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Investigation of the role of dietary myo-inositol hexakisphosphate (phytate) on the relative risk of calcium oxalate kidney stone formation in black and white male South African subjects

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University of Cape Town

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ABSTRACT

Previous studies have shown that calcium oxalate (CaOx) stone-formers have lower urinary concentrations of myo-inositol hexakisphosphate (phytate or IP_6) than healthy individuals, that dietary intake of this substance leads to its increased urinary excretion and that it is an inhibitor of CaOx nucleation and growth. In South Africa it has been reported that the black population has a higher dietary phytate intake than whites. The present study was undertaken to test the hypothesis that South African black subjects have higher urinary phytate levels than their white counterparts and that this contributes to the relative rarity of CaOx kidney stone disease in this population group.

A modified indirect extraction/photometry method to measure urinary IP_6 was designed, developed and tested in the present study. This assay was then used to measure IP_6 in the urine of rural black and urban white subjects while on their free unrestricted diets. In addition, urban black and white subjects each followed IP_6-restricted followed by IP_6-rich dietary protocols for a period of three days. Urines were collected after administration of each protocol and were again analysed for IP_6 using the newly developed assay. Urines were then used in several crystallization experiments to measure the CaOx metastable limit, ^{14}C-oxalate deposition kinetics and inhibition of CaOx crystal aggregation.

The results showed that while on their free diets, rural blacks excreted significantly less IP_6 than urban whites despite their previously reported higher dietary intake of this substance. This suggests that the renal handling of dietary IP_6 is different in the two race groups. Further evidence in support of this observation was obtained upon administration of the IP_6-rich diet, after which black subjects showed a higher uptake of IP_6 than their white counterparts and a statistically significant increase in urinary oxalate. Despite the important latter effect, the tendency towards CaOx crystallization decreased after administration of the IP_6-rich diet and this effect tended to be

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greater in the black group. However, these effects were not statistically significant.

Compelling evidence to support the hypothesis that phytate plays a direct role in contributing towards the rarity of CaOx kidney stones in the black population was therefore not identified. However, the results indicate that phytate may play an indirect role by influencing subtle changes in urine composition in the black population which allow it, for example, to nullify the promotory effect of an increase in urinary oxalate. This provides motivation for future investigations in this area.

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ABBREVIATIONS

^{13}C NMR	^{13}Carbon nuclear magnetic resonance
^{14}C-Ox	^{14}C-oxalic acid
^{1}H NMR	Proton nuclear magnetic resonance
CaOx	Calcium oxalate
COD	Calcium oxalate dihydrate
COM	Calcium oxalate monohydrate
COT	Calcium oxalate trihydrate
GCMS	Gas chromatography mass spectroscopy
HMQC	Heteronuclear multiple quantum correlation
HPIC	High performance ion chromatography
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IP_6	Myo-Inositol hexakisphosphate
MSL	Metastable limit
RS	Relative Supersaturation
SE	Standard Error

University of Cape Town

Table of Contents

Page No.

Acknowledgements i
Conferences ii
Abstract iii
Abbreviations v
Table of contents vi
List of figures and tables xiii

Chapter I

Introduction

1.1 Epidemiology
1.1.1 History 1
1.1.2 Age and Gender 2
1.1.3 Geography and Climate 2
1.1.4 Occupation 3
1.1.5 Drugs 4
1.1.6 Diet 4
1.1.7 Racial Distribution 6
1.2 Mechanism of Stone Formation 8
1.2.1 Supersaturation........ 8
1.2.2 Crystal Nucleation 9
1.2.3 Crystal Growth 11
1.2.4 Crystal Aggregation 11
1.3 Inhibitors 12
1.3.1 Macromolecules 12
Glycosaminoglycans (GAGs) 12
Urinary Proteins 13
Tamm-Horsfall glycoprotein (THP) 13
Osteopontin (OPN) 14
Bikunin (Inter-α-Trypsin Inhibitor) 15
Nephrocalcin (NC) 15
Urinary Prothrombin Fragment 1 (UPTF 1).. 15

University of Cape Town

Chapter I Page No.

1.3.2 Micromolecules 16
Citrate 16
Magnesium 16
Pyrophosphate 17
Phosphonates 17
1.4 Phytate 18
1.4.1 Metabolism, tissue distribution, absorption and excretion 19
1.4.2 Structure 21
1.4.3 Inhibitory activity towards stone formation 21
1.4.4 In Vitro Studies 21
1.4.5 Animal Studies 23
1.4.6 Human Studies 24
1.5 Aims and Objectives 25
1.6 References 26

Chapter II

Development of Method towards Measurement of IP_6 in Urine

2.1 Introduction 46
2.1.1 High Performance Liquid Chromatography (HPLC). 46
2.1.2 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) 47
2.1.3 Gas Chromatography Mass Spectroscopy (GC-MS) 48
2.1.4 High Performance Ion Chromatography (HPIC)...... 48
2.1.5 Modification of an Indirect Extraction/Photometry Method 48
2.2 Purity Verification of IP_6 49

University of Cape Town

Chapter II Page No.

2.2.1 pH Measurement 49
Introduction 49
Method 50
Results and Discussion 50
Conclusion 50
2.2.2 Nuclear Magnetic Resonance
Spectroscopy NMR) 50
Introduction 50
Method 52
Results and Discussion 52
Conclusion 59
2.2.3 Ion Chromatography 59
Introduction 59
Method 59
Results and Discussion 60
Conclusion 61
2.2.4 Hydrolysis 62
Introduction 62
Method 63
Results and Discussion 63
Conclusion 64
2.2.5 Hydrolysis of commercial IP_6 salt for
purity investigation 64
Introduction 64
Method 65
Results and Discussion 65
Conclusion 66

University of Cape Town

Chapter II Page No.

2.3 Test of the Accuracy of Indirect Extraction/Photometry Method 66

2.3.1 Phosphate calibration curve 66

Introduction 66

Method 67

Results and Discussion 67

Conclusion 67

2.3.2 IP_6 Calibration Curves 68

Introduction 68

Method 68

Results and Discussion 69

Conclusion 70

2.4 Anion Exchange Chromatography 70

Introduction 70

Method 71

2.4.1 Determination of optimal HCl concentration Required to elute PO_4^{3-} 72

Method 72

Results and Discussion 73

Conclusion 73

2.4.2 Determination of optimal volume of HCl required to elute PO_4^{3-} 73

Method 73

Results and Discussion 74

Conclusion 75

2.4.3 Determination of optimal H_2SO_4 concentration required to elute IP_6 75

Method 75

Results and Discussion 75

Conclusion 76

University of Cape Town

Chapter II Page No.

2.4.4 IP_6 standard solution application 76

Method 76

Results and Discussion 76

Conclusion 77

2.4.5 Testing the separation and elution of PO_4^{3-} and IP_6 at urinary concentrations 78

Method 78

Results and Discussion 78

Conclusion 79

2.4.6 Use of lower HCl and lower H_2SO_4 concentrations .. 80

Method 80

Results and Discussion 80

Conclusion 81

2.4.7 PO_4^{3-} application onto the column 81

Method 81

Results and Discussion 81

Conclusion 82

2.4.8 IP_6 standard solution application 82

Method 82

Results and Discussion 82

Conclusion 83

2.4.9 Urine 83

Method 83

Results and Discussion 84

Conclusion 85

2.5 References 86

University of Cape Town

Chapter III

Determination of Myo-inositol Hexakisphosphate (Phytate) in Urine

Page No.

3.1 Introduction 89

3.1.1 Concentration of IP_6 in Urine 89

3.1.2 Relationship between Oral Intake and Urinary Excretion 89

3.2 Method 90

3.2.1 Urine Collection and Preparation 90

3.2.2 Dietary protocols 91

3.3 Results 93

3.4 Discussion 98

3.5 References 102

Chapter IV

Crystallization Experiments

4.1 Introduction 105

4.1.1 Crystallisation Experiment used in this study 107

4.2 Metastable Limit (MSL) Determination 107

4.2.1 Introduction 107

4.2.2 Method 107

4.2.3 Results and Discussion 109

4.2.4 Conclusion 110

University of Cape Town

Chapter IV **Page No.**

4.3 Sedimentation Kinetics 110
4.3.1 Introduction 110
4.3.2 Method 111
4.3.3 Results 112
4.3.4 Discussion 114
4.4 ^{14}C-Oxalate Deposition Experiment 115
4.4.1 Introduction 115
4.4.2 Method 115
4.4.3 Results and Discussion 116
4.5 References 118

Chapter V

General Discussion and Conclusion

General Discussion and Conclusion 120
References 122

Appendices

Appendix I: Candidates' Urine Composition 123
Appendix II: Individual Metastable Limit Experimental Results 129
Appendix III: Sedimentation Experimental Graphs 140
Appendix IV: Individual ^{14}C-Oxalate Deposition Experimental Results 148

University of Cape Town

LIST OF TABLES

Chapter II

Development of a method to determine IP_6 in urine

Page No.

Table 2.1: Assignment of ^{1}H NMR spectrum of IP_6 53

Table 2.2: Assignment of the ^{13}C-NMR spectrum of phytate sample 55

Table 2.3: Hydrolysis experiment 66

Table 2.4: Determination of optimal volume of 0.2M HCl 74

Table 2.5: IP_6 concentrations in the three eluates collected after IP_6 application 77

Table 2.6: Application of IP_6 and Na_2HPO_4 mixture onto the anion exchange column 79

Table 2.7: Absorption values for phosphate eluted in fraction 3 after application of various volumes of 0.05M HCl 80

Table 2.8: Recovery of PO_4^{3-} after application of 1 g/l PO_4^{3-} standard solution onto the column 81

Table 2.9: Application of IP_6 standard solution 83

Table 2.10: Urine sample (5ml) application 84

Table 2.11: Application of urine sample dosed with IP_6 onto the column 85

University of Cape Town

Chapter III

Determination of myo-inositol hexakisphosphate (Phytate) in urine

Page No.

Table 3.1: Dietary instructions 92

Table 3.2: Mean nutrient intake of subjects in the present study....... 93

Table 3.3: Comparison of urinary IP_6 concentrations in black and white candidates after following IP_6-restricted and IP_6-rich diet 95

Table 3.4: Mean urinary excretion (mmol/24hr) and physicochemical parameters in black (n = 10) and white subjects' urine (n = 7)... 97

Chapter IV

Crystallization Experiments

Table 4.1: Preparation of NaOx standard solutions......................... 108

Table 4.2: Mean percentage (%) inhibition of aggregation 114

Table 4.3: Average percentage (%) precipitation of $Ca^{14}C$-Ox crystals in the urine of black and white subjects at 120 min.. 117

University of Cape Town

Appendices

Appendix I **Page No.**

Table 1.1: 10 black subjects' urine composition (phytate-restricted diet) 123

Table 1.2: 10 black subjects' urine composition (phytate-rich diet) 124

Table 1.3: 9 rural blacks subjects' urine composition (free diet)....... 125

Table 1.4: 7 white subjects' urine composition (phytate-restricted diet) 126

Table 1.5: 7 white subjects' urine composition (phytate-rich diet) 127

Table 1.6: 9 urban white subjects' urine composition (free diet)...... 128

Appendix II

Table 2.1: Black subjects' metastable limit concentrations 133

Table 2.2: Average particle number at different sodium oxalate concentrations in urine of black subjects (n = 10) 134

Table 2.3: White subjects' metastable limit experimental results ... 138

Table 2.4: Average particle number at different sodium oxalate concentrations in urine of white subjects (n = 10) 139

Appendix III

Table 3.1: Characterization of CaOx crystals using XRD 140

University of Cape Town

LIST OF FIGURES

Chapter I
Introduction

Page No.

Figure 1.1: Kidney stone lodged in the ureter of the urinary system 1

Figure 1.2: Relative supersaturation and CaOx crystal nucleation 10

Figure 1.3: Phytic acid structure 21

Chapter II
Development of a method towards measurement of IP_6 in urine

Figure 2.1: Conformations of phytic acid 51

Figure 2.2: ^{1}H NMR spectrum of phytate sample used in this study.. 52

Figure 2.3: ^{13}C NMR spectrum of phytate used in this study 54

Figure 2.4: $^{1}H - ^{13}C$ HSQC contour plot 55

Figure 2.5: Phosphorus (^{31}P) NMR spectrum of phytate used in this study 56

Figure 2.6: ^{31}P NMR of IP_6 in the presence of sodium as the counter ion 58

Figure 2.7: Ion chromatography of different phytate standard solutions 60

Figure 2.8: Chromatogram of ions contained in a given phytate standard solution 61

Figure 2.9: Hydrolysis of IP_6 to myo-inositol and phosphate 62

Figure 2.10: Structure of phosphomolybdocomplex 63

Figure 2.11: The mean absorbance values of PO_4^{3-} solution after hydrolysis of an IP_6 standard solution 64

Figure 2.12: Phosphate calibration curve prepared using standard phosphate solutions 67

University of Cape Town

Page No.

Figure 2.13: Phytate calibration curve prepared using unhydrolysed phytate standard solutions 69

Figure 2.14: Phytate calibration curve prepared using hydrolysed phytate standard solutions 70

Figure 2.15: Plot of average absorbance vs concentration of HCl for the determination of optimum HCl concentration required to elute PO_4^{3-} 73

Figure 2.16: Plot of average absorbance vs volume of 0.2M HCl......... 74

Figure 2.17: Absorbance as a function of the H_2SO_4 concentration 76

Chapter III

Determination of IP_6 in urine

Figure 3.1.1: Concentration of urinary phytate in black subjects during an IP_6-restricted and IP_6-rich diet 94

Figure 3.1.2: Concentration of urinary phytate in white subjects during an IP_6-restricted and IP_6-rich diet 94

Figure 3.2: Average concentration of IP_6 measured after the black and white participants were on IP_6-restricted and rich diet 95

Figure 3.3: Rural black (n = 9) and urban white (n = 9) candidates' average IP_6 concentration while on an unrestricted diet 96

University of Cape Town

Chapter IV

Crystallization experiments

Page No.

Figure 4.1: Average metastable limit experimental result (10 black subjects) 109

Figure 4.2: Average metastable limit experimental result (7 white subjects) 109

Figure 4.3: X-ray powder diffraction pattern of calcium oxalate crystals used in this study 111

Figure 4.4.1: Typical absorbance vs time graphs observed in the different samples in black candidates 113

Figure 4.4.2: Typical absorbance vs time graphs observed in the different samples in white candidates 113

Figure 4.5.1: Typical graph of % precipitation of CaOx as a function of time in black subjects 116

Figure 4.5.2: Typical graph of % precipitation of CaOx as a function of time in white subjects 116

University of Cape Town

Appendices

Appendix II		Page No.
Figure 2.1:	Metastable limit graphs (black subjects' results)............	129
Figure 2.2:	Metastable limit graphs (white subjects' results)............	135
Appendix III	
Figure 3.1:	Black subjects' sedimentation results	141
Figure 3.2:	White subjects' sedimentation results	145
Appendix IV	
Figure 4.1:	^{14}C-Oxalate deposition experimental graphs (black subjects' results)..	148
Figure 4.2:	^{14}C-Oxalate deposition experimental graphs (white subjects' results)..	150

University of Cape Town

CHAPTER I

University of Cape Town

CHAPTER I

INTRODUCTION

1.1 Epidemiology

1.1.1 History

Urinary stone disease is amongst the oldest recognized medical disorders of mankind and has been acknowledged right through medical history (Drach 1986; Ryall 1993). Stones can occur in the kidneys, ureters or bladder (Figure 1.1). The disease can be traced back 6680 years (Clarke 1968) and the symptoms have been known since ancient times (Grases *et al* 1998b). Urinary stones can occur singly or in large numbers. According to some hospital records in the Scandinavian countries, there has been an upsurge in the incidence of upper urinary tract stone disease in Europe, North America and Japan since the latter years of the 19th century (Andersen 1966, 1969).

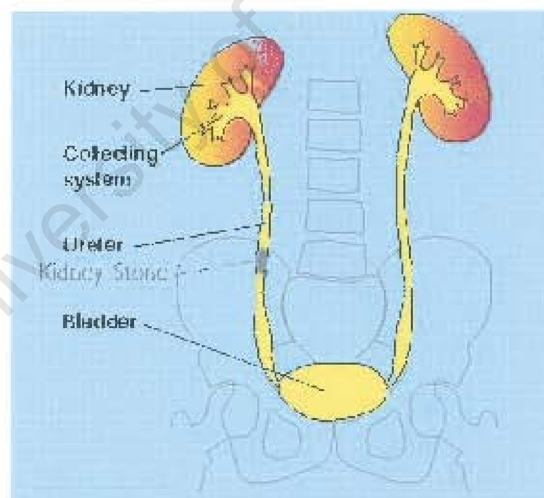

Figure 1.1: Kidney stone lodged in the ureter of the urinary system (www.urosurge.org)

Between 1910 and 1960 a constant increase in the incidence of renal stone was observed (Andersen 1966). However, the increase was interrupted twice by World Wars 1 and 2. Andersen suggested that the decline in stone

University of Cape Town

occurrence during the wars was due to starvation. Despite this observation, Modlin (1981) pointed out that these interruptions coincided with periods of increased ingestion of phytic acid.

1.1.2 Age and Gender

In adults, stones are more common between the ages of 30 and 60 years (Hesse *et al* 1986). This may be attributed to specific metabolic processes which occur during this period and which induce some urinary excretions that influence stone formation. The peak occurrence of calcium oxalate (CaOx) stones in men is at the age of 35 while in women, there are two peaks appearing at 30 and 55 years (Blacklock 1982, Fan *et al* 1999). Menopausal bone reabsorption has been considered to be the cause of stones in women at this latter age (Fan *et al* 1999).

The incidence of stones in males is twice that in females (Blacklock 1982, Robertson and Peacock 1983). In males the occurrence is 7 - 15 % while in women it is 3 - 6% (Scott *et al* 1977, Hesse *et al* 1986). Among the reasons cited for this observation is that men have a larger body mass and therefore have a large amount of calcium, oxalate and uric acid to excrete than women (Blacklock 1982, Robertson and Peacock 1983, Hess and Kok 1996). However, it should be noted that the gap in gender is closing in. An additional suggestion is that sex hormones play a role in this difference (Iguchi *et al* 1999). In females, oestrogen decreases urinary oxalate excretion and calcium oxalate crystal deposition thereby retarding kidney stone formation (Fan *et al* 1999, Heller *et al* 2002). It has also been noted that females generally have higher citrate levels. In contrast, testosterone in men increases the concentration of oxalate (Lee *et al* 1996) and thus the risk of stone formation.

1.1.3 Geography and Climate

The areas where the incidence of renal stone disease per head of population is significantly higher than in any other part of the same country are known as stone belts. An example is the South Eastern stone belt of the USA which

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includes Florida, Georgia and North and South Carolina (Boyce and Strawcutter 1956).

An increased renal stone incidence has been associated with an increase in atmospheric temperature. This observation was made in the south Eastern United States (Prince and Scardino 1960) and Australia (Bateson 1973). In the latter, it was observed that the incidence of renal calculi during the hot summer time was higher than in winter. Owing to extremely high temperatures, people dehydrate and if the lost water is not replenished by taking sufficient fluids then the processes leading to stone formation may be triggered (Blacklock 1982). The stone problem in the tropics is compounded by the hot climate which results in sweating and hence reducing the volume of urine (Robertson 2003). Thus, kidney stones are more likely to form in persons who live in a hot, dry climate, or who exercise strenuously without replacing lost water.

Robertson *et al* (1974) reported a high calcium excretion in troops in the desert. This highlights another factor that contributes to stone formation in tropical and sub-tropical climates, namely increased exposure to ultra-violet radiation (vitamin D), which is believed to increase the intestinal calcium uptake leading to an increased urinary calcium concentration (Parry and Lister 1975). Vitamin D plays an important role in idiopathic hypercalciuria through intestinal absorption of calcium (Giannini *et al* 1993).

1.1.4 Occupation

The observation has been made, especially in the technically developed countries, that stone formation is prevalent in certain occupational groups. Mates (1969) noted that the more professional the occupation, the higher the incidence of stone formation. Also, the more sedentary or inactive the job, the higher the likelihood of forming a stone. In the Royal Navy, the officers and personnel doing predominantly sedentary work appeared to be more prone to the disease (Blacklock 1965).

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Another noteworthy discovery made from the medical records of London bus drivers and conductors was that the former were more afflicted by stone disease than the latter (Boyce and Strawcutter 1956). In light of this, pilots and accountants and many others who have sedentary jobs could be at risk of getting stones (Borghi *et al* 1993; Zheng *et al* 2002). The possible explanation for this could be that the people who do active jobs drink a lot of fluid to replace the water lost through sweating. In contrast, those who have inactive jobs do not ingest adequate amounts of fluid.

1.1.5 Drugs

Certain medications and drugs have been shown to be lithogenic. An example is antacid tablets that contain silica. After the administration of antacids to a number of people, it was discovered that some of the patients formed silica-containing stones (Levison *et al* 1982). Also, indinavir, an anti-HIV drug, has been reported to cause kidney stones (Sarcletti *et al* 2000, Jaradat *et al* 2000).

1.1.6 Diet

Stones form due to a combination of factors amongst which diet plays a major role (Hesse *et al* 1993, Massey *et al* 1993, Goldfarb 1994). The role of diet has been the centre of focus of many researchers for several years. Because many calculi are made up of CaOx, patients have always been advised to avoid oxalate rich foods (Robertson 1987, Lewandowski and Rodgers 2004). There is evidence that there is a direct relationship between ingested and excreted oxalate (Holmes and Kennedy 2000; Holmes *et al* 2001), the mean contribution from diet being from 10 - 20% (Finch *et al* 1981) to 40 - 53 % (Holmes and Assimos 1999; Holmes *et al* 2001; Massey 2003). Some studies indicated that drinking iced tea (rich in oxalate) in the 'stone belt' regions of the USA tie in well with the stone prevalence in these regions (Rodgers 1991).

Owing to the suspected lithogenic role of calcium, dietary restriction of dairy products has been frequently prescribed in patients with kidney stones (Robertson 1987). Dairy products are also known to contain phosphorus,

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which also contributes to stone formation. Although advising CaOx stone patients to reduce their dietary calcium has been common practice for many years (Bleich *et al* 1979, Galosy *et al* 1980, Robertson 1987), it is now accepted that calcium should not be limited in the diet of kidney stone formers (Curhan *et al* 1997, Messa *et al* 1997, Heller 1999). Dietary calcium binds oxalate in the gut and forms CaOx which gets voided in the feaces. This reduces the amount of free oxalate available for absorption and hence reduces oxaluria.

Another important dietary risk factor is animal protein (Hughes and Norman 1992, Curhan *et al* 1993, Hassapidou *et al* 1999, Massey 2003). It has been reported that vegetarians are at a lower risk of having kidney stones (Brockis *et al* 1982, Robertson *et al* 1982, Dwyer 1988). Consumption of animal protein in the form of meat, fish or poultry has been noted to increase urinary calcium which is linked to increased risk of forming kidney stones. In addition to increased urinary concentrations of calcium, protein intake results in increased urinary oxalate, uric acid, phosphate and diminishes the excretion of citrate, all of which are risk factors for stone formation (Robertson 1990, Parivar *et al* 1996).

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Basic physical chemistry dictates that supersaturation is a driving force for crystallization. Supersaturation can only be reduced by increasing the volume of urine by drinking fluids such as water (Robertson *et al* 1980). However, while this practice dilutes the concentration of stone-forming constituents, it also dilutes the concentration of inhibitors. Therefore, chemical composition of drinking water is important. Several studies on soft and hard water have failed to reach consensus (Sierakowski *et al* 1979, Shuster *et al* 1982).

In a study conducted by Rodgers (1997) on a mineral water rich in calcium and magnesium, several risk factors such as excretion of oxalate, relative supersaturation of CaOx, brushite and uric acid, CaOx metastable limit, oxalate: magnesium ratio and oxalate: metastable limit ratio were altered favourably. Based on these results, it was concluded that mineral water containing calcium and magnesium deserves to be considered as a possible

therapeutic or prophylactic agent in calcium oxalate kidney stone disease (Rodgers 1997). Other studies have indicated that bicarbonate-rich mineral water could be useful in the prevention of calcium oxalate stones (Kessler and Hess 2000, Siener *et al* 2004). In addition, Kessler and Hess observed that the effect of bicarbonate-rich mineral water was similar to that of sodium potassium citrate therapy and that it could be useful in the prevention of recurrence of calcium oxalate uric acid stones. In their study Siener *et al* (2004) stated that mineral water rich in magnesium and bicarbonate resulted in favourable changes in urinary pH, magnesium and citrate. The latter two are known to decrease calcium oxalate stone formation by reducing the urinary calcium excretion (Trinchieri *et al* 1999). According to Trinchieri, mild-calcium high-carbonate content water appears to be appropriate for prevention of kidney stone disease.

1.1.7 Racial Distribution

Races in which the disease is rare include the Indians of Mexico, Peru, Ecuador and Bolivia, as well as Eskimos and Aborigines (Bateson 1977). In South Africa, the incidence of renal stone disease in the black population is exceptionally rare while in the white population it is as frequent as that which occurs in other western societies (Muskat 1951, Wise and Kark 1961, Modlin 1967, Whalley 1998).

Blacklock (1982) suggested that as nations become more technically developed, so there is a concomitant increase in the occurrence of renal stone. Indigenous African races appear to be afflicted rarely by upper urinary tract stone whilst living within their tribal environment (Vermooten 1937; Wise and Kark 1961).

In the South African context, Modlin (1981) reported that during the period 1971 – 1979, the incidence of renal stone among white patients admitted to Groote Schuur Hospital in Cape Town was 1 in 510, while in black patients only 1 in 44 298 patients was found to have the disease. Today in the white population the occurrence of kidney stones is between 10 and 15 percent which lies within the range reported for many other countries.

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About forty years ago urolithiasis was hardly detected in black South Africans (Wise and Kark 1961). However, now that significant numbers of this population group are being urbanized, there is a concurrent, yet slender increase in the incidence of stone in urban blacks, but nevertheless it remains less than 1% of the population (Whalley *et al* 1998).

The mystery surrounding the difference in the occurrence of renal stone disease in black and white South Africans has generated various hypotheses. It has not been clear whether dietary differences, racial immunity, or the presence or absence of promoters and inhibitors of renal stone formation are the key factors or not (Whalley *et al* 1998).

Some authors believe that dietary differences can account for the difference in stone incidence between the two race groups (Wise and Kark 1961, Modlin 1967). In Modlin's study, black South Africans were reported to ingest significantly greater quantities of sodium in the form of seasoning or table salt than their white counterparts (Modlin 1967). Modlin attributed low stone incidence in blacks to their relatively higher urinary ratio of sodium/calcium. He hypothesized that sodium ions compete successfully with calcium ions in the formation of the crystal lattice, thereby compromizing the processes of epitaxy and crystal aggregation.

In his study, Modlin (1967) also provided an extensive account of the differences in the 24h urinary composition in healthy black and white subjects. Volume, osmolality and sodium were found to be significantly higher in blacks than whites while pH, calcium, phosphate, citric acid and magnesium were significantly lower. Some of these differences are counterintuitive and have not been satisfactorily explained.

The potential role of diet in the pathogenesis of kidney stone disease on the one hand and its inhibition on the other, provides motivation for the present project. However, it is appropriate to first review the various mechanisms of stone formation.

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1.2 Mechanism of Stone Formation

1.2.1 Supersaturation

Urine is a complicated chemical solution that contains numerous ions. Among the most common inorganic cations and anions are Na^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{3-} and HCO_3^-. In addition, macromolecules (glycosaminoglycans and proteins), micromolecules (myo-inositol hexakisphosphate (phytate)), and dissociated organic compounds such as oxalate, uric and citric acids are also contained in urine (Sohnel and Grases 1995).

Urine is predominantly supersaturated with CaOx monohydrate (COM) and dihydrate crystals (COD). Trihydrate crystals (COT) are, on the other hand, rare (Prien 1963; Heynen *et al* 1985). COM's are the predominant crystals in stones. When examined under high magnification using a scanning electron microscope, urinary crystals appear as tiny deposits of various shapes and sizes (Rodgers 1991). Their presence in urine (crystalluria) does not help differentiate between stone formers and healthy individuals as it is normal to have CaOx crystals in urine (Robertson *et al* 1969; Robertson and Peacock 1972; Fleisch 1978; Werness *et al* 1981). However, it has been reported in some studies that crystalluria is often absent in kidney stone formers (Cifuentes Delatte *et al* 1983). Also, Robertson has reported that there is a unimodal crystal size distribution in urine from healthy controls but in stone formers, a bimodal distribution exists (Robertson and Nordin 1982).

Researchers differ on their findings regarding supersaturation. Kok and Papapoulos (1993) stated that supersaturation values of the two groups are almost the same. On the other hand, it has been reported that stone formers tend to excrete urine that is more highly supersaturated than non-stone formers (Robertson *et al* 1968; Marangella *et al* 1985; Coe and Parks 1986). Despite differences in their findings, researchers agree that supersaturation is a prerequisite for crystallization. However, on its own it is not sufficient for the explanation of the occurrence of stone formation. There are two states of supersaturation: (1) a metastable state in which crystallization does not occur unless it is induced by the addition of seed crystals (Kavanagh 1992); and (2)

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an unstable state in which crystallization occurs spontaneously (Kavanagh 1992).

1.2.2 Crystal Nucleation

The process of crystal nucleation marks the beginning of the phase transformation that leads to the formation of urinary stones (Finlayson *et al* 1984).

An aqueous solution of calcium oxalate can tolerate an increase in the concentration of Ca^{2+} and Ox^{2-} even beyond a relative supersaturation of 1. The solution is then referred to as ideal and is stable (Hess and Kok 1996). However, if more Ca^{2+} and Ox^{2-} are added until the relative supersaturation reaches 80 - 100, then the solution becomes unstable as it passes the upper limit of metastability (formation product). The crystals then start to form spontaneously which is called homogeneous nucleation (Finlayson 1978). This would only occur if Ca^{2+} and Ox^{2-} were in a pure solution.

In a different situation, if the solution contains Ca^{2+}, Ox^{2-} and CaOx crystals, then the formation product (metastable limit) of the solution would be lower. That is, the presence of CaOx crystals induces a change in the relative supersaturation of the solution. Therefore, if the concentration of the two ions continues to be increased beyond the relative supersaturation value, then spontaneous nucleation occurs on the surface of the pre-existing crystals of CaOx. This is called secondary nucleation (Hess and Kok 1996). However, urine is seldom concentrated enough for this process to occur. It is therefore likely that nucleation in urine proceeds via some other mechanism, namely heterogeneous nucleation. This is the process in which COM crystals are formed on crystalline particles of foreign substances that serve as nucleating substrates. These substrates include hydroxyapatite, brushite and uric acid (Lonsdale 1968). If the material of one crystal is precipitated onto another crystal and the lattice dimensions of the two match, then crystallization proceeds in urine. This process is called epitaxy. In urine, the formation product is low because of the presence of many foreign substances.

