

**AN INVESTIGATION INTO THE
NEUROPROTECTIVE PROPERTIES OF THE
NON-STEROIDAL ANTI-INFLAMMATORY
AGENTS TOLMETIN, SULINDAC AND
TURMERIC**

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ABSTRACT

Accumulating evidence suggests that anti-inflammatory agents and antioxidants have neuroprotective properties and may be beneficial in the treatment of neurodegenerative disorders. In the present study, the possible neuroprotective properties of tolmetin, sulindac and turmeric were investigated.

The antioxidant effects of tolmetin and sulindac were determined by inducing free radical generation with quinolinic acid (QA), cyanide or iron (II) in rat brain homogenates or primary hippocampal neurons. Tolmetin and sulindac significantly reduce lipid peroxidation and scavenge the superoxide anion. Metal binding studies were conducted to determine whether metal chelation is a possible mechanism through which these agents reduce QA and iron (II)-induced lipid peroxidation. UV/VIS, infrared spectroscopy as well as electrochemical studies show that both agents bind to iron (II) and/or iron (III).

Histological examination of the hippocampus showed that pre-treatment of animals with tolmetin or sulindac offers protection against intrahippocampal injections of QA. These agents also attenuate QA-induced apoptosis and reduce the loss of neurons in the hippocampus. The co-incubation of primary hippocampal neurons with the NSAIDS also enhanced cell viability which is significantly reduced by QA. Behavioural studies using a water maze showed that the treatment of animals after QA-induced neurotoxicity reduces QA-induced spatial memory loss. Tolmetin and sulindac also reduced glutathione depletion and protein oxidation in rat hippocampus.

Both NSAIDS inhibit liver tryptophan 2,3-dioxygenase activity *in vitro* and *in vivo* and subsequently increased hippocampal serotonin levels. However, both NSAIDS also reduce dopamine levels in rat striatum. Tolmetin but not sulindac increased the synthesis of melatonin by the pineal gland.

The active components of turmeric known as the curcuminoids were separated using preparative thin layer chromatography (TLC). The purity was confirmed by TLC, NMR and mass spectrometry. The environmental toxin lead, induces lipid peroxidation and reduces primary hippocampal neuronal viability. The co-incubation of the neurons with the curcuminoids significantly reduces lead-induced lipid peroxidation and enhances neuronal cell viability in the presence of lead. Lead-induced spatial memory deficit is also attenuated with curcumin, demethoxycurcumin but not bisdemethoxycurcumin. The curcuminoids also reduce lead-induced hippocampal glutathione depletion and protein oxidation. Metal binding studies show that the curcuminoids bind to lead and is another possible mechanism through which the curcuminoids reduce lead-induced neurotoxicity.

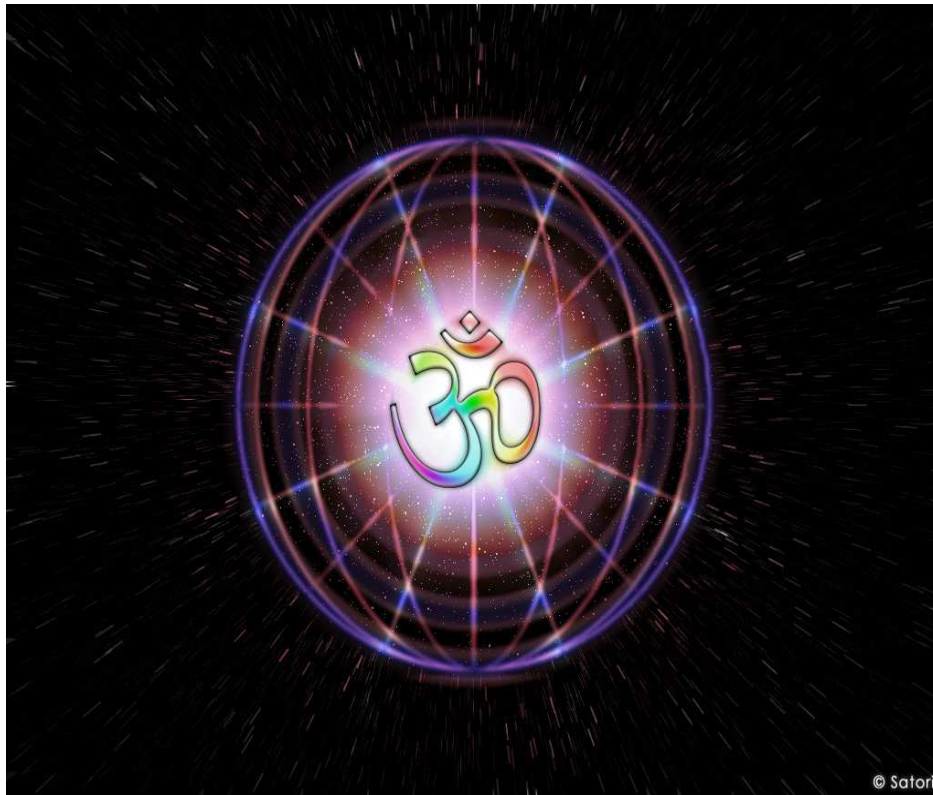
The findings of this study indicate a possible role of tolmetin, sulindac and turmeric in neurodegenerative disorders such as Alzheimer's disease. However, tolmetin and sulindac reduce dopamine levels.

PUBLICATIONS

The article in press is a result of this work.

Amichand Dairam, Prakash Chetty and Santy Daya. Non-steroidal anti-inflammatory agents, tolmetin and sulindac, attenuate oxidative stress in rat brain homogenate and reduce quinolinic acid-induced neurodegeneration in rat hippocampal neurons. *Metabolic Brain Disease*. **In Press**.

Dedicated to my parents and sisters whom I have missed in the past 8 years while studying at Rhodes University.



All scientific work is incomplete-whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us as a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time.

(Sir A. B. Hill. 1965)

TABLE OF CONTENTS

<i>Title Page</i>	i
<i>Abstract</i>	ii
<i>Publications</i>	iv
<i>Dedication</i>	v
<i>Table of Contents</i>	vi
<i>List of Figures</i>	xxv
<i>List of Tables</i>	xxxv
<i>List of Abbreviations</i>	xxxvi
<i>Acknowledgements</i>	xl

CHAPTER ONE LITERATURE REVIEW

1.1	Why the Brain?	1
1.2	The Brain	1
1.2.1	Neuroanatomy	1
1.2.1.1	The Cerebrum	2
1.2.1.2	The Cerebellum	2
1.2.1.3	The Brain Stem	3
1.2.1.4	The Neuron	3
1.2.2	The Nervous System	3
1.2.2.1	The Limbic System	4
1.2.2.1.1	The Hippocampus	4
1.2.2.2	The Autonomic Nervous System	6
1.2.3	The Striatum	6
1.2.3.1	The Substantia Nigra	7
1.2.4	Excitatory Amino Acids	8
1.2.4.1	The NMDA receptor	8
1.2.4.2	The NMDA receptor and Long Term Potentiation	9
1.2.4.3	The NMDA receptor and Neurotoxicity	10

1.3	Mechanisms of Neurodegeneration	11
1.3.1	Generation of Free Radicals	11
1.3.2	Oxidative Stress in the Brain	11
1.3.2.1	Quinolinic Acid	12
1.3.2.2	Cyanide	12
1.3.2.3	Iron	13
1.3.2.4	Lead	13
1.3.3	Neuroinflammation	15
1.4	Measurements of products of Oxidative Stress	15
1.5	Neurodegenerative Disorders	16
1.5.1	Alzheimer's Disease	16
1.5.1.1	Introduction	16
1.5.1.2	Neuropathology of Alzheimer's Disease	17
1.5.1.3	Causative Factors of Alzheimer's Disease	18
1.5.1.3.1	Genetic Component	18
1.5.1.3.2	Free Radicals and Oxidative Stress	18
1.5.1.3.3	Neuroinflammation in Alzheimer's Disease	18
1.5.1.4	Therapeutic Approaches to Alzheimer's Disease	19
1.5.1.4.1	Acetylcholine Esterase Inhibitors	19
1.5.1.4.2	The role of Non-Steroidal Anti-Inflammatory Agents in Alzheimer's Disease	20
1.5.1.4.3	Antioxidants	21
1.5.2	Parkinson's Disease	21
1.5.2.1	Introduction and Clinical Characteristics	21
1.5.2.2	Causative Factors of Parkinson's Disease	22
1.5.2.2.1	Mitochondrial Dysfunction	23
1.5.2.2.2	Genetic Component	23
1.5.2.2.3	Oxidative Stress	23
1.5.2.2.4	Neuroinflammation in Parkinson's Disease	24

	1.5.2.3	Therapeutic Approaches to Parkinson's Disease	24
	1.5.2.3.1	Enhancing Dopaminergic Transmission: Levodopa	24
	1.5.2.3.2	The role of Non-Steroidal Anti-Inflammatory Agents in Parkinson's Disease	25
1.6		Mechanisms of Neuroprotection	26
	1.6.1	Antioxidant Enzymes	26
	1.6.2	Antioxidants	26
	1.6.2.1	Melatonin	27
	1.6.3	Anti-Inflammatory Drugs	28
	1.6.3.1	Tolmetin	29
	1.6.3.2	Sulindac	30
	1.6.3.3	Tumeric	31
	1.6.3.3.1	Curcumin	31
	1.6.4	Chelating Agents	33
1.7		Objectives of This Study	34

CHAPTER TWO LIPID PEROXIDATION

2.1		INTRODUCTION	36
2.2		EFFECT OF QUINOLINIC ACID ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE	39
	2.2.1	Introduction	39
	2.2.2	Materials and Methods	39
	2.2.2.1	Chemicals and Reagents	39
	2.2.2.2	Animals	39
	2.2.2.3	Brain Removal	40
	2.2.2.4	Homogenate Preparation	40
	2.2.2.5	Preparation of Standard Curve	40

	2.2.2.6	Lipid Peroxidation Assay	40
	2.2.2.7	Statistical Analysis	41
2.2.3		Results	41
2.2.4		Discussion	42
2.3		EFFECT OF TOLMETIN AND SULINDAC ON QUINOLINIC ACID INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE	44
2.3.1		Introduction	44
2.3.2		Materials and Methods	44
2.3.3		Results	44
2.3.4		Discussion	45
2.4		EFFECT OF QUINOLINIC ACID ON LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS	47
2.4.1		Introduction	47
2.4.2		Materials and Methods	47
	2.4.2.1	Chemicals and Reagents	47
	2.4.2.2	Routine Cell Culture Procedures	47
	2.4.2.3	Preparation of Culture Reagents	48
	2.4.2.4	Animals	48
	2.4.2.5	Establishment of Primary Hippocampal Neurons	48
	2.4.2.6	Lipid Peroxidation Assay	49
2.4.3		Results	50
2.4.4		Discussion	50
2.5		EFFECT OF TOLMETIN AND SULINDAC ON QUINOLINIC ACID INDUCED LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS	51
2.5.1		Introduction	51
2.5.2		Materials and Methods	51
2.5.3		Results	51

2.5.4	Discussion	52
2.6	EFFECT OF CYANIDE ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE	54
2.6.1	Introduction	54
2.6.2	Materials and Methods	54
2.6.2.1	Chemicals and Reagents	54
2.6.3	Results	55
2.6.4	Discussion	55
2.7	EFFECT OF TOLMETIN AND SULINDAC ON KCN INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE	57
2.7.1	Introduction	57
2.7.2	Materials and Methods	57
2.7.3	Results	57
2.7.4	Discussion	58
2.8	EFFECT OF THE TRANSITION METAL, IRON (Fe^{2+}) ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE	59
2.8.1	Introduction	59
2.8.2	Materials and Methods	59
2.8.2.1	Chemicals and Reagents	59
2.8.2.2	Lipid Peroxidation Assay	60
2.8.3	Results	60
2.8.4	Discussion	61
2.9	EFFECT OF TOLMETIN AND SULINDAC ON Fe^{2+} INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE	62
2.9.1	Introduction	62
2.9.2	Materials and Methods	62
2.9.3	Results	62

2.9.4	Discussion	63
 CHAPTER THREE SUPEROXIDE ANION ASSAYS		
3.1	INTRODUCTION	65
3.2	EFFECT OF QUINOLINIC ACID ON SUPEROXIDE ANION GENERATION IN RAT BRAIN HOMOGENATE	67
3.2.1	Introduction	67
3.2.2	Materials and Methods	67
3.2.2.1	Chemicals and Reagents	67
3.2.2.2	Preparation of Standard Curve	68
3.2.2.3	Homogenate Preparation	68
3.2.2.4	The Nitroblue Tetrazolium Assay	68
3.2.3	Results	69
3.2.4	Discussion	69
3.3	EFFECT OF QUINOLINIC ACID ON SUPEROXIDE ANION GENERATION IN PRIMARY HIPPOCAMPAL NEURONS	70
3.3.1	Introduction	70
3.3.2	Materials and Methods	70
3.3.2.1	The Nitroblue Tetrazolium Assay	70
3.3.3	Results	71
3.3.4	Discussion	72
3.4	EFFECT OF TOLMETIN AND SULINDAC ON QUINOLINIC ACID INDUCED SUPEROXIDE ANION GENERATION IN PRIMARY HIPPOCAMPAL NEURONS	73
3.4.1	Introduction	73
3.4.2	Materials and Methods	73
3.4.3	Results	73

3.4.4	Discussion	74
3.5	EFFECT OF KCN ON SUPEROXIDE ANION GENERATION IN RAT BRAIN HOMOGENATE	76
3.5.1	Introduction	76
3.5.2	Materials and Methods	76
3.5.3	Results	76
3.5.4	Discussion	77
3.6	EFFECT OF TOLMETIN AND SULINDAC ON KCN INDUCED SUPEROXIDE ANION GENERATION IN RAT BRAIN HOMOGENATE	78
3.6.1	Introduction	78
3.6.2	Materials and Methods	78
3.6.3	Results	78
3.6.4	Discussion	79
3.7	SUPEROXIDE ANION INDUCED NADPH OXIDATION: EFFECT OF TOLMETIN AND SULINDAC	81
3.7.1	Introduction	81
3.7.2	Materials and Methods	82
3.7.2.1	Chemicals and Reagents	82
3.7.2.2	Extraction of the enzyme, Superoxide Dismutase	82
3.7.2.3	Conditions for Assay	82
3.7.3	Results	83
3.7.4	Discussion	85

CHAPTER FOUR EFFECT OF TOLMETIN, SULINDAC AND QUINOLINIC ACID ON HIPPOCAMPAL NEURONS

4.1	INTRODUCTION	87

4.2	HISTOLOGICAL INVESTIGATION	89
4.2.1	Introduction	89
4.2.2	Materials and Methods	
4.2.2.1	Chemicals and Reagents	90
4.2.2.2	Animals	90
4.2.2.3	Treatment Regimes	90
4.2.2.4	Surgical Procedures	91
4.2.2.5	Brain Removal	92
4.2.2.6	Histological Techniques	92
4.2.3	Results	92
4.2.4	Discussion	94
4.3	APOPTOSIS	95
4.3.1	Introduction	95
4.3.2	Materials and Methods	96
4.3.2.1	Chemicals and Reagents	96
4.3.2.2	Animals	96
4.3.2.3	Treatment Regimes, surgical procedures and brain removal	96
4.3.2.4	Preparing the brain specimen for TUNEL staining	96
4.3.2.5	Specificity of the TUNEL stain	96
4.3.2.6	The TUNEL staining procedure	96
4.3.2.6.1	Pre-treatment of paraffin embedded tissue	97
4.3.2.6.2	Labelling	97
4.3.2.6.3	Photo-microscopy	98
4.3.3	Results	98
4.3.4	Discussion	99

4.4	EFFECT OF TOLMETIN AND SULINDAC ON INTRAHIPPOCAMPAL ADMINISTRATION OF QUINOLINIC ACID ON ³ H-MK801 RECEPTOR BINDING	102
4.4.1	Introduction	102
4.4.2	Materials and Methods	103
4.4.2.1	Chemicals and Reagents	103
4.4.2.2	Animals and Surgical Procedures	103
4.4.2.3	Preparation of Synaptic Membranes	103
4.4.2.4	Protein Assay	104
4.4.2.5	NMDA Binding Assay Procedure	104
4.4.3	Results	105
4.4.4	Discussion	109
4.5	EFFECT OF QUINOLINIC ACID ON NEURONAL CELL VIABILITY	111
4.5.1	Introduction	111
4.5.2	Materials and Methods	111
4.5.2.1	The MTT Assay	111
4.5.3	Results	112
4.5.4	Discussion	113
4.6	EFFECT OF TOLMETIN AND SULINDAC ON NEURONAL CELL VIABILITY IN THE PRESENCE OF QUINOLINIC ACID	115
4.6.1	Introduction	115
4.6.2	Materials and Methods	115
4.6.3	Results	115
4.6.4	Discussion	116

CHAPTER FIVE BEHAVIOURAL STUDIES

5.1	INTRODUCTION	118

5.2	EFFECT OF QUINOLINIC ACID, TOLMETIN AND SULINDAC ON RAT SPATIAL REFERENCE MEMORY	119
5.2.1	Introduction	120
5.2.2	Materials and Methods	120
5.2.2.1	Chemicals and Reagents	120
5.2.2.2	Animals	120
5.2.2.3	Animal Training: water maze task	120
5.2.2.4	Surgical Procedures	120
5.2.2.5	Performance in the water maze (test trials)	121
5.2.2.6	Statistical Analysis	121
5.2.3	Results	122
5.2.4	Discussion	123

CHAPTER SIX GLUTATHIONE CONTENT

6.1	INTRODUCTION	126
6.2	EFFECT OF QUINOLINIC ACID, TOLMETIN AND SULINDAC ON RAT HIPPOCAMPAL GLUTATHIONE CONTENT	129
6.2.1	Introduction	129
6.2.2	Materials and Methods	129
6.2.2.1	Chemicals and Reagents	129
6.2.2.2	Animals	129
6.2.2.3	Surgical Procedures and Treatment Regime	129
6.2.2.4	Preparation of Standard Curve	130
6.2.2.5	GSH Assay	130
6.2.2.6	Statistical Analysis	130
6.2.3	Results	131
6.2.4	Discussion	132

CHAPTER SEVEN PROTEIN OXIDATION

7.1	INTRODUCTION	133
7.2	EFFECT OF QUINOLINIC ACID, TOLMETIN AND SULINDAC ON RAT HIPPOCAMPAL PROTEINS	136
7.2.1	Introduction	136
7.2.2	Materials and Methods	136
7.2.2.1	Chemicals and Reagents	136
7.2.2.2	Animals	137
7.2.2.3	Surgical Procedures and Treatment Regimens	137
7.2.2.4	Protein Carbonyl Measurement	137
7.2.2.5	Statistical Analysis	138
7.2.3	Results	138
7.2.4	Discussion	139

CHAPTER EIGHT LIVER TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY

8.1	INTRODUCTION	140
8.2	<i>IN VITRO</i> INVESTIGATION OF THE EFFECT OF TOLMETIN AND SULINDAC ON RAT LIVER TDO ACTIVITY	143
8.2.1	Introduction	143
8.2.2	Materials and Methods	143
8.2.2.1	Chemicals and Reagents	143
8.2.2.2	Animals	144
8.2.2.3	Tissue Preparation	144
8.2.2.4	Tryptophan 2,3-dioxygenase Activity	144
8.2.2.5	Statistical Analysis	145
8.2.3	Results	145
8.2.4	Discussion	146

8.3	<i>IN VIVO</i> INVESTIGATION OF THE EFFECT OF TOLMETIN AND SULINDAC ON LIVER TDO ACTIVITY	147
8.3.1	Introduction	147
8.3.2	Materials and Methods	147
8.3.2.1	Treatment Regimes	147
8.3.2.2	Tissue Preparation	148
8.3.2.3	Tryptophan 2,3-dioxygenase Activity	148
8.3.3	Results	148
8.3.4	Discussion	149

CHAPTER NINE EFFECT OF TOLMETIN AND SULINDAC ON BRAIN BIOGENIC AMINES

9.1	INTRODUCTON	150
9.2	EFFECT OF TOLMETIN AND SULINDAC ON BRAIN SEROTONIN AND OTHER NEUROTRANSMITTER LEVELS	150
9.2.1	Introduction	150
9.2.2	Materials and Methods	153
9.2.2.1	Chemicals and Reagents	153
9.2.2.2	Treatment Regimes	153
9.2.2.3	Tissue Preparations	153
9.2.2.4	Instrumentation	154
9.2.2.5	Chromatographic Conditions	154
9.2.3	Results	154
9.2.4	Discussion	156
9.3	EFFECT OF TOLMETIN AND SULINDAC ON THE PRODUCTION OF MELATONIN BY THE PINEAL GLAND	158
9.3.1	Introduction	158

9.3.2	Materials and Methods	158
9.3.2.1	Chemicals and Reagents	158
9.3.2.2	Treatment Regimes	158
9.3.2.3	Instrumentation	159
9.3.2.4	Chromatographic Conditions	159
9.3.2.5	Method Validation	159
9.3.2.6	Extraction Efficiency	159
9.3.2.7	Mass Spectrometry (MS)	160
9.3.2.8	Amount of melatonin produced by the pineal gland	160
9.3.3	Results	160
9.3.4	Discussion	163

CHAPTER TEN METAL BINDING STUDIES: THE INTERACTION BETWEEN TOLMETIN, SULINDAC AND IRON

10.1	INTRODUCTION	165
10.2	UV/VIS SPECTROSCOPY	166
10.2.1	Introduction	166
10.2.2	Materials and Methods	166
10.2.2.1	Chemicals and Reagents	166
10.2.2.2	Technique: UV/VIS Spectroscopy	166
10.2.3	Results	167
10.2.4	Discussion	170
10.3	INFRARED SPECTROSCOPY	171
10.3.1	Introduction	171
10.3.2	Materials and Methods	171
10.3.2.1	Chemicals and Reagents	171
10.3.2.2	Technique: Infrared Spectroscopy	171
10.3.3	Results	172

10.3.4	Discussion	174
10.4	ELECTROCHEMICAL ANALYSIS	177
10.4.1	Introduction	177
10.4.1.1	Adsorptive Stripping Voltammetry	178
10.4.2	Materials and Methods	178
10.4.2.1	Apparatus	178
10.4.2.2	Technique: Adsorptive Stripping Voltammetry	179
10.4.3	Results	179
10.4.4	Discussion	183

CHAPTER ELEVEN THE SEPARATION, ISOLATION AND PURIFICATION OF THE CURCUMINOIDS

11.1	Introduction	184
11.2	Materials and Methods	184
11.2.1	Chemicals and Reagents	184
11.2.2	Separation of the Curcuminoids	185
11.2.3	Isolation of the curcuminoids	185
11.2.4	Determination of the purity of the curcuminoids (TLC)	185
11.2.5	Determination of the purity of the curcuminoids (NMR)	186
11.2.6	Determination of the purity of the curcuminoids (MS)	186
11.3	Results	186
11.4	Discussion	190

CHAPTER TWELVE EFFECT OF CURCUMINOIDS ON LEAD INDUCED NEUROTOXICITY

12.1	INTRODUCTION	192

12.2	EFFECT OF LEAD ON LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS	193
12.2.1	Introduction	193
12.2.2	Materials and Methods	193
12.2.2.1	Chemicals and Reagents	193
12.2.2.2	Lipid Peroxidation Assay	193
12.2.3	Results	194
12.2.4	Discussion	194
12.3	EFFECT OF CURCUMINOIDS ON LEAD INDUCED LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS	196
12.3.1	Introduction	196
12.3.2	Chemicals and Reagents	196
12.3.2.1	Lipid Peroxidation Assay	196
12.3.3	Results	196
12.3.4	Discussion	197
12.4	EFFECT OF LEAD ON NEURONAL CELL VIABILITY	200
12.4.1	Introduction	200
12.4.2	Materials and Methods	200
12.4.2.1	The MTT Assay	200
12.4.3	Results	200
12.4.4	Discussion	201
12.5	EFFECT OF CURCUMINOIDS ON NEURONAL CELL VIABILITY IN THE PRESENCE OF LEAD	203
12.5.1	Introduction	203
12.5.2	Materials and Methods	203
12.5.2.1	The MTT Assay	203
12.5.3	Results	204
12.5.4	Discussion	204

12.6	EFFECT OF LEAD AND THE CURCUMINODS ON RAT SPATIAL REFERENCE MEMORY	206
12.6.1	Introduction	206
12.6.2	Materials and Methods	206
12.6.2.1	Chemicals and Reagents	206
12.6.2.2	Animals	206
12.6.2.3	Animal Training: water maze task	207
12.6.2.4	Treatment Regimes	207
12.6.2.5	Performance in the water maze (test trials)	207
12.6.2.6	Statistical Analysis	207
12.6.3	Results	207
12.6.4	Discussion	208
12.7	EFFECT OF LEAD AND CURCUMINOID TREATMENT ON RAT HIPPOCAMPAL TOTAL GLUTATHIONE CONTENT	211
12.7.1	Introduction	211
12.7.2	Materials and Methods	211
12.7.2.1	Chemicals and Reagents	211
12.7.2.2	Animals	211
12.7.2.3	Treatment Regimes	211
12.7.2.4	GSH Assay	212
12.7.2.5	Statistical Analysis	212
12.7.3	Results	212
12.7.4	Discussion	213
12.8	EFFECT OF LEAD AND CURCUMINOID TREATMENT ON RAT HIPPOCAMPAL PROTEINS	215
12.8.1	Introduction	215
12.8.2	Materials and Methods	215
12.8.2.1	Chemicals and Methods	215
12.8.2.2	Animals	215

12.8.2.3	Treatment Regimes	215
12.8.2.4	Protein Carbonyl Measurement	216
12.8.2.5	Statistical Analysis	216
12.8.3	Results	216
12.8.4	Discussion	217
CHAPTER THIRTEEN	METAL BINDING STUDIES: THE INTERACTION BETWEEN THE CURCUMINOIDS AND LEAD	
13.1	INTRODUCTION	219
13.2	UV/VIS SPECTROSCOPY	221
13.2.1	Materials and Methods	221
13.2.1.1	Chemicals and Reagents	221
13.2.1.2	Technique: UV/VIS Spectroscopy	221
13.2.2	Results	221
13.2.3	Discussion	223
13.3	SPECTROFLUOROMETRY	225
13.3.1	Introduction	225
13.3.2	Materials and Methods	225
13.3.2.1	Chemicals and Reagents	225
13.3.2.2	Technique: Spectrofluorometry	225
13.3.3	Results	226
13.3.4	Discussion	227
13.4	INFRARED SPECTROSCOPY	229
13.4.1	Materials and Methods	229
13.4.1.1	Chemicals and Reagents	229
13.4.1.2	Technique: Infrared Spectroscopy	229
13.4.2	Results	229

13.4.3	Discussion	232
13.5	ELECTROCHEMICAL ANALYSIS	238
13.5.1	Materials and Methods	238
13.5.1.1	Chemicals and Reagents	238
13.5.1.2	Technique: Adsorptive Stripping Voltammetry	238
13.5.2	Results	238
13.5.3	Discussion	241

CHAPTER FOURTEEN SUMMARY OF RESULTS, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

14.1	SUMMARY OF RESULTS	243
	Chapter 2	243
	Chapter 3	243
	Chapter 4	244
	Chapter 5	244
	Chapter 6	245
	Chapter 7	246
	Chapter 8	246
	Chapter 9	247
	Chapter 10	247
	Chapter 11	248
	Chapter 12	248
	Chapter 13	249
14.2	CONCLUSIONS	244
14.3	RECOMMENDATIONS FOR FUTURE STUDIES	251

APPENDICES	254

REFERENCES	264

LIST OF FIGURES

Figure 1.1: The main areas of the brain showing the frontal lobe, parietal lobe, occipital lobe and temporal lobe.

Figure 1.2: A typical neuron with the cell body, axon and dendrites.

Figure 1.3: Diagram of the various structures associated with the limbic system. Although the corpus callosum is not part of the limbic system, its location in the brain is also shown.

Figure 1.4: A Nissl-stained coronal section of the caudal telecephalon of a rat brain showing the major regions in the hippocampus.

Figure 1.5: The areas of the striatum situated within the brain.

Figure 1.6: Diagrammatic representation of the NMDA receptor and its subunits, showing magnesium blocking the narrow channel and preventing the influx of Ca^{2+} .

Figure 1.7: The neuronal model for long term potentiation.

Figure 1.8: Possible mechanisms for dopaminergic neurodegeneration in PD.

Figure 1.9: Illustration of the structure of tolmetin.

Figure 1.10: Structure of sulindac and its metabolites sulindac sulfide and sulindac sulfone.

Figure 1.11: Structure of the curcuminoids.

Figure 2.1: A schematic representation of the steps of lipid peroxidation: initiation, propagation and termination. The end product of lipid peroxidation is malondialdehyde.

Figure 2.2: Reaction between MDA and 2 molecules of TBA to produce a pink complex that can be extracted and measured at 532 nm (Mead et al., 1986).

Figure 2.3: Effect of Quinolinic acid (0-1mM) on lipid peroxidation in rat brain homogenate.

Figure 2.4: Effect of tolmetin and sulindac on QA (1mM)-induced lipid peroxidation in rat brain homogenate.

Figure 2.5: The effect of QA on lipid peroxidation in primary hippocampal neurons.

Figure 2.6: Effect of tolmetin and sulindac on 200 μ M QA-induced lipid peroxidation.

Figure 2.7: Effect of KCN (0-1mM) on lipid peroxidation in rat brain homogenate.

Figure 2.8: Effect of tolmetin on KCN (1mM)-induced lipid peroxidation in rat brain homogenate.

Figure 2.9: Effect of Fe^{2+} (1mM) on lipid peroxidation in rat brain homogenate.

Figure 2.10: Effect of tolmetin and sulindac on Fe^{2+} (1Mm)-induced lipid peroxidation in rat brain homogenate.

Figure 3.1: Effect of QA on superoxide anion generation in rat brain homogenate.

Figure 3.2: Effect of QA on superoxide anion generation in primary hippocampal neurons.

Figure 3.3: Effect of tolmetin and sulindac on QA (200 μ M)-induced superoxide anion generation in primary hippocampal neurons.

Figure 3.4: Effect of KCN on superoxide anion generation in rat brain homogenate.

Figure 3.5: Effect of tolmetin and sulindac on KCN (1mM)-induced superoxide anion generation in rat brain homogenate.

Figure 3.6: Comparison of the effect of NADPH oxidation with and without the presence of tolmetin (0.1mM), active and inactive SOD extract.

Figure 3.7: Comparison of the effect of NADPH oxidation with and without the presence of sulindac or tolmetin (0.1mM) and with sulindac and SOD together in the reaction medium.

Figure 3.8: Percentage of NADPH remaining at 30 minutes. Each bar represents a mean $\pm$ SD (n=5).

Figure 4.1: Basic structure of a neuron with its associated axons and dendrites.

Figure 4.2: Effects of tolmetin and sulindac on QA-induced hippocampal lesions.

Figure 4.3: Effects of tolmetin and sulindac on QA-induced apoptosis in rat hippocampus.

Figure 4.4: Saturation isotherm (Scatchard plot and K_d value inserted) of ³H-MK801 binding to hippocampal synaptic membranes from control rats (n = 3).

Figure 4.5: Saturation isotherm (Scatchard plot and K_d value inserted) of ³H-MK801 binding to hippocampal synaptic membranes from rats that received QA i.h. (no NSAID treatment (n = 3).

Figure 4.6: Saturation isotherm (Scatchard plot and K_d value inserted) of ³H-MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with tolmetin and also received hippocampal injections of QA (n = 3).

Figure 4.7: Saturation isotherm (Scatchard plot and K_d value inserted) of ³H-MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with tolmetin and received hippocampal injections free of QA (n = 3).

Figure 4.8: Saturation isotherm (Scatchard plot and K_d value inserted) of ³H-MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with sulindac and also received hippocampal injections of QA (n = 3).

Figure 4.9: Saturation isotherm (Scatchard plot and K_d value inserted) of ³H-MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with sulindac and received hippocampal injections free of QA (n = 3).

Figure 4.10: Effect of QA (50, 100 and 200μM) on primary hippocampal cell viability.

Figure 4.11: Effect of tolmetin and sulindac on QA (200μM)-reduced cell viability.

Figure 5.1: Performance of the rats in the water maze task.

Figure 6.1: Production of oxygen and nitrogen free radicals and other reactive species within a mammalian cell and the role played by superoxide dismutase, glutathione, vitamin C and vitamin E in counteracting the free radicals.

Figure 6.2: Effect of QA and tolmetin and sulindac treatment on rat hippocampal glutathione content.

Figure 7.1: Formation of protein carbonyls following free radical attack.

Figure 7.2: Effect of intrahippocampal injections of QA and NSAID treatment on rat hippocampal protein carbonyl levels.

Figure 8.1: Graphical representation of the tryptophan-niacin pathway showing the metabolites and enzymes of this pathway.

Figure 8.2: Effect of tolmetin (10 and 20mM) on rat tryptophan-2,3-dioxygenase activity.

Figure 8.3: Effect of sulindac (10 and 20mM) on rat tryptophan-2,3-dioxygenase activity.

Figure 8.4: Effect of tolmetin and sulindac (5mg/kg/bd for 5 days) on rat tryptophan-2,3-dioxygenase activity.

Figure 9.1: Pineal Indole Metabolism.

Figure 9.2: Effect of tolmetin and sulindac on rat hippocampal biogenic amine levels.

Figure 9.3: Effect of tolmetin and sulindac on the rat striatal biogenic amine levels.

Figure 9.4: Mass spectral data of melatonin standard (50 μ M).

Figure 9.5: Mass spectral data of melatonin extracted with chloroform from MEM in which the pineal gland was incubated.

Figure 9.6: Effect of tolmetin and sulindac treatment on melatonin synthesis in the rat pineal gland.

Figure 9.7: Liquid-phase extraction method of indoleamines from rat pineal gland.

Figure 10.1: Spectroscopic analysis of sulindac alone and in the presence of Fe^{2+} and Fe^{3+} . The final concentration of sulindac and the metals were 0.125mM each.

Figure 10.2: Spectroscopic time study of sulindac in the presence of Fe^{2+} (0.25mM each). The interaction was analysed over 5 hours.

Figure 10.3: Spectroscopic analysis of tolmetin alone and in the presence of Fe^{2+} and Fe^{3+} . The final concentration of tolmetin and the metals were 0.0625mM each.

Figure 10.4: Spectroscopic time study of tolmetin in the presence of Fe^{2+} (0.25mM each). The interaction was analysed over 5 hours.

Figure 10.5: Infrared spectra of sulindac alone and in the presence of Fe^{2+} .

Figure 10.6: Infrared spectra of sulindac alone and in the presence of Fe^{3+} .

Figure 10.7: Infrared spectra of tolmetin alone and in the presence of Fe^{2+} .

Figure 10.8: Infrared spectra of tolmetin alone and in the presence of Fe^{3+} .

Figure 10.9: Most dynamic electrochemistry utilizes the classic three-electrode system as shown above.

Figure 10.10: ASV of Fe^{3+} ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of sulindac.

Figure 10.11: ASV of Fe^{3+} ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of tolmetin.

Figure 10.12: Effect of increasing concentrations of tolmetin and sulindac on the potential of $1 \times 10^{-5}\text{M}$ Fe^{3+} .

Figure 10.13: Effect of increasing concentrations of tolmetin and sulindac on the current of $1 \times 10^{-5}\text{M}$ Fe^{3+} .

Figure 11.1: The 3 major curcuminoids within curcumin (65%).

Figure 11.2: TLC plate showing the purity of each curcuminoid that was separated, isolated and extracted from curcumin (65%).

Figure 11.3: Structure of curcumin

Figure 11.4: Structure of demethoxycurcumin

Figure 11.5: Structure of bisdemethoxycurcumin

Figure 12.1: Effect of lipid peroxidation in primary hippocampal neurons exposed to lead (5 and 10 μ M) over a period of time.

Figure 12.2: Effect of the curcuminoids on Pb (10 μ M)-induced lipid peroxidation in primary hippocampal neurons.

Figure 12.3: Donation of a hydrogen atom from the β -diketone moiety of curcumin to a bis-allylic radical.

Figure 12.4: Illustrates curcumin's antioxidant activity deep within the cell membrane

Figure 12.5: Primary hippocampal neuron viability after exposure to lead (5 and 10 μ M) over a period of time.

Figure 12.6: Effect of the curcuminoids on hippocampal neuron viability in the presence of Pb(10 μ M).

Figure 12.7: Performance of the rats in the water maze task. Each point shows the average time taken for 10 rats. Trials were conducted once daily, every day for 14 days.

Figure 12.8: Effect of Pb alone and Pb and curcuminoid treatment on rat hippocampal glutathione content. Hippocampi from animals were analysed after 5 days of treatment (day 1) or at the end of the behavioral studies (day 14).

Figure 12.9: Effect of Pb alone or Pb and curcuminoid treatment on rat hippocampal protein carbonyl levels. Hippocampi from animals were analysed after 5 days of treatment (day 1) or at the end of the behavioral studies (day 14).

Figure 13.1: UV/VIS spectra of curcumin (0.03mM) alone and in the presence of 0.1mM lead.

Figure 13.2: UV/VIS spectra of demethoxycurcumin (0.03mM) alone and in the presence of 0.1mM lead.

Figure 13.3: UV/VIS spectra of bisdemethoxycurcumin (0.03mM) alone and in the presence of 0.1mM lead.

Figure 13.4: The keto-enol tautomerism and quinoid-phenol tautomerism equilibria of curcumin.

Figure 13.5: Spectrofluorometric spectra of CURC (0.03mM) alone and in the presence of 0.1mM lead.

Figure 13.6: Spectrofluorometric spectra of DMC (0.03mM) alone and in the presence of 0.1mM lead.

Figure 13.7: Spectrofluorometric spectra of BDMC (0.03mM) alone and in the presence of 0.1mM lead.

Figure 13.8: Infrared spectra of CURC alone and in the presence of Pb.

Figure 13.9: Infrared spectra of DMC alone and in the presence of Pb.

Figure 13.10: Infrared spectra of BDMC alone and in the presence of Pb.

Figure 13.11: Possible metal coordination to the β -diketone.

Figure 13.12: Possible metal coordination to the quino-phenol system.

Figure 13.13: ASV of Pb ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of CURC.

Figure 13.14: ASV of Pb ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of DMC.

Figure 13.15: ASV of Pb ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of BDMC.

Figure 13.16: Effect of increasing concentrations of the curcuminoids on the potential of $1 \times 10^{-5}\text{M}$ Pb (corrected for baseline).

Figure 13.17: Effect of increasing concentrations of the curcuminoids on the current of $1 \times 10^{-5}\text{M}$ Pb (corrected for baseline).

LIST OF TABLES

- Table 4.1:** Treatment regimes for the histological studies.
- Table 4.2:** Comparison of Bmax and Kd values obtained from the NMDA receptor binding studies.
- Table 5.1:** Treatment regimes for the behavioural studies.
- Table 9.1:** Melatonin assay validation data.
- Table 9.2:** Melatonin extraction efficiency data.
- Table 13.1:** Infrared spectra of the curcuminoids between 1300 and 1100cm⁻¹.

LIST OF ABBREVIATIONS

·OH	hydroxyl radical
5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
ACN	acetonitrile
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AMPA	α -amino-3-hydroxy-5-methylisoazole-4-propionate
ANOVA	one-way analysis of the variance
AP	aqueous phase
Arg	arginine
ASV	adsorptive stripping voltammetry
ATP	adenosine triphosphate
AUFS	absorbance units full scale
A β	beta amyloid
bd	twice daily
BDMC	bisdemethoxycurcumin
BH ₄	(6R)-5,6,7,8,-tetrahydro-L-biopterin
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CA	cornu ammonis
Ca ²⁺	calcium
Carn	carnosine
Cat	catalase
Cd ²⁺	cadmium
Cit	citrulline
CNS	central nervous system
CO ₂	carbon dioxide
COMT	catechol-O-methyl transferase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
Cu ²⁺	copper
CURC	curcumin
CURC ³⁻	curcuminat ion
Cyt C	cytochrome C
DA	dopamine
DMC	demethoxycurcumin
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DNPH	2,4-dinitrophenylhydrazine
DNTB	5,5-dithiobis-(2-nitrobenzoic acid)
DOPAC	dihydroxyphenylacetaldehyde
e ⁻	electron
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation

FeCl ₃	ferric chloride
FeSO ₄	Iron sulphate
Ga ³⁺	gallium
GABA	gamma-aminobutyric acid
GCE	glassy carbon electrode
Glu	L-glutamate
Glu-CySH	glutamyl-cysteine
Gly	glycine
GSH	glutathione (reduced form)
GSH-Px	glutathione peroxidase
GSH-R	glutathione reductase
GSH-T	glutathione S-transferase
GSNO	nitrosylated glutathione
GS-SG	glutathione (oxidised form)
H ₂ O ₂	hydrogen peroxide
HbO ₂	oxyhemoglobin
HClO ₄	perchloric acid
Heme-NO	heme-nitric oxide
His	histidine
HOO·	hydroperoxyl radical
HPLC-ECD	high performance liquid chromatography-electrochemical detector
HSPs	heat shock proteins
i.h.	intrahippocampal
i.p.	intraperitoneal
IDO	indoleamine 2,3-dioxygenase
IR	infrared
K ⁺	potassium
KA	kainate
KBr	potassium bromide
KCN	potassium cyanide
kg	kilogram
kV	kilovolts
LOD	limits of detection
LOH	lipid alcohol
LOO·	lipid peroxyl radical
LOOH	lipid hydroperoxide
LOQ	limits of quantification
LP	lipid peroxidation
LTP	long term potentiation
M	molar
MAO	monoamine oxidase
MDA	malondialdehyde
Mel	melatonin
MEM	Minimum Essential Medium
MeOH	methanol
mg	milligram

Mg ²⁺	magnesium
MIAA	5-methoxyindole acetic acid
ml	milliliter
mm	millimeter
mM	millimole
MnCl ₂	manganese (II) chloride
MPP ⁺	1-methyl-4-phenyl-pyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium
mRNA	messenger ribose nucleic acid
MS	mass spectrometry
MTT	(3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide)
n	sample size
Na ⁺	sodium
NaCl	sodium chloride
NAD ⁺	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NAS	N-acetylserotonin
NAT	N-acetyltransferase
NBD	nitroblue diformazan
NBT	nitroblue tetrazolium
NFTs	neurofibrillary tangles
NF-κB	nuclear factor-κB
ng	nanogram
NMDA	N-methyl-D-aspartate
nmole	nanomole
NMR	nuclear magnetic resonance
NO [•]	nitric oxide
NO ₃ ⁻	nitrate
ns	not significant
NSAIDS	non-steroidal anti-inflammatory agents
O ₂	oxygen
O ₂ ^{-•}	superoxide anion
od	once daily
ONOO ⁻	peroxynitrate
OP	organic phase
OSA	octane sulphonic acid
PA	phosphoric acid
Pb	lead
PC	pentose cycle
PD	Parkinson's disease
PUFAs	polyunsaturated fatty acids
QA	quinolinic acid
R	non-radicals
R [•]	radicals
R5P	ribulose 5-phosphate

RNA	ribose nucleic acid
RNS	reactive nitrogen species
ROO [·]	peroxyl radical
ROS	reactive oxygen species
RSD	relative standard deviation
s	seconds
SD	standard deviation
SH	sulfhydryl groups
SIM	selected ion monitoring
SOD	superoxide dismutase
SPs	senile plaques
sul	sulindac
Tau	taurine
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TDO	tryptophan 2,3-dioxygenase
Tdt	terminal deoxynucleotidyl transferase
TEA	triethylamine
TLC	thin layer chromatography
TNF	tumor necrosis factor
tol	tolmetin
U.S.A.	United States of America
UV/VIS	ultraviolet/visible
v/v	volume by volume
Vit C	vitamin C
Vit C [·]	vitamin C radical
Vit E	vitamin E
Vit E [·]	vitamin E radical
Vs ⁻¹	Volts per second
μA	microampere
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
μmole	micromole

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CHAPTER 1

LITERATURE REVIEW

1.1. WHY THE BRAIN?

Since ancient times, the heart and other organs like the stomach were believed to be the seat of the soul. This may be because we experience visceral sensations, such as increased heart rate or “butterflies” in the stomach, in relation to some emotions. There is no direct record of how the emphasis shifted to the brain however comments of Hippocrates and other old scholars suggest that they may have settled on the brain because of clinical observations. When people suffered internal damage to the head such as a direct blow to the skull in warfare, their minds were altered. Since this occurred often and was witnessed by many medical observers in the ancient world, this made them realise that there is something special about the brain that relates it to the mind in a way that does not occur with any other organ.

Recently the brain and its functions have received much attention in research and many important and life changing discoveries have been made in the field of neuroscience. Since more people are living to an older age, much attention has been focused on the study of age-related dementias, especially Alzheimer’s disease (AD).

1.2. THE BRAIN

1.2.1. NEUROANATOMY

The brain is made of three main parts: the forebrain, midbrain and hindbrain. The forebrain consists of the cerebrum, thalamus and hypothalamus. The midbrain consists of the tectum and tegmentum. The hindbrain is made of the cerebellum, pons and medulla. The midbrain, pons and medulla are often referred to together as the brainstem.

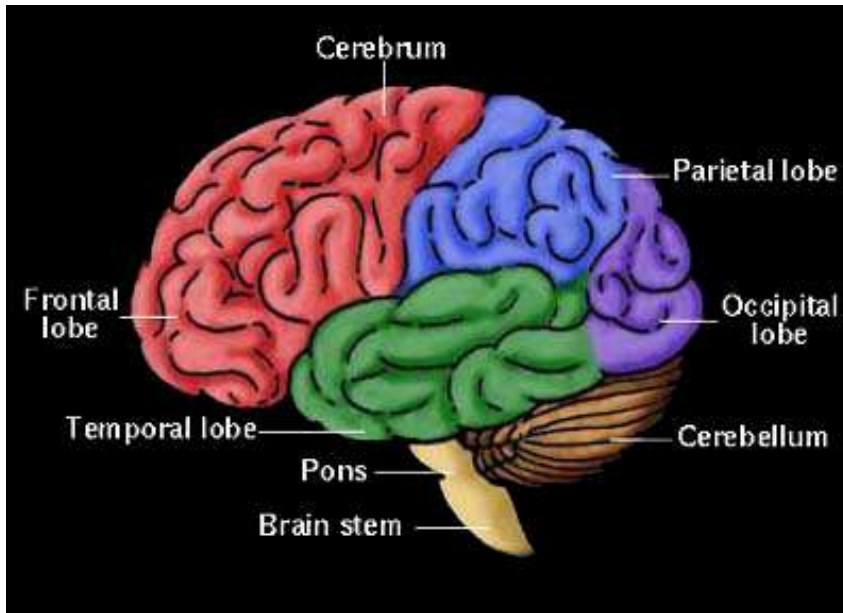


Figure 1.1: The main areas of the brain showing the frontal lobe, parietal lobe, occipital lobe and temporal. (<http://www.sciencebob.com/lab/bodyzone/brain.html>)

1.2.1.1. THE CEREBRUM

The largest part of the human brain is the cerebrum (or cortex). It is associated with higher brain functions such as thoughts and actions. As shown in figure 1.1, the cerebral cortex is divided into four sections or “lobes” which are the frontal lobe, parietal lobe, occipital lobe and the temporal lobe. The cerebral cortex is highly wrinkled to make the brain more efficient. The wrinkles increase the surface area of the brain and the amount of neurons within it. A deep furrow divides the cerebrum into two symmetrical halves, known as the left hemisphere and the right hemisphere. The two hemispheres are connected together by a bundle of neurons known as the corpus callosum (see figure 1.3).

1.2.1.2. THE CEREBELLUM

The cerebellum is similar to the cerebrum in that it also has two hemispheres and has a highly folded surface. It is associated with regulation and coordination of movement, posture and balance.

1.2.1.3. THE BRAIN STEM

The brain stem is located beneath the limbic system. This is also a vital area of the brain since it is responsible for basic life functions such as breathing, heartbeat and blood pressure.

1.2.1.4. THE NEURON

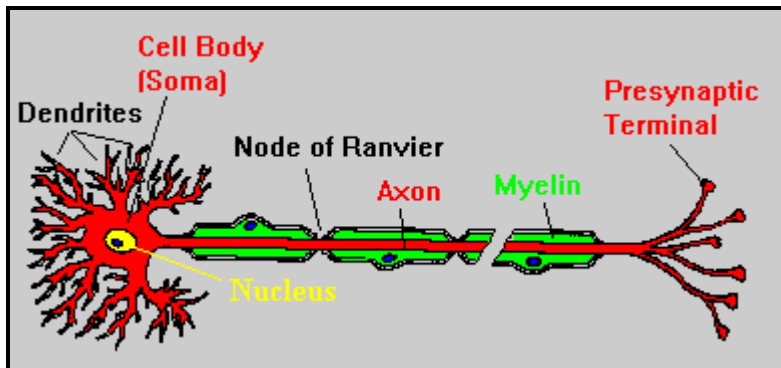


Figure 1.2: A typical neuron with the cell body, axon and dendrites.

(<http://faculty.washington.edu/chudler/cells.html>)

Approximately 10^{11} (hundred billion) nerve cells, or neurons make up man's brain. There are also a large number of glial cells that surrounds, supports and nourishes the neurons. Figure 1.2 shows a typical neuron consists of a cell body from which emanates the axon and a number of fibrous branches, known as the dendrites. The axon may form branches near its beginning but most often branches extensively near its end. The dendrites and cell body receive incoming signals. The cell body combines and integrates the incoming signals and emits outgoing signals. It is the axon that transports the outgoing signals to the axon terminals, which distribute the information to a new set of neurons.

1.2.2. THE NERVOUS SYSTEM

The entire nervous system is responsible for the generation of emotions but there are two parts that are especially important: the limbic system and the autonomic nervous system.

1.2.2.1. THE LIMBIC SYSTEM

The limbic system consists of complex structures that lie on both sides and underneath the thalamus, just under the cerebrum. As shown in figure 1.3, the limbic system consists of the hypothalamus, the hippocampus, the amygdala, the fornix and several other nearby areas that are also responsible for our emotional life.

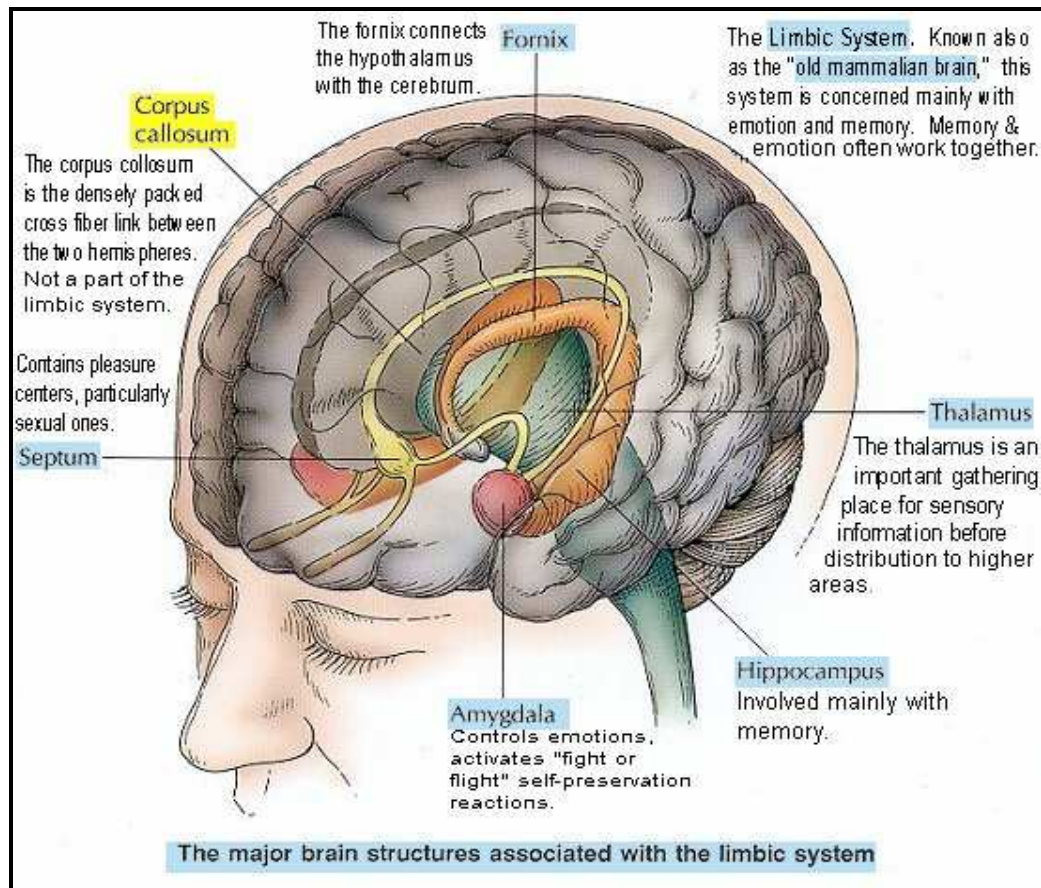


Figure 1.3: Diagram of the various structures associated with the limbic system. Although the corpus callosum is not part of the limbic system, its location in the brain is also shown. (http://www.sruweb.com/~walsh/limbic_system.jpg)

1.2.2.1.1. THE HIPPOCAMPUS

This is the most medial portion of the cerebral cortex developed from the stalk, of the original cerebral vesicle (Smythies 1970). The dorsal half in man is reduced to a vestige by the growth of the corpus callosum but the ventral half adjacent to the inferior horn of the lateral ventricle still remains. Its anterior portion, the pes hippocampi remains an

undulating piece of primitive cortex in the floor of the ventricle, whereas the posterior portions becomes rolled up on itself as the cornu ammonis (CA1-CA3) in the form of the sea-horse. A portion becomes isolated from the rest to cap the cornu as the dentate gyrus. In the neocortex, the cells are arranged in six layers but in the allocortex they form one layer of large pyramidal cells.

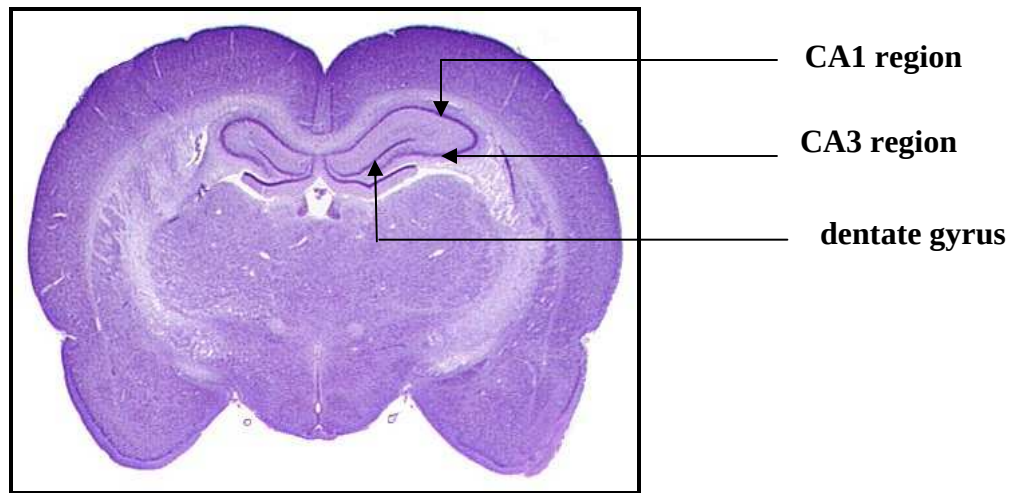


Figure 1.4: A Nissl-stained coronal section of the caudal telecephalon of a rat brain showing the major regions in the hippocampus.

The hippocampal region (the CA field, the dentate gyrus and subicular complex) is important for mammalian memory (Squire 1992). As shown in figure 1.4, the hippocampus is comprised of three major sub-regions which include the CA1, CA3 and the dentate gyrus. Neurons in the CA1, CA3 and dentate gyrus are differentially sensitive to excitotoxic cell death (Ben-Ari *et. al.*, 1980; Golarai *et. al.*, 2001; Olsson *et. al.*, 2003). Damage to these regions impairs performance on a variety of tasks of learning and memory (Eichenbaum & Cohen 2001). single-cell recording and neuroimaging techniques have documented changes in the hippocampal region during both learning and retention (Suzuki & Eichenbaum 2000; Squire *et. al.*, 2004; Fujimichi *et. al.* 2005). The hippocampus is important for both spatial and recognition memory and that the two kinds of memory tasks differ in how much hippocampal tissue is needed to support performance. It has been shown that recognition memory requires more hippocampal

tissue since larger lesions to the hippocampus is required to impair recognition memory (Broadbent *et. al.*, 2004).

The hippocampus is the most adversely affected area of the brain in patients suffering with Alzheimer's disease (Behl *et. al.*, 1997).

1.2.2.2. THE AUTONOMIC NERVOUS SYSTEM

The autonomic nervous system also plays a major part in our emotional life. The autonomic nervous system is composed of two parts, which functions in opposition to each other. The first is the sympathetic nervous system, which starts in the spinal cord and travels to a variety of areas of the body. Its function appears to be preparing the body for the kinds of vigorous activities associated with "fight or flight," that is, with running from danger or with preparing for violence. The other part of the autonomic nervous system is called the parasympathetic nervous system. It has its roots in the brainstem and in the spinal cord of the lower back. Its function is to bring the body back from the emergency status that the sympathetic nervous system puts it into.

1.2.3. THE STRIATUM

The striatum is not considered a part of the limbic system. As shown in figure 1.5, it is a subcortical part of the brain consisting of the caudate nucleus and the putamen. It is part of the basal ganglia and is best known for its role in the planning and modulation of movement in a variety of other cognitive processes involving executive function. In humans the striatum is activated in the presence of stimuli associated with reward, but also in the presence of aversive, novel, unexpected or intense nuclei. The striatum connects to the substantia nigra via the nigrostriatal pathway.

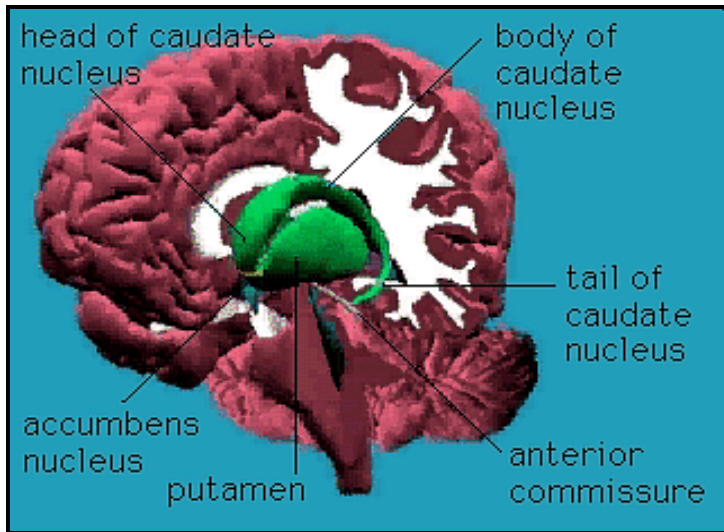


Figure 1.5: The areas of the striatum situated within the brain.

(http://www.med.uiuc.edu/m1/neurosci/Web_Neuro/movies/400.htm)

1.2.3.1. THE SUBSTANTIA NIGRA

The substantia nigra is a portion of the midbrain thought to be involved in certain aspects of movement and attention. It consists of two subdivisions, the pars compacta and the pars reticulata.

The pars compacta contains densely-packed neurons, which in humans are colored black by the pigment neuromelanin. Research suggests that dopamine neurons in the substantia nigra pars compacta are involved in learning to predict which behaviours will lead to a reward. Degeneration of neurons in this region is the principle pathology that underlies Parkinson's disease (PD).

Neurons in the pars reticulata are much less densely packed than those in the compacta. Most of these neurons produce the neurotransmitter gamma-aminobutyric acid (GABA), but some neurons also produce dopamine. The pars reticulata is largely involved in orientation and the control of eye movements. The function of the neurons in this region of the brain is profoundly changed in Parkinsonism and epilepsy. These changes are thought to be mostly secondary to pathology elsewhere in the brain, but may be crucial in understanding the generation of the symptoms of these disorders.

1.2.4. EXCITATORY AMINO ACIDS

Glutamate and aspartate are present in very high concentrations in the central nervous system (CNS). Most neurons that have been tested thus far are strongly excited by both these amino acids. This excitation is caused by the stimulation or activation of receptors that can be divided into two classes. These are the ionotropic and metabotropic receptors. The ionotropic receptors directly gate cation-selective channels and are further divided into three subtypes based on the selective agonists that were first used to identify them. These receptors are the kainate (K), the α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and the *N*-methyl-D-aspartate (NMDA) receptors. The AMPA- and K-activated channels are permeable to sodium (Na^+) and potassium (K^+) ions and for certain subtypes, calcium (Ca^{2+}) as well. The NMDA activated channel is highly permeable to sodium, potassium and calcium ions.

1.2.4.1. THE NMDA RECEPTOR

Memory formation and excitotoxic cell death in the hippocampus involves excitatory glutamatergic neurons, especially of the NMDA type (Haberny *et. al.*, 2002; Silva 2003). As shown in figure 1.6, the NMDA receptor complex consists of four subunits, with at least one NR1 and two NR2 subunits (Dingledine *et. al.*, 1999). The NR1 subunit is expressed throughout the CNS (Monyer *et. al.*, 1994). The four NR2 subunits (A-D) are encoded by four separate genes, whose expression levels are developmentally and regionally regulated, with the NR2A and NR2B subunits being the dominant forms found in the hippocampus of the adult rat (Monyer *et. al.*, 1994). Among the four NR2 subunits, the NR2B is known to be the most important in mediating the learning and memory process since an increase in its expression in the forebrain of transgenic mice have shown an enhancement in learning and memory (Tang *et. al.*, 1999).

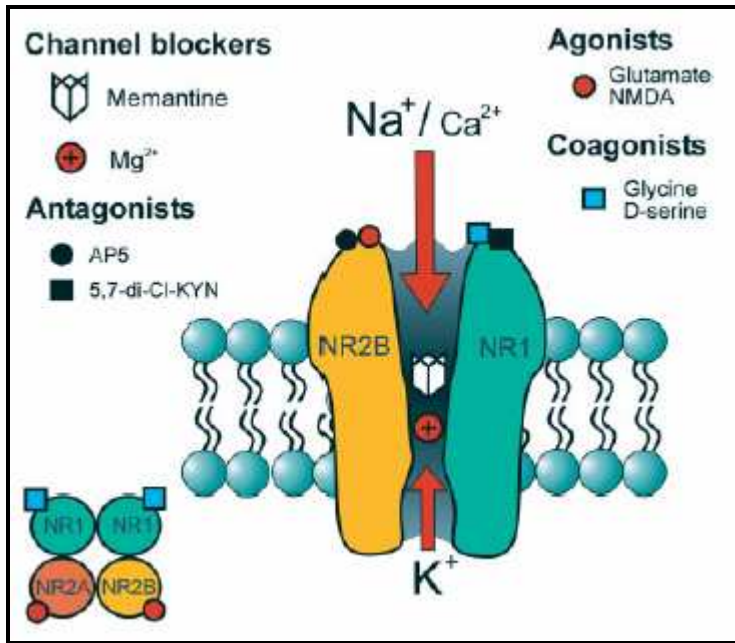


Figure 1.6: Diagrammatic representation of the NMDA receptor and its subunits, showing magnesium blocking the narrow channel and preventing the influx of Ca^{2+} (Danysz & Parsons 2003).

1.2.4.2. THE NMDA RECEPTOR AND LONG TERM POTENTIATION

The NMDA receptor is only activated upon certain conditions. This receptor has three basic features which are: its high permeability to Ca^{2+} ions, voltage dependent blockage by magnesium (Mg^{2+}) ions and slow gating kinetics. These features make the NMDA receptor ideally suited for mediating plastic changes in the brain, such as learning and memory. Long term potentiation (LTP) is an example of plastic changes in the brain and is believed to model basic mechanisms of memory formation. As demonstrated in figure 1.6, LTP can be described by the following sequence of events:

1. A high frequency signal arrives at the glutamatergic synapse leading to a massive release of glutamate.
2. Glutamate binds to both NMDA and AMPA receptors. However, AMPA receptors are initially activated since the NMDA receptor channel is blocked by positively charged Mg^{2+} . Mg^{2+} is attracted into the cell which has a negative resting membrane potential but gets trapped in the narrow region of the NMDA receptor channel (figure 1.7).

3. The continuous activation of the AMPA receptor causes a large amount of Na^+ ions to enter into the cell and leads to a decrease in membrane potential.
4. This depolarization then removes the positively charged Mg^{2+} from the narrow region in the NMDA receptor.
5. It is only at this stage where Ca^{2+} ions can freely enter the cell via the NMDA receptor channel and initiate a number of enzymatic processes that enhance synaptic strength.

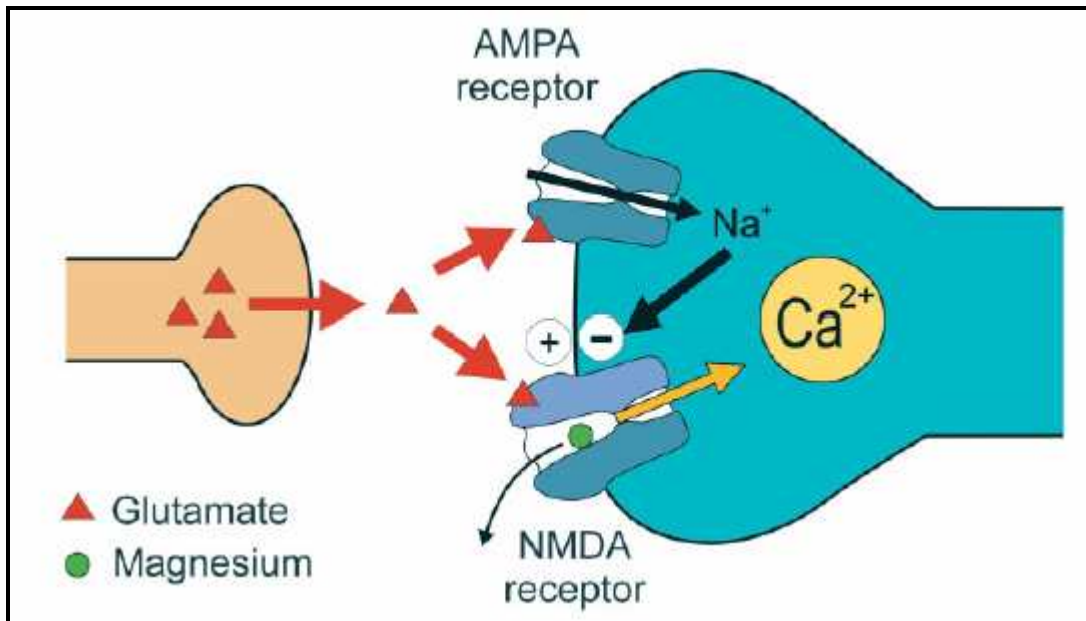


Figure 1.7: The neuronal model for long term potentiation (Danysz & Parsons 2003).

1.2.4.3. THE NMDA RECEPTOR AND NEUROTOXICITY

Although the NMDA receptor has an important role in LTP, excessive stimulation by agonists can induce neuronal dysfunction, cell damage or even death. This type of cell death is termed excitotoxicity and occurs in acute insults such as stroke, trauma and also in chronic neurodegenerative diseases such as AD (Rothman & Olney 1987). This neurotoxicity initiated by the over stimulation of NMDA receptors is due to the large influx of Ca^{2+} into the cell (Choi 1985; Choi 1992; Dubinsky 1993; Lipton & Rosenberg 1994) followed by a cascade of neurotoxic events (Choi 1985). One such example is the Ca^{2+} -dependent depolarisation of mitochondria. This has been suggested to contribute to oxidative stress in neuronal injury through the production of reactive oxygen species

(ROS) such as the hydroxyl radical ($\cdot\text{OH}$), superoxide anion ($\text{O}_2^{\cdot-}$) or nitric oxide ($\text{NO}\cdot$) (Choi 1992; Dykens 1994; Whit & Reynolds 1996).

1.3. MECHANISMS OF NEURODEGENERATION

1.3.1. GENERATION OF FREE RADICALS

Free radicals are unstable, highly reactive molecules that are characterised by the presence of unpaired electrons in their outermost orbital. The reduction of molecular oxygen is the major source of biologically-active radical species such as the $\text{O}_2^{\cdot-}$; singlet oxygen and $\cdot\text{OH}$. Apart from these oxygen derived free radicals, organisms are also exposed to organic free radicals that are derived from the environment (such as herbicides, paraquat and other xenobiotics) or those that are generated within tissue during normal cellular metabolism. The loss of a single electron from a non-radical or the gain of a single electron by a non-radical forms a free radical. A free radical can also be generated when a covalent bond is broken and if one electron from the shared pair remains with each atom. This process is known as homolytic fission.

1.3.2. OXIDATIVE STRESS IN THE BRAIN

Much attention has been focused on oxidative stress in the brain. The brain is particularly vulnerable to oxidative damage due to its high content of easily peroxidisable unsaturated fatty acids, it requires very high amounts of oxygen per unit weight, it has a high content of iron and ascorbate (the key ingredients required for lipid peroxidation), and is not as highly enriched with antioxidant defense mechanisms compared to other organs (Floyd 1999). Brain homogenate is more prone to peroxidation than liver, kidney, muscle or heart homogenates. The brain utilises glucose for its energy source and this probably explains why it consumes such high levels of oxygen per unit weight. The major portion of ROS generated during aerobic metabolism comes from byproducts of the mitochondrial electron transport chain (Hensley *et. al.*, 1999). Studies on isolated mitochondria have shown that approximately 5% of the total oxygen consumed in mitochondrial respiration results in ROS (Papa & Skulachev 1997; Hensley *et. al.*, 1998; Perez-Campo *et. al.*, 1998). These ROS can cause oxidation of important biomolecules

such as proteins, lipids and deoxyribose nucleic acids (DNA), leading to cell injury and death (McCord 2000).

1.3.2.1. QUINOLINIC ACID

In section 1.2.4.3. it was stated that excitotoxicity can occur through the over-stimulation of NMDA receptors. One such agonist which can induce such neurotoxicity is quinolinic acid (QA). QA is a neurotoxic metabolite of the tryptophan-kynurenine pathway (Stone 1993) and has been implicated in many neurologic diseases. Under normal circumstances, the concentration of QA in different brain regions and cerebrospinal fluid (CSF) are less than 100pmol/g and 50nM respectively, in humans, mice and rats (Heyes *et. al.*, 1990; Saito *et. al.*, 1992). There is a substantial increase in brain QA concentrations in patients with a broad spectrum of inflammatory conditions (Heyes *et. al.*, 1991; Heyes *et. al.*, 1992; Vogelgesang *et. al.*, 1996). During immune activation within the CNS, QA is synthesised from L-tryptophan after a large local induction in indoleamine 2,3-dioxygenase activity in microglia, astrocytes and invading macrophages (Heyes *et. al.*, 1992; Heyes & Morrison 1997). The microglia and macrophage infiltrates are considered important sites of QA synthesis within the brain (Heyes *et. al.*, 1992; Heyes *et. al.*, 1996). Intrastratial injections of QA induces neurological damage that resembles that observed in Huntington's disease (Beal *et. al.*, 1986). Intraventricular injections of QA has been shown to induce seizures (Lapin 1978; Schmidt *et. al.*, 2000). Moreover, QA has also shown to induce ROS and induce lipid peroxidation (Rios & Santamaria 1991; Santamaria & Rios 1993; Rodriguez-Martinez *et. al.*, 2000).

1.3.2.2. CYANIDE

The brain is the primary target organ for cyanide toxicity (Gunasekar *et. al.*, 1996). Cyanide produces dopaminergic toxicity that is characterised by a loss of neurons in the basal ganglia which is accompanied by impaired motor function (Gunasekar *et. al.*, 1996). Since cyanide inhibits a number of antioxidant enzymes, it is believed that oxidative stress plays a role in cyanide-induced neurotoxicity (Ardelt *et. al.*, 1989). Several biochemical, morphological and physiological studies have also shown that cyanide-induced toxicity is mediated through histotoxic hypoxia as a result of

mitochondrial dysfunction (Bhattacharya & Lakshmana Rao 2001). Cyanide-induced mitochondrial dysfunction also includes failure of ionic homeostasis, acidosis, elevated Ca^{2+} levels and lipid peroxidation (Maduh *et. al.*, 1988). The elevated brain Ca^{2+} levels and increased free cytosolic Ca^{2+} leads to increased oxidative stress and excitotoxicity (Johnson *et. al.*, 1986).

1.3.2.3. IRON

The transition metals are essential in many biological functions such as protein, DNA and RNA synthesis. They are also cofactors of numerous enzymes, especially those involved in respiration. All metals in the first row of the d-block in the Periodic Table contain unpaired electrons and can therefore qualify to be radicals, with the exception of zinc. Although the transition metals such as iron, copper and magnesium play a role in many important biological functions, abnormal tissue accumulation of these metals can be cytotoxic because changes in the metal homeostasis can result in a variety of cellular disturbances including increased free radical production (Campbell *et. al.*, 2001). Hydrogen peroxide (H_2O_2) is a by-product of oxidases and in the presence of transition metal ions, it can be converted to the reactive hydroxyl radical via the Fenton reaction (Halliwell & Gutteridge 1986; Bielski 1991).

During the breakdown of the blood brain barrier, haemoglobin has been postulated to be the major source for iron in the brain. The increased iron concentrations promotes the generation of ROS, lipid peroxidation, axonal dystrophy and neurodegeneration. These adverse effects of haemoglobin can be blocked by the use of metal chelators such as desferrioxamine, which suggests that free iron is involved (Bergen *et. al.*, 1999). The role of iron in neurodegeneration has been further discussed in chapter 2, experiment 2.8 and chapter 10.

