COLONIC STRUCTURAL AND FUNCTIONAL
RELATIONSHIPS IN HEALTH AND INFLAMMATION

Thesis submitted by
RACHEL ANNE MENON
for the degree of Doctor of Philosophy
in the Faculty of Clinical Science of the University of London

Gastroenterological Unit
Institute of Child Health
30, Guildford Street
London WC1N 1EH
ABSTRACT

In health colonic function includes fluid and electrolyte salvage to produce dehydrated, formed stools. Effective salvage requires the removal of luminal sodium against considerable electrochemical and concentration gradients. This is made possible by the relatively tight structure of colonic epithelium impeding the leakage of absorbed sodium back into the lumen. This generates a transepithelial osmotic gradient necessary for water absorption and faecal dehydration. Colonic structure and function are therefore inextricably linked. The significance of these interrelationships was studied in health and inflammatory conditions of the human colon.

The use of concentration polarisation of impermeant fluorescent dyes and confocal microscopy, to measure colonic crypt concentration capacity in vitro, has been well established in a number of animal species. Data are presented to show the successful adaptation of this technology for use with endoscopic biopsies of human paediatric colon and in the study of colonic function in health and inflammation in children. Structural information, regarding crypt density, architecture and F-actin component of the pericryptal cytoskeleton was also assessed using specific fluorescent probes visualised by confocal microscopy.

Inflammation altered normal colonic structural and functional relationships with the nature of the inflammatory response emerging as an important factor. Eosinophilic colitis was associated with impaired crypt absorptive capacity despite normal structure. Predominant neutrophil infiltration, which featured in ulcerative colitic patients studied, was accompanied by a physical reduction in the number of functioning crypts. Neutrophils cause tissue damage by both oxidative free radical and non-oxidative enzymic mechanisms. Free radical assays revealed elevated levels in inflamed colonic tissue with a positive correlation between superoxide production and neutrophil infiltration.

Maintenance of colonic structure and functional relationships found in health form an important focus for future research in the quest for knowledge regarding the distressing inflammatory bowel diseases of childhood.
ACKNOWLEDGEMENTS

I would like to acknowledge the support of my supervisors, Dr Peter Milla, Dr Keith Lindley and Professor Richard Naftalin. Firstly for allowing me the opportunity of carrying out this research. Secondly for their continued support throughout times of success and frustration. They have taught me much about myself as well as research.

I am grateful to the Dr Hadwen Trust for their generous financial support and funding.

I continue to be indebted to mum and dad for supporting me through yet another venture and for living by the sea.

Finally there are two influences in my life without whom this PhD. would not have been possible and for which I will always be eternally thankful; God and my loving husband, Neil.
CONTENTS

Abstract .............................................................................................................2
Acknowledgements ..........................................................................................3
Contents ............................................................................................................4
Table of contents ..............................................................................................5
Table of tables ................................................................................................14
Table of figures ...............................................................................................15
Abbreviations ..................................................................................................22
Chapters 1 – 9 ........................................................................................25-234
Final discussion ............................................................................................235
Conclusions ..................................................................................................239
Appendix 1 (Statistical methods and notation in this thesis) ......................240
Appendix 2 (Additional patient data from chapters 6 – 9) .........................244
Appendix 3 (Additional methodology information) ....................................266
References ...................................................................................................267
TABLE OF CONTENTS

ABSTRACT ...................................................................................................... 2

ACKNOWLEDGEMENTS ............................................................................... 3

CONTENTS ..................................................................................................... 4

TABLE OF CONTENTS .................................................................................. 5

TABLE OF TABLES ........................................................................................ 14

TABLE OF FIGURES .................................................................................... 15

ABBREVIATIONS .......................................................................................... 22

INTRODUCTION

CHAPTER 1

Human colonic structure and function in health ...................................... 25
  1.1 Introduction ................................................................................... 25
  1.2 Colonic function ................................................................. 26
    1.2.1 Na,K-ATPase .................................................................... 27
    1.2.2 Water ................................................................................. 27
    1.2.3 Sodium and water absorption ....................................... 28
      1.2.3.1 A hypertonic absorbate ....................................... 30
    1.2.4 Chloride and water secretion .......................................... 31
    1.2.5 Potassium transport ......................................................... 33
    1.2.6 Bicarbonate transport ...................................................... 34
  1.3 Colonic structure .......................................................................... 34
    1.3.1 Intercellular tight junctions ............................................... 36
    1.3.2 Extracellular matrix .......................................................... 38
      1.3.2.1 Pericryptal fibroblast sheath ................................38
  1.4 Structural and functional relationships ....................................... 40
1.4.1 An old dogma – shot in the leg ................................................. 40
1.4.2 An old dogma – laid to rest ..................................................... 41
1.4.3 Colonic crypt transport ............................................................ 42
  1.4.3.1 Crypt absorption ................................................................. 42
  1.4.3.2 Crypt secretion ................................................................. 42
1.4.4 Functional significance of crypt absorption ......................... 43
  1.4.4.1 Faecal dehydration ............................................................ 43
  1.4.4.2 A specific role for crypt absorption in faecal dehydration? ................................................................. 44
1.5 Regulation of colonic transport ...................................................... 45
  1.5.1 The enteric nervous system ................................................... 45
  1.5.2 The endocrine system .......................................................... 47
  1.5.3 The immune system ............................................................ 47

CHAPTER 2

Human colonic structure and function in inflammation ............... 48

2.1 Introduction .............................................................................. 48
2.2 The inflammatory process in colitis ......................................... 48
  2.2.1 Inflammatory cell infiltration .................................................. 49
  2.2.2 Inflammatory mediators ......................................................... 50
    2.2.2.1 Platelet activating factor .................................................. 50
    2.2.2.2 Eicosanoid production: prostaglandins and leukotrienes ................................................................. 51
    2.2.2.3 Free radicals ................................................................. 53
2.3 Effects of inflammation on colonic function and structure ...... 53
  2.3.1 Cellular basis for defective function ...................................... 54
  2.3.2 Structural basis for defective function .................................... 56
    2.3.2.1 Tight junction – structural changes ................................ 56
    2.3.2.2 Tight junction – structural plasticity ................................ 58
2.4 Inflammatory bowel diseases ..................................................... 59
  2.4.1 Ulcerative colitis ................................................................. 59
  2.4.2 Crohn’s disease ................................................................. 60
  2.4.3 Epidemiology ................................................................. 62
  2.4.4 Etiology ................................................................. 62
CHAPTER 3

Role of oxygen free radicals in health and inflammatory bowel disease

3.1 Introduction ................................................................................... 67
3.2 Free radical chemistry ................................................................. 67
  3.2.1 Free radical reactions ...................................................... 68
3.3 Free radical sources in health and IBD ...................................... 70
  3.3.1 Phagocyte respiratory burst ............................................. 70
  3.3.2 Eicosanoids: prostaglandins and leukotrienes ............... 72
3.4 Effects of free radicals ............................................................... 73
  3.4.1 In vivo damage potential .................................................. 73
    3.4.1.1 Extracellular matrix – neutrophil-mediated
      ROM damage .................................................................. 74
    3.4.1.2 Cell membranes – lipid peroxidation .................. 75
    3.4.1.3 Proteins ................................................................. 77
3.5 In vivo damage limitation ............................................................ 77
  3.5.1 Catalase ............................................................................ 78
  3.5.2 Glutathione peroxidase .................................................... 78
  3.5.3 Superoxide dismutases ................................................... 79
  3.5.4 Vitamin E ........................................................................... 79
  3.5.5 Ascorbic acid (Vitamin C) ................................................ 80
  3.5.6 Sequestration of metal ions ............................................. 80
3.6 Role of free radicals in IBD ......................................................... 80
  3.6.1 Excess free radical production ........................................ 80
  3.6.2 Decreased defence ......................................................... 82
  3.6.3 Therapeutic treatments .................................................... 82
  3.6.4 Specific known effects on colonic structure and
    function .............................................................................. 83

HYPOTHESES ............................................................................................... 84
AIMS OF STUDY ........................................................................................... 85
METHODS & RESULTS

CHAPTER 4

Measurement of intestinal transport in vivo .........................................................86
4.1 Aims .................................................................................................................86
4.2 In vivo method 1 – agarose gels .................................................................87
  4.2.1 Principles ..............................................................................................87
  4.2.2 Gel production ....................................................................................88
  4.2.3 Measurement of fluid and electrolyte changes ..................................89
  4.2.4 Validation of gels ................................................................................89
    4.2.4.1 Gel concentration ....................................................................89
    4.2.4.2 Gel dimensions ........................................................................89
    4.2.4.3 Hydraulic conductivity ...........................................................89
    4.2.4.4 Measurement of electrolytes .................................................92
  4.2.5 Animal studies ....................................................................................95
    4.2.5.1 Method ....................................................................................95
    4.2.5.2 Results .....................................................................................97
  4.2.6 Human studies ....................................................................................97
    4.2.6.1 Method ....................................................................................97
    4.2.6.2 Results ....................................................................................101
4.3 In vivo method 2 – closed colonic loops .....................................................102
  4.3.1 Method and results .............................................................................102
4.4 Discussion ....................................................................................................103
  4.4.1 Agarose gels as a tool to measure colonic transport in vivo .................103
    4.4.1.1 Practical limitations .................................................................104
    4.4.1.2 Experimental results ...............................................................104
  4.4.2 Gels vs. colonic loops to measure colonic transport in vivo .................105

CHAPTER 5

Measurement of intestinal transport in vitro .................................................108
5.1 Aims .............................................................................................................108
5.2 Principals of confocal microscopy .............................................................108
5.3 Visualisation and measurement of crypt fluid transport in vitro ...............112
5.3.1 Method ............................................................................ 113
5.3.2 Microscopy ..................................................................... 114
5.3.3 Image analysis ................................................................ 114
5.4 Validation of methodology ........................................................ 116
5.4.1 Obtaining an accurate final image – confocal
parameters ........................................................................ 116
5.4.1.1 Fluorescence detection and collection
efficiencies ........................................................................ 116
5.4.1.1.1 The pixelated image ........................................ 116
5.4.1.1.2 Offset ................................................................ 116
5.4.1.1.3 Gain ................................................................ 117
5.4.1.1.4 Pinhole size ................................................... 117
5.4.1.1.5 Experimental protocol ..................................... 118
5.4.1.2 Fluorophore photochemistry .................................. 121
5.4.1.2.1 Photophysical problems related to
high-intensity excitation ................................................. 123
5.4.1.2.1 Proposed experimental protocol ... 125
5.4.2 Tissue viability ................................................................ 130
5.4.2.1 Method ................................................................ 131
5.4.2.2 Validation ............................................................ 131
5.5 Measurement of basal fluid transport in crypts ...................... 134
5.5.1 General method used in functional studies ......................... 134
5.5.2 Crypt concentration profile ............................................. 134
5.5.2.1 Method ................................................................ 134
5.5.2.2 Results ............................................................... 135
5.5.2.3 Problems incurred .............................................. 137

CHAPTER 6

Colonic crypt function in health and inflammation ............... 141
6.1 Aims .................................................................................... 141
6.2 Colonic crypt function in vitro in healthy human colon ....... 142
6.2.1 Basal crypt transport ..................................................... 142
6.2.1.1 Results ................................................................ 144
6.2.2 Nature of crypt FD concentration ................................. 144
6.2.2.1 Results ................................................................. 146
6.2.3 Measurement of extracellular sodium ....................... 146
   6.2.3.1 Method .............................................................. 149
   6.2.3.2 Results .............................................................. 149
6.2.4 Measurement of crypt secretion .................................. 153
   6.2.4.1 Method .............................................................. 153
   6.2.4.2 Results .............................................................. 155
6.3 Colonic crypt function in vitro in inflamed human colon ... 156
   6.3.1 Confocal microscopy ............................................ 156
   6.3.2 Results ............................................................... 156
6.4 Colonic function in vivo - effects of inflammatory mediators .. 161
   6.4.1 Platelet activating factor ....................................... 161
   6.4.2 Phorbol myristate acetate ..................................... 162
   6.4.3 Agarose gels ......................................................... 162
      6.4.3.1 Method ........................................................ 162
      6.4.3.2 Results ........................................................ 163
   6.4.4 Closed colonic loops ............................................ 167
      6.4.4.1 Method ........................................................ 167
      6.4.4.2 Results ........................................................ 167
6.5 Discussion ................................................................. 167
   6.5.1 Crypt function in vitro in health ............................. 167
   6.5.2 Crypt function in vitro in inflamed colon .................. 170
   6.5.3 Effects of inflammatory mediators on colonic transport
      in vivo ................................................................. 173
      6.5.3.1 In vivo methodologies ................................. 173

CHAPTER 7
Colonic structural and functional relationships in health
and inflammation ............................................................. 175
7.1 Aims ........................................................................ 175
7.2 Structural studies ....................................................... 176
   7.2.1 Crypt density ...................................................... 176
      7.2.1.1 Method ....................................................... 176
   7.2.2 Pericryptal F-actin sheath .................................... 177
7.2.2.1 Method ................................................................. 177
7.3 Colonic structure in health .............................................. 178
  7.3.1 Results ................................................................. 178
7.4 Colonic structure in inflammation ..................................... 182
  7.4.1 Results ................................................................. 184
7.5 Effects of inflammation on normal colonic structural and
  functional relationships .............................................. 186
  7.5.1 Results ................................................................. 194
    7.5.1.1 Normal structure and function, abnormal
      histology .............................................................. 194
    7.5.1.2 Normal structure, abnormal function ............... 194
    7.5.1.3 Abnormal structure and function .................... 195
7.6 Cellular nature of the inflammatory infiltrate ..................... 195
  7.6.1 Method ................................................................. 195
  7.6.2 Results ................................................................. 197
7.7 Discussion .................................................................. 197
  7.7.1 Colonic structure in health ..................................... 197
  7.7.2 Colonic structure in inflammation ............................ 198
  7.7.3 Colonic structural and functional relationships in
      health ................................................................. 199
  7.7.4 Colonic structural and functional relationships
      in inflammation ...................................................... 199

CHAPTER 8

Measurement of oxidative stress in vivo and in vitro in
the inflamed colon - Methods ............................................. 202
8.1 Introduction .............................................................. 202
  8.1.1 Measurement of ROM ............................................ 202
8.2 Direct measurement of $O_2^-$ ....................................... 203
  8.2.1 Nitroblue tetrazolium – an $O_2^-$ probe ..................... 204
  8.2.2 NBT – quantification ............................................. 205
8.3 Direct measurement of $O_2^-$ in vivo – using NBT ............. 206
  8.3.1 Agarose gels ....................................................... 206
8.4 Direct measurement of $O_2^-$ in vitro – using NBT .......... 207
8.4.1 Human studies ................................................................................................................. 207
8.4.2 Cellular sources of O$_2^-$ - neutrophil immunoassay .................................................... 208
  8.4.2.1 Method .......................................................................................................................... 208
8.5 Indirect measurement of free radicals in vitro ................................................................. 210
  8.5.1 NAH (3-hydroxy-2-naphthoic acid hydrazide) ............................................................. 210
  8.5.2 Method ........................................................................................................................... 212

CHAPTER 9
Measurement of free radical production in inflamed colon .................................................... 214
  9.1 Aims ......................................................................................................................................... 214
  9.2 Direct measurement of O$_2^-$ in vivo in inflamed colon ....................................................... 214
    9.2.1 Human studies .................................................................................................................. 214
    9.2.2 Animal studies ................................................................................................................. 214
  9.3 Direct measurement of O$_2^-$ in vitro in inflamed colon ..................................................... 215
    9.3.1 Human studies .................................................................................................................. 215
        9.3.1.1 Results ..................................................................................................................... 220
    9.3.2 Cellular sources of O$_2^-$ ............................................................................................... 222
        9.3.2.1 Results ..................................................................................................................... 223
  9.4 Indirect measurement of oxidative stress in inflamed colon ............................................. 224
    9.4.1 NAH .................................................................................................................................. 224
    9.4.2 Initial experiments .......................................................................................................... 225
        9.4.2.1 Results ..................................................................................................................... 229
    9.4.3 Studies in IBD .................................................................................................................. 230
        9.4.3.1 Results ..................................................................................................................... 232
  9.5 Discussion ............................................................................................................................. 232
    9.5.1 Direct detection methods - NBT .................................................................................... 232
    9.5.2 Indirect detection methods – NAH .................................................................................. 233

FINAL DISCUSSION ................................................................................................................... 235
CONCLUSIONS ......................................................................................................................... 239

APPENDIX 1
Statistical methods and notation in this thesis ....................................................................... 240
Parametric data .......................................................................................................................... 240
Non parametric data .................................................................241
Correlation and regression analysis.............................................242

APPENDIX 2

Additional patient data from chapters 5 – 7 & 9 .................244
A.1 Introduction.................................................................244
A.2 Colonic crypt concentration profile...............................244
A.3 Colonic crypt function in vitro in human control colon ........246
   A.3.1 Basal crypt transport .............................................246
   A.3.2 Nature of crypt FD concentration .........................249
      A.3.2.1 Amiloride ..................................................249
      A.3.2.2 Ouabain ...................................................251
   A.3.3 Measurement of crypt secretion ............................252
A.4 Colonic crypt transport in vitro in human inflamed colon ....254
   A.4.1 Ulcerative colitis ...............................................255
   A.4.2 Allergic colitis ..................................................256
A.5 Colonic structure in health .............................................257
A.6 Colonic structure in inflammation ...................................258
   A.6.1 Allergic colitis ...............................................258
   A.6.2 Ulcerative colitis .............................................259
A.7 Effects of inflammation on colonic structural and
    functional relationships ..............................................260
   A.7.1 Normal structure and function ............................260
   A.7.2 Normal structure, abnormal function ....................262
   A.7.3 Abnormal structure and function ..........................263
A.8 Direct measurement of O$_2^-$ in vitro using NBT ..........264
A.9 Measurement of colonic oxidative stress using NAH ± FBB..265

APPENDIX 3

Additional methodology information .................................266

REFERENCES ..............................................................................267-300
TABLE OF TABLES

Table 1,1  Water flow and ionic composition of intestinal contents in humans - comparison of infants with adults ..................................................26
Table 2,1  Electrical profile of human colonic mucosa in control and inflamed tissue, as measured in Ussing chambers ...............................54
Table 2,2  Contrasting patterns of pathological involvement in ulcerative colitis and Crohn's disease ..........................................................61
Table 2,3  Epidemiology of ulcerative colitis and Crohn's disease ..........62
Table 4,1  Clinical details and results of the four patients in which in vivo rectal transport was measured, using agarose gels ......................99
Table 7,1  Patient's clinical details for structural and functional studies ......187
Table 9,1  Histochemical grading for NBF deposits in human colonic biopsies ............................................................................................216
Table 9,2  Scoring assessment for neutrophil and eosinophil infiltration of colonic tissue..............................................................................223
Table A,1  Basal rates of FD concentration by human non-inflamed colonic crypts and adjacent pericryptal areas from a range of colonic regions ........................................................................246
Table A,2  Rate of increase in crypt luminal and pericryptal FD fluorescence intensity in human colonic tissue in the presence of amiloride ...............................................................249
Table A,3  Rate of increase in crypt luminal and pericryptal FD fluorescence intensity in human colonic tissue in the presence of ouabain ........................................................................250
Table A,4  Rates of increase in crypt luminal FD fluorescence intensity in control and inflamed human colonic tissue .......................................254
Table A,5  A) NBT scoring system B) Individual patient NBT scores ..............264
TABLE OF FIGURES

Figure 1,1  Active transport of sodium across an epithelial cell ..................28
Figure 1,2  Cellular mechanisms of sodium and water absorption across human colonic epithelium with the generation of a hypertonic absorbate ...........................................................................................30
Figure 1,3  Cellular mechanisms of chloride, bicarbonate and water secretion across human colonic epithelium ........................................32
Figure 1,4  Anatomical divisions of the human colon..................................34
Figure 1,5  Cellular micro-architecture of the colonic wall .........................35
Figure 1,6  Schematic illustration depicting the location and structure of the tight junction in intestinal epithelial cells ..........................37
Figure 1,7  Schematic representation of the pericryptal fibroblast sheath surrounding individual colonic crypts .................................39
Figure 1,8  Schematic diagram to illustrate intestinal reflex pathways regulating intestinal mucosal function .................................46
Figure 2,1  Process of neutrophil migration across vascular endothelial cells .................................................................................................50
Figure 2,2  Arachidonic acid metabolism via cyclo-oxygenase and lipo-xygenase pathways resulting in prostaglandin and leukotriene production ..................................................................................52
Figure 3,1  Univalent reduction reactions of ground state O₂ to form water ....68
Figure 3,2  Different forms of reactive oxygen showing electron spin restrictions .........................................................................................69
Figure 3,3  Extracellular production of reactive oxygen metabolites via the neutrophil respiratory burst ..................................................71
Figure 3,4  Favourable and detrimental roles of reactive oxygen metabolites leading to either tissue protection or damage .......73
Figure 3,5  Hydrogen abstraction and peroxidation of a polyunsaturated fatty acid .....................................................................................76
Figure 4,1  Agarose gel used in human in vivo intestinal transport studies ....88
Figure 4,2  Set up used to measure hydraulic conductivity of agarose gel ......90
Figure 4.3  Hydraulic conductivity of agarose gel as a function of gel (A) concentration and (B) thickness using 5% gels

Figure 4.4  Accuracy of flame photometry as a tool for the measurement of (A) Na⁺ and (B) K⁺ in deionised water

Figure 4.5  Accuracy of electrolyte extraction from agarose gel cylinders measured by flame photometry

Figure 4.6  In vivo fluid transport in rat descending colon, measured using agarose gel cylinders

Figure 4.7  In vivo Na⁺ transport in rat descending colon, measured using agarose gel cylinders

Figure 4.8  In vivo K⁺ transport in rat descending colon, measured using agarose gel cylinders

Figure 4.9  In vivo rectal fluid transport in four paediatric patients, measured using agarose gel cylinders

Figure 4.10 In vivo rectal Na⁺ transport in four paediatric patients, measured using agarose gel cylinders

Figure 4.11 In vivo rectal K⁺ transport in four paediatric patients, measured using agarose gel cylinders

Figure 4.12 Basal in vivo fluid transport in rat descending colon, measured using closed colonic loops

Figure 5.1  Schematic illustration of the operating principles of confocal and conventional widefield microscopes

Figure 5.2  Complete confocal set-up

Figure 5.3  Typical confocal image of human colon incubated in Tyrode's salt solution containing FITC-dextran

Figure 5.4  Relationship between fluorescence intensity and laser power measured in human colonic tissue incubated in FITC-dextran

Figure 5.5  Relationship between fluorescence intensity and gain measured in human colonic tissue incubated in FITC-dextran

Figure 5.6  Jablonski diagram depicting the energy level system of a typical chromophore

Figure 5.7  Effects of continuous laser / Hg-arc exposure on fluorescence intensity and recovery measured in human colonic tissue incubated in FITC-dextran
Figure 5.8 The effects of laser scan rates and lines averaging on fluorescence intensity in human or rat colonic biopsies incubated in FITC-dextran ...............................................................129
Figure 5.9 The use of supravital dyes, ethidium bromide and acridine orange to assess human colonic tissue viability .....................133
Figure 5.10 Confocal image of human colonic crypts in longitudinal section showing optical sections taken in the z-plane along crypt axis ....135
Figure 5.11 Concentration profile of FD down crypt luminal axis in human non-inflamed descending colon ......................................................136
Figure 5.12 Variations in FD concentration profile along crypt luminal axis with respect to optical level taken as crypt luminal surface ......138
Figure 6.1 Confocal time series showing increase in crypt luminal fluorescence intensity in human ascending colon incubated in FD ......................................................................................................142
Figure 6.2 Time course of FD concentration at constant depth by colonic crypts in vitro in human ascending colon ..........................143
Figure 6.3 The effects of inhibiting active Na⁺ transport on FD concentration by colonic crypts in vitro in human descending colon ..................................................................................................145
Figure 6.4 Chemical structure of Sodium Green .............................................147
Figure 6.5 Fluorescence emission response of Sodium Green in increasing concentrations of Na⁺ .............................................................148
Figure 6.6 Confocal images of human paediatric colon incubated in Sodium Green to show extracellular Na⁺ distribution................151
Figure 6.7 The effects of amiloride on extracellular Na⁺ distribution in human colonic tissue ........................................................................152
Figure 6.8 Time series to show the effects of carbachol on FD concentration by colonic crypts in vitro in human ascending colon ..................................................................................................154
Figure 6.9 Time series to show the effects of carbachol on FD concentration by colonic crypts in vitro in human transverse colon ..................................................................................................155
Figure 6.10 Effects of inflammation on crypt fluid transport in vitro in human descending colon ..............................................................157
Figure 6.11 Effects of inflammation on crypt fluid transport in vitro in human descending colon. A) Control non-inflamed colon ............158

Figure 6.12 Comparison of rates of increase in crypt luminal fluorescence intensity as an index of absorptive crypt transport in descending colon of control, UC and allergic colitis patients ......159

Figure 6.13 Rate of increase in crypt luminal fluorescence intensity in vitro in human descending colon expressed

gs\textsuperscript{+} cm\textsuperscript{-2} min\textsuperscript{-1} ..............................................................................................................................160

Figure 6.14 Model to show the role of PAF in immune-system mediated regulation of colonic water and electrolyte transport...............161

Figure 6.15 The effects of inflammatory mediators A) PAF and B) PMA on fluid transport in vivo in rat descending colon, measured using agarose gels ........................................................................164

Figure 6.16 The effects of inflammatory mediators A) PAF and B) PMA on Na\textsuperscript{+} absorption in vivo in rat descending colon, measured using agarose gels .................................................................165

Figure 6.17 The effects of inflammatory mediators A) PAF and B) PMA on K\textsuperscript{+} transport in vivo in rat descending colon, measured using agarose gels ................................................................................166

Figure 6.18 The effects of PAF on basal fluid transport in vivo in rat descending colon, measured using closed colonic loops ........168

Figure 6.19 Visualisation of colonic crypts using FD and BODIPY stained colonic biopsies from patients with UC ......................171

Figure 7.1 Regional differences in crypt density cm\textsuperscript{-2} in human childhood control colon .................................................................179

Figure 7.2 Comparative confocal images of descending colon to highlight the differences in colonic crypt densities between A) mouse, B) rat and C) human, child aged 9 years ........................................180

Figure 7.3 Confocal images of human control transverse colon to show normal crypt architecture, density and F-actin staining of the pericryptal sheath ........................................................................181

Figure 7.4 The effects of inflammation on regional crypt density throughout the large intestine in human childhood subjects ......183
Figure 7.5 The effects of inflammation due to allergic colitis on the pericryptal F-actin sheath in transverse childhood colon ........... 184
Figure 7.6 The effects of inflammation arising from ulcerative colitis on crypt density in descending colon of children ..................... 185
Figure 7.7 Structural and functional relationships in allergic colitis I .......... 188
Figure 7.8 Structural and functional relationships in allergic colitis II .......... 189
Figure 7.9 Structural and functional relationships in allergic colitis III .......... 190
Figure 7.10 Structural and functional relationships in ulcerative colitis I .......... 191
Figure 7.11 Structural and functional relationships in ulcerative colitis II ....... 192
Figure 7.12 Structural and functional relationships in ulcerative colitis III ...... 193
Figure 7.13 Analysis of cellular inflammatory infiltrate in eosinophilic and ulcerative colitis ................................................................. 196
Figure 8.1 Chemical structure of NBT in A) oxidised colourless form B) reduced blue-black formazan ........................................ 204
Figure 8.2 Schematic representation of neutrophil immunoassay technique ................................................................. 209
Figure 8.3 Scheme of the NAH-FBB reaction ........................................... 211
Figure 9.1 Measurement and quantification of $O_2^-$ production in vivo in rat descending colon, using agarose gels containing INT ...... 215
Figure 9.2 Histology showing NBF deposits in human inflamed colonic tissue sections to illustrate the different individual scoring categories .................................................................................................................. 217
Figure 9.3 Superoxide production in control and inflamed human colonic tissue measured by NBF formation ................................. 219
Figure 9.4 The effects of PAF on $O_2^-$ production in control, UC and allergic patient groups combined ........................................ 221
Figure 9.5 Comparative measurements of lactate dehydrogenase activity from haemolysed red blood cells, colonic biopsies cultured for a). 20 hours at 37°C and b). 2 hours under experimental conditions .......................................................................................................................... 221
Figure 9.6 Detection of neutrophils in human colonic tissue sections by human neutrophil elastase immunoassay .......................... 222
Figure 9.7 Correlation of $O_2^-$ production and inflammatory cell infiltrate ...... 223
Figure 9,8 False colour confocal images of oxidative stress by fluorescent derivativisation of cellular carbonyls with NAH in rat liver ................................................................. 224
Figure 9,9 Visualisation of oxidative stress in animal liver by fluorescent derivativisation of cellular carbonyls using NAH-FBB .................................................. 226
Figure 9,10 Imaging of oxidative stress using NAH, in human control colon peroxidised in vitro .......................................................................................... 228
Figure 9,11 Comparison of fluorescence intensity of reaction products in NAH and NAH-FBB stained human control and peroxidised colonic tissue ......................................................... 229
Figure 9,12 Confocal imaging of oxidative stress in NAH-reacted human control and inflamed colon ................................................................. 230
Figure 9,13 Comparison of measured fluorescence intensity of reaction products in NAH-reacted control vs. colitic human colon ........ 231
Figure 9,14 Comparison of measured fluorescence intensity of reaction products in NAH-reacted controls vs. colitic human colon .... 232
Figure A,1 Concentration profiles of FD down crypt luminal axis in human non-inflamed colon ................................................................. 245
Figure A,2 Time course of FD concentration by non-inflamed human colonic crypts in vitro at constant depth ................................................................. 247
Figure A,3 The effects of amiloride on FD concentration by human colonic crypts in vitro ................................................................. 250
Figure A,4 The effects of ouabain on FD concentration by human colonic crypts in vitro ................................................................. 251
Figure A,5 Time course to show the effects of carbachol on FD concentration by human colonic crypts in vitro ................................................................. 253
Figure A,6 The effects of inflammation due to ulcerative colitis on in vitro crypt transport in human descending colon ......................... 255
Figure A,7 The effects of inflammation due to allergic colitis on in vitro crypt transport in human descending colon ......................... 256
Figure A,8 Human colonic biopsies stained with BODIPY-Phalloidin to show normal colonic structure – crypt density, architecture and pericryptal F-actin sheath ........................................ 257
Figure A.9 Human colonic biopsies stained with BODIPY-Phalloidin to show the effects of inflammation arising from allergic colitis on colonic structure ................................................................. 258

Figure A.10 Human colonic biopsies stained with BODIPY-Phalloidin to show the effects of inflammation arising from ulcerative colitis on colonic structure ............................................................. 259

Figure A.11 Normal structure and function, patients 1 and 2 ......................... 260

Figure A.12 Normal structure and function, patients 4 and 5 ........................... 261

Figure A.13 Normal structure and abnormal function, patients 8 and 9 .......... 262

Figure A.14 Abnormal structure and function, patients 13 and 14 .................. 263

Figure A.15 Measurement of oxidative stress in control and colitic human colonic tissue using A). NAH ± FBB and B) NAH only ...................... 265
ABBREVIATIONS

·OH Hydroxyl radical
5-ASA 5-Aminosalicylic acid
5-HETE Hydroxyeicosatetraenoic acid
5-HPETE Hydroperoxy-eicosatetraenoic acid
AA Arachidonic acid
ABAP 2'2'-azobis-2-amidinopropane hydrochloride
ADC Analogue – to – digital converter
ANOVA Analysis of variance
BSG British society of Gastroenterology
Ca⁺ Calcium
CaCl₂ Calcium chloride
cAMP Cyclic adenosine monophosphate
CCD Charge-coupled device
CD Crohn’s disease
CGD Chronic granulomatous disease
cGMP Cyclic guanylate monophosphate
Cl⁻ Chloride
CO Cycloxygenase
DNA Deoxyribonucleic acid
ENS Enteric nervous system
ESR Electron spin resonance
FBB Fast Blue B
FD Fluorescein isothiocyanate dextran
Fe Iron
FITC Fluorescein isothiocyanate
GSH Glutathione, reduced
GSSH Glutathione, oxidised
Gt Total tissue conductance
H⁺ Hydrogen atom
H₂O Water
H₂O₂ Hydrogen peroxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCO₃⁻</td>
<td>Bicarbonate</td>
</tr>
<tr>
<td>Hg-arc</td>
<td>Mercury-arc</td>
</tr>
<tr>
<td>HMPS</td>
<td>Hexose monophosphate shunt</td>
</tr>
<tr>
<td>HOCI</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HPF</td>
<td>High powered field</td>
</tr>
<tr>
<td>I.P.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>INT</td>
<td>Iodonitrotetrazolium</td>
</tr>
<tr>
<td>Isc</td>
<td>Short circuit current</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LO</td>
<td>Lipooxygenase</td>
</tr>
<tr>
<td>Lₚ</td>
<td>Hydraulic conductivity</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>LSCM</td>
<td>Laser scanning confocal microscope</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotrienes</td>
</tr>
<tr>
<td>mag</td>
<td>Magnification</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified Eagles’ Medium</td>
</tr>
<tr>
<td>MgCl₄</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>Mn</td>
<td>Manganese</td>
</tr>
<tr>
<td>n</td>
<td>Sample numbers</td>
</tr>
<tr>
<td>NaₙK-ATPase</td>
<td>Sodium potassium triphosphatase</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAH</td>
<td>3-hydroxy-2-naphthoic acid 5-aminosalicyclic acid hydrazide</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NBF</td>
<td>Nitroblue formazan</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
</tbody>
</table>
O$_2^-$  Superoxide
OP  Osmotic pressure
PAF  Platelet activating factor
PBS  Phosphate buffered saline
pd  Potential difference
PG  Prostaglandin
PGI$_2$  Prostacyclin
PLA$_2$  Phospholipase A$_2$
PMA  Phorbol myristate
PMN  Polymorphonuclear
PMT  Photomultiplier tube
pO$_2$  Partial pressure of oxygen
r  Correlation coefficient ($r_s$ - Spearman's rank correlation coefficient)
R  Resistance
r$_p$  Pinhole radius
R  Pure epithelial resistance
RI  Refractive index
ROM  Reactive oxygen metabolites
R$_{sub}$  Subepithelial resistance
R$_T$  Total epithelial resistance
SOD  Superoxide dismutase
TBA  Thiobarbituric acid test
TNF$_{\alpha}$  Tumour necrosis factor alpha
TNF  Tumour necrosis factor
TXA$_2$  Thromboxane
UV  Ultraviolet
v/v  Volume to volume
V$_a$  Apical membrane voltage
V$_b$  Basolateral membrane voltage
V$_t$  Basal transepithelial voltage
w/v  Weight to volume
ZO  Tight junction
CHAPTER 1

Human colonic structure and function in health.

1.1 Introduction

The colon is an organ of water, electrolyte and calorie salvage. Its predominant function in health is to dehydrate semi-solid luminal contents to produce formed, solid stools. The basis of electrolyte / ion movements, such as sodium, chloride and potassium ions, are due to membrane proteins forming ion channels and pumps in the epithelial cell barrier lining the gut lumen. Transport via these channels acts to create ion concentration differences across the colonic epithelium between the lumen and blood. These gradients result in water movement from regions of low to high ion concentration by the process of osmosis. Normal colonic salvage function is intrinsically linked to the structure of the colon. The presence / absence of these membrane transporter proteins in conjunction with epithelial cell organisation serve to create a physical structural barrier. This barrier ultimately determines colonic permeability to ions and resulting fluid transport. Characterisation and control of human intestinal transport has previously been studied using a variety of established methodologies. Studies in infants and children with rare gastrointestinal diseases have also provided fruitful insights into the normal physiology of the gastrointestinal tract (Milla 1986).

This introductory chapter outlines our current knowledge of both colonic function and structure in health and the interdependence of these two areas. Novel methodologies directly challenging existing theories of colonic transport are also presented, followed by a brief outline of colonic regulatory mechanisms.
1.2 Colonic function

The primary function of the human colon is the salvage of electrolytes and water to produce dehydrated, formed stools (Read 1982). The colon receives approximately 1.5 litres of fluid per day from the ileum, containing salts at approximately the same concentrations as plasma. Of this fluid 95% is salvaged resulting in the excretion of less than 100ml in stools (Rhoads & Powell 1991).

<table>
<thead>
<tr>
<th>Intestinal Region</th>
<th>Water Flow (ml/kg/24hr)</th>
<th>Ion Concentration (mEq/L)</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Cl⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Entering Duodenum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary</td>
<td>100</td>
<td>30</td>
<td>20</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td>70</td>
<td>3</td>
<td>25</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Gastric juice</td>
<td>70</td>
<td>50</td>
<td>7.5</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Pancreaticbiliary</td>
<td>45</td>
<td>100</td>
<td>3</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>285</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary</td>
<td>35</td>
<td>75</td>
<td>25</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td>30</td>
<td>3</td>
<td>30</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Gastric juice</td>
<td>30</td>
<td>50</td>
<td>7.5</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Pancreaticbiliary</td>
<td>45</td>
<td>100</td>
<td>3</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Entering Caecum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant</td>
<td>60⁺</td>
<td>100⁺</td>
<td>26</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>25</td>
<td>125</td>
<td>9</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>RATIO FLUID:SOLID</td>
<td>20:1 (95% water)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stool</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant</td>
<td>5-10</td>
<td>22</td>
<td>54</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>1-3</td>
<td>32</td>
<td>75</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>RATIO FLUID:SOLID</td>
<td>2:1 (&lt;68% water)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1 Water flow and ionic composition of intestinal contents in humans - comparison of infants with adults (Rhoads & Powell 1991). ⁺ Volume of output from a ‘new’ ileostomy, volume from an established paediatric ileostomy is 25ml/kg/hr (Schwartz et al 1983). † Paediatric ileostomy electrolyte concentrations obtained from Schwartz et al 1983).
Colonic salvage is predominantly characterised by the net absorption of sodium (Na⁺) and chloride (Cl⁻) and net secretion of potassium (K⁺) and bicarbonate (HCO₃⁻, table 1,1, Hawker et al 1978). Both absorption and secretion of these ions results from selective ion channels/pumps present in colonic epithelial apical and basolateral membranes (Field 1989a). Water movement is passive occurring primarily as a consequence of local transepithelial osmotic gradients generated by these ion movements. Evidence for a specific water channel, aquaporin, has recently been presented in colonic tissue. Its functional significance however is undetermined (Hasegawa et al 1994).

1.2.1 Na,K-ATPase
Passive ion movement occurs in response to ion concentration gradients, namely from high to low concentrations, and electrochemical gradients, with positive ions being attracted to regions of negative charge and vice versa. Generation and maintenance of such gradients within the cell is primarily achieved by the Na,K-ATPase exchange pump. This pump is located on the basolateral membrane (vascular side) of intestinal epithelial cells, confirmed by immunohistochemistry (Gramlich et al 1990). Each pump actively directs three Na⁺ ions out of the cell into the intercellular space for every two K⁺ ions it brings in (figure 1,1). This unequal transport of Na⁺ and K⁺ ions serves to create a low intracellular Na⁺, high K⁺ and a negative cell voltage (Rhoads & Powell 1991). Continued maintenance of low intracellular Na⁺ along with a negative cell voltage are crucial to colonic salvage function as they provide the driving force for luminal Na⁺ entry into the cell and ultimately water absorption (section 1.2.3).

1.2.2 Water
Of the fluid delivered to the colon 95% is absorbed to produce stools of ~<68% water content. There is no active transport of water, rather passive transepithelial water movement is always linked to the active transport of solutes via the generation of osmotic gradients. The osmotic pressure difference between mucosal gut lumen and serosal blood exerted by these
gradients is the main force that controls the direction of osmotic water flow (Desjeux 1991).

**1.2.3 Sodium and water absorption**

Human colonic epithelium has the ability to absorb Na\(^+\), from lumen-to-blood, against considerable concentration and electrochemical gradients (Billich & Levitan 1969 and Edmonds 1971). The mechanism of Na\(^+\) absorption involves a two step process resulting from the spatial discrimination between passive and active Na\(^+\) transport processes (figure 1,1). **Step one** - luminal Na\(^+\) concentration is higher than epithelial intracellular Na\(^+\), therefore luminal Na\(^+\) enters the cell by passive diffusion through apical amiloride-sensitive Na\(^+\) channels. **Step two** - Na\(^+\) is then actively pumped out of the cell via the basolateral Na,K-ATPase pumps to the blood side. This serves to maintain a low intracellular Na\(^+\) concentration therefore assisting further luminal Na\(^+\) entry (Desjeux 1991).

![Diagram of sodium transport](image)

*Figure 1,1 Active transport of sodium across an epithelial cell. Step one, luminal Na\(^+\) diffuses down its concentration gradient via Na\(^+\) channels at one cell surface, followed by step two, active Na\(^+\) transport out of the cell across the opposite cell surface.*
The rate-limiting step for Na\(^+\), and therefore water, absorption is the movement of Na\(^+\) into the cell as a result of the low intracellular Na\(^+\) concentration (Wills et al. 1984). Active Na\(^+\) transport via this two-step mechanism is termed ‘electrogenic’ due to Na,K-ATPase pump activity. The unequal transport of 3Na\(^+\) and 2K\(^+\) ions via the pump directly separates electrical charge to produce a potential difference (pd) thereby contributing to the overall cellular pd. Maintenance of normal cellular electrical pd therefore provides indirect evidence that the mucosa is transporting Na\(^+\) by active mechanisms (Bown et al. 1972).

Electrogenic Na\(^+\) transport does not account for all Na\(^+\) transport. Evidence for additional electroneutral Na\(^+\)-Cl\(^-\) transport comes from ionic substitution experiments in which net Na\(^+\) absorption in distal colon is decreased in Cl\(^-\)-free solutions (Sellin & De Soignie 1987). In addition inhibition of both electrogenic and electroneutral Na\(^+\) transport, using Cl\(^-\)-free Ringer solution containing amiloride (inhibits apical Na\(^+\) channels) failed to completely prevent Na\(^+\) absorption with a significant rate remaining. These data suggests another component to Na\(^+\) absorption that is neither Cl\(^-\)-dependent or blocked by amiloride, proposed to be a Na\(^+\)-H\(^+\) antiporter, the NHE-3 isoform (Rajendran et al. 1995 and Dudeja et al. 1996). In proximal colon it is unclear whether the NaCl carrier represents a protein that directly couples the movement of Na\(^+\) to Cl\(^-\) or a combination of Na\(^+\)-H\(^+\) and Cl\(^-\)-HCO\(_3\)\(^-\) exchanges (Mahajan et al. 1996).

Water absorption is a direct consequence of net flow of Na\(^+\) ions plus paracellular solute flow from lumen-to-blood. Concepts of intestinal water absorption are based on a 3-compartmental model, originally proposed by Curran & Maclntosh, in which the lateral intercellular space forms a middle compartment between the lumen and blood (figure 1,2 Curran & Maclntosh 1962). Electrogenic Na\(^+\) absorption results in Na\(^+\) accumulation in the basolateral intercellular compartment rendering it hypertonic. Consequently osmotic pressure (OP) increases and a standing transepithelial osmotic gradient is established separated from the intestinal lumen by the tight junction. This junction, acting as a collection of aqueous pore channels
(section 1.3.1) provides less resistance to hydraulic flow of water than to back diffusion of absorbed solutes therefore intercellular hypertonicity and OP are maintained. The transepithelial OP gradient arising from Na⁺ absorption provides the necessary driving force to draw water from the gut lumen through the tight junction and into the intercellular space. The space dilates and its contents move towards the basolateral membrane and capillaries. Hydraulic water flow across the tight junction entrains solutes contained in the luminal fluid themselves exerting a degree of OP. These solutes are restricted from the narrow pore channels to varying degrees.

Figure 1.2 Cellular mechanisms of sodium and water absorption across human colonic epithelium with the generation of a hypertonic absorbate. Luminal Na⁺ enters the cell via apical Na⁺ channels and electroneutral transporters to be subsequently actively transported out of the cell via basolateral Na⁺K⁺-ATPase pumps. Absorbed Na⁺ accumulates in the intercellular spaces, rendering them hypertonic, to generate a transepithelial osmotic gradient. This causes water to move by osmosis through the tight junctions in the same direction as the transported Na⁺.

1.2.3.1 A hypertonic absorbate

Although this model applies to water absorption throughout all intestinal regions the degree of OP exerted, and therefore water flow across the epithelium, differs dramatically due to specific regional structural and
functional differences outlined below. OP is primarily determined by two factors,

**Firstly** - the osmotic concentration (tonicity) of the contents of the intercellular space, determined by the ratio of Na⁺:water absorbed (absorbate, figure 1,2). For example low water flow through the tight junction increases the Na⁺:water ratio to produce a high absorbate tonicity in the intercellular space. This subsequently exerts a high OP. Conversely high water flow reduces Na⁺:water decreasing both the absorbate tonicity and transepithelial OP gradient.

**Secondly** (regarding structure) - the relative tightness of the epithelium reflected in the tight junction pore size. For example a small pore size impedes the leakage of absorbed solutes back into the lumen therefore contributing to the maintenance of a high OP. In colonic epithelia the small pore size of <2.3Å allows the generation of a high OP, 515mOsm/Kg, essential for faecal dehydration (section 1.4.4.1), compared to leaky ileum in which the larger pore size 8Å results in a lower OP, 325mOsm/Kg (Billich & Levitan 1969 and Hawker et al 1980).

An important function of distal colonic and rectal epithelium is Na⁺ conservation. Although the quantity of Na⁺ absorbed is small compared to that of the proximal colon the work done per micromole transported is very much greater. This is due to higher concentration and electrochemical gradients with faecal fluid distally being of low Na⁺ concentration. As appropriate to an epithelium that maintains ionic gradients, distal epithelium is relatively low in its passive permeability to Na⁺ reflected in the low blood-to-lumen Na⁺ flux rate (Edmonds 1971). Damage to distal regions will therefore be of major importance with regard to Na⁺ conservation and adequate dehydration of luminal contents with diarrhoea as a potential consequence.