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Heterogeneous nucleation, secondary nucleation and epitaxy all take place before the metastable limit value is reached.

The possible conditions under which CaOx nucleation can occur are illustrated in Figure 1.2 below.

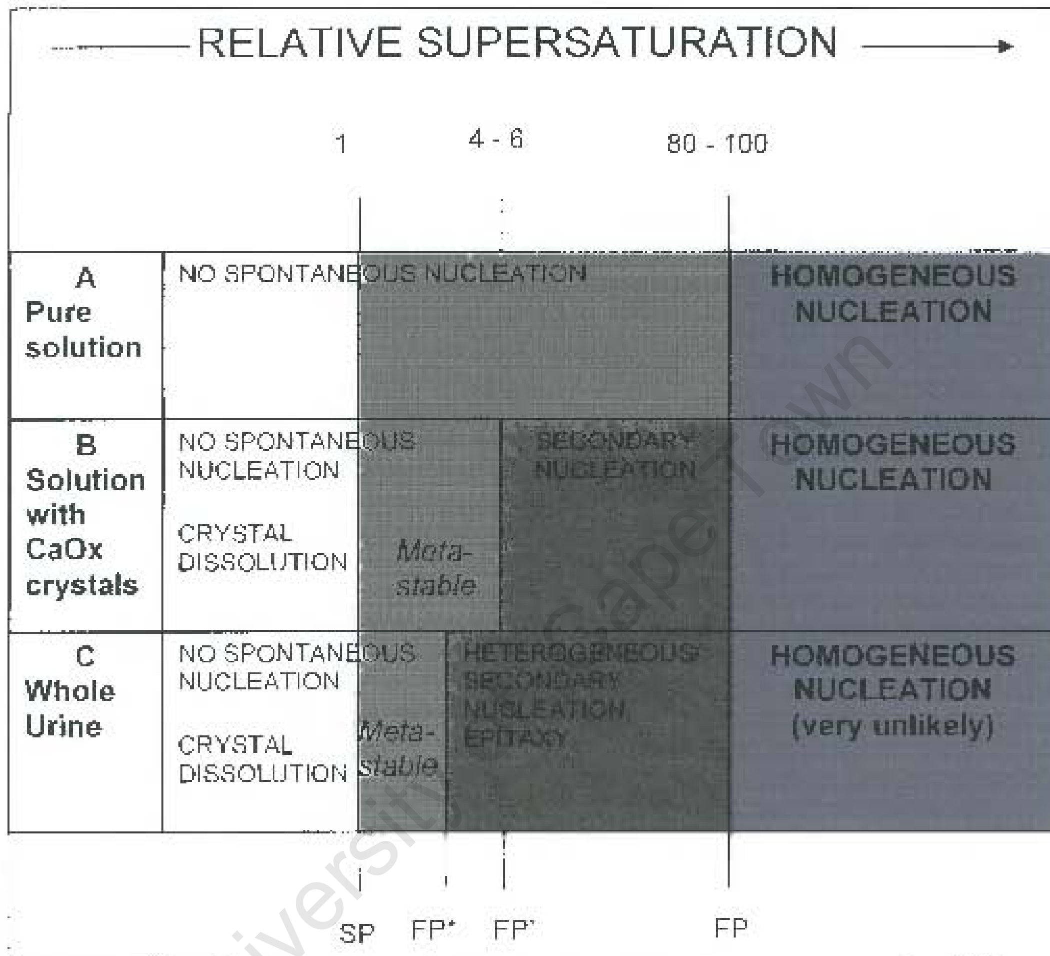

Figure 1.2: Relative supersaturation and CaOx crystal nucleation. A: perfectly clear aqueous solution of CaOx. B: Perfectly clear aqueous solution containing CaOx crystals. C: Whole urine containing foreign surfaces and crystals (Hess and Kok 1996)

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1.2.3 Crystal Growth

Kidney stones can form by either growth or by aggregation of individual crystals, or by simultaneous growth and aggregation of small crystals into large particles of clinically significant size (Finlayson 1978, Fleisch 1978). Crystal growth is the process whereby new crystal components formed in urine add onto an already existing nucleus of a significant size. The incorporation of new crystal components into a crystal lattice is very complicated (Hess and Kok 1996). In order for crystal growth to occur, the newly formed crystal in the urine has to travel through the solution and stick to the crystal. This process is called bulk diffusion. The surface reaction mechanism has been observed to be the one controlling the growth of COM crystals and therefore the hydrodynamic conditions in urine do not have any influence on its growth (Sohnel and Grases 1995).

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1.2.4 Crystal Aggregation

The process whereby crystals stick together to form a large particle is called aggregation. There are two kinds of aggregation mechanisms in kidney stone formation, namely primary and secondary aggregation (Sohnel and Grases 1995). The former is a result of a crystal malgrowth that is found on the surface. Other crystals are then attached to this pre-existing crystal and form an aggregate (Hess and Kok 1996). In secondary aggregation, the free or independent particles suspended in solution collide with each other. This is induced by Brownian motion of particles or shear forces acting in a liquid (Hess and Kok 1996). Colliding particles remain attached or can be separated by hydrodynamic forces. Resulting aggregates are composed of weakly bound, randomly orientated individual crystals and display a disordered structure which is very different from the structure of particles formed by primary aggregation. Normal urine inhibits the aggregation of small CaOx crystal (Fletcher *et al* 1970), thereby suggesting that this is a normal defense against the formation of stones.

If the crystalline solid dissolves then the process of aggregation is defeated and a stone cannot be formed via this mechanism. On the other hand, if the crystalline solid grows into a larger mass then a stone is likely to be formed.

Once it starts, the process of aggregation is very quick (Robertson and Nordin 1982). It can however be slowed down by the repulsive zeta potential. This is the repulsive electrostatic surface charge that keeps the crystalline particles disaggregated (Hess and Kok 1996). In urolithiasis research, an increase in the zeta potential is interpreted as an increase in the tendency to inhibit aggregation (Robertson and Scurr 1986).

1.3 Inhibitors

An inhibitor is any substance, molecule, ion or agent that retards any aspect (nucleation, growth or aggregation) of the crystallization of calcium salts in urine (Kok 1996, Ryall 1997). There have been several major reviews which describe all aspects of structure, classification, mode of action *in vitro* and *in vivo* studies (Worcester 1996, Ryall 1997, Khan and Kok 2004).

Briefly, inhibitors can be classified into two major groups, namely macromolecules (glycosaminoglycans and proteins) and micromolecules (citrate, magnesium, pyrophosphate, phosphonates and phytate).

1.3.1 Macromolecules

Glycosaminoglycans (GAGs)

GAGs are the most abundant heteropolysaccharides (long unbranched polysaccharides containing a repeating disaccharide unit) in the body (www.web.indstate.edu). The most common GAGs include hyaluronic acid (HA), dermatan sulphate, chondroitin sulphate (ChS), heparin, heparin sulphate and keratin sulphate. Among these, ChS is the most prevalent urinary GAG (Roberts and Resnick 1986). Several GAGS, together with proteins are found in the organic matrix which constitutes 2 - 3% of the stone weight (Stapleton and Ryall 1995).

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According to Ryall (1997), there is a lower concentration of GAGS in stone formers than in healthy individuals. This is of interest since different GAGs have been observed to affect different aspects of crystallization of CaOx and hence inhibit the formation of stones. Crawford *et al* (1968) stated that heparin, ChS and HA are known to affect precipitation of CaOx. GAGs, in particular ChS and heparin, are known to inhibit nucleation (Kohri *et al* 1989), growth (Robertson and Scurr 1986) and aggregation (Robertson and Scurr 1986, Scurr and Robertson 1986) of CaOx in urine. Heparan sulphate on the other hand enhances CaOx crystal nucleation (Shum and Gohel 1993) and inhibits aggregation (Suzuki and Ryall 1996) of CaOx crystallisation.

Urinary Proteins

The role of urinary proteins in urolithiasis has been extensively investigated (Worcester 1994, Worcester 1996, Wesson *et al* 2000, Ryall 2004, Chauvet and Ryall 2005, Ryall *et al* 2005). Proteins are produced by renal epithelial cells and gain access to the urine by glomerular filtration (Worcester 1996). Several urinary proteins have been shown to be inhibitors of CaOx crystallization. These include Tamm-Horsfall protein (THP), osteopontin (OPN), bikunin, nephrocalcin and urinary prothrombin fragment 1 (UPTF1). A brief description of these proteins is presented in the discussion below.

Tamm-Horsfall glycoprotein (THP)

THP is an acidic urinary glycoprotein. It is the most abundant protein in human urine (Kumar and Muchmore 1990). Its urinary excretion is 20 to 200 mg/d and has a monomeric molecular weight of 80000 daltons (Robertson and Peacock 1983). Some studies have identified THP in the matrix of calcium stones (Doyle *et al* 1991).

Studies using a spectrophotometric aggregation assay confirmed that THP is a potent inhibitor of crystal aggregation (Ryall *et al* 1991) in a concentration dependent manner, inhibiting aggregation by 50% at a concentration of 10^{-6} mol/L and virtually 90% at 5×10^{-8} mol/l (Hess *et al* 1989). On the other hand, it has been reported that THP has almost no effect on growth or nucleation (Worcester *et al* 1988, Hess 1991). It is also a potent inhibitor of CaOx crystal

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aggregation in undiluted, ultrafiltered urine (Ryall 1997). This is primarily achieved by steric hindrance rather than adsorption to the surface of the crystal (Ryall 1997). Hess has postulated that THP in stone formers has a different molecular structure to that which occurs in normal healthy subjects (Hess 1991).

In South Africa, studies involving THG from black and white candidates' urine have shown that THG inhibits nucleation and aggregation and that the protein obtained from black subjects is a more potent inhibitor of CaOx crystallization than the one from white subjects (Craig *et al* 2001). However, matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF) and amino acid analysis and tryptic finger printing digestion demonstrated that there was no difference in the composition of the protein (Craig *et al* 2000).

Osteopontin (OPN)

OPN is an acidic urinary glycoprotein. Its concentration in urine is 6×10^{-8} mol/l and it has a molecular weight of 32000 daltons (Worcester 1996).

OPN has the ability to retard CaOx crystal growth and in supersaturated solutions it inhibits spontaneous nucleation (Worcester *et al* 1992; Worcester and Beshensky 1995) as well as heterogeneous nucleation of CaOx on apatite. In metastable solutions of calcium phosphate, it inhibits hydroxyapatite crystal formation (Boskey 1995). Nishio *et al* (1999) reported that OPN is a strong inhibitor of CaOx growth. It stereospecifically binds to the surface of the crystals thereby retarding growth (Ryall 1997). Another study reported that the potency of OPN is conferred by the protein's phosphorylation (Robertson and Peacock 1983). Because OPN contains a series of 8 to 11 aspartic acid residues, polyaspartic acid (PA) has also been included as a model inhibitor in some crystallization studies as it exhibits stereospecific binding to the surfaces of crystals (Addadi and Weiner 1985).

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Bikunin (Inter-α-Trypsin Inhibitor)

Inter-α-trypsin inhibitor is a proteoglycan containing a covalently bound glyscosaminoglycan side chain (Dawson *et al* 1998). According to Dean *et al* (2000), the true physiological concentration range is 6.25 - 18nmol/l. Bikunin is the light chain of this larger proteoglycan and its molecular weight by SDS-PAGE is 35000 daltons (Worcester 1996). The source of bikunin is not yet known but it is thought to be derived from serum by filtration at the glomerulus. Bikunin is another glycoprotein found to have inhibitory activity with respect to CaOx crystal nucleation and growth (Atmani *et al* 1993).

Nephrocalcin (NC)

NC is an anionic, phosphorylated glycoprotein, which contains between 2 and 3 residues of γ - carboxyglutamic acid (Gla) per molecule (Ryall 1997). Its urinary excretion is estimated to be in the range of 1 to 20 mg/d. The monomeric molecular weight is approximately 14000 daltons (Worcester 1996).

NC inhibits growth of CaOx crystals in metastable supersaturated CaOx solutions *in vitro* (Worcester 1996). Its ability to inhibit crystal growth is believed to be through blockage of growth sites in the crystal lattice structure. NC also inhibits CaOx crystal nucleation (Worcester 1996). As with most other macromolecules, inhibition of crystal aggregation appears to be related to changes in surface charges that occur when NC binds to crystals.

Urinary prothrombin fragment 1 (UPTF1)

Initial reports showed that UPTF1 was the principal component of CaOx urinary crystals (Doyle *et al* 1991). Its reported molecular mass is 31000 daltons. It is also present in calcium stones (Stapleton *et al* 1996). UPTF1 concentration in urine only rises in response to lithogenic conditions (Ryall 1997). It is known to inhibit both CaOx crystal growth and aggregation in undiluted, ultrafiltered human urine (Ryall *et al* 1995, Webber *et al* 2002) and its effect on CaOx crystal aggregation depends on the concentration of the protein (Ryall *et al* 1995). The inhibitory activity of UPTF1 is imparted by its

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10 γ-carboxyglutamic acid residues which bind Ca in urine (Grover and Ryall 2002).

In recent studies, UPTF1 was isolated from the urine of black and white South African subjects and after being purified, its potency of inhibition of CaOx crystallization was tested in various crystallization experiments (Durrbaum *et al* 2001, Webber *et al* 2002). The results showed that there was quantitatively more UPTF1 in the crystals in the urine of black candidates than that in the white subjects and that UPTF1 from black subjects' urine was more potent than that from white subjects.

1.3.2 Micromolecules

Citrate

Citrate is a potent inhibitor of all aspects of CaOx crystallisation. Citrate has been reported to retard the process of nucleation (Doremus *et al* 1978, Schwille *et al* 1999), growth (Ryall *et al* 1981; Bek-Jensen *et al* 1996) and aggregation (Kok *et al* 1987, Tiselius *et al* 1993a and b). Citrate forms a soluble complex with calcium, which reduces the concentration of free calcium in urine thereby reducing the risk of CaOx crystallization (Rodgers *et al* 2005). Citrate also binds to the surface of crystals and obstructs the attachment of the newly formed deposits in urine (Hallson *et al* 1982, Ryall *et al* 1985).

Owing to its functionality, a number of citrate supplements are now used to remedy the disease. These include potassium citrate (K-Cit) (Pak *et al* 1985, Pak *et al* 1986, Whalley *et al* 1996), calcium sodium citrate (Ca-Na-Cit) (Schwille 1997), sodium potassium citrate (Na-K-Cit) (Schwille *et al* 1987 and Ogawa 1994) and potassium-magnesium citrate (K-Mg-Cit) (Ettinger *et al* 1997).

Magnesium

Owing to the competition between magnesium and calcium to bind oxalate, and to the fact that magnesium oxalate is more soluble than CaOx (Hallson *et al* 1982), the saturation of CaOx in urine is dependent to a certain extent on

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urinary magnesium levels (Lindberg *et al* 1990). *In vitro* and *in vivo* studies conducted by Kohri *et al* (1988), have demonstrated that magnesium is an effective inhibitor of both nucleation and growth of CaOx crystals.

Pyrophosphate

Pyrophosphate is a strong inhibitor of aggregation of CaOx crystals (Robertson *et al* 1973, Robertson *et al* 1974, Felix *et al* 1977). According to Ryall (1997), it also inhibits nucleation, growth and a combination of growth and aggregation in an inorganic solution. It inhibits these processes by binding calcium in its solid phase. Pyrophosphate preferentially and irreversibly binds to COM crystal surfaces and not COD (Shirane and Kagawa 1993).

Phosphonates

These compounds are characterised by P-C-P bonds. One group known as the imidophosphates, however, has a P-N-P bond and has been reported to be a very effective inhibitor of CaOx crystallisation (Robertson and Fleisch 1970). In addition, diphosphonates were also found to possess similar properties after being tested in a number of systems (Francis 1969; Ohata and Pak 1973; Pak 1976).

It is the discovery of polyphosphates (Fleisch and Neuman 1961) and phosphorylated inositols (Thomas and Tilden 1972) that raised the possibility that other inorganic phosphate (Pi) molecules could also have CaOx crystallisation inhibitory capacity and hence be used therapeutically. One such molecule is myo-inositol hexakisphosphate or phytate. Since this molecule is found in fibre-rich foods which have been known for a long time to be consumed by South African blacks while whites ingest refined foods, it is of particular interest in the context of the difference in stone incidence in South Africa's two population groups. As such, it is discussed in detail below.

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1.4 Phytate

Phytate (IP_6) is the salt of phytic acid (myo-inositol hexakisphosphate). It is a substance of relatively low molecular weight (660 g/mol) and is present in blood, urine, interstitial and intercellular fluids (French *et al* 1991, Bunce *et al* 1993, Grases and Llobera 1996, March *et al* 1998). Phytic acid is the principal store of phosphate in plants such as beans and grains. Rich sources include wheat oats, bran and flaxseed, which contain approximately 3% phytic acid (www.Phytochemicals.infor). It constitutes 10 - 30g/kg of the dry matter of cereal, legume and oilseeds and is the major portion of the total phosphorus in the seeds of legumes (Burbano *et al* 1995).

Phytate interacts with basic amino acids, inhibiting a number of digestive enzymes (Reddy *et al* 1982). During digestion in the human gut, phytic acid is enzymatically hydrolyzed by phytases to lower inositol phosphates such as inositol pentaphosphates (IP_5), inositol tetraphosphate (IP_4), inositol triphosphate (IP_3) and possibly the di- and monophosphate (IP_2 and IP_1) (Burbano *et al* 1995). It is not clear how much of IP_6 is freely soluble; however, its concentration is high in some cells (30 - 100µmol/l (French *et al* 1991, Bunce *et al* 1993). In plants, IP_5 has been identified as the immediate precursor of IP_6 (Stephens and Irvine 1990, Brearley and Hanke 1996). It is not known whether a similar situation exists in animal cells.

Some studies have demonstrated that phytate is also naturally present in human urine and normal levels range between 0.5 and 6.0 mg/l (Grases and Llobera 1996, March *et al* 1998). Of considerable interest in the context of urolithiasis is that the urinary concentration found in a group of active CaOx stone-formers was significantly lower than that of a group of healthy people (Grases *et al* 2000a). It has been found that urinary phytate mainly depends on its dietary intake; when phytate is totally eliminated from the diet, the urinary levels immediately fall and after several days become undetectable (Grases and Costa-Bauza 1999, Grases *et al* 2000b). Ingestion of phytate significantly reduces the risk of pathological calcifications such as renal calculi (Grases *et al* 1998a, Conte *et al* 1999, Grases *et al* 2000a).

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A number of important beneficial effects of IP_6 on human health have been noted. It takes part in a number of important processes within the cell (Glennon and Shears 1993, Irvine 1995, Sasakawa *et al* 1995). IP_6 is an important antioxidant (Hawkins *et al* 1993), protects against cancer (Shamsuddin 1995, Shamsuddin *et al* 1997) and heart disease (Jariwalla *et al* 1990), and has hypocholesterolemic effects (Trowell 1972, Trowell 1973).

However, there is still some controversy and uncertainty regarding the biological role of phytic acid (Irvin 1995, Harland and Morris 1995, Sasakawa *et al* 1995). According to Sandberg *et al* (1989), IP_6 (and IP_5) has a negative effect on the bioavailability of minerals. That is, it forms insoluble (chelate) complexes with minerals such as Cu^{2+}, Zn^{2+}, Fe^{3+}, and Ca^{2+} (March *et al* 1998). For this reason, IP_6 has long been considered an antinutrient (Grases *et al* 2001). This antinutrient effect, however, is manifested only when large quantities of IP_6 are consumed in combination with an oligoelement diet (Cullumbine *et al* 1950, Kelsay 1987, Sandstrom *et al* 2000).

1.4.1 Metabolism, Tissue Distribution, Absorption and Excretion

The metabolism of IP_6 was first studied by Sakamoto *et al* (1993). Radiolabelled IP_6 was given to rats and it was observed that phytate was quickly absorbed by the stomach and distributed throughout the body to various tissues. Vucenik and Shamsuddin (1994) also reported that human malignant cells absorb and metabolize IP_6.

Grases *et al* (2000b) carried out a similar study on two groups of six male Wistar rats for a period of 100 days (7 phases) to study the relationship between oral ingestion (absorption) and urinary excretion of IP_6. One group served as the control and the other as the test group. The control group fed on rat chow pellets for the duration of the study while the treated group was at first given an IP_6 free diet. 24h urine samples were collected and analysed for IP_6 every 3 – 5 days. After observing a constantly lower IP_6 urinary concentration in the treated group, IP_6 was then administered to the rats in increasing amounts until a constant IP_6 urinary concentration was attained. Even though the urinary IP_6 concentration increased following ingestion of the

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IP_6-rich diet, it eventually reached a peak excretion level which could not be exceeded despite the ingestion of additional quantities of IP_6.

As a follow up on the rat study, Grases *et al* (2001) administered both an IP_6-poor diet (IP_6-PD) and an IP_6-normal diet (IP_6-ND) to a group of seven healthy volunteers during the first and second period of a pharmacokinetic study. Urinary levels of IP_6 were then determined during the two periods of study. While following IP_6-PD and IP_6-ND for 15 and 16 days, respectively, urine samples were collected every day at 0, 2, 4, 6, 8 hours after an overnight fast to determine the IP_6 urine content. The results showed a dependence of IP_6 urinary concentration on its oral ingestion. The average concentration of IP_6 determined while the candidates were following IP_6-PD was lower than when the same subjects were on IP_6-ND. The urinary levels of IP_6 decreased to around 75 - 80% of the values normally found in humans after an IP_6-ND protocol. Both the disappearance and return of phytate to its normal urinary concentration was noticed to be very slow, requiring more than 10 days.

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1.4.2 Structure

The phytic acid molecule (Fig. 1.3) consists of 6 PO_4^{3-} groups, each of which is bonded to an oxygen atom which is itself bonded to a cyclohexane ring.

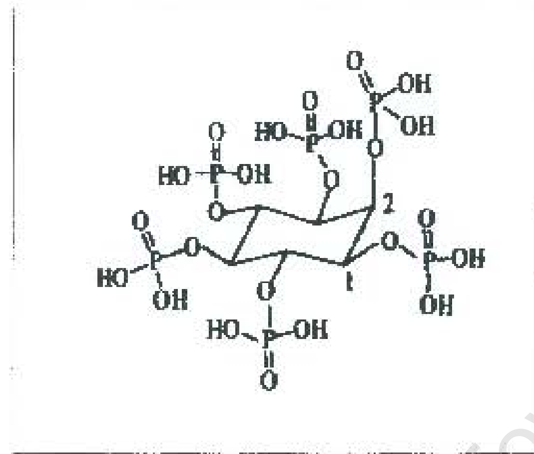

Figure 1.3: Phytic acid structure (Johnson *et al* 1995)

1.4.3 Inhibitory Activity Towards Stone Formation

Phytate has been shown to be an inhibitor of CaOx crystallization. This has been attributed to the affinity of its phosphate groups for calcium ions. Furthermore, the ring structure of phytate causes significant disturbances (steric hindrance) of CaOx and calcium phosphate crystal nucleation and growth (Grases and Costa-Bauza 1999) through adsorption processes. Several in vitro and in vivo studies on phytate have been carried and will be separately described in detail in the following discussion.

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1.4.4 In Vitro Studies

Because kidney stone disease is a multifactorial disease, it has always been difficult to mimic the conditions that lead to the formation of stones in the kidneys. However Grases and Costa-Bauza (1999) set up devices that simulated the conditions closest to the ones in the kidneys in their study when analysing the potency of inhibition of growth of CaOx and $Ca_3(PO_4)_2$ by phytate. In their study they monitored the growth of CaOx and $Ca_3(PO_4)_2$ on pig bladder epithelial tissue in the presence and absence of 1µg/ml IP_6 (1.5µM). One probable reason for using an inert material (pig bladder) was to

eliminate the effect of a heterogenous surface. In the presence of IP_6, a significant and total inhibition of CaOx and $Ca_3(PO_4)_2$ growth was observed. On the other hand, when the study was conducted in the absence of phytate, the amount of crystals of CaOx and $Ca_3(PO_4)_2$ was relatively higher.

Grases and March (1989) investigated crystallization rates in the presence and absence of phytate in urine. The rate of growth of CaOx crystals was high in the absence of phytate and phytate's inhibitory activity was observed to be pH dependent. In the presence of IP_6 and at high pH, the inhibition of crystalline growth was significant. However, when the negative charge on the molecule was reduced by protonation (i.e. at lower pH), inhibition decreased. This can be ascribed to the neutralisation of the negative charge on phytate which would prevent its chelation to Ca^{2+}. The inhibition of CaOx crystallization by IP_6 therefore depends on pH and its concentration.

Chow *et al* (2004) used an *in vitro* model of stone growth to study the effect of phytate. The artificial urine in which the stones were grown was supersaturated with CaOx and dosed with phytate. At final concentrations of 2.5 μM and 5 μM, stone growth was totally prevented, while at 0.5 μM it was inhibited by 50%.

The effect of phytate on CaOx stone growth was again investigated in a subsequent study, in addition to its effect on Ca binding and, CaOx crystallization (Saw *et al* 2005). IP_6, when present at micro-molar concentrations similar to those found in urine, had a negligible effect on Ca in solution and had no observable effect on crystallization. However, CaOx stone growth inhibition was confirmed at physiological concentrations of IP_6. The authors proposed that inhibition of stone growth is achieved through surface effects since IP_6 had no effect on solution calcium.

A recent study showed that phytate enhances the potent effect of trace metals such as Fe^{3+} in the inhibition of CaOx crystallization (Munoz and Valiente 2005). In their study, the authors prepared artificial urine with some trace metals at their physiological concentrations. The inhibitory activity of the latter

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was compared in the presence of some urinary inhibitors such as phytate, pyrophosphate and citrate. In addition to its ability to inhibit CaOx crystallization, Fe^{3+} inhibitory activity became even more enhanced in the presence of phytate and pyrophosphate than in the presence of citrate. This was attributed to the affinity of phytate for the CaOx crystal surface and its ability to form stable complexes with Ca^{2+}, instead of Fe^{3+} in urine. Citrate on the other hand, formed the complex with Fe^{3+}, thereby nullifying its inhibitory properties towards CaOx crystallization.

1.4.5 Animal Studies

Grases and Costa-Bauza (1999) administered ethylene glycol to induce CaOx crystallisation and studied the effect of dietary phytate on urolith development in an animal model (Grases *et al* 1998a). Urinary phytate was determined in control and phytate-treated groups. At the end of the experiment, the kidneys were removed and macroscopically and microscopically examined for possible crystal or stone locations. In the group of rats treated with phytate, the number of calcifications on the papillary tips and the total calcium in the papillary tissue were significantly reduced when compared with the control groups who were treated exclusively with ethylene glycol.

In another study. Grases *et al* (2000c) fed female Wistar rats AIN-76A (rodent diet). There is a purified and non-purified form of this food substance. The former does not contain phytate while the latter does. In their study, Grases *et al* (2000c) divided these rats into three groups of 12. The first group was administered purified AIN-76A, the second group was given purified AIN-76A mixed with 1% phytate dodecasodium salt (1g of phytate per 100g of purified AIN-76A) while the last group was fed non-purified AIN-76A. The animals were fed for a period of 12 weeks. When the measurements on the urinary phytate content were performed, IP_6 could not be detected in the first group while the urinary phytate content in the second and third group was not significantly different, but significantly higher than in the first group. Concentration of calcium and phosphate were also observed to be higher in the kidneys of rats in group 1 than in groups 2 and 3. These observations led

the authors to conclude that absence of phytate in AIN-76A is one of the causes of the renal calcification in female rats.

1.4.6 Human Studies

36 stone-formers (gender not stated) were employed in the study executed by Grases and Costa-Bauza (1999). 17 of the candidates were treated with phytic acid (120 mg/day) for 15 days while the other 19 served as the control candidates. At the end of the 15-day period, urine was collected and analysed. It was demonstrated that deposits of CaOx and calcium phosphate did not form in the phytate-treated group thereby suggesting that the risk of stone formation was greatly reduced.

In another study, (Conte *et al* 1999), seventy-four active calcium oxalate stone-formers (gender not stated) were split into three groups of 19 (group 1), 38 (group II) and 17 (group III). A lithogenic risk test for having calcium stones in the three groups of stone-formers was carried out at baseline. Thereafter, group I was not treated, group II was treated with potassium citrate (6.48 Gm/day) and group III with phytate-rich diet (120 mg per day). After 15 days of treatment, another lithogenic risk test was performed and it was observed that the risk for developing calcium stones in groups II and III was greatly reduced. The risk was reduced in 7% of patients in group I, 52% in group II and 50% in group III.

When the concentration of IP_6 was measured in stone-formers and healthy individuals (Grases *et al* 2000a) it was observed that the stone-formers had lower urinary IP_6 concentration. In the light of these two studies (Conte *et al* 1999, Grases *et al* 2000a) it can be speculated that the lower urinary IP_6 concentration in the urine of the stone-formers is a possible contributory factor to renal stone disease and that dietary IP_6 may have the potential to be used as a conservative therapeutic intervention.

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1.5 Aims and Objectives

As stated earlier, the incidence of kidney stone disease in South African blacks is extremely rare while in the white population it occurs with a frequency of 10 – 15%. Dietary differences have been considered as playing a role in this regard. The black population's diet consists mainly of fibre-rich food while that of the whites is more highly refined (Modlin 1981). Since phytate occurs mainly in fibre-rich foods such as oats, it can be hypothesized that the relatively higher dietary intake of this substance in the black group leads to relatively higher urinary concentration of phytate and hence contribute towards stone rarity in this group. In order to test this hypothesis the following objectives were defined:

i. to test published methods for their efficacy for urinary IP_6 analysis
ii. to design, develop and test a more efficacious protocol, if necessary
iii. to use the new protocol to measure free-diet urinary phytate concentration in rural black and urban white subjects
iv. to investigate the effects on urinary phytate of low and high phytate dietary protocols in black and white urban subjects with a view to establishing whether the two race groups have different handling mechanisms for this substance
v. to investigate the effects on *in vitro* urinary CaOx crystallization processes of low and high phytate dietary protocols in black and white subjects with a view to establishing whether the relative risk of stone formation in the two population groups changes as a consequence of the phytate challenge.