1.3.2.4. LEAD

Lead (Pb) is a heavy metal with no apparent biological function. This heavy metals widespread environmental contamination, its ability to cause a variety of toxic effects and the number of individuals affected makes Pb a public health problem of global interest

(Satcher 2000). Although this ubiquitous neurotoxin has been extensively researched, a detailed explanation of the mechanism(s) by which Pb exerts its toxicity on the CNS is yet to be documented.

Pb toxicity occurs from exposure to contaminated air, food, water or soil. Before 1995, food was contaminated with Pb from the Pb solder in the joints of cans (Goyer 1996). Although rare, exposure to dietary sources of Pb also occurred from ingestion of ethnic foods, dietary supplements and ethnic remedies (Lynch *et. al.*, 2000; USFDA 1998). Drinking water can also become contaminated by Pb. This can happen either at the source due to deposition from environmental sources or in the water distribution system itself. It has been found that some municipal infrastructure distribution systems use pipes made of Pb or components that contain Pb which leaches Pb into the water (Bernard *et. al.*, 2001; Maas *et. al.*, 2002) and this has shown to increase blood Pb levels above 5µg/dl, a level known to be associated with cognitive deficits in children (Canfield *et. al.*, 2003; Lanphear *et. al.*, 2000). Lead emissions from the combustion of leaded fuels and metals smelting also contribute significantly to the accumulation of atmospheric and soil Pb (Mielke 1999).

The developing nervous system is more susceptible to lead toxicity than the mature nervous system (Gonick & Behari 2002). In children the health effects of concern are neurobehavioral and endocrine alterations, such as an increase in hyperactivity and distractibility, delayed puberty and cognitive deficits (Bellinger 1995; Needleman *et. al.*, 1979; Nevin 1995; Selevan *et. al.*, 2003). The current threshold for the clinical treatment of childhood Pb intoxication is 25µg/dl (Markowitz 1996). Chelation therapy has consisted of the administration of chelating agents such as British Anti Lewisite, succimer or ethylenediaminetetraacetic acid (Markowitz 1996; Markowitz 1997). Although these agents rapidly lower the total Pb body levels by mobilizing the Pb and allowing for excretion in the urine (Stangle *et. al.*, 2004), it has been demonstrated that only a few Pb-intoxicated patients responds to chelation therapy (Markowitz 1996) and chelation therapy does not reverse the neurological deficits associated with elevated blood Pb levels (Rogan *et. al.*, 2001). However, it has been demonstrated that it is

advantageous if a metal-chelating agent possesses some degree of antioxidant properties independent of its metal-mobilizing ability, particularly for metals that exerts toxic effects via oxidative stress (Tandon *et. al.*, 2002). Lead's ability to generate ROS will be discussed in chapter 12.

1.3.2.5. NEUROINFLAMMATION

It is well known that several diseases that lead to injury in the CNS are mediated by autoimmunity or inflammatory reactions in the brain. For example, multiple sclerosis is a typical autoimmune disease that is characterised by the breakdown of the blood brain barrier, recruitment of leucocytes and the secretion of antibodies by plasma cells (Matyszak 1998; van der Goes *et. al.*, 1999). The inflammatory mediators such as acute-phase proteins complement factors and cytokines are rapidly induced in the brain but cellular infiltration by peripheral cells in response to inflammation is often weaker or delayed when compared to other tissue (Allan & Rothwell 2001). Although the role of inflammation in neurodegenerative diseases such as trauma, ischemia, PD and AD is not well understood since the triggering events are still obscure, increasing evidence suggests that inflammation does contribute to the pathogenesis of these disorders (Matyszak 1998; Allan & Rothwell 2001; Hartmann *et. al.*, 2003; Hirsch *et. al.*, 2003; van Gool *et. al.*, 2003).

1.3.3. MEASUREMENT OF PRODUCTS OF OXIDATIVE STRESS

Lipid peroxidation (LP) is a process where lipids are attacked by a free radical to form a carbon free radical that reacts with oxygen resulting in a peroxy radical, thus generating lipid peroxides (Gutteridge 1995). The detailed mechanism of lipid peroxidation is discussed in chapter 2. Markers of lipid peroxidation has been the most studied indexes in neurodegenerative disorders such as AD and PD. The extent of LP is measured by a wide range of techniques that quantifies the elevated end products of lipid peroxidation (Moore & Roberts 1998).

Evidence also implicates oxidative stress in protein oxidation (Berlett & Stadtman 1997; Butterfield & Stadtman 1997; Butterfield & Kanski 2001; Stadtman & Levine 2003).

ROS can attack amino acid residues (particularly histidine, arginine and lysine) to produce a carbonyl. The measurement of the carbonyl groups (using 2,4-dinitrophenylhydrazine), incorporated into proteins during protein oxidation, has become the most widely used method in measuring the extent of protein oxidation (Levine *et. al.*, 1990; Reznick & Packer 1994). A detailed mechanism of protein oxidation is provided in chapter 7.

Reactive oxygen species-mediated DNA damage can result in strand breaks, DNA-protein cross linking and base modification. DNA strand breaks can be detected at the tissue level by the alkaline elution assay (Mullaart *et. al.*, 1988) or at the single cell level by the terminal deoxynucleotidyl transferase (Tdt)-mediated *in situ* end-labeling (Mullaart *et. al.*, 1988). The latter has been used in this study and is described in more detail in chapter 4.

1.4. NEURODEGENERATIVE DISORDERS

In a structure as intricate as the human brain, a number of things can go wrong. For most people the brain can function normally for more than 60 years, but disorders can arise in its structural architecture or in its electrical and chemical processes. More than a century ago, pathologists were able to recognise damage in the brain that resulted from hemorrhage, pressure, inflammation, degeneration and atrophy. With the aid of the microscope and chemical stains it was possible to see how morphological damage contributed to degeneration and death of neurons and the interpretation of the pathways connecting them. However, investigations of brain disorders were hampered by the absence of techniques and technology for studying the living brain. Now with modern technology and scientific methods it is possible to study various brain disorders such as AD and PD.

1.4.1. ALZHEIMER'S DISEASE

1.4.1.1. INTRODUCTION

In 1907 Alois Alzheimer published a case report of a 55-year old woman with progressive dementia (Alzheimer 1977). He described the cerebral cortex and pointed out

the presence of abnormal nerve cells that contained tangles of fibers and clusters of degenerative nerve endings. This presenile dementia later became known as Alzheimer's disease.

The most likely explanation of the abnormalities in memory noticed in the early stages of AD is most likely due to the damage to the hippocampal formation seen in the AD patients (Ball 1977; Hyman *et. al.*, 1985). In the hippocampal formation, neuronal loss and abnormal formations with the neurons such as neurofibrillary tangles and neuritic plaques are present in the entorhinal cortex and subiculum. These are the primary pathways that convey information into and out of the hippocampus and abnormalities in these regions produce a functional isolation of the hippocampus (Hyman *et. al.*, 1985; Hyman *et. al.*, 1986). Therefore neuropathological damage to medial temporal lobe structures may be responsible for the marked short-term memory impairment evident in the early stages of AD.

1.4.1.2. NEUROPATHOLOGY OF ALZHEIMER'S DISEASE

AD is the most prevalent cause of dementia in the elderly. The earliest and most prominent clinical feature is memory impairment and impairment to acquire new information remains a hallmark feature throughout the course. Neuropathological study of the AD brain demonstrates that neurofibrillary tangles (NFTs) and senile plaques (SPs) are distributed quite consistently in the brain (Arnold *et. al.*, 1991; Braak & Braak 1991). The development of NFTs in the medial temporal lobe is the earliest and most severe alteration in the AD brain (Arnold *et. al.*, 1991; Braak & Braak 1991; Hyman *et. al.*, 1990). Neurons in layers II and IV of the entorhinal cortex are among the first to be affected and compromises the integrity of crucial neural systems related to memory (Hyman *et. al.*, 1990). As the disease progresses the cognitive deficits increase and more NFTs are present in layers II, II and IV in higher-order associated cortices (Lewis *et. al.*, 1987; Arnold *et. al.*, 1991). The SPs have a broader distribution compared to the NFTs. They appear less in memory-related neural systems such as the hippocampal formation but are present in large amounts in regions such as the primary motor and sensory

cortices and remain clinically silent, even at a later stage in AD (Arriagada *et. al.*, 1992 Arnold *et. al.*, 1991).

1.4.1.3. CAUSATIVE FACTORS OF ALZHEIMER'S DISEASE

1.4.1.3.1. GENETIC COMPONENT

No single factor can be attributed to the cause of AD. It is likely that AD is a multifaceted syndrome caused by many distinct biological aberrations. Some cases can be traced back to genetic defects. The idea that AD has a strong genetic component rests on sound foundations and it is estimated that 30-50% of AD cases are attributed to genetic factors (Breitner *et. al.*, 1988; Lautenschlager *et. al.*, 1996). Mutations in few genes lead to the early onset of the disease but such genetic aberrations account for only a portion of the AD cases (St. George-Hyslop 2000).

1.4.1.3.2. FREE RADICALS AND OXIDATIVE STRESS

The free radical theory of aging suggests that oxidative damage is the major cause of neurodegeneration (Harman *et. al.*, 1976). Age is the primary risk factor for the majority of AD cases. This is consistent with the free radical theory of aging and due to the increasing evidence; oxidative stress has been suggested to play a causative role in the pathogenesis and progression of AD (Melhorn & Cole 1985; Friedlich & Butcher 1994; Ames *et. al.*, 1993; Beal 1995). Although various genetic alterations and mutations have been linked to some familial forms of AD (Sandbrink *et. al.*, 1996; Tanzi *et. al.*, 1996), age is the only reliable risk factor for the non-familial, sporadic forms for the majority of this neurodegenerative disease. Approximately 85% of all AD cases are of such late-onset (after the age of 65) non-familial, sporadic forms (Evans *et. al.*, 1989).

ROS and reactive nitrogen species (RNS) that are generated by various mechanisms intracellularly or extracellularly, are among the major intermediary risk factors than initiate and promote neurodegeneration in AD (Multhaup *et. al.*, 1997; Perry *et. al.*, 2002; Prasad *et. al.*, 2000; Schippling *et. al.*, 2000). Brain tissue from AD patients have shown evidence of oxidative damage since these tissue showed increased amounts of

oxidised proteins (Schippling *et. al.*, 2000, Smith *et. al.*, 1991), advanced glycation end products and 4-hydroxynonenal derived adducts (Ramassamy *et. al.*, 2001; Montine *et. al.*, 1997) when compared to age matched nondemented controls. Lipids are also modified by ROS and RNS and there is strong correlation between lipids peroxides, antioxidant enzymes, the presence of SPs and NFTs in AD brain (Lovell *et al.*, 1995).

1.4.1.3.3. NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

AD tissue is also characterised by neuroinflammatory changes that are observed in both sporadic and familial cases as well as in other neurodegenerative disorders such as PD. The chronic inflammation is not causative but greatly influences the pathogenesis. Neuroinflammation occurs after microglia activation (McGeer & McGeer 1995; Neuroinflammation Working Group 2000) followed by the invasion of the brain by leukocytes (Haymaker & Adams 1982). Significant activation of microglia appears in a very early stage of AD before severe cognitive deficits are noticed (Vehmas *et. al.*, 2003). Lots of activated microglia appear on the SPs and in surrounding tissue (Luber-Narod & Rogers 1988; McGeer *et. al.*, 1996). There is much evidence that highly activated glia produce neurotoxic material (Heyes *et. al.*, 1992; Heyes *et. al.*, 1996). Apart from the oxygen free radicals formed from the accidental leakage from the electron transport chain, the most abundant source of oxygen free radicals in the CNS is from the respiratory burst system of activated microglia. Large amounts of superoxide anions are generated on the microglia external membrane from where they are released into the surrounding tissue and cause oxidative stress (Colton & Gilbert 1987; Klergeris & McGeer 1994).

Cyclooxygenase-1 (COX-1) is a molecule which is constitutively expressed in many tissues including the brain. Moreover, COX-1 positive microglia were observed at beta-amyloid (A β)-rich plaques, and the fusiform cortex of AD brains were more densely populated with COX-1 than non-AD brains (Yermakova *et. al.*, 1999). Cyclooxygenase-2 (COX-2) is much more restricted in its expression than COX-1 but is enriched in the brain (Yamagata *et. al.*, 1993). It is greatly expressed at high levels in the AD brain particularly in the hippocampal pyramidal layer (Ho *et. al.*, 1999). COX-2 is an inducible

enzyme and its levels increase during inflammation (Lipsky 1999), specifically in the microglia cell which mediates the inflammatory process in the brain and are enriched in the SPs of AD patients (Bauer *et. al.*, 1997).

1.4.1.4. THERAPEUTIC APPROACHES TO ALZHEIMER'S DISEASE

1.4.1.4.1. ACETYLCHOLINE ESTERASE INHIBITORS

Much attention has been focused on the cholinergic system since in AD there is dysfunctional cholinergic signaling and transmission in the cerebral cortex and hippocampus (Bartus *et. al.*, 1982; Coyle *et. al.*, 1983). Since these areas of the brain are associated with memory and intellect, any functional imperfections in these regions could impair memory, judgment and compromise intellectual abilities (Nordberg 1982). Due to the vast amount of information suggesting that the cholinergic signaling is impaired, AD treatment targets acetylcholine in an attempt to increase its presence and make it more effective in neuronal transmission (Nordberg 1982; Whitehouse *et. al.*, 1982; Gray *et. al.*, 1989; Amenta *et. al.*, 2001). The most successful approach thus far is by the use of acetylcholine esterase inhibitors, which prevent the breakdown of acetylcholine in the synapse. Tacrine, donepezil and rivastigmine are currently the only acetylcholine esterase inhibitors that are FDA-approved.

1.4.1.4.2. THE ROLE OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS IN ALZHEIMER'S DISEASE

A possible link between arthritis and a protective effect against AD has been identified (Broe *et. al.*, 1990). The link appears that these patients routinely take anti-inflammatory drugs to reduce joint pain. The duration of use of non-steroidal anti-inflammatory drugs (NSAIDS) also have an impact on the relative risk of developing AD (Stewart *et. al.*, 1997). For example, the relative risk of developing AD was much lower in individuals using NSAIDS for more than two years when compared individuals using NSAIDS for less than one year (Stewart *et. al.*, 1997). Inflammation is believed to enhance the progression of AD through two separate mechanisms. Inflammation increases the accumulation of A β deposition to form senile plaques (Walker 1998) and also enhances

neuronal death (Giulian *et. al.*, 1996; Halliday *et. al.*, 2000). Epidemiological studies have suggested that NSAIDS decrease the incidence of developing AD in the elderly population (McGeer *et. al.*, 1996). Clinical studies thus far have also demonstrated that NSAIDS may be beneficial in reducing the incidence of AD or improving cognitive performance (Rogers *et. al.*, 1993; Bruce-Jones *et. al.*, 1994). Adverse gastrointestinal effects have been observed in studies of NSAIDS (Rogers *et. al.*, 1993), therefore the potential use of COX-2 inhibitors in AD has also been considered. These inhibitors are less toxic than the NSAIDS, which is important in the treatment of elder patients. However, COX-1 also contributes significantly to the inflammatory process in AD, therefore COX-2 inhibitors may be insufficient to relieve the symptoms of AD (Cutler & Sramek 2001).

1.4.1.4.3. ANTIOXIDANTS

The neurons high lipid content and abundance of polyunsaturated fats renders it susceptible to oxidative attack (Halliwell & Gutteridge 1985). Low vitamin E levels have been correlated with poor memory (Perkins *et. al.*, 1999) and plasma vitamin E is substantially reduced in AD patients relative to control (Zaman *et. al.*, 1992). Therefore an increased antioxidant concentration may slow down oxidation of the brain and may delay the onset of AD. Similar to vitamin E, vitamin C has also been suggested to be an effective antioxidant in delaying the onset and progression of the disease (Morris *et. al.*, 1998). Ginkgo biloba has also shown modest improvements in cognition in patients with AD (Kanowski *et. al.*, 1996; Le Bars *et. al.*, 1997).

1.4.2. PARKINSON'S DISEASE

1.4.2.1. INTRODUCTION AND CLINICAL CHARACTERISTICS

Parkinson's disease is one of the leading causes of neurologic disabilities in the elderly. The mean age of onset is 55 years of age, and the risk of developing PD increases 5-fold by the age 70. As in the case of other neurodegenerative disorders such as AD, aging is possibly the single most important risk factor for the development of PD. There are two forms of this disease that have been identified. One is a sporadic form which affects

approximately 95% of all PD patients and here the etiology is unknown. The other is the familial form which accounts for approximately 5-10% of all cases, which is linked to mutations in a limited number of genes (Conway *et. al.*, 1998; Athanassiadou *et. al.* 1999; Wszolek *et. al.*, 2004).

PD is a movement disorder characterised by the accelerated loss of dopamine-containing neurons in the pars compacta of the substantia nigra. The symptoms of PD are caused by the loss of more than 50% of the dopaminergic neurons in substantia nigra pars compacta, leading to a loss of more than 80% of dopamine in the striatum (Deumens *et. al.*, 2002). However, cell loss also occurs in other areas of the brain such as the raphe nucleus and locus coeruleus. The cardinal clinical features of PD are bradykinesia, rigidity and resting tremor of the head and limbs. PD patients may also show signs of disturbances in speech and writing, depression, autonomic nervous system dysfunction and respiratory complications. Patients with advanced PD may suffer from severe gait disturbances and postural instability which may progress to complete immobility and institutionalisation.

1.4.2.2. CAUSATIVE FACTORS IN PARKINSON'S DISEASE

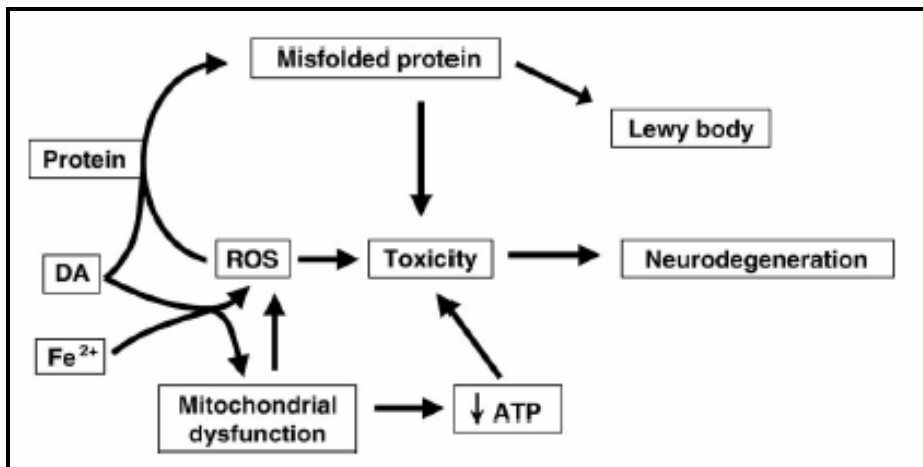


Figure 1.8: Possible mechanisms for dopaminergic neurodegeneration in PD (Hald & Lotharius 2005).

1.4.2.2.1. MITOCHONDRIAL DYSFUNCTION

As shown in figure 1.8, many hypotheses exist to explain the loss of dopaminergic neurons in PD. One theory states that nigral neurons are sensitive to environmental contaminants and that defects in mitochondrial complex I leads to impaired energy metabolism (Orth & Schapira 2002). Mitochondrial dysfunction in PD may also be dependent on dopamine which has been shown to inhibit complex I when injected into the ventricles of rats (Ben Shachar *et. al.*, 1995). It is thought that this toxic effect is caused by H₂O₂ which is produced during dopamine metabolism (Gluck *et. al.*, 2002) or by 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid (an oxidation product of 5-S-cysteinyldopamine, which has shown to irreversibly block complex I (Li & Dryhurst 1997). Moreover, a report by Sherer *et. al.* in 2002 suggested that the dopaminergic neurons have an intrinsic sensitivity to complex I defects since rotenone inhibits complex I throughout the brain but only induced neurotoxicity in the dopaminergic neurons.

1.4.2.2.2. GENETIC COMPONENT

α -synuclein is a protein encoded by a gene mutated in a rare early onset of the familial form of PD. This protein aggregates easily when oxidised (Giasson *et. al.*, 2000). Mutations in α -synuclein have been linked to autosomal-recessive PD. The ubiquitin proteasomal system is responsible for mediating the removal of unwanted ubiquitinated proteins (Lim *et. al.*, 2003) and the 20S proteasome is responsible for the degradation of non-ubiquitinated oxidised proteins (Dunlop *et. al.*, 2002). Oxidative stress severely compromises the normal functioning of the 20S proteasome (Davies 2001) and may also block or overload the ubiquitin proteasomal system and ultimately cause cell death (Chung *et. al.*, 2001).

1.4.2.2.3. OXIDATIVE STRESS

Postmortem studies have shown that oxidative stress occurs not only in healthy neurons, but in diseased nigral tissue as well. Carbonyl modifications, which are indicative of protein oxidation, 4-hydroxy-2,3-nonenal, an aldehyde produced during lipid peroxidation, and 8-hydroxyguanosine, a product of nucleoside oxidation are all elevated

in the substantia nigra of PD brains compared to age matched controls (Yoritaka *et. al.*, 1996; Floor & Wetzel 1998; Zhang *et. al.*, 1999a). Decreased levels of glutathione (GSH) have also been noted in the substantia nigra of PD patients (Pearce *et. al.*, 1997; Sofic *et. al.*, 1992). Furthermore, the metabolism of dopamine by monoamine oxidase (MAO)-A has been shown to increase GS-SG, the oxidised form of GSH (Spina & Cohen 1988). The imbalance in the GSH and GS-SG levels in the substantia nigra reflects the increase in oxidative stress and may also be due to dopamine oxidation (Stokes *et. al.*, 2000; Shang *et. al.*, 2003).

1.4.2.2.4. NEUROINFLAMMATION IN PARKINSON'S DISEASE

Human and animal studies have implicated neuroinflammation in the pathogenesis of PD (Langston *et. al.*, 1999; McGeer *et. al.*, 1988). An increasing number of experimental observations indicate that the inhibition of inflammatory mechanisms could attenuate the degeneration of nigrostriatal dopamine-containing neurons (Liu *et. al.*, 2000; Du *et. al.*, 2001; He *et. al.*, 2001; Tiesmann & Ferger 2001). Microglia, the resident immune cells in the brain, are sensitive to even minor disturbances in the CNS homeostasis and become readily activated during most neuropathological conditions such as PD, AD, multiple sclerosis, trauma and stroke (Kreutzberg 1996; Lui & Hong 2003). As mentioned in section 1.4.1.3.2, microglia are thought to contribute to neuronal damage through the release of pro-inflammatory and neurotoxic substances. These factors include pro-inflammatory cytokines such as tumor necrosis factor α and interleukin 1, ROS, eicosanoids and excitatory amino acids (Merrill & Benveniste 1996; Liu & Hong 2003).

1.4.2.3. THERAPEUTIC APPROACHES TO PARKINSON'S DISEASE

1.4.2.3.1. ENHANCING DOPAMINERGIC TRANSMISSION: LEVODOPA

Levodopa has been used for the symptomatic treatment of PD since the 1960 (Cotzias *et. al.*, 1967). It still remains the most effective drug in alleviating PD symptoms but is frequently associated with long-term complications such as motor fluctuations and dyskinesias (Freese *et. al.*, 1996). Levodopa loses its efficiency and becomes noticeable within a few years after treatment has been initiated (Nutt & Holford 1996). Therefore,

the aim of pharmacological management of PD is to achieve satisfactory symptomatic benefit without any adverse effects. Clinical research in PD and more recently novel therapeutic concepts such as regenerative and neuroprotective therapies have led to improvements in pharmacological management. The primary aim of PD treatment is to enhance dopaminergic transmission in the brain. The use of combination drug therapy such as levodopa and a dopamine receptor agonist relatively early in the course of PD has become an accepted strategy to avoid long-term levodopa-related complications (Stocchi 1994). Apart from levodopa and dopamine receptor agonists, pharmacological management of PD also includes catechol-O-methyl transferase (COMT), MAO inhibitors and anticholinergics.

1.4.2.3.2. THE ROLE OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS IN PARKINSONS DISEASE

Neuroinflammation also contributes to the neurodegenerative process in PD and the use of anti-inflammatory agents in PD has proven to be a promising neuroprotective strategy (Liu *et al.*, 2000; Araki *et al.*, 2001; He *et al.*, 2001; Teismann & Ferger 2001; Sairam *et al.*, 2003; Teismann *et al.*, 2003). Of the NSAIDS, sodium salicylate has been shown to significantly attenuate the 1-methyl-4-phenyl pyridinium ion (MPP⁺)-induced striatal dopamine depletion, an effect that is independent of prostaglandins but is associated with the inactivation of the hydroxyl radicals (Sairam *et al.*, 2003). COX-2 inhibitors have also shown promising neuroprotective results. They have been shown to block the degeneration of dopamine-containing neurons induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Teismann & Ferger 2001; Sairam *et al.*, 2003; Teismann *et al.*, 2003) and lipopolysaccharide (Araki *et al.*, 2001). MPTP is a potent neurotoxin that causes selective nigral dopaminergic lesions, resulting in PD (Burns *et al.*, 1983). It causes depletion of dopamine in rodents when administered in high doses (Heikkila *et al.*, 1984). Although epidemiological and case-control studies have suggested that NSAIDS may be neuroprotective and may reduce the incidence of AD (McGeer & McGeer 2001), a recent study suggests that the protective effect may not be due to the COX-2 inhibition because high doses of some NSAIDS lower A β production independently of COX inhibition (Weggen *et al.*, 2001). Therefore the actual role of

COX and their products in neurodegenerative diseases such as AD and PD needs to be further established.

1.5. MECHANISMS OF NEUROPROTECTION

1.5.1. ANTIOXIDANT ENZYMES

Due to the large amount of free radicals generated within animal cells and the damages inflicted by these free radicals, defenses within the body have evolved. These defenses revolve around the antioxidants. Antioxidants are categorised into those that prevent the generation of free radicals and those that intercept and scavenge free radicals that are generated. Antioxidants are present in both the aqueous and membrane compartments of the cell and may be in the form of either enzymes or non-enzymes (Cheeseman & Slater 1993).

The principal defense systems against ROS are superoxide dismutases (SOD), the reduced form of glutathione (GSH), GSH peroxidases, GSH reductase, catalase and antioxidant nutrients such as vitamin C and vitamin E. Cytochrome C and SOD catalyse the formation of O_2 from O_2^- and the byproduct of the dismutation of the O_2^- is H_2O_2 , which is converted to H_2O by catalase and the selenium-dependent GSH peroxidase. Lipid hydroperoxides are also detoxified by GSH peroxidase to form alcohols (Fang *et al.*, 2002). Since mitochondrial respiration results in a large amount of ROS (Papa & Skulachev 1997; Hensley *et al.*, 1998; Perez-Campo *et al.*, 1998) especially the O_2^- , the mitochondria possesses an efficient antioxidant system which consists of SOD, GSH peroxidase, GSH reductase, GSH, vitamin C and Vitamin E which prevents free radical-induced mitochondrial damage (Sutton & Winterbourn 1989; Gutteridge & Halliwell 1992).

1.5.2. ANTIOXIDANTS

Since free radical injury is important in the initiation or progression of AD and other neurodegenerative diseases, therapy to reduce the oxidative injury and induce endogenous antioxidant defenses that might prevent, delay or even ameliorate the disease

progress and hopefully diminish its human and social consequences (Delanty & Dichter 2000). The use of antioxidants in neurodegenerative diseases has been extensively studied (Braugher *et. al.*, 1989; Halliwell 1992; Ames *et. al.*, 1993; Beal 1995). Numerous antioxidants have been tested for their neuroprotective properties *in vitro* and *in vivo*. Vitamin E (Behl *et. al.*, 1992; Sano *et. al.*, 1997), the pineal hormone melatonin (Reiter *et. al.*, 1997; Reiter 1998), the lazaroids (21-aminosteroids) (Hall *et. al.*, 1997) and Gingko biloba (Oyama *et. al.*, 1996; Le Bars *et. al.*, 1997) are some antioxidants that have shown neuroprotective effects thus far. The efficacy of antioxidants may be enhanced if co-administered with other antioxidants or other neuroprotective drugs with different neuroprotective effects (Delanty & Dichter 2000). For example, vitamin C may help recycle vitamin E, and thus the two antioxidants may be best if they are co-administered (see chapter 6, section 6.1).

1.5.2.1. MELATONIN

Melatonin (N-acetyl-5-methoxytryptamine), the chief secretory product of the pineal gland in all mammals, was first characterised after its isolation from bovine pineal tissue by Lerner and co-workers approximately 50 years ago. It is an indoleamine synthesised from tryptophan and the synthesis of this hormone is under rhythmic control with peak levels produced at night in darkness (Reiter 1987). Melatonin has various biological functions in the body. It is known to be involved in the regulation of circadian (Malpoux *et. al.*, 2001) and seasonal (Reiter 1993) rhythms, immune function (Guerrero & Reiter 2002), retinal physiology (Dubocovich *et. al.*, 1999), tumor inhibition (Blask *et. al.*, 2002) and it is now known to be a powerful antioxidant (Reiter *et. al.*, 2002; Tan *et. al.*, 2002). Melatonin is directly involved in the neutralisation of various ROS and RNS such as the singlet oxygen, H_2O_2 , $\cdot OH$, hypochlorous acid and the peroxyxynitrite anion (Tan *et. al.*, 2002, Allegra *et. al.*, 2003). Melatonin also has indirect antioxidant activity since it also stimulates the synthesis of important intracellular antioxidants such as GSH, superoxide dismutase, GSH peroxidase and GSH reductase (Pablos *et. al.*, 1998; Urata *et. al.*, 1999; Albarran *et. al.*, 2001). It also promotes the enzymatic recycling of GSH so it remains mostly in its reduced form (Reiter *et. al.*, 2000). This neurohormone also reduced the amount of free radicals produced in the mitochondria, by promoting oxidative

phosphorylation which subsequently reduces electron leakage and promotes adenosine triphosphate (ATP) generation. Melatonin has been shown to be neuroprotective in a diverse range of free radical-induced models (Reiter 1998; Pappolla *et. al.*, 2000; Antolin *et. al.*, 2002; Reiter *et. al.*, 2002). Melatonin is metabolised in the liver to its main metabolite, 6-hydroxymelatonin which also has antioxidant properties (Matuszak *et. al.*, 1997). It has been reported that 6-hydroxymelatonin can also scavenge the hydroxyl free radical (Matuszak *et. al.*, 1997).

Moreover, patients with AD have decreased melatonin levels in the periphery and the administration of this indoleamine induced improvements in circadian rhythms and insomnia in these patients (Brusco *et. al.*, 2000; Cardinali 2000; Monti & Cardinali 2000).

1.5.3. ANTI-INFLAMMATORY DRUGS

Several studies have demonstrated that chronic NSAID use reduces the incidence of AD, suggesting that NSAIDS may be neuroprotective (McGeer & McGeer 1995). Although NSAIDS may be neuroprotective, the appropriate agents must be selected. These should act on targets based on correlations between epidemiology and molecular pathology. The need for such correlations has been illustrated by failures in clinical trial of prednisone, the COX-2 inhibitors celecoxib and nimesulide, and hydrochloroquine (Aisen *et. al.*, 2000; Sainali *et. al.*, 2000; van Gool *et. al.* 2001; Aisen *et. al.*, 2002). These agents are very effective in treating arthritic conditions but show little to no evidence in the beneficial use in neuroinflammation. The use of steroids in neuroinflammation should be avoided since these agents themselves cause hippocampal damage (Hoschl & Hajek 2001). Unlike COX-1, COX-2 is highly expressed in normal pyramidal neurons as well in the pyramidal neurons in the AD brain (Yasojima *et. al.*, 1999). Therefore, COX-2 inhibitors will primarily target pyramidal neurons rather than the microglia and since COX-1 is also contributes significantly to the inflammation process, COX-2 inhibitors may be inappropriate. Preliminary clinical studies thus far have suggested that NSAIDS decrease the rate of cognitive decline in AD (Breitner *et. al.*, 1992; Aisen & Davies 1994; McGeer & McGeer 1995; McGeer *et. al.*, 1996). Results have also suggested that

NSAIDS cause a delay in the onset or slow down the progression of AD (Rogers *et. al.*, 1993; Breitner *et. al.*, 1995; Breitner 1996). It has been suggested that the most prominent activity of NSAIDS is the inhibition of COX and lipooxygenases, which may lead to the inhibition of prostaglandin synthesis and subsequently block ROS formation (Breitner 1996; Nikolic & Breeman 2001).

1.5.3.1. TOLMETIN

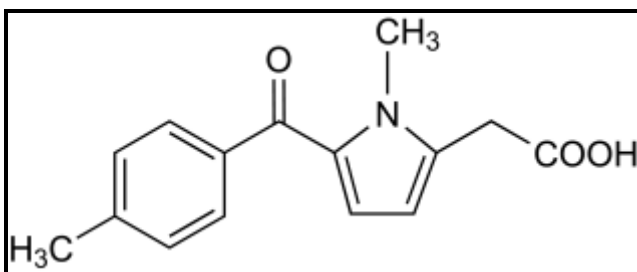


Figure 1.9: Illustration of the structure of tolmetin

(<http://www.medicinescomplete.com/mc/clarke/current/images/clk1627c001.gif>)

The potent anti-inflammatory of indomethacin (indole acetic acid derivative) prompted the synthesis of newer acetic acid derivatives by replacing the indole ring with a pyrrole ring (Carson & McKnistry 1971). This resulted in the production of 1-methyl-5-(p-toluoyl)-1H-pyrrole-2-acetic acid, known as tolmetin (figure 1.9), a NSAID with potent anti-inflammatory, analgesic and antipyretic effects (Carson & McKnistry 1971; Wong *et. al.*, 1973; Freeman *et. al.*, 1977). Tolmetin is used in doses of 600-1200 mg/day. Although gastrointestinal disturbances have been reported, clinical studies have shown that tolmetin was very safe and was well tolerated in geriatric and non-geriatric patients (Ehrlich 1983; O'Brien 1983). However, tolmetin has a short half-life and need to be administered frequently (O'Brien 1983).

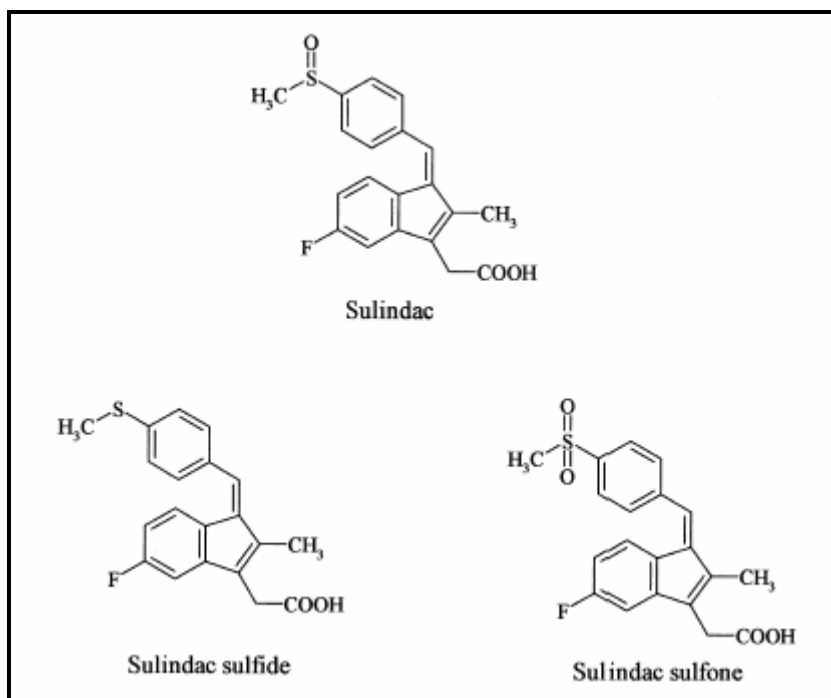
1.5.3.2. SULINDAC

Figure 1.10: Structure of sulindac and its metabolites sulindac sulfide and sulindac sulfone. (Fernandes *et. al.*, 2003).

As shown in figure 1.10, sulindac is a sulfoxide prodrug which is converted *in vivo* to the metabolites sulindac sulfide and sulindac sulfone (Duggan *et. al.*, 1977a; Duggan *et. al.*, 1980). It is the sulindac sulfide metabolite and not sulindac sulfone that blocks prostaglandin synthesis by the nonselective inhibition of COX (Duggan *et. al.*, 1977b). After metabolism, it is excreted in bile and thereafter reabsorbed from the intestine. The enterohepatic cycling prolongs the duration of action to 12-16 hours. It has been recently established that the metabolism of sulindac enhances its free radical scavenging properties (Fernandes *et. al.*, 2003). The authors have demonstrated that sulindac sulfide as well as sulindac sulfone exhibit a more potent antioxidant effect than the parent compound.

1.5.3.3. TURMERIC

Turmeric is a member of the *Curcuma Longa* species of the family Zingiberaceae. The dried ground rhizome of the perennial herb is called turmeric in English, haldi in Hindi, ukon in Japanese and has been used in Asian medicine since the second millennium (Brouk 1975). In addition to its aromatic, stimulant and coloring properties in the diet, turmeric is mixed with other natural compounds such as slaked lime and has been used topically as a treatment for wounds, inflammation and tumours (Thaloor *et. al.*, 1998). In certain parts of South East Asia, turmeric is consumed up to 1.5g per person daily but smaller quantities have been used for medicinal purposes (Eigner & Sholz 1999).

1.5.3.3.1. CURCUMIN

Raw turmeric contains volatile oils such as tumerone, atlantone and zingiberone. Turmeric also contains sugars, proteins, resins as well as a rich source of a group of phenolic compounds known as the curcuminoids (figure 1.11). Curcuminoids can be defined as phenolic compounds derived from the roots from the *Curcuma* spp. The three main curcuminoids in turmeric are curcumin (CURC), demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC). Curcumin was first characterised in 1910 and is generally regarded as the most active constituent of turmeric preparations (Milobedzka *et. al.*, 1910; Heath *et. al.*, 2004). Serum curcumin concentrations peak 1-2 hours after oral intake and gradually decrease within 12 hours (Cheng *et. al.*, 2001).

The primary structure of curcumin (figure 1.11) is a conjugated diene with an adjacent phenolic group that has general antioxidant properties. The addition of the phenolic group, the methoxy group on the phenyl ring and the 1,4-diketone system may be important structural features that contribute to this molecules potent antioxidant properties (Sreejayan & Rao 1996). It has been reported that curcumin is a more potent free radical scavenger than vitamin E (Zhao *et. al.*, 1989). Studies have shown that curcumin is a powerful scavenger of the superoxide anion (Kunchandy & Rao 1990), the hydroxyl radical (Kunchandy & Rao 1989), nitrogen dioxide (Unnikrishan & Rao 1995) and it also protects DNA against singlet-oxygen-induced strand breaks (Subramanian *et. al.*, 1994) and lipids from peroxidation (Sreejayan & Rao 1993). Oral administration of

curcumin has been shown to be centrally neuroprotective (Kaul & Krishnakantha 1997; Rajakrishnan *et. al.*, 1999).

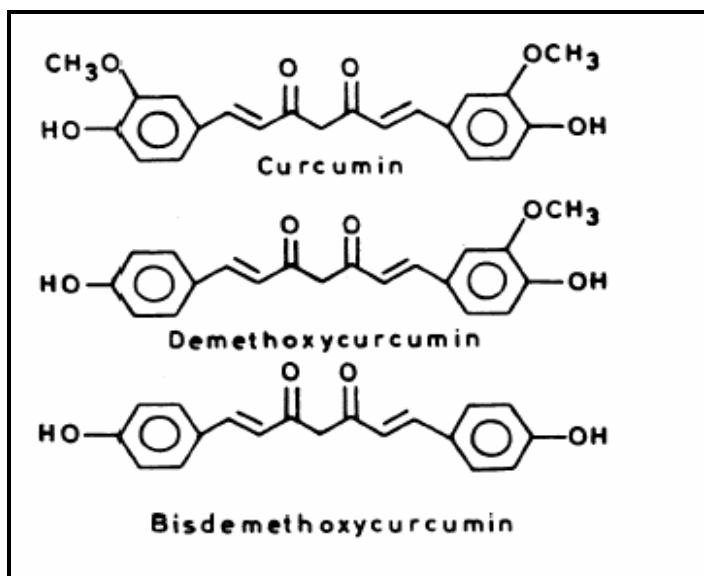


Figure 1.11: Structure of the curcuminoids.

It has long been known that curcumin can inhibit COX activity in rat peritoneal neutrophils and human platelets (Ammon & Wahl 1991). Its ability to inhibit induction of COX-2 gene expression has been demonstrated in oral and colon epithelial cells in vitro (Khafif *et. al.*, 1998; Zhang *et. al.*, 1999). Unlike selective COX-2 inhibitors, which inhibit the catalytic activity of the COX enzyme, curcumin decreases COX-2 expression at the transcriptional level and may also inhibit COX-1 transcription (Plummer *et. al.*, 1999).

The ability of many natural agents derived from the diet to act at more than one level in a cellular pathway is important in the prevention or treatment of diseases with multifactorial aetiologies such as cancer (Gescher *et. al.* 2001). Curcumin has been shown to prevent cancer in the colon, skin, stomach, liver, lung, duodenum, soft palate and breasts of rodents (Conney *et. al.*, 1991; NCI & DCPC 1996; Rao *et. al.*, 1995). Topical administration of curcumin has also been shown to inhibit chemical carcinogenesis of the skin (Conney 2003).

Many clinical studies involving curcumin have been conducted and no reports of any discernable adverse affects have been reported (Deodhar *et. al.*, 1980; Cheng *et. al.*, 2001; Sharma *et. al.*, 2004).

1.5.4. CHELATING AGENTS

Since neurodegenerative disorders such as AD is linked to A β deposition, which may be mediated by abnormal metal interactions as well as metal-mediated oxidative stress, the use of metal chelators has received much attention (Cuajungco *et. al.*, 2000; Richardson 2004). Chelating agents are drugs that are used to prevent or reverse the toxic effects of a heavy metal on an enzyme or other cellular components or to accelerate the elimination of the metal from the body. Chelating agents are usually flexible molecules with two or more electronegative groups that can form a covalent bond with cationic metal atoms. The efficiency of the chelator is partly determined by the number of ligands available for metal binding. The chelating ligands include functional groups such as –OH, –SH and –NH which can donate electrons for coordination during metal binding.

1.7. OBJECTIVES OF THIS STUDY

Since increasing evidence suggests that NSAIDS may be therapeutically beneficial in AD (Rogers *et. al.*, 1993; Bruce-Jones *et. al.*, 1994; McGeer & McGeer 1995) and PD (Liu *et. al.*, 2000; Araki *et. al.*, 2001; He *et. al.*, 2001; Teismann & Ferger 2001; Sairam *et. al.*, 2003; Teismann *et al.*, 2003), this study was conducted to firstly determine the potential neuroprotective effects of the NSAIDS tolmetin, sulindac and the commonly used curry spice, turmeric, and secondly to attempt to elucidate possible mechanism of neuroprotection, should it be occurring.

The possible neuroprotective properties of tolmetin and sulindac were established by various biological assays and inorganic studies. These experiments included:

- Measuring the antioxidant and free radical scavenging potential of these agents in the presence of various neurotoxins in rat brain homogenates as well as in primary hippocampal neurons.
- The effect of these NSAIDS on rat hippocampal neurons were examined histologically after intrahippocampal injections of the excitatory amino acid, QA. Receptor binding and apoptotic studies were also conducted. The spatial reference memory of male Wistar rats was also tested in a water maze. The extent of protein oxidation and glutathione in rat hippocampus was also determined.
- The effect of these NSAIDs on the liver enzyme tryptophan 2,3-dioxygenase was established with the subsequent changes in the biogenic amine levels in the hippocampus and striatum. The amount of melatonin produced by the pineal gland was also determined.
- Metal binding studies involving iron (II) and iron (III) were conducted by employing techniques such as UV/VIS, infrared and electrochemical analysis.

Statistics confirm that there is a reduced incidence of AD among the inhabitants of Indian villagers compared to the western world (Ganguli *et. al.*, 2000; Lim *et. al.*, 2001). It has been thought that this is due to the various antioxidant and neuroprotective components in the spices commonly consumed in the diet of Indians. One such spice is

turmeric. For the purposes of this study, the three main active components of turmeric (curcumin, demethoxycurcumin and bisdemethoxycurcumin) were extracted and tested for their neuroprotective properties against lead (Pb)-induced neurotoxicity.

- Curcumin, demethoxycurcumin and bisdemethoxycurcumin were separated using semi-preparative thin layer chromatography (TLC). The purity of the curcuminoids was confirmed by the use of TLC, nuclear magnetic resonance (NMR) as well as mass spectrometry (MS).
- The antioxidant effects of each of the curcuminoids were determined.
- The ability of the curcuminoids to prevent or reduce Pb-induced memory deficits was measured in the water maze task. Rat hippocampal glutathione levels as well as the extent of proteins oxidised were also measured using known methods.
- The loss of a methoxyl group in each of the curcuminoid in relation to their metal (Pb) binding properties was also determined by the use of various spectroscopic and electrochemical techniques.

It is hoped that this study aids in the further understanding of the use of anti-inflammatory agents in neuroinflammation and determine whether tolmetin, sulindac and turmeric may be beneficial in neurodegenerative disorders such as AD and PD.

CHAPTER 2

LIPID PEROXIDATION

2.1. INTRODUCTION

A major role of oxygen radical-induced cellular injury has been strongly implicated in the pathophysiology of CNS injuries (Braugher & Hall 1989; Braugher *et al.*, 1985). Oxidative degeneration has also been increasingly recognised as a player in Parkinson's disease (Cohen 1986; Jenner *et al.*, 1992), Alzheimer's disease (Subbarao *et al.*, 1990; Smith 1994) and motor neuron diseases like amyotrophic lateral sclerosis (Rosen *et al.*, 1993; Carri *et al.*, 2003).

Proteins, nucleic acids and carbohydrates are all susceptible to oxygen radical damage, but perhaps the most avid targets of oxygen radical-induced injury are cell membrane lipids, including cholesterol and, in particular, polyunsaturated fatty acids (PUFA's). The process of lipid damage by oxygen radicals is known as lipid peroxidation (LP). CNS tissue is the most sensitive to LP. One reason for this is the high content of iron found in many parts of the brain (Zaleska & Floyd 1985). Another reason for the sensitivity of nervous tissue to LP is that it contains a higher proportion of polyunsaturated fatty acids, such as linoleic acid (18:2) and arachidonic acid (20:4), in comparison to other tissue (White 1973).

LP is initiated when a free radical attacks a membrane. As shown in figure 2.1, PUFA's are attacked by the free radical, which removes a hydrogen atom from the PUFA resulting in a lipid-derived free radical. The propagation step begins with the rapid addition of molecular oxygen to the lipid-derived free radical forming a lipid peroxy radical. This lipid peroxy radical can attack other lipids which then generate lipid-derived free radicals and a lipid hydroperoxide. This is a chain reaction and once initiated, termination is extremely difficult (Krinsky 1992). Termination is possible when two free radicals react together to form a non-radical product (Burton & Ingold 1989). The use of antioxidants is another mechanism essential to limit ROS-induced LP (Burton & Ingold 1989).

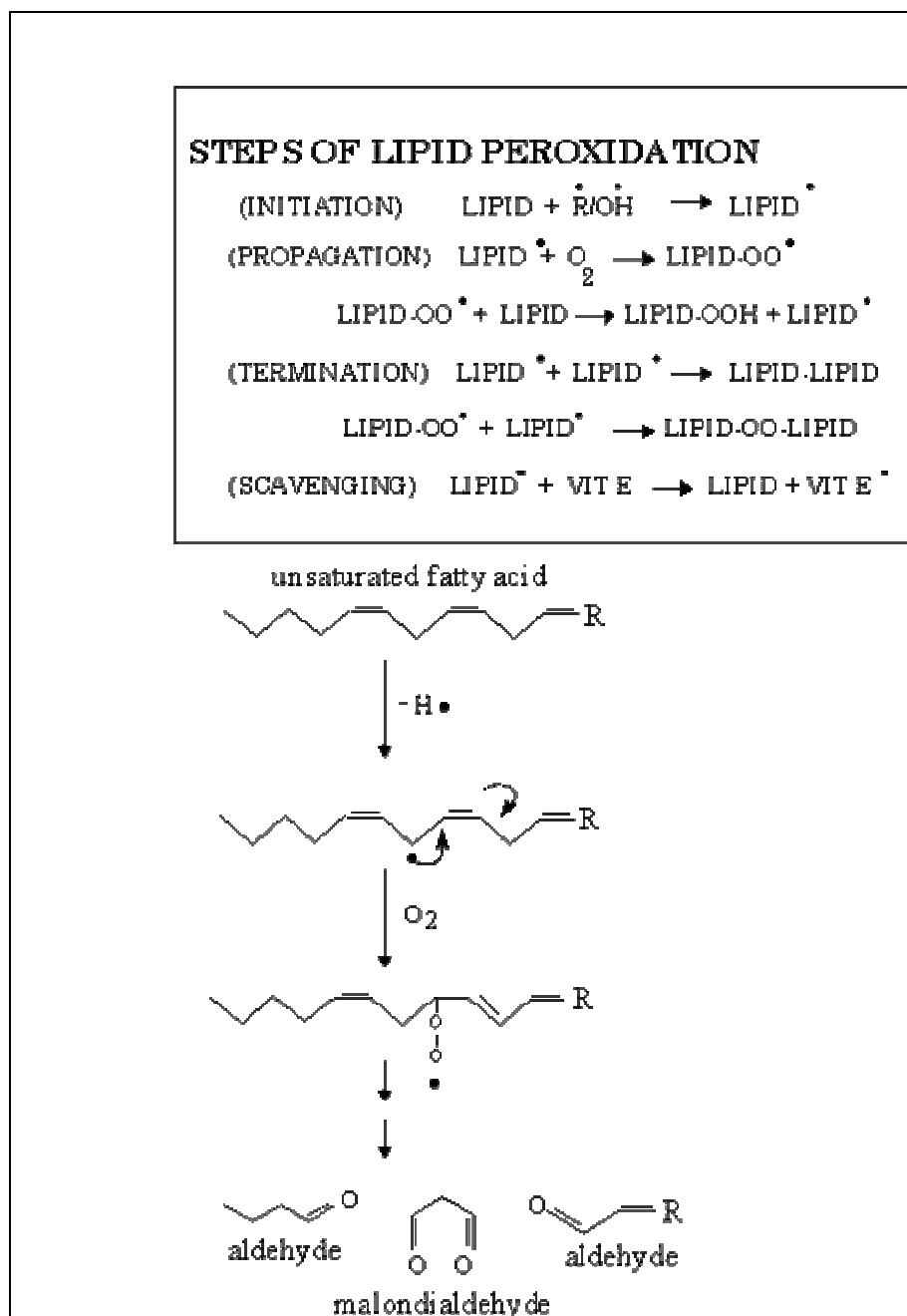


Figure 2.1: A schematic representation of the steps of lipid peroxidation: initiation, propagation and termination. The end product of lipid peroxidation is malondialdehyde. (<http://www.bb.iastate.edu/~jat/image44.gif>).

The measurement of recognised elevated end products of lipid peroxidation in animal material is the evidence most frequently quoted in support of free radical induced tissue

damage (Gutteridge & Halliwell 1990). The thiobarbituric acid test is the most widely used assay for measuring lipid peroxidation (Gutteridge & Halliwell 1990). The assay involves the reaction between malondialdehyde (MDA), an end product of lipid peroxidation, and 2 molecules of thiobarbituric acid (TBA) to yield a pink chromogen (figure 2.2), which is measured colorimetrically at 532 nm using a spectrophotometer (Gutteridge & Halliwell 1990; Southgate & Daya 1999).

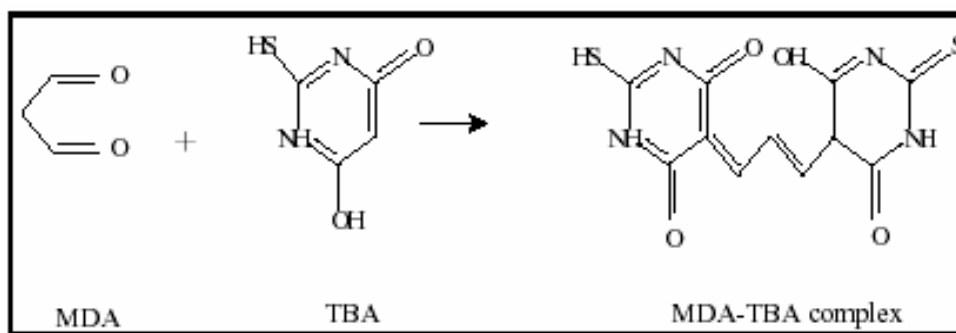


Figure 2.2: Reaction between MDA and 2 molecules of TBA to produce a pink complex that can be extracted and measured at 532nm (Mead *et. al.*, 1986).

As a result of the increasing realisation that LP is probably of vital importance in both acute and chronic neurodegenerative disorders, interest has been growing in the potential treatment of these conditions with antioxidant compounds, i.e. agents that scavenge oxygen radicals and/or directly inhibit LP.

2.2. EFFECT OF QUINOLINIC ACID ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

2.2.1 Introduction

QA is a naturally produced metabolite of tryptophan metabolism (Foster *et al.*, 1984; Heyes & Morrison 1997). QA has been shown to be present in normal postmortem human brains at levels similar to those of other species (Wolfensberger *et. al.*, 1984). QA concentrations have also been shown to increase during the natural aging process in rats (Moroni *et. al.*, 1984).

The following experiment was conducted to investigate the effects of QA on lipid peroxidation in rat forebrain homogenate.

2.2.2. Materials and Methods

2.2.2.1. Chemical Reagents

Quinolinic acid (QA), tolmetin, sulindac, butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and 1,1,3,3-tetramethoxypropane (99%) were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. Trichloroacetic acid (TCA) ethanol and butanol were purchased from Saarchem, Johannesburg, South Africa. All other reagents used were of the highest quality available.

QA, tolmetin, TBA and TCA were prepared by dissolving in Milli-Q water while BHT was prepared by dissolving it in absolute ethanol.

2.2.2.2. Animals

Adult male Wistar rats, purchased from the South African Institute for Medial Research (Johannesburg, South Africa) were used throughout the study. The animals were housed under artificial illumination with a daily photoperiod of 12 hours (lights on at 06h00). The animal-house temperature was maintained at a constant 20°C to 24°C, while an extractor fan ensured the constant removal of stale air. The rats (250-300g each) were

housed four to five per cage with food and water provided *ad libitum*. The Rhodes University animal ethics committee approved all protocols for the experiments.

2.2.2.3. Brain Removal

Rats were sacrificed by neck fracture and decapitated. The brain was exposed by making an incision through the bone on either side of the parietal structure, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain, which was easily removed for use in experiments.

2.2.2.4. Homogenate Preparation

Once the brain was removed, it was weighed and rapidly homogenized in a glass-teflon hand held homogenizer in iced cold 0.1M phosphate buffered saline (PBS), at pH 7.4 to yield a 10%w/v homogenate. This was used immediately for assay.

2.2.2.5. Preparation of Standard Curve

1,1,3,3-Tetramethoxypropane was used as a standard. Varying concentrations of the standard were prepared in reaction tubes using PBS as the diluent. To these tubes BHT (0.5ml) and TCA (1ml) was added. An aliquot of 2ml of this solution was added to TBA (0.5ml) and incubated for 1 hour. A calibration curve was generated by measuring the absorbance at 10 nmole/ml intervals. The absorbance was read at 532nm using a GBC UV/VIS 916 spectrophotometer and plotted against the concentration (Appendix 1).

2.2.2.6. Lipid Peroxidation Assay

Lipid Peroxidation was determined from a modified method of Placer *et. al.*, 1966.

Homogenate (1ml), containing varying concentrations of QA (0; 0.25; 0.5 and 1mM) was incubated at 37°C in an oscillating water bath for 1 hour. At the end of the incubation period 0.5ml BHT (0.5mg/ml in ethanol) and 1ml 25% TCA was added. The tubes were sealed and heated for 10 minutes in a boiling water bath to release protein bound malondialdehyde (MDA), the product of lipid peroxidation. To avoid adsorption of MDA to insoluble proteins, the samples were cooled to 4°C and centrifuged at 2000g for 20

minutes. Following centrifugation, 2ml of the protein free supernatant was removed from each tube and 0.5ml aliquots of 0.33% TBA was added to this fraction. All tubes were heated for 1 hour at 95°C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2ml butanol. The absorbance was read at 532nm and MDA levels were determined from a standard curve generated from 1,1,3,3-tetramethoxypropane. Final results are represented as nmoles MDA/mg tissue. The experiment was replicated five times.

2.2.2.7. Statistical Analysis

The results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p < 0.05$ (Zar 1974).

2.2.3. Results

Figure 2.3 shows that QA significantly induced lipid peroxidation in a concentration-dependent manner in rat brain homogenate.

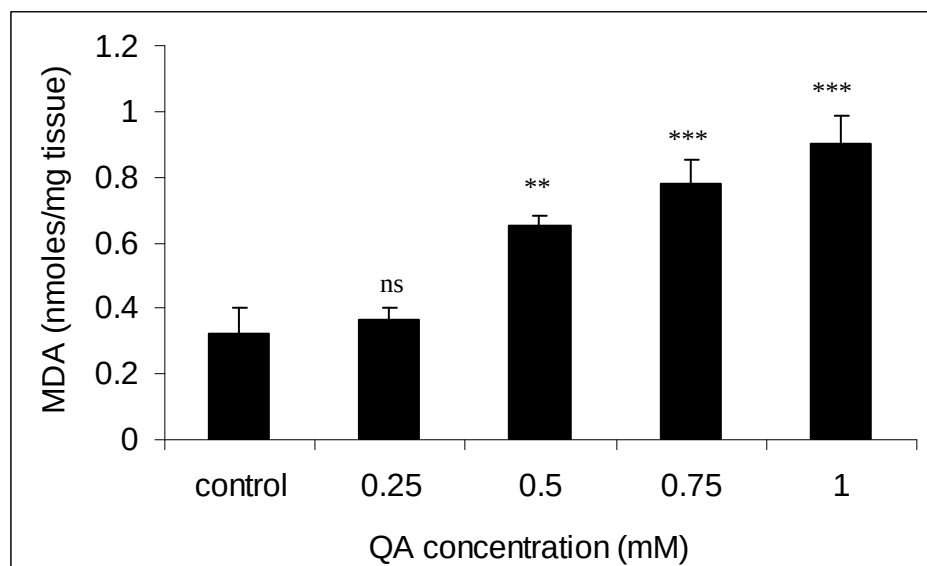


Figure 2.3: Effect of Quinolinic acid (0-1mM) on lipid peroxidation in rat brain homogenate. Each bar represents the mean $\pm$ SD (n=5). ns(p>0.05), ** (p<0.01) and *** (p<0.001) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

2.2.4. Discussion

Research has demonstrated that QA potently induced lipid peroxidation in rat brain homogenates (Rios & Santamaria 1991). These findings are consistent with the results obtained in the present experiment where QA at concentrations of 0.5, 0.75 and 1mM show significant induction of lipid peroxidation in the rat brain homogenate in a concentration-dependent manner.

It has been established that the brain is vulnerable to oxidative damage. One of the many reasons for this is that the brain is rich in peroxidisable fatty acids and contains high levels of transition metals, which can catalyse the formation of the reactive hydroxyl radical (Floyd, 1999). The lipid peroxidation induced by QA could be due to the possibility that QA acts as a pro-oxidant, thus initiating lipid peroxidation. Stipek *et. al.* (1997) showed that the effect of QA on lipid peroxidation in rat brain homogenate is dependent on iron. QA is able to chelate ferrous ions to form a complex, which stimulates

lipid peroxidation. Thus the implication is that QA does not have a direct peroxidative effect *in vitro*, but modulates lipid peroxidation through its interaction with ferrous ions.

2.3. EFFECT OF TOLMETIN AND SULINDAC ON QUINOLINIC ACID INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE.

2.3.1. Introduction

Experiment 2.2 demonstrates that QA, in a concentration-dependent manner, induces LP in rat brain homogenate. This experiment was conducted to determine the effect of increasing concentrations of tolmetin or sulindac (0.25-1mM) on QA (1mM)-induced LP.

2.3.2. Materials and Methods

Experiments were conducted in the exact same manner as in experiment 2.2. In addition, tolmetin or sulindac was also added to the incubation medium at concentrations (0-1mM). All results were analyzed as described in section 2.2.2.7. The experiment was replicated five times.

2.3.3. Results

QA (1mM) significantly induced lipid peroxidation in rat brain homogenate. This phenomenon was reduced by the co-treatment of the homogenate with both tolmetin and sulindac (figure 2.4). Increasing concentrations of tolmetin or sulindac did not demonstrate a concentration-dependent decrease in lipid peroxidation. The NSAIDS alone have no effect on LP.

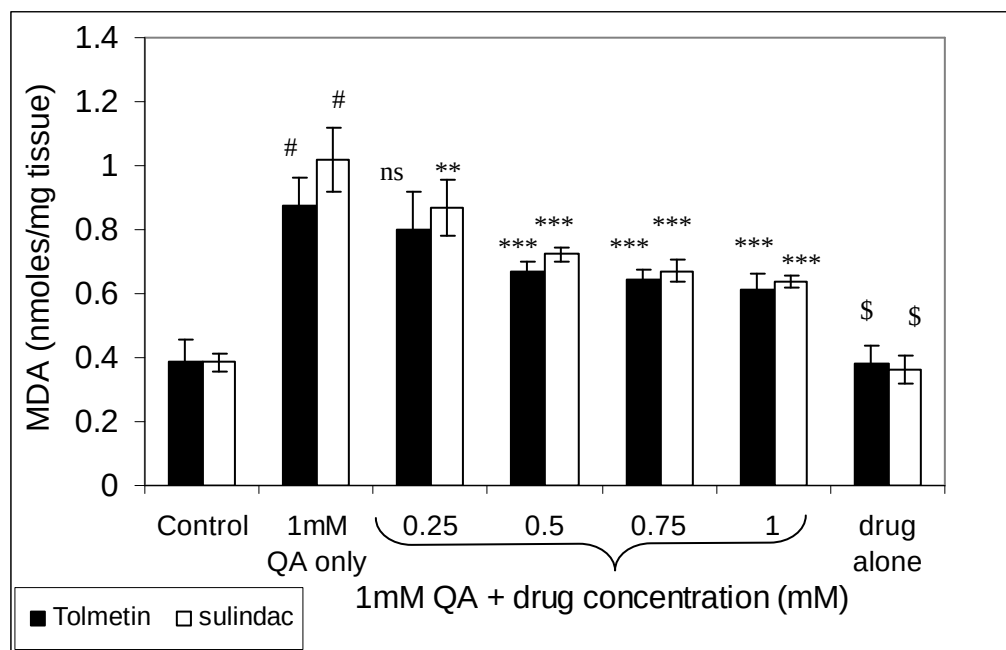


Figure 2.4: Effect of tolmetin and sulindac on QA (1mM)-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean $\pm$ SD (n=5). #($p<0.001$) and \$($p>0.05$) compared to controls. ns($p>0.05$), **($p<0.01$), ***($p<0.001$) in comparison to QA only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

2.3.4. Discussion

It is thought that QA-induces LP is due to the production of free radicals. PUFAs are prone to be oxidized by free radicals and other ROS (Henning *et. al.*, 2001). This can be alleviated by the use of antioxidants such as vitamin C, vitamin E, carotenoids (Evans & Halliwell 2001; Fang 2002) and melatonin (Reiter 1998; Southgate & Daya 1999). Other NSAIDS have also demonstrated the ability to inhibit or reduce lipid peroxidation. Ibuprofen, naproxen, piroxicam as well as indomethacin have been shown to decrease lipid peroxidation in rats pretreated with these agents by interfering with the oxidative stress pathway (Bilodeau *et. al.*, 1995) while some NSAIDS such as ibuprofen and aspirin decrease lipid peroxidation in rat brain homogenate by quenching the free radicals produced (Daya *et. al.*, 2000; Lambat *et. al.*, 2000).

In 1997, Stipek and co-workers demonstrated that QA induces lipid peroxidation in the homogenate by complexing with ferrous ions. This complex induces the LP. In this experiment, the co-treatment of the homogenate with tolmetin or sulindac reduces lipid peroxidation. Although the concentration of the NSAIDS in these experiments is pharmacological, it is necessary to use high concentrations since the concentration of the toxin used to generate the free radicals is also high. Tolmetin and sulindac reduce lipid peroxidation by possibly scavenging the free radicals produced but do not show a concentration-dependent decrease in lipid peroxidation. It is possible that tolmetin and sulindac reduce lipid peroxidation by competing with QA for the ferrous ions thus reducing lipid peroxidation by preventing the QA-ferrous ion complex formation. This possibility has been investigated in chapter 10.

2.4. EFFECT OF QUINOLINIC ACID ON LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS

2.4.1. Introduction

Experiment 2.2 demonstrates that QA has the ability to induce lipid peroxidation in rat brain homogenate. Since rat brain homogenate mostly consists of burst cells, QA possibly induces lipid peroxidation by acting as a pro-oxidant. It has also been shown that the effect of QA on lipid peroxidation in rat brain homogenate is dependent on iron (Stipek *et. al.*, 1997). In these experiments NMDA receptor stimulation by QA is the most likely mechanism through which this excitatory amino acid induces lipid peroxidation.

2.4.2. Materials and Methods

2.4.2.1. Chemical Reagents

Trypsin, trypsin inhibitor, trypan blue and cytosine- β -D-arabinofuranoside were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. Eagle's Minimum Essential Medium (MEM), foetal calf serum, sterile disposable cell scrapers were purchased from Highveld Biologicals, South Africa. Tissue culture flasks (25cm³) and Falcon tubes were purchased from Corning Costar (USA). Sodium benzylpenicillin (Novopen), streptomycin sulphate (Novo-Strep) and amphotericin B (Fungizone) were supplied by Novo Nordisk (Pty) Ltd, South Africa. All other reagents were as per section 2.2.2.1.

2.4.2.2. Routine cell culture procedures

All glassware and equipment used for the cell culture experiments was sterilized by autoclaving for 20 minutes. Following this, glassware and equipment were exposed to ultraviolet light. All cell culture procedures were conducted in a laminar flow bench which had previously been sterilized by regular swabbing with 70% ethanol and exposure to ultraviolet light.

2.4.2.3. Preparation of culture reagents

The incubation medium, MEM (500ml) was supplemented with foetal calf serum to produce a final concentration of 16% (v/v). This was thereafter supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml) and cytosine (10µM final concentration). PBS (pH 7.4) was prepared and sterilized by autoclaving for 20 minutes. This was used as the washing/bathing medium for the neurons and solvent for most chemicals and drugs.

2.4.2.4. Animals

Timed pregnant Wistar rats were purchased from the South African Institute for Medical Research (Johannesburg, South Africa). The animals were cared for as described in section 2.2.2.2.

2.4.2.5. Establishment of primary hippocampal neurons

Primary hippocampal neuronal cultures were established from the brains of one day old rat pups. The heads were decapitated with scissors and the brains were carefully removed. The hippocampus was removed from each pup and transferred to a beaker containing cold PBS. All subsequent steps took place under a laminar flow hood.

The brains were pooled and chopped into very fine pieces before being transferred to sterile 15ml Falcon tubes containing 3ml of 0.1% trypsin. After 10minutes of incubation at 30°C in a shaking water bath, the tissue pieces were dissociated by triturating through narrow fire-polished pasture pipettes. The tissue was then incubated for a further 5 minutes at 30°C in a shaking water bath. Thereafter tissue and dissociated cells were collected by centrifugation at 600 x g for 5 minutes. The supernatant was carefully removed and 3ml of 0.1% trypsin inhibitor was added. After incubating the cells for 5 minutes at 30°C, the cells were collected by centrifugation at 600 x g for 5 minutes. The supernatant was removed and 2-3ml of media was added and the cells were thoroughly suspended. An aliquot of 50µl of the cell suspension was added to 100µl of 0.04% trypan blue* and the resulting suspension was placed on a haemocytometer and counted. The initial cell suspension stock was diluted to obtain a density of 1 000 000 viable cells per

1ml media. To each 25cm³ cell culture flask, 1 000 000 cells in 1ml media was added followed by a further 1.5ml of MEM. Neurons were allowed to attach to the surface of the culture flask for 12-15 hours, after which the media was discarded and replaced with 3ml of fresh MEM. This MEM was supplemented with 5µM. Cell culture flasks were kept in an incubator set at 37°C, the humidity was maintained between 75-80% and the CO₂ was kept at approximately 5-7%.

*Trypan blue is a dye that is used to measure cell viability. Only dead cells are permeable to the dye, as these have damaged membranes. This results in dead cells staining blue, while living cells remain unstained (Dubinsky *et. al.*, 1995).

2.4.2.6. Lipid peroxidation assay

Lipid peroxidation was determined according to a modified method of Placer *et. al*, 1966. Six to eight day old cultures were used for the experiments. Before experimentation, each flask was washed twice with 3ml of PBS. Thereafter 1ml of fresh MEM was added. To this, 100µl of QA dissolved in MEM was added to produce final concentrations of 50; 100 or 200µM. The culture flasks were then kept in the incubator for 3 hours under the conditions mentioned above.

After 3 hours, the cells were scraped off the surface using a cell scraper and transferred to test tubes. After centrifuging for 10 minutes at 600 x g, the media was discarded and the cells were re-suspended in 1.1ml of PBS. A 100µl aliquot was removed for later enumeration using a haemocytometer. To the 1ml of sample that remained, 0.5ml BHT (0.05% in ethanol) and 1ml TCA (25%) were added. The rest of the experiment was conducted as described in section 2.2.2.6. The absorbance was read at 532nm and MDA levels were determined from a standard curve generated from 1,1,3,3-tetramethoxypropane (appendix 2). Final results are represented as nmoles MDA/10⁶ cells and were analysed as described in section 2.2.2.7. The experiment was replicated four times.

2.4.3. Results

Figure 2.5 demonstrates lipid peroxidation induced by QA in a concentration-dependent manner compared to control.

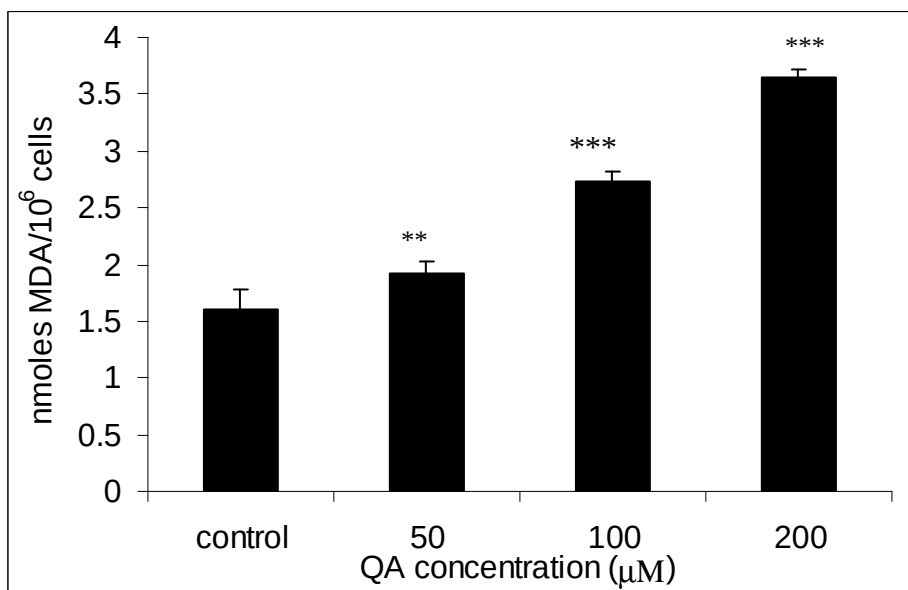


Figure 2.5: The effect of QA on lipid peroxidation in primary hippocampal neurons. Each bar represents the mean $\pm$ SD ($n=4$). **($p<0.01$) and ***($p<0.001$) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

2.4.4. Discussion

High concentrations of the NMDA receptors are found in the cerebral cortex and CA1 regions of the hippocampus (Monaghan & Cotman 1986). QA acts as an agonist on the NMDA receptors in the brain (Stone & Perkins 1981) allowing Ca^{2+} , Na^+ and K^+ to enter the neurons. The influx of these ions can alter the ionic balance of the neurons, leading to swelling and lysis (Coyle & Puttfarcken 1993; Schwarcz *et. al.*, 1983). The influx of Ca^{2+} into the cell can also activate many free radical producing pathways, which can prove toxic to the neurons (Fahn & Cohen 1992). This experiment shows that quinolinic acid has the ability to generate free radicals which in turn can cause lipid peroxidation.

2.5. EFFECT OF TOLMETIN AND SULINDAC ON QUINOLINIC ACID-INDUCED LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS

2.5.1. Introduction

The previous experiment shows that QA induces lipid peroxidation in a concentration-dependent manner in primary hippocampal neurons. Since tolmetin and sulindac both have the ability to reduce the damage initiated by QA in rat brain homogenate (experiment 2.3), this experiment was conducted to determine whether these anti-inflammatory agents can reduce the damage in the primary hippocampal neurons initiated by 200 μ M QA.

2.5.2. Materials and Methods

Experiments were conducted in the same manner as in experiment 2.4. QA (200 μ M) was used to initiate lipid peroxidation. In addition, tolmetin or sulindac was also added to the incubation medium at concentrations of 50; 100 or 200 μ M. Results were analysed as described in section 2.2.2.7. The experiment was replicated four times.

