dense labels in conjunction with immunocytochemical techniques for the electron microscope. Such studies would allow the cellular basis of, for example, cytochrome P-450 induction, metabolic activation or detoxication of xenobiotics to be investigated at a higher resolution than that possible by the immuno-enzymatic techniques described in this chapter.

Figure 7.1 Cytochrome P-450 Localisation in Rat Liver Sections
by MAb 3/4/2

The immunocytochemical localisation of cytochrome P-450 in rat liver cryostat sections was determined using MAb 3/4/2, as described in section 7.2.3 . Sections from (A) control, (B) β NF treated and (C) PB treated animals were analysed, at MAb 3/4/2 dilutions of 1 in 10, 1 in 500 and 1 in 50, respectively.

Magnifications of figures are (A) x 100, (B) x 100 and (C) x 200.
Indicators on figures are : C, central vein; P, portal vein.

A

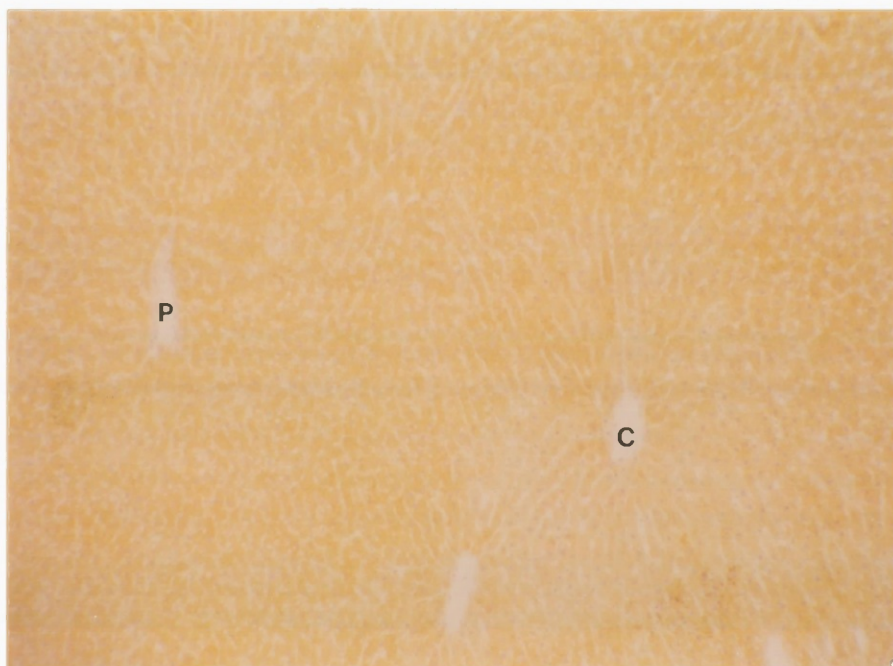
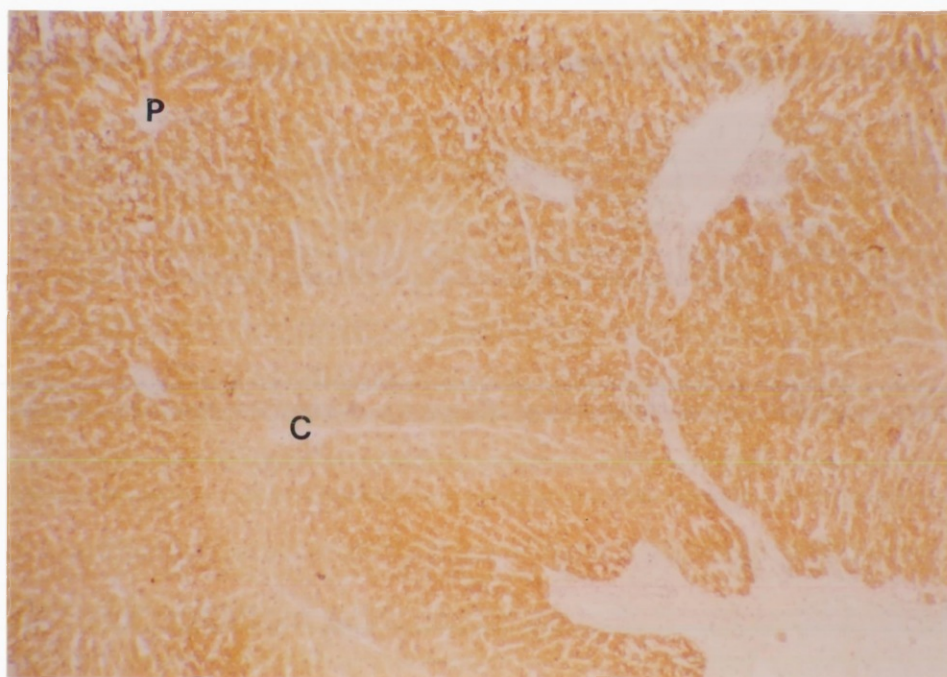