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CHAPTER II

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CHAPTER II

DEVELOPMENT OF A METHOD FOR THE MEASUREMENT OF IP_6 IN URINE

2.1 Introduction

Many methods have been developed for the determination of IP_6 in foods (Talamond *et al* 1998, Talamond *et al* 2000), plants (Burbano *et al* 1995) and biological samples such as plasma (March *et al* 2001) and urine (March *et al* 1998, Grases *et al* 2000, March *et al* 2001, Grases *et al* 2004). These are based on the colorimetric method of Heubner and Stadler (Holt 1955). In this method, IP_6 is extracted and then precipitated using ferric chloride (Davies and Reid 1979, Tangendjaja *et al* 1980). The concentration of IP_6 is then determined by analysing phosphorus or iron in the precipitate. Since these methods cannot distinguish between IP_6 and other partially phosphorylated inositols, they are regarded as inadequate. This has led to the application of other advanced techniques such as high performance liquid chromatography (Talamond *et al* 2000), inductively coupled plasma atomic emission spectroscopy (Grases *et al* 2004) and photometry (March *et al* 1998).

A brief description of various techniques is given in the following paragraphs.

2.1.1 High Performance Liquid Chromatography (HPLC)

This method is used in the direct determination of inositol (Perello *et al* 2004). It is specifically designed for determination of IP_6 in food (Talamond *et al* 1998). As with most other methods used to analyse IP_6, a separation process is required before the actual detection is embarked upon. This is achieved by gradient elution on an anion exchange column using a conductivity detector (Talamond *et al* 1998).

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The study of IP_6 by the HPLC method requires a sequence of mutually dependent steps (Burbano *et al* 1995), namely:

- Effective extraction
- Extract purification
- Separation of individual compounds
- Detection and quantification.

The advantage of this method is that even if different inositols are retained by the column, they can all be analysed separately. Inositol tri- to hexakisphosphates can all be detected and quantified by their relative retention times under isocratic conditions (Burbano *et al* 1995).

According to Scott (cited in Talamond *et al* 2000), the method which is based on separation using anion exchange and a high sensitivity detection system, is regarded as useful for phytate analysis. In addition, the following advantages over other published methods have also been cited (Talamond *et al* 1998):

- There is negligible sample preparation and no derivatisation or precipitation is required.
- Evaporation of sample extract takes less time and precision is improved by reducing the final solubilization volume.
- The retention time of phytic acid is only 6 minutes (Talamond *et al* 1998) as compared to longer retention times on other chromatography methods.

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Finally, the method is sensitive and selective, and can be rapidly and easily performed. It is therefore suitable for routine determinations.

2.1.2 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

This method is used in clinical laboratories for the analysis of IP_6 in urine (Grases *et al* 2004). It primarily requires pre-treatment of urine to achieve separation of PO_4^{3-} from IP_6. This can be accomplished using an anion

exchange resin. When a urine sample is passed over the column, IP_6 is strongly bound to the resin and most of the PO_4^{3-} is eluted. In order to fully separate the two species, the latter is washed out using HCl and the former using H_2SO_4.

Hydrolysis is normally used to dephosphorylate IP_6 by heating in an acidic medium for approximately 36 hours in an oven. The same result can be achieved by heating the IP_6 solution for seven cycles of 2.5 minutes in a microwave oven at 900W (March *et al* 1998). The products of hydrolysis are myo-inositol and PO_4^{3-}. The evaluation of IP_6 is then achieved through analysis of phosphorus in PO_4^{3-} using ICP-AES and an appropriate calibration graph (Grases *et al* 2004).

2.1.3 Gas Chromatography Mass Spectroscopy (GCMS)

Compared to all other published analytical methods, this approach shows the best sensitivity in the determination of IP_6 (March *et al* 2001). Unlike ICP-AES, this method is used to determine phytate through the inositol after derivatisation. Trimethylchlorosilane (Jansen *et al* 1986), heptafluorobutyrylimidazole (Kennington *et al* 1990) and trifluoroacetic anhydride (Haga and Nakajima 1989) are some of the derivatization reagents that have been used.

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2.1.4 High Performance Ion Chromatography (HPIC)

High performance ion chromatography (HPIC) is used to measure IP_6 in food and biological samples. In contrast to most approaches, HPIC does not require prepurification of the samples. It is considered to be precise, simple, sensitive, rapid and reliable in the determination of IP_6 (Talamond *et al* 2000).

2.1.5 Modification of an Indirect Extraction/Photometry Method

The method is called indirect because the evaluation of IP_6 is achieved by the spectrophotometric analysis of PO_4^{3-} using an appropriate calibration curve. After separation of IP_6 from PO_4^{3-} in urine using an anion exchange resin, IP_6 is hydrolysed to release inositol and PO_4^{3-} by heating either in a microwave

oven or a conventional heat oven. One mole of IP_6 releases 6 phosphate groups when fully hydrolysed. Ammonium molybdate is added to form a complex with the liberated PO_4^{3-} ion and tin chloride is used to reduce the phosphomolybdic acid complex to molybdenum blue formation (Vogel 1991). The resulting blue colour is indicative of the presence of PO_4^{3-} and this is used to indirectly determine IP_6 (March *et al* 1998) using a phytate calibration curve.

Owing to the availability of the equipment and simplicity of the indirect method, this approach was adopted for the determination of IP_6 in urine in the present project.

2.2 Purity Verification of IP_6

All reagents used were of analytical-reagent grade. They were all purchased from Sigma-Aldrich. MilliQ water was used throughout.

The commercial IP_6 (Sigma-Aldrich, Germany) employed in the present study was the sodium salt of phytic acid ($C_6H_{12}O_{24}P_6Na_6$). Four methods were used for purity verification: pH, NMR spectroscopy, ion chromatography and hydrolysis.

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2.2.1 pH Measurement

Introduction

The dodecasodium salt of phytic acid was used in this study. The salt is derived from a weak acid (phytic acid) and a strong base (Na). The pH of the solution made from this salt was expected to be greater than 7. A pH value equal to or below 7 would indicate that not all the hydrogens of the phytic acid are being replaced by Na in sodium phytate.

Method

Five milligrams of IP_6 salt were dissolved by stirring in 50 ml of water and the pH was measured (Microprocessor pH meter, Hanna instruments) at room temperature.

Results and Discussion

The pH of the IP_6 salt solution was found to be 5.63. A number of factors could have contributed to this. When this molecule dissociates in water, sodium ions and protons are liberated. The presence of H^+ renders the solution acidic. The lower the concentration of H^+, the higher the pH of the solution. The implication therefore is that some of the phosphate groups of the phytic acid salt are protonated.

However, it should be noted that measurement of pH is not a quantitative way of assessing sample purity as any slight contamination could change the pH drastically when the system is unbuffered. A better method therefore would be to add excess acid and do a back titration with base or to titrate directly with acid.

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Conclusion

The pH of the solution suggests that not all of the protons in IP_6 have been replaced by Na^+ ions. In other words, the sample used in this study is not a pure salt, but a weak acid that dissociates into myo-inositol, Na^+ and H^+ ions in solution. Confirmation of the presence of myo-inositol ions by NMR spectroscopy is necessary as their presence would have to be taken into account in quantitative analyses.

2.2.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

Introduction

There are two possible conformations of IP_6 (Barrientos *et al* 1996) as shown in Figure 2.1. The first (A) is sterically stable and unhindered and this has one axial phosphoric acid and five equatorial (1ax/5eq) phosphoric acids. The

second (B) is hindered and sterically unstable and has five axial and one equatorial phosphoric acid groups (5ax/1eq).

According to the literature (Johnson *et al* 1995, Barrientos and Murphy 1996), the conformation of IP_6 changes with pH. When the pH is below 9.0, five phosphoric acid groups take up equatorial positions while one is axial. However, when the pH is increased above 9 the phosphoric acid groups are deprotonated. Then the molecule flips and adopts a new conformation in which only one phosphoric acid group is in the equatorial orientation.

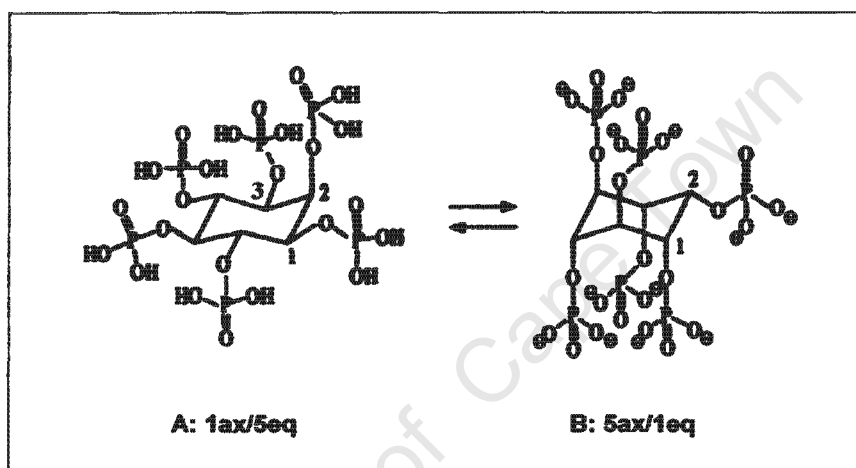

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Figure 2.1: Conformations of phytic acid (Johnson *et al* 1995)

When the phosphoric acid groups are deprotonated, repulsion between the two adjacent charged groups increases. The effect is that the molecule adopts a new conformation with five phosphates in axial positions and one in an equatorial position. Stabilization by coordination with sodium and hydrogen bonding with water takes place in order to minimise the electrostatic repulsion between the five di-anionic phosphate groups (Haga and Nakajima 1989).

The aim of performing NMR spectroscopy using both one and two-dimensional techniques was to identify the conformation of IP_6 and hence establish its purity by comparison with spectra obtained in previous studies.

Method

Twenty milligrams of IP_6 salt was dissolved in 0.6ml of deuterium oxide (D_2O). An NMR spectrum of hydrogen (^1H) was obtained at 300MHz using a Mercury 300 spectrometer (SMM instrumentation, Darmstadt, Germany). Carbon (^{13}C) and the heteronuclear single quantum correlation (HSQC) spectrum of (^1H, ^{13}C) were obtained at 300 MHz while phosphorus (^{31}P) was acquired at 121.472 MHz using a Mercury 300 spectrometer (SMM instrumentation, Darmstadt, Germany). The chemical shifts were reported according to the proton signal of deuterated solvent for ^1H, ^{13}C (referenced to dioxane), ^{31}P (referenced to 85% H_3PO_4).

Results and Discussion

Figure 2.2 presents the ^1H NMR spectrum of the phytate sample used in this study, the signal assignments are displayed in Table 2.1 (with numbering as shown in Figure 2.1).

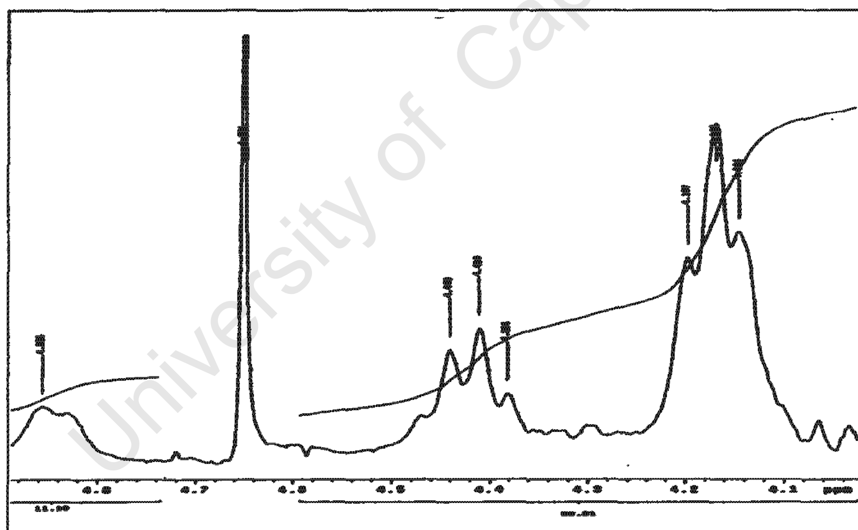

Figure 2.2: ^1H NMR spectrum of phytate sample used in this study

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Table 2.1: Assignment of ^{1}H NMR spectrum of IP_6

Chemical Shift/δ	Multiplicity	J (Hz)	Assignment
4.16	**triplet**	8.0	H1, H3, H5
4.42	quartet	8.7 and 17.7	H4, H6
4.85	broad doublet	-	H2

The equatorial H2 proton at δ 4.85 is in the vicinity of axial H1, H3 and phosphorus (P). Owing to the equatorial/axial relationship between H1 and the H2 and H3 protons, two doublets of the same frequency are produced. However, in addition to these two doublets, H2 couples with ^{31}P to produce the broad doublet observed. H4 and H6, which are magnetically equivalent are respectively adjacent to (H3, H5) and (H1, H5) as well as P. Both H4 and H6 are split into a quartet as they couple with two adjacent axial protons (J_{ax-ax}) and P (J_{H-P}).

The NMR assignments demonstrate that phytate is present in the 1ax/5eq conformation as shown in Figure 2.1A. This is consistent with the conformation reported by Barrientos and Murthy (1996), in which IP_6 adopts this conformation at pH 0.5 - 9.0. This conformation only changes to 5ax/1eq when the pH is above 9.0. The pH of the phytate solution used in the present study was measured and found to be 5.63.

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Figure 2.4 illustrates the complete ^{13}C NMR spectrum of the phytate sample used in this study.

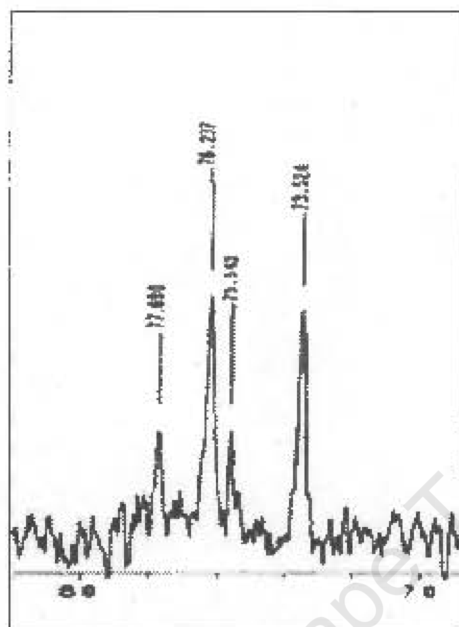

Figure 2.3: ^{13}C NMR spectrum of phytate used in this study

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The proton-decoupled ^{13}C NMR spectrum contains four singlets which were assigned by use of the HSQC plot shown in Figure 2.4.

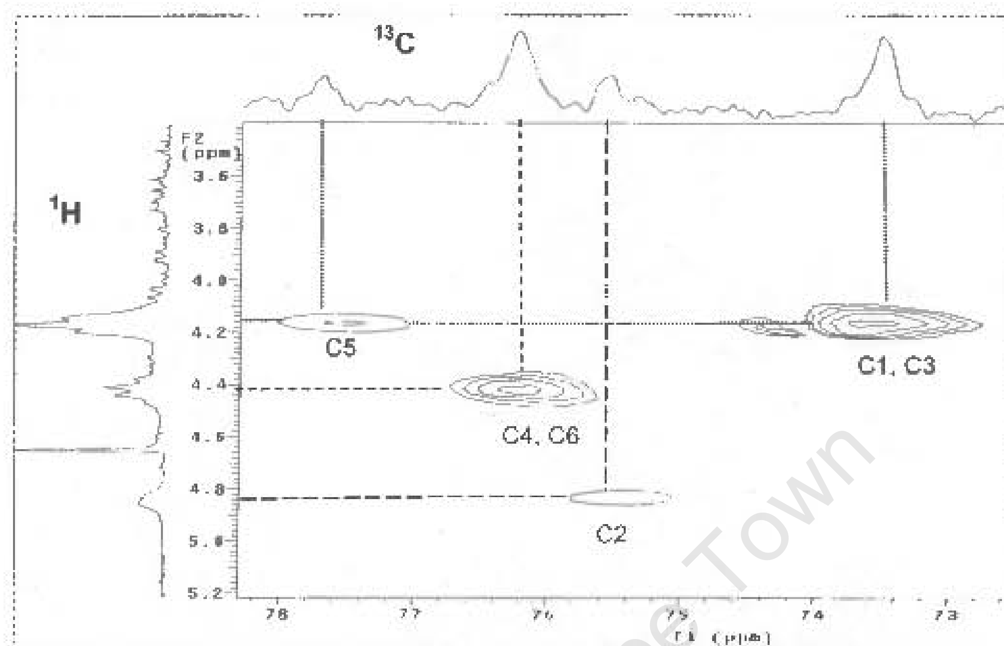

Figure 2.4: ^{1}H - ^{13}C **HSQC contour plot**

The two-dimensional proton - carbon correlation spectrum confirms that the proton triplet at δ4.2 results from protons attached to three carbons (C1, C3 and C5), two of which are equivalent (C1, C3).

Assignment of the carbon atoms in IP_6 is given in Table 2.2 below.

Table 2.2: Assignment of the ^{13}C NMR spectrum of phytate sample

Chemical Shift/δ	Multiplicity	Assignments
73.5	singlet	C1 and C3
75.5	singlet	C2
76.2	singlet	C4 and C6
77.7	singlet	C5

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The chemical shift of the equivalent carbon atoms C1 and C3 is different from the other equivalent pair C4 and C6 (refer to Figure 2.1 for numbering). Both C1 and C3 are adjacent to an axial P at C2, whereas C6 and C4 are adjacent to an equatorial P on C5. This results in greater deshielding of C1 and C3 than C4 and C6 which renders the two pairs of carbon atoms non-equivalent and explains why they resonate at different chemical shift values (Costello *et al* 1976).

The ^{31}P NMR spectrum of IP_6 used in this study together with the assignments is presented in Figure 2.5.

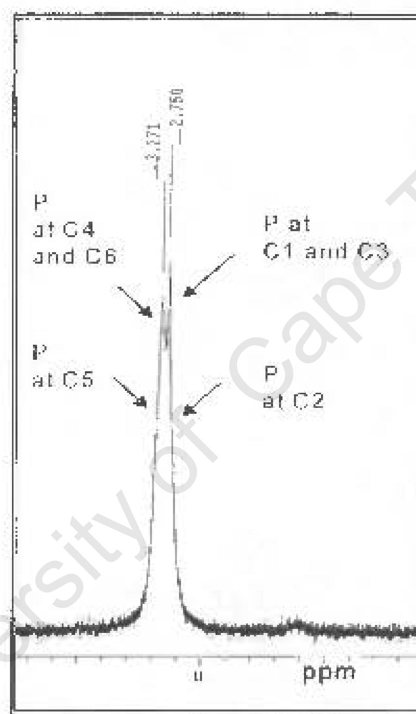

Figure 2.5: Phosphorus (^{31}P) NMR spectrum of phytate used in this study

The ^{31}P spectrum obtained in the present study shows an apparent doublet that appears at 3.00 ppm. Even though the above spectrum appears to be a doublet, it is probably a quartet where overlapping of 2 signals (indicated by arrows) has occurred. This proposal is supported by the observation made by Costello *et al* (1976).

When Costello and co-workers used ^{31}P NMR to study the properties of naturally occurring phosphorus-containing molecules, it was observed that all

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^{31}P NMR spectra differed considerably in position and appearance. This suggested that phytate ^{31}P NMR might be the sensitive to the medium used when conducting the study. It was subsequently shown that the appearance of the ^{31}P NMR spectra differed with the ions contained in the medium. Therefore, in order to validate the comparison, counter-ions present in the medium should also be taken into consideration.

Since sodium hexakisphosphate (myoinositol) was used in the present study, the effect of the counter ion sodium must be considered. When sodium is the counter-ion, two differences are observed:

- The chemical shift values resonate at fields lower than those in the presence of other counter ions such as ammonium.
- The individual signals are grouped more tightly and hence there appears to be a small difference in the chemical shift values leading to overlap of the signals (Costello *et al* 1976).

An example of ^{31}P NMR spectrum of myoinositol hexakisphosphate with sodium as the counter-cation in the pH range of 4 - 10 is shown in Figure 2.6. The pH of the phytate solution used in the present study (5.63) falls within this range.

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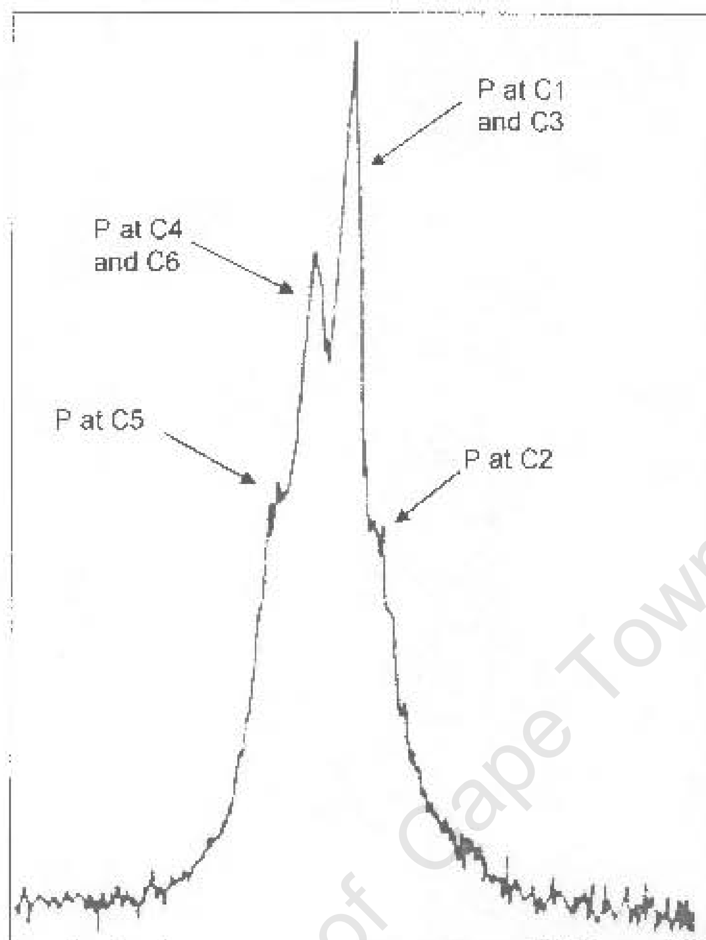

Fig. 2.6: ^{31}P NMR of IP_6 in the presence of sodium as the counter-ion (Costello *et al* 1976)

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The down field signal is due to P at C2 (deshielded) and the high field one is due to C5 P (shielded equatorial) (Johnson and Tate 1969).

The six P groups are dependent on the pH and ionic strength of the solvent, in particular (C1 and C3) and (C4 and C6). The signal pertaining to C1 and C3 was observed to be most affected by ionic strength and least by pH. On the contrary, C4 and C6 signal was affected mostly by pH and least by ionic strength. The two other signals due to P at C2 and C5 were least affected by changes in the ionic strength and pH (Costello *et al* 1976).

Owing to the above-mentioned reasons, it is concluded that what appears to be a doublet in Figure 2.6 is actually a quartet with ^{31}P resonance signals in

the ratio of 1:2:2:1. The two ^{31}P resonance signals due to P at C2 and C5 are not clearly resolved.

^{31}P is a very useful technique to analyse phosphates. However in this case there was severe signal overlap making the analysis difficult. A solution to this problem would be to use lanthanide shift reagent but it was not available in the present study.

Conclusion

All the three spectra (^{1}H, ^{13}C, ^{31}P) and ^{1}H - ^{13}C NMR (contour plot) obtained in the present study supported the previously published analysis and assignments of IP_6. This confirms the identity of the sample characterised as IP_6 and indicates that it is found in the sterically stable conformation with 1 ax/5eq phosphoric acid groups.

2.2.3 Ion Chromatography

Introduction

The purpose of this experiment was to identify ions (impurities) that might be contaminating the commercial IP_6 salt solution. The method was used also to provide quantitative analysis of these anions.

Method

IP_6 salt was dissolved in water to give a solution of concentration 100ppm. The solution was then passed once through Millipore filters (0.45µm) to remove particulate matter.

Samples were analysed by high performance anion exchange chromatography (HPAEC) using a DIONEX BioLC system. This system was coupled with: AS50 autosampler, LC30 oven, EG50 eluent generator, GS50 gradient pump, ED50 electrochemical detector (conductivity mode) and chromelian software. The operating conditions were as follows:

Column: IonPac AS11-HC (2mm x 250mm) analytical column and
IonPac AG11-Guard (2 x 50mm)

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The flow rate was 0.38 ml/min and the total run time was 18 min.

Eluent: Gradient elution 10mM - 75mM NaOH

Conductivity detection with suppression (suppressor current 100 mA)

Sample loop: 25 µl

Results and Discussion

The chromatographs (Figure 2.7) show phytic acid standard solutions of increasing concentrations. The peaks corresponding to PO_4^{3-} and IP_6 are marked in red and green, respectively, on the chromatogram.

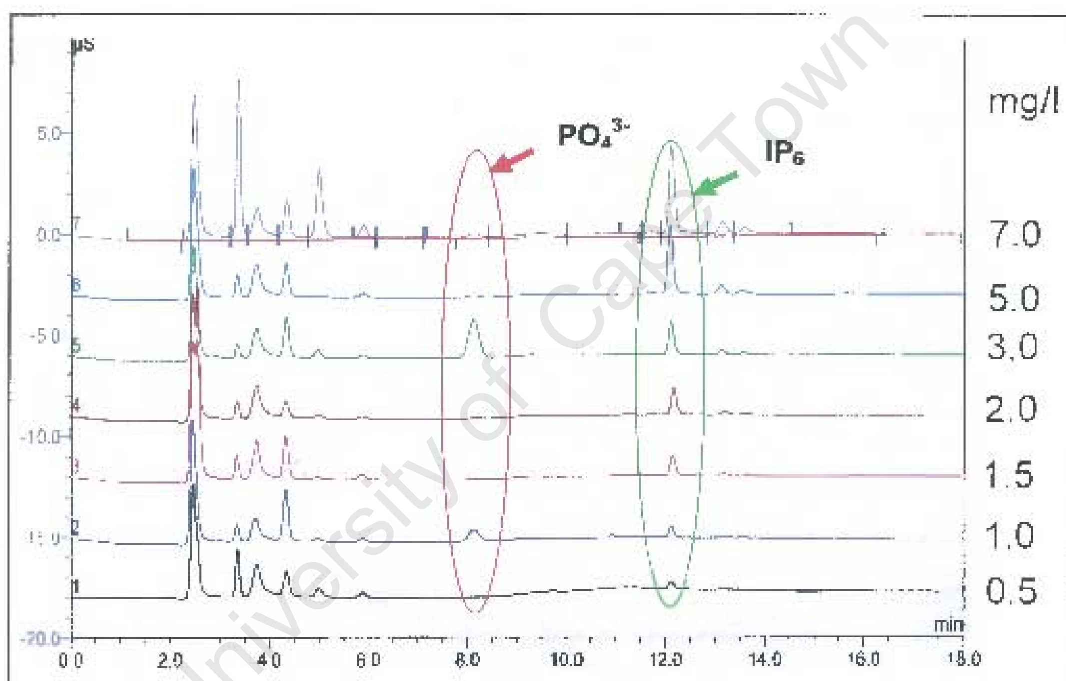

Figure 2.7: Ion chromatography of different phytate standard solutions

The peaks between 2 and 6 minutes represent ions from the solvent (MilliQ water) used because these peaks appear when water is run alone. The identified ones include sulphate, fluoride, chloride, nitrite, bromide and nitrate (identified by comparison with standards of sulphate, fluoride). (Since MilliQ water has high purity, the impurities could have come from the columns or the glassware used). Phosphate ions are present in the phytic acid standard solutions in different concentrations. The only peak showing a proportional

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increase in area is the one representative of the dodeca-anion of phytic acid (12 min). The size of phosphate peaks in Figure 2.7 can be said to be surprising in the sense that it differs in different standard solutions. Its concentration does not increase with the increase in the concentration of IP_6 salt.

Figure 2.8 shows the chromatogram of ions contained in a given phytic acid standard solution. The height of the peak is proportional to the concentration of the respective ion.

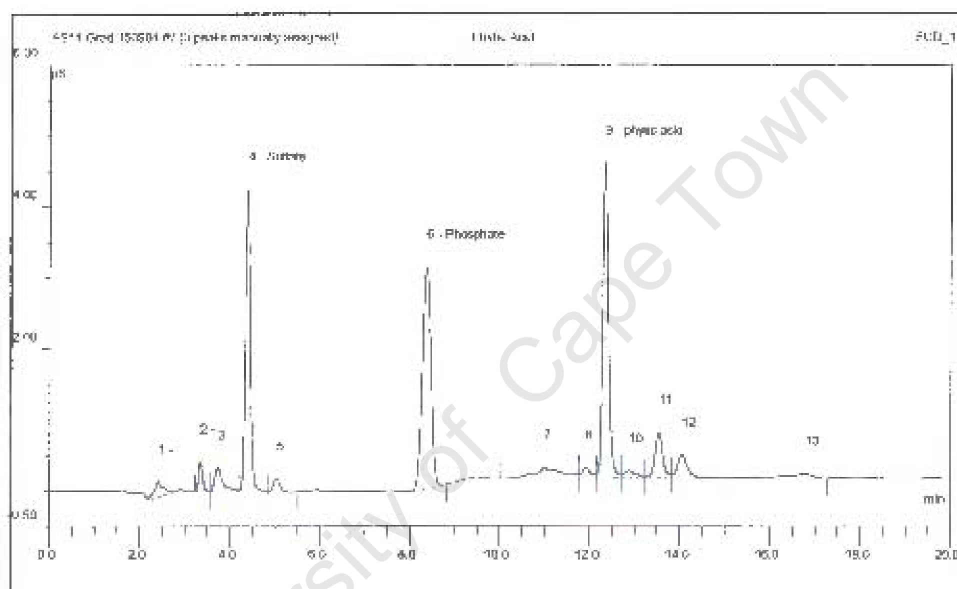

Figure 2.8: Chromatogram of ions contained in a given phytate standard solution

Peaks between 2 - 6 minutes are ions from the solvent (water). Peak at 12.3 minute is phytic acid. Smaller peaks in the same region are probably due to phytic acid isomers. The peak at 8.37 is for the free phosphate ion.

Conclusion

Since the intensity of the impurities does not increase linearly with concentration of phytate (Figure 2.7), it is then concluded that contamination is not in the phytate sample.