2.5.3. Results

As shown in figure 2.6, 200 μ M QA significantly ($p < 0.001$) induces lipid peroxidation. Whilst co-treatment of the neurons with tolmetin or sulindac reduces lipid peroxidation, these agents do not completely prevent QA-induced lipid peroxidation.

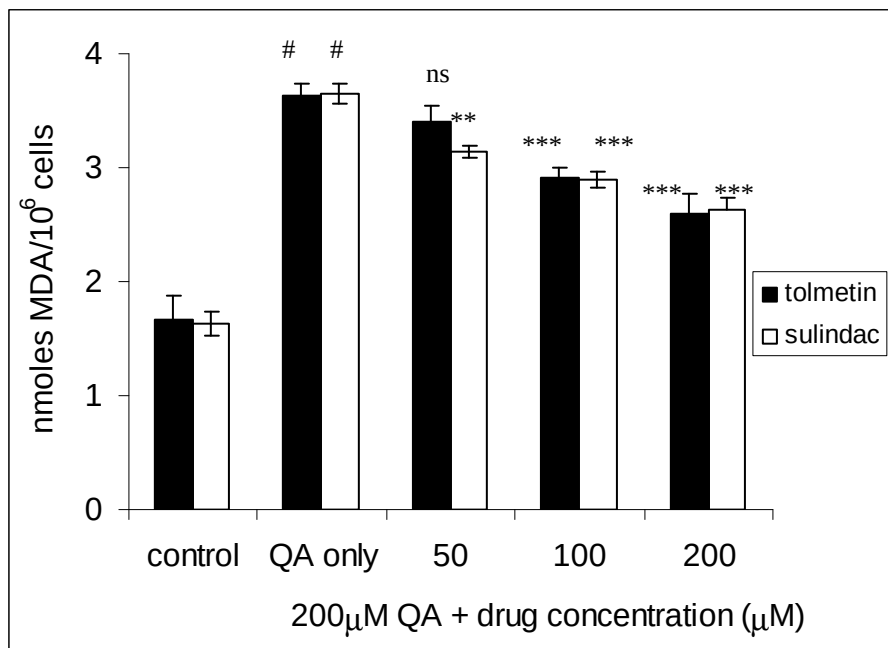


Figure 2.6: Effect of tolmetin and sulindac on 200 μ M QA-induced lipid peroxidation. Each bar represents the mean $\pm$ SD (n=4). # (p<0.001) compared to control. ns (p>0.05), ** (p<0.01) and *** (p<0.001) in comparison to QA only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

2.5.4. Discussion

Fernandes and co-workers have recently shown that other NSAIDs including tolmetin exhibit ROS and RNS scavenging activity. The indole derivatives such as indomethacin, ethodolac and acemetacin, the pyrole derivatives such as ketorolac and tolmetin and the oxazole derivative oxaprozin have shown scavenging activity against the peroxy radical (ROO $\cdot$), $\cdot$ OH, O₂ $\cdot^-$, nitric oxide (NO $\cdot$) and peroxynitrite (ONOO $\cdot$) free radicals. These authors also report that some NSAIDs such as indomethacin, ethodolac and acemetacin have similar if not greater scavenging activity for the ROO $\cdot$, O₂ $\cdot^-$ when compared to endogenous free radical scavengers such as glutathione and ascorbic acid. These authors have also previously reported that sulindac as well as its metabolites demonstrate strong scavenging activity against these free radicals (Fernandes *et. al.*, 2003).

This experiment shows that both NSAIDS offer approximately the same degree of protection, suggesting that these are equipotent antioxidants. Since tolmetin and sulindac reduce LP in rat brain homogenate as well as in primary hippocampal neurons, it is possible that these agents scavenge free radicals generated by QA. This experiment also demonstrates that these agents at lower concentrations have the ability to scavenge free radicals.

2.6. EFFECT OF CYANIDE ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE.

2.6.1. Introduction

Gunasekar and co-workers in 1996 established that the brain is the target organ in cyanide toxicity. Acute cyanide neurotoxicity has been recognized to cause cellular anoxia in the brain (Ballantyne, 1987; Yamamoto & Tang, 1996) and produces tonic and clonic seizures, convulsions (Way, 1985) while in some individuals a Parkinson-like condition may develop as a post toxicity sequel (Utti *et. al.*, 1985). Cyanide also produces dopaminergic toxicity accompanied by impaired motor function (Gunesekar *et. al.*, 1996). Due to a number of antioxidant enzymes being inhibited by cyanide, it is also believed that oxidative stress plays an important role in cyanide induced neurotoxicity (Ardelt *et. al.*, 1989).

The objective of this study was to characterise the effect of cyanide on neuronal membrane lipid peroxidation in rat brain homogenate.

2.6.2 Materials and Methods

2.6.2.1. Chemicals and Reagents

Potassium cyanide (KCN) was purchased from Sigma Chemicals Corporation, St Louis, U.S.A. KCN was prepared by dissolving it in Milli-Q water. All other reagents used were as per section 2.2.2.1.

The animals used for these experiments were cared for as described in section 2.2.2.2. The removal of the brain and homogenate preparation was conducted as described in sections 2.2.2.3 and .2.2.2.4 respectively.

The lipid peroxidation assay was performed the in the same manner as experiment 2.2 except that in this experiment the homogenate was incubated with increasing

concentrations of KCN instead of QA. Results were analysed as described in section 2.2.2.7. The experiment was replicated five times.

2.6.3. Results

KCN, the known neurotoxin, at concentrations 0.25-1mM showed a proportionate and significant ($p < 0.001$) increase in lipid peroxidation when incubated with the rat brain homogenate as shown in figure 2.7.

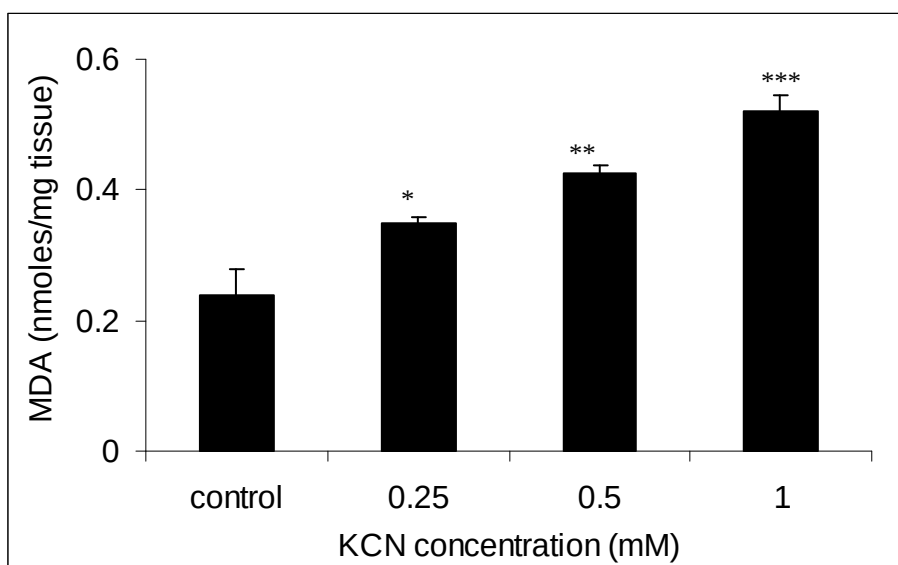


Figure 2.7: Effect of KCN (0-1mM) on lipid peroxidation in rat brain homogenate. Each bar represents the mean $\pm$ SD ($n=5$). *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

2.6.4. Discussion

The study shows that KCN induces lipid peroxidation of rat brain homogenate in a concentration-dependent manner. These results are consistent when compared to past research (Daya *et. al.*, 2000; Lambat *et. al.*, 2000). It has been established that the primary mechanism of action of cyanide involves the inhibition of cytochrome oxidase a_1a_3 , the terminal oxidative enzyme of the electron transport chain (Isom & Way 1984;

Albaum *et. al.*, 1946). These electrons have the capacity to leak out of the mitochondria resulting in the generation of free radicals, which may attack membranes causing lipid peroxidation.

Other studies have shown that cyanide produces elevated levels of brain calcium (Johnson *et. al.*, 1986, Johnson *et. al.*, 1987). Elevated cytosolic calcium is associated with free radical-induced damage of neuronal membranes (Braugher *et. al.*, 1985) and transient ischemic episodes can initiate lipid peroxidation of rat brain via a calcium-dependent process (Watson *et. al.*, 1984).

2.7. EFFECT OF TOLMETIN AND SULINDAC ON KCN INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE.

2.7.1. Introduction

Experiment 2.4 demonstrates that KCN induced LP in a concentration dependent manner. It was therefore decided to determine the effect tolmetin and sulindac would have on KCN (1mM)-induced LP.

2.7.2. Materials and Methods

Experiments were conducted in the exact same manner as in experiment 2.4. In addition, tolmetin was added to the incubation medium at various concentrations (0-1mM). The results were analysed statistically as described in section 2.2.2.7. The experiment was replicated five times.

2.7.3. Results

The homogenate was incubated with tolmetin and sulindac at concentrations 0.75mM and 1mM show (figure 2.8) a significant decrease in MDA produced. Both agents fail to reduce 1mM KCN-induced LP at 0.25mM. The NSAIDS alone have no significant effect on lipid peroxidation when compared to controls.

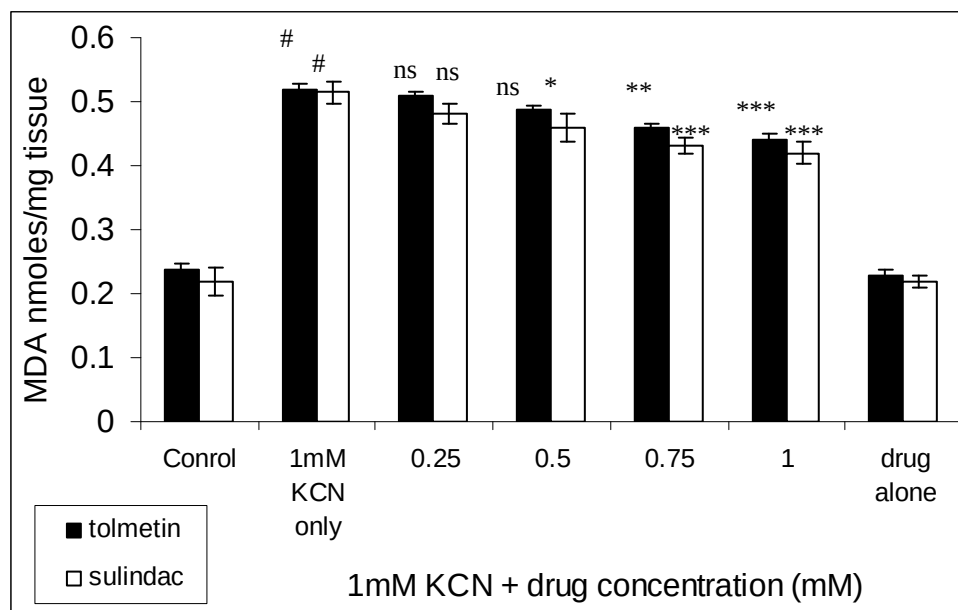


Figure 2.8: Effect of tolmetin on KCN (1mM)-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean $\pm$ SD (n=5). # (p<0.001) compared to controls. ns (p>0.05), * (p<0.05), ** (p<0.01) and *** (p<0.001) in comparison to KCN only.

2.7.4. Discussion

As mentioned earlier, cyanide induced oxidative stress is believed to result from the inactivation of numerous antioxidant enzymes (Ardelt *et. al.*, 1989), which could result in lipid peroxidation. Cyanide at a concentration of 1mM causes the inhibition of many antioxidant enzymes including cytochrome oxidase a_1a_3 in the mitochondria. Therefore higher concentrations of the NSAIDS are required to reduce the free radical damage induced by KCN. Research suggests that anti-inflammatory agents such as diclofenac sodium and mefenamic acid, induce calcium efflux in mitochondria (Pigoso *et. al.*, 1998). If tolmetin and sulindac have this ability, it possibly reduces the calcium levels in the mitochondria and prevents the generation of free radicals. Both NSAIDS may also prevent the inhibition of the enzyme, cytochrome oxidase a_1a_3 . Further studies need to be conducted to determine whether tolmetin and sulindac have the ability to induce calcium efflux or reduce the inhibition of cytochrome oxidase a_1a_3 .

2.8. EFFECT OF THE TRANSITION METAL, IRON (Fe²⁺) ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE.

2.8.1. Introduction

It has been established that the brain is vulnerable to oxidative damage. One of the many reasons for this is that the brain is rich in peroxidisable fatty acids and contains high levels of transition metals, which can catalyse the formation of the reactive hydroxyl radical (Floyd 1999). Increased redox cycling of iron complexes in the affected regions may cause iron-catalysed free radical generation, LP, axonal dystrophy, necrosis and apoptotic cell death which results in neurodegeneration (Chiueh *et. al.*, 1993; Gutteridge 1994).

In biological systems, the $\cdot\text{OH}$ radical is iron-catalysed by the reaction between H_2O_2 and iron or copper in a low valence state (Halliwell & Gutteridge 1986). This reaction is referred to as the Fenton reaction (Halliwell & Gutteridge 1990), which is represented by the equation:



This reaction can be accelerated by ascorbate since ascorbate promotes the redox cycling of tightly bound iron complexes (Rauhala *et. al.*, 1998).

The following experiment was conducted to determine the effect of the transition metal, Fe^{2+} , on LP. The experiment was conducted with ascorbate and hydrogen peroxide in the incubation medium.

2.8.2. Materials and Methods

2.8.2.1 Chemicals and Reagents

Iron sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and ascorbic acid were purchased from Saarchem, Johannesburg, South Africa. H_2O_2 was purchased from BDH Laboratory Supplies, Poole,

England. Ethylenediaminetetraacetic acid (EDTA) was obtained from HOLPRO Analytics, (Pty) LTD, Johannesburg. All other reagents used were as per section 2.2.2.1.

Iron sulphate, EDTA, ascorbic acid and H₂O₂ were prepared by dissolving in Milli-Q water.

The animals used for these experiments were cared for in the exact same manner as described in section 2.2.2.2. The removal of the brain and homogenate preparation was as conducted as described in section 2.2.2.3 and 2.2.2.4 respectively.

2.8.2.2. Lipid Peroxidation Assay

Lipid Peroxidation was determined from a modified method of Placer *et. al.*, 1966.

Homogenate (1.5ml), containing (1mM ascorbate, 100µM EDTA, 1mM iron sulphate and 100µM H₂O₂ was incubated in an oscillating water bath for 1 hour at 37°C. At the end of the incubation period 0.5ml BHT (0.5mg/ml in ethanol) and 1ml 25% TCA was added. The rest of the experiment was conducted as described in section 2.2.2.6. All results were statistically analysed as described in section 2.2.2.7. The experiment was replicated five times.

2.8.3. Results

Figure 2.9 shows that Fe²⁺ significantly (p<0.001) induces lipid peroxidation in the rat brain homogenate.

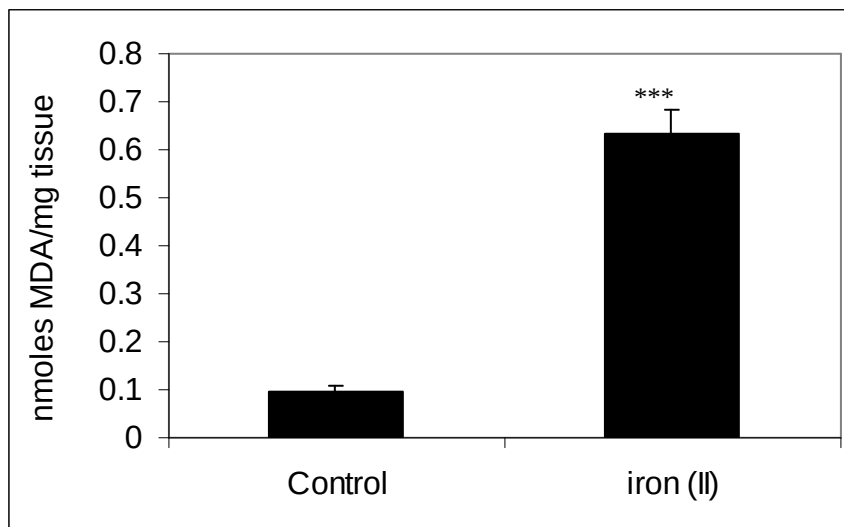


Figure 2.9: Effect of Fe^{2+} (1mM) on lipid peroxidation in rat brain homogenate. Each bar represents the mean $\pm$ SD (n=5). ***($p < 0.001$) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

2.8.4. Discussion

H_2O_2 , a byproduct of oxidases, has been considered a messenger for intracellular signaling pathways (Sziraki *et. al.*, 1998) and is not a toxic molecule. However in the presence of transition metals, most often Fe^{2+} , it is reduced to the $\cdot\text{OH}$ free radical via the Fenton reaction (Halliwell & Gutteridge 1990). The $\cdot\text{OH}$ free radical produced during the Fenton reaction is a very destructive free radical and is known to oxidise adjacent molecules (Halliwell & Gutteridge 1989) within a few Angstroms of its production site. The $\cdot\text{OH}$ radical initiates lipid peroxidation by removing an allelic H^+ from the PUFA and results in the radical chain reaction where lipid peroxidation is self-propagating (Gutteridge & Halliwell 1990). In this experiment Fe^{2+} incubated with H_2O_2 , significantly induced lipid peroxidation in the rat brain homogenate via the Fenton reaction.

2.9. EFFECT OF TOLMETIN AND SULINDAC ON Fe^{2+} -INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE.

2.9.1. Introduction

Experiment 2.6 shows Fe^{2+} significantly ($p < 0.001$) induces LP in rat brain homogenate. In the previous experiments (2.3, 2.5 and 2.7), tolmetin and sulindac have shown to possess antioxidant and free radical scavenging properties. This experiment was therefore performed to determine whether tolmetin could blunt the free radical LP, induced by the transition metal.

2.9.2. Materials and Methods

Experiments were conducted in the exact same manner as in experiment 2.6 except that in this experiment, tolmetin or sulindac was also added to the incubation medium at concentrations (0-1mM). All results were analysed as described in section 2.2.2.7. The experiment was replicated five times.

2.9.3. Results

Rat brain homogenate with Fe^{2+} and hydrogen peroxide shows a significant increase in lipid peroxidation. Homogenates incubated with tolmetin or sulindac (0.75 and 1.0mM) show a smaller degree of LP. Either NSAID alone in the homogenate had no effect on lipid peroxidation when compared to controls.

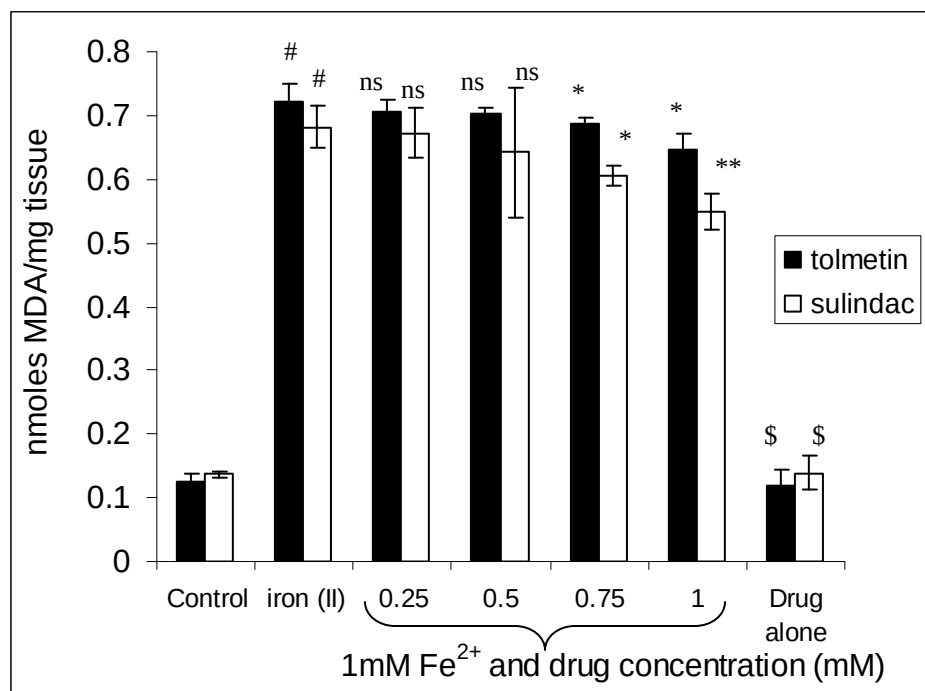


Figure 2.10: Effect of tolmetin and sulindac on Fe^{2+} (1mM)-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean $\pm$ SD (n=5). #($p<0.001$) and \$($p>0.05$) compared to controls. ns($p>0.05$), *($p<0.05$), **($p<0.01$) in comparison to Fe^{2+} only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

2.9.4. Discussion

During the inflammatory process large amounts of H_2O_2 are generated. This is due to the activation of mast cells, macrophages, eosinophils and neutrophils, which generate $\text{O}_2^{\cdot-}$ via NADPH oxidases (Barnes 1990; Hampton *et al.*, 1998). The $\text{O}_2^{\cdot-}$ is then rapidly converted into H_2O_2 by superoxide dismutase and it can easily penetrate membranes of surrounding cells.

The lipid peroxidation is induced by the hydroxyl free radicals generated via the Fenton reaction. Phenolic compounds have the ability to scavenge the $\cdot\text{OH}$ free radical (Hashizume 1993). It may be easily assumed that that hydroxyl bearing compounds such as acetaminophen and sodium salicylate, may act as scavengers of the $\cdot\text{OH}$ free radical.

Although tolmetin and sulindac are not phenolic compounds, in this study these anti-inflammatory agents demonstrate their ability to significantly reduce the transition metal induced free radical-induced lipid peroxidation. Tolmetin and sulindac are likely to reduce the LP by scavenging the $\cdot\text{OH}$ radical produced. In the Fenton reaction, Fe^{2+} is oxidised to Fe^{3+} which in the process causes H_2O_2 to generate the $\cdot\text{OH}$ free radical. Fe^{3+} is reduced to Fe^{2+} in the presence of ascorbic acid. This makes Fe^{2+} and Fe^{3+} both requirements of the Fenton reaction in the presence of ascorbic acid. These NSAIDS may have also reduced the LP by possibly binding to the transition metals (either Fe^{2+} or Fe^{3+}), thereby making them unavailable for the Fenton reaction, since it has been demonstrated that NSAIDS do have metal binding properties (Peterson *et. al.*, 1979; Dendrino-Samara *et. al.*, 1998). It is also possible that both these mechanisms are involved i.e. scavenging the $\cdot\text{OH}$ radical and binding to either Fe^{2+} or Fe^{3+} . The concept of tolmetin and sulindac possibly interacting with Fe^{2+} or Fe^{3+} has been investigated in chapter 10.

The NSAIDS have also been shown to be potent H_2O_2 scavengers (Costa *et. al.*, 2004). The ranking order of potency found with comparison to endogenous antioxidants was: sulindac sulfone > sulindac sulfide > glutathione > sulindac > indomethacin > acetaminophen > etodolac > oxaprozin > ketorolac equipotent as melatonin greater than tolmetin. Therefore another possible mechanism via which tolmetin and sulindac may have reduced Fe^{2+} -induced lipid peroxidation, is by scavenging H_2O_2 and reducing the amount of $\cdot\text{OH}$ radicals formed in the rat brain homogenate.

CHAPTER 3

SUPEROXIDE ANION ASSAYS

3.1. INTRODUCTION

Aerobic lifestyles may be advantageous in many ways but the utilization of oxygen by cells for many of their biochemical reactions results in the formation of highly reactive free radical products (Packer 1991; Di Mascio *et. al.*, 1991). These free radicals and products of free radical reactions can cause reversible and irreversible damage to macromolecular targets such as DNA, proteins and cellular membranes (Dean *et. al.*, 1993; Gey 1993).

Occasionally under normal biological conditions, oxygen manages to steal electrons from other molecules by non-enzymatic autoxidation. Since it cannot accommodate a spin-matched pair, it must settle for ‘stealing’ electrons one at a time. The breaking up of electron pairs results in free radical formation. The one-electron product of oxygen is the superoxide radical, ($O_2^{\cdot -}$) (McCord 2000). The $O_2^{\cdot -}$, which can be generated via multiple enzymatic and non-enzymatic pathways, is often the start of the oxidative stress cascade. The brain derives most of its energy from oxidative respiration through the mitochondrial electron transport chain. Mitochondria are located throughout the cell body and processes of the neurons including the glia. Mitochondria produce adenosine triphosphate (ATP) through the coupling and successive oxidation of NADH to nicotinamide adenine dinucleotide (NAD^+) by reducing O_2 to H_2O via the gradient of redox potential of enzymes within the electron transport chain. (Dawson & Dawson 1996).

There is nothing to prevent the direct reduction of O_2 to $O_2^{\cdot -}$ by non-heme iron proteins, quinones, flavoproteins or cytochrome b in the electron transport chain. However, these reactions are kinetically restricted so that only a small percentage (1-3%) of high energy electrons ‘leak’ in close proximity to O_2 resulting in the generation of $O_2^{\cdot -}$ (Fridovich 1986). The superoxide anion can also be generated chemically by auto-oxidation reactions with catecholamines, tetrahydrofolates and reduced flavins through direct

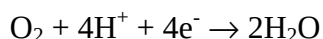
reaction with the $O_2^{\cdot-}$. The $O_2^{\cdot-}$ is also produced by cells involved in the host immune response such as neutrophils, monocytes, macrophages and microglia (Coulton *et. al* 1994). In pH environments of approximately 7.4, the superoxide partially protonates to form the hydroperoxyl radical ($HOO^{\cdot}$) which is a more reactive species (Rao & Hayon 1973). In a study by Antunes *et. al.* (1996) the hydroperoxyl radical was estimated to inflict more than 5 times the damage than the hydroxyl radical. The protective measures that the cells employ in response to such damage are usually sufficient, except when there is excessive ROS produced which causes the biological defenses to be overwhelmed thus leading to oxidative stress (Difiglia 1990).

The cell requires a coping mechanism to prevent this free radical induced damage. Once formed, the $O_2^{\cdot-}$ quickly undergoes dismutation to generate H_2O_2 and this reaction is markedly accelerated by a family of enzymes known as the superoxide dismutases (SOD) (Fridovich 1989). SOD is therefore considered an important antioxidant enzyme. Therefore any agents enhancing the activity of the SOD could aid in the prevention of this free radical induced damage. Another mechanism of defense is by the use of antioxidants that could scavenge the $O_2^{\cdot-}$ free radical limiting the damage that may be caused by this free radical.

3.2. EFFECT OF QA ON SUPEROXIDE ANION PRODUCTION IN RAT BRAIN HOMOGENATE.

3.2.1. Introduction

Of all the organs in the body, the CNS takes more than its share of oxidative abuse (Braugher & Hall 1989). The reasons for this are several-fold. The brain although constituting only a small percentage (in human about 2%) of the body weight, consumes a disproportionately large amount (in human about 20%) of the O₂ inhaled. Well over 90% of the O₂ that enters human cells are used for the production of energy by mitochondrial cytochrome oxidase. During this process, four electrons (e⁻) are added to each O₂ molecule resulting in the formation of two molecules of water (Reiter 1998), as shown in equation 1.



Experiment 2.2 shows that QA induces lipid peroxidation in rat brain homogenate in a concentration dependent manner. It was therefore decided to investigate whether incubating rat forebrain homogenate with increasing concentrations of QA causes an increase in O₂⁻ production.

The nitro-blue tetrazolium (NBT) assay, which is generally accepted (Das *et. al.*, 1990; Saga *et. al.*, 1992) to be a reliable method for assaying for superoxide anions, was used. The assay involves the reduction of the NBT dye by the superoxide anion to the insoluble nitroblue diformazan (NBD), which can be extracted with glacial acetic acid.

3.2.2. Materials and Methods

3.2.2.1. Chemicals and Reagents

Nitroblue diformazan (NBD), nitroblue tetrazolium (NBT) and QA were purchased from Sigma Chemicals Corporation, St Louis, MO, U.S.A. Glacial acetic acid and ethanol was

purchased from Saarchem, Johannesburg, South Africa. All other chemicals used were of the highest quality available from commercial sources.

QA was prepared by dissolving in Milli-Q water. A 0.1% NBT solution was made by dissolving the NBT in absolute ethanol before making up to the required volume with Milli-Q water.

3.2.2.2. Preparation of Standard Curve

NBD was used as the standard. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in glacial acetic acid was prepared. The absorbance was read at 100 μ M intervals at 560nm using a GBC UV/VIS 916 Spectrophotometer. A calibration curve was generated by plotting absorbance against concentration (Appendix 3).

3.2.2.3. Homogenate Preparation

Adult male Wistar rats were used for the experiments. The rats were cared for as described in section.2.2.2.2. Brain removal and homogenate preparation were conducted as described by section 2.2.2.3 and 2.2.2.4 respectively.

3.2.2.4. The Nitroblue Tetrazolium Assay

The method used for this assay was a modification of Das *et. al.*, 1990.

Homogenate (1ml) containing varying concentrations of QA (0, 0.25, 0.5, 0.75, 1mM) were incubated with 0.4ml of 0.1% NBT in an oscillating water bath for 1 hour at 37°C. Termination of the assay and extraction of the reduced NBT was carried out by centrifuging the samples for 10 minutes at 2000g then re-suspending the pellets with 2ml of glacial acetic acid. The absorbance was measured at 560nm and converted to μ moles diformazan using a standard curve generated from NBD. Final results are expressed as μ moles diformazan/mg tissue and were analysed as described in section 2.2.2.7. The experiment was replicated six times.

3.2.3. Results

As shown in figure 3.1, the exposure of whole rat brain homogenate to increasing concentrations of QA, causes no generation of the superoxide anion. The data represented in these experiments is the mean $\pm$ SD of six separate determinations.

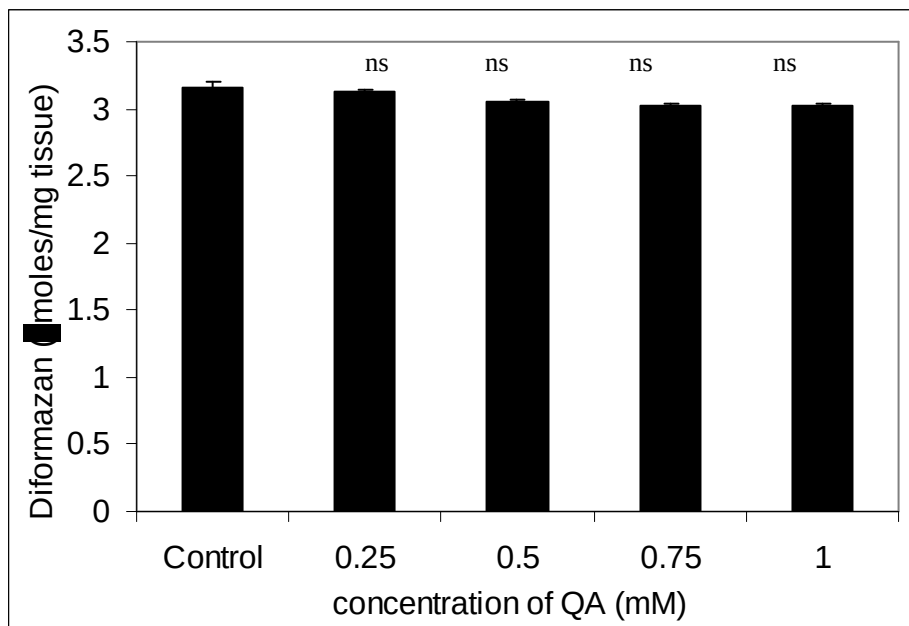


Figure 3.1. Effect of QA on superoxide anion generation in rat brain homogenate. Each bar represents the mean $\pm$ SD (n=6).

3.2.4. Discussion

Although it would have been expected that the exposure of rat brain homogenate to QA would increase the generation of the $O_2^{\cdot-}$, this did not occur. One possible reason for this is that rat brain homogenate was used and if QA had to induce the generation of the $O_2^{\cdot-}$, it would have initiated the process by over-stimulating NMDA receptors or glutamate receptors. This would cause an increase in the intracellular Ca^{2+} levels and subsequently free radical generation (Choi 1992; Choi 1995). By using homogenate the integrity of the cells was damaged therefore any receptor mediated increase in the intracellular Ca^{2+} levels would have been abolished. This would explain why QA did not increase $O_2^{\cdot-}$ generation, even at pharmacological doses.

3.3. EFFECT OF QA ON SUPEROXIDE ANION PRODUCTION IN PRIMARY HIPPOCAMPAL NEURONS

3.3.1. Introduction

In the previous experiment QA did not induce superoxide anion generation in rat brain homogenate. This experiment was conducted using primary hippocampal neurons. Since the integrity of the cells is maintained, receptor stimulation induced neurotoxicity could occur. The over-stimulation of glutamate receptors, and in particular the NMDA subtype of glutamate receptors has shown to initiate cellular processes leading to many neurodegenerative conditions (Rothman & Olney 1986; Mayer & Westbrook 1987; Rothman *et. al.*, 1987; Meldrum & Garthwaite 1990). Some recent studies have suggested that neuronal death involves the participation of ROS like hydrogen peroxide, the O_2^- and singlet oxygen (Zhaung *et. al.*, 2000; Tammariello *et. al.*, 2000; Valencia & Moran 2001). Experiment 2.4 shows that QA induces lipid peroxidation in primary hippocampal neurons in a concentration-dependent manner. This experiment was conducted to determine the effect of QA on the generation of the O_2^- in the neuronal cells.

3.3.2. Materials and Methods

All chemicals and reagents used were as per sections 2.4.2.1 and 3.2.2.1.

3.3.2.1. The Nitroblue tetrazolium assay

Primary hippocampal neurons were established as described in section 2.4.2.5.

Six to eight day old cultures were used for the experiments. Before experimentation, each flask was washed twice with 3ml of PBS. Thereafter 1ml of fresh MEM and 0.4ml of 0.1% NBT was added to each flask. To this, 100 μ l QA dissolved in MEM was added to produce final concentrations of 50; 100 or 200 μ M. The concentrations used were the same as that used for the lipid peroxidation studies. The culture flasks were then kept in the incubator for 3 hours as previously described.

After 3 hours, the cells were scraped off the surface of the culture flasks using a cell scraper and transferred to test tubes. An aliquot of 100 μ l of cell suspension was removed for enumeration using a haemocytometer. The test tubes were centrifuged for 10 minutes at 2000g and the resulting pellet was re-suspended in 1ml glacial acetic acid. The absorbance was measured at 560nm and converted to μ moles diformazan using a standard curve generated from NBD (see appendix 4). Final results are expressed as μ moles diformazan/ 10^6 cells and were analysed as described in section 2.2.2.7. The experiment was replicated four times.

3.3.3. Results

As shown in figure 3.2, the excitatory amino acid, QA induces a significant increase in superoxide anion generation in primary hippocampal neurons. The superoxide was induced in a concentration-dependent manner. The data presented is the mean $\pm$ SD of 4 determinations.

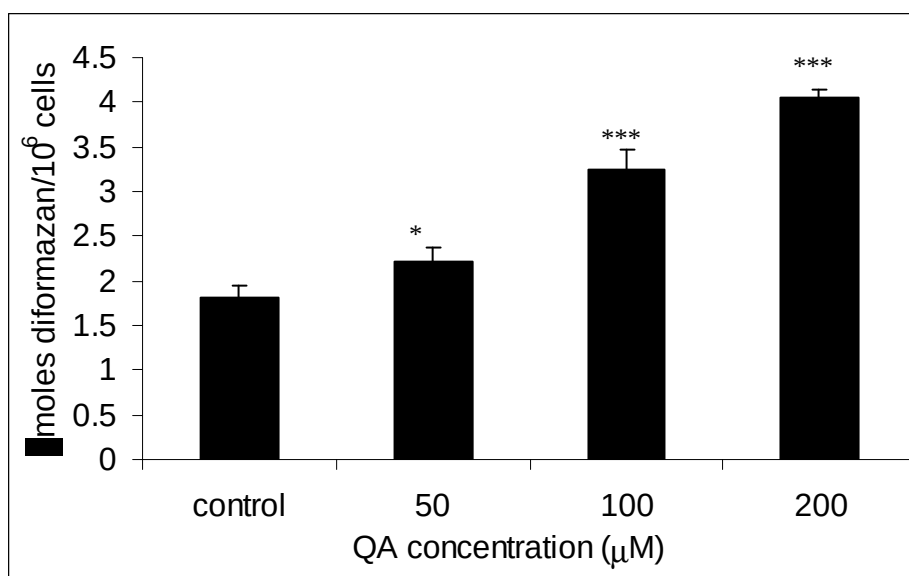


Figure 3.2: Effect of QA on superoxide anion generation in primary hippocampal neurons. Each bar represents the mean $\pm$ SD (n=4). *(p<0.05) and ***(p<0.001) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

3.3.4. Discussion

The results of this study show a concentration-dependent increase in the superoxide anion formation by QA in primary hippocampal neurons. QA did not induce the $O_2^{\cdot -}$ formation in rat brain homogenate. Thus the integrity of the cell/neuron is essential for normal functioning which includes the stimulation of receptors. It has been discussed earlier that the over-stimulation of glutamate receptors especially the NMDA subtype leads to ionic imbalances within the cells (Choi 1985; Choi 1992; Dubinski 1993) and ultimately free radical generation (Choi 1992; Dykens 1994). It has been shown that intrastriatal injections of QA induces ROS generation, GSH depletion, an increase in GS-SG and LP (Rodriguez-Martinez *et. al.*, 2000; Perez-Severiano *et. al.*, 2004). This experiment demonstrates that QA induces the generation of free radicals in primary hippocampal neurons in a concentration-dependent manner. One such free radical that is generated is the $O_2^{\cdot -}$, since it is this free radical that reduces NBT to NBD.

3.4. EFFECT OF TOLMETIN AND SULINDAC ON QA-INDUCED SUPEROXIDE ANION GENERATION IN PRIMARY HIPPOCAMPAL NEURONS

3.4.1. Introduction

Tolmetin and sulindac reduced the LP induced by QA (experiment 2.3 and 2.5). This experiment was conducted to determine whether these agents could reduce or prevent the QA-induced superoxide anion generation in primary hippocampal neurons. The concentration of QA, tolmetin and sulindac used in experiment 2.5 was also used in this experiment.

3.4.2. Materials and Methods

Experiments were conducted in the same manner as in experiment 3.4. In addition, tolmetin or sulindac was also added to the incubation medium at concentrations (50; 100 or 200 μ M). The results were analysed as described in section 2.2.2.7. The experiment was replicated four times.

3.4.3. Results

Figure 3.3 demonstrates the QA (200 μ M)-induced superoxide anion formation was significantly reduced by the co-treatment of the homogenate with tolmetin and sulindac.

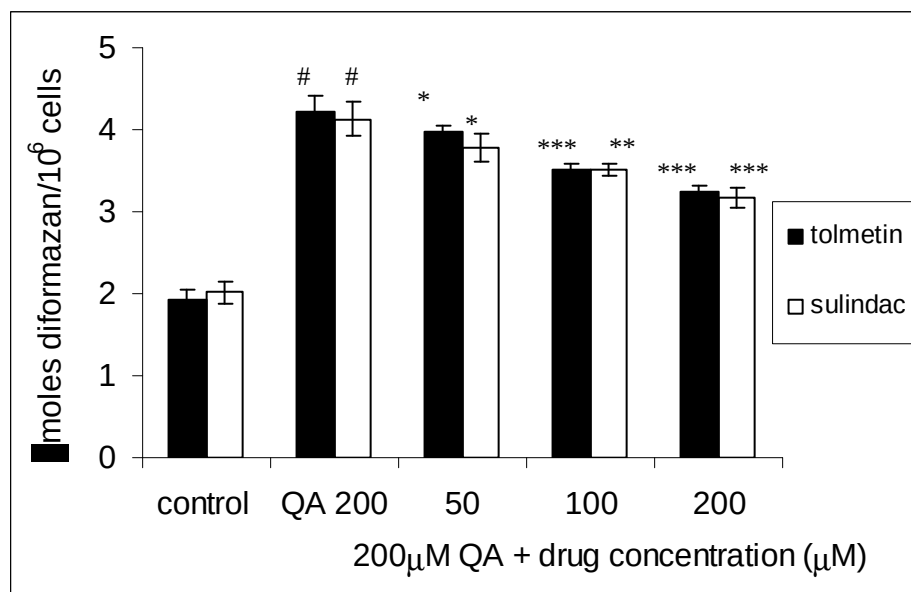


Figure 3.3: Effect of tolmetin and sulindac on QA (200µM)-induced superoxide anion generation in primary hippocampal neurons. Each bar represents the mean $\pm$ SD (n=4). #($p<0.001$) compared to control. *($p<0.05$) and ***($p<0.001$) in comparison to QA only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

3.4.4. Discussion

Both drugs show equal potency by scavenging the superoxide anion thus reducing the conversion of NBT to NBD. Any drug having the ability to prevent the generation of free radicals or scavenge free radicals can play a major role in the protecting cells from oxidative stress and free radical-induced injury.

It has been demonstrated that the superoxide anion decreases cell viability and induces apoptosis in neuronal cultures (Valencia & Moran 2004). Park and co-workers recently showed that NMDA causes an increase in the intracellular Ca^{2+} concentrations, an increase in ROS generation and induces neuronal death. These authors also showed that the co-incubation of these neurons with Zizypus extracts, a herb widely used in Korea and China due to its CNS calming effects, significantly reduces intracellular Ca^{2+} concentrations, reduces ROS formation and enhanced cell viability. This experiment

demonstrates that the NMDA agonist, QA significantly generates the production of the superoxide anion. The co-incubation of the hippocampal neurons with tolmetin or sulindac significantly reduces the generation of the superoxide anion. It is also possible that these NSAIDS may be scavenging the superoxide anion since it has been postulated that NSAIDS may have the ability to inhibit the generation of the superoxide anion as well as scavenge this free radical once generated (Kimara 1997). The effect that QA and the NSAIDS have on primary hippocampal cell viability was investigated in the following chapter.

3.5. EFFECT OF KCN ON SUPEROXIDE ANION PRODUCTION IN RAT BRAIN HOMOGENATE.

3.5.1. Introduction

Experiment 2.4 showed that KCN induces lipid peroxidation in rat brain homogenate in a concentration-dependent manner. It has been discussed in section 2.4.4 that the primary mechanism of action of cyanide involves the inhibition of cytochrome oxidase a_1a_3 , the terminal oxidative enzyme of the electron transport chain (Isom & Way 1984; Albaum *et al.*, 1946). These electrons have the capacity to leak out of the mitochondria and come in close proximity to O_2 resulting in the generation of the $O_2^{\cdot-}$. It was therefore decided to investigate whether incubating rat forebrain homogenate with increasing concentrations of KCN induces an increase in $O_2^{\cdot-}$ production.

3.5.2. Materials and Methods

Potassium cyanide (KCN) was purchased from Sigma Chemical Corporation, St Louis, U.S.A. KCN was prepared by dissolving it in Milli-Q water. All other reagents used were as per section 3.2.2.1.

The animals used for these experiments were cared for as described in section 2.2.2.2. The removal of the brain and homogenate preparation was conducted as described in sections 2.2.2.3 and 2.2.2.4 respectively.

The Nitroblue tetrazolium assay was performed the in the exact same manner as experiment 3.2 except that in this experiment the homogenate was incubated with increasing concentrations of KCN instead of QA. The results were statistically analysed as described in section 2.2.2.7. The experiment was replicated five times.

3.5.3. Results

As shown in figure 3.4, exposure of whole rat brain homogenate to KCN induces a concentration-dependent increase in the superoxide anion generation when compared to

the control. The data represented in these experiments is the mean $\pm$ SD of five separate determinations.

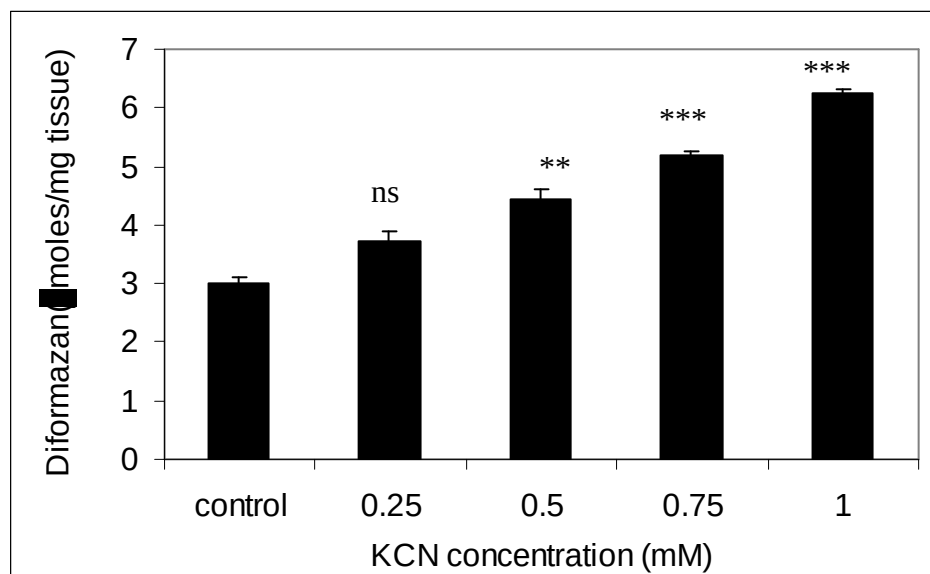


Figure 3.4. Effect of KCN on superoxide anion generation in rat brain homogenate. Each bar represents the mean $\pm$ SD (n=5). ns(p>0.05), ** (p<0.01) and *** (p<0.001) in comparison to control.

3.5.4. Discussion

The results of this study show a concentration-dependent increase in the superoxide anion generation caused by KCN in whole rat brain homogenate. Cyanide is a complex IV inhibitor of the mitochondrial electron transport chain. Distal inhibition of the electron transport chain by cyanide augments reactive oxygen species production (Waypa & Schumacker 2000). Previous research has shown that cyanide elevates brain calcium levels (Johnson *et. al.*, 1986). Elevated calcium levels can lead to activation of numerous neuronal calcium-dependent events and ultimately results in oxidative stress and free radical generation (Braugher *et. al.*, 1985). As mentioned in section 2.6.4, KCN inhibits cytochrome oxidase a_1a_3 , the terminal oxidative enzyme in the electron transport chain (Isom & Way 1984; Albaum *et. al.*, 1946). These electrons can leak out of the mitochondria and reduce O_2 to the O_2^- (McCord 2000). This is a possible mechanism by which KCN induced O_2^- in a concentration-dependent manner in rat brain homogenate.

3.6. EFFECT OF TOLMETIN AND SULINDAC ON KCN INDUCED SUPEROXIDE ANION PRODUCTION IN RAT BRAIN HOMOGENATE.

3.6.1. Introduction

Experiment 3.3 showed that KCN, in a concentration dependent manner, increased the production of the $O_2^{\cdot-}$. This experiment was conducted to determine the effect of tolmetin and sulindac on KCN (1mM)-induced $O_2^{\cdot-}$ generation in rat brain homogenate.

3.6.2. Materials and Methods

Experiments were conducted in the exact same manner as described in section 3.3.2. In addition, tolmetin or sulindac was also added to the incubation medium. A stock solution of tolmetin was prepared by dissolving it in Milli-Q water from which appropriate aliquots were added to the incubation medium to produce final concentrations of (0-1mM). The results were statistically analysed as described in section 2.2.2.7. The experiment was replicated five times.

3.6.3. Results

The superoxide generated by KCN (1mM) was reduced by the co-treatment of the homogenate with the NSAIDS (figure 3.5). Tolmetin and sulindac alone had no effect on superoxide anion formation. The data represented in these experiments is the mean $\pm$ SD of five separate determinations.

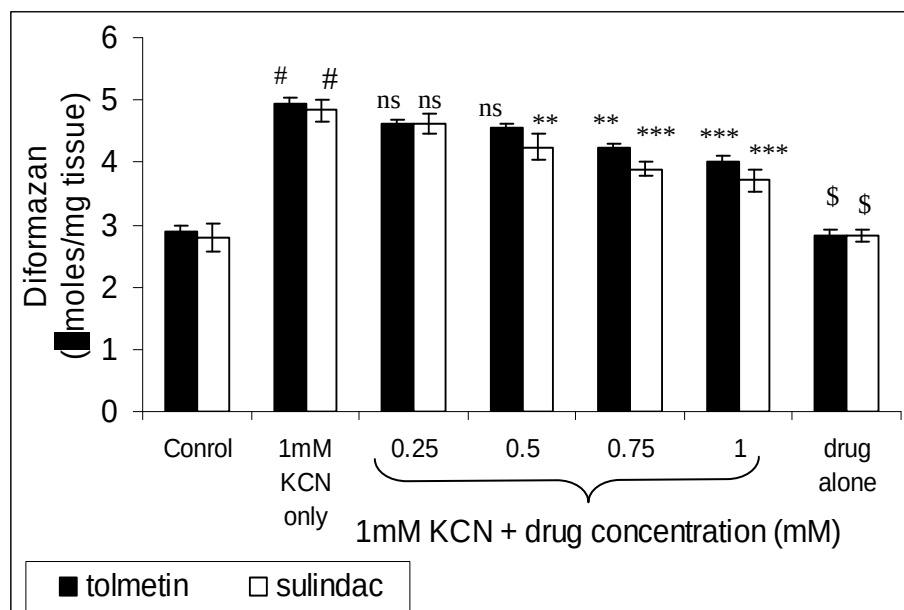


Figure 3.5. Effect of tolmetin and sulindac on KCN (1mM)-induced superoxide anion generation in rat brain homogenate. Each bar represents a mean $\pm$ SD (n=5). # (p<0.001) and \$ (p>0.05) compared to controls. ^{ns} (p>0.05), ** (p<0.01), *** (p<0.001) in comparison to KCN alone. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

3.6.4 Discussion

Kimara (1997) reported that the anti-inflammatory agents, naproxen and flurbiprofen, inhibit the formation of the superoxide anion while sodium salicylate acts to capture the superoxide directly. It has been postulated that the NSAIDS have inhibitory effects on the generation system of the superoxide anion as well as scavenging effects of the superoxide generated (Kimara 1997). Recently it has been stated that the anti-inflammatory agents, ibuprofen (Lambat *et. al.*, 2000) and aspirin (Daya *et. al.*, 2000) have the ability to reduce the generation of the superoxide anion or by possibly scavenging it.

In these experiments tolmetin and sulindac demonstrate the ability to decrease the reduction of the nitroblue tetrazolium dye to the color nitroblue diformazan by the superoxide anion. Tolmetin and sulindac reduced the levels of superoxide anion by

probably scavenging these free radicals. This possibility was investigated in the following experiment (experiment 3.7). The possibility that tolmetin and sulindac may prevent the influx of calcium into the mitochondria and reduce the superoxide anion generation needs to be investigated further. The NSAIDS when incubated with the homogenate and NBT did not induce or reduce superoxide anion generation.

3.7. SUPEROXIDE ANION-INDUCED NADPH OXIDATION: EFFECT OF TOLMETIN AND SULINDAC

3.7.1. Introduction

Oxidative phosphorylation in the mitochondria as well as numerous enzymatic and non-enzymatic mechanisms can generate $O_2^{\cdot -}$ and H_2O_2 (Halliwell & Gutteridge 1989). Moreover, the production of ROS can result from the enzymatic conversion of catecholeamines and indoleamines by monoamine oxidase or from the non-enzymatic autoxidation of catecholeamines (Weyler *et. al.*, 1990; Singer & Ramsay 1995). Superoxide can be produced from lipoxygenases, cyclooxygenases and various flavin oxidases (Cross & Jones 1991; Coyle & Puttfarcken 1993). As the sources of ROS in nerve cells are numerous, the cells have to maintain an effective antioxidant defense system in order to protect themselves against free radical damage. The previous experiment shows that tolmetin and sulindac have the ability to decrease the reduction of NBT to NBD by the $O_2^{\cdot -}$. The mechanism by which this occurs was investigated in the following experiment based on the hypothesis that the NSAIDS directly scavenge the superoxide anion.

The technique employed in this experiment was a modification of the method developed by Paoletti and colleagues in 1986. It is based on the chemical reaction which generates superoxide from molecular oxygen in the presence of EDTA, manganese(II) chloride ($MnCl_2$) and mercaptoethanol. NADPH oxidation is linked to the availability of superoxide anions in the medium. In the absence of the superoxide anion, NADPH absorbencies remain unchanged but when oxidised by this free radical, the absorbance decreases with time. As soon as SOD is added to the assay mixture, it brings about the inhibition of the nucleotide oxidation. Therefore at high concentrations of the enzyme the absorbance at 340nm remains unchanged, while the control (no SOD) absorbance decreases. In this experiment the effect of tolmetin and sulindac in the medium on NADPH oxidation was investigated.

3.7.2. Materials and Methods

2-Mercaptoethanol, MnCl_2 , NaOH, ethanol and chloroform were purchased from Saarchem, Johannesburg, South Africa. Ethylenediaminetetraacetic acid (EDTA) was obtained from HOLPRO Analytics, (Pty) LTD, Johannesburg. NADPH was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A.

3.7.2.1. Chemicals and Reagents

NADPH (7.5mM) and MnCl_2 (100mM) were dissolved in Milli-Q water. EDTA (200mM) was dissolved in Milli-Q water and the pH was adjusted to 7 with 1M NaOH. The EDTA and MnCl_2 stock solutions were combined in a ratio of 1:1 (v/v), and the pH of the resulting mixture was adjusted with 10M NaOH to pH 7. Mercaptoethanol (10mM) was diluted using H_2O .

3.7.2.2. Extraction of the enzyme, Superoxide Dismutase

Animals were cared for as described in section 2.2.2.2. Brain removal and homogenate preparation were performed as described in section 2.2.2.3 and 2.2.2.4 respectively. SOD was extracted using a method employed by McCord and Fridovich in 1969.

Briefly, to 2.5ml of homogenate, 1ml of an ethanol-chloroform solution (2:1 v/v) was added and mixed to obtain a thick precipitate. An aliquot of 2ml of distilled water was added and the mixture was incubated at 37°C for 15 minutes. Following incubation, the mixture was centrifuged for 10 minutes at 3000 x g. The almost colourless supernatant was recovered and dialyzed overnight in the cold room between 4-5°C. PBS was used as the dialysis buffer. The SOD was recovered and diluted so that the final concentration in the reaction medium was 1mg/ml. Protein estimation was done using the method described by Lowry *et. al.* (1951).

3.7.2.3. Conditions for Assay

The following solutions were pipetted into a microcuvette (pathlength 1cm). An aliquot of 0.8ml of PBS, pH 7.4, 40 μl NADPH, 25 μl of the EDTA- MnCl_2 combined solution and 0.1ml of sample (either drug 0.1mM and/or SOD or inactivated SOD). SOD was

inactivated by boiling the enzyme extract. An aliquot of 0.1ml of mercaptoethanol was then added to the medium. The decrease in absorbance was monitored at 340nm over 30 minutes and is reported as the % of NADPH remaining after 30 minutes. Results are a determination of five separate experiments and were analysed as described in section 2.2.2.7.

3.7.3. Results

The superoxide anion was generated non-enzymatically in the solution. Those solutions that contained the nucleotide, NADPH alone in the reaction medium or with inactivated SOD showed an approximate 68% oxidation of the nucleotide. In solutions that contained SOD or a mixture of SOD and tolmetin, the NADPH was only oxidized by approximately 22%. Tolmetin did not affect the functioning of the SOD enzyme. The solution that contained only tolmetin (0.1mM), showed a 46% oxidation of the nucleotide (figure 3.6).

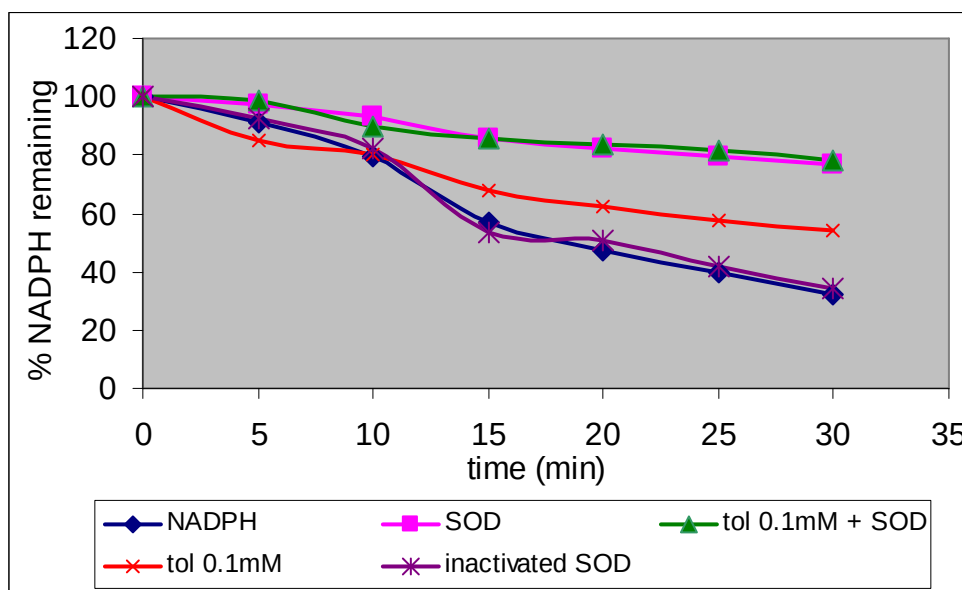


Figure 3.6. Comparison of the effect of NADPH oxidation with and without the presence of tolmetin (0.1mM), active and inactive SOD extract. The amount of NADPH remaining was measured over 30 minutes.

Sulindac also has a very similar $O_2^{\cdot -}$ scavenging profile compared to tolmetin (figure 3.7). The NADPH in the reaction medium that contained sulindac was oxidized by 46% when

compared to the reaction medium with no drug or SOD which was oxidized by approximately 68%. Sulindac also did not affect the functioning of the SOD enzyme.

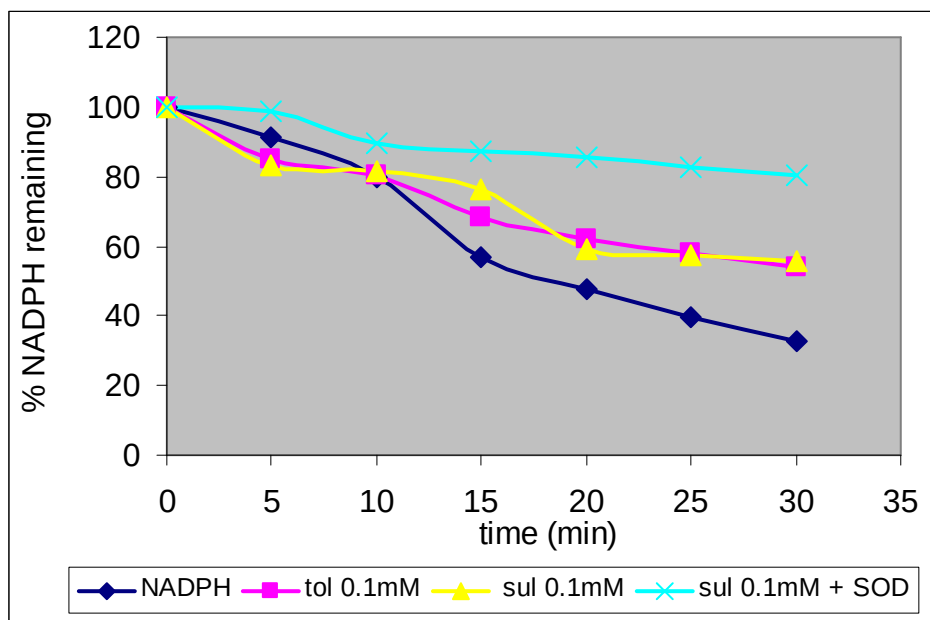


Figure 3.7. Comparison of the effect of NADPH oxidation with and without the presence of sulindac or tolmetin (0.1mM) and with sulindac and SOD together in the reaction medium. The amount of NADPH remaining was measured over 30 minutes.

Figure 3.8 shows the % of NADPH remaining at 30 minutes. At 30 minutes approximately 32% NADPH remains while the reaction mixture with SOD has approximately 78%. The reaction mixtures that contain tolmetin or sulindac (0.1 mM) has approximately 54% and 56% NADPH remaining.

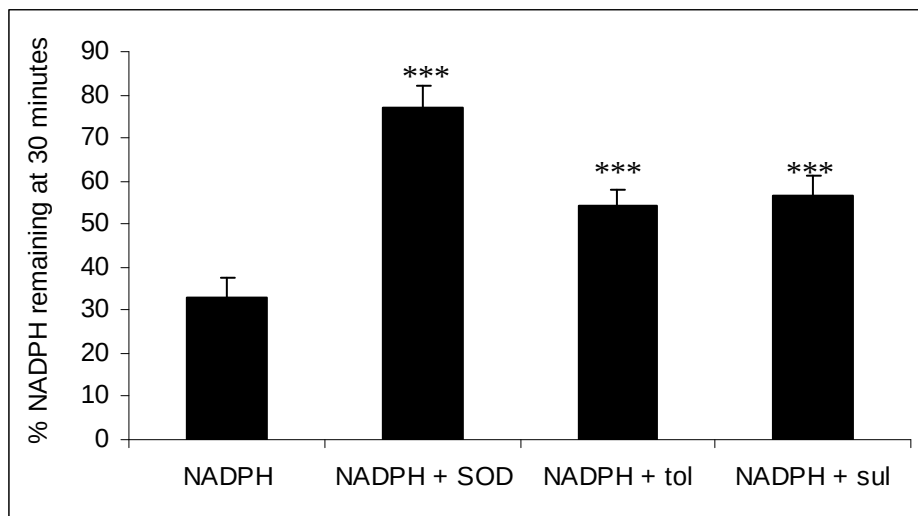


Figure 3.8: Percentage NADPH remaining at 30 minutes. Each bar represents a mean $\pm$ SD (n=5). ***($p < 0.001$) in comparison to NADPH alone. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

3.7.4. Discussion

It is known that the superoxide anion, once produced, rapidly undergoes dismutation by the SOD (Fridovich 1989). In these experiments the superoxide anion brought about the oxidation of the NADPH. Once SOD was added to the solution it brought about the dismutation of the superoxide anion, hence the oxidation of the nucleotide was lower. The SOD that was boiled did not prevent the oxidation to any extent since the enzyme was inactivated. The solution with SOD and tolmetin (0.1mM) or SOD and sulindac (0.1mM) shows no difference in oxidation when compared to the solution with SOD only. As mentioned earlier, the anti-inflammatory agents possess the ability to prevent the generation of the superoxide anion or scavenge it (Kimara 1997). Both tolmetin and sulindac alone in the solution, decreased the oxidation of NADPH. This is only possible if these NSAIDS scavenge the superoxide anion. These findings are consistent with past research, which mentions that the anti-inflammatory agents have the ability to scavenge the superoxide anion (Kimara 1997; Lambat *et. al.*, 2000; Daya *et. al.*, 2000).

Two principle mechanisms of action have been proposed for antioxidants (Burton *et. al.*, 1982; Burton & Ingold 1980). The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present, and becomes a free radical itself but a more stable one (Gordon 1990; Pryor 1994). The second mechanism involves the reaction of a compound with the initiating radical or inhibits the initiating enzyme, or reduce the oxygen level without generating reactive radical species. This compound is known as a secondary antioxidant. It is possible that tolmetin and sulindac are both primary and secondary antioxidants. If these NSAIDS donate a hydrogen atom to the free radicals formed, these drugs could become stable free radicals themselves and therefore could be referred to as primary antioxidants. These drugs also inhibit COX thereby reducing inflammation and the free radicals formed and could also be referred to as secondary antioxidants. This experiment shows that tolmetin and sulindac scavenge the O_2^- and further suggests that these agents could be secondary antioxidants since these agents removed the initiating radical.

CHAPTER 4

EFFECT OF TOLMETIN, SULINDAC AND QUINOLINIC ACID ON HIPPOCAMPAL NEURONS

4.1. INTRODUCTION

Accumulating evidence indicates that excessive formation of free radicals may be involved in the pathophysiology of many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (Beal *et. al.*, 1986; Coyle & Puttfarcken 1993). Oxidative stress is also implicated in acute brain disorders such as ischemia and traumatic damage (Chan 1994). Neurodegenerative diseases have been postulated to have an excitotoxic basis (Rothman & Olney 1987; Shaw 1994). Excitotoxicity induced by glutamate and its agonists, results in the generation of free radicals that can ultimately result in the death of the neurons (Coyle & Puttfarcken 1993).

The neuropathological features of neurodegenerative diseases involve degeneration of a circumscribed group of neurons that may be functionally or neuroanatomically connected. This neurodegeneration is believed to result from over-activation of glutamate receptors, which leads to a rise of intracellular Ca^{2+} that promotes cell damage by both activating destructive enzymes and increasing the formation of reactive oxygen species (Vergun *et. al.*, 1999, Orrenius *et. al.*, 1989; Siesjo & Bengtsson 1989).

QA, a product of tryptophan metabolism, has been detected in both the human and rat brain (Wolfensberger *et. al.*, 1983). The concentration of this metabolite is known to increase with age (Moroni *et. al.*, 1984). This agent has been shown to induce neuronal lesions after intrastriatal and intrahippocampal injections in rat brain (Schwarcz *et. al.*, 1983). These lesions closely resemble those observed in the brains of individuals who have Huntington's disease (Beal *et. al.* 1986; Popoli *et. al.*, 1994).

Chapters 2 and 3 demonstrate the ability of the NSAIDS, tolmetin and sulindac to act as antioxidants and free radical scavengers. The following series of experiments involving histology and apoptotic studies was conducted to determine whether these agents could protect rat hippocampal neurons from QA-induced neurotoxicity. Receptor binding studies and cell viability studies were also performed to provide an indication of the extent of neuroprotection offered by tolmetin and sulindac. It is known that high concentrations of the NMDA receptors are found in the cerebral cortex and CA1 regions of the hippocampus (Monaghan & Cotman 1986). QA acts as an agonist on the NMDA receptors in the brain (Stone & Perkins 1981) allowing Ca^{2+} , Na^{+} and K^{+} to enter the neurons. The influx of these ions can disrupt the ionic balance of the neurons, leading to swelling and lysis (Coyle & Puttfarcken 1993; Schwarcz *et. al* 1983). The influx of Ca^{2+} into the cell can also activate many free radical producing pathways, which can prove toxic to the neurons (Fahn & Cohen 1992).

4.2. HISTOLOGICAL INVESTIGATION

4.2.1. Introduction

Neurotoxicity has been defined as any adverse effect on the structures and functions of the nervous system. Structural (morphological) changes in the nerve cells and tissue can be used as good indices or markers for neurotoxicity assessment. Pathology, including neuropathology, is a visual science where cells and tissues are examined with the aid of optical instruments (microscopes) for the determination of lesions (pathological changes). These optical images are greatly enhanced by the use of dyes and stains which react with the tissue components so that the finally stained tissue is visually detectable. This branch of science is also referred to as histochemistry.

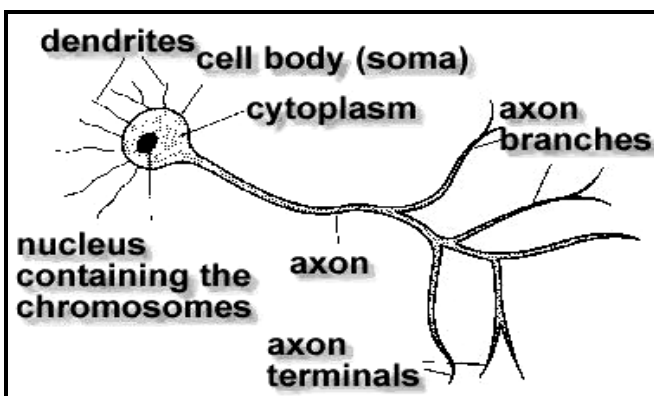


Figure 4.1: Basic structure of a neuron with its associated axons and dendrites.
(<http://www.wutsamada.com/alma/>)

The nervous system is probably the most complex of all biological systems. The neuron (figure 4.1), may be the most complex cell among all parenchymal units. Besides the main cell body, most neurons are associated with other structures such as the axons, dendrites, neurofilaments, Nissl bodies, synaptic terminals and myelin sheaths. The cell bodies as well as its associated structures are altered morphologically during toxic conditions which may give rise to various neurotoxicological conditions.

In this experiment it was decided to investigate whether tolmetin and sulindac could offer neuroprotection against QA-induced intrahippocampal (i.h) lesions in the rat brain. After

treatment, the hippocampus of the rats was sectioned and examined microscopically for evidence of any morphological changes.

4.2.2. Materials and Methods

4.2.2.1. Chemicals and Reagents

Quinolinic acid, tolmetin, sulindac and cresyl violet were purchased from Sigma Chemical Corporation; St Louis, MO, U.S.A. Paraffin wax was obtained from Lasec, South Africa. Sodium pentobarbitone was purchased from Merck, Germany and was at a working concentration of 60 mg/kg. Haupt's adhesive consisted of the following: 1g gelatine, 100ml water, 2g phenol and 15 ml glycerol. Distrene plasticiser in xylene (D.P.X) was a donation from the Rhodes University Botany Department. All other chemicals were of the highest quality available and were purchased from commercial distributors.

4.2.2.2. Animals

Adult male, Wistar rats were cared for as described in section 2.2.2.2.

4.2.2.3. Treatment Regimes

For the purpose of these experiments, rats were divided into various groups as described in the table 4.1. Each group contained seven animals. Animals from each group were also used for the apoptosis and receptor binding studies in experiments 4.3 and 4.4 respectively.

Table 4.1: Treatment regimes for the histological studies

Treatment Regime	Group
Control: PBS (i.h) no drug treatment	Con
QA (i.h) no drug treatment	QA
Tolmetin (5mg/kg/bd for 5 days) and QA (i.h)	tol + QA
Tolmetin (5mg/kg/bd for 5 days)	tol
Sulindac (5mg/kg/bd for 5 days) and QA (i.h)	sul + QA
Sulindac (5mg/kg/bd for 5 days)	sul

Tol and tol + QA rats were injected intraperitoneally (i.p.) with tolmetin, 5mg/kg twice a day for 5 days while sul and sul + QA rats were injected with the same dose of sulindac. Rats were injected every morning between 8: 30am-9: 00am. The second dose was injected between 5: 00pm and 5: 30pm. QA and control rats were injected with vehicle at the same times.

On the 6th day the rats were subjected to unilateral intrahippocampal injections.

4.2.2.4. Surgical Procedures

i. Unilateral Intrahippocampal Injections of Quinolinic Acid

The tol + QA, sul + QA and QA rats were anaesthetised with sodium pentobarbital (60 mg/kg i.p.) and placed in a stereotaxic frame. The rat skull was orientated according to the König and Klippel stereotaxic atlas (König & Klippel 1963). After a sagittal cut in the skin of the skull, the bregma suture was located and unilateral holes were drilled with an electrical drill at the following co-ordinates; 4.4mm anterior, 2mm lateral of the sagittal suture. Care was taken not to damage the meninges. A Hamilton syringe, with a cannula of diameter 0.3mm, was used to inject 0.6 μ moles of quinolinic acid in 2 μ l of PBS, 3mm ventral of the dura. The injection was administered at a rate of 1 μ l per minute and the cannula was left *in situ* for a further 2 minutes following QA injection, to allow for

passive diffusion away from the cannula tip and to minimise spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals were kept warm until recovery from the anaesthesia.

ii. Sham Lesioned Rats

The control, tolmetin (tol) and sulindac (sul) rats were subjected to the surgical procedures outlined in section 4.2.2.4. (i). However, stereotaxic injections into the hippocampus were free of QA, and comprised only of PBS.

4.2.2.5. Brain Removal

The rats were sacrificed 48 hours after QA intrahippocampal injections and the brains were removed as described in section 2.2.2.3.

4.2.2.6. Histological Techniques

Once the brain was removed, it was immediately fixed, processed, sectioned onto slides and stained with 0.1% cresyl violet stain (for a complete overview of the Nissl body staining procedure see appendix 5). Slides were viewed and photographed using a bright light microscope fitted with a digital Olympus camera.

4.2.3. Results

The neurons in the control group (figure 4.2A,B) show optimally sized, pyramidally shaped neurons with clearly observable cell nucleus and continuous cell membranes. The cells in this group are closely packed and form a band-like appearance, which is characteristic of both the CA1 and CA3 regions. Rats, which received intrahippocampal injections of QA, showed degenerated neurons in their CA1 and CA3 regions (figure. 4.2C,D). Neurons appear scattered with little integrity of the cell membranes. There are fewer neurons and these cells display a loss of structure. A loss of the hippocampal formation was also observed. The CA1 and CA3 regions of rat hippocampus from rats treated with tolmetin show a lower degree of damage and degeneration (figure 4.2E,F). Sulindac also shows signs of providing protection (figure 4.2G,H).