Figure 7.1 continued

B



C

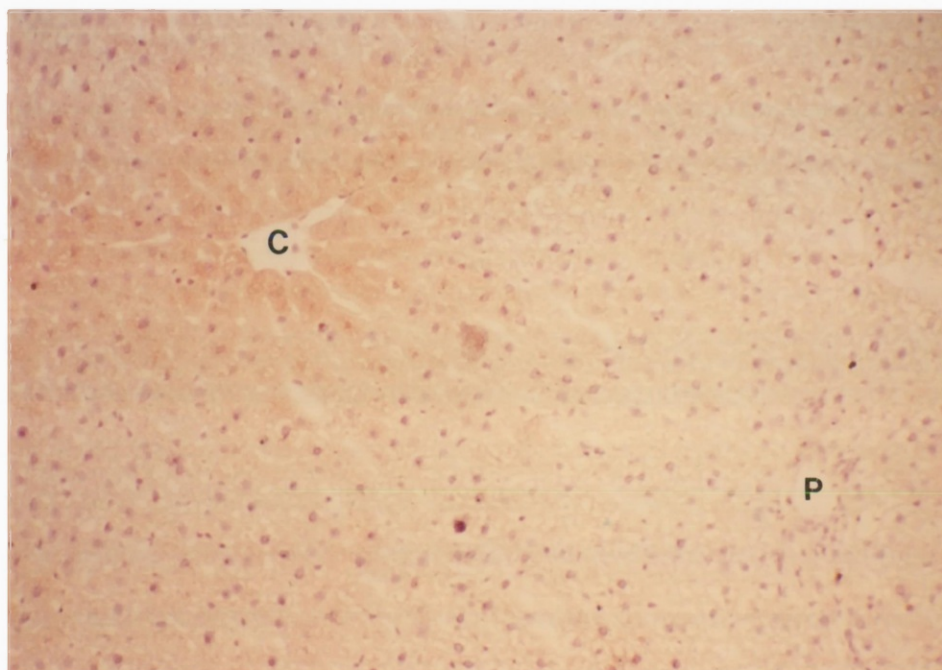


Figure 7.2 Cytochrome P-450 Localisation in Liver Sections from
BNF Treated Rats by MAb 12/2/3/2

The immunocytochemical localisation of cytochrome P-450 in liver cryostat sections from BNF treated rats was determined using MAb 12/2/3/2 at a dilution of 1 in 250, as described in section 7.2.3 .

Magnification of figure is x 100, and indicators on figure are :
C, central vein; P, portal vein.

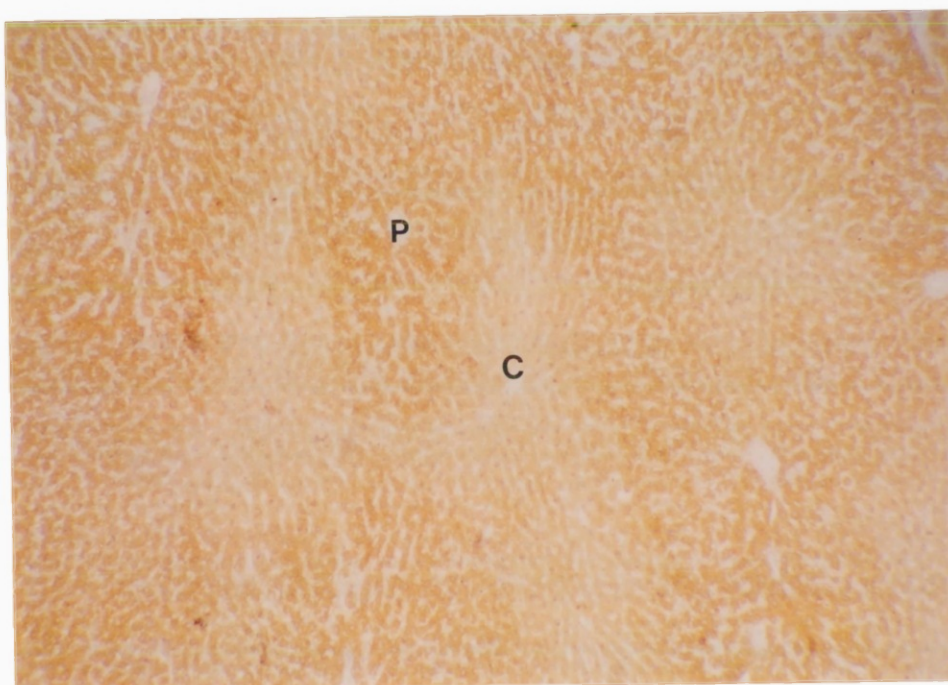


Figure 7.3 Cytochrome P-450 Localisation in Liver Sections from
BNF Treated Rats by MAb B3A3

The immunocytochemical localisation of cytochrome P-450 in liver cryostat sections from β NF treated rats was determined using MAb B3A3 at a dilution of 1 in 10, as described in section 7.2.3 .

Magnification of figure is x 200, and indicators on figure are :
C, central vein; P, portal vein.