### 1.2.4 Chloride and water secretion

Cl⁻ is secreted from blood-to-lumen via transcellular (across cells) and paracellular (between cells) pathways. The paracellular pathway is highly permeable to Cl⁻ whereas apical and basolateral membranes form a
relatively impermeable barrier with transcellular transport possible only via specific anion membrane transporters (Sandle et al 1990). Basolateral electroneutral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporters (inhibited by bumetanide) allow serosal extracellular $\text{Cl}^-$ into the enterocyte. $\text{Cl}^-$ then exits the cell down its electrochemical gradient via apical anion-selective channels (figure 1,3).

Figure 1,3 Cellular mechanisms of chloride, bicarbonate and water secretion across human colonic epithelium. Serosal $\text{Cl}^-$ enters the cell via an electroneutral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter and exits via apical anion-selective channels. $\text{Na}^+$ and water accompany $\text{Cl}^-$ secretion via the paracellular pathway. Bicarbonate ($\text{HCO}_3^-$) secretion occurs via apical $\text{Na}^+$-independent $\text{Cl}^--\text{HCO}_3^-$ exchanger.

The electroneutrality of the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter effectively uncouples $\text{Na}^+$, $\text{K}^+$ and $\text{Cl}^-$ movement from the electrical pd across the basolateral membrane of the cell. Intracellular $\text{Na}^+$ and $\text{K}^+$ are returned to the serosal extracellular fluid via $\text{Na,K-ATPase}$ pumps and $\text{K}^+$-selective channels respectively. Apical $\text{Cl}^-$ channels in the resting state remain closed being opened in response to mediators such as cyclic nucleotides. Ionic events at
the two cell borders conspire to optimize the secretory process. Active Cl⁻ secretion is accompanied by an increase in K⁺ permeability conductance. This moves the cells electrical potential closer to the K⁺ equilibrium potential resulting in a more positive cell interior. The change in pd serves to increase the electrical driving force for Cl⁻ exit. Conversely mediator-induced increases in conductive Cl⁻ permeability partially depolarise the cell moving the electrical potential away from the K⁺ equilibrium so increasing the driving force for K⁺ exit (Field et al 1989a). The rate-limiting step in Cl⁻ secretion is apical membrane permeability with a change in conductive permeability, activated by cyclic adenosine monophosphate (cAMP), cyclic guanylate monophosphate (cGMP) or ionised calcium (Ca⁺), usually forming the initiating event (Donowitz 1986).

Movement of ions across colonic epithelium generates measurable electrical currents. As Na⁺ absorption accounts for virtually all the short circuit current, in the left colon of children measured in Ussing chambers (Jenkins & Milla 1993), net Cl⁻ absorption must be electrically neutral and balanced by either absorption or secretion of an equivalent amount of cation or anion respectively. An electroneutral apical Cl⁻-bicarbonate (HCO₃⁻) exchanger has been proposed. Evidence that outwardly directed OH⁻ and HCO₃⁻ gradients stimulated ³⁶Cl⁻ uptake, blocked by specific anion exchange inhibitors, and stimulation of HCO₃⁻ secretion by the presence of luminal Cl⁻, support this proposal (Mahajan et al 1996 and Hawker et al 1978).

Na⁺ follows Cl⁻ by passage through cation selective paracellular tight junctions in response to the transepithelial pd (Field et al 1989a). Water movement from blood-to-lumen results from the downhill movement of NaCl from the cell into the lumen (figure 1,3).

1.2.5 Potassium transport
K⁺ absorption from lumen-to-blood occurs via both active transcellular and passive paracellular pathways. Active transport occurs via apical K⁺-H⁺ exchange pumps with K⁺ extruded from the cells through basolateral
barium-sensitive K⁺ channels (Wills et al. 1984). Due to the large lumen-negative electrical potential passive paracellular K⁺ secretion is probably the main source of high K⁺ stool content. Active K⁺ secretion occurs via serosal entry through basolateral Na⁺-K⁺-2Cl⁻ cotransporter and Na,K-ATPase pumps with K⁺ exit via apical K⁺-selective channels (Field 1991). Apical K⁺ channels are activated by cAMP and inhibited by epinephrine.

1.2.6 Bicarbonate transport
In distal colon HCO₃⁻ secretion appears to occur via an apical Na⁺-independent Cl⁻-HCO₃⁻ exchanger (Field 1991, figure 1,3). HCO₃⁻ transport has an important role due to its buffering capacity, as demonstrated when induced luminal acidification (reduced from pH 7.6 to 4.2), had no significant affect on Na⁺ and therefore water absorption (Bown et al. 1972).

1.3 Colonic structure.
The colon may be divided anatomically into ascending, transverse, descending and sigmoid segments (figure 1,4). An outer layer of circular and longitudinal smooth muscle surrounds submucosal and inner mucosal layers. Mucosa lining the gut lumen is devoid of villi consisting of a flat epithelial sheet into which thousands of blind-ending tubules are inserted (crypts of Lieberkühn). These crypts extend from the luminal surface to the muscle layer and are separated by thin plates of lamina propria rich in capillaries, lymphatic vessels and leukocytes (Wheater et al 1981). The surface epithelial layer contains three functionally distinct cell types with surface and crypt enterocytes (colonic transport) interspersed with goblet (mucus production) and enteroendocrine (hormone production) cells. Surface enterocytes directly exposed to gut luminal contents are composed of ~40% superficial crypt cells and 60% surface columnar epithelial cells (Naftalin 1994, figure 1,5). The interface between these cells and the underlying tissue is marked by a non-cellular structure known as the basement membrane and a reticular complex composed of collagen fibres enmeshed within a polysaccharide ground substance. Both of these subepithelial layers may constitute a physical barrier to the passage of substances between the epithelium and underlying tissue. The polysaccharide molecules may potentially act as a mechanical sieve whose resistance can be varied by altering the polysaccharide concentration (Kaye et al 1968).
The colonic epithelial layer also presents a physical barrier to both active 
and passive intestinal transport due to its selective permeability and 
structural organisation. Active transport is determined by regional ion 
transporter proteins present in apical and basolateral enterocyte 
membranes. Under certain conditions these become selectively permeable 
to specific ions (Sellin & De Soignie 1987). Passive transport occurs by two 
pathways with solutes either transversing (transcellular pathway) or passing 
between (paracellular pathway) cells. The transcellular route presents two 
biochemical membranes in series, apical and basolateral, each of which is 
very restrictive to leaks of hydrophilic solutes, offering resistance in the 
order of 1,000 – 10,000 Ω cm² (Madara 1989). The majority of passive ion 
transport therefore occurs via the low resistance paracellular route, the 
permeability of which is controlled by tight junctional complexes (section 
1.3.1).

The salvaging function of the colon is ultimately dependent on its ability to 
actively absorb Na⁺ and therefore water, against high ion concentration and 
electrical lumen-to-blood gradients (section 1.2.3). This is possible because 
of the relatively tight epithelial structure, which impedes passive leakage of 
actively absorbed Na⁺ back into the lumen, (Billich & Levitan 1969), and the 
high transepithelial resistance to passive flow of hydrophilic solutes and ions.

1.3.1 Intercellular tight junctions
Adjacent intestinal epithelial cells adhere at focal points by a series of 
junctional complexes namely the zona occludens / tight junction (ZO), 
zonula adherens (ZA) and several desmosomes (figure 1.6 Soergel 1993). 
Fusion sites (‘kisses’) being composed of particulate strands, allow the 
paracellular movement of small ions (<2.3 Å) through the junction with a 
selectivity for cations over anions (Madara 1989). Far from being the 
impermeable gasket as the name suggests the ZO demonstrates a degree 
of plasticity, which largely appears to account for the variance in epithelial 
resistance to passive ion permeation (Powell 1981). This has important 
functional implications regarding the generation of a hypertonic absorbate
Figure 1,6 Schematic illustration depicting the location and structure of the tight junction (ZO) in intestinal epithelial cells. ZO is composed of strands and groves forming a narrow belt that wraps round epithelial cells at the apical pole. The insert displays a speculative model of the molecular substructure of the ZO. Cytoskeletal modulation of the F-actin perijunctional ring, which circumferentially surrounds the cell distal to the ZO, may directly affect ZO structure and therefore barrier function.

necessary for faecal dehydration (section 1.2.3.1). For example a decreased resistance to solute flow permits the leakage of absorbed solutes through the ZO into the lumen. Lumen-to-blood ion concentration gradients are therefore not maintained so osmotic water absorption is effectively reduced. ZO resistance can be increased by intracellular Ca²⁺ and cAMP or decreased by protein kinase C and high concentrations of cytochalasin B. Decreased resistance has been associated with a number of dynamic structural alterations including condensation and contraction of the actomyosin ring accompanied by dilation of intrajunctional spaces and a decrease in strand count per junction. As such, these structural changes in ZO resulting in altered barrier function could form an integral component of
epithelial transport (Madara et al 1987). Given the plasticity of ZO’s in health it is only natural that these crucial barriers might also be affected in disease. Inflammatory conditions of the colon are characterised histologically by small erosions of surface epithelium and macroscopic ulcers. Physical separation of epithelial cells consequently means that these cells will no longer represent a significant barrier to ion diffusion, again eliminating the generation of osmotic gradients and passive water absorption. This is discussed further in chapter 2 (section 2.3.2).

1.3.2 Extracellular matrix
The extracellular matrix is composed of a complex mix of proteoglycans, glycoproteins and fibrous proteins, such as collagens and elastin. It lies below the epithelia surrounding connective tissue cells, serving as extracellular scaffolding with the primary function of maintaining tissue architecture. It also forms an interactive substratum involved in regulating the shape, migration, growth and differentiation of cells. In the event of mucosal injury for example in inflammation, the extracellular matrix plays an indispensable role in the orderly repair of damaged tissues (Weiss 1989 and Donnellan 1965).

1.3.2.1 Pericryptal fibroblast sheath
The pericryptal fibroblast sheath is an important component of the extracellular matrix. Positioned immediately below the surface epithelium separated only by the basement membrane, it serves to communicate between the extracellular matrix and surface and crypt cells (Figure 1,7 Joyce et al 1987). The sheath is formed by a specialised, self-renewing population of actin-rich fibroblasts, which migrate synchronously with crypt epithelial cells from the crypt base to the collagen layer beneath the surface epithelium. At all levels of the crypt a portion of each fibroblast maintains intimate contact with the epithelial cells. At base and mid regions contact is continuous forming a complete fibroblast sheath around each crypt. In contrast at the crypt surface contact occurs via long prolongations extending laterally to produce a fenestrated cellular sheath subtending the absorptive surface epithelium (figure 1,7).
Figure 1.7 Schematic representation of the pericryptal fibroblast sheath surrounding individual colonic crypts. (F1) Fibroblasts in germinating zone at crypt base migrate towards the surface maintaining tight continuous contact with lamina densa. (F2) Differentiated and functioning fibroblasts at crypt surface continue to maintain contact but through long cytoplasmic processes (F3). These form 'pores' between the lamina densa and the collagen table (C.T.) which may play a role in regulating fluid transport. The cell bodies are embedded in the thick collagen table. Epi - surface epithelium.

This arrangement of the pericryptal sheath has both structural and functional implications. Intimate fibroblast-epithelial contact throughout the crypt is important in the maintenance of structure and control of epithelial organisation and differentiation (Halttunen et al 1996 and Berschneider & Powell 1992). Evidence suggests that these actin rich fibroblasts, by F-actin binding to cellular adhesion molecules (integrins and cadherins), may also form a physical pericryptal barrier retarding macromolecular movement from the immediate vacinity of the crypts into the lamina propria. This barrier to interstitial solute movement may be important in the maintenance of high pericryptal Na⁺ concentrations necessary for fluid absorption (Naftalin et al 1995 and Rolfe et al unpublished, section 1.2.3). The F-actin sheath is also involved in the regulation of paracellular permeability via tight junction modulation (section 1.3.1). For example exposure of cultured human
epithelial layers to *Clostridium difficile* toxin A, (known to cause severe inflammation) results in permeability abnormalities such as decreased transepithelial resistance and increased ion fluxes paralleled by disruption of the F-actin cytoskeleton (Hecht et al 1988 and Kaye et al 1968).

### 1.4 Structural and functional relationships

#### 1.4.1 An old dogma - shot in the leg.

Since the 1960's physiologists have upheld the central dogma that structural segregation into surface and crypt cells directly supports a functional segregation of ion transport processes (Powell 1995). The dogma states that absorptive processes are located exclusively in surface epithelial cells and secretory exclusively in crypt cells. This classification resulted from several experimental observations on small intestine, jejunum and colon (Serebro et al 1969, Roggin et al 1972 and Field 1980). Amongst the clearest direct evidence in colonic tissue is that from microelectrode studies recording individual membrane conductances of rabbit colonic crypts. Inhibition of Na\(^+\) absorption (via blocking amiloride-sensitive apical Na\(^+\) channels) decreased membrane conductance but in surface cells only. In contrast serosal application of prostaglandin E\(_2\) stimulated electrogenic cAMP-mediated Cl\(^-\) and fluid secretion in crypt cells only. In addition fluid droplets, seen on oil-covered mucosal surface, formed directly over crypt openings only and were not observed in the presence of frusemide, an inhibitor of Cl\(^-\) secretion (Welsh et al 1982).

However much of the evidence supporting this segregation of ion transport processes arose out of indirect observations of fluid measurement. A novel method, involving the concentration polarisation of impermeant fluorescent dyes visualised by confocal microscopy, demonstrated that colonic crypts in rats exhibit the capacity to absorb fluid (Naftalin & Pedley 1990 and Pedley & Naftalin 1993). Subsequent to this finding the development of a technique permitting direct determination of fluid movement in intestinal crypts, openly challenged the central dogma (Singh et al 1995a). Hand-dissected, perfused isolated crypt preparations, devoid of myofibroblast-containing
pericryptal sheath, exhibited net basal Na\(^+\)-dependent fluid absorption. Addition of secretagogues dibutyryl-cyclic AMP, vasoactive intestinal peptide or acetylcholine to the serosal bathing fluid induced reversible net fluid secretion. From this evidence Singh et al (1995a) speculated that absorptive processes are constitutively expressed in crypt epithelial cells with crypt secretion regulated by one or more neurohumoral agonists. These agents, being released insitu from cells in the lamina propria, such as myofibroblasts, directly into the crypts' local environment, can effectively alter crypt basal function. The major advantage of this technique is the ability to isolate crypt transport processes from surface cells and the surrounding cellular environment. The influence and control of the latter increasingly appears to be responsible for the secretory characteristics previously assumed to be constitutive crypt properties. Both these novel techniques provide conclusive evidence of the dual functional nature of colonic crypts.

The supposition that secretory mechanisms are exclusively located in crypt cells has also been called into question. Köckerling & Fromm (1993) looked at the spatial distribution of cAMP-dependent Cl\(^-\) secretion by measuring local ion conductances in rat colonic crypts and surface epithelium, using voltage-scanning techniques. In control conditions surface and crypt cells contributed 39% and 61% respectively to total ion conductance. Exposure to theophylline, elevating cAMP, increased both crypt and surface epithelial cell conductance. The effects were Cl\(^-\) dependent, as theophylline had no effect in Cl\(^-\)-free buffer and decreased ion conductances were recorded in both cell types when stimulated in the presence of a Cl\(^-\) channel blocker. It was concluded that cAMP-dependent Cl\(^-\) secretion was not confined to crypt cells but also occurred in surface cells in rat distal colon. This has also been shown using the isolated crypt preparation (Greger et al 1997).

**1.4.2 An old dogma – laid to rest.**

Functional segregation within the colonic mucosa no longer holds true. The revised view is that all transporting epithelial cells throughout epithelial layers have the capacity to both absorb and secrete depending on the
nature of the prevailing luminal and submucosal environments (Pedley & Naftalin 1993, Greger et al 1997 and Singh et al 1995a). Further studies, using the isolated crypt preparation in conjunction with morphological studies, have indeed revealed functional heterogeneity's but from within the crypt enterocyte profile itself. Enterocytes at the crypt base constitute poorly differentiated stem cells that frequently divide giving rise to the three major colonic epithelial cell types (section 1.3). Daughter cells migrate over several days to the surface differentiating and, from electrophysiological studies, systematically acquiring various functional transport properties as they travel (Ecke et al 1996a). Consequently several ionic gradients have been observed along the crypt axis. A summary of crypt transport to date arising predominantly from isolated crypts studies on rat distal colon, and the functional significances are outlined below.

1.4.3 Colonic crypt transport

1.4.3.1 Crypt absorption

Colonic crypts in their basal state demonstrate spontaneous Na⁺-dependent fluid absorption (Pedley & Naftalin 1993 and Singh et al 1995a). An apical chloride-dependent Na⁺-H⁺ exchanger with partial amiloride-sensitivity has been demonstrated. It appears functionally distinct from the chloride-independent Na⁺-H⁺ exchanger in surface epithelial cells, which shows comparably greater amiloride-sensitivity (Rajendran et al 1995). In rabbit distal colon crypt luminal cross-sectional surface area was found to increase on exposure to theophylline (a secretagogue) but decrease in control conditions below that seen with octanol (inhibits Na,K-ATPase and therefore water absorption). These findings support the view that in control conditions there is fluid outflow across the crypt wall demonstrated to be generated by a transepithelial osmotic pressure gradient (Bleakman & Naftalin 1990).

1.4.3.2 Crypt secretion

cAMP-mediated Cl⁻ secretion has been proposed to occur in crypt wall but not crypt base cells (Böhme et al 1991 and Siemer & Gögelein 1993). However a recent study recorded cAMP-stimulated Cl⁻ secretion with the most marked response in base cells (Greger et al 1997). From their findings
Greger et al (1997) state that the ability to secrete NaCl appears to be characteristic of colonocytes early in their development being gradually lost and replaced by the ability to absorb NaCl via amiloride-sensitive epithelial Na⁺ channels as they migrate to the surface. Cells in the mid crypt region demonstrated a capacity for both absorption and Cl⁻ secretion suggesting a transitional zone. Conditions and cellular mechanisms involved that suppress or shift crypt cells between these two transport states remain unknown. Greger et al (1997) proposed that the activation of Cl⁻ channels by cAMP itself inactivates Na⁺ conductance in the same cell and maybe one key component in determining the direction of NaCl transport.

1.4.4 Functional significance of crypt absorption
The fundamental driving force for water absorption is a high intercellular Na⁺ concentration generating a transepithelial osmotic gradient (section 1.2.3). Having established the basal absorptive function of colonic crypts, it has been proposed that the specific structure and properties of these crypts confer an ideal microenvironment in which a hypertonic absorbate (high Na⁺, section 1.2.3.1) can be effectively produced (Bleakman & Naftalin 1990 and Naftalin 1994). To understand the potential functional significance of these crypts in providing isolated microenvironments it is necessary to briefly highlight some of the problems of colonic fluid salvage imposed by the specific nature of colonic luminal contents.

1.4.4.1 Faecal dehydration
It is normally assumed that any resistance to fluid movement in the bulk solution is negligible compared with the hydraulic resistance across the epithelium. The properties and nature of luminal contents in the colon however single colonic salvage out as an exception to this assumption. Faeces are composed of very fine particulate matter (forming the skeleton) and fluid (forming pore-water, occupying the spaces between particles), in ratios of fluid to solid 20:1 in ileal fluid, consolidated to 2:1 in dehydrated formed stools (table 1,1). The following steps occur when pressure is applied,
(i) Pore-pressure increases producing hydraulic pressure gradients between gut contents and surroundings.

(ii) Fluid outflow and compression of particulate matter results

(iii) Pore-pressure subsides and the load is taken up by the ‘skeleton’.

Initial reduction in stool water content from 95% to 80% (water to solids 4:1) is relatively easy, requiring a force of only ~ 5 kPa (50cm H$_2$O). This is achieved through mechanical force from colonic smooth muscle contraction. The fine particulate matter of faeces however, infers a composition similar to clay in which there exists an exponential relationship between the force required to compress water out of faeces and its steady state water content (McKie et al 1990). In terms of faecal dehydration this means that as faeces become more solid the hydraulic resistance offered to further dehydration increases exponentially. Dehydration from fluid to solid 4:1, to the final 2:1 of dehydrated formed stools (68% water content) requires the enormous pressure of ~1000kPa (~10 atmospheres). As this amount of pressure is beyond that achievable by compressive muscular forces the only other force available is osmotic pressure. It is these properties of the colonic luminal faecal contents that underlie the essential requirement for the colon to be able to generate a hypertonic absorbate and osmotic pressure to produce dehydrated solid stools. Animals that can not generate this hypertonic absorbate such as cattle only dehydrate faeces to ~83% water (McKie et al 1991).

1.4.4.2 A specific role for crypt absorption in faecal dehydration?

Overall the colonic mucosa is nearly as permeable as the small intestine to both salt and water (Bleakman & Naftalin 1990). However from its capacity to generate a hypertonic absorbate it can be deduced that discrete regions exist within the mucosa that are very impermeant to both salt and water. Indeed hydraulic conductance across the colon has been shown to be heterogeneous (Pedley & Naftalin 1993). It has been proposed and substantiated that luminal surface epithelium is relatively ‘leaky’ with bi-directional passage of water and electrolytes (lumen to blood and blood to lumen). Although this route provides a high capacity absorptive pathway it is unable to produce the high suction pressures necessary for the formation of
solid faeces (section 1.4.4.1). This is because the high water flow reduces the ratio of Na⁺:water absorbed so preventing the generation of a hypertonic absorbate (section 1.2.3.1). By contrast crypt epithelium is very ‘tight’ and accordingly of low hydraulic conductance. Local Na,K-ATPase activity necessary for active Na⁺ absorption is high with cell density approximately eight times that at the mucosal surface. These factors enable colonic crypts to generate and maintain high Na⁺ concentrations as found in the pericryptal space. Colonic crypts are therefore able to generate high osmotic pressure gradients exerting the suction pressures necessary to dehydrate faeces and form solid stools (Pedley & Naftalin 1993 and Naftalin 1994).

1.5 Regulation of colonic transport
Intestinal transport is controlled via three internal regulatory systems - the enteric nervous system (ENS), endocrine and immune systems and an external system - bacterial enterotoxins (Rhoads & Powell 1991). Mediators released by these systems in response to stimuli alter intestinal transport by initiating a chain of events affecting intracellular messenger concentrations to produce functional cellular changes. These messengers include cAMP, cGMP, ionised Ca⁺ and inositol phosphate metabolites - inositol triphosphate and diacylglycerol. They act to activate mediator and substrate specific protein kinases (PK) to transfer the terminal phosphate group from adenosine triphosphate (ATP) to the hydroxyl group on serine, threonine or tyrosine residues of ion channel proteins or associated regulatory proteins. A phosphomonoester bond is formed adding two negative charges to the ion channel protein usually resulting in functionally important conformational and ion permeability changes. One example of this is cAMP-mediated electrogenic Cl⁻ secretion in which normally closed apical Cl⁻ channels are opened. Dephosphorylation to reverse events requires a second PK enzyme (Field et al 1989a).

1.5.1 The enteric nervous system
The first definitive evidence for direct ENS involvement in intestinal transport regulation came from Ussing chamber experiments using whole thickness
sheets of intestine (Hubel 1978). The ENS division of the autonomic nervous system forms an independent, integrative system subdivided into extrinsic nerves, mucosal endocrine cells and the enteric neural plexus (further divided into myenteric, submucosal and mucosal plexus, Rhoads & Powell 1991, figure 1,8).

Figure 1,8 Schematic diagram to illustrate intestinal reflex pathways regulating intestinal mucosal function. Sensory neurons detect texture, fluidity, volume and chemical composition of luminal contents. Information processing and integration occurs within the interneuronal circuitry of the submucosal plexus. Specific motor neurons are subsequently activated releasing neurotransmitters from axon varicosities in the proximity of mucosal effectors including the epithelium, muscularis mucosae and blood vessels.

Most mucosal reflexes involving transport functions appear to be mediated through submucosal and mucosal plexuses, with motility controlled by the myenteric plexus (Bridges et al 1986, figure 1,8). However, myenteric plexus-mediated reflexes have been implicated in Na⁺ secretion induced in
vivo in rat jejunum by luminal D-glucose (See & Bass 1993) and in mucosal *Escherichia coli* STa induced Cl⁻ secretion in vitro across intact unstripped rat ileum via capsaicin-sensitive (afferent?) and nitric oxide-dependent (efferent?) nervous pathways (Rolfe & Levin 1994). In addition neural connections between the two separate plexus suggest they may comprise a single integrative system. Neurons of the mucosal plexus in rat colon appear to be spontaneously inactive requiring continuous stimulation in order to inhibit electrolyte absorption. Once this inhibitory influence is blocked the epithelium returns to a state at or near maximal absorption suggesting that this absorptive state is the functional intrinsic set point of the epithelium (Bridges et al 1986).

1.5.2 The endocrine system

Sources of intestinal endocrine hormones include neuronal and cellular (T/B lymphocytes, polymorphonuclear cells (PMN), fibroblasts and endothelial and smooth muscle cells). Circulating hormones, such as aldosterone, enhance intestinal electrogenic NaCl absorption by increasing the number of Na,K-ATPase pumps, inhibit electroneutral NaCl and stimulate K⁺ secretion in rat distal colon (Foster et al 1983). Glucocorticoids administered systemically also increase rectal Na⁺ and water transport measured in humans (Sandle et al 1986).

1.5.3 The immune system

The primary effector cells of the immune system are phagocytes (macrophages, neutrophils, eosinophils) and mast cells. Immune cell activation has been shown to stimulate electrogenic Cl⁻ secretion and impair neutral NaCl absorption via the release of cyclooxygenase products (prostaglandins, leukotrienes), inflammatory mediators (platelet activating factor) and highly reactive oxygen metabolites (ROM) (chapters 2 & 3). In rat and rabbit colonic enterocytes at least 50% of the secretory response induced by immune cell stimulants/products was attributable to cyclooxygenase product activation of the ENS (Bern et al 1989 and Powell 1992). Similarly neutrophil ROM-mediated secretion has been found to cause secretion via these mechanisms (Karayalcin et al 1990).
CHAPTER 2
Human colonic structure and function
in inflammation.

2.1 Introduction
Inflammation of the colon, known as colitis, affects both colonic structure and function. The severity of impaired function is variable but an inability to effectively salvage colonic fluid is a major cause of morbidity in young children (Jenkins & Milla 1993). This chapter outlines the physiology of the colonic inflammatory process highlighting prominent inflammatory mediators known to be of significance in the pathology of colitis. The effects of this inflammatory process on colonic structure and function are discussed. Finally the main features of the three most common inflammatory conditions affecting intestinal function are briefly presented.

2.2 The inflammatory process in colitis
Activation of both systemic and local intestinal immune systems initiates a cascade of events to produce an acute inflammatory response. Within 30 to 60 minutes circulating inflammatory cells, such as polymorphonuclear cells (PMN’s; neutrophils and eosinophils), lymphocytes and macrophages, are activated in response to chemical mediators. Migration of these cells out of the vascular circulation and infiltration of colonic submucosa and mucosa occurs, with cell density increasing up to three-fold in inflamed colonic mucosa (figure 2,1 Nishida et al 1987). These inflammatory cells release an array of soluble, potent inflammatory mediators, such as platelet activating factor (PAF), prostaglandins (PG’s), leukotrienes (LT’s) and cytokines to act directly or indirectly on enterocytes producing functional and structural changes. This initial inflammatory response becomes amplified by the recruitment of further inflammatory cells in response to these mediators and a damaging cyclical inflammatory response is rapidly established and sustained.
2.2.1 Inflammatory cell infiltration

The migration of large numbers of phagocytes from the subepithelial microvasculature into and across colonic epithelium characterises acute ulcerative colitis (UC) and allergic colitis. Recruitment and control of migration results from a series of interactions between circulating PMN's and endothelial cells (Van Deventer 1997 and Mason & Haskard 1994). The steps involved in phagocyte transendothelial migration are outlined below using neutrophils as an example (figure 2,1, Nash et al 1988).

**Rolling:** At inflammatory sites random contacts between circulating neutrophils and endothelial cells become prolonged due to weak adhesive interactions mediated by selectin molecules (glycoprotein adhesion molecules) and carbohydrate motifs on opposing cells. This initiates ‘rolling’ of neutrophils along the vessel wall.

**Tight adhesion:** Intimate contact between rolling neutrophils and endothelial cells in conjunction with production of endothelial interleukin 8 (Arndt et al 1996) PAF (Casale & Abbas 1991) and tumour necrosis factor (TNF) causes increased expression of neutrophil β₂-integrin (large family of cell surface glycoproteins), in particular CD11b/CD18. Subsequent interaction between neutrophil CD11b/CD18 and its ligand on endothelial cells, ICAM-1 (expressed in inflammatory bowel disease, Koizumi et al 1992) leads to tight adhesion.

**Transmigration:** Transmigration follows via direct physical manipulation of interendothelial tight junctions. Focal intimate contacts between neutrophil and endothelial cells are made, approximately 0.25-0.50μm length, with aggregates of microfilaments (cytoplasmic specialisations) occurring at contact sites. These sites have been speculated to serve as anchoring “footholds” or adhesion plaques from which the neutrophil is able to generate force to open up the tight junction, a prerequisite to transmigration (Madara 1989).
Extension of thin cytoplasmic pseudopodia through the site of endothelial discontinuity results in transmigration.

Figure 2.1 Process of neutrophil migration across vascular endothelial cells. The three steps include (1) neutrophil rolling (2) tight adhesion and (3) transmigration. (IL-8 - interleukin 8).

Transepithelial migration across enterocyte basolateral to apical membranes into the gut lumen also occurs through interepithelial tight junctions by a similar process (Resnick et al 1995). Both activated neutrophils and eosinophils are inherently capable of inducing electrogenic Cl⁻ secretion. One proposed mechanism is the release of neutrophil/eosinophil derived secretagogue which is preferentially active on the apical surface of T84 cells and appears to involve the release of 5'-AMP and its subsequent conversion to adenosine (Madara et al 1992, Traynor-Kaplan & Barrett 1993 and Resnick et al 1993).

2.2.2 Inflammatory mediators
2.2.2.1 Platelet activating factor
PAF is a potent inflammatory mediator not normally present in detectable amounts in control colonic tissue, which is implicated in the pathogenesis of UC and Crohn’s disease (CD) (Eliakim et al 1988 and Kald et al 1990). It is an endogenous membrane-bound phospholipid, synthesised and secreted
by specific cells following their activation with phospholipase A₂ (PLA₂), (Powell 1992 and Prescott et al 1990). Activated immune cells (macrophages, neutrophils and eosinophils) and epithelial cells are capable of synthesising and releasing PAF, with neutrophils exhibiting the highest capacity in humans (Ferraris 1993 and Guimbaud et al 1995). Consequently in patients with acute UC or CD high concentrations of PAF are detectable in mucosal biopsies (Eliakim et al 1988 and Kald et al 1990), faeces (Chaussade et al 1991) and biopsy organ culture medium (Wardle et al 1996). These increased PAF levels in vivo correlate to local injury, inflammation and colonic myeloperoxidase output (a neutrophil enzyme marker). PAF’s actions include stimulation of platelet and neutrophil aggregation, chemotaxis, degranulation and release of inflammatory mediators as well as vasodilation and increases in vascular permeability. These events are accompanied by the release of PG’s and LT’s as well as reactive oxygen metabolites (ROM) as seen in cat ileum where PAF indirectly increased overall mucosal permeability via activating phagocytes to release ROM (Kubes et al 1991). Also PAF released from human inflamed colonic tissue has been shown to be capable of inducing specific electrogenic secretion in rat distal colon in vitro via stimulating the production of eicosanoids which act on colonocytes and the submucosal plexus (Wardle et al 1996 and Bern et al 1989).

2.2.2 Eicosanoid production: prostaglandins and leukotrienes.

PG’s and LT’s form part of a large and complex family of biologically active lipid compounds collectively termed ‘eicosanoids’. They are derived mainly from arachidonic acid (AA) polyunsaturated fatty acid metabolism. AA (5,8,11,14-eicosatetraenoic acid) is a dietary derived fatty acid that normally resides in cell membranes esterified to phospholipid. PLA₂, also present in cell membranes, when stimulated by bradykinin, neurohumoral substances, inflammation, free intracellular Ca⁺ ions or mechanical membrane perturbation, enzymically cleaves AA from its phospholipids. Free AA is then oxidatively metabolised via two major pathways - cyclooxygenase (CO) and lipooxygenase (LO) to produce PG’s and LT’s (figure 2, Eberhart & Dubois 1995, Donowitz 1985 and Hawkey & Rampton 1985). CO, present in all
mammalian cells and LO pathways, present in only a few cells including neutrophils and monocytes, are not equal in all tissues and inhibition of one can lead to up-regulation of the other.

**Figure 2.2** Arachidonic acid metabolism via cyclooxygenase and lipooxygenase pathways resulting in prostaglandin and leukotriene production. ⊕ Denotes stimulation, numbers denote inhibition by – (1) corticosteroids; (2) sulphasalazine; (3) non-steroidal anti-inflammatory agents; (4) vitamin E and (5) 5-aminosalicylic acid. 5-HPETE – hydroperoxyeicosatetraenoic acid.

Eicosanoids exert a vast array of effects important to normal health. Those of possible significance in IBD include stimulation of intestinal secretion, action as potent chemotactic agents, increases in mucosal blood flow and influence on smooth muscle activity (Wardle et al 1993). Inflammation alters the profile of fatty acids stored as phospholipids (Nishada et al 1987 and Pacheco et al 1987) with increasing evidence to implicate a central role for eicosanoids in the pathogenesis of UC (Stenson 1990). For example both AA substrate and metabolite levels are increased in colonic mucosa of UC patients correlating to inflammatory cell infiltrate. PGE₂, PGI₂, LTB₄, LTD₄, thromboxane and 5-HETE (hydroxyeicosatetraenoic acid) form the predominant elevated eicosanoids detectable in colonic and rectal mucosa, serum, urine and stools of patients with active UC compared to control and quiescent disease (Wardle et al 1993, Ligumsky et al 1981 and Stenson...
Increased available substrate in conjunction with elevated phospholipases in UC may therefore be critical in determining eicosanoid synthesising power of colonic tissue and ultimately resulting damage. Sensitisation of quiescent diseased mucosa has also been demonstrated with increased eicosanoid production on exposure to mediators produced during active inflammation (Wardle et al 1992). Further supportive evidence comes from the beneficial therapeutic effects of steroid treatment in IBD, which may be partially related to their inhibition of eicosanoid synthesis via inhibiting free AA formation (Ligumsky et al 1981, section 2.4.5).

### 2.2.2.3 Free radicals
Free radicals also feature in the inflammatory profile of colitis. Their production and specific actions with regard to colonic structure and function are outlined in chapter 3 of this introductory section (page 67).

### 2.3 Effects of inflammation on colonic function and structure
Diarrhoea is the primary presenting symptom of allergic and ulcerative colitis and a major cause of morbidity in young children (Jenkins & Milla 1993). It can be described as,

"The failure of the colon to salvage the amounts of food residue that enter through the ileo-caecal valve. Inadequate/failure to salvage must result in excess stool water – diarrhoea." (Read 1982)

Diarrhoea resulting from inflammation is a consequence of an inability of the colonic mucosa to accommodate a normal load, that is a loss of function as opposed to an increased load. In terms of a 10kg infant with colitis a 50% reduction in volume absorbed, in the face of normal small intestinal function, results in 300ml stool output per 24 hours. That is greater than three times normal stool output (Rhoads & Powell 1991). Inflammation limited to a single colonic segment only can sufficiently impair colonic function to produce a clinical increase in faecal water excretion. Impaired colonic function can be multifactorial arising from a loss of surface area, an increase in colonic ion permeability or stimulation of active secretion. The gross picture is one of impaired net fluid absorption and increased net fluid...
secretion culminating in excess stool water. The exact contributions of cellular based defects in epithelial ionic transport and loss of epithelial barrier structure therefore increasing leakiness to passive ion flow is unknown. These two areas are discussed below.

2.3.1 Cellular basis for defective function

Colonic and rectal epithelium is electrically polarised due to cellular ionic events, with lumen negative in respect to blood. Considerable changes in this electrical profile occur in colitis (table 2,1). Electrophysiological techniques, using Ussing chambers and microelectrodes, have provided detailed information regarding cellular defects underlying defective ion transport in inflamed human colon (Sandle et al 1990 and Archampong et al 1972). Generally inflamed distal colonic tissue was characterised by an overall increase in total tissue ion conductance / permeability (Gt). This signifies a reduction in normal epithelial resistance to ion flow, alterations in Na⁺, Cl⁻ and K⁺ transport mechanisms and/or increased passive paracellular fluxes. Any changes in ionic permeability must ultimately be translated into altered fluid salvage.

<table>
<thead>
<tr>
<th>Electrical Parameters</th>
<th>Transport index</th>
<th>Colonic mucosa</th>
<th>control</th>
<th>inflamed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total tissue ion conductance (Gt)</td>
<td>Index of ionic transcellular and paracellular epithelial permeability</td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Basal transepithelial voltage (Vt)</td>
<td></td>
<td>High</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Resistance (R)</td>
<td>Resistance offered by epithelium to ion flow</td>
<td>High</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Short circuit current (Isc)</td>
<td>Index of electrogenic Na⁺ transport</td>
<td>High</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Apical (Va) Basolateral (Vb) Membrane voltage profile</td>
<td>Reflects “tight” nature of epithelium in which electrogenic Na⁺ transport is present</td>
<td>Vb&gt;Va</td>
<td>Absent</td>
<td></td>
</tr>
</tbody>
</table>

Table 2,1 Electrical profile of human colonic mucosa in control and inflamed tissue, as measured in Ussing chambers. ‘Denotes difference recorded reached statistical significance (Sandle et al 1990).

Na⁺ - specific electrogenic Na⁺ transport was impaired reflected by a reduction in basal transepithelial voltage (Vt) and short circuit current (Isc)
and absence of normal membrane voltage profile (table 2.1). Amiloride-sensitive apical Na⁺ entry was attenuated and basolateral Na⁺ pump activity substantially decreased, ~76%, due to reduction in number of operational pumps, assessed by pump kinetics, rather than Na⁺ binding properties to individual pumps. In addition when Na⁺ entry was inhibited by amiloride only small reductions in Vt occurred in inflamed tissue compared to controls (Sandle et al 1990 and Hawker et al 1980). An early human in vivo study using isolated colonic loops measured a 5% reduction in Na⁺ absorption in inflamed versus control loops (Duthie et al 1964). Rectal dialysis using dialysis solutions as low as 70 m-equiv/l Na⁺ showed an increase in luminal Na⁺ concentration in UC patients again indicating that inflammation prevented the reduction of luminal Na⁺ by normal absorptive processes (Edmonds & Pilcher 1973). Increased luminal Na⁺ concentration has also been reported in the face of normal / slightly increased mucosal to serosal Na⁺ fluxes. This could be attributable to increased back diffusion of normally absorbed Na⁺ via leaky tight junctions, as indicated by the dissipation of transmucosal potential difference (pd) and Isc (Jenkins & Milla 1993, see section 2.3.2). The ability to extract Na⁺ from the lumen against high electrochemical and concentration gradients is crucial as this provides the necessary driving force for water absorption and therefore faecal dehydration (chapter 1 section 1.2.3). Loss of this ability would certainly contribute to the accumulation of excess stool water namely diarrhoea.

Cl⁻ and K⁺ - increases in serosal to mucosal Cl⁻ conductance and K⁺ movement, via active and / or passive routes, also contributes to an increased Gt and water secretion. For example replacement of Cl⁻ ions, with mucosal and serosal gluconate, produced a greater decrease in Gt in inflamed versus non-inflamed (Sandle et al 1990 and Jenkins & Milla 1993). K⁺ serosal to mucosal flux in non-inflamed colon is facilitated by a negatively charged lumen. In inflamed colon considerable serosal to mucosal K⁺ fluxes were recorded when pd was virtually zero. The exact source of increased luminal K⁺ has not been determined as it could arise from blood loss, damaged or dying cells as well as active secretion (Edmonds & Pilcher 1973).
In summary diarrhoea produced in UC and Crohn’s colitis is at least partially attributable to cellular defects in anion, cation and therefore water, transport properties of colonic epithelial cell membranes. The effects of potent inflammatory mediators released in the local pericryptal environment may also switch crypt cells from being absorptive to predominantly secretory in nature therefore contributing to excessive stool water (Greger et al 1997, see chapter 1 section 1.4.1).

2.3.2 Structural basis for defective function
Histological features of UC and CD relating to structural abnormalities include distorted crypt architecture with branching and elongation / atrophy, abscess formation and epithelial cell damage resulting in surface erosions and increased rates of focal cell loss (Delpre et al 1989, Gibson et al 1988 and British Society of Gastroenterology (BSG) guidelines 1997, table 2.2). Colonic epithelium forms a physical structural, as well as functional ionic, barrier against the penetration of antigenic and bacterial contaminants. Consequently inflammation-induced structural defects have been a focus of research regarding possible initiating or causative factors that may lead to the subsequent development of the chronic, recurring inflammatory pattern characteristic of IBD (Gardiner et al 1995).

2.3.2.1 Tight junction – structural changes
As the main determinant in ionic and fluid permeability, structural changes in the epithelial barrier would also contribute to the overall increase in $G_t$ recorded in inflamed colonic tissue, most notably those affecting tight junctional regulation of paracellular passive transport (Nash et al 1988). Ulcerative erosions and extensive dilatation of intercellular spaces result in physical separation of epithelial cells with ZO sites no longer forming significant barriers to lumen-to-blood or blood-to-lumen diffusion (Madara 1989 and Delpre et al 1989). Complete absence of ZO complexes in areas of active disease has been observed in Crohn’s terminal ileum, as well as bizarre fragmented patterns and misalignment of ZO strands (Marin et al 1983). Ultrastructural electron microscopy studies also reveal frequent impalement of colonic epithelium by PMN through these normally occluding
ZO. Corresponding functional studies have recorded a reversible decrease in total epithelial resistance, 83% in 60 minutes in T84 cell model (Nash et al 1987), 61% in human UC tissue (Rohweder et al 1996) with a positive correlation to the number of migrating PMN's. In turn decreased epithelial resistance was accompanied by increased paracellular macromolecule flux localised to the foci of PMN impalement (Almer et al 1993 [UC patient studies] and Gardiner et al 1995 [rat colitis models]). The mechanism of PMN-induced disruption of epithelial ZO and increased permeability has been shown to be primarily due to mechanical force (section 2.2.1). Despite the powerful PMN armoury of free radicals and proteases, all of which are capable of dissolving the extracellular matrix scaffolding (chapter 3 section 3.4.1.1), these were not required for PMN transmigration (Nash et al 1988).

The question arising from these observations concerns the clinical significance of increased permeability arising from PMN-induced decrease in epithelial resistance. Analysis of the ratio of migrating PMN's to epithelial cells in crypt abscesses revealed a ratio of 1:1 in human UC tissue. This would correspond to a PMN transmigration density of $10^6$ PMN cm$^{-2}$ of crypt epithelium and thus be in the range at which maximum alterations in intestinal permeability would be observed (Nash et al 1987). However total epithelial resistance ($R_T$), recorded in the above experiments, is actually the sum of pure epithelial ($R_E$) and subepithelial ($R_{sub}$) resistance. Rohweder et al (1996) in Ussing chamber experiments on stripped mucosa from UC patients, recorded a 61% reduction in $R_T$. $R_E$ however, was depressed by a factor of more than four in UC tissue. This indicated that the defect in barrier function was more dramatic that indicated by $R_T$ measurements alone. The discrepancy was due to the fact that $R_{sub}$ was actually increased in UC, probably due to mucosal oedema and infiltration of inflammatory cells. A large reduction in resistance offered would be translated into a significant increase in ionic and therefore fluid permeability.

The focus on ultrastructural changes mainly involves the study of actively inflamed tissue. Distinctive ultrastructural changes have also been observed in apparently uninvolved, histological and endoscopically normal, parts of
colon thought to be in the inactive stages of UC (Delpre et al 1989). Colonic mucosal involvement may therefore be a universal feature, persisting even during clinical remission.

2.3.2.2 Tight junction – structural plasticity

The effective salvage of colonic fluid is highly dependent on the ‘tight’ nature of colonic epithelium, which not surprisingly, is lost when structural damage occurs. However this does not explain increased epithelial permeability in the face of histologically and structurally normal epithelium, as was the case in the study described above (Rohweder et al 1996). The human intestinal T84 cell line grows as a confluent monolayer of polarised columnar cells that develop high stable baseline resistance to passive ion flow. T84 monolayers also have a ZO subunit structure comparable to native intestinal epithelium and actin-rich perijunctional rings. These cells therefore provide a suitable in vitro model to study inflammation-related structural and functional changes. *Clostridium difficile* Toxin A causes a severe enterocolitis in man and animals, which in part is mediated by acute inflammatory cells. Exposure of T84 cells to toxin A almost abolished resistance, in 6-8 hours, indicating disruption of barrier function. Flux data corresponded to an increase in permeability, restricted to molecules <5Å in Stokes radius. The striking observation, however, was that confluency was maintained with cells remaining abutted to their neighbours and no discernable biochemical or morphological evidence of cytotoxicity. Since toxin A did not specifically increase the permeability of T84 cell plasma membrane to a hydrophilic solute radius 3.6Å, the above alterations were attributed to toxin-elicited alterations in ZO. Analysis of the cytoskeleton showed prominent diminution of F-actin staining in the perijunctional ring confirming structural alterations accounting for functional defects (Hecht et al 1988). Similar findings have been obtained due to the lymphoid cytokine interferon-gamma induced changes in barrier function (Madara et al 1989).