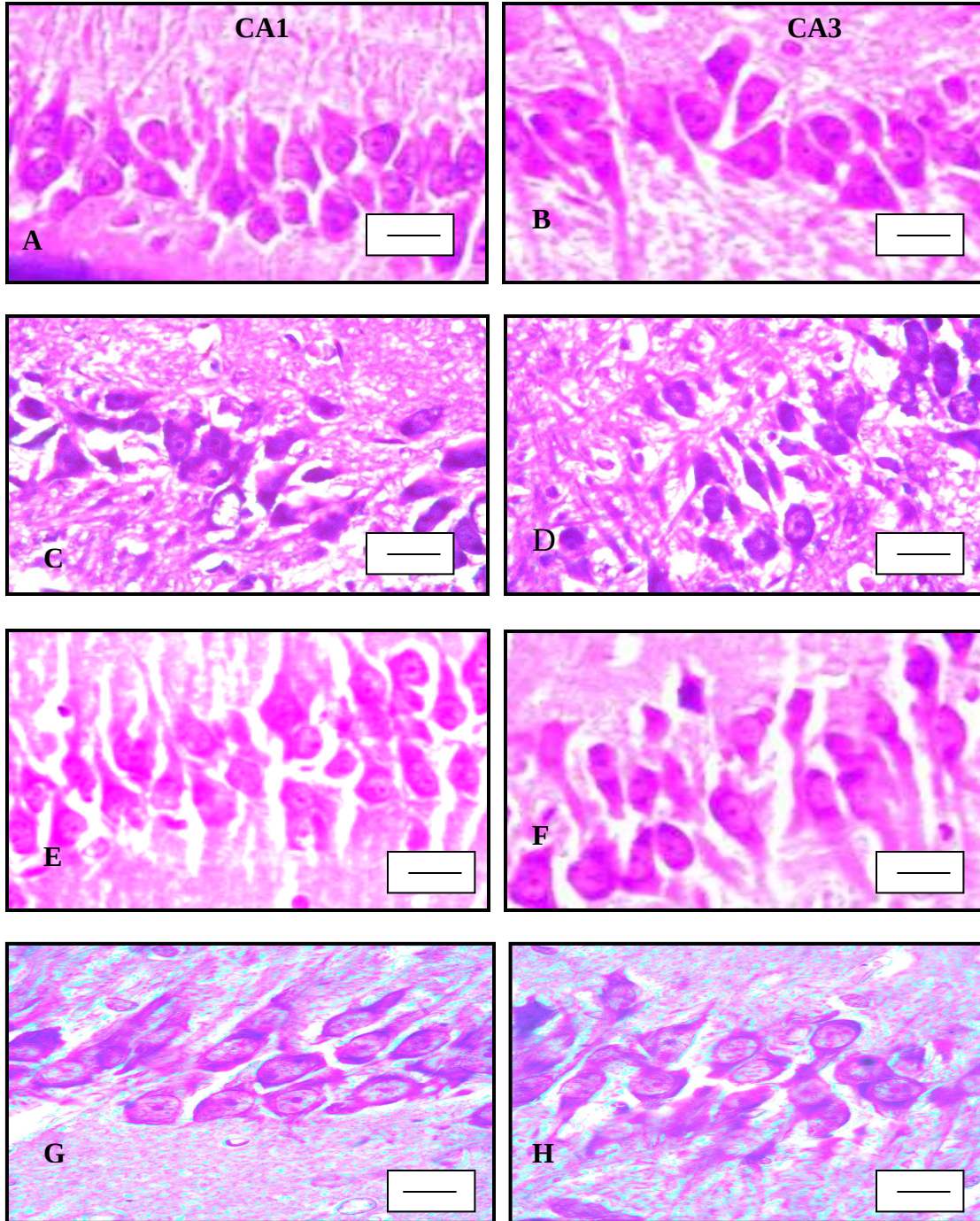


Figure 4.2: Effects of tolmetin and sulindac on QA-induced hippocampal lesions. The left hand panels show the CA1 regions and the right hand panels show the CA3 regions. Fig. A,B represent controls that received 2 μ l of PBS, C,D are the QA injected hippocampi, E,F and G,H are the tolmetin and sulindac treated animals respectively which also received QA. Bar = 10 μ m.

4.2.4. Discussion

It has already been established that QA has the ability to induce neuronal lesions after intrahippocampal injections in rat brain (Schwarcz *et. al.*, 1983). The present study shows that QA induced damage to the rat hippocampal neurons. Fewer neurons were visible in the CA1 and CA3 regions of the rat hippocampus. This can be explained by the excitotoxic effects of QA due to the over-stimulation of the NMDA receptors by QA, which disrupts the Ca^{2+} , Na^{+} and K^{+} ion concentrations within the neuron. The brains of those rats pre-treated with tolmetin or sulindac show less signs of neurodegeneration after QA injections and the CA1 and CA3 regions have more viable neurons. One mechanism via which these agents possibly offer neuroprotection is by scavenging the free radicals that may have formed.

The anti-inflammatory properties of these agents may be another mechanism by which these agents protect neurons. It is also possible that tolmetin and sulindac are indirect anti-oxidants because cyclooxygenase (COX) activity itself produces ROS (Nikolic and Breeman 2001). Therefore, by inhibiting COX activity, the NSAIDS possibly act as antioxidants. It has been shown that NSAIDS improve the circulating SOD levels in human erythrocytes and plasma after NSAID therapy (Nivsarkar 2000) however, the author did not propose a mechanism. Tolmetin and sulindac may have increased the circulating SOD levels and this therefore reduces damage that may have been induced by the superoxide after QA intrahippocampal injections. These anti-inflammatory agents may reduce neuronal damage through the above-mentioned mechanisms.

4.3. APOPTOSIS

4.3.1. Introduction

Apoptosis was originally described by Kerr *et. al.* (1972) based on the examination of tissue specimens from the adrenal gland. Since then, apoptosis has been extensively studied from many medical and biological perspectives.

Apoptosis is a highly ordered process that contributes to the selective elimination of cells in physiologic and pathologic situations. This type of cell death can be recognised by several biochemical and morphological markers. These include chromatin condensation, DNA fragmentation, caspase activation and mitochondrial alterations (D'Mello *et. al.*, 1993; D'Mello *et. al.*, 1997; Nardi *et. al.*, 1997; Nicholls & Budd 2000). Studies have suggested that apoptotic neuronal death involves ROS such as hydrogen peroxide, superoxide anion and singlet oxygen (Wei *et. al.*, 2000; Zhuang *et. al.*, 2000; Tammariello *et. al.*, 2000; Valencia & Moran 2001; See & Lloeffler 2001). It has been suggested that hydrogen peroxide leads to apoptotic neuronal death by involving pro-apoptotic molecules (Wei *et. al.*, 2000), such as initiator caspases (See & Lloeffler 2001). Superoxide anions are claimed to be responsible for the apoptotic cell death of trophic factor-deprived sympathetic neurons (Greenland *et. al.*, 1995a; Greenland *et al.*, 1995b), glutamate-treated cerebellar neurons (Sato *et. al.*, 1998; Ishikawa *et. al.*, 1999) and hippocampal neurons incubated with xanthine oxidase (Guo *et. al.*, 1999; Ishikawa *et. al.*, 1999). Oxidative stress has also been implicated in necrotic cell death and it has been suggested that apoptotic or necrotic cell death can be produced depending on the time of exposure and/or the concentration of the ROS (Manev *et. al.*, 1995; Kruman & Mattson 1999; Cole & Perez-Polo 2002).

This experiment was conducted to determine whether QA induces apoptotic cell death in rat hippocampus and if so, could the NSAIDS prevent or reduce the QA-induced neurotoxicity.

4.3.2. Materials and Methods

4.3.2.1. Chemicals and Reagents

The *in situ* cell detection kit, POD, DNase and Proteinase K was purchased from Roche diagnostics, Mannheim, Germany. All other reagents were as per section 4.2.2.1.

4.3.2.2. Animals

Adult male Wistar rats, weighing between 250g and 300g were used for experiments, and were cared for as described in section 2.2.2.2.

4.3.2.3. Treatment Regimes, surgical procedures and brain removal

Animals were treated as described in section 4.2.2.3 and the surgical procedures that were conducted were described in section 4.2.2.4. The brain was carefully removed as described in section 2.2.2.3.

4.3.2.4. Preparing the brain specimen for TUNEL staining

Once removed, the brain was fixed in formaldehyde, dehydrated, embedded in paraffin wax, sectioned using a rotary microtome and transferred onto slides (for detailed procedures, see appendix 5).

4.3.2.5. Specificity of the TUNEL stain

The TUNEL reaction preferentially labels DNA strand breaks that are generated during apoptosis. This allows for discrimination between apoptosis and necrosis and primary DNA strand breaks induced by cytostatic drugs or irradiation (Gorczyca *et. al.*, 1993; Gold *et. al.*, 1994).

4.3.2.6. The TUNEL staining procedure

Apoptotic cells were detected by immunohistochemistry, using the procedure outlined by the manufacturer. The method that was used is described below.

4.3.2.6.1. Pre-treatment of paraffin embedded tissue

The paraffin embedded tissue had to be dewaxed and hydrated before being exposure to the TUNEL stain. This was achieved as described in appendix 5: procedure for dewaxing and re-hydrating brain sections. However, the tissue sections were not incubated overnight in ethanol but were removed from ethanol after 5 minutes and were rinsed in copious amounts of PBS, pH 7.4. Thereafter, the tissue sections were incubated with Proteinase K (20µg/ml in 10mM Tris-HCl, pH 7.4) for 20 minutes at 37°C in a humidified chamber. Proteinase K digests cross-linked proteins and increases the cell permeability and access to the nucleic acid targets such as the DNA. With the use of Proteinase K, the concentration, time of exposure and temperature has to be optimised for each type of tissue (Gavrieli *et al.*, 1992; Billig *et al.*, 1994). After the 20 minutes incubation, slides were gently rinsed thrice with PBS.

The experimental slides were kept moist with PBS while positive control slides were removed for DNase treatment. Two negative and positive control slides were also included into each experiment. Since DNA fragmentation is characteristic of apoptosis, incubation of control slides with DNase I would generate a positive control. DNase was used at a working concentration of 3000U/ml and was prepared in 50mM Tris-HCl, pH 7.4 containing 1mg/ml BSA. The DNase was applied to 2 control slides which was thereafter incubate for 10 minutes at room temperature.

4.3.2.6.2. Labelling

The TUNEL mixture was prepared during the ten minute incubation time of the positive controls. The kit contains 2 vials:

- (a) vial 1 contains the enzyme solution (Terminal deoxynucleotidyl transferase (Tdt) from calf thymus recombinant in *E-coli*, in storage buffer.), and
- (b) vial 2 contains the labelling solution (nucleotide mixture in reaction buffer).

For the negative controls, 100µl of the labelling solution was removed from vial 2 and kept aside. The TUNEL reaction mixture was prepared by combining the enzyme solution from vial 1 with the rest of the labelling solution that was left in vial 2. This

produces 500µl of TUNEL mixture and is enough for 10 slides. The above procedure was carried out in a darkened room.

After incubation, the positive control slides were rinsed thrice in PBS. Negative controls were stained with the 50µl of labelling solution while the other slides, including the positive controls were stained with 50µl of the TUNEL reaction mixture. The slides were incubated in a humidified chamber (to prevent slides from drying out) for 1 hour at 37°C. The reaction was terminated by thorough washing with PBS.

4.3.2.6.3. Photo-microscopy

Slides were viewed using an Olympus Optical (Model BX60F) fluorescence microscope (made in Japan). Fluorophore excitation was achieved by illuminating the sample with green light at wavelengths between 515 and 565nm. Neurons were viewed in the injected hippocampi and photographs were taken using a digital Olympus camera.

4.3.3. Results

Apoptotic cells can be recognised by the bright green spots against the green background. As shown in figure 4.3A, the positive control (DNase induced apoptosis) there are many bright green spots visible which is completely absent in the negative control (Figure 4.3B). These spots are indicative of DNA fragmentation. Animals that received PBS (i.h.) show no signs of apoptosis (figure 4.3C) while animals that received QA (with no NSAID treatment) display apoptosis (figure 4.3D). Animals dosed with tolmetin and sulindac (5mg/kg/bd for 5 days) show little to no signs of apoptosis (figure 4.3E and 4.3F respectively).

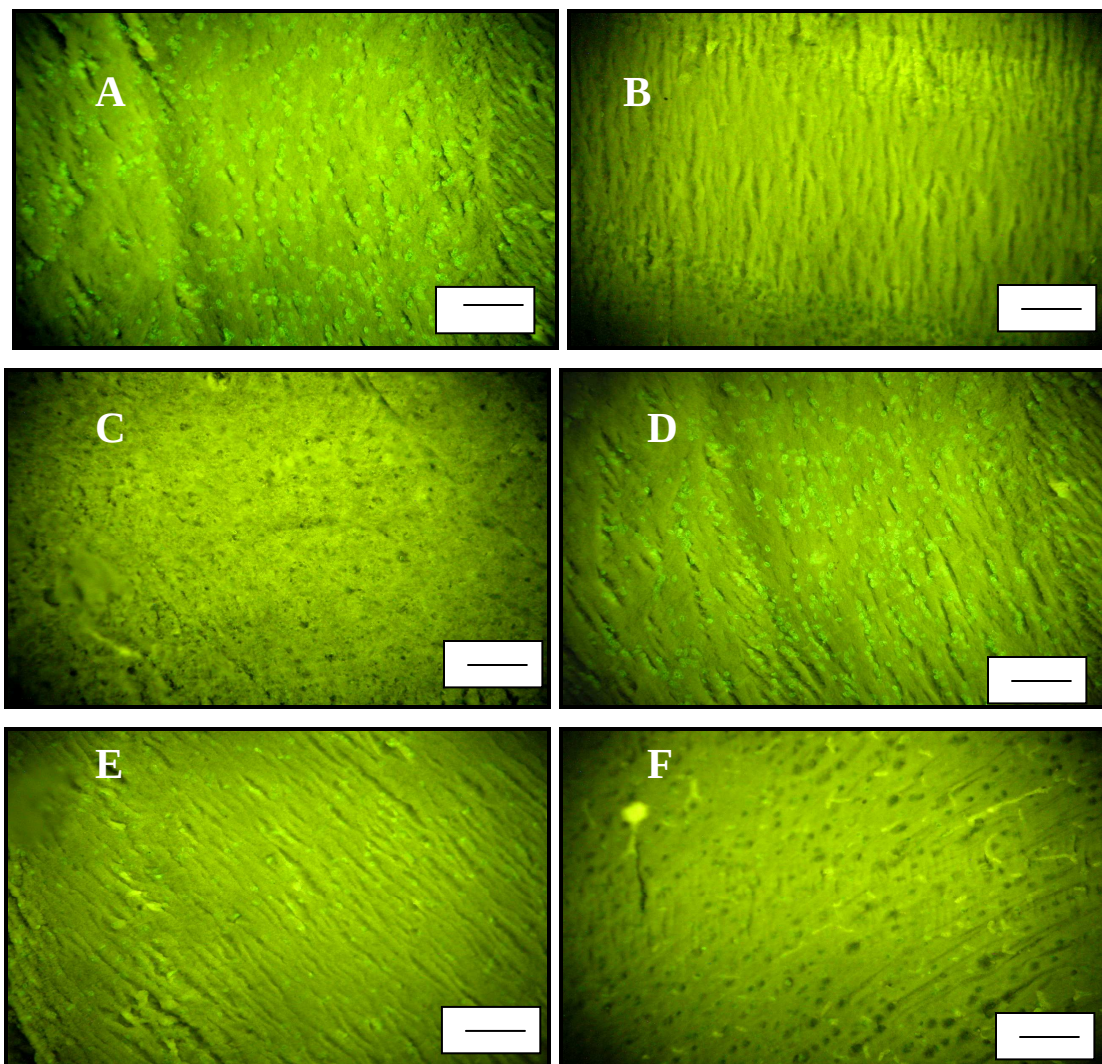


Figure 4.3. Effects of tolmetin and sulindac on QA-induced apoptosis in rat hippocampus. Fig. A & B show the positive and negative controls respectively. Fig. C represents hippocampi from animals that received PBS (i.h.) while Fig. D shows apoptotic cells from animals that received QA (QA i.h. without NSAID treatment). Fig. E & F show hippocampi from animals pre-treated with tolmetin and sulindac respectively. Bar = 100 μ m

4.3.4. Discussion

Excessive stimulation of postsynaptic glutamate receptors, especially those of the NMDA subtype, have shown to contribute to neuronal damage. This damage presents itself as hypoxia and ischemia (Rothman 1984; Simon *et. al.*, 1984), glucose deprivation (Wieloch 1985), trauma (Faden & Simon 1988; Tecoma *et. al.*, 1989) and prolonged

seizures (Clifford *et. al.*, 1989; Garthwaite 1991). Increasing evidence suggests that NMDA receptor mediate nerve cell destruction involves apoptotic mechanisms triggered by oxidative stress (Portera-Cailliau *et. al.*, 1995; Thompson 1995; Qin *et. al.*, 1996). Qin and co-workers showed that NMDA and QA initiate the apoptotic cascade by stimulation of the NMDA but not the AMPA and KA receptors. It has been demonstrated that QA, the known NMDA agonist (Stone & Perkins 1981) induces apoptosis in rat striatum (Nakai *et. al.*, 1999) and this was attenuated with a free radical scavenger, OPC-14117. It has also been shown that the group IIA secretory phospholipase A₂ induced apoptosis in primary cultures of rat cortical neurons and the apoptosis was prevented by the treatment of the neurons with S-2474, a novel anti-inflammatory agent with antioxidant properties (Yagami *et. al.*, 2005). In this study QA induced free radicals and induced apoptosis but this may have been attenuated by the antioxidant and anti-inflammatory properties of tolmetin and sulindac.

In response to environmental and pathophysiological stress, cells induce the synthesis of intracellular proteins that are referred to as heat shock proteins (HSPs) or “shock proteins” (Bukau & Horwich 1998; Sharp *et. al.*, 1999). It has been suggested that HSPs improve cellular recovery by refolding partially damaged functional proteins and by increasing the delivery of precursor proteins to important organelles such as the mitochondria (Rutherford & Lindquist 1998). It is known that HSPs bind to the receptor-interacting protein, a major component of the tumor necrosis factor (TNF) receptor-1 (Lewis *et. al.*, 2000). Receptor interacting proteins play an important role in TNF-mediated nuclear factor- κ B (NF- κ B) activation that may protect cells against TNF-induced apoptosis. Studies have demonstrated that aspirin directly suppresses the NF- κ B inhibitor kinase (Yin *et. al.*, 1998) and results in the inhibition of NF- κ B and neuronal death (Grilli *et. al.*, 1996). Indomethacin, ibuprofen and sulindac have shown to inhibit TNF- α production (Jiang *et. al.*, 1998) and reduce the production of A β 42 but not A β 40 (Lim *et. al.*, 2000; Weggen *et. al.*, 2001). QA may have interfered with the HSPs and triggered a cascade of events which could have induced apoptosis. It is possible that tolmetin and sulindac may have also offered neuroprotection in a similar manner as aspirin, indomethacin and ibuprofen.

This study shows that QA induces apoptosis in rat hippocampus and this is reduced by NSAID treatment. Although other mechanisms may be involved, the antioxidant and anti-inflammatory properties of these NSAIDS are possible mechanisms by which these agents offer neuroprotection. The possibility of tolmetin and sulindac offering neuroprotection at a molecular level needs to be investigated further.

4.4. EFFECT OF TOLMETIN AND SULINDAC ON INTRAHIPPOCAMPAL ADMINISTRATION OF QUINOLINIC ACID ON ³H-MK801 RECEPTOR BINDING

4.4.1. Introduction

In the previous experiments, (4.2 and 4.3), tolmetin and sulindac offer protection against QA induced neurotoxicity in the rat hippocampus. However, the protection offered by tolmetin and sulindac was not quantified.

In an attempt to determine the degree of protection offered by tolmetin and sulindac, receptor-binding assays on hippocampal neurons were conducted. *In vitro* radioligand binding assays has emerged as a powerful technique in the study of excitatory amino acid receptors. With specific ligands and suitable binding conditions, each of the different receptor classes can be selectively examined (Monaghan *et al.*, 1983). Excitotoxicity has been suggested as an underlying mechanism in the neural damage associated with both acute and chronic human disorders (Olney 1989). Intrastratial injections of excitotoxins such as QA have shown to result in heavy neuronal death (Beal *et. al.*, 1986; Beal *et. al.*, 1989; Emerich & Sanberg 1992). In these studies conducted, a reduction in the number of NMDA receptors after intrahippocampal administration of QA implies a reduction in the number of viable neurons in the hippocampus. If it remained constant, it would demonstrate that the number of viable neurons had remained the same after QA injections. Whilst such a relationship cannot be definite, there is a causal relationship between the two since it has been previously demonstrated that neuronal loss results in the loss of receptors (Southgate *et. al.*, 1998). Although this method is not an absolute indication to the amount of protection offered, it was used to give an indication of the number of viable neurons in the hippocampus. Moreover, receptors were quantified *in vitro*, therefore there is no up or down regulation of the NMDA receptors in relation to the ligand MK801 concentration.

4.4.2. Materials and Methods

4.4.2.1. Chemicals and Reagents

Ketamine Hydrochloride was obtained from Kyron Laboratories (Pty) Limited, Benrose, South Africa. [³H]-MK801 was purchased from New England Nuclear (NENTM), Boston, U.S.A. Emulsifier Scintillation PlusTM cocktail and scintillation vials were obtained from Packard Company (USA). Triton X-100 was purchased from Unilab, South Africa. Folin's reagent and Bovine Serum Albumin (BSA) was purchased from Sigma Chemical Corporation; St Louis, MO, U.S.A. All other reagents used were the same as those used in section 4.2.2.1.

4.4.2.2. Animals and Surgical Procedures

Adult male, Wistar rats were cared for as described in section 2.2.2.2. Three animals were put into each group. Treatment regimes were as per section 4.2.2.3. Surgical procedures were performed exactly as per section 4.2.2.4. Briefly, rats were anaesthetised with sodium pentobarbital (60mg/kg i.p). After a sagittal cut in the skin of the skull, the bregma suture was located and holes were drilled with an electrical drill at the following co-ordinates; 4.4mm anterior, 2mm lateral of the sagittal suture. A Hamilton syringe was used to inject 0.6µmoles of QA in 2µl of PBS, 3mm ventral of the dura.

4.4.2.3. Preparation of Synaptic Membranes

Three injected hippocampi from each group were used for NMDA receptor binding studies. Hippocampal neuronal membranes were prepared using a similar method to that described by Yoneda *et. al.* (1991). Briefly, the rats were subjected to cardiac perfusion using 0.9% saline. The rats were decapitated and the injected hippocampi were dissected from the brains. Each hippocampus was homogenised in 40 volumes of 5mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged at 30 000 x g at 4°C for 20 minutes. The pellets were resuspended and washed a further 3 times with 40 volumes of 50 mM Tris acetate buffer (pH 7.4). The pellets were then resuspended in 20 ml of 0.32 M sucrose and rapidly frozen in liquid nitrogen and stored at -70°C until use.

4.4.2.4. Protein Assay

Protein estimation was done using the method described by Lowry *et. al.* (1951). A stock solution of BSA was prepared in Milli-Q water. Aliquots of this stock solution, at 60µg/ml intervals were used to prepare varying concentrations of BSA, to which copper reagent and Folin's reagent was added. The absorbance was read at 500nm and a calibration curve was generated by plotting concentration against absorbance (Appendix 6).

4.4.2.5. NMDA Binding assay procedure

On the day of the binding study, the frozen synaptic membranes were thawed, diluted up to 40 volumes with buffer and centrifuged at 30 000 x g at 4°C for 20 minutes. The pellets were resuspended and treated with 0.05% Triton X-100 for 10 minutes. This was followed by centrifugation at 30 000 x g at 4°C for 20 minutes. The pellets were resuspended in 50mM Tris acetate buffer and the suspension was diluted to a protein concentration of 1mg/ml. The total binding incubation mixtures consisted of 300µl membrane preparation, 35µl 50mM tris-acetate buffer (pH 7.4) and 15µl ³H-MK801 ranging from 0 to 50nM final concentration. The non-specific binding incubation mixtures was conducted in the exact manner as the total binding except here the mixture had 35µl of ketamine (1mM) instead of buffer. The tubes were incubated for an hour at room temperature and the reaction was terminated by the addition of 4 ml of ice-cold Tris-acetate buffer (pH 7.4). This was followed immediately by rapid filtration through Whatman GF/C filters in a 12-place Millipore sampling manifold under negative pressure. The filters were washed a further 2 times with 4 ml of ice cold Tris-acetate buffer, before being dried by negative pressure and placed in scintillation vials containing 5 ml of scintillation cocktail. After 24 hours, the radioactivity in the vials was measured using a Beckman (Model LS 2800) Liquid Scintillation Counter. Results (Bmax and Kd values) are expressed as pmol ³H-MK801 bound per milligram protein. These results were statistically analysed as described in section 2.2.2.7. The experiment was replicated three times.

4.4.3. Results

Saturation isotherms as well as Scatchard plots (figures 4.4 to 4.9) were generated. Each point represents the mean $\pm$ SD of three separate determinations. The Bmax and Kd values were obtained using Graphpad Prism (table 4.2).

The QA rats showed an approximate 37% decrease in the number of NMDA receptors compared to control while those animals that were injected intrahippocampally with QA and were pre-treated with tolmetin only showed an approximate 25% decrease in the number of NMDA receptors compared to the QA rats. The rats pre-treated with sulindac that also received QA show a decrease of 20% in the number of NMDA receptors. The animals treated with either tolmetin or sulindac show an insignificant change in the number of NMDA receptors. No significant differences were noted in the Kd values.

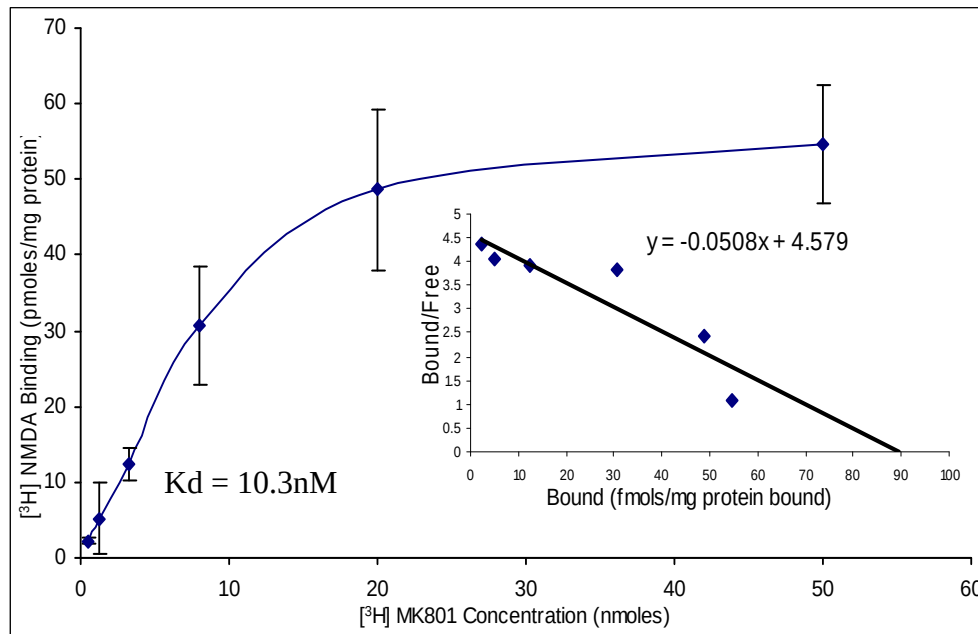


Figure 4.4: Saturation isotherm (Scatchard plot and Kd value inserted) of ^3H -MK801 binding to hippocampal synaptic membranes from control rats (n = 3).

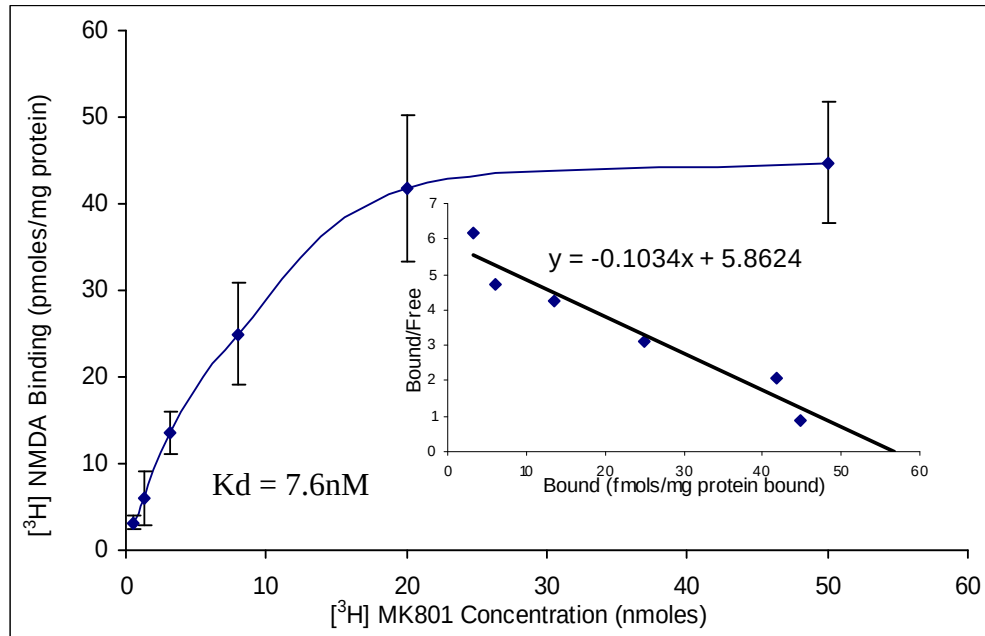


Figure 4.5: Saturation isotherm (Scatchard plot and K_d value inserted) of $[^3\text{H}]$ -MK801 binding to hippocampal synaptic membranes from rats that received QA i.h. (no NSAID treatment) ($n = 3$).

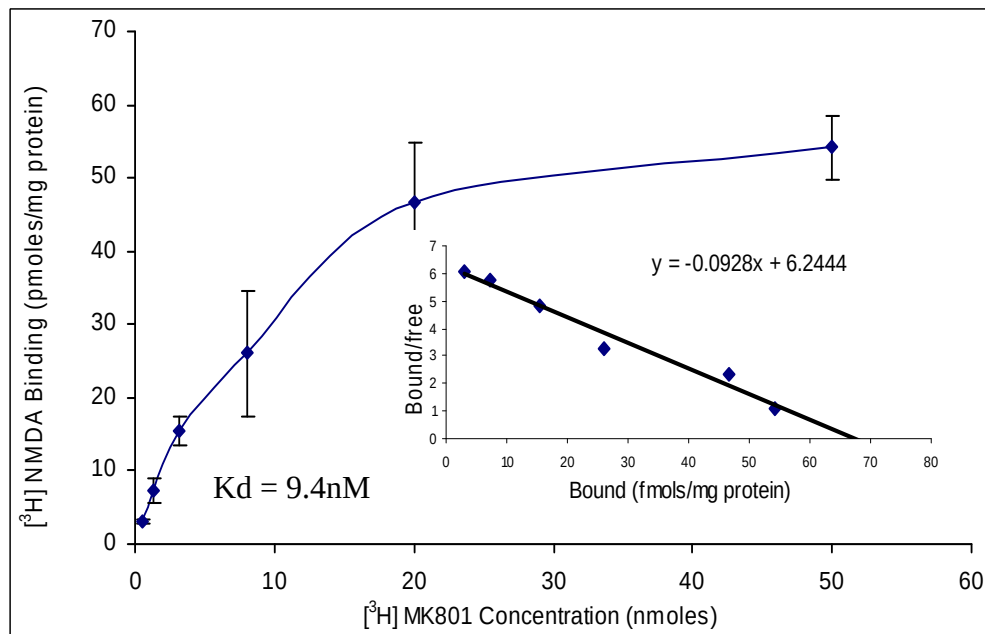


Figure 4.6: Saturation isotherm (Scatchard plot and K_d value inserted) of $[^3\text{H}]$ -MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with tolmetin and also received hippocampal injections of QA ($n = 3$).

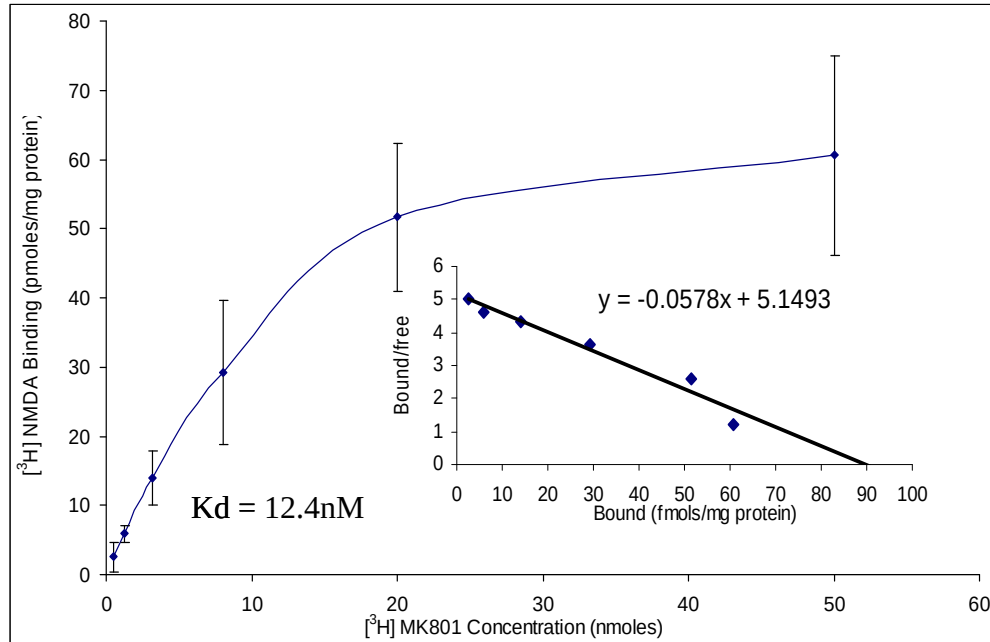


Figure 4.7: Saturation isotherm (Scatchard plot and K_d value inserted) of ^3H -MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with tolmetin and received hippocampal injections free of QA ($n = 3$).

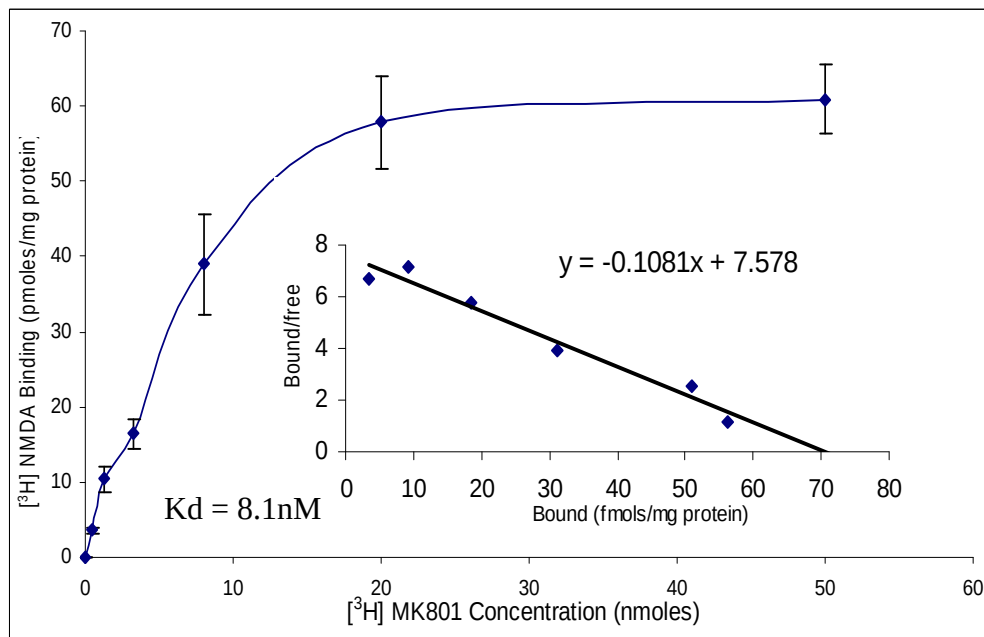


Figure 4.8: Saturation isotherm (Scatchard plot and K_d value inserted) of ^3H -MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with sulindac and also received hippocampal injections of QA ($n = 3$).

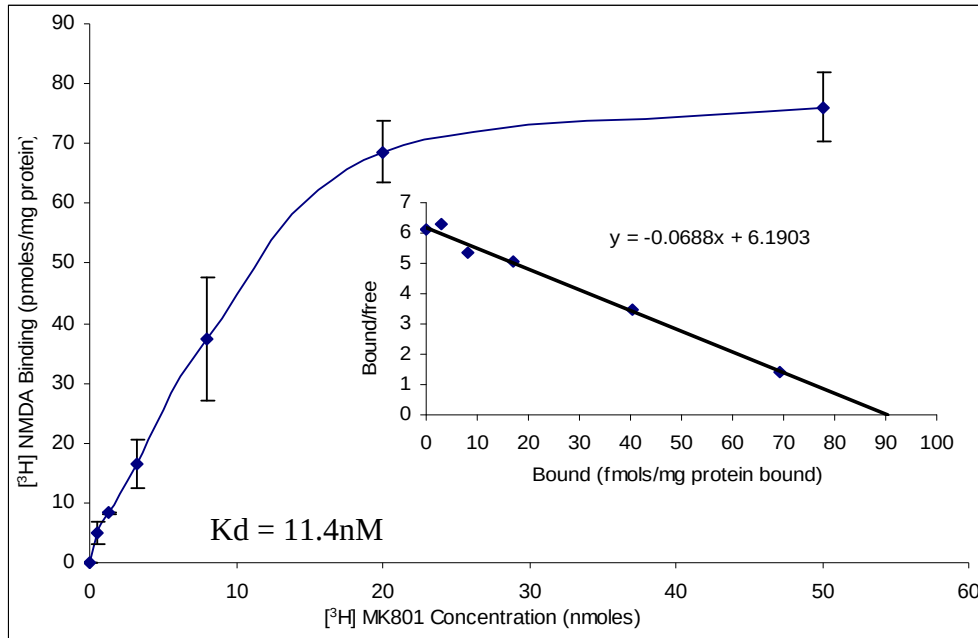


Figure 4.9: Saturation isotherm (Scatchard plot and K_d value inserted) of ^3H -MK801 binding to hippocampal synaptic membranes from rats that were pre-treated with sulindac and received hippocampal injections free of QA ($n = 3$).

Table 4.2: Comparison of B_{max} and K_d values obtained from the NMDA receptor binding studies.

Treatment Regime	B _{max} (pmoles ³ H-NMDA/mg protein)	K _d values (nM)
Control: PBS (i.h) no drug treatment	88.5 ± 3.3	10.3 ± 1.5
QA (i.h) no drug treatment	56.2 ± 3.9 ^{***}	7.6 ± 1.5 ^{ns}
Tolmetin (5mg/kg/bd for 5 days) then QA (i.h)	66.6 ± 3.9 [*]	9.4 ± 1.4 ^{ns}
Tolmetin (5mg/kg/bd for 5 days)	89.2 ± 8.6 ^{ns}	12.4 ± 2.0 ^{ns}
Sulindac (5mg/kg/bd for 5 days) then QA (i.h)	70.9 ± 4.1 [*]	8.1 ± 0.3 ^{ns}
Sulindac (5mg/kg/bd for 5 days)	90.0 ± 8.2 ^{ns}	11.4 ± 1.8 ^{ns}

B_{max} and K_d values are expressed as pmoles ³H-NMDA bound per milligram of hippocampal neuronal membrane protein. Each value represents the mean ± SD (n=3). ^{***}(p<0.001) and ^{ns}(p>0.05) compared to control. ^{*}(p<0.05) compared to QA treatment only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

4.4.4. Discussion

QA has shown to induce neurotoxicity in all the experiments thus far. It can be observed in figure 4.1C&D that QA reduces the number of neurons in the CA1 and CA3 regions in the hippocampus. This reduction was dampened by tolmetin and sulindac treatment.

A previous study by Nakai and co-workers in 1999 demonstrated that intrastriatal infusion of QA reduces the NMDA and DA receptors by about 75% and 79% respectively. This decrease in receptors was attenuated by the co-treatment of the animals with a free radical scavenger, OPC-14117. Another study conducted by Qin and co-worker, demonstrates that glutamate agonists NMDA and QA reduces NMDA receptors in the striatum in a concentration-dependent manner. It has also been shown that neuronal

loss results in loss of receptors (Southgate *et. al.*, 1998). Whilst such relationship cannot be conclusive in itself, there is a causal relationship between the two. The receptor binding data show that QA reduces the number of NMDA receptors without changing the affinity (Kd) of the receptors. As shown in table 4.2, QA reduced the number of NMDA binding sites in the rat hippocampus, which is possibly due to the loss of neurons. The rats treated with either tolmetin or sulindac and which received intrahippocampal injections free of QA, did not show any change in the number of NMDA binding sites. Therefore the increase in the number of NMDA receptors in the animals pre-treated with tolmetin or sulindac and that received QA intrahippocampally could have been possible if these agents prevented the damage or death of the neurons.

There is a large body of evidence indicating that inflammatory processes in the brain result in neurodegenerative disorders (Ames *et. al.*, 1993; McGeer & McGeer 1998; McGeer *et. al.*, 2001). Apart from free radical scavenging activity, these NSAIDS may also offer protection via their anti-inflammatory actions. Tolmetin and sulindac protect neurons and subsequently reduce the loss of receptors by approximately 12 and 17% respectively. This suggests that these agents may protect neurons against free radical-induced neurotoxicity. Since the loss of neurons was reduced, these agents may also prevent QA-induced memory loss. This aspect is investigated in chapter 5.

4.5. EFFECT OF QUINOLINIC ACID ON NEURONAL CELL VIABILITY

4.5.1. Introduction

It has been established that QA is a potent neurotoxin. It is well known that this excitatory amino acid exerts its neurotoxic actions via stimulating NMDA receptors. This affects the ionic balance within the cell which triggers many events that can damage the cell reversibly or irreversibly. Cell death can be brought about in many ways (Orrenius *et al.*, 1989; Siesjo & Bengtsson 1989) of which free radical induced damage is a major cause.

In the previous two chapters it has been shown that QA induces LP and superoxide anion generation in primary hippocampal neurons. QA also induces apoptosis and damage to hippocampal neurons. Thus, this experiment was conducted to determine the effect of QA on the neuronal cell viability.

The general principal involved in the detection of cell growth or kill is indicated by the conversion of the tetrazolium salt to the coloured product, diformazan which can be measured spectroscopically. The formation of the formazan is thought to take place via intact mitochondria although other extracellular locations with dehydrogenase activity may contribute to the total formazan production (Mossmann 1983).

4.5.2. Materials and Methods

MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) and dimethyl sulfoxide (DMSO) was purchased from Sigma Chemicals Corporation, St Louis, MO, U.S.A. All other reagents used were as per section 2.4.2.1.

4.5.2.1. The MTT Assay

The method used for assaying the cell viability was a technique that was originally described by Mossmann in 1983 which was further improved for greater reliability and sensitivity in 1989 by Hansen *et al.*

Primary hippocampal neurons were established as described in section 2.4.2.5.

Six to eight day old cultures were used for the experiments. Before experimentation, each flask was washed twice with 3ml of PBS. Thereafter 1ml of fresh MEM was added. To this, 100 μ l QA dissolved in MEM was added to produce final concentrations of 50; 100 or 200 μ M. The concentrations of QA used were the same as those in the LP and Nitroblue Tetrazolium assays. The culture flasks were then incubated for 3 hours as previously described.

After 3 hours, the cells were gently washed thrice with 3ml of PBS and 1ml of fresh MEM was added. The cells were loaded with 0.5ml of MTT dye at a final concentration of 1mg/ml. The cells were further incubated for 2 hours after which the cells were scraped from the culture flasks using a cell scraper and transferred to a test tube. The culture flasks were rinsed with 1ml ice cold PBS and this was transferred to the test tubes. The test tubes were centrifuged for 15 minutes at 3000g after which the supernatant was carefully poured off and the resulting pellet dissolved in DMSO. The absorbance was read at 570nm. The results were expressed as % viability/ 10^6 cells. The controls (no QA) were taken as 100% viable. All results were analysed as described in section 2.2.2.7. The experiment was replicated four times.

4.5.3. Results

QA exerted its neurotoxic potential in a concentration dependent manner (figure 4.10). QA at a concentration of 200 μ M reduces the cell viability by 20% in 3 hours.

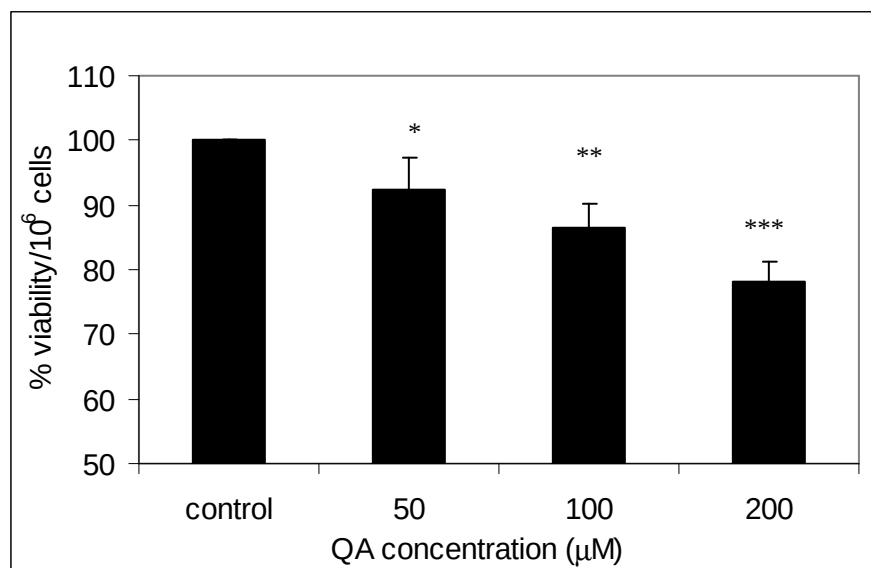


Figure 4.10: Effect of QA (50, 100 and 200μM) on primary hippocampal cell viability. Each bar represents the mean $\pm$ SD (n=4). *(p<0.05), **(p<0.01) and *** (p<0.001) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

4.5.4. Discussion

QA has thus far shown to induce lipid peroxidation and generate the superoxide anion in primary hippocampal neurons. The mechanism of free radical-induced injuries has been discussed. QA induces its neurotoxicity by stimulating NMDA receptors (Stone & Perkins 1981), a subtype of the glutamate receptors, causing an influx of ions into the cell which eventually leads to free radical formation (Choi 1992; Choi 1995). QA is also known to cause ATP exhaustion, γ -aminobutyric acid (GABA) depletion, specific GABAergic and cholinergic neuronal death (Foster *et al.*, 1983; Stone 1993; Santamaria & Rios, 1993). It also causes neuronal excitation (Stone & Perkins 1981), seizure activity (Schwarcz *et al.*, 1984) and neuronal degeneration (Schwarcz *et al.*, 1983). QA induces neuronal degeneration and neurochemical changes similar to those observed in Huntington's disease (Schwarcz *et al.*, 1983; Beal *et al.*, 1986). Research has shown that excitatory amino acids such as NMDA and glutamate increase intracellular Ca^{2+} concentrations, induce apoptosis and decrease the viability of cultured rat cortical

neurons (Black *et. al.*, 1995). This experiment shows that QA reduces the viability of primary hippocampal neurons in a concentration-dependent manner. Although other mechanisms may be involved, the most possible mechanism of QA-induced neurotoxicity may have been by the over-stimulation of NMDA receptors.

4.6. EFFECT OF TOLMETIN AND SULINDAC ON NEURONAL CELL VIABILITY IN THE PRESENCE OF QUINOLINIC ACID

4.6.1. Introduction

QA has been shown to reduce the neuronal cell viability. The antioxidant potential of tolmetin and sulindac have been demonstrated. This experiment was conducted to determine whether these agents could reduce QA-induced damage and increase the cell viability.

4.6.2. Materials and Methods

All chemicals and reagents were as per section 2.4.2.1 and 4.5.2. Experiments were conducted in the exact same manner as in experiment 4.5.2.1. In addition, tolmetin or sulindac was also added to the incubation medium at concentrations (50; 100 or 200 μ M) with or without QA (200 μ M). The concentrations of drugs used were same as those used for the LP and NBT experiments. The results were analysed as described in section 2.2.2.7. The experiment was replicated four times.

4.6.3. Results

As shown in figure 4.11, QA reduces the cell viability to approximately 80% in 3 hours. The treatment of the cells with tolmetin and sulindac improves cell viability. At 200 μ M, both drugs offer the greatest amount of protection to the cells, increasing the cell viability from approximately 80% to approximately 92%.

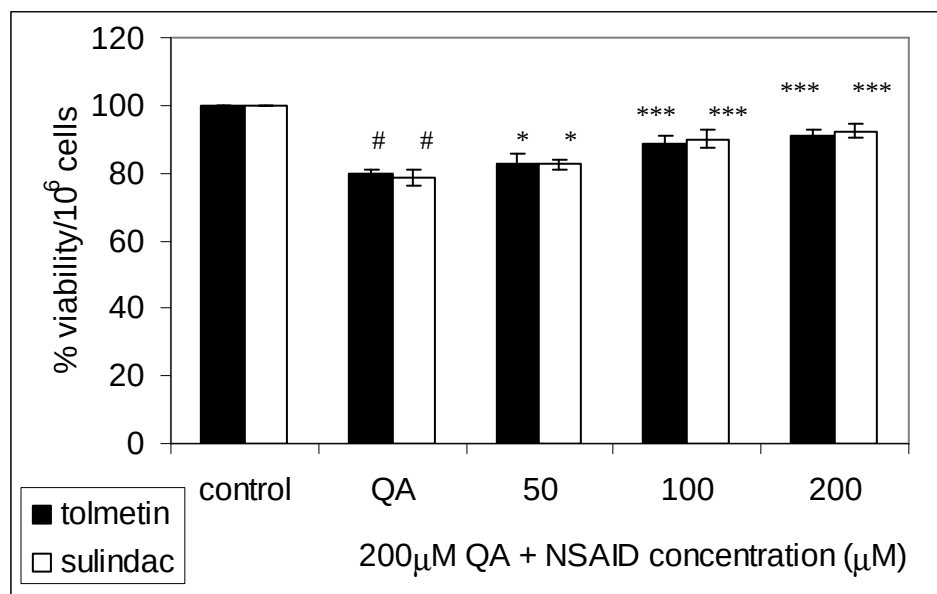


Figure 4.11: Effect of tolmetin and sulindac on neuronal cell viability in the presence of 200 μM QA. Each bar represents the mean $\pm$ SD (n=4). # (p<0.001) compared to control, * (p<0.05) and *** (p<0.001) in comparison to QA only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

4.6.4. Discussion

This experiment shows that tolmetin and sulindac have the potential to protect primary hippocampal neurons against QA-induced neurotoxicity in hippocampal neurons. It is known that QA induces neurotoxicity from the activation of NMDA receptors (Stone 1993) but QA is also known to be a weak NMDA receptor agonist (Schwarcz *et al.*, 1983). However, QA levels are known to rise 10-100 fold in activated microglia and invading macrophages during neuroinflammatory events (Obrenovitch 2001) and can also induce oxidative damage independently of any glutamate receptor stimulation (Behan *et al.*, 1999). As mentioned earlier, QA is not removed from the synaptic cleft and there is no extracellular metabolism of this excitatory amino acid and therefore causes a sustained stimulation of the NMDA receptors, thus making QA a very potent neurotoxin. It was also reported that QA is a pro-oxidant (Santamaria & Rios 1993) and has the ability to affect endogenous antioxidant systems (Cruz-Aguado *et al.*, 2000; Rodriguez-Martinez *et al.*, 2000). It has been shown that the over-stimulation of glutamate receptors

depletes GSH levels in mouse hippocampal cell line and reduces the viability of the cells (Xiao *et al.*, 1999). Park and co-workers demonstrated that the incubation of rat cerebellar granule cells with excitatory amino acids such as NMDA or glutamate increases intracellular Ca^{2+} concentrations, induces ROS generation and reduces cell viability. These authors also showed that the incubation of these cells with an antioxidant increases cell viability. This experiment shows that QA reduces the cell viability and the co-incubation of the neurons with tolmetin or sulindac enhances neuronal viability by approximately 12%. These agents may reduce the damage induced QA by possibly scavenging free radicals or reducing the formation of free radicals. Tolmetin and sulindac may have also prevented the disruption or assisted the endogenous antioxidant defence system and protected the damages induced by QA. This aspect has also been examined in chapter 6.

CHAPTER 5

BEHAVIOURAL STUDIES

5.1. INTRODUCTION

The development and application of behavioural screening tests has received a great deal of attention in recent years and a substantial body of literature has been written on this topic (Alder *et. al.*, 1986; Moser & MacPhail 1990; Tilson & Moser 1992). Screening generally refers to the development of preliminary information on the effect of chemicals on intact animals or some organ systems (Zbiden *et. al.*, 1984). Behavioural screening methods have long been applied in assessing the effects of various compounds such as pharmaceuticals, medicinal compounds and environmental compounds. Recently there has been a considerable increase in screening efforts in the field of neurotoxicology due to a growing recognition that the nervous system is vulnerable to a wide range of chemicals and that most existing chemicals have not been evaluated for their neurotoxic potential (NAS 1984).

There are enormous advantages with the use of behavioural assessments in screening chemical compounds. Behavioural assessments are non-invasive and do not require surgical intervention. Being non-invasive, behavioural tests allow repeated assessment of chemical-treated animals over time. A much more complete description of the onset and progression of a disease state can be provided instead of a static result at the end of dosing. Behavioural assessments also permit determination of the recovery of function following exposure, as well as any compensation for compromised function that may occur during exposure (MacPhail 1994). These features also permit integration of explicit behavioural assessment into a wide variety of ongoing studies and chronic bioassays. It could therefore imply that there is probably no substitute in toxicology that is as versatile and accessible as the behaviour of intact animals.

5.2. EFFECT OF QUINOLINIC ACID, TOLMETIN AND SULINDAC ON RAT SPATIAL REFERENCE MEMORY

5.2.1. Introduction

The standard water maze task, in which a rat is required to localize a submerged platform, measures predominantly spatial reference memory. Reference memory refers to memory for information that remains constant over repeated trials and is trial independent (Olton *et. al.*, 1979). Reference memory is required to learn the general rules of the task, for example, swim to the platform. Studies have shown deterioration of performance in aged rats in the standard water maze task (Fischer *et. al.*, 1989; Fischer *et. al.*, 1991; Frick *et al.*, 1996). These authors suggest that the deterioration of spatial reference memory is due to aging. Working memory refers to memory in which the information to be remembered changes in repeated trials (Olton *et. al.*, 1979). Thus, working memory is trial dependent and can be assessed in versions of water maze tasks which require learning rapidly changing information (Olton *et. al.*, 1979). Some studies have demonstrated that spatial memory is also impaired in aged rats (Frick *et. al.*, 1995; Lindner *et. al.*, 1992).

It has long been known that the hippocampus plays a key role in the formation of new memories (Scoville & Milner 1957) and it has been extensively studied for both its role in learning and memory (Silva 2003) and its susceptibility to excitotoxicity (Ben-Ari *et. al.*, 1980; Golarai *et. al.*, 2001; Olsson *et. al.*, 2003). In this study, we investigated whether unilateral intrahippocampal injections of QA could impair spatial reference memory in rats and if tolmetin or sulindac treatment could attenuate this memory impairment. In the previous experiments (chapter 4), pre-treatment with the NSAIDS offered neuroprotection against QA-induced insults. In these experiments, animals were intrahippocampally injected with QA then treated with NSAIDS. This was to determine whether post-treatment could also offer protection.

5.2.2. Materials and Methods

5.2.2.1. Chemicals and Reagents

All chemicals and reagents were as per section 4.2.2.1.

5.2.2.2. Animals

Adult male Wistar rats were housed and cared for as described in section 2.2.2.2. Control and treatment groups consisted of 20 animals each.

5.2.2.3. Animal training: water maze task

The apparatus consisted of a circular water tank (150cm in diameter and 40 cm high). A platform (12.5 cm in diameter and 31cm high) invisible to the rats, was set inside the tank, which was filled with water maintained at approximately 23°C to a height of 33cm. The platform was submerged 2cm below the surface of the water. The tank was located in a large room where there were several brightly colored cues external to the maze: these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout the study. For each training session each rat was put into the water at one of four starting positions, the sequence of the positions being selected randomly. Training was conducted for 4 consecutive days, twice a day with each session consisting of 4 trials. Animals were trained for 4 days before being subjected to the treatment protocol.

5.2.2.4. Surgical procedures and treatment regimes

For the purpose of these experiments, rats were divided into four groups as described in the table 5.1.

Table 5.1: Treatment regimes for the behavioural studies

Treatment Regime	i.h. injection (2µl)	Received daily dose for 5 days after i.h. injection
Control	PBS	vehicle
QA (i.h.) + vehicle	0.6µmoles QA	vehicle
QA + tolmetin (5mg/kg/bd for 5 days)	0.6µmoles QA	Tolmetin 5mg/kg/bd
QA + sulindac (5mg/kg/bd for 5 days)	0.6µmoles QA	Sulindac 5mg/kg/bd

After 4 days of training, animals were divided into the above 4 groups and received intrahippocampal injections as described above. Surgical procedures were conducted as described in section 4.2.2.4. Thereafter the animals were dosed intraperitoneally with either tolmetin or sulindac at a dose of 5mg/kg/bd for 5 days. On the morning of the 6th day, animals were re-introduced to the water maze task and test trials were conducted for 14 days with animals receiving 1 test trial a day.

5.2.2.5. Performance in the water maze (test trials)

During test trials, rats were placed into the tank at the same starting point, with their heads facing the wall of the tank. The time taken for each rat to find the hidden platform from the starting point was measured by a person unaware of the experimental conditions. After the test trial on the 14th day, animals were sacrificed by neck fracture and the brains were removed as described in section 2.2.2.3. The hippocampi were removed and rapidly frozen in liquid nitrogen and stored at -70°C for analysis of the glutathione and protein carbonyl content (chapters 6 and 7 respectively).

5.2.2.6. Statistical analysis

All results were analysed using repeated measures ANOVA with group as a factor and latency measured over 14 days. Scheffes' test for multiple group comparison was used for post hoc analysis. The level of significance used was $\alpha = 0.05$.

5.2.3. Results

The results show a significant difference in latency to escape between the groups ($F_{3, 36} = 624.4$, $p < 0.001$). Post hoc analyses (Scheffé tests) show that the latency to escape (over 14 days) of rats that received intrahippocampal injections of quinolinic acid (QA) was significantly longer compared to the control group ($p < 0.001$). Tolmetin and sulindac treated animals (that also received intrahippocampal injections of QA) had significantly faster escape times when compared to animals that received intrahippocampal injections of QA and did not receive NSAID treatment ($p < 0.001$). Sulindac treated animals had significant shorter escape latency compared to the tolmetin treated animals ($p < 0.001$).

The results also show a significant difference in latency to escape between days (multivariate Wilks' Lambda = 0.002, $F_{13, 24} = 990.7$, $p < 0.001$; univariate Greenhouse-Geisser: $F_{6.2, 224.8} = 454.4$, $p < 0.001$) and a significant interaction between groups and days (multivariate Wilks' Lambda = 0.002, $F_{39, 71.8} = 12.9$, $p < 0.001$; univariate Greenhouse-Geisser: $F_{18.7, 224.8} = 9.5$, $p < 0.001$).

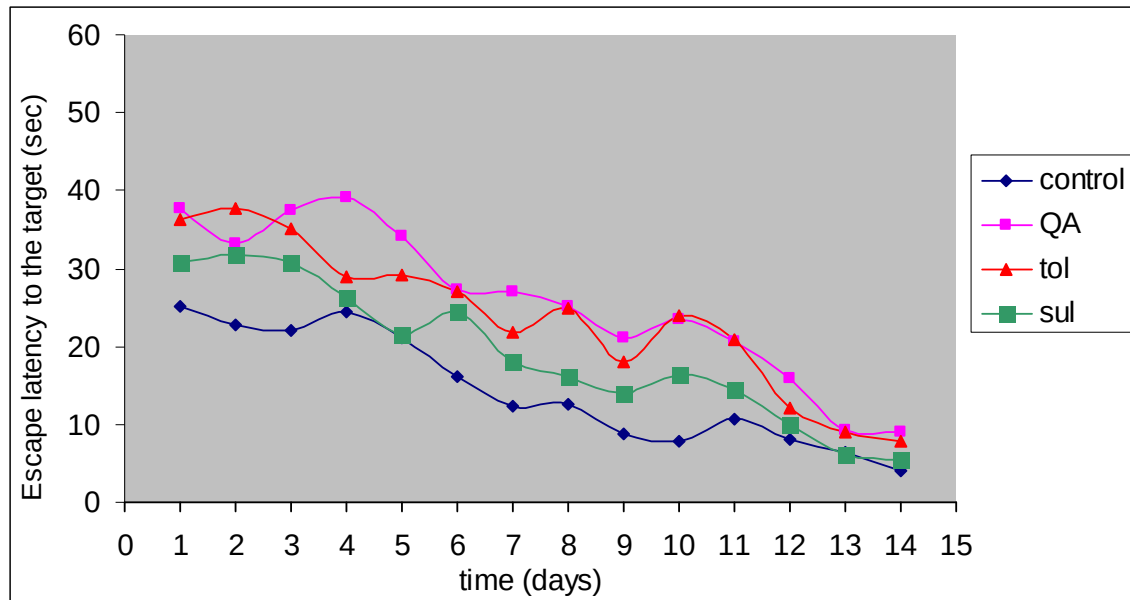


Figure 5.1: Performance of the rats in the water maze task. Each point shows the average time taken for 10 rats. Trials were conducted once daily, every day for 14 days. Results were analysed using repeated measures ANOVA with group as a factor and latency measured over 14 days. Scheffes' test for multiple group comparison was used for post hoc analysis.

5.2.4. Discussion

The hippocampal regions which consist of the CA fields, dentate gyrus and subicular complex, are part of a system of anatomically related structures in the medial temporal lobe that is important for mammalian memory (Squire 1992). Damage to these regions impairs learning and memory (Eichenbaum & Cohen 2001). Single-cell recordings and neuroimaging techniques have documented changes in these regions during learning, memory and retention (Suzuki & Eichenbaum 2000; Squire *et. al.*, 2004). The hippocampus is the final stage of convergence within the medial lobe which receives projections from the adjacent perirhinal and parahippocampal/postrhinal cortices, as well as the entorhinal cortex (Lavenex & Amaral 2000). It has been thought that the hippocampus may be particularly essential for tasks that depend on relating or combining information from multiple sources, as in spatial memory tasks (O'Keefe & Nadel 1978).

It has been reported that hippocampal lesions impairs recognition memory performance in humans (Manns *et. al.*, 2003), monkeys (Beason-Held *et. al.*, 1999; Zola *et. al.*, 2000) and rodents (Clark *et. al.*, 2000, Ckark *et. al.*, 2001; Gould *et al.*, 2002). In the rodent, hippocampal lesions as small as 40% of the total hippocampal volume, markedly impair learning in the water maze (Moser *et. al.*, 1993; Moser *et. al.*, 1995). This study has shown that 0.6 μ moles of QA significantly affects the retrieval of spatial memory. However, treatment of the animals with the NSAIDS tolmetin and sulindac, improved the behavioural response, with sulindac treated animals performing better than the tolmetin treated animals. Although the NSAIDS improved the performance in the water maze, these agents fail to completely suppress the QA-induced memory deficit since these animals have longer escape latencies compared to control animals.

Research has shown that chronic administration of ibuprofen protects against detrimental behavioural effects following i.h. injections of the β -amyloid protein (Richardson *et al.*, 2002). The authors suggest that ibuprofen protection is due to suppression of the inflammatory process. In another study conducted by Mesches and co-workers in 2003, sulindac improved the memory of aged Fischer 344 rats. These authors demonstrated that sulindac increases the NMDA receptor subunits (NR1 and NR2B) and attenuates age-related deficits in learning and memory which was measured using the radial arm water maze. Sulindac also prevents the age-related increase in the pro-inflammatory cytokine, interleukin 1 β (1L-1 β), in the hippocampus (Mesches *et. al.*, 2003).

QA has been associated with inflammatory conditions in the brain (Heyes *et. al.*, 1991; Vogelgesang *et. al.*, 1996). It is possible that tolmetin and sulindac suppress the inflammatory response in the brain as well as scavenge ROS and protect the hippocampus from QA-induced neurotoxicity. It has been established that NMDA receptors are vital for brain function (Forrest *et. al.*, 1994) and that these receptors are central to many of the activity-dependent changes in synaptic strength and connectivity that are thought to underlie the formation of memory and learning (Bliss & Collingridge 1993). The previous chapter (experiment 4.4) demonstrated that QA reduced the number of NMDA receptors in rat hippocampus and this loss was partially curtailed by the

NSAID treatment. These NSAIDS not only reduce the loss of receptors but also prevented the loss of hippocampal neurons thereby preventing the QA-induced spatial reference memory deficit.

CHAPTER 6

GLUTATHIONE CONTENT

6.1. INTRODUCTION

Oxidative and chemical stresses promote degenerative diseases and accelerate ageing of humans and animals (Harman 1994; Halliwell 2001). It has been suggested that free radicals may play a role in this inevitable ageing process and in the development of ageing-associated diseases (Harman 2002). Therefore the removal of free radicals and the balance between oxidant and antioxidant levels within the cells are important. The removal of free radicals is achieved through enzymatic and non-enzymatic reactions (figure 6.1).

The principal defense systems against oxygen free radicals are SOD, glutathione (GSH), GSH peroxidases, GSH reductase, catalase and antioxidant nutrients. Vitamin E can transfer its phenolic hydrogen to a peroxy free radical of a peroxidised PUFA, thereby breaking the radical chain reaction and preventing the peroxidation of the PUFA in membrane phospholipids. Vitamin C reacts with a vitamin E radical to yield a vitamin C radical, thus regenerating vitamin E (figure 6.1). Like the vitamin E radical, the vitamin C radical is not a reactive species since its unpaired electron is energetically stable (Fang 2002). GSH thereafter converts the Vitamin C radical back to Vitamin C.

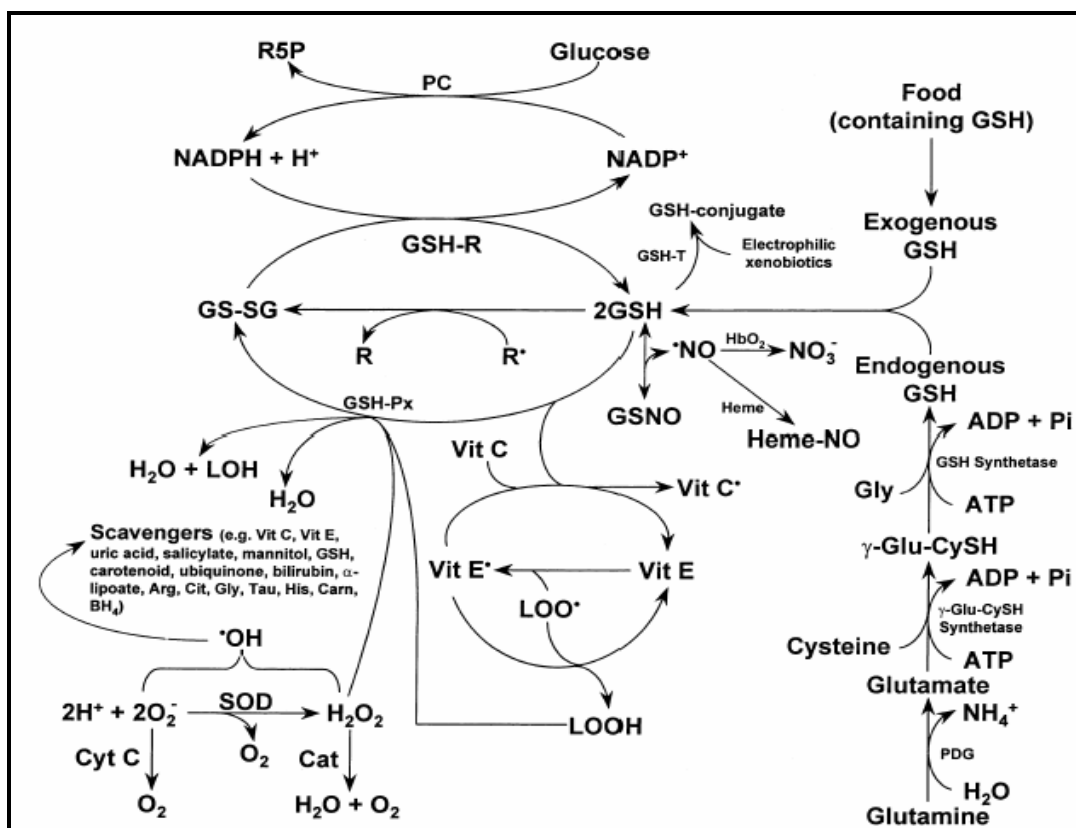


Figure 6.1: Production of oxygen and nitrogen free radicals and other reactive species within a mammalian cell and the role played by superoxide dismutase, glutathione, vitamin C and vitamin E in counteracting the free radicals. Adenosine diphosphate, (ADP); arginine, (Arg); (6R)-5,6,7,8,-tetrahydro-L-biopterin, (BH₄); carnosine, (Carn); catalase, (Cat); citrulline, (Cit); cytochrome C, (Cyt C); L-glutamate, (Glu); glycine, (Gly); glutamyl-cysteine, (Glu-CySH); oxidized glutathione, (GS-SG); glutathione reduced form, (GSH); glutathione peroxidases, (GSH-Px); glutathione reductase, (GSH-R); glutathione S-transferase, (GSH-T); nitrosylated glutathione, (GSNO); oxyhemoglobin, (HbO₂); heme-nitric oxide, (Heme-NO); histidine, (His); lipid alcohol, (LOH); lipid peroxyl radical (LOO•); lipid hydroperoxide, (LOOH); nitric oxide, (•NO); nitrate, (NO₃⁻); superoxide anion radical, (O₂⁻); pentose cycle, (PC); radicals, (R•); non-radicals, (R); ribulose 5-phosphate, (R5P); superoxide dismutase, (SOD); taurine, (Tau); vitamin C, (Vit C); vitamin C radical, (Vit C•); vitamin E, (Vit E); vitamin E radical, (Vit E•). (Fang *et al.*, 2002).

Glutathione is the most abundant thiol-containing substance of low molecular weight in cells. It is synthesised from glutamate, cysteine and glycine. N-acetylcysteine is a stable and effective precursor of cysteine for intracellular GSH synthesis (Sies 1999). Almost all the dietary glutamate is catabolised by the small intestine mucosa in the first pass (Wu 1998). Therefore, the two major sources of plasma and cellular glutamate for GSH synthesis are from the hydrolysis of glutamine to glutamate by glutaminase and the production of glutamate from α -ketoglutarate and branched-chain amino acids via transamination.

GSH, the sulfur-containing tripeptide and the related enzymes are present in most cells. These serve as reducing equivalents for the maintenance of oxidant homeostasis. Lipid peroxides and hydrogen peroxide are reduced by GSH peroxidase (GSH-Px) converting GSH to its oxidized species (GS-SG). Recycling of GSH, concomitant with the oxidation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) is catalysed by GSH reductase (GSH-R). This enzyme is responsible for maintaining brain glutathione almost exclusively (>99%) in its reduced form (see figure 6.1). GSH S-transferases (GSH-T) constitute a multigene family of isoenzymes that catalyzes the conjugation of a wide variety of electrophilic compounds with GSH to render less toxic substances (Dringen 2000). Besides its antioxidant role, GSH has also been suggested to have neurotransmitter and neuromodulatory functions (Bains *et. al.*, 1997, Oja *et. al.*, 2000).

6.2. EFFECT OF QUINOLINIC ACID, TOLMETIN AND SULINDAC ON RAT HIPPOCAMPAL TOTAL GLUTATHIONE CONTENT

6.2.1. Introduction

During metabolism, the brain generates high levels of potentially noxious ROS (Halliwell 1992). GSH in the brain can react with these ROS and detoxify these. The physiological level of GSH is regulated by its biosynthetic pathway (Meister & Anderson 1983; Dringen 2000) and by GSH-R. Exogenous factors, including those leading to the formation of reactive electrophilic intermediates and of ROS, can also react directly with intracellular reduced GSH and result in its depletion.

Chapter 4 shows that QA-induces neurodegeneration in rat hippocampus and the previous chapters demonstrate that the NSAIDS, tolmetin and sulindac reduce QA-induced memory deficits. This experiment was conducted to determine the effect that QA and NSAID treatment has on the total GSH content in rat hippocampus.

6.2.2. Materials and Methods

6.2.2.1. Chemicals and Reagents

5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced glutathione were purchased from Sigma Chemical Corporation; St Louis, MO, U.S.A. All other reagents used were of the highest quality available.

6.2.2.2. Animals

Adult male Wistar rats were housed and cared for as described in section 2.2.2.2.

6.2.2.3. Surgical procedures and treatment regimes

Animals were injected intrahippocampally with QA (0.6 μ moles in 2 μ l of PBS) and dosed with NSAIDS (5mg/kg/bd for 5 days), exactly as described in section 5.2.2.4. However, animals were sacrificed on the morning, the day after the last dose and the brains were removed as described in section 2.2.2.3. The injected hippocampi from each rat was

dissected free and analysed for total GSH and protein carbonyl content (chapter 7). This group of animals are referred to as the day = 1 rats. Animals that were killed on the 14th day, immediately after their test trials in the water maze, are referred to as the day = 14 rats.

6.2.2.4. Preparation of standard curve

Reduced GSH was used as the standard. An aliquot of 0.5ml of varying concentrations of GSH (2-10nmoles/ml) was added to test tubes containing 1.5ml of 0.2M tris HCl (pH 8.2), 0.1ml of 0.01M DTNB and 7.9ml of methanol. This was vortexed and the absorbance was read at 412nm using a GBC UV/VIS 916 spectrophotometer and plotted against the concentration (Appendix 7).