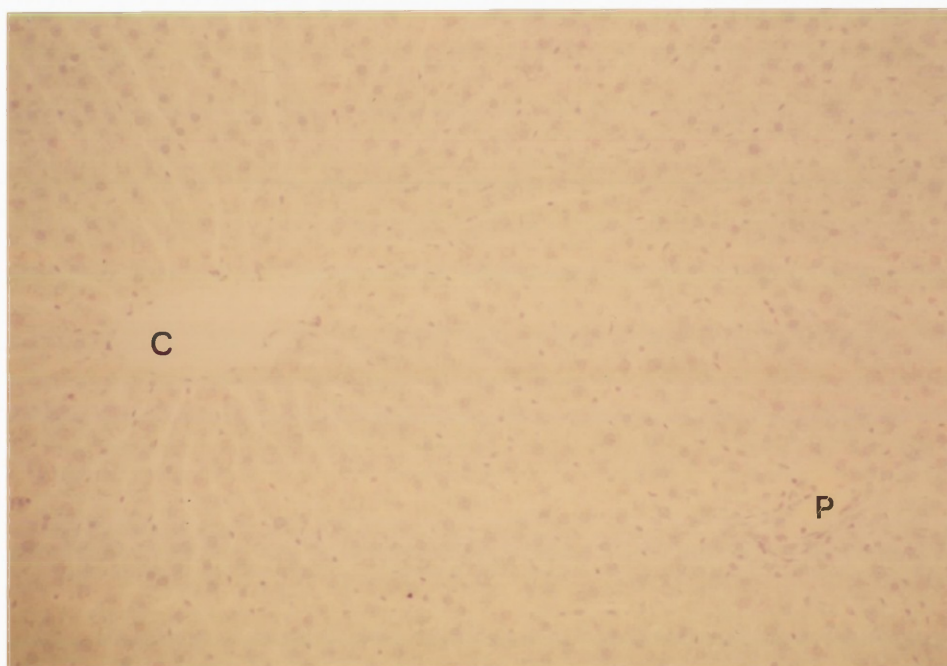


Figure 7.4 Cytochrome P-450 Localisation in Rat Kidney Sections
by MAb 3/4/2

The immunocytochemical localisation of cytochrome P-450 in rat kidney cryostat sections was determined using MAb 3/4/2, as described in section 7.2.3 . Sections from (A) control, (B) PB treated and (C) β NF treated animals were analysed, at MAb 3/4/2 dilutions of 1 in 10, 1 in 10 and 1 in 250, respectively.

All figure magnifications are x 200.

A

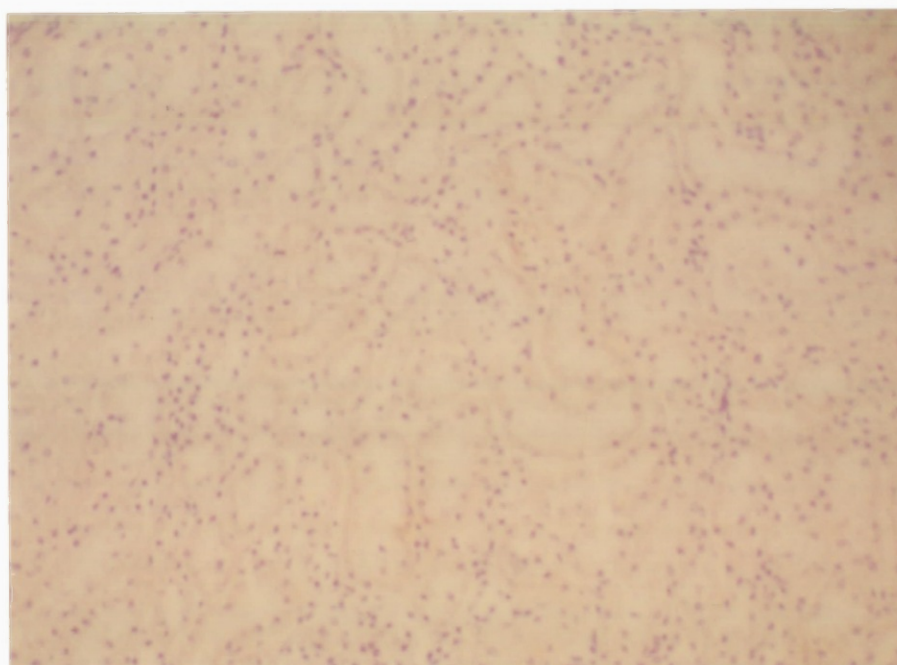
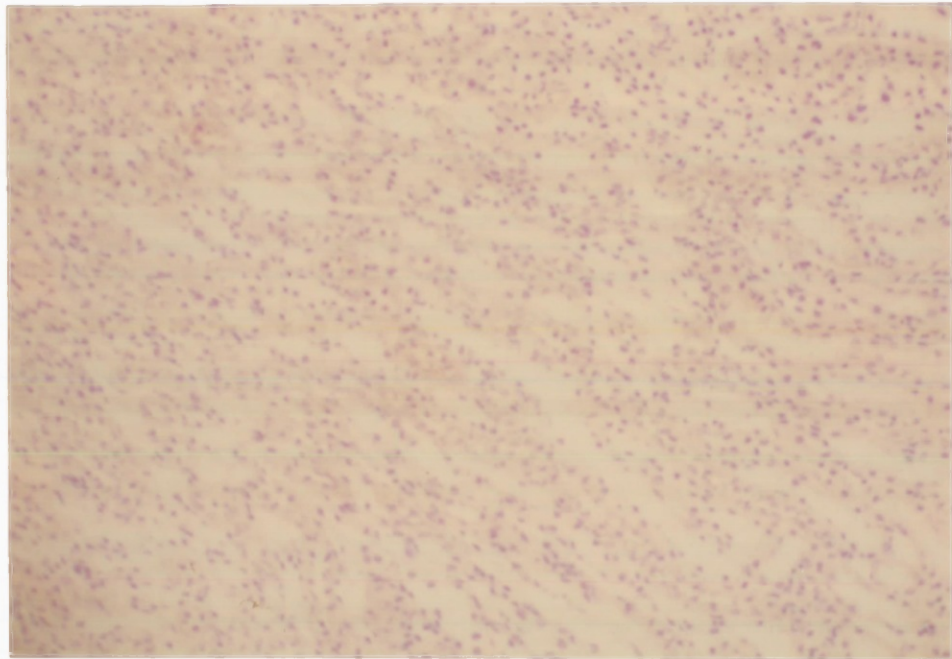


