# KIDNEY STONE RARITY IN SOUTH AFRICA'S BLACK POPULATION: INVESTIGATION OF THE BIOCHEMICAL AND PHYSICO-CHEMICAL PROPERTIES OF TAMM HORSFALL MUCOPROTEIN AS A POSSIBLE CONTRIBUTORY FACTOR.

**TRACY-ANN CRAIG**
BSc (Hons) MSc (Chem) (UCT)



Thesis Presented for the Degree of

**Doctor of Philosophy**

In the Department of Chemistry

University of Cape Town

February 2004

# ABSTRACT

Tamm Horsfall mucoprotein (THP) is a powerful inhibitor of calcium oxalate crystallisation. Since urolithiasis in South African blacks is extremely rare, this study was undertaken to compare the relative inhibitory and biochemical properties of this protein in South African black and white healthy and stone-forming male subjects.

THP was isolated by salt precipitation, purified by column chromatography and verified by electrophoresis on SDS-polyacrylamide gels. MALDI-TOF mass spectroscopy was used to determine the molecular weights of the protein from each group and their respective amino acids were analysed. All four THP groups were subjected to tryptic finger printing digestion and matched those of the database. The secondary structures of the four THP proteins were also determined using circular dichroism. The carbohydrate moeities of the four THP groups were analysed for N- and O-linked oligosaccharides.

THP from each of the four groups were subjected to a series of crystallisation experiments using a crossover design in which the protein was added to the urine from which it had been originally isolated, as well as to the urines from the other three groups. This process generated a 4x4 protein-urine grid for investigation of the inhibitory properties of THP in different urine environments. Calcium oxalate crystallization was induced by administration of aqueous sodium oxalate and was monitored using a Coulter Counter. In another series of experiments $^{14}$C-oxalate was used to initiate crystallization. Monitoring in these experiments was achieved by scintillation counter techniques. Calcium oxalate crystal aggregation was investigated in the presence and absence of THP using zeta potential measurements and crystal sedimentation experiments.

The biochemical characterization revealed that BNTHP was indeed different in its properties when compared to the other three THP groups. It had a larger molecular weight and different percentages of amino acids. Tryptic digestion of the four THP groups indicated that peptides of different masses were present in BNTHP. The circular dichroism revealed that the secondary structure of the BNTHP appeared to be different with smaller percentages of α-helical structures and larger percentages of β-sheet structures than the other three THP groups.

The carbohydrate moities from each THP group were investigated and differences between BNTHP and the other three groups were noted. The complete glycan pool for BNTHP appeared to be different with a lower amount of Manose 6 and after enzymatic digestions with an array of enzymes, the only sugar residues in BNTHP were Manose 3 and 5. The GalNAc residues in BNTHP also appear to have a different pattern, which could be due to differences in the expression levels or activities in the processing enzymes present in the cells. Differences were also noted in the site occupancy between NTHP and SFTHP for both race groups and the NTHP appeared to contain a higher percentage of charged sugar residues as opposed to the SFTHP where these values were greatly reduced or virtually non-existent.

The crystallization experiments involving the various protein-urine combinations revealed that the composition of urine plays a synergistic role in influenceing the behaviour of the THP protein. Urine from black subjects provides a superior milieu than white subjects and appears to induce an inhibitory role upon THP. This is indicated in the urine from white healthy subjects (WNU), where both normal proteins retarded the nucleation rate while in urine from black healthy subjects (BNU) the proteins increased the rate. The latter effect is regarded as favourable as it reduces supersaturation rapidly. Protein from white healthy subjects (WNTHP) promoted growth rates in its own urine (WNU) but inhibited the rate in BNU. Sedimentation data showed that protein from black healthy subjects (BNTHP) is a superior inhibitor of aggregation. Data from zeta potential (Zp) experiments supported these results.

Irrespective of race or the environment, the THP protein was found to be an inhibitor of calcium oxalate crystal deposition as well as a strong inhibitor of crystal aggregation. On the other hand, SFTHP appears to have some abnormality with regards to its properties or structure. When the two race groups were compared, it was determined that the black subjects were superior to that of the whites.

Thus in conclusion, it appears that the above-mentioned findings lend support to the hypothesis that this protein may play a role in the low incidence of kidney stones within the black population.

# CONFERENCE PROCEEDINGS AND PRESENTATIONS

**1). Inhibitory properties of Tamm Horsfall mucoprotein isolated from two different population groups**

T-A. Craig; W.Brandt; A.L.Rodgers
KIDNEY STONES, Proceeding of the 8th European Symposium on Urolithiasis, Institute of Semeiotica Medica, Parma, Italy, 9-12 June 1999, edited by L.Borghi; T.Meschi; A.Briganti; T.Schianchi; A.Novarini, pp261-263.

**2). Comparison of Tamm Horsfall mucoprotein (THP) in normal and stone-forming Caucasian and African males in South Africa**

T-A. Craig; A.L.Rodgers; W.Brandt;
UROLITHIASIS 2000, Proceeding of the 9th International Symposium on Urolithiasis, University of Cape Town, 13-17 February 2000, edited by A.L.Rodgers; B.E.Hibbert; B.Hess; S.R.Khan; G.M.Preminger, pp119-120

**3). Inhibition of Calcium Oxalate Crystallisation by Tamm Horsfall mucoprotein (THP) from two different population groups**

T-A. Craig; A.L.Rodgers; W.Brandt;
EUROLITHIASIS, Proceeding of the 9th European Symposium on Urolithiasis, Erasmus University of Rotterdam, Rotterdam, The Netherlands, 13-15 September 2001, edited by D.Kok; H.C.Romijn; P.C.M.S.Verhagen; C.F.Verkoelen, pp59-60

# ACKNOWLEDGEMENTS

I would like to thank:

- **Prof. Allen Rodgers,** Department of Chemistry, for inviting me into his research laboratory and for his supervision, enthusiasm and guidance throughout this study and the writing of this dissertation
- **Prof George Lindsey and Prof Wolf Brandt,** Department of Molecular and Cell Biology, for their assistance in the research of this study
- **Dr.P.M.Rudd**, Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, UK for the assitance of investigation of the carbohydrate moieties of THP from the four different groups
- **Catherine Radcliffe,** Department of Glycobiology, University of Oxford, for her assistance in the interpretation of the data
- **Dr Dawn Webber,** for help with interpretation of data, writing and proof reading of chapter 3
- **Dr.E.Sturrock,** Department of Medical Biochemistry, for his supervision of the interpretation of the data and assistance in the writing of chapter 3 of this dissertation
- **Dr.N.Ravenscroft,** Department of Chemistry, for his assistance with the NMR analysis and the writing of chapter 3 of this dissertation
- **Prof.H.Klump,** Department of Molecular and Cell Biology, for allowing me to perform CD studies in his laboratory and for his help in the writing of chapter 2.
- **Madhu Chauhan,** Department of Molecular and Cell Biology, for her technical assitance in running the CD spectra

- **Diane Pinnock,** Department of Chemistry, for her help and technical assistance with urinalysis of the urines
- **Pat Thompson,** Department of Molecular and Cell Biology, for her help and technical assistance
- **Rob Karreman,** Department of Molecular and Cell Biology, for his technical assistance with MALDI-TOF
- **Noel Hendricks,** Department of Chemistry, for his technical assistance with the running of the NMR spectra
- **My colleagues and friends in the Kidney Stone Laboratory and Molecular and Cell Biology,** for their encouragement, enthusiasm and provision of a happy working enviroment
- **My husband, John and children, Stephanie and John jnr,** for their patience, assistance and tremendous support and encouragement
- **The NRF, Grant Holders Bursary Program** (courtesy of Prof.A.L. Rodgers) for financial assistance

# ABBREVIATIONS AND SYMBOLS

## GENERAL

| | |
|---|---|
| Å | Angstroms |
| BSA | Bovine serum albumin |
| $^{O}C$ | degrees centigrade |
| CD | circular dichroism |
| COM | calcium oxalate monohydrate crystals |
| COSY | correlated spectroscopy |
| Conc | concentration |
| Da | daltons |
| g | centifugal force |
| GU | glucose units |
| HPLC | high performance liquid chromatography |
| L or lt | litre |
| MALDI-TOF | matrix adsorption laser detection –time of flight |
| mg | milligram |
| min | minutes |
| ml | millilitre |
| mM | millimolar |
| M | molar |
| $M_r$ | relative molecular weight |
| OD | optical density |
| SE | standard error |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SS | supersaturation |
| TCA | trichloroacetic acid |
| BNTHP | Tamm Horsfall mucoprotein from black subjects |
| WNTHP | Tamm Horsfall mucoprotein from white subjects |
| WSFTHP | Tamm Horsfall mucoprotein from white stone formers |
| BSFTHP | Tamm Horsfall mucoprotein from black stone formers |
| Tris | Tris (hydroxlmethyl)methylamine |
| Φg | micrograms |
| Φl | microlitre |
| UV | ultra violet |
| V | volts |

| | |
|---|---|
| Vis | Visisble region |
| WNU | White normal urine |
| BNU | Black normal urine |
| WSFU | White stone-forming urine |
| BSFU | Black stone-forming urine |
| Zp | Zeta potential |

## AMINO ACIDS

| | |
|---|---|
| ala | alanine |
| cys | cysteine |
| glu | glutamine |
| phen | phenylalanine |
| gly | glycine |
| iso | isoleucine |
| lyc | lysine |
| arg | arginine |
| ser | serine |
| theo | threonine |
| val | valine |
| | tryptophan |
| tyr | tyrosine |

## SYMBOLS

| | |
|---|---|
| α | alpha |
| β | beta |
| Φ | micro |

## EXOGLYCOSIDASES

| | |
|---|---|
| **ABS** | (*Arthrobacter ureafaciens sialidase*) releases α 2-6, 3 and 8 linked non-reducing terminal sialic acids (NeuNAc and NeuNGc) |
| **NANI** | (*Sialidase Streptococcus pneumoniae* recombinant in *E.coli*) releases α 2-3 and 8 linked non-reducing terminal sialic acids (NeuNAc and NeuNGc). |
| **NDV** | (Newcastle Disease Virus. Hitcher B1 Strain) release α 2-3 linked non-<br>reducing terminal sialic acids (NeuNAc and NeuNGc) |
| **AMF** | (Almond meal alpha-fucosidase) releases α 1-3 and 4 linked non-reducing<br>terminal fucose residues. Digests outer arm fucose. |
| **BKF** | (Bovine kidney alpha-fucosidase) releases α 1-2 linked non-reducing<br>terminal fucose residues more efficiently than α 1-3 and 4 linked fucose.<br>Digests core fucose α 1-6 and α 1-3 (plants). |
| **BTG** | (Bovine testes beta-galactosidase) hydrolyses non-reducing terminal galactose β 1-3 and β 1-4 linkages. |
| **SPH** | (*Streptococcus pneumoniae* hexosaminidase) will digest GlcNAc β 1-2 Man (if Man is not substitiuted at C6 to a core Man which is linked to a bisecting GlcNAc), β 1-4 and 6 Man (at high concentration i.e. 100 mU/ml), GlcNAc β1-3 Gal and GlcNAc β1-6 Gal. |
| **JBM** | (Jack bean Mannosidase) removes mannose alpha 1-2,6 > 3. After overnight incubation at 37°C, add a further 10µl and incubate for a further 24 hours. |

# TABLE OF CONTENTS

Abstract ii
Publications v
Acknowledgements vi
Abbreviations and symbols viii
Table of contents xi
Index for Tables in thesis xvii
Index for Figures in thesis xviii

**CHAPTER 1: INTRODUCTION**
**1.1. Aetiology of stones 1**
**1.2. Physiocochemical aspects 6**
**1.3. Role of Inhibitors 11**
**1.4. Racial aspects: South African Blacks vs Whites 15**
**1.5. Role of Tamm-Horsfall Glycoprotein (THP) in Urolithiasis 16**
**1.6. Objectives 20**
**1.7. References 21**
**CHAPTER 2: BIOCHEMICAL ANALYSIS**
**2.1. Introduction 24**
**2.2. Objectives 24**
**2.3. Methods 25**
**2.3.1. Subjects, urinalysis and physicochemical risk indicies 25**
**2.3.2. THP Extraction and Purification 25**
**2.3.3. THP Verification 26**
**2.3.4. Biochemical Analysis 26**
2.3.4.a. Matrix Assisted Laser Desorption / Ionisation Time-of Flight (MALDI-TOF) Mass Spectroscopy 26

# TABLE OF CONTENTS

Abstract ii
Publications v
Acknowledgements vi
Abbreviations and symbols viii
Table of contents xi
Index for Tables in thesis xvi
Index for Figures in thesis xviii

**CHAPTER 1: INTRODUCTION**
**1.1. Introduction and aetiology of stones 1**
**1.2. Physiocochemical aspects 6**
**1.3. Role of Inhibitors 11**
**1.4. Racial aspects: South African Blacks vs Whites 15**
**1.5. Role of Tamm-Horsfall Glycoprotein (THP) in Urolithiasis 17**
**1.6. Objectives 21**
**1.7. References 22**
**CHAPTER 2: BIOCHEMICAL ANALYSIS**
**2.1. Introduction 40**
**2.2. Objectives 40**
**2.3. Methods 41**
**2.3.1. Subjects, urinalysis and physicochemical risk indicies 41**
**2.3.2. THP Extraction and Purification 41**
**2.3.3. THP Verification 42**
**2.3.4. Biochemical Analysis 42**
2.3.4.a. Matrix Assisted Laser Desorption / Ionisation Time-of Flight (MALDI-TOF) Mass Spectroscopy 42

2.3.4.b. Tryptic Pepetide Mass Fingerprinting 43
2.3.4.c. Amino Acid Analysis 43
2.3.4.d. Circular Dichroism (CD) 44
**2.4. Results** 44
**2.4.1. Urinalysis and physicochemical risk indicies** 44
**2.4.2. THP Purification and Verification** 48
**2.4.3. Biochemical Analysis** 49
2.4.3.a. Matrix Assisted Laser Desorption / Ionisation Time-of Flight (MALDI-TOF) Mass Spectroscopy 49
2.4.3.b. Tryptic Pepetide Mass Fingerprinting 52
2.4.3.c. Amino Acid Analysis 56
2.4.3.d. Circular Dichroism (CD) 58
**2.5. Discussion** 61
**2.6. References** 65
**CHAPTER 3: CHARACTERIZATION OF THE CARBOHYDRATE MOIETIES OF THP FROM BLACK AND WHITE HEALTHY AND STONE-FORMING SUBJECTS USING $^{1}$H-NMR (1-D) AND *N-GYCOSIDASE F DIGESTION* FOR ANALYSIS**
**3.1. Introduction** 68
3.1.1. Primary Structural Analysis using $^{1}$H-Nuclear Magnetic Resonance (NMR) Spectroscopy 68
3.1.2. Classification and Structure of *N*- and *O*-linked Oligosaccharides 70
3.1.3. Oligosaccharide Analysis 71
3.1.4. Physiological signals of THP glycosylation – Controls vs Stone- formers 72
3.1.5. Structure of THP molecule 74
3.1.6. Carbohydarte Moieties THP - Black vs White 76
**3.2. Objectives** 76

**3.3. Methods** 77

3.3.1. Protein purification 77

3.3.2. Primary Structural Analysis using $^{1}$H-NMR analysis 77

3.3.3. Oligosaccharide analysis - Peptide N-Glycosidase F digestion (PNGase F) 78

3.3.4. Weak Anion Exchange -HPLC 79

3.3.5. Determination of site occupancy- In-solution release of glycans 80

3.3.6. Release of O-glycans by hydrazinolysis 80

**3.4. Results** 81

3.4.1. $^{1}$H-NMR analysis 81

3.4.2a. Oligosaccharide analysis 82

3.4.2b. Site Occupancy 87

3.4.3. GalNAc Structures 89

3.4.4. Charged Structures 90

3.4.5. Sialic Acid linkages 90

3.4.6. O-glycan Analysis 90

**3.5. Discussion** 91

**3.6. References** 98

**CHAPTER 4: INVESTIGATION OF THE EFFECTS OF THP ON CALCIUM OXALATE (CaOx) CRYSTALLISATION PROCESSES IN URINE**

**4.1. Introduction** 104

**CHAPTER 4.1: CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTS IN URINE FROM HEALTHY SUBJECTS: DIRECT AND "SINGLE CROSSOVER" EXPERIMENTS**

**4.1.1. Objectives** 109

**4.1.2. Methods** 110

4.1.2.a. Effect of NTHP on CaOx Crystallisation (Coulter Counter) 110

4.1.2.b. Effect of NTHP on CaOx Crystal Deposition ($^{14}$C-oxalate) 111

**4.1.3). Results** 112

4.1.3.a. Effect of NTHP on CaOx Crystallisation (Coulter Counter)

4.1.3.b. Effect of NTHP on CaOx Crystal Deposition ($^{14}$C-oxalate) 116

**4.1.4. Discussion** 117

**CHAPTER 4.2: CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM STONE-FORMERS: DIRECT AND "SINGLE CROSSOVER" EXPERIMENTS**

**4.2.1. Objectives** 121

**4.2.2. Methods** 122

4.2.2.a. Effect of SFTHP on CaOx Crystallisation (Coulter Counter) and CaOx Crystal Deposition ($^{14}$C-oxalate) 122

**4.2.3. Results** 122

4.2.3.a. Effect of SFTHP on CaOx Crystallisation (Coulter Counter) 122

4.2.3.b. Effect of SFTHP on CaOx Crystal Deposition ($^{14}$C-oxalate) 127

**4.2.4). Discussion** 128

**CHAPTER 4.3: CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTTS IN URINE FROM STONE-FORMERS: "SINGLE AND DOUBLE CROSSOVER" EXPERIMENTS**

**4.3.1. Objectives** 130

**4.3.2. Methods** 131

4.3.2.a. Effect of NTHP on CaOx Crystallisation (Coulter Counter), CaOx Crystal Deposition ($^{14}$C-oxalate) in stone-forming urine from both race groups. 131

**4.3.3. Results** 131

4.3.3.a. Effect of NTHP on CaOx Crystallisation (Coulter Counter) in stone-forming urine from both race groups 131

4.3.3.b. Effect of NTHP on CaOx Crystal Deposition ($^{14}$C-oxalate) 136

**4.3.4). Discussion** 137

**CHAPTER 4.4: CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM HEALTHY SUBJECTS: "SINGLE AND DOUBLE CROSSOVER" EXPERIMENTS**

**4.4.1. Objectives** 141

**4.4.2. Methods** 142

4.4.2.a. Effect of SFTHP on CaOx Crystallisation (Coulter Counter), CaOx Crystal Deposition ($^{14}$C-oxalate) in healthy urine from both race groups. 142

**4.4.3. Results** 142
4.4.3.a. Effect of SFTHP on particle characteristics (Coulter Counter) in healthy urine from both race groups. 142
4.4.3.b. Effect of SFTHP on CaOx Crystal Deposition (14C- oxalate) 146
**4.4.4. Discussion** 147
**CHAPTER 4.5: SEDIMENTATION AND ZETA-POTENTIAL STUDIES ON CALCIUM OXALATE (CaOx) CRYSTAL SLURRIES IN THE ABSENCE AND PRESENCE OF NTHP AND SFTHP**
**4.5.1. Introduction** 149
**4.5.2. Objectives** 152
**4.5.3. Methods** 152
4.5.3.a. Preparation of Calcium Oxalate Crystal Slurries 152
4.5.3.b. Effects of NTHP and SFTHP on Zeta potentials of CaOx crystal slurries
4.5.3.c. Effect of NTHP and SFTHP on Sedimentation of CaOx crystals. 153
**4.5.4. Results** 154
4.5.4.a. Effects of NTHP and SFTHP on Zeta potentials of CaOx crystal slurries. 154
4.5.4.b. Effect of NTHP and SFTHP on Sedimentation of CaOx crsytals. 155
**4.5.5. Discussion** 156
**4.5.6. References** 160
**CHAPTER 5: OVERVIEW AND FUTURE WORK** 164

**APPENDICIES:** CD provided

# INDEX FOR TABLES IN THESIS


**CHAPTER 1: INTRODUCTION**

Table 1: Inhibitors of crystallisation (Marangella, 2000) 13

**CHAPTER 2: BIOCHEMICAL ANALYSIS**

Table 1: Urine composition (mmoles / 24 hrs) and physicochemical data and standard Error. 45

Table 2. Mean values of THP samples obtained from MALDI-TOF Mass Spectroscopy. Values in brackets represent standard error 49

Table 3. Peptides from BNTHP after trypsin digestion that were not found on the Swiss-Prot database. 54

Table 4. Peptide masses for Tamm-Horsfall glycoprotein from Swiss-Prot, covering 97.6% of the sequence together with the peptide masses obtained for the four THP samples after Trypsin digestion. 55

Table 5. Amino Acid Analysis for NTHP and SFTHP. 56

Table 6. Deconvolution into percentage of secondary structures within the four THP samples obtained from CD spectra according to Deleage and Roux, 1987. 60

**CHAPTER 3: CHARACTERIZATION OF THE CARBOHYDRATE MOIETIES OF THP FROM BLACK AND WHITE HEALTHY AND STONE-FORMING SUBJECTS USING $^{1}$H-NMR (1-D) AND *N-GYCOSIDASE F DIGESTION* FOR ANALYSIS**

Table 1. "*Structural-reporter groups*" and chemical shift values that were used for the identification of peaks that were observed in the various THPs. 82

Table 2. Sugar structures and their percentages that appeared to be present in the different groups of THPs. 84

Table 3. Percentage of charged sugars in relation to WNTHP. 90

**CHAPTER 4: INVESTIGATION OF THE EFFECTS OF THP ON CALCIUM OXALATE (CaOx) CRYSTALLISATION PROCESSES IN URINE**

**CHAPTER 4.1: CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTS IN URINE FROM HEALTHY SUBJECTS: DIRECT AND "SINGLE CROSSOVER" EXPERIMENTS**

Table 1: Observed trends in particle characteristics: NU + NTHP. 113

**CHAPTER 4.2: CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM STONE-FORMERS: DIRECT AND "SINGLE CROSSOVER" EXPERIMENTS**

Table 2: Observed trends in particle characteristics: SFU + SFTHP. 124

**CHAPTER 4.3: CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTTS IN URINE FROM STONE-FORMERS: "SINGLE AND DOUBLE CROSSOVER" EXPERIMENTS**

Table 3: Observed trends in particle characteristics: SFU + NTHP. 133

**CHAPTER 4.4: CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM HEALTHY SUBJECTS: "SINGLE AND DOUBLE CROSSOVER" EXPERIMENTS**

Table 4: Observed trends in particle characteristics: NU + SFTHP. 148

# INDEX FOR FIGURES IN THESIS


## CHAPTER 1: INTRODUCTION

Figure 1: Crystallization processes in the urinary tract (Hess, 2001). 10

Figure 2: Distribution of THP in the human nephron is limited to the thick ascending limb and the early distal convoluted tubule. THP is absent from the macula densa (Kumar and Muchmore, 1990). 18

## CHAPTER 2: BIOCHEMICAL ANALYSIS

Figure 1a: Urine composition. 46

Figure 1b: Urine composition (continued). 46

Figure 2: Relative Supersaturation. 47

Figure 3: Tiselius Risk Index 47

Figure 4. 15 % SDS-polyacrylamide gel electrophoresis with the purified THP samples and a Sigma Protein and Peptide Low Molecular Weight Marker in the range of 14 000 – 97 000 daltons visualised using CCB. 48

Figure 5: Histograms representing the mean molecular weight values for the four THP samples. Error bars represent standard error. 49

Figure 6a-6d. Typical MALDI-TOF Mass Spectrum of WNTHP. BNTHP, WSFTHP, BSFTHP. 50 - 51

Figure 7a-7d. Typical tryptic digest spectrum for WNTHP, BNTHP, WSFTHP, BSFTHP. 52 - 54

Figure 8. Histogram representing Amino Acids. 57

Figure 9. CD spectrum of THP from the four groups. 59

Figure 10. Deconvolution into secondary structures of the four THP samples obtained from CD spectra according to Deleage and Roux, 1987. 60

## CHAPTER 3: CHARACTERIZATION OF THE CARBOHYDRATE MOIETIES OF THP FROM BLACK AND WHITE HEALTHY AND STONE-FORMING SUBJECTS USING $^{1}$H-NMR (1-D) AND *N-GYCOSIDASE F DIGESTION* FOR ANALYSIS

Figure 1. Oligosaccharide inner-cores of glycoproteins. 70

Figure 2. A summary of an overall strategy and the new techniques available for oligosaccharide analyses (Rudd, 1997). 72

Figure 3. Schematic representation of THP including specific domains and potential glycosylation sites. 75

Figure 4. Stacked chromatograms representing the variation of the total *N*-glycan pools for the various groups of THPs. 83

Figure 5a. Graphical representations of monosaccharidesand their linkages. 84

Figure 5b. Presents a key for the symbols and GU values used to represent these sugar structures found in Table 2 and histogram, Figure 6. 85

Figure 6. Sugar residues and their percentages that were found in the various THP samples. 86

Figure 7. The percentage of Mannose-3 residue after digestion of the various THPs. 86

Figure 8a. WNTHP and WSFTHP run on a 10% SDS gel visualised using Coomassie Brilliant Blue. 87

Figure 8b. BNTHP and BSFTHP run on a 10% SDS gel visualised using Coomassie Brilliant Blue. 88

Figure 9. WNTHP and WSFTHP run on 10% SDS-PAGE before and after digestion with PNGaseF visualised using Coomassie Brilliant Blue. 88

Figure 10. HPLC profiles after digestion of the various THP. The profile representing each THP group is as follows: WNTHP (a), WSFTHP (b), BNTHP (c) and BSFTHP (d). 89

**CHAPTER 4: INVESTIGATION OF THE EFFECTS OF THP ON CALCIUM OXALATE (CaOx) CRYSTALLISATION PROCESSES IN URINE**

Figure 1. Protocol matrix for crystallisation experiments. 107

Figure 2: Schematic representation of the cross-over design protocol. 108

**CHAPTER 4.1: CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTS IN URINE FROM HEALTHY SUBJECTS: DIRECT AND "SINGLE CROSSOVER" EXPERIMENTS**

Figure 3: Histograms representing the msl of WNU and BNU before and after the addition of NTHPs. The error bars represent standard error. 112

Figure. 4a. Plots of particle number vs time in WNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs 114

Figure 4b. Plots of particle number vs time in BNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs. 114

Figure 5a: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in WNU before and after the addition of NTHP. 115

Figure 5b: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in BNU before and after the addition of NTHP. 115

Figure 6. Mean plots of crystallisation kinetics using $^{14}$C-oxalate in WNU. 116

Figure 7. Mean plots of crystallisation kinetics using $^{14}$C-oxalate in BNU. 117

CHAPTER 4.2: CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM STONE-FORMERS: DIRECT AND "SINGLE CROSSOVER" EXPERIMENTS

Figure 8: Histograms representing the msl of WSFU and BSFU before and after the addition of SFTHP. The error bars represent standard error. 123

Figure 9a: Plots of particle number vs time in WSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs. 125

Figure 9b: Plots of particle number vs time in BSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs. 110 125

Figure 10a: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in WSFU before and after the addition of SFTHP. 126

Figure 10b: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in BSFU before and after the addition of SFTHP.111 126

Figure 11. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in WSFU. 112 127

Figure 12. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in BSFU. 128

CHAPTER 4.3: CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTTS IN URINE FROM STONE-FORMERS: "SINGLE AND DOUBLE CROSSOVER" EXPERIMENTS

Figure 13. Histograms representing the msl of WSFU and BSFU before and after the addition of NTHPs. The error bars represent standard error. 132

Figure 14a: Plots of particle number vs time in WSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs. 134

Figure 14b: Plots of particle number vs time in BSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs. 134

Figure 15a: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in WSFU before and after the addition of NTHP. 135

Figure 15b: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in BSFU before and after the addition of NTHP. 135

Figure 16. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in WSFU. 136

Figure 17. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in BSFU. 137

CHAPTER 4.4: CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM HEALTHY SUBJECTS: "SINGLE AND DOUBLE CROSSOVER" EXPERIMENTS

Figure 18a. Plots of particle number vs time in WNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs. 129 144

Figure 18b. Plots of particle number vs time in BNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs 144

Figure 19a: Particle volume-size distribution (t=80 mins after dosing with NaOx) in WNU before and after the addition of SFTHP. 145

Figure 19b: Particle volume-size distribution (t=80 mins after dosing with NaOx) in BNU before and after the addition of SFTHP. 130 145

Figure 20. Crystallisation kinetic plots using $^{14}$C-oxalate in WCU. 131 146

Figure 21. Crystallisation kinetic plots using $^{14}$C-oxalate in BCU. 147

CHAPTER 4.5: SEDIMENTATION AND ZETA-POTENTIAL STUDIES ON CALCIUM OXALATE (CaOx) CRYSTAL SLURRIES IN THE ABSENCE AND PRESENCE OF NTHP AND SFTHP

Figure 22. Schematic representation of the relationship between energy and distance according to the DLVO theory (Courtsey of Allen). 135 150

Figure 23: Surface charge (zeta-potential) of COM crystals in the absence and presence of NTHP and SFTHP. 140 155

Figure 24: The effect of NTHP and SFTHP on inhibition of COM crystal aggregation. 156

# CHAPTER 1

# AETIOLOGY OF STONES

## 1.1. INTRODUCTION AND AETIOLOGY OF STONES



Urolithiasis, the formation of calculi in the urinary tract, is an age-old disease afflicting mankind and is among the oldest documented medical disorders (Rodgers et al, 1981) which continues to pose a universal health problem today (Aaggarval et al, 2000). It's aetiology is multifactorial resulting from an interaction of epidemiological, metabolic, dietary and genetic factors. In many cases, the disease is idiopathic. Stones occur in several parts of the human body - kidneys, bladder, prostrate gland, gallbladder, salivary glands and panareas (Rodgers et al. 1981). During the last century the incidence of urinary calculi has shifted from the lower tract (bladder, ureter) to the upper tract (kidneys) (Aggarval et al, 2000). This changing pattern is an interesting aspect. Andersen (1973) has reported that the incidence of bladder stones has been on the decline in developed countries. However, in contrast, there has been an increase of stone disease in the first world countries since the late 19th century (Nordin, 1976; Rodgers et al, 1981; Aggarval et al, 2000). The affected areas are Europe, North America (Herring, 1962) and Japan (Robertson et al, 1972, **(a), (b);** Rodgers et al, 1981; Blacklock, 1982). The disease is also shown to affect males more than females with males having double the stone incidence than females (Hesse et al, 1986).

Early recordings of stones date back to the third century BC when the Greek physician, Ammonios used lithotrity to break up bladder stones so that they could be passed in the urine (Rodgers et al, 1981). Two hundred years later, Celsus

gave an account of a surgical procedure, lithotomy, known as cutting for the stone, to give relief. However due to the absence of anaesthetics, side effects such as excruciating pain as well as the risk of death due to infections prompted further research for a potion that would dissolve stones (Rodgers et al, 1981). Towards the end of the 18th century the compostion and cause of renal stones became known from the chemistry of the urine. Rouelle Le Cadet discovered urea in 1773 and uric acid was isolated from urine by Scheete in 1776. In 1810, Wollaston discovered cystine in certain calculi (Modlin, 1967). Alchemists also discovered phosphates. They stimulated the idea that calculi in the bladder could be dissolved by injecting suitable solvents into the bladder and this aroused interest in the possible influence of diet on stone formation (Modlin, 1967). Factors, such as, diet is known to be a contributiong factor in urolithiasis.

The discovery that the "stone" has an organic component dates back to 1684 when Van Hyde found that the calculus had an organic framework, which is now called the matrix (Boyce et al, 1956; Butt, A.J, 1959; Thorne et al, 1983). More than 150 years elapsed before this organic component, the matrix, was proposed as the mechanism for stone formation. The composition of the matrix remained largely unknown until the 1950s when the development of electron microscopy and advances in biochemistry made the investigation possible. The organic matrix, present in most urinary calculi, is a mucoprotein in nature and has been designated as substance 'A' by (King et al, 1963) uromucoid and Tamm Horsfall glycoprotein (THG) (Grant et al, 1973). The matrix is composed mainly of selectively incorporated proteins generally characterised by high glutamic and aspartic acid content and the frequent occurrence of gamma-carboxyglutamic acid, displays a variable and complex composition and shares a few proteins in many stones (Boyce et al, 1956). Analysis of the stone protein extracts suggests that the embryonic stone may first appear in the renal tubules where it can acquire the blood and cell membrane proteins. The combination of supersaturation, an appropriate urine enviroment, the availablity of calcium binding proteins, which may be abnormal and the incorporation

of proteins extracted from leucocytes and cell wall membranes may induce stone formation (Binette et al, 1996).

Urinary tract infection is known to increase the non-dialysable materials in the urine. The matrix is usually the non-dialyzable material (Boyce et al. 1956; Warpehoski et al, 1981; Stocholy et al, 1985) that remains after the crystal component has been dissolved in a solvent (Finlayson et al, 1961). This material contains protein and carbohydrate (Boyce et al. 1956). The mechanism of co-precipitation gives rise to significant amounts of protein in a stone as well as the presence of several protein fractions in a stone matrix (Sugimoto et al, 1985; Fraij, 1989; Jones et al, 1990). Due to this mechanism, the protein can be incorporated into a stone but may not necessarily actively participate in the stone growth (Grant et al, 1973; Leal et al, 1977; Lian et al, 1977; Doyle et al, 1991). The amount of matrix deposited in a stone depends on the concentration of the co-precipitated material and the affinity of the material for stone crystals (Thorne et al, 1983). Thus the amount of matrix in a stone would be due to the summation of these various factors. Short-term fluctuations in conditions influencing co-precipitation of protein would cause variations in the structure of a stone, such as laminations (Finlayson et al, 1961; Thorne et al, 1983).



The variation in the chemical composition of the stone matrix may be due to bacterial infections, which act together with co-precipitation or the bacteria that acts upon the co-precipitated material. It is thought that the matrix participates in stone formation by the possibility of the protein binding the crystal components of the stone together (Finlayson et al, 1961).

Direct inspection of urinary stones can at times afford a remarkable accurate assesment of its composition. Pure calcium oxalate stones may be rounded and polished or may also be "jackstone" variety with dark irregular spikes (Thorne et al, 1983). Calcium oxalate dihydrate crystals are apparent on the surface of some stones. Staghorn calculi are usually related to urinary infections with urease producing bacteria and are composed of carbonate apatite and magnesium ammonium phosphate

but can be composed of other crystalline elements as well. Uric acid stones are usually multiple pale stones and can also be mixed with calcium phosphate and oxalate. Uric acid stones may be yellow or orange in colour due to absorption of urinary pigments (Thorne et al, 1983).

In recent studies (Aggarval et al, 2000) calcium oxalate has been shown as the main component in about 2/3 of all urinary calculi. Calcium oxalate monohydrate (COM) has been found to initate mineralization followed by the deposition of calcium oxalate dihydrate (COD) on it (Elliot, 1973). In addition to CaOx, urinary stones have been found to contain phosphates, uric acid, magnesium ammonium phosphates with apatite and struvites predominating (Aggarval et al, 2000). There are a few factors thought to play a role in urolithiasis (stone formation) namely, climate, fluid intake, diet and racial and hereditary factors (McGeown, 1960). South African blacks, Aborigines, Eskimos and the Indians of Peru, Mexico, Ecuador and Bolivia appeared to be immune to stone disease (Rodgers et al, 1981). This issue will be discussed in more detail in Chapter 1.4.

Diet is thought to also play a role in stone formation (Andersen, 1973; Robertson et al, 1987, 2000). In the past, calcium oxalate stone patients were advised to restrict their intake of dietary calcium (Pak et al, 1980, 1984; Rao et al, 1982; Rose, 1987; Massey et al, 1993; Goldfarb, 1994), as there was a belief that a high calcium intake increased the risk of stone formation (Curhan et al, 1993, 1997). However in a recent study (Curhan et al, 1993) of the relationship between dietary calcium intake and the risk of symptomatic kidney stones in over 45,000 male subjects, it was demonstrated that the converse was true, namely a higher dietary intake of calcium decreases the risk. The authors suggested that this might be due to increased binding of calcium to oxalate in the gastro-intestinal tract, leading to a decrease in urinary oxalate excretion (Curhan et al, 1993, 1997). Since urinary oxalate is more important than urinary calcium in determining stone formation (Finlayson, 1978; Robertson et al, 1978; Rodgers et al, 1981; Borsati, 1991), the risk decreases.

therefore advised to restrict their intake of oxalate rich foods (Andersen, 1951;Curhan, 2000) such as chocolate and tea (Vahlensieck, 1984) and to maintain their dietary intake of calcium (Curhan, 2000). It has also been shown by other workers that a low calcium diet increases the excretion of oxalate (Zarembski and Hodgkinson, 1969; Marshal et al, 1972; Massey, 1993; Jayadevan, 2000). The potential danger of dietary calcium restrictions has also been demonstrated by Bataille, (1984), who reported an increased probability of stones forming in patients with certain types of hypercalciuria when following such a diet (Coe, 1992; Curhan, 1993). These studies suggest an apparent protective effect of dietary calcium. It therefore seems appropriate to advise stone formers to maintain their dietary intake of calcium as previously mentioned or to increase it marginally.

A higher intake of animal protein has been shown to produce an increase in urinary calcium, oxalate and uric acid (Walker, 1970; Robertson, 1979, 1987; Rodgers and Spector, 1981). Therefore it has been suggested that a reduction in dietary animal protein rather than dairy products would be more beneficial for the stone formers (Anderson, 1973; Robertson, 1979, 1987, 2000; Fellstrom, 1981; Curhan, 1993; Borghi et al, 2002). The increase of stone incidence and reoccurrence could be a direct consequence of higher animal protein ingestion when compared to population groups from economically poorer regions in which stone incidence are lower or virtually unseen (Breslau, 1988; Marangella, 1989).

A high fluid intake is necessary for decreasing the risk of stone formation (Blacklock, 1969; Pak, 1980, 1984; Robertson and Peacock 1983, 1987; Ryall, 1989; McCormack, 1991; Borghi, 1996; Parivar, 1996; Rodgers, 1997; Caudarella et al, 1998; Curhan, 1998; Rodgers, 1998). It has been reported that a high fluid intake (Robertson and Peacock, 1983; Pak, 1981; 1984; Rodgers, 1991) is a vital necessity in order to decrease the resident time of crystals in the tubules due to larger flow rates that in turn will lead to a decrease in supersaturation and therefore reduce the risk of stone incidence. There is also a definite correlation between climate and geographical stone belts. It has been shown that exposure to UV radiation increases the calcium uptake in the intestine (Robertson and Peacock, 1983)

while an increase in atmospheric temperature is directly related to stone incidence. Due to high temperatures, dehydration increases thereby increasing the concentration of stone forming salts in the urinary tract, which are unable to pass in the urine (Embon et al, 1990; Schwille et al, 1992).

## 1.2. PHYSICOCHEMICAL ASPECTS

Factors that influence calculosis include the saturation state of the body fluids with stone-forming constituents (Coe et al, 1991), the role of the organic matrix and the presence of various biomolecules (inhibitors and/or stimulators) influencing mineralisation in the body fluids (Robertson et al, 1972 **(a), (b)**; Finlayson, 1978; Boskey, 1981; Aggarwal et al, 2000). The deposition of inorganic crystalline material is called "mineralization" (Hess et al, 2001). In human urine, this above process occurs abundantly. The excretion of small crystals in the urine does not distinguish between kidney stone formers and controls, since this process occurs naturally in both groups (Fleisch, 1978).

Loosely clustered inorganic crystals would never form a dense stone if they were not tightly glued together by organic material (Morse et al, 1988). Thus kidney stones will only develope in the presence of an organic matrix, which forms a precisely structured "framework" for stones (Morse et al, 1988). Kidney stones and single urinary crystals form under the control of macromolecule modulators of crystallisation, which can become included into the crystals and dictate the morphology (Robertson et al, 1972 **(a), (b)**; Khan, 1997; Ryall et al, 1998; Hess et al, 2001).

Two major aspects have to be considered when addressing crystallisation within the urinary tract (Finlayson, 1978; Kok et al, 1990):

a). A thermodynamic process involving high urinary supersaturation during which crystal nucleation occurs,

b). A kinetic process comprising rates of nucleation, growth and aggregation of crystals.

Crystals form only when the solution has become saturated with the relevant component ions (Goldwasser et al, 1986; Marangella et al, 2000). When two or more ions combine to form crystals, as in the case of calcium oxalate or calcium phosphate, the product of the concentration of the two substances governs the level of saturation. The point at which saturation is reached and crystallisation begins is referred to as the solubility product, which is defined as the product of the molar concentration of the two substances at the point when saturation is reached (Goldwasser et al, 1986). Factors that affect the saturation, in addition to the concentration of the constituent ions of the crystals, are the pH and temperature (McQueen, 1966). Since urine varies in pH and body temperature is 37°C, these factors must be considered in the explanation of urolithiasis (Goldwasser et al, 1986).

Saturation and solubility product are easy to define in water but in urine it is more difficult as it is a much more complex solution. Although elements and molecules in urine are suspended in water, the mixture of many electrically active ions in urine causes interactions that change the solubility of the elements (Goldwasser et al, 1986). This solution is referred to as polyionic and the definition of saturation or solubility product of a given substance in this type of solution becomes complex and difficult. Electrical attraction or repulsion of ions in urine is involved in the crystallisation process (Finlayson, 1978; Fleisch, 1978; Scurr et al, 1986 **(a)**, **(b)**) and this type of biological electrical activity is called Zeta potential. Various factors interact to increase the solubility of substances that otherwise might crystallise at the concentrations present in the urine. If a given amount of calcium and oxalate that would crystallise when dissolved in water at a given pH and temperature is added to urine, it will be held in solution. If the amount of calcium and oxalate is increased progressively in the same volume of urine at constant pH and temperature, the calcium and oxalate will remain in solution even though the solubility product has been exceeded, thus creating supersaturation (Goldwasser et al, 1986). The amount of substance in urine can be increased to a point at which it starts spontaneous nucleation (Thorne et al, 1983; Goldwasser et al, 1986). The point at which spontaneous

nucleation of crystals occurs is known as the formation product for urine. The metastable zone is when supersaturation is reached between the solubility product and the formation product (Fleisch, 1978; Goldwasser et al, 1986; Kavanagh, 1995). If the nucleation occurs spontaneously in all parts of the solution at the same time, as may occur in pure solution, the process is called homogeneous nucleation. In urine homogeneous nucleation is unlikely to occur, since different contaminants, such as crystals, macromolecules or cellular debris, may serve as nuclei and cause a secondary nucleation at a much lesser degree of supersaturation, which is referred to as heterogeneous nucleation (Finlayson, 1978; Kavanagh, 2000).

An extension of the theory of heterogeneous nucleation would be epitaxy (Lonsdale, 1968; Thorne et al, 1983; Mandel et al, 1991). If a crystal has a pattern or organisation of ions that is regular and predictable, this structure is called a lattice (Cerini et al, 1999). Heterogeneous nucleation occurs most readily when crystal lattices are structurally and chemically compatible at the zone of contact (Lonsdale, 1968). The process in which crystals of two different substances share sufficient structural similarities to allow atoms of one to organise themselves as the surface of the other to form the primary structure of their own lattice is termed epitaxial growth (Lonsdale, 1968).



The supersaturation theory does not efficiently explain why some people form stones and others do not. It had shown (Robertson et al, 1972, **(a), (b)**; Ryall, 1997) that patients with calcium stones tend to excrete considerably more oxalate and calcium than normal persons. They also showed a moderate overlap in the degree of saturation between normal and stone forming groups (Robertson et al, 1972, **(a), (b)**).

Various researchers (Elliot, 1983; Erwin et al, 1994) have been unable to show differences in the 24-hour excretion of calcium, oxalate and uric acid between patients with stones and normal individuals. They were also unable to demonstrate a difference in the degree of supersaturation of these substances between the two groups (Elliot, 1983).

A possible lack of crystallisation inhibitors (discussed in chapter 1.3) in the urine of patients with stones could provide a possible answer for some individuals forming stones and others not. Citrate (Meyer et al, 1975, 1982; Ryall et al, 1981; Hallson et al, 1983; Kok et al, 1986, 1987; Nicar et al, 1987; Tiselius, 1993; Bek-Jensen et al, 1996; Schwille et al, 1999), pyrophosphate (Sutor, 1969; Fleisch et al, 1972; Welshman et al, 1972; Robertson et al, 1973; Meyer et al, 1975; Doremus et al, 1978; Ryall et al, 1981), chondroitin sulphate (Robertson et al, 1973; Ryall et al, 1981; Burns et al, 1983; Fellstrom et al, 1986; Scurr et al, 1986, **(a), (b)**; Kohri et al, 1989; Ryall et al, 1991), ribonuleic acid (Burns et al, 1983), magnesium (Moore et al, 1964; Doremus et al, 1978; Ryall et al, 1981; Li et al, 1985; Ryall, 1997) and trace elements, such as zinc (Elliott, 1973) have all been positively identified as inhibitors of calcium oxalate or calcium phosphate crystallisation. However, their relative importance or the way in which they interact at the crystal surface remains unclear (Fleisch, 1978; Nicar et al, 1980; Drach et al, 1982; Worcester, 1996; Ryall, 1997; Marangella et al, 2000). The effects of the multi-components are roughly additive and it is likely that patients with calcium stones have deficiency in several of their inhibitors that should be present in the urine (Drach, 1976). This may be a reason as to why patients with stones have low levels of more than one inhibiting element. If this occurs concurrently with a burst of supersaturation of the urine with calcium oxalate at the same time, it is likely that stone formation would begin and proceed rapidly (Goldwasser et al, 1986). In supersaturated urine with a severe lack of inhibitors, theoretically a process of precipitation would commence. However the transit time of urine in the upper urinary tract is 5 to 10 minutes, so even if crystals would form stones, the formation would not be expected to occur in such a short period (Fleisch, 1978). Particle retention (i.e. "fixed particle theory") either by obstruction or adherence to damaged epithelium is an important factor for stone formation (Finlayson et al, 1978; Gill et al, 1981)

The passage of free crystalline particles is very common in healthy people and stone formers alike (Hess et al, 1996; Fleisch, 1978). Simple crystalluria occurs in kidney stone formers and non- stone formers; the formation of kidney stones is thus a pathological incident and reflects a specific form of biomineralization (Boskey, 1981).

In Figure 1 (Hess et al. 2001), the processes, which appear to be most relevant for stone formation in the kidney, are shown. It is noted that the most important is particle retention (Hess et al. 2001), since only retained particles will undergo further growth into full-size stones. Particle retention can occur either because nucleated crystals strongly aggregate and form particles that are too large to pass freely through the tubules ('free particle' theory) (Kok et al. 1994), or because they become abnormally adherent to tubular cell surfaces ('fixed particle' theory) (Mandel et al. 1991; Kok et al. 1994; Hess et al. 2001). Both events require an intimate interaction between newly forming inorganic crystals surfaces and modulating organic macromolecules (Hess et al. 2001).



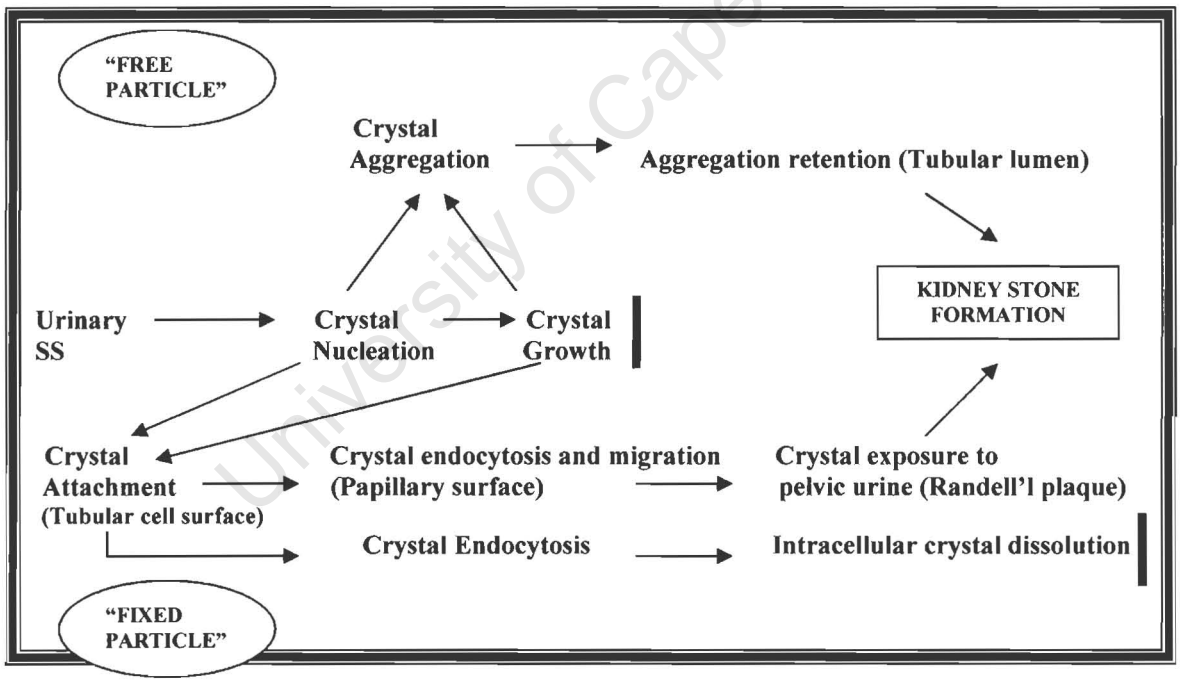



**Figure 1: Crystallization processes in the urinary tract (Hess, 2001). SS = Supersaturation; bars = end of physiological process; single-lined arrow = physiological process. Particle retention occurs due to "fixed and free particle" mechanisms.**

The free particle mechanism predicts that, mainly by the size-increasing effect of crystal aggregation, large particles with sizes that fall within the range of tubules are formed (Blomen, 1982; Kok et al, 1994). They subsequently become retained in the

tubules where they grow out into full-size stones (Riese et al, 1988; Lieske et al, 1992; Hess et al, 1995, 1996; Kok et al, 1994). The fixed particle mechanism implies that urinary crystals strongly and irreversibly adhere to surfaces of tubular cells where they are being endocytosed (Lieske et al, 1993; Mandel, 1994) Subsequently, some of the endocytosed crystals may migrate across the cells and produce a subepithelial calcified lesion (Khan, 1997). This lesion may finally lose its epithelial covering and may then provide a nidus for further stone growth in supersaturated urine (Khan, 1997). Another major theory of stone pathogenesis involves the role of inhibitors and promoters of crystallisation.

## 1.3. ROLE OF INHIBITORS

Supersaturation of urine with respect to calcium salts and protein inhibitors of stone formation play a major role in the natural defense mechanism against nephrolithiasis (Robertson et al, 1976; Worcester et al, 1995; Kavanagh, 1999). The process of crystallisation in urine involves three steps, namely, nucleation, growth and aggregation (Fleisch, 1978; Ryall, 1997; Dussol et al, 1998). The formation of any type of crystal is possible if the solution is supersaturated and proceeds from either homogenous or heterogeneous nucleation on cellular debris or other crystal phases (urate, brushite, calcium oxalate trihydrate) (Finlayson, 1978; Tiselius et al, 1996). A crystal, once formed, may undergo phase transformation, growth and aggregation into larger crystals (Finlayson, 1978). Thus formation and progression of crystal aggregates along the nephron is a vital step for a stone to form, since there is a risk of such aggregates being trapped in the tubules, where they may adhere to the cell membrane molecules which may be altered or abnormally exposed by renal injury and by crystal aggregates themselves (Mandel et al, 1991; Kok et al, 1994; Marangella et al, 2000).

Inhibitors of calcification are found in normal urine but are defficient in that of stone formers (Worcester et al, 1996). This gives rise to speculation that these inhibitory

substances may protect the nephron from pathological crystallisation while allowing normal excretion of urinary salts and implies that abnormal or deficient inhibitor /s may be the cause of nephrolithiasis (Worcester, 1996). So if supersaturation persists and this combines with a reduction in inhibitors of crystallisation, a renal stone will be produced (Ryall, 1997; Coe et al, 1991). A molecule may be referred to as an *inhibitor* if it interfers with any of the previously mentioned stone formation processes in section 1.2 that diminishes the chain of events that a crystal will set in motion which will eventually form a stone (Worcester, 1996).

Urinary inhibitors are naturally occurring components of the urine, which have been shown to prevent or retard the nucleation, growth or aggregation of various crystals (Ryall et al, 1990). Howard and Thomas (1958) were the first to propose that inhibitors played a role in CaOx stone formation and they also postulated that stone-formers had a deficiency of these inhibitors. Other researchers (Robertson et al, 1972, (a), (b); Nakagawa et al, 1985; Worcester, 1996) also supported this hypothesis. Robertson (1971) supported by Dent and Scur (1971) also found that larger sized crystals appeared in the urine of recurrent stone-formers relative to controls and this was due to the absence of inhibitors (Robertson et al, 1969; Hess et al, 1991).