6.2.2.5. GSH assay

The spectrophotometric procedure used was based on the method of Ellman (Ellman 1958; Ellman 1959), who reported that 5,5-dithiobis-(2-nitrobenzoic acid) is reduced by sulfhydryl groups (SH) to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH.

Total GSH levels were measured using the method previously described by Sedlak and Lindsay (1968). Briefly, to 0.5 ml of rat hippocampi homogenates (5% w/v in PBS), 1.5 ml of 0.2M tris-HCl (pH 8.2), 0.1 ml of 0.01M DTNB and 7.9 ml of methanol was added and incubated for 30 minutes at room temperature. Thereafter the incubation mixture was centrifuged at 3000g for 15 minutes and the supernatant was read at 412 nm. The glutathione levels were determined from a standard curve generated from GSH and are expressed as nmoles GSH/mg tissue. The experiment was replicated five times.

6.2.2.6. Statistical analysis

Results were analysed as described in section 2.2.2.7.

6.2.3. Results

Figure 6.2 shows that intrahippocampal injections of QA significantly ($p < 0.001$) reduce the GSH content in rat hippocampus from day = 1 rats. This decrease in GSH content is reduced by the treatment of the animals with tolmetin and sulindac. The GSH content measured 14 days later show that GSH is significantly ($p < 0.01$) lower when compared to controls. Animals treated with NSAIDs had notably higher levels of GSH compared to animals injected with QA and which did not receive NSAID treatment.

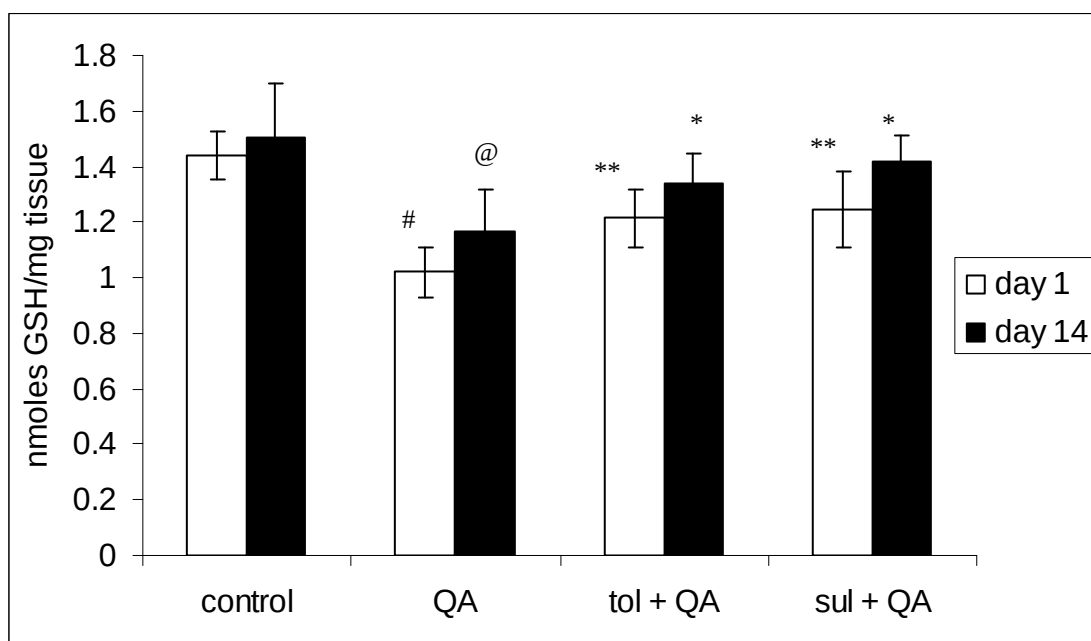


Figure 6.2: Effect of QA and tolmetin and sulindac treatment on rat hippocampal glutathione content. Animals that were sacrificed immediately after the treatment protocol are referred to as the day = 1 rats. Animals that were killed on the 14th day, immediately after their test trials in the water maze, are referred to as the day = 14 rats. Each bar represents the mean $\pm$ SD ($n=5$). [#]($p < 0.001$) and [@]($p < 0.01$) compared to controls. ^{**}($p < 0.01$) and ^{*}($p < 0.05$) when compared to rats that received QA only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

6.2.4. Discussion

ROS are frequently targeted by GSH in both spontaneous and catalytic reactions (Butterfield *et. al.*, 2002a; Drake *et. al.*, 2003). ROS have defined roles in cell signaling events as well as in human disease pathologies, therefore an imbalance in expression of GSH and associated enzymes has been implicated in a variety of pathological conditions (Calabrese *et. al.*, 2003, Butterfield *et. al.*, 2002b). Imbalances between GSH metabolism and diseases such as cancer, neurodegenerative diseases and aging have been shown (Droge 2002). Some authors have shown neurobehavioural impairment in oxidative stressed animals (Avila-Costa *et. al.*, 1999; Watanabe & Satoh 1995). Cruz-Aguado and co-workers recently showed that the GSH status is important during acquisition of spatial memory in maze tasks. Their results support the hypothesis that the oxidant/antioxidant equilibrium is important in the regulation of brain functions.

It has been shown that intrastriatal injections of QA as little as 225nmol reduce GSH-R activity by 20% (Cruz-Aguado *et. al.*, 2000). This decrease is observed at both 1 and 6 weeks after the lesions and that there is a 30% reduction in GSH content 1 week after the lesions. This study demonstrates that QA reduces GSH levels by approximately 30% (measured 1 week after lesion) and more than 20% (measured after 2 weeks) in the hippocampus. NSAID treatment prevents the sharp decrease in GSH levels. It is possible that these NSAIDS also serve as antioxidants and prevent the depletion of GSH in the hippocampus since it has been shown that antioxidants prevent GSH depletion (Patel *et. al.*, 2003). This is another possible mechanism through which these NSAIDS improve performance in the water maze task. Those rats that were sacrificed the day after the last test trial show that the GSH levels had increased. It is possible that the balance between oxidant and prooxidant levels within the neurons. Since NSAID treatment reduces the loss of GSH, this may have also reduced the oxidation of important cellular components such as lipids and proteins. The extent of protein oxidation was investigated in the following chapter.

CHAPTER 7

PROTEIN OXIDATION

7.1. INTRODUCTION

Oxidative stress is an imbalance towards the prooxidant side of the prooxidant/antioxidant homeostasis of a cell and this occurs in many neurodegenerative disorders (Markesbery 1997; Varadarajan *et. al.*, 2001; Butterfield *et. al.*, 2001). Protein oxidation and aggregation occurs in many neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, prion disorders such as Creutzfeld-Jakob disease and disorders with aggregated α -synuclein, such as Parkinson's disease.

The mechanism via which proteins are oxidised is very similar to that of lipid peroxidation. Oxidation of proteins is normally caused by free radicals and is an exothermic process. Although many other free radicals can cause protein oxidation, this process is the most easily initiated by the hydroxyl free radical (Butterfield & Stadtman 1997). The production of the hydroxyl radical has been mentioned in chapter 2, experiment 2.8. Protein oxidation can occur by two possible pathways: (a) backbone oxidation and (b) side-chain oxidation (figure 7.1).

Backbone oxidation is initiated by the abstraction of hydrogen from the α -carbon by a free radical which leads to the formation of a carbon-centered radical. In the presence of oxygen, this radical is converted to the peroxy radical. This can lead to the formation of an alkoxyl radical and subsequent hydroxylation of the peptide backbone (Butterfield & Stadtman 1997). These reactions can be mediated by transition metals, (in the low valence state) or the hydroperoxyl radical ($\text{HOO}\cdot$). Protein oxidation can lead to protein cross-linking and/or peptide bond cleavage via diamide or α -amidation pathways. Cleavage of the peptide bond will result in the formation of carbonyl groups which are

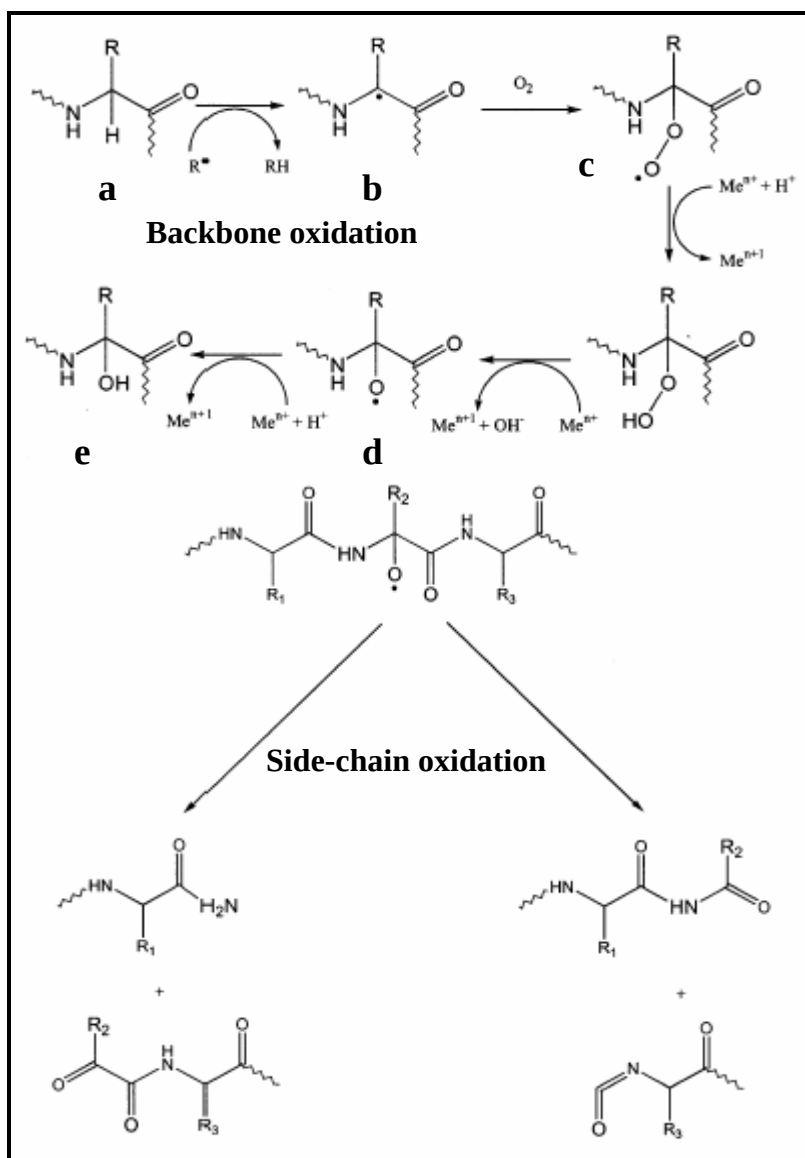


Figure 7.1: Formation of protein carbonyls following free radical attack. (a) protein; (b) carbon-centered protein radical; (c) peroxy radical; (d) alkoxy radical; (e) hydroxylated peptide backbone (modified from Butterfield & Kanski 2001).

often used as a marker of protein oxidation. The peptide-formed radical can also abstract hydrogen from a carbon of the same or another carbon-centered radical, thus propagating the free radical initiated oxidation across and between proteins (Butterfield & Stadtman 1997).

The oxidation of amino acid side-chains greatly depends on their structure. Although most amino acids side-chains are susceptible to oxidation, only oxidation products of some amino acids have been fully characterised (Butterfield & Stadtman 1999).

7.2. EFFECT OF QUINOLINIC ACID, TOLMETIN AND SULINDAC ON RAT HIPPOCAMPAL PROTEINS

7.2.1. Introduction

The previous chapters show that QA depleted GSH in rat hippocampus and adversely affects the spatial reference memory. However, treatment of the rats with the NSAIDS, tolmetin and sulindac, decreases the loss of glutathione and improves the animals' performance in the water maze tests. This experiment was conducted to determine the effect intrahippocampal injections of QA on rat hippocampal proteins and whether the NSAIDS could protect against any damage that may have been induced.

Protein carbonyls are introduced into the proteins via oxidation of the protein backbone, amino acid side-chains, or by reactions with alkenals. These protein carbonyls are used as an index of protein oxidation and can be assayed by the formation of the 2,4-dinitrophenylhydrazone covalent adduct (Butterfield & Stadtman 1997). The reaction of oxidised proteins with 2,4-dinitrophenylhydrazine (DNPH) yields 2,4-dinitrophenylhydrazone which can be measured spectrophotometrically at 370 nm. Prior studies relied mostly on the spectrophotometric quantification of protein carbonyls, but recent studies have also employed immunochemical detection of the DNPH-protein adduct (Butterfield *et. al.*, 1999; Yatin *et. al.*, 1999).

7.2.2. Materials and Methods

7.2.2.1. Chemicals and Reagents

2,4-dinitrophenylhydrazine, guanidine and hepes buffer was purchased from Sigma Chemical Corporation; St Louis, MO, U.S.A. TCA, ethanol and ethyl acetate were purchased from Saarchem, Johannesburg. Streptomycin sulphate was supplied by Novo Nordisc (Pty) Ltd, South Africa. All other chemicals and reagents were as per section 4.2.2.1.

7.2.2.2. Animals

Adult male Wistar rats were housed and cared for as described in section 2.2.2.2.

7.2.2.3. Surgical procedures and treatment regimes

Animals were injected intrahippocampally with QA (0.6 μ moles in 2 μ l of PBS) and dosed with NSAIDS (5mg/kg/bd for 5 days), as described in section 5.2.2.4. However, animals were sacrificed on the morning, the day after the last dose and the brains were removed as described in section 2.2.2.3. The injected hippocampi from each rat was dissected free and analysed for protein carbonyl content. This group of animals are referred to as the day = 1 rats. Animals that were killed on the 14th day, immediately after their test trials in the water maze, are referred to as the day = 14 rats.

7.2.2.4. Protein carbonyl measurement

The assay was conducted according to a modified method of Levine *et al.* (1990).

Rat hippocampi homogenates (10% w/v in PBS) were diluted with buffer to 5 mg/ml. To this, streptomycin sulphate (1% final concentration) was added and gently stirred for 15 minutes, thereafter centrifuged at 11000 x g for 10 minutes at 4°C. The supernatant was collected and diluted to 1mg/ml. TCA was added at a final concentration of 10% to precipitate proteins. Samples were centrifuged at 11000 x g and the pellet was recovered and diluted in buffer to yield a 1 mg/ml suspension. 0.5 ml of 2,4-dinitrophenylhydrazine (2 mM final concentration) was added to each tube and incubated at room temperature for 1 hour with vortexing every 10-15 minutes. Proteins were precipitated with TCA and recovered after centrifuging for 10 minutes at 11000 x g. The pellet was washed thrice with 1ml of a mixture of ethanol and ethyl acetate (1:1) then dissolved in 0.6 ml of 6 M guanidine. The absorbance was read at 370 nm and the carbonyl content was calculated using a molar absorption coefficient of 22000 M⁻¹cm⁻¹ (Johnson 1953). Results were calculated using the equation $A = \epsilon cl$, where $\epsilon = 22000 \text{ M}^{-1}\text{cm}^{-1}$; A = absorbance; c = concentration and l = pathlength. Results are expressed as nmoles/mg protein. Protein estimation was performed using the method described by Lowry *et al.* (1951). The experiment was replicated five times.

7.2.2.5. Statistical analysis

Results were analysed as described in section 2.2.2.7.

7.2.3. Results

Figure 7.2 shows that intrahippocampal injections of QA significantly ($p < 0.001$) increase protein oxidation in rat hippocampus. This increase is also observed 14 days later, after trials in the water maze. Treatment of the rats with tolmetin and sulindac significantly reduce the oxidation of the proteins which was measured immediately after the treatment protocol and after the water maze task.

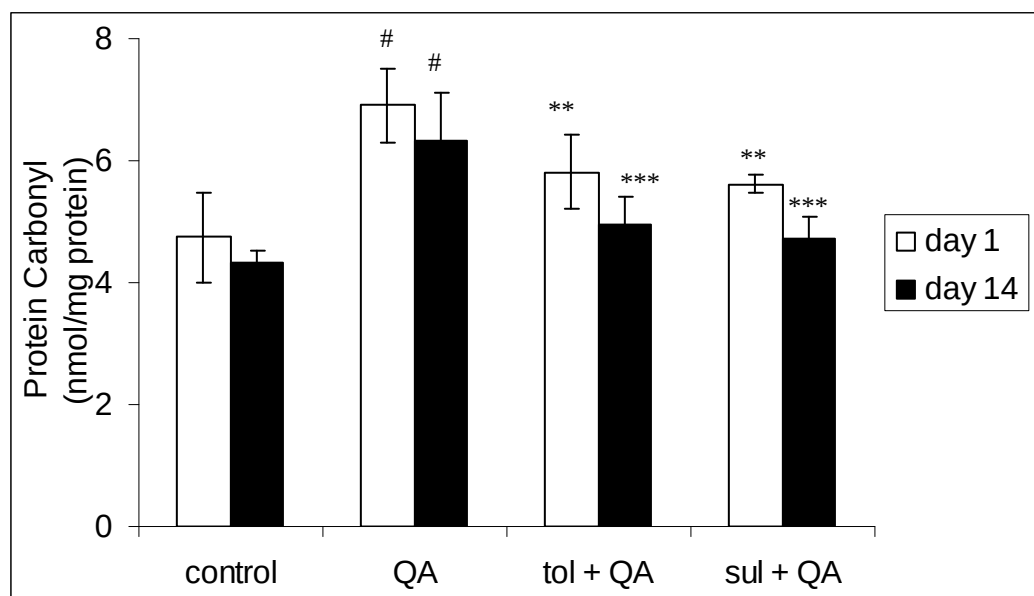


Figure 7.2: Effect of intrahippocampal injections of QA and NSAID treatment on rat hippocampal protein carbonyl levels. Animals were sacrificed immediately after the treatment protocol are referred to as the day = 1 rats. Animals that were killed on the 14th day, immediately after the test trials in the water maze, are referred to as the day = 14 rats. Each bar represents the mean $\pm$ SD ($n=5$). #($p < 0.001$) compared to controls. ***($p < 0.001$) and **($p < 0.01$) when compared to rats that received QA only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

7.2.4. Discussion

Exposure to ROS is unavoidable if an organism exists in an aerobic environment. ROS pose a constant threat to proteins, DNA, RNA and lipids. Increased oxidative stress during aging may lead to accumulation of oxidised proteins (Berlett & Stadtman 1997). As a result, proteins become resistant to proteosomal degradation. However, oxidative stress is also known to enhance protein turnover accompanied by a specific removal of oxidised proteins (Sitte *et. al.*, 1998). The removal of these oxidised proteins has been considered to be impaired during aging since proteosomal activity is decreased (Shringarpure & Davies 2000). Therefore, increased oxidative stress may lead to alterations in both oxidative modification and turnover of proteins. There is evidence that oxidative damage contributes to neurodegenerative disorders such as AD (Odetti *et. al.*, 1998; Pratico 2002) and this oxidative damage has been associated with aggregation of proteins and altered antioxidant function (Butterfield & Kanski 2001; Stadtman & Levine 2003).

This study demonstrates that QA, the excitatory amino acid which increases with age (Moroni *et. al.*, 1984), significantly induces protein oxidation in rat hippocampus and that the treatment of the rats with the NSAIDS reduces protein oxidation. The previous chapter shows that QA reduces the GSH content in rat hippocampus. Since the prooxidant/antioxidant status of the cells are affected, this may have also contributed to the protein oxidation. It has been reported that NSAIDS such as indomethacin, etodolac, acetaminophen, ketorolac as well as tolmetin and sulindac are very effective in scavenging ROS and RNS free radicals such as ROO $\cdot$, $\cdot$ OH, O $_2^{\cdot-}$, NO $\cdot$ and ONOO $\cdot$ (Fernandes *et. al.*, 2003; Fernandes *et. al.*, 2004). Apart from reducing the loss of the endogenous antioxidant GSH, these NSAIDS may have also assisted in the scavenging of the free radicals generated by QA, thereby reducing the amount of protein carbonyls formed.

CHAPTER 8

LIVER TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY

8.1. INTRODUCTION

Tryptophan is an essential amino acid having various important biological functions. Besides being incorporated into proteins, tryptophan is metabolized in mammals along different pathways, one of which is known as the tryptophan-niacin or kynurenine pathway (figure 8.1), this metabolite being the key substance in this process (Musajo & Benassi 1964; Price *et. al.*, 1965; Wolf 1974). In man about 90% of dietary tryptophan is metabolized along this pathway (Wolf 1974).

Tryptophan is first oxidized to formylkynurenine by the enzyme tryptophan 2,3-dioxygenase (TDO) (Knox and Mehler 1950). Formylkynurenine is rapidly converted to kynurenine by formidases (figure 8.1) present in large excess in mammalian liver preparations (Mehler and Knox 1950; Dalgliesh & Tabechian 1956). TDO was first characterized in rat liver by Knox and Mehler in 1950. Thereafter it was found in other mammals including man (Allegri *et. al.*, 1965; Altman & Greengard 1966; Chiancone & Ginoulhiac 1967). Although TDO is the enzyme that initiates the kynurenine pathway, another enzyme can also catabolise tryptophan to produce kynurenine. The enzyme indoleamine 2,3-dioxygenase (IDO) was discovered in the rabbit intestine by Higuchi and coworkers in 1963. However, this enzyme does not show substrate specificity like TDO. It also catalyses the oxygenative ring cleavage of tryptophan and various indoleamine derivatives, therefore it was called IDO (Higuchi & Hayaishi 1967; Yamamoto & Hayaishi 1967; Hirata & Hayaishi 1972).

In the liver, TDO is closely associated with the “free” haem pool, which usually provides sufficient haem to saturate half the available TDO. Consequently, TDO normally exists partly as the holoenzyme and partly as the apoenzyme and thus at half of its full potential (Tanaka & Knox 1959; Hayaishi 1980; Ishimura *et al.*, 1970; Leeds *et al.*, 1993). Haem administration to rats, or incubation of liver homogenates with haemin *in vitro*, results in the saturation of all TDO prosthetic haem binding sites and in the attainment of its

functional potential (Marver *et. al.*, 1966; Badawy & Evans 1975; Bissell & Hammacker 1977). Since TDO activity is modulated by the “free” haem pool, size fluctuations of this “free” haem pool can markedly affect the tryptophan and serotonin turnover in the CNS and other serotonergic tissue (Curzon 1969; Curzon & Bridges 1970; Wurtman 1980).

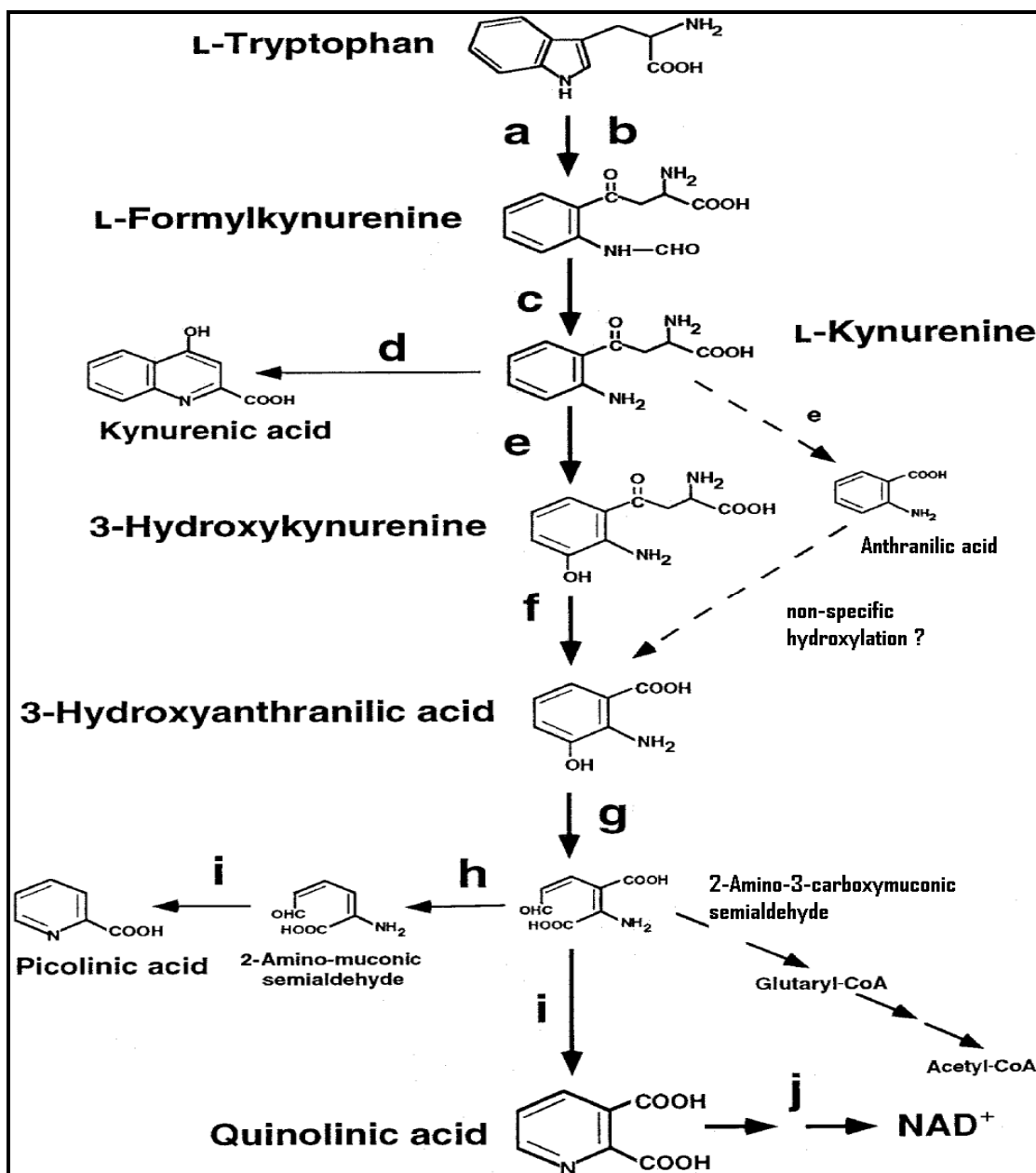


Figure 8.1: Graphical representation of the tryptophan-niacin pathway showing the metabolites and enzymes of this pathway. (a) Indoleamine 2,3-dioxygenase; (b) tryptophan 2,3-dioxygenase; (c) kynurenine formamidase; (d) kynurenine aminotransferase; (e) kynurenine 3-hydroxylase; (f) kynureninase; (g) 3-hydroxyanthranilate 3,4-dioxygenase (3-hydroxyanthranilic acid oxidase; (h) picolinic carboxylase; (i) non-enzymatic cyclisation (spontaneous); (j) quinolinic acid phosphoribosyltransferase. (Heyes *et. al.*, 1997).

8.2. IN VITRO INVESTIGATION OF THE EFFECT OF TOLMETIN AND SULINDAC ON LIVER TDO ACTIVITY

8.2.1. Introduction

Alterations to the activity of TDO may affect the circulating tryptophan levels. It has been shown (Hardeland & Rensing 1968; Walsh & Daya 1998) that the administration of antidepressant drugs, including tricyclic antidepressants, results in the inhibition of liver TDO resulting in the elevation of brain tryptophan and serotonin concentrations. The following experiments were conducted to determine if the anti-inflammatory agents, tolmetin and sulindac, affect TDO activity and alter the amount of metabolites in the tryptophan-kynurenine pathway. These experiments were conducted using liver homogenate.

Changes in circulating levels of tryptophan can be achieved by altering the activity of TDO. This enzyme exists in at least two forms in certain animal species. These two forms are the active reduced holoenzyme, and the predominant, inactive apoenzyme which requires the addition of exogenous haematin for demonstration of its activity (Badawy and Evans, 1975). Both forms of the enzyme are present in the livers of rats, mice, chickens, and human beings. Activation of the apoenzyme *in vitro* involves the conjugation of the apoenzyme and haem to form the oxidised (ferrihaem) holoenzyme. The holoenzyme is then reduced to the active form in the presence of tryptophan. TDO activity is measured by measuring the amount of kynurenines produced. The amount of kynurenines produced is measured at 365 nm spectrophotometrically and calculated using the kynurenine molar extinction coefficient.

8.2.2. Materials and Methods

8.2.2.1. Chemicals and Reagents

Tolmetin, sulindac, haematin and L-tryptophan were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other reagents were purchased from Merk, Darmstadt, Germany and were of the highest chemical purity.

8.2.2.2. Animals

Adult male Wistar rats, purchased from the South African Institute for Medical Research (Johannesburg, South Africa). The animals were cared for as described in section 2.2.2.2.

8.2.2.3. Tissue preparation

Rats were killed by neck fracture. The livers were rapidly removed, perfused with 0.9% NaCl and used immediately for experimentation for the *in vitro* studies. The livers were chopped into fine pieces then homogenised with 60ml 140mM KCl / 2.5mM NaOH using a glass-teflon hand held homogenizer. During the homogenising procedure, care was taken not to allow excessive foaming as this could result in protein denaturation. The homogenate was sonicated for a period of 2 minutes at 30 second intervals for complete removal of enzymes from the cells. Finally 0.2M sodium phosphate buffer, pH 7.0, was used to make up the volume required to yield a 10% w/v homogenate. The entire procedure where possible was conducted on ice.

8.2.2.4. Tryptophan 2,3-dioxygenase activity

The activity of this enzyme was determined according to a modified method described by Badawy and Evans in 1975.

Homogenate (15ml) was added to a flask containing 12.5ml water. Haematin (100 μ l) at a final concentration of 2 μ M (Badawy & Evans 1975) was added to samples that were used to determine the total activity of the enzyme. This was stirred for 1 minute to allow for the activation of the enzyme. Thereafter 1ml test compound (either tolmetin or sulindac) were added at a final concentration of 10 or 20 μ M to both the holoenzyme and total activity flasks. Finally, 2.5ml of 0.03M L-tryptophan was added to all flasks and gently stirred. The rest of the assay was conducted in triplicate. Aliquots of 3ml of the assay mixture was transferred to test tubes, stoppered with carbogen and incubated for 1 hour at 37°C in an oscillating water bath. The reaction was terminated with the addition of 2ml of 0.9M TCA to the reaction mixture and incubated for 2-4minutes. The mixture was filtered through a Whatman no. 1 filter paper. Filtrate (2.5ml) was added to 1.5ml of 0.6M NaOH and vortexed. The kynurenine present in the solution was measured at

365nm spectrophotometrically using the molar extinction coefficient of kynurenine: $\epsilon = 4540 \text{ L.mol}^{-1}.\text{cm}^{-1}$. The blank consisted of 2ml TCA and 1.5ml NaOH. The TDO activity was expressed as nmols/mg protein/h. Protein estimation was conducted according to the method described by Lowry *et. al.* (1951). This experiment was replicated twice.

8.2.2.5. Statistical analysis

The results were analysed as described in section 2.2.2.7.

8.2.3. Results

Tolmetin at both concentrations did not alter the holoenzyme activity (figure 8.2). However, tolmetin reduces the activity of the apoenzyme in a concentration-dependent manner. Tolmetin also significantly ($p < 0.001$) reduces the total enzyme activity at 10 and 20 μM . Sulindac also did not alter the holoenzyme activity but reduces the activity of the apoenzyme at 20 μM (figure 8.3) and the total activity at 10 and 20 μM .

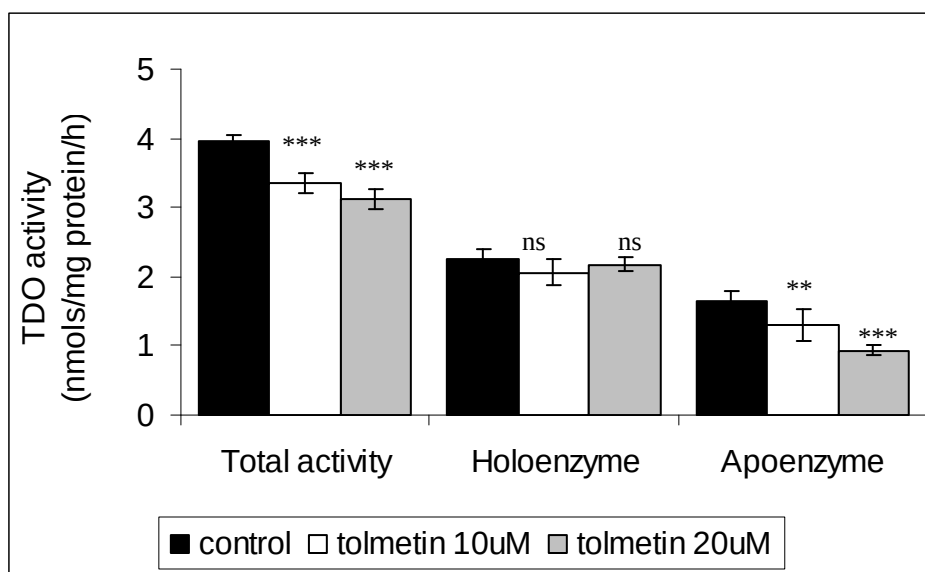


Figure 8.2: Effect of tolmetin (10 and 20 μM) on rat tryptophan-2,3-dioxygenase activity. Each bar represents the mean $\pm$ SD ($n = 6$). ns($p > 0.05$), **($p < 0.01$) and ***($p < 0.001$) compared to controls. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

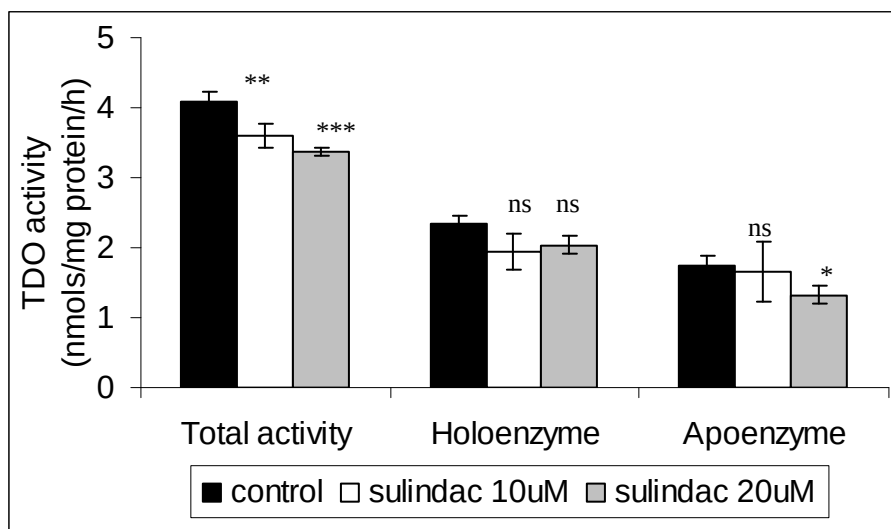


Figure 8.3: Effect of sulindac (10 and 20 μ M) on rat tryptophan-2,3-dioxygenase activity. Each bar represents the mean $\pm$ SD ($n = 6$). ns($p > 0.05$), **($p < 0.01$) and ***($p < 0.001$) compared to controls. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

8.2.4. Discussion

The amount of kynurenine produced in the experiment was markedly reduced by the presence of tolmetin and sulindac in the liver homogenates. It has been previously been shown that antidepressant drugs inhibit liver TDO enzyme activity and cause a rise in brain serotonin levels (Walsh & Daya 1998). The analgesic agent acetaminophen also inhibits liver enzyme TDO and causes an increase in brain serotonin levels (Daya & Anoopkumar-Dukie 2000). Liver TDO is also inhibited by an agent known as 68OC91 ((E)-6-fluoro-3-[2-(3-pyridyl)vinyl]-1H-indole) *in vitro* and *in vivo* (Slater *et. al.*, 1995a; Slater *et. al.*, 1995b). This experiment shows that the co-incubation of the liver homogenate with the NSAIDs tolmetin and sulindac, reduces the enzyme activity. It is possible that the enzyme had a greater affinity for the NSAIDs instead of the L-tryptophan. Tolmetin and sulindac may have also exerted their inhibitory action by preventing the binding of the enzyme to its co-factor, haematin. Since these agents demonstrate TDO enzyme inhibitory properties *in vitro*, it was decided to determine if these agents could also inhibit the TDO enzyme *in vivo*.

8.3. IN VIVO INVESTIGATION OF THE EFFECT OF TOLMETIN AND SULINDAC ON LIVER TDO ACTIVITY

8.3.1. Introduction

The previous experiment tolmetin and sulindac reduce TDO activity and decreases the amount of kynurenine formed. Since this was attained in liver homogenate it was of interest to determine if these agents could also demonstrate this ability in rats injected with these agents.

8.3.2. Materials and methods

Chemicals and reagents were as per section 8.2.2.1. Animals were cared for as described in section 2.2.2.2.

8.3.2.1. Treatment regimes

The animals were treated in a similar manner as described in section 5.2.2.3 except that there were no intrahippocampal injections of QA. Rats were divided into 3 groups, control, tolmetin treated and sulindac treated. Each group consisted of five animals. The rats were injected intraperitoneally with drug, 5mg/kg/bd for 5 days. On the morning of the 6th day the rats were sacrificed by neck fracture. The brains were carefully removed as described in section 2.2.2.3.

The pineal glands were removed and incubated for 24 hours in 50µl of Eagle's Minimum Essential Medium (MEM). The MEM was supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). This experiment will be discussed in chapter 9.

The hippocampi and striata were removed and frozen in liquid nitrogen and stored at -70°C. These were analysed for their indoleamine content (chapter 9).

The livers were rapidly removed, perfused with 0.9% saline then frozen in liquid nitrogen and stored at -70°C until use.

8.3.2.2. Tissue preparation.

The livers were homogenised and prepared as described in section 8.2.2.3.

8.3.2.3. Tryptophan 2,3-dioxygenase activity

The TDO activity was determined as described in section 8.2.2.4. but no drug (tolmetin or sulindac) was added to the incubation mixture. The results were analysed as described in section 2.2.2.7.

8.3.3. Results

As shown in figure 8.4, these agents do not alter the apoenzyme but significantly ($p < 0.05$) reduce the activity of the holoenzyme. Tolmetin (at the same dose of sulindac, 5mg/kg/bd for 5 days) reduces the total activity of the enzyme more than sulindac ($p < 0.05$).

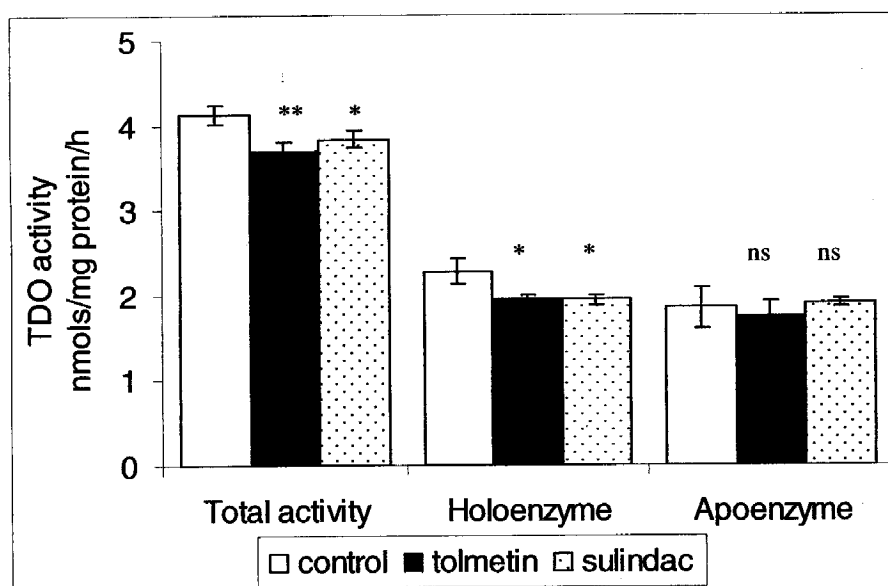


Figure 8.4: Effect of tolmetin and sulindac (5mg/kg/bd for 5 days) on rat tryptophan-2,3-dioxygenase activity. Each bar represents the mean $\pm$ SD ($n = 6$). ns($p > 0.05$), *($p < 0.01$) and **($p < 0.001$) compared to controls. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

8.3.4. Discussion

Liver TDO plays a very important role in determining the levels of circulating tryptophan in the blood (Badawy *et. al.*, 1981). An increase in the activity of this enzyme enhances the conversion of tryptophan to N-formylkynurenine, thus reducing the amount of tryptophan available for uptake into the brain (Badawy 1979). Agents which inhibit the activity of this enzyme increase plasma levels of tryptophan and subsequently induce a rise in brain tryptophan and serotonin levels (Hardeland & Rensing 1968; Daya *et. al.*, 1989). Both tolmetin and sulindac reduce the activity of TDO. This means that there is possibly more tryptophan available for uptake into the CNS for serotonin production. IDO, the other enzyme that is also responsible for tryptophan metabolism, uses the superoxide anion as its cofactor (Hirata & Hayaishi 1975; Taniguchi *et. al.*, 1977). Therefore scavenging the superoxide will reduce the activity of IDO (Hayaishi *et. al.*, 1977) and subsequently amplify circulating tryptophan levels. In Chapter 3 it was established that both tolmetin and sulindac scavenge the superoxide anion. This suggests that these agents may also reduce the amount of superoxide available, thereby reducing IDO activity and possibly increasing circulating tryptophan levels.

Figure 8.1 shows that QA is also a product of the tryptophan-niacin pathway. The inhibition of TDO and IDO could also result in the reduction of circulating levels of this potent excitatory amino acid (Heyes *et. al.*, 1997) since it has previously been shown that the inhibition of TDO does reduce QA in the brain (Reinhard *et. al.*, 1996). Therefore, apart from inhibiting TDO and IDO and increasing circulating tryptophan levels, tolmetin and sulindac may also reduce QA levels.

CHAPTER 9

EFFECT OF TOLMETIN AND SULINDAC ON BRAIN BIOGENIC AMINE LEVELS

9.1. INTRODUCTION

Serotonin (5-hydroxytryptamine) is an important regulator in a wide range of physiological and biochemical processes in vertebrates. Serotonin (5-HT), has been involved in the maintenance of circadian rhythmic functions, in blood pressure regulation, and also acts as a neurotransmitter at central and peripheral regions (Kema *et. al.*, 2000). The largest fraction of serotonin in the body is synthesized by intestinal enterochromafin cells (Kema *et. al.*, 2000). Smaller quantities are produced by the central nervous system, thyroid parafollicular cells, ovaries, pancreas (Kema *et. al.*, 2000) and the skin (Slominski *et. al.*, 2002). Following its release into the blood, serotonin is actively taken up by circulatory platelets and stored in solid granules. Alterations in brain serotonin levels are known to result in mood disorders, particularly depression (van Praag 1982).

In the last several years, the chief secretory product of the pineal gland, melatonin (Reiter 1991) has been found to be both a direct free radical scavenger and an indirect antioxidant (Hardeland *et. al.*, 1995; Reiter *et. al.*, 1995; Reiter *et. al.*, 1997; Hardeland 1997). Because of these actions, melatonin has been pharmacologically tested for its ability to reduce oxidative damage in a variety of experimental neurological processes and has been found highly effective (Reiter *et. al.*, 1997; Reiter 1998). Melatonin (N-acetyl-5-methoxytryptamine) is a product of tryptophan metabolism and is produced in a limited number of mammalian organs including the pineal gland, retina and the gastrointestinal tract (Reiter 1991). Melatonin is both lipid soluble (Costa *et. al.*, 1995) and water soluble (Shida *et. al.*, 1994), although its lipid solubility is greater. It has the ability to traverse almost every organ in the body (Reiter 1995). It has been demonstrated to be a powerful antioxidant and free radical scavenger (Tan *et. al.*, 1993) and reduces oxidative damage in the central nervous system (Reiter 1998).

9.2. EFFECT OF TOLMETIN AND SULINDAC ON BRAIN SEROTONIN AND OTHER NEUROTRANSMITTER LEVELS

9.2.1. Introduction

Brain 5-HT is synthesized from tryptophan which is taken up from the peripheral circulation. The major determinant of the levels of peripheral circulating tryptophan is the haem-dependent liver cytosolic enzyme, TDO.

As shown in figure 9.1, tryptophan is hydroxylated to 5-hydroxytryptophan by tryptophan hydroxylase, which is present in high concentrations in the pineal gland, and this appears to be the rate-limiting step in the synthesis of serotonin (Lovenberg *et. al.*, 1968). However, it is the amount of tryptophan available, and not enzyme activity that influences the rate of the reaction (Deguchi & Barchas 1972; Bensinger *et. al.*, 1974). In the pineal gland there is sufficient enzyme 5-hydroxytryptophan decarboxylase, which decarboxylates 5-hydroxytryptophan to 5-hydroxytryptamine, also known as serotonin.

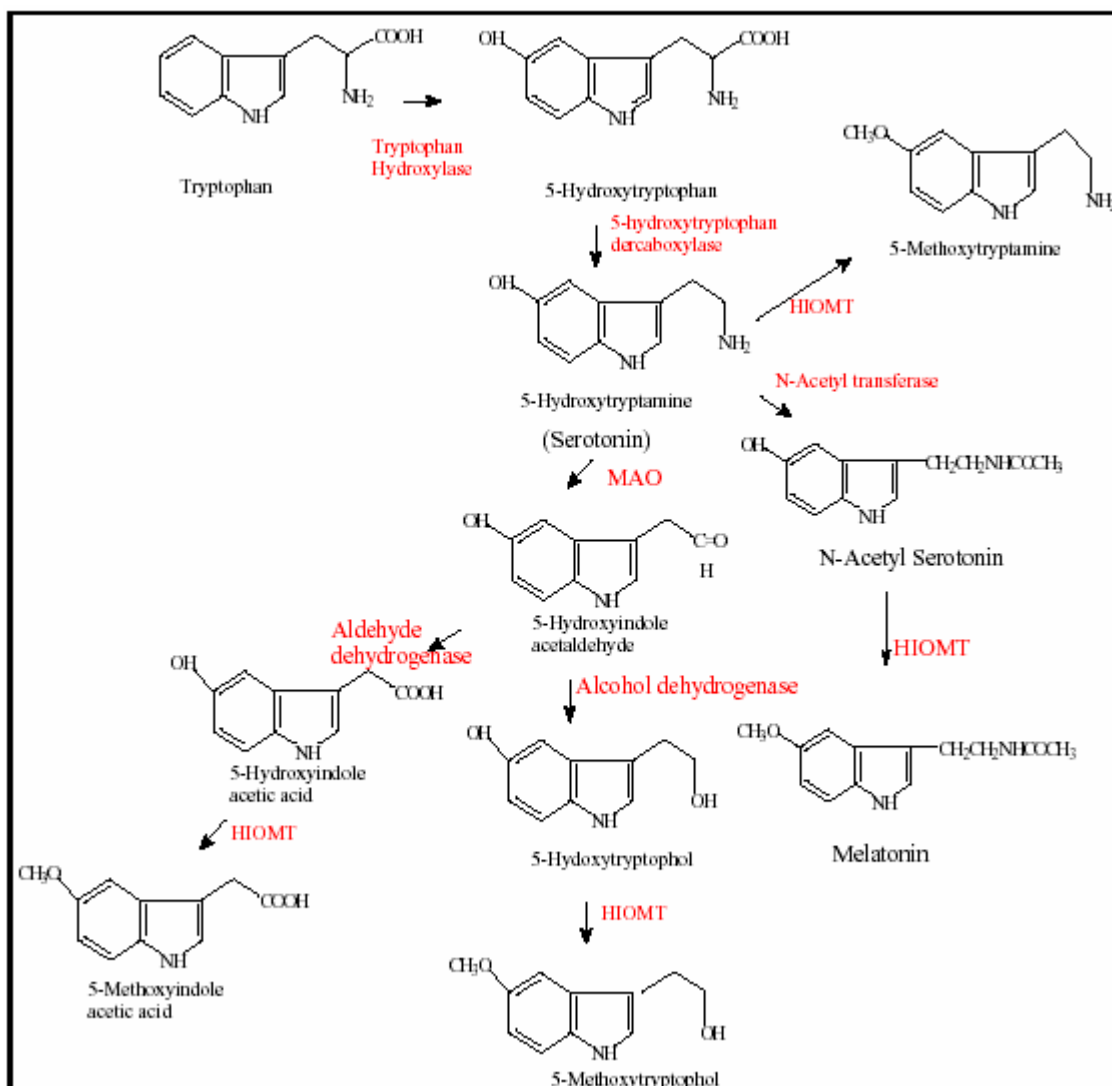


Figure 9.1. Pineal Indole Metabolism (Young & Silman 1982). The product is printed in black and the related enzyme is printed in red.

In the previous chapter, it was demonstrated that tolmetin and sulindac reduced TDO activity. The reduction of TDO activity could have provided more tryptophan to be taken up into the CNS for serotonin production. This experiment was conducted to determine whether the serotonin and other indoleamine levels had changed.

Chromatography involves the separation of the components of a mixture, by virtue of the differences in the equilibrium distribution constant (K) of the components between the

two phases of a chromatographic system, which are the stationary and the mobile phase (Pecsok *et. al.*, 1968; Snyder & Kirkland 1974). The neurotransmitters were separated using a C₁₈ column and were detected using an electrochemical detector set at +0.74V. The method used in this experiment was similar to that described by Muralikrishnan and Mohanakumar in 1998.

9.2.2. Materials and Methods

9.2.2.1. Chemicals and reagents

Octane sulphonic (OSA) acid, EDTA, triethylamine (TEA), phosphoric acid (PA), perchloric (HClO₄) acid, dopamine (DA), serotonin (5-HT), dihydroxyphenylacetaldehyde (DOPAC) and 5-hydroxyindole acetic acid (5-HIAA) were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. HPLC grade acetonitrile (ACN) was purchased from Saarchem, Johannesburg, South Africa. All other reagents were of the highest quality available.

9.2.2.2. Treatment regimes

Animals were cared for and treated as described in section 8.3.2.1. On the 6th day (after receiving tolmetin or sulindac, 5mg/kg/bd for 5 days) the animals were killed by neck fracture, the hippocampus and striatum of each rat was removed and frozen in liquid nitrogen and stored at -70°C. Five brains from each group were used in this study.

9.2.2.3. Tissue preparation

On the day of the experiment, the brain regions were thawed on ice, weighed and individually homogenised (1mg/10µl) in ice-cold HClO₄ (0.1M) containing 0.01% EDTA. This was sonicated at 50Hz for 30 seconds using an ultrasonic cell disruptor and kept on ice for 20 minutes before being centrifuged at 10 000 x g for 10 minutes using a bench top centrifuge. The supernatant obtained was directly injected into the High Performance Liquid Chromatography-Electrochemical Detector (HPLC-ECD) system for analysis. The volume of injection was 10µl.

9.2.2.4. Instrumentation

Samples were analyzed on an isocratic HPLC coupled to an electrochemical detector. The chromatographic system consisted of a Waters Millipore Model 510 pump, Waters electrochemical detector and a Rikadenki chart recorder. The chart speed was set at 15cm/h. Samples were introduced into the system using a Rheodyne Model 772Si fixed loop injector, fitted with a 20 μ l loop.

9.2.2.5. Chromatographic conditions

Separation was achieved using a C₁₈ (Waters Spherisorb®, 5 μ m, 250 x 4.6mm o.d.s.1 column). The mobile phase consisted of 8.32mM OSA, 0.27mM, EDTA, 13% ACN, 0.4-0.45% TEA and 0.2-0.25% PA (v/v) and made to 1000ml using Milli-Q water. Mobile phase was degassed twice using a 0.45 μ M membrane filter prior to use. The mobile phase was recycled but was changed every 3 days. The flow rate was set at 0.8ml/min and the electro detection was performed at +0.74 V. Results are expressed as pmoles/mg tissue and were analysed as described in section 2.2.2.7.

9.2.3. Results

Tolmetin and sulindac treated animals (5mg/kg/bd for five days) show an increase in the levels of serotonin in the hippocampus when compared to controls (figure 9.2). Hippocampal 5-HIAA was much higher in tolmetin treated animals. DA and DOPAC levels in the hippocampus demonstrated no significant changes after treatment. As shown in figure 9.3, in the striatum, both agents significantly reduce DA levels. DOPAC levels were significantly higher in tolmetin treated animals.

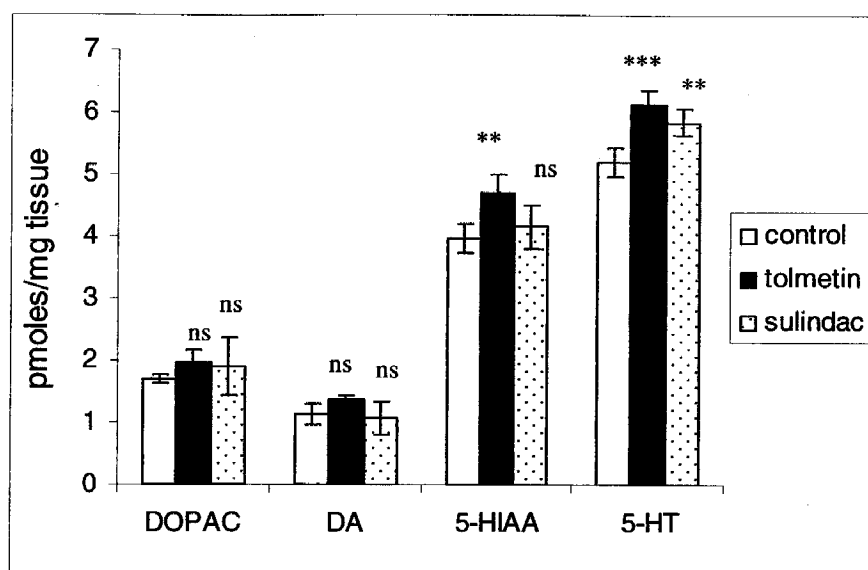


Figure 9.2: Effect of tolmetin and sulindac on rat hippocampal biogenic amine levels. Each bar represents the mean $\pm$ SD ($n = 5$). ns($p > 0.05$), **($p < 0.01$) and ***($p < 0.001$) compared to controls.

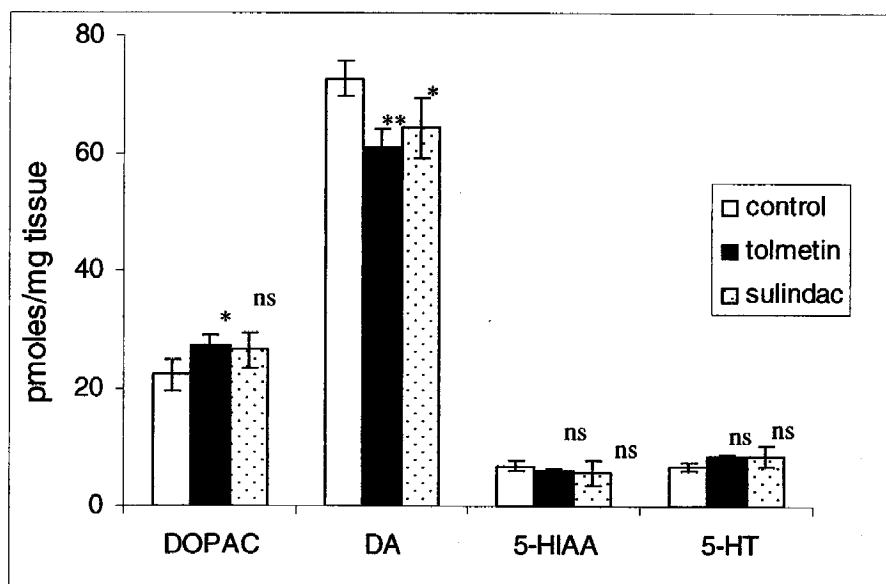


Figure 9.3: Effect of tolmetin and sulindac on the rat striatal biogenic amine levels. Each bar represents the mean $\pm$ SD ($n = 5$). ns($p > 0.05$), *($p < 0.05$) and **($p < 0.01$) compared to controls.

9.2.4. Discussion

Depression is a complex condition involving abnormalities in the sympathetic nervous system as well as the endocrine and immune systems (Szelenyi & Selmeczy 2002). In the present study, much attention was focused on the hippocampus and striatum, where monoamines are present, since these regions play crucial roles in behavioral functions such as learning, memory and emotion, all of which may be affected in depression (van Praag 1982; Butterweck *et. al.*, 2000). It has been shown that the inhibition of liver enzyme TDO causes an increase in brain 5-HT levels (Hardeland & Rensing 1968; Daya *et. al.*, 1989; Slater *et. al.*, 1995a; Slater *et. al.*, 1995b; Daya & Anoopkumar-Dukie 2000). Tolmetin and sulindac have shown to inhibit the liver enzyme TDO (chapter 8). The results of this experiment shows that both tolmetin and sulindac increase 5-HT levels in the hippocampus but not in the striatum implying that these agents may have potential use in patients suffering with depression. The inhibition of the liver enzyme TDO and possibly IDO may be a possible mechanism through which these agents increase the 5-HT levels in the hippocampus.

Dopamine (DA) has received a great deal of attention in the mammalian CNS (Albin *et. al.*, 1989; Williams & Goldman-Rakic 1995; Greengard *et. al.*, 1999). Dopaminergic neurons send abundant axonal projections to the forebrain regions, using DA as the major neurotransmitter. These neurons play important roles in multiple functions such as movement, memory, mental and psychological activities (Albin *et. al.*, 1989; Kalivas & Stewart 1991; Williams & Goldman-Rakic 1995; Knable & Weinberger 1997). Abnormalities of DA signaling may result in several neurological disorders such as Parkinson's disease, epilepsy, schizophrenia and drug abuse (Kalivas & Stewart 1991; Koob 1992; Koob & Le Moal 1997; Nestler & Aghajanian 1997). Both NSAIDS reduce the dopamine content in the rat striatum. Tolmetin and sulindac may therefore have the potential to induce or aggravate the above-mentioned neurological disorders.

Tolmetin may affect the functioning of the enzyme, alcohol dehydrogenase. This would mean that less 5-Hydroxyindole acetaldehyde was converted to 5-Hydroxytryptophol and more was converted to 5-HIAA.

Since the NSAIDS seem increased 5-HT levels, it is possible that melatonin produced by the pineal gland may also increase. This possibility has been investigated in the following experiment.

9.3. EFFECT OF TOLMETIN AND SULINDAC ON THE PRODUCTION OF MELATONIN BY THE PINEAL GLAND

9.3.1. Introduction

After being synthesised, serotonin can be N-acetylated to form N-acetyl serotonin. This reaction is catalysed by the enzyme N-acetyltransferase (NAT). The activity of this enzyme NAT increases from 30 fold to 70 fold at night, and in most circumstances is the rate-limiting step in melatonin synthesis (Klein *et al.*, 1997). The 5-hydroxygroup of N-acetylserotonin is O-methylated in the presence of hydroxylindole-O-methyltransferase to form melatonin (figure 9.1).

Experiment 9.2 shows that there was an increase in the 5-HT levels in the brain. The present study was conducted to determine whether this increase in 5-HT could have also triggered an increase in melatoninsynthesis.

9.3.2. Materials and Methods

9.3.2.1. Chemicals and reagents

Melatonin was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. Minimum Essential Medium (MEM) and all other reagents used to incubate the pineal gland were as per section 2.4.2.1. Chloroform and HPLC grade methanol (MeOH) and acetonitrile were purchased from Saarchem, Johannesburg, South Africa.

9.3.2.2. Treatment regimes

As described in section 8.3.2.1, animals were injected intraperitoneally with tolmetin or sulindac, 5mg/kg/bd for 5 days. On the 6th day, the pineal glands were removed and incubated for 24 hours in 50µl of MEM which was supplemented with antibiotics and antifungal agents as described in section 8.3.2.1. After 24 hours, the pineal glands were removed and the rest of the experiment was conducted as described in section 9.3.2.8.

9.3.2.3. Instrumentation

Samples were analysed on an isocratic HPLC system. The chromatographic system consisted of a Waters Millipore Model 510 pump and a Waters 2487 dual absorbance detector. Samples were introduced into the system using a Rheodyne Model 772Si fixed loop injector, fitted with a 20µl loop. The Breeze software was used to control the HPLC system and to acquire and analyse data.

9.3.2.4. Chromatographic conditions

The column used was a C₁₈ (Waters Spherisorb© 5µm, o.d.s.2, 4.6 x 250mm). The mobile phase consisted of acetonitrile: methanol: H₂O, at a ratio of 4:1:5. Mobile phase was degassed using a 0.45µM membrane filter prior to use. The flow rate was 1ml/min. The UV detector was set at a wavelength of 304nm and the response time was set at 0.1s. The detector sensitivity was set at 2 absorbance units full scale (AUFS). The injection volume was 20µl.

9.3.2.5. Method validation

A stock solution of melatonin (0.1 mg/ml) was freshly prepared for each test by weighing melatonin and dissolving it in mobile phase. Dilutions of this stock solution were made appropriately for the concentration range to be studied. Samples for the assessment of linearity and precision were prepared by serial dilution of the stock solution to yield concentrations over the range 100 – 500 ng/ml. In addition, two concentrations within the calibration range were prepared independently for use as accuracy standards. The limits of quantification and detection were also determined. Specificity was determined by mass spectrometry. Quantitative determination of melatonin was made by comparison of peak heights using known concentrations of melatonin (appendix 8).

9.3.2.6. Extraction efficiency

To 40 µl of MEM, 10 µl of mobile phase containing known amounts of melatonin was added. The final concentrations of melatonin were 200, 300 and 400ng/ml. To this, 1ml of chloroform was added and vortexed. An aliquot of 0.5 ml of the chloroform was

removed and evaporated to dryness on a hot water bath. The residue (melatonin) was dissolved in 50 μ l of mobile phase and 20 μ l was injected onto the column for analyses.

9.3.2.7. Mass Spectrometry (MS)

The HPLC system consisted of a spectrasystem AS300 autosampler, a spectrasystem UV200 detector and a spectrasystem P2000 pump. The mobile phase consisted of A: ammonium acetate made to pH 3 using formic acid, and B: methanol. A linear gradient from 15% to 90% MeOH was run over 17 minutes. Separation was achieved on a C₁₈ (Waters Spherisorb[®], 5 μ m, 250 x 4.6mm o.d.s.2 column). The LC instrument was interfaced with a Finnigan MAT LCQ mass spectrometer equipped with an electrospray ionization (ESI) source. The mass spectrometer was configured in the positive ion mode and to use selective ion monitoring (SIM) for the melatonin parent ion in the *m/z* range 60-240. The mass spectrometer parameters were as follows: the nebulizer nitrogen gas flow rate was set at 80, discharge voltage of 4.5 kV, the capillary temperature was set at 220 °C, the capillary voltage was 20 V and the discharge current was 5 μ A. The LC-MS workstation Xcalibur, version 1.3, was used for system control and data acquisition. The base peak of melatonin that was extracted from the pineal gland was compared to a standard melatonin sample (50 μ M).

9.3.2.8. Amount of melatonin produced by the pineal gland

After the pineal glands had incubated in the 50 μ l of MEM at 37°C for 24 hours, the glands were removed and 1 ml of chloroform was added and vortexed. The rest of the experiment was conducted as described above. Results are represented as amount of melatonin produced per pineal in 24 hours and are shown as uncorrected values. Results were analysed as described in 2.2.2.7. Five pineal glands were used from each group for this study.

9.3.3. Results

Chromatograms of melatonin obtained show symmetrical peaks which were resolved from the solvent front with a retention time of 4.1 minutes. Regression analysis shows

that the concentration versus peak height is linear over the range studied i.e. 100 – 500 ng/ml ($r^2 = 0.9998$). The limits of detection (LOD) and limits of quantification (LOQ) were 5 ng/ml and 15 ng/ml respectively. Inter- and intra-assay precision was determined by calculating the relative standard deviation (RSD) of peak heights of the calibration standards ($n = 5$) and is reported as RSD %. The intra-assay precision revealed RSD % values of 0.86% - 2.28% (table 1) and the inter-assay precision revealed RSD % values of 0.58% - 2.11% (table 1). Table 2 shows that approximately 80% of the melatonin was extracted from 50 μ l of MEM (containing 200, 300 or 400 ng/ml melatonin) using 1 ml of chloroform. The standard sample of melatonin and the extract from the pineal glands showed base peaks at m/z 174 (figure 9.4 and 9.5 respectively).

As shown in figure 9.6, tolmetin significantly ($p < 0.05$) increases melatonin synthesis by the pineal gland while sulindac has no effect.

Table 9.1: Melatonin assay validation data.

Concentration (ng/ml)	Relative standard deviation (%)		
	Intra-assay (n=5)	Inter-assay 1 (n=5)	Inter-assay 2 (n=5)
100	1.77	2.11	0.68
200	0.86	1.08	0.81
300	1.04	1.11	1.44
400	2.28	1.83	1.03
500	0.86	0.58	0.61

Table 9.2: Melatonin extraction efficiency data.

Concentration (ng/ml)	Extraction efficiency (%)	standard deviation
200	81.6	2.19
300	81.1	2.22
400	80.2	3.80

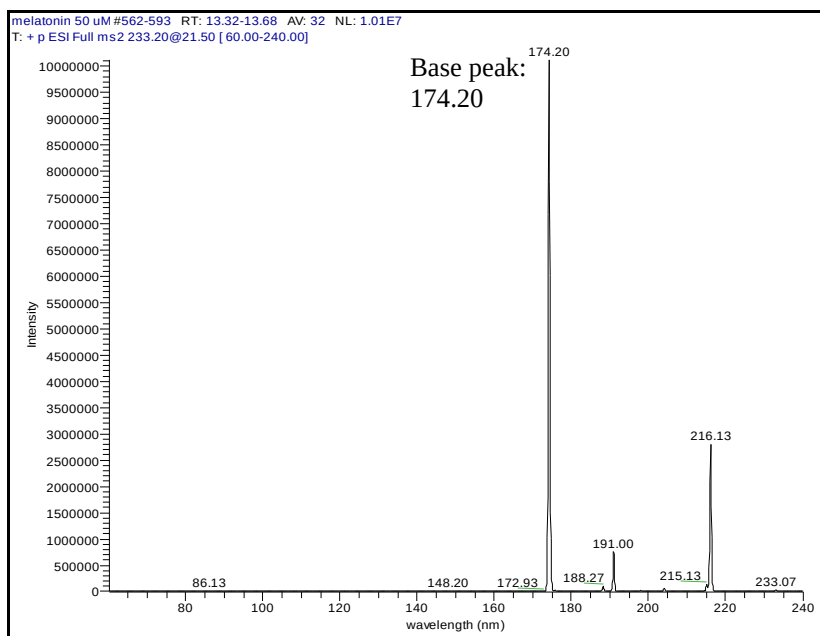


Figure 9.4: Mass spectral data of melatonin standard (50µM)

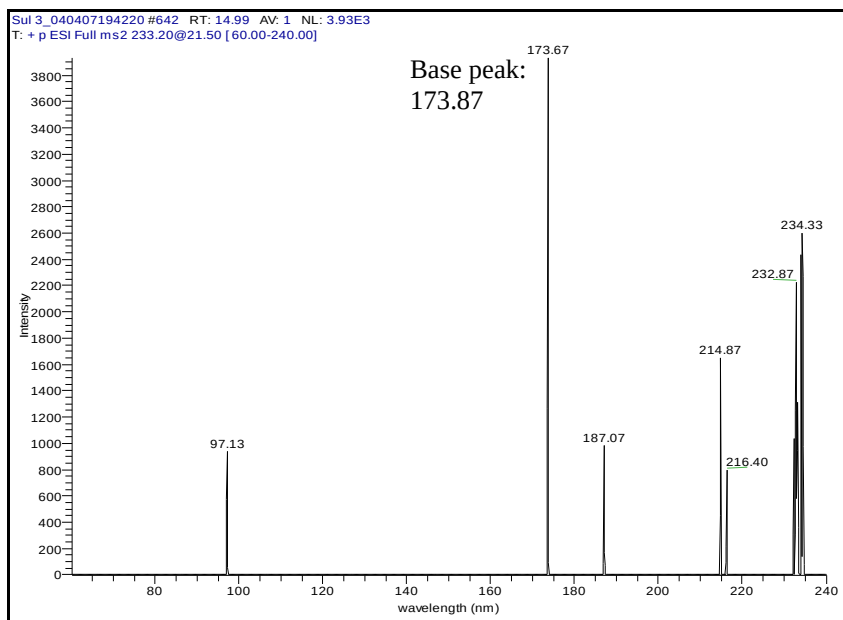


Figure 9.5: Mass spectral data of melatonin extracted with chloroform from MEM in which the pineal gland was incubated.

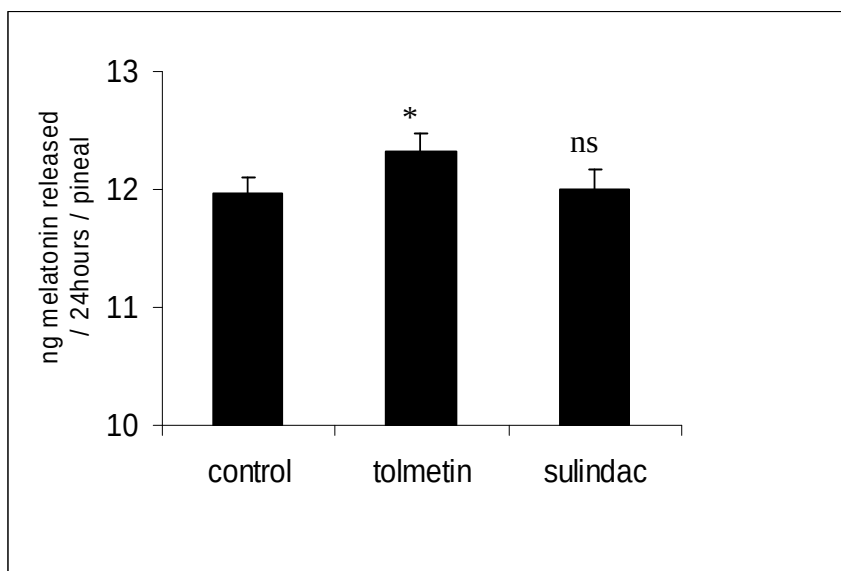


Figure 9.6: Effect of tolmetin and sulindac treatment on melatonin synthesis in the rat pineal gland. Each bar represents the mean $\pm$ SD ($n = 5$). ^{ns}($p > 0.05$) and * ($p < 0.05$) when compared to controls. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

9.3.4. Discussion

Liquid-phase extraction methods have been employed for the separation of indoleamines (Quay 1963; Miller & Maickel 1970). As shown in figure 9.7, the liquid-phase extraction method results in high recovery of 5-HT, Mel and 5-methoxytryptamine (5MT). Chloroform (6ml) extracted approximately 85% of melatonin from the pineal gland. This study demonstrates that 1ml recovers approximately 80% of melatonin. This is lower since a smaller volume of chloroform was used to extract the melatonin. Mass spectrometry confirmed that the extract was melatonin. It has been suggested (Xie *et. al*, 1998), that the base peak observed at 174 m/z is formed by the loss of the $\text{HN}=\text{COH}-\text{CH}_3$ fragment from the protonated parent molecule with the formation of a four-membered ring from the side chain.

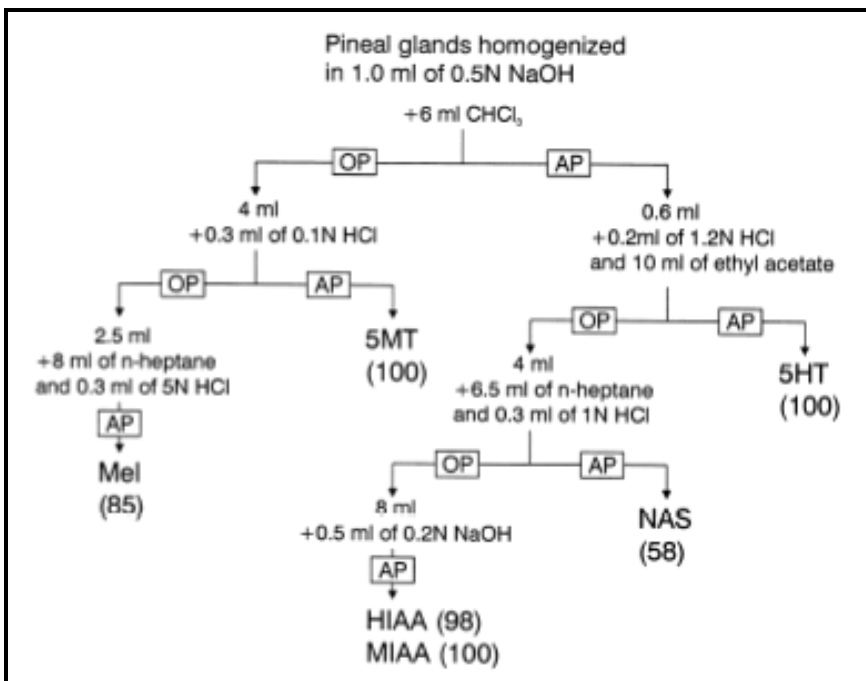


Figure 9.7: Liquid-phase extraction method of indoleamines from rat pineal gland (Miller & Maickel 1970). Organic phase (OP), aqueous phase (AP), melatonin (Mel) 5-methoxyindole acetic acid (MIAA), 5-hydroxyindole acetic acid (5-HIAA) and N-acetylserotonin (NAS). Recovery rates (%) of each indoleamine are indicated in brackets.