Figure 7.4 continued

B



C

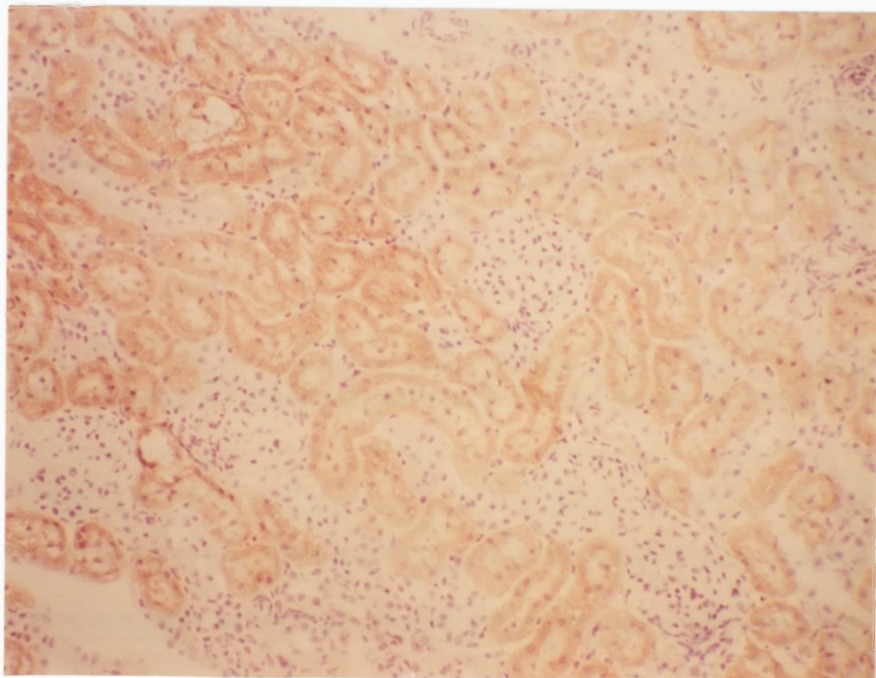
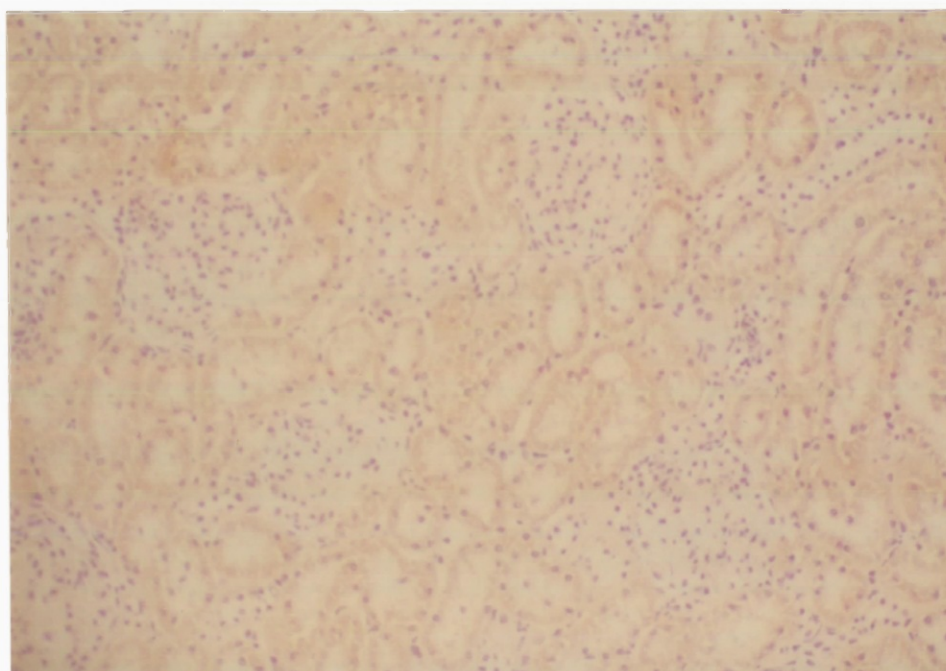


Figure 7.5 Cytochrome P-450 Localisation in Kidney Sections from
BNF Treated Rats by MAb 12/2/3/2

The immunocytochemical localisation of cytochrome P-450 in kidney cryostat sections from β NF treated rats was determined using MAb 12/2/3/2 at a dilution of 1 in 200, as described in section 7.2.3 .

Magnification of figure is x 200.



CHAPTER 8

OVERALL CONCLUSIONS

8.1 Introduction

8.1.1

The Rationale for using Monoclonal Antibodies in this Project

As has been previously described in this thesis, polyclonal antibodies have been used by investigators for many years in studies of cytochrome P-450. At the time this project was initiated, though, MAb's had risen to the forefront of antibody technology in many areas of research because of their specificity and reproducibility. The difference between a polyclonal antiserum and a MAb is simple. In the former, there is a mixture of immunoglobulin molecules directed against different epitopes on the antigen of interest, all of which bind with different affinities. Some of those epitopes may also occur on other proteins, resulting in a cross-reactive antiserum. With a MAb, however, there is an individual immunoglobulin type present, which reacts with a single epitope on the antigen of interest with a specific affinity. That epitope may also be present on other antigens, resulting in a cross-reactive MAb. As each MAb is produced by a single hybridoma cell line, frozen stocks of such cells can be easily prepared and stored for extended periods of time, usually over several years. Recovery of these cells can therefore lead to further production of large amounts of identical MAb. Using such procedures, standardised MAb reagents were prepared throughout the course of this project without the need to re-characterise each separate batch of antibody. In some cases the actual titre of a MAb varied between individual batches, but not its affinity or reactivity profile. With polyclonal antisera, however, the amount of individual specific antibodies present can vary between individual bleeds, more so if the animal has been boosted with antigen. This variation in individual antibodies in different bleeds can have a major effect on avidity. Thus individual batches of an antiserum can vary in their degree of reactivity and cross-reactivity with the various antigens under study.