Inhibitors of crystallisation are divided into two classes: small molecules, namely, citrate and pyrophosphate and macromolecules, such as glycoproteins and glycosaminoglycans (GAGS). It appears that most of the inhibitory activity in urine with respect to calcium oxalate resides in the macromolecules that appear to interfere in some way with the process of stone formation and these macromolecules are active at submicromolar concentrations (Nakagawa et al, 1983). A partial list of inhibitors, classified according to molecular weight is reported in Table 1 (Leal et al, 1977; Doremus et al, 1978; Nakagawa et al, 1983; Addadi et al, 1985; Nishio et al, 1985; Sorensen et al, 1990; Hess et al, 1991; Ryall et al, 1991, 1995; Shiraga, 1992; Stapelton et al, 1993).

| IONIC-LOW MOLECULAR WEIGHT | MACROMOLECULAR: PROTEIN | MACROMOLECULAR: GAGS |
|---|---|---|
| | Albumin | |
| Magnesium | Tamm Horsfall glycoprotein | Heparin |
| Citrate | Nephrocalcin | Hyaluronic acid |
| Phosphocitrate | Uropontin (Osteopontin) | Chrondroitin Sulphate |
| Pyrophosphate | Bikunin (Inter-alpha inhibior of tripsin) | |
| Tartrate | Calgranulin (Calprotectin) | |
| | Urinary prothrombin fragment 1 | |

**Table 1: Inhibitors of crystallisation (Marangella et al, 2000)**

Crystallisation inhibitors are also found in physiological fluids that are saturated with calcium salts, namely, saliva and pancreatic fluids that also contain these macromolecules, which suggests that the production of these substances may be a general strategy for controlling pathological calcification (Coe et al. 1991). The molecules listed in Table 1 have all demonstrated inhibitory activity. However many of them have been found in the protein matrix that forms part of all calcium stones. It is therefore possible that in certain instances (situated in the protein matrix) these macromolecules could provide the site for initiation of crystal formation (Worcester, 1996). Inhibitors act by adsorbing onto the crystal surface, interfering with the formation of the crystal lattice and retarding the attachment of new ions, thus inhibiting nucleation and most importantly, growth and aggregation into larger crystals, which otherwise would form in supersaturated urine (Marangella et al. 2000). Crystal modifiers can inhibit the crystal growth of certain salts and the degree of inhibition is in correlation with the concentration of this compound. Inhibition can be obtained at specific binding sites and therefore the entire surface of the crystal does not need to be covered by the macromolecules (Fleisch, 1978). Inhibitors may act specifically on nucleation or growth or aggregation. Stone formation involves primarily the latter two mechanisms. Inhibition of aggregation is due to a change in the zeta-potential of the surface, which alters the attraction or repulsion between crystals (Fleisch, 1978). Some of the protein inhibitors are produced within the nephron, by a process that can be triggered or enhanced by the crystals themselves

(Pillay et al 1998, Atmani et al, 1999). An increase in their intra-tubular concentration may be seen as a defence mechanism of the kidney against lithogenesis. Anionic inhibitors can prevent adhesion to fixed anionic sites in the tubular cells by absorbing on the crystal surface or modifying the crystal structure (Marangella et al, 2000). To date there is no evidence to indicate which specific inhibitor plays a major role in the pathogenesis of calcium nephrolithiasis, or whether there is one that explains differences between patients and controls.

Inhibitors can retard crystallisation in several ways. Firstly, they can act as chelating agents that bind either calcium or oxalate, thereby reducing supersaturation levels and reducing the probability of precipitation, which in turn prevents nucleation (Cabrera et al, 1958). Secondly, both growth and aggregation can be inhibited by the binding of an inhibitory agent to the newly formed crystal surface (Vermeulen et al, 1964) and at least one protein, Tamm Horsfall glycoprotein, appears to inhibit crystal aggregation not by irreversibly binding to the crystal surface but by simple steric hinderance (Ryall et al, 1991). It should be noted that investigation of an inhibitor's effects depends on the nature of the crystallisation system, the medium and the instrumentation used for monitoring the process (Kavanagh, 1992; Hess et al, 2001).

## 1.4. RACIAL ASPECTS: SOUTH AFRICAN BLACKS VS WHITES

Urolithiasis is extremely rare in South African blacks with an incidence rate of less than 1% (Muscat, 1951; Wise et al, 1961; Whalley et al, 1998). However, in the South African white population, calcium stones form in 15 per cent of the population, with a recurrence rate of 40 to 60 per cent (Whalley et al, 1998). This is comparable with the incident rate in other Westernised countries (Trinchieri, 1996).

The relative immunity shown by black South Africans to kidney stones could be caused by a variety of factors. Formerly, stones formed by this racial group were mainly struvite and occurred secondary to bilharzia or urinary tract infections and were found at times in the bladder (Wise et al, 1961). However, the occurrence of calcium oxalate stones has become more common in the upper urinary tract of the urban black population of South Africa (Beukes et al, 1987), albeit a rare occurrence.

It has been speculated by many researchers whether the difference in stone incidence shown between black and white South Africans is due to racial immunity, dietary differences, a disparity in fluid intake, handling of solute or the presence or absence of promoters and inhibitors for renal stone formation (Whalley et al, 1998). Although several attempts have been made to identify differences in the urinary risk factors of the two race groups that might explain this apparent stone immunity, they have been largely unsuccessful (Modlin, 1967; Meyers et al, 1976; Whalley et al, 1998). Indeed, some of the differences that have been found have been contrary to that which might have been expected. For example, urinary citrate in blacks has been reported as being lower than in whites (Modlin, 1967; Whalley et al, 1998; Lewandowski et al, 2001) while urinary oxalate was found in one study to be higher (Lewandowski et al, 2001). However, urinary calcium has consistently been found to be lower in blacks suggesting that this may be a key factor (Modlin, 1967; Whalley et al, 1998; Lewandowski et al, 2001).

In addition, recent studies have shown that urinary cystine is lower in blacks (Whalley et al, 1998) and that cystine is an *in vitro* promoter of calcium oxalate crystallisation (Martins et al, 2002). This prompted the authors to suggest that low urinary cystine might be a protective factor in South African blacks.

Although the composition of urine in the South African context has received much attention, the potential role of urinary proteins such as THP (Hess, 1992), nephrocalcin (Coe et al, 1991), uropontin (Shiraga et al, 1992), inter-alpha inhibitor (Atmani et al, 1996, Medetogen-Benission et al, 1999) and albumin (Atmani et al, 1999; Cerini et al, 1999) has not been investigated. Since these proteins have been shown to be potential inhibitors of calcium oxalate stone formation, the research group of which the present writer is a member, has undertaken to characterize some of these substances and their relative inhibitory activities in both race groups in South Africa. For example, it has been shown (Durrbaum et al, 2001; Webber et al, 2003) that urinary prothrombin fragment 1 (UPTF1) strongly inhibits CaOx nucleation in both race groups and that a synergistic relationship exists between urine composition and UPTF1 inhibitory activity. Moreover, UPTF1 from blacks is a stronger inhibitor of nucleation than whites. There appeared to be no biochemical differences for this protein for both population groups.

This thesis describes the rigorous investigation and characterization of another well-known urinary protein inhibitor, Tamm Horsfall glycoprotein (THP) in black and white South African subjects.

## 1.5. ROLE OF TAMM-HORSFALL GLYCOPROTEIN (THP)

Tamm Horsfall glycoprotein (THP), also called uromucoid, is the most abundant protein in normal human urine and a major constituent of urinary casts (Kumar et al, 1990). It has been speculated that it plays a role in urolithiasis (Grant et al, 1973; Hess, 1992). THP was first described as "urinary mucoprotein" by Merner in 1789 and further characterised by Tamm and Horsfall (1950, 1952) as a filamentous

molecule. It is synthesized by the kidney and localized in the epithelial cells of the thick ascending limb of the loop of Henle and the early distal convoluted tubules (Fletcher et al, 1970; Kumar et al, 1985; Ronco et al, 1987). THP has a molecular weight of about 80 Kd and a carbohydrate content of about 28-30%. The exact function of THP remains enigmatic (Hoyer et al, 1979; Ronco et al, 1987; Kumar et al, 1990) but several studies have suggested that it might be a regulator of circulating levels and intrerenal bioactivity of cytokines, a major contributor to tubulointestitial renal disease, a trigger of cast nephropathy in multiple myeloma and a natural defense against bacterial infection of the urinary tract (Hess, 1994). In several studies (Grant, 1973; Bichler et al, 1976; Samuell, 1979; Thornley et al, 1985; Hess, 1992; Erwin et al, 1994) there appears to be no difference between the daily urinary excretion of THP in normals and calcium oxalate stone formers, averaging 40 to 50 milligrams.

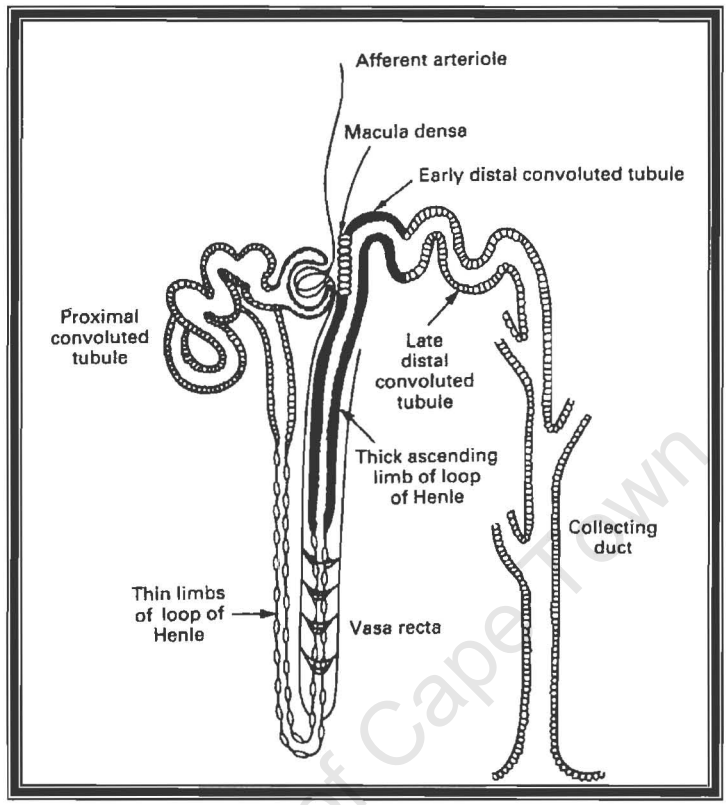



**Figure 2: Distribution of THP in the human nephron is limited to the thick ascending limb and the early distal convoluted tubule. THP is absent from the macula densa (Kumar and Muchmore, 1990).**



In previous studies by various workers, THP has been isolated from urine using a salt precipitation method (Tamm et al, 1950, 1952) and found to be the substance responsible for urinary inhibition of myoxvirus induced haemagglutination (Tamm et al, 1950). It is present in kidneys of all placental mammals but not in marsupials, monotremes and other invertebrates. As stated earlier, it is widely accepted that there are several steps involved in the formation of urinary stones. Spontaneous nucleation is the first step in stone formation and can be induced via a homogeneous or heterogeneous mechanism, either separate or combined. Crystal growth continues until the solubility product of the stone forming salts decreases to the saturation point. Concretion concludes the stone-forming process. The results of various studies of

THP effects on calcium oxalate crystal nucleation, growth and aggregation show that its role varies under different conditions. It has been established that THP affects the nucleation phase as a promoter and the crystal growth phase as a weak inhibitor (Yoshioka et al, 1989). Scurr and Robertson (1986, **(a), (b)**) showed that THP had an inhibitory effect on crystal growth and suggested it could be a potential promoter of crystal aggregation. Kitamura and Pak (1982) noted that THP exerted a slight inhibitory effect on calcium oxalate crystal precipitation and crystal growth. Rose and Sulaiman (1982) showed THP to promote calcium oxalate crystal precipitation, while Sophasan et al (1980) showed no influence of THP on crystal mass formation. Drach et al (1980) revealed the dual potential of THP for calcium oxalate crystallisation, viz an inhibitory effect on crystal growth, as well as an enhancing effect on crystal nucleation. Further evidence (Hess, 1992) that THP plays a dual role in COM crystal aggregation is at higher pH and lower ionic strength (IS), THP is a powerful aggregation inhibitor. Upon lowering pH and raising IS within physiological urinary limits, marked polymerisation of the THP molecules occur. Thus the role of T H P is somewhat controversial and probably has a dual effect on calcium oxalate crystallisation, being both an inhibitor of crystal growth and a promoter of nucleation. Indeed, Yoshioka et al (1989) have speculated that at physiological concentrations the inhibitory activity of THP is so weak, that the promotional activity is predominant.

These paradoxical influences on the various crystallisation processes may be explained by the physicochemical properties of T H P. If the concentration of the protein is increased (McQueen et al, 1966; Hess, 1992), divalent cations like calcium, magnesium (Cleave et al, 1972; Drach et al, 1980; Blomen et al, 1982) sodium (McQueen et al, 1966: Steveson et al, 1971) and hydrogen ions (McQueen et al, 1966) all increase polymerisation of the THP molecules, leading to reversible gel formation (increased viscosity) (Fletcher et al, 1970). These studies have shown that reduced THP inhibition or even promotion of CaOx crystallisation always occurred at lower pH and higher ionic stregth (IS). Comparison of THP from controls and stone formers (Hess, 1992) revealed a molecular abnormality of the THP from stone-formers. THP from this group exhibited an increased tendency to polymerize at lower pH and higher

IS and this is evident from molecular weight determinations and viscosity measurements (Hess et al. 1991).

The mechanism by which THP and macromolecules inhibit crystal growth and aggregation in vitro, involves their binding to the crystals, which induce a more negative surface charge (zeta-potential, ZP) on the crystal surface (Finlayson, 1978; Scurr et al, 1986 **(a), (b)**). When comparing the glycosaminoglycans and RNA to THP, studies have indicated that THP produced a less negative Zp on COM crystals (Scurr et al, 1986 **(a), (b)**; Hess, 1992). However, Hess (1992) found a negative linear correlation between crystal aggregation inhibition and intrinsic viscosity for THP i.e. THP with lower viscosities allowed more crystal aggregation inhibition (Hess et al. 1991). There is further evidence (Hess et al, 1991; Hess, 1992) that THP plays a dual role in COM crystal aggregation. At higher pH and lower IS, THP is a powerful aggregation inhibitor. Upon lowering pH and raising IS within physiological urinary limits, marked polymerisation of THP molecules occurs (Hess et al, 1991). This most probably increases attractive viscous binding forces on COM crystals surfaces, which have been coated with THP. Since the repulsive Zeta potential is not increased any further, the overall forces between crystals become more attractive (Finlayson, 1978), allowing for more crystal aggregation (reduced inhibition). If in addition, physiological calcium concentrations are present at low pH and high IS, stone-forming THPs even become promoters of COM crystal aggregation. Thus it is apparent that THP is a ubiquitious but important urinary protein in calcium oxalate kidney stone prevention on the one hand and stone pathogenesis on the other.

## 1.6. OBJECTIVES

In the South African context, it seems reasonable to speculate that Tamm Horsfall glycoprotein is worthy of investigation as it may be instrumental in providing the black population with enhanced protection against calcium oxalate urolithiasis. With this hypothesis in mind, the following objectives were defined:

1). To extract and purify Tamm Hosfall glycoprotein (THP) from black and white normal and stone-forming male subjects.

2). To use a variety of biochemical techniques to characterize the molecular weight, amino acid and secondary structural composition of all four THP groups.

3). To characterize the carbohydrate moieties from the four THP groups.

4). To investigate the relative inhibitory activity of THP from the four groups in their original urines as well as in the urines derived from each of the other three groups using a cross-over design.

5). To use a variety of crystallisation experiments to achieve objective number 4.

Details of each of these objectives will be presented in the Chapters which follow.

# REFERENCES


- Aaggarval. S., Tandon. C. D., Forouzandeh. M., Singla. S. K., Kiran. R., Jethi. R. K. (2000) Role of biomolecules from human renal stone matrix on COM crystal growth. Molecular and Cell Biochemistry **210**: (1-2). 109.
- Addadi. L., Weiner. S. (1985) Interactions between acidic proteins and crystals: stereochemical requirements in biomineralization. Proc.Natl. Acad. Sci. USA. 82: 4110.
- Andersen. D. A. (1973) "Enviromental factors in the aetiology of urolithiasis" In: "Urinary calculi: Proceedings of international symposium on renal stone research" Madrid 1972 eds: Cifuentes Delatte. L., Rapado. A., Hodgkinson. A., Karger. Basel. pp 130.
- Atmani. F., Mizon. J., Khan. S. (1996) Identification of uronic-acid-rich protein as urinary bikunin, the light chain of inter-α- inhibitor. Eur. J. Biochem. **236**: 984.
- Atmani. F., Glenton. P. A., Khan. S. R. (1998) Identification of proteins extracted from calcium oxalate and calcium phosphate crystals induced in the urine of healthy and stone forming subjects. Urol. Res. **26**: 201.
- Bataille. P., Pruna. A., Gregoire. I., Charransol. G., de Fremont. J. F., Coevoet. B., Galy. C., Fournier. A. (1984) Critical role of oxalate restriction in association with calcium restriction to decrease the probability of being a stone former: Insufficient effect in idiopathic hypercalciuria. Proc. EDTA. **20**: 401.
- Bek-Jensen. H., Fornander. A. M., Nilsson. A-M., Tiselius. H-G. (1996) Is citrate an inhibitor of calcium oxalate crystal growth in high concentrations of urine? Urol. Res. **24**: 67.
- Beukes. G. J., De Bruiyn. H., Vermaak. W. J. H. (1987) Effect of epidemiological factors on the composition and racial distribution of renal calculi. Br. J. Urol. **60**: 1.

- Bichler. K. H., Kirchner. C. H., Ideler. V. (1976) Uromucoid excretion of normal individuals and stoneformers. Br. J. Urol. **47**: 733.
- Binnette. J.P., Binnette. M.B., Gawinowicz. M. A., Kendrick. N. (1996) Urinary stone proteins: An update. Scanning Microsc. **10**: 509.
- Blacklock. N. J. (1969) Patterns of urolithiasis in the Royal Navy. In Hodgkinson. A., Nordin. B. E. C. eds. Renal Stone Research Symposium. London. J and A. Churchill Ltd. pp 33.
- Blacklock. N. J. (1982) "Epidemiology of urolithiasis" in: "Scientific foundations of urology" 2nd ed. Eds: Chisholm. G. D., Williams. D. I., Heineman. London. pp 235.
- Blomen. L. J. M. L. (1982) Growth and agglomeration of calcium oxalate monohydrate crystals. A contribution to the physicochemistry of urinary stone formation. Ph. D. Thesis, University of Leiden. The Netherlands
- Boeve. E., Cao. L.C., Verkoelen. C. F., Romijn. J., De Bruijn. W., Schroder. F. (1993) Glycosaminoglycans and other sulphated polysaccharides. World. J. Urol. **12**: 43.
- Boeve. E., Cao. L.C., De Bruijn. W., Robertson. W., Romijn. J., Schroder. F. (1994) Zeta Potential distribution on calcium oxalate crystal and Tamm-Horsfall protein surface analysed with Doppler electrophoretic light scattering. J Urol 152: 531.
- Borghi. L., Meschi. T., Amato. F., Briganti. A., Navarini. A., Giannini. A. (1996) Urinary volume, water and recurrences inidiopathic calcium nephrolithiasis: a 5-year randomizes prospective study. J. Urol. **155**: 839.
- Borsati. A. (1991) Calcium oxalate nephrolithiasis: defective oxalate transport. Kidney Int. **39**: 1283.
- Boskey. A. 1 (1981) Current concepts of the physiology and biochemistry of calcification. Clin. Orthop. Rel Res. **157**: 225.
- Boyce. W. H., Garvey. F. K., Strawcutter. H. F. (1956) Incidence of urinary calculi among patients in general hospitals 1948 – 1952. J. A. M. A. **161**: 1437.

- Bichler. K. H., Kirchner. C. H., Ideler. V. (1976) Uromucoid excretion of normal individuals and stoneformers. Br. J. Urol. **47**: 733.
- Binnette. J.P., Binnette. M.B., Gawinowicz. M. A., Kendrick. N. (1996) Urinary stone proteins: An update. Scanning Microsc. **10**: 509.
- Blacklock. N. J. (1969) Patterns of urolithiasis in the Royal Navy. In Hodgkinson. A., Nordin. B. E. C. eds. Renal Stone Research Symposium. London. J and A. Churchill Ltd. pp 33.
- Blacklock. N. J. (1982) "Epidemiology of urolithiasis" in: "Scientific foundations of urology" 2nd ed. Eds: Chisholm. G. D., Williams. D. I., Heineman. London. pp 235.
- Blomen. L. J. M. L. (1982) Growth and agglomeration of calcium oxalate monohydrate crystals. A contribution to the physicochemistry of urinary stone formation. Ph. D. Thesis, University of Leiden. The Netherlands
- Boeve. E., Cao. L.C., Verkoelen. C. F., Romijn. J., De Bruijn. W., Schroder. F. (1993) Glycosaminoglycans and other sulphated polysaccharides. World. J. Urol. **12**: 43.
- Boeve. E., Cao. L.C., De Bruijn. W., Robertson. W., Romijn. J., Schroder. F. (1994) Zeta Potential distribution on calcium oxalate crystal and Tamm-Horsfall protein surface analysed with Doppler electrophoretic light scattering. J Urol **152**: 531.
- Borghi. L., Meschi. T., Amato. F., Briganti. A., Novarini. A., Giannini. A. (1996) Urinary volume, water and recurrences inidiopathic calcium nephrolithiasis: a 5-year randomizes prospective study. J. Urol. **155**: 839.
- Borghi. L., Schianchi. T., Meschi. T., Guerra. A., Allegri. F., Maggiore. U., Novarini. A. (2002) Comparison of two diets for the prevention of recurrent stones in idiopathic hypercalciuria. New England Journal of Medicine. **346**: 77.
- Borsati. A. (1991) Calcium oxalate nephrolithiasis: defective oxalate transport. Kidney Int. **39**: 1283.

- Boskey. A. l (1981) Current concepts of the physiology and biochemistry of calcification. Clin. Orthop. Rel Res. **157**: 225.
- Boyce. W. H., Garvey. F. K., Strawcutter. H. F. (1956) Incidence of urinary calculi among patients in general hospitals 1948 – 1952. J. A. M. A. **161**: 1437.
- Breslau. N. A., Brinkley. L., Hill. K.D., Pap. C. Y. C. (1988) Relationship of animal protein – rich diet to kidney stone formation and calcium metabolism. J. Clin. Endocr. Metab. **66**: 140.
- Burns. J. R., Finlayson. B. (1980) A proposal for a standard reference artificial urine in in vitro urolithiasis experiments. Inves. Urol. **18**: 167.
- Burns. J. R., Finlayson. B. (1983) Why do some people have stone disease and others do not. In: Stones: Clinical Management of Urolithiasis. International Perspectives in Urology. Edited by R. A. Roth, B. Finlayson. Baltimore: The Williams and Wilkins Co. 6: chapter 1. pp 3.
- Butt. A. J. (1956) Historical survey of etiology factors in renal lithiasis. In Butt. A. J. ed. Etiology Factors in Renal Lithiasis. Illinois. Charles C Thomas. pp6.
- Cabrera. N., Vermilyea. D. A. (1958) In Doremus. R. H., Roberts. B. W., Turnbull. D. (eds) Growth perfection of crystals. Wiley. New York. pp 393.
- Cao. L. C., Deng. G., Boeve. E. R., de Bruijn. W. C., de Water. R., Verkoelen. C. F., Romijn. J. C., Schroder. F H. (1996) Zeta potential measurement and particle size analysis for a better understanding of urinary inhibitors of calcium oxalate crystallization. Scanning Micrsocopy. **10**: (2). 401.
- Caudarella. R., Rizzoli. E., Buffa. A., Bottura. A., Stefoni. S. (1998) Comparative study of the influence of 3 types of mineral water in patients with idiopathic calcium lithiasis. J. Urol. **159**: 658.

- Cerini. C.,Geider. S., Dussol. B., Hennequin. C., Daudon. M., Veesler. S., Nitsche. S., Boistelle. R., Berthezene. P., Dupuy. P., Vazi. A., Berland. Y., Dagorn. J-C., Verdier. J-M. (1999) Nucleation of calium oxalate crystals by albumin: Involvement in the prevention of stone formation. Kidney International **55**: 1776.
- Cleave.A. J., Kent. P. W., Peacocke. A. R. (1972) The binding of hydrogen and calcium ions by Tamm-Horsfall glycoprotein. Biochem. Biophys. Acta. **285**: 208.
- Coe. F. L., Parks. J. H., Nakagawa. Y. (1991) Protein inhibitors of crystallization. Semin. Nephrol. **11**: 98.
- Craig. T-A., Rodgers. A., Brandt. W. (2003) Inhibition of calcium oxalate crystallisation by Tamm Horsfall mucoprotein from two different population groups. Currently under review.
- Curhan. G. C., Willet. W. C., Speizer. F. E., Stampfer. M. J. (1989) Beverages use and risk for kidney stones in woman. Ann. Intern. Med. **128**: 534.
- Curhan. G. C., Willet. W. C., Rimm. E. B., Stampfer. M. J. (1993) A prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones N. Engl. J. Med. **328**: 833.
- Curhan. G. C., Willet. W. C., Speizer. F. E., Spiegelman. D., Stampfer. M. J. (1997) Comparison of dietary calcium with supplemental calcium and other nutrients as factors affecting the risk for kidney stones in woman. Ann. Int. Med. **126**: 497.
- Curhan. G. C. (2000) Calcium is the most important risk factor for idiopathic calcium oxalate stone formation. Proceedings of 9$^{th}$ International Symposium of Urolithiasis. Eds. Rodgers. A. R., Hibbert. B. E., Hess. B., Khan. S. R., Preminger. G. pp 295.
- Dent. C. E., Sutor. D. J. (1971) Presence or absence of inhibitor of calcium oxalate crystal growth in urine of normals and of stone formers. Lancet. **2**: 775.
- Doremus. R. H., Teich. S., Silvis. P. X. (1978) Crystallization of calcium oxalate from synthetic urine. Invest. Urol. **15** : 469.

- Doyle. I. R., Ryall. R., Marshall. V. R. (1991) The incorporation of urinary proteins into calcium oxalate crystals precipitated from human urine: A highly selective phenonmenon. Clin. Chem. **37**: 1589.
- Doyle I, Marshall V, Dawson C, Ryall R (1995) Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in undiluted human urine in vitro. Urol Res 23: 53.
- Drach. G. W. (1976) Contribution to therapeutic decisions of ratios, absolute values and other measures of calcium, magnesium, urate or oxalate balance in stone formers. J. Urol. **116**: 338.
- Drach. G. W., Thorson. S., Randolph. A. D. (1980) Effects of urinary organic macromolecules on crystallization of calcium oxalate: enhancement of nucleation. J. Urol. **123**: 519.
- Drach. G. W., Sarig. S., Randolph. A. D., Thorson. (1982) The paradox of inhibition and enhancement of the formation of urinary stones. Urol. Res. **10**: 165.
- Durrbaum. D.,Rodgers. A. (2001) A study of crystal matrix extract and urinary prothrombin fragment 1 from a stone-prone and stone-free population. Urol Res **29**: 83.
- Dussol. B., Berland. Y. (1998) Urinary kidney stone inhibitors. What in the news.Urol. Int. **60**: 69.
- Elliot. J. S. (1973) A comparison of the chemical composition of urine in normal subjects and in patients with calcium oxalate calculi. In: Urinary calculi. Int. Symp. Renal. Stone Res Madrid. S. Karger. Bassel. pp 24.
- Elliot. J. S. (1983) Calcium oxalate urinary calculi: clinical and chemical aspects. Medicine. **62**: 36.
- Embon. O. M., Rose. G. A., Rosenbaum. T. (1990) Chronic dehydration stone disease. Br. J. Urol. **66**: 357.

- Erwin. D.T., Alam. J. Kok. D. J., Annalaro. A.Jr., Vaughn. J., Coker. O., Carriere. B., Cole. F. E. (1994) Ultrafiltration of human urine affects calcium oxalate monohydrate (COM) crystallization kinetics, in Ryall. R. L., Bais. R., Marshall. V. R., Rofe. A. M., Smith. L. H., Walker. V. R. (eds) Proceedings of the Seventh International Symposium of Urolithiasis. Cairns. Australia. pp 297.
- Fellstrom. B., Backman. U., Danielson. B. G., Holmgren. K., Ljunghall. S., Wikstrom. B. (1982) (accepted 1981) Inhibitory activity of human urine on calcium oxalate crystal growth: effects of sodium urate and uric acid. Clin Sci. **62**: 509.
- Fellstrom. B., Danielson. B. G., Ljunghall. S. (1986) Crystal inhibition: the effects of polyanions on calcium oxalate crystal growth. Clin. Chim. Acta. **158**: 229.
- Finlayson. B., Vermeulen. C. W., Stewart. R. J. (1961) Stone matrix and mucoprotein from urine. J. Urol. 86: 355.
- Finlayson. B. (1978) Physicochemical aspects of Urolithiasis, Kidney Int, **13**: 344.
- Finlayson. B., Reid. F. (1978) The expectation of free and fixed particles in urinary stone disease. Invest Urol. 15:442-448.
- Fleisch. H., Bisaz. S. (1972) Isolation from urine of pyrophosphate, a calcification inhibitor. Amer. J. Physiol. **203**: 671.
- Fleisch. H. (1978) Inhibitors and promoters of stone formation. Kidney International. **13**: 361
- Fletcher. A. P., Neuberger. A., Ratcliffe. W. A. (1970) Tamm-Horsfall Urinary Glycoprotein. Biochem. J. **120**: 417.
- Fraij. B. M. (1989) Separation and identification of urinary proteins and stone-matrix proteins by mini-slab sodium dodecyl sulphate-polyacrylamide gel electrophoresis. CLIN. Chem. **35**: 658.

- Gill. W. B., Jones. K. W., Ruggerio. K. J., Fromes. M.C. (1981) Calcium oxalate crystallization in urothelial-lined systems. In: Urolithiasis; Clinical and Basic Research. Edited by L. H. Smith., W. G. Robertson., B. Finlayson. New York: Plenum Press, part III, pp 497.
- Goldfarb. S. (1994) Diet and nephrolithiasis. Anna. Rev. Med. **45**: 235.
- Goldwasser. B., Weinerth. J. L., Carson. C. C. (1986) Calcium stone disease: an overview. J. Urol. **135**:1.
- Grant. A. M. S., Baker. L. R. I., Neuberger. A. (1973) Urinary Tamm Horsfall glycoprotein in certain kidney diseases and its content in renal and bladder calculi. Clin. Sci. Med. **44**: 377.
- Grover. P. K., Ryall. R., Marshall. V. (1990) Does Tamm-Horsfall mucoprotein inhibit or promote calcium oxalate crystallisation inhuman urine? Clin Chim Acta 190: 223.
- Grover. P.K., Marshall. V. R., Ryall. R. L. (1994) Tamm-Horsfall mucoprotein reduces promotion of calcium oxalate crystal aggregation induced by urate in human urine in vitro. Clin Sci 87: 137.
- Hallson. P. C., Rose. G.A., Sulaiman. S. (1983) Raising urinary citrate lowers calcium oxalate and calcium phosphate crystal formation in whole urine. Urol. Int. **38**: 179.
- Hering. L. C. (1962) Observations on the analysis of 10000 urinary calculi. J. Urol. **88**: 345.
- Hess. B. (1991) The role of Tamm-Horsfall glycoprotein and nephrocalcin in calcium oxalate monohydrate crystallisation processes. Scan Micro 5: No 3, 689-696.
- Hess. B. (1992) Tamm-Horsfall Glycoprotein-Inhibitor or promoter of calcium oxalate monohydrate crystallisation processes? Urol Res 20: 83.
- Hess. B. (1994) Tamm-Horsfall glycoprotein and calcium nephrolithiasis. Miner Electrolyte Metab 20: 393.

- Hess. B., Nakagawa. Y., Coe. F. L. (1989) Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am J Physiol 257: F99.
- Hess B, Nakagawa Y, Parks JH, Coe FJ (1991) Molecular abnormality of Tamm Horsfal glycoprotein in calcium oxalate nephrolithiasis. Am J Physiol 260: F 569.
- Hess B, Zipperle L, Jaeger P (1993) Citrate and calcium effects on Tamm-Horsfall glycoprotein as a modifier of calcium oxalate crystal aggregation. Am J Physiol 265: 784.
- Hess. B., Meihardt. U., Zipperle. L., Giovanoli. R., Jaeger. P. (1995) Simultaneous measurements of calcium oxalate crystal nucleation and aggregation: impact of various modifiers. Urol. Res. **23**: 231.
- Hess. B., Kok. D. J. (1996) Nucleation, growth and aggregation of stone-forming crystals, in Coe. F.L., Favus. M. J., Pak. C. Y. C. et al. (eds): Kidney Stones: Medical and Surgical Management. Philadelphia. Lippincott-Raven. pp 3.
- Hess. B., Ryall. R. L., Kavanagh. J. P., Khan. S. R., Kok. D. J., Rodgers. A. L., Tiselius. H-G. (2001) Methods for measuring crystallization in urolithiasis research: Why, how, when? Eur. Urol. **40**: 220.
- Hesse .A., Classen. A., Knoll. M., Timmermann. F., Vahlensieck.W. (1986) Dependence of urine composition on the age and sex of healthy subjects. Clinica Chimica. Acta **160**: 79.
- Howard. J. E., Thomas. W. C. (1958) Some observations on rachitic rat cartilage of probable significance in aetiology of renal calculi. Trans. Am. Clin. Climat. Assoc. **70**: 90.
- Hoyer. J. R., Seiler. M. W. (1979) Pathophysiology of Tamm-Horsfall protein. Kidney Int. **16**: 279.
- Israelachvilli. J. N. (1992) Intermolecular and surface forces. Harcourt Brace Jovanovich. New York. pp 246.

- Jayadevan .S., Marickar. Y. M. F., Pillai. R. N. (2000) Correction of dietary intake and urine biochemistry. Proceedings of 9th International Symposium of Urolithiasis. Eds. Rodgers. A. R., Hibbert. B. E., Hess. B., Khan. S. R., Preminger. G. pp 302.
- Jones. W. T., Resnick. M. I. (1990) The characterization of soluble matrix proteins in selected human renal calculi using two-dimensional polyacrylamide gel elctrophoresis. J. Urol. **144**: 1010.
- Kavanagh. J.P. (1992) Methods for the study of calcium oxalate crystallization and their application to urolithiasis research. Scanning Microsc. **6**: 685.
- Kavanagh. J. P. (1995) Calcium oxalate crystallization in virto. In: Khan S. R(ed) Calcium oxalate in biological systems. CRC Press. Bora Raton. pp 1.
- Kavanagh. J.P. (1999) Enlargement of a lower pole calcium oxalate stone: A theoretical examination of the role of crystal nucleation, growth and aggregation, J. Endourol. **13**: (9) 605.
- Kavanagh. J.P. (2000) Suoersaturation: What role does it play in calcific stone formation? Proceedings of 9th International Symposium of Urolithiasis. Eds. Rodgers. A. R., Hibbert. B. E., Hess. B., Khan. S. R., Preminger. G. pp 41.
- Khan. S. R. (1997) Interactions between stone-forming calcific crystals and macromolecules. Urol. Int. **59**: 59.
- King. J. S., Boyce. W. H. (1963) Immunological studies on serum and urinary proteins in urolith. Matrix in man. Ann NY Acad. Sci. **104**: 579.
- Kitamura T, Pak CYC (1982) Tamm and Horsfall glycoprotein does not promote spontaneous precipitation and crystal growth of calcium oxalate in vitro. J Urol 127: 1024.
- Kohri. K., Garside. J., Blacklock. N. J. (1989) The effects of glycosaminoglycans on the crystallization of calcium oxalate. Br. J. Urol. **63**: 584.
- Kok. D. J., Papapoulos. S. E., Bijovet . O. L. M. (1986) Excessive crystal agglomeration with low citrate excretion in recurrent stone-formers. Lancet. i: 1056.

- Kok. D. J., Papapoulos. S. E., Bijovet . O. L. M. (1987) Low inhibition of crystal agglomeration and citrate excretion in recurrent calcium oxalate stone formers. Contr. Nephrol. **58**: 73.
- Kok. D. J., Papapoulos. S. E., Bijovet . O. L. M. (1990) Crystal agglomeration is a major element in calcium oxalate urinary stone formation. Kidney. Int. **37**: 51.
- Kok. D. J., Papapoulos. S. E., (1993) Physicochemical considerations in the development and prevention of calium oxalate urolithiasis. Bone Miner. **20**: 1.
- Kok. D. J., Khan. S.R. (1994) Calcium oxalate nephrolithiasis, a free or fixed particle disease. Kidney Int. **46**: 847.
- Kumar. S., Jasani. B., Hunt. J. S., Moffat. D. B., Asscher. A. W. (1985) A system for accurate immunolocalization of Tamm-Horsfall protein in renal biopsies. Histochem. J. **17**: 1251.
- Kumar. S., Muchmore. A. (1990) Tamm-Horsfall protein-uromodulin (1950-1990). Kidney Int. **37**: 1395.
- Leal. J., Finlayson. B. (1977) Adsorption of naturally occurring polymers on calcium oxalate surfaces. Invest Urol **14**: 278.
- Lewandowski. S., Rodgers. A., Schloss. I. (2001) The influence of a high-oxalate / low-calcium diet on calcium oxalate renal stone risk factors in non-stone-forming black and white South African subjects. B. J. U. Int. **87**: 307.
- Li. M. K., Blacklock. N. J., Garsdie. J. (1985) Effects of magnesium on calcium oxalate crystallization. J. Urol. **133**: 123.
- Lian. J. B., Prien. E. L. Jr., Glimcher. M. J., Gallop. P. M. (1977) Thr presence of protein-bound γ-carboxyglutamic acid in calcium-containing renal calculi. J. Clin. Invest. **59**: 1151.
- Lieske. J. C., Walsh-Reitz. M. M., Toback. F. G. (1992) Calcium oxalate monohydrate crystals are endocytosed by renal epithelial cells and induce proliferation. Am. J. Physiol. 262: F622.
- Lieske. J. C., Toback. F. G. (1993) Regulation of renal epithelial cell endocytosis of calcium oxalate monohydrate crystals. Am. J. Physiol. **264**: F800.

- Lonsdale. K. (1968) Epitaxy as a growth factor in urinary calculi and gallstones. Nature **217**: 56.
- Mandel. N. S., Riese. R. J. (1991) Crystal-cell interactions: Crystal binding to rat renal papillary tip collecting duct cells in culture. Am. J. Physiol. **274**: F958.
- Mandel. N. (1994) Crystal- membrane interaction in kidney stone disease. J. Am. Soc. Nephrol. **5**: 37.
- Marangella. M., Bianco. O., Martini C., Petrarulo. M., Vitale. C., Linari F. (1989) Effect of animal and vegetal protein intake on oxalate excretion in idiopathic calcium stone disease. Br. J. Urol. **63**: 348.
- Marangella. M., Vitale. C., Petrarulo. M., Bagnis. C., Bruno. M., Ramello. A. (2000) Renal Stones: from metabolic physicochemical abnormalities. How useful are inhibitors? J. Nephrol. **13**: S51.
- Marshal. R. W., Cockran. M., Robertson. W. G. et al (1972) The relation between the concentration of calcium salts in the urine and renal stone composition in patients with calcium containing renal stones. Clin. Sci. **43**: 433.
- Massey. L. K., Roman-Smith. H., Sutton. R. A. (1993) Effect of dietary oxalate and calcium on urinary oxalate and risk of formation of calcium oxalate kidney stones. J. Am. Diet. Assoc. **93**: 901.
- Martins. M. C., Meyers. A. A., Whalley. N. A., Rodgers. A. L. (2002) Cystine: A promoter of growth and aggregation of calcium oxalate crystals in normal undiluted human urine. J.Urol. **167**: (1) 317.
- McCormack. M., Dessureault. J., Guitard. M. (1991) The urine specific gravity dipstick: a useful tool to increase fluid intake in stone forming patients. J.Urol.**146**: 1475.
- McGeown. M. G. (1960) Heredity in renal stone disease. Clin. Sci. **19**: 465.
- McQueen. E. G., Engel. G. B. (1966) Factors determining the aggregation of urinary mucoprotein. J. Clin. Pathol. **19**: 392.

- Medetogen-Benission.J., Tardivel. S., Hennequin.C., Daudon. M., Drueke. T., Lacour. B. (1999) Inhibitory effect of bikunin on calcium oxalate crystallization in vitro and urinary bikunin decreas in renal stone formers. Urol. Res. **27**: 69.
- Meyer. J. L., Smith. H. L. (1975) Growth of calcium oxalate crystals. II. Inhibition by natural urinary crystal growth inhibitors. Invest. Urol. **13**: 36.
- Meyer. W. J., Transbol. I., Bartter P. C., Delea. C. (1976) Control of calcium absorption: effect of sodium chloride loading and depletion. Metabolism. **25**: 989.
- Meyer. J. L., Thomas. W. C. Jr. (1982) Trace metal-citric acid complexes as inhibitors of calcification and crystal growth. I. Effects of Fe(III), Cr(III) and Al (III) complexes on calcium phosphate crystal growth. J. Urol. **128**: 1372.
- Modlin. M. (1967) The aetiology of renal stone: A new concept arising from studies on a stone-free population. Ann. Roy. Coll. Surg (Eng). **40**: 155.
- Moore. C. A., Bunce. G. F. (1964) Reduction in frequency of renal calculous formation by oral magnesium administration. Invest. Urol. **2**: 7.
- Morse. R. M., Resnick M. I. (1988) A new approach to the study of urinary macromolecules as participant in calcium oxalate crystallization. J. Urol. **139**: 869.
- Muscat. D. A. (1951) The problem of nephrolithiasis with special reference to the Bantu. S. Afr. J. Clin. Sci. **2**: 18.
- Nakagawa. Y., Abram. V., Kezdy. F. J. et al. (1983) Purification and characterization of the principle inhibitor of calcium oxalate monohydrate crystal growth in human urine. J. Biol. Chem. **258**: 12594.
- Nakagawa. Y., Abram. V., Parks. J. H. et al. (1985) Urine glycoprotein crystal growth inhibitors: Evidence for a molecular abnormality in calcium oxalate nephrolithiasis. J. Clin. Invest. **76**: 1455.
- Nicar. M. J., Holt. K., Pak. C. Y. C. (1980) Ion binding by small molecular weight substances (1000-10000 daltons) in urine. Invest. Urol. **18**: 162.
- Nicar M. J., Hill. K., Pak. C. Y. C. (1987) Inhibition by citrate of spontaneous precipitation of calcium oxalate in vitro. J. Bone and Min. Res. **2** (3): 215.

- Nishio. S., Yakuchi. A. B. E., Wakatsuki. A., Iwata. H., Ochi. K., Takeuchi. M., Matsumoto. A. (1985) Matrix glycosaminoglycan in urinary stones. J. Urol. 134: 503.
- Nordin. B. E. C., Hodgkinson. A. (1967) Urolithiasis. Adv. Intern. Med. **13**: 155.
- Pak. C., Ohata. M., Holt. K. (1975) Effect of diphosphonate on crystallisation of calcium oxalate in vitro. Kidney Int 7: 154.
- Pak. C. Y. C., Galosy. R. A., (1980) Propensity for spontaneous nucleation of calcium oxalate. Quantitative assessment by urinary FPR-APR discriminant score. Amer. J. Med. **69**: 681.
- Pak. C. Y. C., Smith. L. H., Resnick M. I., Weinerth. J. L. (1984) Dietary management of idiopathic calcium urolithiasis. J. Urol. **131**: 850.
- Parivar. F., Low. R. K., Stoller. M. L. (1996) The influence of diet on urinary stone disease. J. Urol. **155**: 432.
- Pillay. S. N., Asplin. J. R., Coe. F. L. (1998) Evidence that calgranulin is produced by kidney cells and is an inhibitor of calcium oxalate crystallization. Am. J. Physiol. **275**: F255.
- Rao P. N., Prendiville. V., Buxton. A., Blacklock. N. J. (1982) Dietary management of urinary risk factors in renal stone formers. Br. J. Urol. **54**: 575.
- Riese. R. J., Riese. J. W., Kleinman. J. G. et al. (1988) Specificity in calcium oxalate adherence to papillary epithelial cells in culture. Am. J. Physiol. **255**: F1025.
- Robertson. W. G. (1987) Diet and calcium stones. Min Electrolyte Metab. **13**: 228.
- Robertson. W. G., Peacock. M., Nordin. B. E. C. (1969) Activity products in stone-forming and non-stone-forming urine. Clin. Sci. **34**: 579.
- Robertson. W. G., Peacock. M. (1972) **(a)** Calcium oxalate crystalluria and inhibitors of crsytallisation in recurrent renal stone-formers. Clin. Sci. **43**: 499.
- Robertson. W. G., Peacock. M., Nordin. B. E. C. (1972) **(b)** Crystalluria: In Finlayson. B. et al. eds: Urolithiasis: Physical Aspects. Washington. D. C. National Academy of Sciences. pp 243.

- Robertson. W. G., Peacock. M., Nordin. B. E. C. (1973) Inhibitors of the growth and aggregation of calcium oxalate crystals. In vitro. Clin. Chim. Acta. **43**: 31.
- Robertson. W. G., Peacock. M., Heyburn. P. J., Marshall. D. H., Clark. P. B. (1978) Risk factors in calcigerous stone disease of the urinary tract. Br. J. Urol. **50**: 449.
- Robertson. W. G., Peacock.. M. (1983) Review of risk factors in calcium oxalate urolithiasis. World. J. Urol. **1**: 114.
- Robertson. W. G., Whitfield. H. N., Unwin. R. J., Mansell. M. A., Neild. G. H., Mostafid. H., Longhorn. S. E. (2000) Proceedings of 9th International Symposium of Urolithiasis. Eds. Rodgers. A. R., Hibbert. B. E., Hess. B., Khan. S. R., Preminger. G. pp 305.
- Rodgers. A. L., Spector. M. (1981) Human Stones. Endeavour. New series **5**: (3) 119. Ronco. P., Brunisholz. M., Legendre. M., Chatelet. F., Verroust. P., Richet. G. (1987) Physiopathological aspects of Tamm-Horsfall protein: A phylogenetically conserved marker of the thick ascending limb of Henle's loop. Adv. Nephrol. **16**: 231.
- Rodgers. A. L. (1997) Effect of mineral water containing calcium and magnesium on calcium oxalate urolithiasis risk factors. Urologia Internationalis. **58**: 93.
- Rodgers. A. L. (1998) The influence of South African mineral water on reduction of risk of calcium oxalate kidney stone formation. South African Medical Journal. **88**: 448.
- Rose. G.A. (1982) Urinary stones: clinical and laboratory aspects. University Park Press. Baltimore. Maryland. pp 10.
- Rose. G.A., Sulaiman. S. (1982) Tamm-Horsfall mucoproteins promote calcium oxalate crystal formation in urine: Quantitative studies. J. Urol. **127**: 177.
- Ryall. R. L., Harnett. R. M., Marshal. V. R. (1981) The effects of urine, pyrophosphate, citrate, magnesium and glycosaminoglycans on the growth and aggregation of calcium oxalate crystals in vitro. Clin. Chim. Acta. **112**: 349.

- Ryall. R. L, Grover. P. K., Doyle. I. R. et al. (1988) The effects of macromolecules on the crystallization of calcium oxalate in human urine. In; Martelli. A., Buli. P., Marchesini. B., eds. Inhibitors of crystallization in renal lithiasis and their clinical application. Rome: Acta. Medica. pp 51.
- Ryall. R. L, Grover. P. K., Harnett. R. M., Hibberd. C. M., Marshal. V. R. (1989) Small molecular weight inhibitors. In: Walker. V. R., Sutton. R. A. L., Cameron. E. C. B., Pak. C. Y. C., Robertson. W. G. eds. Urolithiasis. Plenum. New York. London. pp 91.
- Ryall. R. L., Harnett. R. M., Hibberd. C. M., Edyvane. K. A., Marshal. V. R. (1990) Effects of chrondroitin sulphate, human serum albumin and Tamm Horsfall mucoprotein on calcium oxalate crystallisation in undiluted human urine. Urol. Res. **19**: 181.
- Ryall. R. L, Harnett. R. M., Hibberd. C. M., Edyvane. K. A., Marshall. V. R. (1991) Effects of chondroitin sulphate, human serum albumin and Tamm-Horsfall mucoprotein on calcium oxalate crystallisation in undiluted human urine. Urol Res 19: 181.
- Ryall. R. L, Grover. P. K., Stapleton. A. M. F., Barrell. D. K., Tang. Y., Mortz. R. L., Simpson. R. J. (1995) The urinary F1 activation peptide of human prothrombin is a potent inhibitor of calcium oxalate crystallization in undiluted human urine in vitro. Clin. Sci **89**: 533.
- Ryall. R. L. (1996) Glycosaminoglycans, proteins and stone formation: adult themes and child's play. Pediatr. Nephrol. **10**: 656.
- Ryall. R. L. (1997) Urinary inhibitors of calcium oxalate crystallization and their potential role in stone formation. World. J. Urol. **15**: 155.
- Ryall. R. L, Marshall. V. R. (1998) Mineralis in animals and vegetables (editorial comment). Curr. Opin. Urol. **8**: 298.
- Samuell. C. T. (1979) Uromucoid excretion in normal subjects, calcium stone formers and in patients with chronic renal failure. Urol. Res. **7**: 5.
- Schwille. P. O, Hermann. U. (1992) Environmental factors in the pathophysiology of recurrent idiopathic calcium urolithiasis (RCU), with emphasis on nutrition. Urol. Res. **20**: 72.

- Schwille. P. O., Schmiedl. A., Hermann. U., Fan. J., Gottlieb. D., Manoharan. M. et al. (1999) Magnesium, citrate, magnesium citrate and magnesium-alkali citrate as modulators of calcium oxalate crystallisation in urine: observations in patients with recurrent idiopathic calcium urolithiasis. Urol. Res. **27**: 117.
- Scurr. D. S., Robertson. W. G. (1986) **(a)** Modifiers of calcium oxalate crystallisation found in urine.II Studies on their mode of action in artificial urine. J Urol 136: 128.
- Scurr. D. S., Robertson. W. G. (1986) **(b)** Modifiers of calcium oxalate crystallisation found in urine.III Studies on the role of Tamm-Horsfall mucoprotein and of ionic strength. J Urol 136: 505.
- Shiraga. H., Min. W., VanDusen. W. J., Clayman. M. D., Miner. D., Terrell. C. H., Sherbotie. J. R., Foreman. J. W., Przysiecki. C., Neilson. E. G., Hoyer. J.R. (1992) Inhibition of calcium oxalate crystal growth in virto by uropontin: another member of the aspartic acic-rich protein superfamily. Proc. Natl. Acad. Sci.USA. **89**: 426.
- Sophasan. S., Chatasingh. S., Thanphaichitr. P. (1980) Tamm Horsfall mucoprotein in urine of potential bladder stone formers. J. Urol. **124**: 522.
- Sorensen. S., Hansen. K., Bak. S., Justesen. S. J. (1990) An unidentified macromolecular inhibitory constituent of calcium oxalate crystal growth in human urine. Urol. Res. **18**: 373.
- Stapelton. A. M. F., Seymour. A. E., Brennan. J. S. et al. (1993) **(a)** Immunohistochemical distribution and quantification of crystal matrix protein. Kidney Int. **44**: 817.
- Stapelton. A. M. F., Simpson. R. J., Ryall. R. L. (1993) (B) Crystal matrix protein is related to human prothrombin. Biochem. Bopphys. Res Commun. **195**: 1199.
- Steveson. F. K., Cleave. A. J., Kent. P. W. (1971) The effect of ions on the viscometric and ultracentrifugal behaviour of Tamm- Horsfall glycoprotein. Biochem. Biophys Acta. **236**: 59.