Structural changes therefore, be they physical separation of epithelial cells or alteration in the dynamic properties of ZO complexes, have been shown to be key factors directly affecting epithelial permeability and ion transport.
and as such contributing to impaired colonic salvage and ultimately diarrhoea.

2.4 Inflammatory bowel diseases

Chronic inflammatory bowel disease (IBD) includes two relapsing inflammatory disorders of unknown etiology,

1. non-specific ulcerative colitis (UC)
2. Crohn's disease (CD)

Despite a lack of knowledge regarding initiating factors what is clear is that once inflammation is established the typical disease course follows a series of episodic acute attacks superimposed upon a chronic underlying disease. Both disorders are characterised by recurring bloody diarrhoea resulting from a failure of colonic salvage associated with chronic inflammation. The defining histological features of UC and CD are presented individually with current information regarding epidemiology, etiology and treatment discussed corporately.

2.4.1 Ulcerative colitis

UC was first described in 1875 by Wilks and Moxon as an inflammatory bowel disease distinct from infectious colitis (from Jackson & Grand 1991a). By definition inflammation is limited to the colon and rectum (table 2.2). It is continuous, primarily affecting the mucosa, with varying degrees of ulceration, haemorrhage, oedema and regenerating epithelium which define disease severity. When disease activity is most severe complete destruction of the epithelium can occur with inflammation often extending through the submucosa into the muscularis. Intervening areas of regenerating regranulation tissue and residual epithelium may form islands of tissue termed pseudopolyps.

Early macroscopic features show the mucosa as diffusely erythematous, granular and friable progressing on to contain broad areas of superficial ulceration with overlying mucopurulent exudate. Mucosal histology reveals continuous acute and chronic inflammation with mucosal and submucosal
infiltration by PMN's, leukocytes and mononuclear cells. Colonic crypts show the most characteristic changes with acute changes including goblet cell depletion, cryptitis and crypt abscesses leading to chronic changes of crypt distortion, branching and drop out (Kirschner 1988, Statter et al 1993 and Jackson & Grand 1991a, table 2.2). Clinical assessment of disease activity specific to paediatric patients can be performed using the Lloyd-Still index (Lloyd-Still & Green 1979). A statistical approach to distinguish clinically important changes in rectal biopsies has also been devised (Jenkins et al 1988) and recently the British Society of Gastroenterology have published guidelines for initial diagnosis of suspected IBD from endoscopic biopsy material (BSG guidelines 1997).

2.4.2 Crohn’s disease
CD was first characterised as a unique chronic inflammatory disease by Crohn, Ginzburg and Oppenheimer in 1932 (from Jackson & Grand 1991b). Inflammation, unlike UC, is distinctly transmural, discontinuous and may affect any segment of the gastrointestinal tract, from gums to bum. Discrete segments of diseased mucosa are separated by adjacent intervening normal bowel, leading to the term 'skip' areas. Earliest mucosal changes, often masked by inflammation, appear to be associated with small capillary damage including disruption of basement membranes. This results in small focal intramucosal haemorrhages occurring in the absence of local inflammatory cell accumulation and necrosis of the overlying epithelium. Increased numbers of eosinophilic macrophages beneath the surface epithelium of lamina propria have also been reported (Sankey et al 1993).

Characteristic histopathologic lesions include small superficial ulcerations over a Peyer’s patch (aggregated lymphatic nodules), termed ‘aphthoid’ ulcers (Sankey et al 1993). Focal chronic ulceration extends deep into submucosa and serosa resulting in sinus tracts or fistula formation. Inflammatory infiltrate is predominantly macrophages and lymphocytes. Epitheloid cell granulomas are a predominant and distinguishing feature of chronic inflammation in CD found in 50% of cases. This granulatomas
reaction is assumed to be the result of abnormal phagocytosis and is formed by an aggregate of macrophages (Kitahora et al 1988).

<table>
<thead>
<tr>
<th>Site of Disease</th>
<th>Ulcerative Colitis</th>
<th>Crohn's Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper GI tract</td>
<td>0%</td>
<td>20%</td>
</tr>
<tr>
<td>Ileum alone</td>
<td>0%</td>
<td>19%</td>
</tr>
<tr>
<td>Ileum and colon</td>
<td>Backwash ileitis</td>
<td>52%</td>
</tr>
</tbody>
</table>
| Colon                 | 90% distal colon predominant, 22% left colon *  
                           15% confined to rectum  
                           61% pancolitis (most common) | 9% proximal colon predominant |
| Rectum                | ~100%                               | 50%                              |
| Perianal region       | Rare                                | 25%                              |
| Radiology             | Continuous involvement, foreshortening, loss of haustra, irregular mucosal margins, normal terminal ileum | Segmental involvement, skip regions, mural thickening, stenotic separate loops, abnormal terminal ileum |
| Sigmoidoscopy         | Haemorrhagic mucosa, diffuse continuous inflammation, pseudopolyps | Patchy involvement, skip regions, relative rectal sparing, focal aphthae, linear ulcers |
| Histology             | Mucosal and submucosal inflammation, superficial ulceration, cryptitis, crypt abscess, crypt architecture distortion, goblet cell depletion, Paneth cell metaplasia | Transmural inflammation, granulomas, deep fissure-like ulcerations with sinus tracts, prominent lymphoid tissue, preserved goblet cells, fibrosis |

Table 2.2 Contrasting patterns of pathological involvement in ulcerative colitis and Crohn's disease. * Denotes greater tendency for extension proximal to distal colon in paediatrics than adults 38% v 10% (Jackson & Grand 1991).
2.4.3 Epidemiology

<table>
<thead>
<tr>
<th>Age at onset (year)</th>
<th>Ulcerative Colitis ($n = 465$)</th>
<th>Crohn's Disease ($n = 166$)</th>
<th>($n = 168$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-9</td>
<td>2%</td>
<td>-</td>
<td>2%</td>
</tr>
<tr>
<td>10-19</td>
<td>14%</td>
<td>19%</td>
<td>30%</td>
</tr>
<tr>
<td>20-29</td>
<td>24%</td>
<td>25%</td>
<td>38%</td>
</tr>
<tr>
<td>30-39</td>
<td>25%</td>
<td>16%</td>
<td>13%</td>
</tr>
<tr>
<td>Prevalence in a childhood population (&lt;16yrs)</td>
<td>3.42 per 100,000</td>
<td>16.6 per 100,000</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Epidemiology of ulcerative colitis and Crohn's disease, $n$ – number of cases (Jackson & Grand 1991).

Distribution of age of onset is bimodal with peaks occurring in the second and third decades and again in the fifth and sixth decades. Between 15-40% of all patients with UC present before the age of twenty with peak onset during adolescence. Although though uncommon, UC and CD have been detected in children as young as two years of age (Ojuawo et al 1997). The incidence of CD has more than doubled from 1.30 cases per 100,000 childhood population, <16 years, per year (1983 – 1988) to 3.11 cases per 100,000 (1989 – 1993), while UC remained the same (Cosgrove et al 1996). Both diseases are prevalent among whites and occur three to six times more frequently in Jewish populations regardless of native birth or immigrant history. Male and female are presented equally. Although no specific heritable patterns exists 15-40% of patients may have other family members with IBD.

2.4.4 Etiology

Etiology remains unknown. The familial, racial and ethnic clustering of IBD has supported the concept of genetic predisposition (Statter et al 1993). The functional nature of the intestinal lumen results in its continual exposure to a variety of substances that stimulate local and systemic immune systems. Feedback mechanisms have evolved to regulate this stimulatory process and prevent unrestrained activation of immune and inflammatory responses. The fundamental question therefore regarding the pathogenesis of IBD arises from its characteristic chronic recurrent inflammatory activity and whether this reflects either,
1. an appropriate response to a persistently abnormal stimulus (environmental) or
2. an abnormally prolonged response to a normal stimulus (aberrant immune response)

Many hypotheses have been postulated. The two most serious contenders remaining include an infectious cause and an immunological defect. Extensive studies of immunological profiles in IBD have revealed to date a multiplicity of variable and inconsistent results of both local intestinal and systemic immunity (Fiocchi 1990). These include a tendency towards altered lymphocyte profile (increased B cells and decreased T cells), however no cause and effect relationship has been established. Abnormalities in mucosal mononuclear immunoglobulin production have also been detected. The identification of susceptibility gene loci for both CD and UC has, however, once again focused attention towards the potential genetic basis of IBD (Satsangi et al 1996 and Satsangi et al 1997). Current hypotheses centre on the proposal that these diseases occur as a result of a genetically determined response, which is probably immunologically mediated (Kirschner 1988).

2.4.5 Treatment

Treatment is aimed at restoring colonic structure and function primarily by inhibiting specific arms of the inflammatory response. Anti-inflammatory, steroidal and immunosuppressive agents form the main pharmacological lines of treatment.

Anti-inflammatory amino-salicylates – for example sulphasalazine. Sulphasalazine, the first drug demonstrated to bring about a major reduction in recurrence rate of colitic attacks (Misiewicz et al 1969), is now widely used in the treatment of chronic IBD. It is an azo-bonded combination of sulfapyridine and 5-aminosalicyclic acid (5-ASA). Sulfapyridine prevents absorption in the proximal GI tract and is therefore necessary for therapeutic efficiency. On reaching the colon the 5-ASA moiety is liberated by the action of colonic bacterial azo-reductase and acts topically. The action of 5-ASA manifests itself clinically as an anti-inflammatory agent. Proposed
mechanisms of action include reduction of PG and LT production (Ligumsky et al 1981), modulation of the effects of cytokines (Travis & Jewell 1994), and free radical scavenging (Ahnfelt-Rønne et al 1990, chapter 3 section 3.6.3). A study looking at the effects of sulphasalazine specifically in children with UC showed that clinical remission was achievable without accompanying endoscopic and histological remission (Beattie et al 1996).

**Steroids** - such as hydrocortisone, prednisolone and methylprednisolone. Steroids have been shown to stimulate increases in rectal Na⁺ and water absorption in UC patients (Sandle et al 1986). Cells under the action of glucocorticoid steroid hormones produce lipocortin (from a family of Ca⁺ binding anti-phospholipase proteins) which exerts control over increases in Ca⁺ - normally a powerful trigger for eicosanoid production. Increased synthesis of lipocortin stops many (but not all) cells from generating PG’s and other proinflammatory eicosanoids and may therefore be one mechanism of action of these agents (Halliwell & Gutteridge 1991). Other anti-inflammatory mechanisms include immunosuppression and inhibition of chemotactic factors including PG’s, LT’s, PAF and TNF (Nielsen & Ahnfelt-Rønne 1991).

**Immunosuppression** – azathioprine, 6-mercaptopurine and methotrexate. These are mainly used as adjunctive agents in disease management, maintaining remission and enabling reduction in steroid dosage. Slow onset of drug action precludes their use in the short term for acute symptoms (between 12 – 17 weeks). Possible rapid acting “bridge” treatments to these slow acting agents include cyclosporin and tacrolimus (FK506). Both these immunosuppressant agents selectively block the activation of T helper and cytotoxic lymphocytes by inhibiting the calcium-dependent transcription of interleukin-2 (Sartor 1994). High dose intravenous cyclosporin has been shown to be of rapid clinical benefit in patients with active UC refractory to parental corticosteroid therapy (Lichtiger et al 1994). The requirement for high doses, often with severe side effects, precludes its long-term use. In addition long-term treatment of chronic active CD, in conjunction with low-dose steroids, appears to offer no advantage compared with low-dose...
steroids alone (Stange et al 1995). Tacrolimus has the added advantage of being well absorbed orally, with a preliminary report showing it to be of clinical benefit in the treatment of complicated proximal small bowel and fistulizing CD (Sandborn 1997). However further randomised-controlled trials are required.

Future treatments – the goal of any treatment is the localised inhibition of pathological biochemical events with minimal systemic effects. New treatments reflecting this aim include lipooxygenase inhibitors, superoxide scavengers (chapter 3 section 3.6.3) and inhibition of tumor necrosis factor. 5-lipooxygenase has a unique role in the production of LT's. Zileuton is the first selective 5-lipooxygenase inhibitor, effective in animal studies (Bertrán et al 1996 and Rask-Madsen et al 1992), evaluated for use in the treatment of patients with IBD. The specific effects of zileuton were demonstrated in rectal dialysates from UC patients, with a 75-85% reduction in LTB₄ concentrations but not in PGE₂ (Laursen et al 1990). Clinical trials have demonstrated significantly improved symptom and sigmoidoscopy scores with a trend towards improved histology (Hawkey et al 1994). Tumour necrosis factor-α (TNF-α), a highly pro-inflammatory cytokine found in increased levels in CD, is proposed to play a pivotal role in CD, for example in the promotion of neutrophil-endothelial adhesion and granuloma formation (section 2.2.1, Braegger et al 1992, Murch et al 1993a, Breese et al 1994 and Van Deventer 1997). The therapeutic potential of TNF-α inhibition was shown in a small ten patient open-label study in steroid-unresponsive CD patients given a single infusion of anti-TNF monoclonal antibody. Within four weeks eight out of the ten patients showed complete clinical and endoscopic remission (Van Dullemen et al 1995). A similar study of 16 patients using a different suppressor of TNF-α release, oxpentifylline (PTX) however showed no improvement in clinical disease activity or endoscopic assessment of inflammation (Bauditz et al 1997). A simultaneously published study on PTX's effects on in vitro TNF-α production, by peripheral mononuclear cells and inflamed intestinal mucosa cultures from CD and UC patients, demonstrated a 50% inhibition of TNF-α production (Reimund et al 1997). It would appear from these studies that
PTX could inhibit TNF-α production but that there may be more key mediators in the CD inflammatory process with inhibition of TNF-α alone being insufficient to produce complete clinical remission. Clinical trials using recombinant receptor antagonists against the cytokine IL-1, a potent activator of T&B cells, are also underway (Van Hogezaand & Verspaget 1996).

2.4.6 Allergic colitis
A third commonly presenting colitis in children is that of allergic colitis (Ojuawo et al 1997). This is a separate clinical entity of colitis, which lacks the distinct macroscopic and histological features of UC and CD. The main clinical symptoms include chronic bloody diarrhoea, anaemia and abdominal pain, with histology revealing focal ulceration and gross eosinophilic infiltrate with eosinophils clumping together and degranulating. Macroscopic findings on endoscopy reveal mucosal erythema and aphthous lesions. Inflammation is represented microscopically by a heavy eosinophilic infiltrate without excessive vascularity and an abundance of IgE containing mononuclear cells. The age distribution of 46 children with colitis studied between 1977 and 1981 showed two peaks occurring at <2 years and between 6-13yrs. Food allergic colitis was found to be the major cause of colitis in infancy (< 2 years) with all the children over 6 years suffering from typical UC or CD. It was noted that in older patients eosinophilic colitis could be a precursor of UC (Jenkins et al 1984).
CHAPTER 3
Role of oxygen free radicals in health
and inflammatory bowel disease.

3.1 Introduction
Free radicals are among the most reactive substances known. They are essential in health whilst simultaneously containing the capacity to destroy healthy tissue. A failure to produce free radicals, as seen in chronic granulomatous disease, results in an inability to destroy ingested microorganisms therefore producing repeated bacterial infections and frequent childhood mortality (Ward 1974 and Baehner et al 1976). At the other extreme excessive production is increasingly being implicated in a growing number of inflammatory diseases including chronic rheumatoid arthritis (Blake et al 1987), lung injury (Curran et al 1984) and inflammatory bowel disease (Verspaget et al 1991). In the continuing quest for knowledge regarding possible etiological and exacerbating factors in IBD pathology, research into potential abnormalities in oxidative metabolism has become prominent. Following an outline of basic free radical chemistry and the role of reactive oxygen metabolites (ROM\textsuperscript{1}) in health, this chapter will focus on the current evidence to implicate ROM in IBD.

3.2 Free radical chemistry
Electrons and atoms occupy regions of space known as orbitals, with each orbital holding a maximum of two electrons. Most molecules found in vivo contain paired electrons only with opposite spin directions, forming stable nonradicals. A free radical can be defined as,

\textsuperscript{1}Reactive oxygen metabolites (ROM) is a collective term used to include not only oxygen-centred radicals but also non radical derivatives of oxygen, such as hydrogen peroxide and hypochlorous acid.
“Any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital” (Halliwell et al 1992).

An unpaired electron can be considered as a half open chemical bond inferring an increased reactivity upon a molecule. Free radicals react with nonradicals in a number of ways, such as donating an unpaired electron (oxidation), abstracting an electron from another molecule (reduction) or combining with a second molecule (Simmonds & Rampton 1993). Characteristic of all free radical reactions is the resulting formation of a second highly reactive radical effectively initiating a self-perpetuating chain reaction. Termination can only occur by either interaction with a second free radical to make a nonradical or with an antioxidant molecule such as vitamin E (section 3.5.4). Free radicals therefore form a highly reactive and potentially dangerous species.

### 3.2.1 Free radical reactions

Oxygen (O\textsubscript{2}) is an essential prerequisite for efficient energy production in aerobic organisms. At the same time it is a major promoter of free radical chemistry. In its natural ground state O\textsubscript{2} has two lone electrons each located in different orbitals with parallel spins (therefore qualifying it as a free radical). O\textsubscript{2} requires two electrons of parallel spin to fit into its vacant orbital spaces (figure 3,2). As a pair of electrons in a nonradical molecular orbit always have opposite spins O\textsubscript{2} is effectively restricted to accepting one electron at a time making reactions sluggish (transition metals can overcome these spins restrictions). The reduction of molecular O\textsubscript{2} to form water as its final reduction product illustrates a number of different oxygen-derived species that may be obtained from O\textsubscript{2} (figure 3,1 Halliwell & Gutteridge 1991).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Species</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>O\textsubscript{2} \rightarrow O\textsubscript{2}^- superoxide</td>
<td>(one electron reduction)</td>
<td></td>
</tr>
<tr>
<td>O\textsubscript{2} \rightarrow H\textsubscript{2}O\textsubscript{2} peroxide</td>
<td>(two electron reduction)</td>
<td></td>
</tr>
<tr>
<td>O\textsubscript{2} \rightarrow \cdotOH hydroxyl radical</td>
<td>(three electron reduction)</td>
<td></td>
</tr>
<tr>
<td>O\textsubscript{2} \rightarrow H\textsubscript{2}O water</td>
<td>(four electron reduction)</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 3,1 Univalent reduction reactions of ground state O\textsubscript{2} to form water*
If a single electron is added to a ground state $O_2$ molecule it must enter one of the $\pi^*$ antibonding orbitals, producing the superoxide radical ($O_2^-$). Addition of a second electron to this $O_2^-$ radical produces the peroxide ion, a nonradical (figure 3.1), which at physiological pH will immediately protonate to produce hydrogen peroxide ($H_2O_2$). In biological systems $O_2^-$ production is rapidly and preferentially followed by spontaneous interaction of two $O_2^-$ molecules (dismutation) enzymically catalysed by superoxide dismutase (SOD) to form $H_2O_2$. $H_2O_2$ is potentially an extremely powerful oxidant of relatively low reactivity being able to diffuse considerable distances before decomposing to form the hydroxyl ion ($\cdot OH$), (Blake et al 1987). Interaction of $H_2O_2$ with transition metals reduced by $O_2^-$ can also produce $\cdot OH$, known as the Fenton reaction. The $\cdot OH$ radical is the most reactive species found in biological systems reacting at extremely high rates with almost every type of molecule found in living cells including sugars, amino acids, phospholipids, DNA bases and organic acids (Slater...
Highly reactive free radicals produced in biological systems are largely confined to their immediate vicinity as a direct consequence of their chemical reactivity. As a result their average radius of diffusion will be very small, for example the half life of \( \cdot \text{OH} \) has been calculated as a few microseconds effectively limiting the radius of diffusion from its generation site to less than 100nm (Saran & Bors 1990 and Slater 1984).

### 3.3 Free radical sources in health and IBD
Free radical production in health and disease may occur through several mechanisms including,

(i) normal oxidative metabolism (energy production)  
(ii) microsomal cytochrome P\(_{450}\) activity  
(iii) phagocyte respiratory burst (bactericidal defence activity)  
(iv) eicosanoid production via arachidonic acid metabolism (inflammatory response to injury)  
(v) cytosolic xanthine oxidase  
(vi) auto-oxidation of small molecules such as hydroquinones, leucoflavins, catecholamines and reduced ferredoxins.

The two main sources of ROM in vivo are the phagocytic respiratory burst and eicosanoid production.

#### 3.3.1 Phagocyte respiratory burst
The role of phagocytes in normal defence against pathogens, via the respiratory burst, has been recognised since 1883 (Babior 1978). The respiratory burst describes a metabolic pathway, dormant in resting cells, whose function is to produce a group of highly reactive microbicidal agents by the partial reduction of \( \text{O}_2 \). The pathway is composed of a series of co-ordinated metabolic events initiated when phagocytes are exposed to appropriate stimuli. These events, culminating in ROM production, are outlined using activated neutrophils as an example (figure 3.3; Blake et al 1987, Simmonds & Rampton 1993, Yamada & Grisham 1991, Weiss & LoBuglio 1982 and Maly & Schürer-Maly 1995).
Figure 3.3 Extracellular production of reactive oxygen metabolites via the neutrophil respiratory burst.

1. **Activation** - inflammatory mediators interact with specific surface membrane receptors to activate the latent plasma membrane-associated enzyme, NADPH oxidase indicated by a dramatic increase in O$_2$ consumption within 30-60 seconds.

2. **NADPH oxidase** - once activated shuttles electrons from cytosolic NADPH (electron donor) to O$_2$ dissolved in the extracellular fluid so catalysing the one-electron reduction of O$_2$ to O$_2^·$.

3. **Hexose monophosphate shunt (HMPS)** - O$_2^·$ rapidly undergoes spontaneous dismutation to yield H$_2$O$_2$ with NADP$^+$ produced increasing glucose oxidation via the HMPS (a metabolic pathway oxidising glucose to carbon dioxide and a five-carbon sugar with NADP$^+$ serving as an electron acceptor and rate limiting factor).

4. **Myeloperoxidase (MPO)** - an enzyme simultaneously released from neutrophil phagocytic granules, that catalyses the oxidation of halides (Cl$^-$ preferentially) by H$_2$O$_2$, to yield hypochlorous acid (HOCl). As long as H$_2$O$_2$ is supplied MPO will use plasma Cl$^-$ to generate HOCl for up to 3 hours post activation until the pool of oxidisable targets is used up. The MPO H$_2$O$_2$ - Cl$^-$ system is considered the most potent cytotoxic arm of the neutrophil, with HOCl 100-1000 times more toxic than either O$_2^·$ or
H₂O₂. (Weiss et al 1989). HOCl can further react with certain primary amines to yields N-chloramines.

5. Other reactions - neutrophil derived O₂⁻ and H₂O₂ can also interact via the Fenton reaction to yield the highly reactive ·OH ion.

All events following the initial respiratory burst depend on the activity of the single enzyme system NADPH oxidase with H₂O₂ production, HMPS activation and MPO secretion as secondary consequences. During conditions of severe acute and chronic inflammation this normal defence mechanism however, can be magnified resulting in the prolonged persistent production of a barrage of highly toxic chemicals with the potential to damage healthy as well as diseased tissue.

At this point it is also necessary to mention a second non-oxidative arm in the neutrophil defence weaponry simultaneously released upon activation. Neutrophils also contain cytoplasmic granules storing over 20 enzymes, with three distinctive proteolytic enzymes - serine proteinase, elastase, which cleave protein peptide bonds, and two metalloproteinases, collagenase and gelatinase. The effectiveness of both weapons in causing neutrophil-mediated tissue damage has been demonstrated to be inextricably linked (section 3.4.1.1).

3.3.2 Eicosanoids: prostaglandins and leukotrienes

A second major source of free radicals arises from a normal inflammatory defence reaction, namely the production of PG’s and LT’s via the oxidative metabolism of arachidonic acid in cell membranes. Oxidation occurs in response to mechanical / chemical injury by stereospecific free-radical reactions to produce fatty acyl peroxides (chapter 2 section 2.2.2.2 & figure 2.2). The essential biological functions of PG’s and LT’s include platelet aggregation (TXA₂), smooth muscle contraction, blood vessel tone (PGI₂), localised hormonal actions and regulation of physiological processes. Production is tightly regulated by a number of mechanisms including negative feedback, inactivation to restrain effects locally and strict control of AA release from membranes. These are vital in order to minimise their
destructive capacity to cause excessive inflammation and inappropriate tissue damage (Eberhart & Dubois 1995, section 3.5.4).

3.4 Effects of free radicals
ROM-mediated effects can be favourable or adverse (figure 3,4)

![Effects of reactive oxygen metabolites](image)

<table>
<thead>
<tr>
<th>Favourable</th>
<th>Adverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular respiration</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>Intracellular killing</td>
<td>DNA damage</td>
</tr>
<tr>
<td>Diverse oxidative processes</td>
<td>Protein inactivation</td>
</tr>
<tr>
<td>e.g. arachidonic acid metabolism</td>
<td>Cell activation</td>
</tr>
<tr>
<td>Protection Tissue homeostasis</td>
<td>Cell death Tissue damage</td>
</tr>
</tbody>
</table>

Figure 3.4 Favourable and detrimental roles of reactive oxygen metabolites leading to either tissue protection or damage.

3.4.1 In vivo damage potential
ROMs are toxic not only because of their own intrinsic reactivity but because of their modulation of other inflammatory processes, such as their synergistic action with serum proteases. Neutrophils, as a major source of ROMs, have little intrinsic ability to distinguish between host and foreign antibodies relying on other arms of immune system for such recognition. Inappropriate identification of normal host tissue as foreign or damaged may therefore also result in neutrophil receptor activation and elicitation of the cell's destructive potential (Weiss 1989). ROMs themselves, once released are not designed to act as specific toxins. They are unable to selectively differentiate between nearby targets and excessive production can potentially overwhelm endogenous antioxidant defences. For these
reasons ROM-mediated oxidation of proteins, lipids and carbohydrates is indiscriminative affecting healthy critical cellular processes as well as damaged tissue. This can, therefore, lead to severe structural and functional consequences.

### 3.4.1.1 Extracellular matrix – neutrophil-mediated ROM damage

Neutrophil-mediated tissue damage occurs by both oxidative and non-oxidative mechanisms. OH\(^{-}\), HOCl and N-chloramines can directly degrade / depolymerise important extracellular matrix components, such as hyaluronic acid, collagens, fibronectin and proteoglycans (Greenwald \& Moak 1986 and Yamada \& Grisham 1991). Also combined forces with released proteases effectively alters normal in vivo protease/antiprotease balances enabling intrinsic host-erected defence barriers to be subverted (Weiss 1989). For example oxidation of endogenous tissue antiproteases by extracellular HOCl can decrease the protease:modified-antiprotease association rate thereby increasing protease half life as much as 2000-fold (0.6 milliseconds to 1.2 seconds), whilst also activating proteases released in latent form (Janoff 1985). Using model in vitro inflammatory sites, neutrophils were shown to generate and use HOCl and N-chloramines to oxidise surrounding $\alpha_1$ proteinase inhibitor at a rate that permitted discharged neutrophil elastase to solubilise the extracellular matrix (Weiss \& Regiani 1984). By contrast neutrophils that are unable to generate ROM, from patients with chronic granulomatous disease (CGD), released comparable amounts of collagenase but failed to activate it (Weiss et al 1985). In vivo studies remain to be performed, but fluid and in some cases tissue, recovered from inflamed areas in disease states have be found to contain active neutrophil elastase, collagenase and MPO as well as inactivated $\alpha_1$ proteinase inhibitor (Damiano et al 1986, Cochrane et al 1983 and Edwards et al 1988).

Thus the oxidising potential of ROMs is transformed into enzyme-catalysed degradation of proteins and interstitial, pericellular and basement membrane-associated collagens (Simmonds et al 1992 and Yamada &...
Grisham 1991). In terms of defence, this allows neutrophils to transverse connective tissue barriers and dissolve infected tissues in an attempt to dislodge microbes or participate in abscess formation. In a physiological inflammatory response neutrophils do not continuously degrade host tissues in an interrupted manner as infiltration and activation are tightly regulated, ceasing when the initiating antigen is destroyed. Once the oxidising environment has dissipated the antiproteinase screen can be reconstructed by diffusion of inhibitors from the plasma bed or by locally synthesised antiproteinases. If however inflammatory stimuli are chronically directed against host tissues or not properly down-regulated, prolonged neutrophil invasion could effectively penetrate and subvert normal host defences.

3.4.1.2 Cell membranes - lipid peroxidation
Enzymic lipid peroxidation is a tightly regulated normal metabolic process resulting in the production of stereospecific hydroperoxides and endoperoxides with important biological functions. Lipid peroxidation is also a feature of many types of cell injury in which free radical processes can target esterified cell membrane lipids. It involves two main events, initiation and propagation (figure 3.5).

First chain initiation - free radicals have sufficient reactivity to abstract a hydrogen atom (H\(^+\)) from a methylene carbon in a fatty acid side chain, resulting in the formation of a carbon-centred radical. Most membrane polyunsaturated fatty acids are more susceptible to abstraction due to weak carbon-hydrogen bonds formed between their double bonds and adjacent methylene groups. The resulting carbon-centred radical can have a number of fates of which molecular rearrangement, followed by reaction with O\(_2\) to give a peroxyl radical, is most likely in aerobic cells.

Propagation - peroxyl radicals are also of sufficient reactivity to abstract a H\(^+\) from an adjacent fatty acid side chain, effectively propagating the lipid peroxidation chain reaction. Once combined with the abstracted H\(^+\) a lipid hydroperoxide is formed. Pure lipid peroxides are relatively stable at physiological temperature but in the presence of transition metal complexes
such as iron or copper ions, decomposition is greatly accelerated and may result in a diversity of end products that include epoxides, carbonyls and hydrocarbon gases. The importance of these end products lies in the reactivity of many with membrane proteins.

![Diagram of hydrogen abstraction and peroxidation of a polyunsaturated fatty acid.](image)

**Figure 3.5** Hydrogen abstraction and peroxidation of a polyunsaturated fatty acid.

A single initiation event can therefore result in a self-perpetuating propagated chain of oxidative reactions converting hundreds of fatty acid side chains into lipid hydroperoxides. The length of the propagating chain is dependent on the lipid/protein ratio, fatty acid composition, oxygen concentration and presence of chain-breaking antioxidants (section 3.5.4 Gutteridge & Halliwell 1990). Disrupted or damaged tissues are more susceptible to lipid peroxidation, especially after mechanical disruption causing the release of metal ions from intracellular stores. Lipid peroxidation of biological membranes results in destabilisation decreasing fluidity and barrier function and increasing non-specific permeability to ions,
such as Ca\(^+\). Peroxidation products, hydroperoxides and aldehyde derivatives, can inhibit protein synthesis, cross-link membrane proteins, inactivate membrane bound receptors and alter chemotactic and enzymic activity (Blake et al 1991). Although lipid peroxidation results in significant cellular injury, its occurrence is as a *consequence* of tissue injury, which may significantly contribute to worsening of the initial condition. It is not an initiating / causative factor.

### 3.4.1.3 Proteins

Proteins are susceptible to ROM-mediated oxidation, fragmentation, and modification via cross-linking and aggregation. Oxidation of amino acid residues occurs predominantly at metal binding sites, for example histidine residues of glutamate dehydrogenase, and non-protein thiol groups (Saran & Bors 1990). \( \cdot \text{O}_2^- \) and \( \cdot \text{OH} \) induced modifications of methionine, histidine, cysteine, proline and lysine are among those reported (Blake et al 1987). Cross-linking and aggregation also involve radical-mediated thiol oxidation and inter/intramolecular disulphide bond formation, also including bonds such as dityrosine linkages formed in the absence of \( \text{O}_2 \). ROMs can also bind covalently to membrane enzymes, protein receptors and transporter proteins. Functionally ROM-mediated protein damage results in modification of specific protein activities such as enzymic reactions, membrane receptor interactions and ion transport function. Specific damage to DNA proteins includes chemical alteration of nucleic acids, chlorination of purine bases, induction of DNA-strand breaks and changes in structure resulting in a mutation or cytotoxicity (Slater 1984).

### 3.5 In vivo damage limitation

In view of the damage potential of ROMs tight regulatory mechanisms are present in vivo. \( \cdot \text{OH} \) arising from \( \text{H}_2\text{O}_2 \) is the most reactive of the free radical species therefore regulation is directed at controlling the amount of \( \text{H}_2\text{O}_2 \) that is allowed to accumulate (Halliwell & Gutteridge 1991). Two types of enzymes exist to remove intracellular \( \text{H}_2\text{O}_2 \),
(i) Catalases - convert $H_2O_2$ into water and $O_2$ (ground state)
(ii) Peroxidases - bring about the general reaction, in which $SH_2$ is a substrate that becomes oxidised.

\[
SH_2 + H_2O_2 \rightarrow S + 2H_2O
\]

3.5.1 Catalase
Catalase is present in all major organs of the body with concentrated amounts in liver peroxisomes and red blood cells. It consists of four protein subunits each of which contains a haem (Fe(III)-protoporphyrin) group bound to its active site and usually one molecule of NADPH to aid enzyme stability.

3.5.2 Glutathione peroxidase
This peroxidase is made up of four protein subunits each containing one atom of selenium at its active site. It is present in the cell cytosol and mitochondria. Its substrate is the low molecular weight thiol compound glutathione normally present in vivo in its reduced form (GSH). Glutathione peroxidase catalyses the oxidation of 2 GSH molecules, with $-SH$ groups of cysteine residues forming a disulphide bridge $S-S$, at the expense of $H_2O_2$ to produce GSSG (oxidised GSH) and water. Ratios of GSH/GSSG in normal cells are kept high by glutathione reductase enzymes which catalyse the following reaction, with NADPH provided by the HMPS oxidative glucose pathway.

\[
GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+
\]

Glutathione peroxidase is also responsible for detoxifying organic hydroperoxides such as fatty acid hydroperoxides, nucleotide and steroid hydroperoxides, disulphides and oxidised ascorbate, produced as a result of oxidative stress.
3.5.3 Superoxide dismutases

Intracellular $O_2^-$ concentrations are maintained at very low levels ($10^{-11} M$), despite relatively high rates of cellular production during normal metabolism, by two superoxide dismutase enzymes (SOD). These enzymes are extraordinarily efficient catalysts with virtually every encounter with $O_2^-$ resulting in a reaction (McCord & Fridovich 1978).

(i) Copper-zinc enzymes - are composed of two protein subunits each bearing an active site containing one copper and one zinc ion. They function to greatly accelerate the rate of spontaneous $O_2^-$ dismutation from $\sim 5 \times 10^5 M^{-1} s^{-1}$ (at physiological pH) to $1.6 \times 10^8 M^{-1} s^{-1}$. Copper appears to act in the catalytical role undergoing alternate oxidation and reduction, with zinc maintaining enzyme structural stability (Fridovich 1978). Metallothionein, a ubiquitous metal binding protein, is also important in the regulation of copper and zinc metabolism (as well as holding $\cdot$OH scavenging potential).

(ii) Manganese enzyme - a mitochondrial SOD enzyme with four protein subunits each containing 0.5 or 1.0 ions of manganese (Mn), with Mn undergoing a change in valency during catalyst reactions. Loss of Mn results in loss of catalyst activity of the enzyme.

Tissue levels of these enzymes vary greatly corresponding to the extent of free radical production, with high levels in liver and low in spleen, cardiac and skeletal muscle. Human colonic mucosa, submucosa and muscularis all contain SOD, catalase and GSH peroxidase in significant but low amounts. Most of the protective enzyme activity is localised within the epithelium, leaving the mucosal interstitium comparatively deficient (Grisham et al. 1990).

3.5.4 Vitamin E

Vitamin E (α tocopherol), is a lipid soluble molecule found concentrated in biological membranes with essential antioxidant properties. These include reaction with and removal of singlet oxygen, quenching of $O_2^-$ and reaction with $\cdot$OH. α tocopherol is the major antioxidant mechanism in biological membranes where it acts as a chain breaking antioxidant during lipid
peroxidation reactions by reacting with lipid peroxy and alkoxy radicals
donating labile hydrogen to generate a tocopheryl radical. This radical is
insufficiently reactive to abstract H⁺ from adjacent membrane lipids,
(because the unpaired electron on the oxygen atom can be delocalised to
the aromatic ring so increasing its stability) so terminating the chain
reaction of lipid peroxidation. The tocopheryl radical is then recycled back
to tocopherol within the biological membrane via reduction by ascorbic acid.

3.5.5 Ascorbic acid (vitamin C)
Ascorbate is a water-soluble antioxidant acting as a reducing agent. It is
able to function as a chain breaking antioxidant and a scavenger of both
·OH and O₂⁻. However under the right conditions, such as in the presence
of catalytic metal ions, ascorbate may function as a pro-oxidant so
promoting free radical production.

3.5.6 Sequestration of metal ions
Transition metals in vivo are normally present tightly bound to storage and
transport proteins, either in the circulation or within tissues, rendering them
unreactive and unable to participate in O₂⁻-driven Fenton reactions. Iron (III)
is bound to transferritin in normal human plasma and to ferritin storage
granules in tissues, and copper to caeruloplasmin (Saran & Bors 1991).

3.6 Role of free radicals in IBD
There is a growing body of evidence to suggest that abnormal oxidative
metabolism resulting from a disturbed balance between damage and
protection, is of central importance in active IBD (Verspaget et al 1991 and
Simmonds & Rampton 1993).

3.6.1 Excess free radical production
Acute on chronic inflammatory episodes repeatedly subject colonic mucosa
to significant levels of oxidative stress, mainly produced by infiltrating
activated phagocytes and increased AA metabolism. There is clear
evidence in IBD for elevated ROM levels in both isolated inflammatory cells and inflamed mucosal biopsy specimens. Circulating monocytes and PMN leukocytes from patients with acute IBD demonstrate an increased capacity to generate ROM, with patients in remission showing no such increase (Kitahora et al. 1988 and Shiratora et al. 1989). This increased capacity can be both spontaneous and stimulated, seen in intestinal macrophages from inflamed mucosa compared to controls and non-inflamed tissue from the same IBD patients (Mahida et al. 1989 and Williams et al. 1990). Likewise monocytes from patients with CD are more sensitive to “priming” by bacterial wall products, to which intestinal mucosa is continuously exposed, than normal cells to produce an accentuated release of ROM in response to a specific stimulus (Baldassano et al. 1993). Excessive ROM concentrations measured in IBD are sufficient to induce mucosal damage and increase lipid peroxidation, positively correlating to macroscopic and microscopic disease activity and MPO levels (Ahnfelt-Rønne et al. 1990 and Simmonds et al. 1992). Damage can be primarily attributed to neutrophil infiltration and release of $H_2O_2$ and HOCl (Sedghi et al. 1993 and Keshavarzian et al. 1992). Inflamed colon has been estimated to contain approximately $10^7$ granulocytes per gram wet weight (Sekizuka et al. 1988). Assuming that fully activated granulocytes produce 50-60nmol $O_2^-$ min$^{-1}$ from which 5-10nmol $H_2O_2$ min$^{-1}$ and 5-10nmol HOCl min$^{-1}$ are derived, it has been speculated that even in the face of adequate antioxidant defences colonic tissue may eventually be overwhelmed by these immense levels of oxidants. (Grisham et al. 1990 and Grisham et al. 1984).

In addition to increased capacity and production of ROM the availability of low molecular weight chelated iron, a form accessible to fuel $O_2^-$-driven Fenton reactions, is increased (Babbs 1992). Firstly potential iron sources include released haem iron resulting from microscopic haemorrhage and non haem iron from dietary sources concentrated in faecal material as well as oral iron supplementation therapy for anaemia. Secondly available iron chelators that support Fenton chemistry include,

(i) common bile pigments, abundant in colonic faecal contents
(ii) denatured haem itself from enzymatic digestion of haemoglobin following mucosal haemorrhage
(iii) carboxylic amino acids, aspartate and glutamine liberated by action of proteolytic enzymes from dead/dying neutrophils and mucosal cells.

3.6.2 Decreased defence
Inflamed colonic tissue contains decreased or altered concentrations of endogenous antioxidant enzymes - copper/zinc SOD, metallothionein, glutathione, plasma ascorbic acid and vitamin E, compared to controls (Mulder et al 1991, Inauen et al 1988 and Hoffenberg et al 1997). In addition the normal distribution of these enzymes in the colon is primarily in the surface epithelium leaving the extracellular matrix relatively unprotected. Accumulation of infiltrating phagocytes in the lamina propria would therefore be expected to result in oxidant-induced damage (Grisham et al 1990).

3.6.3 Therapeutic treatments
The role of ROM in IBD has been further strengthened by the efficacy of scavenger enzymes and therapeutic drugs acting on the inflammatory process in the gut (Gross et al 1994). Specific ROM inhibitors, including methoxypolyethylene SOD (O$_2^-$ scavenger), catalase (H$_2$O$_2$ scavenger) and azide (MPO inhibitor), have been shown to be effective in decreasing both phagocyte chemiluminescence (a measurement of phagocytic ROM production) and the severity of colonic inflammation in a rat model of colitis (Keshavarzin et al 1990 and Fretland et al 1991). In human studies a clinical trial using an intramuscular application of SOD to patients with CD has been found to improve the clinical situation, measured by the Crohn's Disease Activity Index, as well as endoscopic demonstration of mucosal restoration and a shortening of relapse duration (Emerit et al 1989). The anti-inflammatory actions of sulfasalazine, effective in the treatment of IBD, have been partly attributed to its free radical scavenging actions suggested to be directly directed at HOCl (Ahnfelt-Rønne et al 1990, Keshavarzin et al
1990 and Weiss 1989). Also the use of free radical scavengers - allopurinol (inhibits xanthine oxidase $O_2^*$ production) and dimethyl sulfoxide (•OH scavenger) in addition to the normal corticosteroid / sulfasalazine regime has been shown to provide the highest therapeutic efficacy in a double-blind trial of patients with recurrent attacks of proctosigmoidal UC (Salim 1992).

3.6.4 Specific known effects on colonic structure and function

Function - both generalised and specific increases in mucosal permeability have been measured in connection with increased ROM levels (Kubes et al 1991 and Gaginella et al 1995). Specific effects include $H_2O_2$, HOCl and NH$_2$Cl induced electrogenic Cl$^-$ and water secretion and inhibition of neutral NaCl absorption, mediated by increased prostaglandin production and the ENS, measured in rat and rabbit colon (Yamada & Grisham 1991, Bern et al 1989, Karayalcin et al 1990 and Powell 1992). Electrolyte secretion in response to monochloramine has been demonstrated in human colonic T84 cells, in part mediated via increased cytosolic Ca$^+$ (Tamai et al 1992). Also in an animal model of vitamin E deficiency in which antioxidant defences were compromised, exposure to oxidative stress resulted in small intestinal hypersecretion and increased lipid peroxidation (Lindley et al 1994).

Structure – the extracellular matrix, with its low levels of antioxidant enzymes, is particularly vulnerable to the dual oxidant / protease attack from infiltrating neutrophil defence armoury. The solubilisation of matrix proteins and higher rates of cell membrane lipid peroxidation serve to destroy both the structural framework, essential for normal colonocyte function, and the repair capacity of the damaged tissue (Ahnfelt-Rønne et al 1990). This is particularly significant regarding the nature of IBD with acute inflammatory attacks superimposed onto a chronic underlying disease process thereby allowing repeated phagocytic infiltration (Weiss 1989). In addition low levels of mucosal trypsin inhibitor (Playford et al 1990) and increased levels of circulating leucocyte elastase (Adeyemi et al 1985) and colorectal collagenase in IBD patients may reflect the activity of HOCl in these patients (Sturzaker & Hawley 1975 section 3.4.1.1).
HYPOTHESES

Inflammatory conditions of the colon disrupt normal colonic structure and function, most noticeably by impairing net fluid absorption and increasing net fluid secretion to produce diarrhoea. Fluid secretion has long been upheld as the sole function of crypt epithelial cells with fluid absorption confined to surface epithelium (Field 1980 & Welsh et al 1992). The discovery of the dual functional nature of colonic crypts dispels this dogma. (Naftalin & Pedley 1990 and Singh et al 1995a, chapter 1 section 1.4.1). Estimations of fluid flow rates into crypt lumina reveal that crypt absorption alone is theoretically sufficient to account for the entire fluid absorption across rat descending colon (Naftalin 1994). This suggests a vital role for colonic crypts in normal fluid salvage. In inflamed colonic tissue normal absorptive function is disrupted and there may be a significant reduction in crypt density (BSG guidelines 1997). One specific arm of the inflammatory response, the production of free radicals, has increasingly been implicated in the pathology of IBD.

This thesis explores two hypotheses.

1. Alterations in normal colonic crypt structural and functional relationships are of central importance in inflammatory conditions of the colon. Novel in vivo and in vitro techniques adapted for use in humans, specifically children, are employed to study these interrelationships.

2. The potential for free radical-mediated damage to colonic tissue suggests that free radical production in IBD forms a significant and integral component of the overall inflammatory response and therefore pathophysiology. The extent of free radical production within inflamed human colonic tissue was assessed in part by visualising oxidative stress using confocal microscopy, a method previously used in conjunction with animal tissue only.
AIMS OF STUDY

♦ To adapt novel methods developed in animals for the study of human colonic fluid transport; in vivo using agarose gels and in vitro by confocal microscopy using endoscopic biopsy material.

♦ To apply the latter confocal methodology to the specific study of crypt functional and structural relationships in health and inflammation.

♦ To assess the contribution of free radicals produced within colonic tissue to the inflammatory process underlying the observed structural and functional changes.
CHAPTER 4
Measurement of intestinal transport
in vivo.

4.1 Aims

Maintenance of near physiological conditions is one of the main advantages in using in vivo methodologies to acquire information regarding human intestinal transport. The simplest non-invasive method involves comparison of ileostomy output composition with that of normal dehydrated stools. Rectal transport has been studied using dialysis bags (Edmonds 1971) and continuous intraluminal perfusion techniques (Devroede & Phillips 1970). More invasive procedures, involving fluid and electrolyte infusion via polyvinyl tubes introduced orally and positioned in the caecum, have provided information concerning relationships between absorption, fluid entry rate into the colon, tonicity and effects of luminal secretagogues (Billich & Levitan 1969 and Debongnie & Phillips 1978). The most invasive technique involves occlusion methods, such as isolated colonic loops performed at the time of surgery, in patients undergoing major colonic resection (Duthie et al 1964 and Bown et al 1972).