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2.2.4 *Hydrolysis*

Introduction

The hydrolysis experiment was used to determine the percentage composition of IP_6 in a given IP_6 salt solution by quantifying the PO_4^{3-} liberated after IP_6 hydrolysis. Myo-inositol hexakisphosphate can be converted to myo-inositol by heating the molecule in an acidic medium. Six phosphate ions are produced as a by-product (Figure 2.9).

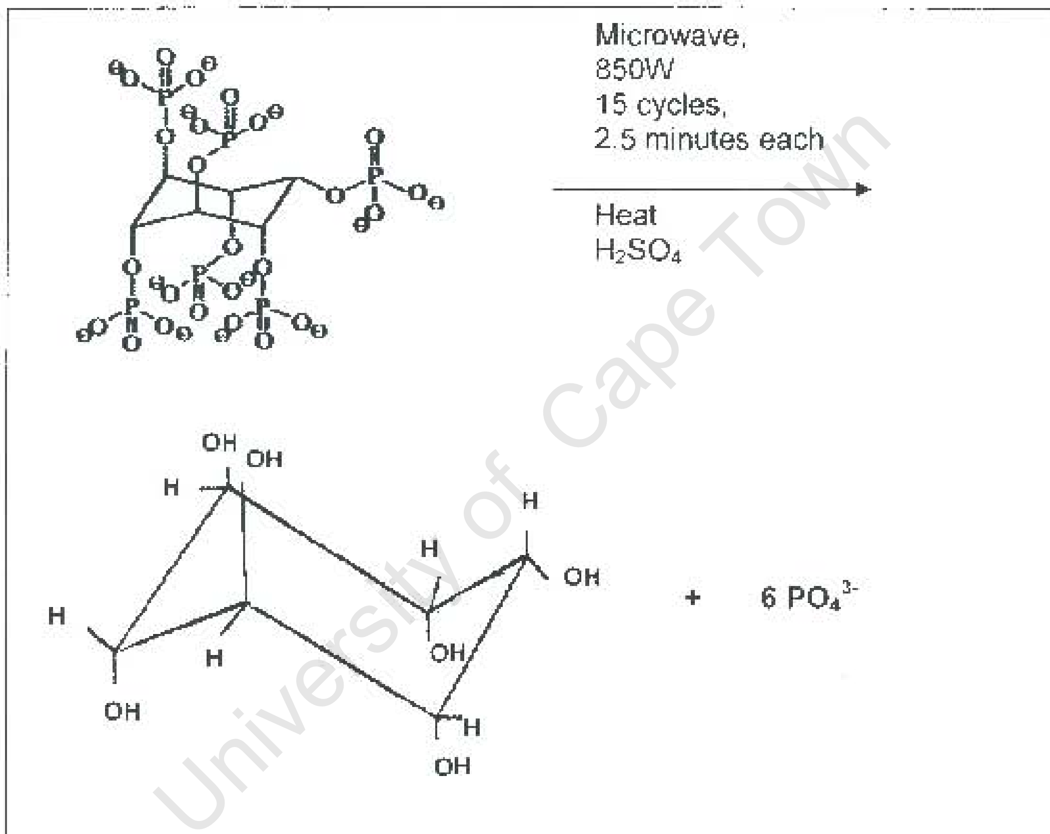

Figure 2.9: Hydrolysis of IP_6 to myo-inositol and phosphate

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When a molybdate solution is added to this mixture (i.e. myo-inositol and phosphate ions), a phosphomolybdic acid complex (yellow) is formed. This complex can be reduced further by addition of $SnCl_2$. The product that results from this reduction process is a blue phosphomolybdate complex (Figure 2.10).

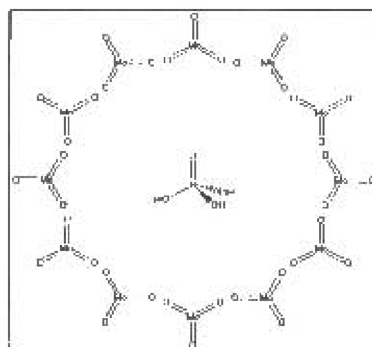

Figure 2.10: Structure of phosphomolybdocomplex (www.science.csumb.edu)

The intensity of the resulting blue solution can be used to measure the concentration of phosphate spectrophotometrically. Absorbance is measured at 715nm and is directly proportional to concentration in accordance with Beer-Lambert's Law.

This method has not only been used to measure the concentration of phosphate, but has also been used to determine indirectly the concentration of other phosphate-containing molecules such as IP_6.

Method

In order to determine the optimum number of cycles required to ensure complete hydrolysis of phytate, eight 10-ml PTFE vials containing 2.6ml of 2.5mg/l phytate were heated in a microwave (850W). Vials 1 and 2 were removed after 7 cycles of 2.5 min each, vials 3 and 4 after nine cycles, vials 5 and 6 after 12 cycles and the last two were removed after 15 cycles. All the vials were then allowed to cool to room temperature and phosphate was determined in each. After measuring the absorbance of the extracted organic solutions, a graph of absorbance versus the number of cycles was plotted.

Results and Discussion

Figure 2.11 shows a graph of absorbance as a function of the number of hydrolysis cycles.

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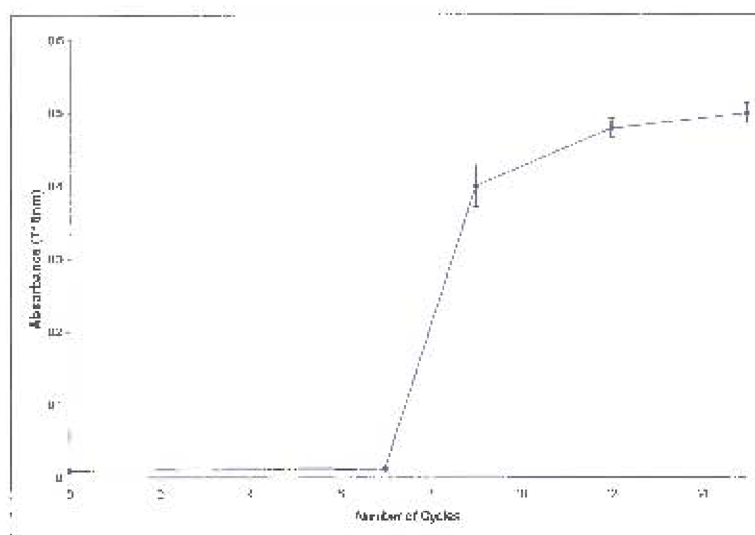

Figure 2.11: The mean absorbance values of PO_4^{3-} solutions after hydrolysis of an IP_6 standard solution

According to the graph, there was virtually no free phosphate detected in the unhydrolysed solution (0 cycles). After seven cycles of hydrolysis, the phosphate concentration was very low as indicated by the very low absorbance value. A sharp increase in phosphate concentration was observed after seven cycles. When the number of hydrolysis cycles was increased to 12, not much difference was noted in the absorbance. Absorbance values tended towards a plateau thereafter.

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Conclusion

Following the observation made in Figure 11, it was concluded that 12 cycles would be sufficient to hydrolyse phytic acid to myo-inositol and 6 PO_4^{3-}. However, even though there was a negligible increase in absorbance when the hydrolysis was performed for 15 cycles, the latter was decided to be the optimum number of cycles to be used in order to ensure thorough IP_6 hydrolysis.

2.2.5 Hydrolysis of Commercial IP_6 Salt for Purity Investigation

Five milligrams of IP_6 salt was dissolved in 1 litre of water. An aliquot of volume 2.6ml of this standard solution was hydrolysed by heating in a polytetrafluoroethylene (PTFE) vial in a microwave (Bauer, 850W) for 15

cycles of 2.5 min each. The vials were allowed to cool for 20 min between cycles to avoid overheating. After the fifteenth cycle, the sample was allowed to cool to room temperature and phosphate was determined as described below (phosphate determination experiment).

Phosphate Determination

The assay described by March *et al* (1998) was used in this study. A standard curve was constructed using concentrations of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 7.0 mg/l solutions of commercial phytate. These were hydrolysed for 15 cycles of 2.5 min each and were treated as described below. The absorbance of phosphate ions liberated from phytate in each aliquot was measured and plotted against concentration.

Two millilitres of PO_4^{3-} containing solution was removed from the PTFE vial, and 0.8 ml of 0.03M ammonium molybdate ($(NH_4)_6Mo_7O_{24}4H_2O$) and 0.2 ml of 9.2 mM $SnCl_2$ were added. After 20 min, 1.8 ml of 1.6 g/l Adogen 464 (Sigma-Aldrich, Germany) in ethyl acetate was added to extract the phosphomolybdate complex formed. Addition of the extractant resulted in the formation of two phases. The top, organic layer was separated from the aqueous bottom layer using a Gilson pipette and the absorbance was measured at 715nm using a UV-spectrophotomer (Spectronic Unicam). The absorbance reading obtained was then used to determine the concentration of IP_6 from the calibration curve of absorbance versus phytate concentration.

This experimentally determined value for the concentration of IP_6 was then compared to the theoretical one and the percentage composition of the commercial IP_6 was calculated. The experiment was performed in duplicate.

Results and Discussion

The commercial IP_6 salt was used in the present study to prepare standard solutions of IP_6. The formula of the salt is $C_6H_{12}O_{24}P_6Na_6$ and its molar mass is 792 g/mol. When solutions of the salt are prepared it dissociates into $6H^+$ and $6Na^+$ and the dodeca-anion of phytate (IP_6). Since the molar mass of IP_6

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is 648 g/mol, the theoretical percentage of phytate ion in the commercial phytic acid salt is 81.8%. The experimentally determined percentage composition value is shown in Table 2.3.

Table 2.3: Hydrolysis experiment

IP_6 salt(mg/l) solution	Ave abs ± SE (715nm)	IP_6 (mg/l) (Experimental)	% Composition
5.00	0.915 ± 0.145	3.79	75.8

The difference between the theoretical value of 81.8% and the experimentally determined value of 75.8% is likely due to the presence of phosphate, sulphate, fluoride, chloride, nitrite, bromide and nitrate anions which were detected by ion chromatography in the present study. This percentage composition would then be taken into consideration when all the relevant calculations are made.

Conclusion

The dodeca-anion of phytic acid constitutes 75.8% of the salt's mass used in any experiment.

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2.3 Test of the Accuracy of Indirect Extraction/Photometry Method

2.3.1 Phosphate Calibration Curve

Introduction

Phosphate concentration can be measured in solution using ammonium molybdate as described earlier. The aim of carrying out this experiment was to determine whether the absorbance of phosphate solutions is directly proportional to the phosphate concentration and hence, whether this method can be used in the present study.

Method

Phosphate standard solutions of concentration 0.0, 3.0, 6.0, 9.0, 12.0, 18.0, 30.0 and 42.0 mg/l were prepared using di-sodium hydrogen phosphate (Na_2HPO_4) (monobasic) in MilliQ water. The phosphate concentration of each standard was determined in duplicate as described above (phosphate determination experiment). A graph of the average absorbance value for each standard was plotted against concentration.

Results and Discussion

Figure 2.12 shows the calibration curve obtained for the determination of phosphate.

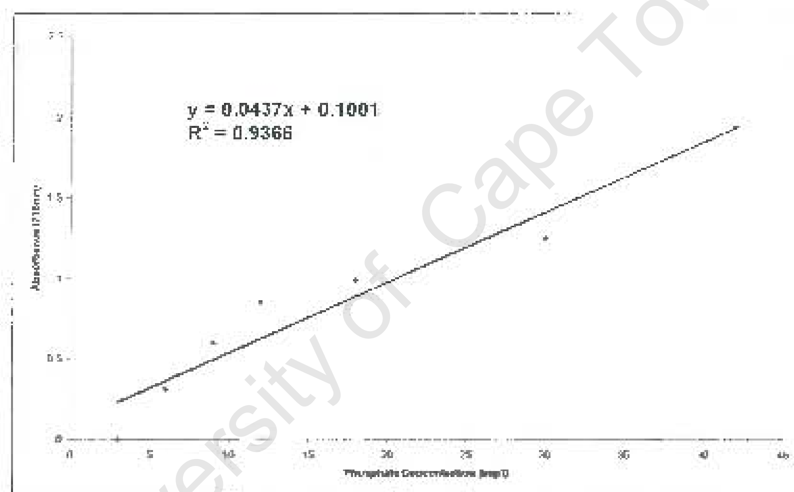

Figure 2.12: Phosphate calibration curve prepared using standard phosphate solutions

Conclusion

The absorbance increased linearly with the phosphate concentration. This demonstrates that the determination of PO_4^{3-} (and hence IP_6) using the method described would be sensitive and accurate.

However, it is necessary to be wary of the fact that urine contains many molecules that may release PO_4^{3-} upon exposure to high temperatures and

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that this contamination may influence the concentration of IP_6 detected by this method.

2.3.2 IP_6 Calibration Curves

Introduction

The first step was to measure the absorbance of the free phosphate in the unhydrolysed IP_6 standard solutions. The second step was to hydrolyse aliquots of the same standards, measure the absorbance and draw a calibration curve. The final step was to use the calibration curve to measure the concentration of IP_6 in urine after hydrolysis.

Method

Non-hydrolysed IP_6

IP_6 standards (0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 7.0 mg/l) were prepared using the commercial sodium salt of IP_6. 2.0ml was withdrawn from each solution and the phosphate concentration was determined as previously described (phosphate determination experiment). The experiment was performed in duplicate. A standard calibration curve was constructed by plotting average absorbance values against concentration.

Hydrolysed IP_6

2.6ml of each IP_6 standard solution was pipetted into a 10ml PTFE vial. These were heated in a microwave oven for fifteen cycles of 2.5min each. After allowing the vials to cool to room temperature, phosphate was determined (phosphate determination experiment). The experiment was performed in duplicate. A standard calibration curve was constructed by plotting the average absorbance values against concentration.

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Results and Discussion

Phytate (Unhydrolysed) Calibration Curve

Figure 2.13 shows the absorbance of the free phosphate ion contained in commercial phytate.

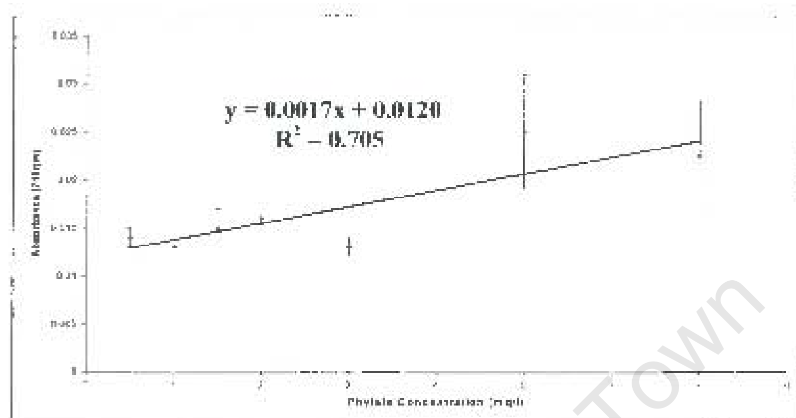

Figure 2.13: Phytate calibration curve prepared using unhydrolysed phytate standard solutions.

The graph demonstrates there was a variation in the content of free phosphate in the different phytate standard solutions. However, all of the absorbances were very low. The expected PO_4^{3-} absorbance in all standards was zero if IP_6 was pure, but the result in Figure 2.11 indicates that the commercial phytate contains a small amount of free phosphate. This result supports the observation made in Figure 2.7 where the amount of PO_4^{3-} determined by ion chromatography differed in a series of IP_6 standard solutions.

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Phytate Hydrolysed Calibration Curve

Figure 2.14 shows absorbance of phosphate ions in solutions of various phytate concentrations after hydrolysis.

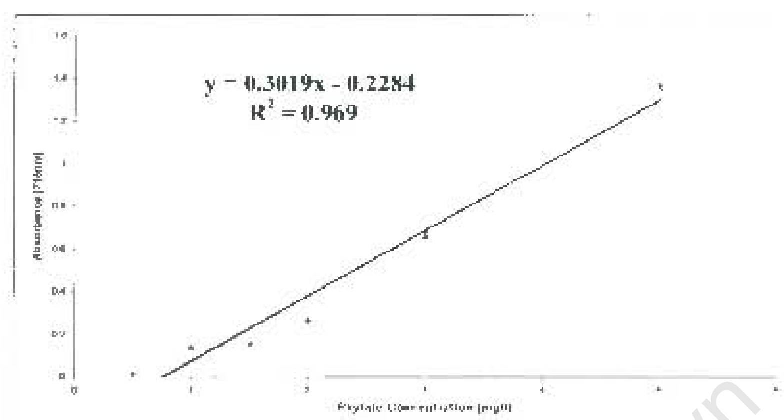

Figure 2.14: Phytate calibration curve prepared using hydrolysed phytate standard solutions

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As expected, the graph shows a linear increase in the mean absorbance values as the concentration of the hydrolysed phytate is increased. Therefore the aliquots after hydrolysis had different concentrations of phosphate ions. On the other hand, a negative absorbance would indicate total absence of phosphate ions (hence phytate) in solution

Conclusion

The method is sensitive to free phosphate only. In order to accurately determine IP_6 concentration using PO_4^{3-}, IP_6 should be fully hydrolysed as no bound PO_4^{3-} groups would be detected by the method.

2.4 Anion Exchange Chromatography

Introduction

When an eluent is applied onto a stationery phase (resin), the ions in the eluent are adsorbed onto the surface of the resin. If the surface of the resin is positively charged (anion exchange column) then all the oppositely charged ions in the eluent are attracted while the ions with a similar charge are eluted. Of the ions of the opposite charge, that is, those retained by the column, the

force with which they are held to the resin depends on the charge they bear. 1 mole of dodeca-anion of phytic acid has a charge of 18- whilst PO_4^{3-} has a charge of 3-. This would result in some IP_6-ion being strongly bound while PO_4^{3-} is weakly bound to the resin. With time, the weakly bound ions are eluted by the mobile phase from the column followed by the strongly retained ions. This property has been used to separate ions contained in a solution and is the principle of anion exchange chromatography.

Method

The resin used in these experiments was an anion exchange Dowex 1 x 8; 200 – 400 mesh Cl, supplied by Sigma-Aldrich. Before packing the column, the resin was dried in an oven for 3 days at 27°C until it was free-flowing (Vogel 1978). 1.5g of the resin was then weighed and used to form a slurry (mixed with 25 ml 0.05M HCl) that was added to a column packed with cotton wool at the bottom. Care was taken to ensure that no trapping of air bubbles or channelling occurred. 2 ml HCl covered the top of the resin bed to prevent drying.

MilliQ water was used as a solvent in all the water-based solutions. HCl was used to wash out PO_4^{3-} ions from the column (0.4ml/min flow rate). H_2SO_4 (0.2ml/min flow rate) was used to elute the more strongly retained IP_6. A peristaltic pump was used to control the flow rate of the column.

Two approaches towards the effective separation and elution of IP_6 from the anion exchange column were tested.

1. Use of a high concentration of HCl to elute the retained PO_4^{3} ions from the column as quickly as possible while retaining the bound IP_6.

   Use of a high concentration of H_2SO_4 for the efficient elution of IP_6 from the column.

   The optimal HCl and H_2SO_4 concentrations and volumes were experimentally determined (as described in the following paragraphs).

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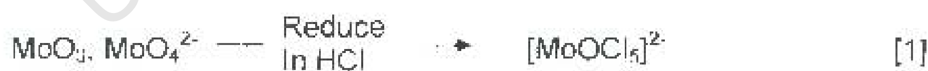
2. Use of a low concentration of both HCl and H_2SO_4 as cited by March *et al* (1998).

2.4.1 Determination of Optimal HCl Concentration Required to Elute PO_4^{3-}

A phosphate solution of concentration 1.0 g/l (approximate concentration of PO_4^{3-} in urine) was prepared by dissolving di-sodium hydrogen phosphate (Na_2HPO_4) in water. Aliquots (5 ml) of this solution were applied to six anion exchange columns. 50ml of HCl standard solutions (0.30, 0.35, and 0.40M) were passed through columns 1 to 3 respectively in order to elute the retained PO_4^{3-}. The final 5ml of the 50ml eluants was collected. Aliquots (2ml) of the latter were used for the determination of PO_4^{3-} (phosphate determination experiment). The experiment was repeated in columns 4 - 6. A plot of absorbance versus HCl concentration was constructed (Fig. 2.16).

It has been reported that the molybdenum oxides (MoO_3 and MoO_4^{2-}) can be reduced in HCl to form $[MoOCl_5]^{2-}$ (reactions 1 and 2 below) (Cotton and Wilkinson 1980). When a suspension of these molybdenum oxides is treated with reducing agents such as stannous (Sn^{II}) ions, an intense blue colour is achieved (www.rsc.org/chemcomm).

When the highest concentration of HCl was used, more of these oxides were reduced making the already blue phosphomolybdate colour even more intense. This will have interfered with the study.


$$MoO_3, MoO_4^{2-} \xrightarrow[\text{In HCl}]{\text{Reduce}} [MoOCl_5]^{2-} \qquad [1]$$

$$[MoOCl_5]^{2-} \underset{HCl}{\overset{H_2O}{\rightleftharpoons}} [Mo_2O_3Cl_8]^{4-} \qquad [2]$$

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Results and Discussion

The average absorbance values of phosphate obtained after elution of PO_4^{3-} from the column using different concentrations of HCl are shown in Figure 2.15. When the trendline was extrapolated to the abscissa, an optimal (lowest concentration of HCl that can be used to elute all the free phosphate from the column) concentration of 0.2M HCl was determined (Figure 2.15).

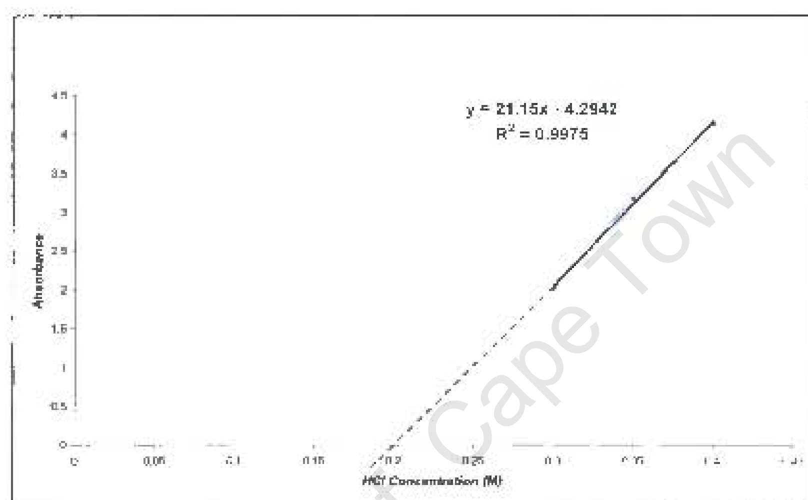

Figure 2.15: Plot of average absorbance vs concentration of HCl for the determination of optimum HCl concentration required to elute PO_4^{3-}

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Conclusion

Following the observation from Figure 2.15, the optimum concentration of HCl to be used is 0.2M.

2.4.2 Determination of Optimal Volume of HCl Required to Elute PO_4^{3-}

Five millilitres of phosphate solution of concentration 1.0 g/l was applied to eight anion exchange columns. Different volumes of 0.20M HCl (optimum HCl concentration required to elute PO_4^{3-}) were then passed through each column: namely 10, 15, 20, and 25ml. Duplicate aliquots of 2ml each were removed from the last 5ml of the acid eluted from each column and phosphate was

determined (phosphate determination experiment). A graph of absorbance versus volume was plotted.

Results and Discussion

Figure 2.16 shows the experimental results to determine the optimum 0.2M HCl volume used to elute PO_4^{3-} from the column.

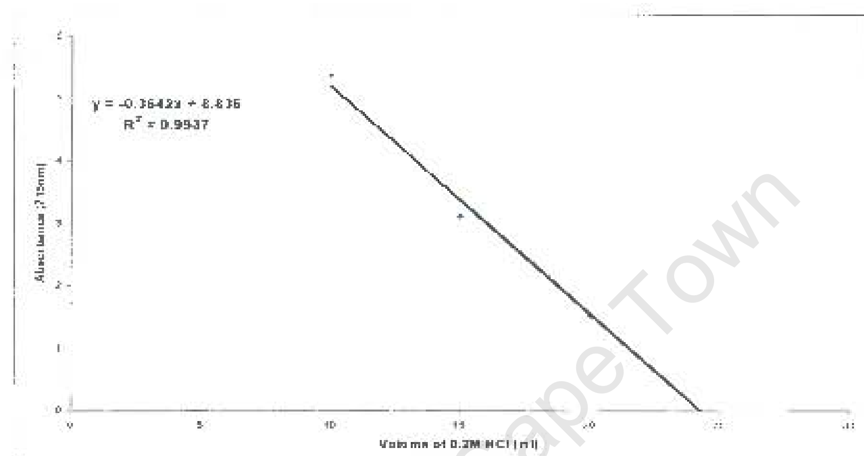

Figure 2.16: Plot of average absorbance vs volume of 0.2M HCl

The trendline crosses the x-axis at a volume of 24.26ml. Thus this is the volume of HCl that was required to elute all phosphate ions from the column. Represented in tabular form (Table 2.4), the absorbance after passing 25ml was found to be –0.17. This shows that there were no phosphate ions retained by the column.

Table 2.4: Determination of optimal volume of 0.2M HCl

0.2M HCl (ml)	Abs (3rd fraction)
10	5.37
15	3.12
20	1.53
25	-0.17

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Conclusion

The volume of 0.2M HCl to be used to elute PO_4^{3-} from the column is 24.26 ml. This volume was approximated to 24.3 ml.

2.4.3 Determination of Optimal H_2SO_4 Concentration Required to Elute IP_6

Sulphuric acid standard solutions of concentrations 2.5, 3.0, 3.5 and 4.0M were prepared. Eight anion exchange columns were set up. Five millilitres of a phytate solution (5.0 mg/l) was loaded onto each column and the eluate (first fraction) from each was discarded. 24.3 ml of 0.20M HCl solution (optimal volume and concentrations determined above) was then applied to each column. The flow through (2nd fraction) from each column was again discarded. Finally, 5ml of each concentration of H_2SO_4 was applied to duplicate columns and the eluants (3rd fraction) were collected.

The 3rd fractions from duplicate columns were mixed and vortexed. From the resulting 10ml solution from each pair, two 2.6ml aliquots were withdrawn and placed in two 10ml PTFE vials. These vials were heated in a microwave for 15 cycles of 2.5min each. After allowing the vials to cool to room temperature, phosphate was determined in each (phosphate determination experiment). The absorbance values at each concentration of H_2SO_4 were then averaged and plotted against concentration.

Results and Discussion

Figure 2.17 shows the plot of absorbance as a function of the concentration of H_2SO_4 which was used to determine the optimum acid concentration required to elute the strongly bound phytate from the column. The trendline crosses the x-axis at a concentration of 4.0M.

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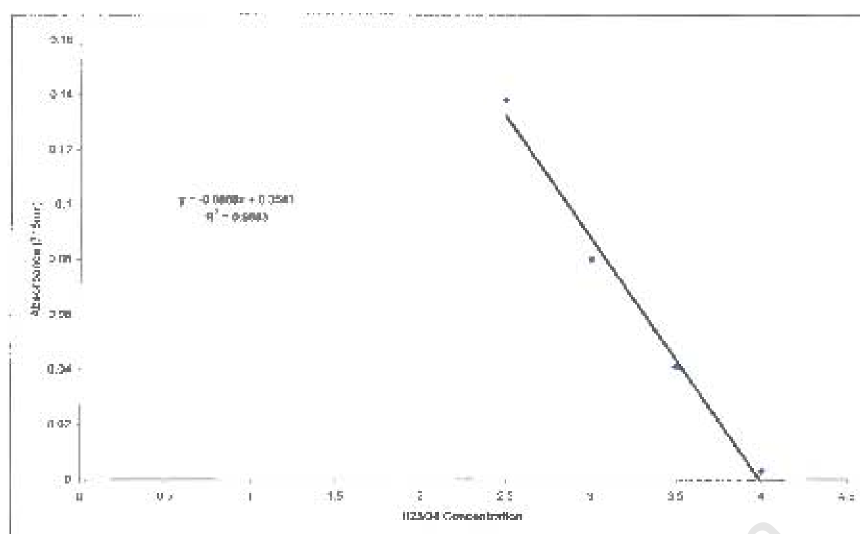

Figure 2.17: Absorbance as a function of the H_2SO_4 concentration

Conclusion

4M H_2SO_4 is the optimal concentration that should be used to elute the strongly bound IP_6 from the anion exchange resin.

2.4.4 IP_6 Standard Solution Application

The efficiency of the derived volume and concentration of HCl and H_2SO_4 solutions was tested. 5 ml of a 6.25 mg/l IP_6 salt solution was loaded onto the column. The first fraction was then collected. 24.3 ml of 0.2M HCl was applied onto the column and the eluate was collected. Lastly, 5 ml of 4M H_2SO_4 was loaded and the flow through was collected. All three fractions were then hydrolysed and phosphate was determined (phosphate determination experiment) and hence IP_6 concentration determined.

Results and Discussion

Table 2.5 shows the composition of the three fractions collected after applying a standard solution of IP_6 (6.25 mg/l) onto the anion exchange column.

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Table 2.5: IP_6 concentrations in the three eluates collected after IP_6 application.

Eluate	*ΔPO_4^{3-} Abs (715nm)	IP_6 (mg/l)	% Composition	
			Theoretical	Actual
1	0.044	0.905		
2	0.236	1.54		
3	0.753	3.25	52.0	**68.6**

*** Difference in mean absorbance of phosphate ion in hydrolysed and unhydrolysed sample**

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Of the three eluates, the third one is the most important. Eluate 1 should contain a minimal amount of phytate ion or be free of IP_6 and the second one should contain impurities separated from IP_6. The third fraction is the one expected to elute retained IP_6 from the column. The amount of IP_6 that is eluted in the third eluate is therefore divided by the amount applied onto the column to calculate percentage composition.

Considering eluate 3 only, the percentage composition of IP_6 was:

$$(3.25/6.25) \times 100$$
$$= 52\%$$

Since IP_6 salt contained 75.8% IP_6, the corrected percentage composition was:

$$52/0.758$$
$$= 68.6\%$$

Conclusion

The percentage yield of 68.6 is low. The effluents (HCl and H_2SO_4) used did not efficiently separate PO_4^{3-} from IP_6. Phytate must have been neutralized by H^+ (because high concentration of HCl was used to prepare the column)

and was not retained in the column. Therefore these concentrations cannot be used in the quantitative determination of IP_6 in urine.