- Stocholy. J., Goldberg E. P. (1985) Microstructural matrix-crystal interaction in calcium oxalate monohydrate kidney stones. Scanning Electron Microsc. II: **781**.
- Sugimoto. T., Funae. H. Y., Rubben. H., Nishio. S., Hautmann. R., Lutzeyer. W. (1985) Resolution of protein in the kidney stone matrix using high-performance liquid chromatography. Eur. Urol. **2**: 334.
- Sutor. D.J. (1969) Growth studies on calcium oxalate in the presence of various ions and compounds. Br. J. Urol. **41**: 171.
- Tamm. I., Horsfall. F. L. (1950) Characterization and separation of an inhibitor of viral haemagglutination found in urine. Proc. Soc. Exp. Biol. Med. **74**: 108.
- Tamm. I., Horsfall. F. L. Jr (1952) A mucoprotein derived from human urine which reacts with influenza, mumps and Newcastle disease viruses. J. exp. Med. **95**: 71.
- Thorne. I., Resnick. M. I. (1983) Urinary Macromolecules and Renal Lithiasis. World. J. Urol. **1**: 138.
- Thornley. C., Dawnay. A., Cattell. W. R. (1985) Human Tamm-Horsfall glycoprotein: urinary and plasma levels in normal subjects with renal disease determined by a fully validated radioimmunoassay. Clin. Sci. **68**. 529.
- Tiselius. H. G., Fornander. A. M., Nilsson. M. A. (1993) The effects of citrate and urine on calcium oxalate aggregation. Urol. Res. **21**: 363.
- Tiselius. H-G., (1996) Solution chemistry of supersaturation, in Kidney Stones: Medical and Surgical Management, edited by Coe. F. L., Favus. M. J., Pak. C. Y. C., et al. Philadelphia. Lippencott-Raven Publisher.
- Trinchieri. A (1996) Epidermiology of urolithiasis. Arch. Ital. Urol. Androl. **68**: 203.
- Vahlensieck. W. (1984) Review: The importance of diet in urinary stones. Urol. Res. **14**: 283.
- Vermeulen.C. W.,Lyon. E. S, Gill. W.B. (1964) Artificial Urinary Concretions. Invest. Urol. **1**: 370.

- Walker. R. N., Linksuiler. H. M. (1970) Calcium retention in the adult human. Clin. Nutr. **23**:63.
- Warpehoski.M., Buscemi. P. J., Osborn. D. C., Finlayson. B., Goldberg. E. P. (1981) Distribution of organic oxalate renal calculi. Calcif Tissue Int. **33**: 211.
- Webber D, Rodgers AL, Sturrock ED (2002) Synergism between Urinary Prothrombin Fragment 1 and urine: A comparison of inhibitory activities in stone-prone and stone-free population groups. Clin Chem Lab Med **40**: 930.
- Welshman. S.G., McGeown. M. G. (1972) A quantitative investigation of the effects on the growth of calcium oxalate crystals of potential inhibitors. Br. J. Urol. **47**: 237.
- Whalley. N. A., Moraes. M. F. B. G., Shar. T. G., Pretorius. S. S., Meyers. A. M. (1998) Lithogenic risk factors in the urine of black and white subjects. Br. J. Urol. **82**: 785.
- Wise. R. O., Kark. A. E. (1961) Urinary calculi and serum calcium levels in African and Indians. S. Afr. Med. J. **35**: 47.
- Worcester EM, Nakagawa Y, Wabner CL, Kumar S, Coe FL (1988) Crystal adsorption and growth slowing by nephrocalcin, albumin and Tamm Horsfall protein. Am J Physiol **255**: F1197.
- Worcester. E. M., Beshensky. A. M. (1995) Osteopontin inhibits nucleation of calcium oxalate crystals. Ann. N. Y. Acad. Sci 760: 375.
- Worcester E. M. (1996) Inhibitors of Stone Formation. Seminars in Nephrology. **16**: (5). 474.
- Yoshioka. T., Koide. T., Utsunomiya. M., Itatani. H., Oka. T., Sonoda. T. (1989). Possible role of Tamm-Horsfall Glycoprotein in calcium oxalate crystallization. Br. J. Urol. **64**: 463.

# CHAPTER 2

# BIOCHEMICAL ANALYSIS

## 2.1. INTRODUCTION

As stated in Chapter 1, Tamm Horsfall glycoprotein (THP) is the most abundant protein in normal human urine and has been shown to play a dual role in modifying crystal aggregation: at high pH and low ionic strength (IS), it appears to be a powerful crystal aggregation inhibitor; when these conditions are reversed, its viscosity increases, leading to reduced inhibition of crystal aggregation (Hess, 1992). Biochemical analysis of the urine and THP from the two race groups might reveal differences which might provide some insight to the stone immunity shown by the black South African population. The latter provides the motivation for the following objectives.



## 2.2. OBJECTIVES

- Analyse 24-hour urines from black and white normal (ie.healthy) male subjects (BNU, WNU) and stone-forming patients (BSFU, WSFU) for routine biochemical and physiocochemical parameters.
- Extract, purify and verify THP from each group (BNTHP, WNTHP, BSFTHP, WSFTHP)
- Characterise THPs using the following techniques:
    1) Matrix Assisted Laser Desorption / Ionisation Time–of-flight (MALDI-TOF) mass spectroscopy to determine molecular weights.
    2) Tryptic Peptide Mass Finger Printing to determine peptide masses.
    3) Amino Acid Analysis to determine differences in the mole percentages of amino acids.
    4) Protein Circular Dichroism (CD)- to determine the secondary structure of the four proteins.

## 2.3. METHODS

### *2.3.1. Subjects, urinalysis and physicochemical risk indicies*

24h urines from healthy normal subjects and stone-forming male patients from white and black race groups were collected in plastic bottles containing 10g of boric acid as a preservative per bottle. All urines were tested using Combur 5 N strips (Macherey-Nagel, Germany) and excluded if found to be nitrate positive. Collections from the same population groups for the healthy subjects were pooled while urines from stone patients were treated individually. Urines were analysed using standard chemical techniques. Compositional data were used to calculate the relative supersaturation (Werness et al, 1985) and risk index for each urine (Tiselius, 1982).

### *2.3.2. THP Extraction and Purification*

THP was precipitated from the pooled urine by addition of sodium chloride (NaCl) to a final concentration of 0.58M (Fletcher et al, 1970). THP was allowed to precipitate overnight at 4 $^{O}$C and the solution was centrifuged at 12000g for 30 minutes. The supernatant was discarded, the precipitate redissolved in distilled water and the solution was centrifuged again at 12000g for 15 minutes. The small coloured precipitate was dicarded. The precipitation process was repeated at least once but with only 0.058M of NaCl added. The final precipitate was dissolved in Milli-Q water, dialyzed exhaustively against distilled water and freeze-dried to yield the crude THP. Crude THP was chromatographed on a Sepharose 4B column (4x80cm), (Pharmacia, Piscataway, NJ) using 0.02 M sodium phosphate buffer, pH 6.8 as the eluant (Appendix 2.1 for preparation of buffer) (Hess et al, 1991). The 230 / 280 nm absorbance of selected fractions were used to determine the fractions corresponding to the first peak of eluted protein, were pooled (Hess et al, 2001). The concentrations of the purified THPs were determined according to Bradford, 1976 (Appendix 2.2 for the Bradford protocol).

### *2.3.3. THP Verification*

The purified THP was verified by electrophoresis on a 15% SDS polyacrylamide gel together with Sigma low molecular weight, 14000 – 97000, markers (Laemmli, 1970) (Appendix 2.3-protocol for Laemmli's gel method). 10µl of purified THP sample dissolved in Milli-Q water with 10µl of sample application buffer was applied to each well on the gel together with a low molecular weight marker (Sigma low molecular weight, 14000 - 97000), which was treated in the same manner as the protein samples. Electrophoresis was at 200 V for 3 hours, after which the gel was stained for 30 min at room temperature in methanol/ water/ acetic acid (5: 4: 1 by vol) containing 1g $l^{-1}$ of CBB R250. The gel was destained in ethanol / water/ acetic acid (9:10:1 by vol) mixture for 1 hour before being scanned using A CanoScan N122OU scanner.

### *2.3.4. Biochemical Analysis*

#### *2.3.4.a. Matrix Assisted Laser Desorption / Ionisation Time-of Flight (MALDI-TOF) Mass Spectroscopy*

Purified THPs were analysed by MALDI-TOF Mass Spectroscopy. An aliquot of 1µl of analyte (a 2mg $ml^{-1}$ solution of purified THP dissolved in 0.1% TFA (Trifluoroacetic acid) is dissolved in a matrix (a weak organic acid) (4 µls of CHCA (CHCA = α - cyno - κ - hydroxcinnamic acid), which is then co-crystallised together. The presence of the matrix prevents degradation of the analyte, reduces intermolecular contacts other than analyte-matrix interactions and acts as a protonating agent. The matrix and analyte mixture was dried onto a sample plate and loaded into the mass spectrometer (Perseptive Biosystems Voyager DE-PRO, USA). A nitrogen laser beam, which operates at wavelenghts at which the analyte is weakly absorbing, excites the matrix / analyte mixture allowing the matrix to absorb most of the laser energy minimising sample damage and ion fragmentation. Once the sample has been vaporised and ionised, it is transferred electronically into a time-of-flight

mass spectrometer (TOF-MS) where it is separated from matrix ions and then individually detected, based on their mass-to charge (m/z) ratios and analysed. Several runs were repeated on various samples from each THP group.

### *2.3.4.b. Tryptic Peptide Mass Fingerprinting*

Purified THP samples were then dissolved in a 2mg $ml^{-1}$ solution of ammonium bicarbonate (ambic buffer) and then digested with trypsin (10ng/µl) (Appendix 2.4 for protocol of trypsin digest). The digested samples were then left overnight at 37 °C and then determined for peptide mass fingerprinting using the MALDI-TOF Mass Spectroscopy protocol. Trypsin digestion breaks down the protein into peptides of specific molecular mass. The trypsin digest protocol was repeated several times on all four THP groups and repeated on gel digests for all four groups (Appendix 2.4 for protocol of (a) solution based digests and (b) in-gel digests). Peptide sequences from each MALDI-TOF Mass spectra were matched via a database and all four spectra were indentified as human uromodulin, P07911 with a pI = 5.05 and molecular weight = 67136.69 daltons.



### *2.3.4.c. Amino Acid Analysis*

Samples were hydrolized in an evacuated sealed glass bottle containg 2 mls of 6N HCl in the bottom of the bottle at 110°C for 24 hours. The hydrolysates were dissolved in sodium citrate buffer, pH 2.0, after removal of the HCl in vacuo. Amino acid compositions were determined with a gas phase Waters HPLC Amino Acid Analyser. The composition of these acids is expressed as mole percentage of amino acid residues (Fletcher et al, 1970).

Cysteine was oxidized by performic acid followed by 24-hour hydrolysis in 6N HCl and then analysed as cysteic acid (Hirs, 1967). Threonine and serine values were obtained by extrapolating at time 0 after plotting 24-hour values on semilogarithmic graph paper.

*2.3.4.d. Circular Dichroism (CD)*

Circular dichroism (CD) spectrum of a protein is a direct reflection of its secondary structure (Hennessey et al, 1981) and provides information on these structural components, for example, α-helix, β-sheets and random coils (Mathews et al, 1999). These structural components provide very different spectra. All protein concentrations were determined as described previously and then dissolved in 0.01M sodium phosphate buffer, pH 6.8. The solutions were dialysed against 1lt of buffer. Solutions were then centrifuged and the supernatant was used for the measurment of the CD spectra. The CD spectra were measured over a range from 195 – 260 nm by using a vacuum ultraviolet (UV) CD spectropolarimeter (JASCO F-810) with a 0.2 cm path length quartz cuvette. The concentrations of the supernatant were again determined by Bradford protocol, as they were required for analysis using the computer software CDNN21 programme. The purpose of this programme is to deconvolute the protein spectra measured by the CD spectropolarimeter.

## 2.4. RESULTS

*2.4.1. Urinalysis and physicochemical risk indicies*

Urine composition values are given in Table 1 and are shown as histograms in Figs 1a and 1b for easy comparison. There are statistically significant differences in some of the urinary risk factors ($p < 0.05$). In healthy subjects, citrate, oxalate, calcium (significantly different) and urate tend to be lower in the black group while magnesium and phosphate excretions tend to be higher (not significantly different). In stone formers, oxalate and calcium tend to be higher in blacks while urate (significantly different), magnesium, citrate and sodium (significantly different) tend to be lower. Also, citrate, magnesium and phosphate excretions are lower in stone formers than in healthy subjects in both race groups. Relative supersaturation values are depicted as histograms in Fig 2. It is of interest that WSFU have apparently lower

values for RS CaOx than WNU ($p<0.05$) while BSFU have higher values than BNU ($p<0.05$). Also of interest is that RS CaOx values for BNU are apparently lower than those for WNU (statistical significantly different) while the opposite is true for the stone formers, viz values for BSFU are higher than those for WSFU (statistical significantly different). RS for brushite is noteworthy for the relatively high value for WNU (statistical different from BNU) and the relatively low value for BSFU (statistical different from WSFU). RS for uric acid is noteworthy for the higher values in stone formers (WNU, BNU are significantly different to BSFU). Histograms for the Tiselius risk index in each group are shown in Fig 3. Of considrable interest is the observed trend which follows the sequence BNU<WNU<BSFU<WSFU (significant difference between WNU and BNU and these normal urines and their respective stone-forming urine).



| PARAMETERS | BNU | | WNU | | BSFU | | WSFU | |
|---|---|---|---|---|---|---|---|---|
| Cit | 1.7 | (0.04) | 2.2 | (0.04) | 1.4 | (0.04) | 1.6 | (0.04) |
| Ox | 0.2 | (0.01) | 0.3 | (0.01) | 0.2 | (0.01) | 0.1 | (0.01) |
| Ca | 2.0 | (0.1) | 3.2 | (0.1) | 2.5 | (0.1) | 1.9 | (0.1) |
| Mg | 3.1 | (0.05) | 2.9 | (0.04) | 2.1 | (0.1) | 2.3 | (0.04) |
| Urate | 2.3 | (0.04) | 2.6 | (0.04) | 1.2 | (0.1) | 2.4 | (0.04) |
| Na | 133 | (3.7) | 123 | (2.7) | 102 | (3.8) | 151 | (3.7) |
| K | 37.5 | (0.9) | 41.4 | (0.7) | 22.3 | (1.2) | 38.3 | (0.9) |
| Phos | 27.4 | (0.6) | 23.7 | (0.3) | 14.2 | (0.7) | 10.1 | (0.1) |
| Creat | 11.7 | (0.2) | 11.1 | (0.1) | 8.4 | (0.3) | 18.5 | (0.3) |
| Cl | 120 | (1.5) | 101 | (1.4) | 94 | (5.6) | 92.3 | (1.6) |
| pH | 6.3 | (0.1) | 6.3 | (0.1) | 5.6 | (0.1) | 6.3 | (0.1) |
| RS CaOx | 2.89 | (0.3) | 6.55 | (1.1) | 5.41 | (1.2) | 2.41 | (0.4) |
| RS BRUSH | 0.91 | (0.1) | 1.96 | (1.2) | 0.38 | (0.1) | 0.91 | (0.2) |
| RS URATE | 0.87 | (0.1) | 1.00 | (0.1) | 1.73 | (0.3) | 1.34 | (0.3) |
| RISK INDEX | 141 | (18) | 259 | (27) | 327 | (57) | 425 | (25) |

**Table 1: Urine composition (mmoles / 24 hrs) and physicochemical data and standard error.**
(Data used to construct Table 1 is located in Appendix 2.5A)

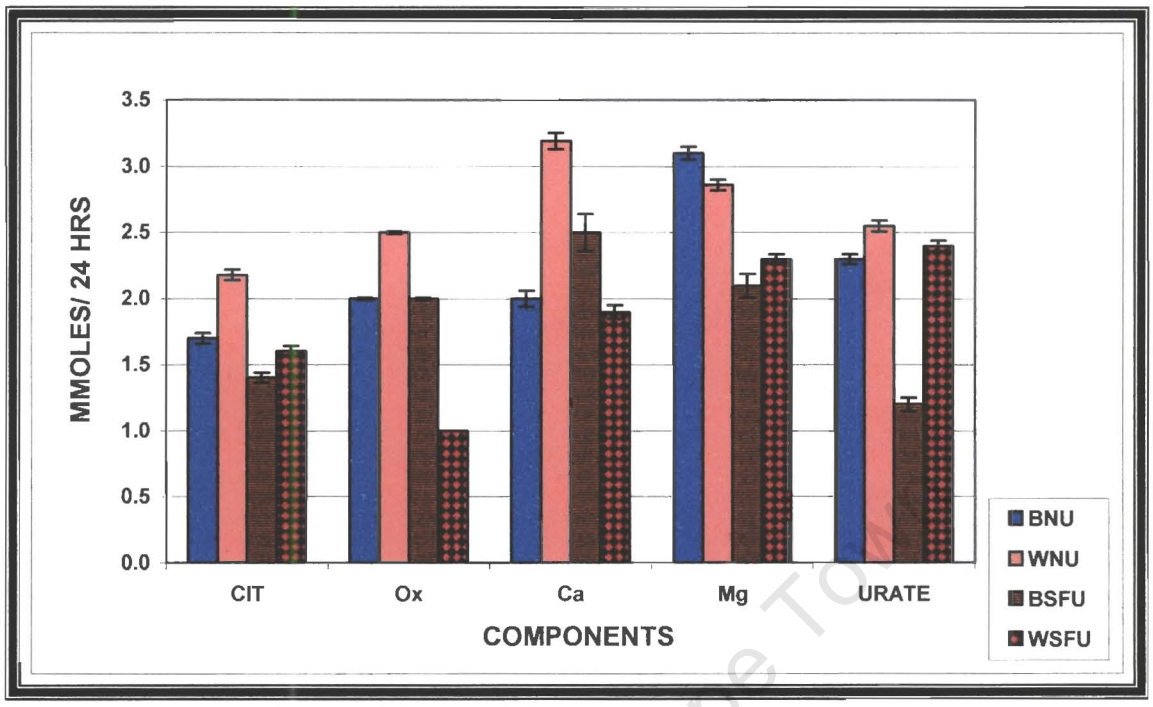



Figure 1a: Urine composition.

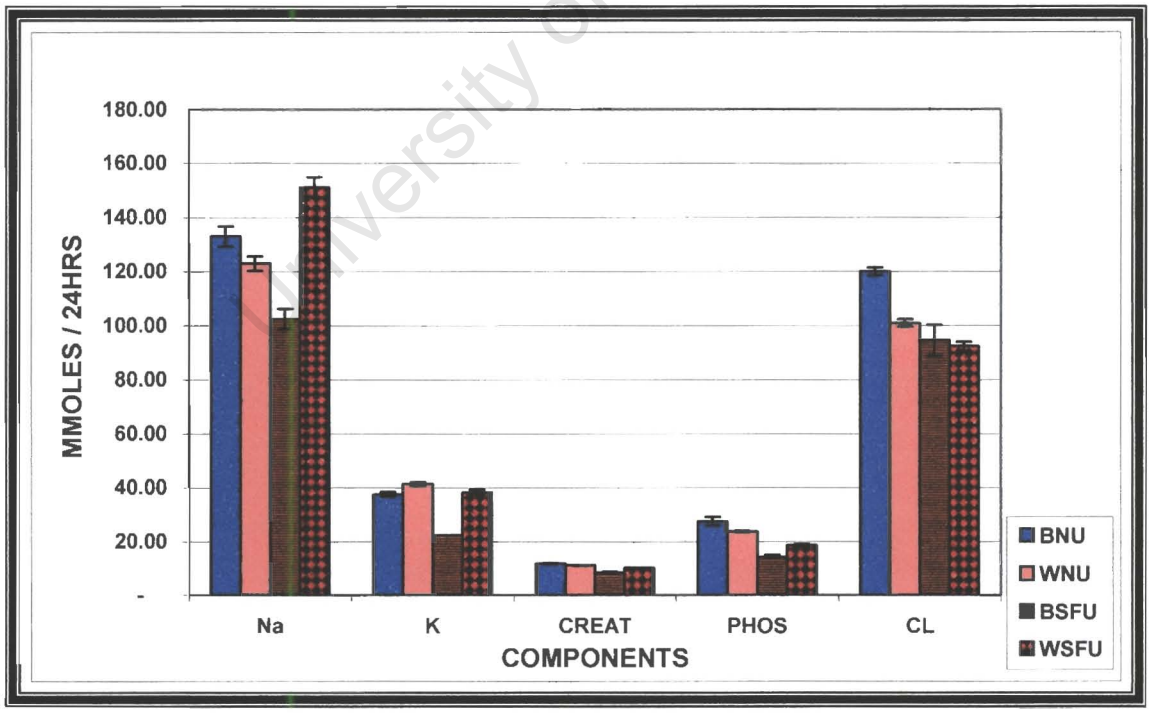



Figure 1b: Urine composition (continued).

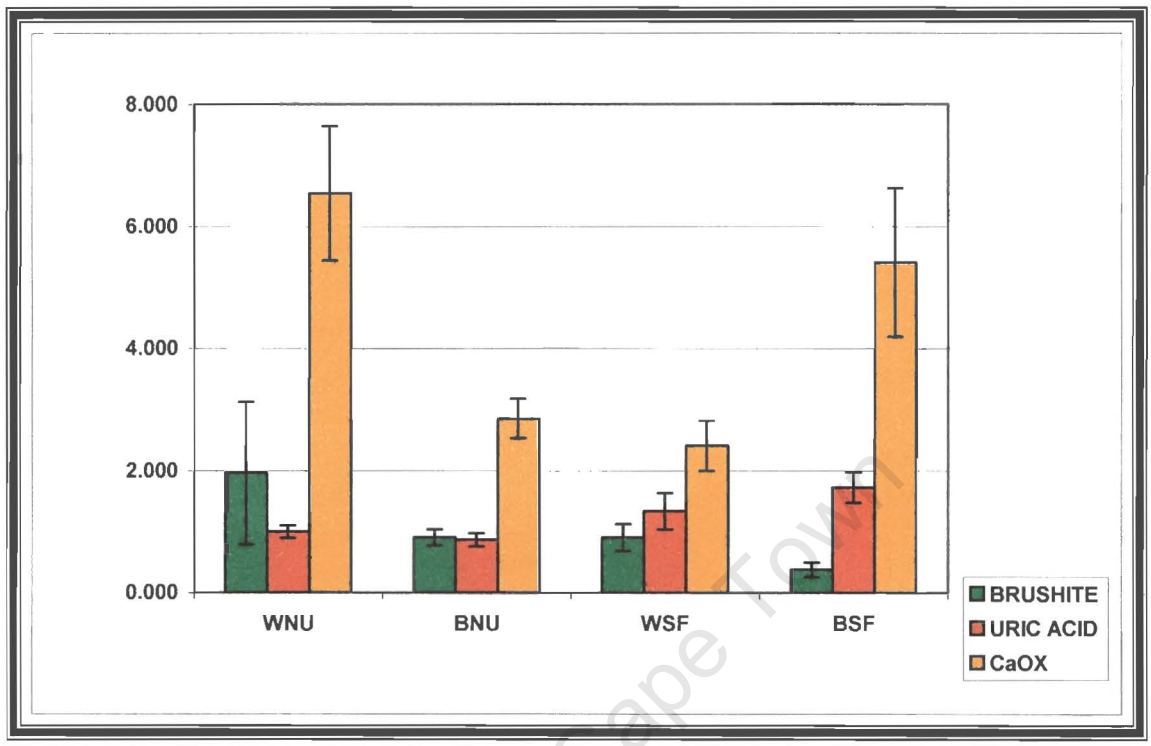



**Figure 2: Relative Supersaturation.** (Data used to construct Figure 2 is located in Appendix 2.5B)

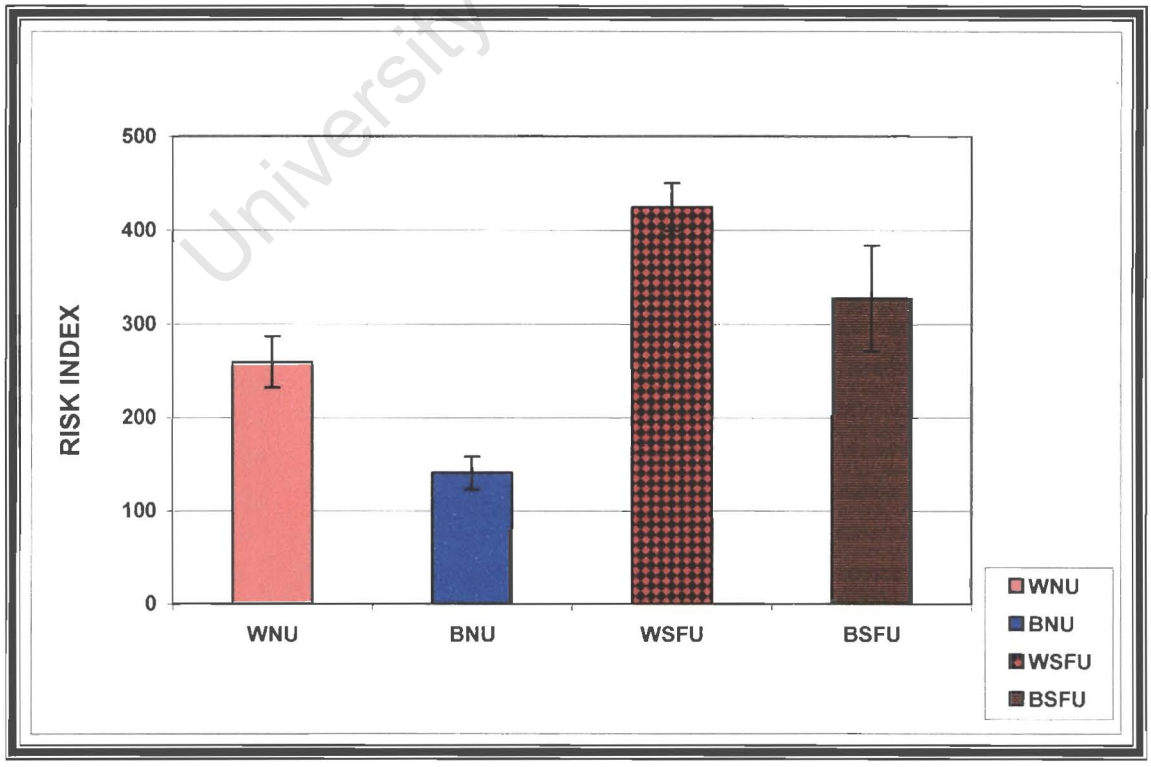



**Figure 3: Tiselius Risk Index.** (Data used to construct Figure 3 is located in Appendix 2.5C)

### *2.4.2. THP Purification and Verification*

Figure 4 is a 15% SDS- polyacrylamide gel together with Sigma low molecular weight markers, 14000 – 97000 daltons. 10µl of a 1mg $ml^{-1}$ solution of each purified THP sample in water and sample application buffer were loaded per well. THP samples were visualised with Comassie Blue stain and indicated a molecular weight of approximately 97kD.

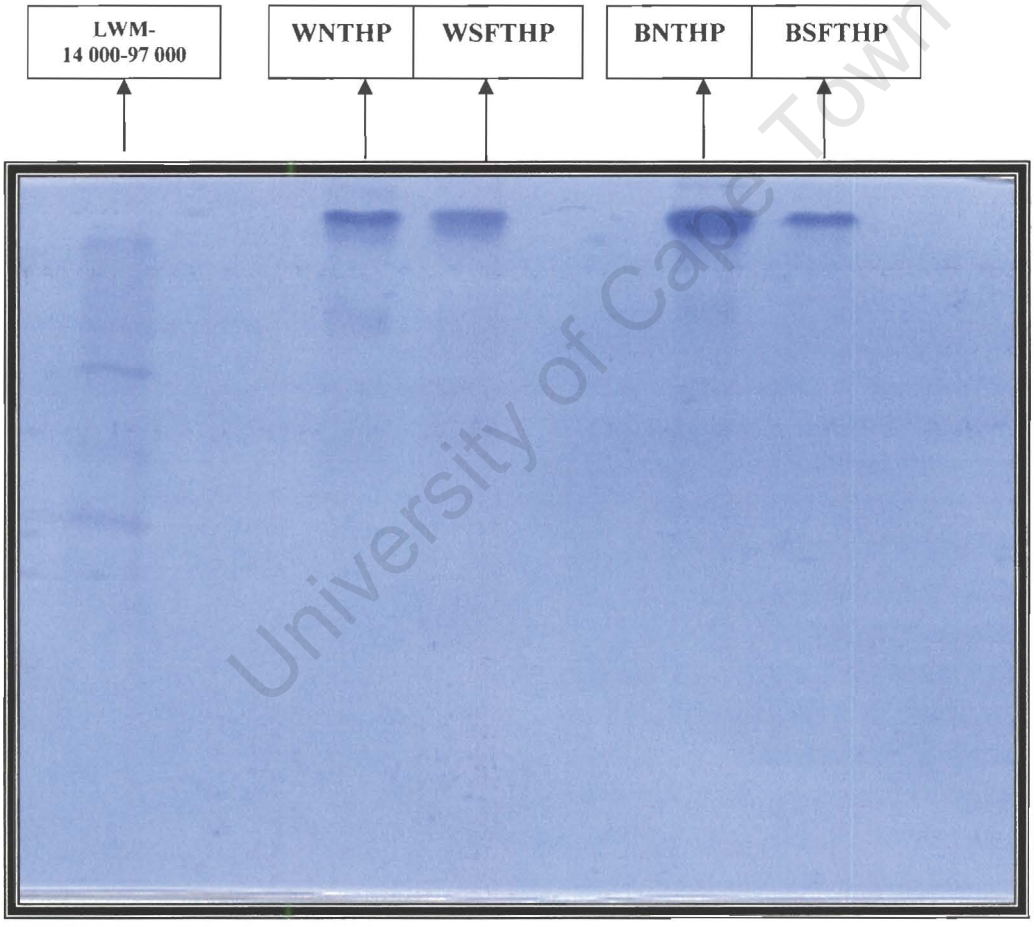





**Figure 4. 15 % SDS-polyacrylamide gel electrophoresis with the purified THP samples and a Sigma Protein and Peptide Low Molecular Weight Marker in the range of 14 000 – 97 000 daltons visualised using CCB.**

### *2.4.3. Biochemical Analysis*

#### *2.4.3.a. Matrix Assisted Laser Desorption / Ionisation Time-of Flight (MALDI-TOF) Mass Spectroscopy*

Several molecular weight determinations were performed on the THP samples. Table 2 provides mean values that were obtained from MALDI-TOF mass spectroscopy, while Fig. 5 presents this data in a histogram. Fig. 6a – 6d present the typical spectra that were obtained for these THP samples. BNTHP appears to have a significantly different molecular weight.

| TYPE OF THP SAMPLE | MOLECULAR WEIGHT (Daltons) |
|---|---|
| WNTHP | 79 968 (544) |
| BNTHP | 80939 (116) |
| WSFTHP | 79753 (189) |
| BSFTHP | 79791 (434) |

**Table 2. Mean values of THP samples obtained from MALDI-TOF Mass Spectroscopy. Values in brackets represent standard error. (Data used to construct Table 2 is located in Appendix 2.6)**

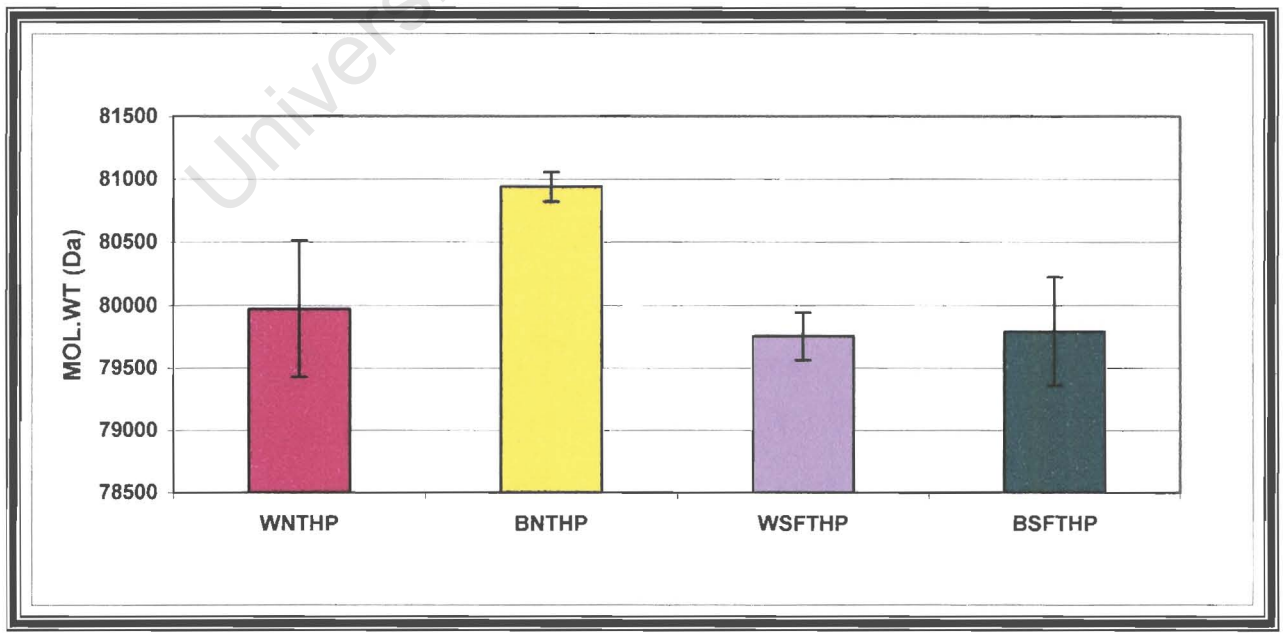



**Figure 5: Histograms representing the mean molecular weight values for the four THP samples. Error bars represent standard error.**

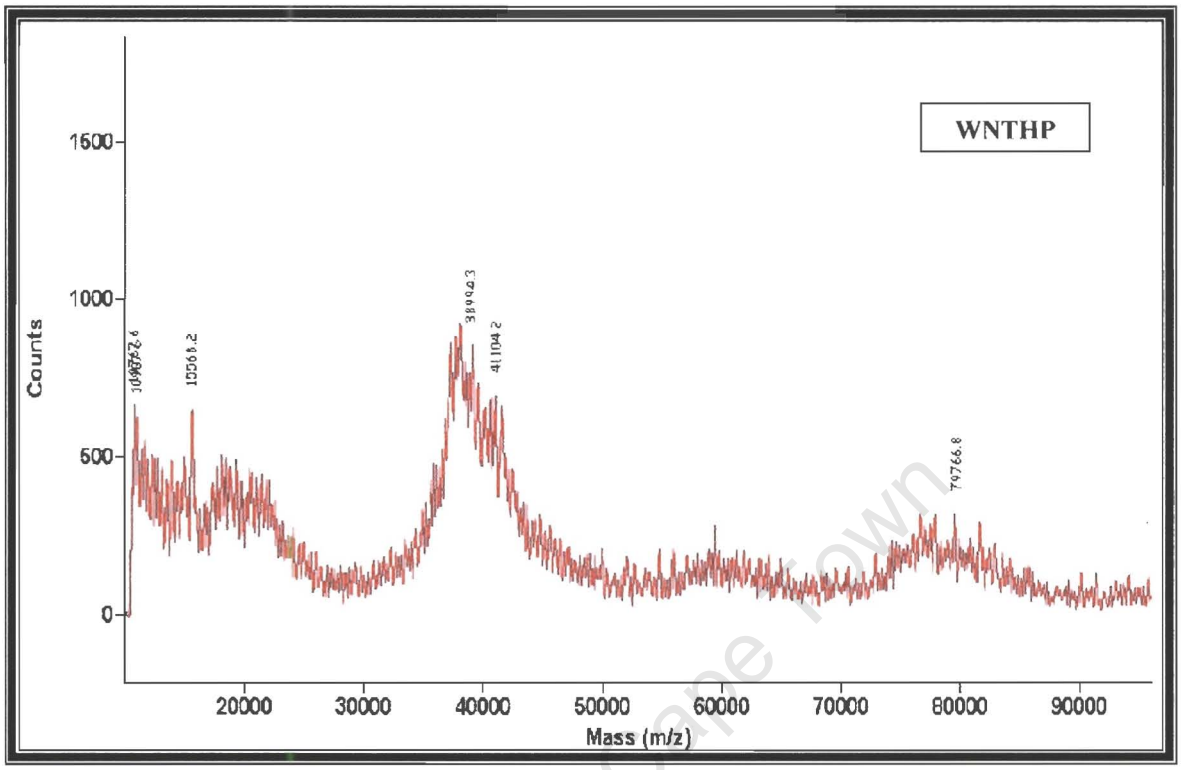



**Figure 6a. Typical MALDI-TOF Mass Spectrum of WNTHP**

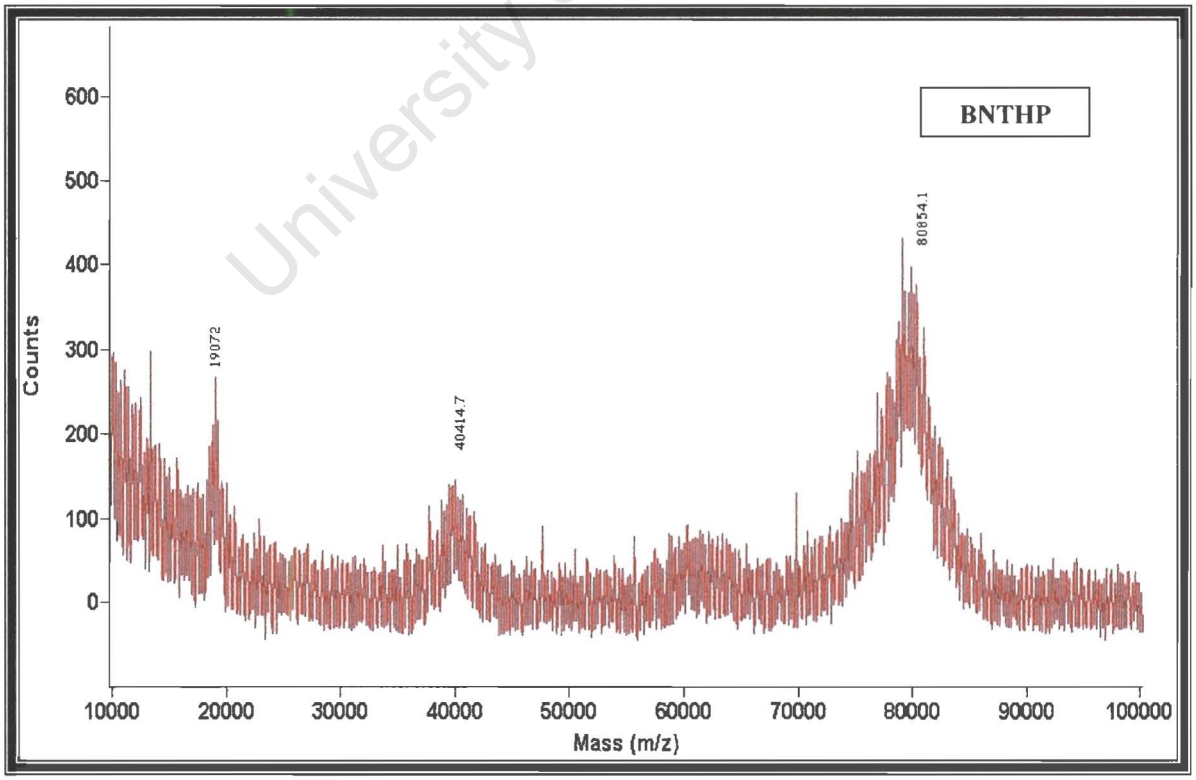



**Figure 6b. Typical MALDI-TOF Mass Spectrum of BNTHP**

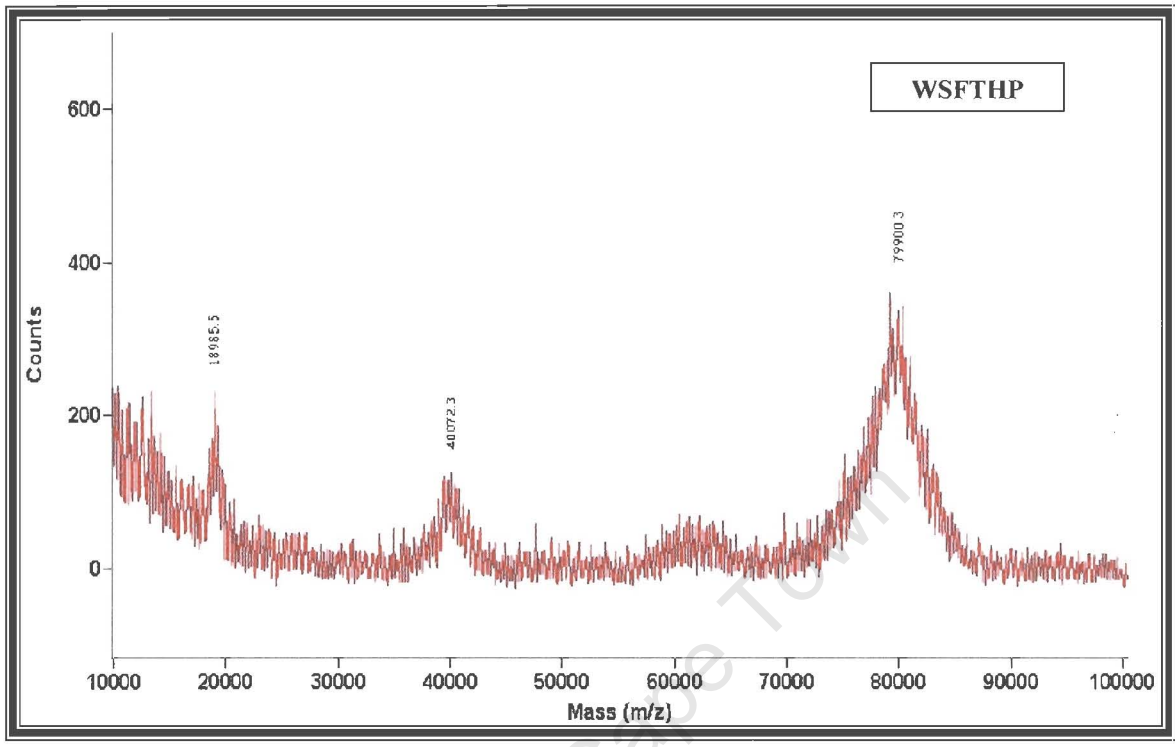



**Figure 6c. Typical MALDI-TOF Mass Spectrum of WSFTHP**

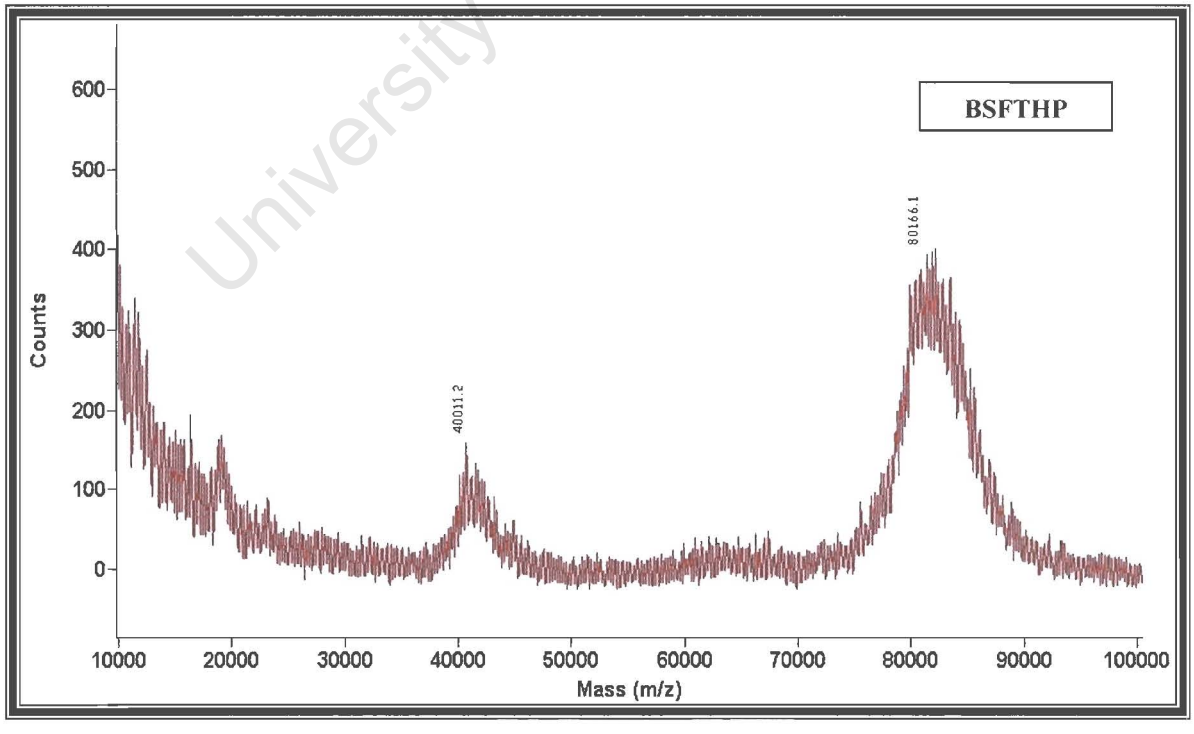



**Figure 6d. Typical MALDI-TOF Mass Spectrum of BSFTHP**

### *2.4.3.b. Tryptic Peptide Mass Fingerprinting*

Fig.7a – 7d are typical spectra obtained after tryptic digestion for each THP sample. Several runs were repeated on various samples from each THP group, including in-gel digestion.