NSAIDS are thought to exert most of their effects via prostaglandin synthesis inhibition (Vane 1971; Flower & Vane 1972) and it is known that prostaglandins markedly enhance melatonin synthesis at night (Cardinali *et. al.*, 1979; Cardinali *et. al.*, 1982; Voisin *et. al.*, 1993). Thus, NSAIDS have the ability to reduce melatonin synthesis. For example, it has been shown that NSAIDS such as aspirin (Murphy *et. al.*, 1996) and ibuprofen (Surrall *et. al.*, 1987; Murphy *et. al.*, 1996) reduce melatonin synthesis in humans. However, in this study, tolmetin, but not sulindac, increases the amount of melatonin produced by the rat pineal gland. It is unlikely that the rise in melatonin induced by tolmetin is a consequence of inhibition of TDO in the liver as it has been shown (Daya *et. al.*, 1989), that such inhibition does not alter melatonin levels. A possible reason for the tolmetin-induced increase in melatonin synthesis could be a direct effect of this drug on the melatonin synthesis pathway in the pineal gland.

CHAPTER 10

METAL BINDING STUDIES: THE INTERACTION BETWEEN TOLMETIN, SULINDAC AND IRON

10.1. INTRODUCTION

Iron is an essential nutritional element because it is the cofactor for enzymes in all life forms. Heme iron also plays critical roles in mitochondrial electron transport and cellular respiration, cell proliferation, and cell differentiation. In addition to its role in hematopoietic cell differentiation, iron also regulates gene expression and brain functions (Connor & Benkovic 1992; Moos *et. al.* 1999; Boldt 1999).

Although essential, most metal species are cytotoxic at sufficiently high doses, resulting in neurochemical abnormalities and psychiatric disorders. Previous biological work (Chapter 2, experiment 2.8) demonstrated that Fe^{2+} induces LP in rat brain homogenate. This occurs through the Fenton reaction. Chapter 2, experiment 2.2 shows that QA also induces LP in rat brain homogenate. It was postulated that the QA-iron complex was responsible for this. Tolmetin and sulindac both demonstrate the ability to reduce the QA as well as the transition metal-induced LP (experiment 2.9). The direct interaction and possible complexation of the transition metal with tolmetin and sulindac was suggested as a possible mechanism through which these agents reduce LP. Tolmetin and sulindac possibly interact with Fe^{2+} or Fe^{3+} making the transition metal unavailable for the Fenton reaction, thus reducing LP. Electrochemical analysis, ultraviolet/visible (UV/VIS) studies as well as infrared (IR) spectroscopy were techniques employed to probe whether any interaction between the NSAIDS and this metal occur.

10.2. UV/VIS SPECTROSCOPY

10.2.1. Introduction

Absorption measurements employing ultraviolet or visible radiation have widespread applications to quantitative and qualitative analysis. Spectroscopic methods are based on the fact that all chemicals and compounds, to some extent, absorb electromagnetic radiation of specific wavelengths. The absorption of ultraviolet or visible radiation generally results from excitation of bonding electrons. The wavelengths of absorption peaks can be correlated with the types of bonds that exist in the species under study. Absorption spectroscopy is, therefore valuable for identifying certain functional groups in a molecule. It can also be used as a selective method for quantitative analysis for compounds containing absorbing bonds. Any change in structure or composition of a compound will result in an electronic change, resulting in a change in the spectrum thus causing a change in the wavelength or a change in the extinction coefficient of the absorbance (Newman 1969).

10.2.2. Materials and Methods

10.2.2.1. Chemicals and Reagents

Iron sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and anhydrous ferric chloride (FeCl_3) were purchased from BDH Laboratory Supplies, Poole, England. FeCl_3 and FeSO_4 solutions were prepared in deaerated Milli-Q water. Tolmetin and sulindac were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A.

Iron sulphate was made immediately before use to avoid the rapid oxidation of Fe^{2+} to Fe^{3+} .

10.2.2.2. Technique: UV/VIS Spectroscopy

The UV/Visible spectra were monitored with a Cary Varian 500 series spectrophotometer. A quartz cuvette with a 0.1cm path length was used for these experiments. The interaction between tolmetin, sulindac and the metals were studied by

comparing the absorption of tolmetin or sulindac alone in solution and then immediately after the addition of the metal solution. A time study was also conducted over 5 hours with the NSAIDS and Fe^{2+} . Various equimolar concentrations (0.0625; 0.125 and 0.25mM) of the drug and either Fe^{2+} or Fe^{3+} were spectroscopically analysed.

10.2.3. Results

As shown in figure 10.1, free sulindac shows absorbance bands at 228nm, 283nm and 326nm. Upon the addition of Fe^{2+} there is an increase in absorbance of all 3 bands and a blue shift of 6nm occurs for the band at 228nm. The time study shows that after four hours there is a decrease in the absorbance of all 3 bands of the complex (figure 10.2). Upon the addition of Fe^{3+} to free sulindac there is also a decrease in absorbance in all 3 bands and a blue shift of 4nm of the band at 228nm (figure 10.1).

As shown in figure 10.3, free tolmetin shows absorbance bands at 259nm and 323nm. Upon the addition of Fe^{3+} there is a decrease in absorbance and a blue shift of 7nm in the 323nm band (figure 10.3). In the presence of Fe^{2+} there is no change in the absorbance or wavelength. The time study shows that the oxidation of Fe^{2+} leads to the subsequent binding of tolmetin to Fe^{3+} (figure 10.4).

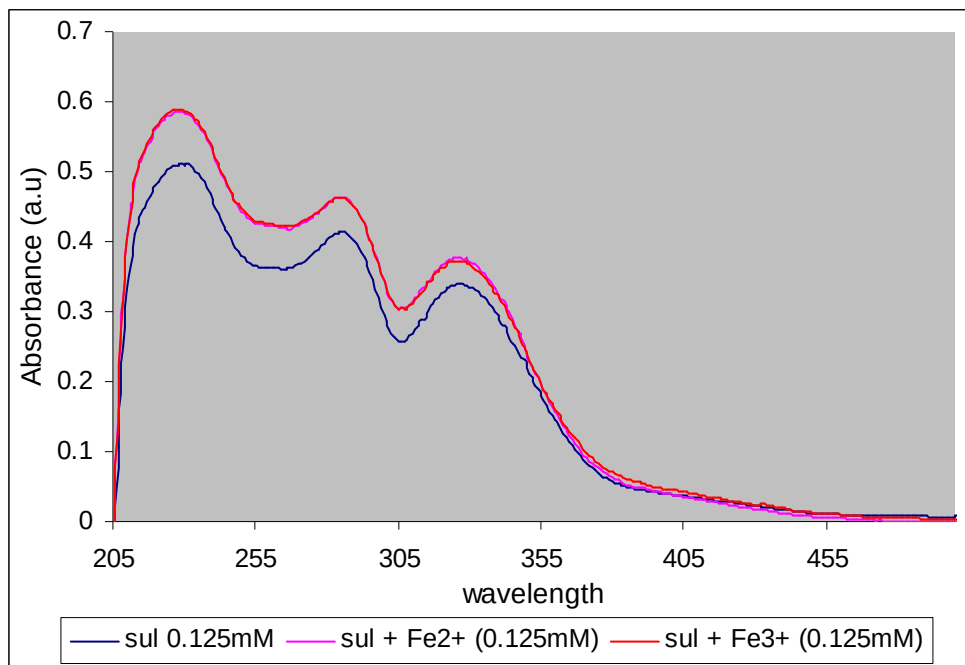


Figure 10.1: Spectroscopic analysis of sulindac alone and in the presence of Fe^{2+} and Fe^{3+} . The final concentration of sulindac and the metals were 0.125mM each. This interaction was also noted at other concentrations.

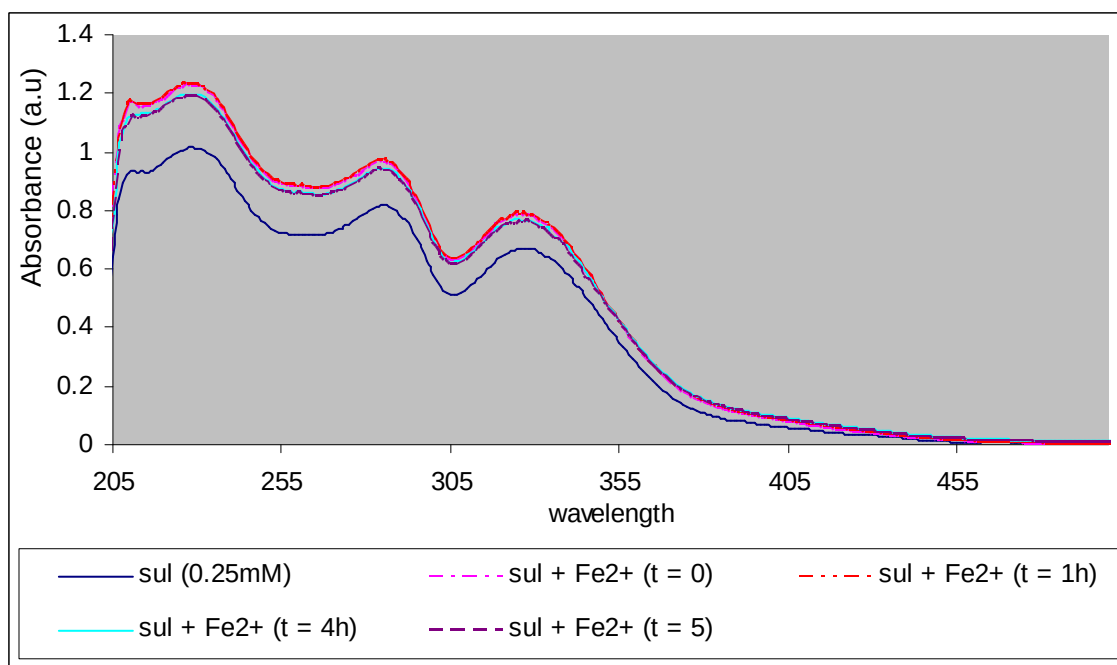


Figure 10.2: Spectroscopic time study of sulindac in the presence of Fe^{2+} (0.25mM each). The interaction was analysed over 5 hours.

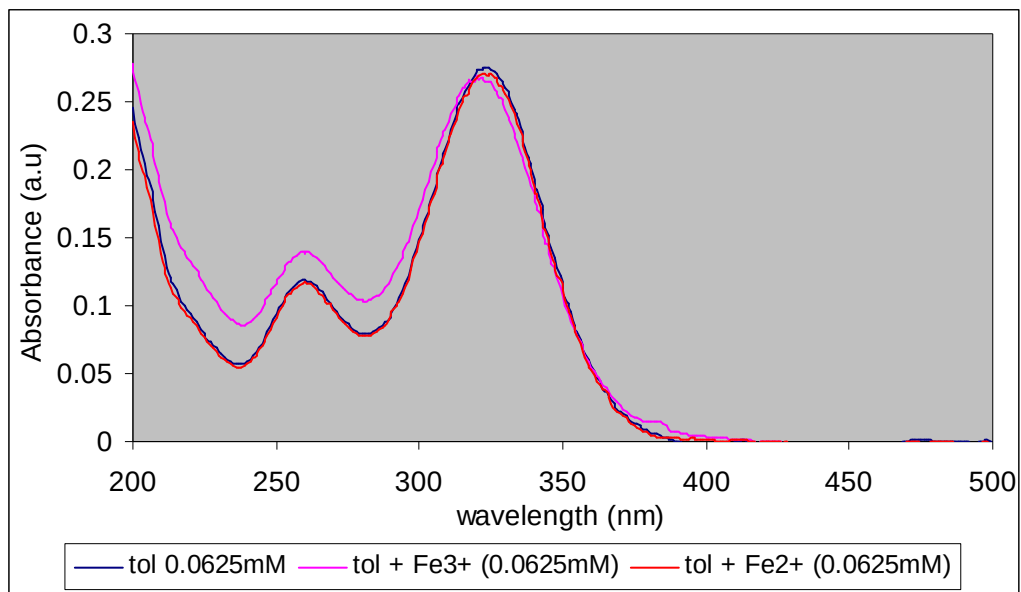


Figure 10.3: Spectroscopic analysis of tolmetin alone and in the presence of Fe^{2+} and Fe^{3+} . The final concentration of tolmetin and the metals were 0.0625mM each. This interaction was also noted at other concentrations.

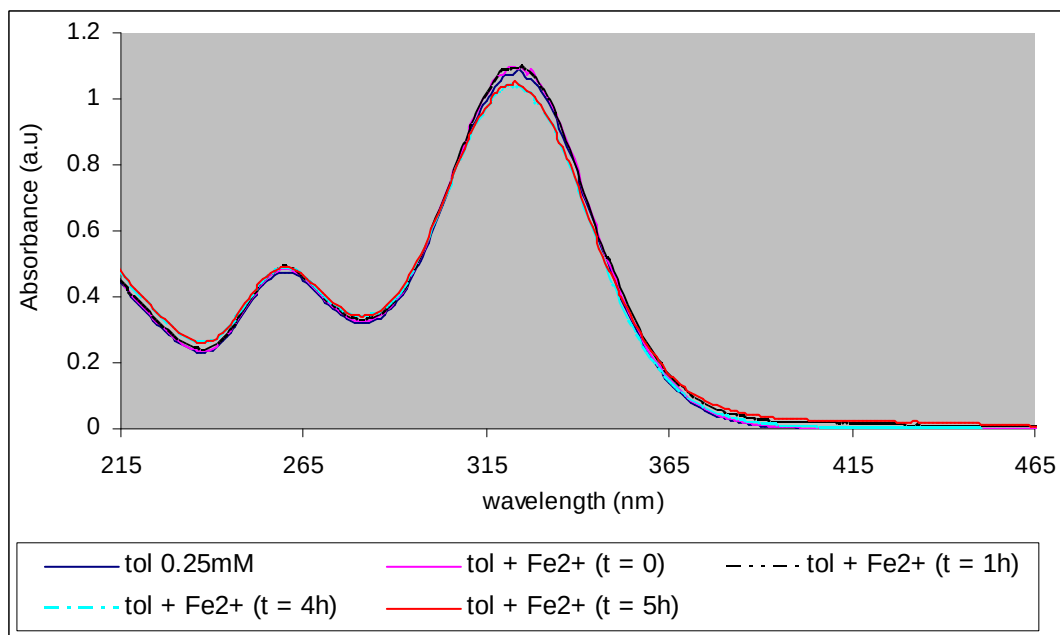


Figure 10.4: Spectroscopic time study of tolmetin in the presence of Fe^{2+} (0.25mM each). The interaction was analysed over 5 hours.

10.2.4. Discussion

Free sulindac shows absorbance bands at 228nm [$n \rightarrow \pi^*$, $>\text{S}=\text{O}$ (Scott 1964)], at 283nm [$\pi \rightarrow \pi^*$, aromatic (Scott 1964)] and at 326nm [$\pi \rightarrow \pi^*$, aromatic (Scott 1964)]. Complexation on addition of Fe^{2+} is evidenced by an increase in the absorbance of all three bands and by a blue shift of 6nm of the $\text{S}=\text{O}$ ligand band at 228nm. A time study shows the slow oxidation of the complex after four hours, with a concomitant decrease in all three absorbance bands. Complexation on addition of Fe^{3+} is similarly shown by the increase in the absorbancies of all three bands and by a blue shift of 4nm of the ligand band at 228nm. The UV/VIS results show that sulindac's interaction with Fe^{2+} is greater than that with Fe^{3+} .

Free tolmetin shows absorbance bands at 259nm [$n \rightarrow \pi^*$, aromatic ketone (Scott 1964)] and at 323nm, [$\pi \rightarrow \pi^*$ aromatic (Scott 1964)]. On the addition of Fe^{3+} complexation is detected by the decrease in intensity of and a blue shift of 7nm in the higher wavelength band. However, in the presence of Fe^{2+} there is no change in either the absorbance or wavelength of either band. A time study shows the slow oxidation of Fe^{2+} and subsequent binding by Fe^{3+} to the ligand (figure 10.4).

The results suggest that tolmetin has an interaction with Fe^{3+} but not with Fe^{2+} , and sulindac has a greater interaction with Fe^{2+} than with Fe^{3+} . However, UV/VIS spectroscopy alone does not identify definite interactions, therefore infrared spectroscopy as well as electrochemistry were other techniques that were also employed to determine the nature of any interaction between tolmetin, sulindac and iron.

10.3. INFRARED SPECTROSCOPY

10.3.1. Introduction

The principal involved in IR analysis is similar to that of UV/VIS. If absorption is accompanied by a transition from one vibrational energy level to another and the radiation is from the infrared portion of the electromagnetic spectrum, then the technique is known as infrared spectroscopy or vibrational spectroscopy (Willard *et. al.* 1958). An IR spectrum is generally displayed as a plot of the wavelength of the IR radiation versus the percent of light transmitted by the compound. The IR spectrum of a compound essentially is the superimposition of the absorption bands of specific functional groups in the compound (Willard *et. al.* 1958). Subtle interactions with surrounding atoms of the molecule also affect the spectrum of the compound (Willard *et. al.* 1958).

10.3.2. Materials and Methods

10.3.2.1. Chemicals and Reagents

Potassium bromide (KBr) was purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. The KBr was heated at 400°C in a furnace to remove moisture. All other reagents were as per section 10.2.2.1.

10.3.2.2. Technique: Infrared Spectroscopy

Tolmetin or sulindac was added to a solution of FeSO₄ and FeCl₃. The final concentrations of drug and Fe²⁺ or Fe³⁺ in solution were 1mM each. Precipitates obtained were thought to be the tolmetin-metal or sulindac-metal complexes. These were collected 24 hours later and washed with ice-cold Milli-Q water under negative pressure then dried for 48 hours at room temperature. The precipitants were stored in desiccators under negative pressure until use. The precipitates were compressed into a pellet with KBr using a metal bolt and nut. The pellets were analysed by mid-range infrared spectroscopy (4000-400cm⁻¹) using a Perkin Elmer Spectrum 2000, FTIR spectrometer.

10.3.3. Results

As shown in figure 10.5, free sulindac shows three bands between 2600-2500 cm^{-1} , strong bands at 1697 and 1007 cm^{-1} , and a medium intensity band at 1270 cm^{-1} . The sulindac- Fe^{2+} complex shows that the bands between 2600-2500 cm^{-1} are lost and the strong band at 1697 cm^{-1} has shifted to a lower frequency of 1599 cm^{-1} (figure 10.5). The medium intensity band at 1270 cm^{-1} is shifted +175 cm^{-1} and is enhanced at 1404 cm^{-1} . There are strong bands at 3439 and 1720 cm^{-1} and the development of bands at 619 and 536 cm^{-1} . The sulindac- Fe^{2+} and sulindac- Fe^{3+} complexes are very similar (figure 10.6). Other bands have also developed in the sulindac- Fe^{3+} complex and these include bands at 3440, 1708, 654 and 545 cm^{-1} . The strong band in free sulindac at 1697 has shifted to 1602 cm^{-1} .

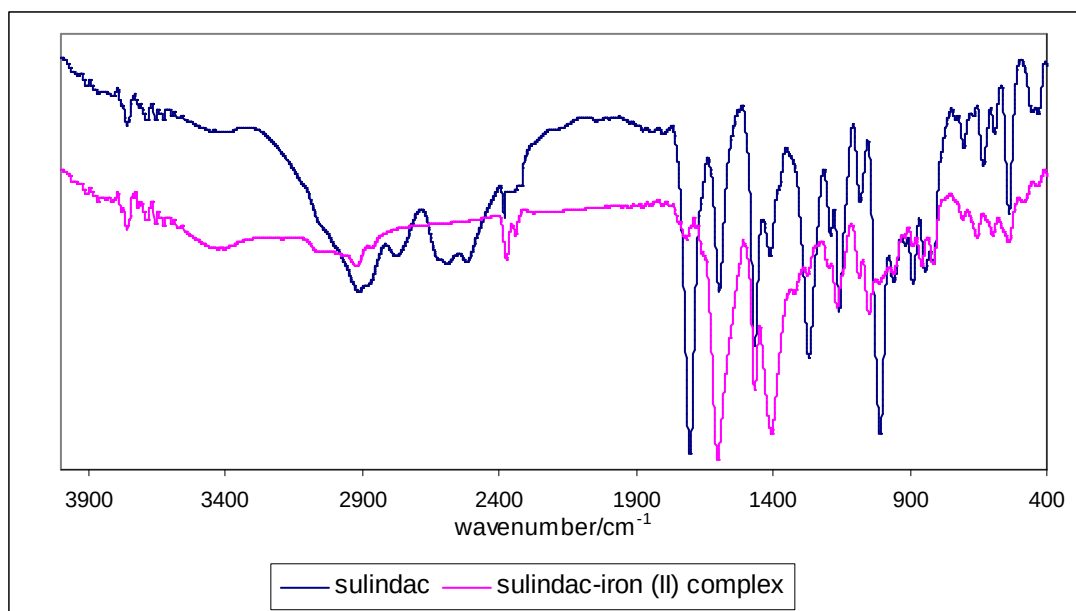


Figure 10.5: Infrared spectra of sulindac alone and in the presence of Fe^{2+} .

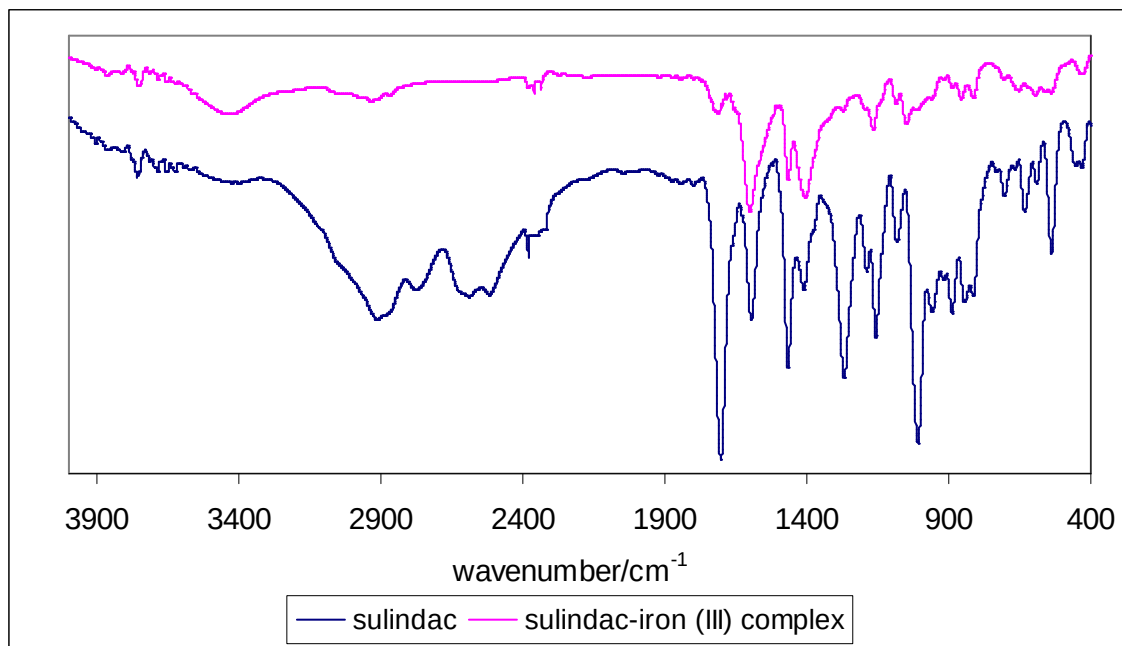


Figure 10.6: Infrared spectra of sulindac alone and in the presence of Fe^{3+} .

As shown in figure 10.7, the dihydrate sodium salt of free tolmetin shows a broad band at 3402cm^{-1} , strong bands at 1587 , 1373 and 1618cm^{-1} . On interaction with Fe^{3+} the band at 1587cm^{-1} shifts by -53cm to 1534cm^{-1} (figure 10.8). The interaction with Fe^{2+} is different since the asymmetric $\text{C}=\text{O}$ stretch is lower by -23cm^{-1} and the band at 1618cm^{-1} in free tolmetin shifts by -11cm^{-1} (figure 10.7).

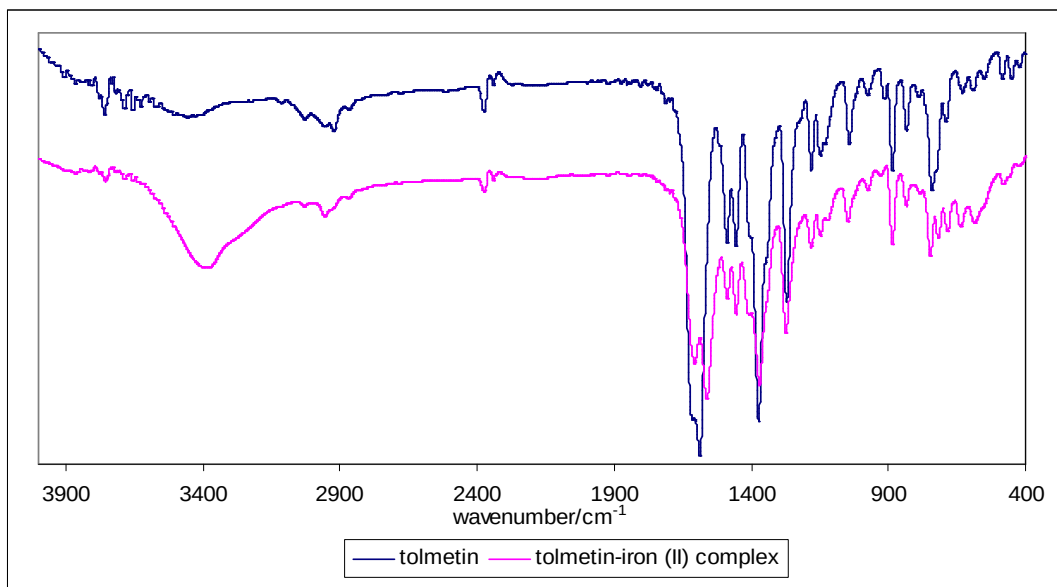


Figure 10.7: Infrared spectra of tolmetin alone and in the presence of Fe^{2+} .

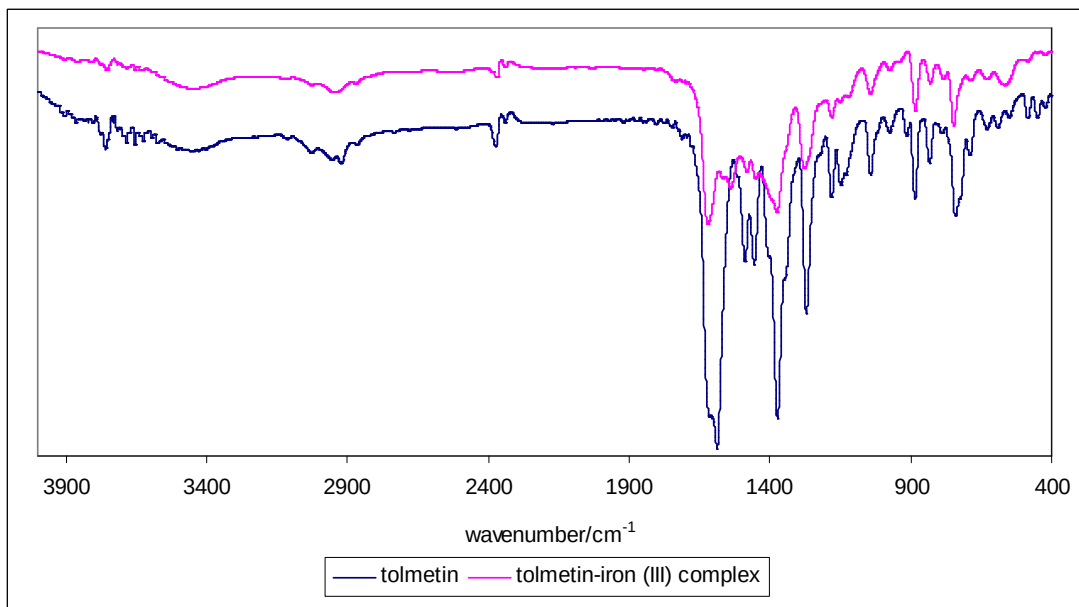


Figure 10.8: Infrared spectra of tolmetin alone and in the presence of Fe^{3+} .

10.3.4 Discussion

Free sulindac shows three bands between 2600-2500 cm^{-1} typical in carboxylic acids of the combination bands between $\nu\text{C}=\text{O}$ and $\delta\text{C}-\text{C}-\text{O}(\text{H})$. The very strong $\nu_{\text{a}}\text{C}=\text{O}$ at 1697 cm^{-1} is typical of the non-ionised carboxylic moiety, while the $\nu_{\text{s}}\text{C}=\text{O}$ is observed as a

medium intensity band at 1270 cm^{-1} (Nakamoto & McCathy 1968; Nakamoto 1977). The $\nu\text{S=O}$ of aryl sulfoxides is typically found between 1050 and 1020 cm^{-1} . The lower frequency of the strong $\nu\text{S=O}$ at 1007 cm^{-1} indicates strong hydrogen bonding by this moiety in the free ligand form (Bellamy 1969).

On interaction with Fe^{2+} the bands between 2600 - 2500 cm^{-1} are lost, and the very strong $\nu_{\text{a}}\text{C=O}$ shifts to 1599 cm^{-1} . While a shift to lower frequencies is commonly taken as evidence for coordination to the carbonyl, the band could also be interpretive of an ionised carboxylic group, expected between 1505 – 1575 cm^{-1} (Nakamoto & McCathy 1968), resulting from the use of a base on synthesis. Similarly, the $\nu_{\text{s}}\text{C=O}$ appearing as a typically strongly enhanced band at 1404 cm^{-1} (a shift of $+175\text{ cm}^{-1}$) has the alternate interpretations. If coordination does indeed occur at the carboxylic group then the difference between the two carbonyl stretches ($\Delta\text{CO}_2 = 195\text{ cm}^{-1}$) would indicate monodentate coordination (Nakamoto & McCathy 1968; Nakamoto 1977). The large shift of the $\nu\text{S=O}$ by $+44\text{ cm}^{-1}$ indicates coordination involves metal-sulfur binding rather than metal-oxygen (Nakamoto & McCathy 1968; Nakamoto 1977), as is to be expected between the soft acceptor (Fe^{2+}) and soft donor (S). The presence of strongly hydrogen-bonded water in the complex is seen by the broad $\nu\text{O-H}$ at $(3439)\text{ cm}^{-1}$, and the $\delta\text{O-H}$ at 1720 cm^{-1} . That there is coordinated water is seen by the appearance of a rocking ($\rho\text{M-OH}_2 = 619\text{ cm}^{-1}$) and a wagging vibration ($\omega\text{M-OH}_2 = 536\text{ cm}^{-1}$, shoulder) (Nakamoto & McCathy 1968; Nakamoto 1977).

The IR spectrum for sulindac on interaction with Fe^{3+} is similar to that of Fe^{2+} , suggesting minor differences in the binding sites. The presence of coordinated water is evident ($\nu\text{O-H} = 3440\text{ cm}^{-1}$; $\delta\text{O-H}$ at 1708 cm^{-1} ; $\rho\text{M-OH}_2 = 654\text{ cm}^{-1}$; $\omega\text{M-OH}_2 = 545\text{ cm}^{-1}$). The very strong $\nu_{\text{a}}\text{C=O}$ occurs at 1602 cm^{-1} and the $\nu_{\text{s}}\text{C=O}$ as an enhanced band at 1404 cm^{-1} . The 3 cm^{-1} difference in $\nu_{\text{a}}\text{C=O}$ between Fe^{3+} and Fe^{2+} shows that the shift from the free ligand must be ascribed to coordination rather than to an ionised carboxylic group, and indicates that for Fe^{2+} a slightly greater back-bonding from the metal into the C=O anti-bonding orbital (resulting in more single bond character) is experienced compared to Fe^{3+} . This suggests the former may be slightly the more stable complex. At 1049 cm^{-1} $\nu\text{S=O}$ for Fe^{3+}

indicates metal-sulfur binding, rather than metal-oxygen coordination (found below 1000 cm^{-1}). This implies a relative local softening of the metal centre on coordination when compared with the binding of Fe^{3+} with diphenylsulfoxide, $\nu\text{S}=\text{O}$ of 931 cm^{-1} (Nakamoto 1977).

The dihydrate sodium salt of tolmetin has a broad hydrogen bonded $\nu\text{O}-\text{H}$ at 3402 cm^{-1} . It shows a typical ionised carboxylic moiety (Nakamoto & McCathy 1968) with a very strong $\nu_{\text{a}}\text{C}=\text{O}$ at 1587 cm^{-1} and an enhanced $\nu_{\text{s}}\text{C}=\text{O}$ at 1373 cm^{-1} . The ketone is identified by a strong band at 1618 cm^{-1} . The low wavelength is characteristic of conjugation in unsaturated ketones, but may also indicate some hydrogen bonding of this moiety with the dihydrate (Bellamy 1969).

On interaction with Fe^{3+} there is no change in either the $\nu\text{C}=\text{O}$ of the ketone or the $\nu_{\text{s}}\text{C}=\text{O}$ frequencies, but $\nu_{\text{a}}\text{C}=\text{O}$ shifts significantly by -53 cm^{-1} , identifying the carboxylic group as the site of metal binding in a unidentate manner. Interaction with Fe^{2+} is substantially different. The asymmetric $\text{C}=\text{O}$ stretch is lowered by only 23 cm^{-1} , while $\nu\text{C}=\text{O}$ of the ketone is also lowered by -11 cm^{-1} . The former indicates a weaker unidentate carboxylate binding than that experienced with Fe^{3+} . The latter implies that, in the solid state, tolmetin either additionally binds to Fe^{2+} via the ketone moiety or, alternatively, that binding to the metal alters the extent of conjugation. The interaction of Fe^{2+} with tolmetin in the solid state is in contrast to its apparent absence in solution.

The IR results demonstrate that there is an interaction between the NSAIDS and Fe^{2+} and Fe^{3+} . The UV/VIS solution study shows no interaction between tolmetin and Fe^{2+} but the IR solid state study demonstrates otherwise.

10.4. ELECTROCHEMICAL ANALYSIS

10.4.1. Introduction

A chemical reaction where one or more electrons are transferred from one species to another is the basis for electrochemical analysis (Pecsok *et. al.* 1968). Recently, electrochemistry was used with success in metal-ligand interactions (Limson *et. al.*, 1998; Matlaba *et. al.* 2000; Daniel *et. al.*, 2004).

The electrochemical cell used in these studies comprised of three electrodes: the working, the reference and the auxiliary electrode as shown in figure 10.9.

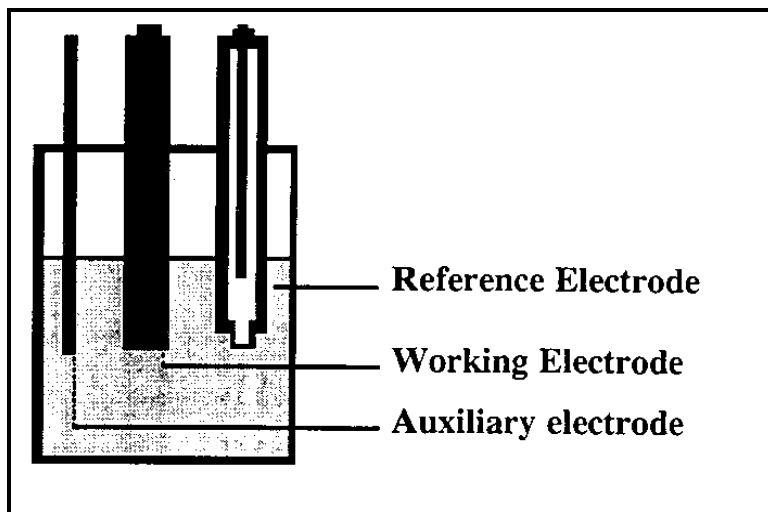


Figure 10.9: Most dynamic electrochemistry utilises the classic three-electrode system as shown above (Limson 1998).

The first electrode is the working electrode. This is where the analyte is oxidised or reduced. In these studies, the glassy carbon electrode (GCE) was used as the working electrode. This GCE provides an ideal surface chemistry for adsorption of analytes. It also possesses excellent mechanical and electrical properties, is chemically inert and exhibits reproducible performance. The reference electrode is the standard electrode against which the potential of the working electrode is referred. The silver/silver chloride electrode was used due to its suitability in working with aqueous solutions. The auxiliary electrode is usually constructed of an inert material such as platinum, and it is used to

prevent a voltage drop across the working and reference electrode. Current passes between the working and auxiliary electrode. Since oxygen is electroactive at almost all electrodes, and seriously interferes with the electrochemical analysis (Sawyer & Roberts 1974), nitrogen or any other inert gas is bubbled through the solution to remove oxygen. In the following experiments, adsorptive stripping voltammetry (ASV) was employed to determine whether tolmetin was interacting with Fe^{3+} .

10.4.1.1. Adsorptive Stripping Voltammetry

This useful technique exploits the natural tendency of analytes to pre-concentrate at an electrode and allows for the gauging of metal-ligand interactions. The electrode is polarised at a more negative potential than the reduction potential of the metal. This then allows the metal to pre-concentrate at the electrode bringing about a characteristic response at a specific potential, with the current being proportional to the amount of metal at the electrode. When a suitable ligand is added to the electrochemical cell, this facilitates the movement of the metal species to the electrode. Theoretically, a ligand that binds to the metal will bring about an increase in the current response as well as a shift in the potential due to the reduction of a new species at the electrode i.e. the metal-ligand complex. The affinity of the ligand to the metal is measured by the increase in the current response of the metal on the addition of the ligand. A decrease in the current response indicates a possible competition of the ligand for binding sites at the electrode or probably the formation of a strong metal-ligand bond which prevents the reduction of the metal upon scanning. A negative potential shift indicates a strong metal-ligand interaction, while a large positive shift is associated with a weaker metal-ligand interaction (Limson *et. al.* 1998; Lack & Nyokong 2001).

10.4.2. Materials and Methods

All chemicals and reagents were as per section 10.2.2.1.

10.4.2.1. Apparatus

Adsorptive stripping voltammograms (ASVs) were recorded on the Autolab PGSTAT 30 voltammeter equipped with a Metrohm VGA cell stand. A 3mm diameter glassy carbon

electrode (GCE) was employed as a working electrode for voltammetric experiments. A silver/silver chloride ($\text{KCl} = 3 \text{ M}$) and a platinum wire were employed as reference and auxiliary electrodes, respectively, for all electrochemical work. Prior to use and between scans, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in Milli-Q water.

For all experiments appropriate concentrations of the metal (Fe^{3+}) ($1 \times 10^{-5} \text{ M}$) and of tolmetin or sulindac ($1 \times 10^{-5} \text{ M}$ - $6 \times 10^{-5} \text{ M}$) were introduced into an electrochemical cell. The electrolyte used was 0.2 M tris-HCl buffer, pH 7.4.

10.4.2.2. Technique: Adsorptive Stripping Voltammetry

For adsorptive stripping experiments appropriate concentrations of Fe^{3+} and of tolmetin or sulindac were introduced into an electrochemical cell. The electrolyte used was 0.2M tris-HCl buffer, pH 7.4. The solution was then deaerated with nitrogen for 5 min, after which a flow of nitrogen was maintained over the solution throughout the measurement. Optimum deposition potential for each metal was identified and applied for 60 s to effect the formation and adsorption of the metal and ligand species onto the GCE. The voltammograms were then scanned in the negative direction from the deposition potential to at least 0.50V beyond the reduction of the metal at the scan rate of 0.10 V s^{-1} to strip the adsorbed metal-ligand species from the electrode. During the stripping step, current responses due to the reduction of the metal-ligand species were measured as a function of potential. All potential values quoted are referenced against the silver/silver chloride reference electrode.

10.4.3. Results

Upon the addition of sulindac ($1 \times 10^{-5} \text{ M}$) to the electrochemical cell containing $1 \times 10^{-5} \text{ M Fe}^{3+}$, a decrease in current response and a shift to a more negative potential was noticed as shown in figure 10.10. Increasing concentrations of sulindac (2×10^{-5} and $4 \times 10^{-5} \text{ M}$) demonstrates a sharp decrease in current response with little change in the potentials. The usual indication of an interaction between a ligand and a target metal would be observed with an increase in current strength of the metal reduction wave

coupled with a shift in potential towards more negative values. Here a decrease in current response upon addition of sulindac is observed with increasing concentrations. It is possible that sulindac binds Fe^{3+} to such an extent that upon the complexation and adsorption of the metal ligand complex, the metal is not easily released, hence the decrease in current strength observed. Further increases in sulindac concentration led to further decrease in current strength as less of the unbound iron became available for reduction.

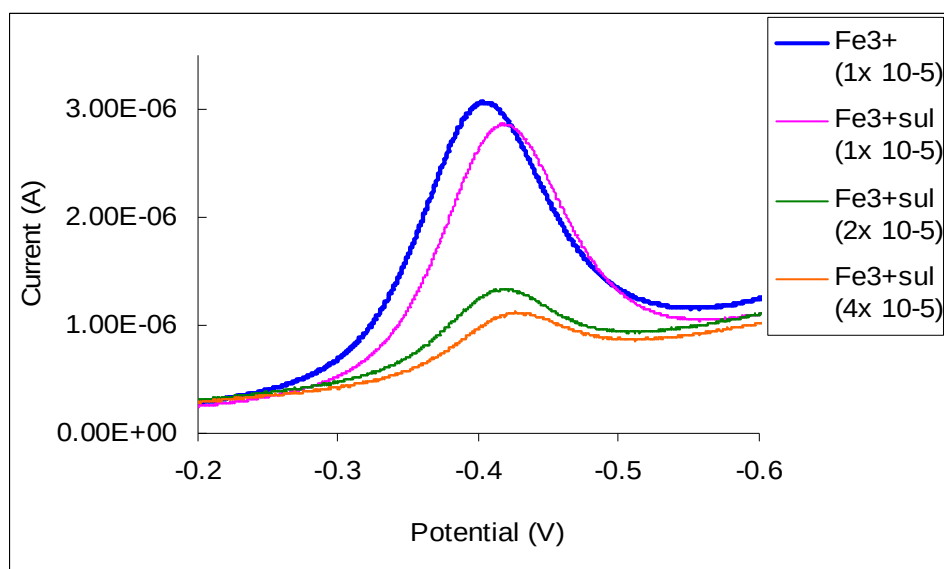


Figure 10.10: ASV of Fe^{3+} ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of sulindac.

The ASV for $1 \times 10^{-5}\text{M}$ Fe^{3+} in 0.2M tris HCl, pH 7.4 buffer shows a peak potential at -0.39V vs Ag/AgCl, as demonstrated in figure 10.11. Initially, upon the addition of tolmetin ($1 \times 10^{-5}\text{M}$), a rise in current and a shift to more negative potential is noticed. Thereafter, increasing concentrations of tolmetin (2×10^{-5} and $4 \times 10^{-5}\text{M}$) show a concentration dependent decrease in current responses (figure 10.11). The shift in potential towards more negative potentials upon addition of tolmetin to Fe^{3+} and slight increases in current strength is indicative of an interaction. However, the decrease in current with increased concentrations of tolmetin is ascribed to the slight stripping of bound complex from the electrode in the presence of excess ligand.

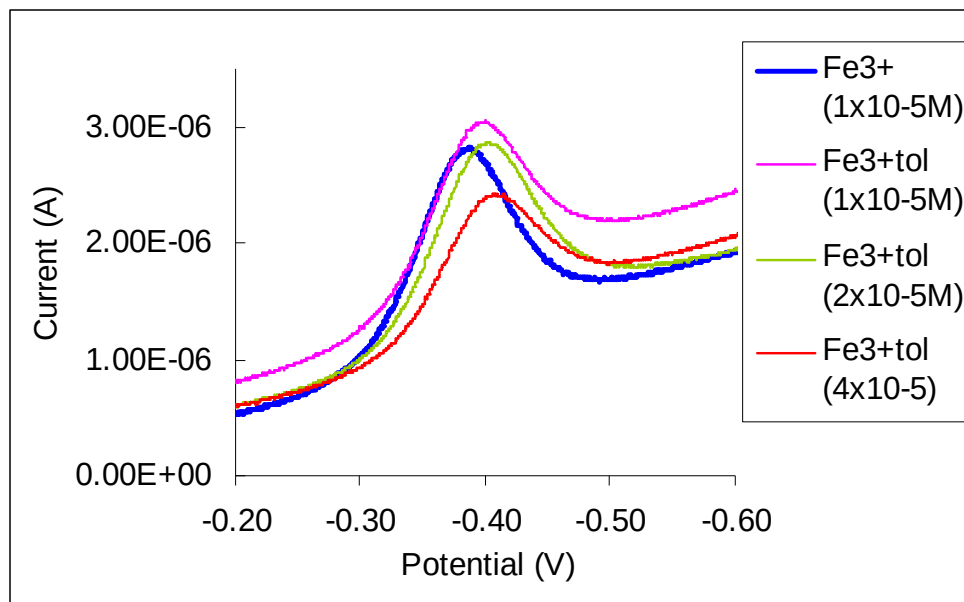


Figure 10.11: ASV of Fe^{3+} ($1 \times 10^{-5} \text{ M}$) alone and in the presence of increasing concentrations of tolmetin.

Coordination between Fe^{3+} and sulindac in solution is shown by a decrease in reduction potential of 0.014 V on addition of the ligand (figure 10.12). The concentration/current plot (figure 10.13) shows that in solution at metal-ligand ratios of 1:1 and 1:2 respectively, sulindac can bind with Fe^{3+} yielding two species of differing glassy carbon electrode adsorption characteristics. In contrast, coordination between Fe^{3+} and tolmetin in solution results in only one metal species, showing only a slight stripping of the complex from the electrode on addition of higher ligand concentrations. With a decrease in reduction potential of 0.013V on addition of the ligand, tolmetin has a similar affinity to Fe^{3+} to that of sulindac.

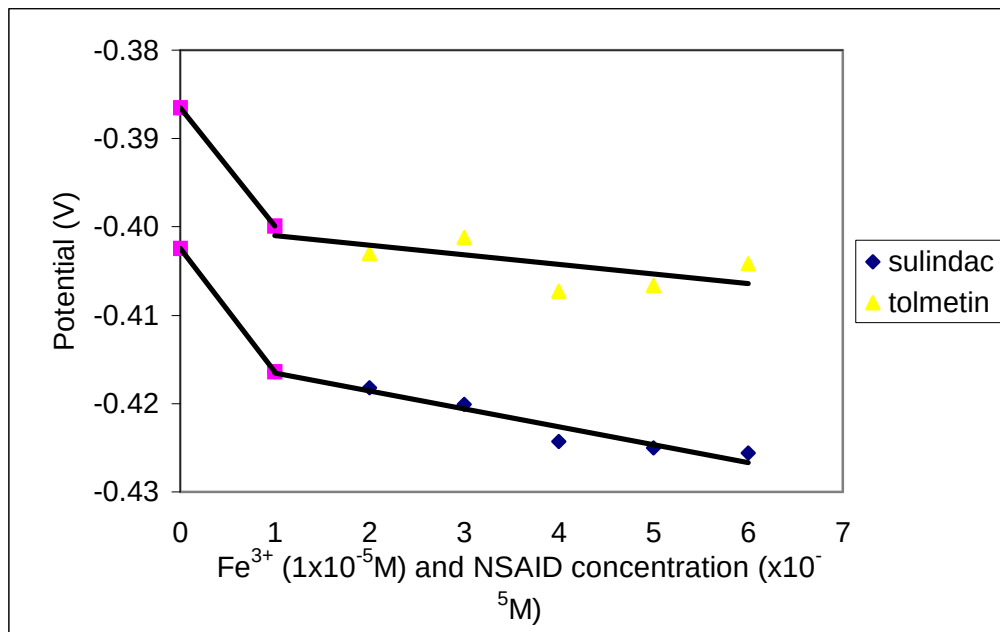


Figure 10.12: Effect of increasing concentrations of tolmetin and sulindac on the potential of $1 \times 10^{-5} \text{M Fe}^{3+}$.

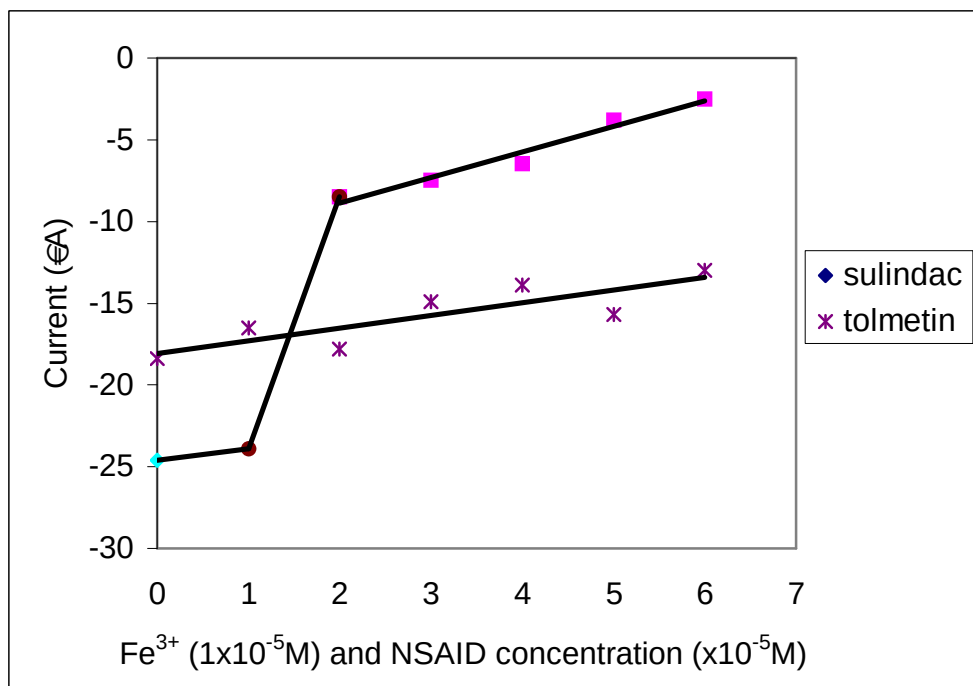


Figure 10.13: Effect of increasing concentrations of tolmetin and sulindac on the current of $1 \times 10^{-5} \text{M Fe}^{3+}$.

10.4.4. Discussion

NSAIDS have shown interaction with metals. Peterson and co-workers in 1979 demonstrated that indomethacin formed a complex with Fe^{2+} and prevented the oxidation of arachidonic acid. These authors also showed that tolmetin and ibuprofen also prevent Fe^{2+} -induced arachidonic acid oxidation and suggest that these agents inhibit the oxidation by possibly forming a complex with Fe^{2+} . Tolmetin and other NSAIDS such as ibuprofen, naproxen and indomethacin also form complexes with zinc, cadmium and platinum (Dendrino-Samara *et. al.*, 1998). The authors suggest that the NSAIDS coordinatively bind to the metal ions through the deprotonated carboxylate group. Another study by Etcheverry and co-workers in 2002 demonstrated that tolmetin, naproxen and ibuprofen form complexes with the vanadium (IV) cation.

This study confirms that tolmetin as well as sulindac interacts with iron. The two compounds show different pathways in that tolmetin is a hard donor ligand, while sulindac contains a soft donor site. In solution tolmetin preferentially binds with Fe^{3+} , while sulindac binds with both Fe^{2+} and Fe^{3+} . With Fe^{3+} tolmetin generates one species in solution while sulindac yields two. This reduces the amount of free iron available for the Fenton reaction and possibly reduces the amount of $\cdot\text{OH}$ radicals and therefore reduces Fe^{2+} -induced lipid peroxidation. The NSAIDS also bind to Fe^{3+} and this prevents the redox cycling of this metal and is also another possible mechanism through which these agents reduce the oxidative damage. These agents also reduce the complexation of QA to iron and also reduce QA-induced lipid peroxidation.

CHAPTER 11

THE SEPARATION, ISOLATION AND PURIFICATION OF THE CURCUMINOIDS

11.1. Introduction

The pharmacologic activities of turmeric have been attributed to the three curcuminoid pigments contained in ethanol extracts (Bong 2000; Jayaprakasha *et. al.*, 2002). These pigments include curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], demethoxycurcumin [1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] and bisdemethoxycurcumin [1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione].

A variety of methods, most being spectrophotometric expressing the total color content, have been employed for the quantification of curcuminoid pigments (ASTA 1985). Commercially available curcumin consists of a mixture of naturally occurring curcuminoids, of which curcumin is the main constituent (Ahsan *et. al.*, 1999; Jayaprakasha *et. al.*, 2002). The curcuminoids vary in structure and it is therefore possible that the chemical and color characteristics, as well as the functional properties will vary among the curcuminoids. Pure curcumin (CURC) is scarce and expensive, and demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) are not commercially available. It was thus important to obtain the pure pigments and to characterize each compound before proceeding onto the biological, and indeed any other, studies. The experiment describes the isolation and separation procedures that were conducted in order to obtain pure curcuminoids that were used throughout the study.

11.2. Materials and methods

11.2.1. Chemicals and Reagents

Curcumin (65%) pure and deuterated DMSO purchased from Sigma Chemicals Corporation, St Louis, MO, U.S.A. Semi-preparative aluminium thin layer

chromatography (TLC) plates coated with silica gel 60, Type F254 (0.25mm) were purchased from Merk, Darmstadt, Germany. Dichloromethane, methanol and acetonitrile (ACN) were purchased from was purchased from Saarchem, Johannesburg, South Africa. All other reagents were of the highest quality available.

11.2.2. Separation of the curcuminoids

Curcumin (65% pure), the starting material, was dissolved in dichloromethane to produce a 0.05g/ml solution. A total of 200µl of this solution was spotted in a line, 1.5cm above the baseline of the TLC plates. The line was dried using a gentle stream of nitrogen. The plates were placed in a saturated TLC tank containing mobile phase. Mobile phase consisted of dichloromethane: methanol in a ratio of 97:3. The plates were allowed to develop, and the plates were thereafter removed and dried using a gentle stream of nitrogen.

11.2.3 Isolation and extraction of the curcuminoids

The yellow bands that separated were cut off the plate using a pair of scissors and kept separately. The silica gel of each strip was scraped and stored. The curcuminoids were re-extracted from the silica using dichloromethane. To each flask containing the silica powder, approximately 40ml of dichloromethane was added and the flask was sonicated for 2 minutes. The flask was allowed to stand for 10 minutes, to allow the silica particles to settle, and the bright yellow supernatant was decanted into another flask. This extraction of the curcuminoids from the silica gel was conducted another 3 times to maximise the yield. The resulting supernatants were combined and centrifuged for 15 minutes at 3000g. The supernatant was gently removed and injected through a 0.45µm membrane filter to remove any solid particles. The dichloromethane was evaporated using an N-EVAP analytical evaporator at 40°C. The yellow residue that remained was the curcuminoids.

11.2.4. Determination of the purity of the curcuminoids (TLC)

A few milligrams of each curcuminoid was dissolved in deuterated DMSO and analysed by NMR for purity (section 11.2.5). Curcuminoids (0.03mM each) were dissolved in

ethanol (50% final concentration) and were used for metal binding studies. The Lambda max of each curcuminoid was also obtained to provide an indication of the purity of the curcuminoid. In addition, a small sample of each curcuminoid was dissolved in dichloromethane and spotted on a TLC plate. The plate was developed as described above. The spots on the plates were viewed at 365nm and photographed using a Sony Cyber Shot digital camera.

11.2.5. Determination of the purity of the curcuminoids (NMR)

Unless otherwise stated, the experimental procedures followed were carried out as follows. The ^1H (400 MHz), ^{13}C (100 MHz), DEPT135, COSY, HMQC and HMBC NMR spectra were recorded on an Avance Bruker 400 MHz spectrometer using standard pulse sequences. Chemical shifts are reported in ppm and referenced to the residual undeuterated solvent resonances (DMSO δ_{H} 2.50, δ_{C} 39.43) and the coupling constants are reported in Hz.

11.2.6. Determination of the purity of the curcuminoids (mass spectrometry)

Analysis of the curcuminoids was also carried out by LC-MS. The HPLC system consisted of a spectrasystem AS300 autosampler, a spectrasystem UV200 detector and a spectrasystem P2000 pump. The isocratic mobile phase consisted 37% ACN and 0.1% formic acid. Separation was achieved using a Waters $\mu\text{Bondipak}^{\text{TM}}$ C_{18} reverse phase column (4.6 x 250mm) and a flow rate set at 1ml/min. The LC instrument was interfaced with a Finnigan MAT LCQ mass spectrometer equipped with an electrospray ionization (ESI) source. Conditions on the LC-MS included: positive ion mode, capillary temperature 240 °C, capillary voltage 14 V, discharge voltage 4.5 kV, tube lens offset 55 V, discharge current 80 μA , octapole 1 offset -2.0 V, octapole 2 offset -8.5 V, interoctapole lens voltage -20.0 V, trap DC offset voltage -10.0 V, with the sheath and auxiliary gasses set to 80 and 20 arbitrary units.

11.3. Results

Commercial curcumin contains approximately 77% CURC, 17% DMC and 3% BDMC (Huang *et. al.*, 1995). Figure 11.1 shows that the mobile phase, dichloromethane:

methanol (97:3) separated the curcuminoids into 3 major bands. The R_f values of the curcuminoids were calculated with curcumin having the greatest R_f value of 0.83, followed by demethoxycurcumin, 0.61 and bisdemethoxycurcumin 0.5. The separated products were pure when compared to the starting material (figure 11.2). Each curcuminoid displayed a single spot and had the same R_f value of the curcuminoid in the starting material. The lambda max obtained for CURC, DMC and BDMC were 430, 422 and 420nm respectively (chapter 13, figures 13.1; 13.2 and 13.3). Infrared spectra of each curcuminoid can also be found in chapter 13.

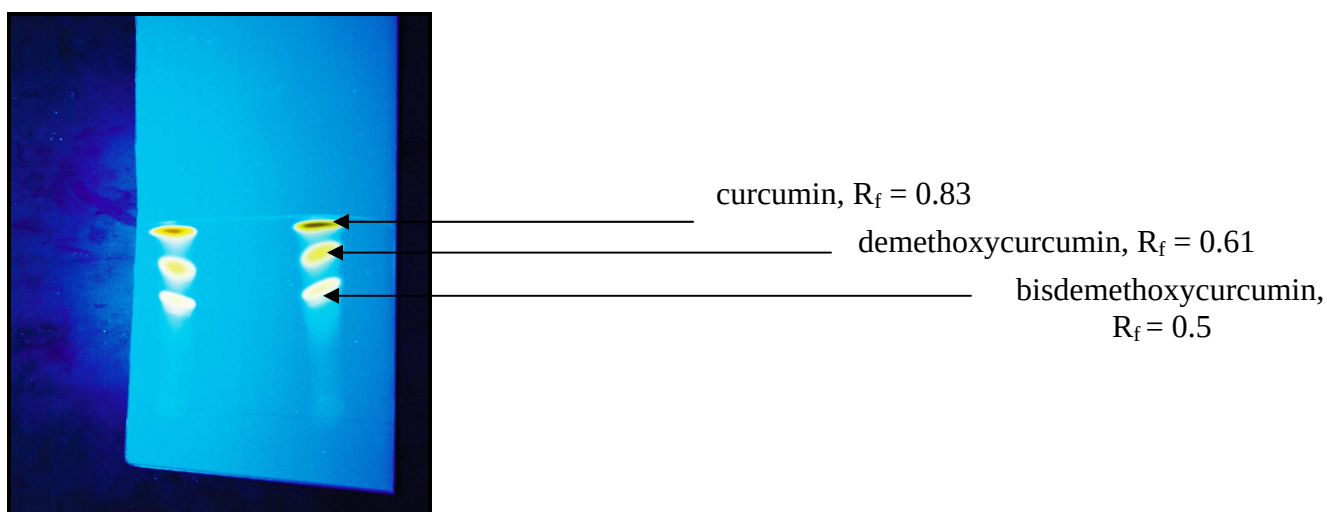


Figure 11.1: The 3 major curcuminoids within curcumin (65%). TLC plates were viewed at 365nm and photographed using a Sony Cyber Shot digital camera.

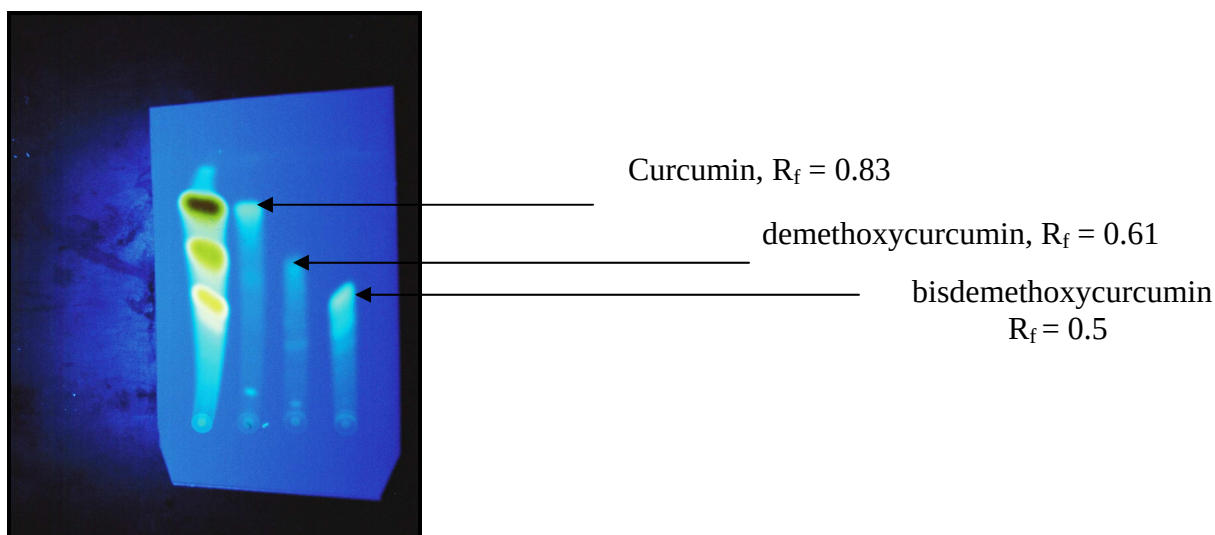


Figure 11.2: TLC plate showing the purity of each curcuminoid that was separated, isolated and extracted from curcumin (65%).

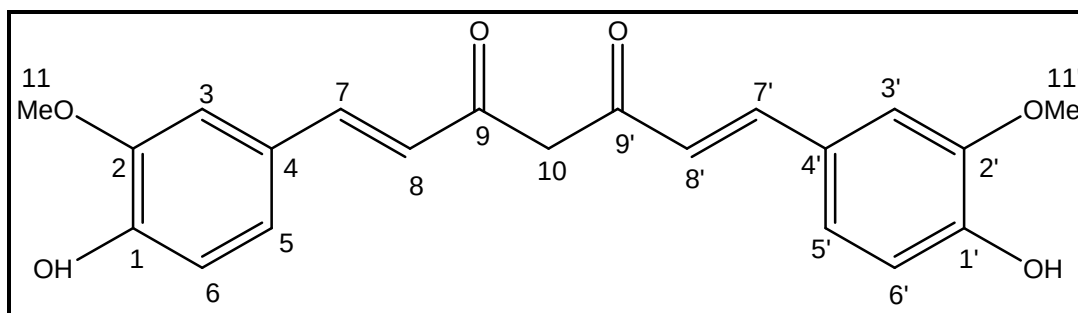


Figure 11.3: Structure of curcumin: ^1H (DMSO- d_6 , 400 MHz) δ 3.83 (6H, s, H-11/H-11'); 4.13 (2H, m, H-10); 6.75 (2H, d, $J = 15.7$ Hz, H-8, H-8'); 6.82 (2H, d, $J = 7.3$ Hz, H-6/H-6'); 7.15 (2H, d, $J = 7.3$ Hz, H-5/H-5'); 7.31 (2H, s, H-3/H-3'); 7.54 (2H, d, $J = 15.7$ Hz, H-7/H-7'); 9.70 (2H, br s, OH).

^{13}C (DMSO- d_6 , 100 MHz) δ 55.6 (C-11/C-11', q); 67.4 (C-10, t); 111.3 (C-3/C-3'); 115.7 (C-6/C-6', d); 121.0 (C-8, d); 123.1 (C-5/C-5', d); 126.3 (C-4/C-4', s); 140.7 (C-7/C-7', d); 147.9 (C-1/C-1', s); 149.3 (C-2/C-2', s); 183.2 (C-9/C-9', s). Low resolution ESI-MS m/z [M+1] 369.4 ($\text{C}_{21}\text{H}_{20}\text{O}_6$).

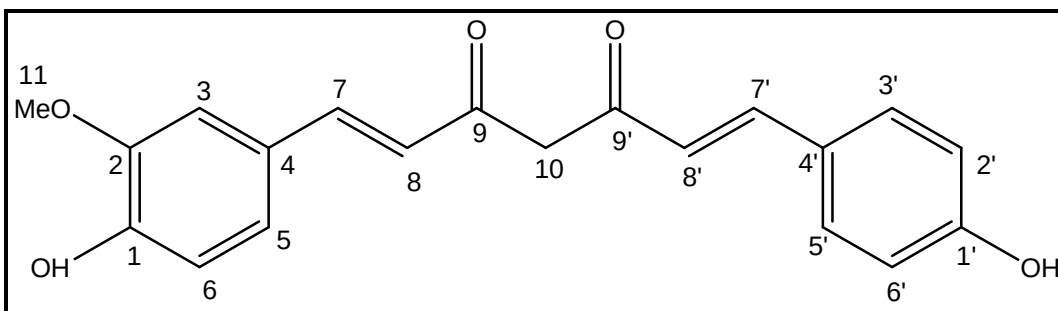


Figure 11.4: Structure of demethoxycurcumin: ^1H (DMSO- d_6 , 400 MHz) δ 3.84 (3H, s, H-11'); 4.13 (2H, m, H-10); 6.69 (1H, d, $J = 15.9$ Hz, H-8'); 6.75 (1H, d, $J = 15.9$ Hz, H-8); 6.83 (2H, d, $J = 8.1$ Hz, H-2'/H-6'); 7.15 (1H, d, $J = 8.3$ Hz, H-5); 7.32 (1H, s, H-3); 7.54 (2H, m, H-7/H-7'); 7.56 (2H, d $J = 8.1$ Hz, H-3'/H-5').

^{13}C (DMSO- d_6 , 100 MHz) δ 55.7 (C-11, q); 67.4 (C-10, t); 111.3 (C-3); 115.9 (C-2'/C-6, C-6', d); 120.8 (C-8'); 121.0 (C-8, d); 123.2 (C-5, d); 125.8 (C-4', s); 126.3 (C-4, s); 140.3 (C-7', d); 140.7 (C-7, d); 147.9 (C-1, s); 149.3 (C-2, s); 159.8 (C-1'); 183.1 (C-9, s); 183.3 (C-9', s). Low resolution ESI-MS m/z [M+1] 339.4 ($\text{C}_{20}\text{H}_{18}\text{O}_5$).

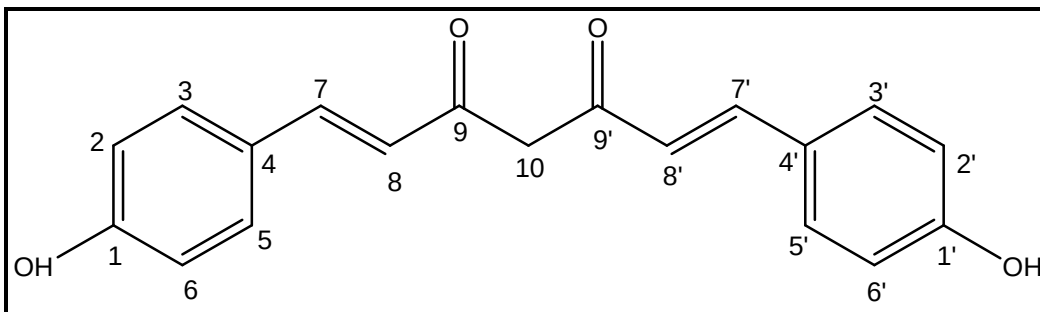


Figure 11.5: Structure of bisdemethoxycurcumin: ^1H (DMSO- d_6 , 400 MHz), *(2H, s, H-10); 6.86 (2H, d, $J = 15.9$ Hz, H-8, H-8'); 6.82 (2H, d, $J = 8.6$ Hz, H-2/H-6/H-2'/H-6'); 7.54 (2H, d, $J = 15.9$ Hz, H-7, H-7'); 7.56 (2H, d, $J = 8.6$ Hz, H-3/H-5/H-3'/H-5'); 10.07 (1H, br s, OH).*(^1H chemical shift for H-10 was not observed for this particular sample).

^{13}C (DMSO- d_6 , 100 MHz) δ 115.9 (C-2/C-6/C-2'/C-6', d); 120.8 (C-8, d); 125.8 (C-4/C-4', s); 130.3 (C-3/C-5/C-3'/C-5', d); 140.3 (C-7/C-7', d); 159.8 (C-1/C-1', s); 183.2 (C-9/C-9', s). The ^{13}C chemical shift for C-10 (t) was not observed for this particular sample. Low resolution ESI-MS m/z [M+1] 309.3 ($\text{C}_{19}\text{H}_{16}\text{O}_4$).

Due to tautomerism, it is difficult to state, unequivocally, the shifts of some of the carbons viz C-10.

11.4. Discussion

Several studies have employed TLC and column chromatography to separate the curcuminoids. The stationary phase most often used was silica gel 60G with different solvent systems such as benzene, ethyl acetate, ethanol, chloroform, acetic acid, hexane and methanol for TLC (Janaki & Bose 1967; Krishnamurthy *et al.*, 1976; Govindarajan 1980) while benzene, water, toluene and ethyl acetate have been used for column chromatography (Srinivasan *et al.*, 1992).

This study shows that a mobile phase consisting of dichloromethane: methanol (97:3), separates the curcuminoids effectively. The lambda max of each curcuminoid is consistent when compared to the literature (Peret-Almeida *et al.*, 2005). The ^{13}C NMR shifts observed at 55.6 and 149.3 ppm, indicative of a methoxy group (C-11/C-11') and an sp^2 carbon atom (C2/C2') attached to an oxygen atom respectively, reveals the presence of curcumin. Curcumin is a symmetrical molecule, thus only half of the signals in the ^1H and ^{13}C NMR spectra were expected to be observed. In contrast, demethoxycurcumin is not a symmetrical molecule and chemical shifts for all carbon atoms were to be observed in the ^{13}C NMR spectra. The relevant shifts of C-11 and C-2 ($\delta 55.7$ and $\delta 149.3$) were observed as for curcumin, but the shift of C-2' was found to be 115.9 ppm, indicative of a double bonded carbon atom with no oxygen atom attached. Bisdemethoxycurcumin, like curcumin is a symmetrical molecule. The methoxy carbon signals present at 55.7 ppm in the carbon spectrum of curcumin are distinctly absent in the NMR spectrum of bisdemethoxycurcumin. The ^{13}C shift of C-2/C-2' present characteristically in curcumin at $\delta 149.3$ is found to be at $\delta 115.9$ in bisdemethoxycurcumin, indicating a non-oxygenated sp^2 carbon atom *i.e.* a carbon atom not attached to an oxygen atom.

The ESI-MS data gave molecular ions at 369.4; 339.4 and 309.3 amu for CURC, DMC and BDMC respectively. The consistent decrease in each spectrum is due to the successive loss of a methoxyl for each of the curcuminoid analogues.

The results indicate that the separation was achieved with pure curcuminoid compounds.

CHAPTER 12

EFFECT OF CURCUMINOIDS ON LEAD-INDUCED NEUROTOXICITY

12.1. INTRODUCTION

Metals play an important role in neural toxicity. Research has shown the involvement of metals in neurodegenerative disorders such as AD, prion disease, mitochondrial disorders, Wilson's and Parkinson's disease (Bush 2000). The heavy metal lead (Pb) is known to be toxic even in low concentrations and exerts extensive damage to the brain, causing severe learning and memory disabilities. Lead has been characterized by the US surgeon general as "one of the greatest environmental threats in America." This was based on the observation that 1 in 20 children under the age of 6 exhibited blood Pb levels greater than 10µg/dl (Satcher 2000). It has been shown that children with these blood Pb levels exhibit deficits in cognitive function and behavior (Banks *et. al.*, 1997). This heavy metal has also been shown to induce oxidative damage (Monteiro *et. al.*, 1995). Recent studies show that lead may be toxic through the disruption of the delicate prooxidant/antioxidant balance that exists within mammalian cells (Monteiro *et. al.*, 1995). *In vivo* studies suggest that Pb is capable of generating reactive oxygen species and so altering the antioxidant defense systems in animals (Lawton & Donaldson 1991).

12.2. EFFECT OF LEAD ON LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS

12.2.1. Introduction

Heavy metals such as Pb and cadmium have shown to induce lipid peroxidation in various tissue (Lawton & Donaldson 1991; Monteiro *et. al.*, 1995; Stohs & Bagchi 1995, Daniel *et. al.*, 2004). This experiment was conducted to determine whether Pb could induce lipid peroxidation in primary hippocampal neurons.

12.2.2. Materials and Methods

12.2.2.1. Chemicals and Reagents

Lead acetate (Pb) was purchased from Sigma Chemicals Corporation, St Louis, MO, U.S.A. All other reagents used for the routine cell culture work was as per section 2.4.2.1. Reagents for the lipid peroxidation assay were as per section 2.2.2.1.

12.2.2.2. Lipid peroxidation assay

Primary hippocampal neurons were established as described in section 2.4.2.5.

Six to eight day old cultures were used for the experiments. Before experimentation, each flask was washed twice with 3ml of PBS. Thereafter 1ml of fresh MEM was added. To this, 100µl of Pb dissolved in PBS was added to produce final concentrations of 5 and 10µM. The culture flasks were then kept in the incubator and the lipid peroxidation assay was performed every 1 hour up to and including those cells that were incubated for 3 hours.

After the respective times, the cells were scraped off the surface of the culture flasks using a cell scraper and transferred to test tubes. The rest of the assay was performed as described in section 2.4.2.6. Final results are represented as nmoles MDA/10⁶ cells and were analysed as described in section 2.2.2.7. The experiment was replicated three times.