To overcome this problem with polyclonal antisera, two strategies are commonly used. The first is to immunise the animal with a pure preparation of antigen. For many abundant proteins this is a feasible procedure. Indeed for many inducible forms of cytochrome P-450, polyclonal antibody preparations have been produced this way (eg. Thomas *et al.*, 1987). In MAb production, however, it does not matter if the immunising antigen preparation is not pure. As each antibody producing cell that is selected by the screening assays is cloned out, individual MAb specificities are selected. In addition, immunisation with an apparently pure protein does not ensure that a polyclonal antiserum so produced will be monospecific and not cross-reactive with similar antigens. Minor contaminants in the protein preparation can elicit a greater amount of antibody response than their relative concentration would appear to warrant. To overcome the effect of these undesirable antibodies, the second of the strategies can be implemented. This is to desorb unwanted antibodies out of an antiserum by incubating it with cross-reactive antigens bound onto a solid surface. In the case of cytochrome P-450 reactivity, for example, an antiserum raised against cytochrome P-450c may have to be desorbed against a cytochrome P-450d coupled matrix to remove anti-cytochrome P-450d reactivity and leave the antiserum monospecific for cytochrome P-450c (Reik *et al.*, 1982). Such a strategy obviously requires access to the cross-reactive antigen in reasonable (mg quantity) amounts. In many cases this will not be feasible, especially with minor or constitutive isozymes. This was certainly the case throughout the course of the project reported in this thesis.

Therefore, for the reasons of the inherent reproducibility of MAb's and the lack of ready availability of purified cytochrome P-450 isozymes for both polyclonal antisera immunisation and desorption, the decision was taken at the start of this project to proceed with the production of MAb reagents.

8.1.2

Production of Monoclonal Antibodies

Over twenty MAb's were prepared against isozymes of cytochrome P-450 during the course of this project. Several of these were selected for further study on the basis of their reactivity profiles generated by the ELISA, Western blotting and other screening programmes employed during the project. A number of control MAb's were also selected for inclusion in all further studies. In particular, MAb B3A3, specific for bovine growth hormone and not reactive with any cytochrome P-450 preparation, was routinely included where possible. MAb's were produced in large quantities by i.p. injection of hybridoma cells into pristane treated mice, and collecting the ascites produced. This was routinely fractionated by ammonium sulfate precipitation. In several studies, however, the immunoglobulin fraction was further purified by ion-exchange chromatography on DEAE Affi-Gel Blue. This allowed immunoglobulins to be purified to greater than 95% purity.

8.2 Summary of Experimental Results

8.2.1

Structural Analysis of Cytochrome P-450 Isozymes

It is the problem of undefined cross-reactivity with both MAb's and desorbed polyclonal antisera which can cause confusion in the interpretation of experimental results. Epitopes present on the protein of interest may occur on other proteins in the tissue under study. With polyclonal antibodies, such reactivity could be also due to incomplete desorption of cross-reactive antibodies, which would require further purification to remove. For a MAb, however, such cross-reactivity is permanent. The reactivity profiles of both MAb's and desorbed antisera

therefore have to be accurately defined. The profiles obtained with the MAb's produced during the course of this project are summarised below.

ELISA results from both microsomal and purified isozyme assays revealed that some MAb's reacted with only a single form of cytochrome P-450 from a single species, whereas other MAb's reacted with several forms of cytochrome P-450 from several species, for example MAb 3/4/2. However, the ELISA assay protocol used was optimised to detect any positive reactivity of a MAb, and as such was not used to quantitate the cross-reactivity of MAb's with different microsomal preparations. A more specific technique is that of Western blotting, and this was performed with a number of the MAb's. MAb's 3/4/2, 3/9/2, 7L6, 12/2/3/2, 12/2/3/5 and 9/20/11, were all raised against rat liver cytochrome P-450c, and all reacted with at least one protein present in liver microsomes from β NF treated rats. MAb's 12/2/3/2, 12/2/3/5 and 9/20/11 reacted with two proteins of 56,000 and 54,000 molecular weight (forms c and d respectively), whereas MAb's 3/4/2, 3/9/2 and 7L6 reacted only with the higher molecular weight (form c) protein. None of the MAb's tested in Western blots reacted with liver microsomes from control rats. However, both MAb 3/4/2 and MAb 1/4 (raised against rat liver cytochrome P-450b) each reacted with a single protein in liver microsomes from PB treated rats. The molecular weight of the respective immunoreactive protein was different, though, for these two MAb's, being 56,000 and 54,000 molecular weight respectively. For MAb 1/4, the molecular weight of this immunoreactive protein corresponds to that of form b and/or e. All other MAb's raised against rat liver cytochrome P-450c were negative with this microsomal preparation. In addition, MAb 3/4/2 reacted with two proteins of 57,000 and 54,000 molecular weight (forms LM6 and LM4 respectively) present in liver microsomes from 3MC treated rabbits, as well as a 54,000 molecular weight protein present in a human liver microsome preparation. MAb's 1/4, 9/20/11 and 12/2/3/2 were all negative with these two microsomal

fractions. Thus, Western blotting studies revealed a complex pattern of MAb reactivity.