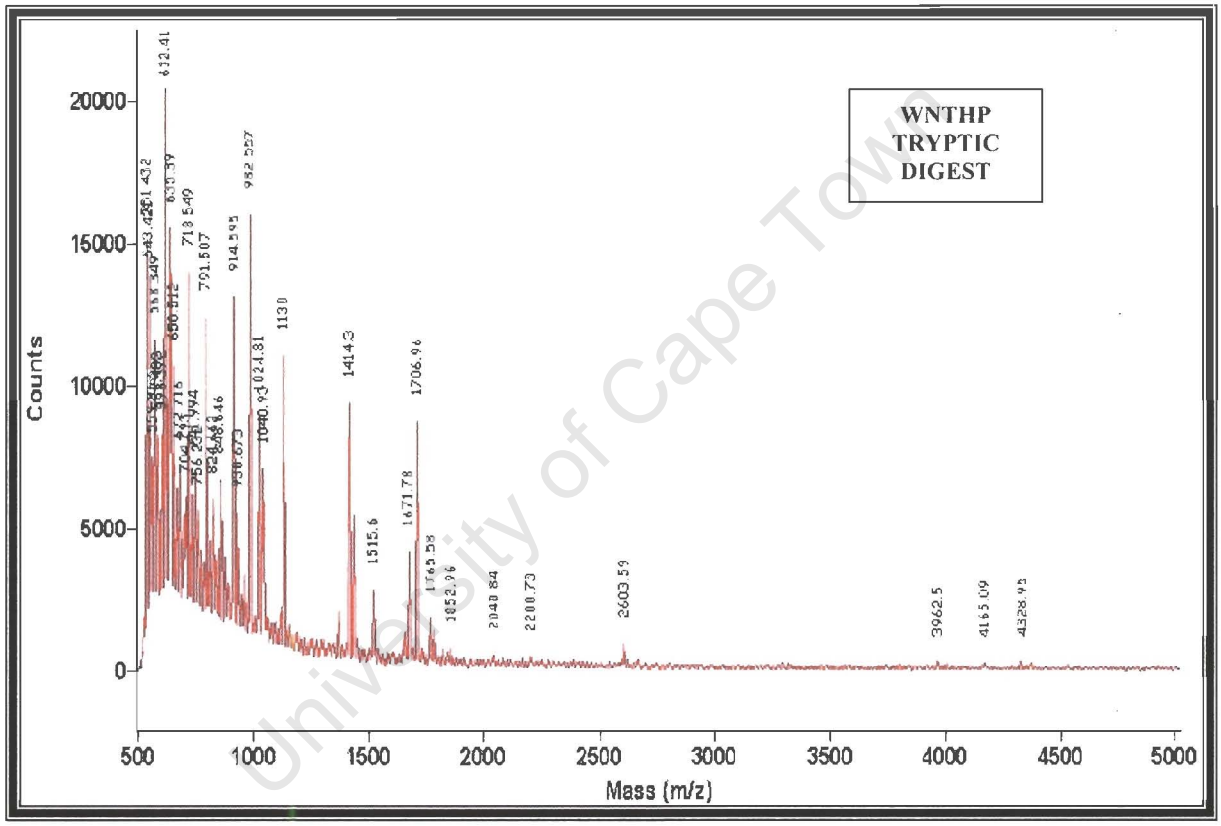





**Figure 7a. Typical tryptic digest spectrum for WNTHP**

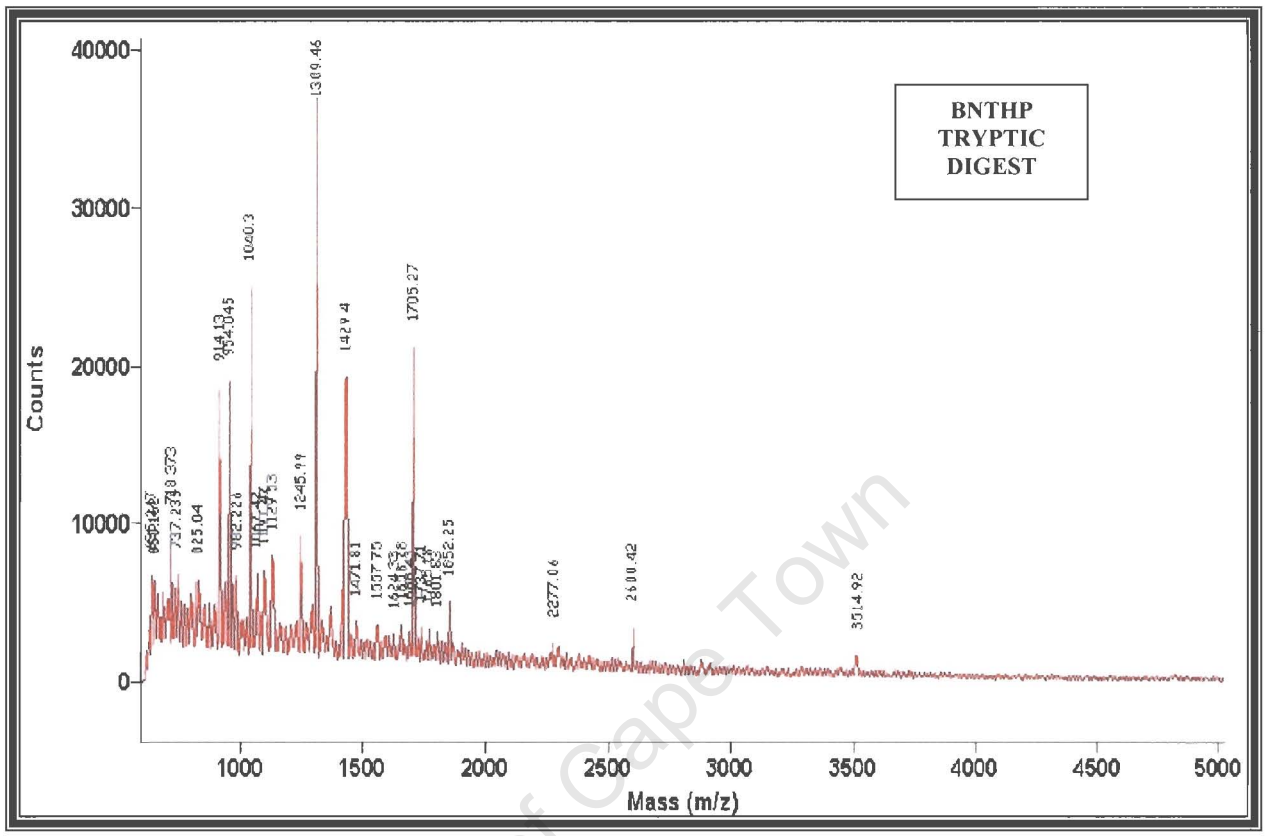



**Figure 7b. Typical tryptic digest spectrum for BNTHP**

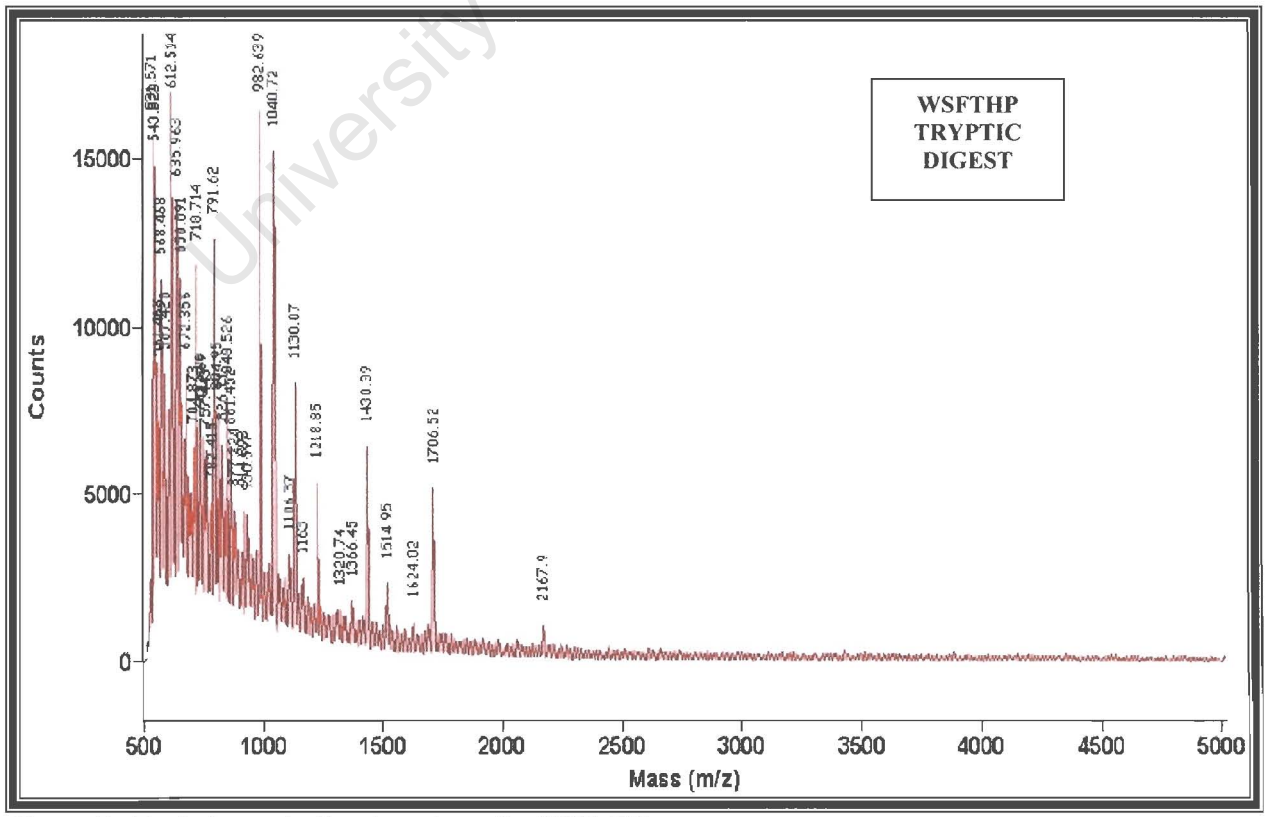



**Figure 7c. Typical tryptic digest spectrum for WSFTHP**

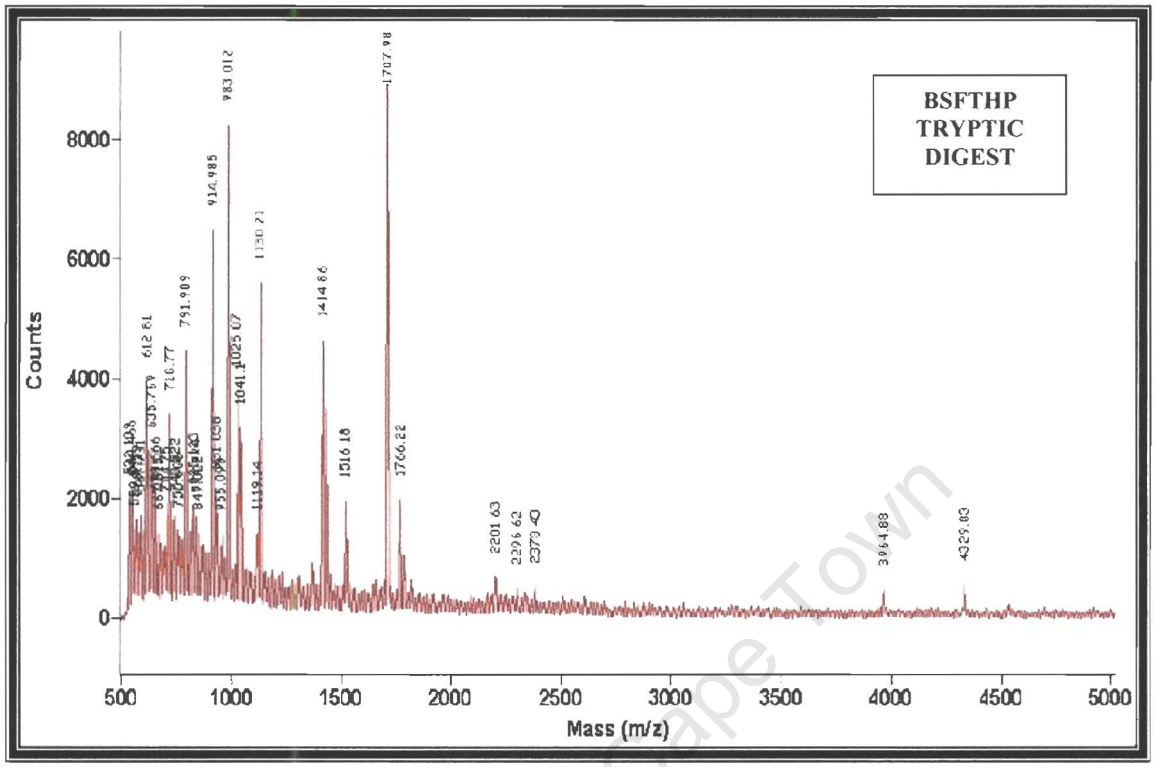



**Figure 7d. Typical tryptic digest spectrum for BSFTHP**



When peptide masses for the various THP samples were tabulated (Table 4) and compared with the Swiss-Prot database, all the peptide masses obtained for WNTHP, WSFTHP and BSFTHP after trypsin digestion agreed with those from the database. However the BNTHP appeared to show some extra peptides of different masses other than those of the database and have been tabulated separately in Table 3.

| PEPTIDE MASS | POSITION | PEPTIDE SEQUENCE |
|---|---|---|
| 1040.3050 | 450-459 | VGGTGMFTVR |
| 1067.4210 | 333-341 | LECGANDMK |
| 1471.8080 | 437-449 | INFACSYPLDMK |
| 1765.1790 | 351-365 | SLGFDKVFMYLSDSR |

**Table 3. Peptides from BNTHP after trypsin digestion that were not found on the Swiss-Prot database.**

| PEPTIDE MASS | POSITION | WNTHP | BNTHP | WSFTHP | BSFTHP | PEPTIDE SEQUENCE |
|---|---|---|---|---|---|---|
| | | | | | | WCSECHSNATCTEDEAVTTC |
| 7169.8200 | 31-99 | | | | | TCQEGFTGDGLTCVDLDECA IPGAHNCSANSSCVNTPGSF SCVCPEGFR |
| 4506.8161 | 266-307 | | | | | ACAGGYYVYNLTAPPECHLA YCTDPSSVEGTCEECSIDEDCK |
| 4379.9684 | 100-142 | | | | | LSPGLGCTDVDECAEPGLSH CHALATCVNVVGSYLCVCPA GYR |
| 4232.0202 | 460-498 | | | | | MALFQTPSYTQPYQGSSVTL STEAFLYVGTMLDGGDLSR |
| 3641.4429 | 143-178 | | | | | GDGWHCECSPGSCGPGLDCV PEGDALVCADPCQAHR |
| 2740.2091 | 555-577 | | | | | FAGNYDLVYLHCEVYLCDTM NEK |
| 2443.1129 | 223-245 | | | | | CNTAAPMWLNGTHPSSDEGI VSR |
| 2337.1721 | 396-415 | | | | | NETHATYSNTL YLADEIIIR |
| 2258.0831 | 499-519 | | | | | FALLMTNCYATPSSNATDPL K |
| 2100.9200 | 247-265 | | | | | ACAHWSGHCCLWDASVQVK |
| 1816.0618 | 625-640 | | | | | VWLPLLLSATLTLTFQ |
| 1705.7987 | 532-547 | $ | # | * | + | DSTIQVVENGESSQGR |
| 1700.8602 | 319-332 | | | | | QDFNITDISLLEHR |
| 1679.6853 | 186-200 | | | | | STEYGEGYACDTDLR |
| 1413.7882 | 437-449 | $ | | * | + | TALQPMVSALNIR |
| 1401.6541 | 421-432 | | | | | INFACSYPLDMK |
| 1129.6000 | 376-385 | $ | # | * | + | DWVSVVTPAR |
| 1118.5696 | 213-222 | | | | + | MAETCVPVLR |
| 1117.5346 | 357-365 | | | | + | VFMYLSDSR |
| 1024.5244 | 450-459 | $ | # | * | + | VGGTGMFTVR |
| 1018.4986 | 386-395 | | | | | DGPCGTVLTR |
| 982.6044 | 598-606 | $ | # | * | + | VLNLGPITR |
| 982.4628 | 179-185 | $ | # | * | + | TLDEYWR |
| 980.4176 | 333-341 | $ | | * | + | LECGANDMK |
| 954.5043 | 520-526 | | # | * | + | YFIIQDR |
| 952.4339 | 578-586 | | | | + | CKPTCSGTR |
| 948.4745 | 589-597 | | | | | SGSVIDQSR |
| 935.5560 | 616-624 | | | | | AFSSLGLLK |
| 914.4553 | 548-554 | $ | # | * | + | FSVQMFR |
| 817.4526 | 608-615 | | | | | GVQATVSR |
| 804.3280 | 313-318 | | | | | WHCQCK |
| 798.3199 | 366-372 | | | | | CSGFNDR |
| 791.4158 | 205-212 | | | | | FVGQGGAR |
| 678.3053 | 25-30 | | | | | DTSEAR |
| 666.3457 | 351-356 | | | | | SLGFDK |
| 613.2875 | 527-531 | | | | | CPHTR |
| 602.3508 | 416-420 | | | | | DLNIK |
| 581.2830 | 201-204 | | | | | GWYR |
| 547.2583 | 308-312 | | | | | SNNGR |
| 503.3187 | 342-346 | | | | | VSLGK |

**Table 4. Peptide masses for Tamm-Horsfall glycoprotein from Swiss-Prot, covering 97.6% of the sequence together with the peptide masses obtained for the four THP samples after Trypsin digestion**

*2.4.3.c. Amino Acid Analysis*

Table 5 presents the percentage moles of amino acids for THP derived from normal subjects and stone-forming patients respectively. This table also provides literature values for normal and stone-forming subjects (Hess, 1991) while values obtained from cDNA sequencing were for normal subjects only (Pennica, 1987). The results in Table 5 are also presented as histograms in figures 8.

| AMINO ACIDS | WNTHP | BNTHP | Hess et al, 1991, N | Pennica, 1987 | WSFTHP | BSFTHP | Hess et al, 1991, SF |
|---|---|---|---|---|---|---|---|
| CYS | 8.27 (1.1) | 10.25 (0.6) | 9.63 | 8.41 | 8.54 (1.1) | 8.75 (0.50) | 6.68 |
| ASP | 12.92 (0.2) | 12.27 (0.4) | 10.97 | 10.90 | 11.54 (0.5) | 11.48 (0.5) | 10.24 |
| THEO | 7.77 (0.3) | 7.31 (0.1) | 6.85 | 7.63 | 7.81 (0.5) | 7.01 (0.1) | 8.27 |
| SER | 9.40 (0.6) | 7.93 (0.2) | 8.39 | 7.86 | 7.78 (0.3) | 7.99 (0.5) | 7.94 |
| GLU | 10.67 (1.1) | 11.77 (0.2) | 8.4 | 8.44 | 12.64 (0.2) | 11.47 (0.3) | 8.72 |
| GLY | 10.92 (0.8) | 15.22 (0.5) | 9.12 | 8.41 | 13.2 (1.8) | 12.12 (0.4) | 8.56 |
| ALA | 8.88 (0.6) | 7.04 (0.1) | 7.12 | 6.79 | 8.41 (1.0) | 9.56 (0.4) | 7.15 |
| VAL | 6.54 (0.1) | 5.33 (0.1) | 4.69 | 6.4 | 6.54 (0.3) | 6.46 (0.4) | 6.66 |
| ISO | 2.61 (0.2) | 2.22 (0.1) | 1.66 | 2.46 | 3.06 (0.2) | 3.10 (0.2) | 2.42 |
| LEU | 8.93 (2.4) | 7.09 (0.2) | 7.28 | 7.58 | 8.02 (0.3) | 8.90 (0.2) | 7.83 |
| TYR | 2.78 (0.3) | 3.37 (0.1) | 7.67 | 3.83 | 1.56 (0.3) | 3.58 (0.2) | 6.25 |
| PHEN | 3.27 (0.3) | 2.77 (0.03) | 2.97 | 3.14 | 3.38 (0.2) | 3.45 (0.2) | 3.05 |
| LYC | 3.03 (0.1) | 3.27 (0.1) | 2.32 | 2.65 | 4.71 (0.3) | 4.10 (0.7) | 3.56 |
| ARG | 5.55 (0.2) | 5.09 (0.1) | 4.34 | 4.49 | 4.41 (0.3) | 6.61 (0.3) | 3.93 |

**Table 5. Amino Acid Analysis for NTHP and SFTHP** (Data from literature (Hess, 1991) and values of amino acids from sequencing data (Pennica, 1987) are included). **(Data used to construct Table 5 is located in Appendix 2.7)**

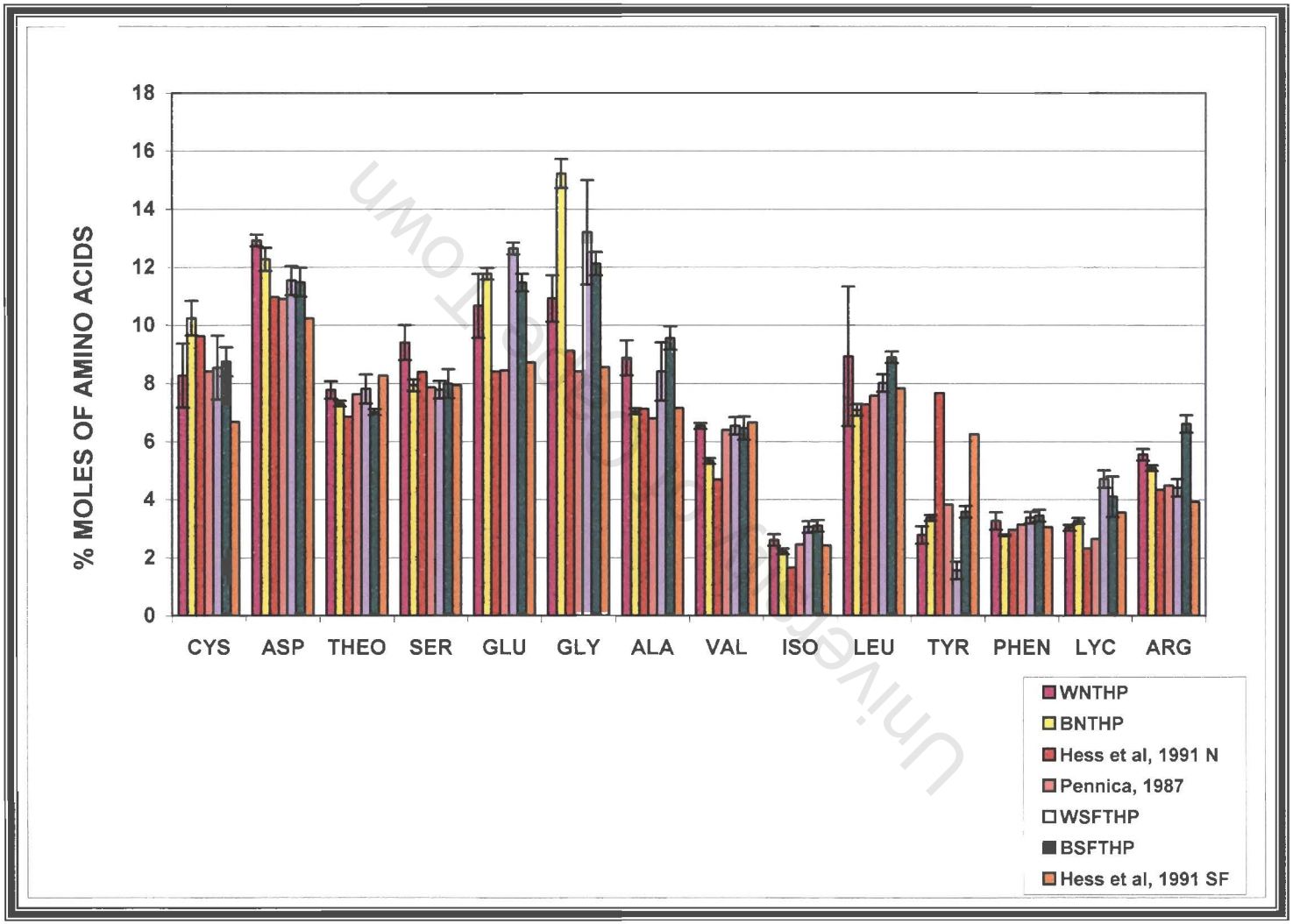





**Figure 8. Histograms representing the Amino Acid Analysis of NTHP and SFTHP. Error bars represent standard error.**

When amino acid analysis was compared between white and black normal THPs, some significant differences were noted and indicated in red type for the following amino acids: Cys, Ser, Gly, Ala, Val, Tyr, Phe (Table 4, Fig. 8). When WSFTHP was compared with BSFTHP some differences were also noted (indicated in green) for the following amino acids: Theo, Glx, Leu, Tyr, Arg. It is of importance to note that amino acid data from black healthy subjects and black stone-patients have not been previously recorded and therefore these results are of considerable interest and importance.

*2.4.3.d. Circular Dichroism (CD)*

The CD spectra were measured over a range from 195 – 260 nm (Hennessey and Johnson, 1981) by using a vacuum ultraviolet (UV) CD spectropolarimeter (JASCO F-810). Figure 10 is a typical CD spectrum obtained for the four THP samples at 25 $^{o}$C. It is noted that a negative extremums occurs between 210 – 218 nm for WSFTHP and BNTHP of approximately –3 and –4 deg.cm $^{2}$ dmole$^{-1}$ respectively and is characterized by a major resolved band between 210 – 218 nm with a rotational strength of –3 x $10^{-40}$ cgs and –4 x $10^{-40}$ cgs units respectively. Similarly, negative extremums occur at 219 and 220 nm for BSFTHP and WNTHP of –6 and –11.5 deg.cm $^{2}$ dmole$^{-1}$ respectively and are also characterized by major resolved bands at 219 and 220 nm with rotational strength –6 x $10^{-40}$ cgs and –11.5 x $10^{-40}$ cgs units respectively (Protocal for CD spectra is available in Appendix 2.8).

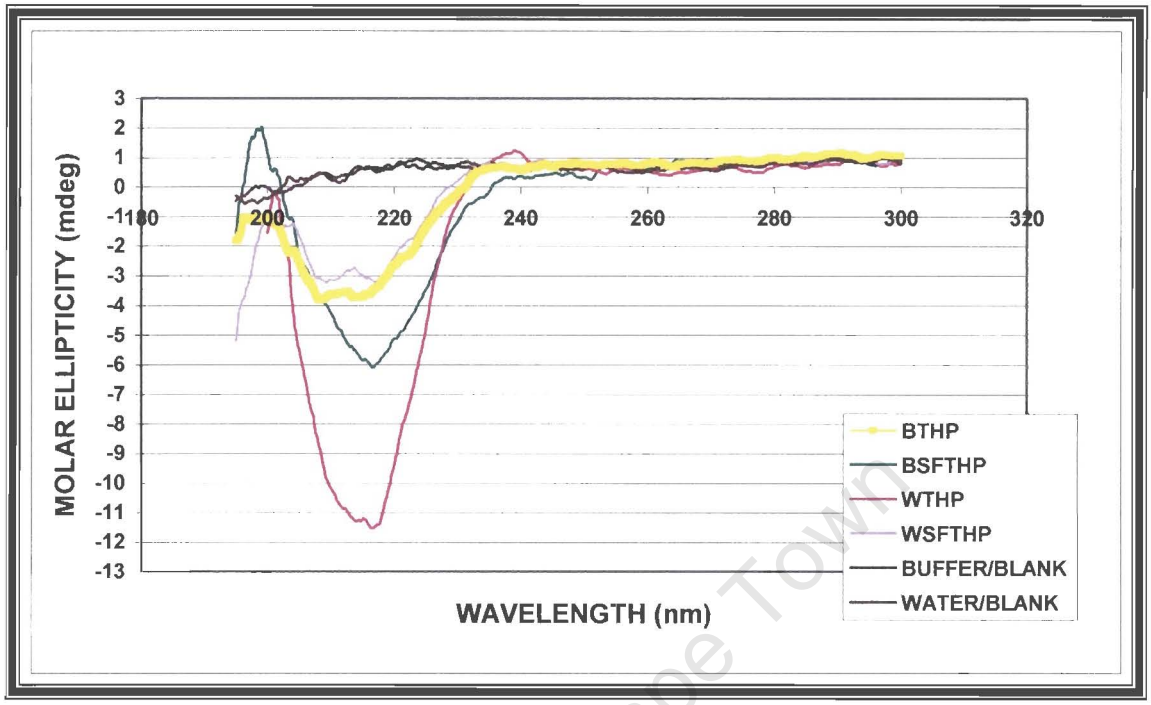



**Figure 9. CD spectrum of THP from the four groups.**



The CD spectra were used to determine the variations of percentages of secondary structural components of the THP samples. All spectra were deconvoluted with the use of computer software CDNN21 programme. It has been established by several researchers that the secondary structural components of the THP appear to be α-helix and β-sheets of approximately 10% and 34% respectively (Jirgensons, 1973; Chen et al, (1974) Biochemistry. 13:3350, cited by Hennesey et al, 1981; Baker et al, (1976) Biochemistry. 15: 629. cited by Hennesey et al, 1981). Similar results were determined from the current data for the WNTHP (Table 6). However WSFTHP and BNTHP had a reduced amount of α-helical structures with a larger amount of β-sheets while BSFTHP had a larger percentage of α-helical structures and a reduced amount of β-sheets. Parallel, Beta-turn and Random Coil structures remained constant for all THPs. In contrast the structure between and within normal and stone-forming THP samples differed from each other. These results are depicted as histograms in Figure 10. Significant differences in the structures are noted within the α-helical and β-sheets structures.

| TYPES OF THP | % α HELIX | % β-SHEETS | PARALLEL | BETA-TURN | RANDOM COIL |
|---|---|---|---|---|---|
| WNTHP | 9.4 (0.5) | 30.3 (0.1) | 5.5 | 19.9 (0.4) | 35.5 (0.1) |
| BNTHP | 4.3 (0.2) | 42.9 (0.9) | 5.4 | 19.5 (0.1) | 35.3 (0.3) |
| WSFTHP | 5.4 (0.3) | 39.1 (0.8) | 5.3 | 19.9 (0.4) | 35.7 (0.2) |
| BSFTHP | 15.9 (0.2) | 22.9 (0.5) | 5.6 | 19.3 (0.1) | 34.9 (0.1) |

**Table 6. Deconvolution into percentage of secondary structures within the four THP samples obtained from CD spectra according to Deleage and Roux, 1987.** (Data used to construct Table 6 is located in Appendix 2.8)

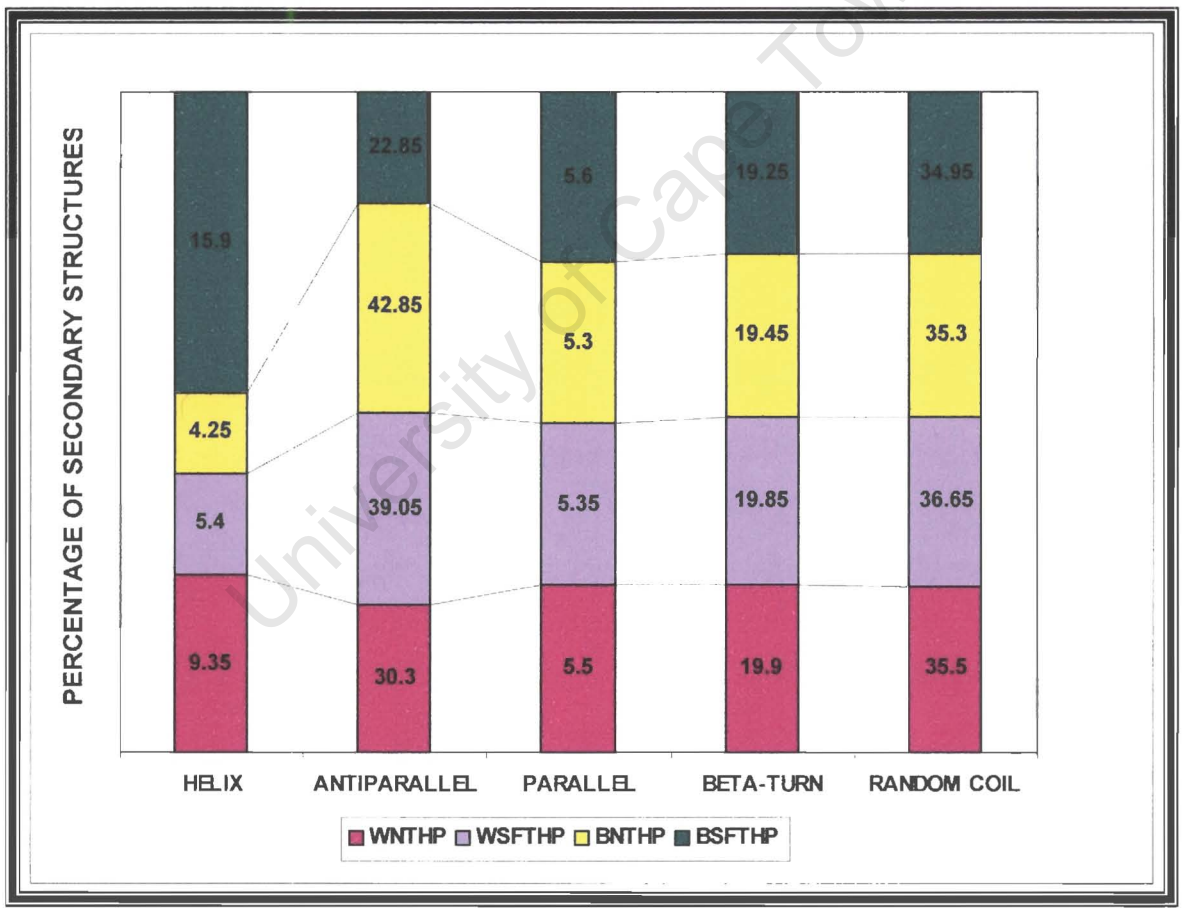



**Figure 10. Deconvolution into secondary structures of the four THP samples obtained from CD spectra according to Deleage et al, 1987.**

## 2.5. DISCUSSION

Forty years ago, urolithiasis was virtually unheard of in black South Africans (Muskat, 1951; Wise et al, 1961; Beukes et al, 1987). Since then there have been a few isolated cases of stone formation among urban blacks, but it is less than 1 percent of the population (Pantanowitz et al, 1973). The low incidence of urolithiasis in the black population has in the past been attributed to dietary habits (Muskat, 1951; Wise et al, 1961). It has been reported that blacks have a decreased intake of calcium and protein which are both risk factors for stone formation (Muskat, 1951; Wise et al, 1961). Robertson (1990) has postulated that an animal-protein-rich-diet is the most important factor in stone formation. A diet high in animal protein increases the urinary excretion of calcium, oxalate and uric acid while the excretion of citrate is reduced (Parviar et al, 1996).

Previous studies which have compared the urine composition in black and white South African subjects have consistently shown that the excretion of calcium and urate in the former group is lower (Modlin, 1967; Whalley et al, 1998; Lewandowski, 2001). This has been confirmed in the present study (Table 1). Interestingly, stone formers of both race groups have lower values for citrate relative to the non-stone forming groups. While this result is not suprising, the lower citrate excretion in healthy blacks is contrary to that which might have been expected as urinary citrate is an universally recognized inhibitor of CaOx crystallization; thus a higher value for this component would have been expected in the black group.

Another suprising result in the present study is the lower oxalate excretion in black normal subjects compared to white normal subjects (Table 1). This has been previously reported in another study conducted in the same laboratory of which the present investigator is a member (Lewandowski, 2001). Thus, it can be speculated that either oxalate is being complexed by calcium in the gut to a greater extent in the black subjects or perhaps the colonization and/ or utilization by oxalate-degrading bacteria in the gut in blacks is greater than in whites.

Relative supersaturation (RS) values for CaOx, brushite and urate in black normal subjects are consistantly lower than those in white normal subjects (Table 1). This is consistent with the lower stone incidence in the black population. However, RS values for stone-formers in both groups do not follow clearly defined trends. Of interest are the values for the Tiselius risk index which clearly demonstrate that, as a group (irrespective of stone-forming status), black subjects have a lower risk than white subjects and that non stone-formers (irrespective of race) have a lower risk than stone-formers. The failure on the one hand of the RS index to successfully emulate stone incidence rates in the two race groups and the success of the Tiselius index on the other hand suggests that the parametes in the latter calculation (Ca, Ox, Mg, Cit, Creat) are more appropriate to the black vs white stone rates in South Africa than those in the RS calculation which incorporate these as well as a host of others.

Biochemical characterization of THP from the four groups revealed interesting results. MALDI-TOF mass spectroscopy indicated that BNTHP had a higher molecular weight than the other three THP groups (Table 2). Thus the possibility exists that BNTHP is either a larger protein or post-synthetically modified; for example the addition of sugar residues would account for the molecular weight difference. Further investigation of the four proteins using tryptic digestion, revealed that different peptide masses were present within the BNTHP and not in the other three groups when compared to the Swiss-Prot database (Table 3). The peptides with different molecular masses could arise from oxidation of methionine to the sulfoxide.

Hess et al (1991) stated that the amino acid data he obtained agreed well with those presented by Pennica (1987) from cDNA sequencing for THP from pooled white urines. However when data from literature were compared with results obtained in the present study for normals and stone-formers, several differences were noted (Table 5). Unfortunately, statistical comparisons could not be made, as such data were not provided in the literature. As such, statistical significance could not be investigated. Nevertheless, some differences were noted between the black and white normals

(indicated in red type in Table 5) and also within the stone formers (in green type in Table 5) for both population groups. In the BNTHP there appears to be a larger amount of cysteine and glycine and a lower amount of alanine, valine, leucine and phenylalanine than in the THP from the other three groups. In BSFTHP there was also notably more arginine than in the other three THP groups. It thus appears that two different genes are possibly being expressed in these two THP groups. These results were obtained from several runs using freshly purified THP samples from each population group for determination; as such the results can be viewed with confidence.

When the secondary structures were investigated by circular dichroism for the four THP groups, the most notable difference occurred in the α-helical and β-sheets structures for the BNTHP. This group had the smallest percentage of α-helical structures (4.3 %) while they had the largest percentage of β-sheets structures (42.9 %). Previous studies elsewhere in the world on THP from normal (white) subjects have shown that there should be about 10 % α-helical and 34 % β-sheets structures (Jirgensons, 1973; Chen et al, (1974) Biochemistry. 13:3350, cited by Hennesey et al, 1981; Baker et al, (1976) Biochemistry. 15: 629. cited by Hennesey et al, 1981). These literature values were given for WNTHP and were also determined in the present study for this particular THP group. However the results for the stone formers did not follow either trend shown by their respective normal THP group. Thus, it may be speculated that the differences noted in peptide masses for BNTHP from MALDI-TOF mass spectroscopy analyses could account for slight sequence differences and this in turn may result in secondary structural variations seen in BNTHP. However further investigation in this field of research is required.

Thus, biochemical analysis has demonstrated that there are differences (molecular weight, peptide masses, amino acids, secondary structures) in BNTHP when compared to the other three THP groups. Therefore, it may be tentatively speculated that these differences may change the physiocochemical properties of BNTHP including its capacity to inhibit urinary crystallizsation of calcium oxalate. Chapter 4

describes experiments to test the relative inhibitory potential of THP from the four groups of subjects.

# REFERENCES


- Beukes. G. J., DeBruiyn. H., Vermaak. W. J. H. (1987) Effects of epidemiological factors on the composition and racial distribution of renal calculi. Br. J. Urol **60**: 1-5.
- Bradford. M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantites of protein utilizing the principle of the protein-dye binding. Anal Biochem **72**: 248-258.
- Deleage. G and Roux. B. (1987) An algorithm for protein secondary structure prediction based on class prediction. Protein Eng. **1**: 289-294.
- Fletcher. A.P., Neuberger. P. A., Ratcliffe. W. A., (1970) Tamm-Horsfall urinary glycoprotein: the chemical composition.Biochem J **120**: 417-424.
- Hennessey. Jr. J. P., Johnson. Jr. W. C. (1981) Information in the Circular Dichroism of Proteins. Biochemistry. **20**: 1085-1094.
- Hess. B. (1991) The role of Tamm-Horsfall glycoprotein and nephrocalcin in calcium oxalate monohydrate crystallisation processes. Scan Micro **5**: No 3, 689-696.
- Hess. B. (1992) Tamm-Horsfall Glycoprotein-Inhibitor or promoter of calcium oxalate monohydrate crystallization processes? Urol Res **20**: 83.
- Hess. B., Nakagawa. Y., Parks. J. H., Coe. F. J. (1991) Molecular abnormality of Tamm Horsfal glycoprotein in calcium oxalate nephrolithiasis. Am J Physiol **260**: F 569.
- Hess. B, Jaggi M, Zipperle, Nakagawa Y (2001) Abnormal urinary and molecular structure of Tamm-Horsfall protein (THP) in calcium renal stone formers with positive family history (CSFFAM). In: Kok DJ, Romijn HC, Verhagen PCMS, Verkoelen CF (eds) Proceedings of the 9th European Symposium on Urolithiasis, Rotterdam, p 135.

- Hirs. C. H. W. (1967) Performic acid oxidation. Methods Enzymol **11**: 197-199.
- Jirgensons. B. (1973) Optical Activity of proteins and other macromolecules. 2nd edition, Springer-Verlag, Berlin, p. 152.
- Laemmli. V. K. ( 1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **256**: 495-497.
- Lewandowski. S., Rodgers. A. L., Schloss. I. (2001) The influence of a high-oxalate / low calcium diet on calcium oxalate renal stone risk factors in non-stone-forming black and white South African subjects. Br. J.Urol Int **87**: 307.
- Mathews and Von Holde (1999) Spectroscopic methods for studying Macromolecular Conformation in solution. Chapter 6A-Tools for Biochemistry. Biochemistry 3rd edition. p. 202-206.
- Modlin. M. (1967) The aetiology of renal stones: a new concept arising from studies on a stone-free population. Ann Roy Coll Surg Engl **40**:155-178.
- Muskat. D. A. (1951) The problem of nephrolithiasis with special reference to the Bantu. S Afr J Clin Scl **2**: 18-38.
- Pantanowitz. D., Pollen. J. J., Politzer. W. M., Van Blerk. P. J. P. (1973) Urinary calculi. S. Afr. Med. J. **56**: 128.
- Parivar. F., Low. R. K., Stoller. M. L., (1996) The influence of diet on urinary stone disease. J Urol 155: 432-440.
- Pennica. D. W. J., Kohr. W. J., Kuang. W. -J., Glaister. D., Aggarwal. B. B., Chen. E. Y., Goeddel. D. V. (1987) Identification of human uromodulin as the Tamm-Horsfall urinary glycoprotein. Science Wash.236: 83-88.
- Robertson. W. G. (1990) Epidemiology of urinary stone disease. Urol Res 18: S3-S8.
- Tiselius. H. G. (1982) An improved method for the routine biochemical evaluation of patients with recurrent calcium oxalate stone disease. Clin Chim Acta 122: 409-418.
- Werness.P. G., Brown. C. M., Smith. L. H., Finlayson. B. (1985) Equil 2: a basic computer program for the calculation of urinary saturation. J Urol 134: 1242-1244.

- Whalley. N. A., Moraes. M. F. B. G., Shar. T. G., Pretorius. S. S., Meyers. A. M. (1998) Lithogenic risk factors in the urine of black and white subjects. Br. J. Urol 82: 785-790.
- Wise. R. O., Kark. A. E. (1961) Urinary calculi and serum calcium levels in African and Indians. S Afr. Med. J 35: 47-50.

# CHAPTER 3

# CHARACTERIZATION OF THE CARBOHYDRATE MOIETIES OF THP FROM BLACK AND WHITE HEALTHY AND STONE-FORMING SUBJECTS.

## 3.1. INTRODUCTION

As mentioned in an earlier chapter, the physiological function of THP is controversial. However it is thought to play a significant protective role in several pathological conditions such as the prevention of infection in the bladder and urinary tract (Orskov et al, 1980) by inhibiting the binding of *Escherichia coli* S fimbriae to epithelial cells (Parkkinen et al, 1988) as well as the prevention of stone formation and interstitial nephritis (Hoyer et al, 1979). It has been speculated that THP from healthy subjects and stone-formers differ with respect to an altered structure in the carbohydrate portion of this protein (Knorle et al, 1994).



### *3.1.1. Primary Structural Analysis using $^1$H-NMR Spectroscopy*

High-resolution $^1$H-Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful method for the determination of primary structures of purified carbohydrate chains of glycoproteins (Van Halbreek et al, (1982) Eur. J. Biochem. **127**: 7; Van Halbreek et al, (1990);In Frontiers of NMR in molecular biology (ed. D. Live. Armitage. I. M., Patel. D.) pp 195. cited by Hard et al, 1993.); (Vliegenhart et al, (1983) Carbohydr. Chem. Biochem. 43: 209. cited by Hard et al, 1993);(Vliegenhart et al, (1988) Pure. Appl. Chem. **53**: 45. cited by Montreuil et al, 1994). NMR is based on the observation that magnetic nuclei such as $^1$H, $^{13}$C, $^{31}$P, and $^{15}$N can absorb energy at characteristic radio frequencies when placed in a strong magnetic field. The resonance frequency of a particular nucleus is called a chemical shift (δ) and is expressed as parts per million (ppm), is sensitive to its chemical environment, making

NMR a valuable technique for structural studies. The resonance lines show fine structure, referred to as spin-spin coupling, originating from interactions with nearby nuclei sharing bonding electrons. Thus a molecule will have a definite NMR profile that can then be used as a fingerprint. Information may also be gained on the dynamic properties of molecules by analysing NMR relaxation processes. NMR spectroscopy can be useful for conformational studies because data can be obtained about distances between two nuclei that are close in space. The non-destructive character of NMR spectroscopy usually makes it the first technique to choose for a structural problem and it is the only method which can in principle give an *ab initio* structure for a novel carbohydrate. $^{1}H$ nuclei are the most commonly used for biologically interesting carbohydrates ((Hard et al. (1991) FEBS. Lett. **287**: 108; Vliegenthart et al. (1993) Carbohydr. Chem. Biochem. 43: 209) cited by Vliegenthart et al. 1993).

Correlated spectroscopy (COSY) was the first 2-D NMR experiment to be proposed and is still one of the commonly used pulse sequences. COSY spectra provide information on directly coupled protons and its main purpose is to establish the connectivity of the spin system. From the fine structure of the cross-peaks in the COSY spectra, information can be extracted about coupling constants, which can be used to estimate dihedral angles in carbohydrates ((Hard et al. (1991) FEBS. Lett. **287**: 108; Vliegenthart et al. (1993) Carbohydr. Chem. Biochem. 43: 209) cited by Vliegenthart et al. 1993).

Vliegenthart et al (1983) developed the concept of "structural-reporter groups" for the interpretation of $^{1}H$-NMR spectra of a carbohydrate chain in terms of primary structural assignments. This means that the chemical shifts of protons resonating at clearly distinguishable positions in the spectrum, together with their coupling constants and the line widths of their signals, bear the information essential to permit assigning of the primary structure.

*3.1.2. Classification and Structure of N- and O-linked Oligosaccharides*

The primary structures of the glycoprotein glycans are divided into families of similar structures and common oligosaccharide sequences (Montreuil et al. 1994). The oligosaccharides of glycoproteins can be classified into two groups, namely, *N*-linked and *O*-linked: -

*N*-glycosylation is a co-translational process that occurs during protein folding and is available to all proteins that contains the sequon asparagine-Xaa-serine / theorine, where Xaa represents any amino acid other than proline. In this process a specific oligosaccharide unit is attached to the nitrogen atom in the side chain of certain asparagine residues.

The addition of *O*-linked glycans is a post-translational process. It occurs when a single monosaccharide is attached to the oxygen atom on an appropriate serine or theonine side chain (Montreuil et al. 1994; Rudd et al. 1997). Fig.1.1 represents the "core 1" *O*-linked glycan.



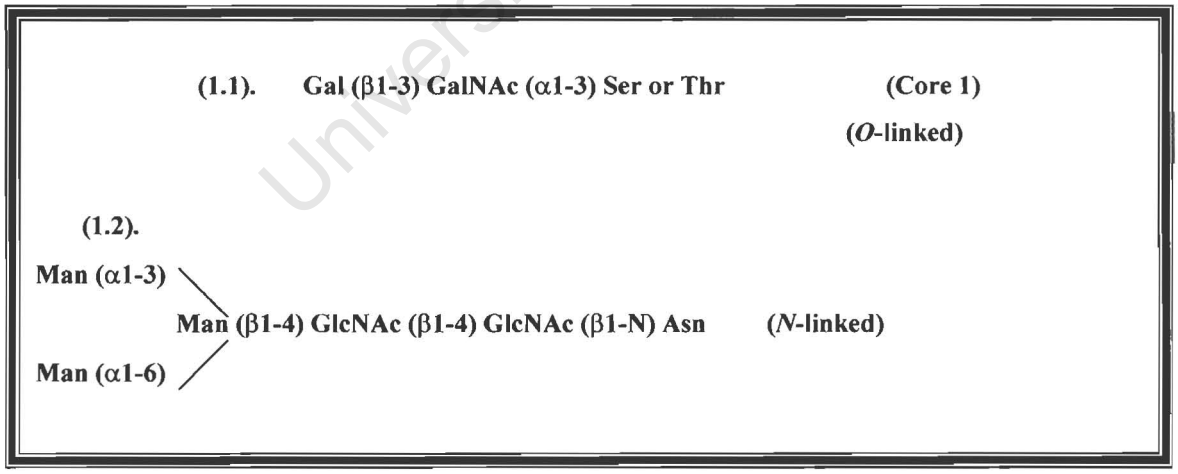



**Figure 1. Oligosaccharide inner-cores of glycoproteins. (1.1) Core 1 exists in the O-glycoproteins. (1.2) The branched structure is the common core to all N-glycoproteins** (Montreuil et al. 1994). Graphical representations of monosaccharides and their linkages can be found in Figure 6a.

1. The concept of the common 'inner-core': - Fig.1.1 represents the carbohydrate moiety for the internal part of the *O*-glycoproteins (Montreuil et al. 1994).

2. The **concept of antenna:** - This has been proposed for the outer variable arms substituting the inner cores of the glycans and takes into account the concept of morphology, flexibility and properties of being a recognition signal for a particular glycan (Montreuil et al. 1994).

3. **Microheterogeneity of glycans:** - Variations of a specific glycan at a specific amino acid in the glycoprotein presents structural heterogeneity, which is produced due to partial substitution of monosaccharide residues on a similar core structure. However microheterogeneity involves the number and position of the most externally situated monosaccharides in the glycan i.e. sialic acid monosaccharides are always found at the termini. Thus microheterogeneity is related to the variation in the level of sialylation or to modification of the number of antennae in *N*-glycoproteins (Montreuil et al. 1994).

### *3.1.3. Oligosaccharide Analysis*

Techniques used in the past were time consuming, difficult to perform and insensitive to glycoproteins available in microgram quantities or less. However Figure 2 shows the major advances in oligosaccharide analyses that have been made using high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and mass-spectroscopy (MS). Due to these advancements, sub-picomole levels of *N*-linked oligosaccharides can be released from 1-5 μg of protein in a band on a polyacrylamide gel. The the entire *N*-glycan pool of sugars may be sequenced simultaneously, from this material using exoglycosidase arrays (Rudd et al. 1997).

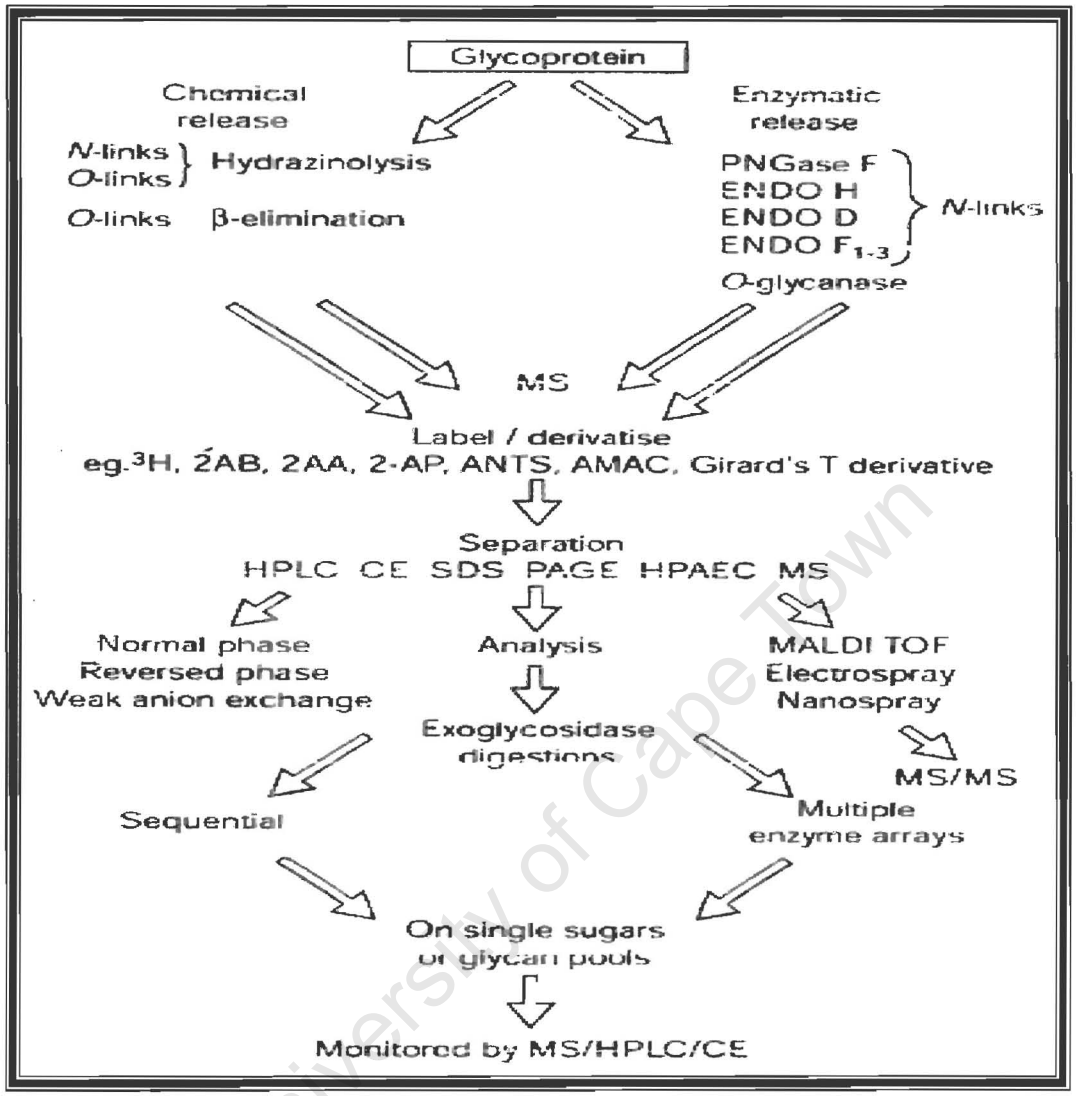



**Figure 2. A summary of an overall strategy and the new techniques available for oligosaccharide analyses** (Rudd et al, 1997).



### *3.1.4. Physiological signals of THP glycosylation – Controls vs Stone-formers*

In 1996, Schnierle et al, using isoelectric focusing (IEF), analysed the isoelectric points (pI) for healthy and stone-forming subjects from the white population and showed that the stone-formers had a pI value of 4.5 to 6 while for controls the pI value was 3.5. They postulated that the different isoelectric points could be a result of altered glycoprotein structure resulting in different chemical composition of the THPs.

They proposed the following causative factors: 1). Different sialic acid contents in these two groups of THP; 2). Different sulphate-group contents; 3). Different contents of carboxy-groups from amino acids.

There appears to be some controversy surrounding the sialic acid content of THP from healthy and stone-forming subjects. Knorle et al (1994), using Fourier-transform infrared spectroscopy (FTIR) and quantitative analysis (thiobarbituric acid assay) for the determination of sialic acid from THPs from white healthy subjects and stone-formers, showed that structural differentiation occurred between these functionally different THPs. They also found a decisive difference in the sialic acid content from quantitative analysis, which showed that the healthy subjects had double the content of sialic acid when compared with that of the stone-formers (white healthy subjects = 51 ± 9 g/kg vs white stone-formers = 21 ± 4 g/kg). They postulated that this decrease could explain the dual role of THP in the precipitation of CaOx and the formation of renal stones and showed the importance of glycosylation in the functioning of this glycoprotein (Knorle et al. 1994).

Hess et al (2001) showed the reverse of the above-mentioned findings. He stated that if THP were of a greater purity when studied, one would find that the urinary excretion and the sialic acid content of THP for stone-formers would be greater than that for healthy subjects. Hess et al also stated that only the stone-formers' THP exhibits a structure-function relationship given that low sialic acid content of THP predicted slight promotion of CaOx crystal aggregation.

The different findings of the two research groups is of interest as they indicate that differences in the content of sialic acid may occur which may provide a partial explanation for the different behaviour of the THPs from healthy subjects and stone-formers. Sialic acid is a generic nomenclature given to a family of compounds that are derived from the 9-carbon sugar neuraminic acid (Gottschalk et al. 1952; Schauer et al. 1982). The major derivative of sialic acid in human tissue is N-acetylneuraminic acid (Neu5Ac). Most sialic acids are present as terminal sugar residues.

In urine, there are two types of sialic acid, namely free and bound. The latter is bound to sialoglycoconjugates, which are components of urinary macromolecules. Very little is known to date about the mechanisms of renal excretion of sialic acid. A considerable amount of free sialic acid, (ie. 30-50% of the total sialic acid), occurs in urine. Free sialic acid is cleared from plasma by the kidneys in a similar manner to creatinine, being filtered by the glomerulus but not reabsorbed by the tubules (Seppala et al. 1990).

### *3.1.5. Structure of THP molecule*

Gottschalk and Odin were the first to investigate the carbohydrate moiety of THP in 1952 and they revealed that THP had a tendency to form aggregates (Hard et al. 1992). The carbohydrate content of THP is about 25-30%. It is comprised of sialylated, sulphated and N-acetylgalactosamine (GalNAc)-containing *N*-linked carbohydrates (Hard et al. 1992).



However in 1987, Pennica and Hession determined from the cDNA sequence analysis that there were in fact eight potential *N*- glycosylation sites (Asn14, Asn 52, Asn 56, Asn 208, Asn 251, Asn 298, Asn 372 and Asn 489) of which at least five sites were occupied (Figure 3) (Alfonso et al. 1981). Out of these eight sites, van Rooijen et al (1999) found that seven were occupied. The potential glycosylation site Asn 14 was close to the *N*-terminus and therefore not occupied. Figure 3 presents the THP molecule with the eight potential glycosylation sites. Glycans at Asn 489 appeared to be terminated with GalNAc4$SO_4$(β1-4)GlcNAc and di- and tri-charged *N*-glycans. The Asn 251 glycosylation site appeared to be the only site that contained oligomannose-type structures ranging from $Man_5GlcNAc_2$ to $Man_8GlcNAc_2$ as well as complex-type *N*-glycans (van Rooijen et al. 1998). It has been speculated that the oligomannose-type carbohydrate chains could possibly be a donor-specific feature playing a role in the immunosuppressive properties displayed by THP (Serafini-Cessi et al. 1979; Muchmore et al. 1985, 1987 (a), (b); Moonen et al. 1988). It also appeared that

the glycosylation site Asn 208 showed a large microhetergeneity resembling that of the total glycan pool of THP (van Rooijen et al, 1998).