A successful, novel in vivo technique to study intestinal transport has recently been developed and validated for use in animals, involving agarose gel cylinders inserted into the bowel lumen. Studies in rat descending colon have provided information regarding the relationship in vivo between hydraulic resistance offered by colonic luminal contents, rate of fluid absorption and tonicity of the fluid absorbate, as well as calculation of colonic dehydrating power (Zammit et al 1994 and Naftalin 1994).

My aims were,
- To adapt this novel in vivo method to study colonic transport and calculate colonic dehydrating power, in humans, specifically children.
To subsequently employ agarose gels to measure the effects of colonic inflammation on transport function and free radical production (chapter 9) in paediatric patients.

To combine agarose gel and colonic loop methodologies to study the effects and possible mechanisms of actions of specific inflammatory mediators on colonic transport in rats (chapter 6).

4.2 In vivo method 1 - agarose gels

4.2.1 Principles

Agarose is a gelling agent of high strength, physical stability and biological inertness. The suitability of agarose gel cylinders as a tool to measure intestinal transport in vivo arises from its ability to mimic certain properties of faeces. The specific composition of faeces, similar to fine clay, imposes an exponential relationship between the force required to compress water out of faeces (dehydration) and faecal steady state water content (McKie et al 1990). In physiological terms initial stool dehydration to reduce stool water content from 95% to 80% (ratio of water:solids falls from 20:1 to 4:1), is relatively easy and achieved by mechanical force from colonic smooth muscle contraction. Hydraulic resistance then imposed by the increasingly solid faecal mass rises, with further reduction to <68% faecal water content (from 4:1 to 2:1) being much harder and requiring ~1000kPa (~10 atmospheres). This amount of pressure cannot be supplied by mechanical force and requires the generation of an osmotic pressure force (chapter 1 section 1.4.4.1). Agarose gels inserted into the gut lumen can mimic this process of faecal dehydration with a decrease in water:solids ratio within the gel producing an exponential rise in hydraulic resistance exerted by the gel. Varying the % agarose composition of a gel can serve to mimic different in vivo conditions. For example low % gels (2.5%) mimic liquid faeces, such as faecal contents of the caecum. The ratio of water:solids is high and hydraulic resistance offered low, so permitting high rates of Na\(^+\) and water absorption. Decreasing the water:solids ratio, \( \geq 10\% \) gels, increases the resistance to dehydration offered by the gel. An osmotic pressure force
would be required to provide the necessary suction force to overcome this resistance.

4.2.2 Gel production

For human studies 5% agarose gels, of known hydraulic conductivity, were made using 5g agarose type II (Sigma Chemical Co.) dissolved in 100ml of a vehicle solution containing 140mM NaCl and 5mM KCl. This solution was heated under pressure for ten minutes, poured into prepared moulds of uniform internal diameter (1.6cm) and left to solidify (<40°C). A length of string was included in the centre of the mould around which the liquid gel was poured to aid gel removal from the rectum. Cooled gels were expressed from their moulds and cut into 5cm lengths. Final gel specifications measured 5cm length (l) x 1.6cm diameter (d) (figure 4,1). Gels used in rats were made as for humans but cast into smaller moulds and omitting the string core. Final gel specifications measured 1cm (l) x 0.6cm (d).

![Figure 4.1 Agarose gel used in human in vivo intestinal transport studies. The gel is composed of 5% agarose in a vehicle solution (140mM Na⁺ and 5mM K⁺), with string core used to aid gel removal.](image-url)
4.2.3 Measurement of fluid and electrolyte changes

Fluid transport was measured by gravimetric means. Gels were weighed pre and post insertion and rate of fluid transport calculated as change in gel weight, expressed in microlitres per square centimetre of gel per hour of exposure. Electrolyte transport was measured by flame photometry. Na$^+$ and K$^+$ were first extracted overnight at room temperature from homogenised gels placed in 0.1M HCl and then measured using a flame photometer (Instrumentation Laboratories (UK) Ltd 943). Control gels were analysed to give basal Na$^+/K^+$ values for each separate batch of gels made. Rate of Na$^+/K^+$ transport was calculated as change in gel Na$^+/K^+$ content compared to controls, expressed in micromoles per square centimetre of gel per hour of exposure.

4.2.4 Validation of gels

4.2.4.1 Gel concentration

5% gels were chosen for use in human and rat experiments. 2.5% gels were not firm enough to facilitate easy insertion into the rectum without disintegration. >10% produced firm usable gels, however the reduction in water:solids ratio would increase the hydraulic resistance exerted by these gels so influencing rates of fluid and electrolyte transport (figure 4.3).

4.2.4.2 Gel dimensions

For human studies 1.6cm diameter is sufficient to allow the gel to pass through the anal sphincter with ease, based on endoscope dimensions. Gels for use in rat experiments, 1cm (l) x 0.6cm (d), were based on previous animal studies (Zammit et al 1994).

4.2.4.3 Hydraulic conductivity

Gel hydraulic conductivity ($L_p$) is required to calculate suction pressure on the gel and power output of the colon. $L_p$ was calculated by measuring pressure-induced water flow through the gel. Discs of agarose gel, 1.6cm diameter, were placed on top of a disc of sintered glass and filter paper, contained within a 10ml syringe, (diameter 1.6cm). The remainder of the
syringe was filled with water, sealed and a constant pressure applied (either 280 or 482 cmH₂O) monitored by a mercury manometer (figure 4.2).

Figure 4.2 Measurement of the hydraulic conductivity of agarose gel.

Fluid flow was determined by weighing the eluant from the syringe after periods of sustained pressure, varying from 50 minutes to 19 hours. Lₚ was calculated as the volume of eluant collected expressed in centimetres per second per centimetre water. The effects of gel concentration 5 - 12.5%, at constant thickness (2mm), and gel thickness 1 - 7mm at constant concentration (5%), on Lₚ were measured (figure 4.3).
Figure 4.3 Hydraulic conductivity of agarose gel as a function of gel (A) concentration and (B) thickness using 5% gels. Results represent mean ± standard error, (n = 3).
L_p decreased as a function of increasing agarose concentration (5%-12.5%) at constant thickness. L_p fell from $1 \times 10^{-8}$ to $8.4 \times 10^{-8}$ cm s^{-1} H_2O. L_p also decreased as a function of increasing gel thickness (1-7mm) at a constant concentration. L_p fell from $1.6 \times 10^{-8}$ to $1.7 \times 10^{-7}$ cm s^{-1} H_2O. From these experiments L_p values can be used to calculate colonic suction pressure exerted by the gels and ultimately estimate rectal suction power (Zammit et al 1994).

4.2.4.4 Measurement of electrolytes

The use of flame photometry as a tool to measure Na^+ and K^+ was assessed by measuring standard solutions containing known Na^+ (0 -140mM) and K^+ (0 - 5mM) concentrations. Measured Na^+/K^+ concentrations were plotted against known theoretical values and degree of correlation assessed using regression line analysis and Pearson's correlation coefficient (r) (figure 4.4). The method of gel electrolyte extraction, using either 0.1MHCI or deionised water as the extraction medium, was assessed by varying Na^+ (0 - 140mM) and K^+ (0 - 5mM) concentrations of the vehicle solution from which 5% agarose gels were made. Measured Na^+/K^+ concentrations were plotted against known theoretical values and the degree of correlation assessed by regression line analysis and Pearson's correlation coefficient (figure 4.5).

A positive correlation was found between Na^+ and K^+ concentrations measured in both standard solutions and extracted from agarose gels (r = 0.99). No difference was found between using water or HCl as the extraction medium (Na^+; HCl, r = 0.99 and water r = 0.97 and K^+; r = 0.95 for HCl and water). Electrolyte extraction by the method described (section 4.2.3) and measurement by flame photometry were therefore used to measure electrolyte transport in all gels studies.
Figure 4.4 Accuracy of flame photometry as a tool for the measurement of (A) Na⁺ (0 - 140mM) and (B) K⁺ (0 - 5mM) in deionised water. Data points at each individual concentration represent mean of 3 readings ± standard error.
Figure 4,5 Accuracy of electrolyte extraction from agarose gel cylinders measured by flame photometry. Na⁺ (0 - 140mM) and K⁺ (0 - 5mM) were extracted using either 0.1M HCl or deionised water. Data points at each individual concentration represent mean of 3 readings ± standard error.
4.2.5 Animal studies

4.2.5.1 Method

Male Wistar rats (200-250g body weight), were anaesthetised (sodium pentobarbitone, 60mg kg\(^{-1}\) body weight I.P.), a midline incision made into the peritoneal cavity and descending colon located. A small incision was made at the distal end of the descending colon through which the luminal contents were flushed out with 0.9% saline. Four pre-weighed 5% agarose gels measuring 1cm (l) x 0.6cm (d) were inserted into the colon and silk ligands applied proximal to the first and distal to the fourth gels ensuring vascular supply to the isolated loop remained intact. Abdominal contents were then replaced into the peritoneal cavity, the incision covered with a plastic sheet to prevent dehydration and the rats placed under a lamp to prevent hypothermia. After 90 minutes the gels were removed, weighed and electrolytes measured by flame photometry (section 4.2.3). Results of basal fluid and electrolyte transport are expressed as mean ± standard error with negative values representing absorption and positive secretion (figures 4, 6 – 4, 8). Rats were killed at the end of the experiment by thoracotomy.

Figure 4,6 In vivo fluid transport in rat descending colon, measured using agarose gel cylinders. Mean ± standard error (n = 6), with negative values representing absorption.
Figure 4,7 In vivo Na⁺ transport in rat descending colon, measured using agarose gel cylinders. (A) Gel Na⁺ concentration as % loss from control gel. (B) Mean rate of Na⁺ absorption. (C) Correlation between Na⁺ and fluid transport, (r = 0.9). Data in A & B represents mean ± standard error (n = 6) and in C raw data.
Figure 4.8 In vivo K⁺ transport in descending rat colon, measured using agarose gel cylinders. (A) Gel K⁺ concentration as % of control gel. (B) Mean rate of K⁺ transport (n = 6).

4.2.5.2 Results

Na⁺ and fluid absorption was measured in rat descending colon using agarose gels (figures 4,6 – 4,8). A positive correlation was found between Na⁺ and fluid transport, ($r^2 = 0.90$, $P<0.01$), with a 75% reduction in gel Na⁺ resulting in fluid absorption at a rate of >50μl cm⁻² hr⁻¹, recorded in the most proximal gel. K⁺ was also absorbed but at much lower rates, 0.1 – 0.6μmol cm⁻² hr⁻¹. These findings are discussed in section 4.4.

4.2.6 Human studies

4.2.6.1 Method

To study in vivo fluid and electrolyte transport in children, 5% agarose gel cylinders measuring 5cm (l) x 1.6cm (d) were used. In order to allow fluid
transport to occur the experimental duration was based on previous in vivo human transport studies using dialysis bags (Edmonds 1971) and set at a minimum of one hour and, in the patients studied, ranged from one to four hours. Following routine colonoscopy one pre-weighed agarose gel was manually inserted into the rectum. Minimal discomfort was experienced as the effects of sedation were still present. The gel was removed using the string if not passed naturally by the patient. Gel weight, including string was recorded and string removed. The difference between wet and dry string weight, dried overnight at 37°C, was subtracted from the initial gel weight post removal. Fluid and electrolyte transport was calculated as previously described (section 4.2.3). A total of six patients were studied, however in two patients the gels were naturally expelled prematurely in less than the minimum experimental duration of one hour and therefore were discarded. Of the four remaining patients experimental and clinical details are shown (table 4.1) and rectal transport presented as raw data values from individual patients (figures 4.9 – 4.11). Electrolyte transport is presented as gel electrolyte concentration expressed as % loss/gain from control gel values ('control' gel representing a 5% gel analysed from the same batch of experimental gels but not used in patient studies), and as rate of electrolyte transport (μmol cm⁻² hr⁻¹). Negative values indicate rectal fluid/electrolyte absorption, with loss in gel weight/electrolytes, and positive indicates rectal secretion, gain in gel weight/electrolytes. Ethical approval and informed consent for each case was obtained.
Table 4.1 Clinical details and results of the four patients, in which in vivo rectal transport was measured, using agarose gels. A = absorption, S = secretion.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Presenting symptoms</th>
<th>Time gel in vivo (hr)</th>
<th>Transport summary Fluid</th>
<th>Na⁺</th>
<th>K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1.5</td>
<td>Diarrhoea, food allergy</td>
<td>1</td>
<td>S</td>
<td>A</td>
<td>S</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>Normal</td>
<td>2</td>
<td>nil</td>
<td>A</td>
<td>S</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>Abdominal pain, diarrhoea</td>
<td>3.5</td>
<td>A</td>
<td>A</td>
<td>S</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>Intermittent constipation, diarrhoea</td>
<td>4</td>
<td>S</td>
<td>A</td>
<td>S</td>
</tr>
</tbody>
</table>

Figure 4.9 In vivo rectal fluid transport in four paediatric patients, measured using agarose gel cylinders. Results expressed as raw data of the individual patients studied with negative values representing fluid absorption and positive secretion.
Figure 4.10 In vivo rectal Na⁺ transport in four paediatric patients, measured using agarose gel cylinders. (A) Gel Na⁺ concentration as % loss from control gel. (B) Rate of Na⁺ absorption. (C) Correlation between Na⁺ and fluid absorption. Results expressed as mean ± standard error of four flame photometry readings per gel.
Figure 4.11 In vivo rectal $K^+$ transport in four paediatric patients, measured using agarose gel cylinders. (A) Gel $K^+$ concentration as % loss from control value. (B) Rate of $K^+$ secretion. Results expressed as mean ± standard error of four flame photometry readings per gel.

4.2.6.2 Results

The measurement of in vivo rectal fluid transport using agarose gels produced varied results in the four patients studied (figure 4.9 – 4.11). Rectal absorption was recorded in patient 3 only, at a rate of $0.24 \mu l \ cm^{-2} \ hr^{-1}$. This patient presented with intermittent constipation and diarrhoea. Routine histology from all colonic biopsies taken including rectal, showed normal crypt architecture with an excess of eosinophils infiltrating the lamina propria, 760 per ten high powered fields. Rectal secretion was recorded in patients 1 and 4 ($1.5 \mu l \ cm^{-2} \ hr^{-1}$ and $0.41 \mu l \ cm^{-2} \ hr^{-1}$ respectively). Patient 1 presented with diarrhoea, with no histological abnormalities. Patient 4 presented with intermittent constipation and diarrhoea, also with no histological abnormalities and no signs of active
inflammation. In patient 2 no rectal fluid transport was detected. Na⁺ absorption was measured in all four patients but no correlation was found with fluid transport, with the highest rates of Na⁺ absorption recorded (16.6µmol cm⁻² hr⁻¹) in the face of minimal fluid absorption (patient 3, figure 4,10). K⁺ secretion was detected in all four patients. These findings are discussed in section 4.4.1.

4.3 In vivo method 2 - closed colonic loops.
Isolated intestinal loops are an established method of measuring fluid transport by gravimetric means. Animal studies, using small and large intestinal loops, have studied the effects of serosal and mucosal agents such as toxins (Serebro et al 1969) and secretagogues (Charney & Dansky 1990) on intestinal transport and the involvement of the enteric nervous system as a controlling factor (Jodal et al 1993 and Nzegwu & Levin 1996). Due to the invasiveness of this technique the few studies performed in human subjects have been limited to patients undergoing major intestinal surgery (Duthie et al 1964 and Bown et al 1972).

Experiments were performed in rat descending colon to compare basal in vivo fluid transport values measured using closed colonic loops with those obtained using agarose gel cylinders. The effects and possible mechanisms of action of specific inflammatory mediators were also studied using the same methodologies (data presented in chapter 6 section 6.4.4).

4.3.1 Method and results
Fluid transport was measured gravimetrically in ligated loops of descending colon in anaesthetised (sodium pentobarbitone, 60mg kg⁻¹ body weight I.P.), male Wistar rats (200-250g body weight). After removal of faecal gut contents within the loop by rinsing with 0.9% saline, loops were instilled with 1 ml 0.9% saline. After 90 minutes loops were removed, weighed full and empty and surface area noted. Rats were killed at the end of the experiment by thoracotomy. Results are shown in figure 4,12. Using this methodology
basal fluid transport was also found to be absorptive in nature occurring at a
mean rate of $14.97 \pm 3.47 \mu l \ cm^{-2} \ hr^{-1}$.

Figure 4.12 Basal in vivo fluid transport in rat descending colon measured
using closed colonic loops. Data expressed as mean $\pm$ standard error, ($n = 6$)
with negative values indicating absorption.

4.4 Discussion
4.4.1 Agarose gels as a tool to measure colonic transport in vivo
Agarose gels have been successfully used as a novel tool to measure in vivo
intestinal transport in animals (Zammit et al 1994 and Naftalin 1994). I
have also demonstrated that basal in vivo fluid and Na$^+$ transport in rat
descending colon is absorptive in nature and can be relatively easily
measured using this novel methodology (figures 4,6 & 4,7). The
effectiveness and limitations of adapting agarose gels for use in human intestinal transport studies are discussed below.

4.4.1.1 Practical limitations
In vivo human intestinal transport studies using agarose gels were limited to the study of rectal transport as manual gel insertion could only be easily performed, incurring minimal patient discomfort, into the rectum. Following insertion gel retention in two patients proved difficult with the gel being naturally expelled before the minimum experimental duration (1 hour). The presence of air in the rectum introduced during the course of the colonoscopy may contribute to this difficulty. Aesthetic reasons, such as gel size and appearance, were in some cases a deterrent to recruiting patient or parental consent.

4.4.1.2 Experimental results
In the four patients studied rectal fluid transport, measured as a change in gel weight, was detectable. However the maximum transport rate recorded was 1.5µl cm\(^{-2}\) hr\(^{-1}\) (fluid secretion), with absorption detected in only one patient at 0.24µl cm\(^{-2}\) hr\(^{-1}\). Average rectal water absorption rates measured in normal adults using dialysis bags have been reported to range from 14.4µl cm\(^{-2}\) hr\(^{-1}\) when luminal Na\(^+\) was high (145m-equiv/l), to 0µl cm\(^{-2}\) hr\(^{-1}\) when luminal Na\(^+\) was low (30m-equiv/l, Edmonds 1971), with variation between patients of SD ± 25%. The lack of significant gel dehydration and therefore recordable rectal absorption may have been attributable to a number of factors including low rectal suction power, arising from a lack of direct mucosal:gel contact, combined with the hydraulic resistance offered by the gel. The presence of faeces or air in the rectum would add to poor mucosal:gel contact. Previous existing transport studies have been on adults therefore patient age (mean = 5.8 years) could also be a factor.

Na\(^+\) absorption was detected in all four patients but showed no correlation to water movement even after 4 hours in vivo, with the highest rate of Na\(^+\) absorption occurring with minimal accompanying fluid absorption (patient 3,
These results suggest that the resistance to fluid movement (hydraulic resistance, $L_p$) exerted by the 5% agarose gels was greater than the osmotic force generated across the rectal epithelium, effectively preventing the paracellular osmotic movement of water from the gels through the tight junctions. Almost zero fluid transport in the face of significant net $Na^+$ absorption has also been documented in rat gel studies using high gel concentrations (12.5 and 15% gels, Zammit et al 1994 and Naftalin 1994). In these situations the ratio of $Na^+$:water absorbed would be high resulting in the generation of a high absorbate tonicity in the intercellular space and therefore high osmotic pressure (chapter 1 section 1.2.3.1). In the rectum generation of a hypertonic absorbate is necessary to overcome the resistance to water movement and faecal dehydration offered by the already solid luminal contents and so enable a final reduction to the 68% water content of dehydrated formed stools.

$K^+$ secretion, seen as an increase in gel $K^+$ concentration compared to controls, was detected in all four patients. These results however should be interpreted with caution, especially the higher values, as gel contamination with blood or faeces was common and unavoidable. As both of these are high in $K^+$ the high levels measured in the gels may not reflect true $K^+$ transport flux.

From the patients studied it can be concluded that agarose gels lack the sensitivity required to detect normal rectal fluid changes in young children. Due to a combination of the lack of sensitivity and practical difficulties experienced, such as gel retention and patient comfort, the use of this novel in vivo method to measure rectal function was not continued in human subjects. Calculation of rectal power output was not possible due to the lack of measurable transport detected using the gels.

### 4.4.2 Gels vs. colonic loops to measure colonic transport in vivo

Basal fluid and $Na^+$ absorption were relatively easily measured in rat descending colon using agarose gels with excellent correlation recorded
between fluid and Na\(^+\) transport rates \((r^2 = 0.9, P<0.01)\). Results were comparable with previous studies performed in rats (Naftalin 1994). Detection of K\(^+\) transport produced less reliable results, reflected in the large standard errors recorded. Blood contamination was often a problem and may partially account for these variations. Basal fluid transport in vivo, measured using closed colonic loops in rat descending colon, was also absorptive in nature.

Comparison of the two methodologies revealed significant differences in basal absorption rates. Rates using colonic loops were lower than those measured in proximal agarose gels (gel 1, \(P<0.0001\) and gel 2 \(P<0.001\)) yet higher than distal gel 4 (\(P<0.006\)). The use of 1cm-gel lengths enabled the specific transport function relating to a 1cm segment of descending colon only to be recorded. As a result a graded absorption profile from the 4cm segment of descending colon was detected (fluid absorption from 56.3 \(\mu\)l cm\(^{-2}\) hr\(^{-1}\) proximal to 1.6\(\mu\)l cm\(^{-2}\) hr\(^{-1}\) most distal). This may reflect regional variations in colonic transport within a distance as little as 1cm. In isolated colonic loops functional fluid absorption rates represented the mean of 3 to 4 cm of descending colon. Any variations therefore in the regional absorptive capacity of the mucosa even within a few centimetres would be masked so accounting for the overall lower mean absorption rate. However, in the gel experiments performed the effects of mucosal trauma on fluid transport rates especially in the most distal regions, due to the repeated insertion of four gels, can not be entirely eliminated. Experiments in which one 1cm gel only per experiment was inserted would reduce this trauma and be necessary to determine the true nature of the graded absorption recorded. Comparison of transport rates from specific 1cm regions could then be more accurately studied.

On the basis of the results presented from these in vivo transport studies the study of functional changes in the human colon induced by inflammation were performed using in vitro methods only (described in chapter 5). Agarose gels and closed colonic loops were limited to use in rats only and
used to study the effects of specific inflammatory mediators on functional fluid transport (chapter 6 sections 6.4.3 [gels] and 6.4.4 [loops]) and free radical production (chapter 9 section 9.2.2 [gels]) in vivo in the non-inflamed colon.
CHAPTER 5

5.1 Aims
My primary aim was to perform comparative studies of human intestinal transport in vitro in control and inflamed colon using endoscopic biopsy material. Ussing chamber studies have provided some human intestinal transport data in children from surgical resection specimens (Jenkins & Milla 1993 and Hardy et al 1993). However such material is limited in supply and usually severely inflamed. Endoscopic biopsies are more freely available but the small size of the tissue presents a physical problem for use with Ussing chambers. I have adapted a novel method involving confocal microscopy, first developed using rat colon (Pedley & Naftalin 1993), to study crypt transport in endoscopic biopsies from human colon. This method measures fluid absorption and secretion in human colonic biopsies by visualising the concentration of an impermeant probe within colonic crypts in a "whole" tissue biopsy using the laser scanning confocal microscope.

This chapter outlines the principles of confocal microscopy and how it can be applied to study fluid transport in human endoscopic colonic biopsies.

5.2 Principals of confocal microscopy
The optical limitations of conventional epifluorescence microscopy restrict its use to in vitro studies of fixed or frozen tissue sections no more than 5 - 10 microns (μm) depth. This limitation arises because the image viewed is the sum of a sharp in-focus region plus blurred images from tissue structures outside the plane of focus. The greater the section depth the more out-of-focus glare occurs rendering the final image useless. This
limitation however, can also occur when viewing sections of only 5μm depth in conjunction with high numerical aperture (NA) objectives. The depth of field using these lenses is restricted to approximately 1μm. Therefore even in 5μm tissue sections the majority of detected light will arise from the out-of-focus regions, resulting in an intense blurred background on which is superimposed the much weaker in-focus image (figure 5,1).

Figure 5,1 Schematic illustration of the operating principles of confocal and conventional wide-field microscopes. Light rays emerging from 3 points in the specimen are shown. Points 1 & 2 emanate from the focal plane, point 1 centrally located (red), point 2 off-axis (blue). Point 3 is on-axis but located below the plane of focus (green). A). Wide-field - specimen is illuminated over an extended region by a light source and condenser. The detector forms an image of the sum of all simultaneously arriving light rays therefore including point 3 which gives only a blurred out-of-focus image on hitting the detector. B). Confocal - 2 pinhole apertures have been introduced. Pinhole 1 restricts illumination to a focused point within the specimen. Pinhole 2 permits only focused light rays from the on-axis in-focus point of the specimen to reach the detector. Therefore only focused light rays emanating from the one focused point in the focal plane fall on the detector (Murray 1992).
The development of the laser scanning confocal microscope (LSCM) by Marvin Minsky in 1955 revolutionised the microscopic study of both fixed and 'living' tissues (Minsky 1988). The confocal imaging geometry provides dramatic optical advantages for fluorescence microscopy. By sectioning tissue optically as opposed to physically, it is possible to discriminate against the out-of-focus background with minimal loss of the image-forming signal. This results in focused, clear, usable images even from specimens of several millimetres depth (Murray 1992). This single property provides an amazing key to open up endless possibilities. For example optical serial sections allow the distribution of structural components throughout tissues or cells to be mapped, possible conventionally only by physically producing literally hundreds of thin serial sections. Optical serial sections can subsequently be combined to produce impressive 3D views at very high resolutions. 'Live' tissues maintained in vitro and cellular dynamics can be studied allowing recording of real-time physiological events. In addition specimens can be viewed in different planes, tilted to and even parallel to the line of sight and clear focused images can be obtained even from deep and light-scattering tissues. This dramatic increase in resolution is achieved by physical means with illumination and detection being confined to a single, diffraction-limited point in the specimen (Murray 1992, figure 5,1).

The first so-called 'pinhole' barrier is placed in the primary image plane to produce a single point of tightly focused laser illumination (figures 5,1 & 5,2). This point is then scanned across the specimen to build up a precise point-to-point image of the optical section within the specimen. The second pinhole barrier lies directly in front of the detector, a photomultiplier tube (PMT), conjugate with the field of pinhole one. This physically restricts detection of emitted light to that arising only from the focal region within the tissue. The laser light falling on the PMT produces a current, which is then amplified and passed directly to the analogue-to-digital converter (ADC). The ADC samples the voltage for a few nanoseconds during the time of each pixel and turns the sensed voltage into a digital number to produce a
Figure 5.2 Complete confocal set-up. Light path - laser light is reflected from a dichroic to a beam scanning system that raster a focused illumination spot over the object plane. Generated fluorescence is collected by an objective lens and directed by scanning and relay mirrors to a detector aperture of radius $r_0$ (pinhole radius). Light that passes through the detector aperture is detected by a photomultiplier tube, and used to form a pixelated image.
final digital, pixelated image (section 5.4.1.1.1). Once in this form the image can be permanently stored in the computer memory for subsequent processing and analysis (Paddock 1991, Fine et al 1988 and Sheppard & Shotton 1997). The plane of focus (z-plane) is selected by a computer-controlled fine-stepper motor which raises and lowers the microscope stage in increments as small as 0.1μm. In addition to single option sections, serial sections scanning down the z-plane can be recorded and subsequently reconstructed into 3D images.

The single main key to the success of this confocal approach is the virtual elimination of glare from out-of-focus structures resulting in increased lateral and axial image resolution (figure 5.1). This is the most powerful advantage of the confocal microscope for imaging thick specimens. It is these optical properties that have made possible the microscopic study of in vitro colonic fluid transport in children using whole endoscopic colonic biopsies. The methodology pertaining to these studies is described below (section 5.3.1).

5.3 Visualisation and measurement of crypt fluid transport in vitro
In conjunction with the LSCM, convective fluid inflow into colonic crypts as a consequence of fluid absorption within the crypts may be estimated by visualising the concentration-polarisation of an impermeant fluorescent dye such as fluorescein isothiocyanate dextran (FD) (Pedley & Naftalin 1993 and Naftalin et al 1995). Fluorescein isothiocyanate (FITC) is a xanthene dye that brightly fluoresces due to its reasonably large extinction coefficient and high fluorescence quantum yield. Conjugates of FITC to a variety of other substances can be easily prepared due to its water solubility. Conjugation of FITC to dextran (FD), yields a high molecular weight (10,000 and 42,000 used), water-soluble, impermeant fluorescent marker that is not readily absorbed across colonic epithelium.
Naftalin (1994) reported that when a sheet of rat colonic mucosa, separated from the underlying muscle, is incubated in a physiological salt solution containing FD, the fluorescent-labelled fluid readily enters the blind ending crypt lumina via diffusion or a convective stream. Water and electrolytes are subsequently absorbed from the crypt across the epithelium with the impermeant FD remaining within the crypt lumen so becoming increasingly concentrated over time. Conversely if fluid is secreted by the tissue into the crypt lumina, luminal FD becomes diluted. These changes in FD concentration can be directly visualised as an increase (fluid absorption) or decrease (fluid secretion) in fluorescence intensity detected using a LSCM (Pedley & Naftalin 1993 and Naftalin et al 1995). Changes in fluorescence intensity can then be quantified offline using image analysis software.

**5.3.1 Method**

Human endoscopic colonic biopsies were carefully removed from biopsy forceps (4mm) and transported in pre-oxygenated Tyrode's salt solution (Sigma Chemicals Co. containing (mM): CaCl_2·2H_2O 1.8, MgCl_2·6H_2O 1.0, KCl 2.7, NaHCO_3 11.9, NaCl 136.9, Na_2HPO_4 0.4, D-Glucose 5.5, pH 7.3 and osmolality mOsm/Kg H_2O 296±5%). One biopsy, measuring approximately 0.7 x 0.3cm, was orientated serosal side uppermost on a glass coverslip using a dissecting microscope. A minute amount of adhesive (superglue) delivered via a capillary tube to the ends of the tissue only, secured the biopsy to the coverslip. This was then inverted and carefully placed onto a perfusion chamber, pre-filled with oxygenated Tyrode's solution, to form an enclosed perfusion system. (Commercial chamber - Clark Electromedical Instruments, Reading, UK Series 20 chamber model RC-21B with heated platform, PH-1). The mucosal surface of the biopsy was now uppermost for direct viewing. A watertight seal between the chamber and coverslip was achieved by pre-applying vacuum grease (Dow Corning High Vacuum grease) to the chamber surface. Heating elements, perfusion and suction tubing were attached to the chamber which was continually perfused with gassed (95% O_2, 5% CO_2) Tyrode's salt solution and maintained at 35°C throughout the duration of all
‘live’ experiments. To measure crypt transport the perifusion solution was then changed to pre-oxygenated Tyrode’s salt solution containing 0.3mM FD. For experiments in which different perifusion solutions were required a multiport perfusion manifold was used in order to prevent any minute movement of the chamber and field of view arising from manually changing the infusion tube, (Clark Electromedical Instruments, Reading, UK manifold number MP-4, with 4 inputs to 1 output).

5.3.2 Microscopy
Within ten minutes of collection the biopsy was mounted in the chamber and ready for viewing using an upright Laser Scanning Confocal Microscope (Leica Beds, UK) with x10 (NA 0.45, NPL Fluotar) or x25 (NA 0.75 NPL Fluotar) oil immersion objectives. The tissue was excited using a Krypton/Argon laser with excitation and barrier filters set for individual fluorophores according to their specific excitation/emission spectra. The emitted light was detected by the PMT tube and converted, via the analogue-to-digital converter, into a digital pixelated image. The detection pinhole was set for use with x10 or x25 objectives accordingly. Offset and gain settings were determined at the start of each experiment and kept constant throughout, with laser power recorded each time (section 5.4.1). Z-axis movement was determined by software controlled stepper motor attached to the focusing control of the microscope. In addition to information from a single optical plane sequential sections along the z-axis of crypt lumina could be recorded to provide a profile of upper and middle crypts regions.

5.3.3 Image analysis
Figure 5.3 shows a typical confocal image of a human colonic biopsy incubated in FD. All images were analysed offline using Sigma Scan (Jandel Scientific Software), and NIH Image (a public domain program written by Wayne Rasband - National Institutes of Health, USA, available from the Internet ftp:\zippy.nimh.nih.gov). In all experiments luminal and adjacent pericryptal fluorescence intensity was measured and background
fluorescence subtracted. Luminal areas were determined by manually defining an area, from 50-200 pixels depending on crypt lumen diameter. Background fluorescence was taken as an area containing pixels of the lowest grey scale values (section 5.4.1.1.1) within a series of images. Data is expressed as either average intensity of each individual crypt lumen or mean ± standard error of 4-8 crypt lumina depending on crypt density per field of view. An increase in fluorescence intensity represents concentration of FD and a decrease represents dilution.

Figure 5.3 Typical confocal image of human colon incubated in Tyrode's salt solution containing FITC-dextran (0.3mM). Mag. x100.
5.4 Validation of methodology

5.4.1 Obtaining an accurate final image – confocal parameters

The accuracy, sensitivity and precision of fluorescence distribution measurements, and therefore of the final image formed, are limited by,

(i) fluorescence detection and collection efficiencies
(ii) fluorophore photochemistry

Factors affecting these parameters image are discussed below (Webb & Dorey 1995 and Ploem 1989).

5.4.1.1 Fluorescence detection and collection efficiencies

5.4.1.1.1 The pixelated image

The initial analogue confocal image is converted into a digital image via the ADC. During digitalisation the image is subdivided into ≥512 units each made of ≥512 picture elements (pixels) to produce the final image in a pixelated form. A pixel is the smallest area of the image that can be represented as distinct from its neighbours. Each pixel has an optical density or brightness that can be accurately represented by a single number, known as its grey level. Grey levels range from 0, representing black, to 256, representing bright white. This 0-256 ‘grey’ scale can also be represented with colours by applying an artificial colour scale (look up table). To obtain the most accurate information from a digital image the full range of grey levels should be used, as determined by the offset, in conjunction with maximum visual contrast, set by the gain control (see below).

5.4.1.1.2 Offset

Offset affects the voltage applied to the emitted signal and defines the low intensity (black) end of the grey scale range. The black level of the PMT amplifier should be set so that the full range of signal can be detected. This is determined at the start of each experiment by scanning while the light is blocked from entering the PMT. The black level is then adjusted using the offset until the scanned area, displayed on the VDU screen, becomes just visible compared to an unscanned area. If the offset is set too high some
intensity values may fall below the voltage corresponding to the zero value of the ADC and therefore will not be recorded. In this situation vital information can be lost and care is needed to utilise the whole range of grey levels in the unprocessed image.

### 5.4.1.1.3 Gain

Practically speaking gain determines image brightness. This is achieved as gain adjusts the accelerating voltage of the PMT so controlling the amplitude of the laser signal. In a fixed tissue, in which fluorescence intensity remains constant, a properly adjusted gain setting should produce bright white pixels in areas of greatest fluorescence intensity in order to utilise the full grey scale range of the data handling system. Care must be taken however, not to saturate the ADC.

### 5.4.1.1.4 Pinhole size

The confocal detector pinhole resides in an image plane of the microscope in front of the photodetector. As a result any point object at the in-focus plane will be focused at the pinhole plane. Light, emitted or scattered into the objective from other planes, will be out of focus on reaching this pinhole and therefore will not pass through to the detector (pinhole 2, figure 5.1). The extent to which this stray light is excluded will determine the characteristics of the single-plane image produced, hence the term ‘optical sectioning’. The diameter of the pinhole aperture is variable, 20 - 400μm, controlling the amount of light that falls on the PMT. Detector radius is an important factor in determining both signal strength (sensitivity), spatial resolution and level of background rejection. Several factors influence the selection of an optimum size (Centonze & Pawley 1995 and Wilson 1995),

(i) strength of signal from the specimen
(ii) noise level in the system
(iii) time available for signal accumulation
(iv) specimen thickness
(v) amount of axial and transverse resolution that can be tolerated.
A small pinhole will improve xy resolution, accepting light from the focal plane only, at the cost of reducing signal strength. As the pinhole is made larger extra light is accepted including that originating from above or below the focal plane effectively reducing the optical sectioning effect of the system. Increased signal strength is therefore obtained at the expense of resolution. For most specimens (with antifade agents) the best compromise between sensitivity and spatial resolution is found by setting the aperture equal to the diameter at which the Airy disc (radius determined by the NA of the lens) reaches 50% of its peak intensity. At this setting 80% of the light from the plane of focus will reach the detector with xy resolution approximately 20% better than that produced by the same optics used in a nonconfocal manner. In experiments performed to measure crypt transport (chapter 6) detection of gross changes in fluorescence intensity within defined regions as opposed to resolution of fine details was the requirement. Therefore signal strength was the main determining factor in setting pinhole size.

5.4.1.1.5 Experimental protocol
At the outset of each experiment optimal pinhole aperture, offset, gain and laser power settings were determined and kept constant throughout the experimental duration.

Optimal pinhole size was defined by the objective magnification used and set according to theoretical calculations, at 40µm for x10 and 60µm for x25. Offset - to control grey scale applied to the PMT, was determined by scanning while blocking the laser light from entering the PMT and adjusting the gain control until the black level in the scanned area displayed on the VDU became just visible compared to an unscanned area.

Gain - to determine image brightness, was dependent on the nature of the experiment. Where fluorescence intensity was constant or at steady state, gain was set at the level at which areas of brightest intensity were 'white', (high end of the grey scale) but avoiding complete saturation at 256. Experiments to measure increases in fluorescence intensity over time, gain
was initially set at the lowest possible level at which areas of brightest intensity were just visible and kept constant thereafter. *Laser illumination setting* – a low laser setting was used for all experiments. During real-time ‘live’ experiments, where changes in fluorescence intensity over time were measured as an index of fluid transport, the initial 0-256 grey scale defined by the offset, was often exceeded rendering any further increases in intensity above this fixed grey scale unrecordable. This was overcome, in the particular Leica LSCM model used, by attenuating laser excitation power manually to produce a corresponding reduction in fluorescent intensity. Further increases could then be recorded. This was possible as a linear relationship exists between laser power, at lower levels, and fluorescence intensity, confirmed by a series of validation experiments (figure 5,4). For these experiments human colonic biopsies were incubated in Tyrode's containing 0.3mM FD. On reaching near steady state images were recorded under the following experimental conditions,

(i) variable laser power, constant gain  
(ii) 50% attenuation of laser power, constant gain  
(iii) variable gain, constant laser power  

Changes in fluorescence intensity from 5-7 random separate areas, approximately 50 - 100 pixels, within one field of view were analysed (figures 5,4 & 5,5). Data from these figures show an approximately linear relationship between fluorescence intensity and laser power over the limited range studied \(r^2 = 0.99, P<0.01\). 50% attenuation of laser power by manual means achieved a 45 ± 0.52% reduction in fluorescence intensity. When reduction in laser power was necessary intensity measurements analysed off line were corrected accordingly. Gain, once set at the lowest possible setting, remained constant, as linearity between gain and intensity was limited over the range tested (figure 5,5).
Figure 5.4 Relationship between fluorescence intensity and laser power measured in human colonic tissue incubated in FITC-dextran (0.3mM). (A) Fluorescence intensity over a range of laser settings, data represents raw intensity measurements of five separate areas from one field of view. (B) Attenuation of laser power by 50% reduces fluorescence intensity by 45%, mean ± standard error of five areas within one field of view, (n = 6).
5.4.1.2 Fluorophore photochemistry

Fluorescence is probably the most important optical readout mode in biological confocal microscopy owing to its increased sensitivity and specificity compared to absorbance or reflectance measurements. Fluorophores are compounds that absorb light and after a short interval re-emit it, a property termed fluorescence. The wavelength of emitted light is usually longer and of lower energy / weaker than that of the absorbed light, known as the Stokes Shift. Crucial limits on LSCM arise from two fundamental features of fluorophore photodynamics,

(i) photobleaching, limits total number of available fluorescence photons
(ii) fluorophore excited-state saturation, limits fluorescence emission rate and thus available imaging intensity.
The theory behind these features can be explained with the help of a Jablonski diagram, which depicts the energy level system of a typical fluorophore (figure 5,6, Sandison 1995 and Tsein & Waggoner 1995). In the absence of radiation, at room temperature the bulk of molecules reside in the ground state (GO) represented by a band of vibrational sublevels. Figure 5,6 depicts two energy “ladders”, singlet and triplet energy states, with triplet states each having a slightly lower energy than the corresponding singlet state.

Figure 5,6 Jablonski diagram depicting the energy level system of a typical chromophore. S0/G0, S1 and S2 represent singlet electronic energy levels; T1 and T2 are the corresponding triplet states. Vibrational sublevels are shown as distinct lines - heavy lines denote radiative transitions and light lines nonradiative interactions. Photon absorption excites ground state (S0/G0) molecules into S1 level. Molecules then relax either returning to G0 by fluorescence (Fl.) or undergoing intersystem crossing (Is.C.) entering T1. From T1 they may relax to G0, again by Is.C. or by phosphorescence (Ph.). Bleaching may occur from either T1 or S1. Abs. – absorption, I.C. – internal conversion.
Upon absorption of a photon, whose energy matches the difference between the G0 and an excited singlet state, a fluorophore will undergo an upward transition to a higher energy level - the excited state (S1). The molecule then relaxes very quickly (t~1-10nsecs) to the lowest vibrational level of S1 by a radiationless process. At this point there are several possible paths by which the molecule may give up energy and return to G0, termed de-excitation. The most desirable is that of fluorescence emission. Fluorescence, however competes with other decay processes including intersystem crossing and photobleaching (figure 5,6),

(i) **Intersystem crossing** (or delayed fluorescence), occurs when an excited electron goes into the adjacent triplet energy state (T1, T2), a process usually orders of magnitude slower than fluorescence emission. Molecules can become trapped in a long-lived T1 state (t~100nsec), so reducing the effective fluorophore concentration (fluorophore excited state saturation).

(ii) **Photobleaching** is a permanent, irreversible chemical change of a dye molecule to a non-fluorescent species. It results in a reduction in the emitted signal intensity therefore limiting the total amount of fluorescent information that may be obtained. Bleaching of excited state fluorophores may occur at S1 or T1.

Fluorophores can be characterised by three parameters
(i) excitation/emission spectrum
(ii) extinction coefficient - quantifies the absorption probability, usually 40,000 - 250,000M\(^{-1}\) cm\(^{-1}\)
(iii) quantum yield - quantifies how likely it is that absorbed light is emitted as fluorescence (determined by the ratio of emitted to absorbed energy). Biologically useful fluorescent molecules have a quantum yield of >0.1

5.4.1.2.1 Photophysical problems related to high-intensity excitation
Bleaching in fluorescence microscopy is an age long problem. In confocal microscopy bleaching often seems to occur at a much higher rate by comparison with conventional epifluorescence microscopy. The main
offending factors are laser illumination and the zoom magnification capacity. A simple lack of understanding as to the potential illuminating power that these two factors confer on the confocal system can often account for the apparent increased bleaching.

Laser illumination is a far more intense source than a mercury lamp (Hg-arc). The confocal system is such that each fluorescent molecule is subjected to brief but intense bursts of excitation as the scanning laser beam sweeps past. The beam dwells on each pixel for approximately 1-10μsec (Tsein & Waggoner 1995). Although the total power striking the specimen may be less in a confocal system, the area of the specimen over which the light is absorbed can be arbitrarily reduced by the zoom magnification, effectively increasing the power per unit area illuminated. The crucial difference therefore between the two microscopical systems is that, with normal epifluorescence the power per area on the specimen is fixed by Hg-arc and collection optics, while in confocal microscopy power per area can be operator controlled and increases with the square of the zoom of magnification. Thus, the extreme intensity of the laser combined with the zoom control makes possible what is impossible in a conventional set-up, namely to see an image that is both large enough to visualise the finest details and bright enough to view by eye - but only for the moment as bleaching occurs. In addition individual scan times maybe short but multiple serial scans to build up a 3D image can also increase the overall exposure.

In practical terms at photoexcitation intensities far below saturation, fluorescence emission rate is proportional to the illumination intensity. When using the zoom control only a few milliwatts (mW) of excitation power can be focused onto a submicron spot, effectively driving most of the fluorophores into the excited state. Fluorescence emission is then limited by the intrinsic fluorescent decay rate (~10^6-10^9 photons/second) and possible transition to the triplet state. It is possible, although in reality difficult, to predict the laser power at which a fluorophore will saturate. This requires an accurate knowledge of,
(i) laser power, in terms of number of photons in the excitation beam
(ii) fluorophore excitation coefficient at the wavelength of excitation
(iii) fluorophore excited-state lifetime

For example when fluorescein is excited by a beam $1.25 \times 10^{-24}$ photons cm$^{-2}$sec$^{-1}$ (produced by just 1mW power at the 488nm line of the argon-ion laser exciting a spot radius 0.25μm, using an objective of 1.25NA), 63% of fluorescein molecules would be in the excited state and only 37% in the G0. This information shows that the emission is nearly saturated and any further increase in excitation intensity could hardly increase the output (Tsien & Waggoner 1995).