2.4.5 *Testing the Separation and Elution of PO_4^{3-} and IP_6 at Urinary Concentrations*

The objective was to test whether the empirically determined optimum concentrations and volumes of HCl and H_2SO_4 would effectively separate PO_4^{3-} from IP_6 as well as elute IP_6 from the column when the latter two components are applied at their urinary concentrations. 5.0 ml of PO_4^{3-} solution (2.0 g/l) was mixed with 5.0 ml of IP_6 solution (10 mg/l). The final concentrations of PO_4^{3-} and IP_6 solutions were 1 g/l and 5 mg/l, respectively (approximate concentrations of PO_4^{3-} and IP_6 in healthy individuals' urine (Grases *et al* 2004)).

5 ml of the mixture was applied to each of two identical anion exchange columns followed by 24.3 ml of 0.20 M HCl (optimal volume and concentration). Finally, 5 ml of 4M H_2SO_4 (optimal concentration) was applied to each column to elute the strongly retained IP_6 from the resin.

In order to monitor the progress of IP_6 through the columns, the first, second and the third fractions were collected. All 3 fractions were treated as above (Optimal H_2SO_4 concentration required to elute IP_6). IP_6 was also determined in each fraction (phosphate determination experiment) and thus the percentage composition of IP_6 in each fraction could be calculated. The experiment was performed in duplicate.

Results and Discussion

Table 2.6 below gives details of the concentration of IP_6 determined in each fraction eluted from the column when a mixture of IP_6 and Na_2HPO_4 was applied.

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Table 2.6: Application of IP_6 and Na_2HPO_4 mixture onto the anion exchange column

Eluate	Δ PO_4^{3-} Abs (715nm)	IP_6 mg/l	% Composition	
			Theoretical	Actual
1	0.101	1.09		
2	0.021	0.83		
3	0.327	1.84	36.8	**48.5**

Percentage composition: (1.84/5.00) x 100
= 36.8%

As stated earlier, commercial IP_6 salt only contains 75.8 IP_6 therefore the actual recovery is

36.8/0.758
= 48.5%

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A possible explanation for the observed high concentration of IP_6 eluted in the first fraction is that the phytic acid anion might have become protonated by the highly concentrated HCl (2.0M) that was used to prepare the column. This would have neutralised the IP_6 ion thereby preventing it from being retained by the positively charged resin.

Conclusion

With regard to the amount of IP_6 eluted in the second eluent (Table 2.6), it is seen that 24.3ml 0.2M HCl failed to separate PO_4^{3-} from phytic acid. A large amount of IP_6 was eluted with PO_4^{3-} indicating that 0.2M HCl is not suitable. In view of the observed low percentage yields obtained, an alternative method was tested. Lower concentrations of HCl and H_2SO_4, namely 0.05M and 2M (March *et al* 1998), were therefore investigated.

2.4.6 *Use of Lower HCl and Lower H_2SO_4 Concentrations (March et al 1998)*

Determination of HCl Volume

5ml phosphate standard solution (1.0g/l) was applied to 7 columns. Thereafter 15, 20, 25, 30, 35, 40 or 50ml aliquots of a 0.050M HCl solution were applied individually to these columns to elute the retained PO_4^{3-}. In order to determine whether all PO_4^{3} eluted efficiently, 5ml of 2.0 M H_2SO_4 was applied to each column immediately after HCl. The 3 fractions collected from each column were tested for the presence of PO_4^{3-} (phosphate determination experiment) and the absorbance values were recorded. The experiment was repeated in columns 8 - 14, *i.e.* it was performed in duplicate.

Results and Discussion

The results of the study in which different volumes of 0.05M HCl were used to elute 1g/l PO_4^{3} from column are shown in Table 2.7. The lowest absorbance in the 3rd fraction indicates the amount of 0.05M HCl that effectively elutes free phosphate from the column.

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Table 2.7: Absorption values for phosphate eluted in fraction 3 after application of various volumes of 0.05M HCl

HCl volume (ml)	PO_4^{3} (Abs) (3rd fraction)
15	0.386
20	0.380
25	0.391
30	0.380
35	0.002
40	0.004
50	0.000

Conclusion

From table 2.7 it can be seen that 50ml of HCl eluted all the PO_4^{3-} that had been initially retained by the column.

2.4.7 PO_4^{3-} Application onto the Column

In order to test whether the derived volume would effect separation of PO_4^{3-} from IP_6 using an anion exchange column, 5 ml of 1 g/l PO_4^{3-} standard solution was applied onto the column. The flow through was collected. This was followed by application of 50 ml of 0.05M HCl. The second fraction was collected and subsequently, 2M H_2SO_4 was loaded onto the column and the third eluate was collected. The three fractions were all analysed for PO_4^{3-} (phosphate determination experiment) and the percentage composition was calculated.

Results and Discussion

Table 2.8 shows the various phosphate concentrations contained in the fractions collected from the column.

Table 2.8: Recovery of PO_4^{3-} after application of 1g/l PO_4^{3-} standard solution onto the column

Eluate	Abs (715nm)	PO_4^{3-} (mg/l)	% Composition
1	2.23	48.7	
2	4.05	904	
3	*Low	-	95.3

* Below detectable range of spectrophotometer

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From the equation of the standard calibration curve (Figure 2.12):

– 0.1001 = 0.0437x

x (concentration) = 48.7

In the same way, 4.05 – 0.1001 = 0.048x

x = 90.39

Since the second eluent was diluted 50 times by the HCl (hence dilution factor =10) then

90.39 x 10 = 903.9

The amount of PO_4^{3-} recovered is therefore, 903.9 + 48.7 = 952.6mg

In terms of percentage, 952.6/1000 x 100 = 95.3 %

The first fraction (i.e. the flow through) contained 48.7 mg/l PO_4^{3}. The majority of the phosphate was eluted from the column by 0.05M HCl. In the third eluate, the concentration of PO_4^{3-} was very low. The percentage composition was 95.3%.

Conclusion

50 ml 0.05M HCl efficiently elutes PO_4^{3-} from the anion exchange resin.

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2.4.8 IP_6 Standard Solution Application

After determining the optimal volume of 0.050M HCl required to elute all of the PO_4^{3-}, 5ml of 5.0mg/l IP_6 was loaded onto two newly prepared columns. The flow through (1st fraction) was tested for the presence of IP_6 (phosphate determination experiment). Fifty millilitres of 0.050M HCl were then passed through the column to elute any PO_4^{3} retained by the resin. The IP_6 concentration was determined in the 2nd fraction. 5ml of 2M H_2SO_4 was passed through the column to elute IP_6. IP_6 was determined in each fraction (phosphate determination experiment) and the percentage composition of IP_6 was also calculated.

Results and Discussion

Table 2.9 shows the amount of IP_6 determined in the three fractions collected from the column after application of 5 ml of a 5.0 mg/l IP_6 solution.

Table 2.9: Application of IP_6 standard solution

Eluate	ΔPO_4^{3-} (Abs)	IP_6 (mg/l)	IP_6 Composition (%)	
			Theoretical	Actual
1	0.002			
2	Low	-		
3	0.89	3.70	74	**97.6**

Percentage composition: (3.7/5.0) X 100

= 74%

Since IP_6 is only 75.8 % pure, the corrected percentage composition is:

74/0.758

= 97.6%

Since 5mg of IP_6 salt contained only 75.8 % IP_6, its percentage composition after passing through the column was 97.6.

Conclusion

50 ml 0.05M HCl efficiently separates PO_4^{3-} from IP_6 and 5ml 2M H_2SO_4 elutes 97.6% of the strongly retained IP_6 ions from the column.

2.4.9 Urine

A urine sample (24-hr) was obtained from a healthy male subject. It was filtered through a pre-filter (0.75µm, Macherey-Nagel, Germany) and 0.45µm filter paper (Millipore Corporation, Bedford). An aliquot of 5 ml was applied to an anion exchange column. The flow through (1st fraction) was collected.

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Then 50 ml of 0.050M HCl was passed through the column followed by passage of 5ml of 2.0M H_2SO_4.and each of these two eluates was collected. Each of the three fractions were analysed for IP_6.

In a separate experiment, a second 24-hr urine sample was collected and treated as the one above. However, on this occasion the urine was dosed with a known concentration of IP_6 (2µl of a 0.758 mg/l phytate standard solution) in order to test whether the measured IP_6 concentration would be the sum of urinary and added IP_6 concentrations. The percentage composition of the standard solution added was calculated.

Results and Discussion

Table 2.10 below shows the results of the application of the method used in determining concentration of phytate in urine using lower HCl and H_2SO_4 concentrations.

Table 2.10: Urine sample (5ml) application

Eluate	$\Delta PO_4^{3-}\Delta$ (Abs)	PO_4^{3-} (mg/l)	IP_6 (mg/l)
1	Low*	-	-
2	Low*	-	-
3	0.28	4.12	**1.68**

***below detectable range of spectrophotometer**

From the phytate calibration curve (Figure 2.14): $0.28 = 0.3019x - 0.2284$

$$0.5084 = 0.3019x$$

$$x = 1.68 \text{ mg/l}$$

The experimental result shows that in the 1st fraction there was virtually no IP_6 as the difference between the unhydrolyzed and hydrolyzed fractions was negligible. The same observation was made for fraction 2. All of the phytate was eluted by 2M H_2SO_4.

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Table 2.11 below shows the reliability and accuracy of the method developed to determine IP_6 from urine.

Table 2.11: Application of urine sample dosed with IP_6 onto the column

Sample	IP_6 added (mg/l)	IP_6 (mg/l)	% Composition	
			Theoretical	Actual
Urine	-	0.96		
Urine + IP_6	0.677	1.40	65.0	**85.8**

IP_6 concentration of the urine increased by 0.44 mg/l (1.4 - 0.96) which may be attributed to the dose of IP_6 administered.

Therefore the percentage composition is: (0.44/0.677*) x 100 = 65.0%
After correcting for the percentage composition: 65/0.758 = 85.8%

Conclusion

The method of indirect extraction/photometry for the determination of IP_6 in urine works efficiently with 0.05M HCl and 0.2M H_2SO_4. The method separates PO_4^{3-} from IP_6, albeit not perfectly. Nevertheless, results are sufficiently reliable to allow interpretation.

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2.5 References

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CHAPTER III

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CHAPTER III

DETERMINATION OF MYOINOSITOL HEXAKISPHOSPHATE (PHYTATE) IN URINE

3.1 Introduction

IP_6 is present in many food substances. It is a naturally occurring plant constituent and forms 10 – 30 g/kg of the dry matter of cereal, legumes and oil seeds (Burbano *et al* 1995).

3.1.1 Concentration of IP_6 in Urine

The normal concentration of IP_6 in urine varies between 0.5 and 6.4 mg/l (Grases and Llobera 1996, March *et al* 1998). It has been observed that recurrent CaOx stone-formers and healthy individuals differ in their concentration of IP_6 (Grases *et al* 2004). Stone-formers characteristically have lower urinary phytate concentrations than healthy individuals. The average concentration of this substance in healthy individuals' urine is 2.94 ± 0.2 mg/l, while in stone-formers it is 1.13 ± 0.17 mg/l (Grases *et al* 2000a).

3.1.2 Relationship Between Oral Intake and Urinary Excretion

The urinary concentration of IP_6 strictly depends on its oral intake (Grases *et al* 2004). Studies have shown that ingestion of IP_6-rich food leads directly to an increase in the urinary excretion of this substance. Conversely, an IP_6-poor diet causes depletion in the concentration of this substance in urine.

After conducting a thorough pharmacokinetic profile study on IP_6, Grases *et al* (2001) showed that the only way to maintain adequate levels of IP_6 in humans is to supply a healthy IP_6-rich diet or IP_6 supplements.

In two studies in which Grases *et al* used a rat model (2000b) and humans (2001) respectively, a mutual relationship between the oral intake and urinary excretion of IP_6 was established. A threshold concentration was found above

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which an increase in urinary concentration of IP_6 could not be induced. This has been demonstrated in rats in which limited absorption of IP_6 by their mucosal areas was observed.

In humans, the amount and rate at which this substance is absorbed varies under different conditions causing the amount of IP_6 excreted in the urine to be significantly less than that ingested. Only 1 - 5% of the administered IP_6 is excreted in urine (Grases *et al* 2000a). In one study, March *et al* (1998) obtained a maximum value of IP_6 urinary concentration of 6.4mg/l in a 24-h sample when the candidates were on their normal diet. However, under IP_6-restricted diet, the concentration of phytate in urine takes 36 hours to significantly drop (about 50%) and eventually become undetectable (Grases *et al* 2000a and b).

After ingestion, phytic acid is normally hydrolysed in the gut. The products of this process vary from inositol mono- to pentaphosphate (Grases *et al* 2004). These phosphorylated inositols feature in a number of biochemical pathways and physiological processes. However, just like IP_6, these molecules may be excreted in urine.

Based on observations made in previous studies that urinary IP_6 concentration depends on its oral ingestion, phytate will be administered to black and white candidates in the present study with the aim of establishing whether handling of this substance is different in the two groups. The urines will subsequently be analysed and subjected to crystallisation experiments in order to establish IP_6's potency towards inhibition of CaOx crystallisation.

3.2 Method

3.2.1 Urine Collection and Preparation

Urine samples (24 hour) were collected (in summer) without preservative from 10 healthy black and 10 healthy white male subjects (only males were used because of the high incidence of the disease in them) in plastic bottles (2.5 *l*) under controlled dietary conditions as described below. The candidates enlisted were aged 19 - 27 years and were students of the University of Cape

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Town. Urinary test strips (Combur 10 test strip, Boehringer Mannheim, Germany) were used to test for the presence of nitrite and blood. All urines that tested positive for either were discarded. Each sample was passed through a 0.75 μm pre-filter (Macherey-Nagel, Germany) to remove flocculants and debris and lastly was passed through a 0.45 μm filter paper.

Urines were analysed for IP_6 as described in chapter 2. In addition, samples were analyzed for calcium, potassium, magnesium and sodium (Fernandez and Kahn 1971, Trudeau and Freier 1967, Willis 1961) using a Varian 1275 Model flame atomic absorption spectrometer. Inorganic phosphorus was determined using ammonium molybdate (Dryer and Routh 1963), creatinine using picric acid (Rock *et al* 1986), uric acid using uricase (Fossati *et al* 1980) and chloride using an ion selective chloride electrode. Oxalate decarboxylase (Chiriboga 1963) was used to determinate oxalate, while citrate was determined by conversion to oxaloacetate using citrate lyase (Gruber and Mollering 1966).

For each sample, relative supersaturation (RS) values of CaOx, brushite and uric acid were computed using the EQUIL program (Werness *et al* 1985).

3.2.2 Dietary Protocols

Dietary intake in ten black and ten white male candidates was controlled for three consecutive days (the rationale was that three days would be sufficient for detection of IP_6 in urine) by means of an instruction sheet which indicated phytate-rich foods to be avoided and included (Table 3.1). On the third day, they were required to collect a 24-hour urine sample. During the following three days, candidates were given a phytate-rich breakfast each morning consisting of 100g of cooked oats (Jungle Oats, Tiger Food Brands Ltd, Sandton, South Africa; IP_6 content not provided) (contains 1.01g IP_6 according to Hidvegi and Lasztity 2002) mixed with 200 ml full cream milk, 2 slices of wholewheat bread, 5g of margarine, 15g of strawberry jam and 200 ml of coffee (with 5g coffee creamer and 10g white sugar) and were advised to avoid any fibre-rich and oxalate rich food, dairy products and all IP_6-containing

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foods (Table 3.1) for their other meals. In addition, all candidates were required to drink 6 – 8 glasses per day of tap water. They were also instructed to refrain from eating before breakfast and for one hour thereafter. 24h urines were collected during the final day of the protocol.

Table 3.1: Dietary instructions

Foods to avoid	Foods to eat instead
Whole wheat bread	White bread
Durham wheat	White bread
Brown rice	White rice/Basmati rice
Corn chips	Potato chips
Peanuts, cashews, almonds	Chocolate
All-Bran flakes	Rice Crispies
Weet-bix	Rice Crispies
Kidney beans	Vegetables
Soya Milk	Cows milk (except on 'high phytate day')
Oats (except oats provided)	Rice Crispies
Legumes (e.g. lentils, beans)	Vegetables

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The candidates were required to complete a food diary to indicate their ingestion during the three days of the experiment. Three candidates from the white group were excluded from the study because their diaries recorded that they had eaten fibre and phytate-rich food substances (*i.e.* pro-nutro, muesli bar and peanut butter) during the IP_6-restricted dietary period. The nutrient content of the IP_6-rich breakfast is given in Table 3.2. The approval for dietary studies was obtained from the University of Cape Town

Means of urinary IP_6 concentrations and physicochemical parameters were statistically analyzed for individual and interactive effects (shown in the appendix) by the method of analysis of variance (ANOVA). A probability of $p < 0.05$ was used for assessing statistical significance at a 95% confidence level. These values were computed using a two-tailed t-test. Average values along with their standard errors (SE) are reported. The reasonable

assumption was made that the two groups had equal variances and thus the variances were pooled.

3.3 *Results*

Table 3.2: Nutrient content of IP_6-rich breakfast

Variables	Mean
Total Protein (g)	22.3
Total Fat (g)	27.5
Total Carbohydrate (g)	116.8
Fibre (g)	14.4
Added Sugar (g)	16.0
Oxalate (mg)	0
Ca (mg)	329
Mg (mg)	223
Phosphate (mg)	787
K (mg)	831
Na (mg)	427
Zn (mg)	4.97
Vitamin A (µg)	160
Vitamin B6 (mg)	0.253
Vitamin C (mg)	4.0
Vitamin D (µg)	0.66
Phytate (mg)	1010

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Figure 3.1.1 and 3.1.2 below show the urinary concentration of IP_6 in each subject from the two race groups on the IP_6-restricted and IP_6-rich diets.

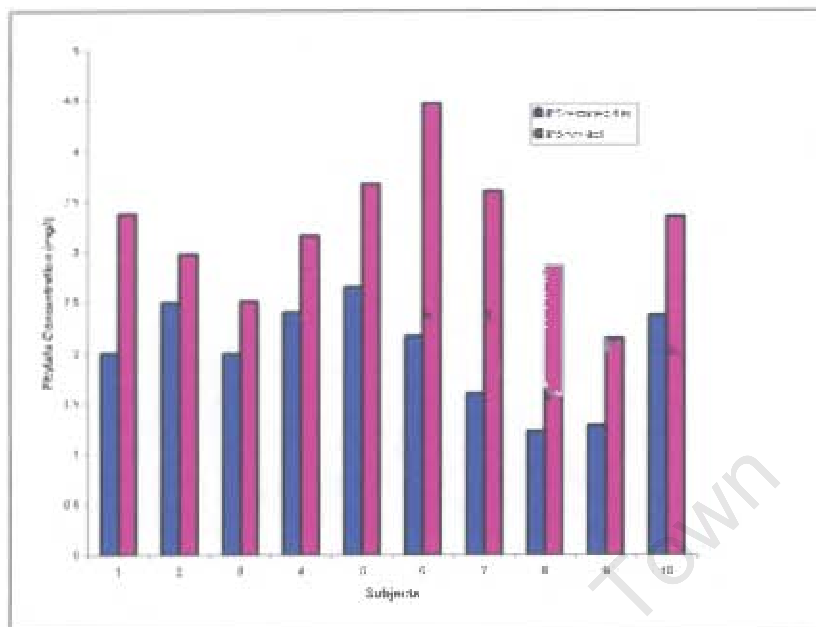

Figure 3.1.1: Concentration of urinary phytate in black subjects during an IP_6-restricted and IP_6-rich diet.

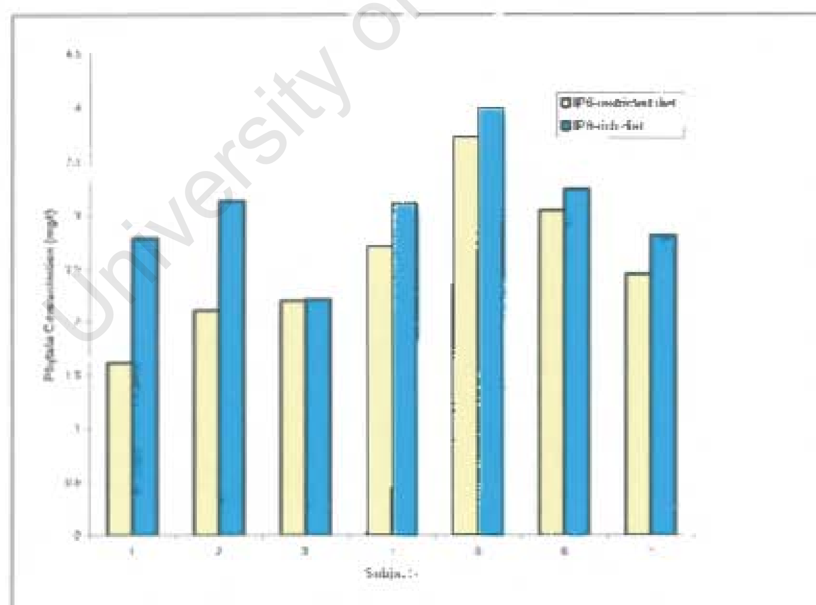

Figure 3.1.2: Concentration of urinary phytate in white subjects during an IP_6-restricted and IP_6-rich diet.

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The corresponding average IP_6 concentrations are shown in Figure 3.2 below.

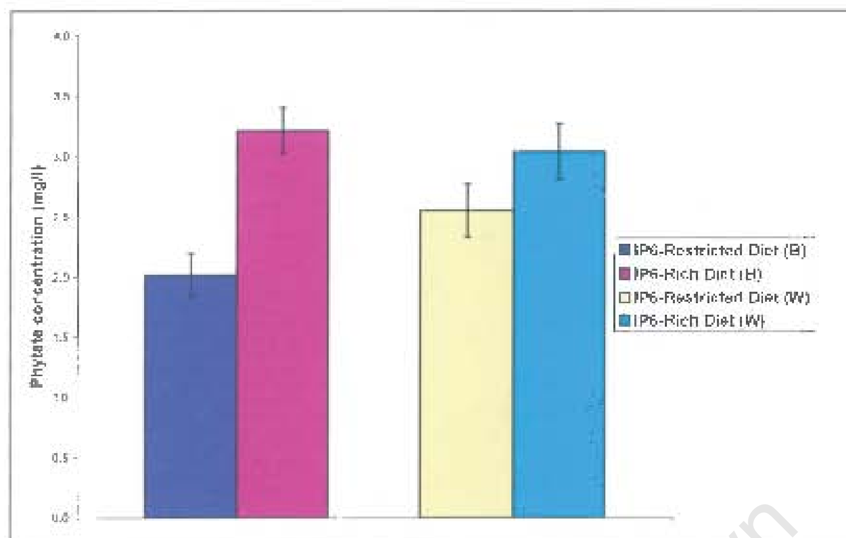

Figure 3.2: Average concentrations of IP_6 measured after the black and white participants were on IP_6-restricted and rich diet.

The standard error (SE) and p-values obtained when the comparisons are made within each race group and when blacks are compared to whites are shown in Table 3.3.

Table 3.3: Comparison of urinary IP_6 concentrations in black and white candidates after following IP_6-restricted and IP_6-rich diet

<table>
<tr><th rowspan="2">Candidates</th><th colspan="4">Average</th><th rowspan="2">p-value</th></tr>
<tr><th colspan="2">IP_6-res. Diet (mg/l)</th><th colspan="2">IP_6-rich diet (mg/l)</th></tr>
<tr><td>B Vs B</td><td colspan="2">2.02 ± 0.19</td><td colspan="2">3.21 ± 0.19</td><td><0.001</td></tr>
<tr><td>W Vs W</td><td colspan="2">2.55 ± 0.23</td><td colspan="2">3.04 ± 0.23</td><td>0.164</td></tr>
<tr><td>B Vs W</td><td>2.02 ± 0.19</td><td>2.55 ± 0.23</td><td colspan="2"></td><td>0.084</td></tr>
<tr><td>B Vs W</td><td colspan="2"></td><td>3.21±0.19</td><td>3.04 ± 0.23</td><td>0.572</td></tr>
</table>

B = Black, W = White, Res. = Restricted

It is noted that there were no inter-group differences which were statistically significant, although blacks tended to have a lower urinary IP_6 than whites

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while on the restricted diet (p=0.084). Of interest is the observation that within the groups, urinary IP_6 increased significantly in blacks but not in whites when switching from the restricted diet to the rich diet.

Figure 3.3 shows the average concentration of IP_6 in the rural black and urban white candidates while on their free, unrestricted diets. It is noted that the black candidates had a statistically significant (p=0.019) lower IP_6 concentration (2.96 ± 0.15 mg/l) than their white counterparts (4.11 ± 0.43 mg/l).

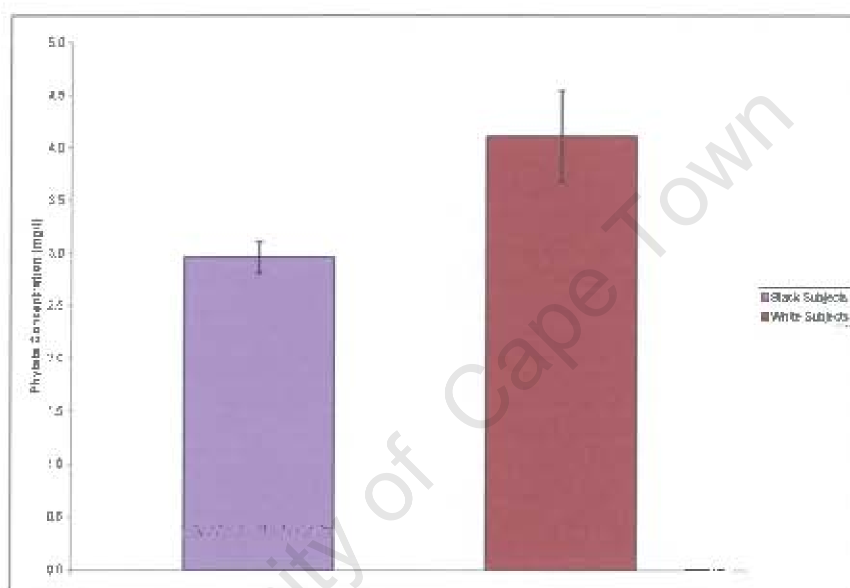

Figure 3.3: Rural black (n=9) and urban white (n=9) candidates' average IP_6 concentration while on an unrestricted diet.

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Urinary composition data parameters (described in the methods section) are presented in Table 3.4 below.

Table 3.4: Mean urinary excretion (mmol/24hr) and physicochemical parameters in black (n=10) and white subjects' urine (n=7)

Variables	Black candidates				White candidates				Black vs White	
	IP_6- Res. Diet	IP_6-Rich Diet	STD E	p- value	IP_6- Res. Diet	IP_6-Rich Diet	STD E	p- value	IP_6 Res. Diet (p-value)	IP_6-Rich Diet (p-value)
RS Uric acid	1.75	1.88	0.328	0.780	2.93	2.83	0.727	0.923	0.161	0.149
RS Brushite	0.571	0.311	0.129	0.064	1.10	1.08	0.321	0.954	0.236	*0.009
RS CaOx	4.86	6.28	0.911	0.287	5.67	5.44	2.55	0.646	0.466	0.399
pH	6.05	5.77	0.120	0.119	5.87	5.99	0.148	0.558	0.380	0.233
Volume (ml/24hr)	1390	2018	187	*0.029	1494	1097	400	0.464	0.837	*0.003
Citrate (mmol/24hr)	2.11	1.83	0.222	0.380	1.97	1.92	0.221	0.883	0.724	0.696
Oxalate (mmol/24hr)	0.298	0.500	0.0420	*0.003	0.451	0.437	0.0700	0.888	0.085	0.379
Calcium (mmol/24hr)	2.67	3.40	0.540	0.347	2.04	2.80	0.596	0.388	0.404	0.510
Magnesium (mmol/24hr)	1.526	3.36	0.362	*0.002	3.62	4.17	0.816	0.641	*0.002	0.427
Sodium (mmol/24hr)	123	145	18.5	0.425	146	134	29.0	0.775	0.555	0.698
Potassium (mmol/24hr)	25.0	44.0	8.18	0.122	103	53.5	11.8	*0.012	*<0.001	0.592
Uric Acid (mmol/24hr)	3.00	3.18	0.287	0.662	3.71	3.89	0.439	0.787	0.101	0.241
Phosphate (mmol/24hr)	23.9	23.1	2.09	0.787	34.1	28.8	3.59	0.312	*0.015	0.180
Chloride (mmol/24hr)	141	145	17.4	0.850	179	123	21.9	0.091	0.257	0.308

* **statistically significant result; $p<0.05$ RS = Relative supersaturation Res. = Restricted STD E = Standard error**

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It is noted that while on the IP_6-restricted diet, urinary potassium, magnesium, and phosphate were significantly lower in black subjects than in white subjects. After administration of the IP_6-rich diet, urinary oxalate increased significantly in black subjects while urinary potassium decreased significantly in whites. The effect of a diet rich in phytate is even more obvious in black candidates if the difference in mean urine volume is taken into account. Comparison of the urinary parameters between the two race groups after the IP_6-rich diet shows that relative supersaturation of brushite is significantly higher in whites.

3.4 Discussion

The observation of significantly lower urinary phytate levels in rural blacks compared to urban whites is surprising. Modlin *et al* (1981) pointed out that the main component of the diet in the former group was high fibre and he attributed the absence of stones to urinary phytate concentration which he speculated would be higher in blacks. It now appears that this hypothesis is incorrect, as the present results demonstrate the converse, namely urinary phytate is lower in the black group, despite the reputed higher intake of phytate in this group. This suggests that dietary phytate is handled differently in the two groups. More particularly, it would appear that absorption of IP_6 from the normal diet occurs to a lesser extent in blacks. Interestingly, when dietary phytate was restricted under controlled conditions, the difference in urinary phytate between the groups was no longer significant indicating a tendency towards a "natural" lower limit in these subjects lying in the range 2.02 – 2.55 mg/l. However, when the IP_6-rich diet was administered immediately thereafter, urinary phytate increased significantly in blacks but not in whites, reaching a concentration that was commensurate with that of rural blacks on their free diet. On the other hand, urinary IP_6 concentration in white subjects while on the IP_6 rich diet (3.21 mg/l) did not reach the level achieved on their free diets (4.11 mg/l). These results again indicate that renal handling of phytate in the race groups proceed via different mechanisms. However, details of these mechanisms remain unclear.