Three of the MAb's raised against rat liver cytochrome P-450c, namely MAb's 3/4/2, 9/20/11 and 12/2/3/2, were selected for further study. As discussed above, two of these MAb's, 9/20/11 and 12/2/3/2, reacted with two proteins present in liver microsomes from β NF treated rats, which are of the same molecular weights as cytochrome P-450 forms c and d. MAb 3/4/2, on the other hand, reacted only with the protein corresponding in molecular weight to form c. However, this was based on separation on SDS-PAGE, which is insufficient evidence for identity of reaction. MAb 9/20/11 was labelled internally with ^{14}C -leucine and used in a competitive binding assay with liver microsomes from β NF treated rats. This revealed that MAb 3/4/2 binds to an epitope spatially distinct from that recognised by MAb 9/20/11, whereas MAb 12/2/3/2 binds to the same epitope as, or to one very close to, that recognised by MAb 9/20/11. From this and other studies, four distinct MAb reactive epitopes were identified on cytochrome P-450c, split into two groups, each containing two overlapping epitopes. One of the epitope groups is also present on cytochrome P-450d.

MAb 3/4/2 was also found to cause a concentration dependent perturbation in the absolute spectrum of rat liver cytochrome P-450c. This effect was similar to that seen with a Type II compound, such as aniline. Aniline did not compete with MAb 3/4/2 for binding to cytochrome P-450, and in fact, the difference spectra produced by each was additive. MAb 3/4/2 did not, however, have any effect on the ability of rat liver cytochrome P-450c to produce a reduced carbon monoxide binding spectrum.

The reactivity of MAb 1/4 appeared to be specific for cytochrome P-450 forms b and/or e. It was not possible to differentiate between these two isozymes by the techniques used in this thesis due to their similarity in molecular weight.

In conclusion, MAb 12/2/3/2 recognised two proteins in β NF treated rat liver corresponding to cytochrome P-450 forms c and d. MAb 9/20/11 also reacted with these two proteins, at the same, or very similar site on the molecule. MAb 3/4/2 also reacts with cytochrome P-450c, but at a different site from the other two MAb's. The MAb 3/4/2 epitope also occurs on rabbit cytochrome P-450 forms LM4 and LM6, a human form of cytochrome P-450, as well as a possibly unique form of cytochrome P-450 induced by treatment with PB, which was not forms b or e. In contrast, MAb 1/4 reacted only with rat liver cytochrome P-450 forms b and/or e.

On the basis of their reactivities, MAb's 3/4/2, 12/2/3/2 and 1/4 were selected for use in a variety of applications, together with the control MAb B3A3. The results of these studies are summarised below.

8.2.2

Inhibition of Cytochrome P-450 Mediated Monooxygenase Activities

MAb 12/2/3/2 produced a concentration dependent inhibition of EROD activity, both in a reconstituted rat liver cytochrome P-450c system, and in an assay system comprised of liver microsomes from β NF treated rats. It also inhibited B[a]P hydroxylase activity in a similar microsomal assay, but did not inhibit this activity when liver microsomes from control rats were used. MAb 3/4/2 had no significant effect on EROD activity in either system, nor did it inhibit control or β NF treated rat liver microsomal B[a]P hydroxylase activities. In addition, neither of these two MAb's were inhibitory toward aldrin epoxidase activity of liver microsomal fractions from either control or PB treated rats. MAb 1/4 was also found to have no inhibitory effect on aldrin epoxidase activity, nor indeed of B[a]P hydroxylase activity, using microsomal fractions from PB treated rats. The control MAb B3A3 had no significant effect on any monooxygenase

activity studied, either in a reconstituted system or with any of the microsomal preparations used.

Thus, of the MAb's tested for their inhibitory properties, only MAb 12/2/3/2 had any significant effect, but only for activities catalysed by those forms of cytochrome P-450 recognised by this MAb, namely forms c and/or d. This effect was apparent both in reconstituted and microsomal assay systems. However, the degree of inhibition was different, being more marked in the reconstituted system. This may well reflect a contribution to BROD activity from forms of cytochrome P-450 other than forms c and/or d present in the microsomal fraction. This MAb should prove useful in determining the contribution of rat cytochrome P-450 forms c and d to the overall microsomal metabolism of a given compound.

8.2.3

Immunopurification of Cytochrome P-450 Isozymes

Several experimental systems were employed during this project to immunopurify isozymes of cytochrome P-450. Initial studies concentrated on the use of MAb 3/4/2, due to its wide cytochrome P-450 specificity. This reactivity could have made MAb 3/4/2 extremely useful for purifying isozymes from several species for further structural and catalytic analysis. Binding and elution studies indicated that this MAb would be suitable for inclusion in immunopurification protocols due to its capability of binding cytochrome P-450 in the presence of commonly used detergents, and also by allowing elution under relatively mild conditions. Using pA-Seph as a solid matrix, MAb 3/4/2 was able to remove, and allow the specific elution of haem containing material from partially purified cytochrome P-450 labelled with ^{125}I . Reconstituted B1a1P hydroxylase activity could also be observed when polystyrene beads were used as a solid matrix with this MAb. A reduced carbon monoxide difference spectrum was additionally obtained

when this MAb was coupled to activated Sepharose as a solid matrix. Unfortunately, no eluted fractions could be resolved on SDS-PAGE analysis, an indication, perhaps, of the amount of cytochrome P-450 able to be bound by this MAb.