The aim when planning experiments involving confocal microscopy, therefore is to optimise light collection efficiencies whilst preserving fluorophore photodynamics. The following parameters require consideration,

(i) laser excitation power
(ii) zoom magnification
(iii) numerical aperture of objective lens
(iv) specific fluorophore characteristics
(v) scanning time regarding relaxation / triple state decay

In the proposed confocal methodology to study human colonic crypt fluid transport in vitro changes in fluorescence intensity were used as an index of this physiological process. Therefore it was necessary to ensure those measured decreases or increases in fluorescence intensity were not attributable to fluorophore dynamics such as photobleaching, recovery from triplet state or saturation. In planning the experimental protocol several sources for potential photobleaching were identified.

**5.4.1.2.2 Proposed experimental protocol**

After mounting the biopsy within the chamber (section 5.3.1) perifusion of Tyrode's salt solution containing 0.3mM FD was commenced at time 0. Initially Hg-arc illumination was briefly used to locate the biopsy within the chamber, confirm correct orientation - mucosa uppermost, and determine
potential optimal scanning areas. Continuous low power laser scanning illumination, using x10 NA 0.45 oil immersion lens, was then used to finalise the field of view from which data would be subsequently obtained, determine scanning depth from mucosal crypt surface (40-60μm) and adjust gain setting to the lowest level at which areas of greatest intensity were just visible. No further excitation was performed until 10 minutes post commencing FD perifusion, after which images were collected at a scan rate of 1 scan per minute (min⁻¹) using line averaging x8, from one field of view only. An increased scan rate was necessary when recording the effects of added secretagogues on crypt transport.

Potential sources of photobleaching within this proposed protocol were identified as,

(i) initial periods of continuous Hg-arc / laser illumination
(ii) scan rates required to measure changes in fluorescence intensity over time
(iii) line averaging of the image

A series of validation experiments were performed to assess the possible contribution of each of these factors to FD saturation, rate of recovery or permanent photobleaching. For all validation experiments human or rat colonic biopsies were incubated in Tyrode's containing 0.3mM FD. On reaching near steady state experiments were carried out under the following conditions,

(i) continuous Hg-arc / laser illumination for 0.5 and 1 minute duration
(ii) different scan rates
(iii) different degrees of line averaging

Images were analysed by measuring the intensity of 4-6 crypt luminal areas of bright white fluorescence and one random area of pericryptal intensity. Data is expressed as mean and standard error with 'n' numbers referring to the number of areas analysed within one field of view.
Results
Continuous laser / Hg-arc illumination for 0.5 minutes did not decrease fluorescence intensity (figure 5,7). Exposure for greater that 0.5 minutes did affect fluorescence intensity with up to 10% reduction from baseline values (taken as 100%). Recovery of loss of intensity occurred at a rate of 2.17 grey scale units (gsu) min$^{-1}$ to within 2% of baseline values in 5 minutes. This recovery suggests that intersystem crossing of fluorophore molecules from the excited singlet state into the triplet state was responsible for the decrease in measured intensity as opposed to entry into the irreversible state of photobleaching.

Figure 5,8 shows the effects of laser scanning rate and image averaging on fluorescence intensity. Scan rate, and therefore image production rate, is dependent on the type of LSCM but more specifically on the scanning rate of the mirrors and computer speed. Generally scan rate is in the range of one full frame (512 x 512 pixels) per second, with dwell time/pixel/frame between 1-10μsec. Faster rates are achievable but require an acoustico-optical modulator or image dissector tube. Scan rate can be reduced by frame or line averaging (figure 5,8). This figure shows the collection of 256 x 256 pixel image frames, line-averaged x8, meaning that each line is averaged eight times per frame collected. Under these scanning conditions one frame takes approximately 8 seconds to collect producing a minimum possible scan rate of 7 scans min$^{-1}$. Increasing line averaging to x16 increases dwell time/pixel/frame, with one frame recorded per 15 seconds, reducing scan rate to 4 scans min$^{-1}$. Image averaging increases the total signal with the advantages of, gradually improving signal-to-noise ratio as well as the time between each scan allowing fluorophores trapped in triplet states to decay back to G0 so available for re-excitation. However irreversible photochemical side effects set limits on the duration to which prolonged repeated exposure to illumination is useful.
Figure 5.7 Effects of continuous laser / Hg-arc exposure on fluorescence intensity and recovery measured in human colonic tissue incubated in FITC-dextran (n = 4).
As figure 5.8 shows, less line averaging at a faster scan rate results in a slower rate of decrease in fluorescence intensity, producing 5% reduction from baseline values in 3.5 minutes (rate of decrease 1.33gsu min\(^{-1}\)). Increasing line averaging to x16 increases the rate of intensity loss by a factor of >2 (rate of decrease 2.77gsu min\(^{-1}\)), resulting in over 10% reduction in the same 3.5 minute time period.

![Graph showing effects of laser scan rates and line averaging on fluorescence intensity](image)

**Figure 5.8** The effects of laser scan rates and line averaging on fluorescence intensity in human or rat colonic biopsies incubated in FITC-dextran (n = 4). Increasing the line average setting by a factor of 2 reduces the scan rate but doubles the rate of decrease in fluorescence intensity, (x16 average, \(y = -2.77x + 99.81\) compared to x8 average, \(y = -1.33x + 98.85\)).

On the basis of these findings, for all time course experiments on live tissue, initial use of continuous Hg-arc and laser illumination was kept to an absolute minimum. In practice Hg-arc exposure was limited to no more than 15 seconds duration and, using short periods of continuous laser exposure only, field of view and scanning depth were determined on average within
four to five minutes of commencing FD perifusion. After this no further excitation was performed until time = 10 minutes following FD perifusion, thereby allowing sufficient time for any fluorophores trapped in triplet states to decay back to G0. Time series data was then collected at a scan rate of 1 scan min⁻¹ with line average x8. Scan rate was increased to 7 scans min⁻¹ for an initial three minute period post addition of secretagogues after which scan rate was reduced back to 1 scan min⁻¹. On analysis of these images any decrease of ≤5% in fluorescence intensity was not attributed to a secretory response due to the potential contribution of saturation at this scan rate (figure 5,8). In addition low laser power with no additional zoom factor was used to minimise the risk of irreversible photochemical damage.

5.4.2 Tissue viability

Tissue viability was assessed using a cocktail of two fluorescent supravital dyes, acridine orange and ethidium bromide (Sigma Chemicals Co.). Acridine orange is a membrane permeable nuclear dye, which on binding to double stranded nucleic acids (DNA) emits a bright green fluorescence. It is widely used as a probe to determine quantity and conformation of nucleic acids in biochemical and cytochemical studies as well as for cellular viability (Nishiwaki et al 1996 and Singh & Stephens 1986). Ethidium bromide by contrast, penetrates intact cell membranes of live cells slowly but rapidly enters dead / damaged cells. On entry it binds to nuclear DNA forming a bright red fluorescent complex (Edldm 1970). Ethidium bromide has been validated for use with several isolated cell types, such as assessment of granulocyte cell membrane integrity (Dankberg & Persidsky 1976) and mycobacteria viability (Jarnagin & Luchsinger 1980) as well as viability of isolated islets of Langerhans (Gray & Morris 1987). The combination of acridine orange and ethidium bromide is routinely used to assess viability of blood cells with regard to tissue typing (The Manchester method, tissue typing laboratory, St Mary’s Hospital Manchester) and has also been used on sheets of endothelium.
5.4.2.1 Method
At the end of ‘live’ in vitro experiments the biopsy, still mounted on the coverslip, was removed from the chamber, rinsed in Tyrode’s solution and placed in phosphate buffered saline containing 0.2mg/ml ethidium bromide and 0.1mg/ml acridine orange for 7-10 minutes, followed by a final rinse. After placing on a routine glass microscope slide the biopsy was viewed using the LSCM. Excitation and emission barriers were set accordingly (ethidium bromide excitation / emission spectrum 510 and 595 and acridine orange 480 and 520, Tsien & Waggoner 1995). Both fluorophores were excited using a single ≤510 line, with the reflected beam split onto two separate photomultiplier tubes individually set with appropriate barrier filters to detect the emitted 595 and 520 wavelengths.

5.4.2.2 Validation
The reliability of acridine orange and ethidium bromide in the assessment of cellular viability in colonic mucosal biopsies and the ability to maintain human colonic biopsies using the chamber set up described (section 5.3.1) were assessed under the following experimental conditions,
(i) ‘live’ biopsy, stained within seconds of removal from biopsy forceps
(ii) ‘dead’ biopsy, incubated in 70% alcohol, heated to 70°C for 30 minutes and stained
(iii) post routine in vitro transport experiments of 30 and 60 minute duration, maintaining biopsies in the heated perifusion chamber.
The results of these validation experiments are shown in figure 5.9. Overall tissue viability was assessed as the % of nuclei stained green estimated in crypt colonocytes and lamina propria. ‘Live’ biopsies demonstrated 100% green nuclear staining throughout the biopsy except around the extreme edges. Here red nuclear staining demonstrated that ethidium bromide was able to access and stain cell nuclei, indicating cell membrane damage. Some peripheral cell damage is predictable and unavoidable due to trauma from the biopsy forceps causing damage to cellular membranes. Dead biopsies demonstrated 100% red nuclear staining of crypt colonocytes, signifying cell death, with red staining in the lamina propria also detectable.
Results from these two extremes indicate that acridine orange and ethidium bromide may be considered as useful tools as indicators of cell viability in colonic tissues biopsies.

Culture in vitro for 30 minutes produced a range of results with ~2% - 10% cell death and after 60 minutes ~35% cell death. These results demonstrate that using the proposed method of incubating colonic biopsies in vitro 90% viability is maintained after 30 minutes and approximately 65% viability after one hour. On the basis of these results the experimental time period from which reliable data could be obtained was set at a maximum of one hour, with most time series data gained within 30 minutes. One difficulty when assessing whole tissue viability as opposed to cell suspensions is ensuring adequate delivery of the dyes to cells in the centre of the biopsy. Stain incubation times were increased to compensate for this.
Figure 5.9 The use of supravital dyes ethidium bromide and acridine orange, to assess human colonic tissue viability. Red stained nuclei are dead and green live.
1) 'Dead' biopsy - incubated in 70% alcohol, heated for 30 minutes and stained (n = 4).
2) 'Live' biopsy, stained immediately following collection (n = 1).
3) Biopsy maintained in the perifusion chamber in vitro for 30 minutes (n = 5) and 4) 60 minutes (n = 2) prior to staining. Mag. 1,2 & 4 x100, 3 x250
5.5 Measurement of basal fluid transport in crypts

Having set up and validated the confocal methodology my initial aim was to establish whether human colonic crypts are capable of concentrating the impermeant fluorescent probe FD before studying colonic crypt transport in both health and inflammation (chapter 6).

5.5.1. General method used in functional studies

Human non-inflamed endoscopic biopsies, as subsequently confirmed by routine histology, from proximal and distal colon were collected following endoscopy and mounted in a heated, perifusion chamber (section 5.3.1). Following mounting the perifusate was changed from plain Tyrode's salt solution to Tyrode's containing 0.3mM fluorescein isothiocyanate labeled dextran (FD) to allow measurement of crypt fluid transport. A confocal image of a transverse optical section through the colonic crypts was then obtained (for protocol see sections 5.4.1.1.5 & 5.4.1.2.2). Filters were set for the specific excitation/emission spectrum of FD, 488nm low pass filter and 515nm long pass filter respectively.

5.5.2 Crypt concentration profile

5.5.2.1 Method

The first series of experiments were designed to establish the ability of human colonic crypts to concentrate FD, representing an index of fluid movement out of the crypts, and locate the region along the crypt axis where concentration was maximal. FD concentration is measured as an increase in luminal FD fluorescence intensity relative to crypt mucosal surface bathing solution. After 30 minutes perifusion to allow concentration of FD, sequential optical sections in the z-plane, were recorded down the crypt axis in 5, 10 or 20μm steps (figure 5,10). Mean fluorescence intensity of 3 - 6 crypt lumina and adjacent pericryptal regions were plotted as a function of depth relative to the fluorescence intensity at crypt mucosal surface (figure 5,11). This experiment was performed using colonic biopsies from proximal and mainly distal regions, n = 8 (ascending n = 2, descending n = 5, sigmoid n = 1)
Figure 5.10 Confocal image of human colonic crypts in longitudinal section showing optical sections taken in the z-plane along crypt axis. L = crypt lumen, P = pericrypt, mag. x100.

5.5.2.2 Results

Human colonic crypts demonstrated the ability to concentrate FD within their lumina (figure 5.11). From the patient data presented concentration was greatest at a depth of 10-40μm with maximal fluorescence intensity reaching approximately 20% relative to the crypt surface. Maximal increases from the total of eight patients studied ranged from 5% to 60% (appendix 2, figure A.1). Based on these results the focal plane used for subsequent functional transport experiments lay 20-50μm from the crypt mucosal surface and was kept constant throughout the experiment. No FD concentration was measured in adjacent pericryptal regions and rate of decrease in intensity was greater compared to crypt luminal intensity.
Figure 5.11 Concentration profile of FD down crypt luminal axis in human non-inflamed descending colon. A). Confocal images showing sequential 5μm optical sections from 0μm (top left) to 45μm (bottom right), mag. x100. B). Fluorescence intensity of crypt lumina and adjacent pericryptal region (n = 3) from one patient, expressed as raw data. Experiment was repeated in eight separate patients (for data see appendix 2 figure A,1).
5.5.2.3 Problems incurred

Defining crypt surface - one problem encountered concerned the subjectivity in defining the optical level designated as crypt mucosal ‘surface’. This was important as false positives regarding crypt concentration abilities could easily be recorded. For example figure 5.12 presents data from three profile scans identical in every aspect - field of view, laser power, gain and offset settings, differing only in the optical level defined as crypt mucosal ‘surface’ (0μm). The surface criteria for scan 1 was taken as, the first optical level at which the mounted tissue (mucosal side uppermost) becomes visible. The crypt luminal intensity profile from scan 1 records an increase in fluorescence intensity of >30% above that seen at the defined surface. Scan 2 and 3, in which crypt surface was taken at the level in which circular crypts formed an intact visible structure, by comparison would suggest that no concentration above that seen at the defined surface occurred. For the purpose of all profile and confocal transport experiments therefore, crypt luminal surface was defined as the first optical level at which crypt surface mucosa became visible forming a clear intact circular structure.

In addition due to the unevenness of the mucosal surface the optical level defined as crypt surface often varied between individual crypts within one field of view. Where this problem arose offline intensity measurements for individual crypts were commenced at different optical levels accordingly as shown in figure 5.12.

Figure 5.12(A) Confocal images (see next page for legend)
Figure 5.12 Variations in FD concentration profile along crypt luminal axis with respect to optical level taken as crypt luminal surface, 0µm. A). Confocal images of respective optical sections designated as 0µm, mag. x100. B). Crypt luminal fluorescence intensity relative to crypt surface, expressed as raw data measurements of the 3 individual crypts studied.

*Depth of field* – the capacity of the confocal microscope to perform optical sectioning of tissue provides dramatic advantage over conventional epifluorescence. Figure 5.11 shows maximum measured fluorescence intensity at 10-40µm beyond which intensity decreases at a mean rate of -1.3gsu min⁻¹. The depth below the surface of the specimen at which useful information can be obtained is dependent on several factors,

(i) optical mismatches within the system
(ii) finite working distance of the objective
(iii) opacity and optical inhomogeneity of the specimen
(iv) limitations of individual LSCM systems
Optical mismatch – a light ray emerging from the objective lens travels through several different mediums – glass coverslip, mounting media and the specimen itself, each of which will refract the ray to different degrees (termed the refractive index, RI). A light ray emerging from an oil-immersion lens that is coupled to the coverslip with the appropriate oil will not be refracted until it hits the glass-medium boundary (in this case glass-fluid boundary). The angle of the ray then changes causing the ray to focus at a different position along the z-axis (defocusing). Spreading of the focus will also mean that the image of the focal spot is spread causing further defocusing with less light penetrating the detector pinhole therefore decreasing the observed intensity. This problem can be lessened by the use of water-immersion objectives and no coverslip. However this imposes practical restrictions such as requiring an open incubation system and the increased potential for movement of the specimen during sequential serial sectioning.

The objective – use of a low NA oil immersion objective, below ~0.85 significantly reduces the loss in intensity when viewing thick specimens. This has to be offset against the lower light collection and resolution.

The specimen – a relatively opaque specimen with the correct optics can be scanned to a depth of several 100μm. However, as with a colonic tissue biopsy, more often than not the specimen is heterogeneous in structure, absorption, RI and thickness. This results in scattering or absorption of the illuminating beam as it passes through the upper layers of the specimen, the extent of which determines the amount of light that reaches or fails to reach the plane of focus located further into the specimen. Therefore any optical inhomogeneity in the specimen above the plane of focus will tend to defocus both the beam of incident illumination and the returning signal. This will increase the effective size of the focused spot that is returned to the detector pinhole again reducing the amount of light reaching the PMT. The use of a low power oil immersion lens of <0.8 NA goes some way to reducing the defocusing effects caused by the specimen.

In the current setup a practical limitation was set by the fixed distance the stage could travel 0-170μm. A low magnification, low NA oil immersion lens
with the appropriate immersion medium was the best option as water immersion lens could not be used with the closed chamber system. Images could be resolved up to a depth of 170µm although fluorescence intensity was attenuated, as would be expected, due to the inhomogeneity of the biopsy material.
CHAPTER 6
Colonic crypt function
in health and inflammation

6.1 Aims
In health the adult colon salvages 1–1.5 litres of fluid per day, delivered through the ileo-caecal valve, with a reserve capacity of a further 3 litres. Paediatric ileostomy and stool output studies indicate that the healthy child’s colon can be challenged by the task of absorbing 2-3 times as much fluid as its adult counterpart (60 vs. 25ml per kg per 24 hours). As such these infants probably absorb at rates near their capacity and so have little reserve (Rhoads & Powell 1991). Failure or inadequate salvage must result in excess stool water, namely diarrhoea. In the inflamed colon diarrhoea is a consequence of an inability of the colonic mucosa to accommodate a normal load, that is a loss of colonic function with impaired absorption and increased secretion as opposed to an increased load (Jenkins & Milla 1993). Inflammatory mediators, such as prostaglandins, leukotrienes and platelet activating factor, have been implicated in the pathophysiology of colitis (Wardle et al 1996). Their effects include stimulation of intestinal fluid secretion and impairment of NaCl absorption, both of which add support to their role as contributory factors in the production of diarrhoea.

The use of concentration polarisation of impermeant fluorescent dyes to measure the concentrative capacity of colonic crypts in vitro has been well validated in a number of animal species. I have adapted and applied this methodology for use with human paediatric colonic mucosa (chapter 5) in order to study colonic crypt function in health and inflammation, namely colitis, in children. In addition, using in vivo methods described in chapter 4 (sections 4.2.5 & 4.3), the effects and possible mechanisms of specific inflammatory mediators on colonic transport in vivo in non-inflamed rat colon were studied.
6.2 Colonic crypt function in vitro in healthy human colon.

6.2.1 Basal crypt transport

Having demonstrated the ability of human colonic crypts to concentrate FD (chapter 5 figure 5,11) basal concentration rates over time were obtained for human non-inflamed colon. Fluid transport was measured in human colonic endoscopic biopsies from different regions as previously described (chapter 5 section 5.5.1). Optical sections from a single constant plane, 20 - 50μm from the defined crypt mucosal surface, were collected at a scan rate of 1 scan min⁻¹ commencing after ten minutes perfusion with FD. Data presented is from the ascending colon of one patient (figures 6,1 & 6,2) with identical experiments repeated in 13 patients (see appendix 2 figure A,2 for all other patient data).

A Confocal time series

![Confocal time series](image)

Figure 6,1 Confocal time series showing increase in crypt luminal fluorescence intensity in human ascending colon incubated in FD. Each successive panel shows fluorescence intensity at 5 minutes intervals, with black representing low fluorescent intensity and white high, mag. x100.
Figure 6.2 Time series of FD concentration at constant depth (~40μm) by colonic crypts in vitro in human ascending colon. A). See figure 6.1 B). Rate of increase in fluorescence intensity expressed as mean ± standard error of 4 crypt lumina and adjacent pericryptal regions with increase intensity representing FD concentration. C). Ratio of crypt lumen to pericrypt intensity. Identical time course experiments were repeated in 13 patients (appendix 2, figure A.2)
6.2.1.1 Results

Increases in luminal fluorescence intensity occurred at a constant rate of 4.75 gsu min\(^{-1}\), six times faster than that measured in adjacent pericryptal areas (figure 6.2). Ratio of luminal:pericryptal rates of increase increased from 3.2 to 5.5 gsu min\(^{-1}\) within 30 minutes. In all 13 patients studied FD was polarised within the crypt lumina becoming concentrated over time - measured as an increase in fluorescence intensity (appendix 2 figure A.2). Mean rates of increase were 5.6 ± 1.2 gsu min\(^{-1}\) proximally (n = 7, caecum to transverse) to 9.36 ± 1.4 gsu min\(^{-1}\) distally (n = 6, descending colon, appendix 2 table A.2).

6.2.2 Nature of crypt FD concentration

The measured increase in luminal fluorescence intensity is proposed to be due to the movement of water out of the crypt with the impermeant FD probe remaining trapped within the lumen becoming increasingly concentrated over time (Pedley & Naftalin 1993 and Naftalin et al 1995). In vivo, net water absorption (mucosa to serosa) is passive depending mainly on active Na\(^+\) transport and generation of an osmotic gradient (chapter 1 section 1.3.3). Inhibition of active Na\(^+\) transport, by amiloride (apical channels, Wills et al 1984) or ouabain (basolateral Na,K-ATPase pumps, Stirling 1972), or dissipation of transepithelial osmotic gradients, reduces the driving force for passive fluid absorption. If FD concentration occurs as a result of fluid movement out of the crypt lumen as proposed, inclusion of either amiloride or ouabain in the chamber perifusate would prevent FD concentration as Na\(^+\), and therefore water, movement is inhibited. Experiments to test this proposal were performed. In human colon amiloride-sensitive electrogenic Na\(^+\) transport has been shown to be graded with minimal effect proximally and maximum distally, therefore endoscopic biopsies from transverse or descending colon only were used (Sellin & De Soignie 1987). Biopsies were immediately transferred from biopsy forceps into pre-oxygenated Tyrode's salt solution containing 1mM amiloride (n = 5) or 1mM ouabain (n = 4, Sigma Chemicals Co.) and incubated for ten minutes. After mounting in the perfusion chamber (chapter 5 section 5.3.1), the biopsy was continuously perifused with oxygenated Tyrode's solution.
containing 0.3mM FD + 1mM amiloride or 1mM ouabain. A time series of confocal images was collected at constant depth (figure 6,3).

**Figure 6,3** The effects of inhibiting active Na⁺ transport on FD concentration by colonic crypts in vitro in human descending colon. A). Inclusion of 1mM amiloride in perifusate. FD polarised within crypt lumina but no increase in fluorescence intensity over time was recorded. B). Inclusion of 1mM ouabain in perifusate. Very little polarisation above that measured in the pericrypt occurred with no increase in intensity over time. Results expressed as mean ± standard error of 4 lumina and adjacent pericryptal regions from one patient. Identical experiments were repeated (n = 4 amiloride and n = 3 ouabain, appendix 2 figures A,3 & A,4).
6.2.2.1 Results

Results in each set of experiments were varied and appeared to fall into two categories (for other patient data see appendix 2 figure A,3). Category one included those in which FD concentration was completely inhibited (n = 3 amiloride, n = 2 ouabain) and category two in which fluorescence intensity did increase but occurred at comparable rates in both lumen and adjacent pericryptal regions (n = 2 amiloride, n = 2 ouabain). Subsequent routine histology from all patient biopsies in this latter category revealed an abnormal infiltration of eosinophils within the lamina propria.

Amiloride - in three of the five patients FD concentration over time was 100% inhibited with no increase in fluorescence intensity measured over 10 – 15 minutes. In two of these patients FD was polarised within crypt lumina at intensities five times greater than measured in adjacent pericryptal regions. In the third patient polarisation was minimal at only 1.5 times above pericrypt. In one of the five patients FD concentration was not completely inhibited but rate of increase was reduced compared to that of a second biopsy from the same patient not exposed to amiloride (rate of increase in luminal intensity, amiloride = 7.71 gsu min⁻¹ control = 11.71 gsu min⁻¹).

Ouabain – in two out of the four patients FD concentration was 100% inhibited with no increase in fluorescence intensity and absence of luminal FD polarisation. The remaining two patients were in category two, as described above (appendix 2 figure A,4).

6.2.3 Measurement of extracellular sodium

In addition to measuring the effects of inhibiting Na⁺ transport on FD concentration, the effects of amiloride on extracellular Na⁺ distribution within colonic tissue were studied. Na⁺ was labeled using the cell impermeant sodium indicator Sodium Green (Molecular Probes), visualised by LSCM. It was not possible to perform both transport and Na⁺ distribution experiments in the same biopsy as the individual fluorophores used have the same excitation-emission spectrums. Sodium Green is a visible light-excitable probe for the fluorometric determination of extracellular Na⁺ distribution and concentration. It consists of a fluorescein analog linked to each of the nitrogens in a crown ether with a cavity size that confers selectivity for the
Na⁺ ion (figure 6,4). Sodium Green exhibits greater selectivity for Na⁺ (41-fold) than K⁺ (18-fold) and displays a much higher quantum yield in Na⁺ containing solutions. Upon binding to Na⁺, Sodium Green exhibits an increase in fluorescence emission intensity. It's dissociation constant (Kd) for Na⁺ is ~6mM at 22°C in K⁺-free solution (figure 6,5) increasing to 21mM in solutions containing both Na⁺ and K.

![Chemical structure of Sodium Green.](image)

Figure 6,4 Chemical structure of Sodium Green.

High Na⁺ concentrations are visualised as regions of increased fluorescence intensity (bright white). This occurs as a result of nonionic diffusion of Sodium Green into the tissue and subsequent binding to Na⁺ (as seen with SBFI, Naftalin & Pedley 1990). The phenomenon of nonionic diffusion can best be described using the secretion of ammonia from kidney tubular epithelial cells as an example. Ammonia (NH₃) as a weak base is able to diffuse easily across the cell membrane into the tubule lumen where it combines with secreted H⁺ to form ammonium (NH₄⁺). NH₄⁺ as a charged molecule remains in the lumen. This removal of NH₃ maintains the concentration gradient that facilitates further nonionic diffusion of NH₃ out of the cell. In the case of Sodium Green, in its unbound form (SG), it does not fluoresce and is uncharged. SG therefore easily diffuses from the bathing fluid into the colonic tissue biopsy where on binding to Na⁺ (SG.Na⁺) it becomes charged, immobile and exhibits an increase in fluorescence emission intensity. Further increases in fluorescence intensity reflect an accumulation of SG.Na⁺ as more SG diffuses into the regions of high Na⁺,
binds to Na\(^+\) and fluoresces. Regions in which fluorescence intensity is greatest, namely pericryptal tissue (figure 6,6), therefore represent maximal SG Na\(^+\) accumulation and indicate areas of high Na\(^+\) concentration.

Figure 6,5 Fluorescence emission response of Sodium Green in increasing concentrations of Na\(^+\) (0 – 200mM). A). Emission measured over range of wavelengths 500-600nm B). Maximum emission at 530mM, insert shows plot of linear range of emission occurring at 0 – 13mM Na\(^+\), \(y = 14x + 193.74\) (\(r = 0.96\)). Each data point represents the mean of 3 readings.
6.2.3.1 Method
Human endoscopic biopsies were transferred from biopsy forceps into pre-oxygenated Tyrode's salt solution containing either 2.5, 5 or 10μM Sodium Green. After 15 minutes incubation the biopsy was mounted on a glass slide, coverslip applied and viewed using the LSCM, with filters set for Sodium Green's excitation / emission spectrum, 488 / 515 respectively. The effects of amiloride were measured using a second biopsy from the same patient, initially pre-incubated for 15 minutes in pre-oxygenated Tyrode's solution containing 1mM amiloride, before being transferred to Tyrode's salt solution containing 5 or 10μM Sodium Green + 1mM amiloride. Areas of Na⁺ accumulation were imaged as regions of increased fluorescence intensity (white) with regions of low fluorescence (black) indicating low / absence of Na⁺. Na⁺ distribution was studied in six separate patient biopsies with paired amiloride studies performed in three of these. Confocal settings remained constant throughout individual / paired experiments.

Extracellular Na⁺ detected using Sodium Green was analysed by taking the ratio of mean background fluorescence intensity, where minimal Na⁺ was detected, (defined as the area of lowest intensity on the 0–256 grey scale) to the mean of areas of brightest intensity, (highest Na⁺ concentrations). A minimum of 4 regions of greatest intensity, usually taken from pericryptal tissue regions, and 1 background reading were measured per image with a total of 6 separate fields of view analysed per biopsy. To determine the effects of amiloride identical measurements were taken with mean fluorescence intensity expressed as the change in intensity from background reading (taken as 100%, figure 6,7). Increases above 100% represent maximum intensity of Sodium Green staining measured within the tissue and therefore regions of highest extracellular Na⁺ accumulation.

6.2.3.2 Results
From figure 6,6 the low power confocal image (A) shows extracellular Na⁺ present throughout the lamina propria (bright white), but absent from the crypt lumina (black). A second patient biopsy (B) shows Na⁺ increasingly localised to the basolateral pericryptal region adjacent to each crypt. This
specific staining is seen more clearly on the high power view of the same field (C). Extracellular Na\(^+\) is visualised accumulating in the lateral and basolateral cells membranes with some detected on the apical crypt membranes but very little staining of crypt luminal fluid. Analysis of fluorescence intensity revealed a four-fold increase in intensity at the basolateral membrane compared to crypt lumen. In the six patients studied ratio of background fluorescence to maximum Na\(^+\) accumulation ranged from 1:2 to 1:4.

The effects of inhibiting crypt luminal Na\(^+\) transport via amiloride-sensitive apical Na\(^+\) channels, and therefore net Na\(^+\) absorption, in the three patients studied were varied (figure 6,7). In patient 1 no Na\(^+\) was detected above the measured background fluorescence indicating that pericryptal Na\(^+\) accumulation, as detected in the paired control biopsy not exposed to amiloride (3.5-fold increase), was inhibited. By contrast in patient 2 minimal inhibition of pericryptal Na\(^+\) accumulation was measured with a % increase above background levels comparative to paired control values. Patient 3, extracellular Na\(^+\) was also detected with a ratio of background:maximum fluorescence 1:2. However the ratio of 1:4 measured in paired control biopsy suggests that amiloride partially inhibited pericryptal Na\(^+\) accumulation.
Figure 6.6 Confocal images of human paediatric colon incubated in Sodium
Green to show extracellular Na⁺ distribution. A) & B) Low power mag. x100.
C) Higher power mag. x250 of image B. Areas of extracellular Na⁺
accumulation visualised as bright (white) fluorescence intensity with black
representing no Na⁺. (n = 6)
Figure 6.7 The effects of amiloride on extracellular Na⁺ distribution in human colonic tissue. A1) Biopsy incubated in 5μM Sodium Green only. A2) Biopsy from same patient incubated in 5μM Sodium Green + 1mM amiloride. Mag. x100. B) Measurement of fluorescence intensity expressed in relation to background (taken as 100%, see text for details), n = 3.
6.2.4 Measurement of crypt secretion.

Colonic crypts have been shown to have a dual functional nature. Under basal conditions crypts demonstrate absorption salvaging Na⁺, water and Cl⁻ ions. In response to stimuli, for example from the immediate crypt microenvironment, crypts also demonstrate a secretory capacity producing a net flow of Cl⁻ ions and water from serosa to mucosa (Singh et al 1996). The prosecretory effects arising from colonic inflammation, due to a variety of stimuli such as neurotransmitters, humoral agents and inflammatory mediators, are well documented (Wardle et al 1996, chapter 2 section 2.3). These agents either act directly on ion channels in colonocyte cell membranes or indirectly via increases intracellular messengers, (cAMP or Ca⁺²) which in turn open apical Cl⁻ channels, for example carbachol (chapter 1 section 1.5, Greger et al 1997 and Rhoads & Powell 1991). Carbachols' secretory effects on human colonic crypts can be visualised in vitro using concentrated luminal FD and the LSCM. The process of fluid secretion into the crypt lumen would effectively dilute the concentrated FD, visualised as a decrease in fluorescence intensity (Pedley & Naftalin 1993).

6.2.4.1 Method

FD concentration over time was initially measured using methodology previously described (section 6.2.1). After approximately 20 minutes or when luminal fluorescence was near maximal intensity as defined by gain and offset settings, the perifusate was changed to pre-oxygenated Tyrode's solution containing 0.3mM FD + 2mM carbachol. Scan rate was increased to 7 scans min⁻¹ for three minutes, line averaging x8, with perifusion commenced on the second scan of the timed series. After collecting images for this initial three minute period scan rate was reduced to 1 scan min⁻¹ to prevent photochemical reduction in fluorescence intensity (chapter 5 figure 5,8). The effects of carbachol were rapid and usually occurred within a few minutes of addition to the chamber.
Figure 6.8 Time series to show the effects of carbachol on FD concentration by colonic crypts in vitro in human ascending colon. Results expressed as mean ± standard error of 4 crypt lumina + adjacent pericryptal regions from 1 patient. Numbers in parenthesis refer to rates of increase in luminal fluorescence intensity. Identical experiments were repeated in 6 patients (appendix 2 figure A,5).
6.2.4.2 Results

Crypt luminal fluorescence intensity increased over time at a rate of 4.75 gsu min\(^{-1}\) in human ascending colon (figure 6,8). Addition of carbachol to the perifusate resulted in an immediate reversal of this increase producing an initial rapid decrease in fluorescence intensity, rate 17.34 gsu min\(^{-1}\). After one minute this rapid decrease plateaued and fluorescence intensity began to increase again, rate 3.94 gsu min\(^{-1}\). Fluorescence intensity decreased by approximately 16% within one minute of adding carbachol and in the second patient shown, by 25% in two minutes (figures 6,8 & 6,9). This experiment was repeated in four patients with reductions in fluorescence intensity ranging from 15% - 25% (see appendix 2 figure A,5 for other patient data).
6.3 Colonie crypt function in vitro in inflamed colon

6.3.1 Confocal microscopy

The effects of inflammation on human colonic crypt fluid absorption in vitro were measured as the concentration polarisation of FD over time, visualised by LSCM (section 6.2.1). Endoscopic biopsies for this study were taken from the descending colon of three histologically distinct patient groups - patients with UC (n = 4), allergic eosinophilic colitis (n = 4) and controls (n = 6), all of which were subsequently confirmed by routine histology. Changes in fluorescence intensity over time were recorded and used as an index of fluid transport with an increase representing absorption and decrease secretion. Results are expressed as mean ± standard error of 4-6 luminal and adjacent pericryptal regions from one field of view, n equals the total number of patients. Figures 6,10 & 6,11 show data from individual patients which is representative of the overall findings (see appendix 2 figure A,6 &7 for other patient data). Differences between groups were statistically analysed using Students t-test with significance level set at P<0.05.

6.3.2 Results

Crypt fluid absorption was measured in both control and UC patients (figure 6,10). Overall mean rate of increase in luminal fluorescence intensity was significantly higher in UC compared to controls, suggesting a greater crypt absorptive capacity in the UC patients studied (overall mean rates, controls 9.10 ± 1.36gsu min⁻¹ and UC 19.6 ± 2.08 gsu min⁻¹, P<0.002, figure 6,12 appendix 2 table A,4). In allergic colitis FD accumulation and polarisation within crypt lumina was measured and was five-fold higher than adjacent pericryptal regions (figure 6,11). However, rate of increase in fluorescence intensity was significantly reduced compared to controls, suggesting impaired crypt fluid absorptive function in those crypts measured (controls 9.10 ± 1.36gsu min⁻¹ allergic 1.55 ± 0.96gsu min⁻¹, P<0.003 figure 6,12 appendix 2 table A,4). Increases in pericryptal fluorescence intensity were small with no difference between the three patient groups observed.
Figure 6.10 Effects of inflammation on crypt fluid transport in vitro in human descending colon. (A) Control non-inflamed colon. (B) Inflamed colon from patient with ulcerative colitis. Changes in fluorescence intensity represent an index of fluid transport with increase indicating absorption. Results expressed as mean ± standard error of 4-6 crypt luminal and adjacent pericryptal regions from one control and one UC patient. Experiment was repeated in n = 5 controls and n = 3 UC (appendix 2 figure A,6).
Figure 6.11 Effects of inflammation on crypt fluid transport in vitro in human descending colon. (A) Control non-inflamed colon (B) Inflamed colon from patient with allergic colitis. Experiment was repeated in n = 5 controls and n = 3 allergic colitis (appendix 2 figure A,7).
Figure 6.12 Comparison of rates of increase in crypt luminal fluorescence intensity as an index of absorptive transport in descending colon of control (n = 6), UC (n = 4) and allergic colitis (n = 4) patients. Results expressed as mean ± standard error. Compared to controls, rate of increase in fluorescence intensity was significantly increased in UC (mean rate, controls 9.10 ± 1.36 and UC 19.6 ± 2.08gsu min⁻¹, P<0.002) and significantly reduced in allergic colitis (mean rate 1.55 ± 0.96gsu min⁻¹, P<0.003).

The average rates of change in fluorescence intensity from combined patient data within each group (figure 6.12) suggest that the differences in the functional changes observed may be related to the type of inflammatory response produced, with increased absorptive crypt capacity in UC and decreased in allergic colitis, compared to controls. However the data provides information regarding crypt transport function only. Colonic inflammation also affects crypt structure often with a reduction in overall crypt number (discussed in more detail in chapter 7). Functional data therefore need to be interpreted in conjunction with structural changes.
Crypt function needs to be presented in terms of increase in fluorescence intensity of functioning crypts within the field of view studied, expressed per cm² of tissue. This would allow the consequences of a reduction in crypt number, as seen in UC, with regard to net fluid absorption per cm² of tissue to be taken into account. As figure 6.13 shows this produces a different overall picture.

![Graph showing rate of increase in crypt luminal fluorescence intensity in vitro in human descending colon expressed gsu cm⁻² min⁻¹. Compared to controls rate of increase was significantly reduced in allergic colitis patients (P<0.01) but not in UC patients.](image)

Figure 6.13 Rate of increase in crypt luminal fluorescence intensity in vitro in human descending colon expressed gsu cm⁻² min⁻¹. Compared to controls rate of increase was significantly reduced in allergic colitis patients (P<0.01) but not in UC patients.

Combined functional and structural data from the three patient groups shows a reduction in rate of increase in luminal fluorescence intensity in both UC and allergic colitis compared to controls. However this reduction only reached significance levels in the allergic colitis group, P<0.01. In UC patients the reduction in crypt number, due to colonic inflammation, was offset by an increased rate of absorption such that the net effect was not significantly different from controls (for further discussion see section 6.5.2). Relationships between colonic function and structure are presented and discussed further in chapter 7.
6.4 Colonic function in vivo - effects of inflammatory mediators

6.4.1 Platelet activating factor.

Platelet activating factor (PAF) is a potent endogenous inflammatory mediator implicated in the pathogenesis of UC and CD (chapter 2 section 2.2.2.1). PAF induces electrogenic Cl⁻ secretion in rat distal colon in vitro via stimulating the production of eicosanoids (Wardle et al 1996) which act on colonocytes and the submucosal plexus (Bern et al 1989, figure 6.14).

![Diagram](image)

Figure 6.14 Model to show the role of PAF in immune-system mediated regulation of colonic water and electrolyte transport. Activation of mast cells/phagocytes results in release of prostaglandins, PAF and free radicals into the lamina propria. These act directly on colonic epithelial cells or preferentially on other immune cells to cause release of PGI₂, which activates the ENS. 50% of immune cell-stimulated Cl⁻ secretion arises via neural activity with inhibition of NaCl absorption also occurring (adapted from Powell et al 1992 and Bern et al 1989).

Previous studies have ignored any contribution due to myenteric plexus dependent neuronal reflexes (Buckley & Hoult 1989 and Travis & Jewell 1992) which are important in the pathophysiology of diarrhoea associated with small intestinal inflammation *in vivo* (Jodal et al 1993). Travis et al (1995) using rabbit distal colon excluding the submucosal neural plexus, suggested that enteric nerves are not essential to PAF-induced changes in epithelial function but may have a regulatory role. Other reported
mechanisms of actions include decreased Na\(^+\) and water reabsorption (Travis & Jewell 1992) and PAF-induced ROM-mediated increases in ileal mucosal permeability (Kubes et al 1991). PAF’s actions arise from serosal applications only, with no effects recorded when added mucosally (Wardle et al 1996). However in vivo, mucosal surfaces are also exposed to a potent cocktail of inflammatory mediators due to the migration of activated neutrophils and eosinophils across epithelial layers into crypt lumina (forming abscesses) and the gut lumen. Using in vivo methods described in chapter 4 (sections 4.2.5.1 & 4.3.1), I have looked at the effects of luminal PAF on fluid transport in vivo in rat descending colon and the involvement of the ENS and ROM production.

6.4.2 Phorbol myristate acetate
Phorbol esters, such as phorbol myristate acetate are highly irritant plant compounds, which act as analogues of diacylglycerol (a lipid produced during membrane phosphatidylositol turnover) to activate protein kinase C. Their effects on electrolyte transport in rat descending colon, when applied serosally, include stimulation of electrogenic Cl\(^-\) secretion (via mechanisms involving extracellular Ca\(^+\), eicosanoid production and cholinergic nerves), and inhibition of NaCl absorption (Donowitz 1986). PMA, a potent surface active agent, is able to trigger the respiratory burst in macrophages and subsequent release of superoxide (Mahida et al 1989b). Macrophages are present in the lamina propria being most prominent close to the epithelial basement membrane in the upper 100-150µm of the mucosa (Donnellan 1965). Macrophages isolated from inflamed mucosa of IBD patients demonstrate increased spontaneous / stimulated (using PMA or zymosan particles) ROM production compared to control and non-inflamed tissues from the same IBD patients, (Williams et al 1990). I have looked at the effects of luminal PMA, on fluid transport in vivo in rat descending colon using agarose gels (chapter 4 section 4.2.5.1).

6.4.3 Agarose gels
6.4.3.1 Method
Gels were made as previously described (chapter 4, section 4.2.2) with 2-10µM PAF or 1µM PMA added to cooling gels (approximately 50° C) to
prevent denaturation of PAF/PMA. At $50^0\text{C}$ the gel remained liquid enough to allow thorough mixing of added chemicals to enable even distribution throughout the gel. Experiments were carried out as previously described and fluid and electrolyte transport calculated (chapter 4 section 4.2.3). Results are expressed as mean ± standard error with negative values representing absorption (loss in gel weight/electrolytes), and positive secretion (gain in gel weight/electrolytes). Statistical analysis was assessed by Student's t-test, with significance level set at $P<0.05$. The degree of association between fluid and electrolyte transport was calculated using the Pearson's correlation coefficient ($r$) (see appendix 1).

6.4.3.2 Results

As measured fluid and $\text{Na}^+$ transport appeared to be graded from proximal to distal gels, for clarity comments are limited to proximal gels 1 and 2 only, other gel data being detailed in figures 6,15 – 6,17. PAF - exposure to luminal PAF (10$\mu$M) caused a reduction in fluid absorption compared to controls (figure 6,15). However PAF-induced inhibition of absorption did not achieve statistical significance. $\text{Na}^+$ absorption was significantly impaired with a positive correlation between fluid and $\text{Na}^+$ transport remaining (2$\mu$M PAF, $r^2 = 0.89$, $P<0.01$ and 10$\mu$M PAF, $r^2 = 0.91$, $P<0.01$, figure 6,16). Measurement of $\text{K}^+$ transport produced variable results with rates of $\text{K}^+$ secretion significantly higher in proximal gels 1 and 2 compared to controls (figure 6,17). PMA – rate of basal fluid absorption was reduced by exposure to PMA compared to control transport rates (gel 2 $P<0.02$). $\text{Na}^+$ absorption was also significantly impaired ($P<0.004$). However no correlation was found between overall fluid and $\text{Na}^+$ transport. $\text{K}^+$ secretion was measured in all gels with $P<0.001$ in gel 2.
Figure 6.15 The effects of inflammatory mediators A) PAF (n = 6) and B) PMA (n = 7) on fluid transport in vivo in rat descending colon, measured using agarose gels. Results expressed as mean ± standard error, negative value indicates fluid absorption and positive secretion. Inhibition of fluid absorption reached significance in PMA gel 2 compared to control gel 2 (P<0.02).
Figure 6.16 The effects of inflammatory mediators A) PAF (n = 6) and B) PMA (n = 7) on Na⁺ absorption in vivo in rat descending colon, measured using agarose gels. Significant differences are indicated by * and refer to comparisons with control values (* = P<0.01; ** = P<0.005 and *** = P<0.0005). Inserts show correlation between fluid and Na⁺ transport, 2μM PAF r = 0.94; 10μM PAF r = 0.95.
Figure 6.17 The effects of inflammatory mediators A) PAF (n = 6) and B) PMA (n = 7) on K⁺ transport in vivo in rat descending colon, measured using agarose gels. Significance levels indicated by * and refer to comparisons with control values (** = P<0.0005).
6.4.4 Closed colonic loops

6.4.4.1 Method
Methodology was carried out as previously described (chapter 4 section 4.3.1). Colonic loops were instilled with either 1ml 0.9% saline (control), PAF (2μM in 0.9% saline), superoxide dismutase (SOD, 100μg/ml in saline), capsaicin (1μM in saline) or combinations of PAF + SOD and PAF + capsaicin. After 90 minutes loops were removed and fluid transport determined gravimetrically. Due to the non parametric nature of the PAF secretory data (loop 2, saline +PAF, figure 6.18) data is expressed as median + interquartile range with negative values representing absorption and positive secretion. Multiple comparisons were made by the Kruskal Wallis one way ANOVA on Ranks and pairwise comparisons by Mann Whitney Rank Sum test based on median rank values. Statistical significance was assumed at P<0.05 (see appendix 1).