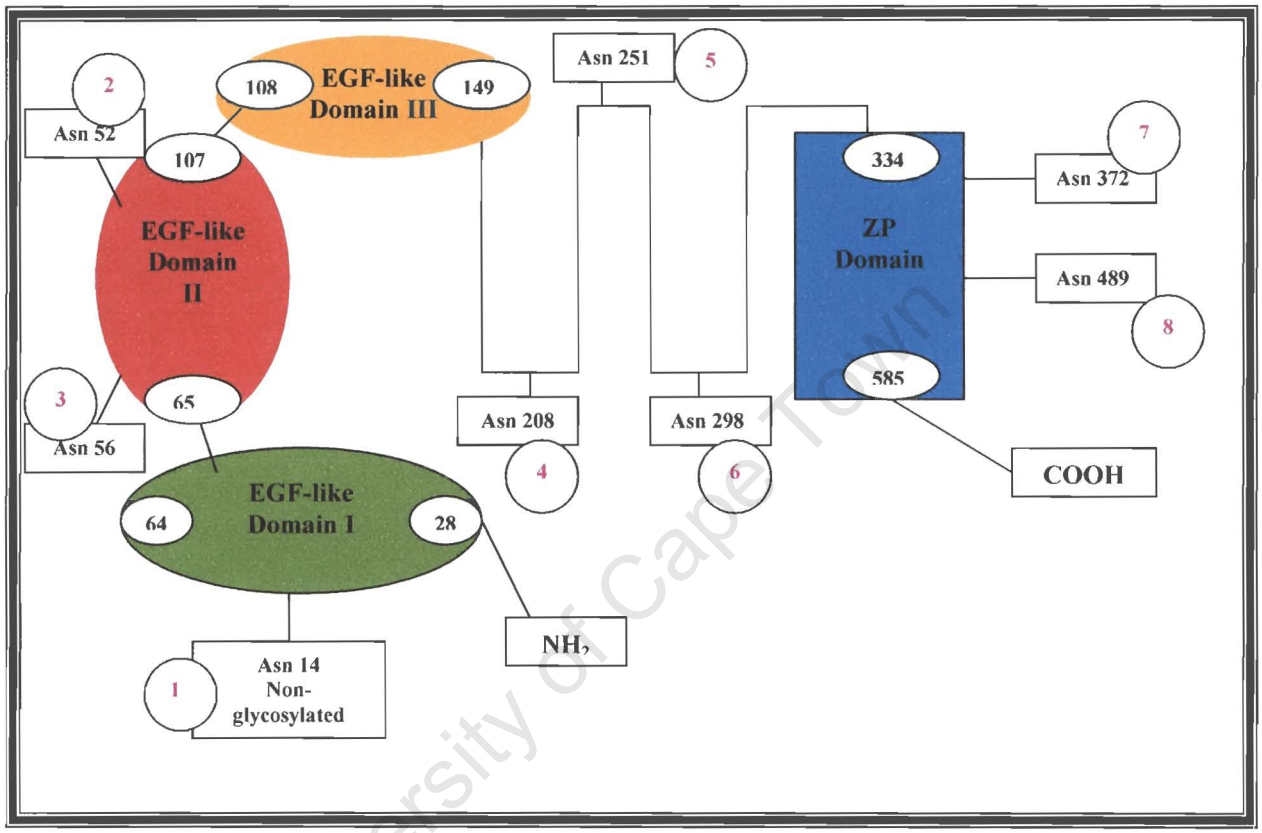



**Figure 3. Schematic representation of THP including specific domains and potential glycosylation sites. THP has eight potential glycosylation sites and only Asn 14 is not glycosylated. There are three EGF-like domains (epidermal growth factor-like domain) and one ZP-domain (zona pellucida domain)** (van Rooijen et al, 1999).

Diantennary, triantennary and tetraantennary structures have been identified in the carbohydrate portion of THP (Williams et al, 1984; Serafini-Cessi et al, 1984; Hard et al, 1992). When THP from pooled urine was analysed, the glycan $Man_6GlcNAc_2$ was found to be the dominant structure (Dall'Olio et al, 1988).

### *3.1.6. Carbohydrate Moieties of THP – Black vs White*

Various studies (van Aswegen et al. 1989; Knorle et al. 1994; Schnierle et al. 1996; Easton et al. 2000; Hess et al. 2001) have shown that differences exist in the carbohydrate portion of THP from normal and stone-forming subjects. The physiological importance of this carbohydrate moiety provided the motivation in the present investigation to characterise differences in the carbohydrate portion of THP isolated from the four groups studied in this thesis, namely: black and white healthy and stone-forming subjects, as it may provide some insight into the low incidence shown by black South Africans to kidney stone formation. Thus the carbohydrate portion of the four groups' THP was investigated.

## 3.2. OBJECTIVES

The overall objective of this particular study was to analyse the *N*- and *O*-linked glycans of THP isolated from black and white healthy subjects and stone-formers with a view to investigating potential differences between the oligosaccharides from these groups. Specific objectives were:

- To screen digests of the four THP samples by $^{1}H$ (1D) and COSY NMR spectroscopy.

- To conduct *N*-linked glycan analyses of the four THP samples.

- To conduct O-linked glycan analyses on the four THP samples. The N- and O-linked glycan analyses were conducted in collaboration with Dr. P. M. Rudd, Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, UK.

## 3.3. METHODS

### *3.3.1. Protein Purification*

THP from black and white healthy and stone forming subjects, (WNTHP, WSFTHP, BNTHP and BSFTHP) was extracted and purified according to the procedure previously described in Chapter 2.

### *3.3.2. $^1$H-NMR Analysis*

Purified THP from black and white healthy and stone-forming subjects was digested with Proteinase K (Boehringer Manneheim). A 100 mM Tris-HCl, pH 8.0, buffer solution was used to dissolve the Proteinase K. A 2 mgml$^{-1}$ Proteinase K solution was used to digest 1 mg of protein or an equivalent ratio. The digested solution was incubated at 37°C for 24 hours (Appendix 3a and 3b for the preparation and digest protocol). The THPs were subjected to this digestion procedure in order to remove most of the peptides. The digested samples were then analysed using MALDI-TOF mass spectroscopy to investigate if the samples had been completely digested. The digested samples were then lyophilized and dissolved in a small volume of $D_2O$. This procedure was repeated three times in order to exchange the labile protons of the hydroxyl and amide groups with deuterium (Hard et al. 1992; Vliegenhart et al. (1983) Carbohydr. Chem. Biochem. 43: 209. cited by Vliegenthart et al. 1993; Vliegenhart et al. (1988) Pure. Appl. Chem. **53**: 45. cited by Montreuil et al. 1994).

$^1$H NMR spectra were recorded on a Varian Unity 400 spectrometer (Department of Chemistry, University of Cape Town) using standard Varian pulse programmes. A standard water suppression programme (PRESAT) was used to prevent the proton spectra from being dominated by the HOD (deuterium) signal. All samples were dissolved in 0.6 ml deuterated $D_2O$ and the 1D and 2D COSY NMR spectra recorded

for each THP group. Each NMR spectra are composed of particular peaks for a specific sample and these are identified with the help of "structural reporter groups". These "structural reporter groups" each have a specific chemical shift (δ) value and are used as a database for assigning unknown structural components of spectra.

### *3.3.3. N-Oligosaccharide Analysis – Peptide N-Glycosidase F Digestion (PNGase F)*

Glycans were released from THP using in-gel deglycosylation techniques. THP from the four groups was resolved by gel electrophoresis according to the method described by Radcliffe et al, 2002. Prior to electrophoresis the protein samples underwent reduction (50mmol/l DTT (dithiothreitol)) and alkylation (10mmol/l IAA (iodoacetamide)) in order to separate the disulphide bonds and to ensure optimal efficiency during the digestion with peptide N-glycanase F (*PNGase F)*. The protein was visualised using Coomassie Blue stain (Radcliffe et al. 2002). The THP protein bands were then removed with the use of a clean scapel, cut into small pieces and transferred to 1.5 ml eppendorf tubes and placed in a freezer for 2 hours before further processing. It has been speculated that freezing assists the digestion process as well (Radcliffe et al. 2002).



The gel pieces were washed with 1ml acetonitrile (ACN) and placed on a shaker for 30 mins at room temperature. Then further washing of the gel pieces occurred, repeatedly and alternatively with 1ml of ACN followed by 1ml of 20mmol/l $NaHCO_3$, pH 7, to remove excess SDS. A final wash was given with 1 ml ACN.

The gel pieces were then dried and the *N*-glycans released by overnight incubation with *PNGase F* (Roche) in a 20 mmol/l $NaHCO_3$, pH 7 buffer at 37° C (12-16 hours). After incubation, the supernatant was removed and the gel pieces underwent a repeated washing procedure with double distilled $H_2O$ and ACN. In order to achieve maximum recovery of the glycans, the washings were combined with the glycans released overnight. Once this procedure had been completed, an AG-50 ($H^+$ activated) resin was added to the samples and they were placed on a shaker for 5 mins

to desalt the samples. The samples were then filtered to remove any remaining resin and dried under vacuum. The released *N*-glycans were labelled with a fluorophore 2-aminobenzamide (2AB; Oxford GlycoSciences, Abingdon, United Kingdom), and incubated for 2 hours at 65 $^{O}$C. The excess 2AB-label was removed by ascending paper chromatography. The labelled glycans were then washed off the paper, dried and dissolved in 200µl of double distilled $H_2O$.

The labelled glycans were then examined by normal phase (NP) HPLC, using a low salt buffer system (Guile et al. 1996). The system was calibrated using an external standard of hydrolysed and 2AB labelled glucose oligomers to provide a dextran ladder from which the retention time for the individual glycans were converted to glucose units (GU). The glucose unit values were compared to a database of experimental values from which preliminary assignments could be made. Appropriate enzymes were used to digest the glycan pools and identify the monosaccharides, their sequences and linkages (Appendix 3.1, enzyme preparation; Appendix 3.2, Tables 1-4). The 2AB-labelled glycans were also analysed by MALDI-TOF mass spectroscopy.



### *3.3.4. Weak Anion Exchange-HPLC*

Glycan pools were separated according to charge by weak anion exchange (WAX)-HPLC according to the modified methodology described by Zamse et al (1998). A bovine serum fetuin standard was included which was previously characterised by Merry et al (2002). Fractions were collected, washed with $H_2O$ to remove solvents, dried under vacuum and analysed by NP-HPLC. Fluorescence was measured at $\lambda_{ex}$ 330 nm and $\lambda_{em}$ 420 nm with16-nm bandwidths. Compounds were retained on the column according to their charge density, the higher charged compounds being retained the longest. These profiles were then compared with the total glycan pool obtained on NP-HPLC. Aliquots of the glycan pools were also analysed by WAX-HPLC following digestion overnight with Abs (converts charged sialylated glycans to neutral glycans).

### *3.3.5. Determination of site occupancy - In-solution release of glycans*

In this protocol, the in-solution release of glycans occurred. This protocol could only be used on pure glycoproteins. In this method, glycans were enzymatically removed from the glycoproteins using PNGase F and then the remaining proteins were analysed by gel electrophoresis. If different molecular weight (MW) glycoproteins resolved to the same MW after deglycosylation, this would indicate different site occupancy.

### *3.3.6. Release of O-glycans by hydrazinolysis*

THP samples were dialysed against 0.1% trifluoroacetic acid, lypholized and then cryogenically dried before hydrazinolysis according to the method of Asford et al (1987). Samples were then incubated with anhydrous hydrazine for 6 hours at 60 °C to release the O-linked glycans (Patel et al. 1993). Excess hydrazine was removed by evaporation and the glycans were re-*N*-acetylated with acetic anhydride in a saturated solution of sodium bicarbonate. Sodium salts were removed with a column containing 5 M binding excess Dowex AG50 x 12(H+) 200-400 mesh (Bio-Rad, Richmond, CA) followed by elution with 5 volumes of $H_2O$. Peptides were removed by descending paper chromatography on prewashed Whatmann 3MM chromatography paper in butanol: ethanol: water (8: 2: 1, v/v) for 48 hours. Glycans were recovered from the paper by washing with $H_2O$. The glycans were concentrated and stored at –20°C prior to labelling with 2AB as for the N-linked glycans.

## 3.4. RESULTS

### *3.4.1. $^{1}$H-NMR Analysis*

"S*tructural-reporter groups*" were used for the interpretation of the $^{1}$H-NMR spectrum of the carbohydrate chain in terms of primary structural assignments. In table 1 there are "S*tructural-reporter groups*" and chemical shifts that were used for the assigment of peaks ((Vliegenhart et al, (1983) Carbohydr. Chem. Biochem. 43: 209. and Hard et al, (1991) FEBS. Lett. **287**: 108; cited by Vliegenthart et al, 1993; Williams et al, 1984; de Waard et al, 1991). In general, the carbohydrate chains have either a α1-6-fucosylated *N, N'*-diacetylchitobiose or a non-fucosylated *N, N'* – diacetylchitobiose unit at their reducing ends. These units are recognised from their anomeric signals.

Due to the limitation of resources and materials, extensive purification of the carbohydrate moieties and structural analysis could not be carried out. The $^{1}$H-NMR spectra revealed more information than the 2D- COSY. The presence of water (HOD peak) was seen in all of the glycoprotein spectra. Thus spectra did not define definite, distinctive peaks clearly indicating different structural reporter groups. Thus as indicated in Table 1, attempts are made to indicate and identify all the peaks that were observed in the spectra from the four THPs according to known "S*tructural-reporter groups*". However under these conditions some differences were noted in the spectra from BNTHP with no reporter groups for NeuAcNAc and GlcNAcNAc and in the WSFTHP there appeared to be no Man-3 H-1 signal.

| STRUCTURAL REPORTER GROUP | CHEMICAL SHIFT (δ) | WNTHP | BNTHP | WSFTHP | BSFTHP |
|---|---|---|---|---|---|
| Fuc CH 3 | 1.264-1.282 | 1.266 | 1.268 | 1.263-1.270 | 1.261 |
| NeuAc H-3a | 1.788-1.818 | 1.815-1.818 | 1.815 | 1.802 | 1.817 |
| NeuAc NAc | 1.860-2.001 | 1.976 | - | 1.960-1.973 | 1.940-1.978 |
| GlcNAc NAc | 1.985-2.001 | 1.989 | - | 1.995 | |
| Ring H | 3.301-4.649 | 3.485-3.722 | 3.490-4.646 | 3.482-4.649 | 3.480-4.646 |
| Man-3 H-1 | 4.770-4.771 | 4.770 | 4.770 | | 4.770 |
| Man 3 H-2 | 4.248-4.253 | | | | |
| Man 4 H-1 | 5.117-5.136 | 5.110 | | | |
| Man 4 H-2 | 4.190-4.215 | | | | |

**Table 1. "*Structural-reporter groups*" and chemical shift values that were used for the identification of peaks that were observed in the various THPs** (Vliegenhart et al, (1983) Carbohydr. Chem. Biochem. 43: 209. cited by Vleigenthart et al, 1993; Hard et al, 1992)



The 2D-COSY spectra contained a crosspeak between the signals 1.2 and 4.0 ppm that could be assigned to the methyl and H5 of fucose. This crosspeak was present in all four glycoprotein 2D-COSY spectra.

### *3.4.2.a. Oligosaccharide Analysis*

THP from all of the four groups exhibited a band by SDS-PAGE at about 90 kDa. Figure 4 shows the stacked chromatograms representing the total *N*-glycan pools for the various groups of THP. The *N*-glycan pool for the BNTHP shows some differences in its profile compared to those of WNTHP, WSFTHP and BSFTHP. The most notable difference is the considerably lower proportion of the mannose 6-sugar residue in BNTHP, represented at the GU value of 7.1 (Figure 4).

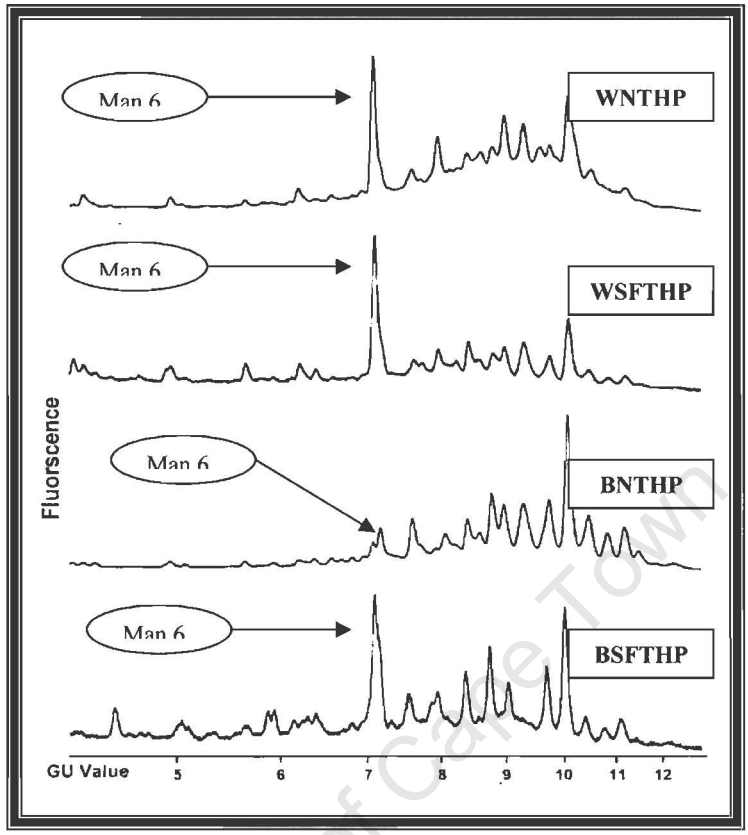



**Figure 4. Stacked chromatograms representing the variation of the total *N*-glycan pools for the various groups of THPs.** These results are supplied courtesy of the Glycobiology Institute, Oxford University.



After enzymatic digestion with an array of enzymes (Appendix 3.1, enzymes; Appendix 3.2, Tables 1-4), sugar structures present in the *N*-glycan pools were identified. All the sugar structures and the percentages detected in the various THP samples are shown in Table 2 and graphical representation of this data is shown in Figures 6 and 7. It has been shown that WNTHP, WSFTHP and BSFTHP contained the same sugar structures while BNTHP only had Man-3 and a small percentage of Man-5 sugars. Comparison of the three THP samples containing the same sugar structures (WNTHP, WSFTHP, BSFTHP) revealed the following significant differences:

(a). Firstly, although sugar structure A1 was present in all three THP samples, a significantly larger percentage was found in the BSFTHP.

(b). Secondly, the percentages of Man 4 and Man 6 were greatly reduced in the BSFTHP.

| SUGAR STRUCTURES | WNTHP | BNTHP | WSFTHP | BSFTHP |
|---|---|---|---|---|
| M 3 | 64% | **99.6%** | 68.1% | 67% |
| A 1 | 2.2% | | 3.6% | 16.9% |
| A 1(S) | 4.0% | | 2.9% | |
| M 4 | 10.3% | | 10.4% | 5.5% |
| M 4(S) | 5.3% | | 4.1% | |
| A1GalNAc | 1.2% | | 0.6% | |
| A1GalNAc (S) | | | 0.8% | |
| A 2 | 1.0% | | | |
| FcA2 (S) | | | | 2.4% |
| M 5 | | | 1.5% | |
| M 5(S) | 3.5% | **0.5%** | 2.6% | 0.5% |
| M 6 | 3.6% | | 4.4% | 1.2% |

**Table 2. Sugar structures and their percentages that appeared to be present in the different groups of THPs. (S) = Likely to be sulphated**



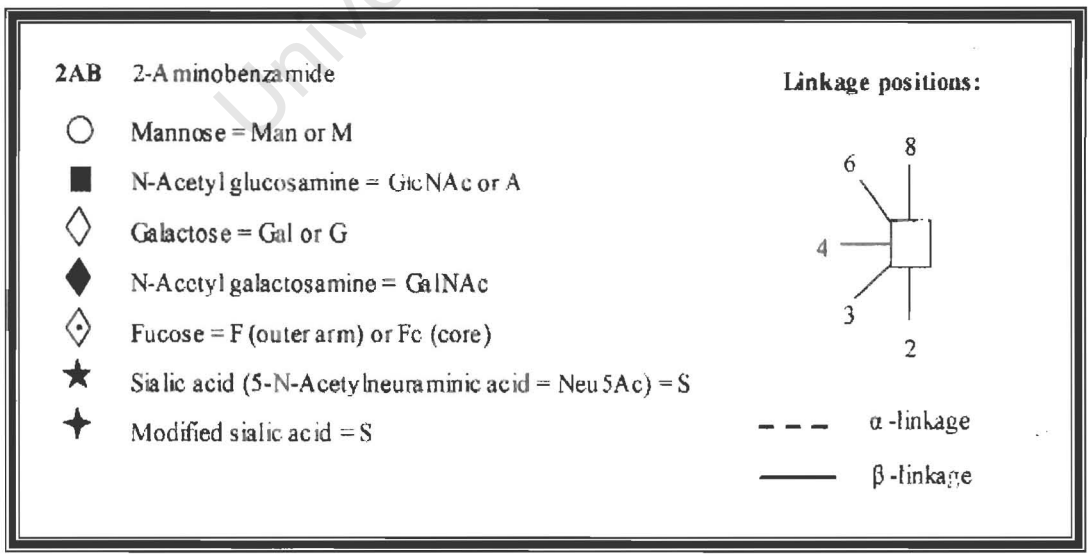



**Figure 5a. Graphical representations of monosaccharides and their linkages**

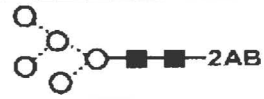
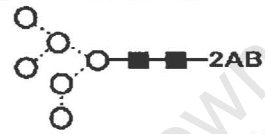
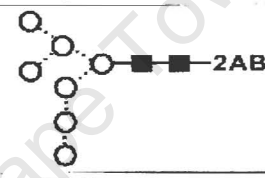
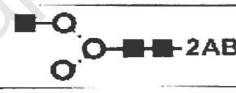
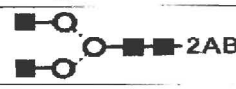
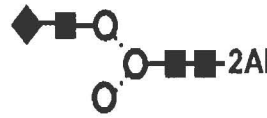
| Name | GU Value | Structure |
|---|---|---|
| M1 | 2.66 |  |
| M3 | 4.43 |  |
| M4 | 5.27 |  |
| M5 | 6.18 |  |
| M6 | 7.05 |  |
| M7 | 7.95 |  |
| A1 | 5.0 |  |
| A2 | 5.5 |  |
| FcA2 | 5.92 |  |
| A1GalNAc | 5.64 |  |



**Figure 5b. Presents a key for the symbols and GU values used to represent these sugar structures found in Table 2 and histogram, Figure 6.** These results are supplied courtesy of the Glycobiology Institute, Oxford University.

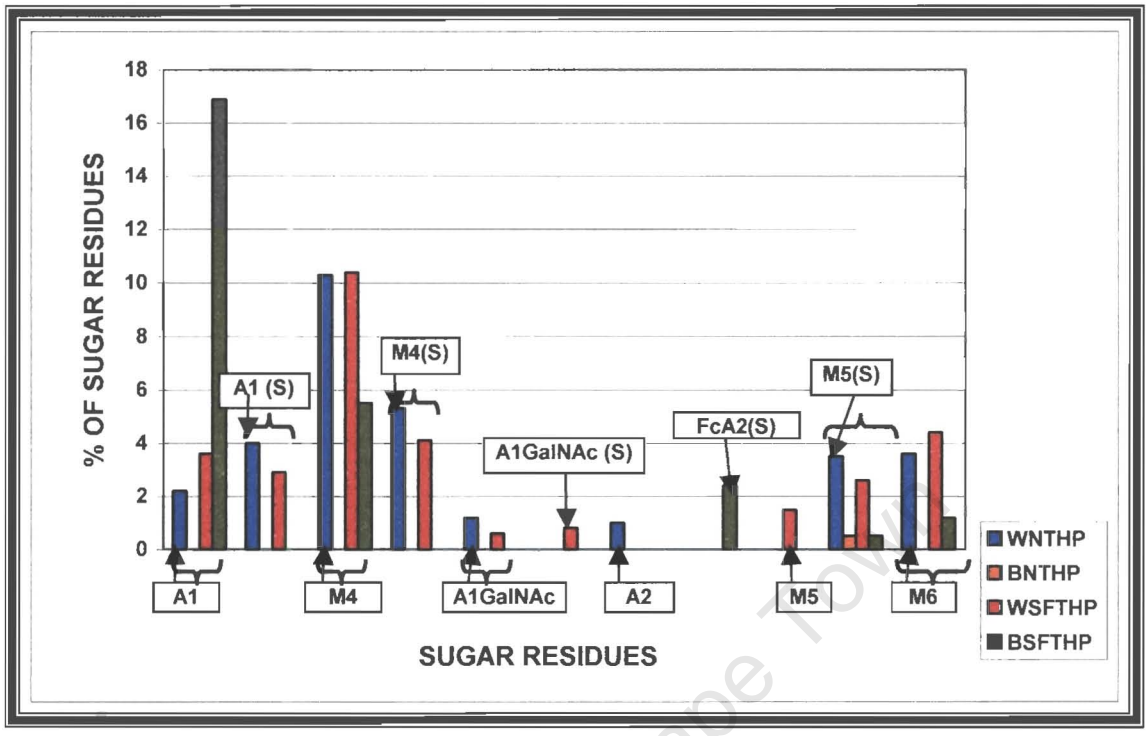



**Figure 6. Sugar residues and their percentages that were found in the various THP samples.**

The main difference seemed to occur in the mannose 3-sugar residue with 99,6% of this residue being present in the BNTHP as opposed to a range of 64 – 67% in the WNTHP, WSFTHP and BSFTHP (Figure 7).

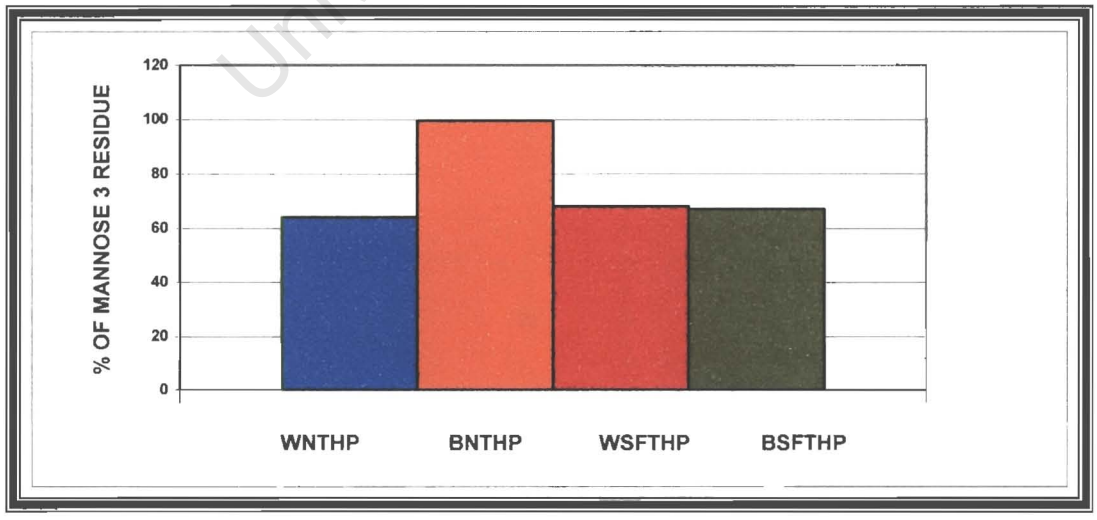



**Figure 7. The percentage of Mannose-3 residue after digestion of the various THPs.**

All four THP samples showed complex-type sugars, which included bi, tri-and tetraantennary glycans. Oligomannose structures were also detected in these samples with WNTHP, WSFTHP and BSFTHP having 11-12% of such structures while BNTHP had only 6%.

### *3.4.2.b. Site Occupancy*

THPs isolated from the four groups were resolved by 10% SDS-PAGE. These gels showed that both NTHPs had a band occurring at a similar position of 97kD. SFTHP from both race groups had similar relative mobilities to each other (84kD) but at a lower position than the NTHP for both race groups (Figures 8a and 8b). The deglycosylated form of WNTHP and WSFTHP both ran at the same position on the gel with a molecular weight of 84kD (Figure 9). BNTHP and BSFTHP were in equivalent positions to those in Figure 9 (data not shown) and the differences of site occupancy demonstrated with the white THP samples are similar to those of the black THP samples.



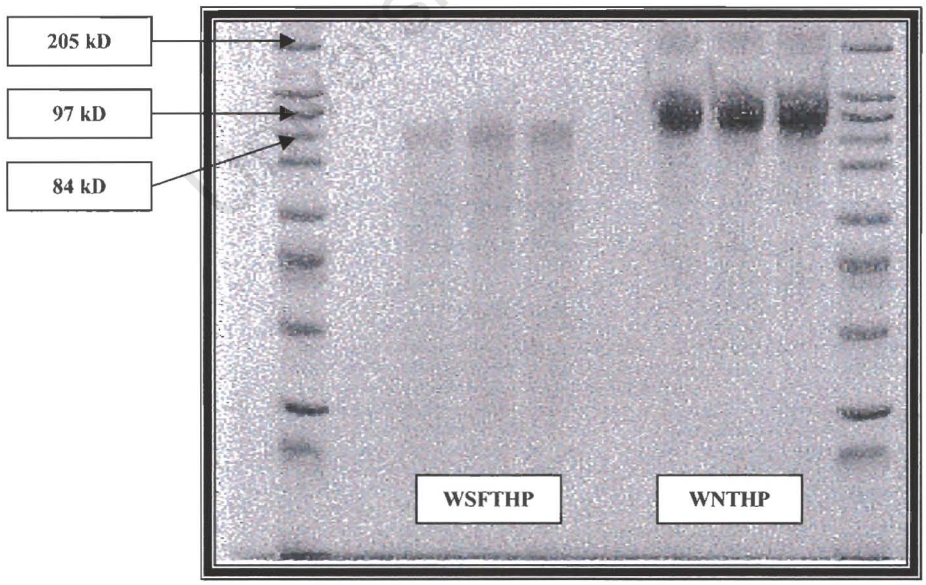



**Figure 8a. WNTHP and WSFTHP run on a 10% SDS gel visualised using Coomassie Brilliant Blue.** (These results are supplied courtesy of the Glycobiology Institute, Oxford University.)

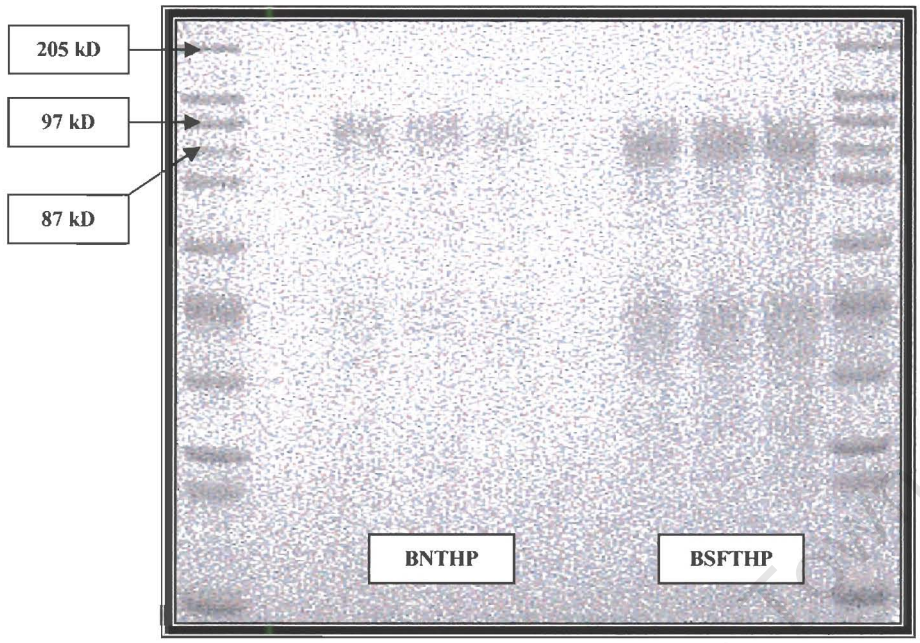



**Figure 8b. BNTHP and BSFTHP run on a 10% SDS gel visualised using Coomassie Brilliant Blue.** (These results are supplied courtesy of the Glycobiology Institute, Oxford University.)

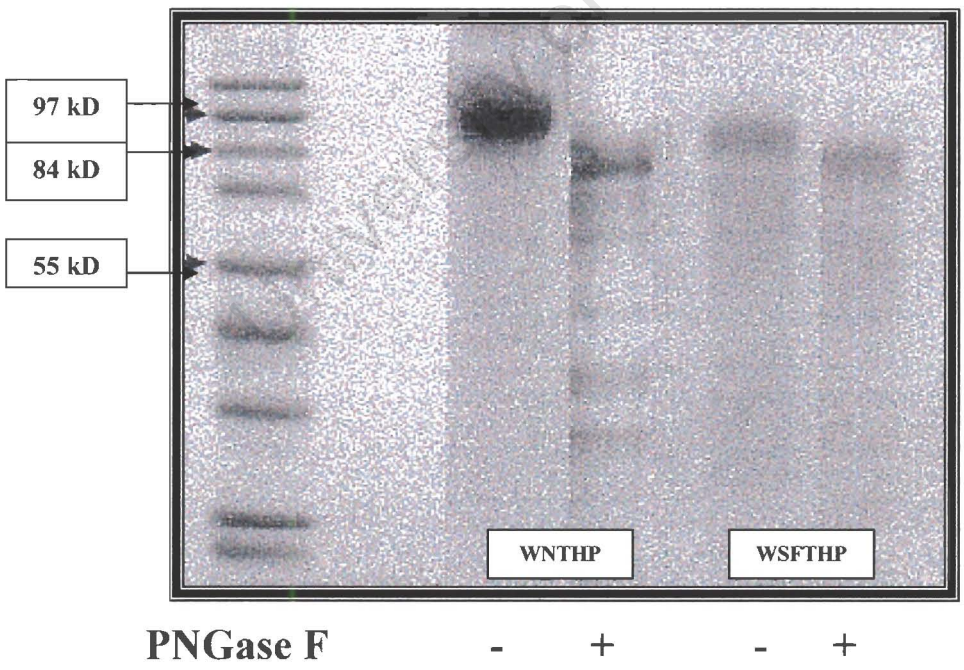



**Figure 9. WNTHP and WSFTHP run on 10% SDS-PAGE before and after digestion with PNGaseF visualised using Coomassie Brilliant Blue.** (Deglycosylated form of WNTHP and WSFTHP ran at the same position on the gel with a molecular weight of 84kD). (These results are supplied courtesy of the Glycobiology Institute, Oxford University.)

### *3.4.3. GalNAc Structures*

GalNAc residues were also found in all four samples. After digestion with a hexosaminidase, BNTHP showed a different NP-HPLC profile when compared to the other three samples (Fig. 10). The complex sugar structures A2, A3 and A4, which have Man 3 cores with only their GalNAc residues remaining, should digest to Man3 (GU=4.4) and possibly also to A1 (GU=5.0). This was seen for WNTHP, WSFTHP and BSFTHP but not for BNTHP (Fig. 10).

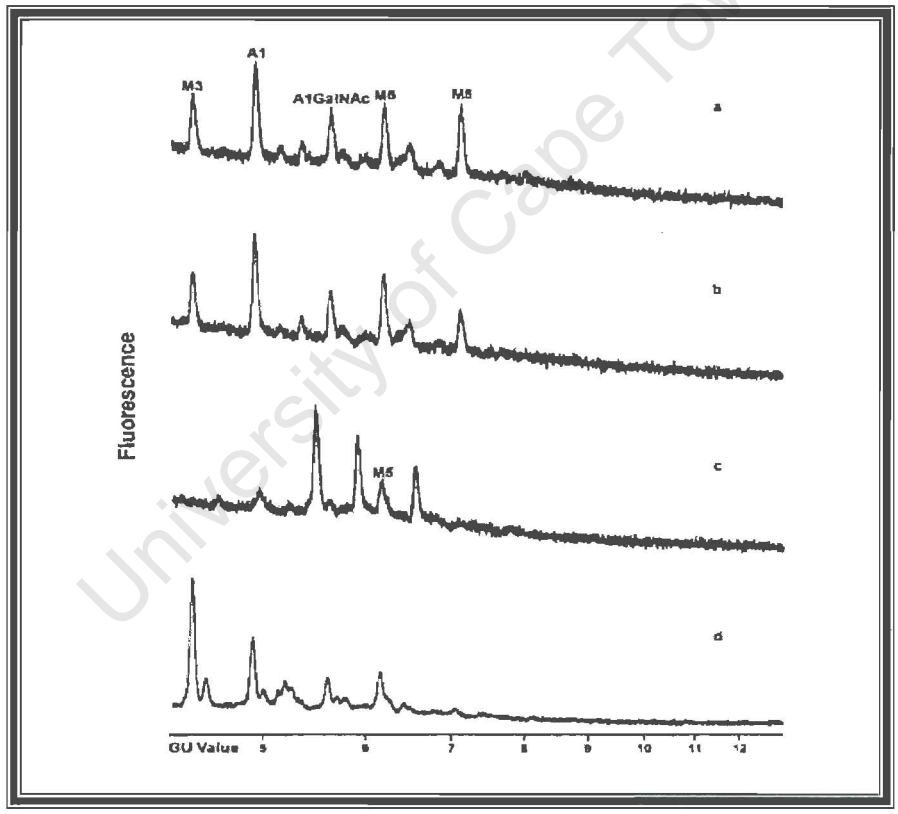



**Figure 10. HPLC profiles after digestion of the various THPs. The profile representing each THP group is as follows: WNTHP (a), WSFTHP (b), BNTHP (c) and BSFTHP (d).** These results are supplied courtesy of the Glycobiology Institute, Oxford University.

### *3.4.4. Charged Structures*

THP from healthy subjects appeared to have more charged groups than their stone-forming counterparts. Table 3 represents the percentage of charged sugars in relation to WNTHP, which had the highest proportion of charged sugars The monosialylated fraction for the stone-forming THP, irrespective of its origin, appeared to be greatly reduced compared with its corresponding healthy population group. Furthermore, no disialylated structures were detected on the stone-forming THP from either race group. The only charged groups present in these samples were sulphates. However NTHP from black and white subjects contained both sulphated and sialylated sugars.

| | % SIALYLATED SUGARS IN RELATION TO THP | |
|---|---|---|
| SAMPLE | MONOSIALYLATED FRACTION | DISIALYLATED FRACTION |
| WNTHP | 100% | 100% |
| BNTHP | 89% | 23% |
| WSFTHP | 5% | - |
| BSFTHP | 2% | - |

**Table 3. Percentage of charged sugars in relation to WNTHP.** These results are supplied courtesy of the Glycobiology Institute, Oxford University.

### *3.4.5. Sialic Acid linkages*

Sialic acids may be attached to THP via three possible linkages, namely α2-3, 2-6 and 2-8. When the THP samples were digested with sialidases specific to these linkages, it was observed that all sialic acids are α2-3 linked. This result is in keeping with the literature (Williams et al. 1984).

### *3.4.6. O-glycan Analysis*

No characteristic O-linked glycan peaks were observed by NP-HPLC. This result was in keeping with two literature reviews (Alfonso et al. 1981; Williams et al. 1984). However Easton et al (2000) revealed for the first time that THP and Uromodulin

(THP from pregnant women) were O-glycosylated. The non-pregnant female and male had a core-1 type O-glycan (Figure 1.1) while the pregnant female had an unusual core-2 type O-glycan linked sugar structure (Galβ 1-3 (GlcNAcβ 1-6) GalNAc). Thus further investigation is required in this area.

## 3.5. DISCUSSION

Previously reported NMR analyses were performed on ultra purified fractions of the carbohydrate chains from THP. However due to a lack of resources (equipment) the purification protocols described in literature could not be repeated and thus a crude digests of the various THP samples were used in the present study. The results obtained from these crude digests were therefore not as comprehensive as those from literature. The spectra for WNTHP, WSFTHP and BSFTHP were similar and the structural reporter groups appeared to be mannose, α2-3 linked Neu5Ac, GlcNAc and fucose structures. However the spectra for the BNTHP appeared to have slight differences as indicated in Table 1. These "structural reporter groups" found in the NMR spectra lend some support to the determination of carbohydrate residues found after enzymatic digestion.

HPLC and MALDI-TOF mass spectroscopy were used to analyse and compare the total glycan pool for each THP sample. In figure 4 the NP-HPLC glycan profiles for these samples are presented for comparison. BNTHP shows a different profile to the other three THPs, with a considerably lower amount of the Man6 structure. After further exoglycosidase digestions Figure 5 shows that there are the same sugars in WNTHP, WSFTHP and BSFTHP. Notably there are differences in the relative proportions of the sugars, namely, Man 4 and Man 6, which are reduced in the BSFTHP glycan pool while the A1 sugar residue is considerably higher in this THP group.

THP from healthy subjects has higher *N*-glycosylation site occupancy than the stone-formers (Figure 9) and this could be due to a difference in the molecular weight of the normal and stone-forming THP. These variations may also be expected in view of previous reports stating that there is a minimum of five *N*-glycosylation sites on THP (Alfonso et al. 1981) and a maximum of eight such sites (Pennica et al. 1987; Hession et al. 1987). However, the approximate 9 kD difference in molecular weight shown by SDS-PAGE corresponds to about two glycosylation sites. Addition of *N*-linked glycans to proteins occurs co-translationally to the appropiate sites bearing the *N*-glycosylation sequon (Asn-Xaa-Ser/Thr) (van Rooijen et al. 1999). Serafini-Cessi et al (1993) showed that the conversion of the precursor form of THP to the mature, glycosylated form of THP is a slow process and depends on the processing of glycans from the oligomannose structures to the complex structures. It appears that the glycan structure of the precursor THP has not been processed by Golgi α1-2 mannosidase, which removes the mannose sugars so that glycosyltransferase can add residues to form the complex glycans (Serafini-Cessi et al. 1993). It is therefore possible that there is a difference in the activity of the Golgi α1-2 mannosidase present in the BNTHP which results in this THP group having a lower amount of the Man 6 structure. The Man 6 structure has been reported to occur at Asn 251 (van Rooijen et al. 1999) (Fig.3) and since the site occupancy for the BNTHP and WNTHP is the same, it can then be concluded that the difference shown by the BNTHP cannot be due to the absence of glycosylation at Asn 251 for this sample.

Easton et al, (2000), noted a similar effect in THP from pregnant women (however this population group is different to the present study) where the Golgi α1-2 mannosidase appears to be more efficient than in non-pregnant women and a difference was also shown in the Man 6 residue. THP from pregnant women (Uromodulin) is also known to have enhanced immunomodulatory effects. The Golgi apparatus is an intracellular organelle in which part of the glycoprotein processing occurs.

Specific folding of the glycoprotein also plays a role in determining normal activity of the Golgi α1-2 mannosidase so that further processing of the carbohydrate chain occurs (Kornfeld et al, 1985). Since Asn 251 is in the region of the polypeptide that does not constitute a conserved protein domain (Kornfeld et al, 1985), it is possible that in the BNTHP there may be a difference in the peptide folding close to this individual *N*-glycosylation site, which may allow increased activity of the mannose enzyme, however this would in turn affect site occupancy as the longer the transit time the greater the opportunity for addition of *N*-glycans. THP is known to have a large number of cysteine residues and a tendency to form aggregates (Hession et al, 1987; Serafini-Cessi et al, 1989). In order to achieve the appropriate folding, the formation of a correct set of disulfide bonds is neccessary (Serafini-Cessi et al, 1993). Thus it is proposed that the BNTHP may have an altered pattern of disulphide bonds, which could account for this reduction of Man 6.

It is postulated therefore that alterations in the site occupancy of THP glycans may play a role in stone formation. Inhibitory THP from healthy subjects is more highly sialylated than promotory THP from stone-formers (Knorle et al, 1994). It has been established that glycosylation is an important factor for the functioning of THP (Hession et al, 1987). In fact van Aswegen et al (1990) noted that the lack of sialic acid was the first step in the conversion of mucosubstances to mineralizable matrix which may lead to stone formation. Knorle et al (1994) also revealed that terminal sialic acid was essential for the inhibitory functioning of THP from healthy subjects. Hess et al (1991) has also postulated that THP from white stone formers has an abnormal structure and is a weak inhibitor or even promoter of crystal aggregation (Hess et al, 1993). This promotory effect of SFTHP correlates well with increased self-aggregation of this THP molecule and could be linked to the abnormal structure predicted for SFTHP, which appears to lack terminally linked sialic acid.

Robertson et al (1986) proposed a mechanism by which urinary macromolecules inhibit crystal growth and aggregation by binding to the surfaces of growing calcium

oxalate crystals and blocking the growing sites. This in turn modifies the attractive or repulsive forces between crystals, thereby impeding or preventing aggregation of the crystals. Edyvane et al (1987) also noted that there were no large crystal clusters that would obstruct the renal tubules and any microcrystals formed due to supersaturation would be flushed out from the urinary tract. Thus it is postulated that sugar structures may affect the binding capacity of THP to CaOx crystal surfaces. Sugars may be altered when exposed to the surrounding urinary environment and therefore modify interactions between THP and CaOx crystals, or block growing sites on the crystals due to their size. Differences in the amount of sugars present could therefore also alter the effect they have on crystal aggregation.

Knorle et al (1994) revealed that THP lacking sialic acid (i.e., fewer negatively charged groups) from stone formers, would no longer bind to the surfaces of calcium oxalate crystals effectively. Thus the unchanged surface of THP from stone formers might act as an additional surface for heterogeneous nucleation and therefore provides a framework for the deposition of stone forming salts. This theory provided by Knorle et al (1994) supports the ideas postulated by Hess et al (1989) that stone formers are no longer fully protected against the formation of larger crystal aggregates which can be deposited in the urinary tract. This loss of THP protective mechanism leads to increasing amounts of the asialo-THP glycoform in the urine and seems to represent a major factor in renal stone formation.

Several researchers have investigated the role of sialic acid in kidney stone formation and have established that there appears to be a considerably lower level of total urinary sialic acid in CaOx stone-formers than in their healthy counterparts (Schauer et al. 1982; van Aswegen et al. 1990; Hess et al. 1991; Knorle et al. 1994; Schnierle et al. 1996; Hallson et al. 1997). The present study seems to support the above findings. Significantly more sialylated and sulphated charged sugars occurred in NTHP from both race groups while less sulphated sugars and the absence or lower levels of sialylated sugars were present in SFTHP. In the current study only small

percentages of monosialylated sugars were present in the SFTHP, namely, WSFTHP (5%) and BSFTHP (2%), while no disialylated sugars were found. A possible explanation for the reduction or lack of sialic acid in the carbohydrate moeity of THP from stone-formers may be due to a defect of an enzyme involved in the processing of the oligosaccharide chains (Knorle et al. 1994). Hess et al (1991) also postulated that recurrent formation of renal stones might be an inherited disease caused by a deficiency of glycosidases or glycosyltransferases. They also postulated that the total urinary sialic acid in stone-formers might be attributed to the rate of excretion of sialic acid by the kidneys.

Studies on asialo-THP have provided another scenario thought to be possible for the reduced amount of sialic acid residues in stone-formers. The results of these studies have indicated that the asialo-THP shows increased viscosity and promotes CaOx crystal aggregation by interactions between crystals and possibly the lining of the urinary tract (Hallson et al. 1997). Thus a reduction in sialic acids could also reduce the level of induced electrostatic potential on surfaces to which the THP binds. This in turn will reduce the repulsive forces between the crystals and therefore allows viscous binding to become dominant. Furthermore, asialo-THP forms a gel-like medium which could adhere to the epithelial cells and provide a suitable environment for the steady growth of large crystals by protecting them from the surrounding medium (Knorle et al. 1994; Mullin. 1993).



A characteristic feature of THP is the presence of GalNAc structures in the *N*-glycans. It is postulated that this monosaccharide is part of the determinant for the blood group $Sd^a$ (Soh et al. 1980, 1989). Exoglycosidase digestions indicated the presence of β-GalNAc residues in the NTHP for both population groups, which is consistent with the occurance of the $Sd^a$ immunodeterminant in the THP molecule (Serafini-Cessi et al. 1993). BNTHP contained a different pattern of GalNAc residues compared to the other three samples. This observation could be due to differences in the expression levels or activities of the processing enzymes present in the relevant

cells of healthy black subjects. Further investigation is required into the relevance of the different pattern of GalNAc residues observed in BNTHP and its relationship to kidney stone formation.

It is also well established that THP is a major human urinary inhibitor of the binding of *E.coli S fimbriae* to epithelial cells of the urinary tract and this interaction with bacteria occurs through its sialyloligosaccharide chains (Parkkinen et al. 1988). These findings were confirmed by Hard et al (1992). showing partial inhibition of this binding occuring in the presence of Neu5Ac(α2-3)Gal(β1-4)Glc (Hard et al. 1992). Krivan et al (1988) has also indicated that pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc(β1-4) Gal found in some glycolipids. Thus, it is postulated that the reduced number of monosialylated sugar residues in SFTHP from both race groups could result in a reduction of the aforementioned GalNAc(β1-4) component and therefore reduced inhibitory properties. Thus NTHP from both population groups bearing the same structural element and it being in abundance in the urine, might play a protective role in preventing adhesion of GalNAc- binding bacteria to the urinary tract (Hard et al. 1992).

Hard et al (1992) states that there are 2 types of sulphate residues found in the carbohydrate moieties of THP, namely, terminal ($SO_4^-$)-4 GalNAc and terminal ($SO_4^-$)-3 Gal. However in this investigation it appears that only the ($SO_4^-$)-4GalNAc residue was found and it was only present in the WNTHP (1.2%) and WSFTHP (0.6%) (Table 4, Appendix 3.2). No terminal ($SO_4^-$)-3Gal residues were found in this investigation which is in keeping with another literature review (de Waard et al. 1991).

In conclusion, it appears that the composition of sugar structures from pooled WNTHP and SFTHP from both population groups match those from literature (de Waard et al. 1991). However, BNTHP appeared to have a different *N*-glycan profile

with a different composition of sugar structures. Thus the structural differences in the NTHP and SFTHP demonstrate the importance of glycosylation for the functional activity of the glycoprotein. Functional differences between the NTHP and the SFTHP may be due to different degrees of sialylation. Thus depending on the glycosylation, THP behaves as an inhibitor or promoter of calcium oxalate precipitation and therefore controls the process of renal stone formation. This fact could therefore be related to the relative immunity shown by the black population to kidney stone formation; however further investigation is required in this field.

# REFERENCES


- Alfonso. A-M. M., Charlwood.P.A and Marshall.R.D. (1981) Isolation and Caracterisation of Glycopeptides from digests of human Tamm- Horsfall Glycoprotein. Carbohydr. Res. 89: 309-319.
- Ashford. D., Dwek. R. A., Welply. J. K., Amatayakul. S., Homans. S. W., Lis. H., Taylor. G. N., Sharon. N., Rademacher. T. W. (1987) The beta 1-2-D-xylose and alpha 1-3-L–fucose substituted N-linked oligosaccharides from *Erythrina cristagalli* lectin: Isolation, characterisation and comparison with other legume lectins. Eur. J.Biochem. 166: 311-320.
- Dall'Olio. F., de Kanter. F. J. J., van den Eijnden. D. H., Serafini-Cessi. F. (1988) Structural analysis of the preponderant high mannose oligosaccharide of human Tamm-Horsfall glycoprotein. Carbohydr. Res. 178: 327-332.
- de Waard. P., Koorevaar. A., Kameling. J. P., Vliegenthart. J. F. G. (1991) Structural determination by 1H-NMR spectroscopy of (sulphated) sialylated N-linked carbohydrate chains released from porcine thyroglobulin by peptide-N4-(N-acetyl-beta-glucoseaminyl) asparagine amidase-F. J. Biol. Chem. 266:4237-4243.
- Easton. R. L., Patankar. M. S., Clark. G. F. Morris. H.R., Dell. A. (2000) Pregnancy-associated Changes in the Glycosylation of Tamm Horsfall Glycoprotein. J. Biol. Chem. Vol 275, No. 29:21928-21928.
- Edyvane. K. A., Hibberd. C. M., Harnett. R. M., Marshall. V. R., Ryall. R. L. (1987) Macromolecules inhibit calcium oxalate crystal growth and aggregation in whole human urine. Clin Chim Acta. 176: 329-338.
- Gottschalk. A. (1952) Carbohydrate residue of a urine mucoprotein inhibiting influenza virus haemagglutination. Nature. 170: 662-663.

- Guile. G. R., Rudd. P. M., Wing. D. R., Prime. S. B., Dwek. R. A. (1996) A rapid high-resolution method for separating oligosaccharide mixtures and analysing sugarprints. Anal Biochem. 240: 210-226.
- Hallson. P. C., Choong. S. K., Kasidas. G. P., Samuell. C. T. (1997) Effects of Tamm-Horsfall protein with normal and reduced sialic acid content upon the crystallisation of calcium phosphate and calcium oxalate in human urine. Br. J. Urol. 80:533-538.
- Hard.K, van Zadelhoff.G, Moonen.P, Kamerling.J.P and Vliegenthart. J. F. G. (1992) The Asn-linked carbohydrate chains of human Tamm- Horsfall glycoprotein of one male. Eur. J. Biochem. 209: 895-915.
- Hard. K., Vliegenthart. . F. G. (1993) Nuclear magnetic resonance spectroscopy of glycoprotein-derived carbohydrate chains. In: Gylcobiology, A practical approach. ed. Fukuda. M., Kobata. A. Oxford University press.
- Hess. B., Nakagawa. Y., Coe. F. L. (1989) Inhibition of calciyum oxalater monohydrate crystal aggregation by urine proteins. Am. J. Physiol. 257:F99-106.
- Hess B, Nakagawa Y, Parks JH, Coe FJ (1991) Molecular abnormality of Tamm Horsfal glycoprotein in calcium oxalate nephrolithiasis. Am J Physiol 260: F 569.
- Hess B, Zipperle L, Jaeger P (1993) Citrate and calcium effects on Tamm-Horsfall glycoprotein as a modifier of calcium oxalate crystal aggregation. Am J Physiol 265: 784.
- Hess B, Jaggi M, Zipperle, Nakagawa Y (2001) Abnormal urinary and molecular structure of Tamm-Horsfall protein (THP) in calcium renal stone formers with positive family history (CSFFAM). In: Kok DJ, Romijn HC, Verhagen PCMS, Verkoelen CF (eds) Proceedings of the 9th European Symposium on Urolithiasis, Rotterdam, p 135.