12.2.3. Results

Lead at 5 and 10 μ M does not induce lipid peroxidation in primary hippocampal neurons after 1 hour (figure 12.1). However, after 2 and 3 hours there is a concentration dependent increase in lipid peroxidation and more MDA is produced with a longer exposure of the cells to lead.

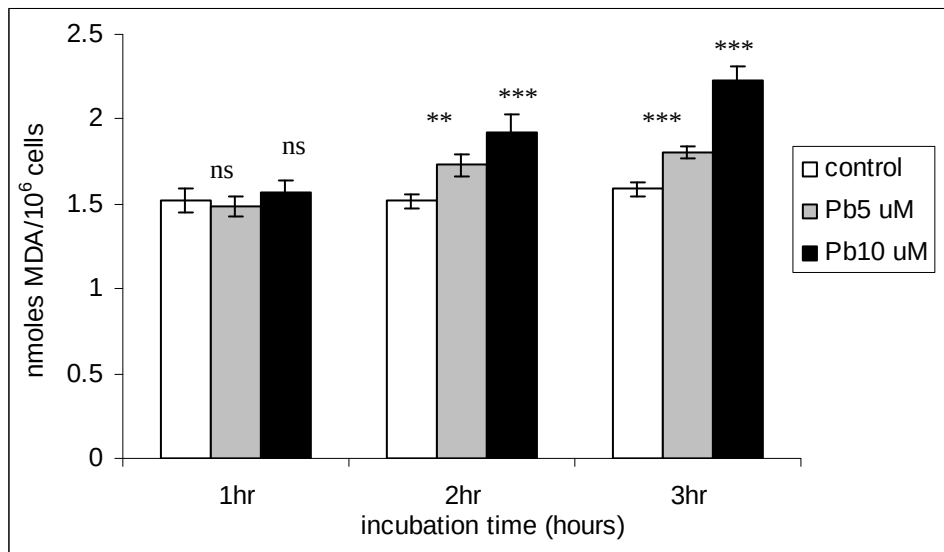


Figure 12.1: Effect of lipid peroxidation in primary hippocampal neurons exposed to lead (5 and 10 μ M) over a period of time. Each bar represents the mean $\pm$ SD (n=3). ns(p>0.05), ** (p<0.01) and *** (p<0.001) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

12.2.4. Discussion

It has been shown that Pb toxicity occurs due to an interaction between iron, zinc and lead. Lead, iron and zinc are postulated to interact with the same proteins, possibly transport proteins, which mediate metal uptake. (Bressler *et. al.*, 1999). Lead ions may directly or indirectly interfere with the integrity of membranes by reacting with sulphhydryl groups and lipids and displacing metals (Nakagawa *et. al.*, 1980). Pb has also shown to potentiate O₂ toxicity in rats (Kiesow 1997), to stimulate oxidative haemolysis of erythrocytes (Gelman *et. al.*, 1978), to decrease erythrocyte superoxide dismutase

activity (Gelman *et. al.*, 1978) and to accelerate the conversion of oxyhaemoglobin to methaemoglobin which results in the production of superoxide and hydrogen peroxide (Ribarov & Bochev 1982). Pb has shown to accelerate the peroxidation of liposomes in the presence of haemoglobin (Ribarov *et. al.*, 1981) and enhance Fe^{2+} initiated lipid peroxidation (Quinlan *et. al.*, 1988). This heavy metal has shown to induce lipid peroxidation in rat brain homogenate (Daniel *et. al.*, 2004). This experiment demonstrates that Pb induces lipid peroxidation in primary hippocampal neurons. Pb does not induce lipid peroxidation after incubating the neurons for 1 hour with either 5 or 10 μM PB. This is possibly because the incubation time was too short and therefore an incubation time of 3 hours was used for all future experiments.

12.3. EFFECT OF CURCUMINOIDS ON LEAD-INDUCED LIPID PEROXIDATION IN PRIMARY HIPPOCAMPAL NEURONS

12.3.1. Introduction

Given the toxicity of free metal species in the brain, the ability of ligands to sequester and bind these metals in a form in which these metals no longer cause neurotoxicity, and/or scavenge the free radicals that may be generated has been the focus in research. The previous experiment demonstrates that Pb induces lipid peroxidation in primary hippocampal neurons. This experiment was conducted to determine whether the curcuminoids could prevent or reduce the Pb-induced lipid peroxidation.

12.3.2 Chemicals and reagents

All chemicals and reagents are as per section 12.2.2.1. The curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) were dissolved in ethanol and made up to volume with PBS. The final concentration of ethanol in the lipid peroxidation experiment was less than 5%. Routine cell culture work was conducted as described in section 2.4.2.2.

12.3.2.1. Lipid peroxidation assay

Experiments were conducted in a similar manner as described in experiment 12.2.2.2. Pb (10 μ M) was used to induce lipid peroxidation and the hippocampal neurons were incubated for 3 hours only. The curcuminoids were also added to the incubation medium to produce final concentrations of 50, 100 and 150 μ M. Controls were free of lead and the curcuminoids but consisted of the same concentration of ethanol as the curcuminoid treated cells. Results were analysed as described in section 2.2.2.7. The experiment was replicated four times.

12.3.3. Results

The curcuminoids show a concentration-dependent reduction in LP (figure 12.2) initiated by Pb (10 μ M). BDMC does not reduce the Pb-induced lipid peroxidation in the primary

hippocampal neurons at 50µM but did so at the higher concentrations. Curcumin reduces lipid peroxidation the most, even at low concentrations.

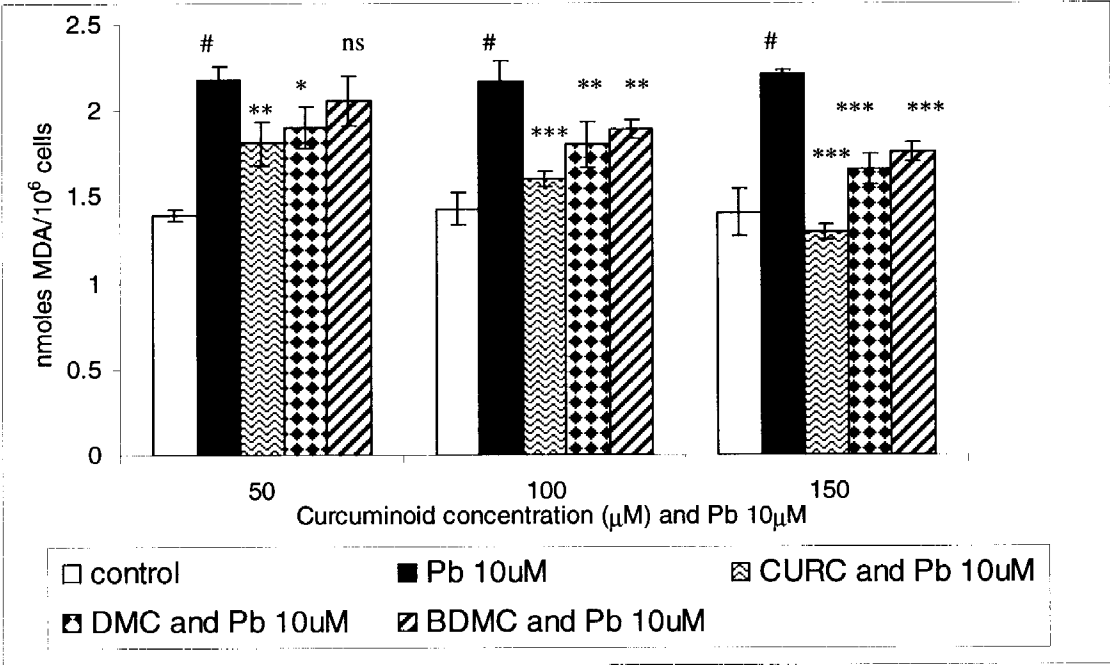


Figure 12.2: Effect of the curcuminoids on Pb(10µM)-induced lipid peroxidation in primary hippocampal neurons. Each bar represents the mean $\pm$ SD (n=4). # (p<0.001) compared to control; ns (p>0.05), * (p<0.05), ** (p<0.01) and *** (p<0.001) in comparison to Pb only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

12.3.4. Discussion

It has been reported that curcumin is a more potent free radical scavenger than vitamin E (Zhao *et. al.*, 1989). Daniel *et. al.* (2004) demonstrated that curcumin reduces Pb-induced lipid peroxidation in rat brain homogenate. Studies have shown that curcumin is a powerful scavenger of the superoxide anion (Kunchandy & Rao 1990), the hydroxyl radical (Kunchandy & Rao 1989), nitrogen dioxide (Unnikrishan & Rao 1995) and it also protects DNA against singlet-oxygen-induced strand breaks (Subramanian *et. al.*, 1994) and lipids from peroxidation (Sreejayan & Rao 1993). It has been thought that the methoxy group on the phenyl ring is one of the functional groups important for this

phenolic compound's antioxidant property (Sreejayan & Rao 1996). This experiment demonstrates that CURC with two methoxy groups has shown to be the most powerful antioxidant while BDMC with no methoxy groups demonstrates to be the weakest antioxidant. Some authors have shown that the donation of a hydrogen atom from the β -diketone moiety to a free radical such as the lipid alkyl or lipid peroxy radical (figure 12.3) is also important for this molecule's antioxidant property (Jovanovic *et. al.*, 2001).

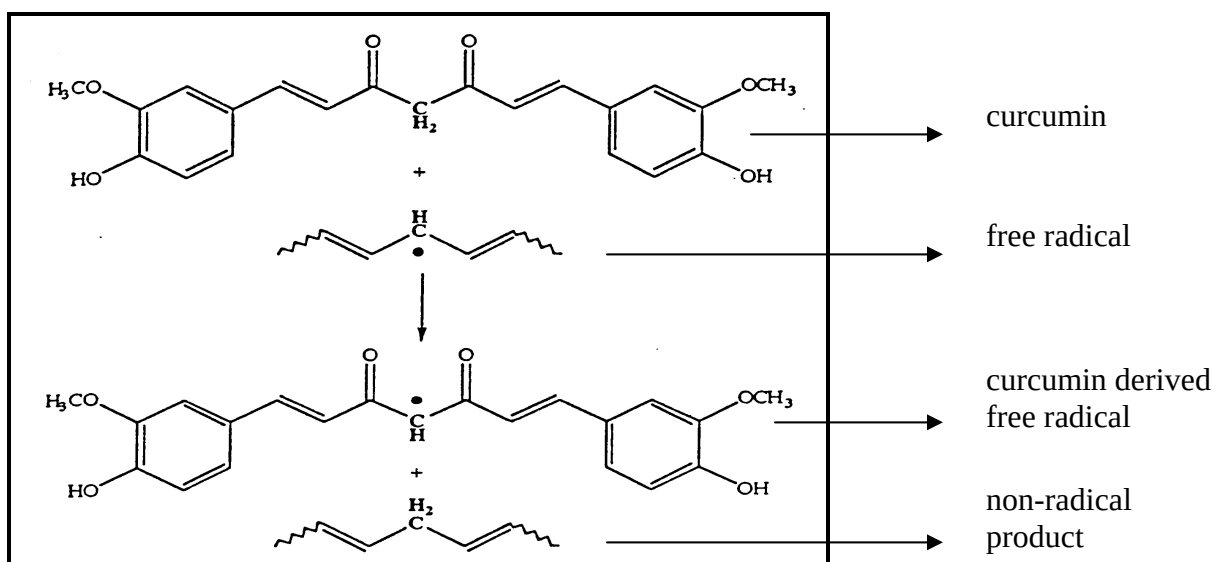


Figure 12.3: Donation of a hydrogen atom from the β -diketone moiety of curcumin to a bis-allylic radical (Jovanovic *et. al.*, 2001).

After the β -diketone moiety donates a hydrogen atom, this results in the formation of a curcumin radical with unpaired electron density between the two oxygen atoms. It is unlikely that this central carbon is oxygenated since this is an unfavorable reaction and the peroxy radical will be formed and this radical itself propagates lipid peroxidation. However, it is possible that the curcumin radicals may react with each other or other radicals and generate stable products such as vanillin, ferrulic acid and curcumin dimers (Masuda *et. al.*, 1999). The main radical that is formed is the phenoxyl radical. (Jovanovic *et. al.*, 2001). Curcumin is a potent lipid-soluble antioxidant and penetrates deep into the cell membrane where it scavenges free radicals and thereby converts itself into the phenoxyl radical (Jovanovic *et. al.*, 2001). Since this radical is more polar than

curcumin, it can move to the membrane's surface where it could be "repaired" by water soluble antioxidants (figure 12.4).

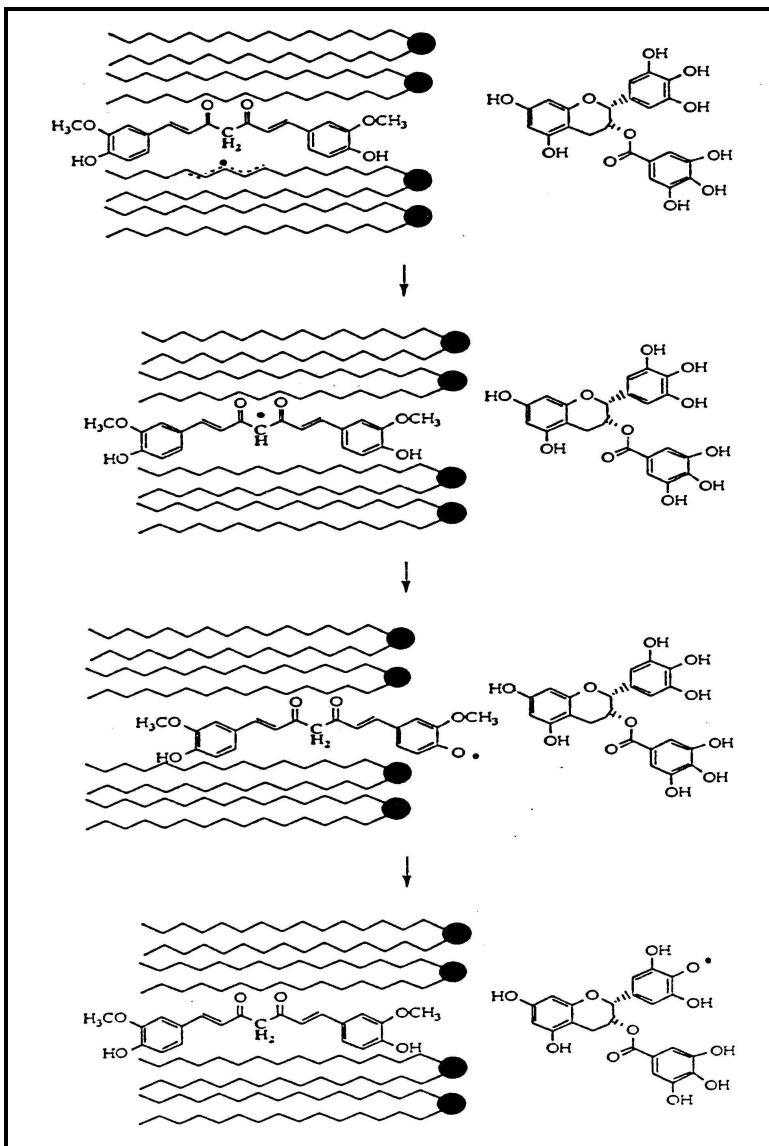


Figure 12.4: Illustrates curcumin's antioxidant activity deep within the cell membrane (Jovanovic *et. al.*, 1999).

Therefore the curcuminoids most likely reduce the Pb-induced lipid peroxidation by scavenging the free radicals that were generated. Another possible mechanism that may be involved is that the curcuminoids have a direct effect on Pb. The curcuminoids possibly bind to Pb and form a complex, thus inactivating and reducing the toxicity of this metal. This has been investigated in chapter 13.

12.4. EFFECT OF LEAD ON NEURONAL CELL VIABILITY

12.4.1. Introduction

Experiment 12.2 demonstrates that Pb induced lipid peroxidation in primary hippocampal neurons. This experiment was conducted to determine the effect Pb has on the neuronal cell viability. The concentrations of Pb and incubation times were exactly the same as described in experiment 12.2.

12.4.2. Materials and Methods

All reagents used were as per section 2.4.2.1. and 4.5.2. Routine cell culture work was conducted as described in section 2.4.2.2.

12.4.2.1. The MTT assay

Primary hippocampal neurons were established as described in section 2.4.2.5.

The cells were exposed to Pb as described in section 12.2.2.2. Six to eight day old cultures were used for the experiments. Before experimentation, each flask was washed twice with 3ml of PBS. Thereafter 1ml of fresh MEM was added. To this, 100 μ l of Pb dissolved in MEM was added to produce final concentrations of 5 and 10 μ M. The culture flasks were then kept in the incubator and the cell viability was assessed every 1 hour up to and including those cells that were incubated for 3 hours.

After the respective incubation times, the cells were gently washed thrice with 3ml of PBS and 1ml of fresh MEM was added. The cells were loaded with 0.5ml of MTT dye at a final concentration of 1mg/ml. The rest of the assay was conducted as described in section 4.2.2. The absorbance was read at 570nm. The results were expressed as % viability/ 10^6 cells. The controls (no Pb) were taken as 100% viable. Results were analysed as described in section 2.2.2.7. The experiment was replicated three times.

12.4.3. Results

Figure 12.5 demonstrates the ability of Pb to significantly ($p < 0.001$) reduce cell viability. Lead at 5 and 10 μM does not reduce cell viability in a concentration dependent manner. However, cell viability decreases with an increase in the time that the neurons were exposed to Pb.

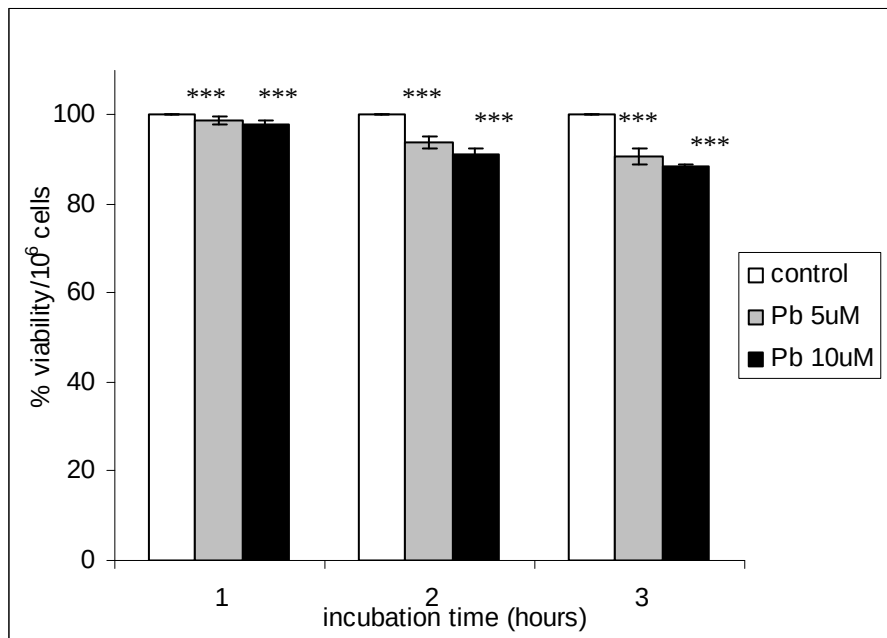


Figure 12.5: Primary hippocampal neuron viability after exposure to lead (5 and 10 μM) over a period of time. Each bar represents the mean $\pm$ SD ($n=3$). ***($p < 0.001$) in comparison to control. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

12.4.4. Discussion

It has been thought that Pb exerts its toxicity by disrupting calcium-dependent mechanisms. Pb and calcium compete for the same binding sites on proteins that belong to a large family of ion binding proteins and lead often substitutes for calcium in such proteins (Habermann *et. al.*, 1983). Evidence exists to support the hypothesis that calcium signaling is altered in Pb exposed rats (Chen *et. al.*, 1999; Nihei *et. al.*, 2001). It has been shown that Ca^{2+} influx during glutamate insults, results in excitotoxicity (Rothman & Olney 1987; Vergun *et. al.*, 1999). Prolonged Ca^{2+} elevations have been correlated with

cell death (Dubinsky 1993; Cheng *et. al.*, 1999). Increased intracellular Ca^{2+} concentrations promote cell damage by both activating destructive enzymes and increasing the formation of reactive oxygen species (Vergun *et. al.*, 1999; Orrenius *et. al.*, 1989; Siesjo & Bengtsson 1989). Pb may have displaced calcium at its binding sites and induced ROS production, which would explain the increase in lipid peroxidation, and also decreased cell viability.

12.5. EFFECT OF CURCUMINOIDS ON NEURONAL CELL VIABILITY IN THE PRESENCE OF LEAD

12.5.1. Introduction

The previous experiment demonstrates that Pb significantly ($p < 0.001$) reduces cell viability. Experiment 12.3 shows that the curcuminoids reduce the Pb-induced lipid peroxidation in primary hippocampal neurons. Therefore, this experiment was conducted to determine whether the curcuminoids could enhance cell viability in the presence of Pb.

12.5.2. Materials and Methods

All chemicals and reagents were as per section 12.4.2. The curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) were dissolved in ethanol and made up to volume with PBS. The final concentration of ethanol in the lipid peroxidation experiment was less than 5%. Routine cell culture work was conducted as described in section 2.4.2.2.

12.5.2.1. The MTT assay

Experiments were conducted in a similar manner as described in experiment 12.4.2.1. 10 μ M of Pb was used to reduce cell viability and the hippocampal neurons were incubated for 3 hours only. The curcuminoids were also added to the incubation medium to produce final concentrations of 50, 100 and 150 μ M. Controls were free of lead and the curcuminoids but consisted of the same concentration of ethanol as the curcuminoid treated cells.

After incubation, the cells were gently washed thrice with 3ml of PBS and 1ml of fresh MEM was added. The cells were loaded with 0.5ml of MTT dye at a final concentration of 1mg/ml. The rest of the assay was conducted as described in section 4.5.2.1. The absorbance was read at 570nm. The results were expressed as % viability/ 10^6 cells. The controls were taken as 100% viable. Results were analysed as described in section 2.2.2.7. The experiment was replicated four times.

12.5.3. Results

Figure 12.6 demonstrates the curcuminoids potential to increase cell viability that was decreased by Pb (10 μ M). DMC and BDMC did not increase cell viability at 50 μ M but did so at higher concentrations. Curcumin increases the cell viability at all concentrations.

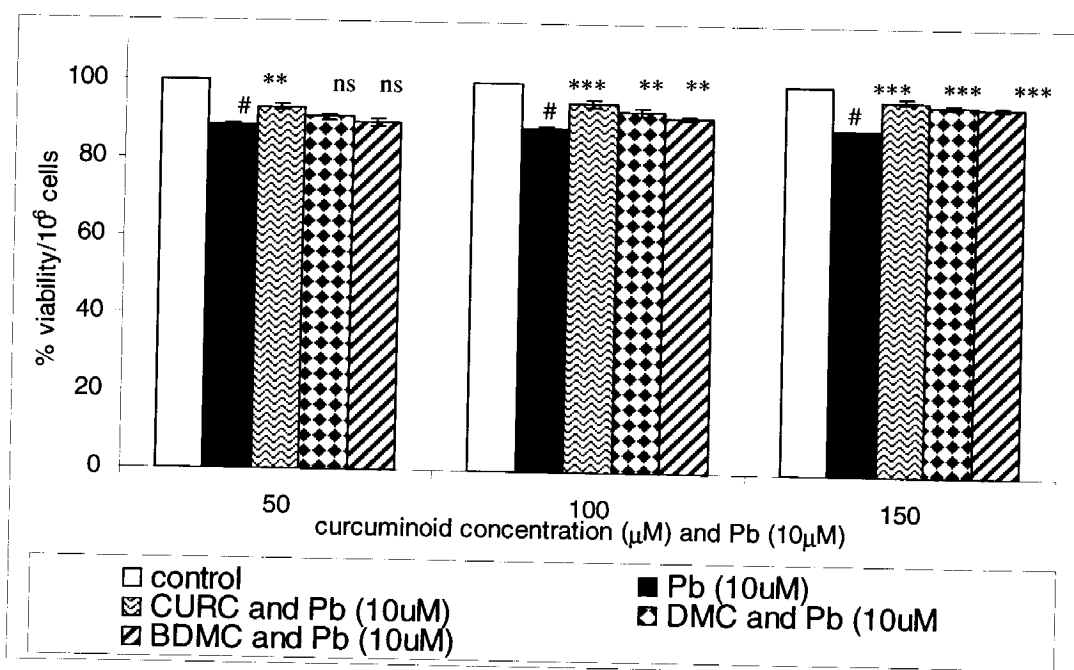


Figure 12.6: Effect of the curcuminoids on hippocampal neuron viability in the presence of Pb(10 μ M). Each bar represents the mean $\pm$ SD (n=4). # (p < 0.001) compared to control; ns (p > 0.05), ** (p < 0.01) and *** (p < 0.001) in comparison to Pb only. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

12.5.4. Discussion

It has been discussed Pb could induce lipid peroxidation and reduce cell viability through the displacement of Ca²⁺ from its binding sites (Habermann *et. al.*, 1983). Increased intracellular Ca²⁺ concentrations are known to induce ROS formation (Choi 1992; Dykens 1994). It has been shown that ROS reduce cell viability (Black *et. al.*, 1995; Park *et. al.*, 2004) and thus the co-incubation of neurons with antioxidants increases cell viability (Park *et. al.*, 2004). The curcuminoids have been shown to reduce lipid

peroxidation and it is established that this is due to their potent free radical scavenging properties (Kunchandy & Rao 1990; Subramanian *et. al.*, 1994; Unnikrishan & Rao 1995). However, DMC and BDMC, both fail at low concentrations to enhance cell viability. BDMC also fails to reduce lipid peroxidation at low concentrations. This suggests that these agents are not as potent free radical scavengers when compared to CURC at the same concentrations. The curcuminoids can scavenge the free radicals formed and thus enhance cell viability. As mentioned earlier, the curcuminoids possibly interact with Pb and reduce free Pb levels reducing the toxic effects of this heavy metal.

12.6. EFFECT OF LEAD AND THE CURCUMINOIDS ON RAT SPATIAL REFERENCE MEMORY

12.6.1. Introduction

The toxic effects of Pb exposure on hippocampal long-term potentiation (LTP) were reported in the early 1990's. LTP is a form of synaptic plasticity that is believed to form the cellular basis for learning and memory in the mammalian brain (Malenka & Nicoll 1999). Among synaptic processes that are NMDA receptor dependent, LTP provides an important model of learning and memory. LTP was described in the hippocampus as a long-lasting increase in synaptic efficacy after brief periods of stimulation of certain synapses (Bliss & Lomo 1973). In the hippocampus, the CA1 and dentate gyrus synapses have been the most extensively studied (Teyler & DiScenna 1987; Madison *et. al.*, 1991; Malenka & Nicoll 1999), although LTP has also been studied in other regions of brain (Tsumoto 1992). The hippocampus is one region in the brain that is responsible for the acquisition and consolidation of some forms of learning and memory, therefore damage to this region may result in learning and memory deficits. It has been shown that Pb exposed rats exhibit deficits in hippocampal LTP (Altmann *et. al.*, 1993; Lasley *et al.*, 1993; Gilbert & Mack 1998; Gutowski *et. al.*, 1998). This experiment was conducted to determine whether Pb affects rat spatial reference memory and whether the curcuminoids could prevent or reduce any memory deficits that may have been induced by this heavy metal.

12.6.2. Materials and Methods

12.6.2.1. Chemicals and Reagents

All chemicals and reagents were as per section 12.2.2.1.

12.6.2.2. Animals

Male Wistar rats, approximately 200-250g each were housed and cared for as described in section 2.2.2.2.

12.6.2.3. Animal training: water maze task

Animals were trained as described in section 5.2.2.3.

12.6.2.4. Treatment Regimes

For the purposes of intraperitoneal injections, the curcuminoids were solubilised in ethyl oleate and injected at a dose of 30 mg/kg/bd for 5 days and lead acetate was dissolved in Milli-Q water and injected at a dose of 20mg/kg/od for 5 days (Daniel *et. al.*, 2004). The curcuminoids were injected every morning between 8:00 – 9:00 and every evening between 17:00 and 18:00. Lead was injected at 12:00. Each group consisted of fifteen animals. Five animals from each group were sacrificed on the morning of the sixth day (these rats are referred to as the day = 1 rats) by cervical dislocation and the hippocampi were analysed for protein carbonyl and GSH content. Animals from the behavioral studies group were sacrificed at the end of the study, 14 days later (these rats are referred to as the day = 14 rats) and the hippocampi were also analysed for the protein carbonyl and GSH content. All brains were rapidly removed, dissected and frozen in liquid nitrogen and stored at -70°C until use.

12.6.2.5. Performance in the water maze (test trials)

Animals performance in the water maze was assessed as described in section 5.2.2.5. After the test trial on the 14th day, animals were sacrificed by neck fracture and the brains were removed as described in section 2.2.2.3. The hippocampi were removed and rapidly frozen in liquid nitrogen and stored at -70°C for analysis of the glutathione and protein carbonyl content (chapters 6 and 7 respectively).

12.6.2.6. Statistical analysis

Results were analysed as described in section 5.2.2.6.

12.6.3. Results

As shown in figure 12.7, the results show a significant difference in latency to escape between the groups ($F_{4, 45} = 21.3$, $p < 0.001$). Post hoc analyses (Scheffé tests) show that the latency to escape (over 14 days) of lead treated animals was significantly longer

compared to the control group ($p < 0.001$). Curcumin and demethoxycurcumin had significantly shorter escape times compared to lead treated animals ($p < 0.001$). The latency to escape of bisdemethoxycurcumin treated animals showed no significant difference compared to lead treated animals. There was no significant difference in the latency to escape between the curcumin treated and demethoxycurcumin treated animals.

The results also show a significant difference in latency to escape between days (multivariate Wilks' Lambda = 0.008, $F_{13, 33} = 323.2$, $p < 0.001$; univariate Greenhouse-Geisser: $F_{3,1, 140.4} = 204.8$, $p < 0.001$) and a significant interaction between groups and days (multivariate Wilks' Lambda = 0.001, $F_{52, 129.9} = 12.0$, $p < 0.001$; univariate Greenhouse-Geisser: $F_{12.5, 140.4} = 6.9$, $p < 0.001$).

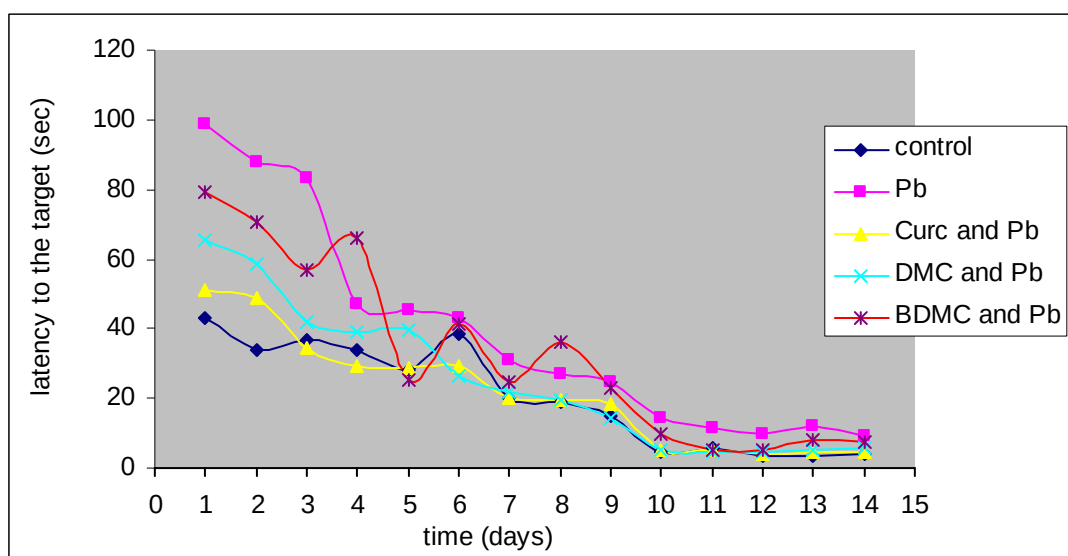


Figure 12.7: Performance of the rats in the water maze task. Each point shows the average time taken for 10 rats. Trials were conducted once daily, every day for 14 days. Results were analysed using repeated measures ANOVA with group as a factor and latency measured over 14 days. Scheffes' test for multiple group comparison was used for post hoc analysis.

12.6.4. Discussion

Previous studies have shown that Pb, even at very low blood concentrations, impair cognitive functions (Satcher 2000; Banks *et. al.*, 1997; Winneke & Kramer 1997). *In vivo*

and *in vitro* studies have shown that Pb interacts with the NMDA receptors and causes an increase (Schulte *et. al.*, 1995; Ma *et. al.*, 1997; Ma *et. al.*, 1998) or a decrease (Rajanna *et. al.*, 1997) in the NMDA binding sites after Pb exposure. Lead exposure has also been reported to affect the hippocampus and cause impairments in LTP and long term depression, thereby reducing the range of synaptic plasticity in these neurons (Ruan *et. al.*, 1998; Zhao *et. al.*, 1999; Ruan *et. al.*, 2000).

Among the subunits within the NMDA receptor, the NR2B is known to be important in mediating the learning and memory process since an increase in its expression in the forebrain of transgenic mice enhances the learning and memory process (Tang *et. al.*, 1999). Lau and co-workers measured the gene expression of NR1 and NR2B subunits in primary cultured cortical and hippocampal neurons after Pb exposure. The results showed that Pb exposure causes an increase in the NR2B mRNA in cortical neurons and significantly decreases both NR1 and NR2B mRNAs in the hippocampal neurons. These authors suggest that Pb may also be toxic by affecting the gene expression of the NR1 and NR2B subunits of the NMDA receptor complex.

As mentioned earlier, Pb is also toxic by disrupting Ca^{2+} -dependent processes (Habermann *et. al.*, 1983) such as protein kinase C (Bressler *et. al.*, 1999). During stimulation by glutamate, NMDA receptors regulate the intracellular Ca^{2+} concentrations (Bading *et. al.*, 1995). It has been suggested that Pb neurotoxicity also results by Pb interfering with zinc and its binding sites in the NMDA complex, which deactivates the NMDA channel and thus affects the intracellular Ca^{2+} concentrations (Guilarte *et. al.*, 1994).

This experiment shows that Pb adversely affects the performance of the rats in the water maze since their escape latencies were much higher compared to controls. The treatment with CURC and DMC prevents the Pb-induced spatial memory deficit. Curcumin has also been shown to prevent the loss of spatial memory induced by intracerebroventricular infusions of the β -amyloid protein (Frautschy *et. al.*, 2001). These authors demonstrated that curcumin is a potent antioxidant and reduces oxidative damage more than ibuprofen

and that curcumin also reduces synaptophysin loss and microgliosis in cortical layers. Experiments 12.3 and 12.5 demonstrate that CURC and DMC reduce lipid peroxidation and enhance cell viability in the presence of lead, more than BDMC. This could be another mechanism by which these agents protect neurons in the rat and reduce the Pb-induced spatial reference memory deficit. The possibility of the curcuminoids binding to lead and preventing it from causing neurotoxicity was investigated in the following chapter. BDMC, being a weaker antioxidant as demonstrated in previous experiments, did not prevent the Pb-induced memory deficits.

12.7. EFFECT OF LEAD AND CURCUMINOID TREATMENT ON RAT HIPPOCAMPAL TOTAL GLUTATHIONE CONTENT

12.7.1. Introduction

Neurotoxicity associated with heavy metals such as mercury has been linked to ROS and depletion of intracellular GSH (Sanfeliu *et. al.*, 2001). Mercury has a high affinity for the thiol (-SH) group on GSH (Sanfeliu *et. al.*, 2003), the tripeptide which consists of cysteine, glutamate and glycine. It is the cysteine moiety of GSH that carries the active thiol group that binds and detoxifies a variety of heavy metals. Normally, GSH is in very high concentrations within the cell (in the mM range) and maintains the balance between oxidant and antioxidant status within the cell (Meister 1995). A depletion of this essential endogenous antioxidant could lead to oxidative stress, cellular functional inactivation and possibly neuronal death. GSH depletion results from compounds that induce GSH oxidation (Plummer *et. al.*, 1981) or from electrophilic compounds that interact with GSH and form adducts (Plummer *et. al.*, 1981). This experiment was conducted to determine the effect of Pb and the curcuminoids on rat hippocampal GSH levels.

12.7.2. Materials and Methods

12.7.2.1. Chemicals and Reagents

All chemicals and reagents used were as per section 6.2.2.1.

12.7.2.2. Animals

Male Wistar rats, approximately 200-250g each were housed and cared for as described in section 2.2.2.2.

12.7.2.3. Treatment Regimes

Animals were treated as described in section 12.6.2.4. Five animals from each group were sacrificed on the morning of the sixth day (day = 1 rats) by cervical dislocation and the hippocampi were analysed for the GSH and protein carbonyl content (experiment 12.8). Animals from the behavioral studies group were sacrificed at the end of the study, 14 days later (day = 14 rats) and the hippocampi were also analysed for GSH and protein

carbonyl content. All brains were rapidly removed, dissected and frozen in liquid nitrogen and stored at -70°C until use.

12.7.2.4. GSH assay

The assay was conducted as described in section 6.2.2.5.

12.7.2.5. Statistical analysis

Results were analysed as described in section 2.2.2.7.

12.7.3. Results

Animals sacrificed immediately after the treatment regime (day =1 rats) and that were dosed with Pb, show a significant decrease in the amount of GSH in the rat hippocampus (figure 12.8). However CURC and DMC, but not BDMC prevent this decrease in GSH content. Animals sacrificed at the end of the study (day = 14), show negligible changes in GSH content in rat hippocampus. The GSH content in Pb only dosed animals has significantly ($p < 0.05$) increased 14 days later. There was no significant difference between the hippocampal GSH content (measured after test trials) in control rats compared to Pb dosed rats or Pb dosed rats compared to Pb and curcuminoid treated rats.

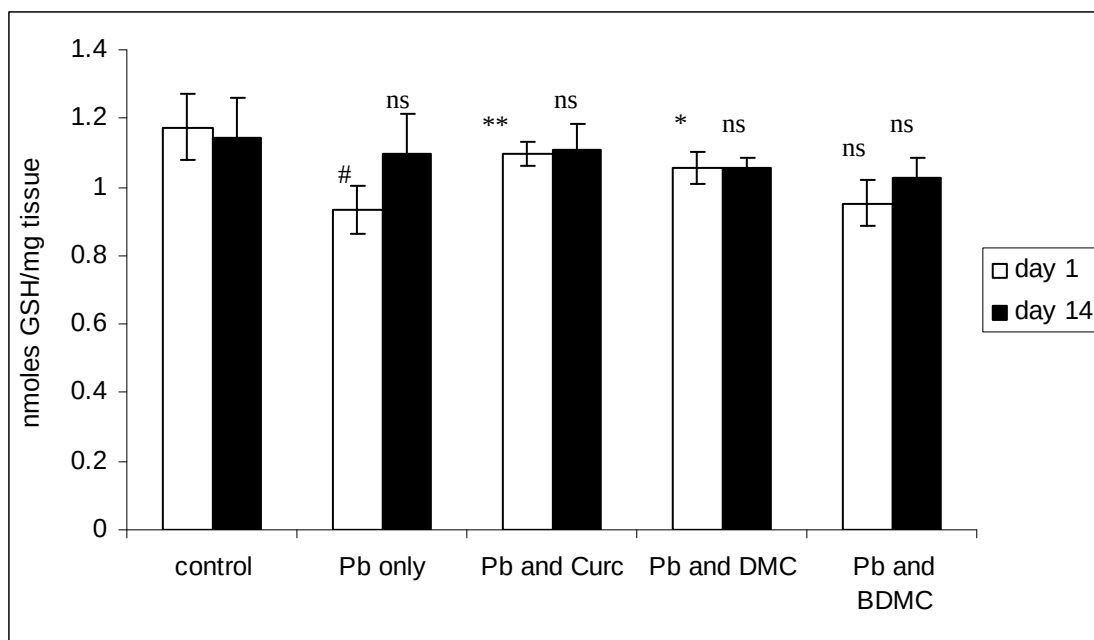


Figure 12.8: Effect of Pb alone and Pb and curcuminoid treatment on rat hippocampal glutathione content. Hippocampi from animals were analysed after 5 days of treatment (day 1) or at the end of the behavioral studies (day 14). Each bar represents the mean $\pm$ SD (n=5). # (p<0.001) in comparison to controls. * (p<0.05) and ** (p<0.01) in comparison to Pb only dosed animals. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

12.7.4. Discussion

The heavy metal Pb is known to cause oxidative damage and disrupt the prooxidant/antioxidant balance within mammalian cells (Lawton & Donaldson 1991; Monteiro *et al.*, 1995). It has recently been shown that the administration of Pb to rats induces LP and reduces antioxidant enzymes in rat liver (Sivaprasad *et al.*, 2004). These authors showed that Pb reduces SOD by 40%, catalase by 45%, GSH-Px by 46%, GSH by 43%, GSH-R by 59% and GSH-T by 42%. The treatment of animals with antioxidants completely ameliorates the Pb-induced toxicity (Sivaprasad *et al.*, 2004). Experiments thus far show that Pb induces oxidative stress in primary hippocampal neurons. It is possible that Pb causes the generation of free radicals and induces GSH depletion. It is known that GSH binds to heavy metals (Plummer *et al.*, 1981; Sanfeliu *et al.*, 2003). GSH may have bound Pb in an attempt to reduce the Pb-induced toxicity and in doing so,

this also causes GSH depletion. The curcuminoids have demonstrated their ability to reduce Pb-induced oxidative stress in primary hippocampal neurons. CURC and DMC can scavenge the free radicals that are generated, reduce GSH oxidation and prevent its depletion. The curcuminoids can also bind Pb and prevent or reduce the amount that binds with GSH. This is also another mechanism by which the curcuminoids may have prevented the depletion of the most abundant endogenous antioxidant. BDMC is a weaker antioxidant when compared to CURC and DMC and possibly offers an insignificant amount of protection which allows Pb to cause GSH depletion. The possibility of the curcuminoids binding to Pb was investigated in chapter 13. GSH measured 14 days after treatment shows no difference between the groups. The prooxidant/antioxidant levels within the cell may have restabilised and GSH levels were restored.

12.8. EFFECT OF LEAD AND CURCUMINOID TREATMENT ON RAT HIPPOCAMPAL PROTEINS

12.8.1. Introduction

Heavy metals such as lead and cadmium are known to induce oxidative damage and adversely interfere with the prooxidant/antioxidant status within the cell (Lawton & Donaldson 1991; Muller 1991; Monteiro *et. al.*, 1995; Stohs & Bagchi 1995; Daniel *et. al.*, 2004). Pb has shown to induce lipid peroxidation in primary hippocampal neurons and this oxidative stress was curtailed by the co-incubation of the neurons with the curcuminoids. This experiment was conducted to determine the effect Pb and curcuminoid treatment on rat hippocampal proteins.

12.8.2. Materials and Methods

12.8.2.1. Chemicals and Reagents

All reagents used were as per section 7.2.2.1.

12.8.2.2. Animals

Male Wistar rats, approximately 200-250g each were housed and cared for as described in section 2.2.2.2.

12.8.2.3. Treatment Regimes

Animals were treated as described in section 12.6.2.4. Five animals from each group were sacrificed on the morning of the sixth day (day = 1 rats) by cervical dislocation and the hippocampi analysed for their protein carbonyl content. Animals from the behavioral studies group were sacrificed at the end of the study, 14 days later (day = 14 rats) and the hippocampi also analysed for protein carbonyl content. All brains were rapidly removed, dissected and frozen in liquid nitrogen and stored at -70°C until use.

12.8.2.4. Protein carbonyl measurement

The assay was conducted exactly as described in section 7.2.2.4. The experiment was replicated five times.

12.8.2.5. Statistical analysis

Results were analysed as described in section 2.2.2.7.

12.8.3. Results

As shown in figure 12.9, animals that were sacrificed immediately (day = 1 rats) after the treatment regime and dosed with Pb have an increased amount of oxidised proteins compared to the control group. Treatment with CURC and DMC but not BDMC, significantly reduces protein oxidation. Animals sacrificed at the end of the behavioural studies (day = 14 rats), reveal that oxidised proteins in the hippocampus are still elevated when compared to controls. The curcuminoid treated animals have lower amounts of oxidised proteins.

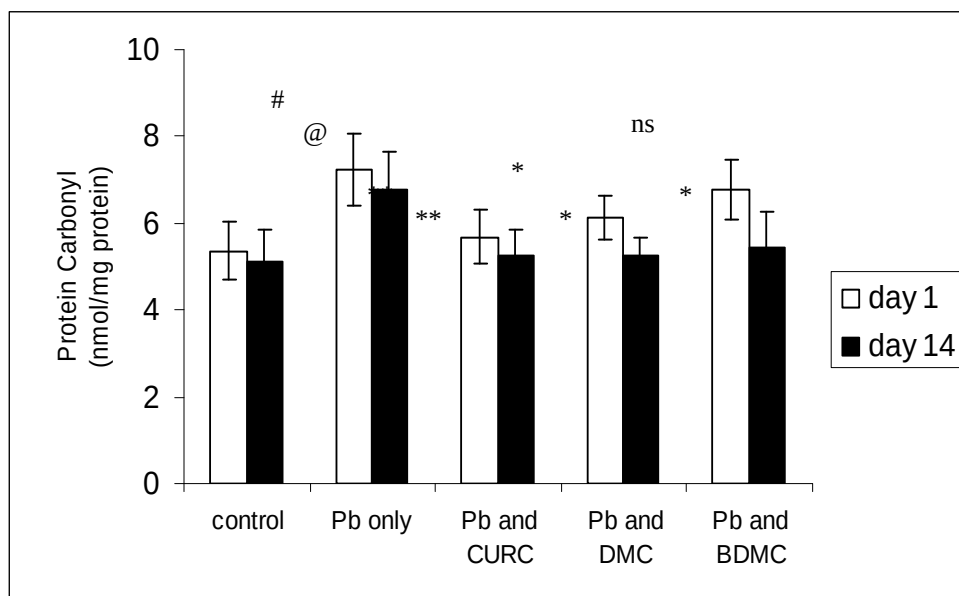


Figure 12.9: Effect of Pb alone or Pb and curcuminoid treatment on rat hippocampal protein carbonyl levels. Hippocampi from animals were analysed after 5 days of treatment (day 1) or at the end of the behavioral studies (day 14). Each bar represents the mean $\pm$ SD ($n=5$). #($p<0.01$) and @($p<0.05$) in comparison to controls. *($p<0.05$) and **($p<0.01$) in comparison to Pb only dosed animals. Results were analysed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

12.8.4. Discussion

The previous experiment demonstrates that Pb causes depletion of the most important intracellular antioxidant, GSH. Research has shown that Pb is capable of generating free radicals (Lawton & Donaldson 1991; Monteiro *et al.*, 1995; Daniel *et al.*, 2004) and thus far Pb has shown to induce lipid peroxidation in primary hippocampal neurons. Therefore, the loss of GSH and the generation of free radicals cause protein oxidation in Pb dosed animals. It has been shown that elevated GSH levels can reduce ROS-induced protein oxidation in rat brain (Pocernich *et al.*, 2000). It has also been shown that the treatment of animals with antioxidants such as vitamin E reduces formaldehyde-induced ROS in rat brain and reduces lipid and protein oxidation in rat frontal cortex and hippocampal tissues (Gurel *et al.*, 2005). The treatment with the curcuminoids provides

protection by their potent antioxidant properties. The curcuminoids reduce GSH depletion and protein oxidation in rat hippocampus and these could be possible mechanisms through which these agents reduce the Pb-induced memory deficit noticed in experiment 12.6.

CHAPTER 13

METAL BINDING STUDIES: THE INTERACTION BETWEEN THE CURCUMINOIDS AND LEAD

13.1. INTRODUCTION

The heavy metal lead has no biological functions. There is evidence from human and experimental animal literature that deficits in cognitive function resulting from exposure to Pb during early childhood lasts well into adulthood (White *et. al.*, 1993; Kuhlmann *et. al.*, 1997).

Chapter 12 demonstrates that Pb induces LP in primary hippocampal neurons and reduces neuronal cell viability. Lead also adversely affects spatial reference memory in rats, reduces GSH levels and increases protein oxidation in rat hippocampus. Blood Pb levels have been correlated with lipid peroxidation in lead-exposed workers (Yiin & Lin 1994; Ye *et. al.*, 1999). The association of oxidative stress with Pb intoxication suggests that an antioxidant may enhance the efficacy of therapeutic agents used in the treatment of Pb poisoning. It has also been reported that the ideal treatment for Pb intoxication should encompass both chelating and antioxidant actions (Tandon *et. al.*, 2002). Chapter 12 also shows that the co-incubation of the neurons with the curcuminoids reduces the oxidative stress induced by Pb and enhances the neuronal viability in the presence of Pb. The curcuminoids CURC, DMC but not BDMC reduces the spatial memory deficits caused by Pb. The curcuminoids also reduces GSH depletion and protein oxidation in rat hippocampus.

The potential for the curcuminoids to reduce heavy metal toxicity in the body will, in part, be dictated by its ability to complex with the specific metal. Lewis acid-base interactions are governed by two independent principles - acid-base strengths, and acid and base hardness (Pearson's HSAB principle). These reflect that strong acids prefer strong bases, and that hard acids prefer hard bases, soft acids prefer soft bases. These two principles may conflict, making metal coordination prediction problematical (Douglas, *et.*

*al.*1994, Wulfberg, 2001). In the curcuminoids there are two set of potential binding sites, the β -diketo moiety, and the methoxy-phenolic moiety. Because of the various tautomeric forms a number of potential coordination forms exist. In terms of HSAB theory these sites will be hard. NMR studies using gallium (Ga^{3+}) have identified the binding site for hard ions to be the β -diketone moiety in the enolic tautomer (Borsari, *et. al.*, 2002), while the borderline copper (Cu^{2+}) has been proposed to bind at both sites (Ahsan, *et. al.*, 1999). Regarding the present Lewis acid, both lead(II) and lead(IV) are soft metals, with the former being slightly harder than the latter. In solution lead is most likely to be found as Pb(II) since Pb(IV) is thermodynamically unstable. Extrapolating from the Pourbaix diagram, depending on concentration and pH, for Pb(II) (Wulfberg, 2001) two possible species could be available for binding to the curcuminoids, these are solvated Pb^{2+} , and solvated $\text{Pb}(\text{OH})^+$. This study is aimed at determining whether lead is involved in coordination, and if so, what possible species exists in solution is, and finally which is the preferred site of binding to lead.

It was thought that apart from the antioxidant properties of the curcuminoids, the curcuminoids bind to Pb and reduce the heavy metal-induced neurotoxicity. Therefore techniques involving UV/VIS, IR, spectrofluorometry and electrochemistry were employed to probe if interactions between the curcuminoids and Pb exist.

13.2. UV/VIS SPECTROSCOPY

13.2.1. Materials and Methods

13.2.1.1. Chemicals and Reagents

The curcuminoids (CURC, DMC and BDMC) were separated and purified as described in chapter 11. These were dissolved in ethanol and made to volume with Milli-Q water. The final concentration of ethanol was 50%. Lead acetate was purchased from Sigma Chemicals Corporation, St Louis, MO, U.S.A. It was dissolved in Milli-Q water.

13.2.1.2. Technique: UV/VIS Spectroscopy

The UV/Visible spectra were monitored with a Cary Varian, 500 Series Spectrophotometer. The interaction between the curcuminoids and lead was studied by comparing the absorption spectrum of the curcuminoids alone in solution and immediately after the addition of lead. Various concentrations of the curcuminoids were analysed in the presence of 0.1mM lead. Curcumin and demethoxycurcumin experiments were conducted in a quartz cuvette with a pathlength of 0.5cm while bisdemethoxycurcumin experiments were conducted in a cuvette with a 1cm pathlength. This reason for this is that curcumin and demethoxycurcumin (the very bright yellow curcuminoids) had very high absorbencies at the concentrations analysed. Therefore by using a cuvette with a shorter path length, lower absorbencies were obtained.

13.2.2. Results

The UV/VIS spectra of CURC, DMC and BDMC in the absence and presence of lead are presented in figures 13.1, 13.2 and 13.3, respectively. Upon the addition of Pb (0.1mM) to CURC and DMC (0.03mM each) there is a decrease in absorbance and a shift to higher wavelengths. Upon the addition of Pb to BDMC, there is no change in either the absorbance or the wavelength. These patterns were also observed at other concentrations.

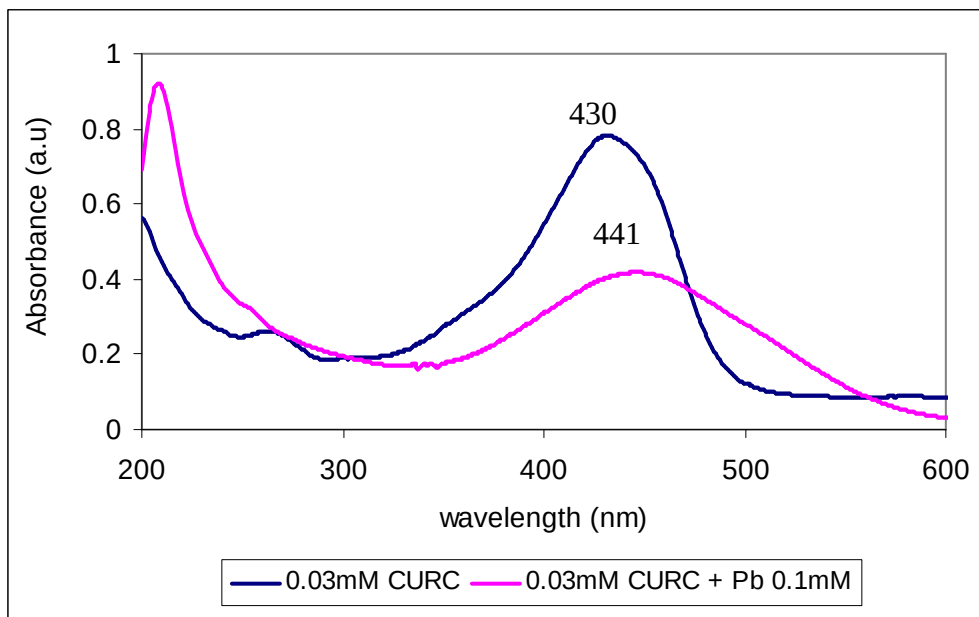


Figure 13.1: UV/VIS spectra of curcumin (0.03mM) alone and in the presence of 0.1mM lead.

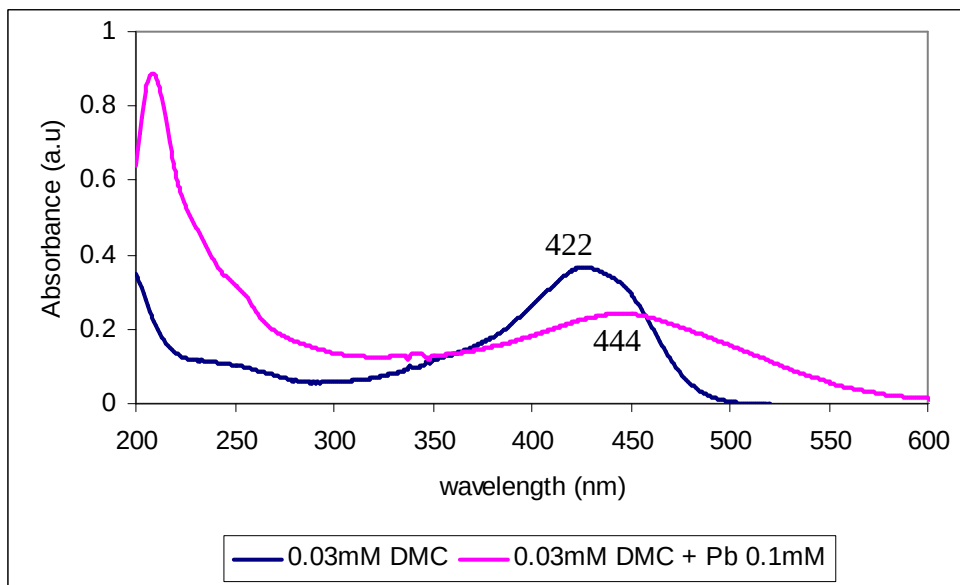


Figure 13.2: UV/VIS spectra of demethoxycurcumin (0.03mM) alone and in the presence of 0.1mM lead.

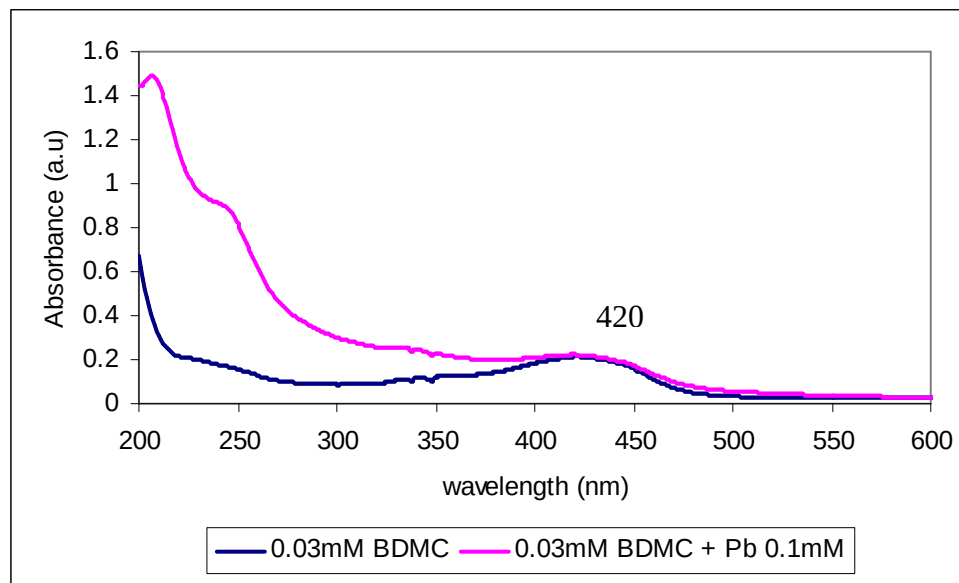


Figure 13.3: UV/VIS spectra of bisdemethoxycurcumin (0.03mM) alone and in the presence of 0.1mM lead.

13.2.3. Discussion

The band at approximately 430 nm is normally associated with the β -diketo moiety (Sundaryano *et. al.*, 2003). On addition of metal to the ligand the behaviour of the UV spectra are similar for CURC and DMC, both showing a reduction in intensity and a red shift of +11nm for CURC and +22nm for DMC indicating that coordination occurs, while that for BDMC shows no change in either intensity or wavelength. This suggests either that no complexation occurs for the latter, or that there are subtle differences in the nature of the complexes.

Curcumin displays two tautomeric equilibria: keto-enol tautomerism and quinoid-phenol tautomerism (Figure 13.4). The position of the equilibria is pH dependent, curcumin having a yellow colour in acidic media and red in basic media. This results in shift of the maximum absorbance from 422 nm to 463 nm, respectively (Bernabé-Pineda, *et. al.*, 2004). Three acid dissociation constants have been identified, the enol proton ($pK_{A1} = 8.38$), the two phenolic protons ($pK_{A2} = 9.88$, $pK_{A3} = 10.51$) (Bernabé-Pineda, *et. al.*, 2004). The curcuminatate ion ($CURC^{3-}$) absorbs at 466 nm, with a hypsochromic shift

occurring on protonation, as the quinoid structure is reduced (Bernabé-Pineda, *et. al.*, 2004).

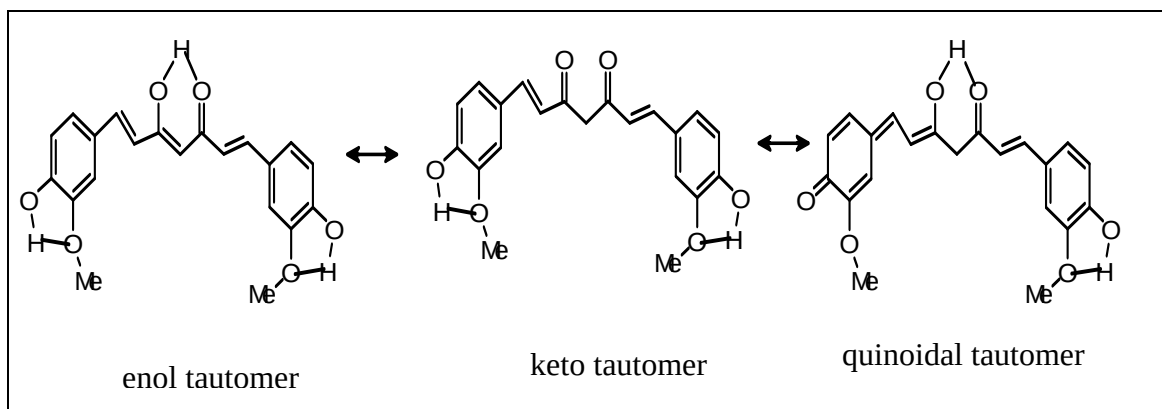


Figure 13.4: The keto-enol tautomerism and quinoid-phenol tautomerism equilibria of curcumin.

The two equilibria are also controlled by the substitution on the ring. The blue shift on demethoxylation (CURC = 430nm, DMC = 422nm, BDMC = 420nm) reflects the weakening of hydrogen bonding to the methoxy oxygen, and the concomitant weakening of the quinoid tautomer.

13.3. SPECTROFLUOROMETRY

13.3.1. Introduction

Some substances may emit radiation on absorption of visible or ultraviolet radiation, that is, they may exhibit photoluminescence. When the emission takes place 10^{-4} seconds after excitation, the phenomenon is known as fluorescence. Emission that occurs at longer intervals is known as phosphorescence. The wavelength of fluorescent radiation is usually longer than that of the exciting radiation. When the emission intensity measured at the maximum of the fluorescence band is plotted against the wavelength of the exciting radiation, an excitation spectrum is obtained. Fluorescent substances are usually organic compounds and are mainly polycyclic, conjugated systems. Several organic complexing agents become fluorescent when they are converted into metal complexes (Pataki & Zapp 1980). On this basis metals and metalloids can be determined very sensitively.

In this method, the sample solution is exposed to radiation and the intensity of the emitted radiation is measured. The fluorescent radiation must therefore be separated from the exciting radiation. The emitted radiation is usually less intense than the excitation radiation and the fluorescent emission is generally observed in a direction that is perpendicular to the incident beam.

13.3.2. Materials and Methods

13.3.2.1. Chemicals and Reagents

All chemicals and reagents used were as per section 14.2.2.1.

13.3.2.2. Technique: Spectrofluorometry

Spectra were captured using a Cary Varian, 500 Series Spectrofluorometer. The interaction between the curcuminoids and lead was studied by comparing the emission spectrum of the curcuminoids alone in solution and then after the addition of lead. The same concentrations of the curcuminoids and lead as described in the UV/VIS study was used in these experiments. A quartz cuvette designed for the use in spectrofluorometry,

with a 1.0cm path length was used for these experiments. The excitation wavelength was set at 390nm and data was captured from 400-800nm.

13.3.3. Results

Curcumin, demethoxycurcumin and bisdemethoxycurcumin have an emission absorbance peak at 545nm, 541nm and 538nm respectively (see figures 13.5, 13.6 and 13.7). These peaks were abolished or reduced upon the addition of lead (0.1mM) with the subsequent enhancement of the peak at 782nm. This pattern was also observed with other concentrations of curcuminoids in the presence of lead 0.1mM.

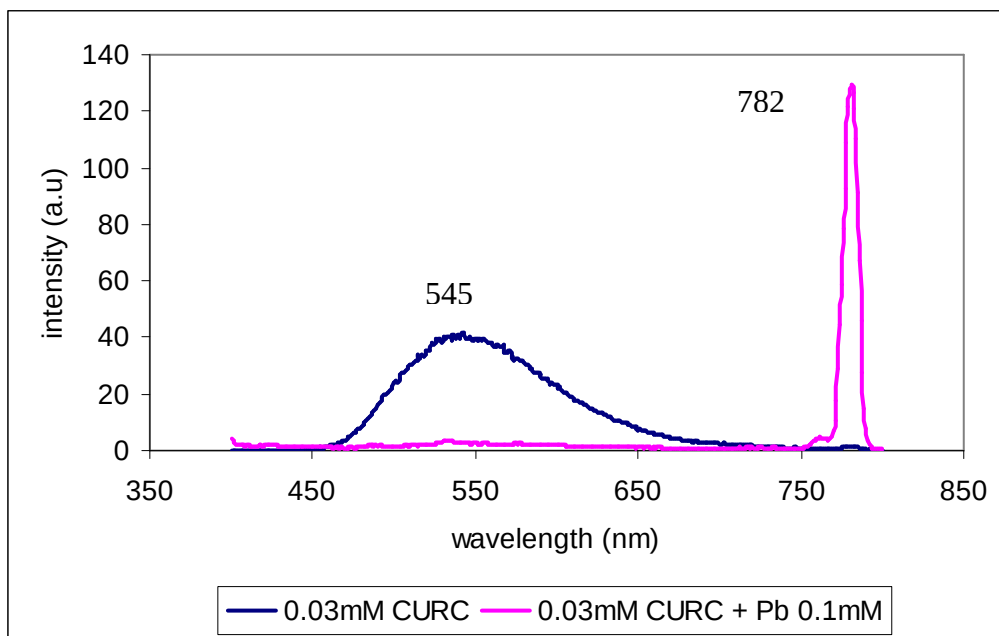


Figure 13.5: Spectrofluorometric spectra of CURC (0.03mM) alone and in the presence of 0.1mM lead.

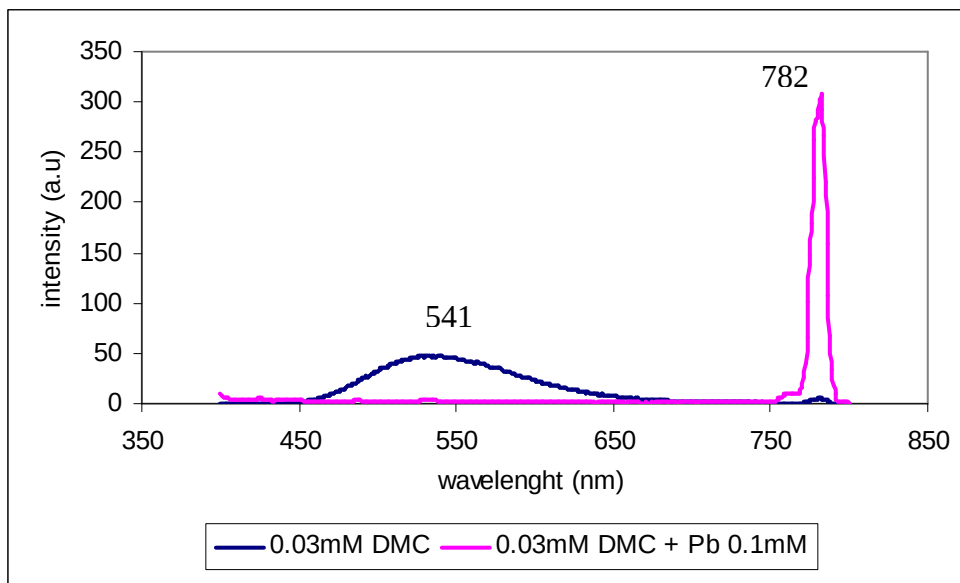


Figure 13.6: Spectrofluorometric spectra of DMC (0.03mM) alone and in the presence of 0.1mM lead.

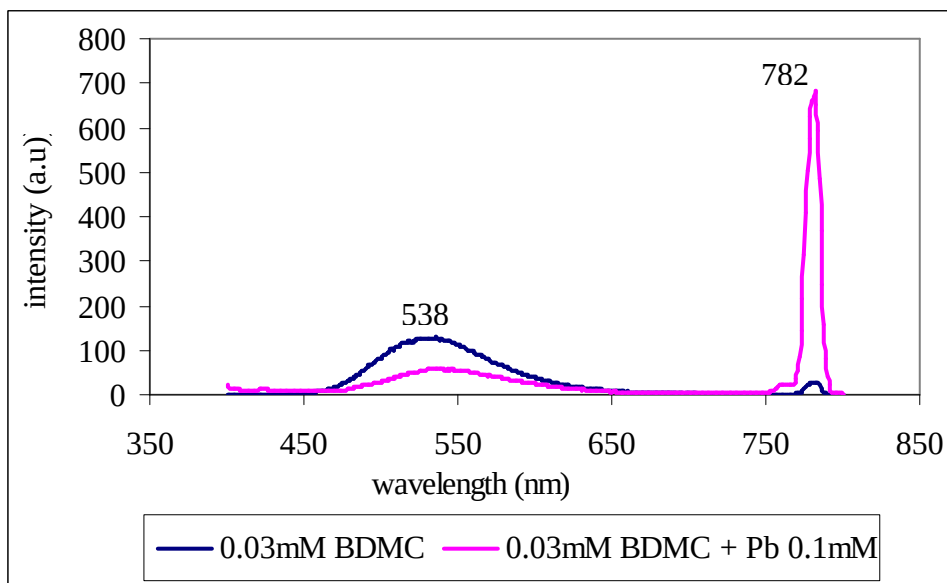


Figure 13.7: Spectrofluorometric spectra of BDMC (0.03mM) alone and in the presence of 0.1mM lead.

13.3.4. Discussion

The broad band at 545nm has been suggested as indicative of the β -diketo moiety (Chignell *et. al.*, 1994), although there is also extensive conjugation within the quinoid

tautomer. Curcumin shows a broad band at 545nm and the appearance of a weak band at 782 nm after the addition of Pb. DMC similarly shows a broad band at 541nm and the appearance of a weak band at 782nm after the addition of Pb. BDMC shows a broad band at 538nm and the appearance of a weak band at 782nm. On the addition of the metal all three show a dramatic decrease in the yellow band and a dramatic increase in the near infrared band, with the changes in BDMC showing the smallest change. This indicates that all three curcuminoids interact with the metal.

13.4. INFRARED SPECTROSCOPY

13.4.1. Materials and Methods

13.4.1.1. Chemicals and Reagents

The curcuminoids (CURC, DMC and BDMC) were separated and purified as described in chapter 11. These were dissolved in ethanol and made to volume with Milli-Q water. The final concentration of ethanol was 50%. Lead was dissolved in Milli-Q water. All other reagents were as per section 7.3.2.1.

13.4.1.2. Technique: Infrared Spectroscopy

Equal volumes of the curcuminoids and lead were mixed together. The final concentration of the curcuminoids and lead in solution was 1mM. Precipitates obtained after 48 hours were collected and the washed with ice-cold Milli-Q water under negative pressure. The rest of the experiment was conducted as described in section 10.3.2.2. The pellets were analysed by mid-range infrared spectroscopy ($4000\text{-}400\text{cm}^{-1}$) using a Perkin Elmer 2000 FTIR spectrometer.

13.4.2. Results

The curcuminoids possess rich spectra in the carbonyl, 1400 and 1100cm^{-1} region, with several strong vibrations (figures 13.8, 13, 9 and 13.10). Carbonyls are also found in the 1660 and 1670 cm^{-1} region with overlapping occurring in the $1660 - 1695\text{ cm}^{-1}$ region. The phenolic C-O stretch is also found between 1150 or 1240cm^{-1} and 1310 and 1400 cm^{-1} region. The O-H stretch is found between 3650 and 2500 cm^{-1} region. The methoxy C-H stretches are found with medium intensities between 2850 and 2818 cm^{-1} region. There is an increase in hydrogen bonding on demethoxylation. This is noticed by the decrease in the frequencies of the curcuminoids, CURC = 3439cm^{-1} , DMC = 3432cm^{-1} and BDMC = 3424cm^{-1} . The β -ketonic carbonyl increases in intensity from CURC to DMC to BDMC. Three strong bands are observed between 1300 and 1100cm^{-1} and these are sensitive to demethoxylation (table 13.1)

Table 13.1: Infrared spectra of the curcuminoids between 1300 and 1100 cm^{-1} .

CURC	1270 cm^{-1}	1204 cm^{-1}	1123 cm^{-1}
DMC	1263 cm^{-1}	1163 cm^{-1}	1119 cm^{-1}
BDMC	1259 cm^{-1}	1159 cm^{-1}	1100 cm^{-1}

On the addition of Pb to CURC, the carbonyl at 1599 cm^{-1} shifts -23 cm^{-1} and the band at 1204 cm^{-1} undergoes a shift of +15 cm^{-1} (figure 13.8). The peaks at 1727 and 1628 cm^{-1} undergoes a shift of -10 cm^{-1} and the band at 1270 shifts by +4 cm^{-1} .

On the addition of Pb to DMC, the carbonyl at 1569 cm^{-1} shifts by +13 cm^{-1} with a decrease in intensity (figure 13.9). The stretch at 1624 cm^{-1} also decreases in intensity. The band at 1510 cm^{-1} undergoes a shift of -15 cm^{-1} with the band at 1163 also shifting -5 cm^{-1} .

On the addition of Pb to BDMC, the carbonyl at 1598 cm^{-1} shifts -23 cm^{-1} (figure 13.10). The bands at 1159 and 1100 cm^{-1} also shift by -11 cm^{-1} and -26 cm^{-1} respectively. Two new intense bands are observed at 1403 and 673 cm^{-1} .