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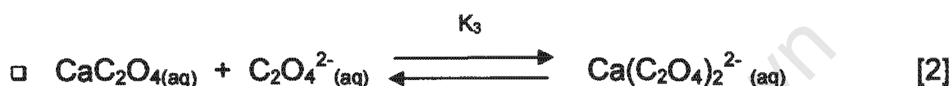
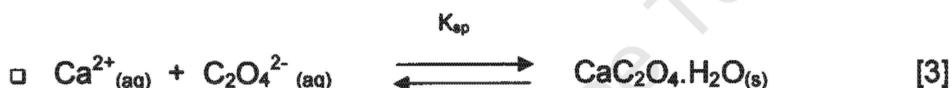
It should be noted that proteins interacts with phytate and may affect its hydrolysis (and hence determination of concentration) so the amount protein could have been measured as well.

The significant increase in urinary oxalate in blacks but not in whites, following the IP_6-rich diet deserves detailed comment. According to Grases and March (1989), the following equilibria (1-4) should be considered in the presence of phytic acid dodeca anion in urine.

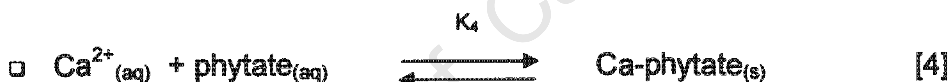
$$Ca^{2+}_{(aq)} + C_2O_4{}^{2-}_{(aq)} \underset{}{\overset{K_2}{\rightleftharpoons}} CaC_2O_{4(aq)} \quad [1]$$



$$CaC_2O_{4(aq)} + C_2O_4{}^{2-}_{(aq)} \overset{K_3}{\rightleftharpoons} Ca(C_2O_4)_2{}^{2-}_{(aq)} \quad [2]$$



$$Ca^{2+}_{(aq)} + C_2O_4{}^{2-}_{(aq)} \overset{K_{sp}}{\rightleftharpoons} CaC_2O_4.H_2O_{(s)} \quad [3]$$



$$Ca^{2+}_{(aq)} + \text{phytate}_{(aq)} \overset{K_4}{\rightleftharpoons} \text{Ca-phytate}_{(s)} \quad [4]$$

In the presence of phytate anions in urine, after ingesting an IP_6 load, more calcium ions are bound by the phytate anion [4]. According to Le Chatelier's principle, the reverse reaction would occur in [1] and [3] in order to counteract the decrease in Ca^{2+} concentration, and maintain the equilibrium in [4]. This results in maintenance of the net Ca^{2+} concentration and a concomitant increase in the concentration of $C_2O_4{}^{2-}$. While urinary calcium did not indeed change in either group, urinary oxalate increased in black subjects but not in whites. This suggests that different equilibria, involving different chemical speciation, might be occurring in the black group. These observations therefore support the hypothesis that the renal handling of phytate is different in the two race groups.

In addition, phytate has been identified as an inhibitor of CaC_2O_4 growth (Grases and March 1989, Grases *et al* 2000a and b). The inhibition is

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attained by binding on the surface of the crystal. Therefore if phytate binds to CaC_2O_4 [1], $Ca(C_2O_4)_2^{2-}$ [2] and $CaC_2O_4.H_2O$ [3], the equilibrium reactions stop. Hence the concentrations of Ca^{2+}, $C_2O_4^{2-}$ and CaC_2O_4 would have been expected to remain constant. Again, the effect in the two groups was different i.e. $C_2O_4^{2-}$ changed in blacks but not in whites.

Regarding urinary phosphate, two changes were expected after giving the candidates a phytate-rich diet. Firstly, that a decrease in relative supersaturation of brushite would occur in both race groups since phytate is an inhibitor of $Ca_3(PO_4)_2$ crystallization (Grases *et al* 2000c and d) and secondly, the degree of decrease in the two race groups was expected to be almost the same since they ingested the same amount of phytate. Both of these expectations were not met, suggesting that there is a different handling mechanism of urinary phytate in the two population groups.

Since the relative supersaturation of brushite was observed to be significantly higher in whites than in blacks after ingestion of the IP_6-rich diet, it might have been attributed to differences in urinary calcium and phosphate in the two groups. However, no such differences were recorded. Hence the observed difference in relative supersaturation of brushite must be due to subtle but undetectable changes in urine composition following the IP_6 challenge. However, it is not possible to identify the exact factors which are responsible.

It should also be noted that while the oxalate values rise significantly in the black subjects, this was paralleled together with magnesium (levels of which reach that of the white subjects). The mechanism for this change could be investigated in the future studies.

Owing to the rarity of kidney stone disease in the black population, the general expectation would be that the concentration of CaOx crystallisation inhibitors in urine of the blacks would be higher than in their white counterparts. Phytate, being an inhibitor of CaOx nucleation and growth, was therefore expected to have higher concentration in the urine of the black candidates in order to justify the abovementioned observation. However, previous studies

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involving different inhibitors in South African black and white subjects have demonstrated that many factors must be brought into consideration whenever the disparity in the incidence of kidney stone disease in the two groups is addressed and that no positive conclusion can be reached based exclusively on the concentration of any particular inhibitor in urine.

In the present study phytate concentration in white subjects were noted to be higher than that in blacks. A possible explanation for this unexpected result could be that in the present study, the candidates were given only three days to ingest the phytate-rich diet before the measurements were performed, whereas in one study Grases and Costa-Bauza (1999) administered 120 mg of phytate per day for 2 weeks and in the other (Grases *et al* 2001), subjects ingested IP_6-normal diet for 16 days. Nevertheless, notwithstanding the relatively short ingestion period, the important point is that differences between the two groups were indeed observed.

Since both black and white candidates followed IP_6-restricted and IP_6-rich diets for the same duration, differences in the urinary IP_6 concentrations after following any of the two diets would suggest that there is a different handling mechanism of phytate in the two population groups. After ingestion of the IP_6-rich diet, there was a significant increase in the urinary phytate concentration in blacks relative to their values on the IP_6-restricted diet, but no such increase occurred in whites. As previously mentioned, this suggests different handling mechanisms in the two groups. More specifically, it may be concluded that the rate of uptake of IP_6 in blacks is higher than in white candidates.

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CHAPTER IV

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CHAPTER IV

CRYSTALLIZATION EXPERIMENTS

4.1 Introduction

Crystallization is a process brought about by deposition of inorganic crystalline materials under normal conditions (Hess and Kok 1996). Though crystallization occurs in all the body fluids, in urolithiasis research, the focus is mainly on studying crystallization of the calcium salts that occurs in the urinary tract. This process is of paramount importance towards formation of calcification (formation of calcium crystals in urine). Formation of crystals in urine is often considered to be a physiological event and therefore cannot be used to distinguish between non-stone formers and the healthy individuals (Hess *et al* 2001). Urine is always supersaturated with CaOx crystals and to a lesser extent with $Ca_3(PO_4)_2$ and the presence of these crystals in urine is referred to as crystalluria (Kavanagh 2001). It is therefore important to know in detail the steps leading to this process as well as the different aspects involved. Crystallization is always studied with respect to CaOx, as the latter is the most common component of human kidney stones (Egan *et al* 2004). Centrifuged and filtered urine is often used to evaluate *in vitro* the crystallization process of CaOx (Guerra *et al* 2004). Conditions considered favourable to trigger this process include the following;

- degree of supersaturation of the calcium salts
- presence of heterogenous nucleants
- presence and concentration of crystallization inhibitors

(Grases and Costa-Bauza 1999)

Many methods have been employed to study calcium oxalate (CaOx) crystallization (Kavanagh 1992). Different approaches enable distinct types of measurements to be made and therefore different mechanisms to be studied. The problem, however, is that due to different conditions under which experiments are conducted, the results concerning CaOx crystallization

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reported in a vast number of publications are apparently conflicting (Sohnel and Grases 1995). Lack of standardized methods may be at least partly responsible for conflicting results reported from evidently similar experiments (Ryall *et al* 1986).

The major aim of crystallization studies is to measure the ability of urine or specific components of urine to hinder or promote some aspect of the crystallization process. This is often achieved by comparing results under test conditions with a control experiment.

No *in vitro* system will ever be accurate enough to mimic what happens in the kidney during renal stone formation (Hess *et al* 2001). However, if the experiments are carefully designed and the appropriate conditions which closely approximate those in the kidney are used, these experiments can be of importance in the urolithiasis research. The conditions such as temperature under which the particular experiment is conducted and the concentration of the substances used to form solutions such as artificial urine should all be taken into consideration.

The methods used to study crystallization of calcium oxalate in urolithiasis can be distinguished by changes in supersaturation. According to Kavanagh (1992), the ability of supersaturation to increase, remain constant or decay has been used to differentiate between the many methods employed in studying crystallization hitherto. Some methods can be used to independently measure crystallization even when nucleation, growth or aggregation may be occurring concurrently.

Depending on how supersaturation varies as the experiment progresses, methods of studying CaOx crystallization can be split into three main categories (Kavanagh 1992). In the first method, crystallization is induced and supersaturation of the solution decreases as crystals of CaOx start to form in solution. Secondly, crystallization is studied by mimicking the way in which the kidney brings about supersaturation. In this approach, initially stable solutions are concentrated to induce crystallization and thus increase

supersaturation. In the third approach, constant supersaturation is maintained by continuously adding calcium and oxalate to a test solution. The latter is thought to be a better model of intrarenal crystallization than a supersaturation decay system (Hess and Kok 1996).

4.1.1 Crystallization Experiments Used in this Study

The following measurements were made to monitor crystallization in this project: metastable limit (Ryall *et al* 1985), sedimentation kinetics (Kavanagh 1992) and ^{14}C-oxalate deposition kinetics (Grover *et al* 1998, Aggarwal *et al* 2000).

For these experiments, two 24h urine samples were collected from each of the 10 black subjects and 7 white subjects mentioned in the previous chapter. The first collection was made after following an IP_6-restricted while the second was collected after an IP_6-rich diet (refer to Chapter 3 for details).

4.2 Metastable Limit (MSL) Determination

4.2.1 Introduction

The MSL is the concentration at which a solution of CaOx is in a state of metastability or supersaturation and above which spontaneous crystallisation will occur (Ryall *et al* 1985). The resistance is measured by addition of increasing concentrations of sodium oxalate solutions. As sodium oxalate is added to the urine, the supersaturation increases to a level where CaOx crystals form in urine. This supersaturation level is called the formation product (Kavanagh 1992).

4.2.2 Method

A sodium oxalate stock solution (0.20 M) was prepared by dissolving 26.8 g of the salt in a liter of distilled water. Thirteen dilutions were prepared from this stock solution as indicated in Table 4.1 below.

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Table 4.1: Preparation of NaOx standard solutions

Vial	Stock Solution (ml)	Distilled Water (ml)	Conc. (mol/dm^3)
1	0	10.0	0
2	0.75	9.25	0.015
3	1.50	8.50	0.030
4	2.25	7.75	0.045
5	3.00	7.00	0.060
6	3.75	6.25	0.075
7	4.50	5.50	0.090
8	5.25	4.75	0.105
9	6.00	4.00	0.120
10	6.75	3.25	0.135
11	7.50	2.50	0.150
12	8.25	1.75	0.165
13	9.00	1.00	0.180
14	9.75	0.25	0.195

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The calcium oxalate metastable limit of each urine was measured using the method described by Ryall *et al* (1985). Briefly, 10 ml aliquots of urine were transferred to thirteen Coulter cups that were incubated in a shaking water bath (100 rpm) at 37^{0}C. Thereafter, sodium oxalate solutions (100µl) of concentrations 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180 and 195 mM, respectively, were added to the cups.

After a 30 minute incubation period the number of particles was counted in each cup using a Coulter Counter I (Beckmann) fitted with a 140-µm orifice (2.8 - 90.0 µm particle size range). A graph of particle number versus sodium oxalate concentration was plotted.

4.2.3 Results and Discussion

Graphs of particle number versus sodium oxalate concentration are shown in Figure 4.1 and 4.2 for black and white subjects respectively.

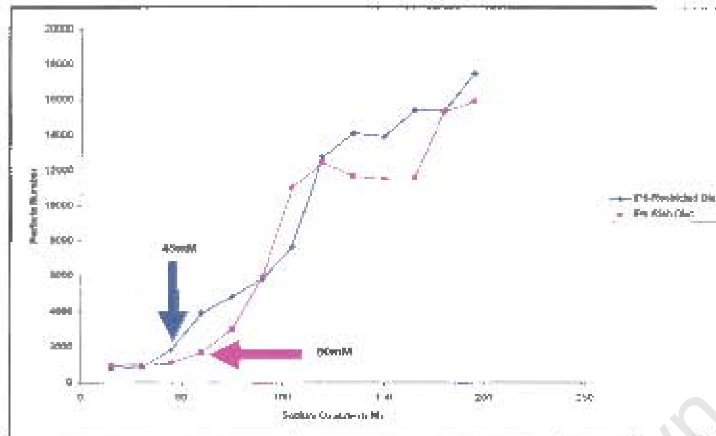

Figure 4.1: Average metastable limit experimental result (10 black subjects)

Figure 4.1 indicates that the average metastable limits in the urine of black subjects while on IP_6-restricted and IP_6-rich diets were 45µM and 60µM, respectively. However, these were not statistically different. Nevertheless, the urine collected after the subjects were on the IP_6-rich diet tended to show a greater resistance to nucleation of CaOx crystals and this is ascribed to the presence of IP_6 in urine. This difference was observed in 9 out of 10 subjects.

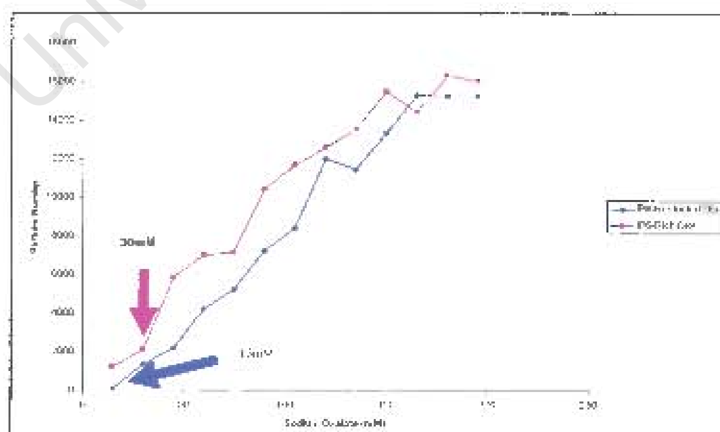

Figure 4.2: Average metastable limit experimental result (7 white subjects)

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The average metastable limit value obtained in the white subjects when analysing the urines collected after administration of IP_6-restricted and IP_6-rich diet was 15 and 30μM, respectively. Though these values appear to be different, statistical analysis reveals that they are not and this is due to the large standard deviations. Nevertheless, it is noteworthy that 6 out of 7 urines had a higher MSL value after the phytate-rich diet.

4.2.4 Conclusion

It can be seen that MSLs are higher in black subjects. Moreover, the average increase in MSL after IP_6-rich diet appears to be greater in white subjects (100% increase) than in black subjects (33%). If this change in the MSL can be attributed to the ingestion of IP_6, it suggests that phytate has a smaller effect in black subjects, possibly because of different renal handling mechanisms in this race group recognising that random errors may also contribute.

4.3 Sedimentation Kinetics

4.3.1 Introduction

The procedure requires constant stirring of a saturated slurry of COM crystals until equilibrium is established. This is ascertained by measuring the optical density of the slurry using a spectrophotometer. A constant OD indicates that equilibrium has been achieved. Thereafter the stirrer is switched off. Spontaneous sedimentation of particles then occurs. Neither nucleation nor growth occurs since the crystals and solution are in equilibrium. However, crystals which were formerly dispersed throughout the suspension begin to aggregate with one another thereby increasing overall particle size. Since the particle are now larger, their rate of sedimentation increases. This manifests itself with a concomitant decrease in OD. The sedimentation rate is determined by measuring the gradient of a graph of OD versus time corresponding to the period during which the stirrer has been switched off. The experiment allows inhibitors in aliquots of urine to be introduced into the slurry thereby enabling aggregation inhibition to be measured. This is achieved by measuring the gradients in a control sample (inhibitor absent)

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and a test sample (inhibitor present) and applying them in the following equation:

% Inhibition of aggregation = 100 – 100(S_c/S_T)

S_c = gradient of control sample (OD vs time)

S_T = gradient of test sample (OD vs time)

4.3.2 Method

Preparation of COM crystals

COM crystals were prepared by mixing calcium chloride (10 mM) and sodium oxalate (10 mM) solutions at room temperature (solutions were made by using filtered distilled water). The solutions were introduced at a constant speed of 1.0 ml/min using a peristaltic pump and the mixture was stirred for a week at 4^{0}C after which it was filtered using 0.22-μm filter paper. The residue was washed with methanol and dried at 95^{0}C for one hour. Its composition was determined by x-ray powder diffraction (Fig 4.3).

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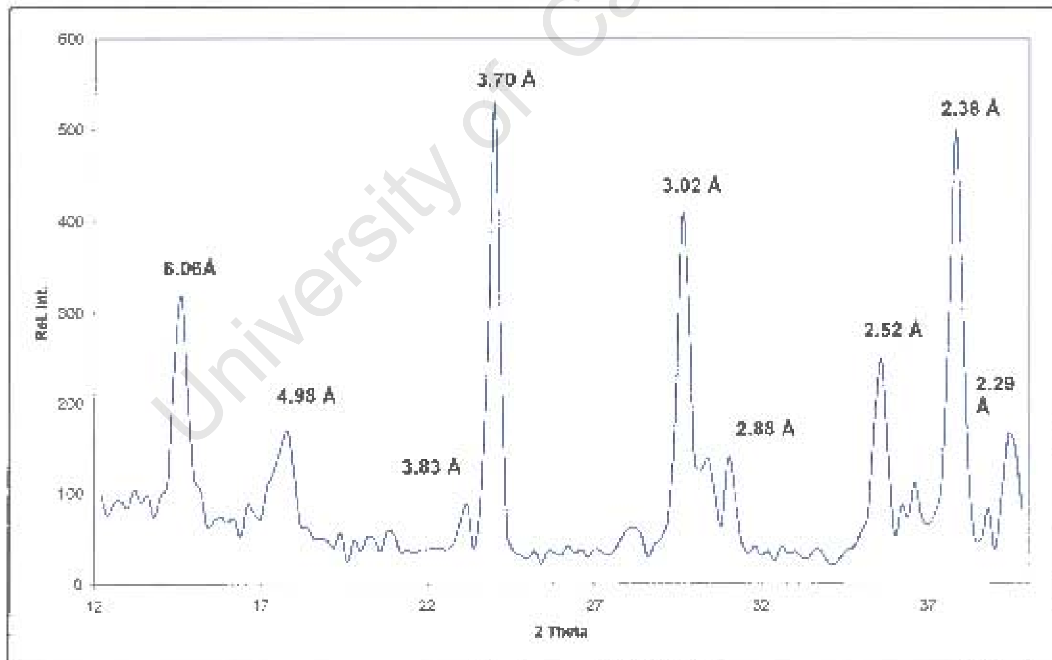

Figure 4.3: X-ray powder diffraction pattern of calcium oxalate crystals used in this study

The d spacings and relative intensities for each reflection correspond with the standard values for COM (Sutor and Scheidt 1968). Characteristic peaks for calcium oxalate dihydrate (COD) and calcium oxalate trihydrate (COT) are absent. Thus, it is 100% COM.

COM slurries were prepared by adding 40 mg of COM crystals to 50 ml of 10mM tris(hydroxymethyl)-aminomethane (Tris)·HCl buffer. The latter was prepared by dissolving 0.79g (10mM) of tris(hydroxymethyl)-aminomethane and 2.63 g (90mM) of NaCl in 500 ml of distilled water with stirring. The pH of the solution was then adjusted to 7.2 using concentrated HCl.

Slurries were equilibrated overnight (16 hours) in a 37^0C water bath under constant magnetic stirring (1100 rpm). After equilibration, slurries (1.6 ml) were incubated for two hours with filtered urine (0.4 ml) collected after candidates were on an IP_6-restricted or IP_6-rich diet at 37^0C, and the mixtures were stirred at 1100 rpm for 2 hours. The urine samples were not dialysed (although this is prescribed by Hess *et al* 1989) to avoid any possible loss of phytate as it is a low molecular weight inhibitor. COM slurries (2.0 ml) were used for control experiments.

After the two hour incubation period, 2 ml aliquots of the crystal-urine slurries were transferred into a 10-mm light path glass cuvette in a cell holder thermostated at 37^0C. The absorbance was measured using a spectrophotometer (Specord 40, United Scientific, Cape Town) interfaced to a computer. Each solution was at first stirred slowly until OD_{620} reached a stable plateau, after which the stirrer was stopped (indicated by arrows in Figure 4.5). The absorbance readings were measured for 900 seconds. The same procedure was followed for the control experiment (COM slurry). The graph of OD_{620} against time was plotted.

4.3.3 Results

Figure 4.4 shows absorbance versus time graphs which were typical for both black (Figure 4.41) and white groups (Figure 4.42) for the different samples:

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(a) urine from controls, (b) urine collected after IP_6-restricted diet and (c) urine collected after IP_6-rich diet.

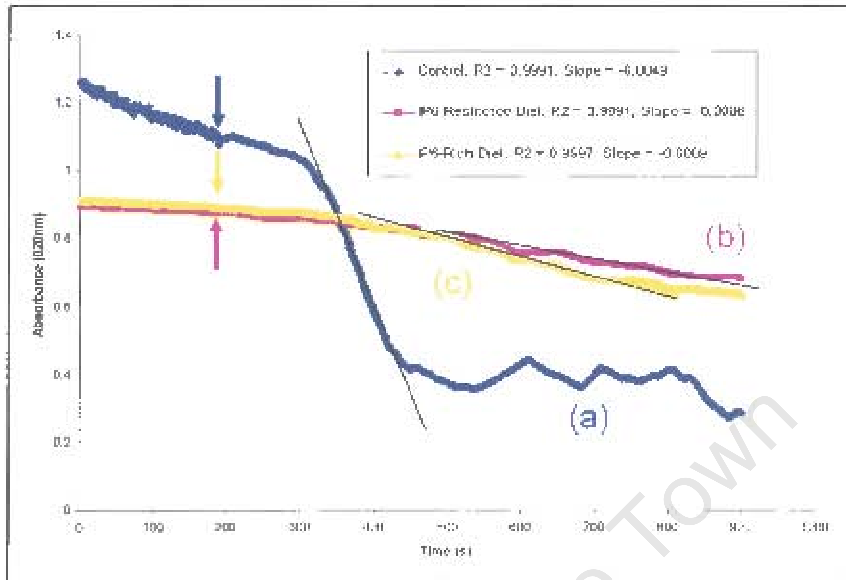

Figure 4.4.1: Typical absorbance vs time graphs observed in the different samples in black candidates. (The arrow indicates the instant at which the stirrer was switched off).

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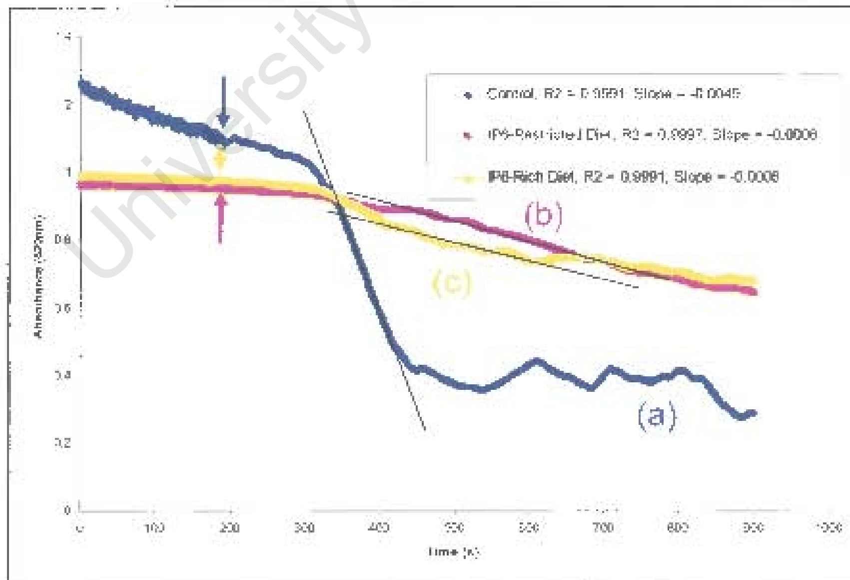

Figure 4.4.2: Typical absorbance vs time graphs observed in the different samples in white candidates. (The arrow indicates the instant at which the stirrer was switched off).

Sedimentation rates and the inhibition of CaOx crystal aggregation were calculated from the gradients of the absorbance vs time decline after the stirrer was switched off. Table 4.2 gives results for both groups.

Table 4.2: Mean percentage (%) inhibition of aggregation

	IP_6-Restricted Diet	IP_6-Rich Diet	SE	p-value
Black	92.5	88.1	1.20	**0.019
Whites	83.1	74.3	5.03	0.230
	black versus white			
IP_6-restricted Diet	92.5 (B)	83.1 (W)	3.20	*0.052
IP_6-Rich Diet	88.1 (B)	74.3 (W)	4.06	**0.026

B = Black Subjects, W = White Subjects

** Statistically significant * Approaching significance

4.3.4 Discussion

Comparison of the results obtained on both IP_6-restricted and IP_6-rich diets (Table 4.2) shows that the percentage inhibition of aggregation was significantly greater in blacks than in whites (p=0.052 and p=0.026 respectively). Since urinary phytate concentrations between the two groups were not significantly different (Table 3.2), differences in the inhibition of aggregation cannot be attributed to this compound. In fact, contra-intuitively, urinary IP_6 concentration tended to be somewhat higher in whites than in blacks on the IP_6-restricted diet (p=0.084, Table 3.2). The urine composition data (Table 3.3) do not provide any clues either, except for a lower urinary oxalate in blacks which tended weakly towards significance on the restricted diet (p=0.085, Table 3.3). However, this is unlikely to be the key factor. The conclusion must therefore be that other unidentified factors in the urine of black subjects are responsible for providing enhanced protection against CaOx crystal aggregation. These factors could be inherently present in the urine of black subjects irrespective of whether IP_6 is restricted or raised in the

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diet, or they might arise as a consequence of the two dietary protocols. The results of the present study are unable to determine which of these possibilities is the appropriate one.

It is noted that relative to the IP_6-restricted diet, inhibition decreased significantly in black subjects following the administration of the IP_6-rich diet ($p=0.019$, Table 4.2) but not in white subjects ($p=0.230$, Table 4.2). This effect can be attributed to the significant increase in urinary oxalate excretion observed in this group ($p=0.003$, Table 3.3).

4.4 ^{14}C-Oxalate Deposition Experiment

4.4.1 Introduction

According to Grover *et al* (1998) and Aggarwal *et al* (2000), CaOx deposition kinetics can be determined in human urine by dosing the latter with radioactive ^{14}C-oxalate.

4.4.2 Method

30 ml of urine from black (n=4) and white (n=5) was transferred to soda-glass bottles and placed in an oven at 37^0C. 15-µl ^{14}C oxalic acid was added to each bottle together with 250 µl sodium oxalate (15 µM above the previously determined metastable limit). The bottles were incubated at 37^0C for various time intervals (0, 30, 60, 90, 120 minutes). Each bottle was shaken throughout the incubation period. At each time point, 2.5 ml of the sample was withdrawn using a Gilson pipette and filtered (0.22 µm) into a vial containing 250 µl of concentrated HCl to quench the reaction. After shaking the vial for about 30 seconds, 1 ml was withdrawn and added to 10 ml of scintillation fluid. The experiment was done in duplicate. Thereafter the samples were placed in a scintillation counter and counts per minute of ^{14}C in solution were determined.

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4.4.3 *Results and Discussion*

Figures 4.5.1 and 4.5.2 show the typical kinetics for calcium oxalate crystal deposition in the urine of black and white subjects respectively.

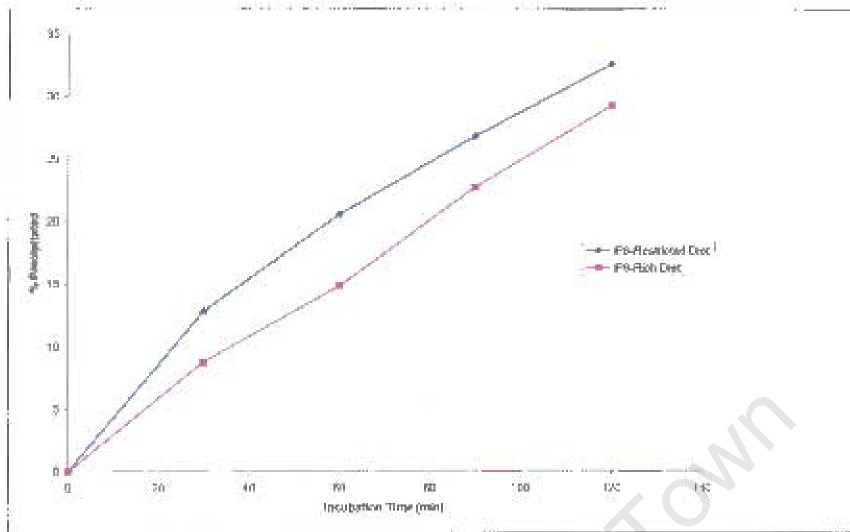

Fig. 4.5.1: Typical graph of % precipitation of CaOx as a function of time in black subjects

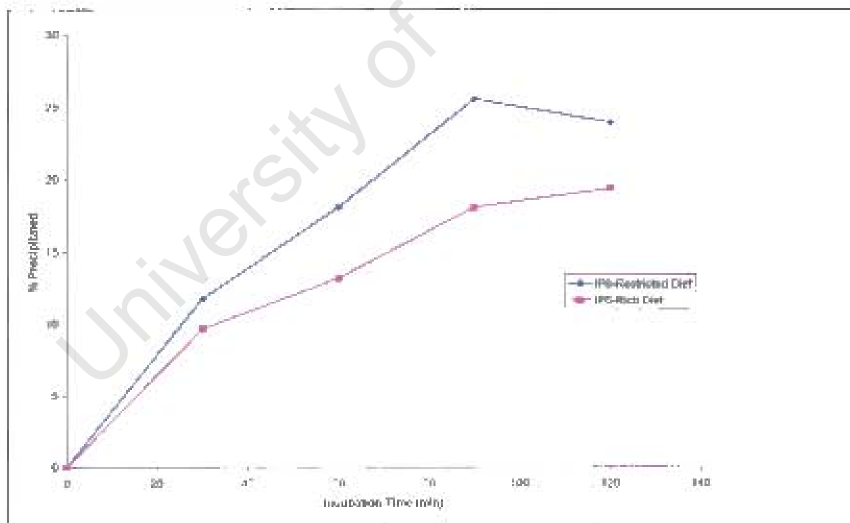

Fig. 4.5.2: Typical graph of % precipitation of CaOx as a function of time in white subjects

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The graphs show that the amount (expressed as a percentage) of CaOx deposited in the urine while the subjects were following IP_6-rich diet was lower than that on the restricted diet. However, statistical evaluation of the results at 120 minutes (Table 4.3) shows that no statistically significant differences were observed in either group after the subjects had followed both IP_6-restricted and IP_6-rich diet nor were there any differences between the groups themselves. This might be explained by the relatively short duration of IP_6 administration in the present study (3 days). Two previous studies in which lower (Grases and Costa-Bauza 1999) and higher (Grases *et al* 2001) IP_6 doses were administered, were carried out for 15 and 16 days, respectively. In the latter studies, after 36 stone-formers were treated with phytic acid (120mg/day) for 15 days, it was observed that deposits of CaOx and calcium phosphate did not form. According to Grases *et al* (2001), stone formers have low IP_6 urinary concentration. Therefore greater inhibition might have been observed in the present study if the period during which IP_6 was ingested had been lengthened.