- Hession. C., Dekker. J.M., Sherblom. A. P., Kumar. S., Yue. C. C., Mattaliano. R. J., Tisard. R. Kawashima. E., Schmeissner. U., Heletky. S., Chow. E. P., Burne. A., Shaw. A., Muchmore. A. V. (1987) Uromodulin (Tamm-Horsfall glycoprotein): A renal ligand for lymphokines. Science. 237:1479-1484.
- Hoyer. J.R., Seiler. M. W. (1979) Pathophysiology of Tamm-Horsfall protein. Kidney Int. 16:279-289.
- Knorle. R., Schnierle. P., Koch. A., Buchholz. N-P., Hering. F., Seiler. H., Ackermann. T., Rutishauser. G. (1994) Tamm-Horsfall Glycoprotein: Role in Inhibition and Promotion of Renal Calcium Oxalate Stone Formation Studied with Fourier-Transform Infrared Spectroscopy. Clin. Chem. 40/9: 1739-1743.
- Kornfeld. R, Kornfeld. S. (1985) Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 54:631-664.
- Krivan. H.C., Roberts. D. D., Ginsberg. V. (1988) Proc. Natl. Acad. Sci. USA **85**: 6157. cited by Hard et al, 1992.
- Merry. A. H., Neville. D. C. A., Royle. L., Matthews. B., Harvey. D. J., Dwek. R. A., Rudd. P.M. (2002) Recovery of intact 2-aminobenzamide-labelled O-glycans released from glycoproteins by hydrazinolysis. Anal. Biochem. 304. 91-99.
- Montreuil. J., Bouquelet. S., Debray. Lemonie. J., Michalski. J.C.,Spik. G., Strecker.G. (1994) Glycoproteins. In Carbohydrate Analysis, a Practical Approach (Chaplin M.F. & Kennedy J.F., eds), 2nd edn, IRL Press, Oxford, 181-293.
- Moonen. P., Gaffner. R., Wingfield. P. (1988) Native cytokines do not bind to uromodulin (Tamm-Horsfall glycoprotein). FEBS Letters. 226: 314-318.
- Muchmore. A. V., Decker. J. M., (1985) Evidence that homogeneous uromodulin via N-linked oligosaccharides. Science 229: 479-481.
- Muchmore. A. V., Decker. J. M., (1987) **(a)** Evidence that recombinant Π 1 α exhibits lectin-like specificity and binds to homogeneous uromodulin via N-linked oligosaccharides. J. Immunol.138: 2541-2546.

- Muchmore. A. V., Shifrin. S., Decker. J. M., (1987) **(b)** In vitro evidence that carbohydrate moieties derived from uromodulin, an 85 000 dalton immunosuppressive glycoprotein isolated from human pregnancy urine, are immunosuppressive in the ansence of intact protein. J. Immunol.138: 2547-2553.
- Mullin. J. W. (1993) Crystallisation. Butterworths, Oxford.3rd Edn. 281.
- Odin.L. (1952) Carbohydrate residue of a urine mucoprotein inhibiting influenza virus haemagglutination. Nature (London) 170:663.
- Orskov. I., Ferencz. A, Orskov. F. (1980) Tamm-Horsfall protein or Uromucoid is the normal urinary slime that traps type 1 fimbriated Eschericia coli. The Lancet. 1: 887.
- Parkkinen. J., Virkola. R., Korhonen. T. K. (1988) Identification of factors in human urine that inhibits the binding of Eschenchia coli adhesions. Infect. Immun. 56: 2623-2630.
- Patel. T., Bruce. J., Merry. A., Bigge. C., Wormald. M., Jaques. A., Parekh. R. (1993) Use of hydrazine to release intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins. Biochemistry 32: 679-693.
- Pennica. D. Kohr. W. J., Kuang. W-J., Glaister. D., Aggarwal. B. B., Chen. E. Y., Goeddel. D. V. (1987) Identification of Human Uromodulin as the Tamm-Horsfall Urinary Glycoprotein. Science. 236: 83-88.
- Radcliffe. C. M., Diedrich. G., Harvey. D. J., Dwek. R. A., Cresswell. P., Rudd. P. M. (2002) Identification of specific glycoforms of major histocompatability, complex class I heavy chain, suggest that class I peptide loading is an adaptation of quantity control pathway involving Calreticulin and ERP 57. J. Biol. Chem. 277: 46415-46423.
- Robertson. W.G., Scurr. D. S. (1986) Modifiers of calcium oxalate growth found in urine. II. Studies on their mode of action in artificial urine. J Urol 136: 128-131.

- Rudd.P.M and Dwek.R.A (1997) Rapid, sensitive sequencing of Oligosaccharides from Glycoprteins, Current opinion in Biotechnnology, 8: 488-497.
- Schauer. R., (1982) Chemistry, metabolism and biological functions of sialic acid. In: Tipson. R.S., Horton. D. (eds) Advances in carbohydrate chemistry and biochemistry. Academic Press, New York, London. 131.
- Schnierle. P., Hering. F., Seiler. H. (1996) Isoelectric focusing of Tamm-Horsfall glycoprotein: a simple tool for recognizing recurrent calcium oxalate renal stone formers. Urol. Res 24: 79-82.
- Seppala. R., Renlund. M., Bernardini. I., Tietze. F., Gahl. W. A (1990) Renal handling of free sialic acid in normal humans and patients withSalla disease or renal disease. Lab. Invest. 63: (2) 197.
- Serafini-Cessi. F., Franceschi. C., Sperti. S. (1979) Specific interaction of Tamm-Horsfall glycoprotein with leucoagglutinin, a lectin from Phaseolus vulgaris (red kidney bean). Biochem.J. 183: 381-388.
- Serafini-Cessi. F., Malagolini. N., Dall'Olio. F. (1984) Tetraantennary glycopeptide from human Tamm-Horsfall glycoprotein inhibits aggutination of desialylated erythrocytes induced by leucoagglutinin. Bioscience Reports 4: 973-978.
- Serafini-Cessi. F., Bellabarba. G., Malagolini. N., Dall'Olio. F. (1989) Rapid Isolation of Tamm-Horsfall glycoprotein (uromodulin) from human urine. J. Immun. Methods 120: 185-189.
- Soh. C. P. C., Morgan. W. T. J., Watkins. W. M., Donald. A. S. R. (1980) The relationship between the N-acetylgalactoseamine content and the blood group Sda activity of Tamm and Horsfall urinary glycoprotein. Biochem. Biophys. Res. Comm. 93: 1132-1139.
- Soh. C. P. C., Donald. A. S. R., Fenney. J., Morgan. W. T. J. Watkins. W. M., (1989) Enzymatic synthesis chemical characterization and Sda activity of GalNAcβ 1-4[NeuAc α2-3]Galβ1-4 GlcNAc and GalNAc Galβ1-4(NeuAc α2-3)Gal β 1-4 Glc. Glycoconjugate J. **6**: 319.

- Van Rooijen. J. J. M., Kamerling. J. P., Vliegenthart. J. F. G. (1998) The abundance of additional N- acetyllactosamine units in N-linked tetraantennary oligosaccharides of human Tamm-Horsfall glycoprotein is a donor-specific feature. Glycobiology 8: 1065-1075.
- Van Rooijen. J. J. M., Voskamp. A. F., Kamerling. J. P., Vliegenthart. J. F. G. (1999) Glycosylation Sites and Site- Specific Glycosylation of Tamm-Horsfall Glycoprotein. Glycobiology 9: 21-30.
- Williams. J., Marshall. R.D., van Halbeck. H., Vliegenthart. J. F. G. (1984) Structural Analysis of the carbohydrate moieties of human Tamm-Horsfall glycoprotein, Carbohydr. Research 134:141-155.
- van Aswegen. C. H., van der Merwe.C. A., du Plessis. D. J. (1990) Sialic acid concentrations in the urine of men with and without renal stones. Urol Res.18: 29-33.
- Williams. J., Marshall. R. D., van Halbeek. H., Vliegenthart. J. F.G. (1984) Structural analysis of the carbohydrate moieties of human Tamm-Horsfall glycoprotein. Carbohydr. Res. 134: 141-155.
- Zamze. S., Harvey. D. J., Chen. Y. J., Guile. G. R., Dwek. R. A., Wing. D. R. (1998) Sialylated N-glycans in adult rat brain tissue: A widespread distribution of disialylated antennae in complex and hybrid structures. Eur. J. Biochem. 258: 243-270.

# CHAPTER 4

## INVESTIGATION OF THE EFFECTS OF THP ON CALCIUM OXALATE (CaOx) CRYSTALLISATION PROCESSES IN URINE

### INTRODUCTION

The initial event for stone-formation appears to be nucleation of microcrystals from supersaturated urine (Finlayson et al, 1973; Kok et al, 1993). Crystal growth and aggregation are responsible for larger particle formation in the renal tubules (Hess et al, 1996). *Crystal growth* of single calcium oxalate (CaOx) crystals in the urine is too slow to produce large particles of clinical significancy (Finlayson et al, 1978; Kok et al, 1994) but *crystal aggregation* (a process of crystals adhering together to form a large mass) allows the formation of larger particles and at a faster rate (Kok et al, 1993; 1994). It therefore appears that the latter mechanism is a very important one in the formation of CaOx renal stones (Kok et al, 1993). The urine of stone-formers has been reported as having larger crystalline particles that could be formed by reduced inhibition or promotion of crystal aggregation (Hess et al, 1996).

Tamm Horsfall glycoprotein has been investigated in several previous studies for its effect on calcium oxalate crystallisation processes in urine and its role has been shown to vary under different conditions. Since THP has been found in variable amounts within kidney stones (Grant et al, 1973), it has been proposed to play a role in nephrolithiasis.

However, THP is not found within urinary calcium oxalate crystals (Doyle et al, 1991) which indicates that its binding to crystal surfaces is not irreversible. As stated earlier *macromolecules*, such as glycosaminoglycans, Osteopontin, Nephrocalcin, Inter-a-trypsin inhibitor (Uronic acid rich protein), urinary prothrombin fragment 1 (Ryall, 1996) and THP itself, may be at low concentrations that will not affect supersaturation but will adsorb onto the surfaces of newly formed urinary crystals thereby altering their surface properties and therefore the rates of crystal growth and aggregation (Hess et al, 1996). THP has been shown to affect aggregation of crystals (Scurr et al, 1986 **(a)**, **(b)**; Hess et al, 1993; Khan et al, 1997). There is controversy whether THP is an inhibitor or a promoter of this crystallisation process (Hess, 1994). The results of various studies of THP effects on calcium oxalate crystal nucleation, growth and aggregation shows that its role varies under different conditions. It has been established that THP affects the nucleation phase as a promoter and the crystal growth phase as a weak inhibitor (Yoshioka et al, 1989). Scurr and Robertson (1986) showed that THP had an inhibitory effect on crystal growth and suggested it could be a potential promoter of crystal aggregation. Kitamura and Pak (1982) noted that THP exerted a slight inhibitory effect on calcium oxalate crystal precipitation and crystal growth. Rose and Sulaiman (1982) showed THP to promote calcium oxalate crystal precipitation, while Sophasan et al (1980) showed no influence of THP on crystal mass formation. Drach et al (1980) revealed the dual potential of THP for calcium oxalate crystallisation, viz an inhibitory effect on crystal growth, as well as an enhancing effect on crystal nucleation. Hess, 1992 found that THP played a dual role in COM crystal aggregation at higher pH and lower ionic strength (IS), THP is a powerful aggregation inhibitor. Upon lowering pH and raising IS

It has also been shown that THP has little or no effect on calcium oxalate crystal nucleation and growth (Hess 1994). It is thus evident that THP's main effect is on crystal aggregation, but that this is dependent on the prevailing urinary conditions. However, all of the previously reported studies have involved white subjects from non South African population groups. Since black and white South African subjects display certain differences in their urinary composition, as described in chapter 2, it is speculated that their THP might have different inhibitory activities in their respective urine environments as well as in those of the other race group and that this might indeed provide some clues to explain the low stone incidence in the black group.

This chapter describes experiments in which the effects of THP on CaOx crystallization processes in urine were investigated. In order to accommodate the variables of race and stone forming status of the subjects from which the proteins and urines were derived, a 4 x 4 matrix involving 16 cells was established for the study (Figure.1). Experiments in which protein was tested in its endogenous urine are termed "direct"; experiments in which either the urine's race group or its stone-former status was switched relative to the protein are termed "single cross-over"; experiments in which the urine's race group *as well as* its stone-former status were changed relative to the protein are termed "double cross-over".

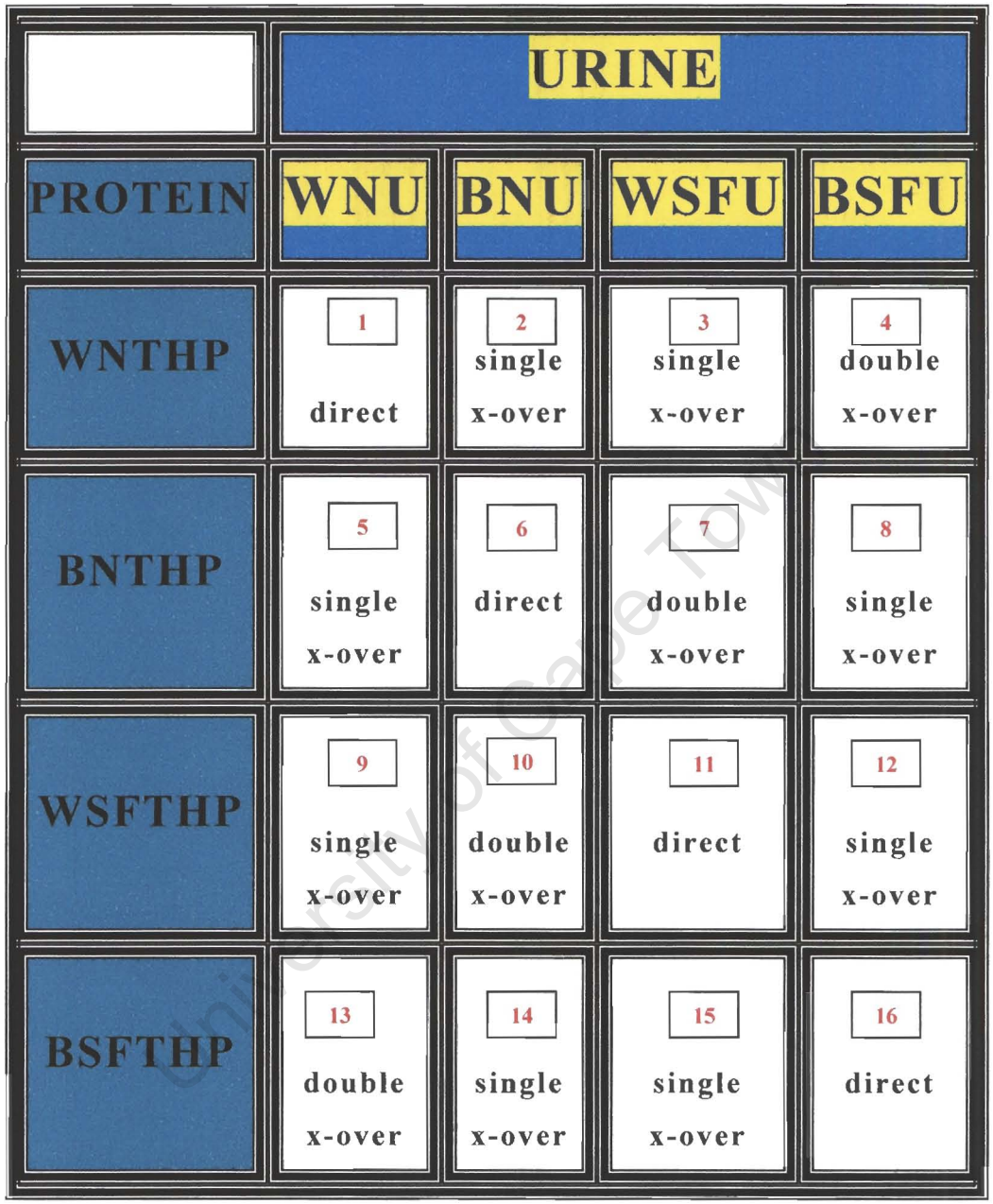



**Figure 1. Protocol matrix for crystallisation experiments**

Within each protein-urine cell (Figure.1), a series of crystallization experiments was conducted. These included determination of CaOx metastable limits, particle formation kinetics, particle volume-size distributions by Coulter Counter and CaOx deposition kinetics using

radioactive $^{14}$C-oxalate. In addition, the effects of the four proteins on the sedimentation rates and zeta-potentials (Zp) of calcium oxalatemonohydrate (COM) crystal slurries were also studied independently of the matrix.

A schematic diagram dipicting the protocol in cells 1, 2, 5 and 6 is given in Figure.2.

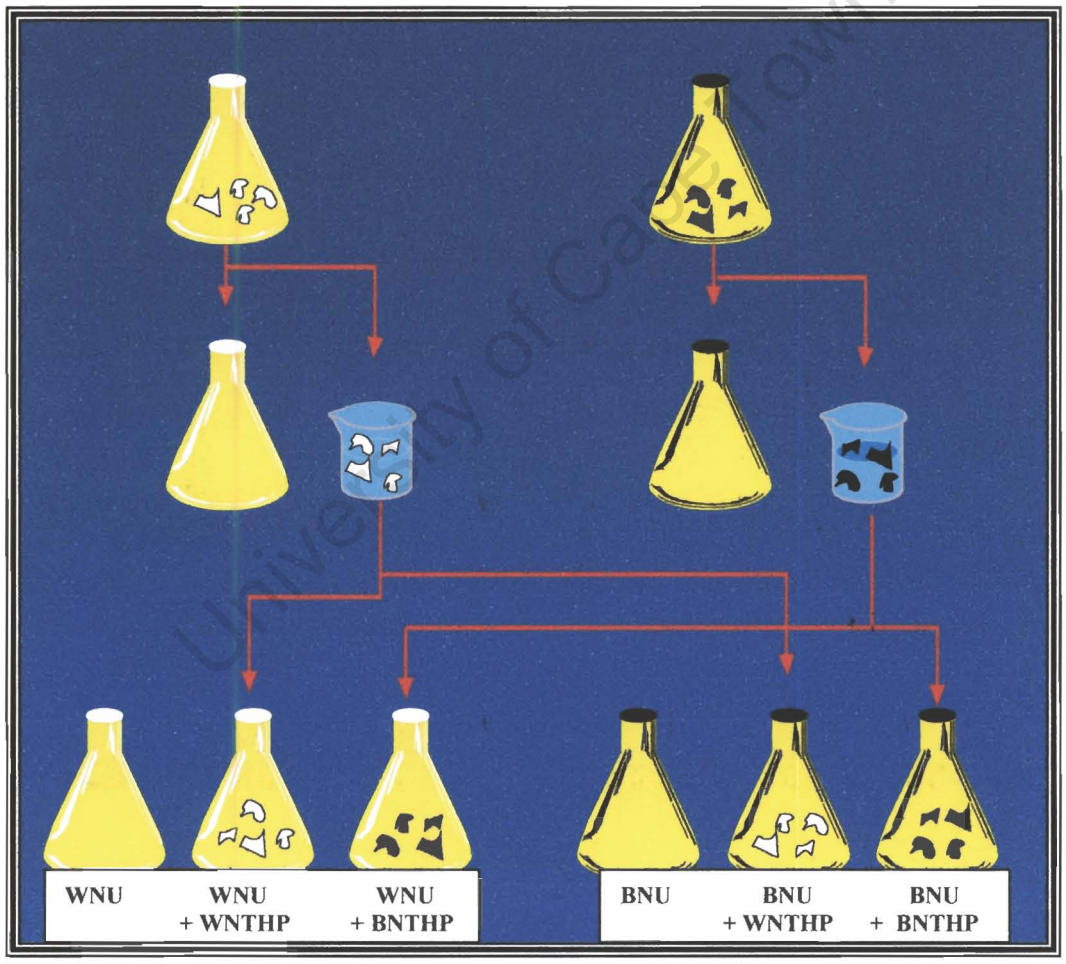



**Figure 2: Schematic representation of the cross-over design protocol.**

# CHAPTER 4.1

## CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTS IN URINE FROM HEALTHY SUBJECTS: DIRECT AND SINGLE CROSSOVER EXPERIMENTS

### 4.1.1. OBJECTIVES

- Determine the CaOx meta-stable limits (msls), particle formation kinetics and particle volume-size distributions in pooled, ultrafiltered urines from black and white healthy subjects (controls)

- Repeat the above experiments in the presence of Tamm Horsfall glycoprotein isolated from the urines of healthy subjects from both race groups (NTHP) in a crossover design involving NTHP from each race group in its own parent ultrafiltered urine as well as in the ultrafiltered urine of the other race group (i.e. Figure.1: cells 1,2,5 and 6)

- Determine the CaOx crystallisation kinetics in the presence and absence of NTHP from both race groups using $^{14}$C-oxalate in the same series of crossover experiments as above in pooled, ultrafiltered urines from black and white healthy subjects

## 4.1.2. METHODS

### *4.1.2.a. Effect oF NTHP on CaOx Crystallisation (Coulter Counter)*

Calcium oxalate metastable limits (msls) and particle formation kinetics were measured separately in the urine from which THP had been removed by successive filtration through prefilters (Macherey-Nagel, Germany, 4.7cm) and 0.45µm nitro-cellulose membrane filters (Millipore, USA). Filtered urines from both groups- normal urine from white subjects (WNU) and normal urine from black subjects (BNU)- were used. Crystallisation experiments were performed in each urine and were then repeated using a crossover design (Figure.2, Chapter 4) in which the protein from each race group (WNTHP, BNTHP), at a physiological concentration of 35 $mgl^{-1}$, was added to its parent filtered urine as well as to the filtered urine from the other race group. The protocol for the measurement of metastable limits and particle formation kinetics was based on that described by Ryall, 1985. In this method, msls were determined by adding aqueous NaOx in increasing concentrations (0 - 0.20M) (protocol for preparation of NaOx standards can be found in appendix 4.1A) to each urine sample until spontaneous crystallisation of CaOx occurred, as detected by a Coulter Multisizer, (Coulter Electronic LTD, England, serial no. 030288). From crystallisation experiments carried out in the laboratory, it was determined that for urine, the 140µm orifice was suitable as the aperture would not become blocked as it would if a smaller orifice was used. The concentration of NaOx corresponding to the initiation of detectable crystallisation was taken as a measure of the msl. Thereafter, 1 ml of the NaOx solution (at the msl concentration) was added to 100 ml filtered urine. The formation rate of the ensuing particles was measured at 10-minute intervals for periods up to 80 minutes, using the Coulter Multiziser. Particle volume-size distributions were determined at the end of the period, using the same instrument. These experiments were conducted

in the presence and absence of protein. Since it is well known that THP interferes with crystal counting using this instrument (Grover et al, 1990), *crystal* formation (as opposed to particle formation) was simultaneously monitored in deposition experiments in which labelled $^{14}$C-oxalate was used to initiate crystallization.

*4.1.2.b. Effect of NTHP on CaOx Crystal Deposition (14C-oxalate)*

Kinetic experiments similar to those described in the previous paragraph were performed using urine samples that had been supplemented with $^{14}$C oxalate solution. Such experiments provide data related to total *crystal* deposition as opposed to *particle* deposition (Grover et al, 1990). A stock solution of $^{14}$C-oxalate (3.125µCi 100 ml$^{-1}$) was prepared from 250 µCi $^{14}$C Oxalic Acid, (Separation Scientific (SA)(PTY) LTD) with distilled water and was stored at –4°C. Aliquots (30ml) of urine from 3 pooled WNUs and 3 pooled BNUs were filtered into flasks through 0.45um Millipore filters in order to remove particulate matter and THP (each pooled urine was made up from 5 subjects). 0.15µl of $^{14}$C-oxalate was added to each flask followed by 0.3ml aqueous sodium oxalate (at a concentration equal to that of the previously determined msl). NTHPs (at physiological concentrations, 35 mg l$^{-1}$) were added in a crossover design as previously decribed in chapter 4. Flasks were incubated at 37°C. Aliquots (2.5ml) were removed from each flask at time intervals corresponding to those of the kinetics experiments, over a period of 80 minutes. Thereafter the aliquots were pumped through a disposable syringe connected to a 0.22µm Millipore filter to trap crystals. The filtrate was collected in a plastic vial containing 250 µl concentrated hydrochloric acid to quench the reaction. The vial was immediately closed and shaken.

Two separate aliquots (1 ml) were pipetted from the vial and transferred to fresh vials containing 10ml scintillation fluid (Zinsser Analytic, Frankfurt, Germany) and each sample was counted for 10 minutes in a Beta scintillation counter (Beckman Scintillation Counter B LS5000 TD).

## 4.1.3. RESULTS

### *4.1.3.a. Effect of NTHP on CaOx Crystallisation (Coulter Counter)*

The average msls for the different protein-urine combinations are given in Figure 3. It is noted that urines from black healthy subjects have significantly higher msls than those from whites (0.068(*0.011*) vs 0.043(*0.005*)). Addition of protein from each race group did not affect the msl significantly.



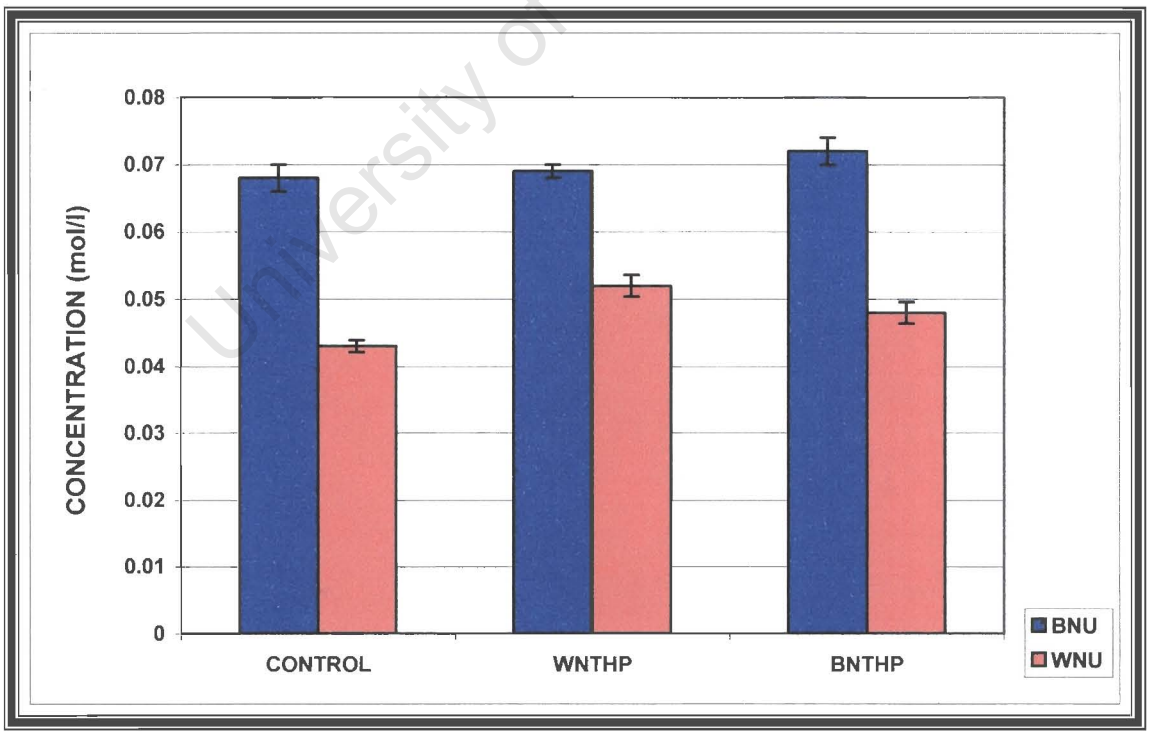



**Figure 3: Histograms representing the msl of WNU and BNU before and after the addition of NTHPs. The error bars represent standard error.** (Data used to construct Figure 3 is available in appendix 4.1B)

Observed trends in particle formation rate, particle size and particle volume are given in table 1 while typical plots for each of these trends are given in Figures 4 and 5. (Due to the Coulter Counter's inability to distinguish between particles and crystals, the former term is used in the present context to represent crystals, THP particles and /or variously sized composites of both).

| URINE INDIVIDUAL | PARTICLE CHARACTER-ISTIC | NO OF URINES DISPLAYING TRENDS | IDENTIFIED TREND | FIGURES |
|---|---|---|---|---|
| WNU (n = 8) | RATE OF PARTICLE FORMATION | 6 | WNTHP < BNTHP < WNU | 4a |
| | PARTICLE SIZE | 5 | WNU = WNTHP < BNTHP | 5a |
| | PARTICLE VOLUME | 5 | WNU < BNTHP <WNTHP | 5a |
| BNU (n= 6) | RATE OF PARTICLE FORMATION | 3 | BNU < BNTHP < WNTHP | 4b |
| | PARTICLE SIZE | 2 | BNU = WNTHP < BNTHP | 5b |
| | PARTICLE VOLUME | 3 | BNU<BNTHP<WNTHP | 5b |

**Table 1: Observed trends in particle characteristics: NU + NTHP.** (Data used to construct Table 1 is available in appendix 4.1C, D, E)

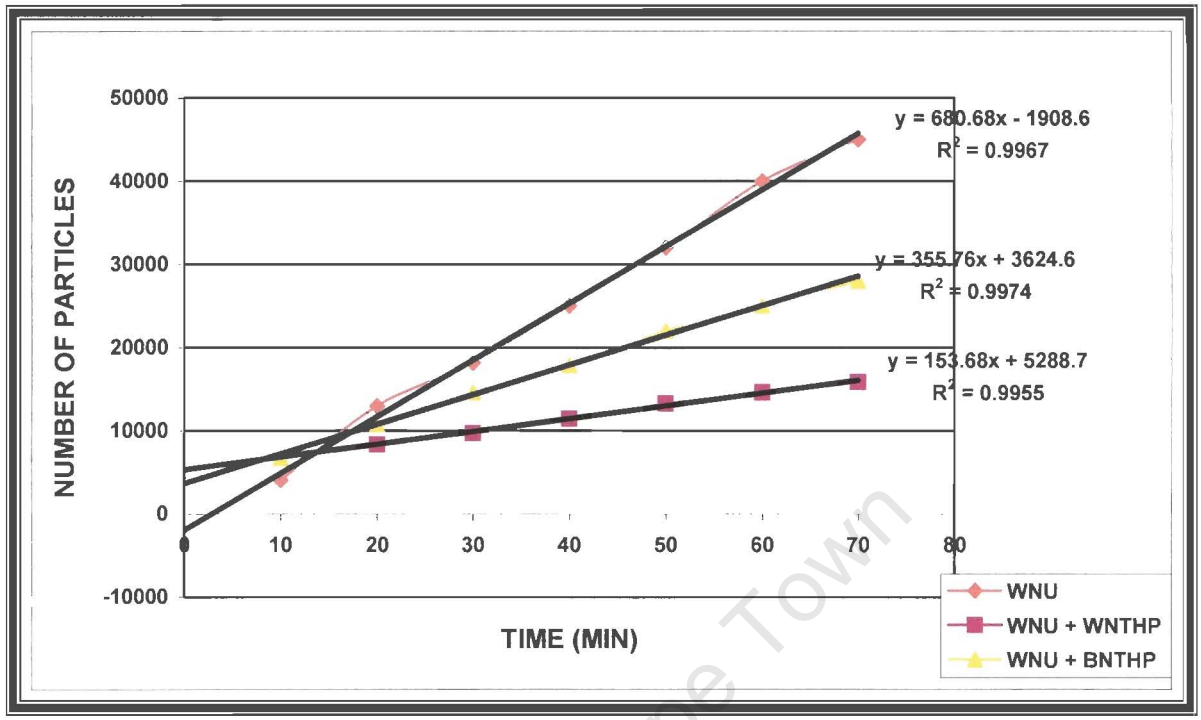



Figure. 4a. Plots of particle number vs time in WNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs

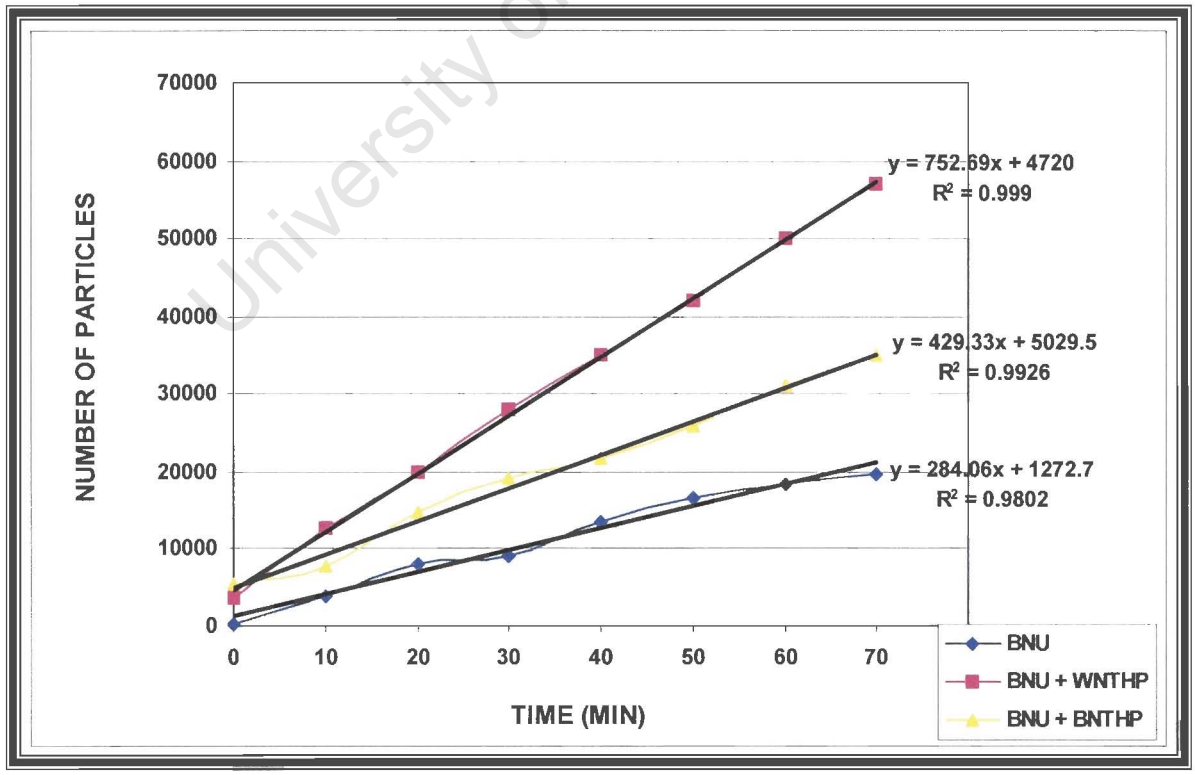



Figure 4b. Plots of particle number vs time in BNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs

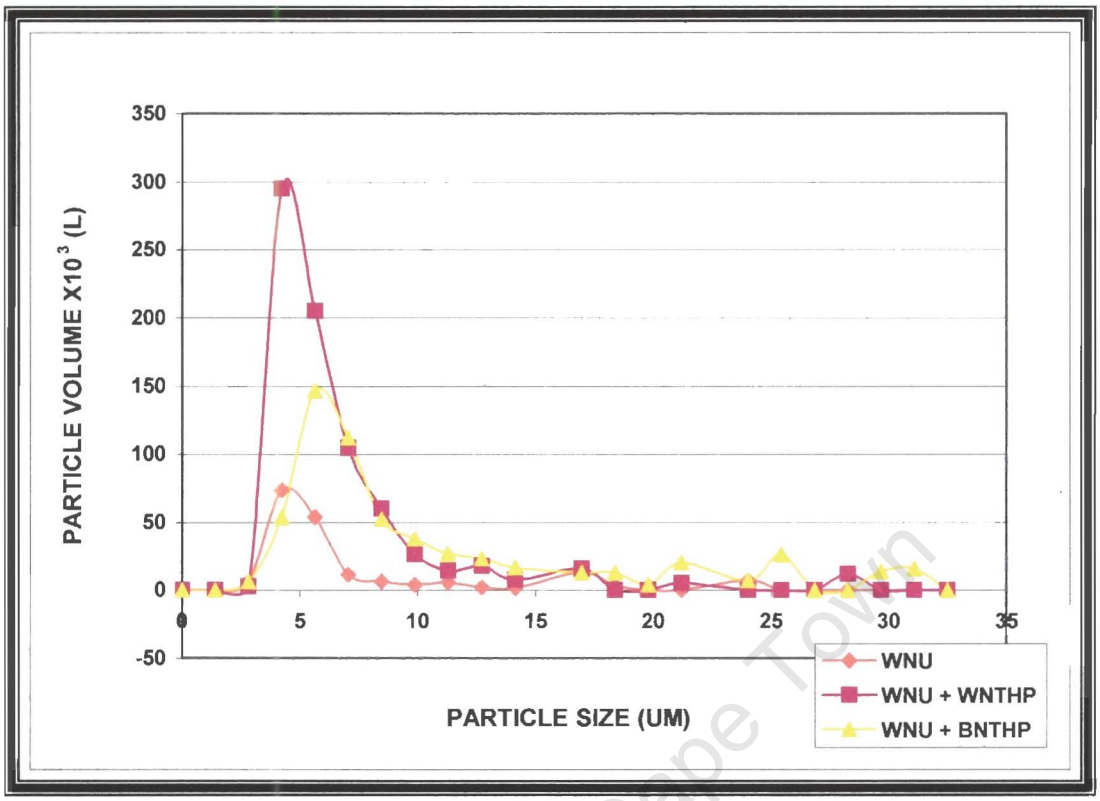



Figure 5a: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in WNU before and after the addition of NTHP.

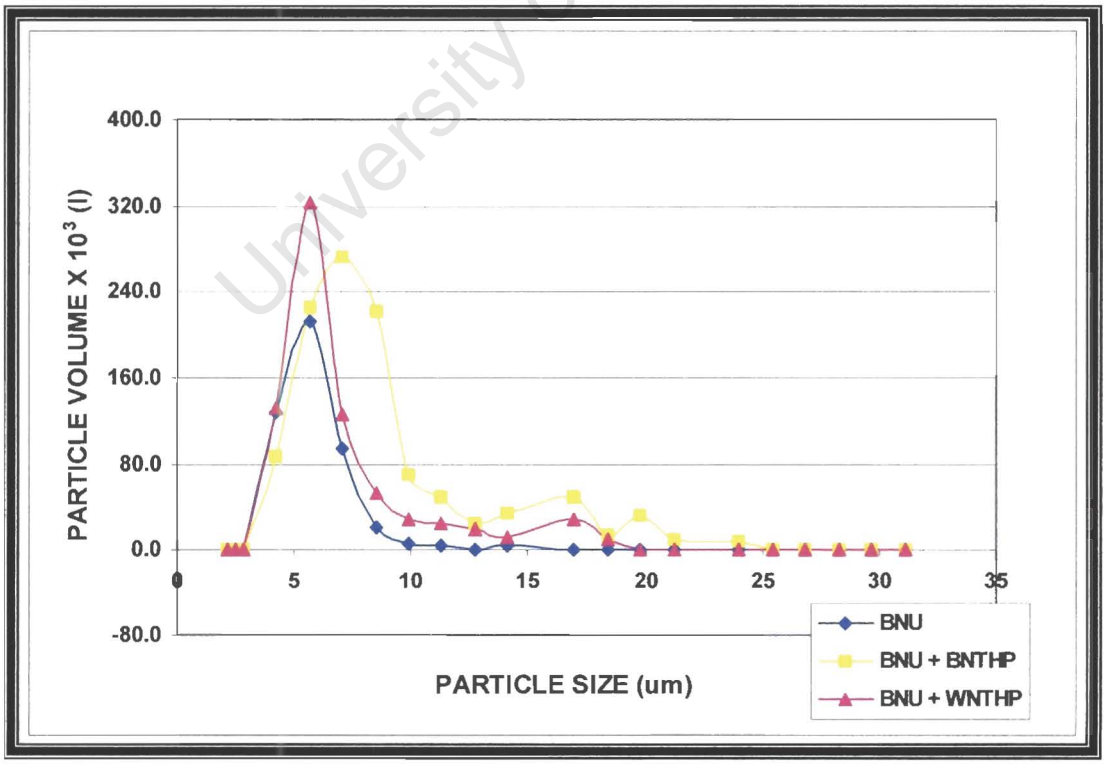



Figure 5b: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in BNU before and after the addition of NTHP.

*4.1.3.b. Effect of NTHP on CaOx Crystal Deposition ($^{14}$C-oxalate)*

Counting the $^{14}$C radioactivity in the filtrate provides a measure of the amount of unbound oxalate. This is plotted as "unprecipitated CaOx" as a function of time as shown in Figures 6 and 7. It is noted that in WCU (Figure.6), the deposition rate follows the trend BNTHP< WNU< WNTHP while in BNU (Figure.7) it is WNTHP< BNTHP < BNU. It is noted that in WNU, addition of NTHP from the two race groups induced *opposite* effects, each of which appears to be significantly different from that observed in the control (Figure.6).



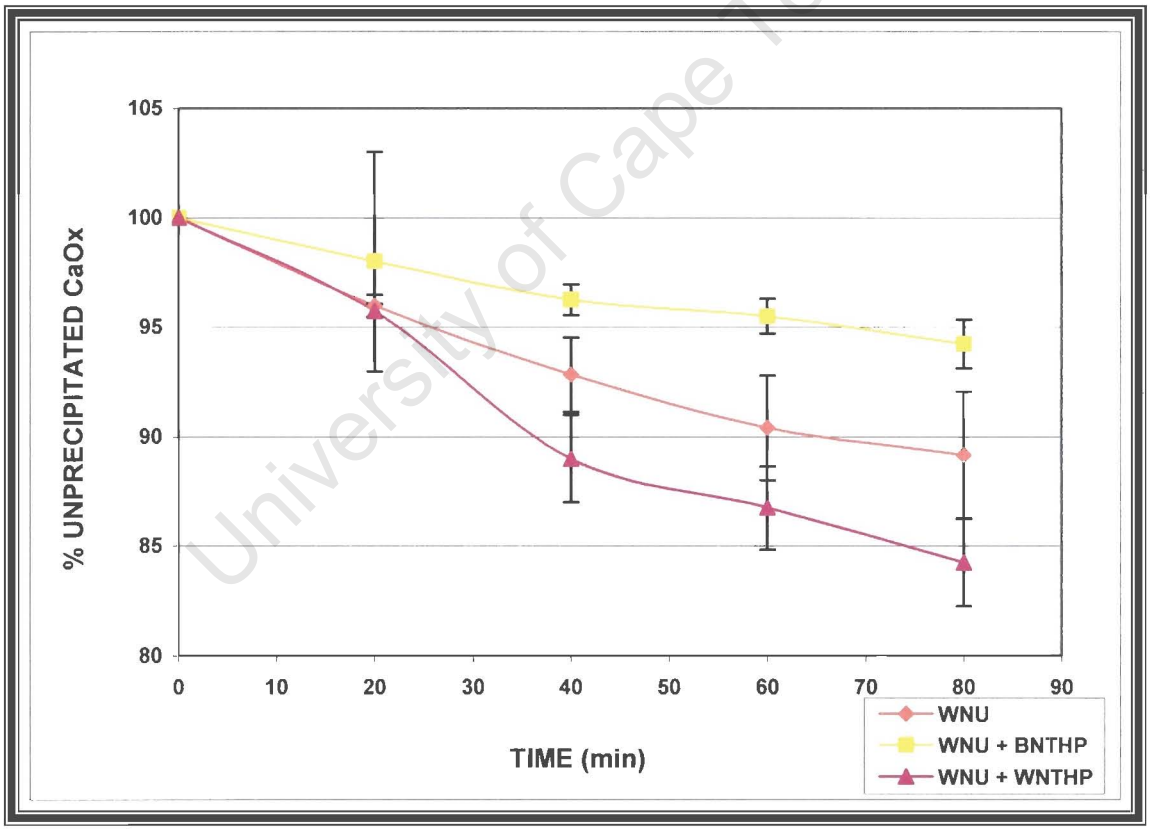



**Figure 6. Mean plots of crystallisation kinetics using $^{14}$ C-oxalate in WNU.** (Data used to construct Figure 6 is available in appendix 4.1F.)

.

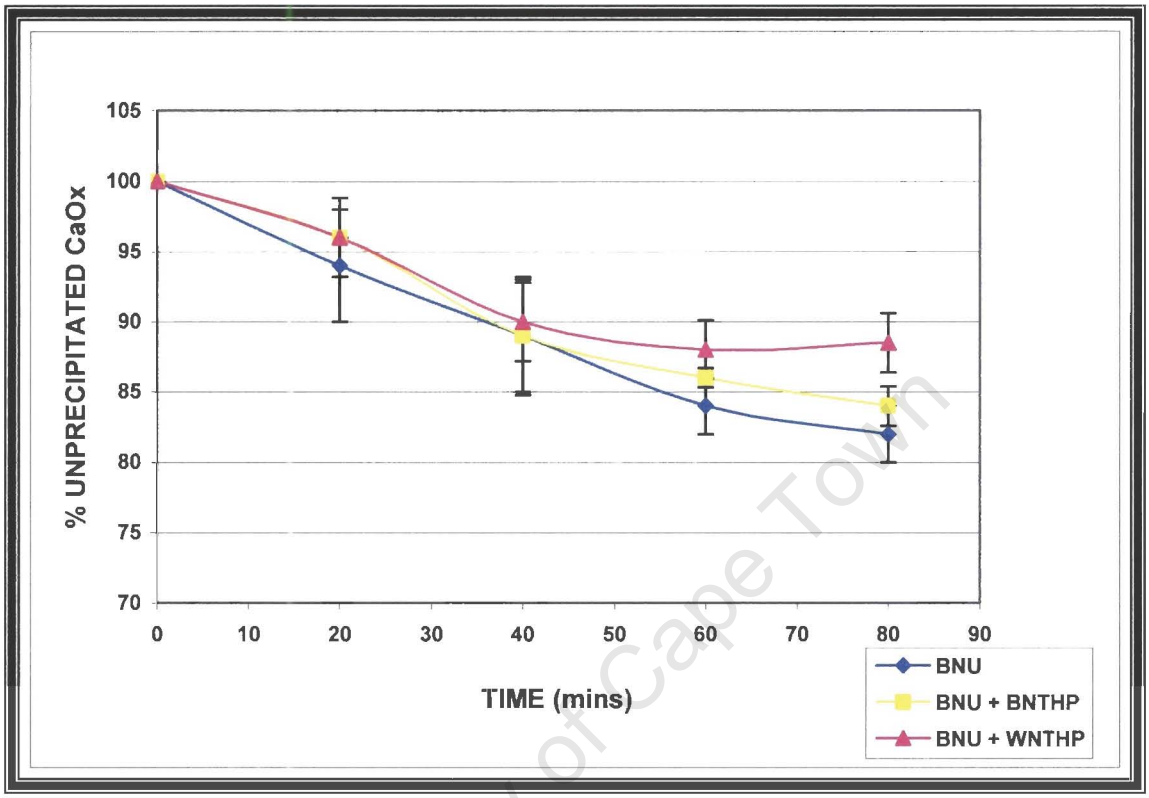



**Figure 7. Mean plots of crystallisation kinetics using $^{14}$C-oxalate in BNU.** (Data used to construct Figure 7 is available in appendix 4.1g.)

## 4.1.4. DISCUSSION

The observation that msl's were not affected by THP indicates that this protein does not play a role in controlling the initiation of crystallisation and is in agreement with the findings reported in other studies (Grover et al, 1990, 1994). However of some interest is the observation of higher msl values in the urines of black subjects. This is commensurate with their significantly lower stone incidence relative to white subjects.

The results for the kinetics of particle formation, irrespective of whether they are crystals, proteins, or both, indicate that THP from

both race groups behaved differently in the two urines. In WNU the proteins retarded the rate of particle formation while in BNU they increased the rate. Hence it is concluded that the nature and composition of the urine environment influence the properties of this protein and the role which it plays in the overall process of stone formation. Since the particles contain calcium oxalate (demonstated in subsequent $^{14}$C-oxalate experiments), it suggests that these results agree with the observations of Hess who found that the role played by THP in influencing calcium oxalate crystallisation processes is dependent on the prevailing chemical conditions within the urine (Hess. 1992).

Of greater significance than particle numbers are the particle volume-size distributions. Size is of pathological importance since it plays a role in determining whether particle entrapment will occur within the urinary tract. On the other hand particle volume, in conjunction with particle size, gives a measure of the quantity of inter-crystalline protein. In the present study, there was no difference in particle size induced by WNTHP in both groups' urines relative to the control, but sizes induced by BNTHP tended to be smaller. However, particle volumes in both urines were larger than those in the control. In general, larger volumes can arise because the total mass of the induced particles increases (by deposition of more crystalline material) or because the density of the particles decreases (by inclusion of more inter-crystalline protein). As explained earlier, the Coulter Counter would "see" an aggregate of crystals and proteins as a single "particle".

$^{14}C$ crystallisation experiments allow one to determine which of these processes are occurring as they measure the deposition of new crystalline material. The results in these experiments show that BNTHP inhibits the deposition of calcium oxalate crystals in the urine of *both* race groups while WNTHP promotes crystal deposition in its own urine but inhibits deposition in urine from black subjects. These data show that the increase in particle volume induced by BNTHP in both groups' urine and by WNTHP in the urine of black subjects arises from the inter-crystal incorporation of more proteinaceous material rather than by deposition of more crystalline material. Indeed in the absence of protein (control sample), particle volumes have their lowest values. Furthermore the observation that the formation rate of particles (Table 1, Figures 4a and 4b) is different to that for *CaOx crystals* (Figures 6 and 7) supports the interpretation that proteinaceous material is being deposited. Thus, the particle volume and $^{14}C$ data indicate that BNTHP (in both groups' urine) and WNTHP (in urine from black subjects) act as inhibitors of calcium oxalate crystal growth. It is noted that THP has been reported previously as a growth inhibitor in varying degrees of efficacies ranging from 38% (Kitamura et al, 1982) to zero % (Worcester et al, 1988) depending on the prevailing chemical conditions. Obviously, the ability of BNTHP to serve in this capacity *in its own urine,* is of some significance. The $^{14}C$ results also suggest that BNTHP is more versatile as a growth inhibitor than WNTHP, as it plays this role irrespective of the urine environment. As such, attention is again drawn to the likelihood of there being inherent differences between the proteins in the two race groups.

The inhibitory performance of WNTHP in urine from black subjects but not in its own, again points to a possible catalytic role-played by the

composition of the former. It is of some considerable interest that a similar synergistic relationship has been reported between inhibitory performance and urine composition of another urinary protein, UPTF1 (Webber et al. 2002).

Thus, the results of the present study have revealed several properties pertaining to Tamm Horsfall protein and urine from black subjects, which are superior to those of white subjects in the context of providing protective mechanisms against calcium oxalate kidney stone formation. Identification and characterisation of the catalysing factors in the urine of healthy black subjects and the critical structural and compositional features in the protein isolated from such urines may have important clinical implications.

# CHAPTER 4.2

## CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM STONE-FORMERS: DIRECT AND SINGLE CROSSOVER EXPERIMENTS

### 4.2.1. OBJECTIVES

- Determine the CaOx msls, particle formation kinetics and particle volume-size distributions in individual, ultrafiltered urines from black and white CaOx stone-forming subjects

- Repeat the above experiments in the presence of THP isolated from the urines of stone formers from both race groups (SFTHP) in a crossover design involving SFTHP from each race group in its own parent ultrafiltered urine as well as in the ultrafiltered urine of the other race group (i.e. Figure 1: cells 11, 12, 15,16)

- Determine the CaOx crystallisation kinetics in the presence and absence of SFTHP from both race groups using $^{14}$C-oxalate in the same series of crossover experiments in individual, ultrafiltered urines from black and white stone-formers

## 4.2.2. METHODS

### *4.2.2.a. Effect of SFTHP on CaOx Crystallisation (Coulter Counter) and CaOx Crystal Deposition ($^{14}$C-oxalate)*

All experiments were performed as described in Chapter 4.1. However because of the rarity of stone formers among the black population, urines were not pooled but were treated individually. Aliquots (30ml) of urine from 5 individual WSFU's and 5 individual BSFU's were used for these experiments.