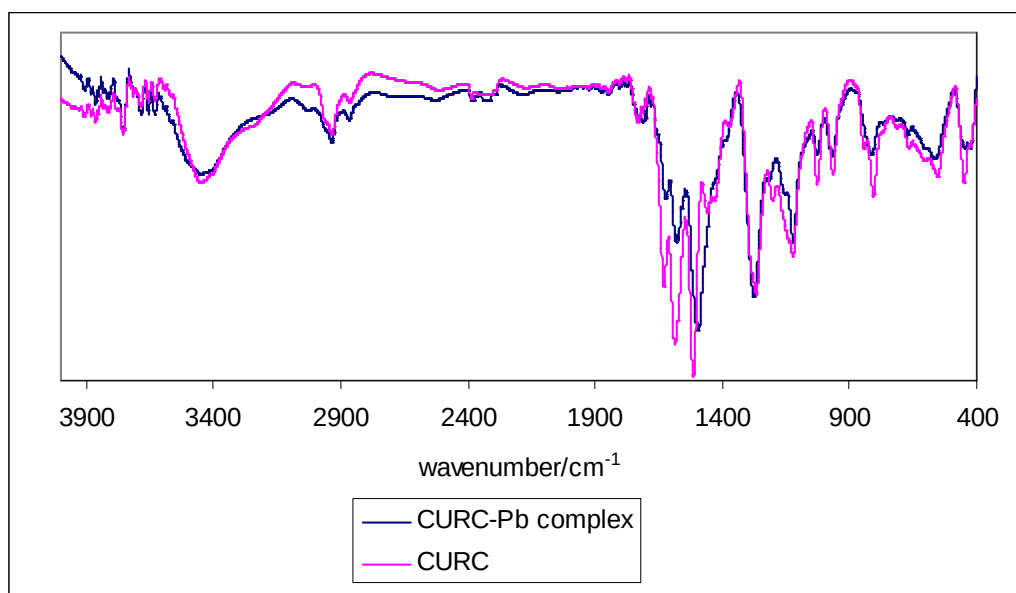


Figure 13.8: Infrared spectra of CURC alone and in the presence of Pb.

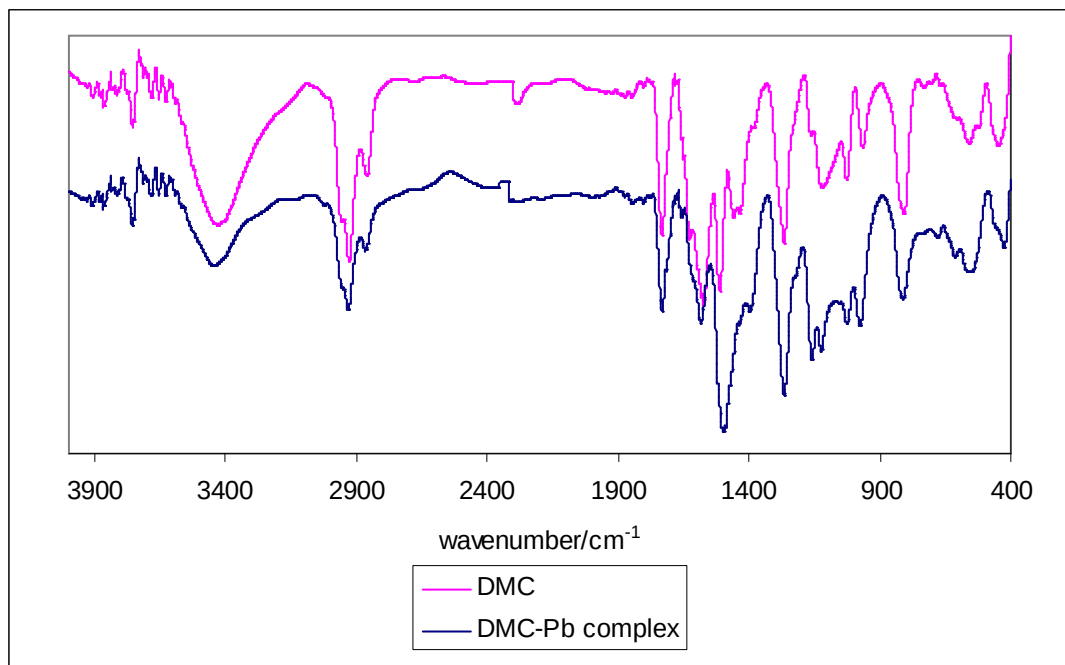


Figure 13.9: Infrared spectra of DMC alone and in the presence of Pb.

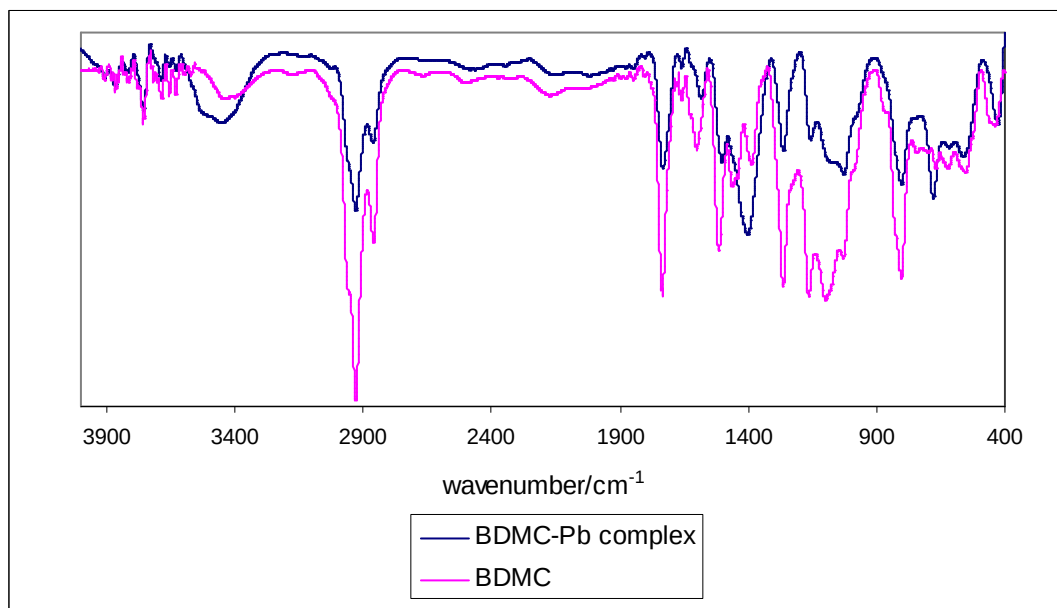


Figure 13.10: Infrared spectra of BDMC alone and in the presence of Pb.

13.4.3. Discussion

Curcuminoids possess a rich spectrum in the carbonyl region, with the presence of several strong vibrations. There is a danger of ignoring the weak bands that reflect minor contributions of the various tautomeric forms. The region for C-O vibrations, between 1400 and 1100 cm^{-1} , is an even more a rich spectrum. A detailed examination allows an insight to the site of metal binding.

The quinoidal form of the phenol moiety would form a cross-conjugated dienone carbonyl between 1660 and 1670 cm^{-1} (Alpert *et. al.*, 1970), over lapping the keto- form of the β -diketo moiety (1660 – 1695 cm^{-1}), but higher than the enolic form (1535 – 1640 cm^{-1}) (Alpert *et. al.*, 1970). The phenolic C-O stretch, $\nu(\text{C-O})$, is similarly a coupled vibration and may be found between 1150 and 1240 cm^{-1} or 1310 and 1400 cm^{-1} (Alpert *et. al.*, 1970), depending on the extent of coupling with the in-plane $\delta(\text{C-O-H})$ deformation. The O-H stretch, $\nu(\text{O-H})$ is typically found between 3650 and 2500 cm^{-1} , depending on the extent of hydrogen bonding.

The methoxy group can normally be distinguished by the appearance of a $\nu(\text{C-H})$ symmetric stretch of medium intensity between 2850 and 2818 cm^{-1} , and the appearance of the degenerate asymmetric and the symmetric scissors between 1470 and 1430 cm^{-1} . These two deformation bands may coincide, and couple with the C-O stretch found between 1140 and 1240 cm^{-1} (Alpert *et. al.*, 1970).

The alkene moiety is characterized by a less intense band at 3019 cm^{-1} (often masked by aliphatic C-H stretches), and by a $\nu(\text{C}=\text{C})$ stretch between 1630 and 1590 cm^{-1} - the lower range is an indication of conjugation to the aromatic ring, with concomitant enhancement of the intensity of the band (Alpert *et. al.*, 1970). It is thus often observed as a shoulder on more intense carbonyl stretches or benzene stretches (Wilson modes 18a and 18b) also found in this region (Vársanyi, *et. al.*, 1969). A strong $\delta(\text{C}=\text{H})$ deformation expected between 980 and 960 cm^{-1} is characteristic of the *trans* alkene. This frequency though is liable to be masked by benzenoid fundamental vibrations (Alpert *et. al.*, 1970).

Substitution on the aromatic ring is normally characterized by the group frequencies found between 950 and 650 cm^{-1} . 1, 4 di-substitution indicated by one strong vibration between 860 and 800 cm^{-1} , 1,2,4 tri-substitution by a strong vibration between 860 and 800 cm^{-1} and a band of medium intensity between 900 and 860 cm^{-1} (Alpert *et. al.*, 1970).

A comparison of the infrared spectra reveals that in the solid state the $\nu(\text{O-H})$ vibration is a broad band showing several shoulders, resulting from its being a composite of the phenolic O-H and the β -ketoenolate O-H stretches. There is a slight increase in the hydrogen bonding on demethoxylation, seen in both the decreasing frequency – CURC (3439 cm^{-1}), DMC (3432 cm^{-1}) and BDMC (3424 cm^{-1}) and in the significant increased intensity. The β -ketonic carbonyl, $\nu(\text{C=O})$ at 1727 cm^{-1} , increases in intensity progressing from CURC to BDMC, indicating that the enolic form is enhanced on addition of the methoxy groups to the ring, and that for the BDMC the β -keto form is more dominant. This is supported by the weakening in intensity of the enhanced coupled $\nu(\text{C=C})$ stretch as conjugation within the system is reduced. And it is also associated with the lost in BDMC of the β -enol carbonyl that is observed at 1599 cm^{-1} in CURC and 1569 cm^{-1} in DMC.

The cross-conjugated dienone carbonyl in the quinoidal tautomer cannot be readily identified, being masked by the strong $\nu(\text{C=C})$ between 1627 cm^{-1} and 1600 cm^{-1} . It is however observed as a weak band at 1653 cm^{-1} in BDMC, where the tautomer is a minor component.

The very strong band at approximately 1510 cm^{-1} in all the curcuminoids is tentatively assigned as the benzene ring vibration, Wilson mode 19a (Vársanyi, *et. al.*, 1969). Its intensity is enhanced by the polar substituents on the ring. Its position and intensity are such as to allow it to be confused with a carbonyl vibration.

Three strong bands between 1300 and 1100 cm^{-1} are sensitive to demethoxylation and as such could be assigned as the coupled phenolic $\nu(\text{C-O})$ stretch. This region also contains the substituent sensitive Kekulé ring vibration (Wilson mode 14), expected about

1270 cm^{-1} (Vársanyi, *et. al.*, 1969), an in-plane $\alpha(\text{C-H})$ bend (Wilson mode 15, expected about 1155 cm^{-1} (Vársanyi, *et. al.*, 1969), as well as the methoxy $\nu(\text{C-O})$ stretch. Based on their behaviour with Pb (particularly that for BDMC) the higher frequency band is assigned as mode 14, the middle frequency could be either mode 15 or a phenolic $\nu(\text{C-O})$ stretch, while the lower is identified as a phenolic $\nu(\text{C-O})$ stretch.

None can be ascribed to the methoxy $\nu(\text{C-O})$ stretch as they are present in all the spectra. Based on the broadening of the band at about 1270 cm^{-1} in curcumin and DMC, compared with its sharpness in BDMC, it appears that the methoxy stretch coincides with mode 14.

Finally, the medium to strong band at 964 cm^{-1} is tentatively ascribed to the trans $\delta(\text{=C-H})$ deformation, while the methoxy sensitive medium strong band between 810 and 800 cm^{-1} (curcumin - 809 cm^{-1} ; DMC - 802 cm^{-1} ; BDMC - 798 cm^{-1}) is indicative of the substitution of the benzene ring, with the appearance of a medium band at 842 cm^{-1} for curcumin confirming 1, 2, 4 trisubstitution.

Four major and other minor forms of metal interaction are known for the β -diketo moiety (Nakamoto 1977), depending on the keto-enol tautomerism, and the two principles governing Lewis acid – base interactions (figure 13.11).

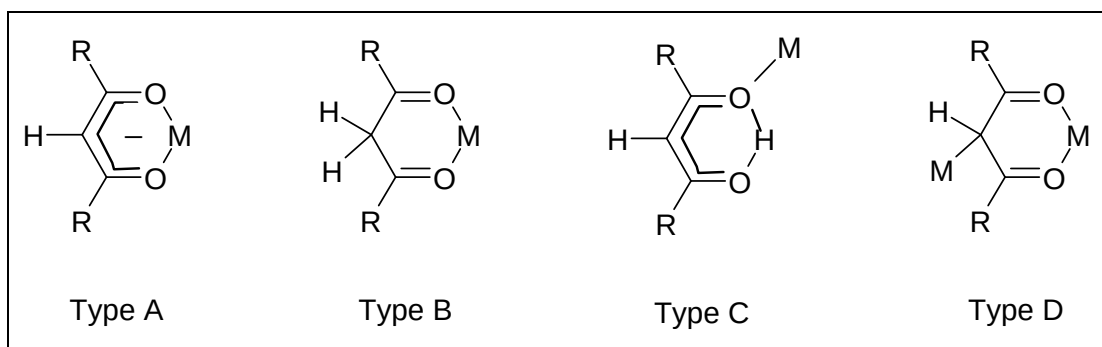


Figure 13.11: Possible metal coordination to the β -diketone system.

Type A is associated with chelation in the ionic enolic form. It is characterised by two strong bands between 1570 and 1525 cm^{-1} and two between 1400 and 1280 cm^{-1} ,

associated with the coupled $\nu(\text{C}=\text{O})$ and $\nu(\text{C}=\text{C})$ stretches, respectively. Type B is associated with chelation in the neutral ketonic form. It is characterized by higher wavelengths of the carbonyls found between 1700 and 1650 cm^{-1} . Type C is associated with the neutral enolic form with only monodentate coordination to the metal. It is characterised by two carbonyl bands, one found at 1630 cm^{-1} (lying between type A and B), and a lower band at 1560 cm^{-1} . Type D is associated with metal binding to the acidic central α -carbon of the ionic ketonic form. It is characterised by two coupled $\nu(\text{C}=\text{O})$ stretches between 1650 and 1600 cm^{-1} , and coupled $\nu(\text{C}-\text{C})$ between 1350 and 1200 cm^{-1} , lower than Type A (Nakamoto 1977).

Metal interaction with the phenolic group could be of Type E, chelation of the ionic methoxy-phenolate; Type F monodentate coordination of the neutral methoxy-phenol; Type G, monodentate coordination of the neutral or ionic demethoxy-phenolate, and either chelation of the methoxy-quinoid, Type H or monodentate coordination of the demethoxy-quinoid, Type I (figure 13.12).

Examining the spectra of the complexes, there are significant differences between BDMC and the other two curcuminoids, as well as subtle differences between the latter.

On addition of Pb to CURC the β -enol carbonyl at 1599 cm^{-1} undergoes a shift of $\Delta-23$ cm^{-1} , while the band at 1204 cm^{-1} undergoes a shift of $\Delta+15$ cm^{-1} . The β -keto carbonyl at 1727 cm^{-1} and $\nu(\text{C}=\text{C})$ stretch at 1628 cm^{-1} both undergo a shift of $\Delta-10$ cm^{-1} and the band at 1270 undergoes a smaller shift of $\Delta+4$ cm^{-1} . The bands at 1617 cm^{-1} and 1576 cm^{-1} are associated with the neutral enolic form with only monodentate coordination to the metal (Type C). The shifts of the β -keto carbonyl and $\nu(\text{C}=\text{C})$ and the methoxy $\nu(\text{C}-\text{O})$ stretch at 1203 cm^{-1} suggest that there is a slight shift in the tautomer equilibria, with an increase in the quinoidal form.

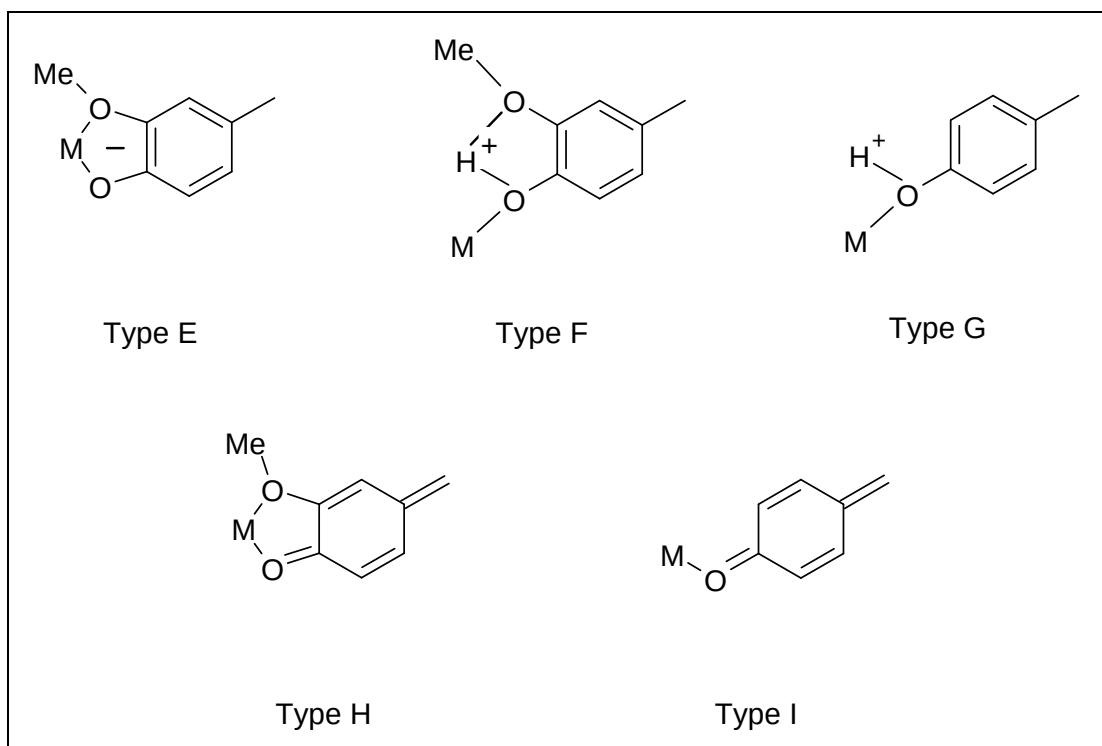


Figure 13.12: Possible metal coordination to the quino-phenol system.

On addition of Pb to DMC the β -enol carbonyl at 1569 cm^{-1} is the only carbonyl to undergo a shift ($\Delta+13\text{ cm}^{-1}$) with a reduction in intensity, indicating the site of interaction is similarly at enolic site. The coupled $\nu(\text{C}=\text{C})$ stretch at 1624 cm^{-1} undergoes a reduction in intensity, and appears as a shoulder on the β -enol carbonyl. Surprisingly, the band at 1510 cm^{-1} (Wilson mode 19a) undergoes a significant shift of $\Delta-15\text{ cm}^{-1}$, with the band at 1163 cm^{-1} undergoing a shift of $\Delta-5\text{ cm}^{-1}$. This behaviour can be explained as a change in the vibrational coupling between the ring mode and a C-H bend (Vársanyi, *et. al.*, 1969), supporting the assignment of the latter as the $\alpha(\text{C-H})$ bend, mode 15. Similarly the trans $\delta(\text{C-H})$ deformation (uniquely amongst the curcuminoids) shows a shift of $\Delta+15\text{ cm}^{-1}$, indicating that a change in the conjugation occurs on complexation.

On addition of Pb to BDMC the medium β -enol carbonyl at 1598 cm^{-1} undergoes a shift of $\Delta-23\text{ cm}^{-1}$ similar to that experienced by curcumin. The two bands at 1159 cm^{-1} and 1100 cm^{-1} undergo a shift of $\Delta-11\text{ cm}^{-1}$ and $\Delta-26\text{ cm}^{-1}$, respectively, with the latter phenolic $\nu(\text{C-O})$ stretch undergoing a reduction in intensity. Two new very intense broad

bands are observed, at 1403 cm^{-1} and at 673 cm^{-1} . This is typical enhancement behaviour found in complexation. The 1403 cm^{-1} is the methoxy (C-O) stretch, shifted to 1100 cm^{-1} . The two different regions associated with its assignment reflect its dependence on the extent of coupling with the in-plane $\delta(\text{C-O-H})$ deformation (Alpert *et. al.*, 1970). In the neutral ligand it is the lower that is regarded as predominantly C-O in character. In the complex it is the higher, as reflected by the gain in intensity of the band. The presence of bands in both regions suggests that metal binding of Type G occurs at only one of the phenolic groups for BDMC.

The infrared spectra therefore suggest that in the solid state there is difference in the nature of metal binding in the curcuminoids. For both CURC and DMC the Pb binds at the β -ketoenolate moiety, with monodentate coordination in the neutral enolic form. For BDMC the Pb binds with monodentate coordination at one neutral demethoxy-phenolate site.

13.5. ELECTROCHEMICAL ANALYSIS

13.5.1. Material and Methods

13.5.1.1. Chemicals and Reagents

The curcuminoids (CURC, DMC and BDMC) were separated and purified as described in chapter 11. These were dissolved in ethanol and made to volume with Milli-Q water. The final concentration of ethanol was 50%. Lead was dissolved in Milli-Q water. pH 4 buffer tablets were purchased from Saarchem, Johannesburg, South Africa.

13.5.1.2. Technique: Adsorptive Stripping Voltammetry

Stock solutions of the curcuminoids and lead were 1mM each. Adsorptive stripping voltammograms (ASVs) were recorded on the Autolab PGSTAT 30 voltammeter equipped with a Metrohm VGA cell stand. The electrochemical analysis experiments were conducted exactly as described in section 10.4.2.2. For all experiments appropriate concentrations of the metal (Pb) and the curcuminoids were introduced into an electrochemical cell. The electrolyte used was prepared by dissolving 1 pH 4 buffer tablet in 100ml of Milli-Q water.

13.5.2. Results

Under the given experimental conditions the system is not well behaved, as evidenced by the variation experienced in the current with respect to different curcuminoid concentrations employed, and to repetition of the experiments. The ASVs of Pb ($1 \times 10^{-5} \text{M}$) with various concentrations of CURC, of DMC and of BDMC at pH4 are presented in figures 13.13, 13.14 and 13.15. A plot of the potential against the curcuminoid concentration as well as a plot of the current response against curcuminoid concentration is presented in figures 13.16 and 13.17 respectively. Upon the addition of CURC or DMC there is an increase in current response and a decrease in the potential. Thereafter increasing concentrations of CURC and DMC show a concentration-dependent increase in current response and decrease in the potential. BDMC initially shows a decrease in the current response and thereafter increasing concentrations causes

and increase in the current. There is initially a sharp decrease in the potential and thereafter increasing concentrations of BDMC shows a gradual decrease in the potential. From the ASV there are two reduction peaks are typically observed on addition of the curcuminoids. The peak observed at -0.92V is the reduction of curcumin and that observed at -1.1V reflects the reduction of the Pb-curcuminoid complex (Daniel *et. al.*, 2004).

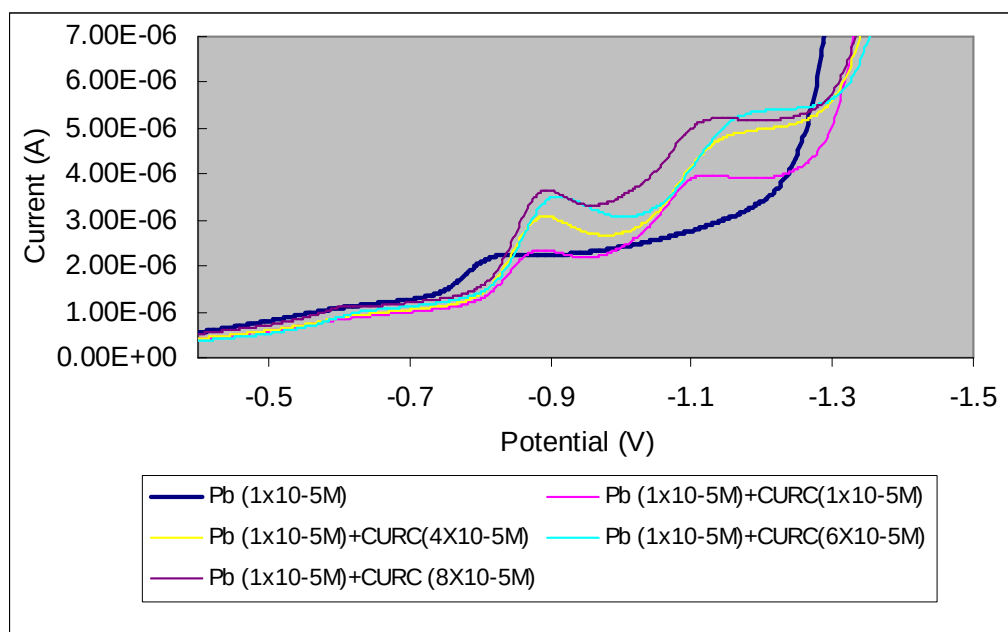


Figure 13.13: ASV of Pb (1×10^{-5} M) alone and in the presence of increasing concentrations of CURC.

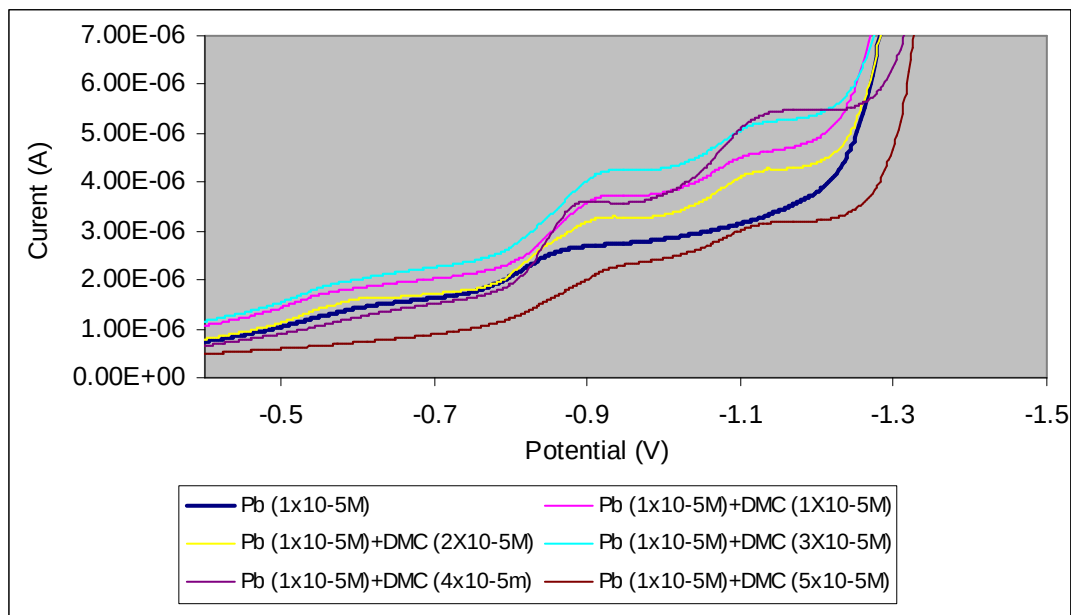


Figure 13.14: ASV of Pb ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of DMC.

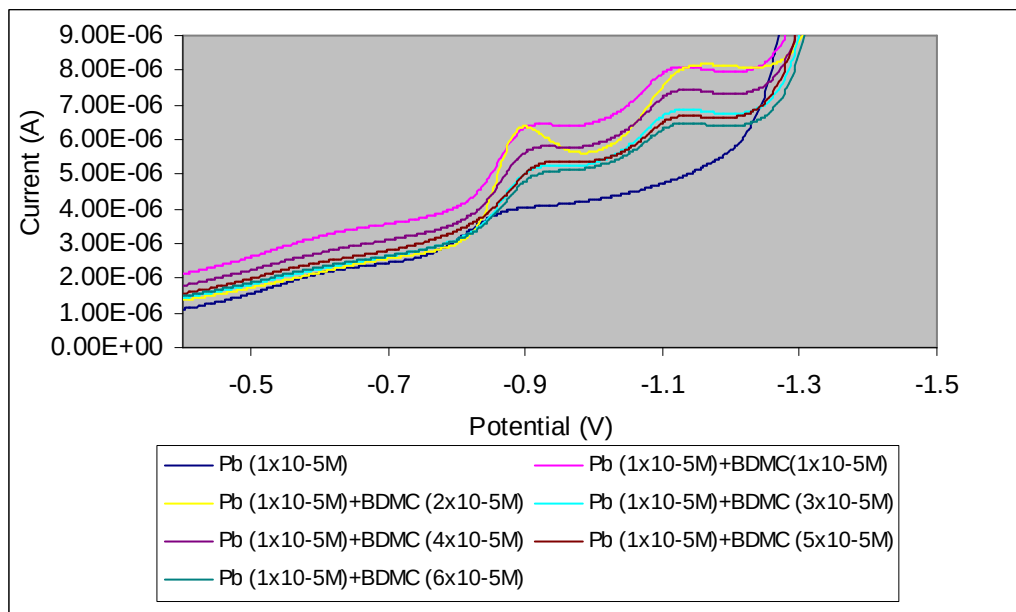


Figure 13.15: ASV of Pb ($1 \times 10^{-5}\text{M}$) alone and in the presence of increasing concentrations of BDMC.

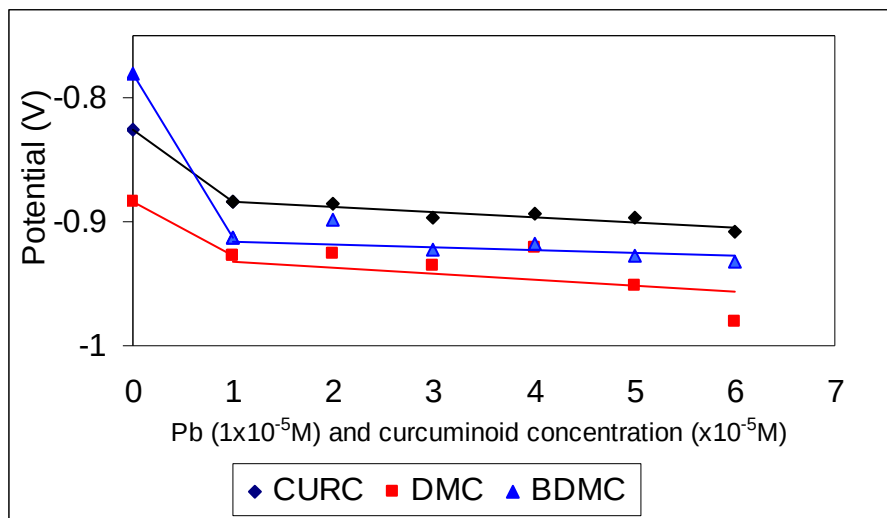


Figure 13.16: Effect of increasing concentrations of the curcuminoids on the potential of 1×10^{-5} M Pb (corrected for baseline).

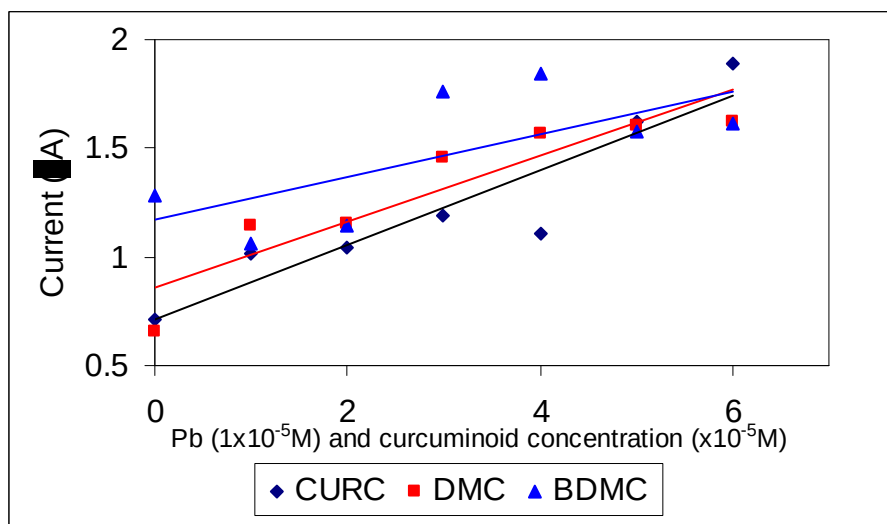


Figure 13.17: Effect of increasing concentrations of the curcuminoids on the current of 1×10^{-5} M Pb (corrected for baseline).

13.5.3. Discussion

Previous NMR and electrochemical studies of curcumin with Ga^{3+} , Fe^{3+} and cadmium (Cd^{2+}) (Borsari *et. al.*, 2002, Daniel *et. al.*, 2004) found a preferred coordination ratio of 1:1, ascribed to solubility or steric considerations associated with curcumin (Borsari *et. al.*, 2002). Using a ten fold larger concentration of Pb with equimolar curcumin (1.2×10^{-5}

⁴M) at pH 4, has previously noted the appearance in the ASV of two new peaks (-1.09 and -1.38 V), indicating the existence of two different species in solution (Daniel *et. al.*, 2004). The large radius of Pb and its possible tetrahedral coordination could possibly favour a coordination ratio of 1:2. Alternatively, with two possible metal species - solvated Pb^{2+} and solvated $\text{Pb}(\text{OH})^+$ - available for binding to the curcuminoids, the most likely complexes in solution could be various protonated forms of PbL^{2+} and $\text{PbL}(\text{OH})^+$. At pH 4 the relative ratio curcumin species ($\text{H}_3\text{L}:\text{H}_2\text{L}^+:\text{HL}^{2+}:\text{L}^{3+}$) would be $1:4.2 \times 10^{-5}:5.5 \times 10^{-11}:1.7 \times 10^{-17}$, favouring the fully protonated species.

At the lower concentrations, even in the excess of curcumin only one metal – complex reduction peak is observed. This suggests that at higher concentration the two observed species must be PbL^{2+} and $\text{PbL}(\text{OH})^+$, and that the species present at $1 \times 10^{-5}\text{M}$ is PbL^{2+} . The appearance, at the lower concentrations, of a curcumin reduction peak even for equimolar concentration, and the difficulties experienced in the metal study indicates a thermodynamic instability of this species at the low concentration, suggesting that the binding to the metal is not particularly strong.

It is apparent that all three of the curcuminoids investigated undergo binding with Pb(II) at pH 4 to form the species PbL^{2+} in solution. The coordination experienced by BDMC is different to those for CURC and DMC. This would account for the different biological properties experience in their ability to reduce the lead depletion of GSH, observed in chapter 12, as well as other biological systems reported in the literature (Ahsan, *et. al.*, 1999; Lee, *et. al.*, 2001; Huang, *et. al.*, 1998). In chapter 12 the neurotoxicity induced by Pb as well as the curcuminoids ability to reduce the neurotoxicity is demonstrated. Apart from the potent antioxidant activity of the curcuminoids, the studies in this chapter show that these agents also bind to Pb, and that the possible action of the curcuminoids to reduce the Pb-induced neurotoxicity may lie in two mechanisms, their antioxidant activity and their metal chelation ability.

CHAPTER 14

SUMMARY OF RESULTS, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

14.1. SUMMARY OF RESULTS

CHAPTER 2: Effect of tolmetin and sulindac on QA, KCN and Fe²⁺-induced lipid peroxidation

The extent of protection offered by tolmetin and sulindac as antioxidants was investigated in chapters 2 and 3. QA, KCN and Fe²⁺ significantly induces LP in rat brain homogenates. Both NSAIDS, tolmetin and sulindac reduce LP induced by these neurotoxins. It is thought that QA induces LP in rat brain homogenate by forming a complex with iron, and it is the QA-iron complex that generates free radicals and causes LP (Stipek *et al.* 1997). The possibility of the NSAIDS binding to Fe²⁺ and Fe³⁺ and reducing the QA and Fe²⁺-induced LP in rat brain homogenate was investigated in chapter 10. Both NSAIDS also reduce the QA-induced LP in primary hippocampal neurons.

CHAPTER 3: Superoxide anion scavenging activity of tolmetin and sulindac

This chapter demonstrates that QA does not induce superoxide anion generation in rat brain homogenate. It is thought that since the integrity of the cell is lost during homogenisation, QA could not stimulate receptors, especially of the NMDA subtype, and generate free radicals such as the superoxide anion. However, QA induces superoxide anion generation in primary hippocampal neurons in a concentration-dependent manner. KCN, a complex IV inhibitor of the electron transport chain induces superoxide anion generation in rat brain homogenates. The co-incubation of these neurons and homogenates with tolmetin or sulindac demonstrate that both NSAIDS were either reducing the generation or scavenging the superoxide anion. The superoxide anion was generated non-enzymatically by the NADPH assay which confirmed that the NSAIDS are scavenging the superoxide anion.

CHAPTER 4: Effect of tolmetin, sulindac and QA on hippocampal neurons

Since chapters 2 and 3 demonstrate that tolmetin and sulindac have antioxidant and free radical scavenging properties, it was decided to determine if these agents could afford protection to rat hippocampal neurons after QA-induced structural damage. Animals were treated with tolmetin or sulindac at a dose of 5mg/kg/bd for 5 days after which they received unilateral intrahippocampal injections of QA (0.6 μ moles in 2 μ l PBS). Histological investigation of neurons in the CA1 and CA3 regions show visible destruction of the structural features of these regions in rats that received QA and significant protection in rats that were pre-treated with either tolmetin or sulindac. QA also induces apoptosis in rat hippocampus but this is attenuated by the treatment of the animals with the NSAIDS. Receptor binding studies were conducted to give an indication of the extent of protection offered by the NSAIDS. In this study, a reduction in the number of NMDA receptors after intrahippocampal administration of QA implied a reduction in the number of viable neurons in the hippocampus. Whilst such a relationship cannot be conclusive in itself, there is a causal relationship between the two. QA significantly ($p < 0.001$) reduces the number of viable neurons in the hippocampus but the treatment of animals with the NSAIDS reduce the neuronal loss. Tolmetin and sulindac alone had no effect on the number of NMDA binding sites. QA also reduces the viability of primary hippocampal neurons in a concentration-dependent manner. The viability of the neurons was enhanced by the co-incubation of the neurons with either tolmetin or sulindac. The antioxidant and anti-inflammatory properties of these agents maintain the integrity of the neurons. It was postulated that the anti-inflammatory properties of these agents also reduce free radical generation and that the pre-treatment with NSAIDS may have also increased the circulating levels of SOD.

CHPATER 5: Effect of tolmetin, sulindac and QA on rat spatial reference memory

In the previous chapter, histological investigations show that the pre-treatment of animals with NSAIDS offer neuroprotection. The following series of experiments were conducted to determine if NSAID treatment after QA insults could also offer neuroprotection. Animals received unilateral intrahippocampal injections of QA (0.6 μ moles in 2 μ l PBS)

and were thereafter treated with tolmetin and sulindac at a dose of 5mg/kg/bd for 5 days. The animal's spatial reference memory was measured in a water tank over 14 days. QA significantly affects the animal's performance in the water maze since animals that receive intrahippocampal injections of QA have longer escape latencies when compared to the control group. Tolmetin and sulindac treated animals have faster escape latencies compared to the untreated animals that also received intrahippocampal injections of QA. Sulindac treated animals had faster escape latencies when compared to tolmetin treated animals. This experiment demonstrates that the NSAIDS offer protection against QA-induced neurotoxicity and reduced the QA-induced memory deficits.

CHAPTER 6: Effect of tolmetin, sulindac and QA on rat hippocampal glutathione content

The previous experiments demonstrate that the NSAIDS offer protection to rat hippocampal neurons. The following experiments were conducted to identify other possible mechanisms through which these agents offer protection and prevent QA-induced spatial memory deficits. Animals were treated as described in chapter 5. Some animals were sacrificed immediately after treatment and were referred to as the day = 1 rats while some animals were sacrificed after monitoring their performance in the water maze. i.e. 14 days later and were referred to as the day = 14 rats. The hippocampi were removed and examined for the glutathione content. The protein carbonyl content was measured in chapter 7. Animals sacrificed immediately after treatment as well as those animals that were sacrificed 14 days later, have less GSH compared to controls. Animals that were treated with tolmetin and sulindac have more GSH in the hippocampus compared to animals that only received intrahippocampal injections of QA. It was thought that the NSAIDS scavenge free radicals that were generated and aids GSH and prevents its depletion in the hippocampus. This may also be a possible mechanism through which the NSAIDS reduced QA-induced spatial memory deficits since it has been shown that GSH is important for the acquisition of spatial memory in maze tasks (Cruz-Aguado *et. al.*, 2000).

CHAPTER 7: Effect of tolmetin, sulindac and QA on rat hippocampal proteins

Animals were treated as described in chapters 5 and 6. The hippocampi were removed and examined for the amount of proteins that were oxidised. Animals sacrificed immediately after treatment as well as those animals that were sacrificed 14 days later, have elevated protein carbonyl levels. Animals that were treated with tolmetin and sulindac have less oxidised proteins in the hippocampus. Thus far the NSAIDS have demonstrated the potential to reduce LP, scavenge the superoxide anion, attenuate QA-induced apoptosis, reduce QA-induced GSH depletion and reduce QA-induced protein oxidation. These may be possible mechanisms through which tolmetin and sulindac protect rat hippocampal neurons and reduce QA-induced memory deficits.

CHAPTER 8: Effect of tolmetin and sulindac on liver enzyme tryptophan 2,3-dioxygenase activity

Liver tryptophan 2,3-dioxygenase is an important enzyme in the metabolism of tryptophan, a precursor of 5HT synthesis. *In vitro* studies demonstrates that co-incubation of liver homogenates with tolmetin (10 and 20 μ M) reduces the apoenzyme and the total activity of the enzyme. Sulindac reduces the apoenzyme activity at 20 μ M and reduces the total activity of the enzyme at both concentrations. The treatment of animals with tolmetin or sulindac (5mg/kg/bd for 5 days) decreases the holoenzyme activity as well as the total activity of tryptophan 2,3-dioxygenase. Since this enzyme activity is reduced, it is possible that more tryptophan is available for serotonin synthesis. IDO is another enzyme responsible for tryptophan metabolism, but this enzyme uses the superoxide anion as its cofactor (Hirata & Hayaishi 1975; Taniguchi *et. al.*, 1977). Since tolmetin and sulindac demonstrate the ability to scavenge the superoxide anion, these agents may also reduce IDO activity, amplify circulating tryptophan levels and cause more serotonin to be synthesised. The amount of serotonin and other biogenic amine levels were measured in the following chapter. The inhibition of TDO and possible inhibition of IDO may also reduce the synthesis of the excitatory amino acid, QA.

CHAPTER 9: Effect of tolmetin and sulindac on brain biogenic amine levels

The hippocampi and striatum from animals that were treated with tolmetin or sulindac in chapter 8 were removed and analysed for the 5HT, DA, HIAA and DOPAC levels. The pineal glands were removed and incubated for 24 hours and the amount of melatonin produced by the pineal glands was also measured. Both NSAIDS increase the production of serotonin in the hippocampus. HIAA is also higher in tolmetin treated animals. The NSAIDS show no effect on DA and DOPAC levels in the hippocampus. Tolmetin and sulindac also significantly reduce DA levels in the striatum. Tolmetin treated animals also show increased amounts of DOPAC in the striatum. The amount of melatonin produced by pineal glands from those animals treated with tolmetin or sulindac show that tolmetin but not sulindac increases that amount of melatonin synthesised by the pineal gland. This increase was thought to be as a result of tolmetin's effect on the melatonin synthesis pathway in the pineal gland rather than the inhibition of TDO.

CHAPTER 10: Fe²⁺ and Fe³⁺ binding properties of tolmetin and sulindac

Chapter 2 demonstrates that both tolmetin and sulindac reduces QA and Fe²⁺-induced LP in rat brain homogenate. The possibility of tolmetin and sulindac binding to Fe²⁺ and reducing the generation of the ·OH radical through the Fenton reaction was proposed as a possible mechanism through which these NSAIDS reduce LP. The UV/VIS data demonstrates that in solution, tolmetin interacts with Fe³⁺ and not Fe²⁺ while sulindac interacts with Fe²⁺ more than Fe³⁺. The IR analysis demonstrates that sulindac has equal affinity for Fe²⁺ and Fe³⁺ while tolmetin has a greater affinity for Fe²⁺ and metal binding occurs in a unidentate manner via the C=O group. The ASVs from electrochemical analysis confirms the metal binding and demonstrates that sulindac binds Fe³⁺ more than tolmetin. Sulindac upon complexation with Fe³⁺ forms 2 metal species on the GCE while tolmetin forms only 1 metal species. This chapter proves that the NSAIDS bind Fe²⁺ as well as Fe³⁺ and prevents the redox cycling of this transition metal and reduces Fe²⁺-induced LP. Tolmetin and sulindac may have also reduced QA-induced LP by binding to iron, thus reducing the formation of the QA-iron complex, and thereby reducing the generation of free radicals.

CHAPTER 11: Separation, isolation and purification of the curcuminoids

The curcuminoids had to be separated out of curcumin (65% pure) since pure curcumin is very expensive and DMC and BDMC are commercially unavailable. TLC was employed to separate the curcuminoids. A mobile phase consisting of dichloromethane : MeOH in a ratio of 97:3 separated the curcuminoids. After isolation, the purity of each curcuminoid was confirmed by TLC, NMR and MS. The lambda max of each curcuminoid was also determined and was consistent with published literature.

CHAPTER 12: Effect of the curcuminoids on lead (Pb)-induced neurotoxicity

Pb induces LP and reduces primary hippocampal neuron viability in a time and concentration-dependent manner. The co-incubation of the neurons with the curcuminoids reduces LP and enhances cell viability. CURC demonstrates to be the most potent antioxidant and BDMC the weakest. The ability of the curcuminoids to bind Pb and prevent this heavy metal from inducing neurotoxicity was investigated in chapter 13. Animals were injected intraperitoneally with Pb (20mg/kg/od for 5 days) while some animals were treated with the curcuminoids at a dose of 30mg/kg/bd for 5 days. Some animals were sacrificed immediately after the treatment protocol (day = 1 rats) while the rest of the animals were sacrificed after the water maze tasks (day = 14 rats). The hippocampi were removed and examined for the GSH and protein carbonyl content. Performance in the water maze task demonstrates that Pb significantly reduces the spatial reference memory of male Wistar rats. CURC and DMC treated animals have significantly faster escape latencies compared to untreated, Pb dosed rats. BDMC does not prevent the Pb-induced memory deficits. Pb also significantly reduces the GSH content in rat hippocampi (measured in day = 1 rats). CURC and DMC but not BDMC reduces the GSH depletion. It was noticed that the GSH levels had normalised 14 days later in all groups. Pb also increases protein oxidation in rat hippocampus while treatment with the curcuminoids reduces the Pb-induced protein oxidation. CURC demonstrates to be the most neuroprotective and BDMC demonstrates to be the weakest. The anti-inflammatory, antioxidant and metal binding properties were considered to be the mechanisms through which the curcuminoids reduce Pb-induced neurotoxicity.

CHAPTER 13: Lead (Pb) binding properties of the curcuminoids

Chapter 12 shows that Pb induces LP and decreases the viability of primary hippocampal neurons in a concentration-dependent and time-dependent manner. Pb also induces spatial memory deficits, causes the depletion of GSH and induces hippocampal protein oxidation. It was thought that the antioxidant properties of the curcuminoids were a possible mechanism through which these agents reduce the Pb-induced neurotoxicity. Another possible mechanism suggested was that, the curcuminoids bound to Pb and prevented this heavy metal from inducing neurotoxicity. The UV/VIS study suggests that CURC and DMC but not BDMC complexes with Pb. The fluorescence spectroscopy demonstrates that all three curcuminoids interact with Pb and the nature of this interaction was confirmed by the IR studies. The electrochemical analysis also demonstrates that the curcuminoids interact with Pb at pH = 4. This chapter demonstrates that the curcuminoids interact with Pb and is also another possible mechanism through which these agents reduce the Pb-induced neurotoxicity.

14.2. CONCLUSIONS

There are multiple factors that contribute to the pathogenesis of AD, PD and many other neurodegenerative disorders. Some of these factors include aging (McNeal *et. al.*, 2001; Floyd & Hensley 2002), excitotoxicity (Choi 1985; Rothman & Olney 1987), free radicals (Gutteridge 1995; Floyd 1999; McCord 2000), neuroinflammation (Matyszak 1998; Allan & Rothwell 2001; Hartmann *et. al.*, 2003) and an imbalance in metal homeostasis (Bush 2000). Environmental factors may also contribute to the pathogenesis of these neurodegenerative disorders (Semchuck *et. al.*, 1992; Liou *et. al.*, 1997; Donato *et. al.*, 2002). There is also a genetic component to neurodegenerative disorders (Breitner *et. al.*, 1988; Lautenschlager *et. al.*, 1996; Conway *et. al.*, 1998).

Thus far there is no cure for these debilitating diseases and current therapeutic strategies aims to delay the onset and slow down the progression of the diseases. This study has demonstrated that anti-inflammatory agents have the potential to be used in the treatment or prevention of neurological disorders.

Tolmetin has shown to possess antioxidant and metal binding properties. The anti-inflammatory effects of this NSAID may have also contributed towards the neuroprotection that this agent has offered. Tolmetin reduces LP, scavenges the O_2^- , reduces GSH depletion, reduces protein oxidation, protects rat hippocampal neurons against QA-induced neurotoxicity and subsequently reduces QA-induced spatial memory deficits. This NSAID also binds Fe^{2+} and Fe^{3+} and reduces Fe^{2+} -induced LP. Sulindac has also shown to possess antioxidant and metal binding properties. Sulindac has shown to offer neuroprotection in the exact manner as tolmetin did. However, this study did not correlate with a previous report that demonstrated *in vitro* that sulindac is a more potent antioxidant than tolmetin (Fernandes *et. al.*, 2004). However, sulindac did demonstrate to reduce QA-induced spatial reference memory deficits more than tolmetin. The treatment of animals before and after QA-induced neurotoxicity offers neuroprotection. Sulindac bound to iron (Fe^{2+} and Fe^{3+}) while tolmetin demonstrated weaker interactions with this transition metal.

Both tolmetin and sulindac inhibit TDO and increase 5-HT levels in the hippocampus. Tolmetin but not sulindac increase melatonin, the well established neuroprotectant produced by the pineal gland. This may be another mechanism through which tolmetin may offer neuroprotection although it was stated that this increase is not due to TDO inhibition. Both these agents also reduced DA levels in the striatum. The inhibition of TDO and possible inhibition of IDO could reduce the synthesis of QA. This may also be a mechanism through which these agents reduce excitotoxicity and subsequent neurodegeneration.

Therefore these NSAIDS may offer neuroprotection and may delay the onset and possibly reduce the progression of most neurodegenerative disorders such as AD. However these agents have to be used with caution in patients suffering with PD. Since these agents are well tolerated by the elderly (Ehrlich 1983; O'Brien 1983; Janke *et. al.*, 1984), they could be used for long periods of time since the chronic use of NSAIDS is known to reduce the risk of AD (Stewart *et. al.*, 1997).

Apart from drug therapy, nutritional approaches must also be considered as an approach in the treatment of neurodegenerative disorders (Deschamps *et. al.*, 2001; Youdim & Joseph 2001). Thus far curcumin, the active agent in turmeric has demonstrated to possess excellent neuroprotective properties. This study also demonstrates that the 3 major active ingredients in turmeric possess antioxidant and metal binding properties. These components of turmeric enhance primary hippocampal neuron viability that was reduced by Pb, reduces GSH depletion and protein oxidation in rat hippocampus. CURC and DMC also reduce Pb-induced spatial memory deficits. It has been thought that the methoxy group on the phenyl ring and the 1,4-diketone system is important for antioxidant activity (Sreejayan *et. al.*, 1996). This study confirms that CURC was the stronger antioxidant and BDMC was the weakest antioxidant. It has recently been reported that a metal chelating agent should preferably possess antioxidant properties as well (Tandon *et. al.*, 2002). The curcuminoids have demonstrated to possess excellent antioxidant and metal binding properties and therefore may be used in metal poisoning especially in Pb poisoning cases. Turmeric may also be used to delay the onset and slow down the progression of neurodegenerative disorders. This spice may also prevent the onset of neurodegenerative disorders.

14.3. RECOMMENDATIONS FOR FUTURE STUDIES

Chapter 2 shows that KCN induces LP in rat brain homogenate and this is reduced by the co-incubation of the homogenate with the NSAIDS. Tolmetin and sulindac may reduce the KCN-induced LP by scavenging the free radicals generated. It was also thought that these agents induce calcium efflux in the mitochondria or prevent the inhibition of the mitochondrial enzyme a_1a_3 . Further research needs to be conducted on the effects of NSAIDS on the mitochondria. This may also demonstrate whether the NSAIDS have the potential to prevent or reduce the generation of the superoxide anion.

The NSAIDS also reduce QA-induced hippocampal neurotoxicity. It is known that excitotoxicity causes intracellular Ca^{2+} levels to be elevated (Choi 1985; Choi 1992; Dubinsky 1993; Lipton & Rosenberg 1994) which subsequently leads to free radical generation (Choi 1992; Dykens 1994; Whit & Reynolds 1996). Apart from scavenging

the free radicals generated, these NSAIDS may also reduce the intracellular Ca^{2+} concentrations and reduce the excitotoxicity. These effects need to be further explored.

Tolmetin and sulindac reduce the DA levels in the striatum. It needs to be established whether this occurs in a dose-dependent manner and the mechanism through which these agents reduce DA levels needs to be established. Tolmetin increases melatonin synthesis. It is unlikely that this is due to inhibition of liver 2,3-dioxygenase but rather a direct effect on the pineal gland. More research needs to be conducted on the effect of NSAIDS on rat pineal indole metabolism.

Another area for further research would be an investigation into the molecular mechanisms by which tolmetin, sulindac and curcumin offer neuroprotection. It has been suggested that aspirin may directly suppress the nuclear factor- κB inhibitor kinase (Yin *et. al.*, 1998) and results in the inhibition of nuclear factor- κB and neuronal death (Grilli *et. al.*, 1996). It has also been shown that indomethacin and ibuprofen affect peroxisome proliferator-activated receptors (Lehmann *et. al.*, 1997) and inhibit tumor necrosis factor- α production (Jiang *et. al.*, 1998). These agents also alter γ -secretase activity and reduce the production of $\text{A}\beta_{42}$ (Lim *et. al.*, 2000; Weggen *et. al.*, 2001). Research needs to be conducted to determine if tolmetin, sulindac and curcumin also have the same effects of the aspirin, indomethacin and ibuprofen. This may provide a clear understanding or a direct mechanism by which the NSAIDS attenuate QA-induced apoptosis.

Although Mesches and co-workers demonstrated that sulindac increases the NMDA receptor subunit levels, this study (experiment 4.4) did not show that this NSAID administered to rats (without i.h. quinolinic acid injections) had any potential to increase the number of NMDA receptors. The effect of NSAIDS on neurogenesis needs to be further explored.

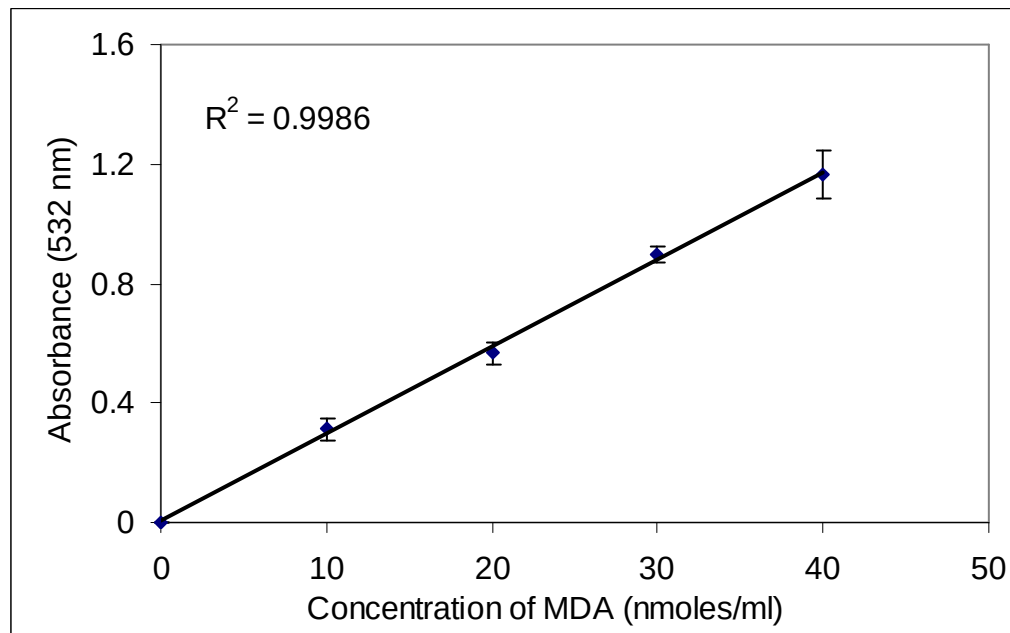
The use of transgenic mouse models may also provide an indication of the possible neuroprotective effects of tolmetin and sulindac since it has been shown that curcumin

reduces oxidative damage and amyloid pathology in Alzheimer transgenic mouse models (Lim *et. al.*, 2001).

Pharmaceutical companies are constantly searching for agents that may aid in the treatment or prevent the onset of debilitating neurodegenerative diseases. Thus far research demonstrates that turmeric possesses potential as a neuroprotectant and the evidence accumulated is sufficient to advocate its advancement into phase II clinical trials. The neuroprotective effects of garlic and ginger, common components of Indian curries, also needs to be further explored.

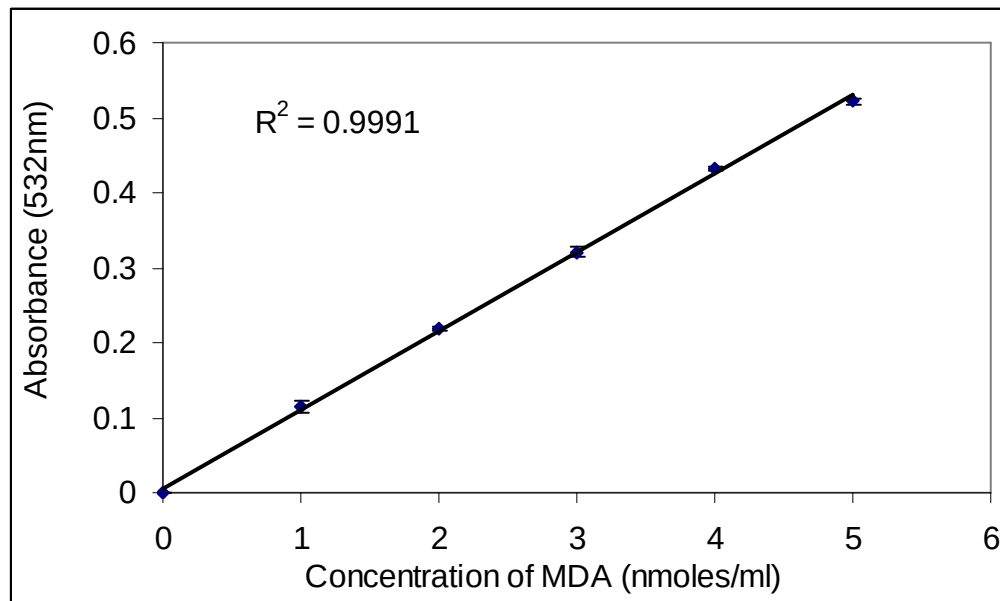
APPENDICES

APPENDIX 1



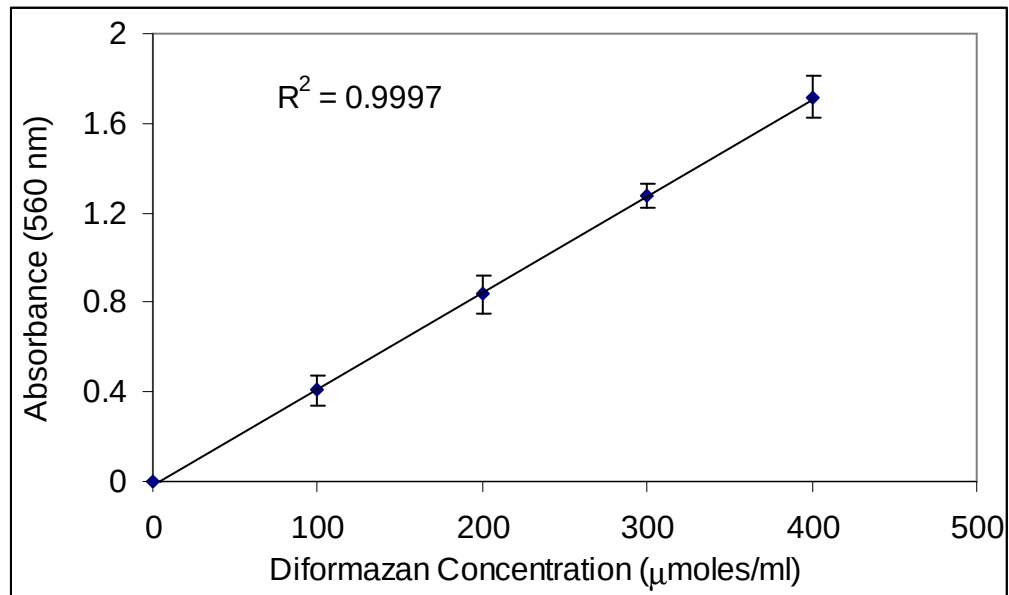
Malondialdehyde Standard Curve (used for rat brain homogenate experiments).

APPENDIX 2



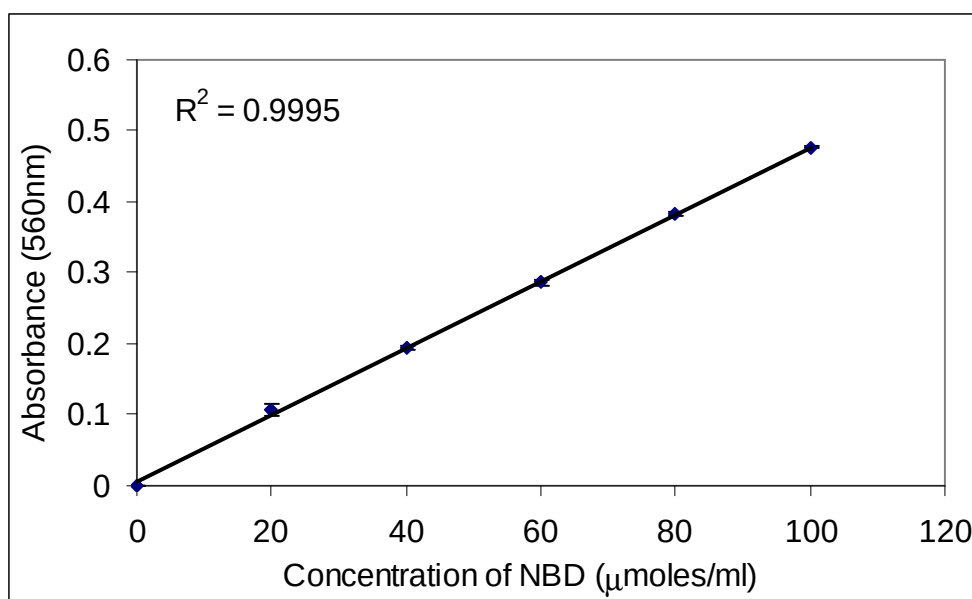
Malondialdehyde Standard Curve (used for cell culture experiments).

APPENDIX 3



Diformazan Standard Curve (used for rat brain homogenate experiments).

APPENDIX 4



Diformazan Standard Curve (used for cell culture experiments).

APPENDIX 5

The following steps fully describe the histological techniques that were performed in chapter 4.

Histological Techniques

i. Fixing the brain

The animals were sacrificed and the brains were removed as described in section 2.2.2.3. The brains were immediately stored in a fixation medium, which consisted of formaldehyde (30%), glacial acetic acid and ethanol (2:1:7 v/v), for 48 hours. Coronal sections were then cut from the rat forebrain.

ii. Tissue Dehydration and Embedding

The tissue was dehydrated (using increasing concentrations of ethanol), followed by the removal of the ethanol using xylene. Finally the tissue was immersed in paraffin wax, which removed the xylene. The steps carried out are shown in the table below.

Procedure for embedding brains in paraffin wax.

Step	Processing Agent	Time
1	70% Ethanol	1 hour
2	90% Ethanol	1 hour
3	Absolute Ethanol I	1 hour
4	Absolute Ethanol II	1 hour
5	Xylene I	1 hour
6	Xylene II	1 hour
7	Melted Paraffin Wax (57°C)	1 hour

iii. Blocking Out

The brain material was fixed into a block so that it could be cut with a microtome blade. An ice tray was used as the mould. This was coated with lubricating gel to prevent the wax block sticking to the ice tray. A little wax (10ml) was added to the ice tray and the surface of the wax was allowed to harden. The brain section was removed from the melted paraffin wax (step 7 above) and gently kept in the ice tray. The brain section was then completely covered in paraffin wax. The ice tray was then immersed in a large dish of cold water. This was to enable the wax to solidify quickly, and prevent the formation of crystals that might have disrupted the tissue. The blocks were left overnight in cold water to ensure that the wax had completely solidified.

iv. Sectioning

The wax block was trimmed with a razor blade so that two of the sides were parallel, while the other two converged slightly. The sides were cut so as to leave about 2-3 mm of wax around the tissue. The wax block was attached to a small wooden block with a small amount of molten wax. Sectioning was done using a rotary microtome. The microtome was set to cut sections of 10 μ m thickness. As sections were cut they would stick to one another, so as to form long ribbons. When the part of the brain containing the hippocampus was reached, sections were removed and placed in a water bath (40°C) using forceps.

v. Transferring Sections to Slides

Microscope slides containing a thin layer of Haupt's adhesive agent were dipped into the water bath so that 4-5 sections at a time were removed onto the slide. The slides were left overnight in an oven at 40°C that was saturated with formalin

vi. Cresyl Violet Staining

The sections were Nissl stained using cresyl violet. This stains Nissl substances intense purple, the nuclei purple, and leaves the background clear (Bauer *et. al.*, 1974). Before the section could be stained, it first had to be dewaxed and rehydrated as the stain is water soluble. This was done as described in the table below.

Procedure for dewaxing and re-hydrating brain sections.

Step	Processing Agent	Time
1	Xylene I	5 minutes
2	Xylene II	5 minutes
3	Xylene/Absolute Ethanol	5 minutes
4	Absolute Ethanol	5 minutes
5	Absolute Ethanol	Overnight at 30°C

Sections were stained by placing the slides in a 0.1% cresyl violet solution for 2 hours. The slides were differentiated rapidly in 95% ethanol by rinsing in a flat dish until the background was clear. Sections were then dehydrated again as shown in the table below.

Procedure for dehydrating brain sections after staining

Step	Processing Agent	Time
1	Absolute Ethanol I	5 minutes
2	Absolute Ethanol II	5 minutes
3	Xylene I	5 minutes
4	Xylene II	5 minutes

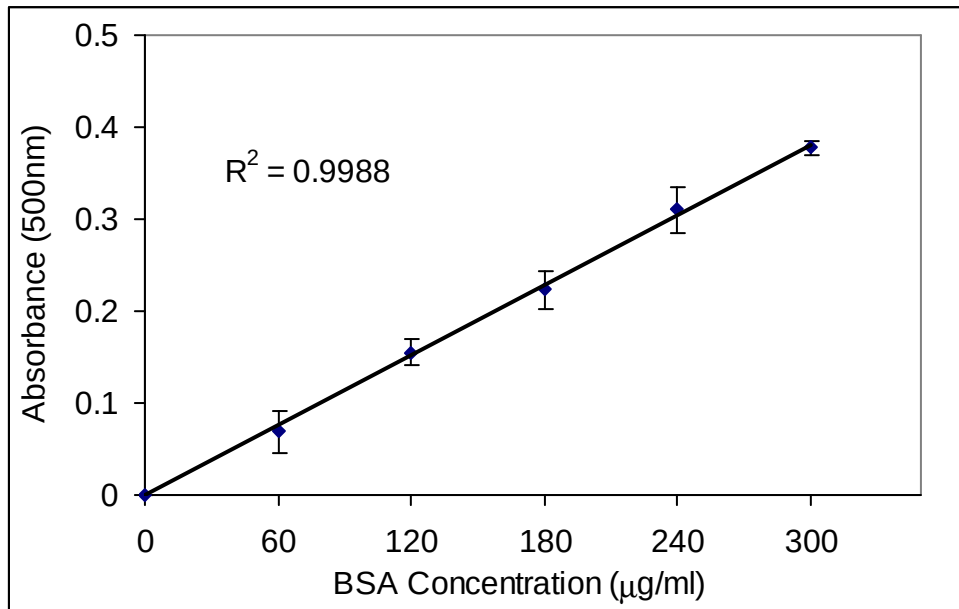
vi. Mounting the Slides

The slides were removed from the xylene (step 4 above) and enough D.P.X was added to cover the stained tissue. A cover slip was placed over the stained tissue and pressed so that all possible air bubbles were removed. The slides were allowed to dry on a flat surface for 48 hours.

vii. Photo-microscopy

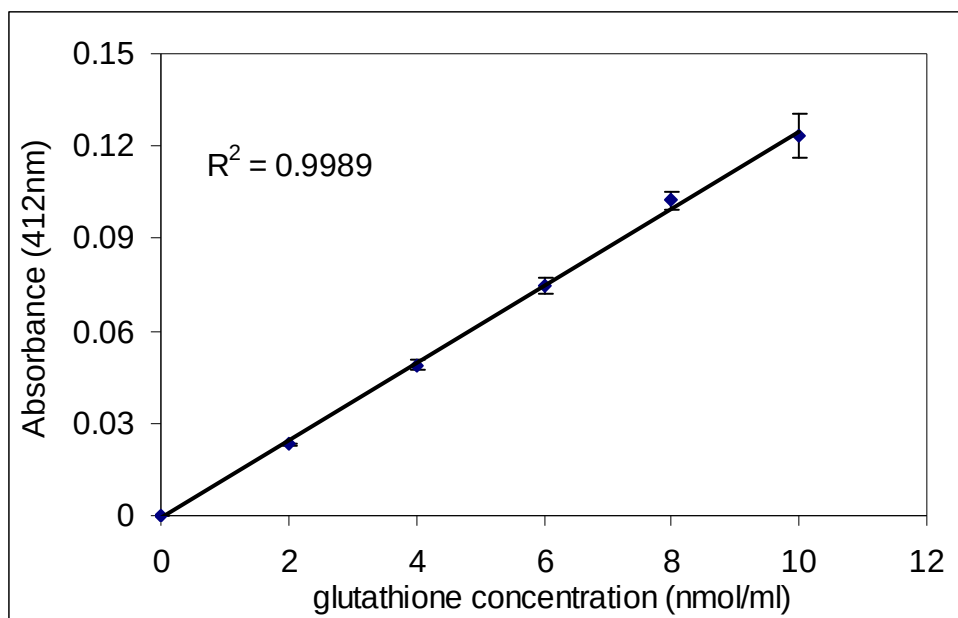
Slides were viewed and photographed using a bright light microscope fitted with a digital Olympus camera.

APPENDIX 6



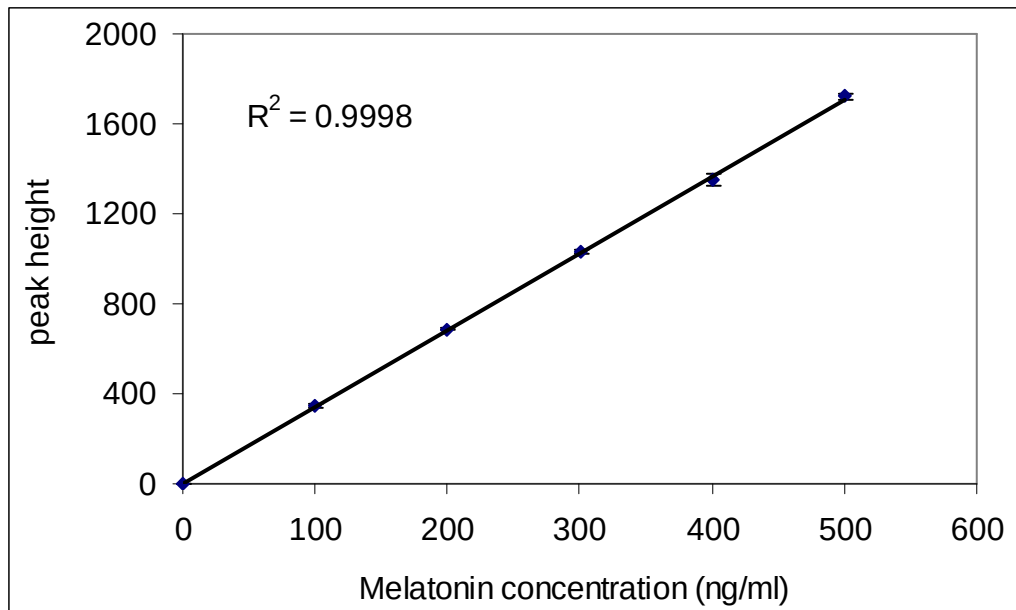
Protein Standard Curve

APPENDIX 7



Glutathione Standard Curve

APPENDIX 8



Melatonin Standard Curve

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