Table 4.3: Average percentage (%) precipitation of $Ca^{14}C$-Ox crystals in the urine of black (n=4) and white (n=5) subjects at 120 min.

	IP_6-Restricted Diet	IP_6-Rich Diet	SE	p-value
Blacks	32.6	29.2	7.06	0.755
Whites	23.9	19.4	8.99	0.731
black versus white				
IP_6-Restricted Diet	32.6 (B)	23.9 (W)	9.25	0.554
IP_6-Rich Diet	29.2 (B)	19.4 (W)	8.13	0.450

B = Black Subjects, W = White Subjects

For blacks, the rate of deposition after ingestion of IP_6-restricted diet and IP_6-rich diet was respectively 0.22 and 0.23 %/min. For their white counterparts, the rate of deposition of CaOx after IP_6-restricted diet was 3.57 %/min and after IP_6-restricted diet it was observed to be 2.33%/min.

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CHAPTER V

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CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

In this chapter, it is appropriate to assess the extent to which the original objectives were achieved.

The first major aim of this project was to design, develop and test an assay to measure the concentration of phytate in the urine of healthy black and white subjects. A considerable proportion of time was spent on attempting to achieve this objective using a commercial IP_6 sodium salt, the purity of which was assessed and confirmed using ion chromatography and NMR spectroscopy. In order to optimise the recovery of IP_6, the method of March *et al* (1998) was modified in two ways: firstly, the use of charcoal to filter the urine was omitted and secondly, HCl was not used as a urine preservative. Both of these procedures were deemed to be sources of error in that they could possibly reduce the concentration of IP_6. The modified method was validated and was shown to be more efficacious than the previously published protocol.

The second objective was to test the hypothesis that the higher dietary intake of phytate in the black group leads to relatively higher urinary concentrations of this substance in this group. Modlin (1981) had previously reported that the black group's dietary intake of fibre-rich foods was greater than that of whites and this was substantiated by a recent study which showed that the total dietary fibre intake of rural black females in South Africa is more than double that of their urban counterparts (no available data for males) (Vorster and Nell 2001). Modlin speculated that rural blacks would have higher urinary phytate levels than urban whites and that these elevated concentrations contribute to the black group's protective mechanism against calcium oxalate stone formation. Despite this important hypothesis, he did not measure urinary phytate. The present study has demonstrated the surprising result that while

on their free diets, the phytate levels detected in the urine of rural blacks are significantly *less* than that of urban whites, thereby refuting Modlin's hypothesis. However, the present results have shown that the absorption of phytate in blacks is higher than that in white subjects. Thus it appears that the renal handling of dietary phytate is different in the two race groups. This result is important when considered in the context of other studies which have shown that renal handling of other dietary challenges (calcium and oxalate) proceed via different mechanisms in South Africa's two race groups (Rodgers and Lewandowski 2002). Furthermore, it also demonstrates the complexity of the "black versus white" kidney stone phenomenon.

The third main objective was to test the CaOx crystallization potential of urines in both groups after administration of IP_6-restricted and IP_6-rich diets. Data indicated that the tendency towards crystallization in both groups decreased after administration of the phytate-rich diet, and that the effect within the black group appeared to be greater although these effects were not statistically significant. It is concluded that dietary phytate may play an indirect inhibitory role in black subjects since the promotory effect of the elevated urinary oxalate observed in this group after ingestion of the IP_6-rich diet was easily overridden. This provides motivation for future studies in this area. However, such studies revise the dietary protocol by extending the period during which a phytate-rich diet is administered.

In conclusion, this study has demonstrated that dietary phytate does not appear to play a direct role in providing the South African black population with their unique protection against the formation of CaOx kidney stones. The results have confirmed that renal handling differences between the two groups do indeed exist for phytate. This should encourage researchers to further investigate renal handling mechanisms of a host of lithogenic and anti-lithogenic agents in the two groups as a general strategy in future studies which address the unique rarity of kidney stones amongst South Africa's black population.

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APPENDICES

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Appendix I

Candidates' Urinary Composition

Table 1.1: 10 black subjects' urine composition (phytate-restricted diet)

Sample No	01	02	03	04	05	06	07	08	09	10
Volume (ml/24hr)	2430	1800	1000	1000	1760	580	1380	1520	1810	620
pH	5.86	5.87	5.9	5.77	6.52	5.94	6.78	5.95	6.14	5.76
Sodium (mmol/24hr)	120.1	54.9	41.0	84.7	234.6	159.7	237.8	158.9	101.2	40.2
Potassium (mmol/24hr)	15.7	29.4	6.46	52.7	33.4	30.6	30.9	25.6	25.3	8.74
Calcium (mmol/24hr)	3.32	5.52	1.98	1.98	3.68	1.87	3.14	4.82	0.36	0.24
Magnesium (mmol/24hr)	1.56	2.48	1.33	1.94	1.08	1.42	2.07	1.68	0.93	0.77
Phosphate (mmol/24hr)	24.1	26.8	13.1	24.1	18.3	27.5	33.1	26.4	29.5	15.8
Oxalate (mmol/24hr)	0.47	0.34	0.27	0.26	0.32	0.18	0.31	0.24	0.39	0.20
Citrate (mmol/24hr)	1.40	2.24	1.08	1.68	3.38	1.30	3.28	3.34	2.27	1.12
Uric acid (mmol/24hr)	3.60	3.40	2.10	3.70	3.50	1.70	3.50	4.20	2.70	1.60
Chloride (mmol/24hr)	191	237	82.0	145	25	131	157	223	164	55.0
RS Brushite	**0.2892**	**0.8033**	**0.5182**	**0.5071**	**0.6669**	**1.3571**	**1.6298**	**0.7924**	**0.0571**	**0.0871**
RS Calcium oxalate	**6.0288**	**8.3824**	**9.3880**	**5.3151**	**3.7221**	**4.9654**	**3.5558**	**5.0937**	**0.5914**	**1.5905**
RS Uric acid	**1.3773**	**1.6972**	**1.8196**	**3.8557**	**0.4764**	**2.2025**	**0.3401**	**2.1003**	**0.8110**	**2.7976**

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Table 1.2: 10 black subjects' urine composition (phytate-rich diet)

Sample No.	01	02	03	04	05	06	07	08	09	10
Volume (ml/24hr)	2610	1980	1490	2560	1300	1380	2540	2600	2420	1300
pH	5.51	5.07	5.69	5.82	6.00	5.64	5.68	5.62	6.7	5.97
Sodium (mmol/24hr)	216.1	90.8	117.2	165	93.7	146.4	163.4	174.4	143.9	135.5
Potassium (mmol/24hr)	27.0	22.1	27.3	34.5	31.8	31.8	84.8	17.3	125.4	44.3
Calcium (mmol/24hr)	5.57	4.81	4.70	2.55	4.05	3.29	2.85	4.48	0.91	0.79
Magnesium (mmol/24hr)	2.98	3.55	2.71	2.78	2.67	2.65	6.99	4.56	3.45	1.26
Phosphate (mmol/24hr)	23.0	20.8	16.1	34.6	19.8	19.0	33.0	23.4	27.6	13.3
Oxalate (mmol/24hr)	0.29	0.46	0.34	0.73	0.35	0.34	0.62	0.71	0.60	0.56
Citrate (mmol/24hr)	1.92	1.65	1.76	1.85	1.44	1.74	1.51	2.64	1.83	1.94
Uric acid (mmol/24hr)	3.1	3.1	3.1	4.6	2.6	2.3	4.3	3.3	3.8	1.60
Chloride (mmol/24hr)	193	97	137	197	150	120	142	140	176	102
RS Brushite	**0.1972**	**0.1159**	**0.4500**	**0.2289**	**1.0200**	**0.3557**	**0.2129**	**0.2004**	**0.2351**	**0.0933**
RS Calcium oxalate	**4.0920**	**9.7343**	**9.3199**	**5.3600**	**10.0334**	**7.2482**	**4.1963**	**8.1851**	**1.3877**	**3.2255**
RS Uric acid	**1.8891**	**4.0118**	**2.5195**	**1.7796**	**1.4040**	**2.1725**	**2.0994**	**1.7446**	**0.2605**	**0.9151**

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Table 1.3: 9 rural blacks' urine composition (Free diet)

Sample No.	01	02	03	04	05	06	07	08	09
Volume (ml/24hr)	2620	2240	1210	1600	1000	1920	1160	2060	900
pH	6.49	5.50	5.32	5.72	5.80	6.01	5.88	5.65	5.94
Sodium (mmol/24hr)	267.3	416.8	285.1	83.5	112.1	139.8	177.7	106.8	37.6
Potassium (mmol/24hr)	8.93	24.4	108.1	13.7	87.4	38.0	109.7	18.3	16.7
Calcium (mmol/24hr)	3.14	3.56	1.19	1.17	7.02	3.47	1.70	1.60	1.20
Magnesium (mmol/24hr)	2.30	3.93	3.65	1.09	4.94	6.59	6.27	1.83	1.64
Phosphate (mmol/24hr)	12.8	25.1	34.6	6.40	26	48.8	40.6	22.9	24.2
Oxalate (mmol/24hr)	0.60	0.55	0.36	0.05	0.21	0.38	0.37	0.61	0.44
Citrate (mmol/24hr)	3.55	2.21	2.05	0.43	2.32	3.56	2.85	4.16	2.09
Uric acid (mmol/24hr)	2.80	3.10	1.80	1.10	4.50	4.80	5.3	3.9	1.5
Chloride (mmol/24hr)	123	215	233	78.0	156	236	92.0	183	36
RS Brushite	**0.2431**	**0.1433**	**0.1134**	**0.0404**	**1.4174**	**0.7858**	**0.5647**	**0.0688**	**0.1465**
RS Calcium oxalate	**4.0754**	**4.6438**	**2.1441**	**0.4400**	**8.5582**	**3.6667**	**3.0523**	**3.5009**	**1.8604**
RS Uric acid	**0.2849**	**2.1715**	**2.8847**	**0.7622**	**6.0737**	**1.7157**	**3.9018**	**2.4641**	**2.9892**

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Table 1.4: 7 white subjects' urine composition (phytate-restricted diet)

Sample No.	01	02	03	04	05	06	07
Volume (ml/24hr)	890	470	4650	840	970	1530	1110
pH	6.25	5.82	4.88	6.07	5.82	6.00	6.26
Sodium (mmol/24hr)	193.9	69.9	227.6	71.3	167.4	236.4	54.8
Potassium (mmol/24hr)	99.4	52.1	158.8	100.3	106.5	89.7	112.4
Calcium (mmol/24hr)	2.43	1.93	2.18	1.47	0.92	1.53	3.84
Magnesium (mmol/24hr)	2.64	2.09	6.53	2.10	4.77	3.27	3.94
Phosphate (mmol/24hr)	27.0	36.0	51.2	30.2	35.3	22.8	36.3
Oxalate (mmol/24hr)	0.45	0.54	0.95	0.24	0.25	0.40	0.33
Citrate (mmol/24hr)	2.11	1.56	2.71	1.04	1.75	2.42	2.17
Uric acid (mmol/24hr)	3.50	3.90	4.60	2.60	3.20	4.40	3.80
Chloride (mmol/24hr)	155	151	265	89.0	246	156	194
RS Brushite	**1.4126**	**2.0606**	**0.0151**	**1.2534**	**0.3366**	**0.2417**	**2.3955**
RS Calcium oxalate	**7.9089**	**21.4155**	**2.0972**	**3.5761**	**1.5719**	**2.5093**	**7.6029**
RS Uric acid	**1.6132**	**7.7254**	**2.8553**	**1.8576**	**3.0818**	**1.9831**	**1.3983**

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Table 1.5: 7 white subjects' urine composition (phytate-rich diet)

Sample No.	01	02	03	04	05	06	07
Volume (ml/24hr)	860	1310	620	690	920	1910	1160
pH	6.44	6.21	5.96	6.18	5.72	5.76	5.71
Sodium (mmol/24hr)	29.9	173.3	207.5	34.4	142.1	209.2	140
Potassium (mmol/24hr)	88.6	93.4	22.5	48.1	25.4	70.1	26.2
Calcium (mmol/24hr)	1.27	3.37	1.38	0.45	6.19	2.48	4.47
Magnesium (mmol/24hr)	1.60	7.62	2.46	0.70	5.35	6.26	5.22
Phosphate (mmol/24hr)	28.1	35.4	22.1	10.8	38.9	28.8	37.2
Oxalate (mmol/24hr)	0.44	0.39	0.38	0.36	0.60	0.53	0.36
Citrate (mmol/24hr)	1.55	2.72	0.96	1.72	1.81	2.61	1.96
Uric acid (mmol/24hr)	3.90	5.70	3.50	1.00	3.60	4.70	4.80
Chloride (mmol/24hr)	123	127	115	28.0	98.0	206	161
RS Brushite	1.1282	1.4657	0.9606	0.1432	2.3776	0.2506	1.2026
RS Calcium oxalate	6.1568	5.2376	7.9494	2.9440	23.6291	3.9480	9.1789
RS Uric acid	1.2780	1.9794	4.0935	0.7280	4.3509	2.6259	4.7501

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Table 1.6: 9 Urban white residents' (Free diet)

Sample No.	01	02	03	04	05	06	07	08	09
Volume (ml/24hr)	960	1590	930	950	1280	950	750	590	890
pH	5.70	6.01	5.77	5.55	5.80	5.43	5.59	5.84	5.79
Sodium (mmol/24hr)	224.8	146.3	280.7	174.8	214.1	111.3	37.6	68.0	69.7
Potassium (mmol/24hr)	29.4	54.9	26.9	45.6	79.2	34.5	16.7	27.8	31.1
Calcium (mmol/24hr)	2.83	2.97	2.23	2.35	4.51	1.77	1.20	287	1.00
Magnesium (mmol/24hr)	6.29	5.00	3.15	3.09	5.76	2.15	1.64	26.6	2.10
Phosphate (mmol/24hr)	30.6	38.8	36.0	34.9	25.8	16.0	24.2	26.6	19.8
Oxalate (mmol/24hr)	0.38	0.49	0.36	0.29	0.45	0.39	0.44	0.38	0.22
Citrate (mmol/24hr)	1.65	2.95	2.75	1.93	1.63	1.22	2.09	2.37	1.07
Uric acid (mmol/24hr)	3.80	3.80	3.90	4.10	5.10	2.20	1.5	3.80	2.20
Chloride (mmol/24hr)	155	187	146	160	107	81.0	36	79.0	124
RS Brushite	**0.8276**	**0.7480**	**0.6995**	**0.5492**	**0.9109**	**0.2082**	**0.3259**	**1.8951**	**0.3096**
RS Calcium oxalate	**7.0325**	**5.5408**	**5.1275**	**5.4594**	**9.3150**	**8.8498**	**10.4807**	**18.0291**	**3.337**
RS Uric Acid	**4.5514**	**1.6307**	**4.2724**	**6.2532**	**3.9225**	**4.0256**	**2.8522**	**5.9100**	**2.5030**

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Appendix II

Individual Metastable Limit Experimental Results

Fig. 2.1: Metastable limit graphs (Black subject's result)

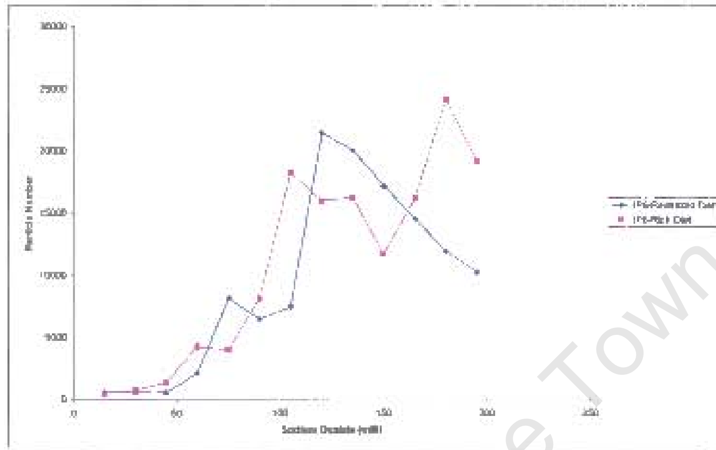

Fig. 2.1.1

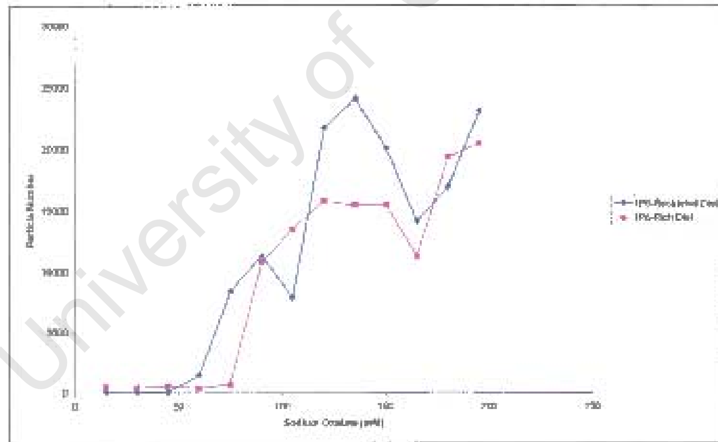

Fig. 2.1.2

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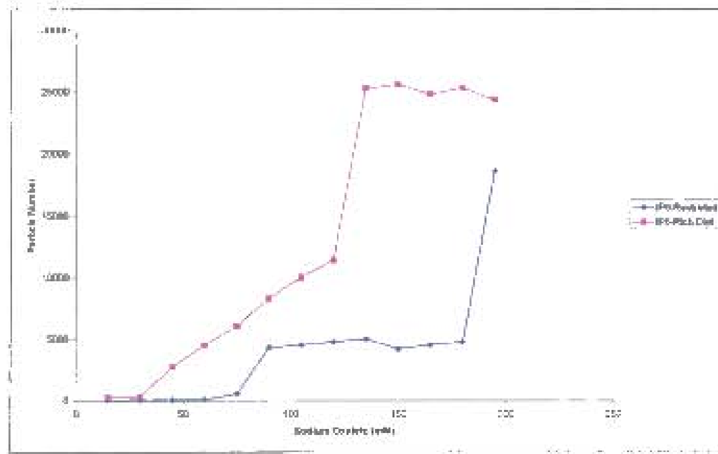

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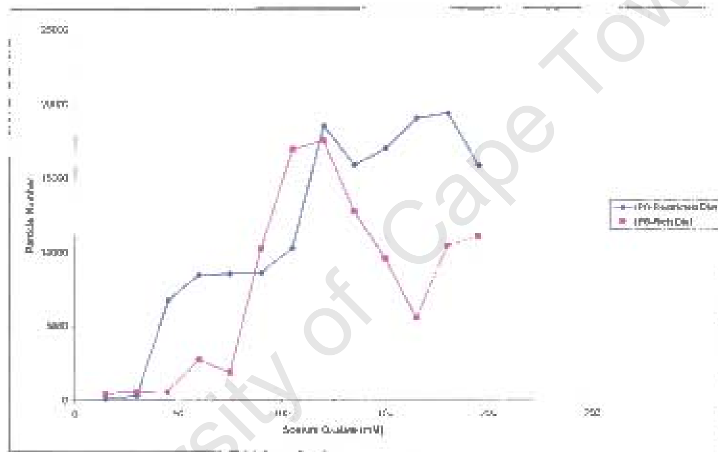

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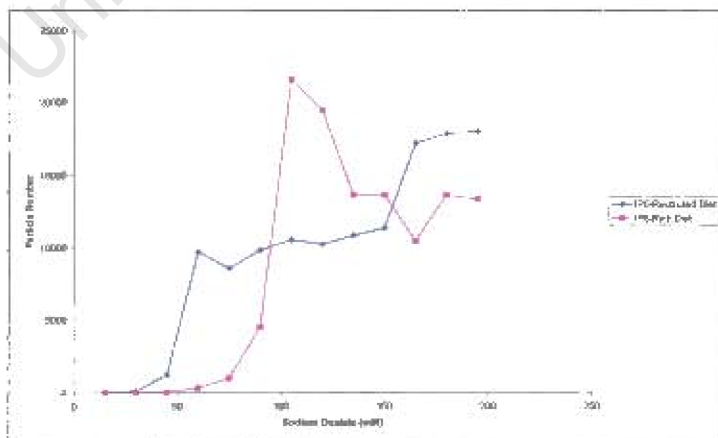

Fig.2.1.8

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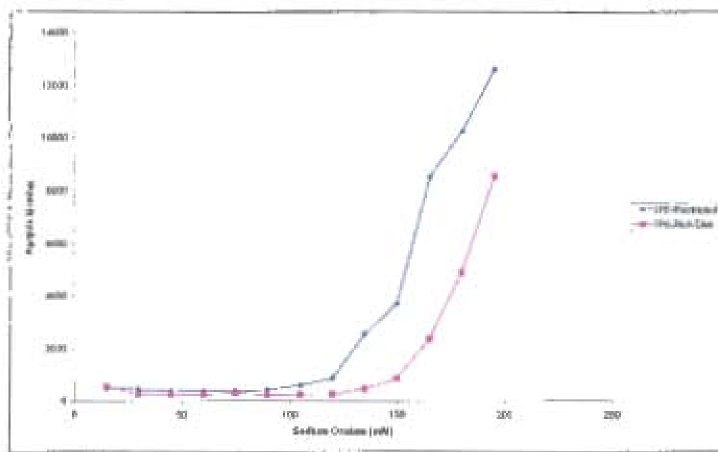

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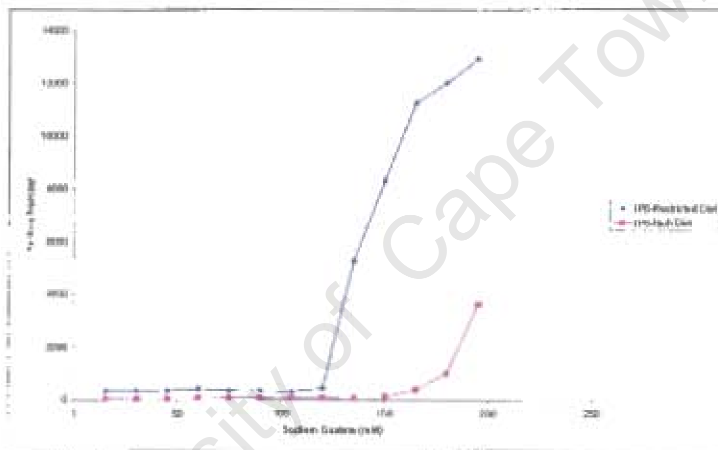

Fig. 2.1.10

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Table 2.1: Black subjects' metastable limit concentrations

Subject	Metastable Limit Concentration (mM)	
	IP_6-restricted Diet	**IP_6-rich Diet**
01	60	60
02	60	75
03	60	75
04	105	135
05	90	45
06	90	105
07	45	60
08	45	75
09	105	135
10	135	165

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Table 2.2: Average particle number at different sodium oxalate concentrations in urine of black subjects (n = 10)

Concentration (mM)	IP_6-Restricted Diet	IP_6-Rich Diet
15	786	918
30	883	998
45	1837	1109
60	3935	1716
75	4884	3018
90	5867	5989
105	7663	11019
120	12768	12431
135	14129	11731
150	13926	11524
165	15414	11592
180	15384	15335
195	17507	15905

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Fig. 2.2: Metastable limit graphs (white subject's results)

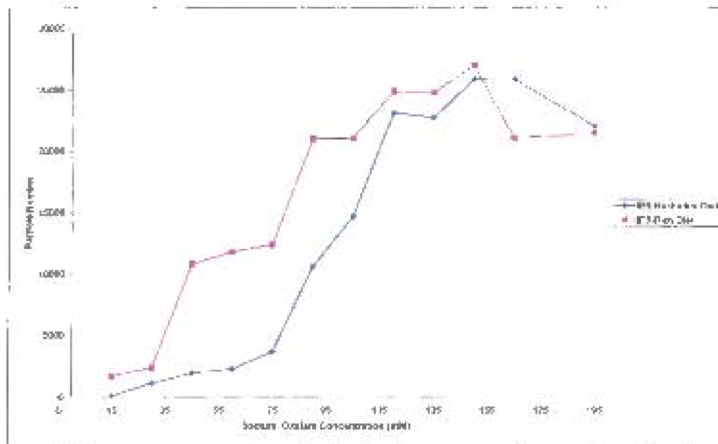

Fig. 2.2.1

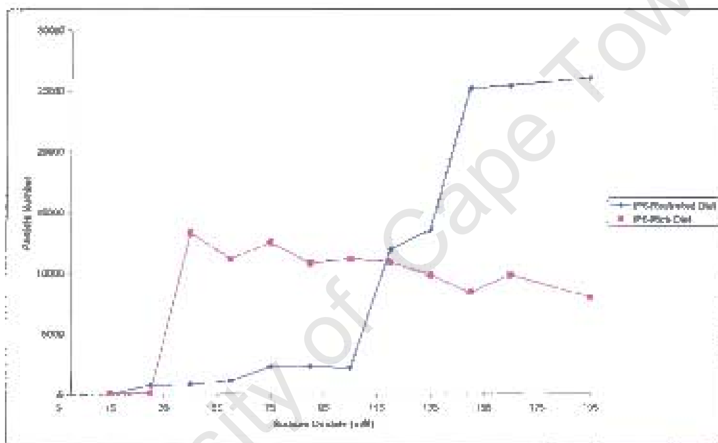

Fig. 2.2.2

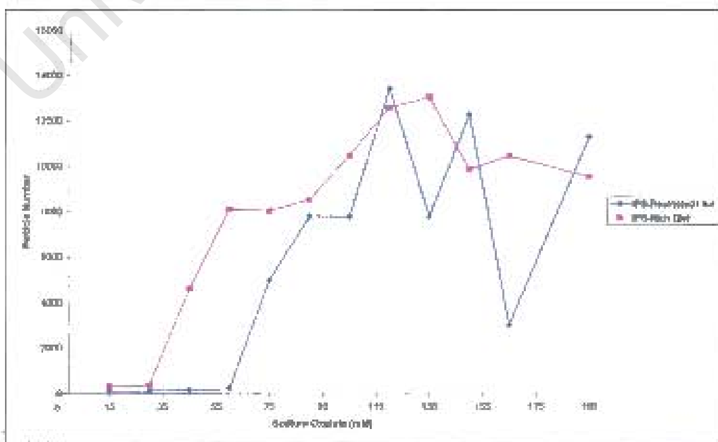

Fig. 2.2.3

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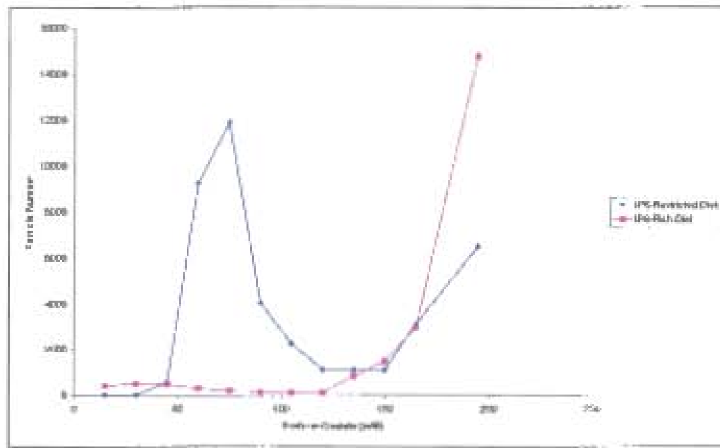
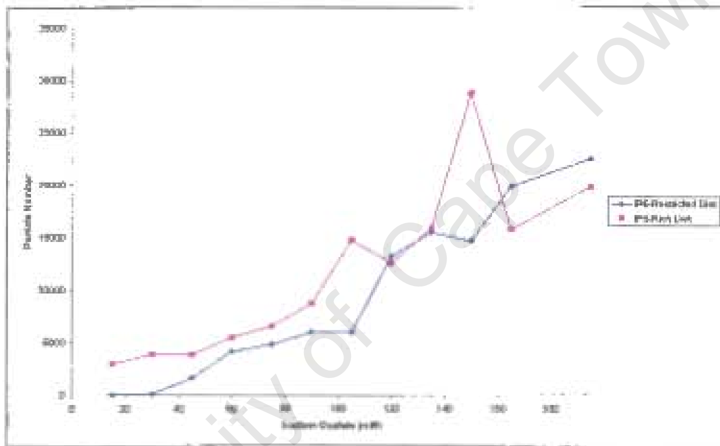


Fig. 2.2.4



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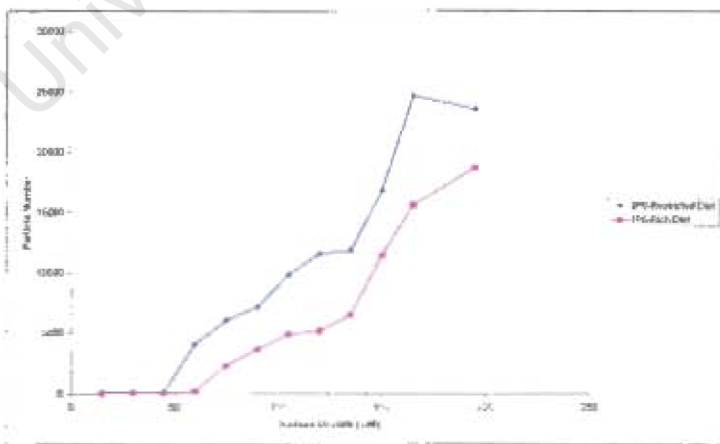