## 4.2.3. RESULTS

### *4.2.3.a. Effect of SFTHP on CaOx Crystallisation (Coulter Counter)*

The average msls for the different protein-urine combinations for stone-forming urines before and after the addition of SFTHP are given in Figure 8. It is noted that urines from white and black stone-formers have msls that are not significantly different (0.036(*0.002*) vs 0.034(*0.002*) repectively). Addition of SFTHP from each race group did not affect the msl significantly. However the msl values in WSFU tended to decrease when SFTHP was added, irrespective of it origin, while that of BSFU tended to increase.

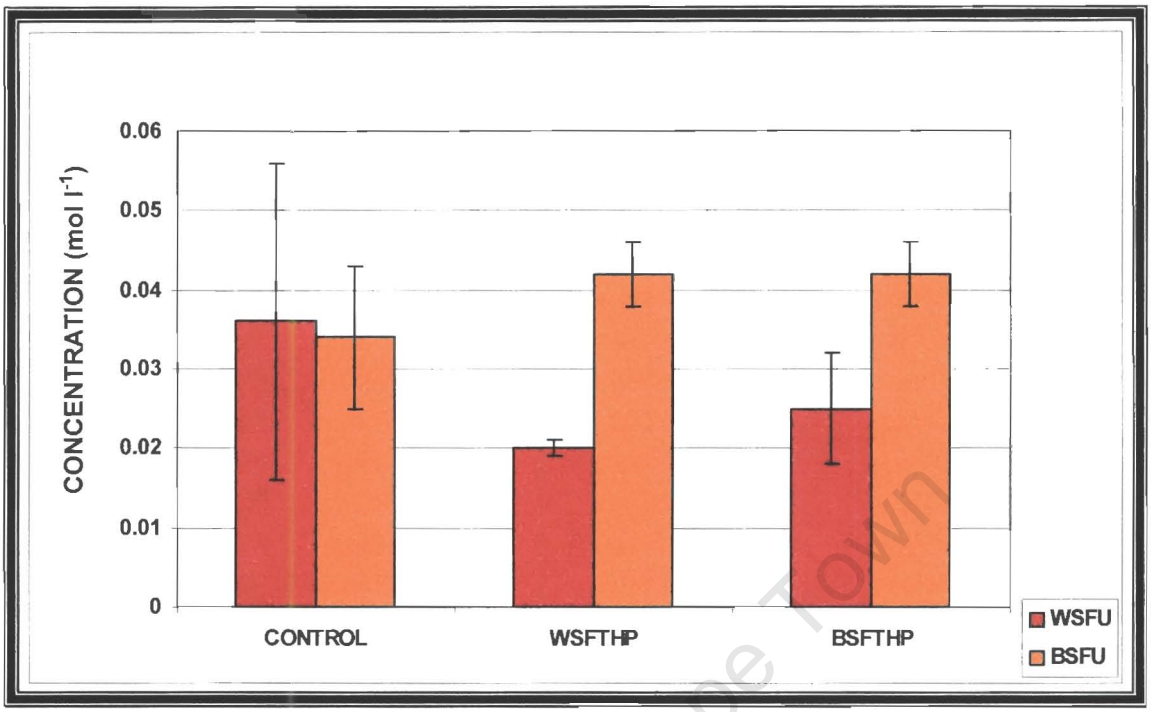



**Figure 8: Histograms representing the msl of WSFU and BSFU before and after the addition of SFTHPs. The error bars represent standard error.** (Data used to construct Figure 8 is available in appendix 4.1A)



Observed trends in particle formation rate, particle volume-size distributions are given in Table 2 while typical plots for each of these trends are given in Figures 9 and 10. Once again the term 'rate of particle formation' is used in the present context to represent crystals, THP particles and /or variously sized composites of both, due to the Coulter Counter's inability to distinguish between particles and crystals.

| URINE INDIVIDUAL | PARTICLE CHARACTER-ISTIC | NO OF URINES DISPLAYING TRENDS | IDENTIFIED TREND | FIGURES |
|---|---|---|---|---|
| WSFU (n = 5) | RATE OF PARTICLE FORMATION | 3 | WSFU = WSFTHP < BSFTHP | 9a |
| | PARTICLE SIZE | 3 | WSFU = WSFTHP = BSFTHP | 10a |
| | PARTICLE VOLUME | 3 | WSFU =WSFTHP < BSFTHP | 10a |
| BSFU (n=5) | RATE OF PARTICLE FORMATION | 3 | BSFU = WSFTHP = BSFTHP | 9b |
| | PARTICLE SIZE | 3 | BSFU = WSFTHP = BSFTHP | 10b |
| | PARTICLE VOLUME | 0 | NO DETECTABLE TRENDS | -- |



**Table 2: Observed trends in particle characteristics: SFU + SFTHP.** (Data used to construct Table 2 is available in appendix 4.2 B, C, D)

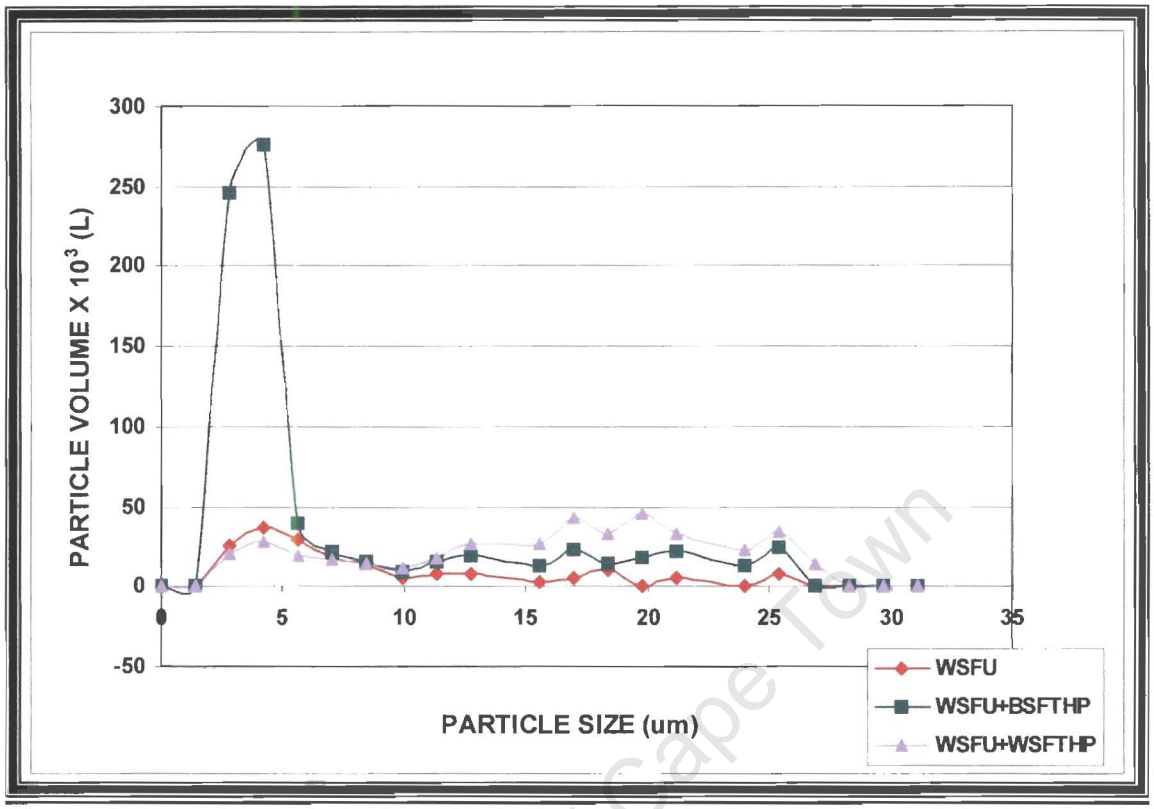



Figure 10a: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in WSFU before and after the addition of SFTHP.

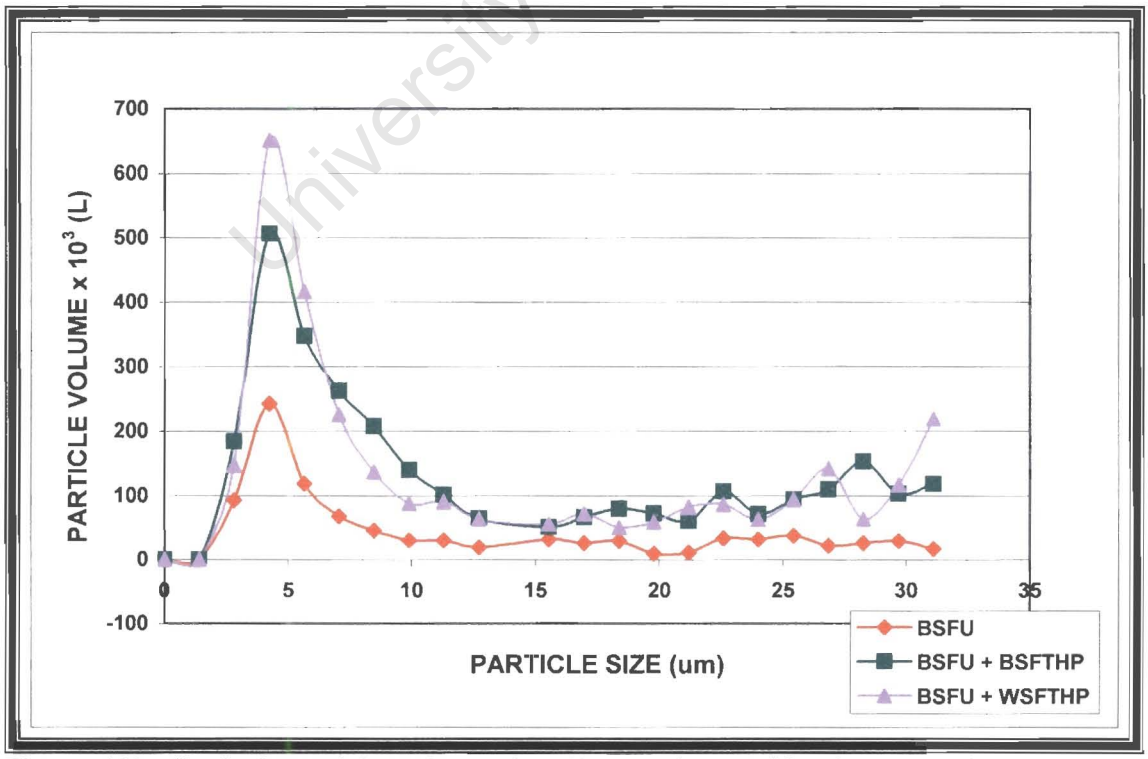



Figure 10b: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in BSFU before and after the addition of SFTHP.

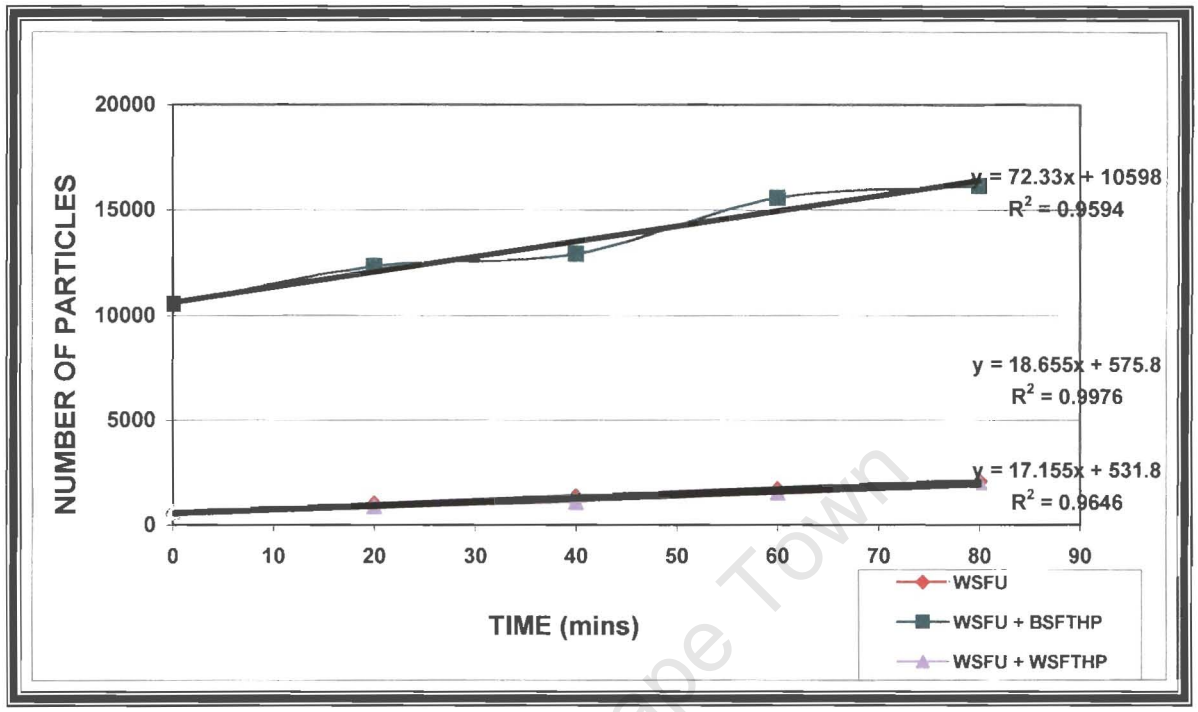



**Figure 9a: Plots of particle number vs time in WSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs.**

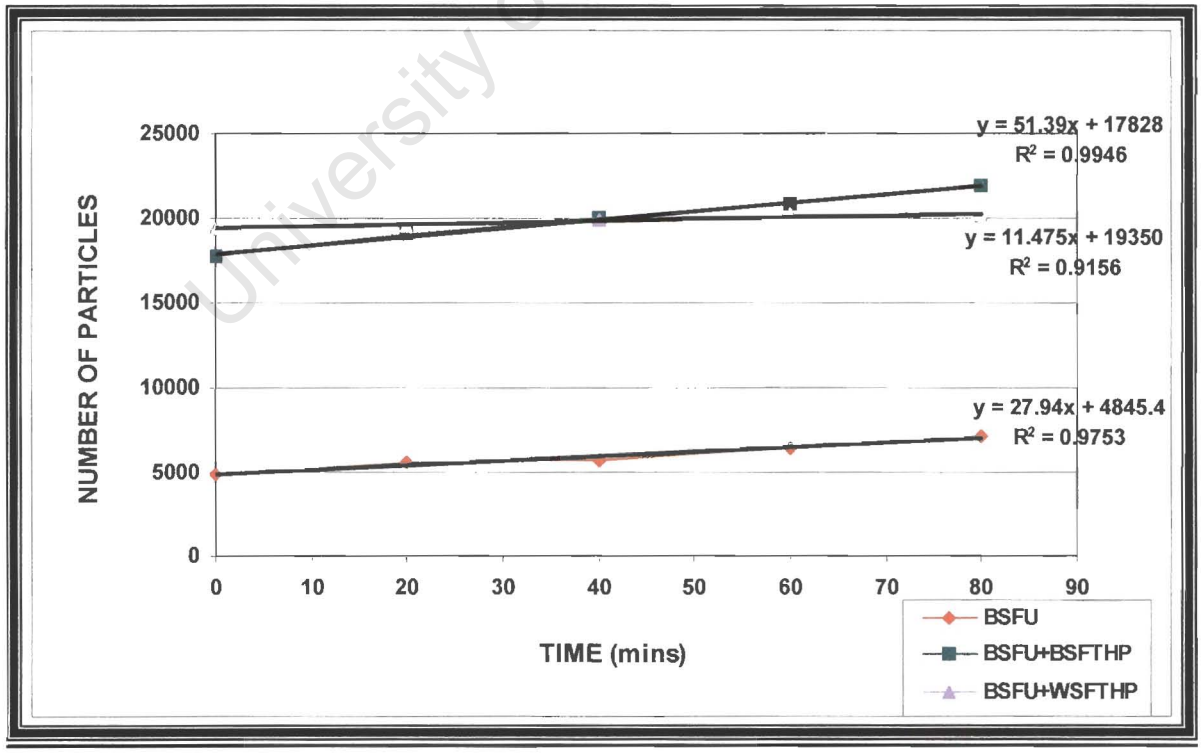



**Figure 9b: Plots of particle number vs time in BSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs.**

*4.2.3.b. Effect of SFTHP on CaOx Crystal Deposition ($^{14}$C-oxalate)*

Figure 11 (WSFU) and Figure 12 (BSFU) are plots of "unprecipitated CaOx" as a function of time. It is noted that in WSFU, the rate of precipitation in the presence of SFTHP from both race groups is minimal and is also far less than the rate of precipitation in the WSFU itself. However in BSFU (Figure 12) significant precipitation does occur in the presence of both proteins; in this case, a greater quantity of precipitate formed in the presence of WSFTHP.

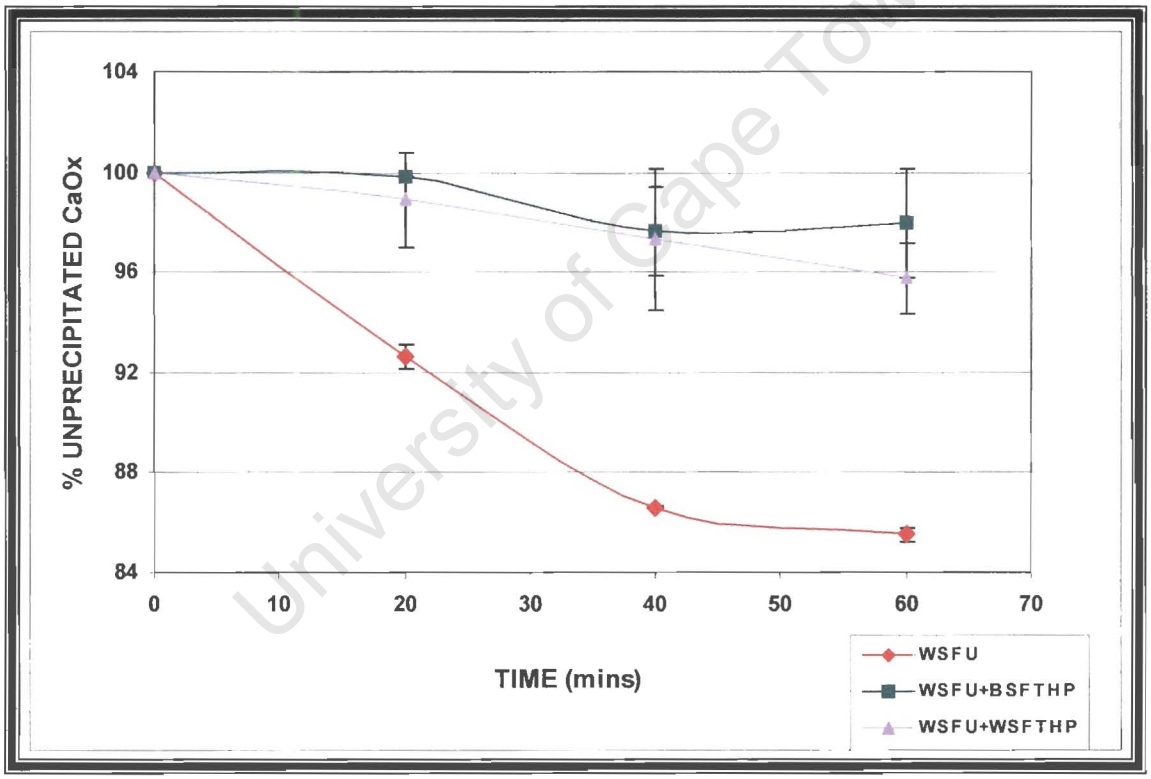





**Figure 11. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in WSFU.** (Data used to construct Figure 11 is available in appendix 4.2E.)

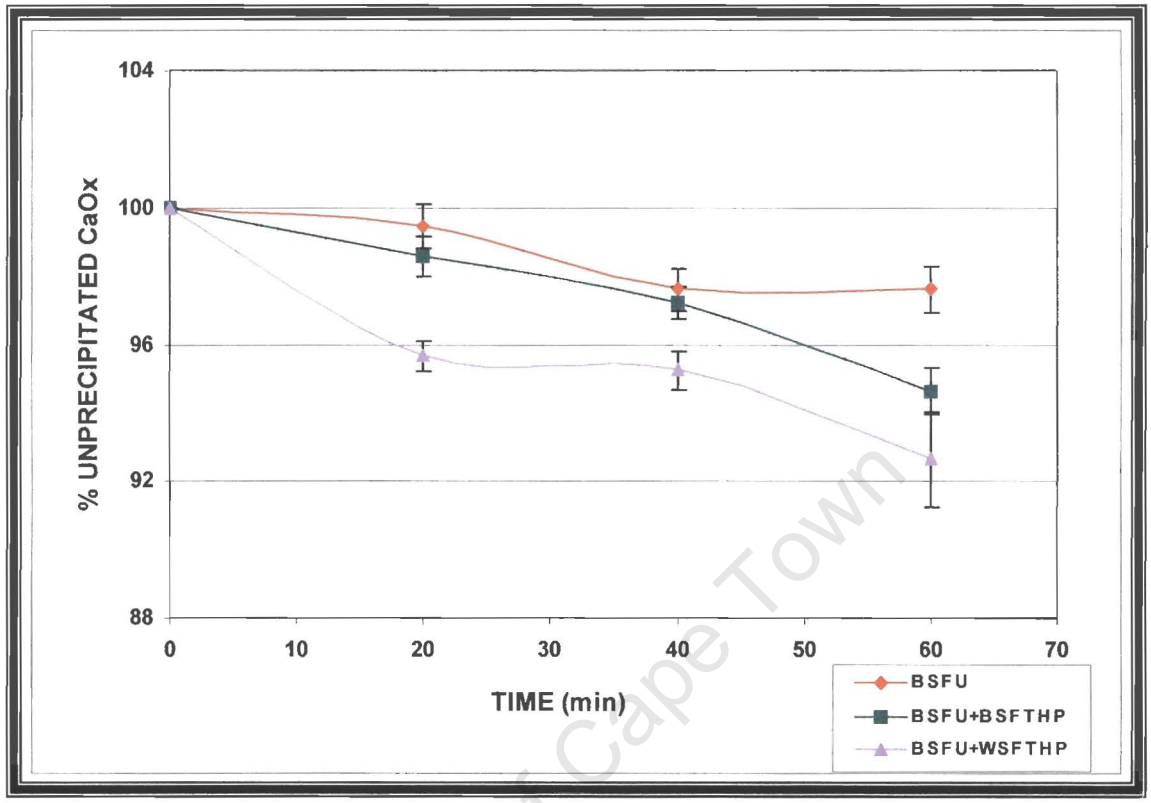



**Figure 12. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in BSFU.** (Data used to construct Figure 12 is available in appendix 4.2F.)



## 4.2.4. DISCUSSION

Since msls were unaffected by SFTHP, it can be concluded that this protein is not an inhibitor of crystal nucleation. Nevertheless, the observation of weak, opposing trends in WSFU and BSFU suggest synergistic interactions between urine composition and protein, which has been described previously (Craig et al, 2003). Moreover, the trend of increasing msls in BSFU indicates that the synergistic effects of the latter are more protective than those in WSFU. It should be noted that msls observed in the present study involving SFTHP and SF urine are lower than those reported in the previous section 4.1 in which NTHP and urine from healthy subjects were involved. This observation is of

some interest, as it appears not to have been reported in any previous study, which has only reported a difference in the msl of white healthy and stone-forming subjects.

The presence of SFTHP has no effect on the kinetics of particle formation, except that in WSFU the rate was increased in the presence of BSFTHP. Thus it can be argued (as in the previous chapter) that the nature of the urine composition influences the properties of the protein. The $^{14}C$ experiments confirm this argument. In WSFU, both proteins inhibited precipitation while in BSFU they promoted it. While particle sizes were unaffected by SFTHP in both urines, particle volumes increased in WSFU in the presence of BSFTHP. As explained in the previous chapter, the cause of the increase in particle volume can be deduced by examination of the $^{14}C$ data which shows that *less* calcium oxalate had in fact been deposited. This leads to the conclusion that the increase in particle volume arises from inter-crystal incorporations of more proteinaceous material rather than by deposition of more crystalline material and is in agreement with the results obtained for the WNU-BNTHP system described in the previous section. Comparison with the results obtained in the previous section is not entirely appropriate, as the urine-protein systems are completely different. However, the interactions between protein and urine appear to be fewer and weaker in the SFU-SFTHP systems.

In conclusion, it is believed that the present study has revealed several interesting findings. Firstly, it has demonstrated that urine composition plays a synergistic role in determining the behaviour of THP. Furthermore, consideration of the particle properties (Table 2) shows that the WSFU-BSFTHP system produced certain unique interactions that did not occur in the other urine-protein combinations.

# CHAPTER 4.3

## CRYSTALLISATION STUDIES OF THP FROM HEALTHY SUBJECTS IN URINE FROM STONE-FORMERS: SINGLE AND DOUBLE CROSSOVER EXPERIMENTS



### 4.3.1. OBJECTIVES

- Determine the CaOx msls, particle formation kinetics and particle volume-size distributions in individual, ultrafiltered urines from black and white CaOx stone-forming subjects (described in Chapter 4.2)

- Repeat the above experiments in the presence of THP isolated from the urines of healthy subjects from both race groups (NTHP) in a crossover design involving NTHP from each race group in individual, ultrafiltered urines from black and white stone forming subjects (i.e. Figure 1: cells 3, 4, 7, 8)

- Determine the CaOx crystallisation kinetics in the absence and presence of NTHP from both race groups using $^{14}$C-oxalate in the same series of crossover experiments as above in individual, ultrafiltered urines from black and white stone-forming subjects

## 4.3.2. METHODS

*4.3.2.a. Effect of NTHP on CaOx Crystallisation (Coulter Counter) and CaOx Crystal Deposition ($^{14}$C-oxalate) in stone-forming urine from both race groups.*

These experiments were performed as described in Chapter 4.1. However, as explained in Chapter 4.2, urines were not pooled but were treated individually (WSFU = 7; BSFU = 5)

## 4.3.3. RESULTS

*4.3.3.a. Effect of NTHP on CaOx Crystallisation (Coulter Counter) in stone-forming urine from both race groups*

Figure 13 represents histograms for the average msls of the different protein-urine combinations. The msl for SFU from each race group did not differ while the addition of NTHP, irrespective of its origin, had no effect.

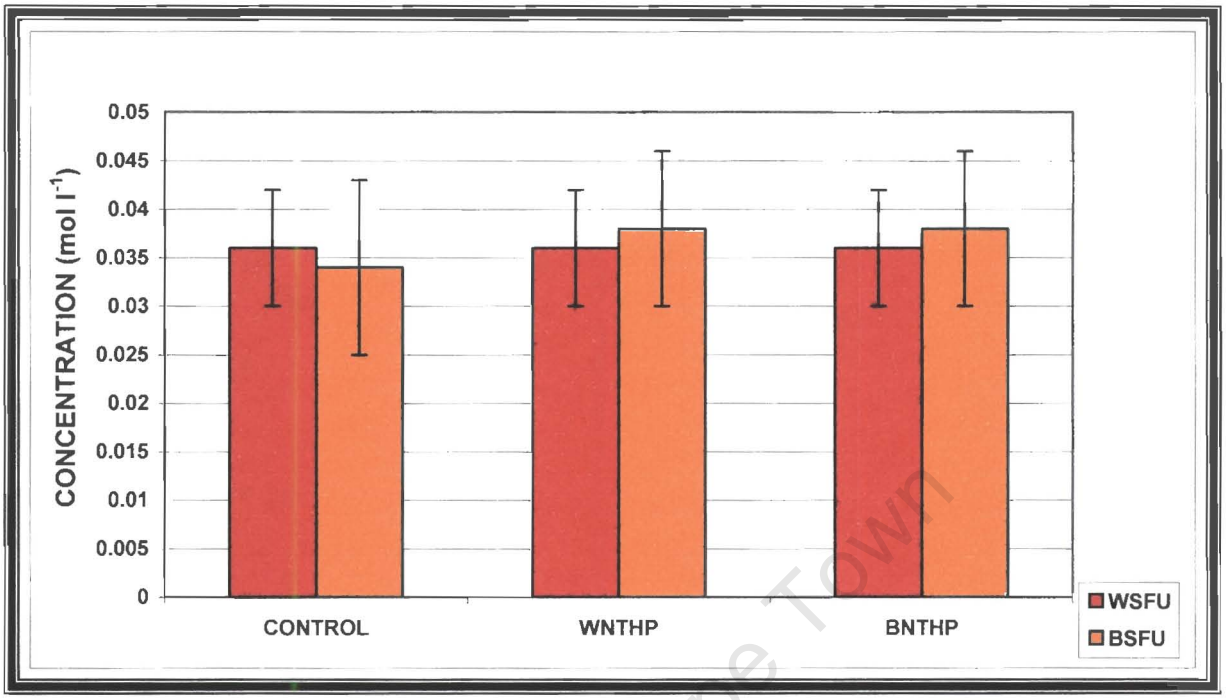



**Figure 13. Histograms representing the msl of WSFU and BSFU before and after the addition of NTHPs. The error bars represent standard error.** (Data used to construct Figure 13 is available in appendix 4.3A)



Observed trends in particle formation rate and particle volume-size distributions are given in Table 3 while typical plots for each of these trends are given in Figures 14 and 15. As explained in Chapter 4.1 the Coulter Counter is unable to distinguish between particles and crystals; as such, the former term is used in the present context to represent crystals, THP particles and /or variously sized composites of both.

| URINE INDIVIDUAL | PARTICLE CHARACTER-ISTIC | NO OF URINES DISPLAYING TRENDS | IDENTIFIED TREND | FIGURES |
|---|---|---|---|---|
| WSFU (n = 7) | RATE OF PARTICLE FORMATION | 4 | WSFU = WNTHP = BNTHP | 14a |
| | PARTICLE SIZE | 6 | WSFU = WNTHP = BNTHP | 15a |
| | PARTICLE VOLUME | 5 | WSFU < BNTHP < WNTHP | 15a |
| BSFU (n=5) | RATE OF PARTICLE FORMATION | 3 | BSFU = WNTHP < BNTHP | 14b |
| | PARTICLE SIZE | 2 | BSFU < WNTHP < BNTHP | 15b |
| | PARTICLE VOLUME | 3 | BSFU < WNTHP < BNTHP | 15b |

**Table 3: Observed trends in particle characteristics: SFU + NTHP.** (Data used to construct Table 3 is available in appendix 4.3 B, C, D)

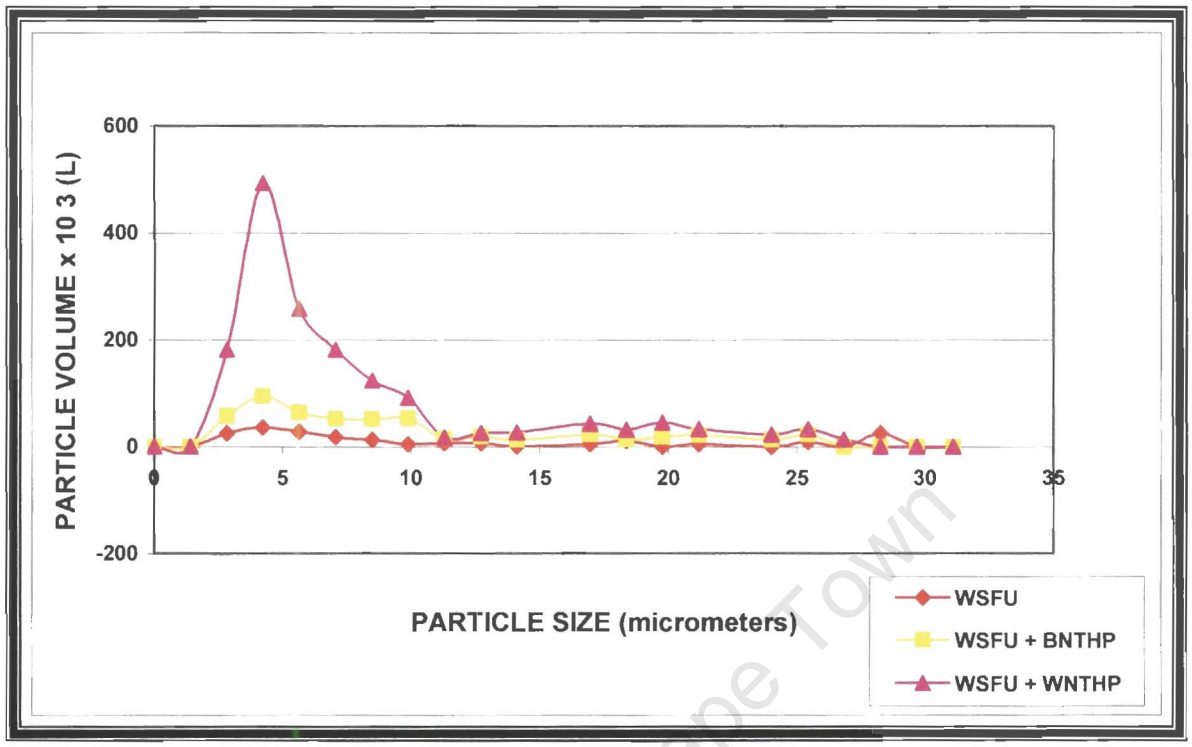



Figure 15a: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in WSFU before and after the addition of NTHP.

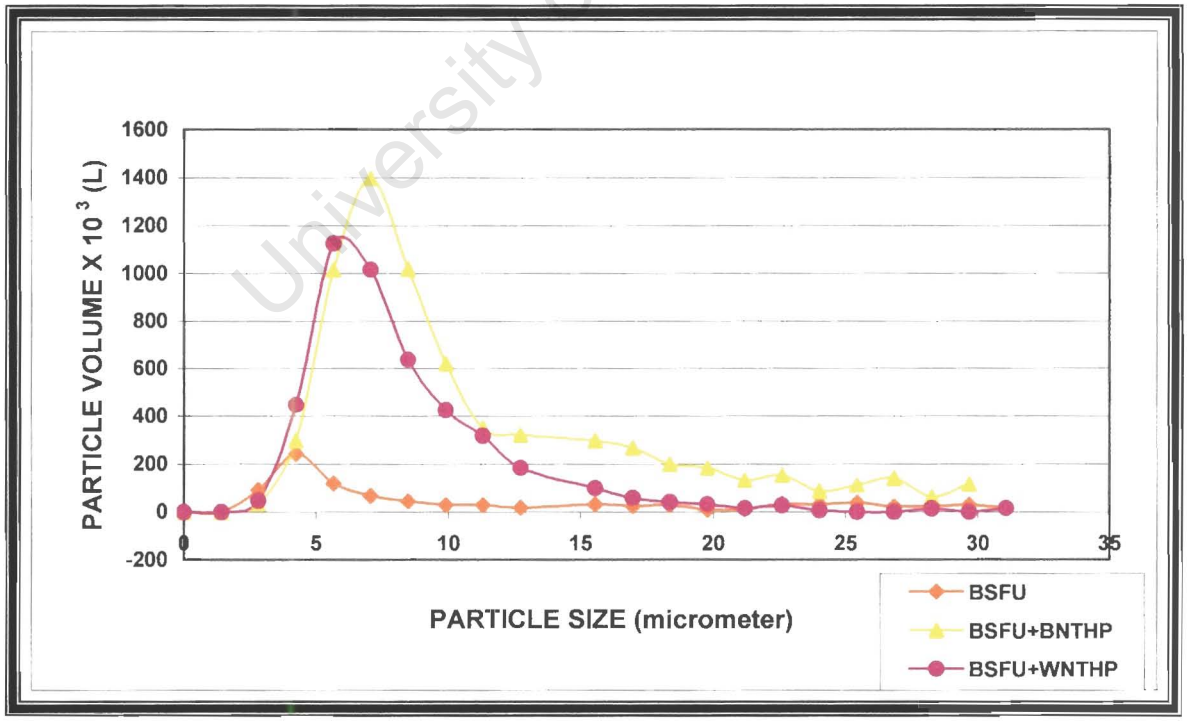



Figure 15b: Typical particle volume-size distribution (t=80 mins after dosing with NaOx) in BSFU before and after the addition of NTHP.

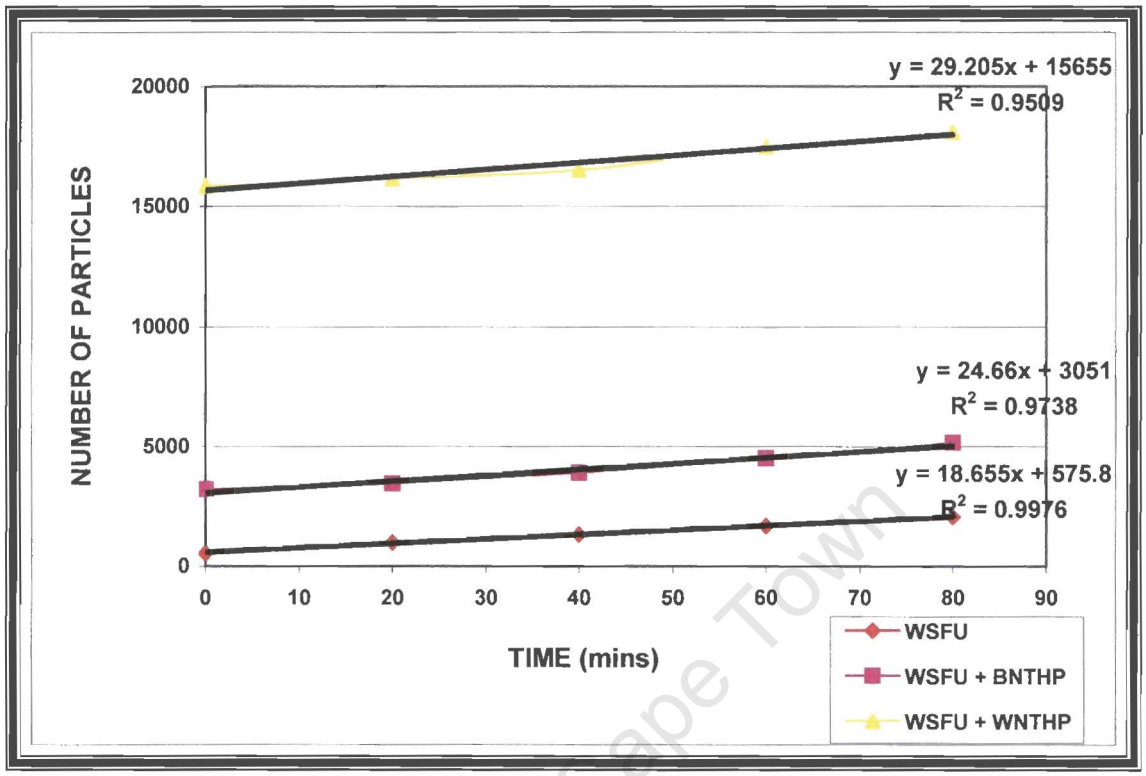



Figure 14a: Plots of particle number vs time in WSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs.

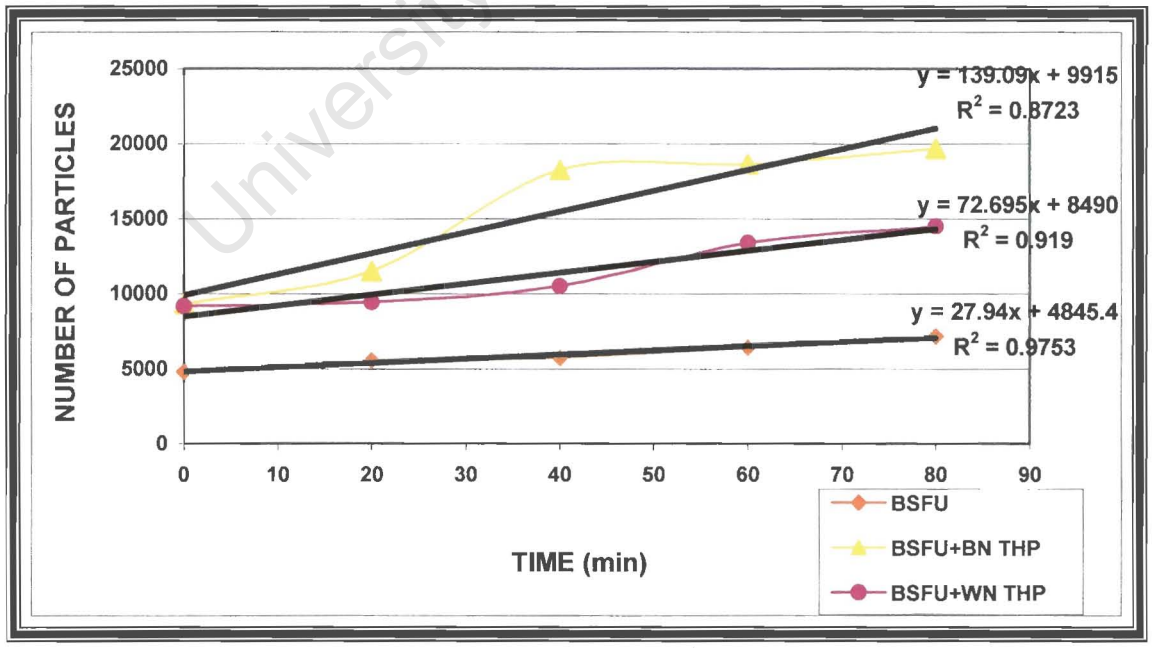



Figure 14b: Plots of particle number vs time in BSFU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs.

*4.3.3.b. Effect of NTHP on CaOx Crystal Deposition ($^{14}$C-oxalate)*

Figures 16 (WSFU) and 17 (BSFU) are plots of "unprecipitated CaOx" as a function of time. It is noted that in WSFU, the rate of precipitation in the presence of WNTHP (given by the gradient of the curve) is virtually non-existent while in the presence of BNTHP precipitation does occur but it is far less than the rate of precipitation in WSFU. Thus a significant difference is apparent in the rates of precipitation after the addition of NTHP, irrespective of its origin, relative to the WSFU. However, in BSFU (Figure 17), there is no difference in the rate of deposition before or after the addition of NTHP.



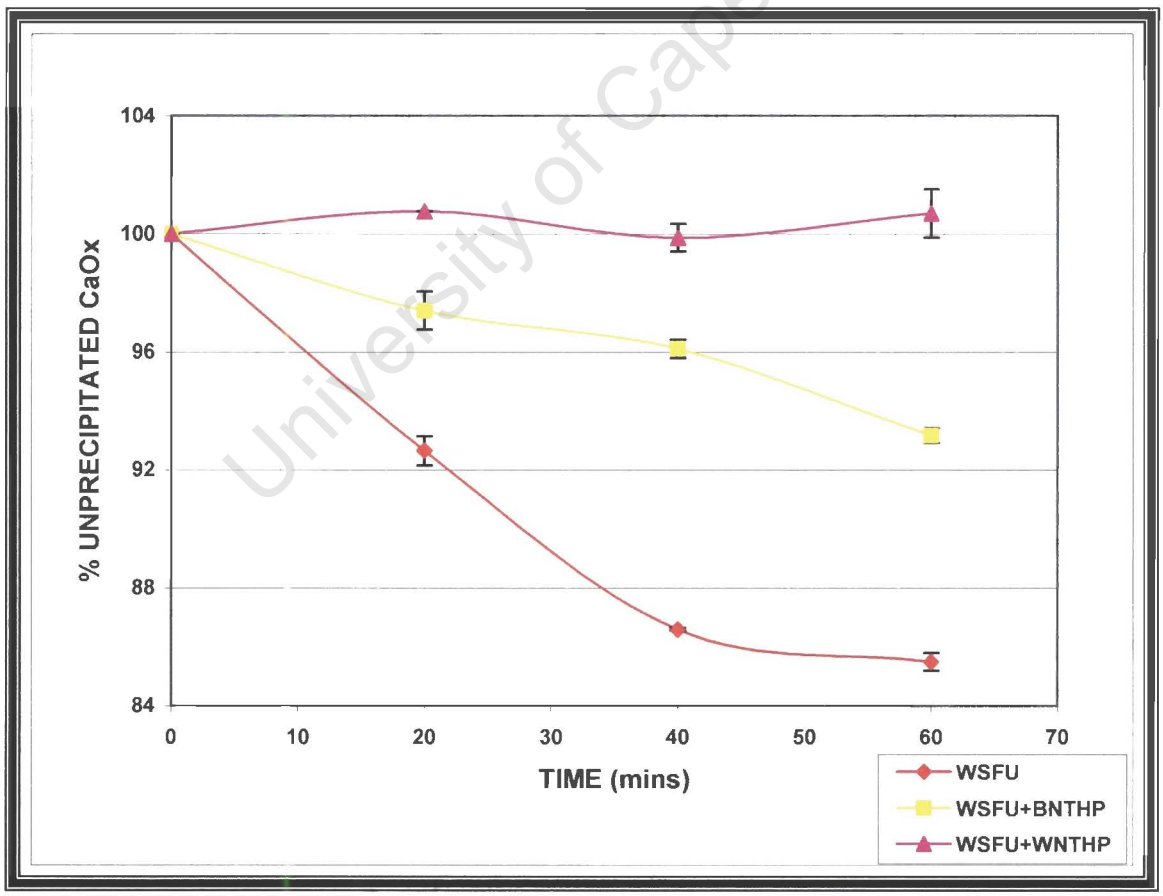



**Figure 16. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in WSFU.** (Data used to construct Figure 16 is available in appendix 4.3E.)

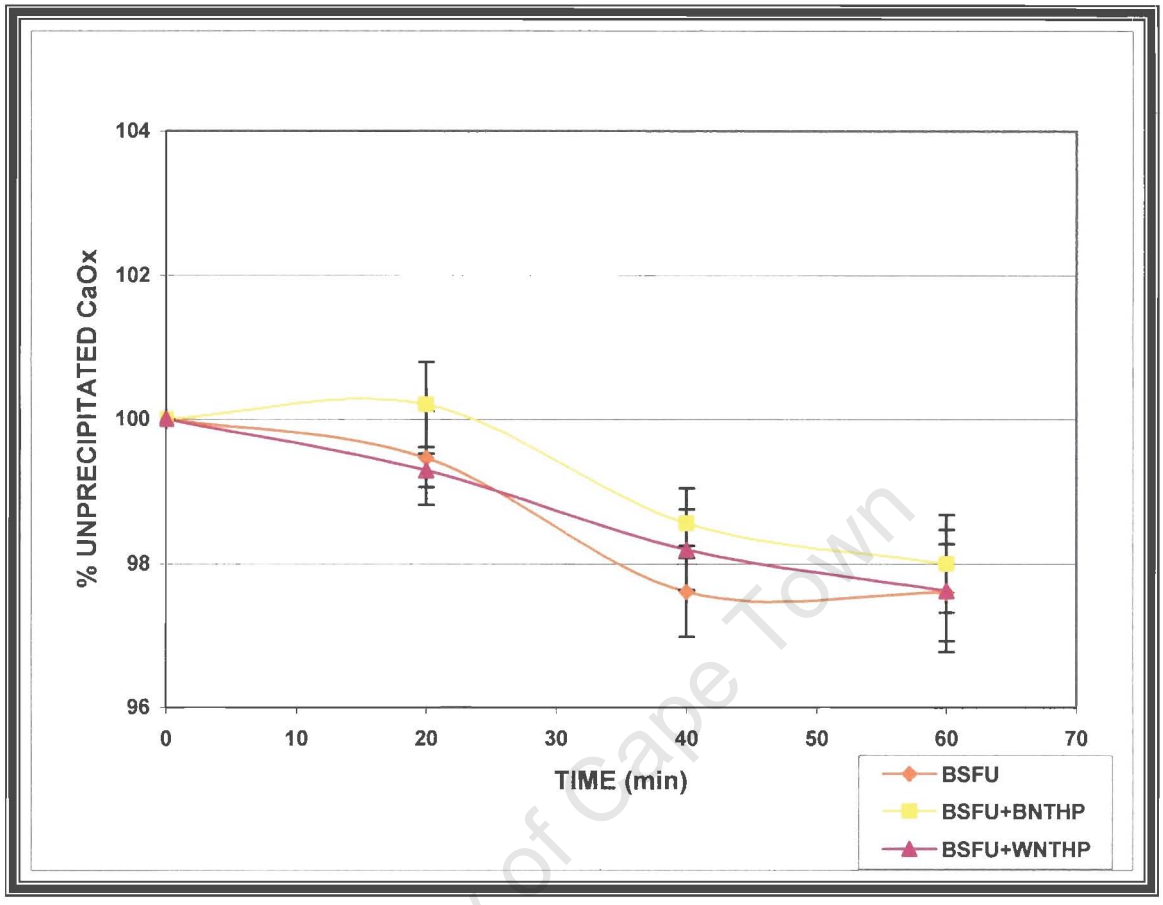



**Figure 17. Mean plots of crystallisation kinetic using $^{14}$C-oxalate in BSFU.** (Data used to construct Figure 17 is available in appendix 4.3 F.)



## 4.3.4. DISCUSSION

The observation that NTHP does not affect the msl is in agreement with the results reported in chapter 4.1. Since the present result was obtained in SFU while the previous result was obtained in healthy NU, it can be concluded that the protein obtained from healthy subjects plays no role in inducing CaOx crystallisation and that its activity in this context is not influenced by urine composition. However, comparison with the results obtained for SFTHP (Chapter 4.2) reveals that THP from stone formers is different to that obtained from healthy controls. In the previous study (Chapter 4.2), evidence was presented

of weak trends which suggested that the activity of SFTHP was indeed affected by urine composition and that msls tended to increase in the presence of BSFTHP and decrease in the presence of WSFTHP. It can thus be speculated that SFTHP might have a different structure to that of NTHP and / or might adopt a different conformation to that of NTHP under the influence of the different urine enviroments. Moreover, it can be further speculated that these structural and / or conformational differences in WSFTHP increase the risk of CaOx crystallisation while in BSFTHP the differences decrease the risk.

It is helpful to interpret the Coulter Counter data for the kinetics of *particle* formation in conjunction with the $^{14}C$ data for the kinetics of *CaOx crystal* formation. In WSFU, particle formation (CaOx crystals and / or proteins and / or both) occurred at a rate which was not affected by the presence of NTHP from either race group. However, the $^{14}C$ experiments show that formation of *calcium oxalate crystals* occurred to a significant extent in the control WSFU but that the respective proteins significantly decreased it. In fact, there was zero precipitation of CaOx in the presence of WNTHP indicating that WNTHP is a potential inhibitor in WSFU and that the particle formation detected by the Coulter Counter (in the presence of WNTHP) corresponds to non CaOx-containing material (probably WNTHP itself).

In the presence of BNTHP, CaOx deposition *did* occur but to a far lesser extent than in the control WSFU, indicating that it too is an inhibitor of CaOx formation. Thus, the particle formation detected by the Coulter Counter (in the presence of BNTHP) corresponds to both CaOx crystals and protein material. In the control WSFU, the particle formation detected by the Coulter Counter corresponds to CaOx crystal formation. Since particle formation was equal in the control and

protein-dosed urines, it seems that there is an inverse relationship between protein precipitation and CaOx formation. Moreover, although particle sizes were unaffected by the addition of protein, the trend dislayed by particle volumes (viz control WSFU< BNTHP <WNTHP) is inversely related to the amount of CaOx deposited. Thus, particle volume increased as CaOx deposition decreased, i.e. particle volume increased as the amount of protein increased.

Thus, these results show that NTHP from both ace groups are inhibitors of CaOx formation in WSFU, but that WNTHP is superior in this regard. The results also show that NTHP is insoluble in WSFU to varying extents but that despite this insolubility, it is still able to exert a substantial inhibitory effect. Finally, the relationship between the properties of NTHP and its urinary enviroment is again evident.

In BSFU, particle formation was accelerated in the presence of BNTHP (Table3, Figure 14b) but formation of CaOx crystal was unaffected (Figure 17). The faster rate of particle formation is attributed to a greater level of insolubility of BNTHP in BSFU (similar to the greater insolubility of WNTHP in WSFU described above). Thus, NTHP from a particular race group appears to be less soluble in SFU from its own race group than in that of the other. The trends in particle size and particle volume support this observation. However, of greater interest is that NTHP from either race group does not display any inhibitory activity in BSFU. This differs from the results in WSFU described above and again highlights the dependence of THP inhibitory properties on urine composition.

In conclusion, this study seems to support the results previously reported in sections 4.1 and 4.2 and is in keeping with those reported in literature (Hess, 1992) that the urinary milieu plays a synergistic role in determining the behaviour of THP and that the properties or structure of THP extracted from SFU has some form of abnormality. This aspect has been explored in Chapter 3.