Studies with MAb 1/4 were more successful. This MAb, when coupled to activated Sepharose, was capable of removing cytochrome P-450 from solubilised microsomes, as determined by the reduced carbon monoxide difference spectra recorded by the gel matrix. Treatment of this matrix with SDS, and analysis by SDS-PAGE, revealed that a 54,000 molecular weight protein had been specifically eluted. The molecular weight of this band corresponded to that of the major forms of cytochrome P-450 inducible by treatment with PB, namely forms b and e.

Investigation of the catalytic activity of the isozyme(s) eluted from the MAb 1/4-SepH matrix was, unfortunately, not possible due to the denaturing effect of the eluting agent used. Further studies with this MAb may identify more suitable eluting agents. Neither were any structural analysis studies performed on the eluted protein. Whilst the protein may be of the expected molecular weight of cytochrome P-450 forms b and/or e, further studies may be required to fully determine its identity.

8.2.4

Immunoquantitation of Microsomal Cytochrome P-450 Isozymes

One application that is well suited to MAb's is in the quantitation of cytochrome P-450 isozymes in different microsomal preparations. This was attempted with several MAb's during the course of this project. Initial attempts were of limited success, however, due to difficulties in labelling the MAb's concerned. Initial studies used MAb 3/4/2 labelled with ^{125}I . It was apparent that although incorporation of ^{125}I into the protein was possible, this resulted in a substantial reduction in the cytochrome P-450

binding capacity of the MAb. In addition, it appeared that a considerable proportion of the MAb, though still able to bind to cytochrome P-450, was unlabelled. As a consequence, iodination was abandoned as a labelling technique and alternatives sought. Although it was possible to internally label MAb 12/2/3/2 by growing the hybridoma cells in culture medium supplemented with ^{14}C -leucine, the resultant preparation had a relatively low specific activity, and was found to be unsuitable for use in a quantitative assay.

One approach that was successful, and which did not require labelling of either MAb or antigen, utilised a competitive solid phase ELISA. Microtitre plates were coated with a fixed amount of microsomal protein from the livers of β NF treated rats. MAb's were incubated with the test antigen, as a microsomal preparation, and then the amount of unreacted MAb was determined by a normal ELISA assay using the precoated plates. Thus, the more immunoreactive antigen present in the test preparation, the lower the readings obtained in the ELISA assay. Initial titration curves were prepared by incubating MAb's with varying amounts of a homologous microsomal antigen preparation.

Of the MAb's studied in this assay, only MAb 12/2/3/2 was found to be suitable, with both MAb's 1/4 and 3/4/2 being unable to produce satisfactory antigen titration curves. Such results are probably due to the reliance of the assay protocol on MAb affinity. A quantitative assay was performed with MAb 12/2/3/2 using a purified preparation of cytochrome P-450c as a standard. The results obtained with indicated that the specific content of cytochrome P-450 reacting with this MAb (namely forms c and d) was 1.193 nmols/mg protein in liver microsomal fractions from β NF treated rats. The specific content of these forms in liver microsomes from 3MC treated rats was 1.333 nmols/mg protein, which is consistent with previously reported observations that 3MC is a better inducer of these forms of cytochrome

P-450 than β NF. Weaker, but still significant, reactivity was obtained with liver microsomes from control and PB treated rats. In the former preparation, the amount of immunoreactive cytochrome P-450 was 0.047 nmols/mg protein, whilst in the latter, the figure was only 0.029 nmols/mg protein. This also agrees with previous observations that the specific content of hydrocarbon inducible forms of cytochrome P-450 increases 20 to 100 fold after such treatment, whilst PB treatment suppresses the expression of these isozymes. A positive reaction was also obtained with liver microsomes from 3MC treated AKR mice, with a specific content estimated at 0.210 nmols/mg protein. This was in contrast to the specific content determined for control AKR mice which was 0.062 nmols/mg protein. The differences in specific content of immunoreactive cytochrome P-450 observed between rat and mouse might well be due to differences in the epitope recognised by MAb 12/2/3/2. Alternatively, whilst MAb 12/2/3/2 reacts with two forms in the rat, it may be that only one of these forms is recognised in the mouse. This would need to be investigated, however, by further Western blotting studies. MAb 12/2/3/2 only reacted with one of the human liver microsomal preparations included in this assay. However, the results obtained for this preparation, and from control guinea pig, were at the limit of sensitivity for this assay. None the less, it was apparent that some MAb 12/2/3/2 immunoreactive isozymes were present in those microsomal fractions. No figure could be obtained for the specific content of immunoreactive isozymes from control pig or 3MC treated rabbits.

The results obtained with MAb 12/2/3/2 in this assay indicate the potential of such an application and assay method. Further studies are obviously required, however, in order to consolidate these results, both with other microsomal preparations and, if possible, with other MAb's.