Fig. 2.2.6

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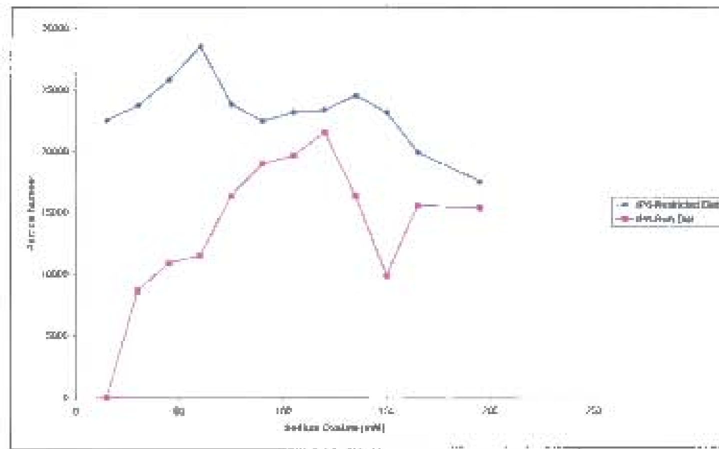

Fig. 2.2.7

Table 2.3: White Subjects' Metastable Limit Experimental results

Subject	Metastable Limit Concentration (mM)	
	IP_6-restricted Diet	IP_6-rich Diet
01	30	30
02	30	45
03	45	45
04	45	135
05	45	60
06	60	75
07	30	30

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Table 2.4: Average particle number at different sodium oxalate concentrations in urine samples of white subjects (n = 10)

Concentration (mM)	IP_6-restricted Diet	IP_6-Rich Diet
15	113	1232
30	1374	2137
45	2213	5875
60	4227	6981
75	5243	7158
90	7243	10454
105	8423	11672
120	12004	12578
135	11438	13534
150	13341	15511
165	15307	14416
180	15241	16377
195	15251	16099

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Appendix III

Sedimentation Experimental Graphs

Table 3.1: Characterization of CaOx crystals using XRD

2 θ	d-Spacing	Assignment
14.6	5.93	COM
17.8	4.99	COM
24.0	3.65	COM
29.6	2.97	COM
35.6	2.48	COM
37.8	2.37	COM

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Fig. 3.1: Black subjects' sedimentation results

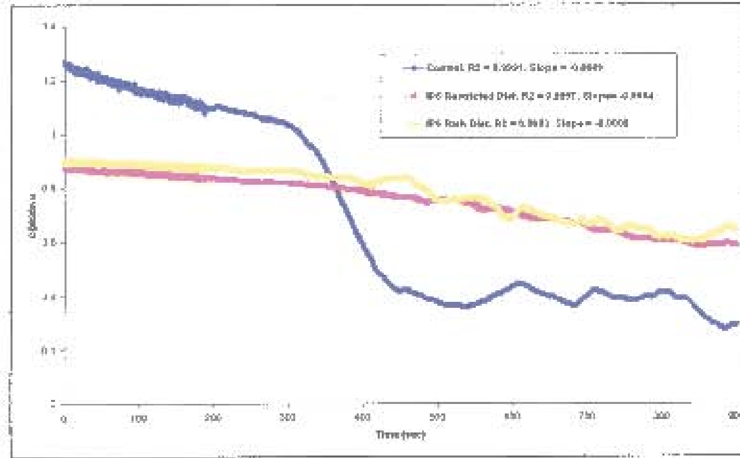

Fig. 3.1.1

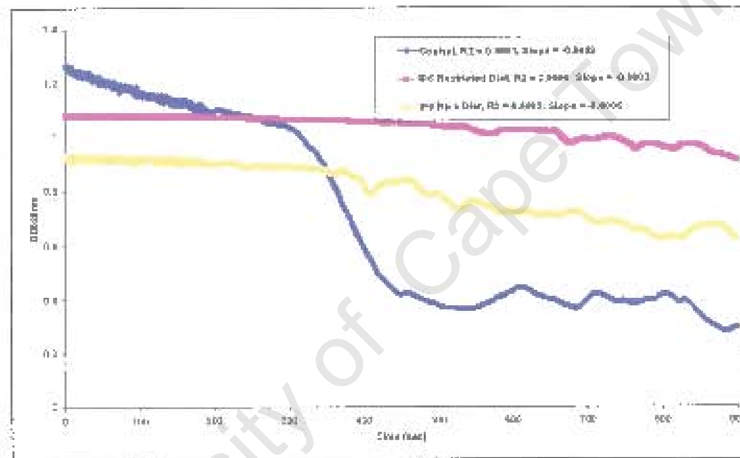

Fig. 3.1.2

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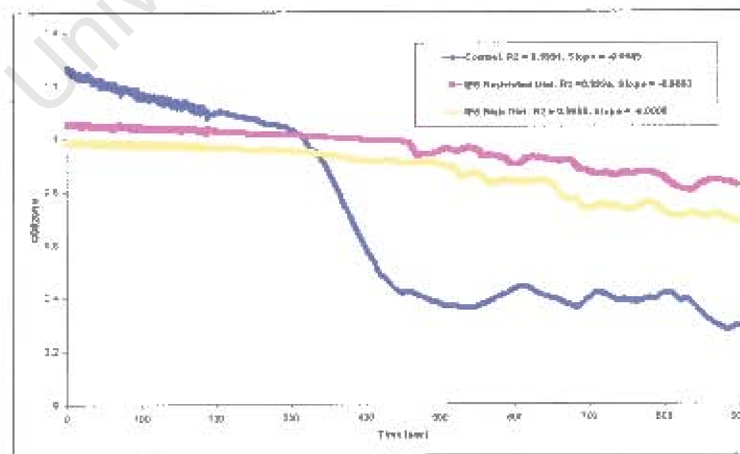
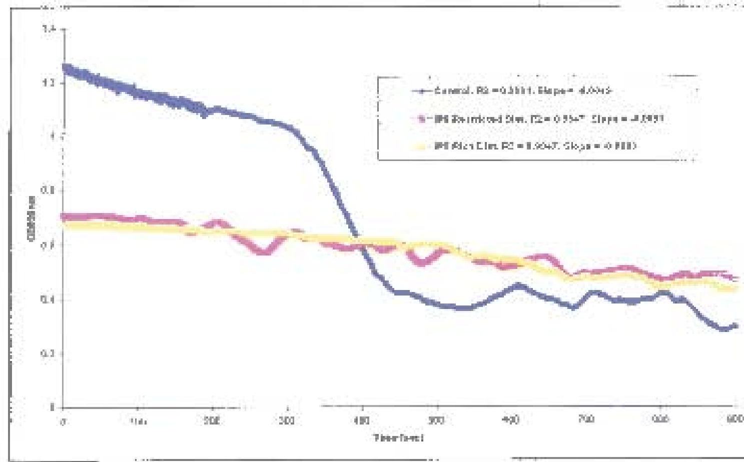


Fig. 3.1.3



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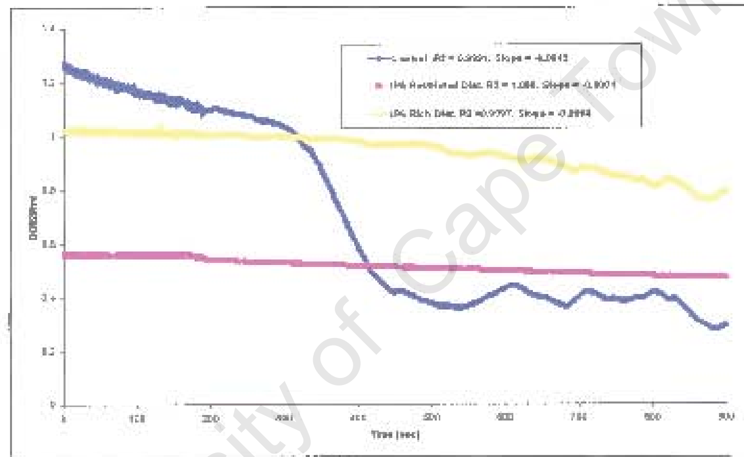

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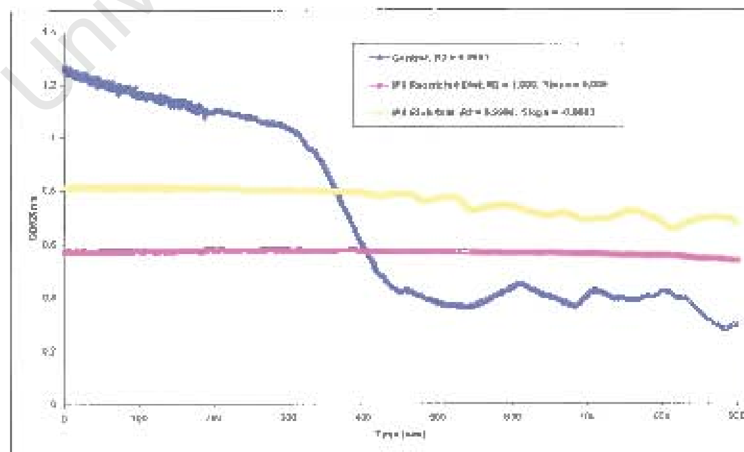

Fig. 3.1.6

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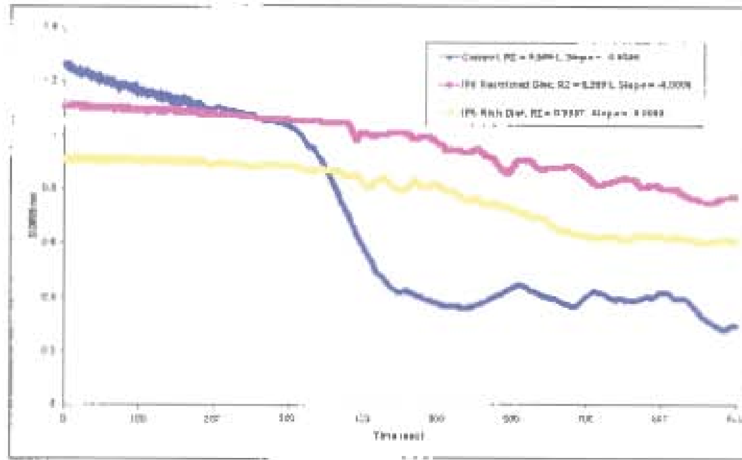
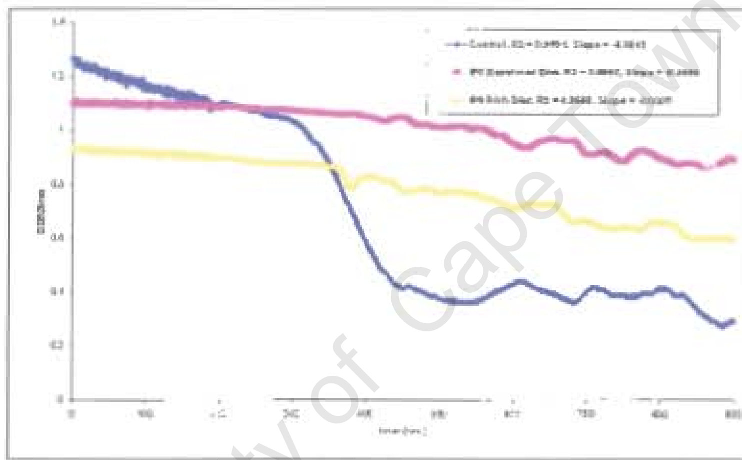


Fig.3.1.7



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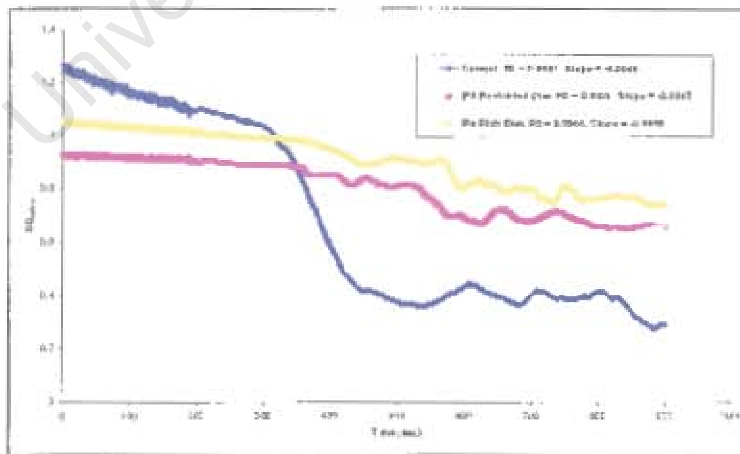

Fig. 3.1.9

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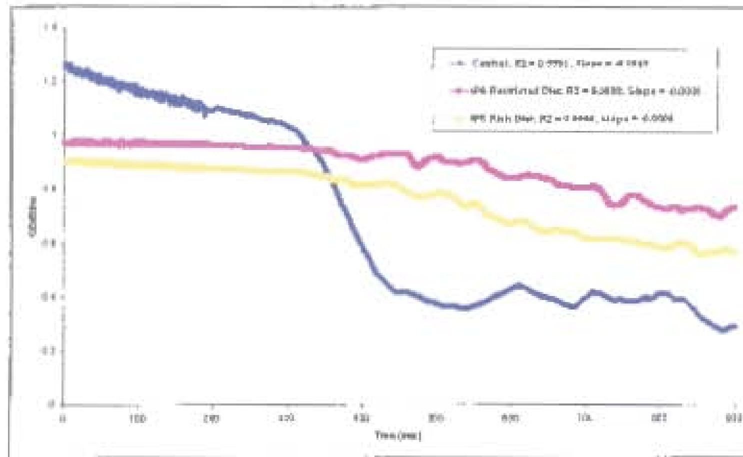

Fig. 3.1.10

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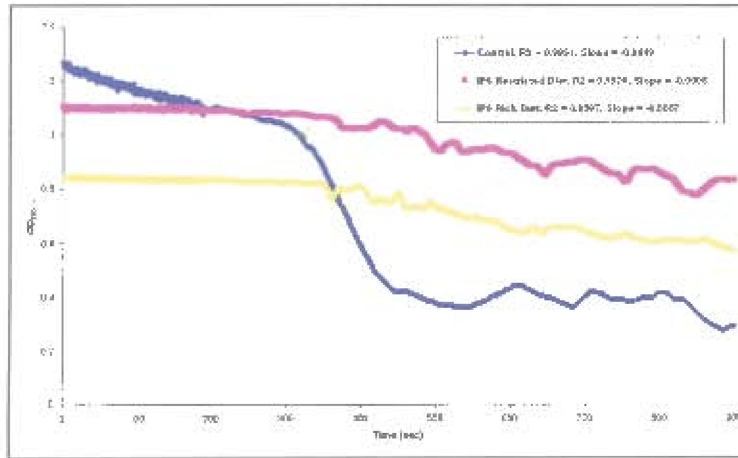
Fig. 3.2: White subjects' sedimentation results



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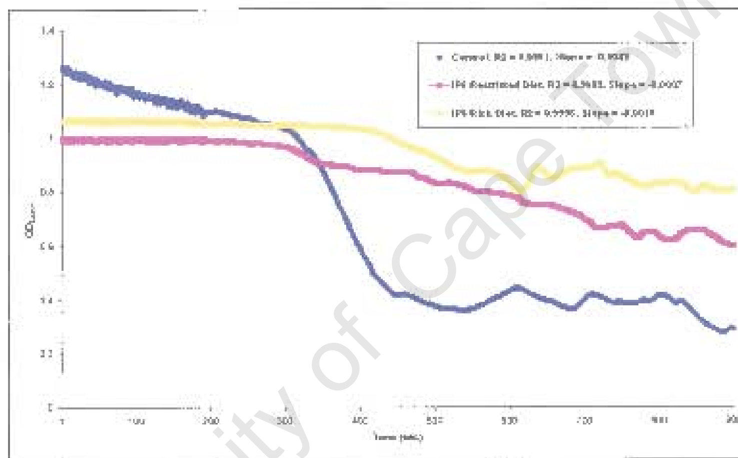

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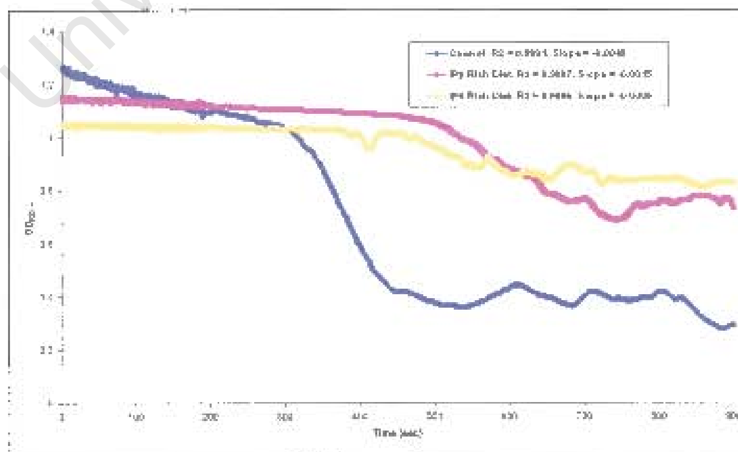

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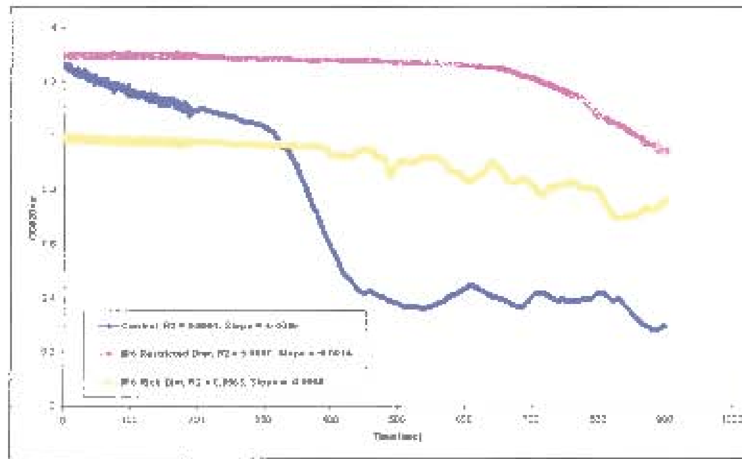

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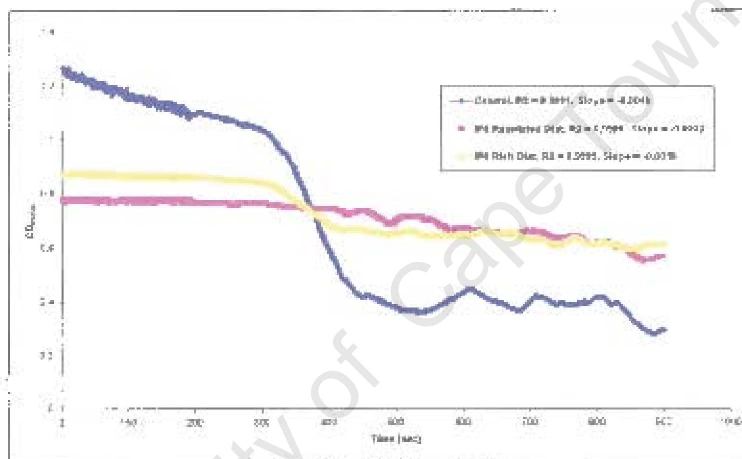

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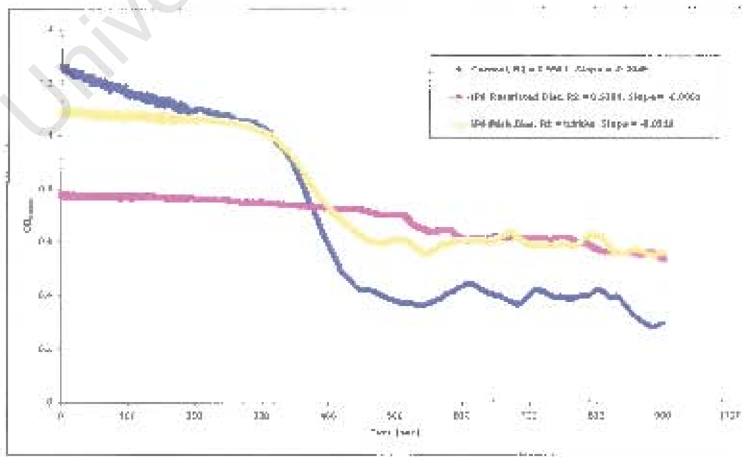

Fig. 3.2.6

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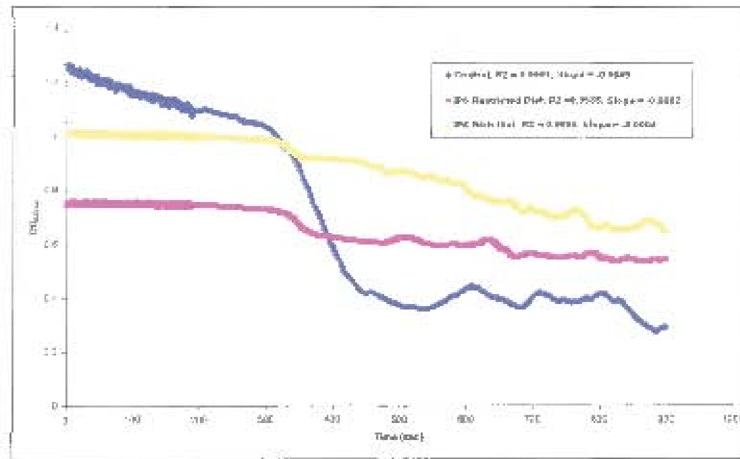

Fig.3.2.7

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Appendix IV
Individual ^{14}C-Oxalate Deposition Experimental Results

Fig. 4.1: ^{14}C-Oxalate deposition experimental graphs (black subjects' results)

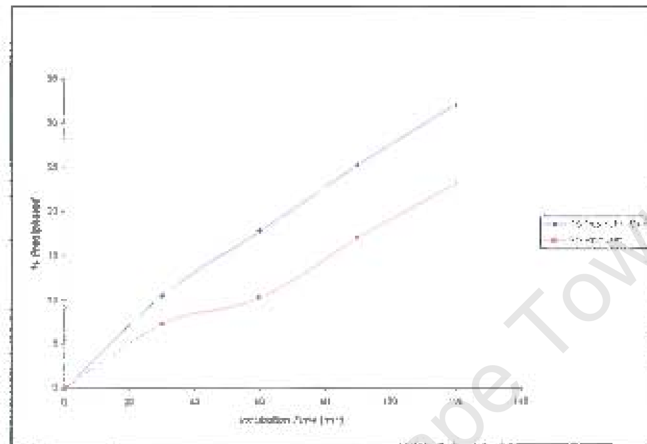

Fig. 4.1.1

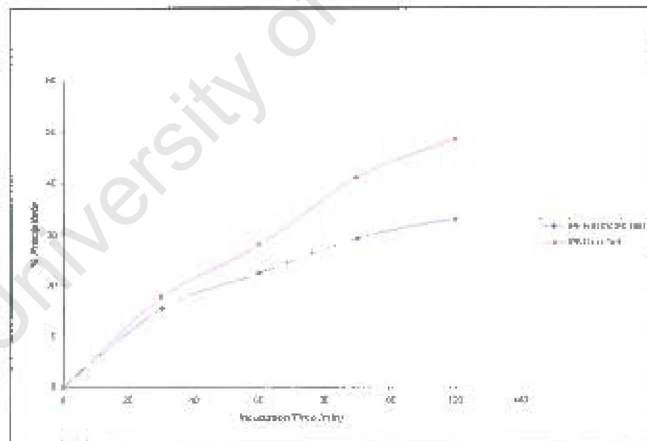

Fig. 4.1.2

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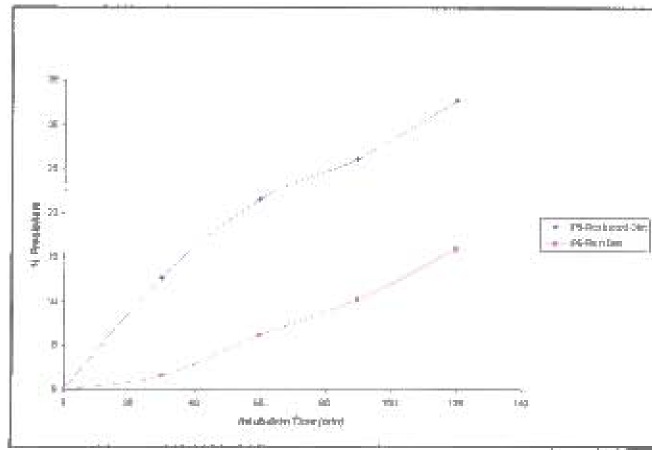

Fig. 4.1.3

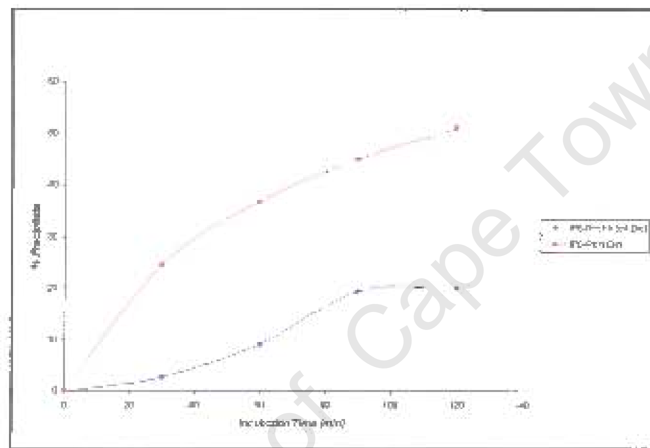

Fig. 4.1.4

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Fig. 4.2: ^{14}C-Oxalate deposition experimental graphs (white subjects)

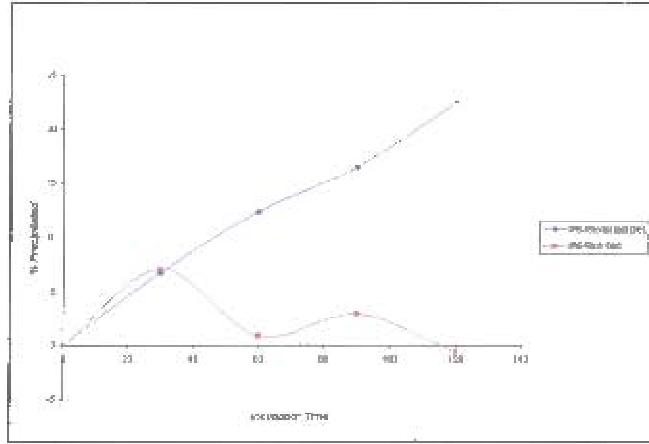

Fig. 4.2.1

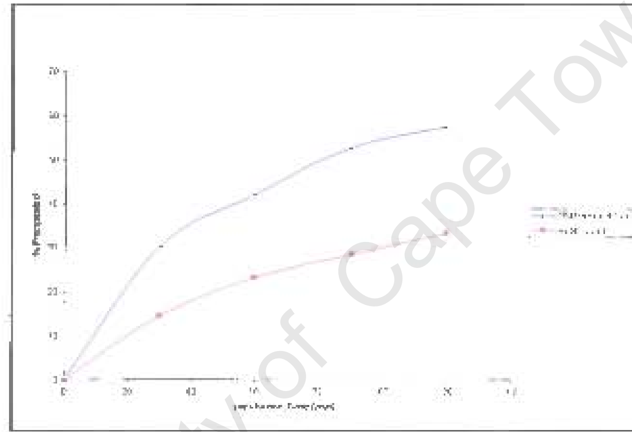

Fig. 4.2.2

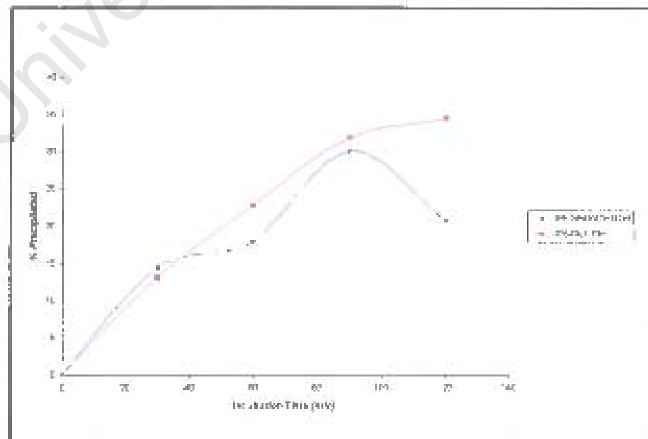

Fig. 4.2.3

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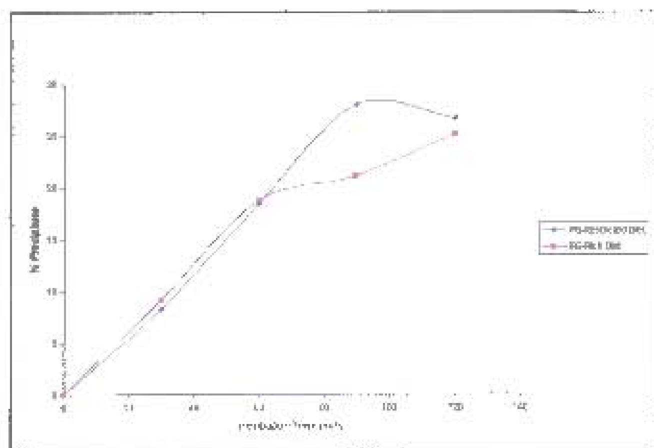

Fig. 4.2.4

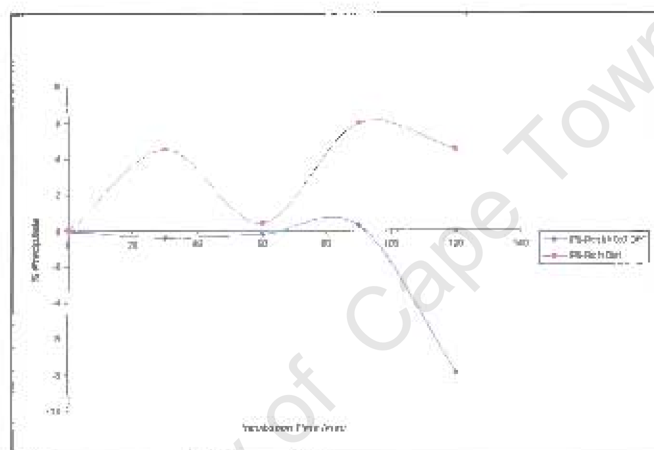

Fig. 4.2.5

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