6.4.4.2 Results
Figure 6.18 shows that basal fluid transport in rat descending colon was absorptive (saline control median = -13.3 ml cm⁻² hr⁻¹ [interquartile range – 8.73 to -20.6], n = 6). Loops containing PAF exhibited net fluid secretion (PAF 19.2 ml cm⁻² hr⁻¹ [46.4 – to 7.39], n = 6, P<0.002 vs. controls). Inclusion of either SOD or capsaicin to PAF-containing loops inhibited the secretory response with a return to net fluid absorption (SOD –9.19 ml cm⁻² hr⁻¹ [-4.85 to -17.7], n = 4, P<0.009; capsaicin –14.3 ml cm⁻² hr⁻¹ [-7.48 to –19.7], n = 5, P<0.004). SOD or capsaicin alone had no effect on basal fluid absorption (SOD –5.31 ml cm⁻² hr⁻¹ [-3.81 to –12.49], n = 5), capsaicin –20.50 ml cm⁻² hr⁻¹ [-10.61 to –33.26], n = 6).

6.5 Discussion
6.5.1 Crypt function in vitro in health
I have shown that in health, paediatric human colonic crypts have the capacity to concentrate the impermeant fluorescent probe FITC-dextran measured over a 30 minute time period (figure 6.2). Passive flux of
Figure 6.18 The effects of PAF on basal fluid transport in vivo in rat descending colon, measured using closed colonic loops. Loops contained saline (n = 6), PAF (n = 6), PAF + superoxide dismutase (SOD, n = 4), PAF + capsaicin (n = 5) and SOD only (n = 5) or capsaicin only (n = 6). Results are medians with interquartile ranges (boxes). P<0.002 refers to PAF vs. saline control.

Luminal FD via paracellular pathways was low, as change in pericryptal fluorescence intensity adjacent to crypts measured was small. The ratio of luminal:pericryptal fluorescence intensity measured over time confirmed that luminal increase was approximately 3 times higher. This supports the evidence as to the restrictive nature of colonic epithelium in forming a physical barrier to the movement of macromolecules as well as ions.

FD concentration by rat colonic crypts has been proposed to be due to the Na^+-dependent movement of water out of the crypt lumen into the
pericryptal region (Naftalin et al 1995). A degree of Na⁺-dependent water absorption could be demonstrated in human colon using the Na⁺ inhibitor amiloride or ouabain (figure 6,3). Data from the patients studied was variable with 100% inhibition of luminal FD concentration occurring in five out of the nine patients studied (n = 3 amiloride, n = 2 ouabain) and partial inhibition produced by amiloride in one patient. In the remaining patients (n = 3) increase in luminal FD intensity was not inhibited but was additionally paralleled by a comparable increase pericryptal fluorescence adjacent to the crypt lumina measured. Subsequent routine histology from these patients revealed increased numbers of eosinophils infiltrating the lamina propria. One possible cause for this observed high pericryptal fluorescence could be an increase in paracellular permeability as a result of mediators released from infiltrating eosinophils, or structural alterations affecting the tight junction complex. A degree of sensitivity to amiloride was also supported by the observation that extracellular Na⁺, visualised using Sodium Green, could be completely or partially reduced in the presence of amiloride (figure 6,7).

Although Na⁺-dependent crypt fluid absorption has been confirmed using an isolated crypt preparation, from rat colon (Singh et al 1995), the exact cellular nature of Na⁺ transport in colonic crypts has not yet been clearly defined. Pedley & Naftalin (1993), measuring intra and extracellular Na⁺ concentrations surrounding rat colonic crypts, have proposed a graded amiloride sensitivity along the crypt axis with deeper parts (>100μm from surface mucosa) possibly possessing an amiloride-insensitive electroneutral Na⁺ transporter. A relatively amiloride-resistant chloride-dependent Na-H exchange has been demonstrated in the apical membrane of rat colonic crypt cells (Rajendran et al 1995). The results presented in this chapter using human colonic tissue suggest that a degree of amiloride-sensitive Na⁺ transport is present in human colonic crypts but that the involvement of other electroneutral Na⁺ transporters could also contribute to crypt water absorption, seen as crypt luminal FD concentration.

Reversible inhibition of FD concentration was also demonstrated in the presence of the carbachol (figure 6,8 & 6,9). Carbachol causes colonic
secretion via increasing intracellular cAMP levels, which act on apical Cl⁻
channels to increase membrane ion permeability. Inclusion of carbachol in
the perifusate caused an immediate rapid decrease in fluorescence intensity
indicating FD dilution by fluid secretion into the crypt lumina, which was
reversible within two minutes. This evidence confirms the dual absorptive
and secretory nature of colonic crypts in human colonic tissue.

6.5.2 Crypt function in vitro in inflamed colon
Using changes in crypt luminal FD fluorescence intensity visualised by
LSCM as an index of crypt absorptive capacity, I have shown that colonic
inflammation alters crypt transport function with the nature of the
inflammatory response emerging as an important contributory factor.
Inflammation arising as a consequence of UC produced an increased crypt
absorptive capacity compared to controls (mean rate of increase in luminal
fluorescence intensity, controls 9.10 ± 1.36gsu min⁻¹ vs. UC 19.64 ± 2.08gsu
min⁻¹, P<0.002 figure 6,12). However, as well as functional changes,
inflammation in UC results in a significant reduction in crypt density such
that overall crypt absorptive function, expressed per cm² (cm⁻²) of tissue is
not significantly different from controls (figure 6,13). Although crypt transport
is only part of the picture, with surface epithelium also responsible for fluid
absorption, this apparently ‘normal’ overall crypt absorption fails to explain
why diarrhoea is still a major clinical symptom in these patients.

One possible explanation involves the methodology used, which enables
the transport functions, both basal absorption and response to
secretagogues, of individual crypts within a biopsy to be visualised and
measured. One difficulty that arose however, is that only those crypts in
which FD accumulation or concentration occurs become clearly visible
(figure 6,19). Other functioning crypts may be present within the field of view
but not visible, for example crypts that are secreting fluid. The effect of crypt
fluid secretion on concentrated luminal FD fluorescence in control tissue,
when exposed to carbachol, is to significantly reduce fluorescence intensity
(figures 6,8 & 6,9). In inflamed tissue crypts in which secretion is stimulated,
due to potent inflammatory mediators, accumulation and concentration of
luminal FD may be prevented altogether therefore rendering these crypts non-visible. This can be seen in figure 6.19 in which the identifiable number of crypts in the FD stained biopsy is much less than that in the bodipy section from the same biopsy. In this situation crypt absorption although detected may not reflect net transport activity occurring within the tissue as a whole.

![Bodipy FITC-Dextran](image)

Figure 6.19 Visualisation of colonic crypts using FD and BODIPY stained colonic biopsies from patients with UC. A & B represent two of the patients studied. Bodipy staining is carried out on the same biopsy once transport studies have been completed. Using FD crypts are only clearly visible when FD accumulates and becomes concentrated within the lumina. Overall crypt density is seen to be much greater when the same tissue is subsequently stained with bodipy suggesting that crypts may be present but non-visible in crypt transport studies using FD.

To obtain a more accurate representation of the predominant fluid flux, secretory data as well as absorptive data would be required and would need to be expressed in terms of the proportion of the total crypt number within
the region studied. A shift from basal crypt absorptive function to secretion would certainly contribute to the diarrhoea accompanying UC (chapter1 section 1.4.3).

Increased transit time of luminal contents through the gut may also contribute to impaired fluid absorption and therefore diarrhoea. The apparent hyperabsorptive capacity (compared to controls) of those crypts in which FD concentration was detected, may arise from an increase in the activity/number of Na,K-ATPase pumps in the basolateral cell membrane. This occurs in response to increased levels of circulating aldosterone and could be determined using immunohistochemical techniques (Gramlich et al 1990).

In contrast to UC, inflammation mounted as a response to an allergen, eosinophilic colitis, impaired luminal FD concentration absorption with patients presenting clinically with watery diarrhoea (mean rate of luminal fluorescence increase controls 9.10 ± 1.36gsu min⁻¹ vs. allergic colitis 1.55 ± 0.96gsu min⁻¹ P<0.003, figure 6,12). Crypt density was not affected. Luminal FD accumulation occurred uniformly in crypts throughout the field of view so enabling the function of the majority of crypts to be visualised and studied. Eosinophils stimulate electrogenic Cl⁻ and therefore water secretion (Resnick et al 1993). Infiltration of eosinophils into colonic tissue may stimulate crypt secretion which would visualised confocally as a reduced rate of increase/no increase in luminal FD fluorescence intensity as concentrated FD becomes diluted (figures 6,11 appendix 2 figure A,7). Lack of FD concentration over time could also be attributable to impaired crypt absorptive function. From the methodology used it is not possible to determine which of these factors or both, produced the observed impaired crypt FD concentration. The reported crypt functional differences arising from UC and allergic colitis indicate that the cause of the mounted inflammatory response may be involved in determining the nature of the altered transport function. This is explored and discussed further in chapter 7.
6.5.3 Effects of inflammatory mediators on colonic transport in vivo

The presence of inflammatory cells and mediators in crypt and gut lumina is common in colitis, yet the contribution of luminal secretagogues to the generation of diarrhoea is ill understood. In non-inflamed rat colon luminal PAF and PMA caused significant in vivo fluid secretion, measured using agarose gels and closed colonic loops, with reduced Na\(^+\) absorption and increased K\(^+\) secretion. PAF-induced secretion was inhibited by the co-presence capsaicin, a highly specific neural toxin for C-afferent nerve fibres (Holzer 1988) or superoxide dismutase. Serosal PAF and ROM activate electrogenic Cl\(^-\) secretion and inhibit electroneutral NaCl absorption in rabbit colon in vitro. This secretion involves synthesis of eicosanoids and activation of the ENS (Bern et al 1989). Mucosal PAF fails to elicit an electrogenic secretory response in vitro in rat colonic mucosa or submucosa preparations, which lack an intact myenteric plexus (Wardle et al 1996). However I have shown that luminal PAF can elicit a secretory response in vivo which involves a capsaicin-sensitive superoxide-dependent neural reflex. Similar neuronal reflexes are implicated in other diarrhoeal states including toxigenic diarrhoea (Nzegwu & Levin 1996) and invasive enteric infection (Jodal et al 1993). Such neuronal reflexes therefore may potentiate the secretory response in inflammatory conditions of the colon in which activated luminal phagocytes are present.

6.5.3.1 In vivo methodologies

The use of novel agarose gels as vehicles to deliver and measure the effects of luminal PAF (2\(\mu\)M) on in vivo colonic fluid and electrolyte transport produced inconsistent results (figures 6,15 – 6,17). Reduction in basal absorptive rates was demonstrated, positively correlating to Na\(^+\) absorption, but failed to reach significance levels despite increased PAF concentration, 10\(\mu\)M (figure 6,16). However luminal PAF (2\(\mu\)M) introduced directly into the gut lumen via closed colonic loops elicited a significant secretory response in vivo clearly reversing basal absorptive values (figure 6,18). Luminal exposure resulted in activation of a basolateral response directly involving C-afferent nerve fibres and the release of superoxide. The dependent nature of fluid secretion on activation of this neuronal reflex can be seen
from experiments performed on preparations lacking an intact myenteric plexus in which no secretory response is seen.

The value of using colonic loops arises from the direct exposure of the intact colonic mucosa to luminal agents, both stimulatory and inhibitory, as well as minimal damage to the surface mucosa. In contrast the lack of a secretory response using agarose gels (with PAF) suggests that delivery/exposure of luminal mediators to the colonic mucosa was inadequate. This may be due to the very small volume added to the liquid gels, 20μl PAF to 20ml gel. Despite thorough rapid mixing it was possible that the chemicals were unevenly delivered to the colonic mucosa or the actual amount of PAF delivered may have been reduced therefore limiting maximal effects on colonic fluid transport. Another factor arises from reported difficulties in stimulating rat intestine using PAF, with in vitro Cl⁻ secretion occurring only at very high doses of PAF, maximal effect around 10⁻⁵M (Powell 1992). This concentration is very much higher than required for maximal stimulation of phagocytes. Increased sensitivity to PAF stimulation of electrogenic Cl⁻ secretion was achieved by rinsing tissue with Ringer-albumin solution. Furthermore a substance extracted from the wash solution was then shown to recreate the loss of sensitivity. From these observations the presence of an endogenous PAF-inhibitor has been speculated. The possibility of an endogenous PAF inhibitor in rat colon in conjunction with inadequate delivery of PAF to the mucosa may provide some explanation as to the lack of a significant in vivo secretory response detected using the agarose gel methodology.
CHAPTER 7
Colonic structural and functional relationships in health and inflammation

7.1 Aims
The colon is an organ of water and electrolyte salvage with a primary function in health of dehydration of luminal contents. The generation of high intramucosal Na⁺ concentrations and transepithelial osmotic gradients provides the driving force for water salvage. The “tight” nature of crypt epithelium, restricting the passive leakage of absorbed Na⁺ back into the lumen, combined with the narrow cylindrical crypt structure, enables crypts to generate these high pericryptal Na⁺ concentrations and transepithelial gradients required to dehydrate faeces (Naftalin 1994 and Pedley & Naftalin 1993, chapter 1 section 1.4.4). As such colonic crypts have been proposed to be a major route of Na⁺ and water absorption. In addition a pericryptal layer of myofibroblast cells may act as a submucosal barrier retarding macromolecular movement from the immediate pericryptal vicinity, therefore potentially contributing to the generation and maintenance of these high Na⁺ concentrations (Joyce et al 1997 and Rolfe et al unpublished).

In health colonic epithelial transport characteristics display regional differences due to the presence or absence of specific membrane transporter proteins (Sellin & De Soignie 1987). Jenkins and Milla (1993) could only show electrogenic Na⁺ transport in distal colon. My advance hypothesis was that as crypts are important for absorption, where high Na⁺ gradients are required crypt density would be expected to be greater. In addition in the inflamed colon, disruption of crypt structure and density may be of significance to the accompanying failure in colonic salvage function. My aims were,

- To assess whether regional crypt densities in non-inflamed colon are significantly different and therefore may potentially contribute to functional regional transport differences.
To record the effects of inflammation on colonic crypt structure, density and pericryptal F-actin sheath
To examine the effects of inflammation on colonic structural and functional relationships.

7.2 Structural studies
Two parameters relating to crypt structure of importance to normal crypt function were studied in control and inflamed human colonic tissue,

(i) crypt density
(ii) presence and integrity of the pericryptal F-actin sheath

7.2.1 Crypt density
Decreased crypt density is a recognised mucosal architectural abnormality associated with IBD (British Society of Gastroenterology (BSG) 1997). Average crypt densities are calculated from fixed tissue sections with accurate assessment requiring adequately sized, well-orientated tissue sections of mucosa and underlying muscularis mucosae. The optical sectioning ability of the LSCM eliminates the need for physical sectioning of tissue and therefore avoids the problems associated with poor orientation. The exact nature and structure of the whole tissue is maintained as seen in vivo. In addition images can be recorded from ‘live’ as well as fixed tissues so avoiding possible artifacts and distortions arising from normal tissue processing procedures such as dehydration, wax impregnation and embedding and physical sectioning.

7.2.1.1 Method
Crypt densities were calculated using both ‘live’ and fixed colonic tissue. Live biopsies - at the end of in vitro transport experiments individual colonic crypts could be clearly identified by the accumulation of luminal FD. To avoid surface mucosa irregularities and unavoidable unevenness of the mounted biopsy, images were collected at depths (~70 - 100μm) from mucosal surface at which crypts formed intact circular structures in an evenly spaced pattern filling the whole field of view. Problems associated
with imaging deep into thick light scattering tissues, such as reduction in fluorescence intensity (chapter 5 section 5.5.2.3) were not an issue as the aim was to image individual crypts only not measure fluorescence intensity. Fixed biopsies – those biopsies stained for F-actin were used (for method see section 7.2.2.1). Images from 'live' and fixed tissues were collected from four random fields of view per biopsy. Crypt number per field of view was counted offline and crypt density per cm² (cm⁻²) calculated, with distances per field of view calibrated using confocal software. Whole crypts and those with ≥ 50% of the crypt lumen visible were included.

7.2.2 Pericryptal F-actin sheath

F-actin rich myofibroblast cells form a major component of the extracellular matrix localised as a prominent continuous perijunctional peripheral ring around the basolateral border of individual colonic crypts. Their functional importance regarding colonic transport is outlined in chapter 1 (section 1.3.2). The F-actin pericryptal sheath can be visualised using the fluorescent probe BODIPY-Phalloidin. Phalloidin is a phallotoxin isolated from the deadly *Amanita phalloides* mushroom. It stains actin in its fibrous form, namely F-actin, at nanomolar concentrations with a similar affinity for both large and small filaments, binding in a stoichiometric ratio of about one phallotoxin molecule per actin subunit. Non specific staining is negligible. To visualise F-actin in human colonic biopsies phalloidin was used in a conjugate form with the long-wavelength fluorophore BODIPY 581/591 (Molecular Probes).

7.2.2.1 Method

Human endoscopic colonic biopsies were fixed in 4% formaldehyde for 24 - 48 hours at room temperature. Following rinsing with phosphate buffered saline (PBS) they were permeabilised using 1% Triton-X for 3 minutes and rinsed twice. 40μl of BODIPY-Phalloidin (dissolved in methanol) was evaporated and reconstituted with 40μl PBS, into which the biopsy was incubated in the dark at room temperature for 30 - 60 minutes. After a final rinse the biopsy was orientated onto a glass slide, mucosal side uppermost, coverslip applied and viewed using the LSCM. The excitation - emission
spectrum for BODIPY-Phalloidin is 584/592 respectively and excitation and barrier filters were set accordingly.

7.3 Colonic structure in health

Crypt density was measured in biopsies taken from all regions of normal non-inflamed paediatric colon and rectum. Four fields of view per patient biopsy were recorded, with number of patients per region studied - caecum \( n = 5 \), ascending \( n = 4 \), transverse \( n = 5 \), descending \( n = 9 \), sigmoid \( n = 4 \) and rectum \( n = 3 \) (mean age \( \pm SD \), 10 \( \pm 4 \) years). In one patient biopsies were collected from the entire colon. Results are expressed as median + interquartile range with regional differences assessed using Mann Whitney Rank Sum Test (figure 7.1). Optical sections from normal mouse, rat and human descending colon are shown to highlight crypt density differences between species (figure 7.2). Experiments were also carried out to visualise the structure, distribution and possible regional differences of the pericryptal F-actin sheath in normal non-inflamed paediatric large intestine \( (n = 8) \) using BODIPY-Phalloidin (figures 7.1 & 7.3).

7.3.1. Results

The data shows that average regional crypt densities from normal childhood colon were approximately 4000 crypts \( cm^{-2} \) of colonic tissue (figure 7.1). A graded decrease was recorded from proximal to distal regions with highest density measured in the ascending colon \( (4300 \, cm^{-2}) \) and lowest in the rectum \( (2975 \, cm^{-2}) \). No significant differences were found between proximal regions (caecum, ascending and transverse) but descending colon revealed significantly lower density compared to ascending and transverse regions \( (P<0.01 \) and \( P<0.02 \) respectively). Greatest decreases were seen in the most distal sigmoidal and rectal regions compared with all other colonic regions \( (P<0.001) \).
Figure 7.1 Regional differences in crypt density in human childhood control colon. A) Confocal images of all regions studied taken from one patient only, mag. x100 B) Collective patient data expressed as median and interquartile range. * Denotes significant difference, see text for P values and patient numbers.
In all regions colonic crypts were arranged in a regular uniform repeating pattern with a central lumen surrounded by an intact circular layer of tissue. Each crypt was tightly abutted to neighbouring crypts on all aspects. The pericryptal F-actin sheath, visualised confocally as a bright ‘white’ fluorescent ring, formed a prominent, intact almost hexagonal band around the basolateral border of each individual crypt (figure 7,3, see appendix 2 figure A,8 for additional patient data). This was detected in all regions of the large intestine. No obvious regional differences were noticed except that the hexagonal shape of the ring was most prominent in the transverse sections (figure 7,1A).

![A) Mouse (B) Rat (C) Human](image)

**Figure 7,2** Comparative confocal images of descending colon to highlight the differences in crypt densities between A) mouse B) rat and C) human, child aged 9 years. All images are of identical magnification (x100) taken from an optical section through whole colonic biopsies fixed and stained with BODIPY-Phalloidin.

Figure 7,2 clearly demonstrates the difference in crypt density between mouse, rat and human descending colon. From this confocal data taken at identical magnifications, childhood human colon has the lowest crypt number, ~4000 crypts cm^{-2}, with rat colon ~8300 crypts cm^{-2} and mouse colon recording the highest density 16200 crypts cm^{-2} – more than four times the density of humans. As crypts are basally absorptive one would predict that, in conjunction with surface epithelial absorption rates, the greater the number of crypts the higher the rate of Na^+ and water absorption. Rates of colonic absorption in vivo using agarose gel cylinders, have been measured as 33.5\mu l cm^{-2} hr^{-1} in mouse distal colon (Rolfe 1996 unpublished data) and 50\mu l cm^{-2} hr^{-1} in rat descending colon (Naftalin
Figure 7.3 Confocal images of human control transverse colon to show normal crypt architecture, density and F-actin staining of the pericryptal sheath. F-actin is stained with fluorescent probe BODIPY-Phalloidin and visualised using LSCM as a bright intact fluorescent ring completely surrounding each individual crypt, mag. x100.
Agarose gels were not sensitive enough to record in vivo fluid absorption rates in human rectum. However, in vivo studies using dialysis methods have estimated rectal absorption rates at 14.4\mu l cm^{-2} hr^{-1} (Edmonds 1971). This data indicates that structural and functional differences are present between species of which crypt density may be a factor contributing to overall fluid absorption rates.

7.4 Colonic structure in inflammation
The effects of inflammation on crypt density were studied in patients with UC or allergic eosinophilic colitis and compared to control values. All patient diagnosis were confirmed by subsequent routine histology. The numbers studied for allergic colitis were as follows (numbers for controls in parenthesis), caecum n = 1 (5), ascending n = 3 (4), transverse n = 5 (6), descending n = 4 (9), sigmoid n = 3 (4) and rectum n = 1 (3), mean age ± SD, 7 ± 2 years (10 ± 4 years). A higher incidence of patients presented with allergic colitis than UC. As such, for UC a crypt density profile of all regions was obtained from one patient and compared to control values with further studies on descending colon only possible in 6 UC patients. Results are expressed as median + interquartile range. Comparisons between colonic regions and groups are made using the Mann Whitney Rank Sum Test with significance level set at P<0.05. The effects of inflammation on the pericryptal F-actin sheath were studied in allergic (n = 8) and UC (n = 8) patients and compared with controls (n = 8). Data is shown in figures 7.4 - 7.6 with additional F-actin patient data presented in appendix 2 (figures A.8 – A.10).
Figure 7.4 The effects of inflammation on regional crypt density throughout the large intestine in human childhood subjects. A) Allergic colitis, crypt density was significantly higher in sigmoid compared to controls (P<0.0001).

B) Ulcerative colitis, all regions except rectum showed a significant reduction in crypt density compared to controls, caecum P<0.03, ascending p<0.003, transverse P<0.014, descending P<0.09 and sigmoid P<0.01.
Control

Allergic colitis

visible spaces between crypts

Figure 7.5 The effects of inflammation due to allergic colitis on the pericryptal F-actin sheath in transverse childhood colon. Biopsy stained with BODIPY-Phalloidin, mag. x100

7.4.1. Results

Allergic colitis - inflammation arising from allergic colitis did not significantly reduce crypt density in any region of the large intestine compared to control values (figure 7.4A). Apart from an apparent increase in the sigmoidal region (P<0.001) crypt density remained constant throughout the large intestine. The pericryptal sheath was visible as an intact brightly fluorescent ring bordering each crypt. In some patients studied the crypts did not appear to be as tightly abutted to their neighbours as seen in controls, with visible gaps appearing between bordering crypts (figure 7.5 and appendix 2 figure A,9)

Ulcerative colitis - the structural effects of inflammation produced in UC were markedly different. Crypt density was significantly reduced in all regions, except rectal, in the patients studied compared to controls. Levels of significance are detailed in the graph legend (figure 7.4B). This initial finding was supported by studies performed in a number of UC patients looking specifically at the descending colon. A 2.5-fold reduction in crypt density was observed in UC compared to controls (P<0.0001). In addition to a physical reduction in crypt number, figure 7.6 clearly shows that crypt structure took on a distorted and ragged appearance. The normally prominent, clearly visible intact pericryptal F-actin sheath was patchy and irregular if detectable at all (figures 7.6 and appendix 2, A,10).
Figure 7.6 The effects of inflammation arising from ulcerative colitis on crypt density in descending colon of children. A) Confocal images, stained for F-actin, mag. x100 B) Crypt density was significantly decreased in UC (n = 6) compared to controls (n = 9, P<0.0001). Results expressed as median + interquartile range.
7.5 Effects of inflammation on normal colonic structural and functional relationships

The generation of high intramucosal Na\(^+\) and transepithelial gradients required for effective salvage function is largely dependent on maintenance of the structural integrity and barrier properties of colonic epithelial mucosa. To investigate the effects of inflammation on these colonic structural and functional relationships a series of experiments were performed in UC (n = 5) and allergic colitis (n = 9) patients. In these studies colonic structure relates to crypt appearance, density and presence of the pericryptal F-actin sheath (section 7.2) and colonic function to the ability of crypts to concentrate FD, an index of crypt absorption, measured by LSCM (chapter 6 section 6.2.1). From the 14 patients studied three trends were observed,

(i) normal structure and function, histological inflammatory changes only
(ii) normal structure with reduced / no absorptive function
(iii) abnormal structure and function

Patient clinical details are outlined in table 7,1, with * identifying those patients whose structural and functional data is presented in this chapter. All other patient data can be found in appendix 2 (figures A,11 - A,14). All structural images are of identical magnification (x100) and unless stated are confocal images of fixed biopsies stained for F-actin. Corresponding patient functional data is expressed as mean ± standard error of luminal fluorescence intensity of 4-6 crypts and adjacent pericryptal regions. An increase in fluorescence intensity represents absorption and decrease secretion with secretory data in response to carbachol presented where performed.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age yrs</th>
<th>Sex</th>
<th>Histology</th>
<th>Diarrhoea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>11</td>
<td>F</td>
<td>Architecture normal, eosinophilia</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>8</td>
<td>F</td>
<td>Architecture normal, large numbers of eosinophils in LP</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 3*</td>
<td>9</td>
<td>M</td>
<td>Architecture normal, eosinophilic colitis</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 4</td>
<td>5</td>
<td>M</td>
<td>Crypt architecture preserved, eosinophilic colitis</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 5</td>
<td>8</td>
<td>M</td>
<td>Crypt architecture normal, excess of eosinophils 760/10 high power fields in LP</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 6*</td>
<td>17</td>
<td>F</td>
<td>Crohn’s disease diagnosed 5 yrs, no crypt atrophy, goblet cell depletion or granulatoma, prominent eosinophilia</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 7*</td>
<td>4</td>
<td>M</td>
<td>Normal architecture preserved, eosinophilic colitis</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 8</td>
<td>12</td>
<td>F</td>
<td>Normal architecture, focal mild increase in eosinophils</td>
<td></td>
</tr>
<tr>
<td>Patient 9</td>
<td>3</td>
<td>F</td>
<td>Normal architecture, eosinophilic colitis</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 10*</td>
<td>14</td>
<td>F</td>
<td>Crypt distortion and reduction in number, active UC</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 11*</td>
<td>10</td>
<td>F</td>
<td>Crypt distortion, branching, some atrophy, dense inflammatory infiltrate of LP, active chronic UC</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 12*</td>
<td>10</td>
<td>M</td>
<td>Occasional crypt abscesses, goblet cell depletion, mucosal ulceration, marked active chronic inflammatory cell infiltration of LP, active chronic UC</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 13</td>
<td>1</td>
<td>M</td>
<td>Focal mucosal oedema, diffuse lymphocytic infiltrate, granuloma, Crohn’s disease</td>
<td></td>
</tr>
<tr>
<td>Patient 14</td>
<td>13</td>
<td>F</td>
<td>Ulcers associated with acute inflammation, as well as diffuse lymphocytic infiltrate, epithelioid granuloma, active Crohn’s dis.</td>
<td></td>
</tr>
</tbody>
</table>

Figure 7.7 Structural and functional relationships in allergic colitis I. A) Confocal image of human ascending colon, normal crypt density, structure and intact pericryptal sheath, mag x100. B) Functional data - FD concentration by colonic crypts, rate of increase in fluorescence intensity 4.97 gsu min⁻¹, with secretory response to carbachol. Data represents mean ± standard error of the luminal intensity of 4 crypts and adjacent pericryptal regions. Increase in intensity represents absorption and decrease secretion. Repeated in 5 patients (see appendix 2 figures A,11 & A,12, for other patient data).
Figure 7.8 Structural and functional relationships in allergic colitis II. A) Confocal image of human descending colon incubated in FD, crypt structure and density normal, pericryptal sheath not stained. B) Functional data - lack of FD concentration by crypts after 15 minutes, with no measurable response to carbachol. Repeated in 4 patients (appendix 2 figure A13).
Figure 7.9 Structural and functional relationships in allergic colitis III. A) Confocal image of human descending colon, normal crypt architecture, density and pericryptal sheath. B) Functional data - limited amount of crypt luminal FD concentration, rate of increase in fluorescence intensity 2.28gsu min⁻¹. Repeated in 4 patients (appendix 2 figure A,13).
PATIENT 10 – Ulcerative colitis

Confocal image, transverse colon

Figure 7.10 Structural and functional relationships in ulcerative colitis I. A) Confocal image of human transverse colon, reduction in crypt density with distorted, irregular crypts, pericryptal sheath not clearly evident B) Functional data - slow rate of increase in fluorescence intensity, $1.59\text{gsu min}^{-1}$, with very little luminal FD concentration after 20 minutes.
PATIENT 11 – Ulcerative colitis

Figure 7.11 Structural and function relationships in ulcerative colitis II. A) Confocal image of human transverse colon, reduction in crypt number, distortion of normal architecture, pericryptal sheath patchy and discontinuous. B) Functional data - concentration of FD in two crypts measured at high rate of increase in fluorescence intensity, 45gsu min⁻¹, small secretory response to carbachol. Repeated in 4 patients (appendix 2 figure A,14)
PATIENT 12 – Ulcerative colitis

Confocal image, descending colon

only two intact crypts clearly visible

pericryptal sheath evident but patchy

B

Figure 7.12 Structural and functional relationships in ulcerative colitis III. A) Confocal image of human descending colon, only two crypts evident per low power field with gross alteration in normal crypt architecture and only patchy F-actin staining. B) Functional data - concentration of FD by crypts measured at rate of increase in fluorescence intensity 14.8gsu min⁻¹. Repeated in 4 patients (appendix 2 figure A,14).
7.5.1 Results
7.5.1.1 Normal structure and function, abnormal histology (figures 7,7, & appendix 2, A,11 & A,12).
Histology from the patient presented documented an eosinophilic colitis with degranulating eosinophils clearly evident invading the lamina propria. Normal crypt and surface epithelium was reported. The confocal image of an optical section through the tissue revealed normal crypt density, compared to controls (3967 cm\(^{-2}\)) and architecture with crypts forming intact circular structures of a regular uniform pattern. F-actin of the pericryptal sheath was present forming a continuous border round individual crypts. Those crypts measured appeared to be functional, demonstrating the ability to concentrate luminal FD, rate of increase in fluorescence intensity of 4.97 gsu min\(^{-1}\). Carbachol caused fluid secretion into the crypt lumina diluting the concentrated FD, visualised as a reversible decrease in fluorescence intensity. FD remained contained within the crypt lumina and very little was detected in adjacent pericryptal regions.

7.5.1.2 Normal structure, abnormal function (figures 7,8, 7,9 & appendix 2, A,13)
Histology from both patients presented reported increased eosinophils within the lamina propria with otherwise normal architecture. The confocal image of patient 6, from transport studies as F-actin stain not performed, clearly showed normal crypt density (4297 cm\(^{-2}\)) intact crypt architecture with FD polarised and contained within crypt lumina. However these crypts were unable to concentrate luminal FD with no recorded increase in fluorescence intensity. No secretory response to carbachol was recorded. Viability, assessed at the end of the experiment confirmed tissue was viable. Patient 7 presented a similar structural picture with normal crypt density, (3667 cm\(^{-2}\) and pericryptal sheath visible bordering each crypt. Functional data showed limited colonic function with a reduced rate of increase in fluorescence intensity compared to control rates from non-inflamed descending colon (mean rate allergic colitis 2.28 gsu min\(^{-1}\) vs. controls 9.1 gsu min\(^{-1}\)).
7.5.1.3 Abnormal structure and function (figures 7,10 – 7,12 & appendix 2, A,14)

Patient 10 had acute UC with clear disruption of normal crypt architecture and density (2645 cm\(^2\)) in the transverse colon. Crypts were unevenly distributed with branching evident. No clear identifiable F-actin sheath could be seen bordering the crypts. Functional data recorded a reduced rate of increase in fluorescence intensity of 1.59gsu min\(^{-1}\) (controls 9.1gsu min\(^{-1}\)). Patients 11 and 12 were both diagnosed to have active chronic UC with histology documenting dense acute and chronic inflammatory cell infiltration of the lamina propria with neutrophils and eosinophils predominant. Patient 11 had evidence of crypt branching and patient 12 crypt abscesses and mucosal ulceration. From the data presented the most striking observation arose from the confocal images depicting crypt structure. Crypt density was significantly and dramatically reduced (1653 cm\(^2\) patient 11 and 611 cm\(^2\) patient 12 vs. controls ~4000 cm\(^2\)) with large areas of lamina propria devoid of identifiable crypt architecture. Crypts were grossly distorted and appeared dilated. In the few intact crypts that were evident there was some degree of F-actin staining but its distribution was patchy. Despite these dramatic structural changes, in the intact crypts studied it was possible to demonstrate an increase in fluorescence intensity over time indicating that these crypts remained functional. However rates of increase, 45 and 15gsu min\(^{-1}\) were actually significantly higher compared to control values (P<0.003). A small response to carbachol was seen in one crypt in patient 11.

7.6 Cellular nature of the inflammatory infiltrate

7.6.1 Method

From the patients on which functional and structural studies were performed the nature of the inflammatory infiltrate was assessed in 5 allergic colitis and 4 UC patients with regard to eosinophils (from routine H&E sections) and neutrophils, (immunostaining for neutrophil elastase, method chapter 8 section 8.4.2.1). Where possible ten high powered fields (HPF) (x400) were
counted per section by two individuals, some sections were too small and in these ≥ 5 fields were counted.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 3</td>
<td>1.57 ± 0.61</td>
<td>21 ± 6.67</td>
</tr>
<tr>
<td>Patient 4</td>
<td>1.37 ± 0.26</td>
<td>14.36 ± 1.44</td>
</tr>
<tr>
<td>Patient 6</td>
<td>2.4 ± 0.5</td>
<td>15.66 ± 4.46</td>
</tr>
<tr>
<td>Patient 8</td>
<td>0</td>
<td>6.5 ± 2.21</td>
</tr>
<tr>
<td>Patient 9</td>
<td>1</td>
<td>7.44 ± 1.48</td>
</tr>
<tr>
<td>Patient 11</td>
<td>15 ± 5.8</td>
<td>2.3 ± 1.3</td>
</tr>
<tr>
<td>Patient 12</td>
<td>&gt; 60</td>
<td>0</td>
</tr>
<tr>
<td>Patient 13</td>
<td>&gt; 60</td>
<td>11.66 ± 4.66</td>
</tr>
<tr>
<td>Patient 14</td>
<td>&gt; 60</td>
<td>13.75 ± 4.21</td>
</tr>
</tbody>
</table>

Figure 7,13 Analysis of cellular inflammatory infiltrate in eosinophilic and ulcerative colitis. Number of eosinophils and neutrophils were counted per 10 high power fields, results expressed as mean ± standard error.
7.6.2 Results
From these results it is evident that neutrophils formed the prominent infiltrating inflammatory cell in UC and eosinophils in allergic colitis (figure 7,13). Neutrophil infiltration of the lamina propria occurred in large numbers, with >100 neutrophils per HPF in one patient, and were considerably higher than the overall number of eosinophils found in allergic colitis. These large numbers of neutrophils were associated with a significant reduction in crypt density and structural damage compared to allergic colitis in which normal crypt architecture was observed despite the presence of inflammatory cells.

7.7 Discussion
7.7.1 Colonic structure in health
Throughout all regions of the human childhood colon colonic crypts were seen as intact circular structures tightly abutted to neighbouring crypts to form a continuous, uniform pattern. Surrounding the basolateral border of each crypt was a continuous ring structure in which F-actin was one prominent component (figure 7,1). This so called pericryptal F-actin sheath was detected in all regions. Crypt density in proximal regions (caecum to transverse) remained constant, approximately 4,200 crypts cm$^{-2}$, gradually decreasing throughout distal regions by approximately 35% to 2600 cm$^{-2}$ in rectal tissue. These figures though slightly lower, are comparable with those obtained from fixed histology sections of adult colon (BSG guidelines 1997). In adults the average number of crypt profiles corresponding to a 1mm length of muscularis mucosae is seven to eight (4900 – 6400 cm$^{-2}$) with a reduction in normal rectal mucosa also detected (actual numbers were not detailed). The data I have presented is on childhood large intestine (mean age ± SD, 10 ± 4 years). To my knowledge no other such comparative studies on crypt density in childhood large intestine have been performed.

The significance, functional or otherwise, of this decrease in crypt density in distal regions is unknown. There are known detectable differences between proximal and distal mucosal transport characteristics that would be expected to reflect functional variations. An important function of distal/rectal
mucosa is Na\textsuperscript{+} conservation yet Na\textsuperscript{+} has to be removed against considerable electrochemical gradients in these regions as the faecal fluid is of low Na\textsuperscript{+} concentration. The specific regional transport characteristics of distal colon/rectum, such as increased amiloride-sensitive electrogenic Na\textsuperscript{+} transport (Sellin & De Soignie 1987) and greater sensitivity to aldosterone (Edmonds & Godfrey 1970) compared to proximal regions, infer a highly efficient active Na\textsuperscript{+} transport mechanism. As such distal mucosa would seem well suited to the specific functional requirements of this region of the intestine. My advance hypothesis was that in addition to regional cellular transporter variations a greater crypt density might also assist salvage function in these regions where resistance to dehydration was increased. The data from normal childhood colon however, shows that distal and rectal crypt densities were actually significantly lower than proximal regions. One possible explanation, especially with regards to rectal tissue, could be the reduced requirement for large volumes of water absorption, as occurs from the more liquid luminal contents of proximal regions, with the rectum functioning more as a storage place prior to final defecation.

Species differences regarding crypt density are pronounced (figure 7.2). Crypt density in rat and mouse colon were 2.5 (rat) and 4-fold (mouse) higher than human childhood colon. This structural difference is accompanied by higher in vivo fluid absorption rates in mouse and rat, measured using agarose gels, vs. humans, measured using dialysis bags. Although these specific methodologies reflect combined crypt and surface epithelial absorption rates the striking differences in crypt density may be a potential contributory factor to the overall normal absorptive capacity of the colon. As such, a change in basal absorptive crypt function to secretion or a physical reduction in crypt number may have implications for colon\textsuperscript{A} function, for example in IBD (section 7.7.3 & chapter 6 section 6.5.2).

7.7.2 Colonic structure in inflammation

Colonic inflammation resulted in structural damage seen as altered crypt architecture, density and pericryptal F-actin sheath. In allergic colitis colonic structure was maintained with crypts forming a regular uniform pattern, crypt
density comparable to control values and evidence of intact pericryptal F-actin sheath (figures 7,4 & 7,5). By comparison inflammation associated with UC revealed a striking reduction in crypt density throughout all regions of the large intestine, except rectal (~2500 crypts cm⁻² UC vs. 4200 cm⁻² controls, figure 7,4). Again this observation has been reported in fixed sections of adult colon (1600 – 2500 cm⁻² BSG guidelines 1997). Crypt structure was distorted with large areas of lamina propria devoid of recognisable crypts and with loss of the normally prominent F-actin sheath (figure 7,6).

7.7.3 Colonic structural and functional relationships in health

Interrelationships between colonic structure and function are essential for effective salvage of water and electrolytes and normal stool dehydration. I have presented functional evidence to support the basal absorptive capacity of human colonic crypts (chapter 6 section 6.2.1). Basal crypt absorption was associated with intact, closely abutted crypts forming a continuous pattern. Each crypt was surrounded by a prominent pericryptal F-actin sheath proposed to be important in the maintenance of high pericryptal Na⁺ concentrations necessary for normal salvage function (Naftalin et al 1995). The importance of a 'tight' epithelium with crypts that can generate and maintain high osmotic pressure gradients, in the production of hard faeces is most clearly illustrated in IBD in which inflammatory-induced changes in colonic structure accompany alterations in colonic function.

7.7.3.1 Colonic structural and functional relationships in inflammation

From the patients studied a number of altered relationships between crypt function and structure due to inflammatory associated changes were identified. An important component of these changes observed concerned the cellular nature of the inflammatory infiltrate.

*Normal structure, abnormal function, (figures 7,8, 7,9 & A,13)*

Firstly it was demonstrated that loss of or impaired crypt absorptive function was possible in the face of apparent normal structure (patients 6 & 7). The confocal structural data presented a picture comparable with controls with a normal crypt density of intact circular crypts forming a uniform pattern.
complete with a prominent F-actin pericryptal ring bordering each crypt. However functional data from these crypts recorded a lack of ability to concentrate luminal FD, seen as a reduction in rate of increase in fluorescence intensity after 15 minutes (all patient data - mean rate $1.55 \pm 0.96 \text{gsu min}^{-1}$ compared to overall mean control rate $9.10 \pm 1.36 \text{gsu min}^{-1}$). Lack of crypt absorptive function could have been attributable to impaired absorption and/or increased secretion, stimulated by high levels of inflammatory mediators, with increased secretion potentially masking underlying basal absorption. From this methodology it is not possible to determine which of these processes may underlie the observed loss of crypt function.

**Abnormal structure and function, (figures 7,10 – 7,12 & A14)**

Secondly altered crypt absorptive function was recorded in conjunction with inflammation-induced structural damage. Structural changes included significant, often severe, physical reduction in crypt density (2.5-fold reduction compared to controls) with crypts taking on a distorted, ragged appearance and the pericryptal F-actin sheath not clearly detectable. Associated with this structural damage was an impaired crypt absorptive function. However a more surprising functional consequence accompanying these structural changes was that in the limited intact crypts that remained and were identifiable an increased absorptive capacity was recorded compared to controls (all patient data - mean rate of increase in luminal FD fluorescence - UC $23 \pm 5.1 \text{gsu min}^{-1}$ vs. controls $9.10 \text{gsu min}^{-1}$). In this situation there is a physical reduction in number of functioning absorptive units as opposed to a malfunction of transport processes themselves.

The structural and functional changes observed appeared to be positively associated with the nature of the cellular inflammatory response. In all cases inflammatory cells, not normally present in control tissue, were detectable in the lamina propria. Of the patients where structure was maintained yet function was impaired, namely allergic colitis, eosinophils were predominant with very few neutrophils present. Eosinophils are known to release a substance that acts to cause colonic secretion (Resnick et al
Greatly increased numbers of eosinophils could potentially switch normal crypt absorptive function to secretion, thereby increasing stool water and contributing to diarrhoea. In cases of active UC where severe structural changes occurred neutrophil infiltration was prominent with frequently over a hundred neutrophils per high power field (figure 7,13). Neutrophils also release a prosecretory mediator (Madara et al 1992). However a prominent arm of their defence weaponry includes the release of an array of ROMs and toxic lysosomal enzymes capable of solubilising major structural components of the extracellular matrix (Yamada & Grisham 1991). For example neutrophils, utilise cellular Cl\(^-\) to produce potent hypochlorous acid providing a protective shield allowing proteases to attack normal healthy cellular components (chapter 3 section 3.4.1.1). Eosinophils are also capable of this process but are less effective in oxidising Cl\(^-\) preferring bromide which is present in much lower quantities (Weiss et al 1986b). Combined with the observation that the overall average number of infiltrating eosinophils in the patients studied was far less than that of neutrophils (figure 7,13) these factors may form one explanation as to why eosinophil infiltration appears to be associated with less structural colonic damage.

From the data presented I propose that normal basal crypt absorptive function of the colon in health is in part dependent on its relationship with crypt structure. Crypt density, intact architecture, absorptive function and nature of the inflammatory infiltrate may all be significant contributing factors to this relationship in health and its disruption in inflammation.
CHAPTER 8
Measurement of oxidative stress in vivo and in vitro in inflamed colon -
Methods.

8.1 Introduction
Reactive oxygen metabolites (ROM) whilst essential to normal metabolism and defence against disease are also among the most highly reactive substances known with a powerful potential to cause damage. In particular superoxide (O$_2^-$) and hydroxyl (OH$^-$) radicals have the capacity to elicit lipid peroxidation, damage DNA and initiate an oxidative cascade perpetuating further ROM production (Verspaget et al 1991, chapter 3 section 3.4.1). Despite extensive endogenous mechanisms to tightly control their production evidence is increasing to implicate a direct pathological role for ROM in a number of inflammatory diseases (Halliwell et al 1992 and Blake et al 1987). In such conditions oxidant stress appears to exceed homeostatic defence mechanisms resulting in the destruction of healthy living tissue. Prerequisites for ROM production, such as large numbers of activated phagocytes and high levels of oxidative enzymes and free iron, are present in colitic mucosa. Evidence of increased ROM production (Keshavarzian et al 1992 and Sedghi et al 1993), reduced concentrations of endogenous ROM scavenging enzymes (Mulder et al 1991), ROM-mediated colonic secretion (Bern 1989) and the effectiveness of pharmacological agents to block ROM release in IBD treatments (Ahnfelt-Rønne et al 1990), all support the proposal that abnormal oxidative metabolism has a central role in active IBD (Simmonds 1993, chapter 3 section 3.6)

8.1.1 Measurement of ROM
Accurate ROM measurement, in order to assess their specific contribution to IBD, has always proved a challenge due to their inherent reactivity, for
example \( \text{OH}^- \) has a half life in biological systems of only a few microseconds (Slater 1984). Existing methods include two approaches,

(i) direct measurement of primary free radicals released

(ii) indirect measurement of the effects of ROMs, such as primary and secondary lipid peroxidation products.