8.2.5

Immunocytochemical Localisation of Cytochrome P-450 Isozymes

MAB's 3/4/2 and 12/2/3/2 were used to localise cytochrome P-450 isozymes in cryostat sections of liver and kidney from control, β NF and PB treated rats. MAB 3/4/2 reactivity with control rat liver was evenly distributed across the liver lobule. Treatment with β NF caused a preferential increase in staining in the periportal region, and staining was observed at a higher MAB dilution. In contrast, treatment with PB caused an increase in staining in the centrilobular region of the liver, though a lower dilution of MAB was required to visualise reactivity. In the kidney, MAB 3/4/2 reactivity was localised to the S_3 segment of the proximal tubule in untreated animals. Treatment with β NF caused a marked increase in staining intensity along the entire length of the proximal tubule (S_1 , S_2 and S_3 segments), again at a higher MAB dilution, whilst PB treatment had no effect on either the intensity or localisation of staining compared to control tissue. The reactivity pattern observed for MAB 12/2/3/2 was analogous to that observed for MAB 3/4/2, indicating that cytochrome P-450 forms c and d co-localise in these tissues, under the induction conditions used in this study. Such results highlight the applicability of MAB's as reagents for studying the localisation of cytochrome P-450 isozymes in both hepatic and extra-hepatic tissues.

8.3 Comparison of Monoclonal Antibody Results with those Possible with Desorbed Polyclonal Antisera

Having decided at the start of this project to proceed with the production of MAB's to isozymes of rat liver cytochrome P-450 rather than polyclonal antibodies, it is perhaps useful to review the results achieved for those MAB's selected for further study (that is MAB's 1/4, 3/4/2 and

12/2/3/2), and to contrast them with the results that could perhaps have been obtained with desorbed polyclonal antisera.

It is clear that the reactivity profiles obtained with those selected MAb's would not have been possible with desorbed polyclonal antisera. The specificity of MAb 12/2/3/2 for distinct epitopes shared by forms c and d, for example, would have been removed by desorption if it had been a polyclonal antiserum. Likewise, the wide ranging reactivity of MAb 3/4/2 would possibly have been attributed to poor desorption. The extensive desorption procedures that would have been required to prove the existence of such a wide cross-species epitope would have been costly, both in terms of reagents and time. The reactivity profile of the MAb's described here illustrate the power of MAb specificity, provided, of course, that extensive characterisation has been performed, such as that described in Chapter 3.

In the studies reported in Chapter 4 on the effect of antibodies on monooxygenase activity, those performed by other workers with polyclonal antibodies (both desorbed and non-purified) have all yielded inhibitory results. Clearly this is an area where the multi-site attachment of polyclonal antibodies is very successful. However, because of this multi-site attachment, there is no single mode of inhibition with polyclonal antibodies. Although not performed in this thesis, the mode of inhibition achieved by inhibitory MAb's can be defined by competition assays performed with cytochrome P-450, MAb and other site specific ligands, such as peptides, NADPH cytochrome c reductase and substrate, to identify MAb attachment sites. Once identified, the location of these sites can be mapped onto other isozymes of cytochrome P-450 through non-inhibitory studies such as ELISA and Western blotting.

Studies on the immunopurification and immunoquantitation of cytochrome P-450 have been described for several desorbed polyclonal antisera in

Chapters 5 and 6 respectively, with results similar to those reported for MAb's 1/4 and 12/2/3/2. For these applications, desorbed polyclonal antisera are viable alternatives to MAb's. The main difference between them would possibly be in the actual assay method used in the studies.

In the field of immunocytochemistry, successful studies on cytochrome P-450 distribution have been reported with both desorbed polyclonal antisera and MAb's, as described in Chapter 7. Indeed, this is an area where polyclonal antibodies may have a wider applicability than MAb's. Although cryostat sections were used in this thesis, many laboratories have access to formalin fixed and paraffin embedded tissue blocks from clinical sources. The process of formalin fixation can be quite damaging to antibody reactive epitopes (Tubbs *et al.*, 1981). Thus, it is possible for a MAb to lose its reactivity with a particular tissue type following formalin fixation. The variability of this type of fixation can also make it difficult to compare MAb results between different tissue preparations. It may be, therefore, that in a clinical situation, or in a retrospective study on clinical material, the multi-site attachment of polyclonal antibodies may provide a more consistent and sensitive result than that possible with MAb's.

In summary, both desorbed polyclonal antibodies and MAb's are suitable reagents for the study of cytochrome P-450 isozymes. For the reasons given in Section 8.1.1 above, the former were not produced during the course of this project. However, the use of MAb's in the studies reported in this thesis has not compromised on the results that could have been obtained if desorbed polyclonal antibodies had been used.

8.4 A Future Strategy for Generating Monoclonal Antibodies Against Isozymes of Cytochrome P-450

During the course of this project, several MAb's have been applied in a number of different areas. It soon became apparent that the properties required of these MAb's varied with the application studied. It would therefore be advantageous to select MAb's with the desired properties for their intended applications as soon as possible after fusion by the use of specifically designed screening assays. Thus, MAb's could be screened for specific cross-reactivities, against, for example, human liver microsomal fractions, in ELISA assays at a very early stage. MAb's directed against the active site of cytochrome P-450 could be screened for by comparing their cytochrome P-450 reactivity in the presence and absence of a specific substrate. MAb's for use in immunopurification studies could be selected by screening for those that bind to cytochrome P-450 in the presence of those detergents required in the purification procedure. In addition, MAb's which can be dissociated from cytochrome P-450 under relatively mild conditions could be identified by screening in the presence and absence of such eluting agents. MAb's directed against different epitopes on the same form of cytochrome P-450 could be selected in a competitive solid phase assay. Western blotting studies should also enable the molecular weights of the cytochrome P-450 forms recognised by MAb's to be determined.

The application of these and similar approaches should enable MAb's of defined specificity and properties to be obtained such that they are suitable for any specified application.

CHAPTER 9

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