# CHAPTER 4.4

# CRYSTALLISATION STUDIES OF THP FROM CALCIUM OXALATE STONE-FORMERS IN URINE FROM HEALTHY SUBJECTS: SINGLE AND DOUBLE CROSSOVER EXPERIMENTS

## 4.4.1. OBJECTIVES

- Determine the CaOx msls, particle formation kinetics and particle volume-size distributions in pooled, ultrafiltered urines from black and white healthy subjects (controls) as described and previously performed in Chapter 4.1

- Repeat the above experiments in the presence of SFTHP isolated from the urines of stone formers from both race groups (SFTHP) in a crossover design involving SFTHP from each race group in pooled, ultrafiltered urines from black and white normal subjects (i.e. Figure.1: cells 9, 10, 13, 14)

- Determine the CaOx crystallisation kinetics in the presence and absence of SFTHP from both race groups using $^{14}$C-oxalate in the same series of crossover experiments as above in pooled, ultrafiltered urines from black and white healthy subjects

## 4.4.2. METHODS

*4.4.2.a. Effect of SFTHP on CaOx Crystallisation (Coulter Counter) and CaOx Crystal Deposition ($^{14}$C-oxalate) in healthy urine from both race groups.*

These experiments were performed as described in Chapter 4.1. However, since the amount of WSFTHP and BSFTHP had been depleted by the experiments described in Chapters 4.1, 4.2, 4.3, and due to the rarity of BSFs, the number of urines in which experiments could be conducted was restricted to n=1 for each race group. Metastable limits were not determined.

## 4.4.3. RESULTS

*4.4.3.a. Effect of SFTHP on particle characteristics (Coulter Counter) in healthy urine from both race groups*

Particle formation rates and particle volume-size distributions are given in table 4 while plots for these are given in Figures 18 and 19 respectively.

| URINE INDIVIDUAL | PARTICLE CHARACTERISTIC | OBSERVED TREND | FIGURES |
|---|---|---|---|
| WNU (n = 1) | RATE OF PARTICLE FORMATION | BSFTHP = WSFTHP = WNU | 18a |
| | PARTICLE SIZE | WSFTHP = WCU = BSFTHP | 19a |
| | PARTICLE VOLUME | WSFTHP = WNU = BSFTHP | 19a |
| BNU ( n=1) | RATE OF PARTICLE FORMATION | BSFTHP < BNU < WSFTHP | 18b |
| | PARTICLE SIZE | WSFTHP = BNU < BSFTHP | 19b |
| | PARTICLE VOLUME | WSFTHP < BSFTHP < BNU | 19b |

**Table 4: Observed trends in particle characteristics: NU + SFTHP.** (Data used to construct Table 4 is available in appendix 4.4 A, B, C)

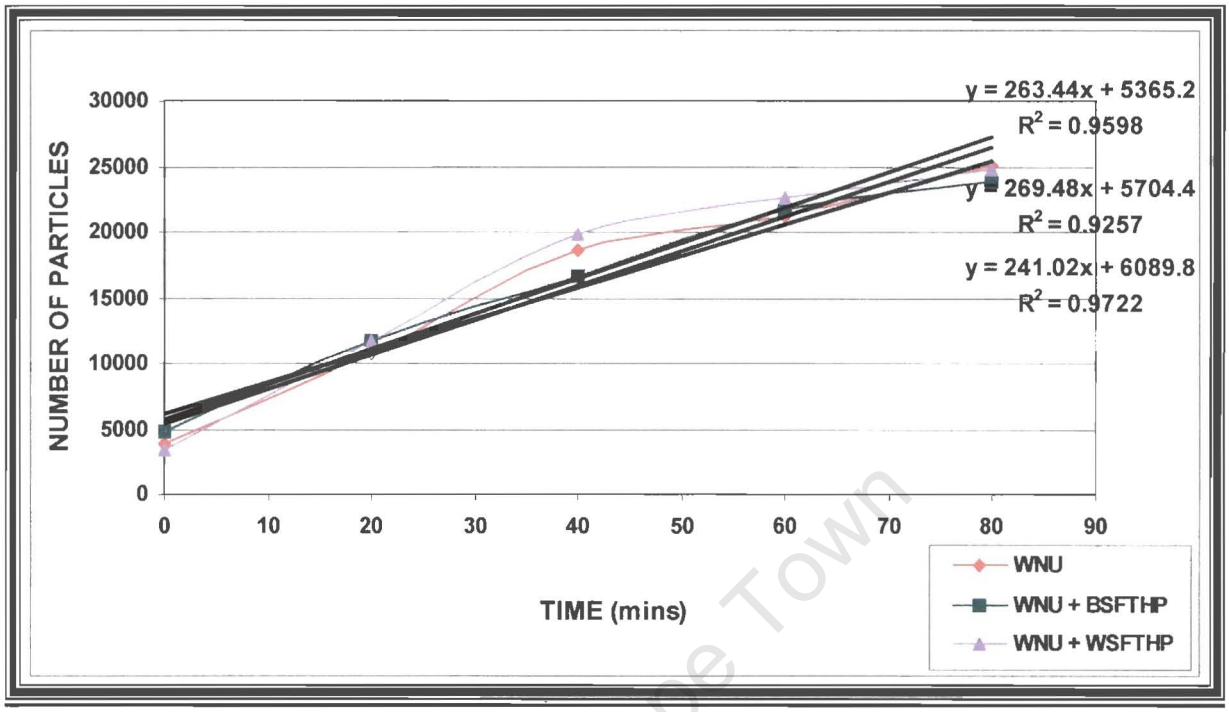



Figure 18a. Plots of particle number vs time in WNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs

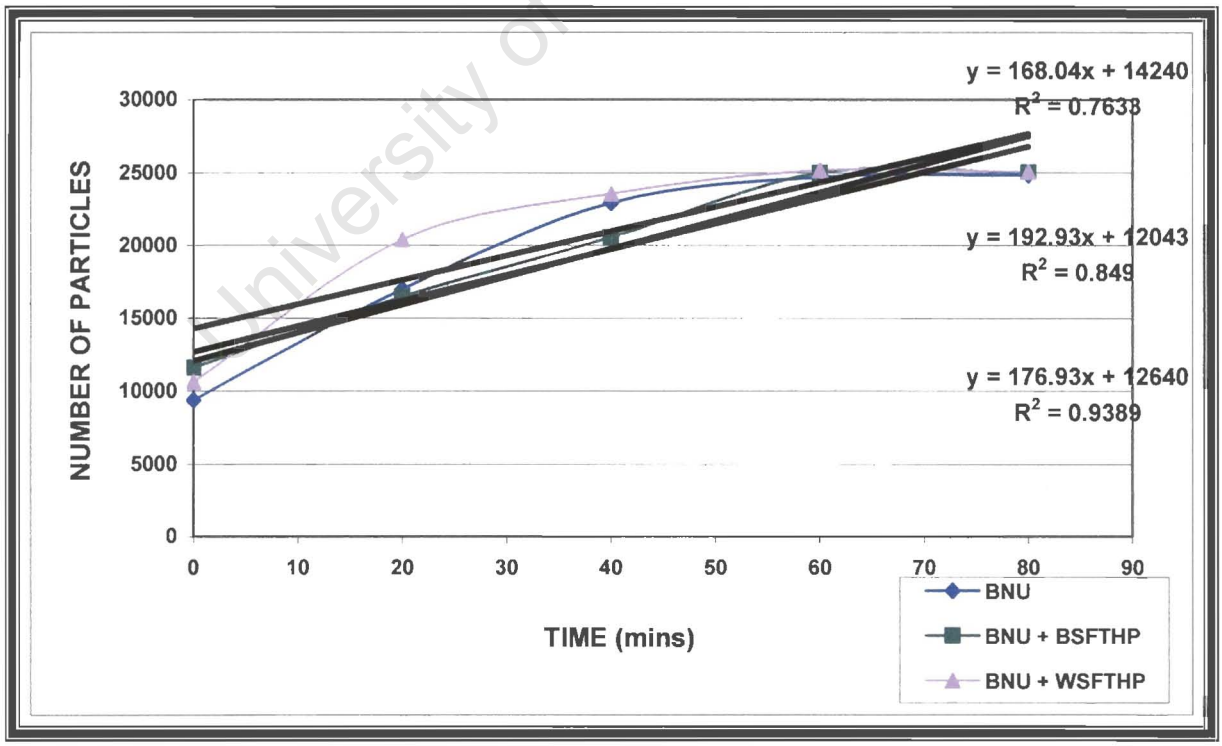



Figure 18b. Plots of particle number vs time in BNU after addition of NaOx. The rate of particle formation is given by the gradient of the graphs

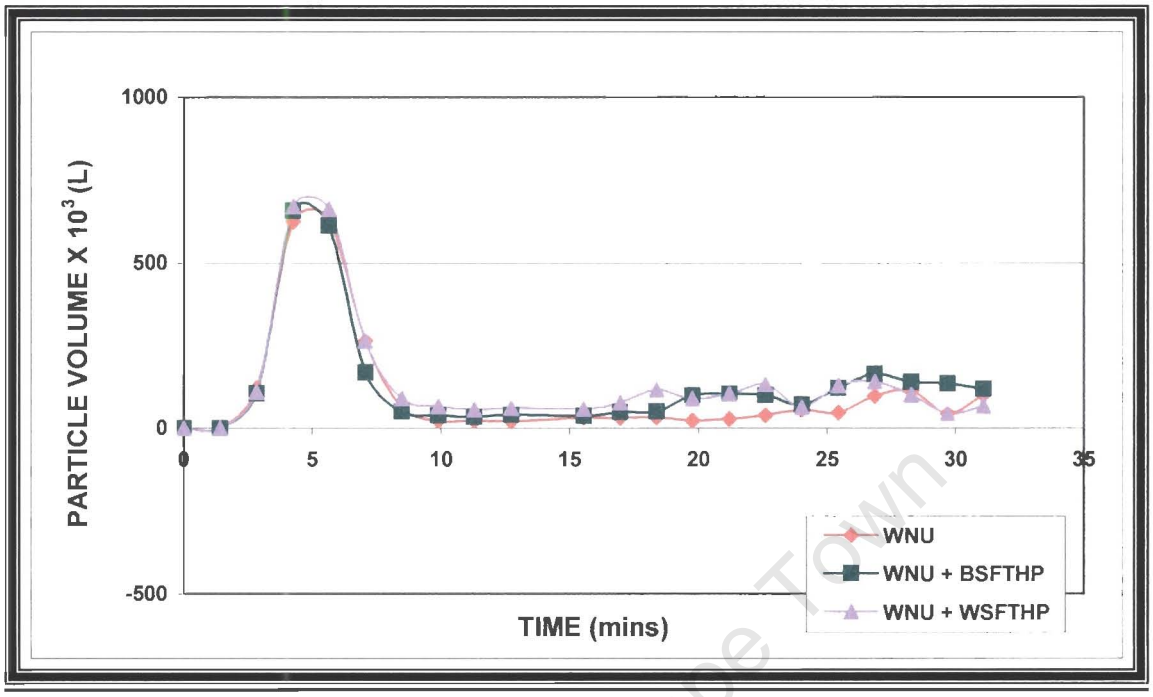



Figure 19a: Particle volume-size distribution (t=80 mins after dosing with NaOx) in WNU before and after the addition of SFTHP.

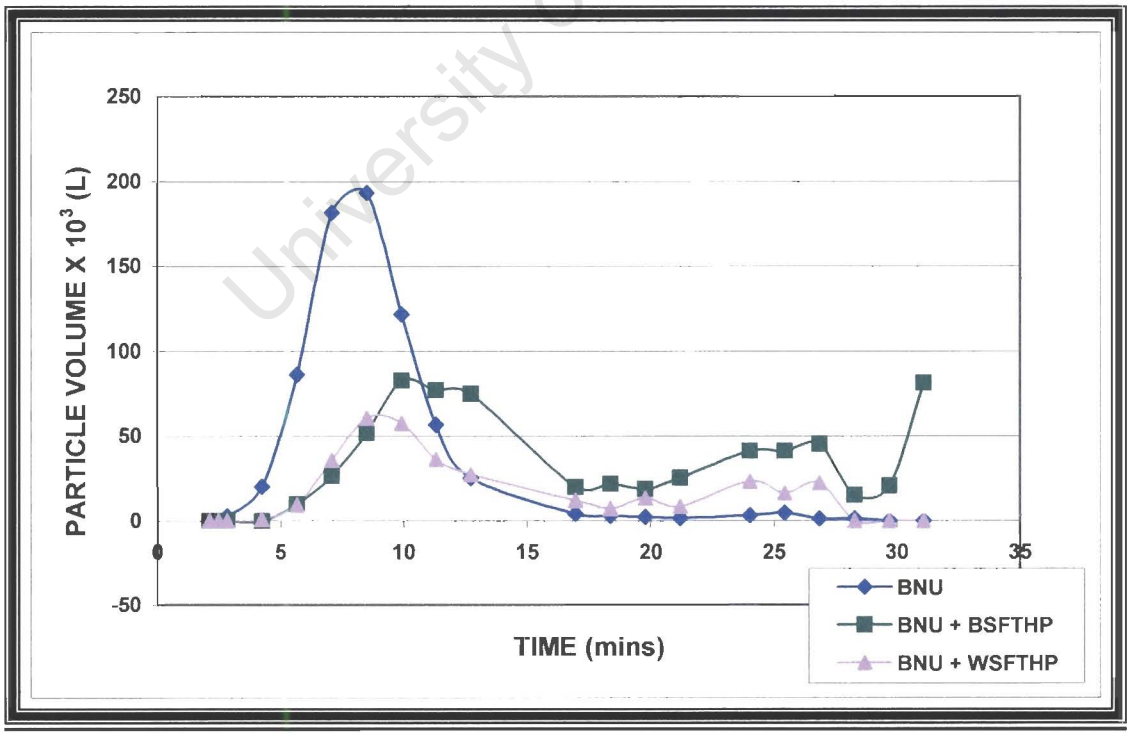



Figure 19b: Particle volume-size distribution (t=80 mins after dosing with NaOx) in BNU before and after the addition of SFTHP.

### *4.4.3.b. Effect of SFTHP on CaOx Crystal Deposition ($^{14}$C-oxalate)*

Figure.20 (WNU) and 21 (BNU) are plots of "unprecipitated CaOx" as a function of time. It is noted that in WNU, the rate of precipitation in the presence of BSFTHP is virtually non-existent (appears to be significantly different) as opposed to the WNU and the WSFTHP, where the rate appears to be the same (Figure.20). However in BNU (Figure.21), there is no difference in the rate of deposition before or after the addition of SFTHP.

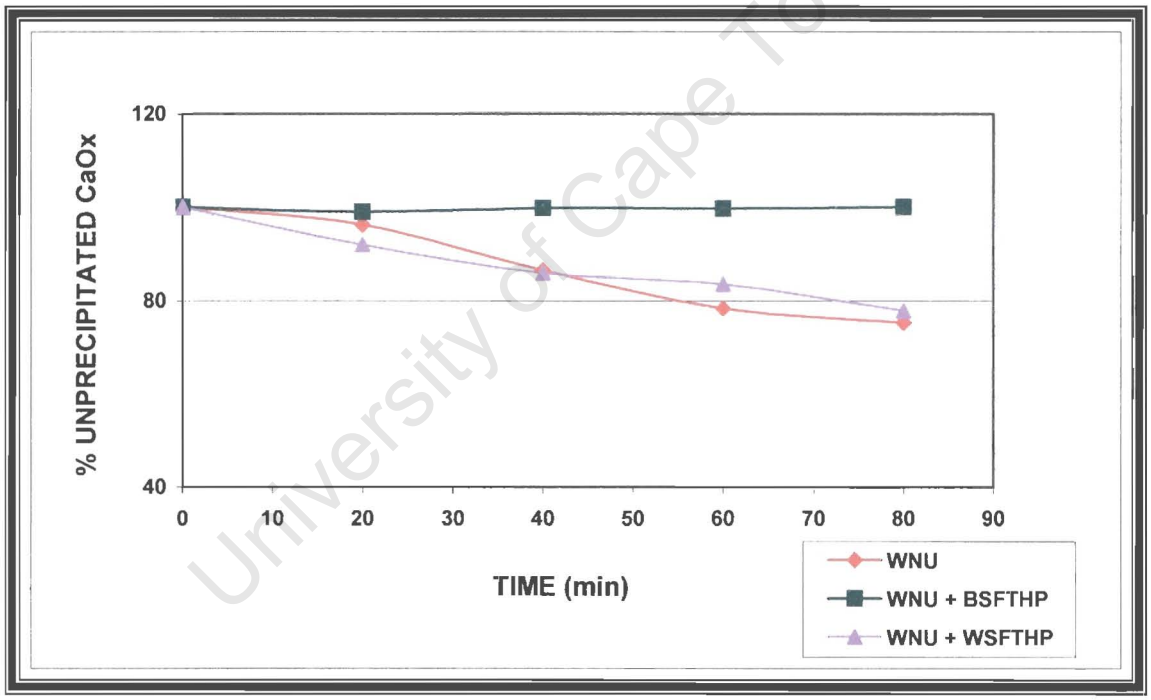





**Figure 20. Crystallisation kinetic plots using $^{14}$C-oxalate in WCU.** (Data used to construct Figure 20 is available in appendix 4.4 D)

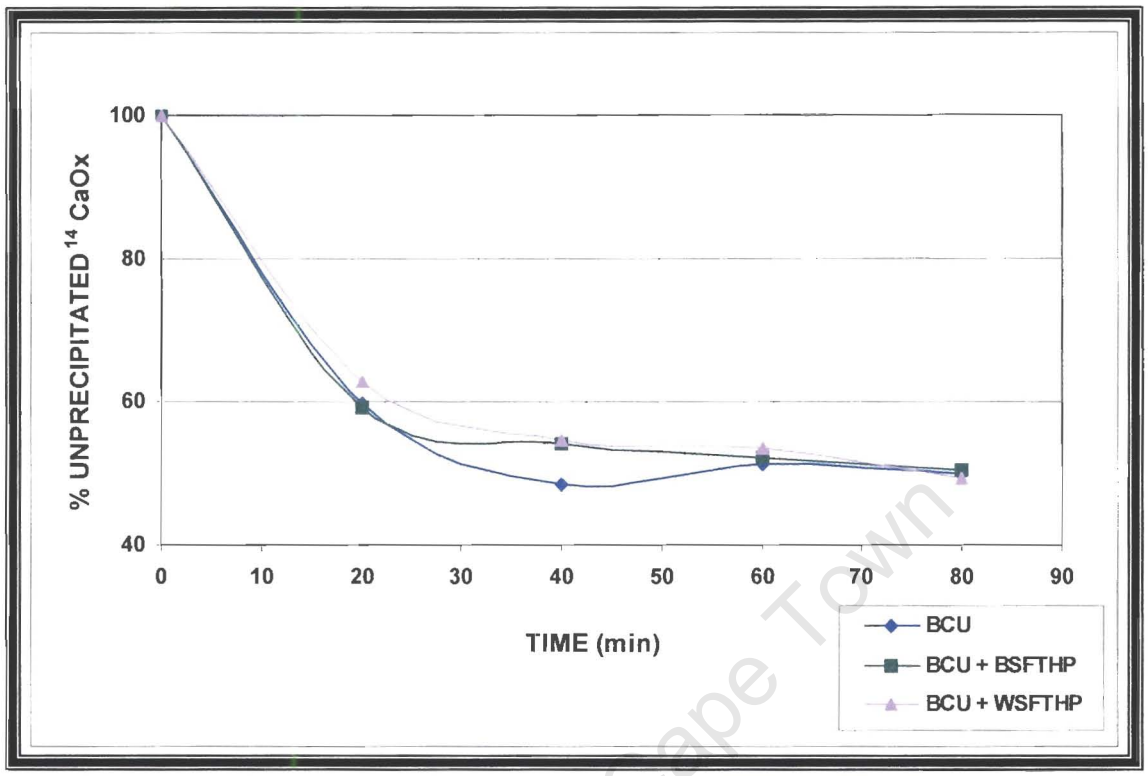



**Figure 21. Crystallisation kinetic plots using $^{14}$C-oxalate in BCU.** (Data used to construct Figure 21 is available in appendix 4.4 E)



## 4.4.4. DISCUSSION

As was the case in the earlier sections of this chapter, it is necessary to interpret the Coulter Counter data for *particle* formation with the $^{14}$C data for *CaOx* formation. In WNU the rate of formation of CaOx in the presence of WSFTHP was the same as in the control (in agreement with the Coulter data) but in the presence of BSFTHP it was virtually zero. This suggests that the "particle" formation observed by the Coulter Counter for BSFTHP was probably proteinaceous material indicative of its low solubility in this particular urine. However, more importantly, the $^{14}$C data shows that BSFTHP is a potent inhibitor of CaOx formation in WNU. Since particle sizes and volumes were not affected by either protein (Table 4, Figure 18a), it seems likely that the

crystallisation mechanism under inhibition here is that of nucleation. Of interest is the observation that the protein, despite its insolubility, is able to exert a strong inhibitory effect. A similar inverse relationship between solubility and inhibitory potential was reported in chapter 4.3. It thus seems that in certain circumstances (pertaining to urinary enviroment), THP is able to perform as an inhibitor of nucleation in the solid state. Although it inhibits aggregation by adsorption onto CaOx crystal surfaces (Hess et al, 1989; Hess, 1994), its effect in the solid phase on nucleation has not been previously reported.

In BNU, formation of CaOx was unaffected by either protein (Figure 21) suggesting that the *particle* formation differences observed by the Coulter Counter (Table 4, Figure 18b) are due to differences in the relative solubilities of WSFTHP and BSFTHP in this urine enviroment. Differences in particle sizes and particle volumes (Table 4, Figure 19b) can also be attributed to solubility differences.

In conclusion, the study described in this section appears to indicate that the urinary milieu and its enviroment as well as the abnormal structure of the SFTHP play a role in determining the properties of THP and its effects on stone formation

# CHAPTER 4.5

## SEDIMENTATION AND ZETA-POTENTIAL STUDIES ON CALCIUM OXALATE (CaOx) CRYSTAL SLURRIES IN THE ABSENCE AND PRESENCE OF NTHP AND SFTHP

### 4.5.1. INTRODUCTION

The adsorption of THP onto calcium oxalate monohydrate crystal (COM) surfaces may cause an inhibitory effect on crystal aggregation (Hess et al, 1989; Hess, 1994). The interaction between two COM crystals coated with THP may depend on the molecular structure of THP and / or its enviroment of the medium used (Boeve et al, 1994; Hess et al, 1989; Hess, 1994; Scurr et al, 1986**(a), (b)**). This interaction occurs as a result of different forces. It has been suggested that Van der Waals forces, shear forces as well as electrostatic repulsion or attraction forces, control aggregation (Finlayson et al, 1978; Marangelle et al, 1989: Sophasan et al, 1980; Hess, 1992; Kok et al, 1993). Aggregation may occur between two different types of materials and is referred to as heterocoagulation (Burns et al, 1980). These two materials will have different surface charges and will be attracted by electrostatic and / or van der Waals attractive forces. Secondary growth and nucleation occurs when a COM particle adheres to the walls of the renal tubules. Firstly, nucleation takes place and then possibly growth due to aggregation of other crystals. Disaggregation occurs when particles have the same charge and the force occuring is repulsion. However shear forces have a dual role.

Firstly, during the stirring of the crystal slurries or the flow of the solvent, this may lead to disaggregation. Secondly, shear forces could lead to increased particle collisions and therefore promote aggregation.

According to the DLVO (Derjaguin, Landau, Verwey, Overbeek) theory (Israelachvili et al, 1992), the sum of the van der Waals forces and electrostatic forces is equal to the total interaction energy between two particles. Figure 22 represents a schematic representation of the relation between energy and distance according to the DLVO theory. The electrolytic concentration and the surface potential determine the behaviour of the two particles and the interaction energy.

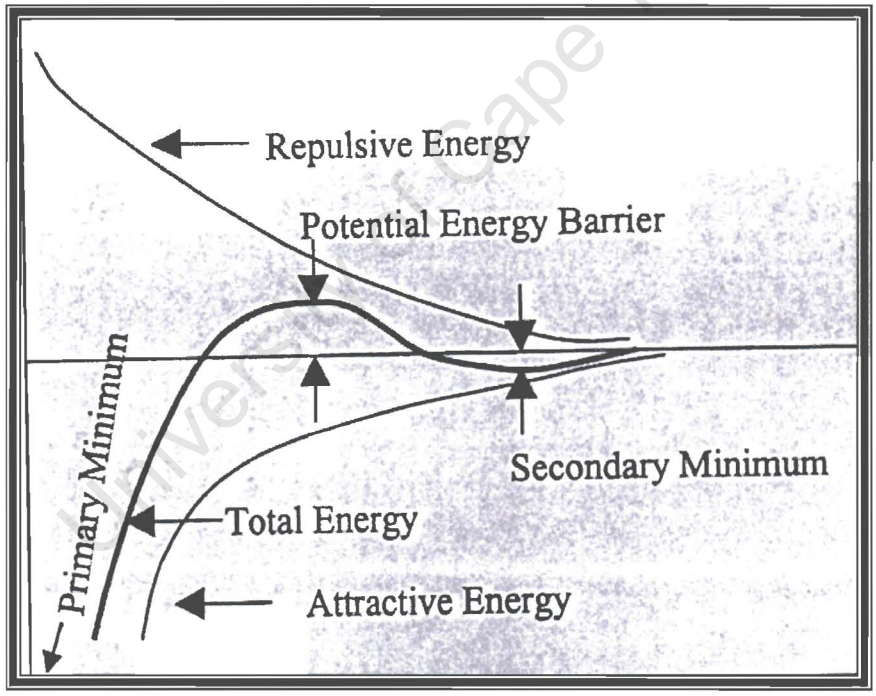





**Figure 22. Schematic representation of the relationship between energy and distance according to the DLVO theory (Hunter, 1986)**

When the surface energy approaches zero, the van der Waals forces dominate and aggregates are formed. When ionic strength is increased then van der Waals forces also dominate (Hunter, 1986).

Urinary macromolecular inhibitors, which adsorbs onto the COM crystal surfaces may have a steric protective effect (steric hiderance) to prevent crystal aggregation (Boeve et al. 1994). The steric effect may be determined by the molecular structure of a substance bound on COM crystals. Thus when a macromolecular substance (such as THP) is transferred from solution to a crystal surface, the DLVO repulsion force and steric hinderances are the dual effects which are expected to influence COM crystallisation (Cao et al. 1996). The most convient way to measure this surface potential is with a zeta potential meter. Zeta-potential (Zp) is the electrical potential at the surface between colloidal particle (solid phase) and it's surrounding solutions (continuous phase) (Boeve et al, 1994). It has been used to study the stability of urinary colloidal systems and the mechanism of crystal agglomeration in the presence of THP (Boeve et al. 1994; Hess et al. 1989; Scurr et al, 1986 **(a)**, **(b)**). A shift to more negative Zp has been interpreted as being indicative of an increase in inhibition of crystal aggregation – the more negative the Zp, the greater the inhibition (Boeve et al. 1994; Hess et al. 1989; Scurr et al. 1986**(a)**, **(b)**).

Clearly, in the context of the present project, measurement of the affects of the THP from the four groups on ZP's in CaOx crystal slurries was warranted. In addition, determining the effects of the THPs on inhibition of CaOx crystal aggregation itself was also justified.

## 4.5.2. OBJECTIVES

- ❖ Determine the zeta-potentials of CaOx crystal slurries in the absence and presence of NTHP and SFTHP from both race groups.

- ❖ Determine the aggregation inhibition from sedimentation rates of CaOx crystal slurries in the absence and presence of NTHP and SFTHP from both race groups

## 4.5.3. METHODS

### *4.5.3.a. Preparation of Calcium Oxalate Crystal Slurries*

Slurries were prepared according to the method of Pak et al, 1975. 10mM solutions of calcium chloride and sodium oxalate were slowly mixed in equimolar amounts at room temperature by simultaneous introduction of the two solutions at a constant rate of 1 ml $min^{-1}$. The precipitate, which formed, was kept in suspension by means of a magnetic stirrer for 7 days at 6°C. The precipitate was then separated by centrifugation, washed in distilled water and methanol and dried at 95°C for one hour. The dry preparation was stored at room temperature before use. It was identified as pure calcium oxalate monohydrate by X-ray powder diffraction (Philips, PW 1752/00, Holland). 0.8 mg $ml^{-1}$ dry crystals were then added to a 10mM tris hydroxymethyl-aminomethane-HCL buffer, pH 7.2 containing 90 mM NaCl.

### *4.5.3.b. Effects of NTHP and SFTHP on Zeta potentials of CaOx Crystal Slurries*

In this study, COM crystal slurries (as previously prepared but with a different buffer viz 0.3 mg $ml^{-1}$ in 10mM sodium acetate buffer, pH 5.70) were equilibrated overnight under constant stirring (1100 rpm) at

25°C. THPs (NTHP and SFTHP) from the two race groups and at the following concentrations, namely, 4.38, 8.75, 17.50 and 35.00 mg $l^{-1}$ were added to the crystal suspensions under constant stirring (1100 rpm) for at least 2 hours. Zp's of the THP-dosed slurries were then determined using a Zeta Meter (Zeta-Sizer 4, Malvern Instruments). Three sets of experiments were performed for both race groups.

*4.5.3.c. Effects of NTHP and SFTHP on Sedimentation of CaOx crystals*

Inhibition of calcium oxalate crystal aggregation was determined according to the sedimentation assay of Hess et al, 1989. Control slurries were stirred at 1100 rpm; after 180 seconds the stirring was stopped and spontaneous sedimentation of particles was monitored for 300 seconds (Hess et al, 1989). The rate of sedimentation in the presence of the protein was measured by recording the decrease in the optical density of the slurry at a wavelength of 620 nm (Spectronic Unicam, Helios Delta, England) as a function of time. Slurries were incubated for 2 hours at 37°C prior to the commencement of each sedimentation experiments (Hess et al, 1989; 1991).

The effects of the protein (NTHP and SFTHP) from both race groups on the sedimentation rate of calcium oxalate crystal slurries were studied at the four previously above-mentioned THP concentrations (4.38, 8.75, 17.50 and 35.00 mg $l^{-1}$) by adding the appropriate quantities of protein to COM crystal slurries (physiological concentration of THP is 35.00 mg $l^{-1}$ (Hess et al, 1989). Three sets of experiments were performed for each population group.

## 4.5.4. RESULTS

### *4.5.4.a. Effects of NTHP and SFTHP on Zeta potentials of CaOx Crystal Slurries*

The zeta potential for the COM slurry was found to be –13 mV. Zp for NTHP and SFTHP became more negative with increasing protein concentration (Figure 23). Values levelled out at about –16 mV for WNTHP and at about –22 mV for BNTHP. The SFTHP had the same magnitude until the physiological level of about 18 mg $l^{-1}$ was reached. At higher concentrations, the value for the ZP of WSFTHP continues to become more negative while the opposite is true for BSFTHP. Since ZP was measure at only one concentration higher than 18 mg $l^{-1}$ (ie. 35 mg $l^{-1}$), it is not possible to confirm whether these trends are real or not. ZP for SFTHP from both race groups was more negative than that of WNTHP over the entire concentration range and was more negative than that of BNTHP until a concentration of 9 mg $l^{-1}$ was reached.

However, the concentration of WNTHP corresponding to the most negative ZP occurred at approximately 18 mg $l^{-1}$, while for BNTHP the negative charge appears to continue to increase with increasing concentration and did not reach a plateau value even at 35 mg $l^{-1}$ which was the highest concentration that was tested.

due to BNTHP and BSFTHP is always greater than WNTHP and WSFTHP.

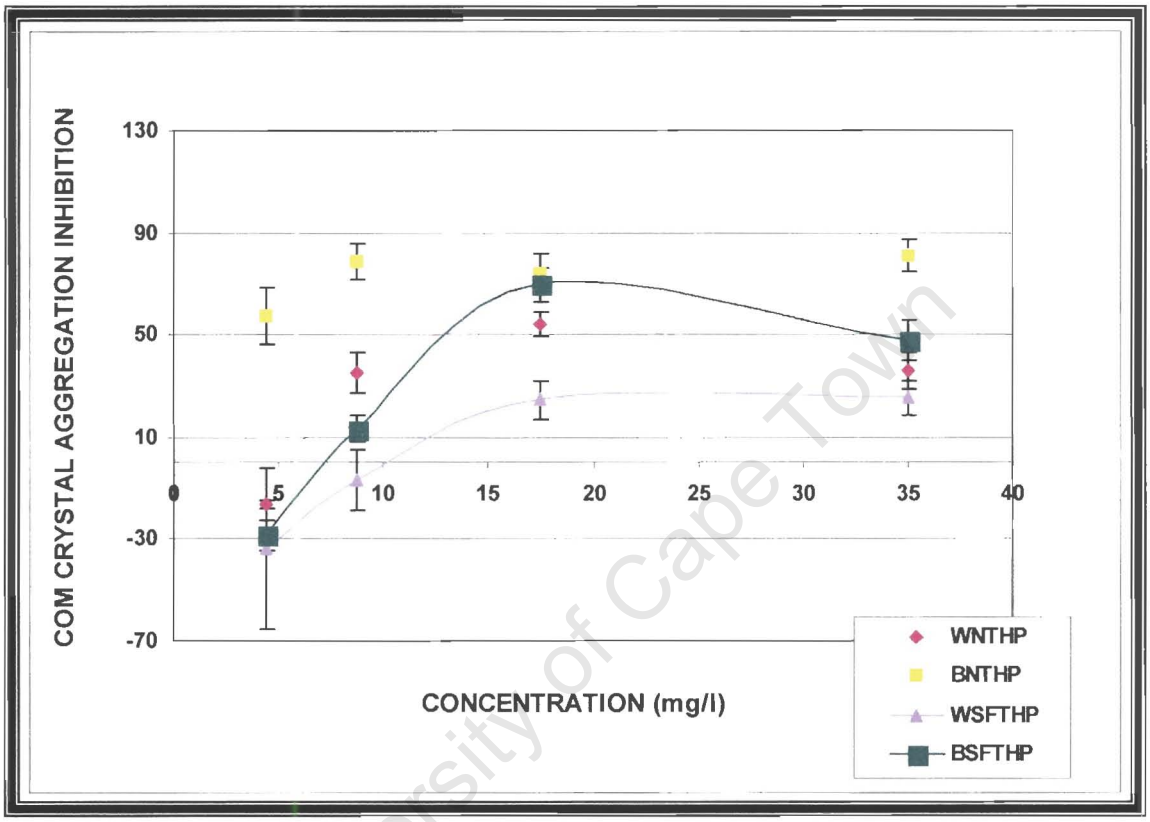



**Figure 24: The effect of NTHP and SFTHP on inhibition of COM crystal aggregation.**
(Data used to construct Figure 24 is available in appendix 4.5 B)



## 4.5.5. DISCUSSION

Figure 24 is a composite plot of data showing the precentage for inhibition of aggregation by NTHP and SFTHP from each race group as a function of their concentration. A shift in ZP to more negative values is an indirect measure of the possibility of inhibition of crystal aggregation. Thus zeta potential data indicate that THP is an inhibitor of calcium oxalate aggregation and is in support of the sedimentation results given above. This confirms the results of several other studies

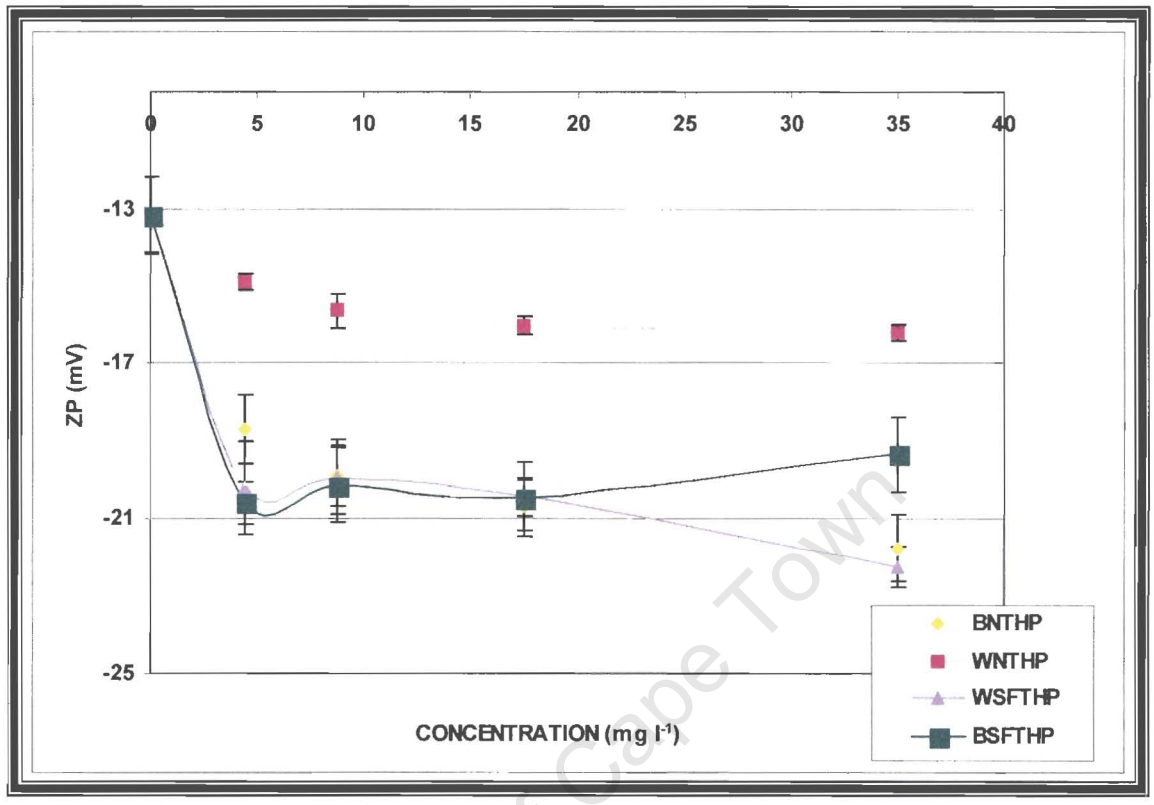



**Figure 23: Surface charge (zeta-potential) of COM crystals in the absence and presence of NTHP and SFTHP.** (Data used to construct Figure 23 is available in appendix 4.5 A)



*4.5.4.b. Effects of NTHP and SFTHP on Sedimentation of CaOx crystals*

Plots of percentage inhibition of calcium oxalate crystal aggregation as a function of protein concentration are given in Figure 24. These demonstrate that % inhibition increased progressively with increasing protein concentration for both NTHP and SFTHP from both race groups but that maximum inhibition occurred at a concentration of 9 mg $l^{-1}$ for BNTHP and at a concentration of 18 mg $l^{-1}$ for WNTHP, BSFTHP and WSFTHP. Interestingly, the % inhibition by WNTHP and SFTHP appears to start to decrease at concentrations greater than 18 mg $l^{-1}$ while inhibition by BNTHP appears to remain constant (Figure 24). In addition, the graphs show that at any given concentration, inhibition

(Hess et al, 1989; Ryall et al, 1991; Scurr et al, 1986(a), (b)). The observation in the sedimentation experiments that inhibition of aggregation by BNTHP is always greater than that by WNTHP and SFTHPs at any given protein concentration indicates that the former is superior in this role. The inhibitory activity trend follows the order BNTHP > BSFTHP > WNTHP > WSFTHP. These results also showed that within either race group, protein from the normal subjects was a stronger inhibitor of aggregation than those of the stone-formers.

It is noted that the sedimentation data suggest a loss of crystal aggregation inhibition by WNTHP with rising protein concentration (Figure 24). This effect has been previously reported (Hess et al, 1993). Moreover, the observation that Zp flattens off for WNTHP (Figure 23) has also been reported elsewhere (Hess et al, 1983; Scurr et al, 1986 (a), (b)). However, of considerable interest in this context, is the observation that BNTHP does not appear to behave in the same way. The sedimentation results for BNTHP do not show any hint of a loss in inhibition with increasing protein concentration (Figure 24) nor do the Zp's for BNTHP flatten out (Figure 23); indeed, to the contrary, Zp's become more negative with increasing concentration. This observation allows one to speculate that the structure of BNTHP may differ to that of WNTHP with respect to the relative numbers of carboxyl groups. It is suggested that there may be additional such groups in the former. This theory of structural difference has previous been proposed as a possible reason for differnces in normals and stone formers (Hess et al, 1993). Thus this would explain the more negative Zp's in BNTHP (Hess, 1991). The zeta potential values for SFTHP show that at concentrations below 9 mg/l, they are more negative than from NTHP. This again lends support to the theory of a greater number of carboxyl groups in the SFTHP.

However, this does not necessarily result in an increase in inhibition of aggregation. Hess has drawn attention to the existence of viscous binding forces that can counteract the repulsive zeta potential forces and may induce promotion of aggregation (Hess, 1994). Indeed, in a previous study in which more carboxyl groups were introduced into THP molecules, crystal aggregation inhibition was reduced (Hess, 1994). However, in the present study using BNTHP, inhibition (as indicated in sedimentation experiments) was unaffected. This suggests that viscous binding forces may be weaker in BNTHP than in WNTHP, thereby allowing the negative repulsive forces to operate relatively unhindered in the former. As a consequence, BNTHP is rendered as a more efficacious inhibitor of calcium oxalate crystal aggregation. The sedimentation results described above have attested to this. However, although the stone formers may appear to have more carboxyl groups at concentrations below 9 mg $l^{-1}$ (ZP more negative) it suggests that the repulsive forces may be stronger but it does not indicate the strength of the adhesive forces, which may indeed be such that the former are overcome. At concentrations greater than 19 mg/l, ZPs in BSFTHP become less negative suggesting the possibility of an unidentified concentration-dependent affect for THP from this particular group.

In conclusion, these results have revealed several properties pertaining to Tamm Horsfall protein and urine from black subjects. Firstly, the sedimentation experiments have demonstrated important differences between the THP's of stone-formers and healthy subjects of both race groups, with respect to their ability to inhibit aggregation, i.e. BNTHP has been shown to be superior in providing a protective mechanism against CaOx kidney stone formation which has not been shown by the other three THPs.

Since stone incidence in black subjects is extremely rare, the observed sequence for inhibition of aggregation provides compelling evidence that this THP-controlled crystallisation mechanism might be the key one for distinguishing between the two race groups with respect to their stone forming potential. Furthermore, it is speculated that the rare occurrence of stone formation in black subjects might occur because of changes in the properties of THP induced by changes in the urine composition. Indeed, the results for BSFU bear testimony to this. Alternatively, stone occurrence in black subjects might occur in cases where the THP structure has some abnormality, perhaps related to the number of carboxyl groups. The zeta potential data provide evidence in support of this view. Thus the identification and characterization of the catalysing factors in the urine of healthy black subjects and the critical structural and compositional features in the protein isolated from such urines may have important clinical implications. Further investigation of the structure of the four THPs and has been discussed in the previous chapter.

# REFERENCES


- Boeve. E., Cao. L., De Bruijn. W., Robertson. W., Romijn. J., Schroder. F. (1994) Zeta Potential distribution on calcium oxalate crystal and Tamm-Horsfall protein surface analysed with Doppler electrophoretic light scattering. J Urol **152**: 531.
- Burns. J. R., Finlayson. B. (1980) A proposal for a standard reference artificial urine in in vitro urolithiasis experiments. Inves. Urol. **18**: 167.
- Cao. L. C., Deng. G., Boeve. E. R., de Bruijn. W. C., de Water. R., Verkoelen. C. F., Romijn. J. C., Schroder. F H. (1996) Zeta potential measurement and particle size analysis for a better understanding of urinary inhibitors of calcium oxalate crystallization. Scanning Micrsocopy. **10**: (2). 401.
- Craig. T-A., Rodgers. A., Brandt. W. (2003) Inhibition of calcium oxalate crystallisation by Tamm Horsfall mucoprotein from two different population groups. Currently under review.
- Doyle. I. R., Ryall. R., Marshall. V. R. (1991) The incorporation of urinary proteins into calcium oxalate crystals precipitated from human urine: A highly selective phenonmenon. Clin. Chem. **37**: 1589.
- Doyle I, Marshall V, Dawson C, Ryall R (1995) Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in undiluted human urine in vitro. Urol Res **23**: 53.
- Drach. G. W., Thorson. S., Randolph. A. D. (1980) Effects of urinary organic macromolecules on crystallization of calcium oxalate: enhancement of nucleation. J. Urol. **123**: 519.

- Finlayson. B., Dubois. L. (1973) Kinetics of calcium oxalate deposition in vitro. Invest Urol. **10**: 429.
- Finlayson. B. (1978) Physicochemical aspects of Urolithiasis, Kidney Int, **13**: 344-360.
- Finlayson. B., Reid. F. (1978) The expectation of free and fixed particles in urinary stone disease. Invest Urol. **15**:442-448.
- Grant. A. M. S., Baker. L. R. I., Neuberger. A. (1973) Urinary Tamm Horsfall glycoprotein in certain kidney diseases and its content in renal and bladder calculi. Clin. Sci. Med. **44**: 377.
- Grover. P. K., Ryall. R., Marshall. V. (1990) Does Tamm-Horsfall mucoprotein inhibit or promote calcium oxalate crystallisation inhuman urine? Clin Chim Acta **190**: 223.
- Grover. P.K., Marshall. V. R., Ryall. R. L. (1994) Tamm-Horsfall mucoprotein reduces promotion of calcium oxalate crystal aggregation induced by urate in human urine in vitro. Clin Sci **87**:137.
- Hess. B. (1991) The role of Tamm-Horsfall glycoprotein and nephrocalcin in calcium oxalate monohydrate crystallisation processes. Scan Micro **5**: No 3, 689-696.
- Hess. B. (1992) Tamm-Horsfall Glycoprotein-Inhibitor or promoter of calcium oxalate monohydrate crystallisation processes? Urol Res **20**: 83.
- Hess. B. (1994) Tamm-Horsfall glycoprotein and calcium nephrolithiasis. Miner Electrolyte Metab **20**: 393.
- Hess. B., Nakagawa. Y., Coe. F. L. (1989) Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am J Physiol **257**: F99.
- Hess B, Nakagawa Y, Parks JH, Coe FJ (1991) Molecular abnormality of Tamm Horsfal glycoprotein in calcium oxalate nephrolithiasis. Am J Physiol **260**: F 569.

- Hess B, Zipperle L, Jaeger P (1993) Citrate and calcium effects on Tamm-Horsfall glycoprotein as a modifier of calcium oxalate crystal aggregation. Am J Physiol **265**: 784
- Hess. B., Kok. D. J. (1996) Nucleation, growth and aggregation of stone-forming crystals, in Coe. F.L., Favus. M. J., Pak. C. Y. C. et al. (eds): Kidney Stones: Medical and Surgical Management. Philadelphia. Lippincott-Raven. pp 3.
- Hunter.R. J. (1986) Foundations of Colloid Science (Oxford University Press Inc, New York), vol **I**.
- Israelachvilli (1992) Israelachvilli. J. N. (1992) Intermolecular and surface forces. Harcourt Brace Jovanovich. New York. pp 246.
- Khan. S. R. (1997) Interactions between stone-forming calcific crystals and macromolecules. Urol. Int. **59**: 59.
- Kitamura T, Pak CYC (1982) Tamm and Horsfall glycoprotein does not promote spontaneous precipitation and crystal growth of calcium oxalate in vitro. J Urol **127**: 1024.
- Kok. D. J., Papapoulos. S. E., (1993) Physicochemical considerations in the development and prevention of calium oxalate urolithiasis. Bone Miner. **20**: 1.
- Kok. D. J., Khan. S.R. (1994) Calcium oxalate nephrolithiasis, a free or fixed particle disease. Kidney Int. **46**: 847.
- Marangella. M., Bianco. O., Martini C., Petrarulo. M., Vitale. C., Linari F. (1989) Effect of animal and vegetal protein intake on oxalate excretion in idiopathic calcium stone disease. Br. J. Urol. **63**: 348.
- Pak. C., Ohata. M., Holt. K. (1975) Effect of diphosphonate on crystallisation of calcium oxalate in vitro. Kidney Int 7: 154.
- Rose. G.A., Sulaiman. S. (1982) Tamm-Horsfall mucoproteins promote calcium oxalate crystal formation in urine: Quantitative studies. J. Urol. **127**: 177.

- Ryall. R. L, Harnett. R. M., Hibberd. C. M., Edyvane. K. A., Marshall. V. R. (1991) Effects of chondroitin sulphate, human serum albumin and Tamm-Horsfall mucoprotein on calcium oxalate crystallisation in undiluted human urine. Urol Res **19**: 181.
- Ryall. R. L. (1996) Glycosaminoglycans, proteins and stone formation: adult themes and child's play. Pediatr. Nephrol. **10**: 656.
- Scurr. D. S., Robertson. W. G. (1986) **(a)** Modifiers of calcium oxalate crystallisation found in urine.Π Studies on their mode of action in artificial urine. J Urol **136**: 128.
- Scurr. D. S., Robertson. W. G. (1986) **(b)** Modifiers of calcium oxalate crystallisation found in urine.Ш Studies on the role of Tamm-Horsfall mucoprotein and of ionic strength. J Urol **136**: 505.
- Sophasan. S., Chatasingh. S., Thanphaichitr. P. (1980) Tamm Horsfall mucoprotein in urine of potential bladder stone formers. J. Urol. **124**: 522.
- Webber D, Rodgers AL, Sturrock ED (2002) Synergism between Urinary Prothrombin Fragment 1 and urine: A comparison of inhibitory activities in stone-prone and stone-free population groups. Clin Chem Lab Med **40**: 930.
- Worcester EM, Nakagawa Y, Wabner CL, Kumar S, Coe FL (1988) Crystal adsorption and growth slowing by nephrocalcin, albumin and Tamm Horsfall protein. Am J Physiol **255**: F1197.
- Yoshioka. T., Koide. T., Utsunomiya. M., Itatani. H., Oka. T., Sonoda. T. (1989). Possible role of Tamm-Horsfall Glycoprotein in calcium oxalate crystallization. Br. J. Urol. **64**: 463.

# CHAPTER 5

# OVERVIEW AND FUTURE WORK

In concluding this thesis, it is appropriate to assess the extent to which the objectives have been achieved and the extent to which the fundamental hypothesis has been tested. This project was based on the hypothesis that the significantly lower incidence of kidney stone disease in the South African black population relative to their white compatriots might be due to the former having urinary inhibitors which are more potent than those of whites. Attention was drawn to the current thinking in stone research that urinary proteins serve as inhibitors of CaOx crystallisation processes and that among these, THP, had been identified as a key role player. Thus this urinary protein was selected for investigation.

The hypothesis which was formulated was that THP in the urine of black subjects might play a role in providing this race group with immunity towards kidney stone disease. In order to test this hypothesis, the protein was isolated from urine of both race groups and tested in a series of crystallization experiments in order to evaluate their relative inhibitory activities. Concurrently with these experiments, the proteins were biochemically characterized with a view to investigating whether any differences exist in their structure and composition.

In the crystallization experiments several important and hitherto unreported results emerged. A feature which was common to all the protein-urine systems studied was the synergistic role played by the composition of the urine in which the experiment was conducted. These experiments showed that urine from black subjects generally

provided a superior milieu for inducing an inhibitory role by THP. For example, in WNU, both proteins retard the rate of particle formation while in BNU they increase the rate. The latter result (i.e. an increase in the rate of particle formation) was interpreted as a favourable process as it rapidly decreases the supersaturation of stone forming salts.

With respect to the protein itself, it was found to be an inhibitor of calcium oxalate crystal deposition irrespective of race and or environment. In particular THP was found to be a strong inhibitor of CaOx crystal aggregation. Comparison of the respective inhibitory capacities on a racial basis consistently revealed that THP from black subjects is superior to that of white subjects. For example, particle volume and $^{14}$CaOx data revealed that BNTHP inhibits the deposition of CaOx crystals in the normal urine from both race groups while WNTHP inhibits the deposition in BNU only and promotes it in WNU.

Thus the crystallisation experiments have shown that there is indeed a difference in the inhibitory capacity of THP in the two race groups. Of considerable interest are the several differences that were identified in the biochemical characterization of the proteins. In particular BNTHP was found to have a larger molecular weight, different percentages of amino acids, a smaller percentage of α-helical structures and a larger percentage of β-sheet structures than the THP in the other three groups. In addition, BNTHP appeared to have a different N-glycan profile which could be due to a different composition of sugar structures. Moreover, the functional differences between NTHP and SFTHP irrespective of race group appear to be due to differences in the degree of glycosylation with sialic acid.

Therefore, based on the results described in this thesis, it seems reasonable to speculate that the biochemical differences identified in BNTHP may influence its inhibitory functionality and that its inhibitory potential could be enhanced. These biochemical differences of BNTHP and its superior ability to inhibit CaOx crystallisation are highly significant findings which support the original hypothesis

that this protein may play a contributory role in the low incidence of stones in black South Africans.