*Electron spin resonance* (ESR) is the only method that can directly "see" free radicals. It is a sensitive method detecting energy changes occurring as unpaired electrons align in response to an external magnetic field. Highly reactive radicals require spin trapping first, allowing the radical to react with a spin trap molecule to produce a long-lived radical from which an ESR can be obtained (Halliwell et al 1992). *Chemiluminescence*, the production of light by activated phagocytes, provides information about levels of phagocyte activation and therefore ROM production (Babior 1978). *The thiobarbituric acid (TBA)* test, the oldest and most frequently used assay, measures amongst other things malondialdehyde (MDA) (a product of lipid peroxidation) to provide an index of ROM production (Gutteridge & Halliwell 1990).

My aims were to measure ROM production quantitatively and qualitatively, in human control and inflamed colon and identify possible cellular sources of \( \text{O}_2^- \) produced. Nitroblue tetrazolium was used to detect and visualise \( \text{O}_2^- \) production directly in vivo and in vitro (Oshitani et al 1993). Naphthoic acid hydrazide was used to detect tissue carbonyls in vitro, a secondary product of lipid peroxidation, as an index of ROMs produced. Methodologies used are outlined in this chapter with data presented and discussed in chapter 9.

### 8.2 Direct measurement of \( \text{O}_2^- \)

Direct measurement of \( \text{O}_2^- \) requires a chemical probe that in the presence of \( \text{O}_2^- \) produces a measurable change in its chemical structure, such as a change in light absorbance properties. The probe needs to be specific for \( \text{O}_2^- \) and the measurement system should ideally be objective, quantitative, and sensitive.
8.2.1 Nitroblue tetrazolium – an $O_2^-$ probe

Nitroblue tetrazolium (3,3'-03,3'-Dimethoxy-4,4'-biphenylene)-bis[2-p-nitrophenyl]-5-phenyl-2H-tetrazolium chloride], is a yellow, water-soluble, electron acceptor dye. It can be rapidly reduced, by activated polymorphonuclear cells (PMN's) in blood or infiltrating tissues, to form a water-insoluble, deep blue-black formazan, nitro-blue formazan (NBF, figure 8,1).

![Chemical structure of NBT](image)

The biochemical basis of PMN-mediated NBT reduction is exclusively due to an oxygen-dependent $O_2^-$ generating oxidase located in the cell membrane. Oxygen dependency is indicated as human PMN's incubated in anaerobic conditions, partial pressures of $O_2$ ($pO_2$) <5mmHg, fail to generate $O_2^-$, measured by reduction of cytochrome C and accompanied by a failure to reduce NBT. In addition deficiency in this oxidase results in an inability to produce $O_2^-$ seen clinically in CGD. PMN's from these patients are unable to reduce NBT despite demonstrating hyperphagocytic activity (Ward 1974). NBT binds with high affinity via its double positive charge to negatively charged groups present on tissue proteins with minimal NBT binding when tissue-binding sites are competitively blocked using 2.5%
protamine (Altman 1969). NBT’s high affinity binding in conjunction with small size of granular formazan deposits, 0.5 x 0.5μm in rat liver sections, results in precise localisation of reduction enzyme activity and O$_2^-$ production (Altman 1976a&b). These findings support the suitability of NBT as a probe to directly measure and locate O$_2^-$ production within tissues.

8.2.2 NBT – quantification

NBT is the tetrazolium salt of choice in determining specific and accurate localisation of O$_2^-$ within tissues or cells. However quantification of reduced NBT produced has always presented a problem due to its insoluble nature and the production of more than one reaction product. Monotetrazolium salts, such as iodonitrotetrazolium (INT), having only one reaction product overcome this later problem but at the cost of poorer localisation. Possible approaches to quantification include,

(i) elution of formazan from the tissue into solution and measurement spectrophotometrically
(ii) measurement of insoluble formazan within the tissue sample.

Elution – NBT’s high affinity binding requires that NBF is first stripped from its tissue binding sites, by changing its molecular nature, after which this altered dye can be eluted and quantitatively measured (Altman 1969). The production of two NBT reduction products, a red half-formazan intermediate and a fully reduced di-formazan with different absorption characteristics, 515 and 715nm respectively (Eadie et al 1970), requires two separate absorption measurements for accurate quantification, from which concentrations of the two products can be calculated. NBF elution has been successfully used to obtain an index of tissue dehydrogenase activity (Altman 1969).

Tissue NBF measurements – include microdensitometry, charge-coupled device (CCD) camera and histological blind scoring methods. Microdensitometry as a means of quantitative analysis, relies on the homogenous distribution of one product within the tissue whose absorption curve has a single, well-defined peak. NBT’s multiple reaction products make quantification in absolute units difficult using this method (Altman
1976). Quantification using a CCD camera is based on measuring the change in colour intensity of NBT staining recorded via fibre optics. It has been successfully applied to measure time-dependent changes in NBT intensity in ischaemic cardiac tissue (Schröder et al 1991). A third option, histological blind scoring, can provide detailed information of both specific microscopic localisation of NBF deposits and qualitative macroscopic measures of NBT staining within reacted tissue sections. The simplest NBT scoring assessment is expressed as positive (blue staining) or negative (absence of blue staining) reactions (Hobson et al 1991), or % number of positive stained cells, as in the NBT test for CGD PMN's (Ward 1974). More detailed scoring systems have also been devised with excellent results (Oshitani et al 1993).

8.3 Direct measurement of $O_2^-$ in vivo – using NBT

8.3.1 Agarose gels

Agarose gel cylinders have been successfully used as vehicles to carry NBT enabling measurement of $O_2^-$ production in vivo in rat descending colon (Taylor & Naftalin unpublished data). Agarose gels were made as previously described (chapter 4 section 4.2.2) with 1mM NBT/INT incorporated, post heating, into cooling yet still liquid gels. Gels used in non-inflamed rat colon also contained 10 – 20μM PMA to stimulate $O_2^-$ release from activated macrophages. Extraction of NBT/INT reduction products have been performed by elution methods dissolving the gel by boiling in deionised water and measuring absorbance spectrophotometrically (Taylor & Naftalin, unpublished data). Using this quantification method at the end of the experiment gels were placed in 4ml deionised water, homogenised and heated to produce a clear non-particulate solution. Absorbance was read immediately at 515nm using a Cecil CE2020 2000 series spectrophotometer, against a heated, liquid, plain gel + NBT / INT unexposed to $O_2^-$. 
8.4 Direct measurement of $O_2^-$ in vitro – using NBT

8.4.1 Human studies

Human colonic endoscopic biopsies from control, UC and allergic colitis patients, were carefully removed from forceps and placed in individual screw capped culture bottles containing 4ml of modified Eagle’s culture medium (MEM, Sigma Chemicals Co.), with 0.1mg/ml NBT (0.122mM). Biopsies were cultured for two hours at room temperature with bubbling air after which they were orientated onto a small piece of foam using a dissecting microscope and fixed overnight at 4°C in freshly made periodate-lysine -2% paraformaldehyde (see appendix 3). Following fixation biopsies were washed in increasing concentrations of sucrose solution (10% and 20%), embedded in optimum cutting temperature compound (O.C.T., Bright instrument Company, Cambs.) and frozen in isopentane cooled in liquid nitrogen. 10µm cryostat sections were counterstained with Kernechtrot’s stain and mounted using glycerine jelly (Oshitani et al 1993). Following culture the MEM was immediately frozen for subsequent assay of lactate dehydrogenase (LDH) content as a measure of tissue viability (Wardle et al 1992). LDH is an intracellular enzyme released by dead and dying cells which catalyses the oxidation of lactate to pyruvate coupled to a reduction in nicotinamide adenine dinucleotide (NADH) measurable as an increase in absorbance at 340nm. For comparison LDH assay was also performed on haemolysed red blood cell samples in which LDH is released due to lysis of the cell membranes and MEM from a colonic biopsy incubated overnight at 37°C.

$O_2^-$ production within the tissues was studied using light microscopy visualised as areas of deep blue-violet NBF precipitate (chapter 9 figures 9,2 & 9,3). As the aim of detecting in vitro $O_2^-$ using NBT was to localise specific areas of $O_2^-$ production within colonic tissue and compare amounts produced in control and inflamed tissues, NBF quantification was assessed using a blind histological scoring system performed by two independent investigators (see appendix 2 table A,5). To look at the possible effects of PAF in potentiating $O_2^-$ production a second biopsy from two patients within
each of the three groups was taken and concurrently incubated in MEM + NBT in addition containing 10\(\mu\)M PAF.

### 8.4.2 Cellular sources of \(O_2^-\) - neutrophil immunoassay

The most prominent sources of ROM in the gut from patients with active IBD are phagocytes via the respiratory burst and AA metabolism (Karacylin et al 1990). Neutrophil ROM-mediated colonic secretion may be an important contributory factor to overall increased fluid secretion and diarrhoea arising in IBD. Neutrophil infiltration can be quantified in histological tissue sections by immunolabeling neutrophil elastase, a specific neutrophil enzyme stored principally in primary azurophilic granules for microbial killing and initiating tissue injury. (Damiano et al 1986, figure 8,2). Using this immunoassay I looked at the relationship between colonic \(O_2^-\) production, detected using NBT, and neutrophil infiltration in UC and allergic colitis patients.

#### 8.4.2.1 Method

Neutrophil elastase was labelled indirectly via an avidin-biotin complex method (figure 8,2). Avidin is a 68,000 molecular weight glycoprotein with an extraordinary high affinity (\(10^{15} M^{-1}\)) for the small molecular weight vitamin, biotin. Binding is rapid and irreversible. This biotin-avidin system can be exploited as avidin has four binding sites for biotin and most proteins, including antibodies (\(A_\beta\)) and enzymes, can be themselves conjugated with several molecules of biotin. Four steps are employed to identify and label the antigen of interest (figure 8,2),

Human endoscopic biopsies were fixed in 4% formaldehyde (24-36 hours) and embedded in paraffin. 5\(\mu\)m sections were dewaxed thoroughly with xylene, hydrated in absolute alcohol and rinsed in Tris-buffered saline (TBS pH7.6). All following incubations were carried out in a humidified, sealed chamber at room temperature. Between each incubation excess medium was tapped off and slides rinsed in TBS (5 minutes).
Figure 8.2 Schematic representation of neutrophil immunoassay technique.

1) Primary (1°) Aₐ against the antigen of interest, human neutrophil elastase, is first raised in another animal. This 1° Aₐ then recognises and binds to any elastase present in the colonic tissue section. 2) The 1° Aₐ is then detected by adding a biotinylated secondary (2°) Aₐ. 3) The 2° Aₐ is labelled using a preformed macromolecular complex between avidin and a biotinylated enzyme, which retains some of its biotin binding sites. 4) Finally a substrate which reacts with the biotinylated enzyme is added, alkaline phosphatase, to produce a visible, coloured, insoluble precipitate. A – avidin, B – biotin, E – enzyme.

**Incubation 1** - blocking serum, 20 minutes. Blocks all non-specific sites to which 1° Aₐ might bind, preventing high background staining.

**Incubation 2** - 1° unlabelled mouse monoclonal Aₐ, 30 minutes. Binds to any human neutrophil elastase in the colonic tissue section.

**Incubation 3** - biotinylated anti-mouse immunoglobulin (2° Aₐ), 30 minutes. Binds to 1° mouse Aₐ.

**Incubation 4** - avidin DH:biotinylated alkaline phosphatase H enzyme complex (APAAP complex), 30 minutes. Avidin portion binds to biotin molecules of the 2° Aₐ.

**Incubation 5** - alkaline phosphatase substrate, Fast Red TR/Napthol AS-MX 5 minutes. Used as a precipitating substrate to detect
alkaline phosphatase activity, with intense red stain produced where elastase complex is present.

After final rinsing in TBS (5 minutes) followed by tap water, slides were counterstained with Karrazi haematoxylin and mounted in aqueous glycerine jelly mounting medium.

8.5 Indirect measurement of free radicals in vitro
8.5.1 NAH (3-hydroxy-2-naphthoic acid hydrazide)

An index of free radical production can also be obtained indirectly via biochemical detection of secondary / end products arising from free radical mediated tissue damage. Such products include a group of highly reactive aliphatic aldehydes possessing a reactive carbonyl group (chapter 3, section 3.4.1.2). Carbonyls within a tissue can be directly visualised histochemically using the reagent 3-hydroxy-2-naphthoic acid hydrazide (NAH) coupled to Fast Blue B salt (FBB, Pompella & Comporti 1991). The hydrazide portion of NAH readily condenses with tissue bound carbonyls with these attachments made visible by azo-coupling to FBB to produce a deep blue violet stain (figures 8,3, 9,8 & 9,9). NAH-FFB has been demonstrated to be a reliable histochemical method of detecting lipid peroxidation in rat liver via light microscopy. NAH-FBB blue staining, quantified using microspectrophotometry, was found to positively correlate with standard biochemical measurements of MDA, taken as an index of lipid peroxidation, released in the incubation medium (Pompella & Comporti 1991). NAH alone is provided with fluorescence enabling its use to be extended to detection by LSCM. The LSCM was found to provide increased sensitivity, earlier visualisation of peroxidation products post induction of peroxidation and subcellular localisation of NAH fluorescence products so indicating specific sites of pathological oxidation (Pompella & Comporti 1993). This method has also been applied to investigate free radical activity in the anterior segment of cats’ eyes (Ishimoto et al 1996). My aim was to assess whether oxidative stress in inflamed human colonic tissue could be
imaged and assessed by LSCM after the fluorescent derivativisation of cellular carbonyls with NAH.

Figure 8.3 Scheme of the NAH-FBB reaction. 1) Reactive carbonyl, 2) 3-hydroxy-2-naphthoic acid hydrazide (NAH), 3) 3-hydroxy-2-naphthoic acid hydrazone, 4) Fast Blue B, 5) monocoupled azo dye and 6) dicoupled azo dye.
8.5.2 Method

Control and inflamed human colonic biopsies and non-inflamed mouse and rat liver were used. Human colonic biopsies were orientated in O.C.T. and immediately snap frozen in isopentane cooled in liquid nitrogen. Liver samples from freshly sacrificed animals, were cut into 5mm² pieces prior to snap freezing. Frozen tissues were stored at −80°C until required.

Fluorescent derivatization of carbonyls - 15μm cryostat sections were air dried (5 minutes), fixed in 5% trichloracetic acid (1 minute) and rinsed with 0.9% saline (4 x 5 minutes). Sections were reacted over night with 0.1% (w/v) NAH solution in 0.9% saline containing 10% (v/v) dimethylsulphoxide (DMSO) and 0.095% (w/v) p-toluenesulfonic acid (pTSA - a catalyst to hydrazone formation, Nöhammer 1989), at room temperature in a humidified chamber to prevent evaporation. Sections were then washed in a 15% (v/v) DMSO solution in 1mM HCl, to remove any unreacted NAH (4 x 5 minutes) followed by running tap water (5 minutes) and saline (5 minutes). Sections were mounted in glycerol jelly and stored at 4°C till examined (within 48hrs, Thomas et al 1994).

Visualisation of carbonyls

a). Confocal microscopy - sections were viewed using an upright LSCM (Leica, Beds, UK) and x10 (NA 0.45 NPL Fluotar) or x25 (NA 0.75 NPL Fluotar) oil immersion objectives. NAH can be maximally excited in the UV range at 267nm and also the visible range at 488nm. Tissue can therefore be excited using a Krypton/Argon laser at 488nm using a low pass filter and emission collected with a long pass 515nm barrier filter. Laser voltage, offset and gain were adjusted to obtain low emissions within the range of the 0-256 grey scale (chapter 5 section 5.4.1) remaining constant throughout individual comparative experiments of control vs. peroxidised sections

b). Light microscopy – for validation experiments confirmation of in vitro lipid peroxidation was performed histochemically by azocoupling NAH fluorescent products with FBB. Coupling was performed following NAH reaction and rinsing, by exposing sections to 0.1% (w/v) FBB solution in
0.1M phosphate buffer, pH 6.5 (20 minutes). After FBB coupling sections were washed in running tap water (5 minutes) followed by saline (5 minutes) before mounting in glycerol jelly (Nöhammer 1989). Visualisation of peroxidation was made by light microscopy with purple staining indicating areas of peroxidation (chapter 9 figures 9,8 & 9,9).

*Enzymic peroxidation* - validation of this novel methodology involved the peroxidation of control animal liver in vitro (chapter 9 section 9.4.2). Two free radical generating systems were used to induce peroxidation, an NADPH-ADP-iron pro-oxidant system and ABAP (2,2'-azobis-2-amidinopropane hydrochloride). ABAP is a water soluble, thermolabile azo compound which, on heating, forms carbon-centred radicals that can rapidly react with O₂ to yield peroxy radicals. These in turn are capable of abstracting H⁺ from membrane lipids so initiating the lipid peroxidation process (chapter 3 section 3.4.1.2). Frozen 15µm sections were incubated for 30mins at 37°C in either:

(i) 50mM Tris maleate buffer (pH 7.4), 0.15mM KCl and 1mM EDTA, (control buffer)

(ii) 50mM Tris maleate buffer pH 7.4, 0.15mM KCl and 0.2mM NADPH, 1mM ADP and 15µM FeCl₂, (pro-oxidant buffer)

(iii) 2mM or 5mM ABAP in PBS, (pro-oxidant buffer)

Following incubation sections were rinsed in a mixture of 50mM Tris maleate buffer and 0.15mM KCl (1 minute). Reaction with NAH ± FBB was then carried out as described above.

*Quantification* – methods used in previous studies include simple positive / negative staining or intensity measurements made by videoanalysis. For the purposes of experiments on human colon intensity measurements were made of defined regions of brightest intensity using Sigma Scan and NIH Image.
CHAPTER 9
Measurement of free radical production in inflamed colon.

9.1 Aims
Using both direct and indirect methods outlined in chapter 8 my aims were,
- to measure $O_2^-$ / oxidative stress in vivo and in vitro in human control and inflamed colon
- to identify possible cellular sources of $O_2^-$ in inflamed colonic tissue.

9.2 Direct measurement of $O_2^-$ in vivo in inflamed colon

9.2.1 Human studies
Agarose gels have successfully been used as vehicles to carry NBT in order to measure $O_2^-$ production in vivo in rat descending colon (Taylor & Naftalin unpublished data). My aim was to adapt this novel method for use in human studies. However tetrazolium salts are known to be toxic. Attempts were made to contain NBT/INT strictly within the agarose gel medium, such as enclosing gels in dialysis tubing to permit the exchange of electrolytes whilst restricting INT movement. This did not prove possible as INT was detectable in the incubating solution in which the INT gels were placed, (demonstrated as a visual / spectrophotometric colour change when an $O_2^-$ generating system was added - 300μM xanthine and 0.01unit/ml xanthine oxidase). This in vivo method was therefore not suitable for use in human subjects and was confined to animal studies only. $O_2^-$ production was successfully measured in human colon using NBT by an in vitro method (section 9.3.1).

9.2.2 Animal studies
My aim was to quantitatively measure $O_2^-$ production in vivo in non-inflamed rat descending colon exposed to PMA (to stimulate macrophage activation and $O_2^-$ release) using agarose gels containing 1mM INT ± PMA (10-20μM).
Elution and spectrophotometry were used to quantify formazan production. Initial experimental results demonstrated a detectable, quantifiable PMA-related increase in $O_2^-$ production in vivo although the increase detected was not statistically significant (figure 9.1). However these initial results proved to be non-reproducible and inconsistent when repeated in further experiments ($n = 5$). Therefore this in vivo method to detect and quantify $O_2^-$ was not pursued further.

Figure 9.1 Measurement and quantification of $O_2^-$ production in vivo in rat descending colon, using agarose gels containing INT. $O_2^-$ measured as a change in absorbance of reduced INT at 515nm. Results expressed as mean ± standard error of 4 gels, with $n = 1$ per PMA concentration used.

9.3 Direct measurement of $O_2^-$ in vitro in inflamed colon

9.3.1 Human studies

$O_2^-$ production in human endoscopic colonic biopsies was detected in vitro using NBT in control ($n=7$), UC ($n=7$) and allergic colitis ($n=10$) patients (age range 6 months to 17 years). The effects of PAF in stimulating $O_2^-$ production from infiltrating phagocytes, was also studied in a second biopsy from 2 patients within each patient group. Tissue viability was assessed by LDH assay (for all methods see chapter 8 section 8.4.1). The aim of detecting colonic $O_2^-$ in vitro using NBT was to localise specific areas where
O$_2^-$ is produced within colonic tissue and to compare amounts produced in control vs. inflamed tissues. Localisation and quantification of deep blue-violet NBF deposits in histological sections using light microscopy was therefore assessed using a blind scoring system performed by two independent investigators (table 9,1 & figure 9,2). Scores from patient groups are expressed as median + interquartile range. Multiple comparisons were made by Kruskal-Wallis one way analysis of variance on ranks. Differences between groups were made using Mann Whitney Rank Sum Test with significance level set at P<0.05 (figure 9,3).

<table>
<thead>
<tr>
<th>Histological Area</th>
<th>Categories</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamina propria</td>
<td>Fine</td>
<td>1</td>
</tr>
<tr>
<td>Positive cells/field of view x10 lens</td>
<td>1-5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5-10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>clumps</td>
<td>4</td>
</tr>
<tr>
<td>Crypt lining</td>
<td>Fine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Heavy/coarse</td>
<td>3</td>
</tr>
<tr>
<td>Surface epithelium</td>
<td>Fine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Coarse</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Patchy</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Continuous</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+tive colonocytes</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 9.1 Histochemical grading for NBF deposits in human colonic biopsies (adapted from Oshitani et al 1993).

See legend for figure 9,2
Figure 9.2 Histology showing NBF deposits in human inflamed colonic tissue sections to illustrate the different individual scoring categories shown in table 9.1. A) Coarse, continuous gut luminal surface epithelial deposits (mag. x250) B) Fine crypt luminal deposits with single positive cells scattered throughout the lamina propria (mag x250) C) Heavy / coarse crypt luminal deposits with large clumps of positive stained cells in lamina propria (mag. x400).
Figure 9,2 (continued) D) Fine luminal NBF deposits along the entire length of the crypt lumen (mag. x 400).
Figure 9.3 Superoxide production in control and inflamed human colonic tissue measured by NBF formation. A) Total NBF scores per patient group. B) Total scores from individual histochemical categories per patient group, with the significantly increased levels of NBF in UC compared to controls and allergic colitis, arising predominantly from an increase in positive NBF stained cells. Controls n=7, UC n=7, allergic colitis n=10.
9.3.1.1 Results

Formazan deposits were localised to the apical border of surface epithelial cells, interstitial cells and crypt lumina (figure 9,2A-D). Minimal $O_2^-$ was detected in control non-inflamed colon being noticeably confined to fine deposits lining crypt lumina only. No positive cells in the lamina propria were detected. Increases in NBF detected in patients with allergic colitis were not significantly different from control tissue. However inflammation arising from UC resulted in significantly increased levels of $O_2^-$ production compared to both controls and allergic colitis ($P<0.001$ and $P<0.004$ respectively). The most striking increase was the large number of strongly positive cells, single and in clumps, distributed throughout the lamina propria (UC v controls $P<0.001$ and UC v allergic $P<0.002$ figure 9,3). These positive cells could be detected migrating through both surface and crypt epithelium. NBF deposits were also denser lining both crypt lumina and surface epithelium of the gut lumen. Inclusion of PAF in the incubation medium produced a measurable increase in $O_2^-$ production but this did not reach statistical significance compared to paired controls (figure 9,4). Tissue remained intact and viable with comparatively low amounts of LDH detected in the culture medium after 2 hours. The small amount of activity detected could be attributable to LDH release from damaged cells due to the trauma of the biopsy forceps (figure 9,5).
Figure 9.4 The effects of PAF on $O_2^-$ production in control, UC and allergic patient groups combined. PAF increased $O_2^-$ production as detected by NBF deposits but this did not reach statistical significance.

Figure 9.5 Comparative measurements of lactate dehydrogenase activity from haemolysed red blood cells ($n = 2$), colonic biopsies cultured for a). 20 hours at 37°C ($n = 1$) and b). 2 hours under experimental conditions ($n = 15$).
9.3.2 Cellular sources of $O_2^-$

Neutrophils and eosinophils, prominent in colitic inflammatory infiltrate, are both capable of producing $O_2^-$ and other ROM products. In order to assess the major cellular source of $O_2^-$ the relationship between $O_2^-$ production (NBF deposits) and neutrophil/eosinophil infiltration in human inflamed colonic biopsies was assessed in control ($n = 5$), UC ($n = 5$) and allergic colitis ($n = 6$) patients. Neutrophils, labeled by elastase immunoassay (chapter 8 section 8.4.2.1) and eosinophils, by routine haematoxylin and eosin (H&E) stain were quantified by two independent investigators as the number of neutrophils/eosinophils per 10 high power ($\times400$) fields. In tissue sections that were too small ≥ 5 high power fields were counted. Mean cell count was then scored (table 9.2). The relationship with $O_2^-$ production and inflammatory cell infiltration was assessed by regression line analysis, with average number of neutrophils/eosinophils plotted against formazan scores from the same patients for each of the patient groups (figure 9,7). The degree of correlation was determined using the Spearman’s rank correlation coefficient ($r_s$, see appendix 1)

![Figure 9.6 Detection of neutrophils in human inflamed colonic tissue sections by neutrophil elastase immunoassay. Counterstained with Karrazzi haematoxylin, mag x100. Large numbers of infiltrating neutrophils can be seen predominantly pericryptally with some migrating through the surface epithelium.](image)
### Average neutrophil count per 10 HPF Score Average eosinophil count per 10 HPF Score

<table>
<thead>
<tr>
<th>Neutrophil Count</th>
<th>Score</th>
<th>Eosinophil Count</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 10</td>
<td>1</td>
<td>1 - 5</td>
<td>1</td>
</tr>
<tr>
<td>11 – 20</td>
<td>2</td>
<td>6 - 10</td>
<td>2</td>
</tr>
<tr>
<td>21 – 30</td>
<td>3</td>
<td>11 - 15</td>
<td>3</td>
</tr>
<tr>
<td>31 – 40</td>
<td>4</td>
<td>16 - 20</td>
<td>4</td>
</tr>
<tr>
<td>41 – 50</td>
<td>5</td>
<td>21 - 25</td>
<td>5</td>
</tr>
<tr>
<td>51 – 60</td>
<td>6</td>
<td>26 - 30</td>
<td>6</td>
</tr>
<tr>
<td>&gt;60</td>
<td>7</td>
<td>&gt;35</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 9.2 Scoring assessment for neutrophil and eosinophil infiltration of colonic tissue.

**Figure 9.7** Correlation of $O_2^-$ production and inflammatory cell infiltrate. A) Neutrophil infiltration, positive correlation observed $r^2 = 0.73$ with $P<0.002$ B) Eosinophil infiltration, no correlation was observed.

### 9.3.2.1 Results

The results show that in human colonic tissue a significant increase in the amount of $O_2^-$ detected occurs as a result of increasing neutrophil infiltration ($r_s = 0.85$, figure 9.7) with this relationship explaining over 70% of the total data variance ($r^2 = 0.73$, $P<0.002$). Amount of $O_2^-$ produced showed no correlation with eosinophil infiltration.
9.4 Indirect measurement of oxidative stress in inflamed colon

9.4.1 NAH

A series of experiments were carried out to assess whether oxidative stress, due to the generation of free radicals in colitis, could be visualised and measured in human colonic tissue after fluorescent labeling of cellular carbonyls with NAH. This method has been successfully applied to animal tissue, namely rat liver (Pompella & Comporti 1993), and cats' eyes (Ishimoto et al 1996) but not as yet to human colonic tissue.

From Pompella & Comporti's (1993) experiments on NAH-reacted liver sections from in vivo CCl₄-intoxicated rats and isolated hepatocytes exposed to CCl₄, lipid peroxidation was detected as areas of increased fluorescence intensity with specific subcellular localisation of these fluorescent reaction products made possible. Fluorescence was greatest in centrilocubular regions with involvement of perinuclear regions, specifically endoplasmic reticulum, and sparing of the nuclear compartment and peripheral cytoplasmic regions (figure 9.8).

Figure 9.8 False colour confocal images of oxidative stress by fluorescent derivativisation of cellular carbonyls with NAH, in rat liver. A) Control and B) 15 minute CCl₄ intoxicated rat. Colour scale bar shows black representing lowest intensity and pale pink highest, mag. x200. An overall increase in fluorescent intensity was detected in peroxidised liver compared to controls with specific increases localised around the centrilocubular region (CV). Nuclear sparing (N) can be clearly seen as blue dots (low intensity). (Taken from Pompella & Comporti 1993).
9.4.2 Initial experiments

My initial aim was to detect oxidative stress using this method by Pompella & Comporti (1993), in mouse and rat liver peroxidised in vitro. In vitro peroxidation and staining were carried out as described in chapter 8 (section 8.5.2). Figure 9B&C shows false colour confocal images of mouse and rat liver peroxidised in vitro using NADPH and stained with NAH ± FBB. Low power mouse images (x100) showed an increase in the intensity of fluorescent reaction products compared to controls. Maximum intensity was located in tissue immediately surrounding the centrilobular region. A higher power image (x250) of rat liver revealed some nuclear sparing, seen as dark areas of minimal intensity, surrounded by regions of increased fluorescence intensity. Peroxidation of tissue by this method was confirmed by light microscopy (9A1&2), visualised as a deep violet stain uniformly distributed throughout the whole tissue section. The same effects were found using ABAP as the peroxidising agent.

One problem encountered concerned the confocal imaging of NAH-reacted sections. Fluorescent derivativisation of cellular carbonyls using NAH alone often produced very low intensity fluorescence staining despite clear evidence of peroxidation from light microscopic detection of violet NAH-FBB staining in sequential reacted sections. However confocal imaging of these \textit{NAH-FBB} coupled sections revealed dramatically increased fluorescence intensity despite FBB’s lack of inherent fluorescence (figures 9,10 & 9,11). In all experiments confocal images were recorded from both NAH and azocoupled NAH-FBB stained sequential sections. Any comparative measurements were always performed on identically stained sections imaged under the same confocal settings. Staining details of images presented are fully outlined in figure legends. All confocal figures have a false colour look up table applied to clarify small variations in fluorescence intensity. Colour scale ranges from black representing lowest intensity through to pink/red highest intensity (for colour scale bar see figure 9,9).
Figure 9.9 Visualisation of oxidative stress in animal liver by fluorescent derivatization of cellular carbonyls using NAH-FBB. A) Light microscopy of rat liver section to confirm lipid peroxidation A1) control (mag. x250) and A2) peroxidised, (mag. x 100). B) False colour confocal images of B1) mouse liver control and B2) peroxidised, mag. x100. C1) Rat liver control and C2) peroxidised, mag. x250. CV = centrilobular region.
Having detected lipid peroxidation in rat and mouse liver, produced by in vitro incubation with an oxygen free radical generating system, using NAH and LSCM, identical experiments were repeated using human control colonic tissue. As the colon is quite resistant to peroxidation the strong prooxidant ABAP was used as the peroxidising agent (chapter 8 section 8.5.2). Results are presented as false colour confocal images and measurements of fluorescence intensity (figures 9,10 and 9,11). Analysis of fluorescence intensity was made by measuring a minimum of five regions of brightest intensity, usually occurring at the colonic crypt epithelial membranes, per field of view with a total of four separate fields analysed per one patient biopsy. Results are expressed as mean ± standard error of the collective total of measurements taken per patient studied. Comparative intensity measurements between control and peroxidised or colitic tissue were performed only when confocal settings were identical, with statistical significance assessed by Student’s t-test.
Figure 9,10 Imaging of oxidative stress using NAH, in human control colon peroxidised in vitro A) Light microscopy with purple staining indicating peroxidation, mag. x100. B&C) False colour confocal images. B1) Control and B2) peroxidised stained with NAH only C1) Control and C2) peroxidised stained with NAH+FBB. All confocal settings for B & C are identical, mag. x 100, c = crypt.
Figure 9.11 Comparison of fluorescence intensity of reaction products in NAH and NAH-FBB stained human control and peroxidised colonic tissue. Results expressed as mean ± standard error. Measured increase was significantly increased in both experimental conditions in peroxidised colon compared to control.

9.4.2.1 Results
Light microscopy of human colonic sections peroxidised in vitro revealed a small localised area of purple staining only, suggesting that lipid peroxidation was not as extensive or uniform as seen in liver sections (figure 9.9A [liver] and 9.10A [colon]). Detection of lipid peroxidation by confocal imaging of NAH and NAH-FBB derived fluorescent reaction products demonstrated significant increases in measured fluorescence intensity in peroxidised colon compared to non-peroxidised control (figure 9.10, NAH P<0.001 and NAH-FBB P<0.01). Fluorescent products were predominantly localised to basolateral epithelial membranes of colonic crypts. Intense white fluorescent delineation of crypt and surface epithelial lipid membranes can be more clearly seen in FBB coupled sections (figure 9.9 C2).
9.4.3 Studies in IBD

Having detected an increase in NAH fluorescent reaction products in human colon peroxidised in vitro, comparative studies on colonic biopsies from IBD patients were performed, UC (n = 4) vs. control non-inflamed colon (n = 6). Following collection biopsies were orientated in O.C.T., immediately snap frozen and stored at -40°C. Biopsies were processed in pairs with each comparative study performed on reacted colonic sections from one colitic and one control patient (n = 6 paired experiments performed). All confocal settings - gain, offset, laser power and magnifications were kept constant throughout these comparative experiments (full method see chapter 8 section 8.5.2). Fluorescence intensity was measured as described previously. Data is presented from one experiment, (figures 9.12 & 9.13) with individual data from the other five studies presented in appendix 2 (figure A.15).

**False colour confocal images of human control and colitic colon**

![Confocal images](image)

Figure 9.12 Confocal imaging of oxidative stress in NAH-reacted human control and inflamed colon. A) Low power mag. x100 A1) control and A2) inflamed. B) High power x250 B1) control and B2) inflamed, c = crypt Experiment repeated on n = 5 paired studies.
Figure 9.13 Comparison of fluorescence intensity of reaction products in NAH-reacted control vs. colitic human colon. Fluorescence intensity under the locus of the straight line is shown as an intensity histogram. Increased intensity in colitic tissue is clearly localised to the basolateral cell membranes. Mag. of confocal images x100.
9.4.3.1 Results

In all six experiments performed, overall measured fluorescence intensity was increased in colitic colonic specimens compared to control reaching significance in 75% of these (figure 9.14 & appendix 2 figure A.15). Fluorescent NAH reaction products were clearly localised to the basolateral membranes of crypt epithelial cells with little fluorescence detected in the lamina propria or apical crypt membranes (figures 9.12 & 9.13).

9.5 Discussion

9.5.1 Direct detection methods - NBT

Qualitative, direct measurement of colonic O$_2^-$ production in vitro using the tetrazolium salt NBT was achieved in children with and without colonic
inflammation. The data confirmed that O$_2^-$ production is significantly increased in inflamed colonic tissue from UC patients compared to both controls and allergic colitis patients (figure 9,3). No significant difference was detected in allergic colitis vs. controls despite the presence of large numbers of infiltrating inflammatory cells seen on routine histology. These detected differences in O$_2^-$ production in inflammation were related to the specific cellular profile of inflammatory cells within the tissue. A positive correlation was demonstrated between the amount of NBF detected within tissue and number of infiltrating neutrophils ($r^2 = 0.73$, P<0.002), in comparison to eosinophil infiltrate which bore no correlation (figure 9,7). Neutrophils are known to be a major source of O$_2^-$ produced in vivo via the respiratory burst and, from the data presented, are in part responsible for the increased levels of ROM detected in colonic tissue from the UC patients studied.

9.5.2 Indirect detection methods - NAH

Derivativisation and subsequent measurement of tissue carbonyls, as an index of oxidative stress, has been successfully carried out using the fluorescent probe NAH in conjunction with LSCM (Pompella & Comporti 1993 and Ishimoto et al 1996). Application of this methodology to assess the contribution of free radicals to colonic inflammation in human tissue was also achieved. Fluorescent intensity was higher in inflamed human colonic tissue compared to controls in 75% of the patients studied with fluorescent reactions products predominantly localised to regions of highest lipid composition (figures 9,13 & appendix 2 figure A,15). Basolateral crypt membranes showed the greatest increase in fluorescent reaction products with little fluorescence detected in the surrounding lamina propria or apical crypt membranes (figures 9,12 & 9,13). In studies to detect oxidative stress in peroxidised rat liver the detection of NAH fluorescence offered a gain in sensitivity over the two step NAH-FBB method, with a clear increase in fluorescence detectable within 15 minutes of peroxidation compared to no stain after 2 hours using NAH-FBB. Liver is an ideal tissue in which to assess oxidative stress due to high concentrations of cytochrome P-450 resulting in high rates of lipid peroxidation. In colonic tissue, relatively low in
P-450, reaction with NAH alone often produced very low intensity fluorescence staining, presenting difficulties when imaging, by comparison to FBB-coupled staining in which overall intensity of staining was much greater. Despite this, significant differences between control and inflamed tissue were detected in both NAH and NAH-FBB stained tissue.

The large numbers of NBT-positive cells infiltrating the lamina propria and the detection of carbonyl peroxidation products localised to crypt basolateral epithelial membranes reveals that the greatest exposure to free radicals occurs in pericryptal and extracellular regions of the lamina propria. Exposure of major structural proteins of the extracellular matrix to high levels of oxidative stress, as well as neutrophils enzymes, has implications regarding maintenance of normal tissue architecture (chapter 3 section 3.4.1.1). For example disruption of tight junctional complexes would impair the generation of a hypertonic absorbate and therefore fluid absorption. Evidence for luminal exposure, of apical crypt and surface epithelial membranes, to oxidative stress varied between the two methods. Little increase in fluorescent products was detected using NAH suggesting minimal exposure, yet NBT deposits were clearly present on both crypt and surface epithelial apical membranes. The formation of crypt abscesses following the serosal to mucosal migration of neutrophils would result in luminal $O_2^-$ release. However one can not entirely dismiss that some degree of the heavier NBT surface and crypt luminal staining may arise as an artifact. Paired studies using the two techniques, with accompanying routine histology on inflamed biopsies from the same region in one patient would be useful in addressing this question further.
This study has examined the effects of inflammation on structural and functional relationships in human colonic tissue, with reference to crypt absorptive capacity, density, architecture and pericryptal F-actin sheath. The contribution of free radicals and oxidative stress in colitis was also examined. The aims of the study were three-fold. Firstly to adapt two novel methods developed in animals, for the study of human colonic fluid transport; in vivo using agarose gels and in vitro by confocal microscopy using isolated perifused endoscopic biopsies. Secondly to apply the latter confocal methodology to the study of crypt structural and functional relationships in health and inflammation. Thirdly to assess the contribution of free radicals produced within colonic tissue to the inflammatory process underlying the observed physiological changes.

The effects of inflammation on the colon are complex with no one single factor being responsible for the subsequent pathophysiology. The focus of this study on the effects of inflammation on colonic crypts forms only part of the story with surface epithelial cells also contributing to overall colonic salvage function. Net rectal fluid and electrolyte fluxes in vivo were measured in children using agarose gels. However although successful in animal studies, these gels proved to lack the sensitivity required to detect normal rectal transport as well as being accompanied by practical limitations when used in children (detailed in chapter 4 section 4.4.1).

I have demonstrated using novel confocal methodology that in health human paediatric colonic crypts exhibit a dual functional nature in vitro, with a capacity for both basal fluid absorption and, in response to stimuli, fluid secretion. Basal crypt function and structure were altered in inflamed colonic tissue. Inflammation arising from ulcerative colitis (UC) was associated with increased crypt absorption compared to controls in the remaining individual intact crypts measured. This initial observation seemingly contradicts the clinical picture of a lack of colonic salvage with diarrhoea that accompanies UC. Two factors need to be taken into account when interpreting these
initial results. Firstly structural studies on these patients reveal a significant reduction in overall crypt density compared to controls. Consequently the potential number of basally absorbing crypts is limited, affecting net fluid salvage. Secondly adequate visualisation of functioning crypts using this confocal methodology relies on the luminal accumulation of fluorescent FD. It is possible therefore that crypts in which no FD accumulates in the lumina would not be clearly visible using this method. One such example could be in the case of crypts stimulated by inflammatory mediators to actively secrete fluid into the crypt lumina, which could serve to prevent luminal FD accumulation. To obtain a more accurate representation of the predominant fluid flux, secretory as well as absorptive data would be required and would need to be expressed in terms of the proportion of the total number of crypts within the region studied. In addition ideally more than one biopsy per patient should be studied where feasible.

In contrast to UC colonic tissue from patients with an allergic inflammatory response retained normal crypt density with luminal FD accumulation occurring uniformly in the majority of crypts throughout the field of view. Initial FD accumulation however was not followed by FD concentration, visualised as increasing fluorescence intensity. This may indicate a smaller crypt secretory response thereby allowing limited luminal FD accumulation, and/or a reduced absorptive capacity. In these patients confocal visualisation of crypt function would be more representative of overall fluid transport as the majority of crypts were visible due to luminal FD accumulation.

This finding that inflammation alters basal crypt function and disrupts crypt architecture is not new or surprising. However my observations further reveal that the distinctive changes in structural and functional relationships were closely associated with the specific cellular nature of the inflammatory infiltrate. The predominance of an eosinophilic infiltrate, termed an allergic colitis, appeared to target crypt function whilst retaining normal crypt density and structure. In contrast a predominantly neutrophilic infiltrate, evident in acute/chronic UC, accompanied maximal structural damage. This clear
distinction maybe an important factor in defining the contribution of these inflammatory cells in mediating pathophysiological intestinal responses.

Both neutrophils and eosinophils elicit adverse effects via a panoply of mediators including secretagogues, reactive oxygen metabolites (ROMs) and lipid mediators. Any one of these may contribute to the reduction in effective salvage function and production of diarrhoea. Free radical assays performed revealed an increased exposure to oxidative stress in inflamed colon with neutrophil infiltration positively correlating with superoxide (O$_2^-$) production. Greatest free radical exposure occurred in pericryptal and extracellular regions of the lamina propria. Large numbers of NBT-positive cells were detected infiltrating the lamina propria and carbonyl peroxidation products were localised specifically to basolateral crypt epithelial membranes, visualised using NAH. The potential effects of neutrophil-released ROMs include lipid peroxidation and solubilisation of extracellular matrix proteins. The data presented would strongly suggest that the pronounced structural disruption associated with a large neutrophil presence, as seen in UC, could be in part attributed to neutrophil ROM-mediated damage. Infiltrating neutrophils also migrate across colonic epithelia into crypt and gut lumina, with the formation of crypt abscesses in severe UC. The presence of luminal O$_2^-$ was confirmed using NBT. In addition to ROM-mediated structural damage, functional changes are also produced. This was demonstrated in in vivo studies on rat descending colon in which luminal exposure to platelet activating factor induced fluid secretion via a O$_2^-$-dependent C afferent fibre reflex. These studies combined confirm that ROM are directly involved in the altered physiology that accompanies colonic inflammation.

The pathology of intestinal damage in colitis is important in providing insight into how disease activity might be modified by specific treatments, for example antioxidants and immunomodulation. In childhood inflammatory bowel diseases (IBD) morbidity and mortality arise as a consequence of both the disease process itself and the side effects of treatment with immunosuppressive and anti-inflammatory agents. Attention has recently
been focused on the beneficial properties of adjunctive fish oil therapies, high in polyunsaturated fatty acids (PUFA) in the treatment of IBD (Stenson et al 1992, Greenfield et al 1993 and Belluzzi et al 1996). Preliminary evidence suggests that their beneficial effects may be attributable to an alteration in the profiles of eicosanoid inflammatory mediators with the production of less proinflammatory products. Neutrophil chemoattractants LTB₄ and 12-HETE, are among those reduced by fish oils therefore providing the potential for decreased free radical production within inflamed colonic tissue (Sperling et al 1993). The methods developed within this project allow the visualisation and measurement of crypt function and free radical production using human endoscopic biopsy material. A future aim arising from this study involves the application of these methods to assess the effects of high dose gamma linolenic acid supplementation (a long chain PUFA) on crypt transport function, structure and free radical production in the colon of children with IBD.
CONCLUSIONS

♦ Normal structural and functional relationships in human colonic crypts are disrupted as a result of colonic inflammation with the specific nature of the inflammatory infiltrate emerging as an important contributory factor.

♦ Exposure of human colon to oxidative stress is significantly increased in the face of inflammation. Detection using confocal microscopy enabled visual localisation of oxidative stress and provides the potential for quantification.

♦ The methodologies developed for visualising and measuring crypt fluid transport and oxidative stress in human colonic tissue may serve as useful tools to assess the effectiveness of potential therapeutic agents on crypt function and structure.
APPENDIX 1
Statistical methods and presentation
adopted in the thesis

This appendix outlines the standard format used to present and analyse data within this thesis. All statistical tests were performed using a computer program entitled SigmaStat by Jandel Scientific software.

1. PARAMETRIC DATA - presentation and analysis
Parametric data is that which is continuous and follows a standard ‘bell’ shaped Normal ‘Gaussian’ distribution. It can be accurately described by two parameters - mean and standard deviation (SD). All parametric data in this thesis is presented as mean with error bars representing standard error of the mean (SEM = SD/√n, with n = sample size) as this improves the clarity of the figures.

Student’s two sample t-test
Comparisons of two independent normally distributed groups were performed using the Student’s two-sample t-test. The t statistic is the ratio of the difference of sample means to the standard error of difference of the sample. First the mean and SD for each sample are calculated (m1, SD1, m2, SD2) from which a common SD is then obtained,

\[ s = \sqrt{\frac{SD_1^2(n_1 - 1) + SD_2^2(n_2 - 1)}{n_1 + n_2 - 2}} \]

s is then used to calculate the common error of difference between the two means

\[ SEM = s \times \sqrt{\frac{1}{n_1} + \frac{1}{n_2}} \]
Finally the t statistic can be calculated and a probability value obtained from t-tables

\[ t = \frac{(m_1 - m_2)}{SEM} \]

A large \( t \) indicates that the difference between the two group means is larger than what would be expected from sampling variability and therefore the null hypothesis, that there is no difference, can be rejected. It should be noted that t-tests do not tell you anything about the size of the difference. Conventionally in the science community probability values are ascribed the following levels:

<table>
<thead>
<tr>
<th>Probability (P) value</th>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 (5%)</td>
<td>*</td>
<td>Significant</td>
</tr>
<tr>
<td>0.01 (1%)</td>
<td>**</td>
<td>Very significant</td>
</tr>
<tr>
<td>0.001 (0.1%)</td>
<td>***</td>
<td>Highly significant</td>
</tr>
</tbody>
</table>

2. NON-PARAMETRIC – presentation and analysis

Samples of continuous data that do not follow a Normal distribution or discontinuous categorical data are termed non-parametric. They are best described by their median and interquartile range – depicted graphically as a box and whiskers plot, which is the format used to present all non-parametric data in this thesis (see below),

Non parametric data requires analysis by either specific non-parametric tests or transformation of the raw data.
Non parametric one way ANOVA - Kruskal-Wallis test
Where comparisons are made between more than one group of ranked data analysis of variance (ANOVA) is assessed first using this test. In essence the complete set of \( N \) observations are ranked from 1-\( N \) regardless of which group they are in and for each group the sum of ranks and the test statistic, \( H \), are calculated. When the null hypothesis is true this statistic follows the Chi-squared distribution. A significant result implies that the null hypothesis, that the groups come from populations with the same median, should be rejected instead concluding that there are real differences between the groups. Two sample Mann Whitney U test is then used to find where these differences lie.

Mann-Whitney U test
The Mann-Whitney U test is used to test the null hypothesis that two samples were not drawn from populations with different medians. It requires that all the observations are ranked as if these were a single sample. The ranks for each group are summed and the rank sums compared. The U statistic is calculated by computer. If there is no difference between the two groups, the mean ranks should be approximately the same. If they differ by a large amount you can assume that the low ranks tend to be in one group and the high ranks in another and conclude that the samples were drawn from different populations so rejecting the null hypothesis.

3. Correlation and regression analysis
Correlation
Correlation is the method of analysis used when studying the possible association between two continuous variables. The degree of correlation, calculated as the correlation coefficient \( (r) \), indicates the strength of the association by measuring the scatter of the points around an underlying trend, the greater the scatter the lower the correlation. \( r \) ranges from \(-1\) to \(1\), with \( r = 1 \) if \( x \) and \( y \) are perfectly correlated and \( r = 0 \) if there is no correlation. The sign of \( r \) indicates if \( y \) increases (+) or decreases (-) as \( x \)
increases. The significance of \( r \) depends on \( r \) and \( n \) and can be round in \( r \) tables with the null hypothesis being one of no association. For continuous data correlation is quantified by the Pearson's correlation coefficient (\( r \)) and for ranked discontinuous data the Spearman's rank correlation coefficient (\( r_s \)), both of which are used in this thesis.

**Regression line analysis**

Regression line analysis describes the relation between two continuous variables and allows the prediction of the value of one variable for an individual when only one set of variables is known. The least squares regression line can be described by the equation \( y = ax + b \) with \( a = \) the intercept and \( b = \) the slope. This line explains a proportion of the variability in the dependent variable \( (y) \) and the amount of unexpected variability. A means of assessing the 'goodness of fit of the line' is to calculate the proportion of the total variation explained by the model. This is usually done by considering the sum of the squares explained by the regression as a percentage of the total sum of squares. This statistic is called \( r^2 \) (being the square of the correlation coefficient \( (r) \) of the two variables). Therefore \( r^2 \) expresses the % variance of the total data that can be explained by the dependent variable being determined. It is an important statistic as often even small \( r \) values prove to be significant, for example \( n = 100, \ r = 0.2 \) with a significance of \( P < 0.05 \). Yet the so called 'significant' relationship explains less than 5% \( (r^2 = 0.04 / 4\%) \) of the total variance and would be described as a confounding variable.
A.1 Introduction
This appendix provides additional confocal patient data to that presented in the chapters 5 – 7 & 9. The reader should refer to the individual chapters for information relating to methods used and general discussion of the cumulative results.

A.2 Colonic crypt concentration profile
Additional patient data to demonstrate the ability of human colonic crypts to concentrate the impermeant fluorescence probe FITC-dextran (chapter 5 section 5.5).
Figure A.1 Concentration profiles of FD down crypt luminal axis in human non-inflamed colon. Data represents mean ± standard error of fluorescence intensity of 3-6 crypt lumina per patient (n = 9 in total, see figure 5.11 for patient 9).
A.3 Colonic crypt function in vitro in human control colon.

A.3.1 Basal crypt transport

Additional patient data to show basal rates of luminal FD concentration over time by colonic crypts obtained in human non-inflamed colon (chapter 6 section 6.2.1).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Colonic region</th>
<th>Rate of increase in fluorescence intensity (grey scale 0 – 256)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Lumen</strong></td>
<td><strong>Pericrypt</strong></td>
</tr>
<tr>
<td>Patient 1</td>
<td>Caecum</td>
<td>$y = 5.9x - 56.2$</td>
<td>$y = 1.62x - 13.6$</td>
</tr>
<tr>
<td>Patient 2</td>
<td>Caecum</td>
<td>$y = 3.11x + 18.4$</td>
<td>$y = 0.12x + 1.4$</td>
</tr>
<tr>
<td>Patient 3</td>
<td>Ascending</td>
<td>$y = 4.75x - 28$</td>
<td>$y = 0.77x - 1.69$</td>
</tr>
<tr>
<td>Patient 4</td>
<td>Ascending</td>
<td>$y = 11.8x - 11.4$</td>
<td>$y = 2.26x - 18.01$</td>
</tr>
<tr>
<td>Patient 5</td>
<td>Ascending</td>
<td>$y = 2.5x + 10.4$</td>
<td>$y = 0.57x + 0.4$</td>
</tr>
<tr>
<td>Patient 6</td>
<td>Transverse</td>
<td>$y = 4.36x - 27.2$</td>
<td>$y = 1.03x - 2.83$</td>
</tr>
<tr>
<td>Patient 7</td>
<td>Transverse</td>
<td>$y = 6.8x - 39.6$</td>
<td>$y = 0.48x - 2.59$</td>
</tr>
<tr>
<td>Patient 8</td>
<td>Descending</td>
<td>$y = 11.79x - 36.4$</td>
<td>$y = 0.6x - 1.7$</td>
</tr>
<tr>
<td>Patient 9</td>
<td>Descending</td>
<td>$y = 10.9x - 17.9$</td>
<td>$y = 3.46x - 22.1$</td>
</tr>
<tr>
<td>Patient 10</td>
<td>Descending</td>
<td>$y = 11.71x - 32.1$</td>
<td>$y = 3.56x - 14.1$</td>
</tr>
<tr>
<td>Patient 11</td>
<td>Descending</td>
<td>$y = 11.55x - 137$</td>
<td>$y = 2.09x - 24.3$</td>
</tr>
<tr>
<td>Patient 12</td>
<td>Descending</td>
<td>$y = 7.04x - 33.3$</td>
<td>$y = 1.74x - 0.2$</td>
</tr>
<tr>
<td>Patient 13</td>
<td>Descending</td>
<td>$y = 3.18x - 25.8$</td>
<td>$y = 0.49x - 2.3$</td>
</tr>
</tbody>
</table>

Table A.1 Basal rates of FD concentration by human non-inflamed colonic crypts and adjacent pericryptal areas from a range of colonic regions. Rates represent rate of increase in fluorescence intensity over time measured in grey scale units (gsu) min⁻¹.
Figure A.2 Time course of FD concentration by non-inflamed human colonic crypts in vitro at constant depth (~40µm). Rate of increase in fluorescence intensity expressed as mean ± standard error of 4-6 crypt lumina and adjacent pericryptal regions with an increase in intensity representing FD concentration (n = 13 in total).
For figure legend see A,2.
A.3.2 Nature of crypt FD concentration

Additional patient data from experiments to study the effects of inhibiting active Na\(^+\) absorption, using amiloride or ouabain, on passive water transport as measured by crypt FD concentration (chapter 6 section 6.2.2)

A.3.2.1 Amiloride

<table>
<thead>
<tr>
<th>Patient</th>
<th>Rate of increase in fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lumen</td>
</tr>
<tr>
<td>Patient 1</td>
<td>y = -0.19x + 59.3</td>
</tr>
<tr>
<td>Patient 2</td>
<td>y = 2.06x + 108.5</td>
</tr>
<tr>
<td>Patient 3</td>
<td>y = -0.89x + 129.1</td>
</tr>
<tr>
<td>Patient 4</td>
<td>y = 8.96x + 69.1</td>
</tr>
<tr>
<td>Patient 5</td>
<td>y = 7.11x - 36.9</td>
</tr>
</tbody>
</table>

Table A.2 Rate of increase in crypt luminal and pericryptal FD fluorescence intensity in human colonic tissue in the presence of amiloride. Rates represent increases in fluorescence intensity over time, measured in gsu min\(^{-1}\).

For figure legend see A, 3.
Figure A.3 The effects of amiloride on FD concentration by human colonic crypts in vitro. Data expressed as mean ± standard error of 4-6 crypt lumina and adjacent pericryptal regions (n = 5 in total).
A.3.2.2 Ouabain

<table>
<thead>
<tr>
<th>Patient</th>
<th>Rate of increase in fluorescence intensity</th>
<th>Pericrypt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lumen</td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>$y = 1.22x + 28.8$</td>
<td>$y = -0.02x + 10.1$</td>
</tr>
<tr>
<td>Patient 2</td>
<td>$y = 0.91x + 19.8$</td>
<td>$y = 0.66x + 2.03$</td>
</tr>
<tr>
<td>Patient 3</td>
<td>$y = 12.5x - 82.6$</td>
<td>$y = 8.31x - 55.1$</td>
</tr>
<tr>
<td>Patient 4</td>
<td>$y = 6.76x - 58.8$</td>
<td>$y = 9.37x - 94.4$</td>
</tr>
</tbody>
</table>

Table A.3 Rate of increase in crypt luminal and pericryptal FD fluorescence intensity in human colonic tissue in the presence of ouabain.

Figure A.4 The effects of ouabain on FD concentration by human colonic crypts in vitro. Data expressed as mean ± standard error of 4-6 crypt lumina and adjacent pericryptal regions ($n = 4$ in total).
A.3.3 Measurement of crypt secretion

Additional patient data showing the effects of carbachol on FD concentration by non-inflamed human colonic crypts (chapter 6 section 6.2.4)

For figure legend see A.5.
Figure A.5 Time course to show the effects of carbachol on FD concentration by human colonic crypts in vitro. Results are expressed as mean ± standard error of 4-6 crypts with increase in intensity representing FD concentration and decrease dilution (n = 6, see chapter 6 figures 6, 8 & 6, 9 for patients 1&2).
A.4 Colonic crypt transport in vitro in human inflamed colon

Additional patient data showing the effects of inflammation on the ability of human colonic crypts to concentrate lumenal FD. Patients studied include those with ulcerative colitis (n = 4) and allergic colitis (n = 4), (chapter 6 section 6.3).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Rate of increase in fluorescence intensity (gsu min⁻¹)</th>
<th>Control</th>
<th>Ulcerative colitis</th>
<th>Allergic colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td></td>
<td>11</td>
<td>20.2</td>
<td>3.96</td>
</tr>
<tr>
<td>Patient 2</td>
<td></td>
<td>7.04</td>
<td>18.56</td>
<td>-0.03</td>
</tr>
<tr>
<td>Patient 3</td>
<td></td>
<td>11.79</td>
<td>25</td>
<td>0.02</td>
</tr>
<tr>
<td>Patient 4</td>
<td></td>
<td>3.18</td>
<td>15</td>
<td>2.28</td>
</tr>
<tr>
<td>Patient 5</td>
<td></td>
<td>10.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patient 6</td>
<td></td>
<td>11.71</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mean± sem</td>
<td></td>
<td>9.10 ± 1.36</td>
<td>19.64 ± 2.08</td>
<td>1.55 ± 0.96</td>
</tr>
</tbody>
</table>

Table A.4 Rates of increase in crypt luminal FD fluorescence intensity in control and inflamed human colonic tissue.
A.4.1 Ulcerative colitis

Figure A.6. The effects of inflammation due to ulcerative colitis on in vitro crypt transport in human descending colon. Results expressed as mean ± standard error of 4–6 crypt lumena (n = 4 patients studied). (Y-axis scale exceeds 0–256 grey scale as it was necessary to attenuate laser power during the experiments (see chapter 5 section 5.4.1.1.5 for details).
A.4.2 Allergic colitis

Figure A,7 The effects of inflammation due to allergic colitis on in vitro crypt transport in human descending colon. Results expressed as mean ± standard error of 4 – 6 crypt lumina (n = 4 patients studied).
A.5 Colonic structure in health

Figure A.8 Human colonic biopsies stained with BODIPY-Phalloidin to show normal colonic structure – crypt density, architecture and pericryptal F-actin sheath, \( n = 8 \).
A.6 Colonic structure in inflammation
A.6.1 Allergic colitis

Figure A.9 Human colonic biopsies stained with BODIPY-Phalloidin to show the effects of inflammation arising from allergic colitis on colonic structure, (n = 8).
A.6.2 Ulcerative colitis

Figure A.10 Human colonic biopsies stained with BODIPY-Phalloidin to show the effects of inflammation arising from ulcerative colitis on colonic structure, \( n = 8 \)
A.7 Effects of inflammation on colonic structural and functional relationships.
See chapter 7 section 7.5 for patients’ clinical details. All results expressed as mean ± standard error of 6-8 crypt lumina and adjacent pericryptal regions, with all confocal magnifications x 100.

A.7.1 Normal structure and function

Figure A.11 Normal structure and function, patients 1 and 2.
Figure A.12 Normal structure and function, patients 4 and 5.
A.7.2 Normal structure, abnormal function

Figure A.13 Normal structure and abnormal function, patients 8 and 9
A.7.3 Abnormal structure and function

Figure A.14 Abnormal structure and function, patients 13 and 14.
A.8 Direct measurement of O$_2^-$ production in vitro using NBT

Tables detailing NBT scoring system used and a breakdown of individual patient scores from experiments to look at the effects of inflammation on colonic O$_2^-$ production (chapter 9 section 9.3.1).

<table>
<thead>
<tr>
<th>Histological Area</th>
<th>Categories</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamina propria</td>
<td>Fine</td>
<td>1</td>
</tr>
<tr>
<td>Positive cells/field of view (x100)</td>
<td>1-5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5-10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>clumps</td>
<td>4</td>
</tr>
<tr>
<td>Crypt lining</td>
<td>Fine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Heavy/coarse</td>
<td>3</td>
</tr>
<tr>
<td>Surface epithelium</td>
<td>Fine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Coarse</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Patchy</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Continuous</td>
<td>3</td>
</tr>
<tr>
<td>+tive colonocytes</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patients</th>
<th>Lamina propria</th>
<th>Positive cells</th>
<th>Crypt lining</th>
<th>Surface</th>
<th>Total score per patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Patient 2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Patient 3</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Patient 4</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Patient 5</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Patient 6</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Patient 7</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Patient 2</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Patient 3</td>
<td>-</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Patient 4</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Patient 5</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Patient 6</td>
<td>-</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Patient 7</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Allergic colitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Patient 2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Patient 3</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Patient 4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Patient 5</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Patient 6</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Patient 7</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Patient 8</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Patient 9</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Patient 10</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>3</td>
</tr>
</tbody>
</table>

Table A,5 A) NBT scoring system, B) Individual patient NBT scores
A.9 Measurement of colonic oxidative stress using NAH ± FBB

Additional patient data from studies to compare oxidative stress in control and inflamed human colon (chapter 9 section 9.4.3)

Figure A.15 Measurement of oxidative stress in control and colitic human colonic tissue using A) NAH ± FBB (n=4) and B) NAH only (n=2). Results expressed as mean ± standard error with comparisons made by Student’s t-test. Fluorescence intensity of NAH reaction products was significantly increased in inflamed tissue compared to controls in four patients.
This appendix outlines any additional methodology details not included in the main text.

Periodate-lysine -2% paraformaldehyde
Freshly made periodate-lysine -2% paraformaldehyde was used to fix human colonic biopsies following incubation with nitroblue tetrazolium in the detection of free radical production (chapter 8 section 8.4.1).

Method
5g paraformaldehyde was added to 250ml of deionised water heated to 70°C. 2 drops of 4M sodium hydroxide were added to assist dissolving the paraformaldehyde in order to produce a clear solution. 125mls 0.1M phosphate buffer pH 7.4 was added to the solution, which was then cooled and filtered. 0.2M lysine-HCl was made by adding 1.83g lysine-HCl to 50mls of distilled water, pH to 7.4 using 0.1M dibasic sodium phosphate and made up to 100mls with 0.1M phosphate buffer pH7.4. Immediately prior to use 33mls of buffered paraformaldehyde was added to 100mls lysine-HCl with solid sodium periodate to a concentration of 10mM.
Bibliography

Circulating human leucocyte elastase in patients with inflammatory bowel
disease.
Gut 26: 1306-1311.

2. Ahnfelt-Rønne I., Nielsen O.H., Christensen A., Langholz E., Binder V., Riis P.
(1990)
Clinical evidence supporting the radical scavenger mechanism of 5-
aminosalicylic acid.
Gastroenterology 98: 1162-1169.

Increased absorption of polyethylene glycol 600 deposited in the colon in active
ulcerative colitis.
Gut 34: 509-513.

The quantitative elution of nitro-blue formazan from tissue sections.
Histochemie 17: 319-326.

5. Altman P.P. (1976a)
The quantification of formazans in tissue sections by microdensitometry II. The
use of BPST, a new tetrazolium salt.
Histochem. Journal 8: 501-506.

6. Altman F.P. (1976b)
The quantification of formazans in tissue sections by microdensitometry III. The
effect of objective power and scanning spot size.
Histochem. Journal 8: 507-511.

Practical statistics for medical research.
Chapman & Hall Press, UK.

Biosynthesis of platelet-activating factor in normal and inflamed human colon
mucosa: evidence for the involvement of the pathway of platelet-activating factor
synthesis de novo in inflammatory bowel disease.

The absorption and secretion of water and electrolytes across the healthy and
the diseased human colonic mucosa measured in vitro.
Role of interleukin-8 on leukocyte-endothelial cell adhesion in intestinal inflammation.
Gut 38: 911-915.

A fluorescence method for the visualization of inflammatory cells in intestinal mucosa.

Oxygen radicals in ulcerative colitis.

Oxygen-dependent microbial killing by phagocytes.

The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes.

Rat enterocyte injury by oxygen-dependent processes.
Gastroenterology 101: 716-720.

16. Baldassano R.N., Schreiber S., Johnston R.B., Fu R.D., Muraki T.,
Crohn’s disease monocytes are primed for accentuated release of toxic oxygen metabolites.
Gastroenterology 105: 60-66.

Treatment with tumour necrosis factor inhibitor oxypentifylline does not improve corticosteroid dependent chronic active Crohn’s disease.
Gut 40: 470-474.

(1996)
Endoscopic assessment of the colonic response to corticosteroids in children with ulcerative colitis.


   A study of water and electrolyte transport by the excluded human colon.

   Tumour necrosis factor alpha in stool as a marker of intestinal inflammation.
   Lancet **339**: 89-91.

   Tumour necrosis factor α-producing cells in the intestinal mucosa of children with
   inflammatory bowel disease.
   Gastroenterology **106**: 1455-1466.

   Mucosal plexus and electrolyte transport across the rat colonic mucosa.
   J. Physiol. **376**: 531-542.

   Guidelines for the initial biopsy diagnosis of suspected chronic idiopathic
   inflammatory bowel disease.

   Platelet-activating factor is a potent colonic secretagogue with actions
   independent of specific PAF receptors.

   Fish oil versus olive oil.
   Lancet **336**: 1313-1314.

35. Calderaro V., Giovane A., Simone B.D., Camussi G., Rossiello R.,
    Quagliuolo L., Servillo L., Taccone W., Giordano C., Balestrieri C. (1991)
   Arachidonic acid metabolites and chloride secretion in rabbit distal colonic mucosa.
   Am. J. Physiol. **261**: G443-G450.

   Detection of mRNAs for macrophage products in inflammatory bowel disease by
   in situ hybridisation.
   Gut **33**: 1214-1219.
Alterations in rat peripheral blood neutrophil function as a consequence of colitis.
Dig. Dis. Sci. **40**: 192-197.

Effects of various barriers on platelet-activating factor-induced neutrophil chemotaxis.

Evidence for the presence of specific high affinity cytosolic binding sites for platelet-activating factor in human neutrophils.

Tutorial on practical confocal microscopy and use of the confocal test specimen.
In: Handbook of biological confocal microscopy. Ed. Pawley J.E. pp 549-569

Additive effects of ileal secretagogues in the rat.
Gastroenterology **98**: 881-887.

The sodium concentration of the lateral intercellular spaces of MDCK cells: a microspectrofluorimetric study.
J. Membr. Biol. **144**: 11-19

Presence of PAF-acether in stool of patients with pouch ileoanal anastomosis and pouchitis.
Gastroenterology **100**: 1509-1514.

44. Chu S., Montrose M.H. (1995)

Neurobiology of the intestinal mucosa.  
Gastroenterology 90: 1057-1081.

The epidemiology of paediatric inflammatory bowel disease.  

A model system for biological water transport.  

Degradation of soluble collagen by ozone or hydroxyl radicals.  

Triphenyltin acetate (TPTA)-induced cytotoxicity to mouse thymocytes.  

Immunolocalization of elastase in human emphysematous lungs.  

52. Dankberg P., Persidsky M.D. (1976)  
A test of granulocyte membrane integrity and phagocytic function.  
Cryobiology 13: 430-432.

Permeability characteristics of human jejunum, ileum, proximal colon and distal colon: results of potential difference measurements and unidirectional fluxes.  
Gastroenterology 83: 844-850.

Data for biochemical research.  

Capacity of the human colon to absorb fluid.  
Gastroenterology 74: 698-703.

Ultrastructural abnormalities in endoscopically and histologically normal and involved colon in ulcerative colitis.  
Am. J. Gastroenterol. 84: 1038-1046.
Transport of water and ions.  
_In: Paediatric gastrointestinal disease pathophysiology, diagnosis, management._  
B.C.Decker Inc, Ontario 1991

Failure of the human rectum to absorb electrolytes and water.  
_Gut_ **11**: 438-442.

Segmental differences along the crypt axis in the response of cell volume to secretagogues or hypotonic medium in the rat colon.  
Pflügers Arch. **426**: 462-464.

Measurement of superoxide release from single pulmonary alveolar macrophages.  
_Am. J. Physiol._ **252**: C677-C683.

61. Donnellan W.L. (1965)  
The structure of the colonic mucosa.  
_Gastroenterology_ **49**: 496-514.

Arachidonic acid metabolites and their role in inflammatory bowel disease.  
_Gastroenterology_ **88**: 580-587.

63. Donowitz M. (1986)  
Ca\(^{2+}\) and cyclic AMP in regulation of intestinal Na, K and Cl transport.  

Intestinal distribution of human Na\(^+/H\(^+\) exchanger isoforms NHE-1, NHE-2 and NHE-3 mRNA.  
_Am. J. Physiol._ **271**: G483-G493.

Serum electrolytes and colonic transfer of water and electrolytes in chronic ulcerative colitis.  
_Gastroenterology_ **56**: 101-109.
Aspects of tetrazolium salt reduction relevant to quantitative histochemistry.

Eicosanoids and the gastrointestinal tract.

68. Ecke D., Bleich M., Greger R. (1996a)
Crypt base cells show forskolin-induced Cl⁻ secretion but no cation inward conductance.
Pflügers Arch. 431: 427-434.

The ion conductances of colonic crypts from dexamethasone-treated rats.
Pflügers Arch. 431: 419-426.

70. Edidin M. (1970)
A rapid quantitative fluorescence assay for cell damage by cytotoxic antibodies.

Measurement of electrical potentials of the human rectum and pelvic colon in normal and aldosterone-treated patients.

72. Edmonds C.J. (1971)
Absorption of sodium and water by human rectum measured by a dialysis method.

73. Edmonds C.J., Pilcher D. (1973)
Electrical potential difference and sodium and potassium fluxes across rectal mucosa in ulcerative colitis.
Gut 14: 784-789.

Immunological detection of myeloperoxidase in synovial fluid from patients with rheumatoid arthritis.

Role of platelet-activating factor in ulcerative colitis.
Gastroenterology 95: 1167-1172.
Phase II trial of copper zinc superoxide dismutase (CuZnSOD) in treatment of
Crohn's disease.

77. Empey L.R., Jewell L.D., Garg M.L., Thomson A.B.R., Clandinin M.T.,
Fedorak R.N. (1991)
Fish oil-enriched diet is mucosal protective against acetic acid-induced colitis in
rats.

78. Escobar E., Galindo F., Parisi M. (1990)
Water handling in the human distal colon in vitro: role of Na⁺, Cl⁻ and HCO₃⁻.

Low-dose cyclosporine for the treatment of Crohn's disease.

80. Ferrante A., Goh D., Harvey D.P., Robinson B.S., Hii C.S.T., Bates E.J.,
Neutrophil migration inhibitory properties of polyunsaturated fatty acids.

81. Ferraris L., Karmeli F., Eliakim R., Klein J., Fiocchi C., Rachmilewitz F.
(1993)
Intestinal epithelial cells contribute to the enhanced generation of platelet
activating factor in ulcerative colitis.
Gut 34: 665-668.

82. Field M. (1980)
Regulation of small intestinal ion transport by cyclic nucleotides and calcium
American Physiological Society 1980.

83. Field M., Rao M.C., Chang E.B. (1989a)
Intestinal electrolyte transport and diarrhoeal disease (part 1).

84. Field M., Rao M.C., Chang E.B. (1989b)
Intestinal electrolyte transport and diarrhoeal disease (part 2).
85. Field M. (1991)  
Intestinal Ion Transport Mechanisms.  

Confocal microscopy: applications in neurobiology.  
*TINS* **11**: 346-351.

87. Fiocchi C. (1990)  
Immune events associated with inflammatory bowel disease.  
*Scan. J. Gastroenterol.* **25**(suppl. **172**): 4-12.

Effects of dietary fish oil supplementation on polymorphonuclear leukocyte inflammatory potential.  

89. Fondacaro J.D. (1986)  
Intestinal ion transport and diarrhoeal disease.  

Corticosteroid alteration of active electrolyte transport in rat distal colon.  

Role of tight-junctional pathways in bile salt-induced increases in colonic permeability.  

Superoxide dismutase (SOD) modulates acetic acid-induced colitis in rodents.  
*Gastroenterology* **100**: A581.

93. Fridovich I. (1978)  
The biology of oxygen radicals.  
*Science* **201**: 875-880.

Investigation of regional differences in water and electrolyte absorption across the human colon by in vivo perfusion method.  
Reactive oxygen and nitrogen metabolites as mediators of secretory diarrhea.

Colitis and colonic mucosal barrier dysfunction.
Gut 37: 530-535.

Ulcerative colitis - a disease characterised by the abnormal colonic epithelial cell?

Active transport of sodium by human colon in vitro.
Gastroenterology 59: 583-588.

Immunohistochemical localization of sodium-potassium-stimulated adenosine triphosphatase and carbonic anhydrase in human colon and colonic neoplasms.

The use of fluorescein diacetate and ethidium bromide as a viability stain for isolated islets of Langerhans.
Stain Tech. 62: 373-381.

A randomized controlled study of evening primrose oil and fish oil in ulcerative colitis.
Aliment. Pharmacol. Ther. 7: 159-166.

Degradation of hyaluronic acid by polymorphonuclear leukocytes.
Inflammation 10: 15-30.

Regulation of ion transport in colonic crypts.

Chlorination of endogenous amines by isolated neutrophils.
J. Biol. Chem. 259: 10404-10413.
Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites.  
Dig. Dis. Sci. 33: 6S-15S.

Oxidant defense mechanisms in the human colon.  
Inflammation 14: 669-680.

Free radicals in inflammatory bowel diseases pathophysiology and therapeutic implications.  
Hepato-Gastroenterol. 41: 320-327.

Intraluminal excretion of PAF, lysoPAF, and acetylhydrolase in patients with ulcerative colitis.  

The measurement and mechanism of lipid peroxidation in biological systems.  

Myenteric plexus destruction alters morphology of rat intestine.  
Gastroenterology 105: 1017-1028.

Free radicals in biology and medicine.  

Free radicals, antioxidants, and human disease: Where are we now?  

Fibroblasts and transforming growth factor β induce organization and differentiation of T84 human epithelial cells.  
Gastroenterology 111(5): 1252-1262.

Electrogenic colonic ion transport in Hirschsprung's disease: Reduced secretion to the neural secretagogues acetylcholine and iloprost.  
Gut 34: 1405-1411.
Some characteristics of Na/K-ATPase from rat intestinal basal lateral membranes.

Absorption and secretion of water and electrolytes by the intact human colon in diffuse untreated proctocolitis.

Extrarenal tissue distribution of CHIP28 water channels by in situ hybridization and antibody staining.
Am. J. Physiol. 266: C893-C903.

Electrolyte transport across colonic mucosa from patients with inflammatory bowel disease.
Gastroenterology 79: 508-511.

Mechanisms of transport of Na, Cl, and K in the human colon.
Gastroenterology 74: 1241-1247.

Prostaglandins and the gastrointestinal mucosa: Are they important in its function, disease, or treatment?
Gastroenterology 89: 1162-1188.

Therapeutic interventions in gastrointestinal disease based on an understanding of inflammatory mediators.
Agents Actions Special conference I: C22-C25.

Efficacy of zileuton, a 5-lipoxygenase inhibitor, in the maintenance of remission in patients with ulcerative colitis.
Gastroenterology 106: A697

Treatment of ulcerative colitis with fish oil supplementation: A prospective 12 month randomised controlled trial.
Gut 33: 922-928.
124. Hecht G., Pothoulakis C., LaMont J.T., Madara J.L. (1988)
Clostridium difficile toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers.

Incorporation of fatty acids from fish oils and olive oil into colonic mucosal lipids and effects upon eicosanoid synthesis in inflammatory bowel disease.
Gut 32: 1151-1155.

Histochemical evidence for generation of active oxygen species on the apical surface of cigarette-smoke-exposed tracheal explants.
Am. J. Path. 139: 573-580.

Circulating antioxidant concentrations in children with inflammatory bowel disease.

The effects of electrical field stimulation and tetrodotoxin on ion transport by the isolated rabbit ileum.

Decreased glutathione (GSH) in colonic mucosa of patients with inflammatory bowel disease (IBD): mediated by oxygen free radicals?
Gastroenterology 94: A199.

Free radical tissue damages in the anterior segment of the eye in experimental autoimmune uveitis.

Ulcerative colitis.
in: Paediatric gastrointestinal disease pathophysiology, diagnosis, management.
B.C.Decker Inc, Ontario 1991
Crohn's disease. 
_in: Paediatric gastrointestinal disease pathophysiology, diagnosis, management. 
B.C.Decker Inc, Ontario 1991

Elastase in tissue injury. 

The use of fluorescein diacetate and ethidium bromide as a stain for evaluating viability of mycobacteria. 
Stain Tech. 55: 253-257.

Food allergy: the major cause of infantile colitis. 

What is colitis? Statistical approach to distinguishing clinically important inflammatory change in rectal biopsy specimens. 

The effect of colitis on large-intestinal transport in early childhood. 

Nerve involvement in fluid transport in the inflamed rat jejunum. 
Gut 34: 1526-1530.

Morphologic and biochemical evidence for a contractile cell network within the rat intestinal mucosa. 
Gastroenterology 92: 68-81.

Novel aspects of Crohn's disease: Increased content of platelet-activating factor in ileal and colonic mucosa. 
Digestion 46: 199-204.


Active oxygen species generated by monocytes and polymorphonuclear cells in Crohn's disease.

Origin of cAMP-dependent Cl- secretion from both crypts and surface epithelia of rat intestine.
Am. J. Physiol. 264: C1294-C1301.

Expression of vascular adhesion molecules in inflammatory bowel disease.
Gastroenterology 103: 840-847.

Platelet-activating factor-induced mucosal dysfunction: role of oxidants and granulocytes.

Changes in colonic antioxidant status in rats during long-term feeding of different high fat diets.

Evans blue permeation of intestinal mucosa in the rat.

Selective 5-lipoxygenase inhibition in ulcerative colitis.

Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and myocyte leukotriene generation and neutrophil function.

Cyclosporine in severe ulcerative colitis refractory to steroid therapy.
Enhanced thromboxane A$_2$ and prostacyclin production by cultured rectal mucosa in ulcerative colitis and its inhibition by steroids and sulfasalazine.
Gastroenterology **81**: 444-449.

Modulation of small-intestinal secretion and absorption in chronic vitamin E deficiency: studies in rat jejunum *in vitro*.
Clin. Sci. **85**: 629-635.

Lipid peroxidation and electrogenic ion transport in the jejunum of the vitamin E deficient rat.
Gut **35**: 34-39.

Measurement of human intestinal secretion and absorption in vitro
London, Greenwich Medical Media (in press)

163. Lloyd-Still J.D., Green O.C. (1979)
A clinical scoring system for chronic inflammatory bowel disease in children.

Alteration of intestinal tight junction structure and permeability by cytoskeletal contraction.
Cell Physiol. **22**: C854-C861.

Loosening tight junctions.

Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers.

Cl$^-$ secretion in a model intestinal epithelium induced by a neutrophil-derived secretagogue


Phagocyte-derived lumenal superoxide induces water secretion in rat colon in vivo.
J. Physiol. **489**: 129P-130P.

J. Physiol. **499**: 109P

Paediatric gastroenterology: Lessons of inborn errors.

Gammalinolenic acid-enriched diet alters cutaneous eicosanoids.

Memoir on inventing the confocal scanning microscope.
Scanning **10**: 128-138.

Effect of oral prostaglandin E1 on intestinal transit in man.
Lancet March **29**: 648-651

Effect of sulphasalazine and its metabolites on the generation of reactive oxygen species.
Gut **28**: 190-195.

Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease.
Gut **32**: 1146-1150.

Location of tumour necrosis factor α by immunohistochemistry in chronic inflammatory bowel disease.
Gut **34**: 1705-1709.

Disruption of sulphated glycosaminoglycans in intestinal inflammation.
Lancet **20**: 711-714.
188. Murray J.M. (1992)
Neuropathology in depth: The role of confocal microscopy.

Stimulation of colonic secretion by lipoxygenase metabolites of arachidonic
acid.
Science 217: 1255-1256.

Video enhanced imaging of the fluorescent Na⁺ probe SBFI indicates that colonic
cysts absorb fluid by generating a hypertonic interstitial fluid.

The dehydrating function of the descending colon in relationship to crypt
function.

Concentration polarization of fluorescent dyes in rat descending colonic crypts:
evidence of crypt fluid absorption.
J. Physiol. 487.2: 479-495.

Effects of polymorphonuclear leukocyte transmigration on the barrier function of
cultured intestinal epithelial monolayers

The selective and superoxide-independent disruption of intestinal epithelial tight
junctions during leukocyte transmigration.
Lab. Invest. 59: 531-537.

Involvement of oxygen-derived free radicals in the pathogenesis of chronic
inflammatory bowel disease.

196. Nishida T., Miwa H., Shigematsu A., Yamamoto M., Lida M., Fujishima M.
(1987) Increased arachidonic acid composition of phospholipids in colonic
mucosa from patients with active ulcerative colitis.
Visualization and quantitative analysis of leukocyte dynamics in retinal microcirculation of rats.

Optimization of the histochemical demonstration of DNA using 3-hydroxy-2-naphthoic acid hydrazide and Fast Blue B.

Luminal capsaicin inhibits fluid secretion induced by enterotoxin E. coli STa, but not by carbachol, in vivo in rat small and large intestine.
Exp. Physiol. 81: 313-315.

Non-infective colitis in infancy: Evidence in favour of minor immunodeficiency in its pathogenesis.

Image sharpness and contrast transfer coherent confocal microscopy.

Location of superoxide anion generation in human colonic mucosa obtained by biopsy.
Gut 34: 936-938.

Increased arachidonic acid levels in phospholipids of human colonic mucosa in inflammatory bowel disease.

204. Paddock S.W. (1991)
The laser-scanning confocal microscope in biomedical research. [Review]

205. Paddock S.W. (1994)
To boldly glow... Applications of laser scanning confocal microscopy in developmental biology.
Role of oxygen-derived free radicals in digestive tract diseases.  
Surgery 94: 415-422.

Handbook of biological confocal microscopy.  

Evidence from fluorescence microscopy and comparative studies that rat, ovine  
and bovine colonic crypts are absorptive.  
J. Physiol. 460: 525-547.

209. Playford R.J., Freeman T., Quinn C., Calam J. (1990)  
Deficiency of a mucosal trypsin inhibitor in patients with ulcerative colitis.  
Gut 31: A1166

Fluorescence microscopy.  
In: Light microscopy in biology – a practical approach. Ed. Lacey A.J. pp163-  
185.  

The use of 3-hydroxy-2-naphthoic acid hydrazide and Fast Blue B for the  
histochemical detection of lipid peroxidation in animal tissues - a  
microphotometric study.  
Histochemistry 95: 255-262.

Imaging of oxidative stress at subcellular level by confocal laser scanning  
microscopy after fluorescent derivatization of cellular carbonyls.  
Am. J. Path. 142: 1353-1357.

213. Poulos A., Robinson B.S., Ferrante A., Harvey D.P., Hardy S.J., Murray  
Effect of 22-32 carbon n-3 polyunsaturated fatty acids on superoxide production  
in human neutrophils: synergism of docosahexaenoic acid with f-met-leu-phe and  
phorbol ester.  
Immunology 73: 102-108.

Barrier function of epithelia.  
Epithelial secretory responses to inflammation. Platelet activating factor and reactive oxygen free metabolites.

Dogma destroyed: Colonic crypts absorb.

Quantitating intestinal ischemia with nitroblue tetrazolium salts.

Platelet-activating factor.
J. Biol. Chem. 265: 17381-17384.

Platelet-activating factor - a possible mediator in the pathogenesis of ulcerative colitis.
Scan. J. Gastroenterol. 25(suppl172): 19-21.

Cytokines and platelet-activating factor in human inflamed colonic mucosa.
Agents Actions Conference issue: C32-C36.

A novel mechanism of sodium transport in colonic crypts.
J. Biol. Chem. 270: 11051-11054.

5-Lipoxygenase inhibitors for the treatment of inflammatory bowel disease.

Diarrhoea: the failure of colonic salvage.

In vitro effects of oxpentifylline on inflammatory cytokine release in patients with inflammatory bowel disease.
Gut 40: 475-480.


The role of marine fish oils in the treatment of ulcerative colitis.

Role of oxygen-derived free radical scavengers in the management of recurrent
attacks of ulcerative colitis: A new approach.

Preliminary report on the use of oral tacrolimus (FK506) in the treatment of
complicated proximal small bowel and fistulizing Crohn's Disease.
Am. J. Gastroenterol. 92(5): 876-879.

Quantitative fluorescence confocal laser scanning microscopy (CLSM)

Effect of glucocorticoids on rectal transport in normal subjects and patients with
ulcerative colitis.
Gut 27: 309-316.

Segmental variability of membrane conductances in rat and human colonic
epithelia.
Pflügers Arch. 410: 173-180.


Early mucosal changes in Crohn's disease.
Gut 34: 375-381.

Radical reactions in vivo - an overview.

Cyclosporine therapy for inflammatory bowel disease.
Contribution of genes of the major histocompatibility complex susceptibility and
disease phenotype in inflammatory bowel disease
The Lancet 347: 1212-1217.

The genetics of inflammatory bowel disease
Gut 40: 572-574.

Application of a CCD linear array camera in the quantification of tissue staining:
NBT detection in ischaemic myocardium.

Sodium needs of infants and children with ileostomy.
J. Paediatrics 102: 509-513.

248. Sedghi S., Fields J.Z., Klamut M., Urban G., Durkin M., Winship D.,
Increased production of luminol enhanced chemiluminescence by the inflamed
colonic mucosa in patients with ulcerative colitis.
Gut 34: 1191-1197.

Glucose-induced ion secretion in rat jejunum: a mucosal reflex that requires
integration by the myenteric plexus.

Inflammation-induced intestinal hyperemia in the rat: Role of neutrophils.
Gastroenterology 95: 1528-1534.

Ion transport in human colon in vitro.
Gastroenterology 93: 441-448.

Inhibition of cholera toxin action in the rabbit by cyclohexamide.
Gastroenterology 56: 506-511.
Signal strength and noise in confocal microscopy: factors influencing selection of an optimum detector aperture.

Confocal laser scanning microscopy

Oxygen-derived free radical generating capacity of polymorphonuclear cells in patients with ulcerative colitis.
Digestion 44: 163-171.

Effects of forskolin in crypt cells of rat distal colon.
Pflügers Arch. 424: 321-328.

Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease.
Gastroenterology 103: 186-196.

Inflammatory bowel disease - a radical view.
Gut 34: 865-868.

Plasma-mediated neutrophil activation during acute myocardial infarction: role of platelet-activating factor.

A novel technique for viable cell determinations.
Stain Tech. 61: 315-318.

Basal fluid absorption and agonist-stimulated secretion in the isolated, perfused colonic crypt.
Gastroenterology 106: A272

Fluid absorption in isolated perfused colonic crypts.


273. Stirling C.E. (1972)  
Radioautographic localization of sodium pump sites in rabbit intestine.  

274. Sturzaker H.G., Hawley P.R. (1975)  
Collagenase activity in rectal and colonic biopsies.  

Rectal biopsy helps to distinguish acute self-limited colitis from idiopathic inflammatory bowel disease.  
Gastroenterology 86: 104-113.

Ca-mediated stimulation of Cl secretion by reactive oxygen metabolites in human colonic T84 cells.  

Direct measurement of the superoxide free radical anion in vivo in the rat colon in the presence of phorbol myristate acetate and superoxide dismutase.  
(unpublished).

Comparison of leukocyte excretion and blood loss in inflammatory disease of the bowel.  
Gut 34: 1535-1538.

NADPH-dependent lipid peroxidation capacity in unfixed tissue sections: characterization of the pro-oxidising conditions and optimization of the histochemical detection.  
Histochemical J. 26: 189-196.

Regional differences in the response to platelet-activating factor in rabbit colon.  

Salicylates for ulcerative colitis – their mode of action.  

Site of action of platelet-activating factor within the mucosa of rabbit distal colon.  
AMP and microamps: Neutrophil-derived chloride secretagogue identified.
J. Clin. Invest. 91: 1857

Fluorophores for confocal microscopy.

Tumour necrosis factor and Crohn's disease.
Gut 40: 443-448.

Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2).

Selective immunomodulation in patients with inflammatory bowel disease – future therapy or reality?

Platelet-activating factor primes neutrophil responses to agonists: Role in promoting neutrophil-mediated endothelial damage.

Reactive oxygen metabolites and colitis; a disturbed balance between damage and protection.

Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure.

Review article: New insights into prostaglandins and mucosal defense.

The eosinophil in inflammatory bowel disease.
The nitroblue tetrazolium test.

The nitroblue tetrazolium test in Crohn's disease and ulcerative colitis.
Digestion 14: 179-183.

Use of coculture of colonic mucosal biopsies to investigate the release of eicosanoids by inflamed and uninflamed mucosa from patients with inflammatory bowel disease.
Gut 33: 1644-1651.

Interrelationships between inflammatory mediators released from colonic mucosa in ulcerative colitis and their effects on colonic secretion.
Gut 34: 503-508.

Platelet activating factor: Release from colonic mucosa in patients with ulcerative colitis and its effect on colonic secretion.
Gut 38: 355-361.

The measurement of oxygen-derived free radicals and related substances in medicine.

The pixelated image

Lab. Invest. 47: 5-11.

Neutrophils degrade subendothelial matrices in the presence of alpha-1-proteinase inhibitor.

Oxidative autoactivation of latent collagenase by human neutrophils.
304. Weiss S.J., Curnutte J.T., Regiani S. (1986a) 
Neutrophil-mediated solubilization of the subendothelial matrix: Oxidative and nonoxidative mechanisms of proteolysis used by normal and chronic granulomatous disease phagocytes. 

305. Weiss S.J., Test S.T., Eckmann C.M., Roos D., Regiani S. (1986b) 
Brominating oxidants generated by human eosinophils. 
Science 234: 200-203.

Tissue destruction by neutrophils. 

Evaluation of activity of putative superoxide dismutase mimics. 
J. Biol. Chem. 268: 23049-23054.

Crypts are the site of intestinal fluid and electrolyte secretion. 
Science 218: 1219-1221.

Functional histology. 
Churchill & Livingstone.

The role of the pinhole in confocal imaging system 

Excitatory and neurotoxic actions of platelet-activating factor on rat myenteric neurons in cell culture. 

Toxic oxygen metabolite production by circulating phagocytic cells in inflammatory disease. 

Apical membrane properties and amiloride binding kinetics of the human descending colon. 
Confocal fluorescence microscopy and three-dimensional reconstruction.  

Role of neutrophil-derived oxidants in the pathogenesis of intestinal inflammation.  

Effects on fluid and Na⁺ flux of varying luminal hydraulic resistance in rat colon in vivo.  
J. Physiol. 477: 